The bacteria: historical introduction

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HISTORY

The problem with writing about history is that developments appear inevitable and indeed when we present a chronological account this must be the case. However, it is as well to remember that at each point in the story the outcome could have been different. The advances in medicine in the twentieth and twenty-first centuries are the result of the demonstration of the validity of the germ theory in the nineteenth century and most significantly the establishment of its functional utility. It is easy to forget how recent these advances are. In 1922, Topley succeeded Sheridan Delépine in the Bacteriology Department of the University of Manchester. Delépine had worked with Pasteur. This was a world before molecular biology, before most immunology, and at the beginnings of antimicrobial chemotherapy. Here, some of the advances are documented, but the choice of what to include is determined by the concerns of the present and the recent past and may not reflect fully the concerns and priorities of the past.

MICROBIOLOGY

Microbiology is the study of living organisms ('microorganisms' or 'microbes'), simple in structure, and usually small in size, that are generally considered to be neither plants nor animals; they include bacteria, algae, fungi, protozoa, and viruses. 'Pure' microbiology concerns the organisms themselves and 'applied' microbiology their effects on other living beings, when they act as pathogens or commensals, or on their inanimate environment, when they bring about chemical changes in it. Thus, microbiology has applications in human and veterinary medicine, in agriculture and animal husbandry, and in industrial technology and even climatology.

Microorganisms were first seen and described by the Dutch lens-maker Antonie van Leeuwenhoek (1632–1723), who devised simple microscopes capable of giving magnifications of c. $\times 200$. In a number of letters to the Royal Society of London between 1673 and his death, he gave clear and accurate descriptions and drawings of a variety of living things that undoubtedly included protozoa, yeasts, and bacteria (Dobell 1932). These striking observations did not lead immediately to great advances in the knowledge of microbes. These were delayed for nearly two centuries, until essential technical advances had been made by workers who nowadays would be described as industrial or medical microbiologists.

When bacteriology started in the nineteenth century the borders of the discipline were uncharted and much of what would now be called virology and immunology was included. More recently bacteriology has contributed to, or perhaps been subsumed by, the emergence of molecular biology. Such categories are largely arbitrary and the concern here is to present an outline that does justice to the roots of the subject.

COMMUNICABLE DISEASES

Long before microbes had been seen, observations on communicable diseases had given rise to the concept of contagion: the spread of disease by contact, direct or indirect. This idea was implicit in the laws enacted in early biblical times to prevent the spread of leprosy. It became less influential in the classical era, when supernatural and miasmatic causes were favored. In the later Middle Ages, there was renewed interest in contagion, reinforced at the end of the fifteenth century by the spread of syphilis in Europe, which was obviously associated with a specific form of contact. Fracastorius (Girolamo Fracastoro), a physician of Verona, published an influential analysis of contagion in 1546:

- by physical contact alone
- by formites, and
- at a distance.

He was led to conclude that communicable diseases were caused by living agents; these he spoke of as 'seminaria' or 'seeds,' but he was unable to give a more definite opinion about their nature.

In the subsequent 250 years, several authors speculated that the agents of contagious diseases were animate, but little evidence for this was produced. Even the recognition of parasitism of animals, e.g. scabies and some forms of helminthiasis (see Foster 1965), appears to have had little impact on thinking about the role of microorganisms as pathogens. Early in the nineteenth century, improvements had been made in the design of microscopes and between 1834 and 1850 numerous accounts were published of morphologically recognizable microorganisms in material from diseased animals or human subjects: of fungi subsequently called *Botrytis* in the silkworm disease 'calcino' by Bassi; of trichomonads in human vaginal discharges by Donné; of ringworm fungi by Schönlein and by Groby; of vibrios in cholera stools by Pouchet; and of large rod-shaped bacteria in anthrax blood by Rayer and Davaine (for references see Bulloch 1938).

THE 'GERM THEORY'

In 1840, Henle affirmed his belief in what came to be called the 'germ theory of disease,' which asserted that certain diseases were caused by the multiplication of microorganisms in the body, but he advanced little supporting evidence for this and his view was hotly disputed.

The lack of a germ theory probably led to the initial rejection by the General Board of Health in 1854 of Snow's explanation of the waterborne spread of cholera in London. In groundbreaking investigations, Snow established that cholera was spread by water contaminated with human feces; however, these findings conflicted with the miasmatic theory and their significance was not fully realized until the germ theory was accepted. As late as 1894 Creighton, in his still unsurpassed 'History of Epidemics in Britain,' based his understanding on the miasmatic theory.

The intellectual and scientific case for the germ theory was established by the studies of Koch and Pasteur, which are set out below, but there was no immediate widespread acceptance. The Hamburg cholera outbreak and the dispute between Koch and Pettenkoffer both publicized and showed the strength of the theory. Drinking water from Hamburg was taken from the river Elbe above the town and sewage was discharged into the river. In Hamburg there were many cases of cholera. Altona, downstream from Hamburg, also drew drinking water form the Elbe; however, this water passed through slow sand filters before distribution. In Altona there were only a few cases of cholera. The demonstration that removal of contamination from the water supplies prevented infection convinced the public and the medical profession of the bacterial etiology of the disease. In spite of this elegant demonstration of the waterborne transmission of *Vibrio cholerae* and the mechanism for control, not all were convinced. Pettenkoffer and Emmerich both drank 1 ml of *V. cholerae* culture. Both survived (Pettenkoffer 1892). This publicity undoubtedly helped to popularize the theory.

FERMENTATION AND PUTREFACTION

In the first half of the nineteenth century, chemists became interested in fermentation: industrial processes in which organic substances underwent changes that yielded useful compounds, such as alcohol and acetic acid. A similar process, termed putrefaction, led to the decay of organic matter, usually with the production of an unpleasant odor and taste. In the 1830s, several observers, notably Cagniard-Latour and Schwann, saw yeasts in liquors undergoing alcoholic fermentation and concluded that these were living organisms and the cause of the process. This view was resisted by leading authorities of the day (Berzelius, Liebig, Wohler), who considered that fermentation was a purely chemical process and that the yeasts were a consequence rather than the cause of it. Between 1836 and 1860 controversy raged but without a clear outcome.

The work of Louis Pasteur

Louis Pasteur (1822–1895) (Figure 1) produced strong experimental evidence that microorganisms were the cause of fermentation and in so doing laid the foundations of microbiology as a science. He was a chemist whose early studies of fermentation aroused his interest in the molecular asymmetry of some of the compounds formed. He concluded that optically active chemical compounds, such as the stereoisomeric forms of tartaric

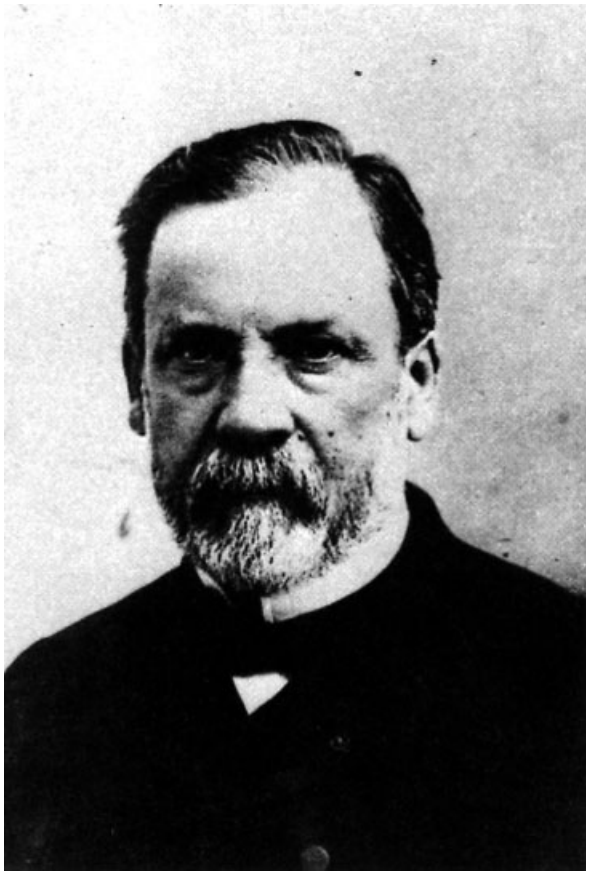

Figure 1 *Louis Pasteur (1822–1895).*

acid and amyl alcohol, never arose from the purely chemical decomposition of sugars but were formed from them by the action of microorganisms; these were always present in fermenting liquors and increased in number as the process continued. Different fermentation processes (e.g. alcoholic, acetic, butyric) were each associated with particular organisms, which were often recognizable by their morphology or requirements for growth.

To maintain that microorganisms caused fermentation, it was necessary to establish that they did not arise de novo. This was contrary to the widely held belief in the spontaneous generation of living things from dead animal or vegetable material ('heterogenesis'). Controversy about this in the eighteenth century had centered around the conditions under which putrefaction developed in organic matter that had been subjected to supposedly sterilizing temperatures in closed containers. This matter was unresolved in 1860. In a series of admirable experiments reported in the next 4 years (see Vallery-Radot 1922–33), Pasteur disposed of many purported instances of heterogenesis by showing that they could be attributed to failure of the initial sterilization or to subsequent recontamination. He emphasized the need for scrupulous sterilization of everything coming into contact with the material under examination and demonstrated numerous sources of contamination from air, dust, and water. He showed that some organisms were not destroyed by boiling. For the sterilization of fluids, he advocated heating to 120°C under pressure, and for glassware, the use of dry heat at 170°C; he showed the value of the cottonwool plug for protecting material from aerial recontamination.

In the course of these experiments Pasteur used various forms of nutrient fluid to grow microorganisms and showed that a medium suitable for one might be unsuitable for another, so for successful cultivation it was necessary to discover a suitable growth medium and to establish optimal conditions of temperature, acidity or alkalinity, and oxygen tension.

The mass of experimental data produced by Pasteur carried general conviction, but a minority of adherents of heterogenesis continued to maintain their position, often supporting this by experiments in which inadequate heating had failed to destroy very heat resistant bacteria. The observations of Tyndall in the early 1870s, that all actively multiplying bacteria were easily destroyed by boiling, led to the introduction of a method of sterilization by repeated cycles of heating interspersed with periods of incubation. This method of 'tyndallization' served to eliminate many of the anomalies reported by the advocates of heterogenesis (see Bulloch 1938).

Pasteur devoted much effort to investigating the troubles of French winemakers, brewers, and vinegar makers. These studies often led him to perform experiments of fundamental scientific importance, as when his involvement in the problems of vinegar making led to valuable observations on the constancy of microbial characters in culture. His general conclusion was that fermentations owed their diversity to the characters of the several organisms responsible for them, but final proof of this was not obtainable until methods of obtaining pure cultures had been discovered.

PATHOGENIC MICROORGANISMS

In about 1865, Pasteur responded to an appeal to investigate a formidable disease of silkworms in southern France (pébrine); by 1869, his experiments had led him to the conclusion that this was a communicable disease transmitted by direct contact or fecal contamination. This, according to his biographer Vallery-Radot (1919), engendered in his mind the idea that communicable diseases of animals and man, like the 'diseases' of wine and beer, might be a consequence of microbial multiplication.

From 1857 onward there had been reports, notably by Brauell and Davaine, of the transmission of anthrax between animals by the injection of blood. At that time there was also much interest in the septic and pyemic diseases of man, including 'surgical fever' (see Bulloch 1938). In 1865, Coze and Felty began to publish a series of papers reporting the presence of bacteria in the blood

of dogs and rabbits that had received injections of purulent material from human patients. In 1872, Davaine, starting with blood from patients suffering from 'putrid' infections, performed serial passage in experimental animals and demonstrated enhancement of virulence.

Joseph Lister (1827–1912) was aware of Pasteur's demonstration that both fermentation and putrefaction might be initiated by airborne organisms. On the assumption that 'putrefying' wounds might be similarly caused, he attempted to prevent surgical sepsis by denying access to wounds of microbes from the patient's surroundings, particularly from the air. His 'antiseptic' regimen, first described in 1867, was strikingly successful and transformed the prognosis of major surgical operations. Lister did not prove that this was due to the destruction of potentially pathogenic microbes, but this was rendered highly probable by the contemporary work of French and German bacteriologists.

In retrospect, the work of another surgeon, Alexander Ogston (1844–1929), who described staphylococci in pus, assumes perhaps equal importance and helped lay the foundations for the study of hospital infection. The history of hospital infection was reviewed by Selwyn (1991) and Ayliffe and English (2003).

The work of Robert Koch

In 1876, while a country physician at Wollstein in eastern Germany, Robert Koch (1843–1910) (Figure 2) published his first scientific work, a study of the anthrax bacillus in experimental animals, of its growth in vitro, and of the formation and germination of its spores. This opened up a new era in bacteriology. In the following year, he described the fixing and staining of bacteria with the newly introduced aniline dyes. In 1878, his study of wound infections explored the role of animal experimentation in establishing the cause of bacterial infections. Then, in 1881, he described means of cultivating bacteria on solid media, thus making it possible to obtain pure cultures by transferring material from a single colony. First he used pieces of potato as his growth medium, then nutrient gelatin, and later agar gel media. In 1882 and 1884, he published classic papers on the tubercle bacillus, and in 1883, he described the cholera vibrio.

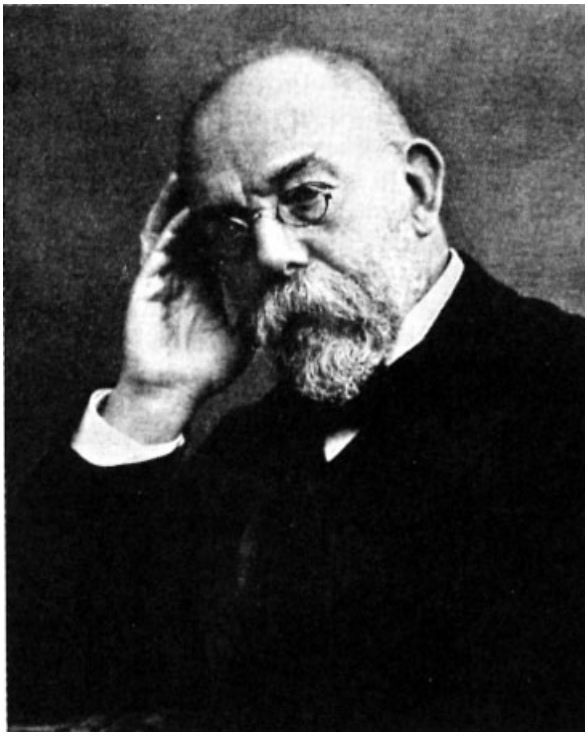

Figure 2 *Robert Koch (1843–1910).*

Koch had now assembled the techniques needed to investigate the bacterial causes of many communicable diseases. He had moved to Berlin, where Loeffler and Gaffky were already his assistants; later came Pfeiffer, Kitasato, Welch, and many others. Koch began to gather round him the group of followers who were destined to introduce his methods into many laboratories throughout the world.

We should remember that not all the techniques were devised by Koch; agar was first used by Hesse at the suggestion of his wife Fannie. In 1887, Petri described his culture dish, which remains a mainstay of bacteriological isolation.

The fruits of this technical revolution appeared with remarkable speed during the years 1876–90, the period described by Bulloch (1938) as 'the heyday of bacterial etiological discovery,' when most of the important groups of bacterial pathogens for man and animals were recognized.

Differential staining

In 1878, Paul Ehrlich had noted differences in the affinity for aniline dyes of various types of living cells, an observation that started him on his long search for chemotherapeutic agents. In 1882, he reported that tubercle bacilli stained with fuchsin retained the dye when subsequently treated with a mineral acid; this property of 'acid-fastness' formed the basis for methods later developed to detect mycobacteria in tissue sections, in sputum and other secretions, and in cultures.

A differential staining method of even wider applicability arose from the observation, reported in 1884 by the young Danish physician Christian Gram, that certain bacteria, when stained with methyl violet and treated with an iodine solution as a mordant, retained the violet dye when washed briefly with ethyl alcohol. The 'gram reaction' proved to be a useful means of dividing bacteria into two categories: 'gram-positive' organisms that were stained violet and 'gram-negative' organisms that lost the violet dye and were stained red by a

counterstain applied after washing with alcohol. This property was later found to reflect differences in cell-wall composition and to be correlated with a number of other characters; organisms that retained the violet stain were in general less susceptible, than those that did not, to various chemical substances and to lysis by complement in the presence of specific antibody.

Establishing the pathogenicity of bacteria

As the number of different bacteria found in constant association with human and animal diseases grew, the question of how to establish their etiological role assumed importance. Already in the 1880s it was being recognized that, though the internal organs were normally sterile or nearly so, many surface sites and body cavities communicating with the outside had a rich bacterial flora, so the presence of an organism here was of little significance. When inflammatory lesions appeared in such places it was often difficult to decide which, if any, of the organisms present was responsible.

Koch's experience with anthrax, wound infection, and tuberculosis led him to place much reliance on the evidence of animal experimentation in establishing relationships between disease and isolate. A set of conditions, all of which must be fulfilled to justify such a conclusion, has been called Koch's postulates. They are as follows (see Topley and Wilson 1931), though Koch did not state them in precisely this form:

- the organism is regularly found in the lesions of the disease
- it can be grown in pure culture outside the body of the host for several generations, and
- such a culture will reproduce the disease in question when administered to a susceptible experimental animal.

It subsequently proved difficult or impossible to fulfill all these criteria in respect of many microbial diseases.

Immunity

Folk medicine had long established that exposure to certain infective agents might engender immunity to them (Parker 1998) and experience with Jennerian vaccination against smallpox had indicated the value of selecting a strain of the agent with low virulence for use as an inducer of immunity. While Koch and his pupils were continuing to characterize more and more pathogens, Pasteur turned his attention to the possibility of inducing prophylactic immunity by injections of live cultures of organisms, the virulence of which had been attenuated by prolonged culture or by growth under suboptimal conditions. Success was reported in 1877 with a live vaccine against the pasteurella of chicken cholera and in 1881 with one against anthrax in animals. In 1886, Pasteur reported on the use of an attenuated live vaccine against rabies. This consisted of a dried suspension of spinal cord from an infected rabbit. Its use was an extension of the original principle of vaccination in that the material was given after infection had taken place. It was used with apparent success to prevent disease in human subjects who had been bitten by a rabid animal. Most of the early attenuated living vaccines caused appreciable morbidity and even some deaths, but in 1886, Salmon and Smith showed that it was possible to protect pigeons against salmonella infection by the injection of heat-killed organisms. Pfeiffer demonstrated in 1889 that immunity conferred by vaccination was usually highly specific, but there were exceptions to this.

At a somewhat earlier date, Metchnikoff had observed the engulfment of bacteria and other microbes by phagocytes; in 1891, he expressed the view that immunity was primarily cellular. This conflicted with growing evidence for the importance of serum factors; the alternative humoral view of immunity was that 'antibodies' appeared in the serum of vaccinated or infected animals and that their specificity corresponded to that of the 'antigens' that elicited them.

Strong evidence for humoral immunity emerged after Roux and Yersin in Paris had demonstrated in 1888 the characteristic lethal effects of broth cultures of diphtheria bacilli on guinea-pigs and shown that these were caused by the liberation of a soluble toxin, an 'exotoxin.' In the following year, Behring in Koch's laboratory observed that chemically sterilized broth cultures of diphtheria bacilli retained their toxicity for guinea-pigs but animals given sublethal doses of them were subsequently immune to diphtheria. He also showed that the pleural fluid of animals dead of diphtheria was toxic but yielded no diphtheria bacilli on culture; however, injections of it rendered other guinea-pigs immune. By 1890, Behring had demonstrated that the blood of immunized guinea-pigs neutralized diphtheria toxin in vitro. Faber demonstrated tetanus toxin in 1889; the following year, Behring and Kitasato immunized rabbits against it and showed that their serum protected mice against tetanus.

By 1890, many of the basic areas of immunology had been outlined, though some concepts that are now considered as basic were discovered surprisingly late. The secondary response was described by Glenny and Sudmerson (1921) and the concept of herd immunity by Topley and Wilson (1923). These events set the study of immunity on a firm foundation and form the basis of the discipline of immunology; the further history can be found in the Immunology volume History.

The chronology of these events is summarized in Tables 1 and 2. Figure 3 shows the Bacteriological

Table 1 *Bacteriology in the nineteenth century*

Year	Event
1834–50	Fungi, protozoa, and bacteria seen in diseased tissues or secretions (see text)
1836–37	Yeasts seen in liquors undergoing alcoholic fermentation
1840	Henle: 'germ theory of disease'
1844–57	Pasteur: studies optically active compounds from fermented fluids
1849-54	Snow: waterborne transmission of cholera
1857–63	Pasteur: reports that anthrax is transmitted by injections of blood from diseased animals
1860–64	Pasteur: experimental evidence that fermentation and putrefaction are effects of microbial growth
1865–67	Pasteur studies 'pébrine' of silkworms; concludes that it is caused by microbial action
1867	Lister: success of 'antiseptic surgery' supports view that microbes cause postoperative sepsis
1876	Koch: demonstrates pathogenicity and sporulation of anthrax bacilli
1877	Koch: staining of bacteria by aniline dyes
1877	Tyndall: heat-resistant bacteria destroyed by repeated cycles of moderate heating and incubation
1877	Pasteur: chicken cholera prevented by injections of live attenuated culture
1877	Soil nitrates replenished by microbial action
1878	Koch: studies of wound infection; use of experiments on animals to establish etiology
1879	Ehrlich: differences in affinity of chemical substances for various sorts of living cells
1880	Ogsten: staphylococci in pus
1881	Koch: use of solid media to obtain pure cultures of bacteria
1881	Pasteur: anthrax prevented by live attenuated vaccine
1882	Ehrlich: acid fastness of the tubercle bacillus
1882	Hesse: use of agar to solidify culture media
1882–84	Koch: etiological role of the tubercle bacillus; 'Koch's postulates'
1883	Koch describes the cholera vibrio
1883–91	Metchnikoff studies cellular defence mechanisms
1884	Chamberland filters
1884	Gram's stain
1886	Pasteur's rabies vaccine
1886	Salmon and Smith: killed bacterial vaccines effective
1887	Petri: double-sided culture dish
1888	Roux and Yersin: diphtheria bacillus forms exotoxin
1888	Nuttall: serum killing of bacteria
1889	Behring: antitoxic immunity to diphtheria
1889	Pfeiffer: specificity of immunity conferred by vaccines
1889	Faber: tetanus bacillus forms exotoxin
1889	Buchner: serum killing of bacteria inhibited by heating the serum
1890	Behring: diphtheria antitoxin neutralizes toxin in vitro
1890	Behring and Kitasato: antitoxic immunity to tetanus
1890	Winogradsky: nitrite- and nitrate-forming bacteria in soil
1892	Tobacco-mosaic disease transmitted by filtered material
1894	Pfeiffer's phenomenon: lysis of vibrios in peritoneal cavity
1895	Bordet: heat-stable and heat-labile factors (respectively, antibody and complement) in immune lysis
1897	Ehrlich: 'side-chain theory' of antibody production
1898	Foot-and-mouth disease transmitted by filtered material

Section of the Congress of Hygiene and Demography, London, 1891.

Bacterial filters and the origins of virology

An important technical advance in the latter part of the nineteenth century was the development of filters that held back bacteria but allowed the passage of smaller microorganisms and biologically important macromolecules: in 1884, Chamberland introduced filters made of unglazed porcelain and in 1891 Nordtmeyer introduced the Berkefeld-type of filter composed of kieselguhr. There were several important consequences of these innovations, as follows.

Filtration provided a convenient means of producing bacteria-free preparations of soluble toxins and thus greatly simplified the task of producing reagents for passive and active immunization against diphtheria and tetanus. It was also an essential preliminary to the

Table 2 *The twentieth century*

Year	Event
1900	Reed: yellow fever virus
1911	Rous: chicken sarcoma caused by a virus
1912	Ehrlich and Hata: Salvarsan for the treatment of syphilis
1915/17	Twort and d'Herrelle: 'bacteriophage'
1921	Glenny and Sudmerson: the secondary response to antigen
1923	Topley and Wilson: herd immunity
1928	Griffith: the transformation of pneumococci
1929	Fleming: penicillin – the first antibiotic
1935	Domagk: prontosil – the first suphonamide
1944	Avery, MacLeod, and McCarty: DNA as the agent of transformation
1944	Schartz, Bugie, and Waksman: streptomycin – the first antituberculosis treatment
1946	Lederberg and Tatum: conjugation in bacteria
1952	Lederberg and Zinder: phage transduction of bacteria
1953	Crick, Franklin, Watson, and Wilkins: structure of DNA
1960	Jacob, Perrin, Sanchez, and Monod: the operon concept
1961	Brenner, Jacob, and Meselson: ribosomes site of protein synthesis
1973	Cohen, Chan, Helling, and Boyer: plasmid vectors
1977	Fox, Pecham, and Woese: molecular systematics (16S RNA)
1977	Woese: Archaebacteria
1977	Gilbert and Sanger: DNA sequencing
1986	Mullis: the polymerase chain reaction (PCR)
1995	Venter, Smith, and Fraser: genome sequence of *Haemophilus influenzae*

purification of toxins and to chemical studies of their constitution.

It soon became apparent that some disease agents passed through bacteria-retaining filters; thus, the first viral pathogens were recognized. In 1892, Iwanowski described the transmission of mosaic disease to tobacco plants and, in 1898, Loeffler and Frosch described the transmission of foot-and-mouth disease in bovines by the injection of filtrates of infective material. In 1900, Walter Reed demonstrated that yellow fever is caused by a filterable agent (Reed 1902). The further history of virology is dealt with in the Virology volume,
A short history of research on viruses.

A later consequence of the use of bacterial filters was the discovery, independently by Twort in 1915 and by d'Herelle in 1917, of bacteriophages, subsequently shown to be viruses that multiply in bacterial cells. Intensive study of the interaction of bacteriophage and bacterium by Delbrück and Hershey in the early 1940s contributed much to the knowledge of viral infections and also led to important developments in bacterial genetics (see Bacterial genetics).

NONMEDICAL APPLICATIONS OF BACTERIOLOGY

The discoveries of Pasteur and Koch had important applications for agriculture and industry. The replacement of nitrates lost from the soil by the washing action of rain had long been a mystery but it seemed to be connected in some way with the decomposition of organic matter. In 1877, Schloesing and Muntz, acting on a suggestion from Pasteur, showed by experiment that the formation of nitrates was due to the action of living organisms. Warington confirmed this in 1878 and 1879 and demonstrated that the process took place in two stages: first, the conversion of ammonia to nitrites, and then the oxidation of nitrites to nitrates. He believed that these two stages were performed by different organisms but failed to prove this. In 1890, Winogradsky isolated and described the nitrogen-fixing bacteria that caused the formation of nodules on the roots of leguminous plants. Later, Winogradsky described a free-living anaerobic organism that fixed atmospheric nitrogen and Beijerinck, some 10 years afterwards, described a large free-living nitrogen-fixing aerobe that he named *Azotobacter*.

The importance of bacteria in maintaining the fertility of the soil has thus been recognized for over a century. A more recent concept is that the chemical activities of primitive ancestral microbial forms may have created the atmospheric conditions essential for the appearance of plants and animals on earth (see Schlegel 1984).

Bacteria cause diseases of plants as well as animals. In 1878, Burrill described the organism responsible for pear blight and, in 1883, Wakker described the bacterial cause of 'yellows' of hyacinths. Recognition of the role of bacteria in the spoilage of foodstuffs and in the production of organic chemicals useful to man led to the entrance of the bacteriologist into numerous industrial fields.

The development of 'pure' bacteriology

Pasteur's studies of fermentation in the early 1860s revealed the physiological diversity of microbes and may be looked upon as the starting-point of 'pure' bacteriological studies. During the 1870s, he became more concerned with the role of microbes as pathogens, but he continued to be interested in their basic properties. For example, in 1878, he described under the name 'Vibrion septique' a pathogenic clostridium responsible for gangrenous conditions in animals and demonstrated that it was an obligate anaerobe. Within a few years it became possible to obtain pure cultures of many sorts of bacteria by colony selection on solid media and then to

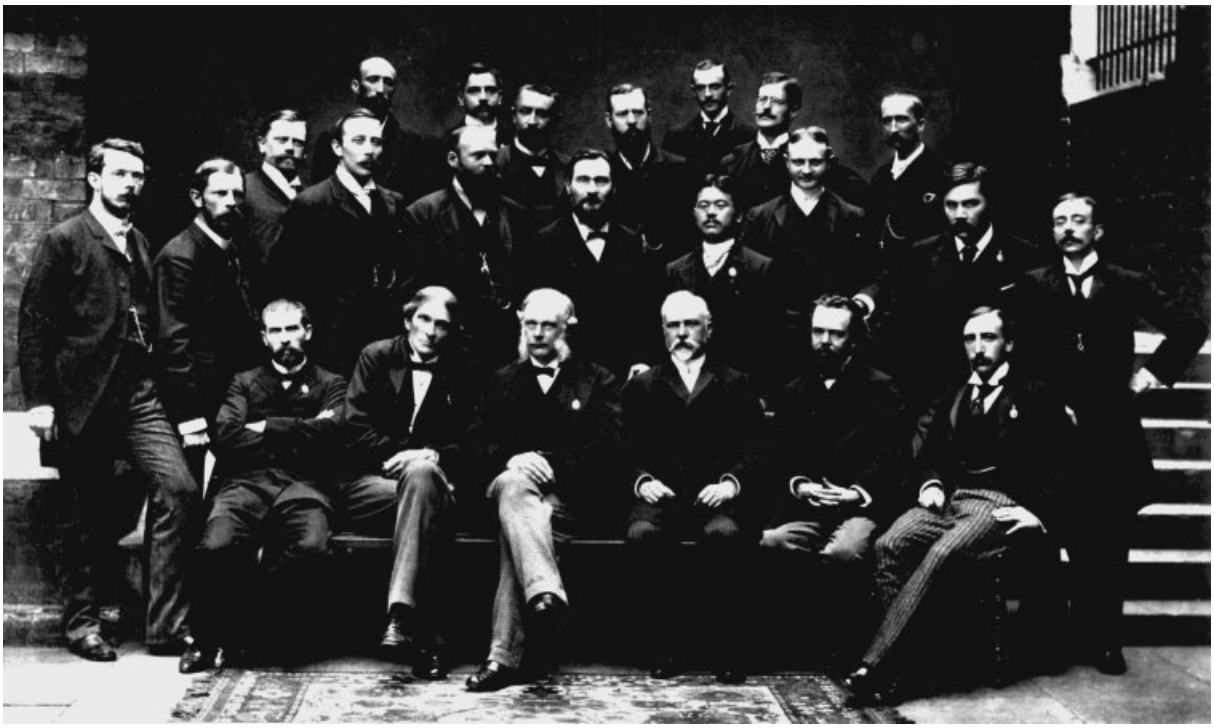

Figure 3 *Bacteriological Section, Congress of Hygiene and Demography, London, 1891.*

collect reliable data about their phenotypic characters. Accounts of their growth on various media under different physical conditions were soon supplemented by information about the range of their fermentative action on organic compounds and the products of fermentation and by the identification of chemical requirements for growth. Thus, the raw materials for systematic bacteriology began to be accumulated and basic studies of bacterial metabolism could begin.

For more detailed accounts of the early history of bacteriology, and for references, see Bulloch (1938), Clark (1961), Lechevalier and Slotorovsky (1965), and Foster (1970).

THE TWENTIETH CENTURY

The last years of the twentieth century were marked by a number of centenary retrospects; in the present context, the most important were those of the American Society for Microbiology founded in 1899 and the *Journal of Hygiene* (now *Epidemiology and Infection*) founded in 1901. Both these events resulted in the consideration of important events in microbiology, some of which are listed in Table 2 (Joklik et al. 1999; ASM 1999).

The twentieth century was the time when both pure and applied microbiology emerged as a science and produced radical developments across the whole field of biology. The most striking development has been the emergence of molecular biology and the way that this has altered our understanding of biology and medicine. This revolution is still at an early stage, but its impacts can be seen clearly in approaches to bacterial classification and typing.

SYSTEMATIC BACTERIOLOGY

Definition of the bacteria

The applied microbiologists of the time of Pasteur and Koch were not much interested in the classification of the microorganisms they considered responsible for fermentation or for communicable diseases. Contemporary biologists recognized two kingdoms of living things, plants and animals, but were uncertain in which to place the bacteria. In 1838, Ehrenberg had used the term ‘bacteria’ to describe rod-shaped organisms visible only with a microscope and considered them animals, but F. Cohn in 1854 claimed them for the botanists and Haeckel in 1866 thought that they should be placed,

along with fungi, algae, and protozoa, in a third kingdom, distinct from plants and animals, the Monera or Protista. Haeckel's view did not receive wide acceptance and for the next 50 years and more the bacteria were in a taxonomic limbo. Then, technical advances, notably the introduction of the electron microscope in 1932 and of the phase-contrast microscope in 1935, led to the recognition, usually associated with the names of Stanier and van Niel (1941), that the bacteria and certain bacteria-like blue–green algae differed from most other microbes in that their genetic material was not separated from the cytoplasm by a nuclear membrane.

This view was later formalized into the concept that there were two sorts of living things differing fundamentally in cellular structure (Murray 1962; Gibbons and Murray 1978):

1. the Prokaryotae, comprising the bacteria (Eubacteria) and the blue–green algae, which were now recognized to be phototrophic bacteria and
2. the Eukaryotae, which included fungi, algae, protozoa, and all the metazoa of the plant and animal kingdoms.

The prokaryotes were characterized by the absence of a membrane-bounded nucleus and also of cellular organelles such as mitochondria and chloroplasts. As pointed out by Woese, the definition of bacteria as nonphototrophic prokaryotes is based entirely on negative characters and provides no grounds for distinguishing the conventional bacteria from the so-called Archaebacteria. These are organisms that inhabit environmentally 'hostile' habitats and include methanogens, extreme halophiles, and thermoacidophiles; they are said to form a coherent group of organisms with characteristic isoprenoid lipids and cell-wall components (see Woese and Wolfe 1985) and to be survivals from an earlier geological era. Woese (1994) considers that genetic evidence (see section on Bacterial genetics) should take precedence over cellular anatomy in defining the relationships of the prokaryotes and eukaryotes to each other and to these primitive forms.

Classification of bacteria

Linnaeus (1707–1778) classified plants and animals according to a hierarchical system based on Aristotle's theory of logical division (see Cain 1962) by placing individuals that were alike in 'essential' characters in the same species and then constructing genera and other higher taxa on the basis of progressively greater differences in characters. The selection of essential characters ('weighting') was at first made according to the intuition of the classifier, but post-Darwinian biologists used the fossil record, often supplemented by embryological evidence, to construct classifications of plants and animals that were wholly or in part phylogenetic. This sort of evidence was not available to microbiologists and the apparent absence of sexual reproduction in bacteria meant that the biologists' favored criterion for the definition of the species, self-fertility, was denied them.

After 1880, the ability to study bacteria in pure culture led to the rapid accumulation of vast amounts of data about their phenotypic characters: colonial appearance, growth on various media, nutritional requirements, biochemical activities, serological relationships, pathogenicity for laboratory animals, and so on. Practical bacteriologists selected sets of key tests that seemed useful in identifying organisms of interest to them and in many cases attached Linnaean binomial epithets to species so characterized. What resulted was not a general classification of bacteria but a series of miniclassifications used by workers in laboratories studying medical, agricultural, or various sorts of industrial problems. There was a great deal of duplication in the naming of species, and the intuitional handling of complex collections of data led to some uncertainties in classification and identification.

NUMERICAL TAXONOMY

An alternative to seeking 'key' characters had been proposed in 1763 by Adanson, a contemporary of Linnaeus, who considered that biological classification should be based on general similarity in phenotypic characters. He rejected 'weighting' and determined, for each possible pair of individuals in a collection, the proportion of all ascertainable characters that were in accord: the so-called 'overall' similarity. Adanson found the manual computation of similarities between large numbers of pairs excessively laborious and his method could not be employed on a large scale until electronic computers became available. Then, the new discipline of numerical taxonomy was developed (Sneath 1957a, b; Sneath and Sokal 1974) and applied to collections of bacterial cultures that had been studied extensively. This made great contributions to bacterial classification at the levels of species and genus by providing an objective measure of the degree of similarity between large numbers of cultures. However, it is remarkable how often numerical-taxonomic studies supported earlier conclusions arrived at by the intuitional recognition of a 'good' classification as one that placed like organisms in the same taxon. Some taxonomists, for example Cowan (1962), expressed the view that the bacterial species was simply a man-made artifact, albeit a useful one, designed to put phenotypic data into manageable form. Numerical taxonomy provided a powerful impetus to the 'anti-essentialist' view of bacterial classification, but the practitioners of two other disciplines, chemotaxonomy and bacterial genetics, continue to search for 'key characters' that might form a basis for a broad classification of microorganisms (see Taxonony and nomenclature of bacteria).

Antigenic specificity and chemotaxonomy

In the early years of the twentieth century the antibody response to bacterial antigens had been studied intensively in vitro (Parker 1998). In the 1920s, evidence began to appear of the chemical nature of some of these antigens. This was investigated eagerly by medical bacteriologists because the antigens in question appeared to have some association with pathogenicity. Thus, a great deal of information accumulated about certain classes of antigenic macromolecules and some of this was of significance for bacterial classification.

From 1923 onwards, Avery and Heidelberger, at the Rockefeller Institute in New York, studied the type-specific capsular polysaccharides of pneumococci and showed that antibodies to them conferred type-specific immunity on experimental animals. Rebecca Lancefield, in the same laboratory, described in 1933 the so-called group polysaccharides from the cell walls of hemolytic streptococci; some of these characterized streptococcal groups that caused disease only in certain species of mammals. These polysaccharides had several characters in common:

- Though determining the specificity of the antibody response, they were unable to elicit it when separated from the bacterial body and purified; in 1921 Landsteiner coined the term 'hapten' for such molecules.
- The specificity of the antibody response to them was relatively limited. Pneumococcal polysaccharides, for example, though defining clear-cut serotypes among pneumococci, cross-reacted widely with polysaccharides of otherwise dissimilar bacteria and even with nonbacterial polysaccharides, as shown by Heidelberger, Austrian, and colleagues. This was explained by the limited repertoire of specificities provided by the sequence of sugars in the terminal part of the polysaccharide chain. The role of the terminal sugars as antigenic determinants was further illuminated by the work of McCarty and Krause on the cross-reactions between streptococcal group antigens.
- Though sometimes clearly associated with virulence, the polysaccharides were not toxic for experimental animals.

In 1943, Rebecca Lancefield described a class of type-specific cell wall protein antigens in *Streptococcus pyogenes*. Like other proteins, they were fully antigenic when extracted and purified. Antibodies to them were highly specific and conferred type-specific immunity. Though nontoxic, these M proteins determined pathogenicity by interfering with phagocytosis.

Certain other cell-bound bacterial constituents proved to have toxic properties when injected into animals. These included the endotoxins of gram-negative bacteria, first described by Boivin and his associates at the Institut Pasteur in Paris in 1932–35. These were complex macromolecules in the cell envelope comprising:

- a polysaccharide responsible for antigenic specificity
- a lipid conferring toxicity, and
- a protein that, when linked to the polysaccharide, rendered it antigenic.

Studies of the amino-acid composition of the cell walls of gram-positive bacteria by Cummins and Harris in 1956 led to the recognition by Ghysen in 1965 of the structure of their main component, the peptidoglycan or mucopeptide. In 1972, Schleifer and Kandler showed that similarities in the cross-linking of the main components of the peptidoglycan molecule were of value in establishing relationships between bacterial genera that would have been difficult to ascertain by conventional serological means. It has since been noted that all eubacterial peptidoglycans contain *N*-acetyl muramic acid but that this is absent from the cell walls of archaebacteria.

Since 1970 the chemical study of bacterial macromolecules has advanced rapidly. New information about the distribution of particular classes of lipids and isoprenoid quinones has provided grounds for establishing relationships between higher taxa of gram-positive bacteria and for distinguishing eubacteria from archaebacteria (for references see Jones and Krieg 1984).

Protein antigens, when studied by conventional serological methods, showed such a narrow specificity as to limit their value to the identification of species or serotypes. Recent studies of the chemistry of some widely distributed classes of protein, e.g. the cytochromes (Jones 1980), have revealed differences relevant to the general classification of bacteria. The increasing ability to determine the sequence of amino acids in individual proteins will add information to that provided by antigenic analysis. If a constant rate of mutation is assumed, then this might be thought to provide evidence of the evolutionary 'distance' between taxa. Such considerations may be attractive to those who favor a phylogenetic approach to bacterial classification.

Bacterial genetics

At the beginning of the twentieth century it was generally recognized that the characters of bacterial strains in culture might vary, either temporarily, in response to changes in the environment ('adaptation'), or permanently, independent of environmental conditions ('mutation'). The latter phenomenon suggested the possession by bacteria of a genetic system analogous to that of larger organisms, but proof of this was lacking in the absence of a distinct nuclear apparatus.

In 1928, F. Griffith demonstrated 'transformation' among pneumococci, that is to say, transfer to a non-capsulate strain of the ability to form capsular polysaccharide of a particular type by contact with a heat-killed culture of a capsulate strain of that type. This indicated a permanent modification of the genetic apparatus of one organism by inanimate material from another. The nature of the transforming material remained unknown until 1944, when Avery, Macleod, and McCarty showed that it was deoxyribonucleic acid (DNA).

In 1946, Lederberg and Tatum demonstrated 'recombination' between two biochemical mutants of a strain of *Escherichia coli*, each lacking different characters of the wild strain; when placed in cell-to-cell contact a strain with all the parental characters was formed. In this process, 'conjugation' had resulted in the transfer of genetic material between cells. In 1953, Hayes discovered that this was a one-way process in which a small amount of genetic material passed from a donor ('male') to a recipient ('female') cell and was thus different from sexual reproduction in plants and animals. The capacity to donate genetic material in this way depended on possession by the donor of an F (for fertility) factor.

Increasing interest in bacteriophages in the 1940s had revealed that their genetic material became incorporated into that of the bacteria they infected, and in 1952 Zinder and Lederberg showed that on occasion they might take a part of the bacterial genome with them when they left one organism and entered another, a process called transduction. Thus, by the early 1950s, it was possible to study the genetics of bacteria by exploiting the phenomena of transformation, recombination, and transduction.

Information gained from these genetic studies, together with physicochemical work on the constitution of nucleic acids, played a major role in Watson and Crick's formulation in 1953 of their hypothesis of the genetic code; according to this, the information that determines the phenotypic characters of an organism is embodied in paired linear strands of deoxyribonucleotides whose sequence in a particular region constitutes a gene. Most bacterial genes form part of a single, circular chromosome, but some are on smaller extrachromosomal closed circles of DNA called plasmids. Plasmid DNA may determine the inheritance of a single character or a few characters; examples are resistance to certain antimicrobial agents or heavy metals, individual biochemical properties, toxin production and virulence. Hayes's F factor, too, is a plasmid, one of a class of plasmids that mediate cell-to-cell transfer of genetic material, usually by means of conjugative pili.

The fact that the code is a sequence of four bases, guanine (G), cytosine (C), adenine (A), and thymine (T), had considerable significance for bacterial classification. In 1950, Chargaff had observed that, whatever the source of DNA, it contained equal amounts (in moles) of G and C, and of A and T. However, the ratio of G to A and C to T varied widely. Thus, the mol% of G + C came to be used as a measure of the genetic unrelatedness of groups of bacterial taxa. This was of most use in drawing attention to groups that were not easily distinguished by their phenotypic characters, e.g. the staphylococci and the micrococci, but had widely differing base compositions. Of course, the converse did not hold: similar base compositions did not necessarily indicate genetic 'nearness.'

Recognition that the base sequence of the DNA determines phenotypic characters suggested that it might provide the ultimate criterion for bacterial classification. At first, such sequences could be determined only in small lengths of genome. However, in 1961, Schildkraut and coworkers showed that it was possible to measure the total similarity in the sequences of the two organisms without knowing the actual sequence in either. This was done by denaturing their DNA into the single-stranded form, mixing preparations from the two organisms under appropriate conditions, and then measuring the amount of reassociation of the two strands. The amount of complementary base pairing is taken as a measure of the relatedness of the organisms. DNA–DNA pairing provided a great deal of information about the relations between organisms at or around the level of species but gave no grounds for relating the degree of homology to a definition of the species or genus. Indeed, the degree of homology within species previously defined on phenotypic grounds proved to be highly variable and proposals have been made to conflate a number of long-recognized species on the grounds of close genetic similarity.

Studies of DNA–DNA homology proved of little value in revealing broader groups among the bacteria. However, some parts of the genome, notably those coding for the production of ribosomal and transfer RNA, have come to be regarded as more 'conserved' than the rest, that is to say, to have evolved more slowly. Thus, taxonomists with a phylogenetic bent consider that studies of the homology of ribosomal RNA or of the oligonucleotide sequence on the RNA cistrons would be decisive evidence of the evolutionary 'distance' between the higher taxa of bacteria and bacteria-like organisms and thus provide a firm basis for classification (Johnson 1984).

In the eighth edition of this book it was thought that the debate between rival concepts of the phenospecies and genospecies (Hill 1990) is unresolved and perhaps unresolvable. The difference between the two schools of thought – the Lockean view that classification is a man-made device for handling information and the Aristotelian concept that the 'essence' of classification is the genetic code – was considered to be perhaps better looked upon as a philosophical rather than a scientific question.

It is a tribute to how knowledge has progressed that we now deal easily with use of molecular chronometers

for the study of relatedness and how molecules other than 16S RNA are considered for this role.

The history of some other aspects of bacteriology – the mechanisms of pathogenicity and of immunity to infection, the antibacterial agents and resistance to them, and laboratory methods of investigating bacterial infections – needs to be reassessed in the light of recent understandings. The early history of bacterial genetics was reviewed in 1990 (Cliffs and Brock 1990), but since then change has accelerated.

Writing in 1938, Bulloch considered 1876–90 as the heyday of bacteriological discovery. It is clear that he underestimated the reach, both intellectually and practically, of our discipline. Pathogens have continued to be discovered. *Legionellae*, *Campylobacter*, and *Helicobacter* are prominent bacterial examples, but it is clear that without bacteriology we would lack much of modern science. The rest of these volumes will show why.

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Taxonomy and nomenclature of bacteria

JOHN T. MAGEE

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This chapter is intended as a brief introduction to the strange mixture of science, philosophy, and formal regulation that guides the division of bacteria into named groups. This is not an unbiased account. In the Alice in Wonderland of taxonomy, nothing is quite what it seems and, as in politics and theology, opinion is an indispensable ingredient. Anyone who reads this set of volumes has reached some viewpoint on bacterial taxonomy, consciously or subconsciously. There is considerable breadth of opinion in key arguments: utilitarian versus 'natural' classification, genomic versus phenotypic versus polyphasic classification, 'splitting' or 'lumping' of taxa (Cowan 1968), and so on. This range guarantees that each microbioilogist will have found some unique position in the spectrum of opinion, and so will disagree with at least some opinions expressed here. Constructive, logical criticism and discussion would be welcome.

AN INTRODUCTORY PERSPECTIVE

The practice of medical bacteriology is founded upon the ability to distinguish between the bacteria that are isolated and to predict their pathogenicity and epidemiology from in vitro characteristics via past clinical observations in infections with closely similar organisms. This predictive capability is essential and flows from the work of many microbiologists over two centuries. Their cumulative efforts have provided the current formal species nomenclature under the aegis of the International Committee for Systematic Bacteriology (ICSB), reviewed regularly in the editions of *Bergey's manual of systematic bacteriology* (currently 2nd edition in five volumes: Garrity 2001), and a wealth of vernacular schemes. Together, these schemes provide much of the technical vocabulary of microbiology and are the basis of interpretation and prediction from laboratory tests.

In a laboratory deprived of all knowledge of the classification of bacteria, their morphological and biochemical characteristics, and the relevance of individual characterization tests, each isolate would have to be regarded as unique. It would be impossible to distinguish between pathogens and commensals, or to assess the cross-infection potential of an isolate, from in vitro tests. All available antibiotics would have to be tested for each isolate. How long would it take, for example, to untangle the characteristics of the fecal pathogens from those of gut commensals, or to rediscover the link between diphtheria and toxin-producing corynebacteria? Clearly, it would be impossible to operate under such

conditions. The laboratory would be forced to search for links between in vitro characteristics and effects on patients, to divide isolates into named groups, and so to reinvent the science of taxonomy.

Despite this key role, many diagnostic microbiologists profess a disregard for taxonomy (Magee 1993a). This attitude may derive from early specialist education, where the subject is often presented as an apparently endless list of genus and species names for rote learning, along with associated laboratory and medical characteristics. The underlying science, philosophy, and history of the subject are rarely taught; when they are, the students are often so inundated by the nomenclature and lacking in a context for their brief contact with taxonomy *per se* that these are soon forgotten. Further conscious contact with the subject is usually when rote learning is overturned by changes in the names of bacteria, for reasons that may appear obscure and irrelevant. It is therefore unsurprising that most medical microbiologists regard taxonomy as an unimportant, dull, and arcane science practiced by academics in isolation for their own edification.

This common view is grossly inaccurate but is also a self-fulfilling proposition. It builds barriers between routine microbiologists and taxonomists that limit communication and cooperation. This breaks the feedback process that is essential to the production of relevant, sensible classifications of the bacteria. Taxonomy and classifications are fundamental to all microbiological work, and an appreciation of the concepts involved is an absolute requirement for all practitioners of microbiology. Equally, taxonomists require the input of other microbiologists to direct them to areas of taxonomic confusion, to supply cultures, to moderate wilder taxonomic excursions, and, more importantly, to supply knowledge of the characteristics of the organisms in the broader context outside the laboratory.

ONE CLASSIFICATION OR MANY? THE IMPORTANCE OF CONTEXT

Cowan (1962) illustrates the range of possible classifications with the example of objects intended to join materials. He divides them first into animal (glues), vegetable (wooden pegs), and mineral (metal nails, bolts, or screws, or plastic adhesives, screws, and pins), then presents an alternative classification into solid devices and liquid glues. A dealer in scrap metals might classify these objects as ferrous, nonferrous, or nonmetal in the context of work, but quite differently in the context of repairing a broken china ornament or a broken chair. Humans have little difficulty in employing a multiplicity of overlapping, but distinct and context-specific, classifications for everyday objects. Microbiologists differ only in that most are trained to acknowledge a single formal scheme and so feel constrained to argue about its minutiae to the exclusion of other possibilities.

However, when presented with a set of clinical isolates, workers revert to practicality, happily employing classifications that have little in common with that embodied in the current edition of *Bergey's manual* (Garrity 2001). At the feces bench, isolates are divided into lactose and nonlactose fermenters, fecal pathogens and nonpathogens, aerobes, anaerobes, and microaerophiles. Staphylococci are divided in three distinct ways when dealing with blood cultures, skin swabs, or urines, reflecting experience of potential pathogenicity and the importance of further identification in the context of the specimen type, the sex of the patient, and catheterization status. Diverse gram-negative and gram-positive species are lumped together under the heading of respiratory pathogens, recognizing the need to extend this definition in the context of special cases: cystic fibrosis patients or patients on ventilators. These vernacular or 'trivial' classifications even intrude into reports in phrases such as 'coagulase-negative staphylococcus' or as multivalued context-specific phrases such as 'commensals only'.

Trivial classifications are as real and valid as those described in the successive editions of *Bergey's manual*. They exist because some worker perceived a new way of dividing bacteria that had practical use or significance, and many other microbiologists recognized the utilitarian value of the division. They reflect unavoidable deficiencies in the flexibility of nomenclature in the formal ICSB scheme, and the fact that a single classification cannot cater for all the myriad ways in which bacteria can be usefully grouped.

These trivial classifications have, by their nature, consensus acceptance and are strongly utilitarian. However, they lack mechanisms for definition and regulation of any nomenclature involved, and so are open to degradation by misuse. Terms such as 'fecal streptococcus' or 'enterobacteria' lack clear definition and are used in any of a range of senses from the all-inclusive to the restricted. Equally, these classifications tend to be restricted by their utilitarian nature to specific branches of applied microbiology.

By contrast, the formal scheme has a strictly defined, regulated nomenclature recognizable by the italicization of the formal species name. It is less robust on consensus acceptance, but it is broadly applicable in all branches of microbiology. The defined nomenclature makes this the vocabulary of choice for international and interdisciplinary communication. However, the rigidity of a defined nomenclature causes problems when changes become necessary (see below). In my view, a second context for the formal system is as a scheme supplying descriptions of the minimum-sized groups of bacteria about which one can make useful generalizations. These species definitions are the building blocks that can be assembled into larger groups in as many ways as utility demands. The ICSB provides an official system of genus and species nomenclature (Sneath 1992); by contrast the

classifications into higher taxa outlined in successive editions of *Bergey's manual* are not official (Staley and Krieg 1984) and tend to change markedly between editions.

However, the formal scheme serves many interest groups. Those who wish the scheme to supply a phylogeny of the bacteria, a 'natural' classification, or to be bent to conform with the results of a specific characterization test, or to suit the requirements of some specialist branch of microbiology would all disagree.

STACK OR FLAT: THE PRACTICAL RELEVANCE OF HIERARCHICAL CLASSIFICATIONS

Classification embraces not only the division into named species but also the further grouping of species into genera, genera into families, and so on through orders, classes, and phyla to kingdoms. This hierarchical approach has much to commend it in the classification of higher organisms. The many morphological and developmental features allow division into higher groupings in agreement with fossil evidence of evolutionary development, and, for the most part, these groups can be neatly packaged into specialist areas relevant to the applied science. However, hierarchical classification has had a more chequered history in bacteriology. Successive editions of *Bergey's manual* have portrayed gross changes in the arrangement and membership of higher taxa. Equally, each of the major applied specialist areas of bacteriology – medical, veterinary, and industrial – deals with a host of diverse bacteria whose membership could not conceivably be grouped into any exclusive 'natural' or phylogenetic higher taxon, but rather forms a diverse portion of the all-inclusive group of 'the bacteria'.

One major difficulty is the lack of nonarbitrary definitions of the taxon concept at any division from species upwards. The definition of a species concept for the bacteria has been in dispute for more than a century, and definitions of the genus, order, etc. are successively more elusive. The value judgments that set these divisions are likely to be as controversial in the new era of phylogenetic classification as they were in the days of van Niel's physiological classifications (Van Niel 1946). However, despite these difficulties, the search for a phylogenetic classification of the bacteria and other prokaryotes is of enormous importance to biology. Results from this search are likely to shape our future views on the origins of life and of the eukaryote and prokaryote forms.

For medical microbiologists, much of the turmoil in the upper reaches of hierarchical classification has remained unnoticed. Changes at genus level have affected the naming of species in laboratory reports, causing considerable inconvenience. However, the higher taxonomic divisions form a province that academics may remap with little fear of criticism from applied microbiologists, who are more interested in nomenclature and divisions at species level than in hierarchical classification.

This highlights an important, but rarely discussed contrast of attitude between academic taxonomy and applied bacteriology. The applied bacteriologist is interested almost solely in divisions at species level, and particularly in those species where identification yields an associated prediction of properties, such as pathogenicity or cross-infection potential, that cannot be examined readily or economically in the laboratory. Their requirements are for a division of the bacteria into readily identified species, each with clear and distinct properties significant to his or her application, and a stable, widely accepted species nomenclature, to allow concise communication of his or her findings. By contrast with the taxonomist, who attempts to group species in a hierarchy determined by phylogeny or similarity, the applied bacteriologist assembles species in a multitude of distinct context-specific classifications of convenience.

The single-tier classification of species nomenclature is common ground for the taxonomist and applied bacteriologist, and deserves a distinguishing name, such as the agnostic, agnarchic (by contrast to hierarchic, from the Greek root *hieros*, sacred, and *arche*, rule), or monadic (Gr. *monados*, unity) classification because of this unique unifying position. Adoption of one of these terms might eliminate the misunderstanding inherent in use of the term 'classification' to cover both the untiered division of bacteria into species, the sense in which many applied microbiologists instinctively interpret the term, and hierarchical schemes.

HISTORY AND OPERATION OF THE FORMAL CLASSIFICATION

In the early days of biology, Linnaeus understood that biologists required a concise, precise, and internationally recognized scheme of species nomenclature. He proposed a scheme of latinized binomials comprising a genus and species epithet, referring to a formal description of the species. This was universally accepted, and replaced the babel of trivial, poorly defined synonyms individual to each language with a defined vocabulary common to all biologists. This technical vocabulary now bridges language and speciality, providing an essential method of communication. Whatever other gulfs of language and misunderstanding may exist, biologists can guarantee that a species name will communicate a complex detailed description of an organism with precision, and in a remarkably concise form.

Microbes, observed earlier by van Leeuwenhoek, were assigned to six species in the class *Chaos* by Linnaeus. Work in the late nineteenth century led to the description of many bacterial pathogens and the first recogniz-

able attempt to classify bacteria by the botanist Cohn (1872). The rapid growth in species descriptions required regulation, and individual international committees were eventually set up to govern the nomenclature of animals and plants, including bacteria. Important landmarks in the advance of bacterial taxonomy were the classifications of Chester (1901), Orla-Jensen (1919), and the work of Buchanan (1918, 1925). Committees for bacterial and viral taxonomy (Murphy et al. 1995) came later, with a breakaway group covering the blue-green algae, claimed originally by the botanists and now by the bacteriologists. Regulations covering validation of new names and changes in nomenclature are embodied in codes for each of these groups, and are supervised by the appropriate international committee.

The ICSB supervises the Bacteriological Code (Buchanan et al. 1948, 1958; Lapage et al. 1973; Sneath 1992), regularly providing lists of recent validly published species names and proposed changes in nomenclature, first in the *International Journal of Systematic Bacteriology* (IJSB) and later in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM). In an effort to rationalize nomenclature, remove the burden of early errors and synonyms, and simplify access to the nomenclature, a list of the approximately 2300 historical bacterial species names considered to be valid was published as the Approved Lists of Bacterial Names (Skerman et al. 1980, 1989). This, supplemented by the subsequent IJSB/IJSEM Validation Lists, defines the current, formal nomenclature of bacterial species.

The status of this scheme is reviewed about every 10 years in successive editions *of Bergey's manual*; the current classifications for each group of bacteria are summarized by experts, and the various genera and species are described. Although there is no formal link between the ICSB and *Bergey's manual*, both clearly draw upon the same pool of expertise, and the nomenclature of the Validation and Approved Lists tend to be closely conserved in *Bergey's manual*.

There are numerous subcommittees of the ICSB, most of which deal with specific groups of bacteria and give recommendations and advice on their area. Meetings of the ICSB and of the subcommittees occur regularly, usually associated with symposia organized by the International Union of Microbiological Societies. Most of the subcommittee meetings are open, allowing participation by any interested parties.

Those wishing to propose new species or changes in systematic nomenclature must follow the rules of the Bacteriological Code if their proposal is to be considered valid. Briefly, the proposal must be published in the IJSEM, or, if it is published elsewhere, a copy of the paper must be submitted to the IJSEM for inclusion of the proposal in the Validation Lists. If a new, culturable species is proposed, then a type culture should be designated and deposited with a recognized service culture collection. Further detailed rules exist, particularly on nomenclature, and it is advisable to read the most recent version of the code (Sneath 1992) in detail before submission of a proposal. In addition, minimal acceptable standards for species descriptions are being formulated (Lévy-Frébault and Portaels 1992; Ursing et al. 1994; ICSB Subcommittee on the Taxonomy of Mollicutes 1995), and the standards for the appropriate group should be consulted. Provisions for proposed species that are (currently) nonculturable but have been characterized by DNA sequencing have recently been clarified (Murray and Schleifer 1994; Murray and Stackebrandt 1995). Recent advice on characterization methodologies and the definition of the species level is presented by Stackebrandt et al. (2002). This paper reiterates the advice of Christensen et al. (2001), who argue that a species proposal should be based on the description of more than one and preferably at least five to ten strains.

THE PROCESS OF CLASSIFICATION

This begins with the selection of a limited series of isolates that appear to show restricted diversity (Figure 1). This is simplification by selection, and requires an intuitive recognition of probable groups. Inclusion of a range of reference and type cultures, and of many strains isolated from a broad range of sources, is advisable. The strains are then characterized in a large number of selected tests, producing descriptions for each. These descriptions are compared, either by calculation of a coefficient of similarity for each pair of isolates in numerical taxonomy (Sokal and Sneath 1963; Sneath and Sokal 1973), or intuitively. From these similarities, the existence of sets of isolates or taxa is postulated. It is desirable that the taxa should be cohesive and differentiable, i.e. that the differences between taxa should be greater than the variation within a taxon.

The taxa are then described and named, according to the rules of nomenclature (Bousfield 1993; MacAdoo 1993), allowing the cohesive (least variable) portions of the description to be communicated in the shorthand form of a name. There are some minor, but irritating problems with the current nomenclatural system. There is no maximum length in characters for a species binomial, but long species names can tax those installing computerized reporting systems. In the absence of an ICSB ruling on this, authors proposing new nomenclature should be mindful of the problem and avoid imposition of this inconvenience on their colleagues. Also, there is no nomenclatural guidance on standards for abbreviation of generic names, e.g. *Str*. for *Streptococcus* or *Streptomyces*, and this may be a problem in wide ranging papers, or general texts.

Finally, new isolates can be identified, i.e. named as being members of one of the postulated taxa, on the basis of their similarity to the taxon description, or to

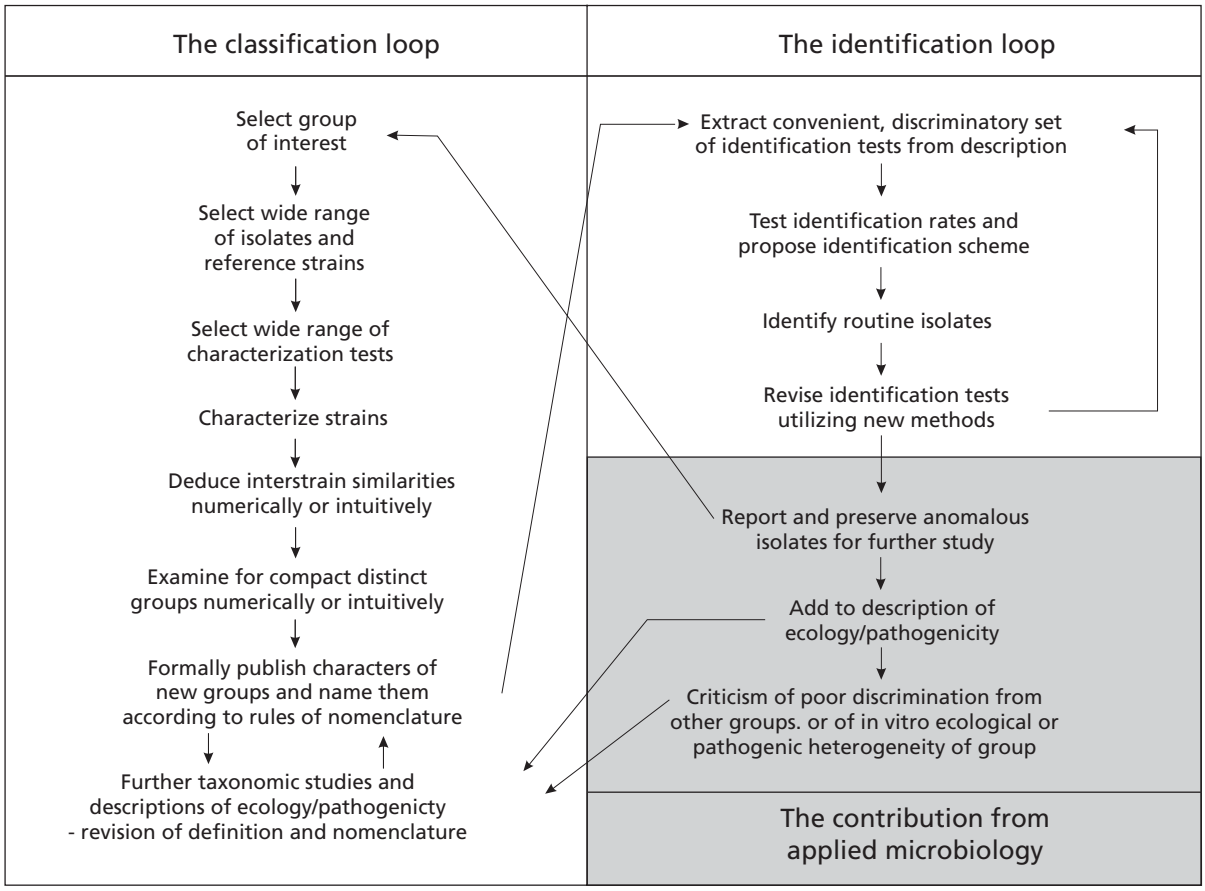

Figure 1 *The taxonomic process represented as a flow diagram.*

exemplar members of the taxon (type and reference strains). The taxon description will often have been enlarged by this stage. In particular, evidence on properties too difficult, time-consuming, or costly to assess in initial tests may have accumulated. In microbiology, this will often include a mass of anecdotal evidence ascribing particular pathogenic, ecological, or spoilage properties to a species. This evidence is a key ingredient in the prediction of significant pathogenic (and other) properties from identification, transforming a new taxon from the arena of solely academic interest to that of significance in applied bacteriology. New tests that are more convenient, reliable, or rapid may have been shown to differentiate the species. Usually, fewer tests are applied in identification than in classification; only those that have proved reproducible, discriminatory, convenient, and inexpensive are used. Note the crucial distinction between classification, which seeks to elucidate a group structure, and identification, which seeks to assign new isolates to an appropriate group within an existing classification.

IDENTIFICATION

There are three broad approaches to identification in general use. The first, and most important, is recognition of colony morphology (and its variation over a range of isolation media) by an experienced microbiologist, sometimes with confirmation by a few rapid, simple tests. This is the basis on which most clinical isolates are divided into commensals or contaminants of no interest, possible pathogens, and probable pathogens. Without this rapid triage of isolates, most diagnostic laboratories would be faced with an overwhelming workload of formal identification. However, the apparent simplicity of the process conceals much complexity and has far-reaching consequences upon the practice of medical microbiology.

Intuitive human pattern recognition is notoriously difficult to document, is impossible to teach in a formal setting, and requires prolonged training, strong perceptive and intuitive talents, and considerable intelligence. This shapes a long training process of one-to-one teaching at the bench, with either tight selection or a high failure rate. Equally, the triage of isolates must be performed or checked by those with great expertise and experience. The screening process is an art, rather than a science; however, it is difficult to justify the expense of the costly but essential training in this art to paymasters. As in the production of hand-crafted furniture, the quality of the reports that a

laboratory generates depends heavily on the expertise, skill, and time that staff can devote to each specimen. Surprisingly, there appears to be no study in the literature that documents the error rate of this process; whether this reflects an unquestionable accuracy, overweening ego, a profound fear of the consequences of documenting errors, or simple omission is difficult to ascertain.

After this triage, formal identification is performed upon potentially significant isolates. In earlier times this often used an 'identification key,' exemplified by the identification scheme for coagulase-negative staphylococci proposed by Kloos and Schleifer (1975). The key is a tree-like logic diagram, with a test name noted at each branch point, and two branches (one for isolates that are positive, and the other for isolates that are negative in that test) indicating the further logic pathway for identification. The investigator follows the logic tree to a terminal branch, where the designer of the key has decided that sufficient similarity has been established for identification and noted the appropriate species name. This was the favored approach in the early editions of *Bergey's manual*, and in the various earlier schemes (Chester 1901; Orla-Jensen 1919), reflecting the lasting botanical influence on early bacterial taxonomy. Keys depend heavily upon invariant characteristic reactions, although the later efforts, particularly the Kloos and Schleifer scheme, make some allowance for test variation. However, the work of numerical taxonomists brought general acknowledgment that bacterial species are polythetic, i.e. any individual property may vary within a species. Only the most sophisticated and complex keys could cope with this variation, and, with the introduction of numerical identification, formal keys have declined greatly in importance.

Numerical identification is based on surveys that define tables of the expected frequency of positivity in a series of tests for each species. The reaction pattern of an'unknown' isolate is compared with this table, and its 'goodness of fit' with each species is determined. This comparison may be intuitive, as in the early schemes (Castellani and Chalmers 1919; Enterobacteriaceae Subcommittee 1958; Cowan 1956) and in the tables of the various editions of Cowan and Steel's classic identification manual (Cowan and Steel 1965), or by formal mathematical comparisons. The latter may be calculated concurrently by computer programs, or the calculations may be performed earlier, giving lists of common reaction patterns with the best-fit identification for each, as in the various identification listings for API identification kits. The underlying mathematical principles are described in the classic paper of Willcox et al. (1980) on numerical identification.

In contrast to classification, where a host of ill-defined mathematical assumptions must be made during data processing, the mathematical identification strategies described by Willcox et al. (1980) rely on only six clear prior assumptions. These are that:

1. the isolate under study cannot be a member of more than one taxon
2. the isolate is a member of a known taxon
3. each of the tests employed yields a result that varies independently of all others
4. the tests have roughly equal within-strain reproducibility
5. this within-strain reproducibility does not vary significantly between species or strains
6. the tests yield clear negative or positive results.

Two important parameters in assessing numerical identifications are the 'strangeness' or absolute likelihood of obtaining the observed pattern, often assessed as the estimated frequency of occurrence, logarithmic probability, or modal likelihood fraction, and the 'equivocality' or relative likelihood of the fit with known result patterns, often assessed as a percentage relative likelihood or 'identification score'. The 'strangeness' index is calculated for each known species (Table 1), and the species with the highest index is chosen as the best-fit identification. Caution is required if the strangeness index is low. This indicates that the observed pattern of reactions is most unusual, even for the best-fit species, and the isolate may well be a 'stranger' – a member of a new species or a species not listed in the table. The equivocality index indicates the level of uncertainty in ascribing the isolate to the best-fit species if it is not a 'stranger'. Again, if this index is low, then the identification is uncertain, split finely between two or more species, and it would be wise to proceed to further tests, particularly if the identification equivocates between species of significantly distinct pathogenic or cross-infection potential.

A common reason for unusual reaction patterns is that the culture is impure; close examination of prolonged subcultures on a wide variety of media, incubated under a range of conditions, will often reveal cryptic contaminants in these cases. Equally, isolates of *Bacillus* or *Clostridium* species that are asporogenous and show a negative gram-staining reaction present an insidious trap for the unwary, who may waste considerable effort under the false assumption that these organisms are true gram-negative bacilli. Conclusive proof of the true nature of such isolates may be difficult to obtain, although colony morphology often provides an indication of the problem.

Ascribing a species name to an isolate should be a carefully considered action. The name will usually communicate an implicit description of many properties that have not been, or cannot be, examined in the laboratory. Once identified, the isolate will be assumed to possess all these implied properties, whether these describe biochemistry, pathogenicity, capability of cross-infection, etc. A poor identification, arrived at with

Table 1 *Mathematical identification: a worked example*

a) Determine reaction pattern of unknown isolate in tests T1 to T7:

Test	T1	T2	T3	T4	T5	T6	T7
Result	+	+	–	+	–	+	+

b) Refer to a reputable identification table

	Known frequency of positive reactions in test:							Maximum
Taxon	T1	T2	T3	T4	T5	T6	T7	likelihood
A	.99	.01	.99	.01	.99	.99	.99	.932
B	.99	.75	.01	.90	.75	.90	.10	.442
C	.90	.85	.10	.95	.05	.99	.99	.609
D	.95	.99	.99	.99	.95	.01	.01	.858
E	.01	.01	.05	.01	.99	.01	.99	.894

The maximum likelihood is calculated for each taxon from the reaction pattern that yields a 'perfect' fit to that taxon (sequentially multiply 1−frequency for tests with frequency ⩽0.5, or frequency for tests with frequency >0.5; see below in c).

c) Calculate the likelihood of obtaining the isolate pattern for each taxon

For each taxon, calculate the likelihood by multiplying the frequencies for each test together, but substituting (1−frequency) for the frequency if the test result is negative

Taxon	Likelihood calculation		Likelihood
A	$.99\times.01\times(1-.99)\times.01\times(1-.99)\times.99\times.99$	=	9.70×10^{-9}
B	$.99\times.75\times(1-.01)\times.90\times(1-.75)\times.90\times.10$	=	$\mathbf{.147\times10^{-1}}$
C	$.90\times.85\times(1-.10)\times.95\times(1-.05)\times.99\times.99$	=	$\mathbf{.609\times10^{-1}}$
D	$.95\times.99\times(1-.99)\times.99\times(1-.95)\times.01\times.01$	=	4.66×10^{-9}
E	$.01\times.01\times(1-.05)\times.01\times(1-.99)\times.01\times.99$	=	9.41×10^{-11}
	Sum	=	.756

d) Calculate the 'strangeness' indices, negative log probability, and modal likelihood score, which indicate how unlikely the test result pattern is for each taxon. Calculate the 'equivocality' indices, identification score, and relative likelihood, which indicate the equivocality of the best-fit result

	Negative log probability			Modal likelihood score		
Taxon	Calculation		Result	Calculation		Result
A	$-\log(9.70\times10^{-9})$	=	8.10	$9.70\times10^{-9}/.932$	=	1.04×10^{-8}
B	$-\log(1.47\times10^{-1})$	=	.83	$1.47\times10^{-1}/.442$	=	**.33**
C	$-\log(6.09\times10^{-1})$	=	.22	$6.09\times10^{-1}/.609$	=	**1.00**
D	$-\log(4.65\times10^{-9})$	=	8.33	$4.66\times10^{-9}/.858$	=	5.42×10^{-9}
E	$-\log(9.41\times10^{-11})$	=	10.03	$9.41\times10^{-11}/.894$	=	1.06×10^{-10}
	Identification score			**Relative likelihood**		
Taxon	Calculation		Result	Calculation		Result
A	$9.70\times10^{-9}/.756$	=	1.24×10^{-10}	$9.70\times10^{-9}/.609$	=	1.59×10^{-6}
B	$1.47\times10^{-1}/.756$	=	**.194**	$1.47\times10^{-1}/.609$	=	**.24**
C	$6.09\times10^{-1}/.756$	=	**.805**	$6.09\times10^{1}/.609$	=	**1.00**
D	$4.65\times10^{-9}/.756$	=	6.16×10^{-9}	$4.66\times10^{-9}/.609$	=	7.65×10^{-9}
E	$9.41\times10^{-11}/.756$	=	1.24×10^{-10}	$9.41\times10^{-11}/.609$	=	1.55×10^{-10}

e) Conclusion

All scores for taxa A, D, and E indicate extremely poor fit. The taxon most likely to yield this result pattern is C. The identification score is poor at .805 (values appraoching .99 are desirable), but this is expected with such a small number of tests that yield mostly variable results for this taxon. Correcting for taxon variability (division by the maximum likelihood for taxon C) yields a modal likelihood score of 1.00, indicating that no better fit can be obtained for this taxon with limited tests chosen. However, the 'equivocality' indices both indicate an equivocal identification, with the fit to taxon B being only one-quarter less likely than the fit to taxon C. The poor identification score and high equivocality indicate that this isolate requires further tests suited to discriminate between taxa B and C to produce a reliable identification result.

much equivocation, can acquire an undeserved aura of precision during the reporting process. In these cases it might well be more accurate to state that the isolate is unusual, and although most similar to the best-fit species, may well not share all the species properties.

Further, the perception of the properties implied in the species name will vary between individuals, according to their knowledge of systematics and the biases imbued in training and specialization. This is a problem of the 'shorthand' communication of data via a species name, which requires good to perfect agreement between the communicating parties on the definition implied in the species name. Clearly, the level of agreement on the implied description may well be unsatisfactory if one side is employing a current nomenclature and description, while the other has little contact or interest in microbiology and is struggling to remember information from a minor part of a syllabus learned decades before. The laboratory cannot assume that clinicians will understand the full implications of a species name for any but the most frequently encountered species and should take every opportunity to expand upon the properties that are intended to be communicated to the recipient. The possibilities of gross distortion of important clinical implications in this communication have become ever greater with reductions in resources, increasing time constraints, and the increasing speed of change in species nomenclature.

CHARACTERIZATION TESTS

The lack of a nonarbitrary, generally accepted definition of the species concept in bacteria (see below) inevitably, but wrongly in my view, directs bacteriologists to definitions based on some particular characterization technology. A broad description of the various technologies is given below and in Table 2. Note that none of these methods yields results that can be considered invariably to give the isologous comparisons of truly independent variables required in taxonomy.

Microscopic morphology

These characters comprise: cell shape, size, arrangement, staining, motility and capsule characteristics, spore position and morphology, and flagellar arrangement. Electron microscopy extends these somewhat to fimbriation, wall and cytoplasmic structures. Early microbiologists of the van Leeuwenhoek era were limited to describing the cell morphology and ecological occurrence of those few groups of bacteria distinguishable on the basis of light microscopy. Macroscopic organisms show a massive range of readily observed distinct morphological characters, sufficient to distinguish a huge number of groups. However, bacteria, at the limit of resolution in light microscopy, can be divided into few groups on the basis of cell morphology.

This may have been a major factor in forming a sharp difference of attitude between bacteriologists and macrobiologists. While eighteenth- and nineteenth-century botanists and zoologists sought to document the diversity of macroorganisms, ranging throughout the world in their search for specimens and describing hundreds of thousands of species, bacteriologists centered their work on groups that were involved in disease or had industrial importance. With few exceptions, bacteriologists did not participate in the eighteenth- and nineteenth-century 'collection fever' phase of biology because bacterial characterization was still primitive. By 1989, only about 2300 bacterial species were cited in the Approved Lists, indicating a combination of the low level of interest in discovery and documentation of new taxa during the previous century, and the difficulty of these tasks.

Table 2 *Characterization methods: features dictating their practical utility*

Method	High speed	Good throughput	Low skill	Automated	Low costs		Differentiation level
					Capital	Running	< Genus Species Type >
Ecological	-	-	-	---	++	--	<<---------->
Morphological	++	++	-	---	+	++	<<----->
Colonial	+++	+++	+	---	+++	+	<<---------->
'Biochemical'	+	++	++	p	++	++	<<---------->
Enzyme detection	+++	++	++	p	++	++	<<---------->
Serology	+++	++	++	p	++	-	<---------->>
Chemotaxonomy	--	--	--	---	-	--	<<---------->
Fingerprinting	+++	+++	+	s	-	++	<<---------->>
DNA base ratios	-	-	-	--	+	++	<-------->
DNA Hybridization	--	--	--	---	+	+	<---------->
16S RNA sequencing	-	+	+	--	++	-	<<---------->
Polyphasic studies	--	++	--	---	++	+	<<----------->>

Key: -, --, ---, increasingly less favorable; +. ++, +++, increasingly more favorable; p, possible; s, some.

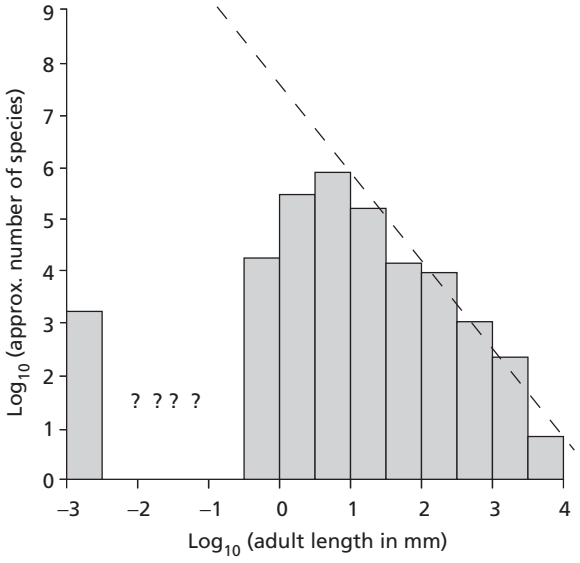

Figure 2 *Histogram representing the number of known species vs adult size. The dotted line indicates proportionality of the number of species with the inverse square of adult body length (modified from May 1988).*

This omission is reflected in the often-cited histogram (Figure 2) of the number of known biological species versus adult size. The diagram shows a maximum number of species at a size corresponding to the limit of resolution of the human eye. This may well be an artifact of limited endeavor. The approximately 5000 currently described species of bacteria probably represent a tiny fraction of bacterial diversity, a contention supported by recent studies of 16S rRNA diversity in natural environments (Stackebrandt et al. 1993; Liesack and Stackebrandt 1992).

Colonial morphology

With the invention of solid media and pure culture techniques came colonial morphological characters. For an experienced bacteriologist, the wealth of data from this source is often sufficient for identification at genus or finer taxonomic levels. Most isolates that are discarded in routine diagnostic work as not clinically significant are identified solely on this basis. However, although often highly discriminatory, particularly after prolonged incubation, the many subtle characters involved are often medium- and incubation-dependent and are notoriously difficult to document. Taxonomists should not ignore this elementary investigation, which will often delineate important divisions within a collection of strains and is crucial to exclusion of impure cultures.

Biochemical tests

Early experiments on optimization of medium composition and incubation conditions led naturally to assessment of nutritional characteristics. The so-called 'biochemical tests' that evolved became an essential tool in bacterial taxonomy and identification. These include tests for fermentation or oxidation of sugars; waste products; metabolism of organic acids, proteins, amino acids, and lipids; temperature, pH, and redox range for growth or survival; and tolerance of various chemical agents. In short, they collectively define the nutritional and physiological interactions of the organism with its environment.

These tests are usually technically simple, are inexpensive, and have high throughput when adapted to multipoint inoculation, commercial test strip, or microtiter plate formats. Most yield bi-state, i.e. positive or negative, results that are convenient to read. However, it should be recognized that these bi-state results usually represent a simplification of a more complex situation.

As an example, the test for production of acid from sugars measures a fall in pH, separating as 'positive' those strains that produce sufficient excreted acidic metabolites to reduce the pH below a threshold where the indicator dye changes color. Measuring the pH of the culture (Figure 3) may reveal more complexity, with distinct groups that produce a large, moderate, or small fall in pH, no change, or even a rise in pH. On further examination, the results of the test depend upon many factors: the threshold at which the indicator changes color; the buffering capacity of the medium; the concentration of the sugar; the amount of acidic products excreted; the strength (pKa) and hence molecular structure of the acidic products; the parallel production of alkaline products (e.g. production of ammonia from amino acids or peptides in *Bacillus* and *Clostridium* spp.); the further metabolism of the acids; and the loss of volatile acids from the medium.

Clearly, the technical simplicity of this test conceals far-reaching complexity. Strains that produce small amounts of acetic acid, and others that produce large amounts of caproic acid, may both yield positive results,

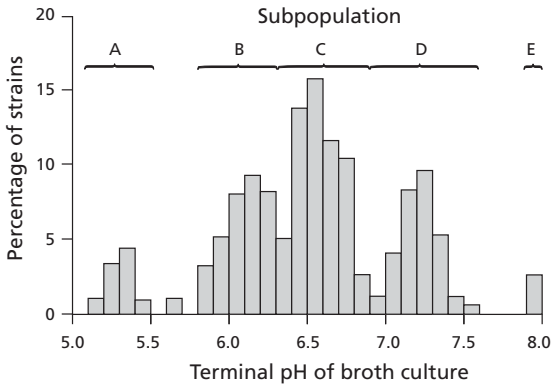

Figure 3 *Terminal pH of sugar-containing media: a histogram for 420 strains of anaerobic gram-negative bacilli showing multiple distinct populations (unpublished data provided by Prof. B.I. Duerden).*

because the quantitative difference is balanced by the qualitative difference in the nature and therefore strength of the acids. Equally, organisms with identical glycolytic pathways, but differing in the presence of a tricarboxylic acid cycle and an electron-transport chain capable of converting stronger acidic products to weakly acidic CO_2, would differ in their acidification of aerobic glucose-containing media. Strains with similar biochemistry may differ in their ability to acidify glucose-containing media, and strains with distinct biochemistry and end-products may yield identical results. In taxonomic studies, comparisons are assumed to be of isologous characters, yet acidification tests may reflect anything from a remote ecological similarity in acidification of sugar-containing environments, to truly isologous biochemistry.

A further problem results from this 'thresholding' of biochemical tests. Equivocal results and test irreproducibility are likely to be a problem if the test threshold does not coincide with a population minimum. In Figure 3, a detection threshold pH of 5.5 coincides with a minimum in the population distribution. A threshold at this level would give few equivocal results. However, a threshold at pH 6.2 would coincide with a subpopulation maximum, giving many equivocal results, and a division into positive and negative groups that would not reflect real underlying similarities in the biochemistry for subpopulation B. In practice, these thresholds are defined by the buffering capacity of the test medium, the pH range for color change in the indicator, and the concentration of the substrate.

Formal proof of adequate thresholding is rare in biochemical tests, but the range of genus-specific basal medium and indicator system formats of, for example, sugar acidification tests clearly indicates intuitive recognition of the problem. Finally, strains that give delayed metabolism of acid products or produce highly volatile acids may yield an early positive result but revert to negative on prolonged incubation.

Morphological, colonial, biochemical, and enzyme detection tests are the basis of current routine identification technology. For routine bacteriologists, it is essential that any species or genus name reaching the Validation Lists should have a description of these characters that allows clear identification and discrimination from similar taxa. Applied microbiologists have a clear interest in ensuring that this principle is a central feature of the minimal descriptions formulated by the ICSB committees to direct taxonomists on a line that is compatible with the current requirements of routine laboratories.

Tests for enzyme activity

Further characterization strategies were investigated. Research showed that some pathogenic effects of bacteria were due to toxins. These toxins included exoenzymes, and an offshoot of this work was a clear lineage of characterization tests based on detection of individual enzymes, from the early tests for proteolytic and lipolytic activity to the modern preformed enzyme tests (Manafi et al. 1991; James 1994). This line produced major advances in biology. Monod's work on the lactose fermentation system of *Escherichia coli* laid the foundations of our understanding of underlying control mechanisms of the cell biochemistry of all living things.

The trend in bacteriology from morphological to physiological and biochemical characterization contrasted with that in macrobiology. Here, morphology still retained practical precedence, but phylogeny, classification based on the postulated evolutionary history of organisms, was becoming a target for academic biologists. Bacteriologists, driven by a paucity of morphological characterization tests, and lacking the fossil material required for phylogeny, resorted to ever more biochemically oriented characterization technology. It is ironic that this technology ultimately led to the precedence of bacteriology in the discovery of DNA as the substance governing inheritance, and in much of the molecular biology that flowed from that event. Bacteriology, which had largely turned its back on phylogeny, provided the basis for the nucleic acid sequencing methods so essential to modern molecular phylogeny.

Chemotaxonomy

Early work on immunology produced serological characterization tests (Bowden 1993). Interest in the chemical basis of these tests, and work on the mode of action of beta-lactam antibiotics, gave a detailed understanding of the chemical structure of bacterial cell walls. The remarkably diverse structures that were found correlated with other properties. Crucially, this clarified grouping of genera into families and laid the foundations of chemotaxonomy, the examination and comparison of cell macromolecules. This approach to characterization has become steadily more important in taxonomy (Goodfellow and O'Donnell 1994).

Methods include comparison of cell-envelope macromolecular structure, particularly the murein polypeptide links, teichoic and teichuronic acids, and polar and nonpolar lipids (Hancock 1994; Embley and Wait 1994); electron-transport-chain components of oxidative and photosynthetic metabolism, the quinones, cytochromes, and bacteriochlorophylls (Collins 1994; Poole 1994; Richards 1994); and overall base ratios of DNA (Tamaoka 1994). For the most part these are costly, slow, low-throughput methods requiring considerable expertise, but they provide detailed chemical information relevant to classification at species, genus, and suprageneric levels.

Fingerprinting

Another characterization technology related to chemotaxonomy is termed 'fingerprinting'. The methods include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins (Vauterin et al. 1993; Pot et al. 1994), pyrolysis mass spectrometry (Hindmarch et al. 1990; Goodfellow et al. 1994), whole-cell matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (Claydon et al. 1996; Lay 2001), and others of less familiar nature (Nelson 1991; Magee 1993b, 1994). These investigations yield results that reflect the chemical composition of the cell, but in comparative terms rather than by elucidation of detailed chemical structure. Fingerprinting methods utilize rapid physicochemical separation methods that produce profiles that can be compared to allow detection of similarities between strains. The profiles are often complex; the underlying nature of differences cannot be deduced from fingerprinting alone; and the methods are often based on costly instrumentation. However, these methods usually have a high throughput, automated processing, and low running costs, and produce many data relevant to cell composition, a facet that is otherwise difficult, slow, and costly to investigate. These data are pertinent at taxonomic levels from subspecies to supragenus.

Molecular genetics

The first inroads into DNA-based bacterial taxonomy came with assays for the ratio of guanine-cytosine (GC) to adenine-thymidine (AT) pairs in DNA (Tamaoka 1994), detecting the frequency of usage for GC-rich codons. The G+C content of bacterial DNA varies widely across the genera (see Tamaoka 1994), but less so within a genus. This is a useful tool to detect heterogeneity at genus level. Although significant differences clearly indicate dissimilarity, close similarity of G+C content does not necessarily indicate close similarity in other properties. Clearly, although the proportion of base pairs may be similar, the base sequence of the DNA may differ widely and code for a distinct metabolic pattern.

Later, DNA-DNA hybridization techniques allowed comparison of base sequence compatibility between strains (Kreig 1988; Johnson 1991; Stackebrandt and Goebel 1994). In this, DNA from a marker strain is annealed with DNA from the strain to be compared, and unbound DNA is removed. The extent of heterologous duplex formation indicates the extent of matching base sequences between the marker and test strain. As a control, 100 percent sequence compatibility is assessed by hybridization of marker and test DNA from the same strain. Annealing is performed under stringent conditions, at a high temperature, ensuring that only fragments with close sequence similarity remain bound, and at a lower, nonstringent temperature, where fragments may bind despite deletions and point-base differences.

Hybridization is currently recognized as the definitive test for similarity in bacteria. Strains showing more than 70 percent binding under stringent conditions with less than 5 percent difference between binding under stringent and nonstringent conditions are considered to belong to the same species (Wayne et al. 1987). Binding at this level indicates about 96 percent sequence identity (Stackebrandt and Goebel 1994). Anomalies between hybridization results and biochemical classifications occur in *Pseudomonas* (Roselló et al. 1991), the *Enterobacteriaceae* (Gavini et al. 1989), and *Bacillus* (Priest 1993).

However, hybridization is a prolonged, low-throughput approach requiring considerable expertise, and hampered by technical difficulties (Sneath 1983; Hartford and Sneath 1988; Johnson 1991) and potential distortion where repetitive sequences occur. Heteroduplex formation decreases markedly at supraspecies levels, and no inferences can be drawn on similarities at genus or further levels. Schleifer et al. (1993) have developed a rapid-lysis blotting hybridization system suited to identification.

Comparison of the base sequence for specific genes, usually that coding for the 16S rRNA of the small ribosome subunit, has become a standard approach. Various methods are available, including the original technique of direct sequencing of the 16S rRNA (Donis-Keller et al. 1977; Peattie 1979; Stackebrandt and Liesack 1993), but most groups currently utilize the polymerase chain reaction (PCR) to amplify a gene segment between two highly conserved sequences suited to PCR priming and DNA sequencing of the product.

The 16S rRNA gene is eminently suited to sequencing by this approach, as it contains 3′ and 5′ sequences that are virtually identical in all bacteria, are a suitable size for priming, and are separated by a sequence of a size (approximately 1.5 KB) that is readily copied by the PCR. Further, it is suggested that much of the sequence is highly conserved and so is unlikely to be subject to strain variation. Databases of known 16S rRNA sequences are available on the world wide web, notably on www.bdt.org.br/structure/molecular.html. Strains of the same species show sequence similarities greater than 97 percent. However, strains showing similarity at or above this level may belong to distinct species as defined by DNA hybridization (Ash et al. 1991; Fry et al. 1991; Fox et al. 1992; Martinez-Murcia et al. 1992; Stackebrandt and Goebel 1994). Advances in the technology have made 16S rRNA sequencing available as an identification method used in reference laboratories for particularly difficult strains. It is considerably less demanding in time, expertise, and cost than DNA hybridization.

Despite its current popularity, there may be several problems with 16S rRNA sequencing. Flawless transcription of a sequence of about 1500 base abbreviations to paper or computer file is no mean feat and the inevitable errors in proofreading these transcripts become embedded in the literature. This is no small source of inaccuracy, as noted elsewhere (Meissner 1995; Bernard et al. 1995). Sequences determined for the same species, or even the same strain, by different groups can differ significantly (Clayton et al. 1995). The source of these differences could be sequencing errors, intraspecies variation, or as many species contain multiple copies of the rRNA operon, interoperon differences. Several methods have been used in sequencing and the copy error level with different enzyme preparations is known to differ. Small differences in error rates or bias in the types of error from either source could lead to significant sequence differences. A level of about 3 percent sequence difference is suggested as a species cut-off, yet 18 percent of sequence pairs determined independently for the same strain differed by more than 1 percent in the study of Clayton et al. (1995), and 8 percent differed by more than 2 percent. Clearly, the overall error level in deposited sequences is close to the natural variation between closely similar species.

The sequence of individual product DNA molecules copied in PCR may differ slightly from that of the original template, or the operon copied may be atypical. With low-error-rate polymerase, these erroneous products are unlikely to affect direct sequencing of the pooled product. However, some groups select a single product molecule by cloning into a plasmid for later sequencing, and there is a possibility that an atypical product may be selected in cloning. A large blind-coded interlaboratory study to define the accuracy of sequence determination for the various methods and modifications and an effort to weed out or confirm any possibly inaccurate sequences in the databases would greatly enhance the utility of this approach.

The 'black-box' approach to numerical analysis that is evident in many papers on 16S rRNA sequencing has also been criticized. Goodfellow et al. (1997), reviewing a single issue of IJSB, found clear deficiencies in the numerical methods section of six papers on sequencing. Numerical analysis of similarity data is a complex subject unsuited to black-box processing. The deficiencies are highlighted by comparison with work on other characterization techniques, where data are often analyzed with four or more distinct numerical approaches and the homogeneity of the proposed consensus groups is then examined to verify the conclusions. Sequence data present greater difficulties in numerical analysis than most other characterization data. There are problems of alignment around deletions and insertions and in the interdependency of base sequences in distinct regions that hydrogen-bond together to form a functional tertiary structure. Sequence data require more effort in numerical analysis, and more caution in interpretation, rather than less. Unfortunately, the rise in availability of numerical taxonomic computer programs has encouraged cursory analysis, without the underlying understanding and caution required for interpretation.

Having said this, gene sequencing has opened the possibility that the evolutionary descent (phylogeny) of the current range of bacterial species may be determined. A phylogeny based on the 16S rRNA gene is now well-advanced (Woese 1987, 1992, 2000). This has had a major influence on the classification presented in the latest edition of *Bergey's manual* (Garrity 2001) and the few studies based on sequences of other conserved genes seem to verify this phylogeny.

Polyphasic taxonomy

Each of these individual characterization approaches has been hailed in its time as the last great advance in taxonomy, the discovery that would put every bacterium in to its proper place in a 'natural classification.' After experiencing four such 'revolutions,' I have come to a more moderate view. Each approach has its individual advantages and disadvantages. Each gives a different, and sometimes contrasting, insight into the differences between bacteria. Each has its place in taxonomic research and identification. Together, the results of all these approaches should give a robust general classification. The deficiencies of low-throughput, high-cost methods can be offset by testing the typical members of clusters detected in high-throughput, low-cost techniques. High-throughput techniques are suited to the search of large strain collections for potential new species.

Taxa that are distinct and cohesive in the large range of current techniques should stand the test of time. Colwell (1970) first suggested this polyphasic approach. However, despite its unarguable logic and repetition of the simple message by many experienced bacterial taxonomists, papers suggesting major taxonomic revisions based on a single technique continue to appear, only to be debunked later. The increasing instability of nomenclature at genus level in some areas of bacterial classification may well reflect the failure of workers to implement this polyphasic approach. A craftsperson uses many tools to fashion a useful object; taxonomists should recognize that each of the many approaches to characterization has an individual appropriate use in construction of classifications. Although a classification can be based on a single characterization method, it is unlikely to be of general use.

VISUALIZING THE RESULTS OF A CLASSIFICATION STUDY

Presentation of results is one of the greatest problems in taxonomy. Most authors choose diagrammatic representations – dendrograms, rooted or unrooted

trees, or occasionally two- or pseudo-three-dimensional ordination diagrams (Figure 4). Each has advantages and disadvantages, but none can represent the full complexity of the data. The alternative tabular presentations (Figure 4) – full or shaded similarity matrices – show the full data but are unwieldy and difficult to interpret. Many applied microbiologists do not have the knowledge to interpret the subtleties of these diagrams, and many would-be taxonomists use the programs that generate these diagrams as black boxes, in ignorance of the principles and shortcomings of the calculations involved (Goodfellow et al. 1997).

The dendrogram (Figure 4a) has a single axis indicating similarity between strains or between groups.

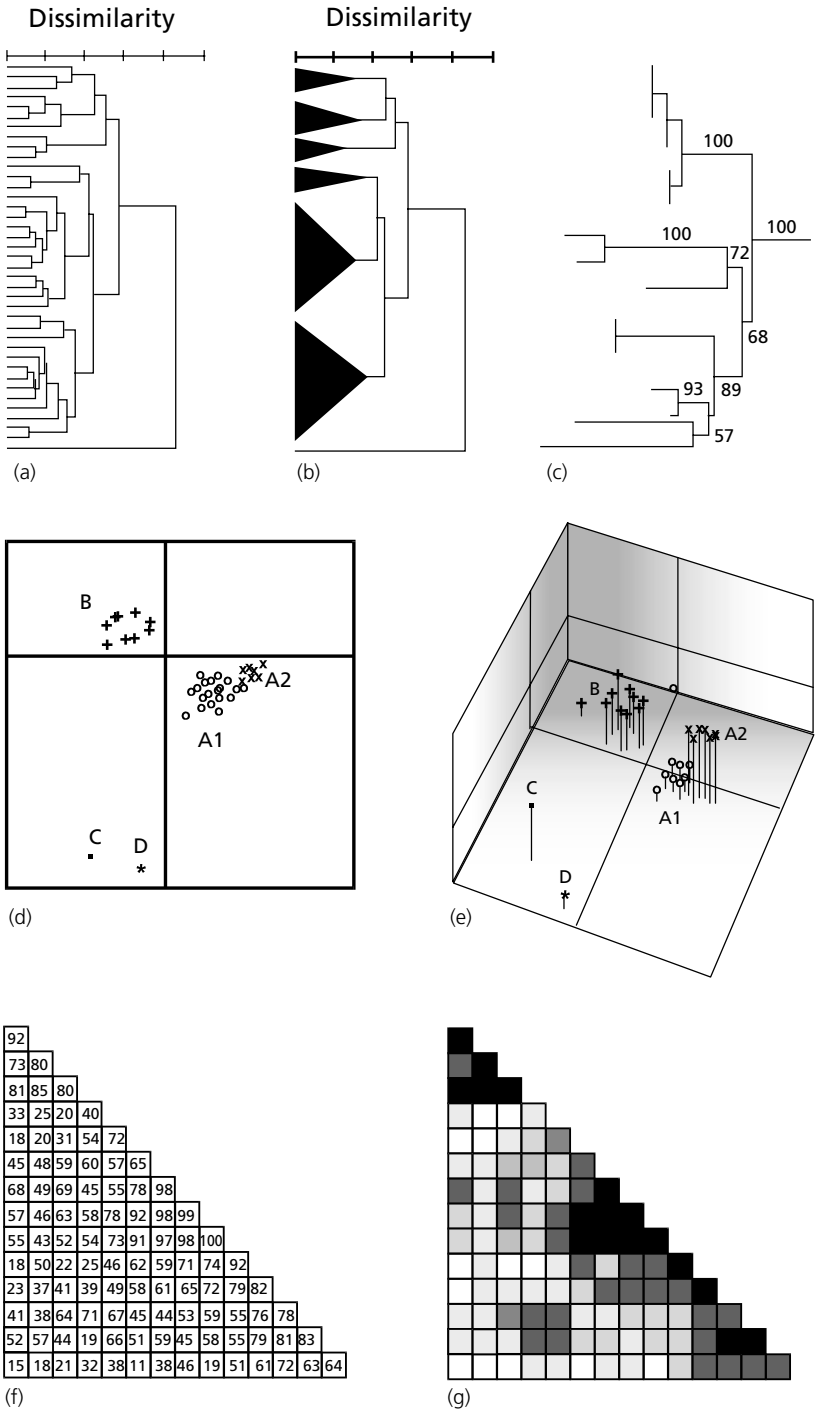

Figure 4 (a–g) *Representations of taxononomic data:* **(a, b)** *dendrograms;* **(c)** *tree (phenogram);* **(d)** *two-dimensional ordination;* **(e)** *pseudo-three-dimensional ordination;* **(f)** *similarity matrix;* **(g)** *shaded, ordered similarity matrix. (Data simplified from various biochemical and pyrolysis mass spectrometry studies by the author and colleagues.)*

The tree-like structure represents fusions of strains or groups, stretching from the multiple tips, each representing a single strain, to the root, a single line representing the fusion of all strains into a single group. Each branch point in the dendrogram represents the formation of a group with the level of intragroup similarity indicated by the position of the join relative to the axis. Where the join is between two single strains, the similarity of the strains is represented without distortion, but fusions between groups of strains become successively less representative of similarities between individual strains in the groups. In the unweighted pair-group method with arithmetic averages (commonly termed UPGMA) (Sneath and Sokal 1973), the most common method for constructing these figures, the mean group properties are compared at these group fusions. The groups formed at low levels of similarity may contain such diverse organisms that the mean group properties have little relevance to the properties of individual members.

Rooted and unrooted trees (Figure 4b) are essentially dendrograms that radiate from a central fusion out to radial tips representing individual strains. In this case the distance from the tip to a group fusion point represents the level of dissimilarity between the strain and the group mean. Often, each fusion point is annotated with a number showing the number (or proportion) of independent 'bootstrap' analyses that produced a similar fusion, indicating the degree of certainty that the fusion reflects a clear discontinuity in the original data.

Ordination diagrams represent interstrain dissimilarity as a distance. In Figure 4c, each strain is represented as a point, and the distance between pairs of points indicates the dissimilarity between the appropriate strains. However, the distance structure is only partially represented, and clusters of points may contain individual strains or small groups that are distant from the main cluster. This is illustrated in Figure 4d, where the data for Figure 4c are replotted on three axes, showing that group A1 is clearly resolved when a larger proportion of the distance structure is represented. The full distance structure from a large taxonomic study cannot usually be represented on fewer than eight axes.

Ordinations represent the distances between major groups well, but distances for some smaller groups and between individual strains are often represented poorly. This is the converse of tree representations, and comparison of the two types of diagram can highlight defects in the representations. The axes of ordination diagrams are usually complex statistically derived functions termed canonical discriminant variates or principal components. For the mathematically inclined, Manly (1994) gives an excellent explanation of these functions. For those averse to mathematics, each function is a composite measure of many properties that collectively distinguish between groups. These are similar to intuitively derived composite measures that are used in descriptions of stature – ectomorphic, mesomorphic, or endomorphic – where the relative measurements of a complex set of features, rather than any single measurable feature, divide the groups. Composite measures are a common feature of languages; 'fair' complexion describes a composite of pigmentation features and 'gangly' describes a combined measure of limb length, posture, and 'slimness,' itself a composite measure.

Similarity matrices and shaded, ordered similarity matrices are shown in Figures 4e and f, respectively. These represent the interstrain similarities as numbers or graded shading in a tabular form, similar to the tables of intercity distances that can be found in road-map atlases. The shaded tables are particularly useful in highlighting intermingling at the edges of clusters and intergroup similarities that may not be apparent in the topology of a dendrogram. It should be noted, however, that the order of strains markedly influences the clarity of these matrices.

These diagrammatic representations all face the fundamental difficulty that it is usually impossible to fully represent the distance structure determined in a taxonomic study in a two-dimensional diagram. Mathematically, it can be shown that if n strains are examined in t discriminatory tests and $n<t$, then the distance structure may require up to $n-1$ dimensions for accurate representation and can be fully represented only rarely in $< (n-1)^{1/2}$ dimensions. For a taxonomic study involving about 100 strains, this would mean that the data would be fully representable only in ten or more dimensions. Clearly, pictorial two-dimensional representations of similarity data are likely to be deficient or misleading in some aspects. Failure to reveal differences may occur when clusters overlap in ordinations because the axes on which they separate are not plotted, or when isolates 'chain' into unsuitable groups in dendrograms.

THE 'TYPE' CONCEPT AND SERVICE CULTURE COLLECTIONS

The type concept originated from botany, where a single typical dead specimen can supply a wealth of morphological data sufficient to describe the species and to identify further examples. Sneath (1995) has suggested that, with modern characterization methods, preserved dead bacterial specimens might eventually prove equally useful. However, for more than a century bacteriologists have preserved cultures of bacteria as viable organisms. Private collections exist in most departments, but the national service collections, which supply preserved viable cultures, are the prime sources of reference and type cultures. Each valid culturable species has a type culture, which Buchanan (1955) considered to be the reference point for the species.

These service collections are of paramount importance to bacteriology. They provide reference cultures

for comparative identification, quality control of characterization tests, teaching, and taxonomic studies. They often also provide focal expertise in taxonomy and identification and an essential service for identification of the more unusual organisms isolated in routine laboratories. Individual routine laboratories have insufficient time and see insufficient material to register rare, unrecognized pathogens as anything other than unusual. The long-standing high throughput of 'unusual' isolates for identification at national centers has repeatedly allowed identification of previously unnamed groups of bacterial pathogens as new species.

The Centers for Disease Control (CDC) laboratories in Atlanta have a notable record for the detection of such groups, recognized in the genus epithet *Cedecea*. It is particularly important that routine laboratories continue to send unusual isolates to such units, along with definitive information on their source and pathogenic effects, despite budgetary constraints. Equally, these units must continue to provide the identification service and maintain and review their collections of unnamed strains if we are to monitor new and rare pathogens adequately.

However, there are problems with these type and reference cultures. Live cultures maintained by serial subculture inevitably undergo selection and adaptation. Experimental evidence in chemostat (Helling et al. 1987) and batch culture (Atwood et al. 1951) strongly suggest that pure cultures undergo virtually complete clonal replacement with mutated variants after about every 100 divisions. Many older type strains were maintained by serial culture in early collections, and it is likely that most collection strains have undergone multiple predeposition culture cycles. Equally, the methods used to avoid continued subculture, freeze-drying and freezing in cryoprotectants, are highly selective. It is rare to recover more than 1 percent of the initial population from these preserved specimens, and such high kill rates must exert some selective pressure. This selection may be particularly severe for obligate anaerobes exposed to air during subculture. Early microbiologists recognized that biological variation was an inescapable consequence of the culture and storage of strains (Rahn 1929) and used the effect in attenuation of the pathogenicity of anthrax and tubercle bacilli. Modern microbiologists would do well to remember their conclusions.

Equally, reference cultures must be treated with the utmost care outside central collections. There have been several incidents of mislabeled reference strains being passed between research laboratories, with consequent errors in published work, notably with *Candida albicans* (Mackenzie and Odds 1991). All reference cultures should be obtained directly from a recognized culture collection and replaced regularly from the same source.

THE SPECIES CONCEPT

The definition of the species concept in bacteriology continues to be a favorite topic of debate, where those of a philosophical bent can argue free from the constraint of experimental data. A criticism is of the lack of enterprise in obtaining data that might elucidate a solution, rather than of the discussion of this central problem in classification.

In general biology, the best known species definition is that of a sexual interbreeding capability with production of offspring capable of further interbreeding. This definition clearly is not applicable to asexual organisms and is extremely difficult to test in the wild. Indeed, there are many examples where interspecies hybrids are viable, particularly in plants (Eckenwalder 1984) but also in animals (Hall 1978). Clearly, the capability of promiscuous genetic exchange in bacteria invalidates these definitions based on interfertility (Jones 1989). Interspecies exchange of chromosomal DNA has been demonstrated repeatedly (Maynard-Smith et al. 1991), and transposons and promiscuous plasmids can transfer large genetic elements across massive taxonomic gulfs (Natarajan and Oriel 1991; Kirby 1990; Arthur et al. 1987). Other species definitions of more general applicability exist (Simpson 1961; Hutchinson 1965; Van Valen 1976; Templeton 1989) but are not helpful in practical delineation of species.

In practice, identification and most classification work in general biology still relies almost exclusively on comparison of phenotypic characters. The various fine shadings of sexual species definitions are discussed with vigor, but their impact on practical aspects is small. Bacteriologists are probably further advanced on the species concept, with reasonable consensus agreement on a practically applicable, though arbitrary, definition based on DNA hybridization (Wayne et al. 1987). Cohan (2002) reviews species definitions and presents an interesting new definition for bacterial species based on the ecospecies concept.

However, even this consensus has been confused by the adoption of a series of terms that fragment the species concept into variants. Ravin (1963) proposed the terms 'genospecies,' encompassing mutually interfertile organisms, 'nomenspecies,' encompassing organisms 'similar' to the type strain, and 'taxospecies,' encompassing groups that share a high proportion of common properties. The term 'genomic species', readily confused with 'genospecies,' encompasses organisms showing high DNA sequence similarity. These concepts have pragmatic merit (Ursing et al. 1995), but they offer a dangerous attraction to an explosive diversification of concepts of convenience, each with its own classification and nomenclature. Medical microbiologists could easily find the concept of a medicospecies, encompassing a classification that they found particularly convenient,

irresistible in the current milieu of rapid nomenclatural change.

The species concept that seems most universally accepted in practice, if not in theoretical discussion, is the taxospecies: a group of organisms that show high similarity in a broad range of characterization approaches and that are delimited from other organisms by an area of character combinations that are rarely found in nature. This definition is implicit in the numerical taxonomic approach and naturally entrains the concept of polyphasic taxonomy. It does not imply any arbitrary breakpoint of similarity in a specific test, but it requires proof of a discontinuity of variation at the species boundary.

Dividing organisms into species is an unavoidable necessity in biology; routine work invariably requires inference of more general properties from a limited number of observed characters. However, the organisms have no knowledge of, nor any obligation to obey, the divisions that we draw. The boundaries between species can often be diffuse, particularly in bacteriology. However, biologists often give the impression that the natural world can be segmented with absolute clarity into the divisions that we desire. Propagation of this erroneous impression in the general population may well encourage the view that humanity itself can be divided with equal clarity, with all the unsavory consequences that ensue. A definition of the species concept that denies the diffuse uncertainty of biological divisions is as undesirable politically as it is scientifically.

SPECIATION AND EVOLUTION

This is a much neglected area of research, but one in which bacteriology could make a considerable contribution to our understanding. In contrast to higher organisms, bacteria reproduce rapidly. It is possible to produce populations comparable with the entire worldwide population of man within a few days in a fermenter of modest size. Further, bacteriologists have evolved sophisticated techniques for detection and selection of variants forming a tiny proportion of a population. This offers the capability of observing evolution in action over short periods and in a level of detail that cannot be approached in macrobiology. It is unfortunate that the potential for useful work in this area has lain fallow for so long. The few studies to date (Atwood et al. 1951; Helling et al. 1987; Lenski and Travisano 1994) have been largely ignored, possibly because they emphasize a level of variability during successive culture cycles that many modern bacteriologists would prefer to ignore.

Equally, bacteriologists have observed evolution in the wild for many years but ignored its implications for evolutionary science. This is particularly true in medical microbiology. The introduction of antibiotics unintentionally produced the largest experiment in evolution ever undertaken by man, but its results have never been analyzed from an evolutionary viewpoint and the period in which a control population could be observed has long passed. What is obvious from this experiment is that bacteria are eminently suited to evolutionary studies. The high selective pressure of antibiotics and the rapid reproduction of bacteria has spawned, for example, at least five distinct successive races of *Staphylococcus aureus* suited to spread in the ecological niche offered by hospitals within 50 years. Fortunately, much material has been stored in culture collections, and this could provide an invaluable resource should sufficient funding and interest become available.

It is singularly unfortunate that the unique combination of the capabilities of bacteriological techniques and the highly selective environments found in hospitals, particularly intensive care units, has not been brought to the attention of evolutionary scientists, population geneticists, and microbial ecologists. The potential for mutual advantage from such a combination of expertise is enormous. However, the beginnings of interest are now obvious in studies of the genetics of evolutionary change in, for example, the acquisition of resistance to penicillin in commensal *Neisseria* spp. (Bowler et al. 1994) and in multilocus sequence typing of methicillin-resistant *S. aureus* (MRSA) (Oliveira et al. 2002) and glycopeptide-resistant enterococci (Woodford 2001). Population geneticists are beginning to study variation in bacteria (Baumberg et al. 1995; Feil and Spratt 2001; Vogel and Frosch 2002), and the impetus provided by the rapidly decreasing effectiveness of antibiotics against hospital-evolved pathogens may lead to funding of such projects.

ORIGINS, EVOLUTION, AND CONTROVERSY

The Earth is thought to have accreted as a planetary body in the solar disk about 5×10^9 years ago and to have cooled to form a solid surface at about 4.2×10^9 years ago. Prokaryotic life appeared less than 0.7×10^9 years later – a remarkably short time, considering that the subsequent appearance of eukaryotes was a further $1.5–2.3\times10^9$ years later. The earliest fossil evidence of life known at present occurs in the Apex chert in Australia (Schopf 1996). Among the diverse microfossils, some appear to be complex, highly evolved organisms that show close similarity in form and diversity to modern blue-green algae, a prokaryote lineage that appears to have originated midway in the bacterial line according to 16S rDNA phylogenies.

These phylogenies postulate three lineages of life (ignoring the viruses, which probably have multiple origins): the Bacteria (including the Cyanobacteria or blue-green algae), the Archaea, and the Eukarya (Woese et al. 1990). The time of origin of the Archaea is controversial (Cavalier-Smith 2002). These are prokar-

yotic organisms similar in morphology to bacteria but differing markedly in many other aspects. No archaeal microfossils have been found that can be unequivocally distinguished from bacteria by current techniques. The various 16S rDNA phyogenies often place the Archaea as diverging early from the bacterial lineage. However, from evidence of cell and biochemical organization, Cavalier-Smith (2002) argues that the Archaea originated later, possibly as late as the Eukarya.

The first unequivocal fossil evidence of eukaryotes occurs in rock about 1.2×10^9 years old, with less convincing evidence back to 1.8×10^9 years (Lipps 1993). The eukaryotic life forms diversified to give various unicellular lineages, the fungi, plants, and animals, with the first fossil evidence of multicellular eukaryotes occurring about 0.9×10^9 years ago. The Eukarya appear to have acquired prokaryotic symbionts early. There is strong evidence that the mitochondria present in most eukaryotes and the chloroplasts in photosynthetic eukaryotes are symbiotic prokaryotic organisms, descended from, respectively, gram-negative bacteria of the alpha-Proteobacteria line and Cyanobacteria (Margulis and Fester 1991). It is interesting to reflect that the dominant bacterium of the human flora is the mitochondrion, an obligate intracellular symbiont that is present in every cell, is essential to life, and represents a cooperative partnership 1 000 000 000 years old. Nature is not controlled solely by tooth and nail competition. Those who propagate the contrary view to politicians and the public should consider the long-standing symbiosis of mitochondria or chloroplasts, or study the wonderfully intricate symbiotic associations between prokaryotes and eukaryotes reviewed by Fenchel (1996) and Douglas (1996).

Beyond this broad picture of evolution of the three divisions of life, there is beginning to be controversy about the linear branching phylogenies that have dominated past thinking on evolution (Doolittle 1999; Jain et al. 2002; Gupta and Griffiths 2002). An increasing number of papers present evidence that widely divergent groups have exchanged genetic material coding for complex biochemical systems (Andersson and Roger 2002; Ragan and Charlebois 2002; Katz 2002). The evidence suggests that significant lateral genetic exchange between the most widely distinct evolutionary branches of life has occurred, possibly on many occasions. This implies that lateral exchange may be a common phenomenon in closely related lines, where similarities in exchange mechanisms and genome composition favor genetic exchange. There is strong evidence from the genetics of antibacterial resistance that sharing of genetic resources across widely divergent lines is a key option for evolutionary adaptation to adverse conditions. The current linear-branching model for evolution covertly assumes that competitive diversification is the sole mechanism involved. As cooperative redistribution of genes becomes recognized as an important additional mechanism, 'tree-of-life' representations of evolution may be replaced by a reentrant ragged 'web-of-life' model. This type of model is likely to gain early favor among bacteriologists, where promiscuous genetic exchange mechanisms are already well documented, and rapid evolutionary processes, such as acquisition of antibacterial resistance, demonstrate the true complexity of evolutionary mechanisms.

Current views of evolutionary hierarchy for the bacterial lineage are based on 16S rDNA sequencing, which divides the following groups (only genera with members potentially pathogenic to man are named; 'others' indicates nonpathogenic genera not normally encountered in medical microbiology; the order of groups reflects order of branching where possible):

Thermotoga group – others
Bacteroides group – *Bacteroides*, *Prevotella*, *Flavobacterium*, *Cytophaga*, *Capnocytophaga*
High G+C gram +ves – *Mycobacterium*, *Nocardia*, *Streptomyces*, *Corynebacterium*, *Propionibacterium*, *Bifidobacterium*, *Gardnerella*, *Arthrobacter*, *Micrococcus*
Fusobacterium group – *Fusobacterium*
Low G+C gram +ves – *Mycoplasm*, *Ureaplasma*, *Acholeplasma*, *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Listeria*, *Staphylococcus*, *Bacillus*, *Peptostreptococcus*, *Eubacterium*, *Clostridium*
Proteobacteria groups:
Gamma group – *Chromatium*, *Francisella*, *Xanthomonas*, *Coxiella*, *Legionella*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Vibrio*, *Aeromonas*, *Haemophilus*, the Enterobacteriaceae + others
Beta group – *Burholderia*, *Alcaligenes*, *Bordetella*, *Neisseria*, *Eikenella* + others
Alpha group – *Wolbachia*, *Rickettsia*, *Brucella* + others + eukaryote mitochondria
Delta group – others
Eta group – *Campylobacter*, *Helicobacter*, *Wolinella* + others
Spirochaetes – *Treponema* + others
Fibrobacter – others
Green sulfur bacteria – others
Planctomyces – *Chlamydia* + others
Cyanobacteria – the blue-green algae + eukaryote chloroplasts

WHAT'S IN A NEW NAME?

Extrapolation of current trends is notoriously unreliable in science, but it is likely that bacterial taxonomy will have to cope with a major increase in the rate of description of new taxa. The background trend is already well established and may well be magnified by new influences. Automated acquisition of characterization data is becoming well established for an ever-wider range of characterization approaches, speeding experimental work, and changing the emphasis of problem-solving effort to data-handling. The international agreements on biodiversity may well increase funding for

taxonomic studies, hitherto poorly funded by industry and public sources. The prospect that species may be patented will clearly lead to increased discovery efforts and provide a financial incentive to the 'splitter' faction. Pending patents have already caused difficulties in the deposition of type strains of commercial significance (Labeda et al. 1995). New technology may well redouble productivity from the molecular biology faction, and DNA sequencing studies of natural environments have already revealed an astounding diversity of previously undescribed and currently nonculturable bacteria, outnumbering described species (Derakshani et al. 2001; Eilers et al. 2000; Paster et al. 2001; Smit et al. 2001).

However, the speed of change in nomenclature has become a problem in applied bacteriology: those who deal with cystic fibrosis patients are, hopefully, aware of the multiple changes in the nomenclature of two major pseudomonad pathogens in the recent past (Hugh 1981; Swings et al. 1983; Yabuuchi et al. 1992; Palleroni and Bradbury 1993). Changes in nomenclature of pathogens generate considerable criticism among medical microbiologists. They are not merely inconvenient; they also increase the problems of communication and may well have unforseen implications in the complex legislation that touches on microbiology in each of the various countries of the world. A further increase in the rate of change could generate a major rift between those who use and those who produce classifications (Magee 1993a).

Key issues for clarification are the date from which proposed changes in nomenclature are to be regarded as active, in the sense that legal proceedings might judge a microbiologist to have erred by using an older nomenclature, and the process of consensus acceptance of change. Most taxonomists and many users regard the date of publication in the IJSB/IJSEM as the date of active change, but many may postpone judgment until the first date of inclusion in an edition of *Bergey's manual*. The latter includes sufficient time to judge the consensus acceptability of the change; the former clearly does not. However, neither solution seems completely acceptable, even at the present rate of change in nomenclature.

If changes in nomenclature are active from their appearance in the IJSEM, then each change is governed by a group comprising the authors of the paper, an editor, and a few referees. Is the judgment of so small a group invariably so reliable, and their insight into the ramifying consequences so broad, that the general community of microbiologists will find the change acceptable? Notwithstanding respect for fellow microbiologists, it is of concern that any group this small can institute changes that may have considerable legal and other consequences on the whole of bacteriology, and that these changes become active, without prior notice, on publication in a journal taken by a minority of applied bacteriologists.

Equally, if active change coincides with mention in *Bergey's manual*, then the interval between editions of about 10 years is too long and the number of concurrent changes to be made is too large. Even here, there is no defined mechanism for judging consensus acceptance of change. One or at most a few experts on each major group write on their subject. There is no guarantee that their interests are so all-inclusive that some change crucial to one branch of the applied science is missed or that their own biases reflect a consensus view of bacteriologists.

The ICSB has a pivotal role in guiding developments in bacterial systematics, including nomenclature, and its members face a new century that is likely to tax their powers of mediation. Negotiating satisfactory compromise between the wishes of applied microbiologists and taxonomists, two groups with widely differing views and requirements, could well require considerable diplomacy. However, the alternative to compromise and cooperation between taxonomists and users is to discard our only internationally accepted scheme of bacterial nomenclature, and this is clearly unacceptable. The solutions to these problems cannot be coercive; the nomenclatural code does not and cannot dictate the nomenclature used by individuals. The wisdom of preserving general acceptance of the formal nomenclature must be convincingly explained to a silent majority, who would much prefer to remain with the nomenclature they were taught years before.

Many of these communication problems could be solved by computerized communication of changes and opinions on the world wide web. One major advance is the availability of lists of all validated names on the world wide web (www.bdt.org.br/cgi-bin/bdtnet/bacterianame). A list of all validated bacterial names of human and veterinary importance is maintained by J.P. Euzéby at www.bacterio.cict.fr/. This site is particularly well maintained and user-friendly and is an essential reference for current names and other taxonomic information. In the interim, efforts are being made to disseminate notification of proposed changes more widely (Frederiksen and Ursing 1995a, b), and this should be supported and encouraged by the community of applied bacteriologists.

An associated problem is the growing gap between the characterization methods used by taxonomists and those available for identification in the routine laboratory. Base ratio determinations, DNA-DNA hybridization, 16S rRNA sequence determination, and chemotaxonomic methods are available in only a minority of routine laboratories and are, as yet, too specialized, tortuous, and slow to have major relevance to identification in routine bacteriology. However, an increasing number of taxonomic papers neglect any mention of characters that can be determined in routine laboratories. The bench microbiologist whose skills lie in identification from colony morphology, pure culture, and

biochemical tests could be excused for regarding this change with horror. This generates a backlash, with regularly heard detrimental comments on the probable capabilities of taxonomists to perform conventional tests, or to examine plate cultures adequately. Clearly, this is an area where medical microbiological input to the formulation of minimal standards for description of new species is essential to our interests. Taxonomists and applied microbiologists must seek mixes of characterization methods that fulfill the requirements of both research and routine work, and revise these as technology advances.

However, medical microbiologists must recognize that they live in a time of great change in bacterial taxonomy. As in all science, taxonomic truth is approached by successive revision in the light of new facts, and the current turmoil in nomenclature is a reflection of growing interest in a subject that forms the basis of routine laboratory work. Nomenclatural change is inevitable and cannot be ignored. Many of the changes may increase the capability to predict important properties from identification results. However, applied microbiologists must also ensure that, when the changes cause problems, these are voiced to the ICSB with sufficiently convincing force, clarity, and representation. The widespread tradition of ignoring nomenclatural change, and restricting critical comment to colleagues in the same field is not helpful to either medical microbiology or taxonomy.

THE FUTURE

The future course of bacterial taxonomy is likely to be interesting and turbulent. We are beginning to discard attitudes and assumptions stemming from the late nineteenth century that have shackled progress for the greater part of the twentieth century. The introduction of numerical taxonomy marked the first major break with the early botanical influence, allowing recognition of the true nature of variation within bacterial species and ridding us of the baggage of monothetic groups and invariant diagnostic characters. Characterization tests now allow comparison of features other than the physiological and nutritional interactions with the environment. Together, these have encouraged the beginnings of exploration of the true diversity of bacteria, which may well produce many unforeseen discoveries of economic significance. Molecular methods are beginning to be used to explore diversification in bacteria, and there is now a possibility that the enormous potential of bacteria in the study of evolutionary processes may be recognized. The phylogeny of bacteria, or at least that of the 16S rRNA gene, is now becoming apparent.

However, these changes are bringing a host of problems. Nontaxonomists are increasingly bewildered by sudden changes in nomenclature and the alienation that this produces must be tackled. The tendency of taxonomists to adopt uniformly a single characterization technique, rather than a polyphasic approach, must be actively discouraged, for it will certainly lead to large, unsupportable swings in nomenclature as techniques fall in and out of style. A major problem of the polyphasic approach is that the full range of characterization techniques cannot be supported in a single laboratory. The consequences of this are that several groups must be involved in each study, and the paper produced must acknowledge a long list of workers in the authorship. The nonscientific problems of contact, cooperation, precedence of discovery, and adequate acknowledgment must be tackled if the polyphasic approach is to become the accepted method of taxonomic advance. Bacterial taxonomy is advancing into the next century in the midst of change. Hopefully, solutions to these problems will be found in a newly matured science that will continue to produce spin-off discoveries of enormous importance in general biology.

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Bacterial growth and metabolism

JAMES P. SHAPLEIGH

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GROWTH OF BACTERIA

Bacterial nutrition and culture media

A minimal medium is one that contains the simplest chemical composition needed to support the growth of an organism. Some bacteria can grow quite well on a minimal medium consisting of distilled water, a carbon and energy source such as D-glucose, and some inorganic salts, for example sodium and potassium phosphates, ammonium sulfate, magnesium sulfate, and ferrous sulfate. Bacteria able to grow in such a medium must be able gain metabolic energy by the catabolism of glucose and synthesize all organic molecules needed for growth from carbon degradation products of the glucose and the inorganic salts. This carbohydrate–salts medium is chemically defined, in that the exact chemical composition is known and can be reproduced. In stating that such a medium is defined, we ignore the requirement for trace elements such as copper, zinc, cobalt, etc., which are required in only minute amounts and are provided as contaminants in the other ingredients.

If an organism lacks the enzymatic capability to synthesize any organic biosynthetic compounds from such a medium, then growth of that organism cannot occur using that medium unless such compounds are provided. If a mutant of *Escherichia coli* loses the ability to synthesize an amino acid, then that amino acid has become a required growth factor and must be added to the medium or growth of that mutant cannot occur. Many bacteria, especially those whose natural environment contains many complex organic compounds, such as pathogens, are unable to synthesize a wide range of biosynthetic intermediates, such as amino acids, nucleosides, vitamins, etc., and these must be supplied to permit growth. Such a growth medium may be complicated and difficult to assemble, but it is still chemically defined in that the exact chemical composition is known. Because a chemically defined growth medium is reproducible, it is often preferred for studies on the metabolism of bacteria.

Complex and rich bacterial growth media such as those made from nutrient broth (beef extract and peptone) or l-broth (peptone and yeast extract) will support the growth of many bacteria and are convenient to use, but such media are undefined in that the exact chemical composition is not known. Complex and undefined media of these types are often used for isolation and growth of bacteria but it should be realized that the exact composition of such media may vary from batch to batch.

Microbiologists have been ingenious in devising selective media that are designed for the isolation from natural sources of a particular type of microorganism. Such media are designed to facilitate growth of organisms targeted for isolation while discouraging, as far as possible, growth of contaminating organisms. Enrichment

cultures are not only designed to possess the proper media for isolation of a desired organism, but growth conditions such as temperature, can be designed to aid in the isolation. A wide variety of media have also been designed to aid in the rapid isolation and identification of microorganisms, especially pathogens.

Growth in liquid culture

A growing bacterial cell increases in size until it separates into two daughter cells. If growth is balanced and the replication of the macromolecules has been carefully controlled, all of the macromolecules of the mother cell must have doubled in mass and each daughter cell is a duplicate of the mother cell. At that time, a cell generation or division cycle has taken place. The growth of bacterial cells may become temporarily unbalanced as a result of environmental stress, but regulatory systems will normally adjust growth rates so that synthesis of macromolecules once again becomes balanced. Continued unbalanced growth can lead to cell death.

Measurement of cell numbers is often performed by viable cell count. With the pour plate method, various dilutions of cells are mixed into tubes of a melted agar-growth medium that has been cooled to just above the temperature of solidification. The tubes are poured into Petri dishes and, after incubation, the number of colonies is taken as the number of viable cells in the original dilution. An alternate method is the spread plate method, where samples of 0.1 or 0.2 ml of the appropriate cell dilutions are spread over the surface of a solidified agar-growth medium plate. The spread plate method is reported to give slightly higher colony counts and has the advantage that individual colonies can be easily sampled for further testing.

Such information as the viable cell count or number is valuable to many microbiologists, especially those concerned with infectious diseases or food poisoning, because these techniques quantify cells capable of growth. However, such techniques really measure colony-forming units, not necessarily individual cells, as clusters of cells in packets or chains will produce a single colony. Also, it takes many generations of growth for a single cell to produce a colony visible to the unaided human eye. With this technique, a bacterial cell could produce hundreds of thousands of descendants and still be classified as nonviable. A direct cell count measures all of the cells present, including those not capable of growth. It is possible to count the total number of cells per unit volume, both living and dead, microscopically, using a special slide. Cell count as well as mass can also be determined by impedance counting, where cells passing through a narrow chamber disrupt the flow of an electrical current.

A problem with using viable or total cell counts to measure bacterial growth is the inability to detect increases in cell mass during the division cycle. Growing bacterial cells must increase their mass before division and an increase in cell mass must be considered as growth, even though the cell number may not have increased. Cell mass can be determined directly by measurement of cell weight after drying a sample of cells in an oven. Cell mass is often estimated by determining the light scattering of a suspension of bacterial cells with a standard curve to relate light scattering to mass.

The amount of bacterial growth that has taken place in a culture can be estimated by a variety of methods that measure a result of growth rather than cell number. The disappearance of a substrate such as glucose can be determined, or the utilization of oxygen during respiration. A product of growth such as cell deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein can be determined, or the amount of acid produced during fermentation might be used as an estimate of cell growth.

The time for a division cycle to be completed is the time for a generation of growth to take place and is known as the generation time. If growth of a bacterial culture is synchronized, all cells are in exactly the same phase of the division cycle, and all will divide simultaneously. With synchronized cell division, the total cell number immediately increases from the initial number (N_0) to $2 \times N_0$. If the cells remain synchronized, another generation will simultaneously raise the cell number to $2 \times 2N_0$. Techniques that have been used for synchronizing cell cultures involve the germination of spores, filtration or centrifugation of cells to obtain a constant size cell, and prevention of initiation of DNA replication by amino-acid starvation. Special mutants have also been used, such as those with temperature-sensitive mutations in genes required for initiation of DNA replication, to prevent cell growth at the restrictive temperature. Upon change to permissive temperature, DNA replication in all cells in the culture will begin simultaneously and all cells will enter the same phase of the division cycle. Synchronization of cell growth can be useful in studying cell chemistry at different phases of the division cycle, but synchronization usually lasts for only a few generations.

CELL GENERATIONS AND GROWTH RATES

Because the total cell number doubles each division cycle or generation (g), the number of cells produced by growth (N) will be equal to the original number (N_o) times 2 to the power g. Therefore:

$$N = N_o \times 2^g. \quad (1)$$

Taking the $\log_{10}$ of both sides of the equation:

$$\mathrm{Log}_{10}N = \mathrm{Log}_{10}N_o + 0.301\ g. \quad (2)$$

Then to solve for g the equation becomes:

$$\frac{\text{Log}_{10}N - \text{Log}_{10}N_o}{0.301} = g. \quad (3)$$

An important value that can be determined from these calculations is t, the generation time. Generation time is the time for each generation to occur so it equals the time elapsed (T) per generation (g), or $t = T/g$. The generation time is also the time required for the cell population to double.

The rate of increase of bacterial number (or mass) is exponential because the population doubles with each generation. If the bacteria are growing at a constant rate, then a plot of cell number or mass against time results in an exponential curve, as shown in Figure 1a. The plot of the $\log_{10}$ of bacterial number/mass against time will give a linear relationship, as shown in Figure 1b, provided the cells are growing at a constant rate. This is the conventional method of plotting bacterial growth.

The rate of increase in total cell number during growth is the initial cell number multiplied by the specific growth rate constant (μ):

$$\frac{dN}{dT} = \mu N. \quad (4)$$

The value of the specific growth rate constant can be derived from the plot of log N versus t, where its value will be given by the slope of the linear plot. The specific growth rate constant is not the same as t but represents the growth rate at a specific instant.

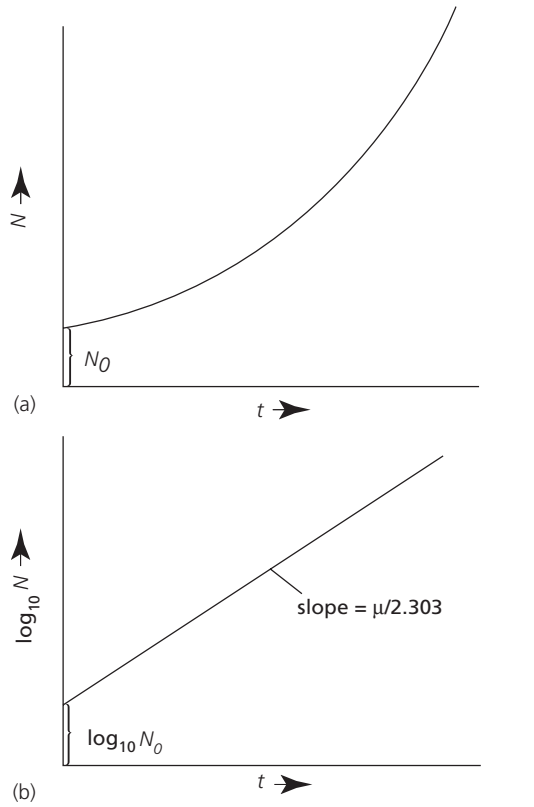

Figure 1 *Theoretical growth curves illustrating instantaneous exponential growth in a bacterial batch culture. Total cell number (*N*, i.e. number of bacterial cells per ml) plotted against time (*t*). N_0, inoculum cell concentration; μ, specific growth rate*

THE GROWTH PHASES AND THE GROWTH CURVE

The lag phase

When an inoculum of cells is transferred to a growth medium, a delay may take place before measurable growth occurs. This lag time is a period where cells are adjusting to new growth conditions, and growth may be temporarily unbalanced during this growth phase. The greater the change in growth conditions, experienced by the cells, the longer the predicted lag phase. Because cells must increase their size before cell division can take place, the exact length of the lag period may depend upon whether cell number or cell mass is being measured. A typical bacterial growth curve, with lag and exponential and stationary phases, is shown in Figure 2.

The exponential (logarithmic) growth phase

Once the cells have adjusted their regulatory systems to producing maximum growth under the new conditions, the culture enters the exponential or logarithmic growth phase where cells have approximately the same generation time. Growth is normally not synchronized and, at any moment, cells will be at different stages of the division cycle. In batch culture the growth rate eventually

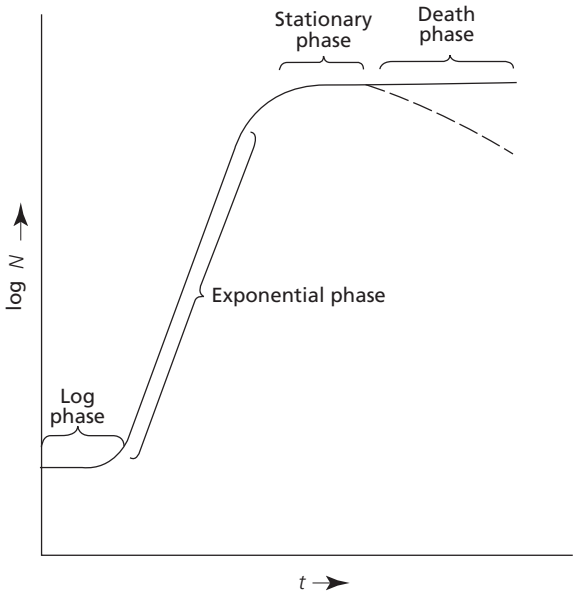

Figure 2 *Idealized growth curve of a bacterial batch culture. Unbroken line, total cell count; dashed line, viable cell count*

must begin to decrease as the availability of some essential nutrient becomes limiting or the accumulation of some product becomes toxic.

The stationary phase

Eventually the stationary phase is reached, a period where measurable growth ceases. Note that if a new viable cell was formed for each cell that lost viability, or if loss and increase in cell mass were balanced, then there would be no measurable increase in cell growth.

If *E. coli* cells are growing on a minimal salts medium containing a carbon and energy source and the energy source becomes exhausted, the pH will remain constant and growth and nitrogen utilization will both stop. However, under conditions where the nitrogen becomes depleted and the energy source is in excess, the cells will continue to use the energy source, even if the energy gained from catabolism is discarded. If possible, the bacteria will increase their internal supply of reserve food material under these conditions, while denying surrounding microorganisms a potential energy source. Traditionally, resting cells of bacteria are those incubated without any added nitrogen source. Such cells are still able to induce enzymes and catabolize carbohydrates, and these types of resting cells have been used for many metabolic studies.

The death phase

Some bacteria retain viability upon reaching the stationary phase, while others rapidly lose viability and enter a death phase. A variety of circumstances, including loss of energy and production of autolytic enzymes, may cause bacteria to lose viability. Reserve food sources that may be accumulated by bacteria, such as poly-β-hydroxybutyric acid and glycogen, are usually used quickly when the cells are starving but once such reserve food materials are exhausted, cellular RNA may serve as its major source of energy. Ribosomal RNA degradation may increase during starvation conditions. For example, *E. coli* can degrade 20–30 percent of its RNA within 4 h of starvation. The degradation of ribosomes will lead to the eventual death of the cell because cells lacking ribosomes are incapable of synthesizing protein. Starvation also increases the rate of protein degradation and peptidase-deficient mutants of *E. coli* have been shown to have poor survival during starvation for carbon and nitrogen. The amino acids produced by protein degradation can be used for the synthesis of new proteins to aid in survival under stress conditions.

A drop in internal energy levels may prevent transport of nutrients across the cytoplasmic membrane so that, upon transfer to recovery medium, the cell is unable to uptake nutrients. The energy charge of a cell has been reported as the total adenosine triphosphate (ATP) plus half of the adenosine diphosphate (ADP) over the totals of the cellular ATP, ADP, and adenosine monophosphate (AMP). Because two ADP are capable of forming one ATP and one AMP, the amount of ATP that can be obtained from ADP is equal to one-half of the ADP present. The equation

$$\text{energy charge} = \frac{\text{ATP} + {}^{1}\!/_{2}\ \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}} \qquad (5)$$

gives the ratio of available ATP to the total adenine nucleotides present. If all of these nucleotides were in the form of the triphosphate, the energy charge would be one. For the normal growth and metabolism of bacterial cells, the energy charge must be maintained in the range of 0.80–0.95; *E. coli* has been reported to grow only when the energy charge is over 0.8, it will maintain its viability when the energy charge is between 0.5 and 0.8, but it will die when the value is under 0.5. There is a certain amount of maintenance energy required just for cells to repair continuing damage but this may vary for different organisms. In experiments with *E. coli*, it was found that once the glucose was exhausted the organism maintained its energy charge at 0.8 by using acetate that had been excreted during growth on glucose. If no other substrates were present, then the capacity for protein synthesis and enzyme induction was lost along with cell viability.

Because a starving cell must maintain a certain level of metabolism and energy to remain viable, many bacteria activate special regulatory systems in response to starvation, regulatory systems that allow them to express 'starvation' genes (Matin 1992). These new proteins improve the cell's ability to use low levels of nutrients, regulate the degradation of their own polymers for carbon and energy, and aid the cells in surviving. Many of the genes encoding for these proteins require the nucleotide cyclic AMP for their induction. The starvation stress regulatory system may overlap with other stress regulatory systems because starved *E. coli* cells have been shown to acquire resistance to heat stress.

In *E. coli*, the nucleotide guanine triphosphate has an essential role in the regulatory adjustments made by cells starved for amino acids. This adjustment to amino-acid starvation is known as the stringent response. In cells with insufficient amino acids, the stringent response will cause a rapid reduction in the synthesis of ribosomal and transfer RNA.

CHANGING GROWTH RATES

The rate of growth of a bacterial strain may be shifted up or down with changes in growth conditions, such as medium composition or temperature. Shift-up experiments refer to a change in growth conditions with an increase in the growth rate of a bacterium. As an

example, an improvement in nutrient conditions can lead to an increase in the internal amino acid concentration, thus resulting in an increase in rate of RNA synthesis, an increase in the rate of protein synthesis, and a more frequent initiation of DNA synthesis. Faster-growing cells tend to be larger, with an increase in the amount of the protein-synthesizing machinery. While the regulatory systems are adjusting to the new conditions, the cell length may continue to increase and the cell may become unusually long. Eventually, the cell length decreases to reach the proper size for the new growth rate (Cooper 1991).

By contrast, a transfer of cells from a complex undefined medium, such as a nutrient broth, to a minimal-salts medium can result in a shift-down or decrease in growth rate caused by a sudden decrease in internal concentration of nutrients, such as amino acids. A decrease in the internal concentration of amino acids leads to a stringent response with a decline in the rate of RNA synthesis, a decline in the rate of protein synthesis, and a less frequent initiation of DNA replication. In fact, DNA synthesis may halt altogether, until activation of biosynthetic pathways can produce the required building block compounds to permit cell growth.

Continuous culture of bacterial cells

The chemostat is a name given to devices that maintain the growth of bacteria in a constant exponential growth rate. A typical chemostat possesses a container containing sterile growth medium that is transferred into the growth chamber at a controlled rate. The growth chamber has an overflow device so that the entrance of new medium results in the overflow and exit of an equal volume of used medium, as shown in Figure 3.

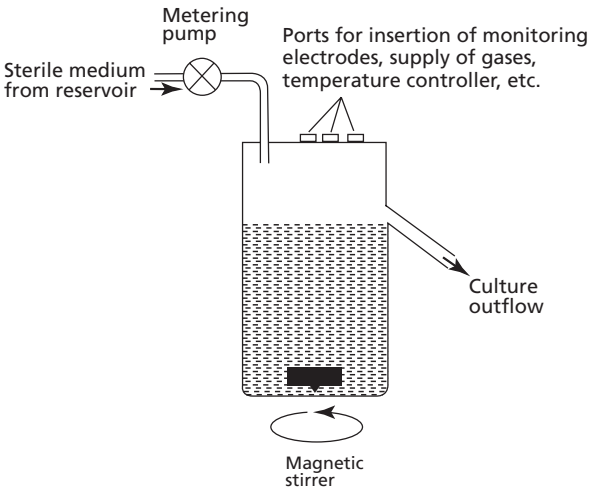

Figure 3 *Principal features of a continuous-flow culture apparatus*

CHEMOSTAT GROWTH RATES

The medium in the growth chamber of a chemostat can be inoculated with a bacterium to give an initial cell concentration of (N). If growth takes place, the rate of increase in cell mass against time can be represented by $dN/dt = \mu N$, where N is the cell number per unit volume and μ stands for the growth rate constant. The rate of increase in cell number can be represented as μN. If the growth rate constant is a positive number, the cell mass in the chemostat will be increasing. However, new medium is introduced into the chemostat at a constant flow rate and old medium is overflowing out of the chemostat at the same rate, removing cells. If the flow rate into the chamber is F volume per unit time period, and the volume of the growth chamber is V, then the fraction of the culture medium that overflows and is replaced each unit time period is represented by F/V (also known as the dilution rate). Therefore, the total amount of cells lost each time period by overflow would be $N \times F/V$. The real rate of change of cell number in the chemostat is the rate of increase resulting from growth (μN) minus the rate of decrease as a result of washout ($N \times F/V$). Therefore, the rate of change in the chemostat is $\mu N - N \times F/V$, or $N(\mu - F/V)$.

If F/V is greater than μ, the cells are being washed out of the chemostat faster than they can grow and eventually all of the cells will leave the chemostat. If F/V is smaller than μ, the number of cells will increase. However, as the cell mass increases, there are more cells competing for a constant food supply, because the flow rate into the growth chamber is constant and this competition causes a decrease in the growth rate constant. Eventually μ will decrease until it equals F/V and then a steady state will exist with μ held constant. In such a manner, by controlling the rate of flow of new medium into the chemostat, the experimenter can control the growth rate of the cells in the chemostat.

Chemostats are usually established with the growth rate limited by a single known factor, such as a limiting supply of a required growth factor or the carbon and energy source. Chemostats are very useful for obtaining mutants, because once a growth rate is established there is strong evolutionary pressure for selection of mutants that have increased their growth rates under the competitive chemostat conditions. However, chemostat experiments must often be terminated because of the selection of 'sticky' mutants that cling to the culture-vessel surface and are not washed out.

CHEMOSTAT CELL POPULATIONS

If the concentration of the limiting nutrient in the reserve chamber is designated as S_r and the unused amount of that nutrient that washes out of the chemostat is S, then the amount of nutrient used by the cells in the growth chamber is $S_r - S$. If Q represents the amount of that limiting nutrient needed to make one bacterial cell, then

the total population of cells will be equal to $S_r/Q - S/Q$. However, S is usually close to zero and then the equation becomes S_r/Q, which represents the total population of cells. The growth rate in the chemostat is controlled by the rate of flow of new medium into the chemostat, but the total population of cells present is determined by the concentration of the limiting substrate.

Growth on solid surfaces

Good growth for a bacterium such as *E. coli*, on rich medium, under aerobic conditions, may range from 1×10^{10} to 5×10^{10} cells per milliliter. The final yield of cells per milliliter in liquid culture can be considerably increased if the cells are placed within a dialysis sack that is suspended in a large vessel of growth medium. The dialysis sack permits waste products to diffuse out of the sack, away from the cells, while fresh nutrients can diffuse into the sac. Cells growing on an agar surface can form colonies with high concentration of cells per unit volume for a similar reason. The agar permits nutrients to diffuse toward the colony, while waste products diffuse away.

The nutritional state of bacteria may also influence their adhesion to solid surfaces. Many bacteria have increased adhesion under starvation conditions but some bacteria actually display the opposite effect. Colonization of surfaces by bacteria may be influenced by motility and cell-surface hydrophobicity as well as by nutritional status (James et al. 1995). In nature, masses of cells commonly exist in films or mats and the cells in such masses may be able to regulate overall behavior by communication systems. Bacteria may grow in a manner that maximize cell-to-cell contact and, perhaps, cellular communication. For this reason, some microbiologists view the bacterial colony as a multicellular 'organism,' an organized population of communicating cells (Shapiro 1995).

STRUCTURE AND ORGANIZATION OF THE BACTERIAL CELL

General composition of bacteria

In order for bacterial cells to grow, they must be provided with all of the chemical elements needed for the synthesis of new cellular material. Once, organisms that could use simple molecules to satisfy their nutritional requirements, such as carbon dioxide (CO_2) as the sole carbon source, were thought of as 'simple' organisms. We now understand that the simpler the nutrients, the more complex the biochemical reactions required by the cell to convert those nutrients into complex cellular material.

The water content of bacterial cells can vary from 75 to 90 percent of the total weight. The dry weight, after removal of water, is composed of about 50 percent carbon, about 10 percent hydrogen, and about 20 percent oxygen. The nitrogen content of the dry weight can vary from 8 to 15 percent, sulfur from 0.1 to 1.0 percent, and phosphorus from 1 to 6 percent. The iron content is also variable and is highest in cells with heme proteins. Other elements found are potassium, calcium, magnesium, chlorine, sodium, zinc, cobalt, molybdenum, manganese, and copper. These and other trace elements total about 0.3 percent of the dry weight.

Cellular polymers

Most of the organic material in the bacterial cell is in the form of large polymers. However, the cell cytoplasm also contains a 'soluble pool' of smaller, unpolymerized organic molecules such as amino acids, nucleotides, carbohydrates, and fatty acids, as well as degradation products from the breakdown of any compounds providing carbon and energy sources for growth. Large organic polymers of the cell include those made of amino acids (proteins), those made of ribonucleotides (RNA), those made of DNA, and those composed of carbohydrates and lipids.

Detailed studies of polymer composition have been carried out in only a few bacteria. In *E. coli* protein represents about 50 percent of the dry weight of a typical bacterial cell. RNA represents about 20 percent of the dry weight of a bacterial cell but can vary depending upon growth conditions. About 90 percent of the RNA is ribosomal RNA (rRNA), which consists of three species, the 23S rRNA, the 16S rRNA, and the 5S rRNA. The transfer RNA (tRNA) represents about 9 percent of the total RNA, with 60 types of tRNA. The messenger RNA (mRNA) represents about 0.2–1 percent of the total RNA. There may be one to four molecules of DNA, depending upon the growth rate, representing about 3.3 percent of the dry weight (Neidhardt 1987). Carbohydrate polymers are present in the cell wall and may also be stored in the cytoplasm as food materials such as glycogen. Lipid material can vary from 1 to 8 percent of the cell dry weight, depending upon growth conditions, and can be found in the cell membrane and wall. Some bacteria, excluding *E. coli*, can store lipid-like substances as reserve food material, often in the form of polyhydroxybutyric acid granules in the cytoplasm. For the synthesis of this storage material, two acetyl-coenzyme A (acetyl-CoA) molecules are converted to acetoacetyl-CoA; this is changed to D-(-)-3-hydroxybutyryl-CoA, which is polymerized to form polyhydroxybutyric acid (Steinbuchel et al. 1991).

Eubacterial cell walls

PEPTIDOGLYCAN MATERIAL

The backbone structure of the eubacterial cell wall is the peptidoglycan material. Peptidoglycan is a polymer of N-acetyl glucosamine and N-acetyl muramic acid, linked together by β-1,4 or 1,6 alternating units and about

12 carbohydrates long. The disaccharide chains are linked together by polypeptide chains, usually from three to eight amino acids long and containing some D-amino acids such as D-alanine and D-glutamic acid. The polypeptide chain is attached by a peptide bond to the carboxyl group of the muramic acid. It normally contains one diamino acid, such as lysine or diaminopimelic acid but not both, and a short peptide bridge joining the free amino group of the diamino acid to the terminal carboxyl group of a similar polypeptide branch from a different disaccharide strand. In such a manner the backbone disaccharide strands are joined together. The cross-links in the peptidoglycan material form a 'web' capable of supporting stress in any direction. During synthesis of the peptidoglycan material, a disaccharide of N-acetylglucosamine and N-acetylmuramic acid is first formed and the amino-acid bridges are then attached with an extra D-alanine on the end of the peptide chain. Teichoic acid and peptide-bridge material may also be added to the unit. To build new wall material, this disaccharide–peptide unit is inserted into the existing cell wall in the space between the cytoplasmic membrane and the cell wall. A membrane-linked transpeptidase removes the final D-alanine unit from one peptide chain or peptide bridge and installs in its place the extra amino group on the diamino acid of another chain. The presence of D-amino acids in the cell wall is believed to give protection to external proteolytic enzymes. Enzymes that attack the bacterial cell wall are termed lysins, and autolysins are lysins, produced by the bacterium itself. Some lysins attack the peptidoglycan backbone, whereas others attack the peptide portion or the point where the peptide chain joins the glycan strands. Lysozyme is a lysin that cleaves at the β-1,4 linkage of N-acetylglucosamine.

GRAM-POSITIVE CELL WALLS

Gram-positive cell walls contain peptidoglycan and may also contain teichoic acid, polymers of glycerol or ribitol, usually about 10 to 50 units long, and linked together through phosphate bonds. Most teichoic acids are covalently linked to the peptidoglycan. Side groups on the teichoic acids can be carbohydrates such as glucose and N-acetylglucosamine and/or amino acids such as D-alanine. Teichuronic acid, a polymer consisting of alternating glucuronic acid and N-acetylgalactosamine, is sometimes found in the cell walls, especially when phosphate is limiting. Lipoteichoic acids are polymers of glycerophosphate covalently linked to a lipid molecule. The bound lipid allows the molecule to be anchored in the lipid bilayer of the cell membrane. However, the glycerophosphate polymer is not linked to the peptidoglycan.

GRAM-NEGATIVE CELL WALLS

The gram-negative cell wall is more complex than that of the gram-positive bacteria. One layer contains peptidoglycan material; the other layer is a convoluted outer membrane consisting of protein and at least two types of lipids: lipopolysaccharides (LPS) and phospholipids. The peptidoglycan material makes up only about 12 percent of the total cell wall material in *E. coli* and the four amino acids usually found in the peptidoglycan material of gram-negative cells are L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanine. LPS consists of three parts: an inner hydrophobic lipid A region, an outer hydrophilic O antigen polysaccharide region, and a core polysaccharide region that connects the A and O regions. The phospholipid and LPS form an asymmetric bilayer, with the phospholipid forming the inner layer and LPS forming the outer layer.

Proteins found in this outer wall include murine lipoproteins. The lipid end of this protein is embedded in the inner face of the bilayer formed by lipid and LPS. The protein end can be bound to the peptidoglycan. The main function of murine lipoprotein may be to help stabilize the outer membrane–peptidoglycan complex. Other important proteins found in the outer wall are porins, which form channels to allow the diffusion of hydrophilic molecules. Porins are sometimes grouped into three types (Nikaido 1992). Type 1 are nonspecific porins that distinguish substrates on the basis of size. A zone of constriction arising from the folding of a stretch of residues into the central pore allows porins to distinguish substrates on the basis of size (Cowan et al. 1992). Examples of this type are OmpF and OmpC of *E. coli*. Type 2 porins are related to type 1; both only permit diffusion of small substrates, but the type 2 porins preferentially transport certain substrates. The LamB porin of *E. coli*, which preferentially transports maltose and maltodextrins, is representative of this type of porin (Schirmer et al. 1995). LamB is also the binding site of the lambda phage. The third types of porins are TonB-dependent porins. TonB-dependent porins specifically transport certain substrates such as vitamin B_{12} or siderophores. These porins are unique in that transport is energy dependent. The energy is supplied through the TonB protein, which resides in both the cytoplasmic membrane and the outer cell wall (Moeck and Coulton 1998).

Between the outer and cytoplasmic membrane of gram-negative organisms is the periplasmic space, which contains many proteins, including transport-binding proteins, scavenging enzymes, detoxifying enzymes, and others. Periplasmic enzymes are synthesized in the cytoplasm and must be transported across the cytoplasmic membrane. Many periplasmic proteins contain a signal sequence of about 20–40 amino acid residues long at their N-terminus (Pugsley 1993). This sequence contains a positive region followed by a hydrophobic region and terminates in a peptidase cleavage site. The signal sequence targets the protein for transport via the Sec transport system (also known as the general secretory pathway). Once transport is completed, the signal sequence is cleaved from the transported protein.

A Sec-independent system for export of periplasmic proteins has been identified in *E. coli* (Weiner et al. 1998). This system recognizes proteins with a 'twin-arginine' motif at their N-termini. Proteins transported by this system appear to be mainly redox proteins containing cofactors. Related proteins are found in a wide range of bacteria, suggesting that this is an important transport system for periplasmic proteins.

Archaea cell walls

The third domain of life, the archaea, have distinctively different cell-wall polymers from the eubacteria (Kandler and Konig 1998). They do not possess the peptidoglycan polymer but may possess a slightly different polymer termed pseudopeptidoglycan. In the genus *Methanobacterium thermoautotrophicum*, this polymer contains N-acetyltalosaminuronic acid and N-acetylglucosamine and only L-amino acids, such as L-lysine, L-alanine, and L-glutamate. The cell wall of *M. ruminantium* has L-threonine in place of L-alanine, and N-acetylgalactosamine in place of N-acetylglucosamine. The synthesis of this polymer is unique to bacteria containing pseudopeptidoglycan. The cell walls in the genus *Methanosarcinaceae* are made of an acid heteropolysaccharide that contains galactosamine, neutral sugars, and uronic acids. No muramic acid, glucosamine, glutamic acid, or other amino acids typical of peptidoglycan are found. Most archaea also contain an S-layer (see below) as a cell envelope. Because the cell walls of archaea are structurally distinct from eubacterial walls, almost all of the antibiotics that target synthesis of components of the wall in eubacteria are ineffective against archaea; for example, β-lactam antibiotics and vancomycin do not inhibit growth of archaea.

Polymers external to the cell wall

Capsule or slime layers may exist as polymers outside the cell wall. They are usually made up of amino acids, carbohydrates, or both; for example, the polypeptide capsule of *Bacillus anthracis* is mostly D-glutamic acid, whereas the *E. coli* K-12 capsule consists of D-glucose, L-fucose, galactose, hexuronic acid, acetate, and pyruvate. Often the extracellular material is not tightly linked to the cell wall. Such loosely adhering material is referred to as a slime capsule. In pathogens surface polysaccharides are an important surface antigen. In *E. coli* the surface polysaccharide gives rise to the K antigens, of which there are more than 80 serotypes (Whitfield and Roberts 1999). Capsules are also important virulence factors. The capsule of *B. anthracis* is critical for cell survival during infection. Strong bonding forces rarely link capsule polymers to one another, although highly acidic types can be cross-linked by divergent metals such as Mg^{2+} or Ca^{2+}.

Another extracellular assemblage is known as the S-layer. The S-layers are made up of protein subunits that are sometimes glycosylated (Sleytr and Beveridge 1999). The unique feature of S-layers is that the subunits spontaneously form higher-order structures with crystalline lattices. S-layers are found in both eubacteria and archaea.

External appendages

Many bacteria contain appendages that extend beyond the outer cell wall. One of these is the bacterial flagellum. Flagella are used to propel bacteria in directions favorable for survival and growth. The flagellar structure can be broken down into three basic units, termed the basal body, the hook, and the filament (Manson et al. 1998). The basal body anchors the flagellum into the cytoplasmic membrane and contains numerous proteins required for assembly and function. The hook is a short curved region that links the basal body to the filament. Attached to the hook is a helical filament made up primarily of a single protein termed flagellin. The filament rotation which propels the cell forward, is driven by the ion gradient across the cytoplasmic membrane. Because the filament is helically shaped its rotation can propel the cell.

Flagella do not just randomly propel the cell through its environment. Cells have external receptors that detect environmental stimuli and then, using a phosphorelay system, this information modulates rotation of the flagellum. In *E. coli* counterclockwise rotation of the flagella produces forward movement termed 'runs,' while clockwise rotation causes cellular 'tumbling.' Stimuli that prolong runs are called attractants and stimuli that shorten the length of a swimming interval are called repellents. Changes in attractant or repellent levels cause changes in flagella rotation and result in movement in favorable directions. Other bacteria, for example *Rhodobacter sphaeroides*, can rotate their flagella in only one direction (Armitage and Macnab 1987). So, instead of runs and tumbles *R. sphaeroides* cells run and then stop and randomly re-orient. There are similarities in the signaling pathways in *E. coli* and *R. sphaeroides*, but the two pathways are not identical.

Many bacteria also possess external protein fibers known as pili or fimbriae composed of protein (pilin). The pili of pathogenic bacteria contain specific proteins that allow the bacteria to attach to and colonize host tissues. Binding of pili to external surfaces may be a key factor in activating expression of genes required for infection in pathogenic *E. coli* (Zhang and Normark 1996). There are other types of pili, referred to as sex pili, that are used by bacteria to attach to each other and exchange DNA.

Cytoplasmic membrane

The cytoplasmic membrane is made up of both lipid and protein and can constitute about 10 percent of the dry

weight of the bacterial cell. In most cases the lipid is primarily phospholipid. The fatty-acid components of the phospholipid can be varied to maintain the appropriate fluidity of the membrane. Cyclopropane fatty acid is also a common modification of the lipids of the cytoplasmic membrane. It has been demonstrated that increases in content of cycloporane fatty acids render cells of *E. coli* more sensitive to acidic pH (Chang and Cronan 1999).

At least 200 different kinds of protein representing up to 6 to 9 percent of all the cellular proteins are located in the cytoplasmic membrane. Some of these proteins are cytochromes, dehydrogenases, NADH-oxidases, enzymes involved in cell-wall synthesis, enzymes involved in electron transport and oxidative phosphorylation, and proteins involved in the transport of specific molecules through the membrane. Typically, membrane-associated proteins are divided into two classes, integral and peripheral membrane proteins. Peripheral proteins associate with the surface of the membrane through ionic interactions. Therefore, they can be removed by treatment of the membrane with high concentrations of salt. Integral proteins are embedded in the lipid bilayer and can be dissociated from the bilayer only by the use of detergents. Integral proteins must contain surface-exposed hydrophobic regions to interact with the hydrophobic regions of the bilayer. In many cases these hydrophobic regions form α-helices and are termed membrane-spanning helices. In *E. coli* integral membrane proteins are targeted to the membrane by the signal recognition particle and the protein FtsY (Ulbrandt et al. 1997). Proteins to be translocated across the membrane by the Sec system are targeted by the SecB protein, differentiating the transport pathways of these two classes of proteins.

The cell membranes of archaea chemically differ from eubacterial membranes. The membrane lipids of archaea are isopranoid alcohols, typically 20 or 40 carbons long, and ether-linked to glycerol. The C20 alcohols can be linked together giving rise to the C40 molecules. The glycerol groups can be modified with a number of different polar head groups. It has been suggested that the use of the novel lipids by Archaea allows them to tolerate extreme environments better (van de Vossenberg et al. 1998).

The cytoplasm

A typical bacterial cell is able to synthesize over 1000 different enzymes to catalyze the many chemical reactions needed for cell growth. Catabolic pathways provide the cell with the energy for growth, while anabolic reactions are the biosynthetic reactions whose purpose is the formation of new cellular material. Although a bacterial cell may possess the ability to synthesize over 1000 different enzymes, its regulatory systems are evolved to control the synthesis of such proteins, and the exact type and quantity of enzyme may be carefully regulated.

Enzymes function as catalysts and rapidly cause the equilibrium of thermodynamically feasible chemical reactions to be reached. Although an enzyme must participate in the reaction it catalyzes, binding substrates and releasing products, it is normally regenerated and can be reused when the reaction is completed. The activity of many enzymes is associated with a relatively low-molecular-weight compound termed a coenzyme (or prosthetic group or cofactor). The catalytic site of the enzyme binds the substrate and the enzyme–substrate complex changes to an enzyme–product complex with the release of the product. The specificity of enzyme action results from the specific recognition of the substrate by the catalytic site. Many enzymes will act upon compounds related in chemical structure to the natural substrate but usually with lower binding affinities. Sometimes such compounds are converted to products and released but some can bind to the enzyme and block activity. Enzyme activity can also be destroyed or inhibited by a variety of chemicals or conditions, including those that alter the structure of proteins in a nonspecific manner.

As increasing concentrations of a substrate are presented to an enzyme, the velocity of the enzyme-catalyzed reaction will increase until, at substrate concentrations sufficient to saturate the available catalytic sites, the rate of reaction will be sustained at some maximum value (V_{max}). Thus, a plot of reaction rate (v) versus substrate concentration [S] frequently approximates to a rectangular hyperbola. An enzyme displaying these kinetic properties is said to demonstrate Michaelis–Menten kinetics and the relationship between the reaction rate and substrate concentration can be described by the maximum velocity (V_{max}) constant and the Michaelis constant (K_m), which is the substrate concentration that limits the rate of the enzyme-catalyzed reaction to be one-half that of V_{max}. Studies of how V_{max} and K_m change under different chemical and physical conditions can provide information on the nature of the substrate–enzyme complex.

The regulatory mechanisms of bacterial metabolism are designed to permit a controlled synthesis of cellular polymers, usually as rapid as possible under the existing environmental conditions. Bacterial cells are not simply a 'bag of enzymes', as was once suggested. Indeed, there is careful organization of many proteins within the bacterial cell. Multienzyme complexes can be found within the cytoplasm and many enzymes and transport proteins are incorporated in the cell membrane or, in the case of gram-negative bacteria, within the periplasmic space. Some proteins involved in anabolic processes themselves are regulated. These allosteric proteins possess a site for binding a low-molecular-weight molecule termed an effector. The effector molecule may

be quite different from the substrate molecule, but its binding to the enzyme results in a conformational change at the active site with a change in the affinity of the active site for the normal enzyme substrate. A positive effector will increase the binding capability of the enzyme for its substrate, while a negative effector will decrease the binding capability. With normal intercellular substrate concentrations, a positive effector will increase the rate of reaction, while a negative effector will decrease the rate of reaction. Proteins with nucleic acid binding sites can also have their binding ability increased or decreased by effector molecules.

BIOSYNTHESIS OF DNA, RNA, AND PROTEIN

The bacterial chromosome and DNA replication

The DNA of the chromosome in a typical bacterial cell can be 500–1 000 times as long as the cell itself. This molecule is compacted by folding and supercoiling. The initiation of chromosomal replication requires a certain cell mass before replication can begin. Replication of the chromosome proceeds bidirectionally from the origin of replication (in *E. coli* this is termed *oriC*). Replication requires that the DNA strands become unwound, forming an open complex. In *E. coli* formation of the open complex requires the protein DnaA and a few other proteins. DNA polymerases then synthesize DNA in the 5′–3′ direction using a 3′-hydroxyl primer. *E. coli* has three DNA polymerases, two of which are required for replication of the chromosome. Polymerase III replicates the chromosomal DNA. Because DNA polymerase III needs a primer to begin DNA polymerization on a single-stranded template, a polymerase is needed first to form an RNA primer. After DNA polymerase has used these primers for the elongation of the DNA, they are replaced by DNA. In the 3′–5′ direction, RNA primers are formed and, after the formation of 1 000- to 2 000-long DNA strands termed Okazaki fragments, destroyed and replaced by DNA using DNA polymerase I. The nicks between the Okazaki fragments are repaired by DNA ligase (Bremer and Churchward 1991). Because replication is bidirectional, the two replication regions (termed replication forks) meet at a region in the chromosome called the termination region. This region blocks progression of the replication forks and allows termination to occur.

Some strains of bacteria have been reported to have more than one chromosome; for example, a strain of *R. sphaeroides* (strain 2.4.1) has been reported to contain two circular chromosomes as well as five plasmids (Suwanto and Kaplan 1989). The two chromosomes are 3.0 million base pairs (Mbp) and 0.9 Mbp in size. Another bacterium with multiple chromosomes is *Deinococcus radiodurans* (Lin et al. 1999). Interestingly this bacterium also contains multiple copies of each chromosome (Hansen 1978). This polyploidy has been suggested to play a role in the radiation-resistance of this bacterium. However, there are probably other factors involved because other bacteria, such as *Azotobacter* (Punita et al. 1989), are polyploid but are sensitive to ionizing radiation.

Protein synthesis

While the DNA contains the sequence information for the various proteins of the cell, the RNA is the machinery that translates that information into the polypeptide chains. The genetic code is triplet, meaning that three nucleotides determine the code ‘word’ for each amino acid. Because there are four nucleotide ‘letters’ (A, T, G, and C in the DNA alphabet and A, U, G, and C in the RNA alphabet), it is possible to have 64 different ‘words’ or codons for the 20 amino acids normally found in proteins. The genetic code is also degenerate in that there may be more than one codon for any one amino acid but no nucleotide triplet will code for more than one amino acid; for example, there are four mRNA codons for serine, UCU, UCC, UCA, and UCG. It has been speculated that the code was once a doublet rather than a triplet and, therefore, the original doublet code for serine might have been UC. Three codons called nonsense codons usually do not code for any amino acid and signal when to end synthesis of a polypeptide chain.

Amino acids are activated before being added to a polypeptide chain by being placed on specific tRNA molecules. An enzyme specific for each amino acid (aminoacyl tRNA synthetase) transfers the amino acid to the tRNA specific for the transfer of that amino acid. These tRNA molecules are about 80 nucleotides in length and each has a site recognized by its tRNA synthetase. The amino acid is first activated using ATP, resulting in the formation of an aminoacyl–AMP complex. This complex is transferred to the tRNA molecule and the tRNA molecule is said to be ‘charged.’ In such a manner, each tRNA can become ‘charged’ at its amino-acid attachment sites with the proper amino acid. The tRNA also possesses a ribosomal recognition site as well as a codon recognition site that will recognize the mRNA codon for that amino acid.

Polymerization of the amino acids takes place with the aid of the ribosome. A bacterial ribosome consists of two subunits, designated 30 and 50S, which combine to form the 70S ribosome. The complete ribosome is made up of three different ribosomal rRNAs and 52 different proteins (r-proteins). The smaller 30S subunit contains 21 proteins and a 16S rRNA, whereas the larger subunit has 21 proteins and two species of rRNA, 23S and 5S. The high-resolution structures of an intact 70S ribosome

(Cate et al. 1999) and the structure of the 50S (Ban et al. 1999) and 30S (Clemons et al. 1999) subunits of the ribosome have been reported. The functional elements, that is the tRNA and mRNA binding sites, of the ribosome are identified in these structures. The channel through which the extending peptide leaves the ribosome has also been visualized. Further analysis of these structures should permit detailed understanding of the chemistry taking place in this complex.

It is known that rRNA is initially transcribed as a single 30S precursor that is then processed into the three final rRNA products. The number of rRNA genes is variable; for example, in *E. coli* K-12 there are seven rRNA operons. Strains of *R. sphaeroides* have between three and five rRNA clusters (Nereng and Kaplan 1999). In *E. coli*, the ribosomal protein L7/L12 is present in four copies per ribosome, while other ribosomal proteins are present in one copy per ribosome. The genes responsible for the synthesis of the 52 r-proteins are organized into at least 20 translational units (operons) containing from 1 to 11 r-protein genes. At least six of the tRNA species are cotranscribed with rRNA species. The rates of synthesis of the 52 ribosomal proteins of *E. coli* and the three rRNA components are balanced so there is normally very little excess ribosomal protein or rRNA in the cells. The translation of ribosomal proteins from an operon can be inhibited by the proteins coded in that operon. These proteins normally bind to rRNAs but if their concentration is higher than required they will bind to their own mRNA, blocking translation. In addition, if sufficient ribosomal proteins are not present, rRNA will combine with other proteins but the complex formed will not function as ribosome (Nomura et al. 1984).

The mRNA represents only 2–3 percent of the total cellular RNA and its synthesis is controlled at the operon level. In translation of the mRNA into protein, a complex is formed between the 30S ribosomal subunit and certain protein initiation factors. The mRNA binds to the 30S subunit as does the first charged tRNA, the 'initiator' tRNA. The eubacterial initiator tRNA is N-formylmethionyl-tRNA, which binds to a site in the 30S subunit, termed the P site. The initiator tRNA appears to be methionine-tRNA for the archaebacteria. The ribosome also contains a binding site for a second charged tRNA (the A site). It is the codons found in the mRNA that determine the order of incorporation of the charged tRNAs. Once the charged tRNA binds at the A site, a peptide bond is formed between its amino group and the amino acid on the tRNA at the P site. The polypeptide chain is attached to the tRNA at the A site and the now uncharged tRNA moves to a third tRNA binding site in the ribosome referred to as the E site. The tRNA at the A site and its polypeptide chain move to the P site (translocation) as the ribosome moves to the next codon on the mRNA. The charged tRNA with the proper anticodon binds to the A site and the process of polypeptide chain elongation continues. The termination codons (UAA, UGA, and UAG) are not charged with any amino acid. When such codons appear on the mRNA, the polypeptide chain is released from the P site. Either the formyl group is removed from the first amino acid or the entire formylmethionine is removed.

PROTEIN FOLDING

The information required for the final structure of the synthesized protein resides in its primary sequence. While many proteins can spontaneously fold into their final tertiary structure, some are apparently assisted by accessory proteins referred to as chaperones. Several bacterial protein-folding chaperones have been studied in great detail. Perhaps the best studied chaperones are the GroEL/GroES chaperones, which have also been given the name chaperonins. The high-resolution structure of the GroEL/GroES complex has been determined. The complex is made up of two seven-membered rings of GroEL, each containing a central cavity (Xu et al. 1997). GroES forms a single seven-membered ring that caps one of the cavities of the GroEL complex. It is unclear exactly how targets of chaperones are recognized but exposed hydrophobic surfaces seem to be important. In many bacteria there are additional chaperones involved in protein folding. Two well-studied examples are DnaK (Hsp70) and DnaJ (Hsp40). These proteins help to limit aggregation of newly synthesized proteins (Fink 1999). They also probably function with the GroEL/GroES complex to carry out protein folding. These protein-folding chaperones also play a role in response to heat stress because they can assist in the refolding of incorrectly folded proteins.

Archaea also contain chaperones but so far have not been found to encode the GroEL/ES type. Archaeal chaperones are known to form stacked rings somewhat similar to the GroEL/ES ring. The high-resolution structure of the complex from the thermophile *Thermoplasma acidophilum* shows these ring structures (Ditzel et al. 1998). Both DnaK- and DnaJ-like chaperones have been found in archaea.

Proteins that are termed chaperones are involved in other cell functions besides protein folding; for example, chaperones are required for the secretion of proteins into or through the cell membrane. The protein SecB is required for recognition of proteins to be transported via the Sec system (Fekkes and Driessen 1998). Once SecB binds to targeted proteins it prevents them from folding and ferries them to the Sec translocation complex.

The eventual fate of almost all proteins is degradation. Interestingly, some proteins involved in the degradation of proteins form complexes similar in structure and function to GroEL/ES. ClpA and ClpP form rings that are probably required for the unfolding of proteins necessary for their degradation (Horwich et al. 1999). Another large complex involved in protein degradation

has been termed the 20S proteasome (De Mot et al. 1999). This is a large structure consisting of four, stacked seven-member rings. This structure has been found in the archaea and eukaryotes but only among the gram-positive actinomycetes in the eubacteria.

PROTEIN SECRETION

It is obvious that bacteria must have systems for the transport of proteins across the cell membrane. The characteristics of some of these systems were discussed above. In addition to transport across the cytoplasmic membrane many proteins are secreted into the surrounding medium. The systems for protein export are referred to as the type I–IV secretion systems. Two of these systems, types II and IV, utilize the previously mentioned Sec machinery. The other two are Sec-independent. These systems are distinct from those that only transport proteins across the cytoplasmic membrane because they also must include pores for transport across the cell wall.

The *E. coli* hemeolysin is an example of a protein transported by a type I secretion system. A unique characteristic of these systems is that transport across the cytoplasmic membrane is driven by an ABC transporter. ABC transporters are a superfamily of proteins that contain an ATP-binding cassette (hence ABC transporter). The type II system is best represented by the pullunase secretion system of *Klebsiella oxytoca*. This system utilizes the Sec system to target proteins but includes additional proteins for transport across the outer membrane. The type III systems have been heavily studied because of their role in pathogenesis. The system used by *Yersinia* species to export a set of proteins known as Yop proteins is a good example of the type III system (Cornelis and Wolf-Watz 1997). Interestingly, the system used to export flagellar proteins is similar to the type III system. It is possible that the type III system may have evolved from the flagella export system. The type IV pathway is not as well studied but appears to include proteins that use the Sec system to cross the cytoplasmic membrane but do not rely on proteins of the type II system to cross the outer membrane.

The regulation of cell division

Cell division requires that cells reach a certain critical mass before division begins. To complete cell division the cell must replicate its chromosome, segregate the chromosome into opposite halves of the cell, and then divide. Replication of the *E. coli* chromosome was discussed above. The mechanisms used to segregate the replicated chromosomes are not yet understood. Once this has been achieved, however, the cell can form a septum at the site where division will occur. Formation of the septum involves the recruitment of certain proteins to the approximate middle of the cell to form a ring. In *E. coli* these ring-forming proteins include MinE and FtsZ. The protein MinC is also required to keep ring formation from occurring at inappropriate sites. The septal rings must then contract to allow two separate cells to form. The process of ring formation and septation is very complex but a number of the proteins responsible for this process have been identified and their roles elucidated (Rothfield et al. 1999).

TRANSPORT OF SOLUTES INTO THE CELL

Simple and facilitated diffusion

Most small solutes can diffuse through the peptidoglycan material and many hydrophilic compounds can pass through the porins of the outer membrane of gram-negative bacteria. However, the cytoplasmic membrane of both gram-negative and gram-positive bacteria is a barrier to hydrophilic compounds, and special transport machinery must be utilized to allow nutrients to pass through this membrane fast enough to allow competitive growth rates. If a compound moves through the cytoplasmic membrane and into the cytoplasm without utilizing the transport machinery this is termed simple or passive diffusion. For most compounds, with the exception of certain hydrophobic compounds, entry via diffusion will be very slow. The rate of entry of a diffusible compound into the cytoplasm by simple diffusion is typically dependent upon its concentration in the medium. Because most compounds are present at extremely low concentrations in the environment, diffusion cannot be used for the uptake of most nutrients required for growth.

One example of diffusion in bacteria is the uptake of glycerol by facilitated diffusion. Facilitated diffusion results when the cell possesses a protein (the facilitator protein) that aids or facilitates the transport of a compound through the membrane. This facilitator protein permits the compound to be transferred down a concentration gradient from one side of the membrane to the other. The process is not directly coupled to metabolic energy and the compound cannot be accumulated against a concentration gradient but it may be altered and trapped to prevent it from leaving once it enters the cell. Once inside the bacterial cell, glycerol is phosphorylated by glycerol kinase and glycerol phosphate can accumulate within the cells. The phosphorylation is essential in order to trap glycerol in the cells, and mutants lacking glycerol kinase are not able to accumulate glycerol. Another example of a facilitator protein is provided by AqpZ, the water channel of *E. coli* (Calamita et al. 1995). This protein is related to other water channels in plants and animals as well as to the glycerol facilitator. Inactivation of aqpZ is not lethal to cells but

does limit cell growth under conditions of low osmolality (Calamita et al. 1998).

Active transport systems

Most solutes are brought into the cell by means of active transport systems that involve the use of metabolic energy. Because energy is used, these active transport systems can accumulate the compound within the cell to a concentration 1000 times greater than the concentration in the surrounding medium, greatly increasing the growth rate of the cell when nutrients are limited.

Substrates can be transported in various bacteria by several different active transport systems. Included among these are the ABC-type carriers. These are also known as periplasmic-binding protein-dependent transport systems or osmotic shock-sensitive transport systems. Examples of another type of active transport system are the chemiosmotic-driven, ion-gradient-linked permeases that use proton- or sodium-motive force to transport nutrients into the cell. The ATP and the proton-motive force systems can be differentiated by the use of inhibitors that prevent ATP from accumulating or by using proton ionophores, such as dinitrophenol (DNP), that collapse the proton gradient. A third type of transport, primarily associated with fermenting bacteria, is the phosphoenolpyruvate phosphotransferase system (PTS), where the compound is phosphorylated during transport through the cytoplasmic membrane.

ATP-BINDING CASSETTE, PERIPLASMIC-BINDING PROTEIN-DEPENDENT TRANSPORT SYSTEMS

These transport systems in gram-negative bacteria are composed of a substrate-binding receptor protein associated with the cell periplasm plus several membrane-bound components. This has led these types of transport systems to be referred to as osmotic shock-sensitive permeases, because a cold osmotic shock causes the loss of the substrate-binding protein, which is loosely attached in the periplasmic space. In gram-positive bacteria, the substrate-binding receptor protein is represented by a lipoprotein fastened to the membrane.

A likely scenario for transport is that substrate binds to the periplasmic-binding protein, which then interacts with the membrane-associated transport complex. This causes the membrane-associated subunits to undergo conformational changes, which, in turn, cause conformational changes in the ATP-binding subunit, which is located on the inner surface of the cytoplasmic membrane. These changes cause ATP hydrolysis, which is used to drive transport. Substrates reported to be transported by this system in *E. coli* include carbohydrates such as L-arabinose, maltose, D-ribose, D-xylose, succinate, and malate, and amino acids such as leucine, isoleucine, valine, histidine, lysine, and ornithine.

A good example of this type of transport system is provided by the histidine permease system of *Salmonella typhimurium*. This system consists of a periplasmic-binding protein, HisJ and a membrane-associated complex of HisQMP2 (Liu and Ames 1998). HisQ and HisM are the membrane-spanning subunits and HisP is the ATP-binding subunit. The high-resolution structure of the HisP subunit has been solved and has provided insight into the functional characteristics of this large family of proteins (Hung et al. 1998).

ION GRADIENT-LINKED OR CHEMIOSMOTIC-DRIVEN TRANSPORT SYSTEMS

Chemiosmotic-driven transport systems use an ion gradient, such as proton- or sodium-motive force, as the energy to drive the transport of the substrate through the membrane. The use of an ion gradient distinguishes them from the binding protein-dependent systems that use the energy of ATP directly. The binding protein-dependent systems are termed primary transport because they are driven by an energy-producing event. The ion-gradient transport systems are termed secondary transport systems because they utilize gradients that typically have been formed by an energy-producing event.

The three basic types of ion-gradient transport are symport, antiport, and uniport. With symport, a single carrier simultaneously transports two substances in the same direction. Antiport involves a common carrier transporting two substances at the same time but in opposite directions. Uniport is the situation where movement is independent of any coupled ion.

Electrochemical ion-gradient permeases are usually composed of a single, very hydrophobic membrane protein. Probably the best studied protein of this type is LacY, the lactose transporter of *E. coli*. Many of the residues in this protein have been mutagenized and only a few have been shown to be essential for transport (Kaback and Wu 1997). Cys-scanning mutagenesis has also been used to provide a better understanding of interactions among the various domains of the protein and to probe structural changes during transport. However, detailed insight into the catalytic mechanism is still lacking at this point.

In some of the thermophilic *Bacillus*, such as *B. stearothermophilus*, Na^+ is used for the coupling ion for amino-acid transport. The proton-motive force as well as an inward-directed Na^+ gradient drives the transport of glutamate and aspartate. Sodium/proton glutamate transporters have been found in a number of thermophilic *Bacillus* species. However, in *Bacillus subtilis*, glutamate uptake was coupled to proton-motive force (Tolner et al. 1995). In *E. coli* and *S. typhimurium*, carbohydrate–sodium cotransport systems have been reported for melibose and proline. Carbohydrate–sodium cotransport systems are common in halophilic bacteria.

THE PHOSPHOTRANSFERASE SYSTEM

PTS uses the phosphate from phosphoenolpyruvate (PEP) for the transport and phosphorylation of incoming sugars. The phosphate group is first transferred from PEP to a nonspecific enzyme component termed enzyme I or EI. The phosphorylated EI (P-EI) transfers the phosphate to histidine residue 15 on a small, heat-stable protein, designated HPr. The phosphate is then transferred from phospho-HPr to a sugar-specific, membrane-bound enzyme II (EII) complex, which catalyzes the transport of the carbohydrate through the membrane as well as its phosphorylation.

The EII complex consists of domains termed IIA, IIB, IIC (and sometimes IID) that may exist as separate proteins or components of the same protein. The IIA and IIB protein domains are associated with the cytoplasmic side of the membrane and are involved in phosphate transfer. If these domains are located in separate proteins, the IIA domain will have the first phosphorylation site and the IIB domain will have the second phosphorylation site. The IIC protein/domain is located in the cytoplasmic membrane, possesses the carbohydrate-binding site, and is responsible for the transport of the carbohydrate through the membrane. The overall effect of the PTS is to both phosphorylate the carbohydrate and transport it into the cytoplasm of the cell.

The EI and HPr components are the nonspecific portions of the PTS system. The other, carbohydrate-specific components of the PTS transport system can be indicated by placing the name of the transported sugar after the enzyme designation. The EII protein/domain that is specific for the transport of D-glucose into the cells as glucose-6-phosphate can be designated as enzyme $EII^{Glucose}$ or II^{Glc}. Some EII PTS transport systems may have activity upon more than one carbohydrate; for example, *E. coli* enzyme II^{Man} has activity for the transport and phosphorylation of glucose, mannose, 2-deoxyglucose, glucosamine, and fructose, in that order (Saier and Reizer 1994).

Because the PTS transport system requires phosphoenolpyruvate as the initial phosphate donor it tends to be found in facultative or anaerobic bacteria that utilize the hexose diphosphate pathway (see below) and produce two PEP molecules for each hexose catabolized. PTS is common in such bacteria as *E. coli* and other enteric bacteria, *Bacillus*, *Clostridium*, *Bacteriodes*, *Staphylococcus*, *Lactobacillus*, and *Streptococcus*. It is not common in aerobic bacteria employing other catabolic pathways, such as *Arthrobacter*, *Azotobacter*, *Micrococcus*, *Pseudomonas*, *Nocardia*, or *Caulobacter*. In *E. coli*, the PTS transport system is used for the transport of such carbohydrates as mannitol, sorbitol, galactitol, glucose, glucose-mannose, fructose, and N-acetylglucosamine. Many gram-positive bacteria transport lactose, sucrose, maltose, and/or pentitols via the PTS. Alternative, non-PTS transport systems for glucose may also be present (Cvitkovitch et al. 1995).

The role of the PTS system in catabolite regulation

When presented with multiple carbon sources, most bacteria will preferentially utilize one of those carbon sources to the exclusion of other available substrates. In *E. coli* the preferred carbon source is glucose. This regulation of carbon metabolism is referred to as glucose repression or catabolite repression. One of the principal proteins involved in regulation of catabolite repression is IIA^{Glc}. When glucose and other sugars transported by the PTS are present, the HPr protein is rapidly being dephosphorylated as glucose is phosphorylated. Nonphosphorylated IIA^{Glc} will interact with, and inhibit the function of, enzymes required for metabolism of other compounds. This phenomenon is known as inducer exclusion. Examples of substrate whose metabolism is affected by inducer exclusion are maltose, lactose, melibiose, and glycerol. There are other causes of catabolite repression besides inducer exclusion. One of the main ways of achieving catabolite repression is via the small molecule, cyclic AMP (cAMP). This regulatory process is discussed below.

The HPr of *Streptococcus pyogenes* can also be phosphorylated on a serine residue (Ser-46) by a specific HPr kinase with ATP as the phosphate donor. This reaction has been found to be widespread among gram-positive bacteria but not gram-negative bacteria and even has been shown to occur in some species lacking a functional PTS. Phosphorylated HPr has some regulatory roles, regulating glucose and lactose permease activity in *Lactobacillus brevis* and inducer exclusion in *Lactococcus lactis*, and is involved in catabolite repression in *B. subtilis* (Thevenot et al. 1995).

BACTERIAL CATABOLIC PATHWAYS

The hexose diphosphate pathway

A very efficient pathway for the production of ATP during the catabolism of D-glucose and similar carbohydrates is often referred to as the EMP pathway to honor three scientists who contributed greatly to its elucidation, Embden, Meyerhof, and Parnas. One of the unique things about this pathway is the formation of a hexose diphosphate as an intermediate. Therefore, an alternative and descriptive name is the hexose diphosphate or HDP pathway (Figure 4).

THE PHOSPHORYLATION OF GLUCOSE

The catabolism of glucose by the HDP pathways begins by phosphorylation at the 6-carbon position. Various mechanisms have been reported for this reaction. If glucose is transported into the cells via the PTS, this

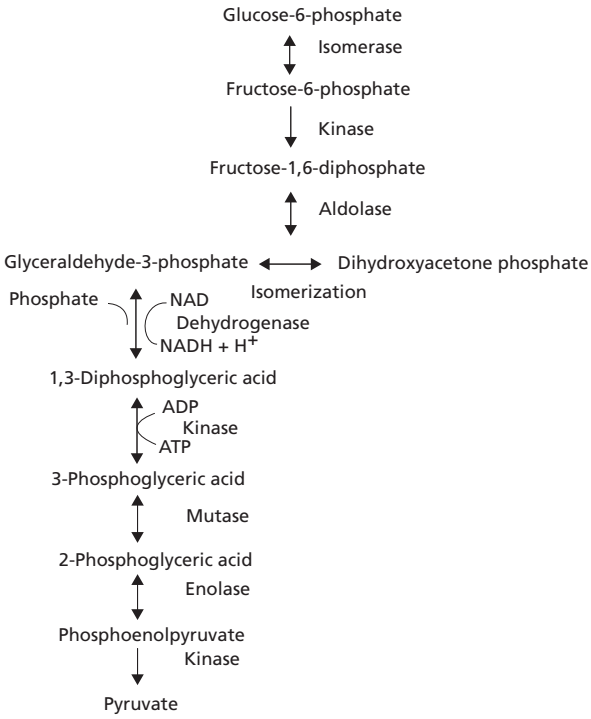

Figure 4 *The hexose diphosphate (HDP) or Embden, Meyerhof, and Parnas (EMP) pathway. Glucose-6-phosphate is converted to fructose-1,6-diphosphate, which is cleaved to produce the 3-carbon triose phosphates. Glyceraldehyde-3-phosphate is oxidized and the product of the oxidation converted to pyruvate.*

efficient transport system will convert glucose to the 6-phosphate upon passage through the cell membrane. If glucose enters the cell by a different transport system or is produced within the cell, it must be phosphorylated by other means. Hexokinase is a nonspecific kinase found in a variety of microorganisms. It can catalyze the phosphorylation of hexoses such as D-glucose, D-fructose, D-mannose, and D-glucosamine to the 6-phosphate, with ATP as the phosphate donor. Some bacteria, such as those in the genera *Staphylococcus*, *Streptococcus*, *Clostridium*, and *Lactobacillus*, possess a glucokinase that is specific for the phosphorylation of glucose to glucose-6-phosphate. At least some bacteria in the genus *Propionibacterium* can phosphorylate glucose using inorganic pyrophosphate as the phosphate donor. Transfer enzymes have been reported, which can transfer the phosphate group from a different hexose phosphate to glucose.

CONVERSION OF GLUCOSE-6-PHOSPHATE TO GLYCERALDEHYDE-3-PHOSPHATE

The reactions of the HDP pathway resulting in the conversion of glucose-6-phosphate to the two triose phosphates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, are illustrated in Figure 4. The first step is the isomerization of glucose-6-phosphate to the 2-ketosugar, fructose-6-phosphate. The enzyme catalyzing the reaction is glucose-6-phosphate (fructose-6-phosphate) isomerase. The equilibrium of this reaction is towards glucose-6-phosphate. However, the equilibrium of the next reaction, the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, is strongly towards product formation. The phosphorylating enzyme is phosphofructokinase (Pfk) and ATP normally is the phosphate donor. Two isozymes for Pfk have been found in *E. coli*. Most (90 percent) of the activity is provided by Pfk-1, an enzyme that is activated by nucleotide diphosphates such as ADP and inhibited by PEP. However, excess ATP and PEP are negative effectors of the enzyme and inhibit activity. Thus, the activity of the enzyme (and the HDP pathway) will be inhibited in cells with high energy charges (high levels of nucleotide triphosphates). The second enzyme, Pfk-2, is inhibited by ATP and the product of the reaction, fructose-1,6-bisphosphate.

Aldolase catalyzes the cleavage of fructose-1,6-bisphosphate to the two triose phosphates, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, as shown in Figure 4. The equilibrium of the reaction is back towards fructose-1,6 bisphosphate so that an accumulation of triose phosphates will lead to an accumulation of fructose-1,6 bisphosphate. The accumulation of this hexose diphosphate often serves as a regulatory signal that the oxidation of glyceraldehyde-3-phosphate is not functioning properly. Dihydroxyacetone phosphate is converted to glyceraldehyde-3-phosphate by the enzyme triosphosphate isomerase.

CONVERSION OF GLYCERALDEHYDE-3-PHOSPHATE TO PYRUVATE

The next reaction of the hexose diphosphate pathway involves the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid. This oxidation is catalyzed by glyceraldehyde-3-phosphate dehydrogenase. The 1,3-diphosphoglyceric acid contains energy in an energy-rich bond between phosphoglyceric acid and phosphate. This reaction is reversible and nicotinamide adenine dinucleotide (NAD) is usually (not always) the electron acceptor. Inorganic phosphate is the phosphate donor.

ATP-phosphoglyceric transphosphorylase, also known as phosphoglycerate kinase, catalyzes the reversible transfer of the energy-rich phosphate at the one carbon of 1,3-diphosphoglyceric acid to ADP, to form ATP and 3-phosphoglyceric acid. Phosphate esterification in this manner, with the phosphate group being transferred to ADP to form ATP, is termed substrate-level phosphorylation. Another enzyme, phosphoglycerate mutase, catalyzes the transfer of phosphate from the 3 to the 2 position of glyceric acid, producing 2-phosphoglyceric acid. Then 2-phosphoglyceric acid dehydrase, also known as enolase, removes water from 2-phosphoglycerate to form PEP, which has the highest group-transfer potential of any intermediate produced by the HDP pathway. Pyruvate kinase transfers the phosphate to ADP to form ATP and pyruvic acid.

Two isozymes of pyruvate kinase have been reported in *E. coli*. They are Pyk-F, activated by fructose 1,6-bisphosphate, and Pyk-A, activated by AMP. Mutants lacking one of these two enzymes can still grow on glucose but mutants lacking both enzymes grow poorly on glucose, except aerobically on substrates transported into the cells by the PTS (Ponce et al. 1995). In the latter case, pyruvate is generated from PEP during substrate transport. The net gain of energy from the HDP pathway is two ATP for each glucose catabolized. This pathway is an efficient pathway for the fermentation of hexoses and is common in fermenting bacteria.

THE PYRUVATE DEHYDROGENASE COMPLEX

The pyruvate dehydrogenase complex (PDH) is a multienzyme complex that catalyzes the decarboxylation of pyruvic acid and an acetyl transfer from pyruvate to coenzyme A. The complex has a structural core of dihydrolipoate transacetylase (E2) components surrounded by independently bound pyruvate dehydrogenase (E1) and dihydrolipoate dehydrogenase (E3). The cofactor requirements of the complex include lipoic acid (dithiooctanoic acid), coenzyme A, NAD, flavine adenine dinucleotide (FAD), and thiamine pyrophosphate (TPP). The products of the reaction of the complex are CO_2, acetyl-CoA, and NADH.

The complex isolated from *E. coli* possesses a chain ratio of 1.0E1/1.0E2/0.5E3 with a core of 24 E2 chains. The genes encoding for the E1, E2, and E3 enzymes (*aceE*, *aceF*, and *ipdA*) are part of the same operon. The E3, the *ipd* gene product, is also a component of the α-ketoglutarate dehydrogenase complex of the tricarboxylic acid (TCA) cycle. The E1 and E3 subunits are assembled around an icosahedral core composed of E2 polypeptides and form the complex with its molecular size of about 10 MDa (Hiromasa et al. 1994).

This pyruvate dehydrogenase from *E. coli* is stimulated by HDP intermediates such as fructose-6-phosphate. It is inhibited by a high-adenylate energy charge but such inhibition is overcome by fructose-1,6-bisphosphate. The ratio of NADH/NAD seems to influence the flow of pyruvate between pyruvate–formate lyase and the PDH complex. The activity of the complex increases with increased NAD^+ concentration within the cells but is inhibited when intracellular NADH levels are high, such high levels indicating a problem in the disposal of electrons (Snoep et al. 1991). For these reasons, utilization of the system tends to increase under aerobic conditions or when the oxidation of NADH is not a problem for the cells.

The tricarboxylic acid cycle or citric acid cycle

The TCA cycle, also known as the citric acid cycle or the Krebs cycle, is a means of oxidizing acetate (as acetyl-CoA produced by the HDP pathway) to CO_2 and electrons. The TCA cycle reactions have both anabolic and catabolic functions in many bacteria. The acetyl group from acetyl-CoA is transferred by citrate synthase to the α-keto, 4-carbon dicarboxylic acid, oxaloacetate, to form citric acid. The methyl carbon of the acetyl moiety is attached to the 2 or α-carbon of the oxaloacetate with the keto-group of oxaloacetate becoming a hydroxyl group. This hydroxyl group is transferred to a different carbon by isocitrate isomerase (aconitase) with the formation of isocitric acid. This hydroxyl group is then oxidized to an α-keto group by isocitrate dehydrogenase with the formation of oxalosuccinic acid. Oxalosuccinic acid is further oxidized with decarboxylation by isocitrate dehydrogenase to form α-ketoglutaric acid, a compound that is important in anabolic pathways. Reactions of the TCA cycle are shown in Figure 5.

In the catabolic TCA cycle, α-ketoglutaric acid is oxidized and decarboxylated by an α-ketoglutarate dehydrogenase enzyme complex, involving coenzyme A and lipoic acid. This complex is similar to the pyruvate dehydrogenase complex described previously, with CO_2 produced and a lipoic succinyl intermediate formed. The succinyl group is transferred to coenzyme A to give succinyl-CoA, which can be converted to succinic acid with the formation of a high-energy bond (ATP) in a manner similar to the formation of acetate from acetyl-CoA. Succinic acid is then oxidized to fumarate by succinate dehydrogenase with the reduction of a FAD^+ to $FADH_2$.

Succinate dehydrogenase (SDA) is a membrane-bound enzyme that is part of the respiratory chain and appears to be present in all aerobic cells. It is composed of two subunits, a flavoprotein (FP) and an iron sulfur

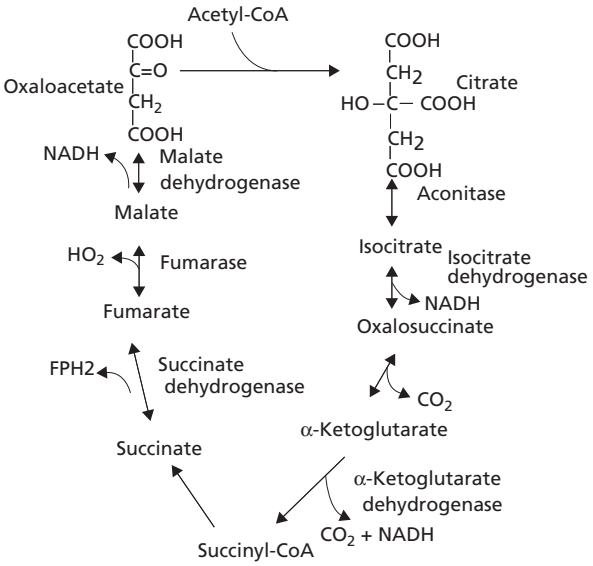

Figure 5 *The tricarboxylic acid cycle, showing the reactions resulting in the oxidation of acetate to carbon dioxide*

protein and, in prokaryotic cells, SDH is fastened to the inner side of the cytoplasmic membrane by a cytochrome. While the high-resolution structure of SDH has not been determined, the structure of fumarate reductase, which catalyzes the reverse reaction and has extensive structural and sequence similarity to SDH, has been determined (Iverson et al. 1999). Fumarate is hydrated by malate dehydrase (fumarase) to form malate, malate is oxidized by malate dehydrogenase to produce oxaloacetic acid, and the cycle is complete. For each acetate oxidized, provided as acetyl-CoA, two molecules of CO_2 and eight pairs of electrons are obtained. Two of these electrons are transported to FAD^+ and six to NAD^+. An ATP can be obtained from the succinyl-CoA.

Succinate and oxaloacetate, as well as α-ketoglutarate, are important compounds in biosynthetic reactions. Even anaerobic bacteria, which do not utilize the TCA cycle for complete oxidation of acetate, may need TCA-cycle enzymes for biosynthetic reactions. However, it would not be efficient for anaerobic bacteria to oxidize acetate completely because they would have difficulty in disposing of the electrons. Such bacteria often lack the α-ketoglutarate enzyme complex. This complex, usually missing in anaerobes, is known as the 'anaerobic lesion' of the TCA cycle.

Growth on acetate as a carbon source

E. coli has two pathways that can be used to convert acetate to acetyl-CoA. One pathway uses acetate kinase and ATP to produce acetyl phosphate, then phosphotransacetylase transfers the acetyl moiety from acetyl phosphate to CoA to form acetyl-CoA. However, this route is believed to function primarily in a catabolic role, generating ATP when acetate is being excreted. This system would be used to activate acetate only when acetate was present in large amounts outside the cells. The second pathway is believed to function by using extracellular acetate that is present in low concentrations. It uses the enzyme acetyl-CoA synthetase to convert acetate and ATP to acetyl-AMP and inorganic pyrophosphate. The acetyl-AMP reacts with CoA to release inorganic phosphate and form acetyl-CoA. Although wild-type *E. coli* cells grow well on acetate at concentrations ranging from 2.5 to 50 mM, mutants lacking acetyl-CoA synthetase grow poorly on low acetate concentrations. Mutants lacking acetate kinase and phosphotransacetylase grow poorly on high concentrations of acetate, and mutants lacking all three enzymes did not grow on acetate at any concentration tested (Kumari et al. 1995).

When growing on acetate as the sole carbon source, bacteria must have the enzyme capability to reverse the HDP pathway and form pentoses and hexoses from these simple 2- and 3-carbon compounds. While some strict anaerobes are able to reverse the pyruvate:ferredoxin oxidoreductase reaction to form pyruvate from CO_2 and acetate, most bacteria that can grow with only acetate as the carbon source incorporate it into TCA-cycle intermediates (see Figure 5) by a series of reactions known as the glyoxylate bypass or glyoxylate cycle. During the glyoxylate cycle the acetyl-CoA produced by acetyl-CoA synthetase is combined with a molecule of existing oxaloacetic acid to form citrate and the citrate is isomerized to isocitrate by normal TCA cycle enzymes. A unique enzyme, isocitrate lyase, cleaves the isocitrate to produce succinate and glyoxylate. The glyoxylate is condensed with acetyl-CoA by the enzyme malate synthase to form malate, as shown in Figure 6. In this manner, one oxaloacetate and two acetyl groups have been converted to malate and succinate. These latter two compounds can be oxidized and converted to two oxaloacetates so the overall series of reactions form two oxaloacetates from one oxaloacetate and two acetates. Oxaloacetate can be used to form other TCA intermediates for biosynthetic purposes or can be decarboxylated to PEP by the enzyme phosphoenolpyruvate carboxykinase.

The genes coding for isocitrate lyase and malate synthase are located in the same operon in *E. coli* and the synthesis of the enzymes is regulated so they are formed when two carbon intermediates are the sole source of carbon. The enzymes of the bypass are induced by acetate and repressed by most other carbon compounds. In *E. coli* the flow of isocitrate through the glyoxylate bypass, rather than the TCA cycle, is controlled by activation and deactivation of isocitrate dehydrogenase. When bacteria are grown on glucose, isocitrate dehydrogenase is fully operational, but when cells are grown on acetate, the activity of the enzyme is decreased by 70 percent (Chung et al. 1988). This decrease is the result of the enzyme being phosphorylated at its active site by isocitrate dehydrogenase kinase/phosphatase (Hurley et al. 1990). The gene encoding the kinase/phosphatase is in the same operon

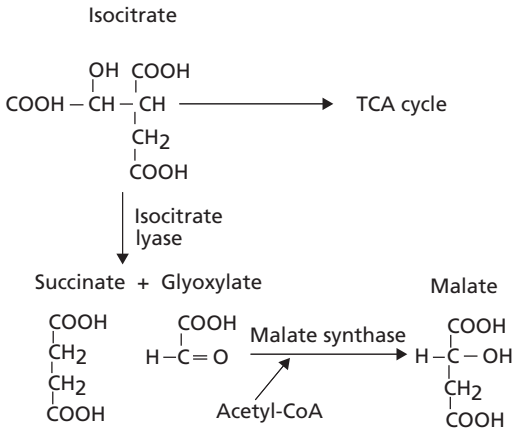

Figure 6 *Reactions of the glyoxylate bypass. Oxaloacetate and acetate are converted to citrate and then isocitrate. The isocitrate is cleaved to succinate and glyoxylate and the glyoxylate is combined with another acetate to yield malate.*

as the genes encoding isocitrate lyase and malate synthase. Some strains of *E. coli* cannot grow on acetate without the expression of this regulatory enzyme (Cortay et al. 1988).

If pyruvate or lactate are substrates, *E. coli* can use the enzyme phosphoenolpyruvate synthase and ATP to convert pyruvate to PEP, with AMP and inorganic phosphate as products. The HDP pathway can then be reversed (glucogenesis) to fructose-1,6-bisphosphate and a phosphatase can cleave phosphate from the 1-carbon position to yield fructose-6-phosphate. The PEP also can be combined with CO_2 (phosphoenolpyruvate carboxylase) to form oxaloacetate, which can be converted to other TCA-cycle intermediates.

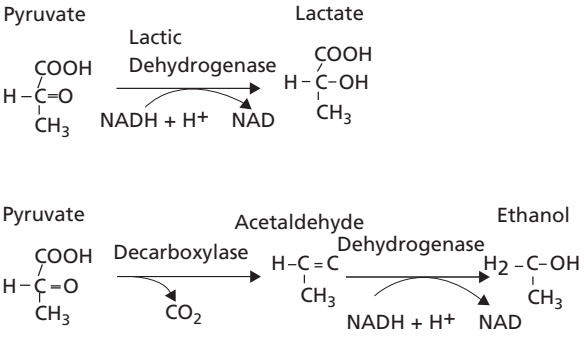

Figure 7 *Formation of lactate and ethanol from pyruvate. Pyruvate can be directly reduced to lactate or decarboxylated to acetaldehyde, which can be reduced to ethanol.*

The glucose fermentations

In microbial physiology and metabolism the term 'fermentation' has a very specific meaning, i.e. the condition where the final electron acceptor of a catabolic pathway is an organic compound. An example of a process where this is important is in the oxidation of reduced NAD^+ produced by the HDP pathway. The NAD^+ reduced by the oxidation of glyceraldehyde-3-phosphate during the HDP pathway must be reoxidized if the pathway is to continue functioning. Therefore, fermentation is a process that can keep the HDP pathway functioning if no other electron acceptors are available. It should be pointed out that in industry the term 'fermentation' is used to denote many metabolic reactions caused by microorganisms, not just the specific definition described above.

THE HOMOLACTIC ACID FERMENTATION

Pyruvate, produced as a result of the HDP pathway, is an excellent acceptor for the electrons produced by the oxidation of glyceraldehyde-3-phosphate and most bacteria possess the metabolic capability of reducing pyruvate to lactic acid (see Figure 7). For some bacteria the reduction of pyruvate to lactate is the most important method of reoxidizing NADH and such bacteria produce large amounts of lactic acid as final products of glucose fermentation. For this reason, such bacteria are termed 'lactic-acid bacteria.' If glucose is the only carbon source provided to the cells, some of the products of glucose degradation by the HDP must be used for biosynthetic purposes to make new cell material. However, because the energy gained as a result of fermentation is relatively low, most of the carbon from the glucose must be converted to fermentation products. The term 'homolactic acid fermentation' means that significantly more than half of the carbon from the glucose fermented is in the form of lactic acid. It is apparent that any bacterium that carries out a homolactic acid fermentation must be able to survive in the presence of a large amount of lactic acid and at low pH.

Lactic acid is the major product of the homolactic acid fermentation but other products may be formed in lesser amounts. Many homolactic-acid bacteria produce such fermentation products as acetate, ethanol, and formate, in addition to lactate. The conversion of pyruvate to formic acid, ethanol, and acetic acid rather than lactose actually increases the energy gained by the fermentation, but it also increases the amount of acid formed. Regulatory systems of homolactic-acid bacteria tend to shift the fermentation to larger amounts of these compounds at higher pH values, where acid production would be less likely to inhibit growth.

The purification of lactic acid dehydrogenase from a homolactic-acid bacterium led to the discovery that this enzyme was an allosteric enzyme with an absolute requirement for a positive effector. If the reduction of pyruvate to lactic acid does not proceed efficiently enough to reoxidize the NADH formed from the oxidation of glyceraldehyde-3-phosphate, the oxidation of the triose phosphate is inhibited. Because of the reversibility of the aldolase-catalyzed reaction, the internal pool of the positive effector of lactic acid dehydrogenase, fructose-1,6-bisphosphate, increases. This results in the activation of more molecules of lactic acid dehydrogenase and the more rapid reduction of pyruvate.

THE DIRECT ALCOHOL FERMENTATION

The direct alcohol fermentation or 'yeast type' of alcohol fermentation permits the oxidation of NADH from the HDP pathway without the formation of large amounts of lactic acid. Pyruvate is directly decarboxylated by pyruvate decarboxylase to yield acetaldehyde and CO_2. The enzyme alcohol dehydrogenase catalyzes the reduction of acetaldehyde to ethyl alcohol with electrons from NADH (see Figure 7). This mechanism of ethanol formation is common in yeast but, although many bacteria are capable of ethanol production, only a few do so by this pathway. Some of those bacteria using this yeast type of pathway for ethanol formation include those in the genera *Zymomonas*, *Acetomonas*, and *Sarcina ventriculi*.

THE FORMIC ACID FERMENTATION

Some bacteria have evolved the ability to obtain extra energy from pyruvate. In one mechanism for pyruvate fermentation, the enzyme pyruvate formate–lyase cleaves pyruvate to formic acid and a 2-carbon fragment that becomes acetyl-CoA. This enzyme is present in either an active or inactive state, with the active state having a free radical at its C-terminus (Sawers and Watson 1998). The cleavage of *S*-adenosylmethionine into methionine and 5′-deoxyadenosine is required for enzyme activation. The active form of the enzyme normally functions only under anaerobic conditions and is rapidly and irreversibly inactivated by oxygen (Sawer and Bock 1988). This enzyme is common in enteric bacteria and also accounts for the formic acid produced during some homolactic acid fermentations.

When pyruvate formate–lyase is active the NADH produced by the HDP pathway cannot be reoxidized by lactic-acid production. Instead, some of the acetyl group on the acetyl-CoA is reduced twice, first to acetaldehyde and then to ethanol. This reduction is catalyzed by a special alcohol dehydrogenase, a CoA-linked alcohol dehydrogenase. The energy of the acetyl-CoA bond is sacrificed in the reaction but two NADH can be oxidized for each ethanol produced. Facultative bacteria, such as *E. coli*, have a single large polypeptide responsible for both reductions. The *adhE* gene of *E. coli*, K-12, encodes a polypeptide of 891 amino acids, equivalent in size to a combined alcohol dehydrogenase and an acetaldehyde dehydrogenase, and may represent an evolutionary fusion product of separate genes. Some obligate anaerobes, such as *Clostridium*, have two proteins to catalyze the reduction of acetyl-CoA to ethanol, a CoA-linked acetaldehyde dehydrogenase and an alcohol dehydrogenase. These two separate polypeptides form a complex so that free acetaldehyde is not released.

The acetyl-CoA that is not needed for the oxidation of NADH can be used for the generation of additional ATP. The enzyme phosphotransacetylase catalyses the reversible transfer of acetate from acetyl-CoA to phosphate with the formation of acetyl phosphate. The enzyme acetate kinase, in a reaction that is also reversible, transfers the phosphate from acetyl phosphate to ADP, to form ATP and liberate acetate. Therefore, two pyruvate and two NADH can result in two formate, one ethanol and one acetate, with the gain of one ATP. This fermentation is also known as the mixed-acid fermentation and the metabolic reactions leading to these products are illustrated in Figure 8.

Mutants of *E. coli* lacking lactic dehydrogenase grow normally under anaerobic conditions, but if these mutants also lack pyruvate formate–lyase they cannot grow by fermenting glucose or other sugars (Mat-Jan et al. 1989). If mutants of *E. coli* lack the ability to produce ethanol from acetyl-CoA, they cannot grow anaerobically on glucose unless provided with some alternative electron acceptor such as nitrate. However, an additional mutation also eliminating the pyruvate formate–lyase enzyme will restore the ability to grow on glucose anaerobically, with the cells now producing lactic acid.

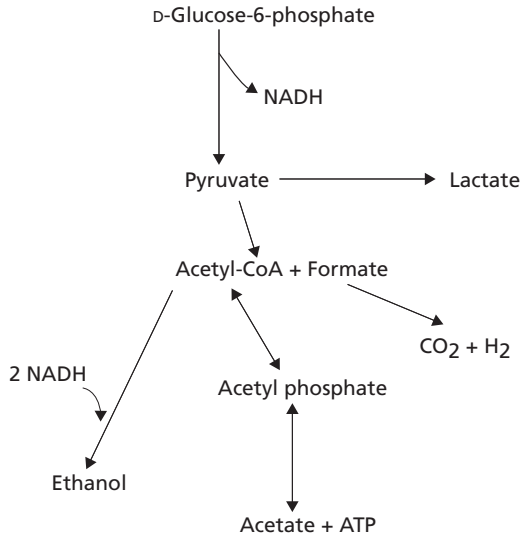

Figure 8 *Possible products of the formic-acid or mixed-acid fermentation. Pyruvate can be cleaved to yield acetyl-coenzyme A (acetyl-CoA) and formate. The acetyl-CoA can be converted to acetate, yielding a high-energy phosphate bond, or reduced to ethanol. If the proper enzymes are present, the formate can be oxidized to yield carbon dioxide and hydrogen gases.*

BUTANEDIOL FORMATION

Some bacteria, for example some bacilli, have developed a pathway for the degradation of pyruvate that produces less acid. An α-acetolactate-synthesizing enzyme with a pH optimum at 6.0 converts two pyruvates to α-acetolactate and CO_2. This enzyme is sometimes named the pH 6.0 α-acetolactate-synthesizing enzyme to distinguish it from a biosynthetic enzyme that carries out the same reaction but possesses a pH optimum of 8.0. During the fermentative pathway the α-acetolactate is decarboxylated by α-acetolactate decarboxylase to produce another CO_2 and acetoin. The acetoin produced can be reduced to 2,3-butanediol by the action of butanediol dehydrogenase and the oxidation of NADH. This system of enzymes appears to be induced by acetate and at high pH values it is inhibited from operating (Zeng and Deckwer 1991). Because this pathway utilizes two pyruvate and results in the oxidation of only one NADH, it cannot be the sole fermentation route of glucose by a microorganism. The advantage of this pathway is the production of neutral products.

BUTYRATE AND BUTANOL FERMENTATION

In many strictly anaerobic bacteria and archaea, the oxidation and decarboxylation of pyruvate are catalyzed

by pyruvate:ferredoxin oxidoreductase, an enzyme that catalyzes the oxidation of pyruvate to form acetyl-CoA and CO_2. The electrons are placed on the electron carrier ferredoxin, which may donate them to a hydrogenase with the eventual formation of hydrogen (H_2) gas. The class of electron carriers known as ferredoxins comprises small (6–13 kDa), iron–sulfur ([Fe-S]) proteins that function in a variety of reactions, including nitrogen fixation and photosynthesis.

The acetyl group from the acetyl-CoA obtained by the action of pyruvate:ferredoxin oxidoreductase can be used for the production of acetate and ATP, via phosphotransacetylase and acetate kinase, or utilized to generate electron acceptors for the NADH formed by the HDP pathway. In some bacteria, such as those of the genus *Clostridium*, an enzyme named acetyl coenzyme A acetyl transferase condenses two molecules of acetyl-CoA to produce one molecule of acetoacetyl-CoA. This latter compound can be reduced by β-hydroxybutyryl coenzyme A dehydrogenase and NADH to produce β-hydroxybutyryl-CoA. This latter compound can be dehydrated by 3-hydroxyacyl coenzyme A hydrolase (crotonase) to form crotonyl-CoA. The double bond in crotonyl-CoA is further reduced by butyl coenzyme A dehydrogenase to butyryl coenzyme A with electrons again coming from NADH. Thus, the overall series of reactions decarboxylates two pyruvates, with the acetyl groups becoming condensed into a four-carbon compound that undergoes two reduction steps to form butyryl-CoA.

The butyryl-CoA can have different fates. Acetyl transferase may transfer a free acetate on to the CoA group, producing acetyl-CoA and yielding free butyric acid. A second possibility is for phosphotransbutyrylase to transfer the butyryl group to phosphate to form butyryl phosphate. The phosphate can be donated to ADP to form ATP. Some clostridia produce free acetoacetate from acetoacetyl-CoA by replacing the acetoacetate with acetate. The free acetoacetate can be decarboxylated by acetoacetyl decarboxylase, producing acetone and CO_2, and the acetone may be reduced by isopropyl dehydrogenase yielding isopropyl alcohol.

The propionic acid fermentations

Some bacteria, for example those in the genera *Propionibacterium*, *Veillonella*, and *Bacteriodes*, produce propionic acid as a fermentation product. These bacteria can ferment lactic acid because reducing reactions involved in the production of propionic acid permit the oxidation of lactic acid to pyruvic acid. Some of the pyruvate formed as a result of fermentation is usually decarboxylated to yield acetate and CO_2. An additional product of this fermentation is succinic acid.

Oxaloacetate is an intermediate in the formation of propionate. To synthesize this four-carbon compound, a carboxyl group is recycled during the fermentation. The carboxyl group is transferred to the three-carbon compound pyruvate. The enzyme catalyzing this reaction is methylmalonyl-CoA-pyruvate transcarboxylase. The reduction of the oxaloacetate to malate, its conversion to fumarate, and the reduction of the fumarate to succinate can be used to dispose of electrons, such as those obtained from the HDP pathway or the oxidation of lactic acid. A CoA-transferase enzyme exchanges the succinate with the propionyl group on a molecule of propionyl-CoA to form free propionic acid and succinyl-CoA. An isomerase then converts the succinyl-CoA to methylmalonyl-CoA, which donates a carboxyl group to the enzyme–biotin complex to form a biotin–ADP–CO_2 complex and a molecule of propionyl-CoA. The activated carboxyl group is used to convert another pyruvate to oxaloacetate while propionic acid is liberated during the activation of another succinate. While this reaction is taking place, pyruvate is also being converted to acetate to balance the overall fermentation. By these reactions, a typical propionic acid bacterium such as *P. pentosoccium* will produce CO_2, acetic acid, propionic acid, and succinic acid as a result of glucose or lactic acid fermentation.

Clostridium propionicum also produces propionic acid as a fermentation product but the pathway differs from those above. Pyruvate is converted to propionic acid by a direct reduction route, involving reduction to lactate that is used to form lactyl-CoA. This latter compound is dehydrated, and reduced to propionyl-CoA. There is no formation of a four-carbon compound during this fermentation.

Another interesting use of oxaloacetate is as substrate for the Na^+-oxaloacetate decarboxylase, which is a membrane-bound Na^+ pump. The free energy of decarboxylation is sufficient to drive the development of a sodium gradient. This sodium gradient can be used to generate ATP by taking advantage of the electrochemical sodium gradient (see below). An example of a bacterium carrying out this reaction is *Propionibacterium modestum*, a strict anaerobe isolated from salt water.

The fermentation of ethanol

Some bacteria such as *Clostridum kluyveri* are able to gain energy by the fermentation of ethanol. Ethanol is oxidized and activated to form acetyl-CoA and the production of H_2 and the formation of fatty acids eliminate the electrons obtained by this oxidation. Two acetyl-CoA molecules are condensed to form acetoacetyl-CoA and this compound proceeds through the metabolic pathways described previously with two reduction steps giving butyryl-CoA. Additional acetyl molecules may be added with the production of larger fatty acids as final products. Pyruvate is synthesized by reversal of pyruvate:ferredoxin oxidoreductase.

The fermentation of pentoses

CONVERSION OF PENTOSES TO INTERMEDIATES OF THE HDP PATHWAY

Some bacteria are able to ferment certain 5-carbon sugars such as D-xylose and L-arabinose. The fermentation route of these pentoses by enteric bacteria generally involves isomerization of the aldopentose to the 2-keto-isomer or pentulose. A pentulose kinase then catalyzes phosphorylation to form the pentulose-5-phosphate. In such a manner, D-xylose is isomerized to D-xylulose and then phosphorylated to D-xylulose-5-phosphate, while L-arabinose is isomerized to L-ribulose and then phosphorylated to L-ribulose-5-phosphate. Epimerization reactions convert the pentulose-5-phosphates to D-xylulose-5-phosphate, which represents the common intermediate in the catabolism of these sugars. The exception is d-ribose, which is usually phosphorylated first and then isomerized to D-ribulose-5-phosphate.

The pentulose phosphates are converted to intermediates of the HDP pathway by a series of reactions known as the transketolase/transaldolase rearrangements. The enzyme transketolase (TK) cleaves 2-keto-sugars such as D-xylulose-5-phosphate between the 2- and 4-carbons and transfers the 2-carbon group to a proper aldehyde receptor, such as D-ribulose-5-phosphate, forming glyceraldehyde-3-phosphate and the seven-carbon carbohydrate sedoheptulose-7-phosphate. Transaldolase (TA) cleaves similar carbohydrates between the 3- and 4-carbons, transferring the 3-carbon moiety to an appropriate acceptor such as glyceraldehyde-3-phosphate. These rearrangements convert the 5-carbon sugar phosphate to intermediates of the HDP pathway such as glyceraldehyde-3-phosphate and fructose-6-phosphates with four- and seven-carbon carbohydrates as intermediates. The structures of some substrates and products of the TK/TA rearrangements are shown in Figure 9.

The conventional HDP pathway enzymes will convert glyceraldehyde-3-phosphate to pyruvate with the production of normal fermentation products. Because these enzyme-catalyzed reactions are reversible, pentose also can be synthesized from HDP pathway intermediates.

THE HEXOSE MONOPHOSPHATE OR PENTOSE PHOSPHATE PATHWAY

Many bacteria, including *E. coli*, can oxidize glucose-6-phosphate at the 1-carbon to form 6-phosphogluconic acid, which is then converted to a 5-carbon compound and metabolized using the TK and TA reactions. The enzyme that oxidizes glucose-6-phosphate, glucose-6-phosphate dehydrogenase, normally uses NADP, rather than NAD, as the electron acceptor. The 6-phosphogluconolactone formed by this reaction is converted to 6-phosphogluconate by a lactonase. The 6-phosphogluconate can be further oxidized and decarboxylated by 6-phosphogluconate dehydrogenase to produce CO_2, D-ribulose-5-phosphate, and, usually, NADPH. In many bacteria, such as the enterics, this is primarily a biosynthetic route, providing the cells with pentose for RNA and DNA synthesis and NADPH for biosynthetic reducing power, and in aerobically growing *E. coli*, about 15 percent of the glucose is diverted to pentoses by this route. However, the pathway can be used for glucose catabolism with the pentose phosphates converted to HDP intermediates via the TK/TA rearrangements. This route of glucose catabolism does not involve a sugar diphosphate as an intermediate and is known as the hexose monophosphate pathway (HMP) or sometimes the pentose phosphate pathway. With an *E. coli* mutant unable to use the HDP, such as a glucose-6-phosphate isomerase-negative mutant, the cells can still grow on glucose using the HMP pathway, but at a slower rate than when the HDP pathway is employed. Some bacteria, such as *Thiobacillus novellus* and *Brucella abortus*, use only the HMP pathway for glucose metabolism.

Because fructose-6-phosphate can be one of the products produced from pentulose phosphate by the TK/TA reactions, the potential exists for some organisms to convert fructose-6-phosphate to glucose-6-phosphate and oxidize this compound to 6-phosphogluconate and then to ribulose-5-phosphate. In such a manner an HMP

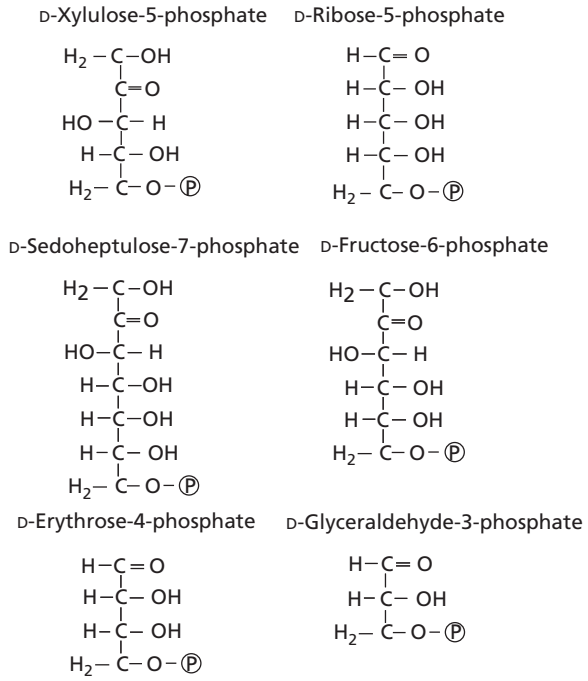

Figure 9 *Carbohydrates involved in the transketolase/transaldolase reactions. D-Xylulose-5-phosphate, D-fructose-6-phosphate, and D-sedoheptulose-7-phosphate can serve as substrates to be cleaved by transketolase or transaldolase. D-Glyceraldehyde-3-phosphate, D-erythrose-4-phosphate, and D-ribose-5-phosphate can serve as acceptors for the 2- or 3-carbon moiety being transferred.*

cycle may exist, with the oxidation of hexoses not requiring the oxidation of glyceraldehyde-3-phosphate.

THE HETEROLACTIC ACID FERMENTATION PATHWAY

A bacterium using the HDP pathway to ferment glucose to pyruvate can gain two ATP per glucose used by substrate-level phosphorylation, but the energy yield is less for the fermentation of a pentose. Theoretically, a homolactic acid fermenter using the HDP pathway could gain six ATP by the fermentation of three glucose to six lactate. However, it could only gain five ATP by the fermentation of three pentoses to five lactate using the pentose phosphate route and the TK/TA rearrangements.

Some lactic-acid bacteria have evolved a more efficient pathway for the fermentation of 5-carbon sugars. Instead of the D-xylulose-5-phosphate being cleaved by TK, it is cleaved by an enzyme termed phosphoketolase in a reaction that results in the esterification of inorganic phosphate and the formation of glyceraldehyde-3-phosphate and acetyl phosphate as products. The glyceraldehyde-3-phosphate can be converted to pyruvate by normal HDP pathway enzymes, yielding a gain of two ATP. The acetyl phosphate can be used to phosphorylate ADP to generate an ATP through the action of acetate kinase. Because one ATP is used to form the pentulose phosphate, the result is a net gain of two high-energy phosphate bonds for each pentose fermented. Bacteria that possess this fermentation route for pentoses normally possess high activity for lactic dehydrogenase and reduce most of the pyruvic acid to lactic acid. Therefore, the fermentation products from 5-carbon sugars are primarily lactate and acetate in equal amounts.

Bacteria possessing phosphoketolase are often lacking key enzymes of the hexose diphosphate pathway such as phosphofructokinase and aldolase and are unable to use the complete HDP pathway for the catabolism of glucose. If provided with D-glucose as a carbon and energy source, they use the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to form D-ribulose-5-phosphate and CO_2. The ribulose-5-phosphate can be epimerized to D-xylulose-5-phosphate, which is cleaved by phosphoketolase to produce glyceraldehyde-3-phosphate and acetyl phosphate. The glyceraldehyde-3-phosphate is converted to pyruvate and lactate in a normal manner for a homolactic acid fermentation. However, in order to dispose of all of the electrons from the oxidation of the six-carbon sugar to the pentulose, the acetyl phosphate must be reduced to ethanol in two reduction steps. Thus, the products of the fermentation of glucose by this route are CO_2, ethanol, and lactic acid and the energy yield is only one ATP per hexose fermented. This fermentation of glucose is termed a heterolactic acid fermentation because the three major products are produced in approximately equal amounts. The CO_2 is derived from the 1-carbon of the hexose, the ethanol from the 2- and 3-carbons, and the lactic acid from the 4-, 5-, and 6-carbons.

Bacteria in the genus *Bifidobacterium* carry out a variation of this fermentation. These bacteria also lack the aldolase of the HDP pathway but possess a fructose-6-phosphate phosphoketolase that converts fructose-6-phosphate to acetyl phosphate and erythrose-4-phosphate. The acetyl phosphate can be used for the production of ATP with acetate being liberated. The transketolase and transaldolase enzymes can act upon the erythrose-4-phosphate and fructose-6-phosphate, producing 7- and 5-carbon carbohydrates and eventually forming some glyceraldehyde-3-phosphate that can be converted to pyruvate. Thus, these bacteria ferment glucose to large amounts of acetic acid with smaller amounts of lactic acid, ethanol, and formic acid.

Alternative routes of carbohydrate catabolism

THE KDPG PATHWAY

An alternative route for the conversion of glucose to pyruvate is known as the Entner/Douderoff pathway or the 2-keto-3-deoxy-6-phosphogluconate (KDPG) pathway, because this latter compound is an intermediate unique to this metabolic route. The two enzymes unique to the KDPG route are 6-phosphogluconate dehydratase and KDPG aldolase. The first reaction of the pathway is oxidation of glucose-6-phosphate to 6-phosphogluconate by the action of glucose-6-phosphate dehydrogenase. The 6-phosphogluconate is dehydrated by 6-phosphogluconate dehydratase, to form KDPG. This latter compound is cleaved by KDPG aldolase, producing pyruvate from the first three carbons and glyceraldehyde-3-phosphate from the 4-, 5-, and 6-carbons. These two reactions are shown in Figure 10.
The pyruvate can be oxidized and decarboxylated to form acetyl-CoA and the acetyl group can be oxidized via the TCA cycle, while the glyceraldehyde-3-phosphate produced by the action of KDPG aldolase can be converted to pyruvate by the normal enzymes of the HDP pathway.

The KDPG route in enteric bacteria

The KDPG route is not normally used by *E. coli* cells catabolizing glucose but can be used when gluconic acid is the carbon source. *E. coli* catabolizes gluconate by first using gluconokinase to phosphorylate it to 6-phosphogluconate. Some of the 6-phosphogluconate may be oxidized to D-ribulose-5-phosphate and CO_2, and degraded by the HMP pentose route. Most, however, is dehydrated by 6-phosphogluconate dehydratase to KDPG, which is cleaved by KDPG aldolase. Because *E. coli* is unable to oxidize glucose to gluconate, the KDPG route is not induced when D-glucose is the substrate.

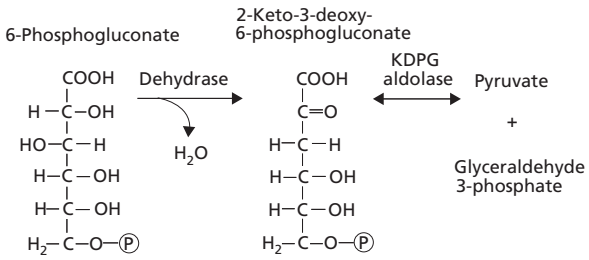

Figure 10 *Reactions of the 2-keto-3-deoxy-6-phosphogluconate (KDPG) route to pyruvate. Two reactions convert 6-phosphogluconic acid to pyruvate and glyceraldehyde-3-phosphate.*

With mutants of *E. coli* deficient in KDPG dehydratase, all gluconate must be metabolized through the pentose phosphate route. If another mutation occurs so that the organism also loses 6-phosphogluconate dehydrogenase activity, the cells cannot grow on gluconate at all.

Why does *E. coli* retain the capacity to use gluconate as a carbon source? One explanation is that it provides an advantage during growth in a specialized environment. Evidence has indicated that one such environment might be the large intestine. Mutants in which the gene encoding KDPG aldolase had been disrupted have been shown to be incapable of colonizing the large intestine of the mouse (Sweeney et al. 1996).

Analysis of the genome of *E. coli* has also revealed that there are two gluconate transporters. It has been shown, however, that one of these transporters is not used for gluconate but for the sugar acid L-idonic acid (Bausch et al. 1998). This compound is apparently transported without phosphorylation and then converted to 6-phosphogluconate. Enzymes of the KDPG pathway then metabolize the 6-phosphogluconate.

GLUCOSE CATABOLISM IN THE GENUS *PSEUDOMONAS*

The phosphorylated routes for glucose catabolism

One pathway of glucose catabolism in the pseudomonads involves phosphorylated intermediates. The phosphorylated route involves the phosphorylation of glucose to glucose-6-phosphate and its oxidation to 6-phosphogluconate. Much of the 6-phosphogluconate formed will be degraded through the KDPG route, although some may be diverted to the HMP route. Glucose-6-phosphate isomerase is usually present, and aldolase may also be present, but phosphofructokinase is often lacking or very weak, so the HDP route usually does not function

Aldolase can be present to form fructose-1,6-bisphosphate from triose phosphates; therefore, some of the pseudomonads have the ability to convert the glyceraldehyde-3-phosphate formed by KDPG aldolase back to fructose-1,6-bisphosphate. Phosphatase activity can cleave phosphate to form fructose-6-phosphate and this can be isomerized to glucose-6-phosphate, which can be oxidized and converted to KDPG. This route, from hexose phosphate to KDPG, to triose phosphate, and back to hexose phosphate, can be considered as a KDPG–hexose cycle. Some mutants blocked in the conversion of glyceraldehyde-3-phosphate to pyruvate can still grow on glucose by using this cycle (Lessie and Phibbs 1984).

In many bacteria, the oxidation of glucose-6-phosphate to 6-phosphogluconate also serves an anabolic or biosynthetic function, producing pentoses and reducing power (NADPH) for biosynthetic reactions. In aerobes where glucose-6-phosphate dehydrogenase is also an important catabolic enzyme, the reaction may need to serve both catabolic and biosynthetic purposes. In some cases, either NAD or NADP may serve as the electron acceptor for glucose-6-phosphate dehydrogenase, depending upon the needs of the cell. In other cases, isozymes may be involved in the reaction, one isozyme reducing NAD and another reducing NADP.

The route of catabolism for 5-carbon carbohydrates by pseudomonads can be similar in many ways to the KDPG route of glucose catabolism. The aldopentose substrate can be oxidized directly to a pentonic acid and a dehydration reaction can follow to form the 2-keto-3-deoxy-pentonic acid. This may be cleaved by an aldolase to yield pyruvate and glycolaldehyde with the glycolaldehyde oxidized to glycolic acid. In this manner D-xylose can be oxidized to D-xylonic acid, which can be dehydrated to 2-keto-3-deoxy-xylonic acid. In a similar manner, D-ribose can be oxidized to ribonic acid, which can be dehydrated to 2-keto-3-deoxy-ribonic acid.

The oxidative pathway for hexoses

In addition to the phosphorylated pathways for the catabolism of sugars described above, many pseudomonads also possess oxidative pathways. In these cases, flavin or cytochrome-linked, membrane-bound oxidase enzymes, located in the periplasmic space, oxidize the sugars with electrons passing from the electron acceptor directly to O_2. Glucose oxidase oxidizes glucose to form gluconate, while other oxidases can convert the gluconate to keto-sugars such as 2-ketogluconate and 2,6-diketogluconate. The glucose oxidase (sometimes called dehydrogenase) of *Pseudomonas fluorescens* was found to be a quinoprotein, whereas the gluconate oxidase was found to be a flavoprotein. Such oxidation reactions, associated with the cell membrane, provide the cells with rapid energy. Pseudomonads lacking glucose oxidase are unable to produce gluconate and cannot induce the KDPG pathway enzymes.

The keto-sugars formed by the oxidative reactions may be later transported into the cells, phosphorylated, and further degraded by phosphorylated routes. Pseudomonads lacking gluconokinase cannot form glucose-6-phosphate from glucose but may utilize the oxidized

products from the periplasm. Some pseudomonads can grow in the absence of oxygen by using nitrate as an electron acceptor. However, then they must use the direct phosphorylated pathways for catabolism because nitrate will not replace oxygen in the oxidative pathways.

If the oxygen concentration is low, the catabolism of glucose in *Pseudomonas aeruginosa* is decreased from the extracellular, direct oxidative route to the intracellular phosphorylative route. The change results in a decrease in the concentrations of glucose dehydrogenase and gluconate dehydrogenase activities and decreases the transport systems for gluconate and 2-ketogluconate, but there is increased activity for glucose transport (Mitchell and Dawes 1982). Global regulatory controls of these strict aerobes differ from those for the enteric bacteria. The major energy supply comes from oxidative phosphorylation reactions and substrates that can be quickly oxidized, such as acetate or TCA-cycle intermediates, cause catabolite repression of glucose catabolism enzymes (MacGregor et al. 1991).

GLUCOSE FERMENTATION BY *ZYMOMONAS*

Bacteria in the genus *Zymomonas* are obligate fermentative bacteria with substrate-level phosphorylation as the only source of energy. They are not, however, strict anaerobes and can grow in the presence of oxygen. The fermentation of glucose produces ethanol, CO_2, and lactic acid as primary products. The pathway of glucose catabolism is unusual in that it is the KDPG route, a pathway with a lower energy yield. The KDPG route is not an efficient route for the fermentation of glucose because only one ATP can be gained per glucose fermented. The pyruvate formed from glucose is oxidized to acetaldehyde and CO_2 by a pyruvate decarboxylase similar to the enzyme common in yeast and the acetaldehyde is directly reduced to ethanol by alcohol dehydrogenase. The pyruvate decarboxylase has an especially efficient promoter and represents about 4 percent of the total cellular protein (Neale et al. 1987). *Zymomonas mobilis* has no TCA cycle and only remnants of a respiratory chain. The KDPG pathway is constitutive and the glucose-6-phosphate dehydrogenase can use either NAD or NADP as the electron acceptor, so reducing power for biosynthetic reactions can be provided.

OTHER BACTERIA

A weak HDP pathway, an active KDPG route, and an active hexose monophosphate pathway characterize the metabolism of bacteria in the genus *Acetobacter*. These bacteria may utilize a hexose cycle with triose phosphate, originating either from the KDPG route or the pentose phosphate route, going to fructose-6-phosphate, glucose-6-phosphate, and 6-phosphogluconate. Bacteria classified as *Gluconobacter* do not possess a complete TCA cycle, a complete HDP pathway, or a KDPG pathway. They can use glucose either by a terminal oxidative route or a pentose phosphate cycle.

Other strictly aerobic bacteria tend to favor the degradation of glucose by the KDPG route and the HDP is rarely used, although some glucose carbon may pass through the pentose phosphate pathway. As examples, bacteria in the genus *Agrobacterium* have been reported to catabolize glucose 55 percent by the KDPG pathway and 44 percent by the pentose route. For bacteria in the genus *Azotobacter* there is no HDP pathway; the pentose pathway is of minor importance but the KDPG route is the major pathway for glucose degradation. Bacteria in the genus *Sphaerotilus* also have been reported to metabolize glucose via the KDPG and pentose phosphate pathways, as have bacteria in the genus *Rhizobium*. For bacteria in the genus *Arthrobacter*, however, an HDP pathway as well as a pentose pathway has been reported. For *Neisseria gonorrhoeae*, about 80–87 percent of glucose goes via the KDPG pathway, while 13–20 percent has been reported to pass through the HMP route.

BIOSYNTHETIC REACTIONS

Small-molecule biosynthesis

AMINO ACIDS

In general, the normal routes of synthesis of amino acids produce the α-oxoacid and a transamination reaction then adds the amino group. The amino acids may be grouped into six families based upon their routes of synthesis (Moat and Foster 1995). Aspartate, threonine, methionine, isoleucine, asparagines, and lysine make up the aspartic acid family, which originates from an intermediate in the TCA cycle, oxaloacetate. Another TCA-cycle intermediate, α-ketoglutarate (α-oxoglutarate), is converted to glutamic acid, glutamine, and arginine. Serine, glycine, and cysteine all require the triose 3-phosphoglycerate in their formation and thus are grouped together. The aromatic acid family comprises phenylalanine, tyrosine, and tryptophan. Erythrose-4-phosphate and phosphoenolpyruvate, both produced during carbohydrate metabolism, are required for the synthesis of the aromatic amino acids. Alanine, valine, and leucine make up the pyruvate family. Alanine is formed directly by transamination of pyruvate, whereas synthesis of valine and leucine requires condensation of two pyruvates to α-acetolactate, which is eventually converted to valine and leucine. Histidine synthesis is independent of the other families and requires ATP and phosphoribosylpyrophosphate.

PURINES AND PYRIMIDINES

The cells must have a supply of phosphorylated ribonucleotides and deoxyribonucleotides for the synthesis of

RNA and DNA. Purines and pyrimidines are synthesized by quite different routes. The purine ring has as its starting material ribose-5-phosphate while the pyrimidine ring originates in aspartate plus carbamoyl phosphate. The pathways of biosynthesis yielding inosine-5′-phosphate (IMP), a critical intermediate in the synthesis of purines, and uridylic acid (UMP), in the pyrimidine pathway, are complex. In bacteria such as *E. coli*, deoxyribonucleoside diphosphates are produced by the reduction of the corresponding ribonucleotide diphosphates in a reaction catalyzed by a ribonucleotide reductase. In a few species of bacteria, vitamin B_{12} is required for the reduction that occurs at the nucleoside triphosphate level. Methylation of the uracil ring to produce thymine occurs at the level of deoxyuridine monophosphatase (dUMP) with methylenetetrahydrofolate usually the donor of the C1 group and the reductant.

FATTY ACIDS AND LIPIDS

Lipids may serve as reserve food materials or as constituents of bacterial membranes. Lipids found in bacteria include phospholipids, lipoproteins, glycolipids, and the lipopolysaccharide of gram-negative bacteria. Most lipids are phospholipids that contain fatty acids ester-linked to glycerol phosphate. Bacteria synthesize fatty acids from acetyl-CoA using an acyl carrier protein (ACP) with a prosthetic group of 4′-phosphopantetheine. Interestingly, synthesis proceeds through the production of a 3-carbon intermediate, malonyl-CoA, produced by the carboxylation of acetyl-CoA. Growth of the fatty-acid chain is achieved by condensation of malonyl-ACP to the extending chain. The newly condensed molecule contains a β-keto group that is removed by reduction of the keto to a hydroxyl; then a dehydration removes the hydroxyl leaving a double bond followed by reduction of the double bond to leave a saturated chain. Synthesis of unsaturated fatty acids from the extended saturated chain can be accomplished in one of two ways. Under anaerobic conditions, the β-hydroxydecanoyl-ACP is acted upon by a special dehydrase to yield a *cis* double bond between carbon atoms 3 and 4. Two-carbon units are added until the proper length is obtained. Under aerobic conditions, molecular oxygen is used to introduce hydroxyl groups, which can be removed by dehydration reactions to produce double bonds.

Degradation of fatty acids is not the reverse of fatty-acid synthesis. Fatty acids are degraded using the β-oxidation pathway. This pathway involves introduction of a double bond into the fatty acid-CoA derivative. A keto group is the introduced β to the carboxyl group by hydration of the double bond and then oxidation of the hydroxyl. The keto group is then attacked by CoA, leading to the production of an acetyl-CoA and leaving the remaining fatty acid-CoA two carbons shorter.

TCA-cycle intermediates as biosynthetic compounds

Bacterial cells need many intermediates for biosynthetic purposes, including 6-carbon sugars for synthesis of cell walls, triose phosphates to make glycerol and lipids, and acetyl-CoA for the synthesis of fatty acids. In aerobic bacteria, the TCA-cycle functions to oxidize acetate and provide energy. However, another major function of the TCA-cycle enzymes is to produce intermediates such as α-ketoglutaric acid and oxaloacetic acid for biosynthetic reactions. During the TCA cycle, one oxaloacetate combines with the acetyl group from an acetyl-CoA to yield citric acid, which is eventually converted back to oxaloacetate and there is no net gain in di- or tricarboxylic acid intermediates. Because some of these intermediates are being used for biosynthetic reactions, mechanisms must exist for their net synthesis. A very important enzyme, especially in enteric bacteria, to replenish oxaloacetate loss as a result of anabolic reactions is phosphoenolpyruvate carboxylase, which forms oxaloacetate from phosphoenolpyruvate and CO_2. The enzyme phosphoenolpyruvate carboxylase is biosynthetic in its regulation and is inhibited by aspartate. Mutants of enteric bacteria that lack the enzyme are unable to grow on a glucose-salts minimal medium unless the medium is supplemented with a compound capable of providing a TCA-cycle intermediate. Pyruvate carboxylase also catalyzes the production of oxaloacetate but uses pyruvate as initial substrate. This enzyme is found in nonenterics such as *B. subtilis* and *Rhizobium etli*. It is also found in eukaryotic organisms.

If the TCA reactions are being used strictly for generation of biosynthetic precursors, oxaloacetate is a key intermediate that is metabolized via two separate pathways. In one pathway oxaloacetate is converted to succinyl-CoA, an essential biosynthetic precursor, by using a reductive TCA pathway. In this pathway malate dehydrogenase and malate dehydrase (fumarase) convert oxaloacetate to fumarate. Then fumarate reductase, instead of the normal TCA cycle enzyme succinate dehydrogenase, reduces the fumarate to form succinate. Succinyl-CoA is formed from succinate using succinate thiokinase. Oxaloacetate and acetyl-CoA can also yield citric acid, which can be converted to α-ketoglutaric acid, another essential precursor, by normal TCA-cycle enzymes. This splitting of the TCA cycle into a reductive and an oxidative branch is common among fermenting bacteria.

Pathways for incorporation of one-carbon compounds into cellular material

Bacteria that can grow on CO_2 as the sole source of carbon are termed autotrophs. However, bacteria that

cannot grow on CO_2 as the sole carbon source also use CO_2 for biosynthesis of cell material; for example, phosphoenolpyruvate carboxylase, which was discussed above, incorporates CO_2 into cellular precursors. Because these reactions are essential for growth under certain conditions, CO_2 is an essential nutrient for most bacteria. However, enzymes like phosphoenolpyruvate carboxylase do not permit growth on CO_2 as the sole carbon source. Autotrophs rely on one of several unique pathways that allow CO_2 to be used as the sole carbon source.

THE CALVIN CYCLE FOR CO_2 FIXATION

The best-studied pathway of CO_2 incorporation for autotrophs is the Calvin cycle. The Calvin cycle, sometimes termed the reductive pentose phosphate pathway, is found in some photosynthetic bacteria and many chemoautotrophs and involves the conversion of a 5-carbon sugar and CO_2 to form two molecules of 3-phosphoglyceric acid. The unique enzymes of the Calvin cycle are phosphoribulokinase, converting ribulose-5-phosphate to ribulose-1,5-bisphosphate, and ribulose bisphosphate carboxylase (sometimes called ribulose-1,5-bisphosphate carboxylase/oxygenase). Ribulose bisphosphate carboxylase converts ribulose bisphosphate and CO_2 to two 3-phosphoglyceric acids. To incorporate the fixed CO_2, the 3-phosphoglyceric acid is phosphorylated, using ATP and phosphoglycerate kinase, to 1,3-diphosphoglyceric acid. This latter compound is reduced by glyceraldehyde-3-phosphate dehydrogenase to glyceraldehyde-3-phosphate, which can be converted to dihydroxyacetone phosphate by triosphosphate isomerase. Aldolase can catalyze the synthesis of fructose-1,6-bisphosphate from the triose phosphates and fructose-1,6-bisphosphate phosphatase can cleave the phosphate from the 1-carbon position to produce fructose-6-phosphate. Enzymatic reactions similar to those of the HMP pathway regenerate the pentose phosphates from hexose phosphates. The final product of these rearrangements is ribulose-5-phosphate, which is converted to ribulose-1,5-bisphosphate by phosphoribulokinase.

THE REDUCTIVE TCA CYCLE FOR CO_2 FIXATION

Bacteria in the genera *Cyanobacteria*, *Rhodospirillaceae*, *Chromatiaceae*, and *Chloroflexaceae* will use the Calvin cycle for CO_2 fixation when growing with CO_2 as the only carbon source. Some bacteria in the genus *Chlorobiaceae*, however, do not possess the enzymes of the Calvin cycle. This family of organisms uses the reductive TCA cycle or the reductive C_4 dicarboxylic acid CO_2 fixation pathway. In this pathway acetyl-CoA is carboxylated by a ferredoxin-dependent pyruvate synthase to form pyruvate. To convert the pyruvate to PEP the enzyme PEP synthetase and ATP are used to form PEP, AMP, and inorganic pyrophosphate. The PEP is carboxylated to oxaloacetate by PEP carboxylase and the normal TCA cycle is reversed, with the oxaloacetate reduced to malate and the malate dehydrated to fumarate. The fumarate is reduced to succinate by fumarate reductase, and the succinate is phosphorylated and changed to succinyl coenzyme A by succinyl-CoA synthetase. The enzyme reaction producing α-ketoglutarate during the normal TCA cycle is irreversible, so a special enzyme is employed during the reductive TCA cycle, an α-ketoglutarate:ferredoxin oxidoreductase. The α-ketoglutarate is changed to citrate by normal enzymes of the TCA cycle, and an ATP-citrate lyase cleaves citrate to form oxaloacetate and acetyl-CoA. Through these reactions, four molecules of CO_2 have been converted to one molecule of oxaloacetate, which can be used for other biosynthetic purposes.

OTHER PATHWAYS FOR INCORPORATION OF ONE-CARBON COMPOUNDS INTO CELLULAR MATERIAL

Methylotrophs are bacteria that can use 1-carbon compounds other than CO_2 as their sole carbon source. Compounds of this type include methane, methanol, formate, and methylamine. When growing on methane it is first converted to methanol by methane monooxygenase (MMO). The methanol is then oxidized to formaldehyde, which can be further oxidized to CO_2 to provide energy or can be converted into cellular material. The MMO exists in either of two forms: a cytoplasmic (soluble) form and a membrane-bound (particulate) form. The soluble form is stable to purification and has been studied in detail. The particulate form is quite labile and little is known about its structure other than that it contains copper.

The incorporation of formaldehyde into cell carbon can proceed by either of two pathways. One pathway is referred to as the serine pathway. This pathway leads to the production of acetyl-CoA from formaldehyde and CO_2. The pathway is known as the serine pathway because the formaldehyde is added to glycine to form serine. This is eventually converted to PEP, which is carboxylated to give oxaloacetate using PEP carboxylase. Through a series of reactions this four-carbon compound is converted into glyoxylate, which is converted to glycine to keep the cycle going, and acetyl-CoA, which can be used for growth.

The other pathway for formaldehyde incorporation is the ribulose monophosphate (RuMP) pathway. In this pathway formaldehyde is added to D-ribulose monophosphate by the enzyme hexulose phosphate synthetase. The product is D-fructose-6-phosphate, which can be converted to other carbohydrates such as glucose-6-phosphate and glyceraldehyde-3-phosphate, or converted back to ribulose-5-phosphate by means of the TK and TA rearrangements. The hexulose phosphate synthetase is specific for D-ribulose-5-phosphate as a substrate but can use other aldehydes, such as glycolaldehyde, in place of formaldehyde (Kato et al. 1988;

Beisswenger and Kula 1991). Most methylotrophs use either the serine or RuMP pathway for incorporation of 1-carbon compounds into cell material.

Methanogens are bacteria capable of producing methane from mainly CO_2 and H_2 (see below). The methane-producing bacteria have the ability to synthesize acetate from 1-carbon compounds. The methyl group of acetate comes from methyltetrahydromethanopterin, which is a folate analog and an intermediate in the reduction of CO_2 to methane. Another molecule of CO_2 is reduced to a bound carbon monoxide by carbon monoxide dehydrogenase and the methane and carbon monoxide are condensed to form acetyl-CoA.

Nitrogen fixation

Many genera of bacteria, including both eubacteria and archaea, are capable of utilizing nitrogen gas (N_2) as the sole source of nitrogen for biosynthetic reactions. The reduction of N_2 to ammonia takes place using an enzyme complex termed nitrogenase. The major nitrogenase of most organisms is a molybdo-iron protein. The active complex has two component proteins, with one termed the iron protein and the other the molybdenum-iron protein (Georgiadis et al. 1992; Chan et al. 1993). The iron protein is a homodimer and the molybdenum-iron protein is a complex with an α-2β-2 structure. The intact complex contains several novel [Fe–S] centers and a novel iron-molybdenum cofactor.

The catalytic reaction involves the iron protein passing an electron to the molybdenum-iron protein, which is the site of nitrogen reduction. This step requires ATP hydrolysis, with a stoichiometry of two ATP per electron transferred. This electron transfer must be repeated six times to reduce the nitrogen to ammonia. The exact mechanism of nitrogen reduction at the iron-molybdenum cofactor remains unclear. In addition to the electrons required for nitrogen reduction there is also obligatory proton reduction during the catalytic cycle, leading to the net consumption of eight electrons per nitrogen reduced. This means that 16 ATP are hydrolyzed per nitrogen reduced.

One of the best studied model organisms for nitrogen fixation is *Azotobacter vinelandii*. This bacterium not only contains the iron-molybdenum nitrogenase but also can synthesize two other nitrogenases. If the growth medium contains molybdenum, then the molybdenum-containing nitrogenase I is synthesized. If the medium is lacking molybdenum but contains vanadium, then nitrogenase 2, which contains an iron-vanadium cofactor in place of the iron-molybdenum cofactor, is produced (Bishop et al. 1982). If the medium does not contain sufficient amounts of either of these two metals, then nitrogenase 3 is synthesized, which contains only iron (Pau et al. 1989). Alternative nitrogenases have also been found in other bacteria.

Nitrogenase activity is normally repressed when other nitrogen compounds are available, and its activity is inhibited by ADP, destroyed by O_2, and repressed by ammonia. In aerobic nitrogen fixers the enzyme must be protected from oxygen. A posttranslational regulation of nitrogenase activity has also been found in many different nitrogen-fixing bacteria (Ludden 1994). It involves the transfer of the ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase by the enzyme dinitrogenase reductase ADP-ribosyltransferase (DRAT). This group can be removed by another enzyme, dinitrogenase reductase-activating glycohydrolase (DRAG), which restores nitrogenase activity.

RESPIRATION

Aerobic respiration

Aerobic respiration is the condition when oxygen is the final electron acceptor of catabolism. In bacteria that can utilize aerobic respiration, the presence of oxygen will result in the repression of any fermentation pathways or anaerobic respiration pathways. A number of different electron carriers are involved in bacterial respiration, including NAD and NADP, flavoproteins, quinones, [Fe-S] proteins, and cytochromes. Flavoproteins are oxidation/reduction enzymes that contain flavin adenine mononucleotide (FMN) or flavin adenine dinucleotide (FAD). Flavins are synthesized from riboflavin and, upon reduction, transport two protons and two electrons. The actual redox potential of the oxidation/reduction reaction of flavoproteins can vary depending upon the protein group, although a range of 0.0–0.2 V is normal. Quinones are hydrophobic electron carriers that can shuttle electrons and protons through the lipid bilayer. The two most common quinones are ubiquinone (coenzyme Q) and menaquinone (vitamin K or MK). Proteins containing non-heme iron- and sulfur-containing clusters, referred to as iron-sulfur proteins, are components of almost all respiratory chains. Some well-studied iron-sulfur proteins are ferredoxin, rubredoxin, and a high-potential iron protein (HiPIP). These [Fe-S] centers carry only one electron and with midpoint potentials ranging from -0.40 to $+0.35$ V can be involved throughout the respiratory chain. Cytochromes are redox proteins that contain heme and transport a single electron. Cytochromes have characteristic light-absorption bands in the visible range and are categorized on the basis of their absorption characteristics. One type of cytochrome, the *c*-type, is additionally distinguished because the heme is covalently attached to the protein by thioether linkages.

The standard mitochondrial respiratory chain serves as a useful model because it was originally derived from the bacterial respiratory chain. The chain is divided into

four protein complexes, complexes I–IV, and additional components required to shuttle electrons. All of the components discussed below are found in bacteria. The major difference between bacterial and mitochondrial electron transport chains is that the bacterial respiratory complexes have a simpler subunit structure.

Complex I is the NADH-ubiquinone oxidoreductase, which oxidizes reduced NADH and transfers electrons to ubiquinone. This complex is made up of at least 20 different polypeptides and contains iron-sulfur proteins and flavoproteins. Electrons passed to the quinone along with two protons convert the quinone to quinol. This can then diffuse to other electron acceptors such as complex III. Complex III is also called the bc_1 complex or the ubiquinol-cytochrome *c* oxidoreductase. In bacteria this complex is made of three major subunits. Electron flow through the bc_1 complex is complex, with the two electrons from quinol eventually flowing to two different acceptors. This flow has been termed the Q cycle. Data from studies of the high-resolution structure of the bc_1 complex have suggested movement of the polypeptides during electron flow is critical for determining the eventual oxidant (Zhang et al. 1998). One of the terminal oxidants in the Q-cycle is a soluble cytochrome *c*. This cytochrome *c* can readily diffuse between different donors and acceptors to facilitate electron flow during respiration. Cytochrome *c* serves as a reductant for complex IV, also termed the cytochrome *c* oxidase, which reduces oxygen to water. This protein is a heme-copper protein whose structure has been solved (Iwata et al. 1995; Yoshikawa et al. 1998). A hallmark of this type of oxidase is the presence of a heme and copper in close proximity resulting in a structure termed a binuclear center. This is the site of oxygen reduction. Cytochrome *c* oxidase is also a vectorial proton pump with residues in and around the binuclear center critical for gating proton flow during turnover. Complex II is succinate dehydrogenase, also termed the succinate-ubiquinone oxidoreductase. This complex serves as an alternative site for entry of electrons into the respiratory chain.

Unlike mitochondria, bacteria can differ greatly in the composition of their respiratory chains. Even in individual bacteria, respiratory chains vary as a function of changes in growth conditions; for example, *E. coli* modulates the beginning and end of its aerobic respiratory pathway depending on available oxygen levels (Gennis and Stewart 1996). *E. coli* has two NADH dehydrogenases. The type I NADH dehydrogenase is the bacterial equivalent of the mitochondrial Complex I. The type II NADH dehydrogenase is a single subunit enzyme. It is a flavoprotein that contains one noncovalently bound FAD per polypeptide chain and also contains [Fe-S] centers. The type II oxidase is primarily expressed when oxygen is not limiting. There are also two terminal oxidases in *E. coli*. With higher levels of oxygen present, a cytochrome *o* oxidase is expressed. This oxidase, which uses quinol as reductant, is a member of the same family of oxidases as the mitochondrial oxidase and contains a heme-copper binuclear center. Under microaerophilic conditions, however, the cytochrome *d* oxidase is expressed. This terminal oxidase is only found in bacteria. This protein contains the novel *d* heme and has an extremely high affinity for oxygen.

Other bacteria have respiratory chains almost exactly like that found in mitochondria. Probably the best studied example is found in bacterium *Paracoccus denitrificans* (Baker et al. 1998). Bacteria also have the capacity to use many compounds as reductants. Compounds ranging from methanol to iron can be used as electron donors and thus would require their own associated oxidoreductase.

Oxidative phosphorylation

It has been known for many years that aerobes gain ATP from the transport of electrons in the cell membrane, a phenomenon called oxidative phosphorylation. During respiration, oxidation/reduction reactions take place in the membrane of the cells so that an electrochemical potential (δp) or proton-motive force (pmf) is created with protons on the exterior side of the membrane. Because the respiratory chain consists of hydrogen carriers and electron carriers in alternating sequences across the cytoplasmic membrane and because the cell membrane was impermeable to OH^- and H^+, as oxidation reactions transferred protons from one side of the membrane to the other, a pH gradient and an electrical potential was established across the membrane (Mitchell 1961). The electrochemical gradient that occurs as a consequence of electron flow through the respiratory chain can be used to make ATP, drive solute transport, drive the rotation of the flagellum, and drive unfavorable electron-transport reactions.

Respiration-dependent ATP synthesis is carried out by the ATP synthase, also termed the F_1F_0 type ATPase. The ATPase is a complex multiprotein system and has been compared to a small organelle. When protons pass from the outside to the inside of the membrane, their passage through the ATP synthase causes inorganic phosphate to be esterified and ADP is converted to ATP.

ATP synthase can be dissociated into two complexes termed the F_0 and F_1. The structure named F_1 contains five different subunits with a ratio of $\alpha 3$, $\beta 3$, γ, δ, and ϵ. The F_1 contains six ATP binding sites, one each on the α and β subunits. However, only those on the β subunits are capable of ATP synthesis. By itself the F_1 complex functions as an ATPase, hydrolyzing ATP to ADP and Pi. The F_0 complex usually consists of three different subunits in bacteria with a stoichiometry of a, b2, and c10–12. The F_0 is an integral membrane complex that

acts as an H^+ channel to translocate H^+ across the cytoplasmic membrane. Using purified protein it has been demonstrated that the complex functions as a rotary motor (Sambongi et al. 1999). The rotor is made up of the c subunits of the F_0 along with the γ and ϵ subunits of the F_1 complex. The remaining proteins are proposed to act as a stator. Models of ATP synthesis require that proton flow through the F_0 causes the rotor to turn. This drives rotation of the γ subunit and as it turns it causes structural changes in the ATP binding sites on the β subunits. The structural changes in the β subunits cause them to switch through low-, medium-, and high-affinity nucleotide binding sites. These shifts lead to the synthesis of ATP. High-resolution structures of both the F_1 and F_0 complexes have been determined and are generally consistent with models of ATPase function (Abrahams and Leslie 1994; Stock et al. 1999).

All bacteria, even strict anaerobes, must maintain an electrochemical gradient of protons across the membrane of the cell. This is needed to support the integrity of the cell membrane, to provide energy for motility, and to transport nutrients into the cell. Under anaerobic conditions, the F_1F_0 complex can function in the reverse direction, hydrolyzing ATP by means of an ATP hydrolase associated with the cell membrane to generate an electrochemical gradient. Some fermenting bacteria are also able to use the excretion of fermentation products through the cell membrane to generate a proton gradient.

In some bacteria, Na^+ substitutes for H^+ as the coupling ion and ATP-driven Na^+ pumps have been described in several genera. All cells appear to transfer energy obtained from external sources to energy stored as either ATP, proton-, or sodium-motive force. These energy sources may be interconvertible and it appears that any one can satisfy all of the energy sources of the cell (Skulachev 1994). An example of a Na^+-dependent respiratory chain was mentioned above in the discussion of oxaloacetate oxidation by *P. modestum*.

Oxygen toxicity

Cells growing in the presence of molecular oxygen produce some very reactive and toxic oxygen species, such as the superoxide radical, the hydroxyl radical, and hydrogen peroxide. These compounds are produced by autooxidation of components of the electron transport chain. In particular, flavoproteins have been shown to produce reactive oxygen species (Messner and Imlay 1999). In order to protect themselves against such compounds, bacteria have evolved special enzymes. Superoxide dismutase (SOD) converts the toxic superoxide anion (O_2^-) to hydrogen peroxide. *E. coli* is known to possess three species of SOD: Mn-SOD, Fe-SOD, and a periplasmic Cu, Zn-SOD (Benov and Fridovich 1994). Catalase and a class of enzymes named peroxidases destroy hydrogen peroxide. Catalase converts hydrogen peroxide to O_2 and water. The synthesis of catalase is normally regulated and the level of activity kept low until needed. *E. coli* encodes two different catalases, hydroperoxidase I and hydroperoxidase II.

E. coli has been an informative model system for the study of how cells respond to oxidative stress. There are several regulons that control the synthesis of proteins responsible for mitigating toxic effects of oxygen radicals in *E. coli*. The two best-studied regulators are the SoxR and OxyR proteins. SoxR responds to increases in superoxide. When activated the SoxR protein activates transcription of SoxS, which activates transcription of target genes in the regulon. SoxR is activated by oxidation of a reduced [2Fe-2S] center in the protein (Hidalgo et al. 1997). It is unclear what molecule oxidizes SoxR but superoxide is a likely candidate. OxyR responds to increases in hydrogen peroxide. It is activated by the formation of a disulfide bond upon exposure to hydrogen peroxide (Zheng et al. 1998). Another important regulator of expression of oxygen-radical tolerance factors is the sigma factor (σ^s), which is synthesized at high levels during stationary phase growth and may control as many as 50 genes.

While the reactivity of the oxygen radicals allows them to react with many different cellular components, there have been a few especially sensitive targets identified. In *E. coli* lacking superoxide dismutase, cellular injury has been determined mainly to occur to a set of dehydrase enzymes (Storz and Imlay 1999). The common factor among this class of enzymes is that they contain superoxide-sensitive [Fe-S] centers. The precise targets of hydrogen peroxide accumulation have not been identified.

Anaerobic respiration

NITRATE RESPIRATION

As mentioned previously, fermentation is the condition in which the final electron is an organic compound. In aerobic respiration, oxygen is the final electron acceptor. Anaerobic respiration is the situation where the final electron acceptor is a compound other than oxygen. There are many compounds that can serve as alternative electron acceptors. One of the most heavily studied is nitrate. Nitrate reduction can proceed via two pathways (Berks et al. 1995). One pathway is the reduction of nitrate to ammonia, termed 'ammonification.' The other pathway is the reduction of nitrate to nitrogen gas, termed 'denitrification.' Both of these pathways allow growth in the absence of oxygen and are termed 'dissimilatory pathways.' Nitrate can also be reduced to ammonia for biosynthetic purposes in an assimilatory pathway. However, the biosynthetic enzyme pathway is not coupled to ATP formation.

Pathways of nitrate reduction

Bacteria that can use nitrate as a terminal electron acceptor for energy production normally will not do so in the presence of oxygen, because aerobic respiration can yield more energy. Possible electron donors for nitrate reduction include NADH formed from a variety of catabolic pathways, formate, H_2, glycerol-3-phosphate, succinate, and lactate. The electron flow is from NAD, through flavoproteins, to cytochromes, and finally to nitrate.

The first step in nitrate reduction is the reduction of nitrate to nitrite. There are two types of dissimilatory nitrate reductases. The enzyme typically associated with nitrate respiration is a membrane-bound three-subunit complex, although the membrane-anchoring subunit (γ or NarI) is sometimes lost during purification (Moreno-Vivian et al. 1999). The largest of the three subunits (α or NarG) contains molybdenum bound by the cofactor molybdopterin guanine dinucleotide and a [4Fe-4S] center. The remaining subunit (β or NarH) contains several [4Fe-4S] centers and a [3Fe-4S] center. The membrane-anchoring subunit typically contains a *b*-type heme. The other type of nitrate reductase is a periplasmically located enzyme. The periplasmic nitrate reductase is a heterodimer with prosthetic groups similar to those found in the membrane-bound nitrate reductase.

The ammonification and denitrification pathways diverge at the level of nitrite. During ammonification a single enzyme catalyzes the six-electron reduction of nitrite to ammonia. In *E. coli* and other enteric bacteria this enzyme is a periplasmically located *c*-type cytochrome (Gennis and Stewart 1996). Ammonia is the terminal product of ammonification so no other enzymes are involved. In denitrifiers, nitrite is reduced to nitric oxide, a gaseous nitrogen oxide (Zumft 1997). There are two types of nitric-oxide-producing nitrite reductases. One contains copper as its redox-active metal and the other contains heme. Both are periplasmically located. Nitric oxide is reduced to nitrous oxide by a membrane-bound nitric oxide reductase containing heme and non-heme iron. Interestingly, this protein is related to the family of proteins that includes the heme-copper binuclear center containing oxygen-reducing oxidases. Nitrous oxide is reduced to nitrogen gas by a copper-containing, periplasmically located enzyme. This enzyme is also related to the terminal oxygen-reducing oxidases.

Dissimilatory nitrate reduction is widespread amongst the bacteria. Ammonification is best studied in the enteric bacteria but is found in other bacteria, for example *B. subtilis* (Nakano and Zuber 1998). Denitrification occurs in both eubacteria and archaea. Ongoing sequencing projects have found portions of the denitrification electron transport chain in several pathogens including *Neisseria meningitidis*, *N. gonorrhea*, and *Corynebacterium diphtheriae*. The role of dissimilatory nitrate reduction in these pathogens has not been determined.

REGULATION OF NITRATE REDUCTION IN *E. COLI*

In *E. coli* several signals are required for the cells to activate expression of enzymes required for the reduction of nitrate. First, oxygen concentrations must be limiting because oxygen is always a preferred oxidant. Low oxygen activates the transcriptional regulatory protein Fnr. Fnr contains an oxygen sensitive [Fe-S] center that undergoes changes in structure in the presence of oxygen (Khoroshilova et al. 1997). Nitrate and nitrite are sensed by 2, 2-component regulatory systems. The interaction of these two systems and their target proteins is complex and is used to activate expression of some genes, such as those encoding nitrate and nitrite reductase, while at the same time repressing expression of other genes (Darwin et al. 1997).

Sulfate reduction

Another commonly used terminal oxidant is sulfate. Dissimilatory sulfate reducers are heterotrophs that are almost always strict anaerobes. Sulfate-reducing bacteria are found in both the eubacteria and archaea. The best studied sulfate reducers belong to the genus *Desulfovibrio*. The first step in the use of sulfur as electron acceptor is its activation on an AMP molecule. The enzyme adenyltransferase (ATP-sulfurylase) uses ATP and sulfate as substrates to form adenyl sulfate (APS) and inorganic pyrophosphate (Hansen 1994). The pyrophosphate is hydrolyzed by inorganic pyrophosphatase with the energy in the pyrophosphate bond lost to the cells. The enzyme APS reductase adds two electrons to APS to reduce the sulfate to sulfite with the liberation of AMP. Next, sulfite reductase reduces sulfite to form sulfide (S_2). A proton-motive force is generated by the appropriate topological arrangement of proton-producing and proton-consuming reactions on either side of the cytoplasmic membrane.

Desulfovibrio desulfuricans is a strict anaerobe that can ferment glucose and pyruvate and can also grow using lactate if sulfate is present to serve as an electron acceptor. Pyruvate, formed by either the HDP pathway or lactate oxidation, is cleaved by pyruvate/ferredoxin oxidoreductase, yielding CO_2, H_2, and acetyl-CoA. The acetyl-CoA can be converted to acetyl-phosphate and then acetate with ATP formation. *Desulfovibrio desulfuricans* can also grow using CO_2, H_2, and acetate if sulfate is present. In this case, electrons are transferred from H_2 to sulfate to gain energy and the pyruvate/ferredoxin oxidoreductase reaction is reversed to make pyruvate for biosynthetic reactions.

Additional terminal electron acceptors

Alternative terminal electron acceptors are not limited to inorganic oxides. Oxidized metals such as iron or manganese can be used as terminal oxidants; for example, members of the genus *Shewanella* can utilize both iron and manganese as terminal electron acceptors (Myers and Nealson 1990). Many carbon compounds can serve as terminal oxidants; examples include dimethyl sulfoxide, trimethylamine oxide, and fumarate. The crystal structure of the membrane-bound fumarate reductase from the microaerophile *Wolinella succinogenes* has been determined (Lancaster et al. 1999). It has also been demonstrated that halocarbons can serve as electron acceptors. Molecular studies of the halorespiring bacterium *Desulfitobacterium dehalogenans* are starting to identify components required for this form of anaerobic growth (Smidt et al. 1999). Some bacteria appear only to be capable of halorespiration (Maymo-Gatell et al. 1997). Another notable carbon compound that appears to serve as a terminal electron acceptor is humic acid (Lovley et al. 1996).

Additional electron donors

In addition to the capacity to utilize a wide variety of compounds as terminal electron acceptors, bacteria also have the capacity to utilize many compounds as electron donors for respiration. In addition to the wide variety of organic compounds that can be used, many inorganic compounds can be used as electron donors. Ammonia and nitrite can be oxidized by a group of bacteria collectively known as nitrifiers. Ammonia oxidizers, such as members of the genus *Nitrosomonas*, can oxidize ammonia to nitrite via the intermediate hydroxylamine (Hooper et al. 1997). Ammonia oxidizers cannot further oxidize nitrite. Nitrite oxidizers, such as members of the genus *Nitrobacter*, oxidize nitrite to nitrate but cannot oxidize ammonia. Reduced sulfur compounds can also be oxidized to provide electrons for respiration. Sulfate oxidation has been heavily studied in the genus *Thiobacillus* (Kelly et al. 1997). Some thiobacilli can couple sulfur oxidation to nitrate reduction, completely eliminating involvement of carbon oxidation or reduction in respiration. This type of metabolism is referred to as 'lithotrophy' and can be found in many other bacterial genera. Other potential reductants include iron, manganese, H_2, and carbon monoxide.

BACTERIAL PHOTOSYNTHESIS

Photosynthesis is the conversion of the energy available in light to chemical energy, which is available for cell growth. Many bacteria are capable of photosynthesis and there are several different bacterial photosynthetic electron-transport systems. In all of these systems light is absorbed (harvested) by accessory pigments and the energy transferred to a reaction center. The reaction center carries out the charge separation that permits light energy to be converted to chemical energy. The different electron-transport systems can be differentiated into three groups based on the types of bacteria having a particular system. The three types of bacteria are:

1. purple photosynthetic bacteria
2. green sulfur photosynthetic bacteria
3. cyanobacteria.

A brief description of the photosynthetic electron-transport chain of each group is presented below.

Purple photosynthetic bacteria

In the purple photosynthetic bacteria both organic and inorganic compounds, such as reduced-sulfur compounds, can be used as electron donors. However, some bacteria are limited in their choice of electron donor. Photosynthesis in the purple bacteria, as is true for most bacteria, is an anaerobic process. Therefore, expression of the components required for photosynthesis occurs when oxygen is limiting. Oxygen limitation causes the most significant increase in photosynthetic gene expression with light having a much smaller effect (Buggy et al. 1994).

Light absorption is carried out by a group of protein-associated pigments organized into light-harvesting (LH) complexes. In most bacteria there are two complexes, referred to as LHI and LHII. High-resolution and modeling studies of LHI and LHII have shown that both are roughly circular and contain both bacteriochlorophyll and carotenoids (Hu et al. 1998). Carotenoids absorb at lower wavelengths, in the 500-nm range, while the bacteriochlorophylls absorb at higher wavelengths (800–900 nm). Current models of the organization of LHII and LHI have many LHII complexes surrounding the LHI complex, which has the reaction center at its center. Therefore, the probable direction of excitation transfer about light absorption is LHII to LHII and LHI and then LHI to the reaction center.

Transfer of excitation energy to the reaction center causes the potential of an electron located at a dimer of bacteriochlorophylls, known as the special pair, to become excited to a lower reduction potential. This electron is then transferred through the electron-transfer components of the reaction center and eventually resides on a quinone molecule designated UQ_A. This quinone is tightly bound to the reaction center complex. UQ_A then transfers the electron to a second quinone referred to as UQ_B. This quinone is less tightly bound and, when fully reduced, can diffuse away from the reaction center complex. These steps are then repeated to reduce the quinone fully, which, after binding two protons, diffuses into the lipid bilayer. Because the special pair is located on the periplasmic surface and the

ubiquinones are located on the cytoplasmic surface, electron transfer is vectorial. This vectorial transfer of electrons is referred to as charge separation because a negative charge is moved to the cytoplasmic side and a positive charge is left on the periplasmic side of the membrane. The details of electron flow through the reaction center are well defined because the high-resolution structure of the reaction center from purple photosynthetic bacteria has been solved (Deisenhofer et al. 1995).

Once UQ_B diffuses into the lipid bilayer it can pass its electrons to the bc_1 complex. Electron transfer through the bc_1 complex leads to the generation of a proton-motive force that can be used for the synthesis of ATP. The electrons are then passed to a periplasmic cytochrome *c* that then passes the electrons back to the reaction center. This type of electron flow during photosynthesis is termed 'cyclic photosynthesis.' Some photosynthetic bacteria use electron donors with reduction potentials higher than the NAD^+/NADH couple so these cells use the proton-motive force to drive reverse electron transport to generate NADH.

Green sulfur bacteria

The basic mechanisms of light harvesting and electron flow through the reaction center are the same in both purple photosynthetic and green sulfur bacteria. The reaction center of the green sulfur also contains a special pair whose reduction potential is decreased upon transfer of excitation energy. The reaction center differs in the nature of its associated cofactors. The green sulfur bacteria reaction center includes iron-sulfur centers, which are not present in the reaction center of the purple photosynthetic bacteria (Oh-oka et al. 1995). In addition, the potential of the excited electron in the reaction center of the green sulfur bacteria is low enough to reduce NAD^+ to NADH. This allows electron transfer to be noncyclic. However, cyclic electron transfer can occur, generating a proton-motive force and ATP production. Sulfide is a common electron donor for the green sulfur bacteria. Its electrons are fed into the electron-transport chain via periplasmic cytochromes.

Cyanobacteria

Cyanobacteria differ from the other photosynthetic bacteria by using water as the source of electrons for photosynthesis. Another difference is that the photosynthetic electron-transport chain of the cyanobacteria is essentially a combination of the purple and green photosynthetic electron-transport chains. The electron-transport chain in cyanobacteria is differentiated into reaction center II (RCII), which is related to the reaction center from purple photosynthetic bacteria, and reaction center I (RCI), which is related to the green sulfur bacteria reaction center. Electrons from water first flow through RCII, allowing formation of a proton-motive force. Electrons then flow to RCI via a copper protein called plastocyanin. The electron is then transferred to $NADP^+$ for biosynthetic purposes or back to RCI generating a proton-motive force. A continual supply of electrons is required because flow through RCII is not cyclic. This is generated by the manganese-containing water-splitting apparatus. The photosynthetic electron-transport pathway of plant chloroplasts is very similar to that of the cyanobacteria.

METABOLISM AMONG THE ARCHAEBACTERIA

Archaebacteria (domain archaea) encompass a diverse array of physiological processes. Probably the most prominent archaea are the methanogens. These bacteria grow by converting a limited set of substrates to methane. In addition to methanogens other archaea carry out aerobic or anaerobic respiration. No photosynthetic archaea have been isolated. However, some halophilic archaea can use light energy to generate a proton-motive force but this reaction is distinct from photosynthesis.

Methanogenesis

All known methanogens are members of the domain archaea and represent the largest group in that domain, with thermophiles, mesophiles, and halophiles included in the group. Methanogens are obligate anaerobes that reduce CO_2 and a few other substrates, such as formate, methanol, methylamines, or acetate, to produce the most reduced organic compound, methane. Most methanogens are autotrophs and the reduction of CO_2 is their only energy-yielding pathway.

The first step of methanogenesis is catalyzed by either a tungsten- or molybdenum-containing formyl-methanofuran dehydrogenase (Thauer 1998). CO_2 is activated and reduced to the level of formyl on methanofuran. The carbon is then transferred to the cofactor tetrahydromethanopterin. The carbon is reduced from the formyl to the methyl state while attached to this cofactor. At this stage the methyl is transferred to the cofactor coenzyme M. Methyl-coenzyme M (methyl-CoM) is reduced by methyl-CoM reductase to methane. The methyl-CoM reductase contains the unusual nickel tetrapyrrole, F_{430}. Methyl reduction also requires cofactor B (7-mercaptoheptanoylthreonine phosphate). During the reaction the cofactors coenzyme M and coenzyme B become linked via a disulfide bond. This bond is cleaved using the membrane-bound enzyme heterodisulfide reductase.

During methanogenesis from methanol about one out of four methanols are oxidized, by reversing part of the

methanogenesis pathway, to provide electrons for the reduction of methanol to methane. Methanogenesis from acetate is more complex, requiring that acetate be first converted to acetyl-CoA using either the combination of acetate kinase and phosphotransacetylase or acetyl-CoA synthetase (Ferry 1999). Carbon monoxide dehydrogenase then breaks the carbon–carbon bond forming a carbonyl and a methyl group. The carbonyl is oxidized to CO_2 and the electrons used to reduce the methyl group to methane.

The energetics of methanogenesis are not completely understood. It seems likely that the heterodisulfide reductase step permits formation of a proton-motive force (Deppenmeier et al. 1991). The coenzyme M–coenzyme B disulfide serves as the terminal electron acceptor, with H_2 being a likely electron donor. It is not clear how the proton-motive force is generated during this reduction but it is possible that scalar production of protons by the hydrogenase is the source. Methanogens do contain ATPases similar in structure and organization to that of the F_1F_0-type ATPases, which are designated A_1A_0-type ATPases (A meaning 'archaeal' here). While there is no direct evidence indicating that the ATPases are ATP synthases, it seems likely they are used for that purpose. The transfer of carbon from tetrahydromethanopterin to coenzyme M may also be an energy-coupling step.

Methanogens must synthesize all cellular organic compounds from simple starting material and some methanogens can grow on a completely minimal medium and convert CO_2 to all required organic material. During growth with CO_2 as the carbon source it is incorporated into acetyl-CoA. This reaction utilizes the carbon monoxide dehydrogenase discussed above. One unit of CO_2 is bound to the carbon monoxide dehydrogenase and then oxidized to carbon monoxide. A second CO_2 unit is then reduced to a methyl group using the same reactions occurring during methanogenesis. The methyl group is then passed to carbon monoxide dehydrogenase where it is combined with the bound carbon monoxide. The dehydrogenase then catalyzes the production of acetyl-CoA. The acetyl-CoA is next converted to phosphoenolpyruvate, which is probably converted to further primary precursors by a modified reductive TCA cycle.

Methanogens such as *Methanobacterium* and *Methanosarcina* assimilate CO_2 but have an incomplete TCA cycle. In *Methanobacterium*, succinate is apparently converted to succinyl coenzyme A and then to α-ketoglutarate. In *Methanosarcina*, oxaloacetate appears to be converted to citrate, which is converted to isocitrate and then to α-ketoglutarate. Although glucose is not a carbon or energy source for growth, there are some reports of methanogens taking up and metabolizing glucose and the distribution of radioactive label that suggested the presence of the HDP pathway. However, there is no report of the presence of phosphofructokinase, a key enzyme of the catabolic HDP pathway.

Nonmethanogenic archaea

The nonmethanogenic archaea are a diverse mix of organisms that include thermophiles, acidophiles, strict aerobes, strict anaerobes, and facultative bacteria. Representatives of many of these groups have been studied in some detail and genome-sequencing efforts are also contributing to our understanding of these bacteria. Strict aerobes are somewhat rare, reflecting the preference of archaea for anaerobic growth. One obligately aerobic genus studied in some detail is the genus *Sulfolobus*. These bacteria are thermophilic acidophiles, some of which can use organic compounds as electron donors, while others use reduced sulfur compounds. The respiratory components have been characterized from some *Sulfolobus* species. These characterizations have shown that the respiratory complexes are orthologs of the eubacterial complexes; however, in many cases they are quite divergent. A good example is provided by the SoxABCD quinol oxidase from the bacterium *Sulfolobus acidocaldarius* (Lubben et al. 1992). Sequence analysis indicated that this complex contains a subunit with a heme-copper binuclear center and other components of binuclear center-containing quinol oxidases. However, in addition to those proteins it has a b-heme-containing protein that is similar to the b-heme-containing protein of the bc_1 complex. This suggests that the complex III and IV components have been modified into a single active complex. The observation that respiratory proteins in eubacteria and archaea are orthologs but that there are no photosynthetic archaea has led some to suggest that respiration arose before photosynthesis and that the archaea and eubacteria diverged before photosynthesis.

The aerobic respiratory chain of halophilic archaea has not been studied as extensively as that of the *Sulfolobus* strains. However, data suggest that the proteins are orthologs of other known respiratory proteins. Some halophilic archaea have been shown to use alternative electron acceptors such as nitrate and fumarate. Several halophilic archaea also have the capacity to use light energy for energy conservation. A single protein termed bacteriorhodopsin carries out this process. Bacteriorhodopsin occurs in high concentration in the membrane and the occurrence of these purple membranes in certain halophiles led to the interest in this protein. The structure and catalytic mechanism of bacteriorhodopsin has been studied in great detail. It is a 26-kDa integral membrane protein with seven transmembrane helices (Pebay-Peyroula et al. 1997). It contains the chromophore retinal covalently attached to the protein at a lysine in one of the transmembrane helices. The absorption of light shifts the *trans*-retinal to

cis-retinal. This structural change begins a reaction scheme that ejects a proton into the periplasmic surface and causes the uptake of a proton from the cytoplasmic side of the membrane. Mutagenesis of bacteriorhodopsin has identified several acidic residues that make up the proton channel. In addition to bacteriorhodopsin these halophiles have several other retinal-containing proteins. One of these, halorhodopsin, is an internally directed chloride pump. The other two are photoreceptors involved in phototaxis.

Many of the nonmethanogenic archaea use proteins and amino acids as carbon and energy sources but a few have been shown to use carbohydrates. *Halobacterium saccharovorum* has been shown to possess a modified KDPG (Entner/Douderoff) pathway (compare with Figure 10, p. 59) where unphosphorylated glucose is oxidized to gluconate by a NAD-dependent dehydrogenase and a gluconate dehydrogenase converts the gluconate into 2-keto-3-deoxygluconate. Next, a kinase, using ATP as the phosphate source, phosphorylates the 2-keto-3-deoxygluconate to form KDPG. KDPG aldolase cleaves this to form glyceraldehyde-3-phosphate and pyruvate as in the normal KDPG pathway, and glyceraldehyde-3-phosphate is converted to pyruvate by the usual HDP pathway enzymes. Pyruvate is converted to acetyl-CoA using pyruvate/ferredoxin oxidoreductase.

Thermoacidophilic archaebacteria such as *Sulfolobus solfataricus* and *Thermoplasma acidophilum* have no phosphofructokinase and, therefore, no HDP pathway. Instead, they have been shown to use another variation of the KDPG pathway, a completely non-phosphorylated pathway. Glucose is oxidized to gluconic acid, which is dehydrated to 2-keto-3-deoxygluconate. This is cleaved by an aldolase to yield pyruvate and glyceraldehyde. The glyceraldehyde is oxidized to glyceric acid, which is phosphorylated to 2-phosphoglyceric acid and converted to phosphoenolpyruvate. The latter compound is cleaved by pyruvate/ferredoxin oxidoreductase. There is no evidence for acetyl phosphate formation as a precursor to acetyl-CoA formation (Danson 1988).

REGULATION OF PHYSIOLOGICAL PROCESSES

Operon organization and expression

Perhaps the best example of operon organization and expression is provided by the set of genes required for lactose metabolism. In order to grow using the disaccharide lactose as a carbon and energy source, *E. coli* requires the presence of the enzyme β-galactosidase (once termed lactase) to cleave lactose into the monosaccharides, D-glucose and D-galactose. This enzyme is inducible, meaning that without the presence of lactose in the growth medium, there is only a very low basal or endogenous level of the enzyme in the cells. However, within minutes of addition of lactose to the medium the differential rate of synthesis of the enzyme, which is the rate of enzyme synthesis compared with the rate of total cell protein synthesis, increases until the amount of enzyme in the cells is 10 000 times higher than previously. If the lactose is removed from the medium, the rate of β-galactosidase synthesis decreases to the previous basal level while at the same time the concentration of enzyme in the cell is diluted by the synthesis of new proteins during cell growth. When cells are provided with lactose, several proteins are induced at the same time, a phenomenon known as coordinate induction. These proteins include β-galactosidase, lactose permease (the protein responsible for the transport of lactose through the cell membrane), and a protein catalyzing the acetylation of certain disaccharides.

During studies on lactose catabolism by *E. coli*, mutants were obtained that changed the regulation of these three enzymes such that they were produced at high levels in the absence of lactose. These mutants where termed constitutive mutants and, because a single mutation affected the regulation of all three proteins at once, the expression of the genes coding for those proteins had to be under the control of a common regulatory gene. Such enzymes are said to be coordinately controlled. The analysis of structural gene and regulatory gene mutations that effected lactose catabolism in *E. coli* led Jacob and Monod (1961) to postulate the operon model of gene regulation. This model proposed that a single regulator controlled expression of all three genes because the genes are cotranscribed on a single transcript. An operon is a region of DNA containing cotranscribed genes and control sequences.

Further studies provided evidence that the regulatory gene produced a product that normally blocked the formation of the enzymes involved in lactose catabolism. This product was termed the repressor. When the lactose operon was induced, an RNA polymerase would bind to the DNA at a site named the promoter region. In the absence of the inducer, however, the repressor protein bound to a DNA site named the operator region and prevented transcription by the RNA polymerase. Thus, the regulation was a negative control system in that the normal function of the regulator gene was to prevent the formation of mRNA for the operon. Any mutation resulting in the loss of function of the repressor (the product of the *lacI* gene) would result in the loss of repression and the constitutive expression of the enzymes of the lactose operon (Jacob and Monod 1961).

THE LACTOSE OPERON OF *E. COLI*

The arrangement of the genes of the lactose operon of *E. coli* is shown in Figure 11. The order of the three

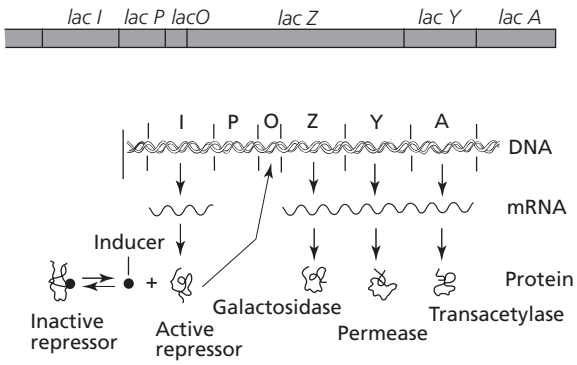

Figure 11 *The lactose operon of* Escherichia coli

structural genes of the *lac* operon is *lacZ*, *lacY*, and *lacA*, encoding β-galactosidase, lactose permease, and thiogalactoside transacetylase, respectively. The promoter (*lacP*) and operator (*lacO*) regions are adjacent to the *lacZ* gene and actually overlap. The regulatory gene (*lacI*) is situated next to the operon. The regulatory gene (*lacI*) is the structural gene for a protein that is termed the lactose repressor. The lactose repressor is an allosteric protein that is normally expressed constitutively and is a DNA-binding protein. When bound to the operator region the repressor blocks binding of RNA polymerase and blocks transcription of the operon. The repressor contains a binding site for the inducer of the lactose operon. The inducer serves as a negative effector and, when bound to the repressor, it decreases the affinity of the repressor for DNA. With the removal of the repressor from the operator region, RNA polymerase can transcribe the operon. The actual inducer molecule is not lactose but an isomer of lactose, allolactose, which is a disaccharide formed by a side reaction of β-galactosidase. Lactose is a D-glucose-D-galactose dimer with a β-1,4 linkage, whereas allolactose differs from this in that it possesses a β-1,6 linkage.

The purpose of the thiogalactoside transacetylase, coded by the *lacA* gene, remained a mystery for a number of years. This enzyme is now believed to be associated with the lactose operon for purposes of detoxification. The enzyme possesses the ability to acetylate a variety of different disaccharides, but lactose is one of the poorest substrates. The lactose permease is able to transport a number of disaccharides other than lactose into the cell and if these disaccharides cannot be further metabolized they accumulate and may be toxic for cell growth. Upon acetylation of these disaccharides by the thiogalactoside transacetylase, they are rapidly excreted by the cells and not transported back into the organism.

THE NATURE OF INDUCERS

The inducer of a catabolic enzyme pathway need not be the initial substrate of the pathway. It may be an intermediate or an end product of a pathway, or even a compound formed by a side reaction of an enzyme in the pathway, as for the lactose operon of *E. coli*, where allolactose is the inducer. If the inducer is produced by the action of the enzyme it induces, some small basal or endogenous level of the enzyme must always be present for the inducer to be quickly synthesized once the pathway substrate becomes available. For the lactose operon of *E. coli*, in addition to the normal promoter site for binding RNA polymerase, there is a second site that permits the synthesis of small levels of permease and β-galactosidase, even in the absence of inducer. Enough of these proteins are present in uninduced cells to permit the rapid synthesis of allolactose when lactose becomes available.

THE LACTOSE OPERON OF *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus possesses a different pathway of catabolism for lactose, using enzyme *IILac* and enzyme *IIILac* of the phosphoenolpyruvate phosphotransferase (PTS) system to transport lactose through the cell membrane. During transport, lactose is phosphorylated to phospholactose, which is cleaved by a phosphogalactosidase enzyme to form D-glucose and D-galactose-6-phosphate. The genes coding for these three enzymes are designated *lacF*, *lacE*, and *lacG*, respectively, which form an operon that appears to be under the negative control of a repressor gene (*lacR*). Interestingly, these genes are in a larger operon including *lacABCD* that encodes genes whose products are involved in the conversion of galactose-6-phosphate to tagatose-6-phosphate and the further metabolism of tagatose-6-phosphate (Rosey et al. 1991). Galactose-6-phosphate is the inducer of the *lac* operon in *S. aureus*.

THE L-ARABINOSE OPERON OF *E. COLI*

While studying the catabolism of the pentose L-arabinose by *E. coli* strain B/r, Englesberg and coworkers found differences from the regulation of the lactose operon (Sheppard and Englesberg 1967). After the transport of L-arabinose into the cells it is isomerized by the enzyme L-arabinose isomerase to the 2-ketopentose, L-ribulose. The enzyme L-ribulokinase phosphorylates L-ribulose to form L-ribulose-5-phosphate, which is epimerized to D-xylulose-5-phosphate by L-ribulose-5-phosphate. The latter compound is further degraded by other cellular enzyme systems. These three enzymes are all coordinately induced when L-arabinose is present and the genes encoding for the three enzymes are termed *araA*, *araB*, and *araD*, respectively. These genes are organized into an operon with a regulatory gene, *araC*, located next to the operon. In contrast to the *lac* operon, deletions of the *araC* gene do not lead to constitutive expression of the enzymes. Instead,

inactivation of *araC* causes the L-arabinose catabolic enzymes to become uninducible. Because the function of the regulator gene is essential for gene expression, this is termed a positive control system.

The regulatory protein, encoded by the *araC* gene, can interact with four different operator regions, O_1, O_2, I_1, and I_2, all connected to the L-arabinose operon. When *araC* binds to both the O_2 and I_1 sites, a loop is formed in the DNA preventing transcription of the operon (Huo et al. 1988). When the inducer, L-arabinose, binds to the allosteric repressor protein, the protein's DNA binding affinity changes and it now binds to operator sites I_1 and I_2, permitting transcription of the operon into mRNA.

REGULATION BY ATTENUATION

Another means of regulating the expression of biosynthetic pathways is by attenuation. The classic example of regulation by attenuation is the L-tryptophan operon in *E. coli* (Yanofsky 1981). Between the location where transcription of the operon initiates and the beginning of the first structural gene of the operon, there is a stretch of DNA termed the leader region. This region contains four stretches of sequence that, when transcribed, have the potential to form three RNA structures termed hairpins. Also located within the region are two L-tryptophan codons and an RNA polymerase pause site. As RNA polymerase proceeds through the leader region it stalls at the pause site. This delay permits a ribosome to bind to the mRNA and proceed towards the RNA polymerase. If internal L-tryptophan levels are high, then the level of tRNA charged with L-tryptophan will also be high and the ribosome can proceed to the stalled polymerase. This allows the formation of a hairpin from the downstream hairpin sites. This hairpin causes transcriptional termination. If L-tryptophan concentrations are low this causes the ribosome to stall at the L-tryptophan codons. This allows the formation of a hairpin from the middle two hairpin regions and prevents formation of the hairpin that terminates transcription. This form of regulation is different from regulation by repressor or activator proteins because transcription is allowed to proceed but the concentration of substrate determines whether the transcription will continue into the region containing the structural genes.

Reports of bacterial operons regulated by transcriptional attenuation mechanisms have been increasing (Yanofsky 2000). The *ilv* and *leu* genes in enteric bacteria are regulated mostly by a translational-dependent attenuation but the *ilv–leu* operon of *B. subtilis* is reported to be regulated, at least in part, by transcriptional attenuation (Lu et al. 1995). Regulation by attenuation has also been reported for the *E. coli* threonine and histidine operons. The *B. subtilis* aminoacyl-tRNA synthetase genes are also regulated by attenuation.

CELL-DENSITY-DEPENDENT GENE EXPRESSION

Certain genes are only expressed when the concentration of bacterial cells reaches a critical level. This cell density-dependent gene expression is termed 'quorum sensing' (Fuqua et al. 1996). The prototype quorum-sensing system is the *lux* system in the bacterium *Vibrio fischeri* (Engebrecht et al. 1983). *Vibrio fischeri* can exist as either a free-living bacterium or in the light organ of certain fishes or squid. When present in the light organs it activates the expression of the *lux* genes, which produce proteins required for bioluminescence. The expression of the *lux* genes only occurs in the light organ because cell density reaches much higher concentrations there than when the cells are present in the open ocean. Cells can sense cell density because they release a compound (the autoinducer) into the surrounding environment and the external concentration of autoinducer increases as the cell population increases. The autoinducer is freely diffusible across cell membranes, so when the concentration of autoinducer in the surrounding medium reaches the proper level, it can interact with a positive regulator in the cell that activates *lux* gene expression. Quorum-sensing systems have been found to be widespread in bacteria.

The autoinducer in *V. fischeri* and in many bacteria is a hydrophobic derivative of n-acyl homoserine lactone (Eberhard et al. 1981). In gram-positive bacteria quorum sensing takes place using a posttranslationally processed peptide (Dunny and Leonard 1997). Typically this peptide functions as a signal for the sensor component of a two-component sensor-regulator regulatory system. Examples of processes regulated by peptide-dependent quorum sensing are competence, the ability to take up DNA in *B. subtilis* and *Streptococcus pneumoniae*, the expression of virulence factors in *S. aureus*, and the production of antimicrobial peptides.

Global control networks

If more than one operon is under the control of a regulatory factor, those operons are said to constitute a regulon. Cells typically contain many overlapping regulons. Another term for a large collection of genes regulated by a common mechanism is global regulatory network. By having such global control networks, cells can quickly respond to sudden changes in the environment by increasing or decreasing the rate of synthesis of entire systems of proteins.

CATABOLITE REPRESSION

A good example of a global regulatory system is the catabolite repression system. It is not uncommon for an organism preferentially to utilize those catabolites most common in its natural environment. Therefore, bacteria given two possible carbon and energy sources simultaneously may not use them at the same time but may use

one before beginning catabolism of the other. An example is the 'glucose effect' first reported in 1942 by Gale and Epps. They found that the synthesis of certain enzymes by *E. coli* was inhibited by the presence of D-glucose in the medium. In 1947, Monod reported that the presence of glucose prevented the induction of many other sugar-catabolic pathways in *E. coli*, especially those sugars catabolized by inducible pathways. If two carbohydrates, such as glucose and lactose, were both present in the growth medium, the growth curve showed two successive growth curves separated by a lag period (Monod 1947). This was termed 'a diauxic growth curve' and it is possible to classify carbohydrates as to whether or not a diauxic growth curve results when they are incubated with glucose.

In 1961, the expression 'catabolite repression' was used to describe this type of repression of enzyme pathways. It was later shown that catabolite repression by glucose of the synthesis of β-galactosidase involves a block in the synthesis of mRNA for the enzyme. Mutants with a *lacI* deletion and, therefore, constitutive for β-galactosidase production, are still subject to catabolite repression by glucose (Magasanik 1961).

Catabolite repression in *E. coli* and other enteric bacteria

In enteric bacteria, the rate of transcription of genes that are subject to regulation by catabolite repression was found to be controlled by the intracellular concentration of a nucleotide, cAMP. The internal level of cAMP in *E. coli* is very low in cultures grown on glucose and addition of cAMP to the medium results in partially overcoming the glucose catabolite repression. Mutants deficient in adenylate cyclase, the enzyme responsible for the synthesis of cAMP from ATP, can synthesize β-galactosidase only when cAMP is added to the medium (Pastan and Adhya 1976).

Mutants were obtained that could not make β-galactosidase or similar catabolite-repressible enzymes even when cAMP was added to the medium. These mutants were found to be deficient in the synthesis of a protein named the catabolite gene activator protein (CAP) or sometimes the cAMP receptor protein (CRP). This protein was found to be a homodimer, with each monomer capable of binding one molecule of cAMP. The cAMP–CRP complex binds to DNA at the operator-distal part of the promoter region of the lactose operon and increases RNA polymerase binding efficiency. Thus, the rate of transcription of the operon is dependent upon the presence of both the CAP protein and the intracellular level of cAMP. In the presence of glucose the internal concentration of cAMP is low and the transcription of those operons requiring the cAMP–CAP complex is repressed.

The CAP protein is a global regulator of gene expression and has been found to activate transcription at more than 50 promoters. The intracellular level of cAMP appears to be regulated at least partially in growing cells by the rate of activity of adenylate cyclase. Under conditions of catabolite repression, adenylate cyclase activity is inhibited and the cellular level of cAMP decreases and, as a result, the activity of the cAMP–CAP-dependent promoters also decreases. When adenylate cyclase is active the cAMP–CAP complex forms and activates transcription through direct contact between cAMP–CAP and RNA polymerase (Busby and Ebright 1999). The cAMP–CAP complex has different affinity for different promoters so the response of separate operons may differ at any concentration of cAMP. The genes controlled by CAP are sometimes referred to as members of the carbon/energy regulon.

The activity of adenylate cyclase in *E. coli* appears to be regulated by interactions with components of the cytoplasmic membrane transport system. In the presence of carbohydrates being brought through the membrane by the PTS, adenylate cyclase is almost inactive and the level of cAMP in the cells is very low. It has been suggested that when glucose becomes limiting the phosphorylated form of enzyme IIA^{Glc} activates adenylate cyclase, resulting in the activation of adenylate cyclase and the synthesis of cAMP (Hogema et al. 1999). Phosphorylated IIA^{Glc} accumulates because no glucose is present to allow dephosphorylation. Conversely, when cells are growing upon glucose, IIA^{Glc} will transfer its phosphate group to the incoming sugar and it will mostly be present in the cell in the unphosphorylated form that does not activate adenylate cyclase.

Other mechanisms of catabolite repression

Catabolite repression also exists in bacteria such as those in the genus *Pseudomonas*. However, several studies have shown that in *P. aeruginosa* and *P. putida*, cAMP is not involved with catabolite repression. Because these bacteria receive most of their energy by oxidative phosphorylation with electrons obtained from the oxidation of acetate by the TCA cycle, it is catabolites such as acetate and TCA-cycle intermediates that cause repression of other catabolic pathways including those for glucose (MacGregor et al. 1991).

The control of catabolite repression in gram-positive bacteria may be controlled by a number of different mechanisms. No cAMP has been detected in *B. subtilis* under aerobic conditions. The addition of cAMP does not effect catabolite repression in that organism and no CAP-like protein has been identified in any gram-positive bacteria (Saier et al. 1996). *Cis*-acting sequences named catabolite responsive elements or CRE seem to be responsible for catabolite repression in *B. subtilis* and these CREs elements also have been found to function in *B. megaterium* and *Staphylococcus xylosus*. A catabolite control protein (CcpA) has been identified and mutations in this protein have been found to prevent

CRE-dependent catabolite repression in both *B. subtilis* and *B. megaterium*. CcpA has been shown either to repress or activate expression of its targets.

The exact mechanism of action of CcpA is still not understood. Repression of gene activity apparently involves the PTS system, but in a different manner than in gram-negative bacteria. During the normal function of the PTS system, the HPr protein is phosphorylated at His-15. However, in a reaction that might be unique to gram-positive bacteria, the HPr protein is phosphorylated at Seryl residue 46 by an ATP-dependent kinase, a reaction that is required for catabolite repression (Deutscher et al. 1994). HPr(Ser-P) acts as a corepressor and may directly interact with CcpA in vivo (Kraus et al. 1998).

SIGMA FACTORS

Bacterial RNA polymerases consist of at least four polypeptides termed α, β, β′, and sigma (σ). The core RNA polymerase consists of two α subunits and the β and β′ subunits, and this core unit will bind randomly to DNA. If the σ subunit is also present, the complex is termed a holoenzyme. The presence of the σ subunit directs the holoenzyme to bind at specific promoter regions and initiate transcription. After the first few nucleotides of the RNA transcript have been synthesized, the σ factor is released and the core enzyme continues the polymerization of the ribonucleotides. By the use of multiple σ factors, the cell can initiate the transcription of different regulons, thereby synthesizing new families of enzymes in response to changing environmental conditions. The RNA polymerase holoenzymes have been designated as E-σ*x*, with E representing the core polymerase and σ*x* the particular σ factor attached.

Sigma factors in *E. coli*

Lonetto et al. (1992) studied the amino-acid sequence of bacterial σ factors and classified them into three broad groups. Group I proteins are the primary σ factors. Primary σ factors from different organisms are very similar and the regions involved in promoter recognition are especially highly conserved. These primary σ factors are required for survival of bacterial cells and are responsible for most RNA synthesis in exponentially growing cells. The primary σ factor in *E. coli* is σ^{70}, which recognizes the promoters of most of the genes expressed during exponential growth. A second group of σ factors are very similar to group I but are not essential for cell growth. The third group consists of the alternative σ factors that are responsible for the transcription of specific regulons. Apparently these σ factors all compete for RNA polymerase.

Sigma factors provide another mechanism for cells to establish global regulatory networks. The ability of the cell to produce a variety of alternative σ factors with different promoter specificity allows σ factors to regulate a wide variety of physiological responses. A good example of the role of σ factors in regulating cellular responses to environmental changes is provided by the heat shock response of *E. coli*. Bacteria have evolved mechanisms that permit them to resist a variety of different environmental stresses, such as heat or cold shock, acid pH, lack of oxygen, and starvation. Bacterial response to stress is to change the expression of groups of genes known under the general term of 'stress genes.' The response of microorganisms to elevated temperatures has been termed heat-shock response. Heat-shock response is a rapid, transient increase in the rate of synthesis of certain proteins (heat-shock proteins (HSP)) within seconds of a shift of the culture to higher temperatures. If the growth temperature of *E. coli* is shifted up, for example from 37 to 46°C, the rate of synthesis of proteins concerned with transcription and translation is decreased. However, the synthesis of HSPs is increased, some by as much as 100 times. If the growth temperature is returned to normal, the rate of synthesis of these proteins returns to normal. HSPs provide the cells with some resistance to high temperatures.

Heat-shock-induced transcription in *E. coli* is associated with the heat-shock-specific σ factor, σ^{32}. The heat-shock regulon consists of over 20 genes and at least 13 promoters are known to be specifically transcribed by the holoenzyme containing σ^{32} ($E\sigma^{32}$). The actual amount of gene transcription depends upon the cellular concentration of σ^{32} and, under heat-shock conditions, σ^{32} levels increase by enhanced synthesis, elevated stability, and increased activity of the σ factor. During normal growth conditions, the σ^{32} polypeptide is very unstable in vivo, with a half-life of about 1 min. When the temperature is shifted from 30 to 42°C, the polypeptide rapidly becomes stabilized and has an eight times longer half-life (Mager and De Kruijff 1995). This change in stability is probably a result of changes in σ^{32} binding to the chaperone DnaK (Bukau 1993). When σ^{32} binds to DnaK it becomes more susceptible to protease degradation. However, when heat shock occurs, resulting in an increased association of DnaK with misfolded proteins, σ^{32} does not bind to DnaK and its turnover time of DnaK decreases. This permits it to bind to core polymerase and activate expression of target genes.

The gene encoding another sigma factor, σ^{E}, also appears to be essential for growth of *E. coli* at temperatures above 43.5°C. The activity of this σ factor appears to be directly modulated by two proteins, RseA and RseB. RseA is a membrane-bound protein that binds σ^{E}, preventing it from interacting with core polymerase. Upon heat stress RseA is probably degraded by a periplasmic protease (Ades et al. 1999). This frees up σ^{E} for interaction with core polymerase and gene activation. This regulatory mechanism suggests that genes activated by σ^{E} are involved in response to heat-shock response in the periplasm. Proteins that inactivate σ factors by directly binding them are termed antisigma factors.

The starvation of bacteria can also result in increased resistance to a variety of different stresses. This starvation effect depends to some extent upon alternative σ factors and involves the expression of starvation genes that code for special resistance factors (Nystrom 1999). This starvation-induced resistance depends upon expression of the regulons controlled by the alternative σ factors, σ^{32} and, a major regulator of the general starvation response in *E. coli*, σ^{38} (σ^{S}). Starvation causes a general response with the synthesis of 30–50 proteins. If cells of *E. coli* in the stationary growth phase are starved for glucose, they also gain an increased resistance to heat, to oxidizing agents such as hydrogen peroxide, and to sodium chloride. The 'starvation proteins' of *E. coli* are also induced by exposure to heat shock or oxidative stresses.

Sigma factors in *B. subtilis*

Another bacterium in which the role of sigma factors has been extensively investigated is *B. subtilis*. The *B. subtilis* σ factor termed σ^{A} (formally σ^{55}) is the primary σ factor present in vegetatively growing cells (Haldenwang 1995). The alternative sigma factor σ^{B} (σ^{37}) controls general stress response but is also active in cells in the stationary phase. Under nonstress conditions σ^{B} is expressed but inactivated by complexing with an antisigma factor. Interestingly, under stress conditions the antisigma factor is inactivated by a separate protein, which acts as an anti-antisigma factor (Vijay et al. 2000). A phosphorelay is involved in activating and inactivating the various factors during σ^{B} activation. Another well-studied σ factor is σ^{D}, which controls the chemotaxis-motility regulon.

Under conditions of starvation, *B. subtilis*, begins the differentiation process that results in the formation of an endospore. After the initiation of sporulation, there is an asymmetrical cell division forming two cells of different sizes. Sigma factors play an essential role in the developmental processes leading to sporulation (Haldenwang 1995). Available evidence indicates there is a cascade of interactions among the various σ factors required for sporulation. During activation of sporulation σ^{F} is the first prespore-specific σ factor, while in the mother cell, a different σ factor, σ^{E}, is the first σ factor activated. It has been suggested that σ^{F} is activated first; it somehow activates σ^{E}, and then sigma factors σ^{F}, σ^{G}, and σ^{K} are activated. The σ^{G} and σ^{K} proteins are required for activation of genes expressed late in the sporulation process.

Two-component signal-transduction systems

External environmental changes are often detected by bacteria using a system referred to as a two-component sensor regulator. This name refers to the fact that in many bacteria this sensing system is made up of a sensor kinase and a response regulator. The protein that detects the external signal is usually a transmembrane protein kinase, which transmits a signal by means of its cytoplasmic domain. Signal transfer involves transfer of a phosphate group from a histidine on the sensor kinase to the response regulator. Binding the phosphate alters the regulator's activity allowing it to bind to its target genes. A phosphatase may later remove the phosphate, restoring the system to its original state. There are many examples of two-component sensor regulator components in bacteria, although not all contain only two components. Some examples have been mentioned previously, including the nitrate-sensing system in *E. coli* and proteins sensing attractants or repellants during chemotaxis.

Altering the catalytic activity of existing enzymes

FEEDBACK INHIBITION

The regulatory processes described previously affect the expression of genes encoding proteins carrying out a particular physiological response. The process named feedback inhibition acts to decrease the activity of previously synthesized enzymatic pathways. Feedback inhibition occurs most commonly where the end product of a pathway inhibits the activity of the first enzyme in the pathway that is specific for the synthesis of that particular end product. The regulated enzyme is usually allosteric, with separate binding sites for substrate and effector molecules. In most cases the product of the pathway acts as a negative effector and, when bound to the enzyme, decreases the affinity of the enzyme for its substrate. To limit substrate flow through a pathway, it is only necessary to inhibit the activity of the first enzyme in the pathway. With the inhibition of that enzyme, the latter enzymes in the pathway will have no substrates and production of the end product must cease.

Feedback inhibition in branching biosynthetic pathways

In many biosynthetic pathways a single reaction can produce a product of which further metabolism leads to the synthesis of two or more different end products. Bacteria have developed different strategies to modulate the activity of these key reactions using feedback inhibition. One mechanism is termed cumulative inhibition. This is where the initial enzyme in the pathway has allosteric sites for each of the products eventually produced by metabolism of the product of the regulated enzyme. However, the enzyme can only be completely inhibited by the presence of multiple effectors. Because multiple pathways are dependent on the activity of the regulated enzyme, no single product should inactivate the enzyme. A variation on this regulation is concerted inhibition, which is when a single end product will not

affect enzyme activity, but when multiple products are present the enzyme is inhibited.

Another type of inhibition is termed sequential inhibition. This occurs when two or more pathways use the same substrate produced by a shared enzyme, and the pathways are subjected to feedback inhibition, resulting in accumulation of the common substrate. If that substrate then causes feedback inhibition of the enzyme producing it, feedback inhibition of the terminal pathways will result in feedback inhibition of the initial common enzyme. An alternative, but very common, method for a bacterium to regulate the activity of an enzymatic reaction shared by several pathways, is for the organism to possess different enzymes to catalyze the same reaction. Different genes encode these isozymes (or isofunctional enzymes) and, therefore, can be both repressed and inhibited separately. Regulation by means of isozymes permits each structural gene coding for an isozyme to be part of a different regulon controlled by a separate end product. The possible disadvantage of the organism having to possess genes to code for two different enzymes catalyzing similar reactions may be offset by the efficiency of regulation.

Enzyme inhibition and activation in catabolic pathways

The regulation of the activity of allosteric enzymes by effectors also functions in catabolic pathways. The effectors may be negative, inhibiting enzyme activity, or positive, activating enzyme activity. An example is the regulation of the activity of lactate dehydrogenase in homolactic acid bacteria (see above). In these bacteria, the reduction of pyruvate to lactate by lactic dehydrogenase permits the rapid oxidation of NADH that has been reduced by the hexosediphosphate pathway. If the rate of NADH oxidation is insufficient to maintain a pool of NAD^+, there will be an increase in the internal pool of fructose-1,6-bisphosphate. Lactate dehydrogenase from these organisms is an allosteric enzyme that has an absolute requirement for fructose-1,6-bisphosphate as a positive effector. The increase in the internal pool of this diphosphate results in the activation of more lactate dehydrogenase enzymes and an increase in the rate of NADH oxidation.

COVALENT MODIFICATION

Another strategy used by cells to modulate the activity of active enzymes is covalent modification. Two examples of this were discussed previously. Enzymes in *E. coli* add a phosphate to the active site of isocitrate dehydrogenase to limit its activity during growth on acetate or other compounds metabolized through acetyl-CoA (Hurley et al. 1990). The other example discussed above was covalent modification in ADP-ribosylation of nitrogenase (Ludden 1994). Modification reduces activity of the nitrogenase.

One of the best-studied examples of covalent modification is the adenylylation of glutamine synthetase in enteric bacteria. Glutamine synthetase is a critical enzyme for the incorporation of ammonia into cellular constituents. This enzyme is used for nitrogen incorporation when nitrogen concentrations are low and uses the energy of ATP to drive the reaction. Because this ATP consumption can put a significant stress on energy levels in the cell, some bacteria, such as *E. coli* and *Salmonella typhimurium*, use covalent modification to fine-tune the activity of glutamine synthetase (Merrick and Edwards 1995). Glutamine synthetase is modified by addition of AMP to the enzyme; because the active enzyme is a dodecamer, as many as 12 AMPs can be added to the active complex (Yamashita et al. 1989). Addition of the AMP reduces enzyme activity, but the active site is not directly modified. A single enzyme, adenylyl transferase/adenylyl-removing enzyme, is responsible for modification of glutamine synthetase. The activity of this enzyme is controlled by another enzyme, designated PII, which is itself covalently modified by addition of UMP. The level of uridylylation of PII is dependent on the level of nitrogen in the cell. The net result is that when fixed nitrogen is high, the activity of glutamine synthetase is reduced to minimize ATP consumption. However, when nitrogen availability is low, glutamine synthetase is deadenylylated so it can be used for the incorporation of nitrogen.

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Bacterial genetics

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INTRODUCTION

Comparison of the current bacterial genetic literature with that of a generation ago reveal a 'sea change' in technical approach. Where once the study of bacterial genetics largely involved isolating mutants that occurred by chance in cell populations, a procedure that often required considerable ingenuity in the design of mutant selections or screens, and investigating the bases of mutation by reversion and complementation analyses, current procedure brings to bear a panoply of powerful molecular genetic techniques that enable an investigator to determine precisely a gene's composition and its mode of expression. These techniques include genomic and proteomic analyses supported by the computational power of bio-informatic databases. This chapter will discuss some of these technologies, and will attempt to indicate the value of both old and new approaches.

The similarities displayed among bacteria and the differences that distinguish one from another reflect the content and expression of the genes possessed by each individual cell. The genes control every property of a cell, including morphology, physiology, biochemistry, and, in general, an exact copy of each one is inherited by the cell's progeny. Genetics is the study of genes, their structures, organizations, and expression.

With the exception of some bacteriophages (see Chapter 5, Bacteriocins and bacteriophages), the genetic information of bacterial systems is encoded in DNA. The characteristics expressed by a cell are referred to as its phenotype. The set of genes found in any particular cell constitutes its genome, which generally consists of a large circular DNA molecule, the bacterial chromosome, and possibly one or more smaller circular DNA molecules called plasmids (Charlebois 1999).

Bacteria are, in general, very adaptable and may alter their phenotype in response to environmental change while the genotype remains unchanged. The ability to adapt the phenotype to the prevailing environmental conditions is determined genetically, i.e. not all the genes of a bacterial cell are expressed all the time. For example, β-galactosidase, an enzyme that hydrolyzes lactose to its constituent sugars, glucose, and galactose, is required by *Escherichia coli* only when lactose is its main source of carbon and energy and normally it is produced only under such circumstances (Jacob and Monod 1961; Prescott et al. 2002). The gene (*lacI*) and associated sequences that control the expression of β-galactosidase are, together with the gene for β-galactosidase itself, *lacZ*, and two other associated genes, *lacY* and *lacA* encoded at essentially the same location on the *E. coli* chromosome. The three genes *lacZ*, *lacY*, and *lacA* together form the *lac* operon, a set of genes that is expressed from a single promoter to produce, in the first instance, a single transcript (polycistronic mRNA) (Chapter 3, Bacterial growth and metabolism). This, in turn, is translated to produce the three gene products of the *lac* operon. This is just one example of the many complex systems that can be switched on or off to allow bacteria to make best use of the nutrients available to them and to adjust their biochemical makeup rapidly in response to changes in their environments. Such switches in gene expression generate phenotypic variation; the phenotype of the cell changes while the genotype

remains unaltered. By contrast, genotypic variation involves a change in the genetic information that determines the phenotype. This type of change can occur by mutation, i.e. an alteration of the nucleotide sequence of DNA, that can occur in one of several ways. Alternatively, gene content can be changed by acquisition of new genes from another bacterial cell, by one of three DNA transfer processes, transformation, transduction, or conjugation.

In general, all the cells in a bacterial culture respond phenotypically in the same way to a change (often nutritional) in their environment. The change in phenotype is normally rapid and reversible, e.g. synthesis of the enzyme β-galactosidase by *E. coli* starts within seconds of the addition of lactose to a suitable culture medium and ceases just as rapidly when lactose is withdrawn (or exhausted). In contrast, genotypic change (mutation) affects the individual cell, rather than the population, and is essentially permanent. There are, however, exceptions to these generalizations and it is sometimes difficult to determine into which category a particular variation fits. For example, lysozyme treatment of a culture of *Bacillus subtilis* converts the cells to protoplasts by removing the cell wall. Removal of lysozyme, however, does not always result in a return to normal cell physiology; the cells may continue to grow as protoplasts or may show mass reversion to the bacillary form, depending upon the conditions of culture. This is an example of a phenotypic change that, in some circumstances, can appear as heritable and permanent. By contrast, physiological changes resulting from mutation may give the impression of being phenotypic because bacteria can multiply so rapidly; a mutant cell, with a particular selective advantage, can overgrow a population of unaltered cells within a few hours. When the culture conditions are reversed, the mutant may be at a selective disadvantage and, in turn, may be overgrown by cells of the original type. If cells are examined on time scales that permit many generations, what is in reality genotypic variation may appear to be phenotypic variation.

Bacteria are haploid organisms with no true nucleus or sexual reproductive cycle. The first great expansion of knowledge of bacterial genetics followed the discovery that genes (DNA) can be passed from one bacterial cell to another by several mechanisms. The second major expansion of activity is now in progress and reflects the introduction of a raft of molecular genetic techniques such as restriction enzyme mapping, gene cloning, DNA sequencing, and site-directed mutation. The bacterium about which most is known is *E. coli* K12, whose single chromosome is a circular DNA molecule of approximately 2 mm, about 1 000 times longer than the bacterial cell in which it is contained. This DNA molecule contains c. 4 700 000 base pairs (bp) or 4 700 kilobases (kb), and accommodates approximately 4 300 genes. Other bacteria also have single circular chromosomes; this is true both of bacteria related to *E. coli*, such as *Shigella* and *Salmonella* spp. (Sanderson 1976), and of unrelated ones such as *Pseudomonas aeruginosa* (Holloway et al. 1979) and *B. subtilis* (Henner and Hoch 1980). Chromosome size varies considerably from one species to another. The smallest recorded to date is that of the intracellular pathogen *Mycoplasma genitalium* at 600 kb, while the largest found so far is the 9 455 kb circular chromosome of *Myxococcus xanthus*, closely followed by that of *Stigmatella aurantiaca* (9 350 kb) (Cole and Saint-Girons 1999). However, circular chromosomes are not a universal feature of prokaryotes. Linear chromosomes have been discovered in several bacteria, including *Borrelia burgdorferi*, the Lyme disease agent (which has a single linear chromosome of approximately 950 kb), *Agrobacterium tumefaciens*, which induces the formation of galls on a number of plants and has one circular chromosome (3 000 kb) and one linear one (2 100 kb), and *Streptomyces* species, sources of many antibiotics, which have linear chromosomes of approximately 8 000 kb (Cole and Saint-Girons 1999; Leblond and Decaris 1999). Circularity of the *E. coli* chromosome was first established by gene linkage experiments, and this provided the paradigm for several other bacteria. In the case of *E. coli* K12, its chromosome was also extracted and visualized (by autoradiography) and shown unequivocally to be circular (Cairns 1963), so confirming the genetic data. In more recent times, the form of a bacterial chromosome has more commonly been established by physical techniques such as restriction mapping. Restriction endonucleases are used to fragment the DNA molecule and the fragments are separated and their sizes determined by pulsed-field gel electrophoresis (PFGE). Fragment contiguity (fragment order) is determined in a number of ways, including cross-hybridization, which involves isolating and labeling a genomic fragment and using it as a probe to identify neighboring sequences. A summary of the different approaches to genome analysis and results obtained have been reviewed by Cole and Saint-Girons (1999). Now, chromosome circularity is much more readily established by complete genome sequencing programs. In addition, many bacteria carry relatively small DNA molecules called plasmids. These may also be circular or linear. Many are itinerant molecules that can be passed from one bacterial cell to another. Examples par excellence of this type are the resistance (R) plasmids responsible for much of the acquired antibiotic resistance displayed by many human bacterial pathogens. However, carriage of some plasmids appears to be a universal feature of a particular bacterial species (Cole and Saint-Girons 1999), in which case they appear to be indispensable and can be thought of as mini-chromosomes. The results of all of these studies have established that circularity is the norm for bacterial DNA, but there are exceptions, as noted.

Bacterial chromosomes must replicate before cell division and be distributed (partitioned) so that each daughter cell has its own copy. A complex organization

exists to ensure that this happens. Plasmids also encode replication functions that allow them to replicate independently of the bacterial chromosome of the host cell and many also encode partition functions, as well as other stabilization mechanisms. DNA molecules that replicate are called replicons (Jacob et al. 1963). The chromosome is the replicon(s) in which are encoded those genes that are essential for the cell's survival. It is also the largest replicon in the cell. However, as indicated in the previous paragraph, not all bacteria carry their essential genes on a single DNA molecule. In a few instances, such as *Brucella melitensis*, *Burkholderia cepacia*, and *Rhodobacter sphaeroides*, the genome comprises two or more large DNA molecules, which are essential to the cell (Cole and Saint-Girons 1999).

MUTATION

Mutation can be defined as any permanent change in the sequence of bases of DNA, irrespective of a detectable change in the cell phenotype. In practice, most mutations are detected by virtue of the change in phenotype generated, although now, with many DNA sequences being determined, it is found that silent mutation is very common, i.e. a change in the nucleotide sequence that has no detectable phenotypic effect. Indeed, silent mutation is a major feature of genetic drift within a bacterial species, and can be used for typing purposes (van Leeuwen et al. 2003). A given gene can exist in a variety of different forms – some functional, some not – as a result of mutation. These different forms are called alleles. The form in which a gene exists in a bacterium as it is first isolated from nature is defined as the wild-type allele of the gene; all other forms resulting from mutation of that gene are, by definition, mutant alleles.

Mutations may occur spontaneously, i.e. in the absence of a specific treatment to generate them, or they may be induced deliberately (the cells of the bacterial culture may be treated in one of several ways so as to provoke changes in the base sequence of the DNA). Classically, induction of mutations has been achieved by chemical treatment with known mutagens such as ethylmethane sulfonate (EMS, $CH_3CH_2.O.SO_2.CH_3$) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG, NTG) or by irradiation with ultraviolet or X-rays. These treatments generate DNA lesions at random and the particular mutation desired is selected by judicious manipulation of the subsequent growth conditions of the culture. In recent years it has become possible to target mutations, i.e. to introduce specific changes into a particular nucleotide sequence, using recombinant DNA technology, a procedure termed site-specific mutagenesis.

The spontaneous nature of mutation was demonstrated by Luria and Delbruck (1943) by an experiment known as the fluctuation test. The experiment exploited the observation that in a large enough population of cells sensitive to a particular bacteriophage (phage) there will be a few cells resistant to the phage. When equal volumes of a broth culture, each containing approximately 10^9 *E. coli*, were spread on a nutrient agar seeded with the phage, the number of colonies arising from resistant cells showed a small fluctuation from plate to plate (Figure 1). In a parallel experiment, small inocula (c. 10^3 cells) of the same culture were used to produce a series of 50 separate broth cultures. When the same volumes of these (i.e. containing approximately 10^9 bacteria) were spread on agar seeded

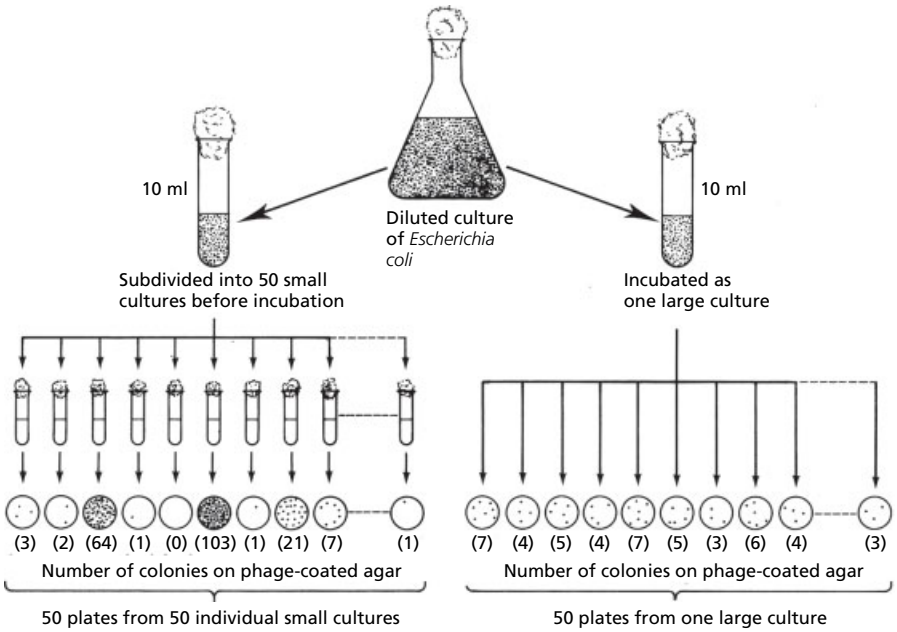

Figure 1 *Proof of the non-directed (spontaneous) nature of bacterial mutation by the 'fluctuation test'*

with the phage, much larger fluctuations were seen in the number of colonies obtained per plate (Figure 1). The results reflected the random nature of the mutations that had occurred in each culture, i.e. mutations arose at different times during the growth of the different cultures (the earlier the mutant appears the greater is the proportion of resistant cells in that population).

That mutations can arise spontaneously in a population of cells, independently of selective forces, was demonstrated directly and elegantly by Ester and Joshua Lederberg (1952). Approximately 10^8 phage-sensitive *E. coli* cells were inoculated onto nutrient agar and the plate was incubated. The resulting colonial growth was replica-plated to nutrient agar and nutrient agar seeded with the phage, where only phage-resistant cells grew to form colonies. Each colony that developed came from an inoculum of a phage-resistant cell (or cells) on the master plate. The locations of these were determined, and approximately 10^5 cells from the identical position on the phage-free nutrient agar were transferred into tubes of broth. From each of the resulting cultures 10^5 cells were spread on individual nutrient agar plates. The colonies that developed were again replica-plated to nutrient agar and nutrient agar seeded with phage and the entire process was repeated several times. By picking only phage-resistant colonies (identified by growth on the replica plate containing nutrient agar seeded with phage) from the phage-free nutrient agar to generate cultures for further testing, it was possible, eventually, to obtain a pure culture of a phage-resistant mutant. During the entire procedure the populations of cells used in the purification steps were never exposed to the phage, demonstrating unequivocally that resistant variants (i.e. mutants) emerged in the complete absence of the selective agent.

Mutagenesis

Much of our understanding of the mechanisms of mutation derives from experiments in which mutations have been induced by chemical agents that react with DNA in a known manner. Experiments designed to study the chemical basis of mutation have been carried out extensively with *E. coli* and several bacteriophages for which *E. coli* is a normal host. The protocols of mutation experiments vary according to the particular mutagen to be tested. For those reagents that react chemically with the constituent bases of DNA, bacteria are mixed with the mutagen and the suspension is incubated. The concentration of mutagen used and the time of incubation are determined pragmatically as those conditions under which approximately 5 percent of the input cells survive the treatment. The survivors are then permitted to grow in normal culture. Among the population that results, the proportion of mutants is high; in practice, however, the abundance of a particular mutation, although considerably enhanced with respect to the untreated starting culture, may still be below the level at which a screen of individual cells in the population would readily detect it. Hence, populations of cells obtained after mutagenic treatment may be subject to culture on selective growth medium, constituted to favor the growth of the desired mutant.

Some chemicals cause mutation because they mimic one of the constituent bases of DNA and may be incorporated into DNA during replication when present in the growth medium. 5-Bromouracil (5-BU), an analog of thymine with a bromine atom at the C-5 position instead of a methyl (CH_3) group, is one such compound. Once part of DNA, such substitutions cause misincorporation of normal bases in subsequent rounds of replication, e.g. 5-BU causes misincorporation of guanine. For these mutagens the cells must necessarily grow during treatment because DNA replication is essential for the effect. The concentration of the reagent has to be chosen so that the level of incorporation is high enough to raise the frequency of mutation but not so high as to kill the majority of cells in the treated population. Again, the conditions of treatment are determined pragmatically.

One easy way to decide if the mutagenic treatment has been successful is to examine the culture for an increase in the proportion of a particular class of mutants, for example auxotrophic mutants or antibiotic resistant mutants. Experimentally, the percentage of auxotrophs generated, i.e. mutants that have acquired a nutritional requirement, or the percentage of antibiotic resistant derivatives generated, is determined. For example, samples of a suitable dilution of a mutagenized culture of *E. coli* are spread onto nutrient agar, so as to give 100–150 colonies per plate after a suitable period of incubation. These colonies are then tested individually, by replica plating, to determine if they can grow on a minimal-salts agar that supports growth of the parent bacterium. A satisfactory mutagenic treatment will generate 5–10 percent auxotrophs that will be unable to grow on the minimal agar. Alternatively, the increase in the proportion of cells resistant to certain antibiotics, e.g. rifampicin, streptomycin, or nalidixic acid, to which the cells in the treated culture were susceptible before treatment, can be monitored.

Various experimental 'tricks' can be used to enhance the chances of success in finding a particular mutant. Two are worthy of mention. The abundance of particular auxotrophic mutations in a population can be increased by penicillin enrichment to the point where they form the majority. Penicillin kills only growing bacterial cells. Non-growing cells survive the treatment, provided the medium is osmotically buffered. Hence, a whole mutagen-treated culture is inoculated into liquid medium lacking the particular growth factor (e.g. amino acid, vitamin, purine, or pyrimidine) needed by the desired auxotroph. After a short incubation (15–20 min), during which time the growth of the mutant type

required is arrested, penicillin is added to the culture. The cells that do not need an exogenous supply of the growth factor grow and most are killed by the penicillin. The auxotrophs, because their growth has been arrested, survive. On removal of the penicillin and inoculation into a growth medium supplemented with the growth requirement, these cell types proliferate, together with any other survivors, to generate a new population of cells, among which the desired auxotrophs constitute an increased proportion. In practice, the procedure is not an all-or-none effect and a single round of treatment rarely increases the proportion of mutants sought by more than 100-fold. However, four or five cycles of treatment can usually enhance the proportion of the auxotroph required to greater than 10 percent of the surviving population of cells (Miller 1972), at which point mutants are easily identified. The procedure can be adapted to recover any mutant type where the mutation creates a conditional inhibition of growth that can be experimentally exploited.

The second technique that has been indispensable in the search for bacterial mutants is replica-plating (Lederberg and Lederberg 1952). Velvet is pressed gently onto the surface of agar on which bacterial colonies of interest have grown. A sample of each colony is imprinted on the velvet, which is then used to inoculate plates of sterile agar differing in composition from that on which the colonies originally appeared. The procedure preserves the pattern of colonies on the original plate. After incubation, the ability of individual colonies to grow under the new conditions can be assessed; e.g. a population of cells can be screened for auxotrophs of a particular type by replica-plating from a medium supplemented with the growth factor in question to a medium that lacks the growth factor but which is otherwise the same. The desired auxotrophs will fail to grow. An experienced person can easily screen 200–300 colonies per plate and, if necessary, several hundred plates per day.

Mutations can be divided into two types, micro- and macrolesions, depending on the extent of the alteration to the base pair sequence of DNA. Microlesions are also known as point mutations.

Microlesions

Point mutations are of two classes: base pair substitutions and frame-shift mutations. The first class comprises those mutants in which a single base pair has been altered, and can be subdivided into transitions and transversions. Such changes may be expressed (i.e. are detected as an altered phenotype) or silent. Frame-shift mutations are those in which one or a few base pairs have been added to or removed from the DNA with the consequence that the translational reading frame of the transcript, i.e. of messenger RNA (mRNA), of the mutated gene is altered. Point mutations are, in general, revertible, i.e. a second mutational event at the same site can precisely reverse the first mutation and restore the original base sequence.

A transition occurs when one purine is replaced by another purine and a pyrimidine is replaced by a different pyrimidine, e.g. a change from a guanine–cytosine (GC) base pair to an adenine–thymine (AT) base pair, or vice versa; in this situation the purine–pyrimidine axis of the double helix at the point of mutation is preserved. Most chemically induced base substitutions are transitions. Transversions are those base pair substitutions in which the purine–pyrimidine axis is changed as a result of the mutation, e.g. a change from GC to CG or to TA. Such changes are much less common than transitions and probably arise as a consequence of occasional mistakes made during DNA replication and by one or more of the cell's DNA repair systems.

All four of the bases in DNA can exist in different tautomeric forms which are related to each other by single proton shifts. The tautomeric forms arise when the keto (C=O) group of a base is changed to the enol (C–OH) form or when the amino ($-NH_2$) group is changed to an imino (=NH) group. The enol and imino groups exist in equilibrium with the keto and amino forms, respectively, but the latter predominate under normal conditions, i.e. at ambient temperatures and at pH values near neutrality. Such tautomeric shifts are important because they can relax the normally strict Watson and Crick complementary base pairing conventions, permitting, for example, G to pair with T instead of with C, while retaining purine–pyrimidine base pairing. Such mistakes do occur during replication but they are kept to a minimum by the intrinsic accuracy of most DNA polymerases together with associated proofreading functions (Kunkel and Bebenek 2000) and by DNA mismatch repair systems (Harfe and Jinks-Robertson 2000; Marti et al. 2002). If these occasional mispairings are not corrected, on replication one daughter chromosome will have, for example, an AT base pair at a particular location instead of a GC base pair, and mutation will have occurred. These different forms are illustrated in Figure 2.

As well as occurring naturally, transitions may be formed by the incorporation of base analogs into the DNA. Among the most important analogs are the halogenated pyrimidines, particularly 5-BU. This compound can be incorporated into DNA in place of thymine. The halogen atom is strongly electronegative and can pull electrons from the oxygen of the keto group of 5-BU, converting it to an enol group. 5-BU and similar analogs, therefore, tautomerize much more readily than the natural base under normal physiological conditions. In its keto form, 5-BU can pair with A and be incorporated into DNA to form a pseudo-AT base pair. If 5-BU then undergoes a tautomeric shift to the enol form, at the next round of replication it will direct the incorpora-

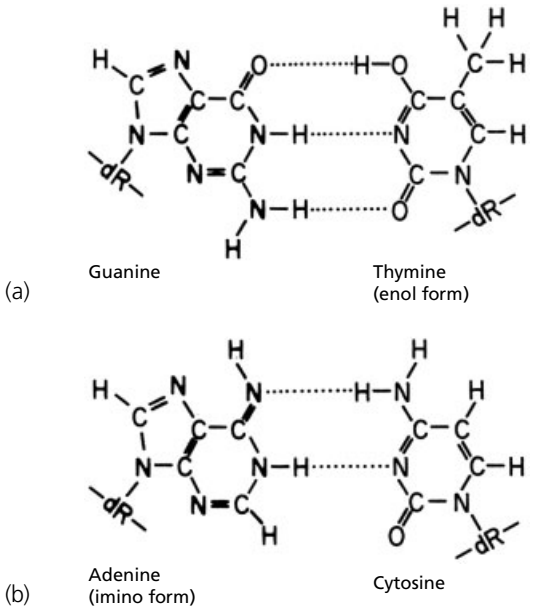

Figure 2 *Changes in base pairing as the result of tautomeric shifts. In the enol form* **(a)**, *thymine forms hydrogen bonds with guanine, instead of with adenine. In the imino form* **(b)**, *adenine forms hydrogen bonds with cytosine, instead of with thymine. Similar shifts in guanine and cytosine will also cause changes in base pairing.*

tion of G rather than A into the newly synthesized strand of DNA. A further round of replication will establish an AT to GC transition. Because they are incorporated into DNA by replication, base analogs are effective as mutagens only in growing cells. By contrast, other chemicals such as nitrous acid and various alkylating agents interact with non-replicating and replicating DNA.

Transitions can be generated by the action of nitrous acid. This chemical reacts with amine groups, converting them to hydroxyl groups (oxidative deamination). Deamination of adenine converts it to hypoxanthine, which can base pair with cytosine. Therefore, upon replication, hypoxanthine may cause misincorporation of C. A further round of replication will then establish the AT to GC transition. In similar reactions, thymine is converted to uracil and guanine is converted to xanthine. The first of these changes can be detected by a dedicated repair system and reversed (Pearl 2000), whereas the latter appears to be lethal rather than mutagenic.

Alkylating agents are powerful mutagens that predominantly generate transition mutations, although some have been reported to generate transversions and frameshifts as well; the mechanisms by which the latter changes are achieved are not fully understood. Alkylating agents all carry one, two, or more reactive alkyl groups. An example, widely used to generate bacterial mutants, is EMS. The action of alkylating agents on DNA is complex. They are known to react with purine bases, predominantly guanine, and the alkylated bases can cause base misincorporation if the damage is not corrected before replication. Bifunctional alkylating agents, i.e. those with two or more reactive alkyl groups, bring about cross-linking of the two strands of the double helix. This reaction is not, of itself, mutagenic but is lethal because cross-linking the strands of the double helix inhibits replication. However, attempts by the cell to repair the damage may result in mutation.

Transversions may arise spontaneously or may be induced at low frequency by some mutagenic treatments. However, no known chemical or irradiation treatment generates transversion exclusively, or even predominantly. Less is known about the mechanisms that generate transversions than about those that generate transitions, but many are probably due to replication or repair errors, rather than to the direct consequence of mutagenic treatment.

Acridine dyes induce frameshift mutations. The properties of these mutations are accounted for by the insertion (+) or deletion (−) of one, or a few, base pair(s) in DNA. Because the information encoded in mRNA is read as a sequence of triplets, insertion, or deletion of bases (other than multiples of three) causes a shift in the reading frame with the result that all codons (triplets) beyond the site of mutation are changed; a second frameshift, of similar magnitude but opposite sign, will correct the reading frame beyond the second mutation. If the amino acid sequence encoded by the base sequence between the two sites of mutation is not critical to the function of the gene product, the two mutations may cancel each other out and an altered but functional peptide may be produced. This is an example of pseudoreversion; the lost phenotype is restored by the second mutation but the original mutation remains. When this occurs, the second mutation is said to be a suppressor of the first and, because it is within the same gene, the phenomenon is termed intragenic suppression. When separated from the primary mutation by recombination, an intragenic frameshift suppressor mutation acts exactly like a primary frameshift mutation. Some frameshift mutations, particularly those that result from nucleotide insertions, will also revert precisely. Although frameshift mutants revert to the wild-type phenotype spontaneously and can be induced to revert at a higher rate by acridine dyes, frameshift mutations are never induced to revert by base analogs, nitrous acid, or hydroxylamine.

One of the best known acridine dyes is proflavine. Because it is a planar (flat) molecule with dimensions similar to that of a purine–pyrimidine base pair, it can intercalate between the stacked bases of DNA. It has been proposed that proflavine exerts its mutagenic effect on DNA undergoing recombination. This idea arose from the observation that while proflavine is a relatively poor mutagen for normal cells of *E. coli*, its mutagenic effect is considerably increased, (1) in the case of *E. coli*

bacteriophage T4 which indulges in a considerable degree of recombination during the course of its development; and (2) with partial diploids of *E. coli* formed during bacterial conjugation as the result of incomplete transfer of the chromosome of one bacterium to another. In the latter system, frameshifts are generated at a higher frequency in that region of the genome that is temporarily duplicated than in the remainder. Recombination of DNA involves breakage and reformation of phosphodiester bonds and it has been proposed that frameshift mutagens act by temporarily stabilizing mispaired sequences that may form following strand cleavage and transient strand separation. All frameshift mutagens are intercalating agents, but not all intercalating agents are frameshift mutagens. Another common intercalating agent, widely used in molecular genetics, which is a frameshift mutagen is ethidium bromide.

In practice, when studying a mutagen or suspected mutagen, it is easier to gauge its effects on the frequency of reversion of a set of known point mutants than to analyze its ability to generate 'knock out' mutations in a particular gene(s). Although reversion frequencies are generally somewhat lower, analyzing reversion offers considerably more sensitivity and convenience. This is the basis of the well-known Ames test, used to test chemicals to determine if they are mutagenic (and so, potentially carcinogenic) (Ames et al. 1973; Ames 1979).

Transition, transversion, and frameshift mutations generate alterations in the sense of the mRNA transcript. A mis-sense mutation gives rise to a codon that specifies an amino acid different from that originally encoded. The effect on the polypeptide product depends on the position of the altered amino acid and the precise nature of the change. In some cases, the polypeptide may show no obvious change in its properties; in others, all protein function may be lost. The change may generate a protein with altered thermal stability such that at a relatively low (permissive) temperature the protein functions more or less normally, whereas at higher (nonpermissive) temperatures the protein is nonfunctional; if this results in cell death the mutation is said to be a conditional-lethal mutation, i.e. the mutation is lethal, but only at the elevated temperature. In the case of most enteric bacteria, such as *E. coli*, the permissive temperature would normally be 30°C and the nonpermissive 42°C. There are many other examples of conditional-lethal mutants in which a potentially lethal mutation can be tolerated under particular physiological conditions. Such mutations can be invaluable when studying a vital component of the cell.

Point mutations may change a codon from one specifying an amino acid to one specifying termination of protein synthesis, namely UAG, UAA, or UGA, when a truncated polypeptide is produced instead of the full-length protein. Such mutant codons, when they appear within a gene, are called nonsense codons because they do not code for an amino acid. The introduction of a nonsense codon into an early gene of an operon may produce an effect known as polarity (Zipser et al. 1970). An operon consists of a set of genes that are transcribed as a single mRNA. A polar mutation is one that results not only in the loss of expression of the mutated gene but also in loss or significantly lower expression of those genes in the operon located distal to (or downstream from) the nonsense mutation, i.e. located on the opposite side of the mutation to that of the operon promoter. Translation of mRNA ceases at a nonsense mutation, the nascent peptide is released and ribosomes are discharged. Some may reattach to the mRNA downstream of the mutation at an appropriate ribosome binding site preceding the next translation start signal. When fewer than normal reattach, polarity results. In some instances the polar effect is so strong that genes distal to the nonsense mutation are barely expressed, if at all. Transcription of most genes is coupled to translation of the transcript. If these processes are uncoupled, transcription may also be terminated prematurely as a consequence of what is termed rho-dependent transcription termination (Landick et al. 1996). Severe polar effects are often related to the location of the mutation close to the start of the mutated gene, when translation is uncoupled from transcription for a prolonged distance. In such instances, mRNA distal to the point mutation is either not synthesized, due to premature transcription termination (attenuation), or is synthesized in considerably reduced quantities (Adhya and Gottesman 1978). This coupling of mRNA synthesis to its translation has evolved in some systems to provide a sensitive mechanism of control of enzyme synthesis termed attenuation (for further details, see Yanofsky 1987; Landick et al. 1996).

Suppression of base substitution mutations

As in the example of frameshift mutations, it is sometimes possible to have a second mutation at a site different from the first, the result of which is restoration of the lost phenotype. Such second site mutations are examples of suppressors, and the double mutants are again referred to as pseudorevertants. Mutations due to base substitutions may be suppressed by either an intragenic mutation or by an intergenic mutation, in which case the second mutation is in an entirely different gene from the first. In the former case, the suppressor mutation alters a second amino acid in the polypeptide; the second substitution neutralizes the damaging effect of the first and restores protein activity. Again, if the second mutation is separated from the first by recombination, it may also impose a mutant phenotype. Such suppressors are quite specific for the original mutation.

Intergenic suppression operates primarily at the level of protein synthesis. Mutations of this type mostly give

rise to altered transfer RNA (tRNA) molecules, although alterations in ribosome components, the so-called *ram* alleles of ribosomal protein genes, have been reported (Andersson and Kurland 1983). The altered tRNA molecules are able to read one, or sometimes two, of the three nonsense codons (UAG, UAA, and UGA) as an amino acid codon (Engelberg-Kulka and Schoulaker-Schwarz 1996). The change in specificity is, in many cases, the result of a base pair substitution in the DNA sequence that determines the anticodon of the tRNA species generating an anticodon to one of the terminating codons. Such suppressors will recognize the cognate codon, i.e. a particular nonsense mutation, wherever it occurs and will translate it. The efficiency with which a nonsense codon is translated by an appropriate suppressor tRNA will be determined by how effectively the mutant tRNA competes with the normal translation termination factors, which will also react to the nonsense codon, and the influence of the nucleotide context, i.e. the surrounding sequences within which the terminating codon appears (Bossi 1983). The affinities of both factors for the nonsense codon are known to alter with the context of the codon.

It was thought initially that suppression must be highly inefficient if the cell is to survive. This is now known not to be true and individual suppressors can translate nonsense codons with efficiencies of 60–75 percent. By contrast, some suppressors are relatively inefficient but, for many enzymes, even a small level of production, say 5 percent of the wild-type level, is sufficient to permit growth of the cell, albeit often with a lower growth rate than the original parent strain.

The generation of a suppressor, i.e. mutation of a tRNA gene, can be tolerated only if the cell produces an alternative tRNA with which it can translate the codons that the mutant tRNA would have recognized before its mutation, otherwise protein synthesis will be fatally compromised (Murgola 1985). In many cases this is possible because there is a redundancy of tRNA species for a number of codons. Hence, one of them can be mutated to recognize a different, but related, codon with little or no damage to the protein synthetic apparatus of the cell.

Since tRNA suppressors may be generated by mutation of the anticodon sequence, it should, in principle, be possible to isolate mis-sense suppressors as well. Several of these have indeed been isolated. They tend to involve minor species of tRNA and their efficiency of suppression is low but effective for the reasons given above. In addition, tRNA frameshift suppressors have also been isolated where the mutations are insertions in the anticodon sequences that increase their sizes from three to four bases (Engelberg-Kulka and Schoulaker-Schwarz 1996).

The importance of tRNA suppressors rests in the fact that they have been used extensively, particularly before gene cloning and sequencing was more or less routine, to demonstrate that mutations were located within nucleotide sequences that encode peptide products, since the effectiveness of such suppressors depends on their abilities to decode mutant codons and restore function to the protein affected by the mutation. Their existence also helped to establish the current model of protein synthesis.

Macrolesions

Alterations of DNA involving large numbers of base pairs are of four types: deletions, duplications, inversions, and additions. Deletion mutations are recognized by their inability to revert and by their failure to recombine with two or more distinct point mutations. A number of mechanisms can be proposed to explain the generation of deletions, including errors in replication, in recombination and in DNA repair. In addition, the activities of transposable elements can and do generate deletions.

Errors in DNA replication (copy errors) involve sequence duplications and slippage of one strand of the replicating DNA in relation to the other. A disruption in the pairing between the parental template strand, DNA polymerase and the newly synthesized daughter strand could lead to either of the two results outlined in Figure 3. After having separated transiently, the two strands re-anneal, but misalign because of the sequence duplication. The misalignment is accommodated by one of the strands looping out part of its sequence. A 'jump ahead' in copying produces a deletion in the daughter strand; a 'jump back' produces a sequence duplication.

Deletions and inversions may result from intra- and inter-replicon recombination, as outlined in Figure 4. These events also require sequence duplications. Recombination between homologous sequences carried in the same orientation on the same DNA molecule results in the loss of one copy of the duplicated sequence and of the sequence between the duplication. Recombination between homologous but inverted sequences inverts the intervening sequence. Inter-replicon recombination between regions that have sequence duplications may result in sequence duplication in one of the DNA molecules involved and deletion in the other (what is lost by one molecule is gained by the other) (Figure 4).

Mutations induced by ultraviolet light

Mutations may be induced by ultraviolet (UV) light. DNA strongly absorbs UV light at an absorption maximum of c. 260 nm. Cells are killed by UV light and there is a high proportion of mutants among the survivors. The most frequent lesion introduced into DNA by UV irradiation is the formation of pyrimidine dimers; covalent bonds are formed between adjacent pyrimidines on the

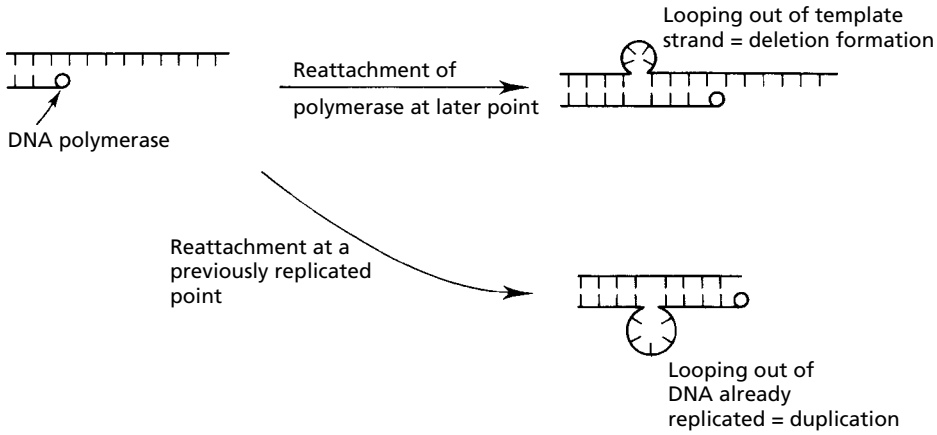

Figure 3 *Errors in DNA replication*

same DNA strand so that the two pyrimidine residues are joined by a cyclobutane ring. The most common are thymine dimers, but cytosine dimers and thymine–cytosine dimers are also generated. The appearance of these dimers distorts the shape of the DNA molecule (i.e. distorts the double helix) by interfering with normal base pairing. Because of the distortion, DNA replication stalls at thymine dimers. Replication may be reinitiated some distance away from the lesion, so generating a gap in the daughter strand. Unless repaired, the generation of a pyrimidine dimer is a lethal event. Not surprisingly, cells, from bacteria to higher eukaryotes, have evolved a number of mechanisms to repair pyrimidine dimers and restore the normal base sequence. If UV-treated bacterial cells are immediately exposed to visible light of wavelength 300–400 nm, both mutation frequency and lethality are greatly reduced, a phenomenon called photoreactivation. The visible light treatment activates an enzyme, photolyase, that hydrolyzes the cyclobutane ring, precisely reversing the reaction mediated by exposure to UV. The mechanism is accurate and error free. In addition, the cell has various repair systems that do not need light stimulation: the dark repair processes. One or more of these systems is error prone and it is the activity of this system (or systems) that can result in mutation (Altshuler 1993; Sinha and Hader 2002). DNA replication is involved and is mediated by DNA polymerases, which are intrinsically error-prone in terms of accommodating non-

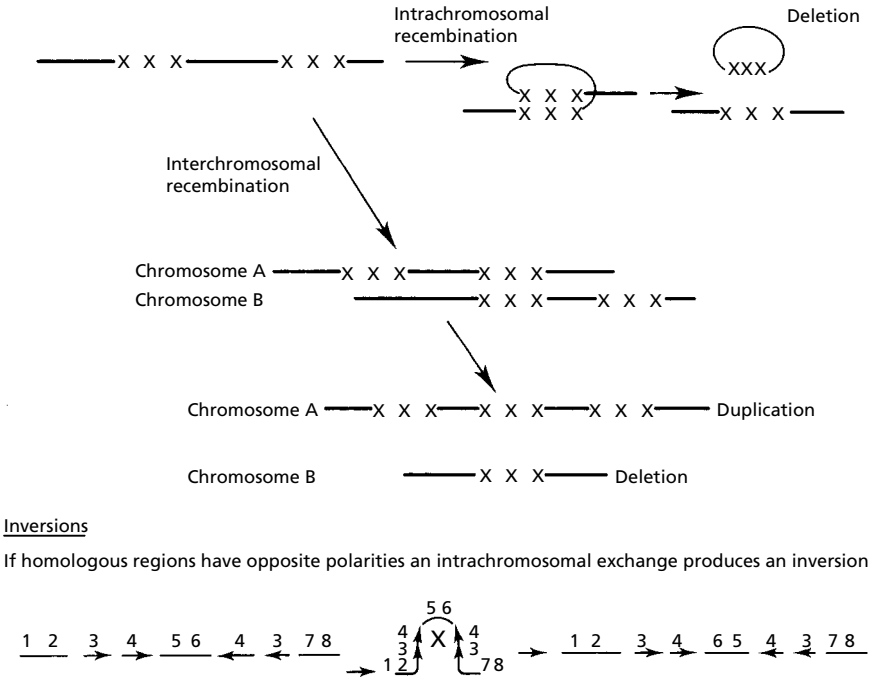

Figure 4 *Inter- and intrachromosomal recombination*

Crick–Watson base pairing during daughter-strand synthesis. This gives them the ability to replicate through damage to DNA, such as the presence of pyrimidine dimers, albeit with a high probability of introducing mistakes at that point (Goodman 2002; Woodgate 2001).

Other types of mutation

Spontaneous mutations may occur in the absence of known mutagenic treatment. There are probably many reasons for this. Various products or intermediates of the cell's own metabolism are demonstrably mutagenic; these include peroxides, nitrous acid, formaldehyde, and purine analogs. Some spontaneous mutations may, therefore, be induced by endogenously generated mutagens. Alkaline pH and elevated temperature have also been implicated in mutation.

Finally, it has been discovered that certain mutations of bacteria themselves predispose the cell to further mutation. Several loci are now known to be involved. They include functions connected with DNA replication and, particularly, with a process termed mismatch repair (Chopra et al. 2003), that operates to maintain the integrity of the genetic message (Schofield and Hsieh 2003).

In these cases the initial mutation diminishes the reliability of either those functions that ensure the fidelity of replication (i.e. the error rate is increased) or one of the DNA repair mechanisms, the purpose of which is constantly to scan the cell's DNA for damage and correct it when found. These mutations generate alleles called mutator (*mut*) genes because they enhance the frequency of mutation (Hutchinson 1996). There is a similar mutator gene in coliphage T4, whose product has been identified as a DNA polymerase. Mutator genes have been reported to induce transversions at relatively high frequencies, consistent with a partial breakdown in the mechanisms that maintain DNA fidelity. Recently, it has been discovered that the mutation frequency may rise significantly when the microbe is subjected to conditions of stress, particularly nutritional stress, which can occur on nutrient exhaustion. The phenomenon has been termed adaptive mutation and can, in part, be attributed to limitation of DNA mismatch repair in stationary phase culture (Notley-McRobb et al. 2003; Rosenberg 1997).

Site-directed mutation

Until the advent of gene cloning, the introduction of mutations into a particular gene was a strictly random process; mutant alleles could be selected or identified, but the location of the mutation could not be controlled. Now, by exploiting DNA synthesis in vitro, a technique known as polymerase chain reaction (PCR) (Mullis et al. 1986; Palmer 2004, see McPherson and Møller 2000 for a more detailed exposition), mutations can be targeted precisely to any base pair that is desired (Primrose et al. 2001), so as to introduce a missense, nonsense, or frameshift mutation. Introduction of a specific macrolesion is also possible. DNA synthesis requires DNA polymerase, a template, deoxyribonucleotide triphosphates, and a priming sequence. In the cell this is usually a short RNA sequence but, in vitro, synthetic oligodeoxyribonucleotide primers of approximately 20 nucleotides are used instead. The gene to be mutated is often cloned into a suitable cloning vector. The construct constitutes the template DNA for the in vitro synthesis. Vectors based on the genome of bacteriophage M13, which is a circular, single-stranded DNA molecule, were initially favored, because the recovery of single-stranded DNA for the reaction is greatly facilitated, in that phage particles contain only one DNA strand (Zoller and Smith 1982). Now, however, the technology is much improved and site-directed mutation is routinely achieved using genes cloned in standard plasmid cloning vectors, or using PCR products.

With M13 based cloning vectors, the primer used is complementary to the sequence at the prospective site of mutation. By incorporating a mismatch into the primer, a targeted mutation can be engineered into the gene of interest. The primer is extended by the DNA polymerase, using the circular single-stranded DNA molecule recovered from phage particles as template until the 5′ end of the primer is encountered, when the newly synthesized DNA circle is closed by DNA ligase, generating a double-stranded DNA replicon with a mismatched nucleotide base pair. When introduced into a suitable bacterial host by transformation, this hybrid molecule is replicated to yield one daughter molecule carrying the original allele of the gene of interest and one carrying the mutant allele. Further replication amplifies both versions of the gene. The plasmids can then be isolated as a mixture and used to transform the same or another suitable host to yield clones carrying one or other of the two recombinant replicons. Clones carrying the mutant gene are identified and separated from transformants with the original allele of the gene of interest. The former clones can then be cultured to provide the mutant gene in quantity. The gene is then resequenced to ensure that the desired mutation is the only one that has been introduced. By using a set of primers, each with a different, but deliberately designed, mismatch in the reaction, a set of mutants with mutations directed to particular nucleotides can be generated. This allows a region of a gene, or indeed the entire gene if so desired, to be subjected to systematic mutation for structure–function studies of the gene product or for analysis of the sequences controlling gene expression. Various modifications of this procedure, designed to make it more efficient and to optimize recovery of the desired mutant, have been developed (see Watson et al. 1992, for simple descriptions)

A multistage PCR method for site-directed mutagenesis now more commonly employed uses double-stranded DNA as template (Figure 5) (see also

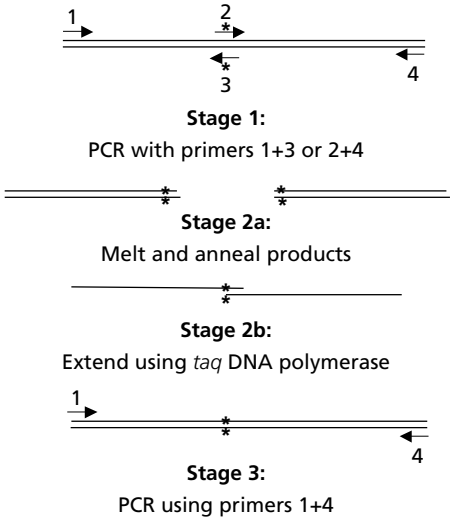

Figure 5 *Schematic representation of PCR site-directed mutagenesis. In stage 1, two separate PCR reactions are used to generate, from the template sequence, two linear, overlapping fragments having a mutation in the overlapping region – with the mutation being the result of primers partially mismatched with their target sequence (noted as stars). In stage 2, the two fragments are melted, mixed, and allowed to anneal to each other. This product is then used as a template for further PCR. The resulting product will comprise copies of the gene of interest with the desired mutation (shown on the diagram).*

Primrose et al. 2001). To begin with, the gene of interest is amplified as two, partly overlapping fragments (stage 1). One fragment is generated using a primer pair with the forward primer a perfect match and targeted to the start of the gene (primer 1) and the reverse primer targeted to the point of mutation and incorporating the desired mutation (primer 3). PCR amplifies a fragment with the mutation. A second fragment is generated in a similar fashion, but with the forward primer (primer 2, the complement of the reverse primer in the first reaction and so incorporating the mutation required) targeted to the point of mutation while the reverse primer (primer 4), perfectly matched to the gene sequence, is targeted to the end of the gene. PCR amplifies the second fragment, with the same mutation within the same sequence as on the first fragment. The two fragments are then mixed and used as a template in a third PCR reaction together with the forward primer from the first PCR reaction and the reverse primer from the second, i.e. primers 1 and 4 (stage 2). When the template is melted and then allowed to reanneal, a proportion of strands from the different fragments anneal across a short common region generating a DNA molecule with a short central double-stranded section (corresponding to the mutational primers in the two initial PCR reactions) and two long single-stranded tails. These then become templates to extend the short double stranded section of DNA in both directions to encompass the full gene. A PCR reaction then amplifies this product (stage 3), namely a full-length version of the mutant gene of interest. This is then recovered by ligation into a suitable cloning vector, where the mutant gene can either be examined directly, or used to mutate the original allele in the strain of origin by allelic replacement. For an up-to-date discussion of in vitro mutagenesis see Braman (2002).

Flip-flop mechanisms

Some functions expressed by bacteria and bacteriophages undergo changes that at first sight appear to be mutations. However, the basis of the changes is more like the operation of a switch than a random change in the DNA. Such changes occur as a result of site-specific recombination mechanisms that invert specific DNA sequences, the consequence of which is to turn off expression of one gene(s) and activate that of an alternative gene(s) (Craig 1988; Nash 1996). Two particularly well characterized systems, encoded by bacteriophages Mu and P1, operate to control the bacterial host ranges of the phages by controlling the production of alternative tail fibers for the phages (Giphart-Gassler et al. 1982). In *Salmonella* Typhimurium flagellar antigen phase variation, which allows the bacterium to evade the immune response of an infected host, is controlled by a very similar DNA inversion mechanism. Two genes, *H1* and *H2*, code for different forms of flagellin. Both proteins can polymerize to produce the cell's flagella. The cell can alternate expression of these two genes by inverting a short segment of the chromosome (Zieg et al. 1977). In phase I, flagellin encoded by *H1* is produced. In phase II, *H2*-encoded flagellin is synthesized and a gene called *rhI* is activated. The product of *rh1* is a transcriptional repressor of *H1*, so that production of flagellin encoded by *H1* is switched off. Hence, expression of *H1* and *H2* is mutually exclusive. The recombination inverts an approximate 1 kb sequence. In phase II orientation, the invertible sequence provides a promoter for *H2* and *rhI*. The sequence also encodes a gene called *hin*, the product of which mediates the inversion. The *hin* gene shows considerable homology with both *gin* and *cin*, the site-specific recombinase genes of bacteriophages Mu and P1, respectively, that mediate host specificity. The products of these three genes are functionally interchangeable.

Each of these three systems operates infrequently, the probability of inversion being about 10^{-8} per cell per generation. However, this low frequency is sufficient, within a population of cells or bacteriophages, to significantly extend the versatility of the organism. The ability of *S.* Typhimurium to switch flagellin production confers some protection against the immune response, whereas the ability to produce two sets of tail fibers broadens the host ranges of both Mu and P1, each set of tail fibers permitting infection of particular bacterial strains.

Other examples of phase variation controlled by invertible DNA segments are *E. coli* type I fimbriae (pili) (Abraham et al. 1985) and the fimbriae of *Moraxella bovis* (Marrs et al. 1988).

MOBILE GENETIC ELEMENTS

Insertion sequences and transposons

In the 1960s, evidence accumulated for the existence of a hitherto undescribed form of mutation. The first examples recognized in bacteria were found by Lederberg, who in 1960 described an unusual class of Gal^- mutants in *E. coli*. They reverted to Gal^+ at frequencies of 10^{-6}–10^{-8}, but the reversion frequency was not enhanced by mutagenic treatments. These results indicated that the mutations were neither deletions nor classical point mutations (base substitutions or frameshifts). Further, when the mutation was in one of the first genes of a set it exerted an unusually strong polarity on expression of subsequent genes in the operon, virtually eliminating expression from them (unlike polar nonsense mutations that merely reduce expression of the distal genes). The new type of mutation was described as a strong polar mutation (Jordan et al. 1968). Molecular genetic analysis of these mutations, using λ*gal* transducing phages, demonstrated that the *gal* operons of the mutants contained extra DNA sequences; revertants to Gal^+ had lost the additional sequences. The conclusion from these studies was that the mutations were generated by the insertion of extra pieces of DNA, called insertion sequence (IS) elements (Jordan et al. 1968), at the point of mutation. IS elements are now known to be but one type of a class of genetic elements called transposable elements. The one characteristic that all such elements possess is the ability to transpose, i.e. they can insert into different sites, usually unrelated, on the same or on different DNA molecules. Unlike host-mediated homologous recombination, transposition requires no homology between the transposable element and its sites of insertion, and the mechanisms are independent of the *recA*+ gene product of the bacterial host.

Since the initial discovery of IS*1*, IS*2*, and IS*3* (Fiant et al. 1972) in *E. coli*, many IS elements have been described from a wide variety of bacteria (Galas and Chandler 1989; Chandler and Mahillon 2002). They are common in bacterial plasmids and are normal components of many, if not all, bacterial chromosomes. They are also cryptic, in the sense that they do not confer on the host bacterium a predictable phenotype. In the past, their presence was usually indicated by mutation, i.e. the genetic damage they cause. More recently, however, many elements have been discovered as a result of bacterial genome sequencing (Chandler and Mahillon 2002).

In 1974, the discovery of a second type of transposable element, one that encoded a recognizable gene product, the TEM-2 β-lactamase determining resistance to several β-lactam antibiotics, including ampicillin (see Chapter 18, Antibacterial therapy), was reported by Hedges and Jacob (1974). It was termed a transposon, and many others carrying various resistance and other genes have since been identified and analyzed (see Berg and Howe 1989; Craig et al. 2002). Because of the ability of transposable elements to insert into a large number of sites, both on the bacterial chromosome and on plasmids, they assumed the popular name of 'jumping genes.' A few of these elements are described in Table 1.

It is now clear that there are several types of transposable element (see Craig et al. 2002). IS elements are generally small (1–2 kb) and encode only those functions needed for their own transposition, whereas transposons are larger, usually 4–25 kb (although considerably larger elements have been described), and they encode at least one function unconnected to transposition that alters the cell phenotype predictably (e.g. by conferring a particular antibiotic resistance). In addition, there are some bacteriophages, such as Mu and D108, that exploit transposition in their life cycles (Toussaint and Résibois 1983).

Bacteriophage Mu, mentioned earlier in relation to its site-specific recombination system, resembles a transposable element in that it inserts more or less at random into many different sites in the *E. coli* chromosome, as a consequence of its mode of replication; hence its name, Mu (for mutator phage). It is now known that phage Mu is a large transposable element that uses one form of transposition for replication.

Because transposition involves the movement of DNA sequences from one site to another, it is clear that whatever the mechanism, it is, by definition, a recombination event. Such events may be referred to as illegitimate recombination, to indicate that they occur independently of a classical recombination system and do not require extensive sequence homology between the sequences involved in the recombination.

The majority of known IS elements have a similar structure, namely a unique central sequence, comprising most of the element, flanked by short perfect or near-perfect inverted repeat (IR) sequences. Terminal IR sequences differ, both in sequence and size, from one element to another (c. 10–40 bp) (Table 1). For those elements that have been analyzed in detail, the unique central section encodes an element-specific transposition function(s). The IR sequences delineate the element, serving as specific sites for recombination mediated by the transposition enzyme(s). The inverted character of the terminal repeats and the asymmetric nature of the sequence ensures that both are retained in the transposed structure, i.e. the same sequence is recognized at both ends of the element and the DNA is cleaved at the same end of the IR sequence in both cases, i.e. precisely at the IS element–host replicon junctions.

Most transposons can be assigned to one of two broad groups, called composite transposons and complex trans-

Table 1 *Some transposable elements in bacteria*

Transposable element	Size (kb)	Terminal[a] repeats (bp)	Target size[b] (bp)	Phenotype[c] conferred	Origin
Insertion sequences					
IS*1*	0.768	18/23	9	None	Gram −ve
IS*2*	1.331	32/41	5	None	Gram −ve
IS*3*	1.258	32/38	3/4	None	Gram −ve
IS*4*	1.426	16/18	11/12	None	Gram −ve
IS*10*	1.329	17/22	9	None	Gram −ve
IS*50*	1.534	12/18	9	None	Gram −ve
IS*903*	1.05	18/18	9	None	Gram −ve
IS*256*	1.35	nd	nd	None	Gram +ve
Composite transposons					
Tn*5*	5.7	IS*50*(IR)	9	Km	Gram −ve
Tn*9*	2.5	IS*1*(DR)	9	Cm	Gram −ve
Tn*10*	9.3	IS*10*(IR)	9	Tc	Gram −ve
Tn*903*	3.1	IS*903*(IR)	9	Km	Gram −ve
Tn*1681*	2.1	IS*1*(IR)	9	Heat-stable toxin[d]	Gram −ve
Tn*4001*	4.7	IS*256*(IR)	nd	Gm Tb Km	Gram +ve
Complex transposons					
Tn*1* family					
Tn*1*, Tn*3*	4.957	38/38	5	Ap	Gram −ve
Tn*21*	19.5	35/38	5	Hg Sm/Sp Su	Gram −ve
Tn*501*	8.2	35/38	5	Hg	Gram −ve
Tn*1721*	11.4	35/38	5	Tc	Gram −ve
Tn*951*	16.5		5	Lac	Gram −ve
Tn*551*	5.3	35/35	5	Ery	Gram +ve
γδ(Tn*1000*)	5.8	36/37	5	None	Gram −ve
Others					
Tn*7*	14	22/28	5	Tp Sm/Sp	Gram −ve
Tn*554*	6.2	None	0	Sp Ery	Gram +ve
Tn*916*	15	20/26	10	Tc, conjugal transfer[e]	Gram −ve
Tn*4291*	nd	nd	nd	Mec	Gram +ve

a) The number of identical base pairs in both terminal repeats/size of terminal repeats. DR, direct repeat; inverted repeat.
b) The size of the direct duplications which are found to flank the transposon in situ.
c) Entries which relate to an antibiotic(s) refer to resistance to that drug(s). Ap, ampicillin; Cm, chloramphenicol; Ery, erythromycin; Gm, gentamicin; Hg, mercuric ions; Km, kanamycin; Mec, methicillin; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Lac, lactose metabolism; Tb, tobramycin; Tc, tetracycline; Tp, trimethoprim. Sm/Sp indicates resistance to both antibiotics conferred by the same modifying enzyme.
d) Heat-stable toxin produced by some enterotoxigenic strains of *Escherichia coli*.
e) See text. nd, not determined.

posons. Composite transposons, as their name implies, are constructed from recognizable simpler units. Their overall form comprises a unique central sequence that encodes the function(s) by which the element is recognized and which lacks transposition functions, flanked by long sequence repeats (1–2 kb) which are arranged as direct repeats or, more commonly, as IRs. These terminal repeats provide transposition functions and, in all cases examined so far, comprise a pair of IS elements. The more common inverted arrangement possibly reflects the fact that it confers greater stability (coherence) on the composite structure than direct repeats where deletion of the transposon's distinguishing feature(s) as the result of recombination between the terminal IS elements, mediated by the host's homologous recombination machinery, is an ever-present possibility (Figure 4).

Complex transposons do not have modular structures. Rather, both transposition and non-transposition functions are located in the bulk of the sequence which is flanked by short (c. 40 bp) terminal IRs. Indeed, complex transposons resemble enlarged IS elements rather than composite transposons. The first transposon identified, Tn*1* (formerly Tn*A*; Hedges and Jacob 1974), typifies this type of element. Its structure is illustrated in Figure 7, p. 94. Tn*1* is one of a family of transposons that are ancestrally related in terms of their transposition functions. This was first recognized because of striking similarities between their IRs. Subsequently, more extensive sequence analysis of several of these

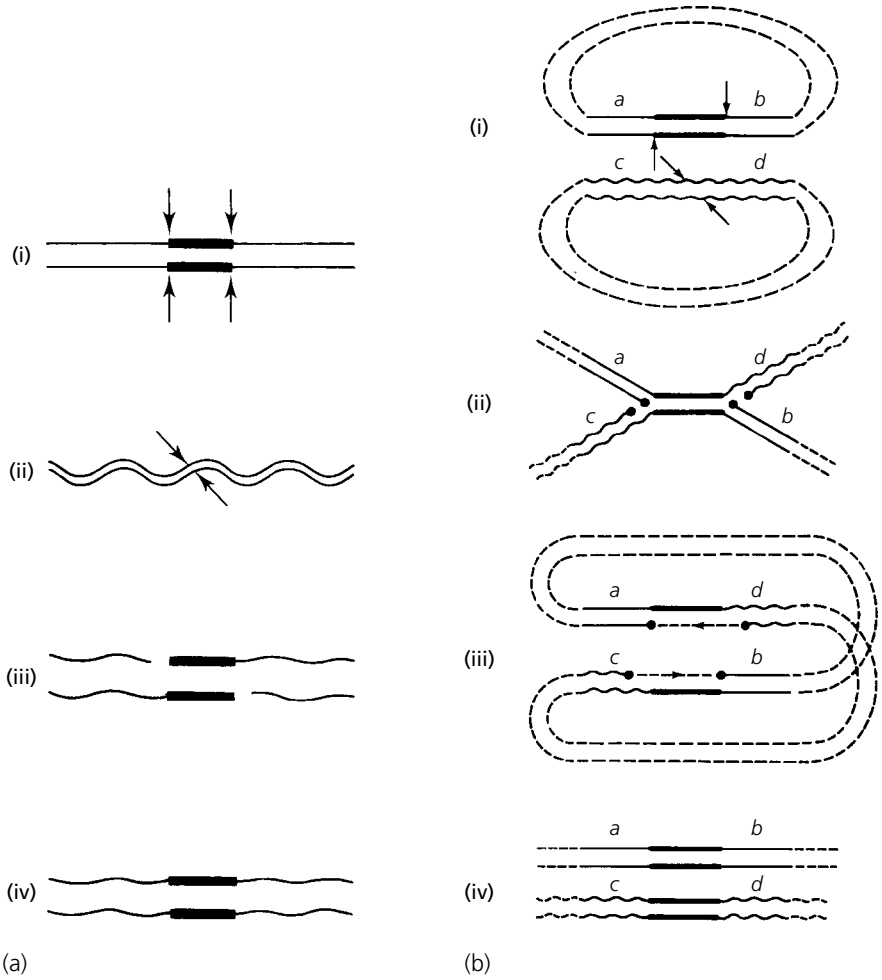

Figure 6 *Models for transposition.* **(a)** *Conservative transposition. (i) Flush double-stranded cuts (vertical arrows) are made at the ends of the transposable element (bold lines) and a staggered cut (diagonal arrows) is made at the target site. (ii) One end of the transposable element is attached by a single strand to each protruding strand at the staggered cut to insert the transposable element into the target site. (iii) Two small gaps, generated as the consequence of the staggered cut, are left on opposite strands, one at each end of the element. (iv) The gaps are filled in by DNA repair synthesis to produce short direct repeats that flank the transposable element.* **(b)** *Replicative transposition. (i) Single-stranded cuts are made at the ends of the transposable element, and a staggered cut of opposite polarity is made in the target DNA. (ii) One end of the transposable element is attached by a single strand to each protruding end of the staggered cut. Two replication forks are thus created, and replication may proceed to copy the transposable element. (iii) Semi-conservative replication has generated two new transposable elements from the original element and a short direct repeat of the target site. If* ab *and* cd *were circles,* a, b, c, *and* d *would now be covalently connected and transposable elements would form the joint regions of a fused replicon. (iv) A site-specific crossover between the transposable elements would resolve the cointegrate into the starting replicon* ab *and the target replicon* cd*, which now has a copy of the transposable element flanked by short direct repeats of the site of insertion (Shapiro 1979).*

elements demonstrated that the entire transposition mechanism of one is related to that of the others. In some cases the evolutionary divergence is small, so that the transposition functions are interchangeable. In other instances, elements are more distant in terms of evolution and analogous functions cannot be interchanged (Sherratt 1989; Grinsted et al. 1990; see also Craig et al. 2002)

Transposable elements have been grouped according to their similarities. Accordingly, IS elements and composite transposons are called class 1 transposable elements, whereas Tn*1* and related transposons are called class 2 transposable elements. Class 3 accommodates the transposing bacteriophages such as Mu. Finally, class 4 at present accommodates those elements that do not naturally fall within classes 1–3. Perhaps two of the best known are Tn*7*, a compound transposon encoding resistance to trimethoprim and streptomycin prevalent in gram-negative bacteria, particularly the Enterobacteriaceae, and Tn*916*, an unusual transposon from *Enterococcus faecalis* that encodes resistance to tetracycline and which is conjugative, i.e. can mediate its transfer not only from one replicon to another by transposition, but also from one bacterial cell to another by a form of conjugation (Salyers et al. 1995). Tn*916* (Franke and Clewell

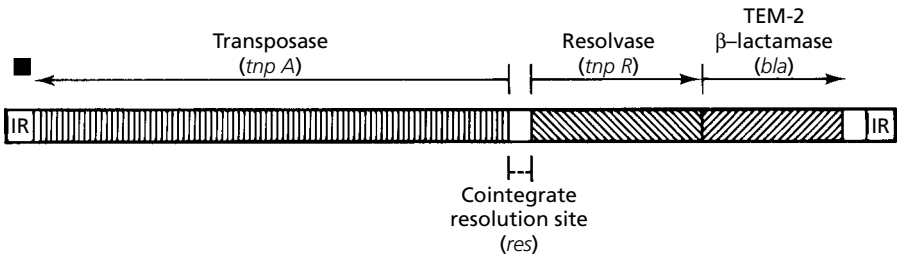

Figure 7 *Map of Tn*1 *showing the location of its three genes. The arrows indicate the direction of transcription. The cointegrate resolution site (*res*) is necessary for resolution of cointegrates, by resolvase, during transposition (see Figure 6). IR, perfect 38 bp inverted repeats*

1981) serves as the archytype for this kind of transposable element. Transposons with conjugative properties have also been found in *Bacteroides* spp. (Salyers and Shoemaker 1995), and, more recently, elements originally thought to be plasmids because they mediate transfer of antibiotic resistance genes between members of the Enterobacteriaceae are now considered to be conjugative transposons (Böltner and Osborn 2004).

Transposable elements not only mediate their own genetic mobility, but can be instrumental in bringing about important rearrangements in DNA molecules. Not only do they provide useful genes, but their transposition activities can generate macrolesions such as deletions, duplications, and inversions. Because many of them insert into DNA molecules more or less at random, they can generate mutation by disrupting the sequence continuity of genes; indeed, it was this consequence that first brought IS elements to the attention of bacterial geneticists. That the insertions are often polar has been noted for IS elements; this is also true for transposons. In addition, they can, in the cases of particular elements, activate previously silent genes by inserting upstream and adjacent to the gene to provide a functional promoter (Jaurin and Normark 1983; Pilacinski et al. 1977; Wood and Konisky 1985; Rasmussen and Kovacs 1991; Podglajen et al. 1994; Chandler and Mahillon 2002). The polar effects of transposable element insertion arise because the elements are, in general, self-contained and encode translational and transcriptional terminators that prevent transcription proceeding far into the element across the junction of the element and the adjacent host DNA. Thus the genes of an operon which are promoter distal to an insertion are separated from their promoter and normally become silent. Only when there is a promoter at the end of the element nearest to the orphan genes that can direct transcription towards that end of the element and beyond, are the remaining genes of the operon (downstream of the insertion) expressed.

IS*2* exerts strong polar effects when in one of its two possible orientations (this was how it was discovered). In this orientation (designated orientation I), a rho-dependent transcription termination site prevents transcription proceeding through the element into adjacent sequences. In the opposite orientation (orientation II), IS*2* can provide a promoter sequence that can switch on downstream genes (Saedler and Reif 1974). Mutations in wild-type IS*2* that create promoters have also been described (Sommer and Saedler 1979). It is of interest to note that cloned genes, either not expressed or expressed poorly from an original construct, may be activated by insertion of a transposable element upstream from them. However, it should be noted that this aspect of transposable element activity appears to be restricted to a minority of specific elements, of which IS*2* is one.

Specificity and mechanism of insertion

When transposable elements insert into new sites, short duplications of recipient DNA sequence are usually generated. These direct repeats, commonly 5 or 9 bp (Table 1), flank the transposable sequence. The size of the duplication is specific to the element. The duplications arise because the recipient DNA at the target site is cleaved across both its strands as a short staggered cut. The transposon is inserted by joining it to the short single-strand extensions created by the cleavage. This generates small single-stranded gaps, on opposite strands, in the recipient DNA at the ends of the element. These gaps are filled in, probably by DNA gap repair synthesis (Sancar 1996) to generate the short flanking duplication. So typical is this arrangement that if, when analyzing a new bacterial nucleotide sequence, a section of DNA is found to be delimited by short IRs and flanked by short direct repeats (DR) then it is immediately considered likely to be a transposable element.

A wide range of insertion specificities has been found for different transposons and some display different characteristics with different DNA molecules. Some (e.g. Tn*1*) insert at many places on different plasmids and into the bacterial chromosome with little recognizable specificity save, perhaps, a preference for AT-rich regions; others appear to be rather more specific, e.g. IS*4* has been found to insert into only one position in the *gal* region of the *E. coli* chromosome. Although

Tn*10* inserts at many different sites, they are all related to the 9 bp consensus sequence, NGCTNAGCN (where N denotes any base). The more closely a site approximates to this sequence the more readily it is used as a site of Tn*10* insertion. The preference for AT-rich regions shown by some transposons suggests that the transposition mechanism used may involve a degree of strand separation at the target, since AT bps are inherently more easily disrupted than GC bps.

Initially, research workers sought to describe transposition in terms of a single mechanism. It is now clear that this notion was misconceived and that there are several different mechanisms. Three fundamentally different strategies have been reported. One, used by several IS elements and their composite transposons, is called conservative, or cut-and-paste, transposition (Figure 6a). In this, the transposable element is cut from the donor DNA as a discrete double-stranded section of DNA with flush ends. The free ends are ligated to the target site using the single strand extensions generated by the staggered cut at the target site. The small gaps that remain are filled in to generate the direct flanking repeats typical of IS element insertions. In this mechanism the entire transposable sequence on one DNA molecule is moved to another DNA molecule (Bolland and Kleckner 1996). It is believed that the donor molecule minus the transposon is degraded (Berg 1989; Kleckner 1989). Some transposons, e.g. Tn*7*, use a variation of the cut-and-paste transposition system (Sarnovsky et al. 1996). Others, e.g. conjugative transposons, are excised from the donor site to generate a circular form of the element. This entity then either inserts into another site on the same or on another replicon in the same cell, or it transfers, by conjugation, to another cell where it is inserted into one of the resident replicons (Salyers et al. 1995). Although these elements display features of a bacterial plasmid, they differ from a plasmid in that they lack their own replication system. They are replicated passively as components of the replicons into which they insert.

A second mechanism of transposition involves semiconservative DNA replication. This was realized when it was found that certain mutants of Tn*1* generated end products that differed from those of the parental transposon. With these mutants, the end product of a transposition from one replicon to another was a cointegrate – a recombinant DNA molecule comprising both the donor and the recipient replicons with directly repeated copies of Tn*1* at the plasmid junctions, i.e. the transposon had been replicated (Figure 6b). These recombinants could then break down in a *rec*$^+$ host, via normal recombination, to yield a replicon indistinguishable from the original plasmid donor and a transposon-carrying derivative of the recipient plasmid, i.e. a product indistinguishable from that generated by the parental transposon. Shapiro (1979) and Arthur and Sherratt (1979) synthesized these observations, and others involving bacteriophage Mu, into the now generally accepted model of replicative transposition (Figure 6b).

For some IS elements a small proportion of transposition events appear to occur via replicative rather than conservative transposition. This observation has been taken to imply that the two forms represent a split pathway proceeding from a common start (Ohtsubo et al. 1981). Consistent with this interpretation is the observation that c. 5 percent of transpositions of Tn*1* appear to be by a direct pathway rather than by replicative transposition (Bennett et al. 1983).

More recently, it has been discovered that members of a novel class of IS elements, of which IS*91* is the type element (Mendiola et al. 1992), and that lack terminal IR sequences, transpose by a third mechanism, RC transposition, that involves a form of rolling circle replication. Insertion is also site-specific, although the target site is small, 4 bp. The transposases of these elements are related to the initiator proteins of certain plasmids of *Staphylococcus aureus* (although the IS elements were isolated from gram-negative bacteria) (Mendiola and de la Cruz 1992). The plasmids replicate via a rolling circle mechanism that generates a single-strand version of the plasmid which is then converted to the double-stranded form. It is likely that this family of IS elements transposes by a mechanism that involves a single-stranded, possibly circular intermediate (Tavakoli et al. 2000; Garcillán-Barcia et al. 2002).

Deletions, duplications, and insertions

Deletion induced by a transposable element was first seen for IS*1*. In the *E. coli* chromosome, the frequency of deletions near a copy of IS*1* was found to be 100- to 1 000-fold higher than that observed elsewhere (Reif and Saedler 1975). Subsequently, other elements and transposons were also shown to stimulate the frequency of deletions in their vicinity. Several studies have demonstrated that the deletions always extend precisely from one end of the particular transposable element, leaving the element itself intact but removing one copy of the direct repeats that originally flanked it. Deletions such as these can, in principle, be formed in one of two ways. First, a second copy of the transposable element can insert into a different position on the same replicon, but in the same orientation as the first. Host-mediated recombination can then occur between the copies, effectively deleting one copy and the intervening DNA sequence (see Figure 4). Second, an element may transpose intramolecularly to a nearby site on the same DNA molecule by replicative transposition. If the insertion at the new site assumes the same orientation as that at the first site, the result is an automatic deletion of one of the copies of the element and the DNA sequences between the original site and the new site of insertion (Arthur and Sherratt 1979; Craig 1996).

DNA inversions may also arise by one of two mechanisms. Following insertion of a second copy of a transposable element into a DNA sequence to generate an inverted repeat, a single host-mediated recombination event across the repeated sequences will invert the intervening sequence (see Figure 4). Alternatively, when an element transposes intramolecularly by replicative transposition to a new site on the same DNA molecule in such a manner as to generate inverted repeats of the element, the intervening sequence between the old and new sites of insertion is automatically inverted, again as a consequence of the mechanics of the transposition (Arthur and Sherratt 1979). In practice, after the event, it is impossible to determine which mechanism generated a particular inversion because extended IRs act as recombination loci periodically to invert and reinvert the intervening sequence. As noted earlier, a similar phenomenon is exploited in the site-specific recombination systems that control P1 and Mu phage host ranges and *S.* Typhimurium flagellar type.

Transposition functions

Several transposons have now been studied in detail and, with very few exceptions, each element encodes its own transposition functions (see Craig et al. 2002). Perhaps the best studied to date is Tn*3* (which is virtually identical to Tn*1*), the class II element that confers resistance to a range of penicillins, including ampicillin and carbenicillin. The element is 5 kb and encodes three proteins: a TEM β-lactamase and two proteins that are necessary for transposition (see Figure 7). The larger of these, called transposase, is encoded by *tnpA* and is indispensable; it mediates cointegrate formation (Figure 6b). The second transposition enzyme, resolvase (encoded by *tnpR*), is normally responsible for resolution of the cointegrate to release the final transposition product. The promoters for both genes are located in the length of c. 100 bp that separates them. The site at which resolvase acts is called the *res* site, also located in the intergenic region between *tnpA* and *tnpR* (Sherratt 1989). A consequence of mutations in *tnpR* is that the frequency of transposition increases by approximately two orders of magnitude, i.e. resolvase functions not only as a site-specific recombination enzyme but also as a transcriptional repressor. This duality indicates a more general feature of prokaryotes that seems to be emerging, namely optimization of the genome. It is not uncommon to find the same DNA sequence performing more than one function.

In a *rec*$^+$ host (Watson et al. 1987), cointegrate resolution can be effected by the host's own recombination system, since the two copies of the element in the cointegrate are direct repeats and provide extensive sequence homology. Hence, *tnpR* mutants of Tn*3* generate cointegrates that are relatively stable in a recombination-deficient host, e.g. one which is *recA*, whereas normal transposition products are generated in a recombination-proficient host. Analyses of other class II transposons, e.g. γδ (Tn*1000*), Tn*21*, *Tn501*, and Tn*1721* (see Table 1), have shown that each encodes a transposase and a resolvase of approximately the same sizes as those of Tn*3*. The transposases are phylogenetically related, as are the resolvases. Some of these analogous enzymes are functionally interchangeable but others are not, indicating different degrees of evolutionary proximity (Grinsted et al. 1990).

Well-studied class I elements are IS*1*, IS*10* (Tn*10*), IS*50* (Tn*5*), and IS*903* (Tn*903*) (see Table 1). Each is genetically distinct and encodes its own unique transposition functions. The transposition of each is also regulated, but the strategies of regulation differ from one element to another. Thus IS*10* regulates both transcription and translation of its single transposition gene, whereas IS*50* regulates transcription of its transposition gene and modulates the activity of the enzyme with another IS*50*-encoded protein (Berg 1989; Kleckner 1989). Bacteriophage Mu, as might be expected of a lysogenic phage, has a variety of control systems to regulate its transposition (i.e. replication) activity (Toussaint and Résibois 1983), which is essentially dependent on a single phage gene designated *A*.

Not all transposition systems have so few element-encoded transposition functions. Tn*7*, for example, has five transposition genes, three of which are needed for all transposition events. Tn*7* displays two types of transpositional behavior. It can insert, more or less at random, into many different sites on plasmids at a relatively low frequency but it can also insert into one specific site on the *E. coli* chromosome at high frequency. Which transposition event occurs is determined by which one of the other two functions is used in the reaction (Craig 1989; Sarnovsky et al. 1996).

In addition to element-encoded functions and irrespective of the transposition mechanism, host functions are also required, such as DNA polymerase and DNA ligase activities. Several others have also been implicated.

Ubiquity of insertion sequences

The importance of insertion sequences and transposons in mediating bacterial variation cannot be overstated. Not only can they add useful genes to an organism directly (e.g. antibiotic resistance) but also, because of their ability to operate in pairs to transpose otherwise non-transposable genes (e.g. the unique central sequences of class I transposons), they can, in principle, effect the transposition of any bacterial DNA sequence from one DNA molecule to another. Indeed, IS elements have been instrumental in mobilizing chromosomal β-lactamase genes, such as the *blaSHV* of *Klebsiella pneumoniae* (Ford and Avison 2004) and the

blaCTX-M genes of *Kluyvera* spp. (Walther-Rasmussen and Hoiby 2002) on to plasmids which have then carried these genes into *E. coli* and other Enterobacteria. These types of elements are not restricted to prokaryotes; indeed, the first reports of transposable elements in higher organisms, specifically maize, preceded description of those in *E. coli* by 10–15 years (McClintock 1953). Transposable elements are now known to exist in a number of eukaryotes, including yeast and *Drosophila* (see Shapiro 1983; Berg and Howe 1989; Craig et al. 2002).

Mutation by transposon insertion

Because of their ability to insert into replicons almost at random, transposons have been exploited as mutagens. They possess the advantage over other random forms of mutation that the site of mutation is grossly altered by the addition of new DNA sequences, which can be physically mapped by restriction enzyme analysis or by sequencing. Because of this, artificial transposons with a range of properties have been created from natural ones to provide tools to facilitate molecular genetic investigations (Berg et al. 1989; de Lorenzo et al. 1990).

Transposon mutagenesis has been adapted in an ingenious way to generate mutations in bacterial virulence genes (Hensel et al. 1995) and to allow negative selection. This technique, signature tagged mutagenesis (STM), is a negative selection method for bacterial virulence gene identification based on the ability to follow, simultaneously, the fates of many different mutants within a single animal. Individual copies of a transposon (mini-Tn*5*, de Lorenzo et al. 1990) were tagged with small, unique nucleotide sequences. The identity of any particular copy of the transposon could then be determined readily by hybridization techniques. These tagged transposons were then used to generate a bank of 1 510 random transposon mutants in a strain of *S.* Typhimurium. Each isolate in the bank was checked to confirm that it had acquired a uniquely tagged copy of the mini-Tn*5*, yielding a slightly smaller bank of 1 152 mutants. Cultures of each isolate were grown in the wells of microtitration trays and 12 mixtures, each with 96 strains, were prepared and used to inoculate BALB/c mice by intraperitoneal injection. Three days later, the mice were killed and the bacteria were recovered from each mouse by plating spleen homogenates onto a suitable medium. Approximately 10 000 colonies recovered from each mouse were pooled and the total DNA was extracted. The transposon tags were amplified and labeled by PCR and colony blots of the original 96 mutants in the subset were probed. The results were compared with the hybridization pattern obtained with a 'tag probe' obtained with DNA from the mixed inoculum of 96 mutants. Colonies which probed positive in the control, but not with the probe prepared from DNA from cells post-passage through mice, were shown to have had a virulence gene inactivated by the mini-Tn*5*. Because these strains were less virulent they survived less well in the mouse and so were poorly recovered from the spleen of the infected animals, in turn giving a much reduced yield of that particular transposon tag in the DNA used to prepare the 'tag probe.' Forty virulence mutants were identified in the bank of 1 152. Among these mutants, approximately half were in genes known to be involved in virulence. The remainder constitute a set of new virulence or colonization survival genes. These results accord well with an estimate of approximately 150 virulence genes in *S.* Typhimurium (Groisman and Saier 1990), of which more than half have been identified.

Integrons and gene cassettes

In addition to plasmids and transposable elements, bacteria have yet other systems for gene dissemination, namely, integrons (Stokes and Hall 1989; Bennett 1999). Integrons are essentially site-specific recombination systems that mediate the movement of small DNA elements called gene cassettes. Most of the gene cassettes described carry a single resistance gene. About 60 antibiotic resistance genes, including those for resistance to aminoglycosides, β-lactams, chloramphenicol, erythromycin, sulfonamides, tetracycline, and trimethoprim have been identified as being on gene cassettes (Recchia and Hall 1995). The most recently identified antibiotic resistance gene cassettes encode the metallo-β-lactamases IMP and VIM (Laraki et al. 1999; Lauretti et al. 1999; Poirel et al. 2000; Riccio et al. 2000; Nordmann and Poirel 2002), encoding resistance to the potent carbapenem β-lactams imipenem and meropenem.

Integrons were first identified on Tn*21* and closely related transposons (Grinsted et al. 1990), but are now known to be more widely distributed (Hall and Collis 1995). Molecular analysis of the resistance genes of Tn*21* and related elements revealed that although different transposons carried different complements of resistance genes, most appeared to be accommodated at a single point on the basic structure, i.e. irrespective of the identity of the resistance gene(s) carried on a particular transposon, the sequences flanking the gene or gene cluster were the same, called the 3′- and 5′-conserved regions. Analysis of the 5′-conserved region revealed a gene, *int*, encoding a protein of the 'integrase' family. This enzyme, called integrase, is a site-specific recombinase. Beside *int* is *attI*, a site that is recognized and utilized by the integrase to capture gene cassettes. The essential components of an integron, located in the 5′-conserved region, are an *int* gene, an *attI* site, and a promoter in the correct orientation to express the genes on captured cassettes, because most cassette genes identified to date lack their own promoters. Within the

3′-conserved region is often found the *sul1* gene, encoding resistance to sulfonamides. These integrons are called type 1 integrons (Hall and Collis 1995). Integrons are classified according to their integrase genes (Hansson et al. 2002; Collis et al. 2002). Members of the same class have closely related integrase genes.

A gene cassette can exist in two forms:

1. as a linear insert within an integron, or
2. transiently as a small double-stranded circle of DNA carrying one (or occasionally two) resistance genes and a recombination site called a 59-base element (Hall et al. 1991).

Integrase-mediated recombination between a 59-base element on a cassette and the *attI* site inserts the cassette gene(s) into the integron and orients it so that it is expressed from the integron promoter (Collis et al. 1993). Several cassettes may be captured in this way to form a resistance gene array in which each gene is separated from the next by a 59-base element. Arrays of five and six resistance genes have been found; 59-base pair elements do not have a unique sequence, but all conform to a consensus, which may be longer than 59 bases (Hall et al. 1991; Stokes et al. 1997).

Movement of gene cassettes is possible because the integration of a cassette into an integron is a reversible process, so what has been captured by one integron can subsequently be released and captured by another (Collis and Hall 1992). In this way the complement of resistance genes can be constantly rearranged and moved from one DNA molecule to another, providing it carries an integron or a suitable integration site.

In principle, there is no limit to the number of gene cassettes that can be incorporated into an integron, but functional constraints, such as reduced expression of genes located some distance from the integron promoter, may limit the number in practice. Nonetheless, class 1 integrons with eight gene cassettes have been reported (Naas et al. 2001), and super-integrons, with dozens of gene cassettes, reported first in *Vibrio cholerae* (Rowe-Magnus et al. 1999; Mazel et al. 1998) as a result of genome sequencing, have since been identified in a variety of bacteria (Nield et al. 2001; Rowe-Magnus et al. 2001; Vaisvila et al. 2001).

ACQUISITION OF NEW GENES

Genetic diversity in bacteria growing in monoculture in the laboratory can occur only by mutation, as described above. In mixed cultures, as are found in nature, diversity can be provided by the transfer of DNA from one cell to another, provided that the new genes can be stably inherited by the progeny of the transcipient cell. Four mechanisms of gene transfer have been described for bacteria, although not all necessarily apply to a particular bacterial species or strain; these are transformation, transduction, conjugation, and cell–cell fusion.

Genetic information (i.e. DNA) acquired by one of these four mechanisms is inherited, provided that one of two situations pertains; either the newly acquired DNA is incorporated into one of the replicons in the recipient cell by some form of recombination, or the newly introduced DNA is self-maintaining, i.e. is a replicon in its own right. In the former case, newly incorporated sequences are substituted for existing ones. The 'invading' DNA is usually linear and homologous to only part of the rescue replicon and is itself not a replicon. Homologous sequences on the acquired DNA and on the appropriate resident replicon (chromosome or plasmid) are efficiently paired and exchanged by recombination (Figure 8). DNA sequences not rescued by recombination are ultimately degraded. The mechanism is one of general recombination and requires that the host be recombination-proficient (Rec^+) (Smith 1988, 1989). In particular, the host must have a *recA*+ gene (Smith 1988), the product of which is essential for homologous recombination. In laboratory studies of this type, gene exchange often involves wild-type genes and their mutant alleles. For example, a length of DNA that includes the lactose fermentation genes (*lac*+) from *E. coli* K12 is introduced into a strain that cannot ferment lactose because of mutation in its *lac* operon. Recombination between the newly acquired chromosomal segment of DNA and the equivalent region on the chromosome of the recipient cell results in the substitution of the wild-type allele for the mutant allele and restoration of the recipient cell's ability to ferment lactose. Allelic variation is seen in naturally occurring bacteria, e.g. the genes that determine the antigens of *Salmonella*.

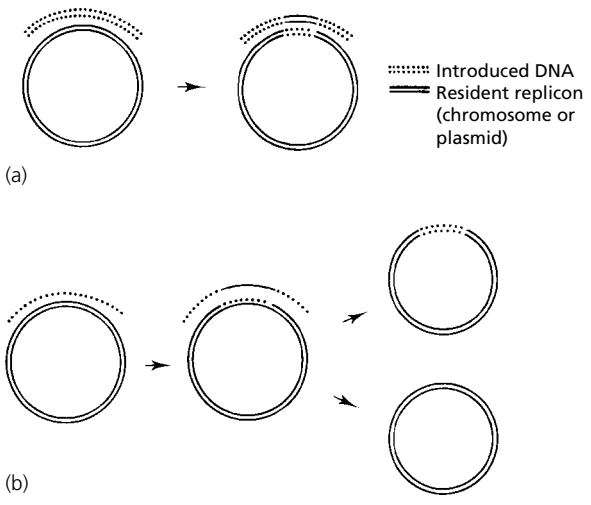

Figure 8 *General recombination. The figure shows two crossovers in homologous DNA having occurred between* **(a)** *two duplex molecules and* **(b)** *a single-stranded and a duplex molecule. Multiple crossovers (not illustrated) usually occur between homologous regions of DNA. The figure does not illustrate the mechanism of crossover. The RecA protein is required and some understanding of the mechanism is emerging (see text).*

When that part of the chromosome of *Salmonella* Paratyphi B that encodes antigen b is transferred into *S.* Typhimurium, recombination can lead to the substitution of the sequence encoding antigen b for the chromosomal sequence that encodes antigen i. In this way, new *Salmonella* serotypes are created. Phylogenetic relationships among Enterobacteriaceae have been reviewed by Sanderson (1976) (see also Chapter 51, The Enterobacteriacea: general characteristics).

DNA may also be added to the genome of the recipient cell. Plasmids (see next section, Plasmids) are autonomous DNA molecules and, to survive transfer, do not need to be rescued by recombination. Transposable elements, as discussed above, although not replicons, may insert into other DNA sequences with no requirement for homology or any loss of recipient DNA (although the insertion may result in loss of a particular function). Conjugative transposons, like plasmids, can transfer by conjugation from one bacterial cell to another, but unlike plasmids must integrate into one of the existing replicons in the new host to survive. Conjugative transposons belong to an extended group of mobile DNA elements called integrative conjugative elements (ICE) (Burrus et al. 2002) that includes elements originally thought to be plasmids (Osborn and Böltner 2002; Böltner and Osborn 2004) and the SXT resistance element of *Vibrio cholerae* (Hochhut et al. 2001; Beaber et al. 2002). In general, these elements integrate into particular sites on the host chromosome mediated by site-specific recombination mechanisms.

It is now known that all four mechanisms of gene transfer apply to both gram-positive and gram-negative bacteria, although not necessarily with any one particular bacterial strain. While some transfer systems that are widely used in the laboratory (e.g. transformation of *E. coli*) are likely to be laboratory artefacts rather than naturally occurring transfer systems, others (e.g. natural transformation of *Neisseria* spp. and *Streptococcus pneumoniae*) are known to have played important roles in gene modification and dissemination in nature (Dowson et al. 1994). For more information, see Grinsted and Bennett (1988).

Transformation

The first observation of bacterial transformation was by Griffith (1928) when studying pneumococcal infection in mice. The virulence of pneumococci depends on the capsular polysaccharide that gives the colonies a smooth appearance (S phenotype). Colonies of nonvirulent strains, without capsules, have a rough appearance (R phenotype). Griffith observed that when a mixture of living avirulent R cells and heat-killed virulent S cells was injected into mice, some of them died, whereas injection of either live R cells or heat-killed S cells was not lethal. From the dead animals, Griffith isolated virulent S cells whose capsular type was the same as that of the injected dead S cells. Griffith concluded that the heat-killed S cells had released something that had been taken up by the live R cells and had transformed them to the virulent S cell type. It was not until 1944 that Avery et al., using fractionated cell components, demonstrated that the transforming factor was DNA.

Cells that are able to take up DNA from their environment and incorporate it into the genome are said to be competent. In the case of some bacteria (e.g. *E. coli*), a state of competence can be developed by washing the cells with certain salt solutions (Saunders and Saunders 1988) but is otherwise absent. In others, competence is a natural feature of the life cycle as, for example, in the pneumococcus. Furthermore, some bacteria are competent at all times, whereas others display competence only at a particular phase of the growth cycle (Saunders and Saunders 1988). In the latter case, competence commonly develops towards the end of logarithmic growth in batch culture. Species that display natural, rather than laboratory-induced competence include *Acinetobacter calcoaceticus*, *Azotobacter vinelandii*, *Bacillus subtilis*, *Haemophilus influenzae*, *Moraxella* spp., *Neisseria* spp., *Pasteurella novicida*, some *Pseudomonas* spp., *Rhizobium* spp., *S. pneumoniae*, and *Xanthomonas phaseoi* (Lorenz and Wackernagel 1994). Species that can be made competent artificially, by one means or another, include *E. coli*, *Pseudomonas* spp., *S.* Typhimurium, and *S. aureus.*

Natural competence

Competence involves the ability to bind DNA to the cell surface and then transport it through the cell envelope into the cytoplasm prior to incorporation into the genome. The first of these activities, particularly in cases of natural competence, may involve specific cell receptors; this idea is supported by studies of interaction of competent pneumococci with DNA, in which the kinetics have been found to follow those of a classic enzyme–substrate reaction, and by the finding that new envelope proteins are synthesized as competence is acquired (Dubnau 1999).

The best characterized naturally occurring transformation systems are those of *S. pneumoniae*, *B. subtilis*, and *H. influenzae.* In all three systems, double-stranded donor DNA binds to the cell surface and is cut into discrete lengths giving fragments of 15–30 kb in the case of *B. subtilis*, 8–9 kb for *S. pneumoniae* and c. 18 kb for *H. influenzae*. The transforming activity of chromosomal DNA is better with large DNA fragments than with small, and there is a minimum size requirement for the donor DNA below which no transformation activity is seen. With *B. subtilis* and *S. pneumoniae* there is a period after DNA uptake during which the acquired DNA is incorporated into the genome. This period has been termed the eclipse phase.

B. subtilis and *S. pneumoniae* take up DNA from unrelated (heterospecific) bacteria as well as from closely related (homospecific) bacteria, whereas *H. influenzae* and *Neisseria gonorrhoeae* efficiently take up only homospecific DNA (Dubnau 1999). A membrane protein detectable only in competent *H. influenzae* and capable of binding DNA has been reported, and Sisco and Smith (1979) found that a specific nucleotide sequence of 8–12 bp mediates binding to the protein. It has been estimated that there are approximately 600 copies of this sequence on the *H. influenzae* chromosome, but it is rarely encountered on heterologous DNA. Such a specific mechanism is unlikely to have been preserved by chance, which implies that transformation plays an important role in the life-style of *H. influenzae*.

The fate of transformed donor DNA depends largely on its source. If the DNA is linear and homospecific, it can be incorporated into the chromosome of the recipient cell by recombination. If the linear DNA is heterospecific, its rescue will depend on whether there is sufficient homology with a resident replicon to permit recombination. Recombination with a resident replicon is not necessary when the donor DNA is itself a replicon, i.e. bacteriophage DNA (in which case the phenomenon is called transfection) or plasmid DNA. In these cases, however, successful transformation appears to require the uptake of more than one copy of the replicon, although complete duplication of sequence is not necessary. This finding indicates that replicon re-establishment also involves recombination (Saunders and Saunders 1988). In systems that rely on natural competence, transformation with homospecific chromosomal DNA is usually much more efficient than with intact heterospecific replicons, probably because of the requirement for sequence duplication to effect recombinational rescue, i.e. in the case of transformation with heterospecific DNA such as a plasmid, uptake of two plasmid molecules is necessary to provide the homology needed for recombinational rescue (unless the cell already contains a replicon with partial homology). Consistent with this interpretation is the finding that transformation with plasmid dimers (i.e. duplicate copies of the same plasmid joined head to tail in the same DNA molecule) is as efficient as transformation with homospecific chromosomal DNA (Saunders and Saunders 1988).

Artificial competence

The ability to transform bacteria is a valuable experimental tool but several commonly used laboratory bacteria (e.g. *E. coli*, *Pseudomonas putida*, *S.* Typhimurium, and *S. aureus*) do not display transformation competence at any stage of their life cycles in laboratory culture. Therefore, methods have been devised that can induce artificial competence. The common feature of these methods is the treatment of bacteria with salt solutions at 0°C. The most commonly used salt is calcium chloride (Saunders and Saunders 1988). The method was first developed to enable *E. coli* to take up noninfective phage DNA, and has since been used extensively to introduce plasmid DNA.

These transformation systems appear to work best with plasmid or bacteriophage DNA, rather than with chromosomal DNA. Indeed, *E. coli* does not transform efficiently with chromosomal DNA unless it is deficient in exonuclease V (encoded by the *recBCD* genes), since this enzyme degrades invading linear DNA; in addition, the cell must also be mutated at another locus, *sbcB*. This mutation restores recombination proficiency which is lost by mutations at *recB* and *recC*. Recombination is necessary to rescue linear homospecific DNA sequences.

Transformation with artificial competence works most efficiently with covalently closed circular (ccc) DNA, i.e. intact replicons such as bacteriophage genomes and plasmids (Cohen et al. 1972). These double-stranded DNA molecules are taken up intact and incorporated efficiently into the genome as autonomous replicating units. Any damage to the ccc form reduces the efficiency of transformation, as does increasing the size of the replicon; small plasmids transform the cell at much higher frequencies than large plasmids. It should be noted that induction of artificial competence is bacterial species-specific. Although very efficient systems have been developed for particular laboratory strains of a particular organism, these systems often do not work efficiently with other types of bacteria, or even with natural isolates of the same bacterium.

DNA may also be induced to enter bacterial cells when cell suspensions are mixed with free DNA and are subject to electrical discharge through the suspension, a procedure termed electroporation (or electrotransformation) (Miller 1994). This is now a commonly used method to introduce DNA molecules into many different bacterial species and electroporation apparatus is readily available commercially.

Conjugation

'Conjugation' is the term used to describe the transfer of DNA directly from one bacterial cell to another by a mechanism that requires cell-to-cell contact. DNA is transferred in a nuclease-resistant form that distinguishes the mechanism from transformation and without the aid of a bacteriophage, which distinguishes the mechanism from transduction.

Bacterial conjugation was discovered by Lederberg and Tatum (1946) when testing pairs of mutant strains of *E. coli* K12 for evidence for DNA transfer and recombination. From a mixture of a strain that required phenylalanine, cysteine, and biotin (a triple auxotroph), and

another that required threonine, leucine, and thiamine, they obtained some clones that were prototrophs, i.e. they required none of the nutrients required by the two parental strains, and other clones with various combinations of these requirements. These recombinant clones could only have arisen as the result of acquisition of chromosomal DNA from one bacterial cell by the other. Further studies, over several years, showed that the recombination was not the result of sexual reproduction in the normal sense of equal participation of both parents in zygote formation. Rather, it appeared that only a small amount of DNA from the 'male' (donor) parent was incorporated into the offspring (recombinants) which otherwise showed all the characteristics of the 'female' (recipient) parent. The capacity to donate genetic information (DNA) in these experiments was shown to depend upon possession of the F (fertility) factor. Strains were found that had become infertile, having lost their F factor, but they could regain fertility by contact with an F-carrying (F^+) strain (Hayes 1953a). At the time of these experiments, the role of DNA and the nature of the F factor were unknown; the F factor is now known to be a conjugative plasmid (see below).

In the first conjugation experiments the bacterial characters transferred were encoded by chromosomal genes. The F factor mediated transfer of part of the donor (F^+) cell's chromosome and this was rescued by homologous recombination into the chromosome of the recipient (F^-) cell, resulting in recombinant clones of bacteria with new assortments of characters. The recombinants remained F^-, i.e. they did not also acquire the F factor.

Conjugation is not an intrinsic function of bacteria but is determined by a variety of plasmids of which the F factor is just one. Conjugation normally functions to transfer plasmids from one bacterial cell to another. Conjugal transfer of chromosomal DNA is a relatively infrequent event, but many different plasmids are able to mobilize chromosomal genes with various degrees of efficiency. Chromosome-mobilizing ability (cma) is a byproduct that arises from certain interactions of the plasmids concerned with the bacterial chromosome, just as transduction of chromosomal genes is a byproduct of phage particle production.

For many years, conjugation was thought to be the prerogative of plasmids found in gram-negative cells, and most of our knowledge derives from these systems (Wilkins 1995). However, conjugation systems have been reported in gram-positive bacteria, notably strains of *E. faecalis*. The mechanics of these systems appear to differ somewhat from those found in gram-negative bacteria, primarily in the way donor and recipient cells are paired (Clewell et al. 1986; Grohmann et al. 2003).

Conjugation determined by F

F was the first conjugative plasmid to be discovered and the functions that mediate conjugation have been analyzed in detail. The genes that determine these functions are arranged as a continuous array, mostly in a single operon on the F factor in a region c. 30 kb in length, called the transfer (*tra*) region (Firth et al. 1996). This cluster, comprising approximately 24 genes, is found not only on the F factor but also on several resistance (R) plasmids, found in members of the Enterobacteriaceae, e.g. Rl and R100. Other plasmids encode unrelated but analogous Tra functions. Several different conjugation systems have now been described (Wilkins 1995; Zechner et al. 2000).

Conjugative pili

For conjugation to occur, donor and recipient cells must be in physical contact. Establishing contact is part random and part directed, in the sense that although encounters between donor and recipient cells happen by chance, the cells are joined by design. In gram-negative systems, the first link is made by thin protein filaments on the donor cell, called conjugative or 'sex' pili. These can be visualized by electron microscopy (Figure 9). Cells that lack conjugative pill cannot transfer DNA. Sex pili are essentially fiber polymers of a protein subunit called pilin. The current view is that a sex pilus effects the initial contact between the donor and recipient cells, via an interaction between a binding ligand at the tip of the pilus and its cognate receptor on the

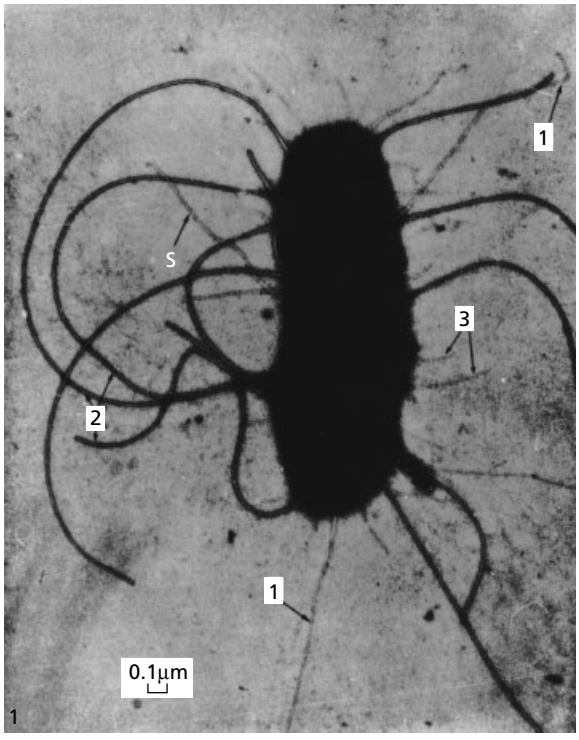

Figure 9 E. coli *K12 F .*The electron micrograph shows three kinds of appendage to the cell: 1, F pili; 2, flagella; and 3, common pili or fimbriae (from Meynell et al. 1968)

surface of the recipient cell. The pilus is then retracted into the donor, by depolymerization, in the process drawing the two cells together until direct contact is made. Pilin protein has been found as a pool of unpolymerized molecules in the outer membrane of the cell.

Conjugative pili may act as receptors for bacteriophages. F^+ cells are therefore susceptible to certain phages that cannot infect F^- bacteria whereas some other phages will infect only cells that carry one of another class of conjugative plasmid. Because these phages are specific for particular pili, they can be used as indicators for the presence of particular types of plasmids (Willetts 1988).

Mating pair formation

Random collision of bacteria is the first stage in conjugation or mating; plasmid transfer is rarely detected at low cell densities (Broda 1979). The initial link between donor and recipient cells, made by the sex pilus, is fragile and easily disrupted (e.g. by the shear force generated by pipetting), so not all donor–recipient encounters are productive. Before plasmid DNA is transferred, cell–cell attachment is strengthened, in the sense that much greater force than that generated by pipetting is needed to separate them. This state reflects the direct cell-to-cell attachment made after pilus retraction, and which is needed for formation of the DNA transfer channel between the participating cells. Such cell pairs can be forcibly split by vigorous treatment, e.g. by vortex mixing or in an electric blender. Once a stable mating pair is formed, initiation of F transfer is rapid and plasmid transfer takes about 2 min. When transfer has been completed, both cells contain F. Thereafter, the cells separate and both can initiate further rounds of F transfer.

High frequency of recombination (Hfr) and F′ formation

The first bacterial conjugation experiments involved transfer of chromosomal genes mediated by F. For this to occur, F must first fuse with the bacterial chromosome to form one large circular molecule. F, like many other plasmids, carries several transposable elements, specifically IS*2*, IS*3*, and γδ (Tn*1000*). Copies of the IS sequences are also found on the chromosome of *E. coli* and so can serve as loci for recombination between F and the chromosome (Brooks Low 1996; Deonier 1996). A single crossover between an IS element on F and a copy of the same element on the chromosome inserts one replicon into the other to form one large conjugative DNA molecule, transfer of which can proceed from the origin of transfer (*oriT*) of the integrated plasmid. Plasmid integration into the chromosome can also result from transposition (by, for example, γδ) with the formation of a transposition cointegrate. Either mechanism can generate integration events that may be transient or relatively stable. *E. coli* clones with the F factor stably integrated into the chromosome are called high frequency of recombination (Hfr) strains because, in matings in which these are donors, chromosomal genes are transferred to recipient cells at high frequency and large numbers of recombinants can be recovered (Hayes 1953b). Although initiation of transfer of DNA from *oriT* is rapid once a stable mating pair is formed, because the chromosome is about 50 times as long as F, complete transfer of the plasmid-chromosome cointegrate takes about 100 min (cf. approximately 2 min for F itself). In practice, mating pairs usually separate before this, so the chromosome is rarely transferred in its entirety.

F can be inserted into the bacterial chromosome at numerous loci and in either orientation. For each Hfr strain the chromosome is transferred to recipients unidirectionally from the *oriT* of the integrated F factor. Genes nearest the transferring end of F enter the recipient first (Figure 10). As a consequence, these donor alleles are recovered in greatest numbers in recombinants. The further a gene is from the leading end of F, the lower the probability that it will be transferred because, as noted above, it is rare that the entire chromosome is transferred; i.e. the longer a mating proceeds, the greater the probability that the participating cells will separate. One consequence is that chromosomal genes are transferred as a gradient from the point in the chromosome where F is inserted. Accordingly, these gradients can be plotted to show the positions of the genes in relation to the *oriT* of the integrated F. The same gradient, more directly related

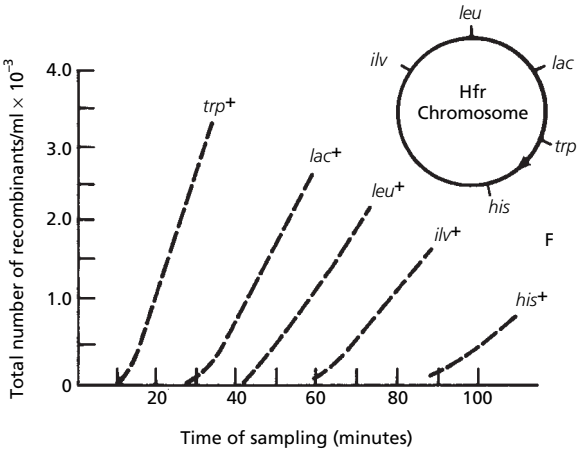

Figure 10 *Interrupted mating experiment. An Hfr strain of* E. coli, *with F inserted at the position shown, was mixed with an F⁻ strain that was* lac *(lactose nonfermenting),* trp, leu, ilv, his *(required tryptophan, leucine, isoleucine–valine and histidine for growth),* str *(streptomycin-resistant). Samples removed from the mixture at times shown were 'whirl-mixed' and plated on medium selective for the respective recombinants and containing streptomycin (to inhibit the Hfr donor).*

to the time of entry of each gene into recipient cells, can be determined from the results of interrupted matings. In these experiments, samples of a mating mixture are removed at intervals and diluted so as to prevent further mating pair formation. Mating pairs are separated by shearing in a blender or mixer and the cell suspension is then plated onto selective media to detect recombinants (Figure 10). When such experiments are performed with several different Hfr strains, the results indicate that the chromosome of *E. coli* is a circular molecule, because linear permutations of the same circular gene array are always obtained. As a consequence, the map of the *E. coli* chromosome is depicted as a circle with 100 min as reference points (Berlyn et al. 1996). Similar experiments with other bacteria, notably *S.* Typhimurium, *Proteus mirabilis*, *P. aeruginosa*, and *P. putida*, using F or a suitable analog, have shown that the chromosomes of these are also circular and similar in size (see O'Brien 1987).

When a plasmid integrates into the chromosome, either by transposition or by recombination between copies of a common sequence (e.g. an IS element), the overall result is the same, namely, formation of a cointegrate in which the plasmid is flanked by direct repeats of the transposable element or of the common sequence. Recombination across these direct repeats will restore the plasmid to its autonomous state. The integrated F of an Hfr strain can be excised from the chromosome in this way. Sometimes, however, excision is not precise and chromosomal DNA adjacent to one side or to both sides of the integrated plasmid is also removed and becomes part of the plasmid; sometimes a remnant of F is left in the chromosome. The derivative plasmids generated by the imprecise excision events are called plasmid primes; e.g. F′*lac* is F with the *lac* operon of *E. coli* incorporated into it. In this case the operon is functional so that when an F′*lac is* transferred to a lactose nonfermenting strain the transconjugants (i.e. the progeny of the cross) are *lac*$^+$ (i.e. are able to ferment lactose).

E. coli K12 strains that carry an F′ plasmid are normally diploid for the chromosomal sequence carried on the plasmid. Recombination between these homologous sequences may be frequent and results in re-entry of the F sequence into the chromosome to generate an Hfr strain with the same origin and direction of chromosome transfer as the Hfr from which the F′ originated. In practice, F′ cultures transfer chromosomal DNA at frequencies between that of a normal F$^+$ culture (with autonomous F) and that of the originating Hfr strain, because the cells in the culture are a mixture of those with the F′ integrated into the chromosome and others in which the plasmid is autonomous, i.e. the integrated and free plasmid forms in these cell lines are in dynamic equilibrium. This phenomenon is not, in principle, restricted to F. Any plasmid can be integrated into the bacterial chromosome by a suitable transposition with subsequent imprecise excision generating plasmid primes that, in turn, can be used in mapping studies, provided that the original plasmid mediates its own transfer.

F or F′ plasmids can be transferred to species other than *E. coli,* including ones belonging to different genera, such as *Yersinia* and *Vibrio* and the *E. coli* genes are expressed in these organisms. In the main, incorporation of chromosomal DNA from *E. coli* into the chromosomes of bacteria of other genera is not observed, unless there is a close phylogenetic relationship as, for example, between *E. coli* and *Shigella* spp., because there is insufficient homology to permit recombination. Even between *E. coli* and *Salmonella*, chromosomal recombination is rare. As might be expected, however, F can insert into the chromosomes of those bacteria in which it can be established, probably via the activities of the transposable elements it carries, to generate Hfr strains, so furnishing a tool for genetic analysis.

Conjugation in other genera

Conjugative plasmids have been identified in many different bacteria including all gram-negative enterobacteria, in species of *Pasteurella*, *Yersinia*, and many nonfermenting gram-negative aerobes such as *Pseudomonas* and *Acinetobacter*, as well as in *Haemophilus*, *Neisseria*, *Campylobacter*, and *Bacteroides* spp., and gram-positive organisms such as *Streptomyces*, *Streptococcus*, and *Clostridium.* Demonstration of conjugation in all these species does not necessarily imply that the mechanisms are the same; for example, conjugative pili have not been identified in all the genera listed above. However, conjugative plasmids in *Pseudomonas* (Holloway et al. 1979), *Streptomyces* (Hopwood et al. 1973), and *Acinetobacter* (Towner and Vivian 1976) spp. mediate chromosomal DNA transfer in ways that appear to be analogous to that of F in *E. coli.*

In some genera, conjugation and transfer of antibiotic resistance determinants have been observed although no plasmid DNA could be detected, before or after transfer (Stuy 1980). In these systems the mobile DNA is normally part of the bacterial chromosome, but the strains do not behave as Hfr strains. Rather, transfer involves a discrete DNA sequence. During transfer the element behaves like a plasmid, i.e. it separates from the chromosome of the donor cell, but it cannot be isolated by conventional plasmid isolation procedures. These transfer systems are now known to reflect the activity of conjugative transposons and other ICEs (see above). One example is typified by the transposon Tn*916* which was first identified in the chromosome of a strain of *E. faecalis*. Tn*916* encodes resistance to tetracycline and accommodates conjugation functions that mediate transfer of the transposon alone, even between Rec$^-$ hosts (Franke and Clewell 1981; Clewell et al. 1988). No plasmid is involved and the element transposes from the

chromosome in the donor cell into the chromosome in the recipient (Salyers et al. 1995).

The conjugative functions of F are naturally derepressed, so that F transfers at high frequency. By contrast, most natural plasmids transfer at rather low frequencies. In some cases, mutant plasmids can be isolated that transfer at greatly increased frequencies, indicating that expression of the *tra* genes on these plasmids is normally genetically repressed. In other cases, genetic derepression has not been found, although the transfer frequency is relatively low; an example is the R plasmid RP4, which originated in a strain of *P. aeruginosa* but which can be transferred to virtually all gram-negative bacteria (Thomas and Smith 1987).

One consequence of regulating the expression of a set of transfer genes negatively, i.e. with a repressor, is that immediately after plasmid transfer expression of the transfer system is transiently derepressed in the recipient and remains so until sufficient repressor has been synthesized to re-establish control. During this time the recipient temporarily becomes capable of high-frequency plasmid transfer. If there are sufficient potential recipient cells available, the plasmid will spread rapidly from one cell to another in a chain reaction (epidemic spread) until the system is saturated, as was seen in early studies on the transfer kinetics of R plasmids such as Rl and R100. This finding has practical significance in the design of experiments to test for plasmid transfer (Broda 1979; Willetts 1988).

Conjugative plasmids in genera other than those of the Enterobacteriaceae may also repress expression of their transfer genes. In *E. coli* the only mechanism known to lift repression, other than mutation, is transfer to a new host. In other genera, however, induction of the conjugation functions has been reported, e.g. conjugation mediated by a tetracycline resistance plasmid in *Bacteroides fragilis* is induced by low concentrations of tetracycline together with induction of resistance to tetracycline (Privitera et al. 1981). This phenomenon does not appear to be widespread.

Conjugation in gram-positive bacteria appears to differ somewhat from that in gram-negative bacteria in the manner in which the donor and recipient cells are brought together. In *E. faecalis*, conjugation is mediated via sex pheromones excreted by potential recipient cells (Clewell 1993). These peptides cause donor and recipient cells to clump to form mating aggregates, a necessary prerequisite to plasmid transfer. Upon acquisition of the plasmid, the recipient promptly ceases to produce the particular pheromone that triggered plasmid transfer. Different plasmids respond to different pheromones and potential recipient cells produce several.

An unusual form of plasmid transfer, called phage-mediated conjugation, has been reported to occur with some strains of *S. aureus*. Lacey (1980) described the carriage of penicillin and cadmium resistance genes by elements with some of the characters of defective phages. Not only were these genetic elements themselves transmissible between non-lysogenic staphylococci, but they could also mediate transfer of unlinked resistance plasmids in mixed culture (but not in conventional transduction experiments).

Transduction

Gene transfer between bacteria can be mediated by some bacteriophages in a process known as transduction. Transduction is the transmission of a piece of host DNA from one cell (the donor) to another (the recipient) by a phage particle. Two forms have been described: generalized transduction (Masters 1996) and specialized transduction (Weisberg 1996). In the first, any part of the donor genome can be transferred to a suitable recipient; in the second, specific sequences are transferred.

Generalized transduction

Phage P1 replicates during infection of its *E. coli* host cell to generate long concatemers (head-to-tail multimers) of DNA that are subsequently cut into new phage chromosomes by the assembling phage particles. Transducing particles of P1 contain a length of the host chromosome, equivalent in size to the phage genome, in place of the phage DNA, indicating that some heads erroneously take in a section of bacterial rather than phage DNA during phage particle development. Such particles are morphologically normal and are released in the lysate along with ordinary phage particles; they can then infect fresh cells, injecting the contents of the head into the cell in the usual manner. Although the length of bacterial DNA transduced is similar to that of a normal phage chromosome, there is conflicting evidence as to whether particular regions of the host chromosome are preferentially transferred. Such transducing particles comprise approximately 1 in 100 000 of the phage particles in a phage lysate. A phage-infected cell has a roughly similar amount of phage DNA at the end of the replication period as there was bacterial DNA at the outset, yet only a small minority of phage particles contain bacterial DNA, indicating that the head-filling process preferentially packages phage rather than host DNA. Transduced genes are recovered by homologous recombination between the transferred DNA and the recipient cell's chromosome, although it is not uncommon for the transduced DNA to persist extrachromosomally but unreplicated in the cell. In this case it is inherited by only one daughter cell at cell division, a phenomenon called abortive transduction. The frequency of transduction of any one bacterial genetic marker is around 10^{-6} per surviving recipient cell.

Insofar as generalized (and specialized) transduction depends on infection by a phage, it is usual to find that bacterial genes can be transduced only to a recipient of

the same species, or perhaps even strain, as the donor; the host range of a phage is generally delimited by the presence of specific receptors on the surface of the cell. Although best studied with phages P1 and P22, two temperate phages, generalized transduction is not a property of temperate phages alone. The lysates of even highly virulent phages, such as those in the T series in *E. coli*, are found to include some particles containing host DNA (Borchert and Drexler 1980; Young and Edlin 1983), but if such particles infect a recipient and establish a transductant, the latter is usually killed by superinfection with other lytic particles. In these cases, transduction is only seen at low multiplicities of infection, i.e. when bacteria outnumber bacteriophage.

Generalized transducing phages can also transduce plasmids, as well as fragments of the donor chromosome, provided these are sufficiently small to be contained within the phage head; where plasmids are too large to be accommodated in the phage head, variants with partial deletions may be transduced, a useful technique for the isolation of plasmid deletion mutants (transductional shortening) before the advent of genetic engineering. The transduction of antibiotic resistance plasmids in staphylococci is of considerable clinical significance (Lacey 1973, 1984). Unexpectedly, the frequency of cotransduction of two plasmids in *S. aureus* is often much higher than would be predicted from the transduction frequencies of each plasmid separately, a result that may reflect cointegrate formation between the two plasmids (Novick et al. 1981, 1986).

Specialized transduction

Phage λ lysogenizes *E. coli* when the phage genome integrates into the *E. coli* chromosome at a specific site by means of a recombination event mediated by the phage function Int and host function IHF (Arber 1983). On induction of the lytic cycle, the integration event is reversed and the phage genome is excised in the presence of an additional phage function, Xis. Both integration and excision are highly specific with respect to the sites in the DNA at which recombination occurs. Occasionally, however, recombination during the excision process occurs between two sites other than the normal attachment regions of phage and host DNA, usually one within the prophage and one just outside (see Figure 11). The consequence is that a ring of DNA is excised that consists primarily of λ genes plus a few host genes from one side of the attachment region. In the presence of other λ DNA molecules undergoing normal lytic replication in the cell, this hybrid DNA can be packaged in a λ head and released in the phage lysate where it is available to infect a further cell and inject into it DNA that contains some host genes.

The gene clusters on the bacterial chromosome that lie closest to the λ integration site and are the most

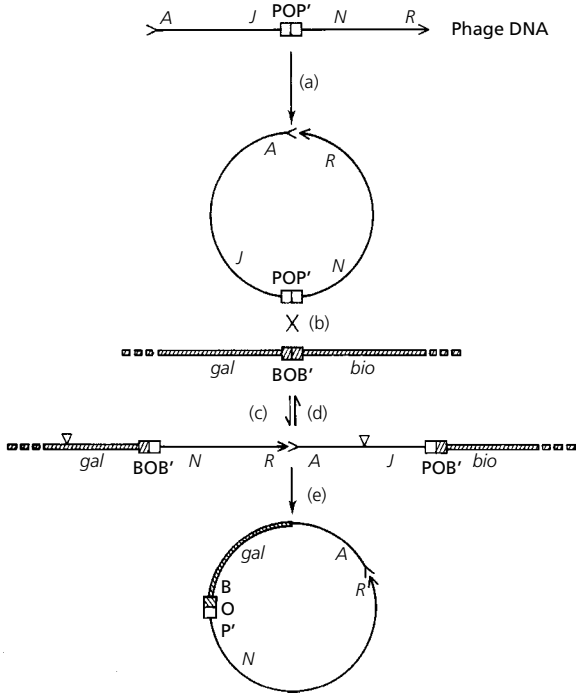

Figure 11 *The Campbell model for prophage λ integration into the bacterial chromosome.* **(a)** *Circularization of λ DNA by attachment of cohesive ends (arrows) followed by ligation.* **(b)** *and* **(c)** *Integration by site-specific recombination between phage (POP′) and host (BOB′) attachment regions; four phage genes (*A, J, N, R*) and two host gene clusters (*gal, bio*) are shown for reference, and phage integrase (*int^+ *product) and integration host factor (IHF) are required. Note that the prophage genes are permuted relative to those in the phage DNA.* **(d)** *Normal excision: integrase and excisase (*xis^+ *product) are required.* **(e)** *Abnormal excision, by recombination between two points (∇) other than the attachment regions, generating a defective λ particle carrying host* gal *genes a particle carrying* bio *genes can be formed similarly.*

easily selected in genetic crosses, i.e. *gal* and *bio* (Figure 11), and hence it is these genes that are readily found in specialized transducing particles represented as λ*gal* and λ*bio*. Lambdoid phages (Campbell 1994) are closely related to λ, but many have a different host integration site and are therefore able to transduce different genes; thus, ϕ80 acts as a specialized transducing phage for the tryptophan genes in *E. coli*. Less closely related phages are exemplified by P22 in *S.* Typhimurium, the prophage of which is integrated within the proline gene cluster; hence P22, which is a generalized transducing phage in *Salmonella*, is a specialized transducing phage for the *pro* region.

There are two constraints on the ability of a chromosomally integrated λ phage to form a specialized transducing particle: the recombination event must not exclude the cohesive end (*cos* site) of the phage genome and the total length of DNA in the particle must not significantly exceed that of wild-type λ itself, i.e. 5 percent increase or less, to allow for packaging in the head. A consequence of the second point is that, if the

phage acquires some host genes, it will normally have to lose some phage DNA to accommodate the acquisition; hence such phage derivatives are often defective in their abilities to propagate themselves and so require a helper phage to supply the missing function(s). With the exception of the limitation on genome size, specialized transducing phage formation is closely similar to the process by which hybrid plasmids such as F-primes are formed (Hu et al. 1975).

Specialized transduction differs from generalized transduction in many respects. Two are significant. The transducible genes are limited to those near the prophage integration site and the transducing particle contains a hybrid of phage and bacterial DNA, rather than consisting entirely of the latter. As a consequence, the transduced genes can be added to the genome of the recipient cell rather than replacing recipient genes, as in generalized transduction of chromosomal markers, because the modified phage genome can integrate into the prophage attachment site of the recipient's DNA. When such transductants are induced to enter a lytic cycle, the integrated hybrid DNA is excised and replicated to generate a large number of copies (a process which may require the presence of superinfecting normal particles to supply missing phage functions), each of which carries bacterial genes; these copies are packaged to form phage particles and if this lysate is then allowed to infect a further population of sensitive cells, the transduction frequency of the phage-associated genes increases about 1 000-fold, giving rise to high-frequency transduction (HFT).

Protoplast fusion

When the cell wall of a bacterial cell is removed, an osmotically fragile form called a protoplast is generated. This remains intact as long as the growth medium is osmotically buffered. Bacterial protoplasts can fuse with one another, a process called protoplast fusion. This generates cytoplasmic bridges that allow mixing of the cytoplasms of the fused cells and exchange of genetic material. Fusions can be obtained between unrelated cells, even between members of different kingdoms, and gene transfer by this mechanism has been termed genetic transfusion.

As in the other forms of gene transfer, plasmid or phage DNA can be transferred interspecifically or intergenerically, but, for recombination between chromosomal DNAs to occur, a close relationship between the participating cells is necessary in practice. In the case of protoplast fusions, two complete chromosomes are brought together to generate an artificial diploid and from such fusions haploid recombinants with many permutations of the traits of the parent cells can be recovered. Protoplast fusion is an artificial system of gene transfer that has little or no demonstrated relevance to gene transfer in nature. However, protoplast (or spheroplast) fusion has proved to be a valuable experimental tool that is likely to continue to prove useful in the advance of molecular genetics (Gokhale et al. 1993).

PLASMIDS

Plasmids are extrachromosomal, autonomous DNA molecules (i.e. replicons) found in many bacterial species (Stanisich 1988; Thomas 2000). Plasmids have also been found in lower eukaryotes, e.g. the 2 μm plasmid of yeast. The term 'plasmid' was proposed by Lederberg (1952) for all extrachromosomal genetic structures that can replicate autonomously. Here, the adjective 'autonomous' means self-governing and separate from the chromosome of the host, rather than an entity capable of separate existence, in that plasmids are obligate endosymbionts of bacteria, using the replication enzymes of their host to ensure their own propagation, but retaining control over replication. They differ from bacteriophages in having no independent, extracellular form. Most plasmids, whether from gram-negative or gram-positive bacteria, are circular elements, but linear plasmids have been reported in *Streptomyces* spp. and in *Rhodococcus* spp. (Hinnebusch and Tilly 1993). As more bacterial genomes have been sequenced, it has become clear that a number of species have multiple chromosomes rather than one (Cole and Saint-Girons 1999), some of which have sizes similar to a large plasmid. This raises the interesting question as to when such molecules should be considered as plasmids and when as minichromosomes. To date, a definition is lacking.

The term 'episome' has, in the past, been used as a synonym for plasmid. Episomes were defined as genetic elements that could exist and be replicated in either of two modes; autonomously or as part of a cointegrated structure with the bacterial chromosome. As it came to be realized that almost any plasmid can be integrated into the bacterial chromosome, the term lost its usefulness as the definition of a particular class of replicon (Novick et al. 1976). However, the adjective 'episomal' may be useful when applied in particular circumstances, e.g. to describe the state of F in an Hfr strain.

Plasmids are widespread (Stanisich 1988), but the practical importance of bacterial plasmids was first recognized in the early 1960s when transferable drug resistance was discovered (Watanabe 1963). Although plasmids represent only a fraction of the prokaryotic gene pool, they have attracted, and continue to attract, much research interest because the genes they carry determine many of the more interesting features displayed by bacteria, e.g. resistance to antibiotics and heavy metals, virulence, symbiosis and nitrogen fixation, exotic metabolic capacity, and many more (Stanisich 1988). They also serve as convenient models, particularly of replication and partition mechanisms (see Thomas 2000).

The extrachromosomal natures of F and of the first R plasmids discovered were deduced from their genetic behaviors. One feature is the tendency for clones in the culture to lose the plasmid, the set of plasmid-determined characters thus being lost simultaneously and irreversibly. The stability of inheritance of plasmids varies from one to another; some are lost very easily, at rates much higher than the rate of mutation, e.g. 1 percent or more of cell progeny may not inherit the plasmid in any one generation, compared with normal mutation frequencies in the range 1 in 10^6–10^8 per generation. Others are very stable indeed and are rarely, if ever, lost. Some plasmids can be eliminated from the host artificially (cured) by treatment with one of a variety of chemicals (curing agents) (Stanisich 1988). The quinolone drugs seem to be particularly effective (Weisser and Wiedemann 1985). However, no single curing agent or treatment can be relied upon; finding an appropriate agent or treatment to cure a specific plasmid is an empirical exercise that may well fail (Caro et al. 1984). The F plasmid is readily eliminated by treatment with acridine orange (Hirota 1960), but few others succumb to this agent. Some plasmids have temperature-sensitive replication systems and so are eliminated by growth of the cell at the nonpermissive temperature (May et al. 1964; Yokota et al. 1969), but this feature is not widespread. It has also been reported that the conversion of some bacteria to protoplasts (spheroplasts), e.g. *B. subtilis* and *S. aureus*, can result in plasmid loss. Again this is not a universal finding. Irreversible loss of a particular character, or set of characters, either spontaneously or after treatment, is still powerful genetic evidence for the existence of a plasmid but the inability to cure a specific feature, for the reasons given above, does not necessarily rule out plasmid involvement.

With the development of methods for isolating plasmid DNA, the presence of a plasmid can be determined directly. Modern small-scale preparative procedures (Sambrook et al. 1989) and the use of agarose gel electrophoresis allow plasmids to be detected in very small culture volumes (0.5–1.0 ml of a stationary phase culture). Plasmid DNA can be purified and reintroduced into bacterial cells by transformation (or electroporation) to establish definitively the connection between a phenotype of interest and the plasmid. However, this is still not always possible, particularly with large plasmids ($\geqslant$100 kb), or when investigating a poorly characterized bacterium, in which case it may be necessary to fall back on classical genetic techniques (conjugation, transduction, curing).

Plasmid genes

The only genes that are formally necessary to a plasmid are those needed for its replication. The amount of DNA devoted to this task is surprisingly small (Scott 1984; Thomas and Smith 1987) and rarely, if ever, exceeds a few kilobases. The minimum requirement is an origin sequence, origin of vegetative replication (*oriV*), at which replication can start, all enzyme functions being provided by the host. Several small plasmids have this arrangement, e.g. ColEI from *E. coli* and the related plasmid, CloDF13 from *Enterobacter cloacae*. In addition to an *oriV*, such plasmids also encode functions that control the extent of plasmid replication, i.e. ensure that the plasmid copy number per cell is maintained within fairly narrow limits specific to the particular plasmid (Sherratt 1986). In these plasmids no more than 1–2 kb is devoted to plasmid replication and its control. In others, more complex arrangements have evolved both to execute replication and to regulate it (Espinosa et al. 2000).

There are basically two modes of replication for circular plasmids: θ (theta) replication and RC (rolling circle) replication (Helinski et al. 1996). In the former, replication proceeds from a fixed origin in a unidirectional or bidirectional manner round the plasmid circle and both strands of the plasmid are replicated simultaneously, in a manner precisely equivalent to *E. coli* chromosome replication. Both strands of the parent plasmid remain intact during the replication process. The type of replication gets its name from the fact that a part replicated plasmid/chromosome resembles the Greek letter theta, θ, if opened out. For most of the plasmids in gram-negative organisms studied, this is the mode of replication utilized.

A few plasmids from gram-negative bacteria and the single-stranded coliphages (Baas and Jansz 1988) have been shown to use the alternative, RC replication mode, as does a family of highly related plasmids in gram-positive bacteria (del Solar et al. 1993). These plasmids always encode an initiator protein which introduces a site-specific nick at the double-strand origin (DSO) to generate a 3′-OH group, which serves as a priming terminus. In some cases, it has been shown that the initiator is covalently attached to the 5′-phosphate exposed by the cut. Replication proceeds by extension from the 3′-OH primer site, using the complementary strand (designated the plus-strand) as template. The old complementary strand (called the minus-strand) is displaced as replication proceeds. Once replication has traveled right round the circle (hence the designation RC) the minus-strand is circularized. Consequently, one double-stranded DNA molecule and one single-stranded molecule result. The circular single-stranded minus-strand is then converted to a double-stranded molecule using a second origin of replication that is specific to the minus-strand. Replication of plasmids, including linear plasmids, has been reviewed by Helinski et al. (1996) and by Espinosa et al. (2000).

Plasmids range in size from c. 1 kb to >400 kb (equivalent to c. 10 percent of the *E. coli* chromosome).

The smallest have only enough DNA to accommodate two or three small genes (e.g. Pl5A; Chang and Cohen 1978). Nevertheless, small plasmids are relatively abundant (Figure 12), though many confer no obvious phenotype on their host. Such plasmids (large or small) are said to be cryptic, a description that is largely an acknowledgment of ignorance of function.

Plasmids capable of mediating conjugation have a minimum size of c. 30 kb. Several have now been analyzed extensively with respect to the plasmid-encoded information needed for replication. Here, again, it is found that relatively little genetic content is devoted to replication. In addition to *oriV*, these plasmids encode one or two specific proteins necessary for their own replication, together with one or more replication-control functions (Scott 1984; Thomas and Smith 1987; see also Thomas 2000). These functions define the essential replicon of the plasmid and can be encoded in 4–5 kb (see Thomas 2000).

The relatively small amount of plasmid-encoded information needed for plasmid replication is a reflection of the fact that most of the replication apparatus is provided by the host. Most of the panoply of functions used to replicate the bacterial chromosome is borrowed to replicate the plasmid. Although not all plasmids have exactly the same requirements, they all use a common core of enzymes, e.g. either all or the bulk of the DNA synthesis is carried out by the main DNA polymerase of the cell, e.g. in *E. coli* DNA polymerase III is used. In *E. coli*, a number of small plasmids (e.g. ColE1 and related plasmids) require DNA polymerase I (product of the *polA* gene) in the early stages of replication, before switching to DNA polymerase III for the bulk of the synthesis. Consequently, such plasmids cannot be established in a *polA* mutant strain of *E. coli.* Most plasmids described to date, however, do not show this requirement.

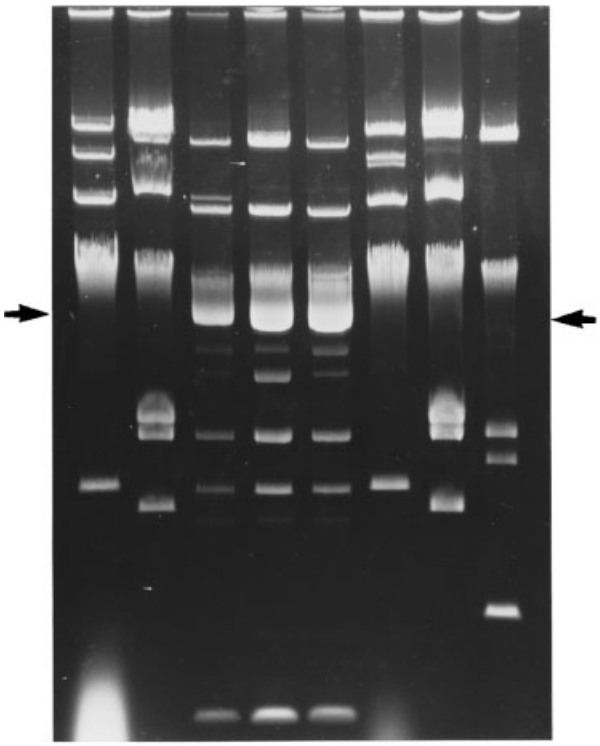

Figure 12 *Plasmid profiles from isolates of* E. coli *obtained from slurry waste from a calf-rearing unit. The diffuse band (arrows) in each track represents DNA fragments (chromosome and plasmid) that arose during the course of the preparation. Plasmid size decreases from top to bottom. The smallest plasmids seen in these preparations have sizes of 1–2 kb.*

F and many other plasmids maintain themselves at a low copy number, i.e. one to four copies of the plasmid per bacterial chromosome. These plasmids have been described as being under stringent replication control. By contrast, other plasmids, usually small ones such as ColEI, maintain themselves at a relatively high plasmid–chromosome ratio (15–20). Plasmids of this type are said to be subject to relaxed replication control because it was initially thought that replication control of these latter elements was less rigorous than for low copy number plasmids. This is now known not to be true; rather, the threshold value at which inhibition of replication operates is set higher. Once activated it is just as efficient.

For each plasmid there is a characteristic copy number, although this can be altered by mutation. Some plasmids also show changes in copy number in response to the prevailing culture conditions; for example, some plasmids continue to replicate when the cells enter stationary phase (when chromosomal replication stops) with the accumulation of large numbers of plasmid molecules per cell. Treatment of a culture of *E. coli* carrying ColE1, or a related plasmid, with chloramphenicol (at a concentration sufficient to inhibit protein synthesis) has the same effect; whereas the initiation of new rounds of replication of the bacterial chromosome (and many plasmids) requires de novo protein synthesis, that of ColE1 does not (see Thomas 2000).

The genes that control plasmid replication include functions that act repressively, i.e. inhibit replication. One consequence is plasmid incompatibility. It was discovered early that two variants of the same plasmid cannot be maintained stably in the same bacterial cell, i.e. they are incompatible. This arises because each directs the production of replication inhibitors that act on the other replicon, as well as on themselves. The consequence is that the content of plasmid molecules in the cell is set to a lower level than the sum of the two individual copy numbers. If the plasmids involved are unit, or low copy number plasmids, plasmid segregation at cell division is a likely result, i.e. clones emerge in which one or other of the plasmids has not been inherited (Novick 1987). Plasmids with unrelated replication control systems do not, in general, interact in this way and so do not destabilize each other; they are said to be compatible. Plasmids that are incompatible are said to belong to the same incompatibility (Inc) group; plasmids that are compatible necessarily belong to different incompatibility groups.

Plasmid partition systems

To be inherited stably, plasmid DNA not only has to be replicated but at each cell division both progeny cells must obtain at least one copy of the plasmid (which will be replicated until the appropriate copy number has been achieved). For low copy number plasmids such as F and some common R plasmids (Rl, R100), plasmid distribution at cell division – namely, plasmid partition – is an active process involving both plasmid- and host-specified products (Hiraga 1992; Draper and Gober 2002).

The former are designated *par* functions. The components comprise a small sequence of a few hundred base pairs, carried on the plasmid, that is believed to function like a primitive centromere to pair plasmids prior to cell division and one or more proteins that interact with it. These may be plasmid- or host chromosome-encoded. The system may direct plasmid DNA membrane association at or near the point of septum formation. If two different plasmids have related partition systems, to the extent that each is unable to distinguish the two plasmids, incompatibility will result. This will reinforce any incompatibility between the two plasmids that arises as a consequence of 'common' replication systems. However, the replication and partition functions on a plasmid act independently, as can be demonstrated by creating synthetic replicons with the replication system of one plasmid and the partition system of an unrelated plasmid. These hybrids are stably maintained, i.e. they are efficiently replicated and partitioned (Austin and Abeles 1983).

In contrast to the active partition described above, small plasmids with high copy number do not encode specific *par* functions. They rely on the random distribution of units to daughter cells. When the copy number is 15–20, the plasmid content of a cell immediately before cell division will be at least 30–40. Assuming random distribution of plasmids throughout the cell, the probability of one daughter cell inheriting the entire complement of plasmid molecules while the other inherits none is negligible (this would not be true for low copy number plasmids). Nevertheless, and somewhat paradoxically, some small, very high copy number cloning vectors generated in the laboratory are not efficiently inherited, in contrast to the plasmid from which they were derived.

If low copy number plasmids, in particular, are to be partitioned accurately, it is desirable, if not essential in all cases, that the molecules be present in the cell as monomers. Cells in a natural environment are recombination proficient. Hence they will actively pair and recombine homologous sequences, including plasmid DNA. A single crossover between two plasmid molecules will fuse them to form a dimer. If this were to happen with a unit copy number plasmid immediately after it had replicated and no steps were taken to reverse the event, at cell division one daughter cell would inherit both copies of the plasmid. The accumulation of plasmid dimers (and higher multimers) is prevented by plasmid-encoded, site-specific recombination systems (Austin et al. 1981; Lane et al. 1986; Dodd and Bennett 1987; Garnier et al. 1987; Krause and Guiney 1991; Eberl et al. 1994; Guhathakurta et al. 1996). These are specialized recombination systems that comprise a specific DNA sequence and a recombination enzyme that acts at the sequence (Smith 1988). The site must be part of the plasmid, but the enzyme can be either a plasmid-encoded function or a host function (Sherratt 1986; Dodd and Bennett 1987). These recombination systems rapidly convert plasmid multimers to their momomeric units, so reducing the chance of plasmid loss at cell division.

Post-segregational killing

Some plasmids have a third type of mechanism that acts to maintain their stability. These systems act after segregational loss of the plasmid to kill the plasmidless cell (Jensen and Gerdes 1995; Yarmolinsky 1995; Gerdes et al. 1997, 2000; Rawlings 1999). Each comprises two genes; one encodes a toxin, the other an antidote. The toxin is more stable than the antidote, which means that to neutralize the toxin the antidote must be produced continuously. This is possible as long as the cell carries a copy of the plasmid. However, in those unfortunate progeny that fail to acquire a copy of the plasmid at cell division, the toxin activity is duly unmasked as the antidote decays and the cell is killed.

A variation on the theme is encoded by the resistance plasmid R1. In this instance, the control exerted over the toxin is at the point of production, rather than inhibition of activity. The control locus, *sok*, overlaps the toxin gene, *hok*, which is encoded on the opposite strand of the DNA duplex. Both sequences are transcribed, but translation of the *hok* mRNA is prevented as a consequence of the two RNA transcripts annealing to one another over the region of overlap, where they are complementary. This prevents ribosome binding to the Hok mRNA. However, the *sok* RNA decays faster than the *hok* mRNA, so if the R1 plasmid is lost then the *hok* mRNA is translated. The toxin produced causes the collapse of the transmembrane potential and the cell dies (Gerdes et al. 1986, 1988).

Plasmid replication functions together with stability functions (*par* and site-specific recombination functions) comprise the minimum requirements of a stable, low copy number replicon, i.e. one that will be inherited efficiently despite there being very few copies of the plasmid. Partition and post-segregational killing mechanisms act in concert to provide stability (Brendler et al. 2004). The whole can be accommodated in a sequence of <10 kb (a stable miniplasmid derived from F, which is 94 kb in size, carries just 9.2 kb of F, in addition to a selectable marker).

Genes determining conjugation

Conjugation determined by F and by many other plasmids is a complex process requiring many genes. Accordingly, conjugative plasmids cannot be very small. The smallest conjugative plasmids in the Enterobacteriaceae are about 30 kb (of which 15–20 kb are devoted to transfer compared with F which devotes approximately 30 kb to transfer functions) (Avila and de la Cruz 1988; Thomas 1989; Zechner et al. 2000). Conjugation can be viewed as a two step process involving synthesis and transfer of DNA, the latter process mediated by a type IV secretion system (Llosa et al. 2002).

Most natural plasmids that are unable to mediate their own transfer can, nevertheless, be transferred conjugally by co-opting the transfer functions of a conjugative plasmid. Such plasmids, e.g. ColEI, have their own *oriT* and encode functions specific to it (mob genes). To transfer by conjugation, these plasmids require a helper plasmid to provide the missing transfer functions; for example, F can serve as a helper plasmid for ColE1 (Dougan and Sherratt 1977). Such plasmids are said to be mobilizable and mainly lack the ability to direct attachment of donor and recipient cells. A few plasmids possess only an origin of transfer, and transfer only when the other necessary transfer functions are provided by helper plasmids; for example, the small plasmid Pl5A, the progenitor of the cloning vector pACYC184 and its derivatives, has its own *oriT*, but to transfer it requires both Mob and Tra functions provided by ColEI and F (or equivalents), respectively.

Other plasmid genes

Replication and its control, maintenance, and transfer functions may be considered as inherent plasmid characteristics. Together, they direct both vertical (inheritance) and horizontal (transfer) transmission in bacterial populations. However, these features alone, no matter how intrinsically interesting, would have been insufficient to attract the degree of interest that bacterial plasmids have engendered. Indeed, until the discovery of transmissible multiple drug resistance (Watanabe 1963), the study of plasmids and their activities was a minority interest. The veritable explosion of interest shown since then reflects the variety of other genes, expression of which can affect the human condition, carried on plasmids.

Antibiotic resistance determined by plasmids was first discovered in Japan in the late 1950s. Until then it had been assumed that antibiotic resistance in bacteria, which had already become a clinical issue, resulted from the selection of chromosomal mutants, multiple resistance resulting from the sequential accumulation of mutations. Then, unexpectedly, epidemiological analysis of drug resistance in *Shigella flexneri* indicated that resistance could be transmitted between bacteria. Bacillary dysentery was common in Japan after the Second World War. By the mid-1950s, most Japanese infections with *S. flexneri* involved bacteria that were resistant to sulfonamides, so antibiotic treatment (now considered inappropriate) was switched to streptomycin, tetracycline, or chloramphenicol (much of it self-prescribed). In due course, a few strains resistant to the four unrelated drugs were isolated; thereafter such strains became progressively more common (Table 2). Isolates resistant to two drugs, which one would expect to see if sulfonamide-resistant clones mutated to resistance to another drug, were rarely isolated. Instead, in outbreaks of infection with a single *S. flexneri* serotype, strains from some patients had the multiple-resistance pattern, whereas others did not. Furthermore, *E. coli* strains with the same pattern of resistance were found in the feces of patients with dysentery. Given these observations, Akiba and, independently, Ochiai in 1959 (cited by Watanabe 1963) tested the hypothesis that multiple antibiotic resistance might be determined by an infective agent analogous to the F factor of *E. coli*. Mixed culture experiments proved this idea correct: multiple drug resistance was transferred from *S. flexneri* to *E. coli.* The infective agents are now called resistance (R) plasmids.

The increasing frequency of drug resistance in *S. flexneri* in Japan was not an isolated phenomenon. The emergence of resistance to antibiotics was recorded in diverse strains of bacteria worldwide: in *Shigella sonnei* in the London area, in *Salmonella* strains in the Netherlands, and in *S.* Typhimurium in England (see Datta and Nugent 1983). These cases differed to some extent from the Japanese experience in that there was a stepwise accumulation of resistance, first to sulfonamides and streptomycin, then to tetracycline, and subsequently (and infrequently) to chloramphenicol (Anderson 1968). Nevertheless, the resistance was primarily plasmid mediated and transmissible; ampicillin resistance then emerged, and within a decade had become common in *Salmonella* and *Shigella* strains throughout Europe (although it remained uncommon in Japan).

Table 2 *Multiple drug resistance in* Shigella flexneri *isolated in Japan*

Year	No. of isolates tested	Percentage multiresistant
1956	4399	0.02
1958	6563	2.9
1960	497	15
1962	6853	23
1964	5388	45
1966	4292	75
1968	1237	64
1970	562	74
1972	824	76

Multiresistant strains were resistant to chloramphenicol, tetracycline, streptomycin, and sulfonamide. Figures from Mitsuhashi (1977).

At the time of the discovery of R plasmids in the Enterobacteriaceae, multiresistant strains of *S. aureus* were a cause of concern in hospital infection. Penicillinase-producing strains of *S. aureus*, first recognized in the 1940s, had become common and had acquired resistance to new antibiotics as each was introduced into clinical practice. In 1963, it was shown that penicillinase synthesis by *S. aureus* was a plasmid-determined characteristic (Novick 1963), as are many other antibiotic resistances in that species (Lacey 1975). Penicillin-resistance plasmids in *S. aureus* have been studied extensively. They encode not only the penicillinase, but also the means to regulate its production which is induced when the cells are exposed to penicillin and other β-lactam agents. The mechanism involves a form of signal transduction, because the inducers (β-lactams) do not penetrate the cytoplasmic membrane (Everett et al. 1990; Bennett and Chopra 1993; Zhang et al. 2001). These plasmids often also encode resistance to mercury or cadmium salts, or both, and sometimes to erythromycin or fusidic acid. Resistance to tetracycline, chloramphenicol, and neomycin is also extrachromosomal in *S. aureus*, but these markers are usually found on separate plasmids.

Not only are many resistance determinants in *S. aureus* encoded by plasmids, but several are also carried on transposons (Murphy 1989). Tn*551*, encoding resistance to erythromycin, was the first to be discovered in *S. aureus* (Novick et al. 1979). Later, a transposon encoding resistance to gentamicin, kanamycin, tobramycin, trimethoprim, ethidium bromide, and quaternary ammonium compounds, designated Tn*4001*, was discovered (Lyon et al. 1984). This element was found in a multiresistant methicillin-resistant *S. aureus* (MRSA) (see Chapter 32, *Staphylococcus*) strain isolated in Melbourne. Strains of this type have caused concern worldwide because they are resistant to all β-lactam agents and many other antibiotics that might be used against them. While many antibiotic resistance genes in *S. aureus* are found on plasmids and transposons, the methicillin-resistance gene, *mecA*, is found on acquired sequences of 21–67 kb (Hiramatsu et al. 2001; Ma et al. 2002), termed SCC*mec* (staphylococcal cassette chromosome *mec*) elements (Katayama et al. 2000), which are integrated into the chromosome of *S. aureus* at a unique site, *attBscc,* found in an open reading frame of unknown function near the origin of replication (Hiramatsu et al. 2001). Integration is mediated by a site-specific recombination mechanism comprising two SCC*mec* genes, *ccrA* and *ccrB*, closely linked to *mecA*. A possible precursor of SCC*mec* elements, SCC_{12263}, that lacks *mecA* has been identified in the methicillin-susceptible strain *Staphylococcus hominis* GIFU12263 (Katayama et al. 2003).

The *mecA* gene encodes a penicillin-binding protein (PBP-2a or PBP-2′) (Utsui and Yokota 1985; Reynolds and Fuller 1986) that is not inhibited by β-lactam agents at clinically achievable concentrations and this new PBP can substitute for the cell's normal PBPs at concentrations of β-lactam agents that would normally be lethal to the cell (Archer and Niemeyer 1994; Berger-Bächi 1994). The drug of choice for these strains is often the glycopeptide antibiotic vancomycin. This inhibits peptidoglycan synthesis by binding to the terminal pair of D-Ala residues in the pentapeptide chain of the sugar–peptide cell wall precursor, sterically impeding its interaction with the transglycosylases which normally incorporate it into the peptidoglycan network. Unfortunately, resistance to vancomycin has emerged in the enterococci. The resistance mechanism is somewhat unusual in that it involves remodeling the pentapeptide of the cell wall precursor. The terminal D-Ala residue of the pentapeptide is changed to D-lactate. The loss of the amino group significantly reduces the affinity of the precursor for vancomycin. Because the D-lactate is cleaved from the precursor in the terminal stage of peptidoglycan synthesis, as the short peptide chains are joined together, the finished product is largely unaffected, although synthesis is slower. Remarkably, expression of vancomycin resistance can be inducible and the mechanism also involves signal transduction, mediated by what is known as a two-component regulatory system (see Hoch and Silhavy 1995). Two genes are involved, *vanS* and *vanR*. The former encodes a membrane protein, VanS, that senses the effect of the drug (Ulijasz et al. 1996) and records the fact by phosphorylating one of its histidine residues. The phosphate group is then transferred to an aspartate residue on the second component, VanR. This converts VanR to an activator specific for expression of the four genes needed for vancomycin resistance (Arthur and Courvalin 1993). A similar mechanism is used to regulate expression of virulence genes in *S.* Typhimurium and several other types of virulence gene (see Salyers and Whitt 2002). As if this degree of complexity were not sufficient, the whole system, vancomycin resistance and regulatory genes, is carried on a bacterial transposon, Tn*1546* (Arthur and Courvalin 1993). Recently, this form of vancomycin resistance has been detected in clinical strains of MRSA isolated in North America (Appelbaum and Bozdogan 2004), a worrying but not unexpected development because it has been shown in the laboratory that plasmids encoding vancomycin resistance transfer readily between enteroccocal strains and *S. aureus*. In addition, vancomycin resistance is evolving in *S. aureus* independently of acquisition of Tn*1546* or similar systems (Walsh and Howe 2002).

Many new antibacterial drugs have been introduced into clinical medicine in the last 25 years and plasmids conferring resistance to most of them have appeared within a relatively short time. Not only has the range of plasmid-determined resistance broadened but also more and more bacterial genera that carry resistance plasmids are being found (Tables 3 and 4). The appearance in the mid-1970s of plasmid-determined β-lactamase production, first in *H. influenzae* and then in *N. gonorrhoeae*, was of prime importance, not only to clinical

Table 3 *Some antibacterial drugs to which plasmids determine resistance*

Antibacterial drugs	
Penicillins, cephalosporins, carbapenems	
Erythromycin, lincomycin, and streptogramin B	
Streptomycin	Tetracyclines
Neomycin	Chloramphenicol
Kanamycin	Fusidic acid
Gentamicin	Sulfonamides
Tobramycin	Trimethoprim
Amikacin	

medicine where the strains presented a significant public health problem, but also to the study of plasmid evolution (Saunders et al. 1986; see also Chapter 25, Bacterial infections of the genital tract and Chapter 48, *Neisseria*), because some of the plasmids in both species were found to be closely related (Laufs et al. 1979). The TEM β-lactamase produced in both these organisms is encoded by a gene, *blaTEM*, carried on various transposons, among them Tn*1* and Tn*3* (Figure 7), which are widely distributed on plasmids. The production of a TEM β-lactamase by clinical isolates of a wide variety of gram-negative bacteria, particularly among the Enterobacteriaceae, of both human and veterinary origin, is common. Undoubtedly, the presence of this gene on a transposon, and its ability to transpose readily to many different plasmids, facilitated its rapid intergeneric spread. The route taken by such a resistance determinant, before it enters a particular bacterial species, may be circuitous, but if acquisition is desirable (i.e. of advantage to the survival of the organism), it is almost certainly inevitable. Indeed, reports indicate that transfer of resistance genes is not restricted to gram-positive–gram-positive and gram-negative–gram-negative exchanges, but can take place between gram-positive and gram-negative bacteria (Mazodier and Davies 1991), and even between bacteria and mammalian cells (Courvalin et al. 1995), although such events seem to be rare and any that do occur are likely to be nonproductive. Trieu-Cuot and Courvalin (1986) reported a kanamycin-resistance determinant encoding an APH(3′)III aminoglycoside phosphotransferase in a strain of *Campylobacter coli* identical to that previously found in streptococcal strains. For an account of antibiotic resistance in clinical practice, see Chapter 18, Antibacterial therapy.

Origin of resistance genes

The origin of resistance genes has been a puzzle for many years. Before the introduction of antibiotics these genes were rare, although plasmids similar to those now carrying resistance determinants were relatively common (Hughes and Datta 1983). Perhaps the most persistent and logical suggestion is that many of them originated in the antibiotic-producing organisms themselves, since these organisms, mostly streptomycetes, are sensitive to the antibiotics and so must necessarily protect themselves. This suggestion has been given credence by the finding that the amino acid sequences of several classes of the APH(3′) aminoglycoside phosphotransferases, which confer resistance to kanamycin and neomycin, from both gram-positive and gram-negative bacteria, are evolutionarily related to another from the neomycin-producing *Streptomyces fradiae* (Trieu-Cuot and Courvalin 1986). An interesting variation on this theme is that in the production of antibiotics, small amounts of DNA from the producing organism remain as a contaminant and this may be the source of some resistance genes seen in clinical strains (Webb and Davies 1993). Clearly, if true, this has considerable significance for future antibiotic production, particularly in terms of quality control measures.

Some, if not all, β-lactamases have probably evolved from the same ancestral gene(s) that produced the β-lactam targets, the PBPs. Recent work has clearly shown the evolutionary relationship between the class A β-lactamase of *Bacillus cereus* and a PBP from *Streptomyces* (Samraoui et al. 1986; Joris et al. 1988, 1991). Furthermore, Ghuysen and his colleagues have shown that the protein structures of several β-lactamases and some PBPs are highly conserved, strongly indicating an evolutionary link (Ghuysen 1991, 1994; Frère 1995). The branches from the ancestral gene that yielded present-day β-lactamases, on the one hand, and PBPs, on the other, almost certainly diverged a long time before the discovery of penicillin, let alone its use in clinical medicine (Massova and Mobashery 1999). Nonetheless, the evolution of β-lactamase genes continues to the present day (Galleni et al. 1995; Bush and Jacoby 1997; Koch 2000; Bradford 2001; Gniadkowski 2001; Ford and Avison 2004).

Although most clinically significant β-lactamases are plasmid-encoded and constitutively expressed (particularly in gram-negative bacteria), β-lactamases are also relatively common chromosome-encoded enzymes in many types of bacteria, not only those causing disease, and their expression can be elaborately controlled; some species even have multiple, coordinately controlled β-lactamases (Imsande 1978; Normark 1995; Rossolini et al. 1996; Hanson and Sanders 1999; Philippon et al. 1998). These findings suggest that protection against β-lactam antibiotics is a common and desirable property in nature, although high concentrations of β-lactam agents in the soil, or in other natural habitats, have not been detected. So, it is not at all clear from where the selection pressure for the evolution, particularly of the inducible β-lactamases, comes. An alternative suggestion is that, although β-lactamases hydrolyze β-lactams effectively, this activity is not the normal function of the

Table 4 *Some genera in which R plasmids have been found*

Genera	
Enterobacteriaceae, i.e. *Escherichia, Salmonella, Shigella, Proteus, Providencia, Klebsiella, Serratia*, etc.	
Pseudomonas	
Acinetobacter	*Staphylococcus*
Vibrio	*Streptococcus*
Yersinia	*Bacillus*
Pasteurella	*Clostridium*
Campylobacter	*Corynebacterium*
Haemophilus	
Neisseria	
Bacteroides	

chromosomally encoded enzymes, some of which, if not all, may be involved in peptidoglycan metabolism (Morosini et al. 2000).

An equally intriguing case is that of trimethoprim. This is a purely synthetic compound that has no known natural analog. Its mode of action is to inhibit dihydrofolate reductase, which catalyzes one of the steps in folic acid synthesis (see Chapter 18, Antibacterial therapy). The enzymes in many bacteria are readily compromised by this antibiotic. Resistance to trimethoprim, e.g. as encoded by Tn*7* (Craig 1989), arises because of the acquisition of a gene encoding a dihydrofolate reductase that is much less sensitive to trimethoprim. The source of the gene is unknown. It may be one that naturally encodes a resistant enzyme; alternatively, it may be a mutant form of a naturally inhibited one. Several trimethoprim-resistance alleles, each encoding a dihydrofolate reductase, are now known (Huovinen 2001; Skold 2001). The speed with which resistance emerged, approximately 10 years after the introduction of the drug into clinical practice, is impressive and gives considerable cause for thought, because on an evolutionary time scale 10 years is but a blink.

The case of trimethoprim is not an isolated example. The emergence of resistance somewhere in the bacterial kingdom, following widespread use of a new antibiotic, and its subsequent dissemination among many bacterial species appears to be the norm rather than the exception, attesting to highly efficient gene transfer processes within populations of bacterial cells (Bennett 1995). The frequency of transfer does not need to be high to achieve efficient spread of a particular resistance gene. Even rare transfer events can be effective if environmental conditions impose a sufficiently strong selection pressure on those few progeny cells that do acquire the gene in question. The presence of the antibiotic in the cells' environment constitutes a powerful selective force. The few resistant cells will then rapidly reproduce, while the majority of their bacterial relatives are unable to do so, to become the dominant bacterial species. There is no reason to believe that the extensive interspecific gene transfer highlighted by the spread of resistance genes is confined to them. Rather, the view that all bacterial genes are potentially available to all bacteria is gaining more general acceptance, epitomized by the movement of some chromosomal β-lactamase genes on to plasmids and then into unrelated bacteria (Philippon et al. 2002; Walther-Rasmussen and Hoiby 2002; Bonnet 2004; Ford and Avison 2004), mediated by IS elements.

Resistance gene evolution by mutation

Resistance to an antibiotic may be the result of acquiring new genetic information in the form of a plasmid or transposon, or it may be due to a mutation in the gene for the antibiotic target that renders it less susceptible to the drug. For some drugs, e.g. streptomycin and rifampicin, this form of resistance is often readily selected in the laboratory; however, it is much less common in clinical experience, probably due in part to the success of plasmid–transposon systems and in part to the fact that alterations to key enzymes often come at a price (Andersson 2003). One clear exception to this is resistance to the fluoroquinolones, antibiotics that interfere with DNA synthesis through inhibition of enzymes called topoisomerases (Drlica and Malik 2003; Ruiz 2003; Sissi and Palumbo 2003). Here, resistance is acquired primarily by mutation of the GyrA component of DNA gyrase or of the ParC component of topoisomerase IV, or on occasion by a mutation that activates an efflux pump that removes the fluoroquinolones from the cell (Elliott et al. 2003; Eaves et al. 2004; Ricci et al. 2004).

There is one bacterial species for which acquisition of resistance by mutation is both the sole option and clinically significant, namely *Mycobacterium tuberculosis* in which plasmids appear to have little or no role to play (Cole and Telenti 1995). Multiple antibiotic resistance in *M. tuberculosis* is acquired by sequential accumulation of mutations in the genes encoding the antibiotic targets (Gillespie 2002; Wade and Zhang 2004). The apparent failure of *M. tuberculosis* to acquire R plasmids may reflect its unusually waxy outer surface that is markedly different from the outer surfaces of most bacteria (see Chapter 46, *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and other slow growing mycobacteria).

Antibiotic synthesis

Plasmids determine the production of bacteriocins and microcins (Pugsley and Oudega 1987; see also Chapter 5, Bacteriocins and bacteriophages). They also play a part in the production of a variety of antibiotics, including

some of those used in medicine (Hopwood 1978; Chater and Hopwood 1989). Plasmid SCP1, in *Streptomyces coelicolor* determines production of the antibiotic methylenomycin. The same plasmid confers resistance to the antibiotic. Plasmids can carry structural or control genes determining production by *Streptomyces* of oxytetracycline, chloramphenicol, and perhaps other antibiotics. The genetics of antibiotic-producing microorganisms is of great interest and of enormous potential importance in the pharmaceutical industry, and rapid progress is now being made in this area (see, for example, McDaniel et al. 1995; Paradkar et al. 2003; Weber et al. 2003).

Resistance to other agents

Plasmids may also confer resistance to antibacterial compounds other than clinically useful antibiotics, such as a variety of metal ions and organometallic compounds. Plasmid-determined resistance to mercuric ions (Hg^{2+}) is very common in bacteria found in soil and water (Bale et al. 1987) such as *Pseudomonas* spp., in members of the Enterobacteriaceae and in *Staphylococcus* spp.; this is frequently linked to antibiotic resistance. In different surveys, 25–60 percent of R plasmids determined Hg^{2+} resistance. In general, high frequencies of antibiotic resistance are found in environments where antibiotic concentrations are high, e.g. hospitals and farms on which livestock are reared intensively. The apparent linkage of Hg^{2+} resistance stems, in part at least, from the finding that a family of large transposable elements encoding clinically significant antibiotic resistance, mediated via integrons, appears to have evolved from an ancestral element that encoded Hg^{2+} resistance. These transposable sequences seem now to be widespread, particularly among the Enterobacteriaceae (Grinsted et al. 1990). Mercury resistance linked to antibiotic resistance may also be perpetuated by the use of dental amalgams that incorporate mercury (Summers et al. 1993).

Mercury resistance is effected by reducing ionic mercury to the pure metal and releasing it in gaseous form (volatilization). The mechanism also involves active concentration of mercuric ions prior to reduction (Barkay et al. 2003). The complexity of the system suggests one of some antiquity rather than one of recent evolution.

Resistance to cadmium, lead, antimony, arsenic, tellurium, and silver compounds are plasmid-determined in staphylococci and gram-negative bacteria (Summers and Jacoby 1977; Summers and Silver 1978; Foster 1983; Silver and Phung 1996; Nies 2003; Silver 2003). Resistance to silver salts was first reported after the use in burns units of silver sulfonamide compounds (McHugh et al. 1975). Resistance to copper salts displayed by bacteria isolated on pig farms where copper is used as a feed additive has also been reported (Tetaz and Luke 1983; Brown et al. 1995). In these cases, the immediate selective conditions are obvious; however, when and where the resistance mechanisms evolved is another question. Resistance to heavy metal ions may be mediated by innate or acquired efflux pumps.

Plasmids may also change the sensitivity of their bacterial host to UV light and other mutagens (Mortelmans and Stocker 1976) and to bacteriocins (Pugsley and Oudega 1987).

The degradation of several toxic organic compounds, such as camphor, naphthalene, octane, and toluene, may be determined by plasmids, particularly in soil bacteria. These bacteria, often *Pseudomonas* or *Flavobacterium* spp., have evolved the ability to use these toxic compounds as nutrients. The use of these and similar bacteria to effect biological detoxification and reverse environmental pollution has been attempted, with some success, but this area of bacterial exploitation is still very much in its infancy (Shannon and Unterman 1993; Janssen et al. 1994; Spain 1995).

Virulence factors

Virulence determinants are genes, the expression of which is necessary for bacteria to establish and maintain an infection (see Chapter 6, Molecular basis of bacterial adaptation to a pathogenic lifestyle and Roth 1988; Dorman 1994; Salyers and Whitt 2002). They include colonization mechanisms, mechanisms to overcome host defense systems, and the production of toxins. All these functions may be encoded by plasmids (Brubaker 1985). For example, enterotoxin production in *E. coli* and *S. aureus* (Betley et al. 1986) and production of surface antigens by *E. coli* that allow colonization of the small intestine in specific mammals, including humans, can both be plasmid-determined (Brubaker 1985). At least one enterotoxin gene found in *E. coli* has been found on a transposon (Tn*1681*) (So and McCarthy 1980).

Strains of *E. coli* isolated from systemic infections in humans and pigs are more frequently hemolytic and more frequently produce colicin V than comparable fecal strains; both characteristics are plasmid-determined. Carriage of a ColV plasmid increases the virulence of *E. coli* strains for calves, chickens, and mice (Smith and Huggins 1976); however, the effect is not due to the colicin itself, but to other determinants encoded by the plasmid. The ColV plasmid encodes functions that facilitate the uptake of iron by the bacterial host (Williams 1979) and others that increase bacterial resistance to serum (Binns et al. 1979). Plasmids other than ColV also confer serum resistance on *E. coli* (Taylor 1983). In one instance this seems to involve the acquisition of an additional outer-membrane protein, which is a component of the plasmid transfer system (Moll et al. 1980). A plasmid that confers serum resistance on *Salmonella* Dublin has also been described (Terakado et al. 1988; Foster and Spector 1995).

The smooth to rough (S→R) variation in *S. sonnei* is accompanied by loss of the form I (S) antigen and loss of virulence. Kopecko et al. (1980) demonstrated that this conversion is determined by a large plasmid, loss of which results in the S→R change. Transfer of the plasmid to *S. flexneri* 2a or *Salmonella* Typhi generated derivatives that were agglutinable both by their own specific antisera and by antiserum specific to *S. sonnei* form I antigen. In *S. sonnei* the form I antigen, and hence the plasmid that encodes it, is one of the requirements for virulence (Chapter 54, *Salmonella*), as demonstrated in the Serény test.

Strains of *S. aureus* responsible for scalded-skin syndrome produce exfoliating toxins that, in some cases, are encoded by plasmids (Wiley and Rogolsky 1977). Some *Staphylococcus* spp. produce enterotoxins that cause the syndrome known as intoxicating staphylococcal food poisoning. Some of the toxin genes responsible for this syndrome are encoded by plasmids (Couch et al. 1988). Other examples of pathogenic properties determined by or associated with plasmids have been described and reviewed elsewhere (Elwell and Shipley 1980; Brubaker 1985; see also Dorman 1994; Salyers and Whitt 2002).

The role of R plasmids as possible virulence factors has been discussed by Elwell and Shipley (1980). Resistant bacteria, such as chloramphenicol-resistant *S.* Typhi, penicillin-resistant *N. gonorrhoeae*, or multiresistant *S. aureus* may sometimes appear to be of unusual virulence simply because the infections they cause do not respond to the first line of antibacterial therapy. This property certainly may facilitate dissemination, but does not necessarily imply increased virulence. Particular R plasmids may well have specific effects upon virulence, either increasing or decreasing it, depending on how expression of plasmid-encoded functions affects other virulence determinants. Indeed Lacey and Kruczenyk (1986) have argued that the plasmid-carrying MRSA, although posing an undeniable clinical problem, are not as virulent as their predecessors. However, generalizations in this area without rigorous experimental evidence are inappropriate and the spread of MRSA into the community would argue differently (Palavecino 2004).

In plants, *Agrobacterium tumefaciens* has long been known as the causative agent of crown gall, a tumorous disease that affects many plants and causes serious losses in fruit growing. The transformation to a tumor-like growth requires transfer from the bacterium to the plant cell of genetic information, part of which is incorporated permanently into the genome of the plant cell. The requisite information is encoded on large tumor-inducing (Ti) plasmids which mediate the transfer. This process is an elegant example of natural genetic engineering since the transformed plant cells produce compounds that can be used as nutrients by the *Agrobacterium* responsible for the transformation (Hooykaas 1989; Hooykaas and Beijersbergen 1994).

Metabolic characters

The metabolic diversity of the pseudomonads is due, in part, to the carriage of plasmids that specify degradation of unusual, often toxic, organic compounds, as already noted (see Harayama and Timmis 1989). Often, the metabolic genes are associated with, or are parts of, transposable elements (Tan 1999). 'Metabolic' plasmids have also been reported in other genera. When they are present in clinical isolates, such plasmids can initially complicate strain identification in the diagnostic laboratory. Lactose fermentation by strains of *Salmonella* (including Typhi) is usually plasmid-determined. Plasmids encoding lactose catabolism have also been found in isolates of *Proteus* and *Yersinia enterocolitica* (Falkow et al. 1964; Cornelis et al. 1976), and *Klebsiella* (Reeve and Braithwaite 1973), although in *Klebsiella* they are not prominent because the chromosome has a *lac* operon. In the study involving *Y. enterocolitica*, the particular genes were not only carried on a plasmid but were also transposable (Cornelis et al. 1978).

Genes for the utilization of other sugars, especially sucrose (*suc*) and raffinose (*raf*) may be plasmid-borne. Plasmids with both *lac* and *suc* genes have been found in *Salmonella* (Johnson et al. 1976), and *raf* genes are associated with those determining the K88 antigen adhesion factor which has been shown to be important in porcine *E. coli* enteritis (Shipley et al. 1978; see also Chapter 52, *Escherichia*). Plasmids determining H_2S production (Ørskov and Ørskov 1973), urease production (Farmer et al. 1977; Wachsmuth et al. 1979), or citrate utilization (Smith et al. 1978) have also been found in clinical isolates. Their preponderance is difficult to assess from phenotypic observation. However, modern molecular genetic techniques such as DNA probing, or PCR analysis, could possibly supply an answer, if needed. Such plasmids are not restricted to clinically important bacteria. Lac plasmids in *Lactobacillus lactis* cheese starter strains are of major importance to the dairy industry (Farrow 1980; see also Chapter 10, The microbiology of milk and milk products).

Phage inhibition

Carriage of certain plasmids can alter the susceptibility of the host to certain bacteriophages, thus changing the phage type of that particular isolate, which may be important in diagnostic and epidemiological investigations (Stanisich 1988). Usually the molecular basis for the change is unknown; however, some plasmids encode one of a class of DNA-degrading enzymes known as restriction endonucleases that fragment foreign DNA as it enters the bacterial cell, rendering it less susceptible to attack by some, if not all, phages. Such plasmids also encode the means to protect the host cell DNA, including the plasmid itself, from similar attack. This usually

involves methylation of particular bases within specific short sequences which are also the sites recognized by the restriction endonuclease (Primrose et al. 2001).

Mapping of plasmids

Because plasmids are small, relative to bacterial chromosomes, they are popular molecules with which to investigate numerous aspects of DNA metabolism, including replication mechanisms (Scott 1984), recombination (Dressler and Potter 1982; Hsu and Landy 1984) and gene expression (Primrose et al. 2001; Thomas 2000). This interest has led to the development of an impressive range of techniques designed to study DNA in general and plasmid DNA in particular (see Grinsted and Bennett 1988; Sambrook et al. 1989). In the past, electron microscopy has been employed extensively to analyze DNA molecules, both as a simple tool to estimate plasmid size and, by means of heteroduplex analysis (Burkardt and Puhler 1988), to examine DNA rearrangements such as insertions, deletions, replacements, and inversions. Electron microscopy has, however, largely been replaced by restriction enzyme analysis and, more recently, by DNA sequencing (Grinsted and Bennett 1988; Brown 2001; Primrose et al. 2001). This last technology has now evolved to the point where DNA sequencing of very large DNA molecules (e.g. human chromosomes) has been achieved. The complete sequences of many bacterial chromosomes, including several human and animal pathogens (e.g. *E. coli*, *H. influenzae*, *Mycoplasma genitalium*, and *Helicobacter pylori*) are now available, as are the complete sequences of several plasmids. These data allow individual genes to be identified and ordered on the plasmid and then to be cloned for further study. By means of in vitro mutagenesis, such genes can be systematically and deliberately altered (Braman 2002; Primrose et al. 2001; see also Watson et al. 1992). These mutations can then be analyzed for their effects in the same way as if they had occurred naturally (Grinsted and Bennett 1988; Brown 2001).

Evolution and classification of plasmids

Plasmids are found in most, if not all, bacterial genera. Their origins are a matter for speculation. Each bacterial genus or group of genera appears to have its set of plasmids. Host specificities of plasmids have not been much studied, with one or two notable exceptions such as RK2 (RP4) (Thomas and Smith 1987) and RSF1010 (Barth et al. 1981; Buchanan-Wollaston et al. 1987). However, R plasmids of *S. aureus* can replicate in *B. subtilis* (Ehrlich 1977) and some R plasmids in *E. faecalis* can transfer by conjugation to *S. aureus* (Engel et al. 1980) and to lactobacilli (Gibson et al. 1979). Plasmids found in the Enterobacteriaceae can usually be transferred between many, if not all, the genera within the family. Some transfer more widely. In general, plasmids resident in gram-positive bacteria appear not to be able to establish themselves in gram-negative bacteria and vice versa.

In classifying plasmids, host range might be expected to be an important criterion, but in practice it is not a very convenient one. When a newly discovered plasmid is not transferred to a new host in laboratory experiments it may be for any one of a variety of reasons, of which an inability to replicate in the new host is one. In practice, a much more convenient criterion is available. As already stated, certain pairs of plasmids cannot coexist stably in the same bacterial cell, a phenomenon termed incompatibility (Novick and Richmond 1965), which is expressed when two plasmids have the same, or very closely related, replication systems or partition systems, or both (Novick 1987). A strong incompatibility reaction generally indicates similarity in the former.

Replication systems and partition systems are not mutually dependent, and perfectly stable replicons with the replication system of one plasmid and the partition system of another can be constructed in the laboratory. In nature, however, it is likely that a particular replication system and a particular partition system will be found in association. Thus, two plasmids with essentially the same replication system are also likely to have related partition systems, reinforcing any incompatibility reaction.

When each plasmid in a collection is tested, in turn, against all others in the set, those which are incompatible can be identified and so the collection can be ordered into incompatibility (Inc) groups. This means of classification has been applied to plasmids in the Enterobacteriaceae, particularly *E. coli* (Jacob et al. 1977), in *Pseudomonas* spp. (Jacoby 1977), and in *S. aureus* (Novick et al. 1977). Plasmids that can replicate in both *E. coli* and *Pseudomonas* spp. are included in both classifications (Hedges and Jacoby 1980), for example, RP4 is IncP when in the Enterobacteriaceae but IncPI when in *Pseudomonas* spp.

Because it principally reflects the primary plasmid maintenance systems (replication and partition), incompatibility is a sound basis for grouping plasmids, and there is good evidence that it reflects evolutionary relationships. Within any one Inc group, plasmids, in general, show extensive DNA homology, i.e. common sequences are not restricted simply to replication and partition functions. By contrast, plasmids of different Inc groups rarely show extensive homology. Exceptions are plasmids of the IncF complex, which can be grouped into at least five Inc groups but which nevertheless display extensive homology because their transfer systems are closely related.

Plasmids that belong to the same incompatibility group in general encode the same or similar sex pili. By contrast, plasmids belonging to different Inc groups are more likely to encode sex pili that differ morphologically and serologically. Sex pili can act as receptors for

bacteriophages. One practical consequence of this is that pili can often be detected because they permit specific bacteriophage adsorption (Figure 13). However, because of the normally repressed nature of a number of plasmid transfer systems it may be necessary transiently to derepress a specific system before its identity can be established by the use of specific bacteriophages (Willetts 1988). In electron microscopy studies of their morphology and serology, Bradley (1980) identified pili determined by conjugative plasmids of many known Inc groups of *E. coli* and several of *P. aeruginosa*. His work shows that, with very few exceptions, all conjugative plasmids within an incompatibility group determine the same type of pilus, a finding consistent with conclusions from DNA homology studies.

Plasmids of the same incompatibility group often share a similar host range, show considerable DNA homology, are of similar molecular sizes, and, with conjugative plasmids, possess related transfer systems. By contrast, other plasmid-encoded properties, in particular antibiotic resistance, are not indicative of a particular incompatibility group. Most antibiotic resistance genes, which can be identified either as DNA sequences or as their protein products, are found on a wide variety of otherwise unrelated plasmids. It seems that present-day plasmids have evolved, and continue to evolve, by the accretion of DNA sequences of transient or lasting value to bacterial cells. These sequences are often components of bacterial transposons and integrons (Wiedemann et al. 1986; Hall and Collis 1995; see also Levy and Novick 1986; Berg and Howe 1989; Thomas 2000).

It is often stated that R plasmids consist of a resistance transfer factor (RTF) (a conjugative plasmid component) linked to a resistance determinant (r-det). The reason is historical (see Novick 1969; Helinski 1973) because the earliest studies of R plasmids involved members of one incompatibility group, IncFII, which display precisely this form. In the light of more diverse studies, it can be seen that the description is specific rather than general and many R plasmids have structures that do not conform to this simple compartmentalized scheme, e.g. RP1 (Figure 14) (Gorai et al. 1979; Villarroel et al. 1983; Thomas 1989).

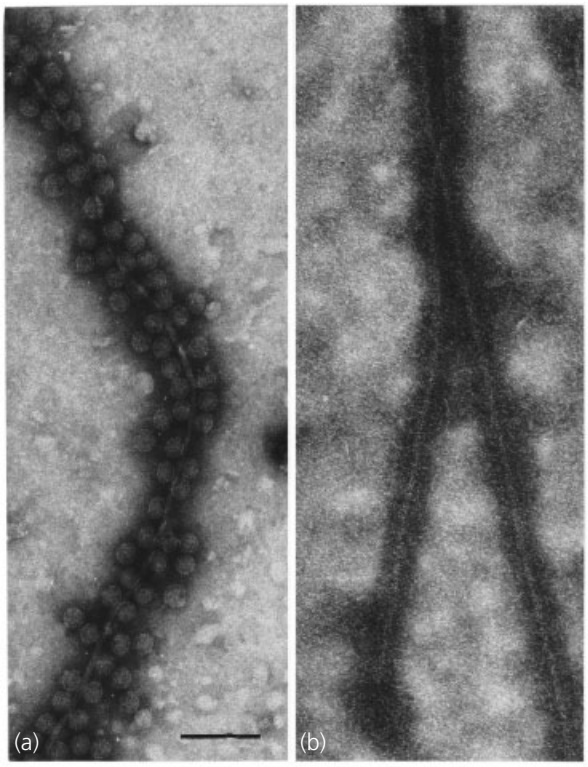

Figure 13 *Conjugative pili labelled with phage or antibody.* **(a)** *F pilus labelled with particles of phage R17. (Reproduced with permission from Bradley 1977).* **(b)** *D pilus labeled with specific antiserum (D.E. Bradley, unpublished). Bar = 100 μm*

BACTERIAL VARIATION AND EPIDEMIOLOGY

In epidemiological studies, the aim is to identify a particular clone within a bacterial species in order to trace its spread and, if possible, to determine its origin. In the past, biochemical and serological characters, phage sensitivities and lysogeny, production of or sensitivity to bacteriocins, sensitivity or resistance to antibiotics and other antibacterial agents, and plasmid profiles have all been exploited to this end. These characters are usually sufficiently stable to be useful when tracing bacterial strains over relatively short periods, but it should be remembered that none of them is immutable and several are encoded by extrachromosomal elements which may be lost over a period of time. Furthermore, new characters can be acquired by acquisition of a plasmid or a transposon. The isolation of variants indicates either genetic change in an epidemic strain or the discovery of a different strain of the same species. It is not always possible to distinguish between these alternatives (Seal et al. 1981), but the more characters used to identify the strain, the greater the chance that these alternatives can be distinguished. Today, molecular genetic techniques such as pulse-field gel electrophoresis (Gardiner et al. 1986; Morrison et al. 1999; Arakawa et al. 2000; Cohen et al. 2001) and multilocus sequence typing (Enright and Spratt 1999; Clarke 2002; Urwin and Maiden 2003; Cooper and Feil 2004) are assuming greater prominence in strain typing. However, these techniques are still technically demanding and relatively expensive, so serological, biochemical, and phage typing systems are still commonly used in the diagnostic laboratory.

Chromosome analysis

With the development of automated high throughput DNA sequencing systems, it has become possible to

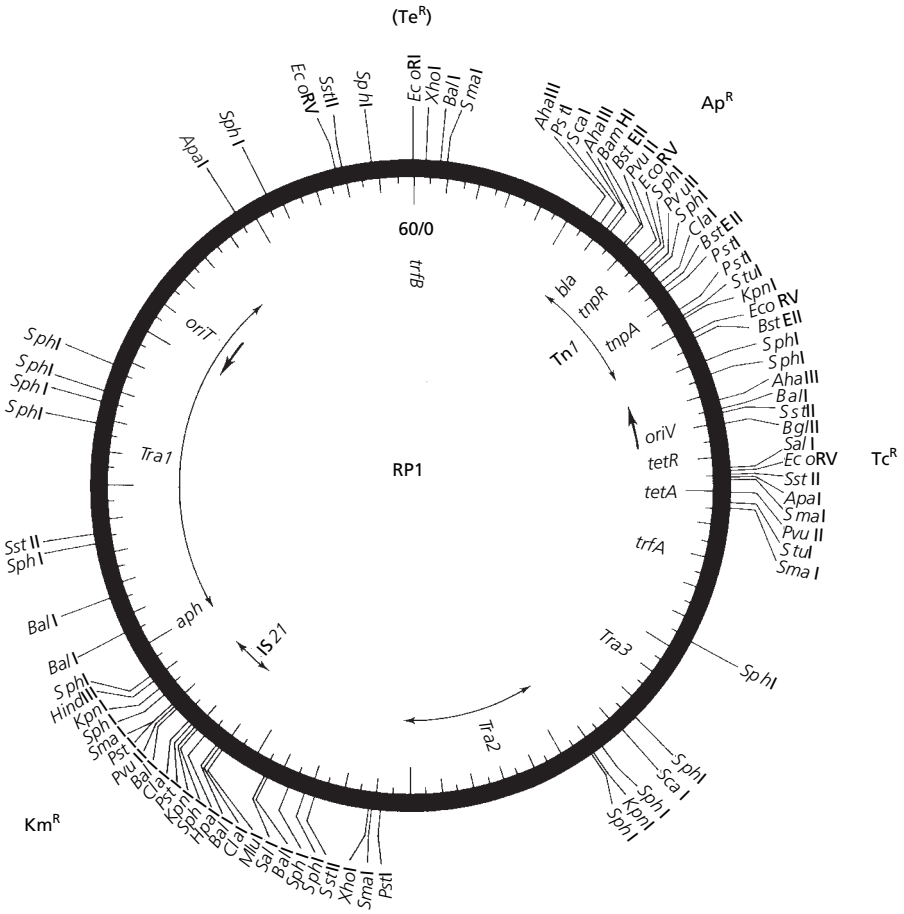

Figure 14 *Restriction and genetic maps of RP1. Plasmid RP1 is a 60 kb IncP conjugative resistance plasmid conferring resistance to ampicillin (Ap), kanamycin (Km), and tetracycline (Tc) that was isolated from a strain of* P. aeruginosa. *Genetic loci:* aph*, aminoglycoside phosphotransferase (resistance to kanamycin and neomycin);* bla*, TEM-2 β-lactamase (resistance to β-lactams including ampicillin and carbenicillin);* tet*, tetracycline resistance genes;* oriT*, origin of conjugative transfer;* Tra*, genes required for conjugative transfer;* oriV*, origin of replication;* trf*, plasmid replication genes;* tnp*, transposition genes of Tn*1 *(Figure 5). Te* ^{R} *indicates the position of a cryptic gene that confers resistance to tellurite when active. The scale indicates plasmid coordinates in kb. The map is redrawn, with permission, from Thomas and Smith (1987).*

sequence entire bacterial chromosomes in a matter of months. Activity in this field has been intense in the last few years and now more than 200 have been completed, with more than 500 in progress (www.genomesonline.org). Analyses of these sequences has led to the realization that most, if not all, bacterial chromosomes are mosaic structures (Lawrence and Roth 1999), comprising a backbone sequence, in which all the genes appear to derive from a common phylogenetic origin, and multiple inserted sequences from diverse sources, such as IS elements, transposons, ICEs, bacteriophage genomes, and genomic islands acquired by horizontal gene transfer.

A subset of genomic islands, detected first in human pathogens and then subsequently in animal and plant pathogens, contains genes for bacterial virulence. These genomic islands are called pathogenicity islands (PAI) (Hacker and Kaper 2000). The origins of some of these are more evident than others in that they are clearly lysogenic phages or their remnants. Examples of virulence genes that are phage components are the cholera toxin genes of *V. cholerae* and the shiga toxin genes of *S. dysenteriae* (Waldor and Mekalanos 1996; Waldor 1998). PAIs encode a diversity of virulence determinants, such as adherence and invasion factors, iron uptake systems, protein-secretion systems, as well as bacterial toxins. Such genomic islands tend to be relatively large sequences (10–200 kb) and are often flanked by short direct repeats reminiscent of the result of site-specific recombination or transposition (Davis and Waldor 2002). Indeed, among the gene complement of a PAI are often found genes encoding putative transposases and integrases. A few PAIs have been shown to be mobile, and in some cases movement has undoubtedly been phage mediated, e.g. PAIs found in *S. aureus* and *V. cholerae* (Lindsay et al. 1998; Karaolis et al. 1999). In the case of the former organism, capture of the PAIs called SCCmecs generates MRSA strains (see above).

It is worth comment that it is possession of different PAIs that distinguishes enterohemorrhagic, enteropathogenic, and uropathogenic *E. coli* from avirulent strains and from each other (Hacker and Kaper 2000). For a more detailed and elegant discourse on the molecular genetics of a variety of human bacterial pathogens, see Salyers and Whitt (2002).

GENETIC MANIPULATION IN VITRO

Molecular genetics, especially recombinant DNA technology (or genetic engineering), provides powerful tools with which to analyze any genetic system. These techniques make it possible, in principle, to isolate any gene from any cell type and manipulate it so that it can be expressed in a bacterial cell such as *E. coli* and then return it, perhaps in a modified form, to the cell line of origin. Gene cloning is undertaken for two primary purposes: to allow the isolation of a desired fragment of DNA in quantities suitable for sequencing, and to enable the experimenter to insert a gene of interest into a suitable cell line where its expression can be studied or in which the gene product is produced in large amounts, so facilitating its isolation and purification.

The bacterial chromosomes of *E. coli* and similar bacteria contain almost 5×10^6 bp. In a human cell the DNA is packed into 46 chromosomes, each containing about $3\,000 \times 10^6$ bp. The problem is, therefore, how one gene a few thousand bp in length can be recovered from such a mass of DNA. Until the mid-1970s such a feat was not possible. This situation was changed by the discovery of the class of DNA endonucleases (type II restriction enzymes) (Primrose et al. 2001) that cleave double-stranded DNA at specific nucleotide sequences, cutting it into much smaller linear pieces that can then be recovered and identified. The target sites for these enzymes normally comprise 4, 5, or 6 bp of specific sequence and are usually rotationally symmetrical, often termed palindromic because the sequence of the site read in the 5′ to 3′ direction on one strand is the same as that read in the 5′ to 3′ direction on the opposite strand (Figure 15). There are two ways in which these enzymes cut double-stranded DNA. Some cut at positions on the DNA strands opposite each other, giving 'blunt' or flush ends. Others give a staggered cut, producing short single-stranded extensions (Figure 15) that, because they are generated from palindromic sites, are the same. Fragments generated by type II restriction enzymes (restriction fragments) can be joined together in vitro by DNA ligase. Ligation of fragments with single-stranded extensions generated by the same restriction enzyme proceeds more efficiently than ligation of fragments with flush ends because the complementary nature of single-strand extensions, sometimes called 'sticky ends,' encourages fragments to align end to end, so facilitating the ligation reaction. The most commonly used ligase commercially available is that encoded by phage T4. After manipulation in the test tube, desired recombinant DNA molecules are recovered by transformation of a suitable bacterial host. The formation of novel hybrid DNA molecules in vitro, coupled with their recovery and amplification in bacteria, is termed cloning (for a more detailed exposition, see Brown 2001; Primrose et al. 2001).

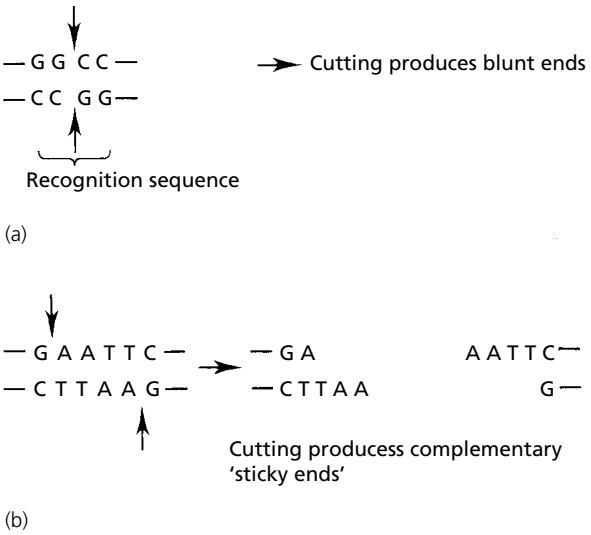

Figure 15 *Type II restriction enzymes.* **(a)** Hae*III from* Haemophilus aegyptius. **(b)** Eco*RI from* E. coli *RY13.*

Cloning vectors

To be perpetuated, cloned genes must become part of a DNA molecule that is replicated in each division cycle. There are two convenient types of genome that can be used for cloning – plasmids and bacteriophages. In bacterial genetics the most commonly used plasmid cloning vectors are small, multicopy plasmids. Many are derivatives of two early vectors, pBR322 and pACYC184 (see, for example, Thompson 1988), constructed in the laboratory from small natural plasmids, pMBI and pl5, respectively. They have been manipulated to possess several desirable features not present on the original elements, such as a choice of suitable cloning sites (preferably associated with insertional inactivation) and a convenient and reliable marker for selection (usually a drug resistance gene); for example, one of the first cloning vectors, pBR322, has approximately 4 200 bp and single sites for a number of restriction enzymes, including *Bam*HI, *Hin*dIII, *Pst*I, and *Sal*I. The single *Pst*I site is within the ampicillin resistance gene which encodes a β-lactamase. Cloning into this site results in the loss of ability to confer resistance to several β-lactam antibiotics, including ampicillin, i.e. cloning into this site causes insertional inactivation of the Amp resistance determinant. Hence, clones containing recombinant plasmids are recognized because

they acquire resistance to tetracycline but not to ampicillin. Similarly, cloning into the single *Bam*HI, *Hin*dIII, or *Sal*I sites creates recombinant plasmids that confer resistance to ampicillin but not to tetracycline.

The next incarnation of pMB1-derived cloning vectors, and one that has stood the test of time, up to the present day, was the pUC series of vectors, whose qualities are best displayed by pUC18, a vector commonly chosen for shotgun cloning for genomic sequencing projects. The size of the vector was reduced by removing the tetracycline resistance gene from pBR322, together with the *rop* region, the function of the latter being to control plasmid copy number. This single change increased the copy number more than 10-fold, greatly facilitating preparation of large amounts of the vector, and improved the transformation efficiency. Then a copy of the sequence encoding the N-terminal 145 amino acid peptide of LacZ (β-galactosidase), the alpha- (α-)peptide, that had previously been subject to in vitro mutagenesis to introduce a set of unique restriction endonuclease sites into the α-peptide coding region, the multiple cloning site (MCS), while retaining α-peptide function was inserted. In a host that produces the remainder of the β-galactosidase protein, the omega- (ω-) peptide, α-complementation occurs to give a functional β-galactosidase. The basis for the screen is the ability of β-galactosidase to hydrolyze the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, known as Xgal, to produce a deep blue, poorly diffusible product. When α-complementation occurs, i.e. the cell produces both the α- and ω-peptides, which combine to create functional β-galactosidase, the colonies are dark blue on agar containing Xgal. In contrast, cells in which there is no α-complementation fail to produce β-galactosidase and the colonies are white on Xgal-containing media. This allows ready identification of vectors containing inserts, because cells carrying such constructs fail to produce a functional β-galactosidase and the colonies are white. The process is referred to as blue-white selection, but it should be noted that the selection refers to experimenter choice, not use of a selective medium. A major strength of the system is that the distinction between blue and white can easily be made on the same plate, so no replica plating is required. Indeed, during construction of a genomic library using pUC18, blue/white screening is used simply to confirm that a high proportion of vectors in the library have inserts. One further development was the replacement of the ampicillin resistance marker of pUC18 with a kanamycin resistance gene to give pK18 (Pridmore 1987) for use when ampicillin selection is inappropriate.

Phages and genetically modified variants are also used extensively as vectors in genetic modification (cloning) experiments in *E. coli* and other organisms (Primrose et al. 2001). Two that have proved useful in this respect are the single-stranded DNA phage M13 (Messing 1991) and the double-stranded DNA phage λ (Brammar 1982), both parasites of *E. coli*.

Phage M13 has a 6.4 kb genome in which a 507 base intergenic region can be used for the introduction of up to 1.5 kb of cloned material. The great value of this particular phage is that, although it replicates as a double-stranded element that can be introduced into a host cell in the same way as plasmids are introduced by transformation (a process termed 'transfection'), its DNA, when packaged, is single-stranded – an ideal element for DNA sequencing and some mutagenesis procedures in vitro (Messing 1991).

Phage λ has a much larger genome (52 kb) than M13 (Arber 1983; Brammar 1982) and, by deleting the inessential integration functions and also sequences in the b2 region, it is possible to accommodate up to 25 kb of cloned DNA into the residue of the phage genome. One consequence of the large genome size is that there are several copies of all the common restriction endonuclease cut sites, so a further step in the design of λ cloning vectors was to remove all but one or two of each; this was accomplished by in vitro mutagenesis and also by selection of variants grown in a host that forms the endonuclease concerned. Two types of λ vector are in common use. Insertion vectors have a unique site (usually *Eco*RI) into which the DNA is cloned, thus inactivating the gene in which the site lies and allowing detection of the vector whose gene is inactivated; λ NM607 has such a site within the *cI+* gene which, when inactivated, eliminates the immunity function and gives clear plaques (Brammar 1979), and members of the λgt11 series (λgt11–λgt23) and λZAP have that part of the *lacZ* gene which encodes the alpha fragment of β-galactosidase and can thus be used in blue-white plaque selection using X-gal (Chauthaiwale et al. 1992). In replacement vectors there are two endonuclease-sensitive sites and the region lying between them is replaced by the material to be cloned (e.g. the λcharon series 4A, 32, 35, 40, and λEMBL3, λEMBL4) (Chauthaiwale et al. 1992). The efficiency of the subsequent transfection step can be improved by utilizing a packaging procedure in vitro rather than introducing the entire vector molecule into its host (Primrose et al. 2001). A further development of λ as a cloning agent is the cosmid (Collins and Bruning 1978). This is a hybrid between λ and a plasmid, in which λ contributes the region containing its cohesive ends and the plasmid supplies an origin of DNA replication together with a gene that allows selection of the desired clone (Wahl et al. 1987). Cosmids do not contain the λ sequences needed for DNA replication and particle maturation and hence there is room for far more DNA to be cloned (up to about 40 kb). The recombinant molecules are packaged in vitro to produce transducing particles which are used to deliver the vector and its insert into a suitable strain of *E. coli*. The cosmid system has been used to construct genomic libraries of several organisms, a set of recombinant clones that between them contain all the DNA present in an individual organism (see, for example, Goodman

et al. 1995; Jacobs et al. 1991; Little 1992). Among the phages specific to other organisms, the λ-like ϕC31 of *Streptomyces* has been used as the progenitor of several cloning vectors (see Pritchard and Holland 1985; Rodicio et al. 1985; see also Primrose et al. 2001).

Specialist plasmid cloning vectors

If a vector has been engineered to carry two origins of replication to allow it to be maintained in at least two different organisms, then it is called a shuttle vector, i.e. one origin supports replication in one cell type while the second supports replication in the other. Such vectors usually carry an origin of replication appropriate to *E. coli* (e.g. from pMB1), a selectable marker such as ampicillin resistance, also appropriate to *E. coli*, together with the *lacZ* α-sequence and multiple cloning site from pUC18, and a selectable marker for the second cell type. The point of incorporating these features is that recombinant plasmids, produced in vitro, can first be recovered by transforming *E. coli*, so maximizing transformation efficiency. Then, large amounts of the correct recombinant plasmid can be purified and used to transform the other target host. Using shuttle vectors, genes can easily be recovered in *E. coli* and then introduced into, for example, a human cell line (where the second origin of replication on the cloning vector often comes from the monkey virus, SV40, which can replicate in human cells), or plant or yeast cells. The purpose of shuttle vectors is to exploit the plethora of molecular genetic techniques that can be used with *E. coli* systems to recover and manipulate DNA sequences before reintroducing the particular sequence into the original cell line or an appropriate surrogate, where the effects of mutation or overexpression can be determined. When coupled with the use of expression systems (below), shuttle vectors can be used to overexpress a protein in a different background, when overexpression in *E. coli* is problematic.

Fulfilling a similar role to shuttle vectors, but generally limited to bacterial systems, broad host range bacterial vectors have a single origin of replication that can be used in many phylogenetically diverse bacteria. Broad host range vectors were originally derived from naturally occurring broad host range bacterial plasmids, which encode their own replication proteins that recognize their own origins of replication (see, for example, Bagdasarian et al. 1981; Morales et al. 1991). As long as the genes encoding the vector-specific replication proteins can be expressed in the target host, the vector will replicate. Broad host range vectors are, by necessity, larger than pUC18, but they have been modified over the years to reduce their size and in some cases, the origin of replication, *lacZ* α-peptide sequence and multiple cloning site from pUC18 have been added to facilitate initial manipulation of the vector in *E. coli*, so optimizing recovery of the sequence of interest, since many potential target bacteria are difficult to transform. Then, sufficient quantities of the correct recombinant plasmid can be produced to facilitate transformation of the final host. In some instances the recombinant plasmid can be mobilized into the final recipient by conjugation from the *E. coli* donor because the vector has also been supplied with an origin of transfer and the functions specific to it. The bulk of the transfer system is usually provided by a helper conjugative plasmid also present in the transformant.

Plasmid vectors for recovery of PCR amplicons

PCR is now the method of choice if what is needed is simple amplification of a DNA fragment. However, PCR cannot easily be used to amplify a section of DNA if the sequences at the ends of the fragment to be amplified have not been determined. Furthermore, to sequence a PCR amplicon is more difficult than to sequence the insert of a recombinant plasmid, particularly if it is more than 1 kb in length. Thus, PCR is generally used to amplify specific, small, known sequences of DNA, for surveys of sequence heterogeneity, and for more complex in vivo techniques, such as allelic replacement by homologous recombination (see below). As a preliminary to these more complex techniques, it is firstly necessary to insert the PCR amplicon into a cloning vector. PCR amplicons are linear, double-stranded molecules, and those produced using the most common form of *taq* DNA polymerase carry single adenine overhangs at the 3′ end of each DNA strand, generated by the inherent terminal transferase activity of DNA polymerase. These adenine residues complicate the task of inserting the PCR product (amplicon) into a cloning vector because they first have to be removed before the fragment can be blunt-end ligated into the vector. However, cloning vectors based on pUC18 are now available where the multiple cloning site contains a restriction site that, when cut, generates single T overhangs at the 3′ end of each strand (for example, see Marchuk et al. 1991). Thus, the PCR amplicon can be ligated directly into the vector, without the need to remove the A overhangs from the amplicon. Once ligated into the 'TA cloning vector,' for example pCR2.1, and replicated in *E. coli*, the insert can subsequently be removed by cleavage at other restriction sites within the multiple cloning site and subcloned into a more specialist vector.

Plasmid vectors for overproduction of proteins in vivo

Modern biochemical studies and biotechnological processes often require large amounts of a particular protein, and the most efficient way to produce proteins

in large quantities is to overexpress recombinant genes in vivo. As with so many other molecular biology processes, *E. coli* was the first to be investigated as a surrogate host for protein production. Gene expression in *E. coli* requires an appropriate promoter sequence to allow transcription of the gene into mRNA, a ribosome binding sequence (RBS) to allow translation of the gene transcript, and an ATG start codon to initiate translation. It is sometimes possible to express a recombinant gene in *E. coli* simply by inserting it, together with its natural promoter, RBS and ATG start codon into a standard cloning vector such as pUC18 and transforming the construct into *E. coli*. In this case, the fact that pUC18 has a copy number of 50–100 relative to that of the *E. coli* chromosome means that the gene of interest can be expressed at levels well above that of a single copy gene. However, this approach is somewhat hit and miss. For many reasons, the expression of a cloned gene may not be optimal in the surrogate host. Because of phylogenetic drift, promoters, RBS sequences, and initiation codons associated with genes not native to *E. coli*, particularly those from eukaryotes and very different bacteria, may not be recognized by the *E. coli* RNA polymerase and ribosomes.

If the promoter or RBS associated with a cloned gene is not active in *E. coli* then, irrespective of the copy number of the recombinant vector, the gene will not be expressed. In such cases, a specialist expression vector can be used, for example one of the pET series of vectors, to provide an optimal *E. coli* promoter, an RBS, and, when necessary, an initiation codon upstream of the inserted gene to promote high-level expression (Studier et al. 1990).

Perpetual high-level expression of a recombinant gene in *E. coli* is rarely successful. For the bacterium to divert large amounts of energy into the production of a single protein tends to be debilitating to the organism as a whole. Furthermore, the presence of large quantities of 'foreign' protein often results in it being sequestered in insoluble aggregates or degraded, thus reducing overall yield. In some cases, the foreign protein directly disrupts the cell's normal physiology, and is said to be toxic. If toxicity is extreme, it may not be possible to transform the surrogate cell with a recombinant plasmid from which the gene encoding the toxic protein is constantly expressed. To help overcome the problems noted above, and to maximize protein yield, it is necessary to control gene expression. This is usually achieved by using an inducible promoter to drive transcription of the cloned gene. This strategy ensures a minimal level of transcription, and hence protein production, during normal cell growth, and allows the cell line to be cultured, but provides the means rapidly to increase production of the protein when the culture has attained a suitable cell density, thus generating large amounts of the protein. That this will also kill the cells is not important, because they are necessarily killed when the protein is harvested.

Many different transcriptional regulatory systems have been characterized, which it would be possible to adapt for incorporation into an expression vector. However, two inducible promoters dominate the field and their regulatory systems were among the first to be discovered. The most common is a derivative of the *E. coli lac* operon promoter, called *ptac*. This is a high efficiency, naturally generated hybrid promoter which is controlled by the *lac* operon repressor, LacI (de Boer et al. 1983). In the absence of an inducer (usually the gratuitous inducer isopropyl-β-D-galactopyranoside (IPTG)) promoter activity is shut off. However, repression is not complete and some escape synthesis of mRNA does occur, resulting in low-level expression of whatever gene is coupled to the promoter. For many cloned genes this level of control is adequate, but for a few even a low level of expression is potentially damaging. In these cases an alternative expression system is necessary.

Some expression vectors (e.g. the pET series) include a stylized promoter, which is recognized by the RNA polymerase of the *E. coli* bacteriophage T7 and not by the *E. coli* RNA polymerase. Regulated expression from this promoter is achieved by regulating production of the T7 RNA polymerase itself. To this end, an *E. coli* cell line carrying the T7 RNA polymerase gene, expression of which is driven by the *ptac* promoter, is used, e.g. BL21 cells (Dumon-Seignovert et al. 2004). Growth of BL21:pET in nutrient broth without IPTG allows little or no expression of the gene cloned into pET, because, whilst repression of transcription at the *ptac* promoter controlling production of the T7 RNA polymerase is leaky, the level of T7 RNA polymerase in the cell is never sufficient to activate transcription of the cloned gene from the T7 promoter. T7 RNA polymerase production is switched on when desired by addition of IPTG to the growth medium, when expression of the cloned gene is also triggered. To enhance production of the desired protein, once the cell has produced T7 RNA polymerase, the antibiotic rifampicin can be added to the medium. This inhibits the *E. coli* RNA polymerase, but not T7 RNA polymerase. So, whilst addition of rifampicin stops any further production of T7 RNA polymerase, because it blocks transcription of all *E. coli* genes by inhibiting the cell's own RNA polymerase, it stimulates transcription by T7 RNA polymerase by eliminating a major competitor for the substrates of RNA synthesis. With this strategy the cell is converted to one that synthesizes only the protein encoded by the cloned gene driven by the T7 promoter.

The second major regulatory system to be exploited for cloning vector development is the Ara system of *E. coli*, which is involved in metabolism of the sugar arabinose (Lee et al. 1974). In this system, a transcriptional activator, AraC, acts on the promoter controlling expression of the *araBAD* operon to enhance RNA polymerase binding and so promote transcription of the operon. Binding of AraC close to the promoter is

absolutely dependent upon arabinose. When there is no arabinose in the cell, the *araBAD* promoter is silent. Genes cloned downstream of the *araBAD* promoter in expression vectors incorporating the *araC* gene, e.g. pBAD (Guzman et al. 1995) are only expressed in the presence of arabinose, and expression is completely shut off in the absence of the sugar, thus minimizing problems associated with toxic protein production.

High-level expression of some proteins in *E. coli* can be difficult to achieve, particularly if the proteins are normally functional in a eukaryotic cell. One reason for this is that eukaryotic proteins are often modified post-translationally; for example, they are processed to remove signal tags associated with protein targeting, or they have molecules added to their backbones, e.g. glycosylation. Without the correct modifications, the protein may lack activity and may even be degraded because it cannot fold properly. The solution to this problem is to express the protein in a eukaryotic host that can perform the required modifications. For this, a shuttle vector is used, which has the necessary promoter and translation signals for expression in the surrogate host, such as insect cells or *Xenopus* oocytes.

Plasmid vectors that allow targeted modification of chromosomal genes

Once a DNA sequence encoding a particular protein has been identified, it is possible to make targeted changes to the DNA, and then determine the effects of such changes on the activity of the protein product. Targeted changes include deletions, truncations, or point mutations in the gene, or in its promoter/operator sequences. Mutations can easily be introduced using PCR technology, as described earlier in the chapter. However, in order to determine the biological significance of a particular mutation, it is necessary to put the mutant gene into the original organism. If the mutant gene phenotype is dominant over that of the chromosomal wild-type allele, this experiment can be performed simply by transforming the original cell line with an appropriate construct carrying the mutant gene. However, in most cases, mutant alleles are recessive, even when present in multiple copies, and their presence is masked by the dominant wild-type chromosomal copy. In this case, the wild-type allele must be replaced with the mutant allele, a procedure termed allelic replacement, using homologous recombination.

Gene replacement by homologous recombination

Homologous recombination involves exchange between two very similar sequences of DNA (Eggleston and West 1996). If a gene knockout is needed, a modification of site-directed mutagenesis can be used to avoid the need to clone the gene of interest when preparing DNA fragments for allelic gene replacement. Multistage PCR is used to create a DNA fragment carrying a version of the gene with a suitably located restriction site (see above). The fragment is cleaved with the restriction endonuclease and the two pieces are ligated to a third fragment encoding an antibiotic resistance gene. This ensemble can then be amplified by PCR to produce ample DNA to use directly to transform the cell line of interest, selecting for antibiotic resistant transformants. To maximize the frequency of transformation, electroporation is used to introduce the mutant allele of the gene of interest into the cell. The desired transformants are created by a double crossover between homologous sequences on the introduced DNA fragment and on the bacterial chromosome, one within each gene segment flanking the antibiotic resistance gene (Datsenko and Wanner 2000). This recombination exchanges a section of the chromosomal gene for its equivalent on the fragment including the interpolated antibiotic resistance gene. The drawback to this technique is that most bacteria are not easily transformed by linear DNA fragments. The fragments tend to be degraded rapidly once inside the cell. To circumvent this problem, the DNA fragment can be circularized before it is used to transform the cell. One way to do this is to clone the mutant allele and then use the recombinant plasmid to effect the transformation. When such a construct is put into a cell, then a single crossover within the sequence common to the recombinant plasmid and the chromosome will fuse the recombinant plasmid with the chromosome, generating in the process a direct duplication of the homologous sequence. A second crossover within the duplicated region, but at another point, will reverse the integration, leaving behind in the chromosome part of the cloned fragment while the equivalent part of the chromosomal copy becomes part of the excised plasmid, thus transferring the mutant allele to the chromosome. However, the system only works efficiently in practice if the recombinant plasmid is unable to replicate in the cell to be mutated (otherwise, excisions that essentially regenerate the mutant allele on the free recombinant plasmid and reconstitute the original chromosomal copy of the gene cannot be distinguished phenotypically from the desired mutants). This problem has been solved by the development of suicide vectors, i.e. cloning vectors that can be propagated only in special cell lines (permissive) and which otherwise are not maintained as extrachromosomal elements (Datsenko and Wanner 2000).

Suicide vectors

Because suicide vectors are designed not to be maintained in most cells, when the DNA is electroporated into cells it will persist simply as nonreplicating circles of DNA, unless rescued by integration into one of the

replicons in the cell. There are many such vectors, which all work in a similar manner, for example pKNG101 (Kaniga et al. 1991) and the pEX18 series of suicide vectors (Hoang et al. 1998). Each of these vectors carries a multiple cloning site, which allows insertion of a mutant allele of the gene of interest. They also possess the R6K origin of replication, ori_{R6K}, which will only function in cells that possess the R6K *pir* gene (Kolter et al. 1978), usually provided in *E. coli* by lysogenization with a specialized λ*pir* transducing phage. In addition, each vector is provided with a selectable marker gene, usually an antibiotic resistance gene, and a copy of the *B. subtilis sacB* gene. This last feature allows selection of derivatives that have lost the suicide vector, because expression of *sacB* is lethal when cells are exposed to sucrose. Finally, each also carries the transfer origin of the conjugative resistance plasmid RK2, which allows the construct to be mobilized easily by conjugation into the target cell line.

When the target host (nonpermissive) is mated with a *pir+ tra+ E. coli* (permissive host) carrying the pEX18 recombinant and a conjugative helper plasmid, the suicide vector and its passenger gene is transferred to the recipient. However, the antibiotic resistance encoded by the plasmid is only acquired if the plasmid is maintained, which is not possible if the plasmid remains autonomous. Accordingly, in order to be replicated, the cloning vector must be rescued by integration into the genome of the recipient cell. This normally occurs by homologous recombination between the cloned gene on the pEX18 recombinant and its homolog on the chromosome. This integrates the plasmid into the chromosome generating an arrangement where the suicide vector is sandwiched between two copies of the target gene, one of which is disrupted by the antibiotic resistance gene cassette. This first recombination event is rare, but because of the plasmid marker, the small number of bacteria carrying the integrated plasmid can easily be selected.

Derivatives with the chromosomally integrated pEX18 recombinant plasmid are then cultured in antibiotic-free medium, if necessary for several passages, to allow a second crossover between the two copies of the target gene. This excises the vector and one copy of the target gene from the chromosome. The excised sequence is lost as the cell multiplies, leaving a single chromosomal copy of the target gene. In a proportion of the derivatives the remaining copy of the target gene will be disrupted by the antibiotic resistance gene cassette. These are easily selected on medium containing sucrose, which kills all those cells that still carry the suicide vector, and the appropriate antibiotic, which kills all those that no longer possess the antibiotic resistance cassette used to disrupt the target gene.

One problem with this technique is that the mutation marker, i.e. the antibiotic resistance gene, remains in the chromosome, so the same antibiotic resistance gene cassette cannot be used to disrupt another gene in the same bacterium. To counter this problem, resistance cassettes have been developed that are flanked by specific recombination sites, so that the antibiotic resistance gene cassette can be excised by the appropriate site-specific recombinase. An inducible form of the gene for this enzyme is introduced into the cell on a helper plasmid and once the allelic replacement has been effected, expression of the recombinase is induced. Removal of the cassette is achieved in such a way that a frameshift mutation is generated in the gene of interest (Datsenko and Wanner 2000).

The technique can also be used to introduce mutations other than antibiotic resistance gene cassettes. Mutant genes are created by site-directed mutation as normal and the fragment inserted into an appropriate suicide vector. Then, the only modification to the final stages is that selection is for sucrose-resistant derivatives only. These are then screened for those carrying the mutant allele of the gene, because only approximately one in two will carry the mutant allele. Screening by change of phenotype may be possible; alternatively, the target gene is recovered by PCR amplification from the chosen clones, the fragments are sequenced and the mutants identified. The major advantage of this approach is that it can be used repetitively to introduce targeted point mutations into different genes of the same cell line or multiple mutations may be introduced sequentially into the same gene.

Transposon-delivery vectors for random insertional inactivation of chromosomal genes

Throughout the history of bacterial molecular genetics, the isolation of loss of function (null) mutants with easily detected phenotypes has been used to study the genetic basis of the particular phenotype. The main reason for selecting null mutants is to locate the mutated gene, and to confirm a role for that gene in a particular phenotype. A classic example is the selection of *E. coli lacI* null mutants, in which production of β-galactosidase is derepressed, i.e. lacZ is expressed at high level irrespective of the provision of an inducer such as IPTG. Although recovery of such mutants is relatively straightforward, locating the site of the mutation, and therefore the *lacI* gene, is somewhat more complicated. Originally this was achieved by classical bacterial genetics using Hfr crosses and recombination analysis, a tedious procedure. Hence, an easier process was developed that combined mutation with acquisition of a selectable marker. One way to achieve this is by transposon insertional inactivation, when a gene of interest is disrupted by an antibiotic resistance transposon. To facilitate this approach, suicide vectors carrying antibiotic resistance transposons, natural and artificial, have been

constructed. The transposons chosen are those which insert more or less at random into any DNA sequence in the cell. Popular elements are Tn*5* and Tn*10* and their derivatives. The suicide vector is used to deliver the transposon into the target cell where it survives only by transposing from its carrier replicon into the host cell chromosome (or other viable replicon in the cell).

Transposition events are selected by plating on medium containing the appropriate antibiotic for the resistance gene on the transposon (e.g. kanamycin for Tn*5* and tetracycline for Tn*10*). The survivors are screened for the knockout mutants of interest. Particular mutants are then chosen and genomic DNA from each of these is isolated, digested with a restriction enzyme that does not cut within the marker gene and no more than once within the transposon as a whole, and the fragments are ligated into a suitable cloning vector, e.g. pUC18, to create a gene bank, which is transformed into *E. coli*. Selection of particular transformants can then be for those that have recombinant plasmids carrying the transposon antibiotic resistance gene. This will have been recovered together with some sequences from the gene disrupted. Analysis of several such transposon mutants, where the transposon is inserted at different locations and in different orientations in the gene of interest, will allow the sequence of the intact gene to be determined.

'One of the most popular transposon delivery vectors is the pUT series (de Lorenzo et al. 1990), which have the same *pir* dependent origin of replication and mobilization functions as pEX18 suicide vectors, but carry the Tn*5* transposase gene, and Tn*5* repeat sequences flanking a selectable marker. These vectors have been designed to include many different antibiotic resistance genes, for use in different bacteria, since some are inherently resistant to certain antibiotics.

Fusion vectors

Derivatives of pUT have also been developed that allow the formation of protein fusions, i.e. hybrid proteins. These are constructed for a variety of reasons, including investigation of the cellular location of a particular protein peptide domain, study of transcription and translation control systems, and to facilitate purification of proteins of interest (de Lorenzo et al. 1990). These systems are typified by the *phoA* fusion system. The *E. coli phoA* gene encodes alkaline phosphatase, an enzyme that is easily assayed. Random insertions of the *phoA* gene into a gene of interest, achieved using a modified Tn*5*, can create hybrid genes, where the reading frames of the disrupted gene and that of *phoA* are in register. These hybrid genes produce proteins that have different lengths of the protein of interest at their N-termini fused to PhoA. If the fusion protein is expressed using the transcription and translation signals specific to the protein of interest, then changes in level of expression can be monitored simply by determining alkaline phosphatase activity. This is a powerful tool with which to investigate production of proteins that lack an easily assayable activity. The *phoA* system is used particularly to study production of membrane proteins and their topologies. Alkaline phosphatase is normally secreted into the periplasmic space of *E. coli*, where it is activated. Enzyme protein that remains in the cytoplasm is generally inactive. Accordingly, alkaline phosphatase activity indicates that the PhoA domain of the fusion protein has been transported to the periplasm. In the study of membrane proteins, this technique is useful for mapping the topology of membrane proteins because, when PhoA is fused to a peptide domain that is normally in the cytoplasm then alkaline phosphatase activity remains dormant. In contrast, when it is fused to a peptide domain that is located in the periplasm, alkaline phosphatase activity is revealed (Hoffman and Wright 1985). The activation of alkaline phosphatase reflects the need for protein chaperones, found in the periplasm, to assist the enzyme to achieve active configuration.

One limitation of pUT vectors is that they can only be used in organisms where the transposase gene can be expressed. However, it is now possible to purchase purified transposon delivery vectors, comprising the Tn*5* terminal repeat sequences flanking an antibiotic resistance gene, complexed with transposase enzyme. In the absence of magnesium ions (i.e. in the buffer supplied) the transposase is not active, but upon transformation of the vector/transposase complex into a bacterial cell, where magnesium ions are present, the transposase is activated and transposition occurs (Goryshin et al. 2000). Another potential use of this system is to introduce a gene of interest into the chromosome of a bacterium. This can be particularly important if the gene under investigation is normally located on a chromosome, when gene copy number is essentially one. The suicide vector pMOD carries Tn*5* terminal repeats flanking a multiple cloning site, allowing the insertion any DNA sequence of interest between the repeats and has been provided with the *pir*-dependent replication origin and so replicates only in *pir*+ *E. coli* strains. The recombinant plasmid is isolated and purified and the plasmid DNA is mixed with purified Tn*5* transposase enzyme, and the mixture is electroporated into the recipient bacterium (*pir*$^{-}$), where the novel Tn*5* construct is transposed into the chromosome of the new host. The suicide vector is lost because it fails to replicate.

One problem arising from the use of transposons to mark genes by insertional inactivation is that it is not always straightforward to clone out the transposon marker together with flanking sequences from the new site of insertion, and hence to identify the gene disrupted. To overcome this limitation, the pMOD vector has had the *pir* replication origin relocated and placed between the Tn*5* repeat sequences, beside the MCS (York et al. 1998). Accordingly, not only is an

antibiotic resistance gene transposed but, also, so is a tightly linked silent plasmid origin of replication. Once appropriate Tn insertion mutants have been identified, the chromosomal DNA of these can be purified, digested with an appropriate restriction enzyme, i.e. one that does not cut the transposed sequence, and the fragments can be end-ligated to circularize them. The circles generated are narrow host-range plasmids, that can be recovered on transformation of a *pir*+ *E. coli* strain. This strategy eliminates the need to construct a gene bank in a cloning vector such as pUC18 and provides a ready source of DNA from which to sequence the gene disrupted by the Tn insertion. This is because gene sequences, both promoter proximal and promoter distal to the point of insertion, are recovered simultaneously. If cleavage with one restriction endonuclease fails to recover the gene of interest in its entirety, then another can be tried.

Gene cloning

Foreign DNA is inserted into a plasmid vector by linearizing the circular plasmid molecule with a particular restriction enzyme. The DNA fragment to be cloned – usually generated by the same restriction enzyme used to cut the cloning vector – is joined (ligated) at both ends to the linearized vector to form a circular molecule. The hybrid DNA is then introduced into a suitable bacterial cell by transformation (see Brown 2001; Primrose et al. 2001). The desired recombinant clones then have to be identified. This may be done by selection if the gene of interest confers a selectable phenotype; otherwise, a set of recombinants called a gene bank is collected and the individual members of the set are screened to identify those with the gene of interest.

In contrast to plasmid vectors, which are circular, λ based vectors are linear DNA molecules. In these cases the vector is cut with a restriction enzyme to generate two (insertion vectors) or three (replacement vectors) fragments. If two, the sequence to be cloned (generated with the same restriction enzyme or one that generates compatible single-strand extensions) is inserted between them. This requires the three fragments to be ligated together in the correct order. The desired recombinants can then be recovered by transfecting a suitable cell line of *E. coli* with the recombinant DNA or by in vitro packaging of the recombinant DNA (i.e. generation of reconstituted λ phage particles in the 'test tube') and subsequent infection of a suitable *E. coli* cell line. The latter process is the more efficient and λ packaging kits are commercially available. If a replacement vector is used, restriction enzyme digestion of the vector produces three fragments, of which the two outside fragments are ligated to the fragment to be cloned and the central fragment, often a stuffer sequence (Chauthaiwale et al. 1992), is discarded. Again, the desired recombinants can be recovered by transfection or by in vitro packaging and cell infection (see Primrose et al. (2001) for a more detailed presentation of gene cloning strategies).

Eukaryotic genes are not transcribed in bacteria from their own promoters because these promoters are not recognized by bacterial RNA polymerases. Hence, to express a eukaryotic gene in a bacterial cell it is necessary to join the gene to a suitable bacterial promoter sequence. A number of cloning vectors with cloning sites close to and downstream from active promoters have been developed for this purpose (see Thompson 1988). Many cloning vectors with a bewildering array of properties are now readily available commercially.

A fundamental problem that arises when genes are cloned on a fragment generated by a restriction endonuclease is that what is recovered may not simply be the gene(s) of interest, but also unwanted sequences flanking it. Expression of genes encoded on these additional sequences may complicate subsequent analysis, so it is frequently desirable that these be removed prior to analysis. This can be achieved by subcloning, i.e. the fragment recovered in the primary cloning is cut into smaller pieces using a different restriction enzyme and these, in turn, are recovered in the same or in a different cloning vector. The desired transconjugant is again detected by an appropriate screening process. If the fragment is still considered to be too large, the reducing process is repeated on the smaller fragment with another restriction enzyme until the smallest fragment that still encodes the intact gene(s) is recovered.

An alternative approach that bypasses the problem completely, and which is suitable for small peptides, is to synthesize the gene chemically and then insert it into a suitable cloning vector. This was done in the early days of cloning for the gene for somatostatin – a pituitary hormone of 14 amino acids, the sequence of which was known (Itakura et al. 1977). The genes for human growth hormone, thymosin and insulin have also been synthesized and cloned. A more recent solution to the problem of eliminating unwanted sequences that accompany a gene of interest recovered from a gene bank, because sequencing is now both straightforward and economical, is to sequence the insert and then amplify the gene of interest by PCR. The synthetic fragment can then be cloned into a specialized cloning vector, e.g. pTOPO, and the required recombinant can be recovered in the normal way by transformation of an appropriate cell line (Ramalingam et al. 2004). Such technology allows, in principle, examination of any system, gene by gene.

A second problem, that does not arise with bacterial genes, is encountered with many eukaryotic genes. Not only are they too large to synthesize with existing techniques but, unlike the genes of bacteria, they may also be discontinuous ('split' genes), the continuity of the coding sequence being disrupted by one or more unrelated sequences (introns) (see, for example, Lewin

2000). Such sequences are removed after transcript synthesis but before translation, a process referred to as RNA splicing, i.e. a functional mRNA is produced by splicing together those sequences necessary for the production of the particular peptide. Another approach must therefore be used if the gene is to be recovered and expressed in a bacterial cell. This involves isolating functional mRNA molecules (already processed), which are used as template to synthesize complementary DNA (cDNA) sequences using reverse transcriptase (an enzyme that synthesizes single-stranded DNA from an RNA template). The single-stranded cDNA is then converted to double-stranded DNA and inserted into a suitable cloning vector. The reconstructed, uninterrupted gene is then recovered by transformation of an appropriate bacterial cell line (Primrose et al. 2001). The necessary mRNA can be prepared by one of a number of techniques; which one is chosen depends largely on the task in hand (e.g. White and Radke 1997; Rosenow et al. 2001).

Several mammalian proteins are now being made in bacteria as the result of gene cloning. They include somatostatin, human growth hormone, insulin, several interferons, and tissue plasminogen activator. Many more can be expected in the future.

Genetic engineering is also likely to make a major impact in the field of vaccines. By making only the antigen against which antibody response is required, the risk associated with the intact pathogen is minimized. This technique is being pursued with respect to many pathogens, notably rabies and hepatitis B viruses, malaria, and *Bordetella pertussis* to name but four. The techniques of molecular biology that have been developed are so powerful, both with respect to unlocking the molecular biology of genetic systems and in furnishing the potential to allow specific manipulation of genes, that they offer the promise of major advances in our understanding of many diseases and of better informed positive intervention.

The power of bacterial genetics has also been harnessed to engineer plants to create novel cultivars with desirable characteristics. These genetic manipulations have resulted in the transfer of bacterial antibiotic resistance genes into these plants. Concern has been raised that large-scale cultivation of these new cultivars might exacerbate the spread of antibiotic resistance genes among bacterial pathogens and so hasten the time when antibiotic therapy is no longer effective. However, although it has been shown that bacteria can transfer DNA into plant cells, the reverse process has never been reported, except when artificial transformation systems have been used. Accordingly, while it is impossible to rule out gene transfer from plant to bacteria, it is exceedingly unlikely that it will have a significant impact on the further development of antibiotic resistance in bacteria (Bennett et al. 2004).

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Bacteriocins and bacteriophages

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INTRODUCTION

Bacteriocins are a group of proteins secreted by bacteria that kill or inhibit competing strains. Bacteriophages are viruses that infect bacteria. Both agents have antimicrobial activity and they share some features, such as the uptake site on their host bacterium. However, examination of their modes of action, reproduction, and genetics reveals differences. The major difference is that only bacteriophages carry a genome (DNA or RNA) enabling them to reproduce in the cells they infect; bacteriocins are only proteins and cannot reproduce. Irradiating a phage will damage its genome irreparably; thus, an irradiated lysate will have no killing activity. Bacteriocins have no genome and thus are insensitive to this level of irradiation and retain their activity fully. Both bacteriocins and phages can kill susceptible cells.

Bacteriocins have for decades been utilized as food additives to limit spoilage. The continued demand for a longer shelf-life of food and other biologically degradable products has meant that these desirable properties of these bioactive agents have been more seriously investigated in recent years when research has shown phages of lactobacilli are a serious problem for the dairy industry. They have been used in the medical arena for phage typing of pathogens such as *Staphylococcus* spp. and as antibacterials. Some are also widely used as recombinant DNA vectors as cloning and expression vectors.

BACTERIOCINS

Microorganisms naturally produce a range of protein components from simple polypeptides to very complex macromolecules, such as antibiotics, toxins, pili, adhesins, siderophores, flagella, etc. Many of these form part of an array that forms a defense system. Bacteriocins are grouped under the term 'toxins' and provide a means of defense against, or a growth advantage over, other microorganisms in the same ecological niche. The term 'colicin' is used to describe those antagonistic compounds produced by *Escherichia coli* (Frederique 1957). The term 'colicin' was coined by Gratia and Frederique (1946) to describe these substances, the effects of which had been observed by Gratia (1925). Jacob and Woolman (1953) introduced the general term 'bacteriocin' to describe substances with similar activity produced by a wide range of bacteria. Tagg et al. (1976) defined bacteriocins, a subgroup of bacterial toxins, as proteinaceous compounds that kill closely related bacteria. Although this is true for most bacteriocins, it is evident that these molecules take many forms and may have bactericidal actions beyond closely related species. Individual bacteriocins are named by attaching 'cin' to the root of the genus or species name. For example, *Pseudomonas aeruginosa* produces aeruginocins and *Bacillus megaterium* produces megacins. The synthesis of most colicins (and this will probably be true of bacteriocins in general) are SOS-inducible (Pugsley 1984a,b). Frequently bacterial species carry genes that encode both the production of one or more bacteriocins and immunity to them on the chromosome or on plasmids (Frank 1994). The significance of the latter location is that the distribution of individual plasmid types in bacterial communities fluctuates under selection, for and against the plasmid and host cell, which provides opportunities for closely related and competing populations to

gain and lose advantage. Loss and acquisition of bacteriocins is a consequence of plasmid stability, incompatibility and transfer. Thus, a great diversity of bacteriocins can be produced within a single species. Bacteriocins have a spectrum of sizes; generally proteinaceous agents, they are sometimes complexed with lipids, carbohydrates, or other distinctive proteins (Lewus et al. 1991; Nissen-Meyer et al. 1992; Jiminez-Diaz and Rios-Sanchez 1993). There are three general classes:

1. microcins, small molecules produced in stationary phase by gram-negative bacteria
2. lantibiotics, small molecules produced by gram-positive bacteria
3. bacteriocins, a group encompassing medium to large phage-like structures, whose synthesis generally appears to be induced by the SOS response.

It has been shown that bacteriocin synthesis provides the strain with a growth advantage over isogenic nonproducing strains (Ruiz-Barba et al. 1994). Since producing these antimicrobials is an energy-costly process, the benefits to the producer must be due to selective pressure. The energy cost must be less than the energy benefit resulting from their synthesis. Viewed in this light their synthesis should be well regulated and expression directed to times when it will be of benefit. Thus, bacteriocin synthesis reflects this by being sensitive to pH, temperature, medium composition, SOS response induction, etc.

These proteins share many characteristics. Many have a low molecular weight, are cationic, are amphiphilic, tend to aggregate, and are benign to the producing organism. They kill sensitive cells in distinct ways, targeting the cell membrane (e.g. phospholipases), affecting DNA tertiary structure (DNA gyrase), or affecting DNA/RNA integrity (ribonuclease or deoxyribonuclease activity). Since their sites of synthesis and modes of action are frequently intracellular, they must also be able to enter susceptible cells to exert their effects. Many are capable of killing activity at extremely low concentrations (one to a few molecules per susceptible cell) and target specific cellular pathways. Death of the cell is a consequence of transport collapse at membrane level, depleting proton-motive force (pmf) and resulting in a rapid reduction in indispensable metabolites or ions, or blocking of macromolecule synthesis (protein or DNA). Thus, due to the specificity of the target, the effect on cell metabolism is catastrophic. There is a further and historical complication in this analysis, as bacteriocins are effectively antibiotics, so there is an activity of structural and biochemical overlap between these molecules. Historically, some were initially regarded as antibiotics and have now been recognized as bacteriocins, and vice versa for others. Thus, their diversity in types and modes of action provides an indication that these molecules have great significance for microbial populations and the dynamics of interactions of these within communities. Bacteriocins are probably present in all bacterial species, and within each species there are probably hundreds of different kinds (Riley and Gordon 1992). For example eubacteria (*E. coli*), the archae (*Halobacteria* Torreblanca et al. 1994), and *Streptomycetes* all commonly produce broad-spectrum antimicrobials (Saadoun et al. 1999). Microbes invest considerable energy in their synthesis. What is less clear is how this diversity arose and what roles these agents serve in microbial communities.

Assay

A simple method for assaying bacteriocin activity is to take the supernate of an overnight culture of the putative bacteriocinogenic strain grown in broth culture, dilute this as a series of doubling dilutions, and place 10-μl drops on the surface of a dry nutrient agar plate prespread with a lawn of the indicator bacterium. After overnight incubation the lawn will have grown and the area containing bacteriocin will show a circular zone of clearing. The further down the dilution series the inhibition zones are found, the more bacteriocin is produced by the strain (Benkerroum et al. 1993). This demonstration of the inhibitory effect is also the basis of bacteriocin typing methods.

Modes of action

A neat and satisfying taxonomy of bacteriocins remains to evolve but may come through an interrogation of bacteriocin sequences. Bacteriocins have been described variously in terms of size, types of activity, and chemical nature. As there is no consistent theme it is difficult to obtain a proper grasp of their relationships. It seems sensible to categorize them through their modes of action, but the problem with this approach is that for most, the mechanisms of activity are unknown. However, the general strategies employed by these antimicrobial agents are illustrated by the examples that follow.

DESTRUCTION OF MEMBRANE POTENTIAL

The membrane is composed of many different molecules in complex functional associations. Bacteriocins that target individual proteins, in associations concerned with membrane potential, lead directly to a loss in pmf. Bacteriocins produced by lactic acid bacteria commonly act by this mechanism, but the role of receptor proteins and the mechanism by which pmf is depleted remain unresolved. Many that act in this way are lantibiotics and are produced only by gram-positive bacteria. The most striking feature of lantibiotics is the occurrence of intramolecular rings, introduced by the thioether amino acids lanthionine and 3-methyllanthionine, and unusual

amino acids such as didehydroalanine and didehydrobutyrine (Bierbaum and Sahl 1993). These smaller bioactive molecules, which in gram-positive strains are 30–60 amino acids in size (Jack et al. 1995), as a group have both broad and narrow spectra of activity. Some are phospholipases that cause susceptible cells to leak intracellular contents through the damaged cell membranes (Kim 1993). In gram-negative bacteria some of the colicins destroy the membrane potential by forming pores in the membrane (Lau et al. 1992). Colicin A is an example of a water-soluble protein that inserts into lipid bilayers and produces this effect (Lakey et al. 1992, 1994).

NUCLEOLYTIC AND RELATED ACTIVITIES

Sano et al. (1993) examined pyocins S1 and S2, S-type bacteriocins of *P. aeruginosa* that have different receptor recognition specificities. The sequence homology suggests that pyocins S1 and S2 originated from a common ancestor of the E2 group colicins. Purified pyocins S1 and S2 make up a complex of the two proteins. Both pyocins cause breakdown of chromosomal DNA, as does colicin E2, as it has endonucleolytic activity (Lau et al. 1992). Colicin E3 has RNAse activity, cleaving the 16S RNA moiety of the 30S ribosome subunit. Microcin B17 inhibits DNA gyrase (Vizan et al. 1991).

INHIBITION OF PROTEIN SYNTHESIS

The large particulate bacteriocins, e.g. the pyocin-R (aeruginocins) produced by *P. aeruginosa* (Hayashi et al. 1994), are derived from defective phage particles; they contain the phage component capable of attaching to the cell surface. This class of agent generally shows a narrow spectrum of specificity, inhibits protein synthesis, but does not lyse the cell. The genes specifying this type of bacteriocin are encoded chromosomally.

Synthesis

The physiological state of the cell affects all cellular metabolism. The biosynthesis of bacteriocins is no exception and thus the yields of bacteriocin depend on local environmental influences on the producer cell physiology and the regulatory mechanisms governing the expression of the bacteriocin. For example, Malkhosyan et al. (1991) showed that levels of DNA supercoiling are influenced by anaerobic metabolism and that this regulates the level of expression of the colicin genes. The expression of the *cea* gene, which encodes colicin El, on the ColE1 plasmid was greatly increased when the cells were grown anaerobically (Eraso and Weinstock 1992). By using *cea–lacZ* fusions to quantitate expression, levels of β-galactosidase produced under aerobic conditions from the fusion were found to be only a few percent of the anaerobic levels. The gene is also induced by the SOS response and subject to catabolite repression.

The majority of bacteriocins, such as colicins, aeruginocins, megacins, etc., are produced as the result of normal gene expression. Their synthesis starts with gene transcription, producing an mRNA, which is then translated into an active protein. This synthetic sequence is mediated by ribosomes (Pugsley 1984a, b). The lantibiotics class of bacteriocins (Hansen 1993) frequently contains unusual amino acids that contribute to their properties and functions. Most are synthesized as peptides by nonribosomal mechanisms (Klienkauf and von Dohren 1990), but some are synthesized by pathways that involve post-translational modification of ribosomally synthesized precursor peptides. Examples of the latter are nicin and subtilisin (Nishio et al. 1983).

RELEASE FROM CELLS

The microcins, e.g. colV, are small (6 kDa) and are not induced by the SOS system; their release coincides with the stationary phase. They do not utilize a lysis protein for release but have a dedicated pathway (Gilson et al. 1990; Van der Wal et al. 1995). The lantibiotics generally use a dedicated secretion pathway (Fath and Kolter 1993).

UPTAKE SITES

It has long been recognized that only a few molecules (perhaps only one) are necessary to kill a susceptible cell, and the receptor targets are known for several bacteriocins, in particular some of the colicins. Susceptible cells bear specific protein receptors in their outer membranes, which explains why colicins attach only to certain strains of bacteria.

'Tolerance' and 'resistance' are two terms that are often incorrectly and synonymously used in medical microbiology. The term 'tolerance' describes a state in which the bacteriocin fails to gain access to the sensitive target site within the cell because the receptor is absent or altered so the colicin remains unattached to the cell; thus, it cannot exert a lethal effect. Thus, tolerance may be produced by mutations (e.g. of *tolA*, *tolB*, or *tolC* genes) that affect the outermembrane proteins and the interactions between the cytoplasmic and outer membranes, which prevent the uptake of the bacteriocin into the cell. Resistance occurs when the bacteriocin can be taken up but, because the site is lost or altered by mutation, the cell remains unaffected. There is often a commonality in receptors, which also have a cellular functional role, utilized by phage and bacteriocins. For example, the site used by colicin κ and bacteriophage T6 is implicated in nucleoside uptake. The colicins E1, E2, and E3, and phage BF2 (phage T5-like) use a glycoprotein receptor involved in vitamin B12 uptake. TonB is a membrane-transport protein utilized by many substances and *tonB*- membrane mutants are tolerant to colV

(Pugsley and Schwartz 1985). This commonality signifies that overlap between resistance to inhibitory molecules can be shown.

Host range

Although the general statement that most bacteriocins have a narrow host range (Jack et al. 1995) is true for many, work on bacteriocins produced by *B. megaterium* (megacins), for example, shows that some have a broad spectrum and attack most strains of this species. These megacins are phospholipases and cause susceptible cells to leak intracellular contents by attacking the membrane phospholipids. The bacteriocins produced by lactic acid bacteria exhibit a relatively broad antimicrobial spectrum and are active against several food-spoilage and health-threatening microorganisms (Kim 1993).

Immunity

The production of bacteriocins is the result of deregulation of one or more genes and, in many instances, the release is by a dedicated export mechanism and not cell lysis (Lakey et al. 1994). Strains harboring plasmid or chromosomally encoded colicins, e.g. colicin E3, have immunity to the lethal activity of the colicin (Riley 1993; Frank 1994).

Clinical, technological, and ecological importance

Applications of bacteriocinogeny have been used as epidemiological tools through bacteriocin typing of clinical strains to aid in the identification or discrimination of opportunist and pathogenic bacterial strains. Bacteriocins that show narrow specificity within individual species or genera, such as the bacteriocins of gram-negative bacilli, have been particularly useful in bacteriocin typing. A strain may be characterized actively by the range of activity of its bacteriocins against a set of indicator strains of the same or closely related species, or passively in which it is characterized by the pattern of its susceptibility to the bacteriocins of a set of indicator strains. The former approach has proved particularly useful in typing isolates of *Shigella sonnei* (see chapter 53, *Shigella*) and *P. aeruginosa* (see chapters 62, Pseudomonas; and 63, Burkholderia spp. and related genera), and a combination of the two approaches has been used for *Proteus* spp. (see chapter 55, *Proteus*, *Morganella* and *Providencia*). In colicin and pyocin typing, strains to be typed are streaked across an agar plate and grown overnight so that their bacteriocins diffuse into the agar. Growth is removed from the surface and remaining viable bacteria are killed by exposure to chloroform, and then the indicator strains are streaked across the original line of growth and again incubated overnight. The bacteriocin type is indicated by the pattern of inhibition of the indicator strains (Gillis 1964).

Bacteriocins have been used in the food industry for a long time. There is a potential for this to increase as more food is required to have a longer shelf-life. Applications stem from their lack of mammalian toxicity and the spectrum of activities available. For example, bacteriocins produced by lactic acid bacteria exhibit a relatively broad antimicrobial spectrum and are active against several food-spoilage and health-threatening microorganisms (Kim 1993) and some produce bacteriocins active against *Listeria monocytogenes*, a food-borne pathogen (Hechard et al. 1993). Nicin, produced by some strains of *Lactococcus lactis subsp. lactis*, was originally described in 1928 and is the most highly characterized bacteriocin produced by lactic acid bacteria. Nicin has been permitted as a food additive in the UK since the early 1960s and is currently an accepted food additive in at least 45 other countries (Harris et al. 1992). One example of its use is in the preservation of liquid egg (Delvesbroughton et al. 1992).

The activity or role of colicins in vivo is not clear. Pugsley (1984a) suggested that high proteolytic activity in the intestinal tract (an anaerobic environment) would degrade colicins and thus lead to an effective loss of their activity. Later Luria and Suit (1987) stated that 'colicinogeny appears an unnecessary complication in the life of coliform bacteria.' It seems a little premature to write off a group of proteins that are expressed from highly organized genetic systems and that selectively target specific pathways in selected species. It appears more likely that they do have an ecological role to promote the growth of the producer against its immediate competitors. These are likely to be strains with a similar metabolism (biochemistry and physiology), and will be related strains and species. As the particular and immediate requirement for bacteriocin activity is to inhibit cells adjacent to the one producing the bacteriocin, it is local spatial activity that is required. An agent that diffuses into larger volumes will help other competitors. This argument is supported by Frank (1994), whose hypothesis was that bacteriocin diversity in species could not occur if the organisms mixed freely. Spatial variation and poor habitats favored both susceptibility and bacteriocin diversity in strains. Bacteriocin producers are favored in good habitats where competition is likely to be high.

BACTERIOPHAGES

Bacteriophages (phages) are viruses that grow in bacterial cells utilizing their biosynthetic systems for reproduction. Their effects were first observed by Twort (1915), who described an infectious agent that distorted the morphology of staphylococcal colonies. D'Herelle (1917) found a filterable agent that sterilized broth cultures of *Shigella* spp., and he believed such agents

might be useful in combating bacterial diseases, as they had no infectivity towards eukaryotic cells. More information about the history and current status of phage therapy can be obtained from a review by Summers (2001).

Phages are extremely common in the environment and can be found wherever their host bacteria are present. They are readily detected for most bacterial species. It is probable that all bacteria are sensitive to one or more phages which thus prey on their specific host strain or strains; it is rare to find one that infects a large number of species. The population density of their host cells is a major component determining the density and distribution of phage (Ogunseitan et al. 1990; Proctor and Fuhrman 1990). For example, high counts of phage occur in biofilms ($>10^8$/cm) (Ewert and Paynter 1980) where bacterial density is high, but are far lower in bulk waters ($10^{3–4}$ cfu/ml) (Saye and Miller 1989) where bacterial density is also low. In sewage, enumeration by electron microscopy (Ewert and Paynter 1980) revealed $10^{8–10}$ phages/ml, compared with about 10^8/ml in freshwater and the open ocean (Bergh et al. 1989). Direct counts of bacteriophage in soil are also high, some 4 percent of the total bacterial counts (Ashelford et al. 2003).

Phage morphology

Examined as a group, phages show considerable morphological diversity (Ackermann 1983; www.virology.net/Big_Virology/BVHomePage.html). Some are filamentous, isometric, and superficially resemble animal viruses, whereas others show complex morphologies. The capsid or the phage head is the structure containing the genome; this can be single- or double-stranded DNA or RNA. Some phages are virulent, killing each cell they infect, whereas others are temperate. In the latter case, the phage resides, as a prophage, in a cell termed a lysogen and replicates synchronously with the cell. When phage replication is completed the particles are released from the cell. They usually contain a phage genome. The phage capsid recognizes an adsorption site on the outside of a susceptible cell, binds to it, and injects the genome, held within the capsid, into the cell. The genome then redirects the cellular metabolism to synthesize more phage. In the lysogenic state, the phage genome does not disrupt bacterial metabolism and replication but may contribute specific characteristics to its host, e.g. toxin production by *Corynebacterium diphtheriae* (see chapter 39, Corynebacteria and rare coryneforms) and *Streptococcus pyogenes* (see chapter 33, Streptococcus and lactobacillus).

Single-stranded RNA phages

These are the simplest of phages in terms of their genomic size and morphology. This is not to imply that the organization of their replicative cycle or their economy in genome size, relative to other phages, makes their biological activities simple, but it reflects an evolutionary adaptation. They rely on the host cell for all metabolic activities except for certain replicative functions to complete their life cycle. The RNA coliphage MS2 (*Leviviridae*), termed male-specific, utilizes the pilus of an F^+ *E. coli* cell as an infection site. The MS2 capsid is icosohedral, largely composed of a single protein species, which encloses a phage genome of 3 569 nucleotides (nt) of linear plus strand (sense) RNA. The capsid binds to the side of the F pilus and the phage genome is transferred into the cell. Once internalized, the phage RNA genome acts as messenger RNA and is translated by the ribosomes. There is no transcriptional control, as promoters and RNA polymerase are not involved, and yet there is temporal control over the expression of proteins. This is achieved by changes in conformation of the RNA genome. Relatively large numbers of coat protein molecules (van Duin 1988) are needed per virion. The RNA-directed RNA polymerase, a lysin gene, and an absorption protein are synthesized in smaller amounts and their expression is regulated by secondary structures formed in the phage genome (Kastelein et al. 1982; Berkhout et al. 1987).

On average, 200 phage particles are produced from one cell infected by one phage and this can be achieved in as little as 22 minutes. To achieve this efficiency, replication proceeds alongside expression and the phage polymerase acts in concert with some host accessory functions. The polymerase initiates repeatedly, at the 3′ end of the RNA (Haruna and Spiegelman 1965), enabling multiple copies (of the minus strand) to be made. Concurrently these full-length minus strands are then copied to yield the plus strands, which are used initially for the expression of phage proteins. Later in the infection cycle the plus strands are individually packaged into a capsid. Cell lysis is induced by the final product, the lysis protein.

Single-stranded DNA phages

The tailless icosoheral phage ϕX174 (*Microviridae*) was the first single-stranded DNA phage to be identified (Hayashi et al. 1988). It has a circular genome (5.4 kb) that codes for ten proteins. Four different monomers are present in the capsid, one of which is an internal protein and is surrounded by a second (60 copies), which forms the icosahedral capsid. The other two monomers form ‘spike’ proteins, which are essential for infectivity. The phage receptor is a lipopolysaccharide that allows entry of the phage genome. This DNA is used as a template for single minus-strand synthesis and is entirely dependent upon the host cell to create a biologically active phage genome (Goulian et al. 1967). Once established as a duplex, the phage genome reproduces by rolling circle

replication (Lewin 1987). This is initiated by a phage replication enzyme expressed from the newly formed duplex. Transcription proceeds in the same direction as replication. As the pools of capsid precursors and replicated genomes increase, the plus strands become encapsulated (Aoyama and Hayashi 1986). The reproductive cycle can be complete within 13 minutes, resulting in cell lysis yielding up to 180 phage particles

Although the replication cycles of the filamentous phages are similar to that of ϕX174, their structure, attachment, and the absence of cell lysis on phage release are different (Lindquist et al. 1993). The genome of phage M13 (*Inoviridae*) is circular (6407 nt) and encapsulated as a single strand by a flexible helical capsid composed largely of a single protein species (Model and Russell 1988). At the ends of this tube-like filamentous phage capsid are the 'minor' proteins. Those at one end are responsible for phage attachment to the F plasmid pilus via which the phage genome becomes internalized. Replication proceeds in the same manner as for ϕX174. The host cell continues to grow and divide and as the phage particles become 'mature' they are released through an intact cell envelope. Thus, phages are continually released after a latent period of 30 minutes, resulting in very high phage yields. The filamentous phages are good cloning vectors because foreign DNA can be spliced into the genome at appropriate sites and transformed into a host bacterial cell to give high yields of cloned material. The splicing of novel genes with the coat protein gives protein chimeras in which the novel peptide component may be exposed on the capsid surface (Barbas et al. 1991). As methods for purifying phages are well established, this provides an easy method for screening for protein activities.

Double-stranded DNA phages

The double-stranded DNA phages are divided into two groups, based on their virulent or temperate nature. Phage T4 (*Myoviridae*) is a virulent and morphologically complex phage; it has an elongated icosahedral capsid composed of one major and several minor (in terms of percentage) proteins (Mathews et al. 1983). The capsid is placed upon a tail comprising a core and contractile sheath on a base plate with six kinked tail fibres. In the capsid the 170-kb DNA genome is linear and double-stranded, with about a 5-kb terminal repeat. Free phage particles recognize a lipopolysaccharide receptor on the cell surface and become anchored to it. The proteins in the baseplate then reorientate to allow the core to be driven onto and through the cell surface as the sheath contracts. The DNA is then extruded from the head into the cell's cytoplasm. Phage replication uses the host polymerase and normal vegetative σ factor (σ_{70}). The phage reproductive cycle, from infection to lysis, is highly organized and complex. For example, transcription is temporally organized such that some genes are expressed early and in the middle of the replicative cycle (Mosig and Eiserling 1988). The late genes are transcribed by a phage-encoded σ factor and also govern DNA synthesis. Immediately after infection this phage degrades the host genome and inhibits host transcription. After replication, the concatomeric DNA is injected into the preformed heads and cut, and then the tail structures are added (Bhattacharyya and Rao 1993). Expression of the late phage genes results in cell lysis and release of ⩾200 mature phage particles 23 minutes after infection. The phage λ (*Siphoviridae*; 48.5 kb) has an icosohedral capsid and is termed a temperate phage. It interacts differently to phage T4 with its host. When carried by the host (λ replicates in-step with the host) it is termed a 'prophage' and the cell is termed a 'lysogen'. Phage replication and consequent cell lysis require phage replication to be induced. This is generally promoted by the SOS response (Ptashne and Gann 2001).

Experimental analysis

Phages may be investigated by a variety of molecular and standard laboratory techniques. Three terms describe key points in the analysis of phages. A plaque-forming unit (pfu) is a phage particle that is capable of forming a plaque. The efficiency of plating (EOP) is the proportion of pfu to total phage particles (enumerated by electron microscopy) in a lysate (Adams 1959). Finally, the multiplicity of infection (MOI) is the ratio of host cells to phage in a mixture.

ASSAY

It is a simple procedure to estimate the number of phage particles in a lysate. As titers are frequently 10^6–10^{11} pfu/ml, 10-fold dilution series of phage lysate is prepared to obtain 10–100 pfu/ml. A small volume (e.g. 100 μl) of diluted lysate is mixed with an equal volume of host bacterial cells in about 3.0 ml of 0.6 percent molten nutrient agar, held at 46°C. This is vortex-mixed and poured on to the surface of a standard nutrient agar plate. After overnight incubation, to allow the plaques to develop in the growing bacterial lawn, the number of plaques formed will reflect the original phage concentration and the dilution of the lysate. Thus, at high dilutions, no phage will be present and no plaques will develop. At a lower dilution, a few plaques (zones of lysis a few millimeter in diameter) will be present in the lawn, and at the next dilution, the plaque count will be high and the plaques may even merge in places. At the next dilution, the MOI will be about 1:1 and confluent lysis will occur, although it is likely that a few bacterial colonies, resistant to the phage, will grow (at a frequency of 10^{-8}). This may be due to phenotypic variation (the lack of expression of a phage uptake site) or to a resistant mutation (leading to a loss of the target

site), or the cell may become infected by a normally lytic phage and yet become lysogenized. In each case, the cell remains unaffected by the phage and continues to grow. The titer of phage is calculated by dividing the dilution assayed into the plaque count from the plate by the highest number of countable plaques.

PLAQUE MORPHOLOGY

Phages can replicate only in an actively metabolizing host cell. A poor nutritional environment will reduce the plaque size and probably also reduce the phage yield. The morphology of the plaque is due to repeated cycles of infection, replication, and lysis of the phage; from the point of infection of a single cell, phages released by lysis infect adjacent cells. This process repeats itself while the bacteria in the lawn are growing, causing a circular plaque to form. Thus, the titer and plaque morphology remain consistent provided the media, incubation conditions, and host are constant. Changes in environmental conditions and the genetic status of the host bacterium or the phage can all influence both plaque morphology and the reproductive success of the phage. For example, virulent phage can display 'a temperate' nature when their host is growing slowly (Ripp and Miller 1998).

TRANSDUCTION

Transduction is achievable by only some phages. For gene transfer to occur, a proportion of the phage particles generated in a lysate have to package some host chromosomal DNA instead of a phage genome. The phage particle acts as a vector for host chromosomal genes, transferring the packaged sequence into the cytoplasm of a recipient cell. Providing the DNA sequence has homology with a recipient sequence (chromosomal or plasmid), recombination can then occur. This is mediated by the host-specified RecA protein, enabling replacement of the host sequence by integration of the transferred sequence. The process is termed transduction. If any gene on the bacterial chromosome may be transferred by the phage, this is termed generalized transduction (Margolin 1987), but if the phage will transfer only a few genes from one site on the genome, this is termed specialized or restricted transduction (Weisberg 1987).

About 2 percent of the phage particles produced by the *Salmonella typhimurium* phage P22 contain host DNA (Ebel-Tsipis et al. 1972), as do about 0.3 percent of *E. coli* phage P1 in a lysate (Lennox 1955). The frequencies of transfer of auxotrophic markers varies between 10^{-5} and 10^{-7} per pfu (Harriman 1971) for both of these phages. However, Schmeiger (1972) has shown that with different genes and hosts the frequencies can vary over 1000-fold.

There are various fates for DNA transduced into a recipient cell. The DNA that is recombined is stably inherited, giving rise to a stable transductant. The displaced strand is degraded and the nucleotides are reutilized. If the DNA sequence fails to become integrated, then it may persist for several generations, producing what is termed 'abortive transductants' (Ozeki 1959). These transductants show unilinear inheritance of the character transferred. This is explained by using a recipient cell that is an auxotroph (carries a mutation in the gene coding for an enzyme required for the synthesis of an amino acid), so it cannot synthesize a particular amino acid. This is transduced to prototrophy by an 'abortive fragment' that contains the prototrophic gene so the cell is able to synthesize the amino acid. When the cell divides, one daughter inherits the wild-type gene and continues to express the enzyme and consequently synthesizes the amino acid and grows. The other cell now has the mutant genotype but is phenotypically wild-type; it retains an active biosynthetic pathway while the enzyme retains its activity. This is lost gradually as it becomes diluted out through growth and cell division; the cell grows, but increasingly slowly. Hence, abortive transductants generate very small colonies, which appear some time after the wild-type has fully grown. The condition is relatively stable as less than 1 percent of cells go on to become complete transductants (Ozeki and Ikeda 1968). Schmeiger (1982) has shown that abortive transduction by P22 is a common phenomenon and varies over a range from 50 percent to less than 1 percent for different loci.

Specialized transduction occurs with *E. coli* phage λ. In this type of transduction the phage enters the chromosome by site-specific recombination at *attB*, using a homologous sequence on the phage termed *attP* (Weisberg and Landy 1983), to form a lysogen. Transducing particles are formed by the illegitimate excision of the phage genome, in which part of the phage sequence remains in the chromosome to be replaced by an adjacent host chromosomal sequence. Thus, genes to one side or the other of the *attB* site may be packaged in the capsid (hence the term specialized). The transducing phage genome is defective and cannot reproduce in the recipient cell. These transducing phages occur at a frequency of about 10^{-6}. If these defective phages coinfect a new recipient with a wild-type phage, 50 percent of the phage in the lysate produced from that cell will be capable of transducing the host gene. The frequency of transduction now is far greater; it can be 100 percent (Lewin 1987). Transducing phages of both types are common in other genera.

ENVIRONMENTAL ROLE

The role of phages in natural bacterial populations remains largely a point of conjecture. They seem to have a dramatic influence on population densities of bacteria in natural habitats (Ogunseitan et al. 1990; Proctor and Fuhrman 1990). Interactions between bacteriophages and planktonic bacteria in aquatic environments have

shown that the abundance of free viruses can exceed that of planktonic bacteria by over an order of magnitude (Bergh et al. 1989). Short-term dynamics imply that bacteriophages can contribute to the control bacterial growth in situ (Bratbak et al. 1990). Transmission electron microscopic (TEM) studies indicate that 1 to 5 percent of planktonic bacteria were visibly infected by phage (Proctor and Fuhrman 1990). Bacterial mortality due to phage reproduction suggests phages can, at times, be entirely responsible for bacterial mortality (Bratbak et al. 1990; Proctor and Fuhrman 1990). Thus, it seems that phages can be significant moderators of microbial populations in a variety of natural environments (Ashelford et al. 2003). These include those in animal and plant as well as populations in water bodies and soil, and between bacterial growth, phage production, and chlorophyll (Cochlan et al. 1993).

Host range has been established by the observation of plaques on putative host strains and on this basis most phages have a narrow host range. There is a widespread assumption that the plating range (i.e. the ability of the phage to form plaques) is a direct reflection of the transduction range. The ambiguity of this has been illustrated with *E. coli* phage P1. Productive infections with phage P1 can be made from *Citrobacter freundii*, *Shigella* spp., *Salmonella* spp., Serratia spp., *Enterobacter liquefaciens*, *Erwinia* spp., *Proteus* spp., *Pseudomonas* spp., and *Klebsiella* spp. (Yarmolinsky and Sternberg 1988). In addition, phage P1 can naturally transduce DNA into *Yersina* spp., *Flavobacterium* sp. M46, *Agrobacterium tumefaciens*, *Alcaligenes faecalis*, and *Myxococcus xanthus*. Goldberg et al. (1974) have reported transduction by phage P1 into *S. typhimurium* even though they were unable to isolate stable lysogens and *Enterobacter amylovora* lysogens did not produce P1 phage. This provides evidence to support the proposal that the transduction and titration ranges for a phage are not unambiguously equivalent and the associations between phage and their host bacteria are not clear-cut. Thus, the contribution of phages to gene exchange remains an open and unanswered question. In addition, the host range can be influenced by the presence or absence of adsorption sites and the mechanism of phage replication. The restriction enzymes possessed by the new host target the phage genome (Roberts 1985). If the phage fortuitously evades the restriction enzymes and becomes modified, to shield the restriction sites, or if these sites are absent, then the phage may be able to replicate.

PHAGE IDENTIFICATION AND TYPING

The specificity and variety of phages that can infect bacterial species have enabled phages to be used for the identification of a species. Strain typing by determining phage sensitivity patterns within a species can be achieved when various phages will attack only a proportion of strains. Phages that lyse all members of a particular species have been used in the identification of *Brucella abortus* and *Bacillus anthracis*. The phages active on *Vibrio cholerae* are specific in their action on serotypes or biotypes. Phage typing within species has been most widely developed and most effectively used in the epidemiological investigation of *Staphylococcus aureus* infection and for strain identification within several *Salmonella* serotypes, including typhi. Typing phages are propagated on a susceptible host strain of the test species to give a high-titer phage suspension, which is then diluted to a concentration (the routine test dilution (RTD)), which gives barely confluent lysis of the propagating culture growing on a solid medium. In phage typing, a set of phages active on only some strains within a species is applied to a lawn of the isolate in question on an agar medium. After incubation, susceptibility is seen as lysis (i.e. plaques) produced by particular phages. The pattern of activity gives the phage type of the organism (Parker 1972).

CONCLUSIONS

Both bacteriocins and bacteriophages are universally present in microbial populations, affecting growth and biochemical relationships within and between bacterial populations (Ashelford et al. 2000). This relationship now clearly extends to interactions, like the pathogenicity endowing genotypes, with higher organisms. Frank (1994) has suggested that the great diversity of bacteriocins produced by a single species cannot occur if the organisms mix freely. His analysis suggests that spatial variation and poor habitats favor susceptible and polymorphic strains, populations with a variety of bacteriocin types. Toxin producers are favored in good habitats since the rate of competition for resources is lower. Although most bacteriocins inhibit the growth of closely related bacteria, some, typified by those produced by lactic acid bacteria, exhibit a relatively broad antimicrobial spectrum and are active against several food-spoilage and health-threatening microorganisms. Thus, there is circumstantial evidence for a role in the regulation of competing bacteria.

The most general use of bacteriophages in the medical sphere has been to phage type bacterial strains of clinical importance. They have been studied since the 1940s and have provided basic information on how DNA and RNA genomes replicate and express genes (Boyd and Brüssow 2002). Because of the relative ease with which they can be grown, purified, and now manipulated, they have become of primary importance (with plasmids) in the development of the molecular biological techniques used to clone and analyze genes and their products. Thus, they have provided a basis for the development of the biotechnological era.

Those debating the risks of recombinant genes being transferred from genetically manipulated bacteria into indigenous bacterial populations now accept that

transfer will occur. Although bacteriophages may be one class of the mediators of such gene exchange, it is still an open question as to how efficient they might be as mechanisms for exchange in situ or in vivo. It will be an interesting step in our ecological appreciation of these genetic determinants when their true ecological value to microbial populations can be demonstrated.

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Molecular basis of bacterial adaptation to a pathogenic lifestyle

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INTRODUCTION

Infection is a complex and dynamic interaction, the outcome of which determines the onset or avoidance of disease. The term 'pathogen' denotes a microorganism that is able to cause disease in an animal, fish, plant, or insect host, while 'pathogenicity' or 'virulence' is the ability of that microorganism to induce disease in one or more target hosts. Virulence can be defined as the capacity of bacteria to cause disease; virulence determinants are those bacterial products or strategies that contribute to virulence, i.e. those cell-surface-associated or extracellular factors that promote tissue colonization or invasion, protect the bacterial cells from innate host immune defenses, and damage the host. While the expression of such virulence determinants is tightly regulated, they are not usually essential for bacterial viability or growth in vitro. The interplay between host- and pathogen-derived factors will determine the final outcome of an infection, which will depend on the relative degree of susceptibility or resistance of the host as well as the intrinsic virulence of the pathogen.

All living organisms are programmed with a biological capability to survive, replicate, and spread. From an evolutionary perspective, genetic modifications that improve any of these attributes will be selected for and these ultimately lead to the enrichment of such organisms within a given environmental niche. For pathogenic microbes, many of which have become highly adapted to a specific host, the death of the host is not necessarily an indicator of pathogen success. This is because it signals the destruction of an environment that has provided nutrition and protection. Pathogenic microorganisms cannot be considered as 'malicious' since they do not 'deliberately' cause disease or the subsequent death of a host. Pathogenic bacteria are unicellular microorganisms that can ensure the survival of some of their number by infecting other hosts. If, during the process of infection, the host dies, pathogen survival will depend on transmission to a new host, a process that may depend on its capacity to survive in diverse environments. The manifestation of disease is very much symptomatic of the exploitation of host resources by the pathogen for promoting bacterial survival, replication, and dissemination. Thus, the lifestyle of pathogenic bacteria revolves around: (1) locating a host; (2) finding a replication niche; (3) initiating an infection; (4) progressing the infection; and (5) dispersal to a new host. For a bacterium to progress from one stage to the next, variations in sensory inputs that signal environmental change must be perceived and acted upon, for example by the induction of gene expression. New environmental signals may derive from movement from one environment to another, may be due to the actions of bacteria within a given environment, or may be a consequence of host responses to the activity of the bacteria. Thus, from a prokaryotic perspective, the successful interaction of bacterial cells with mammalian host tissues depends on a

coordinated response not only to environmental cues such as nutrient availability, bacterial cell population density, temperature, osmolarity, and pH, but also to diverse host cell effector molecules. For example, bacterial pathogens secrete toxins that either release the nutrients from host tissues or alter the host internal environment to optimize bacterial growth and dissemination. The host responds to these changes by attempting to rectify the physiological and biochemical imbalances induced by the pathogen. Together, the damage caused by the pathogen and the processes elicited by the host during infection are manifested as disease.

MECHANISMS OF BACTERIAL PATHOGENICITY

The main characteristics of pathogenic bacteria that underlie the mechanisms by which they cause disease are:

- Colonization and infiltration of host tissues: the colonization of a specific host niche usually represents the first stage of infection. This may result in the carriage state or may lead to infiltration of the host tissues. The latter involves growth on or within host tissues, the ability to bypass or overcome host defense mechanisms, and the production of extracellular tissue-degrading exoenzymes.
- Toxin production: bacteria produce two categories of toxins, termed exotoxins and endotoxins. Exotoxins are released or secreted from bacterial cells and may act at tissue sites distant from the sites of bacterial infection, adhesion, and growth. Endotoxins are cell-associated structural components of the gram-negative bacterial envelope, which may be released from growing bacterial cells as blebs or from lysed cells (see Endotoxins). Hence, bacterial toxins, both soluble and cell-associated, may be transported by blood and lymph and induce cytotoxic effects at tissue sites remote from the original site of infection. Some bacterial toxins may also act at the site of colonization and play a role in promoting tissue invasion.

Colonization

The first stage of bacterial infection is colonization, which is essentially the establishment of the pathogen at a specific body site frequently followed by entry into host tissues. Pathogens usually colonize host tissues that are in contact with the external environment, for example the urogenital tract, the digestive tract, the respiratory tract, and the conjunctiva. Organisms that infect these sites have developed specific tissue-adherence mechanisms alongside the ability to overcome the local innate host defenses (see Host resistance to infection). This stage may, however, be bypassed as a consequence of tissue damage or introduction of medical devices, e.g. catheters.

ADHERENCE TO SURFACES

Bacterial adherence to host tissues involves at least two stages:

1. Nonspecific adherence. This physicochemical process can be considered as reversible attachment of the bacterium to the eukaryotic cell or tissue surface and involves nonspecific attractive forces that allow the bacterium to approach the eukaryotic cell/tissue surfaces. The interactions and forces involved include hydrophobic and electrostatic attractions, atomic and molecular vibrations resulting from fluctuating dipoles of similar frequencies between components of the bacterial and the host cell, Brownian movement, and bacterial cell recruitment or trapping by biofilm polymers (see Biofilms).
2. Specific adherence. This can be a reversible or permanent attachment of the microorganism to the cell or tissue surface. Specific adherence involves irreversible formation of many specific, noncovalent bonds between complementary adhesin and receptor molecules on each interacting surface. The latter must be accessible and arranged in such a way that many bonds form over the area of contact between the two cells. Once the bonds are formed, attachment under physiological conditions becomes virtually irreversible. Direct evidence that receptor and/or adhesin molecules mediate the specificity of adherence of bacteria to host cells or tissues has been derived from the competitive binding of bacteria to isolated receptors or receptor analogs in vitro. Purified bacterial adhesins and adhesin analogs that bind to eukaryotic cell/tissue surfaces have been shown to inhibit the interaction of bacteria with host cells. Such inhibition can also be achieved with purified receptors or antibodies to either the adhesins or to the eukaryotic receptor.

The eukaryotic receptors that have so far been identified are specific surface peptides or carbohydrates, which have functions in the host cell distinct from their functions as receptors for bacterial adhesins. Bacterial adhesins are macromolecular components of the bacterial cell envelope, and adhesins and receptors interact in a complementary and specific fashion.

HOST- AND SPECIES-SPECIFIC TROPISMS

It has long been recognized that bacteria exhibit different degrees of colonization or adherence tropisms. These can be broadly subdivided into (1) tissue- and host-specific tropisms and (2) species-specific tropisms.

1. Tissue and host tropisms. Some bacteria show a preference for certain tissues and specific hosts. For example, uropathogenic *Escherichia coli* adherence

patterns are determined by the binding specificity of the PapG adhesin (a component of the pyelonephritis-associated pili (Pap)) and the differential distribution of the receptor isotypes in different hosts and tissues. Three different *papG* alleles exist in uropathogenic *E. coli* (class I, II, III). Each binds with a different specificity to distinct receptor isotypes, with class II being the allele predominantly associated with pyelonephritis, and class III being correlated with human cystitis. PapG binds to the Gal-α-1-4Gal-containing glycolipids present on the surface of human kidney epithelial cells, allowing the bacteria to gain a foot-hold in the tissue and resist displacement by the mechanical and physical forces operating within the kidney. The glycolipid receptor for PapG consists of a digalactoside core linked via a β-glucose residue to a ceramide group that anchors the receptor within the membrane. This minimum receptor isotype is called globotriasylceramide (GbO3). The various members of this receptor family differ by the addition of sugar residues distal to the Gal-α-1-4Gal core of GbO3. The addition of a single GalNAc sugar to GbO3 creates GbO4 (globoside), whereas the addition of two GalNAc sugars to GbO3 creates the Forssman antigen (GbO5) (Dodson et al. 2001).

2 Species-specific tropisms. Some pathogenic bacteria infect only certain animal species, e.g. *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *E. coli* (expressing CFA I and CFA II), and group A streptococci are limited to infections of humans, whereas enteropathogenic *E. coli* K-88 infections are limited to pigs and *E. coli* K-99 strains predominantly infect calves. Although adhesin type and receptor specificity play an important role, it should be noted that other factors, such as nutritional requirements, may contribute to species-specific tropisms (see Iron scavenging). It should also be noted that certain strains or races within a species are genetically immune to specific pathogens. A good example is the lack of susceptibility of certain pig breeds to *E. coli* K-88 infections (Jann and Jann 1992). Although the differing nutritional or physiological conditions encountered in different animals is likely to influence the course of an infection, the outcome is related much more closely to the structural differences between, or absence of, a given eukaryotic receptor for a specific bacterial adhesin.

EXAMPLES OF BACTERIAL ADHESINS AND THEIR RECEPTORS

The adhesins of *E. coli* are varied and include pili, fimbriae, fibrillar, and afimbrial adhesins. A single strain of *E. coli* may be able to express several distinct adhesins encoded by distinct regions of the chromosome or of plasmids. This genetic diversity permits this organism to adapt to its changing environment and exploit new opportunities presented by different host surfaces. Many of the adhesive fimbriae of *E. coli* have probably evolved from fimbrial ancestors resembling type 1 and type 4 fimbriae. Type 1 fimbriae enable *E. coli* to bind to D-mannose residues on eukaryotic cell surfaces. Although the primary fimbrial subunit (FimA) is the major protein component of type 1 fimbriae, the mannose-binding site is located in a minor protein (FimH) located at the tips or inserted along the length of the fimbriae. By genetically varying the minor 'tip protein' adhesin, the organisms gain the ability to adhere to different receptors. For example, tip proteins on Pap pili recognize a digalactose residue, while tip proteins on S-fimbriae recognize sialic acid. Differential binding can also be achieved by allelic variation of the *fimH* gene, for example, in *Salmonella enterica* serovar Typhimurium (Boddicker et al. 2002). The fibronectin-binding *E. coli* fimbriae known as curli (Olsén et al. 1989) are unusual since they are assembled via an extracellular nucleation precipitation pathway (Smyth et al. 1996). Production of curli increases internalization of bacterial cells by eukaryotic cells and triggers events leading to septic shock. Since curli also promote clumping of bacterial cells in culture and binding to abiotic surfaces, they are important for biofilm formation (Chirwa and Herrington 2003) (see Biofilms). Although *E. coli*, *S. enterica* serovars Typhimurium and Enteriditis, and *Shigella* spp. have the genes required for curli synthesis, the expression of curli is not common to all strains due to insertion sequence disruptions, deletions, or other mutations. Unlike natural isolates of enterohemorraghic, enterotoxigenic, and sepsis *E. coli* strains, enteroinvasive and enteropathogenic (EPEC) strains do not produce curli. EPEC combine the adhesive properties of bundle-forming pili (a type IV fimbria) (Tobe and Sasakawa 2001) with the intimate adherence mediated by effectors secreted by the type III secretion pathway (see Protein secretion) (Delahay et al. 2001). In addition, the flagella of EPEC can function as an adhesin for binding to mammalian cells, although the nature of the eukaryotic receptor(s) is not known (Girón et al. 2002). A fimbrial adhesins include Afa-I and Afa-III, which are made along with the Dr hemagglutinin and fimbrial F1845 adhesin by Afa/Dr diffusely adhering *Escherichia coli* (DAEC). The mechanism by which Afa/Dr DAEC infection is linked to inflammatory bowel diseases is currently unknown, however utilization of a glycosylphophatidylinositol-anchored protein (the complement-regulating decay-accelerating factor (DAF) or CD55) as a receptor for Afa/Dr adhesions offers one possible explanation. Afa/Dr DAEC strains recognize the short consensus repeat 3 (SCR3) domain of DAF, which plays a pivotal role in the regulatory function of DAF. In so doing, they induce a cascade of responses in T84 cells, including interleukin 8 (IL-8) production and polymorphonuclear leukocyte transepithelial migration.

Production of tumor necrosis factor-α (TNF-α) and IL1β follows, which upregulates DAF expression, thus increasing the brush border adhesion of Afa/Dr leading to structural and functional lesions (Bétis et al. 2003).

Pseudomonas, *Vibrio*, and *Neisseria* possess a fimbrial protein subunit that contains methylated phenylalanine at its amino terminus. These type IV pili have been established as virulence determinants of *Pseudomonas aeruginosa* lung infections in cystic fibrosis patients, and the receptor for such fimbriae produced by *N. gonorrhoeae* is the surface glycoprotein CD46 on urogenital cells (Tobiason and Seifert 2001). The major virulence factors of toxigenic *Vibrio cholerae* are cholera toxin (CT), which is encoded by a lysogenic bacteriophage (CTXϕ), and toxin-coregulated pilus (TCP), which was also reported to be encoded on a filamentous phage genome. TCP is a type IV bundle-forming pilus essential for colonization and also serves as the receptor for CTXϕ. Somewhat controversially, TCP has also been proposed to be a coat protein for a novel filamentous phage (Davis and Waldor 2003).

Gram-positive bacteria, such as *Staphylococcus aureus,* possess multiple cell wall proteins (microbial surface components recognizing adhesive matrix molecules (MSCRAMM)) which facilitate binding to host cells and tissues, thus promoting attachment of bacteria to surfaces. These include the fibronectin-binding proteins FnbA and FnbB, which promote adherence to host tissues and are essential for inducing the uptake of staphylococci into epithelial and endothelial cells. Other MSCRAMM can override the loss of fibronectin binding and allow adherence to host cells but not lead to efficient internalization into eukaryotic cells (Bayles and Bohach 2001).

Other gram-postive pathogens, such as Group A streptococci and *Streptococcus pyogenes*, also display cell-surface fibronectin-binding proteins (Bisno et al. 2003). The fibronectin binding proteins (e.g. F, M1 and M3) of *Str. pyogenes* in conjunction with lipotechoic acid (LTA) are involved in promoting attachment to the oral mucosa and buccal epithelial cells, where LTA loosely tethers streptococci to epithelial cells, whereas M proteins secure a much stronger, less reversible association.

Treponema pallidum, the causative agent of syphilis, has three related surface adhesins (P1, P2, and P3), which bind to a four-amino acid sequence (Arg-Gly-Asp-Ser) of the cell-binding domain of fibronectin. It is not clear whether *T. pallidum* uses fibronectin to attach to host surfaces or coats itself with fibronectin to avoid host defenses, such as phagocytic cells (Baughn 1987).

BIOFILMS

In most environmental niches, bacteria survive and multiply not as planktonic cells suspended in liquids but as surface-attached biofilms. These are complex communities of single or multiple species of microorganisms that develop on abiotic (e.g. rocks) and biotic (host mucosal tissues) surfaces. Since biofilms contaminate industrial pipelines, dental-unit water lines, catheters, ventilators, and medical implants, they act as a source of disease for humans, animals, and plants. They also represent a barrier to eradication as both the nature of the biofilm and the physiological state of bacterial cells within the biofilm confers high levels of resistance to antimicrobial agents.

Microbial development on surfaces is a dynamic stepwise process involving adhesion, growth, motility, and extracellular polysaccharide (EPS) production (Figure 1). Five distinct stages of biofilm development (namely (1) reversible attachment; (2) irreversible attachment; (3) maturation 1; (4) maturation 2; and (5) dispersion) (Sauer et al. 2002) culminate in a structured community sometimes characterized by mushroom and mound structures. Underlying this process is a genetically programmed series of events; however, bacteria appear to have evolved more than one pathway, probably due to particular environmental stimuli (growth media or substratum, etc.) triggering different developmental pathways. Despite this, all culminate with the same end point, i.e. a mature biofilm community (O'Toole 2003). For *P. aeruginosa*, biofilm formation involves the production of flagella and pili followed by the production of EPS. To become a member of the biofilm community, the bacterium must then differentiate into a biofilm-associated cell by repressing synthesis of the flagellum (O'Toole 2003; Watnick and Kolter 2000). Ultimately, however, flagella are produced once more to facilitate dispersal (Sauer et al. 2002).

Although redistribution of attached cells to form a structured community can be achieved by motility via flagella, pili, or fimbriae, this is not a prerequisite, since nonmotile organisms readily form biofilms, e.g. staphylococci, streptococci, and mycobacteria. As cells divide, daughter cells spread outward and upward from the attachment point to form cell clusters offering an alternative mechanism of biofilm formation. Surface-associated aggregation can also occur via the recruitment of single cells or cell flocs from the bulk fluid. Bacterial cells in biofilm microcolonies are held together by a slime-like matrix (EPS). This matrix is chemically complex, including polysaccharides, nucleic acids, and proteins. Intercellular adhesion within biofilms of *Staphylococcus epidermidis*, a major cause of medical device-related infections, is mediated by the polysaccharide intercellular adhesin (PIA). Synthesis of this linear glucosaminoglycan composed of *N*-acetylglucosamine in β-1,6-glycosidic linkages containing deacetylated amino groups and succinate and phosphate substituents requires expression of the *icaADBC* operon. These genes are subject to a complex regulatory control mechanism, which involves the alternative sigma factor σ^B, *icaR*, two other regulatory loci, plus phase variation due to IS element insertion. Cessation of PIA produc-

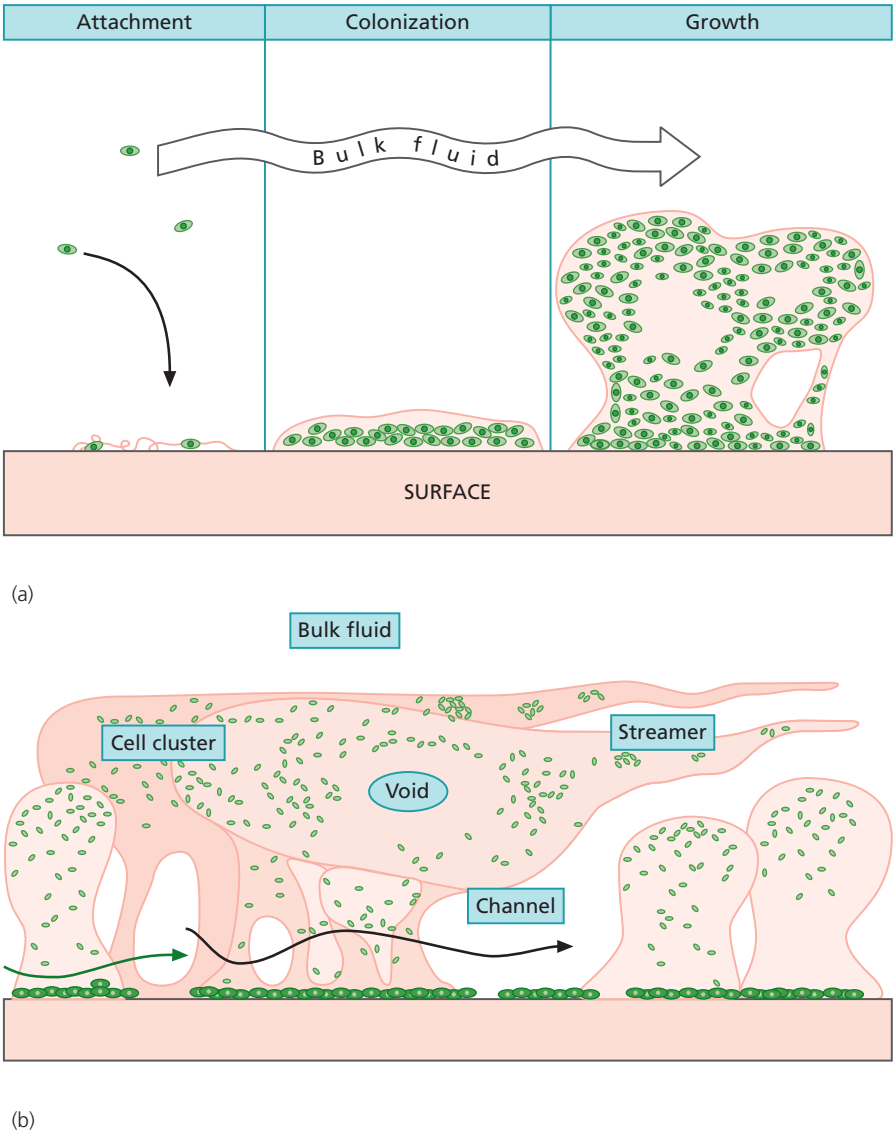

Figure 1 *Biofilm formation:* **(a)** *Bacteria attach to a surface and multiply. An extracellular polysaccharide is produced and the combined influence of this and the physical properties of the environment favor the development of complex structures resembling mounds and mushrooms. The bulk fluid flows between these structures through channels, and, if strong enough, streamer structures develop downstream.* **(b)** *Dispersal of detached bacterial cells finally occurs into the bulk fluid. Redrawn from originals supplied by Dr J.W. Costerton, Montana State University, USA/Artwork PEG DIRCKX from www.erc.montana.edu/Res-Lib99-SW/Image_Library/Structure-Function/default.htm, copyright held by Center for Biofilm Engineering at Montana State University-Bozeman, Bozeman, MT 59717-3980, USA; Tel.: +1-406-994-4770; fax: +1-406-994-6098.*

tion by inactivational IS element insertion allows individual cells to leave the biofilm and colonize new surfaces (Dobinsky et al. 2003). Physical forces acting on the biofilm can also influence its structure, e.g. a faster flow can favor biofilm cell clusters, which tend to elongate in the downstream direction, forming filamentous 'streamers' (Hall-Stoodley and Stoodley 2002).

Proliferation

Bacterial proliferation within a colonization niche and also during the development of infection will depend on an ability not only to resist host defenses but also to acquire essential nutrients. Bacteria have therefore evolved a full complement of mechanisms for nutrient acquisition (e.g. to scavenge for carbon and nitrogen sources, phosphate, sulfur, and metal ions) and for the synthesis of complex organic molecules that cannot be scavenged from the local environment. Specific transport systems or cell-wall components capable of binding limiting substrates and transporting them into the bacterial cell are employed by both pathogenic and nonpathogenic bacteria. Pathogens that are highly host-adapted often dispense with biosynthetic pathways for complex organic molecules that can be obtained directly

from the host. For example, *H. influenzae* requires heme (X factor) and nicotinamide adenine dinucleotide (NAD^+; V-factor) for growth.

For almost all bacterial pathogens, iron is one indispensable nutrient, the lack of which exerts considerable influence on growth in vivo. Iron is a redox reactive metal and is required as a cofactor for many important metabolic enzymes and cytochromes. Although there is an abundance of iron in the extracellular tissue fluids of man and animals, the amount of free ionic iron is of the order of 10^{-9} M, a level that is far too low to support the growth of most microorganisms. The injection of animals with iron compounds results in an increased susceptibility to experimental infection when compared with untreated controls, so that the lethal dose of infecting microorganism required is greatly reduced. Iron is also the only metal cation known to be capable of abolishing the antibacterial activity of body fluids in vitro. The ability of the host to withhold iron thus contributes to host defense against infecting microorganisms and in extracellular tissue fluids depends on the host iron-binding glycoproteins transferrin and lactoferrin (see Iron scavenging). However, most pathogens multiply successfully in body fluids and cause infection in the absence of exogenously supplied iron either because they possess efficient iron scavenging mechanisms capable of removing transferrin-bound iron or by invading host cells where intracellular iron is more freely available.

IRON SCAVENGING

The most intensively investigated bacterial high-affinity iron-transport systems are based on siderophores. These are low-molecular-mass iron chelators that bind iron with high affinity and specificity. They are employed by both gram-negative bacteria such as *E. coli*, *Shigella* spp., *Salmonella* spp., and *P. aeruginosa* and gram-positive pathogens such as the staphylococci, mycobacteria, and corynebacteria. *Shigella* species employ at least three distinct iron-transport systems, two of which involve siderophores (aerobactin and enterobactin) and their associated outer-membrane receptor proteins. A third pathway required for the transport and utilization of iron in the form of heme is employed by shigellae growing intracellularly within epithelial cells. While the enterobactin iron-transport system is found in all *E. coli* strains so far examined, aerobactin production and heme transport are more often associated with pathogenic *E. coli* strains and the corresponding genetic loci are encoded on virulence plasmids, within pathogenicity islands, or on chromosomal regions with the potential for horizontal transmission.

Certain bacterial pathogens and notably *H. influenzae* and *N. meningitidis* use a siderophore-independent receptor-mediated iron-uptake system that involves a direct interaction between a bacterial cell-surface receptor (consisting of two transferrin-binding proteins, TbpA and TbpB) and transferrin. These receptor-based mechanisms are further distinguished from siderophore-mediated mechanisms by their high degree of specificity for the iron-binding glycoprotein of the natural host; humans are the only natural reservoir for *N. meningitidis*, which will only bind human transferrin, while the pig pathogen *Actinobacillus pleuropneumoniae* can use porcine but not human transferrin as an iron source.

Apart from sequestering transferrin-bound iron, many pathogens have also evolved mechanisms for acquiring iron from heme-containing proteins (such as hemoglobin) or from heme- and hemoglobin-binding proteins (hemopexin and haptoglobin, respectively). These involve either specific surface receptors or secreted proteins such as HasA of *P. aeruginosa* and *Serratia marcescens*. The latter consists of an outer-membrane receptor (HasR) and a type I secretion apparatus (see below under Subversion of host cell signal transduction and on protein secretion) in addition to the extracellular heme-binding protein. HasR can bind and transport free heme or heme bound to hemoglobin, in a manner enhanced by the presence of HasA. Thus, it appears that the HasA–HasR interaction, rather than recognition of heme, is important for ligand uptake (Clarke et al. 2001).

For both high-affinity iron- and heme-scavenging systems in gram-negative bacteria that involve outer membrane receptor proteins, a common feature is the requirement for the energy-transducing protein TonB to promote the uptake of iron across the outer membrane together with an ABC transporter for transporting the iron–ligand complex across the periplasm and cytoplasmic membrane. Gram-positives need only the ABC transporter and a binding protein, which is usually a cytoplasmic membrane anchored lipoprotein

Motility

To establish infection in or on a susceptible host, a pathogen must reach a suitable infection site or breach in host tissues. For example, at mucus membranes a pathogen may need to penetrate the mucus blanket and subsequently adhere to the epithelium. Movement through mucus to the epithelial cells may be dependent on chemoattractants present in host tissues, the motility organelles on the bacterium (see below), and possibly mucus-degrading enzymes (see Infiltration of host tissues).

Motility can be achieved through the retraction of surface appendages (type IV pili-mediated twitching motility) (Mattick 2002) or rotation of specialized motors (flagella-mediated swimming) (Josenhans and Suerbaum 2002). Flagella are assembled by a machinery with significant similarity to the type III secretion pathway (see below under Subversion of host cell signal transduction and on protein secretion). The control of this machinery and the structural assembly of the flagella

are subject to a complex hierarchical regulation. The flagellum comprises structurally of a hook filament protruding from the basal body, which anchors it to the cell membranes. Extending from this is the long rigid filament (Aldridge and Hughes 2002). Bacteria may have single or multiple flagella, which can be arranged in a polar fashion or distributed over the entire cell surface. Flagella rotation propels the bacterium, and this forward motion is punctuated with tumbling, during which reorientation may occur to enable bacteria to preferentially spread in certain directions (chemotaxis). The flagella of *E. coli* rotate in a counterclockwise direction to achieve a forward motion, and switch to a clockwise direction to effect stopping and tumbling (Berg 2003). Other bacteria, e.g. *Rhodobacter spirillum*, achieve this control by periodically ceasing flagella rotation (Armitage 1999).

Swarming is a specialized form of bacterial motility that is characteristic of species belonging to genera such as *Proteus, Vibrio*, *Bacillus*, and *Clostridium* but is also exhibited by *Serratia*, *Escherichia*, *Salmonella*, and *Yersinia* spp. Depending on the organism, swarming depends on flagella and pili, as well as surface components such as polysaccharides and surfactants, and involves differentiation of vegetative bacterial cells into hyperflagellate swarm cells that undergo rapid and coordinated population migration across solid surfaces. For pathogens such as *Proteus mirabilis*, swarming facilitates ascending colonization of the urinary tract and entry into host cells (Fraser and Hughes 1999).

Infiltration of host tissues

Motility is not the sole requirement for successful relocation. There are numerous factors that mediate the penetration of bacteria deep into host tissues. These factors function by altering the integrity of the tissues by degrading the extracellular matrix or tissue cells or by travelling directly through cell layers after uptake directly into mammalian cells, a process that leads to either tissue necrosis or programmed cell death (apoptosis). Such determinants are often termed 'spreading factors' and are usually bacterial enzymes that affect the physical properties of tissue matrices and intercellular spaces, thereby promoting the spread of the pathogen (see Table 1).

Once in contact with eukaryotic cells, bacteria may be taken up by the host cell or may mediate their own internalization to proceed further. Bacteria-mediated uptake into nonphagocytic host cells may be achieved by either bacterial surface proteins (invasins), which interact with specific receptors on the host cell (e.g. internalin A and B binding to E-cadherin during uptake of *Listeria monocytogenes*), or bacterial proteins injected into the host cell cytoplasm, which trigger membrane ruffling and concomitant bacterial uptake by macropinocytosis (e.g. *Salmonella* or *Shigella*) (see Subversion of host cell signal transduction). The invasin-mediated 'zipper' mode of entry is achieved by unrelated proteins in different bacteria (e.g. *Listeria* internalins and *Yersinia* invasin), whereas the mediators of the 'trigger' mechanism of uptake used by *Shigella* (Ipas) and *Salmonella* (Sips) are similar. All mechanisms proceed to localization of the bacterium within a membrane-bound vacuole. This vacuolar compartment is acidic, is poor in nutrients, and undergoes progressive vesicle fusion with early endosomes, late endosomes, and lysosomes that contain potent lytic enzymes. Some pathogens have evolved mechanisms to modify the vacuole or its process of maturation and remain localized within the vacuole, e.g. *Mycobacterium tuberculosis* and *Salmonella* spp. *M. tuberculosis* recruits tryptophan-aspartate-containing coat protein (TACO) to the membrane of the phagosome, thus mimicking the plasma membrane and thereby preventing maturation into, or fusion with, lysosomes (Pieters and Gatfield 2002). In contrast, *S. enterica* serovar Typhimurium appears to deliver effector molecules via the type III secretion machinery (*Salmonella* pathogenicity island; SPI-2) (see Subversion of host cell signal transduction) into the cytosol, which disrupt recruitment of the components of the noxious host enzyme NADPH oxidase to the phagosome (Amer and Swanson 2002).

Other pathogens escape from the vacuole into the cytosol, e.g. *Listeria monocytogenes*, *Shigella flexneri*, and rickettsiae (O'Riordan and Portnoy 2002). To escape from phagosomes, *L. monocytogenes* produces the pore-forming cytolysin listeriolysin O (LLO). The combined action of LLO with phosphatidylinositol-specific phospholipase C (PI-PLC) and phophatidylcholine-specific phospholipase C (PC-PLC) disrupts the phagosomal membrane leaflet, although the underlying mechanism employed is uncertain. *Sh. flexneri* appear to use the hemolytic activity of IpaB and possibly also the *icsB* gene product to achieve liberation from the phagosome (Goebel and Kuhn 2000).

Many cytosolically replicating intracellular bacteria possess the ability to spread from the primary infected cell into its neighbors. This ability depends on the polymerization of cellular actin at one bacterial cell pole mediated by a specific bacterial surface protein (ActA for *L. monocytogenes*; IcsA, IcsB, and OpaB for *Sh. flexneri*). *L. monocytogenes*, for example, move ahead of an actin comet tail at a speed of approximately 0.3 μm/s, upon contact with the host cell plasma membrane, a bacterium-containing protrusion is formed, which is internalized by the adjacent cell. The bacterium then lyses the two plasma membranes surrounding it in the newly infected cell to continue the cycle (see Figure 2) (Cossart and Lecuit 1998). Although *Sh. flexneri* spreads in a similar fashion, the proteins that mediate this phenomenon are unrelated to those of listeria (Bourdet-Sicard et al. 2000). This form of dissemination circum-

Table 1 *Spreading factors that aid bacterial invasion of tissues*

Spreading factor	Producing bacteria	Comments
Hyaluronidase	Streptococci, staphylococci, clostridia	Attacks the interstitial cement of connective tissue by depolymerizing hyaluronic acid
Collagenase	*Clostridium histolyticum*, *Clostridium perfringens*	Breaks down collagen, the framework of muscles, facilitating the development of gas gangrene
Neuraminidase	Intestinal pathogens: *Vibrio cholerae*, *Shigella dysenteriae*	Degrades neuraminic acid (sialic acid), an intercellular cement of the epithelial cells of the intestinal mucosa
Streptokinase and staphylokinase	Streptococci and staphylococci	Kinase enzymes that convert inactive plasminogen to plasmin, which digests fibrin and prevents clotting of the blood. The relative absence of fibrin in a spreading bacterial lesion allows more rapid diffusion of the infectious bacteria throughout the tissues of the host
Enzymes that cause hemolysis and/or leukolysis	Staphylococci (leukocidins), Streptococci (streptolysin)	Insert into membranes forming a pore that results in cell lysis, or enzymatically attack phospholipids, which destabilizes the membrane. They may be referred to as lecithinases or phospholipases, hemolysins (if they are able to lyse red blood cells), or leukocidins and streptolysin (lyse phagocytes and their granules)
Phospholipases	*Clostridium perfringens* (α toxin)	Hydrolyze phospholipids in cell membranes by removal of polar head groups
Lecithinases	*Clostridium perfringens*	Destroy lecithin (phosphatidylcholine) in cell membranes, leading to cell lysis
Hemolysins	Staphylococci (α toxin), Streptococci (streptolysin), Clostridia spp.	Channel-forming proteins or phospholipases or lecithinases that destroy red blood cells and other cells, such as phagocytes through cell lysis
Staphylococcal coagulase	*Staphylococcus aureus*	Cell-associated and diffusible enzyme that converts fibrinogen to fibrin, which causes clotting. Cell-associated coagulase may also provide an antigenic disguise by causing clotted fibrin to deposit on the cell surface. Also in the case of a staphylococcal lesion encased in fibrin, e.g. a boil or pimple, bacterial cells may be resistant to phagocytes or tissue bactericides or even antibiotics, which might be unable to reach their bacterial target
Extracellular digestive enzymes	Heterotrophic bacteria	Including proteases, lipases, glycohydrolases, and nucleases. May aid in invasion either directly or indirectly by damaging the cells of the tissue, or contribute to bacterial nutrition or metabolism
Toxins with short-range effects related to invasion	*Bordetella pertussis* (adenylate cyclase), *Bacillus anthracis*, (edema factor component of anthrax toxin)	Immediate effects on host cells that promote bacterial invasion of host tissues by causing increased levels of cAMP and disruption of cell permeability. These toxins may contribute to invasion through their effects on macrophages or lymphocytes in the vicinity, which are playing an essential role to contain the infection or on the cells of the tissue themselves

cAMP, cyclic adenosine monophosphate.

vents both the humoral and complement-mediated host defenses (see Host resistance to infection).

Obligate intracellular bacteria have taken these measures to the extreme. *Rickettsia prowazekii* (the causative agent of epidemic typhus), for example, is maintained in a cycle involving human and body lice (*Pediculus humanus*) cells, and is transmitted through direct contact of humans with contaminated body louse feces. Following entry into cells via induced phagocytosis, *R. prowazekii* escapes from the phagosome into the cytoplasm. Upon cell lysis, spread can continue to neighboring cells in a similar fashion; however, the related *Rickettsia rickettsii* (causative agent of Rocky Mountain spotted fever) achieves intracellular cell–cell spread powered by actin-based motility similar to that of *Listeria* and *Shigella* (Hackstadt 1998).

Avoidance of host immune defenses

During the processes of colonization and tissue invasion, pathogens must avoid host defenses (see Host resistance to infection). While some bacteria adopt an intracellular lifestyle (see Infiltration of host tissues) to evade defense mechanisms by their internal location, others employ

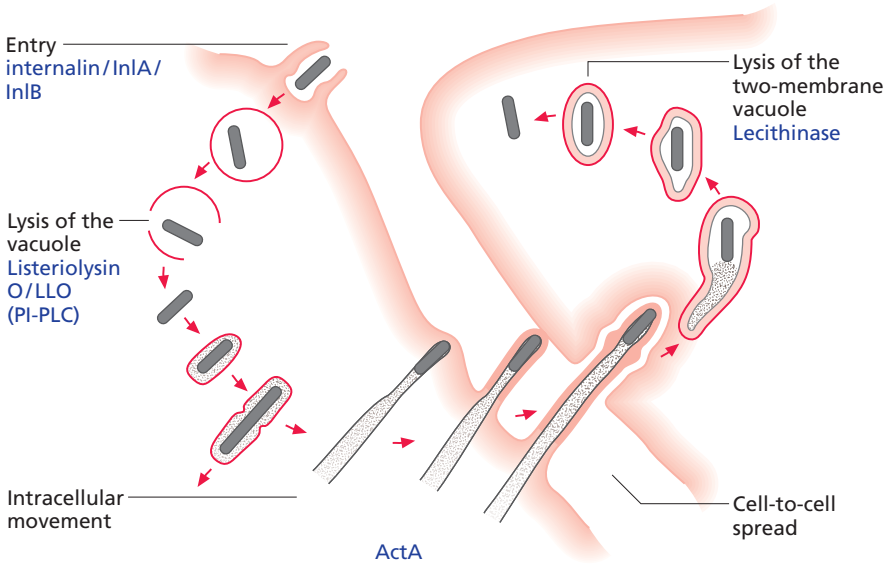

Figure 2 *Schematic representation of the cell infectious process of* Listeria monocytogenes. *Bacterial factors are indicated in blue. (Redrawn from Cossart and Lecuit 1998)*

protective surface coatings, e.g. exopolysaccharides and lipopolysaccharides (LPS) (see Toxins), which can be antiphagocytic and, in the case of surface macromolecules, which mimic those of the host, function as 'camouflage.'

Exopolysaccharides include discrete capsules (e.g. the capsular polysaccharides (sometimes called K-antigens) of *E. coli*, *Klebsiella pneumoniae, Streptococcus pneumoniae*, and *S. aureus*) and the loosely associated slime polysaccharides produced by mucoid bacteria. Exopolysaccharides form highly hydrated water-insoluble gels composed of either homo- or heteropolysaccharides. The former consists of a single sugar monomer (e.g. the levans and dextrans of many oral streptococci), and the latter of repeating oligosaccharide units of more than one monomer. Many exopolysaccharides are acidic due to the possession of carboxyl groups, either from acidic sugars, such as uronic acids or neuraminic acid, or from nonsugar substituents, such as pyruvyl, acetyl, and formyl groups. Their acidic nature is reflected in many of their properties. A few of the bacilli (e.g. *Bacillus anthracis*) have a capsule made up of a single amino acid, poly-D-glutamic acid. In physical chemistry and function, however, it is similar to the polysaccharide capsules.

In the environment, the highly hydrated nature of exopolysaccharides offer bacteria protection from desiccation and a potential mechanism for trapping nutrients, although they are not employed as a reserve carbohydrate food source for use during starvation. Exopolysaccharides do, however, contribute to virulence in two different ways. First, they may contribute to bacterial adhesion. This is particularly obvious in the cariogenic organism, *Streptococcus mutans*. This bacterium can synthesize a dextran-like branched water-insoluble homopolymer of glucose from dietary sucrose. This forms a glutinous layer on the surface of teeth and contributes to the matrix of dental plaque. Second, capsular polysaccharides protect bacteria from phagocytic cells (including amoebae in the environment). This is because capsulate or slime-producing bacteria growing as aggregated microcolonies or biofilms cannot be engulfed for steric reasons, i.e. they are too large to be internalized. Mucoid *P. aeruginosa* strains, which produce the exopolysaccharide alginate in the lungs of infected individuals with cystic fibrosis (CF), provide a good example of this. In CF, lung damage arises in part from the tissue-damaging agents, e.g. elastase released by neutrophils unable to engulf the bacteria. Exopolysaccharides also render bacterial cells more resistant to phagocytic engulfment by increasing surface hydrophilicity, and blocking the action of complement (see LPS and virulence).

Several bacterial pathogens produce exopolysaccharide capsules, the chemical structure of which mimics a host tissue surface antigen. This protects the organism from the immune system by appearing to the host as 'self.' Perhaps the best known examples are the K1 capsule of *E. coli* and the immunologically and structurally identical capsule of *N. meningitidis* group B. These organisms have capsules of α-2,8-linked *N*-acetylneuraminic acid, which is partially *O*-acetylated and immunologically crossreacts with neonatal neural cell adhesion molecules.

Many pathogens have a highly organized proteinaceous surface layer, the S layer. The role of this in pathogenesis has not been fully elucidated, but the S-layer of *Aeromonas salmonicida* can provide resistance to the bactericidal activity of complement, that of *Rickettsia prowazekii* is responsible for humoral and cell-mediated immunity, and that of *Lactobacillus acidophilus* is required for adhesion to the intestinal epithelium (Sara and Sleytr 2000).

Toxins

Toxins are frequently responsible for the tissue damage characteristic of bacterial infections and hence make a significant contribution to pathogenicity. Indeed, for some pathogens, the adminstration of the toxin alone is sufficient to reproduce the same disease as that resulting from infection by the producer organism, e.g. botulism.

PROTEIN EXOTOXINS

Exotoxins are typically proteins secreted or released by bacteria during growth. They are usually produced only by a single bacterial species within a genus. For example, *Clostridium tetani* produces tetanus toxin, whereas *Clostridium botulinum* produces botulinum toxin. Usually, virulent strains of the bacterium produce the toxin while avirulent strains do not, indicating that in many cases the exotoxin is a major determinant of virulence. Bacterial exotoxins are produced by both gram-positive and gram-negative bacteria and have highly specific targets and modes of action. The target may be a component of specific cells, tissues, or organs and usually the site of toxin damage indicates the location of the substrate for that toxin. Terms such as enterotoxin, neurotoxin, leukocidin, and hemolysin are often used to indicate the target sites for protein toxins. Many protein toxins exhibit very specific cytotoxic activities, e.g. tetanus or botulinum toxins attack specific cell types (Table 2). However, other toxins, such as those produced by staphylococci, streptococci, clostridia, etc., have a broad cytotoxicity causing necrotic tissue cell death. Toxins that are phospholipases are particularly nonspecific in their cytotoxicity because they cleave phospholipids (which are components of all host cell membranes), resulting in the death of the cell by leakage of cellular contents. This is also true of pore-forming hemolysins and leukocidins.

A few highly potent protein toxins can kill the host in the absence of the producing organism and are known as lethal toxins. Even though the tissues affected, and the target sites for such toxins (e.g. anthrax toxin) may be known, the precise mechanism by which death occurs is not always understood.

As 'foreign' substances to the host, most protein toxins are strongly antigenic. In vivo, specific antibodies (antitoxins) can neutralize the actions of these bacterial proteins. Also, protein toxins are inherently unstable and can lose their toxic properties while retaining their antigenicity. This was first discovered by Paul Ehrlich who coined the term 'toxoid'. The formation of toxoids can be accelerated by treatment with a variety of chemical reagents, including formaldehyde. The resulting toxoids can be used for immunization against diseases caused by the corresponding pathogen where the primary determinant of bacterial virulence is the toxin.

A + B subunit toxins

Many protein toxins, notably those that act intracellularly, consist of two components termed A and B. Subunit A is responsible for the enzymatic activity of the toxin at the target site. Subunit B facilitates the binding of the toxin to a specific receptor on the host cell membrane and subsequent internalization of the A subunit. The enzymatic component is not active until it is released from the native toxin. Purified A subunits are enzymatically active but lack the ability to bind to host cells. Isolated B subunits may bind to target cells, but they are nontoxic. Toxin subunits may be synthesized and arranged in a variety of ways, for example A-B or A-5B indicates that subunits are synthesized separately and associated by noncovalent bonds, whereas A/B denotes that the subunit domains are formed from a single protein that may be separated only by proteolytic cleavage. The notation A + B indicates that the separate protein subunits interact at the target cell surface to form the toxin. More than one binding subunit is indicated as, 5B for example, which shows that the binding domain is composed of five subunits.

Attachment and entry of toxins

There are at least two mechanisms for toxin entry into target cells. In both cases, a large protein molecule must insert into and cross a membrane lipid bilayer. This activity is reflected by the ability of most A/B native toxins, or their B components, to insert into artificial lipid bilayers, creating ion-permeable channels. For direct entry, the B subunit of the native toxin binds to a specific receptor on the target cell and induces the formation of a pore in the membrane, through which the A subunit is transferred into the cell cytoplasm. The second mechanism relies on the native toxin binding to the target cell and the A + B structure is taken into the cell by the process of receptor-mediated endocytosis. The toxin is internalized in a membrane-enclosed vesicle called an endosome. H^+ ions entering the endosome lowers the internal pH, which causes the A + B subunits to separate. The B subunit effects the release of the A subunit from the endosome by forming a pore, facilitating association with its cellular target in the

Table 2 *Examples of two-component (A-B) exotoxins with intracellular targets*

Exotoxin	Comments
Adenylate cyclase toxin of *Bordetella* spp. Chromosomally encoded	Activated by eukaryotic intracellular calmodulin. Catalyzes conversion of ATP to cAMP, which perturbs cellular activity, inhibiting leukocyte chemotaxis and activity
Anthrax toxin of *Bacillus anthracis* Plasmid-encoded	Comprises three separate proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF) EF + PA causes an increase in intracellular cAMP level, resulting in edema (fluid accumulation) LF + PA is a potent cytolethal toxin causing death of host cells and ultimately death of host
Botulinum toxins of *Clostridium botulinum* Phage-encoded	Comprises seven antigenically distinct toxins designated A to G. Among the most potent of all biological toxins of bacterial origin. The B-subunit binds to neuroreceptor gangliosides on cholinergic neurons, while the A-subunit irreversibly inhibits release of the stimulatory neurotransmitter acetylcholine, at muscle–nerve junctions, resulting in a paralysis and death
Cholera toxin from *Vibrio cholerae* Chromosomally encoded, A-5B structure	The B-subunit binds to GM_1 ganglioside receptors on the surface of small intestinal enterocytes. Reduction of the disulfide bond in the A-subunit activates A_1 fragment that ADP-ribosylates guanosine triphosphate (GTP)-binding protein (G_s) by transferring ADP-ribose from nicotinamide adenine dinucleotide (NAD). The ADP-ribosylated GTP-binding protein activates adenyl cyclase, resulting in an increase in intracellular cAMP, which triggers a profound movement of electrolytes (sodium, potassium, bicarbonate) and water into the lumen of the gut, leading to a profuse life-threatening diarrhea. In addition, the elevated cAMP levels in the gut cells also block the uptake of any further sodium and chloride from the lumen of the small intestine, ultimately resulting in hypovolemic shock and death in the absence of fluid and electrolyte replacement therapy
Diphtheria toxin from *Corynebacterium diphtheriae* Phage-encoded A-B structure	The toxin acts by ADP-ribosylation of intracellular targets, thereby inhibiting cell protein synthesis by catalyzing transfer of ADP-ribose from NAD to elongation factor-2 (EF-2)
Exotoxin A from *Pseudomonas aeruginosa* Chromosomally encoded	Very similar to diphtheria toxin
Heat-labile enterotoxins (LT) from enterotoxigenic *Escherichia coli* (ETEC) Plasmid-encoded A-5B structure	LT comprises two types (LT-I and LT-II). LT-1 is associated predominantly with human disease, while LT-II is produced only by strains isolated from animals. These toxins are very similar to cholera toxin
Pertussis toxin from *Bordetella pertussis* Chromosomally encoded A-5B structure	Different B-subunits where the S2 (B) subunit binds a glycolipid receptor on ciliated respiratory cells while the S3 (B) subunit binds to glycolipids on phagocytes. The S1 or A subunit inhibits cellular signal transduction via ADP-ribosylation of GTP-hydrolyzing protein (G_i), causing unregulated adenylate cyclase and large increases in cellular cAMP levels, resulting in hypersecretion of respiratory secretions, mucus, and paroxysmal cough. It is also known to inhibit leukocyte chemotaxis and activity
Shiga toxin of *Shigella dysenteriae* Chromosomally encoded A-5B structure	This very potent toxin has a similar mode of action to ricin, a toxin derived from castor beans. The B-subunit binds to Gb_3 glycolipid receptor on the surface of target cells. The A-subunit blocks protein synthesis by preventing binding of aminoacyl-transfer RNA through cleavage of the 28S rRNA from 60S ribosomal subunit
Shiga-like toxins of enterohemorrhagic *Escherichia coli* (EHEC) and *Shigella* spp. Phage-encoded A-5B structure	Two forms (SLT-I and SLT-II) in EHEC. SLT-I is identical to *S. dysenteriae* shiga toxin, with the exception of a single amino acid, while SLT-II has approximately 60% homology with shiga toxin. The B-subunit binds to target cell glycolipid globotriaosylceramide. The A-subunit is cleaved and the resulting A_1 fragment binds to 28S rRNA of 60S ribodomal subunit, thereby blocking protein synthesis in the same way as shiga toxin

(*Continued over*)

Table 2 *Examples of two-component (A-B) exotoxins with intracellular targets (Continued)*

Exotoxin	Comments
Tetanus toxin of *Clostridium tetani*Plasmid-encoded	This potent toxin is not secreted but is released upon lysis of the bacterial cell. The binding domain (B) binds to neuroreceptor gangliosides (GD_{1b}). The A-subunit is a zinc-dependent endopeptidase, which is internalized and migrates from peripheral nerves to central nervous system, across the synapses to presynaptic nerve endings this constitutes what is known as retrograde transport, i.e. against the normal direction of nerve impulses. Once at its target location, it accumulates in vesicles and irreversibly blocks the release of inhibitory transmitters. This results in continuous stimulation of muscles by excitatory neurotransmitters, resulting in spastic paralysis (spasms of bulbar and paraspinal muscles) with trismus (lockjaw; spasms of the masticatory muscles), risus sardonicus (spasms of the masseter muscles), and opisthotonos (spasms of back and neck muscles)

ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMP, cyclic adenosine monophosphate; NAD, nicotinamide adenine dinucleotide.

cytoplasm. The B subunit remains in the endosome and is recycled to the cell surface.

Many of the genes encoding toxin production are located on plasmids or on lysogenic bacteriophages. Thus, genetic exchange in bacteria by conjugation and transduction can mobilize these genetic elements between different strains of bacteria and, therefore, may play a role in spreading pathogenic potential to other bacteria; see Table 2 for further examples of two-component exotoxins (Lahiri 2000).

Superantigens (type I toxins)

Conventional antigens are engulfed by antigen-presenting cells (APC), degraded into epitopes, e.g small peptides, and presented to the peptide groove of MHC-II molecules, where they are recognized only by specific T4-helper cells having a receptor (TCR) with a complementary shape. Superantigens (see Table 3), however, bind directly to the outside of the MHC-II molecules and are recognized by T4-lymphocytes. This activation of very large numbers of T4-lymphocytes results in the secretion of excessive amounts of interleukin-2 (IL-2), as well as the activation of self-reactive T-lymphocytes. High levels of IL-2 in the systemic circulation result in fever, nausea, vomiting, diarrhea, and malaise. Excess stimulation of IL-2 secretion can also result in the production of other cytokines such as TNF-α, interleukin-1 (IL-1), IL-8, and platelet-activating factor (PAF) and can lead to the same endothelial damage, acute respiratory distress syndrome, disseminated intravascular coagulation, shock, and multiple organ system failure observed with LPS and other bacterial cell-wall components (see Endotoxins). Activation of self-reactive T-lymphocytes can also lead to autoimmune disease (Llewelyn and Cohen 2002).

ENDOTOXINS

Endotoxins are bacterial cell-envelope components that are not actively secreted by the cell but can be shed as membrane blebs or vesicles into the bloodstream during the course of infection. They are exemplified by the lipooligosaccharides (LOS) and LPS found in the outer membrane of gram-negative bacteria. They are toxic to most mammals and, regardless of the bacterial source, all endotoxins produce the same range of biological effects. The injection of living or killed gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions,

Table 3 *Examples of superantigens*

Superantigen	Associated pathogen	Effect on host
Toxic shock syndrome toxin-1 (TSST-1)	Some strains of *Staphylococcus aureus*	Toxic shock syndrome (TSS): excessive cytokine production leads to fever, rash, and shock
Streptococcal pyrogenic exotoxin (Spe)	Rare invasive strains and scarlet fever strains of *Streptococcus pyogenes* (group A β streptococci)	Toxic shock-like syndrome (TSLS): excessive cytokine production leads to fever, rash, and shock
Staphylococcal enterotoxins (SE)	Many strains of *Staphylococcus aureus*	Food poisoning: excessive IL-2 production results in fever, nausea, vomiting, and diarrhea (vomiting may also be due to these toxins stimulating the vagus nerve in the stomach lining)
Hormone analog (Sta; heat-stable toxin)	Enterotoxigenic *Escherichia coli* (ETEC)	Binds to guanylate cyclase on intestinal epithelial cells, stimulating the overproduction of cGMP and resulting in diarrhea

cGMP, cyclic guanosine monophosphate; IL-2, interleukin-2.

such as fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, and shock. Injection of small doses of endotoxin results in the death of most mammals. The sequence of events follows a regular pattern: (1) latent period; (2) physiological distress (diarrhea, shock); and (3) death. How soon death occurs varies according to the dose of the endotoxin, route of administration, and species of animal. In humans, LPS/LOS levels in plasma and cerebrospinal fluid are associated quantitatively with the production of inflammatory mediators, clinical symptoms, and outcome of disease. Patients with persistent septic shock, multiple organ failure, and severe coagulopathy reveal extraordinarily high levels of LPS in plasma. Mortality related to shock increases from zero to >80 percent with an increase of plasma LPS from 10 to 100 endotoxin units/ml. Although gram-positive bacteria do not produce endotoxins of this type, they can stimulate cytokine release and elicit an inflammatory response identical to that triggered by LPS. Such a response involves cell-wall polymers, such as peptidoglycan and teichoic acid.

Structure and function of LPS/LOS

The gram-negative outer membrane is a permeability barrier that excludes large molecules and hydrophobic compounds and protects the bacterial cell from detergents, such as bile salts. LPS/LOS is located on the outer surface of this membrane and presents an effective barrier to peptidoglycan-degrading enzymes, such as lysozyme, and can confer resistance to bile salts, complement, and phagocytes. As a consequence, LPS plays an important role in host–pathogen interactions. LPS consists of three components or regions, lipid A (region I), an R (rough or core) polysaccharide (region II), and an O polysaccharide (or O-antigen, region III) (Figure 3). LOS characteristically lack the O-antigen.

Region I

The lipid component of LPS/LOS, lipid A, contains the hydrophobic membrane-anchoring region. Lipid A consists of a phosphorylated *N*-acetylglucosamine (NAG) dimer with six or seven fatty acids attached. All fatty acids in lipid A are saturated. Some are attached directly to the NAG dimer, while others are esterified to 3-hydroxy fatty acids. The structure of lipid A is highly conserved among gram-negative bacteria.

Region II

The core (R) antigen or R polysaccharide is attached to the 6 position of NAG. The R antigen consists of a short chain of sugars. For example, KDO–Hep–Hep–Glu–Gal–Glu–GluNAc. Two unusual sugars are usually present in the core region of LPS, heptose and 2-keto-3-deoxy-D-manno-oct-2-ulosonic acid (KDO). KDO is unique and invariably present in LPS and so has been an indicator in assays for endotoxins. With minor variations, the core polysaccharide is common to all members of the same bacterial genus, but it is structurally distinct from other genera. *Salmonella*, *Shigella*, and *Escherichia,* for example, have similar but not identical cores. Loss of the more proximal parts of the core, as in 'deep rough' mutants, makes the strains sensitive to a range of hydrophobic compounds, including antibiotics, detergents, bile salts, and mutagens. This region contains a large number of charged groups and is considered to be important in maintaining the permeability properties of the outer membrane. The LPS of bacteria such as *N. meningitidis, Neisseria gonorrhoeae*, and *H. influenzae* consists only of lipid A and a core polysaccharide and is often referred to as an LOS. The *N. meningitidis* LOS, for example, consists of a symmetrical hexa-acyl lipid A and a short oligosaccharide chain, which can be classified into 11 different 'immunotypes.'

Region III

The O- (or somatic) antigen or O-polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of between three and five sugars. The individual chains vary in length up to 40 repeat units. The O-antigen is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. Substantial variation occurs in the oligosaccharide composition between species and even between strains. At least 20 different sugars are known to be present, and many are unique dideoxyhexoses, which are found naturally only in gram-negative bacterial cell walls. Variations in the sugar content of the O-antigen contribute to the wide variety of antigenic types found in pathogens, such as *Salmonella* spp. and *E. coli*. Specific sugars, confer the immunological specificity of the O antigen and contribute to the 'smoothness' (colony morphology) of the strain. Loss of the O-specific region by mutation results in the strain acquiring a 'rough' colony morphology. Elucidation of the structural organization of LPS has relied heavily on the availability of mutants blocked in specific stages of LPS biosynthesis. Core sugars are added sequentially to lipid A followed by the O side chain, which is linked to the lipid A core polysaccharide as a preassembled unit. Mutants impaired in the assembly of lipid A usually cannot be isolated except as conditional lethal mutants, so this region is essential for cell viability and outer-membrane assembly. For example, *N. meningitidis* mutants deficient in lipid A due to mutation of *lpxA* (which encodes UDP-GlcNAc acyl transferase) require possession of a polysialic acid capsule for growth, since it can apparently substitute for the absent lipid A (Raetz and Whitfield 2002).

LPS and virulence

Lipid A (the toxic component) and the polysaccharide side chains (the nontoxic but immunogenic components) of LPS both contribute to the virulence of gram-negative

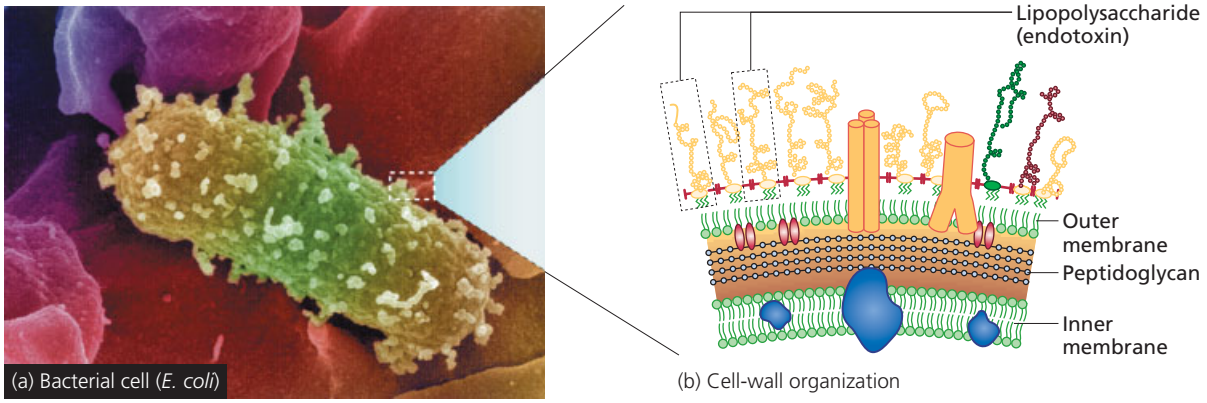

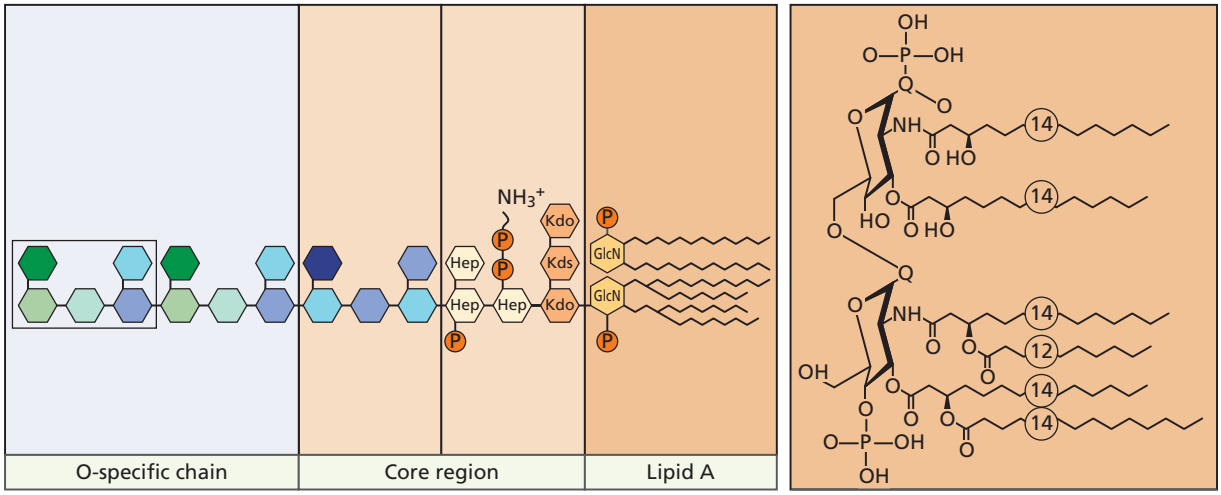

Figure 3 *The structure and localization of lipopolysaccharide (LPS). Electron micrograph of* Escherichia coli **(a)**, *together with a schematic representation of the location of the LPS in the bacterial cell wall* **(b)**, *and the architecture of LPS* **(c)**. *Also shown is the primary structure of the toxic center of LPS, the lipid A component* **(d)**. *GlcN, D-glucosamine; Hep, L-glycero-D-manno-heptose; KDO, 2-keto-3-deoxy-D-manno-oct-2-ulosonic acid; P, phosphate. Redrawn from Beutler and Rietschel 2003.*

bacteria. Loss of the O-antigen increases susceptibility to complement-mediated serum killing. This is because the C5b-9 membrane attack complex (MAC) can much more readily access the bacterial cell membrane of rough rather than smooth bacteria, since in the latter the MAC forms too far from the membrane to damage the cell. Rough strains are also generally more readily phagocytosed than smooth strains. Furthermore, the O-polysaccharide is also the basis of antigenic variation among many gram-negative pathogens, including *E. coli*, *Salmonella*, *Pseudomonas*, and *Vibrio*. The existence of multiple serotypes of the same organism offers opportunities to bypass the acquired immune response to a specific serotype. However, although the O-polysaccharides are highly antigenic, they seldom elicit immune responses that confer full protection to the host against secondary challenge.

The pathophysiological effects of endotoxin are mediated via lipid A. LPS, released into the bloodstream by gram-negative bacterial cells undergoing lysis, is first bound by LPS-binding plasma proteins. These interact with CD14 receptors on monocytes and macrophages and subsequently lead to the induction of inflammation, intravascular coagulation, hemorrhage, and shock through the stimulation of cytokines including IL-1, IL-6, IL-8, TNFα, and platelet-activating factor. These in turn stimulate production of prostaglandins and leukotrienes, which are powerful mediators of inflammation and the septic shock that accompanies endotoxinemia and activate both the complement and coagulation cascades. LPS can also function as a mitogen, stimulating the polyclonal differentiation and multiplication of B cells and the secretion of immunoglobulins, such as IgG and IgM.

Genetic loci involved in LPS biosynthesis

In *E. coli*, where lipid A biosynthesis is best characterized, the genes encoding the enzymes required are scattered around the genome and on the whole are not incorporated into coregulated operons. The genes involved include *lpxABCDHKLMP*. Subsequently, an inner-membrane ABC transporter (encoded by *msbA*) functions to achieve the initial stages of lipid A export. In *Enterobacteriaceae*, the *waa* locus encodes the enzymes required for the sequential assembly of the core oligosaccharide on to the lipid A acceptor. This locus is composed of three operons: (1) the *gmhD* operon directs inner core biosynthesis; (2) the central *waaQ* operon is responsible for the outer core; and (3) the *waaA* operon contains the structural gene for a bifunctional KDO transferase, which is required for the addtion of KDO to the inner core. With few exceptions, the enzymes involved in O-polysaccharide assembly are encoded by genes at the *rfb* locus and are expressed constitutively. There are three currently known pathways for O-polysaccharide biosynthesis, which are distinguished by their respective export mechanisms: (1) dependent on the Wzy-flippase to translocate the assembled polysaccharide across the inner membrane; (2) requiring an ABC transporter to achieve this transport (a pathway confined to unbranched O-polysaccharides); or (3) reliant upon simultaneous extension of the polysaccharide and extrusion across the plasma membrane by an integral membrane synthase (Raetz and Whitfield 2002).

Protein secretion

Many virulence factors are either located on the cell surface or are secreted into the extracellular medium, e.g. adhesins, sensory receptors, and exotoxins. Bacteria have evolved a wide range of mechanisms to translocate proteins across their membranes. It has been estimated that approximately 20 percent of the polypeptides synthesized by a bacterium are destined for a location external to the cytoplasmic membrane, and those in the outer membrane or beyond will be exposed to the host environment.

In gram-negative bacteria, there are three mechanisms for inner membrane translocation: the Sec machinery, the SRP-dependent machinery, and the Tat machinery. The Sec machinery is the most intensively studied, and polypeptides destined for it bear an N-teminal targeting signal, which is cleaved by a peptidase on the periplasmic face of the inner membrane. During Sec-dependent translocation the unfolded polypeptide chain is threaded through a channel (translocon) composed of SecYEG and a number of associated proteins (SecD, SecF, YajC, YidC) powered via SecA by the hydrolysis of ATP (Economou 2002; van Wely et al. 2001). Signal peptide binding to SecA is followed by ATP binding, which induces a profound conformational change in SecA, enabling it to interact with the translocon. ATP is hydrolyzed, reversing the conformational change and directing the release of the polypeptide. Binding of another ATP molecule enables SecA to bind to the polypeptide again, but this time 2.5 kDa nearer its C-terminus. This cycle is repeated many times, shunting the polypeptide through the channel. Some Sec-dependent proteins interact with the cytoplasmic chaperone SecB prior to translocation; others (particularly multiple-membrane-spanning membrane proteins) are recognized by SRP (Economou 1999). The Tat mechanism differs from those described above, since it affects the translocation of folded polypeptides that contain bound cofactors (Palmer and Berks 2003). In common with the Sec and SRP pathways, the Tat system requires an N-terminal targeting signal peptide; however, it differs by the inclusion of two arginines (hence the name twin arginine translocation (TAT)). Beyond this, no specific amino acid consensus has been attributed to signal peptides of any of these mechanisms apart from a general structure composing of a hydrophobic region flanked by a positively charged N-terminal segment and a basic C-terminal sequence contained within an approximately 20 amino acid stretch culminating in the peptidase cleavage site (Pugsley 1993).

Proteins destined for the inner membrane fold within it (Pugsley 1993), whereas those residing in the periplasm are aided in their folding by local chaperones. Outer membrane proteins frequently possess a C-terminal phenylalanine (de Cock et al. 1997), and their localization is usually assisted by periplasmic proteins, e.g. Skp (Buileris et al. 2003; Missiakas et al. 1996). Recently, a highly conserved protein required for outer-membrane protein assembly was shown to be essential in *N. meningitidis* and is likely to play a central role in this process (Voulhoux et al. 2003). In the case of lipoproteins, localization can be achieved by dedicated piloting proteins. Attachment of a lipid is signalled by an LXXC motif contained within the signal peptide adjacent to the peptidase cleavage site. Cleavage results in an N-terminal cysteine residue that is acylated. Inner and outer membrane lipoproteins are then distinguished by the second amino acid of the mature protein. If the second amino acid is not an aspartate, the lipoprotein is recognized by LolCDE and localized on the periplasmic face of the outer membrane (Masuda et al. 2002). LolCDE is an ABC transporter that releases the lipoprotein from the inner membrane, whereupon it interacts with LolA. The LolA–lipoprotein complex crosses the periplasm and interacts with the outer-membrane receptor LolB, which is essential for outer-membrane anchorage (Fukuda et al. 2002).

To secrete proteins across both the inner and outer membrane, gram-negative bacteria utilize one of five main pathways (Figure 4). The type I mechanism

(see Table 4) is well known for its direction of the secretion of the hemolytic toxin HlyA from *E. coli*. This process proceeds in a single step from the cytoplasm using just three proteins. In the inner membrane are the HlyB and HlyD proteins. HlyB contains an ATPase activity and resembles the multidrug resistance protein of eukaryotes. In the outer membrane, and spanning the periplasm, is TolC, which docks into the membrane fusion protein (HlyB) forming a proteinaceous channel through which the unfolded HlyA polypeptide can move directed by its C-terminal targeting signal, which remains uncleaved throughout (Thanassi and Hultgren 2000; Andersen et al. 2001).

Type II secretion (general secretory pathway (GSP)) utilizes two steps. First, proteins use either Sec or Tat to enter the periplasm; then, approximately 14 dedicated GSP proteins form the machinery required to secrete the fully folded protein across the outer membrane. This pathway is used by many bacteria to secrete virulence factors (see Table 5), such as the multisubunit cholera toxin. The targeting signal for the GSP is currently not known, and the machinery is composed of a number of

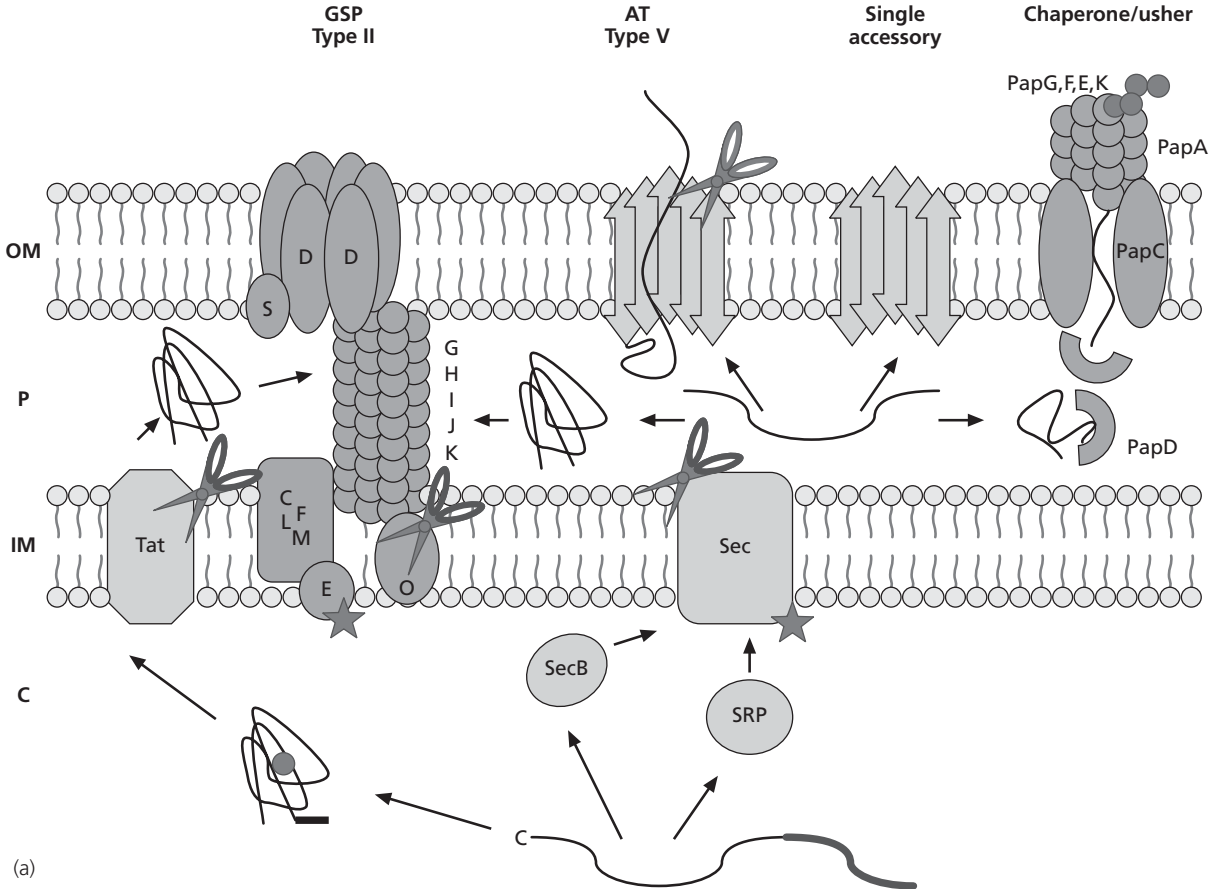

Figure 4 *Schematic representation of bacterial protein secretion machineries.* **(a)** *Depicts those pathways with a distinct step for translocation across the inner membrane. Polypeptides synthesized in the cytoplasm with an N-terminal signal peptide interact with either SecB or SRP and are targeted to the Sec machinery, which translocates them across the cytoplasmic membrane and concomitantly cleaves their signal peptide (as indicated by the scissors). This translocation occurs by threading the unfolded polypeptide across via the action of SecA and is driven by ATP hydrolysis (as indicated by the star). A subset of proteins with an N-terminal signal peptide frequently bearing twin arginines fold around their cofactors and are translocated through the Tat machinery. From the periplasm, a variety of pathways exist to achieve outer-membrane secretion. (1) Autotransported proteins (AT) insert their C-terminal β-barrel domain into the outer membrane to create a channel through which the rest of the polypeptide is threaded. This channel may be formed from multimers or involve Omp85. Cleavage at the cell surface liberates the mature protein, leaving the β-barrel domain attached to the cell. This pathway is also refered to as type V. (2) A similar mechanism is employed by ShlA. However, rather than being contained within a single polypeptide, the transporter domain is a separate protein and thus cleavage is not required (single accessory pathway). (3) Subunits of type IV pili interact with the PapD chaperone, which delivers them to the PapC usher, which in turn directs assembly of the pilus (chaperone/usher pathway). (4) Following folding, some proteins are exported by the general secretory pathway (GSP or type II). This requires 12 or more proteins; the system employed by pullulanase, which is secreted by* Klebsiella oxytoca, *is shown here. The single integral outer-membrane component (GspD) is piloted to its position by the lipoprotein (GspS), together these proteins form a gated channel through which the folded proteins are secreted. This complex is proposed to be linked to the inner-membrane components (GspC, E, F, L, M) by a pilus-like structure composed of GspG, H, I, J, K. The latter are processed by the methylating protease, GspO. GspE is likely to hydrolyze adenosine triphosphate (ATP) and contribute to energizing the system.*

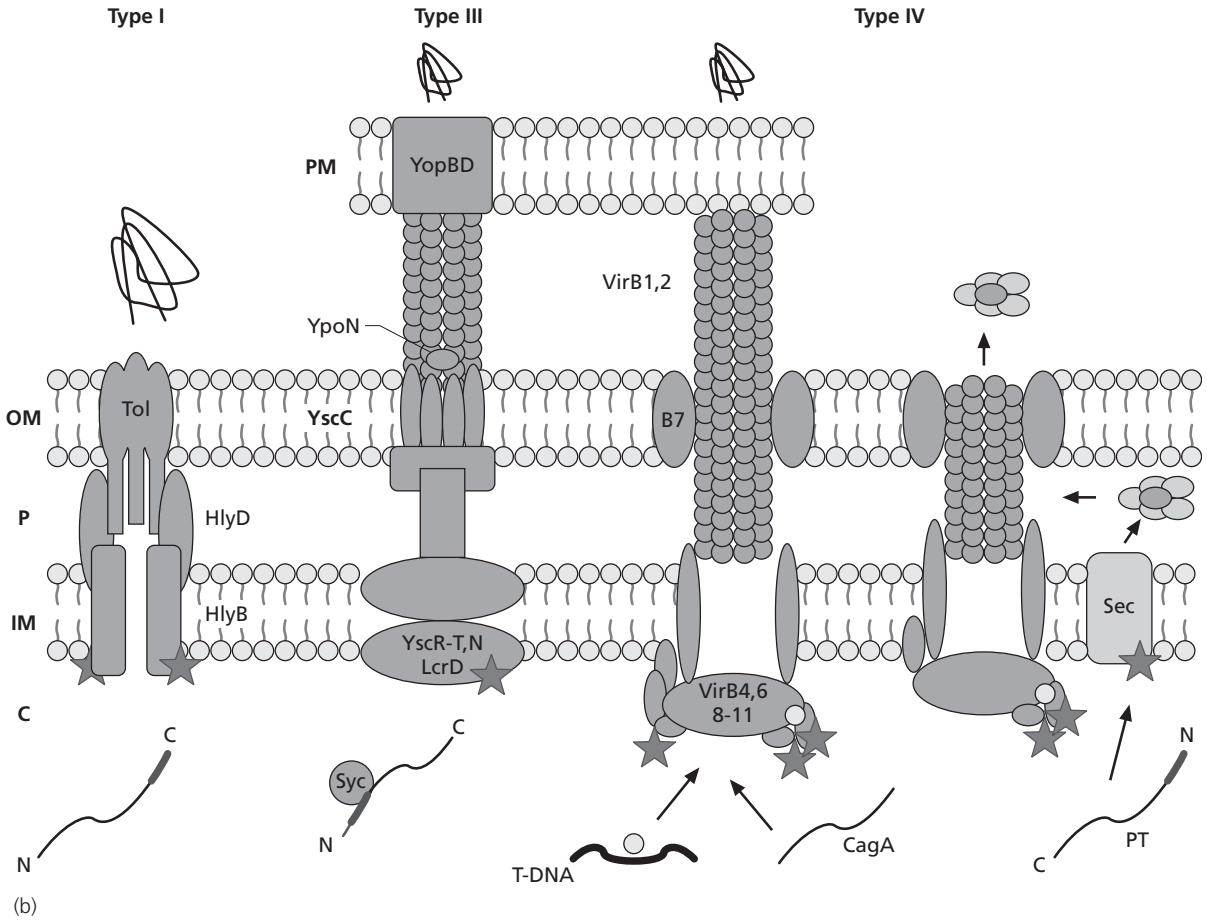

Figure 4 *Schematic representation of bacterial protein secretion machineries (Continued)* **(b)** *A representation of the complex machineries that comprise the type I, III, and IV pathways. Type I-dependent proteins (see Table 5) bear a C-terminal targeting peptide that is not cleaved during translocation of the unfolded polypeptide out of the cell in a single step, which avoids the periplasm. Secretion of the archetypical type I-dependent protein (HlyA from* Escherichia coli*) is achieved through a channel composed of the ATP binding dimer of HlyB, the membrane fusion protein (HlyB), and the outer-membrane protein TolC. The type III machinery uses a needle-like structure that resembles a flagellum to inject proteins directly into eukaryotic cells, and as shown here is used by Yops, which are secreted by* Y. enterocolitica*. Some type III substrates contain two N-terminal signals for targeting, one encoded by the mRNA, and the second by amino acids to which the cytoplasmic Syc chaperones bind. YscR-U and LcrD may form a central secretion apparatus energized by YscN-dependent ATP hydrolysis. The YsdC outer-membrane component resembles the GspD secretin, and the surface-located YopN is thought to act as a channel gate. Translocation of Yops into the target eukaryotic cell may occur via a channel formed in the plasma membrane by YopD and YopD. The type IV secretion pathway can function in alternative ways, depending on the bacterial host. Like the type III pathway, it can achieve direct injection of proteins (e.g. CagA, VirE2) or protein-bound DNA (T-DNA) into eukaryotic cells. Alternatively, proteins that have reached the periplasm via the Sec machinery can assemble and enter the type IV pathway to achieve extracellular localization (pertussis toxin (PT)). The machinery comprises multiple ATPases (VirB4, VirB11) and other inner-membrane structural components (VirB6, VirB8, VirB9, VirB10) plus an outer-membrane component (VirB7) that are linked together by a pilus-like structure composed of VirB1 and VirB2. The relative positions of the bacterial cytoplasm (C), inner membrane (IM), periplasm (P), outer membrane (OM), and the eukaryotic plasma membrane (PM) are indicated.*

inner membrane components, including an ATPase and a set of proteins that resemble pilin subunits (the pseudopilins) and that are proposed to form a retractable pilus, which pushes proteins through the outer membrane pore. The outer-membrane channel is formed by a multimer of a single protein (the GspD secretin) and is likely to be tightly gated (Thanassi and Hultgren 2000).

The type III secretion machinery achieves injection of proteins (see Table 6) with the aid of upwards of 14 components directly into eukaryotic cells (see below), as does the type IV machinery (TFSS). A TFSS is possessed by several pathogens of plants and animals (see Table 7), where the secretion machinery transfers protein substrates intercellularly and appears to be related ancestrally to conjugation systems that have evolved the additional capacity to translocate DNA–protein complexes.

The TFSS machinery comprises 12 components that span the periplasm and membranes through the formation of a pilus, which takes differing forms in different organisms and can directly attach to the eukaryotic

Table 4 *Examples of proteins secreted by type I machinery*

Bacterium	Type I secreted proteins
Escherichia coli	Hemolysin (HlyA)
Serratia marcescens	Metalloprotease
Erwinia chrysanthemi	Metalloprotease
Pasteurella haemolytica	Leukotoxin
Bordetella pertussis	Cyclolysin
Pseudomonas aeruginosa	Metalloprotease
Rhizobium leguminosarum	NodO

target cell (Figure 4) (Christie 2001). The components can be divided into distinct activities: (1) substrate processing (the relaxosome); (2) substrate docking (coupling proteins/chaperones); (3) translocation (channel); and (4) cell–cell attachment (pilus/adhesions). The relaxosome function is not required for those TFSSs that do not transfer DNA since it functions to cleave the DNA strand destined for transfer at the plasmid origin of transfer. The relaxase remains attached to the DNA and may serve to pilot it for translocation. Coupling proteins and chaperones appear necessary for presentation of DNA and also proteins for translocation, and most contain Walker A and B nucleotide-binding motifs suggestive of ATPase activity (the TraG homologues). Other putative ATPases contributing to the function of TFSSs are (1) those showing homology to VirB4, which are proposed to transduce information in the form of ATP-induced conformational changes, across the cytoplasmic membrane to the extracytoplasmic subunits (Dang et al. 1999); and (2) those that form oligomeric structures in the cytoplasm, which are reminiscent of the GroEL family (homologs of VirB11), although it is unclear whether they function for substrate translocation or biogenesis of the translocase. It should be noted that the multisubunit pertussis toxin of *Bordetella pertussis* uses the GSP (Figure 4) to enter the periplasm and, following assembly, is secreted across the outer membrane via the TFSS in contrast to other effectors released via a TFSS, which enter the machinery from the bacterial cytoplasm (Thanassi and Hultgren 2000; Christie 2001).

In common with the type I secretion pathway, the type III secretion process is Sec-independent; proteins

Table 5 *Examples of proteins secreted by type II machinery*

Bacterium	Type II secreted proteins
Klebsiella oxytoca	Pullulanase
Vibrio cholerae	Cholera toxin, protease
Aeromonas hydrophila	Aerolysin toxin, protease, acyltransferase
Erwinia chrysanthemi	Pectate lyases, cellulase
Erwinia carotovora	Pectate lyases, cellulase
Pseudomonas aeruginosa	Exotoxin A, lipases, proteases
Xanthomonus campestris	Cellulase, protease

Table 6 *Examples of proteins secreted by type III machinery*

Bacterium	Type III secreted proteins
Yersinia spp.	Yops
Salmonella typhimurium	Sips, Sops, SptP
Shigella flexneri	Ipas
Pseudomonas aeruginosa	ExoS, ExoT
Enteropathogenic/ Enterohemorrhagic *Escherichia coli*	EspA, EspB (EaeB), EspD, EspF, EspG, Map, EspE (Tir)
Phytopathogens (*Erwinia* spp., *Pseudomonas syringae*, *Xanthomonas campestris*)	Harpins, PopA, AvrB

secreted by it do not accumulate in the periplasm, and nor are they subjected to N-terminal processing. However, the protein signal sequence for type III secretion is considered to reside within the N-terminal of the secreted proteins, since this region appears necessary for secretion and can direct the secretion of hybrid fusion proteins. Exhaustive mutational analysis has revealed a high degree of tolerance for sequence changes within the N-terminus of some secreted proteins, without loss of secretion. Moreover, the N-terminal sequences of proteins secreted via the type III pathway share no recognizable structural similarities that may constitute a common secretion signal. Therefore, it has recently been proposed that the secretion signal may actually reside in the 5′ region of the mRNA that encodes the secreted proteins (Anderson and Schneewind 1997).

The process of protein secretion via this system requires the involvement of small cytoplasmic proteins termed chaperones, which protect the secreted proteins from premature interaction with components of the secretion machinery. Apart from a cytoplasmic, membrane-associated ATPase, and an outer-membrane

Table 7 *Examples of type four secretion system (TFSS) and the effector proteins secreted by them*

Bacterium/plasmid	TFSS effector
Bordetella pertusis	Pertussis toxin
Helicobacter pylori	CagA
Brucella suis	Unknown, probably protein
Brucella abortus	Unknown, probably protein
Bartonella henselae	Unknown, probably protein
Campylobacter jejuni	Unknown, probably protein
Rhizobium etli	Unknown, probably protein
Rickettsia prowazekii	Unknown, probably protein
Wolbachia	Unknown, probably protein
Agrobacterium tumefaciens	T-DNA
pKM101	DNA (conjugal transfer)
R388	DNA (conjugal transfer)
RP4	DNA (conjugal transfer)
F	DNA (conjugal transfer)
Legionella pneumophila	DNA (conjugal transfer)

component that is homologous to the secretin of the type II secretion pathway, most of the components are located in the inner membrane. Interestingly, many of the components of the type III secretion apparatus show homology with those of flagella. In accordance with this, some type III secretion systems assemble supermolecular structures on the bacterial surface (Ginocchio et al. 1994). In contrast to type I secretion, where the secreted enzymes are active in the extracellular space, type III secretion systems appear to be dedicated to the translocation of pathogenicity proteins directly into the cytosol of eukaryotic cells and in some cases appear to be regulated by contact with the surface of the target eukaryotic cell (Charkowski et al. 1997). Type III secretion systems contribute to the virulence of diverse gram-negative pathogens (Table 6). Many of the type III secreted proteins interact directly with host cell components to alter host cell signal transduction, once inside the eukaryotic cytosol (see Subversion of host cell signal transduction).

The type V machinery is perhaps the simplest, since secretion appears to be determined within the primary sequence of the secreted protein, and hence it is often described as autotransportation. This is described later in Evolution of pathogens within the host; however, variations exist whereby the outer-membrane transporter domain is separated from the transported protein (e.g. the hemolysin secreted by *Serratia marcescens*) (Yang and Braun 2000). Finally, another terminal branch of the GSP exists, which is utilized for the formation of Pap pili. This pathway is referred to as the chaperone/usher pathway and involves the interaction of pilus subunits with the PapD periplasmic chaperone, which facilitates their proper folding whilst preventing premature aggregation and guiding them to the PapC usher in the outer membrane, whereupon they assemble into a pilus (Stathopoulos et al. 2000; Thanassi et al. 1998).

Subversion of host cell signal transduction

Many of the proteins injected directly into eukaryotic cells by the type III secretion pathway interfere with signal transduction. For example, through YopH-mediated dephosphorylation of several macrophage proteins whose transient tyrosine phophorylation is required for normal phagocytosis, pathogenic *Yersinia* spp. resist the host's primary immune defenses by preventing their uptake into professional phagocytes. Other effectors are also injected into eukaryotic cells by *Yersinia* spp. and contribute to their cytotoxicity, e.g. by altering the cytoskeleton. In contrast to pathogenic *Yersinia* spp., *Shigella* spp. occupy predominantly intracellular locations, and although the pathogenic strategies and disease caused by these organisms differ entirely, both pathogens use type III secretion systems as a key virulence mechanism. In vivo invasiveness, as well as the induction of membrane ruffles on cultured epithelial cells, depends on the *Shigella* type III secretion system, which delivers four proteins (IpaA, IpaB, IpaC, and IpaD) into eukaryotic cells. Together, these cause protein tyrosine phosphorylation of a number of cytoskeletal proteins, leading to the localized accumulation of a variety of cytoskeletal (F-actin, -vinculin, and -actinin) and signal transduction molecules (the protein tyrosine kinase $pp60^{c\text{-}src}$ and the GTP-binding protein rho) at the site of bacterial attachment. This results in tyrosine phosphorylation of paxillin, cortactin, and the focal adhesion kinase $pp125^{FAK}$. Shortly after internalization, the bacteria lyse the phagocytic membrane and gain access to the cytoplasm and, in macrophages, induce apoptosis. In addition, attraction of neutrophils to the site of bacterial infection occurs (Hueck 1998). Interestingly, salmonellae contain two type III secretion systems, which are encoded by two distinct gene clusters termed SPI-1 and SPI-2 (for *Salmonella* pathogenicity island). These two type III secretion systems appear to play different roles during pathogenesis, with SPI-1 being required for initial penetration of the intestinal mucosa and SPI-2 being necessary for subsequent systemic stages of infection. The effectors delivered by SPI-1 (SopE, SopE2, SopB, SipA, SipC, SptP) induce reversible actin cytoskeletal rearrangements by stimulating the host signal transduction and directing modulation of actin dynamics (Zhou and Galán 2001). The effectors translocated by SPI-2 interfere with vesicular trafficking allowing survival within the eukaryotic cell (see Infiltration of host tissues) (Fang and Vazquez-Torres 2002). The opportunistic pathogen *P. aeruginosa* also employs type III secreted proteins to inflict epithelial cell damage and affect dissemination within infected hosts. Although their precise contribution to pathogenesis is unclear, two exotoxins with ADP-ribosyltransferase activity (exoenzyme S, ExoS; exoenzyme T, ExoT) secreted via a type III secretion pathway by *P. aeruginosa* target members of the H-Ras and K-Ras family of GTP-binding proteins and vimentin (an intermediate filament protein) (Hueck 1998).

Another example of a bacterium that effectively subverts eukaryotic cell signaling is enteropathogenic *E. coli* (EPEC). EPEC belong to a growing family of diarrheagenic *E. coli* strains that cause a characteristic attaching and effacing lesion on infected small-intestine enterocytes and cells grown in culture. This lesion consists of gross cytoskeletal damage to the brush-border microvilli, the deposition of filamentous actin beneath attached EPEC, and the formation of pedestal-like structures. In addition, many classical *EPEC* strains are characterized by their clustered or localized pattern of adherence to epithelial cells, which results in the formation of microcolonies on the cell surface. Intimate attachment, effacing of microvilli, and formation of

pedestals require a bacterial adhesin (called intimin) and EPEC type III secretion. Although intimin is not secreted by the type III secretion pathway, the encoding gene (*eaeA*) is located within the gene cluster that encodes the EPEC type III secretion system, and intimin functions synergistically with type III secretion in pedestal and A/E lesion formation. Intimin specifically binds to translocated intimin receptor (Tir), which is secreted by the type III secretion apparatus. Three other type III secreted proteins, EspA, EspB, and EspD, are required for membrane insertion of Tir. Concomitant with pedestal formation, adherent EPEC strains induce tyrosine phosphorylation of several proteins in the eukaryotic cell, including Tir and phospholipase C-1. While Tir phosphorylation has been thought to facilitate intimin binding, activation of phospholipase C-1 induces inositol triphosphate and Ca^{2+} fluxes, which might be involved in the cytoskeletal rearrangements involved in pedestal formation. Another protein secreted by the type III machinery of EPEC is the mitochondrial associated protein (Map), which targets the mitochondria and disrupts their membrane potential. In addition, via activation of the host GTPase (Cdc42), Map stimulates nonpedestral-related cytoskeletal rearrangement, e.g. filopodia formation (Hueck 1998; Kenny 2002; Vallance and Finlay 2000; Campellone and Leong 2003) (Figure 5).

BACTERIAL SENSORY NETWORKS

Microorganisms inhabit diverse ecological niches where survival will depend on their capacity to sense the local environmental conditions and adapt by regulating the expression of specific genes and operons. Consequently, bacteria have developed multiple, highly sophisticated mechanisms to gather, process, and transduce environmental information, such as pH, temperature, nutrient availability, osmolarity, and cell population density. Pathogenic bacteria are therefore likely to encounter countless different environmental situations as they move through the host or between the host and external environment, and need to continually monitor diverse physical and chemical signals and integrate their responses. Such sensory transduction mechanisms operate at the transcriptional, post-transcriptional, and translational levels and bacteria employ multiple interconnected regulatory circuits to coordinate their responses. The 6.3-MB genome of *P. aeruginosa*, for example, contains around 5 500 genes, some 10 percent of which encode regulatory proteins. These enable the bacterium to express or repress genes as the conditions dictate. For example, if iron is plentiful, the energetically expensive synthesis of siderophores and their associated export and import pathways can be switched off. For bacteria such as *V. cholerae*, switching between an aquatic lifestyle and that required for survival within a human host has highlighted an inverse correlation between motility and virulence gene expression.

Pathogenic bacteria will therefore employ many different signal transduction mechanisms, some of which are required for 'house-keeping,' since they are required for replication, cell division, and the maintenance of cell viability. Others control the synthesis of the colonization factors, toxins, and tissue-degrading enzymes that characterize the organism as a pathogen and that may be required only in certain host compartments.

Given the multistage adaptive process leading to the establishment of infection, bacteria require multiple virulence factors that can be deployed at the appropriate time and place. For example, during the early stages of infection, bacteria express cell-surface adhesins to facilitate attachment to eukaryotic cell surfaces and extracellular matrices. If the infection becomes systemic, the expression of exotoxins and host-avoidance factors is upregulated, with the concomitant downregulation of colonization factors. This is important because virulence factors that contribute to infection in one host compartment may render the pathogen susceptible to host defenses in another. In this section, three main sensory pathways will be considered: iron-dependent repression, two-component signal transduction, and quorum sensing. Although treated as separate environmental parameters, it is important to understand that each is merely one parameter amongst many that a bacterial cell must integrate in order to determine subsequent behavior (Figure 6) (Withers et al. 2001).

Iron-dependent repression

To grow in vivo, bacterial pathogens deploy high-affinity iron-scavenging systems, which depend on either siderophores or surface receptors for host iron and heme-containing proteins (see Iron scavenging). The genes involved in the biosynthesis of siderophores and their transport in and out of the bacterial cell, as well as those encoding for surface receptors, are coordinately negatively regulated in response to the cellular iron status. This is because they are energetically expensive systems that confer an advantage for the bacterium only in environments where iron is not readily available. In *E. coli,* the 17-kDa Fur repressor protein controls transcription from iron-responsive promoters in an iron-dependent manner, i.e. to be active, Fur requires ferrous iron as a corepressor. When iron is plentiful, the Fur–Fe^{2+} complex interacts with an operator sequence called the *fur* box and prevents gene expression. During iron starvation, Fur is stripped of iron by the cell and is no longer able to function as a repressor, thus permitting expression of the iron-repressed structural genes. Homologs of Fur have been described in many different gram-negative pathogens including *Yersinia pestis*, *V. cholerae*,

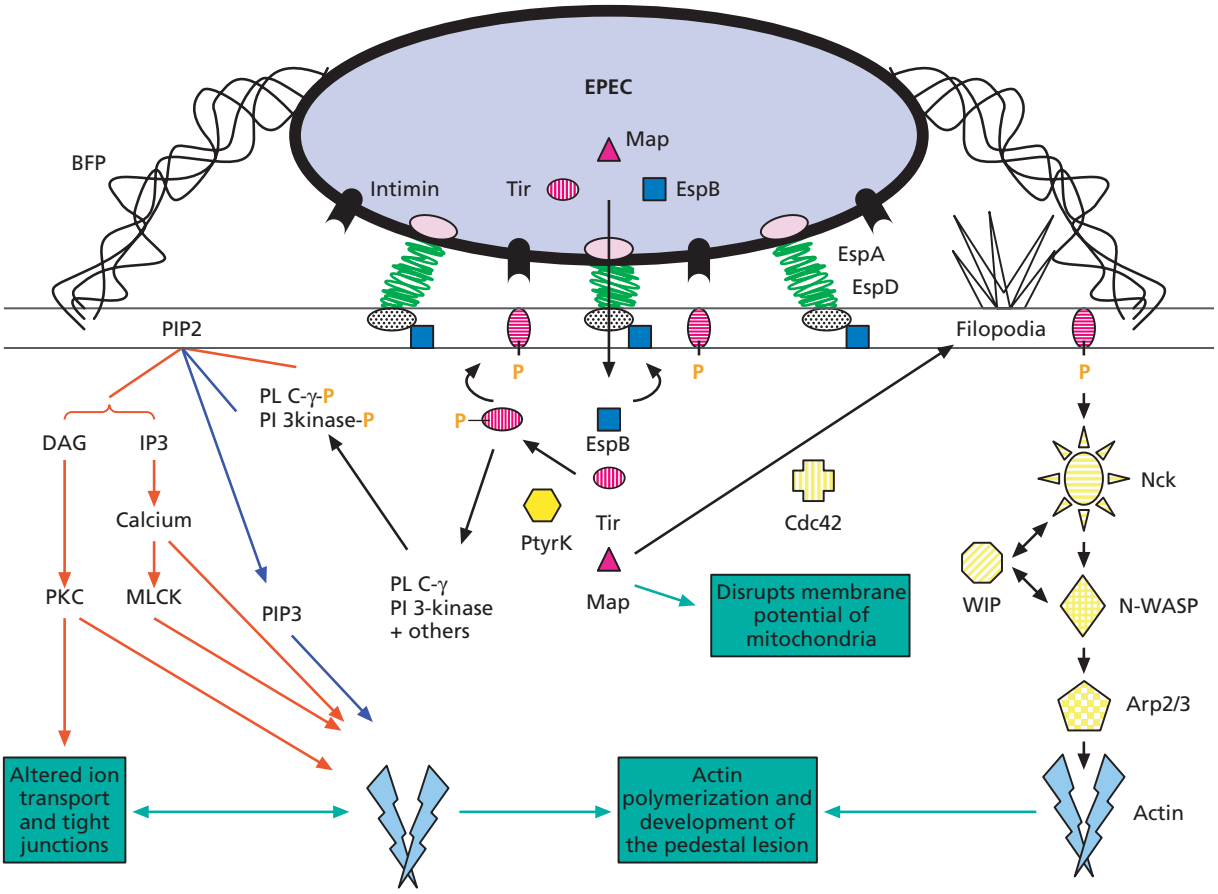

Figure 5 *Subversion of eukaryotic cell signaling cascades by enteropathogenic* Escherichia coli *(EPEC). Bundle-forming pili (BFP) allow initial aderence of EPEC to the eukaryotic cell surface. The type III secretion syringe composed of EspA (green wavy lines) allows injection of EspB (blue square) and EspD (black chequered oval), which form the translocon in the plasma membrane. Other injected proteins include Map (pink triangle) and Tir (red and white striped oval). Map, by direct or indirect mechanisms, activates the small GTPase protein Cdc42, converting it to its GTP-bound membrane-associating form that drives actin polymerization and filopodia formation. It is postulated that Cdc42 activation also leads to the modulation of other undefined cellular processes, again by direct and/or indirect mechanisms. Map is also targeted to mitochondria, where it disrupts their membrane potential. Concurrently, Tir is proposed to undergo host- (PtyrK) and bacteria-dependent modification, leading to its insertion into the plasma membrane. The fully modified membrane-inserted Tir molecule interacts with intimin to trigger signaling events, leading to pedestal formation and other signaling events, such as phosphorylation of phospholipase C (PLC-1) and phosphoinositol 3 kinase (PI3-kinase). Changes in inositol phosphate fluxes lead to calcium release, which may in turn affect actin polymerization. Membrane-embedded tyrosine-phosphorylated Tir binds the adaptor protein Nick, which recruits N-WASP or WIP–N-WASP, complex to trigger activation of Arp2/3 complexes. This leads to actin assembly and pedestal formation with Nick, WIP, N-WASP, and Aro2/3 in its tip, myosin II and tropomyosin in the base, and F-actin, α-actinin, Talin, Exrin, and Villin distributed throughout. DAG, diacylglycerol; MLCK, myosin-like chain kinase; PKC, protein kinase C. See also Kenny (2002) Campellone and Leong (2003), Vallance and Finlay (2000), and www.biotech.ubc.ca/faculty/finlay/EPEC.htm for animations and further illustrations.*

P. aeruginosa, *N. meningitidis*, and *H. influenzae*. In addition to regulating genes associated with iron acquisition, Fur also regulates expression of superoxide dismutases and exotoxins, such as shiga toxin, hemolysin, and exotoxin A. Gram-positive pathogens such as *Corynebacterium diphtheriae*, *M. tuberculosis*, and the staphylococci also possess iron-responsive regulatory proteins related to Fur. In *C. diphtheriae*, for example, DtxR regulates production of siderophores, a heme oxygenase (which enables the organism to strip the ferric iron from heme) as well as diphtheria toxin synthesis. Thus, in addition to regulating high-affinity iron-transport mechanisms, iron-dependent repressors also regulate the expression of virulence genes unrelated to iron metabolism. However, defining their contribution to pathogenicity is not straightforward, since the constitutive expression of target structural genes may either enhance or reduce the fitness of the pathogen. For example, the *S. enterica* serovar Typhimurium *fur* mutant remains virulent in an experimental animal infection model, while many *E. coli* enteropathogens exhibit attenuated virulence when *fur* is inactivated. In other pathogens, e.g. *P. aeruginosa*, attempts to mutate the *fur* gene have not been successful, indicating that in these bacteria, Fur fulfils an essential function (Hantke 2001).

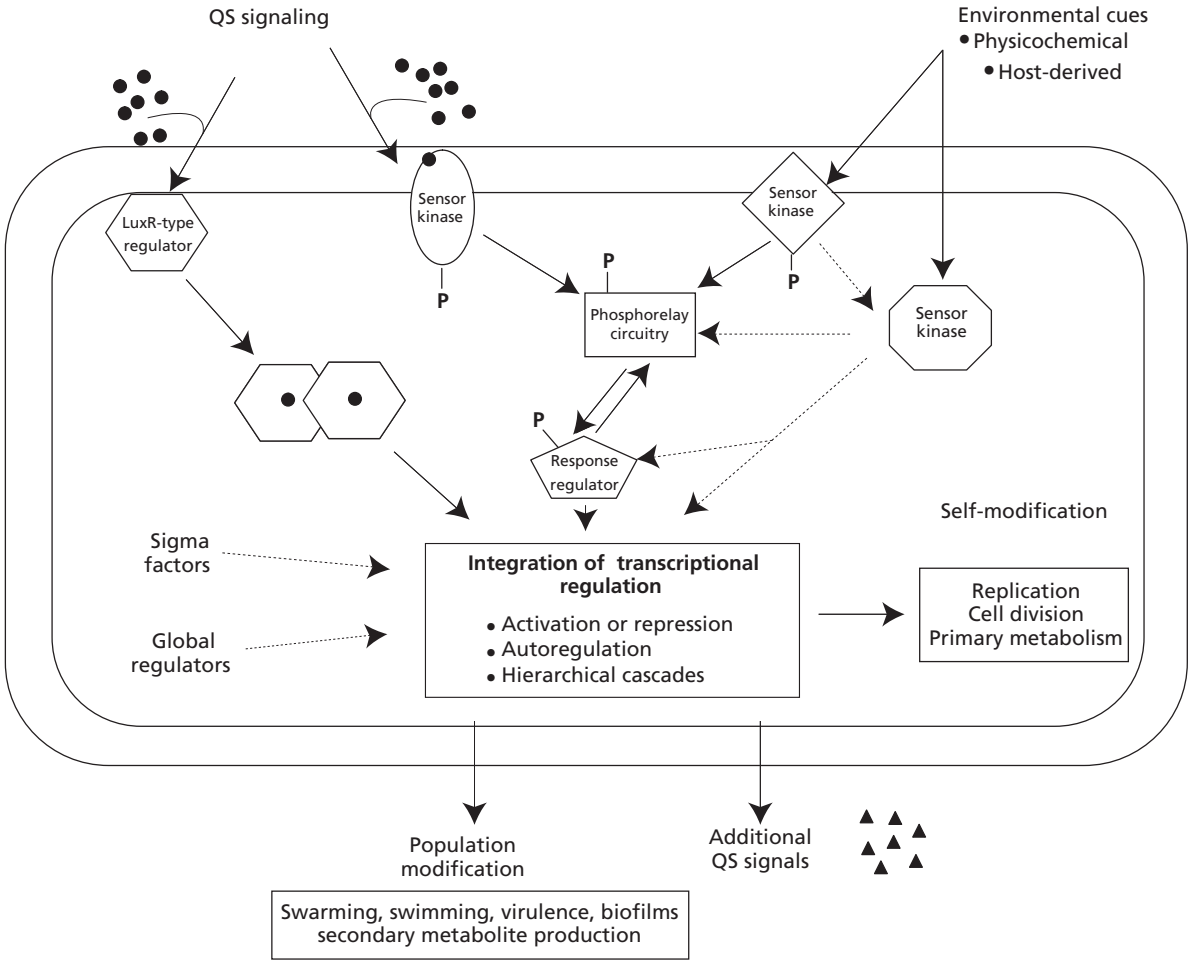

Figure 6 *Integration of quorum-sensing and environmental signals. Quorum sensing is one parameter amongst many that the cell integrates in order to determine subsequent behavior. Quorum-sensing signal molecules and environmental signals are internalized or detected at the cell envelope and initiate signal transduction, leading to gene expression changes. This diverse array of signaling cascades may act in parallel or be organized in a hierarchical manner to create a 'neural' regulatory network. Amended from Withers et al. (2001).*

Two-component signal transduction systems

Bacterial two-component signal transduction systems (TCSTS) are ubiquitous throughout the bacterial kingdom, are abundant within individual bacterial genomes, and have been adapted to regulate an enormous number of genes, including many virulence determinants (Table 8) (Stephenson and Hoch 2002; Dziejman and Mekalanos 1994; Hoch and Silhavy 1995). They consist of two regulatory proteins, a cytoplasmic membrane-associated sensor that samples the environment for a specific signal and a response regulator that mediates the internal response of the bacterial cell. Sensor and response regulator proteins exchange information by transferring a phosphate group from the sensor to the response regulator through conserved domains within the two proteins (Figure 7). In classical TCSTS, perception of an external signal results in the phosphorylation of a histidine in the sensor (a histidine protein kinase). The phosphate group is subsequently transferred to a conserved aspartate within the receiver domain of the response regulator. Most response regulators are transcriptional activators or repressors (or both) that consist of two domains, an N-terminal receiver domain and a C-terminal DNA-binding domain.

TCSTS sense a variety of different factors including oxygen (Aer, *E. coli*), hydrogen (Hox, *Ralstonia eutropa*), quorum-sensing signal molecules (e.g. LuxQO, *V. cholerae*), Mg^{2+} (e.g. PhoPQ, *Salmonella*), and phosphate limitation (e.g. PhoBR, *E. coli*; PhoPR, *Bacillus subtilis*). The number of TCSTS produced by a particular bacterium appears to reflect the range of different environments it encounters during its lifecycle. For example, *H. pylori*, which uniquely inhabits the human stomach, has only three TCSTS pairs, whilst *P. aeruginosa*, which is found in a multitude of different environments, has an extraordinary number of putative two-

Table 8 *Examples of two-component signal transduction systems and their functions in selected pathogens. For more extensive information, see www.genome.ad.jp/kegg/ortholog/tab02020.html and www.uni-kl.de/FB-Biologie/AG-Hakenbeck/Tgrebe/HPK/Table3.html*

Process	Kinase	Regulator	Organism
Virulence/pathogenicity			
Virulence	BvgS	BvgA	*Bordetella*
Virulence	PhoQ	PhoP	*Salmonella*
Extracellular δ-toxin	AgrC	AgrA	*Staphylococcus*
Extracellular toxins	VirS	VirR	*Clostridium*
Alginate	AlgZ	AlgR	*Pseudomonas*
	kinB	AlgB	*Pseudomonas*
Autoinduction			
Competence	ComD	ComE	*Streptococcus*
	ComP	ComA	*Bacillus*
Quorum sensing	LuxQ	LuxO	*Vibrio cholerae*
Vancomycin resistance	VanS	VanR	*Enterococcus*
Catabolism	DegS	DegU	*Bacillus*
Chemotaxis	CheA	CheY	Enterobacteria, *Escherichia coli*, *Helicobacter pylori*, *Listeria*, *Pseudomonas*, *Salmonella*, *Treponema*
Capsule synthesis	RcsC	RcsB	Enterobacteria
Sporulation	Kin A,B,C	SpoOF	*Bacillus*
Nitrogen reduction	NarX	NarL	Enterobacteria
Nitrogen assimilation	NtrB	NtrC	*Salmonella*, *Vibrio*
Phosphate metabolism	CreC	CreB	Enterobacteria
	PhoR	PhoB	Enterobacteria
Citrate fermentation	CitA	CitB	Enterobacteria
	DpiA	DpiB	
Adaptation			
Anaerobic repression	ArcB	ArcB_RR	*E. coli*
Osmosensing	EnvZ	OmpR	*E. coli*, *Salmonella*
Turgor sensing (K+-transport)	KdpD	KdpE	*E. coli*
Cell-wall integrity	CiaH	CiaR	*Streptococcus*
	PmrB	PmrA	*Streptococcus*
Transport			
Hexose-phosphate	UhpB	UhpA	*E. coli*
Phosphoglycerate	PgtB	PgtA	*Salmonella*

component regulators, with 55 sensor proteins, 89 response regulators, and 14 sensor/response regulator hybrids (www.pseudomonas.com/suppdata.asp). The role performed by TCSTS also varies; for example, only three of the 16 TCSTS pairs contained within the genome of *S. aureus* appear to be essential (Throup et al. 2001). TCSTS can perform defined roles, e.g. LuxQO regulates the production of bioluminescence by *Vibrio harveyi*, or they can have a wide-ranging effect, e.g. the PhoPQ system of *Salmonella* governs the expression of at least 40 proteins including: (1) those mediating adaptation to Mg^{2+}-limiting environments; (2) virulence factors; and (3) components of the bacterial cell envelope. Indeed, *Salmonella* strains harboring null alleles of the *phoP* or *phoQ* gene are highly attenuated for virulence. This may be due to an inability to determine that cells have attained a subcellular location through the monitoring of the environmental Mg^{2+} concentration, although PhoPQ can control virulence even in pathogens that do not have an intracellular lifestyle (Groisman 2001).

The BvgAS TCSTS is another example of one that has a major influence on virulence since it controls the expression of nearly all the known virulence genes in bordetella that cause respiratory infections in mammals. BvgAS functions like a molecular switch, mediating a biphasic transition between the Bvg^+ and Bvg^- phases in response to environmental cues, which are currently unknown. The Bvg^+ phase is necessary and sufficient for respiratory infection by *Bordetella bronchiseptica*, whilst the Bvg^- phase is adapted for survival under conditions of extreme nutrient deprivation (Stockbauer et al. 2001).

E. coli contains 36 TCSTS and among these are those that mediate chemotaxis, which are typically transmembrane homodimers composed of an external ligand-binding domain to monitor attractant compounds (such as serine or aspartate), four transmembrane helices, and internal methyl-accepting and signaling domains. These

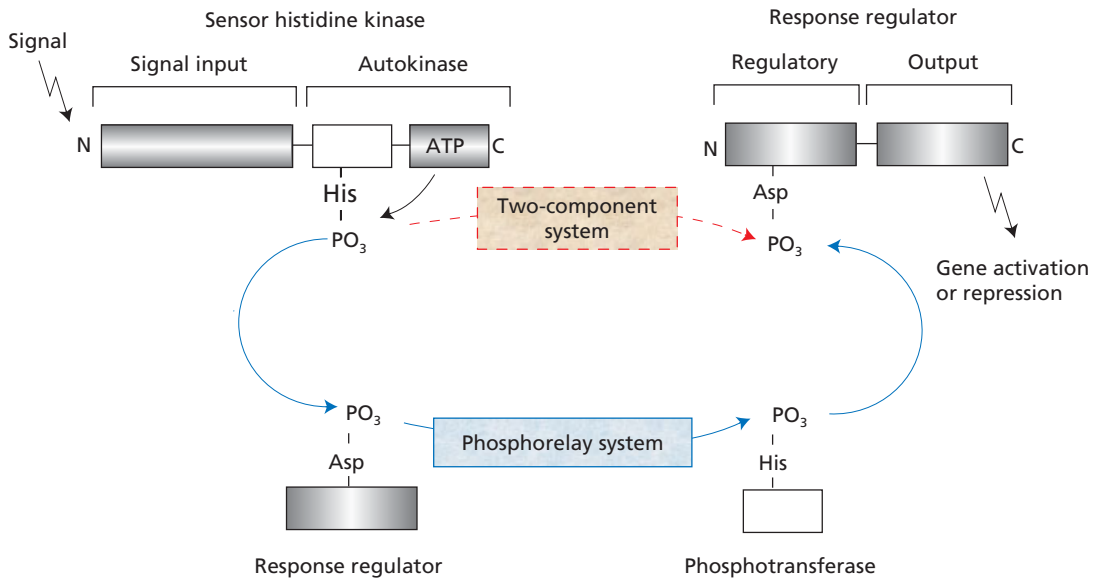

Figure 7 *Cartoon demonstrating the mechanism of two-component signal and phosphorelay signal-transduction systems. Signal ligands stimulate autophosphorylation of the sensor kinase on a histidine residue. In a two-component system (red), the phosphoryl group is subsequently transferred to an aspartate residue of a two-domain response regulator to activate its transcriptional properties. In a phosphorelay (blue), the phosphoryl group is first transferred to an aspartate of a single-domain response regulator that lacks a DNA-binding domain, and then to a histidine residue of a phosphotransferase that subsequently serves as the phosphoryl donor to activate the transcriptional properties of the response regulator. Redrawn from Stephenson and Hoch 2002.*

chemoreceptors (methyl-accepting chemotaxis proteins (MCP)) form higher order aggregates with other signaling proteins at the cell poles and control the phosphorylation state of the histidine protein kinase, CheA, via the linker, CheW, and the two response regulators, CheY and CheB. Phospho-CheY binds to FliM in the flagellar motor to augment clockwise rotation (Armitage et al. 2003; Parkinson 2003).

A family of proteins distinct from TCSTS, but also involved in signal transduction, are the hybrid sensor-regulators related to ToxR and TcpP, which regulate the genes encoding CT and TCP (see Examples of bacterial adhesins and their receptors) of *V. cholerae*. These unusual transcription regulators are localized to the cytoplasmic membrane and also regulate gene expression by binding to DNA and activating transcription. They share a bitopic arrangement with a cytoplasmic amino terminus and a periplasmic carboxy-terminus separated by a short transmembrane stretch of hydrophobic amino acids, and often work in conjunction with an effector protein that is localized on the periplasmic face of the inner membrane, e.g. ToxR interacts with ToxS and TcpP with TcpH. The cytoplasmic DNA-binding domains of ToxR and TcpP bear homology with the transcriptional regulator OmpR and consist of a helix–turn–helix (HTH) domain and two β-strands separated by a loop. Interaction of the loop between the two helices of the HTH domain with the carboxy-terminal portion of the α-subunit of RNA polymerase activates transcription. ToxR and TcpP act together to regulate CT and TCP expression via activation of the *toxT* gene. It should be noted that other factors also influence the production of CT and TCP and that ToxR and TcpP can also regulate other genes in *V. cholerae*, e.g. biofilm formation (Crawford et al. 2003).

Quorum sensing: bacterial cell-to-cell communication

A crucial feature of many bacterial infections is a requirement for the pathogen to attain a cell population density sufficient to overcome host defenses and establish an infection. A mechanism for coordinating the control of multiple virulence genes as a function of population density offers a survival advantage to the pathogen, such that the host is overwhelmed before a defense response can be initiated fully. In this context, the ability of bacterial cells to communicate with each other offers the pathogen a survival advantage by orchestrating collective attack against the host immune system.

Many different gram-negative and gram-positive pathogens are now known to communicate using specific extracellular signal molecules, which facilitate the coordination of virulence and survival gene expression. The term ‘quorum sensing’ is commonly used to describe the phenomenon whereby the accumulation of a diffusible, low-molecular-weight signal molecule (sometimes called an ‘autoinducer’) enables individual bacterial cells to sense when the minimal number, or ‘quorum,’ of bacteria has been achieved for a concerted response to

be initiated (for reviews see Swift et al. 2001; Camara et al. 2002; Williams et al. 2000). The accumulation of a diffusible signal molecule also indicates the presence of a diffusion barrier, which ensures that more molecules are produced than lost from a microhabitat (Winzer et al. 2002b; Redfield 2002). This could be regarded as a type of 'compartment sensing,' where signal molecule accumulation is both the measure for the degree of compartmentalization and the means to distribute this information among the entire population. Similarly, diffusion of quorum-sensing signal molecules between spatially separated bacterial subpopulations may convey information about their physiological state, their numbers, and the individual environmental conditions encountered.

Bacteria employ a number of different quorum sensing 'languages,' and several families of signal molecules have been characterized. These range from post-translationally modified peptides to quinolones, lactones, and furanones (Williams 2002). In addition, siderophores (see Iron-dependent repression), which previously were considered only in the context of iron transport, may also function in the producer organism as signal molecules capable of controlling genes unrelated to iron acquisition (Lamont et al. 2002). Thus, many bacteria produce multiple signal molecules (Fuqua et al. 2001; Withers et al. 2001; Camara et al. 2002) that are components of complex regulatory hierarchies responsible for controlling signal molecule production, detection, and response (Atkinson et al. 1999; Winzer and Williams 2001). At the molecular level, quorum sensing requires a synthase plus a signal transduction system for producing and responding to the signal molecule. The latter usually involves either a response regulator or sensor kinase protein or both (see Two-component signal transduction systems) (Swift et al. 2001; Williams 2002). While there is, as yet, no clear-cut evidence for a molecularly conserved quorum-sensing system throughout the bacterial kingdom, the *luxS* gene and a furanone-related molecule termed AI-2 (for autoinducer-2) have been suggested to fulfill such a role (Bassler 2002). However, many bacteria (e.g. *P. aeruginosa*) do not possess *luxS* and hence do not produce AI-2 (Winzer et al. 2002a). Furthermore, since LuxS is a key metabolic enzyme in the activated methyl cycle responsible for recycling *S*-adenosylmethionine (SAM) (Winzer et al. 2002a, b), phenotypes associated with mutation of *luxS* are often not a consequence of a defect in cell-to-cell communication but the result of the failure to recycle SAM metabolites (Winzer et al. 2002a, 2004).

The most intensively studied quorum-sensing systems in gram-negative bacteria utilize *N*-acylhomoserine lactone (AHL) quorum-sensing signal molecules (Swift et al. 2001; Whitehead et al. 2001; Fuqua and Greenberg 2002). AHLs consist of a homoserine lactone ring attached, via an amide bond, to an acyl side chain. Naturally occurring AHLs containing an acyl side chain ranging from four to 18 carbons in length have been identified, and this chain may be saturated or unsaturated and bear either a hydroxy-substituent or an oxo-substituent, or no substituent on the carbon at the 3 position (see Figure 8a). AHLs are produced by a number of human pathogens, including *Aeromonas*, *Burkholderia*, *Brucella*, *Serratia, Pseudomonas*, and *Yersinia* species (Table 9), but not by *E. coli* or *Salmonella*.

The two central players of AHL-dependent quorum-sensing systems typically belong to the LuxI and LuxR protein families, which function as AHL synthases and transcriptional regulators, respectively (Withers et al. 2001). AHLs with a short acyl side chain passively diffuse out of the cell, whilst those with a long acyl side chain are pumped out. Once in the extracellular milieu, AHLs accumulate until a sufficient concentration is reached, enabling the binding to and activation of the transcriptional regulator LuxR to form a complex responsible for activating or repressing specific sets of target genes. The gene encoding the AHL synthase may itself be activated resulting in a positive autoinduction circuit through which the AHL signal molecule controls its own synthesis (Figure 8b). AHL synthases distinct from the LuxI family have also been identified, e.g. the LuxM family of *Vibrio* species (Milton et al. 2001), and alternative mechanisms for responding to AHLs have also been described (Miller et al. 2002).

MULTISIGNAL QUORUM SENSING IN *P. AERUGINOSA*

In the opportunistic human pathogen *P. aeruginosa*, a complex quorum-sensing hierarchy plays a central role in the regulation of virulence and contributes to the late stages of biofilm maturation (Sauer et al. 2002; Kjelleberg and Molin 2002). *P. aeruginosa* possess two AHL-dependent quorum-sensing systems, termed LasRI and RhlRI. LasI and RhlI are LuxI homologs that direct the synthesis of *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL), respectively (Pearson et al. 1994; Winson et al. 1995). 3-Oxo-C12-HSL and C4-HSL are responsible for activating the LuxR homologs LasR and RhlR, respectively. Together, *las* and *rhl* form a regulatory hierarchy in which the LasR/3-oxo-C12-HSL system regulates the *rhlRI* genes. Moreover, the target genes regulated via *las* and *rhl* overlap considerably (Whiteley et al. 1999), and recently a third LuxR homolog (QscR) has been identified that further modulates their expression (Chugani et al. 2001). The important contribution that quorum sensing makes to the pathogenicity of *P. aeruginosa* has been clearly demonstrated in animal, plant, and nematode experimental infection models. Furthermore, the isolation of AHLs from infected body fluids (e.g. sputa) and tissues has confirmed that AHLs are produced by *P. aeruginosa* in vivo during human infections (Camara et al. 2002). Interestingly, AHLs

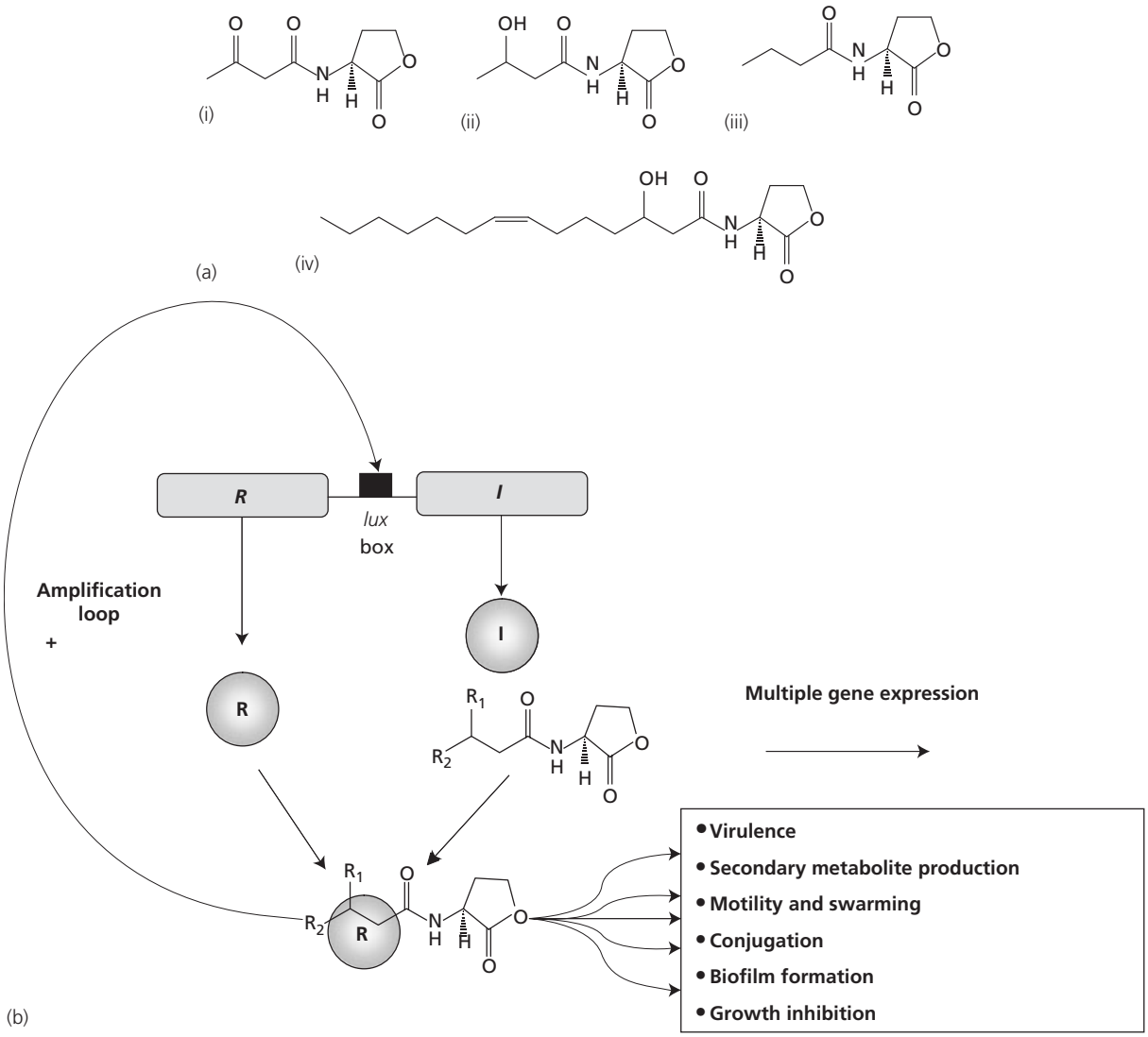

Figure 8 N-*Acylhomoserine lactone (AHL)-mediated quorum-sensing.* **(a)** *Example structures showing diversity of AHLs with either (i) -oxo (3-oxo-C_4HSL), (ii) -hydroxy (3-hydroxy-C_4HSL), or (iii) no substitutent (BHL) at the 3-carbon position of the acyl side chain. An AHL with an unsaturated acyl side chain (3-hydroxy-$C_{14:1}$HSL) is also shown (iv).* **(b)** *LuxRI/AHL-dependent signal circuitry. The 'R' and 'I' genes are homologs of the* Vibrio fisheri luxR *and* luxI *genes, which are arranged differently depending on bacterial species (they can be convergent, divergent, in tandem, or unlinked). The I protein directs the synthesis of AHLs, which exit the cell and are taken up into neighboring bacterial cells. Here, AHLs bind to and activate the response regulator (R) and this complex activates or represses the expression of multiple target genes by binding to conserved DNA sequences (*lux box*). In many cases the R protein/AHL complex also activates the* I *gene, creating an amplification loop resulting in a rapid increase in* I *gene expression and AHL production. Reproduced from Camara et al. (2002)*

such as 3-oxo-C12-HSL may also function as virulence determinants in their own right since they exhibit proinflammatory, immune modulatory, and vasorelaxant properties (Telford et al. 1998; Lawrence et al. 1999; Smith et al. 2001). Since T-cell responses constitute an important component of the host immune defense against *P. aeruginosa*, the 3-oxo-C12-HSL-mediated suppression of T-cell activity is likely to be advantageous to this pathogen.

Apart from AHLs, *P. aeruginosa* also produces a third quorum-sensing signal molecule, which is essential for the expression of many *rhl*-dependent phenotypes as well as biofilm development (Diggle et al. 2003). This is the pseudomonas quinolone signal (PQS) (2-heptyl-3-hydroxy-4-quinolone), which structurally resembles the 4-quinolone antibiotics and several antimalarial drugs (Pesci et al. 1999). PQS, like the AHLs, has been detected in the sputum of CF patients infected with *P. aeruginosa*, indicating that this signal molecule is also produced in vivo during infection. *P. aeruginosa* also produces a number of cyclic dipeptides (diketopiperazines) that are capable of cross-activating or antagonizing AHL-regulated processes, although their physiological function remains unclear (Holden et al. 1999).

Table 9 N-*Acylhomoserine lactone (AHL)-mediated gene regulation in different organisms*

Bacterium	LuxR/I	Major AHL	Phenotypes
Pseudomonas aeruginosa	LasR, LasI	3-Oxo-C12-HSL	Exoenzymes, Xcp secretion apparatus, biofilm maturation, RlhR
	RhlR, RhlI	C4-HSL	Exoenzymes, cyanide, RpoS, lectins, pyocyanin, rhamnolipd
Burkholderia cepacia	CepR, CepI	C8-HSL	Protease, siderophore
Chromobacterium violaceum	CviR, CviI	C6-HSL	Antibiotics, violacein pigment, exoenzymes, cyanide
Yersina pestis	YpeR, YpeI	Unknown	Unknown
Yersina enterocolitica	YenR, YenI	C6-HSL, 3-Oxo-C6-HSL	Motility and swamming
Yersina pseudotuberculosis	YpsR, YpsI	3-Oxo-C6-HSL	Bacterial motility and clumping
	YtbR, YtbI	C8-HSL	Unknown
Aeromonas hydrophila	AhyR, AhyI	C4-HSL	Extracellular protease, biofilm formation
Brucella melitensis	Unknown	C12-HSL	Inhibition of *virR/virS*

The potential contribution of quorum-sensing to the development and maturation of *P. aeruginosa* biofilms has been the subject of much debate and controversy. *P. aeruginosa lasI* mutants, for example, were reported to be unable to form a highly structured biofilm community (Davies et al. 1998), which could be more readily dispersed by detergents than wild-type biofilms. The in vivo relevance of this finding has also been indicated by evaluation of quorum-sensing-dependent biofilm development in the murine model of corneal infection (Zhu et al. 2002). However, it is unlikely that quorum-sensing is the only important effector, since biofilm-associated communities will exploit all available adaptive mechanisms and the corresponding network of regulatory activities (Kjelleberg and Molin 2002). Indeed, mathematical models (Wimpenny and Colasanti 1997; Van Loosdrecht et al. 1997) have illustrated that biofilm formation is a predictable consequence of the physicochemical conditions in the biofilm environment, indicating that the bacterial cells do not perceive that they are within a biofilm but simply respond to local environmental conditions (such as stress and nutrient gradients). Nevertheless, it is clear that many genes important for biofilm development and maturation are subject to AHL-dependent quorum-sensing control in *P. aeruginosa* and in other gram-negative bacteria.

THE STAPHYLOCOCCAL *AGR* SYSTEM

In gram-positive bacteria, quorum-sensing signal molecules are generally small, modified peptides, and the transduction of such signals usually involves a TCST phosphorylation cascade that ultimately triggers target gene expression (see Two-component signal transduction systems). The opportunistic human pathogens *S. aureus*, *Enterococcus faecalis*, and *Str. pneumoniae*, as well as *Bacillus subtilis*, regulate many cellular processes in this way (Novick and Muir 1999; Dunny and Leonard 1997).

The staphylococci, for example, produce quorum-sensing signal molecules termed autoinducing peptides (AIP). These are cyclic peptide thiolactones containing between seven and nine amino acid residues. In *S. aureus* and *Str. epidermidis*, these are produced via the *agr* regulatory locus, which also controls the production of multiple cell-wall colonization factors and exotoxins (Winzer and Williams 2001; Novick and Muir 1999; Otto 2001). The gene *agr* consists of two divergent transcription units, RNAII and RNAIII, controlled via the P2 and P3 promoters, respectively (Figure 9). While RNAIII is the Agr effector molecule, the P2 operon consists of four genes, *agrABCD*. AgrA and AgrC constitute a response regulator and sensor kinase, respectively, while the AIP is derived from an internal fragment of the AgrD protein. The transmembrane protein AgrB is responsible for processing AgrD and secreting the AIP signal molecule.

AIPs interact with the membrane-bound sensor kinase AgrC to activate the response regulator AgrA, located in the cytoplasm. Activation of *agr* occurs through the initiation of a phosphorylation cascade, resulting in the production of more AIP (via the P2 operon) and the effector RNA molecule, RNAIII via P3. At high *S. aureus* cell densities, *agr* represses the expression of genes coding for cell-surface proteins involved in colonization, while activating the expression of genes coding for exotoxins and tissue-degrading exoenzymes, Several experimental models of infection have highlighted a key role for the *agr* locus in the virulence of *S. aureus* (Novick and Muir 1999).

S. aureus strains can be subdivided into four AIP groups (I–IV), in which the AgrD-derived AIP of one group can cross-activate the *agr* response (and thus virulence) in other strains of that group but inhibit the *agr* response of members of other groups (Ji et al. 1997). While the primary amino acid sequence is different for each AIP, they all share a common central cysteine that is located five amino acid residues from the C-terminal amino acid. The AIPs are not linear peptides but contain an intramolecular thiol ester linkage between the sulfydryl group of the

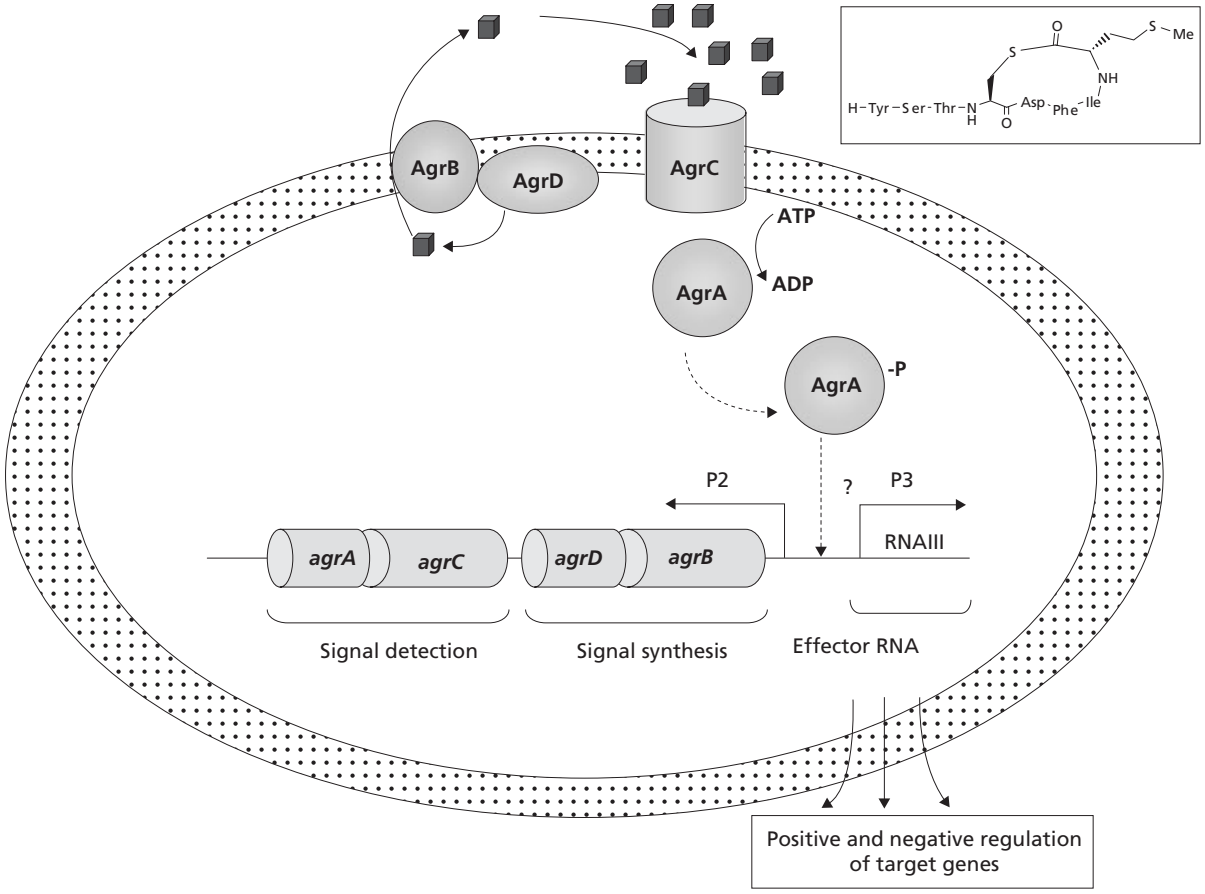

Figure 9 Agr-*mediated quorum-sensing circuitry. The* agr *locus consists of two divergent transcriptional units. One is expressed from the P2 promoter and consists of* agrABCD, *which encodes for the proteins responsible for generating and sensing the peptide signal molecule.* AgrB *acts on the peptide derived from* agrD *to yield the signaling peptide and also to effect its export out of the cell. Once a critical extracellular concentration of the peptide is reached, it interacts with the AgrC membrane-associated sensor protein, causing it to autophosphorylate. This phophate is then transferred to the response regulator, AgrA, which then activates the expression of RNAIII from the P3 promoter. The effector molecule, RNAIII, mediates the changes in expression of multiple target genes, resulting in the* agr *response. An autoinduction feedback loop results in more peptide signal, since* agrBD *are amongst the activated genes. The insert in the top right-hand corner shows the structure of the quorum-sensing peptides produced by group I* Staphylococcus aureus. *Redrawn from Camara et al. (2002).*

conserved cysteine and the α-carboxyl group of the C-terminal amino acid. This thiolactone structure is essential for full biological activity and, consistent with the concept of cell population density sensing, a critical threshold concentration (in the nanomolar range) of the AIP is required to trigger an *agr*-dependent response (Ji et al. 1997; McDowell et al. 2001). AIP antagonists therefore offer a novel means of controlling staphylococcal infections, since they inhibit exotoxin (e.g. toxin shock syndrome toxin, TSST-1) and exoenzyme (e.g. lipase) production. In a mouse subcutaneous abscess infection model, coinoculation of a virulent group I strain with the group II AIP resulted in reduction in the virulence of the group I strain (Mayville et al. 1999). Other staphylococcal species such as *S. epidermidis* and *S. lugdunensis* also produce AIPs that are cross-inhibitory with those of *S. aureus*. Thus, it seems that staphylococci have evolved an innate mechanism for coupling their pathogenic potential with a means to ward off potential competitors.

EVOLUTION OF PATHOGENS WITHIN THEIR HOST

Although pathogens are capable of integrating the diverse environmental signals encountered during the progress of infection to control virulence factor deployment, other strategies are also employed. For example, the capsules of *N. meningitidis* undergo phase variation such that they are expressed at late stages of infection to provide protection against the immune system, and not when their presence would hinder intimate adhesion to host cells, which is required for invasion during the early stages (Deghmane et al. 2002). Phase variation in *N. meningitidis* can occur by several different mechanisms: capsule biosynthesis genes can be acquired or lost allowing antigenic variation, plus their expression can be

regulated by slip-strand mispairing. This mechanism regulates a range of virulence factors of *N. meningitidis* and other bacteria such as *H. influenzae*, and relies upon the presence of an extended nucleotide repeat, which can loop out, resulting in a deletion that results in disruption of the reading frame and loss in gene expression (Moxon et al. 1994). The existence of such mechanisms suggests that the host exerts a certain selective pressure upon the pathogens it harbors. This is perhaps well illustrated by the apparent continuous microevolution of *H. pylori* within its cognate host (Israel et al. 2001). The highly diverse organism *H. pylori* persists in the human stomach and induces chronic gastritis, which may progress to peptic ulceration, noncardia gastric adenocarcinoma, or gastric lymphoma during the life of the host. The differential development of these clinical sequelae probably depends on differentially represented bacterial determinants, host characteristics, and interactions between the host and the bacterium throughout their time of coexistence.

Although *H. pylori* appear to exhibit a clonal population structure over short periods of time, isolates obtained from different individuals exhibit substantial genetic diversity, consistent with extensive recombination and a panmictic (a population derived from a single organism) population structure (Suerbaum et al. 1998). Strains may differ in their possession of point mutations in highly conserved genes (Achtman et al. 1999), the presence of nonconserved and/or mosaic forms of genes (Akopyants et al. 1998; Censini et al. 1996; Peek et al. 1998; Pride et al. 2001), and chromosomal organization (Taylor et al. 1992; Jiang et al. 1996; Alm et al. 1999). These variations may be acquired through horizontal genetic exchange or spontaneous mutation (Suerbaum et al. 1998; Achtman et al. 1999). Comparison of isolates of the sequenced *H. pylori* strain J99, after a 6-year interval from its human source patient, revealed genetic divergence amongst the isolates and between them and the original stain (Israel et al. 2001). Thus, it appears that within an apparently homogeneous population as determined by macroscale comparison (randomly amplified polymorphic DNA, polymerase chain reaction (PCR), and DNA sequencing of four unlinked genetic loci), genetic differences emerged among single-colony isolates of *H. pylori*. The acquisition of genes originating from distinct strains suggests that there may have been an additional subpopulation of *H. pylori* strain J99 possessing these open reading frames (ORF), or that another strain of *H. pylori* or closely related organism was transiently present in the stomach. The implied continuous state of genetic flux may allow *H. pylori* to adapt rapidly to changing conditions in its current host throughout the extended period of coexistence, as well as priming it for colonization of a new host. *H. pylori* strain variation may hinge upon gene mosaicism (see below) or the presence of segments of nonconserved DNA. This is perhaps best illustrated by the gene encoding the vacuolating cytotoxin (VacA) and the cytotoxin associated antigen (CagA), which is encoded on a pathogenicity island (PAI).

VacA is a member of the family of autotransporter proteins (Cover 1996; Telford et al. 1994) (see Table 10 for other members), and as such is produced as a precursor polypeptide in the cytoplasm. An N-terminal targeting domain delivers the autotransporter to the inner membrane and is cleaved during translocation to

Table 10 *Examples of members of the autotransporter family, amended from Henderson and Nataro (2001)*

Bacterium	Autotransporter
Neisseria meningitidis	IgA protease
Neisseria gonorrhoeae	IgA protease
Haemophilus influenzae	IgA protease, adhesin (Hap, Hia, Hsf)
Helicobacter pylori	VacA cytotoxin, BabA adhesin
Shigella flexneri	IcsA
Bordetella spp.	Adhesins (Pertactin, TcfA, Vag8), serum resistant factor (BrkA)
Dichelobacter nodusus	Putative elastases (BprV, BprB, AprV2, BprX)
Moracella catarrhalis	Adhesin (UspA1, UspA2h), serum resistance protein (UspA2)
Pasteurella haemolytica	Ssa1 protease
Pseudomonas aeruginosa	EstA esterase
Pseudomonas fluorescens	Proteases (PspA, PspB)
Ricettsiales	Adhesin (rOmpA, rOMPB)
Salmonella typhiumurium	Esterase ApeE
Serratia marcesens	Proteases (PrtS, PrtT, Ssp-H1, Ssp-H2)
Shigella flexneri	Proteolytic (SepA, SigA), mucinase (Pic), mediator of intracellular motility (IcsA)
Xenorhabdus luminescens	Lipase (PlaA)
Pathogenic *Escherichia coli*	AIDA-I adhesins (AIDA-I, TibA), proteolytic toxin (EspP, Pet, Sat), hemagglutinin (Tsh), mucinase (Pic), biofilm formation (Ag43), EspC.

the periplasm. This translocation may be achieved via the SRP/Sec pathway (Henderson et al. 1998; Sijbrandi et al. 2003). The C-terminal domain is proposed to insert into the outer membrane forming a β-barrel through which the mature protein (or passenger domain) is translocated to the extracellular medium. Cleavage of this C-terminal domain, which may be mediated by the passenger protein or surface proteases (Henderson et al. 1998; Henderson et al. 2000), then releases the mature protein (Figure 4). VacA shows homology to this family both by possession of an extended N-terminal signal peptide and by the presence of a C-terminal domain, which shows homology to other autotransporters and is cleaved during secretion (Cover 1996; Schmitt and Haas 1994). Once secreted, the approximately 90-kDa passenger domain of VacA is processed further, yielding subunits of approximately 58 and 37 kDa. These monomers combine to form dodecamers or tetradecamers (Cover et al. 1997) with a flower-like rosette structure (Lupetti et al. 1996). In acidic conditions, these oligomers dissociate back into monomers, exposing hydrophobic regions and allowing efficient insertion into target membranes (Molinari et al. 1998b).

Once released from the bacterial cell, VacA damages eukaryotic cells, causing mitochondrial damage (Kimura et al. 1999; Galmiche et al. 2000), inducing apoptosis (Galmiche et al. 2000; Kuck et al. 2001), increasing permeability of polarized monolayers (Papini et al. 1998; Pelicic et al. 1999), disrupting antigen presentation (Molinari et al. 1998a), and interfering with vacuolar transport in the late endosomal pathway, resulting in the formation of characteristic vacuoles (Cover and Blaser 1992; Molinari et al. 1997). Although all *H. pylori* strains analyzed to date contain *vacA*, its sequence varies, a phenomenon known as mosaicism. The nucleotide sequence encoding the N-terminal signal region may be one of four variants (s1a, s1b, s1c, or s2), whilst the mature passenger domain may contain a segment of an m1, m2a, or m2b (Atherton et al. 1995; van Doorn et al. 1998) subtype. The s1 and m1 types have been associated with increased virulence of *H. pylori*, since strains bearing this combination are most frequently associated with more serious disease states in humans (Atherton et al. 1995). The more pathogenic strains of *H. pylori* (type I strains) also possess the *cag* PAI, an element lacking in strains associated with milder clinical outcomes (type II strains).

The *cag* PAI encodes a type IV secretion system (TFSS) (see Protein secretion and Figure 4) that facilitates the injection of bacterial proteins directly into eukaryotic cells. Following attachment to the surface of eukaryotic cells, CagA is delivered into the host cell by proteins encoded by other genes on the *cag* PAI (Asahi et al. 2000; Backert et al. 2000; Odenbreit et al. 2000; Segal et al. 1999; Stein et al. 2000). Intracellular CagA is tyrosine phosphorylated by a host cell kinase of the Src family (Selbach et al. 2002; Stein et al. 2002), whereupon it interacts with the SHP-2 phosphatase initiating signaling events that lead to dramatic cellular elongation (the 'humming-bird' phenotype) (Higashi et al. 2002). The precise molecular events occurring throughout this alteration of the actin cytoskeleton remain unclear, although the involvement of small GTPases is possible (Covacci and Rappuoli 2003).

Independent of *cagA*, the *cag* PAI initiates the activation of NF-κB and the expression of proinflammatory cytokines, such as IL-8 (Crabtree 1998). The latter requires that the *cag* PAI encodes a functional TFSS.

HOST RESISTANCE TO INFECTION

Many of the bacterial virulence determinants described above contribute to pathogen evasion of host defenses. Resistance/immunity in higher animals can be divided into innate and acquired (adaptive) immunity. In an evolutionary context, innate immunity is considered to be the most ancient form of defense against invading microorganisms. It is present from birth, functions nonspecifically against diverse microorganisms, and, in contrast to acquired immunity, does not become more efficient on subsequent exposure to the same organisms or antigens. Acquired immunity, on the other hand, is based on the generation of an antibody response towards specific antigens associated with infecting pathogens. It is extremely flexible and is able to mount a response to virtually any antigen presented. A unique feature of the acquired immune response is the memory of previous encounters with an antigen and the capacity to mount an improved response during subsequent exposures.

Innate immunity

Innate immunity refers to the nonspecific defense mechanisms that a host uses immediately or within several hours following exposure to an antigen. There are numerous factors that contribute to the innate immunity of a higher organism and these can be subdivided into: (1) anatomical barriers; (2) mechanical removal; (3) nutritional restriction; (4) bacterial antagonism; and (5) common pattern recognition.

ANATOMICAL BARRIERS

Anatomical barriers are tough intact layers that prevent the entry and colonization by microorganisms. Examples include the skin and the mucous membranes.

The skin, consisting of the epidermis and the dermis, is dry and acidic and has a temperature less than 37°C. These conditions are not favorable for bacterial growth. The dead keratinized cells that make up the surface of the skin are continously being sloughed off, so that microbes that do colonize these cells are constantly

being removed. The hair follicles and sweat glands, which provide potential entry sites, produce lysozyme and toxic lipids that can kill bacteria. Also, beneath the surface, is the skin-associated lymphoid tissue (SALT), which contains cells that kill incoming microorganisms and initiate acquired immune responses by sampling antigens on the skin.

Mucous membranes line body cavities that are open to the exterior environment, such as the respiratory tract, the gastrointestinal tract, and the genitourinary tract. Mucous membranes are probably the most important site of entry of pathogenic bacteria into the host body. Mucosal surfaces are composed of an epithelial layer that secretes mucus, which acts as a physical barrier, trapping microorganisms and preventing deeper penetration into the tissues. Mucus contains the bactericidal protein lysozyme, which degrades bacterial peptidoglycan (leading to the lysis of bacterial cells), as well as secretory IgA, which prevents bacteria from attaching to the epithelial layer of cells. In addition, secretions from mucus membranes contain the iron-transporting protein lactoferrin, which plays a major role in mucosal defence. Lactoferrin not only withholds iron from infecting bacteria (see Motility), but also is capable of disrupting bacterial biofilms (Singh et al. 2002) and degrading bacterial virulence factors (such as the Hap adhesins and IgA proteases of *H. influenzae*) via its serine protease activity (Hendrixson et al. 2003). Lactoferrin also possesses immunoregulatory functions and bactericidal activity and can neutralize the toxic effects of LPS (Caccavo et al. 2002). Mucus also contains lactoperoxidase, which generates toxic superoxide radicals that rapidly kill bacteria. It has also been suggested that the resident normal microbial flora of the mucosa exclude colonization by potentially harmful bacteria. Beneath the mucosal surface is mucosa-associated lymphoid tissue (MALT), which contains cells that destroy invading bacteria and subsequently initiate an acquired immune response.

MECHANICAL REMOVAL OR THE PHYSICAL FLUSHING OF MICROORGANISMS FROM THE BODY

Cilia on the surface of the epithelial cells that line mucous membranes, such as those in the respiratory tract, propel mucus and trapped bacteria upwards towards the throat, where it is swallowed. The acid pH of the stomach breaks mucus down and kills any bacteria present. Coughing and sneezing, in a similar way, removes bacteria from the respiratory tract that have become trapped in the mucus. Although vomiting and diarrhea are considered characteristic symptoms of particular diseases, they also can be considered part of the host defense, since they effectively remove pathogens and toxins from the gastrointestinal tract. It should be noted that the flow of fluids, such as urine, tears, saliva, perspiration, and blood from injured blood vessels, is also important for the mechanical removal of pathogens from the host body.

NUTRITIONAL RESTRICTION

Nutrient-depriving host defense molecules include transcobalamins (which bind vitamin B12), calprotectin (which binds zinc), and lactoferrin (which sequesters iron). Iron is one essential nutrient for both host and pathogen (see Iron scavenging). During the course of infection, the human body makes considerable efforts to make iron unavailable to microorganisms for growth; this is called the hypoferremic response. Much of this is due to production of IL-1α (also known as leukocyte-endogenous mediator (LEM)) which acts to lower free iron in tissues and body fluids. However, there are other factors that contribute to the lowering of available free iron during infection, including: (1) decreased intestinal dietary iron uptake; (2) a reduction in plasma iron levels and increased synthesis of the iron storage protein ferritin; (3) increased synthesis of lactoferrin and transferrin; and (4) release of lactoferrin at sites of microbial infection, such as the mucous membranes.

BACTERIAL ANTAGONISM

Approximately 100 trillion bacteria and other microorganisms reside in or on the human body as the normal body flora. These are considered to exclude potentially harmful opportunistic pathogens by the production of, for example, fatty acids and antibacterial peptides, such as bacteriocins. The normal flora may also influence the growth of invading pathogens by depletion of, and competition for, the available nutrients. An example of the prevention of disease by bacterial antagonism is that of the severe antibiotic-associated colitis caused by *Clostridium difficile*. This anerobe, which cannot grow within a normal gut flora, will infect and colonize when the gut flora microecology is altered by, for example, antibiotics.

COMMON PATTERN RECOGNITION

Common pattern recognition has evolved to identify a few highly conserved molecules present in or on many different microorganisms. The structures recognized are termed pathogen-associated molecular patterns and include LPS, peptidoglycan, LTA, the sugar mannose (which is common in microbial polysaccharides but rare in those derived from higher organisms), bacterial DNA, double-stranded RNA (from viruses), and glucans (from fungal cell walls). Most body defense cells have pattern-recognition receptors (Toll-like receptors (TLR)) for these common pathogen-associated molecular patterns and so, upon infection, there is an immediate response to invading microorganisms. Pathogen-associated molecular patterns can also be recognized by the blood-borne proteins that initiate complement.

The lipid A component of LPS/LOS was the first agonist shown to bind and activate TLRs. These

constitute a family of distinct proteins characterized by an intracellular domain with homology to that of the IL-1 receptor and containing extracellular leucine-rich repeats. TLRs activate the immune system to recognize conserved macromolecular components of pathogens and, through this interaction, activate the immune system. Pathogen recognition leads to the initiation of defensive protective actions, which include the production of reactive oxygen intermediates and the secretion of inflammatory cytokines, such as IL-1, interleukin-12 (IL-12), interferon-γ (IFN-γ), and TNF-α (Raetz and Whitfield 2002). These cytokines activate natural killer cells and also initiate a cascade of signals to cells of the adaptive arm of the immune response, preparing them for the development of antigen-specific immune responses. Activation of TLRs by lipid A also results in the production of defensins, which consist of several distinct families of antibacterial, antifungal, and antiviral peptides (Heine and Lien 2003).

TLRs direct these effects by activating host signal transduction processes, including the transcription factor NF-κB and the MAP kinases, p38, and c-jun. Differences between signals generated by alternative TLRs are emerging. Each TLR is activated by a different microbial surface component, e.g. TLR4 is activated primarily by bacterial LPS and LTA. Both LPS and LTA first bind to the CD14 receptor and are subsequently transferred to the TLR4 complex. LTA can bind directly to CD14, but LPS must be delivered to CD14 by LPS-binding protein (LBP). TLR4 signals through an accessory protein termed MyD88 to activate the transcription factors, NF-κB, Elk-1, and AP-1. TLR6 is also activated by LPS and shares the same signaling pathway as TLR4, as does TLR2 (activated by bacterial lipoarabinomannan (LAM) and peptidoglycans (PGN)). When activated by bacterial lipoprotein (BLP), TLR2 mediates both apoptosis and NF-κB activation. If NF-κB is inhibited downstream of MyD88, then apoptosis occurs through the activation of caspase 8 (Aliprantis et al. 2000). TLR2 is also responsible for the recognition of the yeast cell-wall particle zymosan. It is believed that zymosan acts through the CD14 receptor to influence TLR2, shown by high levels of downstream NF-κB in $CD14^+/TLR2^+$ macrophage exposed to zymosan, as compared with NF-κB levels observed in $CD14^-/TLR2^+$ macrophage exposed to zymosan. TLR2 is internalized after activation by zymosan; the yeast cell is also internalized with TLR2. TLR2 signals the production of TNFα, through NF-κB pathway, from the phagocytosed vesicle (Underhill et al. 1999).

TLR9 is responsible for the recognition of extracellular CpG islands of bacterial DNA. Two mechanisms for TLR9 activation are considered to exist. TLR9 bound to extracellular CpG fragments may be endocytosed, or alternatively phagosome located TLR may interact with phagosome internalized bacteria. The result of either of these is the activation of the NF-κB pathway from the endocytosed vesicle (Hemmi et al. 2000). TLR5 recognizes bacterial flagella and activates NF-κB pathway. The functions of TLR1, 3, 7, 8 and the recently cloned TLR10 (Chuang and Ulevitch 2001) are not yet known.

Acquired immunity

The lymphocyte-generated antibodies comprising the acquired immune system act in different ways. Some antibodies, such as IgG and IgE, function as opsonins, promoting phagocytic engulfment of bacteria. Antibodies, such as IgG, IgA, and IgM, bind to bacterial adhesins, pili, and capsules and block attachment to host cells. IgG and IgM can also activate the classical complement pathway, whilst IgA and IgM crosslink bacterial cells, enabling more efficient phagocytic clearance.

Bacteria evade antibody-mediated clearance in a number of ways (see Avoidance of host immune defenses), including antigenic variation of surface macromolecules, by the synthesis of surface components that mimic host tissue antigens, and by bacterial surface coating with host proteins, such as fibronectin and fibrinogen. By producing immunoglobulin-binding cell-wall proteins that bind the Fc portion of antibodies, bacteria such as *S. aureus* and *Str. pyogenes* can evade opsonization. Some bacteria produce immunoglobulin-degrading proteases, e.g. the IgA proteases of *H. influenzae*, *Str. pneumoniae*, *N. meningitidis*, and *N. gonorrhoeae*.

GENOMICS AND BACTERIAL PATHOGENICITY

Advances in molecular biology and, in particular, the ability to clone, express, and mutate specific genes have proved invaluable for defining the repertoire of virulence determinants employed by a pathogen. This in turn has resulted in the definition of Koch's molecular postulates (Falkow 1988; Joyce et al. 2002; Relman and Falkow 2001) for determining the relationship between a given virulence determinant and disease. The development of specific genetic strategies for identifying structural and regulatory genes that are essential for bacterial life in vivo, but not in vitro (in vivo expression technology (IVET) and signature-tagged mutagenesis (STM)) has provided many novel insights into the lifestyle of diverse pathogens. The information obtained from such experimentation can now be greatly enhanced by the availability of pathogen genome sequence information and the potential offered by DNA microarrays and proteomics for defining the global response of the pathogen to the host environment, and vice versa.

Several approaches can be used to analyze whole-genome sequences for candidate virulence factors, potential vaccine antigens, and novel antimicrobial

targets. By comparing predicted coding sequences with sequences in databases such as GenBank through use of the BLAST program (www.ncbi.nlm.nih.gov:80/BLAST) identifies matches to known genes involved in virulence. Approximately 20 percent of the predicted ORFs in a genome do not match anything in GenBank, while another 10–20 percent match genes of unknown function. Therefore, the comparative approach has been useful for recognizing good candidates for virulence genes among genes whose functions have been previously defined. While this in silico approach has not proved particularly useful for the discovery of novel virulence genes, the availability of such information on easily accessible electronic databases has made it a routine tool for studies of pathogenic microbes and prompted the development of experimental techniques, such as IVET and STM.

STM is a powerful tool for the identification of genes that are required for virulence and in vivo survival. It is a method for identifying mutants that are unable to replicate in vivo and therefore display an attenuated phenotype. A transposon is used for random mutagenesis of the bacterial genome. Each transposon has been prepared to carry a unique index region or signature tag. The unique tag can be amplified by PCR using standard regions flanking the tag and the resulting products used as a hybridization probe to identify the unique transposon that encodes it. The initial set of random insertion mutants are arrayed as a master, pooled, and then grown under either in vitro or in vivo conditions in an experimental animal model of infection. All the mutants that emerge from the in vitro and in vivo growth regimens are collected, their index regions amplified by PCR, and used to hybridize against the master array of original mutants. Mutants in the master array that do not hybridize with the tag sequences amplified from bacteria recovered from in vivo grown bacteria represent mutants that are attenuated and are unable to survive in vivo. Regions flanking the transposon insertions in mutants of interest are sequenced and compared with the genomic sequence databases to identify the inactivated gene(s). This powerful technique has been used to identify novel genes involved in pathogenesis for numerous pathogens, including *Salmonella*, *Neisseria*, and *E. coli* (Tang et al. 2001).

IVET is a method based on trapping promoters. Random genomic fragments are ligated in front of a promoterless reporter gene, the activity of which is used as an indication of transcriptional activity of the fused gene. Reporter genes that have been used include those that can allow auoxotrophy complementation IVET selection (e.g. *purA*), antibiotic IVET selection (e.g. *cat*), and recombinase-based IVET (RIVET), with which screening is achieved using a promoterless *tnpR* allele, as this cleaves a tetracycline resistance gene from elsewhere in the bacterial genome when produced. The suicide plasmids recombine into the chromosome by insertion–duplication, and fusion strains are passaged through an appropriate animal model before being collected from infected tissues and/or fluids. This strategy has been successfully used for *S. entirica* serovar Typhimurium, *Yersinia entercolitica*, *Streptococcus gorndonii*, *V. cholerae*, and *S. aureus* (Merrell and Camilli 2000).

CONCLUSIONS

Pathogenic bacteria exhibit coordinated responses to stimuli received from the external environment and during life on or within the host. These stimuli in turn impose differential selective pressures, which direct pathogen evolution. Certain bacteria have evolved strategies for avoiding the host immune system by invading host cells, while others have adopted measures for immune evasion while remaining adherent to host cell surfaces. Many pathogens produce tissue-damaging exotoxins and exoenzymes, which aid bacterial survival by increasing the availability of nutrients for growth, while simultaneously destroying tissue and host defenses. The coordinated deployment of these multiple virulence determinants is subject to tight regulatory controls through a variety of sensory pathways, some of which involve bacterial cell-to-cell communication. The ongoing unveiling of genomic, proteomic, and metabolomic information is likely to improve substantially our understanding of pathogen adaptive behavior and the host response, which in turn should provide new opportunities for the development of novel anti-infective measures.

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Airborne bacteria

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INTRODUCTION

Bacteria are ubiquitous in the environment, capable of growing on virtually any surface and in most liquids when nutritional and environmental factors are favorable. Their presence in the air is the result of dispersal from a site of colonization and growth. This chapter will review the study of bacteria in the air that impact human health and the environment.

BIOAEROSOLS

A bioaerosol is a collection of airborne biological material consisting of cells, cellular fragments, and by-products of metabolism. This material can be present as particulate, liquid, and volatile organic compounds, and it may have biological activity on other organisms (Cox and Wathes 1995). Bacterial aerosols are generated in a variety of indoor environments and facilities and as a result of a variety of activities (Table 1), including dispersal from heating, ventilation, and air-conditioning (HVAC) systems, water-spray devices (e.g. showerheads, humidifiers), and flooring (Lighthart and Stetzenbach 1994). Bacterial aerosols are also generated by medical (Demers 2001; Nottrebart 1980) and dental (Depaola et al. 2002; Leggat and Kedjarune 2001) procedures, and patient (Shiomori et al. 2001, 2002) and animal care (Kaliste et al. 2002; Kowalski et al. 2002) practices, prompting cautionary measures to protect patients and staff from dispersed airborne bacteria. Talking and coughing generate bioaerosols from building occupants with droplet concentrations of 10^4–10^5 droplets/m^3 of air (Papineni and Rosenthal 1997). Manufacturing practices and biofermentation procedures can also generate bacterial aerosols (Abrams et al. 2000). Outdoors, bacterial aerosols are generated as a result of spray irrigation, wastewater treatment activity, wave splash, cooling towers, air-handling water-spray systems, and agricultural processes (Brandi et al. 2000; Burkhart et al. 1993; Hameed and Khodr 2001; Lighthart and Stetzenbach 1994; Lighthart and Mohr 1997; Pillai and Ricke 2002).

The particulate in a bioaerosol is generally 0.3–100 μm in diameter (Cox and Wathes 1995). Single bacterial cells range in size from 0.5 to 2.0 μm and are commonly spheres (cocci), rods (bacilli), or spirals. However, airborne bacterial cells are often present as aggregate formations of larger particles (Lighthart 1994). Aggregates of cells have different aerodynamic properties compared with single cells and they afford protection of individual cells from environmental stresses such as dessication, ultraviolet irradiation, and exposure to ozone in the atmosphere. Cell aggregates surrounded by a thin layer of water are commonly the result of dispersal from water sources by splash, rainfall, or mechanical disturbance (e.g. cooling towers, fountains). This often aids in the survivability of the cells while airborne. Airborne bacterial cells may also be associated with skin cells, dust, and other organic or inorganic material in a relationship that is commonly referred to as 'rafting.' Rafting cells are often found in bioaerosols generated from agricultural settings (e.g. during harvesting, tilling) and indoor environments. This formation affects the aerodynamic characteristics and the

Table 1 *Published concentrations and populations of airborne bacteria and bacterial components outdoors and in indoor environments*

Facility/activity	Organism or agent	Concentration	Reference(s)
Animal facilities	*Bacillus* species	10^1–10^3 CFU/m3	Wilson et al. 2002
	Corynebacterium species	10^0–10^3 CFU/m3	Wilson et al. 2002
	Culturable bacteria	10^4 CFU/m^3	Blom et al. 1984
		10^5–10^6 CFU/m^3	Chang et al. 2001
		10^3–10^5 CFU/m^3	Curtis et al. 1978
	Endotoxin		Duchaine et al. 2000
			Thorne et al. 1992
		100–500 pg/m^3	Milton et al. 1990
		0.3–41 ng/mg	Andersson et al. 1999
		10^1–10^4 EU/m^3	Duchaine et al. 2000
			Kullman et al. 1999
	Micrococcus luteus	10^1–10^3 CFU/m^3	Wilson et al. 2002
Clean rooms	Viable particles	10^0–10^2 CFU/m^3	Favero et al. 1966
Composting – agricultural or residential	Culturable bacteria	10^3–10^4 CFU/m^3	Hryhorczuk et al. 2001
		10^4–10^6 CFU/m3	Durand et al. 2002
	Endotoxin	0.12–0.41 ng/m^3	Hryhorczuk et al. 2001
	Gram-negative bacilli	10^3 CFU/m^3	Clark et al. 1983
Composting – sludge	Coliforms	<dl	Jones and Cookson 1983
	Culturable bacteria	10^0–10^3 CFU/m^3	Jones and Cookson 1983
Daycare center	Endotoxin	0.43 ng/m^3	Rylander et al. 1992
		5.7 ng/m^3	Wan and Li 1999
Effluent irrigation	Culturable bacteria	10^3 CFU/m^3	Applebaum et al. 1984
	Escherichia coli	10^1–10^3 CFU/m^3	Applebaum et al. 1984
	Fecal coliforms	10^2 CFU/m^3	Teltsch and Katzenelson 1978
	Fecal streptococci	10^2 CFU/m^3	Applebaum et al. 1984
	Total coliforms	10^2 CFU/m^3	Applebaum et al. 1984
Farming, harvesting, bailing, grain storage	Culturable bacteria	10^1–10^4 CFU/m^3	Lighthart 1994
			Swan and Crook 1998
		10^4–10^6 CFU/m^3	Hameed and Khodr 2001
	Endotoxin	10^2–10^3 EU/m^3	Buchan et al. 2002
	Gram-negative bacteria	10^3–10^5 CFU/m^3	Hameed and Khodr 2001
Food processing/ food storage	Endotoxin	0.0125–54.9 Φg/m^3	Dutkiewicz et al. 2000
		0.2–2681.0 Φg/m^3	Dutkiewicz et al. 2001b
		0.011–1893.9 Φg/m^3	Dutkiewicz et al. 2002
Heating, ventilation, and air-conditioning (HVAC) systems	Culturable bacteria	10^2–10^3 CFU/m^3	Hugenholtz and Fuerst 1992
		10^2–10^7 CFU/cm^2	Flaherty et al. 1984
	Endotoxin	NQ	Hugenholtz and Fuerst 1992
Hot-water heaters, hot water systems	*Legionella* sp.	10^0–10^1 CFU/m^3	Bollin et al. 1985
	L. pneumophila	NQ	Helms et al. 1983
Humans (skin fragments)	Culturable bacteria	10^2–10^3 CFU/m^3	Lundholm 1982
Humidifiers (portable)	*L. pneumophila*	10^2–10^4 CFU/ml	Zuravleff et al. 1983
	Pseudomonas	10^2–10^4 CFU/h	Couvelli et al. 1973
Manufacturing	Culturable bacteria	10^6 CFU/m^3	Travers Glass et al. 1991
	Gram-negative bacilli	10^3 CFU/m^3	Lundholm 1982
		10^2–10^4 CFU/m^3	Prazmo et al. 2000
	Endotoxin	10^1–10^3 EU/m^3	Dutkiewicz et al. 2001a
		16.4–234 EU/m^3	Abrams et al. 2000
	Total bacteria	0.02–3.6 ug/m^3	Walters et al. 1994
		10^1–10^3 CFU/m^3	Walters et al. 1994

(*Continued over*)

Table 1 *Published concentrations and populations of airborne bacteria and bacterial components outdoors and in indoor environments (Continued)*

Facility/activity	Organism or agent	Concentration	Reference(s)
Mortuary, necropsies	Coliforms	10^0–10^2 CFU/m^3	Newson et al. 1983
	Gram-positive cocci	10^1–10^2 CFU/m^3	Newson et al. 1983
Office buildings	Culturable bacteria	>10^5 CFU/m^3	Reynolds et al. 2001
	Endotoxin	3.7 ng/m^3	Wan and Li 1999
		0.5–3.0 EU/m^3	Reynolds et al. 2001
Operating room	Culturable bacteria	10^2 CFU/m^3	Tjade and Gabor 1980
	Particles	10^2–10^5 particles/m^3	Seal and Clark 1990
Post office	Endotoxin	0.19 ng/m^3	Rylander et al. 1992
Recycling plant	Culturable bacteria	10^5 CFU/m^3	Reinthaler et al. 1999
Residential	Endotoxin	0.03–5.4 ng/m^3	Gorny and Dutkiewicz 2002
	Gram-negative	<dl–10^2 CFU/m3	Gorny and Dutkiewicz 2002
	Gram-positive	10^2–10^3 CFU/m3	Gorny and Dutkiewicz 2002
Sanitary landfill	Culturable	10^1–10^4 CFU/m^3	Rahkonen et al. 1987
	Total coliforms	<dl–10^3 CFU/m^3	Rahkonen et al. 1987
	Fecal streptococci	<dl–10^4 CFU/m^3	Rahkonen et al. 1987
Schools	Culturable bacteria	7–10^4 CFU/m3	Daisey et al. 2003
	Endotoxin	0.21–0.26 ng/m^3	Rylander et al. 1992
Waste handling	Culturable bacteria	10^4 CFU/m^3	Kiviranta et al. 1999
		10^4–10^5 CFU/m^3	Neumann et al. 2002
	Endotoxin	<10–50 EU/m^3	Neumann et al. 2002
	Gram-negative bacilli	10^3 CFU/m^3	Kiviranta et al. 1999
Wastewater treatment – activated sludge	Coliforms	0.27–5.17 CFU/m^3	Fannin et al. 1985
	Culturable bacteria	10^2 CFU/m^2/sec	Kenline and Scarpino 1972
		10^2 CFU/m^3	Haas et al. 2002
	Endotoxin	0.1–350 ng/m^3	Laitinen et al. 1994
	Escherichia coli	10^2 CFU/m^3	Ranalli et al. 2000
	Gram-negative bacilli	10^1–10^5 CFU/m^3	Laitinen et al. 1994
Wastewater treatment – aeration tanks	Culturable bacteria	10^1–10^3 CFU/m^3	Brenner et al. 1988
			Brandi et al. 2000
			Crawford and Jones 1979
			Keline and Scarpino 1972
	Fecal streptococci	10^2 CFU/m^3	Crawford and Jones 1979
	Streptococcus faecalis	<dl–10^2 CFU/m^3	Crawford and Jones 1979
Wastewater treatment – trickling filter	Coliforms	10^2–10^4 CFU/m^3	Adams and Spendlove 1970
	Endotoxin	<dl–185 ng/m^3	Thorn et al. 2002
	Total bacteria	10^1–10^3 CFU/m^3	Adams and Spendlove 1970

dl = less than detection limit; NQ = not quantitated.

survival of the cells in the bioaerosol (Lighthart and Stetzenbach 1994).

Airborne particulate will remain airborne until settling on to surfaces occurs. Bioaerosols ranging in size from 1.0 to 5.0 μm generally follow the air stream while larger particles are more readily deposited on surfaces (Mohr 2002). The settling of the particles is affected by physical and environmental factors. Air currents, relative humidity, and temperature are the most important environmental parameters while the size, density, and shape of the cell or bacteria-containing particle are the most significant physical parameters (Mohr 2002; Pedgley 1991). These parameters also affect the survivability of the cells and their ability to colonize surfaces or liquid when deposited.

Relative humidity is the most important factor in the survival of bacterial cells with low humidity resulting in desiccation. The bacterial cell membrane is adversely affected by loss of water, resulting in loss of viability (Cox 1987; Israeli et al. 1994), and low relative humidity has been shown to increase the resistance of some bacteria to ultraviolet irradiation (Ko et al. 2000; Mohr 2002). Increased temperatures also decrease the viability of airborne cells (Ko et al. 2000; Mohr 2002).

Vegetative bacterial cells are more susceptible to environmental stresses than bacterial endospores and vegetative gram-negative bacilli are more susceptible than gram-positive cocci. The greater resistance of vegetative gram-positive cocci is due in part to the presence of pigment and the composition of their cell wall. Carotenoid pigments and photoreactivation mechanisms in gram-positive cocci afford protection from sunlight (Henis 1987). The cell wall of gram-negative bacteria is actually an outer membrane composed of mucopeptides linked to lipopolysaccharide with a thin outermost layer of peptidoglycan that is only 5–10 percent of the structure. When exposed to heat, the lipopolysaccharide may be released resulting in injury and/or death to the cell. Gram-positive cells have a peptidoglycan layer that comprises up to 80 percent of the cell wall, affording more protection from drying and heat stress (Marthi 1994). The integrity of the ribosomes in various bacterial genera also contributes to recovery of heat-injured cells (Henis 1987). Several models have been presented to explain the complex interactions of physical, environmental, and physiological factors of bacterial survival in aerosols (Mohr 2002).

Bacterial aerosols can elicit adverse human health effects, including infection, allergic reaction, inflammation, and respiratory disease. The primary route of exposure to aerosols is inhalation. Approximately 10 m^3 of air per day are inhaled by the average human (Lynch and Poole 1979), but some ingestion and dermal exposure may also occur. Large airborne particles are lodged in the upper respiratory tract (nose and nasopharynx) and particles <6 μm in diameter are transported to the lung. One- and two-micrometer-sized particles have the greatest retention in the alveoli of the lungs.

Mycobacterium tuberculosis and *Legionella pneumophila* are recognized as respiratory pathogens that are capable of causing severe disease, but aerosol transmission of other bacteria can also result in illness. Allergic reactions and irritant responses to the foreign proteins of bacterial cells and fragments, and reactions to by-products of bacterial metabolism can result in adverse health. Inflammatory responses (Liu et al. 2000) caused by bacterial fragments present in airborne dust and allergic reactivity to *Pseudomonas* in wood dust (Skorska et al. 2002) have been reported. Disease transmission between farm animals via aerosols is also documented (Brockmeier and Lager 2002).

AIRBORNE BACTERIA ASSOCIATED WITH ADVERSE HUMAN HEALTH EFFECTS

Mycobacterium species

The dispersal of airborne infectious *Mycobacterium tuberculosis* (Kaufmann and van Embden 1993), nontuberculosis mycobacteria (Contreras et al. 1988), and *M. leprae* (Bryceson and Pflatzgraft 1990) are associated with severe illness. The transmission of *M. tuberculosis* via bioaerosols from infected people occurs during coughing, talking, speaking, laughing, and sneezing (Rubin 1991), and exposure to infectious aerosols has been reported during air travel (Driver et al. 1994). Nosocomial *M. tuberculosis* infections have been reported resulting from aerosols generated by the operation of suctioning instruments during treatment, manipulation of tuberculosis ulcers, drainage of necrotic tissue, and exposure during autopsy (Nottrebart 1980). Installation of ultraviolet lights in areas of increased risk of exposure to *M. tuberculosis* and other infectious bioaerosols has been shown to be effective, but efficacy was affected by the number of lights and the air exchange rate and presence of air currents in the area being treated (Ko et al. 2002).

Laboratory experiments with *Mycobacterium terrae* isolated from a water damaged building demonstrated inflammatory responses in mouse lung (Jussila et al. 2002) and mycobacteria of the *M. avium* complex commonly isolated from soil, water, and the air are associated with respiratory infection in immunocompromised patients (Wolinsky 1979). Discharge of 10^8 *M. leprae* bacilli has been reported from the nose blow of an untreated person with leptomatous Hansen's disease (leprosy) (Stewart-Tull 1982).

Legionella

Legionella pneumophila is a vegetative gram-negative bacterium that is ubiquitous in freshwater environments worldwide (Fliermans et al. 1981; Steinert et al. 2002). Exposure to *L. pneumophila* via inhalation of the bacterium in contaminated aerosols and the replication of *L. pneumophila* in macrophages results in severe pneumonia termed legionnaires' disease. The first reporting of this disease followed the outbreak of respiratory illness among American Legion conventioneers in Philadelphia in 1976 (Fraser et al. 1977). Since that time an estimated 10 000–15 000 cases per year are reported in the USA (Marston et al., 1993). Nineteen other *Legionella* species are also human pathogens, often causing disease in immunosuppressed patients (Muder and Yu 2002). Pontiac fever is a mild flu-like syndrome attributed to the inhalation of *Legionella* that cannot replicate in host tissue (Fields 2002; Kaufman et al. 1981).

Fraser (1984) developed a framework for causation of a legionnaires' outbreak that detailed six factors that must occur. These factors are: (1) a reservoir of legionella must be present; (2) amplifying factors must occur to permit the concentration of legionella to increase; (3) the legionella must be dispersed resulting in human exposure; (4) the legionella must be virulent to humans;

(5) the legionella must be delivered to the appropriate site in the human host; and (6) the host must be susceptible to infection by the legionella. Although the organism is present in most freshwaters, it survives as an intercellular parasite of protozoa, thereby resulting in protection for the bacterium.

Exposure to *Legionella*-contaminated aerosols occurs as the result of mechanical air-conditioning systems with water-spray components, cooling towers, and aerosol-generating water devices (e.g. showerheads, faucets, whirlpool tubs, decorative fountains, humidifiers), but no human-to-human transmission has been documented (Mohr 2002).

Other gram-negative bacteria

Airborne gram-negative bacteria are associated with animal facilities with *Escherichia coli*, *Pantoea* (*Enterobacter*) *agglomerans*, *Pseudomonas* spp., and *Acinetobacter* spp. commonly isolated in cow barns, pig houses, and poultry barns (Zucker et al. 2000). Wastewater treatment plants (Brandi et al. 2000; Brenner et al. 1988; Crawford and Jones 1979; Kenline and Scarpino 1972) and recycling facilities (Reinthaler et al. 1999) also disperse gram-negative bacteria into the air. Dutkiewicz et al. (2001b) reported skin-test reactivity of herb-processing workers to extracts of *Alcaligenes faecalis*. These bacteria can cause a variety of health problems, especially to the young, elderly, and immunocompromised.

Endotoxin

Endotoxins are biologically active materials that affect humoral and cellular host mediation systems (Bradley 1979) and affect many organ systems (Hewett and Roth 1993). Endotoxin is an integral component of the gram-negative bacterial cell wall that is released during active cellular growth and after cell lysis (Bradley 1979). It has also been reported that endotoxin is shed in membrane vesicles when the organisms are associated with organic dust (Dutkiewicz et al. 1992).

Exposure to airborne endotoxin is a concern in occupational settings with high concentrations of organic dusts (e.g. cotton-processing facilities, agricultural settings) and industrial environments with water-spray components (e.g. machining fluids) (Gordon 1992). Airborne endotoxin is also a concern in indoor environments with humidified mechanical air-conditioning systems (Dutkiewicz et al. 1988; Flaherty et al. 1984), and the presence of dogs, moisture, and settled dust increases the likelihood of airborne endotoxin in the home (Park et al. 2001).

Symptoms of chest tightness, cough, shortness of breath, fever, and wheezing have been reported by individuals exposed to airborne endotoxin in industrial and agricultural settings (Donham et al. 1984; Lundholm and Rylander 1980; Thelin et al. 1984). Kline et al. (1999) report airflow obstruction and exacerbation of asthma related to airborne endotoxin concentration. Atopic and nonatopic asthma have been associated with endotoxin exposure because of its ability to induce inflammatory reactions (Michel 1999), and repeated inhalation exposure has been shown to induce allergen-specific airway inflammation (Wan et al. 2000). However, childhood exposure to endotoxin in a rural farm setting has been shown to provide some protection from the development of atopic childhood diseases (von Mutius et al. 2000)

Gram-positive nonspore-forming bacteria

Airborne *Staphylococcus* spp. and *Micrococcus* spp. are nonendospore-forming bacteria that are commonly disseminated from the skin, oral and nasal surfaces, and hair of humans (Favero et al. 1966). The presence of these cells in the air increases the likelihood of nosocomial infections in healthcare settings (Schall 1991). Antibiotic-resistant gram-positive bacteria in hospital environments are transmitted among patients through aerosol dispersal from inanimate surfaces, patients, and staff members (Shiomori et al. 2001). Airborne dispersal of pathogens is also a concern in home-care and assisted-living environments, where infection-control practices may not be strictly followed. Actinomycetes are filamentous nonendospore-forming gram-positive bacteria that have been associated with illness in occupants of water-damaged buildings (Hyvarinen et al. 2002). Their presence is reported in association with numerous fungi that colonize following water intrusion and colonization on building materials and furnishings, and concentrations of 10^4 CFU/m^3 have been reported during repair of damaged structures (Rautiala et al. 1996).

Bioterrorism

The threat of purposeful aerosol release of pathogenic bacteria and bacterial toxins as weapons of mass destruction (WMD) has increased the awareness of governmental agencies, the medical community, and the public of the importance of bioaerosols (Sheeran 2002). Several pathogenic bacteria have been listed as possible bioaerosol WMDs, including *Francisella tularensis*, *Yersinia pestis*, and *Bacillus anthracis*, the causative agents of tularemia, plague, and anthrax, respectively. An alert public-health system is needed to recognize these diseases and minimize exposure of the public (Dennis et al. 2001), and additional research is needed to evaluate the risk of exposed populations (Dull et al. 2002).

MONITORING FOR AIRBORNE BACTERIA

Routine monitoring for airborne bacteria is performed in pharmaceutical and food-industry settings, but

monitoring surveys in indoor environments are generally conducted in response to: (1) physician documented illness linked to occupancy of building; (2) visual evidence of biological contamination; (3) occupant complaints after a water damage incident; or (4) occupant complaints that are suggestive of biological contamination. However, documentation of bacteria in the air or on surfaces does not prove that they are responsible for disease in building occupants. Serologic tests or other diagnostic methods may be needed to directly correlate exposure with disease.

Prior to conducting the actual collection of samples, a strategy for testing should be formulated (Burge 1995) and an understanding of the water intrusion/water damage history of the building is needed (Macher 1999). Air sampling is used to document concentrations of airborne contaminants and assess exposure (Macher 1999), but air sampling alone does not provide assurance that a suspect area is free of biological contamination (Higgins et al. 2003). Cells growing on surfaces are aerosolized and settled cells become reaerosolized during routine activity (Buttner and Stetzenbach 1993; Weis et al. 2002). Therefore, surface sampling should be performed in addition to air sampling to assist in determining areas of contamination and in identifying the source of biocontamination. Surface sampling is also used in determining the effectiveness of remediation (Andersson et al. 1997; Buttner et al. 2002; Higgins et al. 2003).

Collection of airborne bacteria involves forced airflow sampling (Buttner et al. 2002). This type of sampling provides a means to determine the concentration of organisms in the bioaerosol per unit volume of air, but the specific collection method and the analysis method(s) used will influence the results. Commercially available forced airflow impaction samplers for use in the collection of bacteria deposit airborne particulate on to a semisolid agar surface. Impingement samplers collect airborne cells and other particulate into a liquid. Both impaction on to agar plates and impingement have been widely used for the collection of airborne bacteria (Buttner et al. 2002). Single-stage impactor samplers operated at 28.3 l/min for 2 min provide a lower detection limit of 18 colony forming units per cubic meter of air sampled (CFU/m^3) and an upper limit of 10^4 CFU/m^3 although at the higher concentrations considerable error is introduced as multiple cells are deposited on the same location of the agar surface. Increased sampling time beyond 3–5 min with agar-based impactor samplers results in drying of the agar surface decreasing the physical collection of airborne particles and decreasing the viability of the bacterial cells (Buttner et al. 2002). Impingement samplers collect cells in a buffered liquid. Commonly used impingement samplers designed for the collection of bacteria are operated at 10–12 l/min, but high-velocity samplers have been developed that collected larger volumes of air over extended sampling times (Radosevich et al. 2002). Collection of airborne cells into buffer permits manipulation of the sample by dilution or concentration to maximize accuracy in quantitation and affords the application of a variety of analytical methods, including culture, microscopy, immunoassay, flow cytometry, and molecular methods (Buttner et al. 2002). However, as observed with the agar-based impactors, extended sampling times may result in increased sampling stress, thereby decreasing the viability of the collected bacteria (Alvarez et al. 1994). The use of filtration sampling for monitoring of bacterial aerosols also results in dessication of bacterial cells, so this technique is generally used for sampling of airborne dust, fungal spores, and pollen (Crook 1995b). The use of settling plates or gravity sampling is not recommended for determination of airborne bacteria as it does not result in a representative sampling of airborne cell due to differential settling of particles from the air (Crook 1995a).

A variety of sampling methods have been used for the collection of surface-associated bacteria. Agar-filled replicate organism detection and counting (RODAC) with a convex sampling surface are used in hospital infection control to validate the efficacy of disinfection of surfaces. RODAC plates and agar-filled surface sampling swatches or strips are commercially available for sampling of smooth, hard surfaces in indoor environments. Sterile swabs are used to sample irregular, nonporous surfaces and they have been used to sample hard surfaces (Buttner et al. 2001; Higgins et al. 2003). Collection of settled dust in porous (e.g. carpeting, upholstery, clothing) and nonporous materials (e.g. flooring, horizontal surfaces, and furnishings) is a means to sample large surface areas and provides material for a variety of analysis methods (Macher 2001). Bulk sampling is a destructive sampling method that necessitates the removal of material from the sample site and, therefore, is used when materials are to be replaced regardless of the outcome of the monitoring (e.g. water-damaged carpet and pad, water-damaged or stained wallboard and wallpaper, and stained ceiling tile). Material collected with vacuum or bulk sampling is processed with a buffer solution containing a surfactant to assist in the separation of microorganisms from the dust and other particulates in the sample.

The agar-based impaction sampling method is limited to culture analysis. The other sampling methods can be analyzed using culture, chemical assay, and/or molecular biology. Limitations of culture-based assay include the requirement that the bacterial cells be viable/culturable at the incubation conditions and on the growth medium selected. For example, a series of media amended with antibiotics and growth factors are needed for the isolation and identification of *Legionella pneumophila* (Fields 2002), and the isolation of *Mycobacterium* species requires incubation for extended periods of time.

Stressed organisms in a bioaerosol may also require resuscitation (Crozier-Dodson and Fung 2002).

Limulus amebocyte lysate assays are used for the analysis of endotoxin (Olenchock 1990), but variations in extraction efficiency and the protocols for analysis result in differences in the results reported (Reynolds et al. 2002). A variety of analytical chemistry methods and flow cytometry can be used to identify and enumerate airborne cells (Spurny 1995). Assays for muramic acid, peptidoglycan, or fatty acids are used to detect markers of bacterial cells and endotoxin (Laitinen et al. 2001; Liu et al. 2000). Polymerase chain reaction (PCR) amplification and quantitative polymerase chain reaction (QPCR) have been shown to provide enhanced detection and rapid identification of specific airborne and surface-associated bacteria (Alvarez et al. 1994; Buttner et al. 2001; Higgins et al. 2003; Pascual et al. 2001). The use of molecular techniques requires the development of primers and probes to unique gene sequences and optimization of protocols, but the increased sensitivity and specificity of these methods, and the rapid reporting of data, provide a means to enhance the capability to monitor for contaminant airborne and surface-associated bacteria in indoor environments.

SUMMARY

Human-source and environmental bacteria can colonize and grow on a variety of indoor and outdoor surfaces when conditions are appropriate. Dispersal of bioaerosols from these surfaces and resulting exposure to bacteria and bacterial components may cause adverse human health effects. The dispersal, transport, and settling of bioaerosols is determined by physical and environmental factors, but health effects depend on the bacterial agent(s) within the aerosol and characteristics of the exposed population.

Monitoring for bioaerosols requires selection of appropriate sampling and analysis methods to determine the concentration and population of airborne bacteria and their components. Monitoring in indoor environments should also include surface sampling to determine the source(s) of bacterial contaminants. New information on human exposure to bioaerosols is being gathered with the assistance of enhanced technologies for collection and analysis, and this knowledge should provide assistance in determining risk of exposure and in understanding the fate and transport of airborne bacteria.

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Bacteriology of soils and plants

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INTRODUCTION

Prokaryotic organisms profoundly affect virtually every facet of life on our planet. While soil- and plant-associated microbes remain unseen and underappreciated by most people, all life on Earth depends on their activities. Indeed, literally billions of years before the arrival of our first animal ancestors, prokaryotes were busy transforming Earth into the habitable planet we now enjoy. Prokaryotic autotrophs were largely responsible for the cooling of our planet by removal of CO_2 from Earth's atmosphere. The evolution of oxygenic photosynthesis in the Cyanobacteria, long before the appearance of plants, created the aerobic conditions that enabled oxygen-using higher life forms to arise. Indeed, the widely accepted theory of serial symbiosis postulates that the first eukaryotic organelles (mitochondria, chloroplasts) arose from prokaryotic symbionts (Margulis 1971). In recent years our comprehension of the abundance and diversity of the soil- and plant-associated microbiota has increased dramatically, but it is clear that good stewardship of Earth's resources will require a still deeper understanding of the microbial communities around and within us.

Microbial ecology, the study of how prokaryotes and other microorganisms interact with their physical and biotic environment, spans a number of component disciplines. Historically, methodologies for the study of soil prokaryotes have evolved in a different framework from the study of plant-associated microbes, often deriving from a different scientific perspective and with different research goals. As Paul and Clark (1989) noted, the primary thrust of soil microbiology has been on the metabolic activities of soil-inhabiting organisms, especially their roles in the biogeochemical energy flow and nutrient cycling, e.g. primary production and carbon mineralization, nitrogen fixation, nitrification and denitrification, sulfur transformations, etc. Less attention has been focused on soils as reservoirs for some animal pathogens. In contrast, approaches to the study of plant-associated microbes have tended to focus on the interspecific interactions between plants and microbes, especially the mutualistic relationships (e.g. mycorrhizae, rhizobia–legume symbioses) and the vast array of plant diseases that are of microbial origin. By definition, plant-associated prokaryotes do not lend themselves to pure culture studies, and there has been relatively less attention paid to the measurement of metabolic activity of plant-associated microbes, compared with those in soil.

SOIL AS A HABITAT FOR PROKARYOTES

Soil quality is the primary determinant of plant productivity in all terrestrial ecosystems, whether they are natural or agricultural. Soils are as much biological

entities as they are physical ones, formed by a variety of interactions that include the weathering of rock parent material, microbial autotrophic primary production, heterotrophic decomposition of plant and animal materials, and myriad mineral transformations and redistribution of materials. Viewed in cross-section, soils typically are described as series of horizons or layers (soil profile), with each layer being approximately parallel to the surface and with a unique set of characteristics. If a layer of organic litter is present it is designated the 'O' horizon; next below that is the 'A' horizon, which represents a zone where plant residues accumulate, and from which materials leach downwards. Below that is the 'B' horizon, which is a zone of accumulation of minerals and humic materials, but typically containing less total organic matter than the A horizon. Finally, the lower 'C' horizon consists of unconsolidated parent material and geologic deposits. There are a number of variations and subdivisions of the above classification scheme, which taken together (and with additional characteristics) provide a useful taxonomy of soils. Bacteria are usually the most numerous organisms in soils, with commonly as many as 10^8–10^9 cfu/g of soil (Metting 1993). In terms of biomass, prokaryote biomass typically is similar to that of fungi in the upper horizons of aerobic soils and may exceed the biomass of plant parts in soil. Typically, prokaryote soil biomass ranges from about 300 to 3 000 kg (wet weight) per hectare (Metting 1993). Microbial biomass in general is very much lower in the lower soil horizons but is dominated by prokaryotic organisms.

A variety of properties determine the suitability of a soil as a microbial habitat. These include, but are not limited to, texture, structure, water content, aeration, pH, mineral nutrient content, and soil organic matter (SOM) content. It is important to realize that each of these properties typically is discontinuous in space, often at a scale of only millimeters or even micrometers. The term 'soil texture' refers to the relative proportions of the three inorganic particulate constituents of soil: clay, silt, and sand. The sand and silt components of soil are relatively large and chemically inert, and by themselves are not sites of significant microbial activity. The nonliving colloidal materials in soil (particles less than 2 μm in size) consist of inorganic clay minerals, composed of aluminosilicate layers, as well as organic humic materials. These colloidal materials have a profound effect on microbial activity. Clay particles may have as much as 8×10^6 cm^2 of surface area per gram, compared with approximately 450 cm^2/g for silt particles and 20 cm^2/g for coarse sand. While the layers of so-called 1:1 clays (consisting of silica + alumina layers) are held together by hydrogen bonds, the layers of 2:1 clays (silica + alumina + silica) are not tightly held together, and thus these clays are highly expandable when water is present and water molecules are interspersed between the layers. Thus, the 2:1 clays have potentially much greater reactive surface area than the 1:1 clays. This is critical for chemical activity at clay surfaces, including microbial biochemical activity, because the negatively charged surfaces of clay minerals are sites of active cation exchange and repulsion of anionic molecules. Humus represents the organic colloidal component of soils; it consists of organic molecules that originated from living organisms but that have been biochemically modified and are no longer recognizable. Humic materials, like clays, have a net negative charge and high cation-exchange capacity (CEC), which enhances their interactions with synthetic organic compounds, metals, nutrients, as well as bacteria.

Soil structure, which is critical to below-ground air and water movement, water-holding capacity, and soil stability, occurs through the formation and spatial arrangement of aggregates. Soil bacteria contribute greatly to soil structure through their production of extracellular polysaccharides and other cementing compounds that help hold aggregates together, although these same compounds may also damage structure by blocking pore spaces. The majority of active bacteria are adsorbed on to particle surfaces in soil or are located at gas–liquid interfaces. Adsorption on to particles provides some degree of protection from predation by protozoa and soil arthropods, and it also places the bacterial cells in close proximity to adsorbed mineral nutrients. Additionally, extracellular enzymes produced by the bacteria also may be adsorbed nearby, so that the products of those enzymes will be more readily available for absorption, rather than being lost by diffusion into the soil solution. It should be remembered that prokaryotes are primarily aquatic organisms, and that at the prokaryote scale, soils often are functionally aquatic habitats. About 30 percent of the volume of a typical well-drained soil is in the liquid phase with another 20 percent in the gaseous phase and the remaining 50 percent as solids. Water in a well-drained soil typically is discontinuous, and its distribution is a function of the soil pore size distribution as determined by soil texture and structure. Through their effects on soil structure, bacteria effectively determine much of their surrounding physical environment. Aerobic and facultative aerobic bacteria also play a role in the spatial distribution of oxygen within and around soil aggregates. Oxygen diffuses only slowly through water, so that water-filled pores form effective seals around portions of aggregates, and oxygen within those zones may be depleted by aerobic respiratory activity and then maintained at low levels by the scavenging of oxygen by facultative anaerobes. In this way, anaerobic microsites are formed and maintained, even in otherwise well-aerated soils. Facultative anaerobes and even strict anaerobes can thus be active within aerated soils, and important anaerobic processes (e.g. denitrification) can take place in these microsites. Even under conditions where microsites become oxic, strict anaerobes may be able to persist

although active growth will not occur. There are two basic ways that strict anaerobes may survive oxic conditions: first, a number of strict anaerobes, for example *Clostridium* spp., form dormant spores. Also, vegetative dormancy may allow cells of a strictly anaerobic species to survive oxic periods. The compounds hydrogen peroxide and superoxide are byproducts of certain oxygen-utilizing reactions, and these compounds are destructive to cells. Aerobic organisms have enzymes that destroy these agents, for example the enzymes superoxide dismutase (which converts superoxide to the products O_2 and H_2O_2) and catalase (which then converts H_2O_2 to water and oxygen). Strict anaerobes lack these enzyme systems; however, because the toxic byproducts are only formed during active metabolism, physiologically dormant anaerobes may escape their effects.

PLANT-ASSOCIATED PROKARYOTES

The metabolic activity of soil bacteria contributes greatly to a soil's fertility and its ability to support plant growth. In turn, plant residues and root exudates are the most important food source for the free-living heterotrophic microbial populations in soils. Also, plant surfaces and interiors are themselves important habitats for microorganisms, and nutrients leaking from aerial plant parts support considerable microbial growth on plant surfaces. Indeed, some microorganisms are able to grow only in association with plants. I will use the terms 'rhizosphere' and 'phyllosphere' as nouns to describe plant-associated habitats and as adjectives to describe the microbial components of those habitats.

The rhizosphere

The term 'rhizosphere' was first used by Hiltner (1904) to describe the interaction between bacteria and the roots of legumes. A somewhat analogous term, 'spermosphere,' refers to the area of increased microbial activity around a seed. Organic and inorganic materials released from roots, leaves, and seeds are important determinants of microbial activity, numbers, and interactions on plant surfaces and in the adjacent soil. In turn, the plant-associated microbial community strongly influences plant health and productivity. Typically, the rhizosphere may extend up to 5 mm or slightly more away from the root surface. As Lynch (1987) noted, the rhizosphere environment actually is comprised of the interacting plant, adjacent soil, and associated microorganisms. Microbial habitats within the rhizosphere are sometimes further classified as endorhizosphere (within the root tissue itself) or rhizoplane (on the root surface). Populations of microbes in the rhizosphere differ quantitatively and qualitatively from those in the bulk soil; their numbers are generally higher, and different populations commonly are represented. Plant roots release compounds, including simple sugars and amino acids, that encourage the growth of specific microbial communities. Because rhizosphere inhabitants rely heavily on organic exudates for their carbon and energy requirements, their metabolism may be closely tied to that of the plant (Bowen and Rovira 1976; Lynch 1987).

Although many rhizosphere microbes form apparently commensal relationships with plant roots, there are numerous well-known relationships involving mutualism and/or parasitism. Roots of many plants form an intimate relationship with certain fungi; the resulting mycorrhizal association results in enhanced uptake of certain mineral nutrients including phosphorus. Legumes and some other plants form symbioses with nitrogen-fixing bacteria. The mutualistic relationship between legumes and *Rhizobium* bacteria allows the plants to grow in otherwise nitrogen-deficient soil and enhances the soil's nitrogen status for other plants. Some microbes in the rhizosphere or phyllosphere are plant pathogens. With the exception of some vectored or seed-borne organisms, most plant pathogens first contact the plant at the phylloplane or rhizoplane. While some fungal plant pathogens are well equipped to breach these surfaces mechanically or enzymatically, many others spend much of their life cycle as nonparasitic rhizosphere or phyllosphere inhabitants and enter a parasitic phase only when conditions are conducive to passive entry and subsequent colonization of plant interiors. In contrast, some microbes in the rhizosphere or phyllosphere are antagonistic to plant pathogens or insect pests, and thus serve as potential biological control agents for agriculture and forestry.

Plant roots modify the surrounding soil environment in various ways. Almost all chemical components of the plant have been found to be lost from roots. Root deposition includes exudates that contain water-soluble compounds such as sugars, amino acids, organic acids, hormones, and vitamins, as well as sloughed whole cells and volatile compounds. It is generally believed that the zone just behind the root tip is the site of maximum root exudation. As roots elongate through soil, cells are sloughed off from the root cap, and root hairs and cortical cells are sloughed from the surface. Deposition from roots provides a readily used energy source for microbes, and spatial variation in the components of rhizodeposition over the root surface greatly influences variability in the composition of the rhizosphere community (Whipps 1990). Other plant influences on the rhizosphere community include the removal of soil water by plant uptake, release of extracellular enzymes, and reduced oxygen tension and the release of CO_2 due to aerobic respiration, with an accompanying localized increase in soil acidity. In turn, microbes associated with plant roots modify the rhizosphere environment by producing extracellular enzymes and plant growth factors and by forming parasitic or mutualistic

relationships with the plant. As a consequence, the quantities and types of available substrates in the rhizosphere differ from those in the bulk soil, resulting in different compositions of the respective microbial communities. This so-called 'rhizosphere effect' reflects the influence of plant roots on the size and composition of the surrounding soil microbial community. One numerical measure of rhizosphere effect is the R/S (rhizosphere/soil) ratio, which compares the total number of microbes in a rhizosphere habitat with the number in the root-free soil. R/S ratios range from as low as 5 to 100 or more but most frequently are from 10 to 20 (Curl and Truelove 1986; Gray and Parkinson 1968; Katznelson 1965). Absolute size estimates of the rhizosphere vary greatly, depending in part on the plant species and spatial location on the root, which is reflective of physiological maturity (Darbyshire and Greaves 1970) as well as soil temperature and moisture content.

The phyllosphere

Aerial plant parts, both surfaces and interiors, are important habitats for bacteria and support both autochthonous and allocthonous microbial populations. The term 'phyllosphere' has a similar etymology to the term 'rhizosphere' and has been applied to the habitats including plant interiors (also called the endophytic habitat) as well as plant surfaces (sometimes referred to as the phylloplane). Microorganisms colonizing the aerial parts of plants are sometimes called epiphytic microbes (from the Greek, *epi* [on top of] and *phytos* [plant]), but unlike the epiphytic plants that derive no nutrition from the plant surfaces they inhabit, epiphytic microbes actively utilize compounds exuded from leaves and other aerial plant parts. In the tropics, species of nitrogen-fixing bacteria are important inhabitants of foliar surfaces. Although noncolonizing (allocthonous) microbes frequently can be found in the phyllosphere, there is substantial evidence of defined microbial succession and the development of specific phyllosphere communities (Blakeman 1981). On new leaves, bacteria predominate in the microbial community, while yeasts and filamentous fungi succeed them and become dominant later in the growing season (Kinkel 1991; Kinkel et al. 1987). The important environmental factors impacting phyllosphere populations include ambient and leaf surface temperature, relative humidity and leaf wetness, and solar radiation. Leaf surfaces can be harsh habitats, since microbes on them are exposed to radiation and the dessicating effects of wind and sunlight, yet populations of 10^6 or more bacteria per square centimeter of leaf surface may be supported. Generally, environmental factors fluctuate more rapidly in the phyllosphere than in soil, so that microbial numbers can differ greatly even between samples taken over a short time period.

With the exception of Cyanobacteria (which are able to fix both C and N) on the foliar surfaces of some tropical plants, plant foliar leachates serve as the primary source of carbon and mineral nutrients for epiphytic bacteria. These leachates include a variety of sugars, organic acids, and amino acids, and their transfer from the leaf interior to the leaf surface takes place via aqueous pores (Blakeman and Atkinson 1981; Tukey 1971). Some epiphytic bacteria may themselves increase leaf wettability by the production of biosurfactant compounds (Bunster et al. 1989). Many epiphytic bacteria appear to be commensals and do not harm the plant upon which they reside. However, aerial plant surfaces may be successfully colonized by virulent strains of phytopathogenic bacteria, as well as by nonpathogenic isolates of the same or related species (Hirano and Upper 1983).

Bacteria in several genera, whether naturally occurring or modified by recombinant DNA techniques, offer promise for foliar application to crops for control of insect pests, plant pathogens, and weeds, or to protect plants from frost injury (e.g. Armstrong et al. 1987; Blakeman and Atkinson 1981; Knudsen and Hudler 1987; Lindow et al. 1988). Research on microbial pest-control agents for foliar application has progressed to where field tests are needed to evaluate their performance. The first genetically engineered microorganisms to be released legally in the USA were strains of *Pseudomonas syringae* and *P. fluorescens* from which the ice nucleation gene was deleted; these were intended to competitively exclude indigenous ice nucleation-active bacteria involved in frost damage. Small-scale field releases of aerosolized ice-nucleation-minus bacteria were undertaken by the University of California at Berkeley, and there was a large investment in time, money, and effort in attempts to determine dispersal patterns and survival of the applied bacteria (Knudsen 1988; Lindow et al. 1988). Concerns about possible environmental effects of genetically engineered microorganisms applied to crop plants include uncertainty about their dispersal, survival, and interactions with indigenous organisms, and this interest has fueled much of the recent research on epiphytic bacteria.

Soil, the rhizosphere, and the phyllosphere are populated by complex communities of organisms, including microbes (bacteria, Archaea, fungi, Protists) but also typically including plant and animal components. The rhizosphere and phyllosphere are defined by the presence of a plant, so it is inherently impossible to characterize microbial activity in these habitats through the study of genetically uniform microbial strains in isolation. Determining microbial contributions to these communities may require a variety of techniques with different areas of focus. For example, it has long been known that microbes may be metabolically active even though they are not culturable (Colwell et al. 1985). Furthermore, quantification of the viable microbial

biomass may help elucidate the potential for specific microbial metabolic activities in a particular habitat, but the nonviable biomass may also be important as a component of the food web, despite its lack of a direct metabolic contribution. Although it is possible and common to make inferences about microbial biomass based on microbial community metabolic activity, it is important to note that these may not be strongly correlated, i.e. high metabolic activity does not necessarily mean an actively growing, dividing microbial community. Also, it is important to remember that microbial physiological activities are often strongly affected by the physical arrangement of the members of a population, especially aggregations such as those that occur in biofilms, on root surfaces, and in soil microsites. Physiological and spatial heterogeneity have important implications for the ecology of microbial populations and communities, and the ability of recently developed molecular and microscopic methods to address heterogeneity in environmental samples is especially exciting.

There has been an explosion of interest in the subject of microbial biofilms in recent years. The majority of microbes in natural ecosystems are not free-floating (planktonic) but instead typically grow attached to surfaces and encased in an adhesive, usually a polysaccharide material, forming complex communities called biofilms (Costerton et al. 1986). Bacteria on plant surfaces such as leaves and roots may form biofilms, and development of plant-surface biofilm communities and regulation of biofilm polysaccharide production has been studied for several root-colonizing bacteria (e.g. Hax and Golladay, 1993; O'Toole and Kolter 1998; Sarand et al. 1998). There is great potential for the use of biofilm bacterial communities on plant roots for bioremediation of soil pollutants (Yee et al. 1998). Techniques used to investigate root biofilm communities have included confocal microscopy with or without targeted ribosomal DNA (rDNA) probing, scanning electron microscopy (SEM) and transmission electron microscopy (TEM), and polymerase chain reaction (PCR)-assisted rDNA restriction fragment length polymorphism (RFLP)/sequence analyses.

BIOGEOCHEMICAL TRANSFORMATIONS BY SOIL- AND PLANT-ASSOCIATED PROKARYOTES

Largely unheralded by humans, the Bacteria, Archaea, and Fungi drive the biogeochemical cycles that make this planet habitable for plants and animals. Entire volumes detail the roles of the prokaryotes in carbon fixation and decomposition or organic compounds, in the cycling of nitrogen, sulfur, phosphorus, and other elements essential to life, and in transformations of metals. We have only started to understand and appreciate the astounding degradative capacity of the prokaryotes and to apply it to remediation of natural and man-made environmental pollutants. Below is a short summary of a few of the major biogeochemical roles that prokaryotes play.

Prokaryote contributions to carbon cycling

Availability of mineral nutrients is an important limitation for most bacteria in soil and on plant surfaces. Available carbon typically is less limiting. Heterotrophy, which is the utilization of fixed organic substrates as a carbon source, is the dominant lifestyle for most soil- and plant-associated prokaryotes. The autotrophic representatives of these habitats (i.e. those organisms that are able to fix CO_2 or carbonates for their carbon needs) include relatively small numbers of the photoautotrophic Cyanobacteria, which inhabit some surfaces that are exposed to light (e.g. some soil and plant surfaces, swampy soils, and exposed surfaces as cosymbionts with ascomycetous fungi in lichens). Several chemoautotrophs are present in soils, most significantly the nitrate- and nitrite-oxidizing nitrifiers, as well as those bacteria that aerobically oxidize reduced forms of sulfur, iron, or manganese for their energy needs. Methanogens, which are members of the Archaea, are able to fix CO_2 and/or carbonates and are important in anaerobic sediments. However, the overall contribution of microbial autotrophy to the carbon balance of soil or plant habitats is relatively minor compared with the importance of heterotrophic decomposition of plant (and animal) materials. Within the constraints of mineral nutrient availability, especially nitrogen, heterotrophic bacteria in soil quickly colonize any organic matter that is added. Because bacteria are generally limited to the surfaces of organic particles, the size of particles (i.e. surface to volume ratio) is critical in determining the extent that this resource can be exploited. Soil arthropods can play an important role here, as their feeding activity shreds and mixes the plant litter, thus exposing it to bacterial activity. Arthropods may also increase available nitrogen by excreting uric acid.

Many bacteria escape limitations imposed by lack of nutrients by dormancy, either in the vegetative state or as spores. When a nutritional source becomes available in soil, typically there is a temporal succession of microbes that are able to utilize the resource, as the quality of the available substrates changes during decomposition. Those first able to colonize the resource usually are r-selected (Andrews and Harris 1986) bacteria such as *Bacillus* and *Pseudomonas*, as well as fungi such as *Penicillium* spp., which are able to rapidly utilize available sugars and amino acids. They are followed by a variety of K-selected (Andrews and Harris 1986) organisms that include prokaryotes such as Cyanobacteria but also a number of fungi. Many of these late-succession organisms either are stress-tolerant or possess

highly competitive mechanisms such as cellulose- or lignin-degrading enzyme systems. Production of antibiotic compounds may also play a role in competition among soil microorganisms, although production of antibiotics in situ, as well as its ecological significance, have been difficult to demonstrate (Williams 1986). Only relatively recently has antibiotic production in soil been definitively shown to occur (Thomashow and Weller 1991), and it is possible that the level of antibiotics is still very low because of adsorption to clay minerals and humic materials. Many of the antibiotics that are so important in the treatment of infectious diseases in medical and veterinary practice, derive from soil microbes, especially the Actinomycetes.

Prokaryote contributions to nitrogen cycling

Nitrogen is a critical element for all forms of life. It is a building block of nucleic acids as well as amino acids, and thus is a major component of the genetics, physiological activity, and structural integrity of all organisms. Although the cellulose and lignin molecules that provide structural rigidity to plants are free of nitrogen, it still is the fourth most abundant element in plant composition, after C, H, and O. It is the mineral nutrient that most often is limiting for plant nutrition. Nitrogen is a cell-wall component of the majority of microbes, part of the chitin molecule found in the higher fungi, and the peptidoglycan molecule found in bacterial cell walls. The ratio of carbon to nitrogen varies among microorganisms, but generally it is significantly lower than in plants or in soil. Typical C:N ratios of fungi range from about 4.5:1 to 15:1, and for bacteria it usually is even lower, in the range of 3:1 to 5:1. By way of comparison, the C:N ratio in a typical arable soil is about 10:1, about 25:1 in green-plant material, and as much as 100:1 in dried plant residues such as straw. For this reason, nitrogen availability is usually limiting when plant materials are being decomposed in soil. The microbiota quickly mineralizes the nitrogen that is present in plant residues and immobilizes much of it in the form of microbial biomass.

Along with its occurrence as a component of organic molecules, the N atom can be found in a number of different inorganic molecules of differing oxidation states. Microbes mediate most of the transitions between these states and thus play essential roles in nitrogen cycling. The NH_4^+ (ammonium) form is the product of microbial mineralization of organic nitrogen compounds in soil and is readily assimilated by both plants and microorganisms. Because it is a cation, the ammonium ion may attach to negatively charged clay minerals and become entrapped or 'fixed' between the layers. This process, ammonium fixation, should not be confused with dinitrogen fixation, which is the transition (most commonly microbially mediated) of atmospheric N_2 into ammonia and subsequently to organic nitrogen in biological systems. Under basic soil conditions, NH_4^+ ions will be deprotonated to NH_3; this form may volatilize and be lost from soil, especially under warm and dry conditions.

The aforementioned nitrifying bacteria are aerobic autotrophs that oxidize ammonium to nitrite (NO_2^-) and then oxidize nitrite to nitrate (NO_3^-), as a source of energy. The latter step occurs readily and nitrite rarely accumulates in soil. Different autotrophic nitrifying bacteria are responsible for each of these steps, e.g. *Nitrosomonas* oxidizes ammonium to nitrite, while *Nitrobacter* oxidizes nitrite to nitrate. Heterotrophic nitrification also occurs and may be especially significant in forest soils. Although heterotrophic nitrification primarily is done by fungi, a number of aerobic bacteria have been shown to nitrify heterotrophically, including species of *Arthrobacter*, *Aerobacter*, *Mycobacterium*, *Streptomyces*, *Thosphaera*, and *Pseudomonas*. Nitrate is a preferred nitrogen source for many plants and thus nitrification may increase soil fertility. However, because NO_3^- is an anion, it is repelled by the negatively charged surfaces of clay minerals and humic materials, and thus it is highly mobile in soil solution. Nitrate leaching and movement into groundwater is a significant health and environmental problem. Excessive nitrate levels in drinking water are the cause of infant methemoglobnemia, or 'blue-baby syndrome', as the infant gut favors formation of nitrite, which in turn binds to hemoglobin and prevents it from accepting and transferring oxygen. Nitrates in drinking water are also linked to formation of carcinogenic nitrosamines. Environmentally, nitrates in groundwater provide a nitrogen input to lakes and ponds and encourage growth of algae and subsequent eutrophication of the water body.

Nitrate is also susceptible to loss from soil through the process of denitrification, in which various facultatively anaerobic bacteria (e.g. *Pseudomonas denitrificans*, *Thiobacillus denitrificans*) utilize NO_3^- as a terminal electron acceptor in anaerobic respiration. The pathway of denitrification is: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The gaseous intermediates NO (nitric oxide) and N_2O (nitrous oxide) are always released during denitrification and are atmospheric pollutants that are involved in catalytic destruction of atmospheric ozone.

DINITROGEN FIXATION

For most plants, the direct acquisition of nitrogen is primarily from uptake of the mineral forms NH_4^+ and NO_3^-, which are derived from organic matter decomposition in soil. However, a significant amount of the nitrogen used by some plants comes directly from the biological fixation of atmospheric nitrogen (N_2). Biological nitrogen fixation, which is the conversion of atmospheric dinitrogen to ammonia, is mediated by the nitrogenase enzyme system, which is unique to prokaryotes. A number of free-living and plant-associated

bacteria fix nitrogen in terrestrial ecosystems. Approximately two-thirds of all the nitrogen fixed globally is through biological dinitrogen fixation.

Nitrogen fixation requires relatively large amounts of energy, either in the form of light energy for the phototrophic Cyanobacteria or as energy stored in the chemical bonds of organic compounds for the heterotrophic nitrogen-fixing bacteria. The symbiotic association with plants is valuable for associative nitrogen-fixers for this reason, and the symbiotic legume–rhizobia and alder–*Frankia* associations are thus especially effective and important in the amount of nitrogen they fix on a global basis. The legume–rhizobia and alder–*Frankia* associations are examples of mutualism, and both partners clearly benefit from the association. In each case, root nodules are formed containing the bacteria, which are only able to fix nitrogen in association with the plant host. Nodule formation is a complex process involving a series of physical and chemical interactions beween the symbiotic partners.

Certain Cyanobacteria also partner with ascomycetous fungi to form lichens, which are common in terrestrial habitats. The prokaryote partner in this mutualistic association is able to fix not only nitrogen but carbon as well, through photosynthesis, while the eukaryote partner obtains mineral nutrients from the substratum. The association allows lichens to thrive in austere habitats such as the surface of rocks, where both organic carbon and mineral nitrogen are extremely limited. Significant amounts of nitrogen are also fixed by free-living bacteria in a variety of habitats, including associated with plant roots (e.g. *Azospirillum* and roots of grasses) or foliage, the latter especially in the tropics. Tropical epiphytic microbial communities are made up of a number of taxonomic groups, including several genera of nitrogen-fixing bacteria, such as *Azotobacter*, *Beijerinckii*, *Klebsiella*, and Cyanobacteria such as *Nostoc* and *Scytonema*.

Prokaryote transformations of sulfur

A variety of primarily chemoautrophic and chemoheterotrophic prokaryotes are responsible for oxidizing sulfur in soils, under aerobic conditions. The most well known genus is *Thiobacillus*, which is found in a variety of habitats (one species, *T. denitrificans*, is also capable of anaerobic growth, using nitrate as a terminal electron acceptor). The phototrophic purple sulfur and green sulfur bacteria, which are dominant sulfur oxidizers in aquatic systems, are mostly absent from soil habitats due to their simultaneous requirements of light and anoxic conditions. However, they may be found in aquatic–soil interfaces, such as rice paddies and wetlands. *Sulfolobus*, a member of the Archaea, is an important sulfur oxidizer, especially in thermal habitats, but its importance in most soils is largely unknown. Soil microbes reduce sulfur compounds in an assimilatory process to meet their sulfur needs. Dissimilatory reduction of sulfate to hydrogen sulfide, by organisms using sulfate as a terminal electron acceptor, occurs primarily in aquatic sediments but also may be significant in anaerobic, waterlogged soils and possibly in anaerobic soil microsites. Release of H_2S by sulfate reducers such as *Desulfovibrio* spp., *Desulfomonas* spp., and *Desulfomaculatum* spp., provides an energy source for nearby sulfur oxidizers in tightly linked microbial communities.

BIOTRANSFORMATION AND BIOREMEDIATION

In the very early twentieth century, the great microbial ecologist Martinus Beijerinck made his famous statement, 'Everything is everywhere, the environment selects.' Beijerinck recognized that microbes and the vast diversity of their metabolic capabilities are almost ubiquitous in the environment and that environmental influences select for certain microbial types. A variety of environmental pollutants are naturally occurring, nonsynthetic materials, such as petroleum products, nitrates, heavy metals, and acid mine drainage. In the latter part of the twentieth century, scientists became more aware of the prescience of Beijerinck's comments, as microbes in natural habitats were observed to successfully degrade a wide range of pollutants. The list of compounds that bacteria may successfully degrade in the environment includes petroleum or hydrocarbon products such as gasoline, diesel, and fuel oil, hazardous crude oil compounds such as benzene, toluene, xylene, and naphthalene, certain polynuclear aromatic compounds, certain pesticides (e.g. the insecticide malathion), coal compounds, some industrial solvents such as acetone, as well as ethers, methanol, methylethylketone, and ethylene glycol.

However, it was soon realized that a large number of organic chemicals synthesized by humans, which we now call 'xenobiotics,' have no close natural chemical analogs and often are able to persist in the environment because appropriate transport mechanisms and catabolic pathways have not evolved in natural microbial communities. Xenobiotic compounds include a number of pesticides and products generated from them, many plastics, industrial chemicals, and manufacturing byproducts, and compounds newly formed in nature from synthetic components. Typically, xenobiotic compounds are not found individually, but rather in simple or complex mixtures. Many of these compounds are toxic at low concentrations, and some are subject to biomagnification through the food chain. The potential for biodegradation of xenobiotic compounds is a subject of intense research. Fortunately, while a number of xenobiotic compounds may not support the growth of any single microorganism, they may nonetheless be degraded in the presence of other natural substrates, a process that is known as cometabolism. Also, a number of xenobiotics

may be degraded through the cooperative efforts of several microbial strains comprising a microbial consortium.

The term 'bioremediation' refers to a variety of technologies that rely on the biodegradative capabilities of microorganisms for the mineralization or inactivation of chemical pollutants in contaminated sites. Complete mineralization, i.e. decomposition of a pollutant to inorganic ions and CO_2, is the most desirable situation because the end products are usually nontoxic. Although soil may be removed and transported from a site for ex situ bioremediation, this usually is prohibitively expensive, and in situ bioremediation (ISB) is the norm. There are two general approaches to bioremediation. The first, sometimes called 'bioaugmentation,' is intended to enhance the degradative potential of microbes already present at the site, by environmental modifications including nutrient application, aeration, mixing, etc. A second approach involves addition of appropriate xenobiotic degraders by seeding. Ideally, bacteria chosen as agents for bioremediation of organic pollutants will have a number of characteristics beyond the ability to digest the particular waste material rapidly and completely; these include the nonproduction of odors or noxious gases, nonpathogenicity to humans or other animals, and competitive ability in the natural environment. For some pollutants, commercial strains of bacteria and fungi have become available. These strains have been selected or in some cases genetically engineered for specific degradative capabilities.

BACTERIA AS PATHOGENS OF PLANTS AND ANIMALS

Despite the fact that prokaryotes are absolutely essential in making our planet a habitable place for plants and animals, most people still are most familiar with the small group of usually specialized bacteria that are pathogenic to humans, other animals, and the crops we grow for food and fiber.

Phytopathogenic bacteria

While the number of plant diseases caused by bacteria is smaller than those caused by fungal pathogens, nonetheless soil-borne and foliar bacterial plant pathogens are responsible for a large number of plant diseases, including various wilts, blights, galls, leafspots, and rots. The taxonomy of phytobacteriology is evolving continually, but pathogens are found in the genera *Pseudomonas*, *Burkholderia*, *Xanthomonas*, *Streptomyces*, *Agrobacterium*, *Erwinia*, *Corynebacterium*, and *Spiroplasma*. Some are obligate pathogens and many are seed-borne. A large number are saprophytic and capable of surviving and reproducing in soil. Pseudomonads especially are common and abundant in soil and the rhizosphere. A number of fluorescent pseudomonads have a permanent soil phase. Xanthomonads, some *Erwinia* spp., and a number of pseudomonads are associated with above-ground plant surfaces and can cause a number of serious foliar diseases. *Pseudomonas syringae*, for example, is responsible for a number of economically significant diseases of vegetables, fruits, and ornamentals.

Phytopathogenic bacteria gain entrance into plants through wounds or natural openings, which include stomata, lenticels, hydathodes, and nectarthodes. Once bacterial cells gain entrance into the plant, they may biochemically elicit plant-defense responses. The recognition phenomena that govern these responses typically are quite specific and are determinants of whether a particular bacterial strain is pathogenic. A number of virulence determinants are encoded by the pathogen genome, and govern the pathogen's 'weapons,' which may include toxins, hormones, extracellular polysaccharides, and enzymes such as cellulases and pectinases that are involved in degradation of plant cell walls.

One unique soil-borne plant pathogen that has proven to be of immense utility is the bacterium *Agrobacterium tumefaciens*, the causal agent of crown gall disease. *Agrobacterium* is a relatively common genus in soils, and the pathogen *A. tumefaciens* is easily disseminated by farm machinery, tools, irrigation, or rain-splashed soil. The pathogen gains entry through wounds and colonizes the wounded plant tissues. Then, a portion of the pathogen's own DNA in the form of a plasmid (Ti plasmid, for 'tumor-inducing') enters into a living host cell and somehow gains entry into the host nucleus. The newly introduced pathogen genes code for production of growth hormones, and large woody galls are formed on the plant roots (or, in some cases, plant stems). The galls form a highly nutritive environment for the pathogen, but the hypertrophic growth may result in collapse of plant vascular tissue and death of the host. Eventually, bacteria are released from decaying galls into surrounding soil or water. The ability of a prokaryotic organism to transfer some of its own DNA into the genome of a eukaryotic host is a unique characteristic. Plant molecular biologists have taken advantage of this ability, by generating variants of *A. tumefaciens* that are able to transfer DNA without the genes required to incite galls in the host plant. A wide variety of genes have been inserted into the bacterial plasmid, allowing plants to be transformed with any number of desirable traits.

Bacteria as biological control agents against plant pathogens

Many or most bacteria associated with the exterior surfaces of plants are nonpathogenic. Among those are a number of organisms that have been found to be

antagonistic to bacterial and fungal plant pathogens, thus potentially acting as biological control (biocontrol) agents. Biological control may be defined as the use of one organism to suppress another organism that is an agronomic pest or parasite, or that is environmentally injurious. A number of mechanisms have been implicated in biological control of plant pathogens by beneficial bacteria, including production of antibiotic compounds, nutrient competition, hyperparasitism, and stimulation of host defenses. A variety of rhizosphere bacteria, which are effective colonizers of plant roots, have been designated plant growth-promoting rhizobacteria (PGPR). A number of PGPR have been identified, and several of these are being promoted as plant-disease-control products (Bahme and Schroth 1987; Kloepper and Beauchamp 1992; Suslow and Schroth 1982). Organisms that have received a great deal of attention include strains of *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Streptomyces griseoviridis*, and they are being used especially for protection of high-value crops, including bedding plants, foliage plants, ornamentals, and some vegetable crops.

Soil- and plant-associated animal pathogens

Humans and other animals generally play a minor role as habitats for bacteria, but the effects on animal populations nonetheless can be devastating. A number of pathogenic prokaryotes are normally soil inhabitants but are facultative parasites of animals. Thus, soil (and to a lesser extent, plants) can serve as a reservoir for these bacterial pathogens.

Pathogens of medical and veterinary significance

Several important animal diseases are caused by pathogens that are indigenous to soil or plant surfaces. Some of the most important causal agents are spore-forming bacteria in the genera *Bacillus* and *Clostridium*, in part because of their ability to survive adverse environmental conditions. Anthrax is an acute infectious disease caused by the aerobic spore-forming bacterium *Bacillus anthracis*. Anthrax most commonly occurs in wild and domestic vertebrates (cattle, sheep, goats, camels, antelopes, and other herbivores), but it can also occur in humans when they are exposed to infected animals or tissue from infected animals. The anthrax organism is endemic in many agricultural regions throughout the world, including South and Central America, Southern and Eastern Europe, Asia, Africa, the Caribbean, and the Middle East. Horses, swine, deer, and humans are less susceptible than are cattle or sheep. Wild ruminants such as deer also may become infected. Infection in ruminants or horses is usually the result of grazing on infected pasture land. The organisms typically enter through the mouth, and less commonly via skin injury. After ingestion or inhalation, the bacteria spread rapidly throughout the body. Anthrax disease in humans typically results from occupational exposure to infected animals or by inhaling anthrax spores from contaminated animal products. Anthrax has become a major public-health issue because *B. anthracis* is considered to be a potential agent for use in biological warfare.

Members of the genus *Clostridium* are also spore-forming bacteria, but they are strict anaerobes. Clostridium is responsible for a number of important animal diseases, including blackleg of cattle and sheep (*C. chauvoei*), red-water disease of cattle (*C. haemolyticum*), enterotoxemias of various mammals caused by *C. perfringens*, botulism of cattle, horses, sheep, and occasionally humans (*C. botulinum*), and tetanus (all mammals) caused by *C. tetani*.

Clostridia are found naturally in soil, aquatic sediments, and animal gastrointestinal tracts. The majority are not pathogenic to animals, and species are significant for their ability to fix nitrogen. *Clostridium perfringens* causes gas gangrene disease, which results from contamination of wounds with soil containing spores of the bacterium. Clostridial food poisoning (botulism) results from eating food that is contaminated with soil or feces containing *C. botulinum*, and which then is stored under anaerobic conditions that permit reproduction of the organism. All forms of botulism can be fatal, although the disease is relatively rare. A number of other genera of soil bacteria, especially gram-negative bacteria in the genera *Pseudomonas*, *Enterobacter*, and *Acinetobacter*, may be of medical importance.

A number of plant- and soil-associated bacteria are pathogens of arthropods, including a number of insect pests. Many, like the gram-negative bacterium *Serratia marcescens*, are facultative parasites that, once ingested, may be able to invade the hemocoel if the integrity of the gut lining is compromised. Two members of the genus *Bacillus* have been widely used as microbial insecticides against a number of important insect pests. *Bacillus popilliae* causes 'milky-spore disease' of beetle larvae in soil. Larvae that ingest spores of this organism cease feeding, turn milky white in color from the profuse sporulation of the bacterium in the hemocoel, and then die. *Bacillus popilliae* has been commercialized and is widely used against Japanese beetle in lawns and turfgrass. More well known is the related organism, *B. thuringiensis*, which is arguably the most successful biological control agent in history. Along with spore formation, *B. thuringiensis* produces a parasporal crystalline protein. The organism is primarily an inhabitant of soil, but it is used primarily for foliar application as a biocontrol agent. When the crystalline protein, also known as the δ-endotoxin, is ingested by the herbivorous larvae of lepidopteran pests, it dissolves in the alkaline

gut of the insect. The toxin disrupts the membranes lining the gut, and septicemia may follow as bacteria proliferate in the hemocoel. The larvae soon stop feeding and die soon thereafter. The organism is easily grown in mass culture, dried, and culture residues containing spores and crystals formulated for spraying on crops and forests. There are several commercial Bt formulations currently on the market, and certain subspecies of the organism have demonstrated efficacy against coleopteran (beetle) and dipteran (flies, including mosquitoes) pests. More recently, the toxigenic gene of Bt has been placed into strains of *Pseudomonas fluorescens* for use as seed or root inoculants, and into the maize endophyte *Clavibacter pyli* for protection against the European corn borer. Genes for toxin production have been inserted directly into crop plants via genetic engineering, and crops with Bt genes are now widely used in commercial agriculture. This approach is controversial, however, as it exerts a strong selection pressure on the pest population to develop Bt resistance. Resistance has already been observed in some important pest species, such as Colorado potato beetle.

Recombinant bacteria

Along with examples noted above, a number of soil- and plant-associated bacteria are candidates for genetic engineering, in order to enhance their environmental fitness or biological control efficacy. Environmental release of engineered bacteria has raised concerns about the possible spread of recombinant DNA sequences to other organisms present in the same environment. Bacterial plasmids and transposons occur in most prokaryotes; they carry the genetic determinants of many important phenotypic properties including antibiotic and heavy metal resistance, production of antibiotics, bacteriocins, and toxins, catabolism of xenobiotic compounds, nodulation and nitrogen fixation, plant tumorigenesis, and insecticidal toxin production. Knowledge about the potential of introduced organisms to transfer genetic information to other bacteria present in a given habitat is essential for assessment of possible environmental risk.

SAMPLING AND DETECTION METHODS FOR SOIL- AND PLANT-ASSOCIATED PROKARYOTES

A wide array of traditional and novel methods is now available to investigate the activities of microbes in soil and plant habitats. Particularly exciting advances have been made in our ability to observe, in situ, many aspects of microbial physiology that previously could only be demonstrated with pure cultures under highly controlled conditions. Various new strategies are available for detection of bacteria, their genes, and their gene products in natural environments. These strategies are equally useful for tracking the fate of introduced organisms and their genetic information in soil and the rhizosphere.

There are two general approaches to the quantitative estimation of microbial populations in the rhizosphere or phyllosphere: direct methods such as visualization techniques, and indirect methods such as dilution plating of root or leaf washings, or most probable number (MPN) estimates (Cochran 1950). Indirect methods have been reported to recover only about 0.01–10 percent of the numbers of bacteria enumerated by direct counts (Campbell and Porter 1982; Colwell et al. 1985; Richaume et al. 1993). However, direct counts are difficult to obtain from an opaque medium such as soil. Also, they may overestimate the number of bacteria in soil because it is difficult to differentiate between living and dead cells unless using a viability stain. In addition, a high level of concentration is needed by the observer; direct counts are labor-intensive if large numbers of samples need to be processed. Another disadvantage with direct counts is that different groups of organisms are difficult to identify unless their morphologies differ. Since precise identification of what constitutes rhizosphere soil is difficult or impossible, researchers are often forced to rely on an operational definition of the rhizosphere. In many studies, the rhizosphere has been defined as the thin layer of soil adhering to the root system after loose soil has been removed by shaking. However, depending on soil texture and moisture content, the amount of adhering soil may vary considerably. Parke et al. (1990) reported that the amount of adhering soil (from 0 to 1200 mg/cm of root) on roots and adhering soil mass (from 25 to 1000 mg) did not significantly affect estimates of root populations of a rhizobacterial strain of *Pseudomonas fluorescens*. If microbial numbers are to be expressed as colony forming units (CFU) per gram of rhizosphere soil, a subsample of the rhizosphere soil should be weighed, oven-dried, and weighed again to determine soil moisture content. For phyllosphere samples there are fewer difficulties, as generally the phyllosphere will be more or less synonymous with the phylloplane (plant surface).

A variety of methods have been used to separate rhizosphere microbes from root surfaces, and phyllosphere microbes from leaf surfaces. These methods include washing techniques (in flasks or tubes on a platform or wrist action shaker), vortexing (with or without additional agitation, for example with added glass beads), sonication, and blending or maceration. The method used for removal of microbes from either the rhizosphere or the phyllosphere affects the recovery of populations (Donegan et al. 1991; Kloepper et al. 1991). Washing or homogenizing diluents commonly include sterile tap or distilled water, saline solution, or buffer solutions. Regardless of the method, consideration must

be taken of whether rhizosphere or phyllosphere microbes might be killed by the process, resulting in artificially low counts, and/or whether growth in the diluent may occur, possibly resulting in inflated counts. Freezing leaf samples for more than 3 days prior to processing is not recommended as recovery of bacteria has been shown to be adversely affected (Donegan et al. 1991).

The dilution plate count is one of the most widely used methods for enumeration of microbes. However, it is well established that because indirect counts take into account only culturable microbes, they generally underestimate actual microbial populations. Typically, only a small fraction (0.01–10 percent) of the number of cells observable by direct microscopy is recovered by the plate-count procedure. Skinner et al. (1952) listed several reasons for inadequacies of the plate count procedure for bacterial isolation; these include (1) clumps of bacterial cells may give rise to single colonies on a plate; (2) antibiosis and competition on plates cause some colonies to fail to develop; and (3) even so-called 'general' growth media are usually selective in some respect. Many cells that are metabolically active do not produce colonies on solid media. Plate counts estimate only those cells that are tolerant of the medium and incubation conditions to which they are subjected (Zuberer 1994). In contrast, the plate-count procedure may provide an apparent overestimation of fungal or actinomycete biomass if clusters of spores are present, since each spore may give rise to a colony on the plate. To accommodate the enormous size of most microbial populations in environmental samples, as well as the microscopic size of individuals, the main principle of enumeration is dilution of the population to countable numbers. Usually, an investigator will add components to the isolation medium which inhibit either fungi or bacteria.

From leaf or root surfaces, it is possible to directly isolate fungal and bacterial propagules by pressing the surface of the leaf or root segment on to an agar surface, then removing it. This method has the advantage of providing some information about the microbial spatial distribution on the leaf surface. It is also possible to pour a small volume of molten agar on to the leaf surface, allow it to solidify, and then to transfer it to fresh medium (Langvad 1980). Testing surfaces for fungal spores is done similarly, but with single-sided tape, and the spores are then identified microscopically (Knudsen and Hudler 1987). Additional information on sampling, including preparation of serial dilutions and plating techniques, is provided in Dandurand and Knudsen (2001) and Zuberer (1994).

QUANTIFYING PROKARYOTE METABOLIC ACTIVITY IN SOILS

Determining the metabolic status of microbial cells in natural habitats is significant, because metabolic status largely determines the effects that the microbes have on their environment. In contrast, determining the identity and distribution of microorganisms can only provide information on their potential environmental activity. Historically, a great deal of research in soil and plant microbiology has focused on biochemical processes, including microbial roles in the processes of biogeochemical cycles, but necessarily many studies were performed in vitro or by using artificially constructed systems. Frequently, the potential metabolic capabilities of microbial populations in natural systems have had to be extrapolated from capabilities observed in the laboratory setting.

Traditional methods, with respirometry being the most notable example, have provided a wealth of information on total microbial metabolic activity in soils but have not always provided a deeper understanding of soil microbial community structure and function. It has long been known that the majority of soil microbes are not amenable to recovery by standard cultural techniques, and often the environmental role of these organisms, if they were detected at all, could only be guessed at. However, recent advances in molecular biology, fluorescent stain technology, and microscopy have provided new and/or improved tools to detect and characterize soil microbes in situ, including their abundance, genetic makeup, and environmental activities. Particularly exciting advances have been made in our ability to observe, under natural conditions and with minimum perturbation, many aspects of microbial physiology that previously could only be demonstrated with pure cultures under highly controlled conditions. Also, by helping to escape the limitations of pure culture methods, these new techniques have provided insight into the roles that individual microbial populations play as contributing members of microbial consortia.

Microbial metabolism can be defined as the sum total of all the chemical reactions that occur in microbial cells, and the seemingly infinite variety of metabolic processes and products suggests potential for a correspondingly vast array of detection methodologies. The scope of available technology for monitoring microbial metabolic activity is increasing so rapidly that it is difficult to catalog the available methods. However, methods can be broadly categorized as those that detect end products or intermediate products of catabolic or anabolic activity (e.g. detection of carbon dioxide produced by aerobic respiration, via respirometry), those that attempt to correlate fluxes in specific structural components of the microbial biomass (e.g. phospholipid ester-linked fatty acids) with metabolic activity levels, and those that are designed to detect the biochemical indicators of metabolism (e.g. RNA, specific enzymes) or the specific products of gene expression.

Soil, the rhizosphere, and the phyllosphere are populated by complex communities of organisms, including microbes (bacteria, Archaea, fungi, Protists) but also typically including plant and animal components. The

rhizosphere and phyllosphere are defined by the presence of a plant, so it is inherently impossible to characterize microbial activity in these habitats through the study of genetically uniform microbial strains in isolation. Determining microbial contributions to these communities may require a variety of techniques with different areas of focus. For example, it has long been known that microbes may be metabolically active even though they are not culturable. Furthermore, quantification of the viable microbial biomass may help elucidate the potential for specific microbial metabolic activities in a particular habitat, but the nonviable biomass may also be important as a component of the food web, despite its lack of a direct metabolic contribution. Although it is possible and common to make inferences about microbial biomass based on microbial community metabolic activity, it is important to note that these may not be strongly correlated; i.e. high metabolic activity does not necessarily mean an actively growing, dividing microbial community. Also, it is important to remember that microbial physiological activities are often strongly affected by the physical arrangement of the members of a population, especially aggregations such as those that occur in biofilms, on root surfaces (Dandurand et al. 1995, 1997), and in soil microsites. Physiological and spatial heterogeneity have important implications for the ecology of microbial populations and communities, and the ability of recently developed molecular and microscopic methods to address heterogeneity in environmental samples is especially exciting. Below, several of the most popular methodologies for quantifying microbial metabolic activity are briefly discussed. In each case, references are provided to original reports and more extensive, step-by-step protocols. The related subject area of bioreporter gene technology, which allows the analysis of in situ gene expression by supplying assayable gene products, and in which the gene product of interest is difficult or impossible to assay, is briefly discussed below. Similarly, biosensor technology is very promising for environmental microbiology; recent reviews on applications of biosensors for environmental microbiology are available (Burlage 1997).

Several methods for estimating metabolic activity levels of microbial populations involve quantifying cellular pools of specific biochemical components, including RNA, DNA, ATP, and total adenine nucleotide (TAN). Because the specific growth rate of bacteria is dependent on cellular amounts and synthesis rates of ribosomal RNA (rRNA), there is a strong correlation between cellular RNA content, or RND:DNA ratio, and growth rate (Kemp et al. 1993). ATP and TAN pool turnover rates can provide estimates of energy flux and specific growth rate in natural microbial populations. Kemp et al. (1993) described procedures to measure cell-specific quantities of rRNA and DNA in order to quantify the frequency distribution of activity among cells. In this procedure, fluorescently labeled oligonucleotide probes are hybridized to complementary 16S rRNA sequences in intact cells. Cell fluorescence, which is proportional to cellular rRNA content, is then measured, and compared with measurements of DNA content obtained by fluorescence of 4′, 6′-diamino-2-phenylindole hydrochloride (DAPI)-stained cells, generating a rRNA:DNA ratio for individual cells. Procedures for measuring rates of stable RNA synthesis have been applied to bacterial cultures as well as aquatic bacteria, based on the uptake and incorporation of radiolabelled adenine into cellular RNA (Karl 1979). Methods for simultaneous quantification of RNA and DNA synthesis rates also have been developed and used for estimating rates of growth, cell division, and biomass in microbial communities (Karl 1981, 1993). Recently, Lee et al. (1999) used a combination of fluorescent in situ hybridization (FISH) performed with rRNA-targeted oligonucleotide probes and microautoradiography, to quantify specific substrate-uptake profiles of individual bacterial cells within microbial communities.

Genetic methods

Recent advances in molecular biology have made it easier to analyze the genomes and quantify the genetic diversity of soil- and plant-associated microbes, as well as to track them in the environment (Bramwell et al. 1995; Louws et al. 1999). Much of this research has been motivated by the need for rapid and accurate detection of plant and animal pathogens, but the methods are applicable to many different aspects of environmental microbiology. Some of the DNA-based microbial characterization methods are dependent on PCR amplification, while others are not. Examples of the latter include DNA–DNA reassociation kinetics, characterization of plasmid profiles, and restriction enzyme analysis of total genomic or plasmid DNA. PCR-based approaches are often more useful because they require smaller amounts of DNA; also, they tend to be faster, more sensitive, and more specific. The main methods for analyzing and quantifying microbial genomes by PCR-based methodology include: repetitive DNA PCR (rep-PCR), rDNA-based PCR, amplified fragment length polymorphism (AFLP) analysis, and arbitrarily primed PCR (AP-PCR) randomly amplified polymorphic DNA (RAPD) (Louws et al. 1999).

A number of technical difficulties are associated with extraction and analysis of nucleic acids from soil; for example, DNA may be tightly bound to humic materials and clays. A number of protocols have been published for direct extraction of nucleic acids from soil, and the technology is rapidly evolving in this area (Levin et al. 1992; Pepper and Dowd 2002). Because PCR-based protocols are so highly sensitive, certain problems may arise. For example, only a very small quantity (e.g. a few molecules) of PCR-generated fragments can be

sufficient to cross-contaminate samples of subsequent PCR runs, resulting in the detection of false positives. A number of recommendations have been suggested to avoid such contamination (Carrino and Lee 1995; Kwok 1990). Because PCR can amplify DNA from dead cells as well as live ones, it may produce false positives. Additionally, PCR cannot directly distinguish viable but not culturable (VBNC) cells from culturable cells. One approach, BIO-PCR, was developed to circumvent this problem when assaying for plant pathogenic bacteria; with BIO-PCR, samples are first plated on to growth medium to culture any viable cells, before PCR analysis (Schaad et al. 1995). This approach has been used successfully for bacteria in soil as well as for plant- and seed-associated bacteria. When PCR is used to detect plant-associated microbes, there is the possibility of the presence of sample-related compounds that can inhibit the polymerase reaction. Wilson (1997) reviewed methods to avoid potential problems with inhibitory compounds.

Use of marker and reporter genes

Recently, genetic engineering of biocontrol agents with reporter or marker genes has provided useful tools for detection and monitoring of introduced biocontrol agents in natural environments (Green and Jensen 1995; Lo et al. 1998; Bae and Knudsen 2000). For example, the selectable hygromycin B phosphotransferase (*hygB*) gene, coding for resistance to this antibiotic, has been used to detect fungal biocontrol agents in the rhizosphere and phyllosphere (Lo et al. 1998). The β-glucuronidase (GUS) marker gene is also a promising tool for ecological studies of biocontrol agents, because of the low background activity of GUS in fungi and plants, the relative ease and sensitivity of detection (Roberts et al. 1989), and the apparent lack of influence of GUS expression on biocontrol efficacy. However, some background GUS activity may be present in unsterile systems or natural soils. The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has also been developed as a reporter for gene expression (Chalfie et al. 1994). It has been successfully cloned and expressed in a variety of different organisms including plants, animals, fungi, and bacteria. GFP requires only ultraviolet (UV) or blue light and oxygen to induce green-fluorescence. An exogenous substrate, such as GUS requires, is not needed for the detection of GFP, thus avoiding problems related to cell permeability and substrate uptake.

Advances in microscopy

A number of methods to quantify metabolic activity and/or biomass of individual microbes, populations, or microbial communities involve direct microscopic observation of cells. These include simple measurement of cell size and size distributions, formation of microscopically observable metabolic products, fluorochromes used as 'physiological stains,' and genetically based marker and reporter systems. Techniques used to investigate root biofilm communities have included confocal microscopy with or without targeted rDNA probing, electron microscopy (SEM and TEM), and PCR-assisted rDNA RFLP/sequence analyses. Most of the wide range of potential microscopic techniques have been attempted for visualization of microbes on root and foliar surfaces, including bright-field, phase-contrast, and differential interference, epifluorescence, scanning and transmission electron microscopy, and atomic-force microscopy. In general, direct visualization for quantitative purposes can be difficult because of opacity of the substrate and the small size of microorganisms; spatial associations are difficult to describe because of the short working distance and the high magnification needed to see them. Phase-contrast or interference microscopy of fresh material is possible, but associated soil and root material can give confusing background for observation of microorganisms. Fluorochromes and other stains are commonly used to observe microbes on plant surfaces. However, autofluorescence of plant tissue can be a problem when using fluorochromes to detect microbes on plant surfaces.

Sampling considerations

A number of special considerations arise when sampling microbes from the rhizosphere and phyllosphere. Parberry et al. (1981), Hirano and Upper (1983, 1986), Kinkel et al. (1995), and Kloepper and Beauchamp (1992) have reviewed some of the difficulties inherent in sampling the phyllosphere and the rhizosphere. Problems include the non-normal distribution of microorganisms, selection of sampling strategy, scale of sample, and sample unit. The choice of sampling strategy, scale of sample, and sample unit can all have a significant effect on the results of an experiment. The type of sampling strategy used in sampling the rhizosphere and the phyllosphere is often dependent on the question that is being asked. It is generally recommended that samples be taken at random so that the assumption of independence of samples for standard statistical tests (e.g. analysis of variance) is met. However, sampling of microbes from the rhizosphere, in many cases, cannot be done randomly. For example, colonization is often determined by taking root segments at a prespecified distance (e.g. 1-cm segments 2 cm below the soil line). Another example, which can result in autocorrelated data, is when roots are sequentially sampled in order to assess the root-colonizing ability of a microbe. In these situations, one must verify that the data meet the assumption of the statistical analysis prior to conducting the tests.

The selection of sample scale and sample unit is based on biological features of plants, reduction of sample variance, ease of processing, and other methodological constraints. When epiphytic bacterial populations were sampled, a high level of variability was reported among sample units at every scale investigated (leaf segments, leaflets, or whole leaves) (Kinkel et al. 1995). In contrast, variability of rhizobacteria was significantly less when whole roots were sampled compared with root segments (Kloepper et al. 1991). Thus, for phyllosphere sampling, Kinkel et al. (1995) suggested that selection of sample scale and unit should not be based only on the assumption that the variance is scale dependent but may be more appropriately based on methodological and biological considerations. Although sample variance may be reduced when sampling whole-root systems rather than root segments, this sample scale may not always be feasible (e.g. when sampling mature plants).

Natural populations and habitats are spatially heterogeneous, i.e. organisms and their resources are usually not uniformly distributed over space or time but are found in different degrees of aggregation. Several theoretical studies have demonstrated the importance of spatial heterogeneity for population and metapopulation persistence and abundance (Hanski 1991). Hirano and Upper (1993) demonstrated changes in frequency distributions of epiphytic bacterial populations on populations of habitats (individual leaflets) within a plant canopy. However, despite continuing demonstration of the theoretical and practical importance of spatial processes to ecology, relatively few mechanistic studies of the factors that create heterogeneous spatial distributions and the processes by which they occur in nature are available. As pointed out by Hirano and Upper (1993), an understanding of factors that regulate epiphytic bacterial population dynamics must address mechanisms that underlie variability.

Most natural environments are spatially structured by various energy inputs that result in patchy structures or gradients (Legendre and Fortin 1989). Thus, biological organisms are rarely distributed in a random or uniform manner. The rhizosphere is a good example of this, since energy input is largely due to root deposition. Spatial variability of nutrient deposition from seeds, as well as the spatial presence of roots, may influence sites of colonization by rhizosphere microbes and the quantity and composition of root deposition varies along the root. Sites may be preferentially colonized by some microbes, such as bacteria in cell junctions (Rovira 1956) or zoospore encystment in the zone of root-cell elongation (Hickman and Ho 1966; Mitchell and Deacon 1986; Zentmeyer 1961). Although the tendency for both phyllosphere and rhizosphere microbial populations to conform to lognormal or similar frequency distributions has been noted (Hirano et al. 1982; Loper et al. 1984; Newman and Bowen 1974), the actual spatial variability of these populations is less well documented. Appropriate characterization of the spatial variability inherent in soil- and plant-associated microbial communities will allow us to improve quantification of the organisms and processes under study.

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Bacteriology of water

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Bacteriology of water continues to be an important component in defining the quality of public health. In recent years, expanding methodology has led to the discovery of additional pathogens as the cause of waterborne outbreaks. As a consequence of these new discoveries, the focus on water quality is being redirected towards better characterization of the microbial risks associated with this vital resource. It is important to note that although water is essential to sustain life, it can be a hostile environment to humans when grossly polluted.

CHARACTERIZING THE BACTERIAL FLORA IN THE AQUATIC ENVIRONMENT

The bacterial flora of water is a composite of organisms indigenous to high-quality water or pristine waters plus all of the contributions from life's many controlled and uncontrolled uses or consumption of water.

Indigenous bacteria

These are naturally occurring bacteria found in waters that are remote from the activities of modern civilization. They are largely saprophytic organisms, some of which belong to the genera *Micrococcus*, *Pseudomonas*, *Serratia*, *Flavobacterium*, *Chromobacterium*, *Acinetobacter*, and *Alcaligenes*. There are indigenous bacteria that are difficult to detect in routine laboratory techniques because of their slow growth or fastidiousness. In the past, these organisms received little attention, but this attitude is changing because of problems with biofilm development in streams, ground-water extraction, water pipe networks, and industrial processes. An additional threat is that some opportunistic pathogens, such as *Legionella*, may be ubiquitous in the water environment.

Contributed bacteria

Rainfall runoff is a major contributor to changes in the bacterial flora found in water. Whereas rain or snow falling over the watershed contains only occasional bacteria acquired from air-borne particles (Table 1), the microbial and chemical qualities of rainwater drastically degrade upon contact with the soil (Geldreich et al. 1968). Various soil and fecal organisms are immediately accumulated into the drainage and merge as the bacterial flora. These organisms, though not normal inhabitants of high-quality water, either adapt to the aquatic environment or die out over time because of various environmental adversities. Predominant among these soil and vegetation organisms are the aerobic spore-forming bacilli, such as *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus mycoides*. Others, such as *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Enterobacter cloacae*, may be found growing in the water- and food-conducting tissue of trees (Knittel et al. 1977), on crops, and on other vegetation. By use of special media, a variety of other specialized bacterial groups can be isolated. In the bottom sediments of

Table 1 *Seasonal variations (median values) for bacterial discharges in rainwater and stormwater from suburban areas, Cincinnati, Ohio*

Source	Period	Total samples	Season	Organisms per 100 ml			Ratios	
				Total coliforms (TC)	Fecal coliforms (FC)	Fecal streptococci (FS)	FC:FS	FC/TC ×100
Rain water	June 1965–February 1967	49	Spring	<1.0	<0.3	<1.0	–	–
			Summer	<1.0	<0.7	<1.0	–	–
			Autumn	<0.4	<0.4	<0.4	–	–
			Winter	<0.8	<0.5	<0.5	–	–
Wooded hillside	February 1962–December 1964	278	Spring	2 400	190	940	0.20	7.9
			Summer	79 000	1900	27 000	0.70	2.4
			Autumn	180 000	430	13 000	0.30	0.2
			Winter	260	20	950	0.02	7.7
Street gutters	January 1962–January 1964	177	Spring	1 400	230	3 100	0.07	16.4
			Summer	90 000	6 000	150 000	0.04	7.1
			Autumn	290 000	47 000	140 000	0.34	16.2
			Winter	1 600	50	2 200	0.02	3.1
Business district	April 1962–July 1966	294	Spring	22 000	2 500	13 000	0.19	11.4
			Summer	172 000	13 000	51 000	0.26	7.6
			Autumn	190 000	40 000	56 000	0.71	21.1
			Winter	46 000	4 300	28 000	0.15	9.4

Data from Geldreich et al. 1968.

streams and lakes where oxygen is restricted, nitrate, sulfate-reducing, and methanogenic bacteria can be detected and impart disagreeable earthy odors and tastes during summer periods of low stream flow.

The microbial flora of sewage is predominantly from fecal wastes including pathogens shed from individuals in the community. These would include species of *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Leptospira*, *Streptococcus*, *Clostridium*, and *Vibrio cholerae* and pathogenic types of *Escherichia coli*. Other significant contributors to the flora of municipal sewage are introduced in combining stormwater runoff, laundry wash water, automatic car-washing water drainage, processing food wastes, and industrial discharges.

Within the complex flora of sewage-treatment sludge are organisms that are responsible for much of the biological breakdown of degradable waste products. Some of these beneficial organisms include the filamentous bacteria *Sphaerotilus natans*, *Haliscomenobacter hydrossis*, *Nostocoida limicola*, *Microthrix parvicella*, *Flexibacter*, *Microscilla*, and *Nocardia* (Eikelboom 1975) and the important floc-forming bacteria (*Zoogloea ramigera*), along with *Proteus* and the anaerobe *Clostridium sporogenes*.

Animal feedlot operations that require the confinement of cattle in small areas create a fecal-waste-removal problem equal to the domestic waste discharges of small cities (Geldreich 1972b). If the animal waste is not discharged to a lagoon or landfill, stormwater runoff over the animal feedlots will bring massive loads of fecal pollution to the drainage basin. While cattle feedlot wastes may be a major source of bacterial pathogens (particularly, *E. coli* O157:H7), poultry-farm fecal wastes can contribute a variety of salmonellae into the drainage basin that could also be a resource for water supply downstream.

Food processing, beet-sugar extraction, and cane-sugar production have a drastic impact on the bacterial flora and the self-purification capacity of receiving waters. Elevated concentrations of nutrient wastes lead to oxygen depletion of the receiving waters plus an increase in environmental coliforms and fecal streptococci, particularly streptococci variants on biotypes (Mundt 1963; Mundt et al. 1966b). Most troubling is the extended persistence of *Salmonella* spp. under these conditions (Geldreich 1972a).

Discharges of poorly treated paper-mill waste to receiving streams or lakes can also have a severe impact on the microbial quality of surface waters. The association of environmental *Klebsiella* with paper manufacturing and the excessive nutrients in paper-mill waste cause a tremendous regrowth of this coliform in downstream waters. Environmental *Klebsiella* spp. colonize plants at flower pollination. As the seed germinates, *Klebsiella* spp. establish a coexistence within the plant by metabolizing nutrients from wood sugars (cyclitols) in the nutrient- and water-conducting tissues during the life of the tree (Seidler et al. 1977). Another byproduct of paper processing is wastewater sulfites, which encourage the growth of biofilm mats of the nuisance organism

Table 2 *Microbial characterization of solid wastes from various cities*

Waste collection site[a]	Number of samples	Organisms per g wet wt[b]			
		Total viable bacteria[c]	Spores	Total coliforms	Fecal coliforms
A	4	110 000 000	270 000	3 000 000	260 000
B	3	450 000 000	110 000	6 700 000	510 000
C	6	78 000 000	38 000	1 600 000	1 200 000
D	3	480 000 000	31 000	1 100 000	630 000
E	4	680 000 000	1 900 000	51 000 000	8 100 000
F	2	54 000 000	35 000	1 3000 000	5 600 000
G	2	4 000 000	25 000	340 000	15 000
H	3	300 000 000	160 000	8 600 000	3 000 000

Data from Peterson (1971).
a) Waste collection sites in Cincinnati, Chicago, Memphis, Atlanta, and New Orleans.
b) Average moisture content was 41 percent.
c) Spread plates (trypticase soy agar + blood) at 35°C for 48 h.

Sphaerotilus natans along the shoreline of receiving streams.

Solid waste, generally referred to as garbage or rubbish, contains a multitude of items such as food discards, garden rubbish, manufactured products (paper, plastic, rubber, leather, textile, wool, metals, glass, ceramics), rock, dirt and ash residues, and also fecal material (Table 2). Much of the fecal material in urban areas is derived from disposable diapers, pet-litter material, and feces of rodents foraging for food in these waste collections (Geldreich 1978). Therefore, it is not surprising that solid wastes contain a large and varied microbial population that includes a wide spectrum of bacteria (aerobes, anaerobes, thermophiles), actinomycetes, and fungi (Cook et al. 1967). If properly disposed of through sanitary landfills, many of the problems (foraging wildlife, odors, unsightliness) common to open dumping are avoided. However, poor placement of landfill sites may result in the migration of leachates into nearby surface waters and groundwater resources.

NATURAL WATERS

Natural waters occur either at or near the surface of the earth. Surface waters are frequently exposed to contamination from soil, stormwater runoff, domestic sewage, agricultural and industrial wastes, and decomposing vegetation. On the other hand, ground waters can be expected to be of excellent bacteriological quality due to the beneficial percolation into underground strata (aquifers). During downward passage to an aquifer, perhaps several hundred meters below the surface, most of the surface contaminants are entrapped in the soil or porous rock layers. One exception is a rock stratum composed of limestone, which is very porous and often results in sink holes and caverns through which surface water passes without effective entrapment of contaminants. In other situations, excessive application of minimally treated wastewaters to land surfaces may overwhelm the natural soil barrier (Kowal 1982). Once the aquifer becomes contaminated, restoration of water purity is very slow, even with accelerated intervention such as pumping the water to a treatment site and then returning it to the aquifer.

Surface waters

Natural water resources are replenished through rain, snow, and hail. This atmospheric moisture comes in contact with particles of suspended dust and smog that immediately contaminate the rain as it falls to the earth. The more dust encountered, the greater is the bacterial contamination. In remote areas away from the plumes of air pollutants, the total number of organisms may not exceed 10–20/l. Springtime dust storms originating in large farming areas may mix with atmospheric vapor to transport one to two total coliforms per liter of rain over great distances. Snow tends to be less pure than rain, probably because the snowflakes have greater surface area on which to collect suspended particles in the atmosphere, and also because their low temperature is conducive to the survival of bacteria. However, in snow on the tops of remote mountains, where the air is clean, very few organisms are present. Apparently hail contains more bacteria than either rain or snow. Hail examined from a storm over Padua during July 1901 was found to contain 140 000 microorganisms belonging to nine different types (Belli 1902). This surprising finding was probably caused by air currents that cycle the raindrops through periods of freezing and thawing as they repeatedly traverse plumes of dust in the air before falling to the ground. The number of organisms in ice depends on the nature of the water from which the ice was formed. With the exception of ice from glaciers, it is generally impure. Various heterotrophic bacteria, including coliforms and pathogens, have been found in ice made with contaminated water.

In remote areas, where human and farm animal populations are sparse, most organisms in water originate from soil with little evidence of contamination except

Table 3 *Indicator correlations with* Salmonella *occurrences in ambient waters (densities per 100 ml)*

Salmonella (%)	Clostridium[a]	Fecal streptococcus[a]	Fecal coliform[a]	Total coliform[a]
0	0	0	0	0
0	13	5	10	100
11	25	50	50	500
21	50	100	100	1 000
33	125	300	1 000	10 000
66	200	1 500	5 000	50 000
99	250	3 000	10 000	100 000
100	2 500	30 000	100 000	1 million
100	6 250	250 000	850 000	2.5 million

Data modified from Nemedi et al. (1984).
a) Densities per 100 ml.

for a few fecal coliforms (<100 organisms per 100 ml) originating from occasional wildlife inhabiting the immediate vicinity. Beaver and muskrats on the river banks, and significant concentrations of deer, elk, and other game animals in grazing plains and forest reserves, are responsible for a residual level of fecal microorganisms in water.

As these waters travel down a watershed, contact with agricultural and industrial activities increases and the river becomes laden with a variety of domestic and industrial wastes. As a consequence, rivers in most countries are heavily contaminated. Some measure of the potential occurrence of salmonellae in Hungarian ambient waters grouped at various average densities for several indicator groups is presented in Table 3. These data support the position that the greater the magnitude of fecal pollution, the greater the chance that some bacterial pathogens may also be present. Absence of any detectable fecal coliforms does not mean that there is no risk from *Giardia*, *Cryptosporidium*, or a virus. These pathogenic agents frequently occur in small densities requiring the testing of larger-volume (>10 liter) samples. For this reason, a surrogate indicator examination at only one sample volume of 100 ml is not going to provide sufficient precision to ascertain the potential for extremely low-density pathogen occurrences.

In a study of surface-water supplies used by 20 cities in the USA serving a total population of seven million people, it was estimated that the minimum wastewater component of the raw source water ranged from 2.3 to 16 percent and increased to predominantly wastewater for several cities during low-flow periods (Swayne et al. 1980). This public-health risk was verified by the finding of various serotypes of *Salmonella* at each water-plant intake. Bacteriological examination of raw water quality at the water treatment plant intakes of Omaha, Nebraska, St Joseph, Missouri, and Kansas City, Missouri, on the Mississippi River (Table 4) frequently revealed fecal coliform densities in excess of 2 000 organisms per 100 ml (Report 1971). This fecal pollution load resulted from inputs of raw sewage, effluents from primary and secondary wastewater treatment plants of differing efficiencies, cattle feedlot runoff, and discharges from meat- and poultry-processing plants. Fecal discharges entrapped on soil also entered the drainage basin by the flushing action of storm events. The major raw-water quality concern is that human pathogens may also be present in surface waters used for water-supply sources or body-contact recreational pursuits.

Similarly high-elevation lakes in remote parts of a watershed will contain high-quality water (unless inhabited by flocks of aquatic birds), whereas those in the lower part of the watershed are fed with surface drainage from metropolitan areas and intense agricultural activity. For large lakes such as the North American Great Lakes, long retention time and vast volumes of water serve to buffer these magnificent water resources from the impact of stormwater runoff. Unfortunately, pollution plumes around waste-water discharges may spread to new areas by wind-driven currents. At greatest risk are the many small lakes surrounded by residential development that ultimately become polluted from sporadic drainage of septic systems and runoff from neighborhood lawns.

Ground waters

Groundwater resources are the major source of water supply for many communities, farms, and individual families world wide. In rural areas, many water supplies consist of dug wells less than 10 m deep. In these wells, source water is influenced by surface-water runoff that percolates through the soil. Since there is no protective bedrock perched on top to seal off surface contaminants, water quality is erratic. Many private wells are in this category, causing the untreated water supply to be an unsafe source of drinking water. Bore holes are often of better quality because casings are driven to depths of at least 30 m and soil depth does provide a barrier to much of the surface contamination. In general, public water supplies that use ground water attempt to reach high-

Table 4 *Fecal coliform densities and pathogen occurrence at Missouri River public water supply intakes*

Raw water intake	River mile	Date	Fecal coliforms (per 100 ml)[a]	Pathogen occurrence
Omaha, NE	626.2	7–18 October 1968	8 300	NT
		20 January–2 February 1969	4 900	NT
		8–12 September 1969	2 000	*Salmonella enteritidis*
		9–14 October 1969	3 500	*Salmonella anatum*
		3–7 November 1969	1 950	NT
St Joseph, MO	452.3	7–18 October 1968	6 500	NT
		20 January–2 February 1969	2 800	NT
		18–22 September 1969	4 300	NT
		9–14 October 1969	NT	*Salmonella montevideo*
		22 January 1970	NT	19 virus PFU
				Polio types 2, 3
				Eco types 7, 33
				3 virus PFU, not typed
Kansas City, MO	370.5	28 October–8 November 1968	6 500	NT
		20 January–2 February 1969	8 300	NT
		18–22 September 1969	3 800	*Salmonella newport*
				Salmonella give
				Salmonella infantis
				Salmonella poona

NT, no test PFU, plaque-forming units.
Data from Report (1971).
a) Geometric mean.

yield aquifers at greater depths, often 300 m or more, to satisfy a greater water demand and reliable source of bacteriologically safe drinking water.

The quality of water flowing from springs depends mainly on their source and surroundings. Unfortunately, in many instances the source is unknown and could be under the influence of surface water, so quality may vary as a result of rainfall events and other activities on the watershed.

Cisterns

This source of water is generally rainfall from some catchment surface, which often is a residential roof or paved hillside for water to drain down into a storage tank. Bacteriological quality is a reflection not only of rainwater and dust particles but also of fecal contamination from birds perched on the catchment surface. Table 5 illustrates the varying densities of several bacterial indicator groups, the standard plate count, and *Pseudomonas aeruginosa* that may be encountered in tropical cistern waters. In some areas of the world, cisterns have become a blend of rainwater augmented by purchased water during the dry season.

Swimming pools and therapeutic baths

Swimming pools most often use treated municipal supplies for filling, but the water may also be derived from thermal springs, mineral springs, or the ocean. Pool operation and construction materials (cement, plastic, and redwood) have a definite effect on microbial growth (Castle 1985; Davis 1985) as does variability in water temperature in both indoor and outdoor pools (Schiemann 1985). Outdoor pools can be contaminated through stormwater drainage, soil, vegetation debris, and pets and wildlife. Microorganisms of major concern are those from the bather's body and mucosa and include those causing infections of the ear, eyes, upper respiratory tract, skin, and intestinal tract (Favero and Drake 1966). Obviously the number of bathers is another serious contributor to the deterioration of water quality. Major microbial contributions are from saliva and sinus drainage, as well as fecal contamination from the defecation of infant bathers. Pool-water disinfection can suppress many microorganisms, but effectiveness is limited by short contact time, disinfectant demand created by bather body oils, particulates, and sunlight exposure.

Bottled water

This water is generally an acceptable alternative drinking water supply and the primary alternative for emergency use (public supply contamination, foreign travel in developing nations), recreational/sports, and use in reconstituting baby formulae. Bottled water

Table 5 *Microbial quality of cistern water, public housing, Virgin Islands*

Cistern site	No. samples	Range of counts per 100 ml			Range of counts per 1 ml	
		Total coliforms	Fecal coliform	Fecal streptococcus[a]	Standard plate count[b]	*Pseudomonas aeruginosa*
1	9	<1–50	<1–3	<1–2	11–24 00	<1–151
2	8	<1–340	<1–8	<1–160	10–5 300	<1–72
3	8	<1–4	<1	<1–14	<1–14 000	<1–112
4	9	<1–11	<1–8	<1–640	2–12 000	<1–799
5	8	<1–100	<1–9	<1	6–2 500	<1–1
6	6	<1–1	<1–1	<1	1–650	<1
7	10	<1–220	<1–27	<1–412	763–30 000	<1–91
8	5	<1–103	<1–20	<1–40	420–3 000	<1–617
9	9	3–1700	3–570	<1–475	400–30 000	1–207
10	9	<1–29	<1–8	<1–972	210–4 400	1–22

Data adapted from Ruskin et al. (1989).
a) Including Enterococcus.
b) SPC agar spread plates, 35°C for 48 h.

sources are most often ground water or springs, but in recent years reprocessed public water supply (distilled, carbon-filtered, deionized, ultraviolet-radiated, or reverse osmosis desalinated) has also become available. Quality of the source water is dependent upon watershed protection, limited access to the area of extraction, and protected housing around well heads and spring outflows. Bottling operations follow prescribed sanitary codes for dispensing a food product and disposable plastic containers are commonly used to avoid possible contamination introduced from returnable glass bottles. As might be anticipated, microbial quality of freshly bottled water (within 48 h of bottling) is usually excellent. The rate of microbial change is related to a variety of factors including assimilable organic nutrients in the water, organisms competing for dominance in the microbial flora, water pH, and storage temperature. High-quality bottled waters undergo a slow rate of change because growth of the indigenous organisms may take hours, not minutes, and the available nutrients are in trace amounts. Waters bottled from sources that have fluctuating microbial populations that exceed 1 000 organisms per milliliter may contain coliforms and some opportunistic pathogens. Such bottled waters are of concern because the higher densities of heterotrophic bacteria may mask the laboratory's ability to detect fecal pollution and pathogens that might be present. Table 6 illustrates the variability in bacterial density and frequency of coliform-positive samples among bottled waters of unknown age purchased from retail outlets.

Although reported disease outbreaks due to contaminated bottled water are rare, the lack of documentation does not lessen the concern posed by use of this alternative. Any contaminated bottled water supply presents a unique hazard because of the widespread distribution of the product, which is not necessarily confined to a single community. In April to November 1974, Portugal had a cholera epidemic that caused 2 467 confirmed cases and 48 deaths (Blake et al. 1977). Most of the country was affected and the source of the infections was traced to the consumption of a brand of commercially bottled water as well as to poorly cooked shellfish. The source water may have become contaminated from cholera-infected individuals living on the watershed or involved in the bottling operation. This outbreak clearly illustrates the importance of watershed protection for ground waters and springs plus the need for effective sanitation practices during bottling operations.

Estuarine areas

These are the areas where fresh water mixes with the salt water environment either through direct discharge to the sea or by tidal flooding of freshwater pools near the ocean. Water-quality protection and enhancement of estuarine waters are particularly important for shellfish cultivation. Release of wastes to shellfish-growing waters brings a variety of organisms to the water, some of which may remain in suspension for varying periods before becoming entrapped in water turbidity. Most of the organisms reaching the area are either in aggregates or adsorbed to fecal cell debris and quickly accumulate in the bottom sediments, aided by the settling action of recirculating bottom silts. This natural deposition process concentrates much of the contaminants at the water–sediment interface, where hard- and soft-shell clams browse for food. For these reasons, it is essential that shellfish-harvesting areas be given a degree of protection from pollution that almost parallels the requirements for drinking water supplies. A zero tolerance for fecal pollution is not achievable in most shellfish-growing waters, so there needs to be a maximum permissible contaminant level recognized for authorized harvesting to minimize the public-health risks from consumption of raw shellfish.

Table 6 *Comparison of standard plate-count variability among brands of bottled water*

Brand	Sample type[a]	No. of samples	Count range[b] (SPC/ml)	Coliform positive samples[c]
A	Fresh	91	<10–25 000	3/91
	Retail	16	<10–28 000	0/16
B	Retail	6	<10–260 000	0/4
C	Fresh	1	<10	0/1
	Retail	11	1 200–160 000	0/11
D	Retail	5	31–650	0/5
E	Fresh	1	<10	0/1
	Retail	2	10 000–390 000	2/2
F	Retail	2	<10–12 000	0/2
G	Fresh	2	<10	0/2
	Retail	1	<10	0/1
H	Retail	9	<10–390	1/9
I	Retail	2	<10	0/2
J	Retail	2	<10–2 000	1/2
K	Retail	2	<10–12	1/2
L	Retail	4	13–4 300	0/4
M	Retail	1	<10	0/1
N	Retail	1	<10	0/1
O	Retail	1	1×10^6	1/1
P	Retail	1	8 600	0/1
Q	Retail	1	<10	0/1
R[d]	Retail	1	<10	0/1
R[d]	Retail	1	12	0/1

Data from Geldreich et al. (1975).
a) Fresh, samples direct from bottler, examined within 24 h; retail, samples of unknown age purchased from retail outlets.
b) Standard plate count (SPC) range values represent average count of bacteria per milliliter calculated from five replicate plates incubated for 72 h at 35°C using plate-count agar.
c) One or more coliforms per 100 ml.
d) Imported bottled water, carbonated.

Coastal waters

Coastal marine waters deservedly receive much attention because of their use as recreational bathing waters and fishery resources. Major sources of pollution are stormwater runoff along the beach areas, offshore dispersion of sewage through outfalls, sanitary wastes from ships in harbor, oil spills, and disposal of municipal garbage outside designated ocean dumping areas. In the sea, off heavily polluted bathing beaches, the coliform content may rise to 200 000 or more per 100 ml and salmonellae are frequently present (Report 1959). Organic enrichment of the sea by pollution enhances the activities of sulfate-reducing bacteria in sediments near the water interface and methanogenesis in the sediments below. After discharge from a sewage outfall, fecal and enteric bacteria rapidly disperse if there is good diffuser design and high tidal energy. At Sidmouth, Devon, which has an outfall discharging 460 m from the high-water mark, the median coliform count at the shore near the outfall was 73 organisms per 100 ml in 1965, equivalent to dilution of the sewage by a factor of 400 000 (Gameson et al. 1967).

PATHOGENIC BACTERIA IN AQUATIC ENVIRONMENTS

Numerous pathogenic agents have been isolated from ambient waters used for water supply, recreational bathing, and irrigation of garden salad crops (Geldreich and Bordner 1971; Geldreich 1972a; Rosenberg et al. 1976; Cordano and Vergilio 1990; Rose 1990). The list of water-borne pathogens in polluted temperate and tropical drinking waters (Table 7) includes many of the same bacterial and viral agents but differs by the additional hazards of a variety of water-borne parasites with alternating life cycles between man and aquatic life forms. The list of water-borne agents will increase as new methodologies evolve to detect the more elusive organisms that cause gastroenteritis or other human illnesses.

The magnitude of pathogen reservoirs in the environment is exacerbated by expanding human populations worldwide. Population crowding and the unregulated development of satellite communities place undue pressures on sanitation infrastructural barriers (sewage collection and treatment, solid waste disposal, potable

Table 7 *Major infectious agents found in contaminated drinking waters world wide*

Bacteria	Viruses	Protozoa	Helminths
Campylobacter jejuni	Adenovirus (31 types)	*Balantidium coli*	*Ancylostoma duodenale*
Enteropathogenic *E. coli*	Enteroviruses (71 types)	*Entamoeba histolytica*	*Ascaris lumbricoides*
Salmonella (1 700 spp.)	Hepatitis A	*Giardia lamblia*	*Echinococcus granulosus*
Shigella (4 spp.)	Norwalk agent	*Cryptosporidium*	*Necator americanus*
Vibrio cholerae	Reovirus		*Strongyloides stercoralis*
Yersinia enterocolitica	Rotavirus		*Taenia solium*
	Coxsackie virus		*Trichuris trichiura*

Data from Geldreich (1990).

water supply processing and its distribution). Add to these problems the mobility of people throughout the world and it becomes apparent that an outbreak can quickly spread by person-to-person contact and through secondary water contamination, reaching epidemic proportions.

Pathogen pathways

Although sewage collection systems have decreased public-health risk in urban centers, this practice only serves to transport the collected wastes to some selected destination, hopefully where treatment is applied prior to release into a watercourse. Raw sewage discharges to receiving waters have often been shown to contain a variety of pathogens. The density and variety of human pathogens released are related to the population served by the sewage-collection system, seasonal patterns for certain diseases, and the extent of community infections at a given time. Some indication of the relative occurrences of various pathogens in raw sewage is given in Table 8 for two cities in South Africa (Grabow and Nupen 1972).

In major river systems receiving discharges of meat-processing wastes, raw sewage, and effluents from ineffective sewage treatment plants, the densities of *Salmonella* spp. may be substantial. It has been calculated that the Rhine and Meuse rivers carry approximately 50 million and 7 million *Salmonella* bacilli per second, respectively (Kampelmacher and Van Noorle Jansen 1973). The Missouri River represents another example of a pollution conduit, transporting a fecal pollution load from raw sewage, effluents from primary and secondary treatment plants of differing efficiencies, runoff from numerous cattle feedlots, and waste discharges from meat- and poultry-processing plants. As a consequence, it is not surprising that various *Salmonella* serotypes and viruses have been detected at the public water supply treatment plant intakes (see Table 4, p. 215).

Cattle feedlots and poultry operations result in an intense concentration of farm animals and their fecal wastes in a confined space. In cattle feedlot operations, the density of beef cattle per square mile may approach 10 000 animals. Under such restrictions, removal of fecal wastes is a major disposal operation. The closeness of farm animals in confined feeding operations invites the spread of disease in a healthy herd or poultry flock. Some farm animal pathogens (*Salmonella*, *E. coli* O157:H7, *Giardia*, and *Cryptosporidium*) are also major human pathogens. Unless contour cultivation and stormwater retention ponds are undertaken to reduce runoff, fecal material transported in stormwater runoff from cattle feedlots and poultry farms becomes a major source of contamination in rural watersheds, polluting streams and lakes in its drainage path. Direct recycling of untreated farm animal wastes and sewage by application to fields as fertilizer can become a serious contributor of pathogens to salad crops and ground water.

Wildlife refuges may also be a significant source of fecal contamination, particularly on a seasonal basis, since many wild animals are migratory by instinct in their search for food. The largest reservoir of wildlife pathogens will be found in warm-blooded animals such as beaver, deer, coyote, ducks, and gulls that are permanent residents of watersheds (forests, prairies, lakes, reservoir impoundments). These animals and others serve as reservoirs for *Salmonella*, *Campylobacter*, *Yersinia*, *Giardia*, and *Cryptosporidium*. Wildlife is also attracted to protected watershed areas (forest reserves, private lands) where human activities over the watershed are more restricted (Walter and Bottman 1967). Protected near-shore water environments are often the location of large beaver colonies, including individual animals infected with *Giardia*. Infected coyotes, muskrats, and voles are other wild animals that may be involved in the shedding of *Giardia* cysts and other pathogens into the aquatic environment. Terrestrial birds and waterfowl can be sources of bacterial pathogens. Songbird populations include individuals that may be infected with *Salmonella*. Seagulls are scavengers that frequent open rubbish dumps, eat contaminated food wastes, and contribute *Salmonella* in their fecal droppings to coastal lakes (Alter 1954; Fennel et al. 1974). In one instance, seagulls were the contributors of *Salmonella* to an untreated surface supply in an Alaskan community, causing several cases of salmonellosis (Anonymous 1954). Pigeons colonizing two water storage towers in Gideon, Missouri, were considered the

Table 8 *Microbial densities in municipal raw sewage from two cities in South Africa*

Organism or microbial group	Average count per 100 ml	
	Worcester sewage	Pietermaritzburg sewage
Aerobic plate count (37°C, 48 h)	1 110 000 000	1 370 000 000
Total coliforms	10 000 000	–
E. coli, type 1	930 000	1 470 000
Fecal streptococci	2 080 000	–
C. perfringens	89 000	–
Staphylococci (coagulase-positive)	41 400	28 100
P. aeruginosa	800 000	400 000
Salmonella	31	32
Acid-fast bacteria	410	530
Ascaris ova	16	12
Taenia ova	2	9
Trichuris ova	2	1
Enteroviruses and reoviruses (TCID50)	2 890	9 500

TCID, tissue culture infective dose.
Source: Grabow and Nupen (1972).

source of *Salmonella typhimurium* that contaminated the water supply, causing 600 illnesses and four deaths (Clark et al. 1996). Wildlife is also believed to be the source of *Campylobacter* that contaminated streams and reservoirs of low turbidity in Vermont (Vogt et al. 1982) and British Columbia (Report 1981). Vacationers to national parks in Wyoming became ill after drinking water from mountain streams, resulting in a 25 percent increase in outbreaks state-wide involving *Campylobacter enteritis* (Taylor et al. 1983).

Pathogen persistence

Upon discharge into the aquatic environment, the persistence of pathogens becomes a variable determined by many factors. For example, *Salmonella* strains were detected with regularity in surface water up to 250 m downstream from a wastewater treatment plant, but never at sample sites 1.5–4 km upstream (Kampelmacher and Van Noorle Jansen 1976). Various *Salmonella* serotypes transported by stormwater through a residential storm sewer and a wash-water drain at the University of Wisconsin experiment farm were isolated with regularity at a swimming beach approximately 800 m downstream (Claudon et al. 1971). Salmonellae were also detected in coastal waters from several Staten Island beaches and from shellfish harvested in New York harbor (Brezenski and Russomanno 1969).

Excessive biochemical oxygen demand (BOD) or total organic carbon (TOC) in a poor-quality wastewater effluent combined with low stream temperatures can also affect pathogen persistence. For example, *Salmonella* were isolated in the Red River of the North (North Dakota, Minnesota), 35 km downstream of sewage discharges from Fargo, North Dakota, and Moorhead, Minnesota, during September (Report 1965; Spino 1966). By November, *Salmonella* strains were found 99 km downstream of these two sites. In January, with the beginning of the sugar-beet-processing season, wastes reaching the stream under cover of ice brought high levels of bacterial nutrients. *Salmonella* were then detected 117 km downstream – a flow time of 4 days from the nearest point-source discharges of warm-blooded animal pollution.

NATURAL SELF-PURIFICATION FACTORS

Natural waters provide a fragile purification buffer to limited amounts of raw wastes and stormwater runoff entering the drainage basin. Every stream, lake, estuary, and water aquifer has some limited capacity to self-purify; surface waters generally have greater capacity than ground waters. Stream self-purification is a complex and ill-defined process that involves bacterial adsorption with sedimentation, nutrients, predation, competitive microbial populations, dilution, aeration, water temperature, water pH, and solar radiation (Geldreich 1986).

Retention of surface water in lakes, impoundments, and streams is an important contribution to water-quality enhancement. Storage and sedimentation of surface water often improves the microbial quality. Many organisms are inactivated by natural self-purification processes, whereas others are removed from the water through natural siltation (Romaninko 1971; Dzyuban 1975; Geldreich et al. 1980). These water-quality improvements are variable and are directly proportional to retention time, dilution, and the number of contributing sources (Geldreich 1991). In temperate zones with pronounced seasonal temperature changes, many lakes and impoundments are subject to periods of thermal stratification in the summer and winter and destratification (water turnover) periods in spring and

autumn. During stratification, water movement in the deeper portion of raw-water reservoirs becomes restricted, generally creating a zone of maximum bacterial contamination in the water layer above the thermocline (Weiss and Oglesby 1960; Collins 1963; Niewolak 1974; Drury and Gearheart 1975). With destratification, water from the bottom and top layers mixes and develops a more uniform water quality. This mixing process causes decaying vegetation and entrapped organisms in settled particulates to re-enter the water column (Weiss and Oglesby 1960; Niewolak 1987). Heterotrophic bacterial populations (including coliform bacteria), turbidity, and humics from partially decomposed vegetation will temporarily increase in the water.

Fresh seawater brings about a fairly rapid decline in fecal bacteria, salmonellae, and viruses. The main inactivating agent is solar radiation at wavelengths less than 400 nm (Gameson and Saxon 1967; Gameson and Gould 1975; Chamberlin and Mitchell 1978; Fujioka et al. 1981). Tidal currents, sedimentation, and sunlight intensity are variable factors that dictate the effectiveness of solar radiation as a force in natural self-purification of coastal marine water contamination.

Biofilms in water

A biofilm is a colonization of various organisms in a slimy matrix of extracellular organic polymers (glycocalyx) at sites that offer surfaces for attachment and protection from the shearing action of water turbulence (Costerton et al. 1978; Characklis and Marshall 1990). Patches of biofilm may be found on submerged rocks in slow-moving streams, within water-supply and wastewater treatment basin structures and their connecting flumes, in the water-pipe environment of sediments and tubercles, and in water-supply-attachment devices used in hospitals and clinics.

The microbial community in a water pipe begins with pioneering organisms in the passing flow of water becoming lodged in sediment, pipe corrosion, and at water sumps created in the design of various water delivery devices (Geldreich and Reasoner 1989). Colonization follows once a critical mass of assimilable organic carbon complexes, nitrogen occurring compounds, and traces of phosphorus become absorbed in associated sediments and tubercles. This growth is further accelerated as water temperatures rise above 15°C (Geldreich and LeChevallier 1999). Over time the consortium grows in complexity, the more fastidious organisms with unique nutrient requirements (*Legionella*) being supported by metabolic byproducts released from other bacteria in the microbial community.

As a biofilm expands in a section of water-distribution pipe, fragments of this bacterial growth are dislodged under certain situations into the bulk movement of water supply: shearing action of water velocity; flow reversals; abrasive action of pipe-cleaning procedures on tubercles; or abrupt shifts in water pH that softens sediment deposits. Among the variety of bacteria sloughed off the pipe surface are coliforms bacteria, particularly *Klebsiella pneumoniae*, *K. oxytoca*, *Pantoea agglomerans* (*Enterobacter agglomerans*), *E. cloacae*, or *Citrobacter freundii*. Coliform releases in biofilm fragments may not be of public health significance per se but should not be ignored because of perpetual concern for a hidden fecal contaminating event (Percival et al. 2000). Biofilms also induce corrosion and increase disinfectant demands and customer complaints of taste and odors. Other bacteria of concern in biofilm include a variety of heterotrophic organisms (*Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Proteus*, *Bacillus*, *Actinomycetes*, and fungi), some of which may be opportunistic pathogens causing nosocomial infections in the hospital environment (Costerton et al. 1981). The bacterial pathogen *Salmonella* has been demonstrated in laboratory research on biofilm development to have a very tenuous persistence because of competition from other organisms in the biological consortium (Camper et al. 1998).

WATER-QUALITY CRITERIA

Bacterial criteria for determining water quality have been directed primarily towards concern for microbial hazards to human health, although some thought has been given to health hazards to farm animals and aqua cultures of fish stocks, shellfish, and turtles (Report 1968, 1985). Indicators of fecal pollution have long been the predominant microbiological tool used to define the microbial quality of a water supply. However, there is growing evidence that water may transport not only intestinal pathogens via ingestion but also respiratory agents by inhalation and skin diseases through body contact. Thus, the microbial risks in water quality may require not only traditional criteria (heterotrophic bacteria, total coliforms, fecal coliforms, *E. coli*, fecal streptococcus, enterococcus) but also special surrogates such as *Clostridium*, aerobic spore formers, bacteriophages, and species-specific pathogens to evaluate health-risk status related to water use.

BACTERIOLOGICAL ANALYSIS

Standard methods for the analysis of a variety of waters are described in specific detail by the World Health Organization (Report 1984, 1992), the American Public Health Association (Report 1998), and the United Kingdom Report on Public Health and Medical Subjects (Report 1994), among other national documents worldwide. These methods relate to drinking water, recreational waters, swimming-pool waters, shellfish-growing

waters, wastewater discharges, recharge waters to aquifers, and other re-use applications.

For monitoring purposes, these indicators and various bacterial pathogens are detected by methods based on four principal procedures: pour or spread plates, selective cultivation in broth (multiple tube tests), membrane filter techniques, and enzyme–substrate reaction. More specific identification of selected heterotrophic organisms may be done by speciation of culture isolates.

Stressed organisms

Microbial detection must be based on methods that yield a high recovery of selected indicator groups or individual species. Recoveries are often 90 percent or better but are subject to some losses, which can be associated with injured bacteria in the water (McFeters et al. 1986; McFeters 1990). In various aquatic environments, competing organisms in the microbial community, toxic metal ions, toxic wastes, and inadequate disinfection are major causes of cell injuries. Many stressed organisms may thereupon become incapable of survival in standard laboratory procedures, a situation that can have a great impact on data interpretation. The public-health concern lies with bathing water, potable supplies, and shellfish beds that have indicator densities ranging below 200 organisms per 100 ml. It is important to note that while some enteropathogenic bacteria may also be susceptible to similar stresses, the potential for virulence remains and the injured cells recover after being injested by exposed indiciduals (LeChevallier et al., 1985; Singh et al. 1986; Singh et al., 1986).

Efforts to reverse these impacts on indicator organisms may include the use of a chelating agent and application of a dechlorinating compound in the sterile sample bottle, initially resuscitating the stressed cells by culturing in an enriched, nonselective medium with incubation for 2 h at room temperature prior to selective incubation on T7 agar at 35°C for the total coliform pupulation (LeChevallier et al. 1985). For recovery of injured fecal coliform cells, a two-layered agar using an overlay of tryptic soy agar on M-FC medium with incubation at 44.5°C provides a gradual sift to more selective nutrients (Rose et al. 1975). A similar principle applies to fecal streptococcus-enterococcus indicator selection (Report 1998). Initial verification of presumptive results for any of these special procedures is essential, using other procedures to establish test validity and to eliminate false-positive results that may break through the recovery protocol (Lin 1974).

Sample integrity is essential. Care must be exercised to collect samples representative of water use and to ensure that the sample does not become contaminated at the time of collection or before examination. Samples should be refrigerated or placed in an insulated cooler and promptly transported to the laboratory. Sample transit time limits for total coliform analysis of drinking water have been established at 24 h to limit any drastic changes in indicator occurrence impacted by antagonistic factors found in polluted natural waters. Sample processing within 8 h of collection should always be a goal for optimum recovery of organisms of significance. A dechlorinating agent (sodium thiosulfate) should be in the sterile sample bottle used to collect treated water supply or wastewater samples that often contain varying concentrations of disinfectant residues.

Heterotrophic bacteria

Heterotrophic bacteria are a diverse group of organisms. They encompass differences in morphology, gram-staining response, and biochemical reactions, but most of these organisms utilize carbon, nitrogen, phosphorus, and various trace elements necessary for the synthesis of living cell materials. The metabolic pathways to achieve these conversions are remarkably diverse, from those that use only inorganic sources of carbon, nitrogen and inorganic salts, to others that grow only when there are complex organics available. Such is the case for *Legionella* spp., which have fastidious nutrient requirements that are only made available in the metabolic biproducts from other bacteria in the aquatic community. Different groupings can be formed from this population of heterotrophs such as those that are pigmented (Reasoner et al. 1989), those that are antagonistic to coliform bacteria (Geldreich et al. 1978), and others that cause taste and odors (Burman 1965). Some of these bacteria are also known to be involved in plasmid transfers of R factors for antibiotics (Armstrong et al. 1982) or disinfection resistance (Ridgway and Olson 1982).

Predominate heterotrophic bacterial genera include *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, environmental coliforms (*Citrobacter*, *Enterobacter*, *Klebsiella*, *Escherichia*), *Flavobacterium*, *Pseudomonas*, *Micrococcus*, and *Moraxella*. Densities of heterotrophic bacteria in drinking water may be in a range from <10/ml to as high as 10 000/ml, while in raw sewage and food-processing wastes discharged to surface waters, densities may be in the millions per milliliter. In high-quality natural waters (protected ground water and springs) most of the organisms are indigenous and of no sanitary significance. In surface water (lakes and rivers), the heterotrophic composition will have varying inputs of contamination from stormwater runoff, domestic and industrial wastes, agricultural activities, wildlife habitation, soil contact, vegetation, and airborne pollutants.

Since the beginnings of bacteriology, the heterotrophic plate count has been used in an attempt to characterize water quality. Obviously, an excellent water was thought to have few bacteria per milliliter, whereas water with more than 1 000 bacteria per milliliter was considered of poor quality. In time, it was concluded that the general density of bacteria in water often had

Table 9 *Effect of incubation temperature and time to recover heterotrophic bacteria from water supply samples by different methods*

Temperature (°C)	Medium	Method	Incubation time (days)			
			2	4	6	7
20	SPC	Pour plate	22	130	570	900
	R2A	Spread plate	90	1 100	4 700	6 100
	R2A	Membrane filter	75	650	3 000	4 900
	M-HPC	Membrane filter	48	400	1 600	2 000
28	SPC	Pour plate	90	640	950	1 000
	R2A	Spread plate	360	2 800	6 700	7 200
	R2A	Membrane filter	160	2 200	3 500	4 000
	M-HPC	Membrane filter	140	1 000	1 700	1 900
35	SPC	Pour plate	22	100	110	115
	R2A	Spread plate	200	340	500	510
	R2A	Membrane filter	41	200	270	280
	M-HPC	Membrane filter	32	140	150	150

Data revised from Reasoner (1990).

little specific relationship to increased health risks and should be replaced by a search for organisms more specific to fecal contamination (Bartram et al. 2003). Characterization and interpretation of heterotrophic bacterial densities now places the focus on using the information to identify changes in the microbial quality of distribution water caused by accumulating pipeline sediments and subsequent loss of a protective disinfectant residue. Most often these organisms are not of immediate public-health significance but upon amplification in a protected pipe habitat become the source of customer complaints of offensive taste/odors or emerge as an opportunistic pathogenic threat to some segment of the population.

Traditionally, most water-plant laboratories have used a nutritive standard plate count (SPC) agar or equivalent formulations for years from which they can provide an extensive database on heterotrophic bacterial densities in various water treatment processes. More recently, the trend is to use a dilute but diverse nutrient formulation such as R-2A agar, which contains a diverse variety of biodegradable materials (peptone, casamino acids, glucose, soluble starch, and sodium pyruvate). Colony densities are often many times greater and consist of a more diverse variety of organisms, including pigmented bacteria (Reasoner and Geldreich 1985). To achieve this improvement, incubation for 5–7 days at a temperature of 28°C is preferred rather than the more conventional time of 48 h at 35°C. This change in cultivation provides more opportunities for the growth of slow-generating bacteria, stressed organisms, and those species that have unique nutrient requirements. Since pour plates limit the growth of obligate aerobes in agar and introduce the risk of heat shock from melted agar (Klein and Wu 1974), attention has turned to surface cultivation of bacteria by spread plate to optimize recovery of these sensitive bacteria.

The membrane-filter procedure provides another approach to surface cultivation of organisms. The unique advantage of this technique is that it permits the analysis of larger sample volumes of high-quality water containing too few organisms to be detected by a 1-ml sample. The only restrictions to the size of sample analyzed are turbidity and colony density on the filter surface.

Some indication of the impact of membrane filter surface cultivation in comparison with pour plates and spread plates can be seen in Table 9. Recovery densities are significantly better on spread plates than by the traditional pour plate method because of agar temperature (Stapert et al. 1962). Limited surface area on the membrane filter for discrete colony formation without developing confluence with the other colonies thereby restricts extended incubation time and is the major drawback for achieving results equivalent to the spread plate technique.

Total coliform bacteria

In an effort to obtain a better focus on the sanitary quality of water, coliform bacteria soon supplanted the heterotrophic plate count as the most widely used indicator for routine analysis. Coliform bacteria consist of an artificial grouping of organisms believed to be associated with fecal pollution, but in reality they may also include environmental organisms from soil, vegetation, and decaying organic matter (Randall 1956; Geldreich et al. 1962; Schubert and Mann 1968). In the aquatic environment, environmental coliforms persist longer than do *E. coli*, the coliform constantly present in large numbers in feces from humans and other warm-blooded animals (wildlife, farm animals, cats, and dogs). Major members of these environmental coliforms include *Klebsiella*, *Enterobacter*, and *Citrobacter*. Although not

perfect, the total coliform concept continues to be a practical tool for providing basic microbial information on drinking-water treatment effectiveness and distribution system integrity.

Fecal coliforms/*E. coli*

Interest in further refining the total coliform group to exclude environmental coliforms, which are of no sanitary significance, led to the development of the fecal coliform (thermotolerant coliform) test based on lactose fermentation within 24 h at 44–44.5°C. The fecal coliform test proved to be a breakthrough with positive correlations of 93–99 percent to coliforms found in the feces of warm-blooded animals (Geldreich 1966).

In polluted waters, fecal coliform measurements relate more precisely to fecal contamination than to total coliforms and are significantly less susceptible to distortions caused by the regrowth characteristics of environmental strains in receiving waters. Essential factors that stimulate bacterial regrowth in polluted waters are nitrogen, carbon, and a warm water temperature (above 15°C). The regrowth phenomenon for fecal coliforms requires excessive nutrient discharges generally associated with poor treatment practices, particularly those used on some food-processing and paper-mill wastes.

Debate on the status of fecal *Klebsiella* and growth of the environmental component in the aquatic environment has led to attempts to search only for *E. coli*. Commercial biochemical kits for the identification of *E. coli* by detection of 4-methylumbelliferyl-β-D-glucuronidase (MUG) in either an amended medium or a chemically defined medium are now being used to further narrow the coliform group to one specific fecal organism – *E. coli*.

VERIFICATION OF COLIFORM BACTERIA

Verification of every positive coliform result is good laboratory practice that eliminates all doubts that the test results are perhaps caused by some analogous false reaction. Coliform analysis of drinking waters has revealed that as much as 30 percent of the samples having positive presumptive test results may also contain *Aeromonas* (Ptak et al. 1974; Leclerc et al. 1977). A few *Aeromonas* strains have been the cause of water-borne gastroenteritis (Moyer 1989), but most are saprophytic organisms in the aquatic environment. This situation illustrates the reason why test results must be confirmed to eliminate false-positive results in all methods even though this delay partly nullifies the rapidity of the results.

Rapid verification of coliform bacteria can be achieved in the demonstration of cytochrome oxidase and β-galactosidase activity. All coliform bacteria are negative for cytochrome oxidase while the enzyme β-glucuronidase appears to be highly specific for *E. coli*. This latter metabolic response has formed the basis of a rapid confirmatory test using the 4-nitrophenyl-β-D-glucuronide (Perez et al. 1986) or MUG (Report 1998).

Coliform speciation is a more in-depth verification process that has been used to demonstrate the equivalence of methods to detect all coliforms. Speciation as a verification tool has its greatest use in the identification of a coliform biofilm event and in further tracing for the existence of a microbial pathway in the water supply distribution system. In either case, further exploration of the original sample may provide information on fecal contamination that might not be present in a repeat sample collected the next day. Most often, repeat samples are negative because the contaminating event is of short duration (Geldreich 1996).

Fecal streptococci

Fecal streptococci are distinguished by their ability to grow at 45°C in the presence of 40 percent bile, and in the concentrations of sodium azide or potassium tellurite that are inhibitory to most coliforms organisms. Methods are available using agar pour plates (KF or PSE agars), multiple tube (azide dextrose broth), and membrane filter (KF agar or broth). The occurrence of fecal streptococci in water generally indicates fecal pollution (Geldreich and Kenner 1969). Although fecal streptococci rarely multiply in polluted waters, they may persist for extended periods in irrigation waters with high electrolyte contents and favorable temperatures (Geldreich 1973). There is also some evidence that *Enterococcus fecalis* (*Streptococcus fecalis*) multiplies in water from vegetable-processing plants (Mundt et al. 1966a). The reason for such contrasting responses is that the fecal streptococcus group includes a wide spectrum of strains that have specific fecal origins and diverse survival rates and includes several environmental biotypes (Mundt et al. 1962; Mundt and Graham 1968). Within the fecal streptococcus group, *Streptococcus bovis* and *Streptococcus equinus* are specific indicators of farm animal pollution. This differential characteristic is particularly useful in pollution investigations involving cattle feedlot runoff, farmland drainage, discharge from meat- and poultry-processing operations, and dairy-plant wastes (Geldreich 1972b). In addition, *S. bovis* and *S. equinus* are the fecal streptococci that die off most rapidly outside the animal intestinal tract. Therefore, the detection of these strains in water indicates very recent farm animal contamination. By contrast, the ubiquitous *E. fecalis* var. *liquifaciens* may affect precision of this indicator system at counts below 100 fecal streptococci per 100 ml, because at these low population levels this biotype generally predominates in high-quality natural waters. Fecal streptococcus counts greater than 100 per 100 ml, however, indicate significant fecal pollution derived from some warm-blooded animal source. The

densities for this indicator group in polluted waters approach the magnitude observed for coliforms, or at times exceed it by a factor of ten, depending upon the source of fecal pollution.

The density difference between fecal coliforms and fecal streptococci in fecal material is a unique relationship that can be useful in defining sources of pollution. The ratio of fecal coliform to fecal streptococcus in human feces and domestic wastes is greater than 4.0. The ratio of fecal coliforms to fecal streptococci in the feces of farm animals, cats, dogs, and rodents is less than 0.7. Such relationships between fecal coliform and fecal streptococci can only be developed from a fecal coliform medium (not an *E. coli* test) and from a fecal streptococcus medium such as KF or PSE agars (not from a medium dedicated to enterococcus). If cultivation of the more restrictive enterococcus subgroup is desired, mE agar or m-enterococcus agar may be considered.

Clostridium perfringens

The genus *Clostridium*, in particular *C. perfringens*, merits some attention as an indicator of fecal pollution because of its significant occurrence in feces (Bishop and Allcock 1960). *Clostridium* spp. spores are resistant to wastewater treatment practices and tolerant to extremes in temperature and environmental stress. *C. perfringens* is an indicator of present fecal contamination as well as a conservative tracer of past fecal pollution (Wilson and Blair 1925). *C. perfringens* has been used in the UK to monitor the quality of ground-water supplies that are examined infrequently, i.e. at intervals of 4–6 months (Wilson 1931). When *C. perfringens* is found in these well waters, sampling is repeated, possibly weekly, using total coliform or fecal coli measurements to search for intermittent fecal contamination and the need for a sanitary inspection to verify adequate well-head protection. The use of *C. perfringens* as a supplemental indicator to measure the efficiency of various water treatment processes has been proposed, but the greater abundance of total aerobic spore formers in raw water may be a more effective system in providing evidence of treatment barrier performance, particularly in reference to pathogenic protozoans(Rice et al. 1996).

The longer persistence of *C. perfringens* in the water environment creates a residual density of this organism, which can obscure the detection of low densities of recent polluting discharges to receiving waters. However, Bonde reported that *C. perfringens* densities in the sediment of marine waters were proportional to pollution discharges and the ratios of spores to vegetative cells of this bacterial species increased with distance from the pollution source (Bonde 1962, 1967, 1968). Thus, vegetative cells of *C. perfringens* may be expected to predominate in raw sewage.

Although *C. perfringens* is an anaerobic organism, it can tolerate up to 5 percent oxygen without significant loss in quantitative recovery (Fulton and Richardson 1971). Therefore, methodology has not been complicated by the restrictions of complete anaerobiosis. On sulfite–alum agar (to reduce available oxygen) incubated at 48°C, 79–100 percent of the black sulfite-reducing colonies recovered from a variety of waters were verified as *C. perfringens* (Bonde 1962). The other black colonies on this medium may be *Salmonella*, *Proteus*, *Bacteroides*, and sometimes *E. coli* (Johnston et al. 1964). This practical procedure has frequently been used to obtain a presumptive quantitation of *C. perfringens* in various fresh and marine waters and sediments. Another approach is to filter an appropriate volume of water sample through a membrane filter, place the membrane filter on a modified m-CP agar (Armon and Payment 1988), and incubate anaerobically for 24 h at 44.5°C. Upon exposure to ammonium hydroxide, the yellow to straw-colored *C. perfringens* colonies turn dark pink to magenta. Confirmation may be done by anaerobic growth in thioglycollate, a positive Gram stain reaction, and stormy fermentation of iron milk.

Pseudomonas species

Pseudomonads are ubiquitous bacteria that are able to flourish in a wide variety of habitats (surface waters, aquifers, seawater, soil, and vegetation). Some pseudomonads are among the prominent denitrifiers while others grow prodigiously in and on tertiary treatment devices such as reverse osmosis and electrodialysis membranes and in sand or carbon filtration beds. Pseudomonads reported in some drinking-water supplies include *Pseudomonas aeruginosa*, *Burkholderia cepacia* (*Pseudomonas cepacia*), *Pseudomonas fluorescens*, *Burkholderia mallei* (*Pseudomonas mallei*), *Stenotrophomonas maltophilia* (*Pseudomonas maltophila*), *Pseudomonas putida*, and *Comamonas testosteroni* (*Pseudomonas testosteroni*) (Geldreich 1990, 1996; Gambassini et al. 1990). To this list can be added *Pseudomonas stutzeri*, *Brevundimonas diminuta* (*Pseudomonas diminuta*), and *Delftia acidovorans* (*Pseudomonas acidovorans*), which have been found in bottled waters at densities ranging from 10^3 to 10^5 organisms per milliliter (Gavin and LeClerc 1974; Hernandez Duquino and Rosenberg 1987). These organisms metabolically adapt to survival on minimal nutrient concentrations typical of protected aquifers and treated drinking water. Routine enumeration of pseudomonads in water supply is not recommended but may be of value in certain industries, such as those manufacturing foods, drinks, and pharmaceutical products, where high-quality water is desirable. Monitoring the hospital building water-distribution system for *P. aeruginosa* is important because this organism is a major opportunistic pathogen

and its presence in this environment is a matter for concern.

PRINCIPLES OF PATHOGEN DETECTION

Detection of pathogens in the aquatic environment requires techniques to concentrate a few organisms from large samples and reduce the interfering flora of many heterotrophic bacteria that are certain to be a factor in contaminated water. For bacterial pathogens, this is generally accomplished through filtration of large volumes of water (1–2 l) followed by cultivation in selective media for suppression of coliforms and other heterotrophic organisms. Another approach is to place gauze pads in the polluted stream for 3–5 days to entrap the pathogenic agent, then express the water from the pad to selective enrichment media (Moore 1948, 1950). A third approach is to filter samples of 1–2 l through diatomaceous earth held in a membrane-filter (MF) funnel by an absorbent pad, and then place sections of the plug in appropriate media for cultivation and species identification.

STRATEGIES FOR RAPID METHODS

Rapid laboratory methods to better characterize water-supply quality are an urgent research priority. The inability of microbiology to provide data within a few hours of sample processing has been a major deterrent to greater utilization of the science in water-plant operations and in bathing-water monitoring. With the discovery that disinfection byproducts may contribute carcinogens and possibly other toxins to the water supply, treatment schemes are being modified to minimize these undesirable formations. In so doing the effectiveness of treatment barriers required to avoid microbial contaminant passages becomes a matter of greater concern. Consequently, the need for rapid assessment of water quality is more urgent than ever before. Such information would also be of singular value as an aid in restoring the quality of drinking water after contamination caused by sudden source-water-quality deterioration, treatment failures, distribution line breaks, poor practices in disinfection line repair, and sudden occurrence of cross-connections.

Any breakthrough in the development of rapid tests must involve specificity, sensitivity, and precision with achievement of a test result within a few hours. Searching for rapid methods in environmental microbiology has revealed a variety of candidate methods that have potential in real-time monitoring (Geldreich and Reasoner 1985). As might be anticipated, rapid tests that can be performed in less than 1 h have little specificity and may possibly include nonviable cells. These limitations are being solved, but the tradeoff is in the need for more costly materials and instrumentation sensitive to trace concentrations of specific metabolic products produced by organisms of interest. One exception to this trend may be seen in a direct membrane-filter method incorporating MUG in a modified medium (M-7h agar), which provides detection of as few as one fecal coliform per 100 ml in 6 h (Berg and Fiksdal 1988).

Efforts to shorten the time required to detect bacterial indicator systems or individual pathogen species in a water sample flora to less than 5 h will require instrumentation capable of detecting viable organisms directly or after a brief period of amplification in a selective enrichment medium. Instrumentation and test materials for these methods will become more readily available to the laboratory in the near future.

Of the rapid test candidates currently in development, gene probes appear to be the most promising (Richardson et al. 1991). Research into microbial genetics has led to techniques that can detect minute quantities of nucleotide sequences unique to a single species of organism, whether bacteria, fungi, virus, or invertebrates. For example, ten enterotoxigenic *E. coli* per milliliter of canal water were detected in the grossly polluted canals in Bangkok, Thailand, by using gene-probe technology (Mosely et al. 1982).

The use of gene probes and automated microbiological techniques will become a major activity in the water plant laboratories of the future (Geldreich 1991). Perhaps development of multiplex probes to detect bacterial pathogens or enteroviruses will become sensitive enough to detect one organism per liter of sample within an hour. At the present time, some probe tests can be completed in 0.5–2 h, but they often require prior culture amplification for 7–18 h to achieve organism densities at levels where acceptable probe detection is possible (Tenover 1988; Report 1998).

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Bacteriology of milk and milk products

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The milk of cows, buffaloes, sheep, goats, donkeys, horses, and camels has been an important item of human food for centuries. Pathogenic microorganisms often contaminate milk, which has been recognized as a vehicle for disease since the late nineteenth century (Department of Public Health 1886). Strategies such as improved hygiene, eradication of tuberculosis and brucellosis from cattle, and heat treatment are now employed in most developed countries to reduce the risk to human health. Liquid milk and milk products such as cheese, yogurt, and fermented milks are often stored for days, weeks, or months and finally consumed without cooking. It is not surprising, therefore, that these items are still implicated in major outbreaks of food poisoning and other communicable diseases (Djuretic et al. 1997; De Buyser et al. 2001; Gillespie et al. 2003a).

Because milk and milk products are such universal items of food, sporadic infections caused by them may go unnoticed. Wilson (1942) discussed the difficulties of ascertaining the frequency of milkborne disease. At that time, pasteurization was not widely practiced, and both milk-associated tuberculosis and undulant fever were major public health problems. Both diseases had long incubation periods, gradual onset, and variable infectivity. It was therefore difficult to prove their relationship with the consumption of milk. Moreover, if, in large explosive outbreaks of disease such as typhoid fever, scarlet fever, or food poisoning, cases were scattered over a wide area, other sources of infection were often suspected. Thus, unless careful epidemiological investigations were made, the association with contaminated milk might be overlooked. Even nowadays, in developing countries, Wilson's observations remain true and where hygiene is poor and animal disease uncontrolled, milk is an important vehicle for various human diseases.

Most developed countries have sophisticated disease surveillance systems in place and resources to investigate outbreaks. In addition, the pathogenic microorganisms likely to contaminate milk differ from those prevalent in 1942 (Bell and Palmer 1983; Milner 1995; Djuretic et al. 1997; De Buyser et al. 2001; Gillespie et al. 2003a); some have decreased as a result of animal disease eradication programs and changes in patterns of human disease, but others have taken their place. It is clear that, although rare, public health problems with milk and milk products remain and where milk products are produced by large commercial establishments there is potential for a major outbreak should anything go wrong.

MILK

Microbiology of cows' milk

The major milk-producing animal is the cow and most of the available information and research into microbiology of milk relates to cows' milk. Microorganisms in raw milk are derived from various sources, including the commensal or pathogenic flora of the udder, teat canals, and skin, which varies depending on whether cattle are housed under cover or in yards or are out in pasture; fecal contamination; and environmental sources such as

milking equipment, storage vessels or the water supply. Commensal or pathogenic organisms from the milkers may also contribute.

The importance of particular organisms depends on whether the milk is to be consumed raw or after heat processing, and on the temperature and duration of storage. In developed countries the commercial production of milk is subject to strict regulations regarding hygiene and refrigeration during transport and storage. The keeping quality and flavor of the heat-treated product is adversely affected by psychrotrophic bacteria (capable of growing at between 2 and 7°C), thermoduric organisms, and post-pasteurization contamination. Some of the enzymes produced by psychrotrophs are heat-tolerant and cause spoilage both before and after pasteurization. Post-pasteurization contamination is the most important factor influencing the quality of pasteurized milk but the quality and hygiene of raw milk also have important effects on the quality of the heat-treated product.

No matter how good the health of the dairy herd, the milking hygiene, and the storage conditions, the safety of raw milk cannot be guaranteed (Sharp et al. 1985; Maguire 1993; de Louvois and Rampling 1998; Jayarao and Henning 2001; Gillespie et al. 2003a). Detailed reviews of the microbiology of raw milk are to be found in specialist publications (Bramley and McKinnon 1990; Gilmour and Rowe 1990). Most of the microorganisms in hygienically produced raw milk with low bacterial counts are derived from the normal flora of the cow's teat canals and skin, and comprise coagulase-negative staphylococci, micrococci, and streptococci. Streptococci belonging to Lancefield group N cause natural souring of raw milk. Commercially cultured strains of these organisms are used as cheese starters in the dairy industry.

The skin of the teats and udder inevitably becomes contaminated by fecal and environmental bacteria, viruses, yeasts, and molds. Cows housed inside during winter have very heavily soiled udders and the bacterial counts in milk are high even after washing the teats. The most effective way of removing contamination is careful washing followed by drying with paper towels or with a disinfectant impregnated cloth. When the cattle are turned out to pasture there is a pronounced decline in the total bacterial count in bulk milk, even compared with milk from carefully washed cows (Bramley and McKinnon 1990). Inadequately disinfected milking machines, pipes, and tanks are also important sources of post-milking contamination with bacteria, which are mainly derived from milk previously in the system. In the UK, the water supply to all areas concerned with milking and milk collection must be potable, but in many countries farms obtain water from boreholes or springs which may at times be contaminated with pathogenic organisms such as *Campylobacter jejuni*, verocytotoxin producing *Escherichia coli* or *Salmonella* serotypes. Contaminated water can also be a source of psychrotrophic organisms such as *Pseudomonas* spp., which cause spoilage of refrigerated milk. Under modern systems of dairy management and marketing, milk is stored in refrigerated farm tanks for up to 48 h prior to collection. Once at the creamery, it may again be stored overnight in large insulated or refrigerated silos. During this time there is considerable scope for multiplication of psychrotrophic organisms.

The level of bacteria in raw milk increases significantly if the cow has mastitis and many of the organisms associated with this condition are potential causes of human infection or intoxication. The most common causes of mastitis are *Staphylococcus aureus* (including enterotoxigenic strains); *Streptococcus agalactiae* (Lancefield group B); *Streptococcus uberis*; *Streptococcus dysgalactiae* (Lancefield group C); and *E. coli*. Less common causes are *Leptospira interrogans* serovar. *hardjo*, *Streptococcus zooepidemicus* (Lancefield group C), *Listeria monocytogenes*, *Bacillus cereus*, *Pasteurella multocida*, *Clostridium perfringens*, *Nocardia* spp., *Cryptococcus neoformans*, *Actinomyces* spp., and *Corynebacterium ulcerans*.

At one time, *Mycobacterium* spp. were an important cause of bovine mastitis in the UK and both *Mycobacterium bovis* and *Brucella abortus* were commonly present in milk even in the absence of mastitis (Galbraith and Pusey 1984). Both these pathogens are now virtually eradicated from cattle in most developed countries. Milkborne scarlet fever due to *Streptococcus pyogenes* (Lancefield group A) was also common at one time (Eyler 1986). Group A streptococci are not natural pathogens of cattle but cows can develop teat lesions and mastitis if infected by a human carrier. Other pathogens such as *L. monocytogenes*, *Coxiella burnetii*, and *S. zooepidemicus* may on occasions be excreted in milk in the absence of udder disease and *Salmonella* serotypes (Marth 1969) and *C. jejuni* occasionally cause clinical or subclinical mastitis (Morgan et al. 1985; Orr et al. 1995). Under natural conditions, fecal contamination is probably the most common source of salmonellae, *L. monocytogenes*, *C. jejuni*, and verocytotoxin producing *E. coli* O157.

A study conducted by the Public Health Laboratory Service (PHLS) in 1996–1997 showed that 41 of 1 097 samples of bottled raw milk were contaminated with potentially pathogenic organisms (de Louvois and Rampling 1998). Of these 41 samples, five contained *Salmonella* serotypes, three *E. coli* O157, 19 *C. jejuni*, 12 *S. aureus*, and two hemolytic streptococci. This study also confirmed that pathogens may be present in raw milk that satisfies other statutory criteria. In a study of bulk tank milk sampled from 131 dairy herds in South Dakota and western Minnesota, USA, 26 percent of samples contained one or more foodborne pathogens (Jayarao and Henning 2001). In this latter study *C. jejuni*, verocytotoxin producing *E. coli*, *L. monocytogenes*, *Salmonella* spp., and *Yersinia enterocolitica*

were detected in 9.2, 3.8, 4.6, 6.1, and 6.1 percent of bulk tank milk samples, respectively. *E. coli* O157 was not detected in bulk tank milk samples in this study. These studies confirm that fecal contamination of raw milk and presence of potential pathogens in raw milk is a frequent event.

Microbiology of ewes' and goats' milk

Milk, and in particular milk products, made from ewes' and goats' milk have become very popular in recent years and the commercial market for cheese and yogurt is expanding. Surveys of the microbiological quality of goats' milk have been performed in Scotland (Hunter and Cruickshank 1984) and England (Roberts 1985; Little and de Louvois 1999). The 1984 and 1985 studies both showed that high standards of hygiene were possible but that problems arose from inadequate disinfection of milking equipment or from inadequate control of storage temperatures at the point of sale.

A study of goats' milk on six farms in Quebec over a one year period showed that the levels of aerobes, psychrotrophs, coliforms, yeasts, and molds increased during the summer months but that this could be controlled by good hygiene, rapid cooling, refrigeration, and frequent collections by refrigerated vehicles (Tirard-Collet et al. 1991). Somatic cell numbers were also measured and these confirmed that, in contrast to the finding with cows, variation with the season and the state of lactation of the animals, high levels of somatic cells were not necessarily an indication of mastitis (Hinckley 1991).

A more recent study undertaken by the Public Health Laboratory Service assessed the microbiological quality of unpasteurised goats' milk sold at retail (Little and de Louvois 1999). Although this was a pilot study, the results indicate that 47 percent (47/100) of goats' milk samples failed to meet the standards required in the Dairy Products (Hygiene) Regulations (HMSO 1995). *C. jejuni*, *E. coli* O157, and *Salmonella* spp. were not detected but *S. aureus*, hemolytic streptococci or enterococci were present in excess of 100 cfu/ml in 6, 2, and 12 percent of samples, respectively.

There are very few publications on the microbiology of sheep milk. Studies in Spain, Bulgaria, and Norway have indicated that significant contamination with Enterobacteriaceae and coliforms may occur during milking and as a result of poor cleaning practices on the farm (Gaya et al. 1987). A very limited study of ewes' milk was reported by Little and de Louvois (1999), and demonstrated that 50 percent (13/26) of ewes' milk failed to meet the standards required in the Dairy Products (Hygiene) Regulations (HMSO 1995). *C. jejuni*, *E. coli* O157, and *Salmonella* spp. were not detected, but *S. aureus*, or enterococci were present in excess of 100 cfu/ml in 11.5 and 27 percent of samples, respectively.

Even when hygiene is good, the risk of milkborne infection from unpasteurized goats' or ewes' milk is similar to that for cows' milk. *Brucella melitensis* and *Brucella ovis* are still common in goats and sheep in the Mediterranean countries, Asia, and Latin America and human infections are associated with the consumption of raw milk or cheese made from unpasteurized milk (Wallach et al. 1994; Report 1995). Other pathogenic microorganisms such as *Salmonella* Typhimurium, *S. aureus*, *Y. enterocolitica*, *C. perfringens*, *B. cereus*, and *C. jejuni* can survive in goats' milk under experimental conditions, and some of these pathogens multiply at temperatures of 22°C or more (Roberts 1985). Outbreaks and incidents of brucellosis, campylobacter infection, salmonellosis, viral tick-borne encephalitis, Q fever, staphylococcal food poisoning, and *C. ulcerans* sore throat have been associated with consumption of untreated goats' milk or goat cheese in Canada, Britain, and the USA (Hutchinson et al. 1985a; Sharp 1987; Fishbein and Raoult 1992; Report 1994a, 1995). There is also a high incidence of toxoplasma infection in goats (Skinner et al. 1987) and there is convincing evidence that consumption of unpasteurized goats' milk can transmit infection to man (Sacks et al. 1982). *E. coli* O157 has emerged as a significant pathogen in milk and dairy products and outbreaks associated with cheese made from unpasteurized goats' milk have been reported from the Czech Republic (Bielaszewska et al. 1997) and from Scotland (Curnow 1999). Both of these outbreaks were associated with infection in children. The outbreak in the Czech Republic was recognized because of a cluster of four cases of hemolytic uremic syndrome. The outbreak in Scotland resulted in 22 confirmed cases of infection in children attending a primary school. The most recent outbreak of *E. coli* O157 associated with unpasteurized goats' milk was reported in British Columbia, Canada (McIntyre et al. 2002). Following a review of infection with *E. coli* O157 in England and Wales and Scotland, it has been recommended that raw ewes' and goats' milk for sale for drinking should be heat treated (Task Force on *E. coli* O157 2001).

Sheep can also carry many microorganisms pathogenic for man, such as *Salmonella* spp., *C. burnetii*, *Campylobacter* spp., *E. coli* O157, *S. aureus*, *Toxoplasma gondii*, and *L. monocytogenes*. *S. aureus* seems to be a common contaminant of both ewes' and goats' milk and more prevalent than in cows' milk (Valle et al. 1990). A high proportion of isolates produce enterotoxin. An outbreak of staphylococcal food poisoning in Scotland was traced to cheese made from unpasteurized sheep milk (Sharp 1987). Although *L. monocytogenes* is a cause of meningoencephalitis in sheep and may also be excreted in the milk and feces of healthy sheep, the risk of contamination of ewes' milk on the farm does not seem to be any greater than for cows' milk. Rodriguez et al. (1994) sampled milk from 1 052 farm bulk tanks on 283

Spanish farms throughout a 1-year period and found that *Listeria* spp. could be detected in 4.56 percent and *L. monocytogenes* in 2.19 percent. This low prevalence was confirmed in the study described by Little and de Louvois (1999). *Listeria* species were not detected by enrichment culture in all 26 samples of ewes' milk and 100 samples of goats' milk. Therefore, the risk of acquiring *L. monocytogenes* infection from these products appears to be low.

Heat treatment of milk

PASTEURIZED MILK

The international definition of pasteurization (Report 1994b) is: 'A heat treatment process applied to a product with the aim of avoiding public health hazards arising from pathogenic microorganisms associated with milk. Pasteurization as a heat treatment process is intended to result in only minimal chemical, physical, and organoleptic changes.' The process was originally introduced by dairies to improve the keeping quality of milk (Wilson 1942). It soon became clear that heat treatment would also destroy pathogenic organisms such as tubercle bacilli and hemolytic streptococci. The public health aspects of the process then assumed the greater importance.

Several time–temperature combinations are effective. The Dairy Products (Hygiene) Regulations (HSMO 1995) for England and Wales state that milk shall be pasteurized by means of a heat treatment involving a high temperature for a short time (at least 71.7°C for 15 s, or any equivalent combination) or a pasteurization process using different time and temperature combinations to obtain an equivalent effect. The product must give a negative alkaline phosphatase test immediately after heat treatment and be cooled as soon as practicable to a temperature of 6°C or lower and stored at or below that temperature until it leaves the treatment establishment.

The high temperature short time (HTST) method is most commonly used both in large commercial dairies and on farms. The HTST plant consists of a plate heat exchanger and a holding tube (Figure 1). Raw milk

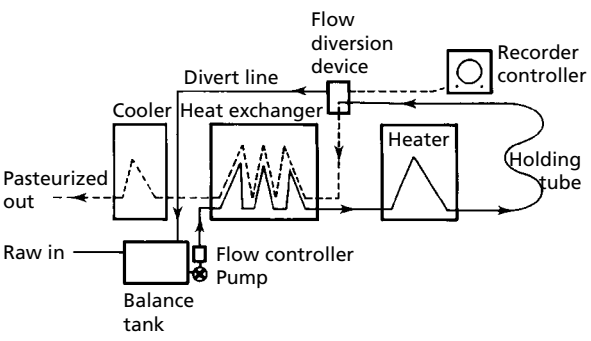

Figure 1 *Diagrammatic representation of milk flow through HTST pasteurizer*

enters the heat exchanger and is warmed by the hot milk leaving the hot section along the other side of the plates. The warmed milk is then heated to the statutory temperature before it runs through the holding tube for at least 15 s. The hot milk then re-enters the heat exchanger, being cooled initially by cold raw milk, then by tap water and finally by chilled water. The law requires that any HTST system has an automatic temperature control and a safety device that automatically diverts milk back for reheating if it has not been raised to the statutory temperature. In addition, there must be indicating and recording thermometers on the plant and an automatic recording device for the safety system.

An alternative method for small quantities of milk is the holder or batch-heating process in which milk is heated to between 62.8 and 65.6°C and held at that temperature for 30 min, followed by rapid cooling to below 10°C. The heating vessel incorporates an insulated outer jacket through which the heating and cooling media can flow, an agitator to ensure uniform heating, thermometers, including a recording thermometer and a temperature control device that automatically regulates the heating of the milk to pasteurization temperature and the subsequent retention at this temperature for the holding period (Report 1994b). Nowadays, the holder or batch method is seldom practiced for the pasteurization of milk but is still used in some small on-farm dairies for the pasteurization of cream.

The combinations of time and temperature used for the heat treatment of milk are designed to kill all pathogenic vegetative cells including *C. burnetii* which is relatively heat-resistant (Zall 1990). Bacterial spores and some preformed bacterial or fungal toxins, however, are not destroyed by pasteurization temperatures. The keeping quality of pasteurized milk depends both on the quality before heat treatment and on the amount of post-pasteurization contamination. Thermoduric bacteria that survive pasteurization belong to the genera *Bacillus*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Streptococcus*, and *Lactobacillus*. The importance of thermoduric organisms depends on the storage temperature. Some thermoduric fecal streptococci, *Bacillus* spp. and *Clostridium* spp. can grow below 7°C and are therefore also psychrotrophic. Most thermoduric organisms are mesophilic with optimum growth between 20 and 32°C. If pasteurized milk is allowed to remain at ambient temperature for several hours, as commonly happens with doorstep deliveries, growth of mesophilic bacteria such as *B. cereus* will soon cause bitterness, sweet curdling, and 'bitty' cream. Contamination with *B. cereus* is very common and yet reports of bacillus food poisoning due to the consumption of milk or cream are rare (Gilbert 1979; Christiansson 1992). This is probably because enterotoxin is not usually produced by *B. cereus* until the concentration of organisms is so high and consequent spoilage so great that the milk is unusable.

The lactic acid-producing streptococci do not survive pasteurization and 'souring' does not therefore take place in pasteurized milk. Repasteurization of returned milk results in enrichment of the thermoduric organisms in the final product and so is bad practice.

The keeping quality and flavor of properly refrigerated pasteurized milk depends on the types and numbers of psychrotrophic organisms. The most important of these are species of *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*, yeasts, and molds. These organisms are not thermoduric but are derived from the post-pasteurization environment. They outgrow any thermoduric psychrotrophs that may be present, and for this reason, hygiene of the pipes, tanks, and bottle fillers is the most important factor influencing the keeping quality of milk stored below 10°C (Schröder 1984). Psychrotrophic organisms are responsible for various 'off' flavors, ropiness, and changes in color.

ULTRA-HEAT-TREATED MILK

According to the Regulations, the description ultra-heat-treated (UHT) may be applied to a continuous flow of milk that has been heated to a temperature of not less than 135°C and retained at that temperature for at least 1 s. The method of heating may be indirect or by direct injection of steam. After treatment, the milk must be packaged in sterile airtight containers that are filled and sealed with aseptic precautions. In practice, the milk is usually heated to more than 140°C for 2–4 s to produce 'commercial sterility' – which means that it has a shelf-life of several weeks because, although small numbers of spores may survive, they are usually unable to grow under the conditions of storage (Lewis 1994).

STERILIZED MILK

The term 'sterilized' is applied to milk that has been filtered, clarified, homogenized, and then heated to 100°C for long enough to denature all casein and whey proteins. Traditionally, sterilized milk is bottled and sealed before heating to 110–116°C for 20–30 min. The process inevitably results in a caramelized flavor and brownish color but this is popular with some consumers.

THERMIZED MILK

In situations where raw milk has to be held under chilled conditions for some time, its keeping quality can be improved by a low temperature heat treatment called thermization. Thermized milk must conform to strict definitions according to European law. It must be obtained from raw cows' milk which, if it is not treated within 36 h of acceptance by the establishment, has a plate count at 30°C prior to thermization which indicates a concentration of 3×10^5/ml or less. The thermization treatment consists of heating raw milk for at least 15 s at between 57 and 68°C so that, after such treatment, the milk still shows a positive reaction in the phosphatase test. This treatment is not sufficient to eliminate pathogenic microorganisms.

Legislation

In most developed countries, the hygiene and composition of milk and milk products are subject to statutory controls, which specify general standards for production, transport, and processing, and specific standards, which set microbiological and chemical limits for each product.

Council Directive 92/46/EEC (as amended) applies to milk from sheep, goats, buffaloes, and cows and covers milk and milk products produced within the European Union. Detailed microbiological standards including absence of pathogenic microorganisms are specified for raw milk, heat-treated milk, and milk-based products, which must be sampled at the production or processing establishments. Nonmicrobiological tests are also specified, including somatic cell count, which is an indicator of mastitis in raw milk from cows and buffaloes (no such standard is specified for milk from sheep and goats because the levels are subject to wide natural fluctuations in these animals), and the phosphatase and peroxidase test for pasteurized milk. Greenwood and Rampling (1995) list the microbiological standards and guideline criteria specified in the Dairy Products (Hygiene) Regulations (HMSO 1995), for England and Wales based on the European Directive (92/46/EEC). Greenwood (1995) has produced details of appropriate methods for the microbiological tests and the phosphatase and peroxidase tests.

Similar standards apply in the USA for milk and milk products from sheep, goats, and cows. The US Food and Drug Administration produces a model code for grade A milk which is specified in the Pasteurized Milk Ordinance and the Dried Milk Ordinance. These cover production, transport, and processing, as well as microbiological and chemical tests. Grade A standards are applicable to all products that are produced in plants that produce milk for shipment between states (more than 95 percent of fluid milk products). Milk is produced on the farm as either 'grade A' or 'manufacturing grade' but most milk for manufacture of dairy products is surplus grade A that has not been sold as liquid milk for drinking. The production of 'manufacturing grade' milk and of products such as cheese, butter, and ice cream is subject to regulatory oversight by the US Department of Agriculture.

QUALITY CONTROL TESTS

The plate count at 30°C and the coliform count form the basis of microbiological quality control tests for milk and milk products in most countries, including the USA. In Europe the plate count at 21°C after pre-incubation at 6°C for 5 days has been adopted as the statutory test for pasteurized milk. This test was designed to assess the

level of psychrotrophic bacteria and thus keeping quality, but it has been subject to much criticism because it is time-consuming, expensive to perform, and has poor reproducibility (Scotter et al. 1993).

The coliform count is universally adopted as an indicator of poor hygiene and of post-pasteurization contamination. High counts in pasteurized milk may also be due to faults in the pasteurizer or contamination of pasteurized milk with raw milk. Pasteurization plants that produce milk which fails this test repeatedly, in spite of attention to cleaning and disinfection routines, should be examined carefully for leaks in the pasteurizer or faulty operation of the plant.

The absence of detectable alkaline phosphatase is the most important test to demonstrate adequate heat treatment of pasteurized milk or cream. Alkaline phosphatase is normally present in raw milk and is destroyed by heat treatment at a temperature slightly above that required to destroy *Mycobacterium tuberculosis* and slightly lower than those employed in the pasteurization of milk. Detection of phosphatase in pasteurized milk may therefore indicate inadequate heat treatment or contamination with raw milk.

There are several internationally recognized methods of testing that may be used for enforcement purposes (Rocco 1990). The statutory test in the Dairy Products (Hygiene) Regulations (HSMO 1995) is an analytical method based on the use of phenyl phosphate as the substrate. Milk that is satisfactorily pasteurized should liberate no more than 4 μg of phenol during a 1 h period. This is equivalent to the presence of 0.1 percent raw milk in pasteurized milk (Greenwood and Rampling 1997). A fluorimetric method is now available commercially which reports results in mU/l of fluorescent substrate released. A reading of 500 mU/l is equivalent to the presence of 0.1 percent raw milk, the statutory test limit of the previously mentioned statutory analytical method. The fluorimetric method is able to detect the equivalent of 0.006 percent raw milk in pasteurized milk and because of this increased sensitivity, it is recommended that a value of less than 100 mU/l should be used as an indicator of effective pasteurization and hence of microbiological safety (Greenwood and Rampling 1997).

It should be noted that not all of the alkaline phosphatase tests have the sensitivity to detect the presence of low levels of raw milk contamination due to small leaks in valves or heat exchange plates. To monitor the process efficiently, the test should be applied to samples taken from the cold milk exit side of the pasteurizer and immediately following an episode of flow diversion to check the integrity of the flow diversion valve.

Alkaline phosphatase in pasteurized milk may be due to any or a combination of residual, microbial, or reactivated alkaline phosphatase. The presence of residual phosphatase indicates inadequate pasteurization or post-pasteurization contamination with raw milk. Microbial phosphatase can occur when the milk has been stored for a long period of time before pasteurization and is the result of psychrotrophic bacteria growing and producing a heat stable alkaline phosphatase that survives the pasteurization process. Reactivated phosphatase may occasionally occur, particularly with cream. Reactivation is mediated by a heat-stable activator in the presence of magnesium and β-lactoglobulin (Lyster and Aschaffenburg 1962). The degree of reactivation depends on the initial concentration of phosphatase and, since the enzyme is adsorbed to the phospholipid of the fat globule membrane, it varies with the amount of fat and is greatest in cream. Statutory phosphatase tests for cream include a test which distinguishes between native (residual) and reactivated phosphatase.

The peroxidase test is a test for excessive heat treatment. If milk is subjected to significantly higher temperatures than those used for standard pasteurization, inactivation of the enzyme will occur. Thus a positive peroxidase test indicates that milk has not been overheated.

RESTRICTIONS ON THE SALE OF UNPASTEURIZED MILK

Most milk for drinking and for the manufacture of milk products is pasteurized but few countries have formal restrictions on the sale of raw milk. In the USA, however, very few milk products can be sold in the unpasteurized state. Some states allow limited sale of raw fluid milk and some hard cheeses may be manufactured from raw milk (Childer R.N., personal communication 1996). In England and Wales, legislation enacted in 1985 and 1986 limited sales of unpasteurized cows' milk to sales by farmhouse caterers, from farm gates, and from local retail deliveries of milk bottled on the farm. In Scotland, legislation in 1983 and 1986 prohibited all sale of unpasteurized cows' milk and cream. There are no restrictions on the sale of raw liquid milk from goats, sheep, or buffaloes or on any milk products made from raw milk anywhere in the UK and no restrictions on the sale of any unpasteurized milk or milk products elsewhere in Europe.

Outbreaks and incidents of infection associated with milk

A recent review of bacterial foodborne disease in France and six other countries estimated that outbreaks associated with milk and dairy products accounted for 1–5 percent of all foodborne outbreaks (De Buyser et al. 2001). A recent review of 69 milk and dairy product associated outbreaks in France during the period 1992–1997 confirmed that 87 percent were linked with cheese, 10 percent with milk, and 3 percent with other products. *S. aureus* was the major problem accounting for 85.5 percent of the outbreaks. Raw milk and raw milk products accounted for 48 percent of the 69 outbreaks. The situation is different in England and Wales where

most of the outbreaks are associated with liquid milk and fewer with dairy products. Milk and dairy products account for 2.5 percent (46/1774) of all foodborne outbreaks in England and Wales (Gillespie, I. CDSC, personal communication). Milkborne outbreaks in England and Wales account for 1.5 percent of all foodborne outbreaks (Gillespie et al. 2003a).

In the UK most outbreaks of infection due to the consumption of liquid milk continue to be caused by pathogens associated with the gastrointestinal tract of animals. *E. coli* O157, *Salmonella* spp. or *C. jejuni* are most prevalent. There have also been incidents due to less common animal-associated organisms such as *Y. enterocolitica*, *L. monocytogenes*, *S. zooepidemicus*, *Streptobacillus moniliformis*, *C. ulcerans*, and *Cryptosporidium parvum*. Table 1 lists outbreaks of infection associated with milk and milk products in England and Wales from 1992 to 2000. In comparison to the data summarizing outbreaks in England and Wales from 1989 to 1994 (Rampling 1998) there has been a significant change in that verocytotoxin producing *E. coli* O157 has emerged as the major cause of outbreaks associated with milk and dairy products. In spite of legislation limiting sales, and even though less than 3 percent of the total milk production is unpasteurized in England and Wales, 53 percent of outbreaks continue to be caused by the consumption of raw milk.

On the other hand, the situation is different in Scotland where, in the face of the epidemiological, microbiological, and research evidence that heat treatment of all cows' milk would be cost-effective, the retail sale of untreated cows' milk was prohibited from August 1983. Thereafter, incidents of infection related to milk were controlled in the general community but a small number persisted in the farming community where farm workers and their families received untreated milk as

Table 1 *Milk- and milk product-associated outbreaks and incidents of infection; reports to the Health Protection Agency Communicable Disease Surveillance Centre, England and Wales 1992–2000*

Year	Organism	Vehicle of infection	Number of persons infected	Evidence
1992	*Salmonella* Enteritidis PT4	Ice cream	7	S
	Salmonella Enteritidis PT4	School milk[a]	44	D
	Campylobacter jejuni	Sold as pasteurized milk	110	S
	Campylobacter jejuni	Raw milk	72	S
1993	*Salmonella* Typhimurium	Raw milk	13	M, D
	Escherichia coli O157	Raw milk	11	M
	Campylobacter jejuni	Raw milk	22	D
1994	*Salmonella* Typhimurium	Raw milk	11	M
	Salmonella Typhimurium	Raw milk	4	M, S
	Salmonella Typhimurium	Sold as pasteurized milk	26	M
	Campylobacter jejuni	Raw milk	23	S
	Streptococcus zooepidemicus	Raw milk	4	M
1995	*Salmonella* Typhimurium	Raw milk	26	M, D
	Campylobacter jejuni	Milk[a,b]	12	S
	Campylobacter jejuni	Raw milk[b]	35	S
	Cryptosporidium spp.	Sold as pasteurized milk	52	S
1996	*Salmonella* Typhimurium	Raw milk	5	M
	Escherichia coli O157	Sold as pasteurized milk	12	D
	Escherichia coli O157	Sold as pasteurized milk and raw milk	6	M
	Campylobacter jejuni	Raw milk	5	S
1997	*Salmonella* Anatum	Milk (infant formula)	17	M, S
	Salmonella Java	Milk (toddler)	14	S, D
	Escherichia coli O157	Raw milk	8	D
1998	*Salmonella* Typhimurium	Sold as pasteurized milk	121	M, D
	Escherichia coli O157	Raw milk	3	M
1999	*Escherichia coli* O157	Sold as pasteurized milk	114	S, D
	Escherichia coli O157	Sold as pasteurized milk	11	S,D
2000	*Escherichia coli* O157	Raw milk	4	M
	Escherichia coli O157	Raw milk	2	M

a) Not known whether raw or pasteurized.
b) Campylobacters: a number of incidents attributed to bird-pecked bottled milk were recorded. D, descriptive; M, Microbiological; S, statistical. Data supplied by the Health Protection Agency, Centre for Infections, London.

wage benefit. Between 1992 and 2000, only four milk-associated outbreaks occurred in Scotland. All four were associated with drinking raw milk and were restricted to farms and did not involve the general community.

In a recent study of milkborne outbreaks in England and Wales, milk sold as pasteurized was associated with 37 percent of all milkborne outbreaks (Gillespie et al. 2003a). Despite the introduction of a British Standards code of practice for pasteurization of milk on farms and in small dairies (BS7771:1994) and guidelines for 'on farm' milk processors (ADAS 1996) there is a concerning trend of outbreaks associated with milk sold as pasteurized. Most of the outbreaks linked to milk sold as pasteurized in Table 1 were associated with farms and in particular those with on farm dairies. Of the nine outbreaks linked to consumption of milk sold as pasteurized, six were associated with failures of the pasteurization equipment and three with post-pasteurization contamination. In a study in the northwest of England, in which farm-produced pasteurized milk was monitored during a $2\frac{1}{2}$ year period from 1999–2001, 18 percent (24/130) of on-farm dairies produced milk that failed the alkaline phosphatase test (Allen et al. 2004). In the absence of timely control checks and regular maintenance of equipment, farm-bottled milk will continue to be a microbiological safety issue (Gillespie et al. 2003a). The study by Gillespie et al. (2003a) also showed a bimodal seasonality with milkborne outbreaks occurring in spring and autumn which the authors highlight may be due to changing animal husbandry practices.

Evidence from a number of outbreak investigations has shown that it is not uncommon for operators to switch HTST pasteurizers to manual/clean mode during running, thereby removing the safety system and preventing flow diversion. This practice occurs most often with small producers who may be unaware of the exact sequence of events during flow diversion. In dairies that experience pasteurization failures, examination of thermograph records may show that soon after the milk temperature drops, initiating flow diversion, the plant has been switched to manual/clean mode, thus preventing the inevitable delay while the correct temperature is re-established. At the same time this has allowed inadequately heated milk to flow forward (Figures 1 and 2). The Dairy Products (Hygiene) Regulations (HMSO 1995) for England and Wales state that HTST pasteurizers must have an automatic safety device to prevent insufficient heating and an automatic recording device that records operation of the safety system. The regulations for Scotland also state that the record must include a record of when the cleaning mode is in operation. Some of the older designs of farm pasteurizers do not always incorporate chart recorders, which include a record of the operation of the safety system.

Information from some of the recent outbreaks associated with milk sold as pasteurized confirms that there

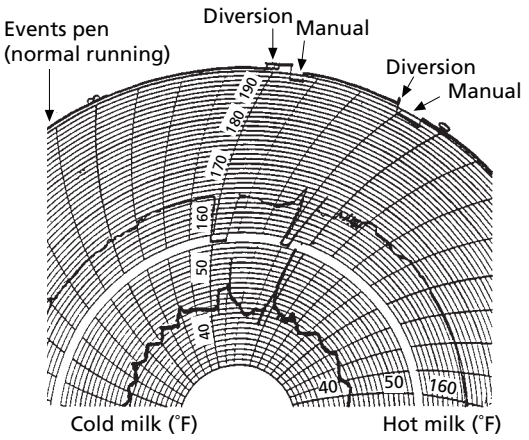

Figure 2 *Part of a pasteurizer thermograph chart demonstrating inappropriate use of manual/clean mode following flow diversion*

are common faults. Integrity of heat exchange plates, problems with flow restriction valves, and incorrect repair of equipment by untrained operatives have contributed to these phosphatase failures. Poor design of dairy plants and addition of other equipment such as homogenizers and cream separators can also alter the efficacy of pasteurization units.

Process control in both large and small plants must include the whole plant. The hygiene of pipes, tanks, bottle or carton fillers, and bottle washers should be monitored and the water supply must be of potable quality. Most dairies use mains tap water but occasionally the supply comes from another source. Whatever the source, water should be sampled regularly to check the microbiological quality. Where bottles are used, the final rinse consists of cold tap water and the bottles are not dried before filling with milk. This could potentially cause recontamination of milk and such a problem occurred many years ago when an outbreak of *Salmonella* Paratyphi B infections that continued for 2 years was eventually traced to river water supplying a pasteurization plant in south Wales (Thomas et al. 1948).

MICROBIOLOGICAL INVESTIGATION OF OUTBREAKS ASSOCIATED WITH ON-FARM DAIRIES

As explained above, most of the outbreaks in England and Wales associated with milk sold as pasteurized are linked to on-farm dairies. Microbiological investigation of these farms is essential to establish the etiology and source of the infecting organism. Microbiological examination should be carried out in conjunction with an evaluation of the process, equipment, and structure of the dairy. Each on-farm producer will have slightly different systems and equipment and therefore each investigation will be different. The microbiological investigations require a team approach and must involve environmental health officers (responsible for licensing

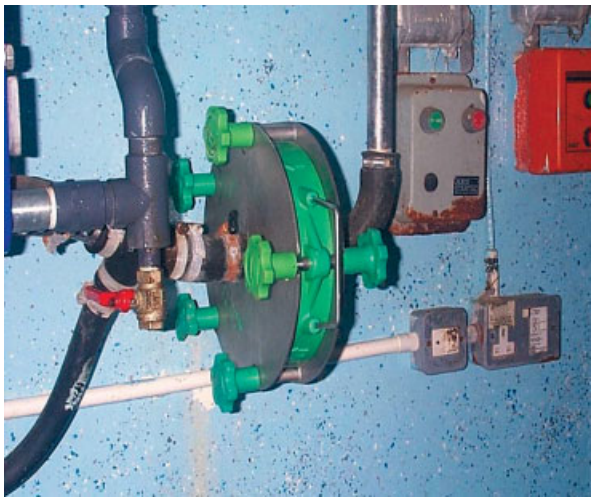

Figure 3 *In-line milk filters sampled during milkborne outbreaks.*

the premises), public health microbiologists, and veterinary microbiologists. The key areas to address are:

- Ensure that the pasteurization and other equipment is functioning correctly and take samples for statutory tests to confirm this.
- Take milk samples for pathogen examination from all pasteurization units, from the bulk milk tank.
- Collect filters or washings from the in-line filter between the milking parlor and the bulk tank (Figure 3). The latter is very important because it is a natural concentration step and will frequently result in isolation of pathogens when other samples are negative.
- Take swabs of any equipment and valve joints that appear to be faulty.
- Environmental samples or swabs from the milking parlor, the processing area and milk bottle/container storage area. Bottle rinses may also be valuable in determining post-pasteurization contamination. Specific sample points can only be decided when visiting the premises.
- Other samples that should be taken from around the farm may include the water supply, water from drinking troughs, samples of soiled bedding, and samples from the midden.
- It may also be necessary to ask a veterinary microbiologist to collect fecal or rectal swab specimens from individual cows. It is important not to limit this to only those cows that are being milked. For example, calves may be the source of the pathogen on an incriminated farm.

SALMONELLA OUTBREAKS

Cattle are the main reservoir of *Salmonella* Typhimurium and it is not surprising that this and other salmonella serotypes are frequently present in raw milk (Threlfall et al. 1980). In the UK study of bottled raw milk, 0.5 percent of samples were salmonella positive and isolates included *Salmonella* Typhimurium phage type DT104, *Salmonella* Dublin, *Salmonella* Virchow PT26, *Salmonella* Anatum, *Salmonella* Havana, and *Salmonella* Ruiru (de Louvois and Rampling 1998). This diversity of serotypes is not unexpected and in a study of 131 dairy herds in the USA Salmonella of O serogroups B, C, D, and E were detected in 6.1 percent of bulk tank milk samples (Jayarao and Henning 2001). These studies confirm that raw milk will from time to time be contaminated with *Salmonella* spp. and will result in outbreaks of infection. *Salmonella* Typhimurium DT104 is now established as a multiresistant clone, although there are subtle differences amongst strains (Threlfall et al. 1997).

During the period from 1992 to 2000, *Salmonella* Typhimurium has been the predominant cause of salmonella associated milkborne outbreaks (Table 1). The growing trend is for these outbreaks to be associated with multiresistant strains of *Salmonella* Typhimurium DT104. Of the nine salmonella outbreaks associated with liquid milk, six were associated with multiresistant *Salmonella* Typhimurium DT104. A large outbreak in 1998 that affected 121 cases was caused by a ciprofloxacin-resistant multiresistant strain of *Salmonella* Typhimurium phage type DT104. There was evidence from molecular typing that this strain developed ciprofloxacin resistance following treatment of cattle in the herd with enrofloxin a few months prior to the outbreak (Walker et al. 2000).

However, large processing dairies produce thousands of gallons of pasteurized milk daily and have the potential for causing major outbreaks if there is an unrecognized fault in the structure or operation of the plant. In 1985 such an outbreak occurred in Illinois, resulting in more than 16 000 confirmed cases of *Salmonella* Typhimurium infection (Ryan et al. 1987; Report 1985).

Salmonella Typhimurium has recently caused a multistate outbreak in the USA (MMWR 2003). Sixty-two cases were linked to consumption of raw milk from a combination dairy–restaurant in Ohio during November 2002 and January 2003. Cases were identified in four states by searching for isolates with a specific pulsed field gel electrophoresis (PFGE) profile. The dairy was the only place in Ohio that sold raw milk in jugs and served raw milk and milk shakes made from raw milk to customers. *Salmonella* Typhimurium with the same PFGE type was also isolated from raw skim milk, butter made with raw milk, and cream. The sale of raw milk is permitted in 27 of the US states and this outbreak has prompted a recommendation that the retail milk regulations should be reviewed and strengthened to minimize the risk from consumption of raw milk.

During the investigation of the multistate outbreak described above, 16/211 workers at the dairy were found to be positive for the outbreak strain. This illustrates the difficulty in determining the role of food handlers in

such outbreaks. There is still no evidence that any milk-associated outbreak was due to asymptomatic carriage of a salmonella by a person. Both the dairy industry and those responsible for enforcing standards should concentrate attention on process control, particularly of pasteurization, instead of screening staff to detect harmless asymptomatic excreters of salmonellae (Editorial 1987).

For further information on salmonella infections, see Salmonella.

CAMPYLOBACTER

Fecal carriage of campylobacters is common in cattle and frequently results in contamination of milk. Typing of isolates from cows within a herd usually reveals that several strains are endemic on the farm and individual cows may excrete one or more strains intermittently. Thus, contamination of milk will reflect the strains circulating within each dairy herd. Several surveys have investigated the prevalence of campylobacters in milk from individual cows, farm bulk tanks, and bottled unpasteurized milk. Between 4.5 and 12.3 percent of untreated milk samples from either bulk tanks or from bottled milk have been shown to be contaminated (Beumer et al. 1988; Humphrey and Hart 1988; Rohrbach et al. 1992). This range of results reflects differences in study design, the volume of milk examined, the sensitivity of the method employed, and on seasonal variations. The most recent microbiological studies of the prevalence of *C. jejuni* in untreated milk report contamination of 2 percent (19/1097) of bottled milk in England and Wales (de Louvois and Rampling 1998) and 9.2 percent (12/131) of bulk milk samples in an American study (Jayarao and Henning 2001). The risk of infection following consumption of unpasteurized milk is substantial and milk has been responsible for large numbers of outbreaks of campylobacter infection in many parts of the world (Skirrow 1991). Robinson (1981) demonstrated on himself that as few as 500 organisms mixed with 180 ml of pasteurized milk were sufficient to induce abdominal cramps, diarrhea, and an antibody response. Thus, a low degree of contamination in a batch of milk could cause a large outbreak. Although *C. jejuni* does not multiply in milk, many strains survive for long periods and can be recovered from milk that has been stored for 14–21 days at 4°C. Pasteurization destroys even large numbers of organisms (Waterman 1982; D'Aoust et al. 1988) and properly controlled heat treatment is the most satisfactory way of ensuring that milk is free from campylobacters.

Most milkborne outbreaks of campylobacter infection reported in the literature have been due to unpasteurized milk (Wood et al. 1992; Morgan et al. 1994b; Orr et al. 1995; Hutchinson et al. 1985b) and the outbreaks listed in Table 1 confirm that in England and Wales from 1992–2000 this is still the case. The outbreaks shown in Table 1 from 1992–1995 have been reviewed by Djuretic et al. (1997) and Peabody et al. (1997) and those from 1996–2000 by Gillespie et al. (2003a). Although the last *C. jejuni* outbreak in England and Wales was reported in 1996, there is evidence from other countries of the continuing risk associated with drinking unpasteurised milk (Kalman et al. 2000; MMWR 2003). Although most milkborne outbreaks are usually caused by a single strain, more than one serotype may be incriminated in a single outbreak. This should not be unexpected if fecal contamination is the source of the organism in the milk.

Another important risk factor for campylobacter infection is consumption of unpasteurized milk during visits to farms for educational, agricultural, or recreational purposes. Wood et al. (1992) identified 20 outbreaks of campylobacter infection amongst children who had consumed raw milk during school field trips and organized youth activities. This mode of transmission is still a problem as highlighted by a recent outbreak in the Netherlands (van den Brandhof et al. 2003). Fifty-seven of 92 children and three teachers drank raw milk provided during the visit to the farm. Forty-seven percent of the 57 children that drank milk developed diarrhea or vomiting and other symptoms. There was a clear dose–response relationship; 17 percent of children that took a mouthful of milk became ill whereas 100 percent of children that drank two cups of milk were ill. In this outbreak, isolates were typed by amplified fragment length polymorphism (AFLP) and they all showed the same pattern. The authors recommend heat treatment of all milk provided during farm visits. Kalman et al. (2000) reported an outbreak in 1998 in Hungary during which they identified 34 confirmed cases of campylobacter infection amongst 500–600 visitors to a farm sale. Similarly, Morgan et al. (1994b) reported the investigation of an outbreak of campylobacter infections in 1992. At a large music festival in southwest England, two local farms had been issued with Temporary Producers Licenses to enable them to sell raw milk at the festival and pasteurized milk was also available for sale. A case–control study based on 72 people with microbiologically confirmed *C. jejuni* infection found that only the consumption of unpasteurized milk was shown to be associated with campylobacter infection. Despite recommendations about the sale of unpasteurized milk, there was another outbreak at the festival the following year that resulted in 22 members of staff being infected (Peabody et al. 1997). Fortunately, on this occasion, unpasteurized milk was not sold to the general public. Campylobacter isolates from cases and the farms collected during both outbreaks were typed by several methods and shown to be indistinguishable. This confirms the endemic nature of pathogenic *C. jejuni* strains within herds for long periods and the potential problem of fecal contamination of milk. In these outbreaks, the supply of unpasteurized milk

resulted in significant but preventable public health problems. It is strongly recommended that visitors to such events and to open farms do not consume raw milk whilst on the premises.

There is also good evidence that *C. jejuni* may cause clinical or subclinical mastitis resulting in the shedding of large numbers of organisms into the milk (Hudson et al. 1984; Hutchinson et al. 1985b; Orr et al. 1995). Outbreaks of infection have also been associated with the consumption of milk from herds in which one or more animals have asymptomatic campylobacter mastitis. In this situation an outbreak could occur even though dairy hygiene is faultless and the milk achieves the microbiological standards for total plate count and coliforms. It is also more likely that milk will be contaminated with greater numbers of campylobacters and hence that the outbreaks will affect more people.

Outbreaks due to improperly pasteurized milk have also been documented on many occasions. In these circumstances, large numbers of people may be put at risk. In 1979, 2 500 children became ill after drinking free school milk supplied from a single milk processing plant in Luton. The campylobacter could not be isolated from the dairy or from raw milk but epidemiological studies strongly indicated milk as a source (Jones et al. 1981). An outbreak of *C. jejuni* enteritis affecting schoolchildren and staff at a boarding school in Vermont in 1986 was traced to inadequate batch pasteurization of milk. Two serotypes of *C. jejuni* were isolated, from both the patients and the dairy cows (Birkhead and Vogt 1988). During 1987, there were two more outbreaks of campylobacter infection in England and Wales, due to improperly pasteurized milk. More than 100 cases were confirmed by culture and serotyping following consumption of milk from a large dairy in East Anglia (Rampling et al. 1987). Another outbreak in 1987 affected 346 students and staff at a field study center in North Wales. Campylobacters were isolated from raw milk prior to pasteurization (Sockett 1991). In 1992 an outbreak in Northamptonshire affected at least 110 people of whom 41 had confirmed infection with *C. jejuni*. Consumption of pasteurized milk from a single farm dairy was implicated by case–control study (Fahey et al. 1995). In all of these outbreaks there was evidence of faulty processing resulting in phosphatase failures. The factors contributing to these outbreaks were: incorrect use of the bypass system (Jones et al. 1981), a leaking valve and inappropriate use of the plant with the safeguards switched off (Rampling et al. 1987), and an incorrectly wired element resulting in plant that had been malfunctioning for some time (Fahey et al. 1995).

Post-pasteurization contamination with campylobacters can also occur as a result of birds pecking milk delivered to the doorstep. This route of contamination was first observed in the late 1980s when magpies and jackdaws were incriminated. This route of transmission was confirmed microbiologically by Hudson et al. (1990, 1991) and epidemiologically by a case control study in south Wales in 1990 (Southern et al. 1990). A further point source outbreak in Exeter in May 1991 affected 11 children at a day nursery and was attributed to consumption of milk that had been pecked by magpies (Riordan et al. 1993). Evidence was obtained from a case–control study and by isolation of campylobacters from two bottles of milk that had been pecked. The most recent outbreak related to bird-pecked milk occurred in 1995 in a residential school in Gloucestershire, England. A retrospective cohort study showed that drinking pasteurized milk from bottles with damaged tops was associated with illness (Stuart et al. 1997). Bird-pecked milk has been suggested as the cause of many of the sporadic cases of campylobacter infection recorded during the annual spring, early summer rise in infections. The demise of doorstep delivery of milk and the growing trend to purchase milk from supermarkets should have an impact on this route of infection.

Peabody et al. (1997) stated that outbreaks of infection are rare events for such a common pathogen and this may in part be due to our inability to recognize that sporadic cases are linked. Two recent epidemiological studies have addressed this issue. A case–control study in Sweden with 101 cases and 198 controls, found that drinking unpasteurized milk was the major risk factor (OR 3.56, 95 percent CI 1.46–8.94) (Studahl and Andersson 2000). Enhanced surveillance of campylobacter cases in England and Wales was launched in May 2000 and analysis of these data also identified that illness in the community was associated with drinking unpasteurized milk (OR 1.55, 95 percent CI 1.06–2.27) (Gillespie et al. 2003b). These studies confirm that the risk associated with drinking unpasteurized milk is still prevalent. It may also be possible that some of the cases currently defined as sporadic cases are in fact part of local unrecognized outbreaks, a question that has been raised by Gillespie et al. (2003b).

For further information on campylobacter infections, see Campylobacter and arcobacter.

VERO CYTOTOXIN-PRODUCING *ESCHERICHIA COLI*

There are many serogroups of *E. coli* that produce verocytotoxins (also called shiga-like toxins) and the bovine is a major reservoir for these organisms (Clarke et al. 1989; Chapman et al. 1993). Not all serogroups of verocytotoxin producing *E. coli* (VTEC) are pathogenic for man, and hence presence of a VTEC in milk or diary product does not necessarily imply a risk to human health. In the UK and North America, VTEC O157:H7 has emerged as the significant public health problem but on mainland Europe and in Australia, non-O157 serogroups are more problematic.

Raw milk contaminated with VTEC is a potential source of human infection. A study in Germany found that 3.9 percent of 127 samples of raw milk and 2.1

percent of 146 samples of certified milk were positive for VTEC (Klie et al. 1997). *E. coli* O157:H was isolated from one raw milk sample, the other isolates comprised five different serogroups with different combinations of virulence markers. The authors concluded that drinking raw milk would continue to be a potential public health problem. Detection of non-O157 VTEC is problematic due to the lack of specific and sensitive culture methods. However, a French study using a PCR-ELISA assay determined that 21.5 percent of 205 raw milk samples contained non-O157 VTEC (Fach et al. 2001).

Most other studies have concentrated on determining the prevalence of *E. coli* O157 in milk. In England and Wales, *E. coli* O157 was detected in 3/1 097 samples of bottled unpasteurized milk (de Louvois and Rampling 1998). However, *E. coli* O157 was not detected in a study in Holland of 1 011 bulk milk samples from different dairy herds (Heuvelink et al. 1998) and in a more recent study in Scotland from 500 samples of raw milk (Coia et al. 2001). Contamination of raw milk is therefore a relatively rare event. Verocytotoxin producing *E. coli* have also been associated with coli mastitis in cows (Stephan and Kuhn 1999). These workers found that 2.8 percent (4/145) of cows with coli mastitis were infected with VTEC but none of the isolates were *E. coli* O157. Although many different VTEC are associated with clinical infection in farm animals, especially in calves and pigs, *E. coli* O157:H7 does not seem to cause clinical infection of animals but has been isolated from healthy cattle and dairy calves (Wells et al. 1991; Chapman et al. 1993). A longitudinal study of infection in the dairy commenced in 1993 and showed that although 29 of 130 cows excreted *E. coli* O157 on one or more occasions during the following year, there was no evidence of clinical disease (Report of the Advisory Committee on the Microbiological Safety of Food 1995).

Growth and survival of *E. coli* O157 in unpasteurized and pasteurized milk has been investigated by several groups of workers. Wang et al. (1997) found that *E. coli* O157 did not grow at 5°C in raw milk and that there was a gradual decrease in numbers after storage for 28 days. In this study, growth was observed at 8°C with an increase of about 1–2 $\log_{10}$ CFU/ml after 4 days and at 15°C about 3–5 $\log_{10}$ CFU/ml was noted after 3 days. In pasteurized milk, *E. coli* O157 continued to grow to populations of greater than 8 $\log_{10}$ CFU/ml at day 7. A report from Holland produced similar findings in that *E. coli* O157 was shown to grow in raw milk at 7 and 15°C (Heuvelink et al. 1998). In a later study, Massa et al. (1999) confirmed that growth at 8°C was strain dependent, some strains producing a 2–3 $\log_{10}$ CFU/ml increase during storage for 9–17 days. The recommendations from all these studies are to store milk at (5°C but, more importantly, to ensure that there is effective pasteurization without the risk of post-pasteurization contamination.

Outbreaks of *E. coli* O157 infection associated with raw milk have been reported from several countries but particularly from the USA, Canada, and the UK. In April 1986, there was a large outbreak of colitis in 60 kindergarten children who had visited a dairy farm in southwest Ontario, where they drank unpasteurized milk (Borczyk et al. 1987). Evidence of infection with *E. coli* serotype O157:H7 was demonstrated by isolating the organism or by detecting verocytotoxin in the feces of 43 patients and by isolation of the organisms from the feces of two cows. Martin et al. (1986) investigated two cases of hemolytic uremic syndrome (HUS) in young children. *E. coli* serotype O157:H7 was isolated from a fecal specimen from one child and also from cattle in both dairy herds which supplied the milk. In December 1992, there was a community outbreak in Oregon, USA which was associated with the consumption of raw milk. Eight people were affected and two were hospitalized (Report of the Advisory Committee on the Microbiological Safety of Food 1995). Keene et al. (1997) described a protracted outbreak, affecting 14 people that occurred over a 2-year period in the Portland area of Oregon, USA. These infections may have been reported as sporadic cases but molecular typing using PFGE demonstrated that isolates from cases had the same PFGE profile. The public health message is clear; raw milk can be contaminated intermittently and unpredictably and this can result in infections in the community from a common source that may go unrecognized as part of an outbreak. Enhanced epidemiological and microbiological surveillance are necessary if such cases are to be recognized.

Table 1 illustrates that *E. coli* O157 has emerged as the most common cause of milkborne outbreaks in England and Wales. Outbreaks are, in the main, associated with drinking unpasteurized milk but have also been linked to milk sold as pasteurized (Gillespie et al. 2003a). The first reported outbreak associated with raw milk in the UK occurred in Sheffield in 1993. Eleven cases, mostly children, were affected with *E. coli* O157:H7, phage type (PT)2, VT2, and three developed HUS (Table 1). The organism was also isolated from farmyard slurry, ten of 105 rectal swabs from dairy cattle, and milk from one rectal culture-positive cow at the farm that supplied the milk (Chapman and Wright 1993). In 1996, cases of infection with *E. coli* O157, PT1 in West Yorkshire were associated with the consumption of unpasteurized milk and the organism was isolated from a bottle of milk (Report 1996). Outbreaks associated with raw milk have continued to occur; one in 1997 resulted in eight cases, another in 1998 in three cases, and of two further outbreaks in 2000, one resulted in four cases and the other two cases (Table 1). The outbreak with four cases occurred in the Oldham area of Greater Manchester and was related to consumption of milk supplied by a single farm. Investigations on the farm recovered *E. coli* O157 from the in-line milk filter

and from 67/127 bovines sampled. All strains from human cases, the milk filter, and the cattle were confirmed as *E. coli* O157 PT 21/28 VT 2 with indistinguishable PFGE profiles. Similarly outbreaks associated with raw milk have been reported in Scotland. One outbreak involving three cases occurred in 1999 and another with two cases in 2000 (Task Force on *E.coli* O157 2001).

Of greater concern are the outbreaks in the UK associated with milk sold as pasteurized that have resulted in large numbers of cases. In May 1994, an outbreak of *E. coli* infection involved a pasteurized milk supply in West Lothian, Scotland (Upton and Coia 1994). One hundred people were affected, 69 had infection confirmed by culture, and one was confirmed by serology. Forty-six were under 15 years of age, and of these, 32 were under 5 years. Almost one-third of patients required hospital admission and HUS developed in nine children. Six required dialysis of whom two now have chronic renal failure. One child who did not have HUS died due to perforation of the large bowel. One elderly woman developed thrombotic thrombocytopenic purpura. More than 90 percent of patients had consumed pasteurized milk from the local dairy and at least seven cases resulted from secondary spread within households. Environmental investigation at the dairy and at the farms that supplied the milk yielded isolates of *E. coli* O157 from fecal specimens from cattle, raw milk from the bulk tank at one of the farms, from a pipe that carried milk from the pasteurizer to the bottling machine, and from a discarded bottling machine rubber at the dairy. The isolates from the patients, the farm, and the dairy were indistinguishable from one another by typing. All were *E. coli* O157:H7, PT2, VT2, and displayed identical PFGE profiles.

There have been four outbreaks in England and Wales associated with drinking milk sold as pasteurized, two in 1996 and two in 1999 (Table 1). One of the outbreaks in 1996 occurred in a rural community in the northwest of England (Clark et al. 1997). Of the 12 cases associated with the outbreak, nine were primary cases that had consumed milk from the farm, and three others were symptomless excreters. Three children <5 years old and one adult were admitted to hospital and one child developed HUS. Investigation at the farm highlighted that a flow restriction valve was faulty on one of the pasteurizers and allowed milk to pass through the heating coil too quickly resulting in inadequate pasteurization of milk. *E coli* O157 was isolated from the in-line milk filter and from two of the cows in the herd. All of the isolates were the same phage type but the isolates from the filter and the cattle had a slightly different PFGE profile than the isolates from the human cases. Distribution of milk from the farm was discontinued and the faulty pasteurization unit repaired. This action prevented further cases. Although no conclusive evidence of the origin of the outbreak was found, the farm was the most likely source. This outbreak highlights the importance of taking preventative action quickly on the basis of good descriptive epidemiological evidence.

The largest milkborne outbreak of O157 infection in the UK occurred in north Cumbria in March 1999 and was associated with drinking milk sold as pasteurized from a local farm dairy (Goh et al. 2002). A total of 114 cases were reported to the outbreak control team and 88 of these were confirmed microbiologically. Twenty-eight (32 percent) of the confirmed cases were admitted to hospital including three children with HUS. A case control study found that illness was strongly associated with drinking pasteurized milk from a local farm dairy ($p = <0.0001$). Microbiological investigations on the farm yielded isolates from calves and the farm environment of *E. coli* O157 PT 21/28 VT2 with a PFGE profile that was indistinguishable from the human isolates. Investigation of the pasteurization unit revealed that at the time of production of the implicated batch, there had been a fault with the heat exchange plates on the pasteurizer and the flow diversion pen on the chart recorder was also broken and hence during routine operation it would have been difficult to ensure that milk was being pasteurized effectively. These outbreaks associated with pasteurized milk raise issues about the effectiveness of current legislation for the prevention and control of milkborne infections.

These milkborne outbreaks, in addition to outbreaks incriminating yogurt and cheese (see later sections), confirm that raw milk, or inadequately pasteurized milk, is an important source of human infection with *E. coli* O157 in the UK. In the light of this evidence, the Advisory Committee on the Microbiological Safety of Food (Report of the Advisory Committee on the Microbiological Safety of Food, 1995) and the Task Force on *E. coli* O157 (2001) have recommended that the government should reconsider banning all sales of raw cows' milk in England and Wales and that raw cows' cream should be subject to the same regulations as raw cows' milk. They also recommend that the industry ensures that the pasteurization of milk and milk products is carefully controlled and that post-pasteurization contamination is avoided.

There is evidence that other serotypes of VTEC that occasionally cause outbreaks of human illness may be derived from milk. In 1994, four people in Helena, MT, USA developed bloody diarrhea due to *E. coli* O104:H21. Further case finding investigations revealed 11 confirmed and seven suspected patients and a case–control study implicated one specific brand of pasteurized milk as the source of infection (Moore et al. 1995). Recently, Allerberger et al. (2003) reported two cases of HUS in Austrian children caused by verocytotoxin producing *E. coli* O26. Both cases, one an 11-month-old boy and the other a 28-month-old girl, had consumed raw milk from a breakfast buffet at the same hotel.

Investigations at the farm supplying the milk resulted in isolation of *E. coli* O26:H- from a cow that had an indistinguishable PFGE profile from the isolates from the two cases.

Non-O157 VTEC characteristically ferment sorbitol and hence culture methods used to detect *E coli* O157 are not suitable for the detection of other VTEC. It is therefore possible that sporadic cases or even outbreaks due to these organisms may be associated with milk, yet go undetected.

For further information on VTEC, see Escherichia.

BACILLUS CEREUS AND *BACILLUS ANTHRACIS*

Spores of *B. cereus* are common in the farm environment and inevitably contaminate raw milk. The extent of contamination can be controlled by careful washing of the udder and teats prior to milking. Post-pasteurization contamination may also contribute to the numbers of spores, and the organism can grow readily and produce toxin in milk. *B. cereus* is a common cause of sweet curdling and bitty cream but documented cases of food poisoning due to *B. cereus* in milk are rare. However, in 1972, an outbreak of abdominal pain and diarrhea of short duration affected 221 schoolchildren in Romania after drinking contaminated milk. Two incidents documented in 1981 in Denmark involved a boy aged 12 months who drank curdled milk and a baby aged 6 months who developed diarrhea and vomiting after drinking contaminated expressed breast milk (Christiansson 1992).

Milk-transmitted anthrax is more of a theoretical than a real danger but difficulties may arise regarding the action to be taken with milk that has been derived from a dairy herd in which anthrax infection is subsequently diagnosed. The chance that such milk could be contaminated is very remote because anthrax bacilli are not excreted in milk until bacteremia develops. This occurs just prior to death at which stage it is likely that milk secretion will have ceased and, furthermore, the animal would be obviously ill and unsuitable for milking. Experiments have also shown that both spores and vegetative cells of *B. anthracis* (unlike those of *B. cereus*) disappear rapidly in milk (Bowen and Turnbull 1992) and infectivity of *B. anthracis* is low by the oral route.

Advice from the Department of Health (London, UK) (PL/CO(95)3 plus appendix and addendum) is that milk from animals known or suspected of being infected with anthrax at the time of milking must be excluded from the rest of the milk supply and sterilized (Anthrax Order 1991). The rest of the milk from the herd or flock must be pasteurized before being offered for sale. Where milk has already entered the public supply or where there is a suspicion that bulk milk may contain milk from an animal that could be suffering from anthrax or has died from the disease, pasteurization should suffice to protect the consumer against the risk of anthrax after consuming the milk.

CORYNEBACTERIUM ULCERANS

Although *C. ulcerans* usually causes only a mild sore throat, it can cause an illness indistinguishable from classic diphtheria. The organism is culturally similar to *Corynebacterium diphtheriae* var. *gravis* but is identified by the production of urease and of the exotoxins of both *C. diphtheriae* and *Corynebacterium ovis*. Cattle seem to be the main reservoir of infection and occasionally excrete the organism in milk. Isolated cases of human infection, including one of classic diphtheria, were identified by Meers (1979) in Devon and Cornwall. He postulated that some of the outbreaks of milkborne diphtheria, which at one time were common in Britain, may have been caused by *C. ulcerans*. Hart (1984) isolated *C. ulcerans* from a patient with a sore throat and from one symptomless carrier, both of whom lived in a farming community. Of the 30 cattle on the farm, eight were excreting toxigenic *C. ulcerans*. Hart also surveyed 52 other dairy herds in north Devon and found toxigenic *C. ulcerans* in milk from four of the herds. The organism does not seem to be prevalent in cattle in the UK nowadays and there have been no new reports of infection associated with milk.

CRYPTOSPORIDIOSIS

An outbreak of acute gastroenteritis due to cryptosporidiosis occurred amongst children at a primary school in Yorkshire in September 1995. There were 52 cases of whom 50 were pupils at the school and two were secondary cases. Initially, water was suspected as the likely source of infection but a case–control study implicated school milk supplied by a small producer with an on-farm pasteurizer. Subsequent investigation at the farm revealed that the pasteurizer had been failing intermittently and had not been working properly at the time of the outbreak (Gelletlie et al. 1997). Cryptosporidia are killed by effective pasteurization and hence this is an important critical control point.

LISTERIA MONOCYTOGENES

The first conclusive evidence that listeriosis was acquired from food was reported by Schlech et al. (1983). Since then it has become clear that many foods, including milk and, in particular, soft cheese, may be contaminated with *L. monocytogenes* and may occasionally cause outbreaks or sporadic cases of human listeriosis (McLauchlin 1993).

L. monocytogenes is prevalent in farm environments, in the feces of cattle, and in raw milk (Slade et al. 1989; Rampling et al. 1992; Sanaa et al. 1993; Fenlon et al. 1995). In addition, the organism survives well in moist environments and multiplies slowly at refrigerator

temperatures. Although it is killed by pasteurization, there may be opportunity for post-process contamination from plant or machinery. Food manufacturers, including manufacturers of dairy products, are very much aware of the problems of *L. monocytogenes* and are now very active in carrying out surveillance for this organism in environments and products.

Samples of milk from farm bulk tanks have shown that a high proportion (up to 16 percent) may be contaminated with *L. monocytogenes* at any one time. However, contamination is usually sporadic and persistent contamination seems to be rare. It can result from fecal contamination or from direct shedding by a cow with subclinical mastitis (Slade et al. 1989; Fenlon et al. 1995). Sanaa et al. (1993) carried out a detailed study of 128 selected dairy farms to assess the risk factors for contamination of milk with *L. monocytogenes*. They confirmed the findings of previous studies that poor quality silage (pH >4.0) was a major risk factor. Other predisposing conditions were infrequent cleaning of the cattle exercise area, obvious fecal soiling of udders, thighs, and anal regions, insufficient lighting in milking barns or parlors, which presumably made it difficult to ensure optimal hygiene during milking, and inadequate disinfection of udder cloths between milkings.

Most of the isolates found in surveys of raw milk belong to serovar 1. More detailed subtyping by multienzyme electrophoresis (MEE) has indicated that many isolates belong to subtypes that are unique to milk or to the environment and are not associated with human or animal disease. However, Fenlon et al. (1995) showed that whereas isolates from 25 of 160 milk producers could be subgrouped into nine different electrophoresis types, most of which were unique to milk, some isolates belonged to a type that had been associated with human infection.

In most instances, liquid milk is probably the original source of *L. monocytogenes* in a dairy product but the environment of dairy plants may also provide a reservoir of contamination from milk and other sources (Cotton and White 1992; Jacquet et al. 1993; Peeler and Bunning 1994). In particular, processing plants that are adjacent to dairy farms are more likely to be contaminated with *Listeria* spp. than processing plants without on-site dairy farms (Pritchard et al. 1994).

The first evidence to suggest that raw milk could be a source of human infection came from Halle in Germany where Reiss et al. (1951) described a pathological condition of stillborn infants which they called *granulomatosis infantiseptica* on account of the distinctive focal necrosis found throughout the bodies. A series of outbreaks of this condition occurred over a long period from 1949 to 1957. Initially, the organism responsible was identified as a new species of *Corynebacterium* but was later recognized as *L. monocytogenes*. These outbreaks were considered to be due to the ingestion of unpasteurized milk (Seeliger 1961).

In 1983, pasteurized milk was implicated in an outbreak in the USA: 49 patients were admitted to hospital in Massachusetts with meningitis, septicemia, or abortion due to *L. monocytogenes* serotype 4b (Fleming et al. 1985). Case–control studies demonstrated that infection was associated with consumption of a specific brand of whole or semi-skimmed pasteurized milk; there had been an outbreak of encephalitis due to *L. monocytogenes* in one of the herds that supplied milk to the dairy. There was, however, no evidence of failure of pasteurization.

Luchansky (1995) reported an investigation in Wisconsin in 1994 which followed recall of four dairy products due to detection of contamination with *L. monocytogenes*. One of these products was a 1 percent low fat chocolate milk which had been stored at inappropriate temperatures and contained 10^9 cfu/ml of serovar 1/2b. It had been implicated in a small outbreak of fever and gastroenteritis in a group of picnickers. The Wisconsin Division of Health then identified four patients retrospectively who had suffered typical invasive listeriosis and had also drunk the recalled chocolate milk.

MYCOBACTERIUM PARATUBERCULOSIS

Mycobacterium paratuberculosis commonly infects dairy cattle, leading to Johne's disease, also known as paratuberculosis, a chronic enteritis in cattle (Collins 1997). *M. paratuberculosis* has been suggested as a possible cause of Crohn's disease in humans but the etiology of Crohn's disease has not yet been established. The association with *M. paratuberculosis* has created significant debate in the last 5–10 years. There is evidence both for and against this theory (Chiodini 1989; Thompson 1994). One of the more convincing cases was reported by Hermon-Taylor et al. (1998). A young boy diagnosed with *M. paratuberculosis* cervical lymphadenitis developed terminal ileitis similar to Crohn's disease 5 years later. The organism was detected by polymerase chain reaction (PCR) in biopsies from both the lymph gland and the inflamed intestine. The infection was treated with a combination of rifabutin and clarithromycin. The debate continues and currently there is no substantial evidence to prove or disprove that infection with *M. paratuberculosis* is a major cause of Crohn's disease (European Commission 2000; Rubery 2001). The possible association with Crohn's disease has also raised the issue that this may be a foodborne disease transmitted in the milk of infected cows. This hypothesis has generated great interest in the organism, its prevalence in dairy herds and milk, and the organism's susceptibility to pasteurization.

Prevalence of *M. paratuberculosis* in dairy herds in Alberta, Canada has been investigated by Sorensen et al. (2003). These workers tested 50 herds for the presence of the organisms by fecal culture and a serum enzyme

immunoassay (EIA). A total of 1 500 cattle from the 50 herds was studied, individual sera were tested by the EIA but fecal samples were pooled so that 500 pooled samples were examined. The true herd prevalence, determined by EIA, was 26.8 percent and that determined by fecal culture ranged from 27.6 to 57.1 percent depending on the number of individual samples positive in each pooled sample.

Grant et al. (2002a) reported the incidence of *M. paratuberculosis* in bulk raw milk and commercially pasteurized cows' milk in the UK. Samples were collected over a 17-month period from March 1999 to July 2000 from 241 approved dairy processing establishments. *M. paratuberculosis* was detected by an immunomagnetic PCR method and a culture method. *M. paratuberculosis* was detected by the PCR assay in 19/244 (7.8 percent) raw milk samples from bulk tanks and in 67/567 (11.8 percent) of pasteurized milk samples. The culture method detected confirmed *M. paratuberculosis* isolates from 4/244 (1.6 percent) of the raw milk samples and 10/567 (1.8 percent) of the pasteurized milk samples. Finding *M. paratuberculosis* in pasteurized milk raises the issue of heat resistance of the organism and its survival after pasteurization.

The effect of pasteurization temperatures and holding times has been the subject of several studies (Grant et al. 1999; Grant et al. 2002b; Lund et al. 2002). Grant et al. 1999 conducted experiments with raw cows' milk spiked with 10^6 CFU/ml of *M. paratuberculosis*. These workers investigated heat treatments ranging from 72 to 90°C for 15 s and also 72°C for 20 and 25 s. Although all treatments achieved a 5–6 $\log_{10}$ reduction small numbers of the organisms (4–16 CFU/10 ml) survived even when heated at 90°C for 15 s. A longer holding time of 25 s at 72°C was more effective and it was concluded that this is more likely to achieve complete inactivation of *M. paratuberculosis* than a higher temperature. In a follow-up study using a commercial-scale HTST pasteurization unit, Grant et al. (2002b) concluded that *M. paratuberculosis* in naturally contaminated milk are capable of surviving commercial HTST pasteurization. Lund et al. (2002) reviewed data from eight studies on the effect of heat treatment of *M. paratuberculosis*. There was significant variation in the strains, culture medium, and methods used which probably explains the differences in the results obtained. These authors highlighted the need for a set of defined performance criteria for pasteurization of milk in relation to *M. paratuberculosis*. Furthermore, information about the heat resistance of this bacterium will enable a decision to be made on the process criterion that is necessary to eliminate the organism in commercially pasteurized milk.

STREPTOBACILLUS MONILIFORMIS

This organism causes a febrile septicemic illness in man, characterized by an erythematous rash, most prominent on the hands and feet, arthralgia, and sore throat. The disease was first described in Haverhill, Massachusetts, where an explosive outbreak affected 86 people and was termed erythema arthriticum epidemicum (EAE). The outbreak was due to consumption of contaminated raw milk (Place and Sutton 1934). In February 1983 an outbreak of EAE affected 130 children attending a boarding school in Chelmsford, Essex. *S. moniliformis* was isolated from the blood of four of the children and unpasteurized milk from a local farm was again the suspected source of infection (Shanson et al. 1983).

STREPTOCOCCUS ZOOEPIDEMICUS

S. zooepidemicus is an animal pathogen commonly associated with infection in horses but occasionally causing clinical or inapparent mastitis in cows. It has been associated with severe milkborne illness in man. In 1968, there was an outbreak of infection among 74 adults and 11 children in a town in Romania, causing sore throat, fever, and lymphadenitis, complicated by post-streptococcal glomerulonephritis in one-third of patients. Consumption of improperly pasteurized milk was implicated and *S. zooepidemicus* was isolated from cows with mastitis (Duca et al. 1969). In one week, three unrelated patients presented to the General Infirmary, Leeds, with *S. zooepidemicus* bacteremia. One patient had meningitis, two had endocarditis, and one subsequently died (Ghoneim and Cooke 1980). Investigations eventually confirmed that unpasteurized milk had been the source of infection in all three cases. In 1983, *S. zooepidemicus* infection was again associated with acute glomerulonephritis in three members of a North Yorkshire family who drank unpasteurized milk from their own cows (Barnham et al. 1983).

The most severe milk-associated outbreak of streptococcal infection in Britain was in West Yorkshire in 1984 (Edwards et al. 1988); 12 people developed *S. zooepidemicus* septicemia or meningitis, and eight died following consumption of raw milk from a single supplier. Three cows in the herd were excreting the organism in the milk. In Australia, a man developed septicemia and glomerulonephritis due to *S. zooepidemicus* and two other members of the family were found to be asymptomatic throat carriers. The source of infection was unpasteurized milk from the house cow and typing demonstrated that the isolates from the three individuals and the cow's milk were indistinguishable from one another (Francis et al. 1993).

In England in 1994, an elderly couple who owned a dairy farm became ill and were admitted to hospital within days of one another (Report 1994c). The man developed fatal meningitis and *S. zooepidemicus* was isolated from his cerebrospinal fluid (CSF). His wife had septic arthritis and the organism was isolated from pus from a knee joint aspirate. Another man who drank milk purchased at the farm gate became ill with

empyema and subsequently died, and a man who had eaten a chocolate cake made with cream from the farm developed endocarditis. *S. zooepidemicus* was also isolated from milk from the bulk tank and from one cow that appeared to have subclinical mastitis. Ribotyping of the isolates from the patients and the milk demonstrated that they were indistinguishable from one another (Burden, personal communication 1995). There have been no further reports in the literature of infections associated with milk in the UK.

It is clear that *S. zooepidemicus* is a rare but extremely serious cause of human illness. When it is diagnosed, milk should be suspected as the source of infection.

For further information on streptococci, see Streptococcus and lactobacillus.

YERSINIA ENTEROCOLITICA

The reported isolation rates of *Y. enterocolitica* from samples of raw milk vary from 14.3 to 48.1 percent. Most strains have the biochemical characteristics of environmental, nonpathogenic strains and are nontypable or belong to serotypes not associated with human illness. A few isolates from milk are pathogenic, however, and yersinia infection has been associated with consumption of raw milk in England and Wales and in the USA.

Y. enterocolitica can survive and replicate at refrigeration temperatures but although one strain of yersinia able to survive laboratory pasteurization has been reported, there is good evidence that HTST pasteurization will destroy all viable organisms (Francis et al. 1980). The presence of pathogenic yersiniae in pasteurized milk implies a fault during pasteurization or contamination afterwards (D'Aoust et al. 1988; Greenwood et al. 1990).

An outbreak in 1976 (Black et al. 1978) resulted in admission to hospital of 36 schoolchildren, 16 of whom had appendectomies. Evidence from a case–control study implicated chocolate milk but chocolate syrup was added to the milk after pasteurization and this may also have been a source of the organism. Case–control studies implicated pasteurized milk in a large multistate outbreak of yersiniosis in the USA in 1982 (Tacket et al. 1984); 172 infections were identified by culture. Enteritis occurred in 148 patients, mostly children, and extraintestinal infection in 24 adults. Appendectomy was performed on 17 patients with intestinal infection. Investigations did not reveal any fault in the pasteurization process but indirect evidence that milk was the source was obtained by isolating the same unusual serotype (O:13,18) from the outside of a milk crate on a pig farm where outdated milk was used as pig feed.

The most recent outbreak associated with *Y. enterocolitica* was reported in the USA (Ackers et al. 2000). Ten patients were infected with *Y. enteroocolitica* O:8, three patients were admitted to hospital and one underwent an appendicectomy. Consumption of pasteurized milk from a local dairy was associated with the illness. Post-pasteurization contamination was the most likely source of the problem originating from either the farm environment, one sample from a pig on the farm was positive, or from contaminated water used to rinse the milk bottles.

MILK POWDER

Commercial drying of milk was introduced at the beginning of the twentieth century. The roller method was developed first followed by the spray method in the 1930s. Both were in operation until the 1950s when the spray method assumed popularity because it gave a more palatable product. For a detailed account of the various processes and designs of plant see Knipschildt and Anderson (1994).

The process is in two stages: preliminary concentration during which 90 percent of the water is removed by evaporation, followed by rapid drying. Before concentration, the milk must be preheated to pasteurization temperatures to kill vegetative pathogenic bacteria. For the spray-drying process, concentrated milk is fed from vacuum evaporators to a balance tank and then through fine jets or rotary atomizers into the top of a large chamber supplied with filtered hot air. Dry powder is collected from the bottom of the chamber.

The development of processing conditions that minimize nutrient change and improve physical and organoleptic properties has increased the microbiological hazards (Lovell 1990). Spray-dried milk has been responsible for many outbreaks of food poisoning due to enterotoxigenic *S. aureus* and to salmonellae. The safety of the process relies on adequate preheating to destroy vegetative pathogenic organisms; if, however, they survive this stage, there is opportunity for them to multiply in the evaporators and the balance tank and thus for heavily contaminated milk to enter the spray-drier. Although hot air enters the spray-drier at over 160°C, cooling is rapid. There is good evidence that salmonellae can survive the drying process (McDonough and Hargrove 1968) and that the heat treatment is insufficient to inactivate *S. aureus* or its enterotoxin. Because of the complexity of the plant, it is very difficult, if not impossible, to eradicate contamination once established in the plant or its environment.

In 1953, several outbreaks of staphylococcal food poisoning in schools near London were traced to powdered milk from the same source. Later that year, another outbreak in Yorkshire was traced to milk powder from a different source (Anderson and Stone 1955). Tests on batches of milk powder from the plant involved in the first outbreak demonstrated that on two days during May the total bacterial concentration was considerably higher than on other days and that there

were approximately 10^4 *S. aureus* per gram. The organism was not detected in batches with low bacterial concentrations. Other studies indicated that toxin was probably produced at some stage before the drying process. Investigations at another plant not implicated in the outbreaks showed that bacteria multiplied in the balance tank throughout the 12 h of continuous processing. There was also evidence that small amounts of contamination remained after cleaning and recontaminated the pasteurized concentrated milk as it passed through the tank (Hobbs 1955). Other studies have indicated that *S. aureus* contamination may occur during manufacture and that isolates from milk powder may not necessarily be the same as those in the raw milk (Lovell 1990). Harvey and Gilmour (1990) examined milk powders from five different processors in Northern Ireland for both *S. aureus* and coagulase-negative staphylococci. They found that low levels of total viable organisms were associated with low total staphylococcal levels but that five of 37 samples had high total levels and high concentrations of coagulase-negative staphylococci. The powders with high levels contained added fat which was mixed into the milk after pasteurization and concentration, but prior to drying. No *S. aureus* or staphylococcal enterotoxins were detected but this study highlighted a potential source of contamination with microorganisms which are able to survive the drying process. Even coagulase-negative staphylococci are undesirable as some strains can produce enterotoxins.

However, staphylococcal intoxication from dried milk does not seem to have caused many problems since the general introduction of refrigerated farm bulk milk tanks, which maintain raw milk at a temperature that inhibits multiplication and toxin production by staphylococci (Morgan-Jones 1987). However, the problem has not totally disappeared. One report from El-Dairouty (1989) describes a series of food poisoning incidents involving 21 patients who consumed non-fat dried milk in Egypt in 1986. The largest outbreak associated with a milk product has recently been reported in Japan (Asao et al. 2003). Over 13 000 cases are likely to have occurred as a result of contaminated powdered milk used as an ingredient in a range of dairy products. Staphylococcal enterotoxin A (SEA) was detected in low fat milk and also in powdered skim milk. The total intake of SEA per capita was estimated at about 20–100 ng. SEA exposed at least twice to pasteurization at 130°C for 2–4 s retained both immunological and biological activity. This outbreak is a reminder that viable *S. aureus* can be eliminated by pasteurization but that the enterotoxin can retain sufficient activity to cause intoxication.

Salmonella infection has been associated with the consumption of powdered milk on several occasions and its occurrence in dried milk is well documented. In 1966, an outbreak of *Salmonella* Newbrunswick infection in the USA was associated with spray-dried milk (Collins et al. 1968). Following this episode, the US Food and Drug Administration instituted a large-scale testing program (Marth 1969) and 156 milk plants in 56 states were examined. Of 2 741 samples tested, 24 samples of non-fat dried milk and 27 environmental samples contained salmonellae. Later, in 1967, 200 factories in 19 states were surveyed: 1 percent of 3 315 product samples and 8.2 percent of 1 475 environmental samples were positive for salmonellae. In total, 13 percent of the plants tested were manufacturing milk powder contaminated with salmonellae. These surveys also demonstrated the value of taking environmental rather than product samples to monitor for pathogenic bacteria.

Outbreaks have continued to occur in the USA. Pickett and Agate (1967) isolated a lactose-positive strain of *Salmonella* Newington from instant non-fat milk powder which had been responsible for nine cases of salmonellosis. *Salmonella* Newport was isolated from dried skimmed milk and from 20 patients with diarrhea and vomiting in Newfoundland (Marth 1969) and an outbreak of *Salmonella* Agona and *Salmonella* Typhimurium infection was traced to powdered milk in Oregon (Furlong et al. 1979).

These episodes in North America are not unique. In England and Wales, *Salmonella* Typhimurium infection has been traced to spray-dried milk on several occasions (Galbraith et al. 1982). In Australia, *Salmonella* Bredeney, harbored in the insulating material of a spray-drier, provided a continuing source of contamination for the milk powder via cracks in the stainless steel lining of the drier (Craven 1978). In Trinidad, approximately 3 000 people, predominantly infants and small children, were infected with *Salmonella* Derby which was traced to contaminated powdered milk produced on the island (Weissman et al. 1977).

In November and December 1985, there was a cluster of *Salmonella* Ealing infections in infants in England and Wales (Rowe et al. 1987). Investigations showed that 76 people, including 48 babies, had been infected and that a smaller cluster of cases had occurred in May, June, and July 1985. Seven of the babies were admitted to hospital and one died. A case–control study implicated dried milk from one manufacturer. *Salmonella* Ealing was isolated from an unopened packet of baby food from the home of a patient and from four other packets from the same batch. Investigations at the factory revealed that the inside of the spray-drier had several minor pinholes and weld cracks and that there was an irregular hole 1×3 cm in the metal lining. Removal of the metal outer casing at the site of the hole showed stained insulation material and a large collection of discolored milk powder. *Salmonella* Ealing was isolated from the insulation material and the milk powder in continuity with the hole, but not from the dry insulation material on the opposite side of the drier. It was thus likely that infected milk had gained access to the insulation material from the inside of the drier

through the hole in the metal lining and had then remained within its wall as a continual source of contamination. *Salmonella* Ealing was also found in the factory vacuum system and in a silo containing waste milk powder and sweepings. Examination of the pasteurizer revealed pinhole defects in the plate heat exchanger which could have been sufficient to allow contamination of the pasteurized milk with raw milk. *Salmonella* Ealing was known to be present in one of the herds that supplied milk to the dairy in April 1985 and the organism may have gained access to the factory at that time.

The many incidents of infection associated with spray-dried milk have caused much concern among manufacturers and public health microbiologists. There has also been controversy about standards for total bacterial levels and their relevance as a method of monitoring quality and the frequency of sampling required. A standard for total concentrations of no more than 5×10^4/g after incubation for three days at 30°C is generally accepted (Lovell 1990) but in practice concentrations of less than 1×10^3/g can be achieved. It is customary to perform quality control tests on each batch of powder before releasing it for sale. As was clearly demonstrated by the surveys in the USA in 1966 and 1967, however, this practice is by no means as sensitive for detecting pathogens as testing the dust and waste powder in the factory environment. An obviously dangerous practice in some milk powder factories is the custom of blending powder with high bacterial levels into powder with low bacterial levels in order to give an apparently satisfactory final product (Rowe et al. 1987).

Mettler (1989) has reviewed the issues raised by the *Salmonella* Ealing and other salmonella incidents. He points out that production of pathogen-free milk powder requires detailed committed attention to good manufacturing practice involving every aspect of the process, including design of equipment, management, staff training and discipline, and control of the environment, including air quality. In addition, sampling programs should be detailed and targeted and include the environment as well as the product; the test methodology should be assessed critically in relation to the detection of pathogens. Regular and thorough crack testing of the spray dryer should also be an essential part of the quality control program. In the long term, it should be possible to design spray-drying chambers that do not have this intrinsic fault and it should be possible to improve the processing equipment at all stages so that a build up of microorganisms does not occur.

An outbreak of *Salmonella* Anatum infection in babies in late 1996 and early 1997 associated with infant dried formula, is a stark reminder of the potential of dried milk products to cause widely dispersed outbreaks. This outbreak was only recognized because of the joint national referral of salmonella isolates to the reference laboratory for typing and the national surveillance system in England and Wales. A case–control study on the first 12 cases strongly associated consumption of infant dried formula. Subsequently, the isolates from the cases were shown to have indistinguishable PFGE profiles. A wider investigation revealed that 22 cases had occurred from September 1996 to January 1997 in four different countries: 13 in England, four in Scotland, four in France, and one in Belgium (Anon 1997). The infant formula was produced at a factory in France and upon further Europe-wide investigation it became apparent that there were also cases in France. Investigation revealed that the spray-dried milk used to make the infant formula may have originated from either a plant in France or the Netherlands. This outbreak illustrates the complexity that has developed in the modern food chain and dairy industry and the essential need for Europe-wide microbiological and epidemiological surveillance.

Although guidelines for the hygienic manufacture of spray-dried milk powders have been issued by the International Dairy Federation (Report 1991a) incidents continue to occur. In 1993, Louie et al. reported that three cases of *Salmonella* Tennessee infection in infants had been traced to the consumption of one particular brand of powdered infant formula and that this organism had also been isolated from production equipment at the spray-drying plant and from cans of powdered milk formula.

Salmonella spp. and *S. aureus* are the pathogens most often associated with dried milk, but other organisms have also caused public health problems. Three cases of neonatal meningitis due to *Enterobacter sakazakii* from infant milk formula were diagnosed in 1986 and 1987 at the National University Hospital, Reykjavik, Iceland (Biering et al. 1989). Two neonates who were normal at birth survived with severe brain damage and a third, who had Down's syndrome, died. *E. sakazakii* was isolated from cerebrospinal fluid from the three babies with meningitis and from the urine of a fourth infant who had no signs of infection. It was also isolated by enrichment culture from five unopened packages of milk powder. All isolates from the babies, and all but one of 23 isolates from the infant formula, had identical biotypes, antibiograms, and plasmid profiles. Other workers have isolated *E. sakazakii* from powdered milk (Muytjens et al. 1988) and powdered infant milk has been suspected in the past as a source of neonatal infection with this organism (Muytjens et al. 1983). These incidents confirm the importance of implementing the strictest possible measures for the control of microbiological quality during the manufacture of milk powder and, in particular, of infant milk. The Reykjavik outbreak demonstrated that even low numbers of *E. sakazakii* were significant perhaps because of the extreme susceptibility of the patients but also possibly because there was opportunity for multiplication in the reconstituted formula.

B. cereus is another contaminant of raw milk that is commonly present in milk powder (Becker et al. 1994; Crielly et al. 1994). The endospores are heat-resistant and able to survive the pasteurization and drying process. Indeed, replication may be encouraged at various stages of the production process and, in the case of infant formula, further contamination may be introduced with added ingredients. Once reconstituted, there is opportunity for germination of spores, growth, and enterotoxin production depending on the temperature of storage. In spite of this, *B. cereus* has rarely been documented as a cause of food poisoning associated with dried milk (Christiansson 1992; Becker et al. 1994). Finally, *C. perfringens* is another sporing organism which is a theoretical risk but has rarely been implicated in food poisoning due to powdered milk (Lovell 1990).

CREAM

Unless hygiene is strict, environmental contamination of cream may be considerable because of the many stages of handling during processing. When cream is separated by centrifugation, most of the bacteria from the whole milk are separated with the cream. In addition, separation is often performed at 40–50°C which is a temperature that encourages the growth of many microorganisms (Milner 1995). However, cream is normally subjected to higher pasteurization temperatures (up to 80°C) than milk and is expected to have a longer shelf-life (Davis and Wilbey 1990).

In 1970, a PHLS working party examined 4 385 heat-treated, 282 clotted, and 517 raw samples of cream (Working Party to the Director of the Public Health Laboratory Service 1971). Bacterial levels were frequently high and, although the unheated creams were the most unsatisfactory, many heat-treated creams also contained large numbers of bacteria including coliforms, thereby indicating either post-pasteurization contamination or inadequate heat treatment. From 3 417 samples examined, *S. aureus* was isolated from 59, 54 of which were unpasteurized. Other pathogens found in untreated samples were one isolation each of *Salmonella* Typhimurium, *B. abortus*, and *E. coli* serotype O126. *C. perfringens* was isolated from one heat-treated sample.

Infections and intoxications associated with cream

Raw cream is as likely as raw milk to contain pathogenic bacteria (Rothwell 1979). It is, however, surprising that few outbreaks have been attributed either to raw or to heat-treated cream. This may be because relatively small volumes are produced and sporadic infections are seldom reported. Furthermore, cream is usually incorporated into desserts, cakes, sauces, etc., and it may be difficult to incriminate the cream alone in the event of an outbreak. Food poisoning due to enterotoxigenic *S. aureus* was a well-recognized hazard associated with cream before the introduction of farm refrigeration tanks and refrigerated storage in shops and homes. When detailed investigations have been made, the source of the organism has usually proved to be a cow or cows with symptomatic or asymptomatic *S. aureus* mastitis. Isolation of the same phage type of *S. aureus* from individual cows, from the cream and from patients with food poisoning has been reported (Steede and Smith 1954).

Raw and pasteurized cream have also been associated with many small incidents of salmonella food poisoning, often related to overt infections in cows or calves on the farms supplying the milk (Vernon and Tillett 1974; Sharp et al. 1985). There have also been incidents of *B. cereus* food poisoning associated with cream (Galbraith et al. 1982; Sockett 1991).

The potential risk associated with the consumption of raw cream was demonstrated in the outbreak of infections with *S. zooepidemicus* which occurred in the home counties of England in 1994. An elderly patient who developed endocarditis due to this organism acquired his infection by eating a chocolate cream cake made with contaminated raw cream (Burden, personal communication 1995). It is probable that he was unaware that the cream was unpasteurized.

Raw cream has also been incriminated as a source of *E. coli* O157. An outbreak in England in 1997 was linked to consumption of raw cream purchased from a farm shop. Descriptive epidemiological evidence indicated that consumption of raw cream from the one outlet was associated with infection in the seven confirmed cases (Task Force on *E. coli* O157 2001).

YOGURT

A wide variety of drinks and semi-solid foods are prepared by the fermentation of milk and yogurt is by far the most popular. Nearly all yogurt is made in large dairies under carefully controlled conditions but there is increasing interest in small-scale farm production, particularly from sheep and goat milks.

There are two systems for commercial manufacture (Robinson and Tamime 1993). In one, incubation and fermentation are performed in the retail container, resulting in *set yogurt*. In the other, fermentation takes place in large tanks and the coagulum is stirred to produce a thick viscous fluid (*stirred yogurt*) which is cooled and packaged by automatic fillers. Fruits or flavorings are added to stirred yogurt after fermentation is completed and to set yogurt before fermentation. Stirred yogurt is sometimes pasteurized to give it an extended shelf-life.

The first stage of manufacture for both products is preparation and standardization of the milk mix. Fresh milk is preheated and separated through a centrifugal

separator. The concentration of total solids in the skimmed milk is increased, either by evaporation to 15–16 percent or by adding milk powder. If a medium or full fat yogurt is required, a monitored amount of cream and sugar is added. Stabilizers, such as pregelatinized starch or plant gums, may also be included at this stage. The mix must be homogenized if cream is added, otherwise separation will occur during incubation. Homogenization also increases the viscosity.

The mix is heat treated to kill pathogenic and spoilage organisms and to expel air in order to produce microaerobic conditions for the fermentation. The optimum heat treatment for improving the physical state of the coagulum is a high temperature, prolonged treatment (85°C for 30 min). This is done in large tanks containing mechanical stirrers and heated by water jackets. The tanks are then cooled to 40–45°C, inoculated with the starter cultures and held at constant temperature until fermentation is complete after $3\frac{1}{2}$–$4\frac{1}{2}$ h. Many dairies find this system too slow and prefer to treat the mix in a UHT pasteurizer (85°C for 2 s) before filling the fermentation tanks or the small volume containers used for set yogurt.

Almost without exception, commercial producers use *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in a ratio of 1:1 as a starter culture for fermenting yogurt. The cultures are grown in milk in bulk starter vats and added to the basic mix to give a final concentration of 1–2 percent (v/v). An incubation temperature of 40–42°C is usually selected for the fermentation. This temperature lies between the optima for acid production by the two species (39°C for *S. thermophilus* and 45°C for *L. bulgaricus*). The two organisms grow symbiotically; *S. thermophilus* produces metabolites that encourage the growth of *L. bulgaricus*. *S. thermophilus* produces most of the lactic acid early in the fermentation, but later, both organisms produce aldehydes and other fermentation products important for flavor. The final acidity is greater if the two cultures are grown together than if either is grown alone. A final concentration of 1 percent lactic acid (pH 4–4.2) should be achieved. Strict quality control is essential to ensure that starters are active and free from bacterial and bacteriophage contamination, and that the milk is free from antibiotics which would inhibit growth of the starter.

Microbiological quality of yogurt

Yogurt mix is heated to very high temperatures before fermentation. It should therefore be free from pathogenic and spoilage organisms but opportunities exist for contamination during fermentation and packaging. Additions such as fruit or flavorings may also carry contaminants. However, it is generally a very safe product because most potential pathogens are inactivated by the high level of acidity and antibiotic substances produced by the starter cultures (Robinson and Tamime 1990). *Campylobacter* spp. and *Salmonella* spp. are inactivated rapidly in yogurt or fermented milks, but under experimental conditions both *Y. enterocolitica* and *L. monocytogenes* can survive for several weeks in the refrigerated product (Schaack and Marth 1988; Ashenafi 1994; Aytac and Ozbas 1994). Between 1988 and 1991, a survey of the occurrence of *Listeria* spp. in milk and dairy products in England and Wales included 180 samples of yogurt. *L. monocytogenes* was isolated from four yogurt samples (one made from cows' milk, two from goats' milk, and one from ewes' milk) (Greenwood et al. 1991). However, yogurt does not seem to have been associated with any cases of human clinical infection due to *L. monocytogenes* or *Y. enterocolitica*.

Provided that yogurt is produced under carefully controlled conditions with the correct starter cultures, pathogenic bacteria, but not their toxins or spores, should die out rapidly. The quality of added ingredients is equally important and these should be subject to the same quality control. An outbreak of staphylococcal food poisoning involving at least 67 people was traced to *dahi* (Indian yogurt) made in a shop in Calcutta (Saha and Ganguli 1957). The suspected source was contaminated milk powder added to the mix just before incubation. It was clear that there was a fault in the fermentation process because it took 36 h to complete. The staphylococcal enterotoxin was presumably produced during this time.

Until 1989 there were no records of outbreaks of human infection associated with yogurt in England or Wales or Europe (Galbraith et al. 1982; Sharp 1987). In June 1989, however, yogurt was associated with the biggest outbreak of botulism in Britain during this century (O'Mahony et al. 1990). Between 30 May and 13 June 1989, 27 cases of botulism, with one death, were traced to the consumption of low fat hazelnut yogurt. *Clostridium botulinum* type B toxin was detected in the contents of an unopened can of the hazelnut purée used to flavor the yogurts, and from unopened cartons of yogurt. The yogurt was made in the northwest of England, and the hazelnut purée was supplied in 6 lb cans by a factory in Folkestone. The hazelnut purée was sweetened with aspartamine, rather than sugar, which might have prevented bacterial growth, and insufficient heat had been used in the manufacturing process to destroy spores of *C. botulinum*.

In the autumn of 1991, an unusual outbreak of *E. coli* O157:H7 PT 49 infections was associated with consumption of a brand of live yogurt made specially for children by a farm producer in the northwest of England (Morgan et al. 1993). Sixteen cases of culture confirmed infection were identified from 1 September to 1 November 1991. Eleven of the children were age 10 years or less and five developed HUS. A formal case–control study confirmed a close association between

consumption of the yogurt and development of *E. coli* O157 infection. However, the organism was not isolated from raw or pasteurized milk on the farm nor from another batch of the implicated brand of yogurt sampled at a later date.

BUTTER

Butter contains approximately 81 percent fat and, under commercial conditions, is prepared from pasteurized cream with or without the addition of starter cultures which increase the acidity and add flavor. Dairy spreads may be defined as products with more than half their ingredients derived from milk. They have a higher water content than butter and low fat spreads contain additional milk proteins which stabilize the product (Wilbey 1994).

Most of the bacteria that are present in raw milk separate with cream and potentially would contaminate butter, so pasteurization is a very important part of the manufacturing process. The physicochemical characteristics of butter appear to inhibit the growth of many microorganisms but it has been shown that under experimental conditions *Salmonella* spp. can grow in butter at room temperature and may survive for long periods at refrigeration temperatures (El-Gazzar and Marth 1992). It has also been demonstrated that *L. monocytogenes* can increase in numbers during refrigerated storage of butter made from contaminated cream and that both *L. monocytogenes* and *Y. enterocolitica* can grow in some refrigerated dairy spreads (Milner 1995).

Spoilage of butter and dairy spreads may occur due to surface growth of *Pseudomonas* spp. or yeasts and molds (Milner 1995). There have been only a few incidents of food poisoning associated with butter and most of these have been due to *S. aureus* enterotoxin. In 1970, 24 customers and staff at a department store in Alabama became ill with acute gastroenteritis soon after consuming whipped butter in the restaurant. The butter had been prepared at the restaurant by whipping milk with softened butter but microbiological tests showed that the butter, not the milk, contained *S. aureus*. The same brand of butter was also implicated in a single case of typical staphylococcal food poisoning in Tennessee a few days later; tests on the butter demonstrated the presence of staphylococcal enterotoxin A (Wolf et al. 1970). A second outbreak of staphylococcal food poisoning, also associated with commercially produced whipped butter, occurred in Kentucky in 1977. More than 100 cases were diagnosed some of whom were admitted to hospital (Francis et al. 1977).

In 1991, an outbreak of food poisoning in California and Nevada involved more than 265 people and was traced to butter blend and margarine that was contaminated with enterotoxin-producing *Staphylococcus intermedius*. Isolates from patients and product produced staphylococcal enterotoxin A and PFGE analysis of restriction enzyme digests of DNA from five clinical and ten food isolates provided evidence that all the isolates were derived from a single strain (Bennett et al. 1994).

Another very serious outbreak of gastroenteritis followed by HUS affected nine children age 1–3 years at a nursery school in Germany (Tschäpe et al. 1995). All nine children required admission to hospital and dialysis and one boy died shortly after the onset of illness. In addition, six children age 4–6 years developed gastroenteritis without HUS. The children had eaten sandwiches prepared with 'green' butter that was made by mixing parsley with the butter. The parsley originated from a garden enriched with pig manure. The organism responsible for the infection was a verocytotoxin producing isolate of *Citrobacter freundii* which was isolated from patients, some symptomless contacts, and from the parsley. The isolates from the patients and the parsley were shown to be toxigenic by specific DNA probes and PCR and to be genetically indistinguishable from one another. This outbreak again demonstrates that the quality and hygiene of added ingredients are as important as those of the dairy product.

ICE CREAM

Ice cream usually contains 8–18 percent fat, 9–12 percent milk solids-not-fat, sugar, small amounts of stabilizer, and emulsifier. If the fat is entirely milk fat the product is known as dairy ice cream. When vegetable oils or other animal fats are used in manufacture the product cannot be described as 'dairy' ice cream.

Ice cream is a complex product and manufacture consists of several stages. Detailed descriptions of the process and plant are given by Rothwell (1990) and Mitten and Neirinckz (1993). Initially, the mix is blended to give a homogenous product. Compositional and heat treatment legislation varies considerably around the world but most countries require pasteurization (Rothwell 1990). Following pasteurization, the mix is homogenized and then cooled to 4°C or below and held in a tank for a variable time ranging from 4 to 12 or more hours to undergo aging. During the aging process, protein is adsorbed to fat globules, fat crystallization occurs, and stabilizers increase in hydration. In large, highly mechanized, and sophisticated ice cream plants, the aging process is carefully controlled and relatively short. However, some processors use a longer aging time of 24 h or more and at this stage there is opportunity for psychrotrophic microorganisms to multiply unless careful attention is paid to hygiene and prevention of post-pasteurization contamination.

Following aging, the mixture is frozen rapidly with agitation and air is incorporated into the mix. It is then extruded and may be dispensed as soft-serve ice cream or packaged into bricks or large containers which are hardened at between −20 and −40°C and sold as hard ice cream. Flavorings are usually added to the mixing or

aging tanks, but pieces of fruit, nuts, syrups, or purées are added to the ice cream after extrusion from the freezer.

Microbiological quality of ice cream

There were many small and large outbreaks of food poisoning due to ice cream in the UK prior to the introduction of heat treatment legislation for commercial ice cream (Rothwell 1990; Nichols and de Louvois 1995). Outbreaks of both *S. aureus* and salmonella food poisoning are recorded. In 1946, a large outbreak of typhoid fever in Aberystwyth in Wales affected more than 100 people with four deaths and was due to contamination of the ice cream by the manufacturer who was a typhoid carrier (Evans 1947). Following this incident, heat treatment regulations were introduced but did not become effective immediately and between 1950 and 1955 there were 19 recorded outbreaks associated with consumption of ice cream and ice lollies (11 due to *Salmonella* spp., two due to *S. aureus*, and six of unknown cause) (Rothwell 1990). Between 1955 and 1984, there were no outbreaks reported in the UK but since 1984 there have been a number due to home-made ice cream. In 1984, an outbreak of *Salmonella* Enteritidis PT4 infection involving 12 people was attributed to home-made ice cream (Barrett 1986). In 1987, six members of a family were ill with *B. cereus* poisoning after eating home-made ice cream containing egg white and fresh cream. The product had $>10^{10}$ *B. cereus* per ml (Sockett 1991).

Several outbreaks of *Salmonella* Enteritidis PT4 infection have occurred due to the use of raw shell eggs in home-made ice cream. In 1988, an outbreak affected 18 of 75 people at a bridge party (Cowden et al. 1989). In 1991 there were three family outbreaks (Report 1991b; Morgan et al. 1994a) and in 1993 another outbreak affected seven staff at a hotel (CDSC 1993, unpublished data). In the UK, it is illegal to use unpasteurized egg in commercial ice cream but it is clear that the continued use of raw egg in domestic homes and by commercial caterers poses a serious threat of food poisoning which may sometimes involve large numbers of people. A large number of outbreaks of salmonellosis in other countries have also been associated with unpasteurized ice cream and the use of contaminated eggs (Nichols and de Louvois 1995). A more recent very large outbreak involving more than 2 000 people in the USA was attributed to the use of eggs in nationally distributed ice cream products (Report 1994d).

Ice cream may also become contaminated with *L. monocytogenes*. In 1986, contamination of ice cream bars in the USA was thought to be responsible for a flu-like illness affecting at least 40 people in four states. This resulted in a recall of the product. Listeria contamination requiring recall of product has also been reported on other occasions in ice cream in the USA and France (Rothwell 1990).

Most countries have strict standards for hygiene and heat treatment requirements for commercial ice cream and it is clear that careful control of both ingredients and the production process is needed to produce a hygienic product and prevent post-pasteurization contamination. In 1992, in the UK, the Ice Cream Federation and the Ice Cream Alliance in collaboration with the Milk Marketing Board produced guidelines for the hygienic manufacture of ice cream and for the safe handling of products by retailers (Report of the Ice Cream Alliance and the Ice Cream Federation 1992a, b). These outline the main principles of quality assurance and attention to premises, raw materials, equipment, and finished products.

A survey of the microbiological quality of 2 612 samples of ice cream, ice lollies, and similar frozen products, was performed in England and Wales in 1993 as part of the European Community Coordinated Food Control Programme (Nichols and de Louvois 1995). The results of this survey demonstrated wide variations in microbiological profile but concluded that, generally, ices were of good quality and unlikely to cause food poisoning. However, nonbranded hard ice creams and soft ice creams were more likely to contain high levels of aerobic bacteria, indicator organisms, and potential pathogens than branded ice cream bars. *L. monocytogenes* was found in four of 1 964 samples of nonbranded hard ice cream and soft ice cream and *Salmonella* Enteritidis PT4 was isolated from a sample of 'real fruit' ice cream.

CHEESE

Cheeses are a heterogeneous group of foods made by coagulating the solids from high or low fat milk by the action of lactic acid and rennet. The curds so formed are treated with varying degrees of heating ('scalding'), cutting, salting, and pressing to produce greater or less acidity and moisture content. The resulting cheese can vary in size, softness, consistency, and degree of ripening. Further modifications depend on the type of lactic acid bacteria used to initiate souring and on whether other bacteria or molds are encouraged to grow within the cheese or on the surface. Survival of pathogens or development of spoilage organisms depends on the physical and chemical characteristics of the cheese and on the degree of maturation. Cheeses may be classified broadly into three main types: hard and semi-hard pressed cheeses, semi-soft cheeses which are usually not pressed, and soft cheeses. Hard cheeses include Parmesan, Gruyère, Cheddar, Derby, Leicester, Caerphilly, Edam, Gouda, and Fynbo. Soft cheeses include Camembert, Brie, Livarot, Cambazola, Cambridge, and Coulommiers, and semi-soft include Munster, Stilton, Roquefort, Gorgonzola, and Mozzarella. There are also

a few unshaped curd cheeses such as cottage and lactic cheese which have high moisture content and acidity, and a very limited shelf-life (Shaw 1993; Tamine 1993).

Hard cheeses such as Cheddar or Swiss are typically large, with low moisture content (c. 39 percent) and a clear rind. The curds are scalded and then matted together while in the vats, 'milled', or chopped, salted, and pressed into molds under heavy weights. Ripening takes 3–9 months and proceeds evenly throughout the cheese. Flavor depends on enzymes from the starter culture and from rennet. The starter is usually a mixture of subspecies of *Lactococcus lactis*. Swiss cheeses such as Emmenthal and Gruyère are manufactured at high temperatures. Thermoduric starters such as *Streptococcus salivarius* subsp. *thermophilus* or *Lactobacillus* spp. are used. In addition, cultures of propionibacteria are added for development of the essential and characteristic flavors.

Semi-hard cheeses such as Caerphilly, Lancashire, and the Dutch cheeses, are lightly pressed cheeses with a moisture content of around 45–50 percent. The textures vary from smooth to crumbly. Most semi-hard cheeses are matured for 1–3 months, but Caerphilly is usually eaten while quite fresh (around 2 weeks old) and Lancashire may be matured for as long as 12 months.

Soft cheeses have a high moisture content of 55–60 percent. The curds are not cut or scalded but are drained into small molds without pressing. Cheeses such as Coulommiers or Cambridge are eaten fresh within a few days but surface-ripened cheeses such as Camembert or Port Salut are surface-ripened for 1–6 weeks by molds (*Penicillium camemberti*) or bacteria (*Brevibacterium linens*).

Internally mold-ripened cheeses such as Gorgonzola or Stilton are semi-soft cheeses with a moisture content of 45–55 percent. They are made with high acid curd to which either *Penicillium glaucum* or *Penicillium roqueforti* is added. The curd is filled into tall molds, which are turned frequently so that it is pressed down by its own weight. After a few weeks, the cheeses are pierced with skewers to admit air and encourage growth of molds. Ripening is complete after 3–6 months, during which the pH rises to 6.0 or more, and lipolysis and proteolysis contribute to the flavor.

Defects and spoilage of cheese

Cheeses made from unpasteurized milk may develop numerous defects depending on the microbial quality of the raw milk and on subsequent contamination from vats and other equipment, and from the curing rooms in which they are matured. The quality of cheese made with pasteurized milk depends mainly on the extent of contamination during manufacture and storage (Chapman and Sharpe 1990). Off flavors and gas production early in manufacture are usually due to lactose-fermenting Enterobacteriaceae or, sometimes, to lactose-fermenting yeasts. Swiss-type cheeses are liable to develop gas some weeks later due to clostridial contamination. Surface growth of yeasts, molds, or proteolytic bacteria can cause softening or discoloration of the rind and spoilage of the cheese. Blue vein cheeses are particularly susceptible to this defect. Growth of yeasts and molds or slime production by psychrotrophic organisms is common in cottage cheese. Production requires a very high standard of plant hygiene.

FUNGAL TOXINS

Cheese-making experiments show that aflatoxin M_1 in milk will survive pasteurization and the cheese-making process, and will not deteriorate on storage. Growth of toxin-producing molds in cheese is also possible. *Penicillium* spp. can produce toxins such as penicillic acid, patulin, cyclopiazonic acid, and roquefortins both at room temperature and at temperatures below 10°C (Chapman and Sharpe 1990). Toxin-producing strains of *Penicillium* have been isolated from both Cheddar and Swiss cheese. There is concern that some of the strains of *P. camemberti* and *P. roqueforti* used for making blue cheese may be toxigenic, but conditions in the cheese tend to be unfavorable for toxin production because of the low content of carbohydrate. Many countries, however, now screen for and select nontoxigenic strains for cheese production. The development of aflatoxin in cheese is probably of less concern because *Aspergillus flavus* and *Aspergillus parasiticus* do not produce toxins at temperatures below 10°C, at which most hard and semi-hard cheeses are ripened.

BACTERIAL TOXINS

Histamine poisoning associated with the consumption of cheese was first reported by Doeglas et al. in 1967. In 1980, a small outbreak occurred in New Hampshire, USA. Specimens of suspect cheese contained 187 mg of histamine per 100 g and a strain of *Lactobacillus buchneri* isolated from the cheese was found to have very high histidine carboxylase activity (Sumner et al. 1985). Staphylococcal enterotoxin has also been the cause of many outbreaks of food poisoning associated with cheese.

Pathogenic bacteria in cheese

The efficient pasteurization of milk should eliminate the risk from all viable pathogenic organisms but many cheese makers still use raw milk or add raw milk to the cheese milk, believing it to be essential for good flavor. Survival of pathogenic organisms in cheese depends on the method of manufacture, the competitive effect of the lactic acid-producing starter bacteria and on conditions of storage and length of maturation of the finished product. Safety cannot be guaranteed if cheese is made from raw milk.

There have been many outbreaks and sporadic cases of infection or food poisoning, including brucellosis and

botulism, associated with the consumption of cheese. Most detailed investigations have demonstrated that the source of contamination was raw milk, inadequately pasteurized milk, or post-pasteurization contamination with organisms originally derived from raw milk. The most recent botulism outbreak occurred in Italy and was linked to eating a dessert made with mascarpone cream cheese (Aureli et al. 2000). The outbreak was caused by *Clostridium botulinum* type A and affected eight young people, one of whom died, having eaten Tiramisu. Type toxin A was detected in five sera and six stools from the patients and in samples of Tiramisu. The authors postulate that a break in the cold storage of the dessert probably lead to germination of the *Clostridium botulinum* spores and toxin production.

Tuberculosis in man has not been traced directly to the consumption of cheese but experimental work has shown that *M. tuberculosis* can survive in many varieties of cheese.

BRUCELLOSIS

Brucellosis is still an important cause of human and animal infection; 500 000 new cases of human infection are reported annually throughout the world. Many cases are associated with consumption of cheese made with raw milk. It has been shown that *B. abortus* and *B. melitensis* can survive and remain viable for many months even in hard cheese such as Cheddar (Chapman and Sharpe 1990).

Wallach et al. (1994) described an outbreak affecting an Argentine family after eating unpasteurized goats' milk cheese. Nine of 14 became ill with *B. melitensis* and blood cultures were positive from seven patients.

Human brucella infection is rare in England and Wales and, with very few exceptions, is acquired abroad after consumption of raw milk and cheese. Two cases of *B. melitensis* were reported in a family who stayed in Malta in April 1995 and ate a locally produced cheese pastry. They were among those affected in an outbreak involving at least 135 people (one death) which was linked to consumption of a soft cheese made with unpasteurized goats' and ewes' milk (Report 1995).

STREPTOCOCCUS ZOOEPIDEMICUS

In 1983, 16 cases of *S. zooepidemicus* occurred in New Mexico and were traced to a home-made cheese made with raw cows' milk which was sold at several stores. A case–control study confirmed the association between *S. zooepidemicus* infection and eating the suspect cheese. In addition, the organism was isolated from multiple samples of cheese and from milk from the cattle at the farm (Espinosa et al. 1983).

STAPHYLOCOCCUS AUREUS

Cheese is the milk product most often associated with food poisoning due to *S. aureus* enterotoxin. This organism is a common cause of mastitis in cattle and common in raw milk. Toxin may be present before pasteurization if the milk has been stored at ambient temperature. If sufficient numbers of *S. aureus* are present at the start of cheese-making, either because raw milk is used or because of post-pasteurization contamination, they may multiply and produce toxin during the cheese-making process (Minor and Marth 1972). Experiments with cheddar cheese-making have shown that even heavily contaminated milk is unlikely to become toxic if the starter culture is working normally. If, however, development of acidity is slow because of the presence of bacteriophage, the numbers of *S. aureus* may attain values of 10^6–10^8/g and produce toxin in the curd. During ripening, the numbers of *S. aureus* decline over the course of a few days or weeks in normally acidic cheese but may continue to multiply in cheese of low acidity during the first few weeks of ripening and survive for months thereafter.

Between 1951 and 1980, cheese was implicated in 16 recorded outbreaks of *S. aureus* food poisoning involving 507 people in England and Wales (Galbraith et al. 1982). Cheddar, Stilton, home-made soft cheese, and Romanian hard cheese were included. In ten outbreaks it was suspected that contamination occurred during manufacture. In the 1950s and 1960s, *S. aureus* intoxication was the most common cause of food poisoning in the USA, and numerous cases were traced to cheese (Hendricks et al. 1959; Walker et al. 1961). Since the almost universal introduction of refrigerated storage for milk, there have been very few incidents. One small outbreak involving only two people occurred in 1983 (Barrett 1986) and in 1987 cheese was suspected as a source of *S. aureus* intoxication in an outbreak involving 28 of 350 people who attended a buffet lunch of a variety of food items (Communicable Disease Report 1987, unpublished). Sharp (1987), in his survey of infections associated with dairy products in Europe and North America from 1980 to 1985, found no other incidents associated with cheese made from cows' milk. Cheese made from contaminated sheep milk was, however, responsible for outbreaks in France and Scotland.

A series of 36 outbreaks of food poisoning in England from November 1988 to January 1989, affecting 155 people, were linked to consumption of Stilton cheese made from raw cows' milk. Although the microbiology and tests for toxin were negative, the clinical illness was typical of staphylococcal intoxication. Control measures included withdrawal of the implicated cheese from sale and subsequent change to use of pasteurized milk for cheese production (Maguire et al. 1991).

For further information on *S. aureus* food poisoning, see Bacteriology of foods, excluding dairy products and 32, Staphylococcus.

SALMONELLA

Salmonellae can multiply rapidly during the cheese-making process and will survive in the curd unless a high

degree of acidity is reached. During ripening, an initial rapid decline in numbers is followed by a relatively stationary phase. Small numbers have been detected even after storage for 6–10 months at refrigeration temperatures (Medina et al. 1982). It may be difficult to isolate the organisms because small numbers are slow to revive and are not distributed evenly through the cheese. Survival tends to be greater in the middle of the cheese and poor near the surface. The infective dose seems to be low. About 10^4 organisms can cause illness, presumably because the high lipid content of the cheese protects the bacteria from the acidity of the stomach (Ratnam and March 1986). Many outbreaks of salmonellosis have been traced to cheeses of all types. It is clear that effective pasteurization of the milk is the only dependable way of ensuring that salmonellae do not contaminate cheese (Fontaine et al. 1980). From 1980 to 1985, outbreaks were recorded in Italy, Canada, Finland, and Switzerland (Sharp 1987).

In 1984, there was a huge outbreak of infection with *Salmonella* Typhimurium PT10 in the four Atlantic provinces of Canada and in Ontario (Ratnam and March 1986). The outbreak was the largest common source outbreak ever to occur in Canada and involved an estimated 10 000 persons. *Salmonella* Typhimurium PT10 was isolated from the factory-packed contaminated lots of Cheddar cheese as well as leftover cheese recovered from homes of human cases. Estimated numbers of *Salmonella* Typhimurium in the cheese were low but the lots of cheese that were positive also contained high levels of coliforms and *E. coli*. Defective pasteurization was shown to be the underlying cause of contamination and the outbreak strain was isolated from milk from a cow on one of the farms supplying the factory. A careful investigation of the pasteurization process revealed that an employee manually overrode the controller and allowed unpasteurized milk to go through to the vats. The pasteurizer was shut down after filling three vats and later restarted to fill the next three vats so that the first and third vats contained *Salmonella* Typhimurium when contaminated raw milk was being pasteurized (Johnson et al. 1990).

In 1989, an outbreak of *Salmonella* Dublin affected 42 people in England and Wales and was traced to imported Irish soft cheese made with unpasteurized cows' milk. *Salmonella* Dublin was isolated from samples of cheese and from the milk of four cows in the milking herd. The cheese was made on the dairy farm (Maguire et al. 1992).

In 1993, *Salmonella* Paratyphi B infection in south-west France was traced to unpasteurized cheese made from goats' milk. *Salmonella* Paratyphi B was isolated from a batch of cheese and from milk from a goat in a herd that supplied milk to the cheese makers (Desenclos et al. 1996).

The most recent outbreak in the UK occurred in the north of England in late 1996. Nineteen cases of *Salmonella* Goldcoast infection were linked by microbiological and epidemiological investigations to consumption of cheddar cheese (Goh, S.K. personal communication). Although the initial cases were in the north of England, additional cases were later identified in other parts of England. The cheddar cheese, made with pasteurized milk, originated from a large commercial cheese manufacturer in Somerset. Microbiological investigations confirmed that batches of the cheese were contaminated with *Salmonella* Goldcoast. Investigations at the factory highlighted process failures during the production of the infected cheese. Veterinary investigation of the farm supplying milk to the cheese manufacturer detected *Salmonella* Goldcoast in cattle on the farm.

Cheese continues to be a possible source of infection with *Salmonella* as demonstrated by an outbreak associated with the consumption of a Tyrolean cheese in Austria. Sixteen cases of *Salmonella* Oranienburg were linked to consumption of the cheese made on a local Alpine farm (Eurosurveillance 2000).

LISTERIA MONOCYTOGENES

The fairly recent association of *L. monocytogenes* infections with soft cheese may be due to changes in marketing practices for dairy products. In recent years, a wider range of dairy products, including many varieties of continental soft cheese, have become popular in Britain and North America and are available in most supermarkets. Many of these products are imported from the country of origin and all have been stored at refrigeration temperatures for days or weeks. *L. monocytogenes* multiplies at low temperatures and minor contamination may increase significantly during storage. *L. monocytogenes* appears to be common in raw milk and many soft cheeses are made from raw milk or have small amounts of raw milk added to them during manufacture. Post-process contamination with *Listeria* spp. may also occur because of the wide environmental distribution of the organism; it can be prevented by strict attention to hygiene and the prevention of cross-contamination during handling and storage (Marfleet and Blood 1987).

In 1985, 86 cases of *L. monocytogenes* infection were identified in Los Angeles and Orange Counties, California (James et al. 1985); 58 cases were in mother–infant pairs. There were eight neonatal deaths and 13 stillbirths out of a total of 29 deaths. Case–control studies implicated Mexican-style soft cheeses from one manufacturer. *L. monocytogenes* serotype 4b was isolated from patients and from packets of cheese. Although most of the cheese milk was pasteurized, about 10 percent was not.

Surveys in the USA and Europe (including the UK) have shown that *L. monocytogenes* may be found in a wide variety of soft cheeses (Gilbert 1987; Pini and Gilbert 1988; Greenwood et al. 1991). Experimental

production of cottage cheese with artificially contaminated milk confirmed that a proportion of the inoculum survived manufacture (which included cooking at 57.2°C for 30 min). Organisms could be recovered from more than one-third of samples by direct plating after storage at 3°C (Ryser et al. 1985). Ryser et al. (1985) also showed that listeria survive during the manufacture of Cheddar cheese and that numbers increase during the first 14 days of ripening. Thereafter, numbers decrease but may persist for months.

Sporadic cases of listeria infection have been associated with the consumption of soft cheeses (Bannister 1987; Azadian et al. 1989; McLauchlin et al. 1990). In 1987, cases of listeriosis in Switzerland were associated with Vacherins Mont d'Or soft cheese and investigations revealed that an outbreak lasting from 1983 to 1987 had involved 122 persons (with 34 deaths) (Bille 1990).

In an outbreak of listerosis reported in France (Goulet et al. 1995), 20 cases were identified between 2 April and 16 May 1995, including 11 in pregnant women, resulting in two spontaneous abortions, four premature births, and two stillbirths. The source of infection was one specific chain of Brie de Meaux (a raw milk soft cheese) production. This is the first outbreak of listeriosis, documented in France, which has been linked to the consumption of a raw milk cheese.

A study to identify the main hazards associated with the spread of *L. monocytogenes* in dairy products in Switzerland reviewed data from 1990 to 1999. Overall, 3 722/76 271 (4.9 percent) of samples were positive (Pak et al. 2002). Cheese-ripening facilities had the highest proportion of positive samples (7.6 percent) followed by small-scale dairies (4.4 percent). Contamination of these products is frequently associated with environmental contamination and in this study 9.5 percent of the samples of water, used for cheese washing, were positive. This may indicate that a preventative step can be introduced. 1 328/3 722 (35.7 percent) of isolates were serotyped with serotypes 1/2a, 1/2b, and 4b, accounting for over 90 percent of isolates. Hard and semi-hard cheeses were more likely to be contaminated with serotype 1/2b and soft cheeses with serotype 1/2a.

An outbreak of listeriosis associated with homemade Mexican-style cheese occurred during October to January 2000 in North Carolina, USA (MMWR 2001). Noncommercial, homemade, Mexican style fresh soft cheese produced from contaminated raw milk from a local dairy was implicated as the source. Once again, this outbreak highlights the risk of using raw milk in the preparation of dairy products.

An outbreak of febrile gastroenteritis following consumption of on-farm manufactured fresh cheese has been reported from Sweden (Carrique-Mas et al. 2003). Thirty-three consumers showed an attack rate of 52 percent. Illness was characterized by presentation of diarrhea (88 percent), fever (60 percent), stomach cramps (54 percent), and vomiting (21 percent) in the cases. Although gene sequences of verocytotoxin producing *E. coli* were detected in six subjects, 27/32 fecal specimens were positive for *L. monocytogenes*. Molecular profiles of *L. monocytogenes* from dairy, fecal, and clinical isolates were indistinguishable. Both microbiological and epidemiological evidence pointed to *L. monocytogenes* as the most likely source of this unusual outbreak. It is a timely reminder that *L. monocytogenes* can cause gastrointestinal symptoms and should be considered as a possible etiology in outbreaks of gastroenteritis associated with dairy products.

ESCHERICHIA COLI

Outbreaks of illness due to pathogenic *E. coli* have been recorded in association with cheese and it has become clear that serious infection with both enteroinvasive and VTEC may be acquired from contaminated cheese.

In 1971, there were 107 episodes of enteroinvasive *E. coli* illness involving 387 people in the USA (Marier et al. 1973). Imported French Brie, Camembert, and Coulommiers cheeses made at a single factory over a period of 2 days were implicated. A slowly lactose-fermenting strain of *E. coli* serotype O124:B17 was isolated from samples of cheese and from the feces of patients. Laboratory tests confirmed that the strain was invasive but not enterotoxigenic. There is no mention of whether the cheese milk had been pasteurized, but river water, used to wash the vats, was the suspected source of infection.

Epidemiological data incriminated French Brie and Camembert cheeses in a large outbreak of enterotoxigenic *E. coli* infection in 1983. This affected more than 3 000 people in the USA (Washington, Illinois, Wisconsin, and Georgia), as well as in Denmark, Holland, and Sweden (MacDonald et al. 1985; Nooitgedagt and Hartog 1988). *E. coli* serotype O25:H20 was isolated from fecal specimens and heat-stable (ST) toxin production was demonstrated. The plasmid profile of isolates from patients in Washington, Illinois, Georgia, and Wisconsin were similar. The illness was associated with cheese from two separate batches made at the same factory 46 days apart. There was no information about pasteurization and the source of contamination was not discovered. Cultures of cheese samples did not grow *E. coli* serotype O27:H20 but organisms binding antiserum to *E. coli* O27 were detected in one cheese specimen by solid-phase radioimmunoassay at a concentration of 10^4 organisms per gram.

There have been several reports of outbreaks of *E. coli* O157:H7 infection due to the consumption of cheese. One cluster of four cases occurred in a rural community of France and was linked to 'fromage frais' made with unpasteurized goats' and cows' milk on a farm. Four children aged from 9 to 15 months were affected, three during the spring of 1992 and one during the spring of 1993. All children had diarrhea, three

developed renal failure, and one child died. Samples taken from the farm yielded isolates of VTEC but not of *E. coli* O157 (Report 1994e). The first outbreak in the UK occurred in Scotland in 1994. Twenty cases of *E. coli* O157:H7 phage type 28 infection, in the Grampian region, were associated with a farm-produced cheese made with unpasteurized milk. Most of the patients had bloody diarrhea and one had HUS. *E. coli* O157 PT28 was also isolated from a sample of the cheese and typing of the isolates from the patients and the cheese by PFGE demonstrated that they were indistinguishable from one another (Curnow 1994). An outbreak associated with a hard cheese made from unpasteurized milk was identified in Lancashire, England in 1997. Five cases were associated with a strain of *E. coli* O157 PT 8 (Aird et al. 2000). Two cases had consumed the cheese, two cases had consumed different cheese from retail outlets that sold the contaminated batch, and there was no apparent connection in the fifth case. All five isolates from the human cases, from the cheese, and from cattle on the dairy farm producing the milk, had PFGE profiles that were indistinguishable. Enumeration of *E. coli* O157 in contaminated cheese revealed that the cheese was contaminated with between 5 and 11 CFU/g (Bolton, F. personal communication). This confirms the low infectious dose for this organism. This outbreak also highlights the potential for cross-contamination of other cheese products at retail outlets.

An outbreak involving four cases in a private house in Scotland in 1998 was also reported to be associated with the consumption of cheese (Task Force on *E. coli* O157 2001). Another outbreak was reported in Wisconsin, USA in 1998, associated with the consumption of fresh cheese curds. A total of 55 cases were linked to the outbreak of which 25 were admitted to hospital (MMWR 2000). Three outbreaks of *E. coli* O157 infection associated with cheese were reported in the UK in 1999. One of these was the outbreak linked to cheese made from goats' milk (see section on Goats' milk). The other two occurred in the north of England. One of these was associated with Cotherstone cheese, a specialist cheese made in the northeast of England from unpasteurized milk (CDR 1999). There were three cases of infection with *E. coli* O157 PT 21/28. Isolates were also obtained from several cheese samples but not from the farm producing the milk. All of the isolates had an indistinguishable PFGE profile. The remaining outbreak in 1999 occurred in the northwest of England. Four cases, one of which was a food handler, were linked to the consumption of a homemade cheese sold at a local restaurant (Aird et al. 2000).

The outbreaks in Scotland prompted a survey of *E. coli* O157 in high-risk foods such as raw meats, raw milk, and raw milk cheeses. The survey examined 739 samples of raw milk cheeses sampled at the point of sale by an enrichment culture-immunomagnetic capture method and did not find any positive samples (Coia et al. 2001). In contrast, a French survey that examined 180 cheeses made from unpasteurized milk, found that for all VTEC 30.5 percent were positive by PCR. None of these were *E. coli* O157 (Fach et al. 2001).

MICROBIOLOGICAL QUALITY CONTROL DURING MANUFACTURE OF CHEESE

In recent years, there has been increasing concern about pathogens in cheese and particularly of the risk of contamination with *L. monocytogenes* and *E. coli* O157 (Report of the Committee on the Microbiological Safety of Food 1990; Report of the Advisory Committee on the Microbiological Safety of Food 1995). It is clear that soft and fresh cheeses that develop pH values in excess of 5.0 should be considered as high risk for *L. monocytogenes* whereas soft cheese which maintains a pH of less than 5.0 is of lower risk. Hard-pressed cheese is also of low risk particularly if there is a long maturation time of 60 days or more, but no cheese is entirely without risk for contamination with *L. monocytogenes* or other pathogens if the hygiene during manufacture is poor or if the cheese has been manufactured from unpasteurized milk.

The industry has taken the problem of hygiene very seriously in the UK. The Creamery Proprietors' Association has published 'Guidelines for good hygienic practice in the manufacture of soft and fresh cheeses' (Report 1988) and the Milk Marketing Board has produced similar guidelines for the manufacture of soft and fresh cheeses in small and farm-based units (Report 1989). In addition, the joint FAO/WHO Codex Alimentarius Commission is at present drafting a code of practice for hygienic practice for the manufacture of uncured, unripened, and ripened soft cheeses (Alinorm 95/13). European Standards (Council Directive 92/46/EEC) and Dairy Products (Hygiene) Regulations (HMSO 1995) reflect the current anxiety about *L. monocytogenes* and are very stringent for soft cheese whether it is made from raw or pasteurized milk. *L. monocytogenes* must be absent from five samples of 25 g taken at the manufacturing premises and each 25-g sample must consist of five pieces of 5 g from different parts of the same product. In the USA, *L. monocytogenes* must be absent from cheese and other milk products, either home-produced or imported, and this is monitored by testing performed by the state and federal authorities.

In early 1995, 13 public health laboratories in England and Wales carried out a survey of the quality of a variety of imported and local soft and semi-soft cheeses purchased from retail outlets such as supermarkets, shops and delicatessens (Nichols et al. 1996). The cheeses were examined by internationally accepted methods according to a standard protocol to see how they compared when tested for *Salmonella* spp., *L. monocytogenes*, *S. aureus*, coliforms, and *E. coli*. Data obtained for 1 437 samples showed that generally the standard was good and that most cheeses would satisfy the legal requirements.

Cheeses made from unpasteurized milk were of a lower microbiological quality than those prepared from pasteurized milk and it was of concern that for 67 percent of the cheeses sampled, there was no information to enable the customer to determine whether the cheese was made from pasteurized milk or not. However, the isolation rate for *L. monocytogenes* was low for all cheeses (1.1 percent overall and 1.4 percent for cheese made from raw milk). This was significantly lower than that found during a previous survey in 1988–89 (Greenwood et al. 1991).

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Bacteriology of foods, excluding dairy products

DIANE ROBERTS

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GENERAL PRINCIPLES OF FOOD MICROBIOLOGY

The field of food microbiology is very diverse, but it can be subdivided into three broad areas of study:

1. the utilization of microorganisms in the production of foods in order to enhance their nutritive value, organoleptic properties (color, odor, flavor, texture, etc.) and shelf-life
2. the control of spoilage of foods by reducing or eliminating their microbial content or by making the food environment unsuitable for the growth of spoilage microorganisms
3. the microbiological safety of foods, protecting of the food supply from those pathogenic bacteria that may be naturally present in foods or that may be introduced during processing and service.

The area of food safety is covered in other chapters of this treatise so will not be discussed in detail here. Local, national, and international food legislation is in place to protect the consumer from food-borne infections and intoxications of both microbial and nonmicrobial origin. These are aimed mainly at protecting the consumer from pathogenic microorganisms, so they will not be discussed here.

Not all microorganisms in foods are harmful and without some of them there would not be the great variety of foods that is enjoyed today. Some of the foods for which microorganisms (bacteria, yeasts, and molds) are essential to their production include cheese, yogurt, fermented sausages, sauerkraut, and a whole array of fermented delicacies (tempeh, tofu, soy sauce). In addition, the production of beer, wines, and other alcoholic beverages is dependent on the fermentation of appropriate substrates by yeasts. Production of a fermented product with the desirable characteristics already mentioned, but without the problems associated with the growth of spoilage organisms and pathogens, requires an intimate knowledge of the microorganisms involved in the three areas. This is particularly true with respect to their behavior in various food systems and their susceptibility to environmental conditions and chemicals. The branch of microbiology that deals with this specific area is called microbial ecology and forms the central focus of this chapter. This topic is addressed in a concise manner with important references for additional information. The two volumes on *Microbial ecology of foods* developed by the International Commission on Microbiological Specifications for Foods (ICMSF) are highly recommended as authoritative sources for additional information on this subject (ICMSF 1980, 1998).

FACTORS INFLUENCING MICROBIAL GROWTH IN FOODS

Foods are very complex biological materials that span a tremendous range in texture, fluidity, and nutritional

content. Nevertheless, there are several universal characteristics that can exert an influence on the growth of microorganisms. The major factors that must be considered are temperature, pH, and water activity (a_w). In addition, redox potential (*E*h) and the presence of organic acids, salts, and other chemicals can all influence the growth of microorganisms in foods. These factors can be considered as two groups, the intrinsic and the extrinsic factors (Mossel et al. 1995; Montville 1997a). Intrinsic factors are those inherent to the food itself such as naturally occurring compounds that stimulate or retard growth, compounds that are added as preservatives, redox potential, water activity, and pH. The primary extrinsic factors, those imposed from the outside, are temperature, humidity, and gas composition (controlled and modified atmosphere packaging).

Extrinsic factors

TEMPERATURE

Although microbial growth can occur over a temperature range of −8 to 90°C, in food microbiology the range of greatest importance is 1–50°C. Within this range the temperature can affect the duration of the lag phase, the rate of growth, the final population of cells, and the nutritional requirements. The temperatures immediately above and below the growth ranges of organisms may just retard growth, but if the temperature exceeds the upper limit, cell injury and cell death occur. The response of microorganisms to freezing temperatures is more complex and will depend on the composition of the microbial cell wall, the final temperature, and, more importantly, the rate of freezing.

Bacteria can be grouped according to their temperature ranges for growth: thermophiles (optimum 55–75°C), mesophiles (30–45°C), psychrophiles (5–15°C), and psychrotrophs (25–30°C). Many food-spoilage and most pathogenic bacteria are mesophiles. Psychrophiles are sensitive to temperatures higher than 20°C; therefore, they are not as important in foods as psychrotrophs, which have temperature maxima in the range of 30–35°C. The ability of psychrotrophs to grow at refrigeration temperatures make them important contributors to food spoilage. Bacteria in the genera *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Enterobacter*, *Flavobacterium*, *Proteus*, *Pseudomonas*, and *Serratia* are important causes of the spoilage of foods, particularly those stored at refrigeration temperatures in order to increase their shelf-life. However, some food-borne pathogenic bacteria (*Listeria monocytogenes*, *Yersinia enterocolitica*) are psychrotrophic and disease outbreaks have occurred from contaminated food products that have been stored at refrigeration temperatures.

Effect of chilling

As the temperature is lowered to freezing point, the lag phase of bacterial growth increases greatly. All except the psychrotrophic group stop growing, thus giving the group a competitive advantage in foods held at low temperatures. Although the generation times of psychrotrophic organisms increase significantly at temperatures near freezing (for example, the generation time for *Pseudomonas fluorescens* at 0.5°C is 6.68 h), during refrigerated storage of foods for several days or weeks their populations can reach several millions. These bacteria grow using the food as their source of nutrients, with the result that the lipids, proteins, and carbohydrates in the food are broken down, producing degradation products of food components and the bacterial metabolic byproducts that are associated with food spoilage.

Effects of freezing

The response of bacteria to freezing ranges from little or no effect to varying degrees of injury and ultimately cell death (see Montville 1997a). Most spores and some vegetative cells can survive with almost no effect, but most non-spore-forming bacteria are affected to some extent. In general, gram-negative bacteria are more sensitive to freezing than gram-positive bacteria. Among those that are sensitive the consequences of freezing are sublethal cell injury and cell death. Cells that are sublethally injured are highly sensitive to the selective agents used in the selective enrichment and plating media used for the isolation and enumeration of food-borne bacteria. The use of these microbiological media to determine bacterial counts in frozen food products is most likely, therefore, to result in an underestimate of the microbial populations because of the failure of these sublethally injured bacteria to produce colonies. However, under appropriate cultural conditions, these sublethally injured bacteria may repair their damage and become fully viable and thus remain a threat to the integrity of the food.

The rate of freezing and the final storage temperature have an influence on bacterial injury and survival with the temperature range of −2 to −10°C being the most detrimental for bacteria. Slow freezing to approximately −10°C will cause maximal damage, whereas rapid freezing to −30°C or lower is likely to cause only minimal damage. Slow freezing also has a detrimental effect on the organoleptic characteristics of foods and is therefore not generally used as a means of bacterial inactivation.

Whether bacteria will grow in frozen foods after thawing will depend on the numbers and types that survived the freezing process and the conditions used for thawing. Uncontrolled thawing may result in a significant zincrease in the numbers of bacteria. When a large block of frozen food is thawed, the areas near

the surface will thaw much more rapidly than the core. Thus, bacteria near the surface of the food may start to grow and go through several generations before the center of the food has completely thawed. This is why it is considered safer to thaw frozen foods, particularly large blocks such as frozen turkeys, either at refrigeration temperature or rapidly by microwave heating rather than at ambient temperature.

Effects of heating

As the temperature rises above the optimum growth range, growth of bacteria ceases and injury and, ultimately, cell death occur. As with the effects of freezing, a mild exposure to heat can cause a sublethal injury in vegetative cells that is repairable. Spores are much more refractile to heat and may be activated to germinate. An exposure of 5 min at 80°C is typically used to inactivate vegetative cells and induce germination of spores. With further increase in temperature there is destruction of the bacterial population at an exponential (logarithmic) rate. Figure 1 shows a typical survivor curve for bacteria. By plotting the logarithm of the number of survivors against the time of heating at a specific temperature, a straight line is obtained because the rate of death is constant at a given temperature and is independent of the initial population of cells. The decimal reduction time (the time required to destroy 90 percent of the cells, *D* value) may be calculated from the

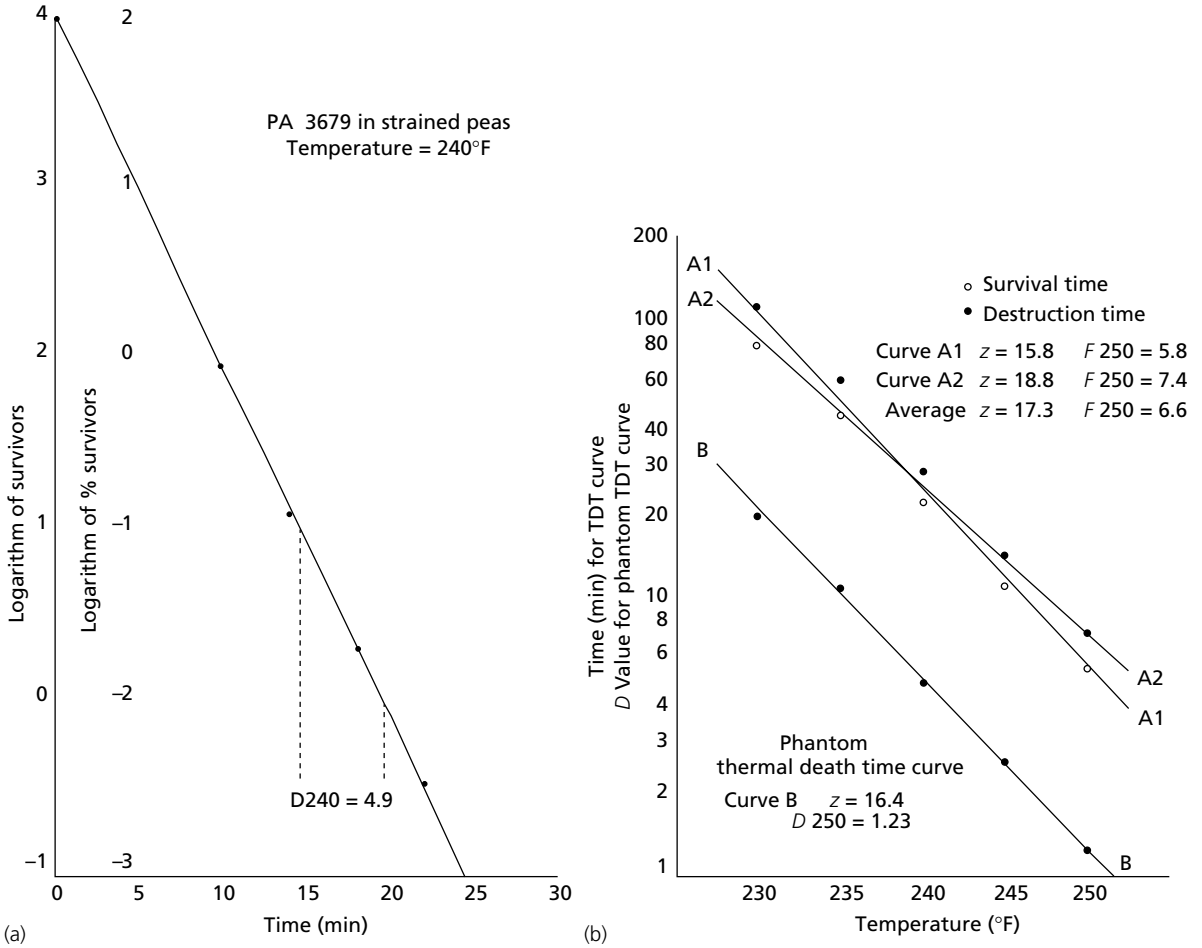

Figure 1 (a) *A typical survivor curve for bacteria. The test organism used to develop this curve is putrefactive anaerobe (PA) 3679, which is a non-toxigenic* Clostridium *species that has heat-resistance characteristics similar to those of* C. botulinum. *Therefore, PA 3679 is often used to determine or verify the thermal process conditions required to inactivate* C. botulinum *in specific foods. In this illustration, the* D *value for PA 3679 at 115°C (240°F) is 4.9 min.* **(b)** *An example of a thermal death time (TDT) curve. The* D *values are plotted on a logarithmic scale against the corresponding temperature on a linear scale to obtain the phantom TDT curves. These lines are described by the* D *value at a reference temperature (usually 60 or 121°C) and the slope of the curve (denoted by* z*).* z *is defined as the temperature that is necessary to bring about a ten-fold change in the* D *value.* F *denotes the time of treatment necessary to destroy microorganisms at a reference temperature, usually 60°C for vegetative cells and 121°C for spores. (Parts a and b based on Figures 7-7 and 7-8 from* Laboratory Manual for Food Canners and Processors*, vol. 1, National Canners Association Research Laboratories, 1968, AVI Publishing Company, Westport, CT, with permission from National Food Processors Association, Washington, DC.)*

survivor curve. The D value for the bacteria in Figure 1 is 4.9 min at 121 C.° The ordinate of the plot is on a logarithmic scale, thus it is evident that the population of bacteria would never be reduced to zero. It follows, therefore, that if a known population of bacteria in each of several unit volumes or containers were exposed to lethal heat treatments, a probability will always remain of a survivor in any one container. Such calculations are used to determine that the heat treatment to be applied to a specific type of food in a specific type of container will result in a probability of survival so low that it is acceptable. For example, a 12D process is generally used in commercial canning operations to assure that canned low-acid foods are free from the most resistant spores of *Clostridium botulinum* and the hazard of botulinum toxin.

The survivor curve gives information on the time of heat treatment required to destroy a certain proportion of bacteria at a specific temperature. The thermal death time curve is used to determine the effect of heat treatment of bacteria suspended in a medium at several temperatures. A thermal death time curve is constructed by plotting the $\log_{10}$ D values against the treatment temperature. The slope of the curve is denoted by the term z, which is the temperature in degrees Celsius required for the thermal death curve to traverse one log cycle. D and z values are used extensively in determining the heating requirement for foods preserved by pasteurization and sterilization.

Many factors influence the heat resistance of bacteria. These may be divided into three broad types:

1. the differences in heat-resistance characteristics of different species within a genus, strains within the same species, or spores versus vegetative cells
2. the environmental conditions to which the bacteria were previously exposed during growth
3. the properties of the food to which they are exposed during heat treatment (pH, a_w, salts and other compounds, type of food).

If these factors are not considered during the development of a thermal treatment process for foods, the outcome may be food spoilage and food-borne infection or intoxication.

GASEOUS ATMOSPHERE

The growth-limiting value of lowered partial pressure of oxygen (pO_2) or redox potential (Eh) is often combined with and reinforced by another inhibitory parameter, such as raised pCO_2. The composition of the gaseous atmosphere in which foods are stored may affect the fate of their microbial association more than by the oxygen partial pressure alone. A number of gases are utilized in the commercial preservation of foods: carbon dioxide, ethylene oxide, propylene oxide, sulfur dioxide, and ozone.

Intrinsic factors

WATER ACTIVITY

Microorganisms, in order to grow and metabolize, require water in an available form. The most useful measurement of available water is water activity (a_w). The a_w of a food or solution is the ratio of the water vapor pressure of the food (p) to that of pure water (p_0) at the same temperature. The a_w of pure water is assumed to be 1. When a solution becomes more concentrated with the addition of more solute (for example, salt, sugar, or other chemical) or the removal of water by drying, the vapor pressure decreases and so the a_w becomes a value of less than 1. The water molecules are oriented about solute molecules or become adsorbed on to insoluble food constituents and are thus less available for reactions. Most bacteria and fungi are unable to grow at an a_w of less than 0.90. Thus the dehydration of foods and the addition of salt and sugar are long-established food-preservation methods particularly in relation to food safety. Freezing can also reduce the a_w. However, some bacteria and fungi can grow at a_w values below 0.90 and are thus the organisms to be considered in preventing food spoilage. They are variously referred to as halophiles, xerophiles, and osmophiles, but these terms strictly should apply only to those organisms that can grow well at reduced levels of a_w but fail to grow at high levels. Gram-negative bacteria generally do not grow below a_w values of 0.97. Between a_w values of 0.98 and 0.93, gram-positive bacteria – Lactobacillaceae, Bacillaceae, and Micrococcaceae – become important. Below 0.93, food spoilage is caused primarily by yeasts and fungi. The only bacterial pathogen growing within this range is *Staphylococcus aureus*. Xerophilic fungi and osmophilic yeasts cause spoilage at a_w values below 0.85. Below an a_w of 0.60, microorganisms are unable to grow but they can remain viable for long periods.

pH

Bacteria have a minimum, optimum, and maximum pH for growth. The cell membrane is only slightly permeable to hydrogen or hydroxyl ions, and the cytoplasmic contents have a buffering effect, thus the pH inside bacterial cells is usually close to 7.0. The minimum pH values at which bacteria can initiate growth do vary. Some bacteria, such as the lactic acid bacteria, create an acidic environment by producing acids as metabolic products; they therefore gain a selective advantage over bacteria that are less acid-tolerant. Most bacteria are unable to grow at pH levels of less than 3.5 and those of importance in food-borne illness are unable to grow below about pH 4.5. High pH values also inhibit microorganisms. Egg white develops a pH of 9 because of the loss of CO_2 after the egg is laid; this protects the egg from bacterial invasion. Proteolytic organisms such as

Pseudomonas spp. can grow in moderately alkaline substrates. The antimicrobial effect of reduced pH depends on interactions with other factors (temperature, a_w, salt concentration).

REDOX POTENTIAL

The redox potential (*E*h) of a biological system is an index of its degree of oxidation and is an important selective factor in the food environment. Redox potential can denote the oxygen relations of a living microbe and may be used to specify an environment in which the organism can produce energy and new cells without recourse to molecular oxygen. Bacteria are classified as aerobic, anaerobic, facultative, or micro-aerobic based on the *E*h (ranging from positive values of about +300 mV (aerobe) to negative values of −420 mV (anaerobes)) required for their growth and metabolism. The *E*h of a food is related to its chemical composition (pH, concentration of reducing substances such as ascorbic acid, mercapto groups in proteins, reducing sugars, etc.) and the oxygen partial pressures existing over the food during storage. Gradients of *E*h may occur from the surface to the depth of a food where gaseous diffusion is restricted.

NUTRIENTS

The nutritional composition of a food has an influence on the type of bacteria that are likely to be present. Also, the nutrient requirements of bacteria may change depending on the environmental conditions in which they are grown. For example, bacteria generally become more nutritionally exacting at higher or lower growth temperatures or at lower a_w values. This may be due to inability to utilize particular nutrients as a result of impaired transport processes or from markedly reduced activity of critical enzymes. In general, most foods provide sufficient nutrients for most microorganisms. However, there is a wide range in nutrient complexity and availability; meats are generally considered highly nutritious and vegetables are considered to provide minimal nutrients.

Spoilage of starchy foods, such as potatoes and cereal grains, is caused primarily by bacteria that are able to break down the complex carbohydrates and that thrive in a minimum nitrogen and salts environment. Fresh vegetables are spoiled primarily by cellulolytic organisms. Bacteria with high lipolytic activity are favored in lipid-containing foods. Highly proteinaceous foods favor the growth of bacteria with complex nutritional needs. Nutrients control the rate of growth only when present at very low levels.

OTHER FACTORS

In addition to the factors discussed above, other factors that influence the growth of bacteria in foods are those natural components of foods, which have antibacterial properties (essential oils, tannins, and lectins). Animal foods contain many inhibitors of bacteria (lactenin in milk, lysozyme, conalbumin, ovomucoid, and avidin in eggs) but they usually have a narrow spectrum of activity and are quite labile. Therefore, they do not play a major role in preventing food spoilage.

Inhibitory factors may also be produced in foods as a result of processing. Sugar syrups undergo a browning reaction during storage, leading to the formation of furfural and its derivatives, which have antimicrobial properties. Similarly, the oxidative chemical deterioration of foods during storage also leads to the formation of products with antimicrobial properties.

FOOD PROCESSING AND PRESERVATION

Most agricultural products used for human food cannot be used directly without some form of further processing. Even fresh produce such as apples, potatoes, tomatoes, and beans must be washed, trimmed, sorted, and packaged. Often, more extensive processing is needed to convert raw agricultural commodities such as beef cattle, wheat kernels, and sunflower seeds into meat, flour and bread, and cooking oil, respectively. Thus, the primary objective of food processing is to convert raw agricultural commodities into processed foods or food ingredients. The food ingredients (flour, sugar, oil, etc.) may then be used in a secondary food-processing operation to manufacture products that are ready for human consumption. Generally, food-processing operations, particularly the secondary operations, integrate food-processing with food preservation. The two objectives of preservation are firstly to prolong the shelf-life of the processed food or food ingredient in order that it may be stored for long periods of time and distributed efficiently over great distances, and secondly to assure the safety of processed foods. Therefore, the food-preservation aspect of food processing deals with the control of microorganisms in foods to enhance the quality of foods, retard their spoilage, and eliminate pathogens. Strategies to achieve these objectives are developed on the basis of our knowledge of microorganisms and their response to the various extrinsic and intrinsic environmental factors discussed earlier (see also Table 1).

Preservation methods can be subdivided into three broad groups:

1. Physical methods involving treatments that inhibit, destroy, or remove undesirable organisms without antimicrobial additives or products of microbial metabolism, e.g. drying, temperature, radiation, high hydrostatic pressure, etc. (Farkas 1997).
2. Addition of chemical preservatives and natural antimicrobial compounds or utilization of their natural presence (Davidson 1997).

Table 1 *Preservation methods used in food processing*

Process	How preservation is accomplished
Well-established processes	
Drying (reduced water activity)	Reducing water activity below the level required for microbial growth
Chilling	Lowering of temperature to a point at which microbial growth is retarded or inhibited
Freezing	Changing of water to a form that microbes are unable to utilize Freezing temperatures also inhibit enzymatic activity and thus growth
Heating 1. Heat sterilization in hermetically sealed containers	Inactivating all viable forms of bacteria followed by storing under conditions that will prevent recontamination and chemical degradation
Heating 2. Pasteurization and other heat processes that do not completely eliminate microorganisms	Reducing spoilage bacteria to a significantly low level so that the shelf-life of the product is extended; vegetative cells of pathogenic bacteria will also be eliminated
Irradiation (ionizing)	a Inactivating all viable bacteria (sterilization) b Eliminating pathogens or prolonging storage time (pasteurization)
Pickling, salting, and sugaring	Reducing the acidity and/or water activity to levels that inhibit the growth of microorganisms
Fermentation	Controlling environmental conditions in order to favor the growth of beneficial microorganisms and retarding or inhibiting the growth of spoilage and harmful microorganisms
New/developing processes	
Gases	
Controlled atmosphere storage	Reducing oxygen content and increasing carbon dioxide content combined with chilled storage inhibits growth of spoilage organisms
Modified atmosphere packaging (MAP)/vacuum packing (VP)	Using combinations of CO_2, N_2, and O_2 to sustain visual appearance and extend shelf-life (MAP) Reducing the pressure of air and thus the O_2 levels to inhibit growth of some organisms (VP) Combined with the use of low-permeability packaging film
Heat treatments:	
Microwaves	Reducing process times and energy and water usage in some food processing areas. Microwaves create internal cellular friction that produces heat. Thermal nonuniformity is a problem
Ohmic heating	Using direct electrical heating by passing an electric current through the product. The heat produced results in microbial death
High hydrostatic pressure	Applying high or ultrahigh hydrostatic pressure that transfers through food instantly and uniformly. Probably alters permeability of cell membranes, leading to cell leakage
Electric-field effects	Applying external pulsed electric field (PEF) causing increase in membrane permeability and leakage of cell contents
Magnetic-field effects	Applying oscillating magnetic field impulses induces changes in ionic drift across the plasma membrane. May affect growth and reproduction of bacteria
Chemical preservatives (traditionally propionates, sorbates, benzoates, nitrites)	Adding to food retards growth of, or kills, microorganisms. Mainly bacterio- or fungistatic so will not preserve food indefinitely
Naturally occurring compounds, e.g. lactoperoxidase system, avidin, lysozyme	Formation of antimicrobial compounds naturally by enzyme activity or interference with transport or causes cell-wall degradation

3 Use of biologically based systems and probiotic bacteria, e.g. use of lactic acid bacteria (Montville 1997b).

Food dehydration

The basic principle of food preservation by dehydration is that microbial growth is inhibited if the water available for growth is removed, i.e. if the a_w is reduced. Among bacteria, gram-negative species have the highest a_w requirements, while the gram-positive, nonsporing bacteria are less sensitive to reduced a_w. Some yeasts and molds have lower a_w requirements than the bacteria.

Removal of moisture from a food by natural sun drying is the oldest food-preservation technique used by humans. The method is still used to preserve large quantities of food in many developing countries, particularly those with tropical climates. In modern food

processing, however, dehydration is accomplished in more controlled environments such as by the use of hot air (spray drying, fluidized bed drying, etc.), by contact with a hot surface (roller drying), or by sublimation of ice from frozen food (freeze drying). In addition to the inhibition of growth of microorganisms, dehydration also reduces the bulkiness of foods, making them more economical to store and transport. However, drying techniques have disadvantages: the acceleration of undesirable chemical reactions that occur in foods during exposure to high temperatures. Some foods withstand dehydration better than others. Freeze drying has the least undesirable effects on the food quality, but it is the most expensive and energy-intensive drying process.

It is important to control carefully the handling of foods both before and after the drying operation in order to prevent microbial spoilage and/or the spread of organisms that would lead to food-borne illness. The ingredients for dried food products must be of good microbiological quality and they should be stored under conditions that minimize microbial growth before the drying process. Some foods, such as meats, require heat treatment prior to drying. Cooking will reduce the moisture content by approximately 20 percent and will also reduce the number of viable bacteria present. Similarly, the blanching of fruits and vegetables or the pasteurization of liquid egg before dehydration will also reduce the total number of viable bacteria in the final product.

The bacterial numbers in dehydrated foods are usually lower than those found in the food before drying due to the inactivation of some, but not all, of the organisms by the process itself. Of the surviving bacteria, a proportion may be sublethally injured. The extent of cell death and cell injury occurring during the process will depend on the type of food (fat content, pH, presence of inhibitors, previous treatment history), the organism (species, strain, physiological age, state of the cells, and the concentration of cells), and the type of drying process.

Freeze drying is the most gentle of freezing methods, so it is likely to result in the greatest survival of bacteria. Thus, for example, the total bacterial levels in freeze-dried shrimps may be reduced by only one or two $\log_{10}$ units. If the shrimp had 10^6 bacteria per gram at the start of the freeze-drying process, the level at the end is likely to be of the order of 10^4 organisms per gram. In general, gram-negative bacteria such as *Pseudomonas* spp., *Escherichia coli*, and *Vibrio* spp. do not survive freeze drying as well as gram-positive bacteria. Cells of *Lactobacillus acidophilus* that survived freeze drying revealed damage to both cell membrane and cell wall (Brennan et al. 1986); cellular DNA may also be damaged.

During spray drying, finely aerosolized liquid food is brought into contact with hot air to remove the moisture. Bacteria present are affected by the aerosolization, heating, and drying. There may be changes in the outer layer of the cell envelope, cytoplasmic constituents may leak out, and they may lack control of ion transport (Banwart 1987). Survival of bacteria in the spray-drying process is related to the outlet temperature (Miller et al. 1972; Thompson et al. 1978). During the spray drying of liquid egg, a lower temperature during drying together with rapid cooling of the product after drying enhance the qualities of the dried egg product. However, the same conditions are favorable for the survival of salmonellae. Over 99 percent of salmonellae cells are killed during spray drying if the inlet temperature is maintained at 121°C and the outlet temperature at 60°C (Banwart 1987). Although dried fish products can be produced with bacterial loads as low as 10^2 cfu/g, commercial dried fish may have bacterial loads as high as 10^7 cfu/g.

All the bacteria present in dried foods are not inactivated during the drying process so growth of the surviving viable and sublethally injured bacteria will be initiated when environmental conditions become appropriate. It is therefore critical to maintain product integrity by storing dried foods under the correct conditions. Dried foods must be stored in moisture-impermeable containers under low humidity to prevent unacceptable increases in the a_w. Salmonellae that survive the drying of eggs are inactivated during the storage of the dried product even at low temperatures. In dried albumen, salmonellae can be inactivated by storage of the product at 50–60°C for up to 7 days. Generally, bacteria survive better in dried foods under vacuum than in the presence of oxygen.

Chilling and freezing

Clarence Birdseye in the USA and Bill Heeney in Canada pioneered the frozen-food industry in North America. The rapid expansion in frozen-food technology paralleled the engineering developments in the refrigeration industry. Although frozen foods are expensive to process and store, food-quality deterioration is greatly diminished by freezing compared with dehydration and heat sterilization.

The obvious major difference between refrigerated food and frozen foods is the temperature at which the food is held. Another important difference is that the water in the refrigerated food is still available for growth of psychrotrophic spoilage bacteria and pathogens, i.e. it still has a high a_w.

The freezing of food makes water unavailable to microorganisms by immobilizing it in the form of ice. The low temperature at which frozen food must be held also deters the growth of microorganisms. Freezing is not effective in inactivating bacteria, although some reduction in bacterial content is achieved. Gram-negative bacteria are more susceptible than

gram-positive bacteria to freezing-induced injury and death.

Several factors influence the effectiveness of freezing as a preservation technique. These include the initial microbial load, the physical dimensions of the product to be frozen, the packaging material, the freezing rate, the time and temperature of subsequent storage, the degree of fluctuation in the storage temperature, and the time–temperature conditions used for thawing the product. The freezing process can be divided into three stages:

1. cooling down from the initial temperature of the food product to the temperature at which freezing begins
2. chilling at the latent heat plateau when there is no temperature change but phase change of water to ice occurs
3. further cooling to the final storage temperature.

The initial cooling of the food may inactivate a proportion of the microorganisms present due to cold shock; the decrease in temperature also reduces the growth rate of psychrotrophic bacteria. During the phase transition from water to ice, bacteria may be killed as a result of mechanical injury to the cell wall and cell membrane through the formation of ice. The growth of most cold-tolerant microorganisms is inhibited during further cooling until it ceases at about −8°C. Slow freezing to a final temperature of −10°C is more lethal to bacteria than rapid freezing to −20°C. During slow freezing, ice crystals form and soluble solids are concentrated that affect the stability of cellular proteins. However, there are also adverse effects on the texture and structural integrity of the food.

The freezing process may cause inactivation of 10–60 percent of the total bacterial population depending on the characteristics of the food and the organisms present and the conditions of freezing. Bacterial spores are unaffected by freezing; and neither are bacterial toxins inactivated. It is possible, therefore, for food intoxication to result from consumption of a frozen food in which the toxin had been produced by bacterial growth prior to the freezing process.

Food preservation by heat treatment

HEAT STERILIZATION IN HERMETICALLY SEALED CONTAINERS

The technology of the conventional canning process may be summarized as filling food into containers of metal, glass, or thermostable plastic, or into multilayered flexible pouches, sealing the containers, and then applying the heat treating process. This process is sometimes called 'terminal sterilization' and is designed to destroy large numbers of *Clostridium botulinum* spores should they be present. It also reduces the chances of survival of spores of spoilage organisms that are even more heat-resistant that *C. botulinum* spores. After heating, the containers must be cooled and handled in a manner that will assure container integrity and avoid contamination resulting from leakage.

The sterilization parameters vary greatly and depend on the food composition. One of the most important factors in determining the correct time–temperature combinations to use for a particular food is the pH of the product. Low-acid foods (pH >4.5) and foods of a_w >0.85 are likely to support the germination and outgrowth of spores of *C. botulinum* and the subsequent production of botulinal toxin. Specific regulations exist, therefore, in most countries for the processing of low-acid foods in hermetically sealed containers. The minimum heat treatment process for these foods is a 12*D* reduction process based on the thermal death times determined for each specific food product. The 12*D* reduction process is sufficient to reduce the probability of survival of *C. botulinum* spores to no more than 1 in 10^{12} cans (containers) processed.

The manufacturing process must include appropriate good manufacturing practices before the heat-sterilization step. The heat-sterilization step will not inactivate preformed heat-resistant toxins. Therefore, the quality of the ingredients must be evaluated and their handling must be designed to prevent bacterial contamination and/or growth. If acidification of the product is possible, i.e. it does not adversely influence the organoleptic characteristics of the food, it should be included because acidified food products require much less heat treatment than low-acid foods to assure their safety. Examples of food products that are amenable to acidification include artichoke hearts, pimientos, and many pickles.

The *D* and *z* values were described earlier in this chapter. Another term that figures in the calculation of heat treatment parameters for foods is the *F* value. This is defined as the time required to inactivate a given number of bacteria at a specified temperature and is usually 121°C for spores and 60°C for vegetative cells. *F*60 is the thermal death time (TDT) at 60°C and *F*121 the TDT at 121°C. In 1922, Esty and Meyer indicated that the *F*121 for *C. botulinum* spores was 2.78 min. Thus, the generally accepted minimum treatment for achieving a 12*D* reduction of *C. botulinum* spores is 3 min at 121°C. The thermal treatments applied to commercially canned cured meat products is well below this because the heat treatment is supplemented by the inhibitory effect of curing salts on the germination of spores of *C. botulinum*. Low-acid canned uncured meats are treated at an *F*121 of 6 min. Using the *D*, *F*, and *z* values, the rate of heat penetration in a specific food, the pH of the food, and the temperature of the pressurized container in which the cans of food are heated, a thermal process for that specific food can be calculated. Experimentally inoculated packs are processed at the calculated thermal process conditions to evaluate the adequacy of the process. *Clostridium sporogenes* strain PA3679 is the usual test organism in inoculated pack

studies because the spores of this non-toxigenic strain have a greater heat resistance than those of *C. botulinum*.

Container integrity is essential for maintaining the safety of commercially sterilized food products and for preventing spoilage. If the cans develop leaks, air or water may enter and introduce spoilage bacteria. In some instances the water may also carry pathogenic bacteria. Effective control measures to prevent post-processing contamination of cans include assuring the proper function of sealing equipment, disinfection of the water used to cool the containers after heat treatment, maintaining the containers in a dry condition as far as possible, and avoiding abuse of the containers.

Heat-sterilized foods in cans are subject to a number of different kinds of spoilage. Spoilage of the food may occur prior to processing or may be due to an inadequate heat treatment or to post-process contamination. Spoilage resulting from inadequate heat treatment is due to heat-resistant spores while inadequate cooling or a high product storage temperature leads to spoilage by thermophilic bacteria. Spoilage following water leakage into the can usually involves nonspore-forming bacteria, yeasts, and molds. The visual signs of spoilage may be bulging of the can at one or both ends, although the growth of the 'flat sour' organisms (*Bacillus coagulans* and *Bacillus stearothermophilus*) does not result in bulging of cans. Occasionally cans may swell due to the effect of acid foods on poorly coated internal can surfaces; the hydrogen produced causes the swelling.

ASEPTIC PACKAGING

In aseptic packaging, the food is sterilized, cooled, and transported under aseptic conditions to the presterilized container. The product is filled into the container and sealed under aseptic conditions. The end product is thus a hermetically sealed container holding a commercially sterile food that can be stored at ambient temperature for prolonged periods.

PASTEURIZATION

Heat treatment of foods below the temperatures needed for sterilization is termed pasteurization. Generally, this type of heat treatment is used to prolong the shelf-life of specific foods that do not withstand the sterilization process. Pasteurization is usually used to inactivate specific groups of bacteria, particularly pathogenic bacteria, and to reduce the numbers of spoilage bacteria. The process is most commonly applied to milk and dairy products, but in addition pasteurization is used to control microorganisms in liquid egg products, alcoholic beverages, smoked fish, and high-acid products (fruit juices, pickles, sauerkraut, and vinegar).

Food regulations in developed countries require that all liquid, frozen, and dried whole egg, yolk, and white be pasteurized to destroy viable salmonellae. However, the temperature and time requirements vary from country to country, ranging from 62°C for 150 s to 68°C for 180 s.

Irradiation

The process of food irradiation uses high-energy electromagnetic radiation (γ-rays) from radioactive isotopes such as caesium-137 or cobalt-60, or electrons from linear accelerators (Banwart 1987). These types of radiation are chosen because they produce the desired effects with respect to the food, they do not induce radioactivity in foods or packaging materials, and they are commercially economical (Farkas 1997). Radiation treatment of foods can have a range of effects depending on the level of the dose applied. Low doses of only 1–2 kGy can inhibit sprouting of vegetables (potatoes and onions) and delay ripening of fruits and vegetables. Higher doses (1–5 kGy) destroy parasites and pathogenic bacteria in meats, poultry, and fish, reduce viable bacteria in dried products such as spices and herbs, and destroy spoilage organisms to extend the shelf-life of perishable and semiperishable foods. The highest doses (20 kGy and above) can produce commercially sterilized foods (WHO 1988).

The ionizing radiation doses required for the destruction of microorganisms in foods will vary widely and are dependent on the type and numbers of cells, type of substrate or suspending medium, moisture content, oxygen tension, temperature, or the presence of protective compounds. Ionizing radiation causes cell death directly by attacking the DNA and causing single- or double-strand breaks or indirectly by the primary water radicals $^{\cdot}H$, $^{\cdot}OH$, and e_{aq}^{-}. The most important in DNA damage is the OH radical: those formed in the hydration layer around the DNA molecule are responsible for 90 percent of the DNA damage (Farkas 1997). The resistance of an organism to ionizing radiation is, in part, dependent on the capacity of the organism to repair the damage to its DNA. Most can repair single-stranded breaks, but the more sensitive organisms (e.g. *E. coli*) cannot repair double-stranded breaks. Bacterial spores are highly resistant to ionizing radiations due to the low water content of the spore protoplast; reported *D* values (decimal reduction dose) for spores of *C. botulinum* type A are 2.18–2.35 kGy in cured ham. Generally, gram-positive bacteria are more resistant than gram-negative bacteria: *Pseudomonas* spp., *E. coli* and *Proteus* spp. are very sensitive to ionizing radiation. Microbial radiation resistance in frozen foods is about two- to three-fold higher than at ambient temperature due to immobilization of the free radicals.

Although ionizing radiation has the potential to enhance the quality of our food supply and to increase its safety, it has not gained wide acceptance for use in

food processing because of the public's fear of anything 'nuclear.' However, its use is permitted in a number of countries with doses up to 10 kGy for extension of refrigerated shelf-life and inactivation of nonsporing pathogens for poultry, pork, and sausages (WHO 1994). Ionizing radiation treatment has been approved in the USA for fruits, vegetables, spices, poultry, and pork, but it is not commercially used. In the UK, regulations (Statutory Instrument 1990a, b) came into force in 1991 introducing food labeling and a licensing system for food-irradiation plants including cover of all irradiated food from the processing plant to the supermarket shelf. The regulations state that food must be of good microbiological quality before it is irradiated.

Hurdle technology

Manipulation of the effect of a number of environmental factors together to inhibit microbial growth, for example pH, salt concentration, and temperature, is the basis of multiple 'hurdle technology.' Instead of setting one environmental factor to the extreme limit for growth, hurdle technology 'de-optimizes' a variety of factors. Whereas growth of food-borne pathogens is limited at a water activity of 0.085 or a pH of 4.6, similar inhibition may be obtained at pH 5.2 and a_w 0.92. In maintaining intracellular pH and accumulating compatible solutes in the low a_w, i.e. in maintaining homeostasis, the cells channel energy needed for biosynthesis. Thus their growth is inhibited. If this energy demand exceeds the energy producing capacity of the cells, they die (Montville 1997b).

BACTERIOLOGY OF SPECIFIC FOOD COMMODITIES

Meats

Reviews of the microbiology of meat and meat products are given by Davies and Board 1998 and ICMSF 1998.

Meat can be defined as the flesh of animals used as food and now tends to exclude poultry and fish. It is primarily the skeletal musculature tissue but commonly includes various organs that are regarded as edible (this may vary between countries). Thus meat comprises the musculature of 'red-meat' mammals, most commonly bovines, ovines, and porcines. Depending upon country and religion, the meat supply may be beef, pig, lamb, goat, camel, buffalo, deer, or horse. Within a species, the proportions of muscle, fat, and connective tissues can vary from one breed to another, and with age and rearing conditions, particularly the energy content and quantity of feed. Young animals deposit mainly protein; fat deposition increases with physiological age at the expense of protein. The amount of connective tissue varies in different muscles: those with little are tender to eat even after mild cooking (e.g. grilling), whereas those containing larger amounts are considered tough unless subjected to more severe and prolonged cooking to gelatinize the collagen. With increasing age the collagen of the musculature becomes more heavily cross-linked. A proportion of cross-links are heat-stable, causing meat from older animals to be less tender unless cooked thoroughly (e.g. by cooking under pressure). Some fat is necessary for maximum eating quality, particularly for succulence, and for the full development of the cooked meat flavor, but there is sufficient intramuscular fat for these purposes in all but unusually lean animals.

The contractile elements of the muscle, the myofibrils, also contribute to meat texture. The ultimate pH of meat is proportional to the amount of lactic acid formed during postmortem glycolysis, which is dependent on the amount of glycogen in the muscles at death. In a muscle rested before slaughter almost all the glycogen is converted to lactic acid (a concentration of approximately 1 percent lactic acid corresponds to pH 5.5), giving a muscle of normal appearance and texture. Low glycogen levels result in muscle of high ultimate pH. Such muscles often appear dark and, in addition, are firm and dry (DFD). In stress-susceptible animals, especially certain cross-bred pigs, excitement or stress before slaughter causes rapid conversion of muscle glycogen to lactic acid, resulting in a low pH before the muscles have cooled. This denatures sarcoplasmic protein and reduces the water-holding capacity of the tissue, giving a pale, soft, exudative (PSE) condition.

Meat comprises contractile myofibrillar elements and soluble sarcoplasmic proteins with up to one-quarter by weight connective tissue and as much as one-third fat. The presence of connective tissue seems unimportant microbiologically, but the relevant properties of the fat differ considerably from those of the muscle.

Intensive rearing of cattle, sheep, and pigs has resulted in a large international trade in meat. This international trade developed initially by transporting carcasses but, in transporting prime cuts, has become predominantly an intermediate stage between the carcass and the retail-sized portion. Muscle contains approximately 75 percent water and a variety of substrates for microbial growth, including carbohydrates, amino acids, lactic acid (Dainty et al. 1983; Lawrie 1985; ICMSF 1998). The a_w of meat is high, approximately 0.99, which makes it a suitable growth medium for most microbes. Only if most of the water is removed (e.g. by drying) will the a_w be reduced sufficiently to affect microbial growth.

Within the normal pH range of meat (approximately 5.4–7.0), values approaching 5.4 are less favorable to the growth of many of the important bacteria; the lower pH values are particularly important in maintaining the microbiological stability and safety of cured meat products.

The redox potential of meat has been claimed to be important in determining the nature of microbial spoilage, but its exact role in permitting or preventing the growth of microbes has not been fully elucidated, probably due to unresolved experimental problems of controlling and measuring redox potential. Tissue respiration of oxygen continues after death; because the supply of oxygen via the blood has ceased, the oxygen content and redox potential of muscle fall, leading to anaerobic conditions even at only a few millimeters below the surface. However, microbial growth in the deep musculature is rare, except where refrigeration is inadequate (Gill 1979; Roberts and Mead 1986).

MICROBIOLOGY AT PRODUCTION

In industrially developed countries, the change in eating patterns and in technological developments in animal husbandry, meat production, food processing, and preservation have led to an enormous increase in the range of meat products available. Novel methods have also been developed to maximize the recovery of meat from the carcass. Many meat products spend weeks, or even months, in distribution and storage before being offered for sale alongside the fresh product. The lower the number of microbes present initially, the longer the shelf-life of the perishable product providing storage conditions are correctly controlled.

Bacterial contamination of carcass surfaces can occur during slaughter and dressing procedures from a variety of sources, such as the hide, intestinal contents, contact surfaces, and handling by workers. Fecal contamination, routinely experienced during the normal slaughtering process, contributes high levels of bacteria (typically >10 000 cfu/cm^2). Good sanitary practices during slaughter, trimming of visible fecal contamination, and washing of carcasses with or without sanitizers are the approaches usually employed to minimize the contamination of carcasses (Dickson and Anderson 1992). The addition of chlorine, acetic acid, or lactic acid to the wash water reduces the bacterial load on the surface but does not significantly increase the shelf-life or completely eliminate pathogenic bacteria (Prasai et al. 1995). Higher water pressures (>13.8 bar) during spray washing are more effective in reducing bacterial loads on the surfaces of carcasses. Also, a two-step washing procedure with a low-pressure, hot-water (72°C) wash followed by a high-pressure, low-temperature (30°C) wash has been reported to be effective in reducing surface bacterial contamination (Gorman et al. 1995; Dorsa et al. 1996).

The microbial flora of the surface of freshly slaughtered carcasses, usually in the range of 10^1–10^3 cfu/cm^2, is primarily mesophilic, having originated in the alimentary tract and on the external surfaces of the live animal. Contamination from the slaughtering environment is also predominantly by mesophiles; psychrotrophs originating from soil and water are also present, but at much lower levels. Usually carcasses are cut into smaller portions in refrigerated work rooms, where most bacteria on the surfaces of processing equipment are psychrotrophic in nature. During refrigerated holding, the microflora of meats begins to shift towards psychrotrophs of the *Pseudomonas–Acinetobacter–Moraxella* group. These organisms are ultimately responsible for the spoilage of refrigerated meats. The spoilage is primarily a surface phenomenon resulting in the formation of slime and off-odor. The shelf-life of refrigerated meats is extended by controlling the factors contributing to the growth of psychrotrophs, namely moisture levels at the surface of the meat, initial load of psychrotrophs, pH, oxygen tension, and temperature. Wrapping meat in oxygen-impermeable films retards surface growth and favors the growth of microaerophilic bacteria, such as lactobacilli and *Brochothrix thermosphacta*, over the *Pseudomonas–Acinetobacter–Moraxella* group. The bacterial load of chilled meats at the retail market may bear no relationship to the load at the processing plant because the psychrotrophs continue to grow during transportation and storage.

The microbiology of meat other than the musculature is relatively poorly researched, with few publications on the microbiology of edible offal such as heart, liver and kidney, despite their increasing use in meat products. The initial contamination of offal is often higher and there is more likely to be contamination with potential pathogens than in skeletal muscle. The chilling process is often done poorly and offal may be packed while still warm before transport to chillers or freezers. Slow cooling results in significant increase in microbial numbers. Contacting surfaces remain moist and may be an important factor in the lower incidence of *Campylobacter jejuni* (*C. coli*) on retail carcass meats compared with offal.

Coliforms, *E. coli*, enterococci, *Campylobacter* spp., *Staphylococcus aureus*, *Clostridium perfringens*, *L. monocytogenes*, *Y. enterocolitica*, and *Salmonella* spp. are often present on fresh tissues because the slaughtering process does not currently include a bactericidal step. The enterohemorrhagic or verotoxigenic strain (EHEC or VTEC) *E. coli* O157:H7 is a recently emerged pathogen that has caused major food-borne disease outbreaks, with undercooked ground beef, particularly as hamburgers, commonly implicated. Enterohemorrhagic *E. coli*, of which *E. coli* O157:H7 is the most frequently encountered serotype (although other serotypes including O26:H11, O103, O104, and O111 have been implicated in cases of bloody diarrhea), have the ability to adhere intimately to human intestinal cells by an attaching and effacing mechanism and produce one or more phage-encoded Shiga-like toxins. In addition to hemorrhagic colitis, *E. coli* O157:H7 may cause serious life-threatening clinical diseases such as hemolytic–uremic syndrome and thrombotic thrombocytopenic purpura

in children and the elderly. *E. coli* O157:H7 has a low infectious dose (estimated to be of the order of 10–100 cfu) and can be spread by person-to-person contact in day-care settings (Griffin 1995).

Cattle are primary reservoirs of *E. coli* O157:H7. The carcass may become contaminated during slaughter and dressing operations. Meat from a single carcass may contaminate large batches of ground beef because of the tendency to mix and blend meat from several animals from different farms during production. Witholding feed, as is normally done before shipping or slaughter, to reduce fecal contamination, can lead to intestinal outgrowth and colonization by both *E. coli* O157:H7 and salmonellae due to the disruption of normal fermentation in the rumen (Rasmussen et al. 1993). Shedding of salmonellae and *E. coli* O157:H7 by feedlot cattle after shipping appears to be directly related to their length of stay in pens (National Animal Health Monitoring System 1995). *E. coli* O157:H7 have also been isolated from lamb products (Chapman et al. 2000).

Few studies have been conducted on the prevalence of pathogenic bacteria at different locations on pork carcasses. Epling et al. (1993) reported that the prevalence of *Salmonella* spp. and *Campylobacter* spp. on shoulder and ham surfaces was as great or greater after 24 h of chilling at 4°C when compared with the numbers at slaughter. Swine are the main reservoir of *Y. enterocolitica* where the organism appears to have a predilection for the tonsils and tongue. Careful removal is needed to prevent carcass contamination. Outbreaks of *Y. enterocolitica* infections have occurred in the USA caused by raw chitterlings (pork intestines); adults handle the chitterlings and then transmit the organisms to their infants and children (Lee et al. 1990). Consumption of undercooked pork in some European countries has also been implicated (Tauxe et al. 1987; Ostroff et al. 1994).

Salmonellae, campylobacters, motile aeromonads, and *Y. enterocolitica* have been isolated from lamb carcasses sampled at commercial abattoirs (Sierra et al. 1995; Chapman et al. 1997). Horizontal transmission of infection and persistent shedding (for 2 weeks) of *E. coli* O157:H7 were demonstrated after oral inoculations of lambs with 10^5–10^9 cfu of the organism. As observed in cattle, withholding of feed from lambs causes increased shedding of *E. coli* O157:H7 (Kudva et al. 1995). In nature, however, sheep appear to harbor *E. coli* O157:H7 and other enterohemorrhagic *E. coli*.

Irradiation treatment has the potential to reduce contamination of meat with pathogens signficantly, while the shelf-life of the meats is also prolonged. The effects will vary with animal species and type of packaging. Irradiation of ground beef in oxygen-permeable packaging with 1 kGy of γ-rays will reduce the numbers of *Salmonella* spp. *Y. enterocolitica*, and *Campylobacter* spp. but may cause objectionable odor, color, and flavor changes, but there are no odor problems with pork. In zvacuum-packaged beef a 2-kGy treatment at 25°C eliminates pseudomonads, enteric bacteria, and enterococci and causes fewer odor and flavor problems (Lee et al. 1996).

SHELF-STABLE RAW SALTED AND SALT-CURED MEATS

Salting of foods is one of the oldest methods of food preservation. Salting of meats was extremely common practice before the introduction of home refrigeration units and raw salted products continue to be produced and sold, especially in predominantly agricultural areas. Salt pork, dry cured bacon, and country-cured hams are still produced in volume. In the process of dry curing, pieces of meat are coated with salt and stored in bins below 10°C. Special care must be taken to ensure that the salt is applied to all folds and crevices in the meat to prevent bacterial growth leading to spoilage. Water is released from the meat by the action of the salt. At intervals, the pieces of meat are recoated with salt. At the end of the salting period, the product contains high levels of salt and is racked and held at ambient temperature until the surface dries. Finally, the meat is rubbed with salt and spices, netted, and sold. With such a high salt content, the product does not require refrigeration. The main microbial flora of these products after curing and drying are members of the Micrococcaceae, with lactic acid bacteria and yeasts as minor components. *Staphylococcus aureus* can be found in low numbers. Products sold as country-cured hams or bacon are similarly processed, except that sodium nitrite is used in combination with a lower level of sodium chloride. The product is hung to dry at ambient temperature for 35–140 days before removal for sale as a shelf-stable product. During the curing process, the salts penetrate and equilibrate in the tissue. The combined action of reduced water activity and the preservative effect of nitrite inhibit or inactivate the bacteria that cause spoilage or human illness. During the subsequent drying period, salt-tolerant enterococci and micrococci begin to grow and predominate; they also suppress the growth of the undesirable bacteria.

PERISHABLE RAW SALTED AND SALT-CURED MEAT

Fresh sausages are the most commonly encountered perishable raw salted meat products, but the salt content used is not sufficient to make the product shelf-stable. The method of packaging is the major factor that determines the predominant spoilage flora. Fresh sausage sold in bulk on trays or stuffed in casings is highly perishable, with a shelf-life of just a few days; psychrotrophic pseudomonads are the primary spoilage organisms. The shelf-life of fresh sausage can be extended to 1 or more weeks by wrapping the sausage in oxygen-impermeable film. The restriction of oxygen favors the growth of lactic-acid-producing bacteria, which impart a tangy

flavor to the product. Large quantities of perishable meat products are cured with salt, sodium nitrite and nitrate, ascorbic acid, and other flavoring materials. Most of these are cooked before shipping from the processing plants, although some are sold with little or no heat treatment and must be cooked before eating. Some raw cured meats are heated or smoked to different degrees to produce dry surfaces and a smoked flavor; these procedures may also extend their shelf-life by reducing the numbers of bacteria.

PERISHABLE COOKED UNCURED MEATS

Most pork products are given a heat treatment sufficient to destroy all nonspore-forming bacteria present, with only spores at a level of approximately 10^2/g surviving. Beef products are usually processed at a lower temperature sufficient to destroy nonspore-forming pathogens, but not necessarily some of the thermoduric bacteria such as enterococci. Microbial levels in the final product depend on the initial microbial load and types of bacteria, the cooking time and temperature, and the storage temperature and time. In meats that are only lightly cooked, i.e. where the center is still essentially raw ('rare'), the center temperature may not have been sufficient to eliminate even relatively heat-sensitive organisms, including those of concern such as *Salmonella* spp., *Campylobacter* spp., *E. coli*, and *Y. enterocolitica*. The color change in meat from red to gray associated with cooking occurs at temperatures approaching 60°C. Freshly cooked meat products may contain only about 10^2 cfu/g, but post-cooking handling and packaging may result in contamination with spoilage bacteria and pathogens. Cooked uncured meat products are ideal substrates for microbial growth. They are highly nutritious, they have favorable pH and a_w, and the cooking process reduces or destroys the competing microflora. Many of these foods are frozen for shipment and distribution. However, if they are held at above freezing point for 1 to several days, spoilage will occur due to the growth of enterococci, pseudomonads, lactic acid bacteria, and other psychrotrophs.

PERISHABLE COOKED CURED MEATS

Precooked cured meats include a variety of popular luncheon meats including frankfurters, bologna, and ham. The heating process applied destroys the normal meat microflora apart from spores and possibly thermoduric bacteria. During chilling, holding, and packaging, exposed surfaces may become contaminated. Salt and nitrite used in the curing process may inhibit the growth of any surviving or contaminating organisms. On prolonged storage at refrigeration temperatures, spoilage with slime formation occurs primarily due to enterococci, micrococci, lactic acid bacteria, and yeasts. If oxygen-impermeable film is used for wrapping the product, spoilage is primarily due to lactic acid bacteria. *Brochothrix thermosphacta* can form a significant part of the spoilage flora of vacuum-packed products, but growth of this organism in relation to lactic acid bacteria is reduced by a lower nitrite concentration, lower pH, and lower packaging film permeability.

CANNED CURED MEATS

Canned cured meats may be shelf-stable or perishable. Shelf-stable products include canned wieners, corned beef, frankfurters, meat spreads, luncheon meat, small canned hams, canned sausages covered with oil, and vinegar-pickled meats. Stability and safety of these products depend on the combined effect of heating and other factors that will inhibit the growth of surviving spores (e.g. salt, nitrite, a_w) and a sealed container to prevent post-process contamination. Some of these products, e.g. canned wieners and frankfurters, are given a 'botulinum cook' under the regulations applicable for low-acid foods packed in hermetically sealed containers. Canned sausages covered in oil do not spoil unless the a_w is higher than recommended (ranging from 0.86 to 0.92, depending on the product) and the vacuum seal is broken. Pickled pigs' feet and pickled sausages are immersed in vinegar-brine and are preserved by low pH, acetic acid, little or no fermentable sugar remaining in the tissue, and/or an airtight package.

Perishable canned cured meats made from pork must be stored refrigerated. They contain nitrite and salt at levels used to prepare shelf-stable products, but their heat treatment is inadequate to inactivate spores. Perishable canned cured meats may be shelf-stable for up to 3 years if properly processed and refrigerated. Spoilage is primarily due to psychrotrophic, thermoduric nonspore-forming bacteria (e.g. enterococci, *Lactobacillus viridescens*) that were present at abnormally high levels or survived an inadequate processing.

FERMENTED AND ACIDULATED SAUSAGES

Fermented products such as Thuringer, summer sausage, pepperoni, Lebanon bologna, Genoa salami, and cervelat depend on lactic fermentation and low water activity for preservation. At the end of fermentation, lactic acid bacteria exceed 10^8 cfu/g. Previously, the primary safety concern for these products was the production of staphylococcal enterotoxins. Improved industrial practices have greatly reduced or eliminated this problem. However, these practices are not adequate to control acid-tolerant bacteria, such as verotoxigenic *E. coli*, which can survive the fermentation and drying process (Glass et al. 1992), as evidenced by outbreaks due both to serotypes O157:H7 (Anon 1995) and O111 (Paton et al. 1996; Desmarchelier 1997).

Use of starter organisms in the fermentation of these meat products allows a greater level of control of the final product. A naturally fermented unheated meat product was the vehicle of infection for an outbreak of

Salmonella serotype Typhimurium infection in the UK when the a_w and pH was insufficient to prevent survival of the organisms present in the raw meat (Cowden et al. 1989).

Spoilage of naturally fermented sausage is due primarily to fungi; *Penicillium* spp. are dominant but species belonging to *Aspergillus* and *Scopulariopsis* are also present (Anderson 1995). Acidulated products are produced by adding acidulants (citric acid, lactic acid, glucono-δ-lactone) to the meat. Because no fermentation is involved, the products require a higher heat treatment than fermented sausages.

DRIED MEATS

Commercial dried meat products, such as beef jerky, include a cooking step that destroys normal vegetative cells and a rapid drying step that reduces the water activity to a level at which microorganisms of concern cannot grow. Thus such products are microbiologically stable at ambient temperature. Some traditionally dried meats such as biltong may include a salting step and a lesser heat process during drying. In such products bacterial numbers, especially micrococci, yeasts, and molds, may be high.

Poultry and poultry products

Poultry production and processing range from small farm or yard operations in developing countries to almost exclusively large-scale, highly integrated, and automated operations in developed countries. Poultry meat is now one of the cheapest forms of meat and consumption has increased dramatically in the past 50 years. It is estimated that 38 million metric tons are produced annually (ICMSF 1998), of which 80 percent is chicken. To meet increased demand, production and processing operations have been integrated and centralized. Intensive production integrates breeding, hatching, rearing, feeding, and slaughtering. The large numbers and close proximity of birds make control of hygiene difficult, and introduction of pathogens into a flock has very serious consequences, although it also provides the opportunity for preventive medicine, efficiencies of sale, and genetic improvements.

In industrialized countries, the microbiological condition of raw poultry at retail sale is a reflection of hygienic measures taken at the breeder farm, at the hatchery, through the growing period, and throughout slaughtering and the subsequent handling and storage.

MICROBIOLOGY AT PRODUCTION

Although several pathogenic bacteria have been associated with outbreaks or sporadic disease caused by poultry products, *Salmonella* spp., *C. jejuni*, and *C. coli* are considered predominant poultry-associated pathogens (Bryan and Doyle 1995), although a thorough examination of raw poultry may yield many other pathogens, including *L. monocytogenes*, *S. aureus*, *E. coli*, and *C. perfringens*. Microbial contamination of the egg can occur during its development in the ovary or later by penetration of the shell after laying. *Salmonella* serotype Enteritidis can infect the ovaries or oviduct of apparently healthy birds, leading to internal contamination of eggs (Coyle and Palmer 1988; Humphrey 1994), but the frequency and extent of such infection are uncertain and the laying of contaminated eggs may be sporadic (Humphrey et al. 1989). Salmonellae may also be present on the egg shell due to fecal contamination and may penetrate the shell during cooling after laying. Chicks and poults may become contaminated from the shell during or after hatching. In contrast, eggs derived from hens that were fecal excreters of *C. jejuni* failed to yield this organism from either the homogenates of yolks and albumen or shell surfaces (Bryan and Doyle 1995). Thus, *C. jejuni* is unlikely to be transmitted by eggs.

Poultry-improvement programs for the genus *Salmonella* identify infected breeder flocks (USDA 1990; EC 1992; Statutory Instrument 1989a, b, 1993a). Infections may be controlled by treatment with antibiotics or by slaughter, particularly if serotype Typhimurium or Enteritidis is identified, and more recently by vaccination programs. Breeder stock should be given feed made free from salmonellae by heating (Williams 1981a, b) or by treatment with organic acids (Hinton and Linton 1988). Other sources of salmonellae, such as rodents and wild birds, should be excluded. New stock should be disease-free and should be quarantined before being mixed with other birds. Contamination of the shell has been reduced by the use of cages and automated feeding systems, belt collection of eggs, and manure removal equipment. Other improvements include in-line washers, mass egg candling, and automated packing equipment (Bell 1995).

The development of the microflora of the live bird is reviewed by Grau (1986) and the origins and composition of the contamination of the carcass by Mead (1980, 1982) and the ICMSF (1998).

Within 24 h of oral inoculation with *C. jejuni* a single chick can contaminate 70–100 percent of fellow chicks held in transport boxes. *C. jejuni* colonizes primarily the lower intestinal tract of chicks, principally the cecum, large intestine, and cloaca. *C. jejuni* are chemotactically attracted to mucin; highly active flagella assist them to move into mucus-filled crypts, where the organisms establish themselves. The colonization of poultry by *C. jejuni* and intervention strategies for prevention of the colonization have been described by Stern (1992).

Salmonellae have been isolated from the yolk sacs of chicks, setters, and chick belts sampled at hatcheries. The cecum is considered the principal site of colonization. Chicks and poults are readily infected with salmonellae from contaminated feeds or drinking water, or by

pecking in contaminated soil or litter; infected chicks may shed as many as 10^8 cfu/g of feces. As they mature, the birds tend to shed fewer salmonellae and fewer birds remain infected unless salmonellae are continuously reintroduced through contaminated feed or other environmental sources (Bryan and Doyle 1995).

Live poultry showing no symptoms of illness may carry and excrete low numbers of salmonellae (symptomless excreters), thereby contaminating the environment. Litter becomes contaminated with droppings, feathers, and soil; with time and use, the water activity, ammonia content, and pH of the litter become unfavorable for the survival of salmonellae.

Transmission of microorganisms between birds continues during transportation from the farm to the slaughtering facilities. Intestinal colonization is the most important factor contributing to carcass contamination. Feed is usually withheld for several hours before transport to reduce the amount of fecal droppings. However, considerable defecation still occurs during transit. Birds stand, walk, and fall on the fecal material present on crates and truck beds, thus allowing fecal bacteria including pathogens to be transferred to the feathers and skin. Stress caused by gathering, transporting, crowding, and holding in crates greatly increases the bacterial load; the levels of *C. jejuni* on unprocessed chicken carcasses increase by 3–4 logs after transportation from the farm (Stern et al. 1995). Cleaning and disinfection of cages and truck beds after each use minimizes the transfer of contamination between flocks.

In countries where birds are killed after purchase by the consumer, the hygienic conditions of preparation may be primitive but the brief time between slaughter and consumption of the cooked product usually prevents most microbiological problems.

Most modern poultry-slaughter operations are largely automated with electrical stunning and mechanical severing of the blood vessels in the neck. Most dressing and processing steps are accomplished with minimal handling. Broilers entering the slaughtering plant are frequently contaminated with *Salmonella* spp., *C. jejuni*, *C. coli*, and *E. coli* (Kotula and Pandya 1995; ICMSF 1998). Dressing procedures are designed to reduce contamination, and controls such as chlorinated rinses of equipment are aimed at minimizing cross-contamination between carcasses.

Carcasses are scalded to facilitate the removal of feathers, the most common method being immersion scalding. Microbes from the exterior surface of the birds and from the intestinal and respiratory tracts are continually released into the scald water. Hot-water sprays, steam, and simultaneous hot-water spray and plucking are alternatives to immersion scalding but are rarely used. Build-up of contamination is prevented by controlled overflow and countercurrent replacement of scald water and by the lethal effects of the high temperature. High scalding temperatures (greater than 60°C) are more effective in eliminating vegetative microorganisms than are the lower temperatures commonly used (50°C), but the lower temperatures are sometimes preferred because they cause less damage to the appearance of the carcass (Slavik et al. 1995).

Plucking (picking) to remove feathers is another process that spreads bacterial contamination between carcasses. Effective cleaning of defeathering machines (pluckers) and the plucking fingers is an important step to prevent transmission of contaminants between carcasses. Spray washing of carcasses after plucking removes only loosely attached contaminating material together with a proportion of the microbial flora, many microbes remaining bound to the surface tissues. Removal of the viscera may lead to further spread of contamination over the carcasses, particularly with intestinal organisms, commonly as a consequence of gut breakage. With time, bacteria become attached more firmly to the skin, so post-evisceration washing should be undertaken as soon as possible and should include washing of both the body cavity and the outer surface of the carcass. Reduction of the levels of bacteria is usually limited to about 90 percent; the remaining bacteria are either entrapped within or adhere to skin and flesh surfaces.

Carcass chilling delays the growth of spoilage bacteria and prevents the growth of food-borne pathogens. Continuous countercurrent immersion chilling, with or without ice, generally decreases microbial numbers on carcasses and minimizes cross-contamination between the carcasses if it is correctly controlled and the water is properly chlorinated. Air chilling using various combinations of temperature, humidity, and time may also be employed. During such dry chilling contact between carcasses is less; however, there is also less dilution of pathogens. Campylobacters, however, are sensitive to drying; therefore, a reduction in the numbers of *C. jejuni* may be expected.

The number of microbes detected on the finished poultry carcass varies with both the site sampled and the method of sampling. Excised skin samples will yield a microflora of approximately 10^3–10^5 aerobic mesophiles/cm, 10^1–10^3 psychrotrophs/cm, and 10^3–10^4 Enterobacteriaceae/cm (Grau 1986; ICMSF 1998). The main causes of spoilage when carcasses are stored in air at approximately 1°C are pseudomonads, *Shewanella putrefaciens*, *Acinetobacter* spp., and *Moraxella* spp. At higher temperatures atypical lactobacilli and *Serratia liquefaciens* (formerly *Enterobacter liquefaciens*) may predominate. *S. putrefaciens* and acinetobacters grow better in leg muscle (pH 6–6.7) than in breast muscle (pH 5.7–5.9), whereas *Pseudomonas* spp. grow well in either. If carcasses are wrapped in oxygen-impermeable films, then *Alteromonas* spp., *B. thermosphacta*, and atypical lactobacilli are the principal causes of spoilage. Atmospheric concentrations of 10–25 percent carbon dioxide delay the growth of pseudomonads and other spoilage

organisms when the product is held below approximately 4°C. The increase in shelf-life is approximately proportional to the concentration of carbon dioxide up to 25 percent, at which point discoloration occurs.

Irradiation of poultry has been extensively investigated with respect to its effects on spoilage and pathogenic bacteria. Treatment of whole eviscerated chickens with 2.5 kGy of γ-rays resulted in an increase in the shelf-life from 10 to 40 days. The same dosage reduces the numbers of salmonellae, campylobacters, and *Y. enterocolitica* by 2.5–4 $\log_{10}$ units. Irradiation is less effective for killing salmonellae on frozen carcasses than on chilled. The US Department of Agriculture issued regulations in 1992 permitting the irradiation of packaged, fresh or frozen poultry and poultry products, including ground and mechanically separated poultry products at a dosage level of 1.5–3.0 kGy (Lee et al. 1996). The UK regulations of 1990 (Statutory Instrument 1990b) permit the sale of specified types of irradiated food (seven types including poultry) where irradiation has taken place under licence. The regulations also specify the limits of overall dose of ionizing radiation which apply.

Eggs and egg products

Since the pioneering study of egg microbiology in 1873 by Gayon, an associate of Louis Pasteur, a number of investigators have shown that the hen's egg is endowed with many chemical and physical defences against microorganisms. The shell and its membranes provide a physical barrier to penetration in addition to possessing bactericidal activity (Board et al. 1994), while the antimicrobial components of the egg white make it an antagonistic medium for microbial growth. The yolk is an excellent medium for bacterial growth; either liquid whole egg or egg yolk permits rapid growth of bacteria if the temperature is appropriate.

Bacterial contamination of eggs can occur at different stages ranging from production to storage, processing, distribution, and preparation (Board and Fuller 1994; ICMSF 1998). Transovarian or 'vertical' transmission takes place when infection occurs during formation of the egg in the hen's ovaries. Horizontal transmission refers to contamination when eggs are exposed to microorganisms that subsequently penetrate the shell (Fajardo et al. 1995). *Salmonella* serotype Enteritidis (*S. enteritidis*) emerged as a transovarian pathogen in 1985 (St Louis et al. 1988) and has caused many egg-related outbreaks of salmonellosis in North America and Europe (Mishu et al. 1991, 1994; ACMSF 1993a, 2001; Humphrey 1994). Phage types (PT) 4, 8, and 13a are the three most frequently isolated phage types of *S. enteritidis*. PT4 causes more severe disease than the other two phage types and during the 1990s essentially displaced the other two phage types in Europe. In the USA the predominant types were PT8 and PT13a, and PT4 did not appear until 1993 (Mason 1994). *S. enteritidis* contamination of eggs may occur by both vertical and horizontal transmission. The frequency of salmonella contamination of shell eggs produced in the UK in 1991 was reported just under 1 percent (de Louvois 1993a, b). Despite extensive and costly measures adopted by the industry to address the problem, this had not improved by 1995 (ACMSF 2001).

The washing of shell eggs does not assure the total removal of bacteria. The temperature of the wash water is not high enough to effect any killing of bacteria, but its pH may affect bacterial growth. Gram-negative bacteria on the egg shell can be resistant to detergents in the wash water; even chlorine rinses of shell eggs may not prevent contamination by salmonellae.

Success in preserving safe and wholesome eggs depends on three steps:

1. preventing spoilage organisms from entering the egg
2. preventing pathogenic bacteria from entering the egg
3. maintaining egg quality by preventing the loss of CO_2 and water.

Refrigeration is the most popular method of preserving egg quality. The low storage temperature retards loss of CO_2 and slows the growth of pathogenic bacteria. Previously the recommended storage temperature for shell eggs in the USA was 13°C. However, *S. enteritidis* grows in eggs stored at 13°C but not at 7°C. Therefore, the 1995 Food Code currently requires the receipt of shell eggs at 5°C (Food Code 1995). The reported decline of infections in the USA (ACMSF 2001) may be explained by the interventions introduced in the egg and or meat/poultry industry (Department of Health and Human Services 1999). In Europe the marketing of eggs is controlled by EC Directives (EC 1989). These allow on-farm storage of eggs for a limited time at ambient temperature not exceeding 18°C before delivery. At retail the directives prohibit storage below 5°C to ensure quality standards are maintained. In the UK, it is recommended that eggs are stored in home refrigeration at less than 8°C (ACMSF 1993b).

Liquid whole egg is a blend of egg albumen and yolk and contains 23–25 percent solids. Liquid egg is used in bakeries, candy makers, and many catering establishments. Contamination of liquid egg products is generally due to contaminants within the shell egg, the cleanliness of the shells on breaking out, or the presence of small egg shell particles that often drop into the liquid. At the time of laying, the egg temperature is above 41°C. The cooling and drying of the moist egg surface, especially the cuticle, and the continued cooling of the contents result in a negative pressure inside the egg. Thus bacterial contaminants are pulled from the surface of the shell through the pores. Shell contaminants therefore make an important contribution to the microbial flora of the liquid egg; washing eggs in water is an effective

method of removing shell contaminants. Spoilage bacteria occurring in liquid eggs are mainly gram-negative rods (*P. fluorescens*, *Alcaligenes bookeri*, *Paracolobacterium intermedium*, *Proteus melanovogenes*, *S. putrefaciens*, *Proteus vulgaris*, *Flavobacterium* spp.). Many *Salmonella* serotypes are associated with egg products. Both *L. monocytogenes* and *Y. enterocolitica* may also be found in liquid eggs (Stadelman 1994).

In the USA and in the European Union, all liquid, frozen, and dried whole egg, yolk, and albumen must be pasteurized or otherwise treated to reduce the number of viable salmonellae to very low levels. The US regulations require that liquid whole egg be heated to at least 60°C and held at that temperature for 3.5 min. The UK regulations specify a heat treatment of not less than 64.4°C for at least 2.5 min or another temperature/time combination that will give the same degree of destruction of vegetative pathogens (Statutory Instrument 1993b; EC 1989). Either temperature–time combination process significantly reduces the number of spoilage bacteria and pathogens without affecting the functional properties of liquid egg. As liquid egg white may have a pH of between 7.6 and 9.3, it should be stabilized by the addition of ammonium sulfate before applying the heating step. Processes using heat and hydrogen peroxide combinations are also acceptable (Baker 1994).

In 1993, a commercially produced mayonnaise was epidemiologically implicated as the source of *E. coli* O157:H7 in an outbreak of bloody diarrhea. However, the evidence suggested that contaminated meat was the more likely source of the organism. Subsequent studies have shown that *E. coli* O157:H7 is more acid-tolerant than other gram-negative enteric bacteria and can survive in refrigerated acidified foods, including mayonnaise. Interestingly, *E. coli* O157:H7 was rapidly inactivated in mayonnaise when it was stored at room temperature (Weagent et al. 1994; Zhao and Doyle 1994; Erickson et al. 1995).

Fish and fish products

The consumption of seafood products has increased dramatically in recent years. This increase has focused greater attention on both the quality and the safety of these products and has led to the development of hazard analysis critical control points (HACCP, see section on New approach to assuring the safety of foods) systems and inspections.

The term 'fish' is used commonly as both a specific term for 'finfish' and also as a generic term covering all edible aquatic and marine finfish, molluscan shellfish, and crustaceans. In this section the individual groups will be referred to by their specific names.

Finfish are generally regarded as more perishable than other high-protein-muscle foods. This high perishability is due primarily to the presence of high concentrations of free nonprotein nitrogenous compounds in fish muscle. These compounds, e.g. ammonia, urea, trimethylamine oxide, creatine, taurine, etc., are utilized actively by bacteria during spoilage, resulting in fishy, ammoniacal smells (Jay 2000). In addition, the perishability of cold-water fish is not effectively reduced by refrigeration because of the preponderance of psychrotrophs in their microflora.

The internal flesh of live, healthy fish is sterile; the natural bacterial flora reside primarily in the outer slime layer of the skin, on the gills, and in the intestinal tract of feeding fish. Bacterial numbers will depend on the temperature of the waters but will range from 10^2 to 10^6 cfu/cm on the skin, from 10^3 to 10^5 cfu/g on the gills, and from very few (nonfeeding fish) to 10^7 cfu/g or greater in the intestines. These initial microflora are directly related to the environment, whereas the total microbial load is subject to seasonal variation. Warm-water fish tend to have a more mesophilic, gram-positive microflora (micrococci, bacilli, coryneforms), whereas cold-water fish harbor predominantly gram-negative psychrotrophs (*Moraxella*, *Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Vibrio*). Regardless of the initial microflora, spoilage of finfish during iced storage is primarily caused by *Pseudomonas* spp. and *S. putrefaciens*.

The level of bacteria present on a fish is not a reliable indicator of its sensory quality or shelf-life. Gram-negative bacteria are the major causes of fish spoilage. Their metabolism produces the unpleasant odors and flavors associated with fish spoilage. *Shewanella putrefaciens* (previously classified in the genera *Alteromonas* and *Pseudomonas*) is the most important fish-spoilage bacterium in marine fish stored at 0°C; it produces H_2S and causes off-odors. Spoilage bacteria belonging to the family Vibrionaceae are important in spoilage of fish stored at temperatures higher than 0°C (Gram 1992).

The only bacteria that are both pathogens for humans and indigenous members of the normal microflora of the marine environment are *C. botulinum* and various *Vibrio* spp. Other bacterial species may contaminate fish taken from waters subject to human or animal pollution; most of the recognized food-borne pathogens have been isolated from finfish or shellfish.

CRUSTACEANS

Crustacean species of commercial importance include crabs, lobsters, shrimps, and prawns. Crabs and lobsters are generally trapped and transported live to the processing plant. Shrimps and prawns are usually captured by trawlers and iced for transport to the processing plant. As crustaceans may be caught close inshore, a proportion will be from polluted waters and will be contaminated with microorganisms derived from sewage.

Shrimps and prawns are common causes of food-borne illness involving *S. aureus*, *Salmonella* and *Shigella* species and *Vibrio parahaemolyticus*. They are harvested

from coastal waters in many parts of the world and may be captured either by fishing from boats or by hand-netting from the shore. Increasingly they are grown in ponds (aquaculture). The microorganisms present will depend on where the crustacean shellfish are caught. There may be a variety of microbial pathogens originating from untreated sewage, such as *Salmonella* and *Shigella* species, or the organisms that are part of the normal flora of the animal, such as *V. parahaemolyticus* or *Vibrio vulnificus*. Crustaceans are extremely susceptible to microbial deterioration and ideally should be harvested from unpolluted water and cooled rapidly to a temperature of −1 to +2°C. The ice used for chilling should be of good microbiological quality; alternatively clean refrigerated seawater may be used. If water quality is suspect, it should be chlorinated or otherwise treated, but excessive use of chlorine or other biocidal agents should be avoided. On fishing vessels, holds and container boxes should be cleaned thoroughly between catches to prevent a build-up of contamination.

MOLLUSCS

Molluscan shellfish include bivalves, such as oysters, mussels, cockles, clams, and scallops, and gastropods such as whelks and periwinkles. With the exception of some oysters and scallops, which are harvested from deeper waters, most molluscs grow in estuarine and nearshore coastal waters where exposure to fecal contamination is both possible and likely. Clams and cockles are usually dug from the sand; oysters and scallops are either raked or trawled from the seabed; and mussels are hand picked. Oysters and mussels are frequently farmed. They are transported live in the shell, often with little refrigeration. Molluscan shellfish are filter feeders and can concentrate microorganisms from the water in their bodies. Hence the need for depuration in clean water, especially if the mollusc is to be consumed raw.

Vibrio spp. are important pathogens often associated with the consumption of clams and oysters. In 1989, a co-ordinated surveillance for human infections caused by *Vibrio* spp. in four US Gulf Coast states (Alabama, Florida, Louisiana, and Texas) revealed 121 cases of molluscan shellfish associated illness, of which 71 had gastroenteritis, 29 wound infections, and 14 primary septicemia. *Vibrio cholerae* non-O1, *V. hollisae*, *V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus*, and *V. vulnificus* were isolated from the patients (Levine and Griffin 1993). *V. vulnificus* causes wound infections and primary septicemia with high mortality in susceptible persons, particularly those with impaired liver function. Shellfish, particularly clams and oysters, taken from contaminated waters have also been incriminated as the source of outbreaks of infectious hepatitis A (Bryan 1980) and may serve to transmit other viruses, including caliciviruses, enteroviruses, noroviruses (Norwalk-type viruses), snow mountain agent, and non-A/non-B hepatitis viruses (Cliver 1971, 1988). Noroviruses may be the most frequent cause of shellfish-borne illness.

Toxins have been responsible for a number of seafood-associated outbreaks of illness in the USA, the UK, and elswhere. The principal intoxications having a microbiologial origin include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP) (or domoic acid poisoning), ciguatera, and scombrotoxic (biogenic amine) fish poisoning (Scoging 1997). PSP, DSP, and NSP are caused by toxins produced by dinoflagellates, ASP by a diatom toxin, and all result from the consumption of bivalve molluscs that have been feeding on toxigenic algae, accumulating the toxin in their organs. Ciguatera toxin originates from toxic microalgae that are passed up the marine food chain from herbivorous to carnivorous fish, magnifying the toxin levels. The toxigenic organisms are not usually detectable in the seafood and identification depends on toxin analysis (Scoging 1997). Scombroid poisoning is caused by consumption of fish containing high levels of histamine (and other biogenic amines) resulting from the histidine decarboxylating activity of bacteria multiplying on fish after catching or during the food processing and preparation chain. The toxins are not denatured by cooking or eliminated by cleansing of shellfish in purification tanks. Hence the only available control measure is to prohibit collection of shellfish when, during periods of dinoflagellate blooms, the toxins in the shellfish approach dangerous concentrations.

Vegetables and fruits

Fresh vegetables and fruits are an essential part of the diet of people worldwide. If land is available, families grow their own fruits and vegetables. Otherwise, produce is purchased from local farmers or retail establishments. In developed countries, the production, processing, storage, and distribution of fruits and vegetables are highly integrated and are consolidated in a few commercial companies. This has resulted in year-round availability of such produce; efficient production and processing have also enabled the industry to meet the increased demand from a health- and nutrition-conscious public. However, these integrated practices have given rise to some new public-health problems.

VEGETABLES

Bacterial contamination of vegetables is a public-health concern because of soil microorganisms and the possibility of contamination from water used for irrigation and the various materials, including night soil, used as fertilizers. Several studies have shown that bacteria of public-health concern can be isolated from whole and prepared

vegetables. Control of the quality of plant foods begins at production with suitable geographical location; choice of seed, fertilizers, and pesticides; effective use of irrigation or drainage systems; and crop rotation. In developed countries there is little obvious association between agricultural practices and food-borne illness. The relatively large scale of many agricultural operations results in minimal human or animal contact with crops and constitutes a useful safety factor. Practices such as prolonged cooking and rejection of bruised or rotted foods also minimize the consumption of potentially hazardous material. In developed countries the control measures for quality of plant foods and for minimizing product loss from bruising, browning, wilting, or rot result in foods with minimal microbiological hazards.

The nutrient content of vegetables is suitable for the growth of molds, yeasts, and bacteria (Jay 2000). The number of bacteria on vegetables upon arrival at a processing plant ranges from 10^4 to 10^7/g (ICMSF 1998); number of fungi on fresh vegetables at harvest ranges from 0 to 5×10^5, and at market from 5×10^3 to 2×10^6 (Brackett 1987). Fresh vegetables are inherently susceptible to spoilage because the a_w is above the minimum (0.90) needed to support the growth of most microorganisms, and the pH of most vegetables is within the range allowing multiplication. Washing can remove up to 90 percent of the surface microorganisms, depending on the smoothness of the surface, but those trapped on the vegetable remain and any residual water facilitates their rapid multiplication. Microorganisms multiply faster in cut produce because of the ready availability of nutrients and water. Further handling of the produce increases the likelihood of additional contamination from the handler, work surfaces, or utensils, which may have been contaminated previously by contact with other foods. Most fungal spoilage is caused by the genera *Penicillium*, *Sclerotinia*, *Botrytis*, and *Rhizopus*. A more complete listing is given by the ICMSF (1998). Fungal spoilage of fresh vegetables is considered more an economic than a health threat because few spoilage fungi produce toxic metabolites. Exceptions include aspergilli, penicillia, and *Alternaria*. Stress metabolites that are sometimes produced by plants in response to fungal infections can affect humans, e.g. furanocoumarin compounds, called psoralens, which are produced when *Sclerotinia sclerotiorum* produces 'pink rot' of celery. The effects of these compounds include blisters or lesions of the skin of consumers and those handling the diseased celery.

Bacteria cause a range of spoilage conditions known as rots, spots, blights, and wilts. Soft rots, which occur during transport and storage, are usually caused by coliforms, *Erwinia carotovora*, and certain pseudomonads. Bacteria causing spoilage other than soft rot include corynebacteria, xanthomonads, and pseudomonads (Lund 1983). Soft rot of potatoes by *Clostridium* spp. has also been reported (Lund 1972, 1986). These organisms break down pectins, causing a soft consistency, sometimes with an objectionable odor and a watery appearance.

Storage

Harvested vegetables and fruits continue to respire actively and produce heat. Handling and storage procedures therefore should minimize respiration and water loss and maintain an environment in which cells of the product remain healthy. Rapid chilling is important for leafy vegetables. Correct temperature and humidity control are essential in order to promote wound healing and to minimize spoilage of root crops. A weak link in the distribution chain for fruits and vegetables is the use of unsuitable containers or packaging material (e.g. large sacks, rough wooden boxes, bamboo baskets), which can result in crushing, bruising, and puncturing of the product. Long journeys, hot weather, unventilated trucks, heavy loads, and unpaved roads in developing countries are responsible for huge losses of fruit and vegetable harvest (Ryall and Lipton 1979; FAO 1981; Proctor et al. 1981).

The only means of control of spoilage microorganisms is by strict sanitation of equipment and control of the temperature, relative humidity, and gas composition of the atmosphere in which the raw vegetable is stored. Further examples of vegetable spoilage are given in Dennis (1987), ICMSF (1998), and Jay (2000).

Modified-atmosphere packaging or vacuum packaging is used to suppress the proliferation of aerobic spoilage microorganisms on vegetables. In addition, these processes reduce the rate of oxidative deterioration, enzymatic degradation, and water loss. Modified-atmosphere or vacuum packaging is used to market fresh-cut packaged vegetables including cabbage, lettuce, onions, green and red peppers, carrots, cauliflower, broccoli, and other leafy salad vegetables. Spoilage of ready-to-use salads is frequently caused by pectinolytic pseudomonads such as *Pseudomonas marginalis*.

Freezing is a preferred means of preserving vegetables, since both the organoleptic and the nutritional quality are better retained than by other processes. Before freezing vegetables it is normal practice to blanch, usually at 86–98°C for several minutes, to inactivate plant enzymes, thereby stabilizing the product during subsequent frozen storage. Blanching commonly reduces the microbial load 10^3–10^5 times. In most frozen vegetables, lactic acid bacteria are numerically dominant, although micrococci and gram-positive and gram-negative rods, including coliforms, comprise a considerable proportion of the total microflora of certain products. Microbial spoilage of frozen vegetables is rare, and they are rarely involved in food-borne illness because nonspore-forming pathogens do not survive

blanching and most frozen vegetables are cooked before consumption.

Dried vegetables are shelf-stable and are rarely involved in food-borne illness although they can be a source of organisms, particularly spores of clostridia and bacilli, when rehydrated or when added in the dried form to other dishes. Modified atmospheres have been used to control fungal spoilage of vegetables, with mixed results. An increased carbon dioxide concentration is fungistatic for many fungi, including *Candida albicans*, *Botrytis cinerea*, *Botrytis parasiticus*, *Penicillium* spp., *Mucor*, and *Aureobasidium pullulans*, but there are examples of some vegetables being either more or less sensitive to increased concentrations of carbon dioxide, or off flavors developing due to the different gases used for preservation.

Vegetables can be contaminated with pathogenic microorganisms during growth, at harvesting, post-harvesting handling and storage, and distribution. Beuchat (1996) has published a comprehensive review on this subject. Food-borne disease outbreaks of bacterial origin are associated more frequently with vegetables than fruits. Among the bacterial pathogens of greatest concern in vegetables are *Shigella* spp., salmonellae, enterotoxigenic, enteropathogenic, enteroinvasive, and enterohemorrhagic *E. coli*, *Campylobacter* spp., *Y. enterocolitica*, *L. monocytogenes*, *Aeromonas* spp., *V. cholerae*, *S. aureus*, *Bacillus cereus*, *C. perfringens*, and *C. botulinum*.

Several large outbreaks of shigellosis have been traced to contaminated vegetables such as shredded or cut lettuce and fresh green onions. With the current popularity of fresh-cut packaged produce, there is a potential for causing large, geographically widespread outbreaks. *Shigella* spp. can survive on vegetables for several months at ambient and refrigerator temperatures.

Vegetables implicated as vehicles of *Salmonella* spp. in food-borne disease outbreaks include raw tomatoes and bean sprouts. *Salmonella* spp. can grow on raw tomatoes (pH 4.0) at 20–30°C. *L. monocytogenes* was established as a food-borne pathogen after a large outbreak of listeriosis in Nova Scotia in 1981 in which contaminated coleslaw was implicated (Schlech et al. 1983). The cabbage that was used to prepare the coleslaw had been grown on fields that were fertilized with contaminated sheep manure. In addition, the cabbage was stored at refrigeration temperatures over winter; this probably allowed the psychrotrophic pathogen to multiply. *L. monocytogenes* is a common natural contaminant of raw vegetables. The ability of the organism to survive and multiply at low temperatures makes it a difficult organism to control in the cool, moist environments of food-processing establishments. The organism can also exist in biofilms, which impede the process of cleaning and disinfection. The cut surfaces and exudate from some chopped vegetables, such as lettuce and cabbage, included in ready-prepared salads, both prepacked and from delicatessen counters, may provide an ideal substrate for the growth of *L. monocytogenes* (Beuchat and Brackett 1990a). The juice of other vegetables, for example carrots, may be inhibitory (Beuchat and Brackett 1990b). Many cases of travelers' diarrhea are attributed to enterotoxigenic *E. coli* acquired from contaminated salads eaten at restaurants. Salad-bar vegetables and lettuce have been implicated in instances of infection with *E.coli* O157:H7.

Vegetables sold in modified-atmosphere packages may pose a threat of botulism. *C. botulinum* type A spores have been isolated from shredded cabbage, chopped green pepper, and a salad mix. There is likely to be a hazard only if they have been prepared or stored under conditions that will lead to spore germination, cell multiplication, and toxin production. The presence of normal spoilage microflora in vegetables packaged under modified atmosphere may actually prevent the growth of *C. botulinum* and production of toxin. Packaging under modified atmosphere does not prevent the growth of *E. coli* O157:H7 on shredded lettuce or sliced cucumbers.

FRUITS

Losses in fresh-fruit harvests due to rots and other defects are usually the result of mold growth. Some molds are able to invade and infect the intact healthy tissue, whereas others can become established only after the fruit has been infected by a pathogen or has been damaged. Several diseases are named after the causative mould (e.g. *Alternaria*, *Botrytis*, and *Fusarium* rots) and others are derived from the appearance of the infected fruit (e.g. brown rot, stem-end rot). In some cases diseases of different fruits may have the same name despite the involvement of different molds (e.g. black rot is caused by *Physalospora obtusa* in apples and by *Alternaria citri* in oranges). The market diseases of fruits, and their causative fungi, are tabulated by the ICMSF (1998). Yeast spoilage of fresh fruits is usually from the fermentative action of, for example, *Hanseniaspora valbyensis*, *Candida* spp., *Candida (Torulopsis) stellata*, *Pichia kluyveri*, and *Kloeckera apiculata*.

At the orchard or vineyard pruning, certain cultural practices and application of fungicides are the main means by which infection and spoilage prior to harvest are minimized. After harvesting at optimum maturity, fruit should be handled gently to prevent physical injury and fruit-contact surfaces should be cleaned regularly and treated to destroy fungal spores. Moldy or bruised fruits are removed during sorting and grading. Fruit may be treated with hot water or with permitted fungicides to control, for example, *Phomopsis*, *Diplodia*, *Penicillium*, and *Botrytis* (Salunkhe and Desai 1984).

Storage

Fruits are stored cooled but at temperatures that do not cause chill injury. The concentration of carbon dioxide in the atmosphere may be increased to inhibit *Alternaria*, *Botrytis*, and *Rhizopus* spp. Fruit products can be preserved by heating to approximately 80–90°C because most aciduric microbes are relatively sensitive to heating. Spoilage of canned soft drinks has occurred because ascospores of *Saccharomyces cerevisiae* or spores of *Kluyveromyces marxianus* survived the heat process. Spoilage of canned-fruit products has occurred because ascospores survived the heating process, e.g. *Penicillium vermiculatum* and *Byssochlamys* (Splittstoesser 1987).

Freezing is often used to preserve fruits and fruit products and results in some reduction in microbial numbers. Growth of yeasts and molds does not occur if temperatures are maintained below −18°C, but exposure to automatic defrost cycles is undesirable in the long term because some multiplication may occur.

Reduction of the water activity is a common means of fruit preservation, either by natural exposure to the sun or by a controlled dehydration procedure. Fungal spoilage of dried fruit does not occur when the water content is below 25 percent. Some high-moisture dried fruits contain 30–35 percent moisture and are therefore heated or treated with potassium sorbate to prevent mold growth. Many fruits are treated with sulfite before drying to prevent browning and this destroys a high proportion of the contaminating microbes. Yeasts and molds able to grow on dried fruit include *Zygosaccharomyces bisporus*, *Zygosaccharomyces rouxii*, *Hanseniaspora*, *Candida*, *Penicillium* spp., *Aspergillus glaucus*, and *Aspergillus niger*. *Monascus bisporus* is one of the most xerotolerant molds and has caused spoilage of dried prunes (Splittstoesser 1987). The relationship between water activity and fungal growth is reviewed thoroughly by Corry (1987).

Pathogenic bacteria are not normally associated with fruit; however they may be present due to fecal contamination. Survival times of enteric pathogens on fruits will depend on pH and temperature. On high-acid fruits, e.g. lemons, it will be shorter than on low-acid types, such as melons. Growth is more likely to occur on cut or damaged surfaces.

Sliced fresh fruits support the growth of *Shigella* spp. and thus may serve as the vehicle of infection. Several outbreaks of salmonellosis have been associated with fresh fruits, particularly raw tomatoes and melons. *Salmonella* serotypes Oranienburg and Javiana have caused outbreaks associated with the consumption of contaminated watermelons; serotypes Chester and Poona have been linked with cantaloupes. Organisms can enter either through fissures or cracks in the fruit surface or via the blade or other implement used to slice the fruit. Outbreaks of *E.coli* O157:H7 were attributed to unfermented apple juice, orange juice, and cantaloupe (ICMSF 1998).

CANNED FRUIT AND VEGETABLE PRODUCTS

Preservation by canning is exploited to increase the shelf-life and availability of fruit and vegetables. The heat processing of these products and the importance of pH are discussed thoroughly elsewhere (Hersom and Hulland 1980; Lund 1986). Most vegetables intended for canning fall into the category of foods known as 'low-acid' because their pH is above 4.6. Low-acid foods must be heated sufficiently to destroy the spores of *C. botulinum*, which are inevitable contaminants of agricultural products, including vegetables. The canning process is a good example of where a high margin of safety is achieved by control of the process rather than by microbiological tests on the raw material and final product. The critical control points in the process, i.e. initial microbial contamination, time between filling and processing, cleanliness of equipment, heat process, cooling cycle and disinfection of cooling water, maintenance of container integrity by seam or seal control, and appropriate handling are outlined in ICMSF (1988). Leaker spoilage of canned vegetables is usually characterized by the presence of a mixture of microbial types, mainly bacteria, while with spoilage due to underprocessing there is often only one species present.

Canned vegetables that have been heat processed to commercial sterility may still contain viable spores that are unable to grow under the normal temperature of storage of the product, e.g. thermophilic spores of *B. stearothermophilus* and *B. coagulans*, which cause flat sour spoilage with production of acid but not gas. Thermophilic anaerobic spoilage may be caused by obligately thermophilic spore-forming anaerobes, such as *Clostridium thermosaccharolyticum*, which forms large quantities of hydrogen and carbon dioxide, or *Desulfotomaculum nigrificans*, which produces hydrogen sulfide, often with blackening of the food if iron is present. Thermophilic spoilage usually occurs after improper cooling of heat-processed vegetables (Hersom and Hulland 1980; Lund 1986; Dennis 1987; ICMSF 1988, 1998).

Cereals, nuts and seeds, and herbs and spices

Cereals are the main staple diet of a large proportion of the world's population, the main ones being wheat, maize, oats, rye, rice, barley, millet, and sorghum. They may be eaten directly after cooking, principally rice, and sometimes maize, or they may be converted into flours for further processing by baking into breads and biscuits or molding into doughs for pasta or noodles. They may be fermented as gruels, manufactured into western-style

cereal products and eaten as breakfast foods, or fermented into beers and wines. The principal microorganisms of concern are fungi and spore-forming bacteria.

The microbiology of cereals is summarized by Pitt and Hocking (1986) and considered in greater detail in ICMSF (1998). Cereals are of near-neutral pH and provide good nutrition for bacteria. Microbial activity is controlled by drying the harvested cereals to a sufficiently low a_w (below 0.70) at which bacteria are unable to multiply, and growth of molds is largely prevented in temperate zones. At the humidity levels common in tropical countries, mold growth is a serious problem. Historically, the molds able to spoil cereals have been grouped into 'field' and 'storage' fungi, but some (e.g. *Aspergillus flavus*) grow in both situations. Field fungi are plant pathogens that invade the crop as a consequence of a combination of favorable circumstances. Particular molds may cause problems in one geographical area, but not in another. For example in Japan an important disease of wheat and barley, (red mold disease) is caused by *Fusarium* spp., predominantly *Fusarium graminearum*, whereas in Australia *Fusarium* spp. appear to cause little problem in wheat, probably due to the drier climate.

Fungi occurring commonly on wheat, barley, and oats in Scotland and England include *Alternaria*, *Cladosporium*, *Epicoccum*, and *Penicillium* spp., while in Egypt wheat carries mainly *Aspergillus* and *Penicillium*, together with *Alternaria*, *Cladosporium*, and *Fusarium*, and barley mainly *Aspergillus* and *Penicillium*, together with *Rhizopus*, *Alternaria*, *Fusarium*, and *Drechslera* (Pitt and Hocking 1986).

Bacteria generally found on cereals are from the Pseudomonadaceae, Micrococcaceae, Lactobacillaceae, and Bacillaceae. The level of fecal contamination will be low in field grains unless there is considerable animal activity. Groups that are nearly always present include psychrotrophic bacteria (10^4 to $>10^7$ cfu/g), actinomycetes (up to $>10^6$ cfu/g), and aerobic sporing bacteria (10^0 to 10^5 cfu/g).

WHEAT

Wheat grows best in fairly dry and mild climates. After harvesting, it is dried and stored and may be transported long distances before final milling. Before consumption, milled wheat is cooked or baked. Spoilage by bacteria is not usually a problem because of the relatively low a_w, but wheat may act as a passive carrier for bacterial pathogens (ICMSF 1998). Some that are part of the normal flora such as *C. perfringens*, *B. cereus* and other *Bacillus* spp. can, under the right set of conditions, become harmful to man when consumed in a final cereal-based dish. Mold growth, spoilage, and the possible presence of mycotoxins are more serious hazards. Under particular weather conditions mycotoxins can be produced while the wheat is growing (Saito and Ohtsubo 1974; Ichinoe et al. 1983) or during storage if the a_w becomes sufficiently high.

Microbiology at production

The diverse surface microflora of wheat in the field does no damage. The grain may, however, be infected by *F. graminearum* to produce wheat scab, red mold disease, or fusarium head blight. Perithecia of *Gibberella zeae* (the perfect stage) maturing in the field liberate ascospores into the air, which infect wheat at the heading stage. Under high relative humidity and low temperatures *F. graminearum* grows and may produce trichothecenes (nivalenol or deoxynivalenol) and zearalenone (Saito and Ohtsubo 1974; Ichinoe et al. 1983; Hagler et al. 1984). There are unresolved geographical differences in the occurrence of trichothecenes, perhaps related to which strains of *Fusarium* occur (Osborne and Willis 1984). *A. flavus* and *A. parasiticus* are common contaminants of certain grains.

The crop is harvested as soon as possible after maturity, before rain can damage the grain (e.g. by causing sprouting). In a single operation, combine harvesters reap, thresh, clean, and load the wheat kernels into trucks for transport to storage. The rate and extent of subsequent drying of the crop depend on the moisture content at harvest (15–22 percent). It is important to harvest mature grain of known moisture content in dry weather without grain breakage and to separate kernels from hull and straw.

The moisture content of harvested wheat may be as high as 22 percent (a_w approximately 0.95), with the temperature reaching nearly 40°C. The grain must then be dried. Also it may need cooling in order to prevent spoilage and mold growth, to lower the respiration rate of the wheat, and to prevent overheating and damage of the grain. Wheat is dried initially to about 13 percent moisture (Zeleny 1971), to an a_w of less than 0.70, and later to 11–12 percent for long-term storage (ICMSF 1998). The higher the moisture content of the grain, the lower the air temperature must be, to prevent heat damage (Kent 1966).

Storage

After drying and before primary storage, wheat is cleaned by screening and aspiration to remove dust, broken and light grain, and foreign material. Broken grain is more susceptible to mold and insect attack (Williams 1983).

When grain is moved from primary storage to long-term storage in terminal elevators by truck or railcar, the vehicles are sources of insects or storage molds (Williams 1983). They may also be a source of contamination by salmonellae if the previous cargo was animals, meat scraps, or meat or fish meal (ICMSF 1998).

Cleaning and fumigation are necessary to keep transport vehicles clean and insect-free. Grain must be protected from rain. On grains at harvest time, mold propagules range from a few to 10^5/g and bacteria from 10^3 to 10^6/g. The bacteria are usually from the families Pseudomonadaceae, Micrococcaceae, Lactobacillaceae, and Bacillaceae. Indicators of fecal pollution are rare unless there has been animal access to fields. The presence of *Bacillus subtilis* and *B. cereus* has been demonstrated and other bacterial spores from the soil should be expected (e.g. *C. botulinum*, *C. perfringens*). Actinomycetes commonly exceed 10^6/g and psychrotrophs 10^4/g. Yeasts occur but do not cause problems during storage. During artificial drying, the temperatures achieved (40–80°C) destroy most mold spores. Sun drying is less effective in this respect.

Flour may be contaminated with spores of *B. subtilis* and *Bacillus licheniformis*, which can give rise to 'ropey' bread particularly if the preservatives commonly added in commercial bakeries are omitted. There is evidence associating large numbers of bacilli other than *B. cereus* with food-borne illness, especially in relation to foods with a flour-based sauce or encased in pastry (Kramer and Gilbert 1989).

RICE

Unlike wheat, rice (*Oryza sativa*) crops are largely grown in relatively small production units without great benefit of mechanization. Also, there is often much human and animal contact with the crop. However, salmonellae and other vegetative pathogens from such sources should be destroyed by the cooking process. Rice is often harvested and stored under hot, humid conditions that increase the risk of fungal attack and present a significant hazard from mycotoxins and spoilage losses. As the a_w is low, bacterial spoilage is rarely a problem. Spores of *B. cereus* survive cooking and have caused food-borne illness (Kramer and Gilbert 1989). The illness that has been recognized as usually associated with the consumption of rice contaminated with *B. cereus* has a rapid onset (1–5 h); the predominant symptoms are nausea, vomiting and malaise and are referred to as the 'emetic syndrome;' occasionally diarrhea may occur. Approximately 95 percent of 'emetic syndrome' episodes have been associated with the consumption of Cantonese-style cooked rice served by Chinese restaurants usually as take-away meals (Kramer and Gilbert 1989). The restaurants store cooked rice for reheating on demand at room temperature for long periods of time. There is a reluctance to store cooked rice in the refrigerator because the grains become sticky and clump together. Heat-resistant spores of *B. cereus* survive the initial cooking period and, during subsequent storage in the warm kitchen, germinate and multiply rapidly and produce toxin. The emetic toxin is extremely heat-resistant (Melling and Capel 1978) and will not be inactivated by the rapid cooking before consumption.

Fungi can invade rice in the field and spoil the developing grain (Fazli and Schroeder 1966; Bhat et al. 1982), while other fungi (storage fungi) attack the rice during drying and storage (Mallick and Nandi 1981; Mossman 1983). A variety of mycotoxins have been found in rice (Jarvis 1976; ICMSF 1998). It is crucial to reduce the a_w of rice quickly to approximately 0.65 to prevent mold growth and to maintain it at that level during storage even in unfavorable climates. Molds can penetrate the endosperm and discolor the kernels, but most mold growth and aflatoxin formation occurs in the bran layers that are removed during milling to produce white rice (Schroeder et al. 1968). Milling also tends to break moldy kernels, which can then be removed, thereby improving the microbial quality of the rice (Ilag and Juliano 1982).

Crop losses caused by plant diseases and insect infestations can be reduced by the planting of resistant varieties and by the application of insecticides (Mikkelsen and DeDatta 1980). Provision of adequate drying and storage facilities for crops harvested in the wet season is critically important (Greeley 1983; Mossman 1983).

Microbiology at production

Rice is harvested when the moisture content is 18–23 percent (DeDatta 1981). Delaying harvest increases field losses (shattering and lodging) and causes subsequent milling losses due to sun-cracking (Chandler 1979; Mikkelsen and DeDatta 1980). Early harvesting gives a more moist grain, which is difficult to hand-thresh and requires more drying.

In traditional tropical operations, rice stalks are pulled out by hand, bundled into sheaves for prethreshing drying, or threshed immediately (Mossman 1983). Manual harvesting causes less damage to the paddy, results in fewer weeds being incorporated into the crop, and allows the gathering of a higher percentage of mature grain. However, the longer the grain is left in the field for prethreshing drying, the greater the risk of cracking and attack by insect molds (Hall 1970). Drying is the most important step in production; if not undertaken or if delayed, the whole crop can spoil. Nevertheless, improper drying can cause grain breakage, partial spoilage, or spoilage losses in storage.

Most rice is sun-dried, and if done correctly this reduces the microbial population (Kuthubutheen 1984). The usual procedure in the tropics is to spread rice on a drying floor exposed to the sun for several days until the moisture level is 14–16 percent (a_w approximately 0.76–0.84). However, at this moisture content, rice cannot be stored safely so further drying during storage is needed. Traditional crops are harvested in the dry season at the time of maximum sun, but this process is often inadequate with increased yields and newer rice varieties that

mature during the wet season. Artificial drying is then necessary, enabling harvests to be dried more uniformly with less loss from grain cracking, and to safe moisture levels (<13 percent moisture or a_w <0.70) regardless of the vagaries of the weather.

Storage

Significant losses can occur during storage if rice is not protected from rodents and insects, or if it is not dry enough to prevent mold growth. Mold attack is exacerbated by the high temperatures and humidities of tropical areas. At the start of storage, up to 85 percent of paddy rice kernels may be internally infected with field molds. During storage, field molds gradually disappear or are replaced by storage fungi (Mallick and Nandi 1981; Kuthubutheen 1984). The type and extent of growth of storage fungi depend on the initial load, temperature, a_w or relative humidity, and time (ICMSF 1998). Paddy rice can be safely stored at 30°C with a constant moisture content of 12.5 percent (a_w 0.67) for 12 months with no fungal growth (Mallick and Nandi 1981). A moisture content of 14.3–14.5 percent (a_w approximately 0.79) produces significant mold growth and deterioration within a few months.

Storage containers such as sacks or baskets give little protection from rodents or insects and may be a source of eggs, larvae, and mold spores (Williams 1983). Some modern rice varieties have thinner husks than traditional cultivars and are more susceptible to insect attack (Greeley 1983). Storage in bins or solid-walled containers provides protection from ingress of rodents, insects, and external moisture from rain or high humidity. However, failure to dry paddy rice to near 12 percent moisture (a_w 0.65) before, or soon after, bin storage causes considerable crop loss. Since rice insulates well, temperature gradients within the bin are readily established and lead to condensation and raised moisture contents in the cooler regions. The resultant increased metabolic activity and mold growth generate heat, carbon dioxide, and more water, resulting in 'hot spots,' which spread upwards and outwards. Bins made of metal are particularly susceptible to temperature gradients resulting from fluctuations in external temperatures.

Insect infestation is controlled by treating sacks, storage areas, and stocks of stored rice with insecticides or fumigants, and by keeping storage areas free of dust, fines, and spilt rice. Paddy rice is not generally transported long distances but transportation should be regarded as an extension of storage, with the same principles of preventing exposure to rain, dew, insects, and rodents.

Milling can improve the microbiological quality of rice. Much of the bacterial and mold contamination and aflatoxin, if present, is on the husk and in the surface layers of the kernel and will be removed by milling in the husk and bran fractions (Ilag and Juliano 1982). Grain heavily infested with mold will also break more readily during milling, yielding discolored, broken rice that is separated in the broken-grain fraction. Much dust is generated during milling; it may absorb enough moisture to allow microbial growth and can be a source of insects. It is essential that dust is controlled by regular sweeping, vacuuming, or aspiration.

The absence of husk makes milled rice more susceptible to insect attack. During storage it must be protected from insects, rodents, and increases in moisture content. Thus the same principles of control apply to milled rice as for paddy. Brown rice may also go rancid as a result of scratches in the bran, allowing lipases access to oils within it (Mossman 1983), possibly because of the presence and activity of a wide range of lipolytic bacteria and molds (DeLucca et al. 1978).

NUTS AND SEEDS

Nuts are dry, one-seeded fruits that are usually enclosed in a rigid outer casing or shell. They mostly grow on shrubs or trees (tree nuts) and include almonds, hazelnuts, pistachios, Brazil nuts, pecans, coconuts, and macadamia nuts. The peanut or groundnut is the only major nut that is a legume, but it tends to be grouped together with tree nuts.

Oilseeds from a range of botanical families are grown primarily for oil production and include palm nuts, rapeseed, sesame, sunflower, safflower, cottonseed, cacao seeds, and maize.

Many types of leguminous seeds are dried, such as a wide variety of beans and also soybeans.

The shells of nuts present an effective barrier to entry of bacteria during growth, and after natural drying the low a_w of most nuts restricts bacterial spoilage or toxin production. Bacterial contamination can occur after harvest, e.g. coconut flesh with salmonellae, with resultant concern over products with high a_w to which nuts are added. The high oil content of nuts and oilseeds results in high susceptibility to attack by lipolytic bacteria and spoilage fungi and possible mycotoxin production. Dried legumes, apart from soybeans, are low in oils so microbiologically are similar to cereals.

Microbiology at production

Tree nuts are usually dried in situ before harvesting. Coconuts are pierced or broken, drained of water, and the kernels cut into slices and sun dried. They may be further shredded as desiccated coconut. Peanuts are pulled from the ground and dried at least partially before threshing. There are few data on the microflora of nuts in the field. The shells provide a strong, protective barrier and tree nuts are essentially sterile before harvest apart from possible systemic fungal infection from the tree. Limited data on peanuts suggest that the flora is mainly field fungi, with *Fusarium* spp., *Lasio-*

diplodia theobromae, and *Macrophomina phaseolina* being dominant (MacDonald 1970).

Primary processing of nuts, seeds, and legumes usually involves natural drying. If there is good sun-drying, the initial microflora may be reduced, but if conditions are adverse, the mycoflora may increase in both kind and numbers and there may be mycotoxin formation. The bacterial flora of raw commodities, as would be expected, reflects the growing and harvesting environment and contamination from soil, equipment, etc. The fungal flora isolated from copra, cashew nuts, peanuts, and dried legumes are listed by the ICMSF (1998).

Storage

Stored dried nuts are very susceptible to spoilage because slight increases in moisture content lead to sharp rises in a_w due to the low levels of soluble carbohydrate and high lipid levels. These increases may be due to uneven storage temperatures; therefore, refrigerated storage is widely used to retard the development of rancidity. Increases in moisture may also lead to growth of spoilage fungi and mycotoxin production. The opening and cutting of coconuts allows contamination with bacteria and also many species of fungi.

A variety of bacteria have been isolated from nuts with damaged cells or contaminated with soil (King et al. 1970) including species of *Bacillus*, *Brevibacterium*, *Streptococcus*, and *Xanthomonas*, as well as *E. coli*. There may also be contamination with bacterial pathogens from animals. Contamination of hazelnuts with *C. botulinum* led to human cases of botulism from yogurt containing inadequately processed hazelnut puree (O'Mahoney et al. 1990). The principal problem with nuts and oilseeds lies with the potential for production of mycotoxins, notably aflatoxins. Peanuts, maize, and cottonseed are the three crops of greatest economic importance affected.

Salmonella has frequently been isolated from desiccated coconut (Gilbert 1982) and has been traced to unhygienic preparation at collection centers at the farms. The salmonella problem has largely been overcome as a result of the hygiene controls that have been implemented. However, a large outbreak of *Salmonella* serotype Java infection traced to a single batch of desiccated coconut was reported in the UK in 1998–99 (PHLS Communicable Disease Surveillance Centre 1999).

The most important step from the microbiological viewpoint in the further processing of nuts is roasting, which results in a large reduction in numbers and types of organisms including enteric pathogens. Control of mycotoxins in nut products consists essentially of control of the raw material or primary processing stages. Sorting and diverting discolored, rejected nuts is an important commonly used means to reduce mycotoxins to acceptable levels in finished products. End-product testing for mycotoxins is widely practiced.

HERBS AND SPICES

Spices are any of a variety of aromatic plant products used to season, flavor, or impart an aroma to foods. They may be fruits (peppers, allspice, coriander), arils (mace), flower buds (cloves), rhizomes (ginger), barks (cassia, cinnamon), or seeds (nutmeg, fenugreek, mustard, caraway, celery, and aniseed). Herbs are generally the leafy parts of soft-stemmed plants (oregano, marjoram, basil, curry leaves, mint, rosemary, and parsley) (ICMSF 1998).

Spices are of microbiological interest for a number of reasons:

- Antimicrobial activity and preservative properties. This activity, due to the essential oils, is of little significance in food preservation. Levels are generally too low to prevent microbial growth but can sometimes augment other microbial activities or act synergistically. Spices containing the most inhibitory of essential oils include cloves, thyme, oregano, cinnamon, allspice, cumin, and caraway. Yeasts are more readily inhibited than bacteria and the gram-positive bacteria are more sensitive than the gram-negative. The activity of a range of essential oils is listed by the ICMSF (1998).
- Stimulation of microbial metabolism.
- Tendency to go moldy at improper humidity and temperature.
- Contain large numbers of microorganisms that may cause spoilage or disease when introduced into foods.

Spices and herbs contain the organisms that are common to the soil and plants in which they are grown and that will survive the drying process. Other sources of contamination include dust, insects, fecal material from birds and rodents, and possibly water. Microbial counts will vary widely depending on original microbial load, opportunity for growth, subsequent die-off, stage of storage, origin of product, etc. Most of the microbial flora consists of aerobic mesophilic sporing bacilli originating from the soil as spores due to their ability to survive the drying process, most commonly *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. brevis*, *B. polymyxa*, and *B. cereus*. A wide variety of nonsporing organisms may be found including coliforms, *E. coli*, fecal streptococci, and, rarely, staphylococci and lactic acid bacteria. The main components of the mold flora tend to be the *Aspergillus glaucus* group, *A. niger*, and *Penicillium* spp.

As the different spices vary widely in form they require diverse harvesting and post-harvesting methods; these will have a variable effect on their microbial content. The most important consideration is to dry the products rapidly to prevent spoilage while maintaining

their desirable characteristics. ICMSF (1998) gives examples of some harvest and post-harvest treatments.

Spoilage of herbs and spices is mainly by molds, chiefly *Aspergillus* spp.; bacterial spoilage is rare. Insect infestation may aid the transfer of mold spores. However, other foods may be spoiled by the addition of herbs and spices bearing large numbers of bacterial spores; the degree of spoilage will depend on the amount of further processing or heat treatment that the final product receives.

Some of the spore-forming bacteria found in herbs and spices are capable of causing gastroenteritis (*B. cereus*, *B. subtilis*, *B. licheniformis*) (Kramer and Gilbert 1989). *C. perfringens* may also be present. As these spores will survive cooking temperatures, spices harboring these organisms must be considered as a potential health hazard if the foods to which the spices have been added are not properly prepared and handled. Salmonellae have also been found in a variety of spices and have been the source of a number of food-borne outbreaks, for example in black and white pepper and paprika. Aflatoxins have also been found.

NEW APPROACH TO ASSURING THE SAFETY OF FOODS

Food is essential to sustain life. People expect safe foods and expect their food supply to be protected and the safety and wholesomeness assured by governments. These expectations are the basis for food laws and food regulations, the inspection programs that monitor compliance with the laws and regulations, and the judicial system that punishes those who violate the laws. Until recently, governments took the responsibility for inspections of foods, food-processing establishments, and their environment. In several countries, government inspectors are assigned to meat, fish, and poultry processing plants. Inspectors of the US Food and Drug Administration (FDA) perform unannounced inspections of packaged or canned food-processing plants and examine finished product to monitor compliance; similar policies exist in other countries.

Currently the approach to assuring the safety of the food supply being implemented in the USA and several other developed countries is the HACCP system, a preventive approach designed in the 1950s to assure the safety of foods in manned space flights (Bernard 1997). The system goes beyond the limited effectiveness of sampling and examining finished goods.

HACCP is a systematic approach to the assurance of safety in food production. It consists of a number of sequential steps:

1. Hazard analysis.
2. Determination of critical control points (CCP).
3. Determination of the critical limits to be met at each identified CCP.
4. Establishment of procedures to monitor critical limits.
5. Establishment of corrective action to be taken when a deviation is identified by the monitoring.
6. Implementation of effective record-keeping-systems.

The concept adopted is based on understanding how a product becomes unsafe. Then control measures can be developed to prevent or detect such failures and keep them from reaching the consumer.

Hazard analysis is an assessment of risks associated with all aspects of food production from growing in the fields to consumption. The hazards may be physical, chemical, or biological. The biological hazard category includes bacterial, viral, fungal, and parasite contamination. Pathogenic bacteria are classified on the basis of the severity of risk: *C. botulinum*, *Salmonella* serotype Typhi, *E. coli* O157:H7, and *V. cholerae* are classified as severe hazards; *L. monocytogenes* and *Salmonella* serotypes other than Typhi are classified as moderate hazards with potential for extensive spread; *B. cereus* and *C. perfringens* are classified as moderate hazards with limited potential for spread. The hazard analysis also includes the ranking of food and its raw materials or ingredients according to six hazard characteristics followed by assigning a risk category to the food and its raw materials based on the hazard rankings.

Critical control point determination is required to control the previously identified hazards. A CCP is defined as any point or procedure in a specific food production system where a loss of control may result in an unacceptable risk.

Critical limits must be established that must be met at each identified CCP. For example, the thermal treatment in a retort in the production of low-acid canned foods is a CCP. The components of the CCP include pressure of the retort, retort temperature and time the food is held at the retort temperature.

Monitoring procedures must be established and also the corrective actions to be taken when a deviation is identified by monitoring critical components of a CCP. Implementation of *effective record-keeping systems* that document the HACCP plan is also an essential requirement of HACCP.

HACCP-based regulations were published first in the USA for low-acid foods in hermetically sealed containers and then later for smoked fish products. In February 1995, the Food Safety and Inspection Service of the United States Department of Agriculture (USDA) published proposed regulations (FSIS 1995). These essentially overhaul the meat and poultry inspection system and change it from a reactive, federal inspection-based approach to a proactive HACCP approach in which the manufacturer or processor assumes greater responsibility for food safety. Both the US FDA and the USDA have published final regulations mandating the development and implementation of

HACCP plans to cover a variety of domestic and imported foods (Bernard 1997).

The US initiatives have been paralleled in other countries. The European Union (EU) has adopted a number of product-specific directives that require specific foods introduced into commerce within EU countries to be produced in accordance with HACCP principles (e.g. fishery products, milk and milk-based products, meat products). In addition the EU has adopted a horizontal directive that requires consistency with HACCP principles for a range of food items (Roberts and Greenwood 2003). Countries outside the EU have also adopted HACCP-based food-safety control systems.

CONCLUSIONS

The processing and regulation of foods are, at the commencement of the twenty-first century, poised to undergo significant changes that will have profound effects on the quality and safety of the food supply. Changes in food-safety regulation are the driving force and changes in the microbiological quality of foods will occur as a result of the changes in the food-safety approach, as an extension of prevention-based HACCP implementation. For example, in July 1996, the US government published a sweeping reform of food-safety rules for meat and poultry (FSIS 1996). The four major elements of the new rules were:

- Every plant must develop and implement a system of preventive controls, known as HACCP, designed to improve the safety of their products. The plant must demonstrate the effectiveness of the HACCP plan, which federal inspectors will continually verify.
- Every plant must regularly test carcasses for *E. coli* of fecal origin to verify the effectiveness of the plant's procedures for preventing and reducing fecal contamination, which is the major source of pathogenic bacteria.
- All slaughter plants and plants producing raw ground meat and poultry products must ensure that the *Salmonella* contamination rate is below the current national baseline incidence.
- Every plant must develop and implement written sanitation standard operating procedures as the foundation for its specific HACCP program.

The time-frame for implementation by large processing plants was 18–42 months depending on the size of the plant.

As systematic programs to reduce and eliminate contamination of products of animal origin are implemented, food-processing plants will critically review their processing operations as part of the hazard analysis component of HACCP. Inevitably steps in their processing will be identified that will affect the keeping quality (related to microbial spoilage) of their products and appropriate control procedures will be implemented to enhance the shelf-life and microbiological quality of the foods.

Despite decades of effort by the food industry and food regulatory agencies, it has not been possible to raise food animals free of pathogenic bacteria. In addition, many more problems of food-borne disease, caused by contaminated fresh fruits and vegetables, are being encountered, possibly as a result of increased worldwide production and movement of these commodities around the globe to meet consumer demands for year-round supplies. The growing conditions for fruits and vegetables vary widely in different parts of the world, thus it may be very difficult to avoid contamination with pathogenic bacteria. At the same time, populations in many developed countries are becoming more susceptible to infectious diseases because of immune deficiencies caused by age, chronic diseases, and immune-depressing infectious diseases. How can consumers be assured of a safe food supply? At least for the foreseeable future, an effective approach seems to be the decontamination of the final raw product. The use of γ-irradiation or electron accelerators for terminal decontamination of food of plant and animal origin may be among the best available options (Corry et al. 1995). Unfortunately, the use of γ-irradiation has been very limited due to public misconceptions about the radiation treatment of foods and the resulting reluctance of the food industry to utilize the process. There are other logistical problems relating to the use of radiation treatment for foods, including the need for transport of foods from processing plants to a central radiation treatment facility, safety problems associated with the use of radioactive materials, and problems associated with the disposal of radioactive wastes. Electron accelerators are more convenient as they do not use radioisotopes. However, their primary limitation is the inability to penetrate more than 1–2 cm below the surface of the food. The ideal decontamination method for uncooked foods still remains to be developed.

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Human microbiota

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GENERAL COMMENTS

For the first 9 months of life, the human fetus lives in a sterile environment protected from microbes, except when pathogens such as cytomegalovirus, rubella virus, or *Toxoplasma* are able to infect the child transplacentally. This state of sterility comes to an abrupt end, however, at the time of birth when the newborn is confronted with the mother's vaginal microbes and environmental organisms. The infant's skin surface is initially colonized and then the oropharynx, gastrointestinal tract, and other mucosal surfaces rapidly become populated. Through the duration of the individual's life, the microbial population evolves, with many organisms, transient colonizers, and others becoming well-established, permanent residents. It is important to recognize that this is a normal phenomenon. Even those microbes with the well-recognized ability to cause serious disease are normally found in and on the human body. Indeed, one hallmark of pathogenesis is not the recovery of a specific organism, but rather the recovery of the organism in a normally sterile site and associated with a pathologic response (e.g. inflammatory cells). For example, *Escherichia coli* is a normal resident in the gastrointestinal tract. To find it there can be anticipated. However, *E. coli* should remain confined to the gastrointestinal tract. If it is found in the abdominal cavity or the patient's blood, this would be considered abnormal. Likewise, organisms such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, and *Haemophilus influenzae* are all capable of causing lower respiratory tract infection. Recovery of these organisms in lower airway secretions would be consistent with their role in disease; however, isolation of the same organisms in a throat washing could be considered insignificant. Naturally, there are certain organisms whose recovery in humans is always associated with clinically significant disease (e.g. *Bacillus anthracis*, *Brucella* spp., *Francisella tularensis*, and *Salmonella typhi*, to name just a few). However, the majority of microbes responsible for human disease are commonly part of the normal microbiota that are able to invade normally sterile tissues and spaces. For that reason, it is important to know the normal habitats of the organisms associated with humans and to understand their capacity for producing disease.

It should also be appreciated that microbes serve a useful purpose in their human hosts. The normal microbiota maintain a protected environment that prevents colonization with potentially pathogenic organisms. For example, *Clostridium difficile* is able to produce gastrointestinal disease ranging from diarrhea to pseudomembranous colitis only when the normal intestinal flora has been reduced or eliminated by antibiotics. The production of proteolytic enzymes by microbes augments host factors in the digestion of foods. Likewise, the disruption of the normal microbial flora can interfere with this process. For example, when the bacteria in the small

intestine are replaced by colonic bacteria following small-bowel stasis (blind loop syndrome), bile salts that are secreted into the intestines are metabolized by *Bacteroides* spp., producing a resultant malabsorption syndrome. Intestinal bacteria can also synthesize vitamins (e.g. biotin, pantothenic acid, pyridoxine, riboflavin, vitamin K), many of which are required for the growth of other bacteria. For example, strains of *E. coli* can produce vitamin K, which is utilized as a required growth factor by *Porphyromonas melaninogenica*, which in turn produces penicillinases that protect other bacteria from penicillin. Thus, a complex relationship among the microbial species can be established for the mutual benefit of each organism and the host.

It is important to define certain terms. The interaction between microbes and humans can result in three general outcomes: disease, transient colonization, and prolonged colonization. Another term for colonization is infection, which does not imply disease but rather the association of the microbe with the human host for a period of time. Disease results when the interaction between microbe (e.g. bacterium, fungus, virus, or parasite) and human host results in a pathological process. This process can be due to microbial factors (e.g. hydrolytic enzymes, toxins) or the host's immune response to the presence of the organism. A few organisms have a high virulence potential and are always associated with disease, whereas most organisms are opportunistic pathogens and cause disease only when the host's immunity is suppressed or when the organisms are introduced into tissues in which they are able to express their virulence potential. The other two outcomes of microbe and host interaction result in colonization, either transiently or prolonged. It should be noted that transient and prolonged colonization implies a distinction based on the duration of the interaction. Although no specific time limit can be used to define colonization, it may be as short as hours to a few days (e.g. organisms passing through the gastrointestinal tract) or extend to weeks, months, or even years. The complex population of microbes that become established in a host are called a variety of names, including natural, indigenous, resident, and normal flora. In previous editions of this text, they were referred to as 'normal microbiota,' a term that has been retained here.

An individual is exposed to numerous organisms that colonize other human hosts and animals or are present in the air that is breathed and food and liquids that are consumed (refer to other chapters in this text that discuss the microbiology of air, soil, water, milk, and food products). Factors that determine whether exposure to a microbe results in transient passage through a human host or prolonged colonization are complex, involving environmental factors, host characteristics, and microbial properties. Nutrients and environmental conditions must favor the survival of microbes. For example, vegetative forms of *Bacillus* and *Clostridium* spp. cannot survive prolonged exposure to desiccation or heat. However, spores of these bacteria can exist in nature for months to years. Gram-positive bacteria have a thick peptidoglycan layer in their cell wall, which promotes osmotic stability and enables these organisms to exist on dry surfaces (e.g. household articles, hospital bed linens, skin surfaces). By contrast, gram-negative bacteria have a relatively thin peptidoglycan layer and a lipoprotein outer membrane. These structures reduce gram-negative bacteria to a more fragile state, restricting their survival to moist, protected areas (e.g. standing water, vegetation, sinks, and showers). Thus, exposure to gram-positive bacteria is frequently by person-to-person contact or exposure to contaminated fomites, whereas gram-negative bacteria are usually acquired following consumption of contaminated food or drink or exposure to a contaminated water source.

Various host factors determine the success of colonization with a microbe. Although the skin forms a barrier, preventing penetration of microbes into the deeper tissues, colonization of the surface is accomplished by organisms that can tolerate the dry surface and that are resistant to fatty acids produced by anaerobic bacteria and from the metabolism of sebum triglycerides. Some organisms are adapted to grow in skin areas with a high moisture content (e.g. axilla, skin folds, perianal area), whereas other organisms survive in hair follicles. The presence of lysozyme in tears and other secretions is highly toxic for many bacteria and restricts the organisms that can colonize these areas. Inhaled organisms can be rapidly trapped in the nasal passages, engulfed with secreted mucus, and either expelled or swallowed. Organisms ingested with food or drinks are frequently eliminated when exposed to the acidic environment of the gastric system or simply passed through the digestive tract. Other organisms fail to survive interaction with locally secreted antibodies, exposure to bacteriocins produced by the normal microbiota, or the localized environment (e.g. acidic pH of the vagina). The age of the host influences microbial colonization. The presence or absence of teeth, hormone secretions that are initiated at the time of puberty or altered in menopause, sexual activity, person-to-person interactions in day-care facilities, the military, or nursing homes, alteration of dietary habits, and many other age-related factors determine which organisms an individual is exposed to and which will become successfully established as a part of the normal microbiota.

The final factors that determine the success of an organism to colonize the human body are properties of the specific organism. For example, the terrain of the oropharynx is diverse, with opportunities for organisms to colonize saliva, the mucosal surface, the tongue, the gingiva above and below the tooth line, and teeth. Extremely oxygen-sensitive bacteria are able to proliferate in the gingival crevices where the *E*h is

appropriately low. Organisms such as *Streptococcus mutans* and *Streptococcus sanguis* adhere to the hard, smooth surface of teeth by producing extracellular polysaccharides (e.g. glucans, dextrans, fructans) from dietary carbohydrates. A pellicle consisting of proteins from saliva and crevicular fluids initially forms on the enamel surface, which forms the anchor for the streptococcal polysaccharides. Subsequent layering of proteins, bacterial polysaccharides, and mixed populations of bacteria leads to the formation of plaque on the tooth surface. Bacteria can also bind to cells lining the oropharynx, intestine, and vagina via specific receptors for the bacterial pili. This ability to adhere can prevent the mechanical elimination of organisms when saliva washes the oropharynx and food and drink pass through the intestines.

With the complex environmental, host, and microbial factors that shape the normal microbiota, it should be appreciated that this population of organisms is constantly changing in an individual. For example, performing tests to determine which microbes colonize an individual's oropharynx provides data for that individual at a discrete point in time. Surveys that estimate the prevalence of colonization with microbes are at best an approximation for the individuals sampled. The ability to apply microbial colonization data from the individual studied to another person is dependent on the similarities between the two individuals. The data collected become more useful if the number of individuals studied is increased and the host factors minimized (e.g. study individuals with a broad age range, include immunocompetent and immunosuppressed patients, etc.). Furthermore, the utility of the data is determined by the thoroughness of the microbiological procedures. Adequate types and volume of specimens must be collected and transported to the laboratory in a manner that preserves the viability of the microbial population; isolation, detection, and identification procedures that will permit recognition of all significant organisms must be used. Unfortunately, studies of the normal microbiota are limited by the numbers of individuals that can be studied using comprehensive microbiological techniques. In addition, refinements in microbiology procedures and changes in the taxonomic classification of microbes make it difficult to compare results of recent studies with those published in the older literature. Finally, molecular techniques have led to the recognition of many bacteria that cannot be isolated in culture. Despite these cautions, extensive literature exists that estimates the prevalence of organisms in different body sites. Table 1 is a summary of this literature for the four major body sites that are populated with the most common bacteria colonizing humans: respiratory tract, gastrointestinal tract, genitourinary tract, and the body surface. The table indicates whether the organisms are commonly present or not present in healthy individuals. The frequency with which these organisms are recovered is discussed in the following text. The source of this information is from the accompanying chapters in this edition, as well as the review articles and reference works that are listed at the end of this chapter (Balows et al. 1988, 1992; Boone and Castenholz 2001; Clarke and Bauchop 1977; Drasar and Hill 1974; Holt et al. 1994; Jousimies-Somer et al. 2002; Maibach and Hildick-Smith 1965; Mandell et al. 2000; Murray 1998; Noble and Sommerville 1974; Rosebury 1961; Skinner and Carr 1974). The taxonomic classification of the organisms listed herein is consistent with the *Manual of clinical microbiology* (Murray et al. 2003) and websites listing currently accepted bacterial nomenclature: www.bacterio.cict.fr (Society for Systemic and Veterinary Bacteriology) and www.dsmz.de/bactnom/bactname.htm.

NORMAL MICROBIOTA OF THE RESPIRATORY TRACT

The respiratory tract can be divided anatomically into the upper airways, which includes the anterior and posterior nares and the nasopharynx, the middle airways, comprised of the oropharynx and tonsils, and the lower airways, with the larynx, trachea, bronchi, and lungs. This classification serves as a useful foundation for examining the dynamics of airway colonization. The structural and physiological differences at each site provide an environment compatible for some organisms and hostile for others.

Nares and nasopharynx

Relatively small numbers of organisms are present in the nares, with *Staphylococcus*, including *S. aureus* and coagulase-negative species, *Corynebacterium*, *Peptostreptococcus*, and *Fusobacterium* species being the most numerous. The carriage rate of *S. aureus* in the anterior nares is higher in preadolescent children than in adults. The recovery of other organisms in the nares is common, but they are usually present transiently and in small numbers. The microbial population in the nasopharynx is more complex, with a predominance of streptococci and *Neisseria* species. The viridans streptococci can be readily recovered in the nasopharynx, with *Streptococcus salivarius* and *Streptococcus parasanguis* most commonly isolated, as well as *Streptococcus pneumoniae*. Many species of *Neisseria*, including *N. meningitidis*, have been recovered in nasopharyngeal cultures. Colonization with *N. meningitidis* varies from less than 10 percent to as great as 95 percent, with the highest incidence in young adults confined to institutions and in the military, where focal epidemics of meningococcal disease are observed. The most commonly isolated species of *Neisseria* are *Neisseria subflava*, *Neisseria sicca*, *Neisseria cinerea*, *Neisseria mucosa*, and *Neisseria*

Table 1 *Human microbiota*

Organism	Site of carriage			
	Respiratory tract	GI tract	GU tract	Skin, ear, eye
Abiotrophia defectiva	+	0	0	0
Acholeplasma laidlawii	+	0	0	0
Acidaminococcus fermentans	+	+	0	0
Acinetobacter spp.	+	+	+	+
Actinobacillus spp.	+	0	0	0
Actinomyces spp.	+	+	+	0
Aerococcus christensenii	0	0	+	0
Aerococcus viridans	0	0	0	+
Aerococcus urinae	0	0	+	0
Aeromonas spp.	0	+	0	0
Alloiococcus otitis	0	0	0	+
Anaerorhabdus furcosus	0	+	0	0
Anaerococcus hydrogenalis	0	+	+	+
Anaerococcus lactolyticus	0	+	+	0
Anaerococcus prevotii	0	+	+	0
Arcanobacterium spp.	+	0	0	+
Bacillus spp.	0	+	0	+
Bacteroides caccae	0	+	0	0
Bacteroides distasonis	0	+	0	0
Bacteroides eggerthii	0	+	0	0
Bacteroides fragilis	0	+	+	0
Bacteroides merdae	0	+	0	0
Bacteroides ovatus	0	+	0	0
Bacteroides splanchnicus	0	+	0	0
Bacteroides thetaiotaomicron	0	+	0	0
Bacteroides vulgatus	0	+	0	0
Bifidobacterium adolescentis	0	+	0	0
Bifidobacterium bifidum	0	+	+	0
Bifidobacterium breve	0	+	+	0
Bifidobacterium catenulatum	0	+	+	0
Bifidobacterium dentium	+	+	+	0
Bifidobacterium longum	0	+	+	0
Bilophila wadsworthia	+	+	+	0
Brevibacterium casei	0	0	0	+
Brevibacterium epidermidis	0	0	0	+
Burkholderia cepacia complex	+	0	0	+
Butyrivibrio fibrisolvens	0	+	0	0
Campylobacter concisus	+	+	0	0
Campylobacter curvus	+	+	0	0
Campylobacter gracilis	+	+	0	0
Campylobacter jejuni	0	+	0	0
Campylobacter rectus	0	+	0	0
Campylobacter showae	+	+	0	0
Campylobacter sputorum	+	0	0	0
Capnocytophaga granulosum	+	0	0	0
Capnocytophaga gingivalis	+	0	0	0
Campylobacter haemolytica	+	0	0	0
Capnocytophaga ochracea	+	+	+	0
Capnocytophaga sputigena	+	0	0	0
Cardiobacterium hominis	+	0	0	0
Centipeda periodontii	+	0	0	0
Citrobacter freundii	0	+	0	0

(*Continued over*)

Table 1 *Human microbiota (Continued)*

Organism	Site of carriage			
	Respiratory tract	GI tract	GU tract	Skin, ear, eye
Citrobacter koseri	0	+	0	0
Clostridium spp.	0	+	0	0
Corynebacterium accolens	+	0	0	+
Corynebacterium afermentans	+	0	0	+
Corynebacterium amycolatum	0	0	0	+
Corynebacterium auris	0	0	0	+
Corynebacterium diphtheriae	+	0	0	+
Corynebacterium durum	+	0	0	0
Corynebacterium glucuronolyticum	0	0	+	0
Corynebacterium jeikeium	0	0	0	+
Corynebacterium macginleyi	0	0	0	+
Corynebacterium matruchotii	+	0	0	0
Corynebacterium minutissimum	0	0	0	+
Corynebacterium propinquum	+	0	0	0
Corynebacterium pseudodiphtheriticum	+	0	0	0
Corynebacterium riegelii	0	0	+	0
Corynebacterium simulans	0	0	0	+
Corynebacterium striatum	+	0	0	+
Corynebacterium ulcerans	+	0	0	0
Corynebacterium urealyticum	0	0	+	+
Dermabacter hominis	0	0	0	+
Dermacoccus nishinomiyaensis	0	0	0	+
Desulfomonas pigra	0	+	0	0
Dysgonomonas spp.	0	+	0	0
Eikenella corrodens	+	+	0	0
Enterobacter aerogenes	0	+	0	0
Enterobacter cloacae	0	+	0	0
Enterobacter gergoviae	0	+	0	0
Enterobacter sakazakii	0	+	0	0
Enterobacter taylorae	0	+	0	0
Enterococcus spp.	0	+	0	0
Escherichia coli	0	+	+	0
Escherichia fergusonii	0	+	0	0
Escherichia hermannii	0	+	0	0
Escherichia vulneris	0	+	0	0
Eubacterium spp.	+	+	0	0
Ewingella americana	+	0	0	0
Finegoldia magnus	0	+	+	+
Fusobacterium alocis	+	0	0	0
Fusobacterium gonidiaformans	0	+	+	0
Fusobacterium mortiferum	0	+	0	0
Fusobacterium naviforme	0	+	+	0
Fusobacterium necrophorum	+	+	0	0
Fusobacterium nucleatum	+	0	0	0
Fusobacterium sulci	+	0	0	0
Fusobacterium russii	0	+	0	0
Fusobacterium varium	0	+	0	0
Gardnerella vaginalis	0	+	+	0
Gemella haemolysans	+	0	0	0
Gemella morbillorum	+	+	0	0
Granulicatella spp.	+	0	0	0
Haemophilus spp.	+	0	0	0

(*Continued over*)

Table 1 *Human microbiota (Continued)*

Organism	Site of carriage			
	Respiratory tract	GI tract	GU tract	Skin, ear, eye
Hafnia alvei	0	+	0	0
Helcococcus kunzii	0	0	0	+
Helicobacter spp.	0	+	0	0
Kingella spp.	+	0	0	0
Klebsiella spp.	+	+	0	0
Kocuria spp.	0	0	0	+
Kytococcus sedentarius	0	0	0	+
Lactobacillus acidophilus	+	+	+	0
Lactobacillus breve	+	0	0	0
Lactobacillus casei	+	0	+	0
Lactobacillus cellobiosus	0	0	+	0
Lactobacillus fermentum	+	+	+	0
Lactobacillus reuteri	0	+	0	0
Lactobacillus salivarius	+	+	0	0
Lactococcus spp.	0	0	+	0
Leclercia adecarboxylata	0	+	0	0
Leminorella spp.	0	+	0	0
Leptotrichia buccalis	+	0	+	0
Listeria monocytogenes	0	+	0	0
Leuconostoc spp.	0	0	+	0
Megasphaera elsdenii	0	+	0	0
Micrococcus luteus	+	0	0	+
Micrococcus lylae	+	0	0	+
Micromonas micros	+	0	0	0
Mitsuokella multiacidus	0	+	0	0
Mobiluncus curisii	0	+	+	0
Mobiluncus mulieris	0	+	+	0
Moellerella wisconsensis	0	+	0	0
Moraxella catarrhalis	+	0	0	0
Morganella morganii	0	+	0	0
Mycoplasma buccale	+	0	0	0
Mycoplasma faucium	+	0	0	0
Mycoplasma fermentans	+	0	+	0
Mycoplasma genitalium	+	0	+	0
Mycoplasma hominis	+	0	+	0
Mycoplasma lipophilum	+	0	0	0
Mycoplasma orale	+	0	0	0
Mycoplasma penetrans	0	0	+	0
Mycoplasma pneumoniae	+	0	0	0
Mycoplasma primatum	0	0	+	0
Mycoplasma salivarium	+	0	0	0
Mycoplasma spermatophilum	0	0	+	0
Neisseria cinerea	+	0	0	0
Neisseria flavescens	+	0	0	0
Neisseria lactamica	+	0	0	0
Neisseria meningitidis	+	0	0	0
Neisseria mucosa	+	0	0	0
Neisseria polysaccharea	+	0	0	0
Neisseria sicca	+	0	0	0
Neisseria subflava	+	0	0	0
Oligella ureolytica	0	0	+	0
Oligella urethralis	0	0	+	0

(*Continued over*)

Table 1 *Human microbiota (Continued)*

Organism	Site of carriage			
	Respiratory tract	GI tract	GU tract	Skin, ear, eye
Pantoea agglomerans	0	+	0	0
Pastuerella bettyae	0	0	+	0
Pasteurella multocida	+	0	0	0
Peptococcus niger	0	0	+	+
Peptoniphilus asaccharolyticus	0	+	+	+
Peptoniphilus lacrimalis	+	0	0	0
Peptostreptococcus anaerobus	+	+	0	0
Peptostreptococcus productus	0	+	0	0
Peptostreptococcus vaginalis	0	0	+	+
Porphyromonas asaccharolytica	0	+	+	0
Porphyromonas catoniae	+	0	0	0
Porphyromonas endodontalis	+	0	0	0
Porphyromonas gingivalis	+	0	0	0
Prevotella bivia	0	0	+	0
Prevotella buccae	+	0	0	0
Prevotella buccalis	+	0	+	0
Prevotella corporis	+	0	0	0
Prevotella dentalis	+	0	0	0
Prevotella denticola	+	0	0	0
Prevotella disiens	0	0	+	0
Prevotella enoeca	+	0	0	0
Prevotella heparinolytica	+	0	0	0
Prevotella intermedia	+	0	0	0
Prevotella loescheii	+	0	+	0
Prevotella melaninogenica	+	0	+	0
Prevotella nigrescens	+	0	0	0
Prevotella oralis	+	0	+	0
Prevotella oris	+	0	0	0
Prevotella oulorum	+	0	0	0
Prevotella tannerae	+	0	0	0
Prevotella veroralis	+	0	+	0
Prevotella zoogleoformans	+	0	0	0
Propionibacterium acnes	0	0	0	+
Propionibacterium avidum	0	0	0	+
Propionibacterium granulosum	0	0	0	+
Propionibacterium propionicum	+	0	0	0
Propionferax innocuum	0	0	0	+
Proteus mirabilis	0	+	+	0
Proteus penneri	0	+	+	0
Proteus vulgaris	0	+	+	0
Providencia rettgeri	0	+	0	0
Providencia stuartii	0	+	0	0
Pseudomonas aeruginosa	0	+	0	0
Retortamonas intestinalis	0	+	0	0
Rothia dentocariosa	+	0	0	0
Rothia mucilaginosa	+	0	0	0
Ruminococcus productus	0	+	0	0
Selenomonas spp.	+	0	0	0
Serratia liquefaciens	0	+	0	0
Serratia marcescens	0	+	0	0
Serratia odorifera	0	+	0	0
Staphylococcus aureus	+	0	+	+

(*Continued over*)

Table 1 *Human microbiota (Continued)*

Organism	Site of carriage			
	Respiratory tract	GI tract	GU tract	Skin, ear, eye
Staphylococcus auricularis	0	0	0	+
Staphylococcus capitis	0	0	0	+
Staphylococcus caprae	0	0	0	+
Staphylococcus cohnii	0	0	0	+
Staphylococcus epidermidis	+	0	+	+
Staphylococcus haemolyticus	0	0	0	+
Staphylococcus hominis	0	0	0	+
Staphylococcus lugdunensis	0	0	0	+
Staphylococcus pasteuri	0	0	0	+
Staphylococcus saccharolyticus	0	0	0	+
Staphylococcus saprophyticus	0	0	+	+
Staphylococcus simulans	0	0	0	+
Staphylococcus xylosus	0	0	0	+
Staphylococcus warneri	0	0	0	+
Streptococcus agalactiae	0	+	+	0
Streptococcus anginosus	+	+	+	0
Streptococcus bovis	0	+	0	0
Streptococcus constellatus	+	+	+	0
Streptococcus criceti	+	0	0	0
Streptococcus crista	+	0	0	0
Streptococcus equisimilis	+	0	0	0
Streptococcus gordonii	+	0	0	0
Streptococcus intermedius	+	+	+	0
Streptococcus mitis	+	0	0	0
Streptococcus mutans	+	0	0	0
Streptococcus oralis	+	0	0	0
Streptococcus parasanguis	+	0	0	0
Streptococcus pneumoniae	+	0	0	0
Streptococcus pyogenes	+	0	0	+
Streptococcus salivarius	+	0	0	0
Streptococcus sanguis	+	0	0	0
Streptococcus sobrinus	+	0	0	0
Streptococcus vestibularis	+	0	0	0
Succinivibrio dextrinosolvens	0	+	0	0
Tissierella praeacuta	0	+	0	0
Treponema denticola	+	0	0	0
Treponema maltophilum	+	0	0	0
Treponema minutum	0	0	+	0
Treponema phagedenis	0	0	+	0
Treponema refringens	0	0	+	0
Treponema socranskii	+	0	0	0
Treponema vincentii	+	0	0	0
Turicella otitidis	0	0	0	+
Ureaplasma urealyticum	+	0	+	0
Veillonella spp.	+	+	0	0
Weeksella virosa	0	0	+	0

GI, gastrointestinal tract; GU, genitourinary tract; skin, includes ears and eyes as well as skin.

lactamica. Multiple species coexist in more than half of all individuals. Related gram-negative coccobacilli that colonize the nasopharynx include *Moraxella catarrhalis*, now recognized as a common cause of respiratory tract infections (e.g. sinusitis, bronchitis), and *Kingella* spp. Unencapsulated strains, as well as occasional encapsulated strains, of *H. influenzae* are commonly found in the nasopharynx, as is *Cardiobacterium hominis*, an organism associated with infections of previously damaged heart valves.

Oropharynx and tonsils

The oropharynx is a complex mixture of ecosystems, each with a distinctive microbial population. Thus, predictable differences in organisms will be found in saliva, gingival crevices, surfaces of teeth, the tongue, and the mucosal lining. Gram-positive and gram-negative cocci predominate in the oropharynx. Overall, anaerobes outnumber aerobic bacteria 100 to 1. The most common anaerobic bacteria are *Peptostreptococcus*, related gram-positive cocci (i.e. *Micromonas*, *Peptoniphilus*), *Veillonella*, *Actinomyces*, and *Fusobacterium*; the most common aerobic bacteria are *Streptococcus* and *Neisseria*.

Relatively small numbers of staphylococci are present, although it has been estimated that as many as 20 percent of individuals are colonized with *S. aureus*. Streptococcal species are more numerous, particularly members of the viridans group. *S. salivarius* is present in high numbers in saliva (as the name implies) and on the surface of the tongue. Tooth surfaces are colonized with *S. sanguis* and *S. mutans*, whereas the oral mucosa is populated with *Streptococcus vestibularis* and *S. sanguis*. Other streptococci that colonize the oropharynx include *S. pneumoniae*, commonly present in children and adults with children, and β-hemolytic streptococci, including groups A, C, F, and G. Group A *Streptococcus* (*Streptococcus pyogenes*, the organism responsible for streptococcal pharyngitis) can transiently colonize healthy individuals or become a more permanent member of the oral microflora. Other gram-positive cocci that are present in the oropharynx include *Rothia mucilaginosa*, *Gemella* species, *Peptostreptococcus anaerobius*, *Micromonas micros*, and *Abiotrophia* species. Both *Rothia* and *Peptostreptococcus* are found in virtually all individuals.

As in the nasopharynx, gram-negative cocci and coccobacilli can colonize the oropharynx. *Veillonella* species are the most numerous gram-negative cocci found in the oropharynx, representing as much as 15 percent of the total bacterial population. *Veillonella atypica* and *Veillonella dispar* are present on the tongue, oral mucosa and in saliva. *Veillonella parvula* is present in subgingival spaces and in dental plaque, in higher numbers in dental caries. Other gram-negative cocci and coccobacilli include *Neisseria*, *Moraxella*, *Kingella*, *Cardiobacterium*, and *Eikenella*. *Eikenella corrodens* can establish residence in the oropharynx by adhering to buccal epithelial cells.

Gram-positive bacilli are also prominent members of the oropharyngeal flora. *Actinomyces* are present in large numbers, comprising 20 percent of the bacterial flora in saliva and on the tongue, 35 percent in gingival crevices, and 40 percent of the bacteria in dental plaque. The *Actinomyces* spp. present in the oropharynx are *Actinomyces israelii*, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Actinomyces meyeri*, *Actinomyces georgiae*, and *Actinomyces gerencseriae*. *A. israelii* and *A. naeslundii* also colonize the surface of the tonsils. The ability of *Actinomyces* spp. to colonize the various surfaces is mediated by the presence of fimbriae that can adhere to mucosal cells and the production of extracellular polysaccharides that form a slime over tooth surfaces, entrapping organisms and preventing their removal by the flow of saliva and food through the oropharynx. Other genera of gram-positive bacilli present in the oropharynx include *Bifidobacterium*, *Corynebacterium*, *Eubacterium*, *Lactobacillus*, *Propionibacterium*, and *Rothia*. *Bifidobacterium dentium* is isolated in dental plaque and species of *Lactobacillus* are prominently associated with carious teeth, thriving in the acid environment produced by the streptococci responsible for the development of caries. *Eubacterium* spp. are isolated from subgingival crevices, dental plaques, and calculus.

The predominant gram-negative bacilli in the oropharynx are anaerobes, including *Fusobacterium*, *Bacteroides*, *Porphyromonas*, *Prevotella*, and *Selenomonas*. *Fusobacterium nucleatum* is the most common fusobacterium in the mouth, *Fusobacterium alocis* and *Fusobacterium sulci* are isolated in the gingival crevices, and the other species are present in smaller numbers throughout the mouth. Many of the anaerobic gram-negative bacilli in the mouth that were formerly classified as *Bacteroides* are now *Prevotella* and *Porphyromonas* spp. However, at least four species found in the mouth have been retained in the genus *Bacteroides*, while multiple species of *Prevotella* and two *Porphyromonas* species colonize the mouth. At least six species of *Selenomonas* are present in the oropharynx, primarily in gingival crevices.

Haemophilus spp. are present in almost all individuals although in small numbers (<5 percent of the microbial population). *H. parainfluenzae* is the most common *Haemophilus* species present in the mouth. *H. influenzae* is less commonly present, with most strains nonencapsulated. Other species are found on the tongue, palate, cheeks, and teeth, associated with dental plaque and periodontal disease. *Actinobacillus actinomycetemcomitans* is commonly isolated in the oropharynx and is an important cause of juvenile periodontitis. Another group of bacteria commonly found in the oropharynx, particularly in the gingival crevices, and associated with periodontal disease are *Treponema* species.

Members of the Enterobacteriaceae and nonfermentative gram-negative bacilli, such as *Pseudomonas* spp. and *Acinetobacter* are present in the oropharynx of healthy individuals, but usually only in small numbers or transiently. This changes in debilitated or hospitalized patients in whom these organisms can become the predominant bacteria in the oropharynx and are frequently responsible for lower respiratory tract disease.

Trachea, larynx, bronchi, and lungs

Colonization of the lower airways is generally transient, with relatively few organisms present at any one time. The only time long-term colonization occurs is when the ciliated epithelial cells are damaged through infection (e.g. with influenza virus) or disease (e.g. chronic obstructive pulmonary disease). This permits drainage of respiratory secretions into the bronchials and lower airways, with subsequent proliferation of the microbes.

NORMAL MICROBIOTA OF THE GASTROINTESTINAL TRACT

As with the respiratory tract, the gastrointestinal tract can be subdivided into distinct anatomical areas, each harboring its own indigenous microbiota. These would include the esophagus, stomach, jejunum and upper ileum, distal small intestine, and large intestine. It should be obvious that within each of these areas, the microbial flora on the mucosa, within crypts, and in the lumen can also be different. Unfortunately, the ability to sample each area without contamination from other sites in healthy individuals is limited. The most complete information has been obtained for the stomach and the large intestine. The microbial flora at other sites is estimated by studying the microbial composition of feces or the specimens collected at the time of intraabdominal surgery.

Esophagus

Insufficient information is available about the microbial flora of the esophagus. However, oropharyngeal bacteria can be isolated from this site, as can the organisms that colonize the stomach. Transient colonization occurs with these organisms in healthy individuals. In the diseased state, bacteria are uncommon causes of esophagitis and esophageal infections, with yeasts (e.g. *Candida*) and viruses (e.g. herpes simplex, cytomegalovirus) playing a more prominent role.

Stomach

The stomach is an inhospitable organ, containing hydrochloric acid and pepsinogen (a precursor of pepsin) secreted by parietal and chief cells, respectively, which line the gastric mucosa. For this reason, the normal microbial microbiota are sparse, primarily associated with the surface epithelium, and protected by secretions of mucus and bicarbonate. The organisms present in the stomach are acid-tolerant *Lactobacillus* spp., *Streptococcus* spp., and *Helicobacter pylori*. Whereas the first two organisms are not associated with gastric disease, *H. pylori* causes gastritis and gastric (peptic) and duodenal ulcers, and is associated with gastric malignancies. Other organisms may be isolated in the stomach, particularly a few hours after a meal, but are believed to represent transient passage through the stomach.

Jejunum and upper ileum

The number of microbes in the upper portion of the small intestine is low (generally fewer than 10^5 organisms per milliliter of fluid) and predominantly anaerobic, consisting primarily of *Lactobacillus*, *Streptococcus*, *Peptostreptococcus*, *Anaerococcus*, *Finegoldia*, *Peptoniphilus*, *Porphyromonas*, and *Prevotella*. If upper-tract obstruction and stasis occur (e.g. blind loop syndrome), then the microbial flora can shift to resemble colonic bacteria (e.g. *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Escherichia*, *Enterococcus*) and lead to a malabsorption syndrome.

Distal small intestine

This is the transition area between the relatively sparse numbers of acid-tolerant bacteria that occupy the upper portion of the intestinal tract and the plethora of microbes that exist in the large intestine. Although it is unclear which organisms colonize this portion of the gastrointestinal tract permanently, it is known that the microbial population is large (approximately 10^8–10^9 organisms per gram of feces) and diverse, with a distinct predominance of strict anaerobes.

Large intestine

This is the most densely populated organ in the human body, with more than 10^8 aerobic bacteria and 10^{11} anaerobic bacteria per gram of feces. The most numerous bacteria in the large intestine are *Bifidobacterium* spp., *Bacteroides* spp., *Eubacterium* spp., *Enterococcus* spp., and *E. coli*. It has been estimated that feces consist of 10^{11} bacteroides per gram. Although *B. fragilis* is the most virulent species, *Bacteroides thetaiotaomicron* is more numerous in the colon. Other *Bacteroides* spp. in the colon include *Bacteroides capillosus*, *Bacteroides coagulans*, *Bacteroides putredinis*, and *Bacteroides ureolyticus*. *Eubacterium* spp. are the second most commonly isolated bacteria in the intestine, with more than 10^{10} organisms per gram of feces and 16 distinct species described. The most commonly isolated species are *Eubacterium aerofaciens*, *Eubacterium contortum*, *Eubacterium cylindoides*, *Eubacterium lentum*, and *Eubacterium rectale*. Ten species of *Bifidobacterium* have been isolated in feces, some species isolated preferentially from infants and others from adults. The most commonly isolated species are *B. bifidum*, *B. longum* and *B. adolescentis*. Many species of *Enterococcus* have been described, most of which are present in the human intestines. *Enterococcus faecalis*,

Enterococcus faecium, *Enterococcus casseliflavus*, and *Enterococcus gallinarum* are most frequently isolated. *E. coli* inhabits the intestine of virtually all humans, establishing intestinal colonization soon after an infant is born. Although it represents a relatively minor position quantitatively (approximately 1 percent of the total bacterial population), it is the most common facultative organism responsible for intra-abdominal infections.

A large number of other organisms have been demonstrated to colonize the large intestine. *Actinomyces* are frequently isolated in fecal specimens, even though intestinal colonization has not been clearly demonstrated. *Streptococcus* spp., including *Streptococcus bovis*, which is associated with intestinal malignancies, have been isolated from fecal specimens.

Gemella morbillorum is a member of the normal intestinal flora, as are a variety of gram-positive anaerobic cocci (e.g. *Anaerococcus prevotii*, *Finegoldia magnus*, *Peptoniphilus asaccharolyticus*). The spore-forming *Bacillus* spp. and *Clostridium* spp. are isolated in fecal specimens. Although this may represent simple transit through the gastrointestinal tract following ingestion with food or drink, most authors believe that *Clostridium* spp. are part of the permanent intestinal population. *V. parvula* can colonize the intestinal tract of humans, although it is generally present in small numbers. Like *E. coli*, other members of the Enterobacteriaceae can establish residence in the intestines. *Citrobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., and various other genera can be consistently isolated in fecal specimens. *Haemophilus* spp. can be recovered in fecal specimens if selective media are used. Other organisms commonly isolated, but in small numbers, include species of *Fusobacterium*, *Porphyromonas*, and *Prevotella*. Uncultivable species of *Treponema* have been observed in fecal specimens.

NORMAL MICROBIOTA OF THE GENITOURINARY TRACT

The genitourinary tract is relatively sterile, with the exception of the female urethra and vagina. Microbes can migrate up the urethra into the bladder, but these are rapidly cleared in healthy individuals by the action of localized antibodies, microbicidal activity of the epithelial cells lining the bladder, and the flushing action of voided urine. The ureters, kidneys, prostate, and cervix are normally sterile. The female urethra is colonized with large numbers of lactobacilli, streptococcal species, and coagulase-negative staphylococci. Fecal organisms such as *Escherichia coli* and *Enterococcus* spp. can also colonize the female urethra but are generally transient and present in small numbers. When these latter organisms migrate up into the bladder, they are able to proliferate in urine and can establish a urinary tract infection.

The microbial flora in the vagina are more numerous and diverse. Lactobacilli are the predominant organisms because they are able to proliferate in the acidic environment. The most commonly isolated species include *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus casei*, and *Lactobacillus cellobiosus*. Other anaerobes commonly isolated in vaginal secretions are *Bifidobacterium*, anaerobic cocci, *Porphyromonas*, and *Prevotella*. Six species of *Bifidobacterium* have been recovered in the vagina; *B. bifidum* and *B. longum* are the most numerous. Likewise, eight species of anaerobic cocci (i.e. *Peptococcus niger*, *Peptoniphilus asaccharolyticus*, *Anaerococcus hydrogenalis*, *A. lactolyticus*, *A. prevotii*, *A. tetradius*, *Finegoldia magna*, and *Peptostreptococcus vaginalis*) reside in the vagina. *Porphyromonas asaccharolytica*, *Prevotella bivia*, and *Prevotella disiens* are also important residents of the vagina. *Actinomyces* spp. are believed to be present in the vagina because they are associated with vaginal infections. However, demonstration of their presence is controversial. *A. israelii* is most commonly associated with genital actinomycotic infections. *Propionibacterium*, particularly *Propionibacterium propionicus*, is also present in the vagina. Finally, *Mobiluncus* spp. are relatively uncommon in healthy women but a significant cause of bacterial vaginosis. Thus, it is likely that this anaerobe is present in the vaginal microbial flora in small numbers.

Common aerobic bacteria present in the vagina include *Staphylococcus* (primarily coagulase-negative species), *Streptococcus*, and *Corynebacterium* spp. The viridans group and β-hemolytic strains (e.g. groups B, C, and G) of streptococci are present in the vagina. *Gardnerella vaginalis* can also colonize the human genital and urinary tract, although it is only present in high numbers in women with vaginosis and their male partners. *Neisseria* spp., including *N. meningitidis*, are recovered in vaginal secretions, as are species of *Haemophilus* (including *H. influenzae* and *H. parainfluenzae*). Although these organisms can be recovered from a large proportion of individuals, they are generally part of the minor bacterial population. Members of the Enterobacteriaceae, particularly *E. coli*, can be found in the vaginal flora, although generally in small numbers. *Weeksella virosa* is almost exclusively isolated in genital specimens, particularly from sexually active women. Three species of nonpathogenic *Treponema* (*Treponema phagedenis*, *Treponema refringens*, and *Treponema minutum*) are isolated in vaginal specimens.

Six species of *Mycoplasma* (*Mycoplasma hominis*, *Mycoplasma genitalium*, *Mycoplasma fermentans*, *Mycoplasma primatum*, *Mycoplasma spermatophilum*, and *Mycoplasma penetrans*) primarily colonize the genitourinary tract. In addition, the related organism *U. urealyticum* is a common inhabitant of this site. The role of these organisms in disease is controversial, but clearly

M. hominis and *U. urealyticum* have pathogenic potential.

NORMAL MICROBIOTA OF THE BODY SURFACE

The surface of the skin is relatively inhospitable in comparison with other body sites. It is exposed to extremes in temperature and moisture and to chemical disinfectants such as soaps and shampoos. It is not surprising, therefore, that the microbial population is less numerous and complex than at other body sites. Despite this observation, many organisms, particularly gram-positive bacteria, are able to establish permanent residence on the skin surface. Transient colonization with a diverse array of environmental and endogenous microbes can also occur.

As with other areas of the body, the skin should not be considered a homogeneous surface, but rather a landscape of mountains and valleys, each with a specific environment and microbial population. Thus, there are relatively dry, hairless areas such as palms and soles, areas with a proliferation of apocrine glands such as the axillae, inguinal, and perineal areas, and areas rich with sebaceous glands such as the forehead and nasolabial folds. Each area is associated with distinct microbes. Most microorganisms proliferate in a moist environment, hence higher densities of microbes are present in areas rich with sweat glands or on occluded surfaces. The skin proximal to the oral cavity (face), gastrointestinal tract (perirectal area), or genitourinary tract (groin) has a more complex microbial flora than at other surface sites, although most of these organisms result from surface contamination and only transiently colonize the skin surface.

Anaerobic bacteria are ten- to 100-fold more numerous on the skin surface compared with aerobic bacteria; gram-positive bacteria predominate over gram-negative bacteria. The bacteria most commonly recovered on the skin surface are members of the genera *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Peptococcus*, *Peptoniphilus*, *Finegoldia*, *Peptostreptococcus*, and *Propionibacterium*. *Staphylococcus epidermidis* is the most frequently isolated bacterium, present on surfaces where the moisture is highest. Other coagulase-negative staphylococci that are found on the skin include *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Staphylococcus warneri*, *Staphylococcus capitis* (particularly on the forehead and face after puberty), *Staphylococcus saprophyticus*, *Staphylococcus caprae*, *Staphylococcus saccharolyticus*, *Staphylococcus pasteuri*, *Staphylococcus lugdunensis*, *Staphylococcus simulans*, and *Staphylococcus xylosus*. *Staphylococcus auricularis* is the most common species found colonizing the exterior auditory canal. The coagulase-positive *S. aureus* can colonize the skin adjacent to the nares and in moist folds and, less frequently, at other sites.

Micrococcus luteus is the most common *Micrococcus* sp. present on the skin, found in virtually all adults and representing as much as 20 percent of the bacterial population on the head, legs, and arms. However, relatively few micrococci are present in areas with a high moisture content and other competitive bacteria (e.g. axillae, nares). Additional gram-positive cocci (formerly classified as *Micrococcus*) that colonize the skin surface include *Dermacoccus nishinomiyaensis*, *Kocuria kristinae*, *Kocuria rosea*, *Kocuria varians*, and *Kytococcus sedentarius*.

Aerococcus viridans, *S. pyogenes*, and various anaerobic *Peptostreptococcus* spp. can also establish residence on the skin surface. *Aerococcus* is an airborne bacterium, so the incidence of true colonization versus contamination and transient colonization is unknown. *S. pyogenes* differ from other streptococci in their tolerance to the dry surfaces of the skin and to the bactericidal fatty acids from sebum and also produced by the anaerobic cocci and bacilli. Thus, they are particularly well suited for dry skin surfaces. Anaerobic cocci survive in the anaerobic niches of hair follicles and skin glands.

Many species of *Corynebacterium* spp. are present on the skin surface, including *Corynebacterium striatum*, *Corynebacterium minutissimum*, *Corynebacterium pseudodiphtheriticum*, *Corynebacterium xerosis*, *Corynebacterium urealyticus* (especially in the groin area), and *Corynebacterium jeikeium* (on moist surfaces rich in apocrine glands in hospitalized patients). The anaerobic counterpart to these aerobic gram-positive bacilli are the *Propionibacterium*. *Propionibacterium acnes* is found in high concentrations in areas rich in sebaceous glands, as is *Propionibacterium granulosum*. *P. acnes* is the predominant species, found in high numbers on virtually all individuals, whereas *P. granulosum* is recovered in small numbers and on fewer than 20 percent of individuals sampled.

Propionibacterium avidum, by contrast, requires an area high in moisture for survival and is most commonly found in axilla and perineum. Other resident gram-positive bacilli include *Dermabacter hominis*, *Brevibacterium epidermidis*, *Brevibacterium casei*, and poorly defined *Brevibacterium* spp. *Turicella otitidis* colonizes the external ear. Some species of *Bacillus* and *Clostridium*, particularly *Clostridium perfringens*, can colonize the skin surface due to their ability to form spores and withstand desiccation and detergents. However, they are generally present in small numbers and only transiently.

Gram-negative bacteria are generally not recovered from the surface of the skin, except during transient colonization. The outer portion of the cell wall of gram-negative bacilli consists of a lipid membrane that is unable to survive exposure to detergents or the dry surfaces of the skin. However, *Acinetobacter* spp. have adapted to survive in moist areas such as toe webs, the groin, and axillae. *Burkholderia* species can also colonize the skin surface but generally is found only transiently.

NORMAL MICROBIOTA OF BLOOD, CEREBROSPINAL FLUID, AND OTHER BODY FLUIDS

The human body is bathed in a variety of fluids, including blood, cerebrospinal, synovial, pleural, pericardial, peritoneal, and other exudates and transudates. All of these fluids are normally sterile or only transiently infected. Whereas microbes from the mouth or gastrointestinal tract can invade the bloodstream in healthy individuals (e.g. during tooth brushing or a bowel movement), these organisms are rapidly removed and generally of little or no significance. Thus, the isolation of an organism from a body fluid should be considered significant unless the specimen is contaminated during the process of collection.

NORMAL MICROBIOTA OF BODY TISSUES

As with body fluids, organ tissues are generally sterile unless they are infected following the systemic spread of an organism in the bloodstream. Some organisms (e.g. *Mycobacterium tuberculosis*) may be disseminated to tissues such as the lungs, liver, or kidneys at the time of initial infection and remain dormant for many years. In this situation it is possible that the organism could be recovered from tissue samples, but even that would be unlikely unless the disease process was active.

CONCLUDING COMMENTS

From the moment of birth until the terminal breath, the human body serves as a home for qualitatively and quantitatively numerous microbes. They cover the skin and mucosal surfaces of the body, occasionally invade into sterile tissues and fluids to produce disease, but more typically coexist with each other and their human host. These microbiota function as a microbial barrier to more virulent microorganisms, supplement the human host's mechanical and enzymatic digestion of food, provide vitamins and other required growth factors for their host, or simply exist as a commensal inhabitant on their host. The complexity of the microbiota is shaped over time by environmental, host, and microbial factors, but it is remarkably predictable for a general population. Certainly the dominant microbial species at each of the major body sites (e.g. respiratory tract, gastrointestinal tract, genitourinary tract, and body surface) are now well known. Variations from the expected population can predictably lead to disease (e.g. replacement of the microbial population of the small intestine with bacteria from the large intestine, leading to a malabsorption syndrome). Likewise, spread of bacteria from their normal habitat into sterile tissues and fluids can initiate well-characterized disease (e.g. colonic perforation leads to a polymicrobic peritonitis and intraabdominal abscess formation by *B. fragilis*). Thus, knowledge of the human microbiota forms a fundamental building block for our knowledge of the normal physiological processes in the human body and our understanding of infectious diseases.

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Epidemiology of infectious diseases

STEPHEN R. PALMER AND MEIRION R. EVANS

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Epidemiology is the study of the patterns of occurrence and causes of disease in populations. It does not stand alone, but is complementary to microbiology and environmental risk assessment methods of investigation. For general accounts of epidemiology see Hennekens and Buring (1987), Beaglehole et al. (1993), Detels et al. (2002), and Rothman (2002). The occurrence of an episode of microbial disease is a result of the interaction of the agent, host, and environmental factors that leads to the exposure of the host to sufficient numbers of the agent in the appropriate transmission mode. Successful investigation and control require that not only the microbiological, but also behavioral, genetic, environmental, and social factors which influence the occurrence and presentation of the disease be taken into account. Close collaboration between epidemiologists, microbiologists, and other public health workers is therefore essential. Accurate laboratory diagnosis is usually a major factor in successful control, though epidemiological methods alone may be sufficient to indicate appropriate interim control measures. For example, the modes of transmission and the risk behavior leading to the acquired immune deficiency syndrome (AIDS) were identified by epidemiological methods 2 years before the causative agent was discovered. Advice to the public about reducing the risk of infection did not need to be revised substantially when the human immunodeficiency virus (HIV) was identified. However, the discovery of HIV was a major advance and allowed the development of diagnostic tools that are now used to study epidemiological characteristics of the infection, e.g. incubation period, progression rate to AIDS, spectrum of clinical disease, period of infectivity. Increasingly, the epidemiological method is seen by microbiologists as a necessary tool in setting priorities for the use of scarce resources. Applications of epidemiology in microbiological disease include population surveillance to monitor trends in disease and infections and to detect case clusters, the investigation of sources and modes of transmission of microorganisms, applying public health measures to control outbreaks of disease, and devising and evaluating preventive and control programs.

CONCEPTS IN THE EPIDEMIOLOGY OF INFECTIOUS DISEASE

Reservoir of infection

This is where the infectious agent normally lives and where it may multiply or survive; it may be human (e.g. in chickenpox), animal (e.g. in brucellosis) or the inanimate environment (e.g. in tetanus).

Source of infection

Infection may be derived from the patient's own microflora (endogenous), or from another human being, or an animal (zoonosis) or an environmental source (exogenous). The source of an exogenous infection may sometimes be different from its reservoir. For example,

in an outbreak of cryptosporidiosis in Maine, the reservoir of infection was a herd of cattle on a farm that supplied apples for making apple cider. The apple cider then became the source of infection for human subjects. *Cryptosporidium* oocysts were detected in the apple cider, on the cider press, and in a stool specimen of a calf on the farm which supplied the apples (Millard et al. 1994). When the source of infection is inanimate, e.g. food, water, or fomites, it is termed the vehicle of infection.

Mode of transmission

The mechanism by which an infectious agent passes from the reservoir or source of infection to the person can be classified as follows.

FOOD-, DRINK-, OR WATER-BORNE INFECTION

Examples of this type of infection are typhoid and cholera. The term 'food poisoning' has in the past sometimes been restricted to incidents of acute disease in which the agent has multiplied in the food vehicle before ingestion (e.g. food-borne salmonellosis), or where it may have formed toxins (e.g. botulism).

DIRECT OR INDIRECT CONTACT

This includes spread from cases or carriers, animals, or the environment to other persons who are 'contacts'. Within this category possible routes include: feces-to-hand-to-mouth spread (e.g. shigellosis); sexual transmission (e.g. gonorrhea); skin or mucous membrane contact (e.g. wound infection, cutaneous anthrax).

PERCUTANEOUS INFECTION

This includes: insect-borne transmission via the bite of an infected insect either directly from saliva (e.g. malaria); or indirectly from insect feces contaminating the bite wound (e.g. typhus); transfusion of contaminated blood and inoculation of blood or blood products from needle stick injuries (e.g. hepatitis B); direct transmission through intact skin (e.g. schistosomiasis), or broken skin (e.g. leptospirosis).

AIR-BORNE INFECTION

Infectious organisms may be inhaled as: droplets (e.g. streptococcal pharyngitis); droplet nuclei (e.g. tuberculosis); aerosols (e.g. Legionnaires' disease); dust (e.g. ornithosis); or spores (e.g. anthrax).

TRANSPLACENTAL INFECTION

Examples include listeriosis, rubella, cytomegalovirus (CMV), and toxoplasmosis.

Occurrence

An infection that is continuously in a population is said to be endemic, whereas an increase in incidence above the endemic level is described as an epidemic, or pandemic when the epidemic is worldwide. Cases may be sporadic, that is, not known to be related to other cases or infections, or clustered in outbreaks which may be defined as two or more related cases or infections, suggesting the possibility of a common source or transmission between cases. Three commonly used measures of occurrence of disease or infection are the incidence rate, the rate of occurrence of new cases in a defined population (e.g. 10 cases per 100 000 persons per year); cumulative incidence or risk, which is the proportion of people who become diseased during a specific period; and prevalence, the proportion of a defined population with the disease at a point in time (point prevalence) or during a defined period of time (period prevalence). The prevalence of a disease depends upon its incidence and duration. In chronic diseases, prevalence may be high although incidence is low, but in short-duration infectious diseases prevalence approximates to incidence.

The attack rate during an outbreak is a type of cumulative incidence, the proportion of the population at risk at the beginning of a time period who became ill during the period. The secondary attack rate is the attack rate in the contacts of primary cases due to person-to-person spread.

Incubation period

This is the time from infection to the onset of symptoms (Richardson et al. 2001). For each organism there is a characteristic range within which infecting dose and portal of entry, as well as host factors such as age (Glynn and Palmer 1992) and immunosuppression, give rise to individual variability.

Host response

This depends upon the dose of the infecting agent and the susceptibility of the host, perhaps influenced by genotype, age, sex, other concurrent disease, immunity, and 'risk factors' such as smoking for Legionnaires' disease. In an outbreak of infection there is often a spectrum of clinical response ranging from no symptoms to fulminant disease and death.

Communicability

The infectious agent may be passed to others over a variable time, the period of communicability (Richardson et al. 2001); in some infections even from symptomless temporary or chronic carriers (e.g. *Salmonella* Typhi).

EPIDEMIOLOGICAL METHODS

Collection of observations

Investigation of the occurrence and distribution of disease requires accurate definition of the disease and its possible determinants and the measurement of their frequency in the population. A clear case definition is essential in epidemiological studies. If serological or cultural confirmation of the diagnosis is not possible, because a specific laboratory test is lacking or appropriate samples were not collected, the case definition will depend upon the clinical features as, for example, with AIDS before the discovery of HIV. During the investigation of an outbreak, laboratory confirmation of the diagnosis may be possible only in a few cases; a clinical definition will then need to be used in further studies. The specificity of the diagnosis in some patients may be doubtful; in these circumstances it is helpful to classify cases as 'definite', 'probable', or 'possible'. When case definitions are based on the presence of symptoms these must be precisely defined, e.g. 'by diarrhea we mean at least $\geqslant$3 loose or watery stools in a 24 h period', so that all respondents understand the questions in the same way and the comparability of different studies can be assessed.

Routine sources of data on infectious diseases are described in a later section. In detailed epidemiological enquiries, however, other sources must be used: medical records, interviews with patients, and questionnaires.

Medical records

Clinical records may contain data on symptoms, investigations performed and their results, and personal details of patients, but for epidemiological purposes clinical records are seldom sufficient. The data recorded are likely to be accurate but incomplete, since they are not normally collected in a standard way from each patient. Scrutiny of these records may be useful in confirming the reported diagnosis and assessing whether the patient meets the case definition, and laboratory records usually provide an invaluable source of microbiological data and data on age, sex, and the geographical distribution of cases. However, to obtain detailed, accurate clinical information, interviews with patients are usually required. For example, in a survey of hydatid disease, a review of clinical records yielded data on the patients' age, sex, and home address, and on the results of clinical investigations and pathological tests. However, data on past history of residence, occupation, and exposure to dogs were seldom recorded; this could be obtained only by questioning the patients (Palmer and Biffin 1987).

Epidemiological interviews and questionnaires

To ensure accurate and comparable records of all persons included in the enquiry and to facilitate analysis, the data should be collected on a carefully designed standard form or questionnaire. Whenever possible, the questionnaire should be tested on a few cases and changes made as necessary before use in the main study. Administration of the questionnaire will often be by direct face-to-face interview by a single investigator or group of investigators trained to administer the questionnaire. Interview by telephone may be useful in obtaining data quickly. When numbers are large and the enquiry is straightforward, a self-administered postal questionnaire is cheaper and quicker to administer, but the response rate and accuracy of the data may be less than those obtained by interview.

When designing questionnaires it is important to take into account the limitations of people's recall of events. For example, in one study, food consumption recalled by people 2–3 days after a luncheon was compared with that observed at the time and recorded on tape (Decker et al. 1986). Only four of 32 patients made no errors in reporting. The sensitivity of the food-history questionnaire was 87.6 percent and its specificity 96.1 percent. Thirteen percent of the respondents reported eating one or both of two food items that were on the questionnaire but not served at the luncheon. Such errors in recall can be reduced by providing background details of events, by design of good questionnaires, and by making use of other sources to check data, such as diaries, menus, discussion with relatives, etc. Random errors in recall are unlikely to give rise to false associations between illness and, for example, an item of food, but they will reduce the power of the study to identify the true vehicle of infection. For some types of data, such as history of immunization, recall by patients or parents is of very limited value; studies of vaccine efficacy usually require validation of vaccine history from medical records.

Descriptive analysis

Variables measured may be:

- fixed or discrete (e.g. sex, occupation, nationality) or
- continuous (e.g. age, white blood cell count).

Analysis of the distribution will usually be by calculation of proportions of people who fall within certain categories or rates of occurrence of disease within subgroups of the population. Analysis of continuous variables is more complex because values obtained from a population will form a continuous distribution, usually approximating to a normal or skewed normal distribution (Figure 1). This distribution may be summarized

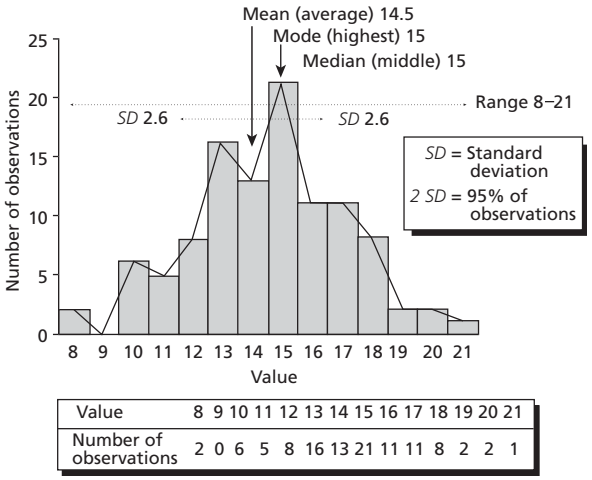

Figure 1 *Frequency distribution and measures of continuous variable*

by the mean, median, mode, and the standard deviation; these measures are commonly used to compare continuous variables in different populations.

The data should first be analyzed within the three classical epidemiological parameters of time, place, and person, taking into account interactions of these variables.

TIME

The epidemic curve is the most useful and immediate means of assessing the type of outbreak (Figure 2). In point-source outbreaks in which all cases are exposed at a given time, onset of symptoms of all primary cases will cluster within the range of the incubation period. For example, in the winter term of 1982, two campylobacter outbreaks were reported in boarding schools in the south of England. In the first of these (Figure 2a), 102 of 780 boys were admitted to the sanatorium with gastrointestinal illness, 46 of them on 1 day; this explosive outbreak was probably due to post-pasteurization contamination of the milk supply on one particular day. The other outbreak occurred in a school supplied with unpasteurized milk and was due to a continuing or recurring source of contamination. In this case the epidemic curve extended over several incubation periods (Figure 2b); 35 of 370 boys were admitted to the sanatorium over the first weeks of the term. In some outbreaks, a point-source of infection may be followed by person-to-person spread as in an outbreak of *Salmonella* Typhimurium, phage-type 10, infection affecting 66 students and one member of staff in a university hall of residence in Bristol in March 1980 (Palmer et al. 1981). The main wave of the outbreak was due to the

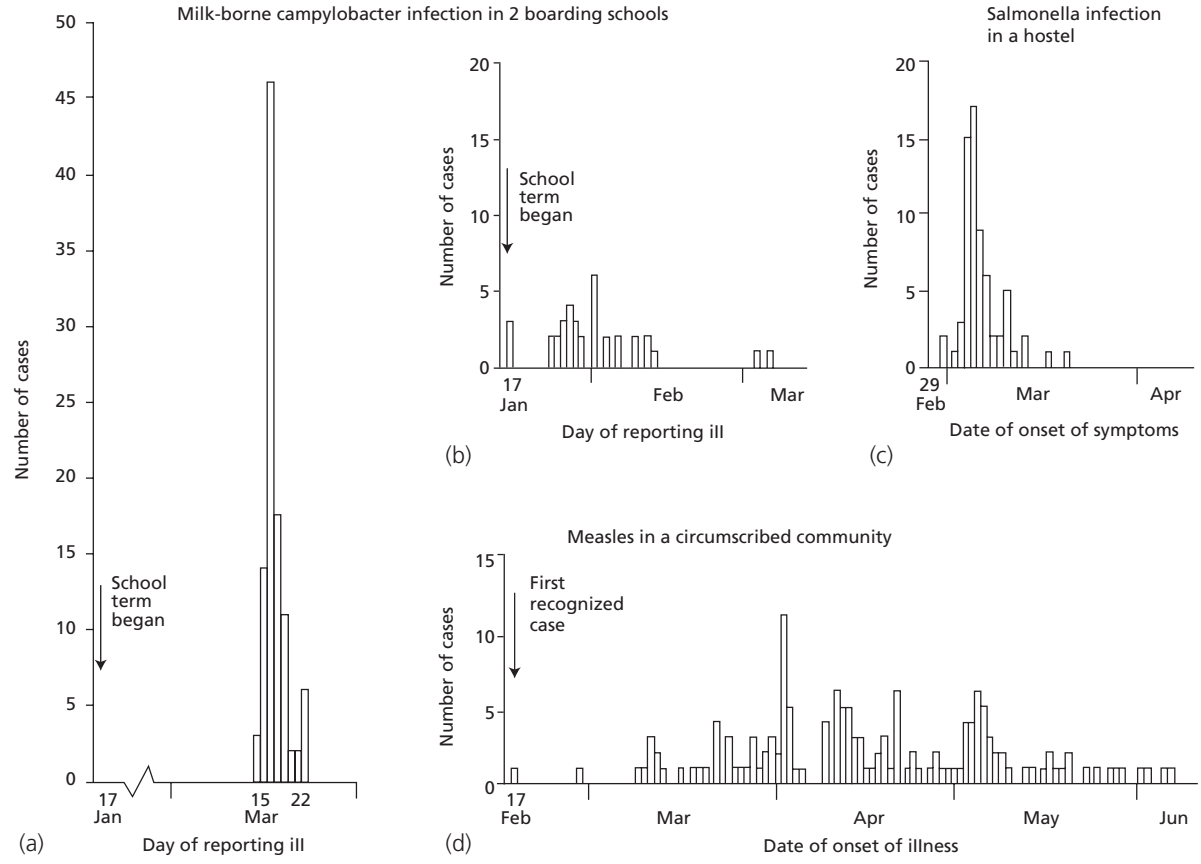

Figure 2 *The epidemic curve*

consumption of contaminated meat pie, but subsequent cases were due to person-to-person spread, prolonging the decline in the epidemic curve (Figure 2c). In outbreaks propagated from person to person the occurrence of cases will be spread over several incubation periods with peaks at intervals of the incubation period. For example, an outbreak of measles affecting 151 persons in a circumscribed rural community in Oxfordshire between February and June 1981 (Figure 2d) showed a smooth epidemic curve but with distinct peaks at 1, 2, 3, and 4 incubation-period intervals after the case (Knightley and Mayon-White 1982). In larger community outbreaks of diseases spread from person to person, the epidemic curve is usually smoother and the peaks at the generation time of the new cases less obvious.

The onset of disease and the epidemic curve should be studied in relation to other events in the environment of the patients; this may draw attention to possible sources of the infection. For example, investigation of a hospital outbreak of Legionnaires' disease in 1983 revealed that a few weeks earlier the domestic hot-water temperatures in the building had been reduced from approximately 55°C at outlets to 45°C, a temperature at which legionellae flourish. Subsequent investigations confirmed that the domestic water supply in the hospital was the source of infection (Palmer et al. 1986).

PLACE

The geographical distribution of disease may provide evidence of its source or method of spread. For example, the crucial factor in the recognition of Lyme disease and the role of tick bites in transmission of *Borrelia burgdorferi* was geographical clustering of presumed childhood rheumatoid arthritis in Old Lyme, Connecticut (Steere et al. 1978). Investigation revealed that the incidence of illness was higher in communities on the east than on the west side of the Connecticut River, and field studies showed that *Ixodes* ticks were particularly abundant in the former area. Case clustering in a particular place of work or neighborhood may indicate the existence of a point-source of infection or of person-to-person spread. In outbreaks of hospital-acquired infection, movement of patients between wards may hide clusters. It is therefore necessary to identify and plot the location of the patients at the time of likely exposure.

PERSON

This includes analysis by age, sex, occupation, and any other relevant characters which preliminary enquiries indicate may be relevant (e.g. food histories, history of travel, leisure activities, and medical or nursing care). For example, food-borne outbreaks of infection due to milk, ice cream, and confectionery characteristically affect children rather than adults. A sudden increase in isolations of *Salmonella* Ealing mainly affecting infants led to the recognition and early control of a nationwide outbreak of salmonellosis due to infant-formula dried milk (Rowe et al. 1987). In contrast, in an outbreak of *Salmonella* Oranienburg infection in Norway in 1981 and 1982, 83 percent of 121 cases were aged 25 years or more, suggesting a food vehicle restricted to adults. This proved to be home-cured meats, the organism originating from contaminated black pepper used as one of the ingredients (Gustavsen and Breen 1984). In another outbreak, of *Salmonella* Cubana infection in hospital patients, the predominance of patients with gastrointestinal dysfunction led to identification of carmine dye, used in investigations, as the vehicle of infection (Lang et al. 1967).

Epidemiological surveys and analytical studies

Descriptive analysis may suggest hypotheses about the source or mode of transmission of an infection, but is not always a sufficient base for introducing control measures. Analytical epidemiology refers to the use of epidemiological techniques to answer specific questions or to test specific hypotheses. The epidemiological approach is complementary to the microbiological. For example, when microbiological investigations reveal legionellae in a cooling tower this does not in itself identify the source of infection because legionellae commonly colonize water systems without causing disease. Epidemiological evidence is necessary to demonstrate an association between exposure to contaminated water and disease.

EXPERIMENTAL AND INTERVENTION STUDIES

Studies of the efficacy of treatments and vaccines are usually undertaken by randomized controlled trials. Random allocation of people to treatment and nontreatment groups is used to overcome bias that can arise if treated and untreated groups differ in underlying factors. For example, if the groups differed in susceptibility to disease, this might produce a favorable result wrongly attributed to the treatment. Treated and untreated groups are then followed to determine the outcome, and incidence rates are compared. With the exception of vaccine trials, most measures of infectious-disease control have not been subject to randomized controlled trials (US Preventive Services Task Force 1996).

Figure 3 shows an epidemiological study design for an experimental study.

PREVALENCE AND INCIDENCE STUDIES

In prevalence or cross-sectional studies (Figure 4) the aim is to measure the proportion of a population with disease or other variable at a point in time. Prevalence studies are often used in descriptive epidemiology, for

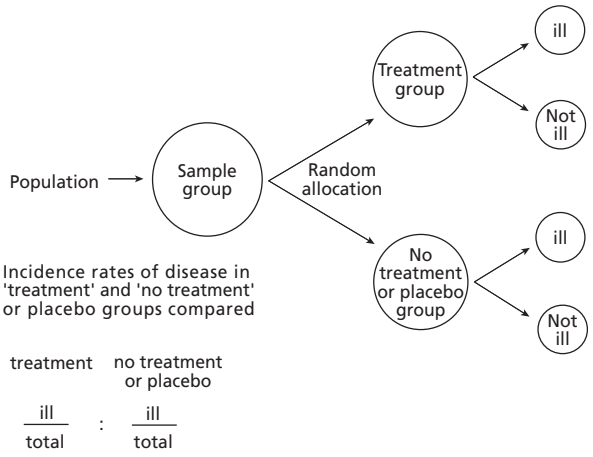

Figure 3 *Epidemiological study design – experimental study*

example, in the surveillance of HIV infection in which the proportions of different risk groups who are HIV-antibody positive are calculated at different times to monitor the spread of infection in the population. Crucial to the success of such studies is the representativeness of the sample of the population studied. For example, studies of AIDS and HIV infection are hampered by an inability to identify the population of homosexuals and drug abusers from which to select a representative sample. Patients attending clinics for sexually transmitted infections (STI) probably represent the extreme end of the spectrum of sexual activity and are likely to have the highest prevalence of HIV infection. On the other hand, high risk patients may attend private medical facilities, so that STI clinic populations cannot be considered necessarily representative even of highly sexually active homosexuals, although they may provide a suitable population for monitoring trends in infection.

The methodology of prevalence studies requires the definition of a population and the study sample. The list

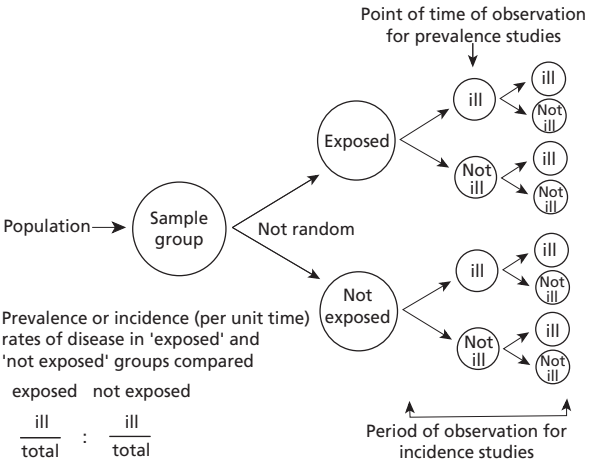

Figure 4 *Epidemiological study design – prevalence and incidence studies*

of names of the population (e.g. electoral register, school register, general practice age-and-sex register) is referred to as the 'sampling frame'; if a sample is to be taken this should be selected either by random, systematic, stratified or cluster sampling (Abramson 1999), so that findings are representative of the practice population. Information on the presence of disease or symptoms is collected by questionnaire or pro-forma, together with other data on personal characteristics or potential risk factors. The proportions of persons with various characteristics or exposures are then compared to identify high risk groups or exposures associated with high prevalence.

Incidence, longitudinal, or follow-up studies measure the rate of occurrence of disease. For these purposes, observations on the population must be made at more than one point in time. Incidence studies may be used descriptively, for example, in following the age incidence of measles to monitor the impact of measles vaccination in a community, in describing the natural history and fatality rates from HIV infection by following cohorts of infected people over several years, or in monitoring crossinfection rates in hospitals. However, they are often also used to test specific hypotheses.

Analytical cohort studies

The analytical cohort study is an application of the incidence study; it attempts to investigate causes of disease by using a natural experiment in which a proportion of a population is exposed and a proportion unexposed. It differs from the true experiment in that exposure is not controlled and may not be random; caution is therefore needed when interpreting the data. If the at-risk population is large, a random sample can be investigated and the results extrapolated to the total population.

Cohort studies may be prospective, when the disease occurs after the study has begun and the characteristics of the population have been identified. For example, in a study to identify risk factors for HIV infection, a serological survey of homosexual men was carried out and seronegative men were enrolled in the study. Baseline data on sexual activity were recorded, and 6 months later the sera of the same men were again tested and seroconversion rates were calculated for groups with a particular sexual behavior. Seroconversion rates were significantly higher in men practicing receptive anal intercourse (Kingsley et al. 1987) than in those who practiced other forms of sexual activity. In a study in the USA of the influence of socio-economic factors on the incidence and outcome of cytomegalovirus infection in pregnancy, two cohorts of women were selected by enrolling those attending a private clinic and those attending a state health department clinic. Seroconversion rates were significantly higher in the lower socio-economic group (Stagno et al. 1986).

Retrospective or historical cohort studies are possible when the population has been defined and identified previously for other purposes. In investigations of food-poisoning outbreaks that have taken place in an institution or after attendance at receptions it is usually possible to identify retrospectively all those exposed and to relate the attack rates to food consumed (food-specific attack rates).

Several possible types of bias may lead to misinterpretation of results of analytical studies and should be considered at the design stage. One potential problem is that of misclassification of cases and non-cases or misclassification of exposure. For example, in the outbreak already described, of *Salmonella* Typhimurium in 1981 in a university hostel, it was not possible to detect the vehicle of infection by comparing food-specific attack rates if a case definition based upon the presence of gastrointestinal symptoms was used. Feces samples were obtained from the whole cohort and only when symptomless excreters had been excluded from the well group did a significant difference in food-specific attack rates emerge (Palmer et al. 1991). To help overcome this problem it is usual to ask all the individuals in the cohort about symptoms over the appropriate time period, so that those who may have been unrecognized cases can be excluded from the analysis or reclassified as cases. The feasibility of serotesting, swabbing, or otherwise testing non-cases to exclude symptomless infected persons should always be considered. To avoid misclassification of exposure, it is desirable to verify exposures by obtaining records from independent sources (such as medical records, employment records) on all or a sample of the individuals in the study. Even quite small errors of classification can lead to misinterpretation of results as in a cryptosporidiosis outbreak in Nevada in 1994 that may have been wrongly attributed to waterborne transmission (Craun and Frost 2002).

Another important possible bias may arise from 'loss to follow-up'. The loss of cases or non-cases from the study because of refusal to be interviewed or failure to trace patients can seriously bias results since exposures in nonresponders may differ from those of responders. A poor response rate may invalidate the results of a study.

CASE-CONTROL STUDIES

The essential difference between a case-control study (Figure 5) and a cohort study is that the former begins with the identification of people with and without the infection and then attempts retrospectively to identify factors associated with disease. In cohort studies, on the other hand, groups of people are identified other than by the presence of disease, and then information on disease occurrence in this group is sought. When the population affected cannot be accurately determined or cases are few, case-control studies are appropriate. Their use should be confined to the testing of specific hypotheses. For example, in the outbreak of *Salmonella* Ealing infection in infants, the hypothesis that the vehicle of infection was infant-formula dried milk was tested by a case-control study that showed a strong association between illness and consumption of one particular brand. Subsequently, *Salmonella* Ealing was cultured from the milk powder and from the factory where it was produced (Rowe et al. 1987). Case-control studies are relatively quick and cheap to perform, but their design and analysis can be complex and special attention must be paid to potential sources of bias.

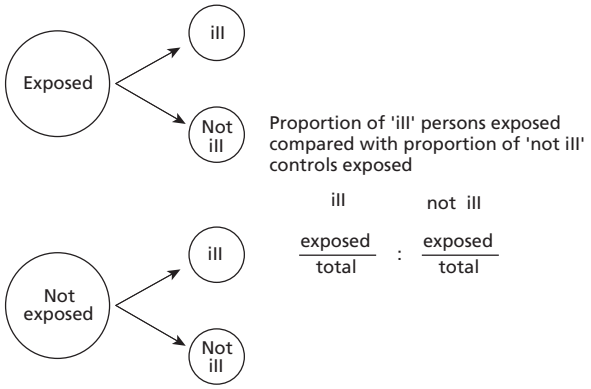

Figure 5 *Epidemiological study design – case-control study*

Cases and controls should be representative of the infected and uninfected population, respectively, from which they came and should have had equal opportunity for exposure to the suspected source. Possible bias in the detection of cases may occur if, for example, only patients admitted to hospital are studied; those who have died of fulminant disease or have only mild illnesses may as a result be excluded. Variables significantly associated with disease in a biased sample may merely reflect the factors that caused the bias, for example, admission to hospital. 'Sampling frames' commonly used to select controls include electoral registers, hospital admissions lists, general practitioner age-sex registers, hotel and reception guest lists, family members of cases, neighbors of cases, acquaintances nominated by cases, and persons investigated by the laboratory, but who were negative for the disease in question. When only a few cases are identified the statistical power of the study can be increased by increasing the number of controls per case up to five before the efficiency of the study falls.

Interpretation of differences in the proportions of cases and controls with a particular variable must take into account the possible effect of confounding factors. A confounding factor is one that is not the source of infection but is associated both with the cases and independently with the suspected source. The association with the occurrence of disease may lead to a misinterpretation of the source of the infection. When

confounding factors can be reasonably predicted they may be excluded by selecting controls that are matched to cases for exposure to those factors. For example, in the first recognized outbreak of hemorrhagic colitis in the USA in 1982, interviews with cases suggested that food eaten at one fast-food restaurant chain were associated with illness. Since exposure to the particular restaurant chain depended upon the location of the restaurants, and probably also on age, controls were matched with cases for neighborhood of residence and age. A significant association between illness and the restaurant chain was found and subsequently frozen hamburger meat from one restaurant yielded the causative organism *Escherichia coli* O:157 H:7 (Riley et al. 1983).

One development of case-control methodology made possible by laboratory typing techniques is case–case comparison to study causation of common infectious diseases (McCarthy and Giesecke 1999). Cases with the same disease can be divided into etiologically meaningful subgroups by subtyping the pathogen. Cases with one organism subtype are then compared with cases with a different subtype of the same organism. This removes several biases since both groups are drawn from the same population and no differential recall of exposures should occur. For example, a comparison of cases of *Campylobacter coli* infection with cases of *C. jejuni* infection from standardized, population-based sentinel surveillance information in England and Wales found that persons with *C. coli* infection were more likely to have drunk bottled water and were more likely to have eaten paté (Gillespie et al. 2002). Similar studies have compared cases of antibiotic-resistant and antibiotic-sensitive infection in order to identify risk factors for antibiotic resistance (Smith et al. 1999).

The major drawback of case-control studies is that accurate and complete data may not be available retrospectively. Medical records are notoriously incomplete and the recall of patients may be faulty. The latter problem is lessened in acute incidents, because there is usually little delay between the event and the interview. A particular problem is that of 'rumination bias'; because of their illness, sufferers will have gone over in their minds possible exposures and recall may be biased by their own preconceptions or by speculation in the press or other 'media'. Cases may also have been interviewed on many occasions; as well as promoting a more detailed recall, this may introduce bias from suggestions made by interviewers.

STATISTICAL ANALYSIS

In both cohort and case-control studies the basic analysis is made by a comparison of proportions. The data can be presented in a contingency table (Table 1).

Table 1 *Contingency table for cohort and case-control studies*

	Case	Not case	Total
Exposed	*a*	*b*	*a+b*
Not exposed	*c*	*d*	*c+d*
Total	*a+c*	*b+d*	*a+b+c+d*

In cohort studies the ratio $a/a + b$ is the attack rate in the exposed. The ratio $[a/(a + b)]:[c/(c + d)]$ is the ratio of the attack rates in the exposed and the unexposed and is called the relative risk. The size of the relative risk is an indication of the causative role of the factor concerned. In case-control studies there are usually no denominators from which risks can be estimated and it is necessary to work with the 'odds' of infection or exposure. The odds of a case having been exposed are a/c and the odds of a control having been exposed are b/d. The odds ratio, ad/bc, or crossproduct ratio, is a very useful measure of association. It approximates to the relative risk when the disease is rare. If there is no association between exposure and infection, the odds ratio will be unity.

In both cohort and case-control studies, dose response effects can be examined if data on different levels of exposure are collected. If the relative risk or odds ratio increases with increasing exposure, the strength of the evidence to determine causality is greatly enhanced. Dose response may be the only way of showing an association between exposure and disease if the exposure is universal (e.g. water consumption).

In analytical studies it is desirable that confidence intervals are presented together with the relative risk or odds ratio, rather than depending solely on p values. The p value is the probability of obtaining a difference between the proportion of cases and non-cases who are exposed, which is as large or larger than that observed in the study if there is no association between disease and exposure. The p value depends not only on the size of the effect but also on the sample size. A study may fail to show an odds ratio or relative risk significantly greater than unity even when a real difference exists because the study size is too small (type II error). Analysis of data sets usually begins by looking at exposure variables one at a time (univariate analysis). Associations between variables and the outcome measures may be causal or they may be due to shared associations with another variable, such as age or sex, known as a confounding variable. The latter can be investigated by stratified analysis in which associations between exposure and disease are examined within subcategories of the confounding variable. The Mantel–Haenszel method is frequently used and logistic regression modelling methods are increasingly used. When matched studies are performed the matching should be preserved in analysis and McNemar's test and the exact binominal probability should be calculated in place of χ^2 and Fisher's exact test. For further discussion of statistical methods see Breslow and Day (1980, 1987) and Altman (1991).

EPIDEMIOLOGICAL SURVEILLANCE

Surveillance needs to be distinguished from research. The former focuses on problem detection and characteristics, whereas research is mainly to do with hypothesis testing. Surveillance systems generate hypotheses and should not be expected to give detailed answers to research questions. Surveillance data provide information for action and as such should stimulate investigation. Emphasis has to be on speed of detection of a potential problem, rather than full and accurate documentation. Consequently, great care has to be exercised in interpreting surveillance statistics. Surveillance systems by definition require ongoing collection of data and consequently rely on minimal data which are often incomplete. National surveillance systems have often gone into decline because data collection has been driven by a desire for full documentation rather than the overriding requirements of timeliness and sensitivity to trends.

One of the earliest recorded surveillance programs was developed in the City of London in the sixteenth and seventeenth centuries to detect the appearance of plague, so that the City administration could decide when to close the theaters of the City to limit the assembly of large crowds of people, and the Royal Court could be advised if and when it was desirable to leave London to escape the disease. The parish clerks of the City were responsible for data collection; each parish appointed two lay searchers to ascertain burials of plague victims, which were then recorded by the clerks along with other burials in the parish burial registers. These data were summated each week by the parish clerks in returns to the Warden of the Hall of Parish Clerks, who then prepared a statistical tabulation of burials by parish. This was published in a weekly bulletin, the 'Bill of Mortality', together with information on the total plague burials compared with the previous week and the number of parishes affected by plague and the number free of the disease (Wilson 1927). The long-term trends in burials in London derived from these Bills of Mortality were studied in 1662 by John Graunt, a City draper, who assessed the validity of the data and demonstrated a higher mortality in towns than in the country by comparing London with Romsey in Hampshire, and drew attention to the high mortality in young children. Greenwood (1948) regarded John Graunt and William Petty, with whom Graunt worked, as the founders of present-day medical statistics.

Thacker and Berkelman (1988) wrote of the USA that: the 'basic elements of surveillance were present in Rhode Island in 1741 when the colony passed an act requiring tavern keepers to report contagious diseases among their patrons. Two years later, the colony passed a law requiring the reporting of small pox, yellow fever and cholera.' Systematic reporting of disease began in the USA in 1874 in Massachusetts, when the State Board of Health introduced voluntary weekly reporting of disease by physicians using a standard postcard.

In the UK, the need for more accurate and complete mortality data led to the introduction of medical certification of death and the civil registration of births, marriages, and deaths in 1836. The General Register Office for England and Wales was established in London, later known as the Office of Population Censuses and Surveys (OPCS) (Nissel 1987) and in 1996 it became part of the Office for National Statistics (ONS). Similar national register offices exist in the other countries of the UK. William Farr, the first Compiler of Abstracts (medical statistician) at the General Register Office, during his 41 years in office, initiated the present international classification of causes of death and developed further the surveillance of communicable disease, establishing a method of influenza surveillance that remains in use today. Dr John Simon, the first medical officer to the Local Government Board (Chief Medical Officer), said of William Farr: 'Eminently he was the man to bring into statistical relief, and to make intelligible to the common mind, whatever broad lessons were latent in the life-and-death registers of that great counting house . . .' (the General Register Office). This provision of readily comprehensible information to those who need it for prompt action remains a crucial component of surveillance.

Surveillance has now assumed even greater importance because of the increased threat of national and international spread of infections arising from the escalating speed, distance, and volume of human travel and the expanding national and international distribution of foodstuffs and other materials which may carry pathogenic organisms as well as from the threat of deliberate release (Lederberg et al. 1992). For example, surveillance may be the only means of detecting outbreaks when the victims have traveled during the incubation period from the place of exposure to many different destinations, or when the vehicle of infection is widely distributed geographically and sometimes also in time. In the face of this threat, national and international surveillance of communicable disease was revived and developed beginning in the 1950s (Langmuir 1963; Raska 1966). More recently, it has been appreciated that increasing human social, technical, environmental, and population change is likely to promote the evolution of new pathogens and facilitate the return of old diseases, now termed 'emerging and re-emerging infections', and that this requires increased surveillance on a global scale to ensure rapid detection, investigation, and control (Heymann and Rodier 1998).

The six main objectives of epidemiological surveillance for communicable disease are:

1 early detection of changes in disease pattern to enable rapid investigation and application of appropriate control measures

2 monitoring long-term trends in disease and infection, including serological surveillance, to assess the need for intervention and to predict future trends
3 determining the prevalent infections in a population so that clinicians may be alerted
4 collation of data about newly recognized or rare diseases at national or international level so that their epidemiology can be described and a basis for research is provided
5 evaluation of disease-control measures and preventive programs
6 planning and costing of health services for the prevention and control of communicable disease.

The method of surveillance remains similar to that of plague surveillance in the seventeenth century, namely:

- the systematic collection of data
- analysis of these data to produce statistics
- interpretation of the statistics to provide information
- timely distribution of this information in a readily assimilable form to all those who require it so that action can be taken
- continuing surveillance to evaluate the action.

The main principles of successful surveillance are simplicity, timeliness, accuracy, and regular analysis and reporting to those who provide data and to those who are responsible for control action (Teutsch and Elliott Churchill 2000). Surveillance is, by definition, an ongoing activity and can be sustained only when the burden placed on the data provider is light. Surveillance data should be limited to the minimum required to meet its specified objectives. Reporting methods should be simple and streamlined. Electronic data collection should be linked to electronic systems for dissemination of high quality surveillance information. For accuracy, surveillance data ideally require clear case definitions as have been developed by the Centers for Disease Control in the USA (Wharton et al. 1990).

Guidelines for the evaluation of surveillance systems have been proposed (Centers for Disease Control 2001). These include the following features:

- a description of the public health importance of the health event including incidence and prevalence, severity of disease as measured by mortality rates and case fatality rates, and preventability
- a description of the system including the objectives, the population under surveillance, case definitions, a flow chart of data collection, details of data transfer, data analysis, and dissemination of information
- a measure of the usefulness of the surveillance system including decisions and actions taken as a result of the information generated
- evaluation of key attributes of the system including: simplicity, flexibility, acceptability, sensitivity, predictive value positive, respresentativeness, and timeliness
- costs of the system.

Data-collection systems

Surveillance data may be sought actively or acquired passively by making use of routinely generated data.

Passive data-collection systems are based upon clinical or microbiological diagnoses which often do not have precise definitions and despite the absence of case definitions, these are invaluable for detecting episodes or cases for further study. For example, in all countries in the UK, cases of typhoid and paratyphoid fevers are detected nationally by statutory notifications, laboratory reports, and referral of cultures of the organisms for identification to the Health Protection Agency (HPA), formerly the Public Health Laboratory Service (PHLS). These three data sources are then linked (Wall et al. 1996). Further active enquiries are made by questionnaire to find out the country where the infection was acquired and possible sources of infection, so that preventive action may be taken. Similarly, cases of Legionnaires' disease are detected by laboratory and incident reports and subsequent active enquiries made, nationally and internationally, to discover cases associated with a common environmental source of infection, so that control measures can be quickly applied.

There is also a surveillance system for general outbreaks of infectious intestinal disease introduced in 1992 by the HPA Communicable Disease Surveillance Center (CDSC). This excludes family outbreaks affecting members of the same private residence only. The CDSC is made aware of outbreaks from laboratory reports, consultants in communicable disease control (CCDC), environmental health officers, and others. Outbreaks are then followed up using a standard request form which documents basic detail which is completed by the local investigator. Participation in the surveillance scheme is entirely voluntary (Wall et al. 1996).

Most active data-collecting systems are based on carefully designed standard case definitions. For example, the surveillance of certain rare childhood disorders, including Reye's syndrome, in the UK is maintained by the British Paediatric Surveillance Unit (BPSU), by monthly mailing of pediatricians to detect cases, followed by subsequent detailed clinical and epidemiological enquiry. These data are then assessed to ensure that all cases meet the standard case definitions before being analyzed to describe the epidemiology of the diseases (Verity and Preece 2002). A unit, similar to the BPSU, was created in 1994 for the active surveillance of neurological disease, the British Neurological Surveillance Unit. A clinical reporting system, set up in 1982 for surveillance of the AIDS epidemic, relies partly on the passive reporting of clinical cases, deaths, and laboratory data for the detection of cases and partly on the active collection of data when these were incomplete. The cases are then scrutinized to ensure that they meet the internationally agreed case definition so that

the changing epidemiology of the syndrome can be accurately described (PHLS AIDS Centre 1991).

MORTALITY DATA

Mortality data on communicable diseases have limited use because they do not usually cause death. However, they can be made available quickly, are usually accurate, and probably nearly complete. They have been used in the surveillance of influenza since the epidemic of 1847, now in combination with morbidity data from general practitioners and laboratory reports (Tillett and Spencer 1982), and in the surveillance of AIDS (McCormick 1994). The death entry is a public document and this may sometimes deter the doctor from entering the correct diagnosis on the death certificate, for example in deaths due to syphilis or AIDS, although the doctor may subsequently provide further information about a death in confidence, after the death entry has been completed.

STATUTORY NOTIFICATION

Notification of infectious disease was first introduced in Huddersfield in 1876 by local act of Parliament; other local authorities followed and in 1899 it became mandatory throughout England and Wales. Weekly summaries of these data began in 1910 and were first published in 1922. The Public Health (Control of Disease) Act 1984, Section 11, states 'if a registered medical practitioner becomes aware, or suspects, that a patient whom he is attending . . . is suffering from a notifiable disease' he/she shall notify forthwith the proper officer of the local authority (usually the CCDC). The CCDC in turn sends each week a return of the number of notifications received in the preceding 7 days to the ONS (except leprosy which is reported in strict confidence to CDSC). Weekly summaries of these data are available on the HPA website and are published electronically by CDSC in the weekly *Communicable Disease Report* (CDR). The data are later corrected and quarterly and annual summaries created. Similar systems operate in Scotland and Northern Ireland. The chief advantages of these data are that they are available quickly, they relate to defined populations so that rates by age and sex can be calculated, and they provide an invaluable means of monitoring trends for diseases that are not often confirmed in the laboratory, for example whooping cough and mumps. The defects of the data are that the clinical diagnosis may not always be correct; the diagnosis may vary between clinicians especially because there are no case definitions (except for ophthalmia neonatorum and food poisoning); under-notification frequently occurs and may vary from place to place and at different times (Doyle et al. 2002). These deficiencies can be partially overcome by training notifying clinicians and by regular feedback of local and national information, but it appears that neither the legal obligation to notify nor the payment of a fee promotes more complete notification (McCormick 1993).

In the USA, systems for the notification of selected diseases such as cholera, smallpox, plague, and yellow fever began to be introduced in 1878. By 1903 notification to local authorities for selected diseases was required in all states. In 1925 all states joined a national morbidity reporting system which was taken on after 1948 by the National Office of Vital Statistics which continued to produce weekly morbidity statistics. These reports have been developed as the *Morbidity and Mortality Weekly Report,* which since 1961 has been the responsibility of the Centers for Disease Control (CDC) in Atlanta. Currently, notifiable diseases are reported by states to CDC through the National Notifiable Diseases Surveillance System using the National Electronic Telecommunications System for Surveillance (Koo and Wetterhall 1996).

LABORATORY REPORTING OF MICROBIOLOGICAL DATA

The routine voluntary reporting of laboratory-diagnosed infections forms the core of communicable disease surveillance in England and Wales and also in Scotland, where a somewhat similar reporting system operates. This reporting system was originally developed by the PHLS in the 1940s and 1950s (Grant and Eke 1993) and comprises confidential electronic reporting each week by medical microbiologists to the director of CDSC in England and Wales and the Scottish Centre for Infection and Environmental Health (SCIEH) in Scotland each week of specified infections diagnosed in their laboratories. At CDSC, data are analyzed within a week of receipt to produce tables and line lists and to compile narrative reports for publication in the CDR. Statistics derived from these data are available on-line to staff in CDSC, and via the HPA website to CCDCs and medical microbiologists. The main benefits of laboratory reports are that they are very precise, in that they are based on laboratory-diagnosed infections and the fine typing of the infecting organisms; they often include clinical and epidemiological details; and they allow for free-text comment. Furthermore, the reporting system is flexible, so that any important, unusual or new infections can be reported, even though they were not necessarily included in the original reporting instructions. However, the reports have some drawbacks: they are limited to infections in which there is a suitable laboratory test; infections which are easily diagnosed clinically tend to be poorly covered; and the reports are not population based, that is the data do not usually have a population denominator, so that incidence rates cannot be calculated. Moreover, as with all routine morbidity reporting systems, the data are incomplete, not all laboratories report, and the completeness of the reports received may vary between laboratories and over time, so that trends are sometimes difficult to interpret.

In order to gain a better appreciation of the true incidence of gastrointestinal infections, a national study, the study of Infectious Intestinal Disease (IID) in England was commissioned by the Department of Health in 1990 (Wheeler et al. 1999; Food Standards Agency 2000). This provides the best data to date of the true incidence of enteric pathogens in the country. The objectives of the study were: first, to estimate incidence and etiology of intestinal infectious disease in people presenting to their general practitioners and from whom stool specimens are routinely sent for laboratory examination; second, to compare these data with the data from the national reporting surveillance system; and third, to estimate the prevalence of asymptomatic infection with these agents. Seventy practices were selected to be representative of the socioeconomic characteristics of the area and cases of infectious intestinal disease were defined as persons with loose stools or significant vomiting lasting less than 2 weeks in the absence of a known noninfectious cause and preceded by a symptom-free period of 3 weeks. Vomiting was considered significant if it occurred more than once in the 24-h period and if it incapacitated the case or was accompanied by other symptoms such as cramps or fever. One component of the study was to draw at random a cohort of people registered with the 70 GPs, who were followed up for a period of 6 months. These volunteers agreed to fill out diary cards every week and return them to the GPs. These cards stated whether the person had suffered gastrointestinal (GI) illness or not. If someone developed an illness, a stool sample was submitted.

Of particular interest in this study was the estimate of the carriage of potential pathogens in otherwise healthy people. Ten out of the 2 264 controls (0.4 percent) were found to be excreting *Salmonella*, 16 out of the 2 264 (0.7 percent) were excreting *Campylobacter*, but none out of 2 264 were excreting *E. coli* 0157. These data confirm that verotoxin-producing *E. coli* are a rare infection in the UK at the moment.

The IID study was also able to quantify the reporting pyramid referred to above using the cohort component of the study. It was estimated that for every case the IID reported to the CDSC, six patients were investigated by routine laboratory tests, 23 presented with GI symptoms to their GP, and there were 136 actual cases in the community. For *Salmonella* cases, however, the ratios were much smaller. For every case reported to CDSC, it was estimated that there were 2.3 cases presenting to their GP and 3.2 actual cases in the community.

GENERAL PRACTICE REPORTING OF CLINICAL DATA

Morbidity data from general practice were studied first in 1955, following which several national morbidity studies have taken place, the last in 1991–1992 (McCormick et al. 1995). Continuously collected clinical data from general practice, which is more useful in communicable disease surveillance, first became available in 1966 when the Royal College of General Practitioners (RCGP) set up a reporting system based on first consultations in a limited number of volunteer practices (Fleming 1999). In 2003 there were 73 participating general practices serving a population of about 615 000 people providing weekly data to the RCGP Research Unit in Birmingham for analysis. These weekly analyses are then sent to the ONS, the CDSC, the Department of Health (DoH), and other organizations concerned with national surveillance. They are published annually by the RCGP Research Unit in the annual reports of the unit. Analogous general practitioner reporting schemes operate in Wales (Palmer and Smith 1991) and some other parts of the UK. These general practitioner reporting systems act primarily as early warning systems providing data rapidly within 10 days of reporting and have the advantages that the data are related to defined practice populations and are unique for some common diseases which are not notifiable and for which laboratory tests are not usually performed, such as chickenpox, herpes zoster, and infectious mononucleosis (Fleming et al. 2002) (see Table 2). In the RCGP system, guidelines to diagnosis are provided and in the Welsh system a set of standard case definitions, but the precise definition of an infection is less important than the speedy recognition of an emerging outbreak which may require prompt investigation. However, there are some deficiencies: reporting may not always be complete; the population covered may not be representative geographically or demographically of the whole country and is too small for the surveillance of less common diseases (Harcourt et al. 2004).

REPORTS OF SEXUALLY TRANSMITTED DISEASES

Legislation in the nineteenth and early twentieth centuries required the registration, regular examination and, if necessary, detention of prostitutes, and compulsory admission to hospital for other sufferers from venereal disease (VD) was recommended. However, when a national VD service came into being in 1916, these legal powers were discontinued and a free confidential service for diagnosis and treatment was established at VD clinics, now genitourinary medicine (GUM) clinics. At the same time, a system of quarterly clinic returns began, providing the number of new episodes of specified infections by gender and for some diseases in age groups, which, with variations and additions, has continued ever since. Sexually transmitted infections, including AIDS, have not been made statutorily notifiable in the UK because of fears that a legal obligation to report individual patient data might lead to some individuals concealing their infections, consequently hindering the control of these infections. The clinic returns are analyzed by the UK departments of health and are collated by CDSC and SCIEH; annual and periodic

Table 2 *The main routine data-collecting systems*

Disease	Data collecting system				
	Death registration	Statutory notification	Laboratory reports	RCGP reports	GUM clinic reports
Anthrax		++	+		
Brucellosis			++		
Chickenpox	+		+	++	
Herpes zoster	+		+	++	
Cholera		+	++		
Diphtheria		++	++		
Food poisoning	+	++	++	+	
Gonorrhea			+		++
Hepatitis A	+	+v	++	+	
Hepatitis B	+	+	++		+
HIV infection (AIDS)	+		++		+
Hydatid disease	+		++		
Influenza	++		++	++	
Infectious mononucleosis			+	++	
Legionnaires' disease			++		
Leptospirosis	+	+	++		
Malaria	+	+	++		
Measles	+	++	++	++	
Meningitis	+	++	+v	+	
Mumps		++	+	++	
Ornithosis			++		
Pneumonia	+		+	+	
Poliomyelitis		++	++		
Rubella	+	++	++	++	
Shigellosis		++	++		
Syphilis	+		+		++
Tetanus	++	++	++		
Tuberculosis	+	++	++		
Typhoid and paratyphoid		+	++		

GUM, genitourinary medicine; RCGP, Royal College of General Practitioners.

reviews of trends are published, the most recent for the decade 1991–2001 (PHLS, DHSS & PS and Scottish ISD(D)5 Collaborative Group 2001). The clinic returns provide data that are probably accurate, being based on specialist clinical diagnosis, often supported by laboratory tests, are unlikely to vary between clinics and over time, and provide a unique set of data extending over nearly three-quarters of a century for some infections. However, the reports record episodes of infection rather than patients, are incomplete because they do not include patients treated outside GUM clinics, and the proportion of patients treated outside the clinics is unknown and may vary by diagnosis, between clinics and over time.

HOSPITAL DATA

Data from a 10 percent sample of hospital discharges and deaths were available from 1955 to 1985, but had limited use in communicable disease surveillance because the data did not become available until about 2 years after collection (Ashley et al. 1991). In 1995, these data were replaced by Hospital Episode Statistics which are currently being assessed for their possible value in communicable disease surveillance. Paget (1897) in his book *Wasted Records of Disease*, referring to hospital records, commented: 'At present these records of disease are rarely utilized for public purposes, and they represent in their present circumstances little more than so much waste of time, material, and intelligence.' Over a century later, a satisfactory method of capturing these data for the surveillance of communicable diseases and other acute diseases has still to be found.

ROUTINE SEROLOGICAL SURVEILLANCE

In 1990 a serological study to measure the spread of HIV infection in the population was begun; it has continued since and become a routine surveillance system. Samples from sera collected for clinical purposes are unlinked from personal identifiers but remain linked to epidemiological information; sera remaining unused

are then tested for HIV infection. This Unlinked Anonymous Prevalence Monitoring Programme has since been extended to include viral hepatitis. The data have helped identify a continuing high rate of transmission among homosexual and bisexual men, and increased hepatitis C transmission in injecting drug users (Report 2002).

OTHER ROUTINE DATA-COLLECTING SYSTEMS

Work-absence data have been used in the surveillance of influenza, but surveillance is now based principally on mortality data, laboratory reports, and general practitioner reports. CCDCs report voluntarily to the CDSC outbreaks occurring in their districts and are required to report serious outbreaks; these reports are usually of food-borne diseases, notably salmonellosis, and provide supplementary data for surveillance of these diseases.

More recently, data from NHS Direct, a national telephone helpline service for health-related enquiries operated by nurses in the UK, have been explored for their potential use in communicable disease surveillance. NHS Direct data on calls for 'influenza-like illness' have been found to correlate well with other routinely available influenza surveillance data (Harcourt et al. 2001). Despite its limitations, the timeliness and population coverage of the service make it a valuable potential source of data for diseases such as influenza or gastrointestinal illness that are mostly treated by primary care (Cooper et al. 2003).

Action consequent on surveillance

Changes in numbers of reports were hitherto usually discovered by examining the data in either numerical or graphical form, but statistical and computer techniques are now often used to assist in the detection of significant variations from previously recorded experience (Tillett and Spencer 1982; Farrington and Beale 1993). Such variations may indicate changes in the incidence of disease, but they may also result from changing interest of disease, altered diagnostic techniques or reporting methods, or fluctuations in the number of microbiologists, laboratories, clinicians, practices, or clinics participating in the reporting system. It is usual, therefore, to compare the data from all the relevant data-collecting systems to validate the observed trends. Often, especially if there is a rapid increase in reports, field investigation is necessary to substantiate an increase and determine its cause.

Routine, regular, and systematic dissemination of information to the providers of the data and to disease control authorities is essential for successful surveillance (Langmuir 1963). In the United Kingdom this is maintained through the weekly CDR and the monthly *Communicable Disease and Public Health*, which are available without charge to professional staff concerned with the control of communicable disease. These bulletins contain narrative reports of newly identified or suspected outbreaks of disease, reviews of prevalent communicable diseases and related topics as well as numerical data on certain organisms, notifiable diseases, and general practitioner reports. More extensive reviews are published in quarterly reviews, annual reports, and in the medical press. Sometimes when an acute episode of infection is identified, more rapid transmission of information electronically, by phone, or facsimile is required.

The uses of epidemiological surveillance may be illustrated by examples that refer to its main objectives.

EARLY DETECTION FOR RAPID CONTROL

In November 2000, an outbreak detection algorithm identified a cluster of 12 cases with *Salmonella* Enteritidis phage type 4b in data from the National Reference Center for Salmonella at the National Institute of Public Health and the Environment (RIVM) in The Netherlands. A rapid descriptive epidemiological study identified chicken, eggs, or bean sprouts as the possible vehicles of infection. These hypotheses were tested by a case-control study which showed a statistically significant association between infection and eating bean sprouts. Two days after the cluster was detected, the company that produced the sprouts voluntarily took several measures including intensified testing, removal of the raw materials from which the contaminated bean sprouts were grown, and disinfection of the factory premises. No further cases of *Salmonella* Enteritidis phage type 4b were reported (Van Duynhoven et al. 2002).

ASSESSING THE NEED FOR INTERVENTION

Notifications of meningococcal disease increased in UK in the early 1990s with a much higher proportion being notified as meningococcal septicemia. Laboratory surveillance also indicated that there was an increase in the proportion of cases due to *Neisseria meningitidis* serogroup C, particularly serotype C2a infections (Ramsay et al. 1997). In response to this, an enhanced surveillance system for meningococcal disease was begun in January 1998 that combined data from statutory notifications, routine laboratory reporting, and the PHLS Meningococcal Reference Unit (Davison et al. 2002a). The greatest burden of disease was in young children and teenagers, and there was some evidence that sequelae were more common than occurred following serogroup B meningococcal disease. This information was provided to the Joint Committee on Vaccination and Immunisation and provided the basis for a decision to introduce a national meningococcal serogroup C conjugate vaccination program in the UK in November 1999, the first country in the world to do so (Davison et al. 2002b).

The vaccine was incorporated into the routine infant immunization schedule and was offered to all under 18 year olds in a catch-up campaign. The vaccine was well accepted with coverage around 90 percent in infants and 85 percent in schoolchildren up to the age of 14 years. The incidence of serogroup C meningococcal disease in the targeted age groups fell by 80 percent in 2000/2001, and the number of deaths in laboratory-confirmed cases under 20 years of age fell from 78 to eight between 1998/1999 and 2000/2001 (Trotter et al. 2002). The incidence of serogroup B disease increased slightly over this same time period, but the reduction in serogroup C disease has been sustained.

DETERMINING PREVALENT INFECTIONS AS AN AID TO CLINICAL CARE

Following the introduction of methicillin-resistant *Staphylococcus aureus* (MRSA) into Wales in the early 1990s, continuous total population surveillance was introduced. The objectives were to gain an understanding of the extent of MRSA, to describe variations in incidence, to measure the burden of disease, and to identify possible risk factors for infection (Morgan et al. 1999). All first isolates of MRSA from both hospital and community settings and all isolates of methicillin-sensitive *Staphylococcus aureus* (MSSA) from blood or cerebrospinal fluid (CSF) were reported electronically by microbiology laboratories, and the data analyzed at CDSC (Wales). The incidence of MRSA was found to be highest in older patients and in men. Blood and CSF isolates were more likely to come from postsurgical patients. The majority of isolates were resistant to at least two antibiotics in addition to methicillin, most frequently erythromycin and the fluoroquinolones. The surveillance system has thus allowed a simple and intelligible picture of the MRSA problem to be determined, and this information has been fed back to hospitals to assist decisions on infection control.

Important developments in communicable disease may be brought to the attention of clinicians when required, by Chief Medical Officer letters, by reports of specialist associations, and by the medical press. The use of electronic communication is increasingly giving clinicians rapid access to communicable disease surveillance information via the Internet.

DETECTING NEW OR RARE DISEASES FOR STUDY

The surveillance of Reye's syndrome began in 1981. This showed that the annual incidence varied between 0.3 and 0.6 per 100 000 in the British Isles with the highest rate in Northern Ireland. No clear seasonal peaks were seen, but 59 percent of patients had an onset of disease in autumn or winter. The median age was 14 years and the sex distribution equal. The national collection of data on cases that met the standard case definition enabled an analytical study to be made of risk factors in the disease. This suggested an association with the use of aspirin, similar to that seen previously in the USA (Starko et al. 1980), and led to the withdrawal of pediatric aspirin preparations and cessation of the general use of aspirin in childhood in 1986. The surveillance scheme continued and demonstrated a decline in the number of cases in subsequent years despite more active data collection (Newton and Hall 1993).

EVALUATION OF PREVENTIVE PROGRAMS

In October 1992, *Haemophilus influenzae* type b (Hib) conjugate vaccine was introduced to infants in the United Kingdom with a catch-up programme for those aged under 4 years. An enhanced prospective survey using data from laboratory surveillance showed that during the prevaccination period the majority of cases of invasive Hib occurred in children under 5 years of age and that it was the most common cause of meningitis in this age group. After the introduction of routine Hib immunization, there was a 16-fold reduction in the annual attack rate of invasive Hib disease recorded in children under 5 years of age, but no decrease in the number of infections caused by other serotypes (Slack et al. 1998).

However, although the rate of invasive Hib disease initially decreased dramatically, surveillance showed a resurgence from 1999 onwards. To identify possible reasons for this increase, the effectiveness of the vaccine was investigated. Vaccine efficacy was found to be lower in children vaccinated during infancy, compared with those who were vaccinated during the catch-up campaign, to decline with time since vaccination and to be lower in children born during 2000–2002 (Ramsay et al. 2003). Lower efficacy during 2000–2002 was subsequently found by means of a case-control study to be due to the introduction of combination vaccines that contained acellular pertussis (DTaP-Hib) (McVernon et al. 2003). In February 2003, the Department of Health announced a second catch-up campaign offering all children between 6 months and 4 years a further dose of Hib vaccine (Trotter et al. 2003).

PLANNING AND COSTING OF HEALTH SERVICES

Surveillance data on AIDS and HIV infection have been used to make future projections of the likely numbers of cases of AIDS and severe HIV disease for planning purposes (Report 1996). By employing surveillance data on salmonellosis Sockett (1995) has assessed the costs of the disease and estimated the cost–benefits of preventive measures.

Surveillance of immunization programs

After licencing and general release of a vaccine, continuing surveillance is necessary, not only to demonstrate the effect of the immunization programs on disease inci-

dence, but also to monitor any subsequent changes in vaccine efficacy under field conditions, to identify groups of susceptible subjects, and to detect unsuspected, rare or new vaccine reactions (Begg and Miller 1990). The main objectives of surveillance of immunization programs are, therefore, the continual measurement of efficacy, safety, and uptake of vaccines.

VACCINE EFFICACY

Potency testing

In the UK, vaccine manufacturers are required by the Medicines Act 1976 to submit to the Medicines and Healthcare products Regulatory Agency (MHRA) a detailed application for licencing of all new vaccines. These applications include information about the quality assurance laboratory tests that they will subsequently carry out on all batches of vaccines that they produce before they are released, to ensure they meet agreed standards of efficacy and safety and conform with World Health Organization (WHO) and European Union standards. The National Institute of Biological Standards and Control (NIBSC), acting on behalf of MHRA, receives samples of all batches before release and verifies that they meet the required standards. After release, samples taken from the field are sometimes retested by NIBSC, for example, in the event of apparent vaccine failure, vaccine-associated disease or problems with the vaccine cold chain. In warm climates special measures are required to ensure the stability of vaccines, particularly live virus vaccines, because of their sensitivity to environmental conditions, and cold-chain monitors (temperature-sensitive color cards) are usually included in vaccine packs to monitor these circumstances. These are now also used in many temperate areas to ensure careful attention to vaccine handling and storage.

Causative organisms

Organisms that cause vaccine preventable diseases need to be continually monitored as they may undergo modifications in antigenic structure that may render vaccines less effective. For example, because of continual small antigenic changes in the influenza A virus (antigenic drift) and occasional larger changes (antigenic shift), viral isolates from all parts of the world are collected and studied in WHO collaborating centers, so that the most appropriate strains may be selected for vaccine production (Pereira 1979). Organisms may also undergo changes in the prevalence of different serotypes. For example, the efficacy of whooping cough vaccine appeared to decrease in the 1960s and was found to be due to a change in the prevalent serotypes of *B. pertussis* (Preston 1965); it was subsequently corrected by the inclusion of the new serotypes in the vaccine.

Serological surveillance

Serological studies provide valuable information about the duration of vaccine-induced immunity in individuals and the extent of immunity in the population. For example, serological surveillance in England and Wales between 1986 and 1991 showed that the proportion of school-aged children who were susceptible to measles was increasing following the introduction of measles, mumps, and rubella (MMR) vaccination in 1988 and, using mathematical models, a major epidemic was predicted in the mid-1990s (Ramsay et al. 1994). A national vaccination program for school-aged children ensued in November 1994 and an epidemic was averted.

Epidemiological studies

Vaccine efficacy (VE) can be calculated from the following equation (where ARu is attack rate in the unvaccinated and ARv is attack rate in the vaccinated):

$$\mathrm{VE} = \frac{(\mathrm{ARu} - \mathrm{ARv})}{\mathrm{ARu}} \times 100$$

The epidemiological techniques for measuring vaccine efficacy in the field were reviewed by Orenstein et al. (1985). There are five main methods:

1. Screening. If a vaccine has a 90 percent efficacy, an attack rate of over 10 percent in vaccinated subjects indicates the need for investigation. Farrington (1993) discusses the method in more detail.
2. Outbreak investigation. The assumption which must be satisfied in the above equation is that both vaccinated and unvaccinated subjects have had equal exposure to infection, an assumption which is likely to be satisfied in outbreaks in confined populations when the attack rate is high (e.g. in institutional outbreaks of measles where case ascrtainment will also probably be complete and immunization records easily available) (Haber et al. 1995). The method can also be applied in community-wide outbreaks in a defined population or in samples of that population.
3. Secondary attack rates in households. This method has been used to determine the efficacy of varicella vaccine, because here again the contacts of the index cases in households are likely to have an equal exposure to infection. By adding together the data from several households, sufficient numbers become available for analysis (Vessey et al. 2001).
4. Routine data-collecting systems. These can be used to estimate vaccine efficacy if the data can be linked with immunization histories. The poportion of cases in the vaccinated (PCV) is related to VE and the proportion of the population vacinated (PPV). Using a variation of the formula:

$$\mathrm{PCV} = \frac{\mathrm{PPV} - (\mathrm{PPV} \times \mathrm{VE})}{1 - (\mathrm{PPV} \times \mathrm{VE})}$$

Orenstein et al. (1985) showed that by means of this formula it was possible to estimate vaccine efficacy when the attack rates in vaccinated and in unvaccinated individuals were not known (Figure 6). For example, analysis of routine surveillance data on hepatitis A infections linked to immunization histories demonstrated the efficacy of routine hepatitis A vaccination to control the disease in a community with recurrent epidemics (Averhoff et al. 2001).

5 Case-control and cohort studies (Smith et al. 1984). Case-control methods have been used to determine measles and pertussis vaccine efficacy by comparing the immunization status of notified cases with that of children on the child-health computer file, which is available in most parts of the UK (Clarkson and Fine 1987).

VACCINE SAFETY

Common, local or systemic vaccine reactions are likely to be identified in large-scale clinical trials before the introduction of vaccines into general use. Post-licensure data on vaccine reactions are collected both passively and actively in the UK. The principal passive data-collecting system relies on medical practitioners reporting suspected reactions following immunization to the Committee on the Safety of Medicines, using specially designed yellow cards which are available in everyedition of the *British National Formulary*. These reports are reviewed regularly by the Joint Committee on Vaccination and Immunization. Other sources of data are complaints to vaccine manufacturers and informal reports to NIBSC, CDSC, and the National Poisons Centres. Unfortunately, none of these passive data collecting systems normally involves the doctors reponsible for immunization, the immunization coordinators, and consequently they are liable to remain unaware of adverse reactions taking place locally in their immunization programs (Miller et al. 1998). Active data collection has been used in the surveillance of smallpox vaccine adverse events in civilians in the United States (Centers for Disease Control 2002) and in special studies of bacille Calmette-Guerin (BCG), measles, and MMR vaccines (Begg and Miller 1990).

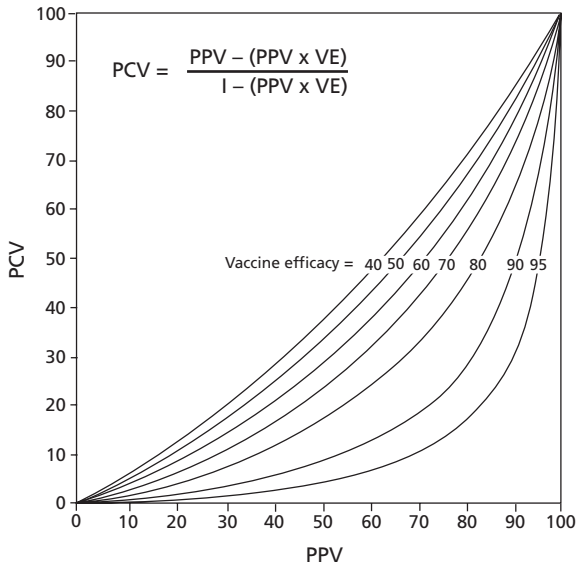

Figure 6 *Relationship between PCV and PPV*

Investigating whether an adverse event is causally related to immunization poses considerable difficulties. The following four methods have been developed:

1 Case clusters. When clusters of disease or reactions are detected in apparent association with imunization, field investigation may discover the cause and lead to prevention of a recurrence. For example, a cluster of cases of aseptic meningitis associated with measles/mumps/rubella vaccine in Nottingham UK, triggered an investigation that led to the withdrawal of Urabe strain mumps vaccine (Miller et al. 1993). Similarly, after a cluster of infant deaths due to severe metabolic acidosis following immunization was reported in a farming village in Egypt, investigations found that the deaths, and other previously unrecognized illness following immunization, were associated with excessive topical application of methanol as an antipyretic and anti-inflammatory agent following injections (Darwish et al. 2002). The practice was subsequently stopped.

2 Etiological investigations. These may detect the agent of a live vaccine in the lesion which has followed immunization, for example, the CSF in meningoencephalitis after mumps vaccination may be shown to contain vaccine virus (Maguire et al. 1991).

3 Case-control studies. The comparison of the vaccine histories of cases experiencing vaccine reactions with those of a control group, matched for age and sex, may reveal a temporal association between vaccination and the reaction, but this association may not be causal. For example, a strict case-control study showed an association between whooping cough immunization and serious acute neurological illness (Miller et al. 1981), but after several years of further studies and continuing debate it was concluded that this association was not causal (Griffith 1989). Cohort studies, comparing disease in a vaccinated group with that in an unvaccinated group, are not suitable for studying rare vaccine reactions because the incidence in the study population is likely to be too low to find significant differences between the two groups.

4 Case series analysis. Farrington et al. (1996) have described a method for estimating the incidence of clinical events after vaccination compared with a control period, based upon data from the cases alone, which may be used for monitoring vaccine safety when a vaccine is in widespread use. This method has the great advantage of reducing the need to follow up

large population cohorts or of selecting and investigating control groups. It has been used, for example, to investigate adverse events after pertussis and MMR vaccinations (Farrington and Pugh 1995) and a possible link between oral polio vaccine and intussusception (Andrews et al. 2001).

VACCINE UPTAKE

Vaccine uptake is measured by four main methods:

1. Vaccine usage gives a coarse measure of vaccine uptake. Despite being inaccurate because vaccine wastage and incomplete courses are not taken into account, the method enables major changes to be recognized quickly, which can then be investigated in the field. This method has been used to investigate variation in pneumococcal vaccine uptake between countries (Fedson 1998).
2. Repeated health surveys can also be used to estimate and monitor uptake. They are useful when data on individual immunization status is not readily available from medical data systems. An example is the Behavioral Risk Factor Surveillance System (BRFSS) used in the United States to estimate coverage of influenza and pneumococcal vaccines (Report 2003).
3. Calculated vaccine coverage gives a much more precise measure but requires accurate data on immunized individuals and up-to-date population denominators. An example is the Cover of Vaccination Evaluated Rapidly (COVER) program used in the UK to calculate uptake in the routine childhood immunization programme. Using a computerized national child-health register, quarterly cohorts of children are studied just after reaching the age of immunization to determine the numbers immunized. Quarterly uptake rates are calculated using the resident population denominators and the results made available quickly to immunization coordinators (Begg et al. 1989).
4. The WHO Expanded Programme of Immunization has developed a variety of techniques for rapid assessment of overall population vaccination coverage. Cluster sampling has been used to randomly select groups of children whose immunization histories are then recorded and used as an estimate of the vaccine uptake of the area sampled. Repeated sampling over time can reveal trends in uptake (Henderson and Sundaresan 1982). Lot quality assessment sampling is a method based on industrial techniques for quality control of manufactured products. It is a form of stratified sampling. Rather than check every item in a lot to determine the number of defective items, a sample of the lot is taken and a predetermined level of risk defined in order to decide whether to accept or reject the entire lot (Hoshaw-Woodward 2001). This method has been used to assess tetanus vaccine coverage in the WHO neonatal tetanus elimination campaign (Cotter et al. 2003).

THE INVESTIGATION OF OUTBREAKS

In the past, the detection of outbreaks of disease relied mainly on the appearance of groups of cases associated in time or place. With the advent of epidemiological surveillance it became possible to search actively for less apparent outbreaks and other variations in disease pattern, recently aided by the use of computer programs (Farrington and Beale 1993). The investigation of outbreaks, or indeed a single case, requires a systematic approach (Goodman et al. 1990) to achieve rapid and effective disease control. This can also be assisted by computer programs, for example, 'Epi-Info' produced by the Centers for Disease Control, Atlanta, now widely used throughout the world. The epidemiological methods used for this purpose were pioneered by John Snow in his famous study of cholera near Golden Square, London in 1854 (Snow 1855) and remain in use today. They may be considered under six main headings:

1. preliminary enquiry
2. management
3. identification of cases and collection and analysis of data
4. control
5. communication
6. further epidemiological and laboratory studies (Palmer and Swan 1991).

It is not always appropriate to follow this sequence of action; the order will depend upon the particular circumstances of an outbreak and often several of the steps are taken at the same time. There is a useful list of sources of information available in the UK for communicable disease control, designed particularly for CCDCs (Morgan et al. 1992). A detailed account of the methods of field investigation can be found in Gregg (2002).

Preliminary enquiry

The objects of the preliminary enquiry are:

- to confirm that there is in reality an outbreak
- to verify the provisional diagnosis of the disease
- to agree a case definition for epidemiological investigation
- to formulate tentative hypotheses of the source and spread of the infection and
- to initiate immediate control measures if required.

CONFIRMING THE OUTBREAK

An increase in the reported number of cases of a disease may not necessarily be caused by an outbreak, but could be due to changes in recognition or reporting. For example, the increase may be related to improved ascertainment of cases following the introduction of new or more sensitive diagnostic procedures, to the need to

detect as many cases as possible because of the availability of a new specific treatment, or to more extensive investigation of a disease because of special interests of new clinicians or microbiologists. Reporting may increase because of changes in the population size or structure, or as a consequence of improved data handling procedures such as computerization, or as a result of false positive laboratory or other tests, or because of the misinterpretation of the original data (Shears 1996).

CONFIRMING THE DIAGNOSIS

The clinical diagnosis can usually be established by a study of the case histories of a few affected persons. Laboratory tests are essential to confirm this in most infections, but epidemiological investigations should begin as soon as possible and not normally be delayed until the laboratory results become available.

CASE DEFINITION

A clear case definition should be agreed at this stage and applied consistently throughout the investigation by all investigators. This is of particular importance in a previously unrecognized disease or one in which there are no satisfactory confirmatory laboratory tests.

TENTATIVE HYPOTHESIS

The preliminary enquiry should include detailed interviews with a few of the affected persons, so that obvious common features may be identified quickly; for example, the association with a specific food, or contact with a particular person or place. Hypotheses can then be developed of the sources and mode of spread of the infection and a questionnaire designed to test these hypotheses in subsequent analytical tudies.

IMMEDIATE CONTROL

It may be possible in some circumstances to take immediate control measures based on the tentative hypothesis before this can be confirmed, so that further cases may be prevented. For example, in serious infections that spread from person to person, such as diphtheria, hepatitis B or poliomyelitis, as soon as the diagnosis is suspected it is necessary to identify individuals who may have been the source of infection so that they are isolated if appropriate, and to identify those who may have been exposed to infection so that they can be traced and given protection by vaccines or chemotherapy. If a common vehicle or source of infection is suspected, appropriate action should be taken to interrupt the spread and control the source.

Management of an incident

If the preliminary enquiry confirms that the outbreak is indeed real, an early decision should be made on the management of the investigation. Small outbreaks will usually be managed by the CCDC with the appropriate and usually essential assistance of a consultant microbiologist and an environmental health officer. In hospitals, an outbreak will usually be managed by the infection control doctor assisted by the infection control nurse (Hospital Infection Working Group 1995). The CCDC is obliged by the Public Health (Infectious Diseases) Regulations 1988 to inform the Chief Medical Officer for England, or for Wales as appropriate, and the CDSC of 'any serious outbreak of any disease' and of cases of disease subject to the International Health Regulations (meaning here cholera, plague, smallpox, and yellow fever) (see also Table 3). He/she should also report directly to CDSC any case of leprosy, malaria, or rabies contracted in Great Britain, and viral hemorrhagic fever. In serious incidents, that is large outbreaks, severe diseases, geographically widespread outbreaks, and those of public interest, an outbreak control team should be formed. This should include, in addition to the CCDC, a consultant medical microbiologist, a CDSC consultant epidemiologist, and a consultant physician in infection. Other agencies may also be involved, for example in suspected water-borne disease these may include the local water companies, river authorities, veterinary services, and the Department of the Environment, Food and Rural Affairs (DEFRA) (Report of the Group of Experts 1990). The control team will require an administrator or nonmedical epidemiologist to manage an 'incident room' where information on the outbreak should be collated and made available to those who require it. The control team should define the responsibilities of its members and allocate to one person the task of spokesperson for the press and other news media, often through the local authority, health authority, or DoH press officers. The control team should meet frequently until the acute incident is over when its tasks may be devolved to the local authorities, health authorities, and the CDSC.

Identification of cases, collection, and analysis of data

The cases first reported in an outbreak usually comprise only a small proportion of the total and may not be a representative sample. Investigation of these cases alone may be misleading for three main reasons:

1. in diseases spread from person to person, such as diphtheria, missed cases or carriers may be responsible for spreading the infection
2. in point-source outbreaks, such as those of Legionnaires' disease or food-borne diseases, the presenting cases may have come to light because of a chance association with a place or potential vehicle of infection
3. without knowing the population from which the cases have come, the denominator of 'persons at risk' is not

Table 3 *Statutorily notifiable diseases*

Notifiable diseases	
Under the Public Health (Control of Disease) Act 1984	
Cholera	Relapsing fever
Food poisoning	Smallpox
Plague	Typhus
Under the Public Health (Infectious Diseases) Regulations 1988	
Acute encephalitis	Ophthalmia neonatorum
Acute poliomyelitis	Paratyphoid fever
Anthrax	Rabies
Diphtheria	Rubella
Dysentery (amoebic and bacillary)	Scarlet fever
Leprosy	Tetanus
Leptospirosis	Tuberculosis
Malaria	Typhoid fever
Measles	Viral hemorrhagic fever
Meningitis	Viral hepatitis
Meningococcal septicemia (without meningitis)	Whooping cough
Mumps	Yellow fever

Notes: 'Viral hemorrhagic fever' means Argentine hemorrhagic fever (Junin), Bolivian hemorrhagic fever (Machupo), Chikungunya fever, Congo/Crimean hemorrhagic fever, dengue fever, Ebola virus disease, hemorrhagic fever with renal syndrome (Hantaan), Kyasanur forest disease, Lassa fever, Marburg disease, Omsk hemorrhagic fever, and Rift Valley disease.
There are minor differences in notifiable diseases in Scotland and Northern Ireland (see Ashley et al. 1991).
Some diseases are notifiable locally, for example, psittacosis in Cambridge.
AIDS is not statutorily notifiable, but clinicians report cases voluntarily, in strict confidence, to the directors of the CDSC in England and Wales and of the SCIEH in Scoland. Advice about reporting is available from these centers from genitourinary medicine physicians.

available to calculate attack rates and so determine whether or not a particular exposure was associated with an increased incidence of the disease.

Identification of the exposed population will enable thorough case finding to be accomplished, for example, by scrutinizing school or hotel registers, lists of institutional residents, pay-rolls, and other occupational records, nominal rolls of travelers, and lists of persons attending functions associated with the disease. If such studies are not possible, as is often the case in community outbreaks, case finding may be undertaken by reviewing routine mortality and morbidity data used for communicable disease surveillance, by individual household enquiry, and by special appeal to medical and lay persons through the press and other news media.

The aim of the enquiry will be to collect data from those affected and those at risk but not affected, by careful questionnaire. The data routinely sought from cases include name, date of birth, gender, occupation, recent travel, immunization history, date of onset of symptoms, description of the illness, and the names and addresses of the medical attendants. Other details will depend on the nature of the infection and possible modes of spread. The case data will then be analyzed by time, place, and person to determine the mode of spread, source of infection, and persons who may have been exposed. The date or time of onset of symptoms of cases should be plotted on a graph so that the type of epidemic curve can be recognized, and on a map to reveal the geographical distribution of the cases. Cases that do not conform to the time or geographical distribution may sometimes provide valuable evidence of the source of infection. For example, in John Snow's classic study of the outbreak of cholera near Golden Square, Soho, in 1854, a widow died of the disease in West Hampstead, several miles from Soho, and provided the most conclusive evidence of the source of infection because she had previously resided in Broad Street and had delivered to her daily a large bottle of water from her favourite supply, the Broad Street pump (Snow 1855). Comparisons of attack rates by age, gender, location, food history, and other appropriate parameters may provide evidence of the source and spread of infection which may then be confirmed by analytical epidemiological studies already described.

Control

Control measures may be directed towards the source of infection, the mode of transmission, people at risk, or a combination of these.

CONTROL OF THE SOURCE

The source may be human, animal, or environmental.

Human source

Infections derived from a human source can be controlled by the physical isolation of cases and carriers

and, if necessary, treatment until they are free from infection, provided that the cases and carriers are easily identified and carrier rates are low. For example, it is possible to control diphtheria and typhoid fever in this way because cases can be recognized clinically and confirmed by laboratory tests and because carrier rates are low and carriers can readily be detected microbiologically. In contrast, meningococcal infection is not susceptible to control by isolation because pharyngeal carrier rates in excess of 20 percent in the population are common during outbreaks and it is usually impractical to detect and isolate all the carriers. The method of physical isolation used depends upon the mode of spread and severity of the disease; for example, negative-pressure plastic isolators are used in the UK for the African viral hemorrhagic fevers, room isolation for diphtheria, and special precautions in the disposal of secretions and excretions (isolation by 'barrier nursing') in typhoid fever. Isolation of human sources of infection can also be accomplished in some diseases by 'ring immunization', that is, by encircling the case or carrier with a barrier of immune persons which blocks the spread of infection to susceptible people outside the 'ring'. This method was first used in Leicester in the nineteenth century and later throughout the UK for smallpox control; when modified and accompanied by meticulous surveillance worldwide it helped to achieve the eradication of the disease (Fenner et al. 1988). It has also been applied in the control of poliomyelitis by mass immunization and in measles by immunization of contacts in school and institutional outbreaks.

Animal source

When animals are the source of an infection, it is sometimes possible to control an outbreak by eradication of that source, for example, in ornithosis by slaughtering the birds and disinfecting the cages and premises. Rabies may be controlled by the destruction of rabid animals and of wild or stray animals that may harbor the virus, and by the muzzling of domestic dogs. Outbreaks of food-borne zoonoses are usually controlled by removing the vehicle of infection: eradication plays a major part in the long-term control of these zoonoses, for example, bovine tuberculosis and brucellosis.

Environmental source

The source of Legionnaires' disease and primary amoebic meningoencephalitis, both recognized in the 1970s, is water in the environment. Outbreaks of Legionnaires' disease are usually associated with water-cooling systems of air-conditioning plants, water-distribution systems in large buildings, or whirlpool spas. Primary amoebic meningoencephalitis is usually contracted from warm unchlorinated natural water sources, such as warm springs, sometimes used to supply swimming pools. Both these infections may be controlled by appropriate cleansing and disinfection. Other pathogens, mainly gram-negative bacteria, may contaminate the inanimate environment of man; for example, salmonellae are found on work surfaces, utensils and equipment in kitchens, and coliform organisms and pseudomonads on surfaces and equipment in hospitals. Control measures include appropriate cleansing, disinfection or sterilization of these environmental sources.

CONTROL OF SPREAD

Food- and water-borne infections can be curtailed by withdrawal from sale of the contaminated product or treatment of the product to render it safe, for example, the recall of a contaminated meat product (Macdonald and Drew 2000), and the pasteurization of a contaminated milk supply (Proctor and Brosch 1995).

Direct contact infections may be reduced by avoiding contact, for example, in cutaneous anthrax by not handling potentially contaminated animal products such as unsterilized bone meal.

Indirect contact infections, for example, staphylococcal infections in hospitals, may be reduced by strict aseptic techniques and meticulous hand washing, accompanied in the case of wound infection by the use of 'no touch' dressing techniques.

Fecal–oral spread, such as in bacillary dysentery and hepatitis A infection, may also be restricted by scrupulous attention to hand washing and by the careful and frequent disinfection of surfaces in lavatories and toilet areas.

Percutaneous spread by insects, such as in malaria, typhus, and yellow fever, may be controlled by vector destruction, protective clothing, and insect repellents. Infections spread by percutaneous inoculation, such as hepatitis B and HIV, may be prevented by measures to avoid accidental inoculation and the contamination of broken skin by infected blood or tissue fluids.

Air-borne spread may be limited by ventilation in buildings and in special circumstances the physical isolation of highly susceptible subjects.

CONTROL OF PERSONS AT RISK

It is sometimes possible to control disease by active or passive immunization or by chemoprophylaxis in persons exposed to risk. For example, active immunization against measles within 72 h of exposure may prevent the disease. Human immunoglobulins are commonly given for passive protection, for example, rabies immunoglobulin in addition to active immunization as part of post-exposure prophylaxis given as soon as possible after exposure, hepatitis B immunoglobulin in post-exposure prophylaxis after accidental inoculation injury and for infants of carrier mothers, and zoster immunoglobulin in the newborn and immunodeficient patients after expo-

sure to chickenpox. Chemoprophylaxis is offered to close contacts of cases of meningococcal disease, such as household contacts and children in the same class at school, and long-term chemoprophylaxis against pneumococcal infection is given to patients after splenectomy.

Communication

Accurate and timely information about an outbreak is of major importance, especially if the outbreak is of national interest and is receiving wide publicity. To achieve this, it is helpful for the CCDC to maintain a check list of authorities and individuals to be informed (Morgan et al. 1992). These would normally include the local consultant physician in infection, microbiologist, chief environmental health officer, and director of public health, but depending upon the type of outbreak might also include the local divisional veterinary officer, employment medical adviser, education officer, and clinicians in hospital and general practice, and similar persons in neighboring areas. In a major incident, national authorities including the HPA and its CDSC, the DoH, the Food Standards Agency (FSA), DEFRA, the Health and Safety Executive, and others may be involved. An initial written report should normally be completed within 24–48 h after the preliminary enquiry and circulated to all the authorities and individuals concerned. A final report suitable for publication should be produced at the end of the investigation and interim reports may be necessary if the investigation is complex or prolonged. In a major incident it may also be necessary for frequent bulletins of updated information to be issued by the spokesperson of the 'outbreak control team' from the 'incident room'.

Further epidemiological and microbiological studies

Analytical studies may be necessary to confirm the association of disease with a particular vehicle or source of infection. Often case-control studies are undertaken early in an enquiry because they can be accomplished quickly and cheaply, but more time-consuming cohort studies may be required to determine the relative risk so that the most cost-effective long-term control measures can be implemented.

An outbreak may suggest a previously unknown source or vehicle of infection and microbiological surveys may be needed to discover the extent, frequency, and mode of transmission so that long-term control measures can be designed and implemented. For example, the discovery that bulk egg products were a vehicle of infection of paratyphoid B and other salmonella infections led to extensive studies of the bacteriology of these products and to improved hygienic methods of production and pasteurization. The identification of Legionnaires' disease and its association with cooling towers of air-conditioning systems and with piped water systems in large buildings led to studies of the growth of legionellae in these systems and subsequently to improvements in design and maintenance to prevent colonization and growth of the organism.

MATHEMATICAL MODELS OF INFECTIOUS DISEASE

The idea of modelling epidemic diseases dates back to a seminal paper published at the beginning of the twentieth century by Hamer (1906), which suggested that cycles of measles infections and deaths were not due to changes in the pathogenicity of the measles virus, but to changes in the number of susceptible and immune individuals over time. Mathematical models have grown in sophistication over the last few decades particularly with increases in computing power; however, all are based on the logical deductions proposed by Hamer. Infectious disease models are used not only to predict epidemics of disease, but also to help understand disease transmission patterns, to measure the burden of infection, and to anticipate the impact of infection control measures and immunization policies.

The potential for an infectious disease to spread from person to person is known as the basic reproductive rate (R_0). It represents the average number of secondary infections produced in a totally susceptible population by a single case during the entire infectious period. The value of R_0 depends on:

- the probability, for a given disease, that an infected person will transmit infection to a susceptible person
- the frequency of contacts with susceptible persons
- the period of time for which an infected person is infectious.

The value of R_0 is normally signified by the equation:

$$R_0 = \beta \times \kappa \times D$$

where β is the risk of transmission per contact, κ is the number of contacts, and D is the duration of infectivity of the case.

When a new disease enters a virgin population, if $R_0 > 1$ there will be an epidemic, if $R_0 = 1$ the disease will become endemic, and if $R_0 < 1$ the disease will eventually disappear. Childhood diseases generally have a high R_0 and confer lifelong immunity, since nearly everyone is exposed to them early in life, but subsequently protected from disease. For example, classic studies by Hope Simpson (1952) on the spread of childhood infectious diseases among household contacts in a rural area of England derived values for attack rates (β) of 0.80 for measles, 0.72 for chickenpox, and 0.38 for mumps. A case of measles is thought to be capable of

infecting 15 other people on average in a susceptible western population, and so has an $R_0 = 15$.

In practice, populations are seldom totally susceptible. Furthermore, if the disease is one that gives rise to immunity and no more susceptible people enter the population, then the number of susceptible people will decline with time and more and more of a person's contacts will be with people who are immune. The chance of spreading the disease gradually becomes less and less, and the actual reproductive rate (R) of the infection decreases (although the basic reproductive rate (R_0) remains the same) until the epidemic eventually dies out.

The steps that are involved in developing and using a model for an infectious disease have been described in detail (Habbema et al. 1996). They are illustrated in Figure 7.

The kind of model structure chosen will depend on the various transition states of the infection, for example, between the categories susceptible, infectious, and immune. A simple model could be represented as follows:

Susceptible → Infected → Infectious → Immune

This model would be appropriate for a disease such as measles. Estimates are required for the transition intervals between each category in order to begin to develop the model. If the model is intended to describe the long-term dynamics of an infection then other parameters such as births, migration patterns, and deaths may need to be incorporated. The model population may also need to be split into subgroups if, for example, the dynamics of infection in different age groups are to be studied.

The next step is to choose the model type, usually either a deterministic or stochastic model. Deterministic or compartmental models aim to describe broadly what happens in the population as a whole. They tend to categorize individuals into subgroups or compartments and describe the transition between categories using average transition rates. They require fewer data and are relatively easy to set up. For example, an age-structured deterministic model showed that routine varicella immunization of preschool children would probably result in a substantial reduction in uncomplicated chickenpox and in cases requiring hospitalization, but that the number and age distribution of cases would depend strongly on the characteristics of the vaccine (Halloran et al. 1994). Stochastic models (or Monte-Carlo simulation models) often try to describe the effect of disease on every individual in the population. They do this by incorporating chance variation into the disease transmission process in order to model a range of possible outcomes. Compared to deterministic models they are more intuitive, they can follow disease dynamics in all individuals in the population, and they can provide estimates of the range within which an outcome may lie, taking account of chance variation. They are, however, laborious to set up and

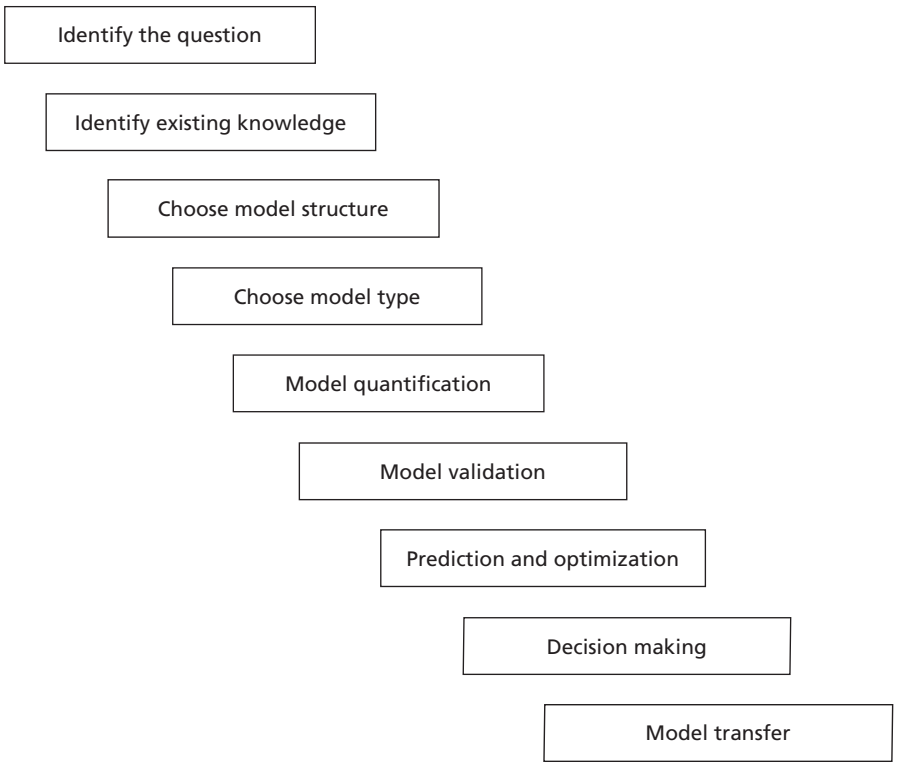

Figure 7 *Steps in developing and using a model for an infectious disease*

many simulations need to be run in order to obtain useful predictions. For example, this approach has been used to model the epidemic of hepatitis C in France based on the temporal pattern of deaths from hepatocellular cancer (Griffiths and Nix 2002).

Once the form of the model has been determined, the rates and distribution of flows between disease transition states need to be quantified. This stage is often problematic because of the lack of reliable data. Key data requirements include the probability that a specific infectious and susceptible individual comes into sufficient contact to transmit infection during a given time period (β), the risk of a specific individual becoming infected per unit time (related to the incidence rate in person years or force of infection), becoming infectious per unit time, and becoming immune per unit time. Sometimes primary data collection is required to make up for deficiencies in the data available. Models then need to be validated by checking outputs against known data, and optimized by testing various model assumptions in a process of sensitivity analysis, before the results of the model can be applied to decision-making and public health practice.

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Theory of infectious disease transmission and control

ANGUS NICOLL, NIGEL J. GAY, AND NORMAN T. BEGG

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INTRODUCTION

Successful control of an infectious disease requires an understanding of the characteristics and behavior of the agent causing disease and its interaction with the hosts it infects and with the environment. This may seem an obvious statement, however history is littered with failed attempts at controlling infectious diseases through ignorance of basic issues such as the mode of transmission, natural reservoirs of infection, and the role of carriers (Table 1).

For example, in the early nineteenth century cholera was thought to be an airborne disease. Control efforts at that time were based on remedies to ward off contaminated atmosphere ('miasma'). It was not until 1854 that John Snow, a British epidemiologist, showed that cholera was a water-borne infection. Snow's discovery was based on the simple observation, during a cholera epidemic, that attack rates were highest in people who obtained their drinking water from one particular pump (Figure 1). His remedy – to remove the handle of the pump and thereby stop the outbreak – revolutionized the control of cholera and presaged a series of public health measures aimed at improving the safety of drinking water. Unfortunately, ignorance about cholera control persists to this day. When the seventh cholera pandemic reached Latin America in 1991, the reaction of many countries was to introduce a requirement for all visitors to be vaccinated. Cholera vaccine may provide some individual protection against disease, but does not prevent cases from excreting *Vibrio cholerae*. As a public health measure it therefore has no value in controlling the spread of infection (Finkelstein 1984).

A particular area of medicine in which knowledge of disease transmission and so how control can be effected, is immunization. There are several characteristics of an infection that determine how readily it may be controlled by vaccines. These characteristics include the infectivity of the disease, the length of the incubation period, the duration of naturally acquired and vaccine-induced immunity, and the presence or absence of subclinical infection and nonhuman hosts (Noah 1988). An especially important concept for immunization is herd immunity, the proportion of a population or group that are immune to a specific infection (Topley and Wilson 1923; Anderson and May 1985b; Last 2001). Mathematical models that incorporate these effects can be used to predict the outcome of alternative vaccine strategies, under varying assumptions about vaccine efficacy and vaccine coverage. This type of information has been pivotal in the design of successful immunization programs against diseases such as smallpox, poliomyelitis, and measles, and predicting the effectiveness or otherwise of proposed interventions.

In this chapter we will describe the basic theory of infectious disease transmission, and consider how the application of theory contributes to the control of communicable disease in man.

Table 1 *Factors affecting transmission and determining means of infectious disease control*

Factors affecting transmission	
Reservoirs of infection	Human
	Animal (Zoonoses)
	Environmental
Modes of transmission	Respiratory – droplets, aerosols
	Oral–gastrointestinal
	Parenteral
	Fomites
	Sexual
	Vectors
	Vertical (mother-to-child)
Period and duration of infectivity	
Subclinical infectious carriers	
Level of infectiousness (R_0 and R)	
Behavior and contact patterns of those infected	
High risk situations and core groups	
Effectiveness of treatments in reducing infectiousness	
Resulting immunity following infection or immunization	
Genetic susceptibility	

HISTORICAL PERSPECTIVE

The theory of infectious disease transmission has its origins in the first decade of the twentieth century. Following the discovery of germ theory in the nineteenth century, scientists tried to understand the cycles in the incidence of many infectious diseases. In particular, they sought an explanation for the regularly recurring epidemics of diseases, such as measles, and why these epidemics peaked and died out before all susceptible persons had been infected. In 1906, William Hamer proposed a model in which he considered the cases to occur in discrete, 2-week generations. The number of cases of disease in one generation was assumed to be a constant multiple of the number of cases in the previous generation and the number of susceptibles. On this simple assumption, Hamer was able to explain the epidemic nature of measles: the regular cycles were caused by the depletion of the number of susceptibles during an epidemic followed by a gradual reaccumulation as more children were born. His argument, however, was not widely accepted at the time. An alternative, empirically derived theory, due to Brownlee (1907, 1909), was based on Farr's (1840) observations of the shape of epidemic curves. According to this theory, the infectivity of the agent was reduced by a constant factor with each successive generation of cases in the course of an epidemic: the decay of an epidemic was due to this attenuation of the infective agent and not to a

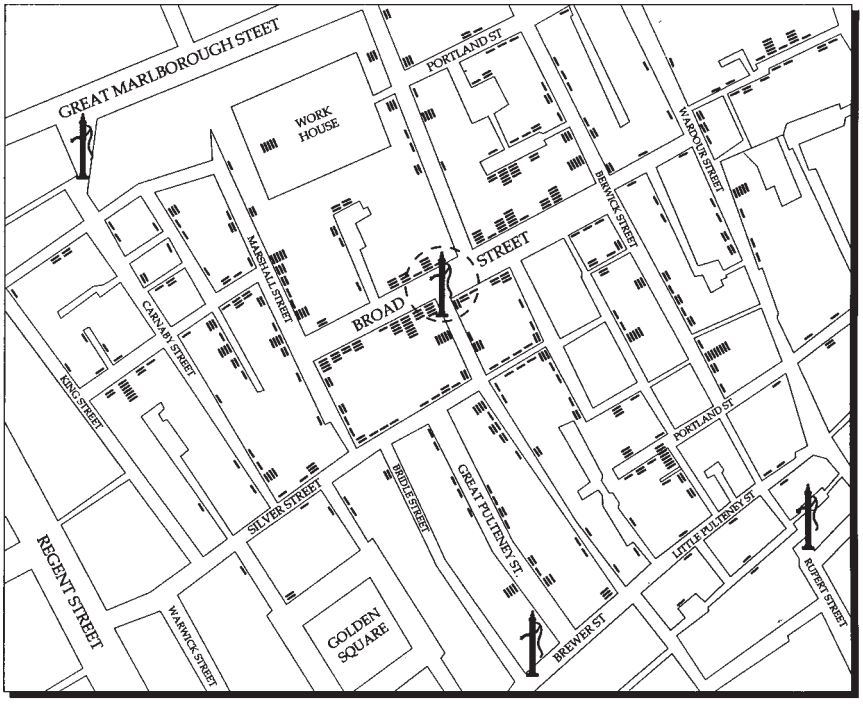

Figure 1 *Distribution of cholera deaths, Golden Square, 1854*

lack of susceptible persons. (An account of Brownlee's work is given by Fine (1979).) Contemporary with this debate, Ross (1909, 1911) proposed a model of malaria transmission along similar lines to Hamer's theory of measles. Both Ross and Hamer were well aware that their models were a great simplification of the heterogeneity of real populations, but realized that they captured the fundamental mechanisms underlying the observed patterns of disease incidence.

It was against this background that Topley and Wilson commenced their classic series of experimental studies of the spread of infection among mice. The carefully controlled conditions in the mouse populations were ideal for testing the competing theories. At the outset of this research Topley (1919a, b, c) set out the main problems to be investigated in his Goulstonian Lectures to the Royal College of Physicians of London. It is clear that he was influenced by Brownlee's ideas, but did not dismiss Hamer's theory: 'Though reasons have been given for believing that the outstanding feature in the subsidence of an epidemic is a loss of infectivity by the bacterial virus [sic], yet the resistance of the host cannot be a negligible factor. It will operate by decreasing the concentration of susceptible individuals, and hence the chances of successful transference.' After 5 years of experiments, Topley and Wilson (1923) were led to the conclusion that 'the question of immunity as an attribute of a herd should be studied as a separate problem, closely related to, but in many ways distinct from, the problem of the immunity of an individual host'. Theirs was the first published reference of the term 'herd immunity'. Its use indicates that the focus of the research was moving away from the properties of the infective agent alone and towards also considering properties of the herd or population. Their most seminal finding was that the addition of a number of susceptible mice to a population in which a bacterial parasite was at equilibrium produced an outbreak of disease (Topley 1923). Similar observations in a human population, on the incidence of diphtheria in a boarding school, were made by Dudley (1926): outbreaks recurred when the admission of sufficient susceptible new boys had removed the herd immunity. Two decades later experiments by Webster (1946) in the United States confirmed this. Gradually, Hamer's theory became accepted as the experimental evidence mounted, while no evidence to support Brownlee's theory of varying infectivity was obtained. A full account of the series of mouse experiments in Britain is given in the final Medical Research Council report (Greenwood et al. 1936).

During this period there was further theoretical progress. Kermack and McKendrick (1927) proved the existence of an epidemic threshold, i.e. that an epidemic would occur if, and only if, the proportion susceptible exceeded a given level. Soper (1929) provided a clear statement of Hamer's model and its continuous time equivalent. He showed that the continuous model produced damped oscillations around the equilibrium solution and derived the period of small oscillations. He also noted the similarity with some laws for chemical reactions, hence the model is sometimes referred to as being of 'mass action'. The inability of this model to yield the undamped epidemic cycles observed for measles was seen as a major flaw, until Bartlett (1956) demonstrated that a stochastic formulation of the Hamer–Soper model overcame this problem. His numerical simulations generated recurrent epidemics with no tendency to the equilibrium. He noted that the size of the population influenced the pattern of epidemics: larger towns had more regular cycles, whereas 'fade out' of infection often occurred in simulations with a small population. This led him to derive a critical community size above which fade out was unlikely (Bartlett 1957), a result which accorded well with observations. Bailey provided the first comprehensive account of 'The mathematical theory of epidemics' in which these developments were discussed (Bailey 1957). The text also included methods for estimating parameters (such as latent and infectious periods) from epidemiological data.

Meanwhile, in the United States, Reed and Frost were developing a model which has become the basis for the study of outbreaks in small populations. This included a nonlinear term to discount the effect of two or more infectious individuals contacting the same susceptible person (Frost 1976). Fox et al. (1971) explored the effects of superimposing family and other structures on a Reed–Frost model, the first study to include heterogeneity in contacts between members of a population. Their stochastic simulations led them to emphasize the important role that small pockets of susceptible individuals could play in sustaining transmission.

The concept of the basic reproduction number (R_0) was introduced to infectious disease epidemiology by Macdonald (1957) in the context of his studies of malaria. R_0 has become a central feature of the theory of infectious diseases since Dietz (1975) and Hethcote (1983) first used it in the study of directly transmitted infections. It provides a simple method of comparing diseases and the difficulty of eliminating them. Dietz (1975) showed how, in a simple homogeneous model, R_0 could be estimated from the average age at infection. Diekmann et al. (1990) provided a rigorous mathematical framework for the definition of R_0 in models of disease transmission in heterogeneous populations. These include the models with age-dependent transmission rates developed by Anderson and May (1984, 1985a, b) and others (Schenzle 1984; Hethcote 1983) that have been used to investigate the dynamics of a wide range of infections and to explore the effects of different vaccination programs (Anderson and Grenfell 1986; Babad et al. 1995; Anderson and May 1985a, 1990; Anderson et al. 1987; Halloran et al. 1994). These considerations are now applied to many other infections apart from those for which effective vaccines are

available and have become increasingly influential on policy decisions relating to control of infectious disease, including when traditional control measures are applied to new infections such as severe acute respiratory syndrome (SARS) and avian influenza (WHO 2003; Anderson et al. 2004). Mollison et al. (1994) has reviewed developments across the entire spectrum of modeling approaches and highlighted areas in which further research is needed. Particular developments took place in the 1980s and 1990s when attention was focused on the epidemiology of human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS). Models for sexually transmitted diseases and other sexually transmitted infections (STI) often require many population subgroups with widely differing activity levels and the long incubation period for AIDS and its changes in infectivity present further complications (Johnson et al. 1992; Boily et al. 2000; Aral 2000). Most recently, field observations were combined with modeling approaches in real time to rapidly determine how best to control severe acute respiratory syndrome (SARS) (WHO 2003).

BASIC PRINCIPLES

Infection in the individual

There are several stages in an episode of infectious disease (Figure 2). The process begins when a susceptible individual is exposed to an infectious case. If transmission of the infectious organism occurs, and the organism begins to multiply in the tissues of the exposed individual, then the individual can be considered to have been infected. Not all exposures result in transmission, and of these only a proportion progress to clinical disease. At this early stage the individual is not yet infectious – this is known as the latent period of infection.

The next stage is when the infected individual begins to shed the organism from the respiratory or gastrointestinal tract or in body fluids, such as blood or urine. At this point the individual is infectious, i.e. capable of transmitting the organism to others. The period of latency has now ended. This next stage marks the beginning of the period of infectivity.

Throughout both the latent period and the early stages of the period of infectivity, the individual has no symptoms. The onset of the first symptom heralds the end of the incubation period, which is defined as the interval between the initial exposure and the onset of symptoms. For some infections, symptoms appear at the same time as the patient becomes infectious. For others, infectiousness rises as symptoms worsen, while for a third group it is more usual for infectivity to precede symptoms by a few days. It is unusual for infectivity to be constant through the infectious period.

The next stage is reached when the individual is no longer infectious. Normally this occurs while symptoms are present. In many infections the organisms are cleared from the body and immunity develops. In some diseases, shedding of the organism can continue after symptoms have resolved. This tends to occur particularly in immunosuppressed individuals. When shedding of the organism becomes persistent, then the individual has become a carrier. Knowledge of carrier states is particularly important in understanding the dynamics of disease transmission. Some patients may be asymptomatic carriers for years, for example as occurs in typhoid fever, while for some persistent viral infections such as hepatitis B and C and HIV, individuals may remain infectious, but without symptoms for many years (Heyman 2004; Aral 2000).

In some infections the symptoms resolve and the patient is no longer infectious, but the organism remains dormant, i.e. the infection re-enters the latent period. The disease may then subsequently reactivate, some-

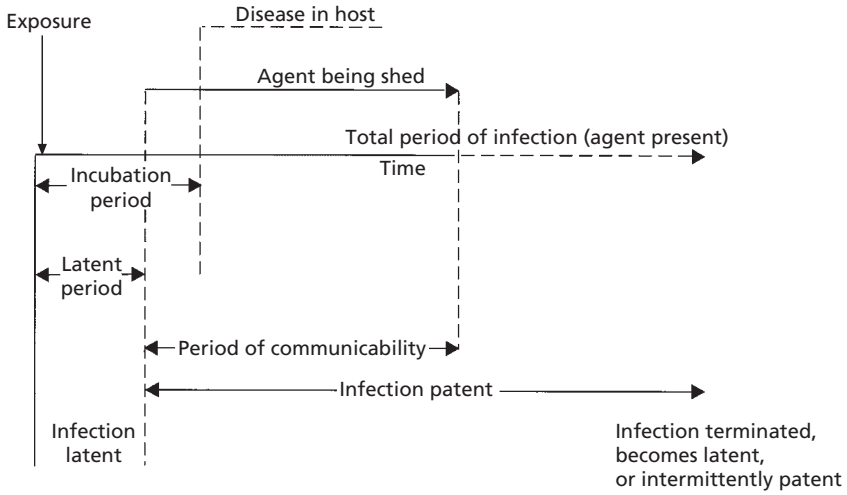

Figure 2 *Stages of infection*

times years later, with shedding of the organism. Varicella and malaria are examples of infections that commonly become latent. In other chronic infections, such as HIV, there may be an initial illness followed by a prolonged latent period with a more serious later illness years later (see Human immunodeficiency virus, in the *Virology* volume in this series). For hepatitis B the infection is commonly cleared, but in some cases the infection and infectiousness becomes chronic perhaps leading to severe disease later (Hepatitis B, in the *Virology* volume in this series).

The duration of the latent and infectious periods are important parameters of mathematical models. Classic epidemiological studies which observed the interval between primary and secondary cases within households enabled these parameters to be estimated for measles and other infections (Hope Simpson 1948; Bailey 1956a, b).

DEVELOPMENT OF IMMUNITY

The immunological basis for the development of immunity to bacterial infections is considered in the *Immunology* volume in this series. From the perspective of infectious disease transmission theory and control, there are a number of key components to be considered.

Some infections do not result in significant natural immunity and consequently can be acquired repeatedly. Gonorrhea and chlamydia are examples (Aral et al. 1999; Stoner et al. 2000). For infections that do stimulate an immune response, the duration of immunity influences the rate at which susceptibles accumulate in a population. Generally speaking, naturally acquired immunity is longer lasting than vaccine-induced immunity and in some infections immunity may be lifelong.

It is particularly important to distinguish between immunity against infection and immunity against disease. For example, live oral polio vaccine protects against both infection and disease, whereas inactivated polio vaccine protects predominantly against disease. This difference has significant implications for control programs – control of a polio epidemic can be achieved much more rapidly, and with lower vaccine coverage, with live vaccine than with inactivated vaccine.

Cross-immunity may occur with one prior infection giving some immunity to another different infection. Breastfeeding reduces susceptibility to a number of infections that affect infants and young children. The influence of maternal antibody which has crossed the placenta in utero is a highly significant factor in infections where transmission is common among babies. For example, the incidence of meningococcal disease in infants bears an inverse relationship to the decline in maternal antibody (Figure 3). The peak incidence is at 6 months of age, coinciding with the nadir in maternal antibody. After 6 months, naturally acquired immunity begins to develop and the disease incidence declines. The significance for control measures is that any vaccine for use in a mass immunization program would need to be effective during the window of susceptibility in early life.

Infection in the population

The course of infection in the individual has a strong influence on the pattern of disease in a population. Diseases that confer permanent immunity after a short infection, such as measles and rubella, occur naturally in a cycle of regularly recurring epidemics. Diseases for which individuals become susceptible to reinfection on recovery (e.g. gonorrhea), or some infected individuals become long-term carriers (e.g. hepatitis B, tuberculosis), do not show such a regular pattern (Noah et al. 1995).

Factors specific to each population, including demographics, behavioral patterns, and environmental factors also affect the incidence of the disease. These determine whether, and at what level, an infection can remain endemic in a particular population. There are many infections that have a widely differing level of endemic infection in industrialized and resource-poor countries. Hepatitis B is commonly acquired in childhood in

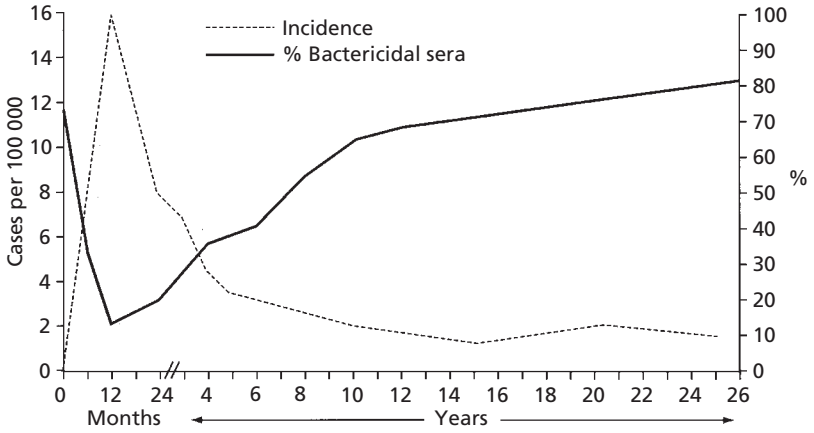

Figure 3 *Relationship between age-specific incidence of meningococcal infection and immunity (after Goldschneider et al. 1969).*

sub-Saharan Africa, but is largely confined to adults in particular risk groups in Northern Europe. Hepatitis A, and other infections transmitted by the fecal–oral route, are acquired at a younger age in countries where sanitation is poor (see Control of disease by hygiene). The zoonoses mostly occur where their specific animal hosts are to be found and humans are exposed, Legionnaires' disease is almost unheard of where its environmental reservoir of piped water is not found (Giesecke 2002). The differences are less striking for infections such as measles and rubella, but these too are acquired at a younger age in less developed countries. The contrasting epidemiology of an infection in different populations may necessitate entirely different approaches to disease control.

BASIC REPRODUCTION NUMBER

The basic reproduction number (R_0) for an infection within a population summarizes the effect of many of these factors in a single parameter. (It is variously termed the basic reproductive/reproduction rate/ratio/number by different authors, but almost always denoted R_0; and pronounced R nought, R zero or R subzero!) It is defined as the number of secondary infections that a typical infectious individual would produce in a completely susceptible population (Anderson and May 1991; Dietz 1993). Thus, R_0 depends both on the properties of the infective organism and on the social and demographic characteristics of the population. It may vary between infections, but also for the same infection in different populations. R_0 can be interpreted as the average number of contacts made by an individual during the infectious period; a contact being defined as any encounter in which an infectious individual would transmit infection to a susceptible individual (a definition that is clearly disease specific). This is a crucial variable for designing rational control programs. Some accepted values of R_0 for different common infections are shown in Table 2. Infections with high values of R_0 are regarded as highly infectious. Vaccine preventable diseases with a high R_0 are much more difficult to eliminate than those with low R_0. For example, a human infectious disease with an $R_0 = 20$ and without an environmental reservoir requires a population immunity of 95 percent or higher to achieve elimination, whereas one with $R_0 = 4$ requires a population level immunity of only around 75 percent.

Where infections have relatively low values for R_0 and are not infectious during the latent period, control may be effected by case finding and isolating those affected or influencing them to reduce infection risk (for example by condom use for sexually transmitted infection). This will be less successful or impossible if R_0 is high and transmission can take place before symptoms appear or immunity emerges. For example compare SARS, sexual acquisition of HIV, influenza, and measles (Table 2).

EFFECTIVE REPRODUCTION NUMBER

In most real situations, not all of the population are susceptible to infection: some are immune either as a result of previous infection or through vaccination. Some of the contacts of an infectious person will not result in new infections but be 'wasted' on immune individuals. The effective reproduction number R is defined as the number of secondary cases produced by a typical infectious individual (Anderson and May 1991). Clearly R depends on R_0 and the susceptibility of the population. For a simple homogeneous model (in which contact between any two individuals is assumed to be equally likely) $R = R_0x$, where x denotes the proportion of the population who are susceptible.

Persistence thresholds

An infection can only establish itself in a population if $R_0 > 1$. Any such infection has an endemic equilibrium state at which $R = 1$, i.e. where a typical infectious individual produces on average one secondary infection. At this equilibrium state only one of the R_0 contacts made by an infective is with a susceptible individual. In the simple model, the proportion of the population who are susceptible is $1/R_0$. The equilibrium at $R = 1$ is a threshold: if $R > 1$ the number of infections increases; if $R < 1$ the number of infections decreases. Many infections demonstrate this threshold behavior in their natural epidemic cycles. During these cycles R oscillates around 1 as the proportion susceptible oscillates around the equilibrium value.

The $R = 1$ threshold is the key to designing vaccination and other control programs. If a vaccination program aims to eliminate a disease in a population, it must maintain $R < 1$, so that the number of infections decreases with each successive generation of cases. The necessary level of immunity could be achieved, in the long term, by immunizing a proportion p of the population at birth,

Table 2 *Selected infections – what stage infection can take place at and some estimated values for* R_0

Infection	At what stage infection generally takes place	Basic reproduction No. R_0
HIV (sexual transmission)	At any stage	<1 (per single sexual act)
Influenza	Prior to or during symptoms	3–4
Measles	Prior to or during symptoms	Around 10
Severe acute respiratory syndrome	Not prior to symptoms, infectiousness peaks in second week	2–3

where $p = 1 - 1/R_0$ (so that only a proportion $1 - p = 1/R_0$ of newborns remain susceptible).

HERD IMMUNITY

The above arguments suggest that it is possible to eliminate a vaccine preventable infection without immunizing every member of the population. It is this concept of indirect protection of susceptible individuals to which the term 'herd immunity' has been attached, although many authors have been deliberately vague in any definition. Fox et al. (1971) discussed a dictionary definition of herd immunity as 'the resistance of a group to attack by a disease to which a large proportion of the members are immune, thus lessening the likelihood of a patient with a disease coming into contact with a susceptible individual'. In a wide-ranging review, Fine (1993) points out the ambiguity of this definition – it fails to distinguish between total protection (when the immunity of the herd is above the threshold and the disease cannot persist) and partial protection (where the presence of some immune individuals lessens the risk to a susceptible). The threshold concept is the more appealing, and it leads to a definition of a population having herd immunity if $R < 1$. An equivalent statement of this definition is 'a population is said to have herd immunity to an infection if a typical primary infection produces less than one secondary infection'. Such a concise definition has the advantage of clarifying a sometimes vague concept; either a population has herd immunity, or it does not. The amount by which the population is above or below the herd immunity threshold is quantified by the effective reproduction number R.

Although the concept of herd immunity has been defined, there is still a need to clarify what is meant by a 'typical' infective. In a simple homogeneous model all individuals in the population are assumed to have the same number of contacts. Every infective is typical. In more complex models, which incorporate heterogeneity in the contact process, the typical infective is defined as some suitably weighted average of all infectives. (The typical infective is determined through a defined mathematical framework as the fastest growing stable distribution of infectives (Diekmann et al. 1990.) The definition of a population having herd immunity if $R < 1$ is applicable to these more complex models. The factors determining the effective reproduction number are the number of contacts made by infectives and the levels of susceptibility. The immunity of the herd does not depend necessarily on the immunity of individuals. For example, a population in which an infection has an $R_0 < 1$ has herd immunity to that infection even if no individuals are immune.

ESTIMATION OF R_0

Dietz (1993) has reviewed the variety of methods that have been used to estimate the basic reproduction number for infectious diseases. Many require data on the endemic state of the disease before the introduction of any control measures. Perhaps the most simple of these is (Anderson and May 1991):

$$R_0 = \frac{N}{B(A - m)},$$

where N is the population size, B is the annual number of births, A the average age at infection and m is theaverage duration of protection from infection provided by maternally derived antibodies. For stable populations this expression reduces to $R_0 = L/(A - m)$, where L denotes the life expectancy. (A brief heuristicjustification of this formula is possible for populations where virtually all individuals are infected during their lifetime: $A - m$ is the average duration of susceptibility so the proportion susceptible $Rx = (A - m)/L$, and at equilibrium $R_0 = 1/x$.) In deriving these simple formulae it is assumed that the risk of infection for a susceptible individual (often called the force of infection) is independent of age. The complications introduced by an age-dependent force of infection, which preclude the derivation of an explicit formula for R_0, are discussed later.

Mathematical modeling

The essential requirement of a mathematical model isthat it describes the mechanism underlying the processof interest. The key to models of infectious diseases is an understanding of the interactions between infectious and susceptible individuals that result in the transmission of infection. These should be described by a set of clearly stated assumptions, which can then be expressed as mathematical equations relating the variables and parameters of the model. The model is simply the means of evaluating the consequences of a set of assumptions.

When formulating a model, a compromise must be reached between simplicity and overcomplication. Simple models, which may have explicit solutions, clearly demonstrate the effect of each parameter, but may grossly oversimplify the complexities of the transmission process. More complicated models provide greater flexibility, but each additional refinement introduces more parameters thereby increasing the task of parameter estimation and making it more difficult to discern the effect of any individual parameter. In general, simple models are most useful for investigating qualitative effects, whereas more detailed models are needed for quantitative predictions.

When interpreting the results of any modeling exercise, it is essential to bear in mind the assumptions on which the model is based. No model can fully describe all the complexities of the transmission of a disease in a population. However, models can greatly enhance the understanding of the processes underlying disease

transmission, and play an important role in the rapid design of control programs, for example, during the control of SARS, modeling, along with observational techniques, was used to quickly estimate the disease's R_0 and this, with other parameters, was used to inform the control strategy (WHO 2003; Anderson et al. 2004).

The examples in this part of the chapter will concentrate on infections for which humans are the only host, that have short latent and infectious periods and confer permanent immunity, as exemplified by measles. A flow diagram illustrates the basic structure of a model for such an infection showing the compartments that may be included (Figure 4). Models for other infections, such as those that confer no immunity or which have a carrier state, require this structure to be adapted accordingly. Anderson and May (1991) present and discuss models with different structures, which are used for a wider range of infectious diseases such as zoonoses, sexually transmitted infections, and infections with environmental reservoirs for which modeling approaches are also used (Anderson and May 1991). Some of these will be considered later in this chapter.

DYNAMIC BEHAVIOR

Numerical simulations of infectious disease transmission proceed in discrete timesteps to describe the changes over time in the number of individuals in each of the model's compartments. The values at each timestep are calculated from the values at the previous timestep, starting from some specified initial values. This procedure is essentially the same for all models, although complex models may have more compartments or further stratification by age, sex, etc.

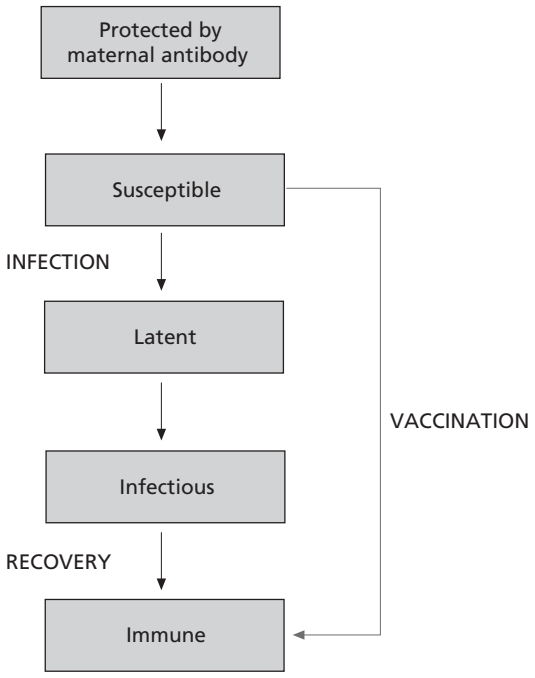

Figure 4 *Flow chart showing the compartmental structure of mathematical models*

The rates at which transitions between compartments occur are determined by the model's assumptions and parameters. For example, the rate at which individuals move from the latent to the infectious compartment is governed by the latent period, and the rate of movement from infectious to immune by the infectious period. The crux of all models of disease transmission is the rate at which susceptibles are infected, referred to as the force of infection, and how this is assumed to depend on the number of infectious individuals.

The mass action assumption

The force of infection, λ, is the rate at which susceptible persons acquire infection. The probability that a given susceptible individual will acquire infection in a short period δt is $\lambda \delta t$. For a directly transmitted infection, the force of infection at any time depends on the infectives present in the population at that time. The simplest assumption is that the force of infection is directly proportional to the total number of infectives. The rate at which new infections occur in the population is then proportional to the product of the number of susceptibles and the number of infectives. This model is known as the mass action model because of its similarity with the model for chemical reactions.

Hamer's discrete time model

The simplest version of a mass action model is that originally used by Hamer (1906) to describe measles epidemics in London. He considered infections as occurring in discrete generations, denoting the number of susceptibles and infectives in the nth generation by S_n and I_n, respectively. The number of susceptibles and cases in the following generation were calculated from the difference equations

$$I_{n+1} = \tfrac{1}{m}\, I_n \mathit{ThickSpace} S_n$$
$$S_{n+1} = S_n - I_{n+1} + \mathit{ThickSpace} b.$$

Thus the number of new infections is assumed to be proportional to the number of susceptibles and the number of infections (the 'mass action' assumption); all infections from the previous generation are removed (by recovery or death). The number of susceptibles is decreased by the number of new infections and increased by the influx of b susceptibles at each timestep; it is assumed that there is no mortality of susceptibles.

The only parameters of the model are the time between generations, the number of susceptibles added at each timestep, and the critical number of susceptibles, m. (The critical number of susceptibles m can be written in terms of the basic reproduction number R_0 and the population size N as $m = N/R_0$. The mass action assump-

tion then has the interpretation that each of the I_n cases makes R_0 contacts, of whom a proportion S_n/N are susceptible; leading to $I_{n+1} = R_0\, I_n\, S_n/N$ cases in the next generation.) Hamer used a 2-week generation time (the sum of the latent and infectious periods for measles), and the values $b = 4\,400$ (there were approximately 2 500 births per week, but 300 of these babies died before losing protection from maternal antibodies at 6 months of age) and $m = 150\,000$ (determined empirically).

The model produces a series of undamped regularly recurring epidemics, whose magnitude and period depend on the parameters and initial conditions. Hamer used initial values of $I_0 = 12\,800$, $S_0 = 150\,000$ to produce a cycle with a period of 18 months (i.e. 39 2-week periods) (Figure 5). He noted that the number of susceptibles was fairly stable during the course of an epidemic cycle, oscillating by just 20 percent around the equilibrium number of 150 000. If the number of susceptibles exceeds this threshold there are more infections in the next generation, i.e. $I_{n+1} > I_n$ if $S_n > m$; if the number of susceptibles is below the threshold, the number of infections decreases. (The effective reproduction number at the nth generation, $R_n = S_n/m$, and oscillates around 1 during the epidemic cycle.)

Continuous time model

The continuous time equivalent of Hamer's model produces damped epidemic cycles (Soper 1929). The period of these cycles, T, can be calculated analytically (Soper 1929) and expressed in terms of the average age at infection, A, and the combined length of the latent and infectious periods D;

$$T = 2\pi\sqrt{AD}.$$

Predictions of the interepidemic period using this formula correspond well with observed values for many infections (Anderson and May 1991).

Reed–Frost model

The Reed–Frost model is preferred to the mass action model for outbreaks in small populations. Again, cases are considered as occurring in discrete generations, and let p be the probability of contact between any two individuals in each timestep. The probability of a susceptible being infected is modeled as the probability of meeting at least one infective, which is $1 - (1 - p)^{I_n}$. In this formulation the number of new infectives is not proportional to the number of infectives in the previous generation, as the model accounts for the possibility that a susceptible may be contacted by more than one infectious individual.

HETEROGENEITY

The models considered so far have been homogeneous models in which all susceptibles in the population experience the same force of infection. This is undoubtedly a gross simplification of most real situations. For infections such as measles, mumps, and rubella, the force of infection is highly age dependent due to heterogeneity in contact patterns (Farrington 1990; Grenfell and Anderson 1985; Anderson and May 1985a); in schools, children have most contact with other children of the same age. Changes in transmission rates as schools open and close can account for the seasonality of measles (Fine and Clarkson 1982a; Schenzle 1984). For sexually transmitted diseases, the degree of mixing between different subgroups of the population may be crucial in determining the spread of infection (Aral 1999; Aral et al. 1999; Gregson et al. 2002). For other infections, spatial factors such as population density may play a role, or genetic differences in susceptibility may exist (Anderson and May 1984). Control of infections spread by intimate contact, notably sexually transmitted infections, have to take into account factors such as sexual networks, partnership patterns, and diverse behaviors with varying risk of infection. Heterogeneity in the

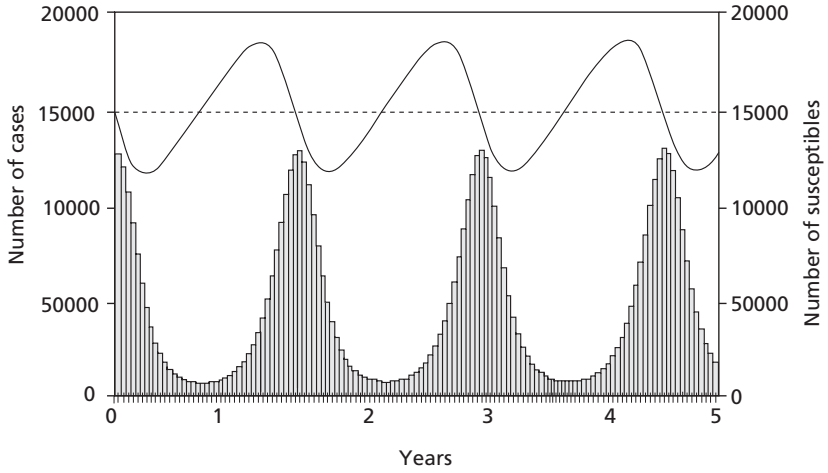

Figure 5 *Number of cases and susceptible individuals predicted by Hamer's discrete time model for measles in London.*

application of control measures such as vaccination may introduce further problems, e.g. pockets of susceptibility from unvaccinated communities or clustering of vaccination failures. The simple homogeneous model can be generalized in order to investigate these effects (Anderson and May 1991).

DETERMINISTIC AND STOCHASTIC MODELS

The transmission of an infection between individuals in a population depends on a number of chance events. In a large population, with many such events, the observed behavior will be close to that expected from averaging random effects. Deterministic models examine the expected behavior of a system; running a deterministic simulation under the same conditions gives the same result every time. This makes them easy to work with.

The major weakness of deterministic models is their behavior in a situation with a small number of susceptibles or infectives, where individual random events may influence the outcome. This may arise from an outbreak in a small population such as a school, or from a very low level of infection in a large population, perhaps as the result of a successful vaccination program. An example of infection outside vaccine preventable diseases is SARS where a small proportion of cases results in superspreading events (WHO 2003; Anon 2003). Stochastic models, which use randomly generated numbers to determine the outcomes of individual events, are better suited to such situations. Stochastic simulations are usually repeated many times to generate a range of results, so that the probability of an outbreak of a particular size or duration can be inferred.

The difference between deterministic and stochastic formulations is illustrated by the case of introducing a single infective into a population in which there is no infection. If $R > 1$, a deterministic model will always generate the same outbreak, whereas with a stochastic model one possibility is that no secondary transmission occurs (this may even be the most likely outcome). Alternatively, if $R < 1$ no significant spread occurs in a deterministic model, whereas a stochastic model has a finite probability of generating a large outbreak.

A combination of stochastic and deterministic approaches may be required at different stages of the same problem. The investigation of the effects of introducing a mass vaccination program in a population is an appropriate application for a deterministic model. If the program was implemented successfully to eliminate endemic infection, stochastic modeling could be used to yield the distribution of the size of outbreaks expected from any infections that come into the population, for example from abroad.

Strategies for disease control

The strategy adopted to control an infectious disease will depend on many factors including the aim of the program, the availability of resources, the acceptability of possible measures, and all the factors around the epidemiology of the disease (Table 1). This section outlines the possible aims, describes the strategies available, and discusses when they are appropriate.

AIM OF A CONTROL PROGRAM

Programs for disease control need to have clearly defined aims. The program can be designed to meet this aim and its success in achieving the aim can be assessed (Hawker et al. 2001). The three broad aims are containment, elimination, and eradication (Noah 1988) and a variety of control measures may be available (Table 3).

Containment

Containment is the most modest control option. Infection and infection risk remain endemic in the population or with an imported infection its spread is limited, but morbidity from the disease is reduced to an 'acceptable' level. An example of a containment aim was the WHO measles target for 1995: a 90 percent reduction in measles

Table 3 *Possible control strategies to interrupt or reduce transmission of infection*

Control strategy	Example
Removal of infections from distribution	Contaminated food source
Controlling infection in a nonhuman reservoir	Immunizing chicken flocks against *Salmonella* species
Isolation or cohorting	Isolating infectious patients in hospital – methicillin resistant *Staphylococcus aureus*
Quarantine	Quarantining smallpox contact
Increasing or inducing immunity	Routine immunization
Treatment of those infected	Whooping cough in early stages
Treatment of those potentially exposed	Immunoglobulin given to newborns born to hepatitis B infectious women
Influencing behavior through education and establishing a facultative environment	HIV – condom use by those infected
Reducing transmission in high risk situations	Protective clothing in operating theaters
Preventing spread between populations	Travel restrictions –SARS

cases and a 95 percent reduction in measles deaths from the levels before vaccine was introduced. With many infections, containment is the only possible aim.

Elimination

Elimination of an infectious disease from a population requires that chains of transmission either do not occur or are readily broken. This will mean that there is no endemic transmission of infection within the population, and that an infection imported into the population produces no more than an isolated outbreak. This may be achieved through the herd immunity of the population or by vigorous application of control measures. Either way the effective reproduction number must be maintained below one. Elimination is generally more difficult to achieve where there are reservoirs of infection outside humans, though it may be achieved if inter-human transmission is inefficient, i.e. R_0 is low.

Eradication

Eradication of an infectious disease requires the global destruction of the pathogen that causes it. There has to be no effective environmental or animal reservoir. It eventually allows the cessation of all control measures, potentially providing huge savings for future generations. Smallpox is the only disease to be eradicated so far. The last case of wild smallpox infection occurred in 1977. A WHO program to eradicate polio is in progress. Elimination from the Americas and the Western Pacific has been achieved and significant advances have been made in all other regions, though as of 2004 the infection is stubbornly persisting in six particular countries in sub-Saharan Africa and South Asia (WHO 2004a).

ROUTINE VACCINATION

Routine vaccination is the most common method for administering vaccine to a population. Individuals (usually children) are vaccinated when they reach a target age. Such a program requires maintenance of a suitable infrastructure (to ensure a regular supply of vaccine, have trained staff, informed families, etc.). If the vaccine provides protection against infection (and not just against the disease), the introduction of a vaccination program will affect the circulation of infection.

Age at infection

The dynamic interaction between susceptible and infectious individuals in the population can lead to counter-intuitive results. A vaccination program that leads to reduced circulation of infection, but not elimination, will cause the average age of infection to rise (Figure 6). Unprotected individuals experience a reduced force of infection and so remain susceptible longer, and thus are older when they eventually become infected. The change in the age distribution of cases is even more marked if transmission rates are higher in older age groups. This effect is beneficial if disease is most severe at a young age (e.g. pertussis, measles in developing countries), but it can lead to an increase in morbidity if disease severity increases with age or if it puts fetuses at risk, for example by shifting the age of infection in females into their childbearing years as is the case with rubella (Anderson and Grenfell 1986). Vaccination programs against diseases such as polio and rubella (see later under Rubella: the prevention of congenital rubella syndrome) must achieve high coverage so that the reduction in circulation outweighs the problem of increasing age at infection.

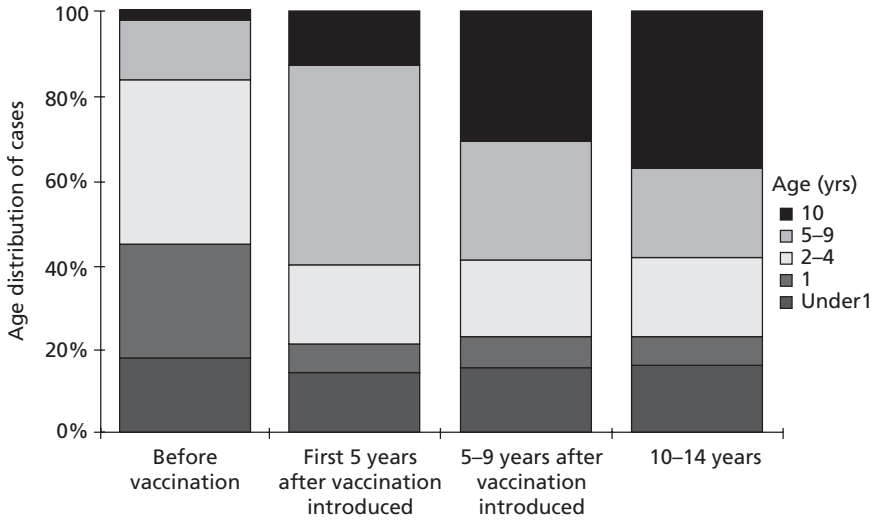

Figure 6 *Modeled change in the age distribution of infection following the introduction of vaccination. Simulation of measles in a developing country before and after the introduction of a routine vaccination program that immunizes 80 percent of children at age 9–12 months. Parameter values: basic reproduction number,* $R_0 = 20$*; life expectancy,* $L = 50$ *years; latent period, 1 week; infection period, 1 week; duration of protection by maternal antibody,* $m = 6$ *months.*

Honeymoon periods

The 'honeymoon period' is the name sometimes given to the period of low incidence that is frequently observed following the introduction of a routine vaccination program, but which (if vaccination coverage or efficacy is not sufficiently high) is inevitably followed by a resurgence of infection (McLean and Anderson 1988; McLean 1995; Chen et al. 1994). When the vaccine is first introduced, many of those above the age of vaccination will be immune through natural infection. Vaccination reduces the proportion susceptible well below the threshold. However, the consequent low force of infection allows unprotected individuals (those who are not vaccinated or in whom vaccination fails to induce immunity) to accumulate. Once the threshold is exceeded, a post-honeymoon epidemic can occur (Figure 7). If the vaccination program is maintained, the infection will settle into a new epidemic cycle with a considerably longer epidemic period than before vaccination.

VACCINATION CAMPAIGNS

Vaccination campaigns aim to vaccinate all children in a target age range and geographical locality within a short period of time (often a day, a week or a month). Campaigns may be used to supplement routine programs (either as a one-off or as a method of giving a second dose) or as an alternative to them. They are often favored in countries that do not have the infrastructure to maintain a high coverage through routine vaccination program, but they are now also used in industrialized countries where it becomes necessary to quickly reduce susceptibility in a particular age group (Gay 1996).

The advantage of a suitably targeted campaign is that it removes most of the susceptible individuals from the population, thus reducing the reproduction number R well below the $R = 1$ threshold. This has a dramatic effect on transmission which may be interrupted if sufficient coverage is achieved. The follow-up program must maintain herd immunity, either through routine immunization or through periodic campaigns. If the latter approach is chosen, the interval between follow-up campaigns should be determined so as to maintain $R < 1$. A campaign is needed whenever the herd immunity threshold is about to be exceeded. Serological surveillance data enable a campaign to be targeted to maximal effect.

This basic strategy is behind the WHO's attempt to eradicate polio, and the attempt of the Pan American Health Organization to eliminate measles from the Americas (de Quadros et al. 1996). The elimination of measles through mass campaigns was pioneered in the Gambia in 1968–1970, based on modeling simulations by Macdonald (Thacker and Millar 1991), and revived in Cuba (Sabin 1991). Elimination of measles from the Americas may pave the way for eventual eradication.

OUTBREAK CONTROL

Outbreak control is a key activity for many types of infections. The techniques and tools that can be used are various and are dependent on the infection and its characterization though it needs to be recognized that the evidence base for a number of the older and more established infections are not as well established as they are for more recently described infections (Tables 1–4). For vaccine-preventable diseases, it may be used to complement or even replace mass vaccination programs when disease incidence is low. However, it is only suitable for infections where outbreaks can be identified at an early stage and/or where the infection is not highly transmissible (a low R_0), so that control measures can be taken before widespread transmission occurs. This requires that cases can be diagnosed rapidly and reasonably accurately (preferably clinically, and there is likelihood that measures are likely to be successful, i.e.

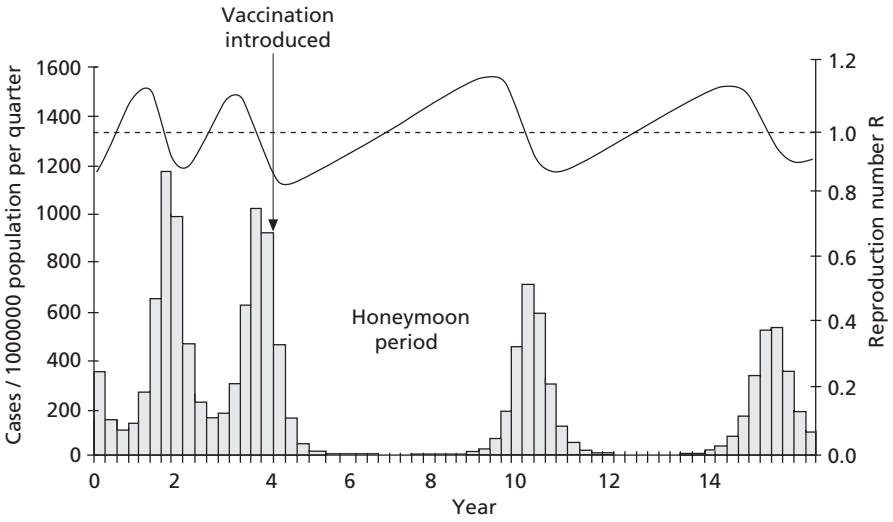

Figure 7 *Modeled number of cases of infection following the introduction of vaccination (for details see Figure 6)*

Table 4 *Techniques used for outbreak control*

Control techniques
Establishing an outbreak control team with defined authorities
Reporting the outbreak
Microbiological investigation of cause
Epidemiological investigation
Determining the appropriate control measures
Case-finding
Contact-tracing
Isolation of those infected and infectious
Quarantine of those exposed for the incubation period
Immunization of those at risk or to induce herd immunity
Source identification and control
Screening to detect those infected or at risk
Treatment of those infected
Protective infection control measures
Cohorting of hospital patients
Informing professionals and public
Reducing risk behaviors
Monitoring application of control measures
Monitoring effectiveness of control measures
Communicating with the authorities, media, and public

without needing laboratory confirmation) and that asymptomatic infections are uncommon. Low transmissibility of infection and a long latent period are also advantageous as they cause outbreaks to develop slowly, permitting time for interventions. As always, the control measures must be acceptable to the population and be within the law.

Aggressive outbreak control was an integral part of the smallpox eradication strategy and for the control of SARS; as both satisfy all the above criteria. However, attempts in the United States to control outbreaks of measles through targeted vaccination proved largely unsuccessful (Davis et al. 1987). Infections for which vaccine is currently used to control community outbreaks include hepatitis A, although it is not ideally suited to such an approach due to the high proportion of subclinical infections, but the low incidence of disease in the population does not justify the expense of mass vaccination.

QUARANTINE AND ISOLATION

Quarantine means to isolate or control the behavior of healthy people who are thought to have been exposed, are probably or possibly infected, and are in the incubation period (Heyman 2004). A quarantine strategy may be used to prevent the introduction of an infection into a population from which it is absent. It is only appropriate if the population is isolated from the potential source of infection. No attempt is made to create herd immunity in the population, so a failure of the quarantine measures could lead to a sizeable outbreak. Quarantine policies should therefore be backed up by outbreak control procedures. Quarantine measures were used extensively in North America in the nineteenth century to prevent ships from Europe introducing infections, such as cholera, smallpox, and typhus. They are still used to exclude rabies from the UK and are part of plans to control viral hemorrhagic fevers (Department of Health 1996). They are also a key part of emergency plans if smallpox should re-emerge and during the 2003 SARS outbreaks were used in some affected areas (Centers for Disease Control 2002; WHO 2003).

Isolation is generally taken to mean separating symptomatic infectious persons from those who are susceptible, as a policy can only be successful if infectives are identifiable and identified before they transmit infection.

CASE-FINDING AND SCREENING

Case-finding is essential to isolation and caring for those infected. However, it is also used more widely in controlling infections where identification contributes to interrupting transmission. It requires that cases are readily identifiable – some diseases, like SARS, pose difficulties because of the problem in distinguishing cases from other diseases causing similar symptoms (WHO 2003).

Case-finding is considered an important part of the strategy to control HIV transmission in industrialized countries where it is used to effect behavior change amongst those infected so as to make them less likely to pass on infection. Another example is when there has been health care worker infections for hepatitis B or C whilst undertaking exposure-prone procedures.

Screening is a special form of case-finding where it is considered useful to test particular population groups for specific infections (Table 4). It may be systematic or opportunistic (testing patients who are attending medical care for another purpose) and usually has to conform to the guidelines applied to screening (Calman 1994). An example of systematic screening is the testing of all pregnant women for infections they may transmit to their babies and for rubella immunity. Donor screening is used to ensure infections are not transmitted to recipients of blood and tissues.

Herd immunity and vaccination programs

Most communicable diseases have a regular cyclical pattern (Noah 1988). The interval between epidemics is determined by the rate at which susceptibles accumulate in the population. If epidemics occur in regular cycles it is possible to predict in advance when the next epidemic will occur. For example, pertussis epidemics in the UK have occurred at 4-year intervals. Other infections are less predictable. Epidemics of meningococcal disease occurred during both world wars, in the early 1970s, and again since the mid-1980s. Influenza epidemics are even less predictable and are associated with major antigenic shift in the influenza virus (see the *Virology* volume in this series). In general, the epidemic cycles of viral infections are more regular than bacterial diseases.

Introducing a vaccine which protects against transmission of infection disrupts the natural epidemic cycle of a disease. Models which simulate the transmission of infection within a population may be used to predict the effects of vaccination programs on the incidence of disease. The predictability of epidemics has been greatly enhanced in recent years by serological surveillance, where the proportion susceptible to an infection by age is determined through large cross-sectional antibody prevalence studies. In the UK, routine serological surveillance for measles, mumps, and rubella antibody was established in 1987, prior to the introduction of combined measles, mumps, rubella (MMR) vaccine (Morgan-Capner et al. 1988) using the unlinked-anonymous technique. The recent application of mathematical models to serological surveillance data enabled the anticipation of a measles epidemic in the UK which was averted by a national immunization campaign (Gay et al. 1997) (see later under Measles: vaccination strategy in England and Wales). Data from other serological surveillance contribute to the control of many other infections (Nicoll et al. 2000; Osborne et al. 2000).

Diphtheria

Diphtheria is an example of an infection where the use of high herd immunity has changed over time. The symptoms of diphtheria are caused by the production of an exotoxin which is produced by the infecting organism, *Corynebacterium diphtheriae*. Naturally acquired antitoxic immunity provides protection which is usually lifelong. The traditional method for measuring diphtheria immunity – the Schick test – is no longer available and has been superseded by the assessment of neutralizing antitoxin in serum. A level of 0.1 units of antitoxin per ml or greater indicates long-term immunity. In the prevaccination era, most babies were born with maternally derived natural immunity, and infection was relatively uncommon in the first year of life. Attack rates were highest in the 1–5 year age group and by early adulthood most individuals were immune to diphtheria (Figure 8).

Close contact is required for transmission of diphtheria and therefore epidemics occurred particularly under conditions of crowding and poor hygiene. The importance of closeness and duration of contact in determining the spread of disease was first described by Dudley in 1926. Children sleeping in the same school dormitory were at greater risk than those in casual contact during working hours. In temperate countries, the disease was most common in the colder winter months, whereas in tropical countries transmission occurred all year round. In tropical countries, the principal source of infection is from cutaneous diphtheria lesions, whereas in temperate countries pharyngeal diphtheria was more common.

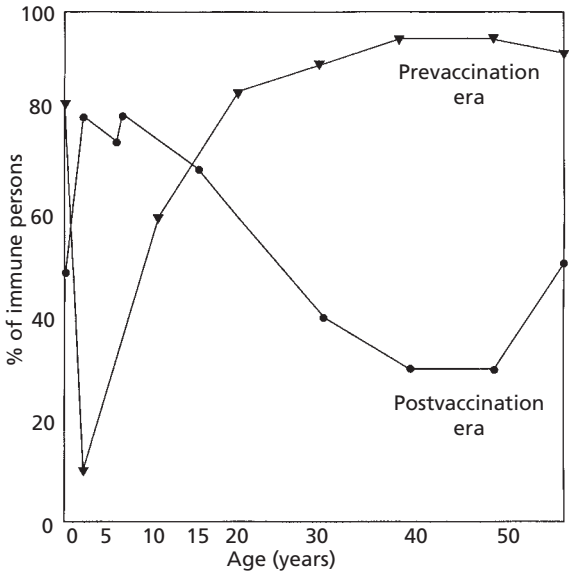

Figure 8 *Diptheria immunity by age in the pre- and post-vaccine eras*

Routine immunization with diphtheria toxoid (which protects against the toxic effects of the disease) was introduced in the 1940s. Immunization very rapidly reduced disease incidence in these countries and by the 1960s diphtheria had virtually been eliminated. As a result, the pattern of immunity in the population has changed completely. Because the organism no longer circulates in the community, adults born since the introduction of immunization rely solely on vaccine-induced immunity, which is generally lower than naturally acquired immunity. Immunization is commenced at 2 months of age so young infants very rapidly acquire vaccine-induced immunity, which is boosted by further immunizations given at school entry and (since 1993) on leaving school. However, older adults, in whom the last dose of vaccine was given many years ago, have waning immunity and up to a third of older adults may now be susceptible to diphtheria in Britain (Maple et al. 1995). Fortunately, this immunity gap does not appear to have led to significant outbreaks despite the fact that cases of toxigenic diphtheria continue to be imported from resource-poor countries (Begg and Balraj 1995). In the states of the former USSR, diphtheria re-emerged in the 1990s. This is only partly explained by waning immunity in adults. Other factors include mass population movements and lower vaccine coverage in young children.

Because vaccine-induced immunity protects against the toxic effects of the disease, one might expect that diphtheria immunization would not reduce circulation of the organism. Nevertheless, isolation of a toxigenic strain of *C. diphtheriae* is very rare nowadays, therefore it appears that vaccine does interrupt transmission as well as prevent disease. This is probably related to the fact that transmission of *C. diphtheriae* is much more efficient from symptomatic cases than from subclinical

carriers. The level of diphtheria immunity in the population that is required to achieve herd immunity is not known precisely, but is generally considered to be between 70 and 90 percent (Simonsen et al. 1987).

Pertussis

Immunity to pertussis is complicated, and many components of *Bordetella pertussis* appear to have a role. At present the serological correlates of immunity are not known, thus population immunity data are of little value in mathematical modeling.

In Britain, pertussis epidemics occurred at 4-year intervals before the introduction of routine immunization in the 1950s (Figure 9). There were over 100 000 reported cases a year and about 1 per 1 000 patients died from the disease. The mortality rate and the risk of complications from pertussis is greatly dependent on age. Most deaths occur in young children under the age of 6 months. After this age the severity of the disease declines progressively and in adults it may be a milder, self-limiting though long-lasting illness. Because the risk of severe disease is greatest in the first few months of life it is important that pertussis vaccines are given as early as possible. For this reason, an accelerated schedule of immunization, at 2, 3, and 4 months of age, was introduced in the United Kingdom in 1992.

Whole call pertussis vaccines were introduced in the 1950s. Coverage was initially high (over 80 percent) and the magnitude of epidemics decreased considerably. In 1972 only 2 000 cases were recorded. However, in the mid-1970s doubts were cast over the safety of the vaccine and vaccine coverage dropped from 80 to 30 percent. This led to a resurgence in epidemics, the size of which had not been seen for over 20 years. Subsequently, vaccine coverage has improved and the epidemics have again decreased in size. In 2000, only 883 cases were notified in England and Wales, the second lowest annual figure ever recorded (the lowest was 712 in 2000).

The interesting feature throughout this period is that despite the introduction of vaccine with great

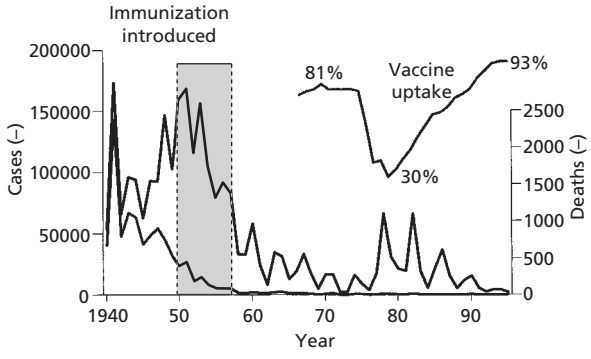

Figure 9 *Whooping cough notifications: cases and deaths, England and Wales 1940–1995 (prepared by CDSC; source OPCS)*

fluctuations in coverage there has been little change in the length of the interepidemic cycle. This led to the suggestion that pertussis vaccine may have no effect on carriage, but simply protects against disease (Fine and Clarkson 1982b). However, a decrease in the incidence of pertussis in children too young to be vaccinated points to some reduction in transmission (Miller et al. 1994). A recent study, which used an age-structured model to simulate the transmission of pertussis in England and Wales, suggested that both these observations are consistent with vaccine reducing transmission by approximately 80 percent (Miller and Gay 1997). In this scenario, the recent improvements in coverage to more than 90 percent would lead to a discernible lengthening of the epidemic period and an increasing proportion of infections would occur in adults. There is increasing evidence that adults with clinically inapparent or mild infection may act as important reservoirs of infection.

The question of vaccine providing protection against the transmission of infection has important implications for the control of pertussis. If the current subunit vaccines do not protect against transmission then elimination of the infection would be impossible. It remains to be seen whether the recently developed subunit vaccines affect transmission. Some preliminary evidence suggests that they may do so. Field trials of efficacy have recently established the efficacy of acellular pertussis vaccines in infants from 2 months of age (Olin et al. 1997).

Rubella: the prevention of congenital rubella syndrome

Most cases of rubella present as a mild illness with rash. Infection in pregnancy, however, can cause congenital rubella syndrome (CRS), resulting in severe malformation of the fetus, including cataracts, deafness, and heart defects, and an outcome of death or severe handicap (Gregg 1941; Miller et al. 1982). The purpose of a rubella vaccination program is to reduce the number of cases of CRS by preventing infections in pregnant women. A number of different strategies has been used to achieve this goal since the vaccine became available in 1970. The risks and benefits of each vaccination program can be evaluated using mathematical models (Anderson and Grenfell 1986), enabling informed policy decisions to be taken.

Before vaccination was introduced in developed countries, rubella epidemics occurred every 4–5 years and the average age of infection was approximately 10 years. About 85 percent of the population experienced the disease by age 16, leaving 15 percent who remained susceptible to infection as adults. A significant number of infections in pregnancy and consequent cases of CRS resulted. In developing countries, rubella is acquired at a much younger age – almost everyone is infected as a

child and cases of CRS are uncommon. However, rubella vaccination may become necessary if social and environmental changes lead to a reduction in transmission rates.

POLICY OPTIONS

The number of rubella infections in pregnancy depends on the number of susceptible pregnant women and the risk of infection in pregnancy. Vaccination programs were designed to reduce the number of infections in pregnancy by reducing one of these contributing factors. The two main options of universal or selective vaccination are often termed the US and UK policies, respectively, after the countries in which they were originally used.

The universal policy

The universal policy was designed to eliminate CRS through the elimination of rubella in the population, achieved by a high vaccine coverage of all children at a young age. Susceptible pregnant women would be protected from infection by the herd immunity of the population.

The selective policy

The selective policy was designed to reduce the incidence of CRS by reducing the level of susceptibility among pregnant women, achieved by vaccinating girls only at age 10–14 and screening all pregnant women for rubella immunity. This would allow the virus to remain endemic, so that many girls would be infected and acquire immunity before they were vaccinated. Vaccination would serve to reduce the number of susceptibles. Further reductions could be achieved by screening pregnant women, and vaccinating those found to be susceptible before their next pregnancy.

RISKS AND BENEFITS

A universal policy offers the greater benefit (the elimination of CRS), but also entails more risk. If vaccination coverage is low, the number of infections in pregnancy may actually be increased by the vaccination program. Vaccination reduces the force of infection acting on the unvaccinated population, causing them to remain susceptible longer until they acquire infection at an older age. The number of susceptible pregnant women may increase. If the increase in susceptibility among pregnant women outweighs the reduction in the force of infection, more cases of CRS will occur. This occurred, for example, in Greece in the 1990s when a universal policy was incompletely implemented (Panagiotopoulos et al. 1999).

In contrast, a selective policy can only eliminate CRS with 100 percent coverage. The risk to susceptible pregnant women is not reduced by the vaccination program. However, the policy does not carry the risk of increasing the incidence of CRS; the reduction in the incidence of CRS is the same as the coverage achieved.

Anderson and Grenfell (1986) used an age-structured model to calculate the number of cases of CRS expected at different levels of coverage for each of the two policies. At the long term steady state under the universal policy, there was an increase in the number of cases of CRS for coverages less than 30 percent, but rubella and CRS were eliminated for coverage greater than 80–85 percent (Figure 10). If coverage exceeded 75 percent, the universal policy prevented more cases of CRS than a selective policy with the same coverage. Short-term dynamic simulations produced similar results.

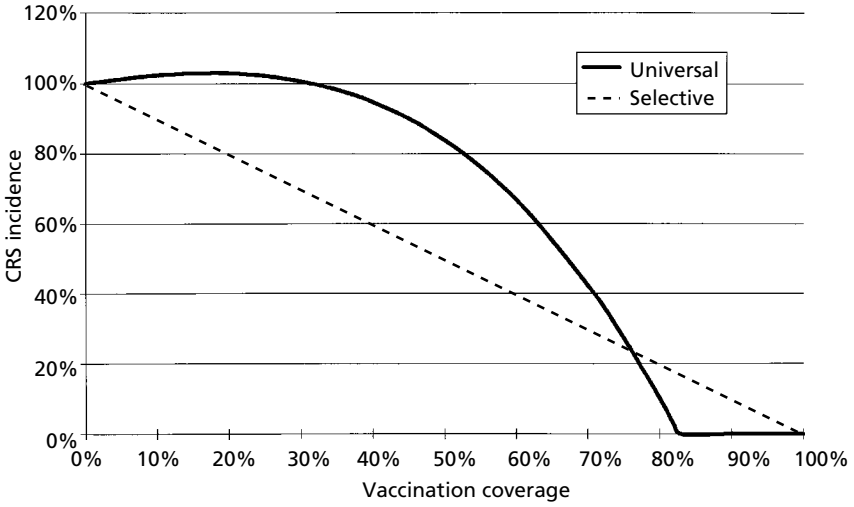

Figure 10 *Predicted equilibrium incidence of congenital rubella syndrome (relative to its incidence in the absence of vaccination) by vaccination coverage for selective and universal rubella vaccination programs (after Anderson and Grenfell 1986)*

Policy implications

The United States introduced a universal rubella vaccination program in 1969. Following a decline in the number of infections during the 1970s and 1980s to very low levels, a resurgence of rubella occurred in 1989–1991 (Lindegren et al. 1991; Centers for Disease Control 1994). Many cases were in unvaccinated young adults (including pregnant women; 56 cases of CRS were reported in 1990–1991) or in children from communities who refused vaccination. Since these outbreaks the incidence of rubella has returned to low levels. The US aimed to eliminate indigenous rubella and CRS by 1996, maintaining herd immunity through high vaccination coverage. To a large extent this has been successful with only 26 infants reported as born with CRS between 1997 and 1999 and 24 of these were in foreign-born mothers who are more likely to have missed out on immunization programs (Centers for Disease Control and Prevention 1994, 2001).

The UK introduced a selective vaccination program in 1970, and achieved 80–85 percent coverage at a time when the coverage of measles vaccination was less than 60 percent. Although this resulted in a considerable reduction in the number of infections in pregnancy, their continuing occurrence prompted a switch to a universal policy in 1988. A vaccination campaign amongst 5–16 year olds in 1994 removed many susceptibles from these cohorts, but in 1995 10–15 percent males aged 18–30 remained susceptible to rubella. This led to a resurgence of rubella in 1996, which included many outbreaks in universities and military barracks. The legacy of the selective vaccination program is that still in the mid-1990s less than 2 percent of women were susceptible to rubella, thus limiting the number of infections in pregnancy (Miller et al. 1996).

Sao Paulo State, Brazil, introduced a universal rubella vaccination program in 1992, following a modeling study to explore strategy options. This is an exemplary approach to vaccination program design. At the start of the program (in which vaccine is given to children at 15 months of age) a one-off vaccination campaign was carried out among older children to prevent the resurgent epidemics experienced in the US. The upper age limit of the campaign was set as 10 years as models suggested that little benefit was gained from including older children, despite the estimated seroprevalence in this age group being only 70–80 percent (Massad et al. 1994). The model's transmission rates were based on an age-specific force of infection estimated from a serological survey that included only 54 samples from persons aged 10 years or more (de Azevedo Neto et al. 1994). The experience of the next 10–15 years following the intervention will determine whether the decision not to include children aged 11–15 years in the campaign is an example of modeling saving unnecessary expenditure, or of too much faith being placed in the predictions of insufficiently robust models.

Measles: vaccination strategy in England and Wales

Measles is the most infectious of the vaccine-preventable diseases (Table 2) and causes significant morbidity and mortality if not controlled by vaccination. In this section we review the history of measles vaccination in England and Wales. In particular, we discuss the role of models in the planning of a national vaccination catch-up campaign conducted in 1994.

BACKGROUND

Measles vaccination was introduced in England and Wales in 1968. Before this time, measles epidemics occurred in alternate years causing an average of 100 deaths per year. Almost everyone experienced measles infection as a young child: 55 percent of notified cases were in those under 5 years, 42 percent in 5–9 year olds, and only 3 percent were in persons aged over 10 years. Vaccine uptake was initially low with only 50 percent of children being vaccinated up to 1980. Coverage then increased steadily reaching 80 percent by 1988. Over this period measles notifications and deaths showed a downward trend, but coverage was sufficiently low for the virus to remain endemic. Children who were not vaccinated became infected, resulting in continuing morbidity and mortality.

In 1988 combined measles, mumps, and rubella vaccine (MMR) replaced single antigen measles vaccine, following which coverage increased to 93 percent by the second birthday. This, together with an MMR catch-up program targeted at preschool children, resulted in a marked reduction in measles incidence in all age groups. Unvaccinated children, therefore, had little opportunity to acquire immunity through infection, and remained susceptible. The aging of cohorts with higher levels of susceptibility caused an increase in the proportion of school children who were susceptible to measles, detected through serological surveillance (Figure 11).

The shift of measles susceptibility was also reflected by a change in the age distribution of measles infections. A study using laboratory salivary diagnosis demonstrated that notifications (which are based on clinical diagnosis) were unreliable, especially in young children (Brown et al. 1994). Most infections occurred in persons aged 10 years or more and outbreaks in secondary schools were becoming more common (Ramsay et al. 1994; Calvert et al. 1994; Morse et al. 1994).

MODELING

Two separate approaches were used to predict the incidence of measles. Both models summarize their results using the reproduction number R. If $R < 1$, the population has herd immunity and no resurgence of disease can occur.

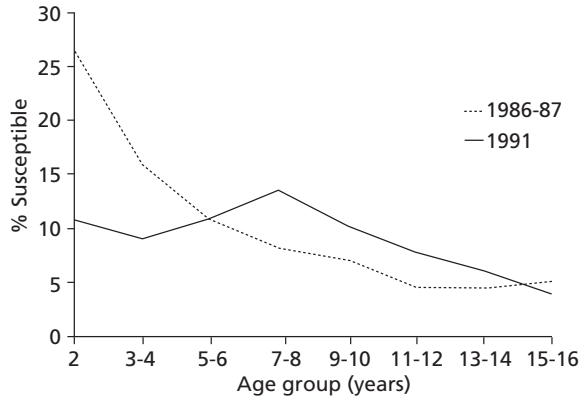

Figure 11 *Serological surveillance data: proportion of samples negative for measles IgG antibody by age, England 1986–1987 and 1991*

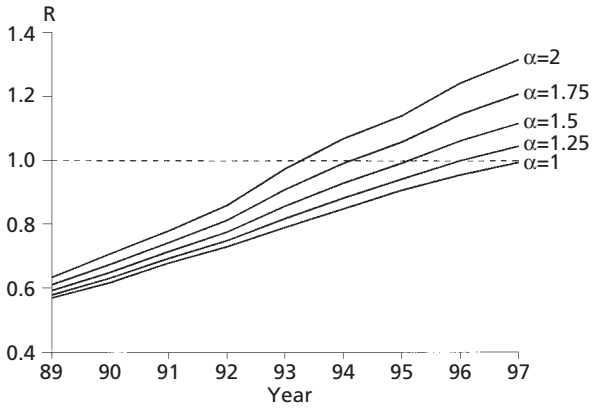

Figure 13 *Calculated value of the reproduction number for measles in England for a range of values of the age-specific transmission rates (Gay et al. 1995)*

The first study used models to interpret the serological surveillance data, to examine whether the increase in susceptibility would be sufficient to exceed the herd immunity threshold (Gay et al. 1995). The model divided the population into five age groups. The reproduction number is determined by the level of susceptibility in each age group and the transmission rates within and between age groups. (This calculation is the age-stratified equivalent of $R = R_0\, x$.) The level of susceptibility expected each year in each age group was projected from the serological data assuming no infection occurred. Transmission rates were derived from age-specific notification data from the prevaccination period (Figure 12). A range of values for the transmission rate within the 10–14 year age group was used due to the difficulty in obtaining a precise estimate. The model predicted that as the most susceptible cohorts moved into the age group with the highest transmission rates (10–14 year olds), the reproduction number, R, would exceed one. This would occur at some point before 1998, depending on the transmission rate assumed in the 10–14 year age group (Figure 13). The scenario that best reflected the observed changes in the age distribution of cases suggested that this would occur sooner rather than later. This provided the potential for a resurgence of measles in 1995/1996 involving more than 100 000 cases with most occurring in persons aged 10 years or more. Such a resurgence would have resulted in a high level of morbidity and mortality, because of the severity of measles in older age groups.

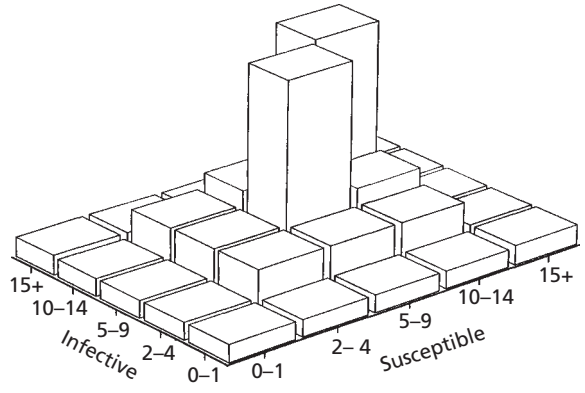

Figure 12 *Age-specific transmission rates for measles in England and Wales (with α = 1) (Gay et al. 1995)*

The second study simulated measles transmission in England and Wales using a dynamic model fed with vaccination coverage statistics (Babad et al. 1995). The model provided a good reflection of historic data. The study concluded that a single dose vaccination policy would not be sufficient to eliminate measles and investigated the options for supplementary vaccination measures. A vaccination campaign covering all children aged 5–16 years would reduce R well below the threshold. The introduction of a second routine vaccine dose at age 4 years, immediately after the campaign, would maintain the herd immunity of the population.

EFFECT OF THE CAMPAIGN

A national catch-up campaign to immunize children aged 5–16 with a combined measles and rubella vaccine was carried out in 1994 (Miller 1994). All children were offered vaccine, irrespective of a previous history of vaccination or disease; 92 percent coverage was achieved. The incidence of measles dropped dramatically following the campaign and the few cases that have occurred are in a pattern consistent with limited spread from imported infections (Gay et al. 1997). The introduction of a second dose into the routine vaccination schedule at school entry should sustain the herd immunity achieved. Continued surveillance is essential to monitor the effect of the program: salivary investigation of all suspected cases enables outbreaks to be identified; serological surveillance monitors the maintenance of herd immunity. Probably the most damaging impact on immunization for measles and mumps have been continuing controversies, much as two decades earlier similar

stories were for pertussis. Though there has been a decline in uptake of MMR in the UK, the fall is much less than that seen for pertussis (Gangarosa et al. 1998; Nicoll 2001; Miller 2003).

ENVIRONMENTAL AND SOCIAL EFFECTS AND POPULATION IMMUNITY

Changes in environmental and social conditions may affect the population in a number of ways. Poor hygiene facilitates spread of gastrointestinal infections, such as dysentery, by the fecal–oral route. Crowded accommodation facilities spread airborne infections, such as tuberculosis and meningococcal disease. Excessive throughput of patients in hospital makes it difficult to attend to infection control and control outbreaks. Temperature and other environmental conditions affect the survival of infectious disease agents. Social conditions, such as sexual behavior, and patterns of occupation influence the likelihood of being exposed to specific infectious agents (Hawker et al. 2001).

Control of disease by hygiene

In populations and settings where hygiene is poor, enteric infections spread easily. In settings such as refugee camps and following natural disasters, intense outbreaks may occur. For example, hepatitis A is endemic in many developing countries where basic hygiene measures such as hand washing and safe disposal of sewage are lacking. Most children become infected in the first few years of life. In this young age group, a large proportion of infections are subclinical and icteric cases are relatively uncommon. Where hygiene is good, infection in young children is relatively uncommon and most reach adulthood with no immunity to hepatitis A (Figure 14). In these countries, most infections occur in adults, at which age symptomatic infection is very common. Two main patterns of disease are observed – slowly evolving community outbreaks lasting for months or even years in which infection is transmitted from person-to-person and explosive point source outbreaks arising from a contaminated food or water source (Tang et al. 1991). Countries in the transition between the highly and lowly endemic states often experience a rapid reduction in the incidence of infection (Perez-Trallero et al. 1994; Lim and Yeoh 1992; Yap and Guan 1993). Models suggest that this may be followed by a substantial resurgence of endemic disease (Gay 1996).

Hepatitis A vaccines have recently become available and are of value in protecting those who are likely to be most at risk, such as travellers to highly endemic areas. No country has yet implemented hepatitis A for routine use (largely on the grounds of cost), however vaccines against hepatitis A, which could be incorporated into other vaccines as combination products, are being developed. In countries with good hygiene, the basic reproduction number for hepatitis A is relatively low (approximately two in England and Wales; Gay et al. 1994) and herd immunity could be achieved with modest vaccination coverage. However, mass vaccination programs may be most appropriate for countries in the transitional phase.

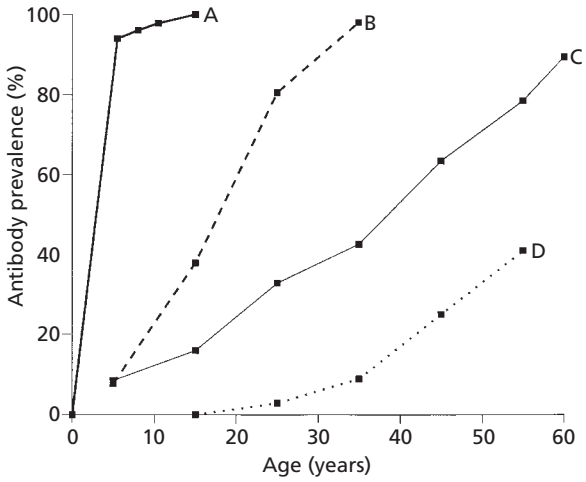

Figure 14 *Prevalence of antibody to hepatitis A in several countries: A, Cameroon, 1989 (Stroffolini et al. 1991); B, Spain, 1986 (Perez-Trallero et al. 1994); C, England, 1987 (Gay et al. 1994); D, Sweden, 1977 (Iwarson et al. 1978)*

Poliomyelitis is another example of a disease that is affected by changes in hygiene. During the early part of this century, when hygiene was poor in Britain, polio circulated widely among preschool children. In this age group paralysis was relatively uncommon and therefore the number of paralytic cases was small. During the 1940s and 1950s, as hygiene improved, and the circulation of wild virus diminished, the average age of infection rose. This led to epidemics of paralytic disease in older children and young adults (Figure 15). Polio vaccines became available in the 1950s, and rapidly reduced the incidence of the disease. Global eradication of polio is now planned by the World Health Organiza-

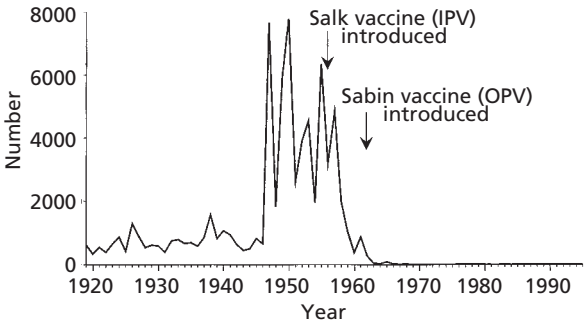

Figure 15 *Poliomyelitis notifications, England and Wales 1919–1995 (prepared by CDSC).*

tion. Eradication of polio is feasible, as there are no known nonhuman reservoirs of the disease, the virus does not survive long in environmental sources, such as sewage, live oral vaccine is able to both interrupt transmission as well as prevent disease, and is relatively cheap and easily delivered. However, at present (2004), it is proving difficult to achieve elimination in some countries experiencing civil strife and certain other populous countries, such as India and Nigeria (WHO 2004a).

Hospital hygiene is a major driver of health care-associated infections, though other factors come into play including surgical techniques undertaken as well as the level of dangerous pathogens in the surrounding population.

Sexually transmitted diseases and blood-borne viruses

Sexually transmitted diseases are an example of infections whose incidence is affected by behavior. The incidence of a disease is affected not only by the mean rate of partner acquisition, but especially by the degree of heterogeneity in behavior within a population. The importance of core groups (groups accounting for disproportionately large amounts of transmission) in the transmission of infection is well documented (Yorke et al. 1978; Jacques et al. 1988). Changes in the behavior of the core groups or their contact with noncore groups can have a dramatic effect on the incidence of infection and close attention to core groups is important for any STI control program.

Core groups are not the same in every country. In industrialized countries, men who have sex with men (MSM) are often important, while in resource-poor countries female sex workers often have a greater role though it may be that MSM are present, but not recognized because of severe stigma. Not all STIs conform to a pattern determined by core groups. Transmission of chlamydia and human papilloma virus (genital warts) is mostly accounted for by contact in the general sexually active population, especially among young adults. Control of STIs has to be through case-finding and treatment and effecting behavior change, including condom use (Mayaud et al. 1997; Samuel et al. 1991; Wawer et al. 1999; Cohen 1995). The blood-borne viruses, hepatitis B and C and HIV, follow a similar pattern of transmission to the STIs, but in addition are spread by equipment sharing among injecting drug users. Control is through prevention of drug injection, targeted promotion of safer injecting (needle exchanges, etc.), and treatment of those addicted.

The studies of gonorrhea by Yorke et al. (1978) and Hethcote and Yorke (1984) were the first detailed investigations of the transmission dynamics of a sexually transmitted disease. They described how changes in behavior affected the level of endemic disease. Since the mid-1980s the public health priority of controlling the HIV/AIDS epidemic has produced explosive growth in the literature. However, the complexity of the interactions between subgroups of the population and the problem of collecting suitable data from which to estimate parameters make modeling particularly difficult. Population-based surveys (Johnson et al. 1992) have provided a basis for initial parameter estimates. However, transmission models remain most useful for assessing the relative contribution of various factors to future trends in the incidence of AIDS, rather than for making quantitative predictions (Williams and Anderson 1994). STI control programs have shown how control of one bacterial infection can in some circumstances reduce transmission of a viral infection, HIV (Wasserheit 1994; Grosskurth et al. 1995; Cohen 1995).

Environmental infections

Some important infections persist in the environment and this is the key to their control. For example, Legionnaires' disease is a persistent problem in large buildings and requires scrupulous attention to water regulations (Giesecke 2002). For other infections, environmental spread via food, water, or the air means careful attention to water sources and food distribution chains are required (Hawker et al. 2001). In these infections par excellence contributions by social scientists and modelers have become crucial to understanding spread and devising control methods (Garnett et al. 1999; Hahn 1991; Klovhdah 1985; Robinson et al. 1995; Morris and Kretzschmar 1997; Schinazi 2001).

Vector control

A variety of infections are transmitted indirectly through vectors, such as insects. One of these, malaria, was the first infection in which population dynamics were studied extensively. Transmission of malaria depends on several factors, but the most important relate to the anopheline mosquito vector. The calculation of the basic reproduction number and eradication threshold are different with reference to vector-borne infections than to directly communicable infections. The contact parameter is a function of the density, survival rate, and feeding behaviors of the vector populations. The estimates of the basic reproduction number for malaria range between 5 and 100. These reflect considerable variations in the epidemiology of malaria, which is observed even within relatively small geographic areas. Macdonald developed a formula for the likelihood of infection based on the proportion of anopheline mosquitoes with sporozoites in their salivary glands. Malaria transmission in Macdonald's model was proportional to the density of the vector, the number of times each day

that the mosquito bites man, and the probability of the mosquito surviving for 1 day. Macdonald's model has been refined in recent years, but it does illustrate certain important points relevant to the control of malaria. Vector longevity is the most important factor in determining transmission and therefore focuses control measures on the adult mosquito. Control programs are most likely to be effective where vector longevity is short and the basic reproduction number is low. This was the situation in Europe where the reproduction level was low in many areas and the vector was found mainly inside houses where it could be attacked with insecticides. There is also considerable variation between different species of anopheline mosquitoes with respect to their ability to transmit malaria (the vectorial capacity). Each vector has its own behavior patterns and breeding grounds. Malaria is often seasonal, coinciding with the rainy season which provides water for mosquito breeding and increased humidity favoring mosquito survival.

Vector-borne human infections are not confined to tropical countries and there are many other organisms that are spread by mosquitoes (Ludwig and Cooke 1975). West Nile virus has, since the late 1990s, appeared and spread extensively from east to west across the USA and is proving very difficult to control (Granwehr et al. 2004).

Current malaria control strategies focus on protecting individuals against bites (bed-nets, etc.) or disease (human chemoprophylaxis). These are not very successful at a population level. Several malaria vaccines are currently under development and these may have individual, as well as population, actions. They are being designed to act at various stages of the cycle of infection. Some of the candidate vaccines may only protect against transmission and not against disease.

GENETIC FACTORS IN POPULATION IMMUNITY

Studies of herd immunity must clearly take into account genetic differences in resistance. Much of the work in this area was done on mice in the 1920s developing so-called Webster mice. These workers were able to show genetic effects in resistance to infection which emerged as a result of inbreeding and other genetic manipulations. The extent to which this work can be extrapolated to man is not clear. Observations in human populations are beset by confounding factors. There is no doubt that some populations are more susceptible to certain infectious diseases than others. Of particular note is the fact that there are sex differences in disease attack rates for many childhood infectious disease, for example mumps is more common among boys than among girls. The incidence of meningococcal disease and other infections caused by bacteria with polysaccharide capsules varies enormously in different parts of the world. Certain populations, for example Australian Aboriginal, have very high rates of disease and respond less well to polysaccharide vaccines than white children (McIntyre et al. 2000).

ZOONOSES AND EMERGING INFECTIONS

A number of the most important human infections have their origins in animals which either act as a continuing reservoir of infections (e.g. salmonellae, *Escherichia coli* O157), or were the original source, but now transmission is established between humans (HIV, measles). For the former, theories of transmission and control rely on animal husbandry, as well as on protecting humans. For example, some countries have been very successful in reducing levels of salmonellae pathogenic to man through vaccinating chicken flocks (Methner et al. 1999).

Zoonoses contribute disproportionately to the continuing stream of emerging infections that threaten human health. Many of these infections are of limited consequence, but a small number, such as HIV, the new viral hemorrhagic fevers, multidrug resistant organisms, and West Nile virus, has posed major threats. These infections require control measures which have to be based on good theoretical work tested against rapidly gathered data. The rapid institution of control measures against SARS shows how modern laboratory and epidemiological science can quickly combine even with an atypical and novel infection (WHO 2003; Anderson et al. 2004). Equally the persistent threat and near occurrence of pandemic influenza from avian influenza in domestic fowls as far away as China and as near as the Netherlands indicates no scope for complacency (WHO 2004b).

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Emergence and resurgence of bacterial infectious diseases

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In recent years, the world has witnessed an emergence and resurgence of infectious diseases. Despite predictions earlier in this century to the contrary, infectious diseases remain the leading cause of death in developing countries, and the potential threats posed by infectious diseases are increasing (Institute of Medicine (IOM) 1992, 2003). The factors contributing to the emergence of infectious diseases are multiple and complex. They include demographic changes, changing lifestyles, political instability, unprecedented global travel and commerce, development of new technologies, ecological changes, microbial adaptation and natural variation or mutation of microorganisms, deterioration of national and international infrastructures for the control of infectious agents, and intent to do harm. These factors may contribute to the emergence of a wide array of microorganisms, including bacteria, viruses, parasites, and fungi.

As broadly defined by the 1992 US Institute of Medicine report, *Emerging Infections: Microbial Threats to Health in the United States*, emerging infections are those whose incidence in humans has increased within the past few decades or whose incidence threatens to increase in the near future. These include the emergence of new agents, the re-emergence of agents that previously had declined in incidence, and the development of antimicrobial resistance. The term also includes the recognition that an established disease has a previously unknown infectious origin.

DEMOGRAPHIC CHANGES

Increasing population density and urban poverty in many areas of the world are major factors favoring the emergence and re-emergence of diseases such as tuberculosis, shigellosis, and cholera. The population of the world, less than two billion at the beginning of the twentieth century, exceeded six billion by the end of the century and by the year 2050 is expected to grow to between 7.3 and 10.5 billion (Figure 1) (Rousch 1994; Lutz and Qiang 2002).

Along with the population surge, the geographical distribution of the world's population is shifting. Since 1950, an increasing percentage of people are living in Asia and Africa. It is predicted that Africa's percentage of the world's population will continue to increase, from 12 percent in 1990 to 23 percent by the year 2050 (Rousch 1994). In turn, the percentage of the world's population living in Europe or North America is declining. There is also a dramatic trend towards urbanization, with most of the population increase expected between 2000 and 2030 to be absorbed by urban areas in developing countries. By the year 2015, the United Nations (2001) projects that six cities will have 20

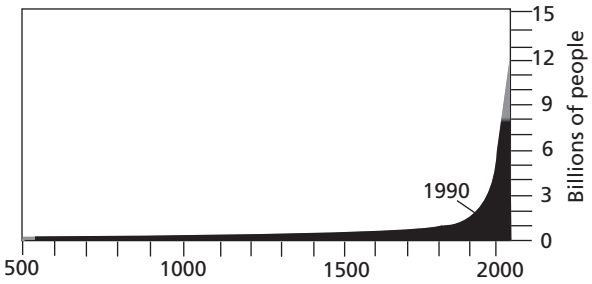

Figure 1 *World population through 1990, with projections by United Nations demographers of high and low scenarios. (Abstracted with permission from Roush, 1994. Copyright 1994 American Association for the Advancement of Science.)*

million population or more, including Tokyo, Dhaka, Mumbai, Sao Paulo, Delhi, and Mexico City. Overcrowding and development of slum areas are growing factors in a complex set of interactions that contribute to disease emergence.

INTERNATIONAL TRAVEL AND COMMERCE

The dramatic increases in international travel and commerce are also playing a significant role in the emergence of infectious diseases. The current volume of global traffic, together with the speed at which destinations can be reached, is unprecedented. In 2000, the number of international trips on commercial airplane flights was estimated to reach nearly 700 million (IOM 2003), and flights carried people with drug-resistant tuberculosis, cholera, and other diseases. Occasionally, the transport vehicles are themselves the sites of dissemination of disease, as with legionnaires' disease on cruise ships (Centers for Disease Control and Prevention (CDC) 1994c; Castellani et al. 1999) or food-borne illnesses or tuberculosis on airlines (Driver et al. 1994; Hedberg et al. 1994; CDC 1995c).

There are many examples of human movement resulting in geographical dispersion of a pathogen; recent advances in molecular epidemiology have been instrumental in documenting the course of a number of pathogens. In the early 1980s, a pneumococcus multiply resistant to penicillin, tetracycline, chloramphenicol, and trimethoprim-sulphamethoxazole was described in Spain, and its spread to the USA was documented in 1992 (Munoz et al. 1991), South Africa in 1994 (Klugman et al. 1994), and South Korea in 1997 (McGee et al. 1997). It is now recognized as a global clone (McGee et al. 2001) and the contribution to this single clone to the burden of penicillin-resistance is remarkable – in three studies, this clone alone comprised 39 percent (Corso et al. 1998), 16 percent (Gherardi et al. 2000), and 18 percent (Richter et al. 2002) of all penicillin-resistant strains from multiple geographic sites across the continental USA. Recently this clone has acquired resistance to the fluoroquinolones. In Hong Kong, which has the highest rate of fluoroquinolone resistance in the world, all the resistant strains belonged to this single clone (Ho et al. 2001).

In the early 1990s, an estimated 20 million people were refugees, a group that may be at higher risk of many infectious diseases (Wilson 1995). More recent estimates are even higher (Summerfield 1997), and tuberculosis, *Shigella*, and cholera are among the diseases causing deaths among refugees. Drug-resistant tuberculosis has been common among refugees entering developed countries from developing countries and has contributed to the resurgence of tuberculosis witnessed in some developed countries.

Acute disasters such as earthquakes and displacement of people through civil strife, may have an impact on food and water supplies and often require temporary living quarters, which frequently resemble slums. Following civil strife in Rwanda in 1994, an estimated 50 000 Rwandan refugees died within the first month as cholera and *Shigella dysenteriae* type 1 swept through the camps in Zaire (Goma Epidemiology Group 1995). This strain spread widely down through Africa, associated with epidemic hemolytic-uremic syndrome (HUS) (Bloom et al. 1994).

Global commerce has also been an important factor in transporting pathogens. Importation of turtles, iguanas, and other reptiles carrying *Salmonella* (CDC 1995b), the carriage of *Vibrio cholerae* in the ballast water of ships, and shipment of fresh fruits and vegetables contaminated with *Shigella* or enterotoxigenic *Escherichia coli* from one geographical area to another (Hedberg et al. 1994) have resulted in outbreaks of disease geographically distant from the source of the pathogen (Wilson 1995).

Although most fruits and vegetables used to be grown and consumed locally, the increasing trend towards global commerce has led to expanded importation of fresh fruits and vegetables from developing countries into more developed countries, and workers within developed countries today are often low-paid migrant workers (Hedberg et al. 1994). These factors increase the potential for products to be contaminated in the field, during packing, and during distribution to retail markets and have contributed to the changes in the epidemiology of food-borne diseases, which has witnessed an increasingly wider array of pathogens including bacteria, parasites, and viruses.

Travel, migration, and commerce allow global mixing of microbial species. Disease emergence may occur if circumstances in the new environment are propitious, allowing survival and proliferation of an introduced microbial pathogen.

SOCIETAL CHANGES

Changes in individual and collective behavior can affect the risk of exposure to infectious agents. For example,

as the association was made between cardiovascular disease risk and consumption of selected foods such as beef, people changed their dietary habits, including the consumption of different food items. In the USA and many other developed countries, the per capita consumption of whole milk and beef declined, and consumption of cheese, poultry, and fresh fruits and vegetables increased (Hedberg et al. 1994). Changes in dietary habits continue as new issues emerge. The concern following the recognition of variant Creutzfeld–Jakob disease related to bovine spongiform encephalopathy led to an abrupt decline in beef consumption in some countries; at the same time, an inceased focus on addressing obesity in the USA has led to increases in the consumption of high-protein foods, including beef. The variety of food has also changed; compared with 50 years ago, a markedly increased number of items are stocked in the average grocery store in developed countries. The place of consumption has also changed; the number of fast-food restaurants has increased, as have such features as salad bars to accompany the changing dietary preferences.

The proliferation of fast-food restaurants with uniform methods of preparation and frequent use of single sources of food has further expanded the opportunities for a large number of people to be exposed to contaminated food. In an outbreak of HUS and hemorrhagic colitis in western USA caused by hamburger contaminated with *E. coli* O157:H7 (Bell et al. 1994), scores of restaurants affiliated with a single fast-food chain were implicated.

In some economies, there has been an expanding participation of women in the workforce and a rising proportion of single-parent families, accompanied by growth in the use of child-care services. In the USA, 90 percent of families with preschool children use full- or part-time child daycare services (Thacker et al. 1992). Because children are frequently in close or direct physical contact and have poor personal hygiene, pathogens may be transferred more readily from one child to another in these facilities. Use of child-care centers has been associated with an increased risk of transmission of many enteric and respiratory pathogens

Outbreaks of diarrheal illness in child-care centers have resulted from many enteric pathogens including *Shigella*, *Salmonella*, *Campylobacter*, and *E. coli* O157:H7 (Thacker et al. 1992). Children attending daycare centers are at a 1.6–3.4 times greater risk of having a diarrheal illness compared with children who receive care at home. The risk is greatest for those not yet toilet-trained. Secondary spread to household and community contacts may also occur.

The incidence of respiratory illnesses, including those caused by *Streptococcus pneumoniae* (Levine et al. 1999) and group A *Streptococcus* (Espinosa de los Monteros et al. 2001), is also elevated for children attending child-care centers. Accumulating evidence suggests that daycare attendees are at greater risk for acute otitis media, which often follows upper respiratory infections, than children cared for at home (Rovers et al. 1999; Baraibar 1997). Increased use of child-care centers may account partially for the marked increase in visits to physicians for otitis media documented in the USA between 1975 and 1990, from approximately ten million visits to over 24 million visits per year (Schappert 1992). Outbreaks of tuberculosis in family daycare homes have also been reported.

Changes in sexual behavior have also affected changes in disease patterns (Wasserheit 1994; Donova 2002). The rates of sexually transmitted diseases are closely associated with changes in the number of sexual partners, use of drugs and adequacy of health-care services. Recent increases in reported rates of chlamydial infection are related not only to improvement in surveillance but also to increases in sexual activity of very young women. High rates of oral contraceptive use may foster rapid transmission of chlamydial infection.

CHANGES IN TECHNOLOGY

Changes in technology have also resulted in the emergence of newly recognized pathogens. New methods of food production have led to new niches for pathogens. Mass production, with its increased complexity of operations, can greatly magnify the public-health significance of microbial contamination. A pathogen present in some of the raw material may contaminate a large batch of final product. For example, there have been dramatic changes in how hamburger meat is produced in the USA. Today, a single hamburger patty typically includes meat from many cattle, and cattle from many different farms (Boyce et al. 1995). Over a quarter of a million hamburgers contaminated with *E. coli* O157:H7 were recalled in one single outbreak (Bell et al. 1994; Berkelman 1994). Contaminated cheese from one plant distributed to four other processors, which subsequently shredded it and thereby contaminated cheeses from other sources, led to a widespread outbreak of salmonellosis (Hedberg et al. 1992).

Medical and technological advances in the medical care of patients have resulted in benefits to many patients, but many of these advances have also been accompanied by an increased risk of infections. The escalation in the use of chemotherapy, radiation, and other immunosuppressive therapy has increased the frequency of opportunistic infections. Bacteremias and fungemias associated with intravascular devices have increased dramatically (Edgeworth et al. 1999). Renal dialysis units expose susceptible patients and personnel to complex equipment that frequently is difficult to decontaminate (Favero et al. 1992). Surgery is performed on patients who are already highly susceptible to infections.

Special attention must be given to the risk of transmission of infection through transfusion of blood or blood products (Kuehnert et al. 2001; Walsh et al. 2002). In the USA, there has been an increase in the number of episodes of sepsis caused by *Yersinia enterocolitica*; prolonged storage of the packed red blood cell units have resulted in high bacterial and endotoxin concentrations in the transfused unit (Tipple et al. 1990; CDC 1991). In addition, there have been more reports of infections with staphylococcal species resulting in sepsis or death associated with contaminated platelets; most of these units were pooled units with long storage times (Zaza et al. 1994).

The use of organ transplants has also grown dramatically in many countries in the past decade. Organ transplants pose special risks: the organ may harbor a pathogen, and the transplantation procedure is accompanied by immunosuppressive therapy (Hibberd and Rubin 1992). The number of opportunistic bacterial, fungal, and other infections has also risen as a result of the emergence of human immunodeficiency virus (HIV). Although the advent of highly active antiretroviral therapy (HAART) has had a major impact on the burden of opportunistic infections in developed countries, these infections threaten to overwhelm health services in sub-Saharan Africa.

Legionnaires' disease has emerged in developed countries as a result of technological change. Cooling towers, air conditioners, whirlpool spas, respiratory therapy equipment, ultrasonic mist machines, and industrial settings in which aerosols are produced have all provided new opportunities for the *Legionella* bacterium to become aerosolized and infect humans (CDC 1994b, Fry et al. 2003).

Technology related to tampon development and use was associated with the emergence of menstrual-associated toxic shock syndrome. This illness is due to in vivo production of a unique toxin, toxic shock syndrome toxin-1, by *Staphylococcus aureus*, and the epidemic of toxic shock syndrome witnessed in 1980 was associated with the introduction and marketing of hyperabsorbable tampons. The molecular basis for increased toxin production in the presence of tampon fibers has been debated (Kass 1987).

LAND-USE PATTERNS AND ECOLOGICAL CHANGE

Ecological changes have contributed to the emergence of tick-borne diseases in many areas of the world. Lyme disease, caused by transmission of *Borrelia burgdorferi* through tick bites, has become the most common vector-borne bacterial disease in the USA, and reported cases of disease continue to increase. The disease is also well recognized across Europe and northern Asia; cases have also been reported from other continents (Berglund et al. 1995). Sites of intense transmission of *B. burgdorferi* often represent newly reforested areas that had been farmed recently. The suburban encroachment on the newly reforested areas, along with increased recreational use of forested areas, has also increased the exposure of humans to ticks.

Human ehrlichiosis was first described in the USA in the mid-1980s (Blanco and Oteo 2002; Dumler and Bakken 1995). *Ehrlichia chaffeensis* causes human monocytic ehrlichiosis, a syndrome characterized by fever, headache, and laboratory findings such as leukopenia and thrombocytopenia, and it may be life-threatening if untreated (Paddock and Childs 2003). It is primarily transmitted by the lone star tick, *Amblyoma americanum*. A closely related organism, *Anaplasma phagocytophilum* (formerly classified in the genus *Ehrlichia equi*), causes a similar syndrome known as human anaplasmosis (formerly human granulocytic ehrlichiosis). People affected in the USA have spent time in tick-infested areas where *Ixodes scapularis* and *Ixodes pacificus* are common. The European vector is the same as that of Lyme borreliosis, *Ixodes ricinus*.

Alterations in the aquatic environment may result in changes in the occurrence of waterborne diseases. The seasonality of cholera has been related to coastal algae blooms (Epstein et al. 1994). Other changes in human ecology, such as urbanization and the development of periurban slums, may also contribute to an increased risk of diseases such as cholera (Levine and Levine 1994).

Both long-term climate changes and weather have been shown to alter the risk of various infectious diseases. Heavy rainfall contributed to a large outbreak of leptospirosis in Nicaragua in 1995, with exposure of humans to flood waters contaminated by urine from infected animals, particularly dogs (Trevejo et al. 1998). El Niño provided unusual conditions that favored growth of *Vibrio parahaemolyticus*, which led to an outbreak of disease related to shellfish (CDC 1998).

The incidence of Buruli ulcer, caused by *Mycobacterium ulcerans*, has dramatically increased in West Africa in recent years (Dobos et al. 1999). The increase is attributed to environmental changes including regional flooding. The natural reservoir is unknown but suspected to be an environmental exposure, since many affected individuals live near slow-moving or stagnant water.

MICROBIAL ADAPTATION AND CHANGE

New pathogens may emerge as a result of a mutation causing enhanced virulence. Such a mutation may explain the emergence of Brazilian purpuric fever in 1984, caused by *Haemophilus aegyptius*; it was the first time that this agent had been shown to cause invasive disease (CDC 1985; Brazilian Purpuric Fever Study Group 1987). Since its recognition in Brazil, this virulent

pathogen has been found in Australia; other parts of the world may be at risk for epidemics.

In 1993, the clonal spread of a novel non-O1 serotype of *V. cholerae* was documented (Ramamurthy et al. 1993); *V. cholerae* O139 was first detected in southern Asia and quickly replaced *V. cholerae* O1 strains in many affected areas. Previous natural infection or receipt of cholera vaccine was found to afford little or no protective benefit.

The development of resistance to antimicrobial agents is also a powerful example of bacteria's capacity to adapt. Almost all major bacterial pathogens acquire antibiotic-resistance genes (Tomasz 1994) and the problem has been amplified by the increased use of antibiotics worldwide.

The introduction of penicillin in the 1940s was soon followed by the detection of resistant bacteria. Bacterial resistance has grown with the addition of large numbers of antibiotics with distinct mechanisms of action. These antibiotics have increasingly had a wider antibacterial spectrum. Antibiotics have been introduced not only for therapeutic use in people but also on a large scale in animal feed. Endtz et al. (1991) demonstrated the rise in prevalence of *Campylobacter* strains that were resistant to ciprofloxacin in both poultry products and in human stools in the Netherlands. Extensive use of fluoroquinolones in the poultry industry between 1982 and 1989 is likely to have resulted in the resistance observed. The ban of antimicrobials for growth promotion in Denmark has led the way to reducing resistance amongst enterococci in that country (Aarestrup et al. 2001).

Once genes for resistance have been acquired, the progeny of these bacteria with their resistance genes tend to spread with great rapidity within the species. Production of β-lactamase by staphylococci was rare before the introduction of penicillin. More than 90 percent of all isolates of *S. aureus* now carry the β-lactamase gene, in both the USA and Europe, and methicillin resistance increased rapidly in the 1980s. Strains with and without genes for resistance demonstrate similar virulence in terms of toxin production. Methicillin resistance is mediated most frequently by the production of a novel penicillin-binding protein. Noble et al. (1992) demonstrated conjugative transfer of high-level vancomycin resistance from *Enterococcus faecalis* to *Staphylococcus aureus* in the laboratory and raised concern that resistance may be transferred to wild-type *S. aureus*. The vancomycin-resistance determinant *vanA* has now indeed spread from the enteroccoccus to *Staphylococcus aureus* to create the first high-level vancomycin-resistant *Staphylococcus aureus* (VRSA) infections (CDC 2002).

The incidence of infections with vancomycin-resistant enterococci has also increased. These strains are now very common in intensive care units, and amongst dialysis units the proportion reporting a patient with vancomycin resistant enterococci (VRE) increased from 11.5 percent in 1995 to 32.7 percent in 2000 (Tokars et al. 2002). Spread of resistant strains within hospitals may be limited by aggressive infection control measures and prudent vancomycin use.

Drug-resistant *S. pneumoniae* was first documented in Australia in the 1960s and multiply resistant strains were reported in South Africa in the 1970s; reports of similar isolation were made with increasing frequency from other parts of the world in the 1980s. By 1998, 24 percent of pneumococcal isolates submitted to CDC were resistant to penicillin and 14 percent were resistant to at least three drug classes. The emergence of penicillin resistance in Europe has been correlated closely with antimicrobial use in those countries (Bronzwaer et al. 2002) (Figure 2). The rapid emergence of drug-resistant strains is complicating the management of pneumococcal infections and demonstrates the need for more judicious use of antimicrobial agents (Heffelfinger et al. 2000).

Penicillinase-producing *Neisseria gonorrhoeae* was first recognized in 1976. By the 1990s, 32 percent of *N. gonorrhoeae* isolates were resistant to penicillin or tetracycline, and resistance to fluoroquinolones was recognized (CDC 1993). Fluoroquinolone-resistant strains now have a global distribution. *N. gonorrhoeae* also demonstrates the increasingly diverse array of mechanisms of resistance, including both plasmid- and chromosomally mediated resistance to the penicillins and tetracyclines.

Antibacterial resistance is also a problem for the developing world. In central and southern Africa, isolates of *S. dysenteriae* 1 have been demonstrated to be resistant to all readily accessible oral antibiotics,

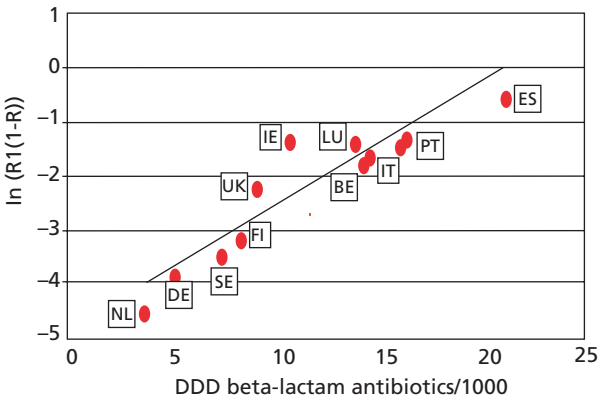

Figure 2 *Association of antibiotic use with resistance in* pneumococci*: The log odds of resistance to penicillin among invasive isolates of* Streptococcus pneumoniae *(ln R/(1-R)) is regressed against outpatient sales of beta-lactam antibiotics in 11 European countries. DDD, defined daily dose; BE, Belgium, DE, Germany; FI, Finland; IE, Ireland; IT, Italy; LU, Luxembourg; NL, the Netherlands; PT, Portugal; ES, Spain; SE, Sweden; UK, United Kingdom. (Redrawn from Bronzwaer et al. 2002, www.cdc.gov/ncidod/EID/vol8no3/01-0192.htm, with permission.)*

including ampicillin, chloramphenicol, nalidixic acid, tetracycline, and trimethoprim-sulphamethoxazole (Ries et al. 1994; Tuttle et al. 1995). The need to monitor, regularly and frequently, the prevailing antimicrobial resistance patterns in different geographical areas has been repeatedly demonstrated. Preventive measures may be more successful than therapy for control of many epidemics.

BREAKDOWN OF PUBLIC-HEALTH INFRASTRUCTURE

Complacency has also resulted in the re-emergence of pathogens previously controlled. Basic tenets of public health, such as close monitoring of disease in a population accompanied by a rapid response for diseases such as shigellosis and salmonellosis, and control programs for diseases such as tuberculosis and diphtheria, have been neglected in many areas of the world as public-health attention towards the control of all but a few selected conditions declined in the 1970s and 1980s (Berkelman et al. 1994). In addition, the emergence of new pathogens such as *E. coli* O157:H7 has been addressed inadequately.

Cases of tuberculosis in the USA increased 18 percent from 1985 through 1991; yet, by 1992, most public-health laboratories had not incorporated the more rapid radiometric methods for routine culture or drug-susceptibility testing of mycobacteria. The erosion of public-health services together with the mistaken perception of tuberculosis as a disease of declining public-health significance resulted in decreased funding for many laboratories in the USA and resulted in the delay in implementation of newer, more rapid diagnostic technologies for tuberculosis (Bloom and Murray 1992; Dowdle 1993; Huebner et al. 1993) as well as a decline in resources for control programs.

In 1994, reports of widespread transmission of pneumonic plague within India resulted in estimated economic losses to India of over $1 billion dollars (Campbell and Hughes 1995). Intense investigation revealed that most reported cases of plague were incorrectly diagnosed and that no transmission of pneumonic plague was identified in any major city except Surat. Effective surveillance coupled with laboratory expertise in plague might have averted some of the economic loss.

The epidemic levels of cholera reached in South America in the early 1990s, after almost a century of absence of the disease from the continent, were attributed, in part, to breakdowns in public-health measures (IOM 1992). Contaminated municipal water supplies contributed to the spread of the epidemic after the pathogen was introduced along the coastal waters of Peru (Blake 1993). As both the population and the percentage of the population in poverty grow in South America, the potential for spread of diarrheal illnesses increases.

In the early 1990s, Russia and many of the newly independent states experienced a dramatic resurgence of diphtheria (CDC 1995a) (Figure 3). Inadequate immunization of the population, crowding and low socioeconomic conditions, and high mobility of the infected individuals may have contributed to this outbreak, which has resulted in thousands of deaths. Also, from 1990 to 1995, tuberculosis rates in Russia increased by 70 percent, with more than 25 000 people dying from the disease each year (Netesov and Conrad 2001). The increased incidence has been compounded by the spread of multidrug resistant tuberculosis.

INTENT TO DO HARM: BIOTERRORISM

The growing threat of intentional attacks using biological agents is a serious one and must be addressed (IOM 2003). CDC has developed three categories of biological agents, prioritized according to their likelihood of bioterrorist use and the severity of the diseases they produce. Three of the six CDC category A (high priority) agents are bacteria or their toxins: *Yersinia pestis*, *Bacillus anthracis*, and *Clostridium botulinum* toxin. Outbreaks may occur as a result of natural occurrence, unintentional release by those working on these agents in laboratories, or intentional use. All of these agents have been weaponized to cause disease when released in aerosol form. Pneumonic plague, inhalation anthrax, and botulism may all occur naturally but are quite rare in developed countries today.

In 1979, an unintentional release of anthrax spores from a Soviet bioweapons facility caused an outbreak of inhalational anthrax, the largest recorded outbreak of inhalation anthrax, with at least 77 cases diagnosed (Meselson et al. 1994). In 2002, 22 cases of anthrax (11 inhalational, 11 cutaneous) were identified as a result of bioterrorism; envelopes containing *Bacillus anthracis* spores were mailed to news media companies and government officials in the USA (Jernigan et al. 2002). No naturally occurring cases of inhalation anthrax had

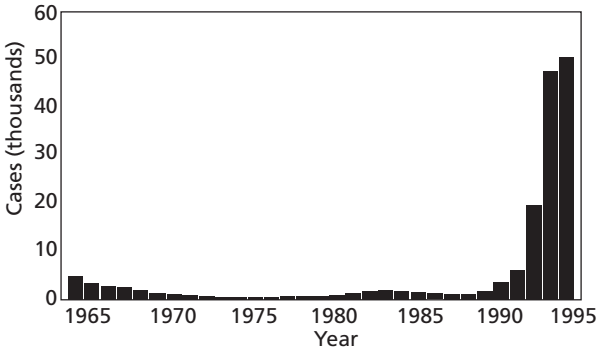

Figure 3 *Reported cases of diphtheria in New Independent States of the Former Soviet Union, 1965–1995. Redrawn from www.cdc.gov/mmwr/preview/mmwrhtml/00043378.htm, with permission.*

been diagnosed in the previous 25 years in the USA. In addition, Salmonella and Shigella have also been used to deliberately contaminate food and cause illness in the USA (Carus 2001; Torok et al. 1997).

INFECTIOUS ORIGINS OF CHRONIC DISEASES

A number of diseases or conditions, previously considered to be noninfectious, have recently been recognized to have an infectious etiology. *Helicobacter pylori* was first isolated from humans in 1982 and is now known to play a central role in the development of diffuse gastritis and duodenal ulceration (Marshall 1989). More than 90 percent of patients with duodenal ulceration are infected with *H. pylori*, and treatment with antimicrobial therapy heals duodenal ulcers; additionally, lower recurrence rates are associated with eradication of *H. pylori*. Presence of *H. pylori*, as determined by serological studies, is also associated with a three- to six-fold higher risk of gastric cancer (Fuchs and Mayer 1995); gastric cancer is the second most common cause of cancer-related deaths in the world. Evidence is still accumulating that *Chlamydia pneumoniae* may have an etiological role in the development of atherosclerosis (Campbell and Kuo 2003).

Guillain–Barré syndrome, a leading cause of acute paralysis, has been associated with infection with *Campylobacter jejuni*; in a study conducted in England and Wales, 26 percent of 96 patients with Guillain–Barré syndrome had evidence of infection with *C. jejuni* (Rees et al. 1995). Peripheral nerves may share epitopes with *C. jejuni*. Other syndromes that are ill-defined and often considered to be immune-mediated may soon also be discovered to have infectious microorganisms playing a central role in etiology, possibly through molecular mimicry (Bolton 1995).

Techniques to identify pathogens that rely on molecular techniques rather than on cultivation have been developed. To identify the bacillus causing Whipple's disease, the bacterial 16S ribosomal RNA sequence from infected tissue was amplified and its phylogenetic relations have been described (Relman et al. 1992; Relman 2002).

SUMMARY

Following the 1992 publication of the US Institute of Medicine report on emerging infections, CDC released a prevention strategy to address emerging infectious disease threats for the USA (CDC 1994a). In 1994, the World Health Organization defined goals to guide implementation of a global effort on emerging infectious diseases (World Health Organization 1994); in 1995, the World Health Assembly passed a resolution calling for attention to the problem of new and re-emerging infections. The original 1992 IOM report was revisited by a new committee in 2003 and its report (IOM 2003) finds that the threat of emerging infections had increased over the past decade. The need to increase global capacity for response, to develop inexpensive and easy diagnostic tests for field use, and to have available the diagnostic reagents, drugs, and vaccines needed has been emphasized, both for naturally occurring diseases and for those diseases likely to result from bioterrorism. As the speed of change in the world continues to accelerate, the challenges confronting us by the re-emergence of old pathogens or the recognition of new ones will likewise be heightened.

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Healthcare-associated infections

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INTRODUCTION

Of the infections dealt with in this volume, very many may be acquired in hospital, but these hospital infections have special features. Hospitals bring together uniquely vulnerable hosts and subject them to particular risks of infection from animate and inanimate sources. Apart from being the cause of morbidity and mortality, infection limits the effectiveness and adds greatly to the cost of medical treatment.

The term 'hospital-acquired infection' (syn. nosocomial infection) is applied to any infection causing illness that was not present or in its incubation period when the subject entered hospital or received treatment in an outpatient or accident and emergency department. The increasing blurring between primary and secondary health care systems, and thus often the lack of clarity about the origin of infection, means that the term 'healthcare-associated infection' is preferably used. It includes not only incidents in which a single microorganism spreads from person to person ('cross-infection') or from a common source in the hospital, but also single and apparently unconnected infections. Sporadic and endemic hospital-acquired infections may be less striking than epidemic ('outbreaks'), but they predominate numerically and their control or reduction present the greater challenge.

Some healthcare-associated infections do not differ from infections with the same microorganism in the general population, but many are profoundly affected by the patient's underlying illnesses or by medical or surgical treatments to which they are subjected while in hospital. The source of the infecting organism may be exogenous, from another patient or a member of the hospital staff, or from the inanimate environment in the hospital; or it may be endogenous, from the patient's own flora, which at the time of infection may include organisms brought into hospital at admission and others acquired subsequently. In either case, the infecting organisms may spontaneously invade the tissues of the patient or be introduced into them by surgical operation, instrumental manipulation, or nursing procedure. The concentration of patients with particular illnesses and undergoing similar treatments in specialist hospital units often creates unique niches for hospital pathogens. Healthcare-associated infection may also affect discharged inpatients, outpatients, and staff, and an episode of hospital infection may be initiated by the admission of an infected patient from the general population. Hospital infection may spill over into the community, necessitating investigation and control in both populations. In all considerations of healthcare-associated infection, the source, the mode of infection, and host susceptibility must all be kept in mind. It is clear from a review of the current literature base that

much modern day infection control practice is unfortunately not evidence based. A step forward in this respect is the production of true evidence-based infection control guidelines, such as The epic project (developing national evidence-based guidelines for preventing healthcare-associated infections) (Pratt et al. 2001).

OCCURRENCE, CONSEQUENCES, AND COST OF HEALTHCARE-ASSOCIATED INFECTION

Information about the extent of hospital infection in general may be obtained from incidence or prevalence studies (see Epidemiology of infectious diseases). The relationship between the results of these two types of survey is complex (Freeman and Hutchison 1980). Most investigations are limited to particular sites of infection, such as surgical wounds, or to individual hospital services, such as intensive care. However, in the USA it has been estimated that more than 2 million, and perhaps as many as 4 million, patients are infected in hospital each year. Study of a random sample of patients from 6 449 acute care hospitals gave an incidence of 5.7 infections per 100 admissions (Haley et al. 1985a, b). Ongoing nationwide surveillance of nosocomial infection in the USA is reported in terms of specific infection sites (National Nosocomial Infections Surveillance (NNIS) System 1995). Recent trends have been an increase in bloodstream infections and pneumonias, and an increase in the rate of infections per 1 000 patient-days because of shorter periods of hospitalization (Weinstein 1998). A large proportion of surgical site infections in particular, present in the community even though they were acquired in hospital. This percentage is likely to increase as hospital stays decrease, and therefore, unless an effective form of post-discharge surveillance is carried out then there will be marked under-ascertainment of some hospital-acquired infections (Weigelt et al. 1992; Mangram et al. 1999).

In Great Britain and Ireland, a cross-sectional prevalence survey (Emmerson et al. 1996) suggested an overall nosocomial infection prevalence rate of 9.0 percent, with significantly higher prevalence in teaching (11.2 percent) than in nonteaching (8.4 percent) hospitals. A previous survey (Meers et al. 1981) found a similar overall prevalence (9.2 percent), but Emmerson et al. (1996) point out differences in the study methods and medical practice in the two periods. For example, earlier patient discharge in the more recent survey implied that many surgical wound infections would be detected after discharge from hospital and were therefore not included (Bailey et al. 1992; Weigelt et al. 1992; Byrne et al. 1994; Emmerson 1995). Nationwide prevalence surveys in Spain (EPINE 1992, 1995) have shown an overall nosocomial infection rate of 8.5 percent in 1990, falling to 7.2 percent in 1994. A similar investigation in Norway found a prevalence of 6.3 percent, which was a fall in comparison with previous years (Aavitsland et al. 1992). A prevalence survey in Hong Kong (Kam and Mak 1993) found 8.6 percent of patients to have a hospital-acquired infection. Caution is needed when comparing these and similar figures, as there may be differences in the spectrum of patients, in medical practice, and in definitions of infection. Indeed, there were important differences between the individual hospitals in all these investigations. A similar body site distribution of infections was seen in the studies, with four sites predominating: urinary tract, respiratory tract, surgical wound, and skin (Figure 1). Improvements in prevalence figures are often due mainly to control of urinary tract infection.

A prevalence of hospital infection between 6 and 9 percent has frequently been observed in recent years and it has been questioned whether the 'irreducible minimum' of hospital-acquired infections has been reached (Ayliffe 1986). This stability may indicate some success, in that an increase in the infection rate might have been expected because modern methods of treatment create more opportunities for infection and highly susceptible patients are surviving in greater numbers. Nevertheless, an examination of many aspects of current hospital practice suggests that the incidence of infection could be reduced.

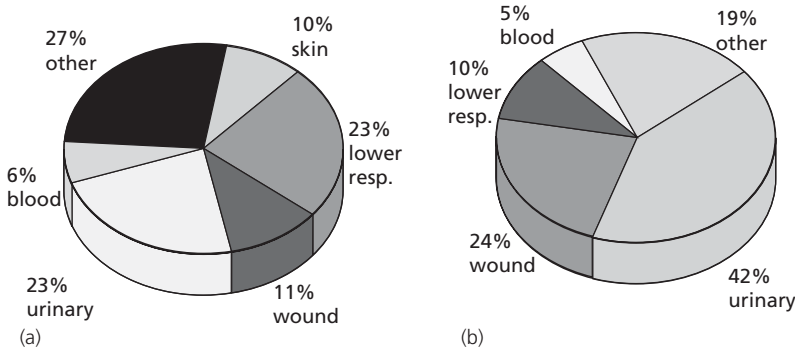

Figure 1 *Occurrence of hospital-acquired infection by site:* **(a)** *UK, prevalence survey (Emmerson et al. 1996);* **(b)** *USA, incidence survey (Haley et al. 1985b)*

Most types of hospital infection cause appreciable morbidity and may lead to residual disability and even death, although a significant worsening of outcome may be difficult to demonstrate statistically except in very large samples of patients (Freeman et al. 1979). Some impression of the impact of hospital infection can be gained from the large prevalence and incidence studies, as can an estimate of the increased cost of care. Martone et al. (1992) suggested that, in the USA, a hospital-acquired infection results in an average of 4.0 extra days of stay. The average extra charges (1992) were $2 100, giving an estimated annual national cost of more than $4.5 billion. It was estimated that more than 19 000 deaths were due directly to hospital infection and that this was contributory to a further 58 000 deaths. Similar figures are not available in Europe. In a 1988 case–control study in England it was established that hospital infection in surgical patients added an average of 8.2 days to hospital stay with added costs of £1 041 (Coello et al. 1993). Antibiotic resistance in the infecting organism adds to costs and reduces the effectiveness of treatment (Holmberg et al. 1987). A socio-economic study of hospital-acquired infection in the National Health Service (NHS) in England in 1994/1995 used modeling based on logistic regression to calculate confidence limits for the estimated increased lengths of stay (Plowman et al. 2001). Extrapolating from measurements in one district general hospital, the calculated annual cost to the NHS was £1 000 million (Plowman et al. 2001). The study examined approximately 4 000 inpatients and identified 7.8 percent as having acquired at least one hospital-acquired infection. Additionally, 30 percent of this hospital-acquired infection cohort, and 19 percent of the remainder reported post-discharge symptoms consistent with the diagnosis of urinary tract, chest, or surgical infections. On average, a patient with hospital-acquired infection spent 2.5 times longer in hospital, and cost £3 000 more to treat than a similar nonhospital-acquired infection patient. This sum is of a similar order of magnitude to other studies, and highlights both the socioeconomic burden of hospital-acquired infections and the potential for savings and/or health gains if even only a small proportion of these can be prevented. Indeed, the control of hospital-acquired infection has been characterized as one of the most cost-effective of health care interventions (Haley et al. 1985b; Chaudhuri 1993; Wenzel 1995).

HISTORY OF KNOWLEDGE OF HEALTHCARE-ASSOCIATED INFECTION

A chapter devoted to hospital-acquired infection appeared for the first time in the seventh edition of this book, reflecting the growth of interest in hospital epidemiology and infection control, but the subject is not new. The need to isolate patients with obviously infectious diseases has been recognized since ancient times and the spread of infection that might ensue from the introduction of such patients into hospitals has been known for centuries. However, segregation of fever hospitals from general hospitals dates only from the early nineteenth century and the usefulness of this measure was not demonstrated statistically until much later. Hospital-acquired infection and the consequent mortality probably reached their peak in the nineteenth century. The following brief account includes only the salient references, but the more recent papers referred to (e.g. Cruickshank 1944; Williams 1956; Ayliffe and English 2003) supply more detail.

The nineteenth century

Urban overpopulation and hospital overcrowding made hospitals places of dread for the poor. There was uncontrolled puerperal sepsis, and surgical sepsis caused death in most cases of compound fracture admitted to hospital. Crossinfection in children's hospitals was associated with mortality rates of 25–40 percent. The now well known work of Semmelweiss (1861) on puerperal sepsis was largely disregarded at the time. He observed its association with medical staff and students who attended patients and also performed autopsies. Semmelweiss deduced that the disease was spread by 'morbid matter' on their hands derived from cadavers or other affected patients. A dramatic reduction in infection rates was achieved by the introduction of hand-washing with chlorinated lime.

At the same time, and to greater immediate effect, Florence Nightingale, after her experience of hospital sepsis at Scutari and her reform of the army medical services, turned her attention to British hospitals. In a much quoted remark in her book *Notes on hospitals* (Nightingale 1863) she states:

> It may seem a strange principle to enunciate as the very first requirement in a Hospital that it should do the sick no harm . . . the actual mortality in hospitals, especially in those of large crowded cities, is very much higher than any calculation founded on the mortality of the same class of diseases among patients treated *out of* hospital . . .

Florence Nightingale established important principles of nursing, hospital design, and hygiene, while remaining sceptical of the germ theory of disease. Further evidence was provided by the survey of the sequelae of amputation by Simpson (1869) which established that sepsis, gangrene, and pyemia were very much more common in large urban hospitals than in rural practice.

At about this time Lister (1867a, b) introduced his 'antiseptic surgery', with extensive use of carbolic acid to pack wounds, especially of compound fractures, to sterilize instruments and sutures, to decontaminate his

hands, as well as use as an air spray. He observed a considerable improvement in the results of treatment of compound fractures and of surgical operations. Lister came to appreciate that the air spray did not add greatly to the effectiveness of the other measures, and that what he was introducing was a barrier between the patients' tissues and infection. 'Antiseptic surgery' was later replaced by von Bergman's 'asepsis' (Schimmelbusch 1894) and by the end of the century, with the introduction of surgical gloves in the USA (see Halsted 1913), the measures in use in a modern operating theater had to a large extent been introduced.

During these years, when many fundamental discoveries in bacteriology were being made, other principles of hospital infection control were established. Flügge (1897, 1899) showed the importance of droplet and aerial spread of tuberculosis and, with Hutinel (1894) and others, established basic isolation systems for diphtheria and other infectious diseases in children's and fever hospitals.

The early twentieth century

The discovery of pathogenic bacteria provided a new basis for the study of hospital infection, and the importance of *Streptococcus pyogenes* was demonstrated during this period, in burn (Cruickshank 1935) and postoperative (Okell and Elliott 1936) infections. Cubicle and barrier nursing was introduced widely (Crookshank 1910) and was shown to be effective in preventing the spread of childhood fevers, except chicken pox and measles.

Aseptic surgery was for a time deemed adequate to keep wound infection to a low level, but this complacency was shattered by the experience of two world wars, when large open wounds readily became infected in the base hospitals (Cruickshank 1944). The local application of mild antiseptics to the wounds proved ineffective (Cruickshank 1944; Williams 1956). The arrival of penicillin in the later years of the Second World War gave considerable benefit (Fraser 1984).

Though Dukes (1929) recognized the importance of the indwelling catheter as a means of introducing infection into the bladder, this was forgotten. The idea was revived much later with the introduction of closed drainage systems (Gillespie 1956; Kunin and McCormack 1966).

The antibiotic era

The introduction of penicillin banished from hospitals the terrible cases of chronic sepsis, mainly caused by *Staphylococcus aureus* (Fletcher 1984). Nevertheless, the era of antibiotics ushered in for the first time a period in which staphylococcal, rather than streptococcal, infection dominated the scene (Williams 1956). Penicillin-resistant, and later multiply-resistant, *S. aureus* strains (Clarke et al. 1952) which had additional properties of transmissibility and virulence, caused serious wound, burn, and other sepsis. Interest in air-borne and dust-borne spread, as well as transmission on the hands of attendants, revived. From this period date many of the established methods of infection control: the supply of clean air for operating theaters, procedures for wound dressings, and the provision of isolation units, for example (Williams et al. 1960). So does the present general system for hospital-infection control: the appointment in the UK of medical control of infection officers (hospital epidemiologists), infection control nurses (nurse practitioners), and control of infection committees (Colebrook 1955, Subcommittee of the Central Health Services Council 1959; Gardner et al. 1962). Similar arrangements were established in the USA, where the first nurse practitioner in the discipline was appointed in 1963 (Osterman 1981).

Antibiotic treatment and hospital infection control are intimately entwined. The use of antibiotics, although not the only influence, has altered the prevailing pathogens in hospital infection. *S. pyogenes* was almost banished and, later, resistant gram-negative bacilli replaced *S. aureus*. Then gram-positive bacteria, *S. aureus*, coagulase-negative staphylococci, enterococci, certain corynebacteria, and *Clostridium difficile*, re-emerged. The emergence of resistant gram-negative bacteria, such as *Klebsiella* spp. with extended spectrum β-lactamases, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*, has occupied much attention recently. Changes in hospital practice may favor particular pathogens irrespective of their susceptibility to antibiotics. An example is the association of coagulase-negative staphylococci of varied antibiotic sensitivity with prostheses and cannulae. The availability of antibiotics has allowed the development of modern medical treatment, for example major surgery and the use of immunosuppression, associated with new infection risks and a new range of pathogens.

Antibiotic resistance may increase the impact of hospital infection that would otherwise be of small importance and may necessitate a change to less satisfactory and more expensive antibiotics. It also appears to worsen the prognosis of infection (Hart 1982; Holmberg et al. 1987; French et al. 1990). On occasion it may exclude the possibility of using antibiotics to remove the source of infection in an outbreak. Hospital infection control measures, such as isolation, are extremely important in preventing the spread of resistant bacteria.

The development of multiple resistance in hospital pathogens, without the prospect of new antibiotics to deal with them, is alarming. There have been reports of infection by strains resistant to all established antibiotics (Murray 1991; Tomasz 1994), accounts of untoward failures of treatment (Armstrong et al. 1995), and trends towards resistance to agents favored for treatment and

prophylaxis, such as the late generation cephalosporins and the fluoroquinolones (Burwen et al. 1994; Jones et al. 1994; Goldstein and Acar 1995). It has been suggested that the end of the antibiotic era may be near, or that the safety of some forms of hospital treatment may be severely compromised (Kunin 1993; Tomasz 1994). Much evidence suggests that particular resistance problems in hospital-acquired infection have resulted from the routine, unthoughtful, or 'blanket' use of antibiotics in courses that are too long. Restraint, enlightened prescribing, and control of antibiotic use in hospitals may prevent a worsening of the problem (Gould 1988; Report 1995; Editorial 1996; Tenover and McGowan 1996). This has been recognized by the medical and scientific community for some years and has also been acknowledged by public bodies. An important landmark was the inquiry into antimicrobial resistance by the House of Lords in the UK. This report called on governments to recognize the threat, educational authorities to move antibiotic resistance up the agenda, and food producers to curb use in animals (Select Committee on Science and Technology 1998).

More recent developments

For a period, the importance of multiply-resistant *S. aureus* appeared to fade (Ayliffe et al. 1979) and interest shifted to gram-negative bacilli: antibiotic-resistant enterobacteria, such as *Klebsiella* and later *Serratia* spp., which caused large outbreaks of colonization, with some clinical infections. Infection by *Pseudomonas aeruginosa* came to prominence with the increasing number of patients rendered susceptible by illness or treatment. The infecting bacteria appeared to be favored by the antibiotics in current use in the hospitals. More recently, possibly as a result of the introduction of new antibiotics, and the extensive use of indwelling medical devices, gram-positive cocci have again become the predominant causes of infection. *S. aureus* strains resistant to even more antibiotics ('methicillin-resistant *S. aureus*', see later under Staphylococcus aureus) have been as difficult to control as those encountered in the 1950s and multiply-resistant strains of *Staphylococcus epidermidis* have been the most common pathogens in some units. *Enterococcus* spp. resistant to all current antibiotics including vancomycin (see later under Enterococcus spp.) have presented problems in specialized units. Outbreaks of diarrhea and colitis caused by *C. difficile* (see section on Clostridium difficile below) have followed the use of broad spectrum antibiotics, such as cephalosporins. The increase in tuberculosis in immunosuppressed patients (see later under Enterobacteriaceae) and the occurrence of multiply-resistant strains have revived the need for isolation facilities and procedures to prevent air-borne spread of infection to patients and staff. On the other hand, the rediscovery of the principles of effective antibiotic prophylaxis in surgery has done much to prevent endogenous wound infection, for example, in colonic surgery.

The spread of viruses from patients admitted to hospital to other already sick patients remains an uncontrolled problem, particularly with respiratory syncytial virus (RSV) and other respiratory viruses in children's hospitals. Spread of viral gastrointestinal infection has been a major problem (see later under Viruses). The larger numbers of immunosuppressed patients has been associated with an increasing incidence and variety of fungal infection (see later under Fungi and protozoa).

Methods of controlling hospital infection established earlier have been questioned. The UK Medical Research Council (Medical Research Council, Subcommittee of the Committee on Hospital Infection 1968) conducted an inquiry into the measures used in operating theaters. In the USA, efforts to establish the cost-effectiveness of infection control measures culminated in the SENIC study (see later under Surveillance) which demonstrated cost savings by the employment of effective numbers of infection control staff and the institution of preventive measures. Methods of surveillance have been further refined and computerization of records has assisted in this process. It has been important to focus the system of surveillance and control of hospital infection on areas of most effective cost benefit. The need to integrate infection control in the hospital with the wider community has been re-emphasized.

Severe acute respiratory syndrome

Following reports in February 2003 of a mystery respiratory illness affecting some 300 people in Guangdong province, mainland China, a new disease entity was described – severe acute respiratory syndrome (SARS). This disease has subsequently been shown to be caused by a novel coronavirus (Ksiazek et al. 2003; Drosten et al. 2003; Peiris et al. 2003). As of 7 August 2003, some 8 422 cases had been notified to the World Health Organization (WHO) of whom 916 had died. The majority of cases occurred in mainland China (5 327 cases, 349 deaths), Hong Kong (1 755 cases, 300 deaths), Taiwan (665 cases, 180 deaths), Singapore (238 cases, 33 deaths) and Canada (251 cases 41 deaths) (Lee et al. 2003; Tsang et al. 2003; Poutanen et al., 2003; Booth et al. 2003; Ho et al. 2003). The deaths were age-stratified: age <24, <1 percent mortality; 25–44, 6 percent mortality, 45–64, 15 percent mortality; >65, 50 percent mortality. Underlying diseases, such as diabetes, were important cofactors in morbidity and mortality. Spread is thought to be predominantly by large droplets and therefore close contacts are most at risk. The possibility of fecal–oral transmission is still debated and for how long patients are infectious remains unknown. It is possible that some patients become 'super spreaders' and are

more infectious than others. There is not much evidence concerning the role of fomites, though studies have shown that the virus can remain viable for over 24 h on inanimate surfaces. Survival in feces may be as long as 96 h, but the relevance of this fact is unknown.

Meticulous attention to infection control procedures were shown to be most effective in preventing the spread of SARS within a healthcare setting (Ho et al. 2003; Seto et al. 2003; Wenzel and Edmond 2003). Ideally, SARS patients should be nursed in an isolation room with negative pressure relative to the surrounding area or a single room with own bathroom and toilet facilities. The personal protective equipment for all healthcare workers includes for routine use, an N95 EN 149:2001 approved respirator/mask or equivalent (95 percent filter efficiency against particles 1 micron in size or smaller, a tight facial seal – less than 1 percent leak); an FFP2 mask with minimum filter efficiency of 92 percent is deemed equivalent. Masks should be fit tested according to the manufacturer's recommendations and fit checked each time the mask is put on. (To check test the mask, the wearer takes a quick, forceful inspiration to determine if the mask seals tightly to the face.) For high risk procedures which generate aerosols and require prolonged very close contact with affected patients (e.g. intubation prior to ventilation, taking respiratory samples, suctioning whilst the patient is being ventilated), a P-100 half face respirator mask (FFP3 is deemed equivalent, minimum filter efficiency, 98 percent). Eye protection (goggles or face shield) when providing direct patient care during cough producing and aerosol generating procedures, and where there is potential for spattering or spraying of body substances. Droplet and contact precautions (including use of gowns and gloves). Instructions are given for removing and discarding masks to avoid transmission of virus during this process. Hand hygiene is also emphasized. More stringent infection control recommendations may be required in specific situations, e.g. outbreak management in a healthcare facility.

MODE OF SPREAD OF INFECTION IN HOSPITALS

Air-borne spread

With the tradition of the spread of disease by effluvium or miasma, it is not surprising that air-borne spread of infection, for example of tuberculosis, was demonstrated early in the scientific era of bacteriology (Flügge 1897, 1899). Interest in this route went into eclipse in the early years of this century (see Williams 1956) to be revived in the later 1930s and the 1940s. The contribution of the air-borne route to common hospital infection remains the subject of controversy. Clearly, the effectiveness of this route depends on the source, on the number of microorganisms present, and the degree of dispersal, whether in droplets, in droplet nuclei or on skin scales; on survival and retention of pathogenicity by the microorganisms in the air or environment (or their death, impairment, or dilution there); on the size of the infecting dose; and on the local or general susceptibility of the persons exposed to infection. Air-borne spread of infection has been reviewed in detail by Ayliffe and Lowbury (1982) (Airborne bacteria).

Bacteria can be counted in air by the slit sampler, which draws in air and impacts the particles on to a moving culture plate. Settle plates, which are agar plates exposed on horizontal surfaces for measured periods of time, capture a limited range of the particles from the air and are not quantitative. Small hand-held samplers (e.g. centrifugal samplers) are now available. They are easier and less disturbing to use in a busy operating theater than the slit sampler, and are adapted to making counts during operations and in different areas near the operation itself. Some, however, extract only some of the particles and give results that are not as accurate as, or comparable with, those obtained with the slit sampler (Whyte 1981). Outside the operating theater, the provision of good quality air for severely neutropenic patients in the absence of high efficiency particulate air (HEPA) filters, especially in the context of preventing aspergillus infection, is the other major indication for occasional air sampling. The absence of agreed standards, however, makes interpretation of air sampling outside the operating theater difficult (Humphreys 1993).

TUBERCULOSIS

Clinical experience and experiments with guinea pigs indicate that air-borne spread of tuberculosis can occur by the transfer of very few microorganisms. Patients differ greatly in their ability to transmit tuberculosis (Riley et al. 1962) and the susceptibility of the recipient is also very significant. Only patients with smear-positive pulmonary tuberculosis are regarded as constituting an infection risk and requiring single room isolation. Infectivity declines rapidly after effective treatment is initiated so that only 2 weeks of isolation during treatment is recommended (but see later under *Mycobacterium* spp.). Multidrug-resistant (MDR) tuberculosis in HIV patients during recent years has been associated with nosocomial spread to patients and healthcare workers and emphasizes the need for early identification of positive patients and patient isolation (Beck-Sagué et al. 1992).

PNEUMOCOCCAL INFECTION

Much infection by *Streptococcus pneumoniae* is endogenous and it has not been customary to isolate patients with pneumococcal pneumonia. The appearance of penicillin-resistant and multi-resistant strains has been associated with significant mortality (Pallares et al. 1995). The ready transmission of these emerging organisms has

demonstrated the need to contain such infections (Gould et al. 1987), but clusters of cases also occur with some more sensitive strains, which appear to have the ability to cause lobar pneumonia in the relatively healthy (Davies et al. 1984). When the disease has been convincingly diagnosed by a Gram stain of sputum, it may therefore be advisable to isolate patients with pneumococcal pneumonia for the first 24 h of treatment. Protection of vulnerable patients with the currently available polyvalent vaccine becomes more important as resistance increases (Austrian 1994).

MENINGOCOCCAL INFECTION

Meningococcal infection acquired in hospital is uncommon, but isolation for the first 48 h of treatment is advised. Among staff, only those who have had particularly close contact with the patient, as in mouth-to-mouth resuscitation, need be offered prophylactic antibiotics.

OTHER BACTERIAL INFECTIONS

The evidence for spread by air of *S. aureus* and *S. pyogenes* is contradictory. These bacteria are carried by many normal people (Williams et al. 1960); streptococci are readily shed from the upper respiratory tract, by coughing, sneezing, and singing (Lidwell 1974), as are staphylococci on skin squames during physical activity. There is considerable variation in the degree of shedding. *S. aureus* is carried particularly in the perineum and nares and males tend to shed more staphylococci than females. Infected lesions on patients provide rich sources of these bacteria and release into the air is brought about by movement of secondarily contaminated articles, such as bedclothes, protective clothing, bed curtains, dressings, and dust. The numbers in the air can be reduced by disinfection with ultraviolet light or chemicals, by such simple measures as damp, rather than dry, dusting or cleaning, and by control of air circulating between infected and susceptible persons. These measures are not, however, always effective in reducing infection rates. Striking results are most common when the prevailing infection rate is very high, as where dressings are handled, or where there is a large exposure of undefended tissue, as in burns. The important experiments in burned patients by Lowbury et al. (1970) suggested that infection by gram-negative bacilli, e.g. *P. aeruginosa*, was almost entirely by contact, infection by *S. pyogenes* by air and staphylococcal infection by both routes. Measures to control ventilation have on occasion resulted in a decline in the rate of nasal acquisition of organisms by patients. Several workers have found it impossible to control the spread of resistant strains of staphylococci by contact barriers alone, but have achieved success with the use of a separate isolation unit or decontamination of the environment (Pearman et al. 1985). It may be that for these strains the selection pressure of antibiotics administered to the recipient is of importance in allowing colonization from very small inocula. These are situations in which air control is likely to yield good results.

Gram-negative bacilli tend to die when desiccated and infection by the aerial route is confined mainly to spread by nebulized spray.

VIRAL INFECTION

Smallpox was notorious as being readily transmitted by very few infective particles by the air-borne route. The most common viral infections transmitted by air in hospital are chicken pox, measles, influenza, and respiratory syncitial virus (RSV). Availability of immunofluorescence techniques for the rapid diagnosis of RSV renders isolation or 'cohort nursing' (placing patients with similar infections in the same ward or area) shortly after admission to hospital is an increasing possibility and it is recommended (Editorial 1992). It seems that transmission of other childhood exanthems requires closer contact. Small round structured viruses (SRSV) (now referred to as noroviruses) are an increasing cause of nosocomial diarrhea and vomiting. Large inocula are present in vomitus and the air-borne inhalation route as well as the fecal route is now recognized as an important means of transmission (Caul 1994).

FUNGAL INFECTION

Dispersal by spores is a feature of most filamentous fungi, but only *Aspergillus* spp. have been shown to be a significant cause of air-borne infection. This may occur after cardiac surgery or in immunosuppressed patients. Outbreaks have been associated with building work in hospitals (Opal et al. 1986) or inexpert filter changing in operating theater ventilation systems. Other fungi occasionally cause clusters of infections by aerial spread, e.g. phycomycetes (del Palacio Hernanz et al. 1983). Exceptionally, other fungi that cause cardiac valve infection are thought to have arrived via air. Effectively filtered air should be supplied to certain classes of susceptible patients (Rhame et al. 1984). *Cryptococcus neoformans* is frequently found in pigeon droppings around hospitals, but there is no convincing evidence of air-borne spread from this source to susceptible patients.

INFECTION IN THE OPERATING THEATER

The pathogenesis of postoperative infection is complex, but most infections arise from the patient's own flora and the remainder are acquired mainly from staff in the operating theater (Ayliffe 1991). At one time 'aseptic surgery' appeared to offer all that was needed to minimize postoperative infection. In the 1940s, partly stimulated by cases of clostridial infection thought to be due to dust, interest was revived in the possibility that improved ventilation of operating theaters and other measures designed to limit air-borne infection would

reduce it further. This was reflected in the principles of modern systems of operating theater ventilation and the design of modern operating areas, including plenum ventilation to ensure flow of air from the theater to the outside rather than the reverse (Humphreys 1993). Satisfactory operating theater clothing, small operating teams with the minimum of movement and restriction of the patient's blankets and dressings to anterooms are other important factors in minimizing postoperative infection, because all these measures reduce the air levels of bacteria in the theater (Dharan and Pittet 2002). Even so, any human activity raises the levels in a conventionally ventilated theater, but these bursts should be quickly suppressed by effective ventilation. Recent publications have addressed the problems of behavior and rituals in operating theaters (Woodhead et al. 2002) and the microbiological commissioning and monitoring of operating theater suites (Hoffman et al. 2002).

The bacteria found in the air of a properly ventilated operating theater are rarely pathogens in the usual sense. For example, they rarely include *S. aureus* or *S. pyogenes*. Indeed, it is difficult to find proof that reducing microorganisms in the air by conventional ventilation systems is beneficial in preventing surgical wound infection, although there have been instances in which the introduction of the systems described above have apparently resulted in a fall from an undesirably high incidence of wound infection (Blowers et al. 1955; Shooter et al. 1956).

It was an early suggestion that in surgical operations on healthy tissues with good defenses, a small degree of contamination with air-borne bacteria is unlikely to result in infection, but that less healthy tissues and areas such as the meninges may not be able to deal with such contamination (Cairns 1939). In the very specialized field of orthopedic surgery, the incidence of infection has fallen with increased expertise, and it is now clear that the spectrum of infecting agents includes *S. epidermidis* and propionibacteria, such as are found in significant numbers in the air of a conventional operating theater. Charnley and Eftekhar (1969) and others found very low rates of clinical infection when ultraclean air was provided, but increased operative skill and the prophylactic use of antibiotics may have played a part in this. A multicenter trial (Lidwell et al. 1982) compared conventional theater conditions, provision of ultraclean air, exhaust-ventilated clothing and, incidentally, use of prophylactic antibiotics, in 8 055 operations (6 781 hip replacements; 1 274 knee replacements). The results (Figure 2) showed a cumulative benefit for the three measures and indicated that antibiotic prophylaxis alone would achieve more than the expensive provision of ultraclean air alone. A close relation was found between the levels of bacteria in the air and the risk of infection, and between staphylococci isolated in infected joints and those found in the patient or operating team at the time of operation. The benefit of very low levels of air-borne bacteria has not yet been proven for other types of surgery, but it would probably apply to the insertion of other prostheses, such as artificial heart valves and cerebrospinal fluid shunts.

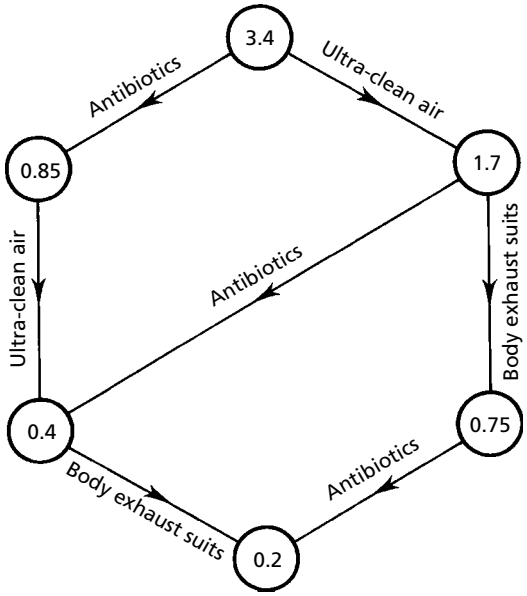

Figure 2 *Reduction of infection in joint prostheses by various measures (percent rate of infection). (Reproduced with permission from Lidwell OM, 1986,* Clin Orthop Rel Res*,* **211***, 91)*

Infection associated with water

LEGIONNAIRES' DISEASE

The Legionellaceae are widespread in water (Legionella), including potable water supplies, and many cases of Legionnaires' disease occur sporadically in the community. In one study in the UK they accounted for 15 percent of pneumonia cases (Macfarlane et al. 1982), but in other studies they were much less common. Hospitals are not spared the occurrence of legionellae in their water supplies (Tobin et al. 1981) and have suffered a series of outbreaks (Bartlett et al. 1986), mainly of infection with *Legionella pneumophila* (particularly serotype 1) but also with *Legionella micdadei*, *Legionella bozemanii*, and *Legionella longbeachae*.

The factors that favor hospital-acquired Legionnaires' disease are as follows. Hospital patients include a number of individuals with an impaired immune system who are more likely to become clinically infected with legionellae and who have a higher mortality than otherwise healthy subjects. Hospital-acquired infection occurs in patients rather than in staff who may nevertheless have circulating antibodies, which suggests exposure. Water in medical equipment that delivers a nebulized spray may become contaminated (Arnow et al. 1982). Hospitals are large buildings with extended hot-water

systems. It has been customary to run these at lower than usual temperatures, for economy and for the safety of patients. Peak demand may lead to a further fall in temperature. Legionellae can tolerate temperatures to 55°C and can grow at 35–45°C and thus are adept at surviving and flourishing in the water systems of buildings (Latham et al. 1992). There have been many examples of the association of cases in hospital with hot-water systems, particularly with those supplying showers (Tobin et al. 1980), or run at a low temperature (Meenhorst et al. 1985). Ancillary systems used only intermittently may discharge water that has stagnated, allowing growth of legionellae in sludge or biofilm, perhaps assisted by substances from plumbing materials (Bartlett et al. 1986). Cases associated with release of stagnant water into the general system have been described (Fisher-Hoch et al. 1982). Hospital ventilation systems often have air-cooling towers in which potentially contaminated water flows over pipes through which air is circulated. Faults in such a system may allow direct access of the water to the air, or drift from the tower may contaminate the air at a later stage. Outbreaks caused by contaminated cooling towers can be explosive, occurring over a short period of time, whereas those due to domestic water systems may be more insidious and may only be revealed after active surveillance.

The source of infection of patients in hospital is thus environmental water; person-to-person spread is unknown. As legionellae are ubiquitous in water systems, regular monitoring is of little value in hospitals that have had no previous problems, but this is more problematical in hospitals where documented cases or outbreaks of nosocomial infection have occurred (Fallon 1994). Prevention depends on the good design, installation, and maintenance of hot-water and ventilation systems and of the use wherever possible of air-cooled condensers rather than water-cooling towers; on keeping domestic hot water at high temperatures (>60°C for storage and >50°C at the point of delivery) unless patients are thereby exposed to risk of scalding, as for example, in mental handicap departments; on flushing those parts of the hot-water system that are subject to stagnation; on chlorination and biocide treatment of water; and on the use of sterile water in nebulizers, etc. A recent outbreak was terminated by a series of measures including the use of copper and silver ions introduced into the water system as disinfectants (Colville et al. 1993). Preventive measures, which should involve clinicians, microbiologists, and engineers, must be accompanied by good surveillance for the occurrence of Legionnaires' disease with adequate diagnostic facilities, and by contingency plans to be brought into action when possible hospital-acquired infections occur. In the UK, the 1995 Approved Code of Practice (ACoP) (L8), The prevention or control of legionellosis (including legionnaires' disease), and the guidance booklet HS(G)70 The control of legionellosis including legionnaire's disease, have both been recently revised and combined into a single document (Health and Safety Executive 2001). In its new form, the document is split into two parts. Part 1 is the ACoP itself, and part 2 of the document then provides guidance specific to particular systems, notably wet cooling systems (systems incorporating cooling towers and evaporative condensers), hot and cold water systems, spa/whirlpool baths, and humidifiers/air washers. In addition, a table in appendix 1 of this document provides a useful list of most other systems known to pose a risk, along with a summary of the main recommended control measures and the frequency of their application. For further information about Legionnaires' disease see Legionella.

OTHER BACTERIA IN WATER AND IN DAMP AREAS IN THE HOSPITAL

Other gram-negative bacteria may be present in hospital water supplies, and their multiplication is encouraged by warmth and the other factors enumerated above. They may then act as opportunist pathogens in hospital patients. *Aeromonas hydrophila*, for example, causes pneumonia and sepsis in immunologically impaired patients and Picard and Goullet (1987) found *A. hydrophila* in maximal numbers in hospital water in hot weather. Risks of infection exist whenever aqueous fluids or damp objects, in or on which gram-negative bacteria have had an opportunity to multiply and have not subsequently been destroyed, come into intimate contact with susceptible patients. Examples include dental water-cooling systems, cooling cycles in autoclaves, pressure-monitoring systems on arterial lines, unsterilized lotions, and irrigation solutions.

Pseudomonas spp. are to be found in mains water supplies and may multiply in any moist area. They have simple growth requirements and often show considerable resistance to antibiotics and may even survive in disinfectants, such as those prepared for multiple use. *P. aeruginosa* is ubiquitous in wet hospital sites. It has been found in food and water, ice machines (also implicated recently with infections due to *Stenotrophomonas maltophilia* in neutropenic patients in the UK), pharmacy preparations, plaster of Paris, mouthwash, dental water units, nebulizers, whirlpools and other hot baths, mattresses, sinks, taps, potted plants, flowers, flower vases, and so on. In some instances, particularly when the organisms have been introduced into the gastrointestinal or respiratory tract or the patients were particularly susceptible, colonization and later infection have been shown to have arisen from such sources. In other instances, however, the strains carried by the patients were different from those found only at inanimate sites in the wards. The patients' strains contaminated the immediate surroundings of the carrier patients and the hands of members of the staff and were occasionally

transferred to other patients (Levin et al. 1984; Allen et al. 1987). A more recent potential hazard that has been highlighted is pseudomonas infection following birth in water baths.

Burkholderia (Pseudomonas) cepacia, and other bacteria such as *Burkholderia pickettii*, possess the attributes that make them suitable for the role of hospital opportunists. Under certain conditions *B. cepacia* grows in distilled water and in the presence of aqueous chlorhexidine and quaternary ammonium compounds and may cause infections in neonates (Kahyaoglu et al. 1995). It has frequently been isolated from badly formulated, unsterilized supplies of these antiseptics, and even from povidone-iodine. Infection from a contaminated disinfectant or other fluid is generally associated with application to an open wound (Bassett et al. 1970), intravenous injection (Phillips et al. 1971), or urethral catheterization (Speller et al. 1971). *B. cepacia* is increasingly recognized as an important pathogen in patients with cystic fibrosis. In this group of patients the most important source is respiratory secretions with subsequent patient-to-patient spread. There is increasing evidence that some genomovars of *B. cepacia* complex are more virulent than others (e.g. genomovar III), and consequently patient segregation may be advisable (Govan et al. 1996; Mahenthiralingam et al. 2002).

Infection acquired from food

As in the general population, gastrointestinal pathogens may be transmitted by foods served to patients. Hospital food can also be a source of antibiotic-resistant bacteria, which may colonize the gut and later cause infection in susceptible patients. Of 1 280 outbreaks of infectious intestinal disease reported to the Communicable Disease Surveillance Centre in England during the period 1992–94 (Djuretic et al. 1996), 189 occurred. Salmonella infections were particularly associated with poultry or eggs, and *Clostridium perfringens* infections with meat. Outbreaks of salmonella infection in hospitals are important because they may cause serious effects in the very young, the elderly, and in patients with impaired immunity (Taylor et al. 1982) and may disrupt the working of the hospital (Kumarasinghe et al. 1982). The catering faults most often responsible are failures of catering staff to follow good practice: incomplete defrosting of frozen meats and poultry, insufficient cooking of large amounts of food, use of raw or insufficiently cooked egg products, inadequate chilling and storage, and contact between food to be consumed without further cooking and raw poultry. Effective processing of all foods, including poultry and eggs, and safe preparation, storage and distribution of food, are important in hospitals, where particularly susceptible patients are exposed to risk (Wilkinson 1988). The system of centralized preparation of food that is immediately chilled to safe storage temperatures and later regenerated in a standard system after distribution ('cook-chill' or variations on this theme) allows, and demands, careful control and monitoring of bacterial content (Wilkinson 1988; Wilkinson et al. 1991; Shanaghy et al. 1993).

Most outbreaks of salmonellosis in hospital are not food-borne, but are caused by person-to-person spread or contaminated fomites. In the 3-year survey by Djuretic et al. (1996), only 19 of 189 hospital episodes were mainly food-borne, whereas 156 were mainly person to person; in seven, both routes were equally involved and in seven the mode of spread was unknown. Similar figures have been reported in the USA (Baine et al. 1973). In a prospective investigation during a period of 2 years, food-borne spread probably accounted for only 11 percent of hospital outbreaks, although these tended to be the larger incidents (Palmer and Rowe 1983). Careful epidemiological investigation is required to determine the mode of spread and to define the population for further study (Palmer and Rowe 1983). There was a recent report by a London hospital of an outbreak of *Salmonella* Enteritidis phage type (PT) 6a. There were 27 hospital-acquired cases over 1 month in 2002. The source was thought to be imported raw shell eggs. A second cluster of approximately 25 hospital-acquired cases predominantly due to S. Enteritidis PT 1 occurred within the following month. During the outbreaks there were six deaths among patients identified as having salmonella, although none of these was directly attributable to salmonella infection (Public Health Laboratory Service 2002).

Hospital food is well known to often be a source of antibiotic-resistant gram-negative bacilli that colonize patients: *P. aeruginosa*, *Escherichia coli*, *Klebsiella* spp., and others (Shooter et al. 1969; Cooke et al. 1970, 1980; Casewell and Phillips 1978). It is possible for dietary components, equipment, and preparation areas to become contaminated with nosocomial bacteria, particularly in facilities for the preparation of individualized diets, including enteral feeds (Casewell 1982). Colonization of the bowel is an important source of infection of other sites, for example of bacteremia in granulocytopenic patients, who may benefit from a diet of low microbial content, with avoidance of foods likely to contain pathogenic or antibiotic-resistant bacteria (Schimpff 1993).

Infection by contact

FROM STAFF

Microorganisms on the hands of staff may be resident (persistent over time and not readily removed by hand-washing) or transient (recently acquired from another source). It is generally accepted that the hands of staff are an important vehicle and that hand-washing makes a significant contribution to the control of hospital-

acquired infection (Reybrouck 1983; Larson 1988). The relevant microorganisms can readily be demonstrated on the hands of staff and may easily be transferred to the skin of others by brief contact (Marples and Towers 1979). Introduction of hand-washing has been accompanied by reduction of the infection rate in many studies, from that of Semmelweiss (1861) to more recent investigations of infection by gram-negative bacilli, e.g. Casewell and Phillips (1977). The literature of more than 100 years was reviewed by Larson (1988), who concluded that the evidence strongly supported a causal connection between hand-washing and control. The effect has been observed in a variety of settings under many different hygienic circumstances (Larson 1988).

The most important microorganisms spread by hand contact are *S. aureus* and gram-negative bacilli, such as *Klebsiella* and *Serratia* spp. Carriage on the hands has been suggested as important in the transmission of antibiotic-resistant enterococci (Rhinehart et al. 1990) and of *C. difficile* (Kim et al. 1981; McFarland et al. 1989), but this has not been a consistent finding. In addition, the route has been described for many other hospital-spread pathogens, including *Candida albicans* (Burnie 1986) and even respiratory pathogens: *Corynebacterium diphtheriae* (in the past) and viruses, such as RSV and rhinoviruses (Pancic et al. 1980). With *S. aureus*, attention has been focused on long-term carriage (Williams et al. 1960); gram-negative bacilli also have been demonstrated to persist for long periods on the hands of some persons (Casewell and Phillips 1977; Adams and Marrie 1982). Transient carriage resulting from recent contact with an infected or colonized patient may often be more important. Casewell and Desai (1983) demonstrated that strains of gram-negative bacilli, particularly of *Klebsiella* and *Serratia*, that had caused outbreaks in which infection was apparently spread by staff from patient to patient, survived longer on the hands of volunteers than did strains capable of causing outbreaks only from a point source in food or the environment. The ability of glycopeptide-resistant enterococci to survive for 1 h on fingers has also been shown (Noskin et al. 1995b).

It should be noted that healthcare workers sometimes erroneously wear gloves as a substitute for hand hygiene. Also, hand hygiene products may degrade gloves, and healthcare workers need to be aware of the need to clean hands after glove removal, because of the inevitable contamination that occurs during degloving. The preparations available for hand decontamination and the methods for assessing them have been reviewed (Ayliffe 1980; Reybrouck 1986). Hand hygiene may be designed for the removal of transient flora (Price 1938), which may be achieved by a simple wash with soap or detergent, or even better by an aqueous preparation of an antiseptic with a detergent. Sometimes a residual disinfectant action is needed, with or without reduction of the resident flora, as in the preoperative preparation of a surgeon's hands, or in high-dependency units in the hospital. Iodophors, the bisguanide chlorhexidine, and trichlorohydroxyphenol and related compounds may all be used and all have some residual activity (Ayliffe 1980). Alcoholic preparations, or alcohols alone, are more rapid in action than aqueous preparations and are more effective (Lowbury et al. 1974). Their drying action on the skin can be prevented by the addition of glycerol or other emollients. Alcohol-based hand rubs are quicker to use and aid compliance with hand hygiene policies, but they are unsuitable for use on soiled hands. A minimum of skin flora cannot be reduced further by these processes (Lilly et al. 1979). Methods should be adopted that are suitable for the particular purpose and that avoid damage to the skin and development of resistant flora on repeated use (Ayliffe 1980; Reybrouck 1986). One clinical trial (Doebbeling et al. 1992) compared different hand-washing methods in consecutive periods in intensive care units. A reduction in nosocomial infection was directly demonstrated when chlorhexidine (rather than soap or alcohol) was used; the effect may have been partly due to better compliance by staff. Poor compliance with the prescribed measures is frequently observed and may vary with the professional group, the hand-washing system prescribed, and its ready availability (Albert and Condie 1981; Doebbeling et al. 1992; Gould 1994; Wurtz et al. 1994; Pittet et al. 1999). Importantly, it has been shown that as the intensity of required patient care increases, the compliance with hand hygiene falls (Pittet et al. 1999). Hand cleaning should be carried out immediately before and after each contact with patient of their close surroundings. Efforts are increasingly focused on how to improve healthcare workers' compliance with hand hygiene. Pittet and colleagues recently examined >20 000 opportunities for hand hygiene and successfully improved compliance by healthcare workers from 48 percent in 1994 to 66 percent in 1997 ($p < 0.001$) (Pittet et al. 2000). This was attributed primarily to the promotion of bedside antiseptic handrubs. Hand hygiene improved significantly among nurses and nursing assistants, but remained poor among doctors. Importantly, the sustained improvement in compliance with hand hygiene coincided with a reduction of nosocomial infections and MRSA transmission.

The clothing of personnel can be shown to become contaminated with potential pathogens, such as *S. aureus* and, less frequently, gram-negative bacilli, particularly after the handling of heavily colonized patients (Babb et al. 1983). The significance of this in the spread of infection is unknown.

FROM THE PATIENT'S ENVIRONMENT

The immediate environment readily becomes contaminated with the bacteria carried by a patient. It has sometimes been difficult to prove that organisms from environmental surfaces cause infection (Maki et al. 1982),

partly because the virulence of the bacteria may become impaired. Nevertheless, if heavily contaminated (Sanderson and Rawal 1987), such surfaces must be regarded as a potential source of air-borne organisms and also of infection by direct contact with another patient or via the hands of staff. Whilst it has been known for many years that staphylococci are desiccation resistant and can survive in the environment, thus prolonging outbreaks, emerging pathogens such as *Acinetobacter baumanni* may also persist. In a recent study the mean survival times of sporadic and outbreak strains on glass coverslips were 21–33 days (Jawad et al. 1998). Particularly in the case of bacteria carried in the bowel, which can survive in the environment for long periods, contamination of the environment or equipment may be responsible for the clustering of cases, as with *C. difficile* (Cartmill et al. 1994) and *Enterococcus* spp. (Karanfil et al. 1992). In a review of intensive care unit design and environmental factors in the acquisition of infection, O'Connell and Humphreys (2000), make the point that the inanimate environment of air, water, food, floors, walls, and ceilings can contribute to the risk of acquisition of infection in intensive care patients, although their actual role can be difficult to quantify in most instances. However the design of the intensive care unit (ICU) should be such as to minimize the entry and persistence of microorganisms into this environment and allow the thorough cleaning of any surfaces which may become contaminated (Dieckhaus and Cooper 1998).

Dancer (1999) found that cleaning in the hospital setting is often a neglected component of infection control and many outbreaks occur secondary to suboptimal cleaning. Hospitals provide a reservoir of microorganisms adapted to the hospital environment including the ability to survive harsh environmental conditions. The immediate environment of patients in sometimes heavily contaminated and may include vancomycin-resistant enterococci (VRE), MRSA, *A. baumanii*, *P. aeruginosa*, and *C. difficile*. The hardy spores of *C. difficile*, an organism that can cause serious outbreaks throughout the hospital, can withstand extremes of temperatures and some disinfectants. They also remain viable for a considerable length of time in the environment and are responsible for many outbreaks of infection in general wards and in the ICU (Spencer 1998). Other nonspore-forming microbes, although not as resilient as *C. difficile*, can survive in areas that are damp and poorly cleaned. Standards for environmental cleanliness in hospitals were published in the UK in 1999 with a view to raising the profile of hygiene in hospitals, developing an audit tool and producing prescriptive recommendations from which standards for cleaning in hospitals could be derived (ICNA/ADM 1999). Cleaning and disinfection of the rooms of infected patients are recommended when the patients leave the room or are discharged from hospital. There are ongoing studies as to whether materials with intrinsic antibacterial activity could potentially be used as finishes for walls, floors, etc., and thus help to control environmental contamination (Talon 1999).

Rampling and coworkers (2001) again highlighted the importance of general hospital hygiene in bringing to an end a prolonged outbreak of MRSA. They showed that a dusty ward was an important source of MRSA for their surgical patients. During the last 15 years in the UK, hospital cleaning services have been targeted for cost-cutting in the NHS (Dancer 1999). However, it is difficult to demonstrate that a dusty or soiled environment can actually be hazardous to patients, with little evidence to support this (Ayliffe et al. 1967; Maki et al. 1982; Collins 1988). Nevertheless, a high standard of hygiene should be an absolute requirement in hospitals for aesthetic, as well as clinical reasons. In the long term, cost-cutting on cleaning service is neither cost-effective nor common sense (Blyth et al. 1998; Plowman et al. 1999; Wilcox and Dave 2000). All this emphasizes that basic hospital hygiene, such as cleaning, remains an important element in preventing infection and attempts at cost containment in this area are likely to be counter-productive.

FROM EQUIPMENT

Infection from surgical instruments is now extremely rare, but other items of equipment, even if they do not penetrate the tissue, may convey infection from one patient to another. Some of these escaped attention because the risks associated with them appeared to be low or had not been perceived. Others are pieces of equipment that are difficult to clean and disinfect adequately, or are expensive and in short supply.

Many items that come into contact with patients usually do not need to be supplied sterile, but patients with impaired immunity may be susceptible to opportunist organisms present on or in these. For example, patients with debilitating illnesses may develop superficial and subcutaneous phycomycosis in macerated areas under adhesive plaster contaminated with *Rhizopus* spp. (Gartenberg et al. 1978). Premature babies have become infected by *Rhizopus* spp. from unsterile wooden tongue depressors, used as limb splints (Mitchell et al. 1996).

Bedpans and urinals have been blamed for the spread of enteric bacteria, such as *C. difficile* and antibiotic-resistant gram-negative bacilli (Curie et al. 1978). The bacterial load may be high if the washing process is not efficient, and bedpan washer-disinfectors often do not maintain an adequate temperature for the intended time. The practice of decontaminating urinals in tanks of phenolic disinfectant is often ineffective because of inaccurate dilution of the disinfectant, or its inactivation by organic matter, or by the survival of bacteria in biofilm on surfaces (Curie et al. 1978). Special decontamination measures may be necessary for particularly resistant pathogens, e.g. *C. difficile* and *Cryptosporidium* spp. Rectal thermometers have been believed to be responsible for

the spread of salmonellae (Im et al. 1981) and enterococci (Livornese et al. 1992); all thermometers should be decontaminated after use (Nystrom 1980).

Many fiberoptic endoscopes for gastrointestinal and bronchial use are difficult to disinfect because they do not withstand heating; also their complex channels are difficult to clean. Numerous species have been transmitted by such endoscopes (Spach et al. 1993). Among the most common have been *Salmonella* spp. and *P. aeruginosa* in the gastrointestinal tract and *Mycobacterium tuberculosis* and other mycobacteria in the respiratory system. Endoscopes are readily contaminated with *Helicobacter pylori* and transmission by endoscopy has been demonstrated (Langenberg et al. 1990). Recent updated guidelines on endoscopy disinfection have increased the disinfection contact times, e.g. from 4 to 10 min for 2 percent gutaraldehyde, to provide a greater margin of safety, and have emphasized the importance of water quality of the final rinse (Report of a Working Party of the British Society of Gastroenterology Endoscopy Committee 1998). In the impaired patient infection of other sites and bacteremia may result (Schimpff 1993); bacteremia is particularly likely to follow biliary endoscopy (Struelens et al. 1993). Hepatitis B has rarely been transmitted on endoscopes although they readily become contaminated with the virus (see Ridgway 1985). Spread of human immunodeficiency virus via endoscopes has not been reported. Such accidents can be avoided by a thorough regimen of cleaning and disinfection with a suitable chemical agent, which may be carried out manually or by special apparatus (Fraser et al. 1993; Bradley and Babb 1995) if sufficient endoscopes can be provided (Anon 1988; Fantry et al. 1995; Babb and Bradley 1995).

The ever expanding use of endoscopic procedures in diagnosis and surgical treatment of patients has reinforced the need for safe and effective methods of cleaning, disinfection, and sterilization of these instruments (Ruddy and Kibbler 2002). Reports on the decontamination of minimally invasive surgical endoscopes and accessories have been published (Ayliffe 2000). As the number of patients undergoing endoscopy has increased, the requirements for the safe use of glutaraldehyde and other potentially irritant or sensitizing instrument disinfectants has led to an increased use of automated washer disinfector systems which provide safe containment of hazardous vapor and can remove toxic residues effectively. These automated systems have been shown to be more effective in removing bacteria than manual methods but they are not without problems, and the following issues need to be recognized:

- Many systems are available on the market. Irrespective of whether or not the system has its own wash cycle prior to disinfection, it is recognized that manual precleaning of endoscopes is essential (Babb and Bradley 1995).
- There is a need to ensure endoscope compatibility with the autodisinfector fittings. Endoscopy staff must follow instructions strictly in order to ensure that all endoscope channels are adequately irrigated with disinfectants. There is a need to ensure endoscopes and washer disinfectors are compatible with the disinfectants used.
- Automated systems require regular and adequate maintenance which is time consuming and expensive. Furthermore, some parts of the decontamination process (e.g. brushing lumens and wiping insertion tubes) cannot be automated and thus manual cleaning is still an essential prerequisite.

The most significant problem which has not been addressed by the increased use of automated systems is that of contaminated rinse water derived from either the internal pipework of the washer disinfector or mains water, as well as intermediate tanks and poorly maintained filters. Recent reports have attempted to address this and give recommendations on possible ways of dealing with this problem (MacKay et al. 2002; Report from a Joint Working Group of the HIS and PHLS 2002).

Infection by inoculation

Since the universal introduction of single-use disposable needles and other devices, and satisfactory procedures for the sterilization of surgical instruments, with a wide safety margin, transmission of infection by this route in the developed world has been infrequent. Few agents can resist the standard sterilization processes. The prions responsible for transmissible spongiform encephalopathies (e.g. Creutzfeldt–Jakob disease (CJD)), previously associated with neurosurgery, are highly resistant to most sterilization procedures and therefore instruments used on patients with these conditions should either be disposed of or subjected to prolonged autoclaving at high temperature (Advisory Committee on Dangerous Pathogens 1994). However, there remain infections (1) transmitted by blood transfusion or tissue donation; (2) resulting from accidental injury from contaminated sharp instruments; (3) from contaminated blood; and (4) from other contaminated infusion fluids.

INFECTION TRANSMITTED BY BLOOD TRANSFUSION AND TISSUE DONATION

Infectious agents may from time to time be transmitted to patients by donors of blood, blood products, or tissue. The risk of transmission of the three most important agents, hepatitis B and C viruses and human immunodeficiency virus (HIV), has been reduced to a low level by a combination of donor selection, screening of donations for antigen or antibody, and heat treatment of blood products, but not before countless cases of 'serum hepatitis' had occurred and a considerable proportion of

hemophilia sufferers received factor VIII infected with HIV (Editorial 1984b). Efforts should also be made to exclude other, less common, blood-transmitted infections such as other forms of hepatitis, including δ agent and possibly hepatitis G in the future, cytomegalovirus, Epstein–Barr virus, parvovirus, HTLV-1, brucellosis, syphilis, salmonellosis, malaria, trypanosomiasis, toxoplasmosis, babesiosis, and filariasis (see Mollison et al. 1987). Acquisition of transmissible spongiform encephalopathies is not considered likely to follow blood transfusion. An epidemiological survey of CJD in the UK revealed that only 16 of 202 cases had a history of blood transfusion and this was not significantly different from controls (Esmonde et al. 1993). However, cases of blood transfusion-acquired CJD have now been reported in England (Llewelyn et al. 2004). Further efforts to exclude infections less common than those already currently screened for in the blood supply must be balanced against the considerable cost of such screening and the impact this might have on blood donation.

Tissue donations have been responsible for the transmission of cytomegalovirus, HIV (L'Age-Stehr et al. 1985), and rabies (Helmick et al. 1987) and may also transmit hepatitis B and hepatitis C. Creutzfeldt–Jakob disease may be acquired during surgical procedures that involve tissue transfer and when this occurs, the incubation period is a matter of months rather than years (Hart 1995). Creutzfeldt–Jakob disease first described by Jakob in 1921, is one of four transmissible spongiform encephalopathies (TSE) found in man. The others being Gerstmann–Straussler–Scheinker syndrome, Kuru (associated with cannibalism in Papua New Guinea), and fatal familial insomnia. TSEs are rare, fatal neurogenerative diseases that cause degeneration of the brain resulting in loss of coordination and faculties. The infectious agents are thought to be unique proteins and are termed 'prions'. Prions replicate by transforming normal cellular prion proteins into abnormal isoform proteins. These abnormal prion proteins, PrP^{res}, then accumulate in the central nervous system causing spongiform changes in gray matter where they trigger neurological symptoms.

Sporadic CJD occurs worldwide. It is rapidly progressive and ultimately results in a fatal disorder of the central nervous system with a long incubation period of 15 months to over 30 years. There are approximately 0.5 to 1 new case per million of the population per year. The similarities between TSEs found in different animal species (e.g. bovine spongiform encephalopathy (BSE)) and those found in man suggest that the disease, in some form or another, may be able to cross the species barrier. Sporadic CJD usually presents in late middle age, mean age 66 years (range, 16–95) with progressive dementia, a duration of illness of <4 months (range, 1–74 months). Diagnosis is based on clinical signs and characteristic waveforms when recording electrical events in the brain (EEG), and as no treatment is available for CJD, medical management remains limited to supportive care.

The new variant of Creutfeldt-Jakob disease (vCJD) was described in the UK in 1996 after the occurrence of ten atypical cases of CJD in patients under 40 years of age, nine of them under 30; median age 28 years (1–74 years). All these patients had common features: young age, a special clinical presentation with psychiatric features at onset, an abnormal duration in the evolution of the disease (14 months on average compared with 2 to 6 months in classical cases of CJD), and a pathognomic neuropathology. Particular neuropathological features were amyloid plaques surrounded by vacuoles (florid plagues) whose distribution in the central nervous system was remarkably similar from one patient to another; a potent PrP immunostaining was identified in the injured areas, and the presence of PrP^{res} was found by Western blot, Moreover, all these patients were homozygous methionine/methionine to codon 129 of the human *PRNP* gene, and did not have a medical history to suggest an iatrogenic or familial origin or disease. In addition, unlike sporadic CJD, no periodical electrophysiological abnormalities were observed; further, the protein 14-3-3, a nonspecific marker usually found positive in sporadic forms of CJD, was not detected. Finally, hyperdense signals to cerebral magnetic resonance imaging (MRI) have been described in the post-hypothalamus area (Pulvinar sign, not seen in sporadic) and can be a helpful diagnostic feature in cases with suggestive clinical picture. This form of CJD was suspected to be the consequence of infection in man with the bovine spongiform encephalopathy (BSE) agent as no other etiological hypothesis could reasonably be considered. The evidence that this is indeed the case is compelling, with BSE manifesting itself as a novel human prion disease – variant Creutzfeldt-Jakob Disease (vCJD), with temporal association with consumption of bovine central nervous system material in food. First exposure was thought to have occurred in 1983, peak exposure 1989, last exposure in 1995. First cases of variant CJD, identified retrospectively, occurred in 1993. Incubation of vCJD is governed by infective dose and methionine/methionine at codon 129. Experimental transmission of BSE and vCJD in bovine transgenic mice have shown similar brain pathology characteristics (possibly satisfying Koch's postulates).

Despite many sensational reports in the media, TSEs although infectious are not easily transmissible as this requires specific material from the affected individual's tissue from or adjacent to their central nervous system. For this reason isolation of sufferers is not necessary. Thorough cleaning and sterilization of surgical instruments is strongly recommended for instruments used on known or suspected CJD cases. Cases of iatrogenic transmission of classical CJD have been reported (corneal grafts, pituitary growth hormone, dura mater grafts). As of 2002 there was no evidence implicating iatrogenic transmission of vCJD (Spencer and Ridgway 2002). This picture has now changed (see above).

INFECTION FROM ACCIDENTAL INOCULATION

In the past, tuberculosis of the skin ('prosector's wart') was a common consequence of inoculation and is once again a potential risk from HIV patients heavily infected with *M. tuberculosis* (Kramer et al. 1993). In the pre-antibiotic era, infection by a needlestick injury with *S. pyogenes* might result in septicemia and death, as observed by Semmelweiss in his colleague, Kolletschka. Such events remain a hazard, though a much reduced one (Hawkey et al. 1980). The main risks of such injuries, however, are infection with hepatitis B and C viruses and HIV.

HEPATITIS B AND C AND HIV INFECTION IN HOSPITALS

Hepatitis B and C viruses and HIV are the pathogens most likely to be transmitted from blood and tissue fluids (Zuckerman 2002). Bodily secretions from infected patients contain much lower concentrations of virus, or none at all. The risk of transmission to staff is greatest for those with the highest exposure to blood and to injuries with sharp instruments, but a history of a definite incident is not always given by hospital staff members who develop hepatitis B. The risk of acquiring hepatitis B from an infectious carrier in an inoculation incident is significant, especially if the patient is e-antigen positive (HBeAg). An investigation following the identification of an HBeAg-positive cardiac surgeon revealed that 13 percent of susceptible patients contracted hepatitis B despite good infection control practice (Harpaz et al. 1996). Until recently it was considered that the risk of nonsocial acquisition from HBeAg-negative staff was low, but a recent report describes spread from HbeAg-negative surgeons with precore mutations. These mutations prevented the expression of HBeAg, but not the assembly of infectious virus and quantitative measurements of HBV DNA may be necessary to fully assess the risk of transmission in surgical and other settings (Incident Investigation Teams and others 1997). Currently, in the UK and Ireland, all healthcare workers performing exposure prone procedures (EPP) are required to be able to demonstrate immunity to hepatitis B. Hepatitis B-infected healthcare workers who are e-antigen negative and who perform EPPs should have their viral loads measured, and those with viral loads exceeding 10^3 genome equivalents per ml should not perform EPPs in future (NHS Executive 2000).

It is clear that the risk of transmission of HIV to staff is very small; of 1 036 subjects included in several series, with adequate epidemiological and serological investigation of percutaneous or mucous-membrane exposure to blood or tissue fluid from known HIV-positive patients, only three (0.3 percent) showed seroconversion (Centers for Disease Control 1987). More recently, only four of 1 103 healthcare workers with percutaneous exposure to HIV-infected blood seroconverted and in one case this occurred following post-exposure zidovudine (Tokars et al. 1993). The risk of a patient acquiring HIV from an infected healthcare worker has been the subject of much debate, some of it ill-informed, but has raised the issue of screening healthcare workers for HIV. A retrospective study of more than 6 400 patients of an infected Florida dentist revealed 28 with HIV infection, of whom 24 had behavioral risk factors. Analysis of the viral gene sequences from the dentist and his patients excluded a possible link with the 24 with behavioral risk factors, but not with the remaining four (Jaffe et al. 1994). A survey of 22 171 patients of 51 infected healthcare workers conducted by the Centers for Disease Control, Atlanta, USA failed to reveal evidence of transmission from healthcare worker to patient (Robert et al. 1995). A cost–benefit analysis of screening surgeons for HIV as a strategy to minimize transmission to patients did not support this approach as cost-effective (Owens et al. 1995). In the UK, recommendations on the investigation of patients who have been cared for by a member of staff who is subsequently identified as HIV-positive, are available (Expert Advisory Group on AIDS and the Advisory Group on Hepatitis 1998).

Post-exposure prophylaxis (PEP) against HIV should be considered if there has been possible exposure to HIV-infected blood or body fluid (Expert Advisory Group on AIDS 2000). The initial risk assessment is based on the potential for viral transmission, i.e. the type of body fluid involved (e.g. blood is high risk), the route (e.g. deep injury by a blood-containing or stained device) or/and severity of exposure (e.g. injury with a needle that has been in the source patient's artery or vein; and/or terminal HIV-related illness in the source patient). Some occupational exposures after careful assessment may not be considered to be significant, that is they do not have potential for HIV transmission, i.e. splashes on to intact skin. In such circumstances PEP is not indicated. If a significant exposure has occurred and the source patient is known to be HIV-positive, PEP should be commenced, the first dose should be given within 1 h of the incident. Based on the results of a HIV test on the donor (with informed consent) the healthcare worker, advised by a specialist clinician can make an informed decision on whether to continue with PEP. However, the exact mechanisms of HIV infection are not fully understood, therefore if more than 1 h has elapsed after exposure this does not automatically preclude starting PEP. It is worth considering starting PEP up to 2 weeks following exposure, however specialist advice should be sought.

Hepatitis C (see Hepatitis C in the *Virology* volume in this series), recognized in the last decade as the major cause of non-A non-B hepatitis following blood transfusion, is encountered worldwide with especially high prevalence in Japan and the southern part of the USA, and may persist in 80 percent of infected patients (van der Poel et al. 1994). Some patient groups have a higher seroprevalence; 3.3 percent

of Dutch hemophilia patients were positive compared with 0.03 percent of blood donors (Schneeberger et al. 1993). Acquisition associated with particular occupations is also described; oral surgeons had a higher incidence (9.3 percent) than dentists (0.97 percent) or unselected blood donors (0.14 percent) in one study (Klein et al. 1991). The risk of seroconversion following a needlestick incident is approximately 3 percent, which is intermediate between HIV (0.3 percent) and hepatitis B (up to 30 percent for hepatitis B eAg positive donors). At present, the risk of a hepatitis C-positive healthcare worker transmitting infection to patients is not certain. An investigation of an incident involving a hepatitis C-positive cardiac surgeon revealed that six of 222 patients followed up contracted postoperative hepatitis; in five patients the viral gene sequences were similar to those of the surgeon (Esteban et al. 1996). Several other reports of hepatitis C transmission from healthcare workers to patients have since occurred (Gunson et al. 2003). The lower risk of transmission of hepatitis C compared with hepatitis B is unfortunately counterbalanced by the greater risk of chronic hepatitis C infection (80 percent), which may in turn lead to cirrhosis or hepatocellular carcinoma. In the UK, healthcare workers entering training to specialities that require practice of exposure prone procedures (EPPs) are now required to be screened for hepatitis C. Healthcare workers that are found to have high levels of hepatitis C RNA in blood are required to cease performing EPPs until RNA levels decline, possibly following interferon/ribivirin treatment. This policy approach is, however, not universal, and in some other countries hepatitis C infected healthcare workers are being allowed to continue to practice after disclosure of their infection status (Barrigar et al. 2001).

Active hepatitis B immunization should be given to those at greatest risk (Mulley et al. 1982) and this and hepatitis B immune globulin (e.g. vaccine staus incomplete/unknown) may be used as post-exposure prophylaxis (Grady et al. 1978). The action needed in the immediate post-exposure period to protect a healthcare worker against hepatitis B is dependent on the vaccination status of the healthcare worker and (where known) the hepatitis B surface antigen status of the source patient. Other measures are clearly to be directed against the risk of inoculation of blood. With the recognition of new agents (e.g. hepatitis C) transmissible by blood and the likelihood of others to follow, control measures have more and more emphasized good practice when handling blood, blood products, and other fluids such as cerebrospinal fluid, irrespective of patient source (Expert Advisory Group on AIDS and the Advisory Group on Hepatitis 1998). These are referred to as universal precautions and include greater awareness of the risks by staff, staff covering cuts or abrasions, careful disposal of sharps and clinical waste, and the use of gloves and gowns when in contact with body fluids (Board of Science and Education 1996; Expert Advisory Group on AIDS and the Advisory Group on Hepatitis 1998). Additional precautions may be taken with known infected patients, or those suspected of being infected (e.g. intravenous drug abusers), but the adoption of universal precautions at all times minimizes the risks to staff and patients when a patient is not known or considered to be in an at-risk group. Guidelines issued in the UK to minimize acquisition of hepatitis B and HIV during surgery, which would also largely apply to hepatitis C, emphasize good practice at all times, e.g. avoidance of passing sharps by hand, with additional precautions such as double gloving when operating on positive patients or those in at-risk groups (Joint Working Party of the Hospital Infection Society and the Surgical Infection Study Group 1992). Continuing education on basic techniques to avoid inoculation risk incidents is important; taking sharps boxes for the disposal of needles to the patient's bedside reduced the number of recapped needles (a practice likely to place the healthcare worker at risk of a needlestick incident) from 33 to 18 percent (Makofsky and Cone 1993).

Testing for hepatitis B surface antigen (HBsAg) has been used to define more closely the patients who present an 'inoculation risk' and identification of HBeAg and antibody (HBeAb) to define the degree of infectivity. Screening of patients for hepatitis C is not usually indicated as an infection control measure. Testing for HIV with consent is not recommended at present, partly because of the implications of detecting seropositivity in a symptomless patient and partly because of the lack of a sufficiently sensitive and specific antigen test to identify infected patients before antibody appears. Testing for hepatitis B, hepatitis C, and HIV is essential before tissue donation and recommended in certain other defined situations.

Containment measures for identified patients should concentrate on the risks associated with invasive procedures and bleeding, and especially with surgical operations and obstetric deliveries. Single-room isolation is required only for certain patients, e.g. those with mental disturbance, uncontrolled bleeding, or dual infection with HIV and another disease, such as tuberculosis. In deciding whether a patient requires single-room isolation, consideration should also be given to the psychological effects this may have. The standard decontamination measures for crockery, bedpans, etc., are adequate. For invasive procedures, limiting the number of staff present, providing adequate protective clothing, including precautions against splash on mucous membranes, limiting and containing blood loss as far as possible, care with sharp instruments, and adequate decontamination of instruments are the principles to apply. Detailed recommendations for the care of HBsAg or HIV risk patients have been formulated (Expert Advisory Group on AIDS and the Advisory Group on Hepatitis 1998; Expert Advisory Group on AIDS 2000).

INFECTION FROM CONTAMINATED BLOOD

Extrinsic contamination of donor blood with small numbers of bacteria is not uncommon and serious consequences of this are rare. Occasionally, stored blood may contain large numbers of bacteria (Braude et al. 1955), often psychrotrophs, and septicemia and shock may occur in the recipient (McEntegart 1956). The range of possible organisms and the risks associated with erythrocytes, whole blood, and platelets together with advice on how to investigate outbreaks and prevent such occurrences have been well reviewed (Wagner et al. 1994).

INFECTION FROM CONTAMINATED INFUSION FLUIDS

Major outbreaks of serious infection and endotoxic shock have been caused by heavily colonized infusion fluids other than blood. Some accidents were due to failure of sterilization during commercial manufacture (Meers et al. 1973), and others to the introduction of microorganisms from cooling water in sterilizers (Phillips et al. 1972) or from contaminated closures which release their bacteria into the infusion fluid during manipulation in the ward. Infusion fluid in reservoirs or administration sets can become colonized during use, from cracks in containers or during manipulations and additions (Duma et al. 1971). Indeed, a significant proportion of containers becomes contaminated with small numbers of bacteria (Maki et al. 1973). Fluids for total parenteral nutrition, such as protein hydrolysate with or without dextrose, readily support the growth of microorganisms, particularly of *Klebsiella* and *Enterobacter* spp., and of *Candida* spp. Disseminated fungal infection has been particularly associated with total parenteral nutrition, but not all episodes have resulted from contamination of fluid in the reservoir or administration set (Goldmann and Maki 1973). The risk can be reduced by care with all manipulations or by having all additions to intravenous fluids made by skilled staff working in controlled environmental conditions. Containers and administration sets should be changed every 48 h; more frequent changing is unnecessary (Buxton et al. 1979). The use of bacterial filters, i.e. in administration sets, has done little to reduce infection (Collin et al. 1973). This may indicate that infection related to the intravascular cannula is more common than colonization of fluid in the rest of the system.

HOSPITAL INFECTIONS AT VARIOUS BODY SITES

Surgical wounds and other soft tissue sites

Surgical site infections (SSI) are important numerically and as a cause of morbidity and prolonged hospital stay. In a prevalence survey (Emmerson et al. 1996) it accounted for 12.3 percent of hospital-acquired infections. In the USA incidence study, SSI accounted for 24 percent of nosocomial infection. In some surveys the definition of SSI was based on a simple, easily observed character such as the presence of pus in the wound and this has the advantages of minimizing observer variability. A more complex definition, making use of several other signs of inflammation (e.g. erythema), can be used with an elaborate scoring system (A.P. Wilson 1995). This may increase the likelihood that significant differences will be observed in comparative trials. The definition should be based on clinical observation. It may be useful to culture all infected wounds for microbiological surveillance purposes, but the presence of pathogenic bacteria in itself does not imply infection. The recorded incidence of infection will also depend on the length of postoperative stay and the degree of follow-up after discharge from hospital (Bailey et al. 1992; Weigelt et al. 1992; Byrne et al. 1994; Emmerson 1995).

Several investigations have been made, in sufficiently large numbers of patients, of the factors predisposing to SSI, to determine where improvements in practice should be directed (e.g. Cruse and Foord 1973). Regression analysis has been employed to separate variables. Surgical procedures may be crudely divided as follows: ‘clean’, when no inflammation is encountered and no colonized body system is entered; ‘clean contaminated’, when a colonized system, e.g. gastrointestinal or respiratory, is entered, but there is no significant spillage; ‘contaminated’, when inflammation, but not pus, is encountered or spillage from a viscus occurs; and ‘dirty’, when a perforated viscus or pus is encountered. The incidence of infection should be less than 5 percent for clean operations; some surgical teams may record a rate of less than 1 percent (Cruse and Foord 1973). On the other hand, operations on the left colon or rectum may be followed by wound infection in more than 30 percent of patients if antibiotic prophylaxis is not given. Refinements of this classification system have been suggested. It may be more useful to consider individual types of operation separately. For example, Cesarean section, though included in ‘clean’ surgery, may have a high infection rate (Beattie et al. 1994; Henderson and Love 1995).

In general investigations, the factors most consistently associated with an increased incidence of postoperative infection are age over 60 years, long preoperative stay in hospital, long duration of operation, pre-existing infection at the site of the wound, and bacteria in the wound at the end of the operation. Underlying disease, such as diabetes, immunosuppression or irradiation, and in some studies, malnutrition and administration of adrenocorticosteroids, are also important. In preoperative preparation, shaving of hair from the site, rather than treatment with depilatories or clipping, has been associated with a much greater frequency of infection (Seropian and Reynolds 1971), but other aspects of skin preparation

appeared not to exert a consistent effect. Factors of significance in some studies but not in others are male sex, emergency operations, and the use of surgical drains. It is generally agreed that good surgical technique is most important, but it is difficult to single out particular surgical practices. The use of diathermy was associated with increased infection in one series (Cruse and Foord 1973).

Overall, *S. aureus* is the dominant species in SSI, followed by the enterobacteria (Meers et al. 1981). UK surveillance data show that approximately two-thirds of *S. aureus* SSIs are caused by MRSA. Even higher MRSA prevalence in SSIs is seen in subspecialities such as vascular surgery (Public Health Laboratory Service 2002). Sternal wound infection after cardiac surgery, though difficult to recognize clinically in the early stages, may later be associated with mediastinitis, osteomyelitis, septicemia, and colonization of prosthetic material, and thus with significant morbidity and mortality. Infection occurs at the time of operation from the many sources outlined above; it is probable that a relatively small inoculum is needed in the inevitably damaged tissue. Prevention may be a major role for the prophylactic antibiotics that are used almost universally in cardiac surgery (Farrington 1986). Among factors that have been cited as predisposing to this type of postoperative infection are long and double procedures, excessive trauma to the sternum, postoperative hematoma formation, and reopening of the wound (Sarr et al. 1984), as well as the features already described as predisposing to surgical wound infection in general. It has been reported that the incidence of infection is greater in coronary artery bypass surgery when leg veins are used, and that the infecting organisms, particularly gram-negative bacilli, may be transferred from the leg. Infection of the leg wound itself is also common (Wells et al. 1983; Farrington et al. 1985).

The microbiological findings vary with the site and type of operation and also with the adequacy of culture methods, which often underestimate the role of anaerobic bacteria, such as *Bacteroides* spp. These, with other gut bacteria, often in mixed growth, are typical of infections after a colonized viscus is entered (Dunn and Simmons 1984). *S. aureus* may occur in all types of wound and is the typical cause of the less frequent wound infection in 'clean' surgery. Most infection of surgical wounds occurs at the time of operation. Evidence from animal experiments (Burke 1961) and studies of the effectiveness of antibiotic prophylaxis (e.g. Stone et al. 1976) suggest that there is a short period after bacterial contamination during which infection is initiated. In the great majority of cases, the origin of the bacteria appears to be the patient's own body flora. Much less often it is from a member of the operating team, but in many instances the origin is obscure. Occasionally a cluster of infections can be related to an organism present in a septic lesion or at a carrier site in a member of the operating team, but this is uncommon. The route by which a patient becomes infected is often not clear. The air-borne route is important in the implantation of prostheses, and in rare episodes in general surgery, which were linked with an identifiable source among the theater staff, the circumstances strongly suggested the air as the vehicle. More usual routes are direct spread from incised organs, and intraoperative contamination of instruments and of surgeons' gloves and clothing. Contamination from apparatus such as that providing an extracorporeal circulation has occasionally been described.

BURNS

Burns provide a suitable site for bacterial multiplication. When this has taken place, the burn is a richer and more persistent source of infection than the surgical wound because a larger area of tissue is exposed for a longer time. The clinical consequences of infection in burns may be very serious; a large proportion of the mortality in burned patients who have survived the initial trauma and shock has been due to infection. Multiple-antibiotic resistance in the infecting agents has for many years presented difficulties in the treatment of burned patients.

S. aureus and *P. aeruginosa* are the most common isolates in most burns units, followed by various enterobacteria and other gram-negative bacilli, such as *Acinetobacter* species *S. pyogenes*, once a cause of serious trouble in burns units (Cruickshank 1935), now colonizes comparatively few patients (Lawrence 1985). Nevertheless, *S. pyogenes* is important both in episodes of acute infection and in causing the failure of skin grafts. As in other hospital areas, the emergence of resistant gram-positive bacteria, including multiresistant enterococci and *S. aureus*, has been seen in burns units (Phillips et al. 1992; Donati et al. 1993).

As in many other sites, colonization without invasion is far more common than invasive infection, but the frequency and clinical severity of septicemic infections caused by most of the potential pathogens, particularly by *P. aeruginosa*, encourage regular monitoring by the culture of swabs from burns. A greater understanding of the relationship between the bacteria present and the host tissues can be obtained by examining punch biopsies (Pruitt and McManus 1992). Septicemia may follow surgical intervention; this may be prevented by systemic antibiotic prophylaxis, as is applied in most burns units (Papini et al. 1995).

Bacteria reach burns mainly by indirect contact. Airborne infection has been demonstrated on occasions and is more important for *S. aureus* than for gram-negative bacilli. Occasionally mattresses, baths, and hydrotherapy pools have been incriminated (Ayliffe and Lilly 1985; Tredget et al. 1992). In some studies, standard infection control measures, including controlled ventilation, have

not been shown to greatly influence the colonization of burns by bacteria, including antibiotic-resistant strains (Ayliffe and Lawrence 1985), although it has been suggested that single-room isolation reduces the problem of infection by gram-negative bacilli (Pruitt and McManus 1992; McManus et al. 1994). It is clear that improvements in the surgical management of burns, including judicious use of excision and timely closure, and the use of topical chemotherapy with agents such as silver sulfadiazine, have considerably reduced infection in many units.

Infection associated with extensive skin disease and major plastic surgery presents problems that are to some extent similar to those with burns.

THE EYE

The cornea is considered sterile, but the conjunctiva is colonized by a number of different bacterial genera, predominantly coagulase-negative staphylococci, *Corynebacterium* spp., and *Propionibacterium* spp. (Willcox and Stapleton 1996). Resident flora and other physiological factors, e.g. immunoglobulins in tears, prevent colonization by more pathogenic bacteria. The use of contact lenses is associated with colonization by *S. aureus* and gram-negative bacilli. Postoperative infections of the eye are uncommon, but may result in considerable disability and are difficult to treat. An etiological diagnosis is usually possible only if material for culture is obtained from within the eye. Preoperative monitoring of the eye flora is not usually indicated unless there are clinical signs of infection on the surface of the eye. It may, however, be useful to examine swabs from the skin round the eye and from the anterior nares for *S. aureus*, particularly if the patient has a skin disease, so that extra care can be taken with skin preparation if *S. aureus* are recovered.

Endophthalmitis is an infection of the intraocular structures and sclera and usually occurs following intraocular surgery or penetrating trauma, or as part of a generalized infection, such as systemic candidiasis. Infection after cataract surgery has declined in recent years and is now less than 0.1 percent (Das and Symonds 1996). Gram-positive bacteria predominate; a survey of 64 centers in France identified *S. epidermidis* as the most important pathogen (Fisch et al. 1991). A similar finding was reported in a UK study (Hassan et al. 1992). The somewhat surprising emergence of *S. epidermidis*, traditionally associated with the immunocompromised host and with foreign body-related infection, may represent improved diagnostic techniques. Various fungi cause occasional infections, presumably by the air-borne route. Many donor corneas yield microbial growth, but few patients develop infection with organisms similar to those found on the cornea. The importance of making a microbiological diagnosis should not be underestimated as management is difficult, partly because antimicrobial agents penetrate poorly into the anterior and vitreous chambers of the eye.

Keratoconjunctivitis is usually community-acquired and is most commonly caused by *S. aureus*, but *P. aeruginosa* may be associated with corneal ulceration in hospital and *Haemophilus influenzae* with infection in children (Limberg 1991). Epidemics caused by adenovirus type 8 are essentially an infection of hospitals and clinics, possibly spread by instruments such as tonometers, but also by the hands of staff, and otherwise only by close, person-to-person, family, or other contact. Scrupulous hand-washing, decontamination of equipment – by chemical means if it is heat-sensitive – and use of individual containers for ophthalmic preparations, will terminate an outbreak (Barnard et al. 1973). Other adenoviruses may also be transmitted in this way (Tullo and Higgins 1980). Hospital-acquired bacterial conjunctivitis is common only in neonatal units, where minor conjuctival infection with *S. aureus* is more common than neonatal ophthalmitis due to *Chlamydia trachomatis* or *Neisseria gonorrhoeae* acquired from the birth canal.

THE PERITONEUM

Peritonitis is one of the classical associations of surgery: a breach in the bowel wall is followed by invasion of the peritoneum by a mixture of enteric bacteria, possibly acting synergistically. Advances in the classification and pathogenesis of sepsis, including peritonitis (Bone 1991), have raised the possibility of new therapeutic strategies. Patients who require intensive care have a mortality of 63 percent; amongst the poor prognostic features are advancing age, the presence of septic shock, failure to clear the source of sepsis and an upper gastrointestinal source (McLauchlan et al. 1995).

With the development of peritoneal dialysis for renal failure, and particularly of continuous ambulatory peritoneal dialysis (CAPD) for its chronic management, a very different form of peritonitis has become common. Short-term peritoneal dialysis, in patients unsuitable for hemodialysis, is often complicated by peritoneal infection caused by gram-negative bacilli. CAPD peritonitis has a different spectrum of etiological agents and coagulase-negative staphylococci, particularly *S. epidermidis*, account for about half the cases (Spencer 1988; von Graevenitz and Amsterdam 1992). Factors important in the pathogenesis of such infections include the size of the bacterial inoculum, adaptation of bacteria to the local microenvironment, especially adherence to plastics, and diminished phagocytic activity in peritoneal fluid (von Graevenitz and Amsterdam 1992). Many factors have been invoked to explain the success of *S. epidermidis* as a pathogen in CAPD and there is increasing interest in bacterial phenotypic variation in peritoneal dialysis fluid. Differences in iron-regulating proteins, cell wall and cytoplasmic membrane protein profiles have

been demonstrated when strains are grown in peritoneal dialysis fluid rather than in nutrient broth (Wilcox et al. 1991). Other gram-positive cocci and other skin bacteria, enterobacteria, and the Pseudomonadaceae, account for most other cases. Anaerobic bacteria and fungi are less common. Special cultural methods may be needed to reveal the presence of intracellular organisms or organisms that for other reasons are difficult to grow. The major cause of infection is a lapse in technique by the patient or attendants in changing the containers of dialysis fluid. Patient motivation is therefore important in minimizing infection. A number of specific measures, including good surgical technique at the time of catheter insertion and the use of occlusive dressings (Ludlam et al. 1989), have been advocated to reduce infections, especially those due to *S. aureus*, which are difficult to treat. The infecting organisms may reach the peritoneum from the abdominal skin, with or without overt 'tunnel' infection. Other sources are the bowel – mixed infection with multiple enterobacteria or anaerobes, which suggests perforation (Editorial 1982) – the bloodstream, and the female genital tract. Episodes of infection may be treated with antibiotics without removal of the catheter (Working Party of the British Society for Antimicrobial Chemotherapy 1987), but repeated infection in association with tunnel infection and certain pathogens, such as *S. aureus*, *P. aeruginosa*, and fungi, make success less likely.

Prevention of postoperative wound infection

The measures taken in the operating suite to prevent postoperative wound infection have been classified into the following categories (Medical Research Council Subcommittee of the Committee on Hospital Infection 1968):

- established
- provisionally established
- rational methods
- rituals.

Since then, some time-honored practices have been discarded. Nevertheless, there is still a lack of firm evidence to justify a number of the 'rational' measures that are taken to protect the patient. This chapter gives only an outline that applies to operations generally and not to special situations, such as orthopedic implants. Many measures can be deduced from the risk factors already outlined. Thus, an operation carried out expeditiously with meticulous technique will have a reduced risk of infection.

In elective surgery, patients may be investigated preoperatively at assessment clinics, to avoid a long period in hospital before the operation. Underlying disease and existing infections should, as far as possible, be controlled. Nasal carriage of *S. aureus* is associated with postoperative infection and a review of recent studies found that the relative risk of infection in *S. aureus* carriers was 7.1 (95 percent, CI 4.6–11.0). von Eiff et al. (2001) recently showed that the great majority of cases of *S. aureus* bacteremia appear to be of endogenous origin. Of 14 patients who had nasal colonization with *S. aureus* and subsequent *S. aureus* bacteremia, 12 (86 percent) had nasal isolates indistinguishable from those recovered from blood cultures 1 day to 14 months later. Preoperative monitoring for pathogens, such as *S. aureus* or *S. pyogenes*, is commonly carried out before high-risk operations, such as those on the heart, but its usefulness has not been established (Ridgway et al. 1990). Recent studies have highlighted the potential use of nasal mupirocin prophylaxis to prevent SSIs, notably in patients undergoing cardiothoracic surgery (Kluytmans et al. 1997; Cimochowski et al. 2001), and possibly upper gastrointestinal surgery (Yano et al. 2000). However, a placebo-controlled study of nasal mupirocin prophylaxis in patients undergoing orthopedic prosthetic implant surgery found a reduction in *S. aureus* nasal carriage, but no effect on SSI incidence (Kalmeijer et al. 1999). Wilcox et al. (2003b) observed a marked decrease in the incidence of MRSA SSIs, from 23/1 000 operations to 3.3–4.0/1 000 operations, following the introduction of empirical perioperative prophylaxis based on nasal mupirocin and also including topical skin disinfection with triclosan. A recent placebo-controlled, randomized study of perioperative prophylaxis with nasal mupirocin failed to demonstrate a reduction in the overall rate of *S. aureus* SSIs, but a significant reduction in *S. aureus* nosocomial infections was seen in patients given mupirocin who were *S. aureus* nasal carriers (Perl et al. 2002).

The value of total-body antiseptic treatment, as with chlorhexidine detergent, is also controversial (Lynch et al. 1992). Antiseptic skin preparation in the operating theater, with alcoholic solutions of chlorhexidine or an iodophor, applied with some friction, is very effective (Davies et al. 1978). There is no evidence that longer applications are beneficial, except perhaps when clostridial spores must be removed (Lowbury et al. 1964). The use of specially impermeable material, whether woven or adhesive plastic, or of disinfectant-impregnated material, for the drapes around the operative field, is not supported by firm evidence (Lewis et al. 1984). Hair should, whenever possible, be clipped or removed by depilatory methods rather than by shaving.

A full discussion of the prophylactic use of antibiotics to prevent surgical wound infection is beyond the scope of this chapter (Leaper 1994; Sheridan et al. 1994). The usefulness of very short perioperative courses of aptly chosen antibiotics has been decisively established for operations with a high incidence of endogenous infection. Examples are colonic surgery, upper gastrointestinal surgery in conditions of bacterial overgrowth, some operations on the biliary system, and vaginal hysterectomy. Prophylactic antibiotics are also justified

for operations with a lower incidence of infection when the consequences would be very serious as, for example, for the insertion of orthopedic and other implants, cardiac surgery, and dental procedures in those at risk of endocarditis. The value of such prophylaxis has been established only for joint replacement surgery (Lidwell et al. 1982). Prophylaxis with cephalosporins, which is associated with a very low complication rate, is beneficial for patients undergoing other categories of 'clean' operations. The degree to which this extension of prophylaxis should be encouraged has been debated (Page et al. 1993; Lewis et al. 1995).

The measures customarily taken to establish a safe environment in the operating theater have been reviewed by Hambraeus and Laurell (1980), and the principles of theater suite design by Humphreys (1993). Briefly, ventilation is provided with clean filtered air sufficient in volume to suppress as far as possible the airborne particles produced by human movement, in particular in the region of the operative field. This is far less important in other areas, such as near the floor. The effectiveness of conventional ventilation systems is readily upset by unsatisfactory building design or by undisciplined human activity. Transfer of bacteria from the operating team to the wound is somewhat reduced by the exclusion of staff with clinical infection and by the wearing of clean, frequently changed clothes and an operation gown. However, if the latter is made of conventional fabric, its effectiveness as a barrier to skin microorganisms is small. Masks to filter or deflect upper respiratory particles and caps to reduce contamination from the hair should be worn by the immediate surgical team (Dineen and Drusin 1973). Hand-washing with chlorhexidine or iodophor solutions before donning gloves should be careful and thorough, but not necessarily prolonged (Larson 1984). Gloves are very commonly perforated during operations, and this may be associated with an increased risk of infection for the patient (e.g. Cruse and Foord 1973) and of blood-borne infection for the surgeon (Palmer and Rickett 1992).

Effective sterilization of surgical instruments, with a large margin of safety (Russell et al. 1992), is now universal, but it was not always so (Howie 1988). Heat-sensitive equipment may give rise to difficulties and the need for a rapid turnover may force the use of less stringent decontamination methods, even for instruments that penetrate tissue.

THE URINARY TRACT

Most healthcare-associated infections of the urinary tract are associated with urethral catheterization. Even the single passage of a catheter is associated with a definite, though usually low, infection risk (Kunin 1987). The need for catheterizations should be carefully assessed in each patient and alternatives considered such as self in–out catheterization in females and external catheter in males (Pearman 1998). With an indwelling catheter eventual colonization of the bladder is almost inevitable, but it may be considerably delayed by the system of closed drainage, first introduced by Dukes (1929) and developed by Gillespie and others (Gillespie 1956; Miller et al. 1958). The closed system is not interrupted for the taking of urine specimens, and irrigation after operation can be provided without opening the system (Miller et al. 1958).

In spite of closed drainage, bladder colonization nevertheless occurs. It may reach about 10 percent of patients per day (Kunin and McCormack 1966). The route of infection is not via the lumen of the catheter, but between the catheter and the urethral wall. Early infection is by local commensals, such as *E. coli*, coagulase-negative staphylococci and enterococci. Later, more resistant hospital-associated gram-negative bacilli such as *Klebsiella*, *Proteus*, *Serratia*, *Pseudomonas*, and *Providencia* spp. may invade, particularly under the selective influence of antibiotics. Careful passage of the catheter by skilled staff under conditions of strict asepsis and scrupulous catheter care thereafter are also important (Falkiner 1993; Pearman 1998). Much attention has been given to the use of disinfectants in preventing infection (Stickler and Chawla 1987). Chlorhexidine may usefully be included in the local anesthetic applied to the urethra, or used as an initial 'flush' after the catheter has been passed. Chlorhexidine irrigations reduce the colonization rate (Kirk et al. 1979), but the prolonged application of chlorhexidine may damage the bladder mucosa. Little evidence supports the use of disinfectants in established infection (Davies et al. 1987) or in the long-term protection of indwelling catheters (Stickler and Chawla 1987). Periurethral disinfection, beyond simple toilet, has not been shown to be beneficial (Burke et al. 1983), nor has the inclusion of disinfectants in the catheter-drainage bag (Gillespie et al. 1983). They may, however, prevent the spread of resistant strains of bacteria (Noy et al. 1982), presumably via the hands of those who empty the drainage bags. Such spread is better avoided by an improved nursing regimen. The bacteria that cause urinary tract infection are variably sensitive to the antiseptics that can be used in contact with tissues (Hammond et al. 1987), especially when the bacteria are present in biofilm on the surface of the bladder (Stickler et al. 1987). It has been suggested, but not proved, that extensive use of chlorhexidine may account for the spread of relatively resistant gram-negative bacilli in certain hospital departments (Dance et al. 1987).

The presence of bladder bacteriuria is benign and symptomless in many patients, but its subtle long-term effects have not been studied, and upper tract infection may itself be silent. There is no doubt that urinary infection prolongs hospital stay and worsens the prognosis (Jepsen et al. 1982). The prevalence of hospital-acquired urinary infection was lower in a recent study in the UK and Ireland when compared with 1980 (Emmerson et al.

1996) and this may reflect improved practice and catheter care. A proportion of patients develop pyelonephritis and other complications, such as epididymo-orchitis. Urinary tract infection is one of the most common causes of bacteremia in hospital. It frequently follows urethral procedures (catheter passing, catheter removal, cystoscopy, transurethral prostatectomy) in patients with colonized urine and may lead to septicemia and even death. Thus, perioperative bacteremia was demonstrated by Murphy et al. (1984) in 60 percent of patients with infected urine undergoing urethral operations and, in similar patients in the same hospital, Cafferkey et al. (1982) recorded 6 percent of clinical septicemia arising in these circumstances. Of 31 patients with postoperative septicemia investigated by Cafferkey et al. (1980), eight suffered severe shock and four died.

The risk of septicemia may be avoided by the preoperative or perioperative administration of suitable antibiotics to patients with colonized urine, especially those due to undergo urological procedures, such as prostatectomy and bladder resection (Amin 1992). There may also be a case for the administration of antibiotics to patients with sterile urine who have had indwelling catheters for some time, because damage to the urethra may encourage significant passage of bacteria into the bloodstream. In a study of prophylactic ciprofloxacin in carefully selected patients undergoing vaginal repairs, there was a reduction in symptomatic and asymptomatic bacteriuria compared with controls (van der Wall et al. 1992), but the long-term implications in terms of cost and the emergence of resistance should be carefully considered before this can be recommended for routine use. Antibiotic prophylaxis has no place in other situations and treatment of urinary infection in the catheterized should be limited to dealing with clinical illness (Gillespie 1986).

Silver impregnated antimicrobial urinary catheters have been proposed as a way of reducing associated infection. However, a recent review of the studies in this area concluded that general poor study design means that claims of efficacy cannot be accepted with confidence (Niel-Weise et al. 2002). The one high quality study identified by the reviewers did not demonstrate superior efficacy of silver coated versus standard catheters (Thibon et al. 2000). Alternatives to bladder catheterization that may be feasible are suprapubic catheterization, which has in some studies been accompanied by less bladder infection than urethral catheterization, and intermittent catheterization, including self-catheterization, which may be successful in patients with neurogenic bladder disorders (Pearman 1984). When catheterization appears necessary to achieve dryness in incontinent patients, external, condom drainage may be used, or effective incontinence pads may be substituted with success (Nordqvist et al. 1984), with other measures to encourage regular micturition.

THE RESPIRATORY TRACT

Infections of the respiratory tract represent a significant proportion of all healthcare-associated infections. In the recent prevalence study, respiratory tract infection accounted for approximately 25 percent of hospital infection and was as common as that of the urinary tract (Emmerson et al. 1996). Mortality is between 25 and 35 percent, but depends on the patient population, the diagnostic approach, and whether the mortality rate is crude or takes into account other factors that contribute to a fatal outcome.

A firm diagnosis of pneumonia is not easy to make and it is even more difficult to establish its microbial etiology. Sputum may be contaminated with bacteria from the upper respiratory tract and cultures may give misleading results. The use of bronchoscopic or alternative nonbronchoscopic approaches in the intensive care unit to obtain representative samples from the lower respiratory tract with quantitation of the organisms isolated is increasingly emphasized (Baselski and Wunderink 1994). The results from brush and lavage specimens correlate with histology even in patients on antimicrobial therapy (Chastre et al. 1995). Bronchoscopy may not, however, always be possible and it requires experience. Nonbronchoscopic lavage, with a catheter inserted through an endotracheal or tracheostomy tube, combined with semiquantitation, can be incorporated into routine practice relatively easily (Humphreys et al. 1996).

The etiology of pneumonia varies according to the patient population, diagnostic approaches used, local practices and procedures, and geographical location. Gram-negative bacilli, especially *P. aeruginosa* and *S. aureus*, account for the majority of cases (Dal Nogare 1994), but *S. pneumoniae* may be responsible for up to 20 percent of nosocomial pneumonia (Berk and Verghese 1989). Resistant enterobacteria, e.g. *Klebsiella*, *Serratia*, and *Enterobacter* spp., *P. aeruginosa*, *Acinetobacter* spp., and methicillin-resistant *S. aureus* (MRSA) pose particular difficulties, especially in high dependency areas of hospitals, because of fewer treatment options and the propensity of some of these organisms to spread. For a more general account of the etiology of pneumonia, see Bacterial infections of the lower respiratory tract.

The general conditions that predispose to pneumonia are similar to those for wound infection: obesity, advanced age, and some underlying diseases. The most important factors, however, are respiratory intubation and tracheostomy. Tracheal intubation removes an important barrier to invasion of the lower respiratory tract, and even brief intubation allows oropharyngeal bacteria to pass into the trachea (Nair et al. 1986). High gastric pH, often induced deliberately to prevent stress ulcers in patients in intensive therapy, encourages overgrowth of potential pathogens that may then pass into the respiratory tract of the intubated patient. It has been

suggested that fibronectin is a barrier to colonization by *P. aeruginosa* and that it is breached by the increased protease content of saliva during illness (Woods et al. 1981). Molecular approaches to understanding the pathogenesis of pneumonia reveal a complex interaction between host factors, such as cytokine release, phagocytosis and lymphocyte activation, and virulence determinants of the pathogen, including lipopolysaccharide and bacterial enzymes, such as elastase. For patients in the ICU this is further complicated by other conditions such as the adult respiratory distress syndrome (Meduri and Estes 1995; Harmanci et al. 2002).

Sporadic cases and outbreaks of pneumonia may be associated with contamination of respiratory equipment, such as anesthetic machines, ventilators, humidifiers, nebulizers, resuscitation equipment, and suction catheters (Hovig 1981). Better design of equipment, improved decontamination methods and the use of filters may lessen the importance of these sources. Breathing circuit change intervals of 7 and 30 days have been found to be associated with lower risks of ventilator-associated pneumonia than a 2-day interval, and the difference yielded marked reductions in morbidity and labor and acquisition costs (Fink et al. 1998). In general, there are two main targets within ventilators where filters may be used: firstly, to protect against contamination of the internal parts of the ventilator itself by microorganisms in respiratory secretions (internal filter), and second, to prevent dissemination of microorganisms in respiratory secretions into the external environment, i.e. filter situated on the exhalation limb of the tubing (external filter). To change filters between operations is expensive and there is no conclusive evidence that this will reduce crossinfection. Also, there are no data to prove whether contamination of ventilator circuits is implicated in the etiology of pneumonia. Both pleated hydrophobic and electrostatic filters can be shown to be permeable to bacteria and viruses in vitro. In short, there are no evidence-based guidelines that support the use of filters, and no recommendations about filter type can be made on infection control grounds alone (Das and Fraise 1997; Thomachot et al. 1999). The importance of maintaining respiratory equipment in good order and of cleaning/decontamination has become better recognized after outbreaks with highly resistant bacteria such as *Acinetobacter* spp. (Vandenbrouke-Grauls et al. 1988; Getchell-White et al. 1989; Hanberger et al. 2001). The causative bacteria may also be spread by indirect hand contact, and from there colonize the oropharynx before causing infection. Antibiotic administration encourages colonization by more resistant gram-negative bacilli.

The above account indicates some of the appropriate preventive measures. These include hand-washing, early removal of nasogastric and endotracheal tubes, maintenance of gastric acidity, and elevating the head of the bed to 30° (Dal Nogare 1994). This conventional approach to prevention, with an assessment of which practices have been validated, has been expanded upon elsewhere (Tablan et al. 1994). Nebulized antibiotics for prophylaxis may at first give satisfactory results, but later lead to problems with resistant bacteria (Feeley et al. 1975). Selective decontamination of the digestive tract (SDD), incorporating the use of topical antimicrobial agents to preserve colonization resistance and the protective effect of normal flora (van der Waaij et al. 1990), has been advocated to reduce ICU-acquired infection, including pneumonia (van Saene et al. 1992). The results have, however, been conflicting and have led some to advise against its routine introduction as a preventive measure particularly because of the potential for selection of multiresistant bacteria, such as MRSA (Hamer and Barza 1993; Hurley 1995). In a recent re-evaluation of SDD, de Jonge et al. (2003) carried out a prospective, controlled, randomized, unblinded study on 934 patients admitted to a surgical and medical ICU who were randomly assigned oral and enteral polymyxin E, tobramycin, and amphotericin B combined with an initial 4-day course of intravenous cefotaxime or standard treatment. They found that SDD use was associated with a significant reduction in ICU and hospital mortality and colonization with resistant gram-negative aerobic bacteria. Importantly, the authors note that their findings may not be applicable to units with endemic vancomycin-resistant enterococci or MRSA.

Though legionellosis (see section on Legionnaires' disease) is predominantly community-acquired, nosocomial cases accounted for 15 percent of cases in England and Wales between 1980 and 1992 (Joseph et al. 1994). Hospital-acquired infection should prompt a search for its source and for active preventive measures, including chemical disinfection, control of circulating water temperature, and surveillance (Hoge and Breiman 1991; Joseph et al. 1994). In young children, RSV and at times other viruses, such as enteroviruses, are significant causes of hospital-acquired respiratory infection. Without special precautions, nosocomial acquisition of RSV can be high, and a combination of cohort nursing and the use of gloves and plastic aprons significantly reduces spread (Madge et al. 1992).

Infection associated with indwelling medical devices

The increasing practice of inserting devices, usually made of various plastics, into the body of patients, and the selection pressure of antibiotics, have shifted the spectrum of significant microorganisms commonly encountered in diagnostic laboratories.

Almost every type of indwelling device may become colonized. The predominant bacteria are coagulase-negative staphylococci, particularly *S. epidermidis*. Othercolonizing organisms are *S. aureus* and other skin organisms such as corynebacteria, including

Corynebacterium jeikeium (see Human microbiota; 32, *Staphylococcus*; and 39, Corynebacteria and rare coryneforms), propionibacteria, streptococci, many of low-grade pathogenicity, various gram-negative aerobic bacilli, yeasts, and filamentous fungi. *S. epidermidis* strains associated with such infections appear to produce greater quantities of extracellular slime than do strains from other sources. The slime consolidates bacteria adherent to the polymer surface and interferes with the body's response to infection (Gristina et al. 1993; Jansen and Peters 1993).

INTRAVASCULAR CANNULAE

Plastic cannulae allow infusions to be given via the same vessel for much longer than do steel needles, but they are also associated with bacterial colonization and resulting infection (Maki et al. 1973). At first it was recommended that the site of infusion should be changed frequently, but with better materials, better cannula design, and careful placement, cannulae such as the Hickman and Broviac catheters may now be left in situ for long periods, albeit with a low but significant infection rate (Darbyshire et al. 1985).

The colonization of intravascular cannulae may remain silent or be associated with a simple fever. However, if it is due to more pathogenic organisms, especially *S. aureus*, it may result in septicemia or disseminated infection. Even with *S. epidermidis*, metastatic infection, particularly of other prosthetic material, endocarditis or even immune-complex nephritis, such as that associated with cerebrospinal fluid shunts, may occur. The rate of colonization of intravascular cannulae is very variable, depending on the site of cannulation, the fluid infused, the plastic used in the cannula, the design of the cannula, and the care to achieve asepsis at insertion. Investigations up to 1973 have been reviewed (Maki et al. 1973). The use of lower limb veins is associated with an increased incidence of infection, as is umbilical cannulation in babies. Subclavian catheters are associated with lower infection rates than those placed in the internal jugular vein. Solutions for total parenteral nutrition readily support microbial growth and, alone or in admixture with circulating plasma near the cannula, they encourage colonization and a high risk of catheter-related bloodstream infection (CRBSI). The use of the same line for infusion of blood products and clear fluids, or its use for pressure monitoring and the taking of blood specimens, also increases the colonization rate.

The cuff of the implanted Hickman or Broviac catheter may prevent infection from tracking between catheter and tissues. The importance for cannula colonization of a side port closely related to the cannula is the subject of controversy. Reports of side port colonization are common, but there seems to be no significant difference in the rate of CRBSI between cannulae with and without side ports (Cheesbrough et al. 1984). Careful adherence to instructions for the setting up and care of the infusion reduces the rate of colonization and subsequent infection. The source of colonizing organisms is usually the skin of the patient. Several studies have shown a correlation between microorganisms on the skin and those recovered from the catheter after removal (e.g. Fuchs 1971). For this reason, careful antiseptic preparation of the skin before cannula insertion, preferably with alcoholic chlorhexidene, toilet of the area, and careful choice of dressings to prevent skin maceration is advocated. The application of antibiotic or antiseptic preparations to the entry site once the catheter is in situ is not recommended and may be associated with candidal overgrowth (Norden 1969). In some circumstances, as with Hickman and Broviac catheters with their cuff and protective tunnel, and other central lines, but also at times with simple peripheral cannulae, contamination of the connections of the cannula with the administration set and other reservoirs appears to be of importance (Sitges-Serra et al. 1983; Cheesbrough et al. 1984; Weightman et al. 1988). Care with manipulation of the apparatus may be important in cannula colonization, as it is in contamination of fluid in the rest of the system. Occasionally, cannulae are infected by the hematogenous route (Maki et al. 1973). Preventive measures and their success, depends on the postulated entry route for microorganisms (Weightman et al. 1988; Parras et al. 1994). The route of infection may determine the preponderant site of colonization, whether the microorganisms are on the outside of the cannula or in the lumen, and thus the best means of culturing the cannula tip after its removal. The technique devised by Maki and colleagues (1977) for peripheral cannulae may not be as suitable for central lines. This approach requires line sacrifice and it is known that the majority of central venous catheters removed on suspicion of CRBSI are ultimately shown not to be infected. Catheter colonization may be detected in lines already being used for the taking of blood, by comparing the microbial concentration in a specimen of blood taken through the cannula with one from a distant vein; a simple pour-plate method will suffice (Weightman et al. 1988). An even simpler approach is to compare the time taken for central versus peripheral blood cultures to become positive with the same microorganism. If the time difference is at least 2 h (in favor of the central blood cultures) then this is highly predictive of CRBSI (Blot et al. 1999; Seifert et al. 2003). Other new approaches to the diagnosis of CRBSI which do not rely on catheter removal include the acridine orange leukocyte centrifugation test and the endoluminal brush (Kite et al. 1999). Luminal colonization of intravascular catheters is probably almost universal, particularly as dwell time increases, and thus it is CRBSI which is the clinically important endpoint and not catheter colonization.

Minocycline-rifampin (MR) and chlorhexidene silver sulfadiazine (CSS) containing catheters have both been

shown to reduce the incidence of CRBSI compared with uncoated/impregnated central venous catheters (CVC). A study comparing MR and CSS catheters found significant differences in favor of the former in terms of both prevention of catheter colonization (22.8 versus 7.9 percent, $p < 0.001$) and CRBSI (3.4 versus 0.3 percent, $p < 0.002$) (Darouiche et al. 1999). The enhanced efficacy of the MR catheters is probably due to the fact that these have antimicrobial coatings on both the outer and inner surfaces, whereas only the outer surfaces of CVCs were coated with CSS. Meta-analyses of studies on the efficacy of antimicrobial impregnated CVCs have reached differing conclusions. A recent review of 11 randomized studies identified several methodological flaws, including inconsistent definitions of CRBSI, failure to account for confounding variables, suboptimal statistical and epidemiological methods, and rare use of clinically relevant endpoints (McConnell et al. 2003). This review also failed to demonstrate any significant clinical benefit associated with the use of antimicrobial-impregnated CVCs for the purpose of reducing CRBSI or improving patient outcomes.

INTRAVASCULAR GRAFTS

A similar spectrum of microorganisms is seen in infected cardiac valve prostheses and patches of polymers, and grafts in vessels (Liekweg and Greenfield 1977). This may be influenced by the antibiotic prophylaxis in use, because this may alter the flora of patients and hospital staff (Archer and Armstrong 1983). A multicenter randomized controlled trial of rifampicin-bonded Dacron grafts in aorto-femoral surgery demonstrated a reduction in total early wound and graft infection rates. However, results after 2 years showed a small, nonsignificant reduction in graft infection (1.7 percent in study group, 2.3 percent in control group) (Pratesi et al. 2001).

Endocarditis on prosthetic valves may be caused by microorganisms implanted at the time of operation or soon afterwards (Marples et al. 1978) when many intravascular lines are in situ and the graft has not yet become endothelialized. It may follow sternal-wound infection (Braimbridge and Eykyn 1987) or it may arise later from hematogenous seeding, as happens with diseased natural valves. This latter gives a microbial spectrum close to that seen in infections of natural valves (Heimberger and Duma 1989). Though the incidence of early prosthetic valve endocarditis is now less than 1 percent, the mortality is high. Perioperative infection may manifest after a delay and the various types of endocarditis cannot be separated by an arbitrary time after the operation (Braimbridge and Eykyn 1987; Freeman 1995). An incidence in the first year of 1.4–3.0 percent has been calculated (Karchmer 1991). Reliance should be placed on general measures to prevent perioperative infection and on antibiotic prophylaxis at critical times when bacteremia is likely (Karchmer 1991).

JOINT PROSTHESES

Infection of total joint replacements is a very serious and costly complication and a variety of preventative measures that contribute to minimizing this have recently been well reviewed (An and Friedman 1996; Gosden et al. 1998). As techniques have improved, the incidence has fallen from approximately 12 percent to less than 2 percent for hip replacements, and from approximately 15 percent to less than 4 percent for knee replacements. The effects of ultraclean air and of prophylactic antibiotics on the incidence of infection have been discussed above. In addition to the use of systemic antibiotics, incorporation of antibiotics in the bone cement is now standard practice for revision operations and is used by some in primary operations. Gentamicin may be used, or alternatively a heat-stable antibiotic can be chosen to correspond to known infecting strains (Strachan 1993). The spectrum of infecting bacteria (An and Friedman 1996) is dominated by *S. aureus* and coagulase-negative staphylococci, with smaller numbers of enterobacteria and other gram-negative bacilli. The prognosis of infections with gram-negative organisms is worse after reoperation than infections with gram-positive organisms (Buchholz et al. 1981). Fungal infection is rare. Some recent series have shown increased numbers of streptococci (especially of enterococci) and of anaerobic cocci. Infection of a joint prosthesis almost always requires its removal, with replacement as a one- or two-stage procedure, under local and general antibiotic cover. Occasionally, with a low-grade pathogen sensitive to oral antibiotics infecting a stable joint, medical treatment may succeed, but this form of management should rarely be undertaken as it is beset with complications.

CEREBROSPINAL FLUID SHUNTS

In ventriculoatrial or ventriculoperitoneal shunts, infection may give rise to systemic illness from bacteremia, to ventriculitis or to shunt blockage. *S. epidermidis* is by far the most common infecting agent (Ersahin et al. 1994). As in the case of cannula infections, there may be a long lag between colonization and clinical illness. Most infecting organisms are probably implanted in the tissues at the time of operation (Pople et al. 1992), but other routes may occasionally be followed (Price 1984; Ronan et al. 1995). These infections are managed by a combination of surgical management, including shunt removal and chemotherapy (Bayston 1985; Walters 1992). There is no consensus about the benefits of prophylactic antibiotic use in shunt insertion (Brown 1993), but a meta-analysis suggests a statistically significant advantage (Haines and Walters 1994).

Bacteremia

Hospital-acquired bloodstream infections constitute a serious health problem and are associated with high

morbidity and mortality which results in increased healthcare costs. Each year, as many as 35 million patients are admitted to hospital in the United States. Of these, at least 2.5 million will develop a hospital-acquired infection with almost 250 000 attributable to bloodstream infections (Correa and Pittet 2000). Extensive studies in the USA have reported hospital-acquired bacteremia in 0.2–0.4 percent of hospital admissions. These account for about half the total bacteremias observed. Much higher rates have been reported for tertiary referral centers than for general hospitals (Bryan et al. 1986).

The microorganisms that cause hospital-acquired bacteremia and the circumstances in which they do this are fully discussed in Bloodstream infection and endocarditis. Briefly, the infecting organism may arise from a focus of infection in another system (secondary bacteremia) or no source may be identified clinically or after additional investigations, such as ultrasound (primary bacteremia). The organisms isolated and their resistance to antibiotics to a large extent reflect those prevailing in the hospital. In a study conducted in a UK tertiary referral center, *E. coli* was the most common cause and accounted for 27 percent of cases, followed by other enterobacteria and *S. aureus* (11 percent); polymicrobial bacteremia was responsible for 7 percent of cases (Ispahani et al. 1987). Between 1980 and 1992, the crude bacteremia rate in a US center increased from 6.7 to 18.4 per 1 000 patient discharges and a considerable proportion of this increase was due to gram-positive cocci (Pittet and Wenzel 1995). An increasing proportion of bloodstream infections due to coagulase-negative staphylococci, enterococci, and *Candida* spp. has been documented in recent years. This may reflect the changing hospital patient population and the increasing use of invasive devices. Central venous lines and blood transfusion were independent risk factors for hospital-acquired bacteremia in Australia (Duggan et al. 1993). Coagulase-negative staphylococci are especially important in the pediatric age group and were responsible for 43 percent of bacteremia in Great Ormond Street Hospital, London, UK (Holzel and de Saxe 1992). They were the third most common bloodstream pathogen after group B streptococci and *S. aureus* in a Swedish neonatal unit (Faxelius and Ringertz 1987).

The increasing proportion of bacteremia due to gram-positive bacteria is seen in immunocompromised patients who may require an in situ intravascular device for a long period and for whom repeated courses of broad spectrum antimicrobial agents are often necessary. Viridans streptococcal bacteremia in neutropenic patients is associated with chemotherapy-induced mucositis as shown by the finding that blood and oral isolates were indistinguishable by ribotyping (Richard et al. 1995). Enterococcal bacteremia, especially that due to *Enterococcus faecium*, is closely linked with the presence of cancer, neutropenia, renal failure, steroids, and recent antibiotics (Noskin et al. 1995a). The risk factors for candidemia are similar, but include recent abdominal surgery (Nielsen et al. 1991). On the other hand, the incidence of bacteremia due to *Bacteroides* spp. has declined in many places since the introduction of effective prophylaxis for operations on the large gut (Young 1982).

Clusters of cases may reflect a hospital outbreak of infection. Thus, nine cases of gram-negative bacteremia involving different organisms and occurring after open heart surgery were traced to an aqueous disinfectant used to clean pressure-monitoring equipment that was assembled before surgery and left open and uncovered overnight in the operating theater (Rudnick et al. 1996). Care must be taken to exclude pseudobacteremia, due to the contamination of blood culture media or their additives, contaminated skin disinfectants, specimen containers or blood-gas analyzers or, in the laboratory, from contaminated apparatus or carriers among the staff (Maki 1980).

Mortality rates depend largely on the age and underlying illnesses of the patients, whether there is a removable or remediable focus of infection, and on the microbial cause. Thus, infection with enterobacteria has a worse prognosis than infection with coagulase-negative staphylococci. Polymicrobial bacteremia also has a poor prognosis. Candidemia is associated with a mortality of 76 percent (Nielsen et al. 1991), which reflects the severity of underlying disease in such patients. In the intensive care patient with severe sepsis and shock, bacteremia is associated with a poor outcome, but the category of organisms appears to be less important (Brun-Buisson et al. 1995). Recent efforts to develop and use monoclonal antibodies directed against the gram-negative bacterial cell wall or cytokine mediators, such as tumor necrosis factor (TNF) in these patients, have been disappointing. A multicenter study in 15 centers revealed that blocking TNF activity with high doses of a competitive inhibitor led to a higher mortality compared with placebo (Fisher et al. 1996). Recently, recombinant activated protein C has been made commercially available for use as adjunctive treatment of severe sepsis accompanied by multiple organ failure. Further work on the pathogenesis of severe sepsis and bacteremia will be necessary before useful and safe immunomodulatory agents can routinely be used in management.

For bacteremia resulting from the contamination of intravenous fluids or additives to these, see the section on Infection from contaminated infusion fluids.

Since April 2001, all English hospital trusts have been obliged to report the number of patients with *S. aureus* bacteremia, along with methicillin/oxacillin sensitivity of the isolate. The current 3-monthly report considers the MRSAs amongst these, and dividing the total by the average daily bed occupancy of each hospital trust produces an index of MRSA bacteremia rates per 1 000 bed-days. The 186 hospital trusts are then ranked

according to their bacteremia rates. At the bottom are a few centers that can congratulate themselves on rates of zero, whilst at the other extreme, high ranking trusts will have to explain MRSA bacteremia rates of up to 0.69 per 1 000 bed-days (Barrett and Spencer 2002).

The wide release of these figures adds to the increasing profile of hospital infections in the English public's awareness, which in turn should be viewed in the context of longstanding media interest in health-service shortcomings. Although the exact relevance of MRSA is debated, collecting MRSA bacteremia data has a certain logic. As a mainly hospital-acquired organism, it can be inferred that MRSA in blood culture suggests that a serious hospital-acquired infection has occurred. However, this does not allow for the unknown but increasing reservoir of common MRSA entering hospitals. In one locality, 17 percent of nursing home residents carried MRSA (Fraise et al. 1997). The higher the rate, by inference, the worse must be the individual hospital trust's problems with hospital-acquired infection – or at least its prevalence of MRSA. Moreover, extracting the numbers from laboratory computers and workload databases is vastly cheaper and quicker than surveillance of infections by clinical documentation. Appropriate surveillance techniques constitute the cornerstone of infection control and make possible the implementation of specific preventive approaches. Furthermore, accurate surveillance may identify risk factors for infection and other adverse hospital-acquired events (Correa and Pittet 2000). Surveillance of hospital-acquired bacteremia may appear straightforward because of the simple definition of the infection. Review of the microbiology laboratory records certainly allows identification of most of the cases. However, clinical sepsis is a recognized entity that may not be associated with positive blood cultures. In 2003, mandatory reporting of glycopeptide-resistant enterococcal bacteremia also began.

HOSPITAL INFECTION AT THE EXTREMES OF LIFE

Geriatric and long-stay facilities

Hospital infection and infection control in aged patients have been somewhat neglected, but their importance is undeniable. Patients older than 65 years account for a disproportionate number of healthcare-associated infections (Smith 1988). The relative frequency of infection at different sites depends, for example, on whether simple colonization of the bladder or of an ulcer is included, but the three most frequently infected systems are the respiratory tract, the urinary tract, and the skin. The last is more common in long-stay units (Darnowski et al. 1991; Jackson et al. 1992) than in acute geriatric units (Hussain et al. 1996).

Particular difficulties may be the declining defenses and multiple underlying chronic diseases in the patients and, in some long-stay hospitals, a lack of facilities, including isolation facilities, low staffing levels, and a comparative lack of access to medical assessment. Patients may be ambulant and have poor hygienic practices, and there may be close contact among patients. This may result in the spread of infections, such as adenovirus conjunctivitis, ringworm, scabies, etc., not seen in acute units. Chronic infections, such as tuberculosis, may spread insidiously. Carriage of MRSA may cause more severe problems when patients are transferred to surgical units or other services with vulnerable patients than in the geriatric unit itself (Combined Working Party of the British Society for Antimicrobial Chemotherapy and the Hospital Infection Society 1995). Infections in nursing homes have been reviewed by Nicolle et al. (1996).

Long-term indwelling urethral catheters are common in the elderly. Most surveys have shown a high prevalence of bacteriuria due to a number of different species. The most common are of *Proteus* or *Providencia* spp. rather than *E. coli* (Standfast et al. 1984; Lee et al. 1992). The clinical significance of most of this bacteriuria may be doubted (Jewes et al. 1988).

Nosocomial pneumonia in the elderly resembles that in younger age groups, in terms of etiology and in risk factors such as tracheal intubation, but it is associated with malnutrition and neuromuscular disease and has a particularly poor prognosis (Craven et al. 1992; Hanson et al. 1992; Woodhead 1994). Infection by respiratory viruses, including influenza A, is common and may cause serious illness and death.

The grave consequences of salmonella infection in aged patients have been reported frequently (e.g. Committee of Inquiry 1986). Clusters of cases of diarrheal disease are common. When an etiological agent is identified it is frequently a Norwalk-like virus (Stevenson et al. 1994; Augustin et al. 1995), but rotavirus outbreaks have also been reported (Holzel and Cubitt 1982).

Over 80 percent of *C. difficile* diarrhea cases in the UK occur in hospitalized patients aged over 65 years. Notably, the frail elderly are more prone to severe *C. difficile* infection (Kyne et al. 1999). Antibiotic use is the prime risk factor, but others linked to severe *C. difficile* diarrhea include functional disability, cognitive impairment, recent endoscopy, and possibly enteral tube feeding. *C. difficile* infection appears to be a marker, but not a cause per se, of increased risk of dying (Wilcox et al. 1996; Kyne et al. 1999, 2002). Further details about *C. difficile* infection can be found below and in *Clostridium perfringens*, *Clostridium difficile*, and other *Clostridium* species.

Neonatal units

Intrauterine and perinatal infections of maternal origin, such as rubella, listeriosis, group B streptococci infection, candidiasis, and infection by HIV will not be

considered here. However, many of these agents may, occasionally, be spread among babies via staff or equipment.

Unlike well-baby nurseries, the prevalence of infection may exceed 20 percent in neonatal intensive therapy units, and on this there may be superimposed outbreaks of infection by particularly virulent and usually antibiotic-resistant strains. The babies in these units, particularly when premature, have undeveloped defenses and lack a normal flora. Ill babies require much handling by staff, and it is therefore difficult to prevent the spread of infection by contact from one to another.

Infection with *S. aureus* was the main concern in the 1950s. Babies in nurseries for the newborn were regularly colonized and some developed septic skin lesions of varying severity that sometimes progressed to fatal generalized infections. The causative staphylococci showed considerable differences in virulence. The problem was ameliorated by standard infection control measures, early discharge of healthy babies from hospital, and prophylactic use of topical hexachlorophane (Alder et al. 1980). A large neonatal outbreak of *S. aureus* identified by routine phage typing required multiple, staged, infection control measures including strict emphasis on hand hygiene, application of topical triclosan solution and hexachlorophane powder, aseptic handling of a skin protectant material, and use of topical mupirocin for staff nasal carriers of the endemic strain and for babies colonized or infected with *S. aureus* (Wilcox et al. 2000b). Withdrawal of hexachlorophane after overdosing had been shown to give rise to neurological damage and was followed by a recrudescence of staphylococcal infections (De Souza et al. 1975; Allen et al. 1994). Control of *S. aureus* in a neonatal unit was maintained when topical umbilical hexachlorophane powder was substituted by 1 percent chlorhexidine powder (Wilcox et al. 2003a). The neonatal unit has often been a focus of infection by MRSA (Reboli et al. 1989; Tam and Yeung 1988). In neonatal intensive therapy units there may occasionally be outbreaks of infection by enterobacteria such as *Klebsiella* spp., *Serratia* spp., and *P. aeruginosa*, sometimes resistant to many antimicrobial agents and with the ability to spread rapidly and cause serious illness (Davies and Bullock 1981; Lewis et al. 1983; Coovadia et al. 1992). Colonization by such bacteria without infection is very common. Routine monitoring of carriage sites contributes little to preventing illness (Jolley 1993), but may help in formulating a rational policy for antibiotic prescribing. Surveillance is better directed towards identifying strains with the potential to spread widely and cause serious illness (Goldmann 1988).

In recent years, *S. epidermidis* or other coagulase-negative staphylococci have been the most frequent cause of infection in many neonatal units (Thompson et al. 1992; Nataro and Corcoran 1994). Such infections are generally more benign than those due to gram-negative aerobic bacilli. The reason for their current prevalence is unknown, but may partly reside in their resistance to commonly used antibiotics and the increased use of intravascular cannulae and respiratory apparatus. Some strains spread within a unit, but there is no evidence of the emergence of particularly virulent or transmissible strains of coagulase-negative staphylococci. Indeed, several different strains may simultaneously infect a single patient (Simpson et al. 1986; Kacica et al. 1994).

Necrotizing enterocolitis has increased in incidence in recent years and has features, such as clustering of cases, that suggest an infective etiology. At times tightening of infection control procedures appeared to abort an outbreak (Willoughby and Pickering 1994). However, attempts to demonstrate a specific microbial cause have been unsuccessful (de Louvois 1986; Gupta et al. 1994).

Outbreaks of virus infection in neonatal units can be serious and difficult to control. Enteroviruses such as echovirus 11 (Nagington et al. 1978), adenovirus 7 (Finn et al. 1988), or adenovirus 8 (Piedra et al. 1992), rotaviruses (Holzel and Cubitt 1982), and RSV (Hall et al. 1979) may be responsible. *Candida* spp. may on occasion cause crossinfection in neonatal units (Finkelstein et al. 1993; Reagan et al. 1995).

THE ROLE OF INDIVIDUAL PATHOGENS

Staphylococcus aureus

This organism has probably always been an important cause of hospital-acquired infection, but it was first seriously investigated in the early years of the antibiotic era (see Williams et al. 1960). Certain strains that could be identified by phage typing (*Staphylococcus*) and were resistant to penicillin, and often also to other antibiotics, became prevalent in hospitals. Some of these multiresistant strains were exceptionally virulent, able to cause severe infections in otherwise healthy persons, and colonized widely. These so-called 'epidemic strains' caused many outbreaks of skin sepsis, most notably in the postnatal period, and were the main cause of postoperative wound infection in the 1950s (Shanson 1981).

During the 1960s, hospital-acquired infection by multiresistant strains became progressively less common (Ayliffe et al. 1979). MRSA strains (*Staphylococcus*) were recognized at this time, but they seldom spread widely. In the late 1970s, however, strains that were methicillin-resistant, and often also resistant to aminoglycosides, became prevalent in many parts of the world and have subsequently spread widely in hospitals. Patients particularly affected are those in hospital for prolonged periods, elderly or debilitated patients, those with wounds or burns, and those who have had recent treatment with antibiotics (Brumfitt and

Hamilton-Miller 1989). Clinical and laboratory evidence suggests that MRSA are not less virulent than sensitive isolates (French et al. 1990) and in severely ill patients under intensive care, mortality from pneumonia may be high (Rello et al. 1994). The financial impact of MRSA is increasingly recognized (Casewell 1995); the additional measures necessary to control an outbreak with a new epidemic strain that involved over 400 patients in England amounted to £400 000 (Cox et al. 1995).

Control of MRSA can be difficult and ward closures can result in the severe disruption of clinical services (Barrett et al. 1993). Nonetheless, active intervention combined with effective surveillance is successful (Hartstein et al. 1995). In the face of endemic infection, control measures are usually based upon a risk assessment of the likelihood of spread and invasive infection, and include selective screening of patients (less so, staff), isolation or cohort nursing, and eradication of carriage by the judicious use of topical antibiotics (Ayliffe 1996; Combined Working Party of the British Society for Antimicrobial Chemotherapy, the Hospital Infection Society and the Infection Control Nurses Association 1998). Recognition of epidemic strains may be assisted by noting the origin of the patient, the clinical illness, the type of infected lesion, the antibiotic-sensitivity pattern, and phage type. Collecting this information and the use of a simple computerized database can provide useful information on local epidemiology (Rossney et al. 1994). The complexity of strains isolated and the presence of phage nontypable strains means that a combination of phenotypic and genotypic typing methods (see later under Epidemiological typing) is now often required to determine source and spread (Tenover et al. 1994). The emergence of MRSA strains with reduced vancomycin susceptibility is of some concern (Hiramatsu et al. 1997). There have been recent reports of outbreaks of community-acquired MRSA infection, particularly in the US, which are caused by strains with a novel methicillin resistance gene and often also a hitherto rare exotoxin, Panton–Valentine leucocidin, that is associated with primary skin infection and pneumonia (Dufour et al. 2002; Mongkolrattanothai et al. 2003; Anonymous 2003). This development raises the specter of the transmission and acquisition of MRSA clones outside the traditional secondary healthcare setting. Basic infection control practice, including the rational use of antimicrobial agents, becomes even more important as we face the possible dissemination of strains for which there are few effective therapeutic agents.

Colonized or infected patients should be treated in isolation, preferably in a single room or designated isolation facility, with precautions against both contact and aerial spread. Contaminated environmental surfaces may be a source for continuation of an outbreak. When large numbers of patients are involved, cohort nursing in separate areas of the hospital may be necessary, but control may be impossible without a dedicated isolation unit (Pearman et al. 1985). Mupirocin seems particularly effective in eliminating carriage (Casewell and Hill 1986), but resistance to this agent can emerge following prolonged or repeated use in MRSA patients and patients with chronic skin disease (Cookson 1990). MRSA carriage may be unresponsive to a variety of antiseptics and increasing numbers of carriers are found in nonacute units and in the community.

Enterococcus spp.

Recent interest in *Enterococcus* spp. as hospital pathogens has focused on unusually antibiotic-resistant strains of *Enterococcus faecium* and *Enterococcus faecalis*. Resistance may be multiple and include high-level resistance to penicillins and to aminoglycosides, such as gentamicin, which are important in providing synergistic bactericidal combinations with penicillins. They may also be resistant to glycopeptides such as vancomycin, which had been regarded as universally active against gram-positive bacteria that may infect humans, apart from intrinsically resistant genera of minor importance such as *Pediococcus*, *Leuconostoc*, and *Lactobacillus* (Woodford et al. 1995; Chavers et al. 2003).

Resistance to vancomycin in enterococci was first reported in a cluster of hospital-acquired infections in the UK (George et al. 1989) and such strains are increasingly found in oncology, renal, transplant, and intensive care units (Hospital Infection Control Practices Advisory Committee 1995). The epidemiology of resistant enterococci is not entirely clear. Treatment, particularly with cephalosporins and glycopeptides, may encourage colonization with enteroccci, which may then spread from patient to patient by indirect contact, via staff and environment, and thus cause persistent problems.

For epidemiological purposes, enterococci may be typed by ribotyping, but pulsed field gel electrophoresis is more discriminating. The strains are heterogeneous, but the evidence suggests that particular epidemiological types spread between hospitals (Chadwick et al. 1996; Morrison et al. 1996). Guidelines for the prevention of spread have been issued in the USA (Hospital Infection Control Practices Advisory Committee 1995) and discussed in the UK (Chadwick et al. 1996). These guidelines are similar to those for the control of MRSA (see above).

Clostridium difficile

C. difficile has emerged during the last decade as a significant hospital-acquired pathogen. It was recognized in the late 1970s as the main cause of antibiotic-associated diarrhea, which may be associated with pseudomembraneous colitis (PMC) (*Clostridium perfringens*, *Clostridium difficile*, and other *Clostridium* species). The number of laboratory reports of *C. difficile* infection sent

to the UK Communicable Disease Surveillance Centre increased ten-fold between 1982 and 1991 (Joint DH/PHLS Working Group 1994). Laboratory reports for *C. difficile* positive samples for England and Wales currently (2002) stand at approximately 29 000 per year (provisional data). This is certainly an under-reporting of the true burden of this disease. *C. difficile* may be part of the normal bowel flora, especially in the elderly, and may also be recovered from the hospital environment. Antibiotics, especially broad spectrum agents, disrupt the normal bowel flora and render patients susceptible to colonization. *C. difficile* rarely, if ever, affects healthcare personnel and spread from patient to staff is unusual.

The increasing numbers of elderly and debilitated patients admitted to hospitals, the misuse of antimicrobial agents and changes in healthcare, including greater diagnostic and therapeutic interventions, have all probably contributed to the increased colonization with *C. difficile*. Whilst most cases are sporadic, clusters or outbreaks also occur. In the UK, a large outbreak involving 175 patients occurred in three hospitals in Manchester in 1992. Most of the patients were over 60 years old and infection was believed to have contributed to 17 deaths (Cartmill et al. 1994). In smaller outbreaks or clusters, epidemiological evidence usually suggests a traceable chain of person-to-person spread between wards or hospitals (Nolan et al. 1987). Apart from significant morbidity and mortality in an already vulnerable population, management and control of this condition have significant financial implications. In a case-control study it was calculated that 94 percent of the additional costs associated with *C. difficile* infection were due to increased duration of hospital stay and the total cost was in excess of £4 000 per case (Wilcox et al. 1996).

Even before the advent of newer typing techniques, clinical and epidemiological evidence suggested that patient-to-patient spread occurred by the fecal–oral route and that precautions were necessary to prevent spread. More than 20 percent of patients may carry *C. difficile* at some stage during hospitalization, especially if they share a room with a positive patient, and environmental and patient isolates are often indistinguishable (McFarland et al. 1989; Samore et al. 1996). Control measures should be aimed at minimizing the risk of antibiotic-associated diarrhea by good antimicrobial prescribing, scrupulous hand-washing, the isolation or cohorting of affected patients, treatment of persistently symptomatic patients, careful disposal of contaminated laundry and, finally, environmental disinfection/decontamination (Joint DH/PHLS Working Group 1994; Gerding et al. 1995; Wilcox 1998). The role of asymptomatic carriers in spread, especially amongst the elderly, remains unclear. Some *C. difficile* strains are more virulent than others. For example, ribotype 1 is epidemic in the UK, being responsible for about 60 percent of reported cases, and posseses several phenotypes that distinguish it from other strains; for example, ribotype 1 sporulates more than comparators, a phenomenon that is enhanced when exposed to detergents (Wilcox and Fawley 2000), and is more resistant to fluorquinolones (Wilcox et al. 2000a). It is known that environmental contamination with *C. difficile* is widespread despite routine detergent-based cleaning (Fawley and Wilcox 2001; Verity et al. 2001). There is some evidence that environmental cleaning with hypochlorite may be more effective than detergent as a control measure for *C. difficile* diarrhea (Wilcox and Fawley 2000; Wilcox et al. 2003b).

Enterobacteriaceae

E. coli is one of the most frequently encountered bacteria in hospital-acquired infection (Jarvis and Martone 1992). It causes urinary tract infection (Bacterial infections of the urinary tract), intraabdominal and gut-related wound infection and bacteraemia (Bloodstream infection and endocarditis and 52, *Escherichia*), but infection is almost always endogenous and sporadic; even resistant strains seldom appear to spread between hospital patients (Hart 1982). However, cross-infection by strains with the potential to cause enteric infection, including verocytotoxin-producing *E. coli* strains, is common in hospitals and other healthcare facilities (Carter et al. 1987; Kohli et al. 1994).

Other Enterobacteriaceae are more important in the spread of nonenteric infection in hospitals. This is due to antibiotic resistance, transmissibility, and virulence, which interact in hospital units that accumulate patients with similar medical problems who undergo similar procedures and receive similar antibiotics. For example, *Klebsiella* spp. have come into prominence in outbreaks caused by multiresistant strains that produce extended spectrum β-lactamases (Johnson et al. 1992; Meyer et al. 1993; Quinn 1994). Ease of transmission from patient to patient, resulting in large-scale epidemics and inter-ward spread rather than small clusters of infections or common-source outbreaks, may be related to better survival on hands (Casewell and Desai 1983) or on inanimate surfaces (Hart et al. 1981), but other factors must be involved (Fryklund et al. 1995). The characteristics of hospital infection by members of the various enterobacterial genera are outlined in Table 1. Infection by *Salmonella* spp. has been mentioned under infection transmitted by food (Bacteriology of foods excluding dairy products and 54, *Salmonella*).

Pseudomonas aeruginosa

P. aeruginosa has been recognized as a pathogen of hospital patients only in the modern era of intensive treatment and antibiotic administration. Its ability to grow in moist conditions with simple nutrients and its comparative resistance to antibiotics and disinfectants have allowed it to become established, often in very large numbers, in fluids and wet places in the hospital

Table 1 *Enterobacteriaceae of importance in hospital infection*

Genus[a]	Source and mode of infection	Circumstances of infection	Antibiotic characteristics of hospital strains	References
Escherichia (*E. coli*)	Endogenous; crossinfection uncommon (except enteric infection)	Common (especially urinary, bloodstream)	Ampicillin R common	Olesen et al., 1995
Citrobacter (*C. freundii*, *C. diversus*)	Infrequent, endogenous infection	Bloodstream, etc.	Often multi-R including cephalosporins	Thurm and Gericke, 1994
Klebsiella (*K. pneumoniae*, *K. oxytoca*)	Endogenous, but crossinfection common, with fecal carriage; transmission via hands	Bloodstream, urinary (especially catheterized), respiratory; ICUs especially neonatal, urological units	Ampicillin R; frequently aminoglycoside R; increasingly R to newer cephalosporins	Johnson et al. 1992; Meyer et al. 1993; Quinn 1994
Serratia (nonpigmented *S. marcescens*)	Crossinfection common, also environmental sources implicated; skin, throat carriage, but fecal carriage less common; hand spread	Urinary, respiratory; ICUs, especially neonatal	R to ampicillin, early cephalosporins; frequently R to aminoglycosides and newer cephalosporins; emergent R during treatment; characteristic polymyxin R	Lewis et al. 1983; Coria-Jimenez and Ortiz-Torres 1994; Luzzaro et al. 1995
Enterobacter (*E. cloacae*, *E. aerogenes*)	Endogenous and hospital infection from common source, e.g. medicaments, food, apparatus; hand spread less common; throat and fecal carriage	Respiratory, urinary, bloodstream; ICUs especially neonatal, burns units	Ampicillin R; frequently R to cephalosporins; emergent R during treatment	Gaston 1988; Weischer and Kolmos 1992
Proteus (*P. mirabilis*, *P. vulgaris*, also *Morganella*)	Endogenous and small clusters; fecal carriage	Urinary, bloodstream; ICUs, urological units	Variable (see *Proteus*, *Morganella*, and *Providencia*)	Williams et al. 1983; Watanakunakorn and Perni 1994
Providencia (*P. stuartii*)	Endogenous; slow transmission among patients; fecal carriage	Urinary, occasionally other sites; geriatric, paraplegia units; mixed infection with *Proteus* spp. common	Aminoglycoside R common	Hawkey et al. 1984; Woods and Watanakunakorn 1996

ICUs, intensive care units; R, resistance or resistant.

a) Only more important species are listed. For *Salmonella*, see Chapter 54.

and to colonize the mucous membranes and skin of patients. Respiratory infection in intensive therapy units, infection of burns, in neonates, in abdominal surgery, and after trauma, are among the most characteristic presentations, but cases may be encountered in any acute hospital unit.

Strains of *P. aeruginosa* can be distinguished by serotyping, pyocine typing, phage typing (*Pseudomonas*) and by genotypic methods. Strains may differ markedly in virulence and some epidemics are caused by particularly virulent strains or by exposure of a sensitive body site, such as the eye, to heavily contaminated fluids or apparatus. Mostly, however, infections are endemic or sporadic, and colonization is more common than clinical disease. Indeed, in some hospital units clinical infection may be rare, though colonization may be almost universal. It may, therefore, be difficult to interpret the significance of the isolation of *P. aeruginosa* from patients' specimens (Davies and Bullock 1981).

Normal subjects may carry *P. aeruginosa* in their bowel. The apparent prevalence depends on the sensitivity of isolation methods (Shooter et al. 1966) and antibiotic treatment encourages such carriage. The importance of strains at inanimate sites in the hospital varies, but their spread to patients has been reported (e.g. Doring et al. 1993). Strains found at such sites are, however, often different from those found in the patients (Kropec et al. 1993). Clearly, there is much greater likelihood of infection when the patient comes into close contact with the infected source or object, such as contaminated medicaments, inhalants, or splashing from sinks. It is, therefore, essential that medicaments and aqueous disinfectants intended for application to patients are supplied sterile and preferably in single-use portions. Patient-to-patient spread may occur when there is a breakdown in infection control measures, such as failure to disinfect urinals and bedpans and failure of hand hygiene (Lowbury et al. 1970). In these situations rectal and other colonization usually precedes infection.

P. aeruginosa is the most common persistent pathogen in chest infection in cystic fibrosis, but the degree to which crosscolonization occurs in hospital units appears to differ widely.

Mycobacterium spp.

In the past, tuberculosis was a serious occupational risk for healthcare staff. This is no longer the case in most clinical situations where tuberculin testing and immunization, supplemented by selective chest X-ray, are available for staff in contact with patients and those handling pathological material (Joint Tuberculosis Committee of the British Thoracic Society 1994). Nosocomial acquisition of tuberculosis by patients in the developed world is also uncommon. Risk factors for transmission to patients and staff include delayed diagnosis, drug-resistant strains and lapses in administrative, engineering, and infection control practices (Menzies et al. 1995). Multidrug-resistant tuberculosis in HIV patients is of increasing concern as presenting a risk to staff and other patients. Previous admission to hospital, failure to isolate positive patients in a single room, and the absence of positive pressure ventilation are associated with spread (Pearson et al. 1992).

The risk of infection depends on the infectivity of the primary source, the duration and proximity of the exposure, and the susceptibility of the recipient (George et al. 1986). Infectivity depends on the amount of cough and the concentration of bacteria in the sputum. Thus, 'three-smear-negative' culture-positive patients with pulmonary tuberculosis and patients with nonpulmonary tuberculosis are usually regarded as noninfective. Sputum infectivity declines rapidly during chemotherapy, including that with rifampicin, with a 99 percent decline in viability after 2 weeks, but scanty bacteria may survive for much longer (Jindani et al. 1980). Isolation for 2 weeks in a well-ventilated side ward, without recirculation of the air to other parts of the hospital, is recommended while patients are infectious (Joint Tuberculosis Committee of the British Thoracic Society 1994). Susceptible individuals are the young and those whose immunity is depressed by disease or treatment. Care should be exercised in the handling of fomites from sputum-positive patients and in protecting highly susceptible patients from exposure (George 1988). This may entail prolonged isolation of smear-positive patients and the segregation of smear-negative or nonpulmonary cases of tuberculosis. Contact tracing should be undertaken after incidents in which staff or patients were exposed to infection (Joint Tuberculosis Committee of the British Thoracic Society 1994). Control measures can halt the transmission of multidrug-resistant strains (Wenger et al. 1995). The use by healthcare staff of HEPA respirators is expensive, inconvenient, and is not believed to be cost-effective (Adal et al. 1994).

Hospital infection, including keratitis and systemic disease, caused by opportunist mycobacteria, such as *Mycobacterium chelonei*, *Mycobacterium gordonae*, and *Mycobacterium fortuitum*, has been observed (Watt 1995). Contributory factors are the ability of these mycobacteria to survive and multiply in moist environments, inadequate decontamination procedures, and poor hygienic precautions in renal dialysis, bronchoscopy, hydrotherapy, and cardiac surgery units (George 1988). Contamination of specimens by the use of tap water to rinse disinfected bronchoscopes has led to 'pseudo-outbreaks' (Nye et al. 1990). The extent to which crossinfection is responsible for the *Mycobacterium avium* complex infections seen in patients with AIDS (*Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and other slow growing mycobacteria) is unknown. The *M. avium* complex may

be recovered from standing water and food sources (Horsburgh 1991) and clusters of cases may therefore reflect acquisition from a common source rather than person-to-person spread.

Other bacteria

The main features of hospital infection by other bacterial species are given in Table 2.

Viruses

The prevalence of nosocomial viral infection is probably underestimated. Most surveys of hospital-acquired infection concentrate on bacteria and the subclinical nature of many viral illnesses together with limited diagnostic facilities partly explains why viruses do not figure prominently. Viral infections are, however, important in neonatal and pediatric patients and in immunosuppressed patients. In pediatrics, rotavirus may be the most common individual pathogen encountered.

Community-acquired infection by respiratory syncytial virus is almost universal in children in the early years of life. In hospital this is a particular danger to premature neonates and children with cardiorespiratory abnormalities. Staff and adult patients may be reinfected, with minor respiratory symptoms, but severe disease may occur in the immunodeficient adult. The virus spreads by respiratory secretions entering the eye or nose, with or without an intervening period on surfaces or fomites. The risks of infection may be reduced by a combination of cohort nursing and the wearing of gowns and gloves (Snydman et al. 1988; Madge et al. 1992). Such a proactive approach is made possible by the availability of rapid antigen detection facilities (Editorial 1992). Similar principles apply to control of the childhood exanthemata, when infected patients are admitted to hospital (Breuer and Jeffries 1990). Staff working with pregnant women should be immune to rubella. Apart from classical influenza, influenza virus A can cause a variety of syndromes in hospital patients, including a particularly dangerous form of pneumonia in the elderly. Selective immunization of patients can be helpful and a case can be made for the routine immunization of some health workers (Heimberger et al. 1995).

Viral gastroenteritis is common in neonatal and pediatric units, in general wards, and in long-stay accommodation for the elderly (Holzel and Cubitt 1982). Rotavirus is the most frequent causative agent in children. In the elderly, outbreaks of diarrhea caused by SRSV, such as noroviruses, and other agents, can affect large numbers of patients. Spread occurs via the fecal–oral and probably also via the air-borne routes. Prevention of spread may be achieved by early diagnosis, for example by electron microscopy, and strict precautions that include wearing of aprons and gloves, strict hand-washing routines, availability of separate toilet facilities, and restriction on the movement of symptomatic patients and staff caring for them, as well as good communications between hospitals to minimize inadvertent spread (Caul 1994; Rao 1995; Chadwick et al. 2000).

Enterovirus infections, though usually subclinical, are also common in hospitals (Dowsett 1988). Besides hepatitis A, coxsackie A and B and echoviruses may cause generalized febrile infections and meningitis, particularly in the newborn, generalized illness with bowel symptoms in immunodeficient persons, and minor infections in staff. Spread may occur by the oral and respiratory routes and by hands, fomites, and such shared facilities as hydrotherapy pools. The same groups of patients are particularly susceptible to the herpes group of viruses. Cytomegalovirus, as well as being transmitted in utero and at birth from mother to baby, may be conveyed in donations of blood or tissue, and infections in the immunosuppressed often result from reactivation. Varicella-zoster may be conveyed by the vesicular exudate or respiratory secretions of patients with chicken pox and, less frequently, zoster. Both diseases necessitate barrier precautions and patients must be segregated from susceptible persons, who may, when appropriate, be protected with immune globulin (Burns et al. 1998). This will also protect healthcare staff, of whom approximately 15 percent may not be immune. A register of staff immunity may be useful, especially in clinical areas where varicella infection may be more common, such as pediatric wards (Breuer and Jeffries 1990). In the USA, vaccination of susceptible healthcare staff likely to be exposed to patients at high risk of complications of chicken pox is recommended (Bolyard et al. 1998). Herpetic whitlows occasionally develop in staff not protected from exposure to the respiratory secretions of patients.

Viral hemorrhagic fevers such as Lassa, Marburg, and Ebola, have been responsible for infections of hospital staff and patients in tropical countries where equipment and staffing are inadequate and medical practice poor (Fisher-Hoch et al. 1995). In the UK, patients are categorized as minimum, moderate, or high risk. Minimum and moderate risk patients may be cared for in an isolation unit, but those in the high risk group should be tranferred to a high security infectious disease unit (Advisory Committee on Dangerous Pathogens 1996). This may not always be feasible and is in contrast to US recommendations which emphasize isolation in a single room with negative pressure ventilation and the implementation of universal precautions, as spread by the air-borne route is considered relatively rare (MMWR 1995).

For other blood-borne viruses (see section Hepatitis B and C and HIV infection in hospitals).

The control and prevention of viral infection amongst hospital staff is best managed by occupational health departments, by staff screening, immunization, and

Table 2 *Other bacteria[a] associated with hospital infection*

Bacteria	Source and mode of spread	Circumstances of infection	Antibiotic characteristics of hospital strains	References
Gram-positive bacteria				
Coagulase-negative staphylococci	Endogenous; 'hospital' strains by contact, air	Implants; intravascular cannulae; sternal wounds; neonates	Variable; often multi-R especially to isoxazolylpenicillins, aminoglycosides; usually S to vancomycin, rifampicin	Stillman et al. 1987; Patrick et al. 1992
Streptococcus				
S. pyogenes (streptococcus Lancefield group A)	Endogenous; uncommon hospital spread from cases and throat, nose, skin, rectal carriers; contact, air	Wounds, burns, skin lesions; parturient women	Invariably S to benzylpenicillin; variable to erythromycin, tetracycline	Garrod 1979; Easmon 1984
S. agalactiae (streptococcus Lancefield group B)	Endogenous (feces, vagina); occasionally contact in neonatal units	Usually congenital in neonates; postpartum infection; skin and soft tissue lesions	Consistently benzylpenpenicillin S	Easmon 1984; Zangwill et al. 1992
S. pneumoniae	Endogenous; occasionally air spread of more virulent or antibiotic-resistant strains	Pneumonia, etc., in respiratory or general impairment; children	Hospital clusters showing penicillin R or intermediate R and multi-R	Millar et al. 1994
Other streptococci: viridans or indifferent; *S. anginosus* (syn. *milleri*); β-hemolytic streptococci groups C and G	Endogenous; rarely contact spread	Abdominal surgery; implants; neutropenic patients	May be relatively R to β-lactams	Editorial 1984a, 1985
Corynebacteria (especially *C. jeikeium*)	Endogenous; skin colonization may precede infection	Implants; cannulae; immune impairment; antibiotic treatment	Multiple R; vancomycin S	McGowan 1988
Clostridium				
C. perfringens, etc.	Usually endogenous (feces); indirect contact spread of enteritic strains	Abdominal surgery; amputations; diabetics: elderly (enteritic)	Benzylpenicillin S	Lowbury and Lilly, 1958; Borriello and Barclay, 1986
C. tetani	In the past, environmental and operative materials; now possibly endogenous	Very rare postoperative infection	Benzylpenicillin S	Parker and Mandal 1984
Bacillus spp.	Environmental; may resist sterilization processes	Opportunistic; soft tissue and occasional systemic infection	Often R to β-lactams	Weber and Rutala 1988
Rhodococcus equi	Unknown, possibly by air from soil, etc.	Pneumonia, etc., in immunocompromised, e.g. HIV infection		Verville et al. 1994

(*Continued over*)

Table 2 *Other bacteria*[a] *associated with hospital infection (Continued)*

Bacteria	Source and mode of spread	Circumstances of infection	Antibiotic characteristics of hospital strains	References
Nocardia asteroides	Inanimate environment; possibly air-borne spread from cases	Respiratory tract infection; immunosuppression		Houang et al. 1980; Javaly et al. 1992
Gram-negative bacteria				
Pseudomonas spp. and similar 'environmental' gram-negative bacilli	Water; damp areas; liquid medicaments; moist equipment; fecal carriage; direct and indirect contact (hands)	Colonization more frequent than infection. Chronic disease; renal failure; immune impairment; major surgery; burns; broad spectrum antibiotic treatment; neonates; implants; cannulae; catheters; respiratory equipment	Often multi-R	*Pseudomonas*
Burkholderia cepacia	Unsterilized aqueous solutions, e.g. disinfectants; nebulisers; patient-to-patient infection, probably air-borne	Various from solutions; late infection in cystic fibrosis	Often R to all classes	Pankhurst and Philpott-Howard 1996
Stenotrophomonas (Xanthomonas) maltophilia	Multiple epidemiological types; source and spread not clear	Bacteremia; device-related infections; broad spectrum antibiotics, including penems	Multi-R; characteristic R to penems	Vartivarian et al. 1994; Laing et al. 1995; Gerner-Smidt et al. 1995; Spencer 1995
Acinetobacter spp. (*A. baumannii*, *A. calcoaceticus*)	Endogenous; also indirect contact staff and equipment	Bacteremia; respiratory infection; ICUs; broad spectrum antibiotics	Increasingly multi-R	Bergogne-Berezin 1995; Mulin et al. 1995; Seifert et al. 1995
Achromobacter xylosoxidans		Uncommon bacteremia, respiratory infection	Aminoglycoside R and trimethoprim S typical	Schoch and Cunha 1988; Legrand and Anaissie 1992
Flavobacterium spp.		*F. meningosepticum* associated with infantile meningitis; others as *Pseudomonas* spp.	Often S to some antibiotics usually thought of as 'anti-gram-positive'	Ratner 1984
Aeromonas spp.		Bacteremia; soft tissue infection; outbreaks and invasive infection uncommon. Toxigenic strains may be enteritic		Mellersh et al. 1984; Murphy et al. 1995
Capnocytophaga spp.	Endogenous; usually from oropharynx	Bacteremia; immunosuppression, neutropenia	Aminoglycoside and trimethoprim R, β-lactam S typical	Kristensen et al. 1995

R, resistance; S, sensitivity.

a) Other than anaerobic gram-negative bacilli, *Legionella* spp., *S. aureus*, *Enterococcus* spp., *C. difficile*, *Mycobacterium* spp. and *P. aeruginosa*.

education programs, preferably working in tandem with infection control personnel.

Fungi and protozoa

Fungal infection continues to increase in hospitals (Pfaller and Wenzel 1992), as more effective and broader spectrum antibacterial agents and immunosuppressive regimens are deployed and increase the number of patients at risk. However, it remains likely that many fungal infections go undiagnosed because of a lack of awareness of risk factors and shortcomings in conventional laboratory techniques, e.g. fungemia is difficult to confirm microbiologically. As has recently been described in the HIV population, where *Candida dubliniensis* is now recognized as an important oral pathogen (Sullivan and Coleman 1998), studies combining molecular techniques and good epidemiology are likely to reveal a higher incidence of nosocomial infection and both new pathogens and new strains. The most common significant fungal isolate is *Candida albicans*, a bowel commensal that in most cases gives rise to endogenous infection. Members of other species, such as *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida glabrata*, are becoming more common. Their source is particularly associated with the skin, and therefore with intravascular cannulae. Less common fungi, such as *Fusarium* and *Trichosporon beigelii* are emerging causes of infection in cancer and bone marrow transplant recipients (Pfaller and Wenzel 1992). Clusters of infections with the same strain of *Candida* with evidence of colonization of the hands of staff, may occur under selective pressure from broad spectrum antibiotics (Burnie et al. 1985). Typing of *C. albicans* for epidemiological purposes is difficult. Phenotypic typing, such as biotyping, may be useful but DNA-based approaches are likely to be increasingly used. *Aspergillus* infection is related to the air-borne spread of spores, particularly when these reach high concentrations, as during building repairs near patients with neutropenia (Rogers and Barnes 1988) and occasionally other vulnerable groups, such as ICU patients (Humphreys et al. 1991). Molecular typing techniques allow the comparison of isolates from patients and the environment (Leenders et al. 1996). Clusters of infections due to non-*A. fumigatus* may indicate an environmental source (Loudon et al. 1996). Strategies for the prevention of aspergillus infection include pre-emptive antifungal therapy. Isolation of especially vulnerable patients in laminar flow ventilated facilities has been recommended.

There is no good evidence for the hospital spread of *Cryptococcus neoformans*. Phycomycetes may be transmitted to susceptible patients by dressings (Gartenberg et al. 1978) or by air (del Palacio Hernanz et al. 1983). Dermatophytes occasionally spread in geriatric units by close contact, or on clothing or apparatus (Peachey and English 1974).

The epidemiology of *Pneumocystis carinii* is obscure. Dormant infection has been said to be ubiquitous and reactivated by immune suppression, as may be infection by *Toxoplasma gondii*. On the other hand, air-borne spread of *P. carinii* can be shown in animals and there have been suggestions of clusters of linked cases (Rhame et al. 1984; Haron et al. 1988).

Cryptosporidium, a coccidian protozoon, is an important cause of debilitating diarrhea in the immunosuppressed, such as AIDS patients (Goodgame 1996). The oocysts are comparatively resistant to disinfection and may survive well in the inanimate environment. There is evidence of case-to-case spread and of transmission to staff and to other patients in hospitals (Martino et al. 1988), presumably by the fecal–oral route. A nosocomial outbreak in Denmark was linked to a ward ice machine (Ravn et al. 1991).

Scabies can be very disruptive in chronic institutions or hospital wards for the elderly (Holness et al. 1992). Infestation in patients with underlying skin disease or Norwegian scabies (extensive hyperkeratotic lesions) are especially contagious (Moberg et al. 1984). Early and reliable diagnosis, effective treatment, for example with permethrin, applied extensively to the body, and decontamination of bed linen and fomites are important to limit spread.

INVESTIGATION AND SURVEILLANCE OF INFECTION

Investigation of outbreaks

An outbreak is defined as two or more cases of symptomatic infection or of symptomatic infection and colonization, suspected on clinical or laboratory evidence. An outbreak may be obvious, such as a number of cases of acute diarrhea occurring in a ward over a few hours, or it may not be apparent for some time, such as deep staphylococcal infection after hip replacement in several patients over a period of months.

Outbreaks may be detected by simple laboratory surveillance with informal sources of clinical intelligence, but more subtle occurrences may be detected only by a more formal investigation. This should, at the same time, follow the epidemiological and the microbiological approaches. A first essential is definition of a case in clinical or microbiological terms. Outbreaks defined by an increase in the infection rate at a single body site may have a multiple bacterial etiology and the question arises whether there is in fact an outbreak. There may simply have been heightened awareness of infective episodes, a series of unlinked but significant clinical events, unrelated cases of infection by similar microorganisms or a ‘pseudo-outbreak’ due to specimen contamination (McGowan and Metchock 1996).

The spread of infection may be:

- from a point-source at one time
- from a point-source with continued exposure
- by person-to-person spread
- from infected persons with secondary contamination of the environment, or
- by a mixture of these.

Outbreaks should be defined epidemiologically in terms of time, place, and persons involved, and an epidemic curve should be plotted where the outbreak is relatively large (Palmer 1989). The shape of this curve and the microbiological cause may yield information for follow-up.

Small outbreaks purely of hospital concern may be dealt with by the Infection Control Team or equivalent (see Organization of hospital infection control) in association with clinical staff and local managers. More serious outbreaks and those with community implications require the involvement of those responsible for infection control in the community, i.e. local public health authorities and public health laboratories. When a major incident is found or suspected that is of potential seriousness for patients, or involves many patients or more than one hospital, or is liable to cause public concern, a formal action group or outbreak committee should be established (Hospital Infection Working Group of the Department of Health and Public Health Laboratory Service 1995). The responsibilities of such a group are to investigate and control the outbreak; to ensure that affected patients are adequately cared for, and that there are sufficient staff and supplies in the wards and laboratory; to disseminate information to patients, staff, relatives, and the general public; to record the episode, prepare a report, and to consider future prevention (Hospital Infection Working Group of the Department of Health and Public Health Laboratory Service 1995).

Depending on the nature of the outbreak (e.g. nosocomial legionellosis) or its extent (e.g. a large number of cases of hospital-acquired food poisoning), national bodies with specialized epidemiological skills such as the Health Protection Agency's Centre for Infections, Communicable Disease Surveillance Centre (UK) or Centers for Disease Control (USA) may need to be involved. Larger outbreaks, particularly those arising from a single source, whether or not microbiological indicators are available, may require formal epidemiological studies, particularly case-control studies (Rothman 1986; see also Epidemiology of infectious diseases). The descriptive epidemiology may have produced hypotheses to be tested. This approach may be more satisfactory than the use of extended case-control studies to investigate a variety of possible factors. However, case-control studies provide a powerful means to study some types of hospital outbreak and may give more rapid answers than microbiological methods, particularly if microbiological diagnosis of the particular infection is difficult or slow, e.g. some forms of viral gastroenteritis. Particularly in point-source outbreaks, 'cohort' studies (Rothman 1986) may be possible. In such studies, two or more groups that differ in exposure to a postulated source of infection may be compared for incidence of the infection. An example is the identification of the responsible food consumed in a food-borne outbreak. Such approaches are less useful in many hospital outbreaks that are due to person-to-person spread.

The increasing numbers of compromised hospital patients, the greater awareness of the hospital environment as a source and means of transmission of infection, and the emergence of more resistant microbes may require the use of considerable laboratory support to identify or confirm the extent of the outbreak and to confirm that preventive measures have been effective. The successful control of an outbreak requires good clinical investigation and in some instances up-to-date typing techniques such as ribotyping, polymerase chain reaction based techniques, etc. (Emori and Gaynes 1993; Wendt and Wenzel 1996).

Epidemiological typing

Epidemiological typing, characterization of isolates to intraspecies level, is useful (Parker 1978; Pitt 1994) to define the extent of an outbreak and to elucidate the sources and spread of infection and the effectiveness of prevention. Established typing methods that permit stable and discriminatory subdivisions are available for some common hospital pathogens, such as *S. aureus* (*Staphylococcus*). However, the continued appearance of new strains that are not typable by the existing systems, and the progress of newly significant species, diminish the value of classically established methods. This is a stimulus for the development of new methods, preferably those of more general applicability. The need is for simple, reproducible and discriminatory methods; fine discrimination can sometimes be attained by applying two or more typing methods sequentially. The aim is to define, with reasonable confidence, the strain responsible for an incident of infection by distinguishing all epidemiologically irrelevant isolates (Anderson and Williams 1956).

Well-established phenotypic methods of typing include serotyping, phage typing (including 'reverse' phage typing; de Saxe and Notley 1978), and bacteriocin typing. Methods in common use for more local studies include biotyping and comparison of antibiotic-sensitivity patterns, which is often the first clue to a common-source outbreak, but it is an unreliable epidemiological tool. Other methods that have proved to be of value for typing particular organisms include morphotyping of *C. albicans* (Brown-Thomsen 1968), the Dienes phenomenon for swarming strains of *Proteus* spp. (

Proteus, *Morganella*, and *Providencia*) and resistotyping (Elek and Higney 1970).

Analysis of DNA and other cellular constituents may provide a means of discriminating between strains that are similar by conventional typing methods, and makes possible the 'fingerprinting' of diverse species for which standard methods have not been established. The ease with which such methods can be applied has sometimes encouraged their use without proper critical evaluation of their suitability (Pitt 1994; Struelens et al. 1996). Electrophoretic analysis of cellular proteins (total cellular proteins, outer-membrane proteins, or essential enzymes) has been applied to several species, with discrimination by pattern recognition or automated mathematical analysis. Electrophoretic demonstration of plasmids, with or without treatment by restriction endonucleases, has been widely used (Mayer 1988). Chromosomal analysis is more difficult because of the larger size of the genome and the many fragments produced by endonuclease action. Pulsed field gel electrophoresis (PFGE) overcomes this problem by separating relatively few large-sized DNA fragments created by rare-cutting restriction endonucleases. PFGE has generally become the gold standard method for fingerprinting bacteria. Nucleic acid probes can also be applied to endonuclease digests (Bingen et al. 1994). The polymerase chain reaction can be used in a wide variety of ways to characterize the genome of microorganisms (van Belkum 1994). The various epidemiological typing and 'fingerprinting' methods have been recently reviewed elsewhere (Struelens 2002), and criteria for their use and evaluation were proposed by a Study Group of the European Society for Clinical Microbiology and Infectious Diseases (Struelens et al. 1996).

Surveillance

Surveillance is the systematic observation and recording of disease. It is an active process and implies the analysis and dissemination of data, so that direct or indirect action can subsequently be taken to improve patient care. Unlike the investigation of an outbreak or epidemic, surveillance is ongoing and the results can be used as an indicator of the quality of care, e.g. surgical wound infection rates (Glenister 1993; Smyth and Emmerson 2000). In his summary of the Proceedings of the First International Conference on Nosocomial Infections, Williams (1971) stated: 'Quite clearly, the first message from this conference is the need for surveillance. It is essential that hospital staff know what is going on in the hospital'. It is important to analyze surveillance activities critically to determine their usefulness in control, rather than to undertake them for their own sake, or with a false idea of their usefulness. The increasing availability of computer databases makes the analysis of data easier, but more traditional and time-consuming methods such as a card recording system can be used equally well. In particular, the use of optical scanning technology has been shown to improve the speed and accuracy of data entry and can greatly facilitate analysis and the dissemination of results (Smyth et al. 1997). In Britain, laboratory-based surveillance, with a variable degree of clinical reporting of infection, especially of organisms such as MRSA, has been the norm. In the USA, partly because of accreditation requirements and the fear of litigation, much staff time is devoted to the continuous gathering of data (Casewell 1980; Wenzel 1986). Justification for the latter approach comes from the Study of the Efficacy of Nosocomial Infection Control (SENIC) (Haley et al. 1985b). This large and expensive investigation determined the infection rates in 1970 and 1976, by a validated retrospective case-paper review, in a large sample of patients from 338 US hospitals. The rates were compared with the very variable infection control activities of the hospitals, expressed in terms of two indices, surveillance and control. The conclusion was that optimal measures would prevent 32 percent of hospital-acquired infections of the urinary tract, the lower respiratory tract, surgical wounds, and the bloodstream.

It should be recognized that comparisons of data collected, for example, in different institutions, are of limited value (Haley 1988). Even the most basic of surveillance activities, the detection of cases of hospital-acquired infection, is difficult and expensive, and many systems give results far below the actual rate. Furthermore, differences in case definitions between centers make reliable comparisons difficult. A comparison of different approaches, with a 'standard' of prospective identification, by an infection control physician who examined patients and records is shown in Table 3 (Freeman and McGowan 1981). Physician self-reporting is the least reliable method of recording infections. In the UK, Glenister (1993) assessed a number of different methods and compared these with a reference method. A laboratory-based telephone method took 3.1 h for every 100 beds, but was only 51 percent sensitive in detecting hospital-acquired infection, whereas a laboratory-based ward liaison method was 76 percent sensitive but more time-consuming.

The dissemination of the results of surveillance is crucial if a beneficial effect on patient care in the form of improved practices and reduced hospital-acquired infection rates are to result. Wound infection rates should be reported to individual surgeons (Cruse et al. 1980). The prevalence of all types of nosocomial infection fell from 10.5 to 5.6 percent over a period of 3 years following surveillance in the form of repeat prevalence surveys accompanied by improvements in infection control policies (French et al. 1989).

It is not always feasible to conduct unit or hospital-wide surveillance. Consequently, targeted or selective surveillance is recommended (Hospital Infection Working Group of the Department of Health and Public

Table 3 *Hospital-acquired infection: sensitivity of methods of case finding*

Method	Sensitivity
Physician self-report forms	0.14–0.34
Fever	0.47
Antibiotic use	0.48
Fever plus antibiotic use	0.59
Microbiology reports	0.33–0.65
Selected chart review with 'Kardex' clues	0.85
Total chart review	0.90
SENIC pilot project	
Prospective data collection	0.52–0.90
Retrospective chart review	0.66–0.80
Standard method	1.00

SENIC, Study on the Efficiency of Nosocomial Infection Control. Standard method, clinical detection of infection by a trained physician, who examined all the patients and reviewed all the data in respect of them, is given a sensitivity of 1.00 by definition. Modified from Freeman J., McGowan J.E. 1981. *Rev Infect Dis*, **3**, 658 (© The University of Chicago) with permission.

Health Laboratory Service 1995; Glynn et al. 1997). Targeted surveillance concentrates on areas where infection is particularly important and where it has a major impact on patient morbidity and mortality, e.g. intensive care and oncology units (Hanberger et al. 2001). Selective surveillance addresses problem areas or units or specialties with potential problems, outside the occurrence of a clearly defined epidemic, where infection rates might be higher than expected, or following the opening of a new specialist unit or facility. As the duration of hospital stay becomes shorter, it is important not to underestimate hospital infection presenting in the community after discharge. With the availability of newer typing techniques (see above), it should become easier to trace the spread of organisms that cause nosocomial infection so that preventive strategies to reduce crossinfection may be based on clearer scientific evidence. The continuing challenge for infection surveillance schemes is to give ownership of infection prevention to clinicians. Surveillance systems need to be cognizant of the time restraints and consequent issues surrounding compliance with data collection. It is therefore important that minimum datasets are used wherever possible to facilitate the process of surveillance. The mandatory *S. aureus* bacteremia scheme in England and Wales referred to above (see Bacteremia) is an example of how a simple collection system can provide easily accessible data, which in turn should allow hospitals to focus resources on problem areas and then monitor the effects.

ORGANIZATION OF HOSPITAL INFECTION CONTROL

Systems for the management of infection control in hospitals developed in parallel in Great Britain and in the USA, and essentially similar systems have been adopted in other European countries and in other parts of the world (Hambraeus 1995; Astagneau and Brucher 2001; Bassetti et al. 2001; Bijl and Vos 2001; Frank et al. 2001; Hryniewicz et al. 2001; Jepsen 2001; Lim 2001; Reybrouck et al. 2001; Rodriguez-Bano and Pascual 2001; Leblebiciogh and Unal, 2002; Melo-Cristino et al. 2002; Reed et al. 2003).

In the UK, the staff who provide infection control expertise in hospitals have been reviewed and guidance has been issued (Hospital Infection Working Group of the Department of Health and Public Health Laboratory Service 1995). Executive action in infection control, though a responsibility of the chief executive (CE) of the unit, is the function of an infection control team (ICT), which must maintain close liaison with the CE, or appointed deputy, and other managers. In its simplest form the ICT consists of an infection control doctor (ICD) and an infection control nurse (ICN) (Gardner et al. 1962). The ICD is the control of infection officer (Colebrook 1955; Subcommittee of the Central Health Services Council 1959) and the leader of the team, and may be formally appointed with responsibility to the medical consultants' committee or to the CE, and may report directly to the Strategic Health Authority or Primary Care Trusts. The ICT may be augmented by the active participation of medical microbiologists, infectious disease physicians, scientists, medical laboratory scientific officers, and others, and should be supported by an Infection Control Committee (ICC) (Hospital Infection Working Group of the Department of Health and Public Health Laboratory Service 1995).

The ICD has a wide-ranging role in hospitals and is the specialist adviser on hospital infection to the executive officers of hospital trusts and to managers and planners. The ICD is expected to develop an infection control program, policies, and procedures and to ensure that all staff are educated in infection control and that suitable audit is carried out. The ICD has the primary responsibility for surveillance and the investigation of outbreaks and advises about infectious disease in hospital staff and its prevention, including immunization policy. The ICD is the leader of the ICT and works closely with and supports the ICN. In the USA, the equivalent is the infection control epidemiologist.

The ICN is responsible for infection control matters to the ICD and undertakes similar activities either with the ICD or independently under his or her general guidance. The ICN may provide clinical surveillance, day by day and in outbreaks, and has particular responsibility for the application of policies and procedures and for the training of staff. The ICN often has close links with various ancillary departments, such as the pharmacy, domestic services, laundry, etc. The counterpart of the ICN in the USA is the infection control practitioner (Goldmann 1986). On 5 December 2003, Sir Liam Donaldson the Chief Medical Officer of England

announced wide ranging proposals in an attempt to reduce the burden of healthcare-associated infections. The plan, 'Winning Ways: Working together to reduce Healthcare Associated Infection in England' means that every NHS trust will get a designated director of infection control – a senior figure with experience in the discipline with the power to impose tough new rules on each hospital in order to reduce infection levels. He or she will lead dedicated infection control teams, charged with ensuring every possible step is taken to minimize the risk of infection. In addition, the NHS watchdog, the Healthcare Commission, will be asked to make infection control a key priority when assessing hospital performance.

The infection control link nurse

All healthcare workers need to ensure that effective infection control practices are implemented in the care of patients to achieve a reduction in healthcare-associated infection. It is important for wards and directorates to develop 'ownership' of infection control. One way of assisting this is by the use of infection control link nurses (ICLN) who have been implemented by infection control teams (ICT) as a method of improving practice at a clinical level (Cooper 2001; Dawson 2003).

ICLNs are a link or intermediary between the clinical areas/wards and ICTs. A key part of their role is to provide information to assist in the early detection of outbreaks of infection and to help increase awareness of infection control issues on their ward. Attention should be drawn to ICNs of changes of practice and equipment which could have implications for infection control (Hospital Infection Working Group, Department of Health and Public Health Laboratory Service 1995; Comptroller and Auditor General, National Audit Office 2000). In some trusts they have been trained to collect surveillance data on healthcare-associated infections for the ICT (Teare and Peacock 1996). It is essential that they have sufficient clinical experience and standing to have authority with managers and colleagues. ICLNs should not be seen as a substitute for the ICN, they are a ward-based resource who act under the supervision of the ICNs as a role model for colleagues. The ideal ICLN should be a keen, enthusiastic, motivated volunteer with a special interest in infection control (Charalambous 1995). The role of ICLNs is one that is still evolving. Many trusts have used them with success and they have been of particular value in the light of controls assurance and clinical governance (Hill et al. 2001). It is important for infection control to become the responsibility of directorates and the use of ICLNs at ward level can assist with this.

The ICC, which includes the augmented ICT, together with representatives of clinical departments and service departments, is not to be confused with the Outbreak Committee (OC), but is usually the body for discussing, monitoring, and approving the activities of the ICT listed above and lends it authority. It produces an action plan for infection control. It also produces general policies, encourages education, and reviews the implementation of the measures (Jenner and Wilson 2000; Naikoba and Hayward 2001).

It is important that the hospital ICT should have strong links with the community. This connection has been responsible for preventing many outbreaks of infection both in hospitals and outside. The consultant for communicable disease control (CCDC) has a general responsibility for infection control in the whole district and provides the main communication channel for the hospital ICT on such matters. In major outbreaks with implications wider than the hospital unit both the ICD and the CCDC will be actively involved. In outbreaks of typical nosocomial infection mainly restricted to hospital patients the ICD will lead the OC, with information to the CCDC.

The present arrangements for infection control in England and Wales were reviewed by Howard (1988) for the Hospital Infection Society and of the 207 Health Districts and London postgraduate hospitals approached, 93 percent responded. They were responsible for 95 percent of 'acute' hospital beds and 85 percent of other beds. Their replies identified 264 ICDs, of whom 82 percent were medical microbiologists; ICCs had been established in 92 percent of districts; 205 ICNs were in post and 93 percent of these worked closely with a medical microbiologist who was usually the ICD.

Thus the ICT, however constituted, has responsibility for the investigation of untoward occurrences and, even more importantly, for establishing microbiologically safe conditions in the hospital, for the provision of necessary supplies, and for ensuring safe practices in the day-to-day care of patients. Written statements of policies and procedures must be provided; the ICC and ICT must oversee the implementation of these and the education of staff in their use and interpretation. For detailed recommendations, the reader is referred to reviews (Bennett and Brachman 1992; Wenzel 1993; Philpott-Howard and Casewell 1994; J. Wilson 1995). Individual procedures may be required for special units, e.g. neonatal, pediatric, obstetric, intensive therapy, dialysis, transplantation, and oncology. In addition infection control is now an important part of risk management (Farrington and Pascoe 2001) and clinical governance (Masterton and Teare 2001).

Matters to be dealt with, in many cases in liaison with other groups of staff, are listed in Table 4.

In spite of the uniformity of approach to infection control in Europe, practices are diverse, as was revealed during a survey of infection in intensive care units (Vincent et al. 1995). In the USA and in Europe, there is an increasing movement towards providing and

Table 4 *General subjects for which infection control policies may be required*

Requirements
Provision of safe air supply (to operating theaters and accommodation for immunosuppressed patients)
Safe provision of water and food
Cleaning and general hygiene
Pest control
Waste disposal
Laundry
Decontamination (cleaning, disinfection, sterilization) of all equipment and materials
Ward procedures, including dressings and minor invasive procedures, urinary catheterization
Disposal of excreta
Venepuncture and vascular cannulation
Operating theater procedures
Antibiotic use, including prophylaxis
Isolation of patients and 'barrier nursing', to prevent spread of infection ('source isolation') or to protect susceptibles ('protective isolation')
Notification of infective disease and other liaison with community officers
Laboratory and pharmacy procedures
Staff health, immunization, and carriage of pathogenic microorganisms

unifying standards in hospital infection control against which practice can be judged and audit can be carried out (Infection Control Standards Working Party 1993; Cookson 1995; Jepsen 1995; McGowan 1995). It has been suggested that these should be 'outcome' rather than 'process' oriented (McGowan 1995). Although the methods available for measuring health benefit are limited, improving the health of the population by reducing the morbidity, mortality, and use of resources incurred by healthcare-associated infection must remain the aim of all the systems outlined above (Ward et al. 1997; Plowman et al. 1999; Comptroller and Auditor General, National Audit Office 2000). The National Audit Office has recently reviewed the extent of progress in the delivery of infection control in England since its original 2000 report (Comptroller and Auditor General, National Audit Office, 2004).

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Microbial susceptibility and resistance to chemical and physical agents

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INTRODUCTION

Chemical and physical agents have, in one form or another, been used for many centuries to destroy microorganisms. In ancient times, methods were empirical and it is only within the last 150 years or so that a scientific basis for them has been developed and their applications expanded. Early studies, considered in part by Hugo (1991a, 1999a) and Block (2001a), form the basis of the scientific consideration of antisepsis, disinfection, preservation, and sterilization.

This chapter will consider various types of chemical and physical agents, their medical and other uses, and the mechanisms of both microbial inactivation and microbial resistance. Because the subject area is so vast, appropriate review articles will be cited frequently together with salient research papers when necessary.

CHEMICAL AGENTS USED IN ANTISEPSIS, DISINFECTION, PRESERVATION, AND STERILIZATION

There are several types of chemical antimicrobial agents (for chemical structures, see Russell and Chopra 1996; Hugo and Russell 1999; Russell et al. 1999). They have a variety of uses, both medical and otherwise; their activity is influenced by many factors (physical, chemical, and biological); their efficacy may be evaluated by various techniques; and their mechanism of action varies from one type of chemical to another.

Terminology

Several important terms (see also BSI 1991; Block 2001b) are in regular use, including the following:

- *Sterilization*: the destruction or removal (by filtration) of all microorganisms.
- *Disinfection*: the destruction of most microorganisms, but not usually bacterial spores. Usually applied to the treatment of inanimate objects, it can also refer to the disinfection of the skin, e.g. before an operation. In some countries, a disinfectant is considered to be a substance that will also destroy bacterial spores.
- *Antisepsis*: the destruction or inhibition of microorganisms on living tissues, thereby limiting or preventing the harmful effects of infection.
- *Preservation*: the prevention of multiplication of microorganisms in formulated products (e.g. food, pharmaceuticals, cosmetics), thereby preventing spoilage or contamination, which could render the product a possible hazard to the consumer.

Other terms are more specific, e.g. those having the suffix 'stat' or 'cide.' A static agent, e.g. bacteriostat, fungistat, or sporistat, inhibits the growth of bacteria or fungi or germination of spores, respectively, whereas a cidal agent, e.g. bactericide, fungicide, sporicide, or virucide, kills bacteria, fungi, spores, or viruses, respectively. The general term 'biocide' refers to a chemical (nonantibiotic) agent that inactivates microbes. The term 'chemosterilant' (or sometimes 'chemosterilizer' or

'sterilant') is used to describe a chemical agent that is used to kill all microorganisms, including spores (Block 2001b).

Types and uses of chemical agents

Antimicrobial agents are widely used for many purposes (Table 1). Comprehensive data are provided by Gardner and Peel, (1991), Ayliffe et al. (1993), Gould (1995), Rutala (1990, 1995), McDonnell and Russell (1999), Russell (1999a, b, c, d, e), and Block (2001b). Fraise (1999) describes factors influencing the choice of hospital disinfectants, Coates and Hutchinson (1994) consider the relevant aspects of a suitable hospital disinfection policy and Rutala and Weber (1999a, b), the role of disinfection and sterilization in infection control, and the disinfection of endoscopes.

PHENOLS, BISPHENOLS, AND CRESOLS

Most phenols used as disinfectants are obtained from tar, a byproduct in the destructive distillation of coal. Fractionation of the tar yields a group of products including phenols (tar acids). A coal tar produced in a low-temperature carbonization process contains (in parentheses, boiling range in °C) phenol (182), cresols (189–205), xylenols (210–230), and high-boiling tar acids (230–310). The combined fraction of cresols plus xylenols is also available commercially as cresylic acid. Noncoal tar phenols are also available, e.g. 2-phenylphenol (*o*-phenylphenol). Phenol itself is now made in large quantities synthetically, as are some of its derivatives (Hugo and Russell 1999).

Para (4)-substitutions in phenols of an alkyl chain up to six carbon atoms in length increase antimicrobial activity. Activity is also improved by halogenation and by a combination of alkyl and halogen substitution; greatest activity is achieved when the alkyl group is in the *ortho*(2) and the halogen *para*(4) positions in relation to the phenolic group. Nitration also increases antimicrobial potency but unfortunately also enhances systemic toxicity; nitrophenols act as uncoupling agents and interfere with oxidative phosphorylation. Phenols are more active in the undissociated forms at acid than at alkaline pH (Russell and Chopra 1996; Hugo and Russell 1999).

Phenols and cresols are bactericidal to gram-positive and gram-negative bacteria, but bacterial spores are resistant at ambient temperatures. 2-Phenylphenol and the black fluids (see below) are particularly effective against mycobacteria, but bisphenols are ineffective. Phenols and cresols, especially when halogenated, possess antifungal activity. Lipid-enveloped viruses may be sensitive to phenolics whereas nonenveloped viruses are more resistant: 2-phenylphenol is active against both types. Triclosan (2,4,4′-trichlor-2′-hydroxydiphenylether) is a chlorinated phenylether (sometimes referred to as a bisphenol) used in some medicated soaps and hand-cleansing gels and as a body wash; it is predominantly bacteriostatic rather than bactericidal, but has a broad spectrum of activity, except for *Pseudomonas aeruginosa* (Schweizer 2001). Hexylresorcinol (HCP) is active mainly against gram-positive bacteria (Russell and Chopra 1996).

Black fluids consist of a solubilized crude phenol fraction prepared from tar acids (boiling range 250–310°C). On dilution with water, black fluids give either clear solutions or emulsions. White fluids consist of emulsified phenolic compounds. On dilution with water, they form weaker emulsions and are more stable in the presence of electrolytes than are black fluids.

ACIDS, ACIDULANTS, AND ESTERS

Many aliphatic and aromatic acids are employed as preservatives, especially in the food industry, and to some extent in pharmaceutical and cosmetic products. They include acetic, benzoic, propionic, and sorbic acids and the methyl, ethyl, propyl, and butyl esters (the parabens) of *para*(4)-hydroxybenzoic acids. They are not sporicidal and the activity of the acids, but not the esters, is very pH-dependent, since activity is associated mainly with the undissociated form. Acids may be added to various foods as acidulants, notably the organic lipophilic acids (benzoic, sorbic, propionic) referred to above. Organic acids of chain length greater than C_{10} or C_{11} are highly effective against gram-positive but not against gram-negative bacteria (Russell and Gould 1988), but their low solubility restricts their use (Sofos and Busta 1999). Lactic acid, an acidulant exhibiting preservative activity, is the main product of many food fermentations.

Citric acid is an approved disinfectant against foot-and-mouth virus. Lactic acid, CH_3-CH(OH)COOH, has been employed as an aerial disinfectant against nonsporing bacteria. Formic acid, HCOOH, and propionic acid have been used for controlling salmonellae in feedstuffs. Two mineral acids employed in veterinary work are hydrochloric and sulfuric acids. The former, but not the latter, is sporicidal and has been used at a concentration of 2.5 percent for disinfecting hides and skin contaminated with anthrax spores. In some countries, a 5 percent solution of sulfuric acid has been used, usually in combination with phenol, for decontaminating floors, feed boxes, and troughs (Russell and Hugo 1987).

ALKALIS

The antimicrobial action of alkalis is related to hydroxyl ion concentration. Sodium hydroxide (caustic soda, lye, soda lye, NaOH) possesses strong alkali properties. It kills most common vegetative bacteria and high concentrations (5 percent and above) are lethal to anthrax spores. Calcium hydroxide (hydrated lime, air-slaked

Table 1 *Summary of uses of some antimicrobial agents*

Group	Use(s)	Example(s)
Acids and esters	Preservation	Organic acids, parabens
	Aerial disinfection	Lactic acid
	Veterinary disinfection	Hydrochloric, sulfuric, citric acids
	Salmonella control in feedstuffs	Formic acid, propionic acid
Alcohols	Working surfaces, equipment, gloved hands (rapid action)	Ethanol, isopropanol
	Preservation	Bronopol, ethanol
Aldehydes	Disinfection/sterilization of thermolabile medical equipment	Glutaraldehyde, succinaldehyde-based products, orthophthalaldehyde
	Virucide in preparation of some human and veterinary vaccines; removal of warts; antiseptic mouthwash	Formaldehyde solution
	Topically; irrigation solutions; treatment of peritonitis	Formaldehyde-releasing agents, e.g. noxythiolin, taurolin[a]
	Cosmetic preservatives	Formaldehyde-releasing agents, e.g. imidazole derivatives
Alkalis	Vehicle disinfection	Sodium carbonate, trisodium phosphate
	Whitewashing surfaces	Calcium hydroxide
Alkylating agents	See Vapor-phase disinfectants	Ethylene oxide, propylene oxide, β-propiolactone
Amphoteric surfactants	Skin 'disinfection;' disinfection of surgical instruments; sanitizers and disinfectants in food industry	Dodecyl-di(aminoethyl)-glycine derivatives
Antibiotic	Food preservative	Nisin
Biguanides	Antiseptic, disinfectant, preservative in some ophthalmic products; antiplaque agent; veterinary teat dip	Chlorhexidine, alexidine
	Swimming-pool disinfection; application to surfaces in food industry; preservation of leather	Polyhexamethylenebiguanide (PHMB), a polymeric biguanide
Bisphenols	Surgical scrubs; medicated soaps; limited uses as preservative in cosmetics	Hexachlorophane
	Surgical scrubs; soaps; deodorants; hand-cleansing gels	Triclosan
Diamidines	Topical application to wounds	Propamidine, dibromopropamidine, as isethionates
Halogen-releasing agents	Disinfection of blood spillages containing HIV or HBV; industrial sanitizing compounds (food, dairy, restaurant, swimming pool); veterinary disinfection	Hypochlorites, dichloro- and trichloro-isocyanuric acids
	Disinfection of hands preoperatively; antiseptics; cleansing of dairy plant; veterinary teat dip	Iodophors (including povidone–iodine)
Heavy-metal derivatives	Algicides; fungicides; wood, paint, cellulose and fabric preservation	Copper derivatives
	Fungicides; bactericides; textile and wood preservation	Organotin compounds
	Pharmaceutical preservation	Organomercurials
	Prevention of infection in burns	Silver nitrate, silver sulfadiazine
Isothiazolones	Preservatives for cosmetics, toiletries and pharmaceuticals, fabrics	Mixture of chloromethyl and methyl derivatives
Peroxygens		
	Oxidizing agent used as antiseptic and disinfectant	Hydrogen peroxide
	Disinfection/chemosterilization of endoscopes	Peracetic acid
Phenols and cresols	Preservation	Phenol, cresol, chlorocresol
	Disinfection	Black fluids, white fluids
	Aerial disinfection	Hexylresorcinol
	Food preservatives	Phenolic antioxidants, e.g. butylated hydroxyanisole, butylated hydroxytoluene

(*Continued over*)

Table 1 *Summary of uses of some antimicrobial agents (Continued)*

Group	Use(s)	Example(s)
Quaternary ammonium compounds	Preoperative disinfection, bladder and urethra irrigation, ophthalmic preservation, skin disinfection, oral and pharyngeal antisepsis, cosmetic preservation of emulsions	Cetrimide, benzalkonium chloride, cetylpyridinium chloride (as appropriate)
Sulfites and nitrites	Food preservation	Sodium metabisulfite, sodium nitrite (carcinogenic?)
Vapor-phase disinfectants	Medical and pharmaceutical sterilization	Ethylene oxide
	Disinfection/sterilization of heat-sensitive materials	Low-temperature steam with formaldehyde
	Veterinary fumigation	Formaldehyde
	Decontamination	β-Propiolactone (carcinogenic?)
	Surface sterilization	Hydrogen peroxide (vapor phase)
	Range of disinfection and sterilization purposes	Ozone

HBV, hepatitis B virus; HIV, human immunodeficiency virus.
a) There is some doubt as to whether taurolin acts as a formaldehyde releaser.

lime, $Ca(OH_2)$) is produced and heat is generated when calcium oxide (lime, quicklime, CaO) is moistened with water. As a 20 percent suspension, calcium hydroxide is effective for whitewashing surfaces, which kills most types of nonsporing bacteria. Sodium carbonate (Na_2CO_3) is used primarily as a cleaning agent, a 4 percent w/v solution being used for washing vehicles before disinfecting them after a shipment of animals. It has also been used extensively as a cleaning agent in outbreaks of foot-and-mouth disease. Trisodium phosphate (Na_3PO_4) has similar properties and uses. Comprehensive data on the veterinary uses of alkalis are provided by Huber (1982) and Linton et al. (1987).

CHLORINE-RELEASING AGENTS

The stability of free available chlorine in solution depends upon a number of factors, in particular chlorine concentration, pH, presence of organic matter, and light (Dychdala 2001).

Hypochlorites and organic *N*-chloro compounds are the two most widely used types of chlorine-releasing agents (CRAs). The hypochlorites have a wide antimicrobial spectrum and are among the most potent sporicidal agents. They are active against enveloped and nonenveloped viruses (Springthorpe and Sattar 1990; Sattar et al. 1994; Maillard 2001; Prince and Prince 2001) but at one time were considered to be rather ineffective against mycobacteria (Croshaw 1971). More recent studies demonstrate, however, that they are mycobactericidal (Favero and Bond 1993; Dychdala 2001).

Two factors with pronounced effects on their antimicrobial action are (a) the presence of organic matter, chlorine being highly reactive; and (b) pH, the hypochlorites being more active in acid than in alkaline conditions: the active factor is undissociated hypochlorous acid, HClO (Dychdala 2001). Hypochlorite solutions gradually lose strength on storage, so fresh solutions must be prepared before use. Methanolic solutions buffered to pH 7.6–8.1 appear to show maximal stability and sporicidal activity and are worthy of further investigation. In addition to their medical uses, hypochlorites are used widely in the dairy industry and as disinfectants of farm buildings, e.g. for concrete floors, walls, and ceilings (Linton et al. 1987). Sodium hypochlorite is normally used for the disinfection of swimming pools.

N-chloro compounds, containing the =N–Cl group, possess microbicidal activity; they include chloramine T, dichloramine, halazone, and the sodium salts of dichloroisocyanuric (NaDCC) and trichloroisocyanuric acids. All appear to hydrolyze in water to produce an imino (=NH) group. Their action is slower than that of the hypochlorites, but this can be increased under acidic conditions. The disinfecting action of chloramine decreases less significantly than that of the other compounds in the presence of organic matter, and it has been used in veterinary practice for washing and spraying surfaces and for soaking items to be decontaminated. The uses of chlorine compounds are summarized in Table 1. NaDCC has been recommended as a disinfectant for use against various body spillages from acquired immune deficiency syndrome (AIDS) patients (Bloomfield et al. 1990; Bloomfield 1996; Dychdala 2001; Prince and Prince 2001).

Chlorine dioxide, a recently introduced instrument disinfectant (Knapp and Battisti 2001; McDonnell 2001), is more active than the hypochlorites at alkaline pH and in the presence of organic matter, although its activity may be reduced under dirty conditions. It is sporicidal, bactericidal, mycobactericidal (including activity against glutaraldehyde-resistant *Mycobacterium chelonae* strains), and fungicidal (Fraise 1999; Griffiths et al. 1999).

SUPEROXIDIZED WATER

Superoxidized water contains a mixture of oxidizing substances, the presence of hypochlorous acid probably contributing to a marked degree to its mechanism of action (Rutala and Weber 2001a, b). It is prepared by the electrolysis of saline solutions, the end product being water. It is rapidly microbicidal to a range of microorganisms in the absence (Selkon et al. 1999) and presence (Shetty et al. 1999) of serum, although activity against *Clostridium difficile* spores is greatly reduced (Shetty et al. 1999).

IODINE AND IODOPHORS

Iodine was first employed for the treatment of wounds some 150 years ago. Normally it is used in aqueous or alcoholic solution but it is only sparingly soluble in cold water; solutions can be made with potassium iodide. Iodine is an efficient microbicidal agent rapidly lethal to bacteria and their spores, molds, yeasts, and viruses. Antiseptic-strength iodine solutions are not sporicidal (Russell 1990a, b; Favero and Bond 1993). Iodine is less reactive than chlorine but, whereas the activity of high iodine concentrations is little affected by the presence of organic material, that of low concentrations is significantly reduced. The activity of iodine is greater at acid than at alkaline pH; the most active form is diatomic iodine (I_2). At acid and neutral pH, hypoiodous acid (HIO) is less bactericidal. At alkaline pH, hypoiodite ion (HIO^-) is even less active and iodiate (IO_3^-), iodide (I^-), and triiodide (I_3^-) ions are all inactive (Trueman 1971; Bloomfield 1996).

Because iodine has certain limitations in use, namely toxicity and staining of fabrics, attention has been turned towards the iodophors (literally, 'iodine carriers'), solutions in which iodine is solubilized by surface-active agents and that retain the microbicidal, but not the undesirable, properties of iodine (Bloomfield 1996). In most iodophor preparations the carrier is usually a nonionic surfactant, whereas in poloxamer iodine formulations the carriers are poloxamers, a series of nonionic polyoxethylene–polyoxypropylene polymers. When an iodophor is diluted with water, dispersion of the micellar aggregates of iodine occurs and most of the iodine is liberated slowly (Gottardi 1985). Dilutions of commercial povidone–iodine solutions may be more bactericidal (Berkelman et al. 1982) or sporicidal (Williams and Russell 1993) than undiluted stock solutions. The reasons are complex but iodine complexation is involved since the concentration of free iodine (I_2) determines activity (Gottardi 1985).

The iodophors are microbicidal with activity over a wide pH range. Provided that the pH does not rise above about 4, iodophors retain their antimicrobial potency in the presence of organic matter. There is a pronounced decrease in activity if solutions are diluted excessively with water that has a high alkaline hardness. The presence of a surface-active agent as carrier improves the wetting capacity. Iodophors are used in the dairy industry; when employed in the cleansing of dairy plant, it is important to keep the pH acidic with phosphoric acid, to ensure adequate removal of milkstone (dried residue of milk). They are also used for skin and wound disinfection. In some countries, alcoholic solutions of iodophors are widely used for disinfection of operation sites. In the veterinary context, iodophors formulated with phosphoric acid are employed as antiseptics, disinfectants, and teat dips (Huber 1982; Russell and Hugo 1987).

SURFACE-ACTIVE AGENTS

Surface-active agents (surfactants) have hydrophobic and hydrophilic regions in their molecular structure. On the basis of the charge or the absence of ionization of the hydrophilic group, these surfactants are classified into anionic, cationic, nonionic, and ampholytic (amphoteric) compounds.

Nonionic surfactants are not antimicrobial, but low concentrations of polysorbates (Tweens) are claimed to affect the permeability of the outer membrane of gram-negative bacteria (Brown 1975). High concentrations, however, neutralize the activity of some types of antimicrobial agents (Russell et al. 1979; Russell 1999a). *Anionic surfactants* usually have strong detergent but weak antimicrobial properties, except at high concentrations, which induce lysis of gram-negative bacteria (Salton 1968). Fatty acids are considerably more active against gram-positive than gram-negative organisms (Russell and Gould 1988).

Amphoteric agents combine the detergent properties of anionic with the antimicrobial properties of the cationic compounds. Activity remains virtually constant over a wide pH range and they are inactivated less readily than cationic surfactants by proteins. Examples of amphoteric surfactants are the Tego series of compounds (Hugo and Russell 1999).

For microbiological use, most important surface-active agents are the *cationic surface-active agents*, quaternary ammonium compounds (QAC) (Hugo and Russell 1999; Merianos 2001). They possess strong bactericidal but, at normal in-use concentrations, weak detergent properties. QACs may be considered as organically substituted ammonium compounds in which the nitrogen atom has a valency of five; four of the substituent radicals (R^1–R^4) are alkyl or heterocyclic and the fifth is a small anion. The sum of the carbon atoms in the four R groups is greater than ten. For a QAC to have high antimicrobial activity, at least one of the R groups must have a chain length in the range C_8–C_{18} (Hugo and Russell 1999). The QACs are primarily active against gram-positive, nonsporing bacteria; at high concentrations they are lethal to gram-negative organisms, although *P. aeruginosa* tends to be particularly resistant (Russell 1999a).

They are sporostatic but not sporicidal, and fungistatic rather than fungicidal. They are active against viruses with lipid envelopes, e.g. herpes and influenza, but are much less so against nonenveloped viruses, e.g. enteroviruses (Narang and Codd 1983; Resnick et al. 1986). A newer QAC product is claimed to have mycobactericidal activity (Nicholson et al. 1995) but no neutralizing agent was included in the test methodology.

The QACs are incompatible with a wide range of chemical agents, including nonionic and anionic surfactants, and phospholipids such as lecithin. Use is made of this property in evaluating the lethal effects of QACs by employing a combination of lecithin and a nonionic surfactant as a neutralizing agent (Russell et al. 1979). Antimicrobial activity is affected greatly by organic matter and by pH. Activity is greater in alkaline conditions because there is an increase in the degree of ionization of bacterial surface groups so that the cell surface becomes more negatively charged.

Organosilicon-substituted quaternary ammonium salts, organic amines, or amine salts with antimicrobial activity in solution are also highly effective on surfaces. One such, 3-(trimethoxysilyl)propyloctadecyldimethyl ammonium chloride, exhibits powerful antimicrobial activity while chemically bonded to a variety of surfaces (Malek and Speier 1982; Speier and Malek 1982). Uses of the QACs are summarized in Table 1.

BIGUANIDES AND POLYMERIC BIGUANIDES

The most important member of the family of N^1,N^5-substitued biguanides is chlorhexidine, which is available as dihydrochloride, diacetate, and gluconate, the last-named being the most water-soluble. Chlorhexidine has a wide spectum of activity against gram-positive and gram-negative bacteria but is not sporicidal or mycobactericidal; it is also generally considered as having a low activity against fungal spores and viruses (Russell and Day 1993; Ranganathan 1996; Hugo and Russell 1999; Russell 1999a, c; Denton 2001). Potency is reduced in the presence of serum, blood, pus, and other organic matter. Because of its cationic nature, activity is also reduced in the presence of soaps and other anionic compounds. Chlorhexidine is more active at alkaline pH because an increase in the degree of ionization of bacterial surface groups renders the cell surface more negatively charged (Hugo 1999b). The main uses of chlorhexidine are as a medical, dental, and veterinary antiseptic, as a disinfectant, and as a preservative in some types of pharmaceutical products (Russell and Day 1993).

Alexidine differs from chlorhexidine in that it posesses ethylhexyl end groups (Russell and Chopra 1996); it is also more rapidly bactericidal and produces a significantly faster alteration in bacterial permeability (Chawner and Gilbert 1989a,b).

Vantocil is a heterodispersed mixture of polyhexamethylene biguanides (PHMB) with a molecular weight of approximately 3 kDa. It is active against gram-positive and negative bacteria (*P. aeruginosa* and *Proteus vulgaris* are less sensitive) but is not sporicidal. Because of the residual positive charges on the polymer, PHMB is precipitated from aqueous solutions by anionic compounds. The activity of PHMB increases with increasing polymer lengths but synergistic effects have been observed in a mixture of polymeric biguanides (Gilbert et al. 1990a, b).

DIAMIDINES

The aromatic diamidines are organic cationic agents that show antimicrobial activity (Hugo 1971). The two most important members are propamidine (4,4′-diamidinophenoxypropane) and the more active dibromopropamidine, both used as the soluble isethionate salts. Gram-positive bacteria are considerably more sensitive than gram-negative organisms and activity decreases at acid pH and in the presence of organic matter. Bacteria cultured in the presence of increasing doses of a given diamidine rapidly acquire resistance to it and to other diamidines (Hugo 1971). The past decade or so has seen the isolation of multiple antibiotic-resistant strains of *Staphylococcus aureus* (Townsend et al. 1984) and *S. epidermidis* (Leelaporn et al. 1994), which possess plasmid-mediated resistance to cationic agents, including propamidine.

ALDEHYDES

Three aldehydes are important, namely the monoaldehyde, formaldehyde (methanal), and two dialdehydes, glutaraldehyde (pentane-1,5-dial) and *ortho*-phthalaldehyde, although other alkdehydes, e.g. succinaldehyde, also possess antimicrobial activity.

Formaldehyde

Formaldehyde (CH_2O) is employed in the liquid and vapor states; use of the latter is described below (see section on Gaseous and vapor-phase disinfectants).

Formaldehyde solution (formalin) is an aqueous solution containing 34–38 percent w/w of formaldehyde (CH_2O). The presence of methyl alcohol (methanol, CH_3OH) delays polymerization. Formaldehyde is lethal to bacteria and their spores (but less so than glutaraldehyde), fungi, and viruses. It combines readily with proteins and is less effective in the presence of organic matter. Formaldehyde is employed as a virucidal agent in the preparation of many human and veterinary vaccines (Russell and Hugo 1987), as an antiseptic mouthwash, for the disinfection of membranes in dialysis equipment, and as a preservative in hair shampoos.

Formaldehyde is often employed in the form of formaldehyde-releasing agents. Examples are (a) noxythiolin (hydroxymethylenethiourea), a bactericidal agent widely used both topically and in accessible body cavities, e.g. an irrigation solution in the treatment of peritonitis (Browne and Stoller 1970); (b) taurolin, in

which the amino acid taurine acts as a formaldehyde carrier (Browne et al. 1976, cf. Myers et al. 1980); (c) hexamine (methenamine), imidazole derivatives, triazines, and oxazolo-oxazoles. For further information, see Hugo and Russell (1999).

Glutaraldehyde

Glutaraldehyde (GTA) is a saturated five-carbon dialdehyde with an empirical formula of $C_5H_8O_2$ and a molecular weight of 100.12. It is highly active, reacting with enzymes and proteins but only slightly with nucleic acids; it prevents dissociation of free ribosomes. Probably because of increased interaction with $-NH_2$ groups, such activities increase with increasing pH, a factor of considerable importance to microbicidal activity (Russell 1994; Power 1997).

GTA possesses high microbicidal activity against bacteria and their spores, mycelial and spore forms of fungi, and various types of viruses, including human immunodeficiency virus (HIV) and enteroviruses (Hanson et al. 1994; Russell 1994). Despite earlier reports to the contrary, GTA is now considered to be mycobactericidal (Russell 1998a), although *Mycobacterium avium–intracellulare* might be of above-average resistance (Hanson 1988) and highly GTA-resistant strains of *Mycobacterium chelonae* have been isolated (van Klingeren and Pullen 1993; Walsh et al. 1999a, b, 2001; Fraud et al. 2001, 2003). In solution, GTA is more stable at acid than at alkaline pH, whereas the converse is true for its antimicrobial activity. In practice, 2 percent (or greater) solutions of GTA are alkalinated when required and are used within a stipulated period. Some formulations are available that appear to have overcome the problem of stability (Babb et al. 1980).

The dialdehyde is an important fixative in leather tanning, in electron microscopy and in biochemistry. It is employed widely for the disinfection or sterilization of medical equipment liable to damage by heat, in particular endoscopes. Nevertheless, there is the possibility of severe toxic reactions arising in personnel and its replacement by other equally microbiologically active agents is being actively pursued.

Ortho-phthalaldehyde

Ortho-phthaladehyde (OPA) (Alfa and Sitter 1994) is a recently inroduced (1998) cyclic dialdehyde. It is used, at a concentration of 0.55 percent and unadjusted pH, predominantly for the disinfection of endoscopes. OPA has potent lethal activity against gram-positive (including mycobacteria) and -negative bacteria (Alfa and Sitter 1994; Gregory et al. 1999; Walsh et al. 1999a, b, 2001; Fraud et al. 2001, 2003). At ambient temperatures, in-use concentrations and unadjusted pH, OPA has a low order of sporicidal activity (Walsh et al. 1999a, b), but sporicidal activity is observed when the concentration is increased and solutions made more alkaline.

Significantly, OPA is lethal towards GTA-resistant *M. chelonae* strains (Walsh et al. 1999a, b, 2001; Gregory et al. 1999; Fraud et al. 2001, 2003).

ALCOHOLS

Generally, alcohols rapidly kill bacteria (Morton 1983), including acid-fast bacilli, but are not sporicidal and have poor activity against some viruses, although HIV type 1 is susceptible to ethanol and isopropanol in the absence of organic matter (van Bueren et al. 1994). The presence of water is essential to the antimicrobial action of ethanol, which is most effective at concentrations of 60–70 percent (Price 1950).

Isopropanol (propan-2-ol) is a more effective bactericide. Benzyl alcohol (phenylmethanol) is a weak local anesthetic and also possesses antimicrobial properties. Phenylethanol (phenylethyl alcohol) is selectively active against gram-negative bacteria in mixed flora and is sometimes used as a preservative in ophthalmic solutions. Phenoxyethanol (phenoxetol) has significant activity against *P. aeruginosa* but less against other bacteria (Hugo and Russell 1999). Bronopol (2-bromo-2-nitropropane-1,3-diol) has a broad spectrum of activity, which is reduced in the presence of serum and especially of sulfydryl compounds. The effect of pH on its activity is complex (Croshaw and Holland 1984). Bronopol is widely used as a cosmetic preservative. Chlorbutanol (chlorbutol, trichloro-*t*-butanol) has been used as a bactericide in solutions for injection, but its instability presents a problem.

ISOTHIAZOLONES

Three isothiazolones have been studied comprehensively: 1,2-benzisothiazol-3-one (BIT), 5-chloro-*N*-methylisothiazol-3-one (CMIT), and *N*-methylisothiazol-3-one (MIT). They are widely used as industrial preservatives. Their activity is rapidly quenched by thiol-containing compounds and by valine and histidine, nonthiol amino acids (Collier et al. 1990).

DYES

Three groups of dyes find application as antimicrobial compounds:

- The acridines, which are heterocyclic compounds and which have been studied extensively. Albert (1979) showed that small changes in their chemical structure cause significant changes in biocidal properties, the most important factor being ionization, which must be cationic in nature. Acridines compete with H^+ ions for anionic sites on the bacterial cell and are more effective at alkaline than at acid pH.
- The triphenylmethane dyes (e.g. crystal violet, brilliant green, and malachite green), which have been used as topical antiseptics. However, their uses were

limited because they are effective only against gram-positive bacteria (Hugo and Russell 1999). This property does, however, have a practical application in the formulation of selective media for diagnostic purposes. Like the acridines, these dyes are more active at alkaline pH.

- The quinones, which are natural dyes imparting color to many forms of plant and animal life. Some members are important agricultural fungicides, notably chloranil and dichlone (Owens 1969; D'Arcy 1971).

HEAVY-METAL DERIVATIVES

The salts of heavy metals are sometimes employed as antimicrobial agents. The main antimicrobial use of copper derivatives is as algicides and fungicides; some copper compounds are used as preservatives in wood, cellulosics, paints, and fabrics (Hilditch 1999; Springle 1999).

Mercury compounds, in the form of organic derivatives (e.g. phenylmercuric nitrate and acetate), are still, despite criticism, sometimes used as preservatives for parenteral and ophthalmic solutions. Thiomersal serves this purpose for various immunological products. They are active against both gram-positive and gram-negative bacteria but are sporistatic, not sporicidal, at ambient temperatures (Russell 1998b; Hugo and Russell 1999).

Organotin compounds are used as biocides (e.g. fungicides, bactericides) and textile and wood preservatives (Hilditch 1999; McCarthy 1999).

Silver and its salts have long been used as antimicrobials. In recent years, silver nitrate has been employed to prevent infection of burns. Lowbury (1992) reviewed the use of this compound, at a concentration of 0.5 percent, for topical antimicrobial prophylaxis, and as a more satisfactory topical prophylactic, silver sulfadiazine (which, however, has the disadvantage that many bacteria are sulfonamide-resistant).

Russell and Hugo (1994) have described the antimicrobial properties and actions of various silver compounds.

PERMEABILIZING AGENTS

A permeabilizing agent is a chemical that increases the permeability of the outer membrane of gram-negative bacteria.

Chelating agents are not usually considered as being antimicrobial agents, but ethylenediamine tetraacetic acid (EDTA) enhances the activity of many antiseptics and disinfectants. The potentiating effects by EDTA on antibacterial agents acting against staphylococci (Kraniak and Shelef 1988) are likely to be the result of sequestration of metal ions present in the growth media rather than an increase in cellular permeability. In medicine, EDTA is used to treat chronic lead poisoning and pharmaceutically as a stabilizer in certain parenteral and ophthalmic preparations (Russell 1999a). In the context of disinfection, EDTA potentiates the effects of many antibacterial agents against gram-negative, but not usually gram-positive, bacteria (Russell and Furr 1977; Ayres et al. 1993, 1998a, b, c, d).

Polyethyleneimine (PEI) is a newer type of permeabilizer (Helander et al. 1997, 1998) that increases the permeability of gram-negative bacteria to several biocides.

ANILIDES

Anilides have the general structure C_6H_5NHCOR. In salicylanilide, R is C_6H_4OH and in carbanilide (diphenylurea) it is C_6H_5NH. Salicylanilide was introduced in 1930 as a fungistat for use in textiles and has also been used in ointment form for treating ringworm. Of the many substituted salicylanilides tested, the tribromo- and tetrachlorosalicylanilides have been the most widely used as antimicrobial agents. Their photosensitizing properties have, however, restricted their use in situations in which they come into contact with human skin. Trichlorocarbanilide, although one of the most potent members of the substituted carbanilides, has the same disadvantage.

QUINOLINE AND ISOQUINOLINE DERIVATIVES

There are three main groups: 8-hydroxyquinoline, 4-aminoquinaldinium, and isoquinoline derivatives. 8-Hydroxyquinoline (oxine) is a chelating agent active only in the presence of certain metal ions (Albert 1979). The 4-aminoquinaldinium derivatives are QACs that contain one or more quinoline ring systems; examples are laurolinium acetate and dequalinium chloride (a bis-QAC), both of which are active against gram-positive bacteria and many species of yeast and fungi (D'Arcy 1971). The most important isoquinoline derivative is hedaquinium chloride, another bisquaternary salt, which possesses antibacterial and antifungal properties.

PEROXYGENS

A peroxygen is a compound containing an –O–O– group. Hydrogen peroxide and peracetic acid are important peroxygens but others also find application.

Hydrogen peroxide

Hydrogen peroxide, H_2O_2, is active against gram-positive and gram-negative bacteria. Because they are unable to produce catalase, anaerobes are particularly susceptible. At high concentrations, hydrogen peroxide is sporicidal; this activity increases as the temperature is raised (Bloomfield 1999; Block 2001b). Activity is less affected by pH than many other biocides and is enhanced in the presence of cupric (Cu^{2+}) of ferric (Fe^{3+}) ions, which promote free-radical oxidation of organic compounds with peroxide. A mixture of peroxide and Cu^{2+}, but neither alone, produces strand

breaks in DNA (Sagripani and Kraemer 1989). It is environmentally friendly because its decomposition products are oxygen and water (Fraise 1999). Some uses are described in Table 1.

New hydrogen peroxide products have been described. A product based on accelerated and stabilized hydrogen peroxide (ASHP) had broad-spectrum activity, the undiluted form being sporicidal (>7/8 log reduction in 6 h), mycobactericidal (>6 log reduction in 20 min), and fungicidal (5 log reduction in 5 min) at 20°C (Sattar et al. 1999). AHSP was shown to be an excellent hard-surface cleaning agent with potent microbicidal activity (Rochon and Sullivan 1999; Alfa and Jackson 2001).

Peracetic acid

The best known example of a peracid is peracetic acid, CH_3COOOH, a strong oxidizing agent that is rapidly lethal for a wide range of microbes, including bacterial spores, viruses, and fungi (Baldry 1983; Broadley et al. 1995; Fraise 1999; Block 2001d). It has greater lipid solubility and more potent antimicrobial activity than hydrogen peroxide. It is sporicidal even at low temperatures and effective in the presence of organic matter (Block 2001c). The lethal species is the •OH radical (Clapp et al. 1994). Peracetic acid is frequently used as a chemosterilant (see section on New low-temperature sterilization technologies).

Performic acid

Performic acid, HCOOOH (see section on New low-temperature sterilization technologies) is a new proprietary liquid sterilant that is rapidly sporicidal (Rutala and Weber 2001a).

Ozone

Ozone, O_3, is an allotropic form of oxygen. Because of its powerful oxidizing properties (Schneider 1998; Weavers and Wickramanayake 2001), ozone is bactericidal, virucidal and sporicidal, although spores are some 10–15 times more resistant than vegetative cells. Gaseous ozone reacts with amino acids, especially those containing sulfur, and with RNA and DNA. In water, ozone is unstable chemically but activity persists because of the production of free radicals, including •OH. Its unstable nature means that ozone has to be produced on site (Schneider 1998).

SULFITES AND NITRITES

Traditional chemical food preservatives include common salt, sucrose, spices, and smoke and its components. Sulfur dioxide has for centuries been used as a fumigant and as a wine preservative. Metabisulfites have been widely used as antioxidants in foods (and in some pharmaceutical products), and sulfur dioxide and sulfites are used to preserve a variety of food products (Gould and Russell 1991; Sofos and Busta 1999).

Nitrite (NO_2^-) and nitrate (NO_3^-) have been used for centuries in meat processing. By reaction with heme proteins, nitrites are responsible for color formation in cured meat. They are antimicrobial, especially against outgrowing *Clostridium botulinum* spores. Nitrates function solely as a source of nitrite. The antimicrobial activity of nitrite is affected by many factors (Roberts et al. 1991). Carcinogenic nitrosamines are formed in some cured meat products cooked under certain conditions.

GASEOUS AND VAPOR-PHASE DISINFECTANTS

Gaseous and vapor-phase chemical agents have long been used to achieve disinfection and sterilization. Sulfur dioxide (obtained by burning sulfur) and chlorine found early application for fumigating sickrooms, but the scientific basis for their use was established only comparatively recently. A brief historical account is provided by Richards et al. (1984). Kaye and Phillips (1949) reviewed early studies on ethylene oxide (ETO).

The most important vapor-phase agents are ETO and formaldehyde.

Ethylene oxide

Ethylene oxide, $(CH_2)_2O$, is a colorless gas that is soluble in water, most organic solvents, and oils and diffuses into rubber in a manner similar to entering a solution (Joslyn 2001). It is inflammable when more than 3 percent is present in air, but this hazard can be overcome by mixing it with carbon dioxide or an appropriate hydrochlorofluorocarbon (HCFC) compound. Chlorofluorocarbons originally used for this purpose are no longer permitted (Alfa et al. 1998) and HCFCs are banned in some countries.

The antimicrobial activity of ETO depends upon several factors, notably relative humidity (RH), temperature, concentration, and time, and especially on the presence of water vapor (Ernst 1974; Joslyn 2001). Its high toxicity must be borne in mind when devising safe sterilization procedures. Furthermore, porous materials absorb the gas to various degrees during the sterilization cycle, so various periods of time after sterilization must be allowed for desorption of residual ETO. ETO is used as a decontaminating agent, for sterilizing ophthalmic and anesthetic equipment, crude drugs, and powders, and in veterinary practice for fumigating egg shells.

Formaldehyde

Formaldehyde gas can be generated by various means, namely evaporation of commercial formaldehyde solution (formalin), addition of formalin to potassium permanganate, or volatilization of paraformaldehyde,

$HO(CH_2)_nH$, where n = 8–100. Its antimicrobial activity depends upon several factors, including RH; it increases with RH up to a figure of 50 percent, but higher RH values confer little further advantage (Nordgren 1939). Formaldehyde is toxic, and inhalation of the vapor may pose a risk of carcinogenesis; adequate precautions should be taken to protect personnel. Low-temperature steam with formaldehyde (LTSF) has been used for disinfecting or sterilizing heat-sensitive materials (Russell 1999a). In the veterinary field, formaldehyde vapor is an important fumigant of animal buildings (Russell and Hugo 1987).

Other gases and vapors

These include

- β-Propiolactone (BPL). Its activity depends primarily on RH, concentration, and temperature. BPL may be carcinogenic, but it has been claimed to have a use in decontamination of animal premises. Liquid BPL is used widely in the preparation of many veterinary viral vaccines.
- Methyl bromide, CH_3Br. This is less active than ETO and is highly toxic but has been used as a fumigant.
- Propylene oxide, C_3H_6O. This is also less active than ETO. It has been used as a decontaminating agent, e.g. for animal feeds.
- Ozone. This is discussed above.
- Carbon dioxide, CO_2. This inhibits the growth of bacteria, including slime-producing bacteria, in soft drinks. Its activity depends upon low temperatures, its addition at an early stage, and its concentration.
- Hydrogen peroxide vapor. This has been used for the sterilization of surfaces in flexible and rigid-wall isolators and small rooms (Groschel 1995) and for endoscopes; it also has dental applications (Schneider 1998).
- Gaseous chlorine dioxide. This is unstable at high concentrations and must thus be generated on site (Schneider 1998).

Gas and vapor-phase plasmas are discussed in the section on New low-temperature sterilization technologies.

AERIAL DISINFECTANTS

An effective aerial disinfectant should be capable of being dispersed so as to ensure its complete and rapid mixing with infected air. An effective concentration should be maintained in the air and the disinfectant must be highly and rapidly active against air-borne microorganisms at different relative humidities. In addition, it should be nontoxic and nonirritant.

Early samples of aerial disinfectants were fumigants, such as sulfur dioxide and chlorine, employed in sickrooms. However, aerosols – which consist of a very fine dispersed liquid phase in a gaseous (air) disperse phase – are the most important form of aerial disinfectant.

Examples of aerial disinfectants are: (a) hexylresorcinol, which is vaporized from a thermostatically controlled hotplate; (b) lactic acid, which is effective but unfortunately an irritant at high concentrations; (c) propylene glycol, which may be used as a solvent for dissolving a solid disinfectant prior to atomization, but which is also a fairly effective and nonirritating antimicrobial agent in its own right; and (d) fumigants such as formaldehyde.

NATURAL ANTIMICROBIAL SYSTEMS

Natural antimicrobial systems (reviewed by Wilkins and Board (1989), Board (1995)) occur in animals, plants, and microorganisms. Major components of natural defense systems are classified broadly into inducible, e.g. complement, and constitutive. Principal representatives of the latter are enzymes, lysozyme (a cell-wall lytic agent, see section on Mechanisms of action of antimicrobial agents) and lactoperoxidase (LP), which mediates the production of compounds toxic to foreign, but not to host, cells. LP is most abundant in bovine cells. With a halide or thiocyanate and hydrogen peroxide, LP forms a potent antimicrobial lactoperoxidase system.

Additionally, transferrin in mammalian blood cells and milk, and lactoferrin in milk, may show antibacterial activity. They both act as iron-binding proteins, inducing lipopolysaccharide loss from the outer membranes of gram-negative bacteria and an increased cellular permeability (Russell and Chopra 1996).

Factors influencing activity

The activity of antimicrobial compounds may be influenced by various extraneous factors, including concentration, pH, time, temperature, type, number, growth phase, and location of microorganisms, and the presence of other agents that may reduce or increase their potency (Russell 1999a). A sound knowledge of these factors is essential for their most effective deployment, e.g. in the design of hospital disinfection policies (Coates and Hutchinson 1994) and in selecting disinfectant agents for particular purposes (Fraise 1999; Rutala and Weber 1999a, b).

CONCENTRATION OF ANTIMICROBIAL AGENT

The effect of concentration, or dilution, on the activity of an antimicrobial compound is not a simple arithmetic one. As was first pointed out by Krönig and Paul (1897), microbial death is not an all-or-nothing response but depends greatly on the period of contact and concentration (Figure 1).

In kinetic studies, the concentration exponent (dilution coefficient, η) is used to express the effect of

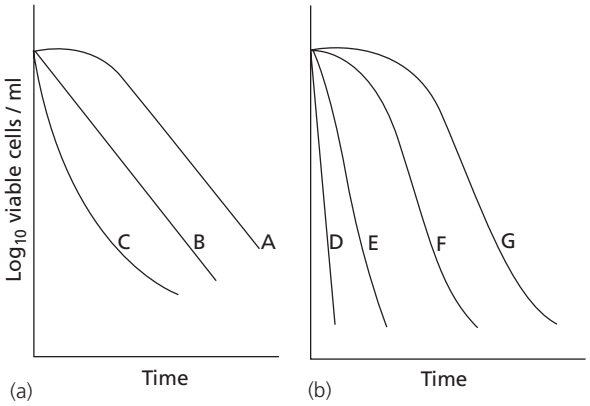

Figure 1 *Examples of time–survivor curves of bacterial suspensions exposed to disinfectants.* **(a)** *Bacterial responses: A, initial 'shoulder' followed by exponential death; B, exponential death; C, exponential death followed by 'tailing.'* **(b)** *Effects of different concentrations (D, highest; G, lowest) of phenol acting on* Escherichia coli. *At the lowest concentration, a sigmoidal curve is produced. This becomes less pronounced as the concentration increases, leading to an apparent straight-line response at the highest concentration.*

changes in concentration (or dilution) on cell-death rate. Its value may be determined by measuring the times necessary to kill the same number of bacteria in a suspension exposed to two concentrations of the antimicrobial agent. If C_1 and C_2 represent the two concentrations and t_1 and t_2 the respective times to reduce the viable population to the same end point, then

$$C_1 \eta t_1 = C_2 \eta t_2 \qquad (1)$$

from which

$$\eta = (\log t_2 - \log t_1)/(\log C_1 - \log C_2). \qquad (2)$$

Alternatively, η may be calculated from the slope of the straight line resulting when $\log_{10}$ death time ($\log t$) is plotted against $\log_{10}$ concentrations ($\log C$) (see Russell 1999a). Examples of η values are provided in Table 2.

A decrease in concentration of compounds with high η values results in a pronounced increase in the time necessary to achieve a comparable kill, other conditions remaining constant. By contrast, agents with low η values are much less affected (Table 2). Concentration is the single most important factor affecting biocidal activity (McDonnell and Russell 1999; Russell and McDonnell 2000).

Knowledge of the effect of concentration on antimicrobial potency is essential:

- in evaluating activity
- in testing medical and pharmaceutical products for sterility
- in ensuring adequate concentrations of preservative in pharmaceutical, cosmetic and food products, and
- in providing adequate concentrations of antiseptics and disinfectants for practical purposes (Russell 1999a).

TEMPERATURE

The activity of a disinfectant or preservative is usually increased when the temperature at which it acts is raised, although compounds vary considerably in response to temperature changes (Table 2).

A useful formula for measuring the effect of temperature on activity is given by

$$\theta^{T2-T1} = k_2/k_1 = t_1/t_2 \qquad (3)$$

in which θ is the temperature coefficient, k_2 and k_1 the rate (velocity) constants at temperatures T_2 and T_1, respectively, and t_2 and t_1 the respective times to bring about a complete kill at T_2 and T_1.

The velocity constant (k) can itself be obtained from a knowledge of the time (t) taken to reduce the initial viable number of cells (N_0) to a value N_t at time t, thus:

$$k = \frac{1}{t}\log_e \frac{N_o}{N_t} = \frac{1}{t} 2.303 \log_{10} \frac{N_o}{N_t}. \qquad (4)$$

The temperature coefficient, θ, refers to the effect on activity per 1°C rise and is nearly always between 1.0 and 1.5 (Bean 1967). It is more meaningful to specify the θ^{10} (also known as Q^{10}) value, which is the change in activity per 10°C rise in temperature (Table 2). At temperatures above about 40°C, there is little, if any,

Table 2 *Effects of concentration and temperature on activity of some antimicrobial agents*

Concentration exponents (η)[a]	Temperature coefficients (Q_{10} [or θ^{10}] values)[b]
Group A (η < 2)	θ^{10} 1.5–5
Hydrogen peroxide, mercurials, chlorhexidine, formaldehyde, QACs	Phenolics, ethylene oxide, β-propiolactone, phenols, cresols
Group B (η = 2–4)	θ^{10} 30–50
Parabens, sorbic acid	Aliphatic alcohols
Group C (η > 4)	
Aliphatic alcohols, phenolics, benzyl alcohol, phenethanol	

QAC, quaternary ammonium compound.
a) Based on Hugo and Denyer (1987).
b) Based on Bean (1967).

difference in activity between acid and alkaline glutaraldehyde, although the latter formulation is less stable (Gorman et al. 1980).

ENVIRONMENTAL PH

pH can influence the activity of antimicrobial agents in the following ways:

- Changes may occur in the molecule. Phenols and benzoic, sorbic, and dehydroacetic acids are effective predominantly in the unionized form and, as pH rises, their degree of dissociation increases. It is claimed that the dissociated form of sorbic acid may make a small contribution to activity.
- Changes may occur in the microbial cell surface. As pH increases, the number of negatively charged groups on the cell surface increases, causing enhanced binding of positively charged molecules, e.g. QACs (Hugo 1999b), dyes (Moats and Maddox 1978), acridines (Albert 1979), and chlorhexidine (Hugo 1999b). It must also be pointed out that the sporicidal activity of sodium hypochlorite is potentiated in the presence of methanol, although there is no simple relationship between activity, stability, and change of pH of the mixture (Coates and Death 1978). Maximal sporicidal activity and stability are achieved by buffering hypochlorite alone or a hypochlorite–methanol mixture to within a pH range of 7.6–8.1 (Death and Coates 1979).

INTERFERING SUBSTANCES

Organic matter

Organic soiling matter may be present as, for example, serum, blood, pus, soil, food residues, dried milk (milkstone), or fecal material; it can interfere with the action of an antimicrobial agent, usually because an interaction results in a reduced effective concentration of the latter (Russell 1999a).

Such reduction in activity is most noticeable with highly reactive compounds such as chlorine disinfectants. Iodine and iodophors, because of their lower chemical reactivity, are influenced to a rather lesser extent. Cationic agents such as QACs and chlorhexidine show a considerable reduction in activity, whereas the efficacy of phenols depends upon the actual phenolic compound; lysol, for example, retains much of its activity in the presence of feces and sputum (Russell 1999a).

Adequate precleaning before employment of a disinfectant, or a combination of disinfectant with a suitable detergent, may overcome the problem caused by the presence of organic soil. However, it should be noted that the activity of disinfectants may suffer considerably in the presence of certain detergents.

Surface-active agents

Cationic bactericides and anionic surfactants are incompatible. Low concentrations of nonionic surfactants such as polysorbates, tritons, and tergitols increase the activity of cationic agents and of parabens (Allwood 1973), whereas at higher concentrations of the nonionic detergents, significantly greater concentrations of the antimicrobial substances are necessary to inhibit or kill microbes. It is believed that below the critical micelle concentration (CMC) of the nonionic compound, potentiation occurs by action on the surface layers of the bacterial cell, resulting in enhanced permeability to the antimicrobial agent. Above the CMC, it either forms a complex with the antimicrobial compound or the latter is partitioned between the aqueous and micellar phases, only the concentration in the aqueous phase being available for microbial attack.

Because of their ability to inactivate various types of antimicrobial compounds, nonionic surfactants are frequently employed as neutralizing agents in sterility testing and in antimicrobial evaluation (Russell et al. 1979; Sutton 1996; Fassihi 2001).

Oils

A problem encountered in the formulation of pharmaceutical and cosmetic creams and emulsions is that the antimicrobial activity of a preservative may be high in aqueous conditions but much less so when an oil is present. The reason for this is that the preservative is partitioned between the oily and aqueous phases (Bean 1972; Fassihi 2001).

Other factors

The hardness of the water with which disinfectants are prepared or diluted is a contributory factor in reducing the effectiveness of QACs and iodophors; black (but not white) fluids are incompatible with hard waters. Partitioning into rubber may cause problems in the preservation of multiple dose parenteral and ophthalmic solutions. Relative humidity has a profound influence on the activity of gaseous disinfectants (see section on Gaseous and vapor-phase disinfectants).

CONDITION, GROWTH PHASE, AND SIZE OF MICROBIAL POPULATION

It is clearly easier for an antimicrobial agent to be effective when there are few microorganisms against which to act. Adequate and thorough precleaning is usually an important prerequisite to a disinfection process. Exponential phase cells of *S. aureus* (Luppens et al. 2002) and *Listeria monocytogenes* (Luppens et al. 2001) are more susceptible to cationic and oxidizing biocides than cells in the decline phase.

The association of bacteria (or other microorganisms) with solid surfaces leads to the production of a biofilm, a consortium of bacteria organized within an extensive exopolymer (glycocalyx). Within the depths of the biofilm, growth rates are likely to be reduced as a

consequence of nutrient limitation (Poxton 1993), which can alter the bacterial cell surface and hence be responsible for modified sensitivity to antibacterial agents (Costerton et al. 1987; Carpentier and Cerf 1993; Stickler and King 1999). This is one reason for the reduced sensitivity of the sessile cells found in biofilms; other reasons include chemical reaction with glycocalyx and prevention by the glycocalyx of access of biocide to the underlying cells. This aspect is considered in more detail below (see Biofilms and resistance).

Types of microorganism

The activity of antimicrobial agents depends greatly upon the type of microorganism present. It is therefore pertinent to examine the sensitivity of various types of microbes which, for convenience, have been classified into the following groups.

GRAM-POSITIVE BACTERIA (COCCI)

Generally, cocci are more sensitive to antimicrobial agents than are gram-negative bacteria (Baird-Parker and Holbrook 1971; Russell and Gould 1988). Cocci are readily killed by halogens, phenols, especially bisphenols, and QACs. However, multiple antibiotic-resistant *S. aureus* and *S. epidermidis* may show reduced sensitivity to cationic-type agents (see Types and uses of chemical agents). Clinical isolates of enterococci (*Enterococcus faecium* and *E. faecalis*) are often antibiotic-resistant and include vancomycin-resistant enterococci (VRE), but do not appear to be more resistant to chlorhexidine than staphylococci (Baillie et al. 1992; Alqurashi et al. 1996). The effects of biocides on antibiotic-resistant cocci are discussed by Day and Russell (1999).

MYCOBACTERIA

The sensitivity of acid-fast bacteria to disinfectants is intermediate between that of other nonsporulating bacteria and bacterial spores (Ayliffe et al. 1993; Favero and Bond 1993; Russell 1998a; Lauzardo and Rubin 2001). QACs and dyes inhibit tubercle bacilli but do not kill them. *Mycobacterium tuberculosis* is also insensitive to chlorhexidine, acids, and alkalis, but moderately sensitive to amphotericsurface-active agents, including the Tego compounds. Of the phenols, 2-phenylphenol is particularly effective, but the bisphenols are inactive. Alcohols, liquid and vapor-phase formaldehyde, formaldehyde–alcohol, iodine–alcohol, and ethylene oxide are all tuberculocidal; despite earlier doubts to the contrary, so is GTA (Favero and Bond 1993). However, *M. avium–intracellulare* (Hanson 1988) and *M. chelonae* (van Klingeren and Pullen 1993) may be resistant to the dialdehyde (see also Terminology).

GRAM-NEGATIVE BACTERIA

Many types of gram-negative bacteria, especially *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *P. aeruginosa*, and *Serratia marcescens*, are increasingly implicated as hospital pathogens. In the veterinary context, bacteria causing zoonotic diseases include *Campylobacter* spp., salmonellae, and brucellae (Linton 1983; Russell et al. 1984). Gram-negative bacteria are usually less sensitive than gram-positive bacteria, and *P. aeruginosa* and *Proteus* spp. may contaminate solutions of QACs (Russell et al. 1986). These bacteria, and others such as *Providencia stuartii*, are also more resistant to chlorhexidine than is *E. coli* (Ismaeel et al. 1986).

Legionella pneumophila is frequently found in domestic water and cooling-water systems of large buildings. Although outbreaks of legionnaires' disease are uncommon, they are associated with high morbidity and some deaths when they occur. Adequate control measures must be taken. Neither raising the temperature of hot-water systems nor continuous chlorination alone is completely effective. Control of the organism in recirculating water systems has also been studied, but extrapolation from laboratory conditions to such systems is often unrealistic because of possible interaction between the biocide and other water-treatment chemicals as well as slime (Elsmore 1999). Wang et al. (1979) found hypochlorite to be effective against *L. pneumophila*, but Kurtz et al. (1982) showed a chlorinated phenol to be more effective than a QAC–tributyl tin oxide mixture or sodium dichloroisocyanurate. Bronopol is effective both under laboratory conditions and in cooling towers (Elsmore 1999).

BACTERIAL SPORES

Many antibacterial compounds have no or little sporicidal activity. Nevertheless, such chemicals are not entirely without effect on spores because they may prevent germination or outgrowth, or both. Chemicals that are sporistatic rather than sporicidal include phenols and cresols, parabens, QACs, biguanides, alcohols, dyes, and mercury compounds. Comparatively few substances are sporicidal and even then the process may be comparatively slow. Examples of sporicides are hydrogen peroxide, hypochlorites, glutaraldehyde, formaldehyde, iodine compounds, ethylene oxide, peracetic acid, and β-propiolactone (Russell 1990a, 1998a, b; Bloomfield and Arthur 1994; Setlow 1994; Bloomfield 1999). Vegetative, rod-shaped cells of *Bacillus* spp. are considerably more sensitive to biocides than the corresponding spore forms (Russell 2003b, c).

MOLDS AND YEASTS

Several species of molds and yeasts are pathogenic. Others are important in spoiling foods and pharmaceutical and cosmetic products.

Many compounds show both antibacterial and antifungal activity, although the latter may be fungistatic rather than fungicidal (Russell and Furr 1996; Russell 1999c). These include phenolics (notably the halogenated members and hexachlorophane), QACs, oxine (8-hydroxyquinoline), diamidines, organic mercury derivatives, and the parabens. Sorbic acid shows significant antifungal activity at low pH values when it occurs in solution mainly in the undissociated form. At higher pH values, activity is lost. Fungal spores are often resistant to chemical disinfectants (Russell 1999c).

PROTOZOA

Several distinctly different types of protozoa, including *Giardia*, *Cryptosporidium*, *Naegleria*, *Entamoeba*, and *Acanthamoeba*, are potentially pathogenic and may be acquired from water. Furthermore, their life cycles may contain a resistant cyst stage (Jarroll 1999). Agents, in ascending order of efficacy, that are cysticidal towards *Giardia muris* cysts are monochloramine, free chlorine, iodine, chlorine dioxide, and ozone (Jarroll 1988, 1999). Chlorine dioxide is more effective against *Cryptosporidium* oocysts than is either free chlorine or monochloramine; these cysts are also sensitive to ozone (Korich et al. 1990).

Acanthamoeba spp. may cause corneal keratitis, sometimes associated with the use of contaminated contact lenses or contact-lens solutions. Whereas trophozoites are readily inactivated by contact-lens disinfecting solutions, cysts are more refractory (Khunkitti et al. 1997, 1998a, b, 1999; Turner et al. 1999, 2000a, b) and the presence of proteinaceous matter is an additional hazard (Seal and Hay 1992).

VIRUSES

Several bactericidal agents are virucidal, but antibacterial activity does not necessarily imply antiviral potency. An excellent comprehensive treatise of virus disinfection is that presented by Grossgebauer (1970). Viruses are classified primarily as to whether they contain DNA or RNA, but the presence or absence of a lipid envelope is in general more important in relation to disinfection. An important hypothesis was put forward in 1963 and modified some 20 years later by Klein and Deforest (1983) (see also Prince and Prince 2001), who classified viruses into three groups:

1. lipid-enveloped viruses, e.g. herpes simplex virus (HSV), HIV, hepatitis B virus (HBV)
2. small nonenveloped viruses, e.g. picornaviruses, parvoviruses
3. larger nonenveloped viruses, e.g. rotaviruses.

The order of these groups, in terms of biocide sensitivity, is $1 > 3 > 2$.

Detailed knowledge of the inactivation of viruses has, in the past, been important in the preparation of vaccines such as inactivated (Salk) poliomyelitis vaccine, in which formaldehyde was used as the virucidal agent. Nowadays, with the current importance of HIV, HBV, and hepatitis A virus (HAV), plus the need to prevent the transmission of viral infection, it is essential to have a sound knowledge of viral inactivation. Important virucidal agents include CRAs, formaldehyde (in inactivated vaccine production), glutaraldehyde at alkaline pH, and peracetic acid (Springthorpe and Sattar 1990; Sattar et al. 1994, 2001; Maillard and Russell 1997; Maillard 2001; Prince and Prince 2001). Bacteriophages have been considered as indicator 'organisms' for assessing the virucidal activity of biocides (Davies et al. 1993; Maillard et al. 1995; Maillard and Russell 1997) (see also Evaluation of antimicrobial activity).

Viruses also cause many diseases of animals. Lists of approved dilutions of approved disinfectants for veterinary use in the UK are frequently published by the Department for Environment, Food and Rural Affairs (DEFRA) (formerly the Ministry of Agriculture, Fisheries and Foods). These are for general use and in connection with certain Statutory Orders applying to viral infections (foot-and-mouth disease, swine vesicular disease) and bacterial disease (tuberculosis). Porcine parvovirus, which causes reproductive failure in swine, is quite resistant to disinfectants, only sodium hypochlorite and sodium hydroxide being rapidly virucidal; by contrast, two enveloped swine viruses (pseudorabies and transmissible gastroenteritis viruses) are highly sensitive to a wide range of disinfectants (Quinn and Markey 1999).

Russell (1990b) has provided a comprehensive list of the susceptibility of different types of viruses to heat and chemical biocides, as well as considering acid stability and sensitivity to the organic solvent ether, which inactivates most lipid-enveloped viruses.

PRIONS

Prions are responsible for a distinct group of unusual neurological diseases, the transmissible degenerative encephalopathies (TDE) or transmissible spongiform encephalopathies (TSE), and are sometimes referred to as unconventional transmissible agents. Examples of TDEs are Creutzfeldt–Jakob disease (CJD) and new variant Creutzfeldt–Jakob disease (nvCJD), scrapie in sheep, bovine spongiform encephalitis (BSE), and kuru.

Prions are composed of an altered protein and are inordinately resistant to most chemical and physical agents, including glutaraldehyde, formaldehyde, organic solvents, chlorine dioxide, iodine, strong acids, normal autoclaving conditions, dry heat, and ionizing and ultraviolet radiations (Taylor 1999; Baron et al. 2001). However, as prions have yet to be purified (Taylor 2004), it is difficult to state whether this is an intrinsic property or to what extent it is influenced by the protective effect of host tissue. They are inactivated by strong

alkali, guanidinium hydrochloride (or isocyanate), and sodium hypochlorite (Baron et al. 2001).

Evaluation of antimicrobial activity

A detailed survey of the numerous published methods for evaluating antimicrobial activity is outside the scope of this chapter. A summary is provided in Table 3 and the following should be consulted for additional information: Quinn (1987), CEN (1996), Quinn and Carter (1999), Reybrouck (1999), and Cremieux et al. (2001).

The evaluation of virucidal activity is not an easy matter (Bellamy 1995). Viruses are unable to grow in artificial laboratory culture media. Another appropriate system, usually involving living cells, must thus be employed. In essence, the principles of testing follow methods of evaluating bactericidal or fungicidal activity in that the virus is exposed to appropriate concentrations of disinfectant in suspension, or on a carrier, e.g. glass coverslips, as used with herpes virus and poliovirus (Tyler and Ayliffe 1987). Inactivation is then tested in an appropriate manner, care being taken to ensure that residual disinfectant is neutralized and that the antimicrobial agent has no toxic effects on host cells.

Estimation of virucidal activity can be made using the following systems (see also Quinn and Carter 1999; Sattar and Springthorpe 2001):

- *Tissue culture* or fertile eggs, which after incubation are examined for signs of viral infection.
- *Plaque-counting* procedures, which have been used for various viruses, e.g. herpes and poliovirus (Tyler and Ayliffe 1987) and human rotaviruses (Lloyd-Evans et al. 1986; Springthorpe et al. 1986).
- Use of an *'acceptable' animal model*, e.g. the chimpanzee, for studying survival of HBV. The discovery of hepadnaviruses that infect ducks and small animals might provide a suitable alternative (Tsiquaye and Barnard 1993).
- *Immunological reactions*: tests for the inactivation of the hepatitis B surface antigen (HBsAg) were at one time used to assess the efficacy of disinfection procedures for this virus. However, since this component is much more stable to chemical and physical agents than those viral parts conferring infectivity, the method is no longer used.
- *Endogenous reverse transcriptase*: HIV is an enveloped RNA retrovirus. After HIV enters a cell, RNA is converted to DNA under the influence of an enzyme, reverse transcriptase. HIV replicates in certain T lymphocytes and in the assay reverse transcriptase activity is not a satisfactory alternative to tests in which infectious HIV can be detected in systems employing fresh human peripheral blood mononuclear cells (Resnick et al. 1986).

Table 3 *Examples of methods used for evaluating antimicrobial activity*

Test/evaluation	Examples of methods available[a,b]	Comments
Bacteriostatic/fungistatic potency (MIC determination)	Broth/agar serial dilution methods; agar diffusion methods	Provide preliminary information only
Bactericidal/fungicidal activity	(1) Suspension tests with determination of numbers of survivors. Include sporicidal and mycobactericidal estimations (AFNOR 1989)	(1) Neutralization of agent essential to prevent 'carry-over' into recovery medium;[a] rapid methods increasingly being studied
	(2) European Suspension Test	(2) 5 Log_{10} reduction in specified period
Phenol coefficient tests	RW, CM, AOAC methods	Compare activity of a test phenolic vs. a standard (phenol)[a]
Capacity-type tests	Kelsey–Sykes test	Refers to capacity of a disinfectant to retain its activity when repeatedly challenged by test organisms
Carrier tests	AOAC use-dilution confirmatory test; DGHM test	Organisms are dried on to appropriate carriers
Virucidal tests	Tissue culture, eggs, plaque counting; animal models; enzyme activity	See text
Skin disinfection	MIC and lethal effects (presence of serum or blood); skin test at 37°C	
Aerial disinfection	Closed-chamber evaluation of microbial population before and after exposure to aerial disinfectant	
Preservative activity	MIC evaluation, lethal activity; challenge testing of preserved pharmaceutical and cosmetic products	See British Pharmacopoeia (2000)

AFNOR (1989), Association Française de Normalisation; AOAC (1990), Association of Official Analytical Chemists; CM, Chick–Martin; DGHM, Deutsche Gesellschaft für Hygiene und Mikrobiologie; MIC, minimum inhibitory concentration; RW, Rideal–Walker.

a) See also text.

b) Suitable tests must be done where relevant to demonstrate adequate neutralization of antimicrobial agents.

- *Use of bacteriophages* as model systems: bacteriophages have been suggested as model systems for human viruses (Davies et al. 1993). Certainly, they have several advantages such as ease of handling and fairly rapid and reproducible results (Maillard et al. 1995). Their resistance to biocides should, of course, mimic a particular virus (or viruses), e.g. coliphage MS2 has the same order of response to some biocides as poliovirus.

Mechanisms of action of antimicrobial agents

Considerable progress has been made in understanding the mechanisms of action of many antibacterial agents (Denyer and Stewart 1998; Denyer 2002, Russell 1998a, b, 2002b, 2003b; Hugo 1999b). The same is not always true when the target organisms are yeasts, fungi, bacterial spores or viruses (Russell et al. 1997; Russell 2003b).

The initial reaction between an antimicrobial agent and a microbial cell takes place at the cell surface. This is followed by penetration of the chemical to reach its site(s) of action, usually within the cell itself, while some act by 'physical' puncture of cell walls. Unlike most antibiotics, however, many biocides have more than one site of action and it is thus often difficult to elucidate the exact mechanism whereby cellular inactivation is achieved. Furthermore, secondary effects may make a significant contribution to the overall process. Conversely, McMurray et al. (1998a) found that the phenylether, triclosan, acts on a specific bacterial target, inhibiting lipid synthesis in *E. coli*.

ACTION ON NONSPORULATING BACTERIA

Uptake of an antibacterial agent by bacteria represents an early manifestation of its effect, although there is little information available about uptake by mycobacteria (Russell 1996, 2003b).

Different agents have different sites and modes of action. Data are presented below, and summarized in Table 4, of the ways in which they act (Russell and Russell 1995; Russell and Chopra 1996). It must again be emphasized, however, that little is known about the mechanisms of inhibition and inactivation of mycobacteria (Russell 1996).

Cell-wall or cell-envelope effects

EDTA is not a powerful bactericide in its own right. Generally, it has little effect on gram-positive bacteria or fungi but is active against certain gram-negative bacteria, especially *P. aeruginosa* (Hugo and Russell 1999). Its usefulness resides in the fact that it is a chelating agent that combines with cations associated with the outer membrane (OM) of gram-negative bacteria with the release of some 30–50 percent of OM lipopolysaccharide (LPS). These changes in the OM render the cells more sensitive to many chemically unrelated compounds such as lysozyme, QACs, dyes, and phenols (Hugo 1999b).

EDTA thus increases the permeability of gram-negative bacteria. Other chemicals that act in a similar manner include sodium hexametaphosphate, gluconic acid, citric acid, and malic acid (Vaara 1992; Ayres et al. 1993, 1998a, b) and PEI displaces Mg^{2+}, thereby opening up the OM of gram-negative bacteria (Helander et al. 1997, 1998).

GTA has several effects on nonsporulating bacteria (Russell 1994). It causes cross-linking of the peptidoglycan in gram-positive bacteria so that lysis of this component is reduced on subsequent treatment with lysozyme (*Bacillus subtilis*) or lysostaphin (*S. aureus*). GTA interacts with protein in the cell envelope of gram-negative bacteria, with consequent reduction of lysis by sodium lauryl sulfate or EDTA–lysozyme.

The dialdehyde increases resistance to lysis of protoplasts and spheroplasts, by stabilizing the cytoplasmic membrane. OPA is much less efficacious as a cross-linking agent (Walsh et al. 1999a, b, 2001).

Membrane-active agents

Various chemical agents effect perturbation of homoeostatic mechanisms in bacteria in one of two ways (Gould 1988; Russell and Hugo 1988). In the first, leakage of intracellular materials is promoted by their physical interaction with the cytoplasmic membrane. In the second, there is a specific attack on the insulatory function of the membrane, such that the proton-motive force (pmf) across it is either discharged or prevented from forming.

Leakage can be considered as being a measure of the generalized loss of function of the cytoplasmic membrane as a permeability barrier, the rate and extent of leakage usually depending on the concentration of the inhibitor and the time and temperature of exposure. Leakage may be related to bacteriostasis but not necessarily to cell death (Denyer and Stewart 1998). Examples of antibacterial agents that induce leakage are cationic, anionic, and polypeptide surface-active agents, phenol, chlorhexidine, parabens, hexachlorophane (hexachlorophene), phenoxyethanol salicylanilides, and triclosan. These effects are not, however, necessarily responsible for cell death. Triclosan, for example, has been shown to have a specific effect on enoyl-ACP reductase (FabI) in *E. coli* (McMurry et al. 1998a, b). Heath et al. (2001) proposed that this is the only target for triclosan in this organism, and that the phenylether does not possess membrane-disruptive properties (cf. McMurry et al. 1998a). FabK and FabL are the respective enoyl-ACP reductase isoforms from *Streptococcus pneumoniae* and *B. subtilis*. FabK is triclosan-resistant

Table 4 *Mechanisms of antibacterial action*[a]

Target site	Example(s)[b]	Mechanism(s)
Outer cell layers (cell wall/ outer membrane)	GTA, OPA	Interaction with $-NH_2$ groups, e.g. proteins, peptidoglycan (OPA less effective than GTA)
	EDTA	Chelation of divalent cations, especially Mg^{2+}
	PEI	Displaces Mg^{2+}
	Lysozyme	β,1–4 links in peptidoglycan
	Cationic biocides	
	CHA, QACs, DBPI, PHMB	Outer-membrane damage, thereby promoting own entry postulated
Cytoplasmic (inner) membrane	Phenols	Generalized membrane damage
	Triclosan	Membrane damage, but at low concentrations inhibits enoyl reductase
	QACs	Generalized membrane damage
	CHA	Low concentrations affect membrane integrity, high concentrations inhibit congeal protoplasm
	Alexidine, PHMB	Phase separation and domain formation of acidic phospholipids
	Hexachlorophane	Inhibits membrane-bound electron-transport chain
	Phenoxyethanol	Proton-conducting uncoupler
	Sorbic acid	Transport inhibitor (effect on pmf); another unidentified mechanism?
	Parabens	Low concentrations inhibit transport, high concentrations affect membrane integrity
	Metallic compounds[c]	
	mercury, silver, copper	Interaction with –SH groups in proteins and enzymes
	Isothiazolones[c]	Interaction with –SH groups in proteins and enzymes
	Bronopol[c]	Oxidizes thiol groups to disulfides
Cytoplasmic constituents	*Alkylating agents*	
	ETO, BPL, PO, formaldehyde	Combination with amino, carboxyl, sulfydryl, and hydroxyl groups in protein; ETO also interactions at N-7 guanine moieties in DNA
	Cross-linking agents	
	GTA, OPA, formaldehyde	Intermolecular protein cross-links; OPA less effective but enters mycobacteria more readily than GTA
	Intercalating agents, e.g. acridines	Acridines intercalate between two layers of base pairs in DNA
	Phenethyl alcohol	Inhibits DNA synthesis
	Oxidizing agents	
	1 Hypochlorites[c]	Progressively oxidize thiol groups to disulfides, sulfoxides, or disulfoxides
	2 Hydrogen peroxide[c]	Formation of free hydroxyl radicals (•OH) causes oxidation of thiol groups in enzymes and proteins
	3 Peracetic acid[c]	Possibly disrupts thiol groups in proteins and enzymes
	Iodine	Interaction with cytoplasmic protein

BPL, β-propiolactone; CHA, chlorhexidine diacetate; DBPI, dibromopropamidine isethionate; EDTA, ethylenediamine tetraacetate; ETO, ethylene oxide; GTA, glutaraldehyde; OPA, *ortho*-phthalaldehyde; PEI, polyethyleneimine; PHMB, polyhexamethylene biguanide; pmf, proton-motive force; PO, propylene oxide; QACs, quaternary ammonium compounds.

a) For further information, see Russell and Chopra (1996).

b) Some agents affect more than one target site.

c) These compounds might affect enzymes and proteins in both the cytoplasmic (inner) membrane and the cytoplasm.

whereas FabL is reversibly inhibited by triclosan. The expression of either FabK or FabL in *E. coli* produces cells with increased triclosan resistance of three orders of magnitude. In *S. aureus*, only FabI is expressed and overexpression results in an increased MIC of triclosan (Slater-Radosti et al. 2001). Suller and Russell (1999, 2000) found that triclosan caused leakage from *S. aureus*. Triclosan and isoniazid inhibit InhA (an FabI analogue) in *M. smegmatis* and *M. tuberculosis*, but unlike isoniazid, triclosan may have a second target in *M. smegmatis* (Heath et al. 2001). Two aspects of triclosan action are important, namely its growth-inhibitory and lethal effects. It is unlikely that inhibition of enoyl reductase is solely responsible for its lethal effect

and membrane damage, including leakage, must play an important role at higher triclosan concentrations.

Chlorhexidine has a biphasic effect on membrane permeability in which an initial high rate of leakage occurs as the biguanide concentration increases, with a progressive decrease in leakage at higher chlorhexidine concentrations as a consequence of coagulation or precipitation of the cytosol (Hugo 1999b).

Both alexidine and the polymeric biguanide PHMB produce lipid phase separation and domain formation of the acidic phospholipids in the cytoplasmic membrane (Broxton et al. 1984, Chawner and Gilbert 1989a, b; Gilbert et al. 1990a, b). Chlorhexidine and QACs also combine with membrane phospholipids but do not bring about lipid phase separation and domain formation.

Ethanol and isopropanol are membrane disrupters, inducing a rapid leakage of intracellular constituents; disorganization of the membrane probably results from their penetration into the hydrocarbon core of the membrane (Seiler and Russell 1991).

Mitchell's (1961) chemiosmotic theory seeks to explain active transport, synthesis of adenosine triphosphate (ATP) and flagellar movement. During metabolism, protons are extruded to the exterior of the bacterial cell, resulting in acidification of the exterior and positivity in charge relative to the cell interior. It may be expressed mathematically as

$$\Delta p = \Delta\psi - Z\Delta pH \qquad (5)$$

in which Δp is the pmf, ψ the membrane electric potential, ΔpH the transmembrane pH gradient, and Z a constant (2.303 RT/F) with a value of 61 at 37°C that is used to convert pH values to millivolts.

Several chemicals that are antiseptics, disinfectants, or preservatives behave in a manner similar to 2,4-dinitrophenol (DNP), i.e. they act as uncouplers of oxidative phosphorylation. Examples include 2-phenoxyethanol, bisphenols, and pentachlorophenol. Tetrachlorosalicylanilide discharges the membrane potential, $\delta\psi$, in *E. faecalis*. The thiobisphenol, fentichlor, behaves very much like DNP, having a direct action on the collapse of a proton potential (Bloomfield 1974). Phenoxyethanol and the alkyl phenols act as proton-conducting uncoupling agents. Pharmaceutical and food preservatives such as the parabens and lipophilic acids (propionic, sorbic, 4-hydroxybenzoic) inhibit the active uptake of some amino and oxo acids in *E. coli* and *B. subtilis*. Sorbic acid affects the pmf in *E. coli* and accelerates the movement of H^+ ions from low pH media into the cytoplasm. Sorbic acid appears to dissipate ΔpH, having a much smaller effect on $\Delta\psi$ (Eklund 1983, 1985a, b; Salmond et al. 1984).

Agents acting on nucleic acids and proteins

Intercalation: The antimicrobial activity of the acridines increases with degree of ionization (Albert 1979). Ionization is the most important factor governing their activity, but it must be cationic in nature, because acridine derivatives that are ionized to form anions or zwitterions are only poorly antibacterial by comparison. The acridines induce filamentous forms in gram-negative bacteria, inhibit DNA synthesis, and combine strongly with DNA, although binding to other sites such as RNA, cell envelopes, and ribosomes has also been reported. Binding to DNA has been studied extensively. The classic studies of Lerman (1961) suggested that the planar drug molecules become intercalated between adjacent base pairs in DNA. Binding to DNA occurs by two distinct mechanisms (Peacocke and Skerrett 1956). In the first, there is a first-order reaction with one proflavine molecule binding to every four or five nucleotides, whereas in the second, there is a slower higher order of reaction with one molecule per single nucleotide.

Alkylation: Alkylation is defined as the conversion of H-X→R-X, where R is an alkyl group. Biological activity of alkylating agents is indicated by reaction with nucleophilic groups.

Epoxides, of which ethylene oxide is an example, interact with amino acids and proteins. Ethylene oxide causes hydroxyethylation of amino acids and combines with the amino, carboxyl, sulfydryl, and hydroxyl groups of proteins (Russell 1976). Alkylation of phosphated guanine in nonsporing bacteria has been proposed as the primary reason for the lethal effect of ethylene oxide (Michael and Stumbo 1970; Russell 1976).

It has been suggested that formaldehyde also acts by its alkylating effect. Binding to RNA is reversible up to a point (Staehelin 1958). Interaction of formaldeyde with T2 bacteriophage DNA and with protein has been described (Grossman et al. 1961).

The reaction of GTA with nucleic acids follows pseudo-first-order kinetics at high temperatures but there is little evidence for the formation of intermolecular cross-links. GTA inhibits synthesis of protein, DNA and RNA in *E. coli*, but this is believed to arise from an inhibition of precursor uptake as a consequence of protein–dialdehyde interaction in the outer structures of the cell.

Interaction with enzymes: Metals such as mercury and silver interact with thiol (–SH) groups on enzymes to form mercaptides (Hugo 1999b). This reaction may be reversed by excess of an –SH compound such as sodiumthioglycollate or cysteine, an important finding since such agents are used to inactivate metals in microbicidal testing (Russell et al. 1979; Russell and Hugo 1994). Bronopol also interacts with –SH groups (Hugo 1999b).

Other effects: Silver salts produce structural changes in the cell envelope of *P. aeruginosa* and the Ag^+ ion reacts preferentially with the bases rather than the phosphate groups in DNA (Russell and Hugo 1994).

ACTION ON BACTERIAL SPORES

Bacterial spores are not inactivated rapidly or readily at ambient temperatures (Bloomfield and Arthur 1994;

Bloomfield 1999) and comparatively few chemicals are sporicidal (Russell 1990a, b, 1998b). The mechanism of action of those that are sporicidal is often poorly understood, although progress continues to be made.

Although it is possible to correlate changes in cell structure with the development of resistance (sporulation) or sensitivity (germination and outgrowth), it is rather more difficult to identify the mechanism whereby death is brought about (Knott et al. 1995). Superoxidized water does not cause DNA damage and dipicolinic acid (DPA) is not released; it is considered that *B. subtilis* spores are inactivated by a modification of the inner membrane, which becomes nonfunctional in germinated spores (Loshon et al. 2001). Hydrogen peroxide-treated spores maintain their permeability barrier, there being no DPA release even after subsequent heat treatment. It is unlikely that significant damage occurs to the spore inner membrane (Melly et al. 2002), although Shin et al. (1994) found that peroxide sensitizes spores to heat damage. Usual targets for peroxide attack on membranes are polyunsaturated acids, which are present in extremely low levels in spores (Melly et al. 2002). Prolonged exposure of spores to 15 percent peroxide at elevated but sublethal temperatures, or to high peroxide concentrations, causes spore lysis with major damage to coat, cortex and protoplast (Shin et al. 1994). Imlay and Linn (1988) are of the opinion that one mechanism of hydrogen peroxide action is the generation of •OH radicals that cleave the DNA backbone. Peracetic acid was found by Clapp et al. (1994) to be the most potent peroxygen in their test series, the lethal moiety being •OH radicals. Reduced transition-metal ions sensitize spores to peracetic acid (Marquis et al. 1995).

Low concentrations (0.01 percent) of alkaline GTA inhibit germination and outgrowth, whereas much higher concentrations must be used for long periods to kill spores (Russell 1990a, 1999c). In fact, as with formaldehyde (Spicher and Peters 1981), it has been shown (Power et al. 1988; Williams and Russell 1993) that it is possible to revive a very small proportion of GTA-treated spores by means of a sublethal heat shock or by sodium hydroxide or other treatment. It is thus possible that the dialdehyde is a less effective sporicide than orginally thought, but that its cross-linking effect on bacterial spores will, under ordinary circumstances, be sufficient to prevent spore germination and subsequent vegetative development. Cabrera-Martinez et al. (2002) provided evidence that GTA does not damage spore DNA but eliminates the spore's ability to germinate and that OPA-treated spores that cannot germinate are not recovered by artificial germinants or by treatment with sodium hydroxide or lysozyme.

The enzyme, lysozyme, hydrolyzes β,1–4-linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the spore peptidoglycan in coatless spores. Sodium nitrite breaks the peptidoglycan chain at the muramic lactam residues unique to spores. Hypochlorites interact strongly with spore coats, but their major site of sporicidal action is believed to be on the cortex (Bloomfield and Arthur 1994; Bloomfield 1999). The alkylating agents, ethylene and propylene oxides, are considered to inactivate bacteria and their spores by combining with amino, carboxyl, sulfydryl, and hydroxyl groups of proteins, as mentioned above.

The general antimicrobial activity of ethylene oxide (CH_2CH_2O) and related substances parallels their activity as alkylating agents (Phillips 1952). Thus, cyclopropane ($CH_2CH_2CH_2$), which is not an alkylating agent, is devoid of antimicrobial activity, whereas ethylene sulfide (CH_2CH_2S) and ethylene imine (CH_2CH_2NH) are potent alkylating compounds that demonstrate antimicrobial properties.

ACTION ON FUNGI

It is often assumed that the same effects as those in nonsporulating bacteria are responsible for fungal inactivation, but in view of the considerable structural and biochemical differences between these microbes this is undoubtedly an oversimplification. However, as with bacteria, it is likely that an initial interaction at the cell surface is followed by passage of a biocide across the fungal cell wall to reach its target site(s). Little information is available about the mechanisms of biocide uptake into fungal cells despite long-standing studies of biocide adsorption to yeasts and molds (Lyr 1987; Gadd and White 1989; Russell 1999c).

Although the fungal cell wall may be a prime target for developing new antifungal antibiotics (Hector 1993), few biocides are likely to have the wall as a sole target. Chitin has been suggested as a potentially reactive site for glutaraldehyde action in yeasts (Gorman and Scott 1977). Glutaraldehyde is also known to cause agglutination of yeast cells (Navarro and Monsan 1976).

Chlorhexidine induces K^+ release from bakers' yeast (Elferink and Booij 1974) and affects the ultrastructure of budding *Candida albicans* with the loss of cytoplasmic constituents (Bobichon and Bouchet 1987). Uptake of chlorhexidine by and membrane damage in *S. cerevisiae* have been described (Hiom et al. 1993, 1995a, b, 1996). The QACs also affect membrane integrity. Heavy metals probably bind to key functional groups of enzymes (Lyr 1987).

Very few relevant studies have been made on other target sites, but DNA and RNA would be expected to be targets for a number of biocides, such as cationic agents that interact strongly with nucleic acids (Hugo 1999b).

ACTION ON VIRUSES

The mechanisms whereby biocidal agents inactivate viruses are still poorly understood, although recent progress has been encouraging. In an excellent paper,

Grossgebauer (1970) discussed the possible effects of biocides on viruses and proposed that (a) water-saturated phenol caused separation of protein from infectious RNA in poliovirus; (b) low formaldehyde concentrations produced an antigenic but noninfectious particle (for use in a vaccine); and (c) higher aldehyde concentrations gave a noninfectious destroyed particle.

Thurmann and Gerba (1988, 1989) considered the possible interactions between viruses and biocides as being:

- adsorption to capsid receptors
- release of infectious nucleic acid
- destruction of capsid leading to release of infectious nucleic acid, or
- nucleic acid rendered noninfectious
- capsid remaining intact, but nucleic acid rendered noninfectious.

They pointed out that CRAs could inactivate viruses by attacking either capsid proteins or nucleic acid.

GTA reduces the activity of HBsAg and especially core antigen in HBV (Adler-Storthz et al. 1983) and interacts with lysine residues on the surface of hepatitis A virus (Passagot et al. 1987). Low concentrations (<0.1 percent) of alkaline GTA act against purified poliovirus, whereas poliovirus RNA is highly resistant to higher concentrations (Bailly et al. 1991). From this, it may be inferred that changes to the capsid are responsible for loss of infectivity. Support for this contention has been obtained by demonstrating that the capsid proteins of poliovirus and echovirus react with low concentrations (0.05 and 0.005 percent, respectively) of the dialdehyde, the ten-fold difference in aldehyde concentration probably reflecting major structural alterations in the two viruses (Chambon et al. 1992). Some biocides (hypochlorite, 70 percent ethanol and cetrimide) induce a rapid loss of the outer capsid layer, whereas chlorhexidine and phenol affect morphology only after extended periods of exposure (Rodgers et al. 1985).

Mechanistic studies of the effects of biocides on bacteriophages have been undertaken. These investigations (Maillard et al. 1995; Maillard and Russell 1997; Maillard 2001) have provided useful information about the interaction of biocides with phage protein and nucleic acid and on the inhibition of transduction, but much remains to be done to understand fully the mechanisms of inactivation.

ACTION ON PROTOZOA

Little is known about the mechanisms of antiprotozoal action of biocides, a disappointing gap in our knowledge and one that needs to be rectified. It has, however, been demonstrated (Khunkitti et al. 1997, 1998a, b, 1999) that chlorhexidine affects the membrane of trophozoites and cysts of *Acanthamoeba castellanii*, although cysts are less sensitive (Furr 1999).

Mechanisms of microbial resistance to biocides

Three levels, low, intermediate, and high, of biocidal activity have been recognized (Favero and Bond 1993). In high level, bacterial spores, fungi, mycobacteria, and viruses are inactivated; in intermediate level, mycobacteria, fungi, and viruses are inactivated; and in low level, nonsporulating bacteria (except mycobacteria) and lipid enveloped viruses are rendered nonviable.

Thus, different organisms vary considerably in their susceptibility to biocides (see Types of microorganism).

RESISTANCE OF NONSPORULATING BACTERIA

Vegetative bacterial cells respond to chemical and other stresses in a variety of ways that include the activation and expression of new groups of genes (Gould 1989). When *E. coli* is subjected to nutrient limitation or to antimicrobial agents, the growth rate decreases and gene expression is markedly altered (Gilbert et al. 1990c). This is essential for the long-term survival of cells and is partly mediated by alternative sigma factors such as σ^s encoded by the *rpoS* gene. Positive regulation of this expression results in σ^s synthesis being activated by guanosine 3′-diphosphate-5′-diphosphate (ppGpp). In an insertional mutant of *E. coli* that cannot produce σ^s, and in *E. coli* and other gram-negative organisms that produce ppGpp, the cells are more resistant to biocides and antibiotics than isogenic mutants (Greenaway and England 1999a, b). Tolerance to stress by hydrogen peroxide arises from intrinsic resistance mechanisms. The oxidative stress response involves the production of neutralizing enzymes to prevent cellular damage, with the SOS defense system response coming into operation when DNA damage takes place (Imlay 2002).

Drug efflux is a major mechanism involved in antibiotic resistance (Poole 2000, 2002; Levy 2002a, b). There are several types of efflux pumps: (1) P-glycoprotein, encoded by a human or rodent gene, mediated by ATP-dependent exporters, and a member of the ATP-binding cassette (ABC) superfamily of transporters, is responsible for resistance to many cytotoxic drugs; (2) small multidrug resistance family (SMR); (3) major facilitator superfamily (MFS); (4) resistance/nodulation/cell division family (RND), unique to gram-negatives and working in conjunction with a periplasmic membrane-fusion protein (MFP) and an outer membrane protein; and (5) multidrug and toxic compound extrusion (MATE) family. Families 2–5 are secondary transporters driven by the protonmotive force.

Enzymatic inactivation occurs at low biocide concentrations (Hugo 1991b) but is unlikely to be a problem with in-use concentrations, although Meade et al. (2001)

consider inactivation to be one mechanism of triclosan resistance in some bacteria.

Bacterial resistance mechanisms fall into two general categories. *Intrinsic resistance* (intrinsic susceptibility) is a natural propert of an organism, whereas *acquired resistance* results from genetic changes to a cell. This general concept (Table 5) has been of value but should perhaps be reconsidered because some biochemical mechanisms of resistance, e.g. efflux, can be associated with both types.

It has been known for many years that repeated exposure of bacterial cells to an antibiotic can result in stable bacterial resistance (Levy 2002a). It has also been demonstrated that bacteria also have this ability to acquire resistance to a variety of biocidal agents (Russell and Chopra 1996; Russell 1998a, 1999d, 2003a, b, c, 2004). The resistance thus developed may be low- to high-level, stable or unstable (in which there is reversion to susceptibility or the adaptive resistance is lost on removal of biocide). The clinical relevance of stable decreased susceptibility to biocides is a matter of conjecture. Cross-resistance to chemically related biocides occurs, but occasionally to chemically unrelated biocides nd to some antibiotics also (Tattawasart et al. 1999a, b, 2000a, b; Thomas et al. 2000; Lambert et al., 2001; Joynson et al. 2002; Lear et al. 2002; see below).

Biocides are generally considered to have concentration-dependent multiple actions on bacteria and other microbial cells. Thus, single mutations are unlikely to lead to large increases in biocide resistance to biocides. However, a mutation in the primary target site, enoyl reductase (FabI) in *E. coli* and *S. aureus*, might be the reason for the reduced susceptibility of these organisms to triclosan (McMurry et al. 1998b) (see also Mechanisms of action of antimicrobial agents).

Mycobacterial resistance

The mycobacterial cell wall is highly hydrophobic with a mycoylarabinogalactanpeptidoglycan skeleton. Thus, hydrophilic agents have difficulty in penetrating the wall in sufficiently high concentrations to achieve a mycobactericidal effect. Low concentrations must, however, traverse the wall because inhibitory concentrations of nonmycobactericidal agents such as chlorhexidine and QACs are generally of the same order as those against other, nonmycobacterial organisms (Russell 1996). The component(s) of the mycobacterial wall responsible for the high level of biocide resistance is (are) unknown, but both the mycolic acids and arabinogalactan may be involved (Broadley et al. 1995; Russell 1996). Acquired mycobacterial resistance by repeated exposure to a biocide is a possibility, but there appears to be no evidence that plasmids are involved.

The new aromatic cyclic dialdehyde, OPA (see Types and uses of chemical agents) is believed to readily traverse the cell wall of GTA-resistant *M. chelonae* strains, whereas the less hydrophobic GTA is unable to do so (Walsh et al. 1999a, b, 2001; Simons et al. 2000; Fraud et al. 2001, 2003).

Resistance of other nonsporulating gram-positive bacteria

The cell wall of gram-positive bacteria is a thick, fibrous layer pressed against the cytoplasmic membrane; the wall consists mainly of an inelastic peptidoglycan interspersed with which may be lipids and teichoic and teichuronic acids (Hammond et al. 1984). The peptidoglycan comprises at least 50 percent of the dry weight of the walls. Mechanisms whereby biocidal agents enter gram-positive bacteria have been little studied, but passive diffusion across the wall is probably a major factor. Thus, organisms such as staphylococci and streptococci are usually highly susceptible to biocides (Russell 1995, 1998b, 1999d, 2003a; Russell and Russell 1995; Russell and Chopra 1996). However, bacteria grown under different conditions may show a wide response to biocides. For instance, fattened cells of

Table 5 *Mechanisms of bacterial resistance to biocides* [a]

Bacteria	Mechanism(s) of Intrinsic resistance	Acquired resistance
Mycobacteria	Permeability barrier associated with hydrophobic cell wall	Not described, unlikely
Other nonsporulating gram-positive bacteria	Cell-wall modulation 1 Peptidoglycan changes 2 Lipid increase	Plasmid-mediated efflux of cationic biocides in multiple antibiotic-resistant *S. aureus*; enzymatic inactivation of Hg compounds
Bacterial spores	Permeability barrier associated with outer membrane	Not described, unlikely
	SASPs and DNA protection	Unlikely
Gram-negative bacteria	1 Reduced uptake associated with outer membrane permeability barrier and efflux	Enzymatic inactivation of Hg compounds; efflux of cationic biocides?
	2 Constitutive biocide-degrading enzymes?	?

a) For further information, see Russell and Russell (1995); Russell and Chopra (1996); Russell (1999a, 2003a) and following text.

S. aureus produced by repeated subculturing in glycerol-containing media are more resistant to benzylpenicillin and higher phenols (Hugo 1999a). Additionally, nutrient limitation and reduced growth rates (Gilbert et al. 1990a; Poxton 1993) can alter sensitivity to biocides by changes in peptidoglycan thickness and cross-linking. Such changes in these two examples can be regarded as the expression of intrinsic resistance brought about in response to a phenotypic (physiological) adaptation.

Acquired resistance is also known in some strains of *S. aureus*. Inorganic mercury (Hg^{2+}) resistance is a common property of clinical isolates of *S. aureus* containing penicillinase plasmids. These plasmids are either narrow-spectrum, specifying resistance to Hg^{2+} and some organomercurials, or broad-spectrum, encoding additional resistance to other organomercurials. The enzymes involved are mercuric reductase (Hg^{2+}) and lyase (hydrolase) and reductase (organo-compounds) (Foster 1983). Resistance to other metals has been described (Silver et al. 1989) and often involves efflux mechanisms. Silver (Ag^{+}) reduction may not form the basis of silver or silver sulfadiazine (AgSD) resistance (Belly and Kydd 1982; Foster 1983; Silver et al. 1989), but may be associated with accumulation (Trevor 1987; Russell and Hugo 1994).

Some methicillin-resistant strains of *S. aureus* (MRSA) containing plasmids encoding gentamicin resistance (methicillin–gentamycin-resistant *S. aureus*, MGRSA) also have increased minimum inhibitory concentration (MIC) values towards such biocides as QACs, chlorhexidine, ethidium bromide (EB), acridines, and propamidine isethionate (PI) (Lyon and Skurray 1987; Cookson and Phillips 1990; Cookson et al. 1991; Day and Russell 1999). Several genetic determinants are responsible for cationic biocide resistance in clinical isolates of *S. aureus*. There is evidence that there is an efflux of cationic agents from such antibiotic-resistant strains (Midgley 1986, 1987). Several genetic determinants are responsible for cationic biocide resistance in clinical isolates of *S. aureus*. These are *qacA*, which specifies resistance to QACs, acridines, EB, propamidine isethionate, and low-level chlorhexidine resistance; *qacB*, which is similar; *smr* (formerly *qacC/qacD/ebr*) encoding QAC and low-level EB resistance; and *qacG* and *qacH*. The corresponding gene products belong to the MFS (qacA, qacB) and SMR (smr, qacG, qacH) efflux families.

Resistance of gram-negative bacteria

Gram-negative bacteria such as pseudomonads and Enterobacteriaceae are usually less sensitive to a variety of chemically unrelated inhibitors than are gram-positive cocci. One reason for this lies in the different structural and chemical composition of the outer layers of the organisms, thereby conferring an intrinsic resistance mechanism on gram-negative bacteria. The cell envelope of gram-negative bacteria is more complex: the inner (cytoplasmic) membrane is adherent to the peptidoglycan which is covalently linked to Braun's elongated lipoprotein. The peptidoglycan–lipoprotein complex partly occupies the periplasmic space that exists between the outer and inner hydrophobic membranes.

The OM acts as a permeability barrier in limiting or preventing the entry of many chemically unrelated types of antibacterial compounds into gram-negative bacteria. Some antibacterial agents, e.g. polymyxins and possibly chlorhexidine and the QACs, damage the OM, thereby promoting their own entry into the cell (Hancock 1984).

Acquired chromosomal resistance by mutation has been described for chlorhexidine resistance with *P. stuartii* (Chopra et al. 1987) and *S. marcescens* (Lannigan and Bryan 1985), alcohols with *E. coli* (Fried and Novick 1973), and cationic bactericides with various organisms (Chopra 1987). Resistance is not always stable. Temporary resistance by phenotypic adaptation to alcohols is described by Chopra (1987), who also considers that, in general, a nongenetic adaptive type of resistance is unlikely to play an important part in determining the long-term survival of bacteria to antiseptics and disinfectants.

Strains of *P. stuartii* isolated from paraplegic patients often harbor plasmids conferring resistance to Hg^{2+} and several antibiotics and the organisms may also express resistance to cationic biocides. However, attempts to transfer chlorhexidine and QAC resistance to suitable recipients have failed and the occurrence of a plasmid-linked association between antibiotic and antiseptic resistance has not been substantiated.

High-level resistance to biocides has been observed in hospital isolates of other gram-negative bacteria but no clear role for plasmid-mediated biocide resistance (Russell 1985, 1997) has emerged in the following instances: (1) chlorhexidine- and antibiotic-resistant *P. mirabilis*; (2) chlorhexidine-resistant strains of *Burkholderia cepacia* and *Alcaligenes denitrificans*; and (3) chlorhexidine- and QAC-resistant *P. aeruginosa* and several members of the Enterobacteriaceae (Russell and Chopra 1996).

'Emerging' gram-negative bacteria that may prove to be of concern include *Acinetobacter* spp. Isolates of these organisms do not, however, appear to date to have a particularly high level of biocide resistance (Higgins et al. 2001; see also Weber and Rutala 1999; Rutala and Weber 2001a, b; Russell 2002a).

BACTERIAL SPORE RESISTANCE

Bacterial spores differ fundamentally in both structure and composition from vegetative cells. In essence, a spore usually consists of a central core (protoplast, germ cell) and germ cell wall surrounded by a cortex, external

to which are an inner and an outer spore coat (Foster 1994; Setlow 1994). Degradative changes take place during spore germination and biosynthetic processes during outgrowth (Paidhungat and Setlow 2002). Vegetative cells thus produced have the typical form and composition of nonsporulating bacteria (Russell 1990a).

Bacterial spores are amongst the most resistant of all microbial forms to inactivation by chemical or physical agents. Several factors contribute to this high resistance (Russell 1990a; Bloomfield and Arthur 1994; Bloomfield 1999; Loshon et al. 2001). These are (a) the thick, proteinaceous coats that limit the uptake of some biocides; (b) the reduced permeability of the spore core/protoplast to hydrophilic agents; (c) the protection of spore DNA by saturation with α, β-type (but not γ-type) small, acid-soluble proteins (SASP); and (d) the repair of DNA damage during germination. SASPs are basic proteins present in the spore core, but are rapidly degraded during germination. They play an important role in determining the response of bacterial spores to biocides. Spores ($\alpha^-\beta^-$) lacking such SASPs are much more sensitive to hydrogen peroxide and hypochlorites than parent spores (Tennen et al. 2000; Loshon et al. 2001).

Several studies (Power et al. 1988; Shaker et al. 1988; Knott et al. 1995; Cabrera-Martinez et al. 2002) have shown that during sporulation, an early resistance develops to formaldehyde, whereas resistance to chlorhexidine and QACs is an intermediate, and to glutaraldehyde and OPA a late, event.

BIOFILMS AND RESISTANCE

A biofilm is a consortium of bacteria or other microbes organized within an extensive exopolysaccharide exopolymer (glycocalyx: Costerton et al. 1987, 1994; Carpentier and Cerf 1993; Denyer et al. 1993; Poxton 1993). Biofilms may consist of monocultures, diverse species, or mixed phenotypes of a given species. Originally envisaged as homogeneous structures, biofilms are considered to be highly structured habitats with spatial and physiological heterogeneity (Wimpenny et al. 2000). Organisms can deposit enzymes within the matrix (Gilbert and McBain 2001a, b; Gilbert et al. 2002a, b) and cell-to-cell signalling (Davies et al. 1998; Swift et al. 2001) is an important feature in biofilm regulation, with the quorum-sensing systems of *P. aeruginosa* providing an excellent example (Wimpenny et al. 2000).

Attachment to surfaces is a vital element in bacterial infections, including those pertaining to medical devices (McBain and Gilbert 2001; Denyer 2002). Sessile bacteria on surfaces or present within biofilms are much less readily inactivated than planktonic cells (Carpentier and Cerf 1993; Poxton 1993; Donlan and Costerton 2002; Dunn 2002). Several reasons have been put forward for this difference. Early stress responses may be involved (see above). Diffusion of biocides into biofilms and interaction with biofilms are both important aspects (Gilbert et al. 2001; Stewart et al. 2001), but are not the only factors responsible. A biocide gradient is produced throughout the biofilm, so that in a thick biofilm there will be an 'in-use' concentration of biocide at the surface, but a greatly reduced concentration as the biocide penetrates into the community (Gilbert and McBain 2001b). Degradative enzymes might be more effective at these lower concentrations within a biofilm than against in-use concentrations acting on planktonic cells

A physiological gradient is also produced such that nutrients and oxygen will be consumed at the periphery of the biofilm with limits to both occurring within the deepest parts of a biofilm. Nutrient-depleted cells are less sensitive to biocides, so that greater numbers of biocide-resistant cells are present towards the interior of a biofilm where they are exposed to lower biocide concentrations. In addition, pockets of surviving organisms may occur as small clusters in biocide-treated biofilms, although neighboring cells have been inactivated (Huang et al. 1995). These might have arisen as a result of the occurrence of biocide and physiological gradients. The clusters might also contain mutants in addition to genotypes with modification in single gene products (Gilbert et al. 2002b).

Furthermore, sublethal treatment with a biocide could induce the expression of multidrug efflux pumps and efflux mutants. Thus, *mar* expression is greatest within the depths of a biofilm where growth rates are lowest (Maira-Litran et al. 2000).

Lewis (2000, 2001) has proposed that the inactivation of a cell does not occur as a result of the direct action of a biocide, but rather from programmed cell death (PCD). Persisters of such a programme are then regarded as cells deficient in PCD that can grow rapidly in the presence of exudate released from lysed community cells.

Thus, many complex factors contribute to the relative insusceptibility to biocides of cells within biofilm communities (Gilbert and McBain 2001a, b; Gilbert et al. 2001, 2002a). In addition to the factors outlined above, cellular impermeability of some types of bacteria will also play a role.

POSSIBLE LINKED BIOCIDE-ANTIBIOTIC RESISTANCE

Biocides and antibiotics share some common mechanisms or sites of action. Acridines, phenylethanol, phenoxyethanol, fluoroquinolones, and some β-lactams all induce filamentation in gram-negative bacteria (Ng et al. 2002), although not necessarily by the same means. However, no cross-resistance was found between the different chemical agents.

Another example occurs with triclosan and the antitubercular drug, isoniazid (isonicotinylhydrazine, INH) (McMurry et al. 1999). Triclosan inhibits enoyl reductase involved in fatty acid synthesis in gram-negative and

gram-positive bacteria (including mycobacteria), whereas INH has a specific effect on *M. tuberculosis* and some other mycobacteria. INH is essentially a prodrug that is converted into an active form by a *katG*-encoded oxidase-peroxidase system in *M. tuberculosis*, but not in other bacteria. Absence of this enzyme is the major mechanism for INH resistance in this organism. In *M. smegmatis*, mutations of the *inhA* gene that encodes a protein target (InhA) of INH involved in mycolic acid biosynthesis confers resistance to both INH and triclosan. Thus, the potential exists for the development of resistance to triclosan in mycobacteria to also be translated into INH resistance. However, it has not been demonstrated that triclosan is responsible for selecting for INH-resistant strains of *M. tuberculosis*.

Possible shared mechanisms of resistance to biocides and antibiotics are impermeability, uptake into gram-negative bacteria of cationic agents, mutations at a specific target site, enzymatic degradation, and extrusion (efflux) (Russell 1995, 2000, 2001c, 2002b, c, 2003a, b, c, 2004; Paulsen et al. 1996; Levy 2000, 2001, 2002a, b; Chuanchen et al. 2001; Health Council of the Netherlands 2001; Sulavik et al. 2001; Schweizer 2001; White and McDermott 2001; Fraise 2002a, b; Koljalg et al. 2002; Loughlin et al. 2002; Walsh et al. 2003a, b). Three biocidal types of agent, namely chlorhexidine, QACs, and triclosan, have been suggested as being involved in antibiotic resistance (Heir et al. 1998, 1999; Sundheim et al. 1998). Lambert et al. 2001 found that biocide/antibiotic-resistance in *P. aeruginosa* occurred especially in hospital, but not in industrial, isolates. However, antibiotic resistance resulted from the selective pressure exerted from antibiotic usage with no evidence that disinfection procedures produced biocide-resistant organisms. In a subsequent study (Joynson et al. 2002), it was demonstrated that an organism, *P. aeruginosa*, that is resistant to a common biocide (the QAC, benzalkonium chloride) could become sensitive to an antibiotic, whereas the converse was not true.

Veterinary clinical isolates of *P. aeruginosa* are resistant to high levels of triclosan, carbenicillin, and tetracycline, all of which are good substrates for the MexAB-OprM efflux system (Beinlich et al. 2001). In an earlier study (Chuanchen et al. 2001), ciprofloxacin resistance had been shown to be possibly associated with triclosan resistance, the phenylether inducing an efflux system. However, this fluoroquine antibiotic is a marginal substrate for this efflux system but a better substrate for MexCD-OprJ and MexEF-OprN (Beinlich et al. 2001).

S. aureus strains are commonly highly sensitive to triclosan (MICs around 1 μg/ml). However, some (but not all) clinical isolates, including MRSA strains, have elevated MICs of 1–2 μg/ml but do not necessarily show antibiotic resistance and are not killed less rapidly than triclosan-sensitive strains by higher concentrations of this compound (Suller and Russell 1999, 2000). VRE (Hiramitsu 1998) are not more resistant to biocides than are vancomycin-sensitive strains (Alqurashi et al. 1996; Day and Russell 1999).

Paulsen et al. (1998) proposed that *qacA* had evolved from *qacB*, that benzalkonium chloride (a QAC) induced the expression of *qacA* and *qacB*, and that their chronological emergence in clinical isolates of *S. aureus* mirrored the introduction and usage of cationic biocides in hospitals. Akimitsu et al. (1999) have claimed that QAC-resistant mutants of MRSA strains show greatly increased resistance to some β-lactam antibiotics.

Ishikawa et al. (2002) suggested that after *E. coli* had mutated to resist a relatively low concentration of a QAC under laboratory conditions, it could be converted to a multidrug-resistant type. However, whilst several authors have shown an association between biocide and antibiotic resistance in laboratory experiments, the 'real-life' situation is more difficult to assess. Currently, it must be stated that the evidence of a linkage between biocide usage and clinical antibiotic resistance is far from proven (Russell 2000; Gerba and Rusin 2001; Gilbert and McBain 2001a; McBain and Gilbert 2001). Antibiotic resistance is far more likely to occur as a result of the inadequate and improper usage of these drugs in clinical practice and their incorporation into animal feed. Many different acquired genes resposible for resistance of gram-negative bacteria to many antibiotics are part of gene cassettes that can move into or out of companion elements (integrons) (Hall and Collis 1998).

The possible role of biocide rotation in preventing biocide resistance from developing in hospitals has been considered by Murtough et al. (2001).

FUNGAL RESISTANCE

Two basic mechanisms of fungal resistance to biocides can be envisaged: intrinsic and acquired (Dekker 1987; Russell 1999d). The fungal cell wall contains various types of polymers, including chitin and chitosan (Zygomycetes), chitin and glucan (mycelial forms of ascomycetes and deuteromycetes), and glucan and mannan (yeast forms of ascomocytes and deuteromycetes). Thus, there is ample opportunity for a cell to exclude biocide molecules. Studies on the sensitivity of *C. albicans* to the polyene antibiotic, amphotericin, suggests that glucan may have a role to play in limiting drug uptake (Gale 1986). Comparable studies with biocides are sparse, but there is tentative evidence that glucan, but not mannan, in yeast cell walls could have a role to play in limiting chlorhexidine uptake (Hiom et al. 1993, 1995a, b, 1996).

There is no evidence linking the presence of plasmids in fungal cells and the ability of the organisms to acquire resistance to fungistatic or fungicidal agents, although acquired resistance of yeasts to organic acids is known (Warth 1986, 1989).

VIRAL RESISTANCE

Conflicting results have been reported about the action of biocides on different types of viruses. In part, this can probably be explained on the basis of different methodologies. However, the penetration of biocides into viruses and phages of different types has not been examined in depth, nor has interaction with viral protein and nucleic acid. It is, therefore, difficult to provide adequate reasons to explain the relative response, or resistance, of different virus types to biocides. Nevertheless, some progress has been made on the basis of the scheme of Klein and Deforest (1983), as described in Types of microorganism. Thurmann and Gerba (1988) suggested that the structural integrity of a virus is altered by an agent that reacts with viral capsids to increase viral permeability. A 'two-stage disinfection' could offer an efficient means of viral inactivation whilst overcoming the possibility of multiplicity reactivation first put forward in 1947 to explain an initial reduction and then an increase in titer of biocide-treated bacteriophage.

RESISTANCE OF PROTOZOA

The cyst form represents the stage in the life cycle that is resistant to biocides. Only a few significant studies have been undertaken about biocide sensitivity or resistance during encystment and excystment and the relative uptake of biocides into cysts and trophozoites. It has been shown that resistance develops as encystment proceeds and is lost during excystment of *Acanthamoeba castellanii*. It may be concluded that the outer regions of cysts limit entry of biocides, thus providing one mechanism of intrinsic resistance (Khunkitti et al. 1997, 1998a, b, 1999; Turner et al. 1999, 2000a, b; Lloyd et al. 2001).

PHYSICAL AGENTS AND OTHER STERILIZATION METHODS

Physical agents (Table 6) are normally used in preference to chemical agents for sterilization, either by microbial destruction or by removal of microorganisms (Dewhurst and Hoxey 1990; Soper and Davies 1990; Kowalski 1993; Russell 1993, 1999d, 2001a, b; Denyer and Hodges 1999; Hoxey and Thomas 1999). It is also practicable, however, to employ cold, desiccation, and freeze-drying as methods of preservation. Traditionally, heat in one form or another has been employed as a sterilization procedure. It still occupies a key role and is the method of choice whenever possible. Filtration is also based on ancient knowledge but suffers several limitations. Ionizing radiation is widely used for the sterilization of single-use, disposable items. Newer procedures employ various types of gas plasmas.

Moist heat

Heating in the presence of water has long been used as a method of sterilization, or sometimes of disinfection. It can be employed in different ways, e.g. at temperatures below, at, or above 100°C.

TERMINOLOGY

Thermal death time (TDT) is the time (in minutes) required to kill all cells in a suspension at a given temperature. Thermal death point (TDP) is the temperature needed to kill all cells in suspension after a

Table 6 *Types and applications of sterilization processes* [a,b]

Process	Application(s)
Moist heat (autoclave)	Sterilization of many ophthalmic and parenteral products, surgical dressings, rubber gloves (high-vacuum autoclave)
Flash sterilization	Modification of conventional steam sterilization
Dry heat	Sterilization of glassware, glass syringes, oils and oily injections, metal instruments; depyrogenation at high temperatures
Infrared radiation	Sterilization of heat-resistant instruments (high temperature responsible)
Ionizing radiation	Sterilization of single-use disposable medical items
Ultraviolet radiation	Poor sterilizing agent; use restricted to air sterilization (in conjunction with air filtration) and water disinfection
	Surface sterilization
Pulsed light (non-ionizing radiation)	
Ethylene oxide	Sterilization of fragile, heat-sensitive equipment, powders, components of spacecraft
Low-temperature steam with formaldehyde (LTSF)	Disinfection/sterilization of some heat-sensitive materials
Filtration	Air sterilization; sterilization of thermolabile ophthalmic and parenteral solutions, and of sera
Gas plasmas	

a) See text for information about most important processes.
b) Chemical agents such as glutaraldehyde and peroxygens are sometimes employed as 'chemosterilizers.'

fixed exposure time, e.g. 10 min. There are many variables associated with these terms, notably inoculum size, and as such they are usually of little value.

The *D* value (decimal reduction time, DRT), is the time (in minutes) needed at a particular temperature to reduce the viable organisms by 90 percent, i.e. to 10 percent or by 1 $\log_{10}$ unit. *D* value is independent of inoculum size and is related inversely to temperature. Examples are given in Table 7.

Inactivation factor (IF) is the degree of reduction in the number of viable cells and is obtained by dividing the initial viable count (N_0) by the final viable count (N_u), i.e. IF = N_0/N_u. An alternative procedure is to use the *D* value approach; IF = $10^{t/D}$ (in which *t* represents the treatment dose, i.e. time). There are, however, pitfalls with this method (Russell 1982).

The *z* value, defined as the number of degrees (°C) to bring about a 10-fold reduction in TDT or *D* value, is obtained from the slope of the curve in which temperature is plotted against time (see Russell 1982). An alternative method is based on a knowledge of the temperature coefficient (Q_{10}) per 10-fold rise in temperature, i.e. $Q_{10} = 10^{10/z}$ from which z = 10/log Q_{10}.

The *F* value is the time in minutes to destroy an organism in a specified medium at 121°C (250°F). F_0 is the *F* value when $z = 10$°C (18°F). Calculations employing F_0 values are used in validating thermal sterilization processes (Soper and Davies 1990).

Exponential rate of inactivation is depicted in Figure 2(A). Deviations from this occur frequently (Figure 2(B, C, D): Cerf 1977; Smerage and Teixeira 1993; Stringer et al. 2000). With convex- or concave-type curves, extrapolation of the line to cut the *Y*-axis gives the *Y*-intercept value (Y_0). If N_0 represents the initial number of cells, then (1) when $Y_0 = N_0$, there is a straight line response; (2) when $Y_0 > N_0$, there is an initial shoulder; (3) when $Y_0 < N_0$, a decreasing death rate is obtained.

The intercept ratio (IR) is a ratio of the two values, i.e. IR = log Y_0/log N_0, and is thus useful in characterizing time–survivor curves.

MICROBIAL SENSITIVITY TO MOIST HEAT

Nonsporulating bacteria are heat-sensitive and are usually destroyed at temperatures of 50–60°C (Russell 2003b). Thermal death times of fungi and protozoa are similar and most viruses are inactivated at 60°C for 20 min (Russell 1999d). *D* and *z* values for some bacterial spores are listed in Table 7.

Bacterial spores are considerably more resistant to moist heat. Among *Bacillus* spp., *B. stearothermophilus* is the most heat-resistant. Considerable variation in sensitivity occurs among *Clostridium* spp., *C. botulinium* type E being far more sensitive than types A, B or C (Russell 1982, 2003d).

Sensitivity to moist heat depends on the conditions of exposure and recovery. In medical, surgical, and veterinary practice, microorganisms are often embedded in organic debris, which may to some extent insulate them from the effect of heat, and thorough cleaning prior to sterilization is essential (Roberts 2001).

TYPES OF MOIST HEAT PROCESSES

Temperatures below 100°C

During his studies on spoilage of French wines, Louis Pasteur employed a temperature of 60°C. Subsequently, this process (60°C for 30 min) was used for the pasteurization of milk. A 'flash' method (71.1°C for 15 s) was introduced later. A procedure similar to pasteurization has been employed in the production of certain bacterial vaccines, e.g. typhoid vaccine, the aim being to kill the organisms while preserving their immunogenicity.

Although some types of spore are killed by moist heat at about 80°C, temperatures below 100°C cannot be relied upon to achieve sterilization.

Temperatures around 100°C

These temperatures cannot be relied upon to kill bacterial spores, and steaming is thus of limited use. Two variations have been used. The first (tyndallization) involved steaming for 30 min (originally 80°C for

Table 7 *Responses* [a] *of some bacterial spores to heat and to ionizing radiation*

	Moist heat		Dry heat		Ionizing radiation (D)	
Bacterial spore	**D (min) at 121°C**	***z***	**D (min) at 160°C**	***z***	**Mrad**	**kGy**
Bacillus stearothermophilus	4–5	8–10	<1	15–25	...	...
B. subtilis	<1	5.5–9.5	1–5	20	0.15	1.5
B. cereus	<1	...	...	20	0.1	1
B. pumilus	...	...	...	25	0.2	2
Clostridium sporogenes	<1	10	2	20	0.15–0.2	1.5–2
C. botulinum type A	<1	9.5	...	...	0.1	1

a) Values are approximate responses to a particular process. The actual response will depend upon several parameters (see text and Russell 1982, where comprehensive details are provided). *D*, time (min) at a specified temperature or radiation dose needed to reduce the viable population by 1 $\log_{10}$ unit; *z*, temperature (°C) needed to reduce the *D* value by 90 percent.

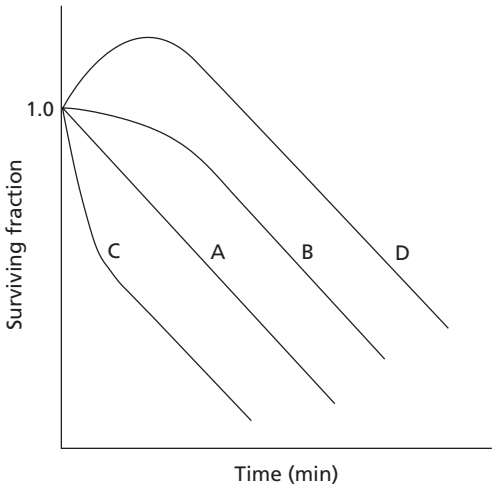

Figure 2 *Thermal inactivation curves of bacterial spores. A, exponential death; B, increasing death rate; C, decreasing death rate; D, heat activation.*

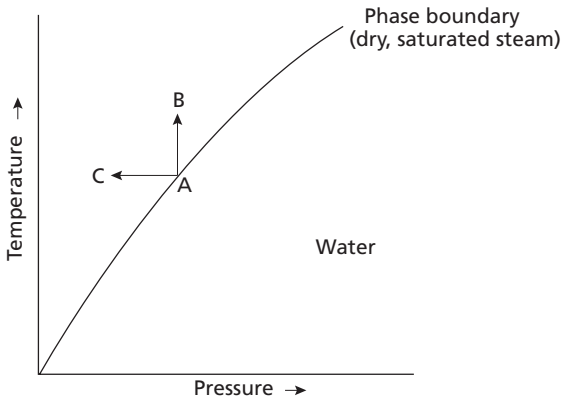

Figure 3 *Phase diagram for water vapor, depicting the phase boundary for dry saturated steam and the production of superheated steam (points B and C). A, any point on the phase boundary; B, pressure kept constant, temperature increased; C, temperature kept constant, pressure increased.*

60 min) on each of 3 consecutive days, the principle being that spores that survived a heating process would germinate before the next thermal exposure and would then be killed. Spores will not, however, germinate in a non-nutrient medium and, although this process was originally an official method for sterilizing injections, it is no longer used. The second variation involved heating to 98–100°C in the presence of a specified antimicrobial agent (the activity of which is considerably potentiated at the high temperature; see Factors influencing activity). This method is no longer permitted in the British Pharmacopoeia (1988, 1993, 1998, 2000) for sterilizing any injectable or eyedrop formulations.

Temperatures above 100°C (autoclaving)

Sterilization by steam under pressure depends upon four properties of dry saturated steam: high temperature, wealth of latent heat, ability to form water by condensation, and instantaneous contraction in volume that occurs during condensation. These properties are optimal only in steam on the phase boundary between itself and condensate at the same temperature (Figure 3). Steam formed at any point on the phase boundary has the same temperature as the boiling water from which it was derived. However, it holds an extra, and relatively heavy, load of latent heat, which, without a drop in temperature, is available instantly and entirely as soon as it wets a cooler surface.

Superheated steam is hotter than dry, saturated steam at the same pressure, the process becoming akin to dry heat, which is much less efficient. Superheated steam behaves as a gas and only slowly yields its heat to cooler objects. In Figure 3, steam at any point A on the phase boundary is saturated: if, however, the temperature is raised to B (keeping pressure constant), then superheated steam is generated. A small amount (5°C) of superheat may be tolerated in practice.

When air is present in a space with steam, the air will carry part of the load so that the pressure of the steam is reduced, creating a condition equivalent to that defined by the horizontal line AC in Figure 3. This can also be deduced from Dalton's law of partial pressures, which, applied to the present context, can be written as $P_{total} = P_{steam} + P_{air}$. The removal of air is, in fact, important in ensuring efficient autoclaving and can be achieved in various ways. In downward-displacement sterilizers, air is displaced downwards, being heavier than steam; this process may, however, be wasteful of steam and time-consuming. Its widest application is in the sterilization of bottled fluids (Owens 1993). Bulky packages of, for example, surgical dressings are, however, air-retentive and the downward-displacement method of removing air is not efficient. Removal of air before admission of steam is far more effective and can be achieved by the use of high prevacuum autoclaves or by means of a pulsed evacuation and steam admission. Thermal processing is also widely employed in the food industry (Brown 1994; Gould 1999). Table 8 lists the time–temperature relationships provided by the British Pharmacopoeia (2000). Sterilizers designed for bottled fluids generally employ temperatures of 121°C and those for porous loads 126–134°C.

Flash sterilization

Flash sterilization is a modification of conventional steam sterilization. In this process, a flashed item is placed unwrapped in an open mesh tray or in a specially designed rigid container to allow for rapid penetration of steam. Either gravity or prevacuum autoclaves can be used for this purpose (Barrett 2001).

Low-temperature steam with formaldehyde

Low-temperature steam (LTS) at subatmospheric pressure was developed originally for disinfecting heat-

Table 8 *Permitted time–temperature relationships in moist-heat and dry-heat sterilization processes* [a]

Process	Temperature (°C)	Holding period (min)
Moist heat (autoclave)	121	15
	126	10
	134	3
Dry heat	Minimum of	Not less than
	160	120
	170	60
	180	30

a) Based on recommendations in British Pharmacopoeia (2000).

sensitive materials. LTS at 80°C was found to be much more effective than water at the same temperature. The addition of formaldehyde to LTS to produce low-temperature steam with formaldehyde (LTSF) achieved a sporicidal effect (Dewhurst and Hoxey 1990; Soper and Davies 1990), and LTSF is thus suitable for sterilizing thermolabile equipment, although there are now doubts about the efficacy of this process.

MECHANISMS OF MICROBIAL INACTIVATION BY MOIST HEAT

Heat induces a multiplicity of injuries in sporing and nonsporing bacteria (Russell 2003b), and every cellular component (outer layers, membranes, enzymes, proteins, RNA, DNA) is likely to be affected to some degree. In thermophiles and hyperthermophiles, enzymes and proteins are much more heat-stable (Madigan et al. 1997) because of critical amino acid substitution in one or a few locations. It is thus unlikely that the lethal effect can be attributed to a single event in an organism.

In nonsporulating bacteria, mild heat treatment (approximately 45–50°C) damages the outer membrane of *E. coli*, rendering cells more sensitive to hydrophobic inhibitors (Hitchener and Egan 1977; Mackey 1983). Lethal damage occurs to the cytoplasmic membrane with RNA breakdown and protein coagulation (Tomlins and Ordal 1976) and damage to the bacterial chromosome (Pellon and Sinskey 1984). Virtually all structures and functions can be damaged by heat, but repair to non-DNA structures can occur only if DNA remains functional, thereby providing the necessary genetic information. DNA is implicated in heat damage, directly or as a result of enzymatic action after thermal injury (Pellon and Sinskey 1984). Using repair-deficient *E. coli* mutants, Mackey and Seymour (1987) proposed that heat-induced DNA damage occurred indirectly via an oxidation mediated by hydrogen peroxide (or free radicals derived from it) in peroxide-generating complex recovery media. Their results also provide a satisfactory explanation for 'minimal medium recovery,' which appears to involve protection from this peroxide-induced effect. By potentiating the effectiveness of DNA repair systems, *Deinococcus radiodurans* (see Physical agents and other sterilization methods) has an extraordinary ability to withstand the lethal and mutagenic effects of DNA-damaging treatments. A relationship exists between bacterial sensitivity to ionizing radiation and mild heat shock, and *D. radiodurans* is considered to be a heat-resistant organism (Battista et al. 2000).

The bacterial cell senses exposure to heat by means of:

- Extracellular alarmones by switching on inherent tolerant mechanisms. Extracellular sensing components (ESC) are directly converted into extracellular induction components (EIC), with very rapid responses occurring at increasing levels of stress (Rowbury 2001).
- Inducible intracellular heat-shock proteins (HSP) Hecker and Volker 2001). In *E. coli*, most genes require the sigma factor σ^{70} for transcription. Genes induced by heat shock have different sequences and RNA polymerase (RNAP) requires σ^{32} to recognize them. The amount of this alternative sigma factor regulates the heat-shock response. σ^{32} is encoded by *rpoH*, which specifically directs RNAP to transcribe from the heat-shock promoters (Polissi et al. 1995; Yura et al. 2000).

In bacterial spores (Figure 2), thermal injury has been attributed to a variety of reasons. These include denaturation of vital spore enzymes, impairment of germination or outgrowth or both, membrane damage (leading to leakage of calcium dipicolinate), increased sensitivity to inhibitory agents, structural damage (as observed by electron microscopy), and damage to the spore chromosome (as evinced by mutations or DNA strand breaks). Effects on DNA are much more pronounced during dry heating (see section on Dry heat); which generates a high level of mutants in spore populations (Gould 1989). DNA is, however, an important target, although the exact mechanism of spore inactivation is unknown. Deficiencies in DNA repair mechanisms are known to render spores more heat-sensitive (Hanlin et al. 1985). SASPs are of two types, the α,β-type associated with spore DNA, and the γ-type, not associated with any spore macromolecule. Spores lacking α,β-type SASPs are more susceptible than wild-type spores to moist heat (Setlow 1995). The DNA in these mutants is more sensitive, with a high frequency of single-strand

breaks (Setlow and Setlow 1996). Spore proteins have also been implicated (Belliveau et al. 1992; Setlow 1994), although the nature of these is unclear.

MECHANISMS OF RESISTANCE OF BACTERIAL SPORES TO MOIST HEAT

The resistance of spores to heat can be manipulated over several orders of magnitude by exposure to extreme pH values and cationic exchange treatment (H-form and Ca-form, respectively) (Alderton and Snell 1963; Alderton et al. 1980). The content and location of water in spores play important roles in their heat resistance. Spores have a low water content (see below) and this is an essential factor in resistance. Spore coats do not contribute to thermal resistance since coatless spores are not more heat-sensitive than ordinary spores. Gould and his colleagues (reviewed by Gould 1989) found that resuspension of newly germinated spores in high concentrations of sucrose or sodium chloride (but not of glycerol) restored resistance to heat and ionizing radiation. In such an osmoregulatory mechanism the cortical structure would have to maintain a uniform pressure upon, and thereby control, the water content of the spore core.

Warth (1985) considered three types of mechanisms that might contribute to the stability of proteins in the spore. They could be intrinsically stable; substances might be present that help stabilize them (there is no strong evidence to support this notion but calcium dipicolinate may play a role); and the removal of water could alter their stability. A direct role for dipicolonic acid (DPA) seems unlikely, but it might help establish and maintain dormancy and heat resistance (Tovar-Rojo et al. 2002).

Several spore properties are, in fact, important for heat resistance, notably protein thermotolerance, dehydration, mineralization, thermal adaptation, and cortex function (Murrell 1981; Beaman and Gerhardt 1986; Beaman et al. 1988, 1989; Gerhardt and Marquis 1989; Marquis et al. 1994, 1995). In addition, SASPs found in the spore core have some role to play. Spores lacking α/β-type SASPs are more sensitive than wild-type spores and it is likely that heat-induced DNA damage is the mechanism involved (Setlow 1994). However, these SASPs are not a major determinant of spore resistance to moist heat because heat resistance during sporulation is attained well after their synthesis (Setlow 1994; Setlow et al. 2001).

The single most important factor contributing to spore resistance to moist heat is the reduced water content of the spore core, with other factors playing a supporting role (Setlow 2000).

Dry heat

Dry heat is a less efficient sterilization process than moist heat. The terminology used in describing moist heat sterilization is also employed here. Bacterial spores are the most resistant organisms to dry heat and D and z values are much higher than for moist heat (Table 7) (Wood 1993; Russell 1999e, 2003d).

TYPES OF DRY HEAT PROCESSES

Normally, dry-heat sterilization takes place in hot-air ovens, a number of combinations of temperature and time being permitted (Table 8). It is essential that the hot air can circulate between objects being sterilized, which must therefore be loosely packed with adequate air spaces to ensure optimum heat transfer. A fan is essential to prevent the wide variation in temperature that would otherwise occur. Industrially, sterilization equipment can also consist of forced-convection ovens, rapid heat-transfer sterilizers, and continuous-belt sterilizers (Wood 1993). Dry-heat sterilization (Table 6) is the method of choice in the pharmaceutical industry for many heat-stable objects and materials (Dewhurst and Hoxey 1990) and at higher temperatures it can also destroy pyrogens (depyrogenation; Wood 1993). The use of infrared ovens has been described (Mata-Portuguez et al. 2002).

MECHANISMS OF MICROBIAL INACTIVATION BY DRY HEAT

Microbial inactivation by dry heat has been considered primarily an oxidation process. Spores heated in oxygen would thus be expected to be more sensitive than when heated in the presence of other gases, but this is not necessarily true (Pheil et al. 1967). Thus, although oxidation may play an important part, other possibilities must be considered.

An effect on DNA is one possibility (Setlow 1994), sublethal temperatures inducing mutants in *B. subtilis* spores (Zamenhof 1960) as a consequence of depurination (Northrop and Slepecky 1967). The water content of spores is an important factor in determining inactivation by dry heat. Rowe and Silverman (1970) postulated that only a relatively small amount of water is needed to protect the heat-sensitive site in spores and that resistance to dry heat depends mainly on the location, rather than on the amount, of water in the spore, and on its association with other molecules.

Inactivation by cold

Growth of microorganisms is retarded and eventually ceases if they are held at reduced temperatures. Some can grow at temperatures approaching 0°C and these psychrophiles may be important food-spoilage organisms. Environmental factors such as nutrient status, pH, salt concentration and a_w (water activity or moisture content) can alter the minimum growth temperature (Herbert 1989).

Freeze-drying involves rapid freezing with subsequent drying in a vaccum and is used widely for preserving microbial cultures and certain foodstuffs, but its use subjects microorganisms to stresses such as freezing, drying, storage, and eventual rehydration (Mackey 1984). Single-strand breaks in DNA and an increase in the frequency of mutation may be caused by freeze-drying (Asada et al. 1979).

Freezing and thawing can inactivate bacteria. Damage to the outer and inner membranes envelope has been shown in *E. coli* (Mackey 1983, 1984). For comprehensive accounts, the papers by MacLeod and Calcott (1976) and Mackey (1984) should be consulted.

Cold shock is a process in which organisms are suddenly chilled without freezing. Gram-positive and gram-negative bacteria, but not yeasts, may be killed (MacLeod and Calcott 1976; Rose 1976). Several factors influence the response of cells, notably the age of the culture (exponential phase, but not stationary phase, cells being susceptible) and the composition of the medium. Divalent cations can protect cells against chilling. Low-molecular-weight materials are released from chilled cells as a consequence of increased membrane permeability caused by a phase transition in membrane lipids (Rose 1976). A modification of this treatment is 'cold osmotic shock,' in which bacteria are suspended in a hypertonic sucrose solution containing EDTA and are then suspended in ice-cold magnesium chloride solution. This treatment does not kill the cells but does induce the release of periplasmic enzymes, including β-lactamases, from gram-negative bacteria.

Hydrostatic pressure

Some organisms can exist at the bottom of deep oceans where they are subjected to high pressure (Dring 1976). Bacterial spores are more resistant to hydrostatic pressure than are germinated spores or nonsporulating bacteria. Temperature profoundly affects bacterial sensitivity to hydrostatic pressure, the rate of inactivation increasing as the temperature rises. This combined effect can be potentiated if an additional heat treatment is given after pressurization (Gould, 1970; Gould and Jones, 1989; see also New low-temperature sterilization technologies). The application of hydrostatic pressure is a useful technique because it enables investigations to be made of germination in the absence of physiological germinants. In view, however, of the occurrence of 'superdormant' spores (Gould et al. 1968), which are resistant to hydrostatic pressure, it is unlikely that this process will find use as a sterilization procedure.

Ionizing radiation

Ionizing radiations, e.g. X-rays, γ-rays, and high speed electrons (β-rays), strip electrons from the atoms of the material through which the radiations pass; essentially all the chemical changes produced in the microbial cell are due to these stripped-off electrons, which initiate a chain of chemical reactions. Ionizations occur principally in water, resulting in the formation of short-lived but highly reactive radicals (hydroxyl, •OH) and protons (H^+). Single-strand, and sometimes double-strand, breakage in DNA ensues. By contrast, ultraviolet (UV) radiation does not possess enough energy to eject an electron to produce an ion, although there is an alteration of electrons within their orbits. It is not therefore an ionizing radiation. Infrared radiations rapidly raise the temperature of objects that they strike and are thus employed for their heating effect. X-rays and γ-rays are of very short wavelengths and are generated by machines or radioactive sources (e.g. ^{60}Co). High-speed electrons were originally produced from radioactive isotopes but had little penetration; various machines have been developed that accelerate atomic particles to give them the energies for penetrating deeply. X-rays and γ-rays have considerable penetrating power. By contrast, α-particles, which consist of helium nuclei ($^4_2$He), have little penetration and thus are not used for sterilization. Industrially, radioisotopes in the form of ^{60}Co (Herring and Saylor 1993) and accelerated electrons (Cleland et al. 1993) are the main sources of ionizing radiation.

The unit of radiation most widely used in microbiology was previously the rad, a measurement of the energy absorbed from ionizing radiation by the matter through which the radiation passes. The newer, more modern (SI) unit is the gray (Gy; 1 Gy = 100 rad), which is defined as the deposition of 1 J/kg energy in tissue.

MICROBIAL SENSITIVITY TO IONIZING RADIATIONS

Bacterial spores are generally more resistant than nonsporulating bacteria, although *D. radiodurans* is the most resistant organism known (Moseley 1989; Russell 1999f). Among the clostridia, *C. botulinum* types A and B are the most resistant, type E being highly sensitive. Among *Bacillus* spp., *B. pumilus* E601 is probably the most resistant (see Table 7). During bacterial sporulation, resistance to γ-radiation develops about 2 h before the onset of heat resistance (Durban et al. 1970). Although spore-coat disulfide bonds were believed to be radioprotective, it is now clear that the –S–S-rich protein structure does not afford resistance, since coatless spores are as resistant as normal spores (Hitchins et al. 1966; Gould and Sale 1970; Farkas 1994).

MECHANISMS OF MICROBIAL INACTIVATION BY IONIZING RADIATIONS

Ionizing radiations induce structural defects in microbial DNA, which, unless repaired, are likely to inhibit DNA synthesis, leading to cell death (Hutchinson 1985). DNA

is not the only target relevant to inactivation, although it is undoubtedly the principal one (Lindahl 1982), with single-strand breaks (SSB) and double-strand breaks (DSB) depending on the intensity of the radiation dose (Moseley 1989).

The state of DNA in the cell is important in relation to bacterial inactivation. Spores are generally more resistant to ionizing radiation than are nonsporing organisms. There are several possible reasons, notably that:

- spores contain a radioprotective substance (but there is no evidence to support this supposition)
- spore coats confer protection, but coatless spores are not less resistant
- DNA is present in a different state in spores.

DNA in bacterial spores exists in the A-form, associated with a low a_w value, and it is certainly true that DNA in the intact spore is more resistant to SSBs and DSBs than DNA in the intact vegetative cell. However, DNA extracted from spores shows the same response in vitro to ionizing radiation as DNA extracted from nonsporulating bacteria. Furthermore, newly germinated spores exposed to ionizing radiation in an environment of high osmotic pressure again become radiation-resistant (Gould 1983, 1984, 1985). Damage to DNA is less extensive under such circumstances.

α/β-type SASPs have some role to play in conferring heat resistance on spores, presumably by stabilizing DNA; however, neither they nor γ-type SASPs are involved in ionizing radiation resistance (Setlow 1994).

MECHANISMS OF MICROBIAL REPAIR FOLLOWING IONIZING RADIATION

Several microorganisms show above-average resistance to ionizing radiation (and to UV radiation, see section on Ultraviolet radiation) because they possess enzymes capable of repairing damage to DNA (Bridges 1976; Moseley 1984, 1989). Repair has been widely studied in *D. radiodurans* and in *E. coli* mutants in which sensitivity to ionizing radiations is conferred by genes such as *rec (A, B, C)*, *polA*, and *lon*.

SSBs in bacterial spores can be repaired during post-irradiation germination (Terano et al. 1969, 1971). In *C. botulinum* 33A, which is highly radiation-resistant, direct repair (rejoining) of SSBs takes place during or after radiation in spores existing under nonphysiological conditions at 0°C and in the absence of germination (Durban et al. 1974). Such repair of SSBs within these dormant, ungerminated spores may result from DNA ligase activity (Gould 1984).

USES OF IONIZING RADIATION

Ionizing radiation has found particular use as a means of sterilizing single-use medical devices (Russell 1999f). Food irradiation has long been considered as a means of sterilizing or preserving food (Russell 1999f) but has met with considerable consumer resistance.

Ultraviolet radiation

UV radiation has a wavelength of approximately 328–210 nm. Its maximum bactericidal effect is documented at 240–280 nm (Sykes 1965). Modern mercury-vapor lamps emit more than 95 percent of their radiation at 253.7 nm, which is at or near the maximum for microbicidal activity. The quantum of energy liberated is low, and consequently UV radiation has less penetrating ability and is less effective than other types of radiation. It is not, therefore, considered as being an effective method of sterilization (Russell 1993, 1999g).

MICROBIAL SENSITIVITY TO UV RADIATION

Bacterial spores are generally more resistant to UV light than are vegetative cells (an exception being *D. radiodurans*: Battista 1997), although mold spores may be even more resistant. Viruses are also inactivated; they tend to be more sensitive than bacterial spores, but are often more resistant than nonsporulating bacteria (Morris and Darlow 1971). HIV is not inactivated by UV radiation (Report 1986).

MECHANISMS OF MICROBIAL INACTIVATION BY UV RADIATION

Exposure of nonsporulating bacteria results in the formation of purine and pyrimidine dimers between adjacent molecules in the same strand of DNA (e.g. the forward reaction in Figure 4a). The photoproduct, 5,6-hydroxydihydrothymine, is also found in *D. radiodurans* exposed to ionizing and UV radiation (Figure 4b). In bacterial spores, another type of photoproduct, 5-thyminyl-5,6-dihydrothymine (TDHT) (Figure 4c) accumulates in DNA. A less well-known and less frequent photoproduct, pyrimidine (6,4) pyrimidone, is the relevant lesion involved in the toxic and mutagenic effects of UV on *E. coli* (Mitchell and Nairn 1989; Koehler et al. 1996). Unless removed, photoproducts form noncoding lesions in DNA and bacterial death results.

MECHANISMS OF MICROBIAL REPAIR IN NONSPORULATING BACTERIA

The SOS network, controlled by the RecA and LexA proteins, is responsible for the expression of genes whose products play roles in radiation-induced excision, daughter strand gap, and DSB repairs. There are three major mechanisms that are involved in repair of UV-induced DNA damage:

1. Photoreactivation (light repair), in which the exposure of UV-irradiated cells to light of a higher wavelength, but below 510 nm, results in the induction of a photoreactivating enzyme that monomerizes thymine

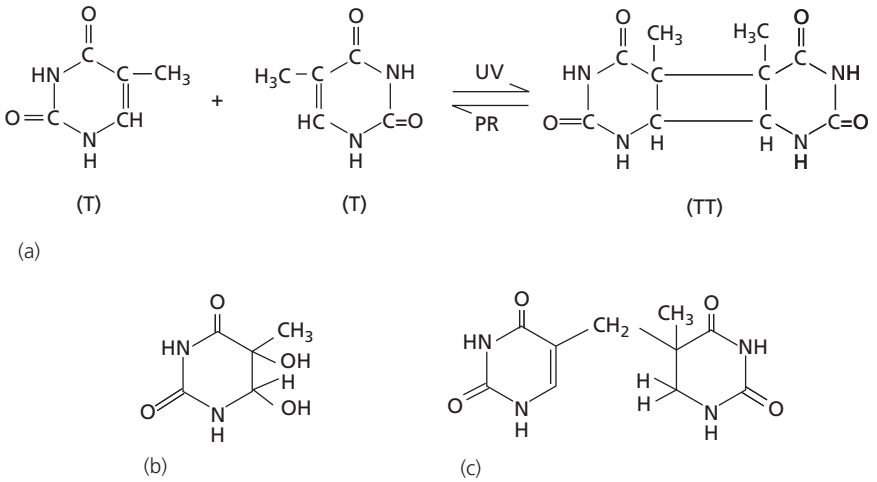

Figure 4 *Ultraviolet (UV)-induced changes in DNA:* **(a)** *pyrimidine dimer formation and reversal (photoreactivation, PR);* **(b)** *5,6-dihydroxydihydrothymine;* **(c)** *5-thyminyl-5,6-dihydrothymine (TDHT). T, thymine; TT, thymine dimer.*

dimers in situ (Figure 4a, reverse direction) and results in the recovery of a large proportion of cells that would otherwise have been inactivated (Moseley 1989; Russell 1999g).

2 Dark repair (excision), in which the stages are first, the action of a dimer-specific endonuclease, which 'nicks' the DNA; second, a dimer-specific exonuclease, which excises the damaged portion together with a number of nucleotides on each side, followed by the action of DNA polymerase I; and, finally, DNA ligase, which is responsible for the final joining together.

3 Dark repair (post-replication recombination), in which replication proceeds normally until it reaches an unexcised dimer, which is a noncoding lesion. A gap is left opposite the dimer and replication recommences at a new initiation site some 800–1000 bases downstream. Because dimers occur in both parental strands of DNA, both daughter strands contain gaps, which are repaired by recombinational insertion of parental DNA from the sister duplex. The process requires a functional recA gene product. Recombination-deficient mutants are abnormally sensitive to UV and ionizing radiations.

For additional information on repair, mutants and error-prone repair, see Bridges (1976), Moseley (1984, 1989), and Sancar and Sancar (1988).

MECHANISMS OF MICROBIAL REPAIR IN BACTERIAL SPORES

The spore photoproduct, TDHT, is identical to one that accumulates in hydrolysates of DNA exposed dry, or as a frozen solution, to UV radiation (Rahn and Hosszu 1968; Varghese 1970). With the exception of *D. radiodurans*, vegetative cells in the frozen state are supersensitive to UV and a photoproduct (presumably TDHT) other than thymine dimers accumulates, which is less susceptible to repair. UV-sensitive mutants of UV-resistant *B. subtilis* spores form the same photoproduct (TDHT) and to the same extent for a given dose of radiation as do the resistant spores. UV resistance is thus linked to the ability to remove TDHT (Munakata and Rupert 1972), and two genetically controlled mechanisms have been described (Munakata and Rupert 1972, 1974):

1 'Spore repair' involving the elimination of TDHT during germination, although vegetative growth is not required

2 Excision repair, in which TDHT disappears slowly from the large-molecular-weight trichloracetic acid (TCA)-insoluble fraction and appears in the TCA-soluble fraction.

PRACTICAL USES OF UV RADIATION

UV radiation has little penetrative power through solids and is extensively absorbed by glass and plastics. Sterilization is achieved only by doses of radiation beyond the limits of practicability. It does, however, have some use as a disinfection procedure. It has been used to disinfect drinking water (Sykes 1965); as a possible means of obtaining pyrogen-free water; and especially for air disinfection, notably in hospital wards and operating theaters, in aseptic laboratories and in ventilated safety cabinets in which dangerous microorganisms are being handled (Morris and Darlow 1971).

Sunlight also has disinfecting properties, rays of wavelengths 254–257 nm possessing the greatest bactericidal activity. It has been claimed that sunlight can inactivate *B. anthracis* spores on wooden and metal surfaces, although only after prolonged exposure. Infectivity of viruses may be reduced on exposure to direct sunlight (Russell et al. 1984).

Sterilization by filtration

Filtration in various forms was used in ancient times, e.g. in attempts to purify water and sewage by allowing it to percolate through beds of sand, gravel, or cinders. These and other applications are considered by Denyer and Hodges (1999) and Jornitz et al. (2002), who also describe the development of modern filtration devices.

TYPES OF FILTER

Several types of filters are used, including (1) *unglazed ceramic filters*, e.g. the Chamberland and Doulton filters, which are manufactured in several different grades of porosity and have been used industrially for the large-scale clarification of water. After use, they can be cleaned with sodium hypochlorite solutions and will withstand scrubbing; (2) *compressed diatomaceous earth filters*, examples of which are the Berkefeld and Mandler filters, which are manufactured in different grades of porosity. They have been used industrially for the large-scale clarification of water. After use, they can be cleaned with sodium hypochlorite but without scrubbing; (3) *asbestos filters*, which include Seitz, Carlson, and Sterimat filters. They have high adsorbing capacities and tend to alkalinate solutions being filtered. There is also a possibility of toxic effects (including carcinogenesis) in handling these filters and they are now practically obsolete; (4) *sintered glass filters*, which are prepared by size-grading finely powdered glass followed by heating. The pore size can be controlled by the general particle size of the glass powder. The filters are easily cleaned, have low adsorption properties, and do not shed particles, but they are fragile and relatively expensive; (5) *membrane filters*, which consist of cellulose esters. They are used routinely in water analysis and purification, sterilization, and sterility testing and are the most suitable for preparing sterile solutions for parenteral use. Membrane filters are available in a range of pore sizes from approximately 12 μm down to 0.5 μm, sterilizing filters usually having an average pore diameter (APD) of 0.2–0.22 μm. The ratio of maximum pore diameter (MPD) to APD is in general 3.5–5. Membrane filters have several advantages over conventional depth filters (Denyer and Hodges 1999).

High-efficiency particulate air (HEPA) filters remove particles of 0.3 μm or larger (and probably particles <0.1 μm) (Denyer and Hodges 1999) and are widely used in air filtration, especially that type incorporating laminar air flow (LAF) (White 1990). LAF units are of two types, horizontal and vertical, depending upon the direction of the air flow.

MECHANISMS OF STERILE FILTRATION

Membrane filters are often described as 'screen' filters in contrast to media such as sintered glass, asbestos fiber, and ceramic, which are believed to retain organisms and particles by a 'depth' process in which particles are trapped or adsorbed within the interstices of the filter matrix. Screen filtration, on the other hand, has been considered to exclude (sieve out) all particles larger than the rated pore size. However, it is now apparent that the filtration characteristics of many membrane filters cannot be accounted for in terms of the sieve retention theory alone, but that other factors are involved, including van der Waals' forces and electrostatic interactions.

Additional information is provided by Lukaszewicz and Melzer (1979), Tanny et al. (1979), and Denyer and Hodges (1999).

APPLICATIONS OF FILTRATION

Filtration can be used for sterilization of thermolabile parenteral and ophthalmic solutions, sterility testing of pharmaceutical products, clarification of water supplies, microbiological evaluation of water purity, viable counting procedures (including estimation of survivors following exposure to antibiotics or disinfectants), and for determination of viral particle size (see also Table 6). For most of these applications, membrane filters are usually employed.

Additionally, filters are used in air sterilization, including LAF cabinets as described above.

New low-temperature sterilization technologies

Several new low-temperature procedures have been described in recent years that have proved to be useful sterilization procedures.

GAS PLASMAS

Low-temperature hydrogen peroxide gas plasmas are generated by applying radiofrequency waves to hydrogen peroxide vapor. The vapor is broken up into reactive free radicals that form the gas plasma (Groschel 1995; Jacobs and Lin 2001). This gas plasma is sporicidal and is eventually broken down to oxygen and water (Rutala and Weber 2001a).

Low-temperature plasma sterilizers that utilize ion plasmas formed from peracetic acid have also been described (Alfa et al. 1998).

LIQUID STERILIZATION SYSTEMS

New liquid sterilization systems have been described that are proving to be of benefit in sterilization of some medical devices. These include automated machines that utilize peracetic acid for the sterilization of lensed flexible and rigid endoscopes (Groschel 1995; Holton et al. 1995; Alfa et al. 1998).

Performic acid, a new proprietary liquid sterilant, is part of a new automated endoscope-reprocessing system

(Rutala and Weber 2001a). It is formed by mixing the two component solutions, hydrogen peroxide and formic acid.

Combined synergistic treatments

Enhanced antimicrobial efficacy is often achieved by combining two or more processes, chemical or physical. There are various types of combined treatment (Gould and Jones 1989).

THERMOCHEMICAL TREATMENT

The antibacterial activity of a compound usually increases as the temperature rises (see Factors influencing activity). This was exemplified by using certain preservatives at high temperatures in the sterilization of certain injectable and ophthalmic products (Russell 1999a), although this process is no longer approved. The gaseous sterilant ethylene oxide is also employed at higher temperatures (approximately 60°C) and LTSF has been claimed to be a useful sterilization procedure. In the food industry, acid–heat treatment is an important method of sterilization (Gould and Jones 1989).

CHEMICAL TREATMENT AND IRRADIATION

The presence of various chemicals during irradiation may either sensitize bacterial spores to, or protect them from, the consequences of ionizing radiation. Spores are more sensitive under oxic than under anoxic conditions. Ketonic agents of widely differing electron affinities sensitize spores suspended in anoxic buffers to subsequent radiation, although the maximum sensitization achieved is only about 40 percent of that in oxygen alone (Tallentire and Jacobs 1972). Sensitizing agents appear to act by removing electrons from the conversion of •OH to OH^-, which is harmless to the spore.

THERMORADIATION

Thermoradiaton is the simultaneous use of heat and ionizing radiation. Careful selection of temperature is, however, important because a 'paradoxical inversion' or 'thermorestoration' occurs at certain temperatures; for example, with *C. botulinum 33A* spores, there is an increase in sensitivity above 80°C (Grecz 1965).

OTHER COMBINATIONS

Other combined processes that have been examined include hydrostatic pressure combined with heat or radiation (Wills 1974), ultrasonic waves with GTA (Boucher 1979), and combinations of chemicals (Russell 1982).

Process validation, monitoring, and parametric release

It is important that strict control measures are employed to validate and monitor the efficacy of sterilization processes. In addition, sterility tests on samples of a batch of treated product are necessary (Favero, 1998; British Pharmacopoeia 2000), although undue reliance should not be placed on such tests alone.

Process validation (Favero 1998) is a means of demonstrating that a process actually works, i.e. that it kills (or removes) microorganisms, thereby achieving what it purports to do (achieve sterilization). For this reason, the choice of a test organism is important: it must possess above-average resistance to the process in question, although it need not necessarily be the most resistant organism known. Biological indicators (BI) in the form (usually) of bacterial spores are used to validate those processes designed to inactivate microbes. *D. radiodurans* is even more resistant to ionizing radiation than spores (see Ionizing radiation). It is not, however, used as a BI for this process because (a) it is not a normal contaminant (bioburden) on pharmaceutical or medical products and (b) its inactivation would require unacceptably high radiation doses that could damage many such products. Examples of BIs are provided in Table 9.

The principle involved in validating sterile filtration is to use a smaller-than-average test organism. If this is retained by the filter, then larger organisms also will not pass through the filter.

Table 9 *Validation and monitoring of sterilization processes*

Process	Validation[a]	Monitoring[b,c]
Moist heat	*B. stearothermophilus*	Temperature recording charts; thermocouples; CI
Dry heat	*B. subtilis* var. *niger*	Temperature recording charts; thermocouples; CI
Ionizing radiation	*B. pumilus*	Dosimeters; CI
Ethylene oxide	*B. subtilis* var. *niger*	Temperature probes; relative humidity; *B. subtilis* var. *niger*
Filtration	*S. marcescens*; *P. diminuta*	Bubble point pressure test

a) In addition to the biological indicators listed, the desired physical and chemical conditions to achieve sterilization must also be validated, e.g. measuring devices for heat, dosimeters for radiation, physical methods for determining filter integrity and temperature, and relative humidity for gaseous sterilization.
b) CI, chemical indicator (used as a visual check that a sterilization process has been undertaken: a color change does not necessarily mean that sterilization has been achieved).
c) Samples taken at random from a batch may also be subjected to sterility testing.

In addition to validating a process with BIs, it is also necessary to measure and record the required physical or chemical conditions reached throughout the sterilization cycle within every part of the load (Habarer and Wallhaeusser 1990).

Routine monitoring of a process is carried out by appropriate physical methods, except for ethylene oxide where difficulties still occur (Table 9). Sterility can then be assessed, except with ethylene oxide, by monitoring only the physical conditions of the process. Such a system (parametric release) is defined as the release of sterile products based on process compliance to physical specification (Dewhurst and Hoxey 1990).

Sterility assurance level (SAL) is a term employed to provide an expected level of safety with a sterilization process. It is generally accepted that terminally sterilized products, i.e. those sterilized in their final containers, have a safety level (SAL) of not more than one unsterile article per 10^6 items processed (Habarer and Wallhaeusser 1990; Bruch 1993; Graham and Boris 1993). SAL is thus based upon a sound knowledge of microbial inactivation kinetics, rather than upon sterility testing, which can detect only gross contamination.

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Antibacterial therapy

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PHILOSOPHY OF ANTIBACTERIAL USE

Prospects for the future

At the start of the twenty-first century, more than 250 antibacterial agents are in use throughout the world. With such an abundance at the prescribers' disposal it might be imagined that bacterial infection has become a minor problem and that bacterial drug resistance would be unable to keep pace with resources available to counteract it. Nothing could be further from the truth. Bacteria appear to possess a limitless ingenuity in avoiding the effects of antimicrobial agents, as well as in finding new ways to invade the compromised host. Moreover, mutations conferring resistance to one antibiotic can, at a stroke, render a whole drug family impotent. Bacteria resistant to one sulfonamide or one tetracycline are usually resistant to all sulfonamides, or all tetracyclines; 'methicillin-resistant' staphylococci are resistant to all β-lactam antibiotics. Even worse, bacteria can assemble resistance genes for unrelated classes of agents on integrons and plasmids that can be readily transmitted between bacterial species or, sometimes, genera.

For these reasons it is unwise to be complacent about our ability to control infection. Resistance is a serious global problem that must be countered by discriminating use of antimicrobial agents by all prescribers, by vigorous application of control of infection measures in health care institutions, and at government level by effective regulation of drug production, distribution, and use (European Union Conference 1998; Gould et al. 2000).

Ecological issues

Antibiotic use is huge with estimates as much as 100 000 tons/year worldwide, the majority of this in farming including veterinary use, growth promotion, crop spraying, and fish farming (Levy 2002; Harrison and Lederberg 1998). Potentially there is significant environmental pollution as much antibiotic is excreted in active form in urine and feces and is stable for prolonged periods (weeks to months) in the environment (Harrison and Lederberg 1998). No doubt this pollution revisits us through the food-chain in the form of antibiotic residues, antibiotic-resistant bacteria, and their resistant-determinate genes contaminating food. The scope of the problem has only been investigated on a very superficial basis.

It seems likely, nevertheless, that with a few notable exceptions, most of the antibiotic-resistant bacteria that cause problems in human medicine are generated by use of antibiotics in human medicine. Within human medi-

cine, the intensity of antibiotic use is greatest in the hospital and this is where (magnified by crossinfection) the most significant problems are seen (Gould 1999, 2002a). Within intensive care units (ICU), these problems of crossinfection and intensity of use are further magnified and even added to, by increasingly invasive and immunosuppressive diagnostic and therapeutic procedures. It is no surprise then that ICUs are considered the genesis units for the creation, maintenance, and dissemination of multiply-resistant bacteria (Gould 2002b).

Community antibiotic use is much less intense, although greater in overall volume (Cars et al. 2001). While the limited data available suggest not much difference in usage patterns between different hospitals across Europe, when case mix is taken into account, community use between different countries differs up to fourfold, tending to be lowest in northern Europe (Harbarth et al. 2002). A European average is approximately one prescription per head of population per year. Some countries have managed to lower community prescribing in the past few years with the help of educational campaigns directed both at doctors and their patients and concentrating on reducing the demand for and use of antibiotics for self-limiting respiratory tract infection which are usually viral in origin (Department of Health 2000).

In most countries of the industrially developed world, antibiotics used in human and veterinary practice are presently available only on prescription. It is most important that this status is maintained if resistance problems are to be kept under control. In developing countries where regulation of pharmaceutical products is generally more lax, more responsible national drug policies need to be enforced. The World Health Organization is actively engaged in promoting rational policies of drug use, but much remains to be done. One important initiative has been the formulation of an essential drug list which is updated at regular intervals (World Health Organization 1995). Only a handful of antibacterial agents appear on this list (Table 1); significantly, there are no cephalosporins or quinolones on the main list, emphasizing the restricted need for these extensively promoted and commonly prescribed drugs.

How use relates to resistance

While it is often said that there is little absolute proof that antibiotic use causes resistance, the link has been well established in numerous studies at individual patient level, group level such as family or day care center, in hospitals, community medical practices, regions, and whole countries (Baquero et al. 2002). The association applies to all antibiotics and bacteria but not to all bug/drug combinations. *Streptococcus pyogenes*, for example, has famously failed to become resistant to penicillins. Even here, however, while strains still yield the same minimum inhibitory concentration (MIC) of penicillin as they did 60 years ago there is some evidence of tolerance developing to the bactericidal activity of penicillins (Slater and Greenwood 1983). The speed of development of resistance and its degree will depend on many things. Probably most important will be the mechanism of action of the antibiotic and the ease with which the bacteria can develop resistance, e.g. bactericidal antibiotics, by eradicating a pathogen, are probably less likely to select resistance than bacteriostatic agents which will merely suppress division (Stratton 2003). If the bacterial strain has a high yield of resistant mutants or resistance spreads easily on plasmids then resistance will, presumably, arise and even spread easily. Intensity of use, spread by crossinfection, high inoculum infections, and immunosuppression are other factors likely to lead to rapid selection and/or

Table 1 *Antibacterial agents (excluding topical agents) on the World Health Organization's list of essential drugs (WHO 1995)*

Main list			
Penicillins	**Other antibacterial agents**	**Antimycobacterial agents**	**Complementary list[a]**
Amoxycillin[b]	Chloramphenicol	Clofazimine	Chloramphenicol (oily suspension)
Ampicillin	Co-trimoxazole[b]	Dapsone	Ciprofloxacin
Benzathine penicillin	Doxycycline	Ethambutol	Clindamycin
Benzylpenicillin	Erythromycin[b]	Isoniazid	Nalidixic acid
Cloxacillin[b]	Gentamicin[b]	Pyrazinamide	Nitrofurantoin
Phenoxymethylpenicillin	Metronidazole[b]	Rifampin	Trimethoprim
Piperacillin[b]	Spectinomycin	Rifampin + isoniazid	Thioacetazone + isoniazid (antimycobacterial)
Procaine penicillin	Sulphadimidine[b]	Streptomycin	
	Tetracycline[b]		

a) For use when drugs on the main list are known to be ineffective or inappropriate for a given individual, or for use in exceptional circumstances (e.g. chloramphenicol oily suspension in epidemics of meningitis) when the health services are overwhelmed. The need for additional reserve agents, e.g. a cephalosporin or vancomycin, is acknowledged.
b) Example of a therapeutic group for which acceptable alternatives exist.

spread of resistance and these are all factors operating in hospitals (Gould 1999; Gould and MacKenzie 2002). Long term, low-dose penicillin therapy has been shown to select efficiently for penicillin resistance in pneumococci (Guillemot et al. 1998). Patients receiving prior trimethoprim or amoxicillin for urinary and chest infections are several times more likely to have antibiotic-resistant infections if they present again within a few months (Steinke and Davey 2001). Within hospitals, most organisms causing problems of hospital-acquired infection (HAI) are selected by intensive antibiotic use; extended spectrum beta-lactamase (ESBL) producing *Klebsiella* by third generation cephalosporins (3GC) (MacKenzie and Gould 1998), derepressed *Enterobacter* and *Citrobacter* by 3GC (Wagenlehner et al. 2002), quinolone-resistant coliforms by quinolones (Scheld 2003; Kern et al. 1994), methicillin resistant *Staphylococcus aureus* (MRSA) by 3GCs, quinolones and macrolides (Monnet 1998; Monnet et al. 2004). In most cases, the organisms are resistant to the antibiotics selecting for them. Things may become more complicated in future as integrons, coding for multiple antibiotic-resistant determinants, are well dispersed in European hospitals (Gruteke et al. 2003; Leverstein-van Hall et al. 2003).

Can resistance be controlled?

It has long been assumed that if the high use of antibiotics is reversed, bacteria will normally return to their susceptible state. There is, however, no clear answer to this as it is usually a multifactorial issue (Austin et al. 1999). There are many examples in the literature, both in communities and hospitals where resistance problems have been reversed (although not necessarily returning to zero) when antibiotic use is controlled (Austin et al. 1999). Generally, resistance problems fall more quickly in hospitals than in the community because of the dilutional effect of patient discharge. Nevertheless, there are examples in the literature where control of use fails to reverse resistance problems and there may be many other cases where such negative results have not been published (Bonhoeffer et al. 1997). Mathematical models and in vitro experiments suggest that bacteria can rapidly learn to carry resistance without too much cost so that in many cases the resistance may be stable, even in the absence of the selection pressure of antibiotic prescribing (Bowler et al. 1994). Similarly, if a virulent clone becomes resistant, it may spread without the need for antibiotic selection pressure. Other factors to consider have been discussed above. Nevertheless, the consensus is that if antibiotic use is inappropriate it is correct to reduce it. If the high use is associated with a resistant problem then it will probably recede along with the reduction in use, but the role of crossinfection should also be addressed, particularly in healthcare institutions.

Antibiotic stewardship

Every healthcare institute and primary care practice should have an antibiotic formulary (list of antibiotics easily available and which the doctors should be familiar with) and antibiotic guidelines (details of diagnosis and treatment for specific infections) (Gould et al. 1994; Gould 2001; Keuleyan and Gould 2001). Within any region it is helpful if the formulary and guidelines are drawn up in consultation between primary and secondary care. Their primary aim is to act as an educational tool for doctors to improve their prescribing and in the UK and USA, most hospitals and primary care centers will have them (Gould et al. 1994; Shlaes et al. 1997; Gould 2001; Keuleyan and Gould 2001). There is also helpful guidance available on design and implementation of guidelines (www.agreecollaboration.org).

The formulary is a good basis for pharmacists to work to, only stocking formulary agents and within this list, monitoring the use of key 'restricted' agents, often with the help of infection specialists with an interest in prescribing. Inappropriate prescriptions can then be targeted with an educational intervention to limit inappropriate use (Solomon et al. 2001). In the near future it is hoped that electronic prescribing will allow easier monitoring of prescriptions (Burke and Pestotnik 1996). Other measures of a restrictive or educational nature that have variable success include special antibiotic order forms, automatic stop orders, therapeutic substitution, antibiotic rotation (or cycling) (Gould et al. 1994; Gould 2001; Keuleyan and Gould 2001; Shlaes et al. 1997), although the evidence base for these measures is often questionable. Currently, there are two Cochrane groups assessing the literature in this area in hospitals and primary care (Arnold 2004; Brown 2004). Help on assessing the effectiveness of interventions is available at www.abdn.ac.uk/hsru/epoc/. There is an increasing tendency to appoint pharmacists and microbiologists with a special interest in antibiotic therapy to monitor quality of use and to create multidisciplinary teams with hospital executive authority (Saizy-Callaert et al. 2003; Fluckiger et al. 2000).

Particular problems of antibiotic use differ between primary and secondary care. In primary care it tends to be unnecessary use for minor, self-limiting bacterial or viral respiratory infections (Steinman et al. 2003a, b; McCaig et al. 2003) . In secondary care it is often too much use of powerful broad spectrum, injectable antibiotics without any evidence of serious infections (Kumarasamy et al. 2003). These are often continued for too long and added to irrationally if the patient does not respond (spiralling therapeutic empiricism) (Kim and Gallis 1989).

Whereas use of the laboratory is relatively rare when treating infection in primary care it could (arguably) be increased (Shackley et al. 1997). Providing specimens

reach the laboratory the same day, it might be possible to give useful preliminary results the next day for the great majority of specimens. In most cases, even where antibiotic is thought to be indicated by the primary care doctor, the patient will not come to any harm waiting overnight to see if culture indicates that one is really needed. With modern electronic links, results can easily be communicated to the doctor the following morning (MacKenzie et al. 2003). In secondary care the laboratory is used much more, but clinicians are often reluctant to act on the results, e.g. streamline successful therapy, even with susceptibility results from clinically significant bacterial growths to guide them (Kumarasamy et al. 2003). They are, generally, even more reluctant to stop therapy early in the presence of negative cultures which may be strong evidence of a nonbacterial etiology for a patient's illness (Lawrence et al. 1973; Bartlett et al. 1991). Much is written about the importance of rapid molecular methods to guide clinicians in the therapy of infectious diseases. Much more efficient use, however, could be made of conventional test results, the great majority of which can be made available in a useful clinical timeframe: antigen tests, Gram stain, microscopy all within an hour, 6-h urine culture, Gram stain of blood cultures – 90 percent positive within 24 h, other specimen preliminary cultures available overnight. Conventional susceptibility testing methods can often give good indications of the best agents to use within 6–12 h, although final results take longer to read (NCCLS, 2000, 2002).

Good clinical liaison by the laboratory is crucial and there is an increasing tendency for microbiologists to undertake clinical consultations and ward visits to report on positive blood cultures, make daily visits to ICU, other high dependency units, and hematology/cancer wards to discuss important results and confirm correct choice of therapy.

General principles of use of antimicrobial agents for treatment and prophylaxis

The general principles underlying the choice of antimicrobial agents in infection are discussed by Lambert and O'Grady (1997), Finch (1995), and Keuleyan and Gould (2001). In brief, antibiotics should be used therapeutically only after thorough clinical assessment of the need, whenever possible on the basis of laboratory evidence of infection. Factors to be considered should include: the type of infection; the age and condition of the patient; the local prevalence of resistance (in the absence of specific laboratory guidance); the pharmacological properties of the agent in its various formulations; the likelihood of adverse reactions; possible interactions with other medications; and cost. Antimicrobial agents should be used for chemoprophylaxis only in individuals in whom the risk of infection is high. The agents chosen should be reliably active against the organisms likely to be encountered and use should be confined to the period of greatest risk (Slack 1995b).

A helpful definition of appropriate antibiotic therapy might include the following: (1) Appropriate antibiotic prescribing should potentially benefit the patient. (2) There should be clinical evidence, supported where possible by laboratory tests of bacterial infection. (3) Sepsis parameters should be documented and should support the need for antibiotic treatment. (4) Critical patients should receive appropriate treatment as quickly as possible. (5) Treatment should be limited to bacterial infections, using antibiotics directed against the causative agent, given in optimal dosage, interval, and length of treatment, with steps taken to ensure maximum patient compliance with the treatment regimen and only when the benefit of treatment outweighs the individual and global risks. (6) Antibiotic therapy should be streamlined at the earliest opportunity using the results of laboratory tests where possible (Gould 2001). There is an increasing number of evidence-based guidelines on the prophylaxis and treatment of infectious diseases now available (www.sign.ac.uk; www.cochcrane.org).

Pharmacodynamics of antibiotics (the effect of antibiotic concentration)

Over the past 20 years great strides have been made in understanding how to optimize antibiotic treatment by varying dose and dosing interval. This not only improves patient outcome, but also reduces the likelihood of selecting resistant strains during therapy. The study of pharmacodynamics (the effect of concentration) developed from a knowledge of the pharmacokinetics of different antibiotics (that is, the concentration achieved at different target sites) and the realization that antibiotics act in different ways. In particular, some antibiotics are primarily concentration dependent in their mode of action, most importantly the aminoglycosides and quinolones (Gould 1998; Drusano 2003). This has led us away from traditional three or four times daily administration of gentamicin to once daily administration to optimize its bactericidal effect. In addition, aminoglycosides and quinolones have a prolonged suppressive effect on regrowth of surviving bacteria (post antibiotic effect (PAE)) which allows a safe period of several hours of subinhibitory concentrations before it is necessary to give the next dose. Conveniently this also appears to ameliorate toxicity which is more dependent on high trough than high peak concentrations, due to the uptake of aminoglycosides by sensitive renal, vestibular, and cochlear cells being concentration limited (Mingeot-Leclecq and Tulkens 1999). Finally, high peak concentrations appear to be important in preventing

selection of resistant mutants which otherwise might arise during therapy and cause treatment failure (Blondeau et al. 2001; Zhao and Drlica 2001; Scheld 2003).

A great deal of work has been carried out recently on the mutant prevention concentration of new quinolones against pneumococci and other bacteria. Although purely theoretical at the moment, it is probable that this work will lead to further reassessment of dosing schedules for quinolones, where mutants selected during therapy appear to be the only way of resistance arising. In the meantime, dosing schedules of quinolones are considered flexible and in general terms can either be given one or two times daily despite widely varying half-lives. The single most important pharmacodynamic predictor of both clinical and microbiological success is achieving an area under the curve inhibitory concentration (AUIC) >125 (AUIC = AUC_{0-24}/MIC) (Thomas et al. 1998).

Other antibiotics, most importantly the β-lactam drugs and macrolides are time-dependent drugs. Once the concentration at the site of infection is above the MIC there is usually little advantage in increasing concentration further. Most of the antibacterial effect will be determined by the duration of exposure. Animal experiments suggest optimal effects if the concentration is maintained above the MIC ($T > \text{MIC}$) for approximately 50 percent of the dosing interval, although this figure is probably lower for most gram-positive infections and higher for gram-negative infections because of the absence of a significant PAE in the latter. For life-threatening or high inoculum infection, such as meningitis, where the host immune response is limited and for more resistant organisms, it is possibly safer to try to ensure $T > \text{MIC}$ for the whole dosing interval and to aim for concentrations around 8× MIC to ensure maximum bactericidal effect.

Antimicrobial drug combinations

The use of combinations of antimicrobial agents is increasing but makes more likely the risk of adverse reactions from the antibiotics themselves and from interactions with other drugs that the patient may be receiving. They include use of combination therapy in tuberculosis and leprosy; use of compounds that interact synergically to achieve improved bactericidal activity in certain serious infections, notably cystic fibrobsis and infective endocarditis; use of combinations to achieve adequate cover of mixed infections or in the 'blind' therapy of serious undiagnosed sepsis and lastly, treatment of infections that are already multiresistant to prevent selection of further resistant mutants. Thus, combinations are frequently used for MRSA infections and particularly when rifampin or fucidin is used as resistant mutants are known to arise easily to these agents when used alone. Such a strategy has been very successful over the years with tuberculosis.

CLASSIFICATION OF ANTIBACTERIAL AGENTS IN CLINICAL USE

Since antibacterial agents exhibit a very wide variety of properties, the simplest way to categorize them is according to their site of action in the bacterial cell: agents acting on the bacterial cell wall; agents acting on bacterial protein synthesis; agents acting on nucleic acid synthesis; and agents acting on the bacterial cell membrane (Table 2).

AGENTS ACTING ON BACTERIAL CELL WALL SYNTHESIS

The bacterial cell wall is a structurally unique feature that is absent from mammalian cells and is thus a prime target for selectively toxic agents. The cell wall of gram-negative organisms is very different from that of gram-positive bacteria and the complex of lipopolysaccharide and lipoprotein that forms the gram-negative outer-membrane confers properties of differential permeability that have a profound effect on the susceptibility to antibacterial agents of all kinds (Nikaido and Vaara 1985;

Table 2 *Classification of antibacterial agents according to their site of action*

Cell wall synthesis	Protein synthesis	Nucleic acid synthesis	Cell membrane
Penicillins	Aminoglycosides	Sulphonamides[a]	Polymyxins
Cephalosporins	Chloramphenicol	Diaminopyrimidines[a]	Gramicidin
Carbapenems	Tetracyclines	Quinolones	Tyrocidine
Other β-lactams	Macrolides	Rifamycins	Valinomycin[b]
Glycopeptides	Lincosamides	Nitroimidazoles	Monensin[b]
Bacitracin	Fusidic acid	Nitrofurans	
Cycloserine	Streptogramins	Novobiocin	
Fosfomycin	Mupirocin		
Isoniazid	Ketolides		
	Oxazolidinones		
	Glycylcyclines		

a) Indirect action on nucleic acid synthesis.
b) Not used in human medicine.

Hancock and Bellido 1992). Within the gram-negative outer membrane are hydrophilic channels (porins) that allow the differential passage of many agents whose target is the underlying peptidoglycan. Permeation of these porin channels by β-lactam antibiotics depends on molecular size and ionic charge and these factors may strongly influence the spectrum of activity and potency (Nikaido 1985). Glycopeptides are too large to penetrate the gram-negative outer membrane and consequently their spectrum of activity is virtually restricted to gram-positive organisms.

β-Lactam antibiotics

STRUCTURE

Antibacterial agents that share the structural feature of a β-lactam ring are now known to be very diverse. The classic penicillins are penams, characterized by a fused heterocyclic structure composed of a β-lactam ring and a five-membered sulfur-containing thiazolidine ring. In the cephalosporins the fused dihydrothiazine ring has an extra carbon with an unsaturated bond between C-3 and C-4, giving a cephem structure. The cephamycins are similar, but the β-lactam ring is substituted with a methoxy group which confers stability to many β-lactamase enzymes. Other structural variants represented among clinically useful compounds are: carbapenems, carbacephems, oxacephems, clavams, sulfones, and monocyclic monobactams (Figure 1).

MODE OF ACTION

All β-lactam antibiotics interfere with bacterial cell wall synthesis, but the effect on gram-positive and gram-negative bacteria is very different because of the differing nature of their cell walls. In the simplest terms, β-lactam antibiotics interfere with the final transpeptidation reaction that forms the crosslink between adjacent peptidoglycan strands and gives the cell wall its essential rigidity. Various forms of transpeptidase exist depending on whether cell wall structure is engaged in extension of the cylinder of rod-shaped cells, forming the poles of the cell, or separating the two daughter cells during the division process. Realization that β-lactam antibiotics affected all of these processes came from studies that showed concentration-dependent effects on the morphological response of gram-negative bacilli (Greenwood and O'Grady 1973a); by observation that certain β-lactam agents were anomalous in the morphological effects they produced (Greenwood and O'Grady 1973b); and by the demonstration of differential affinity of β-lactam agents for penicillin-binding proteins (PBP) in isolated cell membranes (Spratt 1975). The morphological consequences of binding to various PBPs of *Escherichia coli* are shown in Figure 2. PBPs have been investigated in many gram-positive and gram-negative bacteria. Although their number and molecular size vary considerably, they are always present in multiple forms.

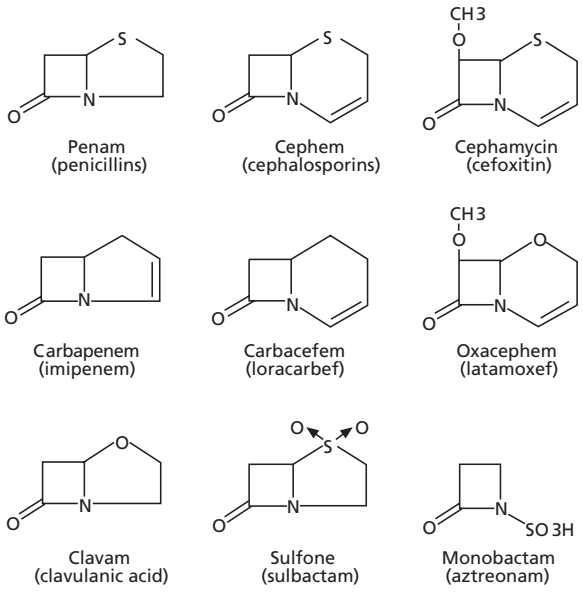

Figure 1 *Core molecular structures of various kinds of β-lactam antibiotics (examples in parentheses). (Redrawn from Greenwood 1995)*

β-Lactam antibiotics are normally bactericidal drugs, but the mechanism of bactericidal activity seems to be different in gram-negative and gram-positive bacteria. In gram-negative rods, cell death can be quantitatively prevented by provision of adequate osmotic protection (Greenwood and O'Grady 1972), so that the mechanism appears to be simple osmotic lysis of bacteria deprived of their normal cell wall. In gram-positive cocci the situation is more complicated: exposure to β-lactam agents causes a loss of lipoteichoic acid from the wall and this seems to remove control from normal autolytic processes that dismantle the peptidoglycan (Tomasz 1979).

Certain strains of gram-positive cocci succumb to the bactericidal effects of penicillin and other β-lactam agents more slowly than usual. Such strains have been dubbed *tolerant* to penicillin (Sabath et al. 1977; Handwerger and Tomasz 1985). Penicillin tolerance may have some relevance in the treatment of bacterial endocarditis or in other situations in which a bactericidal effect is crucial to therapeutic success.

RESISTANCE TO β-LACTAM ANTIBIOTICS

The most common form of resistance to β-lactam agents is caused by enzymes that render the molecules inactive by opening the β-lactam ring. In staphylococci the enzymes involved are inducible exoenzymes that conform to a small number of related types. In contrast, the β-lactamases of gram-negative bacilli vary greatly in their physicochemical characteristics. Indeed, all gram-negative bacilli appear to exhibit some β-lactamase

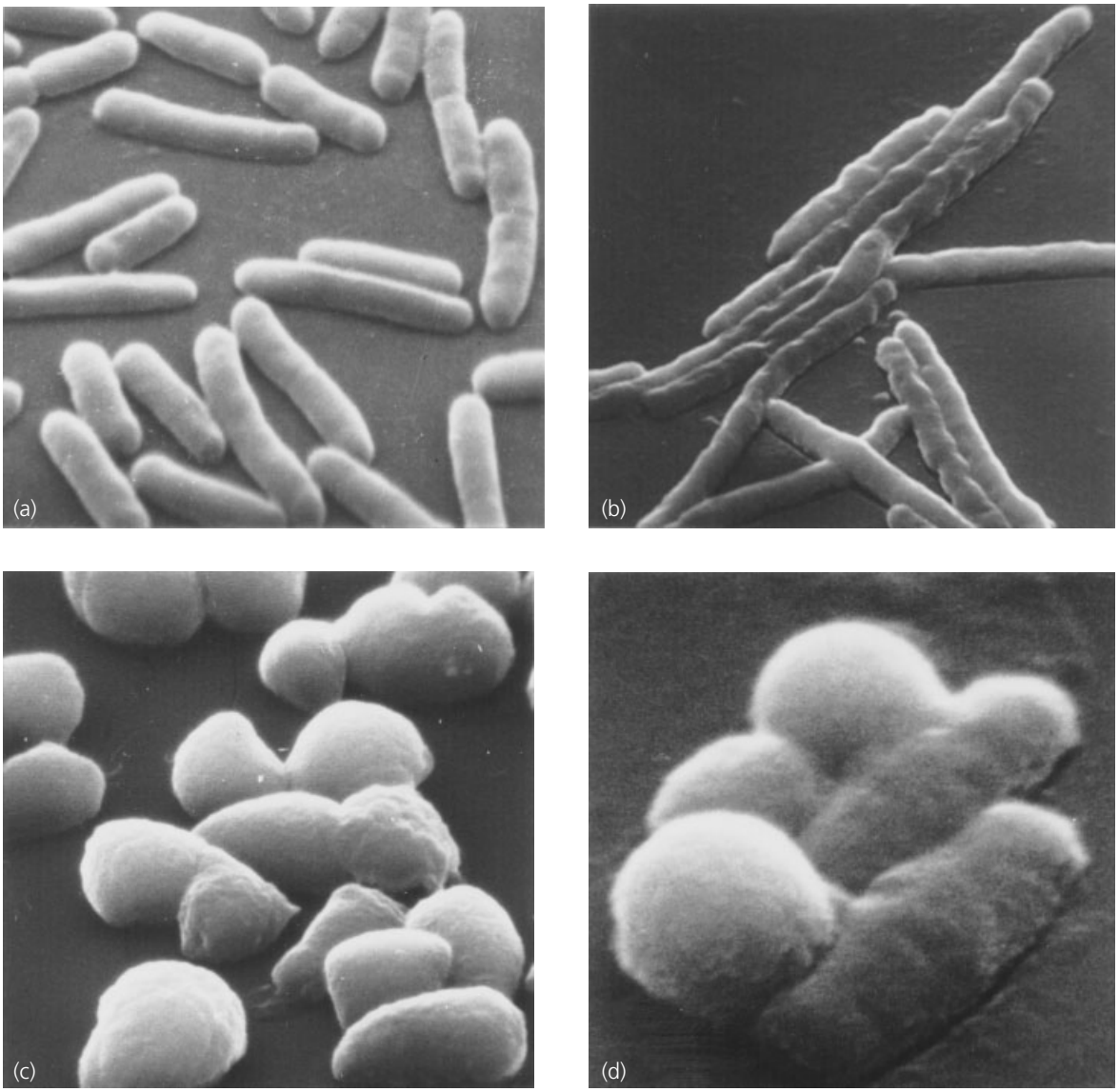

Figure 2 *Morphological effects of β-lactam antibiotics in* Escherichia coli*: **(a)** normal cells (no antibiotic); **(b)** filamentation caused by cephalexin (binding to PBP-3); **(c)** generalized effect on cell wall caused by mecillinam (binding to PBP-2); **(d)** formation of osmotically fragile spheroplasts caused by cephalexin and mecillinam in combination (binding to PBP-2 and PBP-3). Most β-lactam antibiotics, in sufficient concentration, also bind to the PBP-1 complex and cause rapid lysis of susceptible gram-negative bacilli.*

activity as a chromosomally encoded genetic feature. These inherent β-lactamases may be inducible and certain β-lactam antibiotics, notably cefoxitin, are particularly efficient inducers.

There have been various attempts to classify β-lactamases, particularly the many types found in gram-negative rods (Ambler 1980; Richmond and Sykes 1973; Bush 1989). The latest comprehensive classification scheme recognizes four separate molecular types and several functional groupings (Bush et al. 1995).

Although these classification schemes attempt to include all the known types of β-lactamases and are therefore quite complex, the majority of the enzymes that commonly cause problems in clinical isolates of gram-negative bacilli belong to a few related types that share the feature of having serine at the active site and are susceptible to inhibition by clavulanic acid and other β-lactamase inhibitors. Most common of all are the TEM-l and TEM-2 enzymes found widely among enterobacteria and elsewhere, and SHV-l, found predominantly in *Klebsiella pneumoniae*. Many variants of the TEM enzymes have arisen, often by single amino acid substitutions and these may exhibit extended, or idiosyncratic, substrate ranges. Most problematic at present for clinicians are the clavulanic acid-sensitive ESBLs that hydrolyze third and to a lesser extent, fourth generation cephalosporins (Gould 1999) and the clavulanic acid resistant enzymes – the so-called inhibitor-resistant TEM enzymes as they are usually derived from TEM-1 (Chaibi et al. 1999). These enzymes give resistance to

co-amoxiclav and other β-lactamase inhibitors. They may be widespread, but are difficult to test for on a routine basis. TEM-1 itself, and some other β-lactamases, have found their way on to transposons and this has aided their transmission and spread. Bacterial β-lactamases that are able to hydrolyze carbapenems, like imipenem, are still unusual in many countries. They are usually zinc-requiring metalloenzymes that are not susceptible to β-lactamase inhibitors (Payne 1993). They are presently found most commonly in *Acinetobacter* spp., *Aeromonas* spp., and in *Stenotrophomonas maltophilia* and are classified by Bush et al. (1995) as a separate molecular class B (group 3) (see section on Carbapenems).

Resistance to β-lactam antibiotics can arise by means other than β-lactamases. Alterations in porins in the outer membrane of gram-negative bacilli can affect transport of β-lactam antibiotics to their site of action (Nikaido 1985). More importantly, mutations affecting the structure of PBPs can alter their affinity to β-lactam compounds so that inhibitory effects become much less efficient. This, for example, is the mechanism of resistance in methicillin-resistant *Staphylococcus aureus* and in strains of *Streptococcus pneumoniae* that display reduced susceptibility to penicillin (Klugman 1990; Jacoby and Archer 1991).

TOXICITY AND SIDE EFFECTS

Since the β-lactam antibiotics act on a target (bacterial peptidoglycan) that is absent from mammalian cells, they are among the least toxic of all antimicrobial agents. The most troublesome side effect is hypersensitivity, which can range from relatively trivial rashes to life-threatening anaphylactic reactions. Less than 10 percent of patients hypersensitive to penicillins are cross-allergic to cephalosporins (Anne and Resman 1995). Ampicillin and amoxicillin commonly give rise to a maculopapular rash when given to patients with glandular fever; this is not a true hypersensitivity reaction and is not a contraindication to subsequent use of a penicillin. Various other side effects are occasionally encountered. A fuller discussion is provided in the standard texts (Kucers and Bennett 1987; Dollery 1991).

Penicillins

CLASSIFICATION AND SPECTRUM OF ACTIVITY

The various penicillins differ in the nature of the side chain at the C-6 position of the molecule. All except mecillinam (amdinocillin), in which the side chain is joined in an amidino linkage, are acyl derivatives of 6-aminopenicillanic acid (Figure 3).

The original penicillin, benzylpenicillin (penicillin G), has a phenylacetamido group at the 6-amino position. It exhibits exceptionally good activity against the classic pyogenic cocci: staphylococci, streptococci (including pneumococci and enterococci), meningococci, and gonococci. It is also very active against spirochetes, most anaerobes (but not *Bacteroides* spp.), and many gram-positive bacilli. Enterobacteria, *Pseudomonas aeruginosa*, and obligate intracellular bacteria are mostly insensitive.

The chief imperfections of penicillin are: lability to gastric acid, so that it cannot be given orally; an exceptionally short plasma half-life; susceptibility to staphylococcal β-lactamase; and a restricted spectrum of activity. Subsequent developments of penicillin have sought to overcome these shortcomings and presently available compounds can be categorized into several groups with distinctive properties (Table 3).

The long-acting salts of penicillin are poorly soluble depot preparations that are injected intramuscularly and release benzylpenicillin slowly from the injection site. In this way, procaine penicillin can maintain a concentration of penicillin inhibitory to many sensitive organisms for up to 24 h. Benzathine and benethamine penicillin are even less soluble and liberate small amounts of penicillin over several days. An alternative way of sustaining penicillin levels in plasma is by the concurrent administration of probenecid, which competes for the active tubular secretion sites in the kidneys.

Oral derivatives of penicillin, notably phenoxymethylpenicillin (penicillin V), have antibacterial properties very similar to those of benzylpenicillin.

The antistaphylococcal penicillins, of which the isoxazolyl derivatives, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin are most widely used, were designed to overcome the problem of enzymic resistance in staphylococci. They are intrinsically less active than benzylpenicillin and although they retain adequate antistreptococcal activity, their use is largely restricted to infections with penicillinase-producing staphylococci, which now account for more than 90 percent of all isolates in many European countries.

The first successful extension of the spectrum of benzylpenicillin was achieved in 1961 with the β-amino derivative, ampicillin (Rolinson and Stevens 1961). Unlike the parent compound, ampicillin exhibited useful activity against some gram-negative bacilli, including *Haemophilus influenzae*, *Escherichia coli*, *Salmonella* spp., and *Shigella* spp., but not *Klebsiella* spp. or *P. aeruginosa*. Amoxicillin, which is better absorbed when administered orally, followed in 1970. In the meantime, the first penicillin to exhibit (albeit weak) antipseudomonal activity, carbenicillin, the β-carboxy derivative of benzylpenicillin, was described (Knudsen et al. 1967). The thienyl variant of carbenicillin, ticarcillin, and a series of acylureido derivatives of ampicillin (azlocillin, mezlocillin, piperacillin, and apalcillin), all of which display improved antipseudomonal activity, were developed later. None of these compounds is stable to staphylococcal β-lactamase.

PENICILLINS

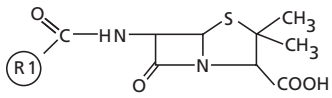

Benzylpenicillin (Penicillin G)

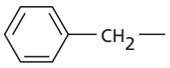

Phenoxymethylpenicillin (Penicillin V)

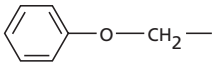

Phenethicillin

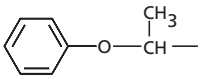

AMINO PENICILLINS

Ampicillin

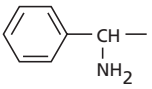

Amoxycillin

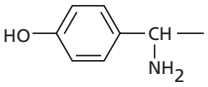

CARBOXY PENICILLINS

Carbenicillin

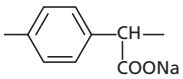

Ticarcillin

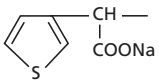

ANTISTAPHYLOCOCCAL PENICILLINS

Methicillin

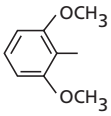

Cloxacillin

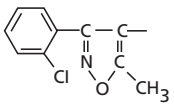

Flucloxacillin

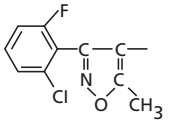

Oxacillin

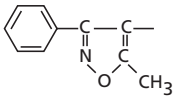

UREIDO PENICILLINS

Azlocillin

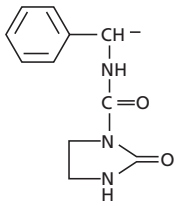

Mezlocillin

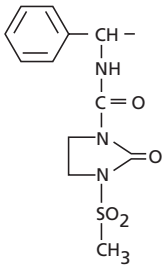

Piperacillin

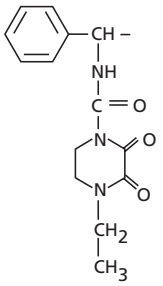

OTHER PENICILLINS (FULL STRUCTURES)

Temocillin

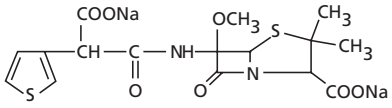

Mecillinam

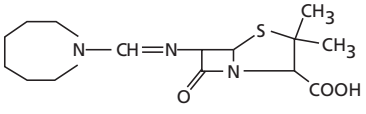

Figure 3 *Structures of the most important penicillins.*

Table 3 *Categorization of the most important pencillins in clinical use*

Most important penicillins in clinical use	Availability
Benzylpenicillin (penicillin G)	Available
Long-acting salts (depot preparations)	
Procaine penicillin	Available via import
Benzathine penicillin	Not available in UK
Benethamine penicillin	Not available in UK
Acid-stable (oral) derivatives	
Phenoxymethylpenicillin (penicillin V)	Available
Azidocillin	Not available in UK
Phenethicillin	Not available in UK
Propicillin	Not available in UK
Antistaphylococcal penicillins (stable to staphylococcal β-lactamase)	
Methicillin	Not available in UK
Cloxacillin	Not available in UK
Flucloxacillin	Available
Oxacillin	Not available in UK
Dicloxacillin	Not available in UK
Nafcillin	Not available in UK
Penicillins with activity against enterobacteria	
Ampicillin (and esters)	Available
Amoxycillin	Available
Mecillinam (and ester)	Available as pivmecillinam
Ciclacillin	Not available in UK
Epicillin	Not available in UK
Penicillins that are stable to enterobacterial β-lactamases[a]	
Temocillin	Not available in UK
Penicillins with activity against *Pseudomonas aeruginosa*	
Carbenicillin (and esters)	Not available in UK
Ticarcillin	Available only with clavulanic acid (Timentin)
Azlocillin	Not available in UK
Mezlocillin	Not available in UK
Piperacillin	Available only with tazobactam (Tazocin)
Apalcillin	Not available in UK

a) Ampicillin, amoxicillin, ticarcillin, and piperacillin are also formulated with β-lactamase inhibitors that confer the property of stability to enterobacterial β-lactamases.

Among penicillins that possess an idiosyncratic spectrum of activity that excludes gram-positive cocci and anaerobes are the amidinopenicillins, mecillinam and temocillin, a compound structurally similar to ticarcillin, but rendered stable to enterobacterial β-lactamases by incorporation of a methoxy group on to the β-lactam ring (compare with cefoxitin and other cephamycins). Neither mecillinam nor temocillin has any useful activity against *P. aeruginosa*.

PHARMACOLOGICAL PROPERTIES

Most penicillins are absorbed erratically when given orally, often because of hydrolysis by gastric acid. Phenoxymethylpenicillin and related compounds are acid stable and are used as oral substitutes for benzylpenicillin. Among isoxazolyl penicillins, flucloxacillin achieves the highest plasma concentrations after oral administration. About 30 percent of an oral dose of ampicillin is absorbed, but this can be much improved by esterification of the carboxyl group. These ampicillin esters, talampicillin, pivampicillin, bacampicillin, and lenampicillin, are inactive prodrugs that are de-esterified during passage through the intestinal mucosa with liberation of ampicillin into the bloodstream. Prodrug esters of carbenicillin and mecillinam have also been produced as oral formulations.

Benzylpenicillin and phenoxymethylpenicillin are extremely rapidly excreted, with a plasma half-life of about 30 min. Most other penicillins exhibit elimination half-lives of around 30 min to 1.5 h. Temocillin has an unusually extended half-life of approximately 4–5 h. Protein binding is generally less than 50 percent; the isoxazolylpenicillins are extensively (>90 percent) protein-bound in plasma, but this does not seem to adversely affect their therapeutic activity.

Penicillins do not penetrate the intact blood–brain barrier well, but in the presence of meningeal inflammation concentrations are often sufficient to treat pyogenic

meningitis and benzylpenicillin (meningococcal and pneumococcal meningitis) and ampicillin (*Haemophilus meningitis*) have been widely used for this purpose in the past.

Cephalosporins

CLASSIFICATION AND SPECTRUM OF ACTIVITY

Since they first became available in the mid-1960s, the cephalosporins have been developed more diversely than any other group of antimicrobial agents. They have overlapping properties that defy any rigid classification, but can usefully be divided into six broad classes (Table 4). In the cephalosporins, the extra carbon of the dihydrothiazine ring carries an additional side chain, the nature of which often affects the pharmacokinetic behavior of the molecule and in some cases, the toxicity. Structures of some of the commonly used cephalosporins are shown in Figure 4. As a group, the cephalosporins have certain properties in common: relative stability to staphylococcal β-lactamase; broad spectrum activity that encompasses most enterobacteria, including *Klebsiella* spp.; and lack of activity against enterococci.

Like the penicillins, cephalosporins interfere with bacterial cell wall synthesis through binding to PBPs. Some cephalosporins, notably cephalexin and cephradine, bind almost exclusively to PBP-3, which has the effect of halting division, but not growth, of susceptible gram-negative bacilli. Consequently, these cephalosporins are much more slowly bactericidal than the others and the build up of endotoxin from filamentation may well have deleterious clinical effects (Gould and MacKenzie 1997).

PARENTERAL CEPHALOSPORINS

The first cephalosporins, cephalothin and cephaloridine, modestly expanded the spectrum of ampicillin (e.g. to include *Klebsiella* spp.) and possessed relative stability to staphylococcal β-lactamase. Some later derivatives offered little, if any, improvement, but cephazolin exhibited the unusual characteristic of achieving enhanced concentrations in bile and cephamandole offered partial resistance to some enterobacterial β-lactamases.

The first major improvement in cephalosporins came with the introduction of compounds that displayed stability to a wide range of enterobacterial β-lactamases. One such compound, cefoxitin, is a semisynthetic derivative of a naturally occurring compound in which the β-lactam ring is substituted with a methoxy grouping that confers stability to the structure. Such compounds are called cephamycins; various examples, including cefotetan, cefbuperazone, cefmetazole, and cefminox, are in use around the world. They are unusual among cephalosporins in their activity against *B. fragilis*.

β-Lactamase stability was achieved in a different way by pharmaceutical chemists who found that addition of a methoximino group to the side chain on 7-aminocephalosporanic acid also protected the β-lactam ring from enzymic attack. The first cephalosporin of this type was

Table 4 *Categorization of cephalosporins in clinical use*

Cephalosporins				
Parenteral compounds	**Oral compounds**			
Cephalothin	Cephacetrile	Ceforanide	Cephalexin	Cephaloglycin
Cephaloridine	Cefapirin	Cefonicid	Cephradine	Cefatrizine
Cephazolin	Cefazedone		Cefaclor	Cefroxadine
Cephamandole	Ceftezole		Cefadroxil	Cefprozil
Compounds with improved β-lactamase stability				
Cefuroxime	Cefmetazole[a]	Cefotiam	**Non-esterified**	**Esterified**
Cefoxitin[a]	Cefbuperazone[a]		Cefixime	Cefuroxime axetil
Cefotetan[a]	Cefminox[a]		Ceftibuten	Cefpodoxime proxetil
			Cefdinir	Cefetamet pivoxil
				Cefteram pivoxil
Compounds with improved intrinsic activity and β-lactamase stability				
Cefotaxime	Cefmenoxime			
Ceftizoxime	Cefodizime			
Ceftriaxone	Latamoxef[b]			
Compounds distinguished by activity against *Pseudomonas aeruginosa*				
Broad spectrum	**Medium spectrum**	**Narrow spectrum**		
Ceftazidime	Cefoperazone	Cefsulodin		
Cefpirome	Cefpimazole			
Cefepime	Cefpiramide			

Adapted from Greenwood (1995).
a) Cephamycins
b) Oxacephem

CEPHALOSPORINS

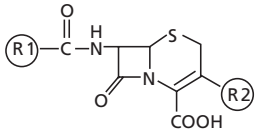

PARENTERAL CEPHALOSPORINS

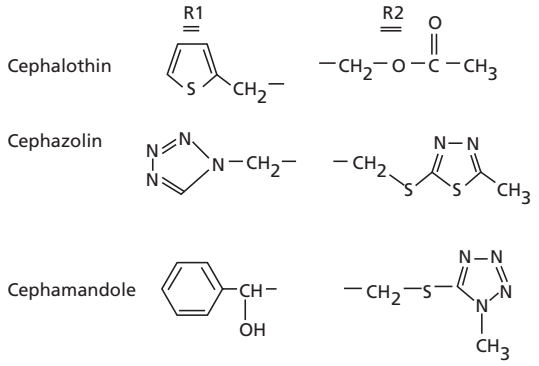

PARENTERAL CEPHALOSPORINS WITH IMPROVED β–LACTAMASE STABILITY

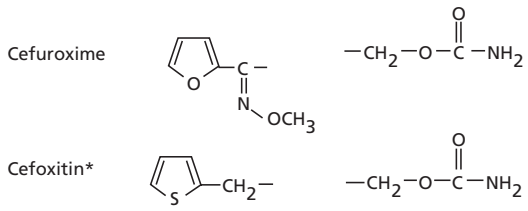

PARENTERAL CEPHALOSPORINS WITH IMPROVED INTRINSIC ACTIVITY AND β–LACTAMASE STABILITY

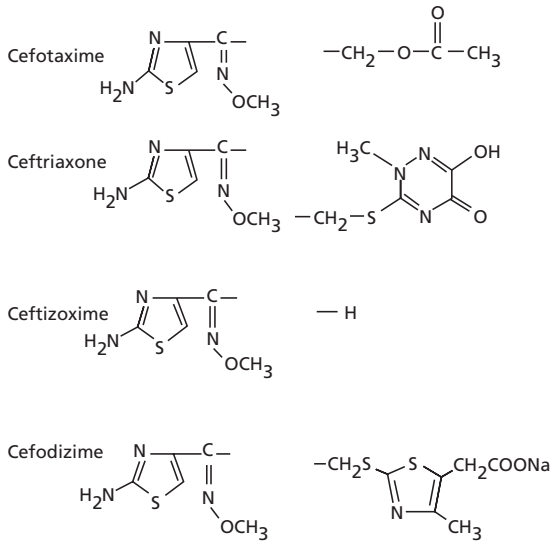

ORAL CEPHALOSPORINS

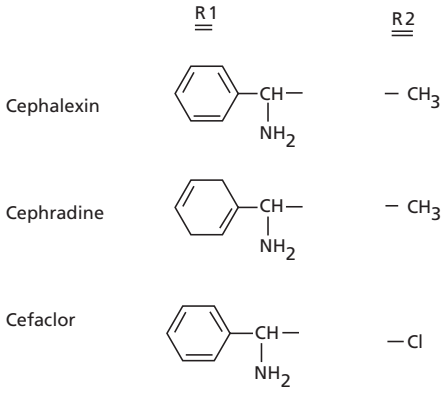

PARENTERAL CEPHALOSPORINS DISTINGUISHED BY ACTIVITY AGAINST *PSEUDOMONAS AERUGINOSA*

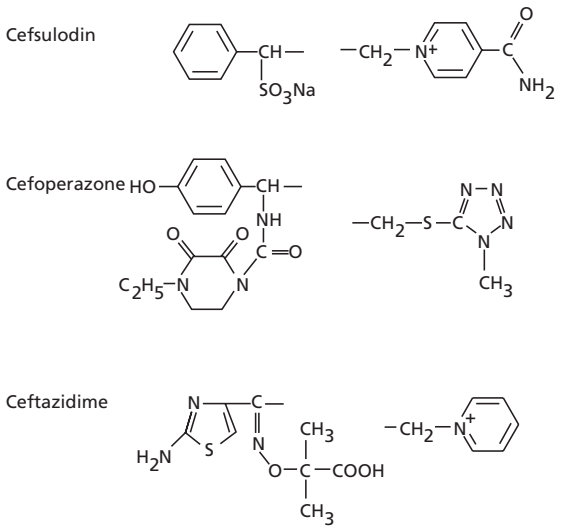

* Cefoxitin (a cephamycin) also has a methoxy group on the β-lactam ring.

Figure 4 *Structures of the most important cephalosporins.*

cefuroxime, but this was quickly followed by a family of compounds, of which cefotaxime was the forerunner, in which the side chain carried an aminothiazole group, as well as the methoximino substitution. The effect of this was not only to provide β-lactamase stability, but also to much improve the intrinsic antibacterial activity, particularly against enterobacteria. Activity of these compounds against *P. aeruginosa* is, however, poor, but some later derivatives, notably ceftazidime, include this organism in the spectrum. Cefsulodin is unique in exhibiting good antipseudomonal activity, but has little or no useful activity against other organisms. Later developments in the form of cefepime and cefpirome not only provide antipseudomonal activity but some resistance to most ESBLs.

ORAL CEPHALOSPORINS

Few cephalosporins are absorbed when administered by the oral route. Cephalexin was the first of those that are well absorbed; it exhibits modest antibacterial activity against a wide spectrum of gram-positive and gram-negative bacteria, but is slowly bactericidal to enterobacteria because of its preferential affinity for PBP-3 (see above). It has no useful activity against *P. aeruginosa*, *H. influenzae* or *B. fragilis*. Most other oral cephalosporins are structurally similar to cephalexin and not surprisingly, share its limitations. Cefaclor is unusual in displaying useful activity against *H. influenzae* and is especially well absorbed. Attempts have been made to improve the intrinsic activity of oral cephalosporins, or to esterify parenteral compounds to enhance their absorption. Cefixime and ceftibuten display much improved activity against enterobacteria, but have some important weaknesses in their gram-positive spectrum, in particular in their poor activity against staphylococci, enterococci, and some streptococci. Cefpodoxime retains better activity against staphylococci and streptococci. None have useful activity against *P. aeruginosa*. Oral cephalosporins have recently been reclassified (Williams et al. 2001).

PHARMACOLOGICAL PROPERTIES

Cephalosporins, like penicillins, are generally excreted rapidly by the kidneys with elimination half-lives that usually range from 1 to 2 h. The expanded spectrum compound, ceftriaxone, is unusual in that its plasma half-life is about 6–8 h. Cephazolin, cefoperazone, and ceftriaxone are notable in achieving significant concentrations in bile. Those cephalosporins that carry an acetoxymethyl group at C-3 (including cephalothin and cefotaxime) are susceptible to hepatic enzymes which deacetylate the molecule to the corresponding hydroxymethyl derivative. This reduces the inherent antibacterial activity, but there is little evidence that this affects the therapeutic potency. Some other cephalosporins, cephamandole, cefotetan, and cefoperazone among them, have a nitrogen-rich tetramethylthiomethyl substituent at C-3. Compounds with this feature have been implicated in hypoprothrombinemia that is reversible by administration of vitamin K (Lipsky 1988).

Cephalosporins penetrate poorly into cerebrospinal fluid after intravenous administration, but some, notably cefotaxime and ceftriaxone achieve sufficient concentration in the cebrospinal fluid (CSF) in the presence of inflammation for them to be useful for intravenous treatment in bacterial meningitis.

Other β-lactam antibiotics

Several variants on the β-lactam theme other than penams (penicillins) and cephems (cephalosporins and cephamycins) are in therapeutic use (Figure 5).

β-LACTAMASE INHIBITORS

Certain molecules that possess a β-lactam ring, including the naturally occurring antibiotic, clavulanic acid and the penicillanic acid sulfones, sulbactam and tazobactam, are noteworthy not for their intrinsic antibacterial activity, but because they have a high affinity for and stability to, bacterial β-lactamases. These agents are formulated with β-lactamase labile partner compounds to secure their activity against otherwise resistant organisms: clavulanic acid with amoxicillin (co-amoxiclav) or ticarcillin; sulbactam with ampicillin (or with cefoperazone in some countries); tazobactam with piperacillin.

CARBAPENEMS, CARBACEPHEMS, OXACEPHEMS

In the mid-1970s, a potent, broad spectrum β-lactam compound, thienamycin, was found as a naturally occurring product of *Streptomyces cattleya* (Kahan and Kahan 1979). The substance, which was found to possess an unusual carbapenem ring structure, was, unfortunately, inherently unstable, but this was overcome by synthesis of the *N*-formimidoyl derivative, imipenem (Kahan et al. 1983) (see Figure 5). Imipenem has exceptionally good activity against most gram-positive and gram-negative bacteria, including organisms like enterococci, *P. aeruginosa*, and *B. fragilis* that are resistant to most cephalosporins. It is very stable in the presence of bacterial β-lactamases, but by a strange trick of nature it is hydrolyzed by a dehydropeptidase located in the brush border of the mammalian kidney and has to be given with a dehydropeptidase inhibitor, cilastatin (Kahan et al. 1983). A related carbapenem, meropenem, is not susceptible to dehydropeptidase and is therefore administered alone. These compounds are not absorbed when given by mouth and are administered parenterally. Resistance to carbapenems is still uncommon, but they are hydrolyzed by naturally occurring metallo-β-lactamases produced by *S. maltophilia*, *Flavobacterium* sp., *Aeromonas* sp., and *B. fragilis*. Methicillin-resistant staphylococci and penicillin-resistant enterococci are

β-LACTAMASE INHIBITORS

Clavulanic acid

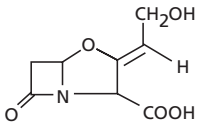

Sulbactam

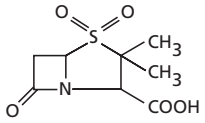

Tazobactam

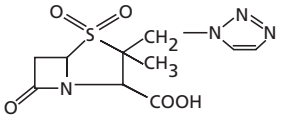

CARBAPENEMS

Imipenem

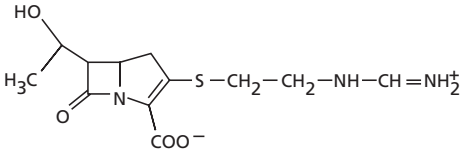

Meropenem

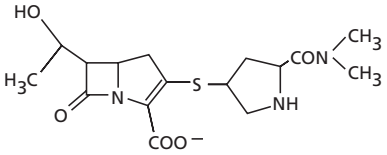

OXACEPHEM

Latamoxef

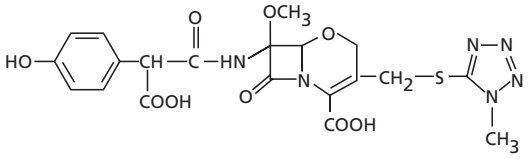

CARBACEPHEM

Loracarbef

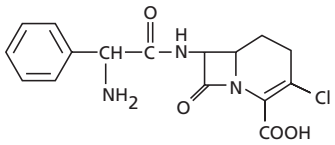

MONOBACTAM

Aztreonam

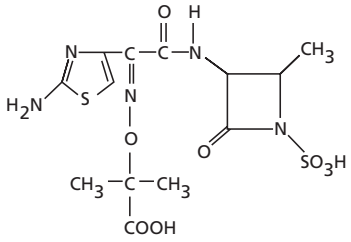

DEHYDROPEPTIDASE INHIBITOR (USED WITH IMIPENEM)

Cilastatin

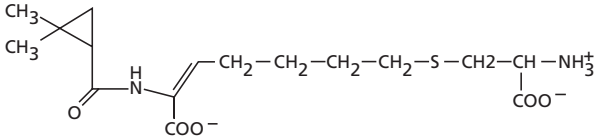

Figure 5 *Structures of important β-lactam compounds other than penicillins and cephalosporins, and of the dehydropeptidase inhibitor cilastatin.*

usually refractory to carbapenems. A new, once daily carbapenem, ertapenem has reduced antipseudomonal activity. Carbapenem resistance in Acinetobacter, Pseudomonas, and some Enterobacteriaceae is becoming an increasing clinical problem. Two types of class B metallo β-lactamase are prevalent: Verona imipenemase (VIM) and IMP. They are now becoming widespread and a VIM-producing *P. aeruginosa* recently spread among 200 patients in Greece (Giakkoupi et al. 2003; Docquier et al. 2003; Jeong et al. 2003). They are very worrying not only because they hydrolyze all β-lactams except the monobactams and are not susceptible to inhibitors, but they can be coded for on integrons.

The possibility of synthesizing carbacephems and oxacephems has also been explored. Two such compounds, loracarbef (a carbacephem) and latamoxef (an oxacephem), have been developed (Figure 5). Loracarbef can be given orally, but does not have the breadth of spectrum or the β-lactamase stability of the carbapenems; its activity and use are similar to those of cefaclor, to which it is related structurally. Latamoxef (also known as moxalactam) is a parenteral compound that exhibits properties similar to those of the expanded spectrum cephalosporins such as cefotaxime, but including *B. fragilis* within the spectrum. It has a methyltetrazole group at C-3 and the associated bleeding problems (see above) have restricted its popularity.

MONOBACTAMS

The idea of developing therapeutically useful compounds in which the β-lactam ring was not fused with another cyclic structure was once thought to be unlikely, if not impossible. Therefore, it came as a surprise when naturally occurring compounds with this feature were described. Only one such compound is in therapeutic use, the semi-synthetic monobactam, aztreonam (Figure 5). In marked contrast to the ultra-broad spectrum of the carbapenems, aztreonam is a narrow spectrum agent, the useful activity of which is restricted to enterobacteria and *P. aeruginosa*. It is administered parenterally. Aztreonam specifically inhibits PBP-3 in *E. coli* and causes filamentation of susceptible gram-negative bacilli, which are killed slowly.

Glycopeptide antibiotics

STRUCTURE AND SPECTRUM OF ACTIVITY

Two glycopeptides, vancomycin and teicoplanin, are in clinical use. A related compound, avoparcin, is used as a growth promoter in animal husbandry. They are complex heterocyclic compounds composed of a heptapeptide substituted with certain sugars, one of which, in teicoplanin, carries a fatty acid chain (Figure 6). The molecule is too large to penetrate the outer membrane of gram-negative bacilli, although some gram-negative anaerobic bacilli are anomalously sensitive, particularly

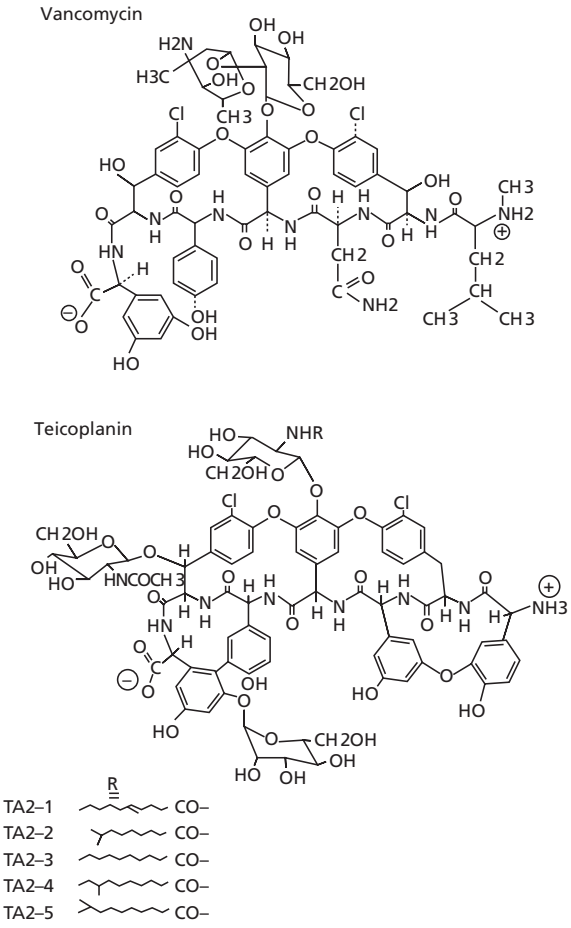

Figure 6 *Structures of the glycopeptide antibiotics, vancomycin and teicoplanin.*

to teicoplanin (Greenwood et al. 1988). Nearly all gram-positive organisms are susceptible, although some genera, including *Lactobacillus*, *Pediococcus*, and *Leuconostoc*, are intrinsically resistant (Ruoff et al. 1988). They are mainly used in serious infection with staphylococci and enterococci that are resistant to other drugs although bactericidal activity is slow. Oral vancomycin has been used in antibiotic-associated colitis in which *Clostridium difficile* is implicated and is still used for refractory cases. New derivatives such as daptomycin are being developed with improved bactericidal activity and daptomycin has already been licensed in some countries.

MODE OF ACTION

Glycopeptides prevent the transfer of peptidoglycan building blocks to the growing cell wall by binding to the acyl-D-alanyl-D-alanine terminus of the pentapeptide side chain (Reynolds 1989).

RESISTANCE

There are many reports of resistance in enterococci (Johnson et al. 1990), and coagulase-negative staphylococci (Sanyal et al. 1993). In enterococci, resistance is

associated with enzymic alteration of the D-alanyl-D-alanine target to D-alanyl-D-lactate (Walsh 1993), but other mechanisms are found in coagulase-negative staphylococci. The most common type of resistance in enterococci (*vanA*) is inducible and transferable (Shlaes et al. 1989; Healy and Zervos 1995); high level resistance to both vancomycin and teicoplanin is conferred. Transfer to *S. aureus* has been reported (Noble et al. 1992) and at the time of writing three clinical isolates of this glycopeptide-resistant *S. aureus* have been described (Chang et al. 2003; Public Health Dispatch 2002; Hiramatsu 2004; Kacia, 2004) Heteroresistant strains are also increasingly described with thickened cell walls probably acting as a sump for both vancomycin and teicoplanin (Manuel et al. 2002; MacKenzie et al. 2003). Some vancomycin-resistant isolates of enterococci retain susceptibility to teicoplanin, but *Staphylococcus haemolyticus* isolates are often more resistant to teicoplanin than to vancomycin.

PHARMACOLOGICAL PROPERTIES AND TOXICITY

Vancomycin has to be administered by slow intravenous infusion, since bolus injection is apt to cause 'red-man syndrome' owing to the release of histamine. Teicoplanin is less likely to cause this complication (Sahai et al. 1990). In addition, teicoplanin does not cause tissue necrosis that is associated with intramuscular injection of vancomycin and it can be safely given by this route. The elimination half-life of teicoplanin (approximately 40 h) is much longer than that of vancomycin (approximately 7 h), and it is much more extensively protein bound (90 versus 50 percent). Renal and ototoxicity may be more common with vancomycin, but serum monitoring of both is recommended to ensure efficacious trough levels.

OTHER CELL WALL ACTIVE AGENTS

Bacitracin

This is a cyclic peptide that prevents the dephosphorylation of the lipid carrier molecule that transfers newly formed peptidoglycan across the cell membrane during cell wall synthesis. It is too toxic for systemic use, but is found in several topical preparations. It is mainly active against gram-positive cocci. The exquisite sensitivity of *S. pyogenes* is exploited in a laboratory screening test for that organism.

Cycloserine

This is an analog of D-alanine, which prevents the racemization of L-alanine and the ligation of D-alanyl-D-alanine. It is quite toxic and is now used only as a reserve agent for drug-resistant *M. tuberculosis*, although it used to be prescribed for prophylaxis of recurrent urinary tract infections.

Fosfomycin

Fosfomycin (epoxypropylphosphonic acid) is a broad spectrum antibiotic that blocks the formation of *N*-acetylmuramic acid from *N*-acetylglucosamine by inhibition of pyruvyltransferase. In vitro activity against *E. coli* and some other gram-negative rods is potentiated by glucose-6-phosphate, which induces a hexose phosphate transport pathway. The sodium salt is used for parenteral administration; for oral use, the earlier calcium salt has been superseded by the much more soluble trometamol formulation, which is particularly suitable for the treatment of urinary tract infection (Reeves 1994). Resistance to fosfomycin emerges readily in vitro, but the drug has been extensively used in some countries without apparent problem. Plasmid-mediated resistance has been reported (Suárez and Mendoza 1991), but is as yet uncommon. A somewhat similar phosphonic acid derivative, fosmidomycin, is available in Japan.

Ethambutol, isoniazid, and pyrazinamide

These are agents used specifically in mycobacterial disease; they have no useful activity against other bacteria. Although the mode of action has not been definitely established, it is likely that they act on the mycobacterial cell wall, which is unusual in its composition. Isoniazid is most active against *M. tuberculosis* against which it exerts bactericidal activity. It is well absorbed when given orally and is eliminated in the urine, largely in an acetylated form. In persons with a genetically determined ability to acetylate the drug rapidly, excretion is hastened and plasma concentrations correspondingly low. Unfortunately, resistance is an increasing problem (Pablos-Mendez et al. 1998; Warburton et al. 1993). Pyrazinamide is also mycobactericidal, but only at an acid pH and after intracellular conversion by a bacterial amidase to pyrazinoic acid (Mitchison 1992). Ethambutol, by contrast, is predominantly mycobacteristatic, but it has a wider spectrum of activity within the mycobacteria, including activity against organisms of the *Mycobacterium avium* complex.

INHIBITORS OF BACTERIAL PROTEIN SYNTHESIS

Many of the naturally occurring antibiotic families that were discovered by mass screening of soil samples in the 1940s and 1950s turned out to achieve their antibacterial effect by interfering with various stages of the process of protein synthesis. Some, but not all, owe their selective

toxicity to the difference in structure between bacterial and mammalian ribosomes.

Aminoglycosides

STRUCTURE AND SPECTRUM OF ACTIVITY

Aminoglycosides are complex heterocyclic compounds that usually possess an aminocyclitol group in addition to one or more amino sugars. Spectinomycin is unusual in being a pure aminocyclitol and has properties, including predominantly bacteristatic activity, that separate it from the aminoglycosides proper. It is used only as a reserve agent in gonorrhea. The remaining aminoglycosides can be divided into two main types depending on the structure of the aminocyclitol ring: those (of which streptomycin and dihydrostreptomycin are the only surviving examples) in which the aminocyclitol is streptidine; and those in which the aminocyclitol moiety is deoxystreptamine. The deoxystreptamine-containing aminoglycosides can, in their turn, be divided into neomycin derivatives, kanamycin derivatives, and gentamicin derivatives (Figure 7).

The neomycins (including framycetin; neomycin B), are mainly used in topical preparations because of their toxicity, but one member of the group, paromomycin, is more conspicuous for its activity against the protozoa *Entamoeba histolytica* and *Leishmania* spp. Most of the remaining aminoglycosides in common therapeutic use, including gentamicin, tobramycin (deoxykanamycin B), netilmicin and the semisynthetic antibiotic, amikacin, share a common spectrum of activity that encompasses staphylococci and enterobacteria, but excludes streptococci, enterococci, anaerobes, and intracellular bacteria. Certain aminoglycosides exhibit activity against *M. tuberculosis* (streptomycin, kanamycin, and amikacin) or against *P. aeruginosa* (gentamicin, tobramycin, netilmicin, amikacin). Members of this last group have been widely used in the management of serious infection, often in combination with a β-lactam agent with which they may interact synergically. Bactericidal synergy with penicillin is also exploited in the therapy of streptococcal and enterococcal endocarditis.

MODE OF ACTION

Aminoglycosides bind to bacterial ribosomes. A single amino acid change in a protein of the 30S ribosomal subunit renders the cells completely resistant to streptomycin, but not to deoxystreptamine-containing aminoglycosides, which bind to both subunits. The mechanism of action is uncertain. Binding induces misreading of messenger RNA so that defective proteins are produced, but it is likely that aminoglycosides also interfere with the formation of functional initiation complexes. Neither of these mechanisms satisfactorily explains the potent bactericidal activity of these compounds and this may follow from membrane-related effects (Davis 1988).

RESISTANCE

Aminoglycosides are taken up by bacterial cells by an active transport process that involves respiratory processes. The absence of such a transport mechanism in anaerobes, streptococci, and enterococci accounts for the relative resistance of these organisms.

Alterations in permeability characteristics of sensitive bacteria may lead to relative resistance to aminoglycosides, and such bacteria are usually resistant to all members of the group. However, acquired resistance in otherwise sensitive species is most commonly due to the production of enzymes that adenylate or phosphorylate hydroxyl groups on the aminoglycoside molecule, or acetylate exposed amino groups. Since these antibiotics vary in the availability of vulnerable groupings on the molecule, they exhibit variable susceptibility to the many aminoglycoside-modifying enzymes that have been described. Some examples are shown in Table 5.

PHARMACOLOGICAL PROPERTIES

Aminoglycosides are very poorly absorbed by the oral route and are administered by intramuscular injection or intravenous infusion. They do not penetrate into cells or cross the blood–brain barrier to enter the cerebrospinal fluid. They are excreted almost entirely in the urine via the glomerular filtrate with an elimination half-life of about 2–4 h. Protein binding is generally low (<25 percent).

TOXICITY AND SIDE EFFECTS

All members of this antibiotic family are nephrotoxic and ototoxic, but they vary in their propensity to cause these adverse effects. For example, gentamicin and tobramycin are less likely to cause vestibular toxicity than is streptomycin, but are somewhat more likely to cause renal damage. Because of the risk of toxicity, it is common practice to assay aminoglycoside levels after intramuscular injection or intravenous infusion ('peak' level) and immediately before the next dose ('trough' level). If single large daily doses of aminoglycosides are used, as is increasingly advocated (Levison 1992), peak levels become difficult to interpret, but trough levels still need to be monitored (Nicolau et al. 1995).

Chloramphenicol

STRUCTURE AND SPECTRUM OF ACTIVITY

Chloramphenicol is a relatively simple antibiotic (Figure 8) that is nowadays synthesized rather than being obtained by fermentation from the producer organism. Modification of the molecule has not been very productive, although thiamphenicol, in which a sulphomethyl substituent replaces the nitro group, is available in some countries. Fluorinated derivatives,

AMINOGLYCOSIDES

Streptomycin

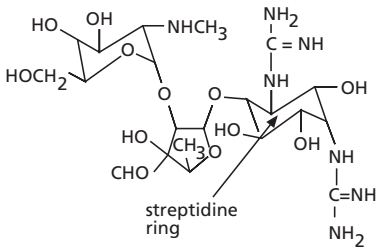

Neomycin

	Neomycin B	Neomycin C
R1	H	CH_2NH_2
R2	CH_2NH_2	H

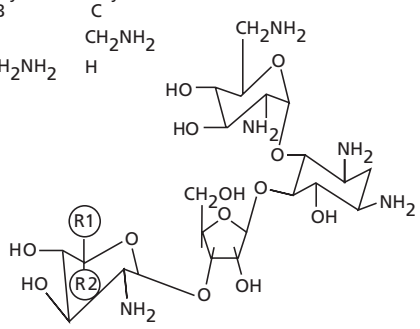

Kanamycin derivatives

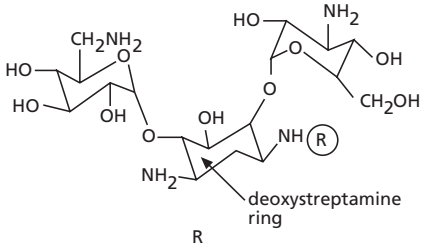

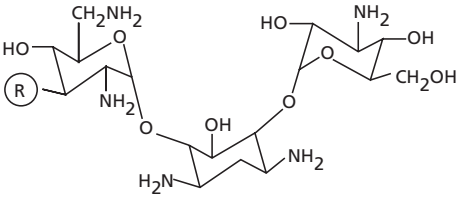

	R
Kanamycin A	H
Kanamycin B	OH
Tobramycin	H

Amikacin

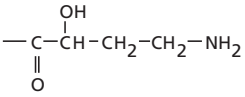

Gentamicin derivatives

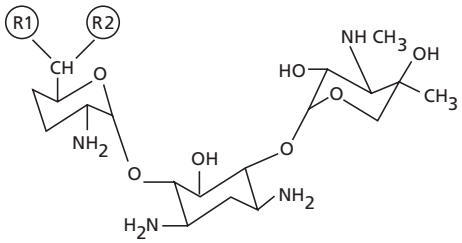

		R1	R2
Gentamicin	C1	CH_3	$NHCH_3$
	C1a	H	NH_2
	C2	CH_3	NH_2

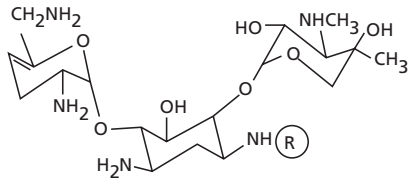

	R
Netilmicin	C_2H_5
Sissomicin	H

Spectinomicin

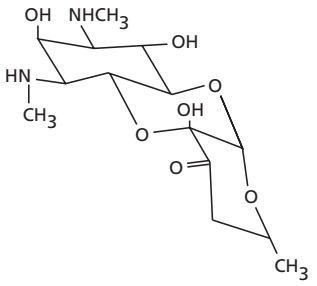

Figure 7 *Structures of the most important aminoglycosides.*

Table 5 *Some common aminoglycoside modifying enzymes*

Enzyme	Preferred substrates	Typical bacterial distribution	
		Gram-positive	Gram-negative
Acetyltransferases			
AAC(3)-I	Gen, Sis	–	+
AAC(3)-II	Gen, Kan, Net, Sis, Tob	–	+
AAC(2′)	Gen, Neo, Net, Sis, Tob	–	+
AAC(6′)-I	Amk, Net, Tob	+	+
AAC(6′)-II	Gen, Kan, Net, Sis, Tob	–	+
Nucleotidyltransferases (adenylyltransferases)			
AAD(6)	Str	+	–
AAD(4′)(4′′)	Amk, Kan, Neo, Tob	+	–
AAD(2′′)	Gen, Kan, Sis, Tob	–	+
AAD(3′′)(9)	Spc, Str	–	+
AAD(9)	Spc	+	–
Phosphotransferases			
APH(6)	Str	–	+
APH(3′)	Kan, Neo	+	+
APH(2′′)	Gen, Net, Sis, Tob	+	–
APH(3′′)	Str	+	+

Amk, amikacin; Gen, gentamicin; Kan, kanamycin; Neo, neomycin; Net, netilmicin; Sis, sissomicin; Spc, spectinomycin; Str, streptomycin.
The figures in parentheses indicate the sites of modification of exposed amino or hydroxyl groups according to the international numbering system for the heterocyclic ring structure of the aminoglycosides. Bifunctional enzymes that act at more than one site have two such numbers. Roman numerals indicate different forms of the enzyme acting at that site.

such as florphenicol, have also been made, but are not used in human medicine.

The spectrum is very broad and includes intracellular pathogens, such as chlamydiae and rickettsiae, as well as most conventional gram-positive and gram-negative bacteria. *P. aeruginosa* and *M. tuberculosis* are usually resistant.

MODE OF ACTION

Chloramphenicol inhibits the enzyme peptidyl-transferase which links new amino acids from aminoacyl transfer RNA to the growing peptide chain. It does not bind to mammalian ribosomes except, perhaps, those within mitochondria.

Chloramphenicol

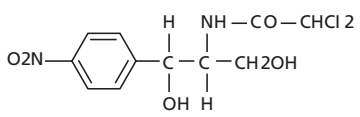

Thiamphenicol

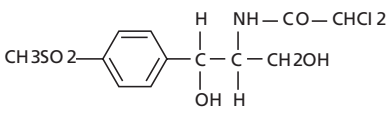

Figure 8 *Structures of chloramphenicol and thiamphenicol.*

RESISTANCE

Target site alterations and reduction in drug uptake have been described, but the most common form of resistance is due to the production of chloramphenicol acetyltransferases. These enzymes acetylate the C-3 hydroxyl group preferentially, but also attack the C-1 hydroxyl; interest in the fluorinated derivatives centers on their resistance to these enzymes. Inactivation of chloramphenicol by a nitroreductase has also been described in *B. fragilis* (Tally and Malamy 1984).

PHARMACOLOGICAL PROPERTIES

Because chloramphenicol is poorly soluble and tastes very bitter, prodrugs are used in therapy: water-soluble chloramphenicol succinate is used for injection and the more palatable chloramphenicol palmitate or stearate for oral administration. These salts release chloramphenicol in the body, but are themselves inactive and unsuitable for laboratory tests. The oral route of administration is very efficient and is generally preferred. The drug is well distributed, with excellent penetration into the cerebrospinal fluid. It is excreted into urine, largely as inactive glucuronide conjugates, with a half-life of about 2–5 h in adults. Plasma protein binding is approximately 50 percent.

TOXICITY AND SIDE EFFECTS

The popularity of this excellent antibiotic suffered a severe setback when it was realized that it occasionally caused an irreversible aplastic anemia. The overall

incidence of this lethal side effect is about 1 in 40 000 courses of therapy, but there may be a genetic component that makes it more likely in some patients. There is concern that sufficient chloramphenicol may be absorbed systemically after topical use, e.g. in eye ointments, for aplastic anemia to be a hazard (Doona and Walsh 1995), but the degree of risk is disputed (Mulla et al. 1995). Thiamphenicol appears to be free of this side effect, but is more likely to cause reversible depression of the bone marrow.

Young infants have a limited capacity to conjugate and excrete chloramphenicol; accumulation of the drug may lead to 'gray baby' syndrome, with circulatory collapse. For this reason chloramphenicol levels should be assayed if the drug is used in neonatal meningitis or other life-threatening conditions in young infants.

Tetracyclines

STRUCTURE AND SPECTRUM OF ACTIVITY

The tetracyclines are a closely related group of naturally occurring and semisynthetic antibiotics that differ according to the nature of chemical substituents on the basic tetracyclic skeleton (Figure 9). They are distinguished more for differences in pharmacokinetic behavior than for variations in antimicrobial activity. The spectrum of activity is broad. They have been widely and successfully used in many types of infection including those in which chlamydiae, rickettsiae, and mycoplasmas are involved. They have some antiprotozoal activity and are sometimes used in drug-resistant malaria.

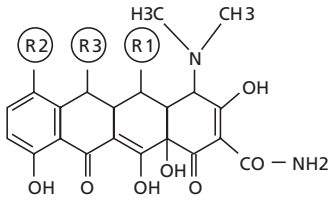

	R1	R2	R3
Tetracycline	—H	— H	CH3, OH
Oxytetracycline	—OH	— H	CH3, OH
Chlortetracycline	—H	— Cl	CH3, OH
Minocycline	—H	— N(CH3)(CH3)	H, H
Doxycycline	—OH	— H	CH3, H

Figure 9 *Structures of the most important tetracyclines.*

MODE OF ACTION

Tetracyclines enter bacterial cells by an active uptake process. They bind to the 30S ribosomal subunit and prevent access of aminoacyl transfer RNA to the acceptor site by a mechanism that has not been fully elucidated (Chopra et al. 1992). Mammalian cells do not concentrate tetracyclines in the way that bacteria do and the ribosomes (other than mitochondrial ribosomes) are relatively insusceptible.

RESISTANCE

The emergence of resistance in gram-positive and gram-negative bacteria has seriously undermined the value of tetracyclines although most epidemic strains of MRSA in the UK retain susceptibility. Resistance is generally plasmid mediated and is commonly found on transposons. Several mechanisms of resistance have been described, but the most prevalent appears to be due to production of a novel cytoplasmic membrane protein that mediates active efflux of the drug so that inhibitory levels are not maintained within the cell (Chopra et al. 1992; Speer et al. 1992).

Resistance usually affects all tetracyclines equally, although minocycline may retain activity against some strains. Efforts have been made to devise tetracyclines that overcome resistance mechanisms. Several candidate molecules have been described, including the glycylcyclines which are under investigation (Tally et al. 1995). One of these, tigecycline is in an advanced stage of development (Milatovic et al. 2003). It is a derivative of minocycline and offers useful activity for problem multiresistant gram-positive cocci such as MRSA, penicillin resistant pneumonia (PRP), and vancomycin-resistant enterococci (VRE).

PHARMACOLOGICAL PROPERTIES

The tetracyclines are usually given orally, but since they form nonabsorbable chelates with divalent cations, administration with milk or other food may interfere with absorption. Doxycycline and minocycline are among the best absorbed; these compounds also have a longer plasma half-life (approximately 16–18 h) than other congeners (generally approximately 6–12 h), but are more extensively (minocycline 75 percent; doxycycline 90 percent) protein bound. They penetrate well into tissues, but not into cerebrospinal fluid. They are excreted by glomerular filtration into the urine and into feces via the bile.

TOXICITY AND SIDE EFFECTS

Diarrhea and other forms of gastrointestinal intolerance are common. Renal failure may occur in patients who already have impaired renal function, but doxycycline, which is predominantly excreted by the hepatobiliary route, may be safely given to such patients. Since tetracyclines are yellow compounds that chelate calcium, the

pigment is deposited in growing bone and teeth. For this reason, tetracyclines should not be given to young children when the dentition is being formed as permanent discoloration of teeth may occur.

Macrolides

STRUCTURE AND SPECTRUM OF ACTIVITY

The macrolides are a family of related compounds that feature a large macrocyclic lactone structure substituted with various unusual sugars (Figure 10). Erythromycin (the oldest and best known member of the group), oleandomycin, clarithromycin (6-*O*-methyl erythromycin), dirithromycin, and roxithromycin have a 14-membered lactone ring. Some others that are used in various parts of the world, including spiramycin, josamycin, midecamycin, kitasamycin, and rokitamycin, possess a 16-membered lactone structure. In azithromycin, the 14-membered structure of erythromycin has been expanded by insertion of a methyl-substituted nitrogen atom; the 15-membered ring thus produced is sometimes referred to as an azalide structure.

Erythromycin and other macrolides are unable to penetrate easily through the outer membrane of enteric gram-negative bacilli, although they exhibit a variable degree of activity against *H. influenzae*, *Legionella pneumophila*, and *Campylobacter jejuni*. They exhibit good activity against staphylococci and streptococci and useful activity against *Mycoplasma pneumoniae* (but not *M. hominis*) and some environmental mycobacteria. Chlamydiae are susceptible and azithromycin is effective in

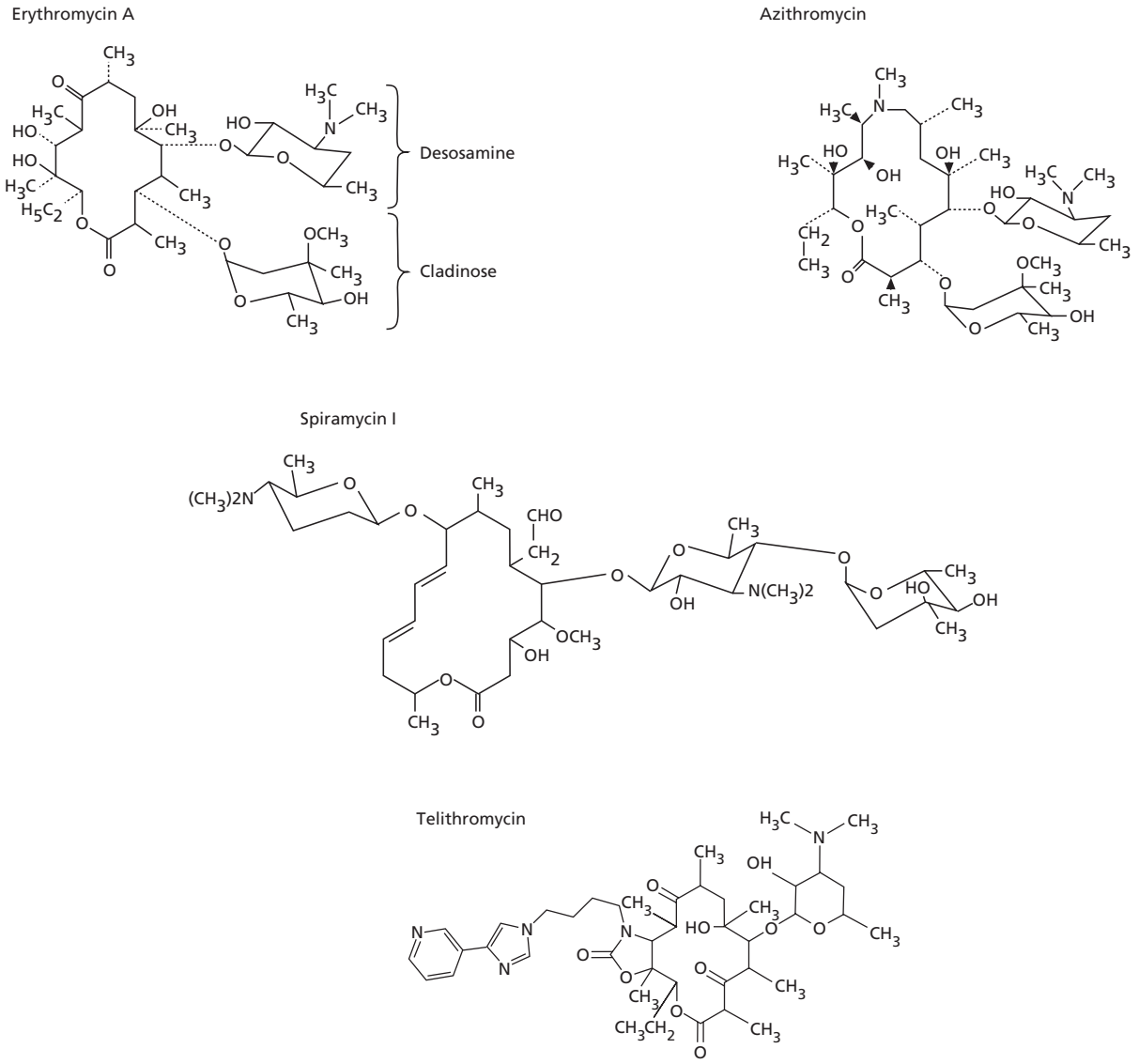

Figure 10 *Structures of erythromycin A (a l4-membered macrolide); azithromycin (a 15-membered macrolide and an azalide); spiramycin I (a 16-membered macrolide); and telithromycin.*

the single-dose treatment of genital infections (Martin et al. 1992; Stamm et al. 1995) and trachoma (Bailey et al. 1993). Spiramycin has been successfully used in toxoplasmosis and clarithromycin has a place in regimens for the eradication of *Helicobacter pylori*. The ketolide derivatives have undergone development recently and one of them, telithromycin is being used clinically. Its spectrum of activity and structure is very similar to clarithromycin, although it is bactericidal rather than static, like traditional macrolides (Fogarty et al. 2003).

MODE OF ACTION

Macrolides bind to 50S ribosomal subunits in bacteria and interfere with the translocation process during synthesis of polypeptides, probably by causing dissociation of peptidyl transfer RNA from the ribosome (Mazzei et al. 1993).

RESISTANCE

Resistance to macrolides is quite commonly encountered in staphylococci and streptococci but the prevalence varies considerably from country to country. Resistance may arise from alterations in ribosomal proteins. It is, though, more commonly due to an inducible, plasmid-mediated enzyme that methylates an adenine residue in ribosomal RNA (Weisblum 1995a) or alternatively, due to active efflux (Alós et al. 2003; Low 2002). The former is due to the *erm (B)* gene which exhibits the MLS_B phenotype, the latter to the *mef (A)* gene which exhibits the M phenotype. This latter mechanism tends to give lower levels of resistance although probably still clinically significant (Lonks et al. 2002). Both efflux and the methylase gene are widespread, but geographical distribution varies. Only 14- and 15-membered ring macrolides are susceptible to efflux but methylase also confers resistance to lincosamides and streptogramins when constituitively expressed although these antibiotics do not act as inducers, so the organisms appear sensitive in laboratory tests, unless erythromycin is also present to induce the enzyme. Variants readily emerge on exposure to lincosamides or streptogramins exhibiting constitutive resistance (Weisblum 1995b). It has been suggested that the newer long-acting macrolides are responsible for recent increases in resistance because of their lack of cidal activity and their prolonged but low tissue levels (Baquero et al. 2002). The ketolides, so far, retain activity against the great majority of erythromycin-resistant streptococci, although not MRSA (Fogarty et al. 2003).

PHARMACOLOGICAL PROPERTIES

Macrolides are mostly irregularly absorbed when given orally; in the case of erythromycin this is because it is unstable at gastric pH. Newer macrolides, such as azithromycin and clarithromycin, are more acid stable and their oral absorption is consequently much improved. Oral formulations of erythromycin are either coated to avoid destruction in the stomach, or are presented as prodrug salts or esters (estolate, stearate, or ethylsuccinate). The lactobionate or gluceptate salts are used for parenteral administration.

All these drugs are well distributed in the body, but azithromycin and clarithromycin are said to achieve particularly good intracellular levels, especially in the lung (Honeybourne and Baldwin 1992). Penetration into cerebrospinal fluid is poor. They are extensively metabolized (except azithromycin) and excreted largely by the hepatobiliary route. The plasma half-life is variable and may be dose-dependent. Azithromycin is unusual in that it has a much extended terminal half-life. The poor serum levels, particularly of azithromycin, make it less desirable for the treatment of bacteremic pneumococcal pnemonia (Lonks et al. 2002).

TOXICITY AND SIDE EFFECTS

Nausea and other gastrointestinal side effects are common, but newer derivatives, including azithromycin and clarithromycin, are better tolerated. Cholestatic jaundice is described as an uncommon complication, particularly with erythromycin formulations. The immunomodulatory and anti-inflammatory properties of macrolides are being investigated in cystic fibrosis and there is good evidence of their benefit in diffuse panbronchiolitis (Krishnan et al. 2002).

Lincosamides

STRUCTURE AND SPECTRUM OF ACTIVITY

The original lincosamide antibiotic, lincomycin, has been largely superseded by the 7-deoxy-7-chloro derivative, clindamycin (Figure 11), which is more active and better absorbed by the oral route. The most potent activity is against staphylococci, streptococci, and anaerobes, including *B. fragilis*. Enterobacteria and *P. aeruginosa* are resistant. Activity against *Propionibacterium acnes* and corynebacteria has led to the use of clindamycin in acne, but its success in this condition may be equally due to the anti-inflammatory and antiphagocytic activity that it is known to possess (Oleske and Phillips 1983) or to effects on skin lipids. Clindamycin possesses some antiprotozoal activity and has been used in malaria and toxoplasmosis.

MODE OF ACTION

The mechanism of action of lincosamides is not known for certain. Early work suggested that these drugs, like chloramphenicol, interfere with the peptidyltransferase reaction (Weisblum and Davies 1968), but later evidence pointed to effects on peptide chain initiation (Pestka 1971; Reusser 1975). Although the structure of lincosamides is completely different from that of macrolides,

Lincomycin

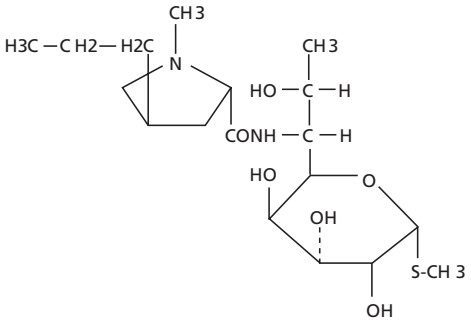

Clindamycin

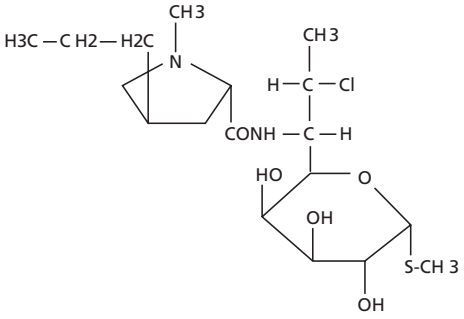

Figure 11 *Structures of the lincosamides, lincomycin and clindamycin.*

the site of action appears to be similar since methylation of an adenine residue in ribosomal RNA confers resistance to both types of antibiotic.

RESISTANCE

As well as resistance caused by ribosomal methylase, described above, resistance may also arise by enzymic modification by nucleotidylation (Russell and Chopra 1996). Resistance emerges readily and is common among methicillin-resistant *S. aureus* (Maple et al. 1989).

PHARMACOLOGICAL PROPERTIES

Like chloramphenicol, clindamycin is poorly soluble and very bitter, so that it has to be administered as inactive prodrugs, clindamycin palmitate (for oral use) and clindamycin phosphate (for injection). It is well absorbed when given orally and after hydrolysis, achieves good concentrations in tissues, but not in the cerebrospinal fluid. It is metabolized in the liver and excreted mainly in bile with a half-life of about 2–3 h. Plasma protein binding is 94 percent.

TOXICITY AND SIDE EFFECTS

Rashes and other occasional adverse events have been overshadowed by the reputation of clindamycin for inducing diarrhea associated with *C. difficile* toxins, which may progress to a life-threatening pseudomembraneous colitis. Other antibiotics, including β-lactam agents, have been implicated in this side effect, but it seems to be more common with clindamycin (Tedesco et al. 1974; Aronsson et al. 1984).

Fusidic acid

STRUCTURE AND SPECTRUM OF ACTIVITY

Fusidic acid is an antibiotic with a steroid-like structure (Figure 12) that does not possess steroid-like activity.
It exhibits modest activity against streptococci, gram-positive and gram-negative anaerobes, *Nocardia asteroides*, and *M. tuberculosis* and good activity against *Corynebacterium diphtheriae*, but its place in therapy hinges on its excellent activity against staphylococci (Greenwood 1988; Verbist 1990). Various interactions with penicillins (synergy, antagonism, and indifference) have been described (O'Grady and Greenwood 1973). Activity against the protozoa, *Giardia lamblia* (Farthing and Inge 1986) and *Plasmodium falciparum* (Black et al. 1985), have been reported. Enterobacteria and pseudomonads are resistant.

MODE OF ACTION

Unlike other inhibitors of bacterial protein synthesis, fusidic acid does not bind directly to the ribosome. It forms a stable complex with guanosine triphosphate and 'factor G', an elongation factor involved in the translocation of the growing peptide chain (Cundliffe 1972).

RESISTANCE

Resistance may be plasmid mediated, but is more commonly due to chromosomal mutation resulting in alteration in the factor G target. This type of resistance emerges readily in vitro, but the general prevalence of resistance in staphylococci has remained low (Shanson 1990). This may have been helped by the fact that

Fusidic acid

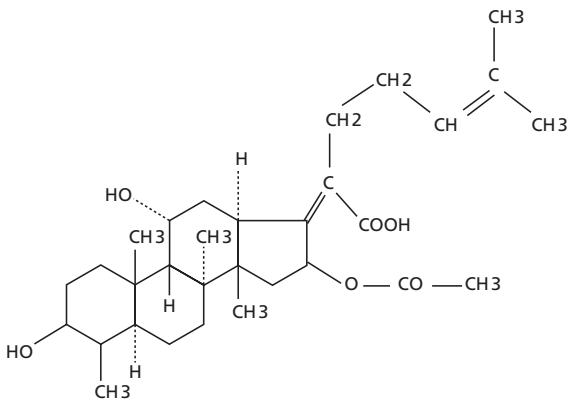

Figure 12 *Structure of fusidic acid.*

fusidic acid is usually given in combination (often with a penicillin) to avoid the emergence of resistance during therapy, but topical preparations are normally given alone and there have been recent concerns that increased resistance has been due to such topical use (Mason et al. 2003).

PHARMACOLOGICAL PROPERTIES

Fusidic acid is very well absorbed by the oral route and penetrates well into tissue, including bone. It is excreted via the bile, mostly as inactive metabolites, with a half-life of about 9 h (Reeves 1987; MacGowan et al. 1989). More than 95 percent is bound to plasma protein.

TOXICITY AND SIDE EFFECTS

A small proportion of patients develop transient jaundice by interference with the metabolism and excretion of bilirubin. It is more common in patients receiving intravenous fusidic acid (Humble et al. 1980). Mild gastrointestinal upsets and rashes are occasionally experienced.

Other inhibitors of bacterial protein synthesis

STREPTOGRAMINS

The streptogramins are a family of antibiotics that consist of two synergically interacting macrolactone components: a polyunsaturated peptolide and a hexadepsipeptide. The bactericidal synergy that results from the combined action of the two components of streptogramins is thought to arise through binding to adjacent sites on the bacterial ribosome (Aumercier et al. 1992). The best known members of the group are pristinamycin and virginiamycin, which are used as antistaphylococcal agents in animal husbandry and, in some countries, in human medicine. Lack of aqueous solubility and poor oral absorption have limited their value, but water-soluble derivatives of the two components of pristinamycin (known, respectively, as quinupristin and dalfopristin) have been synthesized to allow parenteral administration and are now available as a combination for parenteral administration in many countries (Barrière et al. 1992; Etienne et al. 1992) (Figure 13).

Their broad gram-positive spectrum and bactericidal activity ensures their increasing use in difficult to treat cases due to MRSA, PRP, and VRE, but it is only static against *E. faecium* and *E. faecalis* is innately resistant (Eliopoulos 2003). Synergy with glycopeptides and other drugs has been demonstrated (Moyenuddin et al. 2003). Macrolide–lincosamide resistance associated with an adenine methylase also extends to streptogramins (see section on macrolide resistance).

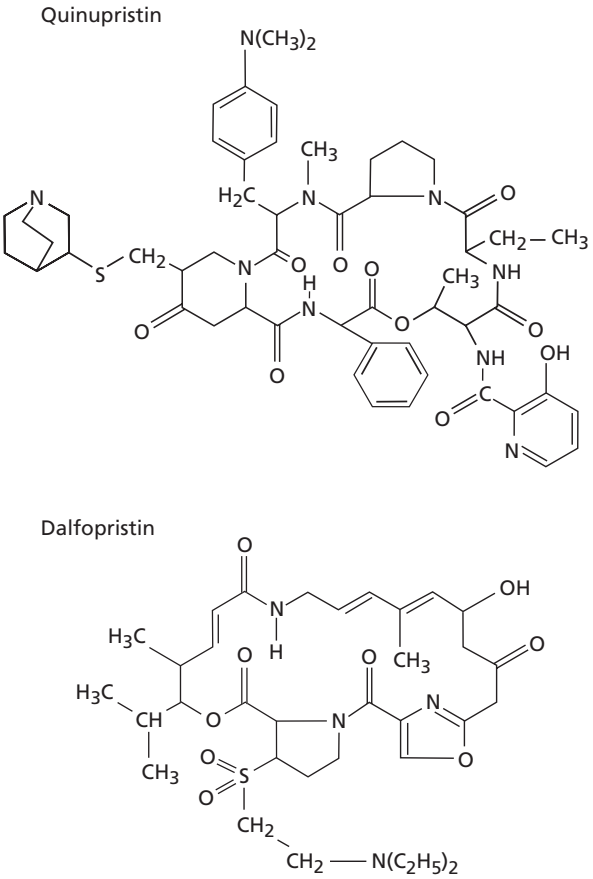

Figure 13 *Structure of quinupristin and dalfopristin.*

OXAZOLIDINONES

This group of drugs are being developed in response to clinical need for new agents active against MRSA and other multiresistant gram-positive organisms. Currently only one, linezolid, has been released for general clinical use (Clemett and Markham 2000). These drugs are the first genuinely new class of antibiotics to be successfully developed for many years (Figure 14). They act at the messenger RNA level where they are thought to interfere with protein synthesis.

As a result of this there is an interesting association with macrolide resistance. To date, when linezolid resistance develops in MRSA it is associated with a reversion to macrolide susceptibility. Development of resistance is, however, very rare to date and associated with long courses of therapy (Wilson et al. 2003; Boo et al. 2003).

Although linezolid is not bactericidal against MRSA, several preliminary reports of randomized studies show it to be more efficacious than glycopeptide monotherapy for serious MRSA infections such as bacteraemia and ventilatory association pneumonia (Cepeda et al. 2004). It seems well tolerated in general, although long-term use has been associated with marrow suppression and neuropathology. There are also some dietary restrictions to be adhered to when it is used because of its inhibitor

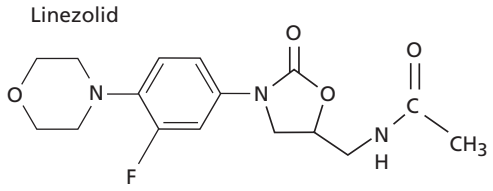

Figure 14 *Structure of linezolid.*

of monoamine oxidase. Currently linezolid should be reserved for serious MRSA infections where conventional therapy has failed. Caution should be exerted in its long-term use, although its excellent bioavailibility makes it a good candidate for oral switch. In vitro studies suggest it is often additive or synergistic in combination with other anti-MRSA drugs and consideration should be given to combinations, e.g. with rifampin in high inoculum or recalcitrant infections (Grohs et al. 2003; Cédrick et al. 2003).

MUPIROCIN

Mupirocin is a naturally occurring antibiotic product of *Pseudomonas fluorescens*. It was formerly known as pseudomonic acid. The structure (Figure 15) is unrelated to other antibiotics and consists of 'monic acid', the distal portion of which is a structural analog of isoleucine and a short fatty acid (nonanoic acid). The useful activity is restricted to gram-positive cocci, in which it presumably halts protein synthesis by binding to isoleucyl transfer RNA synthetase as it does in *E. coli* (Hughes and Mellows 1980).

Although it displays low toxicity, systemic use of mupirocin is precluded by the fact that it is rapidly inactivated in the body. Consequently, it is used only for topical application in skin infections and to eradicate staphylococci from nasal carriers. For the latter purpose, which has been of particular value in carriers of methicillin-resistant *S. aureus*, a paraffin-based formulation is used, rather than the polyethylene glycol-based ointment applied to skin.

INHIBITORS OF BACTERIAL NUCLEIC ACID SYNTHESIS

A surprising number of antibacterial agents achieve their effect by interacting with bacterial DNA in various ways. Sulfonamides and trimethoprim have an indirect effect on other cellular functions, as well as DNA, through their effect on folic acid synthesis, but for convenience are dealt with under this heading.

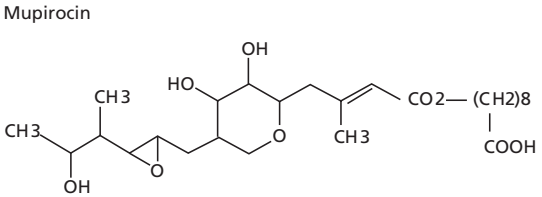

Figure 15 *Structure of mupirocin.*

Sulfonamides

STRUCTURE AND SPECTRUM OF ACTIVITY

The sulfonamides are a large family of compounds, all of which are derived from the original hydrolysis product of Prontosil red, sulfanilamide. They differ in the nature of the substitution on the amino group of the sulfonamide (SO_2NH_2) moiety (Figure 16). The antileprosy drug, dapsone (diaminodiphenylsulphone) and the tuberculostatic *p*-aminosalicylic acid are related substances that are thought to act in a similar way.

They are broad spectrum, predominantly bacteristatic compounds that have a relatively slow effect in halting bacterial growth. Activity in vitro is profoundly affected by the composition of the culture medium, because of the possible presence of interfering substances such as

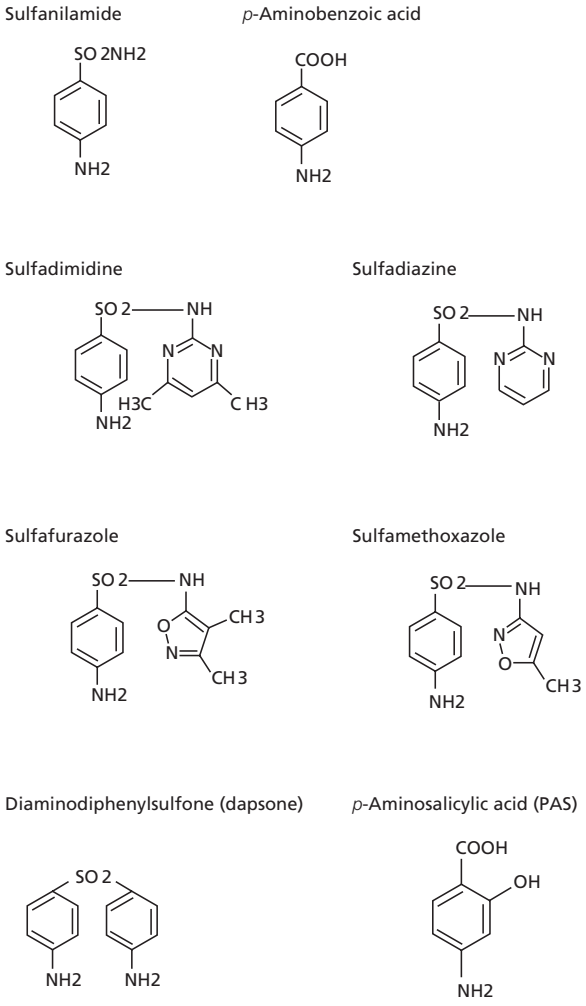

Figure 16 *Structures of some sulfonamides and related compounds.*

folic acid, *p*-aminobenzoic acid, and thymidine. Lysed horse blood, which contains the enzyme thymidine phosphorylase, is commonly added to remove thymidine (Waterworth 1978) and media recommended for sensitivity testing have low levels of this and other sulfonamide antagonists (Report 1991).

MODE OF ACTION

Sulfonamides are analogs of *p*-aminobenzoic acid. They inhibit folic acid synthesis by competitive inhibition of dihydropteroic acid synthetase, the enzyme that brings about the condensation of dihydropteridine with *p*-aminobenzoic acid in the early stage of folate production. Since folic acid is conserved in bacterial cells, the inhibitory effects of sulfonamides become apparent only after several generations of growth when the folate pool has been progressively diluted to below a functional level by distribution to the bacterial progeny.

The selective toxicity of sulfonamides arises because bacteria synthesize folic acid de novo, whereas humans absorb the vitamin preformed. Since they block an early stage of the same metabolic pathway as diaminopyrimidines, sulfonamides interact synergically with those compounds.

RESISTANCE

Resistance to sulfonamides occurs readily and was soon apparent in the early life of these agents. There is complete crossresistance among different members of this drug class. Resistance is commonly plasmid mediated and is usually caused by alterations in dihydropteroate synthetase, leading to less efficient binding of sulfonamides, or bypass of the effects of the agents by a duplicate, insensitive version of the enzyme (Huovinen et al. 1995). Chromosomal resistance due to hyperproduction of *p*-aminobenzoic acid is also recognized (Towner 1992a).

PHARMACOLOGICAL PROPERTIES

Differences in pharmacokinetic behavior are the chief distinguishing characteristics of the various members of the sulfonamide family. In particular, they vary in oral absorption (e.g. phthalylsulfathiazole is very poorly absorbed), protein binding (e.g. sulfadimidine and sulfadoxine are more than 90 percent protein bound) and, above all, plasma half-life, which can vary from about 2.5 h (sulfamethizole) to >100 h (sulfadoxine). The most commonly used sulfonamides, sulfadiazine and sulfamethoxazole (as co-trimoxazole) are well absorbed by the oral route and are excreted into urine, partly as inactive *N*-acetylated metabolites and glucuronide conjugates, with a half-life of about 8–10 h. They are well distributed and penetrate into the cerebrospinal fluid in effective concentrations.

TOXICITY AND SIDE EFFECTS

Crystalluria, with renal blockage, is a problem with some of the less soluble compounds, including sulfadiazine and sulfathiazole, especially if excessive dosage is used. Rashes are common and erythema multiforme (Stevens–Johnson syndrome) is a rare, but potentially life-threatening complication. Serious hematological effects are also seen occasionally. Fears of toxicity have led to major reductions in their use in the UK over the past decade, although this has not been the case in most other countries. Their activity (alone and in combination with trimethoprim) against most epidemic strains of MRSA may well see them being used increasingly in the future.

Diaminopyrimidines

STRUCTURE AND SPECTRUM OF ACTIVITY

This family of synthetic pyrimidine derivatives includes trimethoprim, the most familiar member of the group (Figure 17), and the closely related antibacterial agents, tetroxoprim and brodimoprim which are in use in some countries, but offer little, if any, advantage over trimethoprim. Also related are the antimalarial agents pyrimethamine and cycloguanil (the in vivo metabolite of proguanil), the antipneumocystis agent, trimetrexate and the anticancer drug, methotrexate.

Trimethoprim and its congeners are active against many gram-positive and gram-negative bacteria, but *P. aeruginosa*, *B. fragilis*, chlamydiae, rickettsiae, mycoplasmas, and mycobacteria are outside the spectrum. Although they exhibit excellent activity in their own right, they are often formulated with sulfonamides, with which they interact synergically, at least in vitro. Among such combination products are: co-trimoxazole (trimethoprim + sulfamethoxazole); co-trimazine (trimethoprim + sulfadiazine); co-trifamole (trimethoprim + sulfamoxole), and co-tetroxazine, (tetroxoprim + sulfadiazine).

Trimethoprim

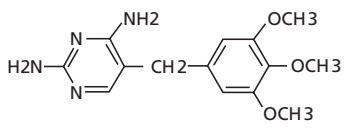

Tetroxoprim

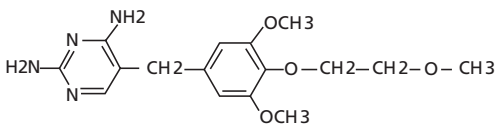

Figure 17 *Structures of trimethoprim and tetroxoprim.*

MODE OF ACTION

Diaminopyrimidines act on the same metabolic pathway as sulfonamides, but at a later stage. They inhibit dihydrofolate reductase, the enzyme that converts the precursor form of folic acid, dihydrofolate, to the active cofactor, tetrahydrofolic acid. The affinity of trimethoprim for bacterial dihydrofolate reductase is several thousand times greater than for the corresponding human enzyme. Tetrahydrofolic acid is an essential carrier molecule in many single-carbon transactions within cells and is usually regenerated unchanged. However, in one such transfer, the production of thymidylic acid from deoxyuridylic acid, diaminopyrimidines act as hydrogen donors, as well as methyl-group carriers and emerge from the reaction in the oxidized form, dihydrofolate. Trimethoprim and its relatives prevent regeneration of tetrahydrofolic acid and thus trap the vitamin in the unusable precursor form. Consequently, these drugs influence bacterial growth more quickly than sulfonamides, which rely on dilution of the folate pool to achieve their bacteristatic effect. In the presence of sufficient diaminopyrimidine to completely halt folate activity, the sulfonamides in combined formulations have no opportunity to interfere with bacterial growth, although they still contribute substantially to the overall toxicity of the mixtures.

RESISTANCE

The prevalence of resistance to trimethoprim has steadily increased since its introduction in 1969. Several mechanisms are recognized, the most common of which is attributable to mutations that lead to the production of altered dihydrofolate reductases (Towner 1992a; Huovinen et al. 1995).

PHARMACOLOGICAL PROPERTIES

Trimethoprim can be given by the oral and parenteral routes. It is widely distributed in tissues, including bronchial secretions. Concentrations achieved in cerebrospinal fluid are about 30–50 percent of the corresponding plasma level. Excretion is almost entirely renal, partly as metabolites, some of which retain antibacterial activity. The plasma half-life is about 10 h and it is less than 50 percent protein bound.

TOXICITY AND SIDE EFFECTS

Trimethoprim is well tolerated and most of the side effects of co-trimoxazole are usually attributable to the sulfonamide component. Nevertheless, trimethoprim itself may give rise to idiosyncratic reactions. The potential for exacerbating folate deficiency can normally be countered with folate supplements, but the drug is not recommended in pregnancy.

Quinolones

STRUCTURE AND SPECTRUM OF ACTIVITY

The quinolones are a large family of compounds (Table 6), the molecular similarity of which is based on the quinolone nucleus, or related naphthyridine, cinnoline or pyridopyrimidine structures (Smith and Lewin 1988) (Figure 18). The first member of the group to be used in therapy, nalidixic acid, is a naphthyridine derivative with a narrow spectrum of activity directed almost exclusively against enterobacteria. With the addition of a fluorine atom at C-6 and a piperazine substituent at C-7, the intrinsic activity and spectrum were substantially altered. Norfloxacin is about 50 times more active than nalidixic acid against sensitive enterobacteria and also exhibits some activity against gram-positive cocci and *P. aeruginosa*. Other derivatives with the 6-fluoro-7-piperazinyl substitutions (now collectively called fluoroquinolones) followed. Ciprofloxacin and ofloxacin offer further improved activity, and recent derivatives such as levofloxacin, moxifloxacin, gatifloxacin, and gemifloxacin have been developed with improved antipneumococcal activity, in response to the world-wide epidemic of PRP. Unfortunately, many other agents in development have dropped by the wayside because of potential neuro, cardiac, and photo-associated toxicity. Certain closely related compounds, including enrofloxacin and sarafloxacin are used in veterinary practice.

Some of the more active quinolones, including ciprofloxacin and ofloxacin, exhibit activity against chlamydiae (Oriel 1989) and mycobacteria (Garcia-Rodriguez and Gomez Garcia 1993), but this does not always translate into therapeutic success (Oriel 1989; Young 1993).

The activity is variably affected by pH (the activity usually being reduced at acid pH values) and by the presence of magnesium and other cations (Smith and Lewin 1988).

MODE OF ACTION

The primary site of action of quinolones is DNA gyrase (topoisomerase II), the remarkable enzyme that engineers the breaking and rejoining of supercoiled DNA. The action mainly involves the DNA gyrase A subunit, although the B subunit can also be affected by some quinolones (Hooper and Wolfson 1989).

RESISTANCE

Resistance to earlier quinolones of the nalidixic acid type occurs readily by chromosomal mutation and restricts the value of these compounds in the treatment of complicated urinary tract infection (Greenwood and O'Grady 1977). Such resistance also affects newer fluoroquinolones, but the reduction of activity is such that variants resistant to nalidixic acid are still inhibited by concentrations achievable therapeutically. Early

Table 6 *Proposed mechanisms of fluroquinolone resistance, by organism*

	Drug-target mutations in DNA gyrase or topoisomerase IV				
	Primary target		Secondary target enzyme		
Class of organisms, organism	Altered enzyme subunit	Quinolone affected[a]	Altered enzyme subunit	Quinolone(s) affected[a]	Active efflux[b]
Gram-positive					
Streptococcus pneumoniae	ParC	Cpfx, Lvfx, Nrfx, Tvfx	Par C	Spfx	Yes
	GyrA	Spfx, Gtfx	–	–	–
	ParE	Cpfx	GyrA, GyrB	Cpfx	-[c]
Staphylococcus aureus	GyrA	All	GyrA, GyrB	All	Yes
Enterococcus faecalis	GyrA	All	–	–	Yes
Gram-negative					
Escherichia coli	GyrA	All	GyrB, ParC, ParE	All	Yes
Salmonella	GyrA	All	GyrB	All	–[c]
Klebsiella	GyrA	All	ParC	All	Yes
Pseudomonas aeruginosa	GyrA	All	GyrB	All	Yes
Neisseria gonorrhoeae	GyrA	All	ParC	All	–[c]
Campylobacter	GyrA	All	–	–	Yes
Helicobacter pylori	GyrA	All	–	–	–
Mycobacterium sp.	GyrA	All	GyrB	All	Yes

Cpfx, ciprofloxacin; Gtfx, gatifloxacin; Lvfx, levofloxacin; Nrfx, norfloxacin; Spfx, sparfloxacin; Tvfx, trovafloxacin.
a) Antimicrobials to which the amino acid alterations in the enzyme confer resistance. For many bacteria, the basic resistance mechanism applies for all quinolone agents. However, in some bacteria, such as *S. pneumoniae*, the resistance mechanism will vary depending on the type of quinolone, as indicated here for Cpfx and Spfx.
b) Several different types of active efflux mechanisms exist; these data indicate only whether an active efflux mechanism of resistance has been found in a bacterial organism.
c) No or unknown (after Goldstein and Garbaedian-Ruffalo 2002).

optimism that this, together with the presence of only two convincing reports of plasmid-mediated resistance (Jacoby et al. 2003; Martínez-Martínez et al. 2003) would reduce the likelihood of resistance to fluoroquinolones becoming widespread, have not been realized. As might be expected, the emergence of resistance is particularly common in bacteria, such as *P. aeruginosa*, *Campylobacter jejuni*, and *S. aureus*, for which the minimum inhibitory concentration of fluoroquinolones does not greatly exceed therapeutically achievable concentrations (Wolfson and Hooper 1989; Peterson 1994; Wiedemann and Heisig 1994). Resistance is usually due to sequential mutations in genes for DNA gyrase, but may also follow from alterations in drug accumulation, sometimes associated with alterations in outer-membrane proteins of gram-negative bacilli (Wolfson and Hooper 1989). Early quinolones, which are innately less active, select well for first-step mutations, as these lead to resistance. Although new quinolones remain active, their MICs are raised, making second-step mutations more likely to occur, giving resistance to these new agents. Consequently, there have been calls to stop using these older agents, but this is probably not realistic because of cost. Also, it suggests a narrow view of the effects of antibiotics to make such calls. The targeted pathogen will not be the only bacteria exposed to the new agents – of necessity the whole bacterial flora will be exposed with unknown consequences in terms of resistance. An outbreak of a nalidixic acid resistant *S. dysenteria* with intermediate susceptibility to cipro-floxacin has recently been reported (Sarkar et al. 2003).

PHARMACOLOGICAL PROPERTIES

Quinolones are generally well absorbed when given orally, but some fluoroquinolones, including ciprofloxacin and levofloxacin, are also available in parenteral formulations. Nalidixic acid and the early congeners are extensively metabolized in vivo and sufficient unchanged drug to achieve an antibacterial effect is found only in urine. Fluoroquinolones are, in general, less susceptible to metabolic changes and since they are well distributed in the body including bile, they are of value in many systemic infections. Plasma elimination half-lives of the fluoroquinolones are prolonged, allowing once or twice daily dosing. Protein binding is low.

TOXICITY AND SIDE EFFECTS

They are generally well tolerated, although rashes, gastrointestinal upsets, and photosensitivity may occur and there are persistent reports of occasional neurotoxic side effects (Hooper and Wolfson 1991; Midtvedt and Greenwood 1994). The observation that

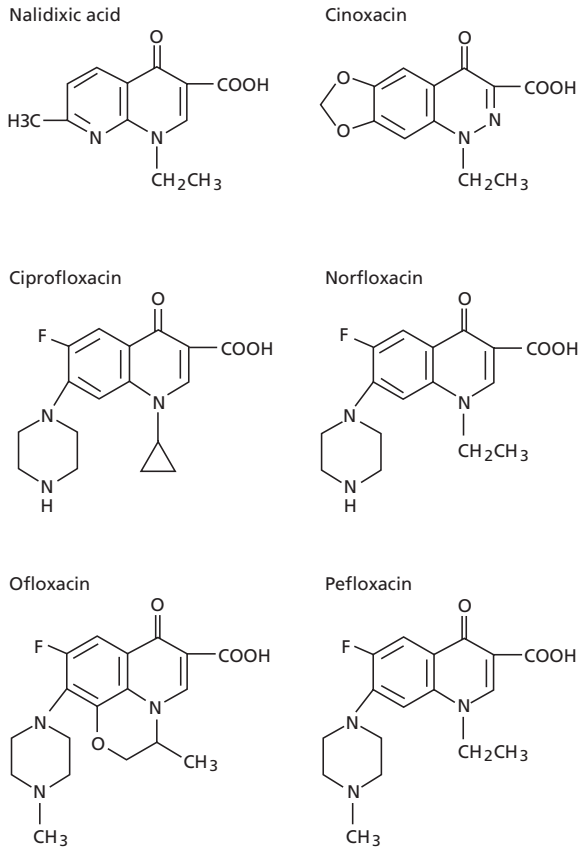

Figure 18 *Structures of some quinolones.*

fluoroquinolones can cause arthropathy in young experimental animals has led to a recommendation that these drugs should be avoided in young children and in women of child-bearing age. Cases of Achilles tendinitis, sometimes with rupture of the tendon, have been reported (Ribard et al. 1992).

Rifamycins

STRUCTURE AND SPECTRUM OF ACTIVITY

The rifamycins that are most widely used clinically, rifampin (Figure 19) and rifabutin (also known as ansamycin), are semisynthetic derivatives of the naturally occurring antibiotic, rifamycin B. Rifampin exhibits good activity against gram-positive and gram-negative cocci, particularly staphylococci, but is more noted for its antimycobacterial activity. Gram-negative bacilli are much less susceptible. Rifabutin has been introduced specifically because of its activity against organisms of the *M. avium* complex. Rifampin is seeing increased use in combination therapy for MRSA, particularly where medical treatment of prosthetic implant infection is being attempted, as it is thought to retain bactericidal activity in this setting (Widmer 2001).

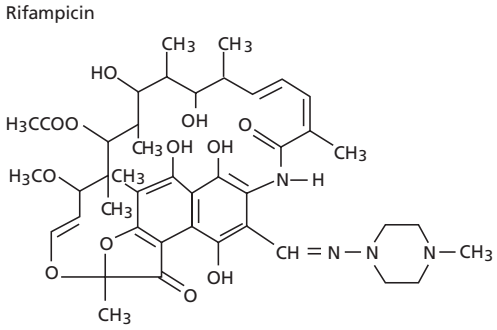

Figure 19 *Structure of rifampin.*

MODE OF ACTION

Rifampin and other rifamycins bind to the subunit of DNA-dependent RNA polymerase and prevent initiation of RNA synthesis (Wehrli and Staehelin 1971).

RESISTANCE

Resistance caused by mutational alterations in the target enzyme arises readily and may be a cause of treatment failure if rifampin is used alone. In tuberculosis and leprosy, in which rifampin is normally used in combination with other drugs, resistance is presently uncommon, although multiply-resistant strains of *M. tuberculosis* are causing concern in some places (Warburton et al. 1993; Young 1993; Pablos-Mendez et al. 1998).

PHARMACOLOGICAL PROPERTIES

Rifampin is very well absorbed when given by mouth, but the bioavailability of oral rifabutin is variable (Skinner et al. 1989). Both drugs are well distributed and excreted primarily by the hepatobiliary route, but also renally. They impart a red color to the urine and to tears, so that they may discolor soft contact lenses. They are potent inducers of liver enzymes, which promote self-metabolism of the antibiotics, as well as that of other drugs, including oral contraceptives and warfarin. There is significant binding to plasma proteins (approximately 70 percent). The terminal half-life of rifabutin (approximately 36 h) is much longer than that of rifampin (approximately 3.5 h).

TOXICITY AND SIDE EFFECTS

Rifampin may cause sensitization when used intermittently, as in some recommended regimens for the treatment of tuberculosis. Gastrointestinal upsets, jaundice, and (particularly with rifabutin) hematological effects are recognized. Induction of microsomal liver enzymes may lead to important interactions with other drugs, including failure of oral contraception.

Nitroimidazoles

STRUCTURE AND SPECTRUM OF ACTIVITY

Azole derivatives of various kinds have wide-ranging antimicrobial activities against fungi, protozoa, and helminths, as well as bacteria. Some may have a role as radiosensitizing agents in cancer therapy. Those that exhibit useful antibacterial activity are 5-nitroimidazoles. Only two, metronidazole and tinidazole (Figure 20), are used in human medicine in the UK, but others, such as nimorazole, ornidazole, and secnidazole, are available elsewhere and yet more are used in veterinary practice. They are primarily antiprotozoal agents, but also exhibit potent activity against anaerobic bacteria. Metronidazole offers a useful alternative to vancomycin in the treatment of *C. difficile*-associated colitis.

Microaerophiles and oxygen-tolerant species, such as *Actinomyces* spp. and *Propionibacterium* spp., are mostly insensitive (Greenwood et al. 1991), though metronidazole is used successfully in infections with *H. pylori* and *Gardnerella vaginalis*.

MODE OF ACTION

The narrow spectrum of activity of 5-nitroimidazoles arises because the antibacterial effect is dependent on reduction of the nitro group under anaerobic conditions. The compounds capture electrons from reduced ferredoxin generated in the course of the decarboxylation of pyruvate by the pyruvate–ferredoxin oxidoreductase complex. The short-lived reduction product kills the cell, probably by inducing breaks in the DNA strands (Edwards 1993a). The anomalous susceptibility of certain microaerophiles remains unexplained, although there is evidence that it is related to unusual metabolic pathways in these organisms (Smith and Edwards 1995).

RESISTANCE

Resistance to metronidazole and other 5-nitroimidazoles remains very uncommon despite the widespread use of these compounds. Some reports have subsequently been shown to have been erroneous because sensitivity tests were carried out under inadequately anaerobic conditions. There are, nevertheless, well documented accounts of resistance in clinical isolates of *B. fragilis* and other anaerobes, usually associated with a decreased ability to reduce the drug (Edwards 1993b).

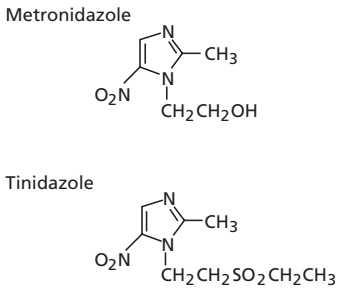

Figure 20 *Structures of metronidazole and tinidazole.*

PHARMACOLOGICAL PROPERTIES

Metronidazole is usually administered by mouth, though intravenous, suppository, and topical preparations are also available for various purposes. Tinidazole is available in the UK only in tablet form. They are virtually completely absorbed by the oral route and are widely distributed, including into cerebrospinal fluid. Plasma protein binding is negligible. Tinidazole has a somewhat longer half-life than metronidazole (12–14 h versus 8–10 h). Both are metabolized and excreted chiefly into urine, partly as glucuronide conjugates.

TOXICITY AND SIDE EFFECTS

Nausea and abdominal cramps occur quite frequently; various other side effects have been reported occasionally. Patients often complain of dryness in the mouth and a metallic taste. Alcohol should be avoided because of a disulfiram-like reaction. Fears that the effect of metronidazole on DNA might lead to mutagenic or teratogenic effects in humans have not been borne out in practice (Morgan 1978; Beard et al. 1979). It is, nonetheless, considered prudent to avoid using these drugs during pregnancy, particularly during the first trimester.

Nitrofurans

STRUCTURE AND SPECTRUM OF ACTIVITY

Various nitrofuran derivatives are in use around the world as antibacterial agents, including furazolidone and nifuratel (which are said to possess antiprotozoal, as well as antibacterial activity) and nitrofurazone. However, the most widely used agent of this type, and the only one available in the UK, is nitrofurantoin (Figure 21), which is used as a urinary antiseptic. Nitrofurantoin is bactericidal to most urinary pathogens at concentrations achievable in urine, although activity against *Proteus* spp. is unreliable, partly because the drug is less active in the alkaline conditions produced by urea-splitting organisms. Many strains of MRSA are susceptible.

MODE OF ACTION

Like the nitroimidazoles, the nitrofurans are susceptible to nitroreductases, though in this case reduction takes place in an aerobic environment. The most likely explanation for the bactericidal action of these drugs is, therefore, that a reduced intermediate causes DNA strand breakage in a manner analogous to that of nitroimidazoles. This suggestion has the attraction of accounting for the known mutagenic effects of these compounds in vitro (McCalla 1977). However, more recently it has been suggested that reactive nitrofurantoin metabolites interfere not with DNA but with protein synthesis

Nitrofurantoin

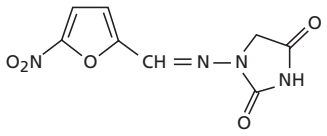

Figure 21 *Structure of nitrofurantoin.*

(McOsker and Fitzpatrick 1994). These authors also suggest an alternative mechanism of action that does not depend on bacterial nitroreductases.

RESISTANCE

Acquired resistance in susceptible bacterial species is uncommon and, even when it does occur, is rarely plasmid mediated. For this reason multiresistant strains of enterobacteria usually remain susceptible to nitrofurantoin.

PHARMACOLOGICAL PROPERTIES

Nitrofurantoin is administered by mouth and is rapidly and almost completely absorbed. It is excreted extremely rapidly into urine (half-life 20–60 min) and such drug, as finds its way into body tissues, is metabolized to the inactive derivative, aminofurantoin.

TOXICITY AND SIDE EFFECTS

Nausea is the most frequent complaint of patients receiving nitrofurantoin, but this is less common with a macrocrystalline formulation. Among less common side effects, pulmonary complications are prominent, but even these are rarely seen. The mutagenic potential of nitrofurans has not prevented their widespread and evidently safe use, even in pregnancy.

Novobiocin

Novobiocin is a naturally occurring antibiotic related to the coumarin anticoagulants. It acts on the B subunit of bacterial DNA gyrase. It is quite active against staphylococci and streptococci, but not Enterobacteriaceae or pseudomonads. It was formerly used principally as an antistaphylococcal agent, but toxicity has limited its value. Multiresistant staphylococci may remain susceptible to novobiocin, but mutations to resistance occur readily.

AGENTS THAT ACT ON THE BACTERIAL CELL MEMBRANE

In contrast to antifungal agents, in which the cell membrane is the most common target, few antibacterial agents act at this level, and those that do are quite toxic. Among membrane-active agents used in human medicine, only the polymyxins have been regularly used systemically.

Polymyxins

STRUCTURE AND SPECTRUM OF ACTIVITY

The polymyxins are a family of antibiotics produced by species of *Bacillus*. They are made up of a polypeptide portion, much of which is arranged in a cyclic fashion, with a hydrophobic octanoic acid tail (Figure 22). Two members of the family are in therapeutic use: polymyxin B and colistin (polymyxin E). Derivatives in which the diaminobutyric acid residues are sulfomethylated are also available. These sulfomethyl polymyxins exhibit reduced antibacterial activity, but they spontaneously break down to the more active parent compounds. Most gram-negative bacilli (with the exception of *Proteus* spp.) are sensitive to polymyxins, but their chief attraction is their activity against *P. aeruginosa*. Gram-positive organisms are much less susceptible.

MODE OF ACTION

The polymyxins act like cationic detergents to destabilize the cytoplasmic membrane. They also act on the outer membrane of gram-negative bacilli by binding to lipopolysaccharide. Polymyxin B nonapeptide, a derivative in which the fatty acid tail has been removed, binds to lipid A of lipopolysaccharide (but not to the cytoplasmic membrane) and has attracted some attention because of its anti-endotoxin properties (Danner et al. 1989).

RESISTANCE

Primary resistance in sensitive species is uncommon. However, adaptation to resistance readily occurs when dense bacterial populations are exposed to the drug (Greenwood 1975). This type of resistance is readily reversible and is apparently due to phenotypic changes in the membrane structure (Gilleland et al. 1984).

PHARMACOLOGICAL PROPERTIES

Polymyxins are nowadays more widely used as topical than as systemic agents. Polymyxin B, in particular, features as an ingredient of a number of topical preparations. They are not absorbed when given by mouth, but oral suspensions of colistin are used in several selective digestive tract decontamination regimens in neutropenic patients (Donnelly 1993). After injection of parenteral preparations, the polymyxins bind to tissue cells, but sulfomethyl derivatives are less readily bound and are excreted more rapidly. They do not penetrate into cerebrospinal fluid. They are excreted renally with a half-life of about 6 h, but tissue binding ensures that much of the dose is retained for much longer periods. Protein binding is low.

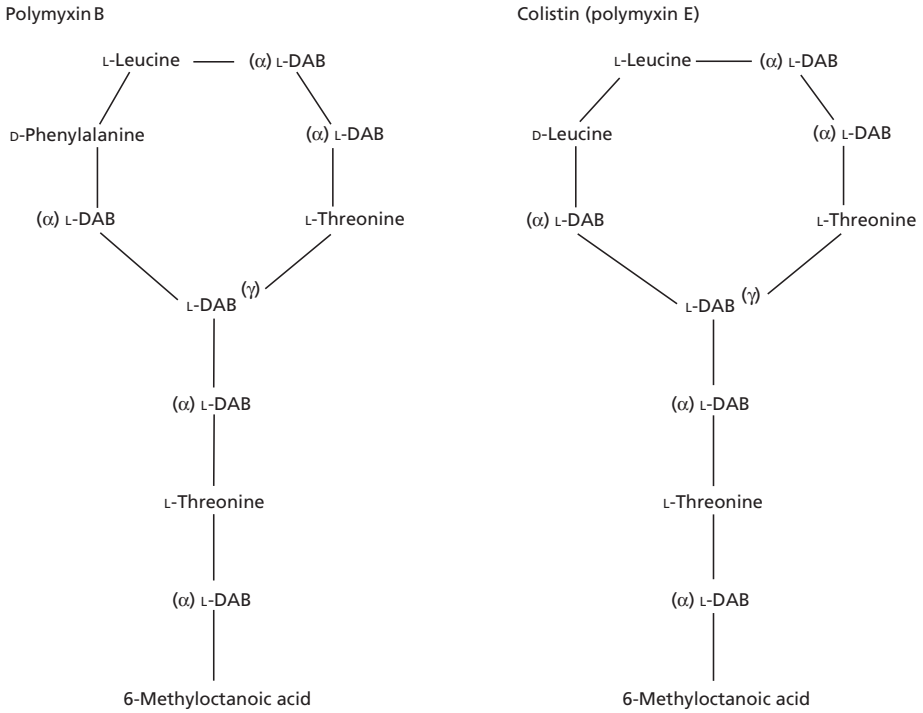

Figure 22 *Structures of polymyxin B and colistin (polymyxin E). In the sulfomethyl derivatives each of the β-linked diaminobutyric acid (DAB) residues may be sulfomethylated.*

TOXICITY AND SIDE EFFECTS

The major toxicity problems of the polymyxins relate to their affinity for cell membranes, including those of mammalian cells. Nephrotoxicty and neurotoxicity occur, but are usually reversible. Sulfomethyl polymyxins are somewhat less toxic than the sulfates. Topical preparations are generally well tolerated.

Other agents acting on bacterial membranes

Several antibiotics that are found in topical formulations, including gramicidin and tyrocidine, interfere with the integrity of bacterial cell membranes. Similarly, a number of agents used in animal husbandry, including valinomycin and monensin, achieve their effect at the membrane level. These agents act as ionophores: compounds that form transmembrane channels with consequent leakage of cellular potassium and other cations (Pressman 1973).

In addition, many antiseptics and disinfectants disrupt bacterial membranes. Naturally occurring antimicrobial peptides that are widespread in nature, such as the magainins, cecropins, defensins, and the lanthionine-containing lantibiotics, also act selectively on cell membranes (Boman et al. 1994). There has been much interest in these and related synthetic oligopeptides, but any possible future for these compounds as therapeutic agents is still some way off.

LABORATORY CONTROL OF ANTIMICROBIAL CHEMOTHERAPY

The performance of antimicrobial sensitivity tests and antibiotic assays are among the most important functions of medical microbiology service laboratories. Similarly, the provision of advice to clinicians and to hospital therapeutics committees on the use of antimicrobial agents forms a crucial part of the role of the clinical microbiologist. Indeed, it is essential that this role is expanded if rational antibiotic use is to be encouraged and the threat of multidrug resistance is to be kept at bay. In addition, the microbiology laboratory provides an important service in monitoring local trends of antibiotic drug resistance and in disseminating this information to prescribers (Greenwood 1993).

Sensitivity test methods

ANTIBIOTIC TITRATIONS

The intrinsic activity of antimicrobial drugs in vitro is usually expressed in terms of the minimum inhibitory concentration (MIC); less commonly by the minimum bactericidal concentration (MBC). These values are derived by titration of the drug against a standard inoculum of a pure culture of an isolated bacterial species in broth or on agar plates. The MIC is the lowest concentration that prevents the development of visible growth after a period of incubation that is usually

16–20 h for most common pathogens, but may be longer for slow-growing organisms. The MBC is assessed from broth titrations by subculture of those dilutions of antibiotic that are above the MIC. The lowest concentration of antibiotic that achieves a 1 000-fold or greater reduction in the original bacterial inoculum (i.e. a fall in viable count of at least 3 $\log_{10}$ colony-forming units) is generally taken as the MBC. The rate of killing may be more important than the overnight MBC value. In this case it is necessary to count the number of viable organisms at regular intervals after exposure to appropriate concentrations of the antimicrobial agent. In assessing viability it is good practice to specify colony-forming units rather than bacterial numbers, since bacteria may grow in clumps or chains that give rise to single colonies on solid media.

Alternatively, the E-test, in which a linear gradient of antibiotic is carried on a special strip applied to a plate inoculum of the test organism, can be used. This method is very simple and appears to correlate well with more traditional methods (Baker et al. 1991; Brown and Brown 1991).

Although MIC titrations provide figures that are widely used in comparing the activity of different antimicrobial agents, the results may be extremely variable depending on the test conditions and on the bacterial inoculum (Amsterdam 1991). Moreover, the factors determining the end point of MIC titrations vary from drug to drug and from organism to organism (Greenwood 1981). Selected MIC values shown in Table 7 should be interpreted in the light of these provisos. Nevertheless, the MIC is the mainstay of modern pharmacodynamic principles on the best antibiotic dosing schedules and is certainly the single most important measure of an antibiotic's activity.

MIC titrations are generally too time-consuming for routine purposes, although prepared microtitration trays containing suitable dilutions of lyophilized antibiotic can be used to simplify the procedure.

DISK DIFFUSION METHODS

Most medical microbiology laboratories use one of the disk diffusion techniques in which antibiotics incorporated into absorbent disks are applied to the surface of culture plates seeded with the test organism (Acar and Goldstein 1991; Brown 1994). Antibiotic diffuses rapidly from the disk into the surrounding medium, setting up a concentration gradient of the drug. The edge of a zone of inhibition is formed where the concentration is able to prevent visible growth of the microorganism (Barry 1991).

In the USA and world-wide, a highly standardized disk method previously known as the Kirby–Bauer test and now known as the NCCLS method (NCCLS 2000, 2002) is widely used. In the UK it is more usual to use Stokes' comparative disk method, in which the test and control organism are both exposed to the gradient of antibiotic under identical conditions on the same plate (Stokes et al. 1993). Stokes' method is particularly useful in conditions such as exist in many developing countries in which refrigeration facilities are unreliable, so that disks are kept in unsatisfactory, often humid conditions. More standardized methods, such as NCCLS, and the recently described BSAC method are increasingly adopted (BSAC Working Party Report 2001). This is because of the increasing need for comparable surveillance data in the battle against antibiotic resistance. If zone sizes are accurately measured then subtle changes that may become significant over a period can be more easily spotted. Steps can then be taken to reverse such trends before they become a clinical problem.

By comparing disk zone sizes and MIC values for many different organisms, a relationship between the two is evident and this can be formalized by regression analysis. However, since the factors that determine the end point of MIC titrations differ considerably from those that operate in the conditions of the disk diffusion test, the relationship is less than perfect. This is particularly true for those organisms that are of intermediate susceptibility, for which there may be wide discrepancies between the two methods.

BREAKPOINT METHOD

Many laboratories with large workloads find it convenient to use the breakpoint method of sensitivity testing, since this allows many isolates of bacteria to be tested together. In this method, antibiotics are incorporated into agar plates at predetermined 'breakpoint' concentrations based on therapeutically meaningful levels. Plates are spot-inoculated with a multipoint inoculation device and incubated overnight. If no growth occurs at the breakpoint concentration, the organism is scored as sensitive; if growth occurs, the organism is regarded as resistant. Sometimes a second, higher breakpoint is also used in order to establish a category of 'reduced susceptibility'. By use of a plate scanner to read the result, the whole method can be semi-automated.

OTHER METHODS OF SENSITIVITY TESTING

Many different mechanized or semi-automated methods have been devised for determining MICs or otherwise estimating the sensitivity status of bacteria (Tilton 1991). Some of these machines produce results much more rapidly than traditional methods, but the correlation is often less than perfect, partly because of the requirement for reading the end point turbidimetrically (Greenwood 1985).

DNA probes that are able to detect the genetic potential for resistance have also received much attention in recent years. These methods need to be refined if they are to be of use as routine methods, since most probes are too specific to detect the wide range of resistance

Table 7 *Activity in vitro of some representative antibacterial agents against selected micro-organisms*

Antibacterial agent	Minimum inhibitory concentration (mg/l)									
	Staphylococcus aureus	*Streptococcus pyogenes*	*Streptococcus pneumoniae*	*Neisseria meningitidis*	*Haemophilus influenzae*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Bacteroides fragilis*	*Chlamydia trachomatis*
Benzylpenicillin	0.1	0.01	0.02	0.02	1	64	R	R	16	R
Amoxicillin	0.1	0.01	0.03	0.06	0.25	2	R	R	16	R
Cephalexin	2	1	2	2	16	8	8	R	R	R
Cefuroxime	1	0.02	0.02	0.06	0.5	2	4	R	R	R
Cefotaxime	1	0.02	0.02	<0.01	0.01	0.06	0.03	16	R	R
Ceftazidime	4	0.1	0.25	<0.01	0.1	0.1	0.1	2	32	
Cefepime	2	0.1	<0.5	. . .	. . .	0.1	0.1	8	R	
Imipenem	0.03	0.01	0.01	0.03	0.25	0.25	0.25	1	0.1	R
Ertapenem	0.12	0.03	<0.03	<0.03	<0.03	<0.03	<0.03	4	0.15	
Vancomycin	1	0.12	0.12	. . .	R	R	R	R	R	. . .
Gentamicin	0.25	8	16	2	0.5	0.5	0.5	1	R	R
Amikacin	1	R	R	R	1	2	2	2	R	
Chloramphenicol	4	4	2	1	0.5	4	1	R	2	2
Doxycycline	0.5	0.25	0.25	2	1	2	4	R	0.25	0.02
Tigecycline	0.25	0.06	0.03	0.015	1	0.12	0.5	8	2	
Erythromycin	0.25	0.06	0.06	1	1	R	R	R	1	0.25
Telithromycin	0.12	0.01	0.004	0.03	0.5	R	R	R		0.01[a]
Fusidic acid	0.06	8	8	0.25	R	R	R	R	2	. . .
Linezolid	0.06	0.5	0.12	R	R	R	R	R	1	
Quinupristin/ dalfopristin	0.03	0.06	0.12	<0.12	1	R	R	R		
Sulfamethoxazole	8	2	16	0.25	2	4	8	R	R	R
Trimethoprim	0.25	0.25	1	8	0.25	0.25	0.5	R	4	R
Nalidixic acid	64	R	R	0.5	1	4	4	R	R	R
Ciprofloxacin	0.5	0.5	1	<0.01	0.01	0.06	0.06	0.25	8	0.5
Moxifloxacin	0.06	0.5	0.25	0.016	0.06	0.06	0.5	8	2	
Rifampin	0.03	0.06	0.06	0.01	0.5	8	16	32	0.25	<0.01
Metronidazole	R	R	R	R	R	R	R	R	0.5	R
Nitrofurantoin	4	4	. . .	. . .	. . .	4	32	R	8	R
Polymyxin B	R	R	R		0.03	0.03	0.03	0.1	R	

These minimum inhibitory concentrations refer to typical values for fully susceptible strains without acquired resistance traits. In using this table it should be borne in mind that inhibitory concentrations may vary considerably depending on the conditions of the test and the bacterial inoculum used. R, intrinsically resistant (agent has no useful activity).

a) *C. pneumoniae*.

mechanisms that may be present. Moreover, the presence of a resistance gene does not guarantee that it will necessarily be expressed (Towner 1992b). Nonetheless, DNA probes offer an attractive solution to the problem of detecting specific resistance mechanisms, particularly in organisms that are difficult to grow and are already in clinical use for rapid detection of MRSA and rifampin resistance in *M. tuberculosis* (Fluit et al. 2001; Holliman and Johnson 1998).

CLINICAL RELEVANCE OF ANTIBIOTIC SENSITIVITY TESTS

A laboratory report indicating in vitro susceptibility of a microorganism to a particular agent by no means ensures that a therapeutic response will be achieved if that drug is used in treatment. Much will depend on the type of infection, the condition of the patient, and the pharmacological properties of the drug. A statement about in vitro resistance is more likely to indicate that treatment with that agent will not influence the course of infection, although the patient may recover for other reasons: for example, through the influence of normal host defense mechanisms, by the surgical drainage of a collection of pus, or by the removal of a catheter.

In vitro susceptibility tests by their very nature offer only a crude approximation to the complex situation that exists in the infected patient. Laboratory reports merely provide information that needs to be interpreted thoughtfully in the light of the limitations of the tests and the condition of the individual patient (Slack 1995a).

Antibiotic assays

INDICATIONS FOR ANTIBIOTIC ASSAY

Aside from the need to establish the pharmacokinetic behavior of antimicrobial drugs in the early stages of their development, indications for the assay of antimicrobial agents in clinical practice are few. The most common grounds for antibiotic assay are provided by those agents, pre-eminently the aminoglycosides, in which the therapeutic range (the difference between an effective concentration and a toxic concentration) is relatively narrow. With such agents, dosage is often initiated by use of a nomogram that takes account of the sex-related creatinine concentration (a measure of renal function), body weight, and age of the patient (Mawer et al. 1974). Assays are subsequently performed 1 h after the dose, to establish that an adequate peak concentration of the drug has been achieved, and just before the next dose ('trough' concentration) to check that drug accumulation has not occurred (Humphreys and Reeves 1991). In once-daily dosing of aminoglycosides a level between 6 and 14 h post dose is recommended. Reference is then made to a monogram to calculate the dosing schedule (Nicolau et al. 1995).

Other antibiotics that may require laboratory assay during drug therapy include the glycopeptides (although the need for this has been disputed: Ackerman 1994; Cantú et al. 1994) and chloramphenicol in young infants who may be in danger of 'gray baby' syndrome through an immature capacity to metabolize the drug.

ASSAY METHODS

Traditional plate diffusion methods of antibiotic assay have largely been superseded by automated immunoassay procedures that are more specific, more accurate, and much more rapid. High pressure liquid chromatography methods are also available for most antimicrobial agents. They are useful for certain purposes, since metabolites can often be identified and quantified by this means, but they are too cumbersome for routine use. The various assay methods that are in use are described in detail by Chapin-Robertson and Edberg (1991) and Reeves et al. (1999).

SERUM BACTERICIDAL TEST

A type of antibiotic assay commonly called back-titration, in which the bactericidal activity of the patient's serum is titrated against the organism responsible for the infection, is sometimes used in conditions in which it is important to achieve a bactericidal effect (usually because of poor host immune response). The test is commonly used in the management of infective endocarditis and is also sometimes recommended in other types of infection, including osteomyelitis, cystic fibrosis, and sepsis in immunocompromised patients. Peak (usually 1 h after administration of the dose) and trough (immediately before the next dose) concentrations are assayed. The bactericidal end point is measured by subculture of dilutions of patient's serum that inhibit growth of the organism during overnight incubation. The result may reflect the activity of more than one agent if combination therapy is being used.

The value of the serum bactericidal test has been questioned, not least because of the many technical variables that may affect the result (Peterson and Shanholtzer 1992). Moreover, clinical correlates with successful therapy have been hard to come by, although a peak serum bactericidal titer >32, and a trough <16 are usually associated with a bacteriological cure. Performance of these tests is no longer recommended in the treatment of infective endocarditis in the UK (Shanson 1998).

CONCLUSIONS

The twentieth century has seen unprecedented advances in the treatment of infection. More than 250 individual compounds are now on the world market for the systemic treatment of bacterial infection. However, the process of drug discovery and development is now so

costly and the return so uncertain that it is unlikely that this abundance will be matched in the next century (Billstein 1994). Already pharmaceutical companies are turning their attention from antibacterial agents to the potentially more profitable areas of antiviral and antifungal compounds. At a recent meeting it was apparent that six major pharmaceutical companies had recently decided to cease their antibiotic programmes and another two to downgrade or sell them off (Shlaes, 2003).

The ability of the drug companies to come up with new and ever more potent agents has, until now, blunted the impact of bacterial resistance and weakened our resolve to preserve the value of antibiotics by circumscribing their use. Such wastefulness can no longer be afforded. The second half of the twentieth century was truly a golden age of antibiotics. The future is largely in the hands of the prescriber.

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Bloodstream infection and endocarditis

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BLOODSTREAM INFECTION

INTRODUCTION

Bloodstream infections (BSI) are a major cause of morbidity and mortality. Based on data from death certificates, these infections represent the tenth leading cause of death in the United States (NNIS 2003), and the age-adjusted death rate has risen by 78 percent over the past two decades (NNIS 2000). The true incidence may even be higher. Assuming that 5 percent of hospitalized patients develop an infection, of which 10 percent are bloodstream infections, with an attributable mortality rate of 15 percent, nosocomial bloodstream infections would represent the eighth leading cause of death in the United States (Wenzel and Edmond 2001). Approximately 250 000 cases of nosocomial bloodstream infection occur annually in the United States (Pittet et al. 1997). Recent studies reported the incidence of BSI to be between 1 percent in intensive care unit (ICU) patients (Warren et al. 2001) and 36 percent in bone marrow transplant patients (Collin et al. 2001). A recent survey reported an overall incidence of six cases of BSI per 1 000 hospital admissions, with pathogen specific attack rates between 0.1 and 1.6 per 1 000 admissions (Wisplinghoff et al. 2004). In ICU patients, incidences between 32 and 54 BSI per 1 000 ICU admissions have been reported (Daschner et al. 1982; Pittet et al. 1996; Darby et al. 1997; Edgeworth et al. 1999). The crude mortality associated with BSI ranges from 12 percent in general hospital populations to 80 percent in ICU patients (Weinstein et al. 1983a; Pittet et al. 1993; Pittet et al. 1994; Digiovine et al. 1999; Warren et al. 2001). The mortality directly attributable to BSI in these populations has been estimated to be between 16 and 40 percent (Pittet et al. 1994; Digiovine et al. 1999). In ICU patients with BSI, the length of stay is prolonged by 7.5 to 25 days and the total hospitalization time by 4.5 to 32 days (Pittet et al. 1994; Digiovine et al. 1999; Warren et al. 2001). Inappropriate empirical antimicrobial therapy is an important predictor of death in patients with BSI (Bryan et al. 1983; Pittet et al. 1994; Kollef and Fraser 2001).

Throughout the 1960s and 1970s, gram-negative organisms were most frequently isolated from patients with BSI. Since then, gram-positive organisms have become increasingly frequent (Edmond et al. 1999; NNIS 2000; Diekema et al. 2002; Wisplinghoff et al. 2004).

Usually bacteremia or BSI originates from a localized infection at a distant site. Such a secondary BSI can

usually only be cured if the site of origin is identified and treated which often requires surgical intervention. Therefore, it is highly important for both clinicians and laboratory personnel to consider and evaluate all possible sources of BSI.

Clinical presentation ranges from benign transient bacteremia with little or no symptoms to fulminant septic shock with high mortality. Transient bacteremia may follow manipulation of or surgery in infected or colonized areas. Intermittent bacteremia is usually seen secondary to abscesses, mostly abdominal or pelvic. Continuous bacteremia is associated with endocarditis and other intravascular infections, but may also occur in the first weeks of typhoid fever or brucellosis. In patients with intermittent or continuous BSI, fever is the most common symptom, followed by other unspecific signs of systemic disease, such as elevated heart and respiratory rate, fatigue, malaise, or failure to thrive in neonates. A substantial proportion of patients with infection may also be euthermic or hypothermic and may present with nondescript clinical conditions. Additional symptoms may develop due to metastatic foci, such as seizures or altered personality or level of consciousness.

In addition to the significant health and economic impact of true BSI, vast resources are also consumed by false-positive blood culture (BC) results. Overall, BCs have a relatively low yield and the proportion of false-positive results is high (Shafazand and Weinacker 2002). Studies in unselected patient populations found that between 7.5 and 12 percent of all BC are positive, and among these about 5 percent are considered contaminated (Weinstein et al. 1983b; Roberts et al. 1991; Darby et al. 1997). Recent studies found about 8.6 percent of blood cultures positive in the ICU, with 3.6 percent being due to contaminants (Schwenzer et al. 1994) while in unselected patient populations 14 percent of all blood cultures were positive with 38 percent of those being due to contaminants (Moser et al. 1998). False positive BCs may increase diagnostic testing, antibiotic use, and prolong hospitalization (Shafazand and Weinacker 2002). Bates et al. (1991) showed that false-positive blood cultures increased the length of stay by a median of 4.5 days, and generated additional hospital, pharmacy, and laboratory charges. Some studies have developed rules to assess the probability of BSI in patients prior to obtaining BCs. The clinical value of these rules remains questionable, since some are retrospective or evaluate subsets of patients only (Shafazand and Weinacker 2002).

DEFINITIONS

Even though definitions for most terms used in the context of BSI are exact, there is still confusion due to imprecise and interchanging usage of these terms. Bacteremia or fungemia denote the presence of viable bacteria or fungi in the blood, with or without clinical symptoms. In contrast, systemic inflammatory response syndrome (SIRS) is a clinical term, defined as the presence of at least two of the following: temperature >38°C or <36°C, respiratory rate >20/min or $PaCO_2$ <32 Torr, heart rate >90/min, and leukocytes >12.0 × 10^9/l or <4.0 × 10^9/l or >10 percent bands. Of note, SIRS is independent of the presence of pathogens in the bloodstream. The combination of SIRS with bacteremia or fungemia is called sepsis, severe sepsis, depending on the additional presence of signs of organ dysfunction, such as hypotension or hypoperfusion, i.e. metabolic acidosis, altered mental status, oliguria, or acute respiratory distress syndrome. For detailed definitions, see Table 1. Septic shock is defined as severe sepsis with hypotension that is unresponsive to fluid resuscitation. To assess the extent of organ dysfunction, other scores such as the sequential organ failure assessment (SOFA) score (Vincent et al. 1998) may also be used. Recently, the Sepsis Definition Conference (Society of Critical Care Medicine, European Society of Intensive Care Medicine, American College of Chest Physicians and the Surgical Infection Societies) proposed the PIRO concept to classify sepsis, which is based on predisposing factors, infection, response, and organ dysfunction (Vincent 2002; Levy et al. 2003a). Bloodstream infection is frequently used interchangeably with sepsis, but is not as exactly defined, denoting bacteremia with clinical symptoms. Septicemia is an outdated term that was previously used to describe the systemic symptoms caused by bacteria, fungi or their toxins in the blood.

A bacteremia or BSI is called primary if the point of entry or focus cannot be determined, or if it originates from an intravascular catheter. The latter is also referred to as catheter-related BSI. Recent studies have recommended differentiating catheter-related bacteremia from both primary and secondary bacteremia (Renaud and Brun-Buisson 2001). If a distant site other than an IV catheter can be established as the point of origin, BSI is referred to as secondary. A distant site is usually confirmed to be the focus, if the same pathogen (with the same resistance pattern) is isolated from the blood culture and the primary site of infection.

Another way to classify BSI is by the time or setting of acquisition, i.e. community-acquired or nosocomial BSI. Usually community-acquired BSI is defined as a BSI that is detected (onset of symptoms) within the first 48 h after admission, while BSI is referred to as nosocomial if detected more than 48 h after admission. In recent years the number of people with frequent healthcare contacts, including those receiving healthcare including IV therapy at home or at a nursing home, has considerably increased. Therefore it may be more accurate to differentiate between nosocomial BSI (detected >48 h after admission), healthcare-associated BSI (detected <48 h after admission or during an outpatient visit in a patient with frequent healthcare contacts) and

Table 1 *Definitions*

Term	Definition
Bacteremia (fungemia)	Presence of viable bacteria or fungi in the blood, irrespective of clinical symptoms
SIRS	Systemic inflammatory response syndrome, defined as presence of at least two of the following: Temperature >38°C or <36°C Respiratory rate >20/min or $PaCO_2$ <32 Torr Heart rate >90/min Leukocytes >12 000/μl or <4 000/μl or >10% bands
Sepsis	SIRS with (proven or suspected) bacteremia (fungemia)
Severe sepsis	SIRS or sepsis with one or more signs of organ dysfunction, hypotension (systolic arterial pressure <90 mmHg) or hypoperfusion such as lactic acidosis (plasma lactate >2.0 mmol/l, altered mental status (GCS of 11 or below), renal failure (acute increase in serum creatinine >180 μmol/l; doubling in admission creatinine level in patients with chronic renal failure; requirement for acute dialysis or ultrafiltration), oliguria (urinary output <0.5 ml/kg per hour for at least 1 h or <30 ml for 2 h), disseminated intravascular coagulation (decrease in platelet count of 25% or more with any increase in prothrombin time), or adult respiratory distress syndrome (PaO_2/FiO_2 <175 with bilateral infiltrates consistent with pulmonary edema in the absence of heart failure; pulmonary capillary wedge pressure <18 mmHg if measured) (Rangel-Frausto et al. 1995)
Septic shock	Severe sepsis with hypotension that is unresponsive to fluid resuscitation and requires the administration of vasopressors
MODS	Multiple organ dysfunction syndrome; dysfunction of more than one organ
Septicemia	Outdated term, previously used for systemic symptoms caused by bacteria, fungi or their toxins in the blood

true community-acquired (detected <48 h after admission or during an outpatient visit in a patient with no or minor healthcare contacts). A recent study proposed to classify BSI as follows: true community-acquired BSI (detected within the first 48 h after admission, in patients who have not been hospitalized within the past 30 days and did not have a recent history of invasive procedures), BSI in recently discharged patients (2–30 days prior to the recent episode of BSI), BSI in patients with a recent history of invasive procedures (including insertion of Foley catheters, placement of intravascular catheters, long-term devices, central venous catheters, or dialysis), nursing home-acquired bacteremia, and true nosocomial BSI (detected >48 h after admission) (Siegman-Igra et al. 2002).

TECHNICAL ISSUES

Indications

Blood cultures are a valuable diagnostic tool and indicate whenever there is clinical evidence of sepsis or an unknown systemic infection (Aronson and Bor 1987). Signs of sepsis are shown in Table 2. Also, clinical suspicion of any infection that may be associated with bacteremia or fungemia such as meningitis, cholangitis, endocarditis, and osteomyelitis warrant the cultural examination of the patient's blood. In addition, blood cultures should be obtained in the following clinical conditions: pneumonia, pyelonephritis, septic arthritis, epiglottitis, and omphalitis (infants and neonates), as well as abscesses, and deep-seated or extended skin and soft tissue infections. Typhoid fever, brucellosis or leptospirosis can also be diagnosed by blood cultures (see below). Since neurological or psychiatric symptoms, such as seizures, acutely altered mental status, or TIA-like symptoms can be due to metastatic foci of BSI or secondary emboli following endocarditis, blood cultures are also highly recommended in these clinical situations. Blood cultures are also imperative in the diagnostic evaluation of fever of unknown origin since this may be caused by a chronic inflammatory process, such as an abscess associated with intermittent BSI. In critically ill patients, blood cultures should be performed in all patients with a new fever, even if the clinical findings do

Table 2 *Signs of sepsis (Vincent and Jacobs 2003)*

Category	Sign
General	Chills, fever or hypothermia, tachypnea or respiratory alkalosis, positive fluid balance
Inflammation	Leukocytosis or leukopenia, elevated CRP, IL-6, procalcitonin
Hemodynamic	Hypotension, tachycardia, centralization (cold extremities), oliguria, hyperlactatemia
Organ dysfunction	Hypoxemia, altered mental status, hyperglycemia, thrombocytopenia, altered hepatic function, altered renal function, nutritional imbalance

not strongly suggest an infectious cause (O'Grady et al. 1998). Furthermore, a substantial proportion of patients with infection are euthermic or hypothermic and may present with nondescript clinical symptoms. These include elderly patients or neonates, patients with open abdominal wounds or extensive burns, patients receiving extracorporal membrane oxygenation, or taking anti-inflammatory or antipyretic drugs (Vincent and Jacobs 2003). In these patients blood cultures can also be a valuable tool for detecting potentially life-threatening infection.

Blood cultures should be considered in an even broader range of situations in compromised patients with underlying conditions that predispose for BSI. This group of patients includes neonates (especially with low birth weight) and elderly patients, but also patients with neutropenia and other immunosuppressive conditions, patients with (artificial) grafts, especially vascular grafts or prosthetic valves, or intravascular access devices (central venous catheters or permanently implanted devices, such as ports or Hickman catheters). Patients in the setting of the ICU and patients hospitalized secondary to severe trauma or severe burns should also be considered in this context.

SPECIMEN COLLECTION AND TRANSPORT

The term 'blood culture' denotes a specific volume of blood which is aseptically drawn by a single venipuncture, inoculated into culture medium or media, and investigated for the presence of microorganisms. Usually an aerobic and an anaerobic vial are used, thus one blood culture – often also referred to as one blood culture set – would be equivalent to two bottles. Each blood culture should be obtained by a separate venipuncture (Smith-Elekes and Weinstein 1993). Obtaining blood cultures through indwelling intravascular catheters is generally not recommended, unless the involvement of the catheter as a source of BSI is to be investigated.

Disinfection

Since the rapid and reliable recovery of even small numbers of bacteria from the blood is the primary goal of blood cultures, the media also provide excellent growth conditions for contaminants and contamination poses a serious problem. False-positive blood culture results may lead to unnecessary, wrong, and potentially harmful antimicrobial therapy and increase length of stay and costs (Bates et al. 1991). In addition, an increasing number of true bacteremia and BSI in immunocompromised patients is caused by bacteria that are part of the common skin flora, such as coagulase-negative staphylococci (CoNS), corynebacteria, and *Bacillus* species. Correct interpretation of such findings largely depends on the assumption that proper aseptic techniques have been used for specimen collection and transport.

Blood must always be collected with a sterile needle and syringe or with a proprietary sterile system of needle and evacuated tube or bottle of blood culture medium. Thorough disinfection of the patient's skin is mandatory to reduce the contamination rate (Strand et al. 1993). For scrubbing of the venipuncture site, 70 percent alcohol or iodine solutions such as 10 percent povidone iodine or a 1–2 percent tincture of iodine or a combination of both has been recommended (Weinstein et al. 1983b; Strand et al. 1993; Dunne et al. 1997; O'Grady et al. 1998). Since none of the disinfectant solutions used to date works instantly, it is generally recommended to let the solution dry for at least 30 s before proceeding. Gloves should be worn by the operator after disinfection of the hands; some institutions even recommend sterile surgical gloves.

Time of collection

To obtain the maximum yield of organisms, blood should be collected immediately before the onset of chills. Since prediction of this event is rarely possible, it is generally recommended to draw blood for culture when the patient's temperature rises, as soon as possible after the onset of fever or chills or in connection with other symptoms that indicate sepsis (see Definitions). There is no evidence to support basing the time of collection on specific temperature thresholds (e.g. 39°C). It has also been recommended to draw blood cultures at arbitrary intervals (Strand and Shulman 1988), but others did not find an increase in sensitivity if blood cultures were obtained simultaneously, instead of spaced over time (Li et al. 1994). Intervals should be based on the patient's clinical condition, and should not result in delaying therapy in critically ill patients (O'Grady et al. 1998). Whenever possible, blood cultures should be obtained before antimicrobial chemotherapy is initiated, because ongoing antimicrobial chemotherapy significantly reduces the yield. If a patient is already receiving antimicrobials, blood cultures should be drawn towards the end of a dosing interval. If possible, antimicrobial therapy should be stopped and cultures should be obtained 24–48 h after the last dose was administered.

Collection site

Usually blood for culture is drawn from a peripheral vein, e.g. vena cubitalis. Contamination is more likely if the femoral vein or a vein in close proximity to an inflammation or skin infection site is used (Washington 1975). In neonates, special blood culture systems can be used to obtain blood from better accessible sites. Arterial blood does not offer any advantage with regard to detection of pathogens, not even for endocarditis or

fungemia (Tenney et al. 1982; Vaisanen et al. 1985). The reliability and diagnostic impact of blood cultures drawn through an intravascular catheter is controversial (Tonnesen et al. 1976; Bryant and Strand 1987). Contamination rates are usually higher and catheters may be colonized with organisms that do not necessarily invade the bloodstream. However, blood from this source, even via different lumens of the catheter, may be used in conjunction with peripheral venous samples to assess the presence of a catheter-related BSI. In this case quantitative blood cultures (lysis centrifugation method) or differential time to positivity when using a continuously monitoring blood culture system (CMBCS) can be used (Blot et al. 1999; Mermel et al. 2001; Rijnders et al. 2001; Gaur et al. 2003; Seifert et al. 2003a).

Volume

In adults, bacteremia is usually characterized by <1 bacteria per ml of blood (i.e. colony forming units (cfu)/ml) (Ilstrup and Washington 1983; Plorde et al. 1985; O'Hara et al. 2003). The volume of blood obtained for culture is therefore one of the most important variables influencing the diagnostic yield (Reller et al. 1982; Wilson et al. 1994). The diagnostic yield of a blood culture is directly related to the volume of blood obtained for culture (Hall et al. 1976; Tenney et al. 1982; Ilstrup and Washington 1983; Plorde et al. 1985) and increases by approximately 3–5 percent per ml blood taken (Hall et al. 1976; Tenney et al. 1982; Mermel and Maki 1993). In clinical practice, usually 10–20 ml of blood per culture is obtained and distributed evenly into an aerobic and an anaerobic culture bottle, leading to a final volume of 5–10 ml blood per bottle (Tenney et al. 1982). According to guidelines, 10 ml per culture are reasonable with an optimal volume being 20–30 ml per culture, depending on the number and type of bottle used (Washington and Ilstrup 1986; Dunne et al. 1997). The ratio between blood and culture media (blood to broth) should be 1:5–1:10, in order to neutralize the bactericidal activity of human serum (see below). For details on the optimal volume of blood per bottle, the instructions of the respective manufacturers should be consulted. Even though the diagnostic impact of the anaerobic vial is controversial (Sharp et al. 1991; Murray et al. 1992), the use of two vials ensures the culture of an adequate blood volume increasing the probability of recovery.

In pediatric patients a smaller volume (1–5 ml) is usually sufficient, because the concentration of microorganisms in these patients is higher (>100 cfu/ml blood) (Dietzmann et al. 1974; Szymczak et al. 1979; La et al. 1981; Reller et al. 1982). Even though less than 1 ml of blood may allow detection of microorganisms in infants if the concentration is high enough (Dietzmann et al. 1974; Neal et al. 1986), the diagnostic yield in these patients is also higher if a bigger blood volume is obtained (Szymczak et al. 1979; Isaacman et al. 1996; Kellogg et al. 2000). Recent studies showed that the detection rate in pediatric patients doubled if 6 ml of blood were obtained instead of 2 ml (Isaacman et al. 1996). Showing that low-level BSI does occur in children, some authors recommend obtaining 4–4.5 percent of a child's blood volume for culture (Kellogg et al. 2000). Specific 'pediatric' blood culture bottles contain a decreased volume of broth, and are supplemented with X and V factors and sodium-polyanetholesulfonate (SPS) to enhance detection of microorganisms. In these bottles, a decreased volume is necessary to maintain an optimal broth:blood ratio. However, data proving the necessity of using these bottles remain rare (O'Hara et al. 2003).

The protective covers of the vials should be removed close to inoculation. The membrane should be disinfected using sterile alcohol swabs. As with any disinfection, contact times should be observed and the membranes must be completely dry before inoculation. Based on the data of a meta-analysis, a new needle should be used to inoculate the vials in order to reduce contamination (Spitalnic et al. 1995). However, others have found no significant increase in contamination rates when a single-needle technique was used and have argued that the benefit of the reduced risk of needlestick injuries of the single-needle technique outweighs the increase in risk of contamination (Mylotte and Tayara 2000b). The atmosphere of aerobic vials used for automated blood culture processing in CMBCS is enriched with oxygen, which eliminates the need for transient venting after inoculation (Rohner et al. 1996).

Number of blood cultures

The detection rate increases with the number of blood cultures obtained. In patients with endocarditis, studies found a sensitivity of 80 percent if one blood culture was used, which increased to 88 and 99 percent if two or three independent cultures, respectively, were obtained over a 24 h period (Washington 1975; Weinstein et al. 1983b). Other studies in patients with BSI found 99 percent sensitivity if only two cultures were drawn (Weinstein et al. 1983b). Therefore, more than three blood cultures do not increase the diagnostic yield, but do increase costs and may cause iatrogenic anemia (Neu 1986). Also, the culture of only a single blood sample should be highly discouraged (Aronson and Bor 1987). There is currently no recommendation for an optimal time difference between the first and the second culture. In patients with suspected endocarditis, two to three blood cultures should be obtained within 24 h. In severely ill patients, it may be necessary to obtain two cultures (with separate venipunctures) in rapid succession in order to start antimicrobial therapy as soon as

possible (Smith-Elekes and Weinstein 1993). A recent study failed to show any benefit in yield of obtaining multiple samples, and recommended obtaining a total volume of 35–42 ml blood for six bottles at the same time (Lamy et al. 2002). However, for interpretation of results more than one separate culture (venipuncture) is needed. In neonates, drawing of a second culture is rarely possible. However, in infants or older children, two vials are mandatory, but the inoculation volume may be as low as 1 ml per vial (see Volume).

Media

Depending on the indication, the patient, and the suspected pathogen, the use of special culture media or a different number of bottles per blood culture may be useful. Commercial media that facilitate growth of almost any pathogen are readily available. Nutrient-rich broth in a variety of formulations can be used to culture microorganisms from the blood. These include brain–heart infusion (BHI) broth, tryptic-soy-broth (TSB), soybean casein digest broth, peptone broth, and Columbia media, which may be supplemented with hemin or NADH. Generally, one vial contains 50–100 ml of media and a 10 percent CO_2 atmosphere in the headspace. Therefore, vials can be used to culture aerobic, facultative, and anaerobic bacteria. An aerobic and an anaerobic bottle are usually obtained, i.e. one containing ambient atmosphere in the headspace, the other various amounts of CO_2. Due to the decrease of BSI caused by anaerobic bacteria over the past decades (Dorsher et al. 1991; Lombardi and Engleberg 1992; Murray et al. 1992; Dunne et al. 1997; Weinstein et al. 1997; Wisplinghoff et al. 2004), some authors have suggested that routine use of anaerobic bottles is not necessary, favoring two aerobic bottles to increase the volume of blood cultured (Murray et al. 1992; Morris et al. 1993; Sharp et al. 1993; Zaidi et al. 1995). However, recent surveys found anaerobes accounting for about 4 percent of BSI (Diekema et al. 2003) and anaerobic blood cultures enhance the isolation of other species, such as streptococci including *Streptococcus pneumoniae*, enterococci, and *Listeria monocytogenes* (Cockerill et al. 1997).

Inhibition of antimicrobial agents

Microbial growth in human blood is inhibited by a number of substances including antibodies, leukocytes, complement, lysozyme, and antimicrobial substances, which about one-third of patients already receive, when blood cultures are obtained (Weinstein et al. 1997). Blood clots also reduce the diagnostic yield. Virtually all commercially available blood culture bottles and media contain SPS (Wilson et al. 1994), an anticoagulant that inhibits lysozomal activity, complement, and phagocytosis, in addition to reducing blood clots (Traub and Kleber 1977; Washington and Ilstrup 1986; Chandrasekar and Brown 1994). Also, the 1:5 to 1:10 dilution of the blood enhances detection of bacteria by neutralizing the endogenous antibacterial activity of the human serum and diluting antimicrobial agents to subinhibitory concentrations. Aminoglycosides and polymyxins are also inhibited by SPS. Side effects of SPS include some inhibition of the growth of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Gardnerella vaginalis*, *Peptostreptococcus anaerobius*, and *Moraxella catarrhalis* and a growth enhancement for gram-positive cocci while gram-negative rods are generally inhibited (Staneck and Vincent 1981; Edberg and Edberg 1983; Reimer and Reller 1985; Reimer et al. 1987). Others found that increasing concentrations of SPS increase the recovery of gram-negative bacteria (Chandrasekar and Brown 1994; Wilson et al. 1994). Despite these effects, no other anticoagulant to date is superior. Antimicrobial agents can also be neutralized by adding unspecific adsorbants such as antimicrobial removal device (ARD) (Peterson et al. 1983), antibiotic binding resins on glass beads (BACTEC) (BD, Sparks, MD), or activated charcoal and Fuller's earth (Ecosorb, BacT/ALERT, bioMérieux Inc., Durham, NC). Supplemented media have shown increased detection rates (Weinstein et al. 1995; Doern et al. 1997), but also increased the number of detected contaminants (Weinstein et al. 1995). Studies of the effects of the antimicrobial removal device have shown conflicting results (Appleman et al. 1982; Peterson et al. 1983; Doern 1994) and routine use is generally not recommended (Shafazand and Weinacker 2002).

Transport

Rapid transport of blood cultures to the microbiology laboratory is mandatory. Blood cultures used in CMBCS can be stored at room temperature if direct transport to the laboratory is not possible. Refrigeration of blood cultures should be avoided. In the laboratory, all blood cultures should be processed immediately. Incubation of blood cultures before transport to the microbiology laboratory is not needed with bottles used in CMBCS. If cultures have been incubated prior to arrival at the microbiology laboratory, Gram stains and blind subcultures of all bottles need to be performed before the cultures are processed in the CMBCS.

SAMPLE PROCESSING

In patients with BSI, the appropriateness of antimicrobial chemotherapy is correlated with survival (Ibrahim et al. 2000). The first notification of positive blood cultures has a high impact on the antimicrobial therapy (Schonheyder and Hojbjerg 1995). Samples should therefore be processed as fast as possible. Proces-

Table 3 *Comparison of blood culture methods*

Method	Aerobic bacteria	Anaerobic bacteria	Yeast	Molds	Mycobacteria
Conventional	+	+	–/+	–	–
CMBCS	++	+/++	+/++	–	++[a]
Lysis centrifugaton	+/++	–	++	++	++

Modified from (O'Grady et al. 1998).
CMBCS, continuous monitoring blood culture systems; –, not recommended; +, acceptable; ++, good.
a) With use of special mycobacterial culture bottles only.

sing of blood cultures generally includes incubation, Gram staining, and subcultures. Manual processing has continuously decreased over the last decade and most laboratories now use automated blood culture systems. Several authors have compared different processing methods and systems. Table 3 compares commonly used blood culture methods.

Manual processing

All manual processing where aerosols could be generated, should be done under a protective hood, both to avoid contamination and to protect laboratory personnel. All blood cultures should be incubated for at least 6–18 h at 36 ± 1°C before processing. Agitation during incubation increases sensitivity and speed of detection (Hawkins et al. 1986; Weinstein et al. 1989; Prag et al. 1991). Afterwards, blind subcultures and microscopic examination of all aerobic cultures should be performed, using approximately 0.2 ml of broth, which should be removed under sterile precautions after gentle shaking. For microscopic examination, Gram or acridinorange stains can be performed. Gram stains are easier to perform, but may lack sensitivity. While acridinorange stains detected 10^4 cfu/ml, Gram stains only detected 10^5 cfu/ml (Seifert et al. 1997). If bacteria are seen, the respective anaerobic (or aerobic) bottle should also be subcultured. Blood-agar, chocolate-agar, anaerobic media, and some differential media (e.g. MacConkey) should be used for subculture of blood cultures and subsequently incubated at 36 ± 1°C in an aerobic atmosphere with 5–10 percent CO_2. Also if signs of turbidity, change of color, or gas production are detected, the respective culture bottle must be processed (i.e. subculture and Gram stain).

Blood cultures do not need to be routinely incubated for more than 7 days. Incubation periods are commonly extended if slow growing organisms or infective endocarditis are suspected, even though some authors noted that in the latter this practice rarely increases sensitivity (Washington 1994). If the cultures remain negative, Gram stains and blind subcultures of the aerobic bottle should be performed again at the end of the incubation period. Blind subcultures of anaerobic bottles do not increase diagnostic yield (Dunne et al. 1997). For special processing requirements, see also individual pathogens.

Automated processing

The first automated blood culture system, BACTEC 460 (Becton Dickinson), was introduced in the 1970s. The use of automated blood culture systems rapidly increased after the introduction of continuous monitoring blood culture systems in the early 1990s. As the earliest systems, most CMBCSs are based on the detection of CO_2, which is produced as microorganisms grow and utilize carbohydrate substrates. While the BACTEC 460 used ^{14}C for detection, the newer instruments of the BACTEC 9000 series use a fluorescence-sensing mechanism. Detection criteria are a linear increase in fluorescence intensity and a change in the increase rate of fluorescence intensity. The BacT/ALERT 3D (bioMérieux) which is the updated version of the BacT/ALERT uses a colorimetric CO_2 sensor system. With emitting and sensing diodes in the incubator units, growth is detected if reflection exceeds an arbitrary threshold, a linear increase or a change in the increase rate are detected. In contrast, the ESP blood culture system (Trek Diagnostic Systems, Inc.) monitors the pressure changes within the bottles' headspace, which changes with the consumption or production of gases (oxygen, hydrogen, nitrogen, CO_2) by growing microorganisms. In all systems cultures are monitored at intervals between 10 and 24 min, 24 h per day, shortening time to detection by 1–1.5 days (Wilson et al. 1992; Morello et al. 1994; Reimer et al. 1997). A 5-day incubation in a CMBCS is usually sufficient to detect most pathogens (Masterson and McGowan 1988; Hardy et al. 1992; Wilson et al. 1993) and even periods of 4 (Dorsher et al. 1991; Johnson et al. 2000) or 3 (Han and Truant 1999; Bourbeau and Pohlman 2001) days have been suggested.

As in manual processing, Gram stains and subcultures must be performed if growth of microorganisms is detected in a culture. Of note, some systems can only detect an increase in CO_2 production, and may not detect a positive culture if the bacterial growth has already entered the static phase. Therefore, if the blood cultures have been incubated previously or obtained more than 24 h prior to processing, it is recommended that Gram stain and blind subculture of both bottles be performed before the culture vials are placed into the system. Blind subcultures of aerobic or anaerobic bottles at the end of

the incubation period do not increase the diagnostic yield (Dunne et al. 1997).

Quantitative blood cultures

The lysis centrifugation system (ISOLATOR, Du Pont) is the only commercial quantitative blood culture system. Processing of cultures is intensive in both time and labor and usually not used for routine diagnostic purposes. Vials contain saponin, EDTA, and SPS and must be subcultured on aerobic and anaerobic media after centrifugation. Samples should be processed within 8–10 h after collection. For detailed procedures the reader is referred to the instructions of the respective manufacturer. However, some advantages of the lysis centrifugation method over qualitative blood cultures have been reported: diagnosis is usually faster than with conventional blood cultures. Also diagnostic yield of the ISOLATOR system is higher in some pathogens such as fungi (see Table 3).

INTERPRETATION OF RESULTS

Interpretation of the clinical significance of positive and negative blood culture results can be problematic. Misinterpretation can lead to antimicrobial therapy which is expensive and may lead to adverse events in the patient. Recovery of *Staphylococcus aureus*, *S. pneumoniae*, *Escherichia coli* and other Enterobacteriaceae, *Pseudomonas aeruginosa* and *Candida albicans* almost always represents true infection. Other microorganisms including *Corynebacterium* species, *Bacillus* species, and *Propionibacterium* species only rarely cause true BSI (Weinstein et al. 1997). The majority of coagulase-negative staphylococcal isolates also represent contamination rather than true infection. To aid interpretation a number of approaches have been suggested. In addition to the identity of the microorganism, the presence of the same organism in more than one independently obtained culture, or the isolation of the same organism from another usually sterile body site may provide valuable aid for interpretation. Correlation of laboratory findings with the clinical status of the patient is mandatory. Generally, clinically significant BSI is likely if two or more separate blood cultures grow the same organism (Weinstein 1996; Kim et al. 2000; McDonald et al. 2001). Interpretation of CoNS isolated from blood cultures is particularly difficult, since 12–15 percent of CoNS bloodstream isolates are the causative pathogens of clinical significant BSI (Weinstein et al. 1997). While in this instance the number of positive cultures in a set is not a reliable criterion (Peacock et al. 1995; McDonald et al. 2001), the number of positive cultures relative to the number of obtained cultures appears to be useful (Peacock et al. 1995; Weinstein et al. 1983b; Weinstein et al. 1997).

PATHOGENS

Almost any pathogen can be isolated from the bloodstream as a causative agent of clinically significant bacteremia, fungemia or BSI, especially in immunocompromised patients. In addition, in patients with a corresponding history (e.g. travel), parasitic and tropical diseases, which may require a different diagnostic approach should also be considered.

The spectrum of pathogens has changed considerably over the last decades (Figure 1). In recent studies, about 13 percent of all episodes were polymicrobial, 60–65 percent were caused by gram-positive and 25–31 percent by gram-negative organisms. Fungi, mainly *Candida* species, were isolated from 7–10 percent of episodes. Anaerobic bacteria accounted for about 1 percent of BSI (Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004). The most common pathogens isolated from BSI are listed in Table 4. Proportions of individual organisms vary with age, gender, underlying conditions, and clinical service.

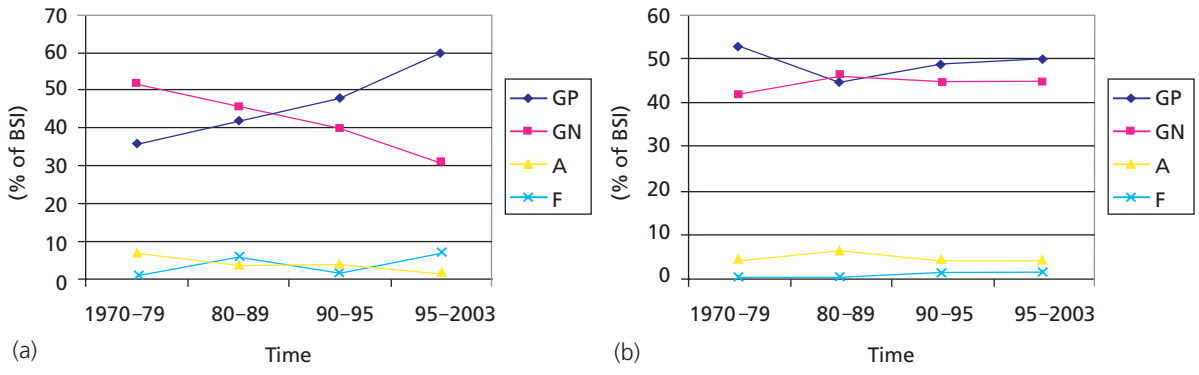

Figure 1 (a) *Trends in pathogens causing nosocomial BSI (data from Eykyn 1998; Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004).* **(b)** *Trends in pathogens causing community-acquired BSI. A, anaerobic bacteria; F, fungi; GN, gram-negative pathogens; GP, gram-positive pathogens.*

Table 4 *Incidence rates and distribution of pathogens most commonly isolated from monomicrobial BSI and associated crude mortalities for all patients, ICU patients, and patients in the non-ICU setting (Wisplinghoff et al. 2004)*

Pathogen	BSI per 10 000 admissions	Percent of BSI [rank]						Crude mortality (%)		
		Total (n = 20 978)		ICU (n = 10 515)		Ward (n = 10 442)		Total	ICU	Ward
CoNS	15.8	31.3	[1]	35.9	[1][a]	26.6	[1]	20.7	25.7	13.8
S. aureus **	10.3	20.2	[2]	16.8	[2][a]	23.7	[2]	25.4	34.4	18.9
Enterococci*	4.8	9.4	[3]	9.8	[4]	9.0	[3]	33.9	43.0	24.0
Candida spp.*	4.6	9.0	[4]	10.1	[3][a]	7.9	[4]	39.2	47.1	29.0
E. coli	2.8	5.6	[5]	3.7	[8][a]	7.6	[5]	22.4	33.9	16.9
Klebsiella spp.	2.4	4.8	[6]	4.0	[7][a]	5.5	[6]	27.6	37.4	20.3
P. aeruginosa	2.1	4.3	[7]	4.7	[5]	3.8	[7]	38.7	47.9	27.6
Enterobacter spp.	1.9	3.9	[8]	4.7	[6][a]	3.1	[8]	26.7	32.5	18.0
Serratia spp.**	0.9	1.7	[9]	2.1	[9][a]	1.3	[10]	27.4	33.9	17.1
A. baumannii	0.6	1.3	[10]	1.6	[10][a]	0.9	[11]	34.0	43.4	16.3

Ward Rank 9: *Bacteroides* spp. (n = 150; 1.4%).
BSI, bloodstream infection; CoNS, coagulase-negative staphylococci.
a) $p < 0.05$ for ICU vs. non-ICU patients; more frequent in patients with neutropenia, *, or without neutropenia, **.

GRAM-POSITIVE ORGANISMS

In the 1980s, gram-positive organisms re-emerged as the leading pathogenic causes of nosocomial bloodstream infections (Figure 1). Today, gram-positive organisms account for about 65 percent of BSI and the three leading pathogens are gram-positive (Table 4). Most gram-positive bacteria causing BSI belong to the genus *Staphylococcus* or *Streptococcus*/*Enterococcus* (Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004).

Staphylococcus aureus

S. aureus is a major cause of morbidity and mortality. Localized infections have a tendency to spread to the surrounding tissue or frequently via the bloodstream to distant sites. Commonly isolated from patients with both community-acquired and nosocomial BSI, *S. aureus* is the second most common cause of nosocomial BSI, accounting for 20 percent of these infections with an incidence of about 1 per 1 000 hospital admissions (Wisplinghoff et al. 2004). Incidence varies with age ranging from about 10 percent in patients under the age of 1 year to about 24 percent in patients over the age of 65 years (Diekema et al. 2002; Wisplinghoff et al. 2004). *S. aureus* BSI usually originates from a localized infection, an intravascular catheter or a contaminated syringe (e.g. in IV drug users). For example, a high correlation has been reported between *S. aureus* BSI and mediastinitis in patients with coronary artery bypass graft (CABG) (Fowler et al. 2003) and isolation of *S. aureus* even from a single blood culture should prompt the search for a primary focus of infection. Clinical manifestations range from benign transient bacteremia with a single episode of fever and chills to fulminant septic shock. Crude mortality associated with nosocomial *S. aureus* BSI ranges from about 18 to 34 percent; in patients with community-acquired *S. aureus* BSI, mortality rates ranging between 12 percent in unselected patient populations and 58 percent in critically ill patients have been reported (Diekema et al. 2003; Valles et al. 2003; Wisplinghoff et al. 2004).

Methicillin resistance, first detected in the 1960s (Barber 1961), emerged as a major clinical and epidemiological problem in hospitals in the 1980s. In hospitals in the United States, the proportion of isolates of methicillin-resistant *S. aureus* (MRSA) have increased from 2.4 percent in 1975 to up to 55 percent in recent years (NNIS 2003). Whether resistance to methicillin is related to transmission or virulence is controversial. Several clinical studies could not identify significant differences in the mortality rate for patients with infections due to methicillin-resistant strains and that for patients with methicillin-susceptible *S. aureus* (MSSA) strains, after correcting for various confounding variables (Hershow et al. 1992; Mylotte et al. 1996; Pujol et al. 1996; Harbarth et al. 1998; Wisplinghoff et al. 2001). In contrast, other studies have suggested that outcome of methicillin-resistant *S. aureus* BSI is significantly worse and that methicillin resistance is an independent predictor of adverse outcome of patients with *S. aureus* infections (Rello et al. 1994; Romero-Vivas et al. 1995; Blot et al. 1998; Conterno et al. 1998; Blot et al. 2002b; Cosgrove et al. 2003). Even though MRSA has been a nosocomial pathogen, there are an increasing number of reports of MRSA BSI in patients with little or no association to the healthcare system (Frank et al. 1999; Fergie and Purcell 2001; Groom et al. 2001; Naimi et al. 2001; Wu et al. 2002; Aires and De Lencastre 2003; Baggett et al. 2003; CDC 2003; Johnson et al. 2003; Said-Salim et al. 2003), suggesting the emergence of true community-acquired MRSA. In recent years there have been a number of

reports of glycopeptide or vancomycin-intermediate *S. aureus* isolates (Rotun et al. 1999; Smith et al. 1999; Wong et al. 2000; Fridkin 2001; Hageman et al. 2001; Fridkin et al. 2003; Naimi et al. 2003) and recently, a *S. aureus* isolate with high-level vancomycin-resistance conferred by the *vanA* gene has been recovered from a catheter tip culture (Johnson et al. 2003).

Small colony variants (SCV), a naturally occurring subpopulation of *S. aureus* have also been identified as a cause of BSI (Seifert et al. 2003b). *S. aureus* SCVs have been implicated in persistent and recurrent infections that give a poor clinical and bacteriological response to standard antimicrobial therapy in patients with BSI, chronic osteomyelitis, cystic fibrosis, and AIDS particularly after prolonged exposure to antibiotics (von Eiff et al. 1997; Kahl et al. 1998; Proctor et al. 1998; Seifert et al. 1999, 2003b). These phenotypic variants are characterized by their fastidious growth and atypical colony morphology on routine media, making recovery as well as correct identification difficult for microbiological laboratories.

Delay of therapy can have deleterious effects on clinical outcomes in patients with *S. aureus* BSI, and appropriate therapy should be initiated without delay (Lodise et al. 2003).

Coagulase-negative staphylococci

BSI caused by Coagulase-negative staphylococci (CoNS) is mostly nosocomial, but CoNS have also been isolated from about 8 percent of patients with community-acquired BSI (Diekema et al. 2003). According to several surveys, CoNS are the most prevalent pathogen isolated from the blood accounting for 15–30 percent of blood culture isolates (Moser et al. 1998; Edmond et al. 1999; NNIS 2000; Diekema et al. 2003; Wisplinghoff et al. 2004). This difference is mostly due to the definitions used in different studies, with lower incidences being reported if only cases were included where CoNS were isolated from two or more separate blood cultures. The fact that CoNS also constitute a major part of the normal microflora makes interpretation of positive blood culture results difficult and if possible detection should be confirmed by more than one blood culture. Although several approaches have been suggested to aid interpretation, this issue remains controversial (see Interpretation of results). *S. epidermidis*, the most frequently isolated species among CoNS has been implicated in a variety of infections, including BSI (Rupp and Archer 1994), mostly associated with intravascular catheters and other prosthetic devices. *S. haemolyticus*, the second most commonly isolated species among CoNS has also been associated with BSI and other infections (Kloos and Bannerman 1994; Rupp and Archer 1994). A variety of other CoNS have also been implicated in BSI, including *S. capitis*, *S. hominis*, *S. lugdunensis*, *S. saprophyticus*, *S. schleiferi*, *S. simulans*, and *S. warneri*. The proportion of CoNS among blood culture isolates seems to decrease with increasing age (Diekema et al. 2002; Wisplinghoff et al. 2004). Crude mortality in patients with CoNS BSI ranges from 14 to 31 percent (Diekema et al. 2003; Wisplinghoff et al. 2004).

Enterococcus species

Enterococcus species are the third most common cause of BSI in the United States (Murray 1990; Schaberg et al. 1991; Moellering 1992; Wisplinghoff et al. 2004), accounting for about 9 percent of nosocomial BSI (Edmond et al. 1999; Wisplinghoff et al. 2004). Enterococcal BSI is mainly nosocomial, and community-acquired BSI due to enterococci should always raise the suspicion of endocarditis. *Enterococcus faecalis* is the most prevalent species, accounting for 80–90 percent of clinical isolates, followed by *E. faecium* (5–10 percent of isolates) (Facklam and Collins 1989; Gordon et al. 1992; Buschelman et al. 1993; Stern et al. 1994). Other enterococci that are rarely isolated from blood include *E. casseliflavus*, *E. gallinarum*, and *E. raffinosus*. As with other gram-positive pathogens, the incidence of nosocomial BSI due to enterococci has been increasing over the past decades (Graninger and Ragette 1992; Maki and Agger 1988; Edmond et al. 1999; Wisplinghoff et al. 2004) and some studies found *Enterococcus* species particularly involved in polymicrobial BSI (Maki and Agger 1988). Primary BSIs are generally seen in highly compromised or immunosuppressed patients and may be due to inapparent bacterial translocation from a gastrointestinal source (Shlaes et al. 1981b; Graninger and Ragette 1992; Linden et al. 1996) or intravascular catheters. Secondary BSI is more frequently seen than primary BSI and usually derives from the urinary or gastrointestinal tract, or from wound infections (Shlaes et al. 1981b; Maki and Agger 1988; Graninger and Ragette 1992). Crude mortality of enterococcal BSI ranges from 34 percent in unselected patients populations to 43 percent in ICU patients (Wisplinghoff et al. 2004). Complications such as septic shock, metastatic infection (except endocarditis, see below) or adverse outcome are rare in monomicrobial enterococcal BSI (Maki and Agger 1988). *E. faecalis* isolates are mostly susceptible to ampicillin and vancomycin, while *E. faecium* isolates are usually susceptible to vancomycin only. Vancomycin-resistant strains emerged in the late 1980s in Europe and have become an increasing nosocomial problem in the United States. Several authors have reported that vancomycin-resistant enterococcal BSI contributes significantly to excess mortality and economic loss (Bhavnani et al. 2000; Lodise et al. 2003; Song et al. 2003) and that early, effective antimicrobial therapy is associated with a significant improvement in survival (Vergis et al. 2001).

Streptococcus pneumoniae

In *S. pneumoniae*, BSI is usually community-acquired and secondary to pneumonia, meningitis or otitis media (Raz et al. 1997). In a recent study about 10 percent of pneumococcal BSI were nosocomial (Canet et al. 2002). The overall incidence of invasive pneumococcal disease is about 15 per 100 000 person-years (Fedson et al. 1998), even though it may be up to ten-fold higher in certain risk populations (Davidson et al. 1994; Torzillo et al. 1995). Neonates and children under the age of 2 years are more likely to develop pneumococcal BSI, as are elderly people over the age of 65 years (Burman et al. 1985; Breiman et al. 1990) with incidences of 160 and 70 per 100 000 person-years, respectively compared to 5 per 100 000 in young adults (Breiman et al. 1990). Incidence of pneumococcal BSI is seasonal with a peak in the cold season (Kim et al. 1996). Meningitis generally occurs in a relatively small number of cases of community-acquired pneumococcal BSI (Gruer et al. 1984), but has been reported in up to 17 percent of patients with BSI by Kuikka et al. (1992). A variety of factors have been identified that predispose to invasive pneumococcal disease, such as immunosuppresion, alcoholism, and chronic lung disease (Burman et al. 1985; Fang et al. 1990; Rahav et al. 1997; Watanakunakorn and Bailey 1997). Severe cases of BSI due to *S. pneumoniae*, often associated with a fatal outcome, occur in children under the age of 2 years or who are physically or functionally asplenic, such as children with sickle cell disease in whom a 100-fold higher incidence of pneumococcal BSI has been reported (Wara 1981; Wong et al. 1992).

While pneumonia and meningitis are the most frequent entities associated with pneumococcal BSI, other manifestations include spontaneous bacterial peritonitis (Wilcox and Dismukes 1987; Gorensek et al. 1988; Shaked and Samra 1988), endo- or pericarditis (Powderly et al. 1986), epidural and brain abscesses, and soft tissue infections (Peters et al. 1989; DiNubile et al. 1991; Grigoriadis and Gold 1997).

Rates of penicillin resistance among peneumococci isolated from the blood increased over the past decade. Recent studies reported a percentage of penicillin-resistant isolates of 25–60 percent with a high degree of variation between individual countries, cities, or hospitals (Doern et al. 1998; Yu et al. 2003). Recent studies in patients with pneumococcal BSI found no correlation between penicillin resistance and outcome (Maugein et al. 2003; Yu et al. 2003).

Other gram-positive organisms

In otherwise healthy adults other gram-positive organisms rarely cause BSI. Among *Streptococcus* species other than *S. pneumoniae*, BSI can also be due to viridans group streptococci as well as serogroups A (e.g. *S. pyogenes*), B (e.g. *S. agalactiae*), D (e.g. enterococci), and G (Facklam 2002). Viridans group streptococci, part of the normal human commensal flora, have been associated with subacute endocarditis (see below), especially *S. gordonii*, *S. mitis*, *S. oralis*, and *S. sanguis* (Douglas et al. 1993; Bouvet et al. 1994). These species have also emerged as important pathogens causing bacteremia and sepsis in neutropenic cancer patients (Awada et al. 1992; Bochud et al. 1994a; Carratala et al. 1995; Richard et al. 1995; Gonzalez-Barca et al. 1996; Bilgrami et al. 1998). Clinical manifestations of viridans group streptococcal bacteremia in these patients may include fever, hypotension, shock, pneumonia, and adult respiratory distress syndrome, with a mortality rate ranging from 6 to 30 percent (Bochud et al. 1997). Serious complications of viridans group streptococcal BSI associated with a high mortality rate occur mainly in patients receiving high-dose antineoplasmatic chemotherapy who develop severe oral mucositis (Marron et al. 2000). The increased frequency of these infections has been attributed to several factors, including high-dose chemotherapy with cytosine arabinoside, oropharyngeal mucositis, and prophylactic therapy with cotrimoxazole or a fluoroquinolone (Elting et al. 1992; Bochud et al. 1994a; Richard et al. 1995). Oral and gastrointestinal mucosal lesions and intravascular catheters have been suggested as the most frequent portals of entry (Elting et al. 1992; Bochud et al. 1994b; Richard et al. 1995; Gonzalez-Barca et al. 1996). In the past, viridans group streptococci were considered to be uniformly susceptible to beta-lactam antimicrobial agents, macrolides, and tetracyclines. However, the emergence of strains resistant to beta-lactams and other antibiotics is a cause of concern and could compromise currently used prophylactic and therapeutic antibiotic regimens (Carratala et al. 1995; Doern et al. 1996; Pfaller et al. 1997; Wisplinghoff et al. 1999). Viridans group streptococcal BSI in both immunocompetent and immunocompromised patients is usually considered to be of endogenous origin.

Streptococci of the *S. anginosus* group (i.e. *S. anginosus*, *S. constellatus*, and *S. intermedius*; previously known as the *S. milleri* group) have been associated with a variety of invasive pyogenic infections. Most episodes of BSI are associated with an identifiable focus (Shlaes et al. 1981a; Kambal 1987; Gossling 1988; Jacobs et al. 1995; Casariego et al. 1996; Salavert et al. 1996; Bancescu et al. 1997), or have been reported in patients with neutropenia or underlying malignancies (Cohen et al. 1983; Awada et al. 1992; Pfaller et al. 1997), but these organisms were also described in neonates and pediatric patients (Hamoudi et al. 1990; Weisman et al. 1990; Raymond et al. 1995). Other clinical features include pulmonary infections, septic arthritis, osteomyelitis, and brain abscesses (Houston et al. 1980; Shlaes et al. 1981a; Brook et al. 1988; Molina et al. 1991; Wong et al. 1995; Jerng et al. 1997).

BSI is relatively uncommon (Weinstein et al. 1983b) in *S. pyogenes* infections. During the 1990s, an increase in frequency and severity of invasive infections due to group A streptococci has been reported (Martin and Hoiby 1990; Demers et al. 1993; Hoge et al. 1993). Patients were predominantly otherwise healthy adults and there has been an association with IV drug abuse (Barg et al. 1985; Craven et al. 1986; Lentnek et al. 1990). Other risk factors include immunosuppression and underlying malignancies, diabetes mellitus, and vascular disease. The clinical course is often fulminant and mortality ranges from 27 to 38 percent with an association of M types 1 and 3 with a more severe clinical course (Francis and Warren 1988; Ispahani et al. 1988; Stevens et al. 1989; Dan et al. 1990; Bucher et al. 1992; Johnson et al. 1992; Demers et al. 1993; Hoge et al. 1993; Martin and Single 1993).

Clinical manifestations of *S. agalactiae* BSI include early and late onset infection in neonates and primary and secondary BSI in adults. BSI is the most common clinical feature of early onset disease (occurring within the first 6 days) which is associated with maternal obstetric complications and infants born at less than 37 weeks. Symptoms are mainly nonspecific and do not allow distinction between infants with and without accompanying meningitis (Baker 1976; Franciosi et al. 1973; Chin and Fitzhardinge 1985). Mortality ranges from 5 to 10 percent and is inversely correlated to birth weight (Yagupsky et al. 1991; Schuchat 1998; Hyde et al. 2002). Occult BSI and meningitis are the most common manifestations of late onset infection (between 7 days and 3 months of age) and mortality is low (Schuchat 1998; Schuchat et al. 1990; Yagupsky et al. 1991; Hyde et al. 2002). In adults, the incidence of group B streptococcal BSI is 0.2 per 1 000 hospital admissions (Opal et al. 1988). About 68 percent of cases are unrelated to pregnancy and there is a predominance of the elderly (Lerner et al. 1977; Gallagher and Watanakunakorn 1985; Verghese et al. 1986; Opal et al. 1988; Schwartz et al. 1991). Predisposing conditions include diabetes, liver disease, neurologic impairment, underlying malignancy, renal failure, cardiovascular disease, and heart failure (Lerner et al. 1977; Gallagher and Watanakunakorn 1985; Verghese et al. 1986; Opal et al. 1988; Schwartz et al. 1991; Farley et al. 1993). Primary BSI accounts for 20–40 percent of cases and is associated with fatal outcome in about 50 percent of cases (Lerner et al. 1977; Opal et al. 1988; Colford et al., 1995; Munoz et al. 1997).

Listeria species, short gram-positive rods, are widely distributed in the environment. *Listeria monocytogenes* is usually seen as a cause of community-acquired BSI and meningitis. Immunocompromised patients including elderly patients are particularly susceptible (Nieman and Lorber 1980; Schuchat et al. 1991). *L. monocytogenes* can cross the placenta, therefore in pregnant woman *L. monocytogenes* BSI can be complicated by placentitis or amnionitis leading to infection of the fetus, abortion or premature birth (Bille et al. 2003). Other clinical manifestations are rare and include cutaneous listeriosis, endocarditis, osteomyelitis, and intraabdominal abscesses (McLauchlin and Low 1994). Early diagnosis is based on maternal blood cultures growing *L. monocytogenes*. Immunoassays based on monoclonal antibodies are also available for rapid detection of *Listeria* species from the blood. Also, polymerase chain reaction (PCR) assays are highly sensitive and specific and can be particularly useful if the patient was receiving antimicrobial therapy prior to culture (Bille et al. 2003).

GRAM-NEGATIVE ORGANISMS

During the 1960s and 1970s, gram-negative organisms were the most frequent blood culture isolates. Today they are isolated from about 30 percent of BSI.

Escherichia coli and other Enterobacteriaceae

Enterobacteriaceae are important causes of both community-acquired and nosocomial infections including BSI. The Enterobacteriaceae combined account for about 17 percent of all BSI (Diekema et al. 1999; Edmond et al. 1999; Wisplinghoff et al. 2004), making them the second most common cause of BSI after CoNS. Besides their involvement in almost any kind of infection, Enterobacteriaceae are an important part of the normal intestinal flora. *E. coli*, the most commonly isolated pathogen in community-acquired BSI (Diekema et al. 2003), accounts for about 7–11 percent of nosocomial BSI to date (Pittet and Wenzel 1995; Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004). Community-acquired BSI due to *E. coli* is usually secondary to urinary tract infection and, less frequently, infection of the gastrointestinal tract (Eisenstein and Zaleznik 2000). *E. coli* strains causing BSI are phenotypically similar to those isolated from urinary tract infections (Johnson et al. 1997; Johnson et al. 2002). In patients with nosocomial BSI, primary sources of infection include IV catheters, the gastrointestinal, respiratory, and urinary tract. A portal of entry is usually absent in patients undergoing chemotherapy, where impairment of the intestinal mucosa may lead to bacterial translocation as a cause of BSI. Metastatic manifestations associated with BSI include septic arthritis, osteomyelitis, septic thrombophlebitis, abscesses in brain and liver, among others (Goldenberg et al. 1974; Saksouk and Salti 1977; Faraawi and Fong 1988; Farstad et al. 2003; Levy et al. 2003b).

Klebsiella species are a frequent cause of gram-negative nosocomial BSI, accounting for about 10 percent of community-acquired BSI and about 6 percent of nosocomial BSI (Diekema et al. 1999; Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004). Similar to *E. coli*, BSI usually originates from a distant site, most

commonly from the urinary tract, followed by the lower respiratory tract, bile duct, and wound infections (Eisenstein and Zaleznik 2000). In patients with nosocomial BSI, crude mortality rates of 25 percent have been reported (Diekema et al. 1999; Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004). A recent survey found no difference in mortality between ICU patients with BSI caused by *Klebsiella* spp. and matched controls after adjusting for severity of underlying disease and acute illness (Blot et al. 2002a).

Enterobacter and *Serratia* species are less frequent causes of nosocomial BSI, accounting for 3 and 4 percent of BSI in recent studies, respectively (Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004). Both are opportunistic pathogens and infections occur in patients with numerous predisposing conditions including immunosuppression, intravascular, intraperitoneal, or urinary catheters or following invasive procedures (Rudnick et al. 1996; Fok et al. 1998; Verghese et al. 1998; Eisenstein and Zaleznik 2000; Choi et al. 2002; Ostrowsky et al. 2002). *Serratia* species have also been associated with bacteremic infections in IV drug users, particularly endocarditis (see Infective endocarditis) (Wyler et al. 1975; Ashby 1976; Mills and Drew 1976). A recent study found that after adjustment for severity of underlying disease and acute illness and in the presence of fast and appropriate antibiotic therapy, *Enterobacter* BSI does not seem to adversely affect outcome in ICU patients (Blot et al. 2003b).

Non-typhoid *Salmonella* species usually cause intestinal infections and have been described as etiological agents of community-acquired BSI in both immunocompromised and previously healthy patients. Overall, BSI occurs in 1–4 percent of patients with gastroenteritis and can be caused by any *Salmonella* serotype. Frequencies are higher in patients with severe underlying conditions, immunosuppression, as well as in neonates and in elderly people (Han et al. 1967; Neves et al. 1971; Wolfe et al. 1971; Celum et al. 1987; Sperber and Schleupner 1987; Wheat et al. 1987; Levine et al. 1991). These patients also have a higher risk for severe invasive disease and extraintestinal manifestations including osteomyelitis, septic arthritis, meningitis, and endocarditis (Warren 1970; Wilson and Feldman 1981; Alvarez-Elcoro et al. 1984; Cohen et al. 1987; Stein et al. 1993; Huang 1996; Huang and Chuang 1997). In children, prolonged fever and infection with specific *Salmonella* serotypes is associated with BSI (Yang et al. 2002). Patients with BSI due to non-typhoid *Salmonella* species frequently have persistent bacteremia at follow-up regardless of the initial antibiotic treatment (Zaidi et al. 1999). *Salmonella typhi* is the etiologic agent of typhoid fever, a serious systemic infection, which is mostly seen in Third World countries. Patients usually suffer from continuous high fever and headache, without diarrhea. Typhoid fever has a generally more benign course in pediatric patients (Mermin et al. 1998). Typhoid fever is now rarely seen in the United States and Europe, and >70 percent of cases are travel-related (Mermin et al. 1998; Mead et al. 1999; Ackers et al. 2000).

Pseudomonas aeruginosa

P. aeruginosa is associated with a wide variety of infections, contributing significantly to morbidity and mortality of hospitalized patients. BSI is mainly nosocomial and usually affects patients with underlying clinical conditions such as malignancies, cardiac, or pulmonary diseases, renal failure or diabetes (El Amari et al. 2001). *P. aeruginosa* accounts for between 4 and 7 percent of nosocomial BSI, but for up to 31 percent of BSI in patients with underlying malignancies (Maschmeyer and Braveny 2000; Diekema et al. 2003; Wisplinghoff et al. 2004). Among gram-negatives, *P. aeruginosa* was the third most commonly isolated pathogen in recent surveys in North America (Weinstein et al. 1997; Diekema et al. 1999; Edmond et al. 1999; Wisplinghoff et al. 2004). The crude mortality of monomicrobial nosocomial *P. aeruginosa* BSI was between 28 and 48 percent in recent studies (Edmond et al. 1999; Diekema et al. 2003; Wisplinghoff et al. 2004). In patients with underlying malignancies crude mortality associated with *P. aeruginosa* BSI varied from 5 to 50 percent. *P. aeruginosa* BSI in IV drug users is relatively common and usually associated with endocarditis (see Infective endocarditis) (Komshian et al. 1990). Nosocomial pneumonia due to *P. aeruginosa* is often associated with BSI in neutropenic patients receiving chemotherapy as well as in adult or pediatric patients with AIDS (Iannini et al. 1974; Kielhofner et al. 1992; Flores et al. 1993). The prognosis of *P. aeruginosa* BSI secondary to nosocomial pneumonia is poor, and a fulminant progression to septic shock and death occurring within 3–4 days after the onset of pulmonary symptoms (Kang et al. 2003) is possible. Resistance of *P. aeruginosa* isolated from the blood is usually high, and therapy should be always based on susceptibility testing.

Acinetobacter baumannii

Acinetobacter species are a heterogenous group of organisms that have emerged as significant nosocomial pathogens mainly affecting patients with impaired host defenses in the intensive care unit setting. Members of the genus *Acinetobacter*, particularly *A. baumannii*, are implicated in a wide spectrum of infections, including nosocomial BSI, nosocomial pneumonia, secondary meningitis, skin and soft tissue infections, and urinary tract infections (Bergogne-Berezin and Towner 1996). The characteristics of *Acinetobacter* BSI have been described by researchers from various parts of the world (Tilley and Roberts 1994; Seifert et al. 1995; Cisneros et al. 1996; Ng et al. 1996; Siau et al. 1999; Wisplinghoff

et al. 2000). Since *Acinetobacter* species are ubiquitous organisms and most species represent contaminants rather than true pathogens, species identification of all BSI isolates should be attempted. While BSI rates as high as 8.4 percent have been reported for *Acinetobacter* species (Seifert et al. 1995), *A. baumannii* accounted for about 1–2 percent of BSI in recent studies (Edmond et al. 1999; Wisplinghoff et al. 2000, 2004), still being among the ten most prevalent causes of BSI. The crude mortality of *A. baumannii* BSI may be as high as 52 percent (Beck-Sague et al. 1990; Seifert et al. 1995; Cisneros et al. 1996; Cisneros and Rodriguez-Bano 2002), but was 34 percent in nosocomial BSI in unselected patient populations (Wisplinghoff et al. 2004). Others reported that *A. baumannii* BSI is not associated with a significantly increased mortality rate in critically ill patients (Blot et al. 2003a). Nosocomial BSI is often catheter-related or secondary to respiratory or urinary tract infections, as well as wound infections, particularly in burn patients. *Acinetobacter* species other than *A. baumannii* may also be associated with catheter-related BSI, but the clinical course in these cases tends to be less severe (Seifert et al. 1994).

Other gram-negative pathogens

Since the implementation of vaccination the number of invasive *Haemophilus influenzae* infections in the United States and Europe has decreased to below 100 cases per annum (Adams et al. 1993; CDC 1999). Systemic infection due to *H. influenzae* is usually community acquired. Virtually all invasive diseases – usually meningitis and BSI associated with acute epiglottitis – are caused by *H. influenzae* serotype b, which remains highly prevalent in unvaccinated children. Invasive disease due to other serotypes is rare, but has been reported for serotype f, mainly in immunocompromised patients (Gatti et al. 2004). All commercial blood culture media have a high recovery rate of *Haemophilus* species. For primary recovery, X- and V-factor enrichment of culture media is required.

Neisseria species

BSI due to *Neisseria* species is usually community acquired. Clinical manifestation of infections due to *N. meningitidis* may range from benign transient bacteremia to fulminant disease with high mortality (Apicella 2000). Transient, intermittent or persistent oro- or nasopharyngeal carriage of *N. meningitidis* has been reported in between 8 and 20 percent of healthy individuals (Ala'Aldeen et al. 2000). BSI due to *N. meningitidis* may be associated with meningitis, but transient bacteremia and severe sepsis have also been described without evidence of meningitis. Clinical presentation includes a maculopapular rash that becomes petechial and may be seen in 50 to 60 percent of patients. In severe sepsis, systemic coagulopathy may lead to ecchymotic, hemorrhagic, or necrotic areas. Purpura fulminans with extensive tissue damage is seen in 10 percent of patients. Septic shock can be the most prevalent symptom in patients with acute meningococcal BSI, but also in patients with meningitis (Apicella 2000; van Deuren et al. 2000). Mortality of meningococcal BSI associated with meningitis is usually between 7 and 10 percent, but can be as high as 30 percent (Dominguez et al. 2004). *N. meningitidis* has also been associated with chronic BSI, usually without meningitis (Apicella 2000). The clinical presentation includes fever, headache, leukocytosis, and arthritis.

Gonorrhea is still prevalent with highest incidences in sexually active teenagers between the ages of 15 and 24 (Sparling and Hansfield 2000). BSI due to *N. gonorrhoeae*, also referred to as disseminated gonococcal infection (DGI) occurs in 0.5–3 percent of cases (Hook and Hansfield 1999; Sparling and Hansfield 2000). Risk factors include female gender (especially during menstruation or the third trimester of pregnancy) and certain complement deficiencies (C7, C8, C9) (Ellison et al. 1987). Complications include gonococcal arthritis (in 30–40 percent of DGI) and rarely meningitis or endocarditis (Hook and Hansfield 1999; Sparling and Hansfield 2000).

FUNGI

Blood cultures are an important tool in the diagnosis of disseminated fungal infections (Weinstein et al. 1983b; Ellis 1991; Geha and Roberts 1994). All CMBCSs provide adequate detection of BSI due to yeasts (Mattia 1993; Prevost-Smith and Hutton 1994; McDonald et al. 2001). However, the lysis centrifugation system appears to be the most sensitive method for the detection of filamentous fungi as well as *Histoplasma capsulatum*, and other dimorphic fungi (Guerra-Romero et al. 1987; Graybill et al. 1990; Lyon and Woods 1995). When fungal BSI or disseminated fungal infection is suspected, maximum blood volumes should be collected multiple times and the use of special media should be considered.

Candida species, particularly *C. albicans*, are frequently isolated from the bloodstream, accounting for up to 10 percent of all nosocomial blood culture isolates. Data on the incidence of *Candida* BSI is controversial. A recent NNIS study (Trick et al. 2002) showed significant decreases in BSI due to *Candida* species and *C. albicans*, and reported a significant increase in BSI due to *C. glabrata* in ICUs between 1989 and 1999. Others, studying about 24 000 cases of nosocomial BSI from the United States, found a significant increase in the proportion of *Candida* species isolated from blood cultures between 1995 and 2002. In the same study, there was an increase in *C. albicans* and *C. parapsilosis* BSI, whereas

BSI caused by *C. tropicalis* and *C. glabrata* decreased (Wisplinghoff et al. 2004). Candida BSI is usually associated with long-term TPN and is most frequently encountered in patients with underlying conditions such as malignancies, neutropenia, or HIV/AIDS. However, blood cultures are frequently (50 percent) negative in patients with disseminated *Candida* infection (or *Candida* species may not be detected in blood cultures from patients that are also bacteremic) (Hockey et al. 1982). Therefore repeated cultures should be obtained if clinical suspicion of disseminated *Candida* infection is present. In patients with blood cultures positive for *Candida* a possible source of infection should be established, and intravascular catheters should be removed and reinserted at a different site (Rex et al. 1994; Rex 1996). Of note, candiduria may occur secondary to BSI and blood cultures should be obtained if candidemia cannot be ruled out clinically. Crude mortality in patients with nosocomial BSI due to *Candida* species is about 48 percent (Diekema et al. 2003; Wisplinghoff et al. 2004).

Although other fungi, particularly *Aspergillus* species, may cause disseminated infections, blood cultures often remain negative.

ANAEROBIC BACTERIA

Anaerobic bacteria account for about 4 percent of community-acquired and 2 percent of nosocomial BSI (Diekema et al. 2003). Gram-negative anaerobic rods are the most commonly encountered anaerobes in clinical specimens. *Bacteroides fragilis* is the predominant cause of anaerobic BSI (Felner and Dowell 1971; Wilson et al. 1972; Weinstein et al. 1983b; Finegold et al. 1985; Brook 1989; Lombardi and Engleberg 1992). Other gram-negative anaerobes commonly isolated from the blood include *B. thetaiotaomicron* and *Fusobacterium* species. BSI commonly originates from intraabdominal infections, infections of the female genial tract, soft tissue infections, the oropharynx, and the lower respiratory tract (Galpin et al. 1976; Finegold et al. 1985; Brook 1989). Anaerobic gram-negative bacteria isolated from blood cultures usually represent true pathogens (Lombardi and Engleberg 1992), and if isolated from the blood without a clinically apparent source, intraabdominal infection should be considered. Crude mortality rates as high as 60 percent have been reported (Chow et al. 1974) in BSI caused by *Bacteroides* spp. The attributable mortality in *B. fragilis* BSI has been reported as 19 percent (Redondo et al. 1995).

Clostridium species accounted for up to 4.5 percent of BSI in neutropenic patients (Mathur et al. 2002). Other studies reported that about 1 percent of all clinically significant blood culture isolates were identified as *Clostridium* species, most commonly *C. perfringens*, but less frequently also *C. septicum*, *C. difficile*, *C. novii*, and *C. tertium* (Brook 1989; Lark et al. 2001). Predisposing conditions include chronic alcoholism, abdominal surgery, bowel necrosis, septic abortions, malignancies, and decubital ulcers (Brook 1989; Gorbach et al. 1998). *C. perfringens* is isolated from a variety of infections, including BSI in various clinical settings and fulminant septic shock associated with post-abortion infections (Gorbach et al. 1998). In patients with clostridial myonecrosis, most commonly caused by *C. perfringens*, blood cultures are positive in 15 percent of patients (Summanen et al. 2002). However, *C. perfringens* BSI is not always associated with a serious underlying condition (Gorbach et al. 1998).

C. septicum is rarely isolated from anaerobic blood cultures and BSI is usually seen in patients with underlying malignancies, especially leukemia and lymphoma, or carcinoma of the large bowel. Underlying malignancies are present in 70–85 percent of patients with *C. septicum* BSI (Bodey et al. 1991; Kirchner 1991). Other associations include neutropenia and enterocolitis (King et al. 1984; Kudsk 1992). However *C. septicum* has also been isolated from BSI in patients with diabetes mellitus, severe arthritis or gas gangrene (Koransky et al. 1979).

BSI due to anaerobic gram-positive cocci, e.g. *Peptostreptococcus* species, is usually associated with obstetric or gynecological infections (Moll et al. 1996). The clinical significance of *Peptostreptococcus* species recovered from blood cultures is not clear and their isolation should be interpreted with caution.

MICROORGANISMS WITH SPECIAL REQUIREMENTS

Brucellosis

Isolation of *Brucella* species from blood, bone marrow, or tissue cultures in the only irrefutable proof of brucellosis (Young 1995; Solera et al. 1999). Organisms were historically cultured in biphasic bottles (Castaneda) containing a liquid and a solid agar phase. Today, commercial biphasic bottles, lysis centrifugation tubes, and CMBCSs, such as Bact/ALERT and BACTEC9000, are used to isolate *Brucella* species from the blood (Arnow et al. 1984; Solomon and Jackson 1992; Bannatyne et al. 1997; Ozturk et al. 2002). While lysis centrifugation is superior to the Castenada bottles, modern CMBCSs, such as the BACTEC9000, achieve detection times that are similar to those of the lysis centrifugation method (Yagupsky et al. 1997). Details on the performance of automated blood culture systems can be found in a recent review (Yagupsky 1999). Biosafety levels 2 and 3 are recommended for handling of specimens potentially containing *Brucella* species and processing of cultures of *Brucella* species, respectively. In addition to blood cultures, serological testing is mandatory.

Leptospirosis

Since leptospiremia only occurs during the acute phase of the illness (usually 4–7 days), cultures drawn beyond day 10 are likely to be of low yield. Dark-field or phase-contrast microscopy may be used to directly examine the blood. Of note, the detection level is 10^4 organisms/ml (Turner 1970). For culture, media with added rabbit serum or albumin and fatty acids are recommended with the addition of SPS. Tubes are incubated for 4–6 weeks in air at 28–29°C and examined weekly by removing a drop of culture from 1–3 cm below the surface of the medium (Dunne et al. 1997). In addition to blood cultures, serological testing is mandatory.

Bartonellosis

Bartonella henselae, the most common cause of cat scratch disease and *B. quintana*, the etiologic agent of trench fever, have both been associated with bacillary angiomatosis and bacteremia. Both species, as well as *B. elisabethae*, have been isolated from the blood, using conventional, lysis centrifugation or CMBC systems (Dunne et al. 1997). Nutrient-rich media, such as chocolate agar, should be used for subcultures, and prolonged incubation for at least 14 days is required (Anderson and Neuman 1997; Maurin and Raoult 1996).

Mycobacteria

While *M. tuberculosis* is rarely isolated from the bloodstream, *Mycobacterium avium* complex (MAC) is responsible for the majority of disseminated mycobacterial infections and can always be regarded as significant when isolated from the blood (Havlik et al. 1992). Rapidly growing mycobacteria can be cultured from the blood using all commercially available CMBCSs (Waite and Woods 1998; Archibald et al. 2000; Pintado et al. 2001). Survival times of MAC are longer if lysis centrifugation (ISOLATOR) tubes are used (Havlik et al. 1993). Today a variety of systems can be used to process mycobacterial blood cultures, including the ISOLATOR system, the radiometric BACTEC 460TB (BACTEC 13A; detection of $^{14}CO_2$), and the nonradiometric BACTEC 9000MB (BACTEC Myco/F lytic), ESP Culture System II (detection of pressure changes; ESP Myco, Trek Diagnostic Systems) or MB/BacT ALERT 3D (colorimetric CO_2 detection; bioMérieux). All systems use modified Middlebrook 7H9 broth or a similar broth supplemented with a variety of growth factors and antimicrobial agents (Pfyffer et al. 2003). The diagnostic yield of the CMBCSs is similar to that of the BACTEC 460TB and superior to conventional solid media (Pfyffer et al. 1997; Woods et al. 1997; Hanna et al. 1999; Whyte et al. 2000; Piersimoni et al. 2001). The average time to positivity is 5 to 14 days for *M. tuberculosis* and below 7 days for other mycobacteria, when using the BACTEC 460TB and similar detection times for the CMBCSs have been reported (Pfyffer et al. 2003). It is not recommended to transport blood before inoculation of blood culture media; however, if necessary SPS, heparin, EDTA or citrate may be used as anticoagulants (Pfyffer et al. 2003) depending on the culture medium used.

MANAGEMENT OF BLOODSTREAM INFECTION

Progression from bacteremia to sepsis, septic shock, and death can be rapid depending on underlying medical conditions of the host, virulence of the pathogen, and treatment parameters. In a recent study, mortality was double when inappropriate empirical antibiotics were administered to ICU patients with BSI (Ibrahim et al. 2000). Therefore, successful management must include early and appropriate antimicrobial therapy in addition to supportive care and the elimination of a removable focus, if present.

Resistance rates to antimicrobial agents have been rising for the last two decades in all predominant organisms, including *S. aureus* (Edmond et al. 1999; Smith et al. 1999; Trick et al. 2002), CoNS (Diekema et al. 2001), enterococci (Doern et al. 1998), and gram-negative pathogens (Diekema et al. 1999, 2002; Heinemann et al. 2000). In the face of emerging multiresistant organisms, antimicrobial prophylaxis and treatment has become increasingly difficult, and timely and accurate epidemiological information is needed for guiding appropriate empirical therapy. Analyses of *Candida* BSIs have shown trends to selection of non-*albicans* species, some of which are difficult to treat with first generation azoles (Trick et al. 2002). In this context, surveillance programs have become important in defining the species distribution and resistance patterns of pathogens causing BSI, thus providing the basis for appropriate empirical therapy.

INFECTIVE ENDOCARDITIS

INTRODUCTION

Infective endocarditis is a microbial infection of the endocardium, generally of a heart valve or valves, but sometimes of the cordae tendineae or at the site of a septal defect or other congenital abnormalities. With the advent of intracardiac valvular prostheses it was soon realized that these, too, or the tissues into which they are sewn, can also become infected. The term *infective endocarditis* covers the various clinical subcategories of the disease and has replaced the old term *bacterial endocarditis* since it became clear that chlamydiae, rickettsiae, fungi, and probably viruses can also be responsible for the disease. However, endocarditis

caused by these latter agents is not within the focus of this chapter.

Historically, infective endocarditis has been classified as *acute*, *subacute*, and *chronic* endocarditis; this classification was related to the usual course of the untreated disease. The *acute* form is a fulminating infection, characterized by high fever, severe malaise, leukocytosis, and rapid destruction of heart valves and is usually caused by a virulent organism, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. pyogenes*, or *Neisseria gonorrhoeae*. Without treatment, the disease was uniformly fatal resulting in the death of the patient in less than 6 weeks, but sometimes in days. *Subacute* endocarditis referred to infection with unspecific clinical findings and an indolent course with death from the disease occurring after 6 weeks to 3 months, and in the *chronic* form after 3 months and up to 2 years. The acute presentation of the disease usually affected patients without a previous cardiac abnormality, whereas the slowly progressing form of the disease was usually caused by relatively avirulent organisms such as the 'viridans' streptococci and occurred in patients with damaged heart valves.

Although this concept is still very useful mainly for educational purposes, it is less helpful nowadays for the individual patient. Currently, the diagnosis of infective endocarditis based on modern imaging techniques is reached earlier in the course of the disease. In addition, there is considerable overlap in clinical manifestation that is influenced by host factors, virulence determinants, and antimicrobial susceptibility of the offending pathogen, and the timely institution of appropriate antimicrobial therapy, as well as surgical intervention. A classification based on the nature of the causative pathogen may be more appropriate particularly with regard to the different treatment regimens recommended for specific pathogens and clinical prognosis. Mortality associated with infective endocarditis has considerably decreased in the past two decades. With modern diagnostic imaging techniques, the use of standardized antimicrobial therapy, and timely surgical valve replacement if necessary, cure rates approach 90–95 percent for streptococcal endocarditis, whereas infective endocarditis caused by enterococci and in particular *S. aureus* still carries a much higher mortality (Fowler et al. 1999; Cabell et al. 2002). Overall mortality of patients with infective endocarditis, however, has decreased to less than 20 percent in recent years (Cabell et al. 2002; Hoen et al. 2002; Wallace et al. 2002).

EPIDEMIOLOGY

Infective endocarditis is an uncommon disease. In a review of 10 large American series, a frequency of about one case per 1 000 hospital admissions with a range of 0.16–5.4 cases per 1 000 admissions was reported (Bayer and Scheld 2000). Several factors have led to dramatic changes in the epidemiologic features and clinical manifestations of infective endocarditis over the past few decades (Finland and Barnes 1970; Watanakunakorn and Burkert 1993). Among these are the increased life expectancy, a shift in the at-risk population with their predisposing valvular lesions illustrated by a decline of rheumatic carditis and an increase in degenerative valvular disease, an increase in nosocomial cases of infective endocarditis, refined diagnostic imaging techniques, and major advances in cardiothoracic valvular surgery. Most cases of infective endocarditis are acquired in the community, but there are now an increasing number of cases of hospital-acquired (nosocomial) endocarditis and this can affect both native and prosthetic valves (Lamas and Eykyn 1998; Gouello et al. 2000). The incidence of infective endocarditis is difficult to determine due to different patient populations surveyed and since the diagnostic criteria may vary in different series. In the United States and Western Europe, the incidence of infective endocarditis has been estimated to range between two and six cases per 100 000 person-years (van der Meer et al. 1992; Berlin et al. 1995; Hogevik et al. 1995; Hoen et al. 2002) with incidences differing between community-acquired (4.5 cases/100 000 patients-years) versus nosocomial (0.94 cases) endocarditis, as well as native-valve versus prosthetic-valve (0.94 cases) endocarditis (Berlin et al. 1995). The increased longevity has resulted in an increased median age of patients with infective endocarditis, a growing number of patients with cardiovascular disease and prosthetic heart valves, and an increased cumulative exposure to nosocomial bacteremia. Among the population with infective endocarditis associated with intravenous drug abuse, in contrast, there is a trend towards younger patients. In the United States, the incidence of infective endocarditis in this group of patients is estimated at 150–2 000 per 100 000 person-years (Frontera and Gradon 2000); it is much lower in most European countries.

There is a well recognized increased incidence of infective endocarditis with age, and over all the infection is more common in males than females (Hoen et al. 2002). Fifty years ago native valve infective endocarditis was mainly a disease of young people: in Cates and Christie's study (1951) of 442 cases of 'subacute bacterial endocarditis' seen between 1945 and 1948, 62 percent of patients were aged between 15 and 35 years. Since then native valve infective endocarditis has become a disease of the middle-aged and elderly (Gagliardi et al. 1998; Dhawan 2003; Gregoratos 2003).

In earlier series, 60–80 percent of patients with native-valve endocarditis had an identifiable predisposing cardiac lesion (Weinstein and Schlesinger 1974; Watanakunakorn 1977). In a recent population-based survey from France, however, 47 percent among 390 patients with infective endocarditis had no underlying valvular disease (Hoen et al. 2002). Almost any type of structural

heart disease that leads to turbulence of blood flow resulting in endocardial damage may predispose to infective endocarditis, including rheumatic heart disease, congenital heart disease, and arteriosclerotic cardiovascular disease. The proportion of cases of infective endocarditis related to rheumatic heart disease, the primary underlying condition in the past has now declined to less than 25 percent (Kaye 1985), however rheumatic heart disease is still the most common underlying cardiac condition in developing countries (Jalal et al. 1998). Congenital heart disease predisposing to infective endocarditis includes bicuspid aortic valve (Lamas and Eykyn 2000), ventricular septal defect, and tetralogy of Fallot. These conditions were seen previously in 6–24 percent of cases, but in the most recent series reported by Hoen et al. (2002) the frequency of underlying congenital heart disease was only 1 percent. The majority of mainly elderly patients with infective endocarditis and without a history of underlying valvular disease have arteriosclerotic cardiovascular disease with calcified nodular endocardial lesions or an intraventricular thrombus following myocardial infarction. Other conditions associated with infective endocarditis include idiopathic hypertophic subaortic stenosis (IHSS) (Morgan-Hughes and Motwani 2002) and mitral-valve prolapse (Baddour and Bisno 1986), in particular with the presence of mitral valve regurgitation. Hemodialysis shunts (Doulton et al. 2003), intracardiac pacemaker wires (Arber et al. 1994), and central venous catheters (Miele et al. 2001) may also predispose to endocarditis.

Nosocomial infective endocarditis is a rare complication of nosocomial bacteremia. Whilst many aspects of nosocomial infective endocarditis are similar to community-acquired infective endocarditis, both entities differ in their predisposing factors, microbial etiology and prognosis. The increasing use of intravascular access devices for hemodynamic monitoring, administration of fluids, and in particular those used for long-term hyperalimentation and dialysis, has brought a parallel increase in associated nosocomial bacteremia and has contributed largely to the increasing number of hospital-acquired cases of infective endocarditis (Gilleece and Fenelon 2000; Gouello et al. 2000). Other predisposing conditions include advanced age, surgical wounds, genitourinary instrumentation, major abdominal surgery, the implantation of prosthetic valves and cardiac devices, and major burn injury (Cartotto et al. 1998; Fernandez-Guerrero et al. 2002; Giamarellou et al. 2002). Nosocomial endocarditis can affect both previously normal and damaged heart valves, as well as prosthetic valves. *S. aureus* in native valve endocarditis and *S. epidermidis* in the presence of foreign bodies are the main implicated pathogens. Fungal endocarditis can also result from infected intravascular catheters with *Candida* spp. as the most usual pathogens. In their review of 21 cases of endocarditis associated with central venous catheterization, Tsao and Katz (1984) found that two-thirds of cases had right-sided infection, suggesting a parallel with the animal model in which catheter-induced traumatic sterile vegetations become infected after bacteremia. In fact, although catheter-related bacteremia may result in right-sided endocarditis, the infection is not invariably right-sided. In the series reported by Gouello et al. (2000), the overall mortality in patients with nosocomial endocarditis was 68 percent ($n = 15$) and mortality was directly associated with endocarditis in 36 percent of cases ($n = 8$).

Intravenous drug users (IVDU) represent a specific risk group for the acquisition of right-sided but also left-sided infective endocarditis usually without predisposing cardiac disease (Brown and Levine 2002; Moss and Munt 2003). Endocarditis was one of the earliest recognized medical complications of intravenous drug abuse and it is the most common cause of bacteremia in IVDUs. Overall, the predominant pathogen is *S. aureus* (including MRSA), but as with endocarditis not associated with IVDU, a wide spectrum of organisms has been reported. These include pyogenic streptococci, pseudomonads, other gram-negative bacilli and fungi, as well as polymicrobial infections. Most cases involve a previously normal tricuspid valve and result from repeated intravenous injection of foreign material and organisms. Left-sided endocarditis is uncommon in IVDUs unless they have previous heart disease, but appears to be increasing (Seghatol and Grinberg 2002). Tricuspid endocarditis presents with the signs of systemic infection and respiratory symptoms from septic pulmonary emboli and not with the 'classical signs' of endocarditis. Such symptoms (and the chest X-ray findings) may be misdiagnosed as pneumonia by clinicians unfamiliar with right-sided endocarditis. The disease is more frequent in HIV-positive or than in HIV-negative IVDUs, but the presentation and clinical manifestations are similar (Wilson et al. 2002). The causative organisms are usually acquired from the patient's own flora although the drug 'works' have been implicated in cases of *P. aeruginosa* infection. Most IVDUs with tricuspid endocarditis have a good prognosis, but the mortality rate is markedly increased in those with very large vegetations and left-sided involvement.

Prosthetic-valve endocarditis accounts for 10–25 percent of cases of infective endocarditis in developed countries (Hogevik et al. 1995; Fefer et al. 2002; Hoen et al. 2002). It has been estimated that between 3 and 6 percent of patients develop prosthetic-valve endocarditis within the first 5 years following surgery (Calderwood et al. 1985; Agnihotri et al. 1995).

PATHOGENESIS

The development of infective endocarditis usually follows a multifactorial course. Alterations of the valve surface and less frequently the epithelial lining of the heart chamber (mural endocarditis) represent the first

step in this series of events. These lesions, induced by high velocity turbulent blood flow across the valve results from a variety of local or systemic stresses, such as a small ventricular defect or a valvular stenosis. The location of valvular jet lesions is determined by the direction of the accelerated bloodstream, i.e. they are usually located on the low-pressure side of the cardiac valves, such as the atrial surface of an insufficient mitral or tricuspid valve and the ventricular surface of an incompetent aortic valve. Any endothelial lesion results in exposure of the underlying extracellular matrix (ECM) proteins, the production of tissue factor, the deposition of platelets and fibrin, and the formation of so-called *sterile vegetation*. These deposits are characteristic of nonbacterial thrombotic endocarditis (NBTE) (Blanchard et al. 1992), a condition also seen to be associated with chronic wasting diseases, particularly malignancy (marantic endocarditis), connective tissue diseases, and intracardiac catheters.

Infective endocarditis occurs when microorganisms are deposited on to the sterile vegetation during an episode of transient bacteremia. Transient bacteremia results from mechanical manipulation of mucosal surfaces colonized with bacteria, such as with dental extractions or other dental procedures (Coulter et al. 1990; Bayliss et al. 1983), endoscopy and/or biopsy of the gastrointestinal (Rigilano et al. 1984; Schlaeffer et al. 1996) or the urogenital tract (Zimhony et al. 1996), and surgical procedures leading to injury of these organs. Such a history, however, is only obtained in a minority of patients with infective endocarditis. Studies have also demonstrated spontaneous transient bacteremia in patients with poor dentition even in the absence of dental procedures whilst chewing or brushing their teeth (Bhanji et al. 2002). These procedures may also induce bacteremia in people with good oral hygiene. Bacteremia is low grade (<10 organisms per ml) and transient, i.e. the bloodstream is usually cleared from the invading bacteria within 15–30 min.

Infective endocarditis can also occur in the absence of a known or identifiable pre-existing valve lesion. This is particularly true for virulent, invasive pathogens such as *S. aureus*. This organism may bind directly to endothelial cells, a property that explains the ability of *S. aureus* to initiate endocarditis on 'normal' cardiac valves, in particular those of the elderly with some degree of degenerative valve sclerosis and IVDUs in whom valvular damage may be induced by repeated injection of impure materials (Clifford et al. 1994).

The adherence of a microorganism to platelets and fibrin is the next critical step in the development of endocarditis. Numerous studies have demonstrated a correlation between the ability of bacteria to adhere in vitro to platelet-fibrin matrices mimicking NTBE and their propensity to induce infective endocarditis in humans and in animal models (Scheld et al. 1978; Ramirez-Ronda 1978). Certain organisms such as viridans group streptococci, enterococci, and staphylococci – the predominant pathogens involved in infective endocarditis – adhere more avidly to normal canine endocardial surfaces in vitro than enteric bacilli, such as *Escherichia coli* and *Klebsiella pneumoniae* (Gould et al. 1975). *S. aureus* and *S. pneumoniae* appear to have a particular propensity to adhere to fibrin-platelet deposits and thus may produce infective endocarditis with a lower inoculum. Adherence is mediated by bacterial surface molecules interacting with host ECM molecules. These are termed microbial surface components recognizing adhesive matrix molecules (MSCRAMM). Streptococci and staphylococci carry multiple MSCRAMMs that mediate binding to NTBE and directly to endothelial cells.

The adherence of oral streptococci to NBTE lesions may be promoted by their ability to produce dextran or glucan, a complex extracellular surface polysaccharide, which in the case of *S. mutans* is also involved in the pathogenesis of dental caries (Gibbons and Nygaard 1968). Glucan production may thus be one of several virulence factors required for infection. Since not all species of streptococci that cause infective endocarditis are glucan producers (including most strains of *S. oralis*, one of the most common of the oral streptococci to cause infective endocarditis), other factors must also be involved in the pathogenesis of infective endocarditis caused by viridans streptococci and enterococci. One example is FimA, a surface adhesin located at the tips of the fimbriae that has been shown to mediate the attachment of *S. parasanguis* to fibrin-platelet matrices (Burnette-Curley et al. 1995). Inactivation of FimA greatly decreased the organisms' ability to induce endocarditis in rats. FimA-like proteins were found in a number of streptococcal species. Another factor involved in the pathogenesis of streptococcal endocarditis is the ability to bind to fibronectin which probably acts as the host receptor within the NBTE (Lowrance et al. 1990).

In contrast to less virulent organisms, such as the streptococci that usually implant only on cardiac sites with pre-existing endothelial lesions (Sullam et al. 1987; Kitada et al. 1997a; McCormick et al. 2000), *S. aureus* can either colonize damaged endothelium or invade physically intact endothelial cells (Yao et al. 1995). The major adhesins involved in the binding of *S. aureus* to NBTE lesions are its fibrinogen-binding proteins such as clumping factor and coagulase (Devitt et al. 1994; Moreillon et al. 1995). The expression of these factors is regulated by the global regulators: accessory gene regulator (*agr*), staphylococcal accessory regulator (*sar*) and probably other determinants (Cheung and Projan 1994). *S. aureus* may also bind directly to and invade endothelial cells, a property mediated by fibronectin-binding proteins FnBPA and FnBPA (Vercellotti et al. 1984; Kuypers and Proctor 1989; Flock et al. 1996; Menzies 2003).

Local bacterial survival and persistence is the next step involved in the development of infective endocarditis. Following bacterial adherence to and colonization of NBTE lesions, the organisms are rapidly covered with more fibrin and platelets which form a protective sheath that shelters the bacteria from host phagocytes and soluble host defenses, as well as from antimicrobials and permits further bacterial proliferation and vegetation growth. Local production of tissue factor (TF) by host monocytes and endothelial cells and aggregation of platelets are mainly involved in this process (Sullam et al. 1996). Additional factors produced by staphylococci, such as hemolysins, may disrupt the endothelial envelope of adjacent cells and allow further tissue invasion and enlargement of the infectious process (Moreillon et al. 2002). These conditions allow for unimpaired bacterial multiplication leading to extremely high colony counts of 10^9 to 10^{11} bacteria per gram of tissue. Bacteria residing deeply within the vegetation can adopt a reduced metabolic state that enables them to resist the attack of beta-lactam antibiotics which exert their action mainly on proliferating bacteria. These fresh vegetations are thus composed of bacterial masses, fibrin, platelet aggregates, rarely red blood cells, and inflammatory cells. The morphology of the vegetations can range from small, flat, granular lesions a few millimeters in size, to large, soft, pedunculated, friable masses several centimeters in size. They may or may not progress to further valvular, perivalvular, or extracardiac complications, resulting from embolic phenomena such as embolization into the coronary arteries or the systemic arterial tree leading to ischemia, infarcts, bleeding, and abscess formation. The same factors involved in both vegetation growth and tissue destruction are likely to be responsible for bacterial dissemination to remote sites where colonization of target tissue probably involves the same infection cycle starting with tissue adherence. During the course of healing, infiltration by polymorphonuclear leukocytes and fibroblasts results in fibrosis and sometimes calcification of the lesion which finally will become covered by endothelium.

Infective endocarditis, like other infectious diseases, is associated with circulating immune complexes that are found in high titers in virtually all patients (Bayer et al. 1976; Bayer and Theofilopoulos 1990). Some of the peripheral manifestations of infective endocarditis, such as Osler nodes and Roth spots as well as glomerulonephritis, may result from deposition of circulating immune complexes.

MICROBIOLOGY

A multitude of microorganisms has been implicated in infective endocarditis, and heart valves, in particular prosthetic valves, can become infected with just about any microbe or fungus. The majority of cases are caused by a fairly limited number of pathogens, i.e. streptococci and staphylococci. The most common etiologic agents are listed in Table 5.

Table 5 *Distribution of etiologic agents in patients with infective endocarditis*

	% of cases	
Microorganism	**Mean**	**Range**
Streptococci	50	35–53
Viridans streptococci	33	17–48
Other streptococci	17	5–33
Enterococci	8	6–10
Staphylococci	30	29–38
Staphylococcus aureus	23	22–31
Coagulase-negative staphylococci	7	6-8
Gram-negative aerobic bacilli	3	1–3
Fungi	1	0–1
Other bacteria	2	1–4
Polymicrobial infections	3	3–4
Culture-negative	8	6–12

Data are compiled from three population-based studies comprising 927 patients with infective endocarditis (van der Meer et al. 1991; Hogevik et al. 1995; Hoen et al. 2002).

Streptococci and *enterococci*

Nearly two-thirds of cases of community-acquired native valve infective endocarditis are caused by streptococci and enterococci: 214 of 326 (65.6 percent) in a Dutch series and 226 of 390 (58 percent) in a recent population-based survey in France (van der Meer et al. 1991; Hoen et al. 2002). However, streptococcal and enterococcal infective endocarditis cannot be considered as a homogeneous entity since both the pathogenesis of the infection and the disease itself differ according to the species responsible.

The largest group are the oral streptococci which are usually referred to as the 'viridans' group streptococci because most produce α-hemolysis (greening) when cultured on blood agar. While clearly predominating in the past, oral streptococci were responsible for less than 20 percent of the cases in more recent series and declined significantly from 7.8 cases per million person-years in 1991 to 5.1 cases per million person-years in 1999 (Hoen et al. 2002). For many years the oral streptococci were known as '*Streptococcus viridans*', but this is inaccurate because this term suggests a species designation. In fact, the oral streptococci constitute a heterogeneous group of different species within the genus *Streptococcus* which are usually nontypable by the Lancefield system. The taxonomy of the streptococci is confusing and has undergone major taxonomic changes. These taxonomic changes are well reviewed by Facklam (2002), but it seems that further reclassification of some species is inevitable. The oral streptococci include *S. gordonii*, *S. mitis*, *S. mutans*, *S. oralis*, *S. salivarius*,

S. sanguinis (previously *S. sanguis*), and the 'nutritionally variant streptococci' (now classified as *Abiotrophia* and *Granulicatella* spp.), as well as some others. *S. morbillorum* is now classified as *Gemella morbillorum*. In Parker and Ball's study (1976) of streptococci associated with systemic disease, the oral streptococci so defined accounted for about two-thirds of the 317 streptococci isolated from the blood in patients with infective endocarditis. Among the 225 streptococci reported in the French series (Hoen et al. 2002), 68 (30 percent) were identified as oral streptococci, while 98 (44 percent) were group D streptococci (including *S. bovis*, *S. gallolyticus*, and *S. infantarius*) and 22 (10 percent) belonged to the pyogenic streptococci. Many of the oral streptococci seldom cause infection other than infective endocarditis. Thus, the presence of these organisms in blood cultures, particularly in more than one set, should raise the suspicion of infective endocarditis even in the absence of any other clinical sign and symptom and prompt appropriate diagnostics such as transesophageal echocardiography (TEE) (see below). In fact, in such cases the microbiologist may often be able to provide the clinician with the necessary clue as to the etiology of the patient's illness and thus initiate an appropriate diagnostic approach and antimicrobial therapy.

The different species of oral streptococci differ in their habitat and in their propensity to cause infective endocarditis. A recent report of the identification of 47 strains of oral streptococci from cases of infective endocarditis found that 31.9 percent were *S. sanguis*, 29.8 percent *S. oralis*, and 12.7 percent *S. gordonii*. Other species were much less common (Douglas et al. 1993).

Cates and Christie (1951) in their study of 'subacute bacterial endocarditis' 40 years ago found that only 12.3 percent of their 187 cases of 'viridans' streptococcal endocarditis had had a tooth extraction less than 3 months before the onset of symptoms, whereas 34.8 percent had dental sepsis or caries. More than 30 years later, Bayliss et al. (1983) reported a similar incidence of 13.7 percent of cases occurring within 3 months of a dental procedure. Even if there is no overt dental focus it can still be assumed that the oral streptococci have originated in the mouth. Transient bacteremia with oral streptococci may also follow procedures such as tonsillectomy or adenoidectomy and (rarely) bronchoscopy or gastroscopy, but there is no evidence that these procedures result in infective endocarditis.

There are currently three recognized species that are traditionally referred to as the *S. milleri* group, *S. anginosus*, *S. constellatus*, and *S. intermedius*. Unlike the viridans group streptococci, organisms of the *S. milleri* group seldom cause native valve endocarditis. These organisms, in particular *S. anginosus*, are commonly isolated from septic lesions at a variety of sites, including abscesses of internal organs, such as the liver and brain, as well as from cholangitis, peritonitis, and empyema, either in pure culture or more commonly in polymicrobial infections with anaerobes (Casariego et al. 1996; Bert et al. 1998). However, *S. milleri* group infective endocarditis does not occur in association with such septic conditions; it seems to be an entirely separate entity (Kitada et al. 1997b; Marinella 1997; Lefort et al. 2002). A Dutch study (van der Meer et al. 1991) is the only published series with data on species distribution within this group. In a total of 326 cases of native valve infective endocarditis they reported 12 cases of *S. intermedius* and three of *S. constellatus* (interestingly no *S. anginosus*), giving an overall incidence for the *S. milleri* group of 4.6 percent. This is similar to the 2–5 percent incidence in the five reports quoted by Douglas et al. (1993), although they included prosthetic infections as well as native. Infective endocarditis caused by *S. anginosus* tends to be a more acute and severe infection associated with intracardiac complications that are more commonly seen with *S. aureus* (Levandowski 1985; Hurle et al. 1996).

Nutritionally variant streptococci (NVS), recently classified as *Granulicatella (Abiotrophia) adiacens* and *A. defectiva*, do not grow on subculture unless specific growth factors are added, therefore isolation and correct identification of these species is often difficult. These organisms accounted for 2 percent of cases of infective endocarditis in the French series (Hoen et al. 2002). The disease usually runs an indolent course and is associated with pre-existing valvular heart disease and illicit drug use (Leonard et al. 2001; Chang et al. 2002). However, complications such as systemic embolization frequently occur and are responsible for the relatively high mortality rate (Christensen et al. 1999).

S. bovis is a normal inhabitant of the gastrointestinal tract and is only seldom found in the mouth. In the past, this organism was often misidentified as *Enterococcus* (previously *Streptococcus*) *faecalis* since the two species share the Lancefield group D antigen and are biochemically quite similar. Other members of the group D streptococci with pathogenic significance include *S. gallolyticus* and *S. infantarius*, previously classified as different *S. bovis* biotypes. With the advent of commercial streptococcal identifiation kits such as the API 20 Strep and Rapid ID 32 Strep (bioMérieux), which are commonly used in many laboratories, correct identification of *S. bovis*, which is important since it involves a different therapeutic approach, has become much easier. *S. bovis* was as common as *S. sanguis* and *S. oralis* (*S. mitior*) as a cause of streptococcal infective endocarditis in Parker and Ball's (1976) series in which it was responsible for 15.8 percent of 317 cases and for nearly a quarter of the cases in patients over 55 years. Comparing the etiologic agents of infective endocarditis between two surveys performed in 1991 and 1999, Hoen et al. (2002) noted an increase of Group D streptococci from 5.3 in 1991 to 6.2 cases per million person-years in 1999. The clinical manifestations of *S. bovis* infective endocarditis are similar to those caused by 'viridans' streptococci (Ballet

et al. 1995; Duval et al. 2001; Pergola et al. 2001). In 1977, Klein et al. reported two patients with *S. bovis* endocarditis and adenocarcinoma of the colon. Since then others have noted an association between *S. bovis* bacteremia and not only colonic cancer but also polyposis and other gastrointestinal disorders (Leport et al. 1987; Waisberg et al. 2002). In their study of 90 cases of *S. bovis* bacteremia during the period 1951–1980, Honberg and Gutschik (1987) identified 15 patients (17 percent) with gastrointestinal cancer and concluded that there was a high frequency of neoplasms in patients with a history of *S. bovis* bacteremia, particularly in those with endocarditis, even several years after the episode of bacteremia. Leport et al. (1987), in a study of 77 cases of group D streptococcal endocarditis, found a significantly higher frequency of colonic polyps and neoplasms in patients with *S. bovis* endocarditis than in those with enterococcal endocarditis and they and others emphasized the need for colonoscopy in any patient with *S. bovis* bacteremia (Klein et al. 1977; Beeching et al. 1985).

In the pre-antibiotic era, *S. pneumoniae* accounted for about 10 percent of cases of infective endocarditis (Powderly et al. 1986), but it is now rarely seen. In a nationwide survey conducted in France between 1991 and 1998, 30 cases of native valve pneumococcal endocarditis were observed; the majority of patients (66.7 percent) had no predisposing cardiac condition (Lefort et al. 2000). The primary focus of infection in one-third of patients was pneumonia. Pneumococcal endocarditis causes an acute fulminating infection, generally of a previously normal valve, most often the aortic valve, and complications such as large arterial emboli, perivalvular abscess formation, and rapid valve destruction are frequent (Aronin et al. 1998; Lindberg et al. 1998; Lefort et al. 2000). The infection tends to occur in alcoholics and these patients usually have concomitant pneumonia and meningitis, a triad called Austrian's syndrome (Munoz et al. 1999). The overall mortality remains high at approximately 25–50 percent, valve replacement is frequently necessary, and penicillin resistance is a cause of concern (Lefort et al. 2000; Martinez et al. 2002).

Alpha-hemolytic pyogenic streptococci of Lancefield groups A, B, C, and G only occasionally cause endocarditis, usually attacking normal valves. The patterns of infective endocarditis caused by these organisms were recently reviewed by Lefort et al. (2002). The group B streptococci (*S. agalactiae*) are by far the most common and their incidence has recently increased (Gallagher and Watanakunakorn 1986; Scully et al. 1987; Baddour 1998; Hoen et al. 2002; Sambola et al. 2002). In the Spanish series (Sambola et al. 2002) *S. agalactiae* accounted for 2.6 percent of native valve endocarditis and a similar figure of 2.8 percent was found in a Dutch series (van der Meer et al. 1991). Although group B streptococci have a well recognized though unexplained predilection for diabetics, most cases of infective endocarditis do not occur in diabetic patients. Other risk factors for group B streptococcal bacteremia and infective endocarditis include carcinoma, alcoholism, hepatic failure, and intravenous drug use (Farley 2001). They are aggressive pathogens and, as in staphylococcal endocarditis, other sites of infection are often found; these include septic arthritis, vertebral osteomyelitis, meningitis, and endophthalmitis. The overall mortality has decreased in the Spanish series from 61 percent in 1975–1992 to 8 percent in 1993–1998 probably due to earlier cardiac surgery. Similar clinical manifestations are seen with group G streptococcal endocarditis with left-sided involvement, frequent complications, and considerable mortality (Venezio et al. 1986; Smyth et al. 1988; Liu et al. 1995). Group A streptococci (*S. pyogenes*) remain a very rare cause of infective endocarditis (Burkert and Watanakunakorn 1991; Liu et al. 1992; Mohan et al. 2000).

In a nationwide prospective survey in Sweden (Olaison and Schadewitz 2002), enterococci accounted for 11 percent of cases of infective endocarditis; in the Dutch series they accounted for 7.4 percent of 326 cases (van der Meer et al. 1991), and a nearly identical figure of 8 percent was reported in the most recent survey from France (Hoen et al. 2002). Enterococcal endocarditis is usually considered a community-acquired disease; however the incidence of nosocomial acquisition appears to be increasing (Fernandez-Guerrero et al. 2002). Enterococci are normal inhabitants of the gastrointestinal tract and occasionally the anterior urethra and they may cause urinary tract infection, particularly after instrumentation. Enterococcal bacteremia has been reported after colonoscopy, proctoscopy, sigmoidoscopy and barium enema, prostate biopsy, and abdominal tract surgery (Caballero-Granado et al. 2001). Most enterococcal bacteremias are nosocomial in origin, associated with invasive procedures, and often polymicrobial. Factors suggesting infective endocarditis in patients with enterococcal bacteremia include community acquisition, underlying valvular heart disease, the absence of another likely portal of entry, and monomicrobial bacteremia. Most cases of infective endocarditis are caused by *Enterococcus faecalis*, but there are occasional cases of other species such as *E. faecium*, *E. avium*, *E. durans*, and *E. gallinarum* (Dargere et al. 2002). Enterococcal endocarditis is most commonly seen in elderly men. Enterococcal bacteruria may be a helpful diagnostic clue. The disease usually runs a subacute course and classic peripheral manifestations are often absent. In cases of enterococcal infective endocarditis, both normal and previously damaged valves, as well as prosthetic valves can be involved (Rice et al. 1991). Enterococcal endocarditis is more difficult to treat than streptococcal endocarditis because enterococci are intrinsically more resistant to antibiotics than streptococci. However, with standard

treatment and the appropriate use of valve replacement, a cure rate of approximately 85 percent can be expected (Megran 1992).

Staphylococci

An ever increasing number of cases of infective endocarditis, both community-acquired and of nosocomial origin, are caused by staphylococci (Cabell et al. 2002). These organisms accounted for nearly 30 percent of infective endocarditis in the large population-based survey in France (Hoen et al. 2002) and their incidence increased from 4.9 cases per million person-years in 1991 to 5.7 cases per million person-years in 1999. Most of these staphylococci are *S. aureus*, but an increasing proportion are now coagulase-negative staphylococci (Hogevik et al. 1995; Cabell et al. 2002; Hoen et al. 2002).

Some 80–90 percent of the staphylococci isolated from cases of community-acquired native valve infective endocarditis are *S. aureus*. This organism can attack 'normal' heart valves (i.e. in patients without detectable cardiac disease) in one-third of the patients or damaged valves in patients with an underlying cardiac condition, producing severe fulminating infection that results in death in approximately 40 percent of patients (Petti and Fowler 2003; Hasbun et al. 2003). The infection arises from a bacteremia which itself stems from a trivial septic lesion such as wound infection, from an episode of catheter-related bacteremia occurring during a previous hospitalization, and often with no detectable focus (Mylotte et al. 1987; Eykyn 1988; Fowler et al., 1998; Mylotte and Tayara 2000a). Infective endocarditis caused by *S. aureus* has been traditionally regarded as a largely community-acquired disease. However, a recent study from Duke University revealed that among 59 definite cases of *S. aureus* infective endocarditis nearly half of the cases were hospital-acquired and were frequently caused by methicillin-resistant *S. aureus* (Fowler et al. 1999). The authors underscore the association of intravascular-device related *S. aureus* bacteremia and the development of infective endocarditis and recommend complete diagnostic evaluation including TEE (see below) for underlying infective endocarditis in any case of nosocomial bloodstream infection caused by *S. aureus*.

The disease often runs a rapidly progressive, *acute* course (Thompson 1982; Roder et al. 1999). Heart murmurs are seldom present initially unless there was a previous cardiac abnormality. The physician is thus confronted with a toxic ill patient with *S. aureus* recovered from a blood culture and sometimes without localizing signs. In about a quarter of cases the urine will contain both pus cells and staphylococci, the result of microabscesses in the kidney, and this can lead to further diagnostic confusion. Embolic phenomena involving the brain, spleen, or kidney, as well as the classic peripheral stigmata of infective endocarditis are more common in staphylococcal endocarditis than in endocarditis caused by other microorganisms. Intracardiac complications such as valve ring or myocardial abscess formation, and purulent pericarditis are frequently observed. Valvular destruction leading to congestive heart failure and death may occur within days (Watanakunakorn et al. 1973; Bayer 1982; Sanabria et al. 1990). This, the classical presentation of staphylococcal endocarditis, was accurately described over a century ago by William Osler in his Gulstonian lectures on 'malignant endocarditis' (1885), but it still seems largely unknown to many clinicians. Any patient who is unwell with a *S. aureus* bacteremia but no obvious site of infection should be assumed to have infective endocarditis and treated accordingly. Native valve infective endocarditis with *S. aureus* may occur at any age, but is more common in the middle-aged and elderly where mortality may exceed 50 percent (Julander 1985; Sanabria et al. 1990); it occurs in the previously healthy, as well as in those with underlying diseases such as diabetes mellitus and end-stage renal disease (Marr et al. 1998; Maraj et al. 2002; Doulton et al. 2003).

S. aureus is also the most common cause of infective endocarditis in intravenous drug users (IVDU) and is often associated with HIV infection (Mathew et al. 1995; Miro et al. 2003). However, since right-sided involvement mainly affecting the tricuspid valve predominates in these patients, the disease tends to be less severe than in non-addicts and usually responds to antimicrobial therapy alone with mortality rates below 10 percent.

Although still regarded by many as pathogens involved in prosthetic rather than in native valve endocarditis, coagulase-negative staphylococci also cause native valve infection and this has become more common, or certainly more commonly recognized, since the early 1980s (Caputo et al. 1987; Etienne and Eykyn 1990; Whitener et al. 1993; Miele et al. 2001). In the French series, coagulase-negative staphylococci accounted for 6.4 percent of cases of infective endocarditis (Hoen et al. 2002), and the Dutch series (van der Meer et al. 1992) reported a similar incidence (4.9 percent). In both studies it was not specified whether the infections were community- or hospital-acquired. Coagulase-negative staphylococci are normal inhabitants of the skin and mucous membranes and different species vary in their distribution throughout the body. Thus, it seems likely that native valve coagulase-negative staphylococcal infective endocarditis is an endogenous infection, even in cases of nosocomial acquisition associated with the use of intravascular catheters. In community-acquired cases a predisposing skin lesion is rarely detected and the oropharynx can be considered a likely portal of entry similar to the case with viridans streptococcal endocarditis. Approximately two-thirds of patients have pre-existing cardiac valvular disease

(Baddour et al. 1986). The clinical course is usually indolent (*subacute*) in onset and complications such as embolic disease and congestive heart failure are rare. Separating infective endocarditis from uncomplicated bacteremia due to coagulase-negative staphylococci or even from contamination may be difficult. Continuously positive blood cultures – in nosocomial cases in particular after intravascular catheters have been removed – may be an important clinical clue to the diagnosis.

The infecting species is most often *S. epidermidis* (Etienne and Eykyn 1990). However, in many reports the designation *S. epidermidis* tends to be used for any unspeciated coagulase-negative staphylococcus. With the advent of commercial identification systems, species identification of coagulase-negative staphylococci has become much easier. Thus, many other species have been reported to cause rare cases of native valve infective endocarditis, including *S. capitis*, *S. caprae*, *S. hominis*, *S. lugdunensis*, *S. saprophyticus*, *S. sciuri*, *S. schleiferi*, *S. simulans*, and *S. warneri* (Singh and Raad 1990; Leung et al. 1999; Sandoe et al. 1999; Hoen et al. 2002). Of these, *S. lugdunensis* appears to cause a more fulminate disease than that due to other coagulase-negative staphylococci (Vandenesch et al. 1993; Burgert et al. 1999; Farrag et al. 2001; Seenivasan and Yu 2003). These organisms are frequently misidentified as *S. aureus* owing to their morphological appearance when cultured on blood agar with yellow pigmentation and complete β-hemolysis.

Gram-negative bacilli

A small proportion of cases – 6.1 percent in the Dutch series (van der Meer et al. 1992), and 1.5 percent in the French series (Hoen et al. 2002) – of community-acquired native valve endocarditis are caused by gram-negative bacilli, the vast majority of these are fastidious, nonenteric organisms that tend to grow slowly on primary isolation from blood cultures or that cannot readily be isolated on conventional culture media. They include species that have a predilection for the heart valve and rarely cause infections other than infective endocarditis, as, for example, those known as the HACEK group (*Haemophilus aphrophilus*, *H. paraphrophilus*, *H. parainfluenzae*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*), whose isolation from blood cultures is virtually diagnostic of infective endocarditis (Das et al. 1997; Feder et al. 2003). The disease usually runs a subacute course and occurs in the setting of pre-existing valvular disease. Large friable vegetations are common, as are frequent emboli, and often the need for valve replacement. However, with early diagnosis and institution of appropriate antimicrobial therapy, prognosis is usually favorable. The slow growth of these organisms in blood cultures necessitates incubation of blood cultures from all patients with presumptive infective endocarditis for at least 3 weeks.

Other fastidious gram-negative rods that have been implicated in infective endocarditis include *Acinetobacter* spp. (Gradon et al. 1992), *Brucella* spp. (Jacobs et al. 1990), *Campylobacter fetus* (Farrugia et al. 1994), *Francisella tularensis* (Tancik and Dillaha 2000), *Haemophilus influenzae* (Geraci et al. 1977), and *Streptobacillus moniliformis* (Rupp 1992). Valve replacement is usually necessary for cure of *Brucella* endocarditis (Jacobs et al. 1990; Keles et al. 2001).

Gram-negative enteric bacilli, although frequently isolated from blood cultures, are only rarely a cause of infective endocarditis (Cohen et al. 1980). Drug addicts, patients with prosthetic heart valves, and patients with liver disease are at increased risk for the development of enterobacterial endocarditis. Sustained gram-negative bacillary bacteremia despite adequate antimicrobial therapy should herald possible infective endocarditis. A fulminate clinical course resulting in congestive heart failure is common and the prognosis usually poor (Carruthers 1977; Geraci and Wilson 1982). *Salmonella* spp., in particular *S. cholerasuis*, *S. typhimurium*, and *S. enteritidis* were most frequently encountered in earlier reports (Schneider et al. 1967), with valvular destruction, intracardiac complications, and rapidly progressive congestive heart failure, commonly observed. *Salmonella* endocarditis has been found in both native and prosthetic valve endocarditis (Goerre et al. 1998) and is also associated with HIV infection (Fernandez Guerrero et al. 1996). High overall mortality was also seen with infective endocarditis caused by *Serratia marcescens* (Mills and Drew 1976), as well as *Pseudomonas aeruguinosa* (Wieland et al. 1986; Komshian et al. 1990). Both organisms have been mainly recovered from patients with infective endocarditis who have abused intravenous drugs. *Pseudomonas aeruginosa* frequently affects normal valves, and complications include embolic phenomena, splenic abscesses, valve ring abscesses, and congestive heart failure. Due to the poor prognosis with medical therapy alone, early surgical intervention has been advocated if left-sided cardiac involvement is present (Wieland et al. 1986; Komshian et al. 1990).

Other bacteria

The nondiphtheriae corynebacteria are major components of the normal flora of human skin and mucous membranes. As such, they are frequently dismissed as contaminants if isolated from clinical specimens. However, these organisms are also uncommon, but increasingly recognized as agents of endocarditis in patients with underlying structural heart disease or prosthetic valves (Ross et al. 2001; Knox and Holmes 2002). Among the species reported are *Corynebacterium amycolatum*, *C. jekeium*, *C. striatum*, and *C. xerosis*.

However, methods that reliably differentiate related species are not available in most routine diagnostic laboratories. Other rare, gram-positive rods implicated in infective endocarditis include *Erysipelothrix rusiopathiae* (Venditti et al. 1990), *Lactobacillus* spp. (Husni et al. 1997), and *Listeria* spp. (Spyrou et al. 1997).

Anaerobes are found as normal inhabitants in the human mouth with or without dental caries and they represent the predominant flora of the bowel. However, these organisms have rarely been reported to cause endocarditis. According to Felner and Dowell (1970), non-streptococcal anaerobic bacteria accounted for 1.3 percent of cases of infective endocarditis and some other authors have reported a higher incidence, especially in IVDUs and polymicrobial infections. *Bacteroides fragilis* was the predominant pathogen in a review of 67 cases published by Nastro and Finegold (1973). Other anaerobes recovered from patients with infective endocarditis include other *Bacteroides* spp., *Clostridium* spp., *Fusobacterium* spp., and *Propionibacterium acnes* (Lortholary et al. 1995; Mohsen et al. 2001; Brook 2002).

Endocarditis due to rare and fastidious bacteria has recently been reviewed by Berbari et al. (1997) and by Brouqui and Raoult (2001). Some 350 cases of endocarditis caused by *Coxiella burnetii*, the etiologic agent of Q fever, have been described in the medical literature. Endocarditis is a recognized, though rare (and very late) sequel of acute *C. burnetii* infection. Most infections occur in middle-aged men usually (88.5 percent) with pre-existing heart disease (Marrie and Raoult 2002). The reservoir of the organism is sheep and cattle, but the source and mode of transmission of this anthropozoonosis to humans often remains unclear. Occupational exposure was reported in 12.5 percent of endocarditis patients. In contrast to most other cases of infective endocarditis, the diagnosis is usually made serologically although *C. burnetii* can be recovered on culture with special techniques (Houpikian and Raoult 2003). In view of this it is likely that *C. burnetii* endocarditis is underdiagnosed and some cases falsely labeled 'culture-negative' endocarditis.

Other organisms occasionally causing infective endocarditis that cannot be isolated in routine laboratories include *Bartonella* spp. (Spach et al. 1995; Raoult et al. 2003), *Chlamydia* spp. such as *C. psittaci*, the agent of psittacosis, and *C. pneumoniae* (Marrie et al. 1990; Brouqui and Raoult 2001), *Mycoplasma* spp. (Popat et al. 1980; Cohen et al. 1989), and *Tropheryma whipplei*, the agent of Whipple's disease (Fenollar et al. 2001; Richardson et al. 2003).

'CULTURE-NEGATIVE' ENDOCARDITIS

Most reported series of infective endocarditis include a variable number of cases that are designated *culture-negative*, that is they have negative blood cultures while other criteria of infective endocarditis such as echocardiographic demonstration of vegetations or histological evidence at surgery (see Clinical manifestations and complications) are met (Pesanti and Smith 1979; Tunkel and Kaye 1992; Hoen et al. 1995; Kupferwasser and Bayer 2000; Lamas and Eykyn 2003; Werner et al. 2003). The reported incidences varied ranging from 12 to 24 percent. Blood cultures remained negative in 9 percent of the cases recently reported by Hoen et al. (2002). Some earlier studies used poorly defined diagnostic criteria and possibly inadequate culture techniques. There are various explanations for the phenomenon of culture-negative endocarditis.

Previous administration of antibiotics will reduce the incidence of positive blood cultures, particularly in streptococcal infective endocarditis. The diagnosis of infective endocarditis is often initially missed in patients who do not present with classical 'textbook' signs, and these will be treated with antibiotics empirically before this diagnosis is being considered and blood cultures are taken. In the series reported by Werner et al. (2003), 45 percent of patients with culture-negative endocarditis had received prior antimicrobial therapy. The duration of previous antibiotic therapy is also an important factor. After only 2–3 days of antibiotic treatment, blood cultures that were initially negative after prolonged incubation often then become positive, but after longer courses they may remain persistently negative. In such cases, the antibiotics should be stopped and repeated blood cultures performed for a period up to 5 days.

Fastidious slow growing organisms are uncommon causes of infective endocarditis, but may take much longer to grow in blood cultures than the more usual 'viridans' streptococci or staphylococci. Examples include the HACEK group, the nutritionally variant streptococci such as *Granulicatella (Abiotrophia) adiacens* and *A. defectiva*, *Legionella* spp. (Chen et al. 1996) as well as mycobacteria (Cope et al. 1990) and some anaerobes. In such cases infective endocarditis only initially appears *culture-negative*, but cultures eventually become positive if they are held for the recommended 3 weeks and/or are subcultured on to appropriate media, respectively.

Organisms requiring special isolation techniques or serological diagnosis include *Bartonella* spp., *Chlamydia* spp., *C. burnetii*, *Mycoplasma* spp., and *T. whipplei* (Raoult et al. 1996; Brouqui and Raoult 2001; Fenollar et al. 2003; Houpikian and Raoult 2003). Although specialized culture techniques are available for some of these obligate intracellular pathogens in certain reference laboratories, most laboratories will still rely on serological diagnosis. Blood cultures also remain negative in over 50 percent of cases of fungal endocarditis such as that caused by *Aspergillus* spp. (Rubinstein et al. 1975).

An incorrect diagnosis of infective endocarditis based on a false interpretation of clinical or echocardiographic

findings may also be the reason for an apparently *culture-negative* endocarditis. This is probably quite common since physicians are rightly concerned not to miss a case of infective endocarditis.

In conclusion, in all cases of defined or suspected endocarditis with negative blood cultures in the absence of previous antimicrobial therapy serum should be analyzed for *Bartonella*, *Coxiella*, and *Chlamydia* species antibodies, and the excized valve should be analyzed by microscopy, culture, and histology. In addition, specialized culture techniques and relevant PCR techniques might be attempted at a reference laboratory (see below).

Clinical manifestations and complications

The clinical presentation of infective endocarditis is determined by the local infection of the valve and its complications, the occurrence of septic embolization of valvular vegetations to distant organs, constant bacteremia that may result in distant foci of infection, and the consequences of circulating immune complexes. The symptoms and signs of infective endocarditis are therefore extremely variable (Table 6), and the differential diagnosis is broad.

Fever is the most common sign but may be absent in severely compromised patients, patients with chronic renal or hepatic failure, infective endocarditis with less virulent organisms and in particular with previous antimicrobial therapy. Fever usually abates within 3–7 days of appropriate antimicrobial treatment, but prolonged fever may be associated with major intracardiac or embolic complications, such as myocardial or splenic abscess, and should therefore prompt further diagnostic evaluation. Nonspecific findings seen especially in subacute cases of infective endocarditis include weight loss, weakness, anorexia, malaise, nausea, and night sweats.

Most patients with infective endocarditis have a heart murmur, that may rarely present as the classic *changing murmur* or *new regurgitant murmur*, but in most cases is a pre-existing murmur. Other classical peripheral findings include splinter hemorrhages (Figure 2), i.e. red to brown streaks below the finger nails or toe nails, petechiae on the skin, the conjunctivae, and oral mucosa that both result from local vasculitis or emboli, and the only rarely seen Osler nodes, small, painful subcutaneous nodular lesions found in the pulp of the digits or in the thenar eminence. Janeway lesions are nontender, hemorrhagic or pustular lesions, often found on the palms and soles, also resulting from peripheral septic emboli (Figure 3). Janeway lesions consist of bacteria, neutrophils, necrosis, and subcutaneous hemorrhage and are

Table 6 *Signs and symptoms of infective endocarditis*

Sign/symptom	% of patients affected
Fever	80
Malaise	25
Chills	40
Weakness	40
Sweats	25
Anorexia/weight loss	25
Myalgia/arthralgia	15
Heart murmur	85
Changing or new murmur	3–10
Skin manifestations	15–30
Janeway lesions	<10
Osler nodes	1–23
Petechiae	20–40
Splinter hemorrhages	15
Roth spots	2–10
Splenomegaly	20–57
Embolic phenomena	>50

Figure 2 *Splinter hemorrhages are usually located under the finger or toe nails.*

Figure 3 *Janeway lesions are nontender, erythematous, hemorrhagic, or pustular lesions, often located on the palm or soles.*

often seen in staphylococcal endocarditis. The offending microorganisms may be readily cultured from these lesions. Roth spots are oval retinal lesions surrounded by hemorrhage occurring only in a minority of cases.

The complications of infective endocarditis have been reviewed recently by Mansur et al. (1992). The leading cardiac complication is congestive heart failure caused by a progressive infection-induced valvular destruction such as perforation of the valve leaflet, the cordae tendineae, or papillary muscle. Severe congestive heart failure was observed in 34 percent of patients with infective endocarditis in the French series (Hoen et al. 2002). Aortic-valve involvement is more frequently associated with congestive heart failure than is mitral-valve involvement in infective endocarditis. Other complications include rupture of the intraventricular septum, myocarditis or myocardial infarction resulting from coronary artery embolization or mycotic aneurysm, and myocardial and valve ring abscesses. Extension of the infection into the septum may cause arrhythmias and conduction abnormalities. Other distant complications mainly result from embolic events and metastatic infections (Millaire et al. 1997). Overall, vascular complications were seen in 44 percent of patients with infective endocarditis reported by Hoen et al. (2002), the vast majority of which were systemic arterial emboli.

Neurologic manifestations usually resulting from cerebral emboli occur in 20–40 percent of cases and often represent the dominating clinical syndrome, especially in staphylococcal endocarditis (Roder et al. 1997; Heiro et al. 2000). A stroke syndrome in a febrile patient with underlying valvular disease suggests the possibility of infective endocarditis. Cerebral manifestations include cerebral infarction, abscesses, intracerebral mycotic aneurysms, cerebritis, meningitis, and intracerebral or subarachnoid hemorrhage. Resulting symptoms encompass hemiplegia, aphasia, sensory loss, headache, seizures, and altered mental status, ranging from slight personality changes to frank psychosis.

Splenomegaly is common among patients with infective endocarditis and mostly seen in patients with prolonged disease. Splenic abscess may develop from bacteremic seeding of a previously infarcted area or directly from an infected embolus. Splenic abscess was among the rarer complications reported by Mansur et al. (1992) with clinical signs or symptoms often absent; this complication was observed in 10 percent of patients with infective endocarditis in the French series (Hoen et al. 2002). However, splenic septic emboli and infarction were reported in 44 percent of patients at autopsy (Weinstein and Schlesinger 1974). Prolonged fever and sepsis may be a clinical clue to this complication and should prompt ultrasound investigation, abdominal CT scan or MRI (Yilmaz et al. 2003).

Renal involvement includes infarction and abscess formation leading to bacteruria, as well as immune complex glomerulonephritis. Renal failure is mainly seen in long-standing disease, but is usually reversible with antimicrobial treatment (Conlon et al. 1998).

Musculoskeletal symptoms in infective endocarditis include diffuse myalgias, low back pain, arthalgia, and septic arthritis.

Mycotic aneurysms may result from direct bacterial invasion of the arterial wall with subsequent abscess formation and rupture, from embolic occlusion of the vasa vasorum, or immune complex deposition into the arterial wall. They are found most commonly at bifurcation points primarily of the cerebral vessels and are often clinically asymptomatic until perforation or rupture occurs. Major vessel emboli are rare in bacterial endocarditis and are more frequently encountered in fungal endocarditis (Pierrotti and Baddour 2002).

Right-sided endocarditis is not associated with peripheral emboli and other peripheral vascular phenomena. Instead, pulmonary findings are usually present, such as pulmonary embolism with and without infarction, pneumonia, pleuritic chest pain, pleural effusion, and empyema (Miro et al. 2003; Moss and Munt 2003). Community-acquired *S. aureus* bloodstream infection in the presence of multiple pulmonary infiltrates should always suggest right-sided infective endocarditis, and in the at-risk person intravenous drug abuse as well. Heart murmurs are frequently absent in right-sided endocarditis.

Diagnosis

DEFINITION OF DIAGNOSTIC CRITERIA

The diagnosis of infective endocarditis requires consideration of clinical, laboratory, and echocardiographic findings. To help the clinician to establish the diagnosis of infective endocarditis and to allow for comparison of published reports, attempts have been made to define hard diagnostic criteria. Von Reyn et al. (1981) introduced the categories of *definite*, *probable*, and *possible* IE but their 'definite' category relied on direct evidence from histology or microbiology at surgery or autopsy. The so-called Beth Israel criteria proved very specific, but less sensitive. Their *definite* category was too restrictive. Over a decade later with the advent and more common usage of echocardiography, Durack et al. (1994) proposed new criteria of *definite* and *possible* infective endocarditis based on a much more comprehensive assessment that included clinical and echocardiographic findings, and these have gained widespread acceptance. The so-called Duke criteria and their suggested modifications (Fournier et al. 1996; Lamas and Eykyn 1997; Li et al. 2000) are built on two major criteria, i.e. microbiologic criteria and clinical or echocardiographic evidence of endocardial involvement.

The microbiologic criteria include a positive blood culture for infective endocarditis as defined by the recovery of (1) a typical microorganism from two sepa-

rate blood cultures in the absence of a primary focus (i.e. 'viridans' streptococci, *S. bovis*, HACEK group, or community-acquired *S. aureus* or enterococci); (2) a persistently positive blood culture defined as the recovery of a microorganism consistent with endocarditis from either blood cultures drawn more than 12 h apart or all three or a majority of four or more separate blood cultures with the first and last drawn at least 1 h apart; or (3) serology for Q fever by IFA showing phase 1 IgG antibodies at >1:800.

Evidence of endocardial involvement includes a positive echocardiogram for infective endocarditis, i.e. (1) an oscillating intracardiac vegetation on valve or supporting structures, in the path of regurgitant jets, or on implanted material; (2) a periannular abscess; or (3) a new dehiscence of a prosthetic valve; or new valvular regurgitation on auscultation (increase or change in preexisting murmur is not sufficient).

Minor diagnostic criteria include (1) a predisposing cardiac condition – such as previous endocarditis, aortic-valve disease, rheumatic heart disease, prosthetic heart valve, complex congenital heart disease, and many others – or injection-drug use; (2) fever $\geqslant 38^{\circ}C$; (3) vascular phenomena such as major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial hemorrhage, conjunctival hemorrhages, or Janeway lesions; (4) immunologic phenomena such as glomerulonephritis, Osler's nodes, Roth spots, or rheumatoid factor; and (5) microbiologic evidence such as a positive blood culture not meeting the major criteria or serologic evidence of an active infection with organism consistent with infective endocarditis, such as *Brucella* spp., *Chlamydia* spp., *Legionella* spp., and *Bartonella* spp.; and (6) echocardiographic findings consistent with infective endocarditis, but not meeting major criteria.

According to the Duke Endocarditis Service, the diagnosis of infective endocarditis is definite (1) when a microorganism is demonstrated by culture or histologic testing in a vegetation; (2) when active endocarditis is histologically confirmed at surgery or at autopsy; or (3) in the presence of two major criteria, one major and three minor criteria, or five minor criteria as outlined above (Durack et al. 1994).

The usefulness of the Duke criteria has been validated and compared to the von Reyn criteria in several subsequent studies (Bayer 1996; Martos-Perez et al. 1996; Heiro et al. 1998; Habib et al. 1999; Perez-Vazquez et al. 2000). Overall, the specificity of the proposed criteria was high (99 percent) and the negative predictive value greater than 92 percent.

MEDICAL HISTORY

The patient's medical history might be an important clue for the etiologic diagnosis of infective endocarditis. A history of contact with cattle or sheep may suggest infection caused by *C. burnetii* (Q fever) or *Brucella* species. Contact with a cat, in particular a history of scratches or bites, suggests *Bartonella henselae* or *Pasteurella* spp. Exposure to birds has been related to infection with *C. psittaci*. Further clues as to patients' medical history related to endocarditis can be found in the review by Brouqui and Raoult (2001).

LABORATORY FINDINGS

Unspecific laboratory findings are usually present in infective endocarditis, but none is diagnostic. Hematological parameters include anemia in 70–90 percent of cases and less often leukocytosis which is present in only 20 to 30 percent of cases of subacute endocarditis, but is more frequent in the acute form. The erythrocyte sedimentation rate (ESR) is elevated in 90–100 percent of cases, so that a normal ESR usually rules out the diagnosis of infective endocarditis. Similarly, the C-reactive protein (CRP) concentration is virtually always elevated. Serial determination of both parameters, ESR and CRP, are useful for monitoring antimicrobial therapy and may also be a diagnostic clue for the detection of clinically silent embolic complications.

ECHOCARDIOGRAPHY

Cardiac imaging, specifically echocardiography, has greatly enhanced the ability of clinicians to effectively diagnose and manage infective endocarditis. Echocardiograms should generally be obtained in all patients suspected of having infective endocarditis, both to establish the diagnosis and to identify complicated cardiac involvement that may warrant surgical intervention (Bayer et al. 1998). Manifestations of endocardial involvement include vegetations, abscesses, aneurysms, fistulae, leaflet perforations, and valvular dehiscence. Transthoracic echocardiography (TTE) is a noninvasive and rapid method with excellent specificity. The sensitivity however, drops to less than 60 percent in patients with obesity, chronic obstructive lung disease, and chest wall deformities. It has therefore been recommended to perform transesophageal echocardiography if TTE is inconclusive. Transesophageal echocardiography (TEE) is more invasive and also more expensive than TTE, but also more sensitive than the transthoracic approach (Shively et al. 1991; Chamis et al. 1999; Roe et al. 2000; Humpl et al. 2003) and currently represents the optimal approach to echocardiographic imaging. Initial use of TEE is more cost-effective and diagnostically efficient in all patients with an intermediate or high pretest probability of infective endocarditis, particularly in the setting of prosthetic valves (Heidenreich et al. 1999). In a prospective study of patients with *S. aureus* bacteremia, Fowler and colleagues (1997) reported that sensitivity of TTE for detecting infective endocarditis was 32 percent, and the specificity was 100 percent. The addition of TEE increased the sensitivity to 100 percent,

but resulted in one false-positive result (specificity 99 percent). Echocardiographic imaging has also been used to guide therapeutic management of patients with infective endocarditis (Harris et al. 2003; Kim et al. 2003). TEE is especially useful for detecting perivalvular extension of infective endocarditis and the presence of an intramyocardial abscess, as well as for evaluation of prosthetic-valve endocarditis.

CULTURE METHODS

The blood culture is the single most important method applied in the diagnostic work-up for infective endocarditis. The bacteremia in patients with culture-positive endocarditis is usually continuous and low-grade. The application of quantitative blood culture techniques has demonstrated that between one and 10 bacteria per ml are present in the blood of patients with infective endocarditis and this bacterial density remains constant during the entire course of the untreated disease (Werner et al. 1967). The details of taking blood cultures have been presented elsewhere in this chapter. It has been recommended that at least 10 ml of blood be obtained for each culture and that at least three blood cultures sets (no more than two bottles per venipuncture) are obtained in a patient with suspected endocarditis (Washington 1982, 1987). If clinical conditions permit, blood cultures should be drawn at least 1 h apart. In cases where the institution of antimicrobial therapy can be postponed for 24 h, another three blood cultures should be obtained the following day. More blood cultures may be necessary if the patient has received antimicrobials in the preceding 2 weeks. Blood cultures should be incubated for at least 3 weeks. An evidence-based approach for sequential testing in infective endocarditis has recently been proposed by Watkin et al. (2003).

If no previous antimicrobial therapy was administered and blood cultures remain negative in a patient with probable or definite endocarditis on clinical or echocardiographic grounds, i.e. culture-negative endocarditis by definition, fastidious organism should be suspected, and special cultivation methods be considered. The simplest approach to isolate *Abiotrophia* spp. is to inoculate the blood culture broth on to blood agar that is cross-streaked with *S. aureus* where these species grow as satellite colonies. Most members of the HACEK group and other fastidious organisms can be recovered after prolonged incubation of blood cultures for 3 to 4 weeks both with conventional, as well as with automated blood culture systems. Prolonged incubation is therefore recommended as a routine procedure in the diagnostic work-up of blood cultures from patients with suspected endocarditis. The isolation of rare pathogens such as *Legionella* spp., mycobacteria, and *Bartonella* spp. requires special techniques recently reviewed by Brouqui and Raoult (2001). These authors also have developed tissue cell cultures for the isolation of obligate intracellular pathogens such as *Bartonella* spp., *Chlamydia* spp., and *C. burnetii*.

Pathogens can also be cultured from resected valves or biopsy specimens by inoculation on to agar or into tissue culture. Histologic examination of resected valve material is mandatory, particularly in the case of culture-negative endocarditis. A discussion of the appropriate staining techniques and immunohistological methods is beyond the scope of this chapter.

SEROLOGY

The most common serologic methods applied to the microbiological diagnosis of infective endocarditis include indirect immunofluorescence for *B. henselae*, the tube agglutination test for *Brucella melitensis* infections, ELISA or indirect immunofluorescence for *Chlamydia* spp., indirect immunofluorescence for *C. burnetii* and *Legionella* spp., and ELISA for *Mycoplasma pneumoniae*. Systematic serologic testing for these organisms is particularly warranted in cases of clinically suspected culture-negative infective endocarditis (Houpikian and Raoult 2003). Among 19 cases of culture-negative endocarditis in the French series, eight microorganisms were identified by serological methods: *Bartonella* spp. ($n = 3$), *C. burnetii* ($n = 1$), *Legionella* spp. ($n = 1$), *Chlamydia* spp. ($n = 2$), and *M. pneumoniae* ($n = 1$).

MOLECULAR METHODS

The advent of molecular amplification methods has enlarged the spectrum of nonculture laboratory methods for the diagnosis of infective endocarditis (Fournier and Raoult 1999; Lisby et al. 2002). Such techniques have been applied to most types of clinical materials including blood and valvular specimens obtained at surgery (Grijalva et al. 2003) or autopsy. Broad-range PCR that incorporates broad-spectrum primers targeting bacterial 16S rRNA allows for the detection of almost all bacteria in a single reaction. This method combined with sequence analysis of the amplified DNA has proved to be a promising tool for patients with culture-negative endocarditis (including instances of prior antimicrobial treatment) and allows the detection of rare, noncultivable organisms (Bosshard et al. 2003). Such novel approaches may lead to more comprehensive patient evaluations and the discovery of new etiologic agents of infective endocarditis. It has therefore been suggested to include positive identification of an organism by molecular biology methods as an additional major criterion in the revised Duke criteria of infective endocarditis (Millar et al. 2001; Bosshard et al. 2003).

In contrast to its use directly on clinical samples, PCR with subsequent sequencing of the amplification product can also be used for identification of bacteria isolated by culture methods in cases when species identification proves difficult by conventional biochemical methods. Such techniques clearly have increased the identification

of rare and fastidious microorganisms recovered from patients with infective endocarditis in recent years.

PROSTHETIC VALVE ENDOCARDITIS

Prosthetic valve endocarditis (PVE) is an uncommon but potentially disastrous complication of cardiac valve replacement. It accounts for 7 to 25 percent of cases of infective endocarditis in most developed countries (Vlessis et al. 1997; Mylonakis and Calderwood 2001; Hoen et al. 2002). The risk of an individual patient developing PVE at any particular time is difficult to determine from reported studies of PVE. As Rutledge et al. (1985) pointed out, to cite a frequency of PVE of 4 percent as many studies do is not very valuable, since the risk of developing PVE is not uniform over time; it is higher during the initial 6 to 12 months after surgery and subsequently decreases. It has been estimated that the cumulative risk developing PVE ranges between 3 and 6 percent by 5 years after surgery (Ivert et al. 1984; Calderwood et al. 1985; Rutledge et al. 1985; Agnihotri et al. 1995). The rate of PVE was higher for recipients of a mechanical valve during the initial 3 months after surgery compared to patients with a bioprosthetic valve, but after 12 months the risk of infection was greater for the latter group (Calderwood et al. 1985). It appears that patients with a prosthetic heart valve carry a life-long risk of acquiring PVE.

PVE has conventionally been considered as *early* and *late*, with 60 days regarded as the time limit for early cases (Dismukes et al. 1973) because of differences between the microbiology and pathogenesis of infection in the two time periods. Early PVE results from perioperative contamination, as well as from bacteremia arising during the early postoperative period, and is thus usually a hospital-acquired infection (Gordon et al. 2000). Early after valve replacement the intracardiac structures involved in the operation are not endothelialized and therefore especially vulnerable to the deposition of fibrin platelet aggregates and subsequent bacterial colonization. Not all these 'early' infections present within 60 days of operation and this has led some investigators to suggest that the time limit for early disease should be extended to 6 months or even a year. PVE occurring later than 12 months after valve replacement is more likely to result from transient community-acquired bacteremia, such as is the case with community-acquired native valve endocarditis. Whatever classification is used, it is clear that the causative microbes of PVE acquired in hospital and the severity of disease differ from PVE acquired in the community, often many years after valve replacement.

The incidence of early PVE has fallen since the advent of valve replacement surgery over 40 years ago, but the most common infecting organisms are still likely to be staphylococci, both coagulase-negative staphylococci (mostly *S. epidermidis*) and *S. aureus*, followed by gram-negative bacilli, enterococci, corynebacteria, and fungi (Wolff et al. 1995). Coagulase-negative staphylococci account for approximately 30 percent of cases. These are skin organisms and may arise both from the patient's own commensal flora, as well as from health care personnel. Interestingly, the majority of these strains are methicillin-resistant (Calderwood et al. 1985).

The pathogenesis of late PVE differs from that of early PVE and is similar to that of native valve endocarditis with bacteria from the bloodstream localizing on the prosthesis or damaged endocardium. As with community-acquired native valve endocarditis, the most common pathogens in late PVE are streptococci, enterococci, and staphylococci, but a larger percentage of cases than in native valve disease are caused by coagulase-negative staphylococci.

Typical features of PVE are large vegetations, embolic complications, and invasive infection extending into the annulus and adjacent myocardium resulting in paravalvular abscess formation and valve dehiscence (Graupner et al. 2002). Invasive infection has been associated with clinical findings of valvular dysfunction, persistent fever despite appropriate antimicrobial therapy, and echocardiographic evidence of abscess formation. Invasive infection is more commonly observed during the first 12 months after surgery.

The Duke criteria, primarily established for the diagnosis of native valve infective endocarditis can also be applied for the diagnosis of PVE (Nettles et al. 1997; Perez-Vazquez et al. 2000). Diagnosis mainly relies on blood cultures and echocardiographic findings. Blood cultures must be obtained frequently and in the absence of antimicrobial therapy, in particular in the febrile postoperative patient. This is of particular importance related to the fact that the organisms most frequently involved in early PVE, i.e. coagulase-negative staphylococci and diphtheroids, are also the most frequently encountered contaminants of blood cultures. The superiority of TEE in comparison to TTE relates to the increased diagnostic sensitivity in detecting periprosthetic leaks, abscesses, and fistulas (Lengyel 1997; Bach 2000; Greaves et al. 2003).

The mortality of PVE is high, particularly in patients with onset of infection within 2 months of surgery and if infection is caused by *S. aureus* (Wolff et al. 1995). Other predictors of mortality are severe congestive heart failure and signs of invasive disease such as persisting fever, despite appropriate antimicrobial therapy. Mortality rates as high as 77 percent have been described in earlier series (Dismukes et al. 1973). Mortality rates ranging from 10 to 30 percent have been observed in more recent surveys, where earlier surgery may have contributed to a more favorable outcome (Akowuah et al. 2003). Overall, 40 to 65 percent of patients with PVE may be candidates for surgical intervention (Lytle et al. 1996; Fowler et al. 1998). Patients that may be treated by medical therapy alone are those with late-onset infection, with infection

caused by less virulent organisms, and those without evidence of invasive disease. The mortality of late PVE is less than that of early infection and not much different than for native valve infection. Patients with late PVE are likely to present to doctors earlier than many of those with native valve infection and hence to be referred to specialist departments earlier. Both these factors are relevant to outcome.

TREATMENT OF INFECTIVE ENDOCARDITIS

In the pre-antibiotic era, infective endocarditis was almost uniformly fatal and no-one can deny that antibiotics have dramatically reduced the mortality of the disease. Antimicrobial therapy should consider some specific conditions in infective endocarditis that are unique among bacterial infections, i.e. the infection involves an area of impaired host defense – the vegetation – with bacteria embedded in a fibrin meshwork where they are sheltered from phagocytic cells, where the bacteria reach enormous population densities, and where they may exist in a state of reduced metabolic activity. Therefore certain general treatment principles should be followed: (1) high-dose parenteral antimicrobial therapy is recommended to reach sustained antibacterial activity; (2) prolonged administration of antimicrobial therapy is necessary to prevent relapse; (3) bactericidal antimicrobial agents are generally preferred over bacteriostatic drugs; (4) antibiotic combination therapy is recommended to produce a rapid bactericidal effect (Le and Bayer 2003). Unless the patient is very unwell, toxic, and septic, antibiotics should preferably be withheld until the blood culture results are available. Many patients with infective endocarditis will have been ill for several weeks or even months, and thus a delay of 1–2 days in starting antibiotics cannot make any difference to the course of the disease.

Wilson et al. (1995) have recently reviewed the treatment of infective endocarditis due to streptococci, enterococci, staphylococci, and fastidious organisms of the HACEK group. Detailed guidelines are also given in the report from the Working Party in Endocarditis of the British Society for Antimicrobial Chemotherapy (BSAC) which has recently been updated (1998).

The recommended approach to 'penicillin-sensitive' streptococcal native valve endocarditis (i.e. oral streptococci and group D streptococci including *S. bovis* with a minimum inhibitory concentration (MIC) for penicillin $\leqslant$0.1 mg/l) consists of administration of aqueous penicillin G, 10–30 million units IV daily, or ceftriaxon, 2 g once daily IV or IM, for 4 weeks. The addition of gentamicin to penicillin exerts a synergistic killing effect on viridans streptococci in vitro and results in more rapid sterilization of cardiac vegetations in an animal model of endocarditis, but has not reached clinical cure rates superior to those obtained with penicillin alone in clinical studies. Short-course therapy of penicillin combined with gentamicin (3 mg/kg IV divided into three daily doses) for 2 weeks has been advocated in uncomplicated cases with comparable cure rates as high as 98 percent. This approach is not recommended for patients with myocardial abscess, embolism, or extracardiac metastatic foci of infection, and impaired renal function. In patients with prosthetic valve endocarditis a 6-week regimen of penicillin together with gentamicin for the first 2 weeks is recommended. Vancomycin therapy, 1 g IV twice daily for 4 weeks is indicated for patients with confirmed immediate hypersensitivity reactions to betalactam antibiotics.

Endocarditis due to viridans group streptococci with an MIC for penicillin >0.1 to 0.5 mg/l usually requires a 4-week course of penicillin together with gentamicin for the first 2 weeks. For patients with endocarditis with viridans streptococci with an MIC for penicillin >0.5 mg/l and nutritionally variant streptococci treatment with the regimen recommended for enterococcal endocarditis is advised (see below).

Enterococci are less susceptible to betalactam antimicrobial agents than streptococci (median MIC for penicillin: 2 mg/l), and in general these agents are bacteriostatic against the enterococci; combination regimens are therefore always recommended for treating enterococcal endocarditis. Cell wall-active antibiotics, such as beta-lactams or glycopeptides plus an aminoglycoside, are synergistic and exert a bactericidal effect in vitro against most enterococci unless high-level resistance to gentamicin (MIC >500 mg/l) or streptomycin (MIC >2 000 mg/l) is present. In these cases, penicillin- or vancomycin-aminoglycoside synergy and bactericidal activity is not apparent. The standard antimicrobial recommendation for enterococcal endocarditis is penicillin (20–30 million units daily) or ampicillin (12 g daily) combined with gentamicin (3 mg/kg per day) administered IV for 4–6 weeks. The 6-week regimen is recommended for patients with duration of symptomatic illness exceeding 3 months, as well as for complicated and prosthetic valve endocarditis. In patients allergic to beta-lactams or infected with penicillin-resistant strains such as *E. faecium*, vancomycin is administered in combination with gentamicin. If endocarditis is caused by a strain exhibiting high-level aminoglycoside resistance, the addition of an aminoglycoside is no longer beneficial, and therapy should be prolonged to 8–12 weeks instead. However, clinical cure is often not obtained without valve replacement. Endocarditis due to vancomycin-resistant enterococci (VRE) is a cause of concern, and the antimicrobial regimen of choice for these cases remains unsettled.

The great majority of staphylococci, acquired either in the hospital or in the community, produce β-lactamase, and they are highly resistant to penicillin. The current recommended regimen for treatment of native valve staphylococcal endocarditis includes a penicillinase-resistant penicillin, nafcillin or oxacillin, 12 g daily IV, or

cefazolin (or another first generation cephalosporin), 6–8 g daily IV, given for 4–6 weeks. The addition of gentamicin induced a synergistic killing effect both in vitro and in an animal model of endocarditis and has also been shown to result in more rapid clearing of bacteremia from the bloodstream in patients. Many authorities therefore recommend the addition of gentamicin for the first 3–5 days of therapy, although the combination did not improve the survival rate in a clinical study involving a small group of patients. In the patient allergic to penicillin or when the isolate is methicillin-resistant (MRSA or MRSE in the case of *S. epidermidis*), vancomycin, 1 g twice daily IV for 4–6 weeks is the agent of choice. However, vancomycin is less rapidly bactericidal than nafcillin in vitro against *S. aureus*, and prolonged bacteremia and increased failure rates have been associated with the use of this agent in infective endocarditis caused by MRSA. Accordingly, caution is advised when considering treatment with vancomycin only for reasons of convenience related to pharmacokinetics such as in hemodialysis patients where once weekly administration of vancomycin is possible. Although routine use of rifampin in native valve endocarditis is not recommended, it has been used, however, as supplemental therapy for patients who did not respond to conventional antimicrobial therapy or those with suppurative complications, such as valve ring abscess, intracerebral abscess formation, and meningitis. However, the adjunctive role of rifampin remains controversial.

Staphylococcal endocarditis in the presence of intracardiac prosthetic material is most often caused by coagulase-negative staphylococci that are usually resistant to methicillin. In these cases, vancomycin and rifampicin are administered for 6 weeks combined with gentamicin for the first 2 weeks of therapy. If the organism is susceptible to methicillin, nafcillin or oxacillin in combination with rifampin and gentamicin is advocated. Based on the experimental finding that rifampin results in the complete sterilization of foreign bodies infected with *S. aureus*, a similar combination therapy of nafcillin or oxacillin in combination with rifampin and gentamicin is also considered the regimen of choice for prosthetic valve endocarditis due to methicillin-susceptible *S. aureus*. If strains are resistant to methicillin or the patient is allergic to penicillin, vancomycin is substituted for the penicillinase-resistant penicillin. If the organism is resistant to gentamicin, another third agent should be chosen based on in vitro susceptibility testing since staphylococci may become resistant to rifampin during combination therapy of prosthetic valve endocarditis.

Treatment options for endocarditis caused by fastidious gram-negative bacilli of the HACEK group include the administration of ampicillin (12 g daily) combined with gentamicin (3 g/kg per day) administered IV for 4 weeks, or preferably ceftriaxone to cover β-lactamase producing strains, 2 g daily for 4 weeks. Therapy should be extended to 6 weeks in the case of prosthetic valve endocarditis.

Treatment regimens for endocarditis caused by less common pathogens such as gram-negative enteric bacilli, *Brucella* and *Bartonella* spp., *C. burnetii*, *Legionella* spp., obligate intracellular pathogens, and fungi is still not adequately defined, and the readers are referred to the most recent review articles for details (Bayer et al. 1998; Brouqui and Raoult 2001; Mylonakis and Calderwood 2001).

The choice of antibiotics for the treatment of infective endocarditis is governed by the susceptibility of the causative organism. Determination of the minimum inhibitory concentration is recommended to define optimal treatment, while standard disk sensitivity testing is unreliable. The determination of the minimum bactericidal concentration (MBC), as well as performance of the serum bactericidal titer (SBT) test is not routinely advocated, the latter correlating poorly with clinical outcome. Since adverse events are rather frequent during prolonged antimicrobial therapy, it is often necessary to revise therapy. For additional testing, the causative organism should therefore be retained in the laboratory until cure has been achieved.

Several studies suggest that combined medical and surgical therapy for infective endocarditis can improve outcome. A major reduction in mortality followed the introduction of valve replacement surgery some 40 years ago, and even a further decline in mortality observed in some of the most recent surveys has been associated with earlier valve replacement and more refined surgical techniques (Cabell et al. 2002; Hoen et al. 2002). Currently, surgical intervention during treatment is required in 25–30 percent of patients with infective endocarditis. The generally accepted indications for surgical intervention during active infective endocarditis include (1) refractory congestive heart failure; (2) more than one serious embolic event; (3) uncontrolled infection or sepsis despite maximal antibiotic therapy; (4) significant valve dysfunction, perforation or rupture as demonstrated by echocardiography; (5) ineffective antimicrobial therapy (e.g. as in Q fever endocarditis, fungal endocarditis, and enterococcal endocarditis caused by high-level gentamicin- or vancomycin-resistant enterococci); (6) local suppurative complications including periannular extension of the infection, perivalvular or myocardial abscess; and (7) most cases of prosthetic valve endocarditis caused by antibiotic-resistant pathogens such as MRSA, gram-negative enteric bacilli, and *P. aeruginosa*. The optimal time for surgical intervention is before severe hemodynamic compromise or extension of the infection to adjacent tissue has occurred. Medical and surgical management decisions can be guided by echocardiographic detection of abscesses, prosthetic valve dehiscence, valvular dysfunction, and obstructive vegetations. If there is evidence of active endocarditis at the time of valve replacement

surgery based on a culture-positive vegetation or histologic evidence of significant polymorphonuclear inflammation, antibiotic therapy should be continued for at least several weeks. Excellent reviews on the indications for surgery during therapy for infective endocarditis are available (Moon et al. 1997; Bayer et al. 1998; Alexiou et al. 2000; Delay et al. 2000; Guerra et al. 2001; Olaison and Pettersson 2003).

PREVENTION OF ENDOCARDITIS

Since infective endocarditis is a disease with significant morbidity and mortality, its prevention is a laudable objective, but how best to achieve this, or indeed whether it can be achieved, is another matter. A variety of preventive measures could theoretically be used to interrupt the sequence of events that finally results in endocarditis. These include elimination or treatment of predisposing conditions, elimination of portals of entry for organisms, e.g. by maintaining good oral hygiene, immunization against bacteria that cause endocarditis, and administration of antibiotics during high risk procedures. Of these, administration of antibiotics has received the most attention. There are numerous national guidelines for prophylaxis and these vary both in their antibiotic regimens and in the range of procedures for which prophylaxis is required. Over the past decade, however, there has been some uniformity of recommendations both within Europe and elsewhere. The diversity of the recommendations serves to emphasize the lack of critical data on which to base them. A detailed discussion of antimicrobial prophylactic strategies is beyond the scope of this chapter, and readers are referred to the above-mentioned guidelines (Simmons 1993; Leport et al. 1995; Dajani et al. 1997).

The guidelines for the prevention of late PVE are essentially the same as those outlined for endocarditis on native valves though some differentiate between the regimens for the two groups (Hyde et al. 1998; Segreti 1999). The prevention of early PVE concerns prophylactic antibiotics at the time of valve replacement. This has been a controversial issue for many years and has been plagued by many small poorly conducted trials. The available evidence suggests that antibiotic prophylaxis reduces the postoperative wound infection in cardiothoracic surgery in general (Kreter and Woods 1992) and, by inference, in valve replacement surgery in particular. It is assumed, though never proved, that a reduction in wound infection will equate with a reduction in PVE and this seems a reasonable assumption. Various antibiotics are used and no single regimen has been shown to be superior. The increasing prevalence of methicillin-resistant staphylococci in hospitals, both *S. aureus* (MRSA) and coagulase-negative staphylococci, has led many to use vancomycin rather than cefazolin and cefuroxime which are the most cost-effective agents for perioperative prophylaxis. However, this issue remains controversial.

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Bacterial meningitis

KEITH A.V. CARTWRIGHT

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INTRODUCTION

More than 2 000 cases of bacterial meningitis, with at least 150 deaths, are notified each year in the UK and similar incidence data are reported by many other countries. The emphasis in this chapter will be on the epidemiological, pathophysiological, and diagnostic aspects of the disease. Fuller accounts of the key organisms can be found in *Streptococcus* and *Lactobacillus*; 37, *Listeria*; 48, *Neisseria*; and 65, *Haemophilus*. The various clinical syndromes are well described in Lambert (1991)).

In meningitis, the membranes that surround the brain and spinal cord become inflamed. Most meningitis is viral, usually with mild illness, but viral and bacterial meningitis may be confused (Maxson and Jacobs 1993).

The commonest cause of bacterial meningitis worldwide is *Neisseria meningitidis*, the meningococcus. In unimmunized populations, capsulate strains of *Haemophilus influenzae* type b (Hib) may cause as many, or more cases, especially in young children. *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* rank next in order of frequency. Many other microbes and organisms, including bacteria, fungi, protozoa, and worms, may also cause meningitis or meningeal inflammation.

In neonatal meningitis, group B streptococci and *Escherichia coli* predominate, whereas *Listeria monocytogenes* causes meningitis in neonates, during pregnancy, and in old age. Fungal meningitis is particularly associated with severe immunosuppression as in the late stages of human immunodeficiency virus (HIV) infection. Many other bacteria, including staphylococci (Jensen et al. 1993), coliforms, and environmental gram-negative bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. (Siegman-Ingra et al. 1993), occasionally cause meningitis, often in associations with inpatient hospital care, severe immunosuppression, neurosurgery, cranial trauma, infection of cerebrospinal fluid (CSF) reservoirs or shunts.

In economically advanced countries, the mortality from bacterial meningitis is less than 10 percent but it may be 30 percent or more in developing countries (Greenwood 1987; Bryan et al. 1990; Bijlmer 1991). The epidemiology is changing swiftly following the development of conjugated polysaccharide vaccines for invasive Hib disease, for meningococcal meningitis caused by serogroups A, C, Y, and W-135, and for pneumococcal meningitis caused by a range of common serotypes. In the UK, following the control of serogroup C meningococcal disease through vaccination, there are now good prospects for further reductions in the incidence of bacterial meningitis in the near future, particularly if serogroup B meningococcal vaccines can be developed, but also if the incidence of pneumococcal meningitis can be reduced through vaccination (Figure 1).

PATHOPHYSIOLOGY

The evolution of bacterial meningitis follows a sequence of exposure to the pathogen, acquisition, and then invasion. Invasion can be subdivided into three processes: mucosal penetration is followed by invasion of the

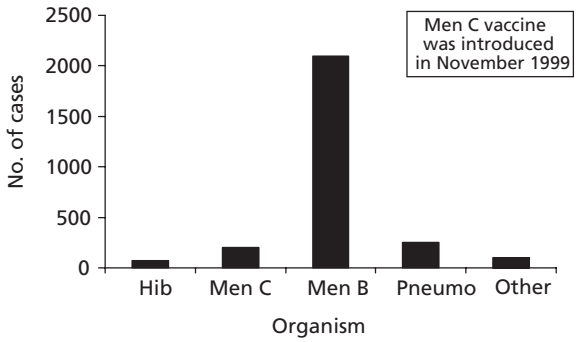

Figure 1 *Estimated incidence of bacterial meningitis and meningococcal disease in the UK, end 2002.*

bloodstream and then invasion of the meninges (Tunkel and Scheld 1993). The human nasopharynx is the natural habitat of the meningococcus, Hib, and the pneumococcus. Exposure is more frequent than acquisition, which is more frequent than bloodstream invasion. Understanding the process of establishment of bacterial meningitis has been greatly assisted by the development of animal models of infection (O'Donoghue et al. 1974; Moxon et al. 1977; Tauber and Zwahlen 1994).

Exposure

Exposure depends on the frequency and intimacy of interactions between colonized and susceptible individuals, the capacity of colonized individuals to disseminate bacteria from the nasopharynx, and the capacity of others around them to become colonized. Surprisingly little is known about these aspects, but dissemination may be influenced by the intensity of colonization, intercurrent viral infections (Gwaltney et al. 1975), and perhaps the ambient temperature and humidity.

Acquisition

Carriage rates reflect both acquisition rates and the duration of carriage. The duality is important; much evidence suggests that if bacterial invasion is to occur, it follows very shortly after acquisition. Individuals colonized for weeks or months are unlikely to develop invasive disease, though anecdotes do exist to show that invasion may sometimes follow weeks after acquisition and colonization (Neal et al. 1999).

With increasing age, the nasopharynx becomes progressively less susceptible to colonization by meningococci, Hib, and pneumococci. This may be due to the development of mucosal immunity.

Mucosal invasion

Bacterial strains vary greatly in their capacity to invade. Meningococci that do not express a capsule are essentially avirulent, while strains of serogroups A, B, and C are more invasive than strains of serogroups X, Y, and W-135 and other groups of meningococci (see *Neisseria*). Similarly, acapsulate strains of *H. influenzae* have little invasive potential, and though there are six capsular serotypes of *H. influenzae*, type b strains account for more than 95 percent of all invasive hemophilus disease in unimmunized populations.

The interaction of bacteria with the nasopharyngeal mucosa and other tissues has been investigated with tissue explants (Stephens and Farley 1991; Read et al. 1995), cultured epithelial and endothelial cells (Virji et al. 1991; Pujol et al. 1997), and the use of bacterial transformants and microarray technology (Grandi 2001). This has allowed comparisons between the behavior of fully virulent bacteria and strains that differ from them by the deletion of single genes or operons.

Capsulate meningococci bind to epithelial cells by adhesins, including fimbriae and the Opc outer-membrane protein (Virji et al. 1991, 1992). They cross the mucosal barrier by direct invasion of epithelial cells (endocytosis), whereas Hib strains migrate between epithelial cells by breaking down intercellular tight junctions (Stephens and Farley 1991). Once in the submucosa, the invading bacteria cross the capillary vessel basement membrane and the endothelium to gain access to the bloodstream.

The three major bacterial meningitis pathogens produce an IgA1 protease, whereas closely related nasopharyngeal 'commensals,' such as *Haemophilus parainfluenzae*, *Neisseria lactamica*, and viridans streptococci, do not (Mulks and Plaut 1978; Mulks 1985). Invasion potential and production of IgA1 protease (and the converse) are so closely associated that a causal relationship has long been sought. One hypothesis is that circulating IgA may block binding sites that would otherwise be accessible to lethal, bacteriolytic complement-fixing IgG or IgM antibodies (Griffiss 1982). Bacteria that invade may be further protected by cleavage of the blocking immunoglobulin molecules by IgA protease.

Respiratory viral infections may play a part in precipitating bacterial meningitis (Rolleston 1919; Krasinski et al. 1987; Moore et al. 1990; Scholten et al. 1999), in particular influenza A, which increases both the risk (Cartwright et al. 1991) and the severity (Hubert et al. 1992) of meningococcal disease. The mechanism in this latter situation is probably postviral immune suppression.

Meningeal invasion

Normal CSF contains few phagocytic cells and very low levels of complement and antibody; the normal CSF:blood IgG ratio is about 1:800. The integrity of the normal blood–brain barrier, and thus of the subarachnoid space, is maintained by a combination of tight intercellular junctions between capillary endothelial cells

of the cerebral microvasculature and by the comparative rarity of pinocytosis by these cells.

The cerebral capillary endothelium of the choroid plexus can bind some of the bacteria that cause meningitis. Bound bacteria may then undergo fimbrial phase variation in order to cross the blood–brain barrier (Saukkonnen et al. 1988). Bacteria may also gain access to the subarachnoid space within host phagocytic cells (Tunkel and Scheld 1993; McNeil et al. 1994).

Once bacteria are established within the subarachnoid space they are transiently sheltered from the host defenses. They can release substances capable of disrupting the blood–brain barrier. In the CSF, *H. influenzae* type b lipopolysaccharide (an endotoxin) rapidly induces the production of inflammatory cytokines, including interleukin-1 and tumor necrosis factor. Pneumococcal cell walls, which contain peptidoglycan and teichoic acid, are more potent than capsular polysaccharide in inducing subarachnoid inflammation (Tuomanen et al. 1985). High CSF cytokine concentrations are reached within 1–2 h of the arrival of bacteria in the CSF (Tunkel and Scheld 1993; Spellerberg and Tuomanen 1994).

Disruption of the blood–brain barrier occurs as a result of breakdown of the intercellular tight junctions of the cerebral microvascular epithelium, permitting an influx of larger molecules from the bloodstream and migration of phagocytic cells into the CSF.

Factors predisposing to bacterial meningitis

Many factors predispose to bacterial meningitis, including age, male gender, winter season, exposure to smokers and smoking, low socioeconomic status, and (probably) stress (Stanwell-Smith et al. 1994). Contact with a case confers a substantially increased risk of meningococcal disease (de Wals et al. 1981; Cooke et al. 1989), though less so for *H. influenzae* meningitis; in contrast, pneumococcal meningitis is not normally regarded as a transmissible infection and clusters of cases of any form of invasive pneumococcal disease (including meningitis) are rare.

Host genetic factors that confer increased susceptibility to bacterial meningitis or to a poor outcome are increasingly the subject of investigation in the context of new investigative methods now becoming available (Hill 2001). This is an area where much information will accrue over the next few years. Specific host factors conferring susceptibility to meningococcal disease are addressed in the relevant section of this chapter.

CLINICAL FEATURES

The presenting features of meningitis are determined largely by the age of the patient and, to a lesser extent, by the causative organism and the route by which it has reached the meninges. In the great majority of cases, the early signs and symptoms are nonspecific. Fever, lassitude, malaise, muscular aches and pains, nausea, vomiting, and headache occur in both viral and bacterial meningitis but are more commonly due to the former. Identification of the symptoms that may give warning of impending serious illness can be very difficult. In temperate countries, the difficulty is compounded by the fact that influenza, viral respiratory infections, and bacterial meningitis all peak in the winter months. Specific symptoms that should immediately raise the suspicion of bacterial meningitis include photophobia, neck stiffness, drowsiness, and fitting. Fever accompanied by pallor, inconsolable crying, a fit, or any evidence of decreased consciousness in a febrile infant or young child are highly suspicious and should warrant immediate careful investigation. The anterior fontanelle may bulge as meningitis progresses in infants.

As bacterial meningitis develops, symptoms become more severe; most patients with bacterial meningitis are much iller than those with viral infections. The highest attack rates of bacterial meningitis are in infants and very young children who cannot give a verbal account of their symptoms. Familiarity with the symptoms, repeated examinations, and a high index of suspicion are the best safeguards for parents and family doctors. The combination of fever and a vasculitic rash (petechiae or purpura), which may herald meningococcal disease, constitutes a medical emergency. Meningococcal septicemia may occasionally progress so rapidly that patients can become moribund within 4–6 h of the first symptoms.

In contrast, tuberculous and fungal meningitis more commonly develop insidiously over days, weeks, or even months.

DIAGNOSIS OF MENINGITIS

Cerebrospinal fluid

LUMBAR PUNCTURE

Examination of CSF has long been considered as routine in the investigation of suspected bacterial meningitis (Gray and Fedorko 1992) but is not without its dangers and drawbacks, especially ‘coning’ (Rennick et al. 1993). If intracranial pressure is raised, lumbar puncture may occasionally be followed by herniation of the tentorium through the *falx cerebri* or of the brain stem through the *foramen magnum* (coning); either may be a fatal event or may lead to catastrophic neurological morbidity. Papilloedema is an insensitive indicator of raised intracranial pressure and clinicians will normally be wary of lumbar puncture in patients with evidence of diminished consciousness or fluctuating neurological status. A computed tomography (CT) scan is helpful in

determining whether there is raised intracranial pressure but does not provide absolute assurance of safety to proceed with lumbar puncture. An increasing proportion of pediatric patients with clinical evidence of meningitis are now managed without lumbar puncture (Cartwright and Kroll 1997; Wylie et al. 1997; Nadel 2001), though the procedure is still recommended in suspected meningitis in adults (Begg et al. 1999) because of the wider range of potential pathogens.

Despite the hazards, examination of CSF still offers the best chance of observing, isolating, and identifying the causative organism in bacterial meningitis (Kaplan et al. 1986b; British Society for the Study of Infection Research Committee 1995). Lumbar puncture is particularly valuable if a dose of a parenteral antibiotic has been given either before or on hospital admission, but if indicated, must be done swiftly, since cultures, even of CSF, rapidly become negative; CSF sterilization times of 2 h for meningococci and 4 h for pneumococci have been suggested (Kanegaye et al. 2001). Even if bacteria cannot be recovered on culture, microscopy is likely to confirm the diagnosis of bacterial meningitis and may indicate the likely causative organism. Lumbar puncture should never be used as a reason to defer commencing antibiotic treatment and other resuscitation measures. The 'door-to-needle' time for patients hospitalized for suspected bacterial meningitis should be less than 1 h.

Polymerase chain reaction (PCR) tests of CSF for the common bacterial meningitis pathogens remain positive for several days after cultures become negative. Thus, if a patient is considered too sick to undergo lumbar puncture on admission, it may be preferable to initiate empirical antibiotic treatment and to reconsider the need for lumbar puncture after 24–48 h, when the patient's condition has stabilized.

A portion of the CSF sample should be examined promptly to determine the number and type of leukocytes present. Bacterial meningitis is usually accompanied by the presence of neutrophils, but about 5 percent of CSF samples subsequently found to be culture-positive contain only a few lymphocytes or no cells at all when first examined. The absence of leukocytes should never be used to justify withholding antibiotic treatment if there is reasonable clinical suspicion of bacterial meningitis. The cell population may consist of a mixture of polymorphs and lymphocytes; this is more likely if there has been a long prodrome or if the patient has been part-treated with antibiotics. A mixture of cells is sometimes seen in viral meningitis. The CSF leukocyte count may be raised in patients who have had a cerebral hemorrhage a few days earlier and in patients who have had recent fits. In these cases, the cells are normally predominantly lymphocytes. Other conditions that give rise to a lymphocytic pleocytosis are listed in Table 1.

The CSF leukocyte concentration falls much more slowly than the concentration of viable bacteria. It is not uncommon for lumbar puncture 1–2 weeks after the onset of bacterial meningitis to show persistence of leukocytes (Chartrand and Cho 1976; Connolly 1979), especially when meningitis has been caused by *H. influenzae* or by a pneumococcus. Persistence of pleocytosis is less likely in meningococcal meningitis.

BIOCHEMICAL ANALYSIS

Estimation of CSF glucose and protein can be helpful. The CSF:blood glucose ratio (normally about 70 percent) is often, though not invariably, reduced in bacterial meningitis, commonly to about 25 percent (Kaplan et al. 1986b), but a normal CSF:blood glucose ratio is common in tuberculous meningitis. The CSF

Table 1 *Conditions causing cerebrospinal fluid lymphocytic pleocytosis*

Infections	Drugs and vaccines	Inflammatory processes	Malignancy
Viral meningitis	Trimethoprim, isoniazid	Postictal, postmyelogram	Meningeal carcinomatosis
Partly treated bacterial meningitis	MMR and polio vaccine	Post-CNS bleed	CNS leukemia and lymphoma
Syphilis	Ibuprofen, NSAIDs	Sarcoidosis, Wegener's granulomatosis	
Parameningeal infection (e.g. abscess, subdural empyema)	Intravenous immunoglobulin	Malignant hypertension, migraine	
Protozoa (e.g. toxoplasmosis)		Kawasaki disease	
Acute and chronic bacterial meningitis		Post-neurosurgery, intrathecal drugs	
Tuberculous meningitis		Multiple sclerosis	
Lyme borreliosis		SLE, Behçet's syndrome	
Fungal meningitis		Benign intracranial hypertension	
Worm infection		Mollaret's meningitis	

CNS, central nervous system; NSAID, nonsteroidal anti-inflammatory drug; SLE, systemic lupus erythemastosus.

protein is often raised, initially reflecting leakage of blood protein into the CSF; later it may reflect local antibody production. CSF lactate concentration may help to differentiate between bacterial and viral meningitis.

Urine reagent strips have been used for the direct examination of CSF (Moosa et al. 1995) to discriminate accurately between viral and bacterial meningitis. This cheap and simple method may have an important role where laboratory facilities are limited or distant.

MICROSCOPY

Direct microscopy of uncentrifuged or centrifuged CSF may reveal the presence of bacteria or fungi and can provide immediate confirmation of the diagnosis. Staining with acridine orange is more sensitive than the Gram stain (Kleinman et al. 1984). The organisms seen on microscopy in antibiotic-treated patients may fail to grow on culture, but the morphology and Gram reaction of the organism, the age of the patient, and the clinical features often permit an educated guess at the identity of the causative organism.

DETECTION OF BACTERIAL ANTIGEN

Tests for bacterial antigen in CSF can provide a quick diagnosis, but they are less sensitive than the Gram stain and do not often alter clinical management (Maxson et al. 1994). Many tests are based on the agglutination of antibody-coated latex particles and work quite well for pneumococci, for meningococci of serogroups A or C, and for group B streptococci. They are less successful for the detection of serogroup B meningococci. The *Limulus* lysate test is a sensitive test for endotoxin from gram-negative organisms but has not found wide acceptance to date.

CULTURE

CSF should be inoculated on to good-quality culture media, always including at least Columbia blood agar and a heated blood agar. Plates should be incubated in five percent CO_2 for a minimum of 48 h. If a ruptured cerebral abscess is suspected, or if meningitis has followed neurosurgery or a history of previous meningeal trauma, a second blood agar plate should be incubated anaerobically for 5–7 days.

The possibility of parameningeal infection, especially subdural empyema and brain abscess, must be considered and pursued actively if CSF shows an inflammatory response, if bacteria are not seen, cultured, or detected by PCR tests, and the patient's condition is not improving. CT scans may sometimes fail to detect intracranial collections of pus. Subdural empyema (or brain abscess) should be suspected if 'meningitis' is diagnosed in a child or young adult in whom there is a recent history of sinusitis or middle ear infection. The importance of not missing these conditions lies in the need for urgent neurosurgical and ear, nose, and throat (ENT) assessment as part of the management protocol. Most cases of subdural empyema are initially misdiagnosed as bacterial meningitis.

POLYMERASE CHAIN REACTION

PCR tests for the detection of meningococcal and pneumococcal DNA in CSF are now used routinely in the UK. Meningococcal PCR is specific and sensitive for the diagnosis of meningococcal meningitis (Borrow et al. 1997) and provides serogroup information in the majority of cases. Amplification of sections of 16S ribosomal RNA, common to most species of pathogenic bacteria, may also prove to be of value (Greisen et al. 1994).

Peripheral blood

BLOOD CULTURE

Blood cultures are positive in only about half of patients with meningococcal disease who have not previously received parenteral antibiotic therapy, and they are almost invariably sterile in those who have. In contrast, in Hib and pneumococcal meningitis the causative organisms are commonly isolated from peripheral blood of untreated patients (British Society for the Study of Infection Research Committee 1995).

MICROSCOPY

When bacteremia is heavy, meningococci and other bacteria may be detected within polymorphs in Giemsa-stained films of peripheral blood (Bush and Bailey 1944) and they can be seen in and grown from tissue fluid obtained from the hemorrhagic skin rash (van Deuren et al. 1993) and from other normally sterile sites such as joints and pericardial fluid.

PCR OF PERIPHERAL BLOOD

PCR tests for the detection of meningococcal DNA (Borrow et al. 1997) are of particular value when the patient has received parenteral antibiotic treatment before hospital admission, an increasingly frequent event. They are now offered routinely in the UK (Kaczmarski et al. 1998), with high sensitivity and specificity (Hackett et al. 2002a). About half of all cases of laboratory-confirmed meningococcal disease in England and Wales are now diagnosed by PCR methodology. This is related to the increasing use of preadmission parenteral antibiotic therapy and to a decline in the use of lumbar puncture, especially in pediatric patients (Wylie et al. 1997). Quantitative meningococcal blood PCR has recently confirmed historical quantitative culture findings, indicating that meningococcal bacterial load at presentation in hospital correlates with disease severity (Hackett et al. 2002b).

Though pneumococcal and Hib meningitis are very frequently accompanied by bacteremia, blood PCR tests for these bacteria are not used widely. Blood PCR tests for pneumococci are only moderately sensitive, and the specificity is low in children (better in adults), either because they fail to differentiate between carriage and invasive infection or, more likely, 'occult' bacteremia (or spillover of bacteria from the nasopharynx) is a frequent occurrence in colonized infants and children.

Serology

Serological tests for meningococcal disease were long hampered by the failure of serogroup B capsular polysaccharide to provoke a good immune response. Second-generation serological tests now available in the UK can reliably detect the presence of acute-phase (IgM) meningococcal antibodies in a single serum specimen obtained after the first week of the illness; alternatively, a rising titer of antibody to meningococcal outer-membrane proteins may be sought (Jones and Kaczmarski 1994).

Serology can be used to confirm invasive Hib infection but antibody responses in pneumococcal infection are highly unreliable because they fail to differentiate between carriage and invasive illness.

Nasopharyngeal swabs

Since the main bacterial meningitis pathogens are nasopharyngeal commensals, their isolation from the nasopharynx in cases of clinical meningitis does not provide evidence of causation. However, when lumbar puncture is not undertaken and blood cultures are negative, a throat swab may give the only chance of obtaining an isolate. Meningococci are rarely recovered from healthy infants and the isolation of a capsulate meningococcus of serogroup B or C from the posterior pharynx of a young child with symptoms of meningitis with or without a hemorrhagic rash is very strong evidence of a meningococcal etiology. Similarly, the isolation of Hib from a child with meningitis aged 12 months or less is unlikely to be coincidental. However, isolation of a pneumococcus from the nasopharynx of a child or adult is sufficiently common that it does not assist in management.

Swabs of the posterior pharynx are ideally taken through the mouth (Olcén et al. 1979). Patients with clinical meningitis are often too ill to cooperate with peroral swabbing, but pernasal swabs are simple to obtain, though an assistant is required to steady the patient's head. A single negative nasopharyngeal swab is an insensitive predictor of freedom from carriage of any specific bacterial meningitis pathogen, and decisions on the prophylaxis of contacts of meningococcal or Hib disease should never be based on the results of nasopharyngeal swabbing of the contacts.

MENINGOCOCCAL DISEASE

Historical aspects

Vieusseux (1805) described an outbreak of meningococcal disease with 33 deaths in the small community of Eaux Vives, near Lake Geneva in Switzerland, in the spring of 1805. Before this, meningococcal disease may have been confused with other spotted fevers, including typhus, which also sometimes occurred in clusters and outbreaks, especially in the military, and was often characterized by a hemorrhagic skin rash. It seems most likely that meningococcal disease has afflicted man for centuries.

August Hirsch (1886) documented many outbreaks of infectious diseases including cerebrospinal fever in Europe and the New World up to 1882. The Italian pathologists Marchiafava and Celli (1884) are credited with the first description of intracellular oval micrococci in a sample of CSF. Three years later Weichselbaum, in Vienna, reported the isolation of an organism he described as *Diplococcus intracellularis meningitidis* from six of eight cases of primary sporadic community-acquired meningitis (Weichselbaum 1887). A period of confusion followed when Jaeger isolated a gram-positive chain-forming coccus from meningitis cases in an outbreak in Stuttgart. Subsequent reports showed that Weichselbaum was correct: Jaeger's gram-positive organism was probably a contaminant.

Eight years later came the first account of lumbar puncture in a living patient (Quincke 1893), and in 1896 meningococci were isolated for the first time from the CSF of patients with meningitis (Heubner 1896). In the same year, meningococci were also isolated from human throat cultures (Kiefer 1896), offering an explanation for the spread of the bacteria in human populations.

Before World War I, German and American bacteriologists were developing serum therapy for meningococcal meningitis in an effort to reduce its extremely high mortality (Kolle and Wasserman 1906; Flexner and Jobling 1908). They were able to reduce mortality from about 80 to 25 percent by repeated intrathecal instillation of immune horse serum (Flexner and Jobling 1908). Not surprisingly, a number of successfully treated patients developed 'serum disease' (serum sickness), with fever and arthritis, and a few developed secondary bacterial meningitis.

In Britain, nationwide notification of a range of infectious diseases including cerebrospinal fever was introduced in 1912. Two years later, during the first winter of World War I, there were several outbreaks of cerebrospinal fever amongst military recruits. Glover (1920) carried out a detailed investigation at the Guards Depot, Caterham, in south London. His report is a masterpiece of early meningococcal epidemiology; he concluded that the disease flared up at times of extreme overcrowding

and was preceded by very high nasopharyngeal carriage rates. Effective interventions, including increasing the amount of sleeping accommodation and the space between beds, fixing open windows in huts, and shortening of parades, brought the outbreaks to a halt, even during the 1918–19 winter in which there was a major influenza epidemic. Later, Dudley and Brennan (1934) and others documented very high nasopharyngeal carriage rates without the occurrence of cases of meningococcal disease. All the major combatant countries in World War I experienced clusters of cases and outbreaks of meningococcal disease in recruit camps, but there was little disease in seasoned troops (Rolleston 1919). At the end of the war, French microbiologists published a serogrouping scheme, which forms the basis for the current classification system (Nicolle et al. 1918).

In the 1930s, Rake and colleagues showed that freshly isolated meningococci were 'smooth' (capsulate), that they produced a polysaccharide capsule and that such strains produced more potent antisera when used to immunize horses (Rake 1931; Rake and Scherp 1933). Rake also carried out studies on the duration of nasopharyngeal meningococcal carriage and showed that, though very variable, carriage could persist for years.

Rake's work, which was leading towards the development of vaccines, was halted by the discovery of sulfonamides. After their first use in the treatment of human meningococcal infections (Schwentker 1937), sulfonamides were used to treat very large numbers of cases of meningococcal disease at the beginning of World War II and, subsequently, also for chemoprophylaxis. In Britain in 1941, there were 11 000 notified cases and more than 2 000 cases in each subsequent year up to 1945 (Figure 2).

Germany, France, and the USA also experienced outbreaks of unprecedented size, in each case shortly after becoming a combatant country. These epidemics occurred in association with mobilization (in 1940–41 in the UK, France, and Germany, and in 1942–43 in the USA) and usually began among recruits in training, later spilling over into civilian populations. At the same time, most noncombatant countries, such as Sweden, did not experience any great change in the incidence of meningococcal disease.

Sulfonamides transformed the treatment of meningococcal disease – mortality rates fell to about 10–20 percent. When penicillin became available later in the war it gradually superseded sulfonamides for treatment of invasive disease, but sulfonamides continued to be used for the treatment of carriers.

Epidemiology

Meningococcal disease remains a worldwide problem, occurring sporadically as clusters of cases and as epidemics. Annual rates of disease in many western countries and in the North American continent are about one to two per 100 000 of the total population, though there are wide fluctuations from year to year and from country to country. Even within countries, the disease rate can fluctuate remarkably, with clustering of cases in small communities or regions, giving high attack rates (5–25 per 100 000) for months or years. Norway experienced high rates of disease, mainly due to strains of serogroup B (ET-5 clone), for almost 20 years from the 1970s to the 1990s (Lystad and Aasen 1991), whereas the disease incidence in Sweden remained low throughout the same period. At the start of a period of increased disease incidence, the average age of cases rises (Peltola et al. 1982) and the severity of disease is greater. These phenomena may reflect the accumulation of susceptible people in the population. New Zealand has experienced hyperendemic serogroup B

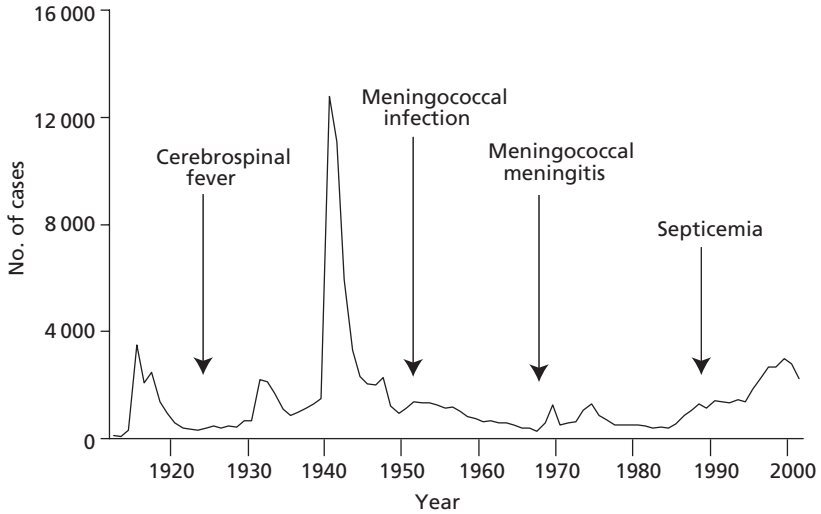

Figure 2 *Notifications of meningococcal disease, England and Wales 1912–2001.*

meningococcal disease since the early 1990s, with particularly high attack rates amongst the Pacific Islander and Maori populations (Martin et al. 1998).

THE 'MENINGITIS BELT'

The pattern of disease is quite different in some tropical countries, with large epidemics of serogroup A disease occurring at irregular intervals, separated by periods of much lower disease activity (Peltola 1983; Moore 1992). Lapeysonnie (1963) drew attention to an area of Africa south of the Sahara and extending almost from coast to coast, where epidemics of meningococcal disease occur every 5–10 years – the 'meningitis belt' (Figure 3). Epidemic waves may last 2 or 3 years.

The boundaries of the meningitis belt are probably defined by climatic factors; continuous high absolute humidity may reduce meningococcal transmission (Cheesbrough et al. 1995). This leads to the situation in sub-Saharan Africa, where countries such as Ghana and Nigeria may experience epidemics of meningococcal disease in their northern regions but have low attack rates in their southern coastal areas.

The disease is highly seasonal in the meningitis belt, with an upsurge during the period of dry, dusty winds ('harmattan'), with abrupt cessation of the epidemic with the start of the rainy season. The numbers of cases are staggering by developed country standards – attack rates of 500 per 100 000 or more have been recorded. The highest attack rate is in the age group 5–15 years; the sexes are affected equally. Multilocus electrophoretic typing (Caugant et al. 1987) and multilocus sequence typing (Maiden et al. 1998), which permit the identification of meningococcal clones, demonstrate that these epidemics are usually due to the spread of new clones, almost always of serogroup A (Olyhoek et al. 1987; Achtman 1990, 1995).

EPIDEMIOLOGY IN TEMPERATE CLIMATES

In temperate and colder climates, the disease is strongly seasonal, with peak rates in the winter months. In Britain, disease rates are highest in December and January, falling to a low point in the autumn months. In western countries, the age-specific incidence shows two peaks, with the first, and larger, in infants and young children. A second smaller, but important, peak occurs in late teenage. There is no obvious relationship between the age-specific prevalence of carriage and the incidence of disease (Figure 4). In most western countries, affected males outnumber females by up to 1.5:1.

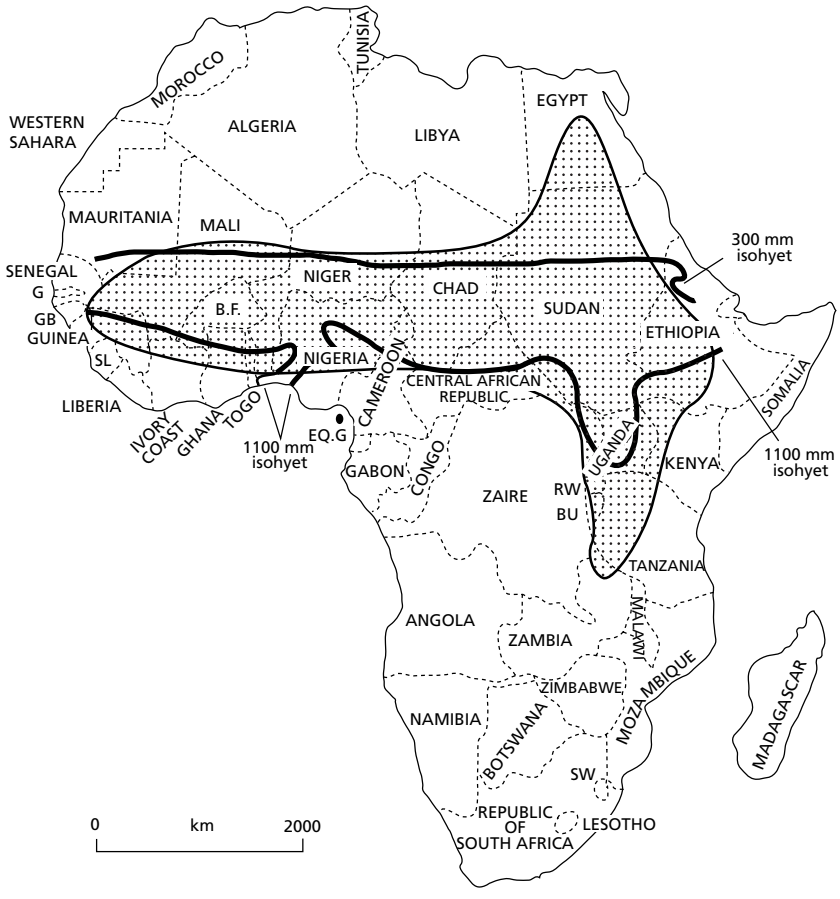

Figure 3 *Africa: the 'Meningitis Belt' (redrawn from Cheesbrough et al. 1995 with permission).*

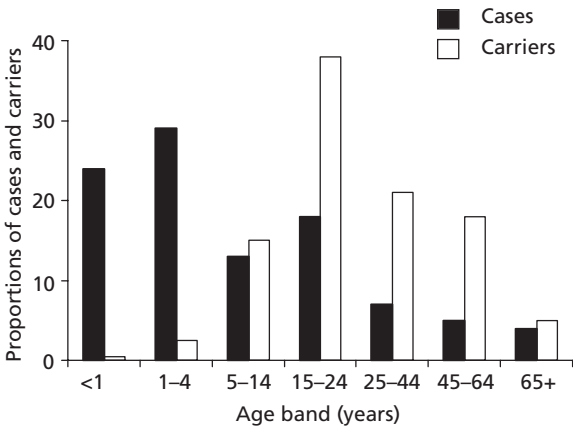

Figure 4 *Proportions of meningococcal cases and carriers within age groups. (Carriage data from Cartwright et al. 1987, with permission; age incidence from Jones and Mallard 1993, with permission.)*

In the UK, prior to the introduction of conjugated meningococcal serogroup C vaccines in 1999, about 70 percent of invasive disease was caused by serogroup B meningococci, a further 25 percent by serogroup C strains, and the remainder by a mixture of other serogroups, including W-135, Y, and X (Jones and Kaczmarski 1994). The large epidemics that occurred during World War I and World War II were caused by strains of serogroup A, with a major change in the epidemiology occurring in the 1950s to the currently observed pattern. During the late 1990s in the UK, the proportion of cases attributable to serogroup C strains has been as high as 40 percent. This was accompanied by a higher incidence of cases in older children and adults and an increase in the number of outbreaks in schools and universities. The introduction into the national childhood immunization schedule in November 1999 of highly effective conjugated serogroup C vaccines has dramatically reduced serogroup C disease in the UK (see below). It has not been accompanied by replacement of serogroup C disease by strains of other serogroups, e.g. serogroup B, either amongst disease-causing strains or amongst carried meningococci (Maiden et al. 2002).

In the UK, the USA, and a number of European countries, there was a notable upsurge in meningococcal disease caused by strains of W-135 amongst Muslim pilgrims making the Haj pilgrimage to Mecca and Medina in 2000 (Taha et al. 2000) and 2001. A requirement by the Saudi Arabian health authorities later in 2001 that all pilgrims should receive quadrivalent A, C, Y, W-135 vaccine as a condition of entry to the country brought these outbreaks of disease to a close. The clone causing this upsurge of W-135 disease was the same as that responsible for most serogroup C meningococcal disease in the UK over the preceding few years (ET-37). This clone is highly virulent and case-fatality rates were high.

Carriage

This subject has been dealt with in two in-depth reviews (Broome 1986; Cartwright 1995). The human nasopharynx is the natural habitat of the meningococcus. Meningococci are not carried by animals; nor are they recovered from the environment. Humans may be selectively colonized because meningococci are only able to acquire iron, an essential nutrient (van Putten 1990), from human (and primate) transferrin and lactoferrin (Schryvers and Gonzalez 1990). Fimbriae facilitate binding to human epithelial cells, but capsulation, essential for invasion, reduces adherence. Colonization by a single strain may last for months or even years. Capsule expression may be downregulated during prolonged carriage.

AGE-SPECIFIC INCIDENCE OF CARRIAGE

Carriage rates are low in infants and young children but rise with age to peak in late teenage and early adult life. They then decline slowly over the next 20–30 years; carriage is rare after the age of 65 years (Greenfield et al. 1971; Cartwright et al. 1987; Caugant et al. 1994). Most nasopharyngeal meningococci have very low invasive potential. Many express little or no capsular polysaccharide and are nongroupable or belong to serogroups of low virulence such as X, Y, or W-135. Exposure to such strains probably boosts levels of antibody against noncapsular surface antigens.

FACTORS AFFECTING CARRIAGE RATES

Throat-swabbing is an insensitive procedure and swabs from individuals and groups underestimate carriage frequency. Swabbing the posterior pharyngeal wall is much more reliable for the detection of meningococcal carriage than swabbing the tonsils or sampling the front of the mouth (J.M. Stuart, unpublished), but a single negative throat swab, even if collected by an experienced sampler, cannot be relied upon as proof of freedom from colonization. Somewhat surprisingly, carriage appears to be unaffected by season (Blakebrough et al. 1982; de Wals et al. 1983) or by intercurrent viral infection. Vaccination with purified capsular polysaccharides also appears to have little effect, but conjugated serogroup C vaccines have reduced dramatically the prevalence of carriage of homologous meningococcal strains (Maiden et al. 2002).

Smoking has a strong dose-related effect on carriage (Stuart et al. 1989), but contact with a case of meningococcal disease is the most important risk factor for carriage. Rates of carriage are high in family members and close contacts of cases of meningococcal disease (Greenfield and Feldman 1967; Munford et al. 1974; Kristiansen et al. 1998). Most (but not all) such isolates are indistinguishable from the index case strain (Olcén et al. 1981). Carriage rates rise rapidly when teenagers

and young adults are brought into close contact and close social interaction, such as that facilitated by entry into a military recruit camp (Riordan et al. 1998) or commencing study at university (Neal et al. 2000), explaining the high rates of disease seen in these groups. For students, high carriage rates and high disease rates are most marked in the freshman (first) year.

Immunity

The best serological correlate of protection from meningococcal disease is the presence of bactericidal antibodies (Goldschneider et al. 1969a) (Figure 5). Opsonizing antibodies are also important (Halstensen et al. 1989). For serogroup A and C meningococci, most bactericidal antibody is IgG directed against the capsular polysaccharide and is capable of causing bacterial lysis through the activation of complement (Gotschlich et al. 1969a). Serogroup B capsular polysaccharide is a very weak antigen for humans, probably because it shares epitopes with host cell antigens (Finne et al. 1983; Azmi et al. 1995). Antibodies present after serogroup B meningococcal disease are directed against a variety of surface-expressed antigens, including outer-membrane proteins and lipopolysaccharide (Kasper et al. 1973; Griffiss et al. 1984).

THE ROLE OF *NEISSERIA LACTAMICA*

Bactericidal antibodies are acquired progressively during childhood (Goldschneider et al. 1969a), beginning at a time when exposure to meningococci is slight. Thus, protection is probably acquired first through exposure to other bacteria that express cross-reacting surface antigens (Glode et al. 1977; Guirguis et al. 1985; Devi et al. 1991).

The carriage rate of *Neisseria lactamica* is high when bactericidal meningococcal antibodies are being acquired. Colonization with *N. lactamica*, a noncapsulate species, generates antibody that is bactericidal against a range of serogroups and serotypes of meningococci (Gold et al. 1978), indicating the presence of cross-protective subcapsular antigenic determinants. Later in life, exposure to avirulent meningococci induces antibodies against a wide range of meningococcal serogroups (Reller et al. 1973), providing further confirmation that some bactericidal antibody is directed against noncapsular antigens. A number of oral and enteric bacteria also express cross-reactive antigens and probably play a part in generating and maintaining immunity.

BLOCKING IGA ANTIBODIES

Complement-dependent immune lysis is not initiated by IgA. However, secretory or circulating IgA antibodies may block meningococcal surface antigens that would otherwise be accessible to IgG or IgM antibodies that activate complement. Blocking IgA antibodies generated in response to cross-reacting enteric bacteria may be important in some cases of meningococcal disease in older children and adults (Griffiss 1995). The timing of exposure to the meningococcus and the cross-reacting enteric bacterium may be critical (Kilian and Reinholdt 1987; Kilian et al. 1988).

HOST SUSCEPTIBILITY

Various congenital or acquired immune deficiency syndromes are associated with an increased risk of meningococcal disease (Figueroa and Densen 1991). Deficiency of any of the terminal complement components (C5 to C9) is associated with an increased risk of meningococcal disease, especially of serotypes not normally regarded as invasive, including X, Y, and W-135 (Fijen et al. 1989). The risk of second and subsequent attacks is also increased but disease tends to be milder (Ross and Densen 1984). Half the individuals with the very rare deficiency of properdin (factor P) develop meningococcal disease, but this tends to occur in older children or adults and is often overwhelming, with high fatality rates (Densen et al. 1987). There are at least three genetic mechanisms leading to properdin deficiency; all three identified to date are inherited in an X-linked manner (Linton and Morgan 1999).

Recent evidence suggests that mannose-binding lectin, part of a phylogenetically older innate immune system,

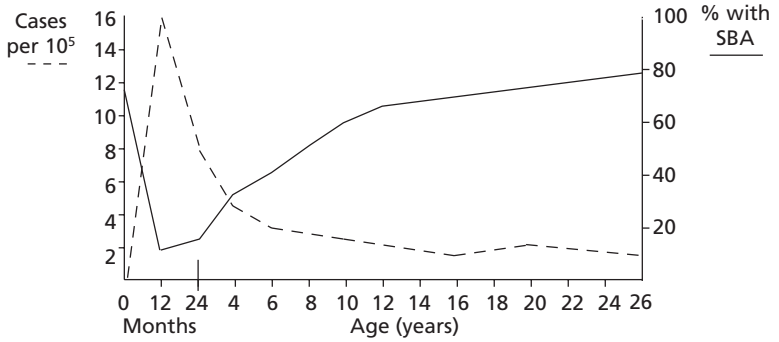

Figure 5 *Meningococcal disease: relationship to bactericidal antibodies (after Goldschneider et al. 1969a, with permission).*

plays an important role in early defense from meningococcal (and other invasive bacterial) infections (Summerfield et al. 1997), and mutations in the mannose-binding lectin gene may be associated with an increased susceptibility to meningococcal infection. There is also evidence that particular patterns of inherited cytokine profile may be associated with a higher risk of a fatal outcome in meningococcal disease (Westendorp et al. 1997). More recently, polymorphisms of genes encoding plasminogen activator inhibitor 1 and IL-1β have been shown to be associated with the development of septic shock or with increased mortality in meningococcal disease (Westendorp et al. 1999; Read et al. 2000), and FcγRIIa (CD32) polymorphisms appear to be associated with susceptibility to, and severity of, meningococcal disease (Bredius et al. 1994; Platonov et al. 1998).

SUSCEPTIBILITY AT DIFFERENT AGES

Meningococcal infection is infrequent in the first 2 months of life, indicating the likely key role of passively transferred maternal antibodies in initial protection. Subsequently, meningococcal disease in infants and young children probably results from exposure of an immunologically virgin child to a virulent meningococcus. Carriage studies suggest that exposure to virulent meningococci is probably uncommon in infancy. This seems less likely in teenagers and young adults in whom there is also a peak of infection (see Figure 4), but at a time when meningococcal carriage is most frequent. Most, if not all, individuals aged 10 years or more will probably have encountered *N. lactamica*, poorly capsulate meningococci, and/or other immunizing bacteria on numerous occasions. Therefore, meningococcal disease in this age group may result from inherited increased susceptibility, a high level of exposure to meningococci, subversion of pre-existing defense mechanisms by environmental factors such as antecedent or intercurrent viral infection, or a combination of these factors.

Meningitis and septicemia

Meningococcal infection most commonly presents as meningitis (75 percent) or septicemia (20 percent), though the two syndromes show considerable overlap; clinical differentiation may be difficult or impossible, and UK clinicians vary in their practice as to what disease they choose to notify; recently, proportionately more cases have been notified as septicemic (Figure 6).

A few patients have other pyogenic infections such as primary septic arthritis, conjunctivitis, and pericarditis. Primary meningococcal pneumonia is usually caused by strains of serogroup Y. Increasing numbers of cases have been reported from the USA in recent years, and this condition may be underdiagnosed in the UK.

The outlook in meningococcal meningitis is very good. Mortality in the UK is less than 5 percent, and the incidence of long-term neurological morbidity, mainly deafness, is low (3–5 percent), though a small percentage of survivors suffer severe or even catastrophic brain damage. Most meningococcal meningitis occurs in infants and young children in whom the symptoms and signs are similar to those of other types of bacterial meningitis occurring at the same age. A common, though not invariable, feature of meningococcal meningitis is the presence of petechiae or purpura in the skin. Though an ecchymotic skin rash may also occur occasionally in Hib or pneumococcal meningitis (and in a wide range of noninfectious conditions), it is seen in more than 50 percent of cases of meningococcal meningitis and in all cases of meningococcal septicemia. The pathology of the skin lesions resembles a series of localized Shwartzman reactions (DeVoe 1982). Nondescript, macular, flat or raised nonvasculitic, evanescent skin rashes are very common in the early stages of development of meningococcal infection in children (Marzouk et al. 1991). They may be mistaken for the rash of viral infection and may cause delay in diagnosis. Such rashes

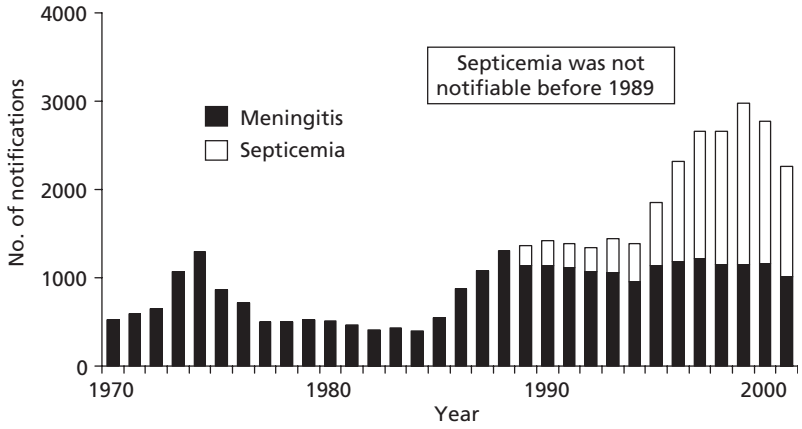

Figure 6 *Meningococcal disease, England and Wales: annual numbers of notifications, 1970–2001*

disappear with the onset of the more characteristic vasculitic rash.

Meningococcal meningitis in older patients usually presents with symptoms of malaise, muscular aches and pains, diarrhea together with vomiting, fever, and headache, progressing if unrecognized to confusion, coma, shock, and circulatory collapse. In the later stages, an ecchymotic skin rash is almost inevitable.

Septicemia in children or adults is a far more serious condition, with a mortality greater than 20–25 percent. It can occur with or without evidence of meningitis. Fever, shock, and an ecchymotic skin rash are the hallmarks of this condition. Onset is frequently abrupt, over a few hours. Multisystem failure is common. In addition to antibiotics, oxygen, aggressive fluid replacement, correction of electrolyte imbalances, inotropes, mechanical ventilation, control of disseminated intravascular coagulation, and hemodialysis may all be required for considerable periods. Survivors of severe septicemia may need amputations of necrotic fingers, toes, or sometimes limbs; a few suffer severe brain damage, probably associated with prolonged periods of hypotension and hypoxia.

Management

ANTIBIOTIC TREATMENT

Benzylpenicillin has traditionally been regarded as the drug of choice in meningococcal disease. The England Chief Medical Officer recommends that general practitioners and casualty doctors should administer a dose of benzylpenicillin to any patient with suspected meningococcal disease, without awaiting the results of laboratory investigations. This course of action may halve mortality in the more seriously ill and is strongly endorsed, though it makes confirmation of the diagnosis somewhat more difficult (Cartwright et al. 1992) (Figure 7). Fortunately, the availability of molecular diagnostic tests on blood and CSF have largely obviated this problem. The combination of fever and a vasculitic (nonblanching) skin rash must be taken to indicate the possibility of meningococcal disease until an alternative diagnosis is established. This combination of symptoms constitutes a medical emergency. As well as administering benzylpenicillin, general practitioners are urged to arrange immediate ('blue-light') transfer of the patient to hospital.

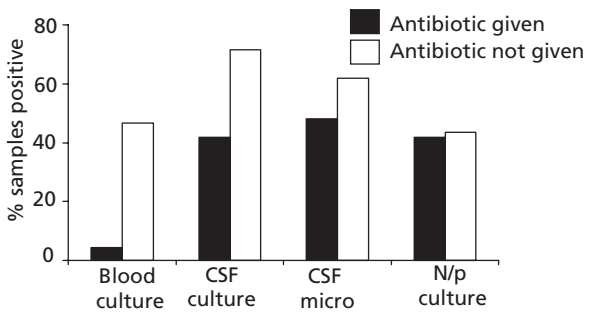

Figure 7 *Effect of parenteral antibiotics on laboratory investigations in meningococcal disease (from Cartwright et al. 1992, with permission).*

Since the etiology will probably not be known for certain at the start of inpatient treatment, it is wise to use a broad-spectrum antibiotic for the first 24–48 h, especially in young children. Cefotaxime, ceftriaxone, or, if cost is a factor, chloramphenicol are suitable alternatives that give high rates of cure; these agents can also be used in patients who are allergic to penicillin. Cefuroxime is inferior. Treatment may be switched to benzylpenicillin when a meningococcal etiology has been confirmed. Wall has argued for the use of cefotaxime or ceftriaxone as agents of first choice for treatment of meningococcal meningitis throughout on grounds of high activity and high penetration into the subarachnoid space (Wall 2001). In view of the very high success rates achieved with benzylpenicillin in this condition, prospective randomized trials would be very difficult or impossible.

'Penicillin-resistant' meningococci

So-called 'penicillin-resistant' meningococci have been reported in a number of countries and regions, including Spain (Sáez-Nieto et al. 1992), South Africa, the UK, and the North American continent. These strains are better described as showing reduced sensitivity to penicillin, and it is likely that such reduced sensitivity results from acquisition of genetic material from related neisserial species including *Neisseria flavescens*, an oropharyngeal commensal, leading to production of an altered penicillin-binding protein 2 (Spratt et al. 1989). Illness caused by these strains responds almost invariably to treatment with adequate doses of benzylpenicillin.

Production of β-lactamase by meningococci isolated from invasive infections has been reported from South Africa and Spain (Botha 1988; Fontanals et al. 1989). Neither strain has survived for detailed investigation. In view of the known transformability of meningococci, it is more than a little surprising (albeit very welcome) that β-lactamase-producing meningococci have not been encountered more often.

Duration of antibiotic treatment

Antibiotic treatment for 7 days usually suffices for children with mild illness (McCracken et al. 1987; O'Neill 1993). Longer periods of treatment, of at least 10 days, are often used in more seriously affected patients, including those with septicemia, even though all meningococci are likely to have been killed soon after the commencement of treatment. Very occasionally, meningococci may survive within the subarachnoid

space in a loculated space after a short period of antibiotic treatment, subsequently to re-emerge and to cause relapse. Normally, they are killed and cleared rapidly from the subarachnoid space (Feldman 1977; Kanegaye et al. 2001).

STEROIDS AND IMMUNOMODULATORS

The role of steroids in meningococcal disease remains controversial. A meta-analysis of trials undertaken in the 1990s showed a reduction in the incidence of neurological sequelae, mainly deafness, in patients with bacterial meningitis when dexamethasone was given before or with the first dose of antibiotics (Feigin et al. 1992; Schaad et al. 1993; Jafari and McCracken 1994). Most patients had Hib meningitis. Since the pathology of neurological damage is probably similar for the three main bacterial pathogens, it seems logical to endorse this addition to the treatment of meningococcal and pneumococcal meningitis. Steroids are ineffective in bacterial meningitis if given more than about an hour after the first parenteral dose of antibiotic. A 2-day course is probably as effective as treatment for 4 days (Syrogiannopoulos et al. 1994). After 48 h the cytokine inflammatory cascade in the CSF should have stabilized.

There is no evidence to support the use of dexamethasone in meningococcal septicemia (Nadel et al. 1995). Various novel therapies for severe meningococcal disease are under consideration or evaluation, including antiendotoxins, anticytokine response agents, leukocyte activation antagonists, cardiovascular support agents, and many others (Nadel et al. 1995). Some encouraging results have been achieved in small uncontrolled studies (Giroir et al. 1997; Goldman et al. 1997; Smith et al. 1997).

Recent evidence indicates that case-fatality rates in meningococcal infection have fallen significantly in centers of excellence (Thorburn et al. 2001; Booy et al. 2001). These analyses indicate that this is almost certainly due to improved clinical management, since the trend to lower mortality was based on children with comparable disease severity scores. The marked trend to lower case-fatality rates that has been observed nationally across the UK could reflect relatively more efficient ascertainment of surviving cases than fatal cases but is also likely to include a number of other factors, including earlier recognition of possible meningococcal infection by parents of young children, the greater use of pre-admission parenteral penicillin, and improved management in hospital. The UK national case fatality rate is now below 7 percent and maintaining a falling trend.

SECONDARY CASES AND CLUSTERS

Close contacts of cases of meningococcal disease run an increased risk of developing disease in the days and weeks after exposure to an index case, especially a first-degree relative living in the same household. The increased risk may be due to exposure to a known pathogenic strain, an inherited susceptibility to meningococcal infection, transient exposure to a common environmental factor (e.g. influenza virus) (Harrison et al. 1991), or a combination of these. An attempt has been made to estimate the proportion of risk attributable to host factors by calculating the sibling familial risk ratio (Haramboulos et al. 2003); these authors estimated that host genetic factors contributed approximately one third of the risk.

The nasopharyngeal carriage rate of meningococci is high in close family contacts of cases (Kristiansen et al. 1998). Rifampicin, ciprofloxacin, and ceftriaxone are all more than 90 percent successful in eliminating nasopharyngeal colonization in contacts (Cuevas and Hart 1993). They also reduce the incidence of secondary cases of disease in the 30–60 days after the illness of the index case. Though there have been no large prospective trials to establish the benefit of chemoprophylaxis, and though there is no evidence to show that the risk of secondary disease is reduced in the longer term, it is accepted policy to offer chemoprophylaxis to defined close contacts (Meningococcal Infections Working Group 1989). If the index case strain is of serogroup A or C, close contacts should also be offered A + C vaccine. Regardless of the causative organism, all contacts should be informed about their increased risk of meningococcal disease, which may persist for many months, especially when chemoprophylaxis has been given.

Clusters of cases sometimes occur in playgroups, schools, universities, military establishments, and other closed and semiclosed communities. Serogroup C strains are more commonly isolated in such outbreaks. Consideration should be given to the value of chemoprophylaxis, with or without vaccination, but each situation must be judged on its merits. The level of public alarm is often at variance with the actual risk (Hastings et al. 1997; Stuart et al. 1997). Cases of meningococcal disease occur occasionally in laboratory workers who have been handling the organism. Safety cabinets must always be used when handling liquid suspensions of virulent meningococci (Boutet et al. 2001).

Multilocus sequence typing (MLST) (Maiden et al. 1998) and other molecular characterization methods will play an increasingly important role in elucidating meningococcal microepidemiology and facilitating rational management of clusters and community outbreaks. MLST can also be applied to other genetically diverse bacterial species such as pneumococci, *Haemophilus influenzae*, etc.

Vaccines

The first vaccines for the prevention of meningococcal disease were developed in 1912 (Sophian and Black

1912). Uncontrolled field trials of a whole-cell meningococcal vaccine took place in England during World War I (Greenwood 1916). In the 1930s, Scherp and Rake (1935) identified capsular material from a meningococcus as a polysaccharide, but work on vaccine development, driven by outbreaks of disease amongst military recruits, waned when sulfonamides and then penicillin became available. With the emergence of sulfonamide resistance in the 1960s, interest in prevention by vaccination was reawakened, stimulated by the inability to control clusters of cases in recruit camps by sulfonamide prophylaxis. In a series of classical papers, Gotschlich, Goldschneider, and their colleagues at the Walter Reed Army Institute of Research, Washington, USA, demonstrated the fundamental importance of bactericidal antibodies in determining protection from invasive meningococcal disease. They went on to develop the first serogroup C polysaccharide vaccine to be properly evaluated in humans (Goldschneider et al. 1969a, b; Gotschlich et al. 1969a, b; Artenstein et al. 1970). Soon after this, a large clinical trial of a serogroup A polysaccharide vaccine was reported from Finland (Peltola et al. 1977), and further serogroup A vaccine studies in Africa followed.

SEROGROUP A AND C VACCINES

Vaccines containing serogroup A and C polysaccharides have been used quite widely in the UK, and quadrivalent A, C, Y, and W-135 vaccine is also available. Their use has declined with the availability of conjugated serogroup C vaccines (see below). Purified polysaccharide (i.e. nonconjugated) vaccines are not immunogenic in infants and give only short-term (3–4 years) protection to older children and adults. They are not suitable for universal use and are employed in a more limited set of circumstances (see Table 2).

A conjugated serogroup A and C vaccine has undergone preliminary evaluation in the Gambia (Twumasi et al. 1995). In the UK, conjugated serogroup C vaccines were evaluated in the latter half of the 1990s, were found to be safe and effective, and were then offered to all infants and older children commencing in November 1999 in the face of a high incidence of serogroup C disease (Miller et al. 2002). Their impact has been immediate and dramatic in the targeted age groups, with a reduction in serogroup C disease incidence now exceeding 80 percent (Figure 8). These conjugated vaccines also reduce the carriage of serogroup C meningococci (Maiden et al. 2002), and an impact on those older people who were not initially offered the vaccine is now becoming apparent, driven by reduced exposure, i.e. herd immunity. The exploitation of herd immunity as a deliberate strategy in the vaccine control of meningococcal disease has been advocated by Stephens (1999).

Table 2 *Uses of meningococcal serogroup A + C nonconjugated vaccine*

Uses
Protection of contacts
Protection of travelers to endemic areas
Control of epidemics
Outbreak control in defined communities
Outbreak control in open communities

Prior to the introduction of conjugated serogroup C vaccines in the UK, most serogroup C disease was caused by a single clone (ET-37). Since it was known that naturally occurring strains of this clone were capable of expressing a serogroup B capsule (as opposed to the much commoner expression of a serogroup C capsule), fears were expressed that though serogroup C disease might be reduced, this could be followed by an expansion of the population of ET-37 strains expressing the serogroup B capsule, with a consequent increase in the incidence of serogroup B disease. Despite very careful ongoing monitoring of both invasive and carried meningococcal strains, to date there is no evidence either of an increase in serogroup B disease overall or of any expansion in the numbers of cases of ET-37 serogroup B disease.

Conjugated meningococcal serogroup A vaccines are now being developed and subjected to clinical trials in the sub-Saharan meningitis belt in a program enjoying substantial funding from a US charitable foundation. The ultimate aim of the program is to offer serogroup A (or possibly A + C) conjugated vaccines to at-risk individuals across the whole meningitis belt, with a view to controlling epidemic meningococcal disease in this region.

Conjugated A, C, Y, and W-135 vaccines are also being developed and could have applications in the USA, where serogroup Y strains contribute significantly to the total burden of meningococcal disease, and also in travelers.

SEROGROUP B VACCINES

Serogroup B meningococcal polysaccharide is a weak antigen in humans and prospects for effective serogroup B vaccines are more distant. Occasionally, serogroup B disease can become hyperendemic, and a single strain variant can become responsible for the great majority of cases. In such circumstances it is possible to develop outer-membrane vesicle vaccines against the causative strain of meningococcus. Such vaccines have been developed in Norway (Bjune et al. 1991) and in Cuba (de Moraes et al. 1992); however, neither was effective in infants and there is some evidence that the duration of protection may be limited. In most countries (including the UK), serogroup B disease is caused by a heterogeneous range of serogroup B strains, and the approach of using a single serogroup B variant as the basis for a vaccine is inapplicable.

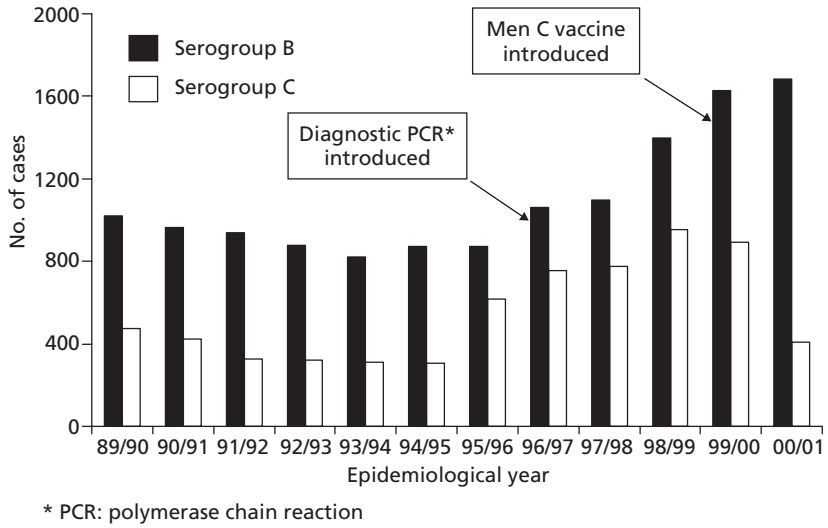

Figure 8 *Laboratory-confirmed cases of meningococcal disease by epidemiological year (July–June), England and Wales, 1989–90 to 2000–01.*

Since bactericidal antibody to serogroup B meningococci is directed against surface components other than the capsular polysaccharide, much effort is being invested in the search for stable, surface-expressed serogroup B antigens or epitopes that will evoke the development of human bactericidal antibodies (Poolman et al. 1995). However, meningococci are very well adapted human colonizers and many outer-membrane proteins have hypervariable regions, presumably to evade the host immune response. A further problem hampering development of serogroup B vaccines is the lack of correlation between the immune responses to candidate antigens in laboratory animals and in man.

Among the antigens currently under investigation are the class 1 outer-membrane protein (van der Ley and Poolman 1992; van der Ley et al. 1993) and iron-binding and iron-regulated proteins (Frasch 1995). Vaccines based on conjugated, chemically modified B polysaccharide are also being explored (Fusco et al. 1997). The recent publication of the entire genetic sequence of a well-characterized serogroup B meningococcus, MC58 (Tettelin et al. 2000) has facilitated the search for genes encoding surface-expressed, well-conserved antigens and may result in the identification of a number of novel targets relevant for vaccine development (Pizza et al. 2000). Progress in the development of serogroup B (and other meningococcal) vaccines has been reviewed by Morley and Pollard (2002) and by Jódar et al. (2002)

PNEUMOCOCCAL MENINGITIS

Unlike Hib meningitis, the incidence of pneumococcal meningitis in the UK has not declined in recent years. In countries that have introduced conjugated Hib vaccines, pneumococci are now the second most common cause of bacterial meningitis. Mortality remains high. Antibiotic resistance, not only to penicillin but also to chloramphenicol and third-generation cephalosporins, is becoming a serious therapeutic problem in many parts of the world (Jacobs and Appelbaum 1995; Tomasz 1995).

Rapid progress in the development of conjugated pneumococcal polysaccharide vaccines offers good prospects for reducing the overall incidence of invasive pneumococcal disease and more modest but still interesting possibilities for reducing the incidence of pneumococcal meningitis. For all these reasons, pneumococcal meningitis is an extremely important condition and will remain so for the foreseeable future.

Epidemiology

Streptococcus pneumoniae causes bacteremia and meningitis at all ages, but attack rates are highest in infants, falling to low levels in children and young adults. Bacteremia rates then rise again to very high levels in the elderly, though rates of meningitis do not (Figure 9).

Meningitis occurs in about one-sixth of all invasive pneumococcal infections. The highest meningitis attack rate occurs in the first week of life, when the invading organisms are probably acquired from the maternal birth canal. Males are affected more often than females and infection is more common in the winter months. In the UK, pneumococcal meningitis attack rates have remained fairly constant over the period 1991–2000 at around 0.5–0.6 per 100 000 of the total population per annum (Aszkenasy et al. 1995; PHLS unpublished data).

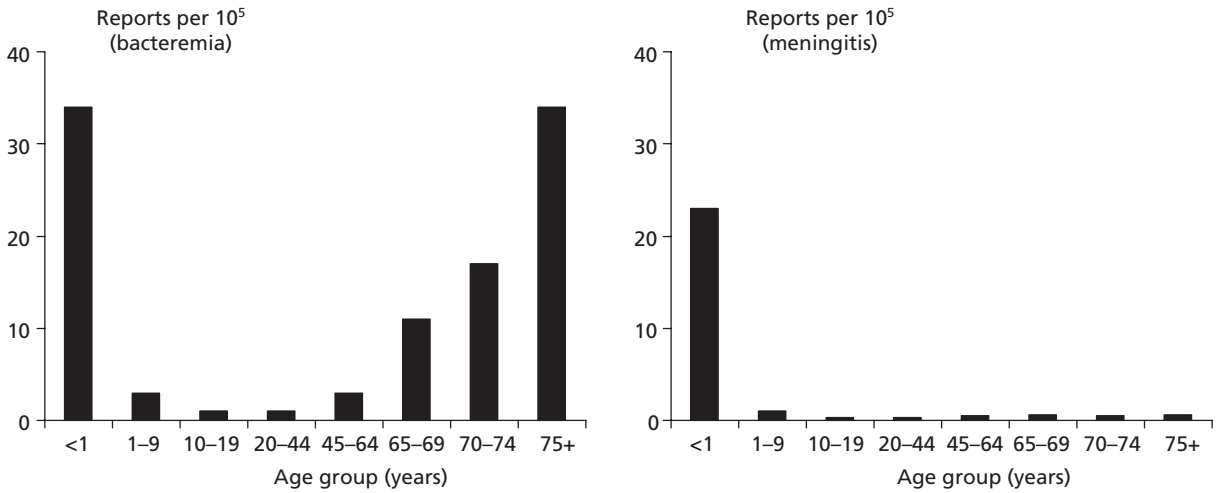

Figure 9 *Age incidence of* **(a)** *pneumococcal bacteremia and* **(b)** *meningitis: England and Wales, 1992.*

RISK FACTORS

Though age is the most important determinant of pneumococcal meningitis, patients with pneumococcal meningitis often have a pre-existing medical condition; recognized associations include splenectomy, thalassemia, sickle cell disease, alcoholism, myeloma, hypogammaglobulinemia, and complement deficiency. In the USA, rates of pneumococcal meningitis are much higher in nonwhites, though whether this is associated in part with socioeconomic factors is not clear.

Though pneumococci usually gain access to the subarachnoid space via the bloodstream, they may also spread directly from adjacent tissues, such as an infected paranasal sinus, the middle ear, or from a more distant focus of infection such as the lung. Recently it has been recognized that the risk of pneumococcal meningitis may be raised in recipients of some types of cochlear implants. Pneumococcal meningitis may follow any breach of the integrity of the subarachnoid space; this occurs most frequently following trauma (road accidents, etc.) resulting in dural tear. Pneumococcal meningitis following extrameningeal pneumococcal infection elsewhere in the body (and particularly pneumonia or endocarditis) carries a poor prognosis (Lepper and Dowling 1951; Bohr et al. 1985).

Only a small number of the 84 pneumococcal serotypes (1–9, 12, 14, 18, 19, 22, 23) are responsible for most invasive pneumococcal disease. Particular serotypes may be associated with central nervous system infection, some with a higher case fatality rate.

Clinical presentations and outcome

Clinical presentations depend on age but are similar to those of other types of acute pyogenic meningitis. Typically the presentation is abrupt, but symptoms may develop over several days. At presentation, patients with pneumococcal meningitis tend to be iller than those with Hib or meningococcal meningitis; fits are commoner (Pedersen and Henrichsen 1983) and there may be focal neurological signs, especially cranial nerve palsies. Comatose or semicomatose patients are more likely to have pneumococcal than meningococcal meningitis (Carpenter and Petersdorf 1962). Petechial rashes, though much less frequently encountered than in meningococcal meningitis, may also occur, especially in asplenic patients. In the neonatal period, clinical presentations are similar to those seen in other types of neonatal bacterial meningitis.

Elderly patients, patients with impaired consciousness or with convulsions on admission, and patients with a distant focus of pneumococcal infection are all more likely to die. Deafness and other permanent neurological sequelae are encountered frequently in survivors of pneumococcal meningitis.

Morbidity and mortality in pneumococcal meningitis are much worse than in Hib or meningococcal meningitis. Sangster and colleagues reported a mortality rate of 25 percent in the UK in the 1970s (Sangster et al. 1982) and mortality rates of 40–50 percent are commonplace in pneumococcal meningitis in tropical Africa (Greenwood 1987). Case fatality rates are still probably in excess of 20 percent in the UK and higher in the elderly. A similar proportion of survivors are left with moderate or severe neurological disability. This may be related in part to the much higher CSF bacterial load in pneumococcal meningitis than in meningococcal meningitis (Hassan-King et al. 1984) and to the long persistence of live bacteria within the subarachnoid space after the commencement of antibiotic treatment (Greenwood et al. 1986), the latter possibly associated with the particular capacity of pneumococci to induce a viscid, gelatinous exudate into which antibiotic penetration is poor.

Treatment

ANTIBIOTICS

Until quite recently, pneumococci were sensitive to penicillin in almost all parts of the world. When the causative strain is fully sensitive (minimum inhibitory concentration (MIC) 0.1 μg/ml), benzylpenicillin in large doses remains the antibiotic treatment of first choice. Schmidt and Sesler (1943) were the first to report penicillin resistance. Multiple antibiotic-resistant strains are now encountered frequently in the USA, Australia, New Guinea, and South Africa (Lister 1995; Schwartz and Tunkel 1995). The prevalence of moderately penicillin-resistant strains (MIC 0.1–1.0 μg/ml) varies widely from country to country; in England and Wales it was 2 percent in 1995 (Aszkenasy et al. 1995) rising to 7 percent in 2000 (PHLS unpublished data), 25 percent in the USA, and 40–45 percent in Spain and South Africa (Friedland and Klugman 1992a; Tomasz 1995); in almost all countries the prevalence of moderately resistant and of highly resistant strains (MIC 2.0 μg ml/1 or greater) is increasing (Breiman et al. 1994). Penicillin resistance may have arisen through transfer of genetic material from strains of *Streptococcus mitis* (Coffey et al. 1995).

Pneumococci resistant to third-generation cephalosporins have been documented in the USA and in South Africa (Friedland and Klugman 1992a; Tenover et al. 1992). Detection of resistance to β-lactam antibiotics (Tenover et al. 1992) is not merely of interest in the laboratory: it is associated with treatment failure (Bradley and Connor 1991; Sloas et al. 1992). In countries with a high prevalence of low-level pneumococcal penicillin resistance, third-generation cephalosporins such as cefotaxime and ceftriaxone are widely used as empirical agents of first choice in bacterial meningitis. However, as the level of resistance to penicillin rises, cephalosporins and chloramphenicol lose their efficacy (Friedland and Klugman 1992b; Lister 1995). Antibiotic treatment options for penicillin and cephalosporin-resistant pneumococcal meningitis have been reviewed (Jacobs and Appelbaum 1995; Lister 1995; Schwartz and Tunkel 1995). Chloramphenicol should not be used, even though strains may appear sensitive in the laboratory. Vancomycin, with or without the addition of rifampicin, becomes the antibiotic of choice, even though it penetrates poorly into the CSF. McCracken's group advocate the use of vancomycin accompanied by cefotaxime or ceftriaxone, and with the addition of dexamethasone for the empirical treatment of suspected pneumococcal meningitis (Paris et al. 1995). To cover meningococci, benzylpenicillin or a cephalosporin should always be incorporated into any empirical treatment regimen for bacterial meningitis when the causative organism has not been identified. Though data are scanty, most experts treat pneumococcal meningitis with parenteral antibiotics for at least 10–14 days (McCracken et al. 1987; O'Neill 1993).

STEROIDS

Dexamethasone reduces morbidity in pneumococcal meningitis. A recent pan-European study has demonstrated significantly improved outcome when dexamethasone is given before or with the first dose of parenteral antibiotic in pneumococcal meningitis in adults (de Gans and de Beek 2002; Tunkel and Scheld 2002). If a number of practical issues can be addressed, this may be advocated more widely as part of management protocols if cases of suspected pneumococcal meningitis in adults can be identified reasonably accurately.

MANAGEMENT OF CONTACTS

Contact with a case of pneumococcal meningitis does not usually confer an increased risk of disease. Neither antibiotic prophylaxis nor vaccination is normally recommended to protect intimate contacts of patients with pneumococcal meningitis.

RECURRENT PNEUMOCOCCAL MENINGITIS

The occurrence of a second episode of pneumococcal meningitis in a patient should raise the strong suspicion of a dural tear and there is usually, but not always, a history of cranial trauma. Such patients should be referred to a neurosurgeon for evaluation. A few patients with a history of recurrent pneumococcal meningitis but without a dural defect respond poorly to pneumococcal capsular polysaccharides; usually they respond to conjugated pneumococcal vaccines.

Prevention

PNEUMOCOCCAL VACCINES

Pneumococcal capsular polysaccharides are major virulence determinants and are immunogenic; vaccines based on capsular polysaccharides have been used for more than 20 years. Multivalent vaccines are used because there are more than 80 serotypes and protection is serotype specific. Vaccines containing 6–14 common serotypes were used in the 1970s but were superseded by 23-valent vaccines in the 1980s. Though the frequency with which the different serotypes cause invasive disease varies from country to country and across different age groups (Scott et al. 1996), the currently available 23-valent polysaccharide vaccines offer a high degree of protection in most, if not all, countries.

Purified pneumococcal polysaccharides do not make ideal vaccines (Mitchell and Andrew 1995). In common with other bacterial polysaccharides, they are high-molecular-weight compounds with repeating structures that stimulate B lymphocytes directly without

Table 3 *Characteristics of T-cell-independent (TI) and T-cell-dependent (TD) antibody responses*

Characteristics	TI	TD
T cells required	–	+
Ontogeny of response	Late	Early
Induction of memory	–	+
Isotype restriction	+	–
Affinity maturation	–	+

Purified polysaccharides are converted from TI to TD antigens by conjugation to carrier proteins, e.g. tetanus toxoid.

stimulating T cells. T-cell-independent antigens have a number of defects as vaccine candidates (Table 3).

In older children and adults, the antibody induced by plain polysaccharide vaccines does not persist, leading to waning of protection over time. The capacity of the 23-valent vaccine to induce good immune responses declines progressively after the age of 60–65 years, particularly in patient groups at increased risk of invasive disease, including those with asplenia, hyposplenism, and a variety of immunodeficiency conditions, including HIV infection. Patients with AIDS and CD4 counts greater than 500/mm^3 respond well, those with lower counts less satisfactorily (Rodriguez-Barradas et al. 1992). Thus, the groups at highest risk of invasive pneumococcal disease are the most difficult to protect with purified polysaccharide vaccines. Nevertheless, the 23-valent polysaccharide vaccine has been recommended for a wide variety of groups at increased risk of invasive or severe disease. These include patients aged over 2 years with functional or anatomical asplenia or hyposplenism (including sickle cell disease), patients with AIDS (regardless of the stage of illness) and other immunodeficiency states, malignancies, chronic respiratory, renal, and liver diseases, and some metabolic diseases such as diabetes (Department of Health 1996). Plain polysaccharide vaccines offer healthy elderly people protection against invasive pneumococcal disease (and may thereby protect against meningitis) but may not protect against nonbacteremic pneumonia.

Vaccination does not protect against recurrent pneumococcal meningitis associated with dural tears; repair of the defect is the only effective treatment known. Revaccination with 23-valent pneumococcal polysaccharide vaccine within 18 months of a previous dose is associated with a higher incidence of side effects (Borgono et al. 1978), but there is no evidence that revaccination after 4–6 years causes increased side effects (Kaplan et al. 1986a; Mufson et al. 1991).

Conjugated pneumococcal polysaccharide vaccines have now been subjected to clinical trials. A study of a seven-valent conjugated pneumococcal vaccine in infants in California showed excellent protection against invasive disease (Black et al. 2000). Similar conjugated vaccines containing a greater number of pneumococcal serotypes are expected to become available shortly. They should give rise to long-lasting, though serotype-specific, protection at almost all ages. Conjugated pneumococcal vaccines also reduce nasopharyngeal carriage of homologous pneumococcal serotypes (Dagan et al. 1996) and thus a herd immunity effect may occur if such vaccines were to be widely used in children. Pneumococcal conjugated vaccines will be expensive and may need to be tailored to the serotypes prevalent in different countries.

Attempts to produce pneumococcal vaccines based on surface-exposed proteins expressed by all pneumococci, regardless of serotype, have so far been unsuccessful. Research is continuing on a number of candidate proteins, including pneumolysin, neuraminidase, pneumococcal surface protein A, and a 37-kDa outer-membrane protein. Such outer-membrane proteins may become constituents of pneumococcal vaccines in the future, but are unlikely stand-alone vaccine candidates (Mitchell and Andrew 1995).

Pneumococcal meningitis in the first month of life is probably not preventable by infant vaccination, even at birth. Alternative strategies, such as maternal immunization during or before pregnancy, or passive protection of newborn infants with immunoglobulin followed by active immunization at a later date, may need to be considered.

ANTIBIOTIC PROPHYLAXIS

Long-term oral penicillin or amoxycillin prophylaxis has been used in an attempt to reduce the risk of invasive pneumococcal disease in patients at increased risk, but there is no evidence that prophylactic antibiotics can reduce the risk of primary or recurrent pneumococcal meningitis.

Haemophilus influenzae type b meningitis

EPIDEMIOLOGY

Before the widespread introduction of effective vaccines, Hib was the commonest cause of bacterial meningitis in children under the age of 5 years in almost all countries; in countries such as the USA, Australia, Brazil, and Zaire, it was the commonest cause of bacterial meningitis in the population as a whole. Within countries, attack rates of invasive Hib disease vary widely. In the UK, attack rates of 24–36 per 10^5 children under 5 years of age were observed in six regions in 1990–92 (Anderson et al. 1995), with almost 90 percent of invasive *H. influenzae* infections occurring in children under 5 years of age.

In contrast, attack rates of Hib disease in 'native American' and Australian aboriginal populations of 150–450 per 10^5 children aged under 5 years, i.e. rates ten-fold higher than in Europeans, have been recorded (Bijlmer 1991). In the USA, higher disease rates in

blacks than whites may have been due to socioeconomic rather than racial factors (Cochi et al. 1986). Though Hib meningitis has a relatively low case fatality rate in economically advanced countries (3–5 percent), high fatality rates (20–30 percent) are common in tropical Africa (Bijlmer 1991).

Before vaccination was introduced, most UK cases of Hib meningitis occurred in infants, with a peak attack rate in the 6–11 months age group (Anderson et al. 1995); in population groups with very high disease attack rates, peak attack rates occurred in children under 6 months old (Bijlmer 1991). A peak of disease at such an early age presented problems in the deployment of vaccines.

The increasing incidence of invasive Hib disease in the USA and the UK in the 1970s and 1980s was probably due to social changes as well as more accurate diagnosis. Attendance at daycare centers or preschool nurseries may have conferred an increased risk of disease; conversely, breast feeding was protective (Redmond and Pichichero 1984; Cochi et al. 1986).

Bacteriology and immunology

Acapsulate strains of *H. influenzae* are part of the human nasopharyngeal commensal flora. They are of low pathogenicity and cause mainly localized infections in the respiratory tract in young children and in patients with pre-existing disease.

A small minority of *H. influenzae* strains possess a polysaccharide capsule, of which structural variants define six serotypes designated a–f. The type b polysaccharide is a polymer of ribose and ribitol phosphate (polyribosephosphate (PRP)). Before vaccination was introduced, type b strains (Hib) were responsible for more than 95 percent of invasive hemophilus disease. Meningitis was the commonest clinical manifestation, followed by epiglottitis, bacteremia without localizing features, cellulitis, pneumonia, and septic arthritis (Anderson et al. 1995). Most, if not all, invasive Hib disease is accompanied by a bacteremic phase.

Hib is carried mainly by children, but the overall carriage rate in unimmunized populations is only 1 percent in children under 6 years of age; the peak of carriage occurs in children aged 24–36 months (Howard et al. 1988).

The early classical investigations of Fothergill and Wright (1933) showed that blood samples from children aged between 3 months and 3 years lacked bactericidal activity against Hib, in contrast to samples from neonates, older children, and adults. Subsequently Alexander et al. (1942) showed that administration of Hib antiserum caused a substantial increase in the phagocytosis of Hib bacteria in the subarachnoid space, suggesting the importance of opsonizing type-specific antibodies.

The carriage rate of Hib strains in young children is so low that many who acquire antibodies to PRP must do so without exposure to Hib strains. Their protective antibodies probably result from exposure to heterologous bacteria that produce polysaccharides with antigenic determinants identical to those on PRP. Amongst several other bacterial species, *Escherichia coli* K100 expresses an almost identical polysaccharide and, if fed to adult volunteers or laboratory animals, induces antibodies that show bactericidal and opsonic activity against Hib (Schneerson and Robbins 1975).

Bacteremia precedes Hib meningitis, epiglottitis, and the other invasive Hib syndromes. Why the bacteria localize in the meninges, or other organs, is not well understood (Moxon 1992). Experiments in an infant rat model indicate that the intensity of bacteremia may be an important factor in the development of meningitis (Moxon and Ostrow 1977).

Clinical features

The disease may develop insidiously over 24–48 h or it may present more abruptly. Most cases are in infants, who often start by being 'snuffly.' Fever and vomiting are common, early, nonspecific symptoms (Haggerty and Ziai 1964) that may be accompanied by failure to feed, pallor, irritability, and persistent crying. As the infection progresses, shock may supervene and the level of consciousness may become clearly depressed with listlessness and inability to maintain eye contact; infants may be extremely ill by the time the diagnosis is made. Seizures occur in a minority of cases (Feigin 1987), more commonly in Hib and pneumococcal meningitis than in meningococcal meningitis.

In older children, the more traditional symptoms of meningitis – headache, neck stiffness, and photophobia – may be encountered.

Treatment

Intravenous chloramphenicol replaced ampicillin when resistance of Hib to the latter due to β-lactamase production began to increase; in 2002 it was 15 percent in the UK but is higher elsewhere. Chloramphenicol fell into disuse partly because of a small but increasing percentage of resistant strains (1–2 percent), but also because of fears of irreversible bone-marrow suppression. It has been replaced by third-generation cephalosporins. Cefotaxime and ceftriaxone are superior to cefuroxime; the latter takes longer to sterilize the CSF and is associated with a higher incidence of hearing loss (Schaad et al. 1990). Ceftriaxone may be given as a single daily dose and, in carefully selected patients with milder illness, it is suitable for outpatient completion of a course of parenteral antibiotic treatment for bacterial meningitis. Antimicrobial treatment of Hib meningitis

should normally be continued for 7–10 days (McCracken et al. 1987; O'Neill 1993).

Administration of dexamethasone to children with Hib meningitis before, or at the same time as, the first dose of antibiotic is given reduces the incidence of neurological sequelae, principally deafness (Lebel et al. 1988; Feigin et al. 1992; Schaad et al. 1993; McIntyre et al. 1997). Steroids probably act by blocking the inflammatory cascade induced by the release of endotoxin into the subarachnoid compartment. If given after the first dose of antibiotic, they are ineffective.

Susceptible contacts of cases of invasive Hib disease run a considerably increased risk of developing Hib disease in the weeks after the contact. The degree of increased risk depends on the intimacy of the contact; household contacts are at greater risk than daycare-center or preschool playgroup contacts. Unvaccinated close contacts aged up to 4 years should be offered rifampicin prophylaxis and an immediate course of Hib vaccine: three doses if aged under 1 year or one dose if aged 13–48 months. Adult contacts do not need Hib vaccine and require chemoprophylaxis only if an unimmunized contact aged under 4 years is in the household (Cartwright et al. 1994).

Vaccines

The first Hib vaccines consisted of purified type b capsular polysaccharide (PRP) and suffered from all the defects of T-cell-independent antigens (see Table 3). In spite of their disadvantages, PRP vaccines were widely used in the USA in the 1980s to immunize children aged over 18 months and they did reduce the incidence of invasive Hib disease albeit modestly.

Conjugated polysaccharides are T-cell-dependent antigens and their introduction as vaccines changed the situation dramatically, since such vaccines are immunogenic even in infants under 3 months of age. They induce high-avidity IgG antibodies and long-term immunity.

The impact of Hib vaccination is almost immediate, because almost all invasive Hib disease occurs in infants and very young children. In England and Wales, Hib vaccines were introduced in October 1992 and by 1994 there was a two-thirds reduction in invasive Hib disease and a continuing falling trend. Invasive Hib disease in unimmunized older people also fell, due to a reduction in the prevalence of Hib nasopharyngeal carriage and the beneficial effect of herd immunity

Hib vaccine is offered to all UK infants aged 2, 3, and 4 months and to selected at-risk groups such as asplenic adults. For financial and logistic reasons, these highly effective vaccines have hardly been used in poorer countries, where the need is greatest (Booy 1998).

Over the past 3 years there has been some evidence in the UK of a slight resurgence of invasive Hib disease (Figure 10) and it is now planned to offer a fourth,

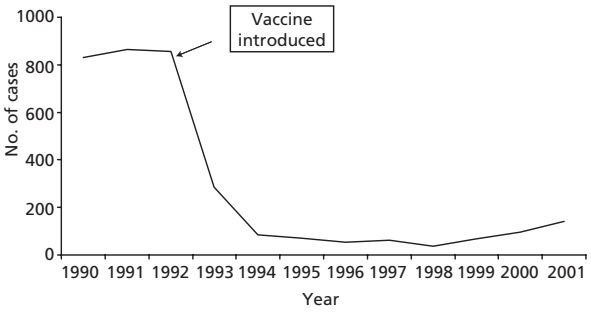

Figure 10 *Invasive* Haemophilus influenzae *type b (Hib) infections, England and Wales: Combined Health Protection Agency (HPA) Haemophilus Reference Unit & HPA Communicable Disease Surveillance Center data.*

reinforcing dose of Hib vaccine to all children. This will be given at the same time as the preschool doses of diphtheria, tetanus, and polio vaccines.

NEONATAL MENINGITIS

Neonatal bacterial meningitis (bacterial meningitis in the first month of life) is rare but serious, with a mortality up to 30–40 percent and permanent sequelae in up to 30 percent of survivors. The causative organisms are different from those of bacterial meningitis at other ages, since most bacterial meningitis in this age group is due to organisms derived from ascending infection in utero or from the birth canal during delivery (de Louvois 1994). Occasionally, outbreaks occur in hospital nurseries. Neonatal bacterial meningitis is almost always preceded by bacteremia. In the UK, the principal causative organisms are gram-negative enteric bacilli and group B streptococci. The latter can cause early-onset or late-onset disease as long as 3–4 months after birth. Other bacteria, including pneumococci, *Listeria monocytogenes*, meningococci, other streptococci, and *Staphylococcus aureus*, can also cause neonatal meningitis.

The overall rate of infection in the UK is about 0.25 per 1 000 live births (de Louvois 1994), whereas in the USA it may be two to three times higher (Riley 1972). Twenty years ago, gram-negative enteric bacteria were the most frequent cause of neonatal meningitis in the USA, but more recently, group B streptococci have predominated. The neonatal sepsis rate with group B streptococci is much higher in the USA than in the UK. In the USA, screening late in pregnancy and treatment of carriers has been advocated, but such a policy presents considerable practical problems (Towers 1995).

Prematurity, low birth weight, prolonged rupture of membranes, and prolonged labor all increase the risk of neonatal meningitis. Babies born to mothers who are pyrexial during labor are also at risk, and some infections are acquired in utero.

It is unclear why particular babies develop meningitis. Almost all strains of *E. coli* that cause neonatal

meningitis express the K1 capsular antigen (Mulder and Zanen 1984). Group B streptococci of type III are more commonly isolated from cases of neonatal meningitis than other group B streptococci, and both neonatal and maternal antibody levels to these organisms are lower in affected than in control babies.

Neonates frequently show little specific evidence of meningeal irritation. They may be 'jittery' or may present with listlessness, pallor, crying that may be high-pitched, failure to feed, vomiting, and sometimes jaundice; respiration, heart rate, and temperature control may become unstable. These nonspecific symptoms may progress to more obvious depression of consciousness. Raised intracranial pressure, as evidenced by a bulging fontanelle, occurs late, and only in a minority of cases.

Blood cultures are part of the standard investigation of any sick baby. If meningitis is suspected, lumbar puncture should be considered at an early stage, though failure to detect an abnormality on initial examination should never lead to the withholding of antibiotic treatment. Infection at another site such as the urinary tract should be excluded.

Once meningitis is confirmed, the choice of antibiotics depends on the causative organism. A combination of benzylpenicillin and gentamicin is often used while the results of blood and CSF culture are awaited and can be continued if group B streptococci are isolated. If a gram-negative bacillus is isolated, a combination of a cephalosporin and an aminoglycoside may be appropriate, but treatment should be guided by the antibiotic sensitivity of the particular organism.

TUBERCULOUS MENINGITIS

Tuberculous meningitis is rare. In the UK there are around 100 cases each year, the majority in people from the Indian subcontinent. After a steady decline over many years in the incidence of tuberculous disease, there has been a rising trend in notifications over the period 1988–2000 (9.4–12.0 cases per 10^5 per annum). In the USA, tuberculous meningitis is a well-recognized late complication of AIDS (Bishburg et al. 1986); atypical mycobacteria are sometimes responsible. So far, there is little evidence of an overlap between tuberculosis and AIDS in the UK.

Tuberculous meningitis probably always arises as a result of dissemination of tubercle bacilli from a distant focus of infection, often the lung. The disease is caused by liberation of tubercle bacilli from subpial tuberculomata into the subarachnoid space (Rich and McCordock 1933). Postmortem examination confirms the gelatinous nature of the exudate, particularly around the base of the brain, consistent with the high incidence of cranial nerve palsies seen in tuberculous meningitis.

The disease almost always presents insidiously with persistent fever and increasing headache over a period of weeks or months, but, rarely, the presentation may be acute. The cellular exudate in the CSF is most frequently lymphocytic, but it may be mixed, and the protein concentration is often very high (2–3 g/l). The CSF:blood glucose ratio is usually, but not always, reduced. In the majority of cases tubercle bacilli are not seen on direct microscopy. The culture of CSF for tubercle bacilli should be extended because about 10 percent of cultures will become positive only after 6 weeks. A mycobacterial PCR test is now available from the Regional Centres for Mycology in the UK for the investigation of suspected tuberculous meningitis.

Plain chest and abdominal radiographs may reveal a primary tuberculous focus, so increasing suspicions of tuberculous meningitis. Computed tomography and nuclear magnetic resonance imaging of the cranium are useful investigative techniques (Berger 1994). Since direct microscopy is insensitive, empirical antituberculous treatment may have to be started if the clinical picture is strongly suggestive. Treatment is usually based on a combination of rifampicin, isoniazid, and pyrazinamide, but the use of steroids is controversial. The prevalence of isoniazid and multidrug resistance has remained stable between 1994 and 1999 (Djuretic et al. 2002). In the UK, tuberculous meningitis is usually managed by chest physicians because of their familiarity with antituberculous therapy.

OTHER TYPES OF BACTERIAL MENINGITIS

In this brief review it is not possible to list all the more unusual types of bacterial meningitis. *Listeria monocytogenes* continues to cause a small number of cases of meningitis, mainly in the elderly and in dependent patients (Calder 1997). Fulminant listeria meningitis is accompanied by a high case fatality rate. Three nonviral causes of aseptic (lymphocytic) meningitis should be briefly mentioned – Lyme borreliosis (Pachner 1995), syphilis (Lukehart et al. 1988), and primary amebic meningoencephalitis (Carter 1968). These may all present acutely, but all are very rare in the UK. The history, such as that of a tick bite in Lyme meningitis, or of swimming in warm water before amebic meningoencephalitis, may provide diagnostic clues.

For cryptococcal and fungal meningitis see the Medical Mycology Volume, Cryptococcosis.

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Other CNS bacterial infections

FIONA E. DONALD

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INTRODUCTION

Focal pyogenic infections of the central nervous system (CNS) are life-threatening conditions that require treatment both surgically and with antibiotics. The most common three conditions are brain abscess, subdural empyema, and spinal abscess; however, these serious infections are uncommon with a cumulative lifetime incidence of approximately 1 percent for brain abscess.

Until the last quarter of the nineteenth century successful treatment for a brain abscess, commonly traumatic in origin, was rare. The first surgeon said to operate successfully on an otitic abscess was a Frenchman, Morand, in 1752 (Canale 1996). Further occasional successes were reported over the next hundred years (Weeds 1872). In 1893, William MacEwan, a surgeon from Glasgow, published a monograph that included details of his cases successfully treated by surgery (MacEwan 1893). Of 19 patients with brain abscess, 18 survived surgery, and this was a remarkable achievement at the time. By the beginning of the twentieth century surgical drainage of brain abscess was accepted as the treatment of choice.

Antibiotics began to play a part in treatment of abscesses as they were discovered throughout the first part of the twentieth century (Canale 1996). Mortality still remained high, however, until antibiotics such as penicillin and chloramphenicol were more widely used. The advent of better imaging techniques such as CT scans, and improved surgical techniques have been responsible for improving the outcome of these serious infections. In the field of microbiology an important advance was the upsurge of interest in obligate anaerobes and their role in human disease (Heineman and Braude 1963). The importance of anaerobic bacteria in CNS infections was emphasized and allowed antibiotic regimens to be tailored accordingly (De Louvois et al. 1977a; Ingham et al. 1977). Further advances which have been made in the last 10 years include the wider availability of MRI scanning which is able to pick up abscesses in the early stages, minimally invasive surgical techniques for abscess drainage, and new antimicrobial agents with better activity and CNS penetration.

INTRACRANIAL ABSCESS (BRAIN ABSCESS)

Epidemiology

Brain abscess accounts for about one in 10 000 general hospital admissions in the UK and USA each year and this incidence has remained relatively stable in the antibiotic era. Approximately four to ten cases are seen annually in neurosurgical units of developed countries each year (Donald et al. 1990; O'Donoghue et al. 1992; Richards et al. 1990). Some series have noted a slight increase in cases in recent years (Neilsen et al. 1982; Chun et al. 1986) that may be accounted for by better diagnostic techniques. Conversely other series have noted a decline in incidence (Nicolosi et al. 1991).

Mortality rates have ranged from 30 to 60 percent until the late 1970s (Garfield 1969) when the overall mortality dropped, and is now 0–24 percent (Seydoux and Francioli 1992; Donald et al. 1990; Ibrahim et al. 1990). This improvement has been attributed to better

radiological techniques to diagnose and localize the abscesses, better surgical techniques, and new more effective antimicrobial agents (Mampalam and Rosenblum 1988).

A male predominance exists in most series, with brain abscesses being two to three times more common in men than in women in most recent series (O'Donoghue et al. 1992; Seydoux and Francioli 1992). The median age is said to be 30–40 years; however, this will vary with predisposing factor and site of abscess. Incidence among other countries varies greatly and is related to differences in predisposing factors and underlying medical conditions (Ibrahim et al. 1990; Lu et al. 2002); for example, in China, 65 percent of brain abscesses are secondary to otitis media, while only 0.5 percent are associated with sinusitis (Yang 1981). In European countries, 20–40 percent occur in association with otitic infections and 15–25 percent with sinusitis (Bradley and Shaw 1983; Van Alphen and Driessen 1976).

Overall about 25 percent of brain abscess occur in children mostly in the 4–7 year age group (Neilsen et al. 1982; Kaplan 1985). Neonatal brain abscess is rare and is usually associated with gram-negative bacillary meningitis (Renier et al. 1988).

Changes in the epidemiology and causative organisms have occurred in recent years with the advent of HIV-related diseases and a larger group of immunosuppressed patients for example, following bone marrow transplant. In a series of bone marrow transplants, brain abscess was found to occur in one of every 49 patients (Hagensee et al. 1994). Solid organ transplant recipients are at increased risk of developing CNS infections – approximately 2–12 percent of solid organ recipients will develop CNS infections including brain abscess (Selby et al. 1997; Conti and Rubin 1988).

Predisposing conditions

The most common predisposing conditions are those which form a contiguous focus of infection, in other words infection in the ear, sinuses, or teeth (Table 1). Ear infections, such as acute or chronic otitis media, or mastoiditis have been most commonly reported in series with numbers ranging from 14 up to 60 percent (Lu et al. 2002; Alderson et al. 1981; Chun et al. 1986; O'Donoghue et al. 1992; Yang 1981; Ariza et al. 1986). The numbers vary with country of origin; overall, ear (otogenic) infections are the most common factor associated with contiguous spread.

Table 1 *Brain abscess: predisposing conditions and site of abscess*

Predisposing condition	Site of abscess
Chronic ear infection, mastoiditis	Temporal lobe, cerebellum
Paranasal sinusitis	Frontal lobe
frontoethmoidal	frontal
sphenoidal	temporal
Dental infection	Frontal lobe
Trauma or post surgical	Related to wound/operation site
Hematogenous spread	Middle cerebral artery distribution

The age incidence of otogenic brain abscesses shows a bimodal distribution with cases peaking in the pediatric age group then again in the over-40s (Small and Dale 1984). Otogenic brain abscess may be decreasing in relative frequency, as shown by a series from Sweden where otogenic abscesses accounted for only 7.4 percent (Schliamser et al. 1988). Areas where otitis media is neglected or therapy is delayed still see the serious complications associated with this condition. A series from Saudia Arabia found that chronic otitis media and sinusitis accounted for 57 percent of cases (Ibrahim et al. 1990).

The majority of otogenic brain abscesses (35–75 percent) are located in the temporal lobe (Figure 1). The cerebellum is the next most common site (20–30 percent) and otogenic infections account for 85–99 percent of cerebellar abscesses. Most of these are solitary lesions (Wispelwey et al. 1997).

Sinus disease is generally the next most common predisposing factor and is reported to account for 10–36 percent of cases in recent series (Alderson et al. 1981; Chun et al. 1986; Lu et al. 2002), but these figures may be showing a decrease as the early use of antibiotics may modify the disease process. Sinus disease is a major predisposing factor for subdural empyema. The age group most commonly affected is 10–30 years (Bradley et al. 1984). Generally cases of frontoethmoidal sinusitis will localize to the frontal lobe, and the rarely seen condition of sphenoidal sinusitis will produce abscesses in the frontal or temporal lobes (Table 1).

Dental infections as a source for intracranial abscess (odontogenic) are much less common but may lead to cavernous sinus thrombosis, subdural empyema, and meningitis. Rarely a brain abscess may complicate cavernous sinus thrombosis, but it is not clear whether the abscess develops as a coexisting event rather than as a consequence of the thrombosis (DiNubile 1988).

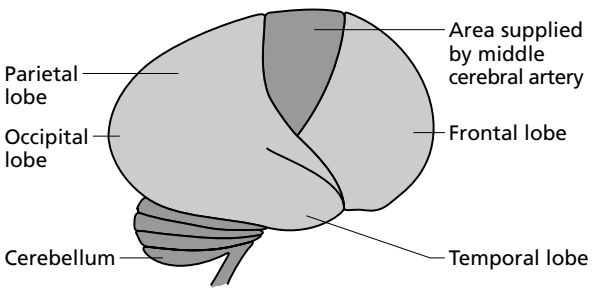

Figure 1 *Diagram of the brain showing the anatomical areas where abscesses occur.*

Odontogenic abscesses were found in six of 45 patients in one series (Chun et al. 1986), and in only five of 81 in another (Small and Dale 1984). Infection of the molar teeth is more likely to lead to intracranial complications as the bacteria can spread between the muscle planes to the base of the skull. Unrecognized dental infection may account for some abscesses where no other clear predisposing factor is evident and the bacteria cultured resemble those of dental origin (Ingham et al. 1978). Odontogenic abscesses are generally found in the frontal lobe. An odontogenic source should be considered in patients presenting with septic cavernous sinus thrombosis and any abscess of uncertain origin.

Hematogenous spread from a distant focus of infection usually causes multiple abscesses in the region of the middle cerebral artery distribution and at the junction of the gray matter and white matter (Figure 1). Hematogenous abscesses account for around 25 percent of cases in most series (Chun et al. 1986; Yang 1981). Endocarditis and pyogenic pulmonary infections such as lung abscess, bronchiectasis, empyema, and cystic fibrosis are common predispositions. Chronic suppurative lung disease has in the past accounted for one half of all hematogenous cases, but now may be becoming less common as a predisposing factor, and a study shows the number of cases dropping from 18 percent in the early 1970s to 2 percent in the 1980s (Mampalam and Rosenblum 1988). Congenital cyanotic heart disease, commonly seen as a predisposing factor in children, and pulmonary arterio-venous (AV) malformations increase the risk of hematogenous spread to brain and may account for 3–13 percent of cases (Park and Neches 1993). A report of 50 cases from India had 18 percent caused by congenital heart disease and 12 percent by pulmonary disease (Lakshmi et al. 1993). Other remote foci of infection which may lead to brain abscess are septic foci in abdomen, pelvis, urinary tract, or bones. Rarely cases have been reported following injection of bleeding varices (Cohen et al. 1985) and following tongue piercing (Martinello and Cooney 2003).

Endocarditis is an important infection that may be complicated by brain abscess; 1–5 percent of cases may go on to develop brain abscess (Lerner 1985). The lesions are mostly multiple. Endocarditis and subsequent central nervous system infection is increasingly being seen in injecting drug users (Tunkel and Pradhan 2002).

Hereditary hemorrhagic telangiectasia is a condition that carries a significant risk for developing brain abscess; this complication may develop in 1–6 percent of patients (Press and Ramsey 1984) most often in the third to fifth decade of life. AV malformations may occur in the brain as a result of this condition, providing a nidus for bacteria.

Trauma, either accidental injury to the brain or post neurosurgical procedure is an important risk factor, but less common than those listed above. Clean neurosurgical procedures, for example, craniotomy not associated with trauma, is complicated by infection in 0.6–1.7 percent of cases (Tenney 1986). Most of these cases are meningitis, about 10 percent are brain abscess.

Cases ascribed to trauma in published series vary greatly and will depend on epidemiological factors and availability of specialist neurosurgical units. Published figures for the incidence of cases related to trauma range from 12 to 37 percent (Schliamser et al. 1988; Lu et al. 2002; Ariza et al. 1986; O'Donoghue et al. 1992) and may be showing an increase as abscesses secondary to chronic lung disease and sinusitis decline. Accidental trauma, such as head injury with skull fractures and penetrating wounds with sharp objects, for example darts, are all reported to cause brain abscess. Abscesses may develop up to 113 days after the injury (Patir et al. 1995).

Bacterial meningitis as a predisposing factor for intracranial abscess is quite unusual, but may predispose to subdural empyema. The exception is in the neonatal age group when gram-negative bacilli may cause focal intracranial sepsis after meningitis (Renier et al. 1988).

Over the last decade there has been an increase in intracranial sepsis seen in immunosuppressed patients. This is an enlarging group of patients who may be immunosuppressed by disease such as HIV-related disease, by hematological malignancies and their treatment, and by immunosuppression following solid organ transplant. A varied range of microorganisms may be seen in these patients to include bacteria, fungi, and protozoa (Calfee and Wispelwey 2000).

In most cases of intracranial sepsis the primary source can be found, but in approximately 15–20 percent of cases no predisposing factor is identified. These are referred to as cryptogenic abscesses.

Pathogenesis and pathophysiology

The factors necessary for establishment of a brain abscess are a source of virulent microorganisms and the presence of ischemic or devitalized brain tissue. There may be a variety of primary foci and there is a close relationship between the primary focus and the bacterial flora isolated. Experimental evidence suggests that infection is difficult to establish in normal brain tissue (Molinari et al. 1973).

There are differences in the ability of microorganisms to produce brain abscess. Animal models of abscess formation studied *Escherichia coli* and *Staphylococcus aureus* injected into skin and brain tissue and found that the brain is much more susceptible to bacterial challenge than the skin with as few as 10^2 colony-forming units (cfu) of either organism resulting in abscess formation (Mendes et al. 1980). Capsular strains of *E. coli* were more virulent (Costello et al. 1983), the capsule of anaerobes such as *Bacteroides fragilis* may play a part in pathogenicity. Bacteria more commonly found in brain

abscess such as *Bacteroides fragilis* or *Streptococcus intermedius* failed to produce infection and abscess formation in an animal model (Onderonk et al. 1979). In most abscesses mixtures of organisms are found and the synergistic interactions undoubtedly play an important role in the pathogenesis of these mixed infections. The aerobic and facultative organisms may aid the anaerobes by using the available oxygen.

The role of other virulence factors in brain abscess formation has not been evaluated. Bacterial lipopolysaccharide has been studied in detail with regard to pathogenesis of bacterial meningitis, but similar studies looking at its effect on pathogenesis of brain abscess have not been done.

The development of a brain abscess from the earliest stages to the development of a capsule has been carefully studied (Britt et al. 1981) using an animal model and CT scanning. These are the stages that were described:

1. Early cerebritis days 1–3: There is a marked inflammatory infiltrate of polymorphonuclear cells, lymphocytes, and plasma cells from capillaries. Bacteria can be seen on Gram stain and there is marked edema of the white matter surrounding the lesion.
2. Late cerebritis days 4–9: A well-formed necrotic center has developed and enlarged and there is new vessel formation around the developing abscess.
3. Early capsule formation 10–13 days: The necrotic center has decreased slightly in size and there is development of a layer of fibroblasts around the center that will eventually form the capsule. The edema is diminishing.
4. Late capsule stage 14 days and later: The process continues with the development of the capsule.

Bacteriology of brain abscess

The range of bacteria isolated from intracerebral abscesses has undergone a marked change during the last 50 years. Several factors may account for this. There have been improvements in microbiology techniques especially anaerobic culture; increased use of antibiotics early in treating predisposing conditions, such as otitis media, may change the flora of the ear or render cultures negative; changes in patient susceptibility, for example, more immunocompromised patients; predisposing factors such as access to neurosurgery; and availability of laboratory facilities and anaerobic culture methods.

In the preantibiotic era the common isolates from brain abscess were *Staphylococcus aureus*, reportedly found in 25–30 percent of cases, streptococci in 30 percent, and coliforms in 12 percent: 50 percent of pus samples were culture negative (De Louvois et al. 1977a; De Louvois 1978). With improvements in anaerobic techniques the role of anaerobes has become apparent (Heineman and Braude 1963). Series from the UK have stressed the importance of anaerobes particularly in otogenic infections (O'Donoghue et al. 1992; Ingham et al. 1977; Donald et al. 1990; Richards et al. 1990). The site of the abscess and the predisposing conditions are important predictors of the likely organisms (Table 2). There seems to be a predominance of aerobic organisms reported but this varies with the center and anaerobes have been reported as being present in 33–60 percent of cases (Seydoux and Francioli 1992; Sofianou et al. 1996; Ariza et al. 1986; Chaudhry et al. 1998), higher numbers

Table 2 *Brain abscess: predisposing condition and likely bacterial isolates*

Predisposing condition	Likely bacterial isolates
Ear disease	Streptococci aerobic and anaerobic
	Bacteroides fragilis and other *Bacteroides* spp.
	Enterobacteriaceae especially *Proteus* spp.
	Pseudomonas aeruginosa
	Actinomyces spp.
Sinusitis	Microaerophilic and anaerobic streptococci
	Bacteroides spp.
	Fusobacterium spp.
	Haemophilus spp.
Dental infection	*Streptococci*
	Fusobacterium spp.
	Bacteroides spp.
	Actinomyces spp.
Hematogenous spread	
Congenital heart disease	Viridans streptococci
	Anaerobic, microaerophilic streptococci
	Haemophilus spp.
Lung disease	*Actinomyces* spp.
	Fusobacterium spp.
Endocarditis	Viridans streptococci
	Staphylococcus aureus
Immunosuppression	
HIV disease	*Listeria monocytogenes*
Neutropenia	Enterobacteriaceae
	Pseudomonas aeruginosa
	Streptococcus pneumoniae
	Nocardia spp.
	Mycobacteria
Trauma surgical	*Staphylococcus aureus*
	Propionibacterium
Trauma by penetrating injury	*Pseudomonas* spp.
	Clostridium spp.
	Enterobacteriaceae
Neonate	*Citrobacter diversus*
	Pseudomonas aeruginosa

being reported in later studies. This may reflect the increasing availability of anaerobic culture facilities and better diagnostic laboratory support. One recent study identified nine anaerobes in 18 patients, these included *Prevotella melaninogenicus*, *Bacteroides preacutus*, *Fusobacterium nucleatum*, and *Peptostreptococcus* spp. (Chaudhry et al. 1998). The samples were received in the laboratory within 30 min, inoculated into anaerobic broth and enriched thioglycollate broth which undoubtedly improved the isolation rate. Four out of six of their patients had chronic suppurative otitis media as the predisposing condition.

The bacteriology of otogenic abscesses can be complex with multiple isolates of bacteria being recorded (Alderson et al. 1981) and represent the microbial flora of a chronically infected ear, but usually with the exception of *Pseudomonas* spp. Isolates include gram-negative aerobic bacilli, such as *Escherichia coli* and *Proteus* spp., aerobic and anaerobic streptococci, and other anaerobes such as *Bacteroides fragilis*, other *Bacteroides* spp., *Eubacterium*, and *Clostridium* spp. It is unusual to find organisms normally associated with upper respiratory tract infection, such as *Haemophilus* spp. and pneumococci. The isolation rate of anaerobes does vary and Seydoux reported almost equal numbers of aerobes and anaerobes in his paper (Seydoux and Francioli 1992). *Fusobacterium* spp. were the most common anaerobes recovered, whilst viridans streptococci and *Streptococcus milleri* were the most common aerobic isolates.

The presence of anaerobes may be detected by noncultural methods such as gas-liquid chromatography (Donald et al. 1990); this investigation showed that 13 of 18 specimens of pus were found to be positive by this method.

Gram stain is an essential part of the investigation of brain abscess pus, particularly if antibiotics have been given. The morphology of bacteria seen on Gram stain will be helpful in choosing antibiotics especially if the cultures are subsequently negative (Lakshmi et al. 1993).

Actinomyces spp. are reported in mixed culture with other aerobic and anaerobic bacteria (Donald et al. 1990; Smego and Foglia 1998) and represent part of the normal flora of the mouth or respiratory tract. Actinomycosis is of course a recognized disease entity and may present with abscess formation in the central nervous system. Usually, in this condition *Actinomyces* spp. would be present either as a single isolate or in conjunction with associate anaerobic organisms.

A series from Taiwan (Lu et al. 2002) looked at isolates over two time periods. During the second time period there was an increase in aerobic gram-negative bacilli. Viridans streptococci and *Klebsiella pneumoniae* were the most common isolates from hematogenous spread. Nosocomial infections and traumatic infections showed an increase in the second time period.

In sinugenic brain abscess the bacteriology resembles that of sinugenic subdural empyema. *S. milleri* is the most common isolate and is often found in pure culture. Mixed cultures are less commonly reported and may be associated with chronic sinusitis.

In general, streptococci are common isolates in brain abscess and are reported in most series. A detailed study of streptococci associated with man reported *Streptococcus milleri* in 13 of 16 streptococcal brain abscesses (Parker and Ball 1976). Streptocci are commonly reported in more than 31 percent of brain abscesses – the actual figure may be higher than this as microaerophilic streptococci may be classified as anaerobes in some series. In some cases the reported figure is as high as 70 percent (De Louvois et al. 1977a; Ariza et al. 1986; Mathisen et al. 1985). The most frequently isolated are those of the *Streptococcus milleri* group (*anginosus*, *constellatus*, *intermedius*), which are well recognized to cause focal suppuration. An evaluation of infections caused by *Streptococcus milleri* found that 10 percent of them were brain abscesses (Molina et al. 1991).

Streptococcus pneumoniae is infrequently found in brain abscess despite being a common cause of meningitis. A review of 23 pneumococcal brain abscesses found that 50 percent of them had a contiguous focus such as sinusitis, otitis media or meningitis (Grigoriadis and Gold 1997).

Abscesses that follow trauma will show a range of bacteria related to the type of trauma. *Staphylococcus aureus* will be a common isolate after surgery, and is isolated in 10–15 percent of cases of postoperative brain abscess. Some of these will be methicillin resistant *Staphylococcus aureus* (MRSA) strains and are most likely to be in pure culture. *S. aureus* is also found in pure culture in intracerebral abscesses as a complication of endocarditis, and has recently been described in injecting drug users (Tunkel and Pradhan 2002).

Aerobic gram-negative bacilli are usually reported in mixed culture. Abscesses associated with chronic ear infections are most likely to yield these organisms, particularly *Proteus* spp. *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp. have also been recorded. *Klebsiella pneumoniae* has been associated with brain abscess in diabetics and produces a gas-forming appearance on X-ray imaging (Lu et al. 2002). Gram-negative bacilli have also been reported following neurosurgical procedures, accounting for 30 percent of cases in one report (Rau et al. 2002). *Pseudomonas aeruginosa* is rarely reported from otogenic abscesses despite its presence in the external ear canal of patients with chronic ear disease. It has been reported as a cause of meningitis and brain abscess in a premature baby (Shah et al. 1999). *Citrobacter freundii* has also been associated with neonatal brain abscess following meningitis (Curless 1980).

Listeria monocytogenes is a recognized cause of meningitis and brain stem encephalitis in the

immunosuppressed patient. Brain abscesses account for approximately 10 percent of cases (Eckburg et al. 2001) and are usually associated with concomitant meningitis and bacteremia in immunosuppressed patients.

Propionibacterium acnes is a true opportunist pathogen and is increasingly seen causing indolent infections in patients with prosthetic material. It is reported to cause post-operative brain abscesses which may be insidious in onset (Berenson and Bia 1989).

Focal intracranial infections with *Salmonella* spp. are rare; a review of the world literature identified only 43 cases, 11 of which were brain abscess mostly in adults (Rodriguez et al. 1986). Patients with HIV disease seem to be more at risk (Aliaga et al. 1997). *Salmonella typhi*, *typhimurium*, and *enteritidis* were the most common isolates. *S. typhi* has been reported to cause a brain abscess in a 2 month old (Hanel et al. 2000).

Nocardia spp. cause single or multiple abscesses and are associated with pulmonary disease or immunosuppression, or indeed as a primary infection in otherwise healthy individuals (Beaman and Beaman 1994). An increase in cases has been seen over the last decade in association with HIV disease. The causative organism is mostly *Nocardia asteroides* (Mamelak et al. 1994).

Rhodococcus equi is an unusual isolate from a brain abscess but was reported first in patients with HIV and recently in an immunocompetent patient (Corne et al. 2002).

Rare causes which have been reported in case reports are *Vibrio cholerae* non-0:1 (Ismail et al. 2001); *Mycoplasma hominis* and *Ureaplasma* (Rao et al. 2002); *Eikenella corrodens* (Asensi et al. 2002); *Capnocytophaga* (Engelhardt et al. 2002); and *Bacillus cereus* (Sakai et al. 2001).

Mycobacterium tuberculosis is a recognized cause of intracerebral lesions (tuberculomas) and the incidence is geographically determined. More cases are seen in Asia and the Far East than in Europe and North America. Acid-fast bacilli can be demonstrated in the pus from these lesions and should be actively looked for if cultures are negative and there are other risk factors for mycobacterial disease, such as immunosuppression and AIDS (Mathisen and Johnson 1997).

Clinical presentation

The clinical manifestations are caused by the presence of a space-occupying lesion in the brain, and the presentation may vary from indolent to fulminant. Symptoms may be nonspecific which can lead to delays in diagnosis. The most common symptom is headache, either hemicranial or generalized, and this may occur in up to 70 percent of patients (Table 3). Sudden worsening of the headache with associated meningism may indicate rupture of the abscess into the ventricle which carries a high mortality rate.

Table 3 *Common signs and symptoms of brain abscess*

Signs or symptoms	Frequency (%)
Headache	70–90
Focal neurology	>60
Fever	45–50
Triad of fever, headache, and focal neurology	<50
Seizures	25–35
Nausea and vomiting	25–50
Neck stiffness	25
Papilledema	25

Other common symptoms are fever, focal neurological signs, nausea, vomiting, and seizures which are usually generalized. If the abscess is near the meninges, neck stiffness may be present. The classic triad of fever, headache, and focal neurology is only seen in <50 percent of cases.

Other symptoms and signs vary with the location, size of the abscess, virulence of the infecting organisms, and the patient's immune status. Fever occurs in <50 percent of patients and the absence of fever should not exclude the diagnosis.

Patients with frontal lobe abscess are more likely to have mental status changes, speech disturbances or hemiparesis with unilateral motor signs. Those with a temporal lobe abscess may have headache, aphasia, and visual field defects. Cerebellar abscesses cause vomiting, ataxia, nystagmus, and dysmetria. Brain stem abscesses cause vomiting, hemiparesis, dysphagia, and facial weakness.

The symptoms caused by the contiguous focus of infection may predominate (for example, discharging ear). The duration of symptoms varies from a few days to as long as 6 weeks. About 75 percent of cases will have symptoms for 2 weeks (Wispelwey et al. 1997). In the immunocompromised patient the presentation may be occult because of reduced inflammatory response (Conti and Rubin 1988).

Diagnostic methods

The diagnosis of intracranial sepsis has been revolutionized by the introduction of CT and MRI scanning. These new techniques have rendered older more invasive diagnostic tests such as angiography and ventriculography virtually obsolete. Scanning methods also allow examination of the paranasal sinuses, mastoids, and middle ear.

The characteristic appearance of a brain abscess on a CT scan is that of a hypodense center with peripheral uniform ring enhancement following injection of contrast material, surrounded by a variable hypodense area of edema (Figure 2). MRI scanning is more sensitive particularly in the early stages and will detect

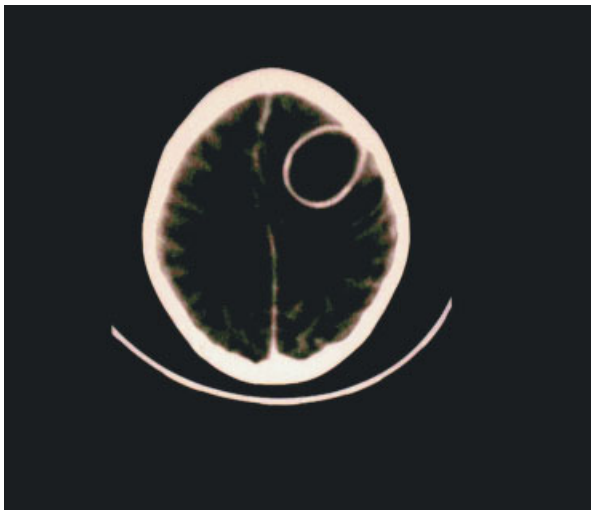

Figure 2 *Contrast enhanced CT scan showing ring enhancing frontal lobe lesion.*

abscesses in the early cerebritis stage (Figure 3). It is also better at defining brain stem abscesses. These forms of imaging are now the main diagnostic tool.

Diagnostic samples may be sent to microbiology; blood cultures will usually be negative. Lumbar puncture to obtain a sample of cerebrospinal fluid (CSF) is contraindicated as there is a significant risk of brain stem herniation (Chun et al. 1986), which outweighs any possible benefit of examining the CSF, which will show a negative or nonspecific result. The CSF may be positive if the abscess has ruptured into the ventricles or subarachnoid space.

Figure 3 *Gadolinium enhanced MRI scan showing temporal lobe abscess.*

There may be a peripheral leukocytosis, but 40 percent of patients will have a normal cell count. Other inflammatory indicators such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) may show elevation. The CRP may be more often raised in intracranial sepsis than in patients with neoplasm and may be useful in differentiating the diagnosis (Hirschberg and Bosnes 1987).

Samples of pus are readily sent for microbiology from surgery and are essential to define the infecting microorganisms, particularly in immunocompromised patients where a variety of bacteria, fungi, or parasites may be causing the infection.

Treatment

ANTIBIOTICS

The availability of antibiotics has undoubtedly contributed to the reduction in mortality seen with brain abscess. Prior to the discovery of antibiotics treatment was only surgical and usually led to death. In the early days of antibiotic use, large amounts of penicillin and streptomycin were instilled into abscess cavities and showed some promising results (Garfield 1969). The availability of more active agents in recent years has shown more improvements and now regimens can be tailored to cover the polymicrobial flora of brain abscesses.

When choosing an antibiotic to treat brain abscess, consideration has to be given to the penetration of the antibiotic into brain tissue. The cerebral capillary endothelial cells constitute the blood–brain barrier, which limits the penetration of numerous substances including antibiotics, into the brain. Inflammation as in meningitis disrupts the tight junctions and allows easier penetration of antibiotics, such as penicillin, into the brain. Other factors regulating antibiotic penetration into the brain include molecular size and structure, lipid solubility, degree of ionization at physiological pH, protein binding, and active transport mechanisms (Everett and Strausbaugh 1980). Antibiotic penetration into CSF has been extensively studied, but the blood–brain and the blood–CSF barriers are not identical and results may not be extrapolated (Gortvai et al. 1987). Therapeutic concerns about abscesses would be whether the results of CSF drug penetration would apply directly to brain abscess therapy, what would be the effect of the abscess environment on activity of antibiotics, and the possibility of negative drug interactions when multiple agents are administered. Certain antibiotics, for example penicillin and chloramphenicol, may be inactivated by abscess pus (Gortvai et al. 1987) and aminoglycosides are ineffective in the acid environment of an abscess. Chloramphenicol may antagonize the effects of penicillin and gentamicin as noted in experimental meningitis models (Tunkel and Scheld 1989). These are of course theoretical concerns

and few studies have addressed these issues in clinical practice.

The key question to ask is what is the concentration of antibiotic in the pus, and overall there have been only a few studies looking directly at this. The earliest study looked at brain tissue, CSF, and serum from 27 patients having a prefrontal lobotomy who had been given a dose of antibiotic preoperatively (Wellman et al. 1954). Tetracycline was detectable in the CSF and brain, but penicillin was not detected in either, perhaps reflected by the low dose that was given. Some other antibiotics were studied (Kramer et al. 1969) in patients having an intracranial neoplasm excised, they were given a bolus antibiotic dose of 2 g, and achieved the following brain:-blood levels: chloramphenicol 9:1, cephalothin 1:10, penicillin 1:23, ampicillin 1:56. On the basis of this study chloramphenicol has been used in the treatment of brain abscess for its excellent penetration into brain tissue; however, it lacks bactericidal activity and has the potential for serious side effects. Black and colleagues (Black et al. 1973) analyzed antibiotic concentrations in brain abscess pus from six patients. Chloramphenicol, methicillin, and penicillin were detectable after standard doses, but nafcillin was not. All the patients deteriorated clinically until surgical drainage was carried out; the cultures were still positive at the time of surgery. This highlighted the importance of surgery as part of the management. Another group analyzed pus from 32 patients (De Louvois et al. 1977b) for various antibiotics. Penicillin G was found to be present, but only if the dose exceeded 24 mega units per day. Incubating the pus for 1 h inactivated the penicillin by 90 percent in four of 22 samples (De Louvois and Hurley 1977). Fusidic acid was found to enter readily but cephaloridine, cloxacillin, and gentamicin showed only low levels. Metronidazole achieves high levels in pus (Ingham et al. 1977) and is widely used in combination to ensure bactericidal anaerobe activity. A single report looked at vancomycin levels in one case and found excellent concentrations (Levy et al. 1986). The first generation cephalosporins have not been recommended for treatment as they have poor CNS penetration, but the second and third generation agents have been shown to have very good CSF penetration and are widely used for treating meningitis. Of course the activity in brain pus cannot be predicted from that. Cefotaxime has been evaluated in brain pus (Sjolin et al. 1991) and it was found that cefotaxime and its active metabolite, desacetylcefotaxime, were present in the pus in concentrations above the minimum inhibitory concentrations (MIC) for the likely infecting organisms. Cefotaxime was evaluated in combination with metronidazole in 15 patients who also had surgery, and the outcomes were good (Sjolin et al. 1993). Ceftazidime, ceftriaxone, and ceftizoxime have all been used successfully in the treatment of small numbers of patients (Green et al. 1989; Donald and Ispahani 1988, 1990). Moxalactam has been shown to reach adequate concentrations in brain abscess pus and has been successful in treatment, but the toxicity of this agent precludes its use in treatment regimens (Yamamoto et al. 1993). Ampicillin/sulbactam was used in 11 patients with brain abscess with success and concentrations in the pus were variable, but adequate (Akova et al. 1993).

Imipenem-cilastatin, a carbapenem antibiotic, has been used successfully in the treatment of brain abscess (Asensi et al. 1996) and also in cerebral nocardiosis (Krone et al. 1989). The drug has a side effect of neurotoxicity so should be reserved for cases of resistant organisms. Meropenem, which is related to imipenem, does not have the same neurotoxic effects. Although limited evidence has been published for its efficacy in brain abscess, it has however been used successfully in treating meningitis. There is one report of successful use in a child with an *Enterobacter cloacae* abscess (Meis et al. 1995).

Cerebral nocardia has also been successfully treated with trimethoprim-sulphamethoxazole combined with surgery (Maderazo and Quintiliani 1974) and this combination may be used for Enterobacteriaceae depending on sensitivities.

Newer antibiotics, such as the quinolones, have not been studied in detail. There is evidence that they have good CSF penetration, and are highly active against the Enterobacteriaceae and Pseudomonads. There are inadequate data to support their use in brain abscess, but anecdotal evidence and case reports provide evidence of their clinical efficacy (Wessalowski et al. 1993). They should be used with some caution as they can lower seizure thresholds.

Undoubtedly some of the newer antimicrobial agents will be useful in the future as they offer additional options for treatment and possibly increased efficacy. There is a growing trend for nonoperative treatment of brain abscess and the new agents may have a part to play.

The antimicrobial regimens recommended for the treatment of intracerebral abscess are largely empiric and are based on the aforementioned studies (Table 4). No controlled trials have been undertaken;
the type of surgery performed also influences the outcomes. The predisposing condition and the site of the abscess is a guiding factor. The initial Gram stain on a pus sample is essential information for guiding empiric antibiotic therapy, which can be modified once culture results are available. Antibiotics should be started as soon as the diagnosis is made: there is no reason to wait for a pus sample before starting therapy, as cultures may still be positive despite antibiotic treatment.

Penicillin remains a mainstay of therapy because of its excellent activity against streptococci; the *Streptococcus milleri* group are commonly found in abscesses. It has a low incidence of side effects, and for many years was combined successfully with chloramphenicol. However, chloramphenicol is associated with side effects and is not

Table 4 *Suggested empiric antimicrobial therapy for brain abscess*

Predisposing condition	Antimicrobial regimen
Chronic ear disease	Third generation cephalosporin plus metronidazole
Sinusitis	Penicillin or third generation cephalosporin plus metronidazole
Dental infection	Penicillin or third generation cephalosporin plus metronidazole
Hematogenous spread	Penicillin or third generation cephalosporin plus metronidazole. Flucloxacillin or vancomycin if *S. aureus* suspected
Trauma or post surgery	Third generation cephalosporin plus flucloxacillin or vancomycin. Consider ceftazidime if *P. aeruginosa* suspected
Immunosuppression	Third generation cephalosporin or meropenem. Ampicillin or meropenem if *Listeria* is suspected
	Meropenem or trimethoprim/sulfamethoxazole if *Nocardia* is suspected

Based on Calfee and Wispelwey (2000) and Tunkel et al. (2000).

bactericidal so should not now be used as a first line agent. Metronidazole is added to give anaerobic cover, as it should be assumed that anaerobes would be present in most cases. The third generation cephalosporins are now accepted as empiric therapy, either cefotaxime or ceftriaxone, and in combination with metronidazole provide an adequate empiric therapy for most sinugenic, otogenic, and odontogenic abscesses (Donald 1990). Adequate coverage for enterobacteria found in otogenic abscesses is provided by the cephalosporins. For abscesses where *Staphylococcus aureus* is suspected, for example, postoperative, then an antistaphylococcal penicillin such as flucloxacillin should be used, in combination with fusidic acid or rifampicin. If MRSA is likely then vancomycin should be used, again in combination with rifampicin depending on local sensitivities.

Local instillation of antibiotics into the brain abscess cavity is not generally recommended as local irritation can occur which will increase the incidence of seizures. Rarely, the local route may be indicated in a difficult, poorly resolving abscess which remains culture positive despite appropriate antibiotics.

SURGERY

Surgical drainage is an important part of the management of these abscesses and is recommended for all abscesses of 2.5 cm or greater. As well as draining pus and relieving the effects of intracranial pressure, a pus sample will provide valuable microbiological information to guide therapy. The procedures commonly used are aspiration by burr hole or open drainage and excision by craniotomy. The technique used will depend on the location of the abscess, whether multiple abscesses are present, and on the clinical state of the patient. A recent technique of closed aspiration using CT guidance is increasingly being used instead of open craniotomy and stereotactic guidance can be employed for greater accuracy (Wispelwey et al. 1997). Follow up CT scans should be done to monitor the size of the abscess.

Sometimes antibiotic treatment alone is used without surgery (Boom and Tuazon 1985). This would be in certain categories of patients, which includes those at high risk for surgery, those with concomitant meningitis or ependymitis, those with hydrocephalus requiring a shunt, and those with inaccessible or multiple lesions (Rosenblum et al. 1980).

Guidance as to the duration of therapy is variable; no controlled trials have been done. Past recommendations have been to give parenteral treatment for 6–8 weeks provided that the infecting organisms are susceptible and that adequate surgical drainage is achieved. Recent studies suggest that 3–4 weeks of parenteral antibiotics may be adequate for those patients whose abscesses have been excised, with 4–6 weeks recommended for those whose abscesses have been aspirated. Those given antibiotics alone should be treated for a minimum of 4 weeks with parenteral antibiotics (Report 2000). The benefit of using oral antibiotics for follow-on treatment is unproven: in practice oral antibiotics based on sensitivity testing may be given for a further 4–6 weeks (Skoutelis et al. 2000). The serum C-reactive protein may be used to monitor resolution of the inflammation (Brown et al. 1994).

Outpatient parenteral antibiotic treatment (OPAT) is used increasingly for treating patients outside hospitals, thus reducing the costs and morbidity associated with inpatient stay. It has been reported to be successful in a carefully selected group of patients with central nervous system infections including 19 with brain abscesses (Tice et al. 1999).

SUBDURAL EMPYEMA AND INTRACRANIAL EPIDURAL ABSCESS

These two conditions will be considered together as they very often occur together in the patient. Subdural empyema is the second most common type of intracranial bacterial infection, followed by intracranial epidural abscess – it is difficult to establish the true incidence. The subdural space lies between the two outer layers of the meninges: the dura mater and the arachnoid mater (Figure 4). There is a large potential space for pus to

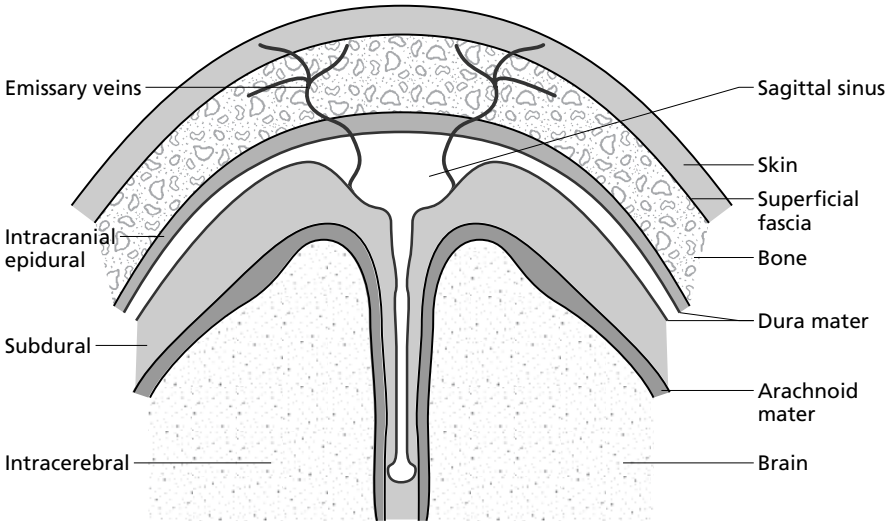

Figure 4 *Diagram of a cross-section through the skull showing the anatomy of the subdural space.*

spread through with only a few anatomical restrictions. Thus subdural empyemas behave as rapidly expanding space-occupying lesions. Overall, subdural empyemas account for 15–25 percent of localized bacterial infections of the CNS (Helfgott et al. 1997).

An epidural abscess represents infection between the outer layer of the dura mater and the overlying skull bone. There is little space there for pus to spread, so they are always very localized and usually associated with osteomyelitis. Subdural empyema is usually found in conjunction as the infection readily crosses the emissary veins. Virtually all cases follow sinusitis, craniotomy, or mastoiditis (Gallagher et al. 1998).

Epidemiology

Before the introduction of effective antimicrobial therapy the mortality from subdural empyema was uniformly 100 percent. After the introduction of penicillin the rate began to drop, and as with other forms of focal intracranial suppuration advances in diagnosis, surgical technique, and antibiotic therapy have brought the mortality rate down to 10–40 percent (Helfgott et al. 1997). Mortality is higher if the patient presents late or is comatose on admission, and in the elderly. Seizures may develop as a late complication (Dill et al. 1995).

Subdural empyema may occur at any age, but is most commonly seen in the second and third decades of life. Men are four times more commonly affected than women (Nathoo et al. 1999); it is seen in children (Smith and Hendrick 1983; Brook 1995). The most common predisposing factor, accounting for up to 60 percent of cases, is sinusitis, either frontal or ethmoidal. The rate may be higher in children. Ear infections account for a smaller number than with brain abscess, about 10–20 percent, but may be a more common predisposing factor in developing countries (Pathak et al. 1990). Hematogenous spread accounts for a small number of cases, around 5 percent, and this route may infect pre-existing subdural hematomas. Other cases have been reported following trauma, burns, and dental procedures. A series of 41 patients with postoperative subdural empyema showed that sinusitis accounted for only 29 percent of cases, 66 percent were postcraniotomy (Hlavin et al. 1994). Meningitis is an important predisposing factor in infants and subdural empyema may occur in 2 percent of infants with bacterial meningitis.

Uncomplicated intracranial epidural abscesses are, however, rare in children and have been reported from a wide age range with a median in the sixth decade. Recent case series suggest that a common predisposition for epidural abscess is surgery, up to 2 percent of craniotomies may be complicated by this infection (Hlavin et al. 1994).

Pathogenesis

Infection may spread directly or indirectly into the subdural space. Direct extension from the sinuses occurs by erosion of the posterior bony wall of the frontal sinus by infection, then further erosion of the dura mater to spread into the subdural space. It is thought that the most likely route is that of indirect spread by infection leading to thrombophlebitis of the emissary veins which connect the facial and dural venous systems. An epidural abscess always arises from infection of contiguous structures, such as sinuses and ear, and osteomyelitis is usually present. Infection from the ears occurs by the same mechanisms, either direct through the bone of the mastoid air cells, or indirect spread via the venous system (Bleck and Greenlee 2000a).

Bacteriology

Many different types of aerobic and anaerobic bacteria have been reported from epidural abscesses (Table 5); however, up to 30 percent may be negative on culture. Many of these patients will have had antibiotics prior to a sample of pus being taken. The types of bacteria grown will reflect the predisposing source. Streptococci are the most common isolates, being found in around 35 percent of cases, and are the most common isolate in children (Smith and Hendrick 1983). Most of these will belong to the *Streptococcus milleri* group. Sinugenic subdural empyemas most often grow streptococci, aerobic or anaerobic, often in pure culture. Staphylococci, in particular *Staphylococcus aureus*, are less commonly reported and account for 12–17 percent of cases. Most of these will be after surgery, trauma or related to hematogenous spread. *Staphylococcus aureus* is a frequent isolate from intracranial epidural abscess. This is due to the higher incidence of postoperative cases.

Skin flora such as *Propionibacterium acnes* have been reported in postoperative cases of subdural empyema (Critchley and Strachan 1996).

Aerobic gram-negative bacilli are less commonly reported, and usually in association with trauma and surgery (Hlavin et al. 1994). *Salmonella* spp. have been reported in neonates and infants (Mahapatra et al. 2002). Other unusual isolates are *Serratia marcescens* (Safani and Ehrensaft 1982) and *Pasteurella multocida* (Stern et al. 1981).

Subdural empyema following meningitis will reflect the causative organism of the meningitis, i.e. *Haemophilus influenzae*, *Streptococcus pneumoniae* or *Neisseria meningitidis* (Jacobson and Farmer 1981).

Polymicrobial infections are much less commonly reported compared to intracranial abscesses, but may be associated with surgery or trauma. As with other intracranial abscesses anaerobes play an important part and may be present either in pure culture or as part of a polymicrobial infection (Yoshikawa et al. 1975).

Mycobacterium tuberculosis is not often associated with subdural empyema despite it being a common cause of other CNS infections (Van Dellen et al. 1998).

Clinical features

In subdural empyema, fever and focal headache are prominent early features, the headache may then become generalized (Table 6). Focal neurological deficits may develop within 24–48 h with vomiting and signs of meningeal irritation. The symptoms can progress rapidly and focal fits can develop in 50 percent of patients. The presentation of localized epidural abscess may be more insidious and the initial presentation will be of the predisposing infection such as sinusitis.

Diagnostic methods

It is important to consider the diagnosis of subdural empyema in patients with sinusitis who go on to develop signs of meningeal irritation, for example, neck stiffness. The investigation of choice is gadolinium enhanced MRI scan which is more sensitive than contrast enhanced CT scan for this condition (Figure 5). Lumbar puncture should not be performed as it is dangerous and will not help in the diagnosis. Blood cultures may be positive in around 10 percent of cases,

Table 5 *Microbial cause and initial antibiotic therapy for cranial subdural empyema and epidural abscess*

Site of primary infection	Probable organism	Suggested initial therapy
Paranasal sinuses	Aerobic, microaerophilic, and anaerobic streptococci (especially *S. milleri* group); *Bacteroides fragilis* and other anaerobes; *Staphylococcus aureus*; *Haemophilus spp.*	Third generation cephalosporin plus metronidazole
Otitis media or mastoiditis	Aerobic, microaerophilic, and anaerobic streptococci (especially *S. milleri* group); *Bacteroides fragilis* and other anaerobes; *S. aureus*; Enterobacteriaceae	Third generation cephalosporin plus metronidazole
Cranial surgery or trauma	*S. aureus*; coagulase negative staphylococci, *Clostridium* spp.; Enterobacteriaceae; *Propionibacterium acnes*	Flucloxacillin or vancomycin plus third generation cephalosporin plus metronidazole
Dental sepsis	*Fusobacterium* sp.; *Actinomyces* sp.; aerobic and anaerobic streptococci	Metronidazole plus penicillin or third generation cephalosporin
Meningitis in infants	*Haemophilus influenzae*; *S. pneumoniae*; *Neisseria meningitidis*	Third generation cephalosporin
Meningitis in neonates	*Salmonella* spp; group B streptococci, Enterobacteriaceae	Third generation cephalosporin

Adapted from Bleck and Greenlee (2000b).

Table 6 *Clinical signs of subdural empyema*

Signs and symptoms	% patients
Fever	77
Headache	74
Hemiparesis	71
Altered consciousness	69
Neck stiffness	63
Fits	48
Other focal neurology	45

Adapted from Helfgott et al. (1997).

particularly in children, and may be helpful in identifying the causative organism.

Treatment

The principles of antibiotic treatment have already been outlined, and it is necessary to choose an antibiotic which will penetrate the blood–brain barrier. The antibiotic choices for subdural and epidural empyema are similar to those for intracranial abscess (Table 5).

Early surgical treatment is essential to remove the pus and relieve the pressure on the brain (Nathoo et al. 1999).

The clinical symptoms, range of bacteria isolated, and management of intracranial epidural abscess mirrors that of subdural empyema. The mortality for uncomplicated intracranial epidural abscess is, however, lower than that for subdural empyema and a recent series showed a mortality rate of 18 percent (Hlavin et al. 1994). A good outcome depends on early diagnosis before irreversible neurological deficits are present.

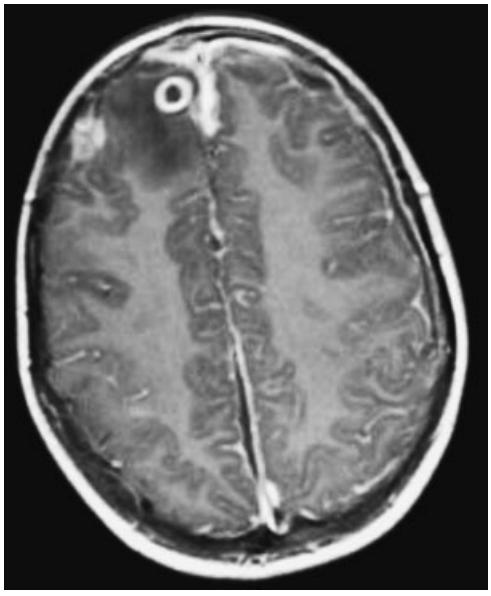

Figure 5 *Gadolinium enhanced MRI scan showing subdural empyema and frontal lobe abscess.*

SPINAL EPIDURAL ABSCESS

A spinal epidural abscess is a localized collection of pus in the paraspinal epidural space. This surrounds the spinal cord between the dura and the bone (Figure 6). This condition is a medical emergency because irreversible damage can be caused to the spinal cord by pressure of the abscess. In the preantibiotic era, these conditions were progressive and universally fatal, but with the advent of antibiotics and better diagnostic and surgical techniques these infections have a better outcome with mortality rates as low as 5 percent (Darouiche et al. 1992); although in some reports the mortality rate remains high at 18–31 percent (Hlavin et al. 1990). The higher mortality rates are thought to be in cases where the diagnosis was delayed; delays in diagnosis also lead to a worse outcome in terms of neurological sequelae.

Epidemiology

Spinal epidural abscess is an uncommon condition. Estimated incidence rates from case series range from 0.2 cases per 10 000 population in the mid 1970s (Baker et al. 1975) to 2.8 cases per 10 000 population more recently as observed in large tertiary care centers (Hlavin et al. 1990). The incidence is reported to be increasing; factors which may account for this are an increase in cases in injecting drug users; more patients with cancer who receive epidural analgesia, and more patients having spinal surgery or invasive diagnostic procedures.

There is a broad age range reported from 14-day-old infants (Rubin et al. 1993) to 78 year olds (Del Curling et al. 1990). It is most common in adults and the average age is now over 50 years of age, reflecting the increasing age of the at risk population. It is a condition which is increasingly seen in injecting drug users who tend to have an average age of 35 years (Koppel et al. 1988).

The male to female ratio has stayed consistent with a male predominance (Reihsaus et al. 2000).

Predisposing conditions

Spinal epidural abscess follows infection elsewhere in the body. Most cases reach the epidural space by the hematogenous route, either by direct seeding of the epidural space or by causing vertebral osteomyelitis which then extends into the epidural space. Infection may also spread from contiguous foci of infection, such as decubitus ulcers, retropharyngeal abscesses, and retroperitoneal infection. Increasingly, cases are seen following direct inoculation from surgery, from spinal procedures, such as epidural catheters for analgesia or

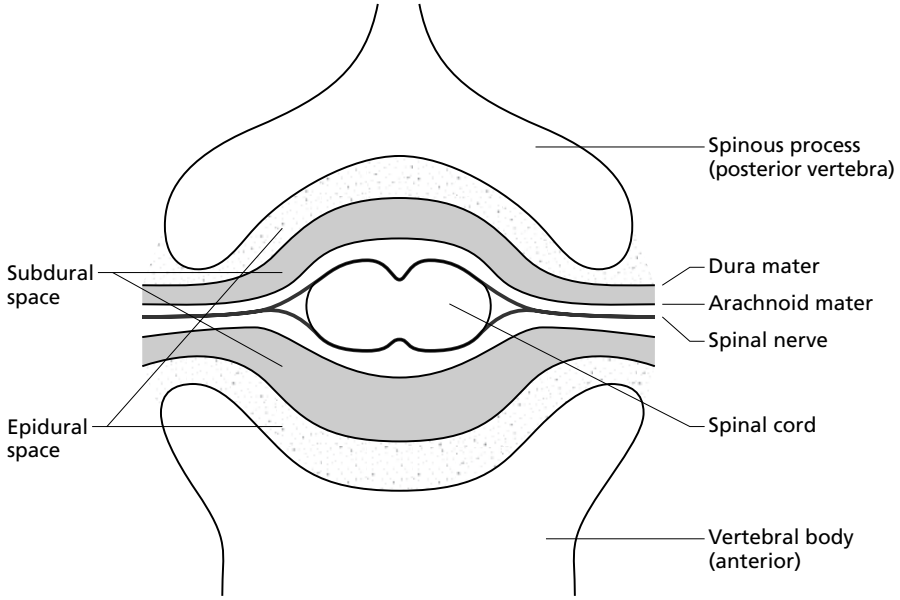

Figure 6 *Diagram showing the anatomy of the spine and the relationships of the epidural space.*

after spinal biopsy procedures (Bleck and Greenlee 2000a). Sometimes there is an underlying medical condition such as diabetes, degenerative joint disease, cirrhosis of the liver, or malignancy. Hematogenous seeding of the epidural space may occur in patients with endocarditis. In children, vertebral osteomyelitis is rare and most cases are associated with direct hematogenous spread.

The primary source is not apparent in 20–40 percent of cases (Gellin et al. 1997).

Pathogenesis

Spinal abscesses may be acute or chronic; chronic abscesses are more often associated with tuberculous osteomyelitis and epidural abscess.

The most common site for an abscess is posterior to the spinal cord; this occurs in 70 percent of cases. Anterior abscesses are more common in tuberculous infections and occur in the thoracic and lumbar regions. The abscess usually occupies about three to six vertebral segments, but rarely may extend more widely up and down the spinal column. Children are less likely to have adjacent vertebral osteomyelitis and usually have abscesses in the cervical or lumbar regions. The thoracic area is most common in adults.

The damage caused by the infection is often out of proportion to the size of the abscess. This may be due to several factors including thrombosis and thrombophlebitis of veins draining the spinal cord leading to edema and venous infarction; focal areas of vasculitis induced by the inflammatory mass; or bacterial exotoxin production particularly that due to *Staphylococcus aureus* (Gellin et al. 1997).

Bacteriology

A microbiological diagnosis is made from blood cultures, which may be positive in up to 90 percent of cases particularly if *S. aureus* is present, or by examination of pus drained at operation. Infections are rarely polymicrobial, only about 10 percent of cases having multiple isolates (Darouiche et al. 1992). *Staphylococcus aureus* is the most common isolate in all series accounting for up to 90 percent of cases; MRSA is also reported (Danner and Hartman 1987; Tang et al. 2002). Aerobic streptococci including *Streptococcus pneumoniae* (Turner et al. 1999) and *Streptococcus milleri* (Gelfand et al. 1991) are reported in up to 18 percent of series. The occurrence of aerobic gram-negative bacilli, including *Pseudomonas aeruginosa*, is on the increase both in postoperative cases and in injecting drug users; up to 37 percent of isolates may be gram-negative bacilli (Gellin et al. 1997). Coagulase-negative staphylococci are associated increasingly with postoperative cases. *Mycobacterium tuberculosis* is a well recognized cause of vertebral osteomyelitis and epidural abscess and is reported in most series.

The increase in infections seen in injecting drug users is represented in most series (Tunkel and Pradhan 2002) and may account for up to 40 percent of cases (Nussbaum et al. 1992).

Clinical features

The initial features may be subtle. There are four characteristic stages described (Gellin et al. 1997). The first symptom, with fever and malaise, is focal back pain. This is followed by nerve root pain; the next stage is

Table 7 *Bacterial isolates and suggested antibiotic therapy for spinal epidural abscess*

Bacteria	Frequency	Suggested antibiotics
S. aureus including MRSA	60–90%	Flucloxacillin or vancomycin plus rifampicin
Aerobic and anaerobic streptococci	18%	Benzylpenicillin plus metronidazole
Aerobic gram-negative bacilli	13%	Third generation cephalosporin or ceftazidime or quinolone
Polymicrobial	10%	Third generation cephalosporin plus metronidazole

spinal cord dysfunction, such as incontinence or sensory loss. The final stage is cord compression leading to paralysis. All this may happen over a short timescale; the progression of symptoms to nerve root pain can be over 3 to 4 days, then signs of cord compression over the next 4 to 5 days. At this stage the neurological effects are usually reversible but urgent surgical intervention is still needed as paralysis can develop rapidly. Acute infections, usually due to hematogenous spread, will progress rapidly in under 2 weeks with prominent signs of local infection, whereas abscesses arising from vertebral osteomyelitis will have a longer duration of back pain lasting more than 2 weeks before neurological symptoms appear. Back pain is a very common symptom, so epidural infection is often not considered as a diagnosis until neurological symptoms appear.

Diagnosis

Routine tests will show that the inflammatory markers, such as ESR and CRP, will be raised, as will the peripheral blood leukocyte count. Cerebrospinal fluid examination is sometimes performed and will show evidence of parameningeal inflammation, in other words raised white cell count and protein. The CSF cultures should be negative unless the abscess was directly sampled in which case the white cell count would be very high and the cultures will yield the causative organism.

Radiological diagnosis is made by MRI scanning using gadolinium enhanced scans which are very specific for this type of abscess.

Management

Surgical drainage to relieve pressure on the spinal cord is essential and is an emergency procedure. Antibiotics should be started as soon as possible to cover the common pathogen, *S. aureus*, with antipseudomonal cover added in postoperative cases and injecting drug users (Table 7). The spinal epidural space is outside the blood–brain barrier which will make antibiotic choice easier. Recommended duration of therapy is intravenous antibiotics for 3 to 4 weeks, then oral agents for a further 2 to 3 months if vertebral osteomyelitis is present. Some studies have recommended a shorter duration of therapy (Del Curling et al. 1990).

Some reports suggest that nonsurgical management may be successful (Wheeler et al. 1992), but the patients were carefully selected and the true success of this is difficult to ascertain. Some patients in another series showed progression of symptoms while on antibiotics alone (Hlavin et al. 1990).

It is well reported that a delay in diagnosis leads to a worse outcome. The preoperative status of the patient is a good predictor of outcome; those that are paralyzed before surgery do worse (Mackenzie et al. 1998).

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Bacterial infections of the eye

SUSAN E. SHARP AND MICHAEL R. DRIKS

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INTRODUCTION

This chapter will focus on bacterial infections of the eye, specifically those associated with conjunctivitis, keratitis, and endophthalmitis. Within each of these infections, the areas of predisposing factors, clinical manifestations, infecting agents, diagnosis, and treatment will be reviewed.

Infections of the eye can be exogenously acquired, contiguous (from infections of the face, eyelids, etc.), or from hematogenous spread (O'Brien 2000). One or more of these routes can be associated with each of the above conditions.

NORMAL FLORA AND COMMON PATHOGENIC BACTERIA OF THE EYE

The bacterial organisms most commonly associated with 'normal flora' and the 'common pathogenic' bacteria of the eye are indicated in Table 1 (Sharp 1999; O'Brien 2000).

BACTERIAL CONJUNCTIVITIS

Predisposing factors

Conjunctivitis is defined as inflammation of the conjunctiva, usually caused by infections, allergens, or irritative substances. Predisposition to bacterial conjunctivitis primarily involves contact exposure to airborne fomites, upper respiratory infections, skin flora on hands, or genital secretions (O'Brien 2000). A patient's age is an important determinant of the likely infecting organism. In children, bacterial conjunctivitis is more common than viral, whereas in adults, the opposite is true (Wishart et al. 1984; Fitch et al. 1989; Woodland et al. 1992). The clinical setting can also determine the microbiology of bacterial conjunctivitis. Institutional outbreaks have been described, presumably due to contact transmission between residents, patients, and medical personnel (King et al. 1988; Schwartz et al. 1988). Community outbreaks of *Haemophilus influenzae* biogroup *aegyptius* occur in children.

Clinical manifestations

All forms of conjunctivitis may be characterized by irritation, itching, foreign body sensation, tearing, and discharge. Bacterial conjunctivitis can be distinguished from other forms by the presence of mucopurulent drainage and a papillary reaction, characterized by many small bumps on the tarsal conjunctiva, creating a velvety appearance (Chung and Cohen 2000). In contrast to viral causes, bacterial conjunctivitis is typically unilateral. In severe cases, there is often significant lid edema with copious purulent drainage, marked conjunctival erythema, chemosis, membrane formation, and associated keratitis (O'Brien 2000). *Neisseria* conjunctival infections cause especially severe inflammation (Valenton and Abendanior 1973; Barquet et al. 1990). Streptococci and *Corynebacterium diphtheriae* are notable for causing membrane formation (Boralkar 1989). Nonpyogenic bacterial organisms, including

Table 1 *Bacterial organisms most commonly associated with 'normal flora' and the 'common pathogenic' bacteria of the eye*

Normal flora	Most common pathogens
Aerobic bacteria	Aerobic bacteria
Corynebacterium	*Streptococcus pneumoniae*
Staphylococcus species	*Staphylococcus aureus*
Micrococcus	*Staphylococcus*, coagulase-negative
Nonpathogenic *Neisseria* species	*Streptococcus* species
Aerococcus	*Pseudomonas aeruginosa* and other gram-negative bacilli
	Haemophilus influenzae
	Moraxella catarrhalis
	Neisseria gonorrhoeae
	Neisseria cinerea
	Bacillus species
	Chlamydia trachomatis
Anaerobic bacteria	Anaerobic bacteria
Propionibacterium	*Propionibacterium*
Clostridium	*Peptostreptococcus*
Peptostreptococcus	

Mycobacterium tuberculosis, *Francisella tularensis*, *Treponema pallidum*, and *Bartonella henselae* can cause an atypical conjunctivitis with unilateral nodules that ulcerate and ipsilateral preauricular lymphadenopathy.

Chlamydial conjunctivitis deserves special mention. Trachoma refers to conjunctival and limbic scarring leading to trichiasis and corneal opacification in recurrent or chronic *Chlamydia trachomatis* infection (Bobo et al. 1997). Trachoma is among the leading causes of blindness in the world. Inclusion conjunctivitis is an acute infection in adults, most often transmitted by contact with genital secretions. In a neonate infected during the birthing process, the disease is grouped together with gonococcal conjunctivitis and referred to as opthalmia neonatorum. As of the 1970s, the incidence of chlamydial conjunctivitis in the newborn was increasing, involving 2.8 percent of all births in one clinic and 35 percent of infants born to mothers with known chlamydial cervicitis (Schachter et al. 1979). The usual incubation period is 5–12 days from birth (Chandler et al. 1977). In infants born to infected mothers, 22–44 percent develop neonatal conjunctivitis (Harrison and Alexander 1990).

Infectingagents

In the healthy and nonimmunocompromised population, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *H. influenzae* are the most common causes of acute bacterial conjunctivitis. However, differences in causative organisms are seen based on the age of the patient.

In the newborn, acute conjunctivitis can be caused by *C. trachomatis*, *Neisseria gonorrhoeae*, *Neiserria cinerea*, or *Pseudomonas aeruginosa*, although *C. trachomatis* and *N. gonorrhoeae* are the most frequently encountered organisms in this population (de Toledo and Chandler 1992; Ratelle et al. 1997). Studies have shown *H. influenzae*, *S. pneumoniae*, and *Moraxella catarrhalis* to be the bacterial agents most often associated with acute conjunctivitis in children (Weiss et al. 1993; Gigliotti et al. 1981; Wald et al. 2001). *H. influenzae* has been reported to be the most prevalent isolated pathogen, accounting for between 54 and 74 percent of infections in children (Gigliotti et al. 1981; Wald 1997; Wald et al. 2001). Ocular *H. influenzae* isolates also appear to be mostly serologically nontypable strains, probably as a result of the widespread use of the *H. influenzae* b vaccine (Alrawi et al. 2002; Robinson et al. 2001). Conjunctivitis in young children may also be caused by *N. cinerea*, *S. aureus*, or *P. aeruginosa* (Trottier et al. 1991; Weiss et al. 1993; Iroha et al. 1998; Shah and Gallagher 1998). In addition, the *Streptococcus* viridans group have been shown to be associated with conjunctivitis in patients aged less than 1 year (Seal et al. 1982).

In adults, the most common causes of bacterial conjunctivitis are *S. pneumoniae*, *S. aureus*, and coagulase-negative staphylococci, while in sexually active teenagers and adults, *C. trachomatis* and *N. gonorrhoeae* can also be found (Seal et al. 1982; O'Brien 2000). Additional bacteria such as those listed at the beginning of this chapter can also be found to cause conjunctivitis in a variety of community and nosocomial settings. For example, hospital- and institutionally acquired cases of conjunctivitis have been associated with infections caused by *Serratia marcescens*, *S. pneumoniae*, *P. aeruginosa*, and *Moraxella* spp. (van Ogtrop et al. 1997; King et al. 1988; Schwartz et al. 1988). Mycobacterial species, especially *M. tuberculosis*, have been shown to be an etiologic agent of atypical conjunctivitis and may be associated with ulcerations of the eye.

Organisms thought to be nonpathogenic can occasionally cause conjunctivitis. However, those bacteria normally considered skin flora may be cultured from diseased eyes without causing infection. For example, 40 percent of specimens in one study were associated with recovery of *S. epidermidis* or mixed skin flora, which also occurred in cultures of normal eyes (Seal et al. 1982). In another study of children, staphylococci, corynebacteria, and alpha-hemolytic streptococci were the predominant organisms recovered from the eyelids of uninfected control subjects (Weiss et al. 1993). In addition, a study by Gigliotti and colleagues found that *S. aureus* was as commonly isolated from normal eyes as from diseased eyes in children (Gigliotti et al. 1981). Thus, care must be taken when interpreting the results of bacterial cultures.

Diagnosis

The value of laboratory diagnostic procedures in mild conjunctivitis is not well established and is therefore not done routinely. Cases of mild conjunctivitis are often treated without the knowledge of bacterial culture information. In severe conjunctivitis, culture of the superior and inferior tarsal conjunctiva is recommended. In all cases of ophthalmia neonatorum, smears and cultures for bacteria, including *Chlamydia*, should be performed.

If culture diagnosis of bacterial conjunctivitis is desired, it can be attempted by performing conjunctiva cultures. Samples of both eyes should be taken with separate swabs that have been premoistened with sterile saline. For routine bacteriology collections, swabs that are used for normal bacteriology specimen collection should be used. If cultures for *Chlamydia* are being collected, cotton swabs should not be used, but dacron or calcium alginate swabs would be acceptable. Topical ointments and analgesics should be avoided prior to collection. Samples are collected by rolling the swabs over each conjunctiva. They should be directly inoculated on to blood agar and chocolate agar plates (warmed to room temperature prior to inoculation) or added to transport media for travel to, and inoculation in, the laboratory. After inoculation, the swabs are rolled across two separate slides for staining purposes. Plates inoculated at the bedside should be transported at room temperature to the laboratory as soon as possible, preferably within 15 min, or stored for up to 24 h at room temperature prior to delivery. If swabs are collected and not directly inoculated at the bedside, they should be placed back into the swab device sleeve, transported at room temperature, and delivered to the laboratory immediately and no longer than 2 h after collection. If samples are being collected for *Chlamydia* culture, appropriate *Chlamydia* transport media should be utilized for delivery of the specimens to the laboratory, again within 2 h of collection. In addition, molecular testing for the presence of *Chlamydia* can be performed if the sample is collected in specimen collection/transfer devices specific for this purpose. Swab samples are normally not appropriate for mycobacterial stain or culture. If *M. tuberculosis* conjunctivitis is suspected, ulcer/lesion scrapings can be collected for appropriate stains and cultures.

As stated earlier, if possible both conjunctiva should be sampled, even if one seems to be uninfected. This will allow the determination of the normal flora of the uninfected eye. This can be used for comparison with the organisms cultured from the infected eye and assist in the determination of possible pathogens. Anaerobic cultures are not performed in cases of conjunctivitis, as the causative bacterial pathogens are aerobic in nature. An exception might exist with *Peptostreptococcus*, an anaerobic gram-positive cocci; however, this is not a frequent cause of conjunctivitis.

The Gram stain can assist in the determination of the causative agent(s) of conjunctivitis, especially if only the infected conjunctiva is sampled. In acute bacterial conjunctivitis, specimens show large numbers of neutrophils, and bacteria may be seen inside or outside of these cells. With chronic conjunctivitis there are more lymphocytes and large mononuclear cells seen in the Gram stain as compared with neutrophils. Organisms seen on the Gram stain are more likely to be involved in the infective process and can be used to help interpret the bacterial culture information. Gram stains of conjunctival scrapings have been shown to provide a rapid means of predicting the causative agent in 90 percent of cases of bacterial conjunctivitis (Weiss et al. 1993).

Treatment

Mild bacterial conjunctivitis is often self-limiting, requiring no treatment. Multiple topical antibiotics, including aminoglycosides for Gram-negative organisms, eythromycin, bacitracin, polymyxin/trimethoprim, or neomycin/polymyxin for Gram-positive organisms, are available in ophthalmic preparations, given every 2–4 h for 7–10 days. Empiric therapy for children should focus on *H. influenzae*, *S. pneumoniae*, and *S. aureus*. Fluoroquinolone solutions offer broad-spectrum activity but have been no more effective than aminoglycosides. Gonococcal conjunctivitis requires parenteral treatment, usually with a single 125-mg dose of intramuscular ceftriaxone. Adult inclusion conjunctivitis and trachoma are treated systemically with doxycycline or macrolide antibiotics. Neonatal chlamydial conjunctivitis is treated with erythromycin. Prophylaxis of both gonococcal and chlamydial opthalmia neonatorum is best achieved with topical erythromycin (O'Brien 2000).

BACTERIAL KERATITIS

Predisposing factors

Keratitis is defined as inflammation of the cornea, produced by infectious and noninfectious causes. Microbial keratitis is a common, potentially sight-threatening infection. Bacterial keratitis accounts for 65–90 percent of all corneal infections (Jones 1981; Asbell and Stenson 1982; Liesegang and Forster 1980). Direct penetration of intact corneal epithelium occurs only by organisms with particular virulence factors. Most organisms gain access via corneal epithelial defects created by mechanical, thermal, or chemical injury or by epithelial erosion secondary to underlying ocular conditions (O'Brien 2000).

Various forms of external trauma, including obvious or subtle injury, contact lenses, trichiasis, entropion, and abnormal lid margins, predispose to infection. Radial keratotomy, now infrequently performed, is associated with infection in 0.25–0.7 percent of cases (Jain and Azar 1996). Infectious complications of photoreactive keratectomy using the excimer laser are rare (Lin and Maloney 1999).

Corneal surface defects may also result from severe dry eyes, exposure, bullous keratopathy, or neurogenic corneal anesthesia. Comatose patients with chemosis that prevents lid occlusion are prone to bacterial keratitis (Parkin et al. 1997). Underlying leprosy or trachoma with lagophthalmos or trichiasis are also at risk.

Clinical manifestations

There are no clinical symptoms or signs that reliably distinguish microbial keratitis from noninfectious inflammatory conditions. Due to the rich nervous innervation of the cornea, the most common complaint is pain. Unlike conjunctivitis, keratitis is usually accompanied by a variable decrease in vision. Examination of the patient is greatly facilitated by use of topical anesthesia. The cornea is assessed for epithelial ulceration and the presence and location of stromal suppuration. Again, unlike conjunctivitis, discharge is uncommon in keratitis. Reflex tearing, photophobia, and blepharospasm are common. Loss of corneal transparency and epithelial defects are best observed with a cobalt blue light after instillation of fluorescein. Neovascularization of the cornea may be seen in severe cases. Slit-lamp examination may reveal associated intraocular inflammation with flare and cells in the anterior chamber and hypopyon formation (O'Brien 2000).

Infecting agents

Both gram-positive and gram-negative bacteria can be associated with infective keratitis, and many organisms can cause keratitis when introduced by trauma or ocular abnormality. The most prevalent causative organisms include *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., and members of the Enterobacteriaceae (O'Brien 2000). Several studies have shown *P. aeruginosa* to be the dominant bacterial pathogen causing bacterial keratitis (Lam et al. 2002; Boonpasart et al. 2002; Martins et al. 2002; Sun et al. 2002), whereas others have found the gram-positive bacteria (staphylococci, *S. pneumoniae*, and other streptococci) to be the most frequent agents of bacterial keratitis (Schaefer et al. 2001; Sun et al. 2002; O'Brien 2000). Other infectious agents found to be associated with bacterial keratitis include *Moraxella* spp., *Serratia marcescens*, *Citrobacter* spp., *Enterobacter* spp., *Proteus* spp., *Morganella morganii*, *Bacillus cereus* (and other *Bacillus* spp.), *Corynebacterium* spp., *N. gonorrhoeae*, *Alcaligenes xylosoxidans*, *Acinetobacter* spp., *H. influenzae*, and *C. trachomatis* (Schaefer et al. 2001; Pinna et al. 2001; O'Brien 2000).

P. aeruginosa is a frequent cause of bacterial keratitis in contact-lens wearers and can cause a quick suppurative course due to the production of proteolytic enzymes that damage the eye (O'Brien 2000). *Streptococcus pyogenes*, also a common cause of bacterial keratitis in adults, may induce a severe inflammatory response due to the production of exotoxins (Bachman and Gabriel 2002). *B. cereus* is recognized as a primary pathogen of ocular infections and is commonly seen in infections associated with post-tramatic events (Tauzon 2000). Extremely serious and virulent ocular infections with this organism can be seen following a foreign body injury (O'Brien 2000).

Staphylococci that normally colonize other parts of the body can occasionally reach the eye and cause corneal infection. *N. gonorrhoeae* may cause keratitis resulting from an inappropriately treated conjunctivitis. Polymicrobic infections can be seen in association with 'bandage' contact lenses in diseased corneas, often associated with a mixture of bacterial and fungal organisms (O'Brien 2000). Several mycobacterial species have also been associated with chronic ulcerative keratitis, including, most commonly, *M. fortuitum* and *M. chelonae* and, much less commonly, *M. gordonae*, *M. avium complex*, and *M. tuberculosis*.

Diagnosis

Superficial keratectomy or corneal biopsy specimens may be necessary for difficult to diagnose nonsuppurative cases. For bacterial culture it is recommended that samples of both the conjunctiva and cornea be collected. Swabs of the conjunctiva should be collected prior to anesthetic applications, whereas corneal scrapings can be obtained afterward. Conjunctiva swab specimens should be collected as described earlier for

conjunctivitis. For routine bacteriology collections, swabs that are used for normal bacteriology specimen collection should be used. If cultures for *Chlamydia* are being collected, cotton swabs should not be used, but dacron or calcium alginate swabs would be acceptable. Again, if possible, both conjunctiva should be sampled, even if one seems to be uninfected to allow the determination of normal flora in the uninfected eye and possible pathogens in the affected eye. After collection of the conjunctiva swabs, instillation of two drops of local anesthetic should be administered. Using a sterile spatula, ulcers or lesions from the cornea are scraped and some of the scrapings are inoculated directly on to culture media (multiple samples from areas of suppurations should be obtained for each stain and medium). Direct culture inoculation should be performed on to brain–heart infusion agar supplemented with 10 percent sheep blood and chocolate agar. All media should be warmed to room temperature prior to inoculation. Anaerobic media is not routinely necessary as the majority of organisms associated with keratitis are aerobic in nature. The remaining material should be applied to two clean glass slides for staining. Inoculated specimens should be held at room temperature and transported to the laboratory within 15 min. If this is not possible, the inoculated specimens should be held at room temperature for not more than 24 h prior to transport to the laboratory. The majority of bacteria that cause keratitis will grow on standard media within 12–48 h.

The sensitivity and specificity of the Gram stain for the diagnosis of keratitis was determined in a study that examined corneal scrapings (Sharma et al. 2002). In this study, the sensitivity of the Gram stain in the detection of bacteria was 36.0 percent in early keratitis and 40.9 percent in advanced keratitis cases. However, the specificity was higher in both groups (84.9 and 87.1 percent, respectively), while predictive values were poor. This study determined that the Gram stain had limited value in determining therapeutic options for bacterial keratitis. However, it is still held that the diagnostic laboratory evaluation of corneal scrapings (including Gram stain and culture) is imperative if there is suspicion of microbial keratitis (O'Brien 2000).

Special methods are necessary for the detection of *C. trachomatis* or the mycobacterial species. Specimens can be inoculated into cell culture or tested by molecular detection techniques for *Chlamydia* detection. If samples are being collected for *Chlamydia* culture, appropriate *Chlamydia* transport media should be utilized for delivery of the specimens to the laboratory, within 2 h of collection. In addition, molecular testing for the presence of *Chlamydia* can be performed if the sample is collected in specimen collection/transfer devices specific for this purpose. For the detection of mycobacterial infections, routine acid-fast bacilli growth media/systems should be utilized.

Treatment

Bacterial keratitis is potentially sight-threatening and demands prompt attention. Corneal perforation with resulting endophthalmitis and eye loss can occur rapidly. Topical antibiotics remain the mainstay of treatment, but the potential for rapid deterioration and the need for high-frequency dosing often requires hospitalization with trained nursing personnel. Several routes of topical administration include drops, ointments, continuous lavage, antibiotic-soaked corneal shields, therapeutic soft contact lenses, and iontophoresis. Subconjunctival injection and parenteral routes are also utilized. Empiric antibiotics are usually begun pending return of diagnostic information. The antimicrobial agent selected should have bactericidal activity against common gram-positive and gram-negative corneal pathogens. A common initial choice includes a topical first-generation cephalosporin (e.g. cefazolin) and an aminoglycoside (e.g. gentamicin, tobramycin, or amikacin). Fluoroquinolone ophthalmic solutions are available for broad-spectrum coverage. Various penicillin, cephalosporin, vancomycin, aminoglycoside, and fluoroquinolone preparations are commercially available or can be formulated and fortified by the pharmacist for directed therapy once a microbiologic etiology is established (Forster 1998). The utility of susceptibility testing is unclear, due to the high local antibiotic concentrations afforded by topical therapy. Response to treatment is based on serial slit-lamp examinations. Decreasing frequency of antibiotic administration and conversion from fortified to commercial strength drops can usually occur within a few days.

BACTERIAL ENDOPHTHALMITIS

Predisposing factors

Endophthalmitis is an inflammatory process involving the deep structures of the eye. Bacteria are the most common cause of infectious endophthalmitis (O'Brien 2000). By far the most common predisposing factor for bacterial endophthalmitis is antecedent eye surgery. The incidence of postoperative endophthalmitis is low (<0.9 percent), but the high frequency of cataract extraction, glaucoma surgery, vitrectomy, and keratoplasty make it an important problem (Aaberg et al. 1998). As for keratitis, radial keratotomy and laser keratectomy are uncommonly complicated by endophthalmitis. Penetrating nonsurgical trauma is another significant presdisposing event, with endophthalmitis developing in 4.2 percent of cases (Duch-Samper et al. 1997). The presence of a distant focus of infection may predispose to endogenous bacterial endophthalmitis, secondary to hematogenous seeding. Cases associated with infection at virtually any site have been described, prominently

including meningitis, respiratory tract infection, abdominal infection, skin and soft-tissue infection, and endocarditis (Barnard et al. 1997). Occasionally, presumed hematogenous endophthalmitis occurs in a previously healthy patient with no apparent focus (Piczenik et al. 1997).

Clinical manifestations

Bacterial endophthalmitis typically develops suddenly and progresses rapidly. Symptoms and signs of ocular inflammation usually manifest within one to several days after surgical or nonsurgical trauma. Patients complain of pain and progressive blurring of vision. Examination reveals intense conjunctival hyperemia, chemosis, lid and corneal edema, hypopyon, and decreased visual acuity. Ophthalmoscopic or slit-lamp examination reveals anterior chamber and vitreous inflammatory cell reaction. Sudden onset of endophthalmitis in an unoperated, nontraumatized eye suggests hematogenous spread. The clinical picture of endogenous endophthalmitis is similar to postoperative cases, except for more prominent involvement of the posterior segment of the eye. These cases normally occur in systemically ill and/or immunocompromised patients (O'Brien 2000). Delayed-onset endophthalmitis can follow cataract extraction with intraocular lens implantation. These patients have a chronic course with low-grade inflammation (Fox et al. 1991; Sawusch et al. 1989).

Diagnosis

Once bacterial endophthalmitis is clinically suspected, prompt diagnostic aspirates should be obtained for thorough microbiologic investigation. Vitreous fluid has the highest yield for microbial pathogens and should be collected for the diagnosis of endophthalmitis along with aqueous aspiration. Microbiological studies should include a Gram stain and appropriate culture media for aerobic and anaerobic organisms. In one study, 78 of 140 specimens (56 percent) collected by vitreous and aqueous paracentesis were positive for a bacterial agent, with the vitreous having the higher yield as compared with the aqueous culture (O'Brien 2000). In another study that cultured vitreous fluid from over 400 patients with endophthalmitis, the overall yields of bacterial organisms recovered from solid media and broth culture were similar. This study also found that a positive Gram stain was highly predictive of a positive culture from the eye; however, a negative Gram stain had little predictive value for the culture result (Braza et al. 1997). Although culture results have little relevance on initial therapy choices, modifications of therapy can be directed by the results of bacterial culture and antimicrobial susceptibility testing. As stated previously, media for both aerobic and anaerobic bacteria should be included in the culture of these specimens; however, the use of broth cultures is not routinely necessary (Braza et al. 1997). The usual pathogens causing endophthalmitis should grow in 24–48 h, but cultures for anaerobic organisms may need to be held for up to 5–7 days. For suspected cases of mycobacterial infection, acid-fast stains and appropriate cultures should be performed.

Polymerase chain reaction (PCR) technology has been studied by several investigators for the diagnosis of bacterial endophthalmitis. Vitreous fluid and aqueous humor have both been shown to be reliable samples for use in PCR followed by sequencing procedures to detect the presence of bacterial organisms, especially in smear- and culture-negative specimens (Therese et al. 1998; Lohmann et al. 1998; Lohmann et al. 1997; Okhravi et al. 2000; Knox et al. 1999). A case of culture-proven *Listeria monocytogenes* endophthalmitis was identified more than 2 days sooner using PCR followed by direct sequencing protocols as compared with conventional culture (Lohmann et al. 1998). PCR was shown to be helpful in identifying *Propionibacterium acnes* in nine of 17 eubacterial genome-positive specimens that were smear- and culture-negative for bacterial organisms (Therese et al. 1998). The study by Lohmann and colleagues tested aqueous humor or vitreous in cases of endophthalmitis (21 eyes) and identified the causative organisms as *P. acnes*, *Staphylococcus epidermidis*, or *Actinomyces israelli* by PCR and comparing the determined sequences with all GenBank entries (Lohmann et al. 2000). The study by Okhravi and colleagues identified *Escherichia coli*, coagulase-negative staphylococci, *B. cereus*, *Pseudomonas* spp., *Comomonas* spp., *Proteus* spp., *Methylobacterium* spp., *Aeromonas* spp., and *Streptococcus faecalis* through detecting bacterial DNA by PCR and identifying sequences by restriction fragment length polymorphism and/or sequencing techniques (Okhravi et al. 2000). In addition, PCR techniques are a useful adjunct to diagnostic techniques, as unlike conventional culture, results are not affected by previously administered antibiotics (Lohmann et al. 2000). Caution must be taken when using PCR or other nucleic amplification techniques to detect 'causative' organisms, especially from nonsterile specimens such as eye collections. Identification of a certain genomic sequence proves that the specimen harbors this piece of nucleic acid (if not contaminated); however, it does not prove that the organism caused the patient's disease or that the organism is viable. Organisms determined to be present in clinical samples can be consistent with infection, colonization, or contamination, thus the results of molecular diagnostic techniques, as well as conventional bacterial culture, must be interpreted with caution. To date, these techniques are expensive and not part of the routine diagnostic microbiology laboratory. It is unclear at this time whether resources will be utilized for the development of PCR and sequencing commercial diagnostic kits for ophthalmic pathogens (Van Gelder 2001).

Treatment

Acute bacterial endophthalmitis is an ophthalmologic emergency, requiring early recognition and immediate and aggressive therapy. Traditional treatment options have included mechanical debridement via vitrectomy, intravitreal, subconjunctival, topical, and/or systemic antibiotics, and steroids by various routes. The Endophthalmitis Vitrectomy Study (Endophthalmitis Vitrectomy Study Group 1995) demonstrated that systemic antibiotics, such as ceftazidime and amikacin, did not improve visual outcome and that early vitrectomy benefited patients with light-perception vision or less on presentation. Patients with better than light perception (hand motions or better) fared equally well with vitreous tap and intravitreal antibiotics, with 50 percent achieving 20/40 or better vision, 75 percent with 20/100 or better, and only 12 percent with severe vision loss (5/200 or worse). Empiric intravitreal antibiotics are aimed at both gram-positive pathogens, including methicillin-resistant staphylococci, and gram-negative pathogens, including *Pseudomonas* spp. Vancomycin and an aminoglycoside is a common therapeutic combination. Amikacin is often chosen due to its possibly decreased retinal toxicity as compared with the other aminoglycosides. Similarly, concern for retinal toxicity has led to a trend toward ceftazidime use as a replacement for the aminoglycosides. Clindamycin is often included in nonsurgical post-traumatic cases because of the risk for *B. cereus* endophthalmitis. Adjunctive subconjunctival and topical antibiotics are often utilized, but their contribution beyond intravitreal antibiotics is unknown. Steroids are commonly added to hypothetically reduce destruction secondary to intraocular inflammation, but their efficacy is not well established. In delayed-onset chronic *P. acnes* endophthalmitis, surgical removal of intraocular lens implants may be necessary to remove sequestered organisms.

Prophylactic antibiotics and antiseptics before, during, and after surgery are widely utilized, but their efficacy is unproven. The low incidence of postoperative bacterial endophthalmitis makes demonstration of efficacy difficult. Povidone–iodine appears to be effective in reducing rates of these infections (Speaker and Menikoff 1991).

CONCLUSIONS

The eye is an anatomically and functionally complex structure, with variable and unique infectious manifestations. Bacterial infections of the eye are common and may be associated with significant morbidity. Predisposing factors, clinical presentation, microbiology, diagnosis, and treatment all depend on the ocular structure involved. Whether dealing with a relatively benign conjunctivitis, or a more threatening keratitis or endophthalmitis, accurate diagnosis and treatment can limit discomfort and disfigurement, and preserve vision.

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Bacterial infections of the upper respiratory tract

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INTRODUCTION

Bacterial infections of the upper respiratory tract are many and varied, ranging from benign pharyngitis to life-threatening epiglottis (Chow 2000; Berman 1995; Peterson and Thomson 1999). In spite of these differences, many concepts pertaining to host defense, pathogenesis, and bacterial virulence mechanisms are shared among diseases. In addition, anatomic features of the upper respiratory tract and normal commensal flora of epithelial surfaces are similar factors in all infections. Finally, infections of the upper respiratory tract may disseminate to distant foci, underscoring the potential seriousness of seemingly simple clinical conditions.

Infections of the upper respiratory tract can be divided into those that occur in the nasal airways and sinuses, middle ear and associated tissues, oral cavity, and in spaces adjacent to the oral cavity as abscesses (Table 1). Descriptions of specific infections will include the etiologies, epidemiology, unique defense mechanisms and pathogenesis, normal flora, a brief summary of clinical features, recommended antimicrobial therapy, and means of prevention. The goal of this chapter is to provide a complete understanding of factors contributing to the development and resolution of upper respiratory tract infection, the accurate and rapid diagnosis of infection, and treatment and prevention of subsequent infection.

ANATOMY

The upper respiratory tract is the area proximal to and including the epiglottis. It includes the middle ear, paranasal sinuses, nasopharynx, and oral cavity. Understanding the anatomy of the upper respiratory tract is necessary in order to understand the pathogenesis and prevention of disease at these sites (Thadepalli and Mandal 1984, 1988). The upper respiratory tract is composed of two distinct types of epithelial surfaces (Thadepalli and Mandal 1984; Thadepalli and Mandal 1988; Chow 2000). A stratified squamous epithelium lines the oropharynx and nasopharynx, and a respiratory epithelium, composed of ciliated columnar cells, goblet cells, and mucous glands, lines the paranasal sinuses and the middle ear. Stratified squamous epithelium is found in areas filled with normal microbial flora, representing sites easy to sample for bacteriologic culture. Epithelium of the sinuses and middle ear is usually sterile and is difficult to access if culture is needed.

The nasal airways and paranasal sinuses include the nasal septum, which divides the nose at the midline, turbinates, and four sinus complexes (Gwaltney 1996;

Table 1 *Upper respiratory tract diseases and etiologies*

Anatomic site/disease	Bacterial etiologies
Oral cavity	
Pharyngitis	*Streptococcus pyogenes*
	Occasionally other beta-hemolytic streptococci,
	Arcanobacterium haemolyticum, *Mycoplasma pneumoniae*
Diphtheria	*Corynebacterium diphtheriae*
Epiglottitis	*Haemophilus influenzae*
	Occasionally *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Staphylococcus aureus*, beta-hemolytic streptococci
Gingivitis	Mixed anaerobes, including spirochetes, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*
Abscess	Mixed anaerobic, and aerobic bacteria, especially *Fusobacterium* spp. and viridans streptococci
Stomatitis	Anaerobaic bacteria, especially *Treponema vincentii*, *Prevotella melaninogenica*, and *Fusobacterium nucleatum*
Ludwig's angina	Streptococci, mouth flora
	Occasionally *H. influenzae*, staphylococci and facultative
	Gram-negative bacilli
Paranasal sinuses	
Sinusitis	*S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, *S. pyogenes*, other streptococci, *S. aureus*, anaerobic bacteria
Rhinoscleroma	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*
Ear	
Otitis media and acute mastoiditis	*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. pyogenes*, *S. aureus*, anaerobes
Malignant external otitis	*Pseudomonas aeruginosa*
Abscesses adjacent to the oral cavity	
Peritonsillar, pharyngeal, retropharyngeal, and parapharyngeal	Mixed aerobic and anaerobic bacteria especially, *S. pyogenes*, *S. aureus*, *S. anginosus* (*milleri*) group
Lemierre's syndrome	*F. necrophorum*, other anaerobes
Suppurative paratitis	*S. aureus*, viridans streptococci, anaerobic bacteria

Wald 1996; Thadepalli and Mandal 1988; Thadepalli and Mandal 1984). The inferior, middle, and superior turbinates are three shelf-like projections that extend from the septum. Beneath the middle turbinate is a meatus that drains the maxillary, anterior ethmoids, and frontal sinuses. Beneath the superior turbinate is a meatus that drains the posterior ethmoids and sphenoid sinuses. The lacrimal duct drains to the inferior meatus. The maxillary sinus has a volume of approximately 15 ml and drains through an ostium high on the medial wall of the sinus. This positioning impedes normal gravitational drainage and, most likely, predisposes to bacterial infection. The tubular passage that connects the ostium to the meatus is called the infundibulum. The ethmoid sinus is composed of multiple cells, each of which drains by a separate ostium into the middle meatus. The frontal sinus is not fully developed until late adolescence. The sphenoid sinuses are located just behind the posterior ethmoids. The sphenoid and frontal sinuses can be a source for the spread of infection to the central nervous system.

The relevant structures of the ear include the external auditory canal, the tympanic membrane (eardrum), middle ear, mastoid, and Eustachian tube (Hendley 2002). The oral cavity includes the nasopharynx, pharynx, epiglottis, tongue, teeth, periodontium, and adjacent salivary and parotid glands (Roscoe and Chow 1988).

DEFENSE MECHANISMS

Protective mechanisms found in the upper respiratory tract include the primary defenses that are constantly active and secondary mechanisms that are activated or recruited at the time of infectious insult (Roscoe and Chow 1988). Primary defense mechanisms include the anatomy of the upper airways, epithelial surfaces, mucociliary clearance, and soluble factors in airway secretions, such as degratory enzymes, complement, and immunoglobulins (Roscoe and Chow 1988). Secondary mechanisms include recruitment of polymorphonuclear leukocytes and lymphocytes and the secretion of chemical mediators of inflammation by these cells (Chow 2000).

During breathing, the turbulence of air flowing through the nasal turbinates, nasopharynx, and oropharynx results in the sedimentation of bacteria within the upper respiratory tract. Tight junctions between epithelial cells provide an efficient barrier to bacterial penetration. The epithelium is continually

replenished if damaged. Throughout the nasal sinuses, middle ear, and Eustachian tubes, mucus-secreting cells secrete a blanket of mucus that is propelled along toward the oropharynx by ciliated cells, where it is coughed out or swallowed. Mucous contains soluble factors that augment the clearance of bacteria from normally sterile areas and prevents invasion past the epithelial layer (Feldman 2001). Other nonspecific defense mechanisms include the continuous cell shedding and turnover of the mucosal epithelium and the constant flow of saliva-containing lysozyme, lactoferrin, beta-lysin, lactoperoxidease, and other antimicrobial constituents (Chow 2000; Roscoe and Chow 1988). Immunoglobulin A (secretory IgA) is the predominant immunoglobulin in the upper respiratory tract. In addition to complement-independent neutralization of respiratory tract viruses, secretory IgA interacts with lysozyme and complement to improve bactericidal activity, augment bacterial adherence to mucus, and inhibit attachment to epithelial surfaces (Bernstein 1992; Roscoe and Chow 1988).

If primary defense mechanisms are unable to eradicate a bacterial pathogen, polymorphonuclear leukocytes and lymphocytes, constituting secondary defense mechanisms, are recruited to bolster the host's response (Roscoe and Chow 1988). Neutrophils migrate from the vascular compartment following the release of chemotactic factors by degraded bacteria. Bacterial antigen is transported to local lymph nodes either within phagocytic cells or free in afferent lymphatic fluid. Antigen processed in lymph nodes results in the expansion and differentiation of specific B and T cell clones. One to two weeks after infection, effector T and B cells migrate from lymph nodes to provide specific antibody locally in the respiratory tract.

PATHOGENESIS

Disease occurs when the balance between host and bacteria tips in favor of the microbes. This results from a defect in host defenses, an overwhelming inoculum of bacteria, or the presence of a novel bacterial virulence mechanism. Host defects range from increased colonization of the respiratory epithelium and failure of epithelial barriers to immunoglobulin deficiency and reduced phagocytic activity. In addition, environmental influences, such as tobacco smoke, may modify host defenses (Obeid and Bercy 2000).

Colonization of the upper respiratory tract is the first step in the pathogenesis of most bacterial infections. Individuals with poor nutrition, particularly vitamin A deficiency, and those with local areas of inflammation, such as gingivitis, are more likely to have increased bacterial colonization densities and a higher concentration of pathogenic species (Enwonwu et al. 2002). Antimicrobial therapy may change the relative numbers of bacterial commensals providing an advantage for some bacteria. It is also known that epithelial cells from patients with severe or chronic illness are more receptive to bacteria, especially gram-negative microorganisms (Niederman 1990).

Compromise of the respiratory epithelium results from trauma, radiation therapy, damage from chemotherapeutic agents, and environmental factors (Niederman 1990). Nasal intubation traumatizes the nasal airway, obstructs normal drainage, and provides a conduit for bacteria to gain access and to persist in deep respiratory spaces (Westergren et al. 1998a, b). Chemotherapy may damage or destroy rapidly dividing epithelial cells resulting in oral ulcers and breaches in barrier defense. Tobacco smoking, secondary tobacco smoke, and air pollution restrict ciliary clearance by direct toxicity, increase mucus production, and attract neutrophils (Obeid and Bercy 2000). Nasal discharge is more frequent in heavily polluted urban areas and more common upper respiratory tract infection is associated with increased pollution (Ng and Tan 1994).

BACTERIAL VIRULENCE MECHANISMS

Most bacteria that are pathogens in the upper respiratory tract are capable of adherence to and colonization of a mucus membrane. Factors that govern colonization are many and varied, including adherence characteristics of bacteria and epithelial cells, oxygen tension and oxidation-reduction potential, pH, age of patient, diet, oral hygiene, smoking habits, antimicrobial therapy, pregnancy, and genetic factors (Chow 2000). Colonization represents a 'period of waiting' for an opportunity to invade deeper tissues following trauma, or to be 'sequestered' in a closed space, such as a paranasal sinus or middle ear, as a result of inflammation caused by viral infection, allergy, or other insult (Bluestone 1996; Saez-Llorens 1994). Once positioned for disease, bacterial characteristics such as avoidance of complement deposition and phagocytosis, toxin production, synthesis of IgA proteases, and avoidance of mucociliary clearance augment disease severity (Kilian 1981).

Most bacteria are pathogenic throughout the upper respiratory tract, rather than one specific site (Table 1). *Streptococcus pneumoniae* resides in the pharynx and nasopharynx and persists in presumably sterile sites such as the paranasal sinuses and middle ear primarily because of an antiphagocytic capsule. *H. influenzae* possess fimbriae that bind to respiratory epithelium and mucin and reduce ciliary beating, which minimizes mucociliary clearance (Rodriguez et al. 2003). Group A streptococcal M protein binds to sialic acid moieties on mucin, followed by local invasion and toxin production that destroy local tissues (Ryan et al. 2001). Anaerobes provide 'colonization resistance,' i.e. prevent introduction or persistence of new bacterial populations, yet they are pathogens if they gain access to subepithelial spaces. The hallmark of anaerobic infection is the abscess. As a rule, multiple

anaerobic species, rather than a single species, are involved in upper respiratory infection. *Corynebacterium diphtheriae* is a specialized respiratory tract pathogen, capable of causing local disease in the pharynx or nasopharynx, yet exerting its most serious damage at distant sites as a result of toxin production (Hadfield et al. 2000). Viridans streptococci are included as part of a mixed etiology in abscesses, yet *Streptococcus mutans*, specifically, is the sole etiology of dental caries (Shaw 1987). Finally, *Staphylococcus aureus* is an abscess-forming, toxin-producing pathogen occasionally involved in infection of paranasal sinus, middle ear, and abscesses of the oropharyngeal spaces.

NORMAL FLORA

The anterior nares are colonized by staphylococci, micrococci, and miscellaneous *Corynebacterium* spp. (Roscoe and Chow 1988; Schuster 1999). The paranasal sinuses are normally sterile. In the mouth, anaerobes colonize periodontal areas along with a variety of other bacteria, including viridans group streptococci, *Haemophilus* spp., *Neisseria* spp., and diphtheroids (Schuster 1999; Roscoe and Chow 1988). The flora of the nasopharynx is much more complex. It may include *Streptococcus* spp., *Neisseria* spp., *Enterobacteriaceae*, *Bacteroides* spp., and *Fusobacterium* spp. (Sutter 1984). In addition, all individuals are colonized intermittently by one or more potential pathogens, such as group A streptococci, *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, and *Moraxella catarrhalis* (Neto et al. 2003). Point prevalence surveys in communities demonstrate the ever-present nature of these pathogens.

Quantitative studies of the oral cavity indicate that certain species predominate in specific sites (Table 2) (Roscoe and Chow 1988; Schuster 1999). Obligate anaerobes constitute a large and important part of the oral flora. As an example, in the gingival crevice of healthy adults, microscopic counts average 2.7×10^{11} bacteria/gram wet weight, with anaerobes outnumbering facultative bacteria by a 8:1 margin (Chow 2000). Identification of specific genera shows *Streptococcus* spp., *Peptostreptococcus* spp., *Veillonella* spp., *Lactobacillus* spp., *Corynebacterium* spp., and *Actinomyces* spp. accounting for more than 80 percent of the cultivatable oral flora (Chow 2000). Other unique niches include *Streptococcus sanguis*, *Streptococcus mutans*, *Streptococcus mitis*, and *A. viscosus*, which preferentially colonize the surfaces of teeth (Chow 2000). *Streptococcus salivarius* and *Veillonella* spp. have a predilection for the tongue and buccal mucosa. *Fusobacterium* spp., *Porphyromonas* spp., *Prevotella* spp., and anaerobic spirochetes concentrate in the gingival crevice (Chow 2000). Facultative gram-negative bacilli are uncommon in the oral cavity of healthy adults; however, this group becomes a more prominent part of the flora in seriously ill, hospitalized, and elderly patients (Lipchik and Kuzo 1996). A number of exogenous factors may disturb the ecology of the mouth and nasopharynx. Antimicrobials can suppress normal flora, allowing yeasts and resistant bacteria to proliferate.

GENERAL CONSIDERATIONS: COLLECTION, TRANSPORT, AND PROCESSING OF SPECIMENS FOR BACTERIOLOGIC EXAMINATION

All specimens for bacteriologic culture should be collected from the specific site of inflammation, as free of contaminating flora as possible (Carroll and Reimer 1996; Peterson and Thomson 1999; Thomson and Miller 2003). In practice, most specimens from the upper

Table 2 *Oral flora*

Site	Predominant bacteria	Bacteria present but not predominant
Gingiva	Viridans group streptococci	Anaerobic gram-negative bacilli
	Lactobacilli	Other aerobic and anaerobic bacteria
	Anaerobic gram-positive rods	
	Anaerobic cocci	
Tongue	Viridans group cocci	Anaerobic gram-negative bacilli
	Lactobacilli	Anaerobic gram-positive cocci
	Anaerobic gram-negative cocci	Anaerobic gram-positive rods
		Other aerobic and anaerobic bacteria
Saliva	Viridans group cocci	Lactobacilli
	Anaerobic cocci	Anaerobic gram-positive bacilli
		Anaerobic gram-negative bacilli
		Other aerobic and anaerobic bacteria
Dental plaque	Viridans group streptococci	Anaerobic cocci
	Lactobacilli	Anaerobic gram-negative bacilli
	Anaerobic gram-positive bacilli	Other aerobic and anaerobic bacteria

Data from Chow (2000), Roscoe and Chow (1988), and Schuster (1999).

respiratory tract will contain at least low numbers of contaminating bacteria. Purulence should be aspirated or gently washed and aspirated. Sterile, nonbacteriostatic saline is an acceptable wash/lavage fluid. Specimens for anaerobic culture are collected by aspiration (Thomson and Miller 2003). Oxygen-free transport vials should be used to hold the aspirate during transport to the laboratory regardless of how quickly this will occur. Specimens for the detection of aerobic and facultative bacteria may be collected with a swab only when absolutely necessary, e.g. pharyngeal swab.

Specimens must be transported to the laboratory as quickly as possible. Receipt within 1 h is optimal (Thomson and Miller 2003; Carroll and Reimer 1996). Longer transport requires a holding medium. Specimens should not be allowed to dry or be frozen. Refrigerated or 'on-ice' temperature (approximately 40°C) is used unless anaerobes or *H. influenzae* are expected. Pathogenic *Neisseria* spp., *H. influenzae*, *S. pneumoniae*, and anaerobes are especially sensitive to extended holding and transport (Thomson and Miller 2003).

Once in the laboratory, processing includes a gram-stained smear, whenever purulence is submitted from an abscess or closed space, and a culture using appropriate media for the pathogens suspected. Gram stain results are used to determine inflammation and the presence of morphotypes suggesting a potential pathogen. Gram stain results are important when selecting empiric therapy, previous antimicrobial therapy inhibits growth, or anaerobes are present but have not survived exposure to oxygen during collection or transport.

Hospital microbiology laboratories are equipped to perform usual aerobic and anaerobic cultures. Staphylococci, streptococci, and nonfastidious gram-negative bacilli as predominant bacterial populations are readily detected. Less common pathogens, such as *Arcanobacterium haemolyticum*, *Neisseria gonorrhoea*, and *Corynebacterium diphtheriae*, can be detected by culture, stain, or molecular methods but only when the laboratory is notified in advance that a specific microorganism is being sought. Multiple anaerobes in mixed culture are not readily isolated and identified with usual methods. Newer selective media and techniques have been introduced to identify specific anaerobic pathogens in oral infections (Moore and Moore 1994; Chow 2000). These are available in a limited number of research, university or commercial settings. A recent, comprehensive review of collection, transport, and processing for bacterial specimens is available.

INFECTIONS OF THE ORAL CAVITY

Streptococcal pharyngitis

Pharyngitis is one of the most common reasons for physician visits among pediatric patients, with the group A streptococcus (*S. pyogenes*) the most likely treatable etiology (Gerber 1998). However, considering all patient groups and etiologies, including viruses, group A streptococci may account for as few as 5 percent of cases of pharyngitis (Carroll and Reimer 1996). Group A streptococcal pharyngitis is important not only because of the morbidity associated with acute disease but also because it may be followed by nonsuppurative sequelae (acute rheumatic fever or acute glomerulonephritis). Accurate detection and treatment of group A streptococcal pharyngitis is necessary to shorten the duration of symptoms, prevent the spread of the organism to other patients, prevent nonsuppurative sequelae, and avoid antimicrobial therapy in patients with viral infections (Gerber 1998). In spite of the frequency of disease and the potential for morbidity, a surprising number of practical questions dealing with the disease and its diagnosis remain unanswered.

Streptococcal pharyngitis is caused by *S. pyogenes*, a Lancefield group A Streptococcus based on cell-wall carbohydrate analysis. Group A carbohydrate is composed of N-acetylglucosamine linked to a rhamnose polymer backbone (Cunningham 2000). Disease is spread by person-to-person contact with infectious nasal or oral secretions, and is more common in situations of crowding. Disease occurs primarily in children between 5 and 15 years of age, mostly during winter and spring months in temperate climates (Gerber 1998). Further identification of group A streptococci can be performed by typing on the presence of M protein in the cell wall or by sequencing the *emm* gene that encodes the M protein (Bisno 1991; Bisno 2001). Currently, more than 80 M protein serotypes have been identified. Typing demonstrates that multiple serotypes circulate in a community at any one time (Haukness et al. 2002). During a 3-month winter period, typing of 63 group A streptococcal strains from three large pediatric practices in the Chicago area identified 16 different pulsed-field gel electrophoresis patterns. The resurgence of invasive, life-threatening streptococcal necrotizing fasciitis, and toxic shock over the past 15 years has stimulated a study of community pharyngeal strains to detect the location and prevalence of potentially invasive strains. Invasive strains constitute 25 percent of pharyngeal pathogens circulating in the community (Haukness et al. 2002).

M protein is the chief virulence factor of group A streptococci (Bisno 1991; Bisno 2001). Strains rich in M protein resist phagocytosis by polymorphonuclear leukocytes. M protein exerts its antiphagocytic effect by diminishing complement activation by the alternative complement pathway, thus limiting the deposition of complement on the bacterial surface (Bisno 1991; Bisno 2001). Immunity to infection is associated with the development of opsonic antibodies to antiphagocytic epitopes of M protein and, with few exceptions, such immunity is type-specific. Immunity lasts for many years, possibly for the life of the patient. In addition,

lipoteichoic acid is expressed on the surface of group A streptococci and is the adhesin responsible for binding of the organism to fibronectin present on the surface of oral epithelial cells (Bisno 1991). The streptococcal cell wall is surrounded by a hyaluronic acid capsule that is also antiphagocytic, serving as an additional virulence factor. Strains with large capsules are mucoid colonies on blood agar plates. Group A streptococcal strains that are both rich in M protein and heavily encapsulated are readily transmitted from person to person and tend to produce severe infections (Bisno 2001).

Patients with group A streptococcal pharyngitis present with sore throat (usually of sudden onset), pain on swallowing, and fever (Gerber 1998). Headache, nausea, vomiting, and abdominal pain (especially in children) also may be present. Clinical examination reveals tonsillopharyngeal erythema, with or without exudate, a red, swollen uvula, petechiae on the palate, and tender, enlarged anterior cervical lymph nodes. Tonsils, if present, are enlarged and erythematous. When a fine, diffuse red rash accompanies streptococcal pharyngitis, the syndrome is called scarlet fever (Gerber 1998).

The gold standard laboratory diagnostic test for group A streptococcal infection is the throat culture (Gerber 1998). If performed correctly, it has a sensitivity of 90–95 percent. Unfortunately, the culture takes 24–48 h to complete. If the patient leaves the physician or healthcare facility office with a prescription for antibiotics, the treatment would then begin before the microbial entity is known. In the age of emerging drug resistance, unnecessary antimicrobial use must be avoided. As a result, rapid streptococcal detection methods were developed to give nearly immediate results allowing for antimicrobial decision making while the patient is in the office or clinic (Peterson and Thomson 1999). Many rapid direct tests for group A streptococci are commercially available, including enzyme immunoassay (EIA), optical immunoassay, and nucleic acid-based probe assays (Thomson and Miller 2003). Although specificities are >95 percent, the reported sensitivities of EIAs and an optical immunoassay vary between 60 and 95 percent but can be as low as 31 percent. The nucleic acid-based assay has a sensitivity greater than 90 percent. When a rapid test is requested, two throat swabs should be collected. If only one swab is received, the culture plate should be inoculated first. Material remaining on the swab is used for the direct test. If the rapid test is positive, the second swab can be discarded, but if the rapid test is negative, the second swab must be used for culture to confirm the negative direct test (Gerber 1998; Thomson and Miller 2003). The nucleic acid-based probe test is considered sensitive and specific enough by many to obviate the need for confirmatory culture (Thomson and Miller 2003). A position paper by representatives of the American Academy of Family Physicians, the American College of Physicians–American Society of Internal Medicine, and the Centers for Disease Control and Prevention, states that rapid tests do not require confirmatory culture when used with specimens from adult patients (Cooper et al. 2001). This recommendation does not hold for specimens from children.

To culture group A streptococci, either sheep blood agar or selective blood agar may be used (Thomson and Miller 2003). Selective agar makes the organism easier to visualize by inhibiting the accompanying flora but may delay the appearance of colonies of *S. pyogenes*. Cultures should be incubated for 48 h at 35°C in an environment of reduced oxygen achieved by incubating anaerobically, in 5 percent CO2, or in air with multiple 'stabs' through the agar surface. Stabbing the agar surface with the inoculating loop pushes inoculum-containing streptococci below the surface, where the oxygen concentration is reduced compared with ambient.

Group A streptococci are uniformly susceptible to penicillin in vitro and the treatment of choice for acute group A streptococcal pharyngitis remains penicillin, as recommended by the American Heart Association, the Committee on Infectious Diseases of the American Academy of Pediatrics, and the World Health Organization (Gerber 1998). Cephalosporins and macrolides, such as erythromycin, are recommended in patients unable to take penicillin. Certain antimicrobials are not recommended for the treatment of group A streptococcal pharyngitis. Tetracyclines and trimethoprim-sulfamethoxazole have been shown to be ineffective in the treatment of this infection. Although in vitro testing is not necessary for penicillin, worldwide surveys show that up to 30 percent of stains are resistant to erythromycin and other macrolides (Alos et al. 2003; Hoban et al. 2003).

During the winter and spring in temperate climates, up to 20 percent of asymptomatic school-aged children may be streptococcal carriers (McEwan et al. 2003; Kaplan et al. 1981; Gerber 1998). These individuals have group A *Streptococcus* in their pharynx but have no evidence of immunologic response to this organism. They may be colonized for several months, and during that period they may experience episodes of intercurrent viral pharyngitis. When tested, these patients have group A streptococci in their pharynx and would seem to be suffering from acute streptococcal pharyngitis. Streptococcal carriers are unlikely to spread the organisms to their close contacts and are at very low risk, if any, of developing suppurative or nonsuppurative complications (Gerber 1998). Streptococcal carriers do not ordinarily require antimicrobial therapy. When eradication of streptococcal carriage is desirable, a short course of rifampin in conjunction with penicillin or a 10-day course of oral clindamycin have been shown to be effective (Gerber 1998).

Non-group A streptococcal pharyngitis caused by beta-hemolytic streptococci groups B, C, G, and F have

been reported (Peterson and Thomson 1999). Added to this list of uncommon causes of bacterial pharyngitis are mixed anaerobes (gangrenous pharyngitis), *N. gonorrhoeae*, *C. diphtheriae*, *Arcanobacterium haemolyticum*, *Yersinia pestis*, *Francisella tularensis*, *Mycoplasma pneumoniae*, *Chlamydophila psittaci*, and *C. pneumoniae* (Peterson and Thomson 1999). The clinical laboratory does not routinely look for any of these other agents in throat swabs, except when specifically requested by the clinician.

Diphtheria

Although much less common than streptococcal pharyngitis, *C. diphtheriae* can still be isolated from patients with a sore throat as well as those with a more serious systemic disease. Epidemic diphtheria re-emerged in the former Soviet Union beginning in 1990, resulting in approximately 125 000 cases and 4 000 deaths between 1990 and 1995 (Markina et al. 2000). The number of new cases began to decrease in 1996 following a World Health Organization strategy to vaccinate 90 percent of people aged 3 years or older (Markina et al. 2000). The introduction of new strains into countries with no endemic disease from exogenous reservoirs can cause outbreaks in developing nations, homeless populations, or in specific communities where vaccination is not accepted (Galazka 2000; Harnisch et al. 1989). The changing epidemiology of diphtheria includes the high proportion of infected adults, a progressive spread of disease from urban centers to rural areas, and transition from well-distinguished outbreaks to a more generalized epidemic (Galazka 2000).

The upper respiratory tract mucosa is the most common site of infection with *C. diphtheriae* in children but was a rare site for localization in adults patients in the epidemic that occurred in the former Soviet Union (Hadfield et al. 2000). In adults with oral mucosal lesions, buccal mucosa, upper and lower lips, hard and soft palate, and the tongue were sites of infection. *C. diphtheriae* localizes in the upper respiratory tract, ulcerates the mucosa, and induces the formation of an inflammatory pseudomembrane. The pseudomembrane is initially white, becoming a dirty gray color over time. Late in the course of infection the membrane may have patches of green or black necrosis. The growth of the organism remains localized, but exotoxin is absorbed and is disseminated through the blood to evoke severe systemic pathology. The exotoxin inhibits cellular protein synthesis by stimulating adenosine diphosphate ribosylation, thus inactivating protein synthesis elongation factor 2 (EF-2) (Harnisch et al. 1989).

The microbiologic diagnosis of diphtheria is designed to provide early and accurate diagnosis, identification of contacts and carriers, and appropriate clinical management (Efstratiou et al. 2000). The recommended microbiologic procedure includes obtaining a clinical specimen from the throat and nasopharynx. If present, membranous material should be sampled, including material from beneath the membrane. Blood agar and selective tellurite medium (Hoyle's tellurite medium is recommended) should be used (Efstratiou et al. 2000). Most normal flora will be inhibited on the tellurite medium allowing only *C. diphtheriae* and some other corynebacteria, staphylococci, and yeasts to be identified by the production of black colonies. The diagnosis of diphtheria on the basis of direct microscopy of a smear is unreliable because both false-positive and false-negative results may occur (Efstratiou et al. 2000). Rapid screening tests recommended for the differentiation of potentially toxigenic species of corynebacteria (*C. diphtheriae*, *C. ulcerans*, and *C. psuedotuberculosis*) are the presence of cystinase and the absence of pyrazinamidase activity (Efstratiou et al. 2000). Although there are four biotypes of *C. diphtheriae* (*C. diphtheriae* var. *gravis*, var. *mitis*, var. *belfanti*, and var. *intermedius*), biotyping is of limited use in epidemiologic investigations because discrimination is poor (Efstratiou et al. 2000). The minimum laboratory information required to report a specimen as positive for *C. diphtheriae* is as follows: Catalase-positive (most biotypes), urea-negative, nitrate-positive (except biotype *belfanti*), pyrazinamidase-negative, and cystinase-positive (Efstratiou et al. 2000). They will also ferment glucose, maltose, and starch (Table 3).

Testing for toxigenicity, which detects the phage-encoded exotoxin, is the most important test and should be carried out without delay on any suspect isolate that

Table 3 *Laboratory identification of* Corynebacterium diphtheriae *and related species*

			Gelatin				
Corynebacterium species	**Nitrate**	**Urea**	**Liquefaction**	**Glucose**	**Maltose**	**Sucrose**	**Trehalose**
C. diphtheriae var. *gravis*	+	–	–	+	+	–	–
C. diphtheriae var. *mitis*	+	–	–	+	+	–	–
C. diphtheriae var. *intermedius*	+	–	–	+	+	–	–
C. diphtheriae var. *belfanti*	–	–	–	+	+	–	–
C. ulcerans	–	+	+	+	+	–	+
C. pseudotuberculosis	–	+	–	+	+	–	–

Data from Efstratiou et al. (2000).

is found by routine screening or while investigating a possible case. The potentially toxigenic species (*C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*) acquire this characteristic when infected by bacteriophage. Although rapid polymerase chain reaction (PCR) assays have been used to detect biologically active fragment A of the toxin, positive results must be confirmed by phenotypic testing with the modified Elek immunoprecipitation test, since strains may possess toxin genes but do not express biologically active toxin (Efstratiou et al. 2000).

Epiglottitis

Acute bacterial epiglottitis, defined as inflammation and edema of the epiglottis, can result in life-threatening airway obstruction (MayoSmith et al. 1986). Inflammation can also occur in the arytenoid cartilage (paired jug or pitcher-shaped structures that move vocal cords apart) or pharyngeal wall, resulting in acute supraglottitis (Donnelly and Crausman 1997). Acute epiglottitis in children is caused by *H. influenzae* type b in most cases. In bacteremic cases an even higher percentage of cases is caused by *H. influenzae* (Bamberger and Jackson 2001). In adults, only 25 percent of cases are associated with *H. influenzae*. Disease caused by *S. pneumoniae*, bete-hemolytic streptococci (groups A, B and C), *S. aureus*, *H. parainfluenzae*, and *N. meningitidis* have been described (Warner and Finlay 1985; Sack and Brock 2002). Unusual pathogens such as *Klebsiella pneumoniae* and *Pasteurella multocida* have been reported in immunocompromised patients (Wine et al. 1997).

Epiglottitis occurs in people of all ages, with most cases found in children 2–7 years of age before widespread use of the *Haemophilus influenzae* type b (Hib) vaccine (Bamberger and Jackson 2001). Annual attack rates of 2–13 per 100 000 children were reported. With the introduction of the vaccine in the mid 1980s, the incidence of acute epiglottis in the pediatric population has decreased to 0.3–0.6 per 100 000. The incidence in adults is reported to be two to three cases per 100 000 and possibly increasing due to greater awareness and recognition of disease in adults (MayoSmith et al. 1986; Bamberger and Jackson 2001). For adults, the mean age at the time of presentation of disease ranges from 42 to 50 years. Cigarette smoking in diseased patients is more frequent than in the general population. Although cases occur in all months, spring and winter seasonal peaks have been reported (Bamberger and Jackson 2001).

Haemophilus species are exclusively adapted to the human mucosal membranes (Kilian 1981). *H. influenzae* is the species most frequently associated with disease in humans. The portal of entry for *H. influenzae* is the nasopharynx following person-to-person spread via respiratory droplets. Upper respiratory tract colonization rates for all species in humans may be as high as 50 percent. Nonencapsulated *H. influenzae* and *H. parainfluenzae* are the species most commonly found as commensals. Colonization by serotype b encapsulated *H. influenzae* strains is uncommon in healthy children and adults (Bamberger and Jackson 2001). The occurrence of disease after acquisition of an *H. influenzae* serotype b strain depends on the ability to colonize the nasopharynx and the ability to infect target tissue. The presence of the polysaccharide capsule and the production of IgA proteases are postulated to play a role in the pathogenesis of serotype b strains.

Epiglottitis in a previously well child is characterized by the sudden onset of high fever, severe sore throat with dysphagia, and drooling (MayoSmith et al. 1986; Ward 2002). In adults, epiglottitis manifests as a sore throat with odynophagia. Inflammation in adult disease is not confined to the epiglottis, as is the case with children, but rather can also affect other laryngeal and oropharyngeal structures. Consequently, a more accurate term than epiglottitis for adults is supraglottitis (Donnelly and Crausman 1997). If the diagnosis of epiglottitis is in question (pediatric or adult patient), radiography may be used but only if personnel experienced in intubation and resuscitation equipment are available, since airway compromise may occur (MayoSmith et al. 1986). Intensive care unit (ICU)-level care for the initial 24–48 h is prudent. In addition, oropharyngeal examination should not be performed, attempts should not be made to lay the patient down, and venipuncture should be avoided until the patient's airway is secured. In one review of acute epiglottitis in adults, two of 44 patients receiving medial treatment with no artificial airway died of sudden respiratory failure (MayoSmith et al. 1986). Epiglottitis must be differentiated from viral laryngotracheitis, bacterial tracheitis, diphtheria, and a foreign body lodged in the larynx. Penetrating pharyngeal injury and parapharyngeal or paravertebral abscess may mimic epiglottitis (Chung 2002).

The likelihood of beta-lactamase-producing *H. influenzae* and the inclusion of multiple etiologies in adults has led to the practice of empiric treatment with a beta-lactamase-resistant, third-generation cephalosporin (Bamberger and Jackson 2001). Most disease in children can be prevented by routine use of Hib conjugate vaccine (McEwan et al. 2003).

Gingivitis/Vincent's disease

Inflammation of the gingiva begins with irritation and microbial invasion of the gums (Fenesy 1998; Chow 2000). Subgingival plaque is always present. Simple gingivitis is characterized by swelling and thickening of the gingiva with a tendency for the gums to bleed after eating or toothbrushing. There is usually no pain. Patients with acute necrotozing gingivitis, also known as Vincent's disease or trench mouth, experience pain in

the gingiva that interferes with normal chewing. Necrosis of the gingiva between the teeth, a grayish pseudomembrane, and a characteristic halitosis with altered taste are present. Fever, malaise, and regional lymphadenopathy may occur (Fenesy 1998).

Vincent described the disease in the late nineteenth century as a fusospirochetal infection due to the prominence of these microorganisms in smears of material removed from the lesion (Loesche and Grossman 2001). Mixed aerobic and anaerobic flora, especially spirochetes, with some that have never been cultured, *Fusobacterium* spp., *Prevotella* spp. and *Porphyromonas* spp., *Actinobacillus acintomycetemcomitans*, and *Eikenella corrodens* are frequently implicated as prominent etiologies of acute necrotozing ulcerative gingivitis and other periodontal diseases (Loesche and Grossman 2001; Darby and Curtis 2001).

Patient defense mechanisms against gingivitis include the highly vascular gingival tissue that presents an oxidative barrier to the penetration of anaerobic flora from the dental plaque (Loesche and Grossman 2001). While certain bacteria, such as *A. actinomycetemcomitans*, can be detected within the tissue, they are rarely able to cause tissue necrosis without the association of other anaerobic bacteria. Conditions that cause vasoconstriction of peripheral arterioles, such as smoking and stress, are risk factors for periodontal disease, most likely because of reduced blood flow allowing invading anaerobes to survive (Loesche and Grossman 2001; Fenesy 1998).

Treatment includes local debridement and lavage with oxidizing agents, which usually brings relief from pain within 24 h. Antimicrobial therapy with penicillin or metronidazole is highly effective (Chow 2000; Duckworth et al. 1966; Loesche and Grossman 2001).

Periodontal abscess

Periodontal disease is a general term that refers to all diseases involving the periodontum (supporting structures of the teeth) (Suzuki 1988). Subgingival plaque and gingivitis precede infection of the underlying supporting tissue, ultimately leading to destruction of the periodontium and permanent loss of teeth. Chronic inflammation of the periodontium is the major cause of tooth loss in adults. Periodontal infections generally drain freely, resulting in little or no discomfort to the patient. The microbial specificity in different periodontal infections has only recently been appreciated (Chow 2000; Moore and Moore 1994). In the healthy periodontum, normal flora is sparse and consists mainly of gram-positive bacteria such as *S. oralis*, *S. sanguis*, and *Actinomyces* spp. In patients with gingivitis, the predominant subgingival flora includes anaerobic gram-negative bacilli, *Capnocytophaga* spp., and *Peptostreptococcus* spp. With progression to periodontitis, the flora increases further in complexity, with the addition of spirochetes (*Treponema denticola*), *Porphyromonas gingivalis*, and *A. actinomycetemcomitans*. With periodontal abscess, the polymicrobial flora also includes *Fusobacterium* spp. and viridans streptococci. Facultative gram-negative bacilli and staphylococci are uncommon in all periodontal infections. This microbial specificity has been defined by improved sampling and anaerobic culture techniques (Moore and Moore 1994; Maiden et al. 1997). The progression from supragingival dental plaque to subgingival dental plaque represents saccharolytic bacteria that can adhere to the tooth versus asaccharolytic bacteria that do not adhere to teeth, respectively. Periodontitis can be treated with systemic tetracycline or metronidazole combined with local treatment involving root debridement and surgical resection of inflamed tissues. Loculated abscesses must be drained (Loesche and Grossman 2001).

Stomatitis

Stomatitis refers to the inflammation of the oral mucosa including the buccal and labial mucosa, palate, tongue, floor of the mouth, and the gingivae. Severe forms occur in those with underlying malnutrition, debility, or immunocompromising disease such as human immunodeficiency virus (HIV) (Wexler et al. 1997). A small gingival ulcer progresses to a necrotic ulcer and then to a painful cellulitis of the lips and cheeks. Sloughing of necrotic soft tissues occurs, exposing underlying bone and teeth. *Borrelia vincentii*, *Prevotella melaninogenica*, and *Fusobacterium nucleatum* are common in the lesions (Chow 2000). Other bacteria are commonly seen in biopsy specimens but can not be cultured and identified. At this time, a comprehensive understanding of the pathogens involved does not exist. Treatment involves debridement, irrigation with a povidone–iodine solution, and systemic antibiotics with anaerobic coverage such as metronidazole, amoxycillin with clavulanic acid, or clindamycin (Chow 2000, Patton and van der Horst 1999).

Ludwig's angina

Ludwig's angina is a cellulitis of the submandibular, sublingual, and submental regions (Hartmann 1999; Spitalnic and Sucov 1995). In most patients, the infection originates from a dental focus. Clinical features include fever, toxicity, and a rapidly progressive edema in the floor of the mouth and the anterior neck. Elevation of the tongue impedes swallowing, and airway obstruction may be fatal. Streptococci and mouth flora are the most common etiologic agents, but *H. influenzae*, staphylococci, and gram-negative bacilli have also been implicated. Broad-spectrum antimicrobial therapy is needed

and tracheostomy may be necessary to preserve the airway (Barakate et al. 2001).

PARANASAL SINUSES AND ASSOCIATED AIRWAYS

Sinusitis

Sinusitis is a common disease with significant morbidity. It is estimated that 20 million cases of acute community-acquired bacterial sinusitis occur each year in the USA (Gwaltney 1996). In addition, bacterial nosocomial sinusitis is commonly recognized. Although bacterial infections of the sinuses are common, they are greatly overdiagnosed by patients and physicians. An understanding of the etiology, pathogenesis, and methods of diagnosis are important factors in preventing overdiagnosis.

Predominant pathogens in adult acute maxillary sinusitis are *S. pneumoniae* and *H. influenzae*, accounting for more than 50 percent of cases. *M. catarrhalis*, other streptococci such as *S. pyogenes* and viridans streptococci, *S. aureus*, and anaerobic bacteria account for the remainder of cases (Table 4). *Moraxella catarrhalis* is more common in pediatric patients, while anaerobes are less common. In spite of comprehensive bacteriologic analysis, cultures are negative from a high percentage of patients with the clinical diagnosis of sinusitis (Gwaltney 1996; Gwaltney et al. 1992). Some of these patients may have had viral infection rather than bacterial, and many may have had ostial occlusion preventing infected inflammatory material from draining and being accessible for culture. Others have suggested that *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* represent likely etiologies when bacterial cultures are negative (Gwaltney 1996). These bacteria have not been confirmed as causes of sinusitis by careful aspiration of sinus material and are not established as true pathogens. Although the relative importance in sinus infection of different bacteria has not changed in decades, there have been important changes in resistance to antimicrobials. The emergence of beta-lactamase production by *H. influenzae* and *M. catarrhalis* and the emergence of multiply resistant pneumococci have focused significant attention on the management of sinus infections (Brook 2002).

Table 4 *Etiology of adult and pediatric acute sinusitis*

Etiology	Approximate percentage of cases	
	Adult patients	Pediatric patients
Streptococcus pneumoniae	30	36
Haemophilus influenzae	20	23
Moraxella catarrhalis	5	20
Staphylococcus aureus	4	0
Streptococcus pyogenes	2	<5
Anaerobic bacteria	6	0
Other bacteria	5–10	<5
Viruses	25	3–5

Data from Bamberger and Jackson (2001) and Gwaltney (1996).

The bacteriology of chronic sinusitis is less clear because many of the patients have had prolonged and varied courses of antimicrobial therapy and culture material has not been collected by endoscopic or surgical drainage that avoids contamination with airway flora. Nevertheless, anaerobic pathogens are common in some studies of adults and uncommon in others (Bamberger and Jackson 2001; Nadel et al. 1998). Bacteria more frequently detected in chronic sinusitis include *S. aureus*, facultative gram-negative bacilli (*Enterobacteriaceae*) and *Pseudomonas aeruginosa*, and nonpneumococcal streptococci (Nadel et al. 1998).

Sphenoid, or frontal sinusitis, differs from acute maxillary sinusitis in that *S. aureus* is a frequent pathogen, in addition to pneumococci, *H. influenzae*, and anaerobic streptococci. Chronic bacterial sphenoid sinusitis is caused most commonly by gram-negative pathogens (Lew et al. 1983).

Bacteria associated with nosocomial sinusitis include usual nosocomial pathogens such as *P. aeruginosa*, *Acinetobacter* spp., enteric gram-negative bacilli, and *S. aureus*. Streptococci and anaerobes are less common. (Westergren et al. 1998a, b). Sinusitis is reported to be present in as many as 68 percent of HIV infected patients, especially those with CD4 cell counts below 200 per mm^3. A variety of bacterial and nonbacterial pathogens can be detected, with *P. aeruginosa* a common bacterial etiology (Rubin and Honigberg 1990). *P. aeruginosa* and *S. aureus* are common in patients with cystic fibrosis or syndromes that compromise ciliary motility (Mak and Henig 2001; Bertrand et al. 2000).

The pathogenesis of sinus infection is dominated by mucosal swelling and mechanical obstruction that occur following upper respiratory tract viral infection (Gwaltney 1996). Seventy-seven percent of patients have occlusion of the infundibulum and 87 percent have sinus cavity disease accompanying the common cold (Gwaltney 1996; Gwaltney et al. 1994). Also important is the fact that rhinovirus infection induces goblet cells to secrete an increased amount of thick mucus that is inadequately cleared by ciliary function. Thus, a major part of the disease process appears to be a function of mucocilliary clearance as a result of increased amounts of viscous material, which occurs in addition to infundibular and ostiomeatal obstruction (Gwaltney 1996). An estimated 0.5–2.0 percent of colds are complicated by bacterial infection of the sinuses arising from normal flora of the nasopharynx and nasal passages. Although the paranasal sinuses are normally sterile, transient inoculation with commensals from the nasal passages

can be effectively cleared by ciliary action within the sinus. Secretions and inflammation following rhinovirus infection, and presumably other viruses causing the common cold, impede effective clearing resulting in trapped bacteria in a 'closed' space (Puhakka et al. 1998; Diaz and Bamberger 1995; Monto et al. 2001).

Other less common causes of bacterial sinusitis include extension of dental infections into the maxillary sinus, ostial obstruction due to allergic rhinitis, anatomic abnormalities including as deviated nasal septum or nasal polyps, and barotrauma due to flying or deep-water diving (Bamberger and Jackson 2001). Immunodeficiency diseases also predispose patients to sinusitis, including HIV infection, diabetes, chronic granulomatous disease, and hypogammaglobulinemias. Risk factors for nosocomial sinusitis include nasogastric tubes and nasoendotracheal tubes (Rubin and Honigberg 1990; Westergren et al. 1998a).

Clinical features of sinusitis represent combined features of viral upper respiratory tract infection and bacterial sinusitis (Andre et al. 2002). Sneezing, rhinorrhea, nasal obstruction, facial pressure, and headache are common sinonasal complaints. In addition, those with confirmed acute community-acquired bacterial sinusitis are described to have purulent nasal discharge, a temperature of $\geqslant 38^{\circ}C$, facial pain or erythema, cough, and a decreased sense of smell (Gwaltney 1996).

Diagnosis of acute bacterial sinusitis is difficult despite modern technology (Gwaltney 1996; Andre et al. 2002). Differentiating allergy from bacterial infection and viral from bacterial etiology, and determining what specific bacterial etiology is involved, can be challenging. Sinus puncture provides definitive identification of etiology in cases serious enough to require invasive diagnostic techniques. Purulent sinus secretions carefully smeared and gram-stained have been shown to improve culture specificity (Jousimies-Somer et al. 1988). Morphotypes seen in the Gram stain, in association with polymorphonuclear leukocytes, predicted pathogens that eventually grew in concentrations above 1 000 CFU/ml on culture media. This suggests that smear, staining, and culture of sinus secretions may help differentiate bacterial from viral acute maxillary sinusitis. In addition, studies have shown that patients with sinus infection who have secretions containing *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* do benefit from antimicrobial therapy (Kaiser et al. 1996).

Appropriate antimicrobials given according to accepted regimens has been shown to reduce bacterial concentrations in the sinus cavity compared with incorrect antimicrobials or regimens (Gwaltney 1996). Because of the possibility of progressive damage to ciliated epithelium in the sinus, early therapy and eradication of a bacterial etiology are beneficial (Gwaltney et al. 1992; Hinni et al. 1992). Although multiply resistant pneumococci are an ever-increasing problem, therapy with amoxicillin-clavulante, an oral cephalosporin with maximal pneumococcal activity, or a newer fluoroquinolone are recommended antibacterial regimens (Pfaller et al. 2001; Poole 1997).

Prevention of acute sinusitis requires preventing upper respiratory tract viral infection and entry of nasal flora into the sinuses (Puhakka et al. 1998). To some extent, colds can be prevented by hygienic measures such as handwashing and covering one's mouth when sneezing. There is no proven measure for preventing secondary bacterial infection of the sinuses.

Rhinoscleroma

Rhinoscleroma is a chronic, progressive, disfiguring, and debilitating granulomatous infectious disease that has affected man for over 1 000 years (Hart and Rao 2000). It predominately affects the upper airways. It is caused by a gram-negative bacterium, *Klebsiella pneumoniae* subsp. *rhinoscleromatis*, most often referred to as *K. rhinoscleromatis*. Although test kits may not reliably identify this subspecies of *Klebsiella*, it can be differentiated from *K. pneumoniae* sensu stricto by a positive methyl red test and negative Voges-Proskauer, urease, citrate, and lysine decarboxylase tests (Toohill 1993).

More than 16 000 cases of rhinoscleroma have been reported since 1960 from most parts of the world (Hart and Rao 2000). It is found predominantly in rural areas and is more common in regions where socioeconomic conditions are poor. In developed countries in temperate zones, cases are detected but are generally imported. The disease has been widely reported from many Middle Eastern countries, tropical Africa, India, Southeast Asia, and Central and South America (Muzyka and Gubina 1971). Females are more commonly affected than males (ratio 13:1) and disease most commonly presents in the second and third decades of life (Hart and Rao 2000).

Disease usually occurs at the junction between epithelial surfaces, such as the interface between the squamous epithelium of the anterior nares and the deeper columnar ciliated epithelium (Hart and Rao 2000). Other explanations of disease prevalence include the fact that iron deficiency leads to squamous metaplasia, suggesting that poor nutrition and pregnancy may play a roll in the preponderance of disease occurring in women of childbearing age (Hart and Rao 2000) (Akhnoukh and Saad 1987).

Rhinoscleroma affects most areas of the respiratory tract (Hart and Rao 2000). The nose is involved in 95–100 percent of cases and the pharynx in up to half of cases. Occasionally, other areas of the respiratory tract are involved, such as the Eustachian tubes, sinuses, mouth, orbit, larynx, trachea, and bronchi. Although it may be considered an opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS), there are no reports of dissemination despite low CD4-lymphocyte (Andraca et al. 1993; Paul et al. 1993). Rhinoscleroma is divided into three stages: catarrhal-

atrophic (referred to as ozaena), granulomatous, and sclerotic (Hart and Rao 2000). The first stage is characterized by a foul-smelling purulent nasal discharge. Most cases are diagnosed in the granulomatous stage as the patient complains of epistaxis and nasal deformity with lesions appearing as bluish-red, rubbery granulomas. Histologic examination during the granulomatous stage reveals Mikulicz cells, which are large macrophages containing vacuoles filled with bacteria.

Treatment involves surgical debridement and prolonged antimicrobial therapy. In vitro, *K. rhinoscleromatis* is susceptible to many antimicrobials. With most therapeutic regimens, relapse is common. Prolonged therapy with fluoroquinolones has produced the best results with low relapse rates (Hart and Rao 2000; Borgstein et al. 1993).

EAR

Otitis media and acute mastoiditis

Otitis media is a common reason for very young children to seek medical treatment. The peak age of disease is between 6 and 15 months of age. By the age of 7 years, 90 percent of children have had at least one episode of otitis media (Klein 1994). Otitis media is categorized as acute, serous, or chronic (Hendley 2002; Klein 1994; Shurin et al. 1983). Acute disease is characterized simply by suppuration in the middle ear, fever, ear pain, and a bulging tympanic membrane. Serous otitis media is defined as the presence of middle ear effusion behind an intact tympanic membrane without acute signs or symptoms. Chronic otitis media occurs when long-standing discharge through a perforated tympanic membrane is noted. It is important to understand that, in spite of the widespread use of antimicrobial therapy for otitis, acute mastoiditis remains the most common suppurative complication of acute otitis media (Klein 1994).

Streptococcus pneumoniae and *H. influenzae* are the most common etiologies detected in all patient groups. Many pneumococcal serotypes are involved and nontypeable strains of *H. influenzae* predominate, similar to strains found as normal upper respiratory tract flora in children and adults. *Moraxella catarrhalis* may be recovered in up to 10 percent of cases (Shurin et al. 1983). Other etiologies occasionally encountered include *S. pyogenes*, *S. aureus*, facultative gram-negative bacilli (Enterobacteriaceae), and anaerobes (Bluestone 1998). *Pseudomonas aeruginosa* is a common etiology in chronic suppurative otitis media (Chan and Hadley 2001). In addition, *Mycobacterium tuberculosis* is an occasional cause of chronic suppurative otitis media (Jeang and Fletcher 1983).

The pathogenesis of otitis media is multifactoral (Bluestone 1996). Prior or concurrent viral infection, host differences, anatomic factors, and environmental exposures all impact the pathogenesis of middle ear infection. Viral infection predisposes the patient to development of acute otitis media by causing Eustachian tube dysfunction and enhancing nasopharyngeal colonization with middle ear pathogens (Saez-Llorens 1994; Faden et al. 1998). Otitis media is more common at a young age, in patients with immature or impaired immunologic status, in those with upper respiratory allergy, in those with a familial predisposition or other siblings in the household, in males, in Native Americans and Australian Aborigines, and in babies who are bottle-fed (Bamberger and Jackson 2001). Otitis media is also more common in children who attend daycare and in those exposed to passive cigarette smoke. The pathogenesis of acute otitis media may be summarized as follows: The patient has an upper respiratory tract viral infection that results in congestion of the respiratory mucosa, including the nasopharynx and Eustachian tube, resulting in obstruction of the tube. Negative pressure in the middle ear develops, and, when prolonged, results in 'aspiration' of potential bacterial and viral pathogens into the middle ear. Because of the obstruction, clearance of infected middle ear fluid is impaired. Fluid accumulates in the middle ear, followed by bacterial growth and resulting inflammation. Suppurative and symptomatic otitis media result. A complete discussion of the pathogenesis of otitis media can be found in a review by Bluestone (1996).

Acute mastoiditis can be a natural extension and part of the pathological process of acute otitis media. In fact, the mastoid air cells are involved in many children who have acute otitis media (Bluestone 1998). Infection of the mastoid air cells usually resolves spontaneously or after effective antimicrobial treatment. The effusion within the mastoid air cells drains into the middle ear and, in turn, into the Eustachian tube. The communication between the middle ear and the mastoid air cells is narrow and is called the aditus-ad-antrum (Bluestone 1996, 1998). Functionally, the mastoid air cell system is a reservoir of gas for the middle ear and the narrow passage makes it difficult for middle ear infection to enter the mastoid. The aditus-ad-antrum can become obstructed as a result of swelling and granulation tissue. This swelling is implicated in the pathogenesis of acute mastoiditis. If the obstruction persists, mastoiditis with periosteitis (infection of the periosteum covering the mastoid process) can develop. This condition can lead to mastoid osteitis and further develop into infection of the temporal bone and intracranial cavity (Bluestone 1998).

Overall, 85 percent of cases of acute otitis media in children will resolve without antimicrobial therapy. Importantly, spontaneous resolution occurs in 90, 70, and 20 percent of cases caused by *M. catarrhalis*, nontypeable *H. influenzae*, and *S. pneumoniae*, respectively

(Faden et al. 1998). It is clear that the pneumococcus is the most important pathogen. It is also clear that pneumococci collected worldwide can be resistant to multiple antimicrobial agents (Zhanel et al. 2003; Muhlemann et al. 2003). Since the widespread use of antimicrobial agents to treat acute otitis media, the incidence of mastoiditis and other complications has decreased from 40 percent early in the twentieth century to less than 0.5 percent at present (Faden et al. 1998). Antimicrobial treatment of acute otitis media is recommended to prevent suppurative sequelae, shorten the time spent with middle ear effusion, and prevent recurrent disease. Amoxycillin remains an appropriate choice for first-line therapy for acute otitis media (Hendley 2002; Klein 1994; Berman 1995; Hoberman et al. 2002). For patients in whom amoxicillin is unsuccessful, second-line therapy should have demonstrated activity against penicillin-resistant *S. pneumoniae* as well as beta-lactamase-producing pathogens. Appropriate options include high-dose amoxycillin-clavulanate and ceftriaxone. Tympanocentesis is useful for identifying persistent and resistant pathogens (Hoberman et al. 2002). The pneumococcal conjugate vaccine was approved recently for use in children and should be administered to all children less than 2 years old. Introduction of this vaccine is likely to have some beneficial effect on the proportion of resistant *S. pneumoniae* isolates by decreasing carriage of vaccine-related serotypes (Pelton 2002). In addition, the possible use of fluoroquinolones in pediatric patients and the introduction of ketolides may impact therapeutic recommendations for acute otitis media (Pelton 2002).

Malignant external otitis

Malignant external otitis is a serious infection caused by *P. aeruginosa* that occurs primarily in elderly diabetic subjects. Infection can progress to a skullbase osteomyelitis with resultant cranial neuropathies. In spite of long-term systemic antimicrobial therapy, the disease is recurrent and may be associated with significant mortality (Rubin and Yu 1988).

Since the natural reservoir of *P. aeruginosa* is water, the accumulation of moisture in the external ear canal is the likely inciting event (*P. aeruginosa* is also the most common etiologic agent of chronic external otitis). Spread from the external canal occurs as infection crosses the cartilaginous-osseus junction into the temporal bone, bypassing the tympanic membrane and middle ear. Infection then passes to the mastoid. Because of its proximity to the initial site of infection, the facial nerve is the first, and often only, cranial nerve to be involved. Small-vessel disease that results in cutaneous hypoperfusion, diminished local host resistance, and increased susceptibility to infection, is a likely reason that most patients have malignant external otitis. The pathogenesis of malignant external otitis in nondiabetic patients appears related to immune dysfunction or a combination of impairments resulting from underlying disease or malnutrition (Rubin and Yu 1988).

Therapy should include surgery for local debridement and excision of foci of infection (Rubin and Yu 1988; Raines and Schindler 1980). *P. aeruginosa* can be cultured from specimens representing material from the external auditory canal and removed during surgical debridement. However, the mainstay of therapy is systemic antimicrobial therapy with two anti-pseudomonal drugs (such as antipseudomonal beta-lactam and aminoglycoside) for up to 8 weeks or more if extensive disease is present.

ABSCESSES ADJACENT TO AND ORIGINATING FROM THE ORAL CAVITY

Peritonsillar, pharyngeal, retropharyngeal, and parapharyngeal abscesses

Infections of the deep spaces of the neck are potentially serious because of the likelihood of spread to critical structures, such as major blood vessels and the mediastinum, leading to thrombosis, mediastinitis, purulent pericarditis, and pleural empyema (Peterson and Thomson 1999; Chow 1992; Parhiscar and Har-El 2001). Peritonsillar abscess is caused by mixed aerobic and anaerobic bacteria, with *S. pyogenes*, *S. aureus*, and *S. anginosus* group streptococci playing a prominent role (Fujiyoshi et al. 2001). Pharyngeal, retropharyngeal, and parapharyngeal abscesses involve structures extending into the neck that may be life-threatening. These infectious sites are associated with anaerobes, which outnumber aerobes three to one, with an average of more than seven bacterial species in the abscess material. As expected, oral flora cause these infections, primarily represented by *Peptostreptococcus* spp., *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., and *Actinomyces* spp. Streptococci, primarily of the viridans group, are also important. When these abscesses develop in the hospitalized patient, *S. aureus* and various aerobic gram-negative bacilli are more likely to be involved (Peterson and Thomson 1999). Immediate surgical drainage and antimicrobial therapy directed at oral anaerobic flora and streptococci are indicated since complications can be life threatening (Parhiscar and Har-El 2001; Matsuda et al. 2002).

Lemierre's syndrome (jugular vein thrombophlebitis)

Lemierre's syndrome is a specialized oropharyngeal infection characterized by secondary septic thrombophlebitis of the internal jugular vein and frequent metastatic spread of infection (Chirinos et al. 2002).

Fusobacterium necrophorum, a normal inhabitant of the oral cavity, is the usual etiologic agent, along with other oral anaerobes that are occasionally detected. The disease progresses most commonly from pharyngitis, to local invasion of the lateral pharyngeal space, to internal jugular vein thrombophlebitis and subsequent metastatic complications involving the lungs, joints, liver, and mediastinal muscle. A swollen and/or tender neck and noncavitating pulmonary infiltrates in patients with recent or current pharyngitis are the most common findings. Blood collected by venipuncture and specimen representing material debrided during surgery, when performed, should be transported and cultured for aerobic and anaerobic bacteria. In the preantibiotic era the outcome was fatal with rapidly progressing septicemia. Mortality with antimicrobials and surgery (if necessary) is approximately 6 percent (Chirinos et al. 2002). Antimicrobial therapy with activity against anaerobic gram-negative bacilli and gram-positive cocci is recommended, such as metronidazole or clindamycin (Chirinos et al. 2002; Williams et al. 1998).

Suppurative parotitis

Acute suppurative parotitis (sialadenitis) is seen in very ill patients, especially those that are dehydrated, malnourished, elderly, or recovering from surgery (Thomson and Miller 2003; Raad et al. 1990). It is characterized by painful, tender swelling of the parotid gland. Purulent drainage may be evident at the opening of the duct of the gland in the mouth. *S. aureus* is the major pathogen, but on occasion viridans streptococci and oral anaerobes may play a role (Brook 2003). A chronic bacterial parotitis has been described that also is caused by *S. aureus*. Less often, other salivary glands may be involved, usually because of ductal obstruction. *Mycobacterium tuberculosis* may involve the parotid gland in conjunction with pulmonary tuberculosis (Mert et al. 2000).

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Bacterial infections of the lower respiratory tract

ROBERT C. READ

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INTRODUCTION

Despite the very large surface area of the lung and airways, few microorganisms colonize this tissue and cause disease. Thousands of microorganisms are inhaled with the air and aspirated with pharyngeal secretions during sleep (Kikuchi et al. 1994); for each of these, there is at least one of four possible outcomes. First, the microorganisms may be rapidly cleared from the lung. Second, they may lodge in the upper respiratory tract and asymptomatically colonize the site. Third, they may persist in regions that are normally sterile, such as below the larynx, as is observed in chronic obstructive pulmonary disease. Finally, they may penetrate epithelium and initiate parenchymal disease, as in pneumonia, or they may invade the bloodstream to cause systemic infection. Microbial load and host defenses are the subject of a constant balancing act. Successful microbes have adapted to avoid rapid clearance, gain nutrients, and survive in the respiratory tract sufficiently to grow and disseminate to other hosts. The aim of host defenses is to maintain the sterility of the gas-exchange areas. Disease results when local defenses are overcome and inflammatory cells are recruited to eradicate the infection.

PULMONARY DEFENSE MECHANISMS

Pulmonary defense mechanisms are of two types (Reynolds 1994): resident defenses, such as airway architecture and mucociliary clearance, which are constantly operative, and recruited defenses, which augment the resident defenses when the host detects danger and result in inflammation with resulting disease.

Resident defenses

AIRWAY ARCHITECTURE

Movements of the glottis and epiglottis during swallowing and the cough reflex are important barriers to bacterial invasion of the airway, especially by organisms aspirated from the alimentary tract.

During nose breathing, particles larger than 5 μm are efficiently removed by impaction on the walls of the nose and nasopharynx as a result of airflow rendered turbulent by the nasal turbinates and the configuration of the nasopharynx and oropharynx. Sedimentation is the main mechanism of deposition of particles, such as bacteria in the size range 5–0.6 μm, and takes place in parts of the lung in which airflow is slow, i.e. between

the fifth bronchial division and the terminal lung units. Invasion of the lung by sedimented particles is prevented by the combination of an efficient epithelial barrier, mucociliary clearance, and phagocytic mechanisms.

THE EPITHELIAL BARRIER

The respiratory tract, from the nose to the respiratory bronchiole, is lined by ciliated, pseudostratified, columnar cells interspersed with occasional mucus-secreting cells (see Figure 1). The remainder is lined by nonciliated epithelium including (1) a small zone of stratified epithelium below the pharyngeal fornix and (2) nonkeratinizing squamous epithelium in the oropharynx, the anterior surface of the epiglottis and the upper half of its posterior surface, the upper half of the aryepiglottic folds, and the vocal cords. Tight junctions between epithelial cells provide an efficient barrier to bacterial penetration. This epithelium is continually replenished; if damaged, fully differentiated ciliated cells are replaced within 2–6 weeks, though basal cells continue to protect the basement membrane.

MUCOCILIARY CLEARANCE

Throughout the airway mucus-secreting cells secrete a blanket of mucus that is propelled along by ciliary action towards the larynx where it is coughed up or swallowed. Inhaled bacteria adhere to mucus and are conveyed along this mucociliary escalator (Figure 2). Efficient mucociliary transport of inhaled foreign bodies depends on coordinated ciliary beating, the depth and constituents of the periciliary fluid that lies beneath the blanket of mucus, and the rheological qualities of the mucus. Viscid mucus, such as that in cystic fibrosis, is difficult for the cilia to clear. Cilia beat at 12–17 Hz, but the frequency is slightly lower in more peripheral airways. In normal airways, clearance times of inhaled aerosols are 30 min from the lobar bronchi, 4–6 h from 1–5-mm airways, and 1 day to several months or more, depending on the substance inhaled, from airways distal to the terminal bronchioles. Clearly, disruption of mucociliary clearance leads to stasis of infected mucus and potentially to disease.

Figure 1 *Micrograph of typical human pseudostratified columnar epithelium within the respiratory tract, showing ciliated cells interspersed with mucus-secreting (goblet) cells. (Courtesy of A. Rutman.)*

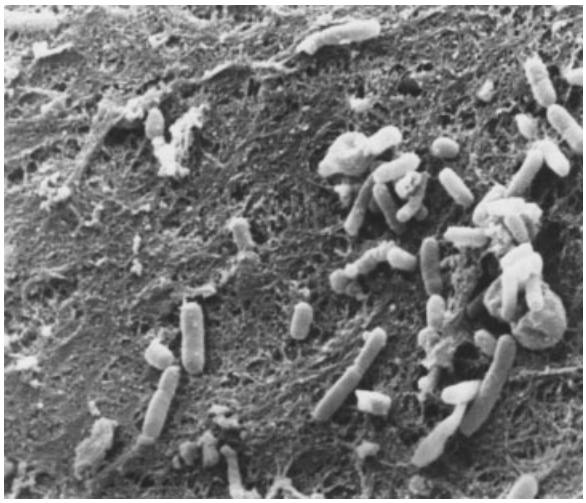

Figure 2 *Scanning electron micrograph of airway mucosa showing* Haemophilus influenzae *attached to mucus. (A. Brain and R.C. Read.)*

SOLUBLE FACTORS IN AIRWAY SECRETIONS

Mucus contains many factors that enhance the clearance of microorganisms from the airway; most of these are plasma proteins and include α_1-antitrypsin, a low-molecular-weight chymotrypsin inhibitor. Neutral protease and elastase are secreted by polymorphonuclear leukocytes and alveolar macrophages. α_1-Antitrypsin deficiency is associated with chronic pulmonary disease, probably because of failure to inhibit the neutrophil products during recurrent inflammatory responses to lung infection.

Lysozyme is secreted by neutrophils and found in most mucosal secretions, where it is directly active against some bacteria. Lactoferrin is secreted by mucosal epithelial cells and neutrophils. Like transferrin, it has a high affinity for iron and inhibits bacterial replication within the airway by restricting iron availability.

Surfactant is secreted by pneumocytes within alveoli and has the vital function of reducing surface tension in gas exchange areas and keeping these patent. Surfactant proteins A and D are significant defense molecules present in alveolar fluid that agglutinate and opsonize a

wide range of bacteria and fungi (Lawson and Reid 2000).

Complement

Many of the components of the complement system are present at very low levels in normal bronchial secretions. They are probably derived directly from serum, but alveolar macrophages also secrete a number of the components. In terms of defense against bacteria, complement has two broad functions. Deposition of C3b on bacteria ultimately results in their recognition by macrophage CR1, CR3, and CR4 receptors, and then phagocytosis. Additionally, there is probably sufficient C5 to initiate bacterial lysis by the terminal membrane attack complex. Complement concentrations rise dramatically during periods of lung damage, e.g. during serious infection of the lung, when airways are relatively leaky for serum proteins.

Immunoglobulins

Immunoglobulins IgA, IgG, IgM, and IgE, including specific antibody, can be recovered from respiratory tract secretions (Lipscomb et al. 1995). IgM is found in lung washings in only very low concentrations.

Secretory IgA (sIgA) is synthesized by lymphoid cells beneath the basement membrane, predominantly in the upper and middle portions of the respiratory tract. Whereas IgA is the predominant immunoglobulin in the upper airways, lower-airway secretions more resemble serum in that the IgG to IgA ratio is much higher. The major function of sIgA is complement-independent neutralization of respiratory viruses. It also opsonizes organisms for phagocytosis by macrophages, but it does not contribute to polymorph opsonization; nor does it activate complement. Secretory IgA interacts with lysozyme and complement to augment their bactericidal activity, mediates bacterial adherence to mucus, and inhibits bacterial adherence to epithelial surfaces. Once challenged by a given agent, the host produces sIgA in response to homologous challenge. However, the period of immune protection is relatively short in contrast to serum IgG and booster responses are variable after later challenge. Certain bacteria, notably *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*, secrete IgA proteases capable of inactivating mucosal IgA and other activities.

IgG gains access to the airways by transudation from the circulation, but it can be produced locally in the lung. It efficiently agglutinates bacteria, mediates bacterial opsonization for macrophages and neutrophils, neutralizes bacterial exotoxins, and activates complement. After a primary challenge, specific antibody is released into blood from lung-associated lymph nodes and transudes into the airway. Subsequently, memory B cells traffic to the lung and produce specific IgG in response to further specific challenge (Bice et al. 1991).

ALVEOLAR MACROPHAGES

Alveolar macrophages are responsible for noninflammatory clearance of microorganisms, including those that sediment in the most distal airways beyond mucociliary clearance (Gordon and Read 2002). They comprise about 80–90 percent of the cell population of fluid obtained by bronchoalveolar lavage. Four populations of pulmonary macrophages can be distinguished:

1. alveolar macrophages
2. interstitial macrophages
3. dendritic macrophages and
4. intravascular macrophages.

Pulmonary macrophages are derived from peripheral blood monocytes; they differentiate after chemotaxis to pulmonary tissue and can replicate in the lung. Alveolar macrophages reside within the airspace, whereas interstitial macrophages are located in the lung connective tissues. Although alveolar and interstitial macrophages both undertake Fc receptor-dependent phagocytosis, they differ in their other functions. Alveolar macrophages are capable of Fc receptor-independent phagocytosis and the production of cytokines, such as tumor necrosis factor α (TNF-α) and interferons α and β, which are released in order to recruit an inflammatory response. Production of oxygen radicals is greatest in alveolar macrophages. Interstitial macrophages are adapted for antigen presentation (Lohmann-Mathes et al. 1994), whereas dendritic cells are predominantly nonphagocytic and specialized for antigen presentation. Macrophages in the vascular compartment are located on the capillary endothelium. They are highly phagocytic and probably remove foreign material that enters the lung via the bloodstream.

Alveolar macrophages possess three classes of surface receptor that facilitate phagocytosis of bacteria. The Fc receptor, of which there are four subclasses with differing affinities, are responsible for the recognition of antibody-coated bacteria. They recognize the Fc region of IgG and IgA on antibody-coated bacteria. The second are complement receptors CR1, CR3, and CR4, which recognize bacteria coated with C3b. The third are lectin-binding receptors such as the mannose-6-phosphate receptor. After phagocytosis, bacteria are enclosed in phagosomes, which ultimately acquire the characteristics of lysosomes (Gordon et al. 2000a). Bacteria are killed by a combination of products of the oxidative burst and bactericidal enzymes and other proteins.

The bactericidal activity of alveolar macrophages depends crucially on the inoculum size. Up to about 10^5 bacteria are eliminated by alveolar macrophage phagocytosis alone, whereas the killing of an inoculum of 10^6 requires a modest influx of neutrophils into the alveoli.

Inocula greater than 10^8 organisms, however, necessitate activation of local T and B lymphocytes in addition to phagocytosis by macrophages and neutrophils. Apart from the number of microorganisms, the species of the bacterium can affect the efficiency of killing by macrophages. *Staphylococcus aureus* can be killed adequately by macrophage phagocytosis alone, but *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* additionally require neutrophils to aid intrapulmonary killing. Other bacteria, such as mycobacterial species and *Legionella pneumophila*, are readily phagocytosed by macrophages but cannot be killed. Killing occurs once the macrophage has been activated by T lymphocyte-derived macrophage-activating cytokines, e.g. interleukin-1.

Alveolar macrophages, in common with other cellular components of innate immune defense, recognize pathogens via a family of Toll-like receptors and cofactors and coreceptors such as MD-2 and CD14. Following activation of these receptors by microbial ligands (e.g. lipoteichoic acid of *S. pneumoniae* or lipopolysaccharide of *H. influenzae*), there is transcriptional activation of a number of important host response genes, including those that encode cytokines and chemokines directly relevant to eradication of bacteria (Read and Wyllie 2001).

Recruited defenses

If the combined effect of mucociliary clearance, complement, and macrophage phagocytosis fails to clear an inoculum of bacteria, or if the infecting organism is particularly virulent, for example some strains of pneumococcus, polymorphonuclear neutrophils and lymphocytes are recruited to augment the host response. Neutrophils migrate from the vascular compartment into the alveolus by chemotaxis. The stimulus for chemotaxis originates within the alveolus and is due to direct generation of chemotactic factors by microorganisms entering the alveoli, and the release of chemotactic factors from alveolar macrophages after phagocytosis. Leukotriene B4 is an important chemotactic factor that also alters pulmonary capillary permeability. This, together with the secretion of TNF-α by macrophages, promotes the accumulation of neutrophils and fluid and other humoral substances in alveoli. Neutrophils kill ingested bacteria very much faster than macrophages with a combination of oxidative metabolites. This process, together with injury due to proteolytic enzymes, e.g. neutrophil elastase, contributes to consolidation of the lung.

Bronchoalveolar lavage fluid from the normal resting human lung contains predominantly macrophages but also lymphocytes (20 percent) including CD4 (helper), CD8 (suppressor), and a few B lymphocytes (Reynolds 1994). There is a complex interplay between lymphocytes, macrophages, and neutrophils in the management and termination of inflammation. Lymphocytes can regulate the activation of macrophages and subsequently coordinate the inflammatory response in a given infection. The resolution of inflammation is accompanied by neutrophil apoptosis, with subsequent consumption by macrophages. This process is conducted by inflammatory macrophages, which employ novel phagocytic recognition mechanisms that fail to provoke a macrophage proinflammatory response (Haslett 1999). Additionally, macrophages may themselves undergo apoptosis following successful phagocytic killing of some organisms, e.g. *S. pneumoniae* (Dockrell et al. 2001).

A host infected with organisms capable of intracellular survival, such as *Mycobacterium tuberculosis*, *Pneumocystis carinii*, and *Legionella pneumophila*, requires cell-mediated immunity to eradicate the infections (Sim 1998).

At the time of primary challenge, antigen is transported to lung-associated lymph nodes either contained within alveolar and dendritic macrophages or recruited neutrophils, or free in the afferent lymphatic fluid (Lipscomb et al. 1995). In the presence of lung inflammation, this process is accelerated. Within lymph nodes, antigen is reprocessed by antigen-presenting cells. Specific T and B cell clones expand and differentiate, and effector T cells and B lymphoblasts migrate out (maximally by 10–14 days), the latter to provide specific antibody locally in lung tissue. Subsequent responses to the same infecting organism are produced by immune memory B cells lodged within the lung parenchyma.

PATHOGENESIS OF BACTERIAL RESPIRATORY INFECTIONS

Disease results when the equilibrium between organisms that enter the respiratory tract and resident host defenses is disturbed. This may be due to a defect in host defenses, an overwhelming microbial challenge, or a combination of the two. A normal host may not be able to deal with the microbial load, due to the large number of organisms or because the organisms possess potent virulence determinants.

Host defense defects

HOST FACTORS THAT PERMIT INCREASED COLONIZATION OF THE UPPER RESPIRATORY TRACT

Colonization of the upper respiratory tract is probably the first step in the pathogenesis of most bacterial pulmonary infections and adherence of bacteria to epithelial cells is a key event in colonization (Woods 1994). Individuals with poor nutrition, particularly vitamin A deficiency, are more likely to have upper respiratory tract colonization. Epithelial cells from patients with severe or chronic illness appear to be particularly receptive to bacteria, especially gram-negative

organisms. This may be due to a reduction in local IgA, local fibronectin production, or a change in the glycoprotein and glycosphingolipid composition of epithelial cell surfaces.

FAILURE OF EPITHELIAL BARRIERS AND REFLEXES

Bypassing the upper respiratory tract with an endotracheal tube or by tracheostomy vastly increases the host dependence on resident host defenses of the lower respiratory tract. The distal airways in intubated patients rapidly become colonized by gram-negative bacteria including *P. aeruginosa* (Johanson et al. 1980). Failure of the swallowing reflex in patients with neurological defects permits aspiration of the commensal oropharyngeal flora and alimentary tract contents.

FAILURE OF MUCOCILIARY CLEARANCE

Ciliary dyskinesia may be due to intrinsic structural abnormalities, including defective dynein arms, defective radial spokes, microtubular transposition, and random orientation of the central tubule (Rutland and de Longh 1990). Such abnormalities can result in complications that range from relatively mild recurrent chest infections and sinusitis to severe clinical problems such as Kartagener's syndrome (sinusitis, bronchiectasis, and malrotation of viscera – *situs inversus*). Respiratory viral infection, particularly influenza, can also result in marked depletion of ciliated epithelial cells, reduced mucociliary clearance, and secondary bacterial infection, for example with *S. aureus* (Shanley 1995).

Cystic fibrosis is due to abnormalities of chloride-ion transport by glandular and ciliated epithelial cells in various tissues, resulting in viscid external secretions. In the airways, this results in inadequate mucociliary clearance, colonization by pathogens with subsequent chronic inflammation, and eventual bronchiectasis. These processes lead to virtually continuous inflammation and infection of the respiratory tract of cystic fibrosis patients from infancy onwards.

IMMUNOGLOBULIN DEFICIENCY

Patients with agammaglobulinemia and dysgammaglobulinemia produce decreased amounts of some or all of the serum immunoglobulins, notably IgG. They are susceptible to respiratory infections, including those due to encapsulated organisms, such as *S. pneumoniae* and *H. influenzae*, and others such as *Mycoplasma pneumoniae* (Buckley 1992). Chronic respiratory infection due to common variable immunodeficiency can lead to bronchiectasis. Selective IgA deficiency, particularly when associated with deficiency of IgG2, is also associated with recurrent respiratory tract infections. The IgG2 includes antibodies against capsulate organisms such as pneumococcus and *Haemophilus* spp., and also to lipoteichoic acid of *Streptococcus* spp. IgG4 deficiency is also associated with recurrent respiratory tract infection.

REDUCED PHAGOCYTIC ACTIVITY

The most common cause of failure of the phagocytic response results from iatrogenic immunosuppression. Neutropenia results in a reduced inflammatory response in the lung and an increased incidence of gram-negative bacillary and fungal infection. Macrophages can also be depleted as a result of immunosuppression, which results in pneumonia due to intracellular microorganisms, including *Legionella* spp.

DISTURBED CELLULAR IMMUNITY

Disorders of cell-mediated immunity can result from hematological malignancies, e.g. leukemia, human immunodeficiency virus (HIV) infection, or from drug therapy, e.g. steroids. The consequence for the lung is that an inflammatory response cannot be adequately recruited or controlled, and macrophages cannot be activated to kill intracellular pathogens. The reduction in CD4 (T helper cells) in acquired immune deficiency syndrome (AIDS) is associated with a variety of respiratory infections by intracellular pathogens, notably *P. carinii* and *M. tuberculosis*.

ENVIRONMENTAL INFLUENCES ON THE HOST

Tobacco smoking is the major cause of airway morbidity that leads to recurrent respiratory tract infection. Smoke reduces ciliary clearance by direct toxicity, oxidizes α_1-antitrypsin, increases mucus production, and is a chemoattractant for neutrophils and macrophages in the peripheral airways. These lead to squamous metaplasia of ciliated epithelium, goblet cell hyperplasia, and intraepithelial inflammation. The consequences are frequent cough, sputum production and intercurrent infection, particularly with *H. influenzae* and *Moraxella catarrhalis* (Floreani et al. 1994).

The effects of air pollution are far more subtle than those of smoking. Upper respiratory tract infections (coryza and nasal discharge) and lower respiratory tract infection (pneumonia and purulent sputum production) are observed more frequently in polluted urban areas (Lunn et al. 1967). High ambient pollutant levels correlate with upper respiratory infections (Ponka 1990). Pollutants, including sulfur dioxide, nitrogen dioxide, acid aerosols, and particulates, reduce mucociliary clearance, damage epithelial cells, and impair phagocytosis in vitro (Schlesinger 1990).

Bacterial virulence mechanisms

Respiratory pathogens produce a variety of virulence factors that promote infection by mechanisms that include:

- increased adherence to mucosal epithelial cells
- avoidance of mucociliary clearance

- increased nutrient acquisition
- avoidance of complement deposition and phagocytosis and
- perturbation of neutrophil and macrophage apoptosis.

Some pathogens produce factors that are directly toxic to the host, such as streptococcal hyaluronidase and *Pseudomonas* elastase, which allow expansion of the ecological niche and damage host connective tissue.

STREPTOCOCCUS PNEUMONIAE

S. pneumoniae produces a protein adhesin that binds to the *N*-acetylgalactose–galactose component of respiratory tract epithelial cell-membrane glycolipids (Krivan et al. 1988). It also produces IgA protease, which inactivates respiratory mucosal IgA. Pneumolysin, a 52.8-kDa protein, is a thiol-activated cytotoxin that shares amino acid homology with bacterial thiol-activated toxins and is released on autolysis. In experimental animals it can induce inflammation in the lungs independently of intact pneumococci (Feldman et al. 1991). Virulent pneumococci have an antiphagocytic capsule, which permits evasion of recruited neutrophils, and some capsular serotypes, e.g. serotype 3, are more virulent than others. This may be due to the relative composition of capsules, particularly their choline content (Bruyn et al. 1992). Pneumococci produce a range of other products important in pathogenesis, including pneumococcal surface protein A, autolysin, choline binding protein A (CbpA), pneumococcal surface antigen A, and neuraminidase (Jedrzejas 2001). Toxic products of *S. pneumoniae* also include hydrogen peroxide (Duane et al. 1993). The cell-wall lipoteichoic acid of *S. pneumoniae* is potently pro-inflammatory in the lung by activation of the alternative complement pathway and, like lipopolysaccharide (LPS), it elicits production of interleukin-1 (IL-1) and TNF-α (Tuomanen et al. 1995). The quantity of strain cell-wall lipoteichoic acid and capsular polysaccharide has been correlated with virulence (Weiser et al. 1996). *S. pneumoniae* expresses a C3-binding protein that elicits interleukin-8 secretion by pulmonary epithelial cells (Madsen et al. 2000), and CbpA also induces chemokine release from these cells (Murdoch et al. 2002). The complete genome sequence of *S. pneumoniae* has been published and contains 2236 predicted coding regions. Approximately 5 percent of the genome is composed of insertion sequences and there are approximately 70 predicted surface proteins (Tettelin et al. 2001).

HAEMOPHILUS INFLUENZAE

H. influenzae possesses fimbriaethat are structurally and serologically related to the P and mannose-sensitive fimbriae of *Escherichia coli* and promote adherence to respiratory epithelium and mucin. *H. influenzae* also appears to bind specifically to GalNAcβ1-4Gal sequences of human lung glycolipids. The organism may also secrete a soluble toxin that reduces ciliary beating and so promotes avoidance of mucociliary clearance (Wilson 1988). Lipo-oligosaccharide of *H. influenzae* is toxic to human respiratory tract epithelium. This organism also secretes IgA protease, which contributes to virulence (Vitovski et al. 2002).

BORDETELLA PERTUSSIS

B. pertussis produces several adhesins including a filamentous hemagglutinin (220 kDa), fimbriae, and an array of toxins, including pertussis toxin (105 kDa), which binds to respiratory tract ciliated cells and macrophages and is clearly important in airway colonization. Release of filamentous hemagglutinin from the surface of individual bacteria within adherent microcolonies facilitates dispersal of the organism through the respiratory tract (Coutte et al. 2003). Pertussis toxin also causes epithelial cell cytotoxicity by increasing host cell cyclic adenosine monophosphate (cAMP) levels (Masure 1992), an action broadly similar to cholera toxin. Tracheal cytotoxin is a peptidoglycan fragment toxic for ciliated cells that directly induces inflammation in animal models (Wilson et al. 1991). *B. pertussis* can survive in human phagocytes by inhibiting phagosome–lysosome fusion, but it does not inhibit the respiratory burst (Steed et al. 1992).

PSEUDOMONAS AERUGINOSA

P. aeruginosa produces fimbrial and nonfimbrial adhesins that mediate binding to epithelial cell gangliosides after its sialic acid residues have been removed by neuraminidase. It also produces a number of toxins, including exotoxin A, which damages respiratory epithelial tissue and inhibits phagocytosis (Coburn 1992), and a number of elastases that act together to damage epithelial cells and blood vessel walls (Galloway 1991). *P. aeruginosa* directly activates NF-kappaB, which in turn activates MUC2 mucin transcription in epithelial cells (Li et al. 1998). A membrane glycolipid of *P. aeruginosa*, rhamnolipid, can reduce ciliary beating and mucociliary clearance (Read et al. 1992), and secreted pigments, including pyocyanin and 1-hydroxyphenazine, also reduce ciliary beating (Wilson 1988). Alginate, which forms a viscous gel around bacteria and gives them a mucoid appearance, functions as an adhesin and prevents phagocytosis. Alginate also appears to contribute to biofilm formation on epithelial surfaces (Nivens et al. 2001), a phenomenon that appears to be regulated by quorum-sensing genes (Davies et al. 1998).

LEGIONELLA PNEUMOPHILA

L. pneumophila appears to survive macrophage phagocytosis and kill human macrophages. It has a 24-kDa outer-membrane macrophage invasion protein that allows invasion of macrophages in the absence of opsonization, but this does not appear to improve intracellular survival once the organism is endocytosed. The

bacterium enters macrophages by coiling phagocytosis (a form of phagocytosis in which the cell envelops the organism by coiling its pseudopodia around it) and then resides within a unique phagosome, which does not become highly acidic. Therein, legionella express stationary-phase proteins that encourage intracellular survival and transmission to new cells (Swanson and Hammer 2000). Several bacterial enzymes, including acid phosphatase, phospholipase C, protein kinases, and superoxide dismutase, potentially enhance intracellular survival. *L. pneumophila* proteases cause lung damage and secretes a metalloproteinase similar to *P. aeruginosa* elastase (Dowling et al., 1992). Adaption and replication of *L. pneumophila* in vivo are enhanced by phase variation of expression of LPS (Luneberg et al., 1998). A 22-kb DNA locus contains at least 16 genes involved in macrophage killing (Segal et al. 1998).

RESPIRATORY TRACT COMMENSALS

The anterior nares are colonized by staphylococci, micrococci, and miscellaneous *Corynebacterium* spp., but the paranasal sinuses are normally sterile.

In the mouth, anaerobes colonize periodontal areas and a variety of other organisms including 'viridans' streptococci (e.g. *Streptococcus mitis*, *Streptococcus sanguis*), *Haemophilus* spp., *Neisseria* spp., and diphtheroids are present in saliva. The practical consequence is that sputum can be heavily contaminated and make laboratory interpretation very difficult.

The flora of the oronasopharynx is much more complex; it may be colonized by *Streptococcus* spp., *Neisseria* spp., coliform bacteria, *Bacteroides* spp., fusobacteria, actinomycetes, and yeasts. From time to time, however, individuals may be colonized by one or more pathogens, such as β-hemolytic streptococci, *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, and *M. catarrhalis*. Periods of asymptomatic colonization may be very short or may last for several months, and although the colonization is asymptomatic, it is generally accepted that stable residence at this site is a prerequisite for invasive disease by such organisms as *H. influenzae*, *S. pneumoniae*, and *N. meningitidis*. Point-prevalence studies in communities indicate the relative prevalence of these pathogens in the nasopharynx of colonized individuals (Table 1).

A number of exogenous factors may disturb the ecology of the mouth and nasopharynx. Antibiotics can suppress oral bacteria and allow yeasts to proliferate and thrush to occur. Smoking and passive smoking increase the prevalence of colonization with pathogens, including *N. meningitidis* (Caugant et al. 1994).

The upper respiratory tract is colonized by an abundant resident microbial flora that varies as a result of changing endogenous and exogenous conditions. Below the larynx, the lower respiratory tract is sterile in normal individuals because inhaled organisms are rapidly removed by clearance mechanisms.

INFECTIONS OF THE UPPER RESPIRATORY TRACT

Infections of the oronasopharynx

SINUSITIS

The paranasal sinuses are normally sterile. Bacteria recovered by sinus puncture before treatment of acute community-acquired sinusitis include *S. pneumoniae*, *H. influenzae*, anaerobes, *Streptococcus* spp., *M. catarrhalis*, and *S. aureus*. In chronic sinusitis, bacteriological cultures are more exotic and polymicrobial than in acute sinusitis and may include anaerobes and *Pseudomonas* spp. (van Cauwenberge et al. 1993). Conservative management of acute sinusitis involves use of appropriate antibiotics such as ampicillin, amoxycillin, trimethoprim–sulfamethoxazole, cefaclor, cefuroxime axetil, amoxycillin–clavulanate, and loracarbef (Gwaltney et al. 1992). An alternative strategy is immediate sinus puncture, which may permit more rapid resolution and provide material for the determination of antimicrobial sensitivities. Chronic disease should prompt allergic and immunological investigation and prolonged therapy, including antibiotics for anaerobic infection. Ultimately, chronic disease may require surgery to establish free drainage.

OTITIS MEDIA

Upper respiratory tract infections, often viral, are most commonly responsible for the events that lead to otitis media. Such infections impair the function of the eustachian tube, creating a negative pressure and transudation into the middle ear. Bacterial contamination results by reflux from the oropharynx, and this leads to further accumulation of fluid and pus.

Table 1 *Point prevalence of nasopharyngeal carriage of pathogens*

	Prevalence (%)
Nontypable *Haemophilus influenzae*	25–48 (Turk 1984)
Haemophilus influenzae type b (prevaccine era)	2–4 (Moxon 1986)
Streptococcus pneumoniae	60–100 (Austrian 1986)
Neisseria meningitidis	5–10 (Broome 1986)

The four stages of otitis media are:

1. Myringitis – inflammation of the tympanic membrane
2. Acute suppurative otitis media, which denotes a middle ear infection behind the reddened tympanic membrane
3. Secretory (serous) otitis media, which refers to chronic middle ear effusion behind an intact tympanic membrane with acute signs and symptoms and
4. Chronic suppurative otitis media – a chronic discharge from the middle ear through a perforation of the tympanic membrane. Chronic disease can lead to loss of aeration of the middle ear cleft, loss of hearing, and speech retardation during infancy.

Otitis media primarily affects children but can lead to lifelong sequelae. In acute otitis media the major organisms are *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*. These organisms can also be recovered in chronic otitis media but in addition, *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and anaerobic bacteria can all be present (Brook and Van de Heyning 1994). Empirical therapy may be given in most cases, including the aminopenicillins and second-generation cephalosporins and aminopenicillins in combination with β-lactamase inhibitors where β-lactamase producing organisms are present. About half the patients recover within 10 days with appropriate therapy, whereas most of the remainder have a residual exudate that resolves over 3 months. About 5 percent develop secretory otitis media. A meta-analysis has suggested that 5 days of short-acting antibiotic use is effective treatment for uncomplicated otitis media in children (Kozyrskyj et al. 1998).

Chronic otitis media requires surgical correction, drainage, and treatment for anaerobic bacteria with agents such as aminopenicillins plus β-lactamase inhibitors. In such cases, the microbiological flora should always be determined to rule out the presence of anaerobes or *Pseudomonas* spp.

RHINOSCLEROMA

This is a chronic granulomatous disease of the nose found in eastern Europe and parts of the tropics. It leads to nasal obstruction and marked secretion with postnasal drip. It is due to *K. pneumoniae* subsp. *rhinoscleromatis* (see Citrobacter, Klebsiella, Enterobacter, Serratia and other enterobacteriaceae).

Infections of the perioral and peripharyngeal spaces

Infections of oral soft tissues are uncommon and caused by endogenous oral bacteria including oral anaerobes. Such infections can lead to pain and swelling of the sublingual and submandibular space (Vincent's angina) and also collections within the lateral pharyngeal space. An extreme form of this is the rare Lemierre's syndrome, which consists of oropharyngeal infection and anaerobic bacteremia and septic thrombophlebitis of the jugular vein, with embolization to the lungs and other areas. *Fusobacterium necrophorum* is usually the cause and prolonged treatment with parenteral benzylpenicillin and metronidazole is necessary.

Peritonsillar abscess can occur in patients with recurrent tonsillitis or inadequately treated pharyngotonsillitis. Bacteria that are recovered include *Streptococcus pyogenes*, *Streptococcus milleri*, *H. influenzae*, and viridans streptococci. Anaerobes, including *F. necrophorum*, *Prevotella* spp., and *Peptostreptococcus* spp., can also be isolated from peritonsillar abscesses. The clinical features are high fever, intense pain in the throat, voice change, and occasionally airway obstruction (Jousimies-Somer et al. 1993). Treatment with needle aspiration and oral penicillin is sufficient for most patients with peritonsillar abscesses (Maharaj et al. 1991). When incipient airway obstruction is present, this is best treated by incision and drainage.

Laryngotracheal infections

Most laryngitis is due to virus infection, but *M. catarrhalis* and *H. influenzae* may be recovered from such patients and some symptoms of laryngitis can be improved with oral erythromycin (Schalen et al. 1992). Tracheitis is usually due to viruses, including respiratory syncytial virus. Bacterial pathogens associated with tracheitis include *H. influenzae*, *S. aureus*, and *Streptococcus* spp., including *S. pneumoniae*.

PNEUMONIA

This is a general term for disease that includes consolidation of the lung parenchyma. There is acute inflammation in the gas-exchanging areas of the lung (pneumonitis), with a polymorphonuclear leukocyte exudate in and around the alveoli and terminal and respiratory bronchioles.

The most useful clinical classification of pneumonia divides cases into community-acquired and nosocomial. Other considerations include whether the pneumonia results from aspiration or follows from an acute viral infection, is acquired in specific geographical settings, or occurs in the context of immunosuppression, including AIDS.

Community-acquired pneumonia

The true incidence of community-acquired pneumonia is not easy to determine, but approximately one per 1 000 of the population is admitted to hospital with pneumonia annually in the UK (Woodhead et al. 1987). In the USA it is estimated that there are 2–3 million episodes of pneumonia per annum (an attack rate of 12 per 1 000 persons per year). Each year the bacterial

pneumonias account for over half a million hospital admissions of patients aged 15 years or older in the USA (Pennington 1994a) and 45 000 deaths (Pinner et al. 1996; Marston et al. 1997). Pneumonia is substantially more common in the winter and affects more males than females (ratio 2–3:1). It is more common amongst older persons; the annual incidence of pneumonia that requires hospitalization of those older than 75 years is 11.6 cases per 1 000; for those aged 35–44 years it is 0.54 cases per 1 000 persons (Marrie 1994).

The symptoms include cough, sputum, dyspnea, and pleuritic pain. Classically the sputum is rusty-colored in pneumococcal pneumonia but is usually mucoid, scanty, or absent, especially at an early stage, and in mycoplasma and legionella infections it may be absent. Extrapulmonary symptoms occasionally predominate and patients may present with severe headache, confusion, and myalgia. Until recently, cases in which these symptoms have predominated have been described as 'atypical pneumonia' and therefore pathognomonic of mycoplasma, coxiella, and legionella infection. It is now accepted that atypical presentations may be seen with pneumococcal pneumonia and the distinction is regarded as unhelpful.

Mortality due to community-acquired pneumonia was markedly decreased by the introduction of antibiotics. In the antibiotic era, mortality from ambulatory pneumonia has been of the order of 1 percent (Woodhead et al. 1987). Mortality in hospitalized patients is about 13–15 percent (Austrian and Gold 1964; Macfarlane et al., 1982). In patients who need intensive therapy, mortality ranges from 22 to 54 percent (Woodhead et al. 1985; Torres et al., 1991). The outcome for patients admitted to hospital with pneumonia can be greatly modified by prompt antibiotic therapy. In a large multicenter study, none of the patients who died of pneumococcal, staphylococcal, or *M. pneumoniae* pneumonia had received appropriate antibiotics before hospital admission (British Thoracic Society 1987). A number of clinical and laboratory features of community-acquired pneumonia are associated with increased mortality (Table 2). Three independent studies have shown a 21-fold increase in the risk of death or the need for intensive therapy when two or more of the following factors are present:

- respiratory rate greater than 30/min
- diastolic blood pressure below 60 mmHg
- serum urea above 7 mmol/l

(British Thoracic Society 1987; Farr et al. 1991; Karalus et al. 1991).

Fine and colleagues have designed a scoring system that enables physicians to identify patients with a low risk of death within 30 days (Fine et al. 1997) and to stratify patients by severity of illness.

Table 2 *Clinical and laboratory features of community-acquired pneumonia associated with increased risk of death*

Clinical features	Laboratory features
Respiratory rate >30[a]	Urea >7 mmol/l[a]
Diastolic BP <60[a] mmHg	Serum albumin <35 g/l
Age >60 years	Hypoxemia Po_2 <8 kpa
Underlying disease	Leukopenia WBC <4 $\times$ 10^9/l
Confusion	Leukocytosis WBC >20 $\times$ 10^9/l
Atrial fibrillation	Bacteremia
Multilobar involvement	

a) Twenty-one-fold increase in risk of death or requirement of admission to an intensive therapy unit if two or three of these factors are present. Data from Farr et al. (1991); Karalus et al. (1991).

Community-treated pneumonia

Accurate epidemiological data for community-treated pneumonia are difficult to obtain. Results of three large studies in which pathogens were identified in 55–67 percent of hospitalized patients are summarized in Table 3. The predominant organisms are *S. pneumoniae*, which is by far the most common bacterial cause, *M. pneumoniae*, and viruses. These series record a preponderance of bacterial causes in hospitalized patients, but it has been estimated that at least half of nonhospitalized cases are due to viruses or mycoplasma (Pennington 1994a).

STREPTOCOCCUS PNEUMONIAE

Epidemiology

S. pneumoniae is frequently isolated from the nasopharynx of asymptomatic individuals (point prevalence 60–100 percent). The American serotyping system assigns serotype numbers in sequence. There are now in excess of 90 serotypes (see Streptococci and lactobacilli). The lower-numbered serotypes are most frequently implicated in pneumococcal diseases. In Papua New Guinea, which has a high rate of pneumococcal disease, serotypes 2, 3, 5, 8, and 14 infrequently colonize the nasopharynx but are likely to cause invasive disease. Other serotypes are commonly carried by the population and cause disease in children, but not as frequently in adults (serotypes 6, 19F, and 23F). The remaining serotypes are carried relatively rarely in the nasopharynx and do not cause disease (Montgomery et al. 1990).

A high incidence of pneumococcal bacteremia occurs in infants under 2 years of age. The incidence is low in teenage children and young adults, but increases again in males and females in their seventies. The incidence of pneumococcal disease in immunocompetent young adults is five per 100 000; the incidence of pneumococcal bacteremia is 1 000 per 100 000 patients with AIDS (Redd et al. 1990). Certain occupational groups, including military recruits, have higher annual rates of infection but the highest recorded incidence is in South African gold miners (Farr and Mandell 1994). Cigarette

Table 3 *Microbial causes of community-acquired pneumonia*

No. of patients	Location	Year of study	Microbial causes (%)	Ref.
236	Nottingham, UK	1984–85	*S. pneumoniae* (36), Viral (13), *H. influenzae* (10), GNB (1.5), *S. aureus* (1), Unknown (45)	Woodhead et al. 1987
359	Pittsburgh, USA	1986–87	*S. pneumoniae* (15), *H. influenzae* (11), *Legionella* spp. (7), *Chlamydia* spp. (6), GNB (6), Unknown (33)	Fang et al. 1990
331	Leiden, the Netherlands	1991–93	*S. pneumoniae* (27), Viral (8), *H. influenzae* (8), *Mycoplasma* spp. (6), *Chlamydia* spp. (3), Unknown (45)	Bohte et al., 1995

After Brown and Lerner (1998).
GNB, gram-negative bacilli.

smoking is a strong independent risk factor for invasive pneumococcal disease in immunocompetent non-elderly adults (Nuorti et al. 2000).

Pathogenesis

The first step in the pathogenesis of pneumococcal disease is believed to be nasopharyngeal colonization. The average duration of carriage is about 6 weeks in adults, but some individuals may carry a strain for more than a year. Most infections occur during the first week of carriage, while sensitization and production of IgG proceed (Musher et al. 1990). People who have antibody against pneumococcal surface protein A appear to be protected against experimental nasopharyngeal carriage (McCool et al. 2002). In normal individuals, with intact mucociliary clearance, there is a much lower risk of pneumococcal disease once colonization is established in comparison with those who have pre-existing pulmonary disease or immunosuppression. However, viral respiratory infections, particularly due to influenza virus, predispose even more individuals to pneumonia. Malnourished individuals, e.g. alcoholics and residents of nursing homes, and those with chronic liver or kidney disease, cancer, or diabetes mellitus, are also vulnerable to invasive disease. Splenectomy renders individuals vulnerable to catastrophic pneumococcal bacteremia.

Highly specific enzyme-linked immunosorbent assay (ELISA) techniques have shown that most healthy young adults lack antibody to the majority of serotype-specific capsular polysaccharides (Musher 1992). Invasive pneumococcal infection probably occurs only in individuals who are deficient of serotype-specific antibody to the capsular polysaccharide of their colonizing serotype.

Clinical features

Pneumococcal pneumonia usually develops over several days with cough and sputum production, dyspnea, pleuritic chest pain, weakness, malaise, and often myalgia. Occasionally a dramatic rigor may be the first symptom; this hyperacute presentation is more common in otherwise healthy young adults. In older patients the presentation is typically more insidious with minimal cough and absence of fever; confusion and hypothermia are often presenting features in this group. Physical examination may reveal evidence of consolidation and chest radiograph commonly reveals an area of infiltration of less than a whole segment (Figure 3), and several areas may be involved (Ort et al. 1983).

Laboratory findings

Most patients have a polymorphonuclear leukocytosis; leukopenia ($<4 \times 10^9$/l) is a poor prognostic indicator. There may be hyperbilirubinemia and elevated liver enzymes.

Identification by Gram stain or culture of pneumococci in appropriate specimens remains the principal microbiological investigation. Examination of sputum requires specimens with at least 15–25 white blood cells

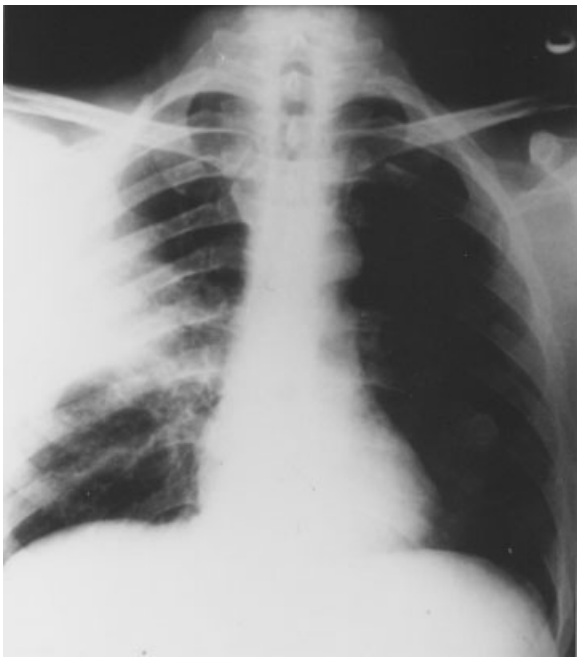

Figure 3 *Lobar consolidation in a male, age 25 years, with pneumococcal pneumonia*

and fewer than ten epithelial cells in a standard microscopic field. If lanceolate-shaped gram-positive cocci (i.e. pneumococci) are seen with more than ten cocci per oil immersion field (×100), the likelihood of culture of pneumococci in the sputum is about 60 percent and the specificity about 85 percent (Rein et al. 1978). The presence of pneumococci can be confirmed by the capsule swelling (quellung) reaction with polyvalent pneumococcal antiserum, but in practice this is rarely used. The success of culture depends on sputum quality – if saliva is present, viridans streptococci may outnumber the pneumococci. Transtracheal aspiration and transcutaneous aspiration are more sensitive but are of value only as research tools.

Pneumococcal polysaccharides can be identified rapidly in sputum, blood, or urine, but these investigations have poor sensitivity and specificity. Countercurrent immunoelectrophoresis (CIE) is positive in approximately 50 percent of patients with nonbacteremic pneumococcal pneumonia when performed on urine and serum, and in 50 percent of bacteremic patients when performed only on serum. Sputum CIE is positive in approximately 75 percent of pneumonia patients with positive pneumococcal culture and remains positive after antibiotic therapy. Latex agglutination, coagglutination and ELISA are as reliable as CIE. A pneumolysin-based ploymerase chain reaction (PCR) assay of whole blood, buffy coat, or plasma has comparable sensitivity and specificity to other antigen tests and remains positive after antimicrobial therapy (Michelow et al. 2002). Clonal origin of pneumococci can be identified by multilocus sequence typing, which is a useful tool in molecular epidemiology (Meats et al. 2003).

Complications

Empyema is the most common complication of pneumococcal pneumonia. A reactive effusion can occur but is trivial; empyema is potentially more serious and is presumably due to bacteria reaching the pleural space via the lymphatics. Clinically, it is signaled by the persistence of fever and leukocytosis after 4–5 days of appropriate antibiotic therapy. Empyema is also suggested by large amounts of pleural fluid seen on the chest radiograph and can be confirmed by ultrasound. Empyema should be drained by repeated needle aspiration or a chest tube; thoracotomy is rarely necessary. Fibrinolytic agents to irrigate the plural space may be helpful. Complications of pneumococcal bacteremia such as endocarditis, pericarditis, peritonitis, and brain abscess are very uncommon in immunocompetent individuals.

Antimicrobial therapy

Penicillin has long been the mainstay of therapy of pneumococcal disease but *S. pneumoniae* has become more resistant to penicillin over recent years (Markiewicz and Tomasz 1989). Strains with intermediate resistance are now prevalent throughout the world and highly resistant isolates are common in certain geographical areas, e.g. Southern Europe, South Africa, and the Far East, where up to 70 percent of isolates are resistant (Felmingham and Gruneberg 2000). For this reason an oxacillin disk is used routinely to screen for penicillin-resistant pneumococci. Such strains are often resistant to other antibiotics, such as chloramphenicol, erythromycin, and clindamycin (Whitney et al. 2000). In practice, pneumococcal pneumonia almost always responds to penicillin or cephalosporins in high doses (Friedland and McCracken, 1994; Pallares et al., 1995), in contrast to meningitis. Organisms highly resistant to penicillin are susceptible to vancomycin. New fluoroquinolones such as moxifloxacin are active against penicillin-resistant pneumococci, but resistance to quinolone-class antibiotics is rising in some parts of the world (Chen et al. 1999).

Prevention

The 23-valent pneumococcal vaccine contains antigens from 23 serotypes of pneumococci: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. These types account for the majority of cases of bacteremic pneumococcal infection and the vaccine produces antibody responses in approximately 85 percent of those vaccinated. It is effective in splenectomized individuals though lower antibody levels are achieved (Giebink et al. 1980) but not in children under 2 years of age. Its use is, therefore, recommended in adults with splenic dysfunction or splenectomy and other chronic diseases, including diabetes mellitus, chronic cardiopulmonary disease, renal failure, nephrotic syndrome, liver disease, and AIDS, but its use in the immunocompromised is controversial (Hirschmann and Lipsky 1994). A large case–control field study revealed a 56 percent efficacy in preventing bacteremic pneumococcal infection; efficacy in immunocompetent patients (61 percent) was higher than in immunosuppressed patients (21 percent) (Shapiro et al. 1991). In contrast, a large randomized control trial among nonimmunocompromised middle-aged and elderly people in Sweden did not demonstrate any effect on prevention of pneumonia overall or pneumococcal pneumonia in middle-aged and elderly individuals (Ortqvist et al. 1998). The vaccines are given by intramuscular or subcutaneous injection. Repeat vaccination is recommended after 3–6 years in certain individuals, e.g. splenectomized adults or children under 10 with nephrotic syndrome or hyposplenism, but local intolerance at the injection site can be a problem. Pneumococcal disease is a particular problem in the African population with AIDS (Gordon et al. 2000b), and in this population the 23-valent vaccine was shown to be ineffective in preventing pneumococcal events (French et al. 2000).

Conjugated polysaccharide vaccines containing seven, nine, and eleven separate polysaccharides possess T-cell-dependent properties and exhibit enhanced induction of B-cell memory and high levels of immunoglobulin G even in very young children (Eskola 2000). Serogroups in the 7-valent formulation (4, 6, 9, 14, 18, 19, and 23) cause 70–88 percent of invasive disease in young children in the USA and Canada, Oceania, Africa, and Europe, but less than 65 percent in Latin America and Asia (Hausdorff et al. 2000). The 7-valent pneumococcal vaccine prevents invasive pneumococcal events in children (Black et al. 2000) and is now routinely used in children from the age of 2 months in the USA (MMWR 2000).

MYCOPLASMA PNEUMONIAE

M. pneumoniae causes tracheobronchopneumonia with severe constitutional symptoms. The clinical syndrome of primary atypical pneumonia was first described by Reimann (1938). *M. pneumoniae* causes a wide spectrum of clinical disease involving the respiratory tract, skin, central nervous system, and blood-forming elements.

Epidemiology

M. pneumoniae spreads with ease in institutions such as schools, universities, and military installations. The highest incidence is during the first two decades of life; along with *C. pneumoniae*, it is a highly significant cause of community-acquired lower respiratory tract infection in children (Principi et al. 2001). The infection can occur during any season but there is generally a 4-year cycle in which 1 year of high prevalence is followed by 2 or 3 years of low incidence (Figure 4). Explosive community outbreaks of pneumonia due to *M. pneumoniae* can occur (MMWR 2001).

Pathogenesis

The organism is carried in the nasopharynx and infection is transmitted by droplets. *M. pneumoniae* binds to epithelial cells via neuraminic acid receptors and subsequently causes damage to cells locally by hydrogen peroxide production. Once the bronchial mucosa is penetrated, a local inflammatory response is initiated. Many of the disease features are a result of a complex immune response to the organism (Tuazon and Murray 1994). Repeated infections result in the generation of sensitized T lymphocytes and autoantibodies, which initiate the pneumonitis. Immune complexes may also contribute to injury of the lung, brain, and synovia and elsewhere. The host-mediated facets of the disease explain why some patients develop only upper respiratory tract symptoms, whereas others may develop pneumonitis with or without severe extrapulmonary manifestations.

Clinical features

The clinical features are at first similar to those of influenza, except that the onset of *M. pneumoniae* infection tends to be gradual over several days or a week (Clyde 1993). Constitutional symptoms initially include headache, malaise, myalgia, and pharyngitis, but rigors are uncommon. The cough, which is initially dry and dominates the disease, is the last symptom to clear. Despite antibiotics clinical relapse can occur a week after the initial response. The physical signs are often minimal and include sinus tenderness, pharyngitis without exudate, and occasionally myringitis. The chest is initially clear, but wheezing and rhonchi may develop in the second week. Although the pneumonia is usually self-limited, occasionally adult respiratory distress syndrome associated with *M. pneumoniae* may develop. Other

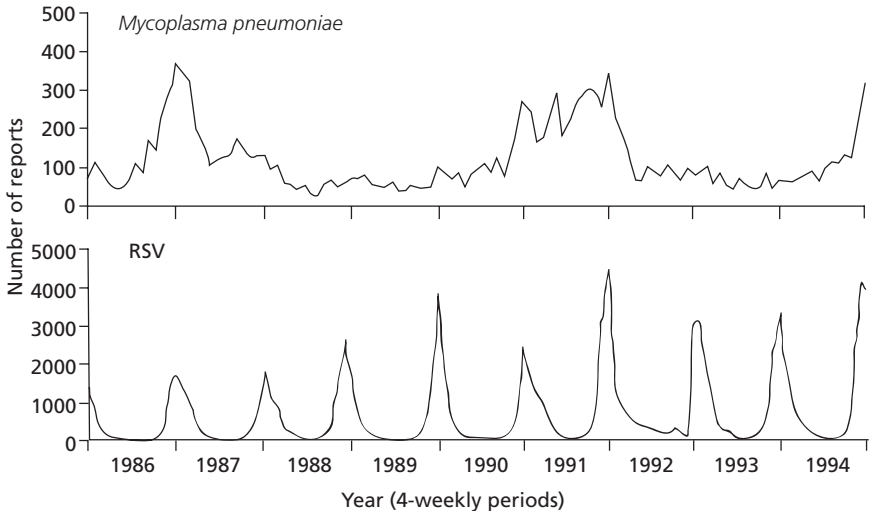

Figure 4 *Periodicity of* Mycoplasma pneumoniae *infection, in comparison with respiratory syncytial virus (RSV). (Reproduced with permission from CDSC 1995.)*

pulmonary complications are unusual (Tuazon and Murray 1994).

Extrapulmonary manifestations

Pneumonia with rash is most commonly due to *M. pneumoniae*. Stevens–Johnson syndrome in association with *M. pneumoniae* is potentially life-threatening and is probably due to the cell-mediated immune response to *M. pneumoniae*, but since the rash often develops after drug therapy, antibiotics, particularly erythromycin, may be responsible (Cherry 1993).

Hemolytic anemia characteristically occurs 2–3 weeks after the onset of illness, is usually self-limiting, and coincides with high cold agglutinin titers. It is complement-dependent and involves IgM antibody to the I antigen of red blood cells (Cherry 1993). Although hemolytic anemia is uncommon, approximately three-quarters of patients with *M. pneumoniae* pneumonia develop high titers of cold agglutinins.

Central nervous system symptoms are present in up to 70 percent of patients with *M. pneumoniae* infection treated in hospital (Koskiniemi 1993). Encephalitis is the most frequent CNS manifestation, but meningitis, myelitis, and polyradiculitis and many other symptoms, including coma, ataxia, and stroke due to cerebral infarction, have been reported. *M. pneumoniae* has not been isolated from brain tissue but has been recovered from the cerebrospinal fluid (CSF). In aseptic meningitis the CSF usually reveals a modest pleocytosis.

Laboratory findings

The white blood cell count is usually greater than 10×10^9 per liter and neutrophilia, lymphocytosis, and monocytosis can all occur, but leukopenia is rare. Routine sputum culture is negative, but *M. pneumoniae* can be grown in broth media in long-term cultures.

A useful bedside test detects the presence of cold agglutinins. A few drops of blood are placed into a citrate tube and placed in a refrigerator for a few minutes. If cold agglutinins are present, then agglutination of red cells will be seen. The IgM cold agglutinins first appear 7–9 days after infection with a peak after 4–6 weeks.

The mainstay of diagnosis is serological. A four-fold increase in complement fixation titer is diagnostic. Unfortunately serology is frequently negative during the acute phase; even laboratory assay for cold hemagglutinins, the first acute-phase marker, is positive in only about 50 percent of acutely ill patients. Some centers offer PCR for *M. pneumoniae*, a technique that is improving in value (Loen et al. 2002). The combination of PCR detection within nasopharyngeal samples together with ELISA-based IgM detection greatly enhances diagnostic yield in children (Ferwerda et al. 2001).

Antimicrobial therapy

The mainstays of treatment for *M. pneumoniae* infection are erythromycin and tetracycline. Recovery with erythromycin is quite slow. Newer macrolides such as azithromycin and clarithromycin are better tolerated and have equivalent activity in vitro. Newer fluoroquinolones are also active against *M. pneumoniae* (Bébéar et al. 1993).

Prevention

Killed and inactivated vaccines do not evoke effective serum or local respiratory tract immune responses (Tuazon and Murray 1994). Future vaccines may be based on recently discovered peptide adhesins.

LEGIONELLA PNEUMOPHILA

This organism is named after an epidemic of pneumonia in legionnaires who attended the American Legion Convention in Philadelphia in the late summer of 1976 (Fraser et al. 1977). Among the 4500 persons attending, 192 cases were observed and 29 proved fatal. The syndrome was characterized by nonproductive cough, pulse temperature dissociation, abnormalities of liver function, diarrhea, hyponatremia, hypophosphatemia, myalgia, confusion, and multiple rigors. The condition became known as legionnaires' disease. A previously unknown pleomorphic gram-negative bacillus was isolated from four of the original patients and named *L. pneumophila*. Some 3 years later an acute, short-lived, febrile illness consisting of myalgia but not pneumonia that affected staff and visitors at the County Health Department at Pontiac, Michigan, was shown to be due to *L. pneumophila*. It had a shorter incubation period and there were no deaths (Broome and Fraser 1979). Subsequently devised serological techniques implicated *Legionella* spp. in pneumonia not only in outbreaks associated with the water facilities of large buildings such as hospitals and hotels but also with sporadic community-acquired infections. Legionella are aerobic, fastidious, gram-negative bacilli, of which 34 species have been characterized (see Legionella).

Rarely, infections may be caused by *L. micdadei*, *L. longbeachae*, *L. dumoffii*, *L. bozemanii*, and other species (Edelstein and Meyer 1994).

Pathogenesis

Infection results from inhalation of *Legionella*-contaminated aerosols, e.g., disseminated by cooling towers, humidifiers, respiratory therapy equipment, showerheads, and cooling sprays. *L. pneumophila* is phagocytosed by macrophages but they fail to kill the organism. In macrophages infected in vitro, legionella can multiply between 100 and 1 000 times in 48–72 h (Yamamoto et al. 1994). Defense against the infection, therefore,

crucially depends on cell-mediated immunity, as illustrated by the susceptibility to legionellosis of patients in whom such immunity is deficient; those with neutropenia do not appear to be susceptible.

Epidemiology

Legionella spp. survive in water for prolonged periods, as intracellular parasites of free-living protozoa (Fields et al. 2002). Although human infection occurs sporadically in the community (Macfarlane et al. 1982), outbreaks are dramatic and associated with water facilities. Of the 129 cases of legionnaires' disease reported in England and Wales in 1993, 51 percent were associated with travel, mainly to Spain, but also to Greece, Turkey, the USA, and within the UK; six were nosocomial and the remainder were sporadic community cases (Joseph et al. 1994a). Outbreaks have occurred in the vicinity of cooling towers (Broome and Fraser 1979), a power station (Morton et al. 1986), and, notably, Broadcasting House, London, in 1988. The role of air-conditioning equipment was demonstrated in Memphis in 1978 (Dondero et al. 1980) and by a large hospital outbreak in Staffordshire, UK (Editorial 1986). Showerheads and hot-water taps spread aerosols contaminated with *Legionella* (Bolling et al. 1985). This has caused outbreaks in hospitals and is the probable cause of disease in holidaymakers returning from Mediterranean resorts. Contaminated showerheads and tap water led to a prolonged and disastrous outbreak in the VA Wadsworth Medical Center, Los Angeles, with a high rate of nosocomial *Legionella* pneumonia, particularly in immunosuppressed patients. The outbreak was terminated by changing showerheads and by hyperchlorination of potable water (Shands et al., 1985). Between 1980 and 1992, 218 cases were reported in the UK, 68 of whom died. Approximately half were sporadic, but three hospital outbreaks contributed to the majority of the remaining cases. Hospital domestic water systems were the source of infection in 19 outbreaks. The cooling tower was thought to be the source in one hospital and probably contributed to two other outbreaks in which the domestic water system was suspected to be the main source (Joseph et al. 1994b). It is considered that contamination of water only presents a risk at a threshold concentration of 10^4–10^5 cfu per liter, though low concentrations can be underestimated because of insensitive culture techniques (Leoni and Legnani 2001).

Clinical features

The majority of patients with *L. pneumophila* infection have a pneumonia virtually indistinguishable from pneumococcal pneumonia (Edelstein 1993; Stout and Yu 1997). Clinical features range from asymptomatic infection and nonpneumonic myalgia (Pontiac fever) to legionnaires' disease with severe pneumonia and extrapulmonary manifestations. After an incubation period of 2–10 days, there is a gradual onset of malaise, lethargy, fever, headache, myalgia, and weakness, with evolution of a dry nonproductive cough after 1–2 days. There may be purulent sputum, which can be bloody (Edelstein 1993). In about 25 percent of patients there may be disorientation, agitation, confusion, hallucinations, ataxia, and seizure. The systemic manifestations are probably due to several exotoxins and, possibly, endotoxins (Edelstein and Meyer 1994). Extrapulmonary manifestations include pancreatitis, peritonitis, cellulitis, myositis, and focal abscesses, and prosthetic valve endocarditis is well recognized. Pneumonia due to species other than *L. pneumophila* is clinically indistinguishable. *Legionella micdadei* infection occurs in immunosuppressed patients and causes nodular pulmonary infiltrates (Pittsburgh pneumonia) (Myerowitz et al. 1979).

Diagnosis

Nonspecific laboratory abnormalities include elevated white blood count (usually with neutrophilia), hyponatremia, elevation of alkaline phosphatase and liver transaminases, and proteinuria. The gold standard for the diagnosis of legionnaires' disease is culture of *Legionella* spp. from sputum samples on charcoal yeast extract medium (BCYE), but this is only possible in patients with productive cough. Antigen can be detected in sputum, respiratory secretions, bronchoalveolar lavage, or lung biopsy using immunofluorescence with genus-specific monoclonal antibodies, even after several days of chemotherapy (Fields et al. 2002). Techniques to detect nucleic acid, for example PCR, are of relatively low sensitivity and are practiced in very few laboratories. Urinary antigen detection has an overall sensitivity of 80 percent (Helbig et al. 2003) but detects only serogroup 1 *L. pneumophila*. Serological diagnosis can be conducted with a variety of techniques, including complement fixation and ELISA, and relies on a four-fold rise between acute and convalescent titres (which can take up to 2 months) or a single titer of $\geqslant$1:256. In culture confirmed cases, the sensitivity of serology is 70–80 percent.

Antimicrobial chemotherapy

In the legionnaires' disease outbreak in Philadelphia in 1976, the case fatality rate was highest for patients treated with cephalosporins and lowest with erythromycin. Erythromycin should be given at a dose of 2–4 g per day, initially intravenously in severely ill patients, for at least 3 weeks. Rifampicin and a fluoroquinalone should be added for patients who are critically ill. Patients usually respond to erythromycin therapy within 2–3 days.

Antimicrobial sensitivity testing is not applicable to *L. pneumophila*. Most of the β-lactam agents are active in

vitro against *L. pneumophila*. However, animal models have shown that these agents are ineffective because they do not enter macrophages sufficiently well. Therefore, the potential efficacy of new agents must be based upon their success in intracellular infection and experimental animal models. Azithromycin, clarithromycin, fluoroquinolone antibiotics, trimethoprim–sulfamethoxazole, tetracyclines including doxycycline, and rifampicin are effective in animal models (Edelstein 1993). In patients with *Legionella* who are severely ill or immunocompromised, a fluoroquinolone must be included in the chemotherapeutic regimen (Edelstein 1995).

Prevention

Person-to-person transmission of legionnaires' disease has not been observed. The most important preventive measure is to render water sources safe. Thirty to 80 percent of hospital cooling towers and water supplies are to some extent contaminated with *Legionella*, and at total viable concentrations $>10^8$ cfu/l the hazard of infection is increased (Edelstein and Meyer 1994). Chlorination, pasteurization, and heating can be used to reduce the *Legionella* burden (Muraca and Stout 1988).

The report of a single case of legionnaires' disease requires investigation to determine whether it is part of an outbreak, which involves tracing the patient's activities during the incubation period. If hospital-acquired infection is suspected, then the hospital water maintenance programme must be reviewed and a search made for associated cases, beginning with the immunosuppressed population, who are most vulnerable to infection (Saunders et al. 1994).

COXIELLA BURNETII

C. burnetii (see Rickettsia and Orienta) is the cause of Q fever, which was first described in Australia in the 1930s in abattoir workers. The organism spreads between farm animals and man by air-borne transmission, by infected milk, feces, and urine, and is probably also transmitted by tick bite. It is highly resistant to drying and in agricultural facilities contaminates dust, which can cause infection by inhalation. After an incubation period of 2–4 weeks there is abrupt onset of high fever, malaise, headache, and neck stiffness. There is dry cough and pleuritic chest pain and on examination there is usually high fever, hepatosplenomegaly, but few chest signs. Some patients develop a transient maculopapular rash, but the exanthem typical of other rickettsial infections is absent. Most illness resolves within 2 weeks but fever can persist for up to 3 months. Q fever can be complicated by extrapulmonary lesions, including endocarditis. There may be hepatitis, which is usually mild but can be severe or even fulminant. *C. burnetii* may cause blood culture-negative endocarditis many months after the primary infection.

Complement fixation and indirect fluorescent antibody tests reach a peak between 1 and 3 months after original infection. Phase 2 antibodies are raised in acute Q fever; phase 1 antibodies are prominent in patients with chronic Q fever, e.g. endocarditis.

Tetracycline or doxycycline is used to treat acutely ill patients and those with persistent symptoms. Long-term treatment with tetracycline is desirable for patients with Q fever endocarditis, but valve replacement may become necessary.

STAPHYLOCOCCUS AUREUS

Although *S. aureus* pneumonia is usually nosocomial, it accounts for a small proportion of community-acquired pneumonia, particularly during influenza epidemics and in certain patient groups, e.g. diabetics. Although 15–30 percent of adults are nasal carriers of *S. aureus*, resulting pneumonia is rare and presumably requires an underlying host defect, e.g. diabetes. Staphylococcal pneumonia is also seen in patients with right-sided endocarditis (e.g. in intravenous drug abusers) and in patients with septic thrombophlebitis or an infected vascular prosthesis (Farr et al. 1989). Patients with staphylococcal pneumonia more commonly have pleural effusions and cavitation on chest radiograph and 25 percent develop abscesses (Waldvogel 1990). Staphylococcal pneumonia secondary to septic embolization from right-sided endocarditis causes multiple small round cavitary lesions.

The appearance of clusters of staphylococci on Gram stain of sputum is diagnostic of staphylococci pneumonia. If this diagnosis is suspected after clinical assessment of a patient, sputum microscopy can rapidly confirm the diagnosis and a penicillinase-resistant penicillin can be added to the empiric antibiotic regimen. Vancomycin should be used only when pneumonia due to methicillin-resistant *S. aureus* is suspected.

HAEMOPHILUS INFLUENZAE

H. influenzae causes community-acquired pneumonia in patients with chronic bronchitis and those with impaired host defenses, such as smokers, alcoholics, or HIV infection. In the pre-Hib vaccine era, it was also a relatively common cause of pneumonia in children, particularly those aged 4 years and younger. Nontypable *H. influenzae* tends to cause mucosal infections, such as those affecting the middle ear and the airway of bronchitics. Capsulate organisms, particularly of type b, cause a significant proportion of invasive parenchymal disease, i.e. pneumonia (Smith 1994). In adults and children the illness tends to be preceded by coryza, and sudden onset of pleuritic chest pain is the predominant chest symptom (Crowe and Lavitz 1987). Microbiological diagnosis is notoriously difficult because of sputum contamination by commensal *Haemophilus* spp. and blood culture is

usually negative. β-Lactamase production by infecting strains may be very frequent in some areas (Felmingham and Gruneberg 2000). If such strains may be involved, second-generation cephalosporins should be used to treat moderately severe pneumonia.

CHLAMYDIA SPECIES

Pneumonia due to chlamydia is common. See Chlamydia.

GRAM-NEGATIVE BACILLARY PNEUMONIAS

Although gram-negative bacteria are a prominent cause of nosocomial pneumonia, they may also cause disease in the community, particularly in alcoholics, diabetics, residents of nursing homes, and patients with underlying diseases, such as malignancy and cardiac or renal failure. The upper respiratory tract becomes colonized followed by aspiration and subsequent pneumonia. The most common gram-negative causes of community pneumonia are *K. pneumoniae*, *E. coli*, *Serratia marcesens*, *Enterobacter* spp., and *Pseudomonas* spp.

K. pneumoniae classically causes pneumonia in debilitated middle-aged males, with sudden fever and rigors, dyspnea, and large volumes of bloody sputum. Often there is a necrotizing process with multiple abscess formation and dramatic lobar consolidation. The sputum contains large numbers of capsulate gram-negative bacilli and blood cultures are often positive. The infection is destructive, often is severe, and has a high mortality (Feldman et al. 1989).

E. coli is the cause of up to 3 percent of community pneumonias in American series (Eisenstadt and Crane 1994). It may result from bacteremic spread from the genitourinary and gastrointestinal tracts and is often bilateral. Other causes of community-acquired gram-negative pneumonia include *Proteus* spp. (*Morganella* and *Providencia* spp.) and *Acinetobacter baumannii*.

Pseudomonas spp. can, rarely, cause dramatic community-acquired pneumonia. *P. aeruginosa* causes pneumonia in patients admitted from nursing homes or individuals with malignancy. The disease is often necrotizing and there is diffuse bilateral consolidation on chest radiograph with occasional multiple abscesses. *Pseudomonas stutzeri* may cause disease in patients with malignancy (Noble and Overman 1994). For a discussion of pneumonia due to *Burkholderia pseudomallei*, the reader is referred to Burkholderia spp., and related genera.

STREPTOCOCCUS SPECIES

Both group A and group B streptococci may cause pneumonia, the latter particularly in elderly debilitated patients with diabetes mellitus, stroke, dementia, and malignancies, and is often associated with other organisms, particularly *S. aureus* and *S. pneumoniae*. Enterococci can cause pneumonia in elderly debilitated patients with multiple chronic problems, often with nasogastric tubes. Treatment is with high-dose penicillin or ampicillin and an aminoglycoside.

BACILLUS ANTHRACIS

Most (95 percent) natural infection by *B. anthracis* results in the cutaneous form of anthrax, but inhalation of spores can result in inhalational anthrax within 60 days of exposure (Friedlander et al. 1993). Inhalation anthrax usually begins with mild fever and malaise, myalgia, and nonproductive cough. Within 2 or 3 days there is rapid evolution of fever, acute dyspnea, cyanosis, and shock. Mediastinal and cervical lymphadenopathy may be sufficiently severe to cause stridor. The chest radiograph reveals mediastinal widening and occasionally parenchymal infiltrates. Microbiological diagnosis of inhalational anthrax is usually made by blood culture of a heavily encapsulated gram-positive bacillus or by PCR detection in tissue samples (Levine et al. 2002). Confirmation can be done by lysis by gamma phage or direct fluorescent antibody staining of cell-wall polysaccharide antigen. Treatment of cases is with ciprofloxacin 400 mg twice daily intravenously (Swartz 2001). Inhalation exposure of individuals without disease can be investigated by nasopharyngeal swabbing and culture or PCR detection. Post-exposure prophylaxis with ciprofloxacin 500 mg or doxycycline 100 mg both orally and twice daily for 60 days is recommended for persons exposed to spores (Inglesby et al. 2002).

FRANCISELLA TULARENSIS

Exposure to as few as one to ten aerosolized organisms may cause tularemia, which may present as pneumonia or in an ulcerglandular or typhoidal form. The incubation period is 1–21 days, with initial symptoms of fever, malaise, and nonproductive cough. The chest radiograph usually reveals pneumonia with or without mediastinal lymphadenopathy. The organism is difficult to culture from blood but serology is positive in 50–70 percent of cases by the second week of illness. Standard therapy is with gentamicin, streptomycin, or doxycycline.

OTHER RARE CAUSES OF BACTERIAL PNEUMONIA

N. meningitidis can cause a typical pneumonia in the absence of the classic or cutaneous rash and without evidence of meningitis or shock. The disease occurs as outbreaks in military and school dormitories (Koppes et al. 1977). The lungs may be involved in up to 20 percent of cases of brucellosis (Lulu et al. 1988) (See Brucella). *Pasteurella multocida*, which causes cellulitis after dog bites, can cause a necrotizing pneumonia, usually in patients with pre-existing respiratory disease and with history of exposure to domestic animals. *Yersinia pestis* can cause pneumonia as part of a septicemic process (see Yersinia).

THE APPROACH TO PATIENTS WITH COMMUNITY-ACQUIRED PNEUMONIA

In most patients presenting with symptoms and signs of pneumonia the likely cause is *S. pneumoniae*. Routine laboratory investigation should include sputum examination and culture, blood culture, acute and convalescent serology to detect antibodies to viruses, *Mycoplasma* spp., *Chlamydia* spp., *Legionella* spp., and *Coxiella burnetii*, urinary antigen detection, and examination of pleural fluid, if available (Carroll 2002), Yersina.

ANTIMICROBIAL THERAPY

The British, European, and American thoracic societies, and also the Infectious Disease Society of America, have each issued guidelines for the management of adult community-acquired pneumonia, and these are summarized in Table 4. Each of these guidelines is opinion-based, as the evidence available to discriminate between therapies is limited (Read 1999). All modern guidelines stratify patients by demographic and epidemiological features and include suggested therapies tailored to the likely pathogens that are seen in more complex clinical settings. The British Thoracic Society guidelines recommend the use of an aminopenicillin (e.g. amoxycillin) in patients with simple community-treated pneumonia who are ambulatory, with erythromycin recommended as an alternative choice. In view of the fact that the greatest cause of mortality is due to *S. pneumoniae*, aminopenicillins are probably adequate in the treatment of the majority of patients with mild community-acquired pneumonia treated in the UK. A problem in southern and eastern Europe, the USA, the Asia-Pacific region, and South Africa has been the emergence of penicillin-resistant pneumococci. Aminopenicillins have not been widely employed in the USA for the management of community-acquired pneumonia, largely because of the perceived frequency of atypical pathogens as microbial etiologies in this condition, and the difficulty in distinguishing, on clinical grounds alone, those patients who are infected with such pathogens. The emergence of penicillin-resistant pneumococci, especially in countries other than the UK, has led to the inclusion of fluoroquinolones as choices in first-line management, even for ambulatory patients. The treatment guidelines issued by the Infectious Diseases Society of America (IDSA) (see Table 4) recommend the use of either doxycycline, a macrolide drug, or a fluoroquinolone for first-line therapy of patients with mild ambulant (outpatient) pneumonia. While such recommendations would probably not be germane in, for example, the UK, in certain parts of the world they are certainly appropriate – the frequency of penicillin resistance in Spain varies from 50 to 70 percent. In such regions, the frequency of macrolide resistance is also high, and clinical failures with this class have been reported.

As can be seen in Table 4, the American Thoracic Society (ATS) has produced a sophisticated set of guidelines in which the likelihood of involvement of penicillin-resistant pneumococci or relatively unusual pathogens in certain patient groups is acknowledged. The ATS recommends the use of either macrolides or tetracyclines for patients who are managed as outpatients and have no pulmonary comorbidity. This approach was validated in the early 1990s (Gleason et al. 1997) in a study of 864 outpatients with community-acquired pneumonia (55.4 percent of whom yielded a positive microbiological diagnosis); 547 younger patients without comorbidity were treated; of these younger patients, 62 percent were prescribed therapy recommended by the ATS – predominantly erythromycin. There were no significant differences in medical outcome between these patients and outpatients who had been erroneously prescribed more sophisticated therapy, but their treatment was associated with a three- to four-fold lower treatment cost.

For outpatients who have pulmonary comorbidities or other risk factors for more exotic flora, including penicillin-resistant pneumococci (e.g. older age), the current ATS guidelines recommend the use of more sophisticated treatments, including oral cephalosporins or a β-lactam/β-lactamase inhibitor combination in addition to a macrolide or doxycycline. An alternative choice for this group is a fluoroquinolone with antipneumococcal activity. The ATS specifies available fluoroquinolones with such activity and ranks them in descending order of antipneumococcal potency as moxifloxacin, gatifloxacin, sparfloxacin, then levofloxacin.

This approach is also followed in the recent guidelines from the IDSA, in which a fluoroquinolone such as moxifloxacin, gatifloxacin, or levofloxacin is recommended in elderly patients or those with underlying disease (see Table 4).

HOSPITALIZED PATIENTS

Patients who require hospitalization are often in groups at risk of unusual pathogens and/or more severe disease. All guidelines recommend broader-spectrum (often combination) therapy for this group (see Table 4), but the ATS guidelines stratify inpatients further into those with or without cardiopulmonary comorbidities or other modifying factors, including nursing-home residency. It is recommended that these inpatients should receive aggressive therapy with an IV β-lactam/macrolide combination or an IV antipneumococcal fluoroquinolone given as monotherapy.

SEVERE COMMUNITY-ACQUIRED PNEUMONIA

All patients with severe community-acquired pneumonia should be managed with adequate ventilatory support and fluid resuscitation, in addition to antibiotics. This group has a high mortality and the pathogens most frequently implicated as the microbial cause include *S. pneumoniae*, *Legionella* spp., *H. influenzae*, enteric

Table 4 *Guidelines for first-line empirical management of community-acquired pneumonia*

Society	Mild ambulant pneumonia (outpatient)	Ambulant pneumonia with risk factors (outpatient)	Inpatient pneumonia (medical ward)	Severe pneumonia (intensive care unit)
European Respiratory Society (1998) (ERS Task Force Report 1998)	Aminopenicillin, or new tetracycline or newer FQ, oral streptogramins, or macrolide	Inpatients with COPD: β-lactam/β-lactamase inhibitor	IV second- or third-generation cephalosporin or IV β-lactam/β-lactamase or IV aminopenicillin and IV macrolide	Either a second- or third-generation cephalosporin and a newer FQ, or macrolide ± rifampicin
Infectious Diseases Society of America (2000) (Bartlett et al. 2000)	Doxycycline or a macrolide, or a newer FQ	Suspected drug-resistant *Streptococcus pneumoniae*, or older patients, or patients with an underlying disease: a newer FQ	A macrolide plus either an extended-spectrum cephalosporin or a β-lactam/β-lactamase inhibitor, or a newer FQ (alone)	Either an extended-spectrum cephalosporin or β-lactam/β-lactamase inhibitor, plus either a newer FQ or a macrolide
American Thoracic Society (2001)	Macrolide (azithromycin or clarithromycin) or doxycycline	With cardiopulmonary disease: β-lactam (oral cefpodoxime, cefuroxime, amoxil, co-amoxiclav) or parenteral ceftriaxone followed by oral cefpodoxime plus macrolide or doxycycline or antipneumococcal FQ monotherapy	Cardiopulmonary disease or nursing home: IV β-lactam (cefotaxime, ceftriaxone, ampicillin/sulbactam, high-dose ampicillin plus IV/PO macrolide or doxycycline or IV antipneumococcal FQ monotherapy, no modifying factors: IV azithromycin or doxycycline plus β-lactam or IV antipneumococcal FQ	No risk of *P. aeruginosa*: IV β-lactam plus IV azithromycin or fluoroquinolone. Risk of *P. aeruginosa*: IV antipseudomonal β-lactam (cefepime, imipenem, pip/tazo) plus IV ciprofloxacin or IV antipseudomonal β-lactam plus IV aminoglycoside plus IV azithromycin or IV FQ
British Thoracic Society (2001)	Amoxycillin or erythromycin	Not specified	Oral amoxycillin plus erythromycin or IV ampicillin plus IV clarithromycin or IV FQ plus IV penicillin	Co-amoxiclav or 2/3 cephalosporin plus clarithromycin ± rifampicin or FQ plus IV penicillin

COPD, chronic obstructive pulmonary disease; FQ, fluoroquinolones (include moxifloxacin, gatifloxacin, levofloxacin); IV, intravenous; PO, orally, 2/3 2nd or 3rd generation.

gram-negative bacilli, *Mycoplasma* spp., and *S. aureus*. There is a paucity of clinical trial data to support individual choices of empirical antimicrobial therapy but, in general, patients should be managed with broad-spectrum therapy: usually an IV cephalosporin with the addition of an IV macrolide. In patients for whom there is clinical or laboratory evidence leading to suspicion of *S. aureus*, IV fluocloxacillin (nafcillin) or vancomycin should be added.

Nosocomial pneumonia

Pneumonia is the second most common nosocomial infection after urinary tract infection (Horan et al. 1986), causes considerable mortality and morbidity, and increases hospital stay and its cost. The US Centers for Disease Control have published the following definition of nosocomial pneumonia:

Onset of pneumonia more than 72 h after hospital admission with lung consolidation or an infiltrate on the chest radiograph plus at least one of the following:

- Infected sputum
- Isolation of a pathogen from the blood, transtracheal aspirate, biopsy, or bronchial lavage specimen
- Isolation of a virus in respiratory secretions
- Diagnostic antibody titers or
- Histopathological evidence of pneumonia (Centers for Disease Control 1988).

According to the US National Nosocomial Infection Surveillance (NNIS) system, nosocomial pneumonia occurs at a frequency of 0.6–1.0 episodes per 100 hospitalizations and in 18 percent of postoperative patients (Horan et al. 1986; Craven et al., 1991). Intubated patients may have rates of pneumonia seven- to 21-fold higher than patients without a respiratory therapy device; the incidence of ventilator-associated pneumonia (VAP) varies from ten to 25/1 000 ventilator days (Chastre and Fagon 2002). Infection rates are twice as high in large teaching hospitals compared with smaller institutions. Factors that lead to nosocomial pneumonia include older age, chronic lung disease, depressed consciousness, mechanical ventilation, the use of H2 antagonists, frequent changes of ventilator circuits, and the winter season (Celis et al. 1988). Mortality in patients with nosocomial pneumonia is high (20–50 percent), but only about one-third of fatalities are due directly to pneumonia (Pennington 1994b).

PATHOGENESIS

Most nosocomial pneumonia results from aspiration of pathogens from the upper airways. Patients admitted to intensive care units become colonized with aerobic gram-negative bacilli within 1 week of entering hospital, and of these about 25 percent develop nosocomial pneumonia; there is a correlation between severity of illness and the likelihood of subsequent colonization with gram-negative organisms (Johanson et al. 1969). Enterobacteriaceaea probably reach the upper respiratory tract by fecal–oral transmission. However, *P. aeruginosa* and *S. aureus* probably colonize the patient from environmental sources and contamination by attending staff, fomites, or supporting equipment (Pennington 1994b). Any factors that reduce gastric acidity may lead to increased oropharyngeal colonization: these include advanced age, achlorhydria, malnutrition, and various pharmacological agents.

MICROBIAL ETIOLOGY

The majority of hospital-acquired pneumonias are caused by aerobic gram-negative bacilli; Enterobacteriaceae cause 40 percent of these pneumonias, *S. aureus* 25 percent, and *P. aeruginosa* 15 percent; *S. pneumoniae*, *H. influenzae*, fungi, viruses, and anaerobic bacteria rarely cause nosocomial pneumonia. The results of three large studies of nosocomial pneumonia are shown in Table 5. Polymicrobial infections are common. Nosocomial pneumonia that occurs during the first 4 days in hospital is usually due to one of *S. pneumoniae* and

Table 5 *Bacteriology from three nosocomial pneumonia studies*

Culture material	Sputum (%)	TTA (%)	Blood (%)
Study	Hughes et al. (1983)	Bartlett et al. (1986)	Bryan and Reynolds (1984)
Pseudmonas aeruginosa	17	9	15
Staphylococcus aureus	13	26	27
Enterobacteriaceae[a]	37	48	42
Fungi	6	ND	ND
Anaerobic bacteria	2	35	2
Streptococcus pneumoniae	<3	31	12
Haemophilus influenzae	<3	17	<4
Viruses	<3	ND	ND

ND, not done; TTA, transtracheal aspirate.
Adapted from Dal Nogare (1994).
a) Includes *Klebsiella pneumoniae*, *Enterobacter* spp., *Escherichia coli*, *Serratia*, and *Proteus*.

H. influenzae. Nosocomial pneumonia occurring later than 4 days after admission is more commonly caused by hospital-acquired, aerobic gram-negative bacilli, *S. aureus*, *Legionella* spp., or, rarely, fungi. A number of host risk factors can predispose to infection with particular pathogens (reviewed by Niederman 1994). Staphylococcal pneumonia is associated with diabetes, renal failure, a recent history of influenza, and recent head injury and trauma, whereas *P. aeruginosa* is seen in malnourished patients and those on steroids or who have chronic structural lung disease or are subject to prolonged mechanical ventilation or tracheostomy. *L. pneumophila* may cause nosocomial pneumonia in hospitals with contaminated water supplies, particularly in patients immunosuppressed by treatment with steroids. Anaerobes are seen particularly in patients with gross aspiration of acid gastric contents and recent thoracoabdominal surgery. *Acinetobacter* spp. are a significant cause of nosocomial pneumonia on surgical intensive care units and are a particular problem because of multiple antibiotic resistance (Fagon et al. 1989). Viruses may contribute to nosocomial pneumonia, particularly influenza A, parainfluenza, and adenovirus (Dal Nogare 1994).

Methods for the diagnosis of nosocomial pneumonia in severely ill patients include transtracheal aspiration, bronchoscopic sampling with or without protective brush, and transcutaneous aspiration of lung material, but in many studies these have not proved superior to clinical evaluation (Pennington 1994b). In patients who are conscious, sputum production may yield gram-positive (*S. aureus*) or gram-negative bacteria. In intubated patients, aspirated respiratory secretions and blood cultures may provide a bacteriological diagnosis without the need for bronchoalveolar lavage.

TREATMENT AND PREVENTION

Appropriate therapy should include antibiotics active against *S. aureus* and gram-negative bacteria, including *P. aeruginosa*; common regimens include a third-generation cephalosporin or fluoroquinolone plus an aminoglycoside. In patients infected with *Acinetobacter* spp., imipenem and tetracycline are among the most active drugs (Vila et al. 1993). A schema for empiric treatment based on severity of disease has been suggested by the ATS (American Thoracic Society 1996) (see Table 6).

Prevention of nosocomial pneumonia depends on prevention of colonization and aspiration and spread of pathogens between patients and staff. Hand washing by staff members should be encouraged. If swallowing is impaired, oral intake should be curtailed and unnecessary nasogastric and endotracheal tubes should be removed. Elevation of the patient's head reduces risk of aspiration (Dal Nogare 1994). Gastric acidity should be maintained by avoidance of H2 antagonists in seriously ill patients.

Aspiration pneumonia

Most aspiration pneumonia is polymicrobial and usually includes anaerobes (Finegold 1994). The latter thrive in lungs injured by chemical pneumonitis. Where there is periodontal disease and gingivitis, anaerobic colonization of the mouth is increased. In hospitalized patients and in nursing homes there is increased oropharyngeal colonization with gram-negative bacilli. A full list of potential organisms that may be involved in aspiration pneumonitis is shown in Table 7.

Pneumonia in patients with a predisposition to aspiration is insidious and mainly affects dependent zones of the lung. The most common site is the posterior segment of the right upper lobe, but the apical segments of the lower lobes can be affected. Lung abscess or empyema with high swinging fever may occur, often with foul-smelling sputum and hemoptysis. Microbiological diagnosis is confounded by the normal anaerobic flora of the mouth and the Gram stain of the sputum is difficult to interpret. Gram stain of empyema fluid or transtracheal aspirate may reveal the characteristic appearance of *Prevotella* spp., *Porphyromonas* spp., and *Fusobacterium* spp. Bacteremia is uncommon in aspiration pneumonia.

Treatment of early uncomplicated aspiration or pneumonia in previously normal individuals is relatively straightforward because the majority of oral anaerobes in this situation are sensitive to benzylpenicillin. In patients with underlying disease or who are hospitalized the frequency of infections with nonpenicillin-sensitive anaerobes is increased, including the *Bacteroides fragilis* group and gram-negative organisms including *Pseudomonas* spp. The possibility of infection with *S. aureus* should also be covered by administration of clindamycin or an aminoglycoside. Alternatively, combination therapy with a broad-spectrum penicillin, such as ticarcillin or piperacillin, plus an aminoglycoside can be given. Newer antibiotics such as imipenem, or a combination of a broad-spectrum antibiotic with a β-lactamase inhibitor, are also effective.

Complications of pneumonia

PLEURAL EFFUSION

Pleural effusions occur very commonly in pneumococcal pneumonia. Usually the effusion is sterile and is absorbed spontaneously within two weeks. Occasionally the effusion is large and requires aspiration. Empyema should be suspected if an effusion persists beyond two weeks, especially if there is continuing fever and pain.

EMPYEMA

Pus in the pleural space leads to symptoms of chest pain, fever, and general malaise. Most empyemas occur as a complication of pneumonia or lung abscess, but 15–30

Table 6 *Summary of clinical setting, organisms, and empiric treatment of nosocomial pneumonia based on severity of disease (adapted in part from the American Thoracic Society 1996)*

Mild or moderate nosocomial pneumonia		
No risk factors	*Core organisms*[a]	*Initial antibiotics*
No unusual risk factors; early onset Early onset	*S. pneumoniae* *H. influenzae* *S. aureus* *E. coli*, *Klebsiella* *Enterobacter* spp. *Serratia* spp.	Second- or third -generation cephalosporin or new fluoroquinolone or β-lactam + β-lactamase inhibitor
With risk factors	*Pathogens*	*Initial antibiotics*
Recent abdominal surgery Witnessed aspiration	Core organisms[a] + anaerobes	Second- or third-generation cephalosporin + clindamycin or new fluoroquinolone alone or β-lactam + β-lactamase inhibitor
High-dose steroids	*L. pneumophila*	Use macrolide or quinolone
Prior hospitalization ICU stay Steroids Prior antibiotics Chronic lung disease	*P. aeruginosa* and MRSA	Treat as severe nosocomial pneumonia
Severe nosocomial pneumonia		
Clinical setting		*Initial antibiotics*
ICU Nursing home Prior antibiotics Respiratory failure Progressive pneumonia Sepsis, shock Organ failure	Core organisms + *P. aeruginosa* + *Legionella pneumophila* + MRSA	Third- or fourth-generation antipseudomonal cephalosporin + quinolone ± vancomycin or third- or fourth-generation antipseudomonal cephalosporin + macrolide ± vancomycin ± aminoglycoside or carbapenem + quinolone ± vancomycin

ICU, intensive care unit; MRSA, methicillin-resistant *S. aureus*.

a) Core organisms are those pathogens commonly implicated in nosocomial pneumonia with no unusual risk factors.

Table 7 *Potential microbial etiology of aspiration pneumonitis*

Anaerobes	Aerobes
Gram-negative bacilli	Gram-positive bacilli
Pigmented *Prevotella* and *Porphyromonas*	*Staphylococcus aureus*
Prevotella aureus, *P. buccae*	*Streptococcus pyogenes*
Prevotella oralis group	'viridans' streptococci
Bacteroides ureolyticus group (especially *B. gracilis*)	Gram-negative bacilli
Bacteroides fragilis group	*Klebsiella pneumoniae*
Fusobacterium nucleatum	*Enterobacter* spp.
Fusobacterium necrophorum, *F. naviforme*, *F. gonidiaformans*	*Serratia* spp.
Gram-positive cocci	*Pseudomonas aeruginosa*
Peptostreptococcus (especially *P. magnus*, *P. asaccharolyticus*,	*Escherichia coli*
P. prevotii, *P. anaerobius*, and *P. micros*	*Proteus* spp.
Microaerophilic streptococci (*Streptococcus intermedius*)	
Gram-positive nonsporing bacilli	
Actinomyces spp.	
Propionibacterium propionicum	
Bifidobacterium dentium	
Gram-positive spore-forming bacilli	
Clostridium (especially *C. perfringens*, *C. ramosum*)	

Modified after Finegold (1994).

percent occur after thoracic surgery and 10 percent occur in association with intraabdominal infection. Empyema following pneumonia is mostly polymicrobial and anaerobes are present in 75 percent of cases. Empyema after thoracic surgery is more likely to be monomicrobial and due to common nosocomial pathogens such as *S. aureus* and aerobic gram-negative bacilli. Progress of pneumonia to empyema is related to delay in appropriate antimicrobial therapy. Once infection is established the empyema rapidly becomes fibrinopurulent with eventual locule formation in the pleural space and fibrous adhesions between the visceral and parietal pleura. Diagnosis is based on demonstration of purulent pleural fluid. Microbiological diagnosis should include Gram and acid-fast stains, wet mount for fungi, and culture for aerobic and anaerobic bacteria, *Mycobacterium* spp., and fungi. Once an empyema has developed, the aim should be to sterilize the space with antibiotics and establish early and adequate pleural space drainage. Diffusion of antibiotics into the pleural space is good, but aminoglycosides and some β-lactams may be inactivated in the presence of pus, a low pH, and β-lactamase enzymes (Hughes and van Scoy 1991). Empirical treatment of empyema should include an effective agent against anaerobic bacteria and aerobic gram-negative bacteria. Metronidazole may not be reduced to its active metabolite in a partially oxygenated environment and so clindamycin in combination with a fluoroquinolone for 4–6 weeks is a suitable combination. Alternatively, imipenem–cilastatin can be used. Drainage of pus is the mainstay of treatment and in the early exudative phase, closed drainage may suffice. If the empyema is loculated, operation can sometimes be avoided by the judicious use of urokinase to break down adhesions (Robinson and Moulton 1994).

NECROTIZING PNEUMONITIS AND LUNG ABSCESS

Pus-filled cavities in the lung can evolve when there is suppurative lung infection together with destruction of lung parenchyma. Necrotizing pneumonitis is arbitrarily defined as multiple cavities of less than 2 cm in diameter; lung abscesses are larger. Aspiration is the most common predisposing factor and in such cases the microbial etiology reflects that seen in aspiration pneumonitis (see Table 5). Tooth decay and gingivitis also predispose to necrotizing infections. Abscess may also occur in patients with necrotic neoplasms, in patients with bronchiectasis, during septic embolization from another source (e.g. endocarditis), as a complication of pulmonary embolus, and as direct spread from the abdomen (e.g. amoebic abscess).

Pathogenesis

Necrotizing pneumonia after aspiration is common because of the destructive nature of gastric contents and the large microbial load. In the unconscious patient, bacteria can replicate in static fluid in dependent lung segments. Some microorganisms produce necrotizing infections by virtue of their virulence factors, including *F. necrophorum*, *K. pneumoniae*, and *S. aureus*. *P. aeruginosa* secretes toxins that create a local vasculitis in addition to pneumonitis. Bronchiectasis can lead to lung abscess by virtue of markedly impaired local airway clearance.

Microbial etiology

In a study of lung abscess caused by transtracheal aspiration, the majority of patients had multiple isolates but in every case one or more anaerobes was recovered (Bartlett et al. 1974). The most common were gram-negative bacilli, including *Bacteroides* spp. and *Fusobacterium* spp., and also gram-positive cocci, including *Peptostreptococcus* spp. and microaerophilic streptococci. The aerobic organisms that can cause necrotizing pneumonitis are *S. aureus*, *S. pyogenes*, *K. pneumoniae*, *P. aeruginosa*, *Proteus* spp., and *E. coli*. Occasionally the more common causes of community-acquired pneumonia such as *S. pneumoniae* and *L. pneumophila* can be involved in a necrotizing process. *Actinomyces* spp., *Arachnia* spp., and *Nocardia* spp. can cause necrotizing pneumonitis as part of a chronic infection. A number of pathogens cause necrotizing pneumonitis in the context of immunosuppression, including *Burkholderia cepacia*, in patients receiving nebulized therapy (Yamagishi et al. 1993). *Legionella* spp. and *Yersinia enterocolitica* can cause necrotizing pneumonias in such patients. Necrotizing pneumonitis is a prominent feature of *B. pseudomallei* infection (melioidosis) and as part of infection with *B. anthracis* in anthrax and *Y. pestis* in pneumonic plague.

Where multiple abscesses are present in the lung, hematogenous spread from an extrapulmonary focus is the probable cause. *S. aureus* abscesses can complicate right-sided endocarditis, particularly in intravenous drug abusers. Enterobacteriaceae can produce multiple abscesses in association with urinary tract or bowel surgery and anaerobes can do so in patients with pelvic infections.

Diagnosis

Microbiological diagnosis can be achieved by transtracheal aspiration, bronchoscopic alveolar lavage, or with a protected brush specimen. Material should be rendered anaerobic as quickly as possible.

Therapy

For patients who are not severely ill, penicillin plus metronidazole or clindamycin is the initial therapy. Treatment must be continued for up to 3 months to achieve cure. In severely ill patients, empirical antibiotic therapy should aim to reduce the burden of β-lactamase producing anaerobes and also *S. aureus* and gram-negative bacilli, including *P. aeruginosa*. Some anaerobic bacteria, including *Actinomyces* spp. and *Propionibacterium* spp., are resistant to metronidazole. Some strains of the *B. fragilis* group are also resistant to the broad-spectrum penicillins, including piperacillin (Finegold 1994). Severely ill patients with necrotizing pneumonia can be given imipenem or a broad-spectrum β-lactam–β-lactamase inhibitor combination.

Chronic pneumonia

Apart from mycobacteria (see Mycobacterium tuberculosis complex, Mycobacterium leprae and other slow growing mycobacteria and 50, Rapidly growing mycobacteria), *Actinomyces* spp., notably *A. israelii* and *Propionibacterium propionicum*, and *Nocardia asteroides* may cause chronic pneumonia (see Actinomyces and related genera, and 45, Nocardia and other aerobic actinomycetes.

Pneumonia in immunocompromised patients

Most pneumonias in non-HIV immunocompromised patients occur in the context either of neutropenia or T-cell defects. These occur in malignancies, particularly hematological malignancies, with and without chemotherapy; they also occur in patients undergoing organ transplantation and attendant immunosuppressive therapy and in other patients receiving steroids. Neutropenic patients are susceptible to pneumonia due to gram-negative organisms, particularly *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Proteus* spp., and *Pseudomonas*. They are also susceptible to gram-positive infections, including *S. aureus* and *Staphylococcus epidermidis* and viridans streptococci (Verhoef 1993). Patients with cellular immune defects such as T-cell defects due to cyclosporin, purine antagonists, corticosteroids, or hematological malignancy are vulnerable to pneumonia mainly due to intracellular pathogens such as *Mycobacterium* spp. and *Legionella* spp., but more importantly to viruses, including cytomegalovirus (CMV), commonly derived from the transplanted organ, fungi (*Candida*, *Cryptococcus*) and *P. carinii* (Hughes 1993). It is difficult to distinguish on clinical grounds alone between pneumonia of bacterial or nonbacterial origin, but in general bacterial infections produce a rapidly advancing clinical course with focal infiltrates on chest radiograph (Fanta and Pennington 1994). *P. carinii* pneumonia in the non-HIV setting can also cause a rapidly advancing pneumonia. Bronchoscopy, with bronchoalveolar lavage, is necessary to identify the agents responsible because several may be involved. A number of unusual bacteria cause pneumonia in the immunocompromised host, including *Nocardia* spp., *Bordetella* spp., and *B. cepacia*. Nosocomial outbreaks of pneumonia in immunocompromised patients can be due to *L. pneumophila* (Carratala et al. 1994) and tuberculosis in renal units (Hall et al. 1994). Fanta and Pennington (1994) have proposed a scheme for initiating empirical therapy in immunocompromised

patients. Patients with a focal infiltrate should be given antibacterial treatment while results of lung sampling are awaited. A combination of antibiotics to cover staphylococci and resistant gram-negative bacilli, including *Pseudomonas*, should be given, for example, a third-generation cephalosporin and an aminoglycoside, or a combination of broad-spectrum penicillin and an aminoglycoside. If *Legionella* is suspected, intravenous erythromycin should be added; if *Aspergillus* is suspected, amphotericin B should also be given. The challenging problem of aspergillosis is discussed in the Medical Mycology volume, Aspergillosis.

Bacterial pneumonia in HIV infection

In late-stage unmodified HIV infection, particularly in patients with CD4 counts $<0.2 \times 10^9$ per liter, lung disease is dominated by infection with *P. carinii*, *M. tuberculosis*, *Toxoplasma gondii*, and *Cryptococcus neoformans*. Patients are also susceptible to conventional bacterial infections. HIV-seropositive individuals have increased susceptibility to bacterial pneumonia, particularly caused by *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *K. pneumoniae*, *N. meningitidis*, *Rhodococcus* spp., and *S. aureus* (Mitchell and Miller 1995). This susceptibility is particularly so in patients with a history of intravenous drug abuse (Caiaffa et al. 1994). Smoking of illicit drugs, such as marijuana and cocaine, is also a risk factor. Patients with AIDS in Africa appear particularly susceptible to pneumococcal disease (Gordon et al. 2002). In patients with relatively normal CD4 lymphocyte counts and no other HIV-related symptoms there is an appropriate response to antibiotic therapy. More advanced immunosuppression increases the risk of bacterial pneumonia (Rosen 1994). In the era of highly active antiretroviral therapy (HAART), there has been a declining morbidity and mortality of patients with AIDS (Palella et al. 1998).

Rhodococcus equi emerged as an important pathogen as a result of the HIV pandemic but is rarely seen in other patients. It is an anaerobic, nonmotile gram-positive organism that causes typical pneumonia which can be necrotizing and associated with pleural effusion. It is sensitive to vancomycin, erythromycin, chloramphenicol, and aminoglycosides. Treatment requires prolonged administration of vancomycin and erythromycin (Guerra et al. 1994). Infection with *H. influenzae* in HIV infection is somewhat unusual in that there is a high frequency of capsulate type b organisms in this group (Casadevall et al. 1992).

HIV-positive individuals have an increased incidence of sinusitis. The organisms involved are similar to those in non-HIV infected individuals, but *P. aeruginosa* is more frequent in the late stages of HIV disease. Occasionally nonbacterial pathogens such as *Cryptococcus*, *Aspergillus*, *Alternaria*, and CMV are responsible. Chronic sinusitis is particularly troublesome in patients with low CD4 counts. Treatment follows general principles.

Patients with HIV infection appear to be more susceptible to chronic bronchitis and bronchiectasis. The most common pathogens responsible for bacterial bronchitis include *H. influenzae* and *S. pneumoniae*, though *Pseudomonas* spp. may also be involved (Verghese et al. 1994). Exacerbations respond promptly to appropriate antibiotic therapy but may recur. Bronchiectasis tends to occur as discrete lesions in single lobes.

PERTUSSIS

Pertussis (whooping cough) is caused by *Bordetella pertussis*. It causes prolonged paroxysms of cough and in the very young can lead to prolonged apnea and death; long-term sequelae include bronchiectasis. There is no effective antibiotic treatment for the paroxysmal stage of the disease and prevention is the lynchpin of management.

Epidemiology

B. pertussis is transmitted by droplets and is highly infectious, with attack rates in susceptible individuals between 50 and 100 percent. Worldwide, there are approximately 50 million cases and 600 000 deaths annually (Crowcroft et al. 2003). Epidemics occur in cycles of 3–5 years, reflecting the appearance of susceptible individuals in a given population. Since 1950 the incidence of pertussis has been modified dramatically by the introduction of the whole-cell vaccine. Vaccination is given to children older than 1 month and provides immunity for approximately 12 years. Before vaccination pertussis was seen mainly in children 1–5 years of age, because of passive protection by maternal antibody in those aged less than 1 year. Adults, including mothers, are generally immune because of natural infection in childhood. In Sweden, where the pertussis programme was terminated in 1979, the incidence of pertussis is 60 cases per 100 000 population; age-specific attack rates per 100 000 have been 630 infants >1 year, 670 in children 1–4 years, and 258 in children 5–9 years (Romanus and Jonsell 1987). In contrast, in Massachusetts, USA, which has an active pertussis programme, the incidence of bacteriologically confirmed pertussis is 0.92 cases per 100 000 population, with a peak incidence in infants aged 1 month (104 per 100 000 population), which then declines to five per 100 000 (Marchant et al. 1994). Vaccination limits disease in children but the peak incidence is now in infants aged 1 month probably because of the absence of passive immunity from mothers in whom vaccine-induced immunity has declined. Even in countries with high coverage, adult pertussis does occur (Gilberg et al. 2002) and there is evidence of

transmission of pertussis from adults to susceptible infants (Mokotoff et al. 1995). During the mid-1970s the general public was alerted by media publicity to the possibility of vaccine-induced brain damage, and this led to a decline in vaccine uptake. Subsequently, there were three large epidemics in the UK at characteristic 4-year intervals in 1977, 1981, and 1985 (Preston 1994). In both Massachusetts (Marchant et al. 1994) and Cincinnati (Christie et al. 1994), there has been a resurgence of pertussis in the past 15 years in the face of vaccination programmes. These are due either to over-reporting or, more worrying, failure of vaccine efficacy.

Pathogenesis

B. pertussis produces a number of virulence factors (see Bordetella). The first step in *B. pertussis* infection is circumvention of mucociliary clearance by binding to ciliated cells and multiplying on their surfaces. This is achieved by two adhesins: filamentous hemagglutinin and pertussis toxin. Filamentous hemagglutinin release facilitates dispersal through the respiratory tract (Coutte et al. 2003). In addition, *B. pertussis* produces serotype 2 and 3 fimbriae, which also facilitate adherence. Colonization of ciliated cells results in their death because of toxin production by the bacterium. This damage to the airway epithelium and direct activity against local neurons by pertussis toxin is responsible for the paroxysmal cough (Salyers and Whitt 1994). *B. pertussis* survives for long periods in phagocytes. Pertussis toxin inhibits phagocyte killing in vitro and the migration of monocytes. The organism can survive in human polymorphs by inhibiting phagosome–lysosome fusion but it does not inhibit the respiratory burst (Steed et al. 1992). Similarly, *B. pertussis* survives for 3–4 days in human monocyte-derived macrophages (Friedman et al. 1992).

Clinical features

After an incubation period of 1–3 weeks a nonspecific catarrhal illness occurs with symptoms of malaise, lethargy, low-grade fever, dry cough, and poor feeding. A week later paroxysms of coughing occur, which increase in pitch before terminating in a deep inspiratory movement accompanied by the typical whoop. Chest examination is usually normal unless pneumonia or lobar collapse has occurred, as it occasionally does in infants. In infants, mortality is due to a combination of apnea and aspiration of vomitus. In adults, the disease tends to be less severe, though typical whooping cough may be seen. Children may be susceptible to bronchial infections for up to a year after pertussis, as the result of reduced host defenses of the airway. Bronchiectasis in some adults has been attributed to severe pertussis in childhood.

Diagnosis

In the presence of the typical whoop, diagnosis presents no difficulty, but isolation of *B. pertussis* by culture is diagnostic. This is most likely during the late catarrhal and early paroxysmal phases, but later becomes progressively less likely. Pernasal pharyngeal swabs should be cultured on Bordet–Gengou agar, supplemented with methicillin or cephalexin to inhibit the normal flora, or patients may be asked to cough directly on to the medium. Cultures must be examined daily for 5–7 days to identify the tiny, slow-growing, hemolytic colonies. In nasopharyngeal smears, *B. pertussis* can be detected directly with fluorescent antibody. Serological tests are not used routinely for the diagnosis of pertussis, but agglutinins and complement-fixing antibodies can be detected in serum and antipertussis secretory IgA can be detected in nasopharyngeal smears. PCR-based detection is also available but is not more sensitive than culture (Lingappa et al. 2002). Laboratory confirmation of pertussis can be difficult, but a cough lasting 2 weeks during community outbreaks is a sensitive and specific marker. Other organisms, including coxsackie viruses, echoviruses, adenoviruses serotypes 1–3, 5, and 7, and *M. pneumoniae*, can cause illness clinically indistinguishable from pertussis.

Management

Treatment during the early stage of the disease can reduce its course and restrict transmission. Erythromycin is recommended for cases and their household contacts, regardless of age or vaccination status. It is important to protect children aged less than 6 months who are too young to vaccinate. Chemoprophylaxis should be given to contacts as early as possible because more than 21 days after first contact it is of limited value (Mokotoff et al. 1995).

Prevention

Whole-cell vaccine can be combined with diphtheria and tetanus toxoids and aluminum-containing adjuvants (DTP vaccine), but an acellular vaccine has been introduced in several parts of the world. The pertussis component of this contains the three major agglutinogens, 1, 2, and 3. It is administered as three primary doses at intervals of 2 months beginning at 6–8 weeks of age. Booster doses are given 6–12 months later, and the third dose is given at 4–6 years of age. The vaccine is believed to have an effectiveness greater than 80 percent in preventing pertussis, but there has been a decline in efficacy in recent years (reviewed by Preston 1994). The

duration of protection is limited and wanes by approximately 12 years after immunization. Vaccine failures have been attributed to omission of one of the three agglutinogens or omission of the adjuvant.

A major problem with whole-cell vaccine has been public reaction to reports of adverse effects. Pertussis incidence has been 10–100 times lower in countries that have maintained higher coverage (Gangarosa et al. 1998). The most common of these are local reactions at the injection site and high fever; convulsions and brain damage are rare. The risk of permanent neurological damage in normal children given a full course of DTP vaccination is approximately one in 100 000, which is less than that of complications from the disease. Vaccination should, however, be avoided in children with a history of convulsions or brain damage in the neonatal period or other neurological disorder and during a febrile illness. A history of idiopathic epilepsy or developmental delay due to neurological disease in first-degree relatives is a relative contraindication that should be individually assessed. Those who have had a serious local or systemic reaction to a dose of vaccine should not be given further doses.

These considerations led to an effort to develop acellular vaccines. Pertussis toxin (PT), filamentous hemagglutinin (FHA), 68-kDa outer-membrane protein (OMP), and fimbrial agglutinogens are the most important antigens that elicit protective immunity against pertussis. FHA and OMP are, together with chemically detoxified PT, the components of some new acellular pertussis vaccines. During a pertussis outbreak in previously immunized children, those with higher serum levels of anti-FHA antibodies remained healthy, whereas those with lower antibody levels developed the disease (He et al. 1994). Formulations of acellular vaccine containing FHA, PT, and agglutinogens have been effective since 1981 in Japan in children 24 months of age and older. Pertussis toxoid alone or in combination with FHA has been tested in Swedish children from 6 months of age and gave partial protection against infection and good protection against severe disease (Ad hoc Group for the Study of Vaccines 1988). Five- and three-component acellular vaccines have been shown to be as effective as the whole-cell vaccine against culture-confirmed pertussis with at least 21 days of paroxysmal cough, though the five-component vaccine was superior to the three-component vaccine in preventing mild disease (Olin et al. 1997). Acellular vaccines can be effectively used as boosters. They are expensive and do not give rise to type-specific agglutinins. However, multicomponent acellular pertussis vaccines do appear to be effective and show fewer adverse effects than whole-cell pertussis vaccines. In areas where whooping cough is more likely to be fatal, it is possible that the higher toxicity of some whole-cell vaccines may be offset by their increased effectiveness (Tinnion and Hanlon 2000).

Infections with *Bordetella* species other than *B. pertussis*

Bordetella parapertussis has been incriminated as a minor cause of whooping cough, particularly a milder form than that caused by *B. pertussis*. Some evidence suggests that *B. parapertussis* may not be a separate species; this includes the isolation of both *B. pertussis* and *B. parapertussis* from whooping cough patients and the apparent conversion of *B. pertussis* to *B. parapertussis* by loss of a prophage. Doubt has been cast on *Bordetella bronchiseptica* as a possible cause of whooping cough (Woolfrey and Moody 1991).

ACUTE BRONCHITIS

Acute bronchitis is manifest as productive cough in a patient without a history of chronic chest disease. The microbial etiology of acute bronchitis includes viruses, particularly adenovirus, influenza virus, respiratory syncytial virus, and parainfluenza virus, the latter two being particularly common in children. *M. pneumoniae* and the pneumococcus are the prominent bacterial causes of acute bronchitis (Pennington 1994a). On balance, antibiotic therapy is regarded as unnecessary (reviewed by Gonzales and Sande, 1995). Randomized double-blind studies have failed to show a major clinical role for antibiotics in uncomplicated acute bronchitis. Some patients with acute bronchitis experience a prolonged episode of bronchial hyperreactivity. Antibiotics do not alter this syndrome.

CHRONIC BRONCHITIS AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic bronchitis is defined as the daily production of sputum for at least three consecutive months in two consecutive years and its essential feature is chronic bronchial mucin hypersecretion. This may be accompanied by bacterial infection, which causes an increase in the purulence of sputum, and also generalized small-airway obstruction. As a result, patients may experience one of three entities of increasing severity. These are simple chronic bronchitis with cough productive of non-purulent phlegm, chronic or recurrent mucopurulent bronchitis with cough, and chronic obstructive bronchitis, in which sputum is mostly purulent and there is airways obstruction (Medical Research Council 1965). Each of these is subject to acute exacerbation with a decline in the clinical state of the patient. The term chronic obstructive pulmonary disease (COPD) describes a range of chronic entities associated with airflow obstruction, including chronic bronchitis, emphysema, chronic asthma, and bronchiectasis. The disease COPD is characterized by episodic exacerbations in which there is fever plus any combination of increased

dyspnea, increased sputum purulence, or increased sputum volume. It is unclear whether exacerbations are associated with accelerated loss of lung function during the natural history of chronic bronchitis (Bates 1973), but following an exacerbation most patients experience at least a temporary decrease in functional status and quality of life (Seemungal et al. 1998).

Pathogenesis

Smoking plays a part in the generation of chronic bronchitis (Floreani et al. 1994). Gradually there is increased mucus hypersecretion, depressed ciliary function, and epithelial cell injury. Bacteria that are not normally present in the airway below the larynx are then able to colonize the diseased epithelium (Read et al. 1991) (Figure 5) and this leads to further damage.

Bacterial infection and exacerbations of chronic bronchitis

Nontypable *H. influenzae* is most commonly present in acute, purulent exacerbations of chronic bronchitis, but *S. pneumoniae* and *M. catarrhalis* can also be isolated. *H. influenzae* adheres to mucus via fimbriae (Barsum et al. 1995) and galactoside sequences on epithelial cells (Krivan et al. 1988). Soluble products of *H. influenzae* reduce ciliary beat frequency (Wilson et al. 1985) and mucociliary clearance. Nontypable *H. influenzae* are present in the lower respiratory tract of most patients with chronic bronchitis and in all patients at some time, even between exacerbations. Molecular typing of strains of *H. influenzae* isolated from sputum of patients with COPD has demonstrated that a single strain may persist for many months within the lower respiratory tract mucosa and remains present even if there have been acute exacerbations of COPD that have been treated with antibiotics (Groenveld et al. 1990). Multiple strains of *H. influenzae* can be present simultaneously in the sputum of an individual with COPD, and these distinct isolates may even vary in their antimicrobial sensitivity (Murphy et al. 1999). Their presence in the sputum during periods of increased symptoms does not necessarily imply a pathogenic role, but such a role for *H. influenzae* has been suggested by Musher et al. (1983), who showed that the serum of patients with acute febrile bronchitis contains opsonizing antibody for their own sputum isolates. There is evidence that acquisition of strain variants of *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* is associated with exacerbated disease, based upon molecular typing of sputum isolates from patients with COPD (Sethi et al. 2002). Virus and mycoplasma infections are responsible for up to a third of the acute exacerbations of chronic obstructive airways disease; the viruses more commonly isolated include influenza A, parainfluenza virus, coronavirus, rhinovirus, and herpes simplex virus (reviewed by Floreani et al. 1994). This suggests that bacteria, such as *H. influenzae*, flourish in the airways when viral infection further damages already compromised host defenses. The role of *H. influenzae* and *S. pneumoniae* in bacterial infection during chronic obstructive pulmonary disease has been reviewed by Murphy and Sethi (1992).

M. catarrhalis is present in pure culture in some transtracheal aspirates and is a recognized pathogen in chronic bronchitis; improvement is seen with specific treatment for *M. catarrhalis* infection. The organism has also been isolated from the blood and pleural fluid of patients with chronic obstructive airways disease (Murphy and Sethi 1992). In patients with more severe chronic bronchitis, a wider range of bacteria, including gram-negative enteric bacilli, *Pseudomonas* spp., and *Stenotrophomonas* spp., can be isolated by protective specimen brush (Soler et al. 1998).

Culture of sputum is of doubtful value because it is likely to be contaminated with the potential pathogens that are also present in oropharyngeal secretions. If such examination is undertaken, care should be taken to ensure that only purulent sputum is examined. This should be homogenized and serial dilutions examined. Examination of mucoid sputum will not yield useful information.

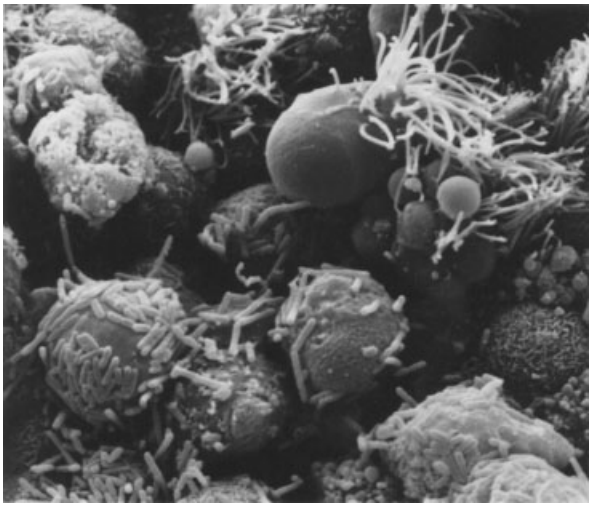

Figure 5 *Scanning electron micrograph of nontypable* Haemophilus influenzae *adhering to damaged areas of airway mucosa. (A. Brain and R.C. Read.)*

Therapy of exacerbations of chronic bronchitis

The use of antibiotics in all but the most severely ill patients is controversial (Saint et al. 1995). Small studies have either shown a positive effect of antibiotics (Berry et al. 1960; Pines et al. 1968), or no effect (Elmes et al. 1965; Nicotra et al. 1982). In a large placebo-controlled study,

Anthonisen et al. (1987) found significant benefit from antibiotics in severe but not in mild exacerbations. Airway function also recovered more rapidly with antibiotic treatment. The use of prophylactic antibiotics is controversial; their use can reduce the frequency and length of exacerbations to a very small degree but is complicated by side-effects of the antibiotics (Black et al. 2002).

It is appropriate to treat severe exacerbations of COPD with antibiotics capable of covering *H. influenzae* and *S. pneumoniae* (Bach et al. 2001). Rates of resistance to aminopenicillins and macrolides of sputum isolates of *H. influenzae* and *S. pneumoniae* are rising throughout the world (Felmingham and Gruneberg 2000). Although antibiotic therapy in patients with mild disease is controversial, appropriate antibiotics include amoxycillin (provided local β-lactamase activity is at low level), co-amoxiclav, or the oral cephalosporins and the macrolides, but erythromycin has relatively poor activity against *H. influenzae*. Newer macrolides such as clarithromycin and azithromycin are better orally tolerated and have greater activity against *H. influenzae* in vitro. New fluoroquinolones are highly active against *H. influenzae* and *S. pneumoniae* and penetrate tissue and sputum well.

BRONCHIECTASIS

Bronchiectasis is permanent abnormal dilation of bronchi accompanied by suppurative inflammation that results in intermittent production of large quantities of purulent sputum. The most common causes are severe parenchymal infections of the lung, including pertussis and measles in childhood and also – most importantly in the developing world – tuberculosis. Foreign bodies, carcinoma, AIDS, allergic bronchopulmonary aspergillosis, and certain congenital abnormalities, including immunoglobulin deficiencies and ciliary abnormalities, can also give rise to bronchiectasis. Cystic fibrosis is associated with bronchiectasis (see Cystic fibrosis). It may also be seen in patients with the α_1-antiprotease deficiencies and is associated with rheumatoid arthritis and inflammatory bowel disease. Once bronchiectasis is present, repeated infections serve to maintain a vicious circle of inflammation and damage to the already compromised airway, which leads to further infection (Cole and Wilson 1989).

The sputum flora of such patients often yields a mixed growth, but *H. influenzae* is probably the most important in sustaining continued infection and inflammation. *S. aureus* and *S. pneumoniae* are also prominent. In established disease, *P. aeruginosa* is associated with a poor prognosis (Angrill et al. 2002). Diagnosis of bronchiectasis is made on the basis of chronic daily production of mucopurulent sputum and confirmed by high-resolution computed tomography (CT), of the thorax (Barker 2002) (Figure 6).

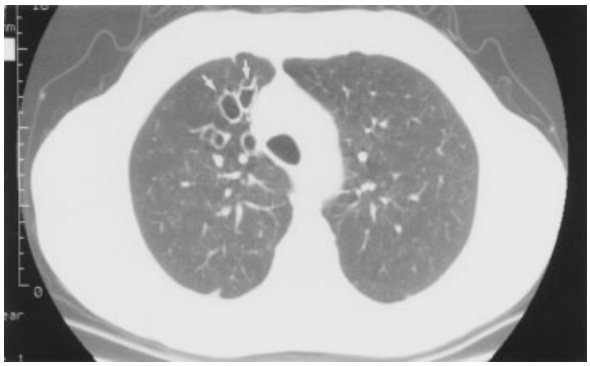

Figure 6 *Computed tomography (CT) scan demonstrating dilated airways and bronchial wall thickening in a patient with bronchiectasis. (Courtesy of A. Nakielny.)*

Management

The mainstay of treatment of bronchiectasis is drainage, which reduces the frequency of exacerbations, and antibiotic therapy. β-Lactam antibiotics do not penetrate diseased tissue at conventional dosages, but high doses of amoxycillin are effective in terminating exacerbations (Cole and Wilson 1989). High doses of oral cephalosporins or quinolones are effective when β-lactamase-producing strains are isolated. Long-term oral antibiotics are well tolerated and lead to symptomatic improvement but ciprofloxacin-resistant *P. aeruginosa* may be troublesome (Rayner et al. 1995). The use of prophylactic aminoglycosides given by nebulizer can reduce bacterial load but does not alter pulmonary function (Barker et al. 2000). In very severe exacerbations the use of intravenous antibiotics effective against *H. influenzae* and *P. aeruginosa* is indicated. Mucolytics have been suggested as adjunctive therapy for bronchiectasis but there is not currently enough evidence to evaluate this. High doses of bromhexine coupled with antibiotics may help with sputum production and clearance (Crockett et al. 2000).

CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians. It is autosomal recessive and caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene located on chromosome 7. Classic CF is characterized by chronic bacterial infection of the airways and sinuses, fat malabsorbtion due to pancreatic exocrine insufficiency, infertility in males due to obstructive azoospermia, and elevated concentrations of chloride in sweat. Patients with partial expression of *CFTR* usually have no overt signs of pancreatic insufficiency and later onset of bacterial infections of the airways (Knowles and Durie 2002). The incidence is approximately one in every 3 300 live Caucasian births. In the airways thick tenacious sputum

causes obstruction, leading to recurrent infection. Patients with CF have airway inflammation that commences at birth (Cantin 1995). The carrier rate is about 5 percent in Caucasians; about 6 000 people have the disease in the UK.

Clinical features

Infants present with meconium ileus at birth or a little later with diarrhea or malabsorption, failure to thrive, or progressive cough. Patients may also present with recurrent pneumonia. Acute exacerbations occur with increased sputum production, cyanosis, dyspnea, fever, and weight loss. Patients also suffer with nasal polyposis and chronic sinusitis.

Role of microbial infection

The first pathogen to colonize children with CF is *S. aureus*. This produces α- and δ-toxins that induce bronchial wall injury and abscess formation. The intense inflammatory response to *S. aureus* leads to further tissue destruction.

Staphylococcal airway injury permits colonization by *P. aeruginosa*. This is the major pathogen implicated in lung function decline and ultimate mortality in CF patients (Lyczak et al. 2002). Patients are at first colonized with nonmucoid strains, but later mucoid variants emerge. The extracellular alginate that they produce increases their adherence to ciliated epithelium (Marcus and Baker 1985). Despite the high antibody levels present against such strains and intensive antibiotic therapy, they cannot be eradicated completely. They reappear after treatment but it is not clear whether this is due to recolonization or acquisition of a new strain (Ojeniyi 1994). Alginate also interferes with antibody coating and inhibits phagocytosis of *P. aeruginosa* (Baltimore and Mitchell, 1980). Bacterial proteases produced by *P. aeruginosa* cause significant damage to the airways. Pseudomonas elastase and alkaline protease are secreted in vivo over prolonged periods in the airways (Suter 1994). *Pseudomonas* elastase increases the permeability of epithelial cells, destroys tight junctions, and induces shedding of epithelial cell-surface heparan sulfate proteoglycans, each of which encourages persistent colonization (Park et al. 2001).

The *Pseudomonas* phenazine pigments pyocyanin and 1-hydroxyphenazine interfere with mucociliary function (Wilson et al. 1987); the membrane glycolipid, the hemolysin rhamnolipid, also reduces mucociliary transport in vivo (Read et al. 1992). Exotoxin A produced by most colonizing strains of *P. aeruginosa* causes tissue injury and is highly immunogenic. Patients become colonized for years with *P. aeruginosa*, often with the same strain, and isolates from sputum relentlessly exhibit multiple antibiotic resistance. It has been demonstrated that the lungs of patients with CF provide an environment ideal for hypermutable strains of *P. aeruginosa* (Oliver et al. 2000).

Noncapsulate *H. influenzae* is most frequently isolated from infants more than a year old. *Burkholderia cepacia* also colonizes CF patients and is transmissible between close contacts. It is occasionally responsible for a fulminating septicemia. This organism produces a number of potential virulence factors, including adhesins, extracellular polysaccharide, lipopolysaccharide, and extracellular enzymes, including proteases and lipases. Some *B. cepacia* genomovars worsen prognosis of patients, whereas others do not (Jones et al. 2001). It is intrinsically highly resistant to antibiotics, including those effective against *P. aeruginosa* (Wilkinson and Pitt 1995), but it is usually sensitive to trimethoprim–sulfamethoxazole and chloramphenicol. *Stenotrophomonas* (*Xanthomonas*) *maltophilia* also colonizes patients with CF and can result in disease, though it does not adversely affect overall prognosis. A range of other microorganisms, including the Enterobacteriaceae, *Candida albicans*, *Aspergillus* spp., and environmental mycobacteria, may also be isolated from these patients, but their significance is uncertain.

Although CF patients produce markedly elevated titers of local and systemic antipseudomonal antibodies, the elastase produced by *P. aeruginosa* interferes with their activity by cleaving off the Fab and Fc fragments of immunoglobulins so that they are unable to interact with receptor sites on pulmonary macrophages or neutrophils (Thick et al. 1985).

Antimicrobial therapy

Antimicrobial therapy of exacerbations should always be guided by sensitivity data from cultured specimens. There is continuing controversy over the use of antibiotics as prophylaxis in CF. Once disease is established, some physicians attempt to suppress sputum colonization with long-term administration of antibiotics. Such antibiotics include cephalexin, oral chloramphenicol, trimethoprim–sulfamethoxazole, and co-amoxiclav, but controlled evaluations of this approach are lacking because of obvious methodological problems with such studies (Chartrand and Marks 1994). The use of oral cephalexin as prophylaxis suppresses *S. aureus* colonization but is associated with increased likelihood of *P. aeruginosa* colonization (Stutman et al. 2002). Most centers treat only acute exacerbations of pulmonary infection, with inclusion of empirical antipseudomonas and *S. aureus* antibiotics. This requires combined antibiotic therapy with a β-lactam plus an aminoglycoside. With the genesis of monotherapy capable of activity against both these pathogens, outpatient administration has flourished. Such antibiotics include ceftazidime, the penems, monobactams, and some β-lactam–β-lactamase

inhibitors. Unfortunately monotherapy can result in the spread of resistant organisms, e.g. *P. aeruginosa*, and this has been demonstrated following the use of ceftazidime (Pedersen et al. 1986). Emergence of *P. aeruginosa* resistance is also seen after monotherapy with imipenem and aztreonam (Chartrand and Marks 1994). The use of oral quinolones has permitted more flexible management of exacerbations. They have been used in children with CF (Schaad et al. 1991) without side effects and their use in moderately severe CF exacerbations can be as effective as traditional intravenous treatment with β-lactams plus aminoglycosides. In vitro susceptibility of isolates returns when the quinolones are withheld for 3 months. The use of aerosolized antibiotics has been advocated but controlled data to support this are limited. Aerosolized *tobramycin* given by nebulizer has been shown to eradicate *P. aeruginosa* (Ramsey et al. 1999) and improve pulmonary function tests (Smith et al. 1989), but emergence of resistance is a problem.

Other forms of therapy

Inhaled corticosteroids are being increasingly used in adults and children with CF to reduce lung damage arising from inflammation, but there is no clear evidence for their efficacy (Dezateux et al. 2000). There is potential to ameliorate the pulmonary disease in CF with somatic gene therapy. The optimal mode of delivery of the normal gene into airway epithelium is under investigation. Adenovirus vectors, DNA–liposome complexes, adeno-associated viral vectors, and DNA–ligand complexes have been used effectively in vitro and have been tested in animals. Adenovirus vectors and DNA–liposome complexes are currently in phase 1 clinical trial. There has been transient correction of electrophysiological defects in human CF nasal epithelium (O'Neal and Beaudet 1994). At present, CF patients face the prospect of lung transplantation as their only salvation once extreme disease ensues. Single-lung, double-lung, and heart–lung transplantation have all been used with success in such patients, and because the transplanted lung retains the physiological characteristics of the donor, there is no tendency for colonization by *P. aeruginosa* over and above that seen in transplanted lungs in non-CF patients.

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Bacterial infections of the genital tract

CATHERINE ISON

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The lower genital tract in women has its own microbial flora and in men is often colonized, although this may be a transient flora following sexual intercourse. In contrast, the upper genital tract in men and women is believed to be sterile. It was Doderlein in 1894 who first described lactobacilli as part of the 'normal flora' of the vagina. He believed that the lactic acid produced by lactobacilli from glucose in the vaginal fluid kept the vagina at an acidic pH, which discouraged colonization by other organisms. Glucose is produced from glycogen by the vaginal epithelium and this is utilized by the lactobacilli producing lactic acid. Glycogen is found on vaginal epithelium after puberty and is under estrogen control, hence is seldom present in premenarchal girls or postmenopausal women, unless on hormone replacement therapy. Lactobacilli are therefore found mostly in the vaginal fluid of women of child-bearing years and are considered 'normal' flora. In latter years it has been evident that the production of hydrogen peroxide (H_2O_2) by some lactobacilli is toxic to other bacteria and that this, in addition to lactic acid, may exert a protective effect in the vagina (Hillier 1998). The prevalence of H_2O_2-producing lactobacilli has been shown to be between 42–74 percent in pregnant women (Hillier et al. 1992, 1993; Puapermpoonsiri et al. 1996) and 65 percent in nonpregnant women (Hawes et al. 1996). In premenarchal girls the prevalence is low, 11 percent (Hill et al. 1995), although postmenopausal women have intermediate levels, 38 percent (Hillier and Lau 1997). Hydrogen peroxide has been shown to be toxic to bacteria associated with bacterial vaginosis, *Gardnerella vaginalis* and *Bacteroides bivius* (Klebanoff et al. 1991) and to the human immunodeficiency virus (Klebanoff and Coombs 1991) supporting the hypothesis that lactobacilli play a major role in protecting the vagina from specific pathogens.

Lactobacillus acidophilus and *Lactobacillus fermentum* have been demonstrated in early studies to be the predominant species in the vagina (Ragosa and Sharpe 1960; Giorgi et al. 1987). However, this may be inaccurate as the use of molecular methods identified *Lactobacillus crispatus* and *Lactobacillus jensenii* as the predominant species (Johnson et al. 1980). It appears that the capacity for the organism to produce H_2O_2 is more important than the individual speciation, being more persistent in the vagina than H_2O_2-negative strains.

GENITAL TRACT INFECTIONS

Disruption of the normal ecology of the genital tract can result by acquisition of a sexually transmitted pathogen such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum* (Table 1) or by increased numbers of bacteria that comprise part of the normal flora, generally in small numbers, such as *G. vaginalis* and group B streptococci (Table 1). Infections due to chlamydia are dealt with in Chlamydia. In many instances the change in ecology will result in symptoms, encouraging the individual to seek care, but

Table 1 *Clinical presentation of bacterial infections of the genital tract*

Organism	Main clinical presentation
Primary pathogens	
Neisseria gonorrhoeae	Uncomplicated infection (*cervicitis*, urethritis, pharyngeal, rectal) Complicated infection (salpingitis, PID, prostatitis, epididymitis)
Treponema pallidum	Genital ulceration Systemic infection
Haemophilus ducreyi	Genital ulceration
Klebsiella granulomatis	Genital ulceration
Secondary pathogens	
Gardnerella vaginalis	Bacterial vaginosis
Prevotella spp.	
Peptostreptococci	
Mobliuncus spp.	
Streptococcus agalactiae	Aerobic vaginitis Asymptomatic carriage
Staphylococcus aureus	Toxic shock syndrome
Clostridium perfringens	Post-partum infection Septic abortion
Actinomyces spp.	Discharge associated with IUCD

in some instances the bacteria remain silent causing asymptomatic infection which may remain undetected. The majority of infections present as mucosal infections of the lower genital tract where the challenge is to diagnose the infecting agent from amongst the resident microflora. However, many of these bacteria can ascend to the upper genital tract, which is normally sterile, to cause more complicated infections such as pelvic inflammatory disease with the possible sequelae of infertility, or, on occasions, can invade the blood causing systemic infections such as toxic shock syndrome. This chapter will first consider those bacteria that are primary pathogens, where their isolation always indicates the need for antimicrobial therapy, and then the bacteria that are secondary or opportunistic pathogens, where isolation needs interpretation together with clinical information before therapy is initiated.

NEISSERIA GONORRHOEAE

Neisseria gonorrhoeae and *Chlamydia trachomatis* (see Neisseria and Chapter 78, Chlamydia) are
the major causes of bacterial sexually transmitted infection in the industrialized world. *N. gonorrhoeae* is primarily a mucosal pathogen that causes infection of the lower genital tract in men and women, occasionally ascends to the upper genital tract to cause complicated infection, and rarely invades the blood to cause disseminated disease. Uncomplicated gonococcal infection (UGI) is predominantly a mucosal infection of the lower genital tract. In men, the primary site of infection is the urethra causing a discharge and/or dysuria and is symptomatic in most of the patients. Rectal infections are common among men who have sex with men and pharyngeal infection can occur in men or women who have oral sexual intercourse and is generally asymptomatic. In women, the primary site of colonization is the cervix, where the organism attaches preferentially to the columnar epithelium rather than the squamous epithelium of the vagina. In contrast to gonorrhea in men, infection in women is often asymptomatic in at least 50 percent of patients. Colonization of the urethra and rectum in women does occur but it is often unclear whether this is a true colonization or is leakage of cervical secretions from the vagina.

N. gonorrhoeae presents as a lower genital tract infection in >90 percent of patients in most parts of the world but can ascend to cause complicated gonococcal infection (CGI) of the upper genital tract. It presents as salpingitis or pelvic inflammatory disease in women and epididymitis or prostatitis in men. Complicated infection occurs more commonly in women than in men, largely because of undiagnosed asymptomatic infection. Disseminated gonococcal infection, where the organism invades into the blood to cause systemic infection, is a separate entity and is rare. It presents primarily as a rash, septicemia, and arthritis and is associated with certain strains of gonococci and is more prevalent in individuals with deficiency in the terminal complement components.

N. gonorrhoeae is an obligate human pathogen with no other natural host, which can cause repeated infection in the same host with no apparent immunity. This is believed to result from its ability for phase and antigenic variation, allowing the organism to appear novel at each infection. *N. gonorrhoeae* is genetically diverse and highly competent for genetic exchange and recombination at all stages of its life cycle, resulting in a nonclonal or panmictic population. In vivo, this probably occurs during mixed infections which are more likely to occur in sexually active individuals with high rates of partner change.

N. gonorrhoeae possess pili, small appendages, which enable the organism to overcome the hydrophobic interactions between the organism and the epithelial cell and hence come into close proximity, allowing specific receptors to attach to the host cell surface. Attachment is then enhanced by a family of outer membrane proteins, Opa proteins. *N. gonorrhoeae* are then believed to adhere to the epithelial cell, mediated by Opa proteins which interact with two receptors on the epithelial cells, CD66 and the heparan sulfate proteoglycans. The bacteria–epithelial cell interaction also induces a proinflammatory cytokine response. Invasion then occurs and the major porin, Por, is believed to play an important

role in this and also induces apototic cell death of both epithelial cells and polymorphs (Naumann et al. 1999).

Diagnosis

PRESUMPTIVE DIAGNOSIS

Microscopy is used for the presumptive diagnosis of gonorrhea in most parts of the world and is simple to perform and gives a result quickly. The presence of intracellular gram-negative diplococci in smears is considered diagnostic and in urethral smears from symptomatic men has a sensitivity of >95 percent. However, in women or asymptomatic men, the sensitivity is between 30–50 percent (Ison 1990; Sherrard and Barlow 1996; Jephcott 1997). This difference is probably due to higher numbers of bacteria being present in symptomatic individuals, which are easily detected by microscopy, compared to considerably lower numbers present in asymptomatic individuals which are below the detection limit. The sensitivity is also lower for rectal smears due to the large numbers of other bacteria present, including other gram-negative cocci, and is really only reliable when specimens have been taken from a lesion through a proctoscope, rather than perianal or blind rectal swabs. Microscopy is not commonly used for examining pharyngeal specimens. The specificity of the Gram stained smear for the presumptive diagnosis of gonorrhea is usually high when performed by experienced personnel (Hook and Handsfield 1999; Knapp and Koumans 1999). Comparison of numbers of positives obtained by Gram stained smears and culture can give an indication of either problems with reading smears or of isolation techniques, the correlation between smear and culture for urethral specimens from symptomatic men should be more than 95 percent.

CONFIRMATORY DIAGNOSIS

Isolation of the causative organism, *N. gonorrhoeae*, is still considered necessary for the confirmation of the diagnosis of gonorrhea, whenever the resources are available. The sensitivity of culture is thought to be high, 80–100 percent, but is dependent on a good specimen and isolation procedure. A nutritious agar base is required for the growth of *N. gonorrhoeae*, which is a fastidious organism, supplements containing an iron source and selective agents. The mostly commonly used agar base is GC agar base, which contains a mixture of peptones, starch, and agar, alternatively Columbia agar can be used. Blood, either lysed, heated (chocolatized) or pure hemoglobin, is often used as a supplement providing both amino acids and iron. Nonblood based supplements include IsoVitaleX or Vitox, which are an equivalent alternative. The use of selective agents to suppress the normal flora is recommended, particularly for specimens taken from the lower genital tract in women or from the rectum. Vancomycin or lincomycin is added to suppress gram-positive cocci, colistin and/or trimethoprim to inhibit other gram-negative organisms, and nystatin or amphotericin to suppress yeasts. Strains susceptible to the vancomycin, *env* mutants, and trimethoprim have been reported and can cause false negative results. In an ideal situation, both nonselective and selective isolation media would be used but there are seldom resources available for both and if a single medium is to be used it should contain selective agents to prevent overgrowth of the *N. gonorrhoeae* by the resident normal flora. Incubation at 36–37°C in a high humidity (>90 percent) and increased carbon dioxide concentration (5–10 percent) should be for a minimum of 48 h before the isolation medium should be discarded as negative.

Preliminary identification of any resulting colonies as oxidase positive gram-negative cocci is a presumptive identification of *Neisseria* spp. In many countries where resources are limited, no further identification is performed but it is still standard practice, where possible, to confirm the identity as *N. gonorrhoeae*. Historically, *N. gonorrhoeae* was identified by its ability to produce acid from glucose but not from other carbohydrates such as maltose, sucrose, and lactose. Carbohydrate utilization has now been combined with other preformed enzymes, such as the aminopeptidases, in identification kits, which give a result within 4 h. These tests will fully speciate the organism under test and are particularly useful for laboratories that encounter only occasional isolates. Biochemical reactions of the clinically important *Neisseria* spp. are shown in Table 2.

Table 2 *Identification of* Neisseria gonorrhoeae *from the clinically important* Neisseria *spp. and* Branhamella catarrhalis

	Acid production from				Aminopeptidase			
Organism	Glucose	Maltose	Sucrose	Lactose	γ-glutamyl	Propyl	DNAase	Butyrate esterase
N. gonorrhoeae	+	–	–	–	–	+	–	–
N. meningitidis	+	+	–	–	+	V	–	–
N. lactamica	+	+	–	+	–	–	–	–
N. cinerea	–	–	–	–	NK	NK	–	–
B. catarrhalis	–	–	–	–	–	–	+	+

NK, not known; V, variable result.

Alternatively, there are immunological reagents available that can identify *N. gonorrhoeae* and eliminate *N. meningitidis* and *N. lactamica* but do not give a full speciation. There are two reagents commonly used which both contain monoclonal antibodies to the major porin, Por. The antibodies are either linked to staphylococcal protein A and used in a coagglutination test (Phadebact Monoclonal GC Test, distributed by Boule in the UK) or linked to fluorescein and used in an immunofluoresence test (Syva Microtrak *Neisseria gonorrhoeae* Culture Confirmation Test, distributed by Launch Diagnostics in the UK). Both these reagents contain mixtures of antibodies to specific epitopes rather than a single antibody to a conserved epitope, hence strains giving false negative results do occasionally occur. Fortunately, the mixtures in each of these reagents differ and so they complement each other, rarely both giving a negative result. The coagglutination test has the advantage that it will also divide gonococcal isolates into two groups, PIA (WI) or PIB (WII/III), allowing a preliminary typing of strains.

MOLECULAR DETECTION

Detection of *N. gonorrhoeae* directly in the specimen can be achieved using probes or by amplification of specific sequences of DNA. The advantage of these tests are that they are highly sensitive and can detect small amounts of DNA and that they can detect nonviable organisms. Unlike previous attempts at antigen detection using immunological approaches, molecular detection methods are also highly specific. This is particularly important for screening low prevalence populations. The disadvantage of using molecular detection for *N. gonorrhoeae* is that susceptibility testing still requires a viable organism.

Molecular detection for gonorrhea has not been as widely used as for chlaymdial infection, largely because existing diagnostic testing has performed satisfactorily and has been less expensive. However, nucleic acid amplification tests (NAAT) has been shown to have a higher sensitivity than culture in some studies, particularly in situations where the resources or facilities for culture are not ideal. They have the added advantage that they can be used with noninvasive samples such as urine or self-taken swabs or tampons. The widespread use of NAATs for chlamydial infection has encouraged the use for gonorrhea as most of the commercial kits can test for both organisms on the same sample.

The nucleic acid hybridization test (Gene Pace 2) has a high sensitivity and specificity when used with endocervical or urethral specimens (Koumans et al. 1998). However because of the lack of amplification, they are likely to be less sensitive than the NAATs. The sensitivity of NAATs using amplification by polymerase chain reaction (Amplicor, Roche) or strand displacement amplification (BD Probtec, Becton Dickinsen) on endocervical specimens (van Dyck et al. 2001) or self-taken swabs or tampons (Knox et al. 2002) is high and often higher than culture. However, they are less satisfactory for detection of *N. gonorrhoeae* in urine from women (Van Doornum et al. 2001). Sensitivity is high when either urethral swabs or urine are used in men (Van Doornum et al. 2001; Palladino et al. 1999; Akduman et al. 2002). The specificty is high with all these tests but confirmation of positive results should be considered, particularly when low prevalence populations are screened.

SUSCEPTIBILITY TESTING

The purpose of susceptibility testing is to predict the outcome of therapy. In many instances for gonorrhea the patient will have been treated before the result of the susceptibility testing is known. The choice of a first line therapy should be based on good surveillance data and should give a high chance of therapeutic success, >95 percent. Surveillance programs mostly test a sample of gonococcal isolates each year using standard methods which detect emergence of resistance and drifts in susceptibility. There are a number of well established programs that have produced longitudinal data (Members of the Australian Gonococcal Surveillance Programme 1984; Schwarcz et al. 1990; Dillon 1992; Van de Laar et al. 1997) that inform therapy. In many parts of the world, penicillin is no longer suitable therapy and in recent years ciprofloxacin resistance has become widespread (WHO Western and Pacific Region Gonococcal Antimicrobial Surveillance Programme 2001).

However, most laboratories continue to test individual isolates on a daily basis in order to categorize strains as susceptible, exhibiting reduced susceptibility or resistance, particularly to the antibiotic used for therapy. Commonly used treatments include ciprofloxacin, ceftriaxone, and in some areas penicillin (or ampicillin). Routine susceptibility testing is largely performed using disc diffusion using a recommended method such as BSAC (British Society of Antimicrobial Chemotherapy) (King 2001) or NCCLS (National Committee for Control of Laboratory Standards 2003). The methods differ in the base medium and the disc content of antibiotic used, but if used according to the standard protocols and with appropriate controls, both give acceptable results. Problems can occur with susceptibility testing for *N. gonorrhoeae* because different strains vary in their nutritional requirements and growth rates, making comparison with control strains difficult. A quick and simple alternative is to use agar dilution breakpoints where growth or no growth is scored on agar plates incorporating one or two concentrations of antibiotic to categorize the isolates. It is a useful method for detecting high level resistance, for example, resistance to ciprofloxacin can be detected using a single concentra-

tion of 0.5 mg/l (minimum inhibitory concentration (MIC) $\geqslant$1 mg/l).

Antibiotic resistance

PENICILLIN

Penicillin or ampicillin was for many years the treatment of choice for gonorrhea until the emergence of chromosomal resistance due to continual usage of the antibiotic and the acquisition of plasmids from *Haemophilus* spp. Chromosomal resistance was the first to appear but was low level and initially responded to the use of increased dosage for treatment. Therapeutic failure due to chromosomally-mediated resistant *N. gonorrhoeae* (CMRNG) was reported in 1985 (Faruki et al. 1985) and became an increasing problem. Chromosomal resistance in *N. gonorrhoeae* is the result of additive effect of mutations at a number of loci including *penA*, *mtr*, *penB*, *penC*, and *ponA1* (Ropp et al. 2002), *penA* affects the affinity of penicillin for penicillin binding protein (PBP)2, *mtr* involves over expression of the efflux pump, *penB* is a mutation in the *por* gene, *ponA1* affects PBP1 and the function of *penC* is unknown. Together, mutations in these loci decrease the permeability of the cell wall, acting as a barrier to the penicillin.

In contrast, plasmid-mediated resistance was not detected until 1976, is enzyme-mediated and high level with a greater chance of therapeutic failure. In many countries the greatest prevalence was also in the 1980s but the prevalence remains high in some areas such as the Western Pacific, and penicillin cannot be used for therapy. Penicillinase-producing *N. gonorrhoeae* (PPNG) were first detected in infections acquired in Africa and Asia and were shown to be carrying plasmids of 3.2 and 4.4 MDa, respectively (Roberts 1989). These plasmids require the presence of a conjugative plasmid to transfer between gonococci and have now become disseminated worldwide. PPNG carrying plasmids of differing molecular weights have been isolated but have not spread so widely. All penicillinase plasmids in *N. gonorrhoeae* encode for the TEM-1 β-lactamase and differences in size reflect deletions in nonfunctional parts of the gene (Pagotto et al. 2000).

CIPROFLOXACIN

Ciprofloxacin, a fluoroquinolone, is an attractive alternative for the treatment of gonorrhea because it is given orally as a single dose, and hence compliance is good and can be observed. When ciprofloxacin was first introduced, *N. gonorrhoeae* were exquisitely sensitive and resistance was unknown. However, probably due to the misuse and overuse, particularly of the earlier generation of the quinolones, resistance due to mutations in the DNA gyrase gene, *gyrA*, and the topoisomerase IV gene, *parC*, has emerged (Knapp et al. 1997). High-level resistance (minimum inhibitory concentration (MIC) $\geqslant$1 mg/l) is due to multiple mutations in both *gyrA* and/or *parC* and may be enhanced by over expression of efflux systems and changes in the porin. Gonorrhea can be treated with other quinolones such as ofloxacin but *N. gonorrhoeae* exhibit cross-resistance between the quinolones and hence these are inactive against resistant strains. The newer quinolones such as moxifloxacin act primarily against *parC* and so are unlikely to be useful alternatives. Currently resistance to quinolones in *N. gonorrhoeae* is solely chromosomally mediated but reports of plasmid-mediated resistance in other organisms is worrying (Martinez-Martinez et al. 1998). Quinolone-resistant *N. gonorrhoeae* (QRNG) are most prevalent in the Western Pacific Region where the prevalence in some countries is above 50 percent. However, the prevalence of QRNG is increasing rapidly in countries in the western world such that ciprofloxacin is unlikely to remain an effective therapy for gonorrhea for very many more years.

CEPHALOSPORINS

Ceftriaxone, a third generation cephalosporin, is a recommended treatment for gonorrhea. It has proved highly active against *N. gonorrhoeae* including PPNG, and hence the original recommended dosage of 250 mg has been reduced to 125 mg in some guidelines (Centers for Disease Control and Prevention 2002a). Therapeutic resistance has not been documented although reduced susceptibility has been detected using in vitro susceptibility testing (Schwebke et al. 1995). This is of concern because cross-resistance has already been noted between cefuroxime, an earlier cephalosporin, and penicillin (Ison et al. 1990), particularly if lower dosages become common and usage increases because of resistance to other agents such as ciprofloxacin. Ceftriaxone is given by injection and some patients may prefer an oral third generation cephalosporin. However, data on efficacy and availability are still limited.

ALTERNATIVE TREATMENTS

Spectinomycin, 2 g intramuscularly, is still recommended as an alternative therapy for gonorrhea. Resistance has been documented and is single-step and high-level but it has occurred sporadically (Easmon et al. 1982; Boslego et al. 1987; Zenilman et al. 1987) and has not spread widely suggesting that it may be a suitable alternative. However, it has not been extensively used in recent years and has become difficult to obtain.

Azithromycin is used more commonly for chlamydial infection but has been suggested as an alternative for gonorrhea particularly when resistance to penicillin and ciprofloxacin is high. The recommended dosage is 2 g, but patients often find this difficult to tolerate and 1 g has been used (Young et al. 1997; Xia et al. 2000). However, resistance has been noted with both dosages

and increased usage is likely to increase the selective pressure.

Tetracycline is also more commonly used as treatment for chlamydial infection but has been used for gonorrhea where resources are limited. Resistance can be both chromosomal and low level (Johnson and Morse 1988) or plasmid-mediated and high-level (Morse et al. 1986). Plasmid-mediated resistance is due to the insertion of the *tetM* determinant, probably from streptococci, into the gonococcal conjugative plasmid, hence the plasmid can mobilize itself between gonococci and other genera. Since its first description in 1985 (Morse et al. 1986), tetracycline-resistant *N. gonorrhoeae* (TRNG) have spread widely, reducing its efficacy as an inexpensive alternative therapy.

Aminoglycosides such as gentamicin and kanamycin have been used in resource poor settings but information on resistance and relationship to in vitro susceptibility testing is lacking (Daly et al. 1997; Lkhamsuren et al. 2001). Co-trimoxazole is also used but the true prevalence of resistance is difficult to determine because of the difficulties in susceptibility testing (West et al. 1995).

Typing

Techniques used to discriminate between strains of *N. gonorrhoeae* from different sources have been used to monitor temporal changes, detect clusters of antibiotic resistant strains, and confirm or dispute contact, largely in cases of child or sexual abuse. Phenotypic methods of auxotyping, differentiation by nutritional requirement (Catlin 1973) and serotyping, reactivity with a panel of monoclonal antibodies directed at specific epitopes on Por, the major outer membrane protein (Knapp et al. 1984), have been used extensively for these purposes. However, these tests lacked discriminatory power and even when used in combination, a few auxotype/serovar classes predominate in most populations. Both auxotyping and serotyping had subjective endpoints presenting some problems of reproducibility, particularly in longitudinal studies. In recent years a range of molecular techniques have been described, all of which have enhanced discriminatory ability compared to the phenotypic methods (Ison 1998). However, many of the techniques examine variation in genes that are exposed to the immune response and hence are subject to greater diversity. While this approach does not give valuable information for studies over long time periods, where change is likely to occur through genetic exchange in mixed infections, it is more useful for identifying sexual contacts, short chains of transmission, and for studying sexual networks (Ward et al. 2000). There is increasing interest in using a molecular approach to identifying clusters of isolates from linked patients to aid public health interventions. Combined studies using epidemiological and molecular data suggest that molecular typing will identify clusters that are larger than clusters identified by epidemiological information alone, which may give useful data to clinicians if used prospectively.

TREPONEMA PALLIDUM

Treponema pallidum subsp. *pallidum* is the causative agent of syphilis, a mucocutaneous infection, which is highly infectious in the early stages and has serious sequelae if left untreated. The primary stage of syphilis usually presents as a painless ulcer, which occurs 9–90 days after exposure, and may be accompanied by a regional lymphadenopathy. The secondary stage has many symptoms including localized or diffuse ulcers, generalized lymphadenopathy, rash, and condylomata lata and presents 6 weeks to 6 months after exposure. Latent syphilis, by definition, has no clinical signs but gives a positive serological reaction and is present either less than (early) or more than (late) 2 years after exposure. Tertiary syphilis is rare when good antibiotic therapy is available but can include gummatous syphilis, neurosyphilis, and cardovascular syphilis.

T. pallidum is a spirochete of approximately 0.2 μm in diameter and 5–15 μm in length. It is an obligate human pathogen and has never been grown successfully on artificial media, the only successful cultivation being in rabbits. The cell envelope structure is unique and unlike other gram-negative organisms, it has an outer membrane, but lacks lipopolysaccharide and porins have not been detected. The organism possesses flagella, for motility, which are located in the periplasmic space instead of externally as is normally found in motile bacteria. It has a very restricted temperature range for growth, 30–35°C, and in rabbits the generation time has been demonstrated as 35 h. The complete genome sequence is known (Fraser et al. 1998) and the genome is determined to be 1.14 Mb, approximately 25 percent of the size of the *E. coli* genome, and encodes for 1040 genes or open reading frames.

Epidemiology

Syphilis is a worldwide public health problem (Gerbase et al. 1998). It is a major cause of genital ulcer disease in the developing world and the prevalence is high in the newly independent states of the former Soviet Union and is a concern because of the strong association with the acquisition of HIV infection. After a peak in incidence following World War II there was a decline in many countries, particularly in the industrialized world, and syphilis became largely a disease of men who have sex with men. Following the advent of AIDS in 1984 and subsequent changes in sexual behaviour, syphilis declined. In the United States there were efforts to eliminate syphilis. In recent years there have been an

increasing numbers of outbreaks of syphilis reported in the USA and many countries in Europe (Doherty et al. 2002). Many of the outbreaks are among men who have sex with men, possibly reflecting an increase in risk behaviour following a perception that HIV infection is now a treatable infection. Penicillin is the mainstay of treatment for all stages of syphilis infection (Centers for Disease Control and Prevention 2002a)

Diagnosis

MICROSCOPY

Examination of lesion exudate or lymph nodes by dark-ground microscopy is a useful presumptive diagnostic tool for infectious (primary) syphilis. However, it should be performed by experienced workers because of the interference with commensal spirochetes that are found as part of the normal flora of the genital and gastro-intestinal tract. It is not considered as suitable for use with rectal or nongenital lesions and is not recommended for oral lesions.

Exudate from the syphilitic ulcer should be collected after cleansing of the ulcer with sterile saline using a gauze swab. The ulcer should then be squeezed to allow sufficient serous fluid to be produced and then this should be collected by a loop and placed onto a microscope slide. It is not necessary to add any saline to the microscope slide. After placing a coverslip onto the fluid it should be examined by dark ground microscopy within 10 min in order to identify the characteristic morphology and motility of *T. pallidum*. If the initial examination is negative, this test can be repeated daily for at least 3 days as long as antibiotics are not given.

SEROLOGICAL DIAGNOSIS

The inability to cultivate *T. pallidum* has led to the use of the detection of the host's immune response as an indicator of exposure to infection. Serological tests for syphilis can be divided into screening tests and confirmatory tests. Historically screening tests employed nontreponemal antigen, which detects antilipoidal antibodies that are produced in response to the tissue damage caused by treponemal diseases including syphilis but also other unrelated diseases, whereas confirmatory tests use a specific treponemal antigen. However, the development of enzyme immunoassays using specific antigen that are automated and can be used to screen large numbers has in some instances replaced the nontreponemal tests for screening.

NONTREPONEMAL TESTS

The nontreponemal tests using the Venereal Disease Research Laboratory (VDRL) antigen, whose main components are cardiolipin, cholesterol, and lecithin, have been widely used for screening for syphilis. Plasma is mixed with the VDRL antigen and if antibody is present flocculation results which can be read microscopically (VDRL test) or macroscopically (Rapid Plasma Reagin (RPR) test), which is applicable for use in resource poor settings with little or no laboratory facilities. The tests differ in the particle size used and the pigment used to enhance visualization of the result. These tests are mostly used qualitatively but if used quantitatively can give a measure of the response to therapy. False positive results do occur, for instance during pregnancy or with autoimmune disease, such that all positive results should be confirmed using a specific treponemal test.

SPECIFIC TREPONEMAL TESTS

Enzyme immunoassays (EIA) using specific treponemal antigen, which has been cloned or sonicated and adsorbed onto a solid phase, are a relatively new approach to detection of antibody in syphilis. The EIAs can detect both IgG and/or IgM but those that detect IgG and IgM together are recommended because they tend to be more sensitive in primary infection (Schmidt et al. 2000). The *T. pallidum* particle assay (TPPA) and the *T. pallidum* haemagglutination assay (TPHA) both utilize a specific antigen which is attached to gelatin beads (TPPA) or erythrocytes (TPHA). The fluorescent-treponemal antibody-absorbed (FTA-ABS) test is an indirect immunofluorescence test which detects antibody that attaches to treponemes coating a microscope slide and the resulting antigen–antibody complexes are detected by a secondary antibody linked to a fluorochrome. The TPHA and TPPA have been widely used as screening and confirmatory tests, although the TPPA has been shown to be more sensitive than the TPHA in primary syphilis. The EIA is attractive because it has the capacity to screen large numbers using a specific antigen in an automated system. The TPHA/TPPA can be used to confirm a positive EIA result and vice versa. The FTA-ABS test is more time consuming and used mostly to confirm discrepant results between other tests. An additional test often used in research laboratories is based on immunoblotting to detect antibody to recombinant antigens. This test has a sensitivity similar to the FTA-ABS test. The choice of screening and confirmatory tests is dependent on workload and facilities available and will differ considerably dependent on the resources available.

HAEMOPHILUS DUCREYI

Haemophilus ducreyi is a fastidious organism which is the causative agent of the sexually transmitted disease, chancroid. It presents primarily as a genital ulcer but can also present as a regional lymphadenopathy with bubo formation. *H. ducreyi* is thought to establish itself through small abrasions, which may occur in the epidermis during sexual intercourse. Once colonization has been achieved, a small papule develops after a short

incubation period of 5–10 days, which may progress to form an ulcer. The ulcers are painful and have ragged and undermined edges, and can persist for many months in the absence of antibiotic therapy. Lesions usually occur on the prepuce and frenulum of men and the vulva, cervix, and perianal region in women. Extragenital chancroid is rare but lesions have been reported on the inner thighs, breasts, and fingers (Lewis 2000). Unlike other sexually transmitted infections, chancroid has few sequelae and has largely been considered a treatable infection. However, the strong association of chancroid, together with syphilis, with the acquisition of the human immunodeficiency virus (HIV) has renewed interest both in providing good diagnostic tests and research studies. This has become more important with the studies which have suggested that aggressive treatment of bacterial sexually transmitted infections (STI), such as chancorid, can reduce the acquisition of HIV (Grosskurth et al. 1995).

Chancroid is one of the most common STIs but is found predominantly in the Tropics and found only occasionally in North America and Europe (Trees and Morse 1995). Chancroid is more common in men than women and there is considerable debate over whether asymptomatic carriage occurs in women, which is largely unresolved. The high prevalence in resource-poor settings raises challenges for diagnosis as good laboratory facilities are often unavailable. Isolation of *H. ducreyi* is still regarded as the gold standard, if available, because microscopy is nonspecific and molecular methods are not commercially available. However, in resource poor settings diagnosis is usually made on the clinical symptoms and signs and treated syndromically.

Isolation of *H. ducreyi*

It is possible to cultivate *H. ducreyi* but it is a fastidious organism that requires a highly nutritious medium. It also appears that different strains of *H. ducreyi* appear to grow preferentially on some culture media, particularly on primary isolation, and so the highest isolation rates (approximately 80 percent) have used multiple media. Two culture media, which include serum, that have been used are GC agar base supplemented with 1 percent hemoglobin, 5 percent fetal calf serum, and 1 percent IsoVitaleX and Mueller–Hinton agar supplemented with 5 percent chocolatized horse blood and 1 percent IsoVitaleX (Dangor et al. 1992). A serum free medium which is less expensive to prepare, which has been used successfully, contains GC agar supplemented with 1 percent hemoglobin, 0.2 percent charcoal, and 1 percent IsoVitaleX (Lockett et al. 1991). Selective agents to inhibit the growth of skin organisms, particularly gram-positive cocci, and allow the growth of *H. ducreyi* have been difficult to choose, but 3 mg/l of vancomycin is usually added.

Isolation of *H. ducreyi* appears most successful if the ulcer is cleaned with a cotton swab to remove superficial pus and the material taken from the undermined edge of the ulcer and inoculated directly from the patient onto the culture medium. Primary isolation media should be incubated at 33°C for optimal growth condition in high humidity with 5 percent carbon dioxide for a minimum of 48–72 h before being discarded as negative. There is no transport medium that has been widely tested although some success has been demonstrated using a thioglycollate-hemin-based medium (Dangor et al. 1993).

After incubation, gray translucent colonies will appear which are gram-negative cocco-bacilli and weakly oxidase positive. The most characteristic sign is friable colonies that can easily be pushed around the plate. These three criteria are highly predictive of *H. ducreyi*, and in most instances no further identification is performed.

Microscopy

Microscopy is often used in clinical settings for the presumptive diagnosis of an STI but has proved poorly specific for the diagnosis of chancroid. The gram-negative cocco-bacilli are described as appearing in sheets, which resemble 'schools of fish' or 'railroad tracks' but this can be very difficult to detect in clinical specimens direct from the patient and is not recommended for the routine diagnosis (Albritton 1989).

Molecular detection

Commercial tests for the molecular detection of *H. ducreyi* are not widely available. However, a multiplex polymerase chain reaction (PCR) assay has been evaluated which gives the simultaneous amplification of DNA from *H. ducreyi*, *T. pallidum*, and herpes simplex virus types 1 and 2 which is more sensitive than existing methods (Orle et al. 1996). Comparison between multiplex-PCR and culture has shown the sensitivity of culture for *H. ducreyi* to be around 75 percent but this is dependent on good isolation procedures and has been less in some studies (Orle et al. 1996; Morse et al. 1997).

Antimicrobial resistance and therapy

H. ducreyi are innately sensitive to most antibiotics but, in a similar manner to *N. gonorrhoeae*, have become resistant to many antimicrobial agents through the acquisition of plasmids or selection of chromosomal mutations. Plasmid-mediated resistance has been reported to tetracycline, chloramphenicol, sulphonamides, aminoglycosides, and β-lactam antibiotics. Chromosomal resistance is less well documented but decreased susceptibilities to trimethoprim, penicillin,

ciprofloxacin, and ofloxacin have been reported (Trees and Morse 1995; Lewis 2000).

Despite the emergence of resistance over the last decade, chancroid is still treatable. Azithromycin, ciprofloxacin, erythromycin, and ceftriaxone form part of the recommendations for treatment from the Centers for Disease Control and Prevention and from the World Health Organization, although the dosage and regimens differ slightly.

Susceptibility testing

Testing the susceptibility of *H. ducreyi* can be difficult because of the fastidious nature of the organism and its tendency to adhere to the agar. Most studies have used agar dilution rather than disc diffusion and have been performed in specialist centers. A prerequisite for susceptibility testing is a viable organism and only a limited number of centers have routine culture established and hence the antibiogram of many strains is not tested. A standardized method has been recommended for determination of the MIC which includes, standardization of the inoculum, use of enriched Mueller–Hinton agar containing a range of antibiotic concentrations, incubation at 35°C in a humid atmosphere containing 5 percent carbon dioxide, and examination of the result after 48 h of incubation (Dangor et al. 1990).

KLEBSIELLA (CALYMMATOBACTERIUM) GRANULOMATIS

K. granulomatosis is a gram-negative bacillus which is the causative agent of donovanosis, which usually presents as genital ulceration but may also be oral or found at extra-genital sites. Genital ulceration is often associated with inguinal lymphadenopathy and if left untreated considerable tissue destruction can occur. Donovanosis is largely considered a tropical disease having not been reported in Europe and the USA although its true prevalence is unknown.

K. granulomatis has been included in several genera and was for a long time called *Calymmatobacterium granulomatis*. However, its similarity with *Klebsiella* species, originally detected using serology (Goldberg 1962) and ultrastructural studies (Kuberski et al. 1980) has been confirmed by molecular studies and the name change to *K. granulomatis* (Carter et al. 1999) proposed, although it remains controversial (Kharsany et al. 1999).

Culture of the organism in vitro is difficult and was originally performed in the yolk sacs of chick embryos but more recently in human peripheral blood mononuclear cells (Kharsany et al. 1997) and in Hep-2 cells (Carter et al. 1997). However, diagnosis is rarely made by culture but usually by the examination of smears stained using a Giemsa or related stain for the characterisitic donovan bodies or more recently by a newly described nucleic amplification test (Carter and Kemp 2000). The lack of a culture has prevented studies on susceptibility to antibiotics although a range have been used for treatment including ampicillin, chloramphenicol, and ceftraixone.

BACTERIAL VAGINOSIS

Gardnerella vaginalis, *Prevotella* spp., *Peptostreptococci*, the anaerobic curved rods, *Mobiluncus* spp. and *Mycoplasma hominis* are closely associated with the syndrome known as bacterial vaginosis (BV) which is characterized by a change in vaginal ecology. These organisms can be found in the 'normal' vagina but in small numbers, which in BV multiply and increase 100–1000 fold to become the predominant flora. These large numbers of bacteria adhere to squamous epithelial cells giving them a studded appearance that led to them being described as 'clue cells', as an indicator of the condition. The pH of the vaginal fluid also increases from below 4.5 to 5.0 or greater. This is almost certainly maintained by the large numbers of bacteria associated with BV that produce amines, but it is unclear which occurs first, the rise in pH or the increase in bacterial numbers. BV was previously known as 'non-specific vaginitis' or *Haemophilus vaginalis* vaginitis (Gardner and Dukes 1955) but in 1983 the term bacterial vaginosis became popular as it reflected the strong association with overgrowth of certain bacteria and the absence of an inflammatory response. Other terms which are commonly used are anaerobic vaginosis and Gardnerella vaginosis or vaginitis.

BV is the commonest cause of vaginal discharge of women of child-bearing years in the western world. BV is a clinical syndrome which presents as an increased vaginal discharge which has a distinctive fishy smell which is often reported as worse after sexual intercourse. The true prevalence is probably unknown but has been reported as being 10–20 percent of sexually active women (Hay et al. 1992). Predisposing factors for BV have been difficult to identify but of the many investigated, the association with sexual activity is the strongest. This is supported by a higher prevalence in sexually active women including those attending specialized clinics for sexually transmitted diseases or for termination of pregnancy (Blackwell et al. 1993). It has also been found to be more common among women using intra-uterine contraceptive devices (Amsel et al. 1983) and in a number of studies among lesbian women (Berger et al. 1995; Skinner et al. 1996). It is generally considered to be a sexually associated condition rather than sexually transmitted. This is supported by its presence in some virgin girls (Bump and Buesching 1988), its high prevalence in lesbian women who have a low incidence of sexually transmitted infections, and the failure to prevent recurrent episodes after treatment of sexual partners (Colli et al. 1997).

G. vaginalis was the first bacterium to be associated with the syndrome of BV, but it became clear in the 1970s, with the improvement of cultural techniques, that a more complex bacterial flora existed including anaerobes and *M. hominis*. It was not until 1984 that *Mobiluncus*, anaerobic curved rods, were described (Spiegel and Roberts 1984) and consists of two species, *Mobiluncus curtisii* and *Mobiluncus mulieris*. *M. curtisii* are small curved rods which are gram-variable and are divided into two subspecies, *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp *holmesii*. *M. mulieris* is a longer curved rod which is gram-negative. Anaerobic curved rods show a strong association with BV with few women without vaginosis apparently being colonized. However, *Mobiluncus* spp. are very difficult to cultivate and more sensitive nucleic acid amplification methods have shown that although a strong association with BV exists, more women without BV are colonized than previously found (Schwebke and Lawing 2001). The strong association between colonization with *Mobiluncus* spp. and BV has led to the hypothesis that they may represent a separate syndrome, but no evidence exists for this.

Diagnosis

For many years a variety of methods were used for the diagnosis of BV, many of which arose from the original description of the condition by Gardner and Dukes (1955). Recognition of the clinical signs and symptoms, presence of 'clue cells' in a wet mount of vaginal discharge, and isolation of *G. vaginalis* were among the methods used. These methods were unsatisfactory because reporting of signs and symptoms is subjective and varies between patients and while 'clue cells' and *G. vaginalis* are good markers for BV they can also be present in small numbers in women without BV.

Since 1983, the accepted standard for the diagnosis of BV has been the use of four composite criteria (Amsel et al. 1983); a thin homogeneous vaginal discharge, elevated vaginal pH, presence of amines detected by a fishy smell on the addition of potassium hydroxide to the discharge, and the presence of 'clue cells'. If three or more of the four criteria are present then the patient is diagnosed with BV, if less than three are detected the patient is considered not to have BV or to be 'normal'. These criteria have formed the basis of the diagnosis of BV for nearly two decades because they are simple to perform, particularly in a clinic or consultation room, and require minimal material with the exception of a microscope. However, the composite or Amsel's criteria do have some disadvantages as they require a vaginal examination and recognition of the vaginal discharge and 'fishy' smell have a subjective endpoint. There has also been a tendency to use only one or at least not all four criteria, hence invalidating the method. Each of the individual criteria are predictive of BV but do not have the same sensitivity and specificity as when they are used in combination.

An alternative approach for diagnosis is the grading or scoring of the Gram stained vaginal smears to detect the change in vaginal ecology and the strong bacterial associations. The method first described divided smears into those with a predominantly *Lactobacillus* morphotype, which was considered normal, and those with a mixed microbial flora and no lactobacilli, which were graded as BV (Spiegel et al. 1983). This initial method was modified to include an intermediate category, where the smears showed a predominantly mixed microbial flora but had significant numbers of lactobacilli (Nugent et al. 1991). These methods quantify the numbers of different morphotypes of bacteria, lactobacilli, *Gardnerella*/*Bacteroides*, and *Mobiluncus*, assign a score for each which when combined gives a number which defines the grading. These methods have been used extensively for research studies and have given valuable data but they require considerable time and skill, which is not always available in clinical or diagnostic situations. Simpler methods have been described where the microbial flora is assessed qualitatively and graded into normal, intermediate or BV (Thomason et al. 1992; Hay et al. 1994). Both the quantitative and qualitative methods have been validated in comparison with the composite criteria and found to have a high sensitivity, specificity, and predictive values for the definition of 'normal' and 'BV' (Schwebke et al. 1996; Thomason et al. 1992; Ison and Hay 2002). However, the inclusion of the intermediate category with BV reduces the specificity and is more frequent in women defined as normal by the composite criteria (Ison and Hay 2002; Taylor-Robinson et al. 2003). This suggests that the vaginal flora associated with the intermediate grade is distinct from that of BV and does not equate with the full clinical syndrome of BV and hence cannot be diagnosed by clinical criteria. A recommended grading system is shown in Table 3 and Figure 1.

Treatment

In the absence of a clear causative agent, the treatment for BV is primarily aimed at the eradication of the bacteria associated with the clinical condition in an attempt to re-establish the normal 'lactobacillary' flora. The inadequacy of antimicrobial agents to effect a cure is highlighted by the high relapse rates but alternative approaches using replacement flora (bacteriotherapy) are not yet widely available.

Metronidazole is the agent of choice by many clinicians and is given either as a 5 day course of 400 mg twice a day or as a single dose, 2 g. The 5 day course may give a slightly higher cure rate but metronidazole causes nausea and the single dose has a higher compliance rate. Topical gels have been used increasingly and

Table 3 *Grading scheme for Gram-stained vaginal smears (Ison and Hay 2002)*

Grade of flora	Definition of flora	Description
0	Normal[a]	Epithelial cells only – no bacteria seen
I	Normal	Lactobacillus morphotype only
II	Intermediate	Reduced lactobacillus morphotype with mixed bacterial flora
III	Bacterial vaginosis	Mixed bacterial flora with few or absent lactobacillus morphotype
IV	Normal in asymptomatic women[b]	Epithelial cells covered with gram-positive cocci with few or absent lactobacillus morphotype

a) Normally found during or immediately post antibiotic therapy.
b) May be a cause of aerobic vaginitis or be of importance in pregnant women.

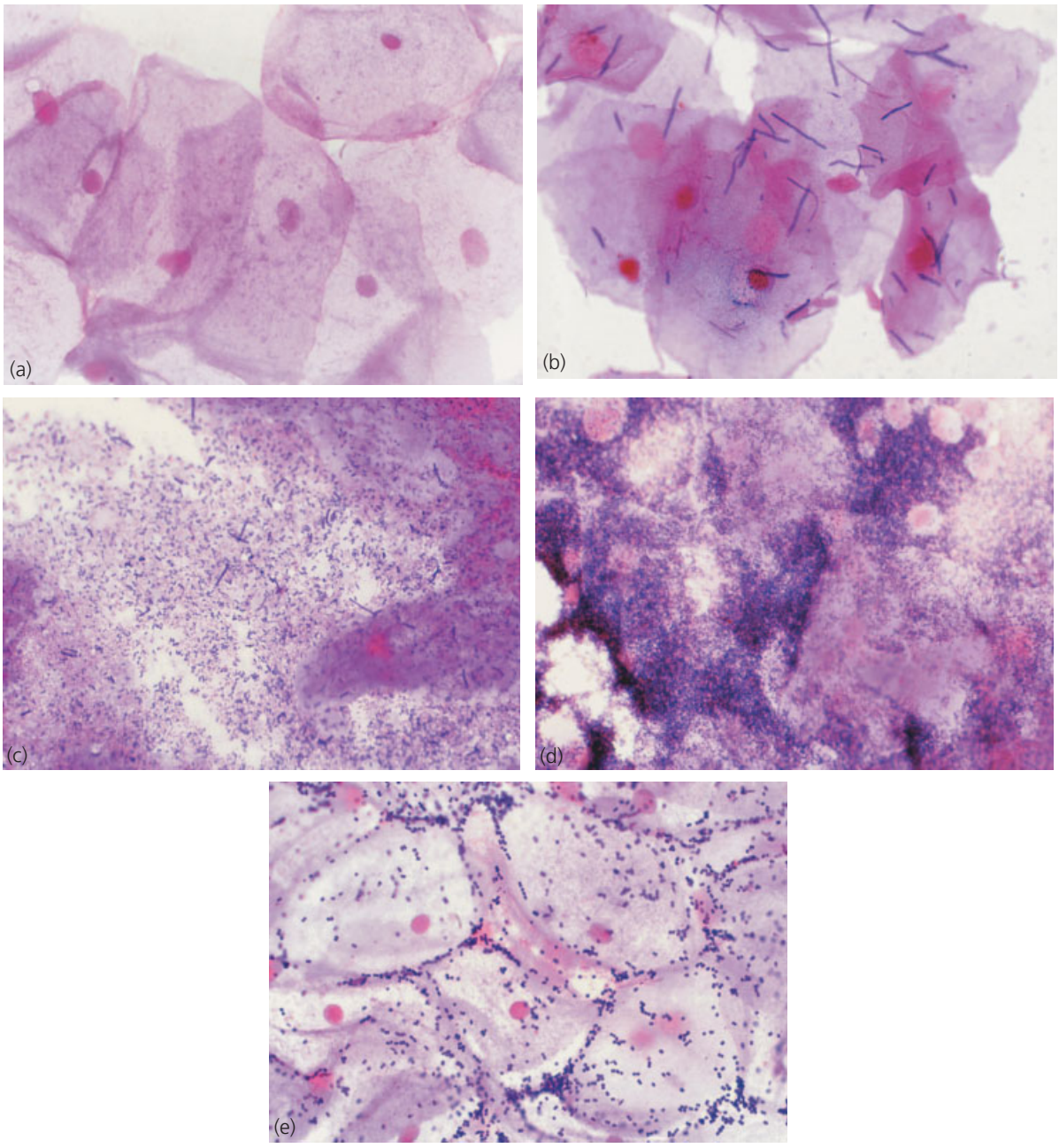

Figure 1 *Examples of grading for Gram stained vaginal smears,* **(a)** *Grade 0,* **(b)** *Grade I,* **(c)** *Grade II,* **(d)** *Grade III, and* **(e)** *Grade IV.*

offer a more acceptable alternative particularly for women with recurrent episodes or for pregnant women. Two agents are widely available, clindamycin 2 percent cream, which is applied twice a day for 7 days or a metronidazole gel, 0.75 percent. Clindamycin is active against lactobacilli and hence it takes a few days for them to re-establish in the vagina. Cure rates with topical treatment are similar to the oral treatment (Hay 1998). An attractive approach is the use of lactobacilli as a probiotic, and has been tried with H_2O_2 producing lactobacilli, but has not been proven to be successful (Hallen et al. 1992) and is not widely available. Treatment of male partners does not appear to improve relapse rates even in double-blind, placebo-controlled trials. Unfortunately, for many women, antimicrobial therapy only offers a short term resolution and recurrence is common with all treatments and probably reflects our ignorance at the underlying cause of BV.

Sequelae

For many years BV was considered a mild if unpleasant condition which had no sequelae and was often not taken seriously as a clinical entity. However, there is now strong evidence that BV is associated with complications in both pregnant and nonpregnant women. Women with BV who are pregnant have an increased risk of late miscarriage and preterm birth (Hay et al. 1994; Hillier et al. 1995), with women at greatest risk being those who have had a previous preterm birth. Complications, such as endometritis, are also more common among women with BV (Watts et al. 1990; Soper 1993; Persson et al. 1996).

STREPTOCOCCUS AGALACTIAE

S. agalactiae (group B streptococci) are known to colonize the vagina of some women, although the prevalence varies between 10–40 percent in different studies (Honig et al. 2002; Bayo et al. 2002). It has generally been regarded as part of the normal flora but its role in vaginitis is unclear. Group B streptococci have been isolated from women with symptoms and it has been suggested as a cause of aerobic vaginitis (Maniatis et al. 1996; Donder et al. 2002) with characteristics distinct from BV (Donder et al. 2002). Its importance lies in its ability to cause neonatal infection, particularly meningitis, which can be acquired from the mother during passage through the birth canal. Of neonates that are colonized, 1–3 percent develop disease caused by group B streptococci, mostly early-onset infection within 24 h of birth. Intervention of vaginal colonization during pregnancy has been found to be ineffective but introduction of guidelines for screening of vaginal and rectal swabs in 1996 at 35–37 weeks gestation followed by treatment of colonized women has reduced incidence (Schrag et al. 2002).

Isolation and identification

Group B streptococci grow easily on enriched culture medium such as blood agar, producing reasonably large colonies and a diffuse β-hemolysis. In women who are colonized, group B stretococci are often found in large numbers, but for screening purposes and for detection of small numbers in mixed cultures, selective media can be useful, blood agar with neomycin (30 mg/l) and nalidixic acid (15 mg/l), neomycin nalidixic acid (NNA) agar or colistin (10 mg/l) and nalidixic acid (10 mg/l), colistin nalixidic acid (CNA) agar. NNA has been reported to be more sensitive than CNA (Dunne 1999). Some studies favor prior incubation in enrichment broth, Todd–Hewitt broth supplemented with 15 mg/l nalidixic acid and 8 mg/l gentamicin, to achieve maximum identification of carriers (Dunne and Holland-Staley 1998; Votava et al. 2001). Detection of group B streptococci using rapid immunological methods such as latex agglutination have only proved useful for specimens from heavily colonized women. However, recent development of real-time PCR has proved a possible alternative to culture (Ke and Bergeron 2001).

Commercial kits, that have good quality control and rarely give false positives, are available for serogrouping β-hemolytic colonies. Other tests for identifying group B streptococci include inoculation of media containing starch and horse serum, which produce a distinctive orange pigment after incubation (Islam 1977), and the Christie–Atkins–Munch-Petersen (CAMP) test, where group B streptococci produce a diffusible extracellular protein (CAMP factor) that acts synergistically with staphylococcal beta-lysin to enhance lysis of red cells, and hydrolysis of sodium hippurate.

Treatment

Treatment of nonpregnant women colonized with group B streptococci who do not have symptoms is not considered necessary. Group B streptococci are not known to be resistant to penicillin and this remains the treatment of choice, if required. Prophylaxis with penicillin is recommended in women colonized when screened at 35–37 weeks gestation. Ampicillin can be used as an alternative (Centers for Disease Control and Prevention 2002b).

STAPHYLOCOCCUS AUREUS

Colonization of the vagina by *S. aureus* is relatively unusual but in menstruating young women, often associated with tampon use, has been linked to toxic shock syndrome. The syndrome presents as an acute febrile illness resulting in shock and organ failure and requires rapid diagnosis for successful treatment. It is caused by *S. aureus* that produce a number of toxins including

toxic-shock syndrome toxin-1 (TSST-1) which can be detected using radio-immunoassays, immunoassays or reverse passive latex agglutination tests.

OTHER BACTERIAL CAUSES OF GENITAL TRACT INFECTIONS

There are a few bacteria that cause infections of the genital tract that are encountered relatively rarely. *Listeria monocytogenes*, which can cause infection in pregnant women and lead to serious infection such as septicemia, may affect the fetus, resulting in stillbirth or neonatal meningitis. It is not routine practice to screen vaginal swabs for *L. monocytogenes* but blood cultures should be taken if clinically indicated. *Actinomyces* spp. are occasionally encountered associated with infections related to the presence of an intrauterine device and *Clostridium perfringens* has been a common cause of infection following delivery of gynecological surgery, which has largely been controlled by good surgical practice.

Mycoplasma spp. are commonly found in the genital tract, particularly *M. hominis*, and their role in infection has been debated. However, *M. hominis* is considered a cause of pelvic inflammatory disease and *M. genitalium* is associated with urethritis in men (see Mycoplasma).

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Bacterial infections of the urinary tract

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Urinary tract infections are amongst the most common infections encountered in clinical practice. About 50 percent of all women experience at least one episode of urinary tract infection during their lifetime. In women, the incidence of symptomatic infections increases with age. In men, urinary tract infections tend to occur more often in young boys or elderly persons and homosexuals. Symptomatic urinary tract infections are characterized by symptoms like dysuria, urgency, painful and frequent voiding, accompanied by the presence of bacteria in the normally sterile urine. Apart from symptomatic infections, there is a condition called asymptomatic bacteriuria that is characterized by the presence of bacteria in the urine but the lack of clinical symptoms. This condition may be a predisposition for symptomatic infection.

It is of utmost importance to distinguish between so-called uncomplicated urinary tract infections, which are characterized by the absence of functional or anatomical obstacles to urinary flow, and complicated urinary tract infections in which such obstacles are present, because patients with complicated urinary tract infections more often develop urosepsis and renal damage and bear a higher mortality. In addition, in complicated urinary tract infections the infecting organisms tend to be less susceptible to antimicrobial drugs.

Urinary tract infections encompass a wide array of clinical entities such as urethritis, cystitis, pyelonephritis, epididymitis, prostatitis, and perinephritic abscess. Most commonly, infections affect the bladder, urethra, and kidney.

ANATOMICAL CONSIDERATIONS

The epithelia of the urinary tract are bathed in a constant flow of urine originating in the kidneys, flowing via both ureters into the bladder where the fluid accumulates until it is voided at intervals via the urethra. Normally, only a minute residual volume of urine remains within the urinary tract after voiding. Anatomical or functional obstacles to urinary flow may cause an increased volume of urine to be retained within the urinary tract. If such residual volume does contain bacteria, the organisms may multiply, attain high bacterial densities, and ultimately, damage the epithelial surface causing an immune response and symptoms. The pressure in the bladder is increased during voiding and reflux into the ureters is normally avoided by the oblique insertion of the ureter into the bladder wall, providing an efficient valve mechanism. Reflux into the kidneys may cause renal scarring and malfunction. A number of conditions may lead to an increased residual urinary volume. These can be pathological conditions that impair voiding such as an enlarged prostate in men, urinary stones, or tumors, as well as neurological or functional conditions such as the neurogenic bladder present in spinal cord injury, multiple sclerosis or cerebrovascular accidents, or the atonic bladder of diabetics.

HOST DEFENSE MECHANISMS

The constant urinary flow and the low residual volume are major factors that counteract bacterial colonization.

In addition, the urine contains protective factors such as immunoglobulins that may interfere with bacterial colonization. Also high concentrations of urea are inhibitory to bacterial growth.

The kidneys produce and secrete a protein, the Tamm–Horsefall protein, which acts as a receptor for bacterial adhesins thus trapping the invading organisms before they reach their cellular targets (Leeker et al. 1997; Pak et al. 2001). It has also been shown that bacterial adhesion to uroepithelial cells causes cells to exfoliate and thus effectively expell the more invasive organisms (Kunin et al. 2002). In addition, adhesion to and invasion of urothelial cells triggers secretion of IL8 which is a signal for transmigration of leukocytes into the urinary tract (Frendéus et al. 2000).

It is known that the distal portion of the female urethra is colonized by bacteria. Colonization becomes less dense in the proximal portions. Several studies have shown that the slope of the curve representing the bacterial counts in different portions of the urethra is steeper in women who do not suffer from recurrent urinary tract infection (UTI) compared to those who do (reviewed in Kunin et al. 2002), suggesting that the urethra has an important function in preventing UTI. The epithelium of the urethra responds to hormonal influences like the vaginal epithelium.

An important defense mechanism is the secretion of blood group antigens that may function as receptors for bacterial attachment. It has been shown that women suffering from recurrent urinary tract infections more frequently do not secrete Lewis blood group antigens than controls (Sheinfeld et al. 1989; Raz et al. 2000).

CLINICAL SYNDROMES

Acute cystitis

Cystitis is the most common presentation of urinary tract infection. It is characterized by dysuria, increased frequency and urgency of voiding, suprapubic discomfort, and a cloudy urine. The condition is far more common in women than in men. In some women, cystitis may be associated with sexual activity (Remis et al. 1987), the use of contraceptive diaphragms (Latham et al. 1985), or spermicides. About one-third of women with cystitis experience a recurrence of the infection.

Urethral syndrome

A striking proportion of women (30–50 percent) who present with symptoms suggestive of a urinary tract infection do not have significant bacteriuria (O'Grady et al. 1970). In some patients the bladder urine obtained by suprapubic aspiration shows that some of these women have cystitis with a low bacterial count, but in the remainder the urine is sterile (Stamm et al. 1980). Various microbial and nonmicrobial etiologies have been suggested to be responsible for the syndrome in these patients. Since the first episodes of urethral syndrome and acute cystitis are virtually indistinguishable in clinical practice, antimicrobial therapy is recommended for both conditions (Baerheim et al. 1999).

Acute pyelonephritis

Patients with acute pyelonephritis often present with loin pain, fever, and a positive urine culture. The microbial spectrum is similar to that of acute cystitis. Acute pyelonephritis is a common complication in pregnant women with previously untreated asymptomatic bacteriuria. In patients with diabetes, upper urinary tract infection is common (Patterson and Andriole 1997) and severe complications such as necrotizing papillitis may occur.

Other infections

Renal abscess is a hematogenous infection most often caused by *S. aureus*. The lesion is localized in the renal cortex and urine culture may be negative even in the presence of pyuria. *Enterobacteria* only rarely cause renal infections via the hematogenous route.

Perinephric abscess does not involve the renal parenchyma, but it may complicate acute pyelonephritis, in which case the causative pathogen derives from the urinary tract.

Acute prostatitis is most often caused by the same spectrum of microorganisms that is resposible for acute cystitis. In chronic prostatitis, bacteria often cannot be isolated by conventional techniques, but the symptoms and the presence of leukocytes suggests infection. In a molecular study, Krieger et al. have found the DNA of fastidious and rarely isolated species in the prostatic fluid of such patients (Krieger et al. 1996). As other authors (Lee et al. 2003) did not find differences of bacterial colonization between men with and without prostatitis, it is unclear at present how the results of Krieger et al. should be interpreted.

Urinary tract infections in special hosts

Diabetics are reported to have a somewhat higher prevalence of asymptomatic bacteriuria. They may also suffer from a nerve dysfunction leading to a lower voiding frequency and an increased residual urinary volume (Patterson and Andriole 1997; Zhanel et al. 1991).

Urinary tract infections in solid organ transplant patients tend to occur more often during the first few months after transplantation, especially after renal transplantation (Muñoz 2001; Takai et al. 1998).

MICROBIOLOGY OF URINARY TRACT INFECTIONS

The vast majority of urinary tract infections is caused by a single organism from a very limited spectrum of bacteria. In uncomplicated cystitis, the organism is almost always *Escherichia coli* or *Staphylococcus saprophyticus*, whereas in complicated cases, the spectrum is more diverse, encompassing several Enterobacteriaceae and gram-positive microorganisms such as enterococci (Table 1). Polymicrobial infections occur virtually exclusively in complicated cases, especially in patients with chronic indwelling catheters. Pyelonephritis is most often caused by *E. coli*, but *Proteus mirabilis* may also be encountered.

Many other species are infrequently or only rarely found (Bottone et al. 1998; Bézian et al. 1990; Cappelli et al. 1999; Fraimow et al. 1994; Funke et al. 1998; Janda et al. 1993; Morgan and Hamilton-Miller 1990; Salahuddin et al. 1996; Snehalatha et al. 1992; Stewart et al. 1993; Sturm 1989; Wong and Yuen 1995). If an infection with an unusual organism is suspected, etiology can be confirmed by repeated culture and/or suprapubic aspiration. However, as most microbiologists only expect the usual bacteria, fastidious or difficult-to-identify bacteria may be overlooked.

In nosocomial infections, gram-positive cocci, especially enterococci are the second most commonly isolated species (Table 1). It is not always clear whether an isolate represents a true pathogen or is merely contamination with an organism selected by chemotherapy. However, urinary tract infections with enterococci are a predisposition for the development of enterococcal bacteremia (Maki and Agger 1988).

PATHOGENESIS

Most often the infecting microorganisms ascend via the urethra into the bladder. Of course, their invasion may be facilitated by the presence of devices such as urinary catheters. Before the bacteria ascend they usually colonize the periurethral area and the vaginal introitus (Bollgren and Winberg 1976b). In addition, rectal colonization is often present during infection episodes. Bacteria that cause infections in otherwise healthy women express distinct virulence properties enabling them to establish an infection, whereas bacterial strains that cause infections in patients with complicating factors do not need to possess the same armamentarium of virulence factors (Dalet et al. 1991; Hull et al. 2002; Sandberg et al. 1988). Although the presence of special virulence factors is often accepted, this theory is not unchallenged. Selection of the infecting strain may just be a consequence of the heavy colonization by the pathogen. However, in recurrent asymptomatic bacteriuria the isolated strains appear to be a random sample of the fecal flora, whereas infecting strains do not appear to be such random samples (Lidin-Janson et al. 1977).

Adhesion

It is commonly accepted that the adherence of *E. coli* to uroepithelium is crucial to counteract the natural clearance of the urinary tract and to allow its colonization (Reid and Sobel 1987; Dalet et al. 1991; Tullus et al. 1991). Two classes of adhesins can be found on the surface of *E. coli*: fimbrial and nonfimbrial adhesins. While fimbrial adhesins carry ligands on their tips that bind to receptors present on cells, the structure and binding domains of nonfimbrial adhesins are less clear. Traditionally, *E. coli* adhesins are grouped according to the specificity of the hemagglutination they cause. Type 1 fimbriae, which are present on many Enterobacteriaceae, are inhibited by D-mannose and various mannosides. Hemagglutination by mannose-resistant fimbriae is not inhibited by D-mannose or mannosides and their receptors contain various carbohydrates.

The best studied of these are P fimbriae that bind to neutral globoseries glycolipids, including globote-

Table 1 *Percentage of bacterial species in urinary samples obtained in different clinical situations*

Species	Community acquired[a]	Nosocomial[b]	Catheter associated[c]	Own data nosocomial[d]
E. coli	69–80	36	30–35	45
Other Enterobacteria[e]	6–26	15	26–30	16
S. saprophyticus	2.3–15			
Enterococci[f]		15–23	12–22	21
Staphylococci[g]		11	8–12	6
Pseudomonas[h]		7–12	11	6

a) Kahlmeter, 2000; Ronald, 2002; uncomplicated urinary tract infections in women.
b) Wagenlehner et al. 2002; Bouza et al. 2002.
c) Wazait et al. 2003.
d) Data from inpatients, 2002 (author's observation).
e) Mainly *Proteus mirabilis*, *Klebsiella* spp., *Enterobacter* spp.
f) Mainly *E. faecalis*.
g) Not *S. saprophyticus*.
h) Mainly *P. aeruginosa*.

traosylceramide and trihexosylceramide, which are antigens of the P blood group system (Leffler and Svanborg-Eden 1981). The minimal receptor to which these fimbriae bind is α-D-Gal-(1→4)-β-D-Gal (Gal-Gal) (Bock et al. 1985; Källenius et al. 1981).

P fimbriae, like many other fimbriae, are synthesized from a polycistronic operon that codes for the structural proteins as well as for chaperone/usher proteins required for assembly of the final pilus. Figure 1 shows a model of the pilus structure. The pilus is anchored into the outer membrane by a protein that functions as an usher during pilus synthesis. The helical shaft is composed of the major structural subunit. The end of the pilus is fibrillar shaped and carries the adhesin (for detailed information see Mühldorfer et al. 2002; Saulino et al. 2000; Soto et al. 1998).

Donnenberg and Welch (1996)) used pooled data from many studies to show that P fimbriae are more often expressed by *E. coli* isolated from pyelonephritis (80.8 percent) than by strains from the fecal flora (relative risk of expression of P fimbriae in pyelonephritis, 6.1). In strains isolated from pyelonephritis complicated by bacteremia, the proportion of P frimbriated strains is even higher (88.9 percent). The prevalence of P-fimbriate *E. coli* in lower urinary tract infections is lower (31.1 percent), but these isolates still produce P fimbriae more often than fecal isolates (relative risk 1.98).

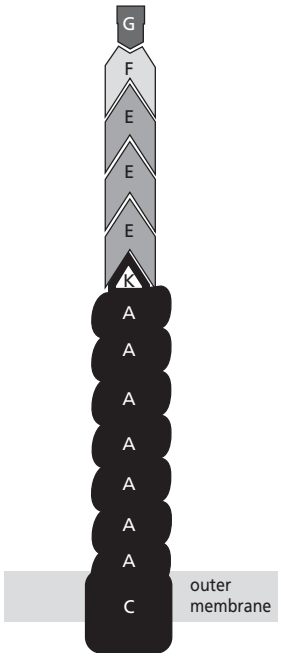

Figure 1 *General structure of* E. coli *fimbriae. The P pilus of* E. coli *consists of a helical shaft that is made up of PapA and a fibrillar structure projecting outwards that carries the adhesin, PapG, at its end. The diverse subunits are assembled by the major usher PapC that also anchors the pilus in the outer membrane. Between the shaft and the fibrillum and between the fibrillum and the adhesin there are adaptors, PapK and PapF. Synthesis of the shaft is terminated by PapH, which is buried in the channel formed by PapC (Soto et al. 1998). Assembly of type 1 pili is similar (Saulino et al. 2000).*

Immunuofluorescence with antibody to P fimbriae was used to show expression of these appendages by *E. coli* in vivo (Pere et al. 1987). In addition, patients with pyelonephritis caused by P-fimbriate *E. coli* mount an antibody response toward these antigens, pointing in the same direction.

Experimental evidence for the importance of P fimbriae comes from in vitro studies showing binding of fimbriated *E. coli* to tissue from human kidney, but not to the bladder epithelium (Donnenberg and Welch 1996) and from experimental infections using suspensions of P-fimbriate bacteria suspended in a solution of a receptor analogue, globotriosylceramide, to show reduced recovery of the infecting organism. In contrast, studies employing isogenic mutants did not always give unequivocal results. Mobley et al. (1993) constructed a mutant that lacked fimbrial components crucial to binding but could not show reduced pathogenicity in experimental infections of mice. Another group used a mutant that produced a truncated adhesin to infect monkeys. The parent and the mutant both established infections but the strain expressing P fimbriae persisted significantly longer (Winberg et al. 1995). These conflicting results may be explained by the presence of additional virulence factors in the strain used by Mobley's group.

Recently, Wullt et al. (2001) used a nonfimbriate clinical isolate and a transformant of the same strain expressing P fimbriae to infect volunteers. Bacteria expressing P fimbriae required lower inocula to reach significant densities within the urine and persisted longer than the wild-type strain. This experiment can be taken as a fulfillment of the molecular Koch's postulates (Falkow 1989) for P fimbriae.

The F1C fimbriae are one additional type of fimbria correlated with uropathogenicity. They interact with two minor glycosphingolipids, galactosylceramide and globotriaosylceramide, isolated from the rat, canine, and human urinary tract (Bäckhed et al. 2002). The authors also showed that binding of F1C-fimbriated *Escherichia coli* to renal cells induced interleukin-8 production, thus suggesting a role for F1C-mediated attachment in mucosal defense against bacterial infections. Strains producing F1C fimbriae are also more often isolated from patients with urinary tract infections than from fecal samples (22.3 percent in pyelonephritis, 13.9 percent in cystitis, and 6.2 percent in fecal samples). This indirect evidence of a role in pathogenesis is corroborated by an experimental infection showing that a mutant devoid of F1C fimbriae colonized the kidneys of rats in lower numbers than the mutant; however, reintroduction of a functional gene did not fully restore virulence (Marre et al. 1986).

Several other fimbrial and nonfimbrial adhesins (e.g. Dr, X, M, G) that confer mannose-resistant hemaggluti-

nation have been described in pathogenic *E. coli*. Association of these adhesins with disease or pathogenicity has not been shown conclusively although adhesins of the Dr series cause internalization of *E. coli* and thus may contribute to persistence of the organism (Goluszko et al. 1997).

Type I fimbriae cause mannose-sensitive hemagglutination. Although they are ubiquitious and can be found in many enterobacterial species and are present in most strains of *E. coli*, they are more often (odds ratio, 1.22) expressed by isolates from cystitis (64.1 percent) than by control isolates. In contrast, there is no such predilection in pyelonephritis (Donnenberg and Welch 1996). Type I fimbriae are known to undergo phase variation that is due to an invertible promoter (Eisenstein 1981; Abraham et al. 1985). Production of fimbriae is selected for by culture in liquid media as opposed to agar. When bacteria were grown under conditions favoring expression of type I fimbriae, the strains colonized the bladder of infected animals in higher numbers than phase variants not expressing fimbriae (Hultgren et al. 1985). Organisms colonizing the bladder were usually fimbriated while those recovered from the kidneys lost their pilation. When a mixture of mutants that expressed either phase constitutively was used to infect animals, the fimbriated strains were recovered in higher numbers than their nonfimbriated counterparts, especially during the early phase of the infection (Gunther et al. 2002). Type I fimbriae bind to uroplakins, major glycoproteins of the urothelial surface (Wu et al. 1996; Zhou et al. 2002). More recently, a study showed that *E. coli* expressing type I fimbriae persist longer and cause more severe symptoms than their nonfimbriated counterparts (Connell et al. 1996). These experiments included epidemiological data, as well as experimental infections. In addition, signature-tagged mutagenesis identified type I fimbriae amongst the most important virulence factors for urinary tract infections (Bahrani-Mougeot et al. 2002). In keeping with these results are observations that type I fimbriae mediate invasion of bladder epithelial cells by *E. coli* facilitating persistence and recurrent infections (Mulvey et al. 2000, 2001; Mulvey 2002). Once within the urothelial cell the bacteria may form intracellular biofilm-like pods in which they are embedded in a polysaccharide (Anderson et al. 2003). Accordingly, recurrent urinary tract infections in infancy seem to be caused by the same persisting strain (Jantunen et al. 2002).

In other uropathogenic bacteria, surface factors necessary for adherence have also been identified. *Proteus mirabilis* produces several fimbriae, some of which have a role in experimental urinary tract infections (Mobley 1996). For *Staphylococcus saprophyticus* a hemagglutinin that recognizes a protein receptor on the erythrocyte has been identified (Gatermann et al. 1992; Meyer et al. 1997). This adhesin has multiple functions as it is also the major autolysin of the species (Hell et al. 1998). It is also responsible for the attachment to uroepithelial cells (Meyer et al. 1996). Enterococci express an aggregation substance that contains an RGDS and an RGDV motif and thus binds to cells expressing appropriate integrins, which are receptors for these motifs (for a review see Gatermann 1996).

Hemolysin

It is a long standing observation that *E. coli* isolated from urinary tract infections more often show hemolysis on blood agar than strains from other infections. Analysis of pooled data from several epidemiological studies showed a higher prevalence of hemolysin-producing strains in pyelonephritis or cystitis than in feces. In addition, two studies used genetically manipulated strains to show that the hemolysin contributes to pathogenicity (Marre et al. 1986; O'Hanley et al. 1991). Recently, it was shown that expression of the hemolysin may contribute to renal failure (Kreft and Pagel 2000).

Cytotoxic necrotizing factor 1

A proportion of 40 percent of urinary isolates of *E. coli* produce this apoptosis-inducing cytotoxin (Caprioli et al. 1987). In experiments by Rippere-Lampe et al. (2001a, b), a isogenic mutant lacking expression of cytotoxic necrotizing factor 1 (CNF1) was less pathogenic in a mouse model than the parent strain. These findings were in contrast to experiments by Johnson et al. (2000). Thus, the function of this toxin remains controversial.

Iron siderophores

Iron is needed by almost all bacteria and free iron is present in exceedingly low concentrations in human serum and tissue. Aerobactin, a siderophore, is more often produced by *E. coli* causing infections, including urinary tract infections, than in fecal isolates (Carbonetti et al. 1986). Recently, Torres et al. (2001) showed that siderophores and TonB, a protein that facilitates transport of iron through the cytoplasmic membrane, are required in experimental infections.

Urease

As early as 1960 it was suggested that bacteria producing urease cause more severe urinary tract infections than urease negative bacteria (Braude and Siemienski 1960). Most studies used urease negative mutants generated by chemical mutagenesis or urease inhibitors to show a contribution of the enzyme to virulence (Griffith and Musher 1975; Griffith et al. 1976; Musher et al. 1975). In 1989, Mobley's group (Jones et al. 1990) as well as that of Gatermann (Gatermann and Marre 1989) used genetically manipulated strains of *Proteus mirabilis*

and *Staphylococcus saprophyticus*, respectively, to show that urease positive strains caused more severe damage to the urinary tract than their urease-negative derivatives. Urease is thought to act by increasing the urinary pH and the concentration of ammonium ions which may lead to precipitation of struvite ($MgNH_4PO_4$) or carbonate-apatite ($Ca_{10}(PO_4)_6 \cdot CO_3$) (Griffith et al. 1976; Johnson et al. 1993). In addition, urease may increase adhesion of bacteria to the urothelial cells (Parsons et al. 1984).

Organization of virulence factors

It has been shown in uropathogenic *E. coli* that virulence factors such as fimbriae and hemolysin are clustered in large segments of DNA, termed pathogenicity islands (Knapp et al. 1986). A pathogen may contain one or more of these islands and a crosstalk between genes encoded by the pathogenicity islands and chromosomal loci has been described (Morschhäuser et al. 1994).

THE PHYSIOLOGICAL FLORA OF THE URINARY TRACT

Normally, the bladder urine is sterile. In adults the distal urethra is colonized by a variety of microorganisms, including coagulase-negative staphylococci (excluding *S. saprophyticus*), viridans streptococci, diphtheroids, nonpathogenic *Neisseria* spp., anaerobic cocci, and lactobacilli. Commensal mycobacteria and mycoplasmas may also be present. In the postpartal period the flora consists predominantly of gram-negative bacilli, esp. *E. coli* (Bollgren and Winberg 1976a). Thereafter, in girls, the flora is replaced by anaerobic bacilli (Bollgren and Winberg 1976b; Bollgren et al. 1979).

Studies have shown that periurethral colonization is a prerequisite for the development of urinary tract infections (Bollgren and Winberg 1976b).

DIAGNOSIS

Sample collection

SUPRAPUBIC ASPIRATION

This technique is considered the diagnostic gold standard. Any species in any number is considered significant in urine obtained by suprapubic aspiration.

MIDSTREAM CLEAN-VOIDED SPECIMEN

As the distal urethra, the periurethral area, and the skin may be colonized by potentially pathogenic bacteria it is of paramount importance to avoid any contamination of the specimen by these bacteria. The most commonly used technique, the midstream urine, requires that the first 10–30 ml of the voided urine be discarded and the second, midstream portion be sampled. The first portion washes away nonadherent colonizing bacteria and the second portion mostly reflects the bacterial colonization of the bladder urine.

In the female patient, adequate periurethral cleansing is necessary to reduce the probability of contamination. For cleaning, water or a soap solution without antibacterial activity should be used as residues of antibacterial soap or disinfectants may impair culture results.

In men, the foreskin should be retracted and the external meatus should be cleansed. There has been some debate whether cleansing is really necessary (Lipsky et al. 1984); however, as it is easily done and decreases the number of contaminated specimens, some authorities recommend this procedure (Schaeffer 1994).

STRAIGHT CATHETERIZATION

Although this procedure yields specimens of a quality similar to that of suprapubic aspiration, it bears the risk of introduction of bacteria into the bladder and bacteriuria after straight catheterization is not uncommon (Eisenstadt and Washington 1996). The technique may be considered if correct clean-voided specimens cannot be obtained and suprapubic aspiration is contraindicated or not feasible.

Specimen transport

Urine should be cultured within 2–4 h. If longer delays cannot be avoided the specimen should be refrigerated to preserve the bacterial density. Transport devices containing boric acid as a preservative have been developed to allow unrefrigerated transport of specimes. However, culture results after prolonged storage of specimens may be altered significantly (Gillespie et al. 1999).

Culture

Bacteriuria is defined as the presence of bacteria within the urinary tract. The central problem in interpreting bacteriuria by the examination of voided urine is to distinguish between contamination originating from the physiological flora and bacteriuria that originates in the normally sterile parts of the urinary tract. Kass (1957) observed that some healthy women have bacteriuria, but the number of colony-forming units (cfu) in their urine was considerably lower than that in urine collected from women with pyelonephritis. In pyelonephritis the urine of 95 percent of patients contained $>10^5$ bacteria per ml, whereas this density was only rarely reached in asymptomatic women (6 percent). The number of 100 000 organisms per ml in a carefully collected sample of clean-voided or midstream urine has since then become the threshold for ‘significant’ bacteriuria.

Later studies have shown, however, that this threshold may not be applicable to cases of uncomplicated cystitis (Stamm 1988), or posterior urethritis (Stamm et al. 1980; Latham et al. 1985). In these infections colony counts as low as 10^2/ml have been found. Therefore colony counts of $<10^5$/ml may be significant when the clinical diagnosis of uncomplicated cystitis has been established, in cases of complicated urinary tract infections colony counts are usually $>10^5$/ml (Naber et al. 2002). However, cases of uncomplicated cystitis are usually treated empirically, without taking urinary cultures (Warren et al. 1999; Le and Miller 2001).

Consequently, many guidelines for microbiological diagnostic workup of clean-voided urine require a species (or isolate) specific enumeration to distinguish contamination from colonization (Clarridge et al. 1987; Gatermann et al. 1997; Kouri et al. 2000; Naber et al. 2002). The diagnostic interpretation is made relative to the clinical diagnosis or situation. In complicated urinary tract infections the number of 10^5 colony-forming units per ml is considered significant, whereas in clearly uncomplicated cases the threshold is 10^2. Specimens from indwelling catheters may present a special situation since colonization with multiple organisms even in high densities is common (Warren et al. 1982).

The diagnosis of urinary tract infections thus requires a method that is capable of simultaneously determining the colony-forming units of all viable bacterial species present in the sample. Traditionally, this has been done by the pour plate method, where a sample of the urine is mixed with a fixed volume of nutritient agar and plates are poured. The number of bacteria per volume can be determined by multiplying the number of colonies with the dilution factor of the urine. Although this method is most precise, it is also cumbersome and time-consuming. Many laboratories, therefore, use the surface streak technique where a calibrated loop is dipped into the urine and the volume held by the loop is applied to the surface of the appropriate agar media. Commercial single-use loops hold 1 or 10 µl of urine, thus the number of cfu per ml is calculated after incubation by multiplying the number of colonies by 1 000 if a 1-µl loop was used or by 100 if a 10-µl loop was used. There is some evidence that 1-µl loops lack precision and underestimate the true number of bacteria in the sample (Frimodt-Møller and Espersen 2001). Most often, media that permit growth of gram-positive bacteria, including β-hemolytic streptococci, e.g. sheep blood agar, and selective media for Enterobacteriaceae, e.g. MacConkey agar, are inoculated. Cystine lactose electrolyte-deficient agar may also be used, although some gram-positive species grow less well than on blood agar.

Simple, semiquantitative culture methods, such as the filter paper method (Kunin and Buesching 2000) and dipslide cultures have also been developed. These techniques are useful as screening techniques but lack sensitivity for fastidious organisms (Eisenstadt and Washington 1996). In addition, technical problems, such as the detachment of the agar from the paddle or dried out agar in dipslide cultures, can occur.

Due to the limited spectrum of bacteria that cause urinary tract infections identification of causative species can usually be done with a reduced set of tests (Baron 2001). However, care must be exercised not to overlook the unusual or rarely encountered species (Damron et al. 1986). Corynebacteria and some gram-positive cocci may require prolonged incubation and other species such as *Haemophilius* or *Gardnerella* or even a fastidious enterobacterial strain may not grow on the routinely used media. It is therefore advisable to be suspicious, especially in cases where UTI is clinically suspected, pyuria is present, but routine cultures are negative. In such cases an infection with mycobacteria, mycoplasmas, or chlamydia should be ruled out and the unusual should be sought.

Some authors recommend testing of urine samples for the presence of antimicrobial substances (Ansorg et al. 1975; Abu Shaqra 2001) which may mask the presence of bacteria in the urine. To this end, a small volume of urine is placed on an agar plate inoculated with an indicator bacterium (e.g. *Bacillus subtilis* or *Staphylococcus aureus*) and any inhibition of bacterial growth is taken as evidence for the presence of antibacterial substances.

Extrapulmonary tuberculosis may affect the kidneys, prostate, epidymis, or testes. Mycobacterial infections of the kidneys often present with pyuria, but fast-growing bacteria cannot be isolated from the urine. Clinical symptoms suggestive of cystitis may be present and the intravenous pyelogram is usually abnormal (El Khader et al. 1997). Diagnosis is based on cultural detection of *Mycobacterium tuberculosis* in urine, if renal tuberculosis or in ejaculate, if epididymitis is suspected.

Infections with mycoplasmas and chlamydia usually affect the urethra but symptoms may suggest cystitis. As expected, bacterial densities are highest in the first portion of a voided urine and suprapubic aspiration is usually negative. Sensitive and specific tests, molecular genetic amplication techniques, have been described that detect chlamydia in genital specimens (Van Der Pol et al. 2001; Vincelette et al. 1999). Mycoplasmas may be grown from urethral samples although a swab offers higher sensitivities than urine cultures. In addition, *M. hominis* is part of the normal flora of the urethra.

In many countries, standardized protocols for the culture and interpretation of urine samples have been developed (Clarridge et al. 1987; Gatermann et al. 1997; Kouri et al. 2000).

Deprecated procedures and unacceptable specimens

Midstream urine or urine obtained via a catheter should not be cultured anaerobically and should not be

inoculated into enrichment broths. Urine should not be centrifuged to culture the sediment.

Foley catheter tips, urine inoculated into broth media, urine sediment, native urine that has been stored unrefrigerated for more than 2 h, or dried dip slides are unacceptable for culture.

Interpretation of urine cultures

For a meaningful interpretation of culture results the clinical situation (complicated, uncomplicated UTI, asymptomatic patient), the patient's sex and the type of the specimen (clean-voided, catheter, suprapubic aspiration) must be available. Also, the number of different bacterial species and their respective number of colony-forming units must be known. Interpretation guidelines aggregate these pieces of information into reasonable laboratory interpretations of culture results and give suggestions for further diagnostic workup, e.g. identification and susceptibility testing. Generally, these guidelines consider pure cultures of typical urinary pathogens to be significant if present in $\geqslant 10^4$ cfu per ml. In acute, uncomplicated cystitis the thresholds are lower, e.g. 10^3 per ml, whereas in complicated infections they may be higher (10^5 per ml) (Clarridge et al. 1987; Gatermann et al. 1997; Kouri et al. 2000; Naber et al. 2002). Repeated isolation of the same microorganism increases the specificity of the result and is absolutely necessary if asymptomatic bacteriuria is to be detected (Pattaragarn and Alon 2003).

In patients with chronic indwelling catheters, cultures often yield more than one potential pathogen regardless of patients' symptoms (Warren et al. 1982). These specimens therefore reflect colonization rather than infection. It is rarely necessary to perform urinary cultures in asymptomatic patients with indwelling catheters because high colony counts may reflect infection or colonization and low bacterial densities usually increase to high ($>10^5$) levels within a few days (Stark and Maki 1984). In symptomatic patients with flank pain or fever, a diagnostic workup of all organisms may be indicated (Gatermann et al. 1997).

Candida spp. is often isolated from the urine of catheterized patients who receive antibiotic treatment and antibiotic therapy is the most important risk factor for candiduria (Lundstrom and Sobel 2001; Weinberger et al. 2003). However, since candiduria may also be a symptom of deep-seated candidal infection, patients should be carefully evaluated to exclude such condition (Fisher et al. 1995).

MANAGEMENT OF URINARY TRACT INFECTIONS

A plethora of literature exists concering the treatment of urinary tract infections. Most current guidelines recommend either trimethoprim/sulfmethoxazol or a fluoroquinolone for empiric therapy of uncomplicated cystitis. These antibiotics are usually given for 3 days. β-lactam antibiotics are less satisfactory, because recurrences are more frequent (Johnson and Stamm 1989) and longer therapies may be needed. During pregnancy trimethoprim/sulfmethoxazol or quinolones cannot be used and a susceptible β-lactam antibiotic is the therapy of choice (Naber et al. 2002). In uncomplicated cystitis in nonpregnant women, the narrow bacterial spectrum permits empiric therapy without the aid of a urine culture. In pregnant women a urine culture may be needed to select the most appropriate agent (Naber et al. 2002).

In patients with acute uncomplicated pyelonephritis, oral therapy can be an alternative to the classical intravenous regimens (Warren et al. 1999; Naber et al. 2002). As with uncomplicated cystitis, a fluoroquinolone, or, if susceptibility has been established or is likely, trimethoprim/sulfmethoxazole is usually appropriate. However, a urine culture should be obtained to guide therapy in poorly responding individuals.

Although most standards recommend the use of trimethoprim/sulfamethoxazole or fluoroquinolones as a first-line treatment (Johnson and Stamm 1989; Warren et al. 1999), the most appropriate therapy is contingent on the local situation, because susceptibility towards antimicrobials may vary widely between geographic regions. For trimethoprim/sulfamethoxazole resistance rates for *E. coli* as high as 45 percent have been described in some regions (Mazzulli 2002; Farrell et al. 2003; Gales et al. 2003) and it has been recommended that fluoroquinolones be used if resistance rates to trimethoprim/sulfamethoxazole exceed 20 percent (Le and Miller 2001). However, resistance to fluoroquinolones is also emerging and resistance rates of 10 percent in *E. coli* are common (Mazzulli 2002; Farrell et al. 2003), and up to 20 percent have been described (Gales et al. 2003).

In complicated urinary tract infections treatment should be based on the results of a urinary culture, because the spectrum of causative organisms is much larger and resistance to antimicrobial agents is frequent (Naber et al. 2002).

Asymptomatic bacteriuria should be sought for and treated in pregnant women (Smaill 2002), because it is a risk factor for pyelonephritis, low birth weight, and early delivery. In contrast, screening for and treatment of asymptomatic bacteriuria in diabetic women (Harding et al. 2002) and patients with renal transplants is probably not indicated (Muñoz 2001).

Prophylaxis

Given the fact that most patients experience only one UTI in their lifetimes, prophylactic measures are needed only for women suffering frequent recurrences. If it has

been established in an individual that episodes of acute UTI frequently occur after sexual intercourse, a prophylactic dose of an antibiotic may be (Stapleton and Stamm 1997) indicated. In some women, for periods of clustering of UTIs a regular dose of an antibiotic may suppress infections. Currently there is no evidence that cranberry juice reduces the frequency of urinary tract infections (Jepson et al. 2002).

There have been many attempts at developing vaccines against UTI with *E. coli* and *P. mirabilis*. Whole cell killed vaccines or vaccines based on single virulence factors have been used in animal models to show that the animals were protected against challanges with strains expressing the respective virulence factor (Langermann et al. 2000; Li and Mobley 2002; Uehling et al. 1994a, b, 1997). Since many different virulence factors contribute to pathogenicity it is unlikely that one single factor will be sufficient to offer protection.

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Bacterial infections of bones and joints

ANTHONY R. BERENDT

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INTRODUCTION

Bone and joint infections pose a number of challenges to the academic and the clinical microbiologist. From the academic standpoint the pathogenesis of musculoskeletal infection, and the pathophysiology of the osteomyelitic lesion or the infected prosthetic joint, remain incompletely understood (Mandal et al. 2002). So too do the molecular mechanisms of infection-related bone destruction (Josefsson and Tarkowski 1999; Nair et al. 2000; Tarkowski et al. 2002) or bacterial persistence in the face of host defenses and antibiotic use.

From the clinical microbiology perspective, there can be significant difficulties to be overcome in the interpretation of microbial cultures, notably because of the role of skin commensals in device-related infection. Additional problems are generated by those clinical scenarios where bone, infected as a consequence of soft tissue loss, is then sampled through the resulting soft tissue defect.

Having isolated bacteria and formed a view about their relevance, the clinical microbiologist is frequently challenged when feeding that information into a management plan. This is partly because of a relative lack of a strong evidence base for recommendations about durations of treatment or the mode of delivery of antimicrobials. It is also because of the strongly surgical nature of the specialty. The microbiologist must exercise skills in multidisciplinary working and to be credible, must demonstrate an understanding not only of his own field, but also of the major orthopedic imperatives and drivers.

Given the very significant morbidity caused by bone and joint infection, and the substantial health gains for patients who are treated successfully, there are major benefits for patients and clinicians (of all disciplines) when the clinical microbiologist can participate in, and if need be lead, effective multidisciplinary working for the management of these problems (Ziran et al. 2003).

CLINICAL MANIFESTATIONS

Musculoskeletal infection, affecting bone and joint, may be acute or chronic. Any part of the skeleton may be affected, and bacteria may reach the tissues of the skeleton through hematogenous or contiguous spread. The range of manifestations most commonly seen is outlined in Table 1.

EPIDEMIOLOGY

The epidemiology of musculoskeletal infection is a reflection of the prevalence, in the population, of underlying risk factors for bacteremia, trauma, and instrumentation on the one hand, and the geographical distribution of pathogens on the other.

In industrialized societies, in which antibiotics are relatively available, child health is good, and orthopedic

Table 1 *Commonly seen range of manifestations*

Manifestation	Site of infection	Presentation	Soft tissue component	Causative pathogen(s)
Septic arthritis	Native	Acute	–	MSSA, BHS, HIB, GC
	Prosthetic		+	MRSA, MSSA, CoNS GNR
	Native	Chronic	–	MTB, Lyme, MSSA
	Prosthetic		++	CoNS, SA, BHS, EC, GNR
Osteomyelitis	Hematogenous	Acute	–	MSSA, BHS, HIB, *Salmonella*
	Contiguous focus		+	MSSA, MRSA, GNR
	Hematogenous	Chronic	++	MSSA, BHS, GNR
	Contiguous focus		+++	MSSA, MRSA, CoNS, BHS, GNR, EC
Vertebral osteomyelitis and discitis	Hematogenous	Chronic	–	MSSA, GNR, MRSA
	Contiguous focus (post surgical)	Acute	–	MRSA, MSSA, CoNS
Fracture-fixation infection	Contiguous focus	Acute	–/+	MSSA, MRSA, GNR
		Chronic	++	MSSA, MRSA, CoNS, GNR
Osteomyelitis 2° to pressure sore including diabetic foot ulcer		Chronic	+++	MSSA, MRSA, CoNS, BHS, GNR, AnO2, EC

ANO2, anaerobic rods; BHS, β-hemolytic streptococci; CONS, coagulase-negative staphylococci; EC, *E. coli*; GC, gonococci; GNR, gram-negative rods; HiB, *H. influenzae* type b; Lyme, Lyme borreliosis; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; MTB, *Mycobacterium tuberculosis*; SA, *S. aureus*.

management includes energetic trauma management and joint replacement, there has been a decline in 'classical' presentations. Advances in fracture management mean that infections of fracture fixations have replaced infections of open fractures (Gustilo and Anderson 1976); childhood hematogenous bone and joint infection is in decline and more frequently presents in a subacute manner (Gonzalez-Lopez et al. 2001; Blyth et al. 2001; Rasool 2001); hematogenous infections of the spine in the elderly are increasing in incidence (Jensen et al. 1997; Chelsom and Solberg 1998; Krogsgaard et al. 1998), as is osteomyelitis secondary to pressure sores (Sugarman 1987). Infections of prosthetic joints are now the most formidable manifestation of septic arthritis (Malchau et al. 1993), a role occupied by tuberculosis some 50 years ago, and by *Staphylococcus aureus* in the pre-antibiotic era.

Much of this remains a contrast to less developed areas of the world, where higher incidences of soft tissue infection, and less universal access to healthcare, perhaps explain the higher recorded incidences of conditions such as acute septic arthritis and osteomyelitis (Gillespie 1990). Limited healthcare resources may restrict access to joint replacement or complex orthopedic instrumentation, but correspondingly make all the more serious the impact of infection on traumatic wounds. Combat and landmine injuries from military conflict are important in this regard, as are injuries from increasing numbers of industrial and road traffic accidents, though systematic data on this are hard to find. As a result, chronic osteomyelitis remains a considerable problem (Museru and Mcharo 2001).

Certain manifestations appear to have a truly worldwide importance. The global epidemic of Type 2 (non-insulin dependent) diabetes is responsible for a dramatic increase in problems due to neuropathic foot ulceration, including osteomyelitis (Lipsky et al. 1990). Because patients with this disease survive for relatively long periods without expensive treatments, developing countries share with the industrialized nations a rising prevalence of diabetics with neuropathy and secondary foot ulceration, frequently leading to osteomyelitis (Abbas et al. 2002).

From a microbiological perspective, although certain pathogens such as *Staphylococcus aureus* are universally important, others are restricted by geographical or occasionally socioeconomic factors. These include the predominant distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) within that group of society in frequent contact with healthcare institutions and nursing homes (Blanc et al. 2002); and the greater prevalence of infections due to *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Brucellosis* spp. in tropical countries (McGill 2003).

Table 2 summarizes available epidemiological data on incidences, prevalence, and risk factors of the various manifestations of musculoskeletal infection.

PATHOGENESIS

The pathogenesis of bone and joint infection, as understood to date, is a multistep process (Mandal et al. 2002). Organisms gain access to musculoskeletal structures and proliferate. This may occur in both planktonic

Table 2 *Epidemiology of musculoskeletal infection*

Manifestation	Incidence	Prevalence	Risk factors
Acute septic arthritis	10–100/100 000/yr	N/A	In Australasia, indigenous race (Gillespie 1990)
Chronic septic arthritis	N/A	Unknown	Delayed diagnosis or inadequate treatment of acute septic arthritis
Acute prosthetic joint infection	1–2% of joint replacements	N/A	Prior joint surgery; adverse wound score, ASA status or prolonged surgery; history of cancer; superficial wound infection (Berbari et al. 1998a)
Chronic prosthetic joint infection	N/A	Unknown	As for acute infection
Acute osteomyelitis		N/A	None proven. Soft tissue infection and bacteremia presumed
Chronic osteomyelitis: hematogenous	N/A	Unknown	Delayed diagnosis or inadequate treatment of acute osteomyelitis
Acute fracture fixation infection	Unknown percentage of high energy tibial fractures without free flap soft tissue cover	N/A	Open fracture; high energy injury; Inadequate debridement; failure to establish early soft tissue cover (Gustilo and Anderson 1976)
Chronic fracture fixation infection	N/A	Unknown	Inadequate management or delayed diagnosis of acute fracture fixation infection
Chronic osteomyelitis due to diabetic foot ulceration	Unknown	Unknown; 4–10% of diabetics have ulcers; 10–40% of ulcers have osteomyelitis	Increasing size, depth and chronicity of ulcer; failure to heal despite correction of ischemia and adequate offloading
Chronic osteomyelitis: other pressure sores	Unknown		Increasing size, depth, and chronicity
Vertebral osteomyelitis		Unknown	Increased age; intravascular catheterization; intravenous drug use

N/A, not applicable.

and sessile states. In each situation, intercellular signaling within the bacterial population orchestrates the expression of genes involved in host tissue damage (Abdelnour et al. 1993) and in the formation of biofilms (Chopp et al. 2003; Kjelleberg and Molin 2002), believed relevant to persistence of infection. Host inflammatory responses have both protective components (in terms of the prevention of septicemia and the long-term preservation of function) and harmful ones (joint damage and bone death) (Josefsson and Tarkowski 1999). Reparative and resorptive responses are seen in bone, the mechanisms of which are beginning to be elucidated.

Access of organisms to bone or joint

Microorganisms can gain access to bone, joint or muscle via hematogenous spread or from a contiguous source of infection or contamination.

Direct inoculation of pathogens via the latter mechanisms, though apparently self-evident, needs a little more consideration. It is noteworthy that it appears to be relatively difficult to infect healthy bone. In many experimental models, it is necessary to injure bone in some way (Norden 1988; Rissing 1990); one of the classic models involves injecting the sclerosing agent sodium morrhuate into the bone marrow prior to a bacterial challenge. Other models have involved surgically creating a cavity, a devitalized segment of a long bone, or a fracture (which can then be plated or pinned), or even resecting a joint and inserting foreign material to mimic joint replacement (Mader 1985; Belmatoug et al. 1996; Smeltzer et al. 1997b; Kaarsemaker et al. 1997; Monzon et al. 2002). In the majority of cases the pathogens are then directly inoculated, though hematogenous challenge can also be employed (Bremell et al. 1991).

These observations appear to find a direct parallel in human disease, in which the majority of contiguous source infections are in contexts where bone is indeed injured. This includes both elective orthopedic and emergency trauma surgery, and infections related to chronic soft tissue defects. Elective surgery usually involves cutting, drilling or instrumenting bone to achieve the desired end, whether that be joint replacement, realignment of the axis of a long bone, fusion of one or more joints, or the removal of a painful bony pressure point or joint. In trauma, fractures may involve one or more bones with variable severity depending on the energy of the injury, and may extend into the joint line, forming a potential route for the spread

of infection. Bone may be directly devitalized by dissipation of the high fracture energies through it, and acutely devascularized by stripping of the soft tissue attachments normally providing the periosteal circulation. This vascular compromise may be compounded by intramedullary nailing (affecting the endosteal circulation) or by further soft tissue stripping performed to gain access to the fracture for its reduction.

Soft tissue loss is also the key initiating element in the pathogenesis of osteomyelitis secondary to decubitus and diabetic foot ulceration. In the former case, immobility, poor prior skin condition, incontinence, and reductions in sensation, all play roles in allowing excess chronic pressure to develop and lead to ischemic soft tissue necrosis. In the case of the diabetic foot, peripheral neuropathy is the key driver (Boulton 1996). Motor neuropathy causes an imbalance of intrinsic and extrinsic musculature, leading to a high arched foot with subluxation at the metatarsophalangeal joints and clawing of the toes. There are also alterations in the compliance of skin and deeper tissues as a result of long-term hyperglycemia and autonomic neuropathy. These changes in the biomechanics of the foot cause excessive pressure on metatarsal heads, heel, tips of clawed toes, and dorsal aspects of interphalangeal joints (on the inner upper surface of footwear) (Murray et al. 1996). Sensory neuropathy causes a lack of protective sensation (without necessarily total numbness) and repetitive loading of the new pressure points leads to ulceration.

Once ulceration develops, it may worsen either through continuing loading, or infection, or both. Once the tissues directly overlying and attaching to bone become involved, the periosteal circulation is compromised and the most superficial cortical layer of bone is at risk. The high numbers of bacteria located in the ulcer base are then situated directly adjacent to injured bone for the initiation of infection.

It is also relevant, in considering infections relating to direct inoculation, to recall the important potentiating role played by devitalized tissue or foreign inanimate matter if present. Since the elegant experiments of Elek and Conen (1957) it has been clear that foreign materials greatly lower the inoculum required to initiate infection, and increase its severity. In elective orthopedic surgery, the use of metal ware and bone cement provides extensive surfaces for colonization with microbes if a breach in sterile technique occurs. In trauma and its management, bony injury also includes variable soft tissue loss and damage, and environmental contamination with dirt, soil, vegetable matter or debris from road surfaces or projectiles. All these factors potentiate infection just as in animal models. They also provide an explanation for the microbiology of this group of conditions, since pathogens are derived from the contaminating external environment or from the skin, and include low-virulence organisms when metal ware is involved (Christensen et al. 1994).

Hematogenous spread is clearly of importance in a number of manifestations. A range of bacteria can, under certain conditions, translocate into a joint space, or set up a focus of infection within bone, usually at the metaphysis just proximal to the growth plate. Although the in vivo mechanisms of this are poorly understood, *Staphylococcus aureus* does display the ability to adhere to and invade cultured human endothelial cells in vitro (Peacock et al. 1999). This is mediated by interactions between the staphylococcal cell wall-anchored fibronectin-binding protein and cell surface-associated fibronectin (Peacock et al. 1999), an interaction involving specific modules within each of these macromolecules (Massey et al. 2001a). Internalization is dependent upon endothelial cell membrane integrins ($\alpha5\beta1$), which appear to generate signals that drive active bacterial uptake on engagement of the fibronectin (Fowler et al. 2000; Massey et al. 2001a). Once within the endothelial cell, bacteria can escape into the cytosol (Bayles et al. 1998), and can persist for prolonged periods of time (several days at least) (Ellington et al. 2003).

The fate of endothelial cells following bacterial uptake varies according to toxin elaboration by the bacteria. If α-toxin is produced, endothelial cell death follows rapidly (Buerke et al. 2002). If cells do not die, they upregulate adhesion molecules and become pro-adhesive for white cells (Beekhuizen et al. 1997). In vivo, this would lead to adhesion of white cells, platelets and the formation of microthrombus; significantly, *Staphylococcus aureus* also has receptors for components of thrombus such as fibrinogen and thrombospondin. If there is no toxin production, persistence or translocation are possible outcomes.

Adhesion is considered to be the next crucial step once bacteria have reached bone or joint (Gristina et al. 1985), the importance of which appears to be borne out by the possession, in the case of *Staphylococcus aureus*, of numerous cell wall-associated molecules that act as proteinaceous receptors for a range of cell matrix or cell-surface associated proteins (Smeltzer et al. 1997a). These include receptors for collagen, fibronectin, bone matrix sialoprotein, and osteopontin (Herrmann et al. 1988; Buxton et al. 1990; Park et al. 1991, 1996; Patti et al. 1992; Switalski et al. 1993; Yacoub et al. 1994). As with direct inoculation of bacteria onto wounded bone, there is a plausible relationship between these adhesive properties and the observation that a proportion of cases of hematogenous infection of bone occur following blunt trauma. In healthy bone the components of the osteoid matrix are not exposed, being covered in mineral, but on injury, the underlying matrix becomes accessible to external agents including bacteria. For *Staphylococcus aureus*, some data support the importance of the collagen binding protein, Cna, in the pathogenesis of bone and joint infection (Patti et al. 1994; Elasri et al. 2002), though other carefully conducted studies of clinical isolates have not confirmed that this specific protein

is either necessary or sufficient (Thomas et al. 1999). The gene *cna*, encoding the collagen binding protein, may however be a more general marker of virulence. It was identified, along with the fibronectin binding protein gene *fnb*, as one of seven genes that appeared to be independently and cumulatively associated with the potential to cause invasive staphylococcal disease (Peacock et al. 2002); in the study concerned, some of the cases certainly had bone and joint manifestations of infection. It is possible that microfracture following blunt trauma produces areas of microscopic thrombus, or exposed bone matrix proteins, that provide a site for bacterial adhesion via Cna or Fnbp.

Adhesion is also of importance when considering infection of orthopedic metal ware of any kind. It has been shown that foreign materials are rapidly 'conditioned', after insertion into the body, by the deposition of plasma proteins such as fibronectin (Delmi et al. 1994). These proteins provide an ideal substrate for bacterial adhesion, though some important device-related pathogens, such as coagulase-negative staphylococci, have relatively weak adhesion mechanisms for fibronectin. They are, however, able to adhere relatively well to unconditioned metal and plastic, via a number of macromolecules, and subsequently establish very strong associations through the production of biofilm (see below) (Christensen et al. 1994).

Quorum sensing

A well-recognized clinical pattern in bone and joint infection is a prodromal illness that may include features suggestive of transient bacteremia, resolving completely, and then followed by acute infection of bone or joint. In such cases there is a high prevalence of bacteremia at the time of presentation, but without clinical evidence to suggest that this has persisted since the initial seeding of the joint. This is compatible with current understanding of the importance of quorum sensing, and global regulation of gene expression, in many pathogens. In *Staphylococcus aureus*, a cyclic octapeptide that is the product of the *agr* locus acts as a density-dependent signal to the whole bacterial population (Ji et al. 1995), ultimately resulting in the transcription of a regulatory RNA molecule, RNA III (Novick et al. 1993). This affects the expression of a wide variety of genes, many responsible for adhesin or toxin production (Novick 2003). Expressed during logarithmic phase, and triggering RNA III expression as the population moves towards stationary phase, *agr* upregulates toxin production and downregulates adhesin expression. Teleologically, as their growth rate slows through nutrient depletion, organisms become less adhesive and more destructive to surrounding cells and tissues, and so more able to disseminate to new sites. This fits with the observed sequence of a clinically unapparent bacteremia that leads to the onset of symptoms within a joint, followed by a secondary bacteremia detected at presentation. The *agr* gene is present in coagulase-negative staphylococci (Van Wamel et al. 1998) where it may be able to interfere with the *agr* expression of *Staphylococcus aureus* (Tegmark et al. 1998). It has been speculated that in this way, coagulase-negative staphylococci could act as negative regulators of the natural virulence of *Staphylococcus aureus*.

Biofilm formation and other mechanisms of persistence

Quorum sensing is also likely to be important within biofilms and during biofilm formation (Davies et al. 1998; Vuong et al. 2003). A biofilm is an adherent consortium of microorganisms embedded in an exocellular polysaccharide (glycocalyx) (Costerton et al. 1999; Gotz 2002). This material protects bacteria from complement and from phagocytes and, within the biofilm, organisms are present that are able to act as 'persisters', with growth states that confer relative resistance to antibiotics and other microbicides. The initial events in the formation of these structures occurs surprisingly rapidly; in *Pseudomonas aeruginosa*, studies have demonstrated how immediately after adhesion to a substrate, bacterial cells begin to express genes involved in alginate synthesis. The genetics of this process are well understood for *Pseudomonas aeruginosa*, and are increasingly so for *Staphylococcus epidermidis* (Heilmann et al. 1996), in which the *ica* gene cluster orchestrates the production of biofilms (reviewed in Gotz 2002). The *ica* operon is also represented in *Staphylococcus aureus*, and is another of the seven virulence factors previously mentioned that are associated with invasive characteristics (Peacock et al. 2002).

Other mechanisms of bacterial persistence have been suggested as potentially relevant. The clinical picture of osteomyelitis includes chronic active sepsis, but also periods of relative or complete remission, with subsequent relapse. Recurrent infection can occur many years after the initial episode, prompting speculation as to the mechanism of such long-term survival. One hypothesis is that small colony variants (SCV), which under certain conditions have been shown to emerge rapidly in populations of staphylococci, might survive for prolonged periods inside long lived cells such as fibroblasts. Certainly SCVs can be demonstrated as a surviving intracellular bacterial population in cultures of endothelial cells when host cell death is prevented through external antibiotic use. *Staphylococcus aureus* can invade a wide range of host cells including fibroblasts, and SCVs are known to have high rates not only of induction, but also of reversion to wild type characteristics. Furthermore, SCVs have been shown capable of causing osteoarticular infection in vivo in animal models

(Jonsson et al. 2003). Finally, a careful study has shown that SCVs can be isolated, in some cases in almost pure growth, from individual cases of relapsed chronic osteomyelitis (Proctor et al. 1995). The general relevance of this observation remains to be demonstrated, but it is an appealing hypothesis that SCVs, located within cells or indeed deep within a biofilm, provide a mechanism for remission and relapse. They may even, through rapid switching to and from wild type, allow populations of bacteria to manifest antibiotic resistance (a feature of SCVs) (Chuard et al. 1997) without the fitness costs usually associated with true antibiotic resistance (Massey et al. 2001b).

Inflammatory responses

BONE RESPONSES

Acute infection in bone and joint leads to acute inflammation. An influx of neutrophils, accompanied by increased vascular permeability and edema, is seen both in infected synovium and in cancellous bone. These responses are important in protection of the host against the secondary bacteremia commonly seen in acute infections, but they also contribute to the immediate damage to the infected part of the skeleton (Josefsson and Tarkowski 1999). The inflammatory response within the joint leads to an outpouring of synovial fluid and a tense effusion. In joints such as the hip and shoulder, where the head of the femur and humerus, respectively, are supplied with blood via recurrent vessels running up the neck of the bone, the high intra-articular pressure may be enough to compromise the circulation in those vessels. Within cancellous bone at any site, edema also leads to high pressures and potential vascular compromise. The procoagulant effects of inflammatory cytokines on vascular endothelium exacerbate this. The consequence is that the inflammatory response frequently leads to areas of bone death, through microvascular thrombosis around the infected focus. If the area of infection is not contained, progressive bone death can result.

The tracking of infection through areas of cancellous or cortical bone leads to other key features of bone infection. Infection originating in the metaphysis of a long bone, the common site for hematogenous infection, can track via the Haversian canals through the cortex and into the soft tissue. Where the periosteum is loosely connected, the accumulation of pus results in a subperiosteal abscess, stripping off the periosteum from the bone. As with traumatic periosteal stripping, this results in further vascular compromise to the bone, already affected internally by the inflammatory focus. A whole segment of bone death can result from the combination of endosteal infection and a circumferentially developing sub-periosteal abscess.

Bone is a highly dynamic structure adapted to respond to biomechanical forces and to preserve function. The response to periosteal stripping is to form new bone beneath the elevated tissue. In a fracture, this would be the beginning of fracture callus, which would then involve the fracture hematoma and so lead to healing. In infection, the periosteal reaction leads ultimately to the formation of new bone that externally bridges the involved segment. Where an area of dead bone would compromise the mechanics of the bone, this is therefore compensated for by the production of the new bony involucrum. Although this may mean that there is dead bone (now relatively trapped within the new bony shell), it does also mean that function, the basic role of the skeleton, is preserved.

Developing in parallel with the process of new bone formation is bony resorption in the areas contiguous with the dead bone. This is a direct consequence of the inflammatory response. There is evidence that inflammation both enhances the activity of professional bone resorbing cells (osteoclasts) and triggers bone resorption by 'semi-professional' cells such as recruited polymorphs. Bone resorption in the area of infection and inflammation acts to separate the dead bone from living bone. The resulting fragment(s) of dead bone, called a sequestrum, lying within the living bone, may now act as a site for permanent infection from a resident population of adherent bacteria, but this phenomenon also opens up the possibility of self-cure. If the sequestrum can be fragmented or is of sufficiently small size (since not all infections cause large areas of bone death), it can potentially be extruded, leaving behind living bone capable of healing. Spontaneous extrusion of sequestra is seen to occur in both chronic hematogenous and contiguous focus osteomyelitis. Bone is discharged either through the soft tissue defect that caused the infection, or through sinus tracts, the formation of which is another characteristic of chronic osteomyelitis. Sinuses occur because of the destructive nature of pus under pressure, which will track along tissue planes (e.g. beneath the periosteum), but will frequently then breach fascial barriers and eventually find a route to the outside world.

Bone resorption is also of key importance in the pathogenesis of bone infections related to orthopedic metal ware. Here, the impact of the infection is not merely how it affects the host systemically and through local damage. It is also that bone resorption, occurring adjacent to the infected orthopedic device, results in mechanical loosening of that device. In infected prosthetic joints, loosening causes additional pain and generates 'wear particles' of metal and acrylic cement, which themselves cause more inflammation (Panday et al. 1996). In infections of fracture-fixations and fusions, loosening leads to instability and increased risk of nonunion. In both situations, loosening also appears to further reduce resistance of the immediate environment to infection, making control of infection more difficult.

MOLECULAR BASIS OF BONE RESORPTION

There have been considerable advances in our understanding of the process by which bone is resorbed in health and disease. Central in this is the osteoclast, which differentiates from monocytic precursor cells in response to a range of endocrine and paracrine signals. Osteoclasts form tight associations with underlying bone, and acidify a physical compartment beneath them, leading to bone resorption (Panday et al. 1996). In vitro, this results in a visible pit on the surface of the bone, and the release of calcium into the medium, either of which can be measured. Using models of this type, the response of osteoclasts to bacterial products has been studied. Cell-associated materials eluted from a number of different bacteria trigger osteoclast activity. In the case of *Mycobacterium tuberculosis*, the chaperonin Hsp 60 is a potent stimulant. Less well-characterized constituents associated with the cell walls of *Staphylococcus aureus* and *Staphylococcus epidermidis* also stimulate resorption, though intriguingly, by different mechanisms as demonstrated by the effects of prostaglandin inhibitors (Meghji et al. 1997).

CELL AND MOLECULAR BASIS OF JOINT DESTRUCTION

Improved understanding in this area owes much to the systematic use of a rodent model of hematogenous septic arthritis induced by tail vein challenge with *Staphylococcus aureus*. This model has proved tractable to manipulation using a range of knockout mice, cytokine antagonists, and varying triggers of articular inflammation (Tarkowski et al. 2002). Clear themes emerging from this large body of work are that the inflammatory response protects the host from septicemic death, but is the major mechanism of articular cartilage destruction. T cells play a major role in this, and their actions are orchestrated by inflammatory cytokines such as TNF. T cell responses are triggered by CpG motifs in bacterial DNA (Deng and Tarkowski 2000) and by peptidoglycan cell wall components (Liu et al. 2001). Downmodulation of inflammatory response protects the infected joint, but only if antibiotics are used to keep the whole animal alive. In addition to demonstrating this in complex situations such as mice defective in adhesion molecules critical for immune function, such as intercellular adhesion molecule-1 (ICAM-1) (Verdrengh et al. 1996), it has also been shown that steroid therapy, if combined with antibiotics, reduces joint destruction, a finding that may be of clinical relevance (Odio et al. 2003).

MICROBIOLOGY

Despite the wide range of bacteria that are known to infect bone, *Staphylococcus aureus* stands as the preeminent cause of these infections (Bouza and Munoz 1999). It is the dominant cause of hematogenous and contiguous focus bone and joint infections. The bulk of hematogenous infections are monomicrobial. In addition to *Staphylococcus aureus*, aerobic gram-negative rods and group B β-hemolytic streptococci play important roles in neonatal infections; in children group A β-hemolytic streptococci, *Kingella kingae* (Birgisson et al. 1997; Dodman et al. 2000) (and in the unimmunized child, *Haemophilus influenzae* type B (HIB) though vaccine use has led to dramatic falls in the incidence of this (Bowerman et al. 1997)); *Salmonella* spp. in those with sickle cell anemia. Chronic infections complicating orthopedic devices have a preponderance of coagulase-negative staphylococci as causes (Christensen et al. 1994), but *Staphylococcus aureus*, including MRSA, remains important in acute device-related infection. In contiguous focus infections secondary to chronic soft tissue loss, infections are commonly polymicrobial, with aerobic gram-negative rods (mainly the Enterobacteriaciae and *Pseudomonas* spp.), anaerobes and enterococci represented alongside *Staphylococcus aureus*. This includes not only osteomyelitis complicating decubitus and diabetic foot ulcers, but also chronic osteomyelitis and prosthetic joint infection when draining sinuses have been present for prolonged periods of time. Gram-negative aerobes also play an important role in vertebral osteomyelitis in the elderly, presumably because of the increased rates of bacteremia secondary to more frequent urinary tract and intra-abdominal infection.

Clearly other pathogens may be of importance. *Mycobacterium tuberculosis* is the commonest worldwide cause of chronic septic arthritis and is of importance elsewhere in the skeleton, notably in the spine (Potts disease). Where endemic, *Brucella* spp. are also relatively common causes of infective spondylitis and septic arthritis (Sankaran-Kutty et al. 1991; Geyik et al. 2002). Brucellosis, Lyme disease (Franz and Krause 2003), and gonococcal infection (Bardin 2003) should always be considered in culture negative cases of septic arthritis. Appropriate epidemiological risk factors should be enquired after in culture-negative cases, or when the pathogens are distinctively unusual. Reports of osteomyelitis caused by *Salmonella arizonae*, linked to pet reptiles, are good examples of how individual risk factors can be identified and used in subsequent public health awareness (Nowinski and Albert 2000; Nowinski 2001).

In addition to the common, there is a long list of rare causes of bone and joint infection, including *Listeria* spp. (Mereghetti et al. 1998), pneumococci (Ispahani et al. 1999; Ross et al. 2003), *Pasteurella* spp. (Shapero and Fox 1999), *Fusobacterium* (Koornstra et al. 1998), and *Tropheryma whipplei* (Lange and Teichmann 2003). This fact is important only because it emphasizes the need for a rational approach to microbiological diagnosis and empiric treatment. Essentially any organism capable of causing a bacteremia or a soft tissue infection can occa-

sionally involve skeletal structures, including atypical mycobacteria (Clark et al. 1997; Ekerot et al. 1998), *Nocardia* spp. (Wilkerson et al. 1985; Ostrum 1993) and *Actinomyces* spp. The compromise to local host responses caused by an implanted device or by debris means that a wide range of environmental or low virulence pathogens are, from time to time, implicated in infections related to prosthetic joints, fracture fixations, and puncture wounds. Although coagulase-negative staphylococci are the prime movers in this regard (Christensen et al. 1994), many other organisms including *Clostridium difficile*, *Pasteurella* (Takwale et al. 1997) *Listeria*, *Brucella* (Malizos et al. 1997; Orti et al. 1997), corynebacteria (von Graevenitz et al. 1998), *Mycobacterium tuberculosis* (Berbari et al. 1998b) and other mycobacteria (Guerra et al. 1998) have been identified. Probably the most common atypical pathogen in this context, and a paradigm for the interaction between environmental contamination and host compromise, would be the high incidence of osteochondritis, progressing to osteomyelitis, caused by *Pseudomonas aeruginosa* in puncture wounds of the foot associated with the wearing of training shoes (sneakers) (Fisher et al. 1985; Niall et al. 1997). High densities of *Pseudomonas aeruginosa* living in the insole allow for inoculation into the weight bearing area of the foot if a nail penetrates the foot through the shoe. There is frequently also some debris present in addition to the bacterial contamination (Chang et al. 2001), and the result is that a pathogen normally only associated with significant host compromise is able to cause a potentially serious infection in an otherwise healthy individual (usually a child).

PREVENTION

Given that no vaccines yet exist against most of the causes of skeletal infection, prevention is geared at restricting the access of organisms to exposed bone or orthopedic implants (Norden 1985). This means taking measures to prevent the breakdown and infection of soft tissue in the at-risk patient; preventing pressure ulcers through risk assessment and risk reduction in hospitalized, bed-bound and other immobile persons, organizing foot care programs for diabetics, and taking measures to prevent surgical wound and deep infection after orthopedic surgery or trauma (Gillespie 1997). The latter include meticulous sterile theater technique, the use of ultraclean air for implant work, prophylactic antibiotics (chosen with regard to local sensitivity patterns among key pathogens) (Glenny and Song 1999), and careful handling of tissues to minimize necrosis and hematoma formation. Preventive measures of this kind involve considerable effort, and regular feedback of results to clinical teams is essential.

Post-operative hematomas need to be drained (Saleh et al. 2002), while leaking and clinically infected wounds should be explored (to prevent the development of deeper infection, or to diagnose it early and minimize the risk of progression to more severe or chronic disease). Traumatic wounds need careful exploration and debridement of devitalized tissue and debris, with priority given to early soft tissue cover of fractures with the involvement of plastic surgeons if necessary (Gustilo and Anderson 1976; Khatod et al. 2003). The relationship of some cases of hematogenous septic arthritis and osteomyelitis to episodes of clinically apparent bacteremia also implies that general measures to reduce staphylococcal bacteremia associated with intravascular catheterization (through appropriate infection control measures) would be valuable, though proof of efficacy would be hard to establish.

There is considerable interest at present in the cell wall-associated adhesins as potential vaccine candidates, hoping that specific antibody might be able to play a role in preventing localization of infection to deeper structures (Flock 1999). Progress is encouraging (Nilsson et al. 1998), but has not yet translated into clinically applied products.

Finally, one specific area for prevention concerns the implantation of prosthetic joints in patients with histories of prior septic arthritis or osteomyelitis, including osteoarticular tuberculosis (Lee et al. 2002; Kim et al. 2003). There is some evidence based on case series that joint replacement can reactivate tuberculosis or pyogenic infection (Wang 1997; Su et al. 1996). Active infection should ideally be treated before joint replacement is undertaken (Hardinge et al. 1979; Jupiter et al. 1981). Where this is impossible, or where the history is recent (raising the suspicion that cultures will in fact be positive), it may be necessary to stage the joint replacement, resecting the infected area, treating according to the results of cultures, and implanting the joint replacement after an interval (Nazarian et al. 2003). However, some centers have described acceptable results using a single stage approach, antibiotic loaded cement, and systemic therapy.

CLINICAL FEATURES

Skeletal infections, as seen above, cause local and sometimes systemic features, together with destruction of bone and joint (irrespective of attempts at preservation of function), and have the potential rapidly to establish chronic active infection. Clinical features are in keeping with these characteristics, notably pain, loss of function, acute inflammatory changes in overlying soft tissue, systemic illness due either to bacteremia or to chronic suppuration, sinus tract drainage, and progressive soft tissue and osteoarticular destruction. There may be other local complications, such as spinal cord or nerve root compromise from vertebral osteomyelitis when there is complicating epidural abscess (Baker et al. 1975), retropulsion of infected disk material, or mechanical instability. Pathological fracture, growth plate distur-

bance, or secondary septic arthritis may complicate osteomyelitis; soft tissue abscesses frequently develop during the evolution of undiagnosed or untreated acute osteomyelitis and are also commonly found in the context of infected metal ware.

When joints are infected, the features are of pain, swelling, and considerable reluctance to undergo active or passive movement. Joint effusion is followed by the development of synovitis, which may persist for weeks after successful treatment of the infection. If the diagnosis is missed or delayed, or treatment unsuccessful, progressive joint destruction ensues, with increased pain and disability.

The evolution of acute to chronic osteomyelitis has been detailed above. Acute osteomyelitis classically presents as a prostrating febrile illness with pain in a long bone. In the young, this may manifest simply as refusal to use the affected limb. There may be little in the way of soft tissue change, though edema and eventually abscess formation will develop once infection tracks from the interior of the bone out through the cortex. Some 50 percent of cases of acute osteomyelitis have an accompanying bacteremia. Chronic osteomyelitis much more commonly has suspicious features visible in the soft tissues, whether this is a chronic wound or ulcer overlying bone, a sinus tract or simply old scars that indicate previous surgery or episodes of drainage. Chronic osteomyelitis presents with fever rarely, unless a collection of pus has been trapped through premature sinus closure, or has not yet discharged since a recurrence began. Bone may be visibly exposed in chronic osteomyelitis, be unexpectedly palpated with a gloved finger on inspection of a pressure sore, or be identified by palpation with a sterile metal probe, which in diabetic foot ulcers is moderately predictive of underlying osteomyelitis (Grayson et al. 1995a).

DIAGNOSIS

General principles underlying diagnosis, including the desirability of understanding the performance and limitations of specific tests, are taken as understood and are dealt with elsewhere in this text. These considerations are of particular importance in relation to imaging studies and the interpretation of cultures, where there are significant problems with sensitivity and specificity to consider. One important point that must be made, however, is that there is a relative shortage of high-quality diagnostic studies, despite a considerable number of publications. Part of the problem is a lack of understanding of the requirements of studies aiming to assess a diagnostic test rigorously. But part too is a lack of consensus definitions of osteomyelitis or prosthetic joint infection, such that although expert clinicians would recognize most of these infections when they saw them, they would not necessarily agree on the formal requirements for making the diagnosis. Imprecision in this matter makes it hard to interpret the majority of studies once the goal is to compare one study with another. This problem besets all the diagnostic modalities that will be discussed below, and must constantly be borne in mind.

Clinical features

Clinical suspicion is of paramount importance in arriving at a diagnosis. Certain features, such as sinus tract formation, are pathognomonic of infection. More commonly, clinical features are nonspecific (pain, loss of function, soft tissue swelling, even fever) and are sometimes insensitive, for example in chronic prosthetic joint or diabetic foot infection (Lavery et al. 1996). It follows that a high level of clinical suspicion for infection must be maintained whenever patients present with acute changes in musculoskeletal function, especially if accompanied by fever; with unexplained pain, stiffness or soft tissue swelling; with destructive bony lesions especially if accompanied by reparative responses; or whenever orthopedic implants fail rapidly with no obvious technical explanation. Night pain is indicative of very significant inflammation and is a warning symptom; pain on first using a prosthetic joint that eases as the joint is used, only to return after each period of rest (so-called 'start-up' pain) is highly suggestive of loosening; and some patients with chronic osteomyelitis will complain that the severity of bone pain is linked to changes in the weather. But none of these features is universal or pathognomonic for infection, and so the diagnosis must rest on recognizing the possibility, and taking the necessary steps, to confirm or refute infection within an appropriate timescale depending on the nature of the diagnosis being entertained. For acute presentations in which early intervention is key in preventing damage and chronicity, diagnosis must be expedited and may even need to follow on from the initiation of treatment. For chronic conditions there is rarely urgency to make the diagnosis or commence treatment unless the differential diagnosis of a tumor is a significant possibility.

Laboratory

Most laboratory studies provide only supportive information alongside the clinical impression. With the exception of serological testing for minority causes of infection such as *Brucella* spp., tests are nonspecific. Measurement of full blood count may reveal anemia of chronic disease. Acute phase responses (elevations of white cell count and C-reactive protein (CRP)) are generally elevated in acute septic arthritis and acute osteomyelitis or if a deep abscess containing a high-grade pathogen has formed. Elevations in the erythrocyte sedimentation rate are often seen, and if no other cause is found, support a diagnosis of osteomyelitis.

Generally, more chronic infections are associated with less perturbation of the inflammatory markers, a feature also commonly seen in diabetic foot infections, where fever and elevations in white cell count are indicative of more severe disease, with many infections not eliciting a strong systemic response (Edelson et al. 1996).

There are no other generally accepted serological diagnostic tests for pathogens prevalent in bone and joint infection. Serodiagnosis of prosthetic joint infection using antibody responses to glyocalyx has been put forward, but has yet to be widely validated and adopted. The majority of pathogens diagnosable by current serological techniques are exceedingly rare causes of bone and joint infection, and so the use of these tests cannot be justified in routine practice, though specific clinical indications may make them relevant.

Imaging

The aims of diagnostic imaging are both to diagnose infection and ultimately to delineate its anatomical extent (Santiago Restrepo et al. 2003). Few single modalities provide all the required information, and all have flaws in their performance depending on the circumstances of the case.

Figure 1 *Plain radiograph of femur with chronic osteomyelitis present. A minor periosteal reaction is seen, but no other changes.*

Plain XR

Plain radiological abnormalities take time to develop; in the case of bone this is several days for sufficient bone to be resorbed or to form to the extent that it becomes visible. The first signs of acute osteomyelitis are soft tissue swelling, followed at 7–10 days by the development of lucency. When periosteal reaction produces new bone, it is not visible for 10–14 days. For this reason, a single normal radiograph does not rule out an acute osteomyelitis. A chronic osteomyelitis is generally identifiable from the mixed picture of lucency and periosteal reaction (Figure 1). Serial films are useful, because osteomyelitis is an aggressive process that generally shows rapid evolution. An evolving mixed destructive and reparative picture is highly suspicious of active infection.

The location of the lesion depends on the chronicity, the age of presentation and the specific manifestation. Acute childhood osteomyelitis is a metaphyseal disease, but the skeletal abnormality will become increasingly diaphyseal as the bone grows, with the growth plate moving away from the old focus of infection. Ongoing activity or episodes of recurrence will maintain a metaphyseal component to the lesion. More active disease is characterized by 'fluffiness' of the periosteal reaction and of the margins of bony lucencies. Healed infection has much 'crisper' margins, with the periosteal reaction being fully organized into involucrum, and lucent areas either being filled in again or becoming more demarcated. Some subacute forms of infection present with obvious cavities within bone, classically surrounded by a sclerotic rim (the whole known as a Brodie's abscess). Even more indolent presentations are found in the form of chronic multifocal osteomyelitis, a poorly understood condition in which cultures are negative, and multiple sites may be involved over a period of time, generally without the kind of progressive destruction usually seen in osteomyelitis, but with intense sclerotic changes in bone and expansion of the bone due to chronic periosteal reaction (Kozlowski et al. 1983; Golla et al. 2002; Huber et al. 2002).

Bony changes in septic arthritis consist of accelerated arthritis with loss of joint space and secondary changes in the underlying bone. In hip or shoulder, there may be avascular necrosis of the femoral or humeral heads, with collapse.

In prosthetic joint infection, the cardinal features are the development of lucency at the bone–cement interface. This reflects loss of bone due to chronic inflammation, and its replacement, at the interface, with connective tissue (so-called 'membrane') that is edematous, hyperemic, and inflamed. As loosening progresses, mechanical failure of one or both components can occur. In hip replacements, one may see evidence of rotation of the acetabular component, or cortical erosion at the tip of the femoral component ultimately leading to femoral fracture. In knee replacement, the tibial component may collapse into valgus or varus.

Although the changes outlined here are consistent with infection, they are rarely diagnostic. In long bones,

the differential diagnosis of tumor must always be borne in mind, while in prosthetic joints it is the accelerated timescale that is particularly suggestive of infection; the changes of loosening are nonspecific and may occur in the absence of infection. In diabetic foot disease, neuropathy can lead to changes in bone and joint; healed fractures, subluxation with secondary degenerative changes, and sometimes of a destructive nature including acute Charcot (neuropathic) arthropathy. The appearances of this diabetic neuro-osteoarthropathy can easily be mistaken for infection.

Ultrasound

Appropriately powered ultrasound can obtain relatively good images of many external parts of the skeleton. Sinus tracts can be identified and followed down to likely points of origin on bone or prosthesis. Collections can be imaged, and periosteal elevation can frequently be seen. Cellulitis or edema within deeper structures (muscle) can be visualized and joint effusions distinguished from synovitis. Ultrasound, if available, can therefore play an important role in the management of acute osteomyelitis, identifying a subperiosteal abscess, the early periosteal edema consistent with the evolution of one, or an abscess that has already tracked out into the soft tissues (Kaiser and Rosenborg 1994; Mah et al. 1994; Cardinal et al. 2001). Diagnostic aspiration under guidance may be possible, and this application is also of great value in attempting to obtain a microbiological diagnosis in suspected or proven prosthetic joint infection, and sometimes in chronic osteomyelitis.

Isotope scanning

The history of nuclear medicine in osteoarticular infection encompasses several isotope scans that have failed to live up to early enthusiasm. The fundamental problem is the relatively nonspecific nature of musculoskeletal responses as currently detected. Noninfective processes such as diabetic neuro-osteoarthropathy, fracture nonunion, and prosthesis loosening, also generate abnormal scans (Turpin and Lambert 2001). The need for high levels of sensitivity and specificity has not yet been met reliably, despite studies with triple-phase Technetium bone scanning (sensitive but very nonspecific) (Schauwecker 1992), Indium-labeled white scan (more specific but less sensitive, and so sometimes combined with conventional bone scan) (Johnson et al. 1996), marrow colloid scan (designed to exclude marrow white cell packing as a confounding cause of false positive white cell scans), labeled immunoglobulins and anti-white cell monoclonal antibody scans (Palestro et al. 2003), and Technetium-labeled white cell scans (Devillers et al. 1995) (designed, in different ways, also to localize the white cells present in inflammatory lesions). Of all currently available isotope scanning protocols, the greatest specificity can be obtained with the combination of indium white cell scanning and nanocolloid scan; alternative labeling techniques for white cells may offer promise. White cell labeling scans are not trivial matters, however, given the need to remove, purify, label, and reinfuse white cells, which opens up obvious possibilities of infection and cross-infection. For prosthetic joint infection, no scan is recommended; for acute osteomyelitis in the otherwise normal skeleton (e.g. in childhood) triple phase bone scan (Figure 2) may be an acceptable confirmatory test that will follow a clinical decision to treat; for chronic osteomyelitis, a labeled white cell scan combined with nanocolloid scanning is acceptable if magnetic resonance imaging (MRI) is unavailable.

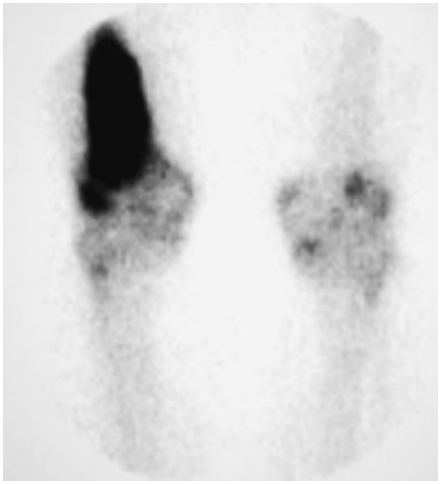

Figure 2 *Triple phase bone scan (bone phase) showing intense uptake*

MRI

MRI is able to produce images in axial, sagittal, and coronal planes, and gives information that is physiologically derived (based on fat and water content of tissues) but anatomically portrayed. With few exceptions, it is the imaging modality of first choice in bone and joint infection. It will show soft tissue edema and fluid collections; cortical breaches in bone; edema (Figure 3 and 4), abscess and gas within bone; and through gadolinium enhancement, will distinguish inflammatory from noninflammatory high signal. By demonstrating intraosseous edema, it can diagnose osteomyelitis before plain radiological changes are evident, and is also of great value in assessing relapse in cases of chronic osteomyelitis, where plain films are already abnormal. In some areas, such as the spine, MRI has transformed the diagnosis of infection, with superior simultaneous resolution of disk space, paravertebral structures, nerve root foraminae, epidural space (important in identifying epidural abscess) and the anterior and posterior bony columns.

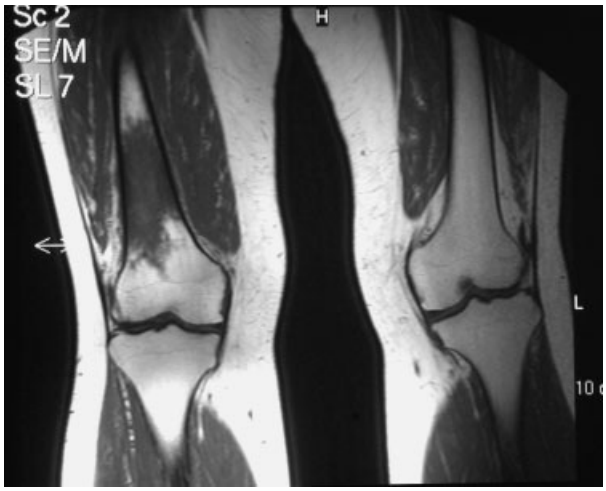

Figure 3 *T1 weighted MRI scan of the same region as in Figure 1, showing intramedullary edema as low signal*

MRI does suffer some pitfalls, notably when there has been previous surgery, when follow-up scans are performed after treatment of infection (images take many months to return to normal after either) or when metal is or has been present. Titanium implants produce minimal signal void, but more standard materials can produce dramatic metal artefact, even when the metal ware has long been removed (small amounts of metallosis in the tissues being enough to disrupt the image).

Figure 4 *T2 weighted MRI scan (STIR) of the same region as in Figure 1, showing intramedullary edema as high signal. Additionally, high signal periosteal reaction is seen.*

There are also problems in the interpretation of diabetic foot osteomyelitis, where underlying neuro-osteoarthropathy can mislead the unwary; some stress-related bone marrow changes and bony fragmentation are to be expected, and do not necessarily indicate infection with as much reliability as in the non-neuropathic patient. Overall, however, when available, MRI is of great value in suspected acute osteomyelitis (as a confirmatory test after the onset of treatment) and in chronic osteomyelitis (assessment of activity and surgical planning). Its role in prosthetic joint infection is minimal because the primary interest is at the bone–cement interface, which is usually affected by signal void, though occasionally distant collections or osteomyelitis can be identified.

Culture

This is clearly the 'gold' or criterion standard in the diagnosis of almost all infections, excepting only those rare cases caused by nonculturable organisms. The major issues that beset bacterial culture are as follows.

LACK OF SENSITIVITY OF STANDARD METHODS OF SAMPLING AND OF CULTURE

Some 15 percent of prosthetic joint infections, and a similar proportion of cases of chronic osteomyelitis, are culture-negative, yet show histological signs of active infection (Atkins et al. 1998). One potential explanation is the very low densities of organisms in some manifestations of infection. Experimental studies in animal models suggest that the density of bacteria in periprosthetic tissues is considerably lower than the density in an acute abscess, and explains why a swab of fluid near the implant or infected bone will not always yield a useful result. To increase sensitivity, a number of pieces of the periprosthetic tissues should be obtained for culture, and the culture routine should include cooked meat broth or an alternative enrichment medium that is supportive to damaged and fastidious organisms (Atkins et al. 1998). To increase specificity, each sample should be taken with a separate set of instruments to avoid cross-contamination between sampling. A carefully conducted prospective study of hip and knee replacements undergoing revision, suggested high levels of both sensitivity and specificity can be achieved through this rigorous multiple sampling approach (Atkins et al. 1998). Other approaches have focused on increasing the yield from cultures by using ultrasonication to dislodge organisms from the surface of the explanted prosthesis. This has suggested that the presence of bacteria is greatly underestimated in loose prostheses, but the work currently awaits independent confirmation.

INTERPRETATION OF POSITIVE AND MIXED CULTURE RESULTS

Many infections are active in the context of chronic wounds that overlie the involved bone or prosthesis. The

wound will acquire a dynamic flora of its own, and irrespective of the original pathogens, secondary ones may establish themselves in the bone after prolonged access via the wound. In our experience, a transition from gram-positive to gram-negative pathogen, or from methicillin-sensitive gram-positive, to methicillin- (and occasionally vancomycin-) resistant gram-positive pathogen is frequently seen. Antibiotic selection is an important cause of this dynamic evolution.

How to interpret microbiology results in this situation is problematic. Studies suggest that superficial cultures are misleading and poorly predictive of the deep flora (Mackowiak et al. 1978). The microbiologist is therefore dependent upon sensible sampling behavior and accurate specimen labeling by the surgeon. Wounds should be thoroughly debrided before cultures are sent, or cultures obtained through a quite separate, uninfected field, as with a percutaneous biopsy. The surgeon must understand the crucial importance of cross-contamination. Tissue samples should be encouraged over swabs, which do not facilitate the isolation of anaerobes. Material must be sent to the laboratory without delay. Results should be fed back to surgeons in person as often as is possible, to allow discussion of therapeutic options and to reinforce joint working with the specialist in infection. Where the pathogens are not distinct from the likely skin or ulcer flora, and the biopsies have had to come through this field, a good rule of thumb is to demand the isolation of an indistinguishable organism (by biochemical identity and antibiogram) in at least two independent samples. Confirmatory histological examination of tissue is also invaluable.

Histology

The majority of pyogenic infections cause an acute inflammatory response. This is in contrast, in the skeleton, with conditions that give more chronic inflammation, where the inflammatory response shows remarkably few neutrophils. It is possible to distinguish infected from uninfected periprosthetic tissue with considerable accuracy, mainly on the basis of the numbers of infiltrating polymorphs (Mirra et al. 1982; Panday et al. 2000). This forms the basis of being able to diagnose infection in a prosthetic joint in real time, using frozen section, during joint revision surgery (Fehring and McAlister 1994; Athanasou et al. 1995; Lonner et al. 1996). It also provides an essential second diagnostic modality to match up with cultures in all contexts (White et al. 1995). The importance of this should be stressed, as it is not unknown for lesions that do not respond to appropriate treatment for infection to turn out to be tumors. In some situations, either because of sampling error, prior antibiotic use or presumed fastidious organisms, histology suggestive of infection may be the only evidence available, because the cultures are negative (White et al. 1995). Histology is also of great importance in the diagnosis of mycobacterial disease, given the slowness of culture results in most mycobacterial infections.

Polymerase chain reaction

The use of polymerase chain reaction (PCR) for diagnosis is appealing given the limitations in sensitivity of conventional culture. There are surprisingly few published studies in this area, suggesting that researchers are encountering significant technical difficulties in achieving a robust and reproducible test. Using a universal primer for the 16S rRNA gene, an early study suggested that a substantial number of culture-negative explanted prostheses had evidence of bacterial DNA on PCR, but with an incomplete correlation between a positive conventional culture and a positive PCR (Mariani et al. 1996; Hoeffel et al. 1999). Other studies have shown detection of bacterial DNA, but have not evaluated this as a formal diagnostic test (Tunney et al. 1999). Although an absolute indicator of infection would be useful to answer certain clinical questions, an identity and preferably an antibiogram are both of importance in definitive treatment planning, so more sophisticated techniques may ultimately have to be employed.

TREATMENT

The fundamental principles of treatment follow on from the special features of pathogenesis outlined earlier. Treatment of acute presentations must be prompt, effective, and predicated on appropriate empiric antibiotics based on clinical suspicion. The aim in acute cases is to prevent the progression to chronicity, via the death of bone, with its correspondingly worse prognosis. Provided treatment is effective and timely, it often does not need to be protracted. The use of clear guidelines and protocols can help ensure consistent treatment approaches to acute infections (Peltola et al. 1997; Levy et al. 2000; Kocher et al. 2003).

Treatment of chronic presentations is based on the principles of establishing a clear microbiological diagnosis, using histology in parallel; excising dead and foreign infected tissue; establishing soft tissue cover and skeletal stability; managing surgical dead space; preserving functional implants where possible while they are necessary; using appropriate antibiotics for often extended periods of time, according to the biology of the infection and the host; and carrying out further reconstructive surgery, usually when infection is controlled. For all forms of chronic bone and joint infection, it is important to set realistic goals and expectations, which must include the possibility of recurrence and plans for the management of such an eventuality.

In treating infected orthopedic implants, the principles are a fusion of the two. For acute infections, urgent appropriate diagnosis and treatment is needed, aimed at preventing chronic infection, often with retention of the implant if still functional. For chronic infections, a more measured approach is required in which realistic goals are set with the patient, reconstructive options considered and a treatment plan delivered as with other chronic skeletal infections.

Surgical treatment

This is a key element of successful treatment in most situations, especially in chronic cases. In acute osteomyelitis and in vertebral osteomyelitis, provided there are no collections or abscesses, surgery can be deferred and may not be needed. Indeed, in acute osteomyelitis, gratuitous windowing or drilling of cortical bone, in the absence of other evidence of intramedullary abscess, is no longer in favor among most surgeons. In all other situations of chronic osteomyelitis, with the possible exception of the diabetic foot with its predominant involvement of small bones, surgical removal of dead infected bone is crucial.

Debridement and excision

Careful exploration of infected tissues allows determination of the extent of infection, scarring, abnormal soft tissue, and bone, and hence permits decisions about the degree of resection needed. The aim should be that at the end of surgery, bone at the resection margins is viable as indicated by bleeding, provided this leaves a limb that is either still functional or is reconstructable. Recurrent infection is often linked to failures of resection. In chronic prosthetic joint infection, the same thinking applies. The prosthesis and all cement, infected soft tissue, and dead bone should be removed, and abscesses drained. Rarely, it is appropriate to attempt salvage of a chronically infected prosthetic joint, if solidly fixed, pain free, and in situations where the expected morbidity of revision surgery exceeds that of ongoing infection and long-term antibiotics (see below). This is a more standard approach in an acute infection with a well-fixed prosthesis, where the damage caused by removal of the implant may be significant, and where studies show success rates of 50–70 percent if the debridement is thorough and timely (Brandt et al. 1997; Mont et al. 1997; Crockarell et al. 1998; Tattevin et al. 1999; Krasin et al. 2001).

Soft tissue cover

Some failures of osteomyelitis management are linked to persisting soft tissue defects or areas of extensive (and by definition relatively avascular) scar tissue. A tissue defect takes a prolonged period of time to heal and in some cases cannot (for example, when it overlies extensive dead bone or metal ware), while scar tissue limits the access of inflammatory mediators and antimicrobials to the infected area. Fortunately, modern plastic surgical techniques allow for the transposition of skeletal muscle with the corresponding vascular pedicle, using microvascular anastomosis, into tissue defects (Kuokkanen et al. 2002). Muscle flaps provide healthy, well-vascularized soft tissue cover and the physical characteristics of muscle make it ideal for laying into a bony defect, to the walls of which it rapidly adheres. Alternatively, muscle can be moved through lateral release or rotation upon the vascular pedicle, in this context most commonly in the case of the gastrocnemius, one head of which can be tunneled anteriorly to provide cover for the knee (McPherson et al. 1997; Casanova et al. 2001). This is of crucial value when wound breakdown compromises the soft tissue over a knee replacement, either acutely to aid implant salvage, or as part of a revision strategy.

Dead space management and reconstruction

Surgical ‘dead spaces’, namely cavities in tissue that are left to fill with blood, exudate or serous fluid, are risk factors for renewed or recurrent infection. Elimination of these dead spaces is an additional priority of surgical technique. Muscle flaps are excellent means of dead space management, but other means are sometimes employed. These include antibiotic loaded acrylic bone cement, which combines antibiotic delivery with dead space management. This can be combined with subsequent bone grafting if required, either for structural indications or to eliminate larger cavities definitively (McNally et al. 1993; Alonge et al. 2002).

Reconstructive options depend upon the specific situation. Fracture non-unions require stabilization; external fixation is generally preferred, to avoid instrumenting an already infected field, but the key priority is effective stabilization, which is essential for comfort, for union to proceed, and it seems, for control of infection.

For prosthetic joints that have been removed, reconstructive options rest with reimplanting a new joint, fusing the joint, or leaving an excision arthroplasty. In the hip, the latter results in a Girdlestone’s pseudarthrosis, which is compatible with function in most patients, albeit with walking aids; for the other major joints, excision is a relatively poor option. For reimplantation, there is significant uncertainty over the choice between one- and two-stage revision surgery (Buchholz et al. 1981; McDonald et al. 1989; Raut et al., 1995; Goldman et al. 1996; Younger et al. 1997; Haddad et al., 2000). The one-stage technique is favored when highly sensitive organisms infect relatively healthy hosts, with two-stage revision favored for less fit hosts and more

resistant bacteria (Hanssen and Osmon 2000; Fisman et al. 2001). Frustratingly, it is precisely in the less fit patient that one stage revision is most inherently attractive, to avoid the considerable morbidities of spending weeks without the function afforded by a functioning prosthesis, and of a second general anesthetic. For many, this limits the usefulness of the one-stage concept (Jackson and Schmalzried 2000), especially in view of difficulties in defining the infecting pathogen before definitive surgery. Some go so far as to take additional samples at the end of the first stage and accompanying antibiotic treatment, to determine whether to carry out additional debridements prior to reimplantation (Mont et al. 1997).

One important advance has been in the management of segmental defects of long bones using the Ilizarov method. This involves external stabilization of the limb using fine wires traversing the bone, attached to circular rings. A number of such rings, arrayed axially along the limb, are linked by longitudinal rods, and the resulting frame stabilizes the limb. The power of this method is that it is possible not only to remove an entire segment of bone, but also to grow new bone. By creating a corticotomy through bone adjacent to the defect, a viable segment can be produced that is itself stabilized by wires and a ring as part of the frame. By altering the relative positions of the rings, this 'transport segment' can be moved through the defect, a few millimeters a day, until it is 'docked' at the far end of the bone defect. The traction on the intact periosteum at the corticotomy site results in new bone formation. Using the method, large segmental defects can be regenerated with healthy bone of normal structure.

Antimicrobial treatment

Although bone infection can be managed without antibiotics, relapse rates were high in the pre-antibiotic era. Furthermore, the prolonged nature of resection and reconstruction surgery would lead to a high postoperative infection rate, highly likely to involve the surgically injured bone. Despite the rise in antibiotic resistance, there are still several good drugs available for use in osteomyelitis. The historical standard of care is prolonged intravenous therapy, but there is growing confidence in the use of shorter treatment regimens and highly bioavailable oral therapies in some situations (Kim et al. 2000; Jaberi et al. 2002; Le Saux et al. 2002; Vinod et al. 2002), alongside technologies that permit early discharge from hospital despite a decision to continue parenteral treatment.

It is notable that relatively few antibiotics have specific licensed indications for either osteomyelitis or prosthetic joint infection. This is more a reflection of the high costs and risks to pharmaceutical companies (in seeking indications for treatment in conditions that require prolonged treatment and may still have appreciable failure rates) than an indication that the drugs inherently will not work. Although the literature frequently raises the issue of 'bone penetration', there are uncertainties about the meaning and relevance of this term. Measuring antibiotic levels in cortical and cancellous bone, accounting for the levels within marrow stroma, and understanding whether the key targets of antibiotics are in the interstitial fluid, the bony matrix, or even the cellular elements within bone are all topics fraught with difficulty.

It has been hard to escape the view that in the antibiotic era, it has not been the development of drugs with bone penetration, but the renewed understanding of the critical nature of adequate surgical resection, and improvements in reconstructive techniques, that have led to improved outcomes for many with chronic infection. Nonetheless, antibiotics are still rightly regarded as a key element of treatment, even though a meta-analysis of published studies concluded that there is insufficient evidence to favor one drug, route of administration or duration over another (Stengel et al. 2001). They do play a pivotal role in treating those manifestations of infection not amenable to surgical excision; acute infections where bone and joint destruction have not yet occurred, and where a decision is made to undertake long-term suppression of the infection, with implant retention (Segreti et al. 1998; Tattevin et al. 1999). This strategy has been useful in acute prosthetic joint infection, and seems particularly likely to succeed when there is a short interval (48 hours or less) between onset of symptoms and the surgical debridement (Brandt et al. 1997; Mont et al. 1997; Crockarell et al. 1998; Krasin et al. 2001).

Systemic: intravenous

The lack of oral bioavailability of many β-lactam antibiotics, aminoglycosides, and glycopeptides, make them good choices for intravenous therapy against many gram-positive organisms. In many situations, such as prosthetic joint infection and some cases of osteomyelitis, courses of intravenous therapy of 4–6 weeks duration are commonplace. This requires the placement of central venous access, most commonly with a tunneled subclavian (Hickman or Broviac) line, but increasingly with peripherally inserted central catheters (PICC) (Parker and Gaines 1995) or shorter lines terminating in the axillary vein, so-called 'mid'-lines. The dependable nature of such vascular access means that they can be used to underpin programs for administration of intravenous antibiotics in the home or outpatient setting (Grayson et al. 1995b). Ceftriaxone and teicoplanin are useful drugs in this regard because of their once-daily dosing regimens, but more frequent dosing is possible, especially if patients can be taught to self-administer,

and various technologies permit the use of drugs that must be delivered by continuous or intermittent infusion if need be. Recent analysis indicates a high success rate in cases of osteomyelitis treated with prolonged courses of ceftriaxone (Tice et al. 2003). Outpatient parenteral antibiotic therapy (OPAT), outpatient and home parenteral antibiotic therapy (OHPAT) and a number of other acronyms describe this mode of treatment, which is in established use in many centers around the world (Bernard et al. 2001). Osteomyelitis and prosthetic joint infection patients are major users of such programs.

Systemic: oral

Although the availability of OPAT transforms the prospects for giving prolonged intravenous treatment (in an era when prolonged hospital stays are, in the main, neither culturally nor financially acceptable), there are obvious issues about the costs and risks of intravenous therapy. There is now a range of highly bioavailable oral therapies, many of which are used in the treatment of osteomyelitis or prosthetic joint infection (Shuford and Steckelberg 2003). With the exception of first-generation cephalosporins, the orally available drugs do not act on the bacterial cell wall but on DNA or protein metabolism. The nature of these targets is, in the main, associated with easier acquisition of resistance, and it is important to bear this in mind in selecting treatment, especially if there is a high bioburden of infection at the start (for example, when debridement surgery is not carried out). Useful drugs include high-dose trimethoprim-sulfamethoxazole, doxycycline, rifampin, and fusidic acid for staphylococci, especially MRSA and MRSE. The published role of linezolid remains anecdotal (Bassetti et al. 2001).

In addition to these drugs, there is a substantial body of evidence supporting the use of ciprofloxacin. This has proved extremely useful for the treatment of gram-negative osteomyelitis (Lew and Waldvogel 1995). It has also assumed a role, in combination with rifampin, in the treatment of gram-positive infections where biofilms are believed to be an important element of the pathophysiology. The use dates from animal models of device-related infection in which cure of staphylococcal infection was observed with fluroquinolone-rifampin combinations. This led to observational studies in orthopedic device-related infection (Widmer et al. 1992; Drancourt et al. 1993), culminating in a randomized controlled trial of ciprofloxacin-rifampin therapy compared to ciprofloxacin monotherapy in patients with staphylococcal infection and a mechanically stable implant, who underwent prompt surgical debridement with retention of the implant (Zimmerli et al. 1998). The results clearly showed the ability of the combined regimen to cure infection with 3–6 months of therapy in selected cases. All the failures were seen in the monotherapy group, with the evolution of resistance in the same bacterial strain, as demonstrated by pulse-field gel analysis of the initial and relapsing isolates. It remains to be seen in larger series of orthopedic device-related infection how generalizable these findings will prove. Applying the same reasoning to chronic osteomyelitis, quinolone–rifampin combinations have also been used in this condition, particularly in diabetic foot osteomyelitis (Senneville et al. 2001). In this condition, several separate studies have shown that antibiotic therapy alone frequently suffices to treat osteomyelitis, though the lack of standardized definitions does cause some difficulty (Venkatesan et al. 1997; Pittet et al. 1999)

Topical

The need for surgical dead space management has already been discussed. A logical extension of this requirement, and arising from a view that if high doses of antibiotics are required these are best provided locally, is the incorporation of antibiotics into a suitable carrier that can be implanted at the site of infection. An ideal carrier would release high levels of active drug for a prolonged period, without generating high systemic levels of the drug. It would not in its own right be systemically or locally toxic, and would be absorbable and support or stimulate the production of new bone. The carrier would be compatible either with one or two agents that singly, or in combination, produce a very broad-spectrum regimen, or with a range of drugs that could be mixed in at the time of application, based on prior microbiological information (Wininger and Fass 1996). In practice, no carriers yet fulfill all these requirements. The most widely used system is polymethylmethacrylate (PMMA) bone cement, which can be loaded with a range of antibiotics (Figure 5). These include cefuroxime, gentamicin, amikacin, tobramycin, clindamycin, and vancomycin. Of these, gentamicin was first developed commercially (in Europe) and can be obtained as a mix for reconstitution, or precast into beads of varying sizes threaded into chains on wire (Wininger and Fass 1996; Walenkamp et al. 1998; Klemm 2001). Antibiotic loaded cements are not available pre-mixed in the USA for regulatory reasons, so surgeons who make use of them there have to hand mix antibiotic powder into standard cement, a procedure that can obviously lead to variations in the properties of the cement and the elution of the drug.

There are extensive data on the elution properties of gentamicin and to a lesser extent other antibiotics from PMMA cement, which indicate that very high local levels of drug are maintained in the immediate environment of the cement and in wound drainage fluid. These are maintained for upwards of two weeks before levels start to fall. Levels are so high that bacteria that are antibiotic resistant may nonetheless be killed. Elution of

Figure 5 *Post-operative radiograph, showing bone loss subsequent to debridement via a cortical bone window, and antibiotic loaded PMMA beads to manage the dead space*

antibiotic is from the exposed surface of the cement, with little drug diffusing out from the depths of the bead or block. This has implications for removal of spacers, which may potentially release bactericidal levels of drug if cracked or otherwise damaged (when new surfaces are exposed), affecting culture results.

In addition to its temporary use in spacers and beads, antibiotic loaded cement has also been used in revision of prosthetic joints. This may be in one- or two-stage exchange, or in rigid or articulating spacers. The latter are designed to preserve some joint movement, in the belief that morbidity is reduced, technical difficulties at reimplantation ameliorated and ultimate range of movement improved (Fehring et al. 2000; Emerson et al. 2002). There are even kits available with molds and internal metal scaffolds around which antibiotic loaded cement can be cast to give a relatively functioning, cement-surfaced implant, the PROSTALAC (prosthesis of antibiotic loaded acrylic cement) (Masri et al. 1994; Haddad et al. 2000; Meek et al. 2003). These may prove invaluable in managing major proximal femoral loss, by preserving function, muscle bulk, and an anatomic space for eventual implantation of a large definitive prosthesis. There are no controlled data on the use of functional spacers compared to static alternatives.

Alternatives to the use of PMMA cement as an antibiotic carrier are available. Gentamicin can also be incorporated into collagen fleeces, which can be packed into cavities and which should, over time, be absorbed (Kollenberg 1998). The kinetics of release differ from antibiotic-loaded PMMA cement, with high levels of antibiotic eluting over a much shorter period of time. Hydroxyapatite can be used (Yamashita et al. 1998; Shirtliff et al. 2002), and 'low technology' versions also exist, in the form of calcium phosphate (plaster of Paris) (Alonge et al. 2002; Santschi and McGarvey 2003), which can be reconstituted, with antibiotics incorporated, into sterile pellets for packing into bone defects (Benoit et al. 1997). This may be particularly valuable in parts of the world where resources are scarce (Alonge et al. 2002; Heybeli et al. 2003). Pellets have also been studied in the treatment of bony defects produced after debridement of diabetic foot ostomyelitis (Roeder et al. 2000).

The evidence base for the use of antibiotic-impregnated PMMA cement is relatively poor, but surprisingly, little different from that supporting the use of systemic therapies. An attempt at a randomized trial of gentamicin-loaded PMMA beads compared to systemic therapy was unsuccessful due to substantial protocol violations and concerns about the randomization (Blaha et al. 1993). Another study was abandoned when nursing staff refused to continue the traditional suction drainage approach in favor of the much simpler management of antibiotic-loaded PMMA beads (Walenkamp et al. 1998). Although systemic and local therapies have therefore never been adequately compared, nor have intravenous and oral therapies except in small studies.

One final concern in the use of antibiotic-loaded cement is the potential for persistence of the infection around the new foreign body. This certainly can occur if the pathogen has absolute resistance to the antibiotic in use (even at the very high doses released) but there is also the potential that small colony variants could be induced by the antibiotic and then persist. Clearly, for this mechanism to be plausible, residual infection would have to be present at the point the beads were used, which would be a risk factor for recurrence anyway. Nonetheless, small colony variants have been recovered from relapsed osteomyelitis in which antibiotic-loaded beads had previously been used (von Eiff et al. 1997).

Duration of treatment

The duration of treatment required varies according to the severity of the disease at the beginning of treatment, a concept that for chronic osteomyelitis at least, includes some sense of the local and systemic host factors that contribute to outcome. Taking this into account, the overall trend of treatment has, in general, been to reserve very prolonged treatment for situations where tissue that is dead or otherwise inanimate is retained in the infected field, and/or where the host has major systemic compromise. Where surgical resection produces remaining tissues that are minimally contaminated and viable, antibiotic treatment durations are reducing. In acute osteomyelitis, overall durations of therapy for uncomplicated infection are also falling (Le Saux et al.

2002; Vinod et al. 2002), in some series to as little as two weeks. Suggestions are given in Table 3, but it must be stressed that these have not been determined by a large series of clinical trials.

Adjunctive treatment

A range of adjunctive treatments has been proposed, the most common one being hyperbaric oxygen (Mader et al. 1990; Stone and Cianci 1997; Bakker 2000; Wunderlich et al. 2000; Wang 1997). This has its strong advocates, and there is laboratory evidence to suggest enhanced bacterial killing with hyperbaric oxygen. It is also believed to support increased function of phagocytes. Despite the existence of these data, the role of hyperbaric oxygen in the patient who has had debridement surgery and antibiotics is unclear. Randomized controlled trials will be crucial in allowing an informed view of the role of this treatment to emerge.

Other commonly advocated adjunctive treatments include Manuka honey, which is believed to have broad antimicrobial activity, and Tea Tree Oil. There is little available information on the value of these agents.

A final modality worthy of consideration is the use of maggots (larvae of the greenbottle fly, *Lucilia serricata*). The larvae of this species eat only dead tissue, and can be reared from eggs on chemically defined, sterile media; furthermore, the eggs themselves are highly resistant to external agents and so can be sterilized of microbiological contamination (Jukema et al. 2002). Larvae reared in this way are thus effectively sterile at the point of application to a wound, and are capable of carrying out efficient debridement. Some believe they also stimulate the formation of healthy granulation tissue. The use of maggots of this sort for the treatment of osteomyelitis was first put forward in the pre-antibiotic era, as an adjunct to sequestrectomy. Their role without surgery, and the extent to which they are necessary in the era of antibiotics and modern plastic surgical methods, is not defined; they certainly have proponents in the diabetic foot world and may be of value in situations where conventional surgery is deemed to carry too high a risk. Microbiologically, maggots develop a bowel flora that includes *Proteus* spp., and it must be assumed that the wound will be contaminated with this. Certainly the maggot secretions and excreta can be highly irritant to skin surrounding the wound, which itself becomes very exudative, and measures must be taken not only to contain the maggots (for obvious therapeutic and aesthetic reasons) but also to manage these wound secretions. In addition, maggots can carry a range of serious pathogenic *Clostridium* spp. in their gut; there were historical accounts of tetanus, botulism, and gas gangrene secondary to maggot use before the need for absolute sterility from egg onwards was identified. Much more recently, outbreaks of septicemia have been associated with the use of other species of maggot, stressing the need for this form of treatment to be subject to careful design and quality control. As with so much else in the management of osteomyelitis, randomized controlled studies are lacking. Even if maggots are effective in the management of diffusely necrotic soft tissue, their role in removing dead bone is open to question; this function is likely to require human intervention in most cases.

PROGNOSIS

The prognosis for acute infections is excellent provided the diagnosis is made promptly and the treatment is appropriate. Cure rates are over 95 percent in modern series of acute childhood hematogenous osteomyelitis. Function is generally good on treatment of infection, but in childhood osteomyelitis, annual follow up is required to look for growth plate disturbance. Acute septic arthritis also carries a good prognosis, with poor outcomes highly associated with delay in diagnosis and treatment (Christiansen et al. 1999). Acute prosthetic joint infection similarly requires rapid intervention for optimal outcomes, with salvage rates from debridement and retention procedures that can reach 70 percent (Karchmer 1998). Certain organisms, for example *Staphylococcus aureus*, confer worse prognosis; this is also seen in prognosis following one-stage revision of prosthetic joints. In relation to cure of infection, suppression (retention of chronically infected joints with long-term antibiotics) fares worse than debridement and retention of acute infections (Deirmengian et al. 2003), which are themselves outperformed by one-, and finally, two-stage revision. These achieve cure rates of 80–95 percent when antibiotic loaded cement is used.

Chronic osteomyelitis has high recurrence rates unless radical excision of dead bone is combined with antibiotic treatment. Recurrence rates in expert series are as low as 10 percent. Diabetic foot osteomyelitis has a poorer outlook, and responds to treatment in only 60–70 percent of cases (Grayson et al. 1994; Venkatesan et al. 1997; Pittet et al. 1999). This is due to the very much less favorable host factors operating, notably degrees of ischemia, and the relatively muted responses well recognized in diabetics (Armstrong et al. 1996). As a result, treatment frequently culminates in minor or major amputation; this is in part due to failure to eliminate the bone infection, but in a substantial number of cases, amputation is precipitated by the soft tissue loss that led to the osteomyelitis. If not fully surgically resected, osteomyelitis in many locations is well known to be able to recur decades after the initial event (Donati et al. 1999). There is no good evidence to suggest that the likelihood of this phenomenon is dictated by the initial treatment; the mechanisms of such prolonged dormancy remain unclear.

Table 3 *Indications for surgery and suggested duration of treatment*

Condition	Indications for surgery	Host biology after any surgery	Duration
Acute septic arthritis	Hip, shoulder, and when antibiotics alone fail. Arthroscopic washout now common	Normal	2 weeks for streptococci, *Neisseria* spp., *Haemophilus* spp.; 3 weeks for *Staphylococcus aureus* and gram-negative rods
		Arthritic joint, exposed bone or devitalized fibro-cartilage	4–6 weeks
Chronic septic arthritis	May need resection and fusion/arthroplasty if joint destroyed	Generally abnormal joint surface, exposed bone, and debris	As chronic osteomyelitis for pyogenic bacteria; *Brucella* spp. 3 months; *Mycobacterium* spp. as for osteomyelitis
Acute prosthetic joint infection	Mandatory, to establish diagnosis, microbiology, debride infected tissue, provide soft tissue cover. Remove implant if loose	Implant removed	6 weeks
		Implant retained	6 months–2 years (varying practice, lifelong treatment sometimes given)
Chronic prosthetic joint infection	Loose implant; pain; sinus or abscess formation	Implant removed definitively	6 weeks
		Implant revised in one stage	3–6 months
		Implant revised in two stages	6–12 weeks
		Implant retained	Indefinite
Acute osteomyelitis	Abscess formation	Infected but viable bone, no abscess	2–4 weeks
		Complicated (surgery required, dead bone)	4–12 weeks
Chronic osteomyelitis: long bone and foot	Dead bone; loss of soft tissue cover; abscess; pain; multiple acute flares	The whole of the involved bone removed (ablative treatment, e.g. amputation), with normal remaining soft tissue	1–3 days
		The whole of the involved bone removed, but with residual soft tissue infection	1–2 weeks
		All dead bone removed, but viable portion of infected bone remains	4–6 weeks
		Residual dead bone currently or previously involved in infected field	12 weeks or more
Chronic osteomyelitis: spine	Abscess; spinal instability; pain; progressive deformity; spinal cord compression	No metal ware	6–12 weeks
		Retained metal in infected field	3–12 months

FUTURE DIRECTIONS

The area of musculoskeletal infection still needs definitive answers to questions such as how best to diagnose and treat infection in the most cost-effective way. The necessary duration, and the favored route of administration (if one exists) are yet to be determined, as is the difference in outcome and morbidity between one- and two-stage revision for prosthetic joint infection. Prediction of successful cure with medical therapy alone remains difficult, and there is yet to be a significant translation into clinical practice of recent advances in understanding the mechanisms of bone loss and bacterial pathogenesis.

CONCLUSIONS

Bacterial infections of bone and joint continue to pose major challenges and questions, which understandings of pathogenesis, diagnosis, and treatment options go only some way to answering. It is to be hoped that improved multidisciplinary working will increase clinical confidence in the management of these problems, and pave the way for the definitive studies that are required to establish a strong evidence base available to all.

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Conventional laboratory diagnosis of infection

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INTRODUCTION

Diagnosis of a microbial infection usually begins with an assessment of the clinical and epidemiologic features, leading to formation of a diagnostic hypothesis. Physical and radiological findings provide clues in determining the anatomic site of infection. This clinical diagnosis suggests a number of possible etiologic agents causing the infection, based on knowledge of infectious syndromes and their usual clinical courses. By applying various diagnostic microbiologic tests on clinical specimens, a specific microbial etiology can be established. Therefore, clear communication between the clinician and the clinical microbiology laboratory is essential to obtain optimal test results for arriving at an accurate microbiologic diagnosis. The clinician must select the appropriate tests and specimens to be processed and suggest the suspected microbial pathogens to the laboratory. The clinical microbiology laboratory must utilize a series of tests that would demonstrate the probable etiologic agents and be prepared to explore other possible agents suggested by the clinical features or findings of the laboratory examinations.

This chapter provides an overview of the appropriate use of the clinical microbiology laboratory and the various common conventional laboratory methods used for establishing the microbial causes of infection. Molecular laboratory methods are discussed separately in Molecular laboratory diagnosis of infection,
while additional specialized tests for identifying specific pathogens are described in the chapters devoted to specific microorganisms. Detailed descriptions of the principles and techniques of these diagnostic laboratory methods are available in several standard textbooks on this subject (Forbes et al. 2002; Murray et al. 2002).

CLINICAL SPECIMENS

Because the objective of most microbiologic laboratory tests is to isolate viable organisms, proper specimens must be collected and delivered promptly in a suitable transport system for processing in the laboratory. The results of these laboratory tests are limited by the quality of specimens and their condition on arrival in the laboratory. Errors in specimen collection and transportation are the most common reasons for failure to ascertain etiologic diagnoses of infections. Therefore, proper collection and transport of clinical specimens from the patient's bedside to the laboratory are the critical first steps in establishing a microbiologic diagnosis.

Collection

Specimens should be obtained from the patient with care to minimize the possibility of introducing extraneous or contaminating microorganisms that are not involved in the infectious process. This precaution is needed especially to distinguish resident (commensal) or 'normal' organisms from those causing the infection. The presence of commensal or normal flora at the anatomic site of infection will mask the true etiological agent, making its isolation more difficult. Of particular

Table 1 *Sites of infection and common sources of contamination*

Site of infection	Source of contamination
Middle ear	External ear canal
Nasal sinus	Nasopharynx
Lower respiratory tract	Oropharynx
Endometrium	Vagina
Superficial wounds/ subcutaneous infections	Skin and mucous membranes
Abdominal fistulae	Gastrointestinal tract
Bladder	Urethra and external genitalia

difficulty are specimens from sites listed in Table 1, which are adjacent to body sites having more resident flora than others. For example, expectorated sputum specimens are frequently contaminated during collection by organisms that are part of the indigenous or normal flora of the oropharynx. Knowledge of the potential contaminating organisms and the probable pathogens to be sought in these 'indirect' specimens is essential to avoid misinterpretation of results. Guidelines to assess the quality of such 'indirect' specimens have been developed to determine the suitability of a specimen for processing. Such an example is the use of microscopic examination of direct Gram-stained smear of expectorated sputum to indicate the high probability of contaminating flora when >10 squamous epithelial cells are present per low-power field (10× magnification).

Use of special techniques or procedures that bypass anatomic areas containing normal flora whenever feasible (e.g. protected brush bronchoscopy for diagnosis of ventilator-associated pneumonia) can prevent false-positive results. Careful skin antisepsis before collection procedures, such as blood cultures and lumbar punctures, will decrease the risk of false-positive findings from contamination by organisms normally present on the skin. Whenever possible, specimens should be collected directly from normally sterile tissues (e.g. lung, liver, bone marrow) and body fluids (e.g. blood, CSF, synovial fluid) by methods ranging from needle biopsy or aspiration to surgical biopsy. Positive findings obtained from these specimens are almost always diagnostic.

Though convenient and most commonly used for specimen collection, the sterile swab absorbs a small volume of specimen material and provides the poorest conditions for microbial survival. If available, infected tissue or needle aspirates of infected fluid should be submitted for microbiologic cultures. An adequate volume of specimen is important for optimal test results because infecting organisms present in small quantities may not be detected in a small sample. Specimens should be collected during the early (acute) phase of an infection and before initiation of antimicrobial therapy, whenever possible.

Transport and handling

Ideally, specimens should be transported to the laboratory as soon as possible, preferably within 30 min of collection. Some microorganisms can only survive briefly outside the body (e.g. *N. gonorrhoeae*) and are susceptible to environmental conditions, such as presence of oxygen (anaerobic bacteria), changes in temperature (*N. meningitidis*), or changes in pH (*Shigella*). To minimize the effects of delay between specimen collection and laboratory processing, various transport media have been developed for specimens that could not reach the laboratory in less than 30 min. These media are usually buffered fluids or semisolid media that contain minimal nutrients and special preservatives to prevent drying, maintain a neutral pH, and minimize bacterial growth during transport. Specialized media providing oxygen-free atmosphere are needed for transporting obligate anaerobes. Detailed comprehensive lists of optimal specimen selection and transport requirements are available in several authoritative references (Miller et al. 2003; Murray 1998).

When received in the laboratory, clinical specimens are processed with priority given to those that are most critical, such as blood, cerebrospinal fluid (CSF), sterile body fluids, and tissue. After macroscopic examination of each specimen to ensure appropriateness and adequate volume for the tests requested, some specimens may need to undergo initial processing by procedures before inoculation on to culture media. These procedures include homogenization (grinding) of tissue, concentration by centrifugation or filtration of large volumes of sterile fluids (ascites, pleural fluid), or decontamination to remove commensal flora that may interfere with recovery of fastidious pathogenic microbes (e.g. mycobacteria, *Legionella*).

DIAGNOSTIC METHODS

The general approaches to laboratory diagnosis of infectious diseases vary with different infective pathogens and infections. The types of conventional diagnostic methods employed for testing clinical specimens are usually a combination of direct examination, culture, microbial antigen detection, and detection of patient's antibody response (serology). In recent years, molecular laboratory methods (see Molecular laboratory diagnosis of infection) capable of directly detecting microbial genomic components have gained increasing importance and popularity for the sensitive and rapid detection of fastidious pathogens that are otherwise difficult to detect by conventional approaches.

Direct examination

Other than some of the parasites, none of the microbial pathogens present in clinical specimens are large enough

Table 2 *Utility of microscopic methods for diagnostic microbiology*

Microscopic methods	Bacteria	Fungi	Viruses	Parasites
Bright-field (light) microscopy	+	+	−	+
Dark-field microscopy	±	−	−	−
Phase-contrast microscopy	−	±	−	±
Fluorescent microscopy	+	+	+	+
Electron microscopy	−	−	±	±

+, commonly used; ±, limited use; −, rarely used.
Adapted from Forbes et al. (2002).

to be visible with the naked eye. Therefore, microscopic examination of clinical specimens is necessary for the initial detection and preliminary or definitive identification of bacterial cells in infected cells. In diagnostic microbiology, five microscopic methods are used with varying utility for each of the four major microbial pathogen groups (Table 2). To better visualize and differentiate these microbes in smears of clinical specimens and histologic sections of tissues, various stains are used commonly in the diagnostic laboratory. As shown in Table 3, these staining techniques are adapted for specific microscopic methods in detecting and identifying certain microorganisms.

LIGHT MICROSCOPY

Direct examination of unstained or stained preparation of clinical specimens by bright-field (light) microscopy is used commonly for detection of bacteria, fungi, and parasites. Due to the minute sizes of bacteria (usually 0.3–0.5 μm wide), oil immersion magnification (500× to 1 000×) is necessary to visualize these organisms. Unstained bacteria are too transparent to be seen directly, but two important staining methods, Gram stain and acid-fast stains, are frequently employed to stain and classify bacteria by light microscopy.

Gram stain

As the principal stain used for microscopic examination of bacteria, the Gram stain divides most bacteria into two groups: gram-positive (purple-colored) when the alkaline dye, crystal violet, is retained by the organism, and gram-negative (pink-colored) when the crystal violet dye is lost with uptake of counterstaining safranin dye. Due to abundant peptidoglycan and teichoic acid cross-linkages in their cell wall, gram-positive bacteria are able to retain the crystal violet dye which is chemically bonded to the Gram's iodine reagent, while resisting alcohol or acetone decolorization in the subsequent

Table 3 *Microscopic preparations and staining methods commonly used for examination of clinical specimens in diagnostic microbiology*

Staining method	Detectable microorganisms
Direct examination	
Wet mount	Parasites and ova (examined by bright-field, dark-field, or phase-contrast microscopy)
10% KOH	Fungal hyphae
Lugol's iodine	*Entamoeba*, other amoeba
Differential stains	
Gram stain	Bacteria, yeast cells
Iron hematoxylin stain	Fecal protozoa
Toluidine blue O stain	*Pneumocystis jiroveci*
Trichrome stain	Fecal protozoa
Wright–Giemsa stain	Blood parasites (filaria, *Plasmodium*, *Babesia*), *Borrelia*, *Rickettsia*, *Ehrlichia*, *Anaplasma*, *Toxoplasma*, *Leishmania*, *Pneumocystis*, viral and chlamydial inclusion bodies
Acid-fast stains	
Ziehl–Neelsen stain	*Mycobacterium* spp.
Kinyoun stain	*Mycobacterium* spp.
Modified acid-fast stain	*Nocardia*, *Rhodococcus*, *Gordonia*, *Tsukamurella*, *Cryptosporidium*, *Isospora*, *Sarcocystis*, *Cyclospora*
Fluorescent stains	
Acridine orange stain	Bacteria cells, fungi elements
Auramine-rhodamine stain	*Mycobacterium* spp.
Calcofluor white stain	Fungal elements, *Pneumocystis*
Direct fluorescent antibody stain	*Chlamydia*, *Bordetella*, *Francisella*, *Legionella*, *Neisseria*, *S. pyogenes*, *Cryptosporidium*, *Giardia*, RSV, influenza virus, HSV, VZV

Adapted from Murray et al. (2002).

staining procedure. Gram-negative bacteria, having a thinner cell wall layer of peptidoglycan, lose the crystal violet dye easily during the decolorization process and appear pink in color from uptake of the safranin counterstain dye. Gram-positive bacteria without an intact cell wall because of cell damage, old age, or antibiotic therapy, may fail to retain the crystal violet dye during decolorization and appear 'gram-variable,' with a mixture of purple- and pink-staining cells.

Acid-fast stain

The acid-fast stain is used specifically for direct light microscopic detection of mycobacteria and related bacteria in clinical specimens. The presence of mycolic acids in their cell wall renders this group of bacteria 'acid-fast' (resistant to acid alcohol decolorization) and difficult to stain with dyes, including Gram stain. However, they can be stained by prolonged application of highly concentrated dyes, with or without the use of heat treatment to facilitate the staining process. Once stained, acid-fast bacteria resist acid alcohol decolorization which removes the same dyes from other bacteria. Ziehl–Neelsen stain is the classic acid-fast stain with heated carbol-fuchsin (red) applied as the primary stain, followed by decolorization with 3 percent HCl–alcohol and counterstaining. A modified method, also known as the Kinyoun acid-fast stain, involves using carbol-fuchsin containing a higher concentration of phenol to facilitate penetration of the primary dye without use of heat.

DARK-FIELD MICROSCOPY

Using special optical features of dark-field microscopy, bacteria that are usually too thin to be visualized by light microscopy can be seen as objects surrounded by a bright halo amid a dark background. This method is used to detect spirochetes, such as *Treponema pallidum* (syphilis) and *Borrelia* spp. in clinical specimens.

PHASE-CONTRAST MICROSCOPY

Phase-contrast is an optical technique that yields visible contrast among microbial cells and cell structures in a given specimen. Objects with different thickness or densities have different refractive indices that produce different light intensities (phases) in an image, allowing better visualization of cell structures in unstained specimens. Whereas microscopic examination of stained specimens permits observation of dead organisms, phase-contrast microscopy offers the advantage of observing viable organisms of interest. This method is used mainly for the identification of fungi and parasites.

FLUORESCENT MICROSCOPY

Using similar optical features as dark-field microscopy, fluorescent microscopy is used to detect microorganisms that are labeled by a fluorescent dye (e.g. acridine orange, calcofluor white, auramine-rhodamine) or by microbial antigen-specific antibody conjugated with a fluorescent dye (immunofluorescence). Against a dark background, the organism of interest appears with a halo emitting color of the fluorescent compound. This method is used to detect organisms that are difficult or slow to grow or to identify those already grown in culture.

ELECTRON MICROSCOPY

With its high magnification of $\geqslant 100\,000\times$, electron microscopy is used mainly for the detection and identification of viruses in clinical specimens and viral cultures. It is also a useful method for the confirmation of microsporidia (e.g. *Enterocytozoon*, *Encephalitozoon*) in clinical specimens. However, the need for specialized equipment limits the availability of this method for routine use in diagnostic microbiology.

Isolation and identification

The most definitive and specific means of diagnosis of an infection is the isolation and identification of the infecting organism in vitro from clinical specimens. To identify and characterize the organism, it must be grown and isolated in the clinical laboratory. Although most bacteria can be cultivated by various artificial media, strict intracellular organisms such as *Chlamydia*, *Rickettsia*, and *Ehrlichia* can be isolated only from cultures of living eukaryotic cells (cell cultures).

Cultivation of bacteria in the laboratory involves inoculating clinical specimens obtained from the infection site into culture media and growing the microorganisms of interest in vitro. Once they are grown in culture, the organisms are present in sufficient amounts to allow further diagnostic laboratory testing to be carried out. Their survival depends on the availability of essential nutrients and appropriate environmental conditions in vitro. The nutritional needs include different gases, water, ions, sources of carbon and nitrogen, and energy. For certain bacteria, the needs are complex, so that exceptional nutrient components must be added in the culture media to support their growth. Pathogens with such growth requirements are said to be fastidious. To date, the growth requirements of certain clinically relevant bacteria, such as *T. pallidum* and *M. leprae*, remain poorly understood such that these pathogens cannot be grown in vitro.

BACTERIOLOGIC CULTURE

Various artificial culture media are employed in the diagnostic laboratory to facilitate the growth of medically important bacteria to be detectable by the naked eye. These media are essentially recipes prepared from digests of animal or plant protein supplemented with nutrients such as glucose, yeast extract, serum, or blood, to meet the metabolic requirements of the organisms (Atlas 1997; Chapin and Lauderdale 2003; Chapin and

Murray 2003). Bacteriologic media can be prepared in solid (agar), semisolid, and liquid (broth) forms to which bacteria or clinical specimens may be added directly. On solid agar plates, bacteria grow as discrete sediment (colonies) or as a film on the agar surface, while their growth is not visible in semisolid and broth media until there are 10^6 to 10^7 bacteria per milliliter of media to produce turbidity or macroscopic clumps.

Compared to broth cultures, solid media offer advantages for diagnostic work, since discrete colonies of bacteria in pure culture grow well separated from one another on the agar surface and arise from a single organism or an organism cluster (colony-forming unit). Bacterial colonies differ significantly among different genera or species, varying greatly in size, shape, color, texture, and other characteristics. For example, colonies of *S. pneumoniae* possessing large polysaccharide capsules are usually mucoid in appearance, while those of organisms that fail to separate after division are frequently granular. These features are usually consistent for colonies derived from the same strain of organism. Therefore, differences in colonial morphology are very useful as clues to bacterial identity and for separating them in mixtures. Broth media offer the advantage of providing a favorable homogeneous environment for enhancing bacterial growth in recovering organisms that are present in minute quantities in clinical specimens. However, broth cultures of clinical specimens frequently yield mixtures of bacteria that are not readily separated for identification.

Bacteriology media may be classified as nutrient, enrichment, selective, or differential media according to their main function in the laboratory. Nutrient media, also known as supportive media, are designed to provide the growth requirements of most nonfastidious bacteria without giving growth advantage to any particular organism. Mueller–Hinton medium and brain–heart infusion in agar or broth are such examples. Enrichment media contain specific nutrients (e.g. trace metals, coenzymes, vitamins) necessary for the growth of certain bacteria. They are used to enhance the growth of a particular bacterial pathogen from a mixture of organisms on the basis of nutrient specificity. Typical examples are thioglycolate broth, chocolate agar (*Neisseria* and *Haemophilus* species), and buffered charcoal-yeast extract agar (*Legionella* spp.). Selective media contain dyes, bile salts, acids, alcohols, or antimicrobial agents that are inhibitory to all organisms except those being sought. Selecting for the growth of certain bacteria to the disadvantage of others, these media are useful for isolating specific pathogenic organisms from clinical specimens containing extensive contaminating flora. *Campylobacter*–blood agar, mannitol salt agar or broth (staphylococci), and phenylethyl alcohol agar (gram-positive cocci) are commonly used selective media. Differential media contain substances that allow colonies of specific pathogens or organism groups to exhibit certain biochemical or other culture characteristics distinguishable from other organisms growing in the same media. Such culture features may be based on changes in pH as a result of carbohydrate metabolism, enhancement of pigment production, or hemolysis of red blood cells present in the media. Examples of such media are bile esculin agar (enterococci), MacConkey sorbitol agar (*E. coli* O157:H7), and sheep blood agar (streptococci). Some media have multiple functions, such as enriched and selective (Thayer–Martin agar for *N. gonorrhoeae* and *N. meningitidis*, Columbia colistin-nalidixic acid with 5 percent blood for gram-positive cocci), enriched and differential (sheep blood agar for hemolytic streptococci), selective and differential (eosin methylene blue agar and MacConkey agar for gram-negative bacilli, Hektoen enteric agar for *Salmonella* and *Shigella* spp., thiosulfate citrate-bile salts agar for *Vibrios* spp.).

BACTERIAL IDENTIFICATION

In addition to Gram-stained smears, conventional methods of bacterial identification involve examination of the cultural characteristics, as well as biochemical and physiologic testing on pure cultures obtained from single colonies. The exact tests and the sequence of testing depend on the suspected group of bacteria and the taxonomic level of identification (e.g. genus, species, subspecies) needed for diagnosis and treatment of the infection. Important properties of bacterial cultures that aid in identification include unique nutritional requirements, pigment production, and the ability to grow in the presence of certain substances (e.g. bile, sodium chloride) or on certain selective or differential media (e.g. MacConkey agar, Hektoen enteric agar), growth at particular temperature, and presence and type of hemolysis (alpha, beta, gamma) on blood agar.

The ability to react with certain chemical substrates and the production of particular metabolites form the basis of biochemical tests for bacterial identification. Such tests include utilization of carbohydrates (e.g. sucrose, glucose, lactose) and citrate, ability to metabolize certain amino acids (e.g. lysine, ornithine, arginine), oxidase reaction, and production of catalase, coagulase, proteinase, urease, indole, and hydrogen sulfide (Chapin and Lauderdale 2003). The results of these tests are analyzed by reference to published algorithms for identification of many bacteria (Ruoff 2003; Funke 2003; Schreckenberger and Wong 2003; Citron 2003; Versalovic 2003).

Bacteria may also be identified by the presence of their antigenic structures, such as capsular polysaccharides, flagellar proteins, and cell wall components. In addition, in vitro detection of specific bacterial toxins, with neutralization of the toxic effect by specific antitoxin, may be necessary to confirm the identity of pathogenic bacteria (e.g. Elek immunodiffusion test for

Corynebacterium diphtheriae toxin) or a clinical diagnosis (e.g. *Clostridium difficile* toxin). Serologic techniques using bacteria-specific antibodies can detect these antigens present on whole bacteria or free in bacterial lysates (soluble antigens). Further details of these methods are discussed in a later section.

Blood cultures

The primary means for establishing diagnosis of bloodstream infections due to bacteria and fungi is by blood culture, which is based on the same microbiologic principles of any culture. A sample of patient's blood is obtained by aseptic venipuncture and inoculated into an enriched broth or agar plate. Once growth is detected by manual or automated methods, the organisms are isolated, identified, and tested for antimicrobial susceptibility. Careful attention must be paid to details of obtaining blood from the patient in order to maximize the yield of blood culture and properly interpret the results.

Proper antiseptic techniques should be used to prepare the skin over the vein for phlebotomy, so that contamination of the blood culture by skin microbial flora is minimized. Blood should not be drawn through indwelling venous or arterial catheters unless it cannot be obtained by venipuncture, or if it is for detection of intravascular catheter-associated sepsis. Many commercially available blood culture media contain an anticoagulant, sodium polyanethol sulfonate (SPS), which is free of antimicrobial properties and has anticomplementary and antiphagocytic activities. SPS also interferes with the activity of some antibiotics, notably aminoglycosides, that are present in the blood of patients receiving antibiotic therapy. Blood cultures obtained from patients after initiation of antimicrobial therapy are not useful unless none was collected before starting therapy or the clinical course of the infection suggests superinfection with a drug-resistant or other microorganism.

Since the number of organisms present in blood is often low (<10 organisms/ml), the yield of blood cultures in a given patient is also dependent on the amount of blood and the number of blood cultures obtained. The diagnostic yield increases as the blood volume drawn is increased. Collection of a minimum 5–10 and 20 ml of blood per culture is strongly recommended for pediatric and adult patients, respectively. Because microorganisms causing infection may enter the blood continuously, intermittently, or transiently, blood infections may not be detectable by a single blood culture. If the blood volume drawn per culture is adequate, two or three blood cultures collected at brief intervals (30–60 min apart) are usually sufficient to yield positive results.

Most diagnostic laboratories currently utilize automated blood culture systems, such as BacT/ALERT® 3D System (bioMerieux, Durham, NC, USA), Bactec™ 9000 Systems (Becton Dickinson Diagnostic Systems, Sparks, MD, USA), and ESP® Culture System II (TREK Diagnostic Systems, Cleveland, OH, USA), for consistent quality control and optimal work efficiency. However, some pathogens, especially intracellular microorganisms such as *Histoplasma*, *Legionella*, and *Mycobacterium* spp., are not easily recovered by these automated culture systems. The lysis centrifugation blood culture method (e.g. ISOLATOR™ Microbial Tubes; Wampole Laboratories, Cranbury, NJ, USA) would be helpful for detection of bloodstream infections due to these pathogens. The ISOLATOR™ tubes contain SPS and saponin which causes lysis of blood cells, releasing intracellular pathogens into the blood. After centrifugation, the supernatant is discarded and the sediment containing the pathogens is inoculated on to solid agar plates capable of supporting the growth of the pathogens of interest. This manual blood culture method provides the following benefits: more rapid and greater recovery of the pathogens, presence of colonies for direct identification and susceptibility testing after initial incubation, and ability to quantify the number of colony-forming units present in the blood.

Results of blood cultures yielding growth of coagulase-negative staphylococci, *Corynebacterium* spp., *Propionibacterium acnes*, *Bacillus* spp. should be interpreted with caution because these organisms are part of the normal skin flora and are frequent contaminants in blood cultures. Growth of multiple species from only one of several cultures would also suggest probable contaminants, since polymicrobial bloodstream infections are uncommon. When the same isolate is recovered from multiple blood cultures obtained at different times or from different anatomic sites, bloodstream infection from this organism is probably real.

Blood cultures obtained for the diagnosis of infections due to fastidious pathogens require special processing in the laboratory, so that special procedures or culture media are used to support the growth of these organisms. Routine automated blood cultures are unlikely to recover such pathogens as *Brucella*, *Borrelia*, *Leptospira*, *Bartonella*, *Mycoplasma hominis*, *Legionella*, *Mycobacterium* spp., and the HACEK group of fastidious gram-negative bacilli. The laboratory should be alerted to process the blood cultures accordingly when one of these pathogens is suspected to cause bloodstream infection in a given patient.

Antimicrobial susceptibility testing

In vitro susceptibility testing of microbial pathogens to antimicrobial agents is an important function of the clinical microbiology laboratory. Testing guidelines, standardized procedures, and interpretative criteria are regularly updated and available from the National Committee for Clinical Laboratory Standards (NCCLS)

in the USA (NCCLS, 2000, 2001, 2002a, b, 2003a, b, c, d, e) and from its counterparts in other countries (EUCAST 2000, 2003). The goal of susceptibility testing is to provide data on antimicrobial resistance in conjunction with other diagnostic information to optimize therapy. Of the methods available to detect and evaluate antimicrobial resistance in bacteria, the conventional methods of directly measuring the activity of the antibiotics against a given infecting isolate are the most widely employed by laboratories. These conventional testing methods include broth dilution, agar dilution, and disk diffusion.

The broth dilution susceptibility test consists of inhibiting the growth of the isolate of interest by a series of doubling broth dilutions (e.g. 0.25, 0.5, 1, 2, 4, 8, 16 μg/ml) of a given antibiotic. The concentration range tested for a particular drug depends on the following criteria: (1) the drug concentration that is safely achievable in a patient's serum (i.e. pharmacokinetic properties of the drug); and (2) the microorganism and its associated antimicrobial resistance features that the test is attempting to detect. The lowest antibiotic concentration that completely inhibits visible pathogen growth in the broth solution is known as the minimal inhibitory concentration (MIC). Broth dilution testing can be performed in two approaches with the same principles: macrodilution and microdilution. The macrodilution method involves testing the pathogen in test tubes containing antibiotic dilution in a broth volume of $\geqslant$1 ml per tube. This approach is cumbersome and labor intensive, and it is rarely used in clinical laboratories. For microdilution testing, the total broth volume is in the range of 0.05 to 0.1 ml for each antibiotic dilution. This method is usually adapted in a convenient, single microtiter tray format with multiple sample wells for several serial dilutions of various antibiotics. Several commercially available manual and automated susceptibility testing systems in use at many laboratories are developed according to this format: MicroScan® WalkAway System (Dade International, Sacramento, CA, USA), BD PHOENIX™ Automated Microbiology System (BD Diagnostic Systems, Sparks, MD, USA), Vitek® 2 System (bioMérieux, Inc., Durham, NC, USA), Sensitive® Microbiology Systems (TREK Diagnostic Systems Inc., Cleveland, OH, USA), and PASCO MIC Panels (PASCO Laboratories, Wheat Ridge, CO, USA).

In agar dilution susceptibility testing, each dilution of a given antibiotic is incorporated into a single agar plate, on which multiple isolates can be inoculated each at a standardized inoculum of 10^4 colony-forming units (cfu). A series of six dilutions of an antibiotic would require using six antibiotic-impregnated agar plates and one positive growth control plate without antibiotic, with $\geqslant$1 isolates tested per plate. After incubation, the plates are examined for growth, with the MIC being the lowest concentration of an antibiotic in agar that completely inhibits visible growth. The main advantage of this method is that MICs can be determined for fastidious organisms, such as *N. gonorrhoeae* and *E. corrodens*, that do not grow well in the broth media used in the broth dilution method. However, preparation of agar dilution plates is labor intensive, so this testing method is limited to use by reference laboratories testing large numbers of isolates daily.

The disk diffusion method is a practical and convenient approach of antimicrobial susceptibility testing, in which antibiotic-impregnated filter paper disks ('antibiotic disks') are placed on the surface of an agar plate that has been inoculated with a lawn of bacteria. Each antibiotic disk contains a known concentration of antibiotic, and the drug diffuses out of the disk into the surrounding agar, creating a concentration gradient around the disk. During incubation, the bacteria grow on the surface of the agar plate, except where the antibiotic concentration gradient is sufficiently high to inhibit growth. Findings of in vitro studies are used to correlate the size (in millimeters) of the resulting zone of inhibition around each disk with the MIC of the drug obtained by broth or agar dilution method.

A commercial susceptibility testing method that combines the convenience of disk diffusion with the ability to determine MIC data is the Etest® (AB Biodisk, Solna, Sweden). It is a unique patented antimicrobial gradient diffusion technique in which a predefined gradient of 15 dilutions of an antibiotic is impregnated on to a single plastic reagent strip. The strip is then placed on to the surface of an inoculated agar plate, allowing a gradient of antibiotic to diffuse from the strip into the surrounding agar. After incubation, the resulting elliptical zone of inhibition intersects the scale of antibiotic dilution markings at the indicated MIC on the strip. Reagent strips of various antibiotic agents are available for testing both fastidious and nonfastidious bacteria and *Candida* spp. The main disadvantage of this method is the relatively high cost of the reagent strips when several antibiotic strips are needed to test a given pathogen isolate.

To interpret test results reliably, antibacterial susceptibility testing methods are standardized and carefully controlled according to the appropriate inoculum size of the organism, conditions of growth medium (pH, calcium and magnesium cation concentrations, blood and serum supplements, thymidine content), incubation atmosphere, temperature and duration, and antibiotic concentrations tested (NCCLS 2003d). Procedural standards and interpretive guidelines have been published for the broth and agar dilution methods (NCCLS 2001, 2002a, b, 2003b) and the disk diffusion testing method (NCCLS 2003a, c). Use of the interpretive guidelines enables laboratories to report susceptibility test results on a pathogen as 'susceptible,' 'intermediate,' or 'resistant' to a given antibiotic. However, in some instances, the MIC value is more valuable in directing therapy than a simple qualitative result because the clinician can

determine the potential success or failure of antibiotic therapy when comparing the MIC to the antibiotic level achievable at the anatomic site of infection. Because no one method can provide adequate testing of all clinically important bacteria, most diagnostic laboratories usually employ a combination of the susceptibility testing methods described above.

Detection of microbial antigens

Immunologic techniques are frequently used to detect, identify, and quantitate antigens of microbes directly in clinical specimens or from microbiologic cultures. These techniques are based on binding interactions between antibody and specific antigen, forming antibody–antigen complexes that can be detected directly by precipitation methods or by labeling of the antibody with a radioactive, fluorescent, or enzyme probe. The complexes can also be detected indirectly by measurement of an antibody-directed reaction, such as complement fixation.

PRECIPITATION AND IMMUNODIFFUSION METHODS

The principle of these tests is based on the formation of precipitate from interaction between soluble antigen and antibody. The precipitate is visible only because the antigen–antibody complexes formed are too large to remain soluble and therefore precipitate. At antigen-to-antibody concentration ratios above and below an equivalence concentration, the complexes are soluble. Further details are described in the section on Serologic methods below.

Immunodiffusion (ID) assays are adaptations of the precipitation technique to determine the identity of an antigen or the presence of antibody. In single radial immunodiffusion tests, antigen is placed into a well and allowed to diffuse into surrounding agar containing a specific antibody. The higher the concentration of antigen, the farther it diffuses in the agar to reach equivalence with the antibody and forms a precipitate ring around the well.

AGGLUTINATION TESTS

When relatively large particles coated with antigen or antibody are allowed to react with the specimen of interest (e.g. serum, CSF, urine), the resulting antigen–antibody reactions are detectable visually by agglutination. Soluble antigen or antibody can be fixed on to the surface of red blood cells or microscopic latex particles, and the assays are known as passive hemagglutination and latex agglutination, respectively. Bacterial antigens present on surfaces of whole bacteria that are large enough to behave as particles may also agglutinate with bacteria-specific antibody (bacterial agglutination). These assays are rapid and technically simple tests for identification of bacteria isolated in pure cultures (e.g. coagglutination test for staphylococcal surface protein A) and for detecting microbial antigens directly in clinical specimens.

IMMUNOFLUORESCENCE ASSAYS

Antigens present on the cell surface or within the cell can be detected by direct or indirect immunofluorescence (IF). In direct IF, antibody to the specific antigen is bonded directly with a fluorescent molecule, usually fluorescein isothiocyanate (FITC), whereas in indirect IF, a second antibody specific for the primary antibody is labeled and used to detect the primary antimicrobial antibody and locate the antigen. Indirect IF assays are usually more sensitive than direct IF tests, but the latter are faster, involving only one incubation step. IF tests are used to identify *Bordetella pertussis* in culture and to detect *Legionella pneumophila* in clinical specimens.

ENZYME IMMUNOASSAYS

Enzyme immunoassays (EIA), also known as enzyme-linked immunosorbent assay (ELISA), utilize antibodies conjugated to enzymes (e.g. horseradish peroxidase, alkaline phosphatase) capable of catalyzing a reaction yielding a visible end product, while the antibody-binding sites remain free to react with their specific antigen. The advantages of such assays are: ability to amplify the antigen–antibody reaction and enhance the sensitivity of detection, relatively long-term stability of the enzyme-conjugated antibodies during storage, and formation of a colored end product that allows both visible detection or automated spectrophotometric measurement. The use of monoclonal antibodies also helps to increase the specificity of these assays. Examples of commercial EIA developed for the direct detection of bacteria in clinical specimens include *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Helicobacter pylori*, and *Chlamydia trachomatis*.

Serologic methods

Immunocompetent humans produce both IgM and IgG antibodies against pathogens during infection. In general, IgM is produced during the first exposure to a given pathogen and is no longer detectable after this relatively short period of time. Over time, production of IgG replaces that of IgM in the immunologic response of the exposed patients. This humoral immune response in the infected hosts is the basis of serologic diagnosis of infection. Serologic testing is commonly used to identify infections due to bacteria that are difficult to be detected by other conventional methods, to evaluate the course of an infection, and to determine the nature of the infection (primary infection versus reinfection, acute versus chronic infection). Serologic results are usually expressed as a titer, which is the inverse of the greatest

dilution, or lowest concentration (e.g. dilution of 1:16 = titer of 16) of a patient's serum that retains the specific antibody–antigen reactivity. Patient's specific IgM or IgG titers can be determined separately through the use of labeled antihuman antibody specific for the antibody isotype in the particular serologic assays.

Serologic diagnosis of infection is confirmed usually by occurrence of seroconversion during a primary infection. Seroconversion is defined as a minimum four-fold increase in antibody titer between serum during the acute phase of infection and that during the convalescent phase ($\geqslant$2 weeks later). Reinfection or recurrent infection later in life causes an anamnestic (secondary or booster) immune response which can be detected as increase in antibody titer.

Most of the immunologic methods for detecting and identifying microbial antigens are also applied to serologic diagnosis by reversing the detection system: using a known antigen to detect the presence of the specific antibody. These serologic methods used in diagnostic laboratories include precipitation, immunodiffusion, agglutination, hemagglutination inhibition, EIA, indirect IF, complement fixation, radioimmunoassay, and Western blot. Some of these methods have been discussed in detail in the above sections.

PRECIPITIN TESTS

As described above, these tests are based on formation of a visible precipitate when antigen and antibody are combined in proper proportions. Two variations of the precipitin tests, flocculation and counterimmunoelectrophoresis (CIE), are available for serologic studies. In flocculation tests, the precipitin forms macroscopically and microscopically visible clumps. An example of such a test is the Venereal Disease Research Laboratory test (VDRL), in which the test antigen (cardiolipin–lecithin-coated cholesterol particles) binds to an antibody-like protein (reagin) in sera of patients with syphilis, causing flocculation of the particles. However, since reagin is not an antibody specific for *T. pallidum* antigens, the test is not highly specific, but is a good screening serologic test. A comparable qualitative serologic test, the rapid plasma reagin (RPR) test, is more widely used than VDRL in laboratories, because of increased specificity and ease of performing the assay.

CIE takes advantage of the net electric charge of the antigen and antibody being tested in a particular test buffer solution, when the two components migrate towards each other in a semisolid matrix under the influence of an electrical current. When the antigen and antibody meet in optimal proportions, a line of precipitation appears. The optimal performance of CIE tests depends on many variables, such as buffer pH, type of agarose gel, amounts of antigen and antibody placed, and amount of electrical current. These tests are difficult to develop and perform and are not commonly used.

COMPLEMENT FIXATION TEST

Complement fixation (CF) assays utilize two properties of complement: complement is inactivated in the presence of antigen–antibody complexes, and complement causes hemolysis of sheep red blood cells (RBC) coated with anti-sheep RBC antibodies (sensitized RBCs). In this assay system, laboratory-derived antigen and patient's serum containing the antibody of interest are allowed first to react in the presence of excess complement, followed by addition of sensitized RBCs to detect residual complement. Hemolysis indicates that complement was present and therefore antigen–antibody complexes were not formed in the reaction. Useful for detecting and quantitating antibody, CF is a test method used in reference laboratories for detecting infections due to *Mycoplasma pneumoniae*, *Chlamydia* and *Chlamydophila* spp., and *Coxiella burnetii*. The CF assay is a technically difficult serologic test and it has been replaced by the use of other simpler methods in most laboratories.

IMMUNOFLUORESCENCE ASSAYS

Due to increased specificity, indirect IF is the preferred technique of IF for detection of specific antibodies in patient serum. In these tests, specific microbial antigens are affixed to the surface of the microscope slide, on which serially dilutions of patient serum are added to form antigen–antibody complexes. After removal of unbound antibodies from the slide surface, fluorescein-conjugated antihuman globulin (directed against human IgG or IgM antibody) is added, and the slide is visualized under fluorescent microscopy. Commercially available indirect IF tests are widely used for detection of antibodies against *Legionella* spp., *B. burgdorferi*, *C. trachomatis*, *M. pneumoniae*, *Rickettsia* spp., and *Ehrlichia* spp.

ENZYME IMMUNOASSAYS

Using specific antigen immobilized on the surface of plastic wells, bead, or some other solid matrix, the antibody of interest in patient serum can be separated from other nonspecific antibodies, captured and quantified according to the intensity of the color produced from the enzymatic reaction. Various EIA are available commercially for detecting specific antibodies to *B. burgdorferi*, *H. pylori*, *M. pneumoniae*, chlamydiae, and other bacteria.

RADIOIMMUNOASSAY

Radioimmunoassay (RIA) is performed as a capture assay, such as that for ELISA, or as a competitive assay. Radioactively labeled antigen or antibody is used to quantitate the amount of antigen–antibody complexes formed. In the competitive assay, the antibody of interest in patient's serum is measured by its ability to compete with laboratory-prepared radiolabeled antibody for the antigen. The antigen–antibody complexes are

precipitated and separated from free antibody, and radioactivity is measured for both fractions. The amount of serum antibody is determined from standard calibration curves obtained from testing known quantities of competing antibody. RIA has been largely replaced by other equally sensitive methods, such as ELISA and indirect IF test, that are not associated with the disposal problems of radioactive reagents and the health hazards of radioactive exposure.

WESTERN BLOT IMMUNOASSAY

This assay involves separating microbial proteins (antigens) by electrophoresis according to molecular weight or electrical charge in a two-dimensional agarose gel matrix. These protein 'bands' are then transferred (blotted) from the gel on to a membrane (e.g. filter paper, nitrocellulose, nylon) to immobilize the proteins. When allowed to react with patient's serum, antibodies will bind to the immobilized proteins and are visualized as bands on the membrane with an enzyme-conjugated antihuman antibody. Western blot assays are mainly used for the confirmation of microbial antigen-specific antibodies in sera of patients who usually produce numerous cross-reacting antibodies in response to certain infections, such as Lyme disease.

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Molecular laboratory diagnosis of infection

ROBIN PATEL

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BACKGROUND

For a variety of reasons, molecular diagnostic tests are being increasingly used for the diagnosis of infectious diseases. Advances in testing formats, including automated specimen preparation instrumentation and rapid amplification and detection technologies, have enabled the introduction of molecular microbiologic testing into even the most routine diagnostic laboratories. For some infections (e.g. herpes simplex viral encephalitis), molecular testing is *the* diagnostic approach of choice. For others, such as hepatitis C virus (HCV) infection, aside from serologic testing (which detects the host's immune response to the infectious agent), molecular diagnostics are routinely used. Even for infectious agents where conventional tests exist (e.g. culture for *Bordetella pertussis*), molecular diagnostic tests are increasingly favored because of improved sensitivity and speed as compared with conventional approaches (Sloan et al. 2002). Besides qualitative detection of microbes in clinical specimens, nucleic acid technologies are also used to quantify microorganisms (e.g. human immunodeficiency virus-1 (HIV-1)) in clinical specimens, to classify microorganisms isolated from clinical specimens, to detect and characterize antimicrobial resistance, and for epidemiology studies.

Nucleic acid amplification technologies have enabled substantial advances in the diagnosis of infectious disease agents because of their ability to detect small amounts of nucleic acid in a wide variety of clinical specimens. Molecular assays are independent of the presence of viable (e.g. able to be cultured) microorganisms. Quantification of microbial nucleic acid in patient specimens enables myriad potential innovations in clinical practice including assessment of disease severity, progression and response to therapy. Quantification of RNA levels is valuable both for measuring organisms with genomic RNA (e.g. HIV-1), and, potentially, for studies of microbial and host gene expression.

The polymerase chain reaction (PCR) was the first nucleic acid amplification technique to be broadly applied to the molecular detection of microorganisms and is a standard method used in clinical microbiology laboratories today. Since the original description of

PCR, numerous technological advances have facilitated its application to routine clinical molecular microbiology diagnostics. Additionally, a number of non-PCR amplification molecular methods are commonly used in clinical microbiology laboratories.

Although molecular tests substantially enhance diagnostic capabilities, as with any laboratory test, results should be interpreted within the clinical context and on the basis of individual assay performance characteristics. Clinical research and strict adherence to guidelines for method validation are needed to compare molecular diagnostic techniques with conventional methods, to validate molecular approaches where comparable conventional techniques are unavailable, and to determine the clinical usefulness of molecular diagnostic testing.

Although molecular diagnostic tests have classically been considered more expensive (i.e. 'boutique' tests) than conventional diagnostic techniques, the ease with which molecular tests can now be performed and the rapid results generated enable timely diagnosis, resulting in overall savings. Cost savings may be realized as rapid diagnoses prevent invasive diagnostic procedures, limit unnecessary or potentially toxic treatment, shorten hospital stays, replace labor-intensive conventional (e.g. cell culture) methods, and/or shorten hands-on laboratory technologist time. Early detection of contagious diseases, such as pulmonary tuberculosis, using molecular techniques would also limit the spread of infection.

ASSAY PERFORMANCE

Molecular techniques developed in research settings have been applied as 'in-house' developed assays in many clinical microbiology laboratories. The reason for this is that for many microorganisms, commercial assays are unavailable. Furthermore, it is relatively easy for laboratories with research and development expertise to develop and validate their own molecular assays based on nucleic acid amplification technology. The downside is that results derived from such assays may not be comparable from laboratory to laboratory, while no universal standards exist for these assays. The National Committee for Clinical Laboratory Standards (NCCLS) published guidelines for molecular diagnostic methods for infectious diseases in 1995 (Enns et al. 1995) and for quantitative molecular methods for infectious diseases in 2001 (Madej et al., 2001). Strategies to provide quality and to standardize molecular diagnostic tests include laboratory requirements for certification, proficiency surveys, and checklists, such as those published by the American College of Pathologists (www.cap.org/html/ftpdirectory/checklistftp.html) (College of American Pathologists 2003). The availability of state-of-the-art Food and Drug Administration (FDA)-approved commercial molecular diagnostic assays substantially contributes to interlaboratory standardization. A limited number of nucleic acid amplification assays for detection of microbes in human specimens are currently available (Table 1).

In order to understand and interpret molecular microbiology diagnostic tests, certain universal assay parameters must be defined (see also Wolk et al. 2001). Analytical sensitivity, also referred to as the lower limit of detection, refers to the lowest number of microorganisms that can be reproducibly detected by the assay. Clinical sensitivity is the proportion of specimens that yield positive test results from patients who have a specified clinical entity. High analytical sensitivity may exist with inadequate clinical sensitivity if false-negative results occur because the target nucleic acid copy number in the clinical specimen is low. Analytical specificity refers to the ability of the test to detect only the microorganism it purports to measure. Clinical specificity is the proportion of specimens that yield negative results from patients who do not have a specified clinical entity.

For quantitative molecular microbiologic diagnostic testing, additional parameters bear consideration. The linear range refers to the quantitative spectrum over which the test provides results detecting a direct relationship between input target concentration and output signal. The upper and lower limits of quantification reflect the upper and lower ends of this linear range. The lower limit of quantification of a quantitative assay may be higher than the analytical sensitivity of a qualitative assay, confusing the interpretation of results. This is exemplified by molecular testing for HCV. The lower limit of quantification of the VERSANT^{TM} HCV RNA 3.0 assay (bDNA) (Bayer Healthcare LLC, Tarrytown, NY, USA), a quantitative HCV RNA assay, is higher than the lower limit of detection of the COBAS AMPLICOR^{TM} HCV Test, version 2.0 (Roche Diagnostic Corporation, Indianapolis, IN, USA), a qualitative HCV RNA assay (Germer et al. 2002).

Precision refers to the agreement between replicate measurements of the same material. Accuracy refers to the ability of a method to reliably determine the true value of the particular target. The tolerance limit is the difference between two results that can be considered to be significantly different and is the sum of the biological variation in quantification and intra-assay variability. For quantitative assays of HIV-1 RNA, for example, increases or decreases in the RNA titer of at least threefold typically reflect biologically relevant changes in the level of viral replication (Wolk et al. 2001).

In an effort to standardize quantitative testing, interlaboratory collaborations with the World Health Organization have established the World Health Organization International Standards, which are standard reference materials with concentrations expressed as international units (IU) per milliliter, which can be used to calibrate,

Table 1 *Commercial amplification assays for molecular detection of microbes in human specimens (United States, 2003)*[a]

Test	Manufacturer	Detection	Application (specimen)
Amplicor™ and Amplicor Monitor™	Roche Diagnostic Corporation www.roche-diagnostics.com/ba_rmd/products.html	Qualitative or quantitative (varies with target) polymerase chain reaction can be automated on the COBAS instrument	*Mycobacterium tuberculosis* (sputum, bronchial specimen)[b] *Chlamydia trachomatis* (urine, cervical, male urethral specimen)[b] *Neisseria gonorrhoeae* (urine, cervical, male urethral specimen)[b] *Mycobacterium intracellulare* (sputum, bronchial specimen) *Mycobacterium avium* (sputum, bronchial specimen) Human immunodeficiency virus-1 (HIV-1) (plasma)[b] Hepatitis B virus (serum, plasma) Hepatitis C virus (serum, plasma)[b] Cytomegalovirus (plasma)
LightCycler®	Roche Diagnostic Corporation www.lightcycler-online.com	Rapid cycle real-time qualitative or quantitative (varies with target) polymerase chain reaction	Hepatitis A virus (serum, plasma) Parvovirus B19 (serum, plasma) *Bacillus anthracis* (multiple) Herpes simplex virus type 1 and type 2 (multiple) Epstein–Barr virus (multiple) *Streptococcus pyogenes* *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. Methicillin-resistant *Staphylococcus aureus* (*mecA*) *Enterococcus faecalis* and *Enterococcus faecium* Vancomycin-resistant enterococci (*vanA/B*) *Pseudomonas aeruginosa* (multiple) *Candida albicans* (multiple)
VERSANT® TMA™	Distributed by Bayer Healthcare LLC under license from Gen-Probe www.bayerdiag.com/products/index.html	Qualitative transcription-mediated amplification	Hepatitis C virus (serum, plasma)[b]
VERSANT® bDNA	Bayer Healthcare LLC www.bayerdiag.com/products/index.html	Quantitative branched DNA assay	HIV-1 (plasma)[b] Hepatitis B virus (serum, plasma) Hepatitis C virus (serum, plasma)[b] HIV-1 (plasma)
NucliSens®	BioMérieux www.biomerieux-usa.com/clinical/nucleicacid/index.htm	Qualitative or quantitative (varies with target) nucleic acid sequence-based analysis	Cytomegalovirus pp67 mRNA (whole blood)[b]
Amplified™ MTD, Amplified CT	Gen-Probe www.gen-probe.com	Qualitative transcription-mediated amplification	*M. tuberculosis* (sputum, bronchial specimen, tracheal aspirate)[b] *C. trachomatis* (urine, cervical, male urethral specimen)[b]
Group A Streptococcus Direct (GASD)	Gen-Probe www.gen-probe.com	Qualitative hybridization protection assay	*Streptococcus pyogenes* (throat swab)
BDProbe Tec™	BD Biosciences www.bd.com/biosciences	Qualitative strand-displacement amplification	*C. trachomatis* and *N. gonorrhoeae* (urine, cervical, male urethral specimen)[b]

(*Continued over*)

Table 1 *Commercial amplification assays for molecular detection of microbes in human specimens (United States, 2003)[a] (Continued)*

Test	Manufacturer	Detection	Application (specimen)
Hybrid Capture®	Digene www.digene.com/lab.html	Quantitative DNA–RNA hybrid capture assay	Human papilloma virus (cervical specimen)[b]
			Cytomegalovirus (peripheral white blood cells)[b]
			Hepatitis B virus (serum)
			C. trachomatis and *N. gonorrhoeae* (cervical specimen)
HCV ASR	Abbott Laboratories www.abbottdiagnostics.com	Real-time quantitative polymerase chain reaction with TaqMan™probe	Hepatitis C virus

Modified with permission from Wolk et al. 2001.
a) Culture confirmation, genotyping and mutation detection assays are not listed.
b) FDA-approved assay as of February, 2003.

validate, and compare different quantitative molecular assays measuring very similar targets in the same pathogen. Currently, standards exist for HIV-1, hepatitis A virus, hepatitis B virus (HBV), HCV, and parvovirus B19. Further information is available from the National Institute for Biological Standards and Controls (www.nibsc.ac.uk).

SPECIMEN SUITABILITY

For each molecular diagnostic assay, criteria for the appropriate specimen, including the optimal specimen source, specimen volume, collection method, transport and storage conditions, specimen stability, and nucleic acid preparation method must be considered. The sensitivity and specificity of an assay may vary considerably if any of these conditions are altered. The choice of specimen plays a key role in the performance and interpretation of test results. Typically, molecular microbiologic assays are clinically validated using one or more specimen sources (e.g. spinal fluid, blood) that may contain a target organism. For blood specimens, sensitivity and specificity may vary depending on the specific fraction (i.e. plasma, serum, whole blood, various leukocyte fractions) analyzed. The viral load, for example, will vary depending on the specific specimen tested; plasma or serum is typically used for detection of HBV DNA, HCV RNA, or HIV-1 RNA. The volume of specimen from which nucleic acid is extracted can also affect the sensitivity and specificity of the assay. Traditional molecular diagnostic tests typically analyze relatively small specimen volumes (e.g. 200 µl or less). For low-copy-number targets, increasing the amount of specimen analyzed may increase sensitivity (Germer et al. 2003).

Inhibitory substances may interfere with various steps in molecular diagnostic assays yielding false-negative results (Wolk et al. 2001). Inhibitors present in the patient specimen (e.g. hemoglobin, lactoferrin, immunoglobulin G, bile salts, polysaccharides, proteinases, urea, leukocyte DNA) or introduced during specimen collection or processing (e.g. ethylene diamine tetraacetic acid (EDTA), heparin, guanidinium HCl, sodium dodecylsulfate) may interact with nucleic acid or critical enzymes (e.g. DNA polymerase) to prevent target amplification or remove reaction components (e.g. metals) affecting enzymatic substrates (Al-Soud et al. 2000, 2001). The presence of inhibitors, and therefore the negative predictive value of the assay, can be assessed in several ways. The patient's specimen can be spiked with known target nucleic acid and the spiked specimen tested along with the native patient specimen. Alternatively, internal amplification controls can be added to the patient specimen and amplified concurrently with the target nucleic acid. Detection of housekeeping genes (e.g. human β-globin, interleukin-2) has been incorporated into some assays to confirm the presence of human cellular DNA in the patient specimen. Although this approach can be used to assess the presence of inhibitors, since the amount of human DNA generally exceeds the amount of microbial DNA, this approach to inhibitor detection may be less sensitive than the use of spiked specimens or internal amplification controls.

SPECIMEN PREPARATION

Specimen preparation techniques are a critical component of clinical molecular microbiologic diagnostics (Wolk et al. 2001). Optimal specimen preparation efficiently releases microbial nucleic acid while preserving its integrity, removes inhibitors, sterilizes the specimen of viable organisms, concentrates the target nucleic acid into a small volume (if appropriate), and places the target into an environment suitable for amplification. For many assays, specimen preparation may be more labor-intensive and time-consuming than actual amplification and detection. Many specimen-preparation

methods exist; some require considerable technical skill, as they are labor-intensive, nonstandardized, and prone to manipulation steps where template cross-contamination may occur. A detailed discussion of available nucleic acid preparation methods for molecular microbiology diagnostic purposes is beyond the scope of this chapter. In general, specimens are treated with alkaline pH, chaotropic agents, sonication, heat, detergents, freeze–thaw cycles, and/or proteolytic enzymes to disrupt the cell membranes and cell walls and to release nucleic acid. Thereafter, nucleic acid may or may not be extracted with organic solvents, adsorbed to silica in the presence of chaotropic agents such as guanidinium salts, and/or precipitated in the presence of salts and alcohol. Nucleic acid capture technologies such as automated silica-based membrane capture technologies or magnetic bead nucleic acid capture can also be used. For specimens that represent a potential biohazard for laboratory personnel (e.g. specimens submitted for testing for agents of bioterrorism), autoclaving the specimen prior to molecular testing (121°C, 15 min, 20 psi) enables inactivation of potentially viable hazardous infectious agents without destruction of nucleic acids (Espy et al. 2002).

Automation of specimen preparation provides rapid, cost-effective, and consistent results. Several automated extraction instruments are available. The ability to interface with high-throughput amplification systems is an important advance of these new automated extraction systems. For example, the MagNA Pure LC (Roche Diagnostic Corporation) integrates with the LightCycler® PCR instrument (Roche Diagnostic Corporation) and can be used to automate or partially automate both extraction and PCR setup processes (Espy et al. 2001).

PROBE HYBRIDIZATION

Nucleic acid probes are short segments of single-stranded DNA or RNA that can be 'labeled' (i.e. with antigen, chemiluminescent substrate, radioisotope, enzyme) and can bind with high specificity to complementary (target) nucleic acid sequences. In situ hybridization uses intact cells (e.g. in formalin-fixed, paraffin-embedded tissue) containing specific DNA or RNA as a target for probe hybridization. The technique allows visualization of infected cells within tissue and thereby association of hybridization results with other morphologic changes. Because in situ hybridization is not an amplification-based technique, sensitivity may be limited. Probe-based commercial kits, such as the PACE® System from Gen-Probe, Inc. (San Diego, CA, USA), which directly detects *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in clinical samples, produce results that are generally equivalent to culture techniques but in much less time. Nucleic acid probes are also an integral component of the amplification techniques described below.

POLYMERASE CHAIN REACTION

PCR is the original and most widely used nucleic acid target amplification technology. A buffered reaction mixture containing the target DNA sequence (template), oligonucleotide primers, thermostable DNA polymerase, deoxynucleotide triphosphates (dNTPs), and magnesium or manganese ions with or without other additives, is placed into a thermal cycler, which heats and cools the components, exposing them to consecutive cycles of alternating temperatures (Wolk et al. 2001). In each cycle, three processes occur:

1. Denaturation: heating to high temperatures to separate double-stranded DNA into single strands.
2. Primer annealing: lowering the temperature to allow for synthetic oligonucleotide primers to anneal to the single-stranded DNA and create a partial double strand.
3. Primer extension: addition of dNTPs to the 3′ ends of the bound primers by DNA polymerase, thereby creating a new synthetic piece of double-stranded DNA, which is complementary to the original template strand.

With the exception of the first cycle, the amount of target DNA theoretically doubles with each cycle, resulting in an exponential increase in the quantity of amplified DNA until the reaction reaches a plateau phase due to depletion of reaction components. The amount of product that accumulates is $N_{\text{total}} = N_{\text{initial}}(1 + Y)^X$, where Y is the efficiency per cycle. Examples of some specific types of PCR are shown in Table 2.

Following amplification, amplified DNA is detected. Many of the classic methods for detection of amplified DNA are manual, requiring manipulation of amplified DNA and adding substantial risk of contamination to subsequent PCR reactions, rendering them suboptimal for use in clinical laboratories. Agarose or polyacrylamide gel electrophoresis use electric current to separate DNA fragments according to molecular size; DNA fragments are visualized by staining with a DNA binding dye (e.g. ethidium bromide). Generally, agarose or polyacrylamide gel electrophoresis (alone) does not provide ideal sensitivity or specificity for clinical microbiology diagnostic testing. To increase specificity, the amplified DNA can be digested with a restriction enzyme prior to electrophoresis; the resultant restriction digest yields a unique pattern of DNA fragments by gel electrophoresis. To increase sensitivity as well as specificity, PCR products may be subjected to Southern blotting followed by probe hybridization. Briefly, DNA from a gel is transferred to a synthetic membrane, on which labeled oligonucleotide probes

Table 2 *Modifications of the polymerase chain reaction (PCR)*

Modification	Principle
Broad-range bacterial PCR	Use of PCR primers targeted to highly conserved regions of bacterial DNA such that, depending on the primer sets used, the target can be amplified from most bacteria
In situ PCR	Amplification of DNA (or complementary DNA) inside an intact cell (e.g. in a paraffin-embedded tissue section) to determine the disposition of a microbe within tissue
Multiplex PCR	Use of two or more primer sets in the same reaction mix to identify multiple microorganisms or multiple nucleic acid targets within one microorganism in a single assay
Nested PCR	Use of one set of primers followed by reamplification with a second set of primers, internal to the first
Quantitative PCR	A technique used to assess the amount of a microbe present in a patient specimen
Semiquantitative or limiting dilution PCR	Titer determination of target template by endpoint specimen dilution prior to PCR
Quantitative competitive PCR	Use of an internal quantitative standard (or calibrator) in the amplification reaction along with the target sequences. The standard competes with target sequences in the PCR reaction. The quantity of internal quantitative standard amplified correlates inversely with the quantity of target
Real-time quantitative PCR	Quantitation of amplified target determined by cycle number at which the amplification product is first detected (using fluorescence detection) in comparison with a quantitative standard
Randomly amplified polymorphic DNA (RAPD) analysis (arbitrarily primed PCR)	Random amplification of segments of target DNA using arbitrary primers that do not have any known homology to the target sequence. Amplified DNA is visualized on a gel following electrophoresis and patterns are compared to one another
Real-time PCR	Simultaneous amplification and amplification product detection using fluorescence detection (Figure 1)
Repetitive element sequence-based PCR	Uses consensus sequence-based PCR primers to amplify DNA sequences located between successive repetitive elements
Reverse transcriptase-PCR (RT-PCR)	Uses reverse transcriptase to create complementary DNA from an RNA target. The complementary DNA is then amplified by PCR Used to detect RNA (either because an infectious agent's nucleic acid is naturally present as RNA, or to detect transcribed microbial DNA as a marker of active infection)

complementary to an internal region of the amplified sequence are subsequently hybridized to the DNA. Hybridized probes are then detected by one of a variety of methods (e.g. autoradiography, chemiluminescence). Another option for detection is incorporation of various chemicals (e.g. digoxigenen-11-dUTP) into the DNA during amplification. Amplified DNA can then be detected on the membrane by a second chemical reaction (e.g. anti-digoxigenin antibody:alkaline phosphatase conjugate and a chemiluminescent substrate).

Besides membrane-based amplified DNA detection methods, a number of other approaches can be used (Wolk et al. 2001). Probes can be bound to microtiter plate wells or beads to capture amplified DNA. A specific 'signal' probe can then be added to react with the captured target nucleic acid. In the PCR-enzyme-linked immunosorbent assay, amplified DNA containing digoxigenen-11-dUTP is hybridized to oligonucleotide capture probes labeled with biotin. Biotinylated DNA hybrids are then bound to a streptavidin-coated microtiter plate so those specific DNA complexes can be detected in a microtiter plate format.

Solution- (liquid-) phase hybridization is another option. An example is the hybridization protection assay wherein chemiluminescent probes hybridize to original target nucleic acid or to amplified DNA, which have been produced by a previous amplification method. Following chemical destruction of the nonhybridized probes, the chemiluminescent label, protected within the hybrid, reacts under specific conditions to produce light, which can be measured by a luminometer.

Other methods that can be used to separate, quantify, or identify amplified DNA include high-performance liquid chromatography, mass spectrometry, and DNA sequencing methods.

Homogeneous or real-time PCR assays incorporate amplification and detection within one closed system. Such assays are being used increasingly in clinical microbiology laboratories because of their rapid turnaround time and the lack of postamplification manipulation and

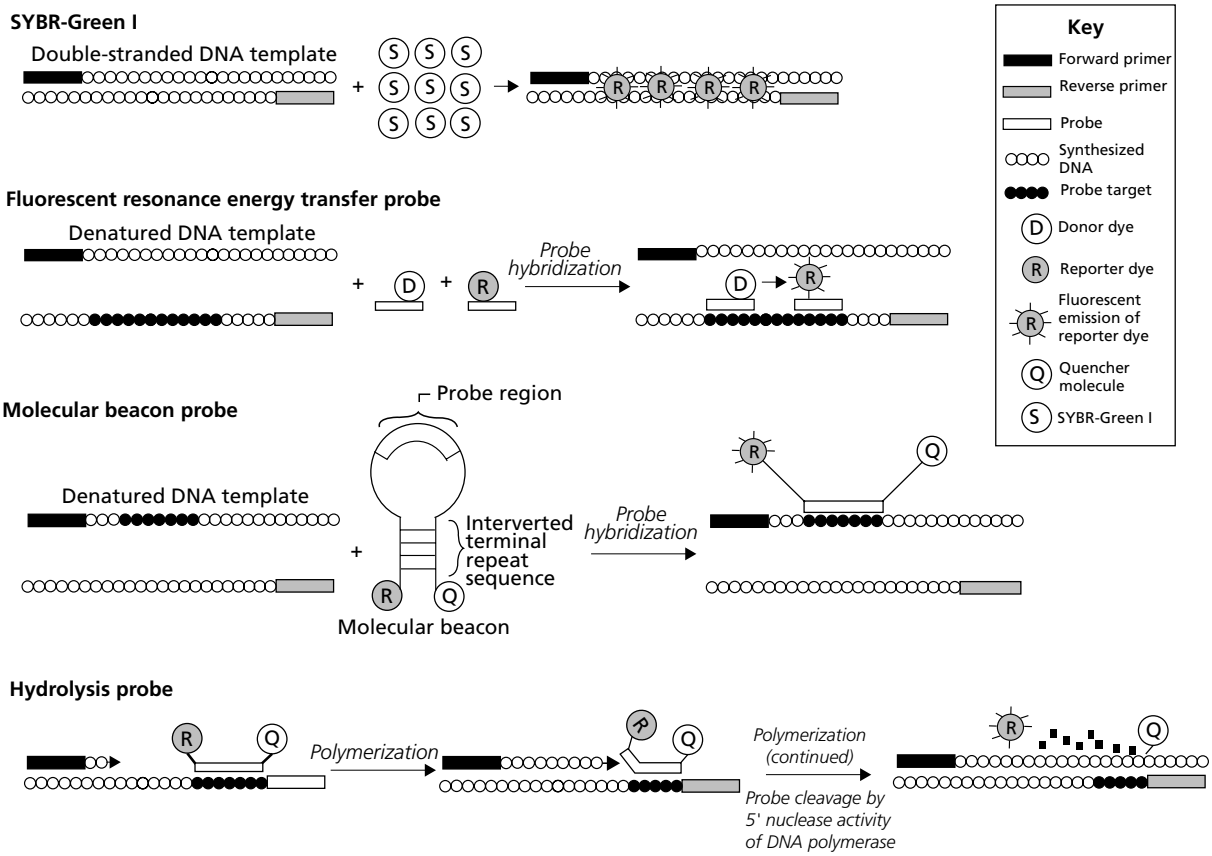

Figure 1 *Real-time polymerase chain reaction detection (see text for details).*

contamination risk inherent to traditional methods. These methods are reviewed in detail below.

CONTAMINATION AND CONTAMINATION CONTROL

The strength of nucleic acid amplification is high sensitivity (Wolk et al. 2001). Because of this sensitivity and the large amount of amplified DNA generated, prevention of nucleic acid contamination of other molecular assays performed in the same laboratory is essential. Sources of contamination include crossover contamination from specimens containing large numbers of target molecules or, most importantly, from manipulation of amplified nucleic acid. Amplified nucleic acid may contaminate reagents, laboratory surfaces, as well as the laboratory personnel themselves. The setup of the laboratory should be such that the movement of patient specimens and personnel proceeds from pre-amplification to postamplification areas. Separate areas (preferably rooms) for reagent preparation, sample processing, and amplification and detection are ideal. Besides these physical approaches to contamination control, enzymatic (e.g. uracil-*N*-glycosylase (UNG)) or photochemical (e.g. isopsoralen) methods of inactivating amplified DNA may be applied (Wolk et al. 2001). For enzymatic inactivation utilizing UNG, dUTP in lieu of dTTP is incorporated into the amplified DNA such that amplified target will contain dUTP (whereas native target contains dTTP). Any dUTP-containing amplification products (i.e. contaminants from prior PCR reactions) are cleaved by UNG prior to amplification in subsequent reactions. For photochemical inactivation, amplified DNA is generated in the presence of isopsoralen. Inactivation occurs by postamplification irradiation of the reaction vessel with ultraviolet light prior to opening the reaction vessel; irradiation causes the isopsoralen to modify the amplified DNA such that the DNA is unable to serve as a template for further amplification but is still capable of hybridization in various detection formats. As discussed above, closed-system PCR reactions can also be used to prevent contamination.

All PCR testing should include a sufficient number of negative controls processed along with patient specimens to provide a measure of assurance that assay contamination is not occurring. For each assay, a positive control in low copy number should also be included.

RAPID REAL-TIME PCR

A significant advance in PCR technology is the development of rapid PCR with simultaneous probe detection,

so-called 'real-time PCR' (Wolk et al. 2001). In real-time PCR assays, amplification and product detection occur simultaneously in the same sealed reaction vessel, tube, or well. A fluorescent signal generated by specific binding of chemicals or probes to amplified DNA is used for real-time monitoring of production of amplified DNA.

Because amplification and product detection are performed in the same vessel, these systems can be closed. Closed PCR systems lessen the risk of contamination by avoiding postamplification manipulation and lessen the needs for extensively engineered molecular laboratory space. Nevertheless, careful attention to unidirectional workflow, amplification product control, and amplification product-inactivation protocols remain important. Template carryover contamination can still occur, and liberation of amplified DNA through broken reaction vessels can lead to contamination events unless DNA-inactivating methods are included in the assay.

Many real-time PCR systems have incorporated technology that enables a dramatic reduction in the time required for assay performance. For example, realtime PCR performed using the LightCycler® instrument (Roche Diagnostic Corporation) can be completed in 30 to 40 min (versus several hours to days for conventional PCR and amplification product detection).

Several fluorescent chemistry approaches can be utilized to detect amplified DNA in real time (Figure 1) (Wolk et al. 2001). Real-time monitoring of nonspecific fluorescent dye (e.g. SYBR-Green I) incorporation can be used (Figure 1). Nonspecific dyes bind to double-stranded DNA generated during PCR and emit fluorescence, which can be monitored by various detection systems. Since the dyes bind to all nonspecific amplification products produced in the reaction, as well as to primer-dimers, dye incorporation alone does not ensure detection of a specific PCR product. To improve specificity, melting-curve analysis can be performed. The amplified product will have a characteristic melting temperature (Tm), the sequence-dependent temperature at which half of the nucleic acid strands become separated in solution, differentiating it from nonspecific amplification products. To further ensure more specific hybridization and product identification, protocols that incorporate specific internal probes are used. Several internal probe formats exist. They include dual hybridization (fluorescence resonance energy transfer (FRET)) probes, molecular beacon probes, and hydrolysis (e.g. TaqMan™) probes (Figure 1).

Dual hybridization uses two probes (one coupled to a fluorescent donor dye and the other to a fluorescent reporter dye) designed to recognize single strands of amplified DNA internal to the primer binding sites (Figure 1). As DNA is amplified, probes hybridize to amplified DNA in close proximity to one another (usually one to five nucleotides apart) during the annealing step. An external light source then stimulates the fluorescent donor dye, and energy emitted from the donor dye excites the fluorescent reporter dye to emit a specific wavelength of light.

Molecular beacons are single-stranded oligonucleotide probes that are created using a specific hybridization probe sequence, which is flanked by two inverted terminal repeat sequences such that a stem-loop (or hairpin) structure is formed (Figure 1). As the molecular beacon probe opens and binds to a denatured DNA template, a quencher molecule (located on one arm) is removed from proximity of a fluorescent reporter dye (located on the other arm), allowing the reporter dye to fluoresce upon stimulation from an external light source. In contrast, while the probes are in a hairpin formation, the reporter dye and quencher molecules are proximal and fluorescence is quenched.

Using FRET-based probe pairs or molecular beacons, melting-curve analyses can be performed by monitoring the Tm at which the probe separates from the amplified DNA. This analysis permits specific identification of the amplified DNA. After PCR is performed, the amplified DNA is heated to its denaturation temperature and diminished fluorescent reporter signal is observed. Because mismatches, such as point mutations or polymorphisms, combined with other amplified DNA characteristics, such as fragment size and percentage GC content, can affect the Tm of the probe:DNA hybrid, Tm curves can be used to distinguish different PCR products including those with as few as 1 bp difference.

TaqMan™ chemistry combines DNA polymerase with 5′-exonuclease activity and a linear oligonucleotide hybridization probe carrying both a fluorescent reporter dye and a quencher molecule (Figure 1). The hybridization probe binds to the denatured amplified target DNA strands. Fluorescence of the reporter dye is prevented (quenched) by its proximity to the quencher molecule. After each annealing cycle, DNA polymerase, with 5′-exonuclease activity, extends the specific target strand and cleaves the hybridization probe, which is specifically bound to the strand. The exonuclease activity fragments the probe, separating the quencher molecule from the reporter dye. The cleaved reporter dye fluoresces when stimulated from an external light source. If specific amplification of target DNA has occurred along with cleavage of the hybridization probe, reporter dye fluorescence increases and accumulates with each PCR cycle. Melting-curve analysis cannot be performed with TaqMan™ probes.

While SYBR-Green I, TaqMan™, FRET, and molecular beacon technology can be used to monitor real-time endpoint (qualitative) PCR, these techniques can also be used to quantify target nucleic acid. The amount of microbe-specific nucleic acid in a given patient specimen is determined by comparing target amplification signal to internal or external quantitative standards and, through mathematical algorithms, determining the exact number of amplification cycles at which amplified DNA is detected above a baseline or threshold value. Real-time

quantitative PCR assays offer advantages over nonreal-time semiquantitative PCR approaches (Table 2), which analyze endpoint amplification products at a point when net synthesis is significantly reduced, inhibitory effects accumulate, and differences in the initial starting template concentrations are masked.

Several automated real-time PCR instruments that use the aforementioned technologies are currently available: LightCycler® (Roche Diagnostic Corporation), SmartCycler® (Cepheid, Sunnyvale, CA, USA), ABI PRISM® Sequence Detection Systems (SDS) (Applied Biosystems, Foster City, CA, USA), iCycler (Bio-Rad Laboratories, Hercules, CA, USA), Mx4000™ Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA), and Rotor-Gene™ (Corbett Research, Sydney, Australia). Real-time PCR assays are run on highly specialized automated instruments that include optical systems to excite fluorescent dyes and detect fluorescent emissions at a variety of wavelengths specific to the dyes used. These systems have the potential to provide rapid results and to facilitate multiplex PCR through the use of different fluorophores.

NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION AND TRANSCRIPTION-MEDIATED AMPLIFICATION

Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are similar isothermal reactions that use a RNA target as the substrate for reverse transcriptase, creating a complementary DNA (cDNA) strand from the RNA template (Figure 2) (Wolk et al. 2001). RNA from the resulting RNA:DNA hybrid is then degraded by RNAse H, and cDNA is used as a template for RNA polymerase, producing multiple RNA copies from the cDNA. Results can be qualitative or quantitative. Modifications of TMA also exist for use with DNA targets.

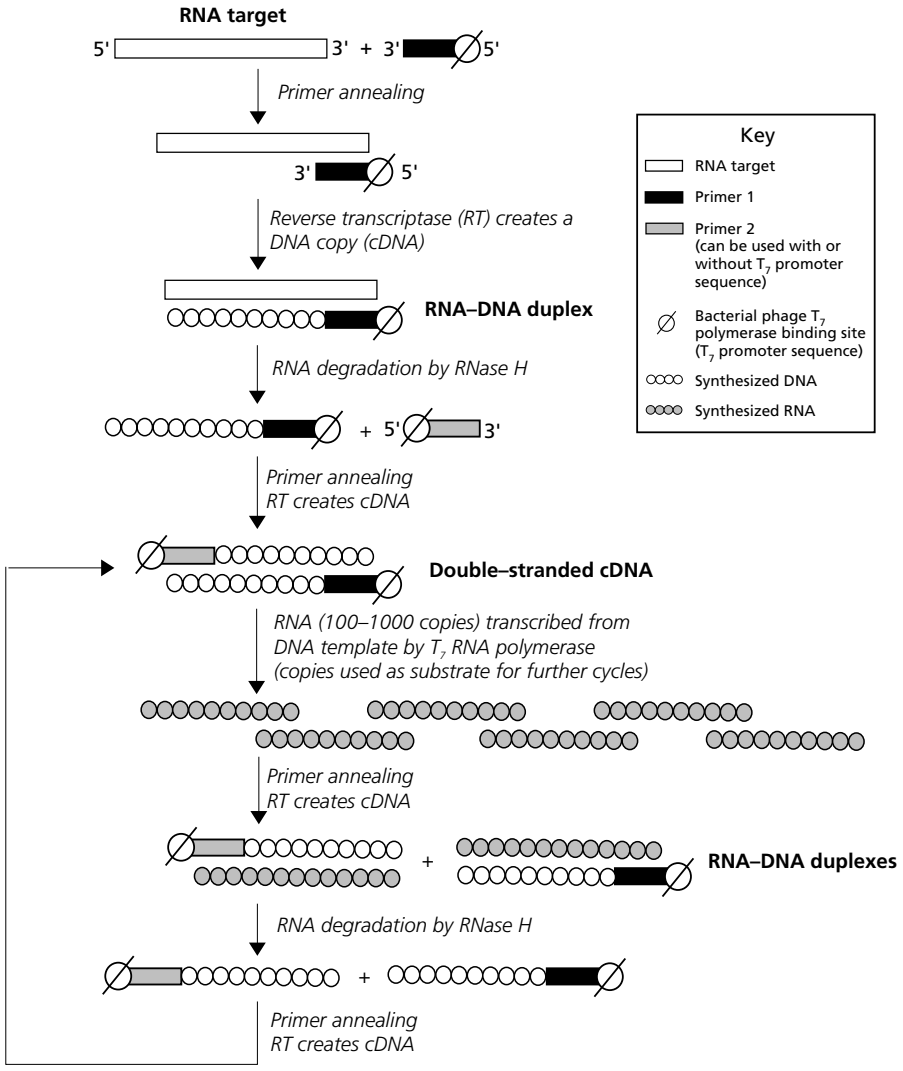

Figure 2 *Transcription-based amplification systems: nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) (see text for details). Reprinted with permission from Wolk et al. 2001.*

NASBA uses separate reverse transcriptase and RNase H enzymes, whereas TMA uses a reverse transcriptase enzyme with endogenous RNase H activity. Gen-Probe, Inc. currently offers commercial TMA assays for detection of *Mycobacterium tuberculosis* and *Chlamydia trachomatis*. A TMA assay for qualitative detection of HCV RNA is available from Bayer Healthcare LLC. BioMérieux (Boxtel, The Netherlands and Durham, NC, USA) offers NASBA testing for quantitative detection of HIV-1 RNA and qualitative detection of CMV pp67 mRNA.

STRAND-DISPLACEMENT AMPLIFICATION

Strand-displacement amplification (SDA) is an isothermal amplification technique in which primers, containing both a target-specific region and a hemimodified restriction enzyme site, combine with a DNA polymerase to create a synthetic double-stranded DNA that contains the restriction enzyme site (Figure 3) (Wolk et al. 2001). A restriction enzyme generates a site-specific 'nick' in one of the two strands of double-stranded DNA. Once the strand is nicked, DNA polymerase initiates synthesis at the 3′ end of the nick site to create a new DNA strand. Because the DNA polymerase used is deficient in 5′ to 3′ exonuclease activity, it does not destroy the existing strand as it polymerizes the new strand; it simply displaces the downstream nontemplate strand. Each displaced strand is then available to anneal with more primers and the process continues with repeated nicking, extension, and displacement of newly formed DNA strands. The entire process results in exponential amplification of the original DNA target.

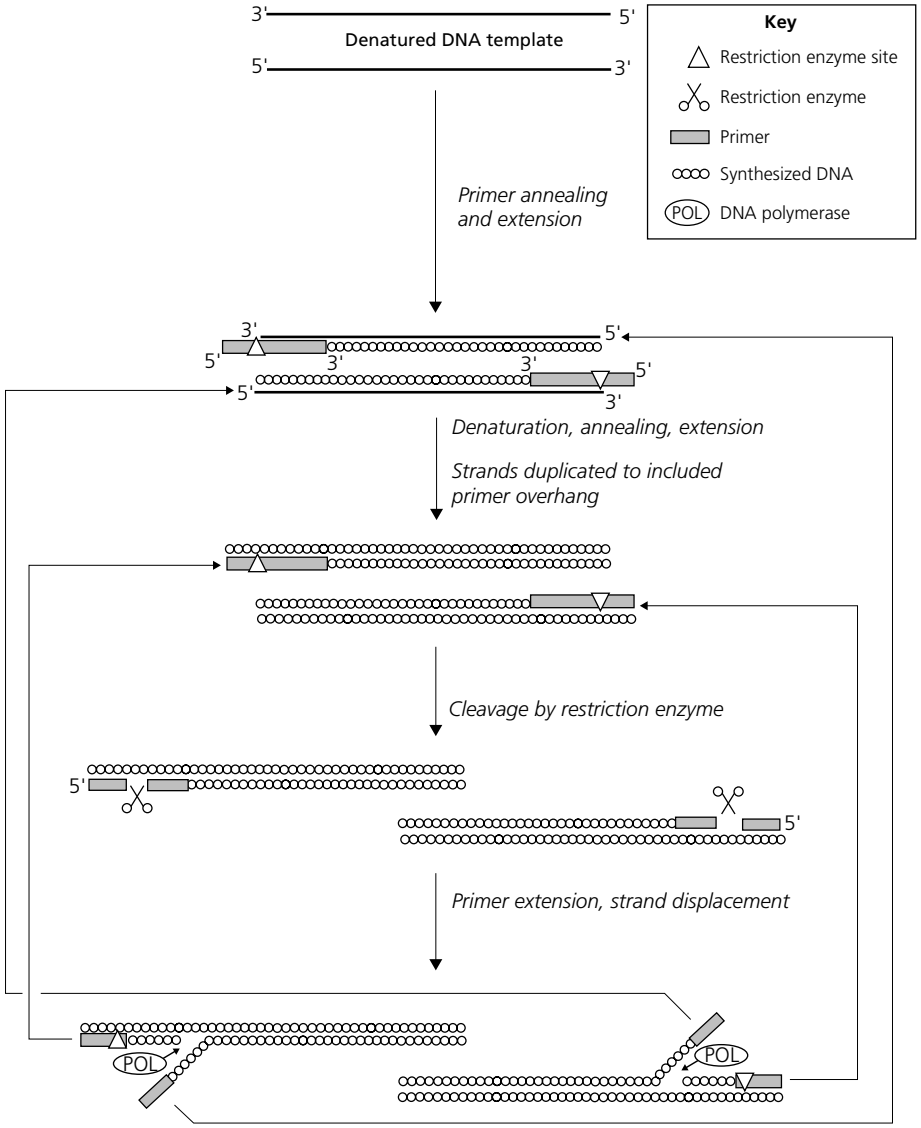

Figure 3 *Strand-displacement amplification (SDA) (see text for details). Redrawn with permission from Wolk et al. 2001.*

HYBRID CAPTURE ASSAY

In the hybrid capture assay, an RNA or DNA probe hybridizes to a DNA or RNA target, respectively (Wolk et al. 2001) (Figure 4) . Hybrids are captured on a solid phase coated with capture antibodies specific for the RNA:DNA hybrids (duplexes). Captured hybrids are detected using chemiluminescence. Digene Corporation (Silver Spring, MD, USA) is a manufacturer for hybrid capture assays for human papilloma virus (HPV), CMV, HBV, *C. trachomatis*, and *N. gonorrhoeae*.

LIGASE CHAIN REACTION

In the ligase chain reaction (LCR), DNA template is denatured and subsequently hybridized to probes that are located next to each other on the target strand (Figure 5) (Wolk et al . 2001). The hybridized probes are ligated (joined) by DNA ligase to form a ligation product that mimics one strand of original target sequence and can serve as a template for ligation of more probes. Successful ligation of the probes depends on the proximal positioning and perfect base pairing of the 3′ end of one probe with the 5′ end of the other to complete a complementary DNA strand. DNA polymerase and dNTPs are used to extend the probe and fill the gap region prior to ligation with DNA ligase. Accumulated ligation products are detected by incorporation of labeled probes.

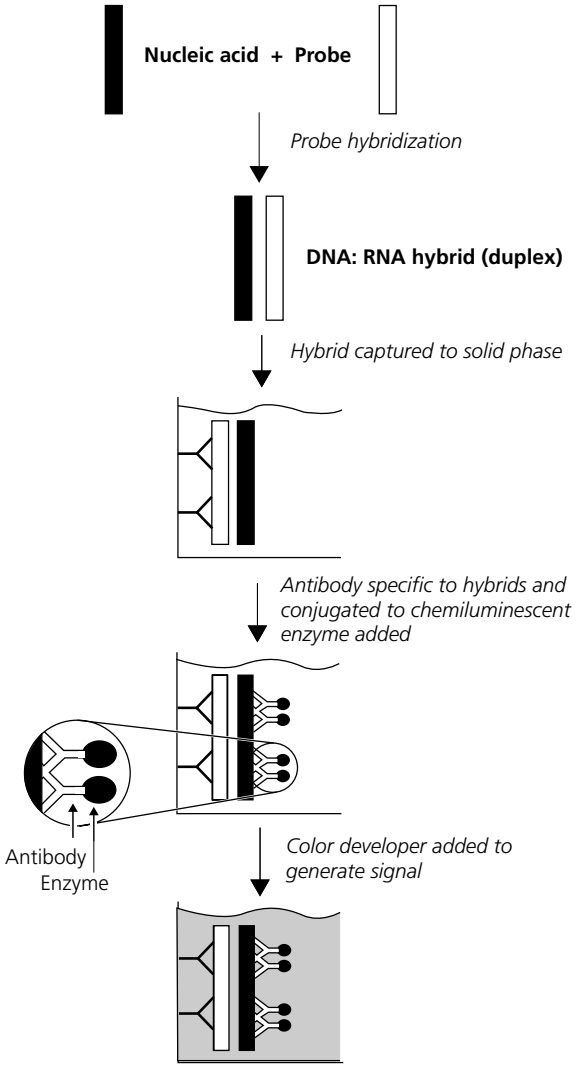

Figure 4 *Hybrid capture assay (see text for details). Reprinted with permission from Wolk et al. 2001.*

BRANCHED DNA ASSAY

Quantitative branched DNA (bDNA) assays are commercially available from Bayer for HBV, HCV, and HIV-1 (Table 1). With bDNA technology, organisms are disrupted, releasing nucleic acid to be captured by multiple capture probes that are bound to a solid surface (Figure 6) (Wolk et al. 2001). Target probes hybridize to both microbial nucleic acid and signal amplification multimer (branched DNA or bDNA). Finally, enzyme-labeled probes hybridize with bDNA structures. Detection occurs via a chemiluminescent process. The amount of signal generated is directly related to the amount of target nucleic acid present in the specimen.

CYCLING PROBE TECHNOLOGY

Cycling Probe Technology (CPT) (Velogene™; ID Biochemical, Vancouver, British Columbia, Canada) is an isothermal process that can be used to detect target DNA (Wolk et al. 2001). Commercial applications of CPT are available as Velogene test kits for methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Briefly, the technology utilizes a synthetic probe, a chimera of DNA-RNA-DNA, which is labeled at the 5′ end with fluorescein dye and at the 3′ end with a biotin molecule (Figure 7). The fluorescein/biotin-labeled probes are designed to anneal with a specific DNA template. Once the probes anneal, RNase H is added to cleave the RNA portion of the bound probe. The shorter, cleaved probe fragments will then dissociate from the target, releasing the target for use in subsequent cycles. In the presence of a specific target molecule, cleaved probes accumulate with each cycle. The test is designed so that a colorimetric reaction will occur in the presence of uncleaved probe; no color will occur in the presence of cleaved probes (specific target). To detect the presence or absence of cleaved probes, an antifluorescein antibody coupled to horseradish peroxidase (Ab/HRP) is added and the reaction is transferred to microtiter wells coated with streptavidin molecules. An alternative test using a nitrocellulose strip has been manufactured for detection of uncleaved probes. All probes will bind to streptavidin, but only the uncleaved probes will bind to the Ab/HRP. A substrate for horseradish peroxidase is added, and

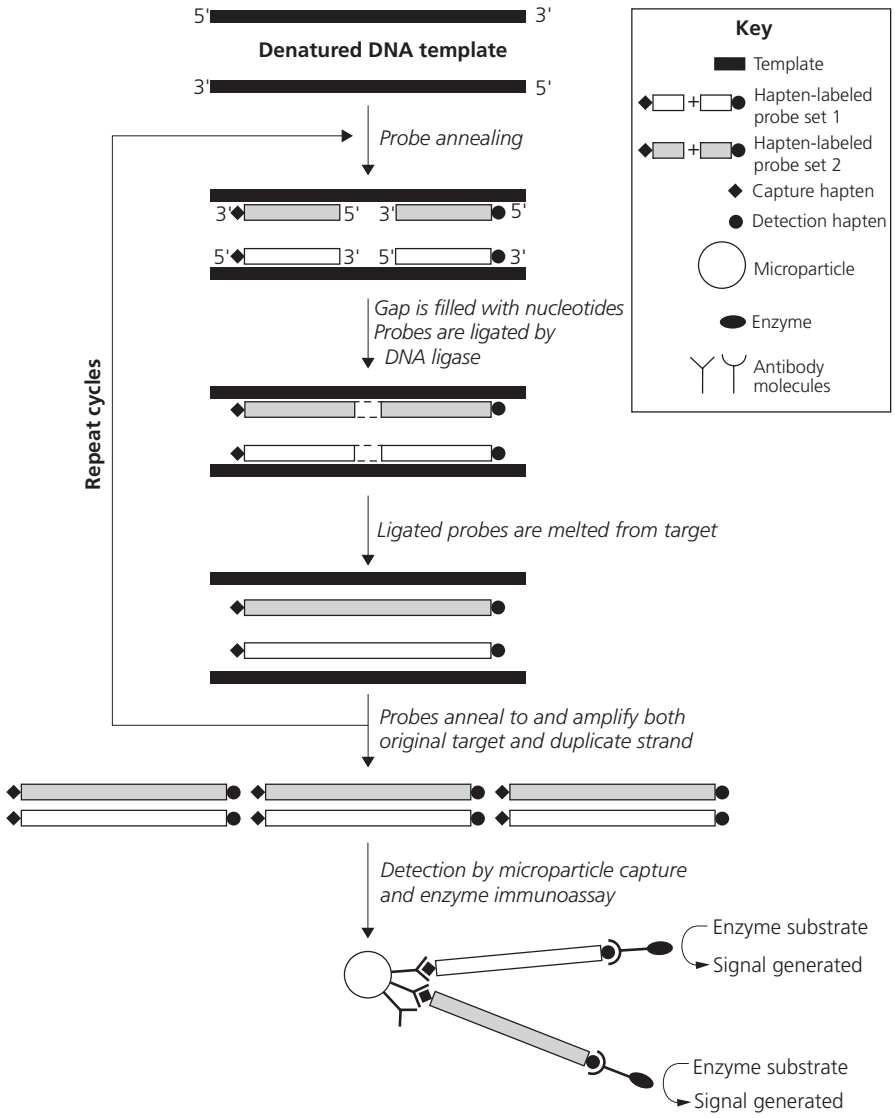

Figure 5 *Ligase chain reaction (LCR) (see text for details). Reprinted with permission from Wolk et al. 2001.*

color development is measured to determine the amount of uncleaved probe present in the well (or in the region of the strip). The color development is inversely proportional to the original amount of target DNA present.

NUCLEIC ACID SEQUENCING

Nucleic acid sequencing has several applications in the clinical microbiology laboratory (Wolk et al. 2001). For example, the technology can be used to determine HBV, HCV, HIV-1, and HPV genotypes, to determine the DNA sequences of bacteria and fungi for microbial classification postculture, and to identify mutations associated with resistance to antimicrobics.

Most DNA sequencing methods are based on a modification of Sanger dideoxynucleotide triphosphate (ddNTP) chain termination chemistry in which synthetic ddNTP molecules that lack 3′ hydroxyl groups normally present in the natural molecules are added to a DNA synthesis reaction (Figure 8) (Wolk et al. 2001). Whenever a ddNTP molecule is incorporated into the growing DNA strand (in lieu of the usual dNTPs), the synthesis reaction is terminated. For dye-terminator cycle sequencing, four different fluorescent dyes are incorporated into each of the four ddNTPs (ddATP, ddCTP, ddGTP, and ddTTP), and the end result is a collection of DNA fragments of different lengths labeled with different dyes depending on the terminal nucleotide. Reaction end products are separated by electrophoresis, excited and detected by a laser. The resulting digital data are processed into electronic DNA sequence data. Sequence data are analyzed by special computer software and are displayed either as linear strings of one-letter nucleotide codes or as a graphic electropherogram.

The generation of large numbers of DNA sequences has created new needs for archiving and updating

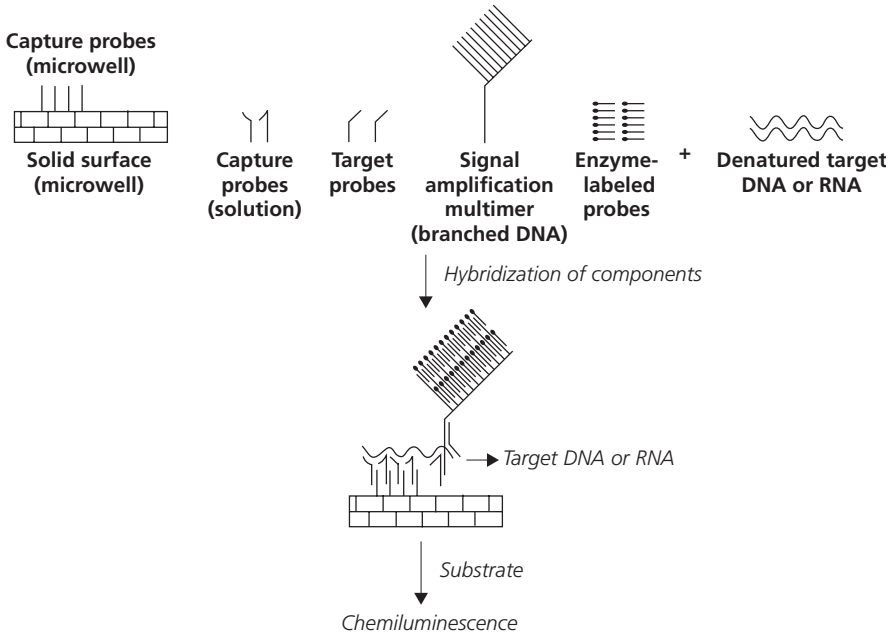

Figure 6 *Branched DNA (bDNA) signal amplification (see text for details). Redrawn with permission from Wolk et al. 2001.*

bioinformatics data and validated sequence information. Although several microbes have been sequenced, the vast majority of microbes, even clinically important microbes, have not yet been sequenced. Furthermore, although microbial genomic databases exist, there are limitations to these databases. The web-based genomic database, GenBank, established in 1988, is available as a national resource of molecular biology information from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Related databases such as the rRNA WWW server (www.oberon.rug.ac.be:8080/rRNA), the Ribosomal Database Project II (www.rdp.cme.msu.edu/html), the Institute for Genomic Research (www.rdp.cme.msu.edu/html), and the Ribosomal Differentiation of Medical Microorganisms (www.ridom.de) provide nonproprietary sources of genomic information (Wolk et al. 2001).

While public databases contain a wealth of information, certain caveats must be noted if the data are to be used by clinical microbiology laboratories (Wolk et al. 2001). The sequence data and the descriptive data included with each entry may not be peer-reviewed, so any use of the information contained in these databases must be made with caution. The quality of the sequence data can vary and some sequences may contain errors. Furthermore, although these are large databases, one cannot be assured that they contain information for every gene for every organism. For this reason, well-validated and comprehensive databases are needed for use in clinical microbiology laboratories. The commercially available MicroSeq® 16S rDNA Bacterial Sequencing kit (Applied Biosystems) combines a PCR and cycle sequencing kit that can be used with the commercially available MicroSeq® software and the commercially available MicroSeq® 16S rDNA database for organism identification. Although this approach is useful to aid in characterization of many organisms, its use is limited by the relatively small size of the MicroSeq® database.

Several commercial systems have been designed for nucleic acid sequencing and sequence analysis. The TRUGENE™ assays (Bayer Healthcare LLC) use dye primer chemistry (as compared with dye terminator chemistry) and a two-dye system in a proprietary (CLIP™) sequencing system. These assays identify mutations in the HIV-1 protease and reverse transcriptase genes, mutations in the HBV surface antigen and polymerase genes, and genotypes of HCV (based on the 5′ NC region) and HBV. Sequence data are obtained and compared with a proprietary sequence. Sequence data are regularly updated and interpreted using proprietary interpretation codes and software.

The line probe assay (VERSANT™ genotype LiPA assay, Bayer Healthcare LLC) is a modification of Southern blot technology in which reverse hybridization occurs with multiple specific nucleic acid probes arranged in a multiline format on a nitrocellulose strip (Mitchell et al. 2002). After PCR is performed using a biotinylated primer, the amplified DNA is hybridized to a strip containing immobilized oligonucleotide probes. Subsequently, streptavidin-alkaline phosphatase is used to detect the hybridized biotinylated amplified DNA. Alkaline phosphatase acts on a chromogenic substrate added to the system to enable color development at locations on the strip where specific DNA is bound. The result is a fingerprint of the hybridization

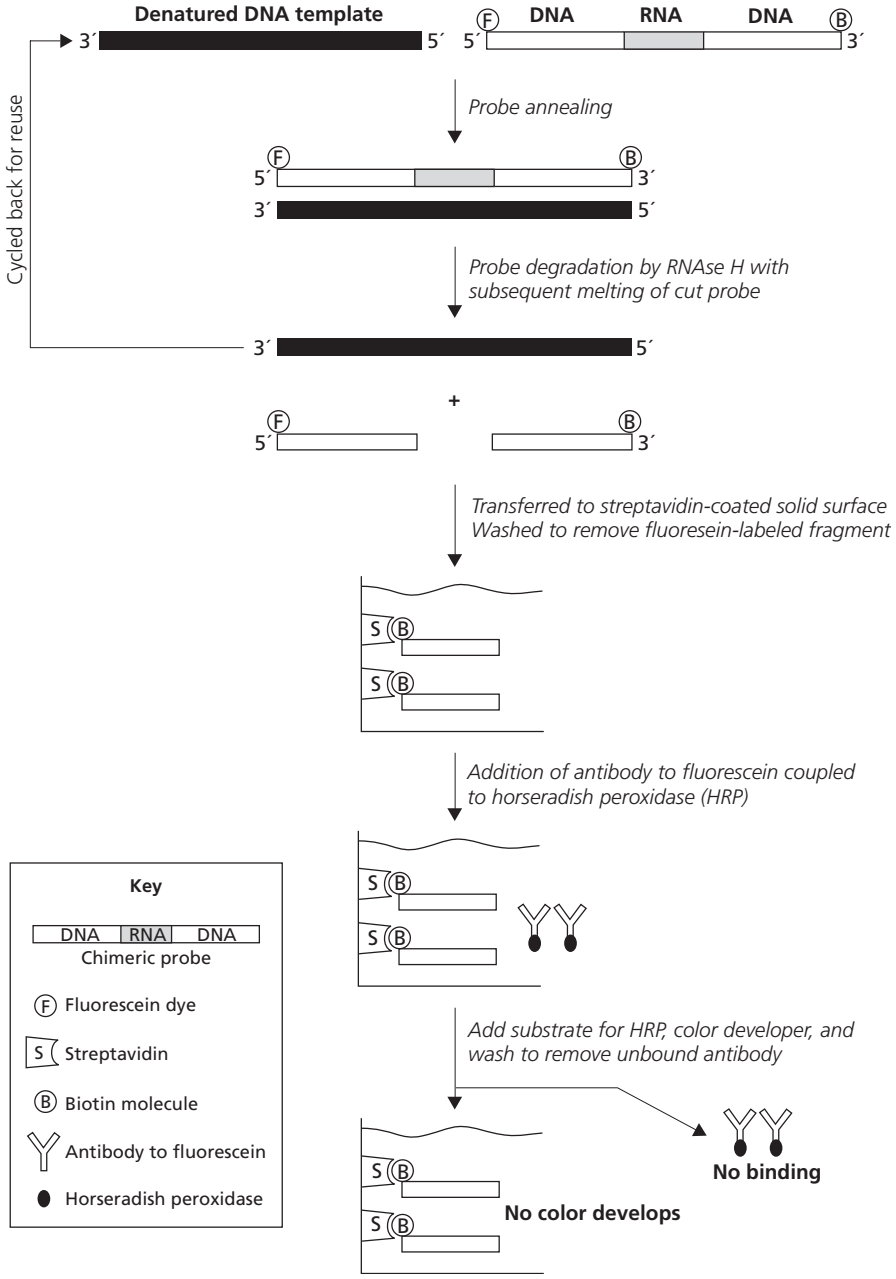

Figure 7 *Cycling probe technology (see text for details). Reprinted with permission from Wolk et al. 2001.*

pattern from the PCR reaction product. Patterns are compared with standard patterns. Kits are currently available for HCV genotyping (based on the 5′ NC region), HIV protease and reverse transcriptase mutation analysis, HBV precore promotor and precore region and polymerase mutation analysis and genotyping, and HPV genotyping.

Pyrosequencing™ (Pyrosequencing, Inc., Westborough, MA and Uppsala, Sweden) is a process by which short fragments of DNA sequence (i.e. 20–30 bases) are determined (Figure 9) (Wolk et al. 2001). With this technique, a sequencing primer is hybridized to a DNA template and incubated with DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5′-phosphosulfate, and luciferin. Complementary dNTPs (dATP shown in Figure 9) are serially added to the reaction mixture. If a dNTP is incorporated into the strand, pyrophosphate is released in a quantity equal to the amount of incorporated dNTP. ATP is generated and drives a luciferase reaction so that light is generated, monitored by a charged-coupled device camera, and converted to graphic data of peaks called a pyrogram. The enzyme apyrase modifies residual dNTPs prior to incorporation of dNTPs in subsequent reactions.

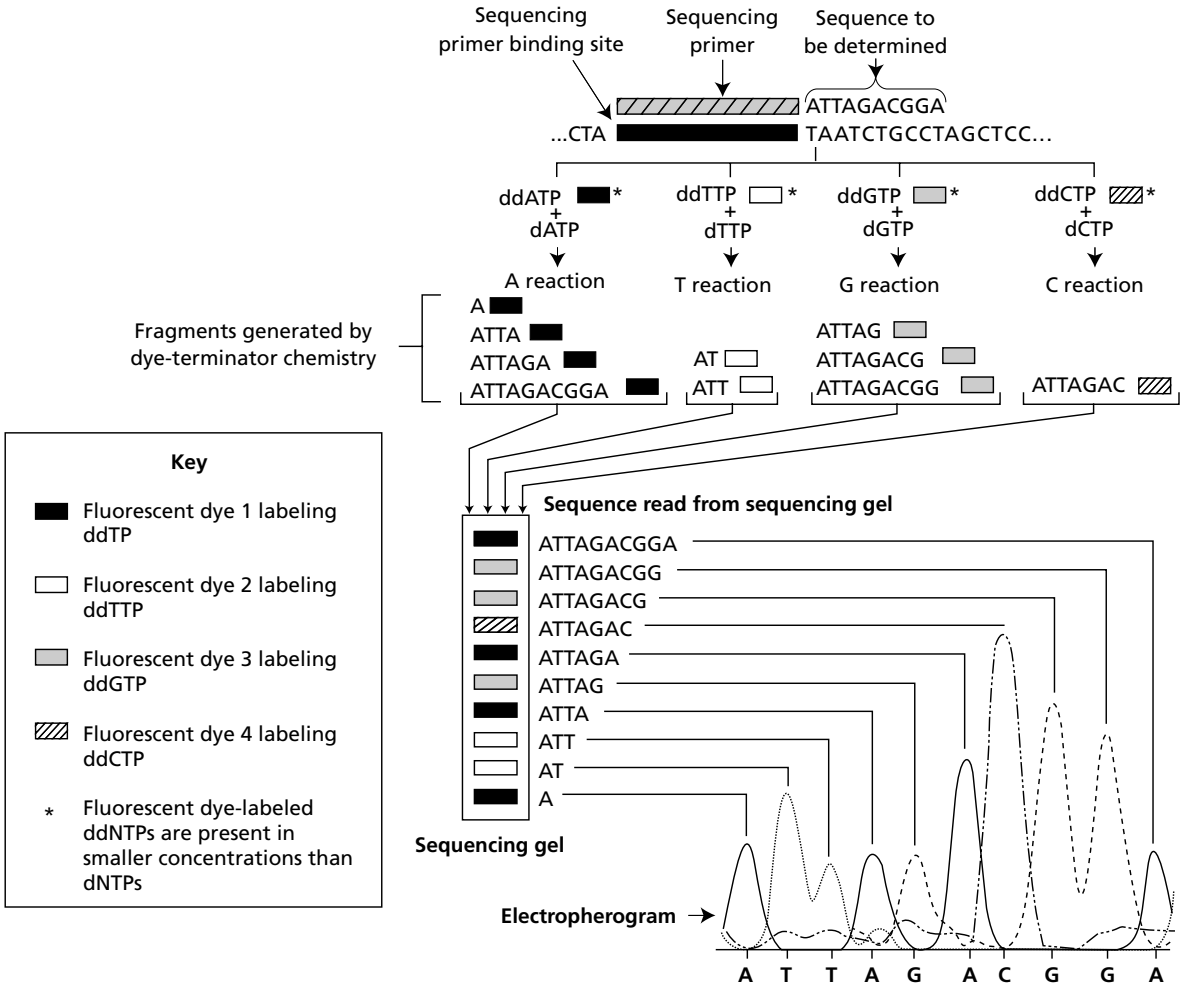

Figure 8 *Dye-terminated cycle sequencing of DNA (see text for details). Redrawn with permission from Wolk et al. 2001.*

MOLECULAR EPIDEMIOLOGY

A variety of molecular techniques for epidemiological strain typing are currently available. Genomic DNA restriction site analysis involves comparison of the number and sizes of DNA fragments produced by digestion of DNA with a restriction enzyme. Because of the specificity of restriction enzymes, complete digestion of an organism's genomic DNA by a specific restriction enzyme provides a reproducible array of fragments that can be separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. Variations in the array of fragments generated by a specific restriction enzyme are called restriction fragment length polymorphisms (RFLP). Changes to RFLP patterns can result from gene sequence rearrangements, insertions or deletions of DNA, or base substitutions within the restriction enzyme cleavage sites. The disadvantage of this technique is that the genomic restriction fragments are usually too numerous and too closely spaced for easy analysis.

To simplify genomic DNA RFLP analysis, several techniques are available to limit the number of fragments for comparison. One approach is to probe the genomic DNA restriction fragments with labeled probes targeting multicopy nucleic acid sequences such as insertion sequences, bacteriophage DNA, or rDNA (ribotyping). The RiboPrinter® System (DuPont Qualicon, Wilmington, DE, USA) is a commercially available ribotyping system that can generate, analyze, compare, and store genetic 'fingerprint' information.

Another approach is to generate large fragments of chromosomal DNA by using 'rare cutting' restriction enzymes that recognize infrequent restriction sites and allow separation of fragments by special electrophoretic procedures. Due to shearing, intact DNA required for the generation of large DNA fragments cannot be isolated using conventional methods. Furthermore, large DNA fragments cannot be efficiently resolved by conventional agarose gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) solves both of these problems. Organisms are embedded in agarose and lysed in situ,

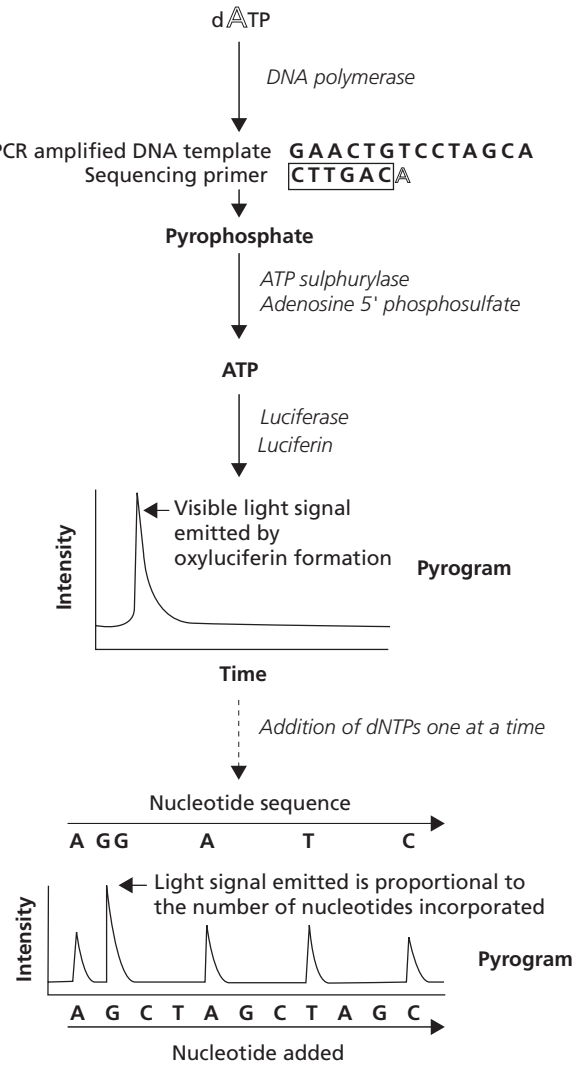

Figure 9 *Pyrosequencing (see text for details). Reprinted with permission from Wolk et al. 2001.*

and the chromosomal DNA is digested with restriction enzymes that cleave infrequently, thereby generating large (macrorestriction) restriction fragments (Wolk et al. 2001). Slices of agarose containing chromosomal DNA fragments are inserted into the wells of an agarose gel. Restriction fragments are resolved into a pattern of discrete bands on the gel by an apparatus that switches the direction of current in a predetermined 'pulsed-field' pattern, resulting in the smaller fragments migrating faster through the gel than the larger ones. The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness. PFGE analysis provides a global chromosomal overview, scanning most of the chromosome, but it has only moderate sensitivity since minor genetic changes may go undetected. Interpretative guidelines for PFGE have been published (Tenover et al. 1995).

AFLP® Microbial Fingerprinting (Applied Bioscience) produces a distinctive DNA fingerprint by selective PCR amplification of restriction fragments of the entire microbial genome. The AFLP® procedure includes the preparation of an AFLP template where genomic DNA is digested with two restriction enzymes ('rare cutter' and 'frequent cutter'), which produce cohesive fragment ends and cut DNA with different frequencies. Following digestion, genomic restriction fragments are modified by ligation (joining) of synthetic, double-stranded oligonucleotide adapters with ends complementary to those of the restriction fragments. Thus, after the ligation step, genomic restriction fragments have termini of known sequences. Such an AFLP template is submitted to highly stringent PCR amplification with primers complementary to the adapter/restriction site sequences. Amplified fragments are separated by electrophoresis and visualized either directly by gel electrophoresis or by the laser detection system of an automated sequencing instrument yielding specific patterns (fingerprints). The different patterns are compared with one another to determine the relatedness of strains.

'Sequence-based' typing involves determining the sequence of portions of several microbial housekeeping genes, a procedure termed multilocus sequence typing (MLST). This technique can generate reproducible isolate profiles that can be compared against standardized databases.

MICROARRAYS

DNA microarray technology has the potential to assess and visualize thousands of nucleic acid templates (targets) simultaneously (Wolk et al. 2001). The underlining principle is nucleotide hybridization, which takes place on a 'chip,' a solid platform (e.g. glass, silica, nylon) containing genetic material (e.g. cDNA, oligonucleotides) arrayed in a predetermined fashion. When the chip is probed with cDNAs that are made from an unknown sample of RNA from a tissue or cell, complementary sequences will hybridize with various sequences already positioned on the chip. By monitoring the hybridization pattern of the unknown cDNA, the identity of the hybridized nucleic acids can be determined. The patterns can indicate which RNAs were in the tissue and therefore which genes are being expressed. Microarray technology can also be used to identify gene sequences and mutations associated with HIV-1 drug resistance (e.g. GeneChip® HIV PRT Plus Probe Array Design for analysis of subtype B protease and reverse transcriptase genes; Affymetrix, Santa Clara, CA, USA), but there is only limited support for its clinical use. Currently, a limitation of microarray technology is the high cost for instrumentation and disposable supplies. In addition, there is an inability to easily analyze the enormous amount of information potentially available through this technology.

CONCLUSIONS

Molecular diagnostic testing is revolutionizing clinical microbiology enabling testing that is faster and more sensitive than conventional methods. The field is rapidly changing as new technology evolves for specimen preparation, nucleic acid amplification, and nucleic acid sequence analysis and interpretation.

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Bacterial immunoserology

ROGER FREEMAN

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INTRODUCTION AND OVERVIEW

Bacterial immunoserology has long been an important component of the diagnosis of infectious diseases. As is so often the case in medicine, the subject is not precisely defined or confined by its title, nor is it always or necessarily underpinned by a thorough scientific understanding of the mechanisms on which it is based. Indeed, rather than immunology being the science out of which bacterial immunoserology emerged, the reverse is often more true. Empirical observations made by early bacteriologists in the pursuit of clinical diagnosis were often the platforms for subsequent scientific immunological exploration.

Bacterial immunoserology is also part of the long tradition in diagnostic microbiology, whereby parallel developments in other medical sciences have been adopted into the subject to satisfy the understandable demands of clinicians for ever more rapid results.

Bacterial immunoserology also illustrates well the need to revisit subject areas in medicine regularly in the light of technological advances. The most recent is the advent of molecular biology. To give two examples: First, the advent of recombinant antigen techniques now allows the use of antigens in amounts well beyond those naturally available, thus unlocking the ability to devise tests which were theoretically, even obviously, attractive in the past but simply impossible to deploy on any useful scale. Obvious examples include antigens not expressed extracellularly (e.g. pneumolysin in *Streptococcus pneumoniae*) or antigens from organisms never or hardly ever cultured successfully (e.g. *Treponema pallidum*). Second, the developments in proteomics, transcriptomics, and microarrays will permit a more logical and precise definition of which antigens (and in which form) might be useful for further study.

All this being so, bacterial immunoserology currently comprises a whole range of methods, often empirical, occasionally paradoxical, but above all practical, deployed to aid in the diagnosis of infectious diseases.

The subject can be divided into two clear areas. First, aiding diagnosis by measuring the response of the patient (or patients), for instance by finding antibodies to a particular organism or its products. Second, by using immunological reagents (usually produced by animals or from another nonhuman source) to detect organisms or their products. These two approaches will be considered separately.

First, however, it is necessary, briefly, to consider the various responses that bacterial invasion evokes. Detailed and comprehensive accounts of the many components of the immune response are found elsewhere in these volumes. What follows is a greatly simplified overview, suitable only for the present purpose.

THE MULTILAYERED RESPONSE TO INFECTION

The immunology of infection is frequently portrayed as a battle in which the aggressors (microbes) gain footholds in the body by overcoming the standing defenses, such as skin, mucous membranes, etc., and then begin to multiply, spreading further, creating more local damage. At the same time, a wide range of microbial products (enzymes, toxins, etc.) are released, which may enter the circulation and cause damage at sites distant from the portal of entry. The body responds in diverse ways, each designed to limit the damage, localize the extent of invasion, and, where necessary, neutralize the toxicity of elaborated bacterial products. Outcomes range from resolution (implying a return to total normality, as though no infection had ever occurred, as for instance in pneumococcal pneumonia) through varying degrees of residual damage (with consequent morbidity) to death.

There is an order and a chronology to these events, which allows bacterial immunoserology to be applied.

THE ACUTE-PHASE RESPONSE

The acute-phase response (APR) is the body's generic response to any significant injury, but its magnitude is greatly enhanced if infection is involved or supervenes. There are three pathological components: the development of local hyperemia as blood vessels dilate, the local exudation of certain plasma proteins, and the attraction and diapedesis of white blood cells (predominantly neutrophils) into the tissues at the site of damage. This is not a passive process. Indeed, it is the consequential responses of the body in augmenting the white cell response and the plasma protein response that make the APR a valuable phenomenon in the diagnosis of bacterial infections.

As neutrophils are attracted to the site and consumed, the bone-marrow neutrophil reserves are immediately released into the circulation and the bone-marrow stem cells respond by producing more neutrophils. This results in a peripheral leukocytosis with a pronounced neutrophilia, containing predominantly juvenile neutrophils, the so-called 'shift to the left.' In inflammation due to infection, these events are considerably exaggerated, probably due to the ability of most bacteria to degrade complement (a prominent acute-phase protein), the degraded or 'split' products of which are an extremely powerful chemotactic stimulus for neutrophils.

Within 2–4 h of injury/infection, the liver begins to synthesize and release large amounts of a range of plasma proteins (acute-phase proteins), some of which are normally found in the plasma in small amounts (e.g. complement) and some of which only appear under these circumstances, e.g. C reactive protein (CRP). The magnitude of this response (i.e. the levels of these easily measured proteins) gives an index of the degree and extent of the inflammatory damage being undergone by the body, from which inferences can be drawn. Thus, an apparently inappropriately large APR response (for instance, a very high white blood cell count, a very pronounced neutrophilia, and a very high CRP level) would imply that either a noninfective injury was much larger or more severe than originally suspected, for instance having gone on to necrosis, or, commonly, that infection was present.

The ease and speed with which both the leukocytosis and CRP can be measured and their equally rapid response to successful control of the APR, for instance by correct chemotherapy of an infection, make them useful and versatile tools in clinical microbiology. They are especially useful when the patient has not responded as well as expected to antibiotic therapy, say by continuing to be febrile. There being many other reasons for the fever, a normal or rapidly declining leukocytosis and CRP might eliminate lack of control of the infection as the reason. Conversely, if both remained high, it might be wise to alter therapy, take further samples, or even to contemplate superinfection with a different organism.

A major disadvantage of the APR is that it is nonspecific, i.e. the organism is not identified, or not, at any rate, by this means. However, the APR can be very specific indeed in a negative sense. Thus, if an organism is isolated from a blood culture or another pathologically very significant site, such as cerebrospinal fluid, it will be very unusual indeed if all parameters of the APR, and particularly the peripheral white blood cell count (WBC) and the CRP, are entirely normal.

This very combination of events occurred many times several years ago, when the organism *Stenotrophomonas maltophilia* (then called *Psedudomonas maltophilia*) was isolated from blood cultures in apparently well, or at least apparently nonsepticemic, patients. The clearly negative APR parameters led to a search for another explanation and it emerged that blood culture samples were being cross-contaminated with *Ps. maltophilia* from nonsterile biochemistry sample tubes, filled at the same venepuncture. The condition dubbed 'pseudobacteremia' was thus identified (Jumaa and Chattopadhay 1994). Similar intelligent uses of the APR have undoubtedly saved many patients from unnecessary antibiotic therapy.

Problems in interpretation of the APR

Problems may be encountered with interpreting the APR under several circumstances.

First, if either of the major contributing organ systems is grossly deranged, then production of the APR components may be inappropriately low or even absent. Thus, the WBC response may be abnormally low in marrow

failure. Acute-phase proteins may not be produced in normal amounts in patients with liver disease, whether acute or chronic. Rare conditions in which complement or its components are not manufactured may greatly dampen the amplitude of the APR.

Second, there are rare instances in which organisms, especially *Staphylococcus aureus*, can survive often for many years, in a 'privileged site,' immured from the APR. A good example is the so-called Brodie's abscess (Stephens and McAuley 1988) in which the infection survives as an indolent localized pocket of infection in a sclerosed area of bone, often the residue of a childhood bacteremia that did not progress to acute osteomyelitis. After a period the APR declines, although the CRP is often not entirely normal. However, the patient very occasionally has a febrile attack or even a rigor. If blood cultures are taken, they may be positive. Brodie's abscesses are easily seen on X-rays. The erythrocyte sedimentation rate (ESR) is often raised, but this is as a result of the dysglobulinemia and secondary anemia (often called the 'anemia of inflammation') due to longstanding production of humoral antibody (see below), rather than the presence of any acute-phase protein.

Third, in severe infection due to gram-negative bacilli, such as *Escherichia coli*, the WBC may fall precipitately rather than rise. This effect is thought to be due to the direct effect of endotoxin (lipolysaccharide (LPS)) on the neutrophils, causing their degranulation and resulting in some of the features of disseminated intravascular coagulation (DIC). Prompt and effective therapy of the presumptive gram-negative septicemia will often result in a rapid and gratifying resolution of the leukopenia.

Recent developments in measuring the acute-phase response

There has been a longstanding wish to find a component within the APR that is specific to infection and, therefore, will allow the distinction to be made between noninfective inflammation and sepsis without recourse to the subjective estimates of magnitude alone. In any case, it is frequently the case that infective and noninfective values overlap. This desire is particularly strong in those caring for infants and children (Nuntnarumit et al. 2002) and those dealing with intensive care patients of any age, in all of whom physical signs are notoriously unreliable. There have been two lines of investigation.

First, several candidate acute-phase proteins have been put forward as being disproportionately raised or even raised at all in bacterial infection. The most recent candidate is procalcitonin (PCT). The evaluation of PCT has parallelled that of many other candidates over the years, in that it seems that PCT does indeed rise higher in bacterial inflammation than in noninfective syndromes (or viral infection), but still not exclusively so (Balc et al. 2003; Schuttrumpf et al. 2003).

Second, it is known that one component of the APR does behave very differently in infection. This is the neutrophil. When neutrophils phagocytose a live bacterial cell, they must first kill the bacterium prior to being able to digest it. The intracellular killing is achieved by a respiratory energy burst fuelling an oxidative microbicidal system, which results in the organism being killed by halides (probably by iodination). This efficient system will kill *S. aureus* within approximately 20 min (Klebanoff 1980).

It is possible microscopically to identify such 'activated neutrophils' by supravital staining methods wherein tetrazolium (for instance, nitroblue tetrazolium (NBT)) salts are reduced to insoluble formazan, which is visible as a deposit in the neutrohil phagosome. It was, therefore, hoped that estimation of the percentage of peripheral blood neutrophils reducing NBT would be much higher in patients with severe infection. In general, this is so, but extensive evaluation concluded that, once again, the test was not specific enough for the purpose, although it retains an important place in the testing of neutrophil function (see below). Attempts to find an 'infection-specific' component of the APR will doubtless continue. The prize is immense.

Final comments on the APR

Clinicians and microbiologists evaluate the APR in most patients daily without formally recognizing the exercise. The mundane nature of the APR is, in fact, one of its most important aspects. The ability rapidly to detect inflammatory damage, including that due to, or complicated by, bacterial invasion, rapidly to deploy an effective systemic response to the site that can begin to impede the progress of the damage and moreover deliver effector cells that can kill pathogenic bacteria and scavenge debris, is a triumph of evolution. This mechanism deals, mostly successfully, with the vast majority of infective assaults on the human body. Its manifestations range from small pustules on the skin to large areas of suppuration, such as peritonitis. Studies of neutrophil function and of other aspects of the APR suggest that low-level ongoing activity of this system, outside the occasions when it is recognizably evoked, is probably essential to the maintenance of the integrity of mucosal surfaces and the skin. The development of 'mucositis' in patients in whom neutrophil function or numbers is depleted also suggests this.

If the APR is successful, invading organisms do not, largely, survive to present an ongoing threat and, hence, do not require further response. There are, however, organisms that survive the APR.

THE GRANULOMATOUS RESPONSE

In an immunocompetent host, the granulomatous response is the exception rather than the rule and is induced by organisms that can survive intracellularly. Thus, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Brucella* spp. will be the organisms predominantly involved in temperate countries. Other species will be more common in other climates and environments.

The unity of the above hypothesis is strikingly supported by the occasional example of what happens when immune defects in, for instance, the neutrophil bactericidal mechanisms, detected by a demonstrable inability to reduce NBT, even in the presence of live bacteria, force the body to react to unkilled common organisms, such as staphylococci, as though they were intracellular bacteria, such as mycobacteria. This results in granulomatous reactions to these mundane bacteria and produces a clinical disease called chronic granulomatous disease (CGD) (Gallin and Fauci 1983). CGD was for some time thought to be an atypical form of tuberculosis, but the discovery of the intracellular killing system and of defects within it showed that affected children were forced to deal with all bacteria in a way in which their healthy peers reserved for rarities. Other examples of deficits, such as myeloperoxidase deficiency, and so on, serve to emphasize the pivotal importance of the neutrophil bactericidal pathway in determining how the body reacts to bacteria.

There are two consequences of the granulomatous response relevant to bacterial immunoserology.

First, the surviving organisms will enter the active specific immunity system, antigen will be processed, and both cellular immunity and humoral immunity will result. Thus, it will be possible to devise tests based on (1) humoral antibody detection and/or (2) evocation of specific cellular immune responses to antigens to diagnose infection with these organisms.

Second, it is highly possible that the bacteria involved will remain alive within the body, albeit localized and contained by the granulomatous reaction. Frequently, it seems, these contained organisms adopt a different physiological and metabolic state, and this may even extend in some species to a viable but noncultivable state. In terms of the infection or disease associated with the organism, it is often described as a latent phase. Latent infections may and do reactivate, often when an intercurrent illness, medical procedure, acquired immunoincompetence, or simply advancing age depresses the immune system below the level required for continued containment of the organism. Thus, it will be possible not only to devise tests based upon (1) humoral antibody detection and/or (2) evocation of specific cellular immune responses to diagnose infection with these organisms, but also, under certain circumstances, to identify latently infected but seemingly healthy individuals who will be at risk of subsequent symptomatic disease. Pre-emptive therapy may therefore be possible.

Humoral antibody production

Figure 1 is a diagrammatic and idealized depiction of humoral antibody production after the introduction of pathogenic bacteria into the body, subsequent bacterial infection, and development of active acquired immunity in a patient never before exposed to this particular organism.

All specimens for serological studies must be seen as individual static 'snapshots' in a dynamic process, the natural history of the disease. This is easier to appreciate in virology since viral diseases have, until recently, run

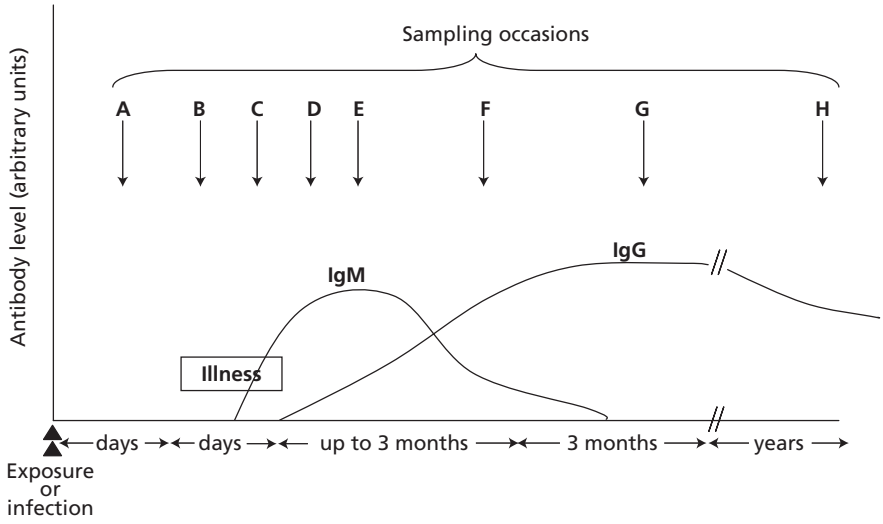

Figure 1 *Schematic representation of humoral antibody response following a single point exposure to an intracellular infectious agent.*

their course, there being no possibility of intervention. Nonetheless, the same view of many nonpyogenic, granulomatous bacterial diseases is equally important.

Before considering the differing significance of results from specimens taken at the sampling occasions given in Figure 1, it is important to clarify two matters of simple practical importance.

First, this account will shortly be referring to antibody 'titers,' a measure of the amount of antibody/unit volume determined by serial dilution (titration) of a sample to the endpoint of reactivity. These will be referred to as, for example, 'a titer of 1 in 32 (or 1/32).' This is a scientifically incorrect statement that has become correct by common usage. Strictly, titers are the *reciprocal* of serum dilutions, so the preceding should be 'a titer of 32,' the titer being a measure of the strength of the reaction studied, in this case 32 units/volume. It is a way of comparing the amounts of antibody in two or more different samples. This is an essential concept in the quest to study and compare serial samples, but it can be confusing. Despite many laudable efforts to correct this problem, it persists. To summarize, a sample with a titer of 1/32 has a stronger reaction (i.e. contains more antibody/unit volume) than one with a titer of 1/8.

Second, the technical reproducibility of doubling dilution is one dilution either side of the intended value. Hence, a serum with a titer of 1/32 may, some of the time give a titer of 1/64 and some of the time 1/16. In order, therefore, to be certain that two titers are significantly different, it is insisted that they differ by at least two dilutions. This leads to the stipulation that a significant rise in antibody titer between two samples must be at least four-fold, say 1/8 to 1/32, or 1/10 to 1/40.

Figure 1 shows that after infection, there is often a short period in which the patient remains ostensibly unaffected and well (the incubation period). Next follows the clinical illness, during which it is often possible to directly detect the causative agent and so make the diagnosis without recourse to bacterial immunoserology. However, if it is assumed that the organism cannot be cultured, or that inadvertent antibiotic therapy has rendered culture useless, or for any other reason diagnosis cannot be made by direct detection, bacterial immunoserology may be useful.

It is, therefore, important to note that IgM is the first antibody produced by the host, often whilst the clinical illness is still ongoing. IgM is the classical first response and, because of its large molecular size, remains confined to the bloodstream even after its production declines. It can usually be detected for at least 3 months after its first appearance. Unfortunately, however, IgM is a notoriously difficult immunoglobulin with which to contend in assays, mainly because it is so avid or 'sticky' that it frequently reacts with antigens other than that which evoked it. Nonetheless, its appearance (and, therefore, detection) strongly suggests both a first infection with the appropriate organism and its recency.

Shortly thereafter, specific IgG becomes detectable. IgG is a much more easily assayed immunoglobulin and assays based upon it are usually very specific. However, its later appearance and the fact that it is the 'memory' immunoglobulin (i.e. the antibody that persists for years, perhaps for life) make its detection difficult to interpret in a single specimen. To settle a judgment of de novo production (and, therefore, recency) on IgG levels requires evidence of increasing levels over a short period of time. This is the classical immunoserological concept of the rising titer.

Some recent work has drawn attention to the differences in avidity for the same antigen between the IgG produced for the first time and that produced later or after rechallenge with the same antigen. These differences can be demonstrated in technically simple ways. It may, therefore, be possible to devise IgG assays that will also indicate recency of infection. However, although this interesting approach has been pursued in a small number of instances (Prince and Leber 2002), it has not yet gained wide support.

Turning, therefore, to the details in Figure 1, it is now possible to discuss the results of assays for both IgM and IgG at the different sampling occasions and to interpret the significance of the results. It will already be evident that the same assay result (especially for IgG) may have an entirely different significance, depending on when it was taken in relation to the disease.

SAMPLE A

No antibody of any sort is detected. Absence of antibody in the incubation period is to be expected, implying that if known to have been exposed, the patient is likely to become ill soon. This is not often a useful observation in bacterial infection, but it is of immense use in the childhood viral exanthema, allowing isolation.

SAMPLE B

Early in the illness and no antibody is yet detectable. Although such samples will hardly ever themselves make a diagnosis, such so-called 'baseline' or 'acute' samples are vital for subsequent comparative tests (see below). Many experienced investigators simply store such samples until later samples are obtained.

SAMPLE C

In later illness, specific IgM is detected. Provided that the specificity of the assay is beyond question, the diagnosis can be made, the presence of specific IgM being evidence of both a novel antibody and of a recently produced antibody. However, further samples should be requested to confirm subsequent IgG production so that specificity is confirmed (see below).

SAMPLE D

In late illness or early convalescence, both IgM and IgG are detected, the specificity of the IgG complementing and confirming the implications of the IgM result. This is a common format for commercial test kits. However, the IgG levels found at this stage may border on the limits of detectability.

SAMPLE E

In established convalescence, IgM can still be detected and IgG is plentiful. It is also now possible to explore an alternative and highly specific way to make the diagnosis. If it can be shown between successive samples that the levels of IgG are significantly increasing with time, this is good evidence that the infection is ongoing or recent. Thus, if the IgG antibody level is greater in E than in D, this will be so and will secure the diagnosis. It will be even more certain if E is compared with B, since B contains no antibody. Hence, the prudence in taking samples early in the illness, though they alone cannot make the diagnosis (see above).

It might be argued that samples taken prior to A might also serve. For instance, serum may be available from an unrelated event in previous years. However, all that can then be said is that the infection has occurred some time between the sample dates of 'pre-A' and E. If this is a long time (months or even years), the significance might be lost in terms of any one specific episode of illness if several have occurred in the interval.

SAMPLES F AND G

IgM is declining to low levels and its index of recency is becoming eroded. Low levels of IgM antibodies (and IgM-like antibodies) can be found in normal states. However, the relatively high levels of specific IgG make recent infection likely, but unless earlier samples are available for comparison, the results on a single sample taken at F might be misleading. Since IgG production is now nearly maximal, comparison of the titer at F with that at G may not help. It might be observed that the need to make the diagnosis is now long past and, therefore, the exercise is pointless. However, it is occasionally very important to establish the nature of an illness undergone several months before, perhaps in a distant location, to ascertain the need to test contacts and to gauge prognosis and the need for further, perhaps invasive, investigations. In earlier days, a common such example was enteric fever.

The serological picture has now gone full circle because similar questions can now be asked as were also asked at point A, although the answers will be quite the opposite. If antibody is detectable during the incubation period, the patient is immune, assuming the antibody detected correlates with protection. It is remarkable how often a previous serum specimen, often submitted for a totally unrelated reason, such as, for instance, antenatal screening, can be recalled and tested to solve a current problem. Consequently, storage of sera is good practice.

SEROEPIDEMIOLOGY

A very important aspect of humoral antibody testing is the use of the results from individual patients being viewed collectively as those of a population. This is the basis of the very important subject of seroepidemiology, whereby antibody testing of carefully structured populations is designed to address specific questions in relation to the incidence and prevalence of a given illness within the population. Good evidence can be produced to assess the extent of disease, those groups most likely to contract it, and, therefore, those groups most likely to benefit from interventions such as vaccination. This is yet another reason for sera to be stored over a longer period. If storage of all sera is impracticable, the preservation of a collection representative of the wider population and extending through a period of several years is a useful surrogate and will greatly inform epidemiology studies.

Three other points require to be addressed before progressing to look at the application of these principles in real examples.

First, in disease the antigen persists. The situation is not precisely analogous to the single antigen injection given under experimental conditions on which Figure 1 is based. Patients may receive both primary and further 'doses' of antigen as the illness continues. Therefore, the speed and magnitude of antibody production will be greater than in experimental observations.

Second, in clinical disease the host is required to respond to a variety of antigens of differing immunogenicity, both polysaccharides and proteins. A spectrum of antibodies results, each directed against a different antigen. These different antibodies may exhibit markedly different degrees of persistence, some being detectable for years, others for only a few months, perhaps reflecting the different immunogenicities of the antigens which elicited them. Whatever the explanation, these phenomena can be exploited to good practical effect in bacterial immunoserology, an excellent example being the contrasts between the O and H antibodies in enteric fever (see below). A side issue of this point is the realization that antibodies evoked by weak (poor) or incomplete immunogens may not only be transient in their appearance in the circulation but also may escape immunological memory and be re-evoked in subsequent infective episodes.

Third, there is little true knowledge of the functions of the many antibodies which can be detected. The point re-emerges that bacterial immunoserology is an empirical, albeit useful, practice. What follows are several examples of the practical application of humoral antibody testing in diagnostic bacteriology.

Enteric fever (infection with *Salmonella* Typhi) is here used as the paradigm. Infections with *Ss. paratyphi A*, *B*, and *C* behave similarly but are omitted for clarity.

S. Typhi exhibits three well-studied antigens. H, or flagellar, antigens, are protein in nature and unique to *S.* Typhi; O, or somatic, antigens, are polysaccharides and more widely distributed within the *Salmonella* species than in *S.* Typhi alone. The Vi, or virulence, antigen is thought to be capsular in nature and is found only in recently isolated strains.

It is relatively straightforward to produce suspensions of *S.* Typhi that exhibit either only H or only O antigens at a time (the details are unnecessary). Since these suspensions will be particulate, antibody can be detected by demonstrating the ability of the serum to agglutinate the suspension (see below). The test to be described (and many variations on this theme) is often called the Widal reaction.

Typhoid fever follows the ingestion of the organism and has an asymptomatic incubation period of 7–10 days, during which the organisms multiply within the gut lymphoid tissues (Peyer's patches) and subsequently invade the bloodstream to give rise to a particularly serious form of gram-negative septicemia, often characterized by a leukopenia. Before ingestion of *S.* Typhi and during the incubation period, no antibodies will be detectable. As the fever breaks, diarrhea ensues and at this time, or shortly afterwards, both O and H antibodies may be detectable. As the disease continues and, hopefully, thereafter subsides, both antibodies can be detected, although typically O antibody may be more plentiful than H. In late convalescence the H titer continues to rise, but the O titer plateaus. A year later, the H antibody will remain easily detected, but O antibody may have become undetectable once more.

The preceding commentary serves to explain these results. O antibodies rise first but, more importantly, decline quickly after the illness, perhaps because of their nonprotein nature. Though less specific than H antibodies they serve as a good index of recency. Their specificity, however, must be confirmed by the copresence of the highly specific H antibodies, which also serve as the memory antibody in future years, when O antibody is gone. Four-fold or greater rises in antibody titers in either antibody indicate ongoing production. Thus, it is possible to distinguish between a nontyphoidal enteritis, early typhoid fever, recent typhoid fever, and past typhoid fever. No IgM detection was typically employed in the Widal reaction, although it might well have been in the same way as that for the brucella test (see below).

Vaccination against typhoid (and paratyphoid) fever has been commonly practiced since the days of Almroth Wright. Most such vaccines were intended to evoke H antibody, which is at least partially protective. However, such antibodies can confuse the interpretation of the Widal reaction, but in cases of doubt antibodies to the Vi antigen can be sought. This antigen was traditionally excluded from the vaccine but is present in great amounts in a natural and ongoing infection.

The Widal reaction has fallen from favor for three reasons. Modern cultural and noncultural detection methods make it increasingly outmoded, vaccine changes make it increasingly difficult to interpret, and, most of all, the development of antibiotics capable of being used widely and effectively (especially fluoroquinolones) limits the necessity to make serological and retrospective diagnoses.

BRUCELLOSIS

Brucellosis is a zoonosis. Humans at risk include farm workers, vets, slaughter men, and those in other occupations concerned with livestock and farms. The organism can also be transmitted via the ingestion of unpasteurized milk.

Brucellosis is a disease with discernible clinical stages. It begins with an acute bacteremic phase in which the patient has chills and rigors, is usually severely debilitated, and sometimes has a rash. This phase may then remit and then recur. Sometimes this cycle is repeated several times, the characteristic temperature pattern earning the disease its alternative name of undulant fever. Brucella organisms can usually be isolated from the blood at this stage but successful culture of this very fastidious bacterium is very difficult and cultures are often pronounced negative. Additionally, culture is often discouraged because of a high risk of laboratory-acquired infection. Untreated, the acute phase eventually peters out and the organisms localize into the reticuloendothelial system, principally into the liver and the spleen, together with involvement of bones and joints, especially of the spine. Unsurprisingly, as the infection becomes chronic, the patient becomes debilitated with anemia, low-grade fever, weight loss, and increasing problems with arthritis, especially of the spine. If still unsuspected the disease continues, eventually shortening the life span and occasionally ending in brucella endocarditis.

The difficulties in culturing the organisms make the diagnosis of brucellosis an ideal opportunity for bacterial immunoserology, and it is a particularly instructive example. The main technique used is that of agglutination of whole brucella organisms. Serum taken in the acute phase will contain high levels of specific agglutinins, and this result will of itself strongly suggest the diagnosis of acute brucellosis, the antibody being unusual in the general population. Since at this stage the organism is predominantly in the blood, it also follows that the agglutinins will be largely of IgM. IgM is a pentameric immunoglobulin rich in disulfide bonds, and exposure to 2-mercaptoethanol (2ME) destroys its activity. If, therefore the agglutination test is performed in parallel, first using normal saline as diluent and

second using saline plus 2ME, a substantial drop in the agglutination titer in the second test strongly suggests that the agglutinins are IgM in nature and confirms that the patient has acute brucellosis. This is doubly important since the disease responds better and more completely to antibiotic treatment in the acute phase.

As the disease (if undiagnosed and untreated) enters the chronic phase, the titer of agglutinins will fall, but not usually to an undetectable level. However, faced with a patient with a suggestive clinical picture but only very modest levels of antibody in the agglutination test, the diagnosis may be difficult to sustain by this means alone. Brucella organisms (or, perhaps more accurately, processed brucella antigens in the tissues) evoke IgG antibodies in high titers, which react in a complement-fixation test (CFT). Thus, the detection of low-titer agglutinins, the activity of which is not abolished by mercaptoethanol, and a strong reaction to high titer in the brucella CFT is the classical immunoserology of chronic brucellosis, a condition requiring very prolonged antibiotic therapy with drugs that will penetrate granulomata and one that is difficult to declare cured even after many years. Thus, bacterial immunoserology can not only diagnose but also stage the disease and, thereby, give useful guidance to therapy and cure.

There is one caveat. As will be seen below, it is a characteristic of intracellular infection that both cellular immunity and hypersensitivity will be evoked, especially in the chronic phase. Repeated exposure to brucella antigens can result in such hypersensitivity without the necessity of preceding infection with the organism. This can be manifest as a positive 'Brucellin' skin test, which is performed and read in a way analogous to the Mantoux test in tuberculosis (see below). Low levels of agglutinins can accompany this, although the typically negative brucella CFT will suggest an absence of genuine chronic infection. The hypersensitivity nature of the phenomenon can be positively shown by the detection of specific IgA in the serum.

Thankfully, acute brucellosis is now a rare human disease in most advanced countries due to the eradication of the infection in animals by a slaughter and restocking approach. However, patients with chronic brucellosis will continue to be discovered for some time to come. In many laboratories, a single screening test using an enzyme immunoassay technique (see below) is now used.

SYPHILIS

The history of the serological diagnosis of this medically and socially very important disease is almost the history of bacterial immunoserology in microcosm. The protean clinical manifestations of syphilis, resulting in its nickname of the 'Great Imitator,' made the need for an objective and impartial laboratory test essential for differential diagnosis in illness and for the detection of a latent phase of indeterminate duration (up to decades).

The first tests were based on the empirical observation that a liver extract from syphilitic fetal liver appeared to fix complement in the presence of sera from patients with syphilis. The obvious, but erroneous, assumption was that antigens from the then (and still) uncultivable *Treponema pallidum* were the basis of the test, subsequently universally dubbed the Wassermann reaction (WR). In fact, even some of the early observations hinted at two anomalies with the WR. These were the finding of false-positive results (reactors who clearly did not have syphilis) and occasional claims that the test worked almost as well if nonsyphilitic tissue was used as the antigen. Finally, it emerged that the relevant antigen (called cardiolipin) could be found in other normal tissues and was eventually able to be synthesized. It also emerged that the substance reacting with cardiolipin was not, in fact, a true antibody but a serum component evoked, possibly due to tissue damage, in syphilis and some other states and called reagin. (Note: this substance must be clearly distinguished from the reaginic antibodies encountered in allergic states.)

Reagin is nowadays detected by a slide or tube flocculation technique (this latter being a virtually unique serological appearance in which 'agglutination' of a much coarser than normal nature is produced) using a cardiolipin preparation produced to a standard defined by the Venereal Disease Research Laboratories (VDRL) several years ago. This test is inevitably referred to as the 'VDRL test' or, simply, the VDRL.

As experience grew with cardiolipin tests, it became obvious that specificity was a major problem and that false-positives were common. These so-called biological false-positives (BFP) were of two sorts. Many were transient and, interestingly, often associated with diseases or states in which liver function was temporarily deranged. Common examples included viral hepatitis, glandular fever, and pregnancy. When the associated condition resolved, these acute BFPs became negative. A timeframe of 3 months usually sufficed for this. Conversely, chronic BFPs by definition of more than 3 months' duration were frequently associated with much more serious and chronic diseases, typically of the autoimmune variety, such as systemic lupus erythematosus (SLE).

Nonetheless, the VDRL was also still positive in almost all cases of syphilis and retained and still retains its place in the diagnosis and management of the disease because the VDRL titer is an extremely useful index of the activity of syphilis. It has been justifiably called 'the ESR of syphilis.' Its retention, however, made mandatory the devising of other much more specific tests to set the VDRL result in a proper context.

First efforts centered on attempts to grow *T. pallidum* and thereby prepare a highly specific antigen for (at that time) the inevitable CFT. An early partial success was the isolation of what came to be called Reiter's strain. This is not *T. pallidum* but at least the extracted antigen (Reiter protein) was more specific than cardiolipin and

conferred a treponemal group status to any antibody detected. This was the basis of the Reiter protein CFT (RPCFT).

Although *T. pallidum* was, and yet remains, uncultivable, the organism can be made to infect and multiply within rabbit testes and on this basis artificial serial passage can be accomplished. The resultant suspensions of live *T. pallidum* so produced were used in a form of neutralization test, whereby the characteristic motility of these live spirochetes was shown under dark ground microscopy to be inhibited by the presence of immune serum. This very laborious and expensive assay was the *Treponema pallidum* immobilization (TPI) test. Immunological technical developments have since allowed the *T. pallidum* suspension to be used as the basis of an indirect immunofluorescent test (fluorescent treponemal antibody (FTA) test). An even more specific version of this approach was then devised, whereby treponemal group antibodies were removed from the sample, prior to testing, by adsorption with the Reiter protein antigen so that any remaining antibodies must be to antigens found on *T. pallidum* only. This is the principle of the FTA (abs) test. *T. pallidum* material was also made to adhere to red cells, allowing the development of a passive hemagglutination test (the TPHA).

In terms of *T. pallidum*-specific tests, it is now possible, using genomics, proteomics, and recombinant antigen technology, to produce a range of tests, utilizing a range of different formats (most commonly enzyme immunoassay assay (EIA)), which detect antibodies specific to only a few epitopes of *T. pallidum*. As will be appreciated, many of these kit-based tests will also be capable of being made specific for IgM antibodies, if needed. Thus, over 100 years of immunoserological development has furnished the tools to decipher the diagnosis of syphilis, but it is testimony to the practicality of the bacterial immunoserology approach that the oldest, least specific test and one that in retrospect never detected an antibody at all, the VDRL, is still an essential component of the syphilis testing portfolio.

Tests of hypersensitivity and cellular immunity

In intracellular infections, a range of immunological phenomena occurs in addition to the production of humoral antibodies. The granulomatous nature of the histological response has been mentioned already. Two further characteristics can also be capitalized upon to devise diagnostic tests. These are the behavior of lymphocytes and other cells in response to an appropriate antigen and the development of hypersensitivity phenomena.

Other chapters will discuss these matters in detail. As always in bacterial immunoserology, this section will concentrate on the practical applications. This account will deal almost exclusively with tests in tuberculosis, which is currently by far the major disease in which these tests are used. Hypersensitivity tests were the first to be developed.

HYPERSENSITIVITY

The fundamentally different immunological processes involved in tuberculosis, the archetypal model of cell-mediated immunity, were recognized from the outset. Koch and other early workers were able to show that primary infection with *M. tuberculosis* could be shown to have occurred by the alteration in the body's response to intradermal injection of a protein extract of the organism (originally, Koch's old tuberculin; later purified protein derivative (PPD)). Before infection, no reaction occurred, whereas after infection, an area of redness and induration within the skin appeared 72 h after injection. It was also noticed that reinfection with *M. tuberculosis* in animals with a positive skin test provoked a brisk, even necrotic, reaction at the site of reinfection (Koch's phenomenon), again indicating that the body's reaction to the organisms was altered forever. The intradermal test was later standardized and done by differing techniques (Mantoux test, Heaf test), all of which can variously be described as tuberculin skin tests (TST).

It is important to note that this 'tuberculin conversion,' i.e. the acquisition of hypersensitivity to tuberculoproteins, occurs whether or not the primary TB infection progresses to manifest clinical illness (progressive primary tuberculosis, including tuberculous meningitis and miliary tuberculosis). If conversion occurs and the subject remains healthy, including nowadays no radiological evidence of disease, the supposition is that the subject has latent TB infection (LTBI). The clinical significance of these different outcomes and the results of reactivation of LTBI to produce 'adult-type' tuberculosis in later life are found elsewhere in these volumes. From the bacterial immunoserology viewpoint, the significance is that TSTs can act as a quasi-serological marker that infection has occurred.

It is remarkable that a test devised in the nineteenth century is still in daily use in the twenty-first century. It is also testimony to the dearth of any better methods for the diagnosis of primary tuberculosis. It should be remembered that over 90 percent of cases of primary tuberculosis are asymptomatic and that in symptomatic primary disease the bacillary load in the body is so small that even the most sensitive of modern cultural techniques are usually unhelpful.

TSTs were the only method until very recently, but there are considerable problems with them. First, they are cumbersome to perform, require an injection (particularly unpopular with children, the most tested group), require a second visit for the 'reading,' and are subjective. Second, infection due to nontuberculous

mycobacteria, such as *M. avium-intracellulare*, *M. malmoense*, and other environmental species, especially in childhood, can also elicit positive TSTs. Third, BCG vaccination, which is, after all, an artificial primary mycobacterial infection, will also give positive TSTs, rendering the TST approach virtually useless for the detection of primary tuberculosis in a universally vaccinated population after the age of screening. Fourth, since the TST itself introduces the test antigen to the immune system, regular and repeated TSTs can produce false-positive results, the so-called 'booster effect.' Lastly, whereas it was at one time thought and, indeed, observed that once TST conversion has occurred (including by BCG vaccination) the TST would be positive for life, it is now thought that in low-incidence countries, the lack of repetitive contact with the disease allows hypersensitivity to fade. Hypersensitivity ceases, thereby, to be a reliable surrogate for cell-mediated immunity, itself a marker of past primary infection. Nonetheless, despite these many drawbacks, TSTs have proved very helpful in investigating, diagnosing and controlling tuberculosis.

EVOKED GAMMA INTERFERON ASSAYS

It is against this background that a new approach to the 'serodiagnosis' of primary infection with *M. tuberculosis* has been adopted. The new method makes use of the fact that the 'sensitized' or primed T cells, especially those of certain subsets, will produce and release gamma interferon in the presence of the appropriate antigen. The production of gamma interferon can then be assayed or otherwise detected and thus confirm or deny the previous exposure to the antigen.

Unfortunately, the composition and comparative composition of mycobacterial cell walls is ill understood. Consequently, although these evoked gamma interferon assays (EGIA) can more precisely, reliably, and objectively assess responses to tuberculin and, moreover, do so without injection and at a single patient visit and be endlessly repeated without affecting the test result, differentiation between TB and infections due to nontuberculous mycobacterial species is difficult. A common approach is to expose the cells to a range of 'tuberculins' from different mycobacteria and allocate species specificity to that eliciting the highest response.

The most desirable goal is to differentiate between responses due to BCG and other members of the *M. tuberculosis* complex. Fortunately, it is known that the molecular hallmark of BCG is the lack of the RD1 region in its genome. It is likely, therefore, that if proteins coded for by this region (for instance, early secretory antigen type 6 (ESAT6)) elicit a gamma interferon response, then the primary exposure was to *M. tuberculosis* or *M. bovis* and not to BCG (Pollock et al. 2001).

Extensive studies have shown excellent correlation between TSTs and at least one EGIA (Mazurek et al. 2001) and assessments of the later *M. tuberculosis*-specific EGIAs are under way. Assays based on this technology will benefit many patients with LTBI and others. They may, in fact, be the key to the eradication of tuberculosis rather than its control (Institute of Medicine 2002).

As will be clear from the earlier section on the granulomatous response, tuberculosis may well not be the only disease in which these assays can be applied with benefit.

PRIVILEGED SITE INFECTIONS

Both the APR and the granulomatous reaction require access to the site of infection for cells. A blood supply to the infected site is, therefore, essential for these reactions.

Very rarely, examples occur whereby organisms gain access to the body, typically as a result of bacteremia, and lodge in sites that either physically or functionally are beyond the normal immune system. The nidus of *S. aureus* infection encased within sclerotic bone that persists for years, even decades, known as Brodie's abscess has already been mentioned, but the best naturally occurring example is subacute bacterial endocarditis (SBE).

In SBE, classically the organism lodges on a previously damaged area of a heart valve. The organisms involved are typically of very low pathogenicity and would normally be extinguished rapidly by the APR/pyogenic response, but the almost avascular nature of the distal third of a heart valve cusp, exacerbated by fibrosis due to previous scarring, means that the site is 'privileged,' i.e. beyond the reach of the blood-delivered components of the pyogenic response, and, because cells of all kinds are denied access, even of the granulomatous response. Organisms multiply unhampered and a lesion comprising fibrin, platelets, and bacteria grows at the site – literally, a vegetation. Occasionally a fragment of the vegetation embolizes into the tissues, where the indolent organisms in the embolus are killed almost immediately. This paradox – a septic embolus resulting in a sterile infarct – illustrates well the unusual but typical nature of a privileged site infection. Accounts elsewhere in these volumes discuss the details of SBE. For the present purposes, it is important to note that the release of soluble antigens from the vegetation (bacterial cell-wall material, toxins, and other extracellular products) will stimulate humoral antibody production and this will typically go on over many weeks, if not months, before the true diagnosis is made by blood culture. This hyperglobulinemia is often unaccompanied by a significant APR (there is not the usual stimulus) and, for instance, the CRP is not very high (but it is raised) in SBE. Similarly, there may not be a leukocytosis of any note, although the dysglobulinemia usually

induces a so-called 'secondary' or sideropenic anemia. Prolonged IgG production may eventually result in damaged or abnormal IgG being circulated, and this may provoke an IgM response, leading to the classic setting for production of rheumatoid factor (RF). A positive RF is found in approximately 60 percent of patients with SBE.

Since much of the IgG produced in SBE is nonspecific and also because the antigenic analysis of streptococcal cell walls is little studied, the humoral antibody response in SBE is hardly ever used for etiological diagnosis. Fortunately, blood culture is a very reliable test under these circumstances and little is therefore lost. However, serology can occasionally be useful, typically when it is either difficult or dangerous to isolate the organism from the blood. The best illustration is the diagnosis of Q fever endocarditis, since this obligate intracellular Rickettsia-like organism is both difficult to grow and very highly infectious to laboratory workers. However, the serological diagnosis of Q fever has an additional merit that, once again, illustrates the practical utility of bacterial immunoserology.

It has been found that *Coxiella burneti*, the causative agent of Q fever, can exist in two quite distinct antigenic phases. Serial passage of the organism in guinea pigs, results in a preparation said to be in phase I, whereas culture in fertile hens' eggs yields an organism in phase II. In uncomplicated Q fever infection, which usually presents as an atypical pneumonia, only antibodies to phase II antigens can be detected by CFT. However, in either of the two recognized chronic complications of Q fever infection, endocarditis or granulomatous hepatitis, antibodies to phase I antigens also appear (Raoult et al. 2000). Thus, bacterial immunoserology, in this case, will confirm Q fever infection, say in a respiratory infection serology screen, but will also alert the investigator to the possibility of complications.

From the above account, it should now be clear that much bacterial immunoserology depends on the detection, characterization, and quantitation of humoral antibody. It is now necessary to discuss in outline how this is accomplished.

THE TECHNOLOGY OF ANTIBODY DETECTION

Whenever tests are said to 'detect antibody,' they are in fact techniques that allow us to visualize or to infer, often indirectly, that an antigen–antibody reaction has taken place. With a few exceptions (antibody-mediated lysis and serum bactericidal tests, for instance, neither of which are routinely used in bacterial immunoserology), the test-tube event does not replicate an in vivo event.

It is common practice for all serological tests to be conducted on an initial dilution, even if only for screening. This is because neat human serum contains low titers of a wide range of nonspecific antibodies of no diagnostic significance, and these are best disposed of by a simple initial dilution step. Thus, serological tests are often reported as 'titer <1/8,' rather than 'negative,' but the meaning is the same. There are, however, exceptions, the most common being the VDRL, which is reported as 'positive/negative in neat serum or at a specific titer.' However, it should be remembered that reagin is not an antibody in any case.

The following brief account gives a range of examples of techniques commonly used, but it does not give other than outline details.

Direct visualization of an antigen–antibody reaction

Agglutination is one of the oldest techniques and depends on the simple observation that whole bacterial cells will form aggregates in the presence of an antibody specific to a cell-surface antigen, the lattice formed by the action of the divalent antibody and the bacterial particles becoming a visible aggregate. Classically, antibodies to flagellar (H) antigens produce a delicate floccular appearance, those to somatic (O) antigens a granular appearance, and those to capsules a disk-like appearance within the fluid. The appearance of the supernatant fluid (after settling) is said to be 'water clear' in contrast to the milky and smooth appearance of the original bacterial suspension. The Widal test best exhibits these different agglutination phenomena. Agglutination can be demonstrated in test tubes (special tubes such as Dreyer's tubes were devised for this) but can also be shown on slides. However, for the latter, the antibody levels in the serum must be very high and the bacterial suspension must be very dense. It will still be necessary to do a tube test to assess the titer, but slide agglutination is often used as a rapid preliminary screening technique.

Variations on the agglutination theme include passive (heme)agglutination in which an antigenic extract of the organism is coated (by various chemically assisted processes) on to the surface of inert carrier particles, most commonly red cells, which will then (heme)agglutinate in the presence of the appropriate antibody. Tests can be made very precise indeed if a recombinant antigen is used. Other commonly used inert particles include latex and gelatin.

A new, very exciting, and highly sophisticated development of these relatively simple principles is the binding, severally, of a range of different antigens to the surfaces of a matched range of differently colored inert particles, the chromophores used being detectable by fluorescence. Antibody-induced or antibody-dependent changes in any one or more of the subpopulations of particles of a specific 'color' can be rapidly, accurately, and objectively detected using multiplex differential flow analysis (Opalka et al. 2003). In this way, antibody to one or more antigens within a panel of a much greater

number can be measured in one reaction. There are now several commercial systems available. The system can be reversed, so that the particles are coated with diverse antibodies, thus allowing the detection of more than one antigen in clinical material within a single screening test.

Lastly, the agglutination tests described thus far all detect divalent antibodies (IgG and IgM) in which the antibody links two (or more) particles to thus build up the lattice. Agglutination tests can also be used to detect monovalent antibody (IgA), which will ordinarily only coat the antigen-bearing particles, whether bacterial cells or carrier particles, but not cross-link them and, therefore, not result in visible agglutination. If, however, following washing of the IgA-coated suspension, antihuman (e.g. mouse, rabbit, goat, and so on) divalent IgG is added, a lattice will form. The resultant agglutination will indicate the presence of specific IgA.

Precipitation results when a soluble antigen meets the appropriate antibody. It can be demonstrated simply by allowing two liquids to meet at an interface whereupon a precipitate will form.

If both liquids are allowed passively to diffuse towards each other in a solid but transparent matrix, for instance clear agar or agarose gel, a visible precipitin line will form where they meet. This passive (immuno)diffusion, or double diffusion, has two additional properties. First, it will reveal the presence of more than one antigen–antibody system within the same reaction since they will form separate lines. Second, double diffusion will confirm the identity/nonidentity of any two systems if they meet. If they differ, the lines will not merge, but if they are identical, the lines will smoothly merge (the so-called 'reaction of identity'). This is because the precipitate will always redissolve in an excess of either reactant (antigen or antibody). These two reactions and the third possible outcome indicating partial identity are all well illustrated in the classical double diffusion method for the detection of diphtheria toxin production, the Elek plate, although it is a test for antigen and not antibody.

Precipitation methods can be speeded up considerably by applying an electric field across the gel, so driving the antigen and presumed antibody towards one another (immunoelectroosmophoresis (IEOP) or countercurrent immunoelectrophoresis (CIE)).

Indirect visualization of an antigen–antibody reaction

The general principle used here is that a secondary visible system, itself an antigen–antibody reaction, is used to infer that the sought for and invisible reaction has occurred.

CFT is the oldest and still the most widely used example. In this method the patient's serum and the antigen are mixed and allowed to react, but in the presence of complement. If the (invisible) reaction occurs, complement will be consumed ('fixed'). The fact that the complement has or has not been consumed can be demonstrated by the addition of a second system comprising animal red cells coated ('sensitized') with an antibody to the red cells raised in another (animal) species. If complement remains, the red cells will undergo complement-mediated immune lysis; if it has been consumed, no lysis will follow and, commonly, the red cells sediment to a button. No lysis therefore implies that the antibody sought has been detected and lysis implies that it is absent.

CFTs are inexpensive and popular because the reagents are relatively cheap and easily accessible. However, CFTs can be very difficult to standardize and there are many pitfalls in their use. Nonetheless, the CFT approach was the first semiautomated and generic antibody detection and screening system in microbiology.

Indirect immunofluorescent antibody tests (IFAT) utilize a similar principle to CFTs but confirm that immunoglobulin has been bound to the antigen by detecting it using an antihuman immunoglobulin tagged with fluorescein. The antigen is almost always solid, being cells from a culture or even a sample of tissue within which it is known that the whole organism can be seen. It is important to ensure that all unbound traces of serum are washed from the preparation prior to applying the detection system. Antihuman antibodies can be raised that are specifically of either the IgM or IgG class, thus enhancing the usefulness of the technique.

Obvious disadvantages of this method are the fading of the fluorescence after some time, making standardization and archiving very difficult. Quantitation of the antibody detected, even by titration to a poorly defined endpoint of fluorescence extinction, is subjective. The method, however, has the advantage of detecting exactly where in the tissues the antibody can be found.

EIAs take the indirect detection principle in a direction that solves many of the problems outlined above. Although there are many variations on the theme, the essence of EIAs is the production of a sandwich comprising (1) the antigen (whether a crude organism preparation, an extract of the organism or, increasingly, a recombinant antigen); (2) the patient's serum as a putative source of the antibody; and (3) an antihuman globulin tagged with an enzyme. As with IFATs, if the antigen–antibody reaction occurs, the enzyme-tagged antibody will become attached. The enzyme is then made to convert an appropriate substrate to a visible product. Enzymes commonly used include alkaline phosphatase and peroxidase. In both cases, the color intensity can be read on a colorimeter and the amount of antibody (a value equivalent to the 'titer') can be determined and compared with that of other samples.

EIAs have several distinct advantages and have become one of the workhorses of bacterial immunoserology. First, the 'antiantibody' nature of the detector system makes it possible to produce EIAs specific for IgM, IgG, or IgA antibody detection. Second, the stability, reproducibility, and standardizability of the end-product make EIAs excellent assays for automation, in turn making them quicker, cheaper, and less subjective.

However, EIAs remain individual reactions, capable of detecting only one antibody at a time. The multiplex differential flow analysis technology outlined above is, therefore, likely to be a strong competitor, able to detect several antigen–antibody reactions in one assay.

Other antibody assay techniques

Other, rarer, methods have been devised to detect antibody. Many are historical curiosities and can be dismissed, having been replaced by one or more of the above techniques. Nonetheless, they demonstrate the basis of bacterial immunoserology as a practical response to a clinical need and so will be briefly mentioned.

Toxin neutralization tests allow the detection of an antibody, in this case literally an antitoxin, by showing that the patient's serum will abolish the toxicity of the antigen. Thus, a cytopathic effect (CPE) of a toxin on a tissue culture monolayer will be prevented by the addition of immune patients' sera. Other examples include the inhibition of hemolysis due to a wide range of hemolytic extracellular toxins (such as streptolysin O, staphylococcal α-lysin), intradermal tests in animals, and even tests designed to show that immune serum will prevent a specific lethal or morbid reaction in an animal (such as the paralytic effects of tetanus or botulinum toxins, or the lethality of diphtheria toxin).

Thankfully, almost all of these animal assays have been successfully replaced by modern assays in which animals are not used, although equivalent sensitivity has not always or easily been achieved. In those bioassays that remain, it should be obvious that control reactions are absolutely vital, more so than in any other assays, because of the immense biological variation that there must inevitably be in the assay components.

Immobilization assays make use of the fact that many microbes have the property of locomotion (motility) and an immune serum will immobilize them. The TPI has been discussed above. Immobilization assays are prone to the particular problem that other inimical substances in the patient's serum, most commonly antibiotics, can produce an identical result to that of antibody.

Metabolic assays are a derivative of toxin and mobility assays since they capitalize on the fact that immune serum will either kill the organism or, as in this case, prevent it expressing a particular and easily measured characteristic. Thus, antibody to *Mycoplasma pneumoniae* can be detected by showing that the patient's serum will prevent the mycoplasma growing and metabolizing glucose, this property being manifest by a color change in the reaction tubes. Clearly, antibiotics will produce an identical effect.

COMMON APPLICATIONS OF ANTIBODY ASSAYS IN ORGANISM GROUPS

Staphylococci

In general, because of the pyogenic nature of staphylococcal infection, immunoserology is not needed. There are two exceptions. First, in suspected long-standing and deep-seated infection (see Brodie's abscess, above) in which samples for culture are difficult to obtain, recourse is made to the antistaphylolysin test, a hemolysin inhibition test, utilizing the α-hemolysin of *S. aureus* as antigen. When antibody is detected, the test is very predictive of chronic infection with *S. aureus*. Unfortunately, a significant minority of *S. aureus* strains do not produce the α-hemolysin, so negative results do not exclude infection. EIAs have also been devised testing for antibodies to the universal *S. aureus* antigen, ribitoltechoic acid (Ayyagari and Pal 1991), and seem promising.

Second, the propensity of coagulase-negative staphylococci to cause chronic infections of prosthetic material (a 'privileged site infection'), especially in replacement joints, but also in cerebrospinal fluid (CSF) shunts and indwelling intravascular devices, has led to the development of EIAs for antibodies to the glycocalyx ('slime') (Elliott et al. 2002) that such strains produce in abundance. These assays have the extra merit of confirming or denying the likely significance of isolates of these otherwise ubiquitous contaminants in tissue and blood cultures.

Streptococci

The need to relate recent *Streptococcus pyogenes* infection to postinfective sequelae, such as rheumatic fever, acute type 1 nephritis, and rarer events such as erythema nodosum and Henoch–Schoenlein purpura, has provoked the development of a range of assays, all originally based on the principle of neutralizing the effect of an extracellular product of *S. pyogenes*.

The most common by far is the antistreptolysin O titer (ASOT). Since, however, *S. pyogenes* infections of the throat and middle ear are common and the number of different (M protein) types is so large, some background level of ASOT is almost inevitable in any population. Thus, a 'positive' ASOT is usually defined as a value in excess of the norm for the population from which the individual to be tested comes. Alternative

tests such as antiDNAse B and antihyaluronidase are more rarely performed but remain necessary since *S. pyogenes* infections of skin (streptococcal pyoderma), which can be followed by nephritis but not rheumatic fever, rarely give rise to a raised ASOT, whereas the other antibodies will rise (Ferrieri et al. 1970). *S. pyogenes* throat infections, however, reliably provoke ASOT rises. Unrelated infections and, rarely, infections due to streptococci of other Lancefield groups can also occasionally provoke a raised ASOT, giving another useful role for the second line tests. Nowadays, especially with the availability of recombinant antigen technology, these antibodies are more commonly detected by passive particle agglutination tests or EIAs rather than the classical neutralization approach.

Much recent interest has been targeted on devising a reliable antibody test for infection due to *S. pneumoniae* (the pneumococcus). New vaccines offer the prospect of broad and enduring protection but will need accurate evaluation. The diversity (>90 types) of the capsular polysaccharide antigens makes simple tests unlikely, although the multiplex differential flow analysis technology may greatly help, and attention has centered on the universal pneumococcal product, pneumolysin, which is of the same family of thiol-activated toxins as Streptolysin O. Progress has been hampered until recently because it is found only intracellularly and cannot, therefore, be produced in large amounts. However, recombinant protein technology has solved this problem and assays are being developed (Lankinen et al. 1999). Other targets are also under study.

Corynebacteria

The principal immunoserological need is for the detection of diphtheria antitoxin prior to, and subsequent to, immunization. Although EIAs have been developed, experience suggests that (in contrast to the detection of the toxin itself; see below) they are insufficiently sensitive. The standard assay presently consists of a toxin neutralization assay using the CPE in Vero cells. This assay has replaced the subjective and complex skin test previously used, the Schick test.

Clostridia

The highly virulent exotoxins of *C. tetani* and *C. botulinum* produce the characteristic diseases with which they are associated at concentrations below those necessary to immunize – a conundrum that should lead all doctors to remember that their last duty to a patient recovering from clinical tetanus is to immunize them with tetanus toxoid prior to discharge. Therefore, antitoxin assays are not used in diagnosis. However, assays to detect high titers of *C. tetani* antibodies following repeated immunization with toxoid have been devised to select donors for the production of human tetanus immunoglobulin for treatment purposes.

Neisseria

Antibodies to *Neisseria meningitidis* are not commonly assayed for diagnostic purposes, although they have occasionally helped in retrospective diagnosis of the serogroup involved in an outbreak. The most common circumstance requiring a noncultural approach, the failure to culture *N. meningitidis* from blood or CSF, is now addressed through polymerase chain reaction (PCR) of these specimens. PCR is quicker, more revealing, and more sensitive than immunoserology.

However, antibody tests for *N. meningitidis* have been invaluable in assessing the efficacy of vaccines, particularly those for *N. meningitidis* serogroup C. Although many different assays for antibodies to many different antigens have been devised, the classical bactericidal antibody test remains the specified method for vaccine licensing purposes.

There are no currently useful assays for antibodies to *N. gonorrhoeae*. The gonococcal complement fixation test (GCFT) continues to be requested and even promoted in some textbooks. However, many investigations have shown that it is very rarely helpful and often is misleading in an area of diagnosis that has considerable medicolegal potential.

Enterobacteria (here taken to encompass most nonfastidious aerobic gram-negative bacilli)

The Widal reaction for enteric fevers (typhoid and paratyphoid) has already been discussed (see above). It should be emphasized that these methods cannot and should not be applied to nonenteric fever *Salmonella* species. The sharing of antigens (especially O antigens) between species makes the results impossible to interpret. No useful routine antibody assays are in current use for infections with shigellae.

The recent recognition of serious infection with *Escherichia coli* O157 has stimulated the development of an EIA to detect antibody to the lipopolysaccharide antigen of its cell wall, incorporating the O antigen. Judicious use of this Reference Laboratory test has given invaluable help in identifying unrecognized cases prior to an outbreak and in rapidly determining the true extent of an outbreak by testing apparently unaffected subjects (Chart 1999).

Although numerous antibody tests have been developed from time to time for various research studies, there are few other established assays used for diagnosis of infections with the remaining organisms within this group. However, an assay for antibodies to *Campylobacter jejuni* is frequently requested for a tangential

purpose. It has emerged that the most common preceding event to the onset of Guillian–Barre syndrome is *C. jejuni* infection. The relationship is thought to be based upon cross-specificity of *C. jejuni* antigens and certain epitopes in neural tissue, a situation dubbed molecular mimicry (Schwerer 2002).

Parvobacteria (fastidious gram-negative cocco-bacilli)

Serology is still used for the diagnosis (using paired sera) of infection with *Bordetella pertussis*, but as with *N. meningitidis* (see above), the most common reason for the test is for confirmation of clinically suspicious but culture-negative cases, especially in adults, older children, and 'partially vaccinated' children. As with *N. meningitidis*, this need is increasingly being met by PCR-based testing. The demand for serology, which is complex and the results of which are difficult to interpret, is likely to diminish.

Apart from research settings and studies for the evaluation of vaccines, serology is little used in the diagnosis of infections with *Haemophilus* spp. The immunoserology of *Brucella* was discussed earlier. There is an occasional rare need for antibody assays in melioidosis, and a passive hemagglutination test using *Yersinia pestis* Fraction 1 antigen is used in the serodiagnosis of plague. In both diseases, isolation of the causative organism is much the most common diagnostic method.

In tularemia, however, diagnosis is commonly made by demonstration of a rising antibody titer, since the clinical appearances are often confusing. Cross-reactions with *Proteus* spp. (see below) and *Brucella* spp. antigens can occur. Such is the complexity and diversity of *Francisella tularensis* strains and their antigens that interpretation is an expert matter. Nonetheless, serodiagnosis is an attractive option given the highly infectious nature of *F. tularensis* and the danger of laboratory-acquired disease.

Legionella

Antibodies to *Legionella pneumophila* were originally detected by IFAT using a formalized yolk-sac culture of the organism on a slide as the antigen. High and, preferably, rising titers are diagnostic. It was noted from the start that antibody might take up to 3 weeks to appear after the onset of the illness.

EIAs have now largely replaced the IFAT, and PCR of tracheal secretions or bronchoalveolar lavage fluid and urine antigen assays have supplemented serology. However, all these tests are specific for *L. pneumophila* infections, which account for nearly all cases of legionnaires' disease. Investigating infections with one or more of the nearly 40 'other legionella species' (sometimes called fluorobacters) in highly immunosuppressed patients is a task for an expert.

Low and unchanging levels of antibodies to *L. pneumophila* can be found in Pontiac fever, an extrinsic alveolitis (but not a pneumonia) triggered by inhalation of *L. pneumophila* antigens from colonized or contaminated air conditioning systems and, as such, one of many forms of 'sick building syndrome' (Friedman et al. 1987).

Mycoplasma pneumoniae

Interest in the serology of this common organism is three-fold. The technology is rather mundane, most cases being diagnosed by a rising antibody titer using a CFT or by detection of specific IgM using an EIA.

The first point of note is the association between serological evidence of infection with *M. pneumoniae* and various neurological conditions, most notably Guillian–Barre. As with the previously mentioned association with *C. jejuni* infection, there is good evidence that cross-reacting antibodies are the basis of this problem (molecular mimicry) (Kusunoki and Kanazawa 2001). Second, there is a similar association with acute pancreatitis.

The third point of interest harks back to the early empirical days of bacterial immunoserology. It was noticed many years ago that patients with 'atypical pneumonia,' a condition not then known to be due to *M. pneumoniae*, often exhibited cold agglutinin antibodies (CAA) in the early stage of the disease, the titer of which appeared to correlate with the severity of the illness. In fact, the CAAs are IgM class antibodies produced in the acute stage of the infection. Their specificity and evocation are still uncertain. They do not, for instance, react with the antigen used in the CFT. They are, however, relatively specific, being found only in pneumonias and nearly always only in those due to *M. pneumoniae*. Their very early appearance in the illness can be capitalized upon in a very simple bedside test (Davidsohn and Wells 1962).

Freshly drawn blood is mixed four parts to one with citrate in a small glass tube, the anticoagulated blood sample then being placed in a refrigerator or on ice for a few minutes. If CAAs are present, large aggregates will form and can be seen if the blood is run over the glass surface. These readily disappear on hand-warming of the tube and can be redeveloped by its return to the cold. Samples with this result can be taken to contain CAA titers of at least one in 80 and a presumptive diagnosis can be made. Other causes of cold agglutinins must also, obviously, be considered in certain settings.

Rickettsia

Diagnosis of rickettsial disease is usually by IFAT, but this may not distinguish between the different forms of

typhus (epidemic louse-borne, murine, scrub, etc.). Washed and adsorbed antigens can be used to achieve these distinctions, which are of both diagnostic and epidemiological importance. However, recourse can still be made to yet another empirical bacterial immunoserology test, the Weil–Felix reaction. This relies on the observation that the serum of patients with these differing rickettsial infections will agglutinate different suspensions of correspondingly different strains of *Proteus vulgaris*. Thus, sera from patients with epidemic louse-borne typhus agglutinate *Proteus* OX-19 strains, whereas sera from patients with scrub or mite-borne typhus agglutinate *Proteus* OXK strains. These easily maintained antigens were invaluable before rickettsial culture was possible and still retain their usefulness on occasion today.

The usefulness of bacterial immunoserology in the diagnosis and management of Q fever infection has already been discussed above.

Spirochetes

Serology is a common method for the diagnosis of leptospiral infection, since the best opportunity to see leptospira in clinical material, such as urine, is usually gone by the time the diagnosis is considered and microscopy (and culture, if attempted) is notoriously difficult.

At a group identity level, CFT can be used but it is increasingly overtaken by EIA, which can offer IgM-specific testing. However, microscopic agglutination tests with specific leptospiral antigens are necessary to identify the particular species involved, for instance *L. canicola*, *L. interrogans*, *L. icterohaemorrhagicae*, and so on. Borrelioses such as relapsing fever (*B. recurrentis*) are not usually diagnosed by serology.

The complex serological approach to syphilis (*T. pallidum*) has already been discussed in some detail. It should be added here that the same tests are used to investigate suspected infection with *T. pertenue* and *T. carateum*, the agents of, respectively, yaws and pinta, although serology cannot discriminate between them.

In recent years, Lyme disease has been added to the list of major spirichetal diseases. Lyme disease can be diagnosed by serology using EIAs with *Borrelia burgdoferi* antigens. Like syphilis, Lyme disease evolves through recognizable acute, latent, and chronic stages. Whilst humoral antibody assays are invaluable in diagnosis and following the disease, they may be unpredictable in both the time and magnitude of their appearance. EIAs are particularly troublesome in chronic Lyme disease and/or treated cases. There is a need to develop an equivalent to the VDRL to act as an index of activity. The advent of the evoked gamma interferon assays measuring cell-mediated immunity may be helpful in this.

Chlamydiae

Until recently, it was held that only two groups of *Chlamydiae* existed. These were group A (now known as *Chlamydia trachomatis*) and group B (*C. psittaci*). Since *C. trachomatis* largely caused infections of mucosal surfaces (trachoma in the eye, inclusion conjunctivitis, and nongonococcal urethritis), the need for serology was limited. Occasional invasive infection was recognized in the forms of salpingitis and infant pneumonitis and a specific subgroup of *C. trachomatis* was held responsible for the granulomatous genital infection known as lymphogranuloma venereum (LGV).

C. psittaci was accepted as an uncommon but well-recognized cause of atypical pneumonia (psittacosis or ornithosis), and almost all such cases were diagnosed serologically. It was convenient, therefore, to use a common CFT antigen, a mixture of an LGV strain and a *C. psittaci* strain, to detect all antibodies, the likely specificity being decided by the markedly different clinical circumstances. The recent discovery of a third species, *C. pneumoniae*, which causes pneumonia, appears to be of purely human origin, and antibodies to which may not always react with the old antigen (Blasi et al. 1998), has spurred the development of species-specific antibody tests, which are usually of a microIFAT basis.

Other comments

SALIVA TESTING

It is important to note that along with all the many advances in assay technology, there has also been an advance in specimen technology. This account thus far has assumed that all tests are carried out on serum (excluding those concerned with cytokine evocation from cells). Recently, there has been extensive investment in detecting antibodies of all immunoglobulin classes in ‘saliva’ or, more precisely, crevicular fluid (Akingbade et al. 2003). Special oral devices allow adequate volumes of sample to be obtained. Salivary testing is convenient, noninvasive, and particularly suitable for children and others with aversions to venepuncture. It is being taken up with increasing frequency.

NEAR-PATIENT TESTING

Unsurprisingly, the development of sophisticated systems for the direct or indirect visualization of antigen–antibody reactions, the ever-increasing precision and robustness of these technologies, and the benefits of modern mass-production techniques have all combined to promote near-patient testing kits. In addition to simply refining the methods outlined above, commercial houses have introduced some truly innovative approaches. For instance, it is possible to tag

detection monoclonal antibodies with gold particles, so that when the antibody localizes (having reacted with an immobilized antigen), its presence becomes macroscopically visible. All the necessary reagents can be offered in a cartridge format, including control reagents, and the addition of the sample under tightly specified circumstances can give a result within minutes, with no laboratory involvement.

Even more sophisticated approaches include embedding antigens or antibodies within membranes, the optical properties of which will alter if a reaction occurs, thus giving a visual signal of the event.

The potential variations on this theme are myriad and need not be listed here. However, whatever the technology employed, it is important to address three areas of concern:

1. Safety. Many of the near-patient kits require venepuncture, needle-stick, or other means of drawing blood. Since the instant diagnosis of diseases such as hepatitis B or HIV in the field is one of the attractive settings for such kits, it is important to attend to waste disposal of the kits (often catered for by the manufacturer) and the attendant materials (not always catered for).
2. Controls. Although all reputable kits come with control reagents and the results obtained will be reproducible if the instructions are carefully followed, all serologists and laboratory diagnosticians will be aware that these alone are an inadequate control framework. Diagnostic tests need to be continually challenged with archived and current 'wild' samples, thus allowing for possible events such as the effects of target mutation, epitope loss, and so on. These checks are part of laboratory systems but are unlikely to be available to individual clinicians. Arranging for patients to be cohorted or even to attend a clinic for testing destroys the essence of near-patient testing.
3. Inappropriate use in inappropriate contexts. The devolution of bacterial immunoserology testing to the bedside implies that a test will be performed by interested and well-motivated nonlaboratory personnel, the experience of whom will likely be confined to one medical specialty. Occasionally, therefore, a near-patient test system may be used outside the testing scenario for which it was designed. Under these conditions, very misleading results can occur, the consequences of which can be both unfortunate and embarrassing.

With careful planning and forethought and, most importantly, the ready cooperation of the conventional laboratory, all these problems can be resolved. Done well, near-patient testing will be an increasing boon to clinical care (Borriello 1999).

Lastly, it must also be remembered that the need to avoid sending specimens some distance to a laboratory can also be avoided by careful clinical assessment and the development of algorithms, which eventually may result in no specimens being required at all. An excellent example is seen in the long story of the attempts to optimize treatment of the 'strep throat' (Needham et al. 1998; Bisno et al. 2002; McGinn et al. 2003).

METHODS FOR THE DETECTION OF ORGANISMS AND THEIR PRODUCTS AND FOR THE CHARACTERIZATION OF ORGANISMS BY IMMUNOSEROLOGICAL MEANS

In this aspect of bacterial immunoserology, the principles, and largely, the technologies remain as before, but the purpose of the assays is to detect the antigen using the antibody as the tool. First, it is important to recognize the settings within which this noncultural approach is useful and then to understand the limitations and the alternative, nonimmunoserological means by which it can also be attempted.

Noncultural methods of diagnosis

Broadly, noncultural methods are used because of their speed or specificity or because they give useful information in circumstances in which culture is not possible. In this latter instance, this might be because the organism has never been successfully cultured or, more commonly, because prior antibiotic therapy has rendered culture useless.

Until recently, the major approach for noncultural diagnosis was by detecting a component of the organism by immunological means. Since antibodies were the essential reagent, it followed that the target had to be an antigen and this almost invariably dictated the choice of a surface component such as a capsule, flagellum, somatic antigen, and so on, or a secreted antigen, such as an exotoxin. Medical microbiologists continue to use a very large number of such assays. The technology used to detect the antigen–antibody reaction comprises the whole range described above (agglutination, precipitation, EIA, etc.), but there are some notable additional aspects.

Advances in antibody production and application

MONOCLONAL ANTIBODIES

In many cases a test used will have begun as a method using a polyclonal antibody raised in animals. The polyclonal antibody is now almost always replaced by a monoclonal antibody. This change has several important advantages but one important limitation. The advantages include the 'immortalization' of the antibody, or at least the hybridoma, the standardization of the antibody, thus removing batch variation problems at one stroke,

and the potential to generate antibodies of such potency that the sensitivity of the test can be exquisite and well beyond that of its polyclonal antibody-based predecessor.

However, since the monoclonal antibody is, by definition, directed towards only one epitope of the target antigen, the whole assay can be vitiated if this epitope is not universally present or, if, as occasionally happens, the organism mutates and loses it (Smith et al. 1996). The considerable and well-deserved reputation for the accuracy of monoclonal antibody assays can then be a hindrance to good practice. It is, therefore, often recommended that very important results, for instance identifications for medicolegal purposes or in the diagnosis of life-threatening conditions, be confirmed by alternative pathway tests or by a second monoclonal assay directed towards a different epitope.

COAGGLUTINATION

Protein A on the surface of well-characterized strains of *S. aureus* binds to the Fc component of IgG molecules, making it possible to coat suspensions of the inactivated organism with monoclonal antibody. The resultant preparation becomes a particulate suspension in which each particle exhibits on its surface the free Fab component of the IgG used. Thus, in the presence of the appropriate antigen (in whatever physical form), the staphylococcal suspension will agglutinate, a lattice having been formed by the antigen and Fab receptors, which also brings down the suspension. This assay format, known as coagglutination, is widely used. Suspensions can be stained in different colors for different Fab specificities and then mixed so that several antigens can be tested for at one time. In some assays, protein A is itself coated on to inert or passive particles (for instance latex or gelatin).

PHAGE ANTIBODY DISPLAY (PINI AND BRACCI 2000)

Phage antibody display (PAD) works on a simple principle, which is that it is possible to insert recombinant DNA sequences coding for antigen-binding fragments (Fab) of IgG into bacteriophages, which will then express the appropriate Fabs on their protein coats. In this way, a 'library' of such modified phages can be produced, which will contain an almost limitless variety of Fab specificities. Selecting one individual phage and cultivating it in a bacterial host becomes akin to growing a monoclonal antibody, but without the rest of the immunoglobulin in the final product.

The Fab-bearing phage can then be tagged with fluorescein to give the equivalent of a fluorescein-tagged monoclonal antibody for microscopy assays. Alternatively, immobilization of the phage on a surface, such as in the wells of an assay plate, can form the basis of an EIA. The details of the process whereby the appropriate phage is selected from within the original library are complex but are analogous to the old serological method of making a polyclonal antiserum more specific by serial adsorption of antibodies to closely related but ultimately undesirable antigens (technically described as 'panning'). This offers the prospect of producing equivalent reagents to monoclonal antibodies without the use of animals and more quickly.

However, PAD offers one further, potentially revolutionary advantage. The epitopes and 'antigens' to which PAD 'antibodies' can be 'raised' need not be constrained by their acceptability, or not, to any human or animal immune system. Thus, antigenicity and immunogenicity are irrelevant. As importantly, self- or autoantigens are not excluded as is the case in conventional raising of antibodies. The only real prerequisite is that the chemical group can be recognized by a Fab grouping on one of the phages.

Consequently, as yet unrealized differences in surface structures between two populations of bacterial cells can be explored as the basis of their reliable distinction, and reagents can be produced to do this reliably, quickly, and relatively cheaply. In the future, we may refer to nonimmunoserology tests.

APTAMERS (JAYASENA 1999; OSBORNE ET AL. 1997)

Aptamers are nucleic acid molecules that bind to specific ligands. Consequently, they can be used to link oligonucleotides to specific sites. If a pair of aptamers is targeted on to a protein (for instance, an epitope or an antigen), the PCR can be used to amplify a stretch of DNA or RNA corresponding to the nucleotide sequence that bridges the 'gap' between the aptamer-linked oligonucleotides. By this means, assays detecting zeptomolar (10^{-21} mol) amounts of the target can be devised.

Since the aptamers themselves can be synthesized, this system can rival antibody-based assays, yet, as with PAD, animals are not necessary and diversity is not necessarily confined by antigenicity or immunogenicity of the target.

COMPETITION WITH MOLECULAR ASSAYS

The major new change in recent years has been the influence of molecular biology, especially PCR. The ability to detect small amounts of DNA and the development of assays in which the PCR detects specific sequences indicative of certain organisms or their characteristic products has challenged the previous primacy of the antigen-detection immunoassays. PCR assays are, of course, also very quick, sensitive, and helpful if the organism cannot be cultured, is dead, or its growth is inhibited by antibiotics. However, it should be remembered that PCR assays also have problems. The reaction is very vulnerable to the presence within clinical material of substances inimical to the Taq polymerase enzyme ('inhibitors'). These are relatively common

(heparin, IgG, and so on), whereas immunoassays do not suffer these problems.

Furthermore, the genomic assay can detect the gene that confers on the organism the ability to produce a given product, e.g. a toxin, but the gene may not be expressed. In diseases such as diphtheria, which is defined almost totally by the actions of the toxin, mere detection of the gene is insufficient to make the diagnosis. It is the phenotype that infects and/or causes disease.

It seems likely that the immunoassays will be needed for some time to come and that the two approaches should be complementary rather than competitive.

COMMON APPLICATIONS OF IMMUNODETECTION ASSAYS IN ORGANISM GROUPS

Staphylococci

The never-ending search for a test with the simplicity and speed of the slide coagulase reaction and the accuracy of definition of the tube coagulase reaction in this most ubiquitous of human pathogens has led to the development of a whole range of assays. Almost all of the recognized formats have been deployed. Since speed was an important objective, reverse passive hemagglutination, passive particle agglutination, and coagglutination, have been especially popular. These assays have variously targeted coagulase itself (here being detected as an antigen and not an enzyme), capsular antigens, and fibronectin receptors, amongst others. Almost all such tests are very nearly as accurate as the definitive tube coagulase test (Summers et al. 1998).

A widely used innovation has been the identification of methicillin resistance in *S. aureus* by EIA or passive particle agglutination immunodetection of penicillin binding protein (PBP) 2A on its surface by monoclonal antibodies, thus identifying methicillin-resistant *S. aureus* (MRSA) in a few minutes (Naratomi and Sugiyama 1998).

Streptococci

One of the classical applications of the precipitin reaction was the Lancefield grouping of β-hemolytic streptococci in which the extracted 'C substance' (a carbohydrate) was identified by layering a series of group-specific antisera on to aliquots of the extract in capillary tubes and observing a precipitation reaction at the appropriate interface. Nowadays, expectedly, this has been replaced by a passive particle assay, usually by slide agglutination, using particles coated with a series of group-specific monoclonal antibodies.

S. pneumoniae is commonly identified by a series of rapid tests, one of which is a slide agglutination test targeting a C substance common to all pneumococci.

Pneumococci also provide a good example of an unusual form of the precipitin reaction. They can be typed by the capsule swelling or Quellung reaction. This is based on the observation that if pneumococci in broth culture are microscopically observed before and after the addition of the homologous capsular antiserum, the capsule appears to swell dramatically in size. In fact, the capsule is at least 90 percent water and continually dissolving into the surrounding medium. It is a soluble antigen that precipitates when in contact with the appropriate antibody. This increases its refractivity and thus it appears to swell in the presence of the antiserum.

Pneumococcal infections illustrate another use of immunodetection. During invasive pneumococcal disease, it is often possible directly to detect capsular antigen in CSF, blood and urine, by passive particle agglutination (usually latex) or by EIA. The diversity of the capsular antigens (>90 serotypes) and their polysaccharide nature mitigate against a monoclonal antibody approach so that antigen detection is not as sensitive as culture. It has the merit of being suitable when culture is likely to be unrewarding. Students of the potential pitfalls of immunoserology will realize that antigen will be detected in the urine of the recently vaccinated.

Corynebacteria

Mention has already been made of the Elek double diffusion plate method for the detection of diphtheria toxin production in isolates of *Corynebacterium diphtheriae* or, more rarely, in some strains of *C. ulcerans*.

More recently, toxin production has been assayed by EIA. In either instance, it is important to understand that these tests demonstrate toxin synthesis, whilst the PCR often used to screen strains merely demonstrates the presence of the gene (see above).

Clostridia

The many exotoxins of clostridia are highly antigenic and are a potentially rich field for immunoserology. However, many of those of human interest, classically tetanus toxin and botulinum toxins, are still assayed when required by animal tests, although EIAs are being increasingly applied. These are Reference Laboratory tests. However, routine bacterial immunoserology includes two clostridial assays.

The first is the Nagler reaction. In this unusual format, the production of a lecithinase by an organism thought to be *Clostridium perfringens* is demonstrated by streaking the organism on to an egg-yolk medium. The further demonstration that the lecithinase produced is

the α toxin of *C. perfringens* (there are other lecithinases) is shown by the specific neutralization of the effect of the enzyme on the egg yolk on one half of the medium that was pretreated with specific antitoxin. The organism will grow on both halves of the plate. The Nagler reaction thus permits a secure identification of *C. perfringens* overnight. It should be noted that mere demonstration of lecithinase activity without neutralization is not the Nagler reaction.

Second, detection of the toxin(s) in the liquid stool of an affected patient is an essential prerequisite for the diagnosis of *Clostridium difficile*-associated disease (CDAD). Originally, this was accomplished by detecting a CPE in cell cultures (several were used, the most common being Vero cells) and then confirming that the CPE was neutralized by appropriate antitoxin. Although *C. difficile* antitoxin can be produced, it is common practice to use *C. sordellii* antitoxin, which was first used. There is a very high degree of homology between the two toxins. It is also important to note, contrary to widespread belief, that the neutralized cytotoxin assay so produced detects both *C. difficile* toxins, A and B.

Although the neutralized cytotoxin assay remains the gold standard, its possible subjectivity and relatively long turnround time have stimulated the search for more standardized and quicker tests. These are usually in EIA format. An adequate replacement assay for the cytotoxin test must detect both toxins A and B by an immunological pathway.

Neisseria

There are several monoclonal antibody-based tests using a variety of formats, which can confirm the identity of a suspect isolate as *N. gonorrhoeae*. Over-reliance on any one such assay, the accuracy of which is, after all, vulnerable to a change in only one epitope, can be problematic in practice. Gonococcal identity should, therefore, be investigated in parallel by an alternative pathway test, for instance sugar fermentation tests. A second monoclonal test (directed to a different epitope) might also be necessary on occasion.

Rapid serogrouping of isolates of *N. meningitidis* is very important since it influences case management, especially the decision to vaccinate close contacts. It is usually performed with assays similar to those used in streptococcal grouping or the identification of pneumococci (see above).

Enterobacteria

The use of antibodies to analyze the antigenic make-up of Enterobacteria is very widely used. A classic application is the identification of salmonellas by analysis of their O and H antigens (the Kaufmann–White scheme). Shigellas can be identified in an analogous way, although since these nonmotile organisms do not display H antigens, it is necessary to use adsorbed O antisera and some supplementary biochemical tests.

Within the species *Escherichia coli*, there are several identification schemes based on serological analysis. Thus, the identification of enteropathogenic *E. coli* is based on O antigen analysis. Serological analysis plays a part in identifying enteroinvasive and other pathogenic strains.

It is in identifying *E. coli* O157 isolates, however, that serology is most commonly used. Colonies of putative *E. coli* O157 growing on a selective medium can be screened for further investigation by slide agglutination with an O157 antiserum. However, before being accepted as the cause of disease, the organism must next formally be confirmed to be *E. coli* and, secondly, to be elaborating one or both of the verocytoxins (VT1 and/or VT2). Final proof is obtained by toxin gene PCRs and an EIA for VT1 and VT2. This process re-emphasizes the important principle that O antigens are shared within and even between species of Enterobacteria. Serological analysis must, therefore, always be assessed within a context.

Parvobacteria

Serology can be used to identify and type *Brucella*, *Yersinia*, *Francisella*, and other Parvobacteria, but in common practice it is *Haemophilus influenzae* that attracts the most attention. *H. influenzae* is divisible into six capsular serotypes, A–F, often called Pittman types. These can be identified in capsule swelling (Quellung) reactions using specific antisera. *H. influenzae* capsule type B, often abbreviated to HiB, is responsible for the overwhelming majority of severe human infection, which is predominantly in childhood. HiB meningitis, septicemia, pneumonia, osteomyelitis, and epiglottitis are all life-threatening diseases. Recently, HiB vaccination has successfully controlled these diseases, but it is necessary to maintain vigilance. All isolates of *H. influenzae* from frankly pathogenic circumstances merit examination for the B antigen. Serotyping can now be performed in simpler formats than capsule swelling.

It should be noted that 'noncapsulate' *H. influenzae* is very commonly isolated from the sputum of adult patients with exacerbations of chronic bronchitis and bronchiectasis, but this is not the setting for HiB.

Meningitis screening

It is common practice to examine samples of CSF, especially those samples with a pleocytosis or other markers of bacterial meningitis, for capsular antigens of *N. meningitidis*, *S. pneumoniae*, and HiB. Whilst these tests are occasionally valuable and the effort is laudable, it should be remembered that these tests are designed

primarily to identify organisms, i.e. under circumstances in which antigen is in plentiful supply.

In contrast, in CSF for instance, it has been estimated that up to 10^6 organisms/ml may be needed to detect antigens reliably (West and Nasrallah 1988). Therefore, negative results do not exclude the disease. It is in such circumstances that the new approach of PCR is often preferable, being more sensitive and, when necessary, also capable of pointing to other properties of the causative organism, such as antibiotic sensitivity or resistance (Kearns et al. 2002).

Legionella

The slow growth of these organisms in culture and the unusually slow appearance of detectable antibody in the patient's blood have been stimuli to develop alternative diagnostic means. The detection by EIA of a cryptic antigen of *L. pneumophila* in the urine of infected patients has proven to be a very successful approach. These tests (several are available) have greatly enhanced patient management in individual cases. Their simplicity and rapidity have also assisted outbreak investigation.

Two caveats must be applied. First, the tests are effectively restricted to diagnosing infections with *L. pneumophila*. These tests cannot, therefore, assist in the diagnosis of infections, usually of highly immunosuppressed patients, with other *Legionella* species, such as *L. bozemanii* (Humphreys et al. 1992). Second, the antigen may continue to be excreted for extraordinarily long periods after first becoming detected, even if the clinical course has been benign and response to treatment has been good.

Monoclonal antibodies are used in Reference Laboratories as an important tool in identifying and typing legionella species.

CONCLUDING REMARKS

The above account is not exhaustive, but it gives some idea of the extent, diversity, and utility of current bacterial immunoserology practice. Although, inevitably, future technical advances will continue to be incorporated into this area of diagnostic microbiology, the principles remain the same. It is likely that bacterial immunoserology will continue to be an important element in medical practice.

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Biological safety for the clinical laboratory

MICHAEL NOBLE

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INTRODUCTION

Laboratory biosafety is the active, assertive process based on evidence-based principles to ensure safety from microbial contamination or infection, or toxic reaction for workers, the public, and the environment as a result of the active manipulations of live microorganisms or their products while pursuing academic, research, industrial, and clinical investigations. The goals are to prevent laboratory-acquired infections in workers and to prevent accidental releases of live agents that can potentially endanger and have severe negative impact on humans, animals, and plants. Laboratory safety involves all aspects of the laboratory cycle, starting from before microorganisms arrive in the facility, through to the training of personnel, and the establishment and monitoring of safe working practices, through the proper use of reagents, materials, and equipment, through the safe storage and transport of agents, and ultimately to the terminal sterilization and destruction of microorganisms.

EPIDEMIOLOGY OF LABORATORY-ACQUIRED INFECTIONS

Laboratory-acquired infections are generally defined as infections that likely occur while handling offending organisms during the analytic phase of laboratory testing. Usually, this is an epidemiological conclusion, based on the presumption of low likelihood of a concurrent community exposure, although with molecular or other identifying techniques the likelihood of another source can be reduced or eliminated. It is important to appreciate that the testing cycle begins well before the sample actually reaches the laboratory (the preanalytic phase of laboratory testing). In one of the original studies of laboratory-acquired infections, infections acquired during the collection of some samples were included if it could be ascertained that the collection was solely for the purpose of a laboratory investigation. Infections experienced by phlebotomists as a result of needlestick injuries are now routinely included as laboratory acquired infections. Arbitrarily, phlebotomist infections with chicken pox acquired while collecting samples in patients rooms are often not included (Collins and Kennedy 1999).

Although difficult to date precisely, the first microbiology laboratories were active by 1840–60, with the laboratories run by pioneers including Pasteur and Koch. The first report of a laboratory-acquired infection was in 1899, with the reporting of Mediterranean fever.

The study of laboratory-acquired infections was not carried out in a systematic fashion until 1951 with the most extensive and historic survey of 5 000 American laboratories by Sulkin and Pike (Collins 1983). This work was extended by three additional series (Sulkin

1964; Pike et al. 1965; Pike 1976). Through these reports, the authors cited 3 921 laboratory infections dating between 1930 and 1974, with a mortality of 164 (4.1 percent).

Of note, 2 307 (58.8 percent) of the infections were reported from research facilities, 677 (17.3 percent) were from diagnostic facilities, 134 (3.4 percent) were from the generation of biological products (industry), and 106 (2.7) were from teaching facilities. The remaining 697 (17.8 percent) were unspecified by source.

From four series performed in the UK between 1971 and 1991, it was noted that within clinical facilities, the majority of infections were reported from workers in the microbiology laboratory, with the second most common department being morbid anatomy. Of note, over the 20-year period, using the format of single-year surveys, the number of infections reported dropped over 80 percent, from 104 to only 17.

All studies are hampered, however, by having access only to those laboratory-acquired infections that are officially reported, either in literature or through other database systems. In no jurisdiction is there open access to required laboratory-acquired infections reporting. It is assumed that even complete listings reflect an immeasurable minority of infections that indeed occur (Sewell 1995).

Microorganisms are transmitted by a limited number of routes, which includes generation of aerosols of respirable size affecting the lungs or nonrespirable size resulting in contamination of external surfaces. Contamination of surfaces also result from spills, leaks, and untidy work practices. Ingestion results from contamination of edible or nonedible oral substances that reach the mouth as a consequence of contaminated hands, contaminated foods, or unsafe practices such as mouth pipetting. Laboratory accidents, especially involving the use and abuse of sharp instruments or breakable glass, result in puncture wounds of potentially fatal outcome. Inadvertent exposures resulting from inadequately identified or improperly stored reagents and materials, can result in serious, preventable infection. A laboratory risk assessment recognizes the potential routes and limits their ability to function.

While laboratory factors including aerosols, splashes, and accidents are described as major causes of laboratory-acquired infection, so too are human factors. In four series between 1952 and 1976 (Pike and Sulkin 1952; Reid 1957; Pike 1976; Harrington and Shannon 1976), it was repeatedly demonstrated that most workers who developed infection had had prior laboratory training. While at first blush this would appear to be problematic, it is also this group that would be performing the vast majority of hands-on activity. Disturbingly, it has also been pointed out that three of four surveys have indicated that 7–8 percent of laboratory infections occur in clerical and maintenance personnel (Collins 1983).

Briggs Phillips used a matched case–control study of 33 laboratory workers who experienced a laboratory associated injury over a 2-year period (Briggs Phillips, 1986). No differences were noted related to age, length of employment, years of formal education, wearing of glasses, use of prescription medications, off-the-job accidents, or driving record. On the other hand, accident-involved people were significantly more likely to have had a laboratory accident or laboratory infection prior to the 2-year study period and were significantly more likely to have a low opinion of laboratory safety programs. When the conditions surrounding the accidents were examined, 36 percent occurred when the employee was working too quickly, either just before lunch or at the end of the day. In 30 percent of accidents, the employee acknowledged a breech in safety regulations. In summary, in this study, attitudes and work habits were important contributing factors to laboratory accidents. Of note, to date there have been no published studies that confirm or refute these findings.

Quality assurance materials

Clinical laboratory workers are aware of the risks and hazards associated with handling clinical samples; however, laboratory-acquired infections in clinical laboratories can occur from improper handling not only of clinical samples but also of quality control materials, especially proficiency testing samples. In 1980, Blaser and Feldman (1980) reported 24 cases of infection with *Salmonella typhi* resulting from national proficiency testing program samples. A similar experience was repeated by Chin (1988) with an infection in a worker with *Corynebacterium diphtheriae*. In 2002, Richardson et al. (2002) reported two workers in separate laboratories infected with *Shigella flexneri* from external quality assessment samples that clinically simulated fecal samples. All materials within a laboratory, regardless of source, must be handled with all required safety and diligence.

BIOSAFETY QUALITY MANAGEMENT

Risk assessment

Laboratory biosafety programs may be driven by the recognition of good practices, in some jurisdictions may be directed by the application of established international standards, and in others may be driven by regulatory requirements. All laboratorians should regularly survey the facilities for potential hazards and implement corrective plans. Assessments should be formal, systematic, and planned, and cover all laboratory elements, including safety manual policies and procedures, training requirements and performance,

supervision, maintenance of hazardous materials and substances, first aid services and equipment, personnel safety, and health reports (ISO 2002).

Laboratory safety officer

All laboratories should designate a knowledgeable, trained, and experienced individual with the responsibility of ensuring laboratory safety. The position may require only part-time activity or may be a full-time responsibility. The responsibilities and authorities of the safety officer vis-à-vis the laboratory workers and management, and their role with respect to reporting and ability to initiate action, should be clearly defined. The laboratory safety officer should execute their activities on behalf of the laboratory management and at the same time have both the authority and responsibility to act independently and effectively. The roles of a laboratory safety officer may vary depending on the size and complexity of the facility; however, as a minimum, they should include (WHO 2003a):

- establishing and monitoring a laboratory safety program to a process of regular audit;
- performance of regular risk assessment, and development of recommendations for laboratory safety improvement;
- participating in investigation of accidental exposures, in order to prevent their occurrence;
- identifying the training needs of laboratory workers, and assisting with training implementation;
- ensuring that laboratory procedures for decontamination and disinfection are followed.

CLASSIFICATION SYSTEMS TO CLARIFY RISK

Three commonly recognized classification schemes are integral to defining the degree of risk associated with the handling of infectious materials and agents. The first is the classification of organisms, the second is the classification of environments, and the third is the classification of behaviors.

Classification of organisms

Given the variety of experiences and possible exposures, it is accepted that some microorganisms are more pathogenic than others. This may in part be due to the broad range of virulence factors that affect attachment, invasions, tissue tropisms, and production of exotoxins. While laboratorians need to have cautionary respect for everything they handle, it is accepted that some are inherently more dangerous and need to be addressed with an increased level of safety procedures and concern.

In order to designate increasing risk, national bodies including the Health Canada Bureau of Biosafety, Centers for Disease Control, National Institutes of Health, European Union, and Australia (ABSA 1998), have developed and harmonized a classification system for microorganisms that is subclassified across the spectrum of virulence. Classifications of microorganisms have traditionally been based on four stages:

Category 1: Microbes not known to consistently cause disease in healthy adults. Examples of category 1 organisms include *Bacillus subtilis* and *Naeglaria gruberi*. Microorganisms assigned to category 1 can be safely addressed in all categories of laboratories.
Category 2: Microbes that may commonly cause disease as a consequence of injury or ingestion. Therapy is readily available. Organisms tend not to spread or represent a significant community concern. Examples of category 2 organisms include *Staphylococcus* spp, most *Enterobacteriaceae*, pseudomonads, and hepatitis B virus.
Category 3: Microbes that have the potential for aerosol transmission. Therapy may be less readily available. May be indigenous or exotic agents. Examples of category 3 organisms include *Mycobacterium tuberculosis* and *Brucella* spp.
Category 4: Microbes that are exotic and dangerous, and that pose a high risk of life-threatening disease. May be transmitted by aerosol spread. Examples of category 4 agents include tick-borne encephalitis virus, Marburg virus, and Ebola fever virus.

While the classification of most agents is relatively straightforward, some organisms, such as rabies virus, dengue virus, and *Bacillus anthracis*, may be classified differently by different agencies. In these situations, appropriate handling of these agents depends on the circumstances, the availability of settings, and, above all, the practice behaviors. Note that complete listings of organisms can be obtained through examination of the tables drafted for the original documents

Classification of laboratory environments

Having established a microbial risk-classification system, it is appropriate to develop parallel and coordinated systems for addressing the handling of microorganisms safely. All laboratory workers require a safe working environment; however, it is critical that the intensity of the safety is seen to be appropriate to the inherent level of risk to avoid on the one hand undue potential of exposure to dangerous organisms and on the other hand unreasonable interference in work practices when handling commonplace pathogens. Classification of laboratory environments is relatively straightforward in research and reference laboratories where the workload and pathogen awareness are predictable. Specific requirements are more problematic in the clinical

laboratory setting because of the potential for a broad range of samples and microorganisms; that being said, even in this setting, there exists a high probability that most microorganisms processed are of category 2. While infrequent, clinical laboratories do receive risk group 3 pathogens, including *Mycobacterium tuberculosis* and *Francesella tulerensis*, and need to be prepared for the possibility.

Clinical and research laboratories can also be distinguished based on the types of organisms likely to be handled or processed. Biosafety level (BSL) 1 requirements are the most basic and general and are required for all laboratories. Each subsequent BSL builds upon the level below. BSL 4 provides the highest level of sophistication and safety.

BSL 1 facilities should be separated from public access by a lockable door. A biohazard sign should be posted at the entrance. There should be handwashing sinks readily available. Work surfaces should be constructed of materials that allow for proper disinfection. There should be equipment available for the safe decontamination of wastes prior to disposal. Special containment equipment need not be available; however, mechanical pipetting devices are mandatory for all pipetting procedures. Personal protective equipment, including laboratory gowns, coats, gloves, and eyewear, are required to be available for appropriate use. Laboratories designated as BSL 1 would be appropriate for undergraduate or secondary education and training but would be inappropriate for work involving samples of human blood or other tissues or body fluids.

BSL 2 facilities need to adhere to the requirements as above. In addition, door signage should include the name and contact information of the primary responsible person. Biological safety cabinets and other containment equipments are recommended to be available if procedures involving large inocula are to be performed, and emergency eyewash equipment should be available. While regional regulations would vary, most clinical microbiology laboratories would be required to meet the requirements of BSL 2 facilities.

BSL 3 facilities are designed to a higher level of security. A higher degree of door security is required, often under key, key card, or electronic access. BSL 3 laboratories are maintained under negative pressure, and access to the laboratories is through a vestibule or anteroom in order to protect the integrity of the pressure gradient. Exhaust air pressure handling systems is monitored and controlled. All interior surfaces, including walls, floors, and ceilings, need to be impact-resistant and without separations that would allow microorganisms to reside. Office areas are not allowed within BSL 3 facilities.

BSL 4 facilities are designed for the handling of dangerous and exotic organisms. Often, BSL 4 facilities are in separate buildings built to the required specification. In addition to the BSL 3 requirements, access to the laboratory must be through an anteroom with interlocking airtight doors. The anteroom area provides space for changing containment suits and for both chemical and water showers. Air supply into the laboratory is filtered with high-efficiency particulate air (HEPA). Outbound air passes through a two-stage HEPA filter process. Autoclaves need to be of a double-door design with interlocking doors to prevent accidental release of waste. All plumbing drains must pass through an effluent sterilization system.

Classification of laboratory practices

Just as handling of organisms and materials with increasing levels of risk requires increased levels of laboratory security, the practices of laboratorians can be scaled to risk.

General practices for all laboratory practice requires that all personnel working within a facility are trained and knowledgeable of the potential hazards to which they may be exposed, at least to the level of their competence and expertise. Eating and drinking and similar practices are not allowed within the laboratory workspace. The laboratory environment should be maintained tidily. Oral pipetting is prohibited. Long hair should be tied back or otherwise restrained. Protective garb and equipment should be available and be worn and used properly. Use of sharps should be strictly limited. All injuries should be reported without delay. All equipment, and especially autoclaves, should be maintained in good working order in order to prevent leaks, spills, and release of viable organisms. There should be an up-to-date laboratory safety manual that is available to all. The manual should include information on hazards within the specific laboratory, required safety information including material safety data sheets, and policies and procedures for all safety practices. Handwashing should be a mandatory practice when hands are either known or suspected to be contaminated and when gloves are removed.

In addition, level 2 practices would ensure that entry to the laboratory is restricted to only those on official business and that hazard warning signs are clearly posted at all entry locations. A health and medical surveillance program should be provided for all those working in the facility. An emergency procedure for spills and accidents should be written and accessible.

Level 3 practices would ensure that, in addition, there is a designated biosafety officer with the appropriate authority to oversee practices, and that all persons have successfully completed training courses in the specific requirements for added precaution. All employees should not only follow required procedures but also certify in writing that they understand the protocols. Personnel need to understand that when performing level 3 practices, the laboratory should be locked and

they must stay within the laboratory in order to maintain traffic at a minimum. Personal items are not brought into the laboratory. Personal clothing and jewelry should not be worn while performing level 3 practices. Laboratory-specific protective equipment and footwear should not be worn outside the laboratory.

In most situations and most facilities, practices and facilities would be in sync with the most common agents that pass through the facility. On some occasions, where a heightened level of concern exists, the level of recommended practices would raise. A case in point are the recommendations for laboratories likely to receive and process samples from patients with severe acute respiratory syndrome (SARS) containing coronavirus SARS-CoV. This agent, first recognized in the spring of 2003, was noted to transmit by both aerosols and contact and was often associated with a severe atypical pneumonia. While routine handling of diagnostic samples for serology or bacteriology and mycology agents was acceptable, it was deemed appropriate to recommend that workers performing procedures likely to result in aerosols follow practices more consistent with level 3, which included wearing disposable gloves, a mask or respirator, and eye protection, or a full-face shield (WHO 2003b). Despite this, there have been laboratory-acquired infections recorded while working with live strains of SARS-CoV in level 3 facilities.

At the highest level of security, level 4 practices entail all the aforementioned, but also require establishment of protocols to cover emergencies to cover possible damage to positive pressure suits. Staff should be highly trained and knowledgeable about all entry and exit procedures. They should be knowledgeable and experienced in working with all small and large animals, as may be required. They must keep a detailed log book of all agents handled, including date and time and procedures performed. A daily log should be maintained for all workers to monitor and report any and all illnesses (Health Canada 1996).

PREVENTION OF INFECTIONS

Biosafety measures to prevent laboratory-acquired infections are a combination of ready awareness, application of knowledge and education, proper use of personal protective equipment, devices, and procedures, and application of specific public-health measures. Breakdown in any domain can result in significant injury and illness.

Respiratory protective devices

Arguably, the most dangerously formed structure in the laboratory is the microbially contaminated airborne aerosol of respirable size. Microbially contaminated droplets larger than 5 μm settle rapidly, and while they tend not to be inhaled, they may be associated with contamination of the environment. Droplets smaller than 1–5 μm remain suspend in air longer and are more likely associated with inhalation (Hatch 1961).

Individual procedures tend to be associated with aerosols of certain size. Protection from droplets may be addressed by a variety of equipments. Large liquid splatters may be contained by splash guards or face masks. Smaller aerosols are far better contained by a properly functioning biological safety cabinet. The decision with respect to the most appropriate equipment would need to take into consideration the procedure being performed, potential agents, possible concentration, and availability of equipment.

Masks that are properly worn, covering the nose and mouth, provide the most direct respiratory protection. Disposable masks are constructed of a variety of materials and styles, either molded or pleated. Additional changes may include the application of hydrophobic coating to make the mask more water-resistant or the application of a face mask to provide eye protection. Application of masks may be by ear-loops, tie-on straps, or snug elastic bands.

Masks can be quantitatively measured to determine their ability to block the penetration of particles, including bacteria. N95 masks are measured to be at least 95 percent efficient. Many surgical masks, dependant upon their material and composition, may meet the filtering level of 95 percent efficiency; however, in order to be effective, masks must be snug to the face in order to prevent unfiltered air penetrating around the periphery. Beards, hair, or a thin face may result in gaps that impede snugness. Simulation studies based upon head models with external spray and upstream microbial and particle capture units clearly indicate the efficiency of form-fitted masks. Masks can be fit-tested to ensure the most effecting fitting. In many settings, disposable masks that provide nontested fitness can be considered as providing a sufficient level of safety, although in situations of higher security concerns, fitted masks are more appropriate. Unfortunately, observation studies indicate that many procedures are performed in the absence of any respiratory protection. There is ample real case evidence to indicate that the absence of wearing any personal protective equipment can result in the acquisition of infectious disease. Evidence that demonstrates the clinical inferiority of surgical masks in comparison to NIOSH approved N95 masks is more difficult to cite. In the absence of approved N95 masks, it is appropriate to use surgical masks as an alternative.

Biological safety cabinets (BSC) are units to protect the operator and the environment from aerosol contamination. BSCs prevent contamination through the presence of a combination of a physical glass containment barrier, an air curtain of 75 linear feet per minute face velocity or greater on the front access point, negative air flow, and a process of exhaust filtration.

Table 1 *Classification of biological safety cabinets (BSCs)*

Class	Front	Air curtain (linear feet per minute)	Supply air through HEPA filter	Exhaust air through HEPA filter	Ducted exhaust
I	Open	75	No	Yes	No
IIa	Open	75–100	Yes	Yes	No
IIb	Open	100	Yes	Yes	Yes
IIb[a]	Open	100	Yes	Yes	Yes
III	Closed	Not required	Yes	Double or followed by incineration	Yes

HEPA, high-efficiency particulate air.
a) IIb BSCs are subclassified as IIB-1, IIB-2, and IIB-3 based upon the complexity of air supply.

BSCs are classified by their degree to protection. Class 1 and class 2A units are designed for laboratories working with low or moderate risk. As class 2B and class III units are exhausted through ducts to outside the laboratory, they can also be used with volatile chemicals. Class III units are the most secure and are the only acceptable BSCs for use with risk group 3 and risk group 4 agents (see Table 1).

In many laboratories, small, inexpensive equipment can be readily available and significantly reduce the exposure of workers to biological hazards. Splashguards of plastic or glass can provide an effective physical barrier to block workers eyes from aerosols when opening blood tubes. This same function can be served well by full-face protectors or goggles or other eye protectors. Mechanical or electrical pipetting aids must always be used for all pipetting procedures. Mouth pipetting, once common practice, is completely unacceptable regardless of reagent or chemical or body fluid. Mouth pipetting not only creates the opportunity to inhale or swallow dangerous materials while applying suction, but it also puts others at risk by inhalation or contamination as large amounts of aerosols are generated with each clearing blow. Homgenizers, vortex stirrers, blenders, shakers, and centrifuges are all important laboratory equipment, but all create great risk for generation of aerosols unless they are used with sealed vessels. For some equipment, it is appropriate that it be housed only within a biological safety cabinet. For others, such as large centrifuges, it is critical that all tubes and vials are used with sealed protective units that are opened under a biological safety cabinet. In the microbiology laboratory, sterilization of platinum loops by thrusting the tip into a Bunsen burner flame can result in aerosol splatter. This activity can be avoided in many laboratories by the use of disposable loops or the use of microincinerators.

PERSONAL PROTECTIVE CLOTHING AND EQUIPMENT

Exposing personal street clothing to splashes and accidents can result in damage or contamination that could endanger the user or others that have contact with soiled laundry. Workers should be in the practice of wearing protective garb while performing all activities within the laboratory. Protective garb should be, as a minimum, long enough to cover the lap while sitting, be fluid-resistant, be closed at the neck, and have long sleeves, preferably with cuffs. Workers should not leave the laboratory wearing their gowns. Gowns can be used throughout the day, provided there have been no spills or obvious contamination, and should be hung in a fashion to prevent contamination when not being used. Gowns should be laundered regularly, in many facilities at the end of each day's use. It is inappropriate to take home soiled gowns for personally laundering.

Glove use is a valuable technique to protect hands from contact exposure to microbial agents, provided that the gloves are selected properly and used appropriately. Readily available gloves are made of a variety of materials including vinyl, latex, and nitrile.

The quality of gloves can be affected by a wide range of variables, including composition, manufacturer, design, degree of manipulation, chemical exposure, and type of glove analysis performed.

For most tasks within the laboratory, the most appropriate gloves to wear are disposable, made of light-weight materials that allow the greatest degree of flexibility and dexterity, and yet provide a safe protective barrier from microorganisms.

While there was little evidence to suggest that individuals wearing vinyl gloves had developed seroconversion to human immunodeficiency virus (HIV), early studies demonstrated that vinyl gloves were significantly more likely to leak than latex gloves, especially after being exposed to isopropyl alcohol. The impact of these studies contributed to a rush on latex gloves and the recognition of glove use contributing to latex allergy. Thermoplastic elastomer materials were implemented for glove use as an alternative to both vinyl and latex. Studies based upon the penetration by bacteriophage phi X174 have suggested that thermoplastic elastomer gloves provide a superior mechanical barrier to either vinyl or latex.

While gloves can provide a barrier for the user, the exterior surface does become contaminated. While some will tolerate exposure to isopropyl alcohol, most will not,

and glove disinfection is not considered an appropriate practice. Glove use should be task-specific, and they should be removed and discarded before performing tasks that can lead to environmental contamination of keyboards, telephones, and other equipment.

For many tasks, protection from injury due to either sharps or chemical irritation requires more durable gloves. Fabric undergloves can be worn along with a disposable glove to provide improved grip compared with double-gloving with disposable gloves and some protection from disposable-glove tears. Metal-mesh gloves provide substantial protection when using cutting tools and other sharps, although they compromise dexterity. Household rubber and rubber-blend gloves can provide added protection against a variety of chemical irritants.

Regardless of the glove materials, or the reason for which they are worn, removal of gloves should always be followed by adequate handwashing.

HANDWASHING

Handwashing is commonly recognized as the single most important procedure to prevent the acquisition of infection. One might conclude that handwashing is sufficiently commonplace; however, it has been the focus of study and guidelines for many years.

The single most important procedure for the prevention of infection in oneself and in others is to wash hands. The wisdom of handwashing was first demonstrated and reported in 1847 by Semelweis (Lechevalier and Solotorovsky 1974), and yet occult observation studies within both the hospital and community consistently show poor handwashing practices by all professional groups (Muto et al. 2000; Eckmanns et al. 2001). The reasons for inappropriate lack of handwashing have a degree of justification: excessive work load, lack of time, poor access to handwashing sinks and materials, concerns for skin irritation, and other problems associated with frequent handwashing (Pittet 2001).

Laboratories tend to rely primarily on a brief list of agents for handwashing, including plain soap, chlorhexidine- or triclosan-containing compounds, and more recently liquid or gel forms of alcohol, either ethanol or isopropanol.

Plain soap with running water is an effective technique to remove soil and organic substances but is not effective for the removal of bacterial pathogens.

Chlorhexidine is a chlorine-containing cationic organic compound with broad-spectrum antimicrobial activity. It is inhibited by anionic soaps but is insensitive to interference by organic materials.

Triclosan is an organic phenyl ether also with broad antimicrobial activity, although its activity against gram-negative bacilli is reduced. It is also insensitive to organic matter.

Replacing plain soap with antimicrobial soaps that contain antiseptics such as chlorhexidine, chloroxylenol, hexachlorophene, quaternary ammonium compounds, and triclosan, are more effective for the removal of pathogens, although individual products may have their own concerns. Chlorhexidine, triclosan, and quaternary ammonium compounds can be contaminated with gram-negative bacteria. Skin irritation is recognized with higher concentrations of chlorhexidine and free iodine concentrations with iodophors. Hexachlorophene can manifest neurotoxicity when bathing neonates.

On some occasions, especially in older facilities, ready access to handwashing sinks is not immediately available. Waterless alcohol-based antiseptics are handwashing agents. Alcohols used in these products include ethanol, isopropanol, n-propanol, or combinations, all usually between 60 and 90 percent alcohol. Alcohol preparations are available in both gel and liquid formats.

Alcohols are germicidal against a broad range of microorganisms, including gram-positive and gram-negative bacteria and lipid-enveloped viruses. The activity is rapidly germicidal through the denaturation of proteins. Bacillus species tend to be resistant to alcohols. Alcohols do not have persistent activity, although bacterial regrowth following alcohol wash tends to be slow. Activity is dependent upon alcohol concentration, volume, and contact time. Frequent use can lead to skin dryness if glycerol is not incorporated as a conditioning agent. It is important to remember at all times that all alcohols are flammable and the potential for flash fires may exist.

Ultimately, the agent that is the most successful in reducing carriage and transmission of microbial pathogens may not necessarily be the most antimicrobially active agent but the one that is used most effectively by the greatest proportion of individuals. Indeed, there is little information that clearly links a specific mean log-reduction in bacterial count associated with any specific product and a correlate reduction in nosocomial or occupationally related infection. In-use comparison of 2 percent chlorhexidine versus 61 percent ethanol has demonstrated no significant differences in bacterial counts but less skin irritation with the alcohol and a significant reduction in required wash time with the alcohol (Larson et al. 2001).

In vitro studies of handwashing have to address many issues, including gender, age, underlying condition of skin, presence of jewelry, test organisms, inoculum, method and duration of application, and retrieval and enumeration methods. Campaigns to encourage improved handwashing unfortunately tend to have short-lived efficacy and require persistent energy and activity (Muto et al. 2000).

In many institutions, the number and placement of handwashing sinks has been found to be less convenient

than desirable. As an alternative to expensive plumbing alterations, ready access to alcohol hand rubs has been associated with increased compliance with hand hygiene (Bissett 2002).

Immunization

A regular and maintained program of protective immunization is an essential part of a biosafety program. As a minimum, laboratory workers should be protected from diphtheria, hepatitis B, measles, mumps, poliomyelitis, rubella, tetanus, and typhoid fever. Many workers will have protection from either childhood infection or an immunization program. For the remainder, an adult immunization program is appropriate. While laboratory-acquired influenza does not occur more frequently than community-acquired infection, annual influenza immunization provides protection from loss of work activity.

All workers in laboratories that may handle specimens or other samples containing *Mycobacterium tuberculosis* should participate in a regular skin-test surveillance program. Chest X-rays need not be preformed other than at the time of skin-test conversion or at the onset of symptoms suggestive of respiratory disease. While BCG immunization is available and used in many countries, others find that BCG obscures the interpretation of skin tests without providing long-term protection and as such is often no longer provided.

As a minimum, all laboratory workers required to work with animals, especially including large and small mammals, should receive pre-exposure human diploid cell rabies vaccine. Rabies vaccine is not required for workers handling fish, birds, amphibians, and small rodents.

Laboratories of special interest in other specific organisms including, but not limited to, *Neisseria meningitidis*, *Haemophilus influenzae*, *Coxiella burnettii*, *Francisella tularensis*, and *Yersinia pestis*, may consider providing additional specific immunization. Vaccinia vaccine can be used postexposure to prevent clinical manifestation of human monkey pox as a result of handling prairie dogs and exotic rodents (Wilson 2003).

Sharps injuries and blood-borne infection

Blood-borne infection is a frequent cause of occupation acquired infection in healthcare workers. It is estimated that between 800 and 1 000 healthcare workers acquire hepatitis B on an annual basis, along with an additional 500–1 000 who acquire hepatitis C. Worldwide since 1978, fewer than 60 healthcare workers have acquired HIV from occupational exposure (Sewell et al. 2001). This disproportionate distribution is related to the concentration of the virus found in blood samples and the established risk of infection following a known single occupational percutaneous exposure (see Table 2).

Blood-borne agents are most often transmitted through sharps injuries; however, mucus-membrane exposure and contact with apparently intact skin are also associated. While blood splatter from accidents and spills may lead to skin or mucus-membrane exposure, there are no descriptions of occupationally acquired hepatitis or HIV associated with aerosols. Hepatitis B can be transferred from common environmental surfaces, although studies have clearly demonstrated the absence of transmission associated with cardiopulmonary resuscitation training manikins (Glaser and Nadler 1985)

Laboratory workers, including phlebotomists, are second only to nurses as the most common professional group to have occupationally acquired HIV infection.

While antiviral therapy may play a role in the clinical suppression and treatment of blood-borne disease, clearly avoidance and prevention are superior alternatives. Diligence while handling all samples, and assuming that all samples are contaminated, provides a mental framework for preventing infection in oneself and others.

It is instructive to note that the first paper citing laboratory-acquired infections, published in the *Lancet* in 1899, described two laboratory workers who acquired Mediterranean fever (brucellosis) by needlestick injury (Birt and Lamb 1899). As much as possible, sharps instruments should be avoided when handling blood and body fluids. Recapping of sharps is almost always inappropriate. Where recapping is not avoidable, glove-use procedures to recap single handedly make procedures safer.

BIOTERRORISM AND LABORATORY SAFETY

The intentional use of infectious agents long predates the actual knowledge and history of microbiology or laboratories. The deliberate use of biological agents used for war has been documented through the twelfth century (Marty 2001). The use of laboratories for the deliberate development of biological agents for warfare has been documented in many countries.

Table 2 *Relative risk factors with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis virus C (HCV)*

	HBV	HBC	HIV
Concentration in blood particles per ml	10^8–10^9	10^2–10^3	10^0–10^4
Risk per percutaneous exposure (%)	18	1.8	0.3
Healthcare cases per year	800–1 000	500–1 000	Fewer than 2

In the latter part of the twentieth century, several key events were associated with biowarfare agents. From 1941 to 1969, the USA, the UK, Canada, and others formed national programs of biological warfare research. In 1979, at least 66 Soviet citizens living downwind of a top-secret biological warfare research center in Sverdlovsk died as a result of an accidental release of *Bacillus anthracis*. In 1984, Iraqi forces were accused of deploying biological weapons against Iran.

The poignancy of biological risk was again heightened with the spread of *B. anthracis* through the postal service in the USA in 2001. Events at this time have resulted in a new and active level of laboratory awareness with respect to safety and security, and with the detection and proper handling of samples with specific identified biological warfare agents.

While virtually any microorganism deployed creatively could be used for bioterror or biowarfare, the predominant microorganisms of concern include *B. anthacis*, *Escherichia coli* O157:H7, *Francisella tulerensis*, *Yersinia pestis*, and smallpox.

The Laboratory Response Network (LRN) within the USA asserted that hospital-based clinical microbiology laboratories should be prepared to respond to events through the collection, preservation, transport, and testing of human specimens that potentially contain agents of biowarfare potential. A four-tier system was developed and categorized from front-line facilities (level A), through local public health laboratories (level B), and state health laboratories with advanced capability (level C), to federal high-level security facilities (level D). Level A laboratories are recommended to have developed bioterror response plans with standard operating procedures. Level A laboratories would be expected to have the technical capability to rule out or refer suspicious isolates to the next level. It is expected that all hospital-associated laboratories would be able to meet the requirements of BSL 2 and also have access to a properly functioning biological safety cabinet. The LRN system is predicated upon the workers in a level A facility having access to guidance and assistance from a level B facility.

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Staphylococcus

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HISTORICAL PERSPECTIVE

Rosenbach provided the first formal description of the genus *Staphylococcus* and divided the genus into two species, *Staphylococcus aureus* and *Staphylococcus albus*. Passet added a third species, *Staphylococcus citreus*. Cell morphology and type of cell aggregation provided the criteria for genus classification, and colony color was used for species classification. Zopf placed the staphylococci and a group of saprophytic, tetrad-forming micrococci back into the genus *Micrococcus* and Flügge rearranged the cocci and separated the genus *Staphylococcus* from the genus *Micrococcus*. He differentiated the two genera primarily on the basis of their action on gelatin and on the symbiotic relationship to their hosts. Evans et al. (1955) proposed separating staphylococci from micrococci on the basis of their relation to oxygen using a standard oxidation–fermentation (OF) test for glucose fermentation. The facultative anaerobic cocci were placed in the genus *Staphylococcus* and the obligate aerobes were placed in the genus *Micrococcus*. The separation of the genera based on the OF test continued until it became apparent that this division was not absolute. A clear distinction could be made between staphylococci and micrococci on the basis of DNA base composition (Silvestri and Hill 1965); however, this property could not be determined in the routine diagnostic laboratory. Surveys of gram-positive, catalase-positive cocci indicated that staphylococci have a G + C content of DNA of 30–39 mol%, whereas micrococci have a G + C content of 63–73 mol%. Although both genera were placed in the family Micrococcaceae (Prévot 1961), the very wide divergence in DNA base composition indicates that they are not significantly related. More recent systematic studies have distinguished staphylococci from micrococci and other bacteria on the basis of cell wall composition (Schleifer and Kandler 1972), cytochromes (Faller et al. 1980), menaquinones (Collins and Jones 1981), cellular fatty acids, polar lipids (Nahaie et al. 1984), DNA–rRNA hybridization (Kilpper et al. 1980; Schleifer 1986), and comparative oligonucleotide cataloging of 16S rRNA (Ludwig et al. 1981). It is now recognized that the genus *Staphylococcus* belongs to the broad *Bacillus–Lactobacillus–Streptococcus* cluster consisting of gram-positive bacteria that have a low G + C content of DNA. These organisms are now tentatively arranged together with staphylococci in the family *Bacillaceae* of the order *Bacillales* (Cato and Stackebrandt 1989). The closest relatives of staphylococci are the macrococci (Kloos et al. 1998a), and on the basis of partial oligonucleotide sequencing of 16S rRNA and rDNA, staphylococci are also related to salinicocci, enterococci, planococci, bacilli, and listeriae (Ludwig et al. 1985; Stackebrandt

et al. 1987). The closest relatives of the micrococci are the arthrobacters, renibacteria, rothias, dermatophili, and stomatococci (Koch et al. 1994). The genus *Micrococcus* has, more recently, been dissected into six genera (*Micrococcus*, *Kocuria*, *Nesterenkonia*, *Kytococcus*, *Dermacoccus*, and *Arthrobacter*) on the basis of 16S rDNA sequence relationships and several chemotaxonomic characters (Koch et al. 1995; Stackebrandt et al. 1995).

Early medical bacteriologists placed major emphasis on distinguishing the pathogenic species *S. aureus* from the presumed commensal staphylococci, referred to as *S. albus*, *Staphylococcus epidermidis albus* (Welch 1891), or *Staphylococcus epidermidis* (Evans 1916). The distinction was of considerable importance since *S. aureus* was a major cause of morbidity and mortality and clinical specimens often carried both types of organisms. Von Darányi (1925) was the first person to draw attention to the practical value of the coagulating principle (coagulase) test to identify *S. aureus*, and it remains one of the most important tests used by the clinical laboratory to identify this species. Baird-Parker (1963, 1965a) attempted to subdivide the genera *Staphylococcus* and *Micrococcus* by using many classical morphological, physiological, and biochemical tests. He first divided staphylococci into six subgroups (I–VI) and micrococci into eight subgroups (1–8). In subsequent schemes, Baird-Parker (1965b, 1974) recognized the species *S. aureus*, *S. epidermidis*, and *Staphylococcus saprophyticus*, and divided the latter two species into several biotypes. His schemes were widely used for more than a decade, especially by medical and food diagnostic laboratories. Meyer (1967) and Hájek and Maršálek (1971) divided the species *S. aureus* into several biotypes on the basis of physiological, biochemical, and phage typing properties, and host preferences.

DEFINITION OF THE GENUS

Members of the genus *Staphylococcus* are gram-positive cocci (0.5–1.5 μm) that occur singly, in pairs, tetrads, short chains, and irregular 'grape-like' clusters. They are nonmotile and nonspore-forming. Most species demonstrate catalase activity and are facultative anaerobes, growing better under aerobic than anaerobic conditions; the anaerobic species *Staphylococcus saccharolyticus* and subspecies *S. aureus* subsp. *anaerobius* are exceptions. Most species grow in the presence of 10 percent sodium chloride and at 18–40°C. Staphylococci are susceptible to furazolidone (100 μg furazolidone disk) and resistant to low levels of bacitracin (0.04 unit TAXO A bacitracin disk). They are susceptible to lysis by lysostaphin (some species more than others) and are relatively resistant to lysis by lysozyme. The cell wall contains peptidoglycan and teichoic acid. The diamino acid present in the peptidoglycan is L-lysine except in the ancient species *S. succinus* where it is *meso*-diaminopimelic acid; the interpeptide bridge of the peptidoglycan consists of oligoglycine peptides that are susceptible to the action of lysostaphin. Depending on the particular species, some glycine residues may be substituted by L-serine or L-alanine. The teichoic acids of staphylococci are either of the poly(polyolphosphate), poly(glycerolphosphate-glycosylphosphate), or poly(glycosylphosphate) class. Glycerol or ribitol or both occur as typical components of poly(polyolphosphate) teichoic acids. Species contain either *a*- and *b*-type cytochromes or *a*-, *b*- and *c*-type cytochromes. Staphylococci contain unsaturated polyisoprenoid side chains in their menaquinones. The major isoprenologues are MK-6, MK-7 or MK-8. The principal cellular fatty acids are $C_{16:0}$, $C_{18:0}$ and $C_{20:0}$ and *iso*- and *anteiso*-methyl-branched $C_{15:0}$ and $C_{17:0}$. The G + C content of DNA is 30–40 mol%.

Investigations on cell structure, cell wall composition, G + C content of DNA, DNA–DNA hybridization, 16S rRNA sequence similarities, and the genome sizes of *Staphylococcus caseolyticus* (Schleifer et al. 1982) and the newly introduced species *Staphylococcus bovicus*, *Staphylococcus carouselicus*, and *Staphylococcus equipercicus* have indicated that this group of four species should be placed into a separate, but closely related genus, which has been given the name *Macrococcus* (Kloos et al. 1998a). Gram-stained cells of these organisms are generally larger (1.3–2.5 μm diameter) than the cells of staphylococcal species. *S. caseolyticus* (renamed *Macrococcus caseolyticus*) has a small amount of an unusual cell wall teichoic acid and the other species of this genus fail to demonstrate teichoic acid. The G + C content of DNA is 38–45 mol%. The estimated genome size of the members of the genus *Macrococcus* is 1 500–1 820 kbp, which is significantly smaller than the genome size of 2 000–3 000 kbp shown for *Staphylococcus* spp.

CLASSIFICATION OF SPECIES AND SUBSPECIES

Species

The currently recognized species in the genus *Staphylococcus* are shown in Table 1. *Staphylococcus succinus* is considered to be an ancient species as it was recovered viably from 25–35 million year old Dominican amber free of cracks and fissures (Lambert et al. 1998). The two strains isolated had *meso*-diaminopimelic acid instead of L-lysine in their cell wall and contained tuberculostearic acid characteristic of coryneform bacteria. *Staphylococcus* spp. are currently classified on the basis of DNA–DNA hybridization (DNA relatedness) as determined by the relative binding of DNAs in reassociation reactions conducted at nonrestrictive (optimal), restrictive (stringent) conditions, or a combination of both (Kloos 1980; Schleifer 1986; Kloos et al. 1991). Members of the same species demonstrate relative

Table 1 *Currently recognized* Staphylococcus *species*

Species	Reference
S. arlettae	Schleifer et al. 1984
S. aureus	Rosenbach 1884
S. auricularis	Kloos and Schleifer 1983
S. capitis	Kloos and Schleifer 1975a
S. caprae	Devriese et al. 1983
S. carnosus	Schleifer and Fischer 1982
S. chromogenes	Devriese et al. 1978; Hájek et al. 1986
S. cohnii	Schleifer and Kloos 1975
S. condimenti	Probst et al. 1998
S. delphini	Varaldo et al. 1988
S. epidermidis	Winslow and Winslow 1908; Evans 1916; Schleifer and Kloos 1975
S. equorum	Schleifer et al. 1984
S. felis	Igimi et al. 1989
S. fleurettii	Vernozy-Rozand et al. 2000
S. gallinarum	Devriese et al. 1983
S. haemolyticus	Schleifer and Kloos 1975
S. hominis	Kloos and Schleifer 1975a
S. hyicus	Devriese et al. 1978
S. intermedius	Hájek 1976
S. kloosii	Schleifer et al. 1984
S. lentus	Kloos et al. 1976; Schleifer et al. 1983
S. lugdunensis	Freney et al. 1988
S. lutrae	Foster et al. 1997
S. muscae	Hájek et al. 1992
S. pasteuri	Chesneau et al. 1993
S. piscifermentans	Tanasupawat et al. 1992
S. saccharolyticus	Foubert and Douglas 1948; Kilpper-Bälz and Schleifer 1981
S. saprophyticus	Shaw et al. 1951
S. schleiferi	Freney et al. 1988
S. sciuri	Kloos et al. 1976
S. simulans	Kloos and Schleifer 1975a
S. succinus	Lambert et al. 1998
S. vitulinus	Webster et al. 1994
S. warneri	Kloos and Schleifer 1975a
S. xylosus	Schleifer and Kloos 1975

DNA-binding values of generally 70 percent or greater when reactions are performed at the optimal criterion. Organisms representing different species demonstrate relative DNA-binding values of less than 70 percent at the optimal criterion and have a much lower relative binding percentage at the stringent criterion. A dendrogram depicting the DNA relationships of most of the species is shown in Figure 1. The thermal stability ($\Delta T_{m(e)}$) of the DNA duplexes formed in reassociation reactions conducted at nonrestrictive conditions supports the conclusions drawn from the relative DNA-binding studies, although not all species have been investigated by this approach. Comparative immunological studies of catalases (Rupprecht and Schleifer 1979; Schleifer et al. 1979) and fructose-1,6-biphosphate (FBP) aldolases (Fischer et al. 1983), using microcomplement fixation techniques have, essentially, indicated the same relatedness as DNA–DNA hybridization.

Chemical properties of the cell wall, especially the amino acid composition and sequence of the interpeptide bridges of the peptidoglycan (Schleifer 1983, 1986) and teichoic acid composition (Endl et al. 1984), provide useful chemotaxonomic markers for the classification of species. Penta- and hexaglycine interpeptide bridges are found in the peptidoglycan in approximately half the species, whereas in the other half a minor portion of the glycine residues are replaced by L-serine. In three species (*Staphylococcus sciuri*, *Staphylococcus lentus*, *Staphylococcus vitulinus*), an L-alanine residue, in place of glycine, is bound to lysine of the peptide subunit. Most species have cell wall teichoic acids of the poly (glycerolphosphate) and/or poly(ribitolphosphate) type that are substituted with various combinations of sugars and/or *N*-acetylamino sugar residues. Some strains of *S. sciuri*, *S. vitulus*, and *Staphylococcus hyicus* have the poly(glycerolphosphate-*N*-acetylglucosaminylphosphate) type and *Staphylococcus auricularis* has the poly(*N*-acetylglucosaminylphosphate) type, where the *N*-acetylamino sugar residues form an integral part of the polymer chain. Certain other biochemical properties of staphylococci are useful for the differentiation of species including production of lactic acid when grown under anaerobic conditions (Schleifer 1986), or the lactate isomer formed and the occurrence of NAD-dependent L- and/or D-lactate dehydrogenases. Many of the staphylococcal species can also be distinguished on the basis of their relative percentages of cellular fatty acid components, as determined by gas–liquid chromatography (Durham and Kloos 1978; Kotilainen et al. 1991). Some of the biochemical and physiological properties of staphylococci can be determined by simple conventional tests and rapid identification systems. These include various enzyme activities, hemolysins, oxygen requirements, products of glucose metabolism, utilization of substrates, production of acid from carbohydrates, and intrinsic resistance to certain antibiotics (Kloos et al. 1991; Kloos and Bannerman 1995). Colony morphology and pigment can serve as secondary characters for the classification of species and are valuable markers for the initial identification of staphylococci growing on primary isolation plates (Kloos and Bannerman 1994). Specific combinations of the above phenotypic characters have been quite accurate in predicting the identification of species based on DNA relatedness.

Molecular approaches have also been used for species classification. The comprehensive ribotyping studies of Webster and colleagues (Webster et al. 1994) have identified conserved, chromosome *Eco*RI fragments containing portions of the rRNA operons that are species-specific. PFGE studies have identified conserved,

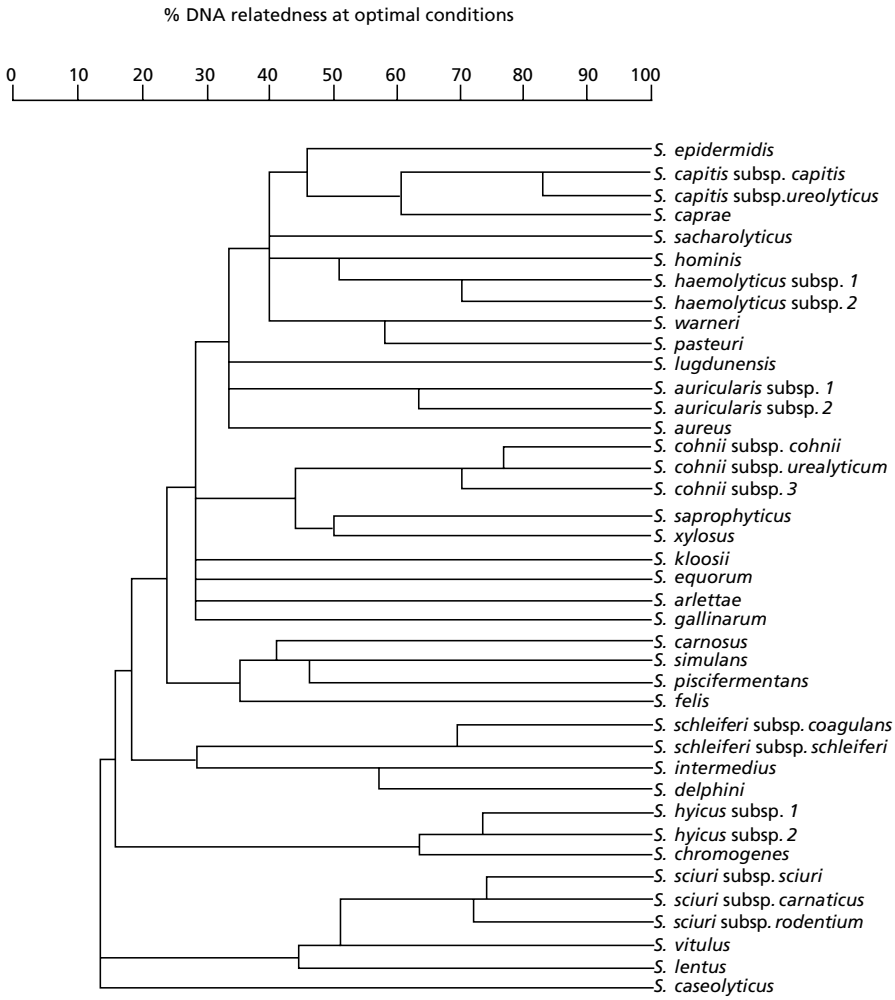

Figure 1 *Dendrogram of the DNA relationships of* Staphylococcus *species and subspecies based on the relative percent of DNA–DNA hybridization (reassociation). Optimal DNA reassociation conditions were performed at 55°C (29–31°C below the T_m of the DNA) (Kloos and Wolfshohl 1979).*

chromosome *Sma*I fragments that are species-specific (George and Kloos 1994; Bannerman et al. 1995; Shimizu et al. 1996). Polymerase chain reaction (PCR)-amplified ribosomal DNA spacer and tDNA intergenic spacer fingerprinting has also been applied to species identification (Welsh and McClelland 1992; Jensen et al. 1993). Goh et al. (1997) have demonstrated that a 600 bp region of the chaperonin 60 genes from 28 staphylococcal species could be amplified by PCR with a pair of degenerate primers and the resulting amplification products used as species-specific probes. However, DNAs from the closely related species *S. intermedius* and *S. delphini* crosshybridized. Sequencing of whole genomes and comparative genomics will ultimately provide the opportunity to reexamine the current taxonomic scheme and will provide new insights into what defines a species. Seven *S. aureus* genomes are known to have been sequenced to date, five of which have been reported in the published literature (Kuroda et al. 2001; Baba et al. 2002; Holden et al. 2004). A recent comparative genomics study of five *S. aureus* genomes reported that around 80 percent of the genome is highly conserved between strains (Holden et al. 2004). The genome sequence of one strain of *S. epidermidis* has been published (Zhang et al. 2003). Publication of comparisons between the *S. aureus* and *S. epidermidis* genomes, and between the *S. haemolyticus* genome and other available staphylococcal genomes are expected in the near future. A comprehensive taxonomic overview using this approach will require sequencing of a large number of species, together with sufficient numbers of strains within a given species to detect the full spectrum of divergence. This may ultimately provide the framework for the development and validation of more rapid sequence-based approaches for the study of taxonomy.

Subspecies

Staphylococcal subspecies (Table 2) have been identified on the basis of phenotypic characters and DNA

Table 2 Staphylococcus *subspecies*

Subspecies	Reference
S. aureus subsp. *aureus* and *S. aureus* subsp. *anaerobius*	De la Fuente et al. 1985
S. auricularis subsp. 1 and *S. auricularis* subsp. 2	Kloos 1986a, 1986b
S. capitis subsp. *capitis* and *S. capitis* subsp. *ureolyticus*	Bannerman and Kloos 1991
S. caprae subsp. 1 and *S. caprae* subsp. 2	George and Kloos 1994
S. carnosus subsp. *carnosus* and *S. carnosus* subsp. *utilis*	Schleifer and Fischer 1982; Probst et al. 1998
S. cohnii subsp. *cohnii*, *S. cohnii* subsp. *urealyticum*, and *S. cohnii* subsp. 3	Kloos and Wolfshohl 1983, 1991
S. equorum subsp. *equorum* and *S. equorum* subsp. *linens*	Schleifer et al. 1984; Place et al. 2003
S. haemolyticus subsp. 1 and *S. haemolyticus* subsp. 2	Kloos and Wolfshohl 1979
S. hominis subsp. *novobiosepticus* and *S. hominis* subsp. *hominis*	Kloos and Schleifer 1975a; Kloos et al. 1998b)
S. hyicus subsp. 1 and *S. hyicus* subsp. 2	Phillips and Kloos 1981; Shimizu et al. 1997
S. saprophyticus subsp. *saprophyticus* and *S. Saprophyticus* subsp. *bovis*	Shaw et al. 1951; Hájek et al. 1996
S. schleiferi subsp. *schleiferi* and *S. schleiferi* subsp. *coagulans*	Igimi et al. 1990
S. sciuri subsp. *sciuri*, *S. sciuri* subsp. *rodentium* and *S. sciuri* subsp. *carnaticus*	Kloos et al. 1997
S. succinus subsp. *succinus* and *S. succinus* subsp. *casei*	Lambert et al. 1998; Place et al. 2002

relatedness (Igimi et al. 1990; Bannerman and Kloos 1991; Kloos et al. 1997), ribotyping (De Buyser et al. 1992; Kloos et al. 1997) and pulsed field gel electrophoresis (PFGE) (George and Kloos 1994). Members of the same subspecies demonstrate relative DNA-binding values of, generally, 80 percent or greater when DNA–DNA hybridization reactions are performed at either optimal or stringent conditions. Different subspecies demonstrate relative DNA-binding values of, generally, 65–80 percent at the optimal criterion, but have significantly lower relative DNA-binding values, in the range of 30–70 percent, at the stringent criterion. *S. pasteuri* (Chesneau et al. 1993) is a borderline species–subspecies, as it demonstrates relative DNA-binding values of 50–67 percent at the optimal criterion and relative DNA-binding values of 19–36 percent at the stringent criterion when its DNA is hybridized to *S. warneri* DNA (Kloos and Wolfshohl 1979).

Host range

Many of the staphylococcal species and subspecies living on mammals demonstrate host specificity (Table 3).
The host range may be narrow to wide, depending on the particular species or subspecies. With the exception of staphylococci that prefer the single host species *Homo sapiens*, preferences are either at the host subfamilial, familial, or ordinal levels. Generally, staphylococci from closely related hosts are more closely related to one another than staphylococci from distantly related hosts. A comparison of the dendrogram of resident mammalian staphylococcal species and subspecies, based on DNA–DNA hybridization data (Kloos et al. 1991; Webster et al. 1994), and proposed consensus trees of mammalian orders, based on amino acid sequence data (Goodman et al. 1985; Prothero 1993) and nucleotide sequence data (Goodman et al. 1985; Saccone et al. 1991; Milinkovitch 1992) indicates a close alignment between the position of most staphylococcal taxa and their corresponding mammalian hosts. These observations suggest the possibility that staphylococci, like the eukaryotic parasites of mammals, may have coevolved with their hosts (Kloos and Wolfshohl 1979; Kloos 1980; Brooks and Glen 1982; Kim 1985). *S. xylosus*, the principal resident species of rodents, is an exceptional species in that populations are very widespread in nature. Its extensive distribution appears to be primarily the result of host and environmental transfer rather than by descent, perhaps as a consequence of the very large population size and geographical range of rodents. *S. aureus* and *S. caprae*, resident species of primates, are also exceptional in that distinct ecovars can be found living on various domestic animals.

HABITAT

Cutaneous habitats, excluding the nose

Staphylococci are one of the major groups of bacteria inhabiting the skin, skin glands, and mucous membranes of mammals (Noble and Somerville 1974; Kloos 1980, 1986a; Devriese 1986; Kloos et al. 1991). Noble and Somerville (1974) proposed three categories of skin microbes according to their host relationship, a useful framework when considering the habitats of staphylococci. *Residents* are natural inhabitants of the skin, *temporary residents* are contaminants that multiply and persist for short periods of time, and *transients* are contaminating organisms that do not multiply. Resident staphylococcal species and subspecies are represented by at least several different strains on an individual host,

Table 3 *Host range of* Staphylococcus *species and subspecies*

Species and subspecies[a]	Natural host	Temporary (recent) host
S. epidermidis	Humans	
S. capitis subsp. *capitis*	Humans	
S. capitis subsp. *ureolyticus*	Primates	
S. caprae subsp. 1	Humans	
S. caprae subsp. 2		Domestic goats
S. saccharolyticus	Humans	
S. hominis subsp. *hominis*	Humans	
S. hominis subsp. *novobiosepticus*	Humans	
S. haemolyticus subsp. 1	Humans	Domestic artiodactyls and horses
S. haemolyticus subsp. 2	Primates	
S. warneri	Humans	Domestic artiodactyls and horses
S. pasteuri	Primates	Domestic artiodactyls and horses
S. lugdunensis	Humans	
S. auricularis subsp. 1	Humans	
S. auricularis subsp. 2	Primates	
S. aureus subsp. *aureus* (A)	Primates	Domestic artiodactyls, poultry
S. aureus subsp. *aureus* (B)		Domestic pigs, poultry
S. aureus subsp. *aureus* (C)		Domestic artiodactyls
S. aureus subsp. *aureus* (D)		Lagamorpha, Rodentia
S. aureus subsp. *anaerobius*		Domestic sheep
S. saprophyticus subsp. *saprophyticus*	Primates, Scandentia	Rodentia
S. saprophyticus subsp. *bovis*	Domestic cattle	
S. cohnii subsp. *cohnii*	Humans	
S. cohnii subsp. *urealyticum*	Primates, Scandentia	
S. cohnii subsp.3	New-world monkeys	
S. xylosus	Rodentia	Mammals and birds
S. kloosii	Rodentia	Domestic artiodactyls
S. equorum subsp. *equorum*		Domestic horses and cattle
S. arlettae		Poultry
S. gallinarum		Poultry
S. simulans	Carnivora	Domestic artiodactyla, humans
S. carnosus subsp. *carnosus*		Domestic artiodactyla
S. felis	Carnivora	
S. lutrae	Carnivora (otters)	
S. intermedius	Carnivora	Domestic horses, poultry
S. schleiferi subsp. *schleiferi*	Carnivora	Humans
S. schleiferi subsp. *coagulans*	Carnivora	
S. delphini	Cetacea	
S. hyicus subsp. 1	Artiodactyla (Suidae)	
S. hyicus subsp. 2	Artiodactyla (Bovidae)	Poultry
S. chromogenes	Artiodactyla, Perissodactyla	
S. sciuri subsp. *sciuri*	Rodentia, Artiodactyla, Perissodactyla, Cetacea, Marsupialia	Poultry
S. sciuri subsp. *rodentium*	Rodentia, Cetacea	
S. sciuri subsp. *carnaticus*	Artiodactyla, Cetacea	
S. lentus	Artiodactyla, Perissodactyla, Cetacea	
S. vitulinus	Artiodactyla, Perissodactyla, Cetacea	

a) The host range of *S. muscae*, *S. piscifermentans*, *S. condimenti*, *S. succinus*, *S. fleurettii*, *S. carnosus* subsp. *utilis*, and *S. equorum* subsp. *equorum* is unknown. *S. aureus* subsp. *aureus* biotypes are designated by the letters (A–D). From Kloos et al. (1991) and Kloos and Bannerman (1994, 1995)

many of which may persist for a period of several weeks to several years (Kloos 1986a; George and Kloos 1994; Kloos and Bannerman 1994).

The commonest resident staphylococcal species on human skin in terms of population size is *S. epidermidis*. The largest populations of cutaneous staphylococci (c. 10^4–10^6 cfu/cm^2) are found in regions of the skin of mammals supplied with large numbers of pilosebaceous units and sweat glands and on the skin and mucous membranes surrounding openings to the body surface. The microenvironment of a given host site strongly influences the predominant species present. For example, *S. epidermidis*, *S. hominis*, and *S. haemolyticus* are the major staphylococci found on the scalp of preadolescent children. An increase in sebaceous gland activity at puberty is associated with an increase in the *S. capitis* population which becomes the predominant species. *S. capitis* subsp. *capitis* has a strong preference and *S. capitis* subsp. *ureolyticus* has a moderate preference for the human head. The external auditory meatus of the ear also provides a unique habitat. The outer two thirds of the canal contain sebaceous and ceruminous glands, but no eccrine sweat glands. Nutrients are supplied to the area by the processes of keratinization, sebaceous secretion, and ceruminous secretion. Although amino acids and proteins are present, there is only a very small amount of urea. The major species and subspecies found living in the external auditory meatus of adults, *S. auricularis* and *S. capitis* subsp. *capitis*, do not exhibit urease activity. *S. auricularis* has a strong preference for this habitat and reaches a maximum population in young adults, following the onset of ceruminous gland activity. The face provides several different habitats, the predominant species for which is *S. epidermidis*.

Other important anatomical sites for colonization of the human host are the axilla and groin. The axilla contains a rich supply of both eccrine and apocrine glands and is characterized by high humidity, rich supply of nutrients, near-body temperature, and a higher pH (6.8–7.9) than is found on the general skin surface, factors that enhance the growth of staphylococci and certain other bacteria. Populations of staphylococci reach very high densities in this habitat (c. 10^4–10^6 cfu/cm^2). The major species of the axillae and groins are *S. epidermidis* and *S. hominis*. *S. schleiferi* is part of the axillary flora in some individuals, while *S. lugdunensis* is predominantly a 'below the belt' colonizer. Populations of urease producing *S. capitis* subsp. *ureolyticus* also reach a relatively large size in the groin of some adults. *S. aureus* and *S. saprophyticus* are sometimes found in the inguinal and perineal area. The environment presented by the arms and legs is different in that it is relatively dry to moderately moist and is bathed in an emulsion of lipids and eccrine sweat containing lactic acid–lactate, amino acids, urea, and electrolytes. These habitats support small populations of staphylococci (c. 10–10^3 cfu/cm^2). The major resident is *S. epidermidis*, but members of the axillary, inguinal, and perineal flora are often dispersed to adjacent areas of skin. A wide variety of transient staphylococcal species may also be isolated from the arms and legs.

The anterior nares

The nose provides a major habitat for *S. epidermidis*, and is the dominant ecological niche for *S. aureus*. Three *S. aureus* carriage patterns have been described in the healthy adult population, with approximately 20 percent of individuals being persistent *S. aureus* carriers, about 60 percent intermittent carriers, and 20 percent persistent non-carriers (Kluytmans et al. 1997). This is probably an oversimplification, but there is sufficient data to support the idea that some individuals within the population are never, or hardly ever, positive for *S. aureus*, others are infrequent carriers and the remainder are usually carriers. This pattern of carriage suggests the possibility of a host genetic influence. Consistent with this is the observation that carriage rates vary between different ethnic groups (Noble 1974), together with a community-based carriage study that demonstrated a family predisposition to nasal carriage of *S. aureus* that could not be explained solely on the basis of exposure to a common reservoir of bacteria (Noble et al. 1967). Variation in host phenotype in the nares has also been demonstrated, in which nasal secretions from three subjects colonized with *S. aureus* lacked antimicrobial activity against *S. aureus* in vitro, whereas fluid from noncarriers was bactericidal (Cole et al. 1999). The number or nature of host receptors in the nares may also vary, one study demonstrating that adherence of *S. aureus* to desquamated epithelial cells from carriers was significantly greater than that for noncarriers (Aly et al. 1977). Genetic studies of host susceptibility for carriage of *S. aureus* have been conducted, but their findings are inconclusive (Hoeksma and Winkler 1963; Aly et al. 1974; Kinsman et al. 1983).

The host may also modulate carriage through immune regulation. In a human experimental inoculation study, those susceptible to the smallest initial *S. auteus* inoculum were colonized for the greatest period of time (Ehrenkranz 1966). After spontaneous nasal elimination, survival of the same strain on repeated reinoculation was uniformly brief, but duration of colonization of a second strain of *S. aureus* mirrored that of the primary inoculation. These data suggest an acquired immune response. Studies examining the potential role of the local immune IgA response, such as differences between persistent carriers and noncarriers of *S. aureus*, have not been reported. Other circumstantial evidence for the involvement of the immune system comes from carriage rates in patient groups who are immunocompromised. Rates of *S. aureus* carriage are higher in those infected with human immunodeficiency virus compared with both

health care workers and patients with a range of chronic diseases (Weinke et al. 1992). Carriage rates are also higher in individuals with insulin-dependent diabetes, in patients on continuous ambulatory peritoneal dialysis and hemodialysis, and in intravenous drug abusers when compared with the healthy population (Kluytmans et al. 1997).

Unlike other bacterial species that colonize nasopharyngeal tissue, *S. aureus* is predominantly localized to the anterior nares. The epithelium in this region is stratified, keratinized, nonciliated epithelium, contrasting with the rest of the nasal cavity and respiratory tract which is lined with ciliated columnar epithelium. *S. aureus* attaches to a specific region of moist epithelium devoid of hair and cilia (Cole et al. 2001). Some individuals carry *S. aureus* in the pharynx as detected by throat swab, but scanning electron microscopy using human nasal tissue obtained from surgical specimens has demonstrated that adherence of *S. aureus* to ciliated epithelium is poor (Shuter et al. 1996). Other sites such as the axilla and perineum may become colonized, but *S. aureus* appears to be a temporary resident here since elimination of nasal carriage by topical antibiotics generally leads to loss of carriage in other sites (Reagan et al. 1991). The nature of the host receptor recognized by *S. aureus* during attachment and colonization of the nose is unclear. Possible sites of *S. aureus* adherence include stratified epithelial cells, or mucus or serum constituents associated with these cells. Epithelial cells express a complex array of glycoproteins, glycolipids, and proteoglycans and the presence/absence or heterogeneity in one or more of these molecules could be important in *S. aureus* carriage. An association has been described between blood group A and nasal carriage (Veselov and Malyshkina 1988), and between adhesion of *S. aureus* to buccal epithelial cells and expression of the Lewis^a blood group antigen (Saadi et al. 1993). Adherence of *S. aureus* to gangliosides and asialo-GM1 extracted from corneal epithelial cells has been described (Schwab et al. 1996), and asialoganglioside 1 has been identified as a receptor for *S. aureus* in a cystic fibrosis bronchial epithelial cell line (Imundo et al. 1995). *S. aureus* also adheres to glycolipids (fucosylasialo-GM1, asialo-GM1, and asialo-GM2) extracted from lung tissue (Krivan et al. 1988). To date, there are no published studies evaluating the role of these host receptors in adherence of *S. aureus* to nasal cells.

S. aureus adhere to desquamated epithelial cells harvested from the anterior nares by a swab or cytobrush, and this has been utilized to study bacterial-host cell interactions in vitro. Squamous epithelium undergoes a process of maturation from basal layer to the exposed surface involving a change of shape, loss of nucleus, and increasing keratinization. Bacterial adherence has been compared between squamous epithelial cells of different maturity. Adherence appears to be related to the age of the cell and state of keratinization, the greatest adherence occurring to the most mature cell type (Bibel et al. 1982). Adherence is reduced if epithelial cells are pretreated with high concentrations of ribitol teichoic acid or lipoteichoic acid extracted from the cell wall of group A streptococci (although this may represent a steric effect) (Aly et al. 1980; Carruthers and Kabat 1983). Mucin may enhance adherence since *S. aureus* has been shown to adhere to ferret airway mucus, bovine submaxillary gland mucin, and purified human mucin in vitro (Sanford et al. 1989; Shuter et al. 1996). The bacterial adhesin for mucin has not been characterized. The surface expressed *S. aureus* protein clumping factor B (ClfB) was the first bacterial determinant to be ascribed a role in nasal carriage. Initially described as a fibrinogen-binding protein, this multifunctional adhesin also interacts with epidermal cytokeratins and desquamated nasal epithelial cells in vitro (O'Brien et al. 2002a). The *S. aureus* surface protein SasG, identified previously by in silico analysis of genome sequences, and two homologous proteins, Pls of *S. aureus* and AAP of *S. epidermidis*, also promote bacterial adherence to nasal epithelial cells (Roche et al. 2003).

Colonization resistance is likely to influence the strain of *S. aureus* carried. During the late 1950s it was recognized that colonization of newborn infants with staphylococci of the 52/52A/80/81 phage group complex was often followed by disease in infants and their contact (Schaffer et al. 1957), and that the presence of *S. aureus* in the nose before contact with phage type 80/81 strains prevented colonization with this strain. This was attributed to bacterial interference, which was further verified by a study in which newborn infants were inoculated with the 'non-pathogenic' *S. aureus* strain 502A, the effect being a reduction in colonization with a second strain (Shinefield et al. 1963). The relevance of interactions between *S. aureus* and other microbial species present in the anterior nares to carriage status is undergoing investigation. One study has reported a low incidence of *S. aureus* carriage in individuals whose nose swabs were positive by culture for *Corynebacterium* sp. (Uehara et al. 2000). *S. aureus* carriage was interrupted in 12 of 17 persistent carriers after an average of 12 inoculations of *Corynebacterium* sp. (the actual species was not defined) (Uehara et al. 2000). The presence of viridans streptococci has been reported to prevent colonization with methicillin-resistant *Staphylococcus aureus* (MRSA) in newborns, possibly as a consequence of H_2O_2 production by the streptococci (Uehara et al. 2001a, 2001b). The bacterial aerobic nasal flora of 216 healthy volunteers was defined to identify potential competitive interactions among different species (Lina et al. 2003). The *S. aureus* colonization rate correlated negatively with the rate of colonization by *Corynebacterium* spp. and non-aureus staphylococci, especially *S. epidermidis*, suggesting that both *Corynebacterium* spp. and *S. epidermidis* antagonize *S. aureus* colonization. The effect of other components of the normal nasal

flora on the development of *S. aureus* carriage was examined in 157 consecutive infants over the first 6 months of life (Peacock et al. 2003). Negative associations (putative bacterial interference) between *S. aureus* and other species occurred early in infancy but were not sustained. An increasing antistaphylococcal effect was observed over time, but this was not attributable to bacterial interference. These studies suggest that complex interactions are likely to occur both within the polymicrobial human flora, and between the flora and host.

CELL MORPHOLOGY AND CULTURAL CHARACTERISTICS

Light microscopy and staining reactions

Gram stained cells of staphylococci are uniformly gram-positive in young (18–24 h) cultures and appear spherical with an average diameter of 0.5–1.5 μm on light microscopy. The cells of old cultures (>48 h) are often gram-variable to nearly gram-negative. Cells divide in more than one plane to form irregular clusters and aggregates of pairs, tetrads, and short chains, depending on the species. For example, *S. aureus* mainly produces irregular clusters of cells; *S. hominis*, *S. haemolyticus*, and *S. capitis* mainly produce aggregates of tetrads and pairs; and *S. saprophyticus* and *S. cohnii* produce loose aggregates of single cells and pairs. Cell wall defective or deficient (L-form) cells have been described for *S. aureus* (Kagan 1972) and *S. epidermidis* (Acevedo et al. 1985). These organisms fail to take Gram strain, are osmotically sensitive, and are not easily cultured on the usual isolation media. As a result, their frequency and relevance to medical practice are not known. They may be induced by benzylpenicillin, methicillin, and lysostaphin. Most of the L-form strains isolated from clinical specimens have been associated with chronic infections, malignant tumours and body fluids of immunodeficient patients.

Colony morphology

Staphylococci produce distinctive colonies on a variety of commercial, selective, and nonselective agar media. The commonly used selective media include mannitol–salt agar, lipase–salt–mannitol agar, phenylethyl alcohol agar, Columbia colistin–nalidixic acid (CNA) agar, and Baird–Parker agar base supplemented with egg yolk tellurite enrichment. These media inhibit the growth of gram-negative bacteria, but allow the growth of staphylococci and certain other gram-positive bacteria. Baird–Parker agar base, when supplemented with egg yolk tellurite enrichment, is widely recommended for the detection and enumeration of *S. aureus* in foods (Schwab et al. 1984). This medium and the other selective media were designed to distinguish *S. aureus* from other species on the basis of colonial and biochemical properties; however, with the discovery of additional staphylococcal species that share some properties with *S. aureus*, it is now recognized that such a distinction cannot be made accurately. Schleifer–Krämer agar (Schleifer and Krämer 1980) is used by some laboratories for the selective isolation and enumeration of staphylococci from foods and other heavily contaminated sources. Incubation of cultures on selective media should be for at least 48–72 h at 35–37°C for colony development.

P agar (Kloos et al. 1974) and tryptic soy agar have been widely used in the primary culture and subculture of staphylococci from cutaneous habitats (Kloos et al. 1991). Colonies of each of the recognized species and subspecies can be distinguished from one another on these media with considerable accuracy, provided reference strains of each of the taxa are included for comparison. Detailed descriptions (and in some cases color photographs) of colonies have been reported in the original taxonomic descriptions of the species and subspecies. Pigmentation and pigment sectoring patterns are usually more pronounced in colonies growing on these media than on blood agar. When pigment is present, colonies are usually different shades of cream-yellow, yellow, yellow-orange, or orange, depending on the particular species and strain. Some strains of *S. epidermidis*, *S. intermedius*, and *S. lentus* demonstrate a subtle violet, pinkish, or brownish pigment. All staphylococcal species grow well on tryptic soy agar, but *S. vitulus*, *S. lentus*, *S. caseolyticus*, and some strains of *S. sciuri*, grow poorly on P agar. These species should be cultured on tryptic soy agar or tryptic soy agar supplemented with blood. The anaerobic species *S. saccharolyticus* and subspecies *S. aureus* subsp. *anaerobius* grow well on the above nonselective media under anaerobic conditions, but grow very poorly under aerobic conditions.

Colonies of the same strain generally exhibit similar features of size, consistency, edge, profile, luster and pigment, but some strains may produce two or more morphotypes. The mechanisms for this are undergoing investigation, particularly for *S. epidermidis*; one explanation for this phenomenon is variable expression of the *ica* operon, a gene cluster encoding the production of polysaccharide intercellular adhesin (PIA) which mediates intercellular adherence of bacteria and the accumulation of a multilayered biofilm. Several unusual morphotypes of *S. aureus* have been described that depart significantly from the normal colony morphology, and include certain encapsulated strains (Wiley 1972), L-forms (Kagan 1972) and small colony variants (SCV) (Quie 1969). SCVs are believed to have a defect in electron transport and are resistant to aminoglycosides by virtue of poor drug uptake (Wilson and Sanders 1976; Proctor and Peters 1998). These variants are characterized by a slow growth rate, absence of pigmentation, a reduced range of carbohydrate utilization, and failure to

express several putative virulence factors. There are two classes of SCVs distinguished on the basis of whether they are stable following serial passage or revert back to wild type (McNamara and Proctor 2000). SCVs have been reported to arise after uptake of bacteria by endothelial cells in vitro (Vesga et al. 1996), following exposure to gentamicin in broth culture, and during human infection or experimental models (Proctor et al. 1995; von Eiff et al. 1997; Kahl et al. 1998; Abele-Horn et al. 2000; von Eiff et al. 2001a). SCVs also persist within endothelial cells in vitro (Balwit et al. 1994). It has been proposed that SCVs represent a small subpopulation of organisms that become auxotrophs following the acquisition of a mutation in genes involved in the electron transport chain. However, recent work suggests that reversible phenotypic switching can occur between *S. aureus* SCV and the parental phenotype (Massey and Peacock 2002). Switching between gentamicin resistant SCV and gentamicin sensitive wildtype may circumvent the lasting fitness cost of antibiotic resistance associated with permanent genetic mutation. The significance of SCVs for human infection is that they are associated with cases of *S. aureus* infection that prove resistant to antibiotic cure as manifest by persistence or relapse (Proctor et al. 1995; Kahl et al. 1998; Abele-Horn et al. 2000).

Growth in broth and semisolid media

Staphylococci grow well in a variety of commercial broth media, including tryptic soy broth, brain–heart infusion broth, nutrient broth, and tryptose phosphate broth, with or without the addition of blood. Most of the species are butyrous and easy to emulsify, but the *S. sciuri* species group and the strong slime-producing strains of *S. epidermidis* are glutinous and difficult to emulsify. Many of the staphylococcal species can produce abundant anaerobic growth in a semi-solid Brewer's thioglycollate medium within 24–72 h at 35–37°C (Kloos and Schleifer 1975b; Kloos and Bannerman 1995). Species such as *S. aureus*, *S. epidermidis*, *S. lugdunensis*, and *S. schleiferi*, which are among the most pathogenic species, produce abundant anaerobic growth with overnight incubation. Some species such as *S. hominis*, *S. auricularis*, *S. kloosii*, *S. equorum*, *S. arlettae*, *S. vitulinus*, and *S. lentus* fail to grow, or grow only very slowly, in the anaerobic portion of this medium.

METABOLISM

Carbohydrate metabolism

Glucose and other reducing sugars are present in very small amounts in the sweat and aqueous surface film of human skin and is probably not a major exogenous source of carbon for staphylococci growing in their primary habitat. Higher levels of glucose are present in certain host tissues (e.g. blood, liver, kidneys, muscle), and may be available to invading staphylococci. Lactic acid and lactate are major sources of carbon for staphylococci which can readily and reversibly convert these end products into pyruvic acid, thereby allowing them to enter the pathways of pyruvate metabolism and assimilation. These compounds are derived mainly from glycolysis in host tissues and the breakdown of glycogen in the sweat glands. Staphylococci are capable of using a variety of carbohydrates as carbon and energy sources. The carbohydrate can be taken up and accumulated inside the cell without a change in its form, or it can be covalently modified during uptake. In the latter type of carbohydrate uptake, the carbohydrate is phosphorylated during the transport process mediated by the phosphoenolpyruvate (PEP)–carbohydrate phosphotransferase system (PTS). Glucose, mannose, glucosamine, fructose, lactose, galactose, mannitol, *N*-acetylglucosamine, and β-glucosides are taken up by the PTS (Reizer et al. 1988). The lactose-specific PTS of *S. aureus* is composed of four enzymes, enzyme I (EI), Hpr, enzyme III $(EIII)^{lac}$ and enzyme II $(EII)^{lac}$, that appear to form a membrane-bound multienzyme complex (Kalbitzer et al. 1981). Some carbohydrates are not taken up via the PTS, such as the pentoses ribose, xylose, and arabinose, and the corresponding pentitols. These are taken up unsubstituted in the species *S. saprophyticus* and *S. xylosus* (Lehmer and Schleifer 1980).

The Embden–Meyerhof–Parnas (EMP) (glycolytic) pathway and the oxidative hexose monophosphate pathway (HMP) are the two central routes used by staphylococci for glucose metabolism (Strasters and Winkler 1963; Blumenthal 1972). *S. aureus* and *S. epidermidis* metabolize glucose mainly by glycolysis, and to a limited extent by the HMP (Sivakanesan and Dawes 1980). The major end product of anaerobic glucose metabolism in *S. aureus* is lactate (73–94 percent); smaller quantities of acetate (4–7 percent) and traces of pyruvate are also formed. Under aerobic conditions, only 5–10 percent of the glucose carbon appears as lactate and most of it appears as acetate and CO_2. In *S. epidermidis*, lactate is the major end product of anaerobic glucose metabolism. The other end products are acetate, formate, and CO_2, which are formed in trace amounts. *S. saccharolyticus* appears to be an exceptional species in that it ferments glucose mainly to ethanol, acetic acid, and CO_2; only small amounts of lactic and formic acid are produced (Kilpper-Bälz and Schleifer 1981). In members of the *S. saprophyticus* species group and *S. intermedius*, *S. capitis*, *S. haemolyticus*, and *S. warneri*, lactose and galactose are metabolized via the Leloir pathway whereby galactose-1-phosphate is epimerized to glucose-1-phosphate (Schleifer et al. 1978; Schleifer 1986). *S. aureus*, *S. epidermidis*, *S. hominis*, *S. chromogenes*, *S. sciuri*, and *S. lentus* metabolize lactose and galactose via the tagatose-6-phosphate pathway, whereby galactose-6-phosphate is isomerized to taga-

tose-6-phosphate, which is further converted to tagatose-1,6-diphosphate and then cleaved to triosephosphates (Schleifer et al. 1978; Schleifer 1986).

Respiration

Cytochromes and unsaturated menaquinones form the membrane-bound electron transport system. The major cytochromes of the respiratory chains of staphylococci are a_{602}, b_{557}, and o_{555} (previously b_{555}) (Faller et al. 1980). It has been suggested that cytochrome *a* does not play a major role in the respiration of at least some staphylococci. The minor cytochromes b_{552}, b_{560}, and b_{566} are widely distributed in staphylococci, although b_{552} is not found in *S. sciuri* and *S. lentus*. Cytochrome b_{557} appears to be an intermediate electron carrier of the cytochrome *b* or b_1 type. Members of the *S. sciuri* species group have cytochromes of the *c* type, in addition to cytochromes *a* and *b*. Cytochrome o_{555} binds carbon monoxide and is probably the major terminal oxidase. Menaquinones (MK-6 through MK-9) are the sole isoprenoid quinones of staphylococci and play important roles in electron transport and oxidative phosphorylation (Collins and Jones 1981). Most staphylococcal species contain MK-7 as the major isoprenologue. MK-8 is a major isoprenologue in *S. aureus* and *S. lugdunensis* and MK-6 is a major isoprenologue in *S. hyicus*, *S. equorum*, and members of the *S. sciuri* species group (Collins 1981; Nahaie et al. 1984; Schleifer and Kroppenstedt 1990).

Amino acid biosynthesis and nitrogen requirements

Most staphylococcal species are capable of synthesizing a large proportion of the different amino acids needed for growth. The genetic control of histidine (Kloos and Pattee 1965), isoleucine and valine (Smith and Pattee 1967), lysine (Barnes et al. 1971), tryptophan (Proctor and Kloos 1970), leucine (Pattee et al. 1974) and alanine, threonine, tyrosine, and methionine (Schroeder and Pattee 1984; Mahairas et al. 1989) biosynthesis has been studied extensively in *S. aureus*. The histidine (*hisEABCDG*), isoleucine–valine (*ilvABCD*) and leucine (*leuABCD*), lysine (*lysOABFG*) and tryptophan (*trpABFCDE*) genes of *S. aureus* are each clustered in what appear to be individual operons. Many naturally occurring strains have mutations in one or more of the amino acid biosynthesis genes, resulting in certain amino acid requirements. The amino acid requirements of approximately half the recognized species of staphylococci have been determined in vitro with the use of chemically defined media (Tschäpe 1973; Emmett and Kloos 1975, 1979; Hussain et al. 1991). Members of the *S. epidermidis* species group have numerous (c. 5–13) amino acid requirements (Emmett and Kloos 1975). All require arginine, the principal amino acid of human sweat and one that is present in moderate amounts (10–18 mg per 100 ml) (Heir et al. 1946). Most of the strains of species in this group also require isoleucine–valine and proline, and with the exception of *S. epidermidis*, most require histidine. *S. capitis* generally requires more different amino acids than other members of the group. *S. aureus* requires arginine and has either an absolute or partial requirement for proline. Most strains of this species require isoleucine–valine and have either an absolute or partial requirement for cysteine and leucine. Different ecovars of *S. aureus* demonstrate some differences in their amino acid requirements (Tschäpe 1973). *S. simulans* requires arginine, and most strains of this species also require proline, isoleucine–valine, leucine, and alanine. Members of the *S. saprophyticus* and *S. sciuri* species groups require fewer amino acids than the other species and some do not even require an amino acid or an organic nitrogen source. Some strains of *S. saprophyticus* have an absolute or partial requirement for proline and isoleucine–valine and some strains of *S. cohnii* require arginine and proline. Most strains (89–95 percent) of *S. xylosus*, *S. kloosii*, and *S. sciuri* do not require an organic nitrogen source (Emmett and Kloos 1979). They can grow well with $(NH_4)_2SO_4$ as the nitrogen source. Relatively high levels of ammonia (3–78 mg per 100 ml) and urea (24–620 mg per 100 ml) are present in the sweat of humans (Rothman 1954) and certain animals (Montagna and Parakkal 1974), making them available to staphylococci as additional sources of substrate nitrogen. These nitrogenous compounds may also reach the skin surface of mammals from urine. Many of the amino acid requirements of staphylococci exhibit spontaneous reversion to independence. *S. capitis* is an exceptional species in that it has a large number (9–13) of amino acid requirements and, of these, only requirements for alanine, glycine, proline, and cysteine have been known to exhibit reversion, either spontaneously or in response to alkylating mutagens (Emmett and Kloos 1979).

Vitamin requirements have been determined for approximately a third of the recognized staphylococcal species. Nicotinic acid and thiamine are usually required by *S. aureus* and members of the *S. epidermidis* and *S. saprophyticus* species groups (Tschäpe 1973; Cove et al. 1983). Biotin, or pantothenic acid, or both, are also required by many strains of species in the *S. epidermidis* species group (Emmett and Kloos 1975). Some strains in the *S. saprophyticus* species group may also require pantothenic acid.

CELLULAR COMPONENTS

Cell membrane

The cell membrane appears to be a typical lipid–protein bilayer, being composed mainly of phospholipids and

proteins. It represents a selective barrier and fulfils many functions, including electron transport, active transport, participation in septum formation, and segregation of DNA. The cytochromes and menaquinones bound to cell membranes are important components of the electron transport system. Phospholipids, glycolipids, menaquinones, and carotenoids make up the major lipid components of the membrane. The major polar lipids are phospholipids and glycolipids (Shaw 1975; Nahaie et al. 1984). The major phospholipids are phosphatidylglycerol, diphosphatidylglycerol (cardiolipin), and phosphatidic acid. Lysylphosphatidylglucose is also a major component of the cell membrane of *S. aureus* and *S. intermedius*. The glycolipids are monoglucosyl and β-diglucosyl diglycerides and phosphatidyl glucose. Various proteins have been isolated from the membranes of *S. aureus*, such as adenosine triphosphatase (Gross and Coles 1968), polyprenolphosphokinase (Sandermann and Strominger 1972), various oxidases and dehydrogenases (Theodore and Weinbach 1974), and several penicillin-binding proteins (PBP), which catalyze terminal reactions of peptidoglycan biosynthesis (Hayes et al. 1981; Hartman and Tomasz 1984). Some PBPs are species- or species group-specific (Canepari et al. 1985; Pierre et al. 1990). The pigments of staphylococci are triterpenoid carotenoids having a C_{30} chain (Marshall and Wilmoth 1981a). In *S. aureus*, carotenoids range from colorless polyisoprene compounds such as squalene, diapophytoene, and diapophytofluene, to the yellow, orange, and red triterpenoid carotenoids. The main pigment is staphyloxanthin, a compound in which glucose is esterified with both a triterpenoid carotenoid carboxylic acid and an *anteiso*-methyl-branched C_{15} fatty acid (12-methyl tetradecanoic acid).

Certain iron-regulated cell membrane proteins are expressed under iron limitation in *S. aureus* (Domingue et al. 1989) and members of the *S. epidermidis* species group (Smith et al. 1991; Wilcox et al. 1991). Two siderophores are known to be produced extracellularly by staphylococci: staphyloferrin A (Meiwes et al. 1990) and staphyloferrin B (Haag et al. 1994). Staphyloferrin A, B, or both, are produced by a wide range of staphylococcal species. A 42 kDa cell wall protein that binds human transferrin, the major iron-binding protein in serum, has been detected in *S. aureus* and certain members of the *S. epidermidis* species group (Modun et al. 1994) and a protein that binds human lactoferrin, an iron-binding protein found in milk, tears, saliva, and some other body fluids, has been identified in *S. aureus* (Naidu et al. 1992).

Cell wall

Peptidoglycan and teichoic acid are the major components of the staphylococcal cell wall. The peptidoglycan amounts to approximately 50–60 percent of the dry weight (Schleifer and Kandler 1972; Schleifer 1983). The cell wall teichoic acid amounts to approximately 30–50 percent of the dry weight, and it is covalently linked to peptidoglycan. The peptidoglycan is the main structural polymer in the wall and it plays an important role in maintaining the spherical shape of the cell. It is a heteropolymer consisting of glycan chains that are cross-linked by short peptides. The glycan moiety is made up of alternating β-1,4-linked units of *N*-acetylglucosamine and *N*-acetylmuramic acid. Some of the C-6 hydroxyl groups of muramic acid are phosphorylated and the resulting muramyl-6-phosphate residue represents the attachment point between peptidoglycan and teichoic acid (Kojima et al. 1985). The carboxyl group of muramic acid is substituted by an oligopeptide (peptide subunit) containing alternating L- and D-amino acids. The peptide subunits are cross-linked by the insertion of an interpeptide bridge that extends from the COOH-terminal D-alanine in position four of one peptide subunit to the ϵ-amino group of L-lysine in position three of an adjacent peptide subunit (Schleifer 1973). Interpeptide bridges consist of oligoglycine peptides, though some glycine residues may be replaced by L-serine or L-alanine. The biological activities of peptidoglycans include endotoxin-like properties (pyrogenicity, complement activation, generation of chemotactic factors, aggregation and lysis of animal blood platelets), inflammatory skin reactions, inhibition of leukocyte migration, adjuvant activity, mitogenic activity, and induction of immunosuppressive cells (Schleifer 1983). Peptidoglycan antibodies can be found in normal human and animal sera. The cell wall teichoic acids of staphylococci are water-soluble polymers composed of alditol (glycerol, ribitol), sugar and/or *N*-acetylamino sugar, and sometimes D-alanine (Schleifer 1983). Most staphylococcal species contain ribitol, or glycerol teichoic acids, or both. Cell wall teichoic acids are at least partially exposed to the cell surface where they contribute to the binding of antibodies (Knox and Wicken 1973), bacteriophages (Schleifer and Steber 1974) and lectins (Reeder and Ekstedt 1971).

Cell surface associated and secreted adhesins

A range of *S. aureus* cell wall-associated proteins have been characterized and shown to promote adherence to extracellular matrix proteins and/or soluble plasma components (Patti and Allen 1994) (Table 4). These adhesins may play a central role in colonization and invasive disease. A total of 21 genes encoding surface proteins that are anchored to the peptidoglycan cell wall have been identified by in silico analysis of six *S. aureus* genome sequences. The best characterized of these are protein A SpA (Forsgren et al. 1983; Uhlén et al. 1984;

Table 4 S. aureus *cell surface associated and secreted proteins*

Bacterial determinant	Putative function	Ref.
Fibronectin binding proteins A and B (FnBPA and FnBPB)	Adhesin for fibronectin FnBPA also shown to recognize fibrinogen	Switalski et al. 1983; Rydén et al. 1983; Signas et al. 1989; Jönsson et al. 1991; Greene et al. 1995
Clumping factor A and B (ClfA and ClfB)	Adhesins for fibrinogen ClfA and ClfB mediate platelet activation and aggregation ClfB mediates binding to epidermal cytokeratin	McDevitt et al. 1994; Ni Eidhin et al. 1998; O'Brien et al. 2002a, b; Siboo et al. 2001
Collagen binding protein (Cna)	Adhesin for collagen	Patti et al. 1992; 1994
Protein A (SpA)	Binds Fc domain of Ig and von Willebrand factor	Forsgren et al. 1983; Uhlén et al. 1984; Hartleib et al. 2000
Members of serine-aspartate multigene family (SdrC, SdrD, SdrE)	Function of SdrC and SdrD unknown; putative adhesins SdrE mediates platelet activation and aggregation	Josefsson et al. 1998
Bone sialoprotein-binding protein (Bbp)	Member of the Sdr family Allelic variant of SdrE Adhesin for bone sialoprotein	Rydén et al. 1989; Tung et al. 2000
Elastin-binding protein (EbpS)	Adhesin for elastin	Park et al. 1996; Downer et al. 2002
Map/Eap	Map (cell associated) described as an MHC class II analog protein Eap (secreted protein) has a broad binding specificity; involved in adherence to fibroblasts, and enhances internalization into eukaryotic cells in vitro; serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes	McGavin et al. 1993; Jönsson et al. 1995; Palma et al. 1999; Flock and Flock 2001; Hussain et al. 2001, 2002; Chavakis et al. 2002; Haggar et al. 2003
Plasmin-sensitive protein (Pls)	Associated with *mecA* Large surface protein that may interfere with bacterial interactions with host proteins including fibronectin and immunoglubulins	Savolainen et al. 2001
Biofilm-asociated protein (Bap)	Present in 5% of bovine strains but absent from 75 human isolates tested May interfere with bacterial attachment to host tissues and cellular internalization	Cucarella et al. 2001, 2002
Extracellular matrix-binding protein homolog (Ehb)	Very large cell wall-associated proteinBinds human fibronectin	Clarke et al. 2002

Hartleib et al. 2000), collagen-binding protein Cna (Patti et al. 1992), the fibrinogen-binding proteins clumping factor A and B (ClfA and ClfB) (McDevitt et al. 1994; Ni Eidhin et al. 1998), and the two fibronectin-binding proteins FnBPA and FnBPB (Switalski et al. 1983; Rydén et al. 1983; Signas et al. 1989; Jönsson et al. 1991; Greene et al. 1995). Other less well characterized proteins include three members of the serine-aspartate multigene family (SdrC, SdrD, and SdrE) (Josefsson et al. 1998); the bone sialoprotein-binding protein Bbp (an allelic variant of SdrE) (Tung et al. 2000); the plasmin-sensitive protein Pls (Savolainen et al. 2001; Huesca et al. 2002), and biofilm-associated protein (Bap) (Cucarella et al. 2001, 2002). These proteins have features characteristic of gram-positive bacterial surface-expressed proteins, including a secretory signal sequence at the N-terminus, and at the C-terminus a positively charged tail, a hydrophobic transmembrane domain and a wall-spanning region with an LPXTG (Leu-Pro-X-Thr-Gly) motif. The latter represents the target of a specific membrane-associated enzyme (sortase, SrtA) which cleaves the motif between the threonine and glycine residues. The protein is then covalently anchored to the peptidoglycan cell wall (Schneewind et al. 1995; Ton-That et al. 1999; Mazmanian et al. 1999; Ilangovan et al. 2001). A second sortase (SrtB) has been described which is required for anchoring a surface protein with a NPQTN motif. The gene encoding SrtB is part of an iron-regulated locus called iron-responsive surface determinants (*isd*), which also contains a ferrichrome transporter and surface proteins with NPQTN and LPXTG motifs (Mazamnian et al. 2002). Two cell wall proteins

designated FrpA and FrpB have also been reported to be expressed under iron-restricted growth conditions. FrpA may be involved in adhesion of *S. aureus* to plastic in vitro, and both proteins are regulated by the ferric uptake regulator Fur (Morrissey et al. 2002).

S. aureus also expresses bacterial adhesins that lack an LPXTG motif. These include Efb (formally Fib) (Boden and Flock 1994), the elastin-binding protein EbpS (Park et al. 1996; Downer et al. 2002), the major histocompatibility complex class II analog Map and the highly similar extracellular adhesin protein Eap (McGavin et al. 1993; Jönsson et al. 1995; Palma et al. 1999), and Ehb which is a very large cell wall-associated fibronectin binding protein (Clarke et al. 2002). Localization studies have shown Ebh to be cell envelope associated, and is proposed to form a specialized surface structure involved in cellular adhesion (Clarke et al. 2002).

These proteins are variably present in *S. aureus* isolates associated with carriage and disease. For example, some are ubiquitous (or almost so); this includes FnBPA, ClfA, ClfB, SdrC, SpA, and Map/Eap, while Bbp, SdrD, and SdrE are present in less than half of isolates (Peacock et al. 2002a). They are often named in response to the first identified ligand, but it is becoming clear that many proteins are multifunctional. Furthermore, there is considerable redundancy both within a given protein, (for example, FnBPA has multiple binding sites for fibronectin (Massey et al. 2001)), and for the bacterium as a whole which has multiple mechanisms for adhering to host proteins such as fibrinogen and fibronectin. This is likely to reflect the biological importance to the bacterium of adhering to, or becoming coated with, and perhaps masked by, host proteins. The observation that FnBPA has many ligand binding sites may be explained by the mechanism by which *S. aureus* interacts with fibronectin, in which attachment occurs via a tandem zipper (Schwarz-Linek et al. 2003).

Protein expression is influenced by at least three major regulatory systems, termed the accessory global regulator (Agr), staphylococcal accessory regulator (Sar) and Sae (Novick 2000). Other factors can affect expression; for example, a *hem*B mutant defective in hemin biosynthesis and exhibiting a small colony variant phenotype was reported to have increased expression of genes encoding ClfA and fibronectin binding proteins (Vaudaux et al. 2002). FnBP expression is also altered in vitro on exposure to subinhibitory ciprofloxacin (Bisognano et al. 2000). Clinical isolates with grlA and gyrA mutations (encoding altered topoisomerase IV and DNA gyrase, respectively) exhibited significant increases in attachment to fibronectin-coated surfaces after growth in the presence of one-quarter the ciprofloxacin (MIC) of ciprofloxacin. This phenomenon may be explained by the observation that ciprofloxacin activates the *fnbB* promoter.

Coagulase-negative staphylococci also express surface adhesins, although fewer have been described to date. *S. epidermidis* SdrF, SdrG, and SdrH are members of the Sdr family of proteins (McCrea et al. 2000). SdrF and SdrG are cell wall-associated proteins as determined by the presence of an LPXTG motif, but Sdr H lacks this motif. SdrG (Fbe) is a fibrinogen binding adhesion molecule (Hartford et al. 2001), but the ligands for the other two proteins are unknown. A fibronectin-binding protein has also been described for *S. epidermidis* (Williams et al. 2002). *S. schleiferi* and *S. lugdunensis* bind to fibrinogen and fibronectin, although the adhesins have not been fully characterized to date (Hebert 1990; Vandenesch et al. 1994).

Exocellular material

Staphylococcal capsules are located external to the cell wall and they are primarily composed of antigenic (capsular) polysaccharides (Sutherland 1977). The capsular polysaccharides of *S. aureus* are made up of different combinations of either amino uronic acids (*N*-acetyl-D-aminoglucuronic acid, *N*-acetyl-D-aminogalacturonic acid, or *N*-acetyl-D-aminomannuronic acid) or nonamidated uronic acids (glucuronic acid or galacturonic acid), and *N*-acetyl-D-fucosamine or galactose, depending upon the particular strain (Wilkinson 1983). Some *S. aureus* strains also contain taurine in their capsule which is linked to the carboxyl group of *N*-acetyl-D-aminogalacturonic acid via an amide bond (Liau and Hash 1977; J.C. Lee et al. 1987). *S. aureus* strains can be assigned to at least 11 capsular serotypes by means of polyclonal rabbit antiserum specific for their associated capsular polysaccharides. Of these, serotypes 5 and 8 are associated with microcapsules and are the ones most frequently encountered in bacteremia (Arbeit et al. 1984) and a variety of other infections (Hochkeppel et al. 1987; Albus et al. 1988). Highly encapsulated *S. aureus* isolates of serotypes 1 and 2 are more virulent in mouse lethality tests than isolates of serotypes 5 and 8 (J.C. Lee et al. 1987, 1993). Furthermore, they are relatively resistant to phagocytosis, are not phage typable, and have negative clumping factor reactions and diminished coagulase reactions. The capsule acts in varying degrees as a diffusion barrier, molecular sieve, and adsorbant. Phagocytosis may be prevented by the inability of encapsulated staphylococci to attach to phagocytic cells.

Slime is a complex extracellular substance produced in varying amounts by many, if not most, staphylococci. The amounts of slime produced by a particular strain may vary widely according to genetic factors and growth conditions. Many strains of *S. epidermidis* and some strains of *S. capitis* subsp. *ureolyticus* produce copious amounts of this substance (Peters et al. 1987; Kloos et al. 1992). Other members of the *S. epidermidis* species group such as *S. capitis* subsp. *capitis*, *S. hominis*, *S. haemolyticus*, and *S. warneri* generally produce much

smaller amounts of slime, and although many strains of these species may fail to produce detectable quantities of slime by standard tests (Christensen et al. 1985), it can usually be detected by transmission electron microscopy. Slime appears to be rather loosely bound to the cell and is water-soluble. It can be observed trailing from the cell surface and also detached from the cell. Continued production of slime by a growing clone of cells attached to a polymer surface (e.g. an implanted catheter) results in encasement and formation of connective cell-slime clusters (biofilm) (Peters et al. 1987). Once established, the bacterial biofilm may act as a penetration barrier to antibiotics (Gristina et al. 1987; Peters et al. 1987). Crude slime is a very heterogeneous substance, which is usually composed of a variety of monosaccharides, including mannose, galactose, glucose, glucosamine, and glucuronic acid, and proteins and small peptides, the chemical composition of which may vary according to the medium used for cell propagation. Under some growth conditions, components of teichoic acids may be present in slime (Hussain et al. 1991). Some strains of *S. epidermidis* and *S. capitis* subsp. *ureolyticus*, especially those from foreign body infections or demonstrating a long-term persistence on their host, produce a slime containing PS/A that is rich in galactose (Tojo et al. 1988; Kloos et al. 1992; Muller et al. 1993). PS/A appears to enhance the very early stages of colonization of these species on biomaterials and perhaps also on the surfaces of their natural habitat. It is highly immunogenic in its purified form and may play a role in protective immunity. Somewhat similar polysaccharide antigens have been described that appear to be important for the surface proliferation of cells (Christensen et al. 1990; Mack et al. 1992). Most *S. epidermidis* strains can be serotyped and belong to either polysaccharide surface antigen type 1 (14 percent) or type 2 (77 percent) (Fattom et al. 1992). There is growing evidence that slime interferes with host defense mechanisms such as opsonization and phagocytosis (Gray et al. 1987; Johnson et al. 1989; Stout et al. 1992). The material which embeds sessile bacteria adherent to prosthetic surfaces is now commonly referred to as biofilm. The mechanism by which coagulase-negative staphylococci (CoNS) attach to prosthetic material and elaborate biofilm is being increasingly understood, and is clearly a complex and multistep process (reviewed in Mack 1999 and von Eiff et al. 1999, 2002). One important element in this pathway is the *ica* operon, a gene cluster encoding the production of PIA which mediates intercellular adherence of bacteria and the accumulation of multilayered biofilm (Heilmann et al. 1996).

Extracellular proteins

Staphylococci produce a variety of different extracellular proteins acting as either toxins, nontoxic enzymes, or enzyme activators (Table 5). The toxins can attack or regulate cellular components of the host, thus presumably facilitating the successful invasion and proliferation of staphylococci within the host. Some of the nontoxic enzymes and polypeptides provide staphylococci with an ability to survive or defend themselves in the presence of competing microorganisms. Other enzymes are capable of degrading various macromolecules such as lipids, nucleic acids, proteins, and polysaccharides, thereby providing low molecular weight nutrients for growth. Most of the extracellular proteins that have been investigated in detail have been derived from *S. aureus*, a species that produces a wide range of such proteins.

The hemolytic toxins (hemolysins) attack the membranes of erythrocytes, as well as some other cells, from various host species. *S. aureus* produces at least four types of hemolysins known as alpha-, beta-, delta-, and gamma-toxin. Alpha-toxin has been studied extensively, and the wide range of information obtained on its structure and function has been reviewed by Bhakdi and Tranum-Jensen (1991). A high percentage (86–95 percent) of human *S. aureus* strains produce alpha-toxin, although many produce only small amounts. Alpha-toxin-defective mutants have reduced virulence in animal models compared with the wild-type parental strain (Foster et al. 1990). Beta-toxin has species-dependent activity, with goat, cow, and sheep erythrocytes being most sensitive, human erythrocytes having intermediate sensitivity, and dog and mouse erythrocytes being resistant (Bohach and Foster 2000). Around 85 percent of human strains are positive. The gene encoding beta-toxin carries the attachment site for group F-converting phages and is disrupted by prophage integration in lysogens (Coleman et al. 1986). Delta-toxin is produced by a high percentage (86–97 percent) of *S. aureus* strains of human and animal origin. Gamma-toxin and Panton–Valentine leukocidin (PVL) are bicomponent toxins encoded by the *hlg* and *luk-PV* loci, respectively (Bohach and Foster 2000). The toxins contain two synergistically acting proteins, one S component (HlgA, HlgC, or LukS-PV), and one F component (HlgB or LukF-PV). Any S component can combine with either F component, leading to six combinantions in strains that carry both loci (less that 2 percent of human isolates), or two combinations in strains positive for *hlg* alone (>95 percent). Gamma-toxin is strongly hemolytic but weakly leukocytic, while PVL can lyse neutrophils and macrophages. However, the effect of a given toxin will be modulated depending on the component pairing. For example, Luk-PV paired with HlgA or HlgC results in a toxin that is weakly hemolytic but which has strong leukocytic activity. The recently described community-acquired methicillin-resistant strains (CA-MRSA) commonly carry the PVL locus (Vandenesch et al. 2003); this is likely to result in a rise in the proportion of *S. aureus* positive for PVL.

Table 5 *Staphylococcal extracellular proteins*

Protein	Biological activities	Gene	Gene location	Species distribution	References
Membrane-active toxins					
Alpha-toxin	Cytolytic pore-forming toxin	*hla*	Chromosome	*S. aureus*	Bhakdi and Tranum-Jensen 1991
Beta-toxin	Neutral sphingomyelinase Acts as a type C phosphatase, hydrolyzing sphingomyelin to phosphorylcholine and ceramide	*hlb*	Chromosome	*S. aureus* *S. intermedius*	Doery et al. 1965; Smyth et al. 1975; Coleman et al. 1989
Delta-toxin	Surfactant on various cells: erythrocytes, leukocytes, bacterial protoplasts	*hld*	Chromosome	*S. aureus* *S. epidermidis* *S. haemolyticus*	Turner and Pickard 1979; Fitton et al. 1980; Gemmell and Thelestam 1981; Scheifele et al. 1987
Gamma-toxin	Bicomponent toxin Cytolytic for erythrocytes and leukocytes. Can form active combinations with Panton–Valentine leucocidin components	*hlgA*, *hlgB*, and *hlgC*	Chromosome	*S. aureus*	Fackrell and Wiseman 1976; Cooney et al. 1988; Prevost et al. 1995
Panton–Valentine leucocidin	Cytolytic for leukocytes	*lukS-PV* and *lukF-PV*	Chromosome	*S. aureus*	Noda et al. 1980; Rahman et al. 1991; Prevost et al. 1995
Pyrogenic toxin superantigens					
Enterotoxins SEA-SEE, and SEG-SEQ	Ingestion of preformed toxin in food results in nausea, vomiting and diarrhea	*sea* – see seg – seq	Varies between enterotoxins	*S. aureus*	Reviewed by Dinges et al. 2000
Toxic shock syndrome toxin-1 (TSST-1)	Toxic shock syndrome Mitogenic activity for T cells	*tst*	Accesory genetic element	*S. aureus*	Schlievert et al. 1981; Kreiswirth et al. 1993; Chu et al. 1988
Exfoliative toxins					
Exfoliative toxin A (ETA)	Staphylococcal scalded skin syndrome Causes intra-epidermal blisters Has superantigen activity	*eta*	Chromosome	*S. aureus*	Melish and Glasgow 1970; Rogolsky 1979; C.Y. Lee et al. 1987; Smith et al. 1989; Dancer et al. 1990
Exfoliative toxin B (ETB)	Staphylococcal scalded skin syndrome Causes intra-epidermal blisters Has superantigen activity	*etb*	Plasmid	*S. aureus*	Jackson and Iandolo 1986; O'Toole and Foster 1986; C.Y. Lee et al. 1987

(*Continued over*)

Table 5 *Staphylococcal extracellular proteins (Continued)*

Protein	Biological activities	Gene	Gene location	Species distribution	References
Fibrin-forming and fibrinolytic enzymes					
Staphylocoagulase (coagulase)	Clots plasma in the absence of Ca^{2+} Coagulase reacts with coagulase-reacting factor (CRF) and the resulting complex (staphylothrombin) converts fibrinogen to fibrin	*coa*	Chromosome	*S. aureus* *S. intermedius* *S. hyicus* *S. delphini* *S. schleiferi* subsp. *coagulans*	Drummond and Tager 1963; Phonimdaeng et al. 1988, 1990
Staphylokinase (SAK)	Binds to plasminogen and activates it to become the fibrinolytic enzyme plasmin	*sak*	Temperate phage phi 13	*S. aureus*	Papke and Blobel 1978; Sako and Tsuchida 1983; Iandolo et al. 2002
Bacteriolytic enzymes					
Endo-β-*N*-acetylglucos-aminidases	Bacterial cell lysis Cleaves β1–4 glucosaminidic bonds of *Bacillus* and *Micrococcus* cell wall peptidoglycan Interferes with phagocytosis			*S. aureus* Many species of staphylococci	Wadström and Hisatsune 1970; Varaldo and Satta 1978; Valisena et al. 1982; Guardati et al. 1993
Lysostaphin endopeptidase (lysostaphin)	Staphylococcal cell lysis Cleaves glycyl–glycyl bonds of interpeptide bridge of staphylococcal cell wall peptidoglycan Zinc metalloenzyme	*end*	Plasmid	*S. simulans*: biovar staphylolyticus	Schindler and Schuhardt 1964; Sloan et al. 1982; Heath et al. 1987; Recsei et al. 1987
Hydrolytic enzymes					
Lipase	Hydrolyzes triglyceride,Tweens, phosphatidylcholines, lysophospholipids	*geh*	Chromosome	*S. hyicus* *S. aureus* Many other species	Götz et al. 1985; Lee and Iandolo 1986, 1988; van Oort et al. 1989
Staphylococcal nuclease (thermonuclease)	Hydrolyzes RNA and DNA to 3′-phosphomono-nucleotides Phosphodiesterase requiring Ca^{2+}	*nuc*	Chromosome	*S. aureus* *S. intermedius* *S. hyicus* *S. schleiferi*	Anfinsen et al. 1971; Davis et al. 1977; Shortle 1983
Urease	Hydrolyzes urea to ammonia and carbamate Nickel requirement Plays role in invasiveness of *S. saprophyticus* in urinary tract	*ure*	Chromosome	*S. saprophyticus* *S. aureus* *S. xylosus* Many other species	Gatermann et al. 1989; Jose et al. 1991; Jose et al. 1994; Schafer and Kaltwasser 1994

(*Continued over*)

Table 5 *Staphylococcal extracellular proteins (Continued)*

Protein	Biological activities	Gene	Gene location	Species distribution	References
Hyaluronate lyase (hyaluronidase)	Hydrolyzes hyaluronate at 1–4 hexosaminidic bonds Aids in invasiveness by breakdown of hyaluronate-rich tissue barriers	*hysA*	Chromosome	*S. aureus* *S. hyicus*	Rautela and Abramson 1973; Farrell et al. 1995
Proteases	Staphylococcal serine protease (V8 protease; SspA) Cysteine protease (SspB) Metalloprotease (aureolysin; Aur) Staphopain (Scp) Cleave and degrade host proteins	*sspA* *sspB* *aur* *scp*	Chromosome	*S. aureus*	Drapeau 1978; Karlsson and Arvidson 2002

S. aureus produces a group of functionally related pyrogenic toxins that cause fever and shock in their hosts. These toxins are classified as superantigens (SAg) and include the enterotoxins (SE) and TSST-1, which have in common a potent mitogenic activity for T lymphocytes of several host species (Iandolo 1989; Fleischer 1994). Exfoliative toxins are also reported to have superantigenic activity. Positivity of human isolates for the genes enocoding enterotoxins varies between toxins; for example, 23, 8, and 60 percent of 333 human isolates tested in one study were positive for *sea*, *seb*, and *seg*, respectively (Peacock et al. 2002a). TSST-1 causes approximately 75 percent of all staphylococcal TSS cases (Bergdoll et al. 1981; Schlievert et al. 1981). Approximately 90 percent of *S. aureus* strains isolated from the vagina of patients with TSS produce TSST-1. The incidence of TSST-1 is much lower (in the region of 25 percent) in *S. aureus* strains isolated from other types of patients or healthy individuals, and it has not been detected in other staphylococcal species. The *S. aureus* epidermolytic toxins (ET) known as exfoliatin A (ETA) and exfoliatin B (ETB) are recognized as the cause of staphylococcal scalded skin syndrome (SSSS), a disease associated with severe blistering of the skin, especially in young children (Rogolsky 1979). ETs are produced by a low percentage of *S. aureus* strains (14 percent and 2 percent for *eta* and *etb*, respectively, in one study) (Peacock et al. 2002). Staphylococcal superantigens are encoded by accessory genetic elements (reviewed in McCormick et al. 2001 and Novick et al. 2001). The first five to be described (SapI 1–4 and SapI bov) are pathogenicity islands that each encode one or more of the superantigens (Novick et al. 2001). A further six novel pathogenicity islands have been identified in the genome sequences of two *S. aureus* isolates (N315 and Mu50) (Kuroda et al. 2001).

Coagulase clots plasma in the absence of Ca^{2+} but requires a plasma constituent known as coagulase-reacting factor (CRF). CRF, perhaps a derivative of prothrombin, reacts with coagulase, and the resulting coagulase–CRF complex (staphylothrombin) converts fibrinogen to fibrin (Drummond and Tager 1963). A very high percentage (98–99 percent) of *S. aureus* strains exhibit coagulase activity. Coagulase activity is also exhibited by the species *S. intermedius*, *S. hyicus*, *S. delphini*, and *S. schleiferi* subsp. *coagulans*. Staphylokinase (SAK), a protein that exhibits fibrinolytic activity indirectly by binding to plasminogen, is produced by a relatively high percentage (60–95 percent) of human *S. aureus* strains of biotype A, but by only a small percentage (5–10 percent) of bovine *S. aureus* strains of biotype C (Hájek and Maršálek 1969).

Various lipases (and esterases) are produced by staphylococci (Saggers and Stewart 1968; Zimmerman and Kloos 1976). Staphylococcal lipases hydrolyze a wide range of substrates, including both water-soluble and water-insoluble triglycerides, as well as oxyethylene-sorbitan fatty acids (Tweens), phosphatidylcholines, and lysophospholipids (Rollof et al. 1987; van Oort et al. 1989). Both heat-stable and heat-labile nucleases are produced by staphylococci. A large percentage (95–100 percent) of the strains of *S. aureus*, *S. hyicus*, *S. intermedius*, and *S. schleiferi* produce detectable levels of the heat-stable nuclease (or thermonuclease) (Devriese et al. 1978; Freney et al. 1988). Some staphylococcal species exhibit proteolytic activity on casein and gelatin, especially *S. aureus* (Abramson 1972), *S. sciuri* (Kloos et al. 1976) and *S. hyicus* (Devriese et al. 1978). *S. aureus* produces four major extracellular proteases (Karlsson and Arvidson 2002). These are: staphylococcal serine protease (V8 protease; SspA), cysteine protease (SspB), metalloprotease (aureolysin; Aur), and staphopain (Scp). All four are secreted as proenzymes which become proteolytically cleaved to become mature enzymes. Synthesis is activated by the regulator *agr* and suppressed by *SarA*. A survey of 92 clinical isolates demonstrated that protease genes appear to be conserved, but the level of protease production is determined by the level of SigB-dependent expression of protease repressor *sarA* (Karlsson and Arvidson 2002). Their role in human disease pathogenesis is unknown. Urease activity is exhibited by many staphylococcal species, and it apparently plays a role in the invasiveness of *S. saprophyticus* in the urinary tract (Gatermann et al. 1989). Hyaluronate lyase (hyaluronidase) is a glycoprotein produced by *S. aureus* and *S. hyicus* that hydrolyzes the mucopolysaccharide hyaluronate (Rautela and Abramson 1973).

Gene regulation

S. aureus gene expression is under the control of regulatory systems that respond to changes in environmental conditions. This is reflected by gene expression of *S. aureus* grown in vitro. As a generalization, synthesis of surface proteins occur very early in exponential phase; these are downregulated at the transcriptional level later in the exponential phase. This is followed in the post-exponential phase by the transcription of a large number of genes encoding extracellular proteins, synthesis of which ceases in stationary phase. These observations can be explained by the coordinated action of several regulatory systems. The best characterized of these are Agr (accessory global regulator) and Sar (staphylococcal accessory regulator), with a third regulator termed Sae (Novick 2000). Readers are referred to publications by Richard Novick and colleagues, and Ambrose Cheung and colleagues for indepth bodies of work on Agr and Sar, respectively. The *agr* locus consists of two divergent transcription units driven by promoters P2 and P3. The four genes within the P2 operon are termed *agrB*, *D*, *C*, and *A*; all are necessary for transcriptional activation of the *agr* system and activate the two promoters P2 and

P3. The P3 transcript is RNAIII, the effector of gene regulation which acts predominantly at the level of gene transcription. This is a density-sensing system which is autoinduced by a short peptide processed from a propeptide encoded by *agrD*. Four *agr* groups have been defined based on nucleotide differences, and the autoinducing peptides have been demonstrated to exhibit crossinhibitory responses. Many of the secreted exoproteins are downregulated and the cell surface proteins upregulated in *agr* mutants. *S. lugdunensis* also produces an RNAIII-like transcript; it is produced from the *lgr* locus (Vandenesch et al. 1993). The RNAIII transcripts from *S. epidermidis*, *S. warneri*, and *S. simulans* are very similar to that of *S. aureus*, and they can equally repress transcription of protein A in *S. aureus*; however, their ability to stimulate transcription of the α-toxin and serine protease genes in *S. aureus* was less than that of *S. aureus* RNAIII (Tegmark et al. 1998). The mode of action of *sar* appears to be at the transcriptional level (Cheung and Ying 1994). The *sar* locus upregulates transcription of *agr*, and also affects the expression of certain exoprotein genes directly. A large body of data exist on regulation of *S. aureus* gene expression in vitro. Regulation in vivo is poorly defined, but studies addressing this area indicate that regulation does not always mirror that seen under laboratory conditions (Goerke et al. 2000).

Bacteriocins

Lantibiotics are low molecular weight bacteriocins produced by a wide range of gram-positive bacteria including staphylococci, lactococci, bacilli, and streptomycetes (Jack et al. 1995). They are small polypeptides of 19–34 amino acids and have several sulfide rings consisting of two unusual amino acids, *meso*-lanthionine and 3-methyllanthionine (Schnell et al. 1988). Epidermin (staphylococcin 1580) and Pep5 are lantibiotics produced by *S. epidermidis* (Allgaier et al. 1986; Kellner et al. 1991). The 52 amino acid epidermin is encoded by the gene *epiA* contained within an operon located on a 54 kbp plasmid (Schnell et al. 1988, 1992). The epidermin operon contains a minimum of six genes (*epiABCD* and *epiQ*, *epiP*). The epiP gene product is homologous to serine proteases and may be involved in the cleavage of the 30 amino acid leader from the epidermin prepeptide molecule. *pepA* encodes the 60 amino acid Pep5 and is contained within a gene cluster (*pepI*, *pepT*, ORF X and *pepA*, *pepP*, *pepB*, *pepC*) found on a plasmid (Kaletta et al. 1989; Reis et al. 1994). The *pepI* gene product is essential for Pep5 immunity. Gallidermin is produced by *S. gallinarum* (Kellner et al. 1988). The activity of these lantibiotics is directed mainly against gram-positive bacteria, many of which interact or compete with staphylococci in their natural habitats. These lantibiotics depolarize bacterial and planar lipid membranes in a voltage-dependent manner and they can also form pores (up to 1 nm in diameter) in the membrane (Kordel et al. 1988). Some strains of *S. haemolyticus* produce one or more small, 44 amino acid peptides (sharing 65–75 percent sequence homology) that exhibit antibacterial activity, especially antigonococcal activity (Frenette et al. 1984; Watson et al. 1988).

GENETIC MECHANISMS

Genetic exchange mechanisms

Transduction has been demonstrated in both laboratory and natural populations of *S. aureus* (Novick and Morse 1967; Richmond and Lacey 1973). Transduction methods were used for the fine structure mapping of amino acid biosynthesis genes (Kloos and Pattee 1965; Smith and Pattee 1967; Proctor and Kloos 1970; Barnes et al. 1971; Pattee et al. 1974). The generalized transducing phages of *S. aureus* belong to the serological group B (Pattee and Baldwin 1961). The size of the chromosomal DNA packaged into the phage head is similar to that of the phage DNA (Beryhill and Pattee 1969). Transducing DNA fragments appear to be highly uniform in size (Kloos and Pattee 1965; Pattee et al. 1968). Small plasmids of *S. aureus* (1–5 kbp) may be transduced by being packaged in a phage head as a phage genome-sized plasmid concatemer, whose formation requires plasmid-specific initiation of replication (Novick et al. 1986). The plasmid concatemer is processed to the monomeric form in the recipient. The cotransduction of different plasmids can occur via a stable cointegrate formed between the plasmid DNAs, which is apparently not resolved in the recipient cell (Novick et al. 1981). The cointegration involves recombination at specific sites on certain small class I plasmids (Novick et al. 1984). The larger class II plasmids may also be transduced, though plasmid replication is not required (Novick 1989). Transduction of plasmids and their maintenance are affected by the host restriction–modification system, which may limit the distribution of plasmids in staphylococcal communities to some extent. Transduction of chromosomal markers and plasmid DNA has also been demonstrated in *S. epidermidis* (Olson et al. 1979).

Transformation of chromosomal and plasmid markers was first described in *S. aureus* strain NCTC 8325 by Lindberg and coworkers (1972). The ability of this strain to become competent for transformation (and transfection) depends on the presence of high concentrations of Ca^{2+} and the presence of certain bacteriophages of serotype B (e.g. ϕ11, ϕ14, 83A, 80α) (Rudin et al. 1974; Thompson and Pattee 1981). The involvement of bacteriophage 80α in conferring competence is believed to be due to an interaction of externally supplied phage components with the surface of the cell (Birmingham

and Pattee 1981). Transformation has played a major role in determining the genetic map of the chromosome of *S. aureus* strain NCTC 8325 (Pattee 1993). Transformation by plasmid DNA occurs at a low frequency (10^3–10^4 transformants/μg plasmid DNA), and it is lower for plasmids than for chromosomal markers (Lindberg et al. 1972; Lindberg and Novick 1973). The transformation frequency is greatly influenced by the restriction–modification system of the recipient cell. Heterologous plasmids (isolated from a different *S. aureus* strain or species) are associated with very low transformation frequencies (Sjöström et al. 1975).

Relatively high transformation frequencies (10^6 transformants/μg plasmid DNA) can be obtained with protoplast transformation of *S. carnosus* by plasmid DNA (Götz and Schumacher 1987). Protoplast transformation is inefficient for *S. epidermidis* and other species that are not very susceptible to lysostaphin. Transformation of *S. aureus*, *S. epidermidis*, *S. carnosus*, and *S. simulans* by plasmid DNA has been accomplished using electroporation techniques, which provide low to moderate transformation frequencies (10^3–10^5 transformants/μg plasmid DNA) (Augustin and Götz 1990).

Plasmid mediated conjugative transfer has been demonstrated both in laboratory and natural populations of *S. aureus*, *S. epidermidis*, *S. hominis*, and *S. haemolyticus*, and includes both intra- and interspecies transfer (Archer and Johnston 1983; Forbes and Schaberg 1983; Goering and Ruff 1983; McDonnell et al. 1983; Naidoo and Noble 1987). Most of the conjugative plasmids are class III plasmids that encode resistance to gentamicin and certain other aminoglycosides, such as tobramycin and kanamycin. Many also encode resistance to ethidium bromide and quaternary amines, whereas some also encode resistance to penicillin and trimethoprim. These plasmids are relatively large (30–60 kbp) and have a conjugative transfer (*tra*) region that is often flanked by directly repeated 900 bp IS*257* elements (Thomas and Archer 1989a). They are also capable of mobilizing or cotransferring certain smaller coresident plasmids which are not independently transferable. Class I plasmids that can form DNA–protein relaxation complexes can be mobilized, whereas nonrelaxable plasmids cannot be mobilized or can be mobilized only at a very low frequency (McDonnell et al. 1983; Projan and Archer 1989). Conjugative gentamicin-resistance plasmids from *S. aureus* and *S. epidermidis* strains isolated from different geographical regions in the USA are very closely related in their structure and may have had a clonal origin (Jaffe et al. 1982; Goering et al. 1985). Two other types of conjugative plasmids have been detected in *S. aureus* (Townsend et al. 1986; Udo et al. 1987, 1992). One of these appears to be unable to mobilize other plasmids and encodes for the production of a diffusible pigment, in addition to encoding resistance to one or more antibiotics. The other type is cryptic except that it is conjugative and can mobilize nonconjugative plasmids. Transposon-labeled derivatives of these plasmids have been transferred from *S. aureus* to *S. epidermidis* and *E. faecalis* and back to *S. aureus* (Udo and Grubb 1990).

Fusion of lysostaphin–lysozyme-prepared, staphylococcal protoplasts can be accomplished by treatment of the protoplast mixture with polyethylene glycol (Götz et al. 1981). By this method, plasmids can be transferred between strains of the same species and between strains of different species. In fusions between different species, the frequency of transfer is reduced somewhat from that of intraspecific fusions. A very low frequency of recombination of chromosomal genes by protoplast fusion has been demonstrated for strains of *S. aureus*.

Plasmids

Plasmids are facultative extrachromosomal genetic systems (elements) which are common in natural populations of most staphylococcal species (Lacey 1975; Kloos et al. 1981; Weinstein et al. 1982). They are rare (<2 percent of strains) in the species *S. auricularis* (Kloos 1990) and relatively uncommon (⩽20 percent of strains) in *S. schleiferi* and members of the *S. sciuri* species group (Kloos et al. 1981). The characteristics of many of the plasmids from *S. aureus* have been reviewed by Lyon and Skurray (1987) and Novick (1989). *S. aureus* plasmids have been classified into three general classes, I through III. Class I plasmids are of small size (1–5 kbp), have a high copy number (10–55 copies per cell), and usually encode a single antibiotic resistance or are cryptic. These plasmids are the most widespread throughout the genus *Staphylococcus*. Novick (1989) has subdivided class I plasmids into four subgroups or families on the basis of their nucleotide sequence and functional organization. Plasmids in this class are largely composed of cassettes or specific segments that are not transposons. They represent at least ten different incompatibility groups and replicate by an asymmetric rolling-circle mechanism (Ruby and Novick 1975; Iordănescu and Surdeanu 1980). The pT181 family is made up of a group of small (4–4.6 kbp) plasmids that usually encode tetracycline or chloramphenicol resistance, and is defined by homologous minimal replicons, consisting of an initiator (Rep) protein cistron containing the leading-strand replication origin (Novick 1989). All major transcription units are codirectional with replication. Tetracycline-resistance plasmids belonging to this family are highly conserved and are very common in a wide range of staphylococcal species (Kloos et al. 1981; Cooksey and Baldwin 1985; Wells and Kloos 1987). The pC194 family contains plasmids with homologous Rep protein cistrons and replication origins, though the functional organization of plasmids and their phenotype varies considerably. Some members of this family are very closely related to certain *Bacillus* plasmids (Polak and

Novick 1982; Muller et al. 1986) and *E. faecalis* plasmids (Perkins and Youngman 1983). The pSN2 family plasmids have a direction of replication that appears to be opposite that of the Rep protein coding frame. Most plasmids of this family encode erythromycin resistance and contain the recombination site RS_A. Some, such as pSN2, are cryptic. The erythromycin-resistance plasmids belonging to this family are usually 2–2.5 kbp in size and except for variation in the control region of the *ermC* gene, are highly conserved (Iordănescu and Surdeanu 1980; Lampson and Parisi 1986; Catchpole et al. 1988; Kloos et al. 1992). They, like the tetracycline-resistance plasmids of the pT181 family, are very common in a wide range of staphylococcal species. A naturally occurring plasmid found in *B. subtilis* is a member of this family (Projan et al. 1987). The pE194 family is currently represented by the 3.7 kbp plasmid pE194 of *S. aureus* that contains an *ermC* gene, which is nearly identical to the *ermC* of the pSN2 family, but the plasmid functional organization is similar to certain pT181 family plasmids. Somewhat similar erythromycin-resistance plasmids have also been isolated from members of the *S. saprophyticus* species group.

Class II plasmids of *S. aureus* have been subdivided into two major families on the basis of incompatibility (Novick and Richmond 1965). These plasmids, commonly referred to as penicillinase or β-lactamase plasmids, are relatively large (15–33 kbp), have a low copy number (4–6 per cell), and replicate using the θ replication mechanism (Sheehy and Novick 1975). They carry various combinations of antibiotic and heavy metal resistance genes, many of which are located on transposons (Shalita et al. 1980; Lyon and Skurray 1987). Class II plasmids have been identified in many other staphylococcal species but, unlike class I plasmids, they are very uncommon in the species *S. hyicus*, *S. intermedius*, *S. simulans*, and *S. lugdunensis* (Kloos et al. 1981; Götz et al. 1983; Etienne et al. 1990). Class III plasmids include the large conjugative plasmids discussed above. Like class II plasmids, they appear to be assemblages of transposons and transposon remnants (Gillespie et al. 1987).

Bacteriophages

For several decades since its inception in 1952–1953, typing of *S. aureus* strains by an official International Basic Set of phages found wide use as an established method for epidemiology (Williams and Rippon 1952). By 1970, it became apparent that the Basic Set could not type *S. aureus* strains isolated from a wide range of animal species, so it was redesigned and limited to a Basic Set of Typing Phages for Human Strains of *S. aureus*, which could identify many strains from human sources as belonging to phage groups I, II, III or V. A separate set of typing phages was established for bovine strains. Some unofficial sets of *S. epidermidis* phages have been used for the typing of *S. epidermidis* strains (Verhoef et al. 1971; Pulverer et al. 1976). The use of phage typing in epidemiology is now being reevaluated in the light of the newer molecular typing methods (Bannerman et al. 1995). In general, phage typing appears not to be as discriminatory or as reproducible as PFGE, ribotyping or multilocus sequence typing. Most strains of *S. aureus* and *S. epidermidis* (and probably many other species) are multiply lysogenic and the phages can usually be induced by UV and mitomycin C (Verhoef et al. 1971a; Pulverer et al. 1976). The temperate phages of *S. aureus* can be subdivided into three main serological groups, A, B, and C. Serological group B contains most of the known transducing phages and phages that can confer competence in transformation. Members of this group exhibit considerable DNA sequence homology (Inglis et al. 1987). The group B transducing phage ϕ11 has been studied extensively (Löfdahl et al. 1981; Chapple and Stewart 1987). It has a 45 kbp genome that is circularly permuted and terminally redundant with a blunt end. Examples of lysogenic conversion include the prophage carriage of genes encoding SEA and SAK. The phage ϕ11 genome has also been shown to acquire the transposon Tn*554* from its primary chromosomal site during lytic infection. It may transfer Tn*554*, via transduction, directly or through a plasmid intermediate, to the chromosome of the recipient cell (Murphy et al. 1981). Phage S1 can exhibit recombination with the tetracycline-resistance gene of a class I plasmid, producing a hybrid phage–plasmid element (Inoue and Mitsuhashi 1976). Negative lysogenic conversion has been demonstrated for the *geh* (Lee and Iandolo 1986) and *hlb* (Coleman et al. 1986) genes, which contain phage attachment sites within their sequence. Several *S. epidermidis* (Verhoef 1971a, b) and *S. saprophyticus* phages have been characterized, but not to the extent of the *S. aureus* phages.

S. aureus genome sequence

The genome of *S. aureus* is around 2.8 Mb and contains in the order of 2 500 genes. Whole genome sequences of *S. aureus* were first reported in the published literature in 2001 (Kuroda et al. 2001). Two related *S. aureus* strains (N315 and Mu50) were sequenced; N315 is a MRSA strain isolated in Japan in 1982, and Mu50 is a MRSA strain with intermediate-level resistance to vancomycin isolated in Japan in 1997. An important observation arising from this study was that many genes seem to have been acquired by lateral gene transfer, and that most of the antibiotic resistance genes were carried either by plasmids or by mobile genetic elements including a unique resistance island. Three classes of new pathogenicity islands were identified in the genome; a toxic-shock-syndrome toxin island family, exotoxin

islands, and enterotoxin islands. The analysis also identified 70 candidates for new virulence factors. Sequencing of strain MW2 was subsequently undertaken by the same research group (Baba et al. 2002). This strain represented a virulent community-acquired MRSA isolated from a 16 month old girl in the USA with fatal septicemia and septic arthritis. The genome of this strain was compared with that for N315 and Mu50. The methicillin resistance gene (*mecA*) in MW2 was carried by a novel allelic form (type IVa) of staphylococcal cassette chromosome *mec* (SCC*mec*), by contrast with type II in N315 and Mu50. Type IVa SCC*mec* did not carry any of the multiple antibiotic resistance genes reported in type II SCC*mec*. Nineteen additional virulence genes were recorded in the MW2 genome, all but two of which were carried by one of the seven genomic islands of MW2. Sequencing of two further islolates by The Wellcome Trust Sanger Institute, UK, provided an opportunity to undertake comparative genomics for the five genomes. The strains used were an methicillin-sensitive *Staphylococcus aureus* (MSSA) which is phylogenetically close to MW2 (MSSA476), and a hospital-acquired representative of the epidemic methicillin-resistant E-MRSA 16 clone (MRSA252), which is phylogenetically divergent from any other sequenced strain. Comparison of the five *S. aureus* whole genome sequences showed that around 80 percent of the genome was highly conserved between strains. Genes in this core region were often 98–100 percent homologous at the amino acid level, and the gene order was conserved (Holden et al. 2004). In contrast, the remaining 20 percent of each sequenced genome consisted of highly variable genetic elements that spread horizontally between strains at high frequency. A total of 166 genes (6 percent) in the phylogenetically divergent MRSA252 genome were not found in the other published genomes. Publication of the genome sequence of two further strains (COL and 8325) are imminent. A DNA microarray giving more than 90 percent coverage of the *S. aureus* genome has been used to characterize and compare the genomes of 36 strains of divergent clonal lineages (Fitzgerald et al. 2001). Genetic variation was found to be very extensive, with approximately 22 percent of the genome being comprised of what was termed dispensable genetic material. Eighteen large regions of difference were identified, ten of which contained genes that encode putative virulence factors or proteins mediating antibiotic resistance.

The whole genome sequence of *S. epidermidis* ATCC 12228 was published in 2003 (Zhang et al. 2003). The genome consists of a single chromosome (approximately 2.5 Mb) and six plasmids. The chromosomal G + C content is 32.1 percent and 2 419 protein coding sequences are predicted, among which 230 are putative novel genes. This genome contains a lower number of putative virulence determinants compared with *S. aureus*. Toxins were apparently absent with the exception of delta-toxin and beta-toxin, although many adhesins were intact in ATCC 12228. The *ica* operon coding for the enzymes synthesizing intercellular adhesin is absent in ATCC 12228.

RESISTANCE TO ANTIMICROBIAL AGENTS

The rising level of resistance to a wide range of antibiotics by both *S. aureus* and CoNS represents a significant threat to future treatment efficacy. The various major antibiotic and heavy metal resistance mechanisms described for staphylococci are outlined in Table 6. The most significant events in recent medical history are the epidemic rise in methicillin resistance, the recognition of strains with intermediate resistance to vancomycin, and the emergence of *S. aureus* that is fully resistant to vancomycin.

Resistance of staphylococci to β-lactam antibiotics

Penicillin-resistant *S. aureus* strains began emerging shortly after the introduction of penicillin in medicine in the early 1940s. Today the percentage of penicillin-resistant strains has risen to 75–95 percent, with the highest rates being found among hospital strains. Similar percentages have been observed for penicillin-resistant strains of the *S. epidermidis* species group. Most penicillin-resistant staphylococcal strains produce β-lactamase which hydrolyzes the β-lactam ring of the antibiotic. β-Lactamase genes (*bla*) are most often found on class II plasmids that are distributed among many staphylococcal species (Baldwin et al. 1969; Archer and Scott 1991).

Resistance to methicillin and other β-lactamase-resistant penicillins was first observed in *S. aureus* soon after methicillin was introduced into clinical use in Britain (Jevons 1961). The methicillin-resistant strains isolated in Britain at that time came from hospitalized patients, were multiply antibiotic-resistant, belonged to phage group III, and their resistance to methicillin was heterogeneous, affecting only a minority of the cell population (Barber 1961). After the mid-1970s, large outbreaks of infection by MRSA were recorded in many hospitals in Australia (Pavillard et al. 1982), the USA (Schaefler et al. 1981), Britain (Shanson et al. 1976; Cookson and Phillips 1988), and Ireland (Cafferkey et al. 1983). Many of these outbreaks appear to have been caused by a single epidemic strain that was transferred between hospitals by the movement of patients (Duckworth et al. 1988). Many clones of MRSA have now been described throughout the world. A nomenclature has been developed to differentiate strains associated with epidemic spread (E-MRSA) from those associated with sporadic infections which do not appear to have the same degree of transmissibility. MRSA is now responsible for around 30 percent or more of all serious *S. aureus* infections,

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Penicillin (β-lactams)					
Penicillinase (A–D) (β-lactamase)	Inactivation of penicillin and cephalosporin by hydrolysis of the β-lactam ring	*blaZ*	Plasmids (mainly class II and III) >chromosome	*S. aureus*	Dyke 1979; Murphy and Novick 1979; Weber and Goering 1988; Wang et al. 1991
	A, B and C require induction by penicillin or its analogs		*blaI-blaR1-blaZ* region is often on transposon (Tn *552*, Tn *4002*; Tn *4201*)	*S. epidermidis* Most species of staphylococci	
β-Lactamase repressor BlaI	Transcriptional repressor for the system	*blaI*			
β-Lactamase antirepressor BlaR1	Receptor for β-lactams and the derepression of *blaZ*	*blaR1*			
Methicillin (β-lactamase resistant penicillins)					
Penicillin-binding protein PBP 2a (PBP2′)	Low-affinity penicillin-binding protein substituting activities of essential PBPs	*mecA*	PBP2a encoded by *mecA* which is part of a mobile genetic element termed staphylococcal cassette chromosome mec (SCC*mec*).	*S. aureus*	Sjöström et al. 1975; Hartman and Tomasz 1986; Mathews et al. 1990; De Lencastre and Tomasz 1994
	MIC range: 1.5–1 500 mg/ml			*S. epidermidis*	
	Variation not solely *mecA*, but also *fem* and other antibiotic response genes		Four types (I–IV) of SCC*mec* described to date	*S. haemolyticus* Several other species	
Methicillin-resistance repressor MecI	Transcriptional repressor for the system	*mecI*			
Methicillin-resistance MecR1 protein (or antirepressor)	Derepression of *mecA*BlaI and BlaR1 can substitute for MecI and MecR1	*mecR1*			
Aux (auxiliary) or Fem (factors essential for expression of methicillin resistance) (A–D)	A and B: biosynthesis of pentaglycine interpeptide bridge of peptidoglycan	*femA*, *femB*	Chromosome	*S. aureus*	Berger-Bächi et al. 1992; De Jonge et al. 1992, 1993; Ornelas-Soares et al. 1993; De Lencastre et al. 1994; Gustafson et al. 1994
	C: amidation of α-carboxyl group of D-glutamic acid in peptide subunits of the peptidoglycan	*femC*	Chromosome	*S. aureus*	
	D: controls rate of synthesis of unsubstituted disaccharide pentapeptide precursor	*femD*	Chromosome	*S. aureus*	

(*Continued over*)

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci (Continued)*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Macrolide, lincosamide, and streptogramin B (MLS)					
23S rRNA methylases (or methyl-transferases) A–C	N^6, N^6-dimethylation of adenine-2058 residues at peptidyl transferase center of 23S rRNA, resulting in a decreased affinity of 50S ribosomal subunit for MLS antibiotics	*ermA*	Chromosome: on transposon Tn *554* which prefers a single *att* site	*S. aureus* *S. epidermidis* Frequently found in *mecA* strains Several other species	Murphy 1985a; Murphy et al. 1985; Thakker-Varia et al. 1987
	Require induction by erythromycin or are made constitutively Regulation by translational attenuation	*ermB*	Plasmids and chromosome: on transposon Tn *551* which can transpose to many sites	*S. aureus* *S. intermedius* *S. hyicus* *S. xylosus* Several other genera	Novick et al. 1979; Khan and Novick 1980; Luchansky and Pattee 1984; Eady et al. 1993
		ermC	Plasmids (mainly class I) > chromosome	*S. aureus* *S. epidermidis* Most species of staphylococci *Bacillus subtilis*	Iordănescu and Surdeanu 1980; Horinouchi and Weisblum 1982; Weisblum 1985; Lampson and Parisi 1986; Kloos et al. 1992
Macrolide and streptogramin B (MS)	Plays a role as ATP-dependent efflux pump to remove MS antibiotics and requires a transmembrane complex				
ATP-dependent efflux protein MsrA (member of ATP-binding ABC cassette transporter superfamily)	Possibly inactivate macrolides	*msrA*	Chromosome and plasmids	*S. hominis*, *S. cohnii*, *S. epidermidis* Several other species	Jenssen et al. 1987; Ross et al. 1989, 1990; Eady et al. 1993; Wondrack and Sutcliffe 1995
Lincosamide					
3-Lincomycin, 4-clindamycin *O* -nucleotidyl-transferase	Inactivates lincosamides. Requires a nucleoside 5′-triphosphate as nucleotidyl donor and Mg^{2+} as cofactor	*linA*, *linA′* (*lin* gene group)	Plasmids (class I)	*S. haemolyticus* *S. aureus* *S. hominis* *S. cohnii* Several other species	Leclercq et al. 1985, 1987; Brisson-Noël et al. 1988; Kloos et al. 1992

(*Continued over*)

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci (Continued)*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Tetracycline					
Tetracycline efflux proteins (metal–tetracycline/H^+ antiporters)	Plays role as energy-dependent efflux pump to remove tetracycline	*tetK*	Plasmids (class I) > chromosome	*S. aureus*	Iordănescu et al. 1978; Kloos et al. 1981; Cooksey and Baldwin 1985; Wells and Kloos 1987
	Uses antiport mechanism of transport exchanging a proton for a metal–tetracycline complex			*S. epidermidis* Many other species of staphylococci *Bacillus subtilis*	
	Requires induction by very low amounts of tetracycline	*tetL*	Plasmids	*S. hyicus*	Schwarz et al. 1993
	Regulation by translational attenuation			*Bacillus* species	
Tetracycline and minocycline					
Ribosomal protection protein TetM	Permits productive binding of aminoacyl-tRNAs to ribosomes in the presence of tetracycline and minocycline	*tetM*	Chromosome Chromosome: Tn *916* and Tn *916* -like transposons	*S. intermedius* *S. aureus* Other gram-positive genera	Schaefler et al. 1976; Wells and Kloos 1987; Nesin et al. 1990; Burdett 1993; Schwarz et al. 1994
	Requires tRNA modification (miaA) activity				
	Homology with elongation factors G and Tu and has GTPase activity		Plasmids	Gram-negative genera	
Aminoglycosides					
Aminoglycoside-modifying enzymes:					
Aminoglycoside 6′-acetyltransferase-aminoglycoside 2″- phosphotransferase [AAC(6′) + APH(2″)]	Bifunctional: 2 domains AAC(6′) acetylates amino groups of tobramycin, netilmicin, amikacin, and gentamicin C at 6′ position APH(2″) phosphorylates 2″-hydroxyl groups	*aac(6′)-aph(2″)*	Plasmids (mainly class III) and chromosome on transposons (Tn *4001* and Tn *4001*-like, Tn *4031*; Tn *3851*)	*S. aureus* *S. epidermidis* *Enterococcus* and some other gram-positive genera	Townsend et al. 1983; Townsend et al. 1984; Ubukata et al. 1992; Rouch et al. 1987; Thomas and Archer 1989b
Aminoglycoside 3′-phosphotransferase [APH(3′)-III]	Phosphorylates 3′-hydroxyl groups of kanamycin, neomycin, paromomycin, amikacin and gentamicin B	*aph(3′)-IIIa (aphA)*	Plasmids and chromosome	*S. aureus* *Enterococcus*	Gray and Fitch 1983; Coleman et al. 1985; El Solh et al. 1986

(*Continued over*)

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci (Continued)*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Aminoglycoside-aminocyclitol-4′- adenylyl-transferase (aminoglycoside 4′-adenylyltransferase) [ANT(4′,4″)-I]	Adenylylates tobramycin, amikacin, isepamicin, paromomycin, kanamycin, neomycin and gentamicin A at 4′-hydroxyl groups and also dibekacin at the 4″-hydroxyl group	*ant(4′)-Ia (aadD)*	Plasmids	*S. aureus* *S. epidermidis*	Schwotzer et al. 1978; Thomas and Archer 1989b
Aminoglycoside 9-adenylyltransferase [ANT(9)-I]	Adenylylates spectinomycin	*ant(9)-Ia (spc)*	Chromosome: on transposon Tn *554*	*S. aureus* *S. epidermidis* Frequently found in *mecA* strains Several other species	Murphy 1985b; Thakker-Varia et al. 1987
Trimethoprim					
S1 Dihydrofolate reductase (S1 DFHR)	Marked reduced affinity for trimethoprim High-level resistance: MIC >1000 mg/ml Phe→Tyr change at position 98 (compared with native DHFR) is major determinant of resistance	*drfA*	Plasmid > chromosome: on transposon-like Tn *4003* in an operon with *thyE* and ORF	*S. aureus* *S. epidermidis* *S. hominis* Several other species	Coughter et al. 1987; Tennent et al. 1988; Rouch et al. 1989; Dale et al. 1995
Chloramphenicol					
Chloramphenicol acetyltransferase (CAT)	Acetylates chloramphenicol via acetyl coenzyme A to yield mono- and diacetylated derivatives unable to bind to the ribosome Requires induction by chloramphenicol Regulated by translational attenuation	*cat*	Plasmids (class I) Chromosome: with *cat* plasmid integrated	*S. aureus* *S. intermedius* Several other species	Iordănescu et al. 1978; Shaw 1983; Schwarz et al. 1989, 1990; Lovett 1990; Cardoso and Schwarz 1992; Schwarz et al. 1995

(*Continued over*)

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci (Continued)*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Fluoroquinolones					
DNA gyrase subunits [DNA topoisomerase (ATP-hydrolyzing)]	Subunit A: Ser→Leu or Phe change at position 84 and Glu→Lys change at position 88 result in moderate to high fluoroquinolone resistance Subunit B: mutations may result in novobiocin resistance and a slight reduction in fluoroquinolone susceptibility	*gyr AgyrB (nov)*	Chromosome Part of operon: *recF-gyrB-gyrA*	*S. aureus* *S. epidermidis* *Many other species* *Bacillus subtilis*	Sreedharan et al. 1991; Goswitz et al. 1992; Margerrison et al. 1992; Brockbank and Barth 1993; Ito et al. 1994
Multidrug efflux protein (or transporter) NorA	Plays role as energy-dependent efflux pump to remove hydrophilic fluoroquinolones and some other unrelated antibiotics Related to tetracycline and *Bacillus*-Bmr efflux proteins Change in NorA or increased transcription rate of *norA* can result in resistance	*norA* or its regulatory region *flqB* may affect level of expression of *norA*	Chromosome Infrequently on plasmids	*S. aureus* *S. epidermidis* *S. haemolyticus* Many other species	Ohshita et al. 1990; Yoshida et al. 1990; Kaatz et al. 1993; Ng et al. 1994
DNA topoisomerase IV subunit A	Subunit A: Ser→Phe or Tyr change at position 80 results in either high or low fluoroquinolone resistanceSubunits A and B are closely related to subunits A and B of DNA gyrase *grlA* mutations may be prerequisite for resistance by *gyrA* mutations	*grlA* (*cfxB*, *ofxC* of *flq* locus) *grlB*	Chromosome Part of operon: *grlB-grlA*	*S. aureus*	Trucksis et al. 1991; Ferrero et al. 1994; Ferrero et al. 1995
Rifampin					
DNA-dependent RNA polymerase β subunit	Mutations in rifampin binding area (area 1) of β subunit may result in rifampin, rifamycin, and streptovaricin resistance Specific amino acid changes have not been documented	*rpoB* (*rif*)	Chromosome Part of operon: *rpL-rpoB-rpoC*	*S. aureus* *S. epidermidis*	Aboshkiwa et al. 1992; Aboshkiwa et al. 1995; Deora and Misra 1995

(*Continued over*)

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci (Continued)*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Glycopeptides					
Intermediate-level resistance to vancomycin (VISA)	Alterations in cell wall	Unknown	Unknown	*S. aureus*	Hiramatsu et al. 1997a
Heteroresistance to vancomycin (hVISA)	Alterations in cell wall	Unknown	Unknown	*S. aureus*	Hiramatsu et al. 1997b; Hiramatsu 2001
Vancomycin-resistant *S. aureus* (VRSA)	Alteration in composition of the terminal dipeptide in muramyl pentapeptide cell wall precursors leading to decreased binding affinity to glycopeptides, and high level-resistance to vancomycin and teicoplanin	*vanA*	Plasmid	*S. aureus*	CDC 2002a, b
Cadmium and zinc					
Cadmium-efflux ATPase (an E_1 E_2 cation-translocating ATPase)	Exports Cd^{2+} and Zn^{2+} and low levels of Pb^{2+} and Bi^{3+} to the outside of the cell	*cadA*	Plasmids (class II) In operon: *cadC-cadA* Infrequently on chromosome	*S. aureus*	Nucifora et al. 1989; Endo and Silver 1995
Cadmium efflux system accessory protein	Transcriptional regulatory protein for system Induction by cadmium, bismuth and lead	*cadC*			
Cadmium-binding protein CadB	Binds Cd^{2+} and Zn^{2+}	*cadB*	Plasmids (class I and II) Infrequently on chromosome	*S. aureus* *S. lugdunensis*	Smith and Novick 1972; Shalita et al. 1980, El Solh and Ehrlich 1982; Poitevin-Later et al. 1992
Mercury and organomercurial compounds					
Mercuric reductase	Reduces Hg^{2+} to the volatile Hg^{o}	*merA*	Plasmids (class II) Occasionally on chromosome	*S. aureus*	Weiss et al. 1977; Novick et al. 1979
Organomercurial lyase (alkylmercury lyase)	Cleaves the carbon–mercury bond of organomercurials The Hg^{2+} product is subsequently reduced by mercuric reductase	*merB*	Located in *mer* operon: *merR* -ORF3-ORF4-ORF5-*merA-merB*		Laddaga et al. 1987; Lyon and Skurray 1987; Chu et al. 1992
Mercuric resistance operon regulatory protein	Transcriptional regulatory protein for system Induction by mercury	*merR*	Mer region is provisionally noted as Tn *4004*		

(*Continued over*)

Table 6 *Major antibiotic and heavy metal resistance mechanisms for staphylococci (Continued)*

Resistance phenotype/ responsible protein	Major activity or mechanism	Gene	Gene location	Species distribution	References
Arsenic and antimony					
Arsenical pump membrane protein (arsenite efflux protein)	Exports arsenite [As(III)] and antimonite [Sb(III)] to the outside of the cell	*arsB*	Plasmids (class II) Located in *ars* operon: *arsR-arsB-arsC*	*S. aureus* *S. xylosus*	Novick and Roth 1968; Silver et al. 1981; Götz et al. 1983; Ji and Silver 1992; Rosenstein et al. 1992; Bröer et al. 1993; Ji et al. 1994; Rosenstein et al. 1994
Arsenate reductase	Reduces arsenate [As(V)]to arsenite [As(III)]	*arsC*			
Arsenical resistance operon repressor (DNA-binding negative regulatory protein)	Transcriptional repressor for the system Induction by arsenate, arsenite and antimonite	*arsR*			

although there is considerable variability in this figure worldwide. Until relatively recently, MRSA was mostly confined to the hospital setting and MRSA colonization of those discharged into the community rarely persisted except when associated with prosthetic material such as catheters, or defects in the integrity of the skin (for example, wounds, ulcers, and bed sores). However, this situation continues to evolve, with the description of community-acquired MRSA associated with both colonization and infection (Daum et al. 2002; Okuma et al. 2002). These strains are resistant to fewer non-beta-lactam antibiotics than the majority of previously defined MRSA strains.

Methicillin resistance is not confined to *S. aureus*. During the 1980s, attention was drawn to the high incidence (20–85 percent of strains) of methicillin-resistant *Staphylococcus epidermidis* (MRSE) in nosocomial infections (Karchmer et al. 1983; Jones et al. 1989; Schaberg et al. 1991). Methicillin resistance has also been detected in several other staphylococcal species, including *S. haemolyticus*, *S. hominis*, *S. capitis*, *S. warneri*, *S. caprae*, *S. simulans*, *S. saprophyticus*, and *S. sciuri* (Archer and Pennell 1990; Pierre et al. 1990; Murakami et al. 1991; Suzuki et al. 1992).

The methicillin resistance gene *mecA* is carried by the chromosome of MRSA and MRSE. This encodes an altered low affinity penicillin-binding protein termed PBP 2a. This gene is part of a mobile genetic element termed staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama et al. 2000). SCC*mec* has been sequenced in its entirety, analysis of which describes a genomic island which includes two site-specific recombinase genes designated *ccrA* and *ccrB* (cassette chromosome recombinase) that are responsible for the movement of SCC*mec*. A further three types of SCC*mec* have since been described. Comparison of the first three elements (SCC*mec* types I–III) demonstrated substantial differences in the size and nucleotide sequences, but they all share conserved terminal inverted repeats and direct repeats at the integration junction points, conserved genetic organization around the *mecA* gene, and the presence of *ccr* genes (Ito et al. 2001). Evaluation of 38 epidemic MRSA strains isolated in 20 countries (published in 2001) showed that the majority possessed one of the three typical SCCmec elements on the chromosome (Ito et al. 2001). SCC*mec* type IV was subsequently defined in community-acquired MRSA (Daum et al. 2002). This element differs from SCC*mec* types I–III in its small size and absence of non-beta-lactam genetic-resistance determinants. SCC*mec* type IV has been found in community-acquired isolates with diverse genetic backgrounds, many of which are resistant only to beta-lactam antibiotics (Daum et al. 2002). Multiple MRSA clones carrying type IV SCC*mec* have been identified in the community-acquired MRSA strains of both the United States and Australia (Okuma et al. 2002).

Chromosomal DNA of *S. sciuri* hybridizes with *mecA*-specific DNA probes, suggesting the presence of an intrinsic gene homolog in this species that is closely related to the *mecA* gene of methicillin-resistant strains of *S. aureus*. This property raises the possibility that *S. sciuri* may serve as an important reservoir of genetic determinants of methicillin resistance. More recent studies have indicated that two major homologs of *mecA* may be found in *S. sciuri* (Kloos et al. 1998b; Wu et al. 1998). They share approximately 79–80 percent base pair similarity. The *mecA* homolog present in MRSA and MRSE, as well as in some other staphylococcal species and some strains of *S. sciuri*, has been designated homolog A. The other *mecA* homolog which is native to the species *S. sciuri*, has been designated homolog B. Strains carrying only this homolog are susceptible or only uniform borderline-resistant to methicillin. Sequences of the homologs and their flanking regions support the speculation that they may be evolutionary relatives and that the homolog native to *S. sciuri* may be a precursor of the homolog present in MRSA (Wu et al. 1998). *S. hominis* subsp. *novobiosepticus* appears to have the *mecA* homolog A present in a high percentage of strains (17 of 17 strains tested) and it is perhaps intrinsic to this subspecies (Kloos et al. 1998b). A genetic element that is structurally very similar to SCC*mec* except for the absence of *mecA* has been defined in a methicillin susceptible strain of *S. hominis*. Homologs of the type 1 *ccr* genes were localized on the element (Katayama et al. 2003). An SCC element that lacks *mecA* has also been defined during sequencing of MSSA 252 (Holden et al. 2004). Investigation of the role of this element in genetic transfer between and within staphylococcal species represents an exciting and important challenge.

A DNA microarray giving more than 90 percent coverage of the *S. aureus* genome has been used to examine 36 strains with divergent clonal backgrounds (Fitzgerald et al. 2001). The findings indicated that *mecA* has been horizontally transferred into distinct *S. aureus* chromosomal backgrounds at least five times, demonstrating that methicillin-resistant strains have evolved multiple independent times, rather than from a single ancestral strain. The population genetic structure of an international collection of 912 MRSA and MSSA isolates has been defined using multilocus sequence typing. Eleven major MRSA clones were identified within five groups of related genotypes. The likely evolutionary origins of each major MRSA clone, the genotype of the original MRSA clone and its MSSA progenitor was resolved using an algorthim termed BURST (based upon related sequence types). The conclusion of this study was that major MRSA clones have arisen repeatedly from successful epidemic MSSA strains, and that isolates with decreased susceptibility to vancomycin are arising from some of these major MRSA clones (Enright et al. 2002).

Some *mecA*$^-$ *S. aureus* strains, which harbor β-lactamase plasmids and belong to phage group 94/96, exhibit borderline susceptibility to methicillin and other β-lactamase-resistant penicillins (McMurray et al. 1990; Barg et al. 1991). The slight increase in resistance of these closely related strains (oxacillin MIC, 1–2 μg/ml) is partly due to the hyperproduction of β-lactamase type A, resulting in some inactivation of β-lactamase-resistant penicillins (McDougal and Thornsberry 1986; Chambers et al. 1989). A low-level intrinsic mechanism has been proposed to explain the moderately increased methicillin MICs (4–8 μg/ml) of *mecA*$^-$, borderline methicillin-resistant strains of *S. aureus* (Berger-Bächi et al. 1986; De Lencastre et al. 1991). Although the mechanism is not fully understood, it at least partly involves the presence of modified PBPs with decreased β-lactam affinity (Tomasz et al. 1989).

Resistance of *S. aureus* to vancomycin

The glycopeptide antibiotics teicoplanin and vancomycin bind to the peptidyl-D-alanyl-D-alanine termini of peptidoglycan precursors and prevent the transglycosylation and transpeptidation steps of cell wall peptidoglycan synthesis. Since this mode of action is different from that of β-lactams, glycopeptides are being used to treat severe infections caused by methicillin- and other β-lactam-resistant staphylococci.

Three categories of *S. aureus* resistance to vancomycin have been described since 1997:

1. *S. aureus* with intermediate-level resistance to vancomycin (VISA)
2. *S. aureus* with hetero-resistance to vancomycin (hVISA)
3. vancomycin-resistant *Staphylococcus aureus* (VRSA).

Three criteria have been developed by the (CDC) for the identification of VISA. These are:

1. growth within 24 h on commercial brain heart infusion agar screen plates containing 6 μg/ml vancomycin
2. E-test vancomycin MIC $\geqslant$6 μg/ml
3. broth microdilution MIC of 8–16 μg/ml (Tenover et al. 1998).

A variety of screening methods have been described to detect hVISA, but the optimal method is still under debate (Liu and Chambers 2003). VRSA is defined by an MIC $\geqslant$32 μg/ml (NCCLS 1997).

S. aureus with intermediate-level reistance to vancomycin (VISA) was first detected in Japan in 1996 (Hiramatsu et al. 1997a). This strain (Mu50) was isolated from an infant who had undergone surgery to correct a congenital cardiac defect, and who had developed a sternal wound infection postoperatively that was refractory to treatment. This strain was reported to have an MIC of 8 mg/l (Hiramatsu et al. 1997a). Since then, patients infected with VISA have been identified worldwide, although such strains are currently rare in clinical practice. Most appear to evolve from MRSA strains in patients who have received prolonged vancomycin treatment. The first hVISA (Mu3) was also identified in Japan from the sputum of a patient with MRSA pneumonia following surgery (Hiramatsu et al. 1997b). This strain was reported to have an MIC of 3 μg/ml (Hiramatsu et al. 1997b). Typing showed that Mu3 and Mu50 had the same PFGE pattern, and serial passage of Mu5 in increasing concentrations of vancomycin gave rise to subpopulations with levels of resistance comparable to Mu50 (Hiramatsu et al. 1997b). hVISA has since been reported from around the world and appears, at least based on the number of literature reports, to be more common than VISA (Hiramatsu et al. 1997b; Hiramatsu 2001; Liu and Chambers 2003). The first VRSA was reported in Michigan in July 2002, with a second apparently unrelated case in Pennsylvania two months later (CDC 2002a, 2002b).

The mechanisms of vancomycin resistance for VISA and VRSA are currently under investigation. Vancomycin-resistant enterococci were reported in 1988, and the emergence of vancomycin-resistant *S. aureus* resulting from transfer of the genetic element encoding vancomycin has been awaited since. This was achieved in the laboratory in the early 1990s when *vanA* was transferred from *E. faecalis* to *S. aureus* by conjugation, with the concomitant expression of vancomycin resistance. This represents the mechanism of resistance in the two VRSA isolated in the USA which contain the *vanA* gene. This gene encodes a ligase that effects an alteration of composition of the terminal dipeptide in muramyl pentapeptide cell wall precursors, leading to decreased binding affinity to glycopeptides and high level resistance to both vancomycin and teicoplanin (Fraimow and Courvalin 2000). The mechanisms of vancomycin resistance for VISA and hVISA are less clear. These strains do not carry the enterococcal vancomycin-resistance genes *vanA*, *vanB*, or *vanC* 1–3 (Hanaki et al. 1998a). Comparison of the peptidoglycan compositions of Mu50 and Mu3 has demonstrated that Mu50 has increased amounts of glutamine-nonamidated muropeptides and decreased crosslinking of peptidoglycan, with a greatly decreased dimer/monomer ratio of muropeptides (Hanaki et al. 1998b). The peptidoglycan of Mu50 also binds 1.4 times more vancomycin than that of Mu3 (Hanaki et al. 1998b). It has been proposed that these factors may contribute to vancomycin resistance by increasing the consumption of vancomycin by the cell wall of Mu50 and reducing the amount of vancomycin reaching the cytoplasmic membrane. Both strains produce three to five times the amount of PBPs 2 and 2′ when compared with vancomycin-susceptible *S. aureus* control strains with or without methicillin resistance (Hanaki et al. 1998a). Transmission electron microscopy has shown a doubling

in the cell wall thickness in Mu50 compared with the control strains, although Mu3 did not show obvious cell wall thickening (Hanaki et al. 1998a). Microarray transcription analysis of two clinical VISA isolates was compared with two derivatives with MIC of 32 μg/ml after passage in the presence of vancomycin (Mongodin et al. 2003). Of the 35 genes with increased transcription, 15 involved purine biosynthesis or transport and the regulator of the major purine biosynthetic operon was mutant (Mongodin et al. 2003). It has been hypothesized that increased energy (ATP) is required to generate the thicker cell walls that characterize resistant mutants (Mongodin et al. 2003). The genome sequence of Mu50 was published in 2001 (Kuroda et al. 2001) The genome sequences of Mu50 and vancomycin-susceptible MRSA strains N315, EMRSA 16, and COL have been compared (Avison et al. 2002). This revealed several loss-of-function mutations in Mu50, affecting important cell wall biosynthesis and intermediary metabolism genes.

Resistance has also been detected in other staphylococcal species. *S. haemolyticus* isolates with teicoplanin resistance (MIC >16 μg/ml) and/or intermediate-level resistance to vancomycin have been detected, especially in methicillin-resistant strains of this species (Goldstein et al. 1990; Bannerman et al. 1991a). Strains of *S. haemolyticus* and *S. epidermidis* that are moderately resistant to teicoplanin (MIC 16 μg/ml) are relatively common. Vancomycin and teicoplanin resistance can be selected in vitro in *S. haemolyticus* (Schwalbe et al. 1990; Herwaldt et al. 1991).

Resistance to macrolide, lincosamide, and streptogramin (MLS)

Erythromycin-resistant staphylococci often have cross-resistance to macrolides (erythromycin, oleandomycin, spiramycin, clarithromycin, azithromycin), lincosamides (lincomycin, clindamycin) and streptogramin type B antibiotics (designated MLS-resistant). The different types of MLS antibiotics bind to the 50S ribosomal subunit at overlapping binding sites, and binding interferes with transpeptidation and translocation reactions needed for peptide chain elongation. These sites are protected by the N^6,N^6-dimethylation of an adenine-2058 residue at the peptidyl transferase centre of 23S rRNA, a reaction performed by an rRNA methylase (Weisblum 1985). Three distinct rRNA methylase genes have been detected in staphylococci: *ermA* (Murphy 1985a), *ermB* (Novick and Murphy 1985; Leclercq and Courvalin 1991), and *ermC* (Horinouchi and Weisblum 1982; Projan et al. 1987). Even though each of the *erm* gene classes can be distinguished by hybridization, the amino acid sequences of the encoded methylases are highly conserved, which suggests that they evolved from a common ancestor (Arthur et al. 1987). The chromosomal *ermA* gene is common among methicillin-resistant (*mecA*) strains of *S. aureus* and *S. epidermidis*, but it is relatively uncommon in *mecA*$^-$ strains and usually accounts for only 6–11 percent of those resistant to MLS antibiotics (Thakker-Varia et al. 1987; Kloos et al. 1992; Eady et al. 1993). *ermB* is usually not found in staphylococcal species indigenous to primates; however, it appears to be relatively common in MLS-resistant strains of *S. intermedius* from dogs, in which expression can be either inducible or constitutive, and in MLS-resistant strains of *S. hyicus* and *S. xylosus* from pigs, in which expression is constitutive (Eady et al. 1993). The *ermC* gene is most often located on class I plasmids of the pSN2 and pE194 families (Iordănescu and Surdeanu 1980; Parisi et al. 1981; Thakker-Varia et al. 1987; Kloos et al. 1992). These plasmids are present in very high frequencies (85–98 percent of strains) in most staphylococcal populations following treatment of the host with tylosin (a member of the spiramycin group), eythromycin, or clindamycin (Kloos et al. 1992). Constitutive *ermC* genes have been selected in laboratory populations of *S. aureus* by the growth of inducible strains in the presence of tylosin (Horinouchi and Weisblum 1981) and in natural populations of staphylococci exposed to clindamycin. On the skin of outpatients receiving topical clindamycin for acne vulgaris, the percentage of total staphylococcal strains represented by constitutive MLS-resistant strains increased from 1 percent, prior to treatment, to 60 percent after 1 month of treatment, and to 71 percent by 2 months of treatment (Kloos et al. 1992). Approximately 95 percent of these strains had the constitutive *ermC* gene. As an exceptional species, *S. capitis* responded very slowly to clindamycin pressure, with only 12–15 percent of its strains expressing constitutive MLS resistance. This species also responds slowly to penicillin, erythromycin, or tetracycline pressure and it is seldom multiply resistant to antibiotics (Wells and Kloos 1987). Clinical isolates of constitutive MLS-resistant staphylococci are continuing to increase in frequency and this trend may be a reflection of the increased clinical use of clindamycin (Jenssen et al. 1987).

The inducible macrolide and streptogramin (MS) resistance phenotype involves erythromycin cross-resistance to other 14–16-membered ring macrolides and streptogramin type B, but not lincosamides (Jenssen et al. 1987; Ross et al. 1989; Wondrack and Sutcliffe 1995). MS resistance appears to be most prevalent in members of the *S. epidermidis* and *S. saprophyticus* species groups, especially in *S. hominis* and *S. cohnii* (Kloos et al. 1992; Eady et al. 1993).

Resistance to tetracycline

This is widespread among staphylococcal species and ranks along with β-lactam and MLS resistance as one of the most frequent types of antibiotic resistance found in

natural populations of staphylococci (Cooksey and Baldwin 1985; Wells and Kloos 1987; Bismuth et al. 1990; Archer and Scott 1991; Schwarz et al. 1993). There are two mechanisms of tetracycline resistance recognized in staphylococci. The most common one involves an energy-dependent pumping (efflux) of tetracycline and doxycycline from the cell, so that levels of these antibiotics are reduced below that required to inhibit the ribosome. The efflux protein is most often encoded by the inducible gene *tetK* which is located on class I plasmids of the pT181 family. The second mechanism, one that is controlled by the gene *tetM*, involves ribosome protection such that protein synthesis is unaffected by the presence of tetracycline, doxycycline, or minocycline (Schaefler et al. 1976).

Resistance to aminoglycosides

There are three major mechanisms responsible for aminoglycoside resistance in staphylococci. One mechanism involves changes in ribosomal proteins as a consequence of certain mutations in their structural genes, such that ribosomes can no longer bind streptomycin. A second mechanism involves the energization and permeability of the cell membrane. Some energy-deficient, small-colony mutants of *S. aureus* have been shown to be resistant to aminoglycosides due to a diminished uptake of the antibiotics (Miller et al. 1980). The third and most common mechanism of resistance involves modification of aminoglycosides by aminoglycoside-modifying enzymes so that the antibiotics are no longer capable of binding to ribosomes. Genes encoding these enzymes are either located on plasmids (e.g. gentamicin-resistance plasmids and neomycin- and kanamycin-resistance plasmids) or on the chromosome (Jaffe et al. 1982; Lyon et al. 1984; Coleman et al. 1985; El Solh et al. 1986; Thomas and Archer 1989b).

Resistance to trimethoprim

Trimethoprim resistance is either mediated by alterations in the expression of the intrinsic chromosomal *dfr* gene (e.g. *dfrB* of *S. aureus* or *dfrC* of *S. epidermidis*), possibly resulting in overproduction of the native dihydrofolate reductase (DHFR) or a reduced affinity of the native DHFR for trimethoprim, or by the acquisition of a second chromosomal or plasmid *dfr* gene (e.g. *dfrA* in *S. aureus*, *S. epidermidis*, and *S. hominis*) that encodes a trimethoprim-resistant DHFR capable of rescuing the reduction step leading to tetrahydrofolate in the presence of trimethoprim (Galetto et al. 1987; Rouch et al. 1989; Dale et al. 1995). In Australian *S. aureus* strains, the *drfA* gene is usually carried by non-conjugative plasmids, whereas in the USA *S. aureus* strains, the gene is frequently carried by both conjugative and nonconjugative plasmids, and is occasionally found on the chromosome.

Resistance to fluoroquinolones

Three mechanisms for fluoroquinolone resistance have been postulated for staphylococci. One mechanism found in *S. aureus* and *S. epidermidis* involves mutations in the chromosomal gene *gyrA*, encoding the DNA gyrase subunit A, so that its function is no longer inhibited by the antibiotic (Sreedharan et al. 1991; Goswitz et al. 1992). A second mechanism found in *S. aureus*, *S. epidermidis*, and *S. haemolyticus* involves mutations in the chromosomal gene *norA* (or its regulatory region) that encodes a membrane efflux protein for hydrophilic fluoroquinolones and other unrelated antibiotics (Yoshida et al. 1990; Kaatz et al. 1993). A third mechanism found in *S. aureus* involves mutations in the chromosomal gene *grlA* that encodes the A subunit of DNA topoisomerase IV (Trucksis et al. 1991; Ferrero et al. 1994, 1995). Clinical isolates of *S. aureus* that have mutations in both *gryA* and *grlA* genes generally exhibit higher levels of ciprofloxacin resistance (MIC 16→128 μg/ml) than isolates with only mutations in *grlA* (MIC 2–16 μg/ml). Wide use of the fluoroquinolone ciprofloxacin has resulted in a steady increase in the incidence of fluoroquinolone-resistant (MIC $\geqslant$8 μg/ml) staphylococci, especially among clinical isolates (Kotilainen et al. 1991; Bannerman et al. 1991b; Archer and Climo 1994). The newer fluoroquinolones such as fleroxacin, levofloxacin, and clinafloxacin are more active than ciprofloxacin against ciprofloxacin-susceptible staphylococci by in vitro testing; however, the problem still remains that fluoroquinolone-resistant mutants emerge following exposure to these antibiotics. Fluoroquinolone resistance has been identified in the species *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. warneri*. The *S. saprophyticus* and *S. sciuri* species groups exhibit an intermediate susceptibility (MIC 1–4 μg/ml) to fluoroquinolones and this property may be related to their intrinsic nalidixic acid resistance (MIC 64–1 024 μg/ml) and novobiocin resistance (MIC 1.6–64 μg/ml) (Schleifer 1986; Magni and Soltész 1986), the latter, presumably due to modifications of the *gyrB* gene and/or its regulation.

Resistance to other compounds

Some strains of *S. aureus*, *S. lugdunensis* and members of the *S. epidermidis* and *S. saprophyticus* species groups demonstrate resistance to various combinations of the heavy metal ions: cadmium, zinc, bismuth, lead, mercury, arsenate, arsenite, and antimony (III) (Novick and Roth 1968; Weiss et al. 1977; Götz et al. 1983; Poitevin-Later et al. 1992). The genes controlling heavy metal resistance are usually carried by plasmids, but

they may be integrated into the chromosome either as a result of transposon insertion or integration of the resistance plasmid (Novick and Roth 1968; Novick et al. 1979; Shalita et al. 1980; Silver et al. 1981; Witte et al. 1986). Cadmium ions enter the cell via a specific, energy-dependent transport system that normally takes up manganese ions (Tynecka et al. 1981). One of the main toxic effects of cadmium is the inhibition of respiration caused by its binding to sulfhydryl groups on essential proteins. Both cadmium and zinc resistance in *S. aureus* are determined by the same mechanisms. One mechanism involves the *cadA* gene product, which is a membrane-translocating ATPase that prevents internal accumulation of cadmium and zinc by exporting these cations to the outside of the cell (Nucifora et al. 1989; Endo and Silver 1995). Mercury and organomercurial compounds are toxic primarily because of their ability to bind to the sulfydryl groups of enzymes, resulting in their inactivation. Resistance to inorganic mercury is determined by the gene *merA*, which encodes a mercuric reductase capable of reducing the Hg^{2+} ion to the volatile, metallic Hg^0 (Weiss et al. 1977). Resistance to organomercurials is determined by the gene *merB* that encodes an organomercurial lyase which cleaves carbon–mercury bonds, resulting in the liberation of Hg^{2+} ions. These ions can then be acted upon by mercuric reductase for volatilization. The other three genes of the *mer* operon encode hydrophobic proteins involved in mercury uptake, presumably allowing the entry of mercury into the cell to permit detoxification by MerA. Arsenicals are toxic to staphylococci and other bacteria. Arsenate is an analog of phosphate and therefore inhibits kinases and can interrupt energy transfer during glycolysis; arsenite can bind to the cysteine residues of proteins and inhibit many enzymes with essential thiol groups. The primary mechanism for arsenic resistance is plasmid mediated and involves an energy-dependent arsenic efflux pump (Silver et al. 1981; Silver and Keach 1982). The gene *arsB* encodes a membrane efflux protein specific for arsenite and antimonite (III), and *arsC* encodes a thioredoxin-dependent arsenate reductase that reduces arsenate to arsenite (Bröer et al. 1993; Ji et al. 1994).

LABORATORY ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

This section gives an overview of the isolation and identification of staphylococci. Identification tests are described in brief; readers wishing to perform such tests are referred to dedicated texts on the topic detailing standard operating procedures. The methods used to speciate bacteria isolated from a clinical specimen will be dictated by the global setting and the investment in technology. For example, a resource-poor setting relying on inexpensive tests is likely to aim for identification to the point where it was possible to distinguish between *S. aureus* and other staphylococci. Laboratories in affluent countries will differ depending on inward investment, but are very likely to be able to speciate with some precision using either commercially available kits or fully automated technology.

Laboratory isolation of staphylococci

Culture of clinical specimens should be performed as soon as possible to minimize changes in the microbial composition from that originally present. Direct microscopic examination of normally sterile fluids (e.g. cerebrospinal fluid, joint aspirates, and pus aspirated from deep sites) may provide a rapid, presumptive report of gram-positive cocci resembling staphylococci. Isolation of staphylococci from primary clinical specimens is usually performed using blood agar (e.g. tryptic soy agar supplemented with 5 percent sheep blood), following an incubation period of 18–24 h in air at 35–37°C. Most staphylococcal species will produce abundant growth and well isolated colonies will be 1–3 mm in diameter, circular, smooth and raised, with a butyrous consistency. Colonies of most of the species and subspecies cannot be distinguished from one another on this medium. Screening for the presence of *S. aureus* in mixed cultures such as nasal swabs is often performed using mannitol-salt agar; *S. aureus* ferments mannitol, resulting in a change in the color of the medium from pink to yellow. Blood agar containing antibiotics that inhibit gram-negative organisms should be used for specimens containing mixed flora, but again colonies of most of the species and subspecies are indistinguishable.

Colony morphology can be used by experienced operators to define presumptive staphylococci. The presence of gram-positive cocci on Gram stain and a positive catalase test will provide further positive indicators if there is doubt about colony appearance. Each discernible staphylococcal morphotype in a given specimen should be analyzed. The next step in a clinical diagnostic setting is to distinguish between *S. aureus* and the remaining staphylococcal species. This is commonly determined on the basis of results from two or three simple tests. The rationale for performing more than one test is that rare strains of *S. aureus* occur which are negative for one or more test. Furthermore, some strains of coagulase-negative staphylococci are positive for one or more of these tests (see Table 7). These species are rare in clinical practice, and any uncertainty can be resolved by full speciation. The coagulase test detects the production of coagulase by *S. aureus* during growth. A well isolated colony is mixed with plasma, incubated at 37°C for 4 h, and observed for clot formation. Tests which are negative at 4 h are incubated and observed again for clotting at 24 h. Clumping factor can be detected by a standard slide test performed by making a heavy suspension of cells in distilled water, stirring the

Table 7 *Identification of clinically significant* Staphylococcus *species and subspecies*

Species and subspecies	Characteristics[a]													Acid (aerobically) from						
	Colony pigment	Anaerobic growth	Staphylocoagulase	Clumping factor	Thermonuclease	Alkaline phosphatase	Pyrrolidonyl arylamidase	Ornithine decarboxylase	Urease	β-Glucuronidase	β-Galactosidase	Acetoin production	Novobiocin resistance	D-Trehalose	D-Mannitol	D-Mannose	D-Turanose	Maltose	Sucrose	*N*-Acetylglucosamine
S. epidermidis	–	+	–	–	–	+[b]	–	(d)	+	–	–	+	–	–	–	(+)	(d)	+	+	–
S. capitis subsp. *capitis*	–	(+)	–	–	–	–	–	–	–	–	–	d	–	–	+	+	–	–	(+)	–
S. capitis subsp. *ureolyticus*	(d)	(+)	–	–	–	–	(d)	–	+	–	–	d	–	–	+	+	–	+	+	–
S. hominis subsp. *hominis*	D	–	–	–	–	–	–	–	+	–	–	d	–	d	–[c]	–	+	+	(+)	d
S. hominis subsp. *novobiosepticus*	–	–	–	–	–	–	–	–	+	–	–	d	+	–	–	–	ND	+	(+)	–
S. haemolyticus	D	(+)	–	–	–	–	+	–	–	D	–	+	–	+	d	–	(d)	+	+	+
S. warneri	D	+	–	–	–	–	–	–	+	D	–	+	–	+	d	–	(d)	(+)	+	–
S. lugdunensis	D	+	–	(+)	–	–	+	+	d	–	–	+	–	+	–	+	(d)	+	+	+
S. aureus	+[d]	+	+	+	+	+	–	–	d	–	–	+	–	+	+	+	+	+	+	+
S. saprophyticus	D	(+)	–	–	–	–	–	–	+	–	+	+	+	+	d	–	+	+	+	d
S. simulans	–	+	–	–	–	(d)	+	–	+	D	+	d	–	d	+	d	–	(±)	+	+
S. felis	–	+	–	–	–	+	ND	–	+	–	+	–	–	+	+	+	ND	–	d	+
S. intermedius	–	(+)	+	D	+	+	+	–	+	–	+	–	–	+	(d)	+	d	(±)	+	+
S. schleiferi subsp. schleiferi	–	+	–	+	+	+	+	–	–	–	(+)	(+)	–	d	–	+	–	–	–	(+)
S. schleiferi subsp. *coagulans*	–	+	+	–	+	+	ND	–	ND	–	(+)	+	–	–	d	+	–	–	d	ND
S. hyicus	–	+	D	–	+	+	–	–	d	D	–	–	–	+	–	+	–	–	+	+

a) Symbols: +, 90 percent or more strains positive; ±, 90 percent or more strains weakly positive; –, 90 percent or more strains negative; d, 11–89 percent of strains positive. Parentheses indicate a delayed reaction. All species listed are negative for the modified oxidase test, arginine arylamidase, esculin hydrolysis and acid (aerobically) from D-xylose, D-cellobiose, L-arabinose and raffinose. The characteristics of *S. capitis* subsp. *capitis* are shown for making a comparison with the clinically significant subspecies *S. capitis* subsp. *ureolyticus*.

b) Alkaline phosphatase activity is negative for 6–15 percent of the strains of *S. epidermidis*.

c) Acid is produced aerobically from D-mannitol in 5–12 percent of the strains of *S. hominis*.

d) Approximately 6–11 percent of *S. aureus* strains fail to produce detectable pigment on commercial agar media. From Kloos and Bannerman (1995).

mixture to a homogeneous composition, and then adding a drop of plasma. The mixture should be examined for clumping within about 10 s. This detects the expression of ClfA in *S. aureus*. The majority of *S. aureus* and 10–15 percent of *S. intermedius* strains are clumping factor-positive. *S. lugdunensis* and *S. schleiferi* subsp. *schleiferi* are usually clumping factor-positive if human plasma is used in the slide test (Freney et al. 1988). A number of commercial tests that are based on latex agglutination are commonly used in diagnostic laboratories which detect one or more of clumping factor, protein A, capsular polysaccharide and group-specific antigens (van Griethuysen et al. 2001). The DNase test determines the ability of an organism to produce deoxyribonuclease. This is detected by streaking bacterial isolates onto solid agar containing DNA. After 24 h incubation, hydrochloric acid is added to the surface of the plate which precipitates unhydrolyzed DNA to produce a white opacity. The production of deoxyribonuclease results in hydrolysis of DNA, detected by a zone of clearing around the bacterial growth. An organism which is positive by two or three tests in a routine diagnostic laboratory will usually be considered to be *S. aureus*. If doubt remains, this can usually be resolved using a commercially available rapid identification kit. Some isolates from clinical samples remain difficult to speciate after this process, when use should be made, where available, of a reference laboratory.

Identification of *Staphylococcus* species and subspecies

The most prevalent staphylococcal species and subspecies in human infections are *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*, followed by *S. hominis*, *S. warneri*, *S. lugdunensis*, *S. schleiferi* subsp. *schleiferi*, *S. capitis* subsp. *ureolyticus*, and *S. simulans* (Kloos and Bannerman 1994). Those of special veterinary interest include *S. aureus*, *S. intermedius*, *S. hyicus*, *S. felis*, and *S. schleiferi* subsp. *coagulans* (Devriese 1986; Igimi et al. 1989, 1990). The above staphylococcal species and subspecies can be distinguished on the basis of the minimal key characters shown in Table 7.

Several manufacturers have developed rapid identification kits and automated systems requiring only a few hours to one day for completion of tests. With these products, identification of most species and subspecies can be made with an accuracy of 70–90 percent (Kloos and Wolfshohl 1982; Crouch et al. 1987; Kloos and George 1991; Bannerman et al. 1993). Currently, *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. capitis* subsp. *capitis*, *S. saprophyticus*, *S. cohnii* subsp. *cohnii*, *S. simulans*, *S. intermedius*, and *S. sciuri* can be identified reliably by most of the commercial systems available. Some additional testing may be required to increase the accuracy of identification to above 90 percent. The rapid identification systems now available include the following: RAPIDEC Staph and API STAPH kits and the fully automated Vitek system, which utilize a gram-positive identification (GPI) Card (bioMérieux Vitek Inc., Hazelwood, MO, USA); MicroScan Pos ID panel (read manually or on MicroScan instrumentation), MicroScan Rapid Pos ID panel (read by AutoScan-W/A), and the Pos Combo and Rapid Pos Combo panels, which combine species identification and antimicrobial susceptibility tests (Dade-Behring Inc., West Sacramento, CA, USA); Crystal Rapid Gram-Positive Identification System, Sceptor *Staphylococcus* MIC/ID panel; Sceptor Gram Positive Breakpoint/ID Panel and the frozen Pasco MIC/ID Gram-Positive Panel (Becton Dickinson Microbiology Systems, Sparks, MD, USA); GP MicroPlate test panel (read manually using Biolog computer software for interpretation or automatically with the Biolog MicroStation) (Biolog, Hayward, CA, USA); Microbial Identification System (MIS) that automates identification by combining cellular fatty acid analysis with computerized high-resolution gas chromatography (MIDI, Newark, DE, USA) and the fully automated RiboPrinter Microbial Characterization System (Qualicon, Inc., Wilmington, DE, USA), based on ribotype pattern analysis. Rapid identification of the species *S. aureus* can be made using the AccuProbe culture identification test for *S. aureus* (Gen-Probe Inc., San Diego, CA, USA). This species can also be identified by an immunoenzymatic assay based on a monoclonal antibody prepared against the *S. aureus* endo-β-*N*-acetylglucosaminidase (Guardati et al. 1993). These specific tests are especially useful in identifying coagulase-negative, protein A-negative, and/or clumping factor-negative mutants of *S. aureus*, that would otherwise be misidentified by coagulase and latex agglutination tests.

The oxacillin agar screen test has been a mainstay for the detection of MRSA in diagnostic laboratories over many years. However, this test has been reported to be ineffective for CoNS (Tenover et al. 1999), and its use for this bacterial group is no longer recommended. Readers are referred to NCCLS recommendations for a detailed description of methodology for the reference methods (broth microdilution, disk diffusion, oxacillin agar screen) (NCCLS 2000a, b). A wide range of other tests is available for the identification of methicillin resistance which can be grouped together according to the technology used. Genotypic testing for detection of the presence of *mecA* can be performed using the PCR (Swenson et al. 2001; Jonas et al. 2002; Grisold et al. 2002; Fang and Hedin 2003). This has become a gold standard for the comparison of sensitivity and specificity of other tests. Some investigators include PCR detection of a second gene such as *femA*, *femB*, or the *S. aureus* specific marker gene *nuc*, and multiplex PCR can be performed to detect several genes associated with resistance to different antibiotic groups (Perez-Roth et al. 2001). PCR methodology has also been described for the

detection of MRSA directly from swabs (Jonas et al. 2002), including those taken from colonized sites (Francois et al. 2003). The latter involved an initial enrichment step for *S. aureus* using immunomagnetic separation (Francois et al. 2003). The use of real-time PCR has also been developed for the detection of MRSA (Fang and Hedin 2003; Grisold et al. 2002). Gene probe hybridization assays have also been devised and are commercially available. One example is the EVIGENE MRSA detection kit, which has been evaluated for *S. aureus* and coagulase-negative staphylococci using blood cultures or bacteria from agar plates (Levi and Towner 2003; Poulsen et al. 2003). This appears to be an accurate test when valid test results are produced, although readable results are not always produced. For example, eight of 59 tests for CoNS were not valid in the study reported by Poulsen et al. (2003). Detection of PBP 2a can be performed using a latex agglutination test (for example, MRSA-Screen test (Horstkotte et al. 2001)). This has a lower sensitivity and specificity compared with PCR (Yamazumi et al. 2001; Jureen et al. 2001). Latex tests may represent an alternative for resource-poor laboratories that cannot afford PCR technology, but are not sufficiently accurate to replace PCR as the gold standard method in well funded laboratories.

Studies that compare different technologies are a useful source of information when deciding on the relative usefulness of a range of tests that have been individually reported in the literature. One such study used a set of 55 *S. aureus* challenge organisms including several borderline oxacillin resistant strains that were *mecA* positive (Swenson et al. 2001). Comparison was made between six routine methods (three reference methods (broth microdilution, disk diffusion, oxacillin agar screen), two commercial automated systems (MicroScan conventional panels and Vitek cards), the MicroScan rapid panel), and two new rapid methods, Velogene (cycling probe assay) and the MRSA-Screen (latex agglutination)). PCR for *mecA* was used as the gold standard. On initial testing, the percentage of correct results (percent sensitivity/percent specificity) were reported as: broth microdilution, 100/100; Velogene, 100/100; Vitek, 95/97; oxacillin agar screen, 90/92; disk diffusion, 100/89; MicroScan rapid panels, 90/86; MRSA-Screen, 90/100; and MicroScan conventional, 74/97. The MRSA-Screen sensitivity improved to 100 percent if agglutination reactions were read at 15 min.

In summary, there are now many methods described and commercially available for the rapid identification of MRSA. The method used for detection of methicillin resistance in any given laboratory will be dictated by funding and availability of technical expertise. Conventional microbiological methods are likely to persist in many laboratory settings. PCR is highly sensitive but requires careful standardization and quality control to maintain reproducibility and to prevent false positives. Many of the other technologies do not offer the same degree of sensitivity as PCR, but may be chosen in particular laboratory settings.

Interpretation of the clinical significance of cultures positive for staphylococci

Staphylococci are normal members of the human commensal flora. They are ubiquitous and widely distributed on the human body, and microbiological sampling without contaminating the specimen can be difficult. However, it is often crucial to the diagnostic process of an individual suspected of having bacterial infection that interpretation is accurate. This has caused problems to microbiologists and clinicians for more than 100 years. This section briefly highlights some factors that can be useful in this process.

The first essential step is to have an appreciation of the quality of the specimen. As a rule of thumb, good quality samples are those taken from normally sterile sites (such as cerebrospinal fluid, pus from joints or deep sites obtained by aspiration or during theater procedures, and blood cultures). Some samples will inevitably contain contaminating bacteria, such as those from patients with suspected urinary tract sepsis or ventilator-associated pneumonia.

Once an organism has been isolated, the next step is bacterial speciation and application of this information within the clinical context. *S. aureus* is a rare contaminant of specimens taken from normally sterile sites. Patients with invasive *S. aureus* disease usually have obvious clinical manifestations of disease, and a positive culture often confirms a strong clinical suspicion. Interpretation of *S. aureus* from colonized samples such as urine and bronchial lavage is more complex. Defining the significance of coagulase-negative staphylococci from any specimen always required careful consideration (Archer 1985). Contamination of the clinical sample with CoNS may occur during even the most carefully conducted procedure (such as in the theater setting). Further factors that can be taken into account when interpreting these culture results fall into three broad categories: (1) microbiological, (2) clinical, and (3) other investigations.

1 Microbiological evidence for infection versus contamination can be gathered from data on quantitative bacteriology. The number of bacterial organisms in a defined volume of sample such as urine or bronchial lavage can help distinguish between a contaminated sample and one representative of true infection. Quantitation of staphylococci in blood cultures has also been used, on the basis that very low bacterial numbers are more likely to represent skin contaminants. Bacterial quantitation is time consuming in a routine laboratory where surrogates of the number of colony forming units per ml of sample are commonly

used, including time of blood culture to positivity (using automated machines), or the number of blood culture bottles positive versus the number taken. For example, one blood culture bottle positive for a coagulase-negative staphylococcus out of a total of four taken reflects a very low bacterial inoculum and often represents contamination, assuming that all samples have been taken in the absence of antibiotic treatment. Blood cultures that contain more than one strain or species of coagulase-negative staphylococcus usually represent contaminated samples. Useful information can also be derived from the reproducibility of microbial culture, that is, repeated culture of the same organism from either multiple samples from the same site (as may be taken during orthopedic procedures to define bone sepsis), or serial sampling over time (such as blood cultures). Repeated sampling following a positive culture of dubious significance can be extremely helpful if taken before the start of antibiotics, where this is clinically feasible. Culture of the same organism from a different sample may be pivotal. For example, culture of the same bacterium from blood and from the tip of an intravenous device which has been removed indicates a high probability of intravenous device-associated bacteremia.

2 Clinical information is always crucial to the correct interpretation of positive microbiological specimens. This includes a full medical history and findings on clinical examination. Determining whether a patient has risk factors for a given infection is extremely helpful. For example, coagulase-negative staphylococci are not considered to be virulent pathogens and usually cause opportunistic pathogens in the immunocompromised or in association with prosthetic material. Relevant symptoms and signs of infection will depend on the type of infection in question.

3 Other investigations may prove very helpful. These include the broad tests such as white cell count and C-reactive protein, and the targeted tests focusing on specific suspected diagnoses, for example, echocardiography in suspected endocardtis and MRI in the investigation of bone and joint sepsis.

Some specimens verge on the uninterpretable. For example, isolation of *S. aureus* in swabs from wounds, ulcers and other skin defects may reflect colonization or infection, and the diagnosis of superficial tissue infection is best judged using clinical criteria.

TYPING METHODS

Typing techniques can be used to study both the macro-epidemiology and micro-epidemiology of staphylococci. A wide range of typing techniques have been described. These differ in their discriminatory ability, and in reproducibility and portability, factors which will dictate which method to use for a given question. Phage typing was the mainstay of typing for many years but has been replaced by alternative methods. These include ribotyping, PFGE, multilocus enzyme electrophoresis (MLEE), plasmid profiling (reviewed by Kloos and Bannerman 1994; Arbeit 1995) and multilocus sequence typing (MLST) (Enright et al. 2000). Plasmid profiling and plasmid restriction endonuclease analysis have been applied to all the recognized species and subspecies (Kloos et al. 1981, 1992; Archer et al. 1982; Parisi 1985; Wells and Kloos 1987; Etienne et al. 1990). PCR techniques for random amplified polymorphic DNA (Welsh and McClelland 1991; Marquet-Van Der Mee et al. 1995) and specific gene sequences, e.g. *mecA* sequences combined with variable DNA sequence motifs (van Belkum et al. 1993) or *coa* sequences and subsequent enzyme digestion of the PCR product(s) (Goh et al. 1992), have been used to identify certain strains of *S. aureus*. The PCR-based methodology applied to multiple-locus variable numbers of tandem repeat (VNTR) analysis has recently been applied to MRSA. Five VNTR loci (*sdr*, *clfA*, *clfB*, *ssp*, and *spa*) were subjected to analysis; this scheme was reported to be equivalent to pulsed-field gel electrophoresis in terms of discriminatory power and reproducibility (Sabat et al. 2003).

The commonest method currently used to type staphylococcoci worldwide is PFGE. This technique has been hampered by problems of reproducibility both within and between laboratories. Large collaborative studies have recently been reported both in Europe and Canada which attempt to standardize methodology for typing of MRSA and thereby improve reproducibility (Mulvey et al. 2001; Murchan et al. 2003). MLEE has been used to examine *S. aureus* strains from a wide range of sources (Musser and Selander 1990; Musser and Kapur 1992) but is technically demanding. MLST is derived from the principles of MLEE but is sequenced-based (Enright et al. 2000); this is suited to the study of macro-epidemiology by virtue of the fact that this documents genetic change of highly conserved housekeeping genes. PFGE and MLST have been compared in a micro-epidemiological study conducted in a regional renal unit (Peacock et al. 2002b). This demonstrated that they have very similar discriminatiory abilities in this setting. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has been evaluated for its ability to monitor the bacterial fingerprints expressed by two *S. aureus* strains over time, and to define whether a profile for MRSA could be defined (Bernardo et al. 2002). Bacterial fingerprint data remained strain-specific data for subcultures analyzed over a period of 3 months, and after changing the growth media from Mueller–Hinton to blood agar. A uniform signature profile for MRSA could not be identified using clinical isolates, although the bacterial fingerprints obtained were specific for any given strain. The utility of this technique in epidemiological studies is currently undergoing investigation.

S. AUREUS DISEASE

Determinants of *S. aureus* infection

Determinants of infection can be divided into those associated with host and bacterial factors; whether a given individual develops *S. aureus* infection is likely to depend on a complex interplay between the two. This topic is very poorly understood overall. Little experimental work has been performed to examine host genetic susceptibility to either *S. aureus* carriage or disease. However, it is clear that host factors are important since individuals with disorders of the immune system affecting neutrophils have increased susceptibility for *S. aureus* infection. For example, a variety of *S. aureus* infections occur more frequently in association with chronic granulomatous disease, leukocyte adhesion deficiency syndromes and Job's syndrome (hyperimmunoglobulin E syndrome) (Nauseff and Clark 2000). A role for host determinants can also be inferred from the known risk factors for specific manifestations of *S. aureus* disease. For example, the onset of septic arthritis after closed joint trauma is well recognized. Possible explanations include facilitation of bacterial transmigration across the endothelial cell following upregulation of endothelial cell receptors, and extension of infection following bacterial seeding of local hematoma, but neither has been subjected to study. Similarly, there is an increased risk of *S. aureus* septic arthritis in those with inflammatory arthritides such as rheumatoid arthritis, but the mechanism is unclear and may be multifactorial. Diabetes is also associated with a higher risk of septic arthritis and other types of *S. aureus* infection, but the relative importance of host factors such as the higher *S. aureus* carriage rate, increased frequency of *S. aureus* skin sepsis, repeated needle puncture, and immunodeficiency is uncertain.

Acquired host factors also influence the likelihood of *S. aureus* infection and fall into two broad categories. The first is immune dysfunction, such as that present in patients undergoing hemodialysis who have decreased granulocytic chemotaxis and lower immunoglobulin, complement, and T cell levels (Greene et al. 1976; Waterlot et al. 1985), or in those undergoing chemotherapy for malignancy. The second is the presence of prosthetic devices or surgical incisions that breach the normal host defenses and give direct access to normally sterile body sites. Prosthetic material also provides a site of colonization to which staphylococci are well adapted. The importance of prosthetic material as a determinant of infection is reflected in the variable rates of sepsis associated with different types of hemodialysis vascular access. Endogenous arteriovenous fistulae are less prone to infection than prosthetic shunts (Bonomo et al. 1997), while intravenous hemodialysis catheters have the greatest associated risk and are a leading cause of *S. aureus* bacteremia in patients requiring renal replacement therapy (Hoen et al. 1998).

Bacterial determimants of infection have undergone extensive study using in vitro, ex vivo, and animal models. Signature-tagged mutagenesis was used to identify *S. aureus* genes required for virulence in a murine model of bacteremia. Transposons were inserted in genes of unknown function in half of the mutants identified, while the remaining genes were predominantly involved in nutrient biosynthesis and cell surface metabolism. Three mutants were found with transposon insertions in different positions in *femA*, and one mutant had an insertion in *femB*. Both *femA* and *femB* are involved in the formation of cell wall peptidoglycan pentaglycine cross-bridges. A further mutation occurred in a previously unknown gene that shares significant similarity to *femB*. Mutations were also obtained in *recA* and *lsp* (encoding the *S. aureus* prolipoprotein signal peptidase (Mei et al. 1997)). Signature-tag transposon mutants have also been screened in four complementary animal infection models including mouse abscess, wound, bacteremia, and rabbit endocarditis. The largest gene class identified encoded peptide and amino acid transporters, some of which were important for *S. aureus* survival in all animal infection models tested (Coulter et al. 1998).

Studies have been also been performed to examine the role of adhesins in disease pathogenesis. Fibrinogen and fibronectin binding proteins confer the ability to adhere to host proteins deposited on artificial surfaces (Vaudaux et al. 1984, 1989, 1993, 1995; Herrmann et al. 1988), and are likely to be important in the process of *S. aureus* device-related infection in humans. A role has been demonstrated for ClfA in the process of experimental *S. aureus* endocarditis (Moreillon et al. 1995; Que et al. 2001), while ClfB has a limited effect in this animal model (Entenza et al. 2000). Evidence for the involvement of FnBP in endocarditis is conflicting. Rats inoculated with a transposon mutant defective in fibronectin binding had fewer bacteria on the heart valve (Kuypers and Proctor 1989). A second study was unable to find a difference in valve bacterial counts between wild type *S. aureus* strain 8325-4 and an isogenic mutant defective in fibronectin binding (Flock et al. 1996). However, the nonpathogenic *Lactococcus lactis* engineered to express FnBPA required a lower infective dose to induce endocarditis (Que et al. 2001). Experimental septic arthritis has also been used widely to study disease pathogenesis. ClfA and Cna have been reported to be determimants of experimental septic arthritis (Patti et al. 1994; Josefsson et al. 2001), and Cna has been implicated as a virulence determinant in osteomyelitis (Elasri et al. 2002). FnBP was shown to be involved in adherence to human airway epithelium (Mongodin et al. 2002), although FnBP expression was associated with decreased virulence in a rat model of pneumonia (McElroy et al. 2002). A study comparing the presence

of 33 putative virulence determinants in a large number of *S. aureus* isolates associated with carriage and disease showed that the genes for three surface proteins (*fnbA*, *cna*, *sdrE*) were more commonly detected in invasive isolates (as were genes encoding enterotoxin J, gamma toxin, and intercellular adhesion (*ica*)). All seven factors contributed independently to virulence and no single factor predominating as the major predictor of virulence, their effect appearing to be cumulative (Peacock et al. 2002a).

The fibronectin binding proteins confer bacterial attachment to, and invasion of, a range of host cells in vitro (Ogawa et al. 1985; Vercellotti et al. 1984; Peacock et al. 1999; Sinha et al. 1999, 2000; Dziewanowska et al. 1999; Lammers et al. 1999; Fowler et al. 2000; Massey et al. 2001). Cell invasion is an active process and appears to involve host cell integrins (Sinha et al. 1999; Fowler et al. 2000; Dziewanowska et al. 1999; Massey et al. 2001). The suggestion that *S. aureus* can exist as a facultative intracellular pathogen in vivo where it can avoid host cell defenses and the effect of antibiotics is attractive but currently unproven. The relevance to human disease of experimental data on putative bacterial virulence factors is uncertain.

CLINICAL SYNDROMES

S. aureus causes a wide range of infections. These can be broadly divided into community- and hospital-acquired sepsis. Community-acquired infections include the following: toxin-mediated disease (for example, food poisoning and toxic shock syndrome); infections affecting the skin and soft tissues (such as boils, impetigo, cellulitis, and myositis); infection of bones and joints; infection relating to other deep sites (endocarditis, abscess formation in liver, spleen, and other sites), and infections of the lung and of the urinary tract. Meningitis also occurs, although this is rare as a single presentation and usually occurs in the setting of endocarditis complicated by septic embolization. This wide range of conditions occur both in those with known risk factors, and in individuals who were previously fit and well. Nosocomial or hospital-acquired infections can include the diseases described above, but are more commonly represented by surgical wound infection, ventilator-associated pneumonia, bacteremia associated with intravenous devices, and infection associated with other types of prosthetic material such as cerebrospinal fluid shunts, prosthetic joints, and vascular grafts. The rise in use of intravenous devices for treatment of individuals in the community blurs the distinction between community and hospital infection, since nosocomial types of infection are increasingly occurring in people living at home. The commonest deep site infections are endocarditis, and bone and joint infections (Fowler et al. 2003).

Bacteremia and endocarditis

Around two thirds of patients with *S. aureus* bacteremia are nosocomially acquired, most of which are associated with intravenous-device related infection; the remainder represent community-acquired bacteremia. Fever is common at presentation, but other clinical features will be influenced by the presence and site of foci of infection at other sites. The overall condition of the patient can range from good to one of profound septic shock. A major element in the management of patients with *S. aureus* bacteremia is to distinguish between those with uncomplicated versus complicated bacteremia (that is, bacterial dissemination from the bloodstream to other sites). This is a common event; a recent study of 724 adult patients presenting to hospital with *S. aureus* bacteremia identified complications in 43 percent (Fowler et al. 2003). Presence of complications will determine length of treatment and the need for investigations and additional interventions. A careful clinical examination should be conducted for features of deep infection including signs of endocarditis, septic arthritis, and pain suggestive of bone involvement. However, these are often not apparent, as demonstrated by a recent study of the clinical features of *S. aureus* endocarditis over a 10 year period in Denmark in which the diagnosis was not suspected and was first detected at autopsy in 32 percent of patients (Roder et al. 1999). Serological testing is not useful in this setting (nor indeed in any other in relation to staphylococcal disease) to confirm the diagnosis, or to define complicated bacteremia or extent of disease. A study of clinical identifiers of complicated *S. aureus* bacteremia represents significant progress (Fowler et al. 2003). A scoring system based on the presence or absence of four risk factors (community acquisition, skin examination findings suggesting acute systemic infection, persistent fever at 72 h, and positive follow-up blood culture results at 48–96 h) was able to accurately identifiy complicated *S. aureus* bacteremia. The findings of this study allow readily available clinical variables to be used in the identification of patients requiring further investigation and prolonged therapy.

Bone and joint sepsis

Staphylococcus aureus is the leading cause of primary septic arthritis and osteomyelitis in all ages except neonates (Baker and Schumacher 1993; Lew and Waldvogel 1997). These conditions may be complicated by disabling joint destruction, sequestration of necrotic bone leading to chronic pain and sepsis, or spinal destruction that may lead to deformity, paralysis or death. The essential sequence of events during these diseases is bacteremia, followed by seeding from the

bloodstream to bone or joint, bacterial proliferation and the development of arthritis/osteomyelitis, and finally consequences in the form of tissue damage. Chronicity may ensue, particularly if the infection is partially or untreated. Initial access of bacteria to bone or joint occurs either via hematogenous spread or directly, due to some breach in the overlying soft tissues. In the case of direct spread, the process that injures the overlying tissues often also injures the bone or joint itself, or rapidly sets up conditions that harm exposed bone or cartilage. These conditions include open fractures (where the bone is exposed), surgery involving the bone (including joint replacement or internal fracture-fixation), and chronic ulceration down to bone or joint (for example, in the diabetic foot, or in a bed-bound patient with a pressure sore). Often, however, no such direct communication is established and in these cases it appears that organisms have successfully entered the bone or joint from the circulation. Once present in the bone or joint, bacterial multiplication triggers a host inflammatory response, the features of which are increased blood flow, increased capillary permeability to fluids and cells, and the recruitment of inflammatory cells, initially neutrophil polymorphs. If this immune response is unsuccessful in controlling infection, the result is intense inflammation surrounding an area with high densities of bacteria and the development of an abscess. This presents as a swollen and exquisitely painful, immobile joint or a bone that is so painful that normal function is impossible. Secondary bacteremia is common at this point in the absence of antibiotic therapy. On presentation, fever is common but once again the overall condition of the patient can be highly variable, and symptoms and signs will depend on the site affected.

Pulmonary infections

S. aureus infection may involve the lung parenchyma, pleural cavity or both. Infection arises either due to inhalation of the organism from a site of colonization (community-acquired), via an endotracheal tube in ventilated patients, or more rarely because of hematogenous spread in a bacteremic patient. No clinical or radiological features are typical of *S. aureus* pneumonia. Microbiological diagnosis is complicated by the fact that *S. aureus* is a normal colonizer of the upper respiratory tract (predominantly the nose) in a proportion of normal individuals. As a result, interpretation of *S. aureus* in sputum culture is difficult, and more invasive procedures such as bronchoscopy and lavage may be necessary. A syndrome of rapidly progressive *S. aureus* necrotizing pneumonia affecting previously healthy children and young adults has recently been described (Gillet et al. 2002). The strains responsible were positive for Panton-Valentine leuokocidin, and pneumonia was often preceded by influenza-like symptoms.

Toxin-mediated staphylococcal diseases

Food poisoning caused by staphylococcal enterotoxins results from ingestion of preformed toxin present in food (Dinges et al. 2000). It is a result of poor standards of food handling in which *S. aureus* is inoculated from a skin lesion into food under conditions where bacteria can multiply before human consumption. The mechanism of action of the toxin is still under study, but it is thought to initiate the emetic response through interactions with the emetic reflex located in the abdominal viscera, with subsequent activation of the medullary emetic center in the brain stem which is stimulated via the vagus and sympathetic nerves (Dinges et al. 2000). The incubation period is between 2 and 6 h, which is followed by nausea and vomiting. Abdominal pain and diarrhea are also common features. The disease is self-limiting and usually resolves within 24 h. Diagnosis is made on clinical grounds; suspected food can be cultured for the presence of *S. aureus*. Management is centered on replacement of fluids and electrolytes, and antibiotic treatment is not necessary.

Staphylococcal scalded skin syndrome (SSSS) is caused by the effect of exfoliative toxins produced by *S. aureus*. Readers are referred to a review by Ladhani et al. (1999). This disease primarity affects neonates and young children. Severity varies from localized skin infection (bullous impetigo) to generalized involvement of the skin. SSSS usually follows a localized infection in sites such as the umbilical stump, ear or conjuntiva. Skin lesions in SSSS are formed within the epidermis, with midepidermal splitting. Affected individuals present with fever, lethargy, poor feeding, and irritability, followed by a tender red rash which can spread to involve the entire body within a few days. Blisters develop which are fragile, thin roofed, and rapidly rupture to leave denuded areas. Patients develop poor temperature control, suffer large fluid losses, and may develop secondary infection. Diagnosis of SSSS is a clinical one, aided by Nikolsky's sign in which the skin wrinkles on gentle pressure. Culture of blister fluid in SSSS is usually negative since the blisters have developed as a result of toxin formed by *S. aureus* at a distant site (although culture is often positive in cases of bullous impetigo). Fluid and electrolyte replacement forms the mainstay of treatment. Antibiotics are given to eradicate the toxin-producing *S. aureus* isolate. Affected neonates should be isolated and strict barrier precautions applied to prevent crossinfection. Fever usually settles after 2 or 3 days and no new bullae form, although it takes 2–3 weeks for the skin lesions to resolve completely.

Toxic shock syndrome (TSS) is a severe disease characterized by high fever, hypotension, diffuse erythematous rash with subsequent desquamation 1–2 weeks later, and involvement of three or more organ systems.

Readers are referred to a review by Dinges et al. (2000). An epidemic of TSS in young women in the early 1980s associated with menstruation and the use of high absorbancy tampons was caused by TSST-1 producing organisms (Dinges et al. 2000). The number of cases associated with tampons has declined as a result of modifications in tampon manufacture. Nonmenstrual TSS is also well recognized, the associated isolates being positive for TSST-1 in 50 percent of bacterial strains, and positive for SEB and SEC in 47 and 3 percent, respectively. Nonmenstrual cases encompass a wide range of *S. aureus* disease types including invasive *S. aureus* infection; noteworthy inclusions in this group are patients with wound infection in which clinical features of wound sepsis are not evident, and influenza-associated cases. Clinical features of TSS may include mucous membrane hyperemia, myalgia, vomiting and diarrhea, renal and hepatic impairment, altered level of consciousness, coagulopathy and low platelet count. The diagnosis is made on clinical grounds. The CDC have devised a clinical case definition to assist in this process (CDC 1997). Patients may have features consistent with TSS but fail to meet the CDC diagnostic criteria (Dinges et al. 2000). The results of laboratory tests can be helpful in reaching a diagnosis in these cases (Parsonnett 1998). These are: isolation of *S. aureus* from a mucosal or normally sterile body site; production by this organism of TSST-1 or other superantigen known to be associated with TSS; absence of antibodies to the relevant toxin at time of illness; and seroconversion to this toxin with detection of antibodies during convalescence. Management often includes care in a high dependency setting. The presence of tampons should be determined and removed, and possible sources of infection in nonmenstrual cases considered. Cultures should be taken for isolation of *S. aureus* and testing of these isolates for toxin production, together with serum samples for serological testing in the convalescent phase in patients in whom the diagnosis is in doubt. A β-lactamase resistant anti-staphylococcal antibiotic should be given to eradicate the toxin-producing strain of *S. aureus*. Clindamycin has been advocated for use as an adjunct to penicillin for the treatment of streptococcal toxic shock syndrome because of its ability to reduce toxin production in vitro (Mascini et al. 2001; Sriskandan et al. 1997). Evidence for the value of clindamycin in TSS caused by *S. aureus* is scanty. One study compared the effect of clindamycin, flucloxacillin, or flucoxacillin plus gentamicin on growth and toxin production in vitro. All three inhibited toxin production during logarithmic growth, with inhibition of 75, 30, and 75 percent of the control, respectively, during stationary phase growth (van Langevelde et al. 1997). Clindamycin was bacteriostatic for logarithmic phase cultures, while flucoxacillin alone or in combination with gentamicin was rapidly cidal. The relevance of these data to human disease is unclear, but it is common practice in many centers to administer a β-lactamase-resistant anti-staphylococcal antibiotic plus clindamycin. The case fatality rate is reported to be 3 percent, although this reflects the outcome of individuals treated in affluent nations; the outcome for people in developing countries is not known.

Management of other *S. aureus* infections

Basic principles for the management of infection apply. Pus should be drained, and infected joints washed out in a theater setting by an orthopedic surgeon to reduce damage and prevent poor joint function. Use of investigations should be guided by features and clinical suspicion. Tools which aid definition of disseminated infection or extent of disease includes transthoracic and transesophageal echocardiography, ultrasound, CT scan, and MRI. Prosthetic material associated with infection should be removed where possible, for example, temporary intravenous devices. Infected prosthetic material of a more permanent nature such as heart valves and vascular grafts may be salvaged by long courses of antibiotics. As a broad generalization, skin and soft tissue infections are treated until clinical cure; bacteremia without features of seeding requires two weeks of therapy; infection of deep sites require four weeks or more of treatment, depending on the type and extent of infection. Endocarditis involving prosthetic valves requires six weeks of therapy. The antibiotic used will depend on susceptibility of the infecting strain. Flucloxacillin is the drug of choice for MSSA, while MRSA treatment will depend on the presence of resistance to other drugs but usually relies on, or includes, a glycopeptide such as vancomycin. Those involved in management of this potentially complicated patient group are referred to published guidelines and more detailed text for an indepth review.

PREVENTION OF *S. AUREUS* INFECTION

Prevention of *S. aureus* disease falls into three main categories:

1. eradication of *S. aureus* carriage;
2. hospital infection control measures to prevent nosocomial infection, including perioperative antibiotic prophylaxis; and
3. vaccination strategies.

Eradication of *S. aureus* carriage

There is considerable evidence to indicate that nasal carriage of *S. aureus* and the development of staphylococcal infection are linked. Rates of infection are higher in carriers than in noncarriers in a range of

clinical settings, including the development of postoperative wound infections (Weinstein 1959), in patients undergoing continuous ambulatory peritoneal dialysis and hemodialysis (Yu et al. 1986; Luzar et al. 1990), and in those infected with HIV (Weinke et al. 1992). Studies comparing carriage and infecting isolates of *S. aureus* show that individuals are usually infected with their own carriage isolate (Yu et al. 1986; Luzar et al. 1990; Nguyen et al. 1999). This has been clearly shown in a large study in which greater than 80 percent of isolates from *S. aureus* bacteremic infections were identical to strains carried by these individuals (von Eiff et al. 2001b). The temporary eradication of carriage by the use of topical mupirocin has been reported to result in a reduction in nosocomial infection in dialysis patients and in those undergoing surgery. Several strategies have been used to eradicate *S. aureus* carriage; none result in a permanent loss of *S. aureus* carriage and treatments need to be repeated. The most common approach is to use topical antibiotics such as mupirocin applied to the nose or to exit sites of prosthetic devices such as intravenous dialysis catheters and peritoneal dialysis catheters. This antibiotic blocks protein synthesis by competitively inhibiting isoleucyl tRNA. Lysostaphin is currently undergoing evaluation; this is a peptidoglycan hydrolase secreted by *Staphylococcus simulans* which cleaves the polyglycine interpeptide bridges of the *S. aureus* cell wall. Lysostaphin has been shown to have efficacy in the treatment of experimental *S. aureus* endocarditis (Climo et al. 1998; Patron et al. 1999), and keratitis (Dajcs et al. 2000). Exposure of *S. aureus* to lysostaphin alone has been reported to result in the emergence of lysostaphin-resistant mutants in vitro and in vivo (Climo et al. 2001). These mutants could be suppressed in an animal model of experimental endocarditis by the coadministration of antibiotics (Climo et al. 2001). A single application of 0.5 percent lysostaphin formulated in a petrolatum-based cream has been shown to eradicate *S. aureus* nasal colonization in 93 percent of animals in a cotton rat model. A single dose of lysostaphin cream was more effective than a single dose of mupirocin ointment in eradicating *S. aureus* nasal colonization in this animal model (Kokai-Kun et al. 2003). Topical lysostaphin could become an important agent in clinical practice, but studies of efficacy, safety, and emergence of resistance during use in humans are awaited.

Most research on eradication of nasal carriage has been carried out in patients requiring renal replacement therapy. This is a high risk group in whom *S. aureus* disease represents an important cause of morbidity and mortality. Eradication of nasal carriage in hemodialysis patients using topical mupirocin to the nose has been shown to reduce the number of episodes of *S. aureus* infection per patient year (Boelaert et al. 1989, 1993; Holton et al. 1991; Kluytmans et al. 1996). Efficacy of nasal *S. aureus* carriage eradication has also been evaluated for patients receiving peritoneal dialysis. Overall, evidence for the efficacy of nasal mupirocin in preventing morbidity in continuous ambulatory peritoneal dialysis (CAPD) patients is not convincing. Prophylactic intranasal application of mupirocin has been assessed in a large randomized, double-blind, placebo-controlled trial of patients undergoing surgery to examine effect on nasal carriage and the effect on rates of postoperative *S. aureus* infection. Prophylactic intranasal application of mupirocin did not significantly reduce the rate of *S. aureus* surgical-site infections overall, although it did significantly decrease the rate of all nosocomial *S. aureus* infections among the patients who were *S. aureus* carriers (Perl et al. 2002). The effect of topical mupirocin applied to the dialysis catheter exit sites has also been assessed. The peritoneal dialysis catheter exit site is the most frequent colonizing site of strains causing peritonitis (Amato et al. 2001), and two studies have shown that topical application of mupirocin to the exit site was associated with a reduction in exit-site infection and peritonitis (Bernardini et al. 1996; Thodis et al. 1998). However, the routine application of mupirocin to the exit site of all patients has potentially important implications for the emergence of drug resistance.

An evidenced based review has recently been published to critically appraise the published evidence regarding the efficacy of intranasal mupirocin for eradication of *S. aureus* nasal carriage and for prophylaxis of infection. Sixteen randomized, controlled trials were appraised, of which nine trials assessed eradication of colonization as a primary outcome measure, and seven assessed the reduction in the rate of infection. Mupirocin was generally highly effective for eradication of nasal carriage in the short term, but this effect did not convert into clinical benefit overall. The prophylactic treatment of patients with intranasal mupirocin in large trials did not lead to a significant reduction in the overall rate of infections. However, subgroup analyses and several small studies revealed lower rates of *S. aureus* infection among selected populations of patients with nasal carriage treated with mupirocin. The authors concluded that although mupirocin is effective at reducing nasal carriage, routine use of topical intranasal mupirocin for infection prophylaxis is not supported by the currently available evidence (Laupland and Conly 2003). A Cochrane review focused on MRSA has been performed to examine the effects of topical and systemic antimicrobial agents on nasal and extra-nasal MRSA carriage, adverse events, and incidence of subsequent MRSA infections. There was no demonstrated superiority of either topical or systemic therapy, or of combinations of these agents. It was noted that potentially serious adverse events and development of antimicrobial resistance can result from therapy (Loeb et al. 2003).

Hospital infection control and antibiotic prophylaxis

Hospital infection control measures can prevent a proportion of nosocomial infections. Central to this process is handwashing which reduces transmission of pathogens between individuals, and from the hands of a given individual to sites such as dialysis catheters that are at increased risk of infection. Perioperative antibiotic prophylaxis is also important in preventing surgical sepsis. There are a wide range of other measures which can be used; implementation will depend on the global setting and health care infrastructure. In resource-rich countries, hospital infection control is implemented by a team of individuals who devise policies, educate staff, monitor hospital infections either through a diagnostic microbiology laboratory or by active ward-based surveillance, and implement outbreak procedures where necessary. Medical staff are educated and actively encouraged to follow guidelines which reduce infection rates in given settings. For example, high quality care of intravenous devices includes the use of protocols for line insertion and line care, training and education of staff regarding line insertion and after care, and good mechanisms for recording line insertion, removal, and infective complications to facilitate audit.

Vaccination

Potential applications for an effective *S. aureus* vaccination are broad. Patients undergoing medical treatments such as hemodialysis or other forms of therapy requiring the use of intravenous devices are growing in number in both hospital and community settings. This group are at high risk of developing *S. aureus* bacteremia, and are likely to derive considerable benefit from a vaccine that prevents disease, or else complications of *S. aureus* bacteremia such as endocarditis and bacterial seeding to distant sites including bones, joints, and solid organs. An argument could also be made for vaccine use in many other patient groups with increased risk of *S. aureus* disease. For example, patients undergoing surgery such as joint replacement or cardiothoracic surgery where infection is a disaster could be targeted, or vaccine could be applied more widely to the operative group. Finally, vaccination could be extended to the healthy population to reduce or prevent serious community acquired invasive disease.

At the time of writing there is no licensed anti-staphylococcal vaccine. The most significant human anti-staphylococcal vaccine study published to date used a single dose of a conjugate vaccine comprising *S. aureus* type 5 and 8 capsular polysaccharides conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A. Evaluation by a randomized trial of 1 804 adult patients at 73 hemodialysis centers demonstrated that this conferred partial immunity against *S. aureus* bacteremia for approximately 40 weeks, after which protection waned as antibody levels decreased (Shinefield et al. 2002).

Many studies have been conducted to examine the effect of DNA vaccines in experimental animal models. These can be roughly divided into vaccine targets against bacterial determimants that are always, or almost always, present such as ClfA and FnBP, and those which are variably present in populations of clinical isolates including Cna, enterotoxins and PBP2a. Vaccination against ClfA has been examined by several investigators, with mixed results. Mice immunized with recombinant ClfA and challenged with *S. aureus* developed less severe arthritis than controls, and passive immunization of mice with rat and rabbit anti-ClfA antibodies protected against *S. aureus* arthritis and sepsis-induced death (Josefsson et al. 2001). A second group reported that a DNA vaccine directed against ClfA did not protect mice against an intraperitoneal challenge of *S. aureus*, despite a strong and specific antibody response (Brouillette et al. 2002). Vaccination using fibronectin binding proteins as the target has also been examined. FnBP has been shown to reduce the bacteria count on an injured heart valve in an animal model of endocarditis (Schennings et al. 1993), to lower the bacterial count in rabbit blood and excised organs compared with controls after intravenous inoculation (Park et al. 1999), and to lead to a more rapid bacterial clearance from mouse peritoneum and liver after peritoneal inoculation (Rozalska and Wadstrom 1993). FnBP vaccination also reduced disease severity in a mouse mastitis model (Mamo et al. 1994). This line of investigation has subsequently been hampered by the observation that blocking of the interaction between FnBPs and fibronectin is limited to 50 percent inhibition in vitro. One explanation for this is that antibodies raised in vivo are predominantly to epitopes that are presented by the ligand–FnBP complexes, that is, after binding has occurred. These have been termed ligand-induced binding sites. Mapping of immunodominant epitopes has been performed (Rozalska et al. 1994; Sun et al. 1997), and current research directions include the generation of antibodies using recombinant FnBP protein with preserved antigenic activity but reduced ligand-binding activity (Rennermalm and Li 2001; Brennan et al. 1999; Huesca et al. 2000).

Vaccination of mice against PBP2a (the penicillin binding protein expressed by methicillin resistant strains) followed by a sublethal dose of MRSA reduced the *S. aureus* counts in the kidney compared with nonimmunized controls (Ohwada et al. 1999; Senna et al. 2003). Crossprotection against the PBP of MSSA would be required for good clinical efficacy. Vaccination using a recombinant fragment of the *S. aureus* collagen adhesin or passive transfer of collagen adhesin-specific

antibodies protected mice against sepsis-induced death caused by inoculation of the Cna-positive strain Phillips (Nilsson et al. 1998). Potential clinical application may be restricted since only around half of clinical isolates carry the gene encoding Cna. Toxins have also been evaluated as target vaccine candidates. Immunization of mice with recombinant staphylococcal enterotoxin A (SEA) devoid of superantigenic properties provided good protection against *S. aureus* sepsis (Nilsson et al. 1999), and vaccination against staphylococcal enterotoxin B (SEB) provided protection from the effect of subsequent toxin challenge given intraperitoneally or mucosally (Stiles et al. 2001). However, many clinical isolates do not express SEA or SEB. *S. aureus* synthesize poly-N-succinyl beta-1-6 glucosamine (PNSG) as a surface polysaccharide during human and animal infection, although few strains expressed PNSG in vitro. All *S. aureus* strains examined carried genes for PNSG synthesis, and immunization protected mice against kidney infections and death from strains that produced little PNSG in vitro (McKenney et al. 1999). An approach to the identification of *S. aureus* vaccine candidates has been reported in which a comprehensive list of immunologically relevant proteins is identified. In brief, *S. aureus* proteins are displayed in vitro using an expression library and screened using antibodies in sera from patients. A large number of antigenic antigens have been identified for further evaluation (Etz et al. 2002; Weichhart et al. 2003).

Coagulase-negative staphylococcal infection

Overall, CoNS have a low pathogenic potential, and infection usually arises in the severely compromised or in association with prosthetic material. Important patient groups affected by CoNS bacteremia are preterm low birth weight infants, and individuals who are immunocompromised by diseases such as cancer and the chemotherapy which renders them neutropenic. The latter patient group almost invariably require intravenous devices for treatment, and these are usually implicated as the source of the bacteremia. CoNS bacteremia can also affect many other patient groups with intravenous devices, although the severity of clinical manifestations is often less severe. CoNS can become involved in infection relating to any other type of prosthetic device, those commonly encountered in clinical practice including heart valves, pacemaker lines and boxes, cerebrospinal shunts, and prosthetic joints. CoNS are also the leading cause of peritonitis associated with peritoneal dialysis. Some CoNS infections occur in association with native tissue in apparently healthy individuals, an important example of which is endocarditis. It is also a signifcant cause of community-acquired urinary tract infection, *S. saprophyticus* being the most common member of the group implicated.

Clinical manifestations of CoNS infection will be dictated by host factors (such as prematurity and immunocompromise), the site affected, and the presence or absence of prosthetic material. CoNS infection involving prosthetic material is often associated with a relative lack in the host inflammatory response compared with that seen with *S. aureus* infection, and profuse pus production is rare. Device-related CoNS infection in an immunocompetent individual will usually result in mild local signs and symptoms, and bacteremia is extremely unusual with the exception of individuals with infected devices positioned within the vascular tree, such as a prosthetic heart valve or vascular graft. Those who are immunocompromised may present with a systemic response, most often in relation to a bacteremia. Diagnosing CoNS infection is often difficult; factors that can be taken into consideration during this process have been discussed in detail above. A central part of patient management is removal, where possible, of the colonized device. Treatment duration will depend on the persistent presence of prosthetic material and the underlying condition of the host. Choice of antibiotics will be guided by the results of antibiotic susceptibility testing, but hospital-acquired strains are commonly methicillin-resistant. Cure may require prolonged antibiotics if prosethetic material persists and relapse in such cases is not unusual. This has been attributed to poor penetration of antibiotics into bacterial biofilms; some antibiotics such as rifampicin are added on the basis that they have been shown to penetrate bacterial biofilms in vitro. A multidisciplinary team approach for the management of patients with these often complex conditions can make an important contribution towards high standards of care.

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Streptococcus and *Lactobacillus*

MOGENS KILIAN

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The genera *Streptococcus* and *Lactobacillus* belong to the lactic acid bacteria, which is a taxonomically diverse group of gram-positive, nonspore-forming cocci and rods defined by the formation of lactic acid as a sole or major end-product of carbohydrate metabolism (Wood and Holzapfel 1995). Based on 16S and 23S rRNA sequence data, gram-positive bacteria form two lines of descent. The typical lactic acid bacteria, which are characterized by a G + C content of less than 50 mol%, belong to the '*Clostridium* branch' (Schleifer and Ludwig 1995). For many years the genera *Streptococcus* and *Lactobacillus* were both allocated to the family *Lactobacillaceae* but now belong to separate families *Streptococcaceae* and *Lactobacillaceae*.

STREPTOCOCCUS

GENUS DEFINITION

Streptococci are spherical or ovoid cells, arranged in chains or pairs. They are gram-positive. All species are nonmotile, nonsporing, and catalase-negative, with complex nutritional requirements. All are facultatively anaerobic; most will grow in air but some require the addition of carbon dioxide for growth. All species fail to reduce nitrate. They ferment glucose with the production mainly of lactic acid, never of gas. Many species are members of the commensal microflora on mucosal membranes of humans or animals, and some are highly pathogenic. The G + C content of the DNA is 36–46 mol%. The genus, named according to its typical morphology (from the Greek *streptos*, pliant; *coccos*, a grain or berry) currently includes 53 species that are listed in Table 1. The type species is *Streptococcus pyogenes*.

INTRODUCTION AND HISTORICAL PERSPECTIVE

The term *Streptococcus* was first applied by Billroth (1874) to a chain-forming coccus he saw in infected

Table 1 *Species of the genus* Streptococcus*: serological and hemolytic reactions, peptidoglycan type, and main habitat*

Phylogenetic group	Lancefield serogroup	Serotype antigen	Hemolysis	Peptidoglycan type	Main habitat
Pyogenic group					
S. pyogenes	A	M– and T–antigens (protein)	β	Lys–$Ala_{1–3}$	Humans
S. agalactiae	B	Capsular polysaccharide	β (CAMP+)	Lys–$Ala_{1–3}$(Ser)	Humans, cattle
S. canis	G		β (CAMP+)	Lys–Thr–Gly	Many animals, humans
S. didelphis	–		β	ND	Opossums
S. dysgalactiae					
subsp. *dysgalactiae*	C		α, β,–	Lys–$Ala_{1–3}$	Pigs, cattle
subsp. *equisimilis*	C, G, L, A		β	Lys–$Ala_{1–3}$	Humans
S. equi					
subsp. *equi*	C		β	Lys–$Ala_{1–3}$	Horses, donkeys
subsp. *zooepidemicus*	C		β	Lys–$Ala_{2–3}$	Many animals
S. hyointestinalis	–		α	Lys–Ala(Ser)	Pigs
S. iniae	–		β, α	Lys–$Ala_{1–3}$	Freshwater dolphins
S. parauberis	–, E		α	ND	Cattle
S. phocae	–, F, C		β	ND	Seals
S. porcinus	E, P, U, V		β (CAMP+)	Lys–$Ala_{2–4}$	Pigs
S. uberis	–, E		α	Lys–$Ala_{1–3}$	Cattle
S. urinalis	–		–	ND	Humans
Anginosus group					
S. anginosus	–, F, C, G, A		α, β	Lys–$Ala_{1–3}$	Humans
S. constellatus				Lys–$Ala_{1–3}$	Humans
subsp. *constellatus*	–, F, C, G, A		–, α, β		
subsp. *pharyngis*	C		β		
S. intermedius	–, F, C		α, β	Lys–$Ala_{1–3}$	Humans
Mitis group					
S. mitis	O		α	Lys–direct	Humans
S. oralis	–		α	Lys–direct	Humans
S. pneumoniae	O	Capsular polysaccharide	α	Lys–Ala_2(Ser)	Humans
S. infantis	O		α	ND	Humans
S. gordonii	H1, H2, –[a]		α	Lys–$Ala_{1–3}$	Humans
S. sanguis	H1, –		α	Lys–$Ala_{1–3}$	Humans
S. parasanguis	–		α	ND[b]	Humans
S. australis	–		α	Lys-Ala-Gly	Humans
S. crista	–		α	ND	Humans
S. peroris	–		α	ND	Humans
S. sinensis	–		α	ND	Humans
Salivarius group					
S. salivarius	H2, K, –		–	Lys–$Ala_{2–3}$, Lys–Thr–Gly	Humans
S. vestibularis	–		–	ND	Humans
S. thermophilus	–		–	Lys–$Ala_{2–3}$	Milk, dairy products
Bovis group					
S. equinus/'*S. bovis*'	D		α	Lys–Thr–Ala	Horses, other ruminants
S. alactolyticus	D		α, –	ND	Pigs, chickens
S. gallolyticus	D, –		–, α	ND	
subsp. *gallolyticus*			–, α	ND	Marsupials and mammals (cows, horses, pigs, dogs, guinea pigs, sheep, humans)
subsp. *macedonicus*			–, α	ND	Dairy products

(*Continued over*)

Table 1 *Species of the genus* Streptococcus: serological and hemolytic reactions, peptidoglycan type, and main habitat (*Continued*)

Phylogenetic group	Lancefield serogroup	Serotype antigen	Hemolysis	Peptidoglycan type	Main habitat
S. infantarius	–		α	ND	Humans
S. lutetiensis	–		α	ND	Humans
S. pasteurianus	ND		α	ND	Humans
Mutans group					
S. mutans	–, E	Cell wall carbohydrate c, e, f	–, β, α	Lys–$Ala_{2–3}$	Humans
S. cricetus	–	a	–, α	Lys–Thr–Ala	Hamster, rats, humans
S. sobrinus	–	d, h, g, (or–)	α, –	Lys–Thr–Ala	Humans
S. downei	–	h	–	Lys–Thr–Ala	Monkeys
S. rattus	–	b	–	Lys–$Ala_{2–3}$	Rats, humans
S. macacae	–	c	–	ND	Monkeys
S. ferus	–	c	–	Lys–$Ala_{2–3}$	Rats, pigs
S. orisratti	–	ND		ND	Rats
Unclustered species					
S. suis	D[c]	Capsular polysaccharide	β, α, –	Lys–direct	Pigs, cattle
S. acidominimus	–		α	Lys–Ser–Gly	Cattle
S. intestinalis	–, G		β	ND	Pigs
S. gallinaceus	–		α	ND	Chickens
S. ovis	–		α	ND	Sheep
S. entericus	D		α	ND	Cattle
S. hyovaginalis	ND		α	ND	Pigs
S. pluranimalium	ND		α	ND	Cattle, goats, cats, canaries
S. thoraltensis	ND		α	ND	Pigs

a) H1, Reaction detected in antiserum against strain Blackburn ('European group H'); H2, Reaction detected in antiserum against strain F90A ('American group H').
b) ND, Not determined.
c) Previously designated group antigens R, S, and T are capsular polysaccharide types 2, 1, and 15, respectively.

wounds. The first good accounts of *S. pyogenes* appear to be those of Pasteur (1879) and Rosenbach (1884) who described its morphology, cultural appearance, and virulence for mice and rabbits. Since then, the genus has accommodated a broad spectrum of bacteria from benign organisms used in the dairy industry to true pathogens of humans and several animal species. As a result of more recent taxonomic studies, this complex situation has been resolved to some extent with the transfer of the dairy species to the new genera *Lactococcus* and the motile *Vagococcus*, the enteric streptococci to the genus *Enterococcus* (*Enterococcus*), the anaerobic streptococci to *Peptostreptococcus* (Gram-positive anaerobic cocci), and some species to the genus *Gemella*. The so-called 'nutritionally variant streptococci,' which for many years were considered variants of recognized *Streptococcus* species, were included in a new genus *Abiotrophia* (Kawamura et al. 1995b). Some *Abiotrophia* species were subsequently transferred to a separate genus, *Granulicatella* (Collins and Lawson 2000). The two latter genera will be discussed in this chapter. For a comprehensive discussion of other catalase-negative gram-positive cocci, the reader is referred to reviews by Facklam and Elliott (1995) and Ruoff (2003).

The phylogenetic relationships between the genus *Streptococcus* and the numerous other genera of catalase-negative, gram-positive cocci have been studied extensively by Collins and his coworkers and others (Aguirre et al. 1993; Collins et al. 1989; Williams et al. 1989; Stackebrandt and Teuber 1988; Ludwig et al. 1985). Schleifer and Ludwig (1995) have published a comprehensive discussion of this topic.

HABITATS

All the *Streptococcus* species are obligate parasites of mucosal membranes and, for some species, tooth surfaces of humans and several animals. Many of the species are life-long and dominant members of the commensal microflora on mucosal membranes of the upper respiratory tract and some colonize the intestinal and genital tracts of humans and various animals. A few species exclusively colonize tooth surfaces and are present only after tooth eruption. Most of these

members of the resident flora may cause infection when introduced into normally sterile compartments of the body or in immunocompromised patients. Other species are true pathogens that spread from patient to patient and cause infection in nonimmune individuals.

MORPHOLOGY

Streptococci are spherical cells that may be more or less elongated and arranged in pairs or chains of up to 50 cells or more. Chain formation is most pronounced in broth media. Individual cells are typically 0.5–1.0 × 1.0–2.0 μm. Growth occurs by elongation on the axis parallel to the chain. Cross-walls form at right angles to the chain and after division an appearance of pairing may remain (Figure 1). Some species may develop rod-like cells depending on the growth conditions (Clarke 1924; Rosan and Eisenberg 1973). Members of the genus are nonmotile in the conventional sense, although a twitching motility associated with polar fimbriae has been described for *S. sanguis* isolates (Henriksen and Henrichsen 1975).

A variety of proteinaceous surface fibrils and filaments, sometimes organized in tufts, have been described in some *Streptococcus* species (Swanson et al. 1969; Handley 1990; Kawamura et al. 2000). Some of these structures form a 'fuzzy coat' on the entire surface of the cells and appear to play a role in adherence and in some cases as a layer protecting against phagocytosis.

Capsulation is not considered a regular feature of streptococci but a distinct polysaccharide capsule has been identied and characterized in several of the more virulent species. Recent studies of polysaccharides associated with the cell wall of the commensal *S. mitis* identified a structure which is likely to constitute a variable (type-specific) capsular polysaccaride (Bergström et al. 2000, 2003). This conclusion is supported by the fact that the genome of *S. mitis* contains an operon, which includes all the genes required for synthesis and transport of a capsular polysaccharide (www.tigr.org/tdb/ufmg/). This suggests that capsule production may be more widespread among streptococci than hitherto believed.

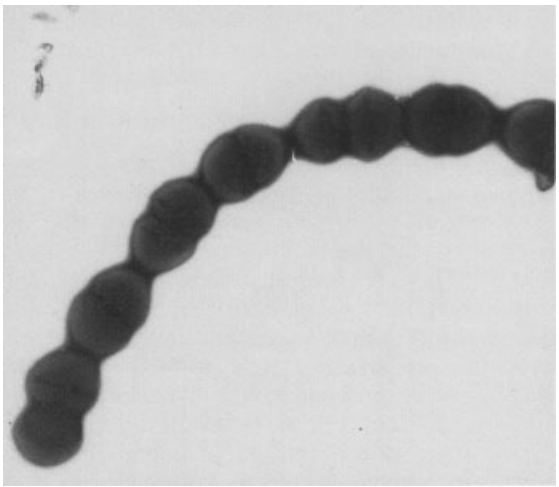

Figure 1 *Electron micrograph of* S. pyogenes *M-type 2 from a broth culture (×12 500) (kindly supplied by Dr C.A.M. Fraser and Mr A.A. Porter).*

Several of the oral streptococci form extracellular polysaccharides when grown in the presence of sucrose. These are simple polysaccharide structures that are synthesized on the cell surface and do not become covalently linked to the cell wall. Some of these extracellular polysaccharides are soluble and others are insoluble and play a role in forming the matrix of biofilms on tooth surfaces (Guggenheim 1970).

Streptococci stain readily with ordinary dyes and are almost frankly gram-positive although old cultures may show variable staining.

CULTURAL CHARACTERISTICS

Growth on ordinary nutrient media is generally poor in contrast to that of enterococci. On media enriched with blood, serum, or a fermentable carbohydrate, growth is more profuse, but colonies on blood agar seldom exceed 1 mm in diameter after 24 h at 37°C. Some species characteristically form relatively large colonies whereas others form colonies of pinpoint size. Growth on agar media by some strains of *S. pneumoniae*, the mutans streptococci, and *S. anginosus* requires a supplement of carbon dioxide to the incubation atmosphere. Many streptococci grow more readily under anaerobic conditions than in air plus carbon dioxide.

In general, colonies of streptococci on blood agar are nonpigmented. The exception is the majority of members of the species *S. agalactiae*, which form an orange–red pigment, which presumably is a β-carotenoid (Tapsall 1986).

When cultivated on sucrose-containing agar media, such as Mitis Salivarius agar, some of the viridans streptococci and bovis group streptococci produce extracellular polysaccharides, which may facilitate their recognition (Figure 2). The polysaccharide will in some of the species (*S. sanguis*, *S. gordonii*, *S. oralis*, and the mutans streptococci) result in coherent and adherent colonies that may be difficult to subcultivate on to agar plates or into fluid media.

The yield of cells from broth cultures is increased by the addition of a fermentable carbohydrate but the rapid fall in pH becomes inhibitory and the bacteria eventually die. In buffered media containing glucose, such as Todd–Hewitt broth, or in media with a high glucose content in which a neutral pH is maintained by the regulated addition of alkali, a heavy yield of streptococci is obtained.

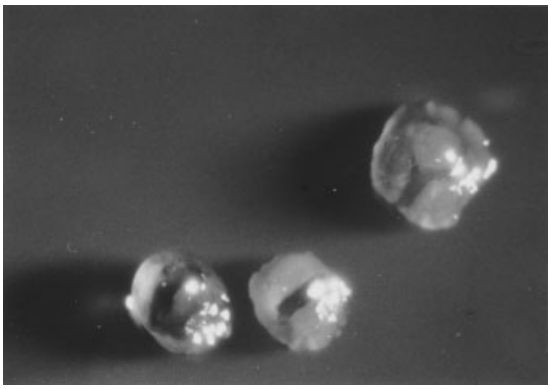

Figure 2 S. mutans *grown on sucrose-containing Mitis Salivarius agar (×10). Incubated in N_2 (90 percent) + CO_2 (10 percent), 24 h, 37°C and then in air, 24 h, room temperature*

Hemolysis

The ability of certain clinically important streptococci to induce zones of complete hemolysis (β-hemolysis) around colonies on blood-containing agar media was one of the first characters to be recognized and used for distinguishing between isolates. Other isolates of streptococci induce a zone of greenish discoloration (α-hemolysis) on blood agar, and yet others cause no detectable changes to the red blood cells. β-hemolysis is caused by well-characterized hemolysins (see below) whereas α-hemolysis is due to hydrogen peroxide released from the colonies and inducing oxidation of hemoglobin to methemoglobin. The latter phenomenon is most clearly detected on chocolate agar because heating of the added blood destroys its inherent catalase activity.

METABOLISM

Members of the genus *Streptococcus* are facultatively anaerobic with a preference for anaerobic conditions. They obtain energy, as well as some precursors for the synthesis of cellular material, strictly via fermentation of carbohydrates and are incapable of respiratory metabolism, either aerobically or anaerobically (Poolman 1993). They are unique amongst bacteria able to grow aerobically in that they do not synthesize porphyrins, cytochromes, or catalase, and are not capable of forming ATP via an electron transport system. Detailed information about the metabolic characteristics of several *Streptococcus* species is now available from analyses of complete genome sequences (Tettelin et al. 2001; Ferretti et al. 2001; Hoskins et al. 2001; Glaser et al. 2002; Tettelin et al. 2002; Ajdic et al. 2002).

Carbohydrate metabolism

Streptococci degrade glucose via the Embden–Meyerhof glycolytic pathway, which is driven by constitutive enzymes. Most of the streptococci are able to ferment various other sugars and sugar alcohols by means of inducible enzymes that are synthesized only in the presence of each of the carbohydrates and in the absence of glucose. Available biochemical and genome data suggest that *S. mutans* is capable of metabolizing a wider variety of carbohydrates than any other gram-positive bacterium examined in detail (Ajdic et al. 2002).

Active transport of several monosaccharides, disaccharides, and sugar alcohols through the cytoplasmic membrane into the cytoplasma takes place with the help of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) transporters. This is a high-affinity 'group translocation' process which utilizes phosphoenolpyruvate as an energy source and results in the transport and phosphorylation of the sugar to the inner surface of the cell (Reizen and Peterkofsky 1987). In addition, the energetically less-efficient ATP-binding cassette (ABC) transporters are used for the import of some sugar species (Hoskins et al. 2001; Glaser et al. 2002). Analysis of the genome of *S. agalactiae* identified 17 sugar-specific PTS enzyme II complexes and four sugar-specific ABC transporters, in addition to three glycerol permeases and one glycerol-phosphate permease (Glaser et al. 2002). In *S. pneumoniae* more than 30 percent of the transporters were predicted to be sugar transporters, which is the highest percentage observed to date in any sequenced prokaryote (Tettelin et al. 2001).

The metabolic characteristics of streptococci mentioned emphasize the importance of sugars in their lifestyle. A significant source of these sugars is likely to be host glycoproteins. Thus, streptococci produce various glycosidases that enable them to take advantage of the sugar residues in glycoproteins present in the secretions in their natural habitat (Pinter et al. 1969; Bradshaw et al. 1994). However, some of the streptococci, in particular oral streptococci, may encounter considerable fluctuations in the amount of carbohydrate available in their natural habitat and adjust their metabolism accordingly. When glucose or other fermentable carbohydrate is supplied in excess, the vast majority is converted to L-lactate through pyruvate. However, with low sugar levels, other more energy efficient pathways of pyruvate conversion are operating. Under anaerobic conditions, streptococci use the pyruvate–formate lyase pathway and generate formic and acetic acids and ethanol as endproducts (Figure 3). Under aerobic conditions, mutans streptococci use pyruvate dehydrogenase to convert pyruvate to acetic acid and ethanol, while some of the other oral streptococci use pyruvate oxidase with the formation of acetic acid and hydrogen peroxide as endproducts (Carlsson and Hamilton 1994).

In times of carbohydrate excess streptococci may synthesize intracellular polysaccharide (glycogen), which is utilized to provide maintenance energy when

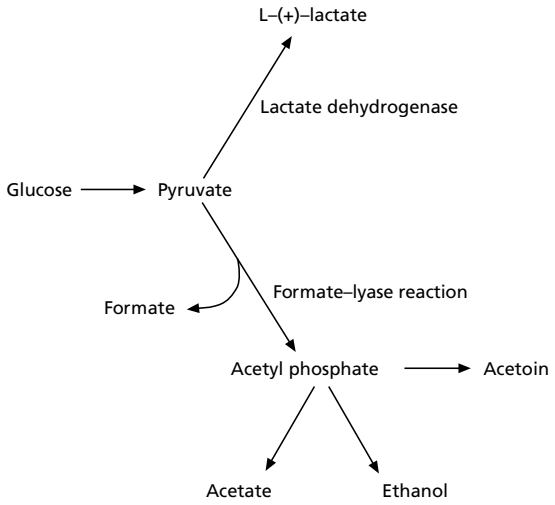

Figure 3 *Pathways of pyruvate metabolism in streptococci*

exogenous sources are absent. In addition, some of the species synthesize extracellular polysaccharides but only from sucrose (see Table 3). Some of these polysaccharides may function as storage of carbohydrate, whereas others seem to perform other functions.

Some of the oral streptococci, in particular *S. mutans* and *S. sobrinus*, show a remarkable degree of acid tolerance. This is based primarily on the presence of a membrane-bound, acid-stable, proton-translocating F_0F_1 ATPase that can maintain the intracellular pH at 7.5 (Quivey et al. 2001; Ajdic et al. 2002).

Other nutritional requirements

The nutritional requirements of streptococci in general are complex and include amino acids, peptides, purines, pyrimidines, vitamins, and salts in addition to the energy source (Milligan et al. 1978b). From genome analyses it is clear that *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* completely lack the triocarboxylic acid (TCA) cycle, depriving them from the ability to synthesize the precursors of most amino acids (Ferretti et al. 2001; Tettelin et al. 2001; Glaser et al. 2002). However, some of the oral streptococci will grow in the presence of sugar, vitamins, and salts with ammonia as the sole nitrogen source (St. Martin and Wittenberger 1980). Accordingly, all amino acid biopsynthetic pathways and a partial TCA cycle were identified in the *S. mutans* genome. In addition to being able to synthesize glutamine *S. mutans* also possesses five predicted glutamine ABC transporters, emphasizing the importance of this amino acid as a principal source of nitrogen and a substrate for amino acid biosynthesis in this species (Ajdic et al. 2002).

In their natural habitats the requirements for nitrogenous compounds may be satisfied by amino acids excreted by other members of the resident microflora or released from local secretions or tissues by bacterial proteinases. The utilization of peptides depends upon adequate mechanisms for transport across the cytoplasmic membrane and intracellular peptidases capable of hydrolyzing the peptides to amino acids. Genome analyses of various streptococci have demonstrated a variety of ATP- and ion-driven amino acid transporters, as well as transporters for polyamines and, in some cases, choline, which is an important component of the cell wall of some streptococci (see below) (Ferretti et al. 2001; Tettelin et al. 2001; Glaser et al. 2002). Some of the streptococci are able to degrade arginine by the arginine deaminase system, which is an energy-yielding reaction. Accordingly, animal experiments have demonstrated that arginine in the diet favors the selection in the mouth of arginine-utilizing streptococci such as *S. sanguis* and *S. anginosus* (van der Hoeven et al. 1985; Rogers et al. 1990).

The growth requirements of streptococci are usually met in blood agar, which combines meat extract, peptones, and blood. However, the nutritionally variant streptococci (*Abiotrophia* and *Granulicatella* spp.) will grow only in media that contain supplements of cysteine or pyridoxal hydrochloride.

GENETIC MECHANISMS

Streptococcal genetics has a long history and has been of paramount importance in the developments of genetics and biology in general. Genetic transformation of a bacterial property (capsule production) was first demonstrated in pneumococci by Griffith in 1928. These studies subsequently led to the identification of DNA as the genetic material ('transforming principle') (Avery et al. 1944). Recently, streptococcal genetics and knowledge of streptococci in general have been significantly boosted by the complete genome sequencing of three *S. pyogenes* strains, two *S. agalactiae* strains, two *S. pneumoniae* strains, and one strain each of *S. mutans*, *S. mitis*, *S. sanguis*, *S. gordonii*, *S. equi*, *S. suis*, and *S. uberis* (Ferretti et al. 2001; Hoskins et al. 2001; Tettelin et al. 2001; Ajdic et al. 2002; Beres et al. 2002; Glaser et al. 2002, Smoot et al. 2002a; Tettelin et al. 2002; www.tigr.org/tdb/ufmg, www.sanguis.mic.vcu.edu; www.sanger.ac.uk/projects/microbes). Several additional genome sequences of streptococci are available in the commercial domain. Application of genome sequence data to gene chips has been employed in studies of global gene expression in *S. pyogenes* during active infection and intracellularly in neutrophils (Graham et al. 2002; Voyich et al. 2003).

There is substantial evidence that horizontal gene transfer and recombination play an important role in the short-term evolution of *Streptococcus* species and in the generation of genetic diversity within natural populations of streptococci (Simpson et al. 1992; Hollingshead

et al. 1994; Whatmore et al. 1994). Such processes, including the rapid spread of genes determining virulence factors and antibiotic resistance, appear to be responsible for fluctuations observed in the prevalence of serious infections due to pyogenic streptococci and in the spread of antibiotic resistance from commensal to pathogenic species (Musser et al. 1993a, b; Dowson et al. 1990, 1993). Furthermore, horizontal transfer plays a significant role in the spread of drug resistance genes between streptococci and between streptococci and other bacteria. However, the genetic mechanisms involved differ, as does the frequency of horizontal gene transfer in the natural population of the individual species.

Comparison of the three available genome sequences of *S. pyogenes* strains (M1, M3, and M18) and screening of multiple other strains clearly reveals that lysogenization with temperate phages is the predominant source of genetic diversification within *S. pyogenes*. Four to six complete prophage genomes present in the M1 and M3 strains made up approximately 10–12 percent of the genomes. Variations among strains range from total absence of prophages to differences in the gene contents of the prophages (Ferretti et al. 2001; Beres et al. 2002). In general, 90 percent of *S. pyogenes* isolates are lysogenic. Genes that are phage-associated and may be horizontally transferred between strains by transduction encode several virulence factors (see below) including hyaluronidase, superantigenic toxins, bacteriocin determinants, DNases, and phospholipase A (Zabriskie et al. 1972; Malke 1972; Tagg et al. 1976; Totolian 1979; Banks et al. 2002; Wagner and Waldor 2002; Canchaya et al. 2003). Although phage-associated virulence genes are not regularly expressed in broth cultures of clinical isolates, growth in mice or in coculture with human pharyngeal cells induces expression via a small heat-stable factor released from host cells (Kazmi et al. 2001; Broudy et al. 2001).

The temperate phages, which lysogenize streptococcal strains, have a relatively narrow host range (Maxted 1964). However, horizontal spread of phage-associated genes among several species of pyogenic *Streptococcus* species is suggested by the presence of sequence-identical genes (Canchaya et al. 2003). The great majority of clinical isolates of *S. pneumoniae* carry prophages of two types (Siphoviridae and Myoviridae), but no particular function has been identified (Ramirez et al. 1999). However, the genomes of neither strain R6 nor strain TIGR4 showed evidence of complete prophages (Hoskins et al. 2001; Tettelin et al. 2001). Two platelet-binding surface proteins (PblA and PblB) expressed by *S. mitis* strains were shown to be encoded by phage DNA (Bensing et al. 2001). No prophage genome was detected in *S. mutans* strain UA159 (Ajdic et al. 2002).

Streptococcal phages also play a role in mobilizing nonphage-encoded antibiotic resistance genes. There is evidence that streptococcal phages have transferred resistance to tetracycline, chloramphenicol, macrolides, lincomycin, clindamycin, and streptomycin (Wagner and Waldor 2002).

Transformation appears to be the principal mechanism of horizontal gene transfer in *S. pneumoniae*, *S. gordonii*, and other species belonging to the mitis and anginosus phylogenetic groups (see below). Competence for genetic transformation is a transient state that is induced in exponentially growing cultures by quorom sensing, and enables streptococcal cells to take up extracellular DNA and incorporate it into their genomes by homologous recombinantion. Competence is controlled by the competence stimulating peptide (CSP), a product of the gene *comC*, and includes activation of a CSP secretion apparatus (ComAB) and a two-component regulatory system (ComDE) (Hui and Morrison 1991; Håvarstein et al. 1995; Pestova et al. 1996; Håvarstein and Morrison 1999). CSP will induce competence when its concentration in the growth medium reaches 1–10 ng/ml by triggering the signal transduction pathway consisting of the CSP receptor histidine kinase ComD and its cognate response regulator ComE (Håvarstein et al. 1996). Competence for transformation in at least some strains of *S. infantis* appears not to be dependent on a competence pheromone (Ween et al. 2002). Although a significant portion of the transformation mechanism appears to be present in *S. pyogenes*, the crucial late genes for expression of competence, comABC cannot be identified in the genome (Ferretti et al. 2001).

Many *Streptococcus* species contain conjugative as well as nonconjugative plasmids of several sizes (LeBlanc and Lee 1979; Simpson and Tagg 1984) that may encode antibiotic resistance traits. However, early reports demonstrated the en bloc transfer of resistance to several antibiotics by a mechanism unrelated to plasmids (Buu-Hoi and Horodniceanu 1980; Horodniceanu et al. 1981, 1982). Clewell and coworkers have provided a molecular basis for plasmid-free resistance transfer with the characterization of a streptococcal tetracycline resistance conjugative transposon (Gawron-Burke and Clewell 1982).

Conjugative transposons and insertion sequences are widespread among streptococci and play a significant role in the dissemination of drug resistance (Clewell and Gawron-Burke 1986; Horaud et al. 1991). One of the transposon families found in streptococci, Tn*916*/Tn*1545* displays a remarkably broad host range which encompasses a large number of both gram-positive and gram-negative bacteria. Tn*916* is an 18 kb element originally discovered in *Enterococcus faecalis* and Tn*1545* a 25.3 kb element first detected in *S. pneumoniae*. The transposons are transferred between bacterial cells by a conjugation-like mechanism requiring contact between donor and recipient. They become integrated in the bacterial genome by an excision/insertion process that

can take place at numerous AT-rich target sites. In this way, transposons are capable of disrupting the expression of particular genes, a property that provides an important method for the development of streptococcal strains deficient in defined properties and for identification of genes (transposon mutagensis). Tn*916*-like chromosomal elements have been reported to exist in *S. pyogenes*, *S. agalactiae*, *S. equisimilis*, *S. anginosus*, *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguis*, and '*S. bovis*' and in multiple other gram-positive and gram-negative species (Clewell et al. 1995).

Insertion sequences (IS) of multiple types are abundantly present in all streptococcal genomes that have been analyzed. In *S. agalactiae*, disruption of the gene encoding hyaluronidase by an IS element explains the lack of this enzyme activity in some clinical isolates (Granlund et al. 1997).

While the relatively unrestricted recombination of genomic DNA sequences transferred by transformation among pneumococci (and possibly some of the closely related commensal streptococci) results in an epidemic population structure (panmictic population with successful spread of seleted clones), a basically clonal population structure is maintained in *S. pyogenes* and *S. agalactiae*, because horizontal transfer only to a limited degree affects house-keeping genes outside prophages and other mobile genetic elements (Lomholt 1995; Hauge et al. 1996; Enright and Spratt 1998; Enright et al. 2001; Jones et al. 2003).

CELL-WALL COMPOSITION; ANTIGENIC STRUCTURE

The cell wall of streptococci consists of the shape-forming peptidoglycan (murein), various carbohydrate structures including teichoic acids, and a number of proteins, which form an interwoven complex.

Reliable methods for the preparation of cells walls from streptococci have been described by Salton and Horne (1951). The bacterial cells are broken by rapid oscillation with small glass beads. Adherent intracellular debris and cell membranes are then removed by washing with buffers or detergents followed by enzymatic digestion. Extraction of the isolated walls with hot formamide removes the cell wall carbohydrate. The insoluble residue after formamide extraction is the peptidoglycan. Extraction with trichloroacetic acid is the classical method for extraction of teichoic acids from the cell wall, but the extract is not necessarily representative of the various forms present (Knox and Wicken 1973). A lysozyme-like lytic enzyme (phage-associated lysin), which is produced by phages infecting group C streptococci, is able to degrade the cell wall peptidoglycan of *S. pyogenes* and has been extensively employed in procedures for isolation of wall-associated proteins in that species (Fischetti 1989).

Cell wall peptidoglycan

As in other gram-positive cell walls, the peptidoglycan consists of multiple glycan chains that are cross-linked though short peptides (Schleifer and Kandler 1972). The glycan moiety is composed of alternating β-1,4-linked units of *N*-acetylglucosamine and *N*-acetylmuramic acid. The carboxyl group of muramic acid is substituted by an oligopeptide that contains alternating L-and D-amino acids, which in streptococci always include lysine (Figure 4). Adjacent oligopeptide chains from parallel glycan chains are cross-linked either directly or via an interpeptide bridge between the ϵ-amino group of lysine in one peptide and the D-alanine of a second chain. The type of bridge is consistent within most species and shows some characteristic differences (Table 1). The most common interpeptide bridge in streptococci consists of 1–4 L-alanine residues (Lys-Ala$_n$). In some species (e.g. members of the bovis and mutans groups) the alanine residues can be partly replaced by L-threonine and/or L-serine. The two species *S. oralis* and *S. mitis* lack an interpeptide bridge and have directly linked oligopeptide chains (Lys-direct). Detailed studies of the pneumococcal peptidoglycan have disclosed several types of cross-linking, one of which resembles that found in gram-negative bacteria (Fischer and Tomasz 1985; Garcia-Bustos et al. 1987). Most of the enterococci and lactococci, which are now allocated to separate genera, have different types of interpeptide bridges (Lys-D-Asp) with the exception of *E. faecalis* (Lys-Ala$_{2–3}$) (Schleifer and Kilpper-Bälz 1987). The methods that can be employed for the determination of the composition and structure of peptidoglycans are outlined by Schleifer and Seidl (1985).

Cell wall carbohydrates

The streptococcal cell wall contains two forms of polysaccharides, one of which constitutes the Lancefield group carbohydrate. Although initially believed to form a continuous layer outside the peptidoglycan, ultrastructural studies reveal that the group polysaccharide traverses the wall and may be found both on the inner and the outer surfaces of isolated cell walls (Rýc et al. 1989; Sørensen et al. 1988; Wagner and Wagner 1985). In addition to the group polysaccharides, all streptococcal cell walls contain lipoteichoic acid which is initially attached in the cell membrane, but may become associated with various parts of the cell wall. In addition to the group carbohydrate and lipoteichoic acid, a capsular polysaccharide is present in several species and usually copurifies with the cell wall as it is covalently attached to the wall peptidoglycan.

The group carbohydrates of the *Streptococcus* species are complex, branched polysaccharide structures. Many of them contain phosphodiester bonds and, accordingly,

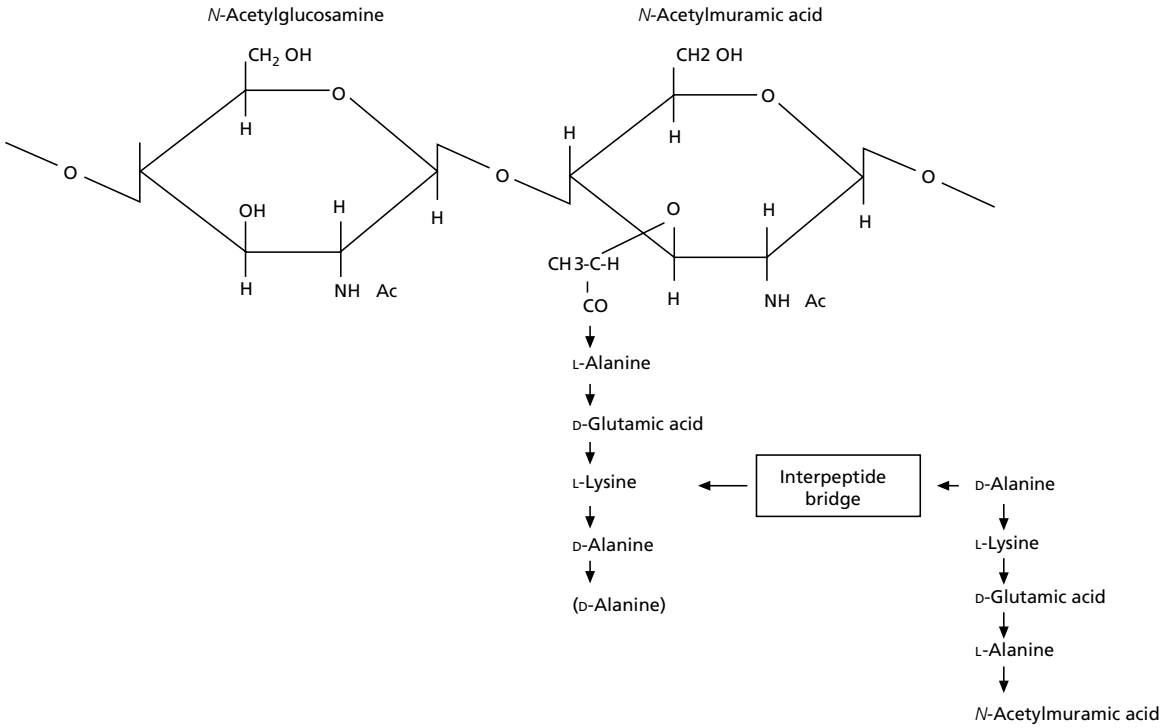

Figure 4 *Repeating unit of cell wall peptidoglycan of* Streptococcus *species. The major variability between species is in the structure of the interpeptide bridge (see Table 1).*

may be characterized as teichoic acids. Rhamnose is a major constituent in the majority of species (Slade and Slamp 1962; Colman and Williams 1965). The exception are the four closely related species, *S. pneumoniae*, *S. oralis*, *S. mitis*, and *S. infantis*, in which ribitol is present (Colman and Williams 1965; Rosan 1978; Gillespie et al. 1993; Bergström et al. 2000, 2003). Rhamnose also appears to be missing in some strains of serogroups K, R, and S, but it is unclear how the variation in these serogroups corresponds with named species (Slade and Slamp 1962).

Structural studies show that the polysaccharides of groups A (*S. pyogenes*) and C (*S. dysgalactiae* and *S. equi*) contain an identical backbone consisting of α-1→2- and α-1→3-linked L-rhamnopyranose molecules, but differ in their limited side branches: the group A polysaccharide carries *N*-acetylglucosamine residues linked β-(1→3) to alternate rhamnose rings and group C disaccharides of *N*-acetylgalactosamine, which are the immunodominant substituents (McCarty 1959; Coligan et al. 1978; Höög et al. 2002). The so-called group A-variant polysaccharide, which may be detected in strains of *S. pyogenes* after several mouse passages, is not substituted (Coligan et al. 1978). The group B polysaccharide of *S. agalactiae* consists of a backbone composed of rhamnose, glucitol, and phosphate, and trisaccharide side chains composed of rhamnose, galactose, and *N*-acetylglucosamine that are linked to the 4-position of a rhamnose in the backbone of the polysaccharide (Pritchard et al. 1984).

The group polysaccharides in members of the species *S. oralis*, *S. mitis*, *S. sanguis*, and *S. gordonii* likewise contain different hexa- or heptasaccharide repeating units linked end to end by phosphodiester bonds (Ward 1981; McIntire et al. 1988; Abeygunawardana et al. 1989, 1990, 1991; Reddy et al. 1993, 1994). In the case of *S. pneumoniae*, *S. mitis*, *S. oralis*, and *S. infantis* ribitol is one of the constituents (Colman and Williams 1965; Rosan 1978; Bergström et al. 2000). The group polysaccharide of *S. pneumoniae* and *S. mitis* is identical and consists of repeating units of 6)-β-D-Glc*p*-(1→3)-α-AAT*p*-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-ribitol-5-*P*-(O→ (where AAT is 2-acetamino-4-amino-2,4,6-trideoxy-D-galactose), and contains one or two phosphocholine residues per repeat attached to the 2-acetamido-2-deoxy-β-galactose (D-Gal*p*NAc) residues. Minor variations in the backbone structure were also described (Jennings et al. 1980; Kulakowska et al. 1993; Karlsson et al. 1999; Bergström et al. 2000). This polysaccharide constitutes the Lancefield group O antigen and is referred to as C-polysaccharide in *S. pneumoniae* (Bergström et al. 2000). In occasional strains of *S. mitis*, phosphocholine is substituted by ethanolamine (Bergström et al. 2003). The wall polysaccharides of many of the oral species contain the lectin recognition determinant involved in adhesion to other microorganisms (coaggregation) (Glushka et al. 1992; Cisar et al. 1995; Holmes et al. 1995; Xu et al. 2003).

Presumably all streptococcal species contain lipoteichoic acids in the cell wall in addition to the group

polysaccharide described above. In most species the lipoteichoic acid is a glycerol teichoic acid. These structures are linear polymers of 1→3-linked glycerol phosphate residues and may include glycosyl substituents and D-alanine on O-2 of the glycerol moiety. They are covalently attached to a terminal hydrophobic region comprised of fatty acids, which is integrated in the cell membrane (Fischer 1997). The glycerol teichoic acid of *S. pyogenes* is composed of a chain of 25–30 glycerol phosphate units joined by phosphodiester linkages with ester-linked molecules of D-alanine of the peptidoglycan layers or fatty acid of the plasma membrane. The closely related species *S. pneumoniae*, *S. oralis*, *S. mitis*, and possibly *S. infantis*, contain ribitol teichoic acids instead of glycerol teichoic acid (Rosan 1978; Hogg et al. 1997). The chain structure of the ribitol lipoteichoic acid in *S. pneumoniae* (referred to as the F-antigen) is identical to that of its group polysaccharide (see structure above) (Fischer et al. 1993). It is conceivable that this also is the case for *S. mitis* and *S. oralis* (Hogg et al. 1997).

The lipoteichoic acid of streptococcal cell walls is synthesized in the cytoplasmic membrane where it may remain anchored though the glycolipid tail. However, in the model proposed for *S. pyogenes*, lipoteichoic acid is not anchored in the cytoplasmic membrane but remains associated with the cell surface by interactions with M protein (Beachey and Simpson 1982). Similar models have been proposed for oral streptococci (Shockman et al. 1986). In the case of some of the oral streptococci, lipoteichoic acid may become associated with extracellular polysaccharide (Rölla et al. 1980; Hogg and Lightfoot 1992) and considerable amounts are released into the environment depending on the growth conditions (Jacques et al. 1979; Wicken et al. 1982). Quantitative studies have shown that lipoteichoic acid represents only 2–4 percent of phenol extractable contents from *S. sanguis* (Hogg and Old 1993).

Although a definitive function for lipoteichoic acid has yet to be described, there has been considerable interest in its potential as a mediator of bacterial adhesion, particularly in the case of *S. pyogenes* (Ofek et al. 1975; Beachey and Courtney 1987; Hasty and Courtney 1996), *S. agalactiae* (Nealon and Mattingly 1984) and *S. mutans* (Rölla et al. 1978). The model that has been proposed for the involvement of lipoteichoic acids in adhesion requires the mentioned release of the amphipathic lipoteichoic acid from the cytoplasmic membrane and subsequent orientation at the cell surface with the fatty acid moieties available for interaction with hydrophobic domains on the surface of the epithelial cell (Ofek et al. 1982). This initial phase is followed by a secondary phase during which the glycerolphosphate backbone of the lipoteichoic acid interacts with epithelial cells (Nealon and Mattingly 1984). It is notable that the lipoteichoic acids of strains of *S. pyogenes* may vary significantly with regard to chain length, structure, and biological activities (Leon and Panos 1990).

Cell wall proteins

Streptococci produce an array of proteins associated with the cell wall (Kehoe 1994; Navarre and Schneewind 1999). They are generally exposed on the outer surface of the cell wall. Many of them protrude as filamentous structures of different length and may even protrude through the capsule as in *S. pyogenes* and *S. agalactiae* (Wagner and Wagner 1985; Fischetti 1989). Predictions based on two genomes of *S. pneumoniae* suggest that this species expresses at least 69 different surface-exposed proteins (Tettelin et al. 2001; Hoskins et al. 2001). The biological functions and structure of some of the surface exposed proteins in pyogenic streptococci and pneumococci are discussed under Pathogenicity and virulence factors .

Antigenic structure

Antigenic analysis of cell wall components and capsules has played a major, and sometimes excessive, role in the classification of members of the genus *Streptococcus* and as an epidemiological tool. Historically, the cell wall carbohydrates have received most attention as a result of the original work of Rebecca Lancefield on pyogenic streptococci isolated from infections. The cell wall carbohydrate that can be extracted from whole streptococcal cells by a number of techniques including treatment with hot hydrochloric acid (Lancefield extraction) (Facklam and Washington 1991) will in some cases show a specific precipitate with antisera prepared by injecting whole cells into rabbits (Lancefield 1933). Such carbohydrate antigens are the Lancefield group antigens and are termed A–W (with the exception of I and J). Although serological grouping remains a valuable means of characterizing streptococci of medical importance, attempts to equate the Lancefield groups with species have not been universally successful. Therefore, accurate species identification should not be based on the results of serogrouping alone (Brandt et al. 1999). The association of group antigens with streptococcal species is shown in Table 1. As shown in this table the group A antigen is characteristic of *S. pyogenes* but occasional strains of other species may also react with group A antisera. The group B antigen is an exclusive characteristic of *S. agalactiae*, whereas several species may carry antigens reactive with group C, F, and G antisera. Reactivity of extracts of strains of *S. pneumoniae* with a Lancefield group C antiserum is due to cross-reactivity between the group C carbohydrate and lipoteichoic acid (F antigen) of that species (Sørensen and Henrichsen 1987). The group D antigen, which is a teichoic acid, is associated with several species in the bovis group and with streptococci now allocated to the genus *Enterococcus*. Likewise, the group N antigen, also a teichoic acid, is associated with streptococci now allocated to the genus *Lactococcus*.

The group antigens can be demonstrated by various techniques. Thus, group-specific, precipitating, agglutinating, and fluorescently labeled antibody preparations, which can be used to examine extracts, cell suspensions, and spent broth media, are commercially available. Most valuable are group A, B, C, F, and G antisera for characterization of isolates of β-hemolytic streptococci. Detection of the group A antigen taken in swabs directly from the respiratory tract is successfully used in commercial kits for identification of throat infections with *S. pyogenes* (Facklam and Washington 1991). There is some confusion concerning the group H antigen as two different strains with distinct carbohydrate antigens have been used for the production of group H antisera. One is referred to as 'American group H' and the other as 'British group H' (Cole et al. 1976) (see Table 1).

A similar serotyping strategy has been applied to differentiate among the mutans streptococci although there has been no attempt to include this in the grouping system (Bratthall 1970; Perch et al. 1974; Beighton et al. 1981) (see Table 1).

The highly diverse filamentous M and T protein antigens of *S. pyogenes* (see below) form the basis of serologic typing of this species, which has played a significant role in epidemiological studies (see below) (Colman et al. 1993). Likewise, the diverse polysaccharide capsules produced by members of the species *S. pneumoniae*, *S. agalactiae*, and *S. suis* form the basis of valuable typing systems for these bacteria (see below).

CLASSIFICATION

Since its original description, the genus *Streptococcus* has undergone numerous revisions. Several subgroups of species have been transferred to new genera, new species have been added and existing species have been redefined or deleted. The major part of this development occurred within the last 20 years (Hardie 1986a, b, c; Rotta 1986), and is a result of the application of molecular and chemotaxonomic approaches to this group of bacteria. A comprehensive discussion of the early history of streptococcal taxonomy has been provided by Jones (1978).

The slow development of a satisfactory taxonomy of streptococci is due to several factors. One problem has been an overriding belief in serological reactions and hemolytic activities as reliable taxonomic criteria. Although serological grouping based on cell wall carbohydrates has been a valuable tool when applied to the more pathogenic streptococci, as originally intended by Lancefield (1933), this approach was less successful and sometimes misleading for the rest of the streptococci, in which group-specific antigens may be absent or shared by several distinct taxa. The same limitations apply to hemolysis. Although type of hemolysis is a useful marker in the initial recognition and examination of clinical isolates (Table 1) it is important to recognize that differences occur within species and that reactions depend on incubation procedures, and the origin of the blood incorporated in the agar. Another problem, which mainly applies to commensal streptococci, is that the extreme genetic diversity that exists within this group has been difficult to appreciate until DNA sequencing became a taxonomic tool.

Since the publication of the last edition of this book in 1998, the number of *Streptococcus* species has been increased by more than 50 percent. Many of these new species are difficult to identify by phenotypic characterization and require DNA techniques for unequivocal identification.

Intrageneric structure

Comparative analyses of 16S rRNA sequences of members of the genus *Streptococcus* have revealed interesting phylogenetic relationships between species and an intrageneric structure with correlation to pathogenic properties and ecology (Whiley et al. 1990b; Williams and Collins 1990; Bentley et al. 1991; Kawamura et al. 1995a). The subdivision of the genus into five major clusters and a more diffuse group based on 16S rRNA gene sequences (Figure 5) corroborates results of studies based on nucleic acid hybridization, and numerical taxonomic analyses (Carlsson 1968; Colman and Williams 1972; Coykendall and Specht 1975; Coykendall and Munzenmaier 1978; Bridge and Sneath 1983; Kilpper-Bälz and Schleifer 1984, 1987; Kilpper-Bälz et al. 1985; Coykendall et al. 1987; Whiley and Hardie 1989; Whiley et al. 1990a; Whiley and Beighton 1991).

The five clusters are:

1. the pyogenic group consisting predominantly of β-hemolytic species that are pathogenic in humans or animal species
2. the anginosus group of streptococci found in the human oral cavity, gastrointestinal and genital tracts and sometimes isolated from infections
3. the mitis group which includes the pathogen *S. pneumoniae* and several oral streptococci
4. the salivarius group consisting of both dairy streptococci and species found in the human oral cavity
5. the bovis group of species inhabiting the intestinal canal of several animal species and sometimes humans.

In addtion to these well-defined clusters, the term 'the mutans group' of streptococci is used for eight phenotypically similar species (Table 1) which, however, are genetically rather diffuse (Figure 5). Members of these species mainly colonize tooth surfaces of humans and a number of animal species.

Several species are not related to any particular phylogenetic cluster (Table 1, Figure 5). The so-called

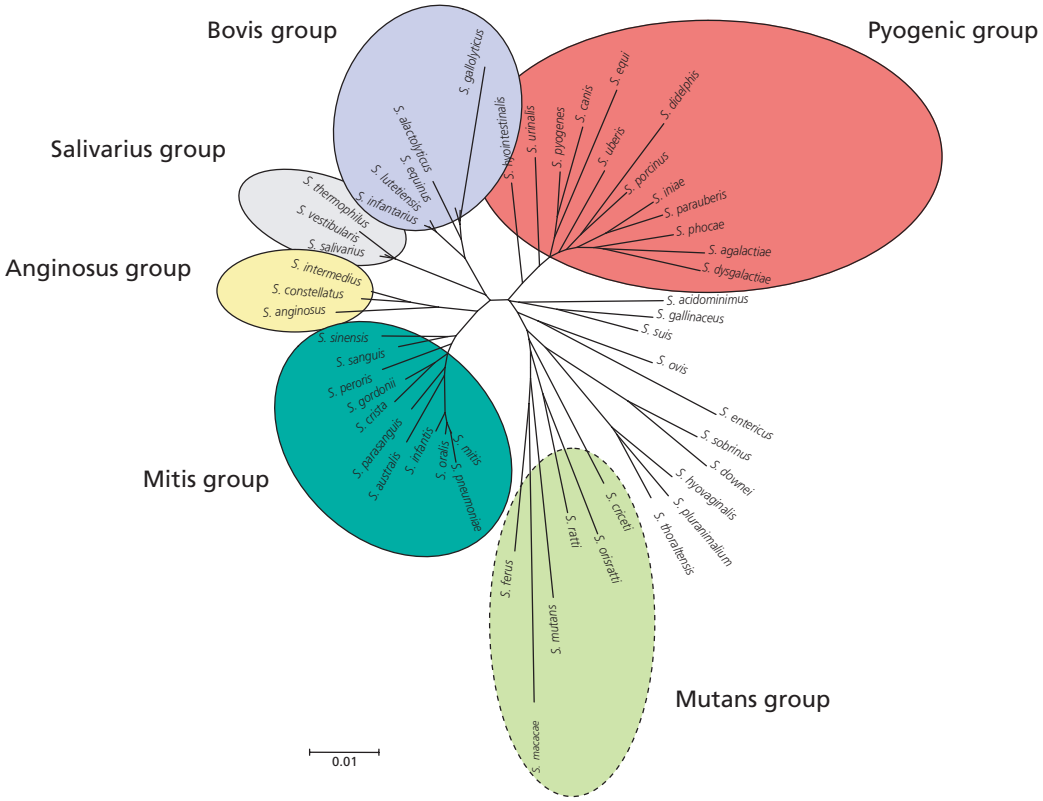

Figure 5 *Phylogenetic relationships of* Streptococcus *species based on complete 16S rRNA gene sequences. The tree was prepared by the neighbor-joining (NJ) method using MEGA2 (Kumar et al. 2001).*

S. pleomorphus is only distantly related to the other species and is incorrectly placed in the genus *Streptococcus*.

It has been proposed to change the names of several well-established *Streptococcus* species because the specific epithet is grammatically incorrect (Trüper and de'Clari 1997, 1998). Those species affected are *Streptococcus sanguis*, *Streptococcus parasanguis*, *Streptococcus crista*, and *Streptococcus rattus*, which were amended to '*Streptococcus sanguinis*', '*Streptococcus parasanguinis'*, '*Streptococcus cristatus*', and '*Streptococcus ratti*.' This has been challenged as unnecessary and potentially confusing (Kilian 2001), although the amended names have already been adopted by some authors. The Judicial Commission of the International Committee on Systematic Bacteriology recently ruled (minutes of the meeting published in vol. 50, p. 2239, of the *International Journal of Systematic and Evolutionary Microbiology* 2000) that priority be given to the stabilization of nomenclature over orthographic correctness and that validly published names should not be changed on grammatical grounds. Accordingly, the original and validly published names are maintained in this chapter.

PYOGENIC GROUP

Thirteen species can be distinguished within the pyogenic group of streptococci, most of which are characterized by β-hemolytic activity. Accordingly, the species associated with man are also simply referred to as hemolytic streptococci. The latter include the classic pathogens *S. pyogenes* and *S. agalactiae* which are well-defined species that present no taxonomic problems. *S. canis* is also a well-defined species proposed by Devriese et al. (1986) for streptococci isolated from dogs and cows and possessing the group G antigen. Occasional infections in humans have been described (Whatmore et al. 2001).

The taxonomic situation of several of the other species in this group has been confused. *S. dysgalactiae* was originally proposed for certain distinct streptococci causing bovine mastitis (Diernhofer 1932). For reasons that remain unclear the species was not included on the 'Approved lists of bacterial names' published in 1980 (Skerman et al. 1980), but the name was later revived to accommodate exclusively β-hemolytic group C strains of bovine origin (Garvie et al. 1983). Several studies have demonstrated that the species should also include human isolates of group C streptococci previously designated '*S. equisimilis*' as well as certain streptococci that possess the group G (large colony type) or L antigens (Feltham 1979; Farrow and Collins 1984b; Kilpper-Bälz and Schleifer 1984). Historically, the name '*S. equisimilis*' was given to human isolates resembling *S. equi*, but the name has had no formal standing since 1980

(Skerman et al. 1980) although it is still often used in the literature and by many clinical microbiologists. Thus, the species *S. dysgalactiae*, as currently defined, includes streptococci both from humans and from various animal species. However, it has been clear that such strains, although genetically closely related, may differ, not only in their ecological preferences, but also in their biochemical activities (Devriese 1991; Efstratiou et al. 1994). This situation has recently been resolved by the demonstration of two mutually distinct and genetically homogeneous clusters consisting of animal and human isolates respectively (Vandamme et al. 1996). The two clusters have been proposed as separate subspecies, i.e. *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis*. Subspecies *dysgalactiae* accommodates strains of animal origin, with group C or L antigens, and β-, α-, or no hemolysis, whereas subspecies *equisimilis* includes human isolates with group C or G antigens and is invariably β-hemolytic. The adaptation of the latter subspecies to the human host is reflected in the fact that it shows streptokinase activity on human plasminogen and proteolytic activity on human fibrin in contrast to animal isolates belonging in subspecies *dysgalactiae*. The two subspecies can, furthermore, be differentiated by whole cell protein electrophoretic patterns and biochemical properties. One may argue that it would be practical and in agreement with current clinical usage to maintain the two taxa as separate species, *S. dysgalactiae* and *S. equisimilis*.

Like '*S. equisimilis*', the species '*S. zooepidemicus*' was not included on the 'Approved lists of bacterial names' (Skerman et al. 1980). This is justified on the grounds that '*S. zooepidemicus*' is genetically closely related to *S. equi*, which also carries the group C antigen, and has been proposed as a subspecies of that species (Feltham 1979; Farrow and Collins 1984b).

As described, streptococci that react with Lancefield group C antiserum may belong to *S. dysgalactiae* (both subspecies) and *S. equi* (both subspecies). These are often referred to as 'large colony-forming group C streptococci' in contrast to members of the anginosus group, some of which also carry the group C antigen, but form small ('minute') colonies. Likewise, streptococci with the group G antigen may belong to *S. dysgalactiae* subsp. *equisimilis*, *S. canis*, or *S. intestinalis*, which form large colonies, but also to members of the anginosus group, which form small colonies. Although it is clear that streptococci belonging to these groups cause various diseases in humans and animals (Jelinková and Kubin 1974; Carmeli and Ruoff 1995), the exact role of the individual species, in particular in human infections, remains obscured by the failure to differentiate between them in most clinical studies (Woo et al. 2001). The species *S. porcinus* was established to accommodate β-hemolytic pyogenic streptococci with a distinct phenotypic profile that are usually associated with infections in swine (Collins et al. 1984). Although it is infrequently isolated from human infections, recent findings suggest that it may become more prevalent in infections with a genitourinary source (Facklam et al. 1995). Porcine isolates may carry Lancefield group E, P, U, V or other nonrecognized antigens, whereas human isolates appear to be serologically distinct. Streptococci now included in the species *S. porcinus* have previously been referred to as '*Streptococcus infrequens*', '*Streptococcus lentus*', '*Streptococcus subacidus*', or 'Lancefield group E, P, U, or V streptococci'.

S. uberis is one of the important etiologic agents of bovine mastitis, first recognized by Diernhofer (1932). Subsequent studies demonstrated that the species included two distinct genetic groups ('*S. uberis* type I and II') which are now recognized as *S. uberis* and *S. parauberis*, respectively (Williams and Collins 1990). In contrast to most other streptococci in the pyogenic group, *S. uberis* and *S. parauberis* are α- or nonhemolytic. Some strains have been shown to react with Lancefield group E, P, or G antisera (Garvie and Bramley 1979).

S. hyointestinalis is the name proposed by Devriese et al. (1988) for α-hemolytic streptococci that may be isolated from the intestinal tract of pigs and are phenotypically distinct from both *S. porcinus* and *S. intestinalis* which have the same ecological niche.

S. iniae is the name given to pyogenic streptococci isolated from abscesses on the thorax and abdomen of freshwater dolphins (*Inia geoffrensis*) living in the Amazon river (Pier and Madin 1976) but has been found to be pathogenic for other animals also. The name '*S. shiloi*', which was proposed to accommodate streptococci causing septicemia in fish, has been shown to be identical to *S. iniae* and the name may be considered a junior synonym of *S. iniae* (Eldar et al. 1995). From infected organs of seals, Skaar et al. (1994) isolated β-hemolytic streptococci that constitute a new species named *S. phocae*. Among 22 isolates originally examined, some reacted with antisera against group F or C antigens while the major part failed to show reactivity with antisera against any of the known group antigens.

S. didelphis was isolated from cases of suppurative dermatitis and liver fibrosis in oppossums (*Didelphis virginiana*) in the United States. Like many other species in the group of pyogenic streptococci, *S. didelphis* isolates are β-hemolytic, but quite surprisingly they are described as showing strong catalase activity on initial passage on blood-free agar media after growth on blood agar (Rurangirwa et al. 2000). The genetic basis of this should be examined.

S. urinalis was described by Collins and his coworkers (2000) to accommodate a single nonhemolytic streptococcal isolate from the urine of a patient suffering from cystitis. Thus, there is limited information about the properties and occurrence of this taxon. Similarities of 16S rRNA gene sequences suggest that the actual isolate belongs in the pyogenic group of streptococci

(Figure 5), and its whole-cell protein pattern most closely resembles those of *S. iniae* and *S. parauberis* (Collins et al. 2000).

ANGINOSUS GROUP

The anginosus group has been a source of considerable confusion, partly as a result of a lack of international consensus on nomenclature (Facklam 1984) and partly because it has been difficult to identify reliable phenotypic differences between taxa within the group (Jones 1978; Kilian et al. 1989; Taketoshi et al. 1993; Whiley et al. 1999). European and Japanese microbiologists have used the term '*Streptococcus milleri*' to embrace all members of the group, whereas North American workers have used various terms such as '*Streptococcus MG-intermedius*' and '*Streptococcus anginosus-constellatus*' (Facklam 1984). Dependent on methods employed, DNA–DNA hybridization studies have concluded that the group includes 1–3 distinct species (Welborn et al. 1983; Coykendall et al. 1987; Schleifer and Kilpper-Bälz 1987; Whiley and Hardie 1989). The currently recognized proposal, which is supported by 16S rRNA gene sequence data, and to some extent by phenotypic differences, is that the group includes three distinct species: *S. anginosus*, *S. constellatus*, and *S. intermedius* (Whiley and Hardie 1989; Whiley and Beighton 1991; Bentley et al. 1991), although there is still discussion about the validity of this differentiation (Vandamme et al. 1998). As currently defined the three species show surprising antigenic heterogeneity and include strains that react with group A, C, F, or G antisera (Table 1). Such strains are often referred to as 'minute (or small) colony type' strains of the respective serological groups. Some strains require anaerobic conditions for growth.

DNA–DNA reassociation studies (Kilpper-Bälz et al. 1984; Whiley and Hardie 1989) and 16S rRNA gene sequence data (Bentley et al. 1991) reveal that *S. constellatus* and *S. intermedius* are closely related (Figure 5). *S. intermedius* is phenotypically quite distinct, whereas *S. constellatus* and *S. anginosus* may be difficult to differentiate on the basis of biochemical reactivities (Whiley et al. 1990b). Two subspecies of *S. constellatus* have been identified, *S. constellatus* subsp. *constellatus* containing strains that may possess a variety of Lancefield group antigens (F, G, C, A or none) and *S. constellatus* subsp. *pharyngis* that includes group C strains. The two subspecies are well separated by phenotypic characters (Whiley et al. 1999). Whiley and coworkers (1999) also demonstrated a putative subspecies of *S. anginosus* composed of group C or G strains.

It is likely that future studies will disclose additional distinct species within this group of streptococci. Thus, further taxonomic heterogeneity has been suggested by differences observed by both phenotypic and genotypic analyses (Cookson et al. 1989; Whiley and Hardie 1989; Doitt et al. 1994; Bergman et al. 1995; Whiley et al. 1995, 1997; Jacobs et al. 2000).

The three recognized species are members of the resident microflora of the human mouth, and gastrointestinal and genital tracts (Frandsen et al. 1991; Whiley et al. 1993; Paster et al. 2001). Collectively, the group has been referred to by various terms such as 'the milleri streptococci' or '*Streptococcus milleri* group (SMG).' Use of the term 'anginosus group' as suggested by Kawamura et al. (1995a) is to be preferred as it may eliminate much of the nomenclatural confusion.

MITIS GROUP

Although *S. pneumoniae* has previously been placed among the pyogenic streptococci (Rotta 1986) 16S rRNA gene sequences and nucleic acid hybridization data show that it is closely related to *S. oralis* and *S. mitis* (Welborn et al. 1983; Kilpper-Bälz et al. 1985; Bentley et al. 1991; Whatmore et al. 2000), in agreement with the distinct cell wall composition of the three species among all the streptococcal species. In addition to these three species, the mitis group includes *S. sanguis*, *S. gordonii*, *S. parasanguis*, *S. crista*, *S. australis*, *S. infantis*, *S. peroris*, and *S. sinensis* (Table 1). All species are primarily associated with humans, but closely similar, though not necessarily identical, species may be isolated from the upper respiratory tract of various animal species (Dent et al. 1978).

For many years this group of streptococci has been refractory to satisfactory taxonomic solutions. The individual species have been poorly defined and several synonyms have been applied to the same species. A further source of confusion in many published studies was that the type strain of *S. mitis* did not conform with the description of the species (Welborn et al. 1983; Kilian et al. 1989). The strain was subsequently identified as an atypical *S. gordonii* and has been replaced by another type (Kilian et al. 1989). For a historical account of the nomenclature of the recognized species and a list of synonyms applied to them, the reader is referred to previous publications (Coykendall 1989; Kilian et al. 1989).

The present taxonomy of members of the mitis group is the result of numerous phenotypic and genetic studies (Carlsson 1968; Colman and Williams 1972; Coykendall and Specht 1975; Coykendall and Munzenmaier 1978; Welborn et al. 1983; Kilpper-Bälz et al. 1985; Schmidhuber et al. 1987; Kilian et al. 1989). However, as reflected by the constant introduction of new species, the full diversity of this group of bacteria is still incompletely appreciated.

S. sanguis was originally described to accommodate clinical isolates from cases of subacute bacterial endocarditis (White and Niven 1946). Among the salient characteristics of this species are synthesis of extracellular polysaccharide (glucan) when supplied with

sucrose, the ability to hydrolyze arginine and in most cases esculin, fermentation of inulin, and IgA1 protease activity. Many of the 'classical' *S. sanguis* strains, including strain Challis, are now included in *S. gordonii*, which shares many phenotypic characteristics of *S. sanguis* but lacks IgA1 protease activity (Kilian et al. 1989). Three other species, present in the human oral cavity, resemble *S. sanguis* in many ways but do not produce extracellular polysaccharide and IgA1 protease. *S. crista* was based on isolates that are characterized by laterally positioned tufted fibrils (Handley et al. 1991). These bacteria were previously referred to as '*S. sanguis* I with tufted fibrils', the 'CR group,' or the 'tufted-fibril group.' *S. parasanguis* and *S. australis* are genetically distinct (Whiley et al. 1990a; Wilcox et al. 2001) but information about their ecology and other characteristics is still limited (Table 3).

The two species *S. mitis* (Kilian et al. 1989) and *S. oralis* (previously referred to as '*S. mitior*' or '*S. sanguis* II') (Bridge and Sneath 1983) form genetically distinct populations but share many phenotypic traits and are notoriously difficult to distinguish. Most strains of *S. oralis* produce extracellular polysaccharide (glucan). Although phenotypically homogenous *S. mitis* is a species of extreme genetic diversity, and may more appropriately be referred to as a cluster of species (Kilian, Poulsen and Sørensen, in preparation). Strains previously referred to as *S. mitis* biovar 2 (Kilian et al. 1989) belong to *S. infantis*, *S. crista*, and *S. parasanguis* (Kilian, Poulsen and Sørensen, in preparation). The two latter species are difficult to distinguish on the basis of phenotypic traits (Ruoff et al. 2003).

S. infantis and *S. peroris*, defined primarily by DNA–DNA homology data, include streptococci isolated from the human upper respiratory tract (Kawamura et al. 1998). *S. infantis* is closely related to the *S. pneumoniae–S. mitis–S. oralis* cluster and shares several properties of these species, in particular *S. mitis* (Kilian, Poulsen and Sørensen, in preparation). Recent studies using species-specific probes indicate that *S. infantis* is a numerically important member of the oral microflora (Kazor et al. 2003) and it is conceivable that many streptococci previously referred to as *S. mitis* belong in this species. There is very limited information about *S. peroris* and the recently described *S. sinensis*. Description of the latter species was based on a single isolate from subacute bacterial endocarditis (Woo et al. 2002). Recently, an additional species from dental plaque was described as *Streptococcus oligofermentans*. The species is most closely related to *S. sinensis* (Tong et al. 2003).

SALIVARIUS GROUP

The three species in the salivarius group, *S. salivarius*, *S. thermophilus*, and *S. vestibularis* are genetically closely related and are usually nonhemolytic. *S. salivarius* is a member of the resident microflora of mucosal membranes of the human oral cavity and pharynx (Carlsson 1967; Frandsen et al. 1991). It produces characteristic large mucoid colonies (2–3 mm) on sucrose-containing media as a result of the production of extracellular fructan (levan). It usually does not colonize tooth surfaces like other oral streptococci (Nyvad and Kilian 1990), but occasional strains which produce insoluble glucan in addition to fructan may do so. These strains show characteristic large rough colonies on sucrose-containing media.

S. salivarius is genetically homogenous but phenotypically heterogenous (Coykendall and Gustafson 1985; Beighton et al. 1991). It is distinct from *S. thermophilus* which may be isolated from milk products (Bridge and Sneath 1983) although the latter species was temporarily included in the species *S. salivarius* as 'subspecies *thermophilus*' on the basis of their close genetic relationship (Farrow and Collins 1984a).

The species *S. vestibularis* (Whiley and Hardie 1988) resembles *S. salivarius* in its ecological preferences and biochemical characteristics but does not produce extracellular polysaccharide.

BOVIS GROUP

The bovis group of streptococci is currently undergoing comprehensive taxonomic reorganization, and certain questions remain unresolved. '*S. bovis*' is the name widely used in human clinical microbiology for certain streptococci that occur in the human intestinal tract and occasionally is isolated from blood of patients with endocarditis with an underlying colonic disorder, such as malignancy or peridiverticulitis. Similar streptococci that occur in the intestinal tract of cows, horses, sheep, and other ruminants has been referred to as *S. equinus*. Both phenotypic and genetic analyses of the type strains of *S. bovis* and *S. equinus* have demonstrated their close relationship, indicating that the two species should be combined in a single species, *S. equinus*, which has priority (Farrow et al. 1984; Ludwig et al. 1985; Bentley et al. 1991; Schlegel et al. 2003). However, so far this has not been adopted in clinical microbiology and the name '*S. bovis*' remains widely used. Recent observations show that this should end.

It has long been recognized that the strains referred to as '*S. bovis*' were both genetically and phenotypically very heterogenous. Two biotypes have been recognized based on several biochemical differences (Facklam et al. 1984; Farrow et al. 1984; Knight and Shlaes 1985; Ruoff et al. 1989). 'Biotype II' included the type strains of '*S. bovis*' and *S. equinus* which, as mentioned, now represent the single valid species *S. equinus*. However, even among 'biotype II strains of *S. bovis*' a considerable degree of genetic heterogeneity exists, and recent studies demonstrate that only rare human isolates belong in the species *S. equinus* (Clarridge et al. 2001). The majority of human isolates belong in the newly

described species *Streptococcus pasteurianus* (Poyart et al. 2002), which corresponds to '*S. bovis* biotype II.2,' and *Streptococcus infantarius* (Bouvet et al. 1997; Schlegel et al. 2000), which corresponds to '*S. bovis* biotype II.1.' According to the proposal by Schlegel et al. (2000) the species *S. infantarius* includes two subspecies, subsp. *infantarius* and subsp. *coli*. However, subsequent phylogenetic analyses based on sequences of the gene *sodA*, which encodes the manganese-dependent superoxide dismutase, suggested that subspecies *coli* deserved recognition as a separate species, for which the name *S. lutetiensis* is proposed. The specific recognition of this taxon is supported by limited DNA–DNA hybridization data (Poyart et al. 2002).

In contrast to 'biotype II', 'biotype I' strains have been considered genotypically homogenous. Nevertheless, during the 1990s, four new species within this group were proposed, *Streptococcus gallolyticus* (Osawa et al. 1995), *Streptococcus macedonicus* (Tsakalidou et al. 1998), *Streptococcus waius* (Flint et al. 1999), and *Streptococcus caprinus* (Brooker et al. 1994). Subsequent studies convincingly demonstrate that *S. gallolyticus* and *S. caprinus* are subjective synonyms and also that *S. waius* is a later synonym of *S. macedonicus*, both containing isolates from dairy products (Sly et al. 1997; Poyart et al. 2002). In fact, genetic data suggest that the four taxa constitute a single species, which should carry the name *S. gallolyticus* (Poyart et al. 2002). A recent report proposed recognition of three subspecies within *S. gallolyticus*, subsp. *gallolyticus*, subsp. *macedonicus*, and subsp. *pasteurianus* (Schlegel et al. 2003). The inclusion of strains previously included in *S. pasteurianus* as a subspecies of *S. gallolyticus* requires additional supportive data as the genetic relationship between the two, as revealed by DNA–DNA hybridization, is below the limit usually considered necessary for including strains in the same species.

S. gallolyticus, subsp. *gallolyticus*, has been isolated from the feces of a variety of animal species (Table 1). The species had also been isolated from bovine mastitis (Osawa et al. 1995; Sly et al. 1997). Most human isolates were from feces and blood and included isolates from cases of endocarditis associated with colonic cancer (Schlegel et al. 2003).

A well separated member of of the bovis group is *S. alactolyticus* proposed by Farrow et al. (1984) for porcine and chicken isolates with resemblance to '*S. bovis*.'

MUTANS GROUP

The mutans group includes eight species which exclusively colonize tooth surfaces in humans and in some animal species (*S. mutans*, *S. sobrinus*, *S. cricetus*, *S. downeii*, *S. macacae*, *S. ferus*, *S. rattus*, and *S. orisrattii*). The name *S. mutans* was first applied by Clarke (1924) to strongly acidogenic streptococci isolated from carious lesions in humans. These streptococci were able to induce carious lesions in extracted teeth and were considered by Clarke to be the cause of dental caries. Attempts to subdivide the species by serological means based on cell wall carbohydrates disclosed eight serotypes (a–h) (Bratthall 1970; Perch et al. 1974; Beighton et al. 1981). This was followed by the discovery of an unexpected degree of genetic heterogeneity (Coykendall 1971) which eventually led to the recognition of seven separate species including species encompassing strains isolated from monkeys and rodents (Table 1) (Coykendall 1977, 1983; Beighton et al. 1981; Whiley et al. 1988; Zhu et al. 2000). The seven species and their primary habitat are listed in Table 1. Phenotypically, the mutans group of streptococci is relatively homogenous, but species-associated differences in cell wall carbohydrates reflected in serotypes, cell wall peptidoglycan type (Table 1), and other characteristics have been described (Hamada et al. 1986).

OTHER *STREPTOCOCCUS* SPECIES

S. suis is an important pathogen of pigs. The species was proposed by Elliott in 1966 (see Kilpper-Bälz and Schleifer 1987) and includes streptococci that carry the group D or related antigens. It is encapsulated, and more than 30 capsular serotypes have been detected. The antigens previously referred to as Lancefield group R, S, and T antigens are capsular polysaccharides that characterize serotypes 2, 1, and 15, respectively (Perch et al. 1983; Gottschalk et al. 1989). *S. suis* may cause meningitis, septicemia, arthritis, and bronchopneumonia in pigs, and may also be found in the nasal cavity and tonsils of healthy pigs. It is a zoonotic cause of rare cases of meningitis in humans, most of which occur in slaughterhouse workers (Hampson et al. 1993).

DNA–rRNA hybridization and 16S rRNA gene sequence studies have confirmed that *Streptococcus acidominimus* is a member of the genus *Streptococcus* but it does not cluster with any of the other species in the genus (Kilpper-Bälz et al. 1985; Bentley et al. 1991). The species is associated with cattle and differs from other *Streptococcus* species by being weakly acidogenic, but is otherwise poorly defined.

The two species *Streptococcus hyovaginalis* and *Streptococcus thoraltensis* both include isolates from sows. They are both found in the genital tract, but the capnophilic species *S. thoraltensis* may also occur in the intestinal tract of pigs. They differ from *S. suis* by giving a positive Voges–Proskauer reaction (Devriese et al. 1997).

Streptococcal isolates from subclinical mastitis, from the genital tract and tonsils of cattle, from tonsils of a goat and a cat, and from the crop and the respiratory tract of canaries form the basis of a species desribed as *Streptococcus pluranimalium* (Devriese et al. 1999). The species is phenotypically heterogeneous but shows a

characteristic whole-cell protein pattern. It is most closely related to *S. hyovaginalis*.

Based on seven clinical isolates from sheep Collins et al. (2001) described a new species *Streptococcus ovis*, which is α-hemolytic and not closely related to any of the other *Streptococcus* species. It may be distinguished from other species by a characteristic 16S rRNA gene sequence and whole-cell protein pattern.

LABORATORY ISOLATION AND IDENTIFICATION

All the *Streptococcus* species, except for *S. thermophilus*, grow well on blood agar incubated at 37°C in air plus carbon dioxide or anaerobically. Species of clinical significance will usually predominate even when members of the resident microflora are present. Sensitive detection of *S. agalactiae* by initial inoculation of a selective broth medium has been introduced in the United States for prophylactic screening of vaginal carriage by pregnant women (Centers for Disease Control and Prevention 1996). A semiselective medium that allows quantitative detection and presumptive identification of *S. agalactiae* based on the CAMP reaction is described by Hansen and Sørensen (2003). Detection of pneumococci in nasal or pharyngeal swabs is facilitated on blood agar plates with 5 mg/l gentamicin (Gray et al. 1977).

The pyogenic streptococci are detected initially by their hemolytic activity. Colonies of β-hemolytic streptococci on blood agar may resemble those of *Haemophilus parahaemolyticus*, but the two are easily distinguished upon subculture and Gram staining. Colonies of the α-hemolytic pneumococci are smooth and may vary in size according to the amount of capsular polysaccharide produced; those of serotype 3 are usually larger than the rest and have a watery or mucoid appearance. During prolonged incubation, the center of the colony becomes depressed as a result of autolysis.

Once it has been established that the isolates are catalase-negative, gram-positive cocci with a tendency to form chains, they are identified by a combination of serological and biochemical examinations. The essential discriminatory tests for the pyogenic group of streptococci and *S. suis* are summarized in Table 2. An additional test that is helpful in the identification of *S. pyogenes* is the bacitracin sensitivity test. *S. pyogenes* is uniformly sensitive, but sensitivity can also be detected in some hemolytic strains of groups C, E, G, and L, in

Table 2 *Differential characteristics of the species of the pyogenic group and* S. suis, S. hyovaginalis, *and* S. thoraltensis

Species	Lancefield serogroup	Hemolysis	Hydrolysis of				Presence of				Fermentation of					
			Hip	Aes	Arg	VP	PYR	βG	AP	Rib	Man	Sor	Lac	Tre	Inu	Raf
S. pyogenes	A	β	–	V[c]	+	–	+	V	+	–	V	–	+	+	–	–
S. agalactiae	B	β (CAMP+)	+	–	+	+	–	V	+	+	–	–	V	+	–	–
S. equi																
subsp. *equi*	C	β	–	–	+	–	–	+	+	–	–	–	–	–	–	–
subsp. *zooepidemicus*	C	β	–	V	+	–	–	+	+	V	–	+	+	–	–	–
S. dysgalactiae																
subsp. *dysgalactiae*	C	α, β, –[a]	–	–	+	–	–	+	+	+	–	+	+	+	–	–
subsp. *equisimilis*	C, G, L	β	–	V	+	–	–	+	+	+	–	–	V	+	–	–
S. canis	G	β (CAMP+)[b]	–	V	+	–	–	–	+	+	–	–	V	+	–	–
S. phocae	C, F, –	β	–			–	–					–				
S. iniae	–	α,β	–	+	NT[d]	NT	NT	NT	NT	NT	+	–	–	+	–	–
S. didelphis	–	β	+	–	V	–	–	+	NT	+	–	–	V	+	–	V
S. porcinus	E, P, U, V	β (CAMP+)	–	+	+	+	+	+	+	+	+	+	V	+	–	–
S. uberis	–, E	α	+	+	+	+	+	+	V	+	+	+	+	+	+	–
S. parauberis	–, E	α	V	+	+	+	+	–	+	+	+	+	+	+	V	V
S. urinalis	–	–	–	NT	+	+	+	–	+	+	–	–	–	+	–	–
S. hyointestinals	–	α	–	+	–	+	–	–	+	–	–	–	+	+	NT	V
S. suis	D	β, α, –	–	+	+	–	V	+	–	–	–	–	+	V	V	V
S. hyovaginalis	NT	α	+	NT	–	+	–	+	+	V	+	+	+	+	–	–
S. thoraltensis	NT	α	V	NT	+	+	–	+	V	+	+	V	+	+	+	V

Aes, aesculine; AP, alkaline phosphatase; Arg, arginine; βG, β-glucuronidase; Hip, hippurate; Inu, inulin; Lac, lactose; Man, mannitol; VP (Voges–Proskauer), production of acetoin; PYR, pyrrolydonyl-amylamidase; Raf raffinose; Rib, ribose; Sor, sorbitol; Tre, trehalose.

a) Bovine strains are never β-hemolytic.

b) Only canine strains are CAMP positive.

c) V, variable.

d) NT, not tested.

S. suis and in *S. phocae*. If the end-point is taken as absence of any zone of inhibition, the disk should contain 0.04 units of bacitracin. *S. pyogenes* will give a zone of diameter ~11 mm around a 6 mm disc containing 0.1 unit of bacitracin. The bacitracin sensitivity test should not be performed on primary culture plates. Comprehensive lists of the characteristics of pyogenic and other streptococci have been compiled by Hardie and Whiley (1995). Caution is recommended with regard to the use of identification tables compiled from different publications (this also applies to the tables in this chapter) because different methods for testing biochemical activities of streptococci often yield different results.

Pneumococci are not readily identified by biochemical tests but in most cases can be identified on the basis of their sensitivity to optochin, bile solubility, and autolysis. The majority of pneumococci are sensitive to optochin (ethylhydrocupreine) which provides a valuable means of distinguishing pneumococci from other α-hemolytic streptococci. The test can be performed conveniently with a paper disk containing 5 μg optochin. Some of the oral streptococci may show small zones of inhibition.

All capsulate pneumococci, but not all noncapsulate variants, are lysed by bile. Recent studies suggest that at least some of the latter, which sometimes are referred to as atypical pneumococci (Pease et al. 1986) are *S. mitis* clones that carry some of the genes otherwise considered characteristic of pneumococci (Whatmore et al. 2001). The bile, or other detergents, activates the autolysin *N*-acetylmuramyl-L-alanine amidase which results in the lysis of cultures of pneumococci by attacking the peptidoglycan. Presumptive identification of pneumococci may furthermore be achieved by the demonstration of a reaction in so-called 'omni-serum' which is a concentrate of pooled typing sera (Lund and Henrichsen 1978).

Oral streptococci are often examined on the selective medium Mitis Salivarius agar on which some species produce extracellular polysaccharide from sucrose in the medium resulting in characteristic colonies. *S. salivarius* produces large (2–3 mm) soft and glossy colonies which collapse after prolonged incubation. Mutans streptococci produce hard adherent colonies resembling frosted glass. Drops of extracellular polysaccharide often accumulate on the top or at the side of the colonies (Figure 2). Colonies of the polysaccharide-producing *S. sanguis*, *S. gordonii*, and *S. oralis* are regular, dome-shaped, smooth, hard and adherent, and indistinguishable. The species that do not produce extracellular polysaccharides (*S. mitis*, *S. parasanguis*, *S. crista*, *S. peroris*, *S. infantis*, *S. australis*, *S. anginosus*, *S. intermedius*, *S. constellatus*, *S. vestibularis*) form flat or raised uncharacteristic colonies. Biochemical reactivities important for the identification of oral streptococci are summarized in Table 3. Characteristics that allow differentiation of biovars of *S. sanguis* and *S. gordonii* have been published by Kilian et al. (1989), and tests that allow differentiation of species within the mutans group and genotypes within *S. bovis* have been compiled by Hardie and Whiley (1995). Whiley and Beighton (1998) have published a comprehensive review of the current classification and identification of oral streptococci.

Commercial kits such as Rapid Strep 32 are available for identification of *Streptococcus* species. Evaluations demonstrate good agreements of the final identification with traditional methods, although individual tests do not generally give identical results in different versions. Furthermore, newly described species are often overlooked because of incomplete or lacking data in the identification databases (Freney et al. 1992; Kikuchi et al. 1995). Identification of streptococci may be achieved by comparing partial DNA sequences of 16S rRNA genes or selected housekeeping genes with those of appropriate type strains (note that many strains in databases are mislabeled according to the present state of taxonomy). To some extent, identification may also be achieved with DNA probes that hybridize exclusively with the respective species (Schmidhuber et al. 1988; Bentley and Leigh 1995; Jacobs et al. 1996; Kawamura et al. 1999).

BACTERIOCINS

Many strains of streptococci produce bacteriocin-like inhibitory substances (BLIS) which inhibit growth of a wide range of bacteria (Tagg 1992). One example is the intensely investigated nicin produced by some *Lactococcus lactis* strains. Nicin has a long history of application in food preservation, especially of dairy products (Jack et al. 1995). BLIS activity among *Streptococcus* species associated with humans and animals are believed to play a role in their ability to interfere with growth of other bacteria in their local environment along with other released inhibitory substances such as hydrogen peroxide and lactic acid (Rogers et al. 1978; Jack et al. 1995).

BLIS activity has been detected in several *Streptococcus* species, with the highest prevalence among *S. salivarius* and other oral streptococci (Kelstrup and Gibbons 1969; Dempster and Tagg 1982). A survey of clinical isolates of *S. pyogenes* revealed BLIS activity in approximately 10 percent of strains (Tagg et al. 1971). The activity is confined to strains of particular M types, and the overall occurrence within these M types is very high (50–100 percent). The BLIS of *S. pyogenes* inhibited a wide range of gram-positive bacteria and a single strain of *Prevotella intermedia* (Hynes and Tagg 1985). BLIS activity has also been demonstrated in occasional strains of *S. agalactiae*, *S. dysgalactiae*, and *S. equi* (Schofield and Tagg 1983). The commensal *S. salivarius* produces bacteriocins salivaricin A and salivaricin B, which have strong inhibitory activity

Table 3 *Differential characteristics of species of the* mitis, anginosus, salivarius, *and* mutans *groups*

Species	Production of EP[a]	Hydrolysis of		Presence of									Fermentation of				
		Aes	Arg	VP	H_2O_2	IP	Ne	AP	βG	αF	αG	Hy	Amy	Arb	Inu	Man	Sor
S. mitis	–	–	–	–	+	V	V	V	–	–	V	–	–	–	V	–	–
S. infantis	–	–	–	–	+	V	V	V	+	NT[b]	+	–	–	–	–	–	V
S. oralis	G/–	–	–	–	+	V	+	+	+	–	+	–	–	–	–	–	–
S. pneumoniae[c]	–	V	–	–	+	+	+	–	V	V	+	V	–	–	+	–	–
S. crista	–	–	+	–	+	–	–	–	+	+	–	–	–	+	NT	–	–
S. sanguis	G	V	+	–	+	+	–	V	–	–	–	–	–	+	+	–	V
S. gordonii	G	+	+	–	+	+	–	–	+	+	+	–	–	+	+	–	–
S. parasanguis	–	V	+	–	+	–	–	V	+	V	+	–	V	V	–	–	–
S. peroris	–	–	–	–	+	–	+	V	–	NT	NT	NT	–	–	–	–	–
S. australis	–	+	+	–	+	–	–	+	–	NT	NT	NT	NT	NT	NT	–	–
S. anginosus	–	+	+	+	V	–	–	+	–	–	+	–	+	+	–	V	–
S. constellatus	–	+	+	+	–	–	–	+	–	–	–	V	V	+	–	–	–
S. intermedius	–	+	+	+	–	–	+	+	+	+	V	+	V	+	–	–	–
S. sinensis	NT	+	+	+	+	NT	NT	–	–	NT	NT	NT	NT	NT	–	NT	–
S. oligofermentans[d]	NT	–	–	–	+	NT	NT	V	NT	NT	NT	NT	NT	NT	–	–	–
S. salivarius	F	+	–	V	–	–	–	+	–	–	+	–	+	+	V	–	–
S. vestibularis	–	V	–	–	–	–	–	–	–	–	V	–	V	V	–	–	–
S. thermophilus	NT	-	–	V	–	–	NT	–	–	–	V	NT	–	V	–	–	–
S. mutans	G	-	–	+	V	–	–	–	–	–	+	–	+	V	+	+	+
S. sobrinus	G	+	–	+	NT	–	NT	NT	–	NT	–	–	V	V	V	–	V

Aes, aesculine hydrolysis; αF, α-l-fucosidase; αG, α-n-glucosidase; Amy, amygdalin; AP, alkaline phosphatase; Arb, arbutin; Arg, arginine hydrolysis; βG, β-glucosaminidase; EP, extracellular polysaccharide; Inu, inulin; IP, IgA1 protease; Ne, neuraminidase; Hy, hyaluronidase; Man, mannitol; Sor, sorbitol; VP, production of acetoin.

a) G, glucan; F, fructan; V, variable.

b) NT, not tested.

c) *S. pneumoniae* is soluble in bile in contrast to all other species. Opthochin susceptibility is also a characteristic, which in most cases will differentiate *S. pneumoniae* from other species listed in this table.

d) *S. oligofermentans* is reported to hydrolyze hippurate in contrast to most other species listed in this table.

against *S. pyogenes* strains and a variety of other bacterial pathogens (Upton et al. 2001; Walls et al. 2003). The majority of BLIS characterized from *Streptococcus* are relatively small (<5 kDa) heat-stable proteins termed lantibiotics because they contain the unusual amino acids lanthionine and/or 3-methyllanthionine (Jung 1991; Hynes et al. 1993; Ross et al., 1993b; Jack et al. 1995). Some appear to be considerably larger (30 kDa) such as the so-called zoocin A produced by some strains of *S. equi* subspecies *zooepidemicus* (Simmonds et al. 1995), the streptococcin A-M57 of *S. pyogenes* M-type 57 (Simpson and Tagg 1983), and a bacteriocin detected in *S. mutans* (Paul and Slade 1975; Fukushima et al. 1983). Although bacteriocins in many gram-positive bacteria are encoded by plasmid-born genes, neither of the currently documented lantibiotic-producing strains of *S. pyogenes* and *S. salivarius* appears to contain plasmids (Simpson and Tagg 1984). The finding that different bacteriocin activities in *S. mutans* strains reflect distinct phylogenetic lineages support the notion that the genes have evolved over time along with the genome in general (Balakrishnan et al. 2002).

Salivaricin SalA production in *S. salivarius* in encoded by the *sal* genetic locus which comprises the lantibiotic structural gene, *salA*, genes encoding peptide modification and export machinery proteins (salBCTX), and genes encoding a putative immunity protein and two-component sensor-regulator proteins (salYKR). The production of SalA and the related lantibiotic in *S. pyogenes* are autoregulated, enabeling them to act as intra- and interspecies signaling molecules (Upton et al. 2001).

Bacteriocin production and sensitivity were successfully used as a fingerprinting method in epidemiological studies of streptococci before DNA techniques became available (Kelstrup et al. 1970; Tagg 1985; Ragland and Tagg 1990).

ROLE IN NORMAL HUMAN FLORA

Soon after birth, streptococci comprise a considerable proportion of the commensal microflora of human mucosal membranes of the oral cavity and pharynx. Almost half of the bacteria that can be isolated from oral mucosa and from saliva are streptococci. These are primarily the streptococci of the mitis (except *S. pneumoniae*) and salivarius groups. Each of the species in these groups shows a characteristic predilection for specific sites. While *S. salivarius* predominates on the tongue surface and in the pharynx, others such as *S. sanguis*, *S. oralis*, *S. mitis*, and *S. infantis*, colonize tooth surfaces and the buccal mucosa (Carlsson 1967; Carlsson et al. 1970; Gibbons and van Houte 1971; Hardie and Bowden 1974; Nyvad and Kilian 1990; Frandsen et al. 1991; Pearce et al. 1995; Kazor et al. 2003). The three species of the anginosus group are found in dental plaque and in the intestinal canal (Whiley et al. 1992). The mutans streptococci (*S. mutans* and *S. sobrinus*) exclusively colonize tooth surfaces and are not found in the oral cavity until after tooth eruption (Caufield et al. 1993). Being particularly acid tolerant they can become the predominant species during periods of high sugar consumption and may be found in high proportions in dental plaque associated with caries lesions. The extensive literature on the mutans streptococci and their etiological implications in dental caries has been reviewed by Hamada et al. (1986).

An individual usually carries multiple clones of the oral streptococcal species. While clones of some species (i.e. *S. mutans* and *S. sobrinus*) persist over many years (Alaluusua et al. 1994; Emanuelsson and Thornqvist 2000) clones of other species (i.e. *S. mitis* and *S. oralis*) show remarkable fluctuations particularly during early childhood (Hohwy and Kilian 1995; Hohwy et al. 2001; Fitzsimmons et al. 1996; Alam et al. 2000). The forces driving these fluctuations are unknown.

The commensal streptococci of the respiratory tract play an important role in preventing colonization by potentially pathogenic bacteria including *S. pyogenes*, presumably owing to their secretion of bacteriocins and hydrogen peroxide (Ross et al., 1993a; Jack et al. 1995). Some of these commensal streptococci (*S. sanguis*, *S. oralis*, and *S. mitis*) release IgA1 protease and may theoretically interfere with the protective function of secretory IgA. Significantly increased proportions of IgA1 protease-producing *S. mitis* have been demonstrated in the pharynx of infants who develop atopic disease (Kilian et al. 1995).

S. pyogenes is almost totally restricted to humans. The carrier rate of *S. pyogenes* in the respiratory tract is only a few percent in adults and just over 10 percent in children attending kindergarten. It may be considerably higher before or during an epidemic (Hoffmann 1985).

The intestinal tract and the perianal skin area appears to be the primary habitat of *S. agalactiae* and the main source of vaginal colonization (Ross 1970; Jelinková 1977). In a recent longitudinal study using a sensitive detection method, a mean vaginal and anorectal carriage rate at individual observations during pregnancy was 36 percent and the cumulative rate over the entire pregnancy period 54 percent. While some women appear to be transient carriers only, others are persistently colonized by the same clone for periods exceeding 1 year (Hansen et al. 2004).

Pneumococci may be detected in the nasopharynx of 10–20 percent of healthy adults but constitute only a minor part of the microflora. When infants are studied prospectively, nearly all are found to be colonized with pneumococci on at least one occasion in the first 2 years of life (Gray et al. 1980). During episodes of disease, for example otitis media, pneumococci often predominate in the microflora of the upper respiratory tract. In developing countries rates exceeding 90 percent have been documented.

PATHOGENICITY AND VIRULENCE FACTORS

The description here of streptococcal virulence factors focuses mainly on those demonstrated in the principal human pathogens *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*. Closely related factors are produced by several of the other species in the group and will only be mentioned. For a complete historical account of the virulence factors the reader is referred to the 8th edition of this book (Colman 1990).

S. pyogenes

S. pyogenes is among the most prevalent human pathogens, capable of causing various severe suppurative skin and mucosal infections, necrotizing fasciitis, scarlet fever, and nonsuppurative sequelae such as streptococcal glomerulonephritis and rheumatic fever. Since the mid-1980s, resurgence in severe streptococcal infections and a high mortality rate due to streptococcal sepsis and toxic shock-like syndrome has been reported (Veasy et al. 1987; Colman et al. 1993). Numerous virulence factors that are potentially involved in the different types of disease have been described. Many of these virulence factors are involved in evasion of components of the innate and adaptive immune system of the human host, while others degrade host tissues or are directly toxic to host cells.

THE M PROTEIN FAMILY

Early studies by Lancefield (1928) showed that the ability of *S. pyogenes* to persist in infected tissues is due primarily to the cell surface-exposed M protein, a molecule which confers to *S. pyogenes* the ability to resist phagocytosis or intracellular killing by polymorphonuclear leukocytes in the absence of specific antibodies. Resistance to infections by *S. pyogenes* appears to be related to the presence of antibodies in secretions and sera to the M protein molecule (Lancefield 1959; Bessen and Fischetti 1988). However, as there are more than 100 different serotypes of M proteins (M1, M2, M3, etc.), though not all with antiphagocytic properties, individuals may suffer from recurrent *S. pyogenes* infections with strains expressing different serotypes of the M protein.

For many years it was assumed that *S. pyogenes* strains express a single type-specific M protein. However, it is now clear that the M proteins discovered by Lancefield are members of a family of surface proteins, collectively termed the M protein family or just M proteins. They are structurally similar and share functional properties including antiphagocytic activity, although these properties vary among the individual proteins (Frithz et al. 1989; Heath and Cleary 1989; Kehoe 1994). The individual proteins of the M protein family are encoded by tandemly arranged genes located within the so-called *mga* regulon between the regulatory gene *mga* (previously *mry/virR*) and the coregulated *scpA* gene encoding C5a peptidase (see below) (Caparon and Scott 1987; Simpson et al. 1990; Perez-Casal et al. 1991; Caparon et al. 1991). Most strains contain 1–3 genes that have evolved through gene duplication followed by sequence and functional divergence (Hollingshead et al. 1994; Kehoe 1994). The 'classical' M protein is encoded by the *emm* gene, which is present in all strains of *S. pyogenes*. The two other genes encoding proteins of the M protein family, *mrp* and *enn*, are variably present with some association with other properties. Thus, strains expressing the opacity factor (OF^+ strains, see below) usually have all three genes, whereas the majority of OF^- strains only have the *emm* gene. Some OF^- strains, however, contain two or three genes (Figure 6).

All members of the M protein family form fibrillar structures that protrude 50–60 nm outwards from the bacterial cell (Swanson et al. 1969; Fischetti 1989). The proteins are anchored in the cytoplasmic membrane and span the entire cell wall to which they are anchored through an LPXTG motif. This wall-spanning region shows homology with several proteins in other

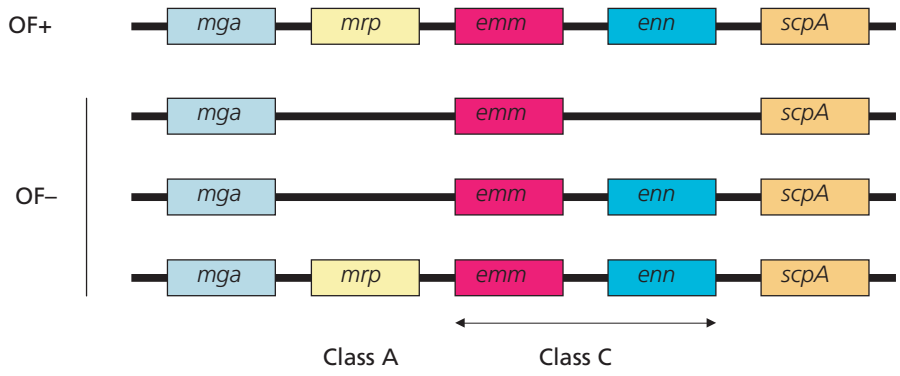

Figure 6 *Structure of the* mga *regulon in* S. pyogenes *with genes for regulation (*mga*), proteins of the M protein family (*emm, enn, *and* mrp*), and C5a peptidase (*scpA*).*

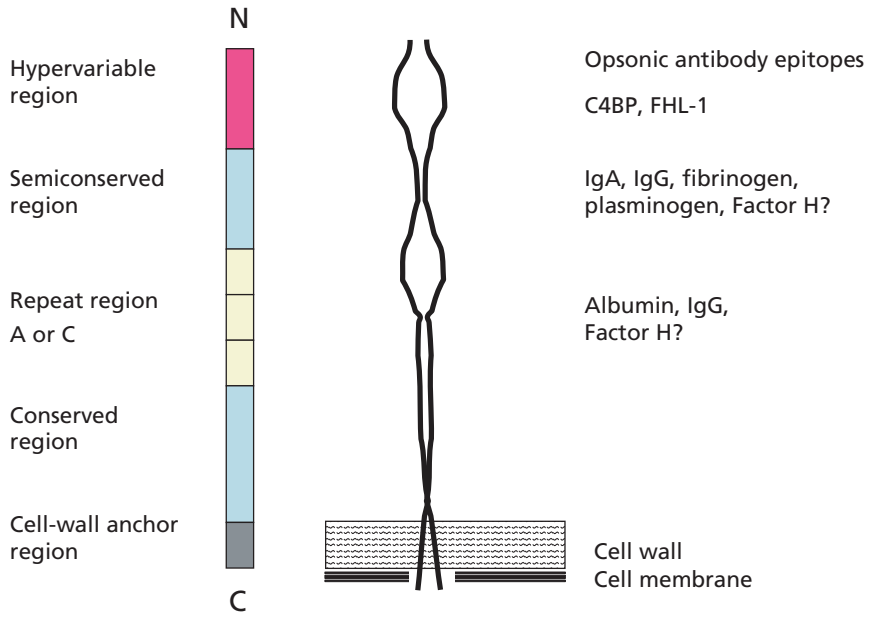

Figure 7 *Structure of an M protein and its gene. The diagram to the left illustrates the gene encoding the M6 protein with the characteristic A and C repeats. The location of the functional domains in the protein are indicated on the protein shown to the right.*

gram-positive bacteria such as staphylococcal protein A (Fischetti 1989).

Each fiber consists of a dimeric M protein molecule, which, with few exceptional domains, has an α-helical coiled-coil structure with a 7-residue periodicity (Fischetti 1989). The central part of the proteins is composed of tandemly arranged sequence repeats of two different types, A or C (Figure 7). Each repeat has a length of 35 or 42 amino acids and the number of repeats varies. A-type repeats are found in Mrp proteins whereas C-type repeats are found in both Emm and Enn proteins. Proteins with type C repeats may be subdivided into two major classes. Those of Class I share an epitope that can be detected with a monoclonal antibody, whereas Class II M proteins lack this epitope (O'Toole et al. 1992). Class CII proteins are associated with OF^+ strains (Bessen et al. 1989). Although both classes are associated with suppurative infections and glomerulonephritis, the majority of strains associated with outbreaks of rheumatic fever express M proteins of Class I (Bessen and Fischetti 1988). This association is supported by the recent observation that rheumatic fever patients display elevated levels of serum IgG antibodies to the Class I-specific epitope (Bessen et al. 1995).

The N-terminal of the M protein family molecules, which is distal to the cell surface, represents a hypervariable region. The sequence variation, which is most pronounced in Emm proteins (Bessen and Hollingshead 1994; Whatmore et al. 1995; Thern et al. 1998), is the result of accumulation of nonsynonymous mutations combined with recombination involving related genes within the genome or horizontal transfer between strains. In OF^- strains, the N-terminal region is longer and contains variable numbers of short repeats adding to the structural and antigenic polymorphism (Hollingshead et al. 1987; Héden and Lindahl 1993; Whatmore and Kehoe 1994; Kehoe et al. 1996). Antibodies to the N-terminal part of the molecule effectively opsonize the streptococci for phagocytosis.

FUNCTIONAL PROPERTIES OF M FAMILY PROTEINS

The M family of proteins are generally regarded as important virulence factors. This is supported by animal experiments although results have varied depending on host species and infection route (Schmidt et al. 1997; Moses et al. 1997; Ashbaugh et al. 1998; Boyle et al. 1998). The ability of *S. pyogenes* strains to survive and grow in nonimmune fresh human blood has been attributed to an antiphagocytic activity of M proteins.

The M proteins share the ability to interact with numerous host proteins but specific properties vary between individual proteins (Kehoe 1994). (Note that M proteins from different strains have been described using a variety of designations such as 'protein PAM,' 'protein Arp,' 'protein Sir,' or alternatively 'Emm53,' 'Emm4,' or 'Emm22' with reference to the M serotype of the strain in which these proteins were identified. The latter is highly preferable.) Some display receptors for human fibrinogen, fibronectin, β_2-microglobulin, α_2-macroglobulin inhibitors of complement activation (e.g. factor H, factor H-like protein-1 (FHL-1), C4b-binding protein (C4BP)), the Fc region of IgA and IgG of various subclasses, H-kininogen, N-CAM, and serum albumin (Kronvall et al. 1979; Wagner et al. 1986; Rýc et al.,

1989; Frithz et al. 1989; Heath and Cleary 1989; Lindahl 1989; Lindahl and Åkerström 1989; Gomi et al. 1990; Bessen and Fischetti 1992; Horstmann et al. 1992; O'Toole et al. 1992; Frick et al. 1994; Johansson et al. 1994; Johnsson et al. 1994, 1998; Retnoningrum and Cleary 1994; Åkesson et al. 1994; Natanson et al. 1995; Thern et al. 1995). It is conceivable that most of these interactions serve immune escape purposes, in particular opsonization, ensuing phagocytosis, and intracellular killing (Lindahl et al. 2000; Staali et al. 2003). Recently, Lindahl and his coworkers have provided convincing evidence that explains the M protein-mediated resistance to phagocytes (Carlsson et al. 2003). In contrast to the direct antiphagocytic effect of bacterial polysaccharide capsules, which prevent deposition of C3b, at least some M proteins exert their activity by concentrating two inhibitors of complement activation, C4BP and IgA, on the bacterial surface (Carlsson et al. 2003). Recent studies have suggested that M-protein, in addition, may prevent intracellular killing (Staali et al. 2003).

Additional putative functions of interactions with host proteins include acquisition of plasminogen, which upon activation with streptokinase (see below), confers proteolytic activity to the bacteria (Berge and Sjöbrink 1993), capture of nutrients, molecular mimicry, and environmental sensoring (Cleary and Retnoningrum 1994). It has also been suggested that some of these interactions facilitate adhesion to epithelial cells including keratinocytes (Courtney et al. 1992; Okada et al. 1995). Studies using M-positive and M-negative isogenic strains have demonstrated that one mechanism may be promotion of bacterial accumulation at the site of infection as a result of coaggregation between bacterial cells mediated by the interactions with host proteins (Caparon et al. 1991).

Proteins structurally and functionally related to M proteins have been demonstrated in several other pyogenic streptococci such as *S. equi* (group C) and *S. dysgalactiae* (group G) (Woolcock 1974; Bisno et al. 1987; Jones and Fischetti 1987; Fischetti 1989; Simpson et al. 1992; Ben Nasr et al. 1994; Schnitzler et al. 1995) and in *S. sanguis* (Demuth et al. 1990). Other species like *S. agalactiae* also bind various serum proteins via surface-exposed proteins, but the functional similarities appear to be the result of convergent evolution rather than shared phylogeny (see below).

CROSS-REACTIONS WITH HOST TISSUES

M proteins share physicochemical properties and significant sequence homology with a number of mammalian fibrillar proteins. For example, the M5, M6, M12, and M24 proteins have up to 30 percent overall sequence homology with myosin heavy chain and type I keratins, and M5, M6, and M24 share a similar level of homology with human α-tropomyosin (Fischetti 1989). Potential common epitopes provide a theoretical basis to explain the induction of cross-reactive antibodies associated with post-streptococcal autoimmune disease in genetically predisposed hosts (Khanna et al. 1989). There is a strong association between autoimmune sequelae and particular M types of *S. pyogenes*. However, there is no clear correlation between rheumatogenic or nephritogenic potential and the pattern of tissue cross-reactivity of antibodies to a particular M type (Robinson and Kehoe 1992).

CAPSULE

Some strains of *S. pyogenes* form a capsule composed of hyaluronic acid, which results in their forming mucoid colonies on blood agar. The capsular material is not immunogenic, presumably because it is indistinguishable from the hyaluronic acid of mammalian connective tissues (linear polysaccharide composed of β-1–4-linked repeating disaccharide units of glucuronic acid β-1–3 linked to *N*-acetylglucosamine). The capsule may, in this way, serve antigenic mimicry purposes. The notion that the capsule is a virulence factor is supported by the observation that the capsule is formed by 3 percent of isolates from patients with uncomplicated pharyngitis, 21 percent of isolates from severe streptococcal infections, and 42 percent of rheumatic fever isolates (Johnson et al. 1992). Among the frequently isolated serotype M1 the prevalence of capsule production is even higher (6, 22, and 80 percent, respectively). Capsule-producing strains are highly virulent in animal models. Recent studies of capsule-deficient strains constructed by transposon mutagenesis have confirmed that it is an important antiphagocytic factor but that the relative significance of the M protein and the capsule differs among strains (Wessels et al. 1991b; Dale et al. 1996).

Capsule production is encoded by the *HAS* operon, which contains at least three genes (*hasA*, hyaluronate synthase; *hasB*, UDP-glucose dehydrogenase; *hasC*, UDP-glucose pyrophosphorylase). The operon appears to be present in all strains of *S. pyogenes* and is also detected in strains of group C streptococci (Crater and van de Rijn 1995) which sometimes express a hyaluronic acid capsule (*S. equi* but not '*S. equisimilis*'). The capsule is produced in vitro only during exponential growth, but the regulatory mechanisms determining expression of the operon have not been identified.

PROTEIN F

Interaction with host fibronectin has long been considered the mechanism by which *S. pyogenes* adheres to epithelial cells. This notion is supported by the observation that exogenous fibronectin inhibits adherence and that the organism preferentially adheres to fibronectin-coated epithelial cells (Simpson and Beachey 1983; Abraham et al. 1983). Cloning and mutagenesis studies reveal that fibronectin binding is mediated by a surface protein (MW 120 kDa) known as protein F (protein F1/SfbI), which may be considered a major adhesin of

S. pyogenes (Hanski and Caparon 1992). There is also evidence that protein F plays a role in the ability of strains to invade epithelial cells (Molinari et al. 1997; Jadoun et al. 1998). However, it is notable that the presence of the gene encoding protein F is less prevalent among serotypes responsible for invasive disease and rheumatic fever, but usually present in strains that successfully persist in the throat after treatment for tonsillitis (Natanson et al. 1995; Neeman et al. 1998). Protein F also binds fibrinogen (Katerov et al. 1998).

STREPTOCOCCAL PYROGENIC EXOTOXINS AND OTHER SUPERANTIGENS

Early studies demonstrated that the intradermal injection of culture filtrate of *S. pyogenes* is followed, in some individuals, by the development of an area of erythema. This reaction (the Dick test) is the direct and indirect effect of one or more streptococcal pyrogenic exotoxins (SPE) produced by *S. pyogenes*. The SPEs represent a long array of small highly mitogenic proteins that are produced as monomeric precursor molecules with typical signal peptides (Betley et al. 1992). The mature secreted toxins have superantigen activity. Superantigens share the ability to bind to major histocompatibility complex (MHC) class II molecules to form a complex that interacts with T-cell subsets bearing particular Vβ regions on their T-cell receptors. This causes antigen-nonspecific T-cell proliferation and the release of cytokines such as interleukins (IL-1, IL-2), tumor necrosis factor α and interferon γ, which may induce hypotension, fever, and shock. The streptococcal exotoxins with superantigen include SPE-A, -B, -C, -G, -H, -I, -J, -K (so far only identified as a pseudogene), -L, and –M, streptococcal superantigen (SSA), and several allelic variants of streptococcal mitogenic exotoxin Z (SMEZ). The genes are often located on mobile DNA elements such as a bacteriophage which explains why they are variably present in strains and that identical toxins may be expressed by different evolutionary lineages of *S. pyogenes* (Mollick et al. 1993; Reda et al. 1994; Proft et al. 1999, 2003; Smoot et al., 2002b). The fact that a genetically diverse array of *S. pyogenes* clones expressing many different combinations of the pyrogenic exotoxins is recovered from contemporary invasive disease episodes suggests that no single toxin is responsible for toxic shock-like syndrome (Musser et al. 1991).

The streptococcal superantigens are members of a family of bacterial superantigens, which share biological and biochemical properties (Johnson et al. 1986; Hynes et al. 1987). Other members include staphylococcal enterotoxins, staphylococcal pyrogenic exotoxins, and staphylococcal toxic shock syndrome toxin 1. Superantigens nearly identical to some of those found in *S. pyogenes* are produced by some strains of *S. equi* (group C streptococci) (Artiushin et al. 2002; Proft et al. 2003).

SPE-A AND SPE-C

SPE-A and SPE-C are both referred to as erythrogenic exotoxins A and C, and are believed to be responsible for the skin and mucosal rash observed in patients with scarlet fever. When administered to young rabbits, together with a protein to which they have been sensitized, a skin reaction resembling that seen in scarlet fever patients develops; injection of anti-exotoxin antibodies prevents this reaction (Schlievert et al. 1979). Besides being pyrogenic, SPE-A and -C enhance susceptibility to lethal shock by endotoxins (Kim and Watson 1970), streptolysin O (Schwab et al. 1955), and other substances.

Both toxins are variably present among *S. pyogenes* in accordance with the fact that the genes are carried by temperate bacteriophages and that toxin production depending on lysogeny is responsible for their production (Zabriskie et al. 1972). The *spe-A* gene was detected in 40 percent of isolates from cases of scarlet fever in several countries and 16 percent of isolates from other conditions (Ferretti et al. 1987; Musser et al. 1991).

SPE-B

SPE-B is different from other pyrogenic exotoxins in a number of ways. It is released from cells of virtually all *S. pyogenes* strains as a 398 amino acid protein (M_r c. 40 kDa). The protein is subsequently converted into a 253 amino acid protein by proteolysis or autocatalytic truncation which requires reducing conditions (Gerlach et al. 1983; Hauser and Schlievert 1990). The truncated protein is a cysteine proteinase which cleaves a number of host proteins including fibronectin and vitronectin (two abundant extracellular matrix proteins), IgG, and human interleukin-1β precursor to generate biologically active IL-1β, suggesting an important role in inflammation and shock (Kapur et al. 1993a, 1993b; Eriksson and Norgren 2003). Purified proteinase injected into experimental animals produces myocardial necrosis and death (Kellner and Robertson 1954). Its role in the pathogenesis of *S. pyogenes* infections is supported by several observations:

- an inhibitor of cysteine proteinases (*N*-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone) can cure mice from an otherwise lethal dose of *S. pyogenes* (Björck et al. 1989)
- immunization of mice with the cysteine proteinase generates protection of mice against intraperitoneal challenge (Kapur et al. 1994)
- in patients with recent invasive infection there is a significant association between lack of antibody to SPE-B and more severe disease course (Holm et al. 1992).
- genetic inactivation of SPE-B decreases resistance to phagocytosis and dissemination to organs (Lukomski et al. 1998).

However, other studies of the effect of inactivating the cysteine protease gene *speB* on virulence in mouse models have yielded conflicting results (Ashbaugh and Wessels 2001).

The gene encoding SPE-B is highly conserved in the *S. pyogenes* population (Kapur et al. 1993b). Although it is debated if SPE-B is a true superantigen that exerts indirect toxic effects on the host, its proteolytic activity may be directly responsible for the extensive tissue destruction (cellulitis, fasciitis, and myositis) observed in many patients with severe invasive and toxic shock-like syndrome (reviewed by Rasmussen and Björk 2002). SPE-B has previously been referred to as streptococcal proteinase precursor and interleukin-1β convertase.

STREPTOLYSINS

S. pyogenes and many other pyogenic streptococci produce two distinct hemolysins, one which is hemolytic in the reduced form and the other is oxygen stable and released in serum-containing media. They have been termed streptolysin O (oxygen labile) and streptolysin S (serum soluble).

Streptolysin O is one of a family of lysins formed by various bacteria including pyogenic streptococci, *S. pneumoniae* (pneumolysin), *Clostridium tetani* (tetanolysin), *C. perfringens* (θ-toxin), *Bacillus cereus* (cereolysin) and *Listeria monocytogenes*. All are activated by sulfydryl compounds and inhibited by cholesterol and related sterols. They are antigenically related (Cowell and Bernheimer 1977), although the similarity is not evident from sequence comparison of the respective genes encoding these toxins (Kehoe and Timmis 1984). The O streptolysin is a protein of approximately 60 kDa (Kehoe et al. 1987).

There is good evidence that streptolysin O binds to cholesterol on the cell surface (Alouf 1980). Erythrocytes of all readily available animal species except the mouse are lysed, as are polymorphonuclear leukocytes and platelets.

The exact biological significance of streptolysin O is not known. However, intravenous injection of streptolysin O preparations into mice, rabbits, and guinea-pigs causes death within seconds. Death is the result of an acute toxic action on the heart (Halbert et al. 1961). It has been suggested that streptolysin O plays a role in the development of rheumatic fever (Gupta et al. 1986). Serum antibodies to streptolysin O can be demonstrated after streptococcal infection, with a frequency that increases with the severity of the infection. The highest titers are found in patients with rheumatic fever.

Streptolysin S is responsible for the β-hemolysis around colonies of most pyogenic streptococci on aerobically incubated blood agar. Most of these oxygen-stable hemolysins of pyogenic streptococci have similar properties, but that produced by *S. agalactiae* is distinct (Marchlewicz and Duncan 1980). Expression of Streptolysin O is governed by a contiguous mini-gene locus (*sagA* to *sagI*) which is conserved among *S. pyogenes* strains regardless of M protein type (Nizet et al. 2000).

STREPTOKINASE

Strains of *S. pyogenes* produce a streptokinase also referred to as fibrinolysin. It binds to human plasminogen and triggers a conformational change and self-cleavage that results in the formation of the active serine protease plasmin which is responsible for cleavage of fibrin and fibrinogen (Wulf and Mertz 1968; Ringdahl et al. 1998). Streptokinase has been referred to as streptococcal spreading factor because of its ability to induce lysis of clots and fibrin deposits that limit areas of infection.

Streptokinase activity has also been detected in *S. dysgalactiae* isolates from infections in humans and various animal species and in isolates of *S. equi*, *S. uberis*, and *S. porcinus* (group E). Although these enzymes all bind human plasminogen, their ability to activate is restricted to plasminogen from their own host. *S. dysgalactiae* subsp. *dysgalactiae* (group C) activates equine or porcine plasminogen depending on its origin, whereas strains of *S. dysgalactiae* subsp. *equisimilis* (group C and G) activate human plasminogen (McCoy et al. 1991; Vandamme et al. 1996). The streptokinases produced by *S. porcinus* and *S. uberis*, similarly, act only on porcine and bovine plasminogen, respectively (Ellis and Armstrong 1971; Leigh 1993).

The streptokinase from *S. pyogenes* is a 46 kDa extracellular protein encoded by the *ska* gene (Ohkuni et al. 1992). There is substantial diversity of the *ska* gene, giving rise to antigenic and chemical diversity (Gerlach and Köhler 1979; Huang et al. 1989; Reed et al. 1993).

C5A PEPTIDASE (SCP)

C5a peptidase is a large (over 1 100 amino acids) surface-associated serine proteinase produced by all members of *S. pyogenes*. The gene encoding the enzyme (*scpA*) is part of the *mga* regulon (Figure 6). C5a peptidase specifically cleaves human C5a, one of the principal chemoattractants of polymorphonuclear leukocytes (Wexler et al. 1985). The enzyme is produced as a precursor protein of approximately 128 kDa which includes membrane and wall-spanning domains (Chen and Cleary 1990). It cleaves a specific Lys-Asp peptide bond near the C-terminus of C5a, thereby destroying its ability to attract polymorphonuclear leukocytes (O'Connor and Cleary 1987). Neutralizing antibodies to the C5a peptidase are opsonic and can be detected in sera and secretions of patients recovering from *S. pyogenes* infections (O'Connor et al. 1991). Recent studies suggest that, in addition to the protease activity, the large protein is one of several potential invasins (Cheng et al. 2002).

Highly related C5a peptidases are produced by all strains of *S. agalactiae* (gene *scpB*) and by group C and G streptococci of human origin, presumably *S. dysgalactiae* subsp. *equisimilis* (Cleary et al. 1991; Chmouryguina et al. 1996). The *scp* gene is highly conserved but some

sequence variation with potential functional significance has been detected in the 3′ end in *S. pyogenes* (Koroleva et al. 2002).

HYALURONIDASE

Hyaluronidase activity can be detected in virtually all strains of *S. pyogenes*, and antibodies can be detected in human convalescent sera. The activity in some M types of *S. pyogenes* (M12 and M49) is phage-associated (Hynes and Ferretti 1989). Hyaluronidase activity is also present in some strains of *S. agalactiae*, *S. equi*, *S. dysgalactiae*, *S. uberis*, *S. suis*, *S. intermedius*, *S. constellatus*, and *S. pneumoniae*, and is often bacteriophage-associated (Schaufuss et al. 1989; Homer et al. 1993; Hauge et al. 1996; Granlund et al. 1997). Although the enzyme is believed to be a spreading factor favoring invasion of tissues, there is no direct evidence to support this.

NUCLEASES

All strains of *S. pyogenes* form at least one DNase. Of the four enzymes (A–D) that have been detected (Gray 1972), the B enzyme is the predominant nuclease in *S. pyogenes*. Antibodies to the B nuclease appear after most infections with *S. pyogenes*. Similar nucleases have been demonstrated in strains of group C streptococci.

NEURAMINIDASE

Neuraminidase (sialidase) activity has been detected in *S. pyogenes*, *S. dysgalactiae*, *S. pneumoniae*, *S. oralis*, *S. intermedius*, and in some strains of *S. mitis* (Hayano et al. 1969; Kilian et al. 1989; Whiley et al. 1990b). In contrast to neuraminidases of *Vibrio cholerae* and clostridia, the streptococcal enzyme acts also on *O*-acetylated sialic acids (Varki and Diaz 1983). The enzyme is a candidate colonization factor for pathogens and commensals that survive on mucosal surfaces.

SERUM-OPACITY FACTOR

Many strains of *S. pyogenes* grown in a medium consisting of three parts horse serum to one of broth induce an opalescence of the serum, which is associated with the expression of an apoproteinase (Saravani and Martin 1990). Isolates of *S. pyogenes* can be divided into two major groups on the basis of presence or absence of the opacity factor (OF), which is strongly correlated to M-types (Top and Wannamaker 1968; Widdowson et al. 1976). Subsequent studies have shown that the factor is produced exclusively by strains expressing M protein of class II (Bessen et al. 1989). OF is extractable with hot acid from M-positive strains but not from M-negative variants of them. Purification of M antigen from positive strains yields material progressively richer in opacity factor but the two substances can be partly separated (Hallas and Widdowson 1983). The exact biological function of OF is not known but the protein binds fibronectin (Rakonjac et al. 1995) and mutated strains lacking OF show reduced pathogenicity in a mouse model (Courtney et al. 1999). Neutralizing antibodies can be detected in some human convalescent sera.

TYPING OF *S. PYOGENES*

The variation in the M protein molecule between different strains is the basis of the Lancefield M serotyping scheme for *S. pyogenes* (Lancefield 1962). Typing is usually performed as a precipitation reaction with extensively absorbed antisera prepared against whole bacteria. The antigenic determinants of the M-typing procedure are resistant to heat and acid, are precipitated by ethanol, and destroyed by trypsin. It is conceivable that M-typing based on polymerase chain reaction (PCR) sequencing of M protein genes (*emm*) or using sequence-specific oligonucleotide probes will gradually replace serological typing (Scott et al. 1985; Kaufhold et al. 1992; Beall et al. 1996). This method may also be applied to isolates that do not react with available antisera (Relf et al. 1992). More than 120 *emm* sequence types are recognized among *S. pyogenes* and at least 30 *emm* types have been detected among group C and G isolates (Facklam et al. 2002, www.cdc.gov/ncidod/biotech/strep/strains.html). For the majority of M types, a direct correlation between the M-typing reaction and a specific M protein has not been established and it remains possible that serologically defined type specificity might reflect variation in more than one of member of the M protein family (Fischetti 1989; Kaufhold et al. 1992, 1994; Whatmore et al. 1994; Musser et al. 1995). Strains expressing the same M protein are not necessarily genetically related. Thus, studies of large numbers of strains by multilocus enzyme electrophoresis and DNA-based techniques demonstrated substantial genetic diversity within single M-types (Norgren et al. 1992; Haase et al. 1994; Seppälä et al. 1994; Musser et al. 1995). The lack of concordance between *emm* type and overall genetic relationship is particularly striking among group C and G streptococci (Kalia et al. 2001).

The second typing system developed by Lancefield is based on the T antigens, which are less resistant to heat and acid, are not precipitated by ethanol, and are trypsin resistant (Kehoe 1994). It was recognized from the beginning (Lancefield 1940) that M and T antigens are not necessarily separate chemical entities. Occasionally, strains with the same M type show different T type reactivity. T typing has been a powerful adjunct to M typing and may provide markers for typing of strains in which no recognized M antigen can be detected (Gaworzewska and Colman 1988).

More recently, a highly sensititive typing system based on partial sequencing of seven gene loci encoding housekeeping enzymes (multilocus sequence typing (MLST)) was established. The system is generally accessible for reference at www.spyogenes.mlst.net (Enright et al. 2001).

S. agalactiae

S. agalactiae or group B streptococci (GBS) are important causes of mastitis in cattle and, since 1960, have been recognized as the leading cause of early- and late-onset septicemia and meningitis in neonates and as an important cause of invasive infections in elderly or immunocompromised adults. Its ability to survive in the host is, to a high degree, dependent on its ability to resist phagocytosis.

Although phylogenetically related to *S. pyogenes* only 55 percent of the predicted genes of *S. agalactiae* have an ortholog in *S. pyogenes*, representing a conserved backbone between these two pathogenic species. Among the remaining genes in *S. agalactiae*, 50 percent are clustered within 14 islands, which contain known and putative virulence genes mainly encoding surface proteins. Many of these genes are associated with mobile elements, including bacteriophages, transposons, and insertion sequences, which suggests acquisition of specific virulence traits from other species (Glaser et al. 2002; Tettelin et al. 2002).

CAPSULE

Clinical isolates of *S. agalactiae* produce a polysaccharide capsule. A total of nine different capsular serotypes (Ia, Ib, and II–VIII) has been demonstrated (Lancefield 1934, 1938; Perch et al. 1979; Kogan et al. 1994, 1995, 1996). They are all polysaccharides composed of galactose and glucose, combined with 2-acetamido-2-deoxyglucose, rhamnose, or *N*-acetylglucosamine, and with terminally positioned sialic acid which gives them a net negative charge (Jennings et al. 1983; Schifferle et al. 1985; Wessels et al. 1987, 1991a; DiFabio et al. 1989; Kogan et al. 1994, 1995, 1996). The serotype designation Ic is no longer used. The c antigen is a protein which is present in nearly all strains of type Ib, some of Ia, and also in other serotypes. Strains containing the c antigen are now designated Ib/c, II/c, III/c, NT/c, etc. (NT indicates that the strain is nontypable with recognized antisera) (Henrichsen et al. 1984).

Most of the serotypes constitute separate evolutionary lineages that correlate with the expression of other properties such as α, β, and Rib proteins and hyaluronidase activity (Stålhammar-Carlemalm et al. 1993; Hauge et al. 1996; Wästfelt et al. 1996). Two separate evolutionary lineages, which seem to differ in pathogenic potential, express serotype III capsular polysaccharide, suggesting horizontal transfer of the capsular biosynthesis genes (Musser et al. 1989; Hauge et al. 1996). Recent studies show that a single gene in the capsule gene locus converts serotype Ia to III (Chaffin et al. 2000).

The sialic-acid containing capsular polysaccharides are essential virulence factors (Rubens et al. 1987). In the absence of specific antibody they prevent alternative pathway complement activation, and ensuing phagocytosis, by limiting deposition of the C3-convertase complex. This effect is eliminated by removal of sialic acid residues, and serum lacking antibodies to the complete antigen is not opsonic (Edwards et al. 1982; Wessels et al. 1989). Although invasive infection may be associated with all serotypes, strains with serotype III capsule predominate among isolates from neonatal infection (Baker and Barrett 1974; Dillon et al. 1987; Musser et al. 1989). During recent years, successful spread of a serotype V clone has been observed in both North America and Europe (Harrison et al. 1998; Lin et al. 1998; Le Thomas-Bories et al. 2001).

CAMP FACTOR AND HEMOLYSIN

Most strains of *S. agalactiae* isolated from human sources give a narrow and indistinct zone of β-hemolysis. Bovine strains are more often nonhemolytic. However, practically all strains, whether hemolytic or not, give a positive CAMP reaction: they produce a diffusible substance that completes the lysis of sheep erythrocytes exposed to a sphingomyelinase C such as staphylococcal β-toxin or the α-toxin of *C. perfringens* (Sterzik and Fehrenbach 1985).

Purified CAMP factor is lethal to rabbits when injected intravenously (Skalka and Smola 1981). Furthermore, its role as a virulence factor is supported by its ability to bind immunoglobulins G and M of humans and several animal species via the Fc part (Jürgens et al. 1987). It has also been referred to as protein B.

The hemolysin of *S. agalactiae*, which has been cloned and sequenced (Conrads et al. 1991), is not related to the streptolysins of *S. pyogenes*. It has been considered a virulence factor, but isogenic strains with and without expression of the hemolysin show no significant difference in virulence in a neonatal rat model (Weiser and Rubens 1987).

OTHER VIRULENCE FACTORS

Like *S. pyogenes*, strains of *S. agalactiae* produce several surface proteins which bind various host proteins, a C5a peptidase and hyaluronidase, although strains differ in their expression of the respective factors (Musser et al. 1989; Stålhammar-Carlemalm et al. 1993; Hauge et al. 1996; Wästfelt et al. 1996). Earlier studies demonstrated enhanced neuraminidase production in *S. agalactiae* isolates from babies with invasive disease compared to those from colonized infants (Milligan et al. 1978a; Musser et al. 1989). However, subsequent studies have shown that *S. agalactiae* does not have neuraminidase activity and that its detection was an artefact due to contamination of experimental commercial substrates with hyaluronic acid (Pritchard and Lin 1993).

TYPING OF *S. AGALACTIAE*

Capsular typing of isolates by serological means is technically difficult and satisfactory typing sera are not commercially available. Recently, serotype identification was achieved by PCR and sequencing of *cap* genes (Kong et al. 2002). DNA-based techniques may be used for differention of clones. An MLST system developed for *S. agalactiae* can be accessed at www.sagalactiae.mlst.net (Jones et al. 2003).

S. pneumoniae

Pneumococci are diplococci, hence the former name '*Diplococcus pneumoniae*.' Although genetically closely associated with oral streptococci, *S. pneumoniae* is an important pathogen which, to a high degree, is ascribed to its polysaccharide capsule.

CAPSULE

The capsule of *S. pneumoniae* is a critical virulence factor. It enables the bacteria to resist phagocytosis either by resident pulmonary macrophages or by recruited polymorphonuclear leukocytes. Antibodies to the capsule confer protection. The sizes of pneumococcal capsules vary depending on the strain, the capsular type and the growth conditions (MacLeod and Krauss 1950; Sørensen et al. 1988). The capsular polysaccharides can be isolated by alcohol precipitation but most preparations, including commercial vaccine preparations, contain contaminants of cell wall polysaccharide (C polysaccharide) which cannot be eliminated owing to covalent linking through the cell wall petidoglycan (Sørensen and Henrichsen 1984; Sørensen et al. 1990).

A total of 90 distinct capsular serotypes have been demonstrated (Kaufmann et al. 1960; Henrichsen 1995). Although they were identified by serological techniques, the chemical structures of many have been determined (for reviews see Kenne and Lindberg 1983; van Dam et al. 1990). Two different numbering systems have been used. The American system numbered all distinguishable types in order of discovery. The Danish system places antigenically closely related types together in groups (for example, Danish group 19 includes types 19F, 19A, 19B, and 19C which in the American system were types 19, 57, 58, and 59, respectively) (Henrichsen 1979, 1995; van Dam et al. 1990). The Danish method of numbering has prevailed. Six of the pneumococcal capsular polysaccharides (24A, 27, 28A, 28F, 32A, 32F) cross-react with C polysaccharide (see below) because they contain phosphorylcholine in their structure (Sørensen 1986). A few may be characterized as teichoic acids as they include phosphodiester linkages.

The genes essential for synthesis of the individual capsular polysaccharides are currently being mapped (Guidolin et al. 1994; Garcia et al. 2000; Hoskins et al. 2001; Tettelin et al. 2001). Recent data confirm the original observations by Griffith (1928) that changes in capsular type by recombination occur and that this may be a relatively frequent event which possibly will be intensified by the selective pressure exerted by vaccination against a limited number of serotypes (Coffey et al. 1998).

TEICHOIC ACIDS AND PHOSPHOCHOLINE

The cell-wall polysaccharide common to all pneumococci, which is the equivalent of the Lancefield group polysaccharides of other streptococci, is called the C polysaccharide (C substance) (Tomasz 1981). It is composed of the chemically defined wall teichoic acid linked to fragments of peptidoglycan. The wall teichoic acid is a ribitol teichoic acid associated with one or two phosphocholine residues per repeating unit (Jennings et al. 1980; Behr et al. 1992; Fischer et al. 1993; Karlsson et al. 1999). The C polysaccharide is identical to the group O antigen that is chacteristic of *S. mitis* (Bergström et al. 2000).

The lipoteichoic acid of pneumococci is also referred to as the F antigen (Forsman antigen) (Briles and Tomasz 1973). It is designated F antigen because is can evoke heterophilic antibodies that cross-react with antigens of the Forsman series (e.g. on sheep erythrocytes). Antibodies against the F antigen cross-react with Lancefield group C polysaccharide (Sørensen and Henrichsen 1987).

Phosphocholine is a crucial component of both lipoteichoic acid and the cell-wall teichoic acid (C polysaccharide). The phosphocholine residues are required for transformability, activity of autolysins, separation of daughter cells after cell division, and for the anchoring of many of the surface-exposed proteins (choline-binding proteins) that play important roles in pneumococcal infections (Fischer 2000).

IGA1 PROTEASE AND OTHER ZINC-METALLOPROTEINASES

Pneumococci of all serotypes secrete a highly specific endopeptidase which cleaves a Pro–Thr peptide bond in the hinge region of human IgA1, which is the principal mediator of specific immunity in the upper respiratory tract and the predominant IgA subclass in human serum. Cleavage of IgA1 in the hinge region abolishes the cross-binding activity and all secondary effector functions associated with the Fc portion of the antibody molecules. In addition, it has been hypothesized that the bacteria mask surface epitopes with the released monomeric Fab fragments which also inhibit complement activation (Kilian et al. 1996). Recent studies indicate that coating with released Fab fragments of IgA1 antibodies enables pneumococci to negate the hydrophilic properties of the capsule and to adhere to epithelial cells (Weiser et al. 2003). Several comprehensive genome-based screenings have confirmed that the IgA1 protease is a crucial viru-

lence factor of pneumococci even in experimental infections in mice whose IgA is not susceptible to cleavage (Polissi et al. 1998; Chiavolini et al. 2003).

The pneumococcal IgA1 protease shows significant antigenic diversity (Lomholt 1995). Genetically and functionally related IgA1 proteases are produced by *S. oralis*, *S. mitis*, and *S. sanguis* and functionally identical but genetically unrelated IgA1 proteases are secreted by strains of *Haemophilus influenzae*, *Neisseria meningitidis*, and *N. gonorrhoeae* (Kilian et al. 1996; Poulsen et al. 1996; Wani et al. 1996).

The IgA1 protease is one of several (usually two to three) zinc-metallo-proteinases produced by pneumococci (Tettelin et al. 2001; Chiavolini et al. 2003). The function of the other proteases, ZmpB and ZmpC, is incompletely elucidated. ZmpC cleaves and inactivates human matrix metallo-proteinase 9 and ZmpB induces production of tumor necrosis factor α in the respiratory tract (Oggioni et al. 2003; Blue et al. 2003).

OTHER VIRULENCE FACTORS

Pneumococci produce an intracellular hemolysin (pneumolysin) which is liberated by autolysis. Like streptolysin O of *S. pyogenes*, pneumolysin belongs to the family of thiol-activated toxins, which lose their activity on oxidation (Smyth and Duncan 1978). The two lysins are very similar in structure and contain a single cysteine residue (Walker et al. 1987); they are irreversibly inactivated by cholesterol and are immunogenic during infection (Kanclerski et al. 1988). Pneumolysin inhibits neutrophil chemotaxis, phagocytosis, and the respiratory burst and, furthermore, inhibits lymphocyte proliferation and immunoglobulin synthesis (Paton and Ferrante 1983; Ferrante et al. 1984).

Pneumococci carry numerous surface proteins of which several have been studied in detail (reviewed by Jedrzejas 2001). Pneumococcal surface protein (PspA) shows some similarities to M proteins of *S. pyogenes* (Yother et al. 1991). PspA is an inhibitor of factor B-mediated complement activation and binds lactoferrin (Hakansson et al. 2001; Hammerschmidt et al. 1999). Although PspA shows considerable antigenic diversity, immunization of mice with a recombinant PspA from one pneumococcal serotype conferred protection to challenge with different serotypes (McDaniel et al. 1991). PspC (synonyms: SpsA and CbpA) is a surface protein present in approximately 75 percent of clinical isolates. It binds secretory component of secretory IgA (S-IgA), and has been reported to interact with human lung cells and endothelial cells, and binds factor H of the complement system (Hammerschmidt et al. 1997; Brooks-Walter et al. 1999; Dave et al. 2001). PavA is a surface protein that binds fibronectin like the fibronectin-binding protein of *S. pyogenes* and the CshA protein of *S. gordonii* (Courtney et al. 1996; McNab et al. 1996; Holmes et al. 2001).

The autolysin (LytA) of pneumococci when activated, breaks the peptide cross-linking of the cell-wall peptidoglycan, enabling the release of pneumolysin (Lee and Liu 1977). In addition, cell-wall fragments released during autolysis lead to a self-perpetuating inflammatory response which is an important part of the pathogenesis of pneumococcal pneumonia and meningitis (Johnson et al. 1981; Tuomanen et al. 1985).

Like several of the pyogenic streptococci, pneumococci produce neuraminidases (NanA and NanB), and a hyaluronidase which are believed to contribute to the virulence of pneumococci. The neuraminidases strip sialic acid from the lung mucosa, thus exposing receptors for pneumococcal adherence (McCullers and Bartmess 2003). Proteolytic activity resulting in degradation of complement factor C3 is associated with a protease called PhpA (Angel et al. 1994; Zhang et al. 2001). A comprehensive review of virulence factors of *S. pneumoniae* has been published by Jedrzejas (2001).

TYPING OF *S. PNEUMONIAE*

Serotyping based on capsular polysaccharides has been the traditional typing method for pneumococci and will remain important because of its relevance for monitoring the success of vaccination which is based on a limited selection of the 90 capsular types. For other purposes various DNA based techniques are more discriminatory. An MLST system developed for *S. pneumoniae* can be accessed at www.spneumoniae.mlst.net (Enright and Spratt 1998).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Members of the genus *Streptococcus* are naturally susceptible to a wide range of therapeutically useful antibiotics. The pyogenic streptococci and some, but not all, pneumococci and other viridans streptococci are inhibited by readily achievable blood levels of benzylpenicillin, although moderate penicillin tolerance was recently described in *S. pyogenes* (De Melo et al. 2003). Streptococci are more or less resistant to all the aminoglycoside antibiotics (Horodniceanu et al. 1982).

As mentioned above, streptococci may be resistant to multiple antibiotics such as tetracycline, streptomycin, erythromycin, and chloramphenicol as a result of the acquisition of resistance genes on mobile genetic elemements. Of special clinical concern is the increasing prevalence of penicillin resistance of *S. pneumoniae* strains in several parts of the world. Penicillin resistance is often associated with resistance to multiple other antibiotics (Appelbaum 1992; Doern et al. 1996). Likewise, in parts of Asia, all clinical isolates of *S. pyogenes* became resistant to erythromycin within less than six months (Gerber 1995). Vancomycin resistance has been

described in clinical isolates of '*Streptococcus bovis*' (Poyart et al. 1997).

Penicillin resistance in *S. pneumoniae* is due to alterations of penicillin-binding proteins, the enzymes that assemble the cell-wall peptidoglycan layers (Tomasz 1987). Penicillin resistance among pneumococci has been primarily associated with strains of serotypes 6B, 14, 19A, and 23F, which have been successfully spreading to several countries with a high level of antibiotics usage. In several countries, 50–80 percent of pneumococcal isolates are penicillin-resistant (Crook and Spratt 1998). Genetic studies indicate that resistance to pencillins, cephalosporin, and some other antibiotics evolved by accumulation of point mutations in relevant chromosomal genes in commensal species such as *S. mitis* and *S. oralis*, which serve as a genetic reservoir for *S. pneumoniae* (Dowson et al. 1990, 1993; Sibold et al. 1994; König et al. 1998; Janoir et al. 1999). In addition to beta-lactam antibiotics, resistance to erythromycin, fluoquinolones including levofloxacin, gentamicin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and vancomycin has been described in clinical isolates of *S. pneumoniae*, *S. mitis*, and *S. oralis* (Klugman 1990; Kaufhold and Potgieter 1993; Krcmery et al. 1996; Gonzalez et al. 1998; Entenza et al. 1999; Wisplinghoff et al. 1999; Novak et al. 1999).

Differences in susceptibility to certain agents are useful for the presumptive identification of particular streptococci: for example, sensitivity to bacitracin and optochin, which can be detected by means of standardized disk tests. The frequent resistance of leuconostocs and pediococci to vancomycin is similarly of value in distinguishing them from streptococci.

Streptococci are destroyed by the usual strengths of disinfectants but may survive for months in dry dust in buildings. *Streptococcus* species are killed by moist heat at 55°C for 30 min, in contrast to the enterococci and some of the lactic streptococci and the exceptional *S. thermophilus*.

ANTIMICROBIAL SUSCEPTIBILITY TESTING OF STREPTOCOCCI

The broth microdilution method is the method recommended and considered the reference for susceptibility testing of pneumococci and other *Streptococcus* species (National Committee for Clinical Laboratory Standards 2000, 2002). This method, which may be used for all relevant antimicrobial agents, employs cation-adjusted Mueller-Hinton broth with 2–5 percent lysed horse blood (CAMHB-LHB) as growth medium and incubation for 24–24 h in ambient air. As an alternative, a disk diffusion method performed on Mueller–Hinton agar with 5 percent sheep blood incubated in 5–7 percent CO_2 may be used. However, the method does not work for the β-lactam antibiotics when applied to pneumococci and other viridans streptococci unless oxacillin is used (National Committee for Clinical Laboratory Standards 2000, 2002). Details of interpretative criteria have been compiled by Hindler and Swenson (2003). It is noteworthy that the recommended breakpoints that define the degree of susceptibility, mainly to penicillin, differs between pneumococci and other streptococci.

Several commercial systems are available for determining minimal inhibitory concentrations (MIC) of antimicrobial agents for streptococci, although most have been evaluated only for pneumococci. The most widely used and evaluated option is the E-test (AB Biodisk, Solna, Sweden) (Jorgensen et al. 1994; Tenover et al. 1996). However, penicillin MICs determined by the E-test are slightly lower than those determined by the standard broth microdilution procedure (Jorgensen et al. 1994). Other FDA approved methods (for pneumococci) are Pasco (Difco, Wheatridge, CO), MicroScan (Dade MicroScan Inc., West Sacramento, CA), MicroTech (Aurora, Colorado), Sensititre (Trek Diagnostic Systems Inc., Westlake, OH), and Vitek 2 (bioMérieux Inc, Hazelwood, MO).

Susceptibility testing of the fastidious streptococci of the genera *Abiotrophia* and *Granulicatella* can be accomplished by adding 0.001 percent pyridoxal HCL to the growth medium (Ruoff 1991).

ABIOTROPHIA AND *GRANULICATELLA*

Members of the two genera *Abiotrophia* and *Granulicatella* constitute the so-called nutritionally variant streptococci (NVS). They grow as satellite colonies around other microorganisms and in complex media only when supplemented with sulfydryl compounds such as cysteine. They have previously been referred to as satellite or symbiotic streptococci, thiol-requiring streptococci, vitamin B_6- or pyridoxal-dependent streptococci or NVS. They were previously considered nutritional variants of other streptococcal species, in particular *S. mitis*. Bouvet and coworkers (1985, 1989) demonstrated two distinct nutritional variants originally proposed as '*Streptococcus adjacens*' and '*S. defectivus*' respectively. Subsequent comparisons of 16S rRNA gene sequences revealed that they are quite distinct from other species in the genus *Streptococcus*, which led to the proposal that they be placed in a new genus *Abiotrophia* as *A. adiacens* and *A. defectiva*, respectively (Kawamura et al. 1995b). Subsequently, three additional species were described, *A. elegans* and *A. paraadiacens*, and *A. balaenopterae* (isolated from a minke whale) (Roggenkamp et al. 1998; Lawson et al. 1999; Kanamoto et al. 2000). Based on phylogenetic analysis Collins and Lawson (2000) transferred three of the species to a new genus *Granulicatella* as *Granulicatella adiacens*, *G. elegans*, and *G. balaenopterae*.

Members of the two genera form minute α-hemolytic colonies on sheep blood agar supplemented with 10 mg/l

pyridoxal hydrochloride or 100 mg/l cysteine. Their cell morphology depends upon growth conditions and phase. When cultivated in pyridoxal- or cysteine-supplemented complex media, they are pleomorphic with chains that include cocci, coccobacilli, and rod-shaped cells. A tendency towards rod formation is observed in the stationary growth phase. In a semisynthetic medium (CDMT) they form small ovoid cocci (diameter 0.4–0.55 μm) which occur singly, in pairs, or in chains of variable length. They do not produce extracellular polysaccharides from sucrose and may be easily distinguished from the mitis group of streptococci by their growth characteristics and the production of pyrrolidonyl-arylamidase (Bouvet et al. 1989). A comprehensive study of their enzymatic activities have been presented by Beighton et al. (1995) and Christensen and Facklam (2001).

Abiotrophia and *Granulicatella* species (with the exception of *G. balaenopterae*) form part of the resident microflora of the human oral cavity (Mikkelsen et al. 2000; Paster et al. 2001; Kazor et al. 2003) and other parts of the upper respiratory tract, and may be isolated from vaginal and intestinal tracts. Like most members of the mitis group of streptococci they have been isolated from various human infections including subacute endocarditis, brain abscesses, and wound infections, and from urine (Ruoff 1991; Christensen and Facklam 2001).

LACTOBACILLUS

GENUS DEFINITION

Lactobacilli are straight or curved rods of varying length and thickness, with parallel sides, arranged singly or in chains, sometimes filamentous or pleomorphic, without branching, clubbing or bifid formation. They are gram-positive and nonsporing. Colonies on agar media are usually small. They have complex nutritional requirements. Growth is favored by anaerobic or microaerobic conditions and by carbon dioxide. Energy is obtained by the fermentation of sugars; glucose is fermented, and either lactic acid alone or lactic acid along with other volatile acids and carbon dioxide is formed. Most strains have cell wall bound proteinases and peptidases. There is no production of catalase, oxidase, or indole and no reduction of nitrate. The organisms are readily killed by heat but unusually tolerant of acid. Lactobacilli are widely distributed in fermenting vegetable and animal products and in the alimentary tract of humans and animals. They are rarely pathogenic for humans. The G + C content of DNA is 32–53 mol%. The genus includes more than 90 recognized species (www.dsmz.de/bactnom/nam1537.htm; Figure 8). The type species is *Lactobacillus delbrueckii*.

INTRODUCTION AND HISTORICAL PERSPECTIVE

The type species of the genus *Lactobacillus*, *L. delbrueckii*, was originally isolated from milk by Leichmann (1896). A similar bacillus was observed by Döderlein in 1892 in the vaginal secretion of women, but the identity of this organism is in doubt (Sharpe 1981). In 1900, Moro cultured a slender gram-positive bacillus, *L. acidophilus*, from the feces of breast-fed babies. Lactobacilli from cheese were named *L. casei* by Orla-Jensen (1904) and Heinemann and Hefferan (1909) isolated lactobacilli from human saliva, gastric juice, soil, and various foods. An organism isolated from carious teeth and named *L. odontolyticus* (McIntosh et al. 1922, 1924) is probably the same as *L. plantarum* which was described by Pederson (1936). For other references on early work with lactobacilli see previous editions of this book.

The decision to describe *Streptococcus* and *Lactobacillus* in the same chapter follows Orla-Jensen's (1919, 1943) concept of a cluster of 'lactic-acid bacteria.' Orla Jensen's primary division was between the homofermentative thermobacteria and streptobacteria on one hand and the heterofermentative betabacteria on the other. The streptobacteria will grow at 15°C and most thermobacteria at 45°C. One organism currently classified as a streptobacterium, *L. casei* var. *rhamnosus*, will grow at either temperature.

The species of lactobacilli described here include those isolated most commonly in medical laboratories. Kandler and Weiss (1986), Sharpe (1981), Hammes et al. (1991), and Hammes and Vogel (1995) offer more comprehensive descriptions and review their classification. The review by Hammes et al. (1991) provides a comprehensive survey of the isolation, ecophysiology, identification, and application of lactobacilli. Schillinger and Lücke (1987) give an account of the lactobacilli present in meat and meat products.

There is renewed interest in lactobacilli in human medicine because of the probiotic effects of some species. The complete genome sequence of a strain of *Lactobacillus lactis* subsp. *lactis* has been determined (Bolotin et al. 2001).

HABITATS

Lactobacilli are found where rich carbohydrate-containing substrates are available; they live in a variety of habitats such as on mucosal membranes of humans and animals (oral cavity, intestine, and vagina), in plant materials such as silage, and in foodstuffs and agricultural products, particularly milk, cheese, and fermented milk products and in fermented beverages such as wine and cider. In some of these products the multiplication of lactobacilli brings about desirable changes, in others it causes spoilage. In the body flora, lactobacilli are present in moderately large numbers in

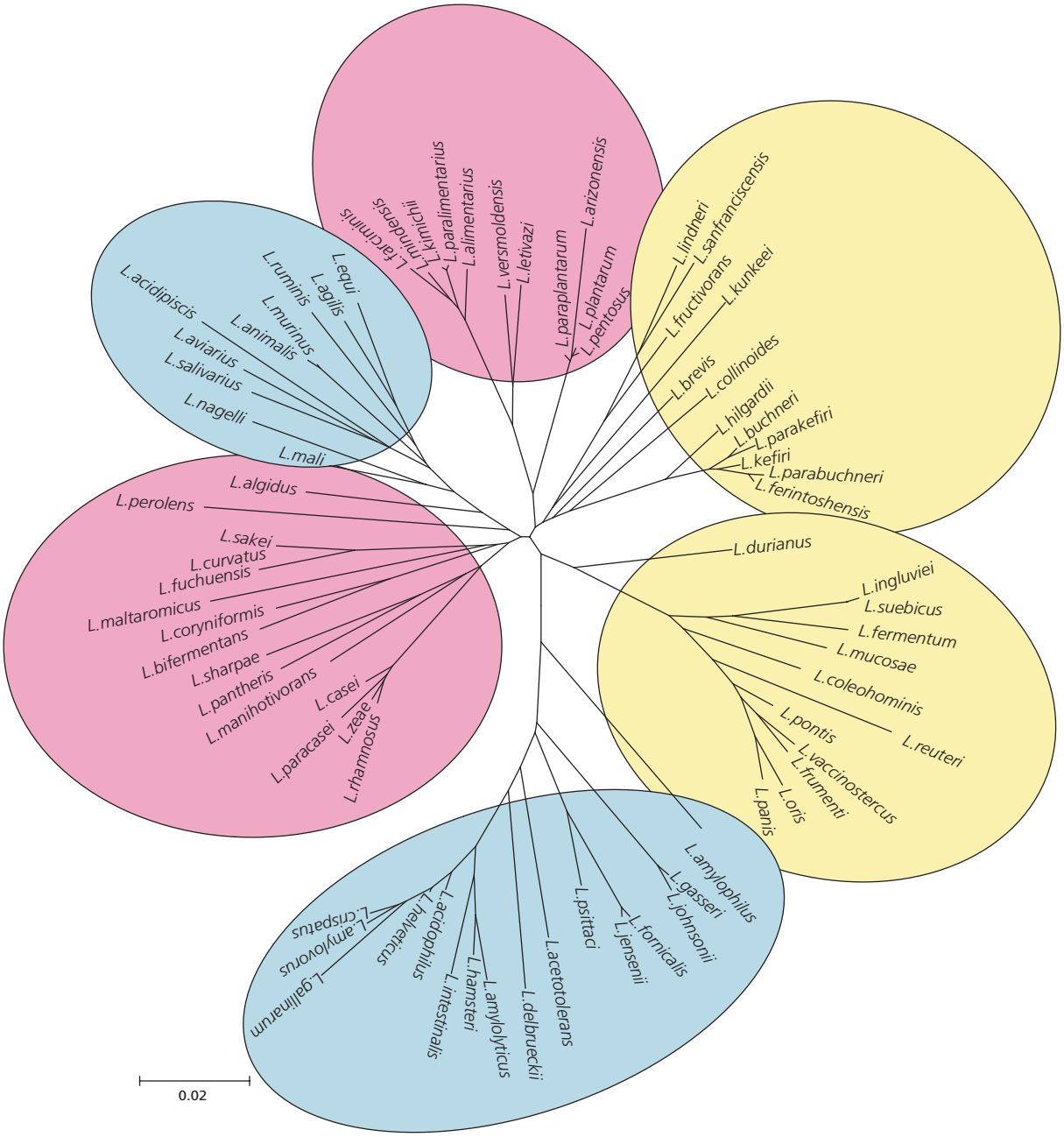

Figure 8 *Phylogenetic relationships of* Lactobacillus *species for which complete 16S rRNA gene sequences are available. The tree was prepared by the neighbour-joining (NJ) method using MEGA2 (Kumar et al. 2001). With few exceptions, species within light blue circles are obligately homofermentative, within yellow circles obligately heterofermentative, and within pink circles facultatively heterofermentative.*

the mouth, gut, and vagina but seldom predominate (Salminen et al. 1993). Members of several species of lactobacilli are found at each of these sites. In general, those most often present in the body flora are: in the mouth, *L. casei*, *L. fermentum*, *L. brevis*, and *L. acidophilus* (Rogosa et al. 1953); in the small intestine, *L. acidophilus*, *L. fermentum*, *L. salivarius*, and *L. reuteri* (Molin et al. 1993) and in the vagina primarily *L. crispatus*, *L. jensenii*, *L. gasseri*, and to a smaller degree, *L. acidophilus*, *L. fermentum*, *L. casei*, *L. cellobiosus*, *L. coleohominis*, *L. mucosae*, *L. paracasei*, *L. reuteri*, *L. rhamnosus*, *L. ruminis*, and *L. vaginalis* (Sharpe 1981; Antonio et al. 1999; Nikolaitchouk et al. 2001; Pavlova and Kilic 2002). These species belong in several evolutionary clusters within the genus (Figure 8).

MORPHOLOGY

Lactobacilli are in general fairly large nonsporing, gram-positive rods, but they vary in length and breadth and in

old cultures tend to be gram-negative. A few strains are motile by peritrichous flagella. Metachromatic granules are prominent in some species, notably *L. lactis*, *L. leichmannii*, and *L. bulgaricus*. Some members form long chains with cells coiled or twisted. The thermobacteria are usually large, thick, and often filamentous. Among the streptobacteria, *L. casei* is a short square-ended rod, forming chains of varying length. *L. plantarum* varies in length from coccoid to short filamentous forms. Two variants of streptococci previously classified as '*Streptococcus lactis*' with a tendency to form elongated cells, have been reclassified as *L. xylosus* and *L. hordniae* (Garvie et al. 1981; Schleifer et al. 1985).

CULTURAL CHARACTERISTICS

All media for the isolation of lactobacilli are complex. A widely used nonselective medium, at pH 6.2–6.4, is the MRS medium of de Man et al. (1960). For selective isolation the acetate medium (SL) of Rogosa et al. (1951) is the medium of choice particularly when prepared in the manner described by Sharpe (1981). The presence of Tween 80 stimulates the growth of many lactobacilli and a high content of acetate at pH 5.4 is selective for them. Sharpe (1981) recommends additional media for the isolation of lactobacilli from foods and beverages because those from specialized environments may require more specific supplements.

Colonies on agar media are usually small, 1–3 mm in diameter, with entire margins. Some species form rough colonies. Rogosa and Sharpe (1959) made the general observation that colonies of streptobacteria are smooth and those of thermobacteria are rough. Some strains isolated from foodstuffs form slime.

METABOLISM

In the homofermentative species, the streptobacteria and the thermobacteria, glucose is broken down to lactic acid almost exclusively by the Embden–Meyerhof pathway. The heterofermentative species, the betabacteria, possess the 6-phosphogluconate pathway in which the end products are carbon dioxide, acetic acid, ethanol, and lactic acid (see Kandler 1982). Some species are facultatively heterofermentative. They ferment hexoses almost exclusively to lactic acid by the Embden–Meyerhof pathway. In addition, the organisms possess both aldolase and phosphoketolase, and are capable of fermenting pentoses (and often gluconate). In the presence of glucose, the enzymes of the phosphogluconate pathway are repressed (Hammes and Vogel 1995). Because carbon dioxide is soluble in water, the conventional Durham tube is inapplicable and other methods, such as a shake culture in MRS agar in a tube with a plain agar overlay as a seal, are required to demonstrate carbon dioxide production. Practically all of the streptobacteria and betabacteria, but none of the thermobacteria, ferment ribose.

The lactobacilli are acidophilic and grow best in medium at about pH 6. They are aciduric and the final pH in glucose broth with some species can be as low as 3.5. For testing the fermentation of carbohydrates, Rogosa and Sharpe (1959) recommend a medium with an initial pH of 5.5–6.0. Kits in which patterns of fermentation are determined against many different carbohydrates are often employed for identification (Hammes et al. 1991).

Most of the lactobacilli will grow in air but grow best in an atmosphere lacking oxygen but supplemented with carbon dioxide (Rogosa and Sharpe 1959). A few are strict anaerobes. The catalase test is nearly always negative and the occasional weakly positive reaction can be attributed to a pseudocatalase action because negative benzidine tests indicate the absence of a cytochrome system (Sharpe 1981).

The temperature at which growth occurs varies with the species. Thermobacteria grow best at 37–40°C; none grows at 15°C and most will grow at 45°C. The optimum for streptobacteria is about 30°C; all grow at 15°C. Among the betabacteria, *L. brevis*, *L. buchneri*, and *L. viridescens* resemble streptobacteria, *L. fermentum* resembles thermobacteria, *L. cellobiosus* is variable in this characteristic.

Other nutritional requirements

The nutritional requirements of lactobacilli are complex and varied but are normally met by media which, in addition to fermentable carbohydrate, contain peptone, meat, and yeast extract. Supplements that are stimulatory, or even essential, include tomato juice, manganese, acetate, and oleic acid esters, and Tween 80 in particular. Requirements for vitamins are scattered throughout the species and vitamin-dependent strains are used for bioassays. Thiamine is necessary for the growth of nearly all the heterofermentative organisms but not those that are homofermentative (Ledesma et al. 1977). Requirements for amino acids and peptides seem to be met by a combination of cell-wall-bound proteinases and peptidases and mechanisms for active transport across the cell membrane (Law and Kolstad 1983).

CELL-WALL COMPOSITION; ANTIGENIC STRUCTURE

The chief amino acid in the peptidoglycan of most species of lactobacilli is lysine, but in some it is diaminopimelic acid, and in others ornithine. All of the thermobacteria considered here have interpeptide bridges of the L-lysine–D-aspartate type. Among the streptobacteria, *L. casei* has a peptidoglycan bridge of

Table 4 *Group antigens of lactobacilli*

Species	Group	Antigen	Location	Determinant
L. helveticus	A	Glycerol teichoic acid	Wall–membrane	α-D-Glucose
L. casei	B	Polysaccharide	Wall	α-L-Rhamnose
L. casei	C	Polysaccharide	Wall	β-D-Glucose
L. plantarum	D	Ribitol teichoic acid	Wall	α-D-Glucose
L. lactis	E	Glycerol teichoic acid	Wall	α-D-Glucose
L. fermentum	F	Glycerol teichoic acid	Membrane	α-D-Galactose

Based on Knox and Wicken (1976).

the L-lysine–D-aspartate type and *L. plantarum* one of the *meso*-diaminopimelic type. Three types of peptidoglycan are found in the species of betabacteria described here: (1) L-lysine–D-aspartate in *L. brevis* and *L. buchneri*; (2) L-ornithine–D-aspartate in *L. fermentum* and *L. cellobiosus*; and (3) L-lysine–L-alanine–L-serine in *L. viridescens* (Schleifer and Kandler 1972).

Many lactobacilli share an antigen present in the cell membrane (Sharpe et al. 1973). This corresponds to the 1–3-linked glycerol phosphate units of the membrane glycerol-teichoic acid. This antigen is distinct from the group antigens described by Sharpe (1955), which she identified by precipitation reactions between hot-acid extracts and antisera. The chemical composition of these antigens was reviewed by Knox and Wicken (1976) and is shown in Table 4. It will be noted that the *L. casei* strains, except those of subspecies *rhamnosus*, may possess one of two group antigens, and that the group E antigen occurs in members of four species, two of them thermobacteria and two betabacteria.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

The lactobacilli have no particular resistance to heat and are destroyed by exposure to 60 or 65° for 30 min. They are, however, especially resistant to acid and are able to grow in concentrations of acid that are fatal to most other bacteria. The tolerance to bile varies and has been used to distinguish between species (Sharpe 1981).

Lactobacilli of several species can be resistant to many antibiotics including vancomycin, the peptide antibiotics, the macrolides, tetracycline, and the aminoglycosides. In these strains, loss of plasmids was usually accompanied by a change to sensitivity with several antibiotics (Vescovo et al. 1982).

Lactobacilli are refractory to transformation and transduction, but the transmission of the plasmid that determines the ability of *L. casei* to ferment lactose does occur naturally.

CLASSIFICATION

Orla-Jensen (1919, 1943), whose monographs have influenced all subsequent workers, laid particular stress on fermentative ability and the type of lactic acid produced from glucose in his classification of lactobacilli. His three primary divisions of the lactobacilli into thermobacteria, streptobacteria, and betabacteria were followed for many years, though not his proposal to consider them as separate genera. Recent comparative studies of 16S rRNA gene sequences revealed that the three groups lack phylogenetic foundation. Furthermore, such studies indicate that lactobacilli are phylogenetically intermixed with members of the genera *Leuconostoc* and *Pediococcus* despite differences in morphology and fermentation patterns (for review see Ludwig and Schleifer 1994). Sequence studies also revealed that several taxa were erroneously placed in the genus *Lactobacillus*. As a result some were transferred to the genus *Clostridium* and two anaerobic species '*L. uli*' and '*L. rimae*' isolated from human gingival crevices (Olsen et al. 1991) were transferred to the new genus *Atopobium* (Collins and Wallbanks 1992). Some of the obligately heterofermentative species isolated from fermented sausages (i.e. '*L. confusus*,' '*L. fructosus*,' '*L. halotolerans*,' '*L. viridescens*,' '*L. kandleri*,' and '*L. minor*') are now transferred to a new genus *Weissella* (Collins et al. 1993).

At least six phylogenetic groups are evident based on analysis of 16S rRNA gene sequences. To a high degree these groups of species correlate with fermentation patterns (Figure 8). Species that are regularly isolated from humans are distributed over several groups.

Hammes and Vogel (1995) provide details about cell-wall composition and identification criteria. It is remarkable that for an unequivocal identification of a *Lactobacillus* isolate it is not always sufficient to use the classical physiological and biochemical tests. A review by Pot et al. (1994) provides a discussion of methods for identification of lactobacilli including a critical evaluation of their limitations.

PATHOGENICITY

The pathogenic potential of lactobacilli is very limited. In spite of their ubiquitous presence in the digestive tract and in the vagina, lactobacilli are only occasionally isolated from cases of bacteremia or endocarditis with the highest prevalence in elderly individuals and immunocompromised patients (Husni et al. 1997; Salminen

et al. 2002; Arpi et al. 2003). Various *Lactobacillus* species are often part of the mixed microflora in samples from orofacial infections, periapical infections associated with teeth with necrotic pulp tissue, and appendicitis, but there is no evidence that they contribute to the infectious process (Moncla and Hillier 2003). The relationship of lactobacilli to dental caries was the subject of considerable interest for many years. However, its increased proportions in dental plaque of caries-active individuals may merely be due to its acidophilic properties (for review see Hardie and Bowden 1974). Some clinical isolates of lactobacilli are reported as being resistant to vancomycin (Holliman and Bone 1988; Felten et al. 1999).

ROLE IN NORMAL HUMAN FLORA

Some species of lactobacilli are members of the commensal microflora of human mucosal membranes in the mouth, intestines, and vagina, although they usually comprise a minor part of the flora (London 1976). In the oral cavity, lactobacilli usually amount to less than 1 percent of the microflora although their proportion increases in individuals with a frequent intake of sugar. Studies of the intestinal lactobacillus flora of piglets have demonstrated a rapid turnover of clones (Tannock et al. 1990).

Due to production of bacteriocins, hydrogen peroxide, and to their acidogenic potential, which reduces the pH in the local environment, lactobacilli play an important role in inhibiting the establishment of potential pathogens on mucosal surfaces (Roach and Tannock 1979; Hentjes 1983). Although the probiotic effect of lactobacilli is an old concept described by Metchnikoff in 1901 (see review by Bibel 1988), there is considerable renewed interest in this topic. Recent studies have demonstrated that lactobacilli administered orally to patients with viral and bacterial intestinal infections augment mucosal immune responses and promote recovery (Kaila et al. 1992; Perdigon et al. 1995). Furthermore, it has been demonstrated in a rat model that lactobacilli increase the barrier functions of the gut mucosa (Isolauri et al. 1993). More recent studies demonstrated that feeding of infants with lactobacilli significantly reduced subsequent development of atopic disease (Kalliomaki et al. 2003).

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Enterococcus

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GENUS DEFINITION

The members of the genus *Enterococcus* are gram-positive cocci that occur singly or arranged in pairs or as short chains. Cells are sometimes coccobacillary when Gram stains are prepared from growth on solid media. Cells tend to be ovoid and in chains when Gram stains are prepared from growth in thioglycolate broth. The enterococci are facultative anaerobes that grow at temperatures ranging from 10 to 45°C, with optimum growth at 35°C. They grow in media containing 6.5 percent NaCl and hydrolyze esculin in the presence of 40 percent bile salts (bile–esculin (BE) medium). Some species are motile. Most enterococcal species hydrolyze L-pyrrolidonyl-β-naphthylamide (PYR) by producing pyrrolidonyl arylamidase (pyrrolidonase [PYRase]): the exceptions are *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus pallens*, and *Enterococcus saccharolyticus*. All strains hydrolyze leucine-β-naphthylamide by producing leucine aminopeptidase (LAPase). Enterococci are not able to synthesize porphyrin precursors and therefore do not express cytochrome enzymes (Devriese et al. 1992; Huycke et al. 1998; Huycke 2002). However, cytochrome activity is sometimes expressed when strains of *Enterococcus faecalis* are grown on blood-containing media, and a weak effervescence is observed in the catalase test. Positive catalase testing was also reported for strains of *Enterococcus haemoperoxidus*, a recently described species (Svec et al. 2001). Nearly all strains are homofermentive, gas is not produced, and lactic acid is the end product of glucose fermentation. Most strains produce a cell-wall-associated glycerol teichoic acid antigen that is referred to as the Lancefield's serologic group D antigen. The guanine and cytosine (G+C) content of the DNA ranges from 37 to 45 mol% (Schleifer and Kilpper-Balz 1984).

The tests listed in Table 1 can be used to identify and distinguish most enterococci from other catalase-negative gram-positive cocci (Facklam and Elliott 1995).

The phylogenetic analysis of genera of catalase-negative gram-positive cocci based on the comparison the 16S rRNA gene sequences has revealed that the genus *Enterococcus* is more closely related to the genera *Vagococcus*, *Tetragenococcus*, and *Carnobacterium* than to the *Streptococcus* and *Lactococcus* (Figure 1), genera to which it has been phenotypically associated (Devriese et al. 1992; Facklam et al. 2002).

HISTORICAL PERSPECTIVE

Early documentation of microorganisms that are now included in the genus *Enterococcus* is mainly related to the 'streptococci of fecal origin' or 'enterococci.' The term 'enterococcus' probably originated with the

Table 1 *Phenotypic characteristics used for the differentiation of facultatively anaerobic, catalase-negative, gram-positive cocci*

Genus or category	Cell arrangement[a]	Phenotypic characteristic[b]								
		VAN	GAS	BE	PYR	LAP	NaCl	10°C	45°C	HEM
Enterococcus group[c]	ch	S/R	–	+	+	+	+	+	+[d]	α/γ
Leuconostoc/Weisella[e]	ch	R	+	V+	–	–	V+	V–	V–	α/γ
Streptococcus	ch	S	–	–[f]	–[g]	+	–[h]	–	V–	α/β/γ
Nutritional var. Strep[i]	ch	S	–	–	V+	+	–	–	V–	α/γ
Unusual Strep/Genera[j]	ch	S	–	V+	V+	V+	V+	V–	V–	α/γ
Pediococcus	cl/t	R	–	V+	–	+	V–	–	V+	α
Tetragenococcus	cl/t	S	–	+	–	+	+	–	+	α
Aerococcus[k]	cl/t	S	–	V+	V+	V–	+	–	V–	α
Helcococcus	cl/t	S	–	–	+	–	+	–	–	γ
Gemella	cl/t/ch	S	–	–	+	+	–	–	–	γ
Salt-tolerant *Gemella*-like[l]	cl/t/ch	S	–	–	+	+	+	–	–	γ

a) Cell arrangement in Gram stain: ch, chains; cl, clusters; t, tetrads.
b) VAN, vancomycin susceptibility screening test; S, susceptible; R, resistant; GAS, gas production in MRS broth; BE, hydrolysis of esculin in the presence of bile; PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; NaCl, growth in broth containing 6.5 percent NaCl; 10 and 45°C, growth at 10 and 45°C; HEM, hemolytic activity on Trypticase soy 5 percent sheep blood agar; β, beta-hemolysis; α, alpha-hemolysis; γ, no hemolysis; +, 85 percent or more of the strains are positive; –, 15 percent or less of the strains are positive; V+, variable positive (50–84 percent of the strains are positive); V–; variable negative (16–49 percent of the strains are positive).
c) *Enterococcus* group includes all *Enterococcus* species, *Vagococcus* species and some *Lactococcus* species.
d) Some strains of lactococci and vagococci grow very slowly when incubated at 45°C.
e) *Leuconostoc* and *Weisella* are often coccobacillary, sometimes appearing rod-like in chains.
f) All the *Streptococcus bovis* strains and approximately 10 percent of the 'viridans' streptococci are bile–esculin-positive.
g) All the *Streptococcus pyogenes* strains are PYR-positive, and most of the other streptococci are PYR-negative.
h) Some strains of β-hemolytic but none of the non-β hemolytic streptococci grow in 6.5 percent NaCl.
i) Nutritional variant streptococci are now identified in two different genera: *Abiotrophia* and *Granulicatella*.
j) Unusual strep/genera includes species of streptococci usually found in animals and *Globicatella sanguinis* and *Dolosicoccus paucivorans*.
k) *Aerococcus* species includes *A. viridans*, *A. urinae*, *A. sanguicola*, and *A. urinehominis*.
l) Salt-tolerant *Gemella*-like bacteria include *Alloiococcus*, *Dolosigranulum*, *Facklamia*, and *Ignavigranum* species.

discovery of the first organism of this group. Thiercelin (1899) used the term to describe bacteria seen in pairs and short chains in human feces. In the same year, MacCallum and Hastings described a case of acute endocarditis caused by a bacterium that was 'very hard and tenacious of life,' which was named *Micrococcus zymogenes*. These authors also mentioned that a similar 'micrococcus' had previously been isolated from a cesspool. A few years later Gordon reported the isolation of fecal streptococci in a broth exposed to air probably contaminated with animal feces, and Houston (cited in Wade 1997) noted the abundance of streptococci in sewage, proposing that they might be useful for detecting contamination of water by human feces. In 1906, the name *Streptococcus faecalis* was used by Andrews and Horder to identify an organism of fecal origin that clotted milk and fermented mannitol and lactose, but not raffinose. Orla-Jensen (1919) described a second organism of this group, *Streptococcus faecium*, which differed from the fermentation patterns of *S. faecalis*. A third species, *Streptococcus durans*, proposed by Sherman and Wing (1935, 1937) was similar to *S. faecium* but had less fermentation activity. The term 'enterococcal group' was used by Sherman (1937, 1938) to describe streptococci that grew at 10 and 45°C, grew in broth with pH adjusted to 9.6 and in broth containing 6.5 percent NaCl, and survived heating to 60°C for 30 min. In 1967, Nowlan and Deibel (1967) added *Streptococcus avium* to the enterococcal group. In 1970, Kalina proposed that a genus for the enterococcal streptococci be established and suggested that, based on cellular arrangement and distinguishing phenotypic characteristics, *S. faecalis* and *S. faecium* be named *Enterococcus*. No action on this proposal was ever taken and the use of the genus name *Streptococcus* continued. For a long time the enterococci remained as a major division of the genus *Streptococcus* differentiated by their higher resistance to chemical and physical agents, and accommodating most of the serological group D streptococci. In the past decades, however, the enterococci have undergone considerable changes in taxonomy, which started with the splitting of the genus *Streptococcus* and the recognition as a separate genus. Definite evidence that *S. faecalis* and *S. faecium* were sufficiently different from the other members of the genus *Streptococcus* to merit allocation into a separate genus, named *Enterococcus*, was provided by Schleifer and Kilpper-Balz, in 1984, on the basis of DNA–DNA and DNA–rRNA hybridization studies, as well as 16S rRNA sequencing. Thereafter, the other enterococcal species were transferred to the new genus, and several new species were described and proposed for inclusion in the genus *Enterococcus*. Table 2 lists the species proposed to be included in the genus *Enterococcus*.

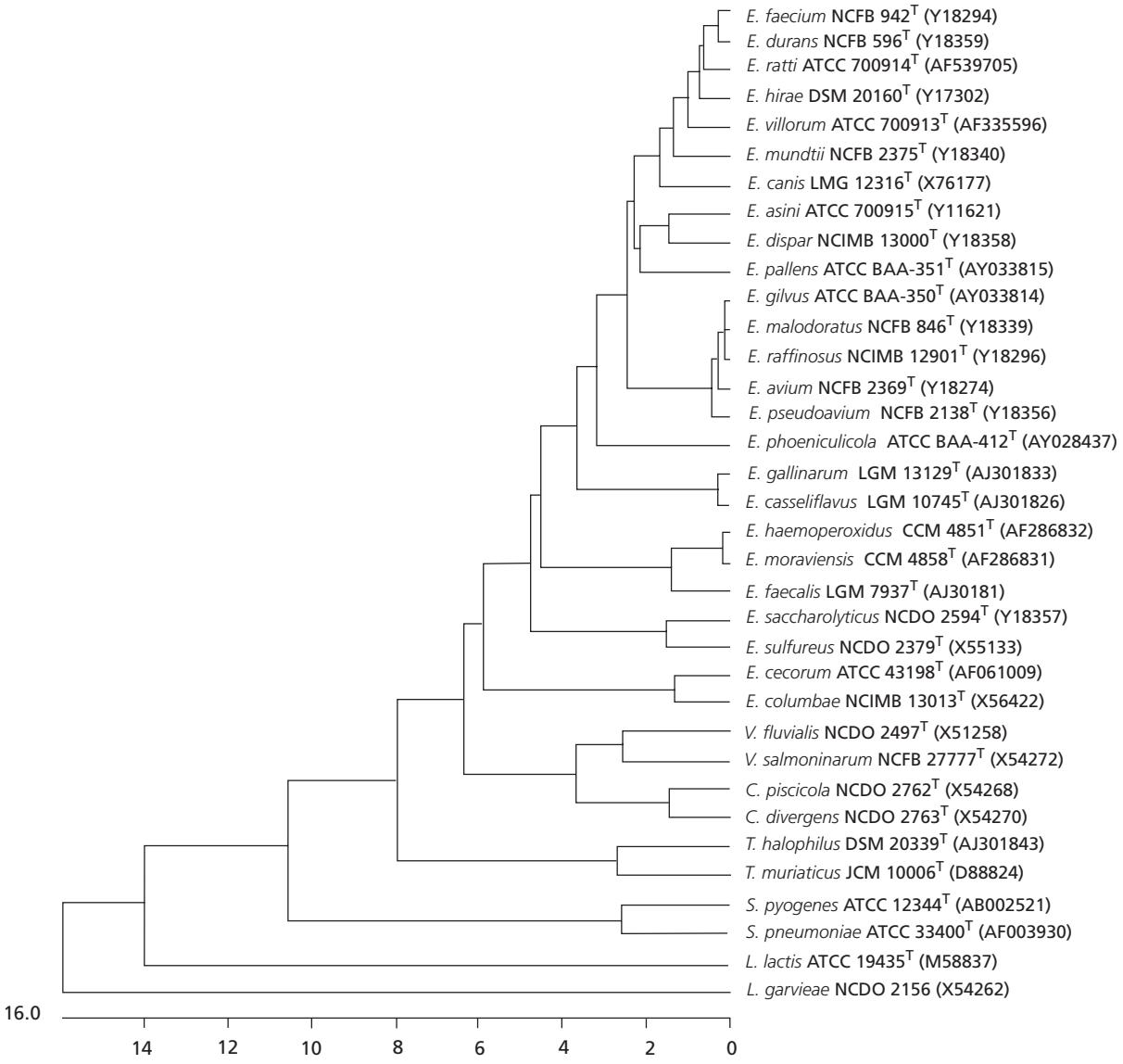

Figure 1 *Phylogenetic relationships among species of* Enterococcus *and some related species of other catalase-negative gram-positive cocci, based on analysis of 16S rRNA gene sequences. The dendrogram was constructed by the ClustalW method using the DNASTAR program. The scale units indicate evolutionary distance values between sequence pairs.*

Current criteria for inclusion into the genus *Enterococcus* are based on a combination of results of DNA–DNA reassociation experiments, 16S rRNA gene sequencing, analysis of whole-cell protein (WCP) profiles, and conventional phenotypic tests. While DNA–DNA reassociation is considered the gold standard for species definition, 16S rRNA gene sequencing and WCP profile analysis correlate very well with this gold standard (Merquior et al. 1994; Teixeira et al. 1995; Teixeira et al. 1997a; Patel et al. 1998; Tyrrell et al. 2002). The long-chain fatty-acid composition of enterococcal cells, as revealed by gas–liquid chromatography analysis, is also of taxonomic value and has been used to discriminate species (Schleifer and Kilpper-Balz 1984; Hsueh et al. 2000; Tyrrell et al. 2002). By using these techniques some of the proposed species have not been validated as new *Enterococcus* species (Table 2). *Enterococcus seriolicida* was shown to be homologous to *Lactococcus garvieae* (Teixeira et al. 1996). Since *L. garvieae* was named first, it was retained as the species name. *Enterococcus casseliflavus* and *Enterococcus flavescens* were also shown to be closely related at the species level (Teixeira et al. 1997a). Since *E. casseliflavus* was described first it is the senior subjective synonym of *E. flavescens* and it was retained as the species denomination. *Enterococcus solitarius* was shown to be more closely related to the genus *Tetragenococcus* than to any species of the *Enterococcus* by 16S rRNA gene sequence analysis (Williams et al. 1991). There is also evidence that two of the most recently proposed

Table 2 *Proposals of species to be included in the genus* Enterococcus

Species	References
E. faecalis	Schleifer and Kilpper-Balz 1984
E. faecium	Schleifer and Kilpper-Balz 1984
E. avium	Collins et al. 1984
E. casseliflavus	Collins et al. 1984
E. durans	Collins et al. 1984
E. gallinarum	Collins et al. 1984
E. malodoratus	Collins et al. 1984
E. hirae	Farrow and Collins 1985
E. mundtii	Collins et al. 1986
E. raffinosus	Collins et al. 1989
E. solitarius[a]	Collins et al. 1989
E. pseudoavium	Collins et al. 1989
E. cecorum	Devriese et al. 1983; Williams et al. 1989
E. columbae	Devriese et al. 1990
E. saccharolyticus	Farrow et al. 1984; Rodrígues and Collins 1990
E. dispar	Collins et al. 1991
E. sulfureus	Martinez-Murcia and Collins 1991
E. seriolicida[a]	Kusuda et al. 1991
E. flavescens[b]	Pompei et al. 1992
E. asini	De Vaux et al. 1998
E. villorum[c]	Vancanneyt et al. 2001
E. haemoperoxidus	Svec et al. 2001
E. moraviensis	Svec et al. 2001
E. ratti	Teixeira et al. 2001
E. porcinus[c]	Teixeira et al. 2001
E. gilvus	Tyrrell et al. 2002
E. pallens	Tyrrell et al. 2002
E. phoeniculicola	Law-Brown and Meyers 2003
E. canis	De Graef et al. 2003

a) DNA reassociation studies and/or 16S rRNA sequencing studies indicate that these two species do not belong to the genus *Enterococcus*. *E. seriolicida* is related at the species level to *L. garvieae* and *E. solitarius* is more related to the genus *Tetragenococcus*.
b) DNA reassociation studies showed that *E. flavescens* and *E. casseliflavus* are related at the species level and constitute a single species.
c) Reports describing *E. villorum* and *E. porcinus* were published only 3 months apart; whole-cell protein profiles and DNA reassociation studies indicate that these two species are the same.

enterococcal species (*Enterococcus porcinus* and *Enterococcus villorum*) constitute a single species. Two independent groups of investigators examined the taxonomy of these strains and named the species differently (Teixeira et al. 2001; Vancanneyt et al. 2001). The reports describing *E. villorum* and *E. porcinus* were published only 3 months apart, with *E. villorum* coming in first.

Figure 1 shows the phylogenetic relationship of the 25 species of *Enterococcus* recognized at this time.

HABITAT

Several intrinsic characteristics of the enterococci allow them to grow and survive in harsh environments and to persist almost everywhere. Enterococci are widespread in nature and can be found in soil, plants, water, food, and animals, including mammals, birds, insects, and reptiles (Devriese et al. 1992; Blaimont et al. 1995; Aarestrup et al. 2002). In humans, as in other animals, they are predominantly inhabitants of the gastrointestinal tract and are less commonly found in other sites such as the genitourinary tract and the oral cavity. The occurrence of enterococci in natural nonhuman reservoirs (Aarestrup et al. 2002), as well as members of the intestinal microflora of humans (Tannock and Cook 2002), has been extensively reviewed. The prevalence of the different enterococcal species appears to vary according to the host, and is also influenced by age, diet, and other factors that may be related to changes in physiologic conditions, such as underlying diseases and prior antimicrobial therapy. *E. faecalis* is one of the most common bacteria isolated from the human gastrointestinal tract. Other species, such as *E. faecium* as well as *E. casseliflavus*, *E. durans*, and *E. gallinarum*, are also found in variable proportions in the gastrointestinal tract of humans (Devriese et al. 1992; Endtz et al. 1997; Ieven et al. 1999; Strausbaugh and Gilmore 2000; Tannock and Cook 2002). Even though the same enterococcal species can be found in several different animal species, the information available on the distribution of distinct enterococcal species in other sources indicates differences from the distribution in humans (Devriese et al. 1992; Stern et al. 1994; Aarestrup et al. 2002; Tannock and Cook 2002). Several aspects of the ecology of the enterococci merit further evaluation, especially in the light of the changing classification and taxonomic approaches of the genus.

CULTURAL CHARACTERISTICS AND MORPHOLOGY

Enterococcal cells may be spherical, oval, or coccobacillary and occur singly or arranged in pairs or short chains. All species are gram-positive and most are nonmotile. *E. gallinarum* and *E. casseliflavus* are motile. After growth on blood agar media for 24 h, colonies are usually between 1 and 2 mm in diameter, although some variants may appear smaller. Some (about one-third) cultures of *E. faecalis* may be β-hemolytic on agar containing rabbit, horse, or human blood but nonhemolytic on agar containing sheep blood. Some cultures of *E. durans* are β-hemolytic regardless of the type of blood used. All other species are usually α-hemolytic or nonhemolytic. Strains that appear α-hemolytic are actually nonhemolytic strains that produce peroxide. This 'greening' of the agar is due to peroxide action on the blood cells in the medium and not to the production of an α toxin. *E. casseliflavus*, *E. gilvus*, *E. mundtii*, *E. pallens*, and *E. sulfureus* produce a yellow pigment on blood-agar medium. The pigment can be detected by using a white cotton swab to pick up the growth on the swab and examining the swab for a yellow color. The

colonies of enterococci on selective media will depend on the chemicals used in the medium. For example, on media containing esculin, such as bile–esculin–azide medium (Sabbaj et al. 1971) and Enterococcosel agar (a bile–esculin–azide medium supplied by Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), the colonies will appear gray-white and surrounded by a black halo, whereas on agar containing tetrazolium salts the center of the colony will appear brick-red (Barnes 1964).

METABOLISM AND GROWTH REQUIREMENTS

The nutritional requirements of the enterococci are complex and these microorganisms are recognized by their metabolic adaptability in order to fulfill diverse roles as commensals and as pathogens. The capacity to utilize a variety of carbohydrates, as well as the characteristic tolerance to a diversity of physical and chemical conditions, including various pH, temperatures, salt concentrations, detergents, and antimicrobial compounds, are likely to enhance enterococcal competitiveness in many harsh environments. For a long period, a major source of information on the enterococcal metabolism was a review published in 1964 (Deibel 1964). More recently, a comprehensive review on the physiology of the enterococci was provided by Huycke (2002), emphasizing topics such as central carbon metabolism, respiration, ion transport, pyrimidine and folate pathways, stress responses, and the metabolism of reactive oxygen species. Most studies addressing the growth requirements and physiology of the enterococci have been done with *E. faecalis* strains and in some cases with misidentified strains. Growth requirements include the B vitamins, nucleic acid bases, and a carbon source, usually glucose. The results of a work by Murray et al. (1993) indicate that all 23 strains of *E. faecalis* tested had an absolute requirement for histidine, isoleucine, methionine, and tryptophan, while some strains required arginine, glutamate, glycine, leucine, and valine and others did not. This implies that there are strain-to-strain differences in growth requirements. Intensive antimicrobial pressures have created atypical growth requirements, such as vancomycin-requiring strains (Fraimow et al. 1994). It is likely that the different species of enterococci have different growth requirements.

IMPORTANT GENETIC MECHANISMS

The enterococci are capable of transmitting genetic information by both plasmid and transposon exchange (Clewell and Dunny 2002; Weaver et al. 2002). The genetic material included on enterococcal plasmids and transposons may be drug-resistance determinants and/or virulence genes. A variety of plasmids and transposons have been identified and characterized in enterococci, mainly in *E. faecalis* and *E. faecium*. Three classes of plasmids are known to be capable of replication in the enterococci: the rolling circle replicating (RCR) plasmids, the Inc18 plasmids, and the pheromone-responsive plasmids. While RCR and Inc18 plasmids are capable of replication in a broad range of bacteria, the replication of pheromone-responsive plasmids appears to be restricted to the enterococci, primarily to *E. faecalis*. Such plasmids are characterized by the ability of their hosts to form readily visible mating aggregates when mixed with plasmid-free (recipient) cells in broth. Thus, in *E. faecalis*, exchange of these plasmids is unique and involves the production of sex pheromones (Clewell and Weaver 1989; Clewell 1993a). Plasmid-free recipient strains produce extracellular pheromones. The pheromone-induced mating response results in the production of a proteinaceous substance on the outer surface of the donor cells called aggregation substance. The aggregation substance then reacts with a binding substance on the surface of the recipient cell. The plasmid exchange occurs when the cells are clumped together. The pheromone-induced transfer increases the frequency of transfer of plasmids 10^5- to 10^6-fold (Clewell 1993b). Other species of enterococci have the capacity to acquire and exchange plasmids, but the mechanism of exchange is not pheromone-induced.

The enterococci are also capable of genetic exchange by conjugative transposons. The process requires cell-to-cell contact. The excised DNA can be viewed as being transferred to the recipient cell by a 'plasmid-like' process, followed by insertion into the target chromosome (Clewell and Flannagan 1993). The transposons frequently carry determinants coding for resistance to antimicrobials, such as tetracycline, erythromycin, gentamicin, kanamycin, and other aminoglycosides (Clewell 1990; Su and Clewell 1993). Transposons have a very broad host range and are commonly found in several *Enterococcus* species, as well as species of *Streptococcus*, *Lactococcus*, and other gram-positive bacteria. The contribution of movable enterococcal elements to the emergence and dissemination of antimicrobial resistance and enhanced virulence is evident, and it appears that certain conjugation-related molecules may even contribute to pathogenicity, as they may play an immunomodulatory role during infection (Clewell and Dunny 2002).

CELL-WALL COMPOSITION AND ANTIGENIC STRUCTURES

Three main constituents of the enterococcal cell wall are generally reported: peptidoglycan, teichoic acid, and polysaccharides. Sometimes, proteins are also mentioned. Comprehensive reviews of the enterococcal cell-wall chemistry were reported by Schleifer and Kilpper-Balz (1987) and more recently by Coyette and

Hancock (2002). However, because of the changes in enterococcal taxonomy, the identification of the strains used in previous studies is not clear, but a general picture can be drawn. As in other gram-positive bacteria, peptidoglycan polymers are the main structural and shape-maintaining components of the cell wall. The mesh of the peptidoglycan net is made of parallel glycan chains held together by small peptide cross-links. The glycan moiety is composed of alternating β-1,4-linked units of N-acetylglucosamine (GlcNac) and N-acetylmuramic acid (MurNAc). Short-stem peptides are connected to the glycan chains through amide linkages between the carboxyl groups on muramyl residues and the terminal amino groups of the peptides. As in many other gram-positive bacteria, the sequence of the enterococcal stem peptide is L-Ala-D-Glu-L-Lys-D-Ala. The D-glutamic acid is bound through its amino group to the L-Ala carboxyl group and its γ-carboxyl group to the amino group of the L-Lys residue. Adjacent stem peptides are cross-linked by interpeptide bridges.

The structural composition of many accessory (nonpeptidoglycan) cell-wall polymers is still largely unknown. Much of the information available is derived from investigations on the serological grouping scheme developed by Lancefield (1933) to differentiate the streptococci. Whereas most groups of streptococci were categorized on the basis of immunoreactivity of a predominant cell-wall carbohydrate, the basis for defining the group D streptococci stemmed from the serological recognition of their distinct lipoteichoic acid (LTA). Chemical analysis showed that the group D antigen was a teichoic acid polymer of glycerol phosphate containing a high proportion of glucose attached to the glycerol units. The lipid component of the LTA was shown to be a 1-kojibiosyl diglyceride, with the sugars linked to the diglyceride by a phosphatidyl substituent. This glycolipid was found to be a membrane component. The linkage of LTAs to the cell membrane is in contrast to the attachment of cell wall teichoic acids, which are supposed to be anchored to the N-acetylmuramic acid in the peptidoglycan. The so-called 'streptococcal group D antigen' is usually associated with the *Enterococcus* species, but it is not exclusive. This antigen is also found in the strains belonging to the *Streptococcus bovis* complex, as well as in some *Leuconostoc*, *Pediococcus*, and *Vagococcus* strains (Facklam and Elliott 1995; Teixeira et al. 1997b; Barros et al. 2001). Type antigens, carbohydrate in nature, are also present on the surface of the cells of many of the strains of *E. faecalis*, *E. faecium*, and *E. durans* (Sharpe and Shattock 1952; Barnes 1964; Sharpe 1964).

CLASSIFICATION AND IDENTIFICATION OF SPECIES

After Schleifer and Kilpper-Balz (1984) suggested the establishment of the genus *Enterococcus*, composed initially by *E. faecalis* and *E. faecium*, the transfer of the other known enterococcal species to the new genus was proposed and several new species have been described. DNA–DNA reassociation experiments, which are considered the gold standard for establishing bacterial identification to the species level, have been carried out for most of the enterococcal species. Sequencing of the 16S rRNA gene is also a very powerful tool in aiding in the description of bacterial species. This procedure should not be used in itself as a validation tool for defining new species, but together with results of DNA reassociation experiments or WCP analysis and phenotypic characterization it can be very useful, especially in relating one species to the other. 16S rRNA gene sequencing has been performed on all enterococcal species. Recently, authoritative journals have required that the sequence of the 16S rRNA gene be deposited in the GenBank when describing a new species.

At this time there are 25 distinct species included in the genus *Enterococcus* (Table 3). Accurate presumptive identification of a catalase-negative gram-positive coccus as an *Enterococcus* can be accomplished by demonstrating that the unknown strain is positive for BE, PYR, and LAP tests and grows in the presence of 6.5 percent NaCl and at 45°C. Because strains of *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Vagococcus* with phenotypic similarities have been isolated from human infections (Facklam and Elliott 1995; Teixeira et al. 1997b), the presumptive identification of enterococci based only on BE reaction and growth in 6.5 percent NaCl broth can be erroneous. Confirmation that a strain is an *Enterococcus* requires complete identification to the species level. Demonstrating the presence of group D antigen by serological reaction may be helpful in the identification, although antigen is detected in only about 80 percent of the enterococcal strains. On the other hand, pediococci and leuconostocs (Facklam and Elliott 1995; Barros et al. 2001), as well as some vagococcal strains (Teixeira et al. 1997b; Teixeira et al. 1999) can also react with anti-group D serum. Reactivity with the AccuProbe *Enterococcus* genetic probe manufactured by GenProbe, Inc. (San Diego, CA, USA) can also be used to confirm an unknown strain as an *Enterococcus*. This probe is based on a DNA oligomer having a structure complementary to a segment of enterococcal rRNA (Enns 1988; Daly et al. 1991). Most strains of known *Enterococcus* species react positively with this probe. However, *Vagococcus* strains also react (Teixeira et al. 1997b; Teixeira et al. 1999).

Once it is established that an unknown catalase-negative gram-positive coccus is a member of the genus *Enterococcus* or of a closely related genus (*Lactococcus* or *Vagococcus*), the tests listed in Table 3 can be used to identify the species. The recognition, in recent years, of different phenotypic variants of *E. faecium* (Teixeira et al. 1995), *E. faecalis*, *E. casseliflavus*, and *E. gallinarum* (Teixeira et al. 1997a), and physiologically

Table 3 *Phenotypic characteristics used for the identification of* Enterococcus *species and some physiologically related species of other gram-positive cocci*

	Phenotypic characteristic[a]											
Species	**MAN**	**SOR**	**ARG**	**ARA**	**SBL**	**RAF**	**TEL**	**MOT**	**PIG**	**SUC**	**PYU**	**MGP**
Group I												
E. avium	+	+	–	+	+	–	–	–	–	+	+	V
E. gilvus	+	+	–	–	+	+	–	–	+	+	+	–
E. malodoratus	+	+	–	–	+	+	–	–	–	+	+	V
E. pallens	+	+	–	–	+	+	–	–	+	+	–	+
E. pseudoavium	+	+	–	–	+	–	–	–	–	+	+	+
E. raffinosus	+	+	–	+	+	+	–	–	–	+	+	V
E. saccharolyticus[b]	+	+	–	–	+	+	–	–	–	+	–	+
Group II												
E. faecalis	+*	–	+*	–	+	–	+	–	–	+*	+	–
E. haemoperoxidus[b,c]	+	–	+	–	–	–	–	–	–	+	–	+
Lactococcus sp.	+	–	+	–	–	–	–	–	–	V	–	–
E. faecium	+*	–	+	+	V	V	–	–	–	+*	–	–
E. casseliflavus	+	–	+*	+	V	+	–*	+*	+*	+	V	+
E. gallinarum	+	–	+*	+	–	+	–	+*	–	+	–	+
E. mundtii	+	–	+	+	V	+	–	–	+	+	–	–
Group III												
E. dispar	–	–	+	–	–	+	–	–	–	+	+	+
E. durans	–	–	+	–	–	–	–	–	–	–	–	–
E. hirae	–	–	+	–	–	+	–	–	–	+	–	–
E. ratti	–	–	+	–	–	–	–	–	–	–	–	–
E. villorum[d]	–	–	+	–	–	–	–	–	–	–	–	–
Group IV												
E. asini[b]	–	–	–	–	–	–	–	–	–	+	–	–
E. cecorum[b]	–	–	–	–	+	+	–	–	–	+	+	–
E. phoeniculicola[b]	–	–	–	+	–	+	–	–	–	+	–	+
E. sulfureus	–	–	–	–	–	+	–	–	+	+	–	+
Group V												
E. canis[b]	+	–	–	+	–	–	–	–	–	+	+	+
E. columbae[b]	+	–	–	+	+	+	–	–	–	+	+	–
E. moraviensis[b]	+	–	–	+	–	–	–	–	–	+	+	+
Vagococcus fluvialis	+	–	–	–	+	–	–	+	–	+	–	+

a) MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; TEL, 0.04 percent tellurite; MOT, motility; PIG, pigment; SUC, sucrose; PYU, pyruvate; MGP, methyl-α-D-glucopyranoside; +, 90 percent or more of the strains are positive; –, 10 percent or less of the strains are positive; V, variable (11–89 percent of the strains are positive); *, occasional exceptions occur (<3 percent of strains show aberrant reactions).

b) Phenotypic characteristics based on data from type strains.

c) Late mannitol positive (5 days' incubation)

d) Phenotypic characteristics of *E. villorum* are identical to those of *E. porcinus*. These two recently described species correspond to a single taxon.

related species of *Lactococcus* and *Vagococcus* isolated from human sources (Facklam and Elliott 1995; Teixeira et al. 1997b; Teixeira et al. 1999), as well as the description of several new species, made it necessary to expand the species identification scheme. It should be pointed out that most of the information presented here is related to the phenotypic characteristics of enterococci, lactococci, and vagococci isolated from humans. The characteristics of strains of these microorganisms isolated from nonhuman sources may be different. The enterococcal species can be separated into five physiological groups based on acid formation from mannitol and sorbose and on hydrolysis of arginine (Table 3). All the tests listed in Table 3 are conventional test-tube tests and have been previously described (Facklam and Collins 1989; Facklam and Elliott 1995; Facklam et al. 1999). The species of each group are then identified according to the results of additional testing. The grouping in this manner is based on key phenotypic characteristics and does not necessarily conform to the grouping by 16S rRNA sequencing or by other molecular techniques. The grouping by conventional phenotypic tests can be performed in any laboratory with responsibilities for identifying the species of enterococci. Identification of enterococcal species by conventional tests is not rapid and may require incubation of the tests

for up to 10 days. However, most identifications can be made after 2 days of incubation.

Group I consists of *E. avium*, *E. malodoratus*, *E. pseudoavium*, *E. raffinosus*, *E. saccharolyticus*, *E. gilvus*, and *E. pallens*. These species form acid from mannitol and sorbose but do not hydrolyze arginine. They are differentiated by reactions in arabinose and raffinose tests, the capacity to utilize pyruvate, and whether or not the strains are pigmented (Table 3). Production of acid from methyl-α-D-glucopyranoside (MGP) also helps to differentiate some of these species from each other.

Group II comprises *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum*, *E. mundtii*, and *E. haemoperoxidus*, a more recently described species. These species form acid from mannitol and hydrolyze arginine but fail to form acid from sorbose. Atypical strains fail to hydrolyze arginine or form acid from mannitol. *Lactococcus* sp. is also listed in this group because the phenotypic characteristics of some strains can lead to the misidentification as an *Enterococcus*. If nonmotile variants of *E. casseliflavus* and *E. gallinarum* are encountered, production of acid from MGP can be used to help in the identification of these species. *E. casseliflavus* and *E. gallinarum* form acid from MGP while *E. faecium* and *E. mundtii* do not. In all likelihood the mannitol-positive strains of *E. haemoperoxidus*, a recently described species (Svec et al. 2001), will also fall into group II enterococci.

Group III enterococci hydrolyze arginine but do not form acid from mannitol or sorbose. This group consists of *E. dispar*, *E. durans*, *E. hirae*, and two new species, *E. ratti* and *E. villorum* (*E. porcinus*). The new species in this group are difficult to identify and differentiate from *E. durans*. *E. durans*, *E. ratti*, and *E. villorum* have very similar phenotypic profiles in the tests listed in Table 3. They can be differentiated by reactions in litmus milk, hydrolysis of hippurate, and acid formation from trehalose and xylose. *E. durans* forms acid and clot, *E. villorum* forms acid but no clot, and *E. ratti* does not form acid or clot litmus milk. *E. durans* hydrolyzes hippurate, while *E. villorum* does not hydrolyze hippurate. *E. ratti* is variable in the hippurate hydrolysis test. *E. durans* forms acid from trehalose but not from xylose, *E. villorum* forms acid from both trehalose and xylose, and *E. ratti* does not form acid from either trehalose or xylose. The other members of this group are easily identified by the reactions in the pyruvate, arabinose, raffinose, and sucrose tests. Uncommon mannitol-negative variant strains of *E. faecalis* and *E. faecium* resemble species in this group. However, *E. faecalis* strains are positive in the pyruvate test but not for acid formation from arabinose, raffinose, or sucrose, and *E. faecium* variant strains form acid from arabinose. If the results of conventional tests compare to the results of testing with the API system originally reported (Svec et al. 2001), the mannitol-negative strains of *E. haemoperoxidus*, a recently described species, will be grouped with the enterococci in group III. In addition, although similar to *E. dispar* it appears that the mannitol-negative strains of *E. haemoperoxidus* can be differentiated from the other group III enterococci.

Group IV is composed of four species (*E. asini*, *E. cecorum*, *E. phoeniculicola*, and *E. sulfureus*) that do not form acid from mannitol or sorbose and fail to hydrolyze arginine. These four species can be differentiated by acid formation from arabinose, raffinose, and sorbitol and pigmentation tests (Table 3). *E. phoeniculicola* and *E. sulfureus* form acid from MGP.

Group V consists of *E. canis*, *E. columbae*, *E. moraviensis*, and *Vagococcus*. Variant strains of *E. casseliflavus*, *E. gallinarum*, and *E. faecalis* that fail to hydrolyze arginine resemble the microorganisms included in this group. However, these variant strains have characteristics similar to the strains that hydrolyze arginine and can be differentiated by these same phenotypic tests. *Vagococcus fluvialis* is listed here because the phenotypic characteristics of this species are very similar to those of the genus *Enterococcus* and some strains may be identified as enterococci (Teixeira et al. 1997b; Teixeira et al. 1999). It appears that *E. moraviensis*, another newly described species, will fall into group V enterococci and if the results of conventional test are similar to those of the API testing originally reported (Svec et al. 2001), *E. moraviensis* will be easily differentiated from the other group V enterococci.

There are several commercially available miniaturized, manual, semiautomated, and automated identification systems for the identification of *Enterococcus* species. Since their introduction, these systems have been updated to improve their performance characteristics and expand their identification capabilities, as investigators have become more aware of inaccuracies (Wilke et al. 1997; Hamilton-Miller and Shah 1999; Garcia-Garrote et al. 2000; d'Azevedo et al. 2001). In general, these systems are reliable for the identification of *E. faecalis*, and to a lesser extent, *E. faecium*. Accurate identification of other species, by most systems, depends on additional testing, although improvements have been observed with updated formats and data banks. Commercial systems now available for the identification of enterococcal species include: the API 20S and the API Rapid ID32 STREP systems (bioMérieux, Hazelwood, MO, USA), the Crystal Gram-Positive and the Crystal Rapid Gram-Positive identification systems (Becton Dickinson Microbiology Systems), the Gram-Positive Identification Card of the Vitek system (bioMérieux), and the Gram-Positive Identification panel of the MicroScan Walk/Away system (Dade MicroScan, West Sacramento, CA, USA). The accuracy of identification by some of these systems in comparison with identification by molecular techniques was reported by Angeletti et al. (2001). In general, approximately 80 percent of all enterococcal isolates will be accurately identified by any one of these systems. However, the

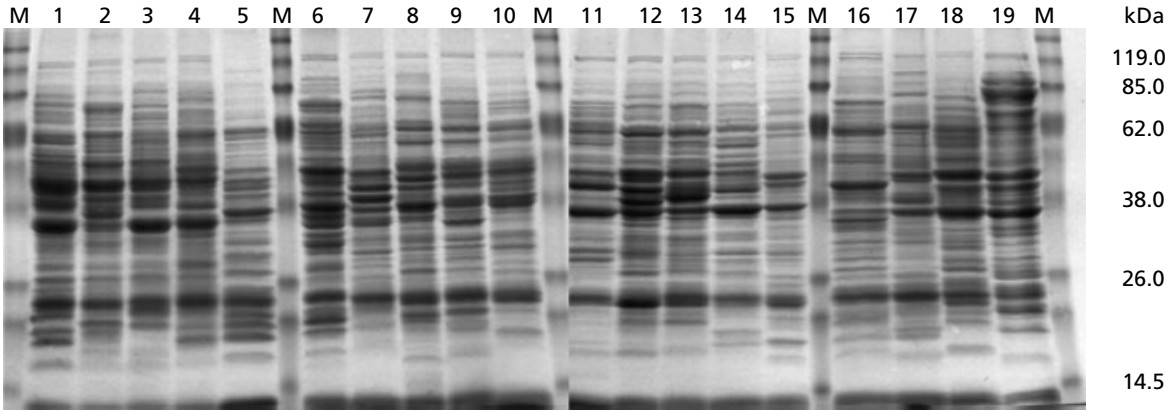

Figure 2 *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of whole-cell protein extracts of* Enterococcus *species. Lanes M, molecular mass markers; lane 1,* E. avium*; lane 2,* E. malodoratus*; lane 3,* E. pseudoavium*; lane 4,* E. raffinosus*; lane 5,* E. saccharolyticus*; lane 6,* E. faecalis*; lane 7,* E. faecium*; lane 8,* E. casseliflavus*; lane 9,* E. gallinarum*; lane 10,* E. mundtii*; lane 11,* E. durans*; lane 12,* E. hirae*; lane 13,* E. villorum*; lane 14,* E. ratti*; lane 15,* E. dispar*; lane 16,* E. asini*; lane 17,* E. cecorum*; lane 18,* E. sulfureus*; lane 19,* E. columbae.

accuracy will be dependent on the distribution of species found in each specific setting. Identification of unusual species by a commercial system should be confirmed by a reference method before being reported.

Molecular methods based on the analysis of different target molecules present in the enterococcal cells, such as DNA–DNA hybridization and sequencing of the 16S rRNA genes, have been primarily used for taxonomic purposes in special laboratories. In the past 10 years, however, the application of molecular techniques for the identification of *Enterococcus* species has expanded dramatically. The range of molecular procedures proposed for the identification of enterococcal species has been reviewed and summarized (Facklam et al. 2002). They include: analysis of WCP profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); vibrational spectroscopic analysis; proton magnetic resonance spectroscopic analysis; randomly amplified polymorphic DNA (RAPD) analysis; sequencing analysis of the 16S rRNA gene; fragment-length polymorphism analysis of amplified 16S rDNA; broad-range amplification (BR-PCR) of the 16S rDNA; sequencing of the domain V of the 23S rRNA gene; amplification of the tRNA or the rRNA intergenic spacers, or of the D-Ala:D-Ala ligases (*ddl*) and the vancomycin resistance (*van*) genes; sequencing of the *ddl* genes or of the manganese-dependent superoxide dismutase ($sodA_{int}$) gene; sequencing and hybridization of the chaperonin 60 (*cpn60*) gene; amplification and probing of the *Enterococcus* protein A (*efaA*) genes or of the *E. faecalis* adhesin for collagen (*ace*) gene; and amplification of the elongation factor EF-Tu (*tuf*) gene, or the *pEM1225* gene.

Among the molecular techniques proposed to identify the enterococcal species, SDS-PAGE analysis of WCP profiles is the only one that has been extensively evaluated and meets a stage of being recommended for general use. This method was shown to be a reliable for the differentiation and identification of typical and atypical *Enterococcus* strains, since WCP profiles are species-specific (Figure 2). Apart from minor qualitative and/or quantitative differences among strains, each of the known enterococcal species corresponds to a unique WCP profile (Merquior et al. 1994; Teixeira et al. 1995; Teixeira et al. 1997a). WCP profiles of related species of *Lactococcus* and *Vagococcus* are also unique and different from those of the enterococci (Teixeira et al. 1996; Teixeira et al. 1997a; Teixeira et al. 1997b). Analysis of WCP using a standardized technique has revealed a correlation between WCP profiles and DNA homology studies. Table 3 depicting the phenotypic characteristics of the *Enterococcus* species is based on correlations between the WCP profiles and the phenotypic tests and, in some occasions, in conjunction with DNA–DNA reassociation experiments. Sequencing the 16S RNA gene has been performed on all species of enterococci and the sequences are available via GenBank for comparison purposes. Among the other molecular methods, only a few have been performed in more than one laboratory or have been evaluated for the majority of the *Enterococcus* species. Some do not appear to be practical for routine use in clinical laboratories. On the other hand, some of these recently developed molecular procedures deserve consideration for expanded testing and future improvement.

LABORATORY ISOLATION

Trypticase–soy–5 percent sheep blood agar, brain–heart infusion–5 percent sheep blood agar, or any blood agar base containing blood supports the growth of enterococci. Some strains of *E. faecalis* are beta-hemolytic on agar bases containing rabbit, horse, or human blood but nonbeta-hemolytic on the same base media containing

sheep blood. Some strains of *E. durans* are beta-hemolytic regardless of the type of blood used. The other species are usually alpha-hemolytic or nonhemolytic. Enterococci grow at 35–37°C and do not require an atmosphere containing increased levels of CO_2, although some strains grow better in this atmosphere. If the sample is likely to contain gram-negative bacteria, bile–esculin azide (Sabbaj et al. 1971), Pfizer selective enterococcus (Isenberg et al. 1970), and some other commercially prepared medium containing azide, such as Enterococcosel agar, are excellent primary isolation media. The azide inhibits the gram-negative bacteria and the enterococci appear as black colonies because of hydrolysis of esculin. The use of selective media for the isolation of enterococci has been reviewed (Sabbaj et al. 1971; Facklam 1976; Devriese et al. 1992). Since then, several new media have been described, including those containing chromogenic substrates for the isolation and presumptive identification of enterococci from urine (Merlino et al. 1996) and the cephalexin-aztreonam-arabinose agar for the isolation of *E. faecium* from heavily contaminated sites (Ford et al. 1994). Another medium that has been used to improve the isolation rates of enterococci from polluted waters is oxoline–esculin agar (OOA) (Figueras et al. 1998). OOA has not been used in a clinical setting.

The increasing incidence of vancomycin resistance among the enterococci has raised the importance of selective isolation of vancomycin-resistant enterococci (VRE). Early identification of VRE is necessary for controlling the spread of these organisms (Hospital Infection Control Practices Advisory Committee 1995). Several different selective agar and/or broth media have been employed for the isolation of VRE from sources containing normal flora, such as stool samples and rectal swabs (Landman et al. 1996; Sahm et al. 1997; Satake et al. 1997; Ieven et al. 1999). Although there is not a single generally accepted screening method at this point, the use of a selective-enrichment broth to enhance the recovery of VRE seems the most effective procedure. Enterococcosel broth has been used in a number of studies as the base medium supplemented with different concentrations of vancomycin, 6 μg/ml being the most common concentration. In some of these studies, vancomycin and other antimicrobials, such as aztreonam and clindamycin, have been used in the enrichment broth as well as in the subculture medium. It would be prudent, however, to use a nonselective agar as well for subculturing from a selective enrichment broth, as some strains may be inhibited to a point of very poor or no growth. Satake et al. (1997) have evaluated several selective media for the isolation of VRE from fecal specimens before and after broth enrichment. They found that culturing with Enterococcosel agar containing 6 μg of vancomycin per milliliter after enrichment in supplemented Columbia colistin–nalidixic acid (CNA) broth at 35°C was the most sensitive method.

In some circumstances, it may be necessary to recover VRE from environmental surfaces for epidemiological studies. Isolation of the organisms from these surfaces is accomplished by swabbing the surfaces with premoistened swabs and placing them either into an enrichment broth or on to agar plates. Alternatively the Rodac imprint method may be employed by applying the agar surface directly to the environmental surface to be cultured (Hacek et al. 2000).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most of the enterococcal species. Moreover, the majority of the information available is based on studies with *E. faecalis* and *E. faecium*, the two species that are more frequently associated with human infections. Antimicrobial resistance can be classified as either intrinsic or acquired. Intrinsic resistance is related to inherent or natural chromosomally encoded characteristics present in all or most of the enterococci. Furthermore, certain specific mechanisms of intrinsic resistance to some antimicrobial agents are typically associated with a particular enterococcal species or group of species. In contrast, the occurrence of acquired resistance is more variable, resulting from either mutation in existing DNA or acquisition of new genetic determinants found in plasmids or transposons (Murray 1990, 1998; Leclercq 1997; Huycke et al. 1998; Strausbaugh and Gilmore 2000; Kak and Chow 2002).

Enterococcal intrinsic resistance involves two major groups of antimicrobial therapeutic drugs: the aminoglycosides and the β-lactams. Because of the poor activity of several antimicrobial agents against enterococci due to intrinsic resistance, the recommended therapy for serious infections (i.e. endocarditis, meningitis, and other systemic infections, especially in immunocompromised patients) includes a combination of a cell-wall-active agent, such as a β-lactam (usually penicillin) or vancomycin, combined with an aminoglycoside (usually gentamicin or streptomycin) (Murray 1990; Huycke et al. 1998; Murray 1998; Strausbaugh and Gilmore 2000). These combinations overcome the intrinsic resistance exhibited by the enterococci and a synergistic bactericidal effect is generally achieved since the intracellular penetration of the aminoglycoside is facilitated by the cell-wall-active agent.

In addition to the intrinsic resistance traits, enterococci have acquired different genetic determinants that confer resistance to several classes of antimicrobials, including chloramphenicol, tetracyclines, macrolides, lincosamides and streptogramins, aminoglycosides, β-lactams, glycopeptides, and, more recently, quinolones. A variety of antimicrobial resistance patterns involving

different mechanisms that can be found in enterococcal isolates are presented in Table 4.

Over the past decades, the occurrence of acquired antimicrobial resistance among enterococci, especially high-level resistance (HLR) to aminoglycosides, β-lactams, and resistance to glycopeptides (especially vancomycin), has been increasingly reported. These resistance traits are of particular clinical relevance as they confer resistance to agents used in the treatment of serious enterococcal infections and can abolish the activity of the therapeutic regimens with proven bactericidal activity against enterococci. Isolates that are resistant to the cell-wall-active agent or have HLR to aminoglycosides are resistant to the synergistic effects of combination therapy and constitute an even more serious problem concerning the effective management of enterococcal infections. Therefore, the detection of resistance to these groups of antimicrobial agents is important to predict the likelihood of synergy by using antimicrobial association as a therapeutic strategy.

Enterococcal isolates exhibiting HLR to one or more aminoglycosides have been described with increasing frequency (Murray 1990, 1998; Antalek et al. 1995; Leclercq 1997; Huycke et al. 1998; Strausbaugh and Gilmore 2000) and are now present in large proportions in several geographic areas. Strains expressing acquired HLR to aminoglycosides usually have minimal inhibitory concentrations (MIC) >2000 μg/ml and cannot be detected by diffusion tests with conventional disks. Special tests using high-content gentamicin and streptomycin disks (Sahm and Torres 1988), as well as a single dilution method, were developed to screen for this type of resistance (Swenson et al. 1995). Strains exhibiting HLR to penicillin and ampicillin due to altered penicillin-binding proteins have also disseminated widely in the past several years (Murray 1990, 1998; Boyce et al. 1992; Huycke et al. 1998; Strausbaugh and Gilmore 2000), and strains producing β-lactamase have been identified (Murray 1990; Gordon et al. 1992).

The emergence of vancomycin resistance as a therapeutic problem in enterococcal strains was first documented in western Europe and in the USA (Kaplan et al. 1988; Leclercq et al. 1988; Uttley et al. 1988). Thereafter

Table 4 *Major patterns and mechanisms of resistance to antimicrobial agents in enterococci*

Pattern of resistance	Mechanism of resistance
High-level resistance to aminoglycosides[a]	Enzymatic (Production of aminoglycoside-modifying enzymes, AMEs)[b]
Gentamicin	AAC(6′)-Ie + APH(2″)-Ia
Kanamycin	AAC(6′)-Ii
Streptomycin	APH (2″)-Ib; APH (2″)-Ic; APH (2″)-Id
	APH(3′)-IIIa
	ANT(3′)-Ia; ANT(4′)-Ia; ANT (6′)-Ia
	Alteration of the target (leading to decreased ribosomal binding)
Resistance to glycopeptides[c]	Alteration of the target (modification of the peptidoglycan biosynthetic pathway)
Vancomycin	Van A
Teicoplanin	Van B
	Van C (Van C1, Van C2, and Van C3)
	Van D
	Van E
	Van G
Resistance to β-lactams[d]	Alteration of the target (altered penicillin binding proteins, PBPs)
Penicillin	Enzymatic (production of β-lactamase)
Ampicillin	
Resistance to quinolones	Alteration of the target (changes to the subunit A of DNA gyrase)
Resistance to chloramphenicol	Enzymatic (production of chloramphenicol acetyl transferase, CAT)
Resistance to the MLS group	Enzymatic (production of methylating enzymes)
Macrolides (erythromycin)	
Lincosamides (clindamycin)	
Streptogramin B	

a) Found in increasing frequencies in *E. faecalis* and *E. faecium*.
b) AAC, acetyltransferases; ANT, nucleotidyltransferases; APH, phosphotransferases.
c) *vanA* and *vanB* genotypes are usually found in *E. faecalis* and *E. faecium*: *vanA* has occasionally been found in several other enterococcal species; *vanC1* is usually associated with *E. gallinarum* and *vanC2* and *vanC3* with *E. casseliflavus*. VanD resistance has been described in *E. faecium* strains while VanE and VanG were found in *E. faecalis*.
d) Found in *E. faecalis* and *E. faecium*. Also described among *E. raffinosus* strains.

the isolation of VRE has been continuously reported in diverse locations (Huycke et al. 1998; Murray 1998; Cetinkaya et al. 2000). VRE strains have been classified by phenotypes and genotypes. Six types of glycopeptide resistance have already been described among enterococci. Three of them are the most common: the VanA phenotype, with inducible high-level resistance to vancomycin, as well as to teicoplanin, encoded by the *vanA* gene; the VanB phenotype, with variable (moderate to high) levels of inducible resistance to vancomycin only, encoded by the *vanB* gene; and the VanC phenotype, with noninducible low-level resistance to vancomycin. The VanA and VanB phenotypes are considered the most clinically relevant and are usually associated with *E. faecium* and *E. faecalis* strains while the VanC resistance is a intrinsic characteristic of *E. gallinarum* (*vanC1* genotype) and *E. casseliflavus* (*vanC2* and *vanC3* genotypes) strains (Clark et al. 1998; Huycke et al. 1998; Murray 1998; Cetinkaya et al. 2000). The remaining three types of enterococcal glycopeptide resistance seem to occur rarely and are encoded by genetic determinants that were recently recognized, named *vanD* (Perichon et al. 1997; Ostrowsky et al. 1999), *vanE* (Fines et al. 1999), and *vanG* (McKessar et al. 2000).

The diversity and, in some cases, species specificity of emerging antimicrobial resistance traits among enterococcal isolates created an additional need for accurate identification at the species level and for in vitro evaluation of susceptibility to antimicrobial agents. Because of the diverse antimicrobial resistance mechanisms, successful treatment and control of enterococcal infections are becoming increasingly difficult. On the other hand, several of such special drug-resistance characteristics acquired by enterococcal strains are not adequately detected by the routine susceptibility tests most commonly used in the clinical microbiology laboratories, so they require modifications of the usual procedures. Early detection of resistant strains would be of great value for the selection of the more appropriate therapy to be prescribed for the treatment of serious enterococcal infections. Therefore, a better patient management will be achieved since treatment failure, selection, and spreading of resistant strains can be minimized.

For several years, controversies and confusion existed regarding antimicrobial susceptibility testing of enterococcal isolates, particularly about the reliability of phenotypicmethods for detection of HLR to aminoglycosides and resistance to vancomycin. Results of studies devised for evaluation of different methods and interpretative criteria, have led to the proposal of more accurate procedures. Updated guidelines for the selection of antimicrobial agents that should be considered for routine testing and reporting, as well as performance and interpretative criteria of susceptibility testing for enterococci, have been published by the National Committee for Clinical Laboratory Standards (2001). The in vitro methods for detecting antimicrobial resistance in enterococcal isolates have been reviewed and summarized (Facklam et al. 2002).

Molecular methods have been used to detect specific antimicrobial-drug-resistance genes (Swenson et al. 1995; Endtz et al. 1997; Patel et al. 1997; Sahm et al. 1997; Satake et al. 1997; Clark et al. 1998; Jayaratne and Rutherford 1999; Ostrowsky et al. 1999; Kariyama et al. 2000; Papaparaskevas et al. 2000; Portillo et al. 2000; Werner et al. 2000; Duh et al. 2001) and have substantially contributed to the understanding of the spread and genetics of acquired enterococcal resistance, especially resistance to vancomycin. However, because of their high specificity, molecular methods will not detect antimicrobial resistance that is due to a mechanism that is not included in the testing or due to an emerging resistance mechanism.

TYPING METHODS

Because enterococci are a leading cause of nosocomial infections and frequently exhibit multiple antibiotic resistance, there has been an increasing need to type and subtype isolates as a means of assisting infection control and epidemiological studies both within and among various medical institutions. Furthermore, the evidence supporting the concept of exogenous acquisition of enterococcal infections has generated an additional need for typing the isolates. Therefore, the investigation of nosocomial outbreaks along with the dissemination of enterococcal strains harboring antimicrobial resistance markers is of major interest, particularly in the light of the increasing occurrence of VRE. Besides outbreak analysis, the methods used for epidemiological investigation of enterococcal isolates must be able to track enterococcal dissemination in different environments and hosts and the evolution of multiresistant strains. Early epidemiological investigations of enterococcal infections were based on phenotypic characteristics and have been hampered by the lack of simple, highly reproducible, and sufficiently discriminatory typing systems. Classic phenotypic typing methods used to investigate the diversity among isolates of a given enterococcal species have included biotyping and antibiotyping, based on physiological and/or antimicrobial resistance characteristics (Coudron et al. 1984; Luginbuhl et al. 1987; Smyth et al. 1987; Murray 1990, 1992; Kuhn et al. 1995), serotyping (Sharpe and Shattock 1952; Sharpe 1964; Smyth et al. 1987; Maekawa et al. 1992), bacteriocin typing (Pleceas et al. 1972; Sadatsune et al. 1978; Kuhnen et al. 1987; Kuhnen et al. 1988), and bacteriophage typing (Pleceas and Brandis 1974; Caprioli et al. 1975; Brandis et al. 1985; Kuhnen et al. 1987; Smyth et al. 1987; Kuhnen et al. 1988). Although these approaches have occasionally yielded useful information, they are generally time-consuming and difficult to reproduce and/or interpret. Some of these techniques frequently fail to

adequately discriminate among strains and, therefore, they have limited value in epidemiological studies. On the other hand, the use of phenotypic typing methods in association with more recent molecular techniques can contribute valuable information (Willey et al. 1994; Kuhn et al. 1995, 2000; Morrison et al. 1999; Teixeira et al. 2000)

The introduction of various molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into epidemiological aspects of enterococcal infections. As a result of the use of more discriminatory typing methods, it has been possible to demonstrate that strains can be exogenously acquired by direct and indirect contact among patients (Zervos et al. 1986; Livornese et al. 1992; Woodford et al. 1993; Willey et al. 1994; Pegues et al. 1997). Intrahospital transmission and interhospital spread has also been documented for antimicrobial resistant enterococci (Chow et al. 1993a; Sader et al. 1994; Willey et al. 1994). The first molecular techniques developed for typing of enterococci were the analysis of plasmid profiles and the restriction enzyme analysis (REA) of genomic DNA by conventional electrophoresis (Luginbuhl et al. 1987; Lacoux et al. 1992; Savor et al. 1998; Quednau et al. 1999). These techniques may be helpful in some instances, but problems have been encountered with their use. A remarkable contribution to the ability to discriminating among enterococcal strains was noted with the use of techniques involving the analysis of chromosomal DNA restriction endonuclease profiles by pulsed-field gel electrophoresis (PFGE) by either field-inversion gel electrophoresis (FIGE) (Green et al. 1995) or, ideally, counter-clamped homogeneous electric-field electrophoresis (CHEF) (Murray et al. 1990; Gordillo et al. 1993; Green et al. 1995; Plessis et al. 1995; Pegues et al. 1997; Perlada et al. 1997), which is the basis for most of the recent PFGE studies. Multilocus enzyme electrophoresis (MLEE) (Carvalho et al. 1997; Tomayko and Murray 1995), ribotyping (Gordillo et al. 1993; Woodford et al. 1993; Kühn et al. 1995; Plessis et al. 1995; Endtz et al. 1997), and the polymerase chain reaction (PCR)-based typing methods, such as the random amplified polymorphic DNA (RAPD)-PCR assay and the repetitive element sequence (REP)-PCR have also been used to investigate the genetic relationship among enterococcal strains (Barbier et al. 1996; Issack et al. 1996; Descheemaeker et al. 1997; Malathum et al. 1998). Sequencing of PCR products and restriction fragment length polymorphism (RFLP) analysis of PCR products have been used to trace and to determine differences among specific resistance genes in enterococci, therefore representing additional tools for typing resistant strains (Patel et al. 1997; Donabedian et al. 2000; Kawalec et al. 2000; Willems et al. 2000; Lee et al. 2001).

Results from a number of investigations indicate that analysis of *Sma*I restriction digests of genomic DNA by PFGE is widely useful for studying enterococcal species (Chow et al. 1993a; Gordillo et al. 1993; Thal et al. 1993; Antalek et al. 1995; Plessis et al. 1995; Tomayko and Murray 1995; Carvalho et al. 1997; Descheemaeker et al. 1997; Pegues et al. 1997; Perlada et al. 1997; Clark et al. 1998; Malathum et al. 1998; Murray 1998; Bischoff et al. 1999; Reinert et al. 1999), showing definite advantages in strain discrimination. PFGE is currently the single most useful and reliable typing method, being considered as the gold standard for the epidemiological analysis of enterococcal infections. Several protocols for performing PFGE analysis of enterococcal strains have been published. However, the development of standardized protocols as a result of collaborative studies is needed in order to allow for interlaboratory comparison in future investigations. Most of the information accumulated over the past years is related to *E. faecium* (Plessis et al. 1995; Privitera et al. 1995; Bischoff et al. 1999; Morrison et al. 1999; Ostrowsky et al. 1999; Reinert et al. 1999; Willems et al. 2000) and *E. faecalis* (Thal et al. 1993; Antalek et al. 1995; Privitera et al. 1995; Ma et al. 1998). Although PFGE has been more discriminatory than other methods, epidemiological interpretation of PFGE profiles is not always clear-cut. The occurrence of genetic events can be associated with substantial changes in the PFGE profiles, leading to problems in clonality assessment (Savor et al. 1998; Morrison et al. 1999; Kawalec et al. 2000). The more appropriate interpretation in such cases remains a controversial issue, as it may be difficult to define which level of polymorphism is acceptable. In consequence, there is not a single definitive typing technique for enterococci; a strong match of the results of several typing techniques, particularly those based on different genomic polymorphism, however, can be used as indicative of high relatedness. The use of PFGE in conjunction with at least an additional typing technique or independent PFGE analysis using different restriction enzymes is highly recommended to help in clarifying epidemiological interpretation. General principles proposed for the interpretation of molecular typing data, based on fragment differences (Tenover et al. 1995), are usually applied to interpret PFGE profiles obtained for enterococcal strains.

BACTERIOCINS AND BACTERIOPHAGES

Bacteriocins are bacteriostatic or bactericidal proteins or peptides that are produced by a number of bacteria and prevent the growth of other bacteria. These bacterial products exhibit a relatively narrow spectrum of antimicrobial activity and are usually active only against bacteria closely related to the producer strain. The ability to produce bacteriocins has been shown to confer ecological advantages. Among the enterococci, *E. faecalis* (Salzano et al. 1992; Villani et al. 1993; Balla

et al. 2000; Del Campo et al. 2001), *E. faecium* (Kato et al. 1993; Laukova et al. 1993; Cintas et al. 2000; Del Campo et al. 2001), *E. hirae* (Siragusa 1992), and *E. mundtii* (Kawamoto et al. 2002) are known to produce bacteriocins. Several enterococcal bacteriocins have been described, but only relatively few have been characterized at the molecular level. The best-characterized inhibitor substances produced *by E. faecalis* strains are the bacteriocin/hemolysin (or cytolysin), encoded on a conjugative plasmid (pAD1) and associated with strain virulence (see Pathogenicity and virulence factors); the cyclic peptide antibiotic AS-48 encoded on the pMB2 plamid; bacteriocin 21 encoded on plasmid pPD1, and bacteriocin 31 encoded by a conjugative plasmid (pY117) (Ike et al. 1990; Martinez-Bueno et al. 1990; Tanimoto et al. 1993; Gilmore et al. 1994; Tomita et al. 1996, 1997; Balla et al. 2000; Del Campo et al. 2001). Bacteriocins characterized in *E. faecium* include enterocin A, enterocin B, enterocin I , enterocin P, and enterocins L50A/L50B (Aymerich et al. 1996; Casaus et al. 1997; Cintas et al. 1997, 1998, 2000; Floriano et al. 1998; Del Campo et al. 2001). In recent years, additional interest in bacteriocins has been generated due to their potential use as food preservatives to improve food safety and quality and even as alternatives to antibiotics for some medical applications (Kato et al. 1993; Twomey et al. 2002). The enterococcal bacteriocins are inhibitory to several spoilage and food-borne pathogenic bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringes*, and *Lactococcus* and *Enterococcus* species.

The detection of bacteriocin production, which is based on the use of various species of bacteria as indicators, has also been applied as a typing system for enterococci (Pleceas et al. 1972; Sharma and Shriniwas Bhujwala 1976; Sadatsune et al. 1978).

Phage typing of enterococci has been performed in several laboratories. In some cases phage typing has been combined with bacteriocin typing to discriminate between strains (Herman and Hoch 1971; Hoch and Herman 1971; Caprioli et al. 1975; Kuhnen et al. 1987, 1988).

ROLE IN NORMAL FLORA OF HUMANS

As outlined in Habitat, the enterococci are primarily members of the gastrointestinal microflora of humans, occurring in numbers as high as 10^8 colony forming units acronym>CFU per gram of feces of adult individuals (Noble 1978; Huycke et al. 1998). Enterococcal populations in the intestinal tract fluctuate in size according to the age and physiological condition of the human host, being more numerous during early life (Tannock and Cook 2002). Diet also seems to affect the numbers of enterococci in fecal samples. Although *E. faecalis* appears to be the enterococcal species most commonly detected in human feces, in all likelihood most *Enterococcus* species are normal inhabitants of the gastrointestinal tract of humans. Since the enterococci are opportunistic pathogens, the incidence of each species found in human infections probably reflects the distribution of the different *Enterococcus* species in the human gastrointestinal tract. This site is believed to represent an important reservoir for strains associated with disease; from this location they may migrate to cause infections and can also disseminate to other hosts and environmental surfaces. *E. faecalis* is usually the most frequent enterococcal species isolated from human clinical specimens, representing 80–90 percent of the isolates, followed by *E. faecium* which is found in 5–10 percent of enterococcal infections (Facklam and Collins 1989; Gordon et al. 1992; Buschelman et al. 1993; Stern et al. 1994; Mondino et al. 2003). The other enterococcal species are identified less frequently. However, clusters of infections with *E. casseliflavus* (Nauschuetz et al. 1993) and *E. raffinosus* (Chirurgi et al. 1991; Wilke et al. 1997) have been reported. Therefore, the distribution of species varies with each clinical setting. Although less frequently or even rarely, several of the other enterococcal species, including *E. avium*, *E. cecorum*, *E. dispar*, *E. durans*, *E. gallinarum*, *E. gilvus*, *E. hirae*, *E. mundtii*, *E. pallens*, and *E. faecalis* variant strains, have also been isolated from human sources (Facklam and Collins 1989; Collins et al. 1991; Gordon et al. 1992; Stern et al. 1994; Facklam et al. 1999; Teixeira et al. 2000; Tyrrell et al. 2002; Mondino et al. 2003). *E. columbae*, *E. haemoperoxidus*, *E. malodoratus*, *E. moraviensis*, *E. porcinus*, *E. pseudoavium*, *E. ratti*, *E. saccharolyticus*, and *E. sulfureus* have not been isolated from human sources.

The comparison of data from different publications and the evaluation of the real incidence of the different species of enterococci as members of the intestinal microflora or as members of the microflora in other body sites have been impaired due to differences in the methodology used and the changes in the taxonomy of the genus.

PATHOGENICITY AND VIRULENCE FACTORS

The enterococci are commensal microorganisms that act as opportunistic pathogens, particularly in elderly patients with serious underlying diseases or in other immunocompromised patients who have been hospitalized for prolonged periods, use invasive devices, and/or have received broad-spectrum antimicrobial therapy. Although the enterococci can be a cause of infections in humans in the community and in the hospital, these microorganisms began to be recognized with increasing frequency as common causes of hospital-acquired infections in the late 1970s, paralleling the increasing resistance to most currently used antimicrobial agents. As a result, enterococci have emerged as one of the leading

therapeutic challenges when identified as a cause of serious or life-threatening infections. The ubiquitous nature of enterococci, however, requires caution in establishing the clinical significance of a particular isolate.

The variety of infections in which enterococci are involved has been thoroughly reviewed and summarized (Murray 1990; Hancock and Gilmore 2000; Strausbaugh and Gilmore 2000; Malani et al. 2002). Enterococci have become the second most common agent recovered from nosocomial urinary tract infections (UTI) and wound infections and the third leading cause of nosocomial bacteremia in the USA (Murray 1990; Schaberg et al. 1991; Moellering 1992; Hancock and Gilmore 2000). UTIs are the most common of the enterococcal infections: enterococci have been implicated in approximately 10 percent of all UTIs (Felmingham et al. 1992) and in 16 percent of nosocomial UTIs (Schaberg et al. 1991). Enterococcal bacteriuria usually occurs in patients with underlying structural abnormalities and/or in those who have undergone urologic manipulations (Moellering 1992). Intra-abdominal and pelvic infections are the next most commonly encountered infections. However, cultures from patients with peritonitis, intra-abdominal or pelvic abscesses, biliary tract infections, surgical-site infections, and endomyometritis are frequently polymicrobial, and the role of enterococci in this setting remains controversial. Enterococci are being recovered from wound infections at an increasing rate, which likely results from increased antibiotic usage and emerging resistance among these organisms (Moellering 1992; Murray 1990; Schaberg et al. 1991; Fridkin and Gaynes 1999). Enterococci have been considered as an important cause of endocarditis, since early descriptions, and are estimated to account for about 20 percent of the cases of native valve bacterial endocarditis and for about 6 to 7 percent of prosthetic valve endocarditis (Megran 1992; Hancock and Gilmore 2000). Whereas endocarditis is a serious enterococcal infection, it is less common than bacteremia. Enterococcal endocarditis is more common in the elderly and in patients with degenerative valvular diseases and patients with genitourinary conditions. Enterococcal infections of the respiratory tract or the central nervous system, as well as otitis, sinusitis, septic arthritis, and endophthalmitis may occur but are rare (Murray 1990; Hancock and Gilmore 2000; Strausbaugh and Gilmore 2000).

Several potential virulence factors have been identified in enterococci, as reviewed recently (Jett et al. 1994; Huycke et al. 1998; Hancock and Gilmore 2000; Mundy et al. 2000; Strausbaugh and Gilmore 2000; Eaton and Gasson 2001; Gilmore et al. 2002), but none has been established as having a major contribution to virulence in humans. The enterococcal virulence traits include secreted factors, such as a cytolysin that displays both hemolytic and bacteriocin activity and proteolytic enzymes with gelatinase and serine protease activity; adhesins, such as the aggregation substance (a surface-localized protein encoded by pheromone-responsive plasmids, the enterococcal surface protein (Esp), a collagen-binding adhesin of *E. faecalis* (Ace), and an immunodominant antigen named EfaA (*E. faecalis* antigen A); and capsular and cell-wall polysaccharides. Most of the information available on enterococcal virulence factors is related to *E. faecalis*. The production of gelatinase, cytolysin, aggregation substance, and adhesins are the most frequently studied. Despite the significance of *E. faecium* in human infections, the potential virulence factors of this species remain poorly characterized.

The proteolytic activity of some enterococcal strains was early recognized and used for classifying *E. faecalis* into subspecies. Such activity is easily demonstrated by gelatin liquefaction tests and the enzyme involved is usually referred to as gelatinase. Animal models of experimental endocarditis have suggested a possible role for gelatinase in virulence (Gutschik et al. 1979). Recently, the *fsr* locus was described and associated with *E. faecalis* virulence in animal models (Qin et al. 2000). This locus is comprised of three genes and has been shown to regulate the transcription of at least two genes: those for gelatinase (*gelE*) and serine protease (*sprE*). Additional studies are needed to determine the exact mechanism of regulation of protease gene expression and also to define the potential role or *fsr* in regulating other enterococcal virulence factors.

The cytolysin is a bacterial toxin expressed by some strains of *E. faecalis* that is distantly related to streptolysin O and also to members of the bacteriocins known as lantibiotics (Hancock and Gilmore 2000). It is either encoded within pheromone-responsive plasmids or on chromosome within pathogenicity islands (Gilmore et al. 2002). The production of cytolysin (hemolysin) was reported to be more common in strains of *E. faecalis* isolated from human infections than in strains isolated from fecal sources of healthy people (Ike et al. 1987). Hemolysin production by *E. faecalis* has also been associated with enhanced virulence in mice (Ike et al. 1984) and was found to contribute to the severity of *E. faecalis* experimental endophthalmitis (Jett et al. 1992; Stevens et al. 1992).

The production of aggregation substance is induced by a pheromone (small linear peptides), and the immediate effect of this substance is the aggregation of plasmid-free and plasmid donor cells. Then, exchange of plasmids carrying the hemolytic–bacteriocin genes as well as antimicrobial-resistance genes is conducted through efficient conjugal transfer (Clewell 1993b). Aggregation substance has been shown to enhance colonization in tissue-culture models for adherence to urinary epithelium (Hirt et al. 1993). Olmsted et al. (1994) reported that aggregation substance facilitated the *E. faecalis* internalization by host epithelial cells. However, Coque et al. (1995) were unable to find an increase in the inci-

dence of production of aggregation substance or hemolysin in *E. faecalis* isolated from patients with endocarditis and patients without endocarditis or normal flora of healthy individuals.

The mechanism by which hemolysin and aggregation substance enhance virulence is not completely understood. It is conceivable that these substances have an effect on colonization, invasion, platelet aggregation, or generation of fibrin (Chow et al. 1993b). Aggregation substance can act as an adhesion capable of promoting adherence of *E. faecalis* to epithelial cell surface, a necessary first step in colonization, and of promoting internalization and survival within intestinal macrophages and polymorphonuclear neutrophils (PMNs). Aggregation substance and hemolysin act synergistically to enhance virulence by facilitating achievement of a quorum and activating the quorum-sensing mode of hemolysin regulation, resulting in tissue damage and invasion (Gilmore et al. 2002).

Properties involved in the adherence to host tissues are considered important virulence factors for bacteria to establish infections. Guzman et al. (1989) reported that *E. faecalis* strains isolated from urinary tract infections adhered to urinary tract epithelial cells more efficiently than to human embryonic kidney cells or Girardi heart cell lines. *E. faecalis* strains isolated from endocarditis strains preferentially adhered to Giriadi heart cell lines. These findings indicated that strain-specific adhesins may be necessary to establish initial colonization. Direct experimental evidence for the role of a novel cell-surface-localized protein named Esp as an adhesin involved in colonization of the urinary tract was recently published (Shankar et al. 2001).

The investigation of hemagglutination properties can be a convenient approach to detect the presence of adhesions on bacteria. Results of a study by Carvalho and Teixeira (1995) indicated that the surface structures related to the enterococcal adhesion are diverse. Most of the enterococcal strains analyzed were shown to possess hemagglutinins, both proteins and nonproteins, that are involved in the attachment to sialic acid-containing receptors on the surface of rabbit erythrocytes. Other investigators have demonstrated that an adhesin similar to that found in oral streptococci is produced by *E. faecalis* (Lowe et al. 1995) and postulated that this molecule may function as an adhesin in endocarditis. Guzman et al. (1991) described a nonprotein molecule that promoted the adhesion of *E. faecalis* to human Giradi heart cell lines.

Enterococcal invasive properties under certain circumstances have been investigated. Evidence on the capacity of enterococci to translocate across intact intestinal mucosa in a murine model of antibiotic-induced superinfection was provided (Wells et al. 1990). Translocation of enterococci across normal intestinal epithelium occurred in mice orally inoculated with *E. faecalis* and given broad-spectrum antimicrobials that favor enterococcal overgrowth, resulting in disseminated infection.

Neutrophils (polymorphonuclear leukocytes) are one of the primary participants in host defense response to invading bacteria. Complement-dependent killing of enterococci has been demonstrated with strains of *E. faecalis* (Harvey et al. 1992; Arduino et al. 1994a). Neutrophil-mediated killing of *E. faecium* was ineffective in some cases, which led the investigators to postulate that resistance to neutrophil-mediated killing could be an additional virulence factor (Arduino et al. 1994b). Recent evidence indicates that polysaccharides on the enterococcal surface are involved in hindering leukocyte killing of enterococci (Teng et al. 2002).

Overall, the studies on the pathogenesis of enterococcal infections are beginning to identify specific enterococcal and host traits that contribute to the balanced commensal relationship, as well as those that destabilize it. The results indicate that we are only beginning to understand the pathogenicity and the virulence mechanisms of the enterococci.

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Gram-positive anaerobic cocci

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INTRODUCTION

The first description of 'anaerobic streptococci' was by Veillon, who isolated an organism that he named *Micrococcus foetidus* from a case of bartholinitis in 1893. He noted that the organism could be recovered only under conditions of rigorous anaerobiosis. Veillon subsequently showed this organism to fulfill Koch's postulates. Other workers soon cultured similar organisms from a variety of infections, notably puerperal fever, abscesses, and gynecological sepsis. However, their classification has never been satisfactory. Previous schemes relied on inconsistent morphological characteristics and almost certainly included capnophilic strains, which are now placed in other genera such as *Streptococcus* (Holdeman Moore and Moore 1974; Cato 1983) and *Staphylococcus* (Kilpper-Baelz and Schleifer 1981). Watt and Jack (1977) defined anaerobic cocci as 'cocci that grow well under satisfactory conditions of anaerobiosis and do not grow on suitable solid media in 10 percent carbon dioxide (CO_2) in air even after incubation for 7 days at 37°C.' This definition is valuable for distinguishing obligate anaerobic cocci from microaerophilic organisms and has been followed in this chapter.

Gram-positive anaerobic cocci (GPAC) are now recognized as a major part of the normal human flora of the mouth, gastrointestinal tract, female genital tract, and skin. They are often isolated from clinical specimens but seldom in pure culture, making it difficult to determine their importance in human disease. However, the recovery of these organisms from almost any body site coupled with the sheer weight of numbers recovered would suggest their clinical relevance in many cases. They have received less study than some other anaerobic groups and most aspects of their biology are poorly understood. For the early history of these organisms see Wilson and Miles (1964) and Finegold (1994).

CLASSIFICATION

There have been several attempts to develop a satisfactory classification. The genera *Peptococcus* and *Peptostreptococcus* were proposed by Kluyver and van Neiel (1936), who separated them by morphological characteristics: peptococci were arranged in clumps and peptostreptococci in chains. This distinction is unreliable because it is influenced greatly by many variables, in particular composition of the medium. Prevot (1948) divided anaerobic cocci into eight genera on the basis of their microscopic appearance. Individual species were differentiated by tests such as indole production, liquefaction of gelatin, and growth in litmus milk, which are now recognized to be of little value. Hare and coworkers (1952) distinguished ten groups of anaerobic cocci by fermentation and gas production from carbohydrates and inorganic acids, and consequently they did not add more names to a classification already confused by poorly defined species. Rogosa (1971) created the family Peptococcaceae for *Peptococcus*, *Peptostreptococcus* and *Ruminococcus*. Shortly afterwards, Holdeman Moore

and Moore (1974) proposed the genus *Coprococcus* and assigned it with *Sarcina* to the Peptococcaceae, which then contained five genera classified together purely on morphological grounds. Some authorities considered that the Peptococcaceae were too heterogeneous to be considered a valid taxon (Holdeman Moore et al. 1986; Holdeman Moore and Moore 1986) but this term was still used by others (Ezaki et al. 1994).

In recent years, a wide range of analytical techniques has been used, including DNA G + C content, DNA–DNA homology studies, comparison of 16S rRNA sequences, analysis of cell-wall fatty acids and peptidoglycan structure, whole-cell composition by pyrolysis mass spectrometry (PMS), analysis of 16S rDNA sequences, and the analysis of 16S-23S intergenic ribosomal RNA polymorphisms. These studies have emphasized the heterogeneity of GPAC and, consequently, major changes at the genus level have recently taken place (Murdoch and Shah 1999; Wade et al. 1999; Ezaki et al. 2001).

Changes at the genus level

The results of DNA–DNA homology studies led Ezaki et al. (1983) to propose that most species in the genus *Peptococcus* should be placed in the genus *Peptostreptococcus*. However, similar studies by Huss et al. (1984) suggested that these organisms were considerably more heterogeneous. The genus *Peptococcus* now includes only one species, *Peptococcus niger*, with a G + C content of 50–51 mol%, but left 13 species in the genus *Peptostreptococcus*, with a G + C range of 27–37 mol%. Comparisons of 16S rRNA sequences indicate that *Pc. niger*, the type species of the family Peptococcaceae, is not related to other members of the family (Ezaki et al. 1994) and *Peptostreptococcus anaerobius*, the type species of its genus, is related more closely to some members of the genus *Clostridium* than it is to other peptostreptococci (Lawson et al. 1993; Paster et al. 1993; Collins et al. 1994; Ezaki et al. 1994). It became clear that the genus *Peptostreptococcus* is not a genetically valid taxon (Lawson et al. 1993; Ezaki et al. 1994). Li et al. (1994) recommended that it be divided into at least seven genera. A recent study of whole-cell composition by PMS agreed with these conclusions (Murdoch and Magee 1995).

Ezaki et al. (1994) proposed moving *Peptostreptococcus productus*, a strongly saccharolytic species with a G + C content of 45 mol%, and *Streptococcus hansenii*, another obligately anaerobic coccus, to the genus *Ruminococcus*. They also suggested that members of the genera *Coprococcus* and *Ruminococcus* might be better classified in a single genus. Unfortunately, this study did not include the type species of the genus, *Ruminococcus flavefaciens*. Studies using 16S rRNA sequence comparisons (Collins et al. 1994; Rainey and Janssen 1995) confirm that the genus *Ruminococcus* contains at least two genetic clusters. It is likely that *Ruminococcus productus* will be placed in a new genus. *Streptococcus parvulus*, an obligately anaerobic coccus previously in the genus *Peptostreptococcus* (Cato 1983), has now been placed in a new genus *Atopobium* together with some species formerly in the genus *Lactobacillus* (Collins and Wallbanks 1992). Recent taxonomic studies have now begun to resolve these problems. Murdoch and Shah (1999) using 16S rRNA sequence analysis proposed two new genera, thus reclassifying *Peptostreptococcus magnus* as *Finegoldia magna* and *Peptostreptococcus micros* as *Micromonas micros*. The other three genospecies suggested by Ezaki and coworkers have now been given the generic names *Anaerococcus*, *Gallicola*, and *Peptoniphilus* (Ezaki et al. 2001) on the basis of 16S rDNA sequence analysis. *Peptostreptococcus heliotrinreducens* is only distantly related to other anaerobic cocci (Li et al. 1994; Murdoch and Magee 1995) and appears closer to some organisms at present in the genus *Eubacterium*.

Peptostreptococcus heliotrinreducens has therefore been reclassified as *Slackia heliotrinreducens* by Wade et al. (1999) on the basis of 16S rRNA gene analysis.

In spite of all this work Hill et al. (2002) using intergenic ribosomal RNA polymorphisms have shown that, using both type strains and wild strains, there is still some heterogeneity within certain species (e.g. *Anaerococcus vaginalis*).

In summary, there has been much progress since the previous edition of this book, but clearly there are still issues awaiting resolution. The currently accepted genera of the anaerobic gram-positive cocci are summarized in Table 1.

Recent and new species of anaerobic gram-positive cocci

It has become increasingly clear that *Peptoniphilus (Peptostreptococcus) asaccharolyticus* and *Anaerococcus (Peptostreptococcus) prevotii* are genetically heterogeneous (Ezaki et al. 1983, Harpold and Wasilauskas 1987; Murdoch and Mitchelmore 1991; Ng and Dillon 1991; Murdoch and Magee 1995). Several new species have recently been described: *Gallicola (Peptostreptococcus) barnesae* (Schiefer-Ullrich and Andreesen 1985), *Anaerococcus (Peptostreptococcus) hydrogenalis* (Ezaki et al. 1990), *Anaerococcus (Peptostreptococcus) vaginalis*, *Anaerococcus (Peptostreptococcus) lactolyticus*, and *Peptoniphilus (Peptostreptococcu) lacrimalis* (Li et al. 1992), but there is good evidence from DNA hybridization and PMS studies (Li et al. 1992; Murdoch and Magee 1995) that further species await description. *Finegoldia (Peptostreptococcus) magna*, *Micromonas (Peptostreptococcus) micros*, and *P. anaerobius*, the other species most often isolated from human material,

Table 1 *Currently recognized genera of anaerobic gram-positive cocci*

	G + C %	Fermentation of carbohydrates	Major VFA	Cell-wall diamino acid	Interpeptide bridge	Peptone as major energy source
Anaerococcus	26–34	+	B[a]	L-Lysine	D-Glutamic acid[b]	+
Atopobium	46	+	A	nd	nd	+
Coprococcus	39–42	+	B	m-DAP	None	–
Finegoldia	32–34	–	A	L-Lysine	Glycine	+
Gallicola	32–34	–	A	Ornithine	D-Aspartic acid	+
Micromonas	28–30	–	A	L-Lysine	D-Aspartate	+
Peptoniphilus	25–35	–	B	Ornithine	D-Glutamic acid	+
Peptococcus	50–51	–	B,IV,C	L-Lysine	D-Aspartate	+
Peptostreptococcus	34–36	+	A,IC	L-Lysine	D-Aspartate	+
Ruminococcus	39–46	+	A	m-DAP	None	–
Sarcina	28–31	+	A	LL-DAP	Glycine	–

A, acetic acid; B, butyric acid; C, caproic acid; DAP, di-aminopinelic; IC, iso-caproic acid; IV, iso-valeric acid; nd, no data; VFA, volatile fatty acid.
Data compiled from Ezaki et al. (2001); Murdoch and Magee (1995) and Murdoch (1998).
a) Except *An. octavius* where major VFA is caproic acid.
b) Except *An. octavius* where it is D-aspartate.

appear to be valid and homogeneous taxa (Ezaki et al. 1983; Murdoch and Magee 1995). There are some data from protein electrophoretic studies (Cato et al. 1983; Taylor et al. 1991) suggest that ATCC 14955, the type strain of '*Peptococcus variabilis*,' is distinct from *F. magna*. Most workers have found no evidence that *F. magna* is heterogeneous.

Three further species of anaerobic gram-positive cocci have been identified recently. *Peptoniphilus (Peptostreptococcus) harei*, *Peptoniphilus (Peptostreptococcus) ivorii*, and *Anaerococcus (Peptostreptococcus) octavius* were all described by Murdoch et al. (1997) based on genotypic and phenotypic methods. All isolates were recovered from human clinical material.

Two other groups of anaerobic gram-positive cocci, as yet not given species status, were described by Murdoch (1998). The 'trisimilis' group and the 'β-Gal' group are both recovered from human clinical material. The 'trisimilis' group are saccharolytic and weakly proteolytic cocci and are phenotypically distinct from other groups. This group of cocci are slow-growing.

The 'β-Gal' group are a heterogeneous group. Analysis of 16S rRNA of two strains show a close relationship to *Anaerococcus lactolyticus*. They are slow-growing and some strains reveal satellite growth around other microorganisms.

Currently recognized species of GPAC are listed in Table 2.

LABORATORY ISOLATION AND MAINTENANCE

Most of the anaerobic gram-positive cocci are strict anaerobes in that they need an anaerobic atmosphere for multiplication (Watt and Jack 1977), but the limited data available suggest that most clinical strains are moderately aerotolerant. The 14 clinical strains studied by Tally et al. (1975) all survived 8 h or more of exposure to oxygen; nine (63 percent) survived more than 72 h. Murdoch (1998) has shown in investigations of fresh clinical isolates that 1 percent of cells of *F. magna* and *M. micros* were still viable after exposure to air for 48 h. Most workers consider that anaerobic conditions are necessary during specimen transport, though opinions differ (Smith et al. 1986a; Ezaki et al. 1992). Growth is best in the temperature range 35–37°C and is enhanced by the presence of 10 percent CO_2 in the atmosphere (Watt and Smith 1990). If evacuation and replacement jars are used, a palladium catalyst should be present to remove traces of oxygen. The newer non-catalytic sachets for generating anaerobic atmospheres are satisfactory for the recovery of clinically important anaerobes including the anaerobic cocci (Brazier 1995). The anaerobic gram-positive cocci require complex but ill-defined nutrients; consequently, commercial non-selective media vary in their ability to support the growth of fresh clinical isolates (Heginbothom et al. 1990). Tween 80 (final concentration 0.02 percent) enhances growth of some species (particularly *P. anaerobius* and *F. magna*) but is not essential, nor are vitamin K or hemin supplements (Holdeman Moore et al. 1986). Opinions differ concerning the need for pre-reduced media for isolation (Holdeman et al. 1977; Ezaki et al. 1992). Viability in chopped-meat broth depends on the commercial source since some preparations do not support growth at all. Solid media without blood can also be used (Ezaki et al. 1992).

Gram-positive anaerobic cocci may fail to signal in commercial blood culture media. Terminal subculture should be considered when there is suspicion of these organisms in clinical bacteraemia (van der Vorm et al. 2000). The observation that some strains show satellitism around other species of GPAC would suggest that the growth requirements of some anaerobic cocci are not yet fully known (Murdoch 1998).

Table 2 *Currently recognized species of anaerobic gram-positive cocci*

Current classification	Previous approved name	Other previous designations
A. hydrogenalis	*Pst. hydrogenalis*	
A. lactolyticus	*Pst. lactolyticus*	
A. octavius	'*Pst. harei*'	
A. prevotii	*Pst. prevotii*	*Pc. prevotii*
A. tetradius	*Pst . tetradius*	*Gaffkya anaerobia*
A. vaginalis	*Pst. vaginalis*	
F. magna	*Pst. magnus*	*Pc. magnus, Pc. variabilis*
G. barnsae		
M. micros	*Pst. micros*	
Pe. asaccharolyticus	*Pst. asaccharolyticus*	*Pc. asaccharolyticus*
Pe. indolicus	*Pst. indolicus*	*Pc. indolicus*
Pe. ivorii	'*Pst. ivorii*'	
Pe. lacrimalis	*Pst. lacrimalis*	
Pc. niger	Pc. niger	
Pst. anaerobius		
R. productus	Pst. productus	
S. heliotrinreducens	Pst. heliotrinreducens	
'β-Gal' group		
'*Pst. trisimilis*'		

A., Anaerococcus; F., Finegoldia; G., Gallicola; M., Micromonas; Pc., Peptococcus; Pe., Peptoniphilus; Pst., Peptostreptococcus; R., Ruminococcus; S., Slackia.

Ruminococci, coprococci, and sarcinae (see below) are more sensitive to oxygen and will grow only in well-maintained anaerobic conditions (Ezaki et al. 1992). Carbohydrates stimulate the growth of coprococci and are essential for ruminococci and sarcinae. Few strains of these genera are likely to grow on standard commercial media and are therefore unlikely to be recovered from clinical material. Bryant (1986) and Ezaki et al. (1992) give excellent guidelines for their isolation and culture.

Pus or the aspired fluid material is preferred for the isolation of anaerobes and gives a better recovery of these organisms including the anaerobic cocci (Wren 1991; Mangels 1994). Anaerobic transport systems are necessary for optimum recovery when swabs are used (Smith et al. 1993).

The development of selective media has been neglected. This is an area that deserves active study as clinical isolates grow slowly on standard media and are usually present in mixed culture. Overgrowth by more rapidly growing organisms on non-selective media occurs, thus many clinical studies will give a falsely low estimate of their frequency. However, GPAC are so heterogeneous that any single selective medium is unlikely to be satisfactory for all. Wren (1978) found that nalidixic acid-Tween agar was superior to an enriched blood agar containing either neomycin or kanamycin, the traditional isolation media for anaerobic bacteria from clinical material. These findings were confirmed in his survey of selective media for the recovery of anaerobes from clinical samples (Wren 1980). Anaerobic gram-positive cocci are very sensitive to most antibiotics but are usually resistant to bicozamycin (Watt and Brown 1983; Smith et al. 1986a); however, this agent is no longer easily available. Ezaki et al. (1992) recommend the use of phenylethylalcohol blood agar but this medium may not recover some species of cocci (Turng et al. 1996). A specific medium for *M. micros* has been described (Turng et al. 1996).

Media should be incubated under continuous anaerobic conditions for 48 h before examination followed by a further 3 days of incubation to allow maximum growth of the slower growing species.

The gram-positive anaerobic cocci retain viability well at −70°C or in liquid nitrogen. For long-term storage, Holdeman Moore et al. (1986) recommend lyophilization of cultures in the early stationary phase of growth in a medium containing less than 0.2 percent fermentable carbohydrate. The use of commercially produced cryobeads stored at −70°C is a satisfactory alternative for long-term storage.

For short-term storage, most strains survive well on the open bench in high-quality chopped-meat broth; however, regular subculture is necessary to maintain the viability of many strains.

LABORATORY IDENTIFICATION

Identification to the genus level

An important early step in the identification of anaerobic cocci is their differentiation from the strains of microaerophilic and capnophilic organisms at present

classified in the genera *Streptococcus*, *Staphylococcus*, and *Gemella*. Although such species may appear only on anaerobic plates on primary isolation, they will grow in 10 percent CO_2 on repeated subculture, particularly strains of *S. asaccharolyticus*, *S. anginosus*, and *G. morbillorum*. The detection of large amounts of lactic acid in the absence of other acids by gas–liquid chromatography (GLC) would indicate a member of the *Streptococci*. *Staphylococcus saccharolyticus*, a normal part of the skin flora, will usually grow only in an anaerobic atmosphere on primary incubation. Strains ferment several carbohydrates, produce urease and catalase, and reduce nitrate (Evans et al. 1978; Ezaki et al. 1992). The species of anaerobic gram-positive cocci of major medical importance are at present classified in the genera *Anaerococcus*, *Finegoldia*, *Micromonas*, *Peptoniphilus*, and *Peptostreptococcus*, but the definition of Watt and Jack (1977) also embraces *Peptococcus*, *Ruminococcus*, *Coprococcus*, *Sarcina*, and *Atopobium* (Table 1). Isolates of these genera have rarely been reported from human clinical specimens, perhaps because techniques for their isolation and identification are difficult and currently used routine diagnostic media are unsatisfactory.

Antibiotic disk susceptibility tests have been widely used for the presumptive identification of gram-negative anaerobic rods (Sutter and Finegold 1971) but not for GPAC. Watt and Jack (1977) and Murdoch and Mitchelmore (1991) used sensitivity to metronidazole as a simple screening method for separating GPAC from facultative organisms. Anaerobic cocci gave a zone of inhibition greater than 15 mm in width around a 5-μg disk. Metronidazole resistance has been reported but such strains have been incompletely characterized (Edson et al. 1982; Wexler and Finegold 1988; Sanchez et al. 1992; Sheikh et al. 1993). Many isolates of GPAC retain Gram's stain poorly and can be confused with gram-negative organisms especially *Veillonella*. Sensitivity to vancomycin is a simple and reliable method of separation (Murdoch and Mitchelmore 1991). The presence of elongated cells in Gram-stained smears of older cultures may also lead to confusion.

Identification to species level

Identification based on microscopical appearance, detection of volatile fatty acids (VFAs) by GLC, ability to ferment carbohydrates, and standard biochemical tests such as nitrate reduction and indole production have been the traditional standard methods for identification of the anaerobic cocci (Holdeman et al. 1977; Sutter et al. 1985; Summanen et al. 1993; Hillier and Moncla 1995). The results of GLC are of limited discrimination. They permit a useful if arbitrary division into groups based on the major terminal volatile fatty acid (Murdoch and Mitchelmore 1991). However, with the current reclassification resulting in new genera this grouping by GLC does not necessarily correlate with any specific genus. GLC remains useful as part of the full identification process.

Production of non-volatile fatty acids (NVFAs) has been little studied, except by Ezaki et al. (1983), but appears to be of little value except to exclude streptococci. Few species of GPAC produce acid from carbohydrates or are active in standard biochemical tests (Ezaki and Yabuuchi 1985; Holdeman Moore et al. 1986; Summanen et al. 1993). Therefore most species have in the past been identified on the basis of negative reactions. *P. anaerobius* can be presumptively identified by sodium polyanethol sulfonate (SPS) disk testing (Graves et al. 1974, Wideman et al. 1976). Murdoch and Mitchelmore (1991) found that this test had a sensitivity and specificity approaching 100 percent. A few strains of *Pe. ivorii* may also be SPS-sensitive.

The products of protein decomposition are a major energy source for most species of GPAC (Rogosa 1974; Holdeman Moore et al. 1986). Ezaki and Yabuuchi (1985) showed that tests for amidase and oligopeptidase activity clearly and reproducibly distinguished between most species of 'peptostreptococci' and microaerophilic streptococci. Commercial preformed enzyme kits for the detection of proteolytic and saccharolytic enzymes (Karachewski et al. 1985; Murdoch et al. 1988a; Murdoch and Mitchelmore 1991; Wren 1996) have now made rapid, reliable identification straightforward for routine diagnostic laboratories (Table 3). Between 10 and 16 percent of isolates still cannot be identified to recognized species (Murdoch et al. 1994; Wren 1996). The heterogeneity that still exists within some species is the likely reason for most identification failures.

DNA probes provide a sensitive and specific method for the rapid identification of single bacterial colonies. ^{32}P-Labeled probes for the identification of *P. anaerobius* (Yasui et al. 1989) and *M. micros* (Yasui 1989) are limited by the short half-life of the radioisotope and problems with their disposal. The development of a 'cold' digoxigenin-labeled probe for *M. micros* (Gunaratnam et al. 1992) could be of considerable value, particularly in oral microbiology.

The use of the polymerase chain reaction (PCR) for the detection of *M. micros* in destructive dental infections (Riggio et al. 2001) and for the detection of *Ruminococcus species* in human and animal feces (Wang et al. 1997) has been described.

Two 16S rRNA targeted probes have been developed and used for the study of the *Atopobium* cluster in human intestinal flora by Harmsen et al. (2000).

ROLE IN NORMAL FLORA OF HUMANS

The GPAC are a major part of the normal flora of skin and the mucosal surfaces of the mouth and upper respiratory, gastrointestinal, and genitourinary tracts

Table 3 *Identification of the clinically relevant anaerobic gram-positive cocci*

	An. hydrogenalis	*An. lactolyticus*	*An. octavius*	*An. prevotii*	*An. tetradius*	*An. vaginalis*	*F. magna*	*G. barnsae*	*M. micros*	*Pep. asaccharolyticus*
Major VFA	B	B	C	B	B	B	A	A	A	B
Indole	+	–	–	–	–	v	–	w	–	v
Urease	v	+	–	+	+	–	–	–	–	–
ALP	–/w	–	–	–	–	–/w	v	–	+	–
ADH	–	–	–	–	–	+	v	–	–	–
Carbohydrate fermentation										
Glucose	+	+	+	w	+	+	–/w	–	–	–
Lactose	+	+	–	–	–	–	–	–	–	–
Mannose	+	+	+	+	+	v	–	–	–	–
Raffinose	+	–	–	+	–	–	–	–	–	–
Ribose	–	–	+	+	–	–	–	–	–	
Saccharolytic enzymes										
α-Gal	–	–	–	+	–	–	–	–	–	–
β-Gal	–	+	–	–	–	–	–	–	–	–
α-Glu	v	–	–	+	+	–	–	–	–	–
β-Gur	–	–	–	+	+	–	–	–	–	–
Proteolytic enzymes										
ArgA	–	+	–	+	+	+	+	–	+	+
ProA	–	–	+	–	–	–	–	–	+[a]	–
PheA	–	–	–	–	w	–	–	–	+[a]	–
LeuA	–	–	–	–	+	+	+	–	+	v
PyrA	–	–	w	+	w	–	+	–	+	–
TyrA	–	–	–	w	w	–	–/w	–	+	v
HisA	–	–	–	+	w	+	w	–	+	w
GGA	–	–	–	–	–	–	–	–	+[a]	–
SPS sensitivity	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

(*Continued over*)

Table 3 *Identification of the clinically relevant anaerobic gram-positive cocci (Continued)*

	Pep. harei	*Pep. indolicus*	*Pep. ivorii*	*Pep. lacrimalis*	*Pc. niger*	*P. anaerobius*	*R. productus*	*S. heliotrinreducens*	'trisimilis' group	'β-Gal' group
Major VFA	B	B	IV	B	C	IC	A	A	B	B
Indole	v	+	–	–	–	–	–	–	+	–
Urease	–	–	–	–	–	–	–	–	–	–
ALP	–	+	–	–	–	–	–	–	v	w
ADH	–	–	–	–	–	–	–	+	–	v
Carbohydrate fermentation										
Glucose	–	–	–	–	–	+	+	–	+	+
Lactose	–	–	–	–	–	–	+	–	w	+
Mannose	–	–	–	–	–	w	v	–	+	+
Raffinose	–	–	–	–	–	–	+	–	–/w	–
Ribose	–	–	–	–	–	–	v	–	–/w	+
Saccharolytic enzymes										
α-Gal	–	–	–	–	–	–	+	–	–	–
β-Gal	–	–	–	–	–	–	–	–	v	w/+
α-Glu	–	–	–	–	–	+	+	–	–	–/w
β-Gur	–	–	–	–	–	–	–	–	–	–
Proteolytic enzymes										
ArgA	+	+	–	+	–	–	–	V	–	+
ProA	–	–	+	–	–	+	–	+	–	–
PheA	–	+	–	+	–	–	–	+	–	–
LeuA	–/w	+	–	+	–	–	–	+	–	+
PyrA	–	–	–	–	–	–	–	–	+	w
TyrA	w	w	–	v	–	–	–	w	–	–
HisA	+	+	–	+	–	–	–	w	–	–
GGA	–	–	–	–	–	–	–	–	nd	nd
SPS sensitivity	R	R	R/S	R	R	S	R	R	R	R

Data compiled from: Murdoch and Mitchelmore (1991); Murdoch (1998); Wren (1996); Ng et al. (1994); Ezaki et al. (2001).
An., *Anaerococcus*; *F.*, *Finegoldia*; *P.*, *Peptostreptococcus*; *Pc.*, *Peptococcus*; *Pe.*, *Peptoniphilus*; *R.*, *Ruminococcus*; *S.*, *Slackia*; +, positive in >90 percent; –, negative in >90 percent; nd, no data; v, variable; VFA, volatile fatty acid; w, weak; A, acetic; B, butyric, IC, iso-caproic, IV, iso-valeric, C, caproic; ALP, alkaline phosphatase; ADH, arginine dihydrolase; α-Gal, α-galactosidase; β-Gal, β-galactosidase; α-Glu, α-glucosidase, β-Gur, β-glucuronidase; Arg, arginine; Pro, proline; Phe, phenylalanine; Leu, leucine; Pyr, pyroglutamyl; Tyr, tyrosine; His, histidine; GG, glutamylglutamyl; SPS, sodium polyanethol sulfonate (liquoid); S, sensitive; R, resistant; V, variable.
Results of tests for indole, urease, ALP, ADH, and saccharolytic and proteolytic enzyme reactions are based on results from a commercial kit API ID32A (Biomerieux, Basingstoke, Hants, UK).
Reactions given for *An. prevotii* and *An. tetradius* are of the type species only (see text for discussion).
a) Eight of 11 genital tract isolates of *M. micros* did not produce ProA, PheA, or GGA. See Ng et al. (1994).

(Neut et al. 1985; Bartlett 1990; Summanen et al. 1993) but reports have usually not attempted identification to the species level. Many commensals of low pathogenicity are poorly studied and cannot be placed in recognized species (Murdoch and Mitchelmore 1991; Summanen et al. 1993). There is considerable variation between reports, partly because of genuine differences between the populations studied and partly because of difficulties with nomenclature and identification. In particular, organisms described in earlier reports as *Pe. asaccharolyticus* and *An. prevotii* are likely to be heterogeneous. As the endogenous flora is believed to be the source of most anaerobic infections (Finegold and George 1989), further studies of commensal GPAC are required, with identification when possible to the species level.

The GPAC constitute 1–5 percent of the normal oral flora (Sutter 1984), the major species being *M. micros* and, according to some reports, *P. anaerobius* (Neut et al. 1985; Holdeman Moore et al. 1986; Rams et al. 1992). Dental plaque and the gingival sulcus seem to be the main sites of colonization. The gastrointestinal tract hosts most species of GPAC, with considerable numbers of little known commensals such as coprococci, sarcinae, and ruminococci (Holdeman Moore and Moore 1974; Ezaki et al. 1992), which have rarely been isolated from infectious processes. *Ruminococcus productus*, *M. micros*, *An. prevotii*, and *F. magna* are reported as being particularly common. Furthermore it has been demonstrated that the composition varies with diet (Finegold et al. 1974; Neut et al. 1985). The female genitourinary tract contains high numbers of GPAC, including *F. magna*, *An. prevotii*, *Pe. asaccharolyticus*, *P. anaerobius*, and *M. micros* (Bartlett 1990; Summanen et al. 1993) but its detailed composition at the species level appears to have been little studied. *Finegoldia magna*, *Pe. asaccharolyticus*, and *An. vaginalis* are likely to be present in large numbers as they are the predominant species in associated superficial wound infections and abscesses (Murdoch et al. 1994). Levels of GPAC in the female genitourinary tract may vary with stages of the menstrual cycle and with pregnancy (MacDonald et al. 1994).

CLINICAL IMPORTANCE

The GPAC are the second commonest group of anaerobes cultured from human clinical material. They consistently account for 20–35 percent of all anaerobic isolates (Holland et al. 1977; Wren et al. 1977; Rosenblatt 1985; Brook 1988a; Murdoch et al. 1994). They can produce disease and be cultured from a wide variety of sites, particularly gynecological, oral, cutaneous, gastrointestinal, and upper respiratory tract infections (Brook 1995), and are commonly recovered from a variety of skin diseases (Higaki et al. 1999). They constitute a significant proportion of the infecting flora in sinusitis (Mitchelmore and Tabaqchali 1999). GPAC are also major causes of serious obstetric infections, particularly puerperal fever (Schwarz and Dieckman 1926; Colebrook and Hare 1933; Hare and Polunin 1960; Hillier et al. 1990). Isolation from the blood is uncommon except in obstetric cases (DiZerega et al. 1980; Topiel and Simon 1986; Brook 1989a). Endocarditis is rare (Felner and Dowell 1970; Cofsky and Seligman 1985). Central-nervous-system infections usually present as abscesses (Brook 1981; Murdoch et al. 1988b) and meningitis has been reported after head and neck surgery (Brown et al. 1994). Careful investigations have shown that GPAC are common in superficial infections (Wheat et al. 1986; Brook 1989b; Edmiston et al. 1990; Brook et al. 1992). Most infections are mixed, particularly abdominal wounds and deep-organ abscesses, which may yield a characteristic flora of anaerobes and microaerophilic streptococci (Murdoch et al. 1988b). However, they are sometimes isolated in pure culture from serious infections, in particular from splenic abscess (Loualidi et al. 2001), renal abscess (Casullo et al. 2001), prosthetic valve endocarditis (Ubeda et al. 1998; van der Vorm et al. 2000), paraspinal abscess (Leder and Barlam 2000), destructive knee-joint infection (Riesbeck and Sanzen 1999), other osteoarticular infections (Bourgault et al. 1980; Fitzgerald et al. 1982; Murdoch et al. 1994; Felten et al. 1998), spondylitis (Rousseau and Harle 1998), ovarian abscess (Mikamo et al. 1998) and meningitis (Korman et al. 1997). Prosthetic joints provide potential foci for low-grade infections, which can easily be overlooked (Fitzgerald et al. 1982; Davies et al. 1988).

M. micros may be involved in the etiology of acute dentoalveolar abscess and in adult periodontitis (Riggio et al. 2001).

The species of greatest clinical importance appear to be *P. anaerobius*, *Pe. asaccharolyticus*, *M. micros*, and, in particular, *F. magna*. These species constituted 77 percent of all GPAC isolates in two recent series (Murdoch et al. 1994; Wren 1996). The anaerobic coccus most frequently isolated in pure culture is *F. magna*, (Bourgault et al. 1980; Murdoch et al. 1994). Previous series reported *An. prevotii* as one of the commonest pathogens but these organisms are likely to have been heterogeneous.

The anaerobic gram-positive cocci are important pathogens in infections in pediatric patients, particularly in brain abscess, acute otitis media, chronic sinusitis, aspiration pneumonia, lung abscess, and osteomyelitis. In many of these infections GPAC are the most common anaerobes present (Brook 2002).

PATHOGENICITY AND VIRULENCE FACTORS

The pathogenicity of the GPAC has been little studied, with clinical observations suggesting that they are most often expressed via a synergistic interaction with other

anaerobes, facultative or obligate. However, as most infections are mixed, it is difficult to evaluate the relative importance of different organisms. Synergy of GPAC with facultative and anaerobic organisms has been demonstrated in an animal model, with increased mortality, increased ability to induce abscesses, and enhanced growth of the bacterial components (Brook 1988b). The ability to produce capsules is an important virulence mechanism in this model (Brook 1986). Few other virulence factors have been described. Tam and Chan (1983) isolated hyaluronidase-producing peptostreptococci (not fully identified) in higher numbers from diseased periodontium than from healthy periodontium. Murdoch et al. (1988b) noted the strong proteolytic activity of *M. micros* and suggested that it might contribute to the formation of deep-organ abscesses. Krepel et al. (1991, 1992) studied the production by *F. magna* of proteolytic enzymes, particularly collagenase and gelatinase, and demonstrated that strains from nonpuerperal breast abscesses and diabetic foot infections had significantly greater enzymic activity than those from intra-abdominal sepsis. They proposed that these proteolytic enzymes could be important virulence factors in the causation of soft-tissue infections by *F. magna*.

Elastase activity of strains of *F. magna* and *M. micros* recovered from the amniotic fluid of patients with preterm premature rupture of membranes has been reported (Mikamo et al. 1999).

Protein L is a multidomain cell-surface protein formed by some strains of *F. magna* that can bind to the κ-light chain variable domain of human immunoglobulin (Ig) molecules (Nilson et al. 1992); its expression has been linked to clinical infection (Kastern et al. 1990). The production of an albumin-binding protein (PAB) related to protein L and the protein G produced by some hemolytic streptococci has been reported in some strains, which are identifiable by both ribotyping and pulse-field gel electrophoresis (Ng et al. 1996).

Deoxyribonuclease, RNAase, coagulase, and hemolysins have been reported in some strains of GPAC (Marshall and Kaufman 1981). Adherence mechanisms may be important for certain anaerobic cocci, particularly strains of *M. micros* (Kremer et al. 1997, 1999).

Commensal symbiosis in batch and continuous culture between *P. anaerobius* and *Prevotella bivia* may hold potential significance to the pathogenesis of bacterial vaginosis (Pybus and Onderdonk 1998). Of similar interest is the finding that four of seven strains of *F. magna* recovered from the vaginal flora of women with bacterial vaginosis expressed protein L (Kastern et al. 1990).

TYPING METHODS

There have been several attempts to devise a serologically based classification of GPAC but they have had little success (Porschen and Spaulding 1974; Wong et al. 1980; Collins et al. 1989). Several antigens are shared between strains of *P. anaerobius* (Smith et al. 1986b; Collins et al. 1989). Bacteriophage and bacteriocin typing schemes have not been developed. Neither nucleic-acid-based nor molecular typing methods have yet been devised for GPAC.

SUSCEPTIBILITY TO ANTIBIOTICS

The anaerobic gram-positive cocci are usually susceptible to a wide range of antibiotics commonly used for the treatment of anaerobic infections (Garcia-Rodriguez et al. 1995). This predictablility has led some authorities (Finegold 1988) to suggest that routine susceptibility testing is required only in limited, well-defined circumstances. Guidelines for quantitative susceptibility testing have been published (King and Phillips 1988; National Committee for Clinical Laboratory Standards 1990). Almost all strains tested have been susceptible to vancomycin, rifampicin, chloramphenicol, broad-spectrum penicillins, cefotaxime and cefoxitin, imipenem, and combinations of β-lactam with β-lactamase inhibitor (Watt et al. 1979; Edson et al. 1982; Watt and Brown 1986; Wexler and Finegold 1988). Most studies have found that strictly anaerobic cocci are highly susceptible to metronidazole (Watt et al. 1979; Panichi et al. 1990; Wexler et al. 1993). Edson et al. (1982) and Sanchez et al. (1992) reported resistance rates of 10–15 percent but did not give the species identity of their strains and it is likely that some may have been microaerophilic streptococci. Wren (1996) found no resistance to metronidazole or chloramphenicol in 169 strains of anaerobic cocci.

Penicillin G is usually considered to be highly effective but Panichi et al. (1990) reported a resistance rate of 10 percent. Twenty-four percent of the strains tested by Greenwood and Palfreyman (1987) showed penicillin minimum inhibitory concentrations (MICs) of greater than 1 mg/l but did not exhibit β-lactamase activity. *F. magna* and *M. micros* had penicillin resistance rates of 16 and 8 percent, respectively, in the study reported by Wren (1996). Four strains of *P. anaerobius* studied by Reig and Baquero (1994) had a modal MIC to amoxicillin of 8 mg/l; MIC was unaffected by the addition of β-lactamase inhibitors. These observations suggest that β-lactam resistance may be mediated via modified penicillin-binding proteins. The literature concerning the production of β-lactamases is confusing (Aldridge et al. 1983; Sturm 1989; Murdoch 1998) but it would appear that their production in GPAC is rare. Sensitivity of GPAC to the cephalosporins is dependent upon the class of drug (Panichi et al. 1990). The carbapenems are fully active (Wexler et al. 1993).

Ciprofloxacin has borderline activity (Watt and Brown 1986) but recently developed quinolones are more active, for example gatifloxacin and gemifloxacin (Wexler et al. 1993; Goldstein et al. 1999a, b).

Susceptibility to tetracycline, erythromycin, and clindamycin appears to be variable (Edson et al. 1982; Reig et al. 1992; Sanchez et al. 1992). Importantly clindamycin resistance rates may be as high as 11–12 percent (Wren 1996; Finegold et al. 2002). In one study clindamycin resistance was reported to be as high as 16 percent (Hecht et al. 1999). Selection of clindamycin-resistant strains following clindamycin therapy has been described (Ohm-Smith et al. 1986). Inducible macrolide-lincosamide resistance has been demonstrated (Reig et al. 1992; Sanchez et al. 1992), though regrettably the species identities of the relevant strains were not reported. In the study by Reig et al. (2001), 21 of 26 anaerobic gram-positive cocci resistant to the macrolides possessed the *erm*TR gene encoding a methyltransferase enzyme. It was shown by these workers that resistance was transferrable from *F. magna* to *Streptococcus pyogenes*. There is the possibility that the circulation of this resistance determinant among the oropharyngeal flora may occur. Sanchez recommended testing erythromycin susceptibility so that inducible clindamycin resistance can be detected. Wexler et al. (1999) showed clarithromycin to be the most active of the macrolides against the anaerobic cocci.

Resistance to glycopeptides has not been reported. Teicoplanin is reported to be more active than vancomycin (Greenwood and Palfreyman 1987).

Recently published studies have shown high rates of sensitivity of GPAC to ertapenem and faropenem (Aldridge 2002; Wexler et al. 2002), daptomycin (Goldstein et al. 2003), quinopristin/dalfopristin (Ling et al. 2001), linezolid (Goldstein et al. 1999c), amoxicillin-clavulanate (Goldstein et al. 1999d), cefminox (Hoellman et al. 1998), a new semisynthetic glycylcycline, GAR-936, (Goldstein et al. 2000), and a new ketolide ABT-773 (Finegold et al. 2002).

As GPAC are usually isolated from polymicrobial infections (frequently with other anaerobes, aerobes, and microaerophilic streptococci) therapy should consist of a suitable antibiotic in combination with metronidazole; a β-lactam with a β-lactamase inhibitor is a reasonable alternative. More work would be valuable, particularly on the frequency of resistance to antimicrobial agents. Currently, reports of significant antibiotic resistance in GPAC to many agents are rare and as identification of most strains is now straightforward such isolates should be identified to the species level.

GENUS AND SPECIES DESCRIPTIONS

Genus *Anaerococcus*

Strictly anaerobic gram-positive non-motile cocci. Cells are found in pairs, tetrads, irregular clusters, or chains. Peptones and amino acids are metabolized. The major terminal volatile fatty acid is butyric acid. Most species weakly ferment carbohydrates. The diamino acid of the cell wall is L-lysine and the interpeptide bridge is D-glutamic acid (except in *An. octavius*, where it is D-aspartate). The G + C content of the DNA is 26–34 percent. The type species is *Anaerococcus prevotii*.

ANAEROCOCCUS HYDROGENALIS

An. hydrogenalis cells are 0.5–1.8 μm in diameter and occur in clumps, diplococci, and tetrads, sometimes in short chains. Colonies are usually 2–3 mm, gray-white, low convex, entire, and circular after incubation for 5 days, often with an unpleasant smell. They produce indole and most strains are positive for alkaline phosphatase (ALP) but not coagulase. Urease production is variable and they do not reduce nitrate. *An. hydrogenalis* is strongly saccharolytic but weakly or non-proteolytic. Some strains produce α-glucosidase. No arylamidases are produced. Butyric acid is the terminal volatile fatty acid (VFA) produced. *An. hydrogenalis* has been isolated from human feces, vaginal discharge (Ezaki et al. 1990), infected superficial sites (Murdoch et al. 1994), ulcers, and urine. Pathogenicity is unknown. The G + C content of DNA is 30–31 mol% and the type strain is DSM (Deutsche Sammlung von Mikroorganismen) 7454. *An. hydrogenalis* is synonymous with Hare group III (Thomas and Hare 1954; Murdoch and Magee 1995).

ANAEROCOCCUS LACTOLYTICUS

The type strain of *An. lactolyticus* is slow-growing, forming translucent colonies 1 mm in diameter after 5 days. Cells are 0.5–1.5 μm in diameter and occur in short chains or clumps. *An. lactolyticus* produces urease but not ALP or indole; it is strongly saccharolytic but weakly proteolytic (Li et al. 1992). Strains are positive for β-galactosidase and arginine arylamidase. Butyric acid is the terminal VFA produced. Two strains described by Li et al. (1992) were from vaginal discharges. The G + C content of DNA is 34 mol% and the type strain is DSM 7456.

ANAEROCOCCUS OCTAVIUS

An. octavius cells are 0.7–0.9 μm in diameter, occurring in clumps. Colonies after 5 days are 1–2 mm in diameter, yellow-white, glistening, circular, raised, and entire (Murdoch and Mitchelmore 1991). Indole, urease, and ALP are not produced. Strains of *An. octavius* are strongly saccharolytic but weakly proteolytic. No glycosidases are produced. Strains are positive for proline arylamidase and give a weak reaction for pyroglutamyl arylamidase. They produce large quantities of butyric acid and some isovaleric acid. The terminal VFA is *n*-caproic acid. Fifteen strains studied by Murdoch et al. (1994) came from nasal flora; their pathogenicity is unknown. The type strain is DSM 11663.

ANAEROCOCCUS PREVOTII

An. prevotii cells are 0.6–1.2 μm in diameter, usually occurring in clumps and tetrads. Colonies are matt grey with whiter centers, low convex, circular, entire, 2 mm in diameter after 5 days, but showing marked variation in size and shape. The type species *An. prevotii* produces urease but not indole or ALP. There is weak saccharolytic and proteolytic activity. The strain produces α-galactosidase, α-glucosidase, β-glucuronidase, and arginine, pyroglutamyl, and histidine arylamidases. Weak activity occurs for tyrosine arylamidase. Butyric acid is the terminal VFA. *An. prevotii* is a constituent of the normal flora but its pathogenic potential is unclear. The G + C content of DNA is 29–33 mol% and the type strain is NCTC (National Collection of Type Cultures) 11806 (= ATCC [American Type Culture Collection] 9321).

Clinical isolates of this species show considerable variation in identification tests (Murdoch 1998; Wilson et al. 2000).

Taxonomic note

The original description of this species (Foubert and Douglas 1948) was based on six strains, some saccharolytic and some asaccharolytic. Rogosa (1974) considered that '*P. prevotii*' was a 'composite description of two organisms,' one similar to '*Gaffkya anaerobia*' (now *An. tetradius*) and the other to indole-negative strains of *P. asaccharolyticus*; he recommended that the name should be rejected. The type strain is weakly saccharolytic and closely related to *An. tetradius* (Ezaki et al. 1983; Li et al. 1994). Until recently, identification manuals and clinical papers described most or all indole-negative, butyrate-producing strains of GPAC as *An. prevotti*. However, strictly defined isolates are infrequently isolated from human clinical material (Murdoch et al. 1994); strains previously reported as *An. prevotti* probably belong to a variety of species such as *An. vaginalis*.

ANAEROCOCCUS TETRADIUS

An. tetradius (formerly *P. tetradius*; 'Gaffkya anaerobia') cells are 0.5–1.8 μm in diameter, occurring in clumps and tetrads. Colonies after 5 days are usually 2 mm in diameter, matt gray with whiter centers, low convex, circular, and entire, often showing marked variation in size and shape. The type strain of *An. tetradius* produces urease but not indole or ALP; nitrate is not reduced. The species is strongly saccharolytic but proteolytic activity is very weak. α-Glucosidase and β-glucuronidase are produced, as are arginine and leucine arylamidase. Variable and weak reactions occur in many other tests, probably owing to the heterogeneity of the species. Butyric acid is the terminal VFA. *An. tetradius* is probably a constituent of the normal vaginal flora; it is rarely isolated from human clinical specimens but has been little studied. The G + C content of DNA is 30–32 mol% and the type strain is ATCC 35098 (= DSM 2951).

Considerable variation occurs in tests performed on clinical isolates of this species (Murdoch 1998; Wilson et al. 2000).

ANAEROCOCCUS VAGINALIS

An. vaginalis cells are 0.5–1.5 μm in diameter and usually occur in clumps or tetrads. Colonies are 2–3 mm, gray-white, low convex, and circular after incubation for 5 days, often with an unpleasant smell. *An. vaginalis* produces arginine dihydrolase (ADH) but not urease or indole. ALP production is variable. There is moderate saccharolytic and proteolytic activity. No glycosidases are produced but strains are positive for arginine, leucine, and histidine arylamidase. Butyric acid is the terminal VFA produced. *An. vaginalis* is part of the vaginal flora (Li et al. 1992) and a significant human pathogen, accounting for six percent of all isolates of GPAC in one survey (Murdoch et al. 1994). It has been cultured from pleural empyema and superficial and post-surgical wound infections, sometimes in pure culture. The G + C content of DNA is 28–30 mol% and the type strain is DSM 7457.

Genus *Finegoldia*

Obligately anaerobic non-motile gram-positive cocci. Cells occur in pairs, clusters, or short chains.

Carbohydrates are not fermented but peptones and amino acids are metabolized. The major terminal volatile fatty acid is acetic acid. The diamino acid of the cell wall is L-lysine and the interpeptide bridge is glycine.

The G + C content of the DNA is 32–34 percent. This genus contains only one species *F. magna*, the type species.

FINEGOLDIA MAGNA

F. magna cells are 0.8–1.6 μm in diameter and arranged in clumps. In direct smears of clinical material they resemble staphylococci. The colonial morphology is highly variable; they are entire, circular, low convex, and most commonly translucent but color varies from white to grayish or yellowish. The surface is glistening or matt. Colonies measure 2 mm diameter after 5 days but marked variation in size may occur. Occasional strains are β-hemolytic. *F. magna* does not produce urease or indole or reduce nitrate. Activity in ADH and ALP is variable. There is weak saccharolytic activity, which may be enhanced by the presence of tween 80 and marked proteolytic activity. Glycosidase enzymes are not produced. *F. magna* is positive for arginine, leucine, and pyroglutamyl arylamidase and gives a weak reaction for histidine arylamidase. The terminal VFA is acetic acid.

F. magna is part of the normal flora of the gastrointestinal and female reproductive tracts and probably of the skin. It is the commonest species of anaerobic cocci isolated from human pathological specimens (Bourgault et al. 1980; Wren et al. 1977; Wren 1996) and is frequently cultured from soft-tissue infections (Bourgault et al. 1980; Murdoch et al. 1994), septic joints, particularly in the presence of prosthetic material (Fitzgerald et al. 1982), diabetic foot infections (Sanderson 1977; Wheat et al. 1986), breast abscesses (Edmiston et al. 1990) superficial wound infections (Brook 1989b; Murdoch et al. 1994), and ulcers (Wren 1996). It is often isolated in pure culture and can be a primary pathogen (Cofsky and Seligman 1985; Phelps and Jacobs 1985; Panagou et al. 1991; Pouedras et al. 1992; Brown et al. 1994). Collagenase (Krepel et al. 1992), protein L (Nilson et al. 1992), and albumin-binding protein (Ng et al. 1996) may be virulence factors. The G + C content of DNA is 32–34 mol% and the type strain is NCTC 11804 (= ATCC 15794). *Peptococcus variabilis* is probably a synonym.

Genus *Gallicola*

Obligately anaerobic non-motile gram-positive cocci. Cells occur singly or in pairs. Carbohydrates are not fermented and indole is negative. The diamino acid of the peptidoglycan is ornithine and the interpeptide bridge is D-aspartic acid. The major terminal volatile fatty acid is acetic acid. The G + C content of the DNA is 27–34 percent. The type species is *G. barnsae*.

GALLICOLA BARNSAE

G. barnesae cells are 0.5–0.9 μm in diameter, usually occurring in pairs. Colonies of the type strain are white and up to 1 mm in diameter after incubation for 5 days. Growth is enhanced by bile salts. *G. barnesae* forms indole weakly but not urease, ALP, or ADH. Nitrate is not reduced. *G. barnesae* is asaccharolytic but utilizes purines and glycine as the only amino acid (Schiefer-Ullrich and Andreeson 1985). No glycosidases or arylamidases are produced. Acetic acid is the terminal volatile fatty acid. *G. barnesae* has been isolated from chicken feces. The type strain is DSM 3244.

Genus *Micromonas*

Obligately anaerobic non-motile gram-positive cocci. Cells occur in pairs, chains, and clusters.

Carbohydrates are not fermented but peptones and amino acids are metabolized. The major terminal volatile fatty acid is acetic acid. The diamino acid of the cell wall is L-lysine and the interpeptide bridge is D-aspartate. The G + C content of the DNA is 28–30 percent. This genus contains only one species, *M. micros*, which is the type species.

MICROMONAS MICROS

M. micros cells are 0.3–0.7 μm in diameter and arranged in chains and clumps. Colonies grow slowly and are usually distinctive, being glistening, white (sometimes gray), domed, entire, circular, 1 mm in diameter after incubation for 5 days, and often surrounded by a yellow-brown halo of discoloration in the medium about 2 mm wide (Murdoch and Mitchelmore 1991). A rough morphotype possessing fibrillar structures form dry, white, hemolytic colonies (Van Dalen et al. 1993). *M. micros* produces ALP but not indole, urease, or ADH. It is asaccharolytic but very strongly proteolytic; it can be distinguished from *F. magna* by production of proline, phenylalanine, and glutamylglutamyl aminopeptidases. *M. micros* also produces arginine, leucine, pyroglutamyl, histidine, and tyrosine arylamidase, though Ng et al. (1994) reported that some of these tests were not reliable for vaginal strains of *M. micros*. Glycosidases are not produced. *M. micros* produces acetic acid as the terminal VFA or, with some strains, no VFAs. It is a normal part of the mouth flora, associated with other anaerobes in periodontitis (Moore et al. 1982; Socransky et al. 1988; Rams et al. 1992), peritonsillar abscess (Jokipii et al. 1988; Murdoch et al. 1988b), and endodontic abscesses (Williams et al. 1983). It is mostly isolated from infections in close proximity to the respiratory tract but it is probably under-recorded; it can be cultured from a wide range of human polymicrobial abscesses, including the brain (Murdoch et al. 1988b). The type strain is NCTC 11808 (= ATCC 33270). *Peptococcus glycinophilus* is a synonym (Cato et al. 1983).

Genus *Peptoniphilus*

Strictly anaerobic non-motile gram-positive cocci. Cells may occur in pairs, short chains, tetrads, or small clusters. Peptone and amino acids are metabolized. Carbohydrates are not fermented. The major terminal volatile fatty acid is butyric acid. The cell-wall diamino acid is ornithine and the interpeptide bridge is D-glutamic acid. The G + C content of the DNA is 30–34 percent. The type species is *Peptoniphilus asaccharolyticus*.

PEPTONIPHILUS ASACCHAROLYTICUS

Pe. asaccharolyticus is a genetically heterogeneous species (Ezaki et al. 1983; Holdeman Moore et al. 1986; Murdoch and Magee 1995). Typical strains form cells 0.7–0.9 μm in diameter that vary little in size, occur in clumps, and retain Gram's stain poorly. Colonies after 5 days are 2–3 mm in diameter, glistening, lemon-yellow, low convex, circular, entire, and often with a musty smell. Between 80 and 90 percent of strains form indole but urease, coagulase, and ALP are not produced. The species is asaccharolytic and moderately proteolytic. Glycosidases are not produced. Arginine arylamidase is produced. Variable results occur in leucine and tyrosine

arylamidase. Weak activity occurs for histidine arylamidase. The terminal VFA is butyric acid. *Pe. asaccharolyticus* is a normal part of the human flora of the gastrointestinal tract; the species is commonly isolated from clinical specimens, sometimes in pure growth (Murdoch et al. 1994). The G + C content of DNA is 30–34 mol% and the type strain is NCTC 11461 (= ATCC 14963). On microscopy, atypical strains show marked variation in cell size and shape (cell diameter 0.5–1.5 μm), with oval and elliptical forms; they grow more slowly-forming colonies that are 1 mm in diameter, translucent, matt, flat, and entire after 5 days. Indole production is weak. Atypical strains can be isolated from human pathological specimens, occasionally in pure growth (Murdoch et al. 1994). The identification of the indole negative strains is currently problematic.

PEPTONIPHILUS HAREI

Murdoch and Mitchelmore (1991) distinguished two groups within *Pe. asaccharolyticus* based on phenotypic properties. Analysis of strains by pyrolysis mass spectrometry and analysis of 16S rRNA led to the proposal that the so-called type B strains of *Pe. asaccharolyticus* be reclassified as *Pe. harei*.

Cells of *Pe. harei* show considerable variation in size (0.5–1.5 μm) and appear in clusters. Growth is very slow, colony size being only 1 mm in diameter after 5 days of incubation. Colonies are flat and transluscent. They are asaccharolytic; glycosidase enzymes are not produced. Indole is variable and urease, ALP and ADH are negative. Arginine and histidine arylamidase are produced and weak activity is detected for tyrosine arylamidase. Some strains give a weak reaction for leucine arylamidase. The terminal volatile fatty acid is butyric acid. The type strain is DSM 10020.

PEPTONIPHILUS INDOLICUS

Pe. indolicus cells are 0.7–1.6 μm diameter and occur mainly in clumps, often retaining Gram's stain poorly. Colonies are usually 3–4 mm (though with considerable variation), raised, yellow-buff, circular, entire, and smooth after incubation for 5 days. They produce indole but not urease. Biochemical activity is similar to typical *Pe. asaccharolyticus* but *Pe. indolicus* is distinguished by the production of coagulase (Ezaki et al. 1983) and ALP (Murdoch and Mitchelmore 1991) and, in some test systems, reduction of nitrate to nitrite (Ezaki et al. 1983). *Pe. indolicus* is asaccharolytic but strongly proteolytic. Arginine, phenylalanine, leucine, and histidine arylamidase are produced by all strains but tests for tyrosine arylamidase show weak activity. Butyric acid is the terminal volatile fatty acid. *Pe. indolicus* is a species of veterinary importance, causing bovine summer mastitis (Madsen et al. 1992), but it is rarely isolated from human clinical specimens. The type strain is NCTC 11088 (= ATCC 29427).

PEPTONIPHILUS IVORII

Pe. ivorii cells are 0.4–1.5 μm in diameter, occurring in clumps. Colonies grow slowly; they are 1–2 mm in diameter, whitish, low convex, entire, and circular after 5 days. The organism does not produce indole, urease, ADH, or ALP. The four strains tested by Murdoch and Mitchelmore (1991) are asaccharolytic and weakly proteolytic. No glycosidases are produced. Proline arylamidase is produced. The terminal volatile fatty acid produced is isovaleric acid but large quantities of butyric acid are also formed. Some strains are inhibited by sodium polyanethol sulphonate. *Pe. ivorii* has been isolated from the normal flora of humans (Murdoch et al. 1994); its pathogenicity is unknown. The type strain is DSM 10022.

PEPTONIPHILUS LACRIMALIS

Cells of the type strain *Pe. lacrimalis* are 0.5–0.7 μm in diameter and occur in short chains or clumps. After 5 days colonies are pink-white and 1–2 mm in diameter, but with considerable size variation. They are asaccharolytic and inactive in classic biochemical tests, using peptones and oligopeptides as energy source (Li et al. 1992). Glycosidase enzymes are not produced. Arginine, phenylalanine, leucine, and histidine arylamidase are produced. Variable reactions occur for tyrosine arylamidase. Butyric acid is the terminal volatile fatty acid. Both strains described by Li et al. (1992) were isolated from human eyes. The type strain is DSM 7455.

Genus *Peptococcus*

Obligately anaerobic non-motile gram-positive cocci. Cells occur singly or in pairs and clusters. Peptones and amino acids are metabolized but carbohydrates are not fermented. The major terminal VFAs are butyric, caproic, and isovaleric acids. The cell-wall diamino acid is L-lysine and the interpeptide bridge is D-aspartate. The G + C content of the DNA is 50–51 percent. The genus now contains only the type species, *Pc. niger*.

PEPTOCOCCUS NIGER

Pc. niger cells are 0.3–1.3 μm in diameter, occurring in singles, pairs, and clumps. The type strain grows slowly to form raised, circular, entire colonies 1 mm in diameter after incubation for 5 days. Black pigmented colonies may be seen on initial isolation but become gray on exposure to air. Pigment is often absent after several laboratory transfers (Wilkins et al. 1975). The type strain forms catalase but is inactive in other tests including glycosidases and arylamidases (Ezaki and Yabuuchi 1985; Murdoch 1998). The distinctive VFA profile includes butyric, isovaleric, and terminal

n-caproic acids. The neotype and other strains were isolated from human umbilical flora by Wilkins et al. (1975). Very rarely the organism is isolated from pathological specimens (Ezaki et al. 1992; Murdoch et al. 1994). The G + C content of DNA is 51 mol%. Although it is the type species of the family Peptococcaceae, *Pc. niger* is related only distantly to other members (Ezaki et al. 1994). The type strain is NCTC 11805 (+ ATCC 27731).

Genus *Peptostreptococcus*

Obligately anaerobic nonmotile gram-positive cocci. Cells occur in pairs, chains, and clusters. Carbohydrates are weakly fermented. Peptones and amino acids are utilised. The terminal VFA is isocaproic acid in the majority of strains. In a few strains the terminal acid is isovaleric acid. The diamino acid in the cell wall is L-lysine and the interpeptide bridge is D-aspartate. The G + C content of the DNA is 34–36 percent. The genus now contains only one species, the type species *Pst. anaerobius*.

PEPTOSTREPTOCOCCUS ANAEROBIUS

Pst. anaerobius cells are 0.5–0.9 μm in diameter, often coccobacillary, and with a variety of pleomorphic forms; cells usually occur in pairs or chains. *Pst. anaerobius* is the most rapidly growing species in the genus. Colonies reach 1 mm in diameter after incubation for 24 h; after 5 days they reach 2−4 mm, and are gray, flat, entire, and with slightly raised whitish centers and a distinctive, sickly sweet 'plasticine-like' smell. Growth is inhibited by polyanethol sulfonate. This species does not produce indole, urease, ADH, or ALP. It is a weak fermenter of carbohydrates. The organism produces α-glucosidase and proline arylamidase. Various volatile fatty acids are formed, usually terminating in isocaproic acid, but 5–10 percent of strains produce a terminal isovaleric peak. *Pst. anaerobius* is a normal part of the human flora of the genitourinary and gastrointestinal tracts, commonly isolated from human clinical specimens, particularly from abdominal sites, but rarely in pure culture. The G + C content of DNA is 33–34 mol% and the type strain is NCTC 11460 (= ATCC 27337). Recent taxonomic studies suggest a closer relationship to some clostridia than to other genera of anaerobic gram-positive cocci.

Further detailed information on all of the above species can be obtained from the excellent review by Murdoch (1998).

Genus *Ruminococcus*

Analysis of 16S rRNA sequences shows that *Ruminococcus* spp. are genetically heterogeneous. They are gram-positive coccbacilli (though often retaining stain poorly) with ends that can be rounded, flat, or pointed. They occur in pairs and chains. They are obligate anaerobes and do not form spores; they require carbohydrates, which are fermented to form various proportions of acetate, formate, succinate, lactate, ethanol, carbon dioxide, and hydrogen. Amino acids and peptides are not fermented; most strains require ammonia as their nitrogen source. They have the ability to digest cellulose. They do not grow well on ordinary laboratory culture media. The cell-wall diamino acid is m-DAP with no interpeptide bridge amino acid. The G + C content of DNA is 39–46 mol% and the type species is *Ruminococcus flavefaciens*.

Most strains have been isolated from the gastrointestinal tract of animals, including humans, and appear to be of low pathogenicity (Bryant 1986); however, the culture methods used in diagnostic laboratories are unlikely to lead to their isolation (Ezaki et al. 1992). Bryant (1986) listed eight species; Ezaki et al. (1994) have since proposed that *P. productus* and *S. hansenii* should be transferred to the genus *Ruminococcus*. Collins et al. (1994) and Rainey and Janssen (1995) disagree; Collins et al. (1994) place *R. productus* as a major part of the human anaerobic flora and can be readily identified using preformed enzyme kits (Murdoch and Mitchelmore 1991).

RUMINOCOCCUS PRODUCTUS

R. productus cells are elliptical, (0.6–0.9) × (0.8–2.0) μm; they occur in pairs or short chains and will grow on enriched blood agar to form circular, convex, glistening gray colonies, 1–3 mm in diameter, after 2 days. They are strongly saccharolytic, forming acid from many carbohydrates, but have no proteolytic activity. Nitrate reduction, urease, ALP, and indole production are all negative. Aesculin is weakly fermented. α-Galactosidase and α-glucosidase are produced, but not arylamidases. The terminal VFA produced is acetic acid. The organism has rarely been isolated form human clinical specimens. The type strain is NCTC 11829 (=ATCC 27340).

Genus *Slackia*

Obligately anaerobic nonmotile gram-positive cocci. Cells occur as cocci, coccobacilli, or short bacilli. They are bile-sensitive, hydrolyse arginine, are catalase-, urease-, and indole-negative and do not hydrolyse aesculin. Growth is stimulated by 0.5 percent arginine.- Carbohydrates are not fermented. The G + C content of the DNA is 60–64 percent. The type species is *S. exigua*.

SLACKIA HELIOTRINREDUCENS

S. heliotrinreducens cells are 0.3–0.6 μm in diameter and occur in pairs, chains, and clumps, often with elongated pleomorphic forms. Cells are easily decolorized. They are very slowly growing, forming translucent, glistening,

low convex, entire, circular colonies 1 mm in diameter after incubation for 5 days. *S. heliotrinreducens* strains produce ADH but not ALP, thus distinguishing them from *M. micros*; they do not produce urease. Nitrate may be reduced. *S. heliotrinreducens* strains are asaccharolytic but strongly proteolytic (Murdoch and Mitchelmore 1991). Glycosidases are not produced. Positive tests occur for proline, phenylalanine, and leucine arylamidase. Weak tyrosine and histidine activity occur. The terminal VFA is acetic acid. The type strain was isolated from sheep rumen (Lanigan 1976); similar isolates cultured from human polymicrobial anaerobic abscesses (Murdoch and Mitchelmore 1989) may belong to a different species. Two of these isolates were shown to be different from the type species (Goodacre et al. 1996). The type strain is NCTC 11029 (= ATCC 29202).

Species awaiting description

There are some species that still await description, particularly in the butyrate group. The following have been well characterized (Murdoch 1998).

'PEPTOSTREPTOCOCCUS TRISIMILIS'

'*P. trisimilis*' is similar to *An. hydrogenalis* (Hare group III; Murdoch and Magee 1995); cells are 0.8–1.5 μm in diameter, occurring in clumps and tetrads. Colonies are gray with whiter centres, entire, circular, and 1–2 mm in diameter after 5 days. '*P. trisimilis*' produces indole but not urease. ALP activity is variable. The organism is strongly saccharolytic. Some strains produce β-galactosidase, and all are pyroglutamyl arylamidase positive. '*P. trisimilis*' has been isolated from blood, infected mastoid, and sternotomy wounds.

THE 'β-GAL' GROUP

Murdoch and Mitchelmore (1991) described a loose group of 11 strains with characteristic arylamidase patterns. These strains were strongly saccharolytic and most produced β-galactosidase and ALP but not indole or urease. Arginine and leucine arylamidase were produced, together with weak pyroglutamyl activity. The cells of these organisms appear in clusters and colonies were 1–2 mm in diameter after 5 days of incubation. The terminal VFA is butyric acid. Strains isolated in the study by Murdoch et al. (1994) were mostly recovered from abscesses of the breast and buttock.

Other genera

COPROCOCCUS

This genus was proposed by Holdeman Moore and Moore (1974) to include gram-positive obligately anaerobic cocci for which fermentable carbohydrates are required or are stimulatory to growth and that form butyric acid. They do not form spores and are differentiated form *Ruminococcus* by production of butyric acid. However, Ezaki et al. (1994) suggested that coprococci and ruminococci might be better classified together. Coprococci are unlikely to grow on routine media; methods for their isolation have been described (Holdeman et al. 1977; Ezaki et al. 1992). There are three species, all described from the human gastrointestinal tract; they have not yet been reported from clinical specimens (Holdeman Moore and Moore 1986). The G + C content of DNA is 39–42 mol%.

SARCINA

This is a little-studied genus of gram-positive obligately anaerobic cocci characteristically arranged in packets of eight. The organisms can form endospores, and 16S rRNA sequence data indicate that the *Sarcina ventriculi* is closely related to *Clostridium perfringens* and *P. anaerobius* (Collins et al. 1994; Ezaki et al. 1994). *S. ventriculi* is placed by Collins et al. (1994) in *Clostridium* cluster I. The species require fermentable carbohydrate, will grow at pH range 1.0–9.8, and can be selected out using bifidobacterium medium (Ezaki et al. 1992). Two species have been described, both isolated from the human gastrointestinal tract and from soil. *S. ventriculi* has been cultured from human vomitus (Ezaki et al. 1992) and in the feces of patients with gastrointestinal pathology. The G + C content of DNA is 28–31 mol%.

ATOPOBIUM

The genus *Atopobium* was proposed by Collins and Wallbanks (1992) on the basis of 16S rRNA sequence data. The organisms are gram-positive obligately anaerobic cocci or rods occurring in pairs or short chains. Cells may be elongate but they do not form spores. The major metabolic product is lactic acid. *Atopobium parvulum*, formerly in the genus *Peptostreptococcus*, later *Streptococcus* (Cato 1983), is weakly saccharolytic and proteolytic. The type strain will grow slowly on commercial media to form tiny translucent colonies after 5 days but its growth can be stimulated by Tween 80. *Atopobium parvulum* has been recovered from human clinical material taken from infections of the abdomen, abscesses, and from high vaginal swabs (Wren et al. 1977). Its principal habitat is unknown. The G + C content of DNA is 35–46 mol% and the type strain is ATCC 33793.

Three other species of *Atopobium* (*A. vaginae*, *A. minutum*, and *A. rimae*) have been described and may be differentiated from each other and *A. parvulum* using glycosidase and arylamidase reactions (Rodriguez Jovita et al. 1999). They are rarely recovered from human material but *A. rimae* and *A. parvulum* have been reported in dental abscesses and *A. minutum* from abdominal wounds and pelvic abscess (Rodriguez Jovita et al. 1999).

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Bacillus anthracis, *Bacillus cereus*, and other aerobic endospore-forming bacteria

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DEFINITION

Although the production of resistant endospores in the presence of oxygen remains the defining feature for *Bacillus* and the new genera derived from it, the definition was undermined by the discoveries of *Bacillus infernus* and *B. arseniciselenatis*, which are strictly anaerobic (Boone et al. 1995; Switzer Blum et al. 1998), and spores have not been observed in *B. halodenitrificans*, *B. infernus*, *B. selenitireducens*, *B. subterraneus*, and *B. thermoamylovorans*.

Nonetheless, those species of *Bacillus* and related genera likely to be isolated in a clinical laboratory are rod-shaped, endospore-forming organisms which may be aerobic or facultatively anaerobic. They are usually gram-positive (in young cultures), but sometimes gram-variable or frankly gram-negative. They are mostly catalase-positive, and may be motile by means of peritrichous flagella. Most species are mesophilic, but *Bacillus* contains some thermophiles and psychrophiles, *Aneurinibacillus* and *Brevibacillus* both contain thermophilic species, and *Paenibacillus* contains one psychrophilic species.

TAXONOMY

Bacillus has been divided into more manageable and better-defined groups on the basis of 16S rRNA gene sequencing studies. So far, ten new genera have been proposed: *Alicyclobacillus* (Wisotzkey et al. 1992), which contains six species of thermoacidophiles; *Paenibacillus* (Ash et al. 1993), containing 49 species and including organisms formerly called *B. polymyxa*, *B. macerans*, *B. alvei*, and the honey bee pathogens *B. larvae* and *B. pulvifaciens* (now both subspecies of *P. larvae*); *Brevibacillus* (Shida et al. 1996), containing 12 species and including organisms formerly called *B. brevis* and *B. laterosporus*; *Aneurinibacillus* (Shida et al. 1996), with *A. aneurinilyticus* and three other species; *Virgibacillus* (Heyndrickx et al. 1998) with *V. pantothenticus* and four other species; *Gracilibacillus* and *Salibacillus* (Wainø et al. 1999), which each contain two species of halophiles; *Geobacillus* (Nazina et al. 2001) with ten species of thermophiles including *Bacillus stearothermophilus*; *Ureibacillus* (Fortina et al. 2001) with two round-spored, thermophilic species, and single-membered *Marinibacillus* (Yoon et al. 2001b). *Salibacillus* has subsequently been merged with *Virgibacillus* (Heyrman et al. 2003). *Sporosarcina* contains the motile, sporeforming coccus *S. ureae*, which is closely related to *B. sphaericus*, and five rod-shaped, round-spored species, including *Bacillus pasteurii*, that have now been placed in this genus (Yoon et al. 2001a). Other genera of aerobic endospore formers, not derived from *Bacillus*, are: *Sulfobacillus*,

Amphibacillus, *Halobacillus*, *Ammoniphilus*, *Thermobacillus*, *Filobacillus*, *Jeotgalibacillus*, and *Lentibacillus* (Golovacheva and Karavaiko 1978; Niimura et al. 1990; Spring et al. 1996; Zaitsev et al. 1998; Touzel et al. 2000; Schlesner et al. 2001; Yoon et al. 2001b, 2002). *Alicyclobacillus*, *Ammoniphilus*, *Amphibacillus*, *Filobacillus*, *Gracilibacillus*, *Halobacillus*, *Jeotgalibacillus* , *Lentibacillus*, *Sulfobacillus*, *Thermobacillus*, and *Ureibacillus* strains are unlikely to be encountered in a clinical laboratory, and clinical isolates of *Geobacillus* and *Sporosarcina* have not been reported, so these genera will not be considered further.

Bacillus continues to accommodate the best-known species such as *B. subtilis* (the type species), *B. anthracis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, and *B. thuringiensis*. It still remains a large genus, with over 90 species, as losses of species to other genera have been balanced by proposals for new *Bacillus* species (four species await transfer to *Geobacillus* and *Paenibacillus*). The pathogenic members of the *B. cereus* group, *B. anthracis*, *B. cereus*, *B. thuringiensis*, are really pathovars of a single species (Turnbull et al. 2002).

Unfortunately, taxonomic progress has not revealed readily determinable features characteristic of each genus. They show wide ranges of sporangial morphologies and phenotypic test patterns. Many recently described species represent genomic groups disclosed by DNA–DNA pairing experiments, and routine phenotypic characters for distinguishing some of them are very few and of unproven value. New species and subspecies of aerobic endospore formers are regularly described (47 in the three years January 2000–January 2003, during which period no proposals for merging species had been made), but often on the basis of very few strains.

IMPORTANCE OF THE AEROBIC ENDOSPORE-FORMERS

Most species of aerobic endospore-forming bacteria appear to have little or no pathogenic potential and are rarely associated with disease in humans and other animals. The main exceptions to this are *B. anthracis* (anthrax), *B. cereus* (food poisoning and opportunistic infections), *B. thuringiensis*, *P. larvae* and its subspecies, *P. lentimorbus* and *P. popilliae* (all pathogenic to invertebrates), but a number of other species, particularly *B. licheniformis*, have been implicated in food poisoning and other human and animal infections. The resistance of the spores to heat, radiation, disinfectants, and desiccation also results in *Bacillus* species being troublesome contaminants in the operating room, on surgical dressings, in pharmaceutical products, and in foods.

Aerobic endospore-formers have been, and continue to be, important in many fields of basic research, and long-term research into the sporulation process in *B. subtilis* has led to its being probably the best understood developmental system. The genomes of four *Bacillus* species, *B. subtilis*, *B. halodurans*, *B. anthracis*, and *B. cereus*, have been sequenced (Kunst et al. 1997; Takami et al. 2000; Read et al. 2003; Ivanova et al. 2003). The restriction endonucleases and DNA polymerases of several *Bacillus* species are of considerable importance as research tools for better understanding of disease and improved diagnosis. *Bacillus* species lend themselves well to host-vector systems for production of bioengineered therapeutic products.

Aside from the pathogenicity of some species, several aerobic endosporeformers are of clinical or health importance in very positive ways: in the production of antibiotics (bacitracin from *B. licheniformis* or *B. subtilis*, gramicidin from *Brevibacillus brevis*, and polymyxin from *Paenibacillus polymyxa*) or vitamins (e.g. vitamins B_{12} and B_2 from *B. megaterium*, biotin and riboflavin from *B. subtilis*); as the bases of antibiotic assays (*B. cereus*, *B. circulans*, *B. megaterium*, *B. pumilus*, *B. subtilis*, and *G. stearothermophilus*); in the validation of disinfectants (*B. cereus*), or the monitoring of fumigation (*B. subtilis*), heat sterilization (*G. stearothermophilus*) or radiation (*B. pumilus*) processes; and in various clinical tests (such as a uric acid assay using *B. fastidiosus*, a *Chlamydia* detection assay employing a variant of subtilisin from *B. subtilis*, or a blood screening test for phenylketonuria utilizing *B. subtilis*). A number of *Bacillus* species are the active ingredients of probiotics for animals and humans.

Bacillus species are also of considerable commercial importance in the production of industrial enzymes. The largest consumer is the detergent industry, accounting for one third of the global market in such products, and the proteases and amylases it uses are all of *Bacillus* origin. *Bacillus* amylases, proteases, and glucanases are also widely used by the food and potable alcohol industries (Outtrup and Jørgensen 2002). *Bacillus thuringiensis* is preeminent as a biopesticide, even though the global market for *Bacillus* insecticides is quite small, representing less than 2 percent of the global insecticide market (Bishop 2002). The genes for the δ-endotoxins of *B. thuringiensis* have been in the forefront of the development of transgenic crop plants (Van Rie 2002). Strains of several species show promise as agents to control fungal diseases of plants (Chanway 2002).

Sporeformers are also important deleterious agents and contaminants in the food industry. The very enzymes that are of commercial interest can also be responsible for spoilage of several food products. *B. coagulans*, *B. licheniformis*, *B. subtilis*, *G. stearothermophilus*, and *P. macerans* have all been implicated in flat sour spoilage of evaporated milk, in which fermentation occurs without gas production. *P. macerans* and *P. polymyxa*, on the other hand, produce both acid and gas, which can lead to canned food swells and cheese defects. *B. cereus* causes problems in the dairy industry – it is a regular contaminant of milk, and it causes 'bitty cream defect' and 'sweet

curdling'. *B. subtilis* causes 'rope' in bread, which appears as slimy stringiness when the loaf is cut.

HABITATS

Most aerobic endospore-formers are saprophytes widely distributed in the natural environment, but some species are opportunistic or obligate pathogens of animals, including humans, other mammals, and insects. The main habitats are soils of all kinds, ranging from acid to alkaline, hot to cold, and fertile to desert, and the water columns and bottom deposits of fresh and marine waters. Their spores readily survive distribution in soils, dusts, and aerosols from these natural environments to a wide variety of other habitats, and Nicholson (2002) has considered the roles of *Bacillus* spores in the natural environment. In studies of indoor and outdoor air and dusts, *Bacillus* species commonly dominate the cultivable flora or form a large part of it. Some species appear to be ubiquitous contaminants of man, other animals, their foodstuffs, water, and environments: natural, domestic, industrial, and hospital. Dried foods such as spices, milk powders, and farinaceous products are often quite heavily contaminated with spores.

Endospores show much greater resistance to physical and chemical agents, such as heat, cold, desiccation, radiation, disinfectants, antibiotics, and other toxic agents, than do their counterpart vegetative cells, and they can survive for very long periods. They are typically more resistant to heat than vegetative cells by a factor of 10^5 or more, while resistance to UV and ionizing radiation may be 100-fold or more. If protected from radiation, spores may show remarkable longevities. Striking claims, which require independent confirmation, include the isolation of a viable strain of *B. sphaericus* from an extinct bee preserved in 25- to 40-million-year-old amber (Cano and Borucki 1995), and the recovery of an aerobic endosporeformer related to *Salibacillus* and *Virgibacillus* from a water droplet trapped in a Permian salt crystal for an estimated 250 million years (Vreeland et al. 2000). *B. infernus* was isolated from Triassic shales, lying at depths of around 2.7 km below the land surface, that may have been hydrologically isolated for 140 million years, and although they have not been demonstrated for this organism, endospores are likely to have contributed to its survival (Boone et al. 1995). Another organism from a deep subsurface environment is *B. subterraneus*, which was isolated from the Great Artesian Basin of Australia, a thermal aquifer up to 2 km deep (Kanso et al. 2002); spores have not been demonstrated for this species either.

Bacillus anthracis is, to all intents and purposes, an obligate pathogen of animals and humans. Some authorities consider that microcycling of the organism in soil is very rare, and that the highly conserved nature of the organism is consistent with it having infrequent multiplication phases in animals, but others believe that multiplication may occur in certain soils (Cherkasskiy 1999). Its close relative, *B. cereus*, is now well established as an opportunistic pathogen, and other aerobic endospore-formers can also, from time to time, be opportunistic pathogens. Six organisms are important as insect pathogens: *B. thuringiensis* (another close relative of *B. anthracis*), *B. sphaericus*, the two *P. larvae* subspecies, *P. lentimorbus* and *P. popilliae*. The commonly isolated species, such as *B. subtilis* and *B. cereus* are very widely distributed worldwide; *B. thuringiensis* has been isolated from all continents, including Antarctica (Forsyth and Logan 2000).

Most known species of *Bacillus* and related genera are heterotrophic organisms which have been isolated on complex organic media. Relatively few attempts have been made to isolate aerobic endosporeformers that can utilize inorganic sources of carbon and energy, or to demonstrate that established heterotrophic species are capable of facultative autotrophy. The two thermophiles *B. schlegelii* and *B. tusciae* remain the only species in *Bacillus* shown to be facultatively chemolithoautotrophic. Nitrogen fixation is well established for certain species in *Paenibacillus* (*P. azotofixans*, *P. macerans*, *P. polymyxa*), and less is known about *Bacillus* species utilizing atmospheric nitrogen. However, several studies have demonstrated nitrogen fixation by strains of *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. sphaericus*, and unidentified strains (some of which may have been *Paenibacillus* species) isolated from rhizospheres and phylloplanes, and from endophytic sites and mycorrhizae. Nitrogen-fixing *Bacillus* and *Paenibacillus* growing in the rhizosphere may help to promote plant growth; and aerobic endosporeformers may promote plant growth in other ways (Chanway 2002), including the production of phytohormones, increasing nutrient availability, interactions with symbiotic bacteria and fungi, enhancement of root nodulation, and biological control of plant pathogens by various mechanisms including the production of antibiotics.

LIFE CYCLE

Under suitable nutritional, temperature, pH, gaseous and other conditions, *Bacillus* cells will grow and divide by binary fission, with the dividing septum traversing the middle of the cell. Depending on species, strain, and cultural conditions, daughter cells may separate so that the culture appears to be composed of single cells and pairs of dividing cells when viewed by phase-contrast microscopy. In other cases, daughter cells may remain attached to each other, so that chains of cells are seen. Filaments may also be observed, and these can often be symptomatic of a stressed culture. An organism that exists predominantly as regular rods in optimal growth conditions may produce swollen, pleomorphic, unhealthy-looking cells when stressed.

Aerobic endospore-formers show a very wide range of colonial morphologies (Figure 1), both within and between species, and medium composition and other incubation conditions do of course have strong influences. Despite this diversity, however, colonies on routine media are not generally difficult to recognize, and some species have characteristic, but seemingly infinitely variable, colonial morphologies (see Diagnosis sections, below).

L-form *Bacillus* cells have been reported from humans, other animals, and plants. Several authors have found L-forms in the blood of normal and arthritic persons in association with erythrocytes (Bisset and Bartlett 1978), in other body fluids such as synovial fluids of arthritic patients (Pease 1969), in association with neoplasms (Livingston and Alexander-Jackson 1970), and in chickens and turkeys with infectious synovitis. These organisms may revert to small, acid-fast diphtheroids, and on prolonged culture or subculture in the presence of agents known to stimulate reversion of L-forms, some increase in size and become gram-positive endospore-forming rods. The fully reverted isolates of Bisset and Bartlett (1978) were phenotypically similar to *B. licheniformis* in other respects, and they named them '*B. licheniformis* var. *endoparasiticus*'. Symbiotic associations between L-form bacteria and plants have been

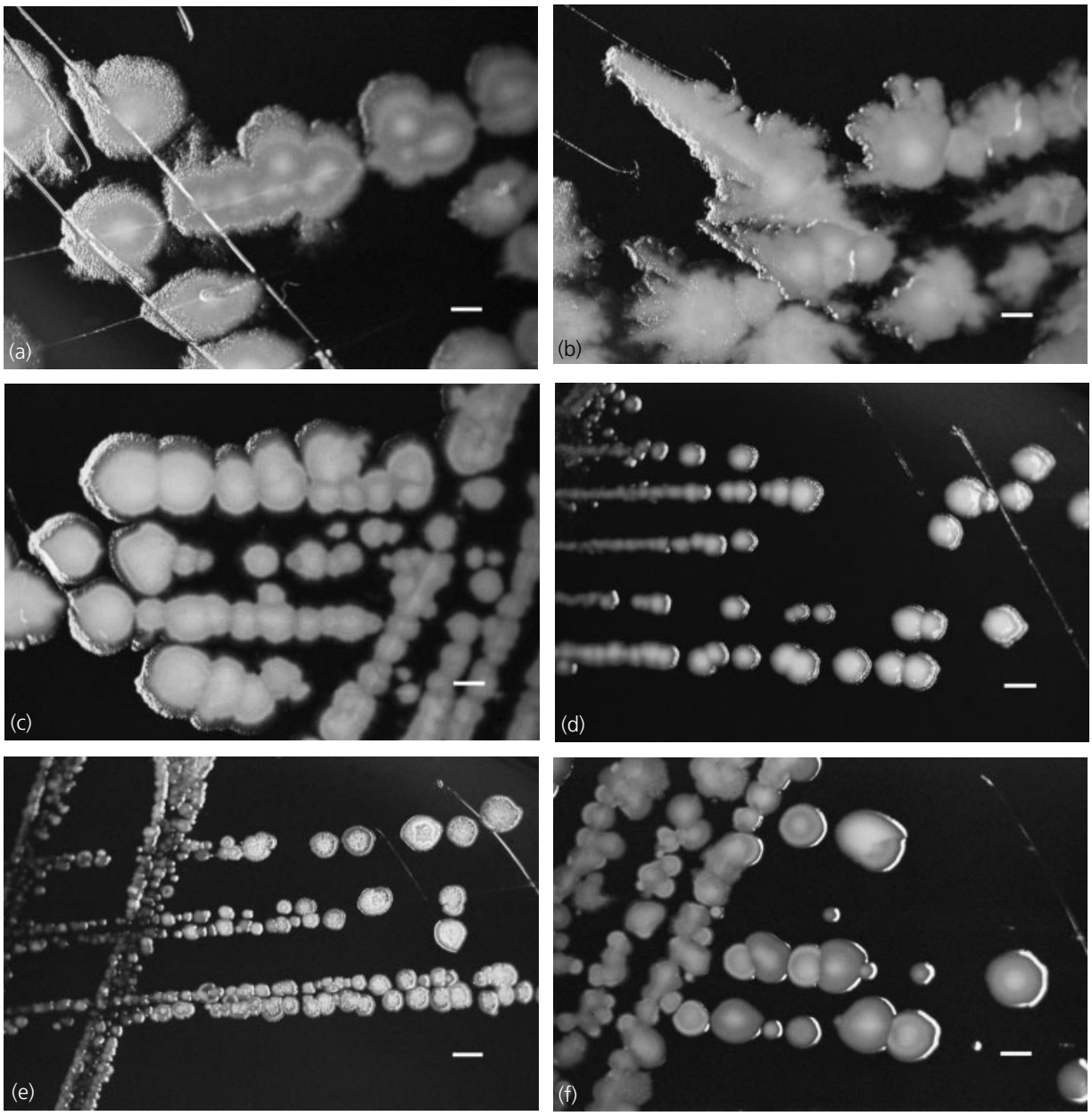

Figure 1 *Colonies of endospore-forming bacteria on blood agar* **(a–i)** *and nutrient agar* **(j–l)** *after 24–36 h at 37 C.°Bar markers represent 2 mm.* **(a)** B. anthracis, **(b)** B. cereus, **(c)** B. thuringiensis, **(d)** B. megaterium, **(e)** B. pumilus, **(f)** B. sphaericus. *(Continued over)*

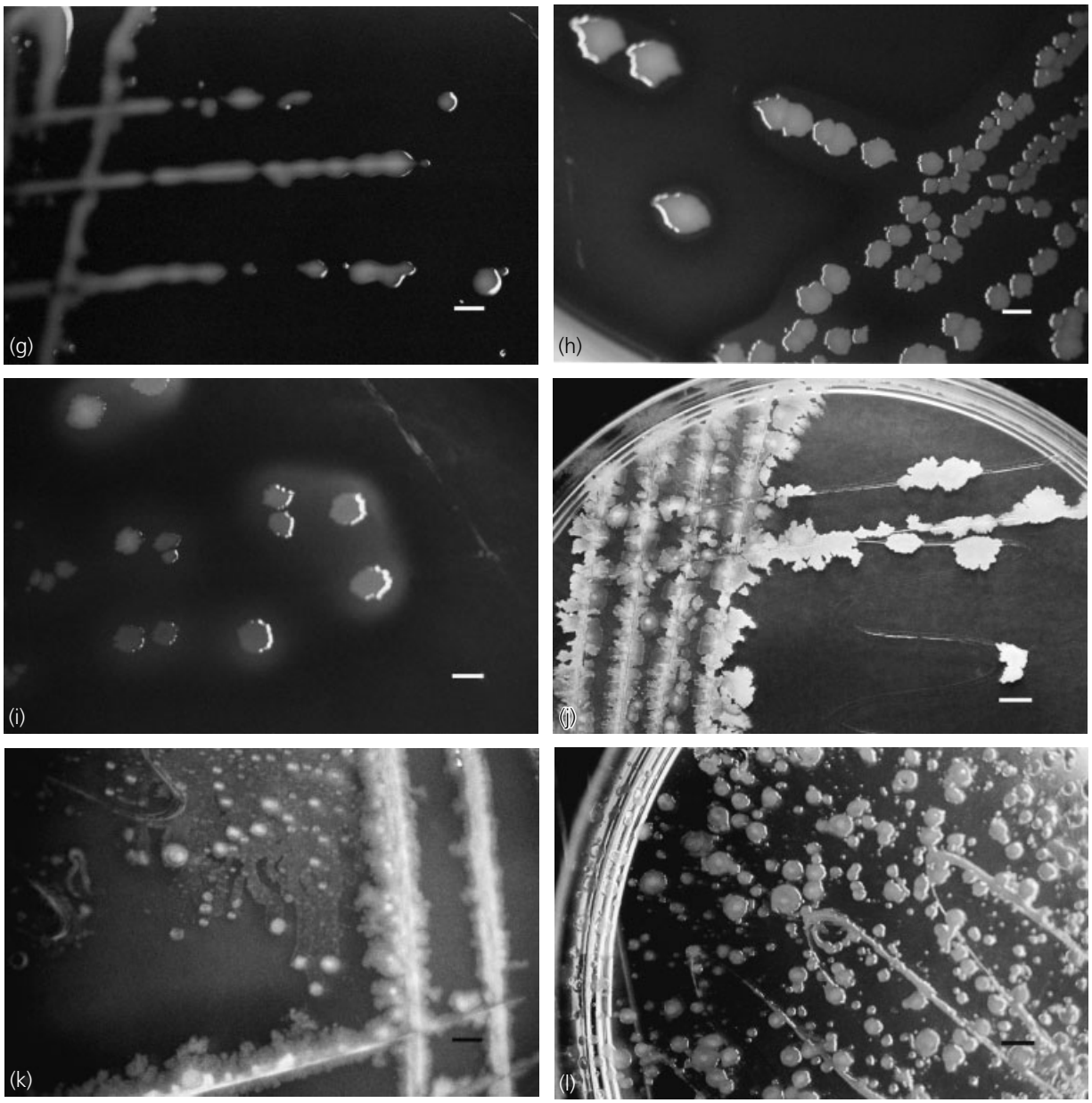

Figure 1 *Colonies of endospore-forming bacteria on blood agar* **(a–i)** *and nutrient agar* **(j–l)** *after 24–36 h at 37 C.°(Continued) Bar markers represent 2 mm.* **(g)** Brevibacillus brevis, **(h)** Brevibacillus laterosporus, **(i)** Paenibacillus polymyxa, **(j)** B. subtilis, **(k)** *spreading colonies of* Paenibacillus *sp.; see comments on* B. circulans *in Identification below.* **(l)** Paenibacillus alvei.

observed (Paton and Innes 1991), and a stable L-form of *B. subtilis* in Chinese cabbage seedlings has been shown to inhibit the germination of *Botrytis cinerea* conidia (Walker et al. 2002).

THE ENDOSPORE

It was independently discovered by Cohn (1876), Koch (1876), and Tyndall (1877) that some bacteria could spend part of their lives as the dormant cellular structures we now call endospores. The first two of these authors recognized the significance of these structures in the epidemiology of anthrax, and Koch's study of the life history of *B. anthracis* proved the germ theory of disease and so marked the birth of clinical bacteriology. The ability to form endospores in aerobic conditions has been a defining character of the genus *Bacillus* since the 1920s.

Endospores are so named because they are formed intracellularly, and they differ from their parent vegetative cells in many ways: they are optically refractile, and are highly resistant to chemical and physical stresses that are lethal to vegetative cells. These properties are conferred by the spores' special chemical composition and ultrastructure, and much effort has been expended over many years in order to elucidate the processes of

spore formation and germination, and the molecular mechanisms that make endospores the hardiest form of life known on Earth. Although endospores are to be found in other genera, it is the spores of *Bacillus subtilis* that have been the most intensively studied, especially those of strain 168, which was also the first *Bacillus* strain to have its genome sequenced (Kunst et al. 1997).

Endospores are formed at the end of exponential growth phase, and at least two kinds of environmental factors have been implicated in the induction of sporulation. It should be appreciated that spores will not be formed under all cultural conditions, and that cultures may die if conditions are not conducive to sporulation. Under phase-contrast microscopy, such cells can be seen to have lost their phase-darkness and become 'ghost cells'. Some strains may lose the ability to sporulate upon repeated subculture. One trigger for sporulation is nutritional deprivation, so that sporulation may be initiated by transferring an actively growing culture from a rich to a poor growth medium, but many environmental factors are known to affect endospore formation. These include: growth temperature, environmental pH, aeration, presence of certain minerals, and carbon, nitrogen and phosphorus sources and their concentrations. A second influence is population density, and sporulation is poor at low cell densities, even though the cells may be starved. As the mass of a culture increases, there is an extracellular accumulation of a secreted peptide (competence and sporulation factor (CSF)), which acts as an autoinducer for quorum sensing (Miller and Bassler 2001). When this peptide reaches a concentration that relates to a particular cell density, high intracellular levels of CSF lead to an increase of the phosphorylated form of a response regulator (SpoOA), which leads to derepression of various stationary phase genes, some of which are needed for sporulation (Sonenshein 2000). Studies of *B. subtilis* biofilms have shown that the cells do not behave as strictly unicellular organisms, but that sporulation is also tightly linked with the spatial development of the microbial community; motile cells may form aligned chains of attached cells that produce aerial structures that can be seen as primitive fruiting bodies, as they are the preferred sites of sporulation (Branda et al. 2001).

Sporulation is closely tied to the cell cycle, and a round of DNA replication must be initiated as a prerequisite for the sporulation pathway being activated (Michael 2001). The cell division of vegetative growth is symmetrical, and yields two similar cells. During sporulation, however, cell division is asymmetrical and two quite different kinds of cells, the small forespore and the larger mother cell, are produced, each with its own copy of the chromosome. The two different kinds of division are believed to use essentially the same protein machinery (Errington 2001). At the beginning of sporulation, the chromosomes form an elongated structure called the axial filament, with migration of a specific region of the chromosomes towards the poles, and polar septation bisects one end of this filament so that only part of the nucleoid lies within the forespore. The remainder of the chromosome is then transferred into the forespore from the mother cell (Levin and Grossman 1998; Errington 2001). The process of sporulation may be divided into seven morphologically-recognizable stages following vegetative growth:

1. preseptation, with the DNA forming the axial filament;
2. asymmetric septation, in which the membrane of the developing spore surrounds the spore protoplast and becomes detached from the membrane of the mother cell;
3. the forespore so formed becomes surrounded by the cytoplasm of the mother cell and so is contained within two membranes of opposing polarity;
4. spore cortex formation commences, with a primordial cell wall being laid down between the membranes, next to the forespore inner membrane; the cortex (a thicker layer of electron-transparent peptidoglycan, unique to bacterial endospores) is laid down on the outside of this primordial cell wall; the exosporium, a thin and delicate proteinaceous outermost covering, appears at this time;
5. proteinaceous spore coats are synthesized and begin to be deposited outside the cortex;
6. the spore matures, and acquires its refractility and heat resistance;
7. the sporangium lyses and releases the mature spore (Foster 1994).

In a laboratory culture of *B. subtilis*, the whole process of sporulation may take about 8 h.

Endospores are metabolically extremely dormant and do not contain adenosine triphosphate (ATP); this dormancy is the key to their resistance to many agents, including heat, radiation, and chemicals, and their survival over long periods. Spore structure, resistance, and germination are reviewed by Atrih and Foster (2002). The spore cortex is essential for spore dehydration (down to 10–30 percent of the water content of the vegetative cell) and so for the maintenance of dormancy, and for the spore's heat resistance; the temperature of sporulation influences the mature spore's heat resistance (Nicholson et al. 2000). The mechanisms of spore resistance to chemical agents and radiation are not well understood, but saturation of the chromosome by protective small acid-soluble proteins (SASP) is believed to play a part, while the spore coats are believed to prevent access of peptidoglycan-lytic enzymes to the spore cortex, and are also known to protect from hydrogen peroxide and UV radiation (Riesenman and Nicholson 2000); coat assembly and composition are reviewed by Takamatsu and Watabe (2002). SASPs appear to be more important than the low core water

content in protecting DNA from heat and oxidative damage (Setlow 1995). Pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) is a unique and quantitatively important spore component (comprising 5–14 percent of the spore dry weight), and Ca^{2+} and other divalent cations are chelated by it, but precisely how it contributes to spore resistance is unclear (Slieman and Nicholson 2001). DPA may be used as a marker for detecting the presence of spores by Curie-point pyrolysis mass spectrometry and by Fourier transform infrared spectroscopy (Goodacre et al. 2000). Mechanisms of spore resistance have been reviewed by Nicholson et al. (2000) in the contexts of survival both in extreme terrestrial conditions and during travel through extraterrestrial environments.

Conversion from the dormant spore to vegetative cell involves the three steps: activation, germination, and outgrowth. Dormancy may be broken by heat treatment at a time and sublethal temperature appropriate to the organism concerned, and by aging at low temperatures, but endospores of many species do not require such activation. The heat treatment procedure used to assist the isolation of *Bacillus* species, by destruction of all kinds of vegetative cells, is often effective in activation. Following dormancy, with or without activation, the spore may encounter conditions that trigger germination; the cortex is rapidly hydrolyzed, SASPs are quickly degraded, and refractility is lost in a matter of minutes. The germinated spore protoplast then outgrows: it visibly swells owing to water uptake, biosynthesis recommences (taking advantage of the nutrients released by germination as well as those available in its new environment), and a new vegetative cell emerges from the broken spore coat; another period of vegetative reproduction ensues. It seems incredible that a metabolically dormant spore can monitor its external environment in order to trigger germination within seconds in suitable conditions, and that this triggering mechanism can escape the constraints of dormancy while being resistant to damaging agents. Germination can be induced by exposure to nutrients such as amino acids and sugars, by mixtures of these, by nonnutrients such as dodecylamine, and by enzymes and high hydrostatic pressure. For many species, L-alanine is an important germinant, while D-alanine can bind at the same site as L-alanine and acts as a competitive inhibitor (Foster and Johnstone 1990; Johnstone 1994). The mechanism of germination has been most studied for *B. megaterium*. Although spore structure is very similar between species, and cortex peptidoglycan structure is highly conserved (Atrih and Foster 2001), there are many different germinant receptor specificities. Nonetheless, the underlying mechanism of germination may be universal (Foster and Johnstone 1990). Recent developments in the understanding of the spore germination process and the spore components required for it are reviewed by Moir et al. (2002).

ANTIGENS

Despite the promising findings of some early studies of somatic, flagellar, and spore antigens of a range of species, and the potential taxonomic value of spore precipitinogens, serological studies have been taken little further for classification and identification of members of this genus. Paradoxically, however, although the *B. cereus* group appeared to be one of the least tractable in these early studies, where species-specific antigens were sought, the H-antigens of *B. cereus* and *B. thuringiensis* are now used with considerable success for typing purposes; *B. anthracis* is nonmotile and so has no H antigen. Smith et al. (1952) reviewed the earliest work, following their own disappointing results with antisera to vegetative cells of a range of species, and Berkeley et al. (1984) reviewed the application of serological methods to the identification of *Bacillus* species.

Somatic antigens

These have been little used for the identification of *Bacillus* strains. Investigations into the O-antigens of *B. cereus* and *B. licheniformis* (Norris and Wolf 1961) found them too strain-specific to be of taxonomic value. Walker and Wolf (1971) and Wolf and Sharp (1981) found the O-antigens of *B. stearothermophilus* (now *Geobacillus stearothermophilus*) to show some correlation with the three biochemical and physiological subgroups of this species that they recognized. Serotyping of *B. thuringiensis* strains has been attempted on the basis of extracellular heat-stable somatic antigens (HSSA) (Ueda et al. 1991; Ohba et al. 1992), by observing the formation of immunoprecipitin haloes around colonies on antiserum-agar plates; they found a lack of correlation with H-antigen serogroups, while field isolates showed little HSSA variation within a single H-serovar. Concerns about the potential of *B. anthracis* as a biological weapon have emphasized the need for a rapid method for its identification and the diagnosis of anthrax. Polyclonal antibodies lack the desired specificity, because they react with other members of the *B. cereus* group. Phillips and Ezzell (1989) were able to identify *B. anthracis* by raising polyclonal antibodies against extracted vegetative cell antigens, absorbing with *B. cereus* and *B. thuringiensis*, and detecting reactions by immunofluorescence or immunoblotting. A monoclonal antibody specific to the *B. anthracis* cell wall polysaccharide antigen is effective in identification (Ezzell et al. 1990), but this antigen may be masked in vivo by the organism's poly-γ-D-glutamic acid capsule. De et al. (2002) therefore developed a two-component direct fluorescent-antibody assay that allows rapid, sensitive, and specific detection of the cell wall and capsule of *B. anthracis* in clinical specimens.

Flagellar antigens

H-antigens have been more widely used in *Bacillus* typing than any other kind of antigen, as they provide the highest strain specificity, and valuable serotyping schemes have been developed for *B. thuringiensis* and *B. cereus*. High frequencies of H-antigen sharing between *B. cereus* and *B. thuringiensis* have been reported; in one study of *B. cereus* strains from soils, phylloplanes, and animal feces, the seropositivity of the isolates with *B. thuringiensis* H-antisera was 60–77 percent (Shisa et al. 2002). The common flagellar antigen of *B. cereus* has been shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot assay to be due to a 61 kDa protein, and monoclonal antibody studies showed that the common antigenic epitope of this protein also exists in the flagella of *B. thuringiensis* (Murakami et al. 1993).

Sixty-nine serotypes and 13 sub-antigenic groups of *B. thuringiensis* have been recognized, giving 82 serovars (Lecadet et al. 1999). New strains of the species are screened by reference H-antisera, and antisera are prepared against any strains that do not agglutinate. New antisera are then screened with all the known H-antigens, and a new serovar is recognized if cross-reactions do not occur or if new subfactors can be demonstrated by the antiserum-saturation technique (de Barjac 1981). New serovars are registered at the International Entomopathogenic *Bacillus* Centre (IEBC) Collection at the Institut Pasteur, Paris, France; this laboratory has been the international reference for *B. thuringiensis* since 1965. Serotyping services for this species are available at the IEBC and at Abbott Laboratories, North Chicago, Illinois, USA. Distinct serovars of *B. thuringiensis* are given names and abbreviations, such as finitimus (FIN, H-antigen 2), sotto (SOT, H4a4b), tolworthi (TOL, H9) and pirenaica (PIR, H57). The first two of these two names were formerly used as the specific epithets of distinct species (Gordon et al. 1973). Although these serovar names have often been informally regarded as subspecific epithets, they do not represent validated subspecies of *B. thuringiensis* and should instead be regarded as varieties. These varieties do not, unfortunately, show much correlation with toxicity to invertebrates; for example, serovar morrisoni (MOR, H8a8b) includes strains pathogenic to mosquitoes (Diptera), Coleoptera and Lepidoptera (Lecadet et al. 1999), while the invertebrate toxicity, if any, of many serovars (especially the recently recognized ones) is unknown.

A strain differentiation system for *B. cereus* based on H-antigens is available at the Food Hygiene Laboratory, Central Public Health Laboratory (Colindale, London, UK), for investigations of food-poisoning outbreaks or other *B. cereus*-associated clinical problems (Kramer and Gilbert 1992). This system was developed by Taylor and Gilbert (1975) for the investigation of food poisoning outbreaks and recognized 18 serovars, but many strains from outbreaks were untypable (Gilbert and Parry 1977). Terayama et al. (1978) extended the system and recognized further serovars in foods. Some of the serotypes show some correlation with pathogenicity and biotype; the distinction is not absolute, however, and it is probable that some organisms can produce both diarrheal and emetic toxins. Serovar 1, 3, 5, and 8 strains comprise a biotype distinguishable from other *B. cereus* serovars and untypable strains, and these former four serovars have often been isolated in connection with cases of the emetic form of food poisoning (Logan et al. 1979; Logan and Berkeley 1984), a form especially associated with cooked rice. Gilbert and Parry (1977) found that strains of serovar 1 are found more frequently in cooked rice than in uncooked rice, while strains of serovar 17 are commoner in uncooked rice than they are in cooked rice; they later showed that serovar 1 strains form more heat resistant spores than do serovar 17 strains (Parry and Gilbert 1980).

Flagellar serotyping was developed for *B. sphaericus* (de Barjac 1981; de Barjac et al. 1985) independently of the recognition of DNA relatedness groups, so that the DNA relatedness group IIA, the group in which the mosquitocidal strains of this species lie, is divided into nine nonconsecutively numbered serovars. The serotyping scheme for *B. sphaericus* shows good agreement with a phage typing scheme (Yousten 1984) for this species, but, as with *B. thuringiensis*, the types do not always concur with pathogenicity (Priest 2002).

The study of H-antigens of *B. subtilis* (Simon et al. 1977) by agglutination tests was complicated by the tendency of the cells to clump spontaneously, and so double-diffusion in agar or complement fixation methods were applied. At least five distinct serovars were recognized, and although cross-reactions made the establishment of a practical serotyping scheme difficult, this approach was considered to be potentially useful in taxonomic studies.

Spore antigens

As spores are so characteristic of the genus *Bacillus*, it is not surprising that their antigens have attracted much interest. Early workers (reviewed by Norris 1962) recognized:

1. that any attempt to use living spores as antigens might be complicated by their germination in the host animal, giving rise to antisera against vegetative cells as well as spores;
2. the need for a rapid method to overcome any problems of germination during the agglutination test itself;
3. the tendency of spores to auto-agglutinate owing to their hydrophobic surfaces; and

4 the need to remove vegetative cell debris from spore suspensions – by using media promoting complete sporulation, or by autolysis with lysozyme or thiomersalate, or by autoclaving.

Once these problems are overcome, spore antigens can be useful for serological identification, and a revival of interest has been stimulated by the need to detect the spores of *B. anthracis* in the contexts of biowarfare and bioterrorism (Iqbal et al. 2000).

In order to circumvent cell-clumping problems, Kim and Goepfert (1972) developed a fluorescent antibody technique for confirming identifications of *B. cereus* from food poisoning cases, but could not distinguish between spores of this species and *B. thuringiensis*; it has subsequently been recognized that strains of the latter species may also cause food poisoning (Damgaard et al. 1997). Phillips and Martin (1983a, 1983b) used radiolabeled polyclonal antibody to probe for *B. anthracis* spores attached to solid supports. Spores would not attach reliably to microtitre plates, but although attachment to glass slides was better, the background signal in the immunoradiometric assay (IRMA) was higher; the best sensitivity was achieved with an indirect assay, but specificity was only moderate. Fluorescein-conjugated polyclonal antibodies to *B. anthracis* spore surface antigens were found to cross-react with spores of several other *Bacillus* species, but these cross-reactions could be absorbed with strains of *B. cereus*; however, spores of the Vollum strain (= type strain) did not react with antibodies to spores of most of the other strains of *B. anthracis* tested (Phillips and Martin 1988). A monoclonal antibody to viable and heat-killed spores of *B. anthracis* was used in an immunofluorescence assay and achieved higher specificity but lower sensitivity than the IRMA approaches, but the epitope recognized by the antibody appeared to be unstable in spores stored for long periods (Phillips et al. 1988).

PATHOGENICITY

Bacillus anthracis

Anthrax is a disease of great antiquity, and descriptions are found in ancient literature such as Virgil's *Georgics* (36–29BC). It is primarily a disease of herbivores, and before an effective veterinary vaccine became available in the late 1930s, it was one of the foremost causes worldwide of mortality in cattle, sheep, goats, and horses. An outbreak in Iraq in 1945 killed 1 million sheep. The development and application of veterinary and human vaccines together with improvements in factory hygiene and sterilization procedures for imported animal products, and the increased use of man-made alternatives to animal hides or hair, have resulted over the past half century in a marked decline in the incidence of the disease in both animals and humans. Sites where these natural materials were formerly handled, such as disused tanneries, may be sources of infection when they are disturbed during redevelopment. Likewise, anthrax carcasses can remain infectious for many years, even when buried with quicklime. Nonendemic regions must be constantly on the alert for the arrival of *B. anthracis* in imported products of animal origin; the disease continues to be endemic in several countries of Africa, Asia, and central and southern Europe, particularly those that lack an efficient vaccination policy.

Natural infectious cycle

The cycle of infection is as follows: a grazing animal ingests the spores which may then gain access to the lymphatics, and so to the spleen, through abrasions in the alimentary canal. After several days of the organism multiplying and producing toxin in the spleen, the animal suffers a sudden and fatal septicemia and collapses; hemorrhagic exudates escape from the mouth, nose, and anus and contaminate the soil, where the vegetative cells sporulate in the air. In an infected herd of herbivores, not all the animals develop clinical disease, and there is serological evidence that mild and symptomless infections occur (Turnbull et al. 1992a). The spores may remain viable in soil for many years and so the persistence of the organism does not depend on animal reservoirs; *B. anthracis* is therefore exceedingly difficult to eradicate from an endemic area. The organism continues to be generally regarded as an obligate pathogen, with its continued existence in the ecosystem appearing to depend on a periodic multiplication phase within an animal host, and its environmental presence reflecting contamination from an animal source at some time. Excepting cases of carnivorous scavenging of meat from anthrax carcasses, direct animal-to-animal transmission within a species is very rare; however, such scavengers can show transient carriage of the organism (Lindeque and Turnbull 1994).

Human anthrax

In public consciousness, *B. anthracis* is associated more with warfare and terrorism than with a disease of herbivores, and it is feared accordingly. It has long been considered a potential agent for biological warfare or bioterrorism. Its first use is believed to have been against livestock during World War I (Christopher et al. 1997; Barnaby 2002). It has been included in various development and offensive programs in several countries since (Zilinskas 1997; Alibek 2003; Mangold and Goldberg 1999; Miller et al. 2001; Barnaby 2002) and it has also been used in terrorist attacks (Christopher et al. 1997; Lane and Fauci 2001). Apart from such artificial attacks, humans almost invariably contract anthrax

directly or indirectly from animals. It is a point-source type of disease, and direct human-to-human transmission is exceptionally rare. Indeed, circumstantial evidence shows that humans are moderately resistant to anthrax as compared with obligate herbivores; infectious doses in the human inhalational and intestinal forms are generally very high (LD_{50} 2 500 to 55 000 spores). Naturally acquired human anthrax may result from close contact with infected animals or their carcasses after death from the disease, or be acquired by those employed in processing wool, hair, hides, bones, or other animal products. Most cases (about 99 percent) are cutaneous infections, but *B. anthracis* meningitis and gastrointestinal anthrax are occasionally reported. In industrial settings, inhalation of spore-laden dust used to occur, but cases of clinical disease were rare; anthrax weapons are normally intended to cause the inhalational form, but are likely to cause cutaneous cases as well. There have been a few reports of laboratory-acquired infections, none of them recent (Collins 1988), but a major outbreak of anthrax occurred in April 1979 in the city of Sverdlovsk (now Yekaterinburg) in the Urals as a result of the accidental release of spores from a military production facility; 77 cases were recorded and 66 patients died (Meselson et al. 1994).

CUTANEOUS INFECTION

The organism is not invasive, and cutaneous infections occur through breaks in the skin, so the lesions generally occur on exposed regions of the body. Before the availability of antibiotics and vaccines, 10–20 percent of untreated cases were fatal, and the rare fatalities seen today are due to obstruction of the airways by the edema that accompanies lesions on the face or neck, and sequelae of secondary cellulitis or meningitis.

INHALATIONAL INFECTION

Inhalational (formerly called pulmonary) anthrax cases are more often fatal, because they go unrecognized until too late for effective therapy, but undiagnosed, low-grade infections with recovery may occur. The number of recorded cases of inhalational anthrax is lower than might be expected from the high profile given to this condition. In the 20th century there were just 18 reported cases (two of them laboratory-acquired) in the USA, 16 (88.9 percent) fatal (Brachman and Kaufmann 1998); figures in the UK showed a similar picture. In 11 confirmed cases of inhalational anthrax that followed a bioterrorist attack in the USA, in which spores were delivered in mailed letters and packages, early recognition and treatment helped a survival level of 55 percent to be achieved (Jernigan et al. 2001; Bell et al. 2002).

GASTROINTESTINAL INFECTION

Oropharyngeal and gastrointestinal anthrax are not uncommon in endemic regions of the world where socio-economic conditions are poor, and people eat the meat of animals that have died suddenly; such cases are greatly underreported. Gastrointestinal infections are mainly characterized by gastroenteritis, and asymptomatic infections and symptomatic infections with recovery are not uncommon. The symptoms of oropharyngeal infections are fever, toxemia, inflammatory lesions in the oral cavity and oropharynx, cervical lymph node enlargement, and edema, and there is a high case-fatality rate (Sirisanthana and Brown 2002).

MENINGITIS

Meningitis can develop from any of the forms of anthrax. The emergence of clinical signs is rapidly followed by unconsciousness, and the prognosis is poor. Outbreaks of primary anthrax meningoencephalitis have been reported from India and elsewhere (George et al. 1994).

Pathogenesis

The spore is the primary infectious form of the organism (Hanna and Ireland 1999), and it enters the body from the environment. Spores are rapidly phagocytosed by macrophages, some of which undergo lysis, and in cases of inhalational anthrax the surviving macrophages are carried towards the mediastinal lymph nodes by the lymphatics. Phagocytosed spores may not germinate for up to 60 days, and so incubation of the inhalational form of the disease may take between 2 days and 6–8 weeks; this latency does not appear to occur in the cutaneous form of the disease. By analogy with other *Bacillus* species, germination is presumed to be triggered by a specific chemical germinant, but this remains unidentified. In several *Bacillus* species L-alanine is a germinant, and its binding to a receptor causes loss of spore refractility and resistance, the cortex swells, and metabolic activity commences; triggering of the germinant receptor is believed to activate endogenous proteolytic activity which converts the proenzyme of a germination-specific cortex-lytic enzyme to its active form which allows hydrolysis of the cortex, uptake of water, and all the other events associated with germination. The elevated CO_2 level and body temperature of the host cause the organism to transcriptionally activate the capsule and toxin genes. These genes are carried on two plasmids: plasmid pX01 encodes the toxin genes, and plasmid pX02 encodes the capsule genes; loss of either plasmid effectively renders the organism avirulent. Spores germinating in the presence of serum and elevated levels of CO_2 release blebs of capsular material through openings in the spore surface; the capsule of poly-γ-D-glutamic acid is purported to resist phagocytosis by virtue of its negative charge (Ezzell and Welkos 1999). The anthrax toxin complex comprises three components: edema factor (EF), protective antigen (PA), and lethal factor

(LF), none of which is toxic alone; EF and LF are active in binary combinations with PA and have different activities. PA molecules bind to molecules of a particular host cell membrane protein (anthrax toxin receptor (ATR)); Bradley et al. 2001) and form ring-shaped prepores of heptameric oligomers (aggregates of seven); PA is then cleaved and so activated by a furin-like protease on the surface of the cell under attack. An active PA heptamer can then bind one or more molecules of EF, LF or both. The complex passes into the cell by receptor-mediated endocytosis and into an acidified endosome; following conformational change of the heptamer in the low-pH environment, the complex escapes directly to the cytosol by insertion of the heptamer into the endosomal membrane. The PA-EF binary toxin interacts with the abundant host protein calmodulin (CaM; the major intracellular calcium receptor) and becomes an active adenylyl cyclase in most cell types; this elevates levels of the secretogogue cAMP and leads to hypovolemic shock. The crystal structure of EF in complex with CaM has been elucidated (Drum et al. 2002). The PA-LF binary toxin is a zinc metalloprotease which cleaves members of the mitogen-activated protein kinase kinase family, so affecting certain signaling pathways, and levels of shock-inducing cytokines. This toxin primarily affects macrophages, so that removal of macrophages from mice using silica renders them insensitive to the toxin (Hanna 1999); however, the process which leads to macrophage lysis is unclear (Pannifer et al. 2001). The importance of any interaction between EF and LF awaits clarification.

Depending on the host, there is a rapid build up of the bacteria in the blood over the last few hours to terminal levels of 10^7–10^9/ml in the most susceptible species.

Clinical presentation

The incubation period in cutaneous anthrax is generally 2–3 days (with extremes of approximately 12 h to 2 weeks). A small pimple or papule appears, and over the next 24 h a ring of vesicles develops around it, and it ulcerates, dries, and blackens into the characteristic eschar. Differential diagnoses include other cutaneous and subcutaneous infections (Hart and Beeching 2002). The eschar enlarges, becoming thick and adherent to underlying tissues over the ensuing week, and it is surrounded by edema, which may be very extensive. Pus and pain are normally absent; their presence, or the presence of marked lymphangitis and fever, probably indicates secondary bacterial infection. Oropharyngeal and gastrointestinal anthrax are essentially cutaneous anthrax occurring on the oropharyngeal and gastrointestinal mucosas. Symptoms of gastroenteritis may occur prior to onset of systemic symptoms.

In inhalational anthrax the inhaled spores are carried by macrophages from the lungs, where there is no overt infection (thus 'pulmonary' anthrax is a misnomer), to the lymphatic system where the infection progresses. Germination and initial multiplication begin within the macrophages while in transit to the lymph nodes (Hanna and Ireland 1999). The vegetative cells kill the macrophage and are released into the bloodstream where they continue to multiply and give rise to fatal septicemia. Analysis of ten of the cases associated with the bioterrorist events of 2001 (Jernigan et al. 2001) revealed a median incubation period of 4 days (range 4–6 days) and a variety of symptoms at initial presentation including fever or chills, sweats, fatigue or malaise, minimal or nonproductive cough, dyspnea, and nausea or vomiting. All patients had abnormal chest X-rays with infiltrates, pleural effusion, and mediastinal widening. Mediastinal lymphadenopathy was present in seven cases.

In fatal cases of any of the forms, the generalized symptoms may be mild (fatigue, malaise, fever and/or gastrointestinal symptoms) and are followed by sudden onset of acute illness with dyspnea, cyanosis, severe pyrexia, and disorientation followed by circulatory failure, shock, coma, and death, all within a few hours.

Specimens and safety

SPECIMENS FROM HUMANS

In all cases, specimens from possible sources of the infection (carcass, hides, hair, bones, etc.) should be sought in addition to specimens from the patients themselves. Swabs are appropriate for collecting vesicular fluid from cutaneous anthrax. Adequate material should be submitted for both culture and a smear for visualizing the capsule. In the case of inhalational infection, anthrax will only be suspected if the patient's history suggests the possibility. If the patient is not severely ill, immediate specimen collection is likely to be unfruitful and the person should be treated and simply observed. Paired sera (when first seen and >10 days later) may be useful for confirmation of diagnosis. In the severely ill patient, blood smear and culture should be carried out. Again, results will depend somewhat on previous treatment. Postmortem blood collected by venipuncture (a characteristic of anthrax is nonclotting blood at death) should be examined by smear (for capsule) and culture. Any hemorrhagic fluid from nose, mouth or anus should be cultured. If these are positive, no further specimens are needed. If negative, specimens of peritoneal fluid, spleen and/or mesenteric lymph nodes, aspirated by techniques avoiding spillage of fluids, may be collected for smear and culture. Histology is only of academic interest. As with inhalational infection, esophageal and gastrointestinal anthrax will only be suspected if an adequate history of the patient is known. If the patient is not severely ill, a fecal specimen may be collected. However, isolation of *B. anthracis* may not be successful. If the patient is severely ill, their blood should also be

cultured, and although isolation may not be possible after antimicrobial treatment, treatment should not await laboratory results. A blood smear may reveal the capsulated rods or, if treatment has started, capsule 'ghosts'.

SPECIMENS FROM ANIMALS

Anthrax should be considered as the possible cause of death in herbivorous animals which have died suddenly and unexpectedly, particularly if hemorrhage from the nose, mouth or anus have occurred and if death has taken place at a site with a history of anthrax (perhaps several decades previously). With carcasses 1–2 days old, owing to the nonclotting nature of the blood of anthrax victims, it is usually possible to aspirate a few drops of blood from a vein for (1) M'Fadyean-stained smear, and (2) direct plate culture on blood agar. Pigs frequently do not develop the enormous terminal bacteremia seen in herbivores and the capsulated rods may not be visible in blood smears. When cervical edema is present, smears and cultures should be made of fluid aspirated from the enlarged mandibular and suprapharyngeal lymph nodes. In porcine intestinal anthrax, possibly only obvious at necropsy, the rods are usually visible in stained smears made from mesenteric lymph nodes. *B. anthracis* competes poorly with putrefactive organisms, and so after 2–3 days in putrefying carcasses it may not be seen in smears, and culture becomes necessary for diagnostic confirmation. Sections of tissue, or any blood-stained material, should be collected. If the animal has been opened, spleen or lymph node specimens should be taken. With putrefied and very old carcasses, swabs of the nostrils and eye sockets are likely to yield *B. anthracis*, but the best specimens may be samples of contaminated soil from beneath the nose and anus.

OTHER SOURCES

Tests for the presence of *B. anthracis* may be requested for a variety of specimens such as animal products (e.g. wool, hides, hair, bonemeal) from endemic regions, soil or other materials from old burial sites or from sites of tanneries or laboratories due for redevelopment, or other environmental materials associated with outbreaks (e.g. sewage sludge). At present, culture by the selective agar techniques described below is the only available approach. Suitably equipped laboratories are beginning to use polymerase chain reaction (PCR) techniques for rapid detection of *B. anthracis* in such samples, but it remains prudent to confirm positives with conventional methods.

SAFETY

Anthrax is not highly contagious. Cutaneous anthrax is readily treated and is only life-threatening in exceptional cases. The infectious doses in the human inhalational and intestinal forms (also treatable if recognized early) are generally very high, and, generally speaking, precautions need to be sensible, not extreme. However, nonclinical materials associated with an attempt at deliberate release of *B. anthracis* spores may be very hazardous and no attempt to process them should be made without the appropriate instructions from the correct authorities. The laboratory examination of clinical specimens from suspected or potential victims of such an attack will be as described for cases of naturally acquired anthrax. When collecting environmental specimens related to suspected natural anthrax, disposable gloves, disposable apron or overalls, and boots which can be disinfected after use should be worn. For dusty samples that might contain many spores, the use of head-gear and dust masks should be considered.

Discard disposable items into suitable containers for autoclaving followed by incineration. Nonautoclavable items should be immersed overnight in 10 percent formalin (5 percent formaldehyde solution). Glutaraldehyde (5 percent) is also effective. Items that cannot be immersed should be bagged and sent for formaldehyde fumigation. Ethylene oxide and hydrogen peroxide vapor are also effective fumigants, but the latter is inappropriate if organic matter is being treated. The best disinfectant for specimen spillages is again formalin. Where this is considered impractical, 10 percent hypochlorite solution can be used although its limitations should be appreciated; it is rapidly neutralized by organic matter, and it corrodes metals. Other strong oxidizing agents, such as hydrogen peroxide (5 percent) and peracetic acid (1 percent) are also effective but with the same limitations in the presence of organic matter.

For further information, see Turnbull et al. (1998) and visit www.bt.cdc.gov and www.hopkins-biodefense.org.

Isolation

Isolation and presumptive identification of *B. anthracis* can be performed safely in the routine clinical microbiology laboratory, provided that normal good laboratory practice is observed, and vaccination is not required for minimal handling of the organism (Jackson et al. 1998). If aerosols are likely to be generated, the work should be performed in a safety cabinet.

In specimens submitted for isolation of *B. anthracis* from old carcasses, animal products or environmental specimens, the organisms will mostly be present as spores. Heating at 62.5°C for 15 min will both heat-shock the spores and effectively destroy nonspore-forming contaminants (solid samples should first be emulsified in sterile, deionized water, 1:2 [w/v]). Direct plate cultures are made on blood, nutrient, or selective agars, as appropriate, by spreading up to 250 µl volumes from undiluted, and 10- and 100-fold dilutions of the treated sample.

There is no effective enrichment method for *B. anthracis* in old animal specimens or environmental samples; isolation from these is best done with polymyxin-lysozyme EDTA-thallous acetate (PLET) agar (Knisely, 1966; see Appendix). Aliquots (250 µl) of the undiluted and 1:10 and 1:100 dilutions of heat-treated suspension of the specimen are spread across PLET plates which are read after incubation for 36–40 h at 37°C. Roughly circular, creamy-white colonies, 1–3 mm diameter, with a ground-glass texture are subcultured on (1), blood agar plates to test for gamma phage and penicillin susceptibility, and for hemolysis, and (2) directly or subsequently in blood to look for capsule production using M'Fadyean's stain (see Appendix). PCR-based methods are increasingly being used for confirming the identity of isolates (see Diagnosis below).

Diagnosis

Although Gram staining is considered to be of limited value in anthrax diagnosis because it does not reveal the capsule (Figure 2), the first examinations of smears and cultures will probably be with the Gram stain nontheless, and, as with the American bioterrorism victims in 2001, in a well-developed country it is unlikely that large numbers of gram-positive bacteria in the blood at death are going to be anything but *B. anthracis*, particularly when this is supported by the recent history of events. In other circumstances, and in animals in particular, the blood or other specimen may not be collected soon after death and before the rapid appearance of putrefactive organisms, and *B. anthracis* may be indistinguishable without the use of the proper capsule stain.

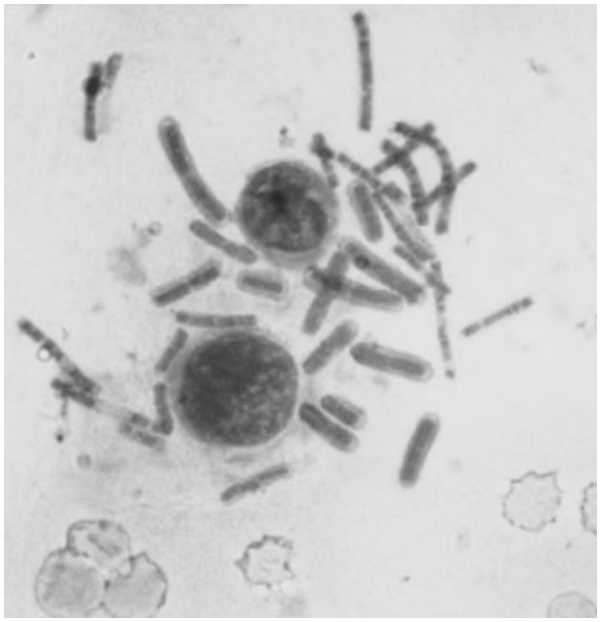

Figure 2 *Capsulated* Bacillus anthracis *cells around two blood mononuclear cells. Polychrome methylene blue stained blood smear from a guinea pig that died of anthrax (M'Fadyean stain). The cells, occasionally single but usually in short chains of two to a few cells and frequently square-ended ('boxcar' shaped), are blue-black; the surrounding capsule is pink. Oil immersion (×100) objective. Note that smears of artificially grown* B. anthracis *would show the cells in endless strings and, unless cultured under special conditions, lacking any capsule.*

Colonies of *B. anthracis*, *B. cereus*, and relatives (Figure 1a–c) are very variable, but readily recognized: they are characteristically large (2–7 mm in diameter) and vary in shape from circular to irregular, with entire to undulate, crenate or fimbriate edges; they have matt or granular textures. Smooth and moist colonies are not uncommon, however. The optimum growth temperature is approximately 37°C with minima and maxima of 15–20°C and 40–45°C, respectively. Although colonies of *B. anthracis* and *B. cereus* can be similar in appearance, those of the former are generally smaller, non-hemolytic, may show more spiking or tailing along the lines of inoculation streaks, and are very tenacious as compared with the usually more butyrous consistency of *Bacillus cereus* and *B. thuringiensis* colonies, so that they may be pulled into standing peaks with a loop.

It is generally easy to distinguish virulent *B. anthracis* from other members of the *B. cereus* group. An isolate with the correct colonial morphology, white or gray in color, nonhemolytic or only weakly hemolytic, nonmotile, susceptible to the diagnostic 'gamma phage'[1] and penicillin, and able to produce the characteristic capsule (as shown by M'Fadyean staining) is *B. anthracis*. Routine characters for distinguishing between *B. anthracis* and other aerobic endospore-formers are shown in Table 1.

An isolate with the characteristic phenotype but unable to produce capsules may be an avirulent form lacking either or both its capsule or toxin genes (Turnbull et al. 1992b) and should be referred to a specialist laboratory; such isolates are generally found in environmental samples and are frequently identified in routine laboratories as *B. cereus* and discarded. Primer sequences are now available for confirming the presence of the toxin and capsule genes (Jackson et al. 1998), and hence the virulence of an isolate. Molecular studies are giving valuable insights into genetic profiles among the *B. cereus* group (Keim et al. 2000; Turnbull et al. 2002), and the genomes of *B. anthracis* and *B. cereus* have been sequenced (Read et al. 2003; Ivanova et al. 2003).

The capsule of virulent *B. anthracis* can be demonstrated on nutrient agar containing 0.7 percent sodium bicarbonate incubated overnight under 5–7 percent CO_2 (candle jars perform well). Colonies of the capsulated *B. anthracis* appear mucoid, and the capsule can be visualized by staining smears with M'Fadyean's polychrome methylene blue or India ink (Turnbull et al. 1998) or by indirect fluorescent antibody staining.[1] More simply, 2.5 ml of blood (defibrinated horse blood seems best;

Table 1 *Characters for differentiating some species of aerobic endospore-forming bacteria.*

Characteristics[b]	*B. subtilis*	*B. amyloliquefaciens*	*B. licheniformis*	*B. pumilus*	*B. anthracis*	*B. cereus*	*B. cereus* (emetic biotype)[a]	*B. mycoides*	*B. thuringiensis*	*B. megaterium*	*B. circulans*	*B. coagulans*	*B. sphaericus*	*Brevibacillus brevis*	*Br. agri*	*Br. laterosporus*	*Geobacillus stearothermophilus*	*Paenibacillus polymyxa*	*P. alvei*	*P. macerans*	*Virgibacillus pantothenticus*
Motility	+	+	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell diameter >1.0 μm	–	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
Spores																					
Ellipsoidal	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
Cylindrical	–	–	–	+	–	d	–	–	d	–	–	–	–	–	–	–	–	–	d	–	–
Spherical	–	–	–	–	–	–	–	–	–	d	–	–	+	–	–	–	–	–	–	–	d
Borne terminally	–	–	–	–	–	–	–	–	–	–	d	d	+	–	–	–	–	–	–	d	+
Swell sporangia	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+
Parasporal crystals	–	–	–	–	–	–	–	–	+	–	–	–	(+)[c]	–	–	–	–	–	–	–	–
Parasporal bodies	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Anaerobic growth	–	–	+	–	+	+	+	+	+	–	+	+	–	–	–	+	–	+	+	+	+
Arginine dihydrolase	–	–	+	–	+	d	d	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Voges–Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	–	d	+	+	+	+	+	d	–
Indole production	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Acid from:																					
L-arabinose	+	d	+	+	–	–	–	–	–	+	+	d	–	–	–	–	d	+	–	+	–
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+

(*Continued over*)

Table 36.1 *Characters for differentiating some species of aerobic endospore-forming bacteria. (Continued)*

Characteristics[b]	*B. subtilis*	*B. amyloliquefaciens*	*B. licheniformis*	*B. pumilus*	*B. anthracis*	*B. cereus*	*B. cereus* (emetic biotype)[a]	*B. mycoides*	*B. thuringiensis*	*B. megaterium*	*B. circulans*	*B. coagulans*	*B. sphaericus*	*Brevibacillus brevis*	*Br. agri*	*Br. laterosporus*	*Geobacillus stearothermophilus*	*Paenibacillus polymyxa*	*P. alvei*	*P. macerans*	*Virgibacillus pantothenticus*
Glycogen	+	+	+	–	+	+	–	+	+	+	+	d	–	–	–	–	d	+	d	+	–
D-mannitol	+	+	+	+	–	–	–	–	–	+	+	d	–	+	+	+	+	+	–	+	–
D-mannose	+	d	+	+	–	–	–	–	d	–	+	+	–	–	–	+	+	+	d	+	+
α-methyl-D-mannoside	–	–	–	+	–	–	–	–	–	–	–	d	–	–	–	–	–	d	–	+	d
Salicin	+	+	+	+	–	+	–	d	d	+	+	+	–	–	–	+	d	+	d	+	+
Starch	+	+	+	–	+	+	–	+	+	+	+	+	–	–	–	d	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	–	–	d	+	+	+	d	+	+
D-xylose	+	d	+	+	–	–	–	–	–	+	+	d	–	–	–	–	d	+	–	+	–
Gas from carbohydrates	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–
Hydrolysis of:																					
Casein	+	+	+	+	+	+	+	+	+	+	–	+	d	+	+	+	d	+	+	–	+
Gelatin	+	+	+	+	+	+	+	+	+	+	–	d	d	+	+	+	+	+	+	d	+
Starch	+	+	+	–	+	+		+	+	+	+	+	–	–	–	–	+	–	+	+	+
Egg yolk reaction	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	+	–	+	d	+	d	+	d	d	–	–	+	–	+	d	+	–	–	d
Growth at:																					
50°C	d	+	+	d	–	–	–	–	–	d	–	+	–	–	d	–	+	–	–	d	d
65°C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–

Symbols and abbreviations: +, >85 percent positive; d, reactions differ between strains (16–84 percent strains are positive); –, 0–15 percent positive.

a) Reactions for the *B. cereus* biotype isolated particularly in connexion with outbreaks of emetic-type food poisoning and for strains of serovars 1, 3, 5, and 8, which are commonly associated with such outbreaks.

b) Arginine dihydrolase, indole production, gelatin hydrolysis, and nitrate reduction reactions were determined using tests in the API 20E strip. Acid from carbohydrate reactions were determined using the API 50CHB System.

c) Mosquitocidal *Bacillus sphaericus* strains produce parasporal crystals which, although smaller than those of *B. thuringiensis*, can be visualized by phase-contrast microscopy.

horse or fetal calf serum are quite good) can be inoculated with a pinhead quantity of growth from the suspect colony, incubated statically for 6–18 h at 37°C, and M'Fadyean stained. The M'Fadyean stain is preferable to other capsule staining methods, as it is more specific for *B. anthracis* capsules; it dates from 1903 and has proved a remarkably successful rapid diagnostic test over the decades. However, reliable stain and adequate quality control of its performance are becoming hard to guarantee. A rapid immunochromatographic on-site test has been developed (Burans et al. 1996) but is not commercially available.

Real-time PCR assays, which use primer and fluorescently-labelled probe systems to allow the rapid and sensitive detection of genes specific for *B. anthracis*, have been developed in several laboratories. Makino and Cheun (2003) described an assay that targeted genes for capsule and PA and allowed a single spore to be detected in 100 l of air in 1 h. Drago et al. (2002) outlined an assay targeting fragments of a chromosomal gene (*rpo*) for detecting the organism in clinical samples. Hoffmaster et al. (2002) evaluated and validated a three-target assay, with primers for capsule, PA, and *rpo*, in order to test suspect isolates and to screen environmental samples during the outbreak that followed the 2001 bioterrorist attack in the USA.

Monoclonal antibody tests based on specific spore cortex or cell wall epitopes, for reliable differentiation of *B. anthracis* from close relatives, are not yet available. Breakthroughs have recently been made in the long-standing problem of differentiating strains of *B. anthracis* for epidemiological and strategic purposes using amplified fragment length polymorphism (AFLP) analysis (Jackson et al. 1998; Keim et al. 2000).

The three protein components of anthrax toxin (PA, LF, and EF) and antibodies to them can be used in enzyme immunoassay systems. For routine confirmation of anthrax infection, or for monitoring response to anthrax vaccines, antibodies against protective antigen alone appear to be satisfactory; they have proved useful for epidemiological investigations in humans and animals. In human anthrax, however, early treatment sometimes prevents antibody development (Turnbull et al. 1992a). PA is available commercially from List Biological Laboratories, Inc (www.listlabs.com).

In countries of the former USSR, a skin test utilizing *Anthraxin*, a heat-stable extract from a noncapsulate strain of *B. anthracis*, which has been licensed for human and animal use since 1962, is widely acclaimed for the retrospective diagnosis of anthrax (Shlyakhov and Rubinstein 1994). The delayed-type hypersensitivity is interpreted as indicating cell-mediated immunity to anthrax and can be used not only to diagnose anthrax retrospectively, but also to evaluate the vaccine-induced immune status after periods of several years. *Anthraxin* does not contain highly specific anthrax antigens and depends on the nature of anthrax rather than the specificity of the antigens involved. This is also true of the Ascoli test which, dating from 1911, must be one of the oldest antigen detection tests in microbiology. It is a precipitin test using hyperimmune serum raised to *B. anthracis* whole cell antigen to provide rapid retrospective evidence of anthrax infection in an animal from which the material being tested was derived. The test is still in use in eastern Europe and central Asia.

Treatment and prophylaxis

Most strains of *B. anthracis* are susceptible to penicillin, and until recently there were few authenticated reports of resistant isolates (Lalitha and Thomas 1997); consequently this antibiotic was the mainstay of treatment and there were few studies on the organism's sensitivity to other antibiotics. Mild and uncomplicated cutaneous infections may be treated with oral penicillin V, but the treatment usually recommended is intramuscular procaine penicillin or benzyl penicillin (penicillin G). In severe cases, and gastrointestinal and inhalational infections, the recommended therapy has been penicillin G by slow intravenous injection or infusion until the fever subsides, followed by intramuscular procaine penicillin; the organism is normally susceptible to streptomycin, which may act synergistically with penicillin (Turnbull et al. 1998). The use of an adequate dose of penicillin is important, as Lightfoot et al. (1990) found that strains grown in the presence of subinhibitory concentrations of flucloxacillin in vitro became resistant to penicillin and amoxicillin. The study of Lightfoot et al. (1990) on 70 strains, and that of Doganay and Aydin (1991) on 22 isolates, found that most strains were sensitive to penicillins, with minimal inhibitory concentrations of 0.03 mg/l or less; however, the former authors found that two resistant isolates from a fatal case of inhalational infection had a minimum inhibitory concentration (MIC) in excess of 0.25 mg/l. *B. anthracis* is resistant to many cephalosporins. Coker et al. (2002) found that of 25 genetically diverse mainly animal and human isolates from around the world, five strains were resistant to the 'second generation' cephalosporin cefuroxime, and 19 strains showed intermediate susceptibility to this agent; all strains were sensitive to the 'first generation' cephalosporins cephalexin, and cefaclor, and three were resistant to penicillin but were negative for β-lactamase production. Mohammed et al. (2002) studied 50 historical isolates from humans and animals and 15 clinical isolates from the 2001 bioterrorist attack in the USA; the majority of their strains could be regarded as nonsusceptible to the 'third generation' cephalosporin ceftriaxone, and three strains were resistant to penicillin. Genomic sequence data indicate that *B. anthracis* possesses two β-lactamases: a potential penicillinase (class A), and a cephalosporinase (class B) which is expressed (Bell et al. 2002). Tetracyclines, chloramphenicol, and gentamicin are suitable for the

treatment of patients allergic to penicillin; tests in primates showed doxycycline to be effective, a finding confirmed by Coker et al. (2002), and indicated the suitability of ciprofloxacin (Turnbull et al. 1998). Mohammed et al. (2002) found that most of their strains showed only intermediate susceptibility to erythromycin. Esel et al. (2003) found that ciprofloxacin and the newer quinolone gatifloxacin had a good in vitro activity against 40 human isolates collected in Turkey, but that for another new quinolone, levofloxacin, high MICs were needed for 10 strains.

Because human cases tend to be sporadic, clinical experience of alternative treatment strategies was sparse until the bioterrorist attack occurred in the US in late 2001. The potential and actual use of *B. anthracis* as a bioweapon has also emphasized the need for post-exposure prophylaxis; recommendations include ciprofloxacin or doxycycline, with amoxycillin as an option for the treatment of children and pregnant or lactating women, given the potential toxicity of quinolones and tetracyclines; however, β-lactams do not penetrate macrophages well, and these are the sites of spore germination (Bell et al. 2002). Combination therapy, begun early, with a fluoroquinolone such as ciprofloxacin and at least one other antibiotic to which the organism is sensitive, appears to improve survival (Jernigan et al. 2001). Following the 2001 outbreak in the US, the recommendation for initial treatment of inhalational anthrax is ciprofloxacin or doxycycline along with one or more agents to which the organism is normally sensitive; given supportive sensitivity testing, a penicillin may be used to complete treatment. The same approach is recommended for cutaneous infections (Bell et al. 2002). Doxycycline does not penetrate the central nervous system well, and so is not appropriate for the treatment of meningitis.

The Sterne attenuated live spore vaccine, based on a toxigenic but noncapsulate strain, was introduced for animal vaccination in the late 1930s and spores of this strain remain in use as the basis of livestock vaccines in most parts of the world today. As this vaccine can show some slight virulence for certain animals, it is not considered suitable for human protection in the West, but live spore preparations are used in China and Russia. The former USSR vaccine was developed in the 1930s and 1940s, and licensed for administration by injection in 1959. The UK vaccine is an alum-precipitated culture filtrate of the Sterne strain; it was first formulated in 1954, introduced for workers at risk in 1965, and licensed for human use in 1979. The current human vaccine in the USA is an aluminium hydroxide-adsorbed vaccine strain culture filtrate containing a relatively high proportion of PA and relatively low amounts of LF and EF; it was licensed in 1972 (Turnbull 2000). Concerns about the lack of efficacy and safety data on the long-established UK and US vaccines, especially following the Sverdlovsk incident, and allegations that anthrax vaccination contributed to Gulf War syndrome in military personnel, have led to demands for new vaccines that would necessarily undergo stricter testing than was customary in the past. Favoured active ingredients of these next-generation vaccines are whole-length recombinant PA or a mutant (nontoxic) portion of this molecule (Turnbull 2000).

OPPORTUNISTIC PATHOGENS

Apart from *B. anthracis*, the majority of *Bacillus* species are common environmental contaminants, and isolation from a single clinical specimen is generally not sufficient basis for incriminating one of these organisms as the etiological agent. Moderate or heavy growth of aerobic endospore-formers from wounds is usually significant, however, and *B. cereus* infections of the eye are emergencies which should always be taken seriously and reported to the physician immediately.

In the clinical laboratory, the most important questions to ask about an aerobic sporeforming isolate are: Was it isolated in pure culture or at least apparently dominating the flora? Was it isolated in large numbers? Was it isolated more than once? A repeatedly isolated aerobic endospore-former, found in large numbers in pure culture, particularly from blood cultures, is unlikely to be a mere contaminant. Histopathology and tests on the toxigenicity of the isolate may also help decide the relevance of the isolate.

Low-level contamination of foodstuffs by aerobic endospore-formers is common, as is asymptomatic transient fecal carriage by human and animal populations. Therefore, in foodborne illness investigations, qualitative isolation tests are insufficient. The ideal criteria for establishing that an aerobic endospore-former is the etiological agent are the isolation of significant numbers ($>10^5$ cfu/g) of the organism from the epidemiologically incriminated food (and, in the case of suspected *B. cereus* food poisoning, detection of emetic toxin and/or enterotoxin) together with recovery of the same strain (biovar, serovar, phage type, plasmid type, etc.) in significant numbers from acute phase specimens (feces or vomitus) from the patients. In practice, it is rare that these ideal criteria can be met. A complete set of the appropriate food and clinical specimens is seldom available and the epidemiological aspects of the outbreak, such as incubation times, clinical symptoms, the types of food implicated, the time lapse between the episode and specimen collection, and the manner in which the food was stored during this period, must all be considered along with the laboratory findings in forming conclusions as to the etiology.

Specimens and isolation

Clinical specimens for isolation of species other than *B. anthracis* can be handled without special precautions,

and they will normally survive transport in freshly collected specimens or in a standard transport medium. All these species of aerobic endospore-forming bacteria that may be isolated from clinical specimens can be handled safely on the open bench.

All the clinically significant isolates reported to date are of species that grow, and often sporulate, on routine laboratory media, at 37°C. It seems unlikely that many clinically important, but more fastidious, strains are being missed for the want of special media or growth conditions. Maintenance is simple if spores can be obtained, but it is a mistake to assume that a primary culture or subculture on blood agar will automatically yield spores if stored on the bench or in the incubator. It is best to grow the organism on nutrient agar containing 5 mg/l manganese sulfate for a few days, and refrigerate when microscopy shows that most cells have sporulated. For most species, sporulated cultures on slants of this medium, sealed after incubation, can survive in a refrigerator for years. Alternatively, cultures (preferrably sporulated) can be frozen or lyophilized.

In specimens submitted for *B. cereus* food-poisoning investigations, the organisms will mostly be present as spores. Heating at 62.5°C for 15 min will both heat-shock the spores and effectively destroy nonsporeforming contaminants (solid samples should first be emulsified in sterile, deionized water, 1:2 [w/v]). Direct plate cultures are made on blood, nutrient, or selective agars, as appropriate, by spreading up to 250 µl volumes from undiluted, and 10- and 100-fold dilutions of the treated sample.

Enrichment procedures are generally inappropriate for isolations from clinical specimens, but when searching for *B. cereus* in stools $\geqslant$3 days after a food-poisoning episode, nutrient or tryptic soy broth with polymyxin (100 000 U/l) may be added to the heat-treated specimen. Several media have been designed for isolation, identification, and enumeration of *B. cereus*. They exploit the organism's egg-yolk reaction positivity and acid-from-mannitol negativity; pyruvate and polymyxin may be included for selectivity. Three satisfactory formulations (see Appendix) are MEYP (mannitol, egg yolk, polymyxin B agar), PEMBA (polymyxin B, egg yolk, mannitol, bromthymol blue agar) and BCM (*Bacillus cereus* medium) (van Netten and Kramer 1992). There are no selective media for other bacilli, but spores can be selected for by heat treating part of the specimen, as described above; the vegetative cells of both sporeformers and nonsporeformers will be killed, but the heat-resistant spores will not only survive but may be heat-shocked into subsequent germination. The other part of the specimen is cultivated without heat treatment in case spores are very heat-sensitive, or absent. Heat treatment is not appropriate for fresh clinical specimens, where spores are usually sparse or absent.

Bacillus cereus

Bacillus cereus is next in importance to *B. anthracis* as a pathogen of humans (and other animals), causing food-borne illness and opportunistic infections, and its ubiquity ensures that cases are not uncommon.

Food-borne illness

B. cereus is the etiological agent of two distinct food poisoning syndromes (Kramer and Gilbert 1992):

1. the diarrheal-type, characterized by abdominal pain with diarrhea 8–16 h after ingestion of the contaminated food and associated with a diversity of foods ranging from meats and vegetable dishes to pastas, desserts, cakes, sauces, and milk; and
2. the emetic-type characterized by nausea and vomiting 1–5 h after eating the offending food, predominantly oriental rice dishes, although occasionally other foods such as pasteurized cream, milk pudding, pastas, and reconstituted infant-feed formulas have been implicated.

One outbreak followed the mere handling of contaminated rice in a children's craft activity (Briley et al. 2001), and fulminant liver failure associated with the emetic toxin has been reported (Mahler et al. 1997). Both syndromes arise as a direct result of the fact that *B. cereus* spores can survive normal cooking procedures. Under improper storage conditions after cooking, the spores germinate and the vegetative cells multiply. In diarrheal illness, the toxin(s) responsible are produced by organisms in the small intestine (infective doses 10^4–10^9 cells per gram of food), while the emetic toxin is preformed and ingested in food (about 10^5–10^8 cells per gram in order to produce sufficient toxin). Variations in infective dose of the diarrheal illness will reflect the proportion of ingested cells that are sporulated, and so can survive the acid barrier of the stomach. The capacity of the strain concerned to produce toxin(s) will, of course, influence the infective or intoxicating dose in both types of illness. It is likely that cases showing both diarrheal and emetic symptoms are caused by organisms producing both diarrheal and emetic toxins.

The toxigenic basis of *B. cereus* food poisoning and other *B. cereus* infections has begun to be elucidated, and a complex picture is emerging (Beecher 2001; Granum 2002). *B. cereus* is known to produce six toxins: five enterotoxins, and the emetic toxin. The enterotoxins are: (1) Hemolysin BL (Hbl), a 3-component proteinaceous toxin which also has dermonecrotic and vascular permeability activities, and causes fluid accumulation in ligated rabbit ileal loops; Hbl is produced by approximately 60 percent of strains tested (Granum 2002), and it has been suggested that is a primary virulence factor in *B. cereus* diarrhea, but the

mechanism of its enterotoxic activity is unclear (Granum 2002); (2) Non-hemolytic enterotoxin (Nhe) is another 3-component proteinaceous toxin which is produced by most strains tested (Granum 2002), and whose components show some similarities to Hbl; (3) and (4) Enterotoxin T (BceT) and Enterotoxin FM (EntFM) are single-component proteinaceous toxins whose roles and characteristics are not known; (5) Enterotoxin K (EntK) is similar to the β-toxin of *Clostridium perfringens* and was associated with a French outbreak of necrotic enteritis in which three people died (Lund et al. 2000). The genetics of toxin production are summarized by Granum (2002).

The emetic toxin, cereulide, is a dodecadepsipeptide comprising of a ring of four amino- and/or oxy-acids: [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val] thrice repeated; chemically speaking, it is closely related to the potassium ionophore valinomycin (Agata et al. 1994). It is resistant to heat, pH and proteolysis, but it is not antigenic (Kramer and Gilbert 1989). Cereulide is probably an enzymatically synthesized peptide rather than a direct genetic product; it is produced in larger amounts at lower incubation temperatures, its production does not appear to be connected with sporulation (Finlay et al. 2000), and it is not produced in anaerobic conditions (Finlay et al. 2002). Its mechanism of action is unknown, but it has been shown to stimulate the vagus afferent through binding to the 5-HT_3 receptor (Agata et al. 1995). The earliest detection system for emetic toxin involved monkey-feeding tests (Logan et al. 1979), but a semi-automated metabolic staining assay has now been developed (Finlay et al. 1999).

Other infections

Bacillus cereus is also a destructive ocular pathogen. In the case of posttraumatic endophthalmitis, *Bacillus* species, particularly *B. cereus*, may be the second most commonly isolated organisms after *Staphylococcus epidermidis* (Davey and Tauber 1987). For relevant reviews, see Logan (1988) and Berkeley and Logan (1997). Endophthalmitis may follow penetrating trauma of the eye or hematogenous spread and evolve very rapidly. Loss both of vision and the eye is likely if appropriate treatment is not instituted very early (Davey and Tauber 1987; Das et al. 2001). *B. cereus* keratitis associated with contact lens wear has also been reported (Pinna et al. 2001). Other *B. cereus* infections occur mainly, though not exclusively, in persons predisposed by neoplastic disease, immunosuppression, alcoholism, and other drug abuse, or some other underlying condition, and fatalities occasionally result. Reported conditions include bacteremia, septicemia, fulminant sepsis with hemolysis, meningitis, brain hemorrhage, ventricular shunt infections, endocarditis, pneumonia, empyema, pleurisy, lung abscess, brain abscess, osteomyelitis, salpingitis, urinary tract infection, and primary cutaneous infections. Wound infections, mostly in otherwise healthy persons, have been reported following surgery, road traffic, and other accidents, scalds, burns, plaster fixation, drug injection (including a case associated with contaminated heroin; Dancer et al. 2002), and close-range gunshot and nail bomb injuries; some became necrotic and gangrenous. A fatal inflammation was caused by a blank firearm injury; blank cartridge propellants are commonly contaminated with the organism (Rothschild and Leisenfeld 1996). Neonates also appear to be particularly susceptible to *B. cereus*, especially with umbilical stump infections, and respiratory tract infections associated with contaminated ventilation systems have also occurred (Van der Zwet et al. 2000).

Bacillus cereus also causes infections in domestic animals. It is a well-recognized agent of mastitis and abortion in cattle, and can cause these conditions in other livestock (Blowey and Edmondson 1995).

Bacillus thuringiensis

This species is closely related to *B. cereus*, and is distinguished principally by the ability of its members to produce crystalline, proteinaceous, parasporal bodies within the sporangia; the insecticidal activities of many of these δ-endotoxins have made the organism (often referred to as Bt) one of the most widely produced and studied bacteria in biotechnology. The applications and development of Bt pesticides have been reviewed by Bishop (2002), while Van Rie (2002) has reviewed the development of transgenic crop plants. Strains of *B. thuringiensis* may in addition produce the *B. cereus* diarrheal toxin, and *B. thuringiensis* has indeed been implicated in cases of gastroenteritis (Damgaard et al. 1997). There have also been reports of wound, burn, and ocular infections with *B. thuringiensis* (Damgaard et al. 1997). Cases of illness caused by *B. thuringiensis* may have been diagnosed as caused by *B. cereus*, as the former may not produce its characteristic insecticidal toxin crystals when incubated at 37°C, owing to the loss of the plasmids carrying the toxin genes. The safety of using *B. thuringiensis* as a biopesticide on crop plants has been reviewed by Bishop (2002); Bishop et al. (1999) found that the main pesticide strains that they assayed produced low titers of enterotoxin, and there is as yet no evidence of infections associated with the use of this organism as an insecticide.

Diagnosis

Colonies of *B. cereus* and its relatives are very variable, but readily recognized (Figure 1, and see Diagnosis of *B. anthracis*, above). The key characteristics for

recognizing and distinguishing the *B. cereus* group are: colonial morphology; large cells with rounded or squared ends, often in chains, producing ellipsoidal spores not swelling the sporangia (Figure 3a, b), usually within 48 h and often apparent after 24 h; facultative anaerobes; egg yolk reaction (i.e. lecithinase) positive. Negative or very weak hemolysis and lack of motility distinguish *B. anthracis* and *B. mycoides* from *B. cereus* and *B. thuringiensis*. *Bacillus cereus*, *B. mycoides*, *B. thuringiensis* and, to a lesser extent, *B. anthracis*, synthesize lecithinases, forming opaque zones of precipitation around colonies on egg yolk agar as the colonies grow (i.e. usually after overnight or perhaps 24 h incubation). Recognition of *B. thuringiensis* is largely dependent on observation of its cuboid or diamond-shaped parasporal bodies in sporulated cultures (after 2–5 days) by phase-contrast microscopy (Figure 3b), or by staining with malachite green counterstained with carbol fuchsin or safranin. *Bacillus mycoides* produces characteristic rhizoid or hairy-looking, adherent colonies which readily cover the whole agar surface.

A strain differentiation system for *B. cereus* based on flagellar (H) antigens is available at the Food Hygiene Laboratory, Central Public Health Laboratory (Colindale, London, UK), for investigations of food-poisoning outbreaks or other *B. cereus*-associated clinical problems (see Antigens, above). *B. thuringiensis* strains are also classified on the basis of their flagellar antigens; 82 serovars have been recognized (see Antigens, above). This is carried out at the Pasteur Institute, Paris, France, and at Abbott Laboratories, North Chicago, Illinois.

The enterotoxin complex responsible for the diarrheal type of *B. cereus* food-poisoning has been increasingly well characterized (Granum 2002). Two commercial kits are available for its detection in foods and feces, the Oxoid BCET-RPLA (Oxoid Ltd, Basingstoke, UK, Product Code TD950) and the TECRA VIA (TECRA Diagnostics, Roseville, NSW, Australia, Product Code BDEVIA48). However, these kits detect different antigens, and there is some controversy about their reliabilities. Other assays, based on tissue culture, have also been developed (Fletcher

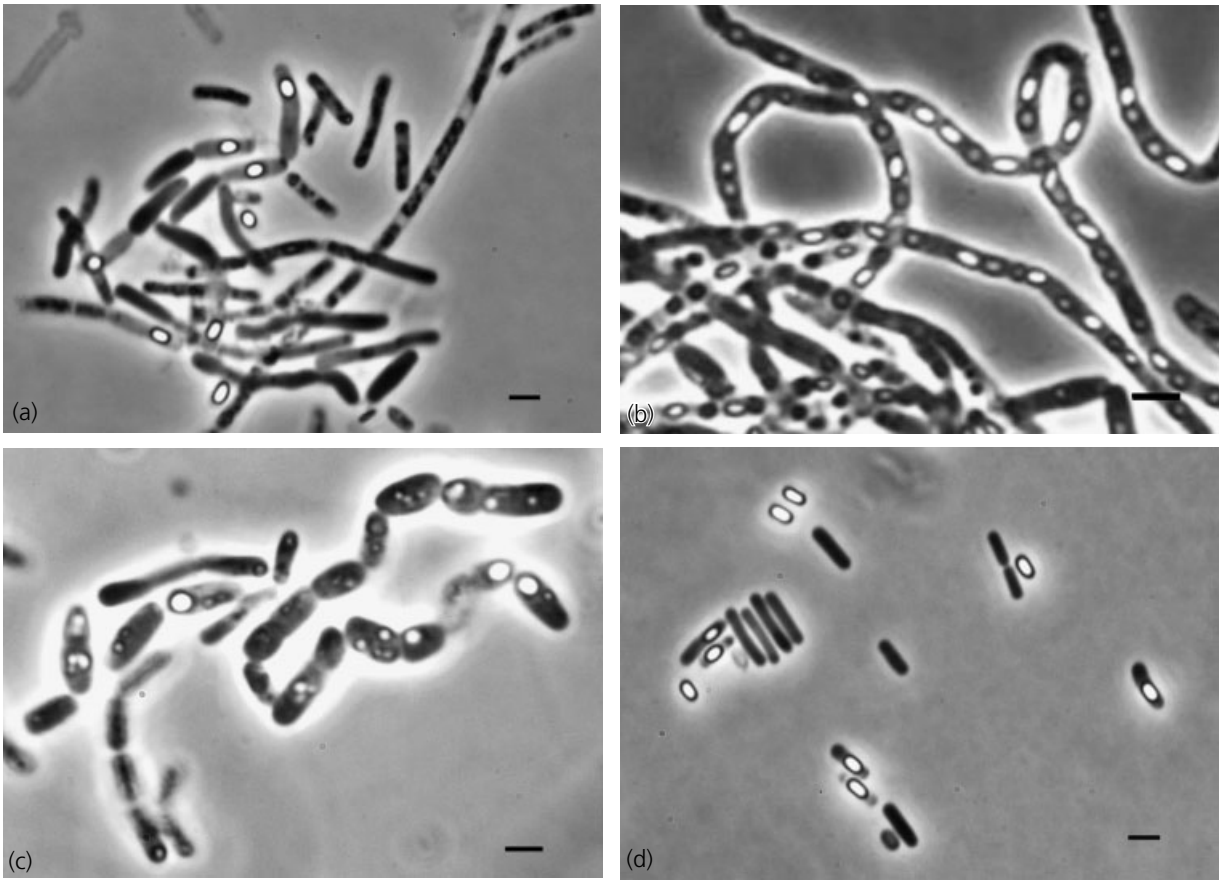

Figure 3 *Photomicrographs of endospore-forming bacteria viewed by phase contrast microscopy. Bar markers represent 2 μm.* **(a)** B. cereus*, broad cells with ellipsoidal, paracentral and subterminal spores, not swelling the sporangia and showing some poly-β-hydroxybutyrate inclusions, which are smaller and less phase-bright than the spores;* **(b)** B. thuringiensis*, broad cells with ellipsoidal, subterminal spores, not swelling the sporangia, and showing parasporal crystals of insecticidal toxin, which are less phase-bright than the spores;* **(c)** B. megaterium*, broad cells with ellipsoidal to spherical, subterminal and terminal spores, not swelling the sporangia, and showing poly-β-hydroxybutyrate inclusions, which are smaller and mostly less phase-bright than the spores;* **(d)** B. subtilis*, ellipsoidal, central and subterminal spores, not swelling the sporangia. (Continued over).*

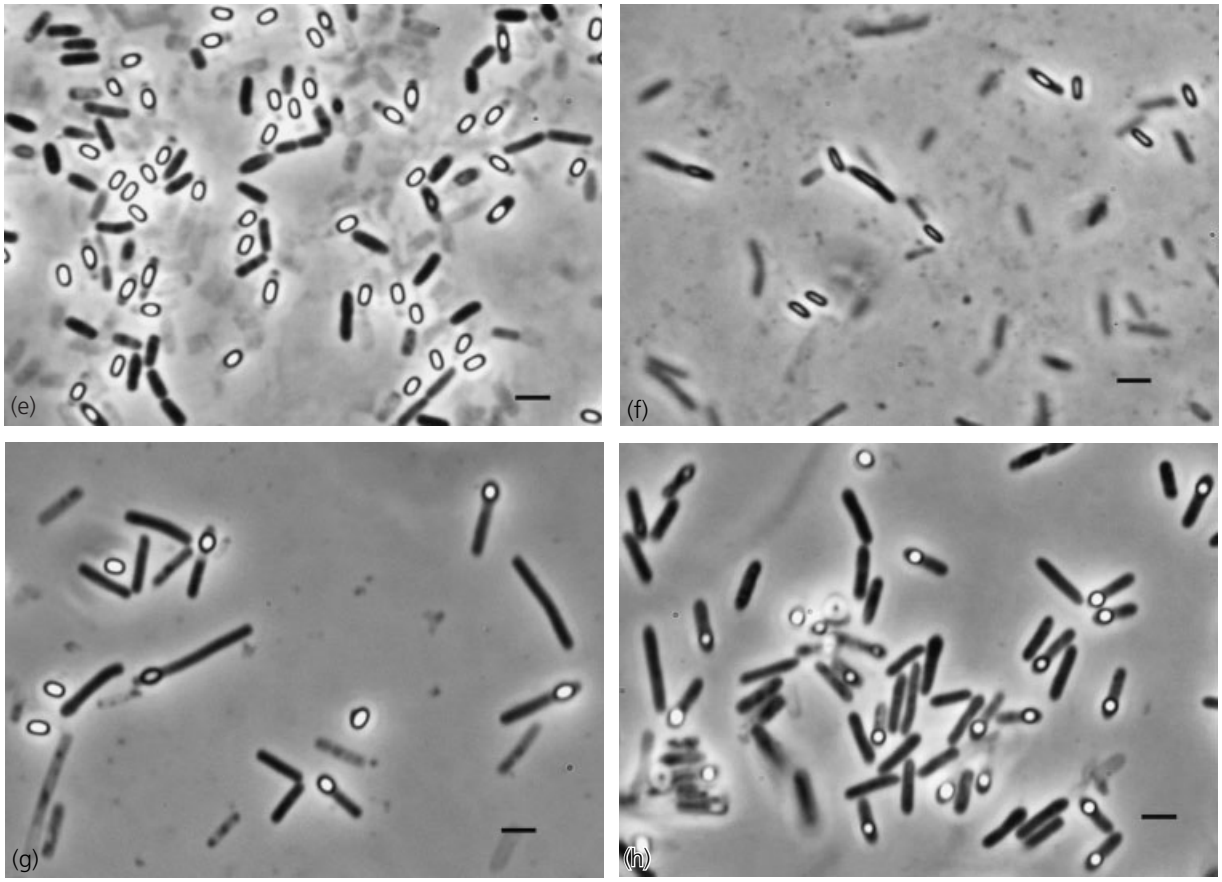

Figure 3 *Photomicrographs of endospore-forming bacteria viewed by phase contrast microscopy (Continued) Bar markers represent 2 μm.* **(e)** B. licheniformis*, some chaining of cells evident; ellipsoidal, central and subterminal spores, not swelling the sporangia;* **(f)** B. pumilus*, slender cells with cylindrical subterminal spores, not swelling the sporangia;* **(g)** B. circulans*, ellipsoidal, subterminal spores, swelling the sporangia;* **(h)** B. sphaericus*, spherical, terminal spores, swelling the sporangia. (Continued over).*

and Logan 1999). The emetic toxin of *B. cereus* has been identified, and it may be assayed in food extracts or culture filtrates using HEp-2 cells (Finlay et al. 1999).

Routine characters for distinguishing between members of the *B. cereus* group are given in Table 1.

Treatment

Despite the well-established importance of *Bacillus cereus* as an opportunistic pathogen, there have been rather few studies of its antibiotic sensitivity, and most information has to be gleaned from the reports of individual cases or outbreaks. *Bacillus cereus* and *B. thuringiensis* produce a broad spectrum β-lactamase and are thus resistant to penicillin, ampicillin, and cephalosporins; they are also resistant to trimethoprim (however, an Antarctic *B. thuringiensis* isolate was penicillin sensitive; Forsyth and Logan 2000). An in vitro study of 54 isolates from blood cultures by disk diffusion assay found that all strains were susceptible to imipenem and vancomycin and that most were sensitive to chloramphenicol, ciprofloxacin, erythromycin, and gentamicin (with 2, 2, 6, and 7 percent strains, respectively, showing moderate or intermediate sensitivities), while 22 and 37 percent of strains showed only moderate or intermediate susceptibilities to clindamycin and tetraclycline, respectively (Weber et al. 1988); in the same study, microdilution tests showed susceptibility to imipenem, vancomycin, chloramphenicol, gentamicin, and ciprofloxacin with MICs of 0.25–4, 0.25–2, 2.0–4.0, 0.25–2, and 0.25–1.0 mg/l respectively. A plasmid carrying resistance to tetracycline in *B. cereus* has been transferred to a strain of *B. subtilis* and stably maintained (Bernhard et al. 1978).

Although strains are almost always susceptible to clindamycin, erythromycin, chloramphenicol, vancomycin, and the aminoglycosides and are usually sensitive to tetracycline and sulphonamides, there have been several reports of treatment failures with some of these drugs: a fulminant meningitis which did not respond to chloramphenicol (Marshman et al. 2000); a fulminant infection in a neonate which was refractory to treatment that included vancomycin, gentamicin, imipenem, clindamycin, and ciprofloxacin (Tuladhar et al. 2000); failure of vancomycin to eliminate the organism from cerebrospinal fluid in association with a fluid shunt infection

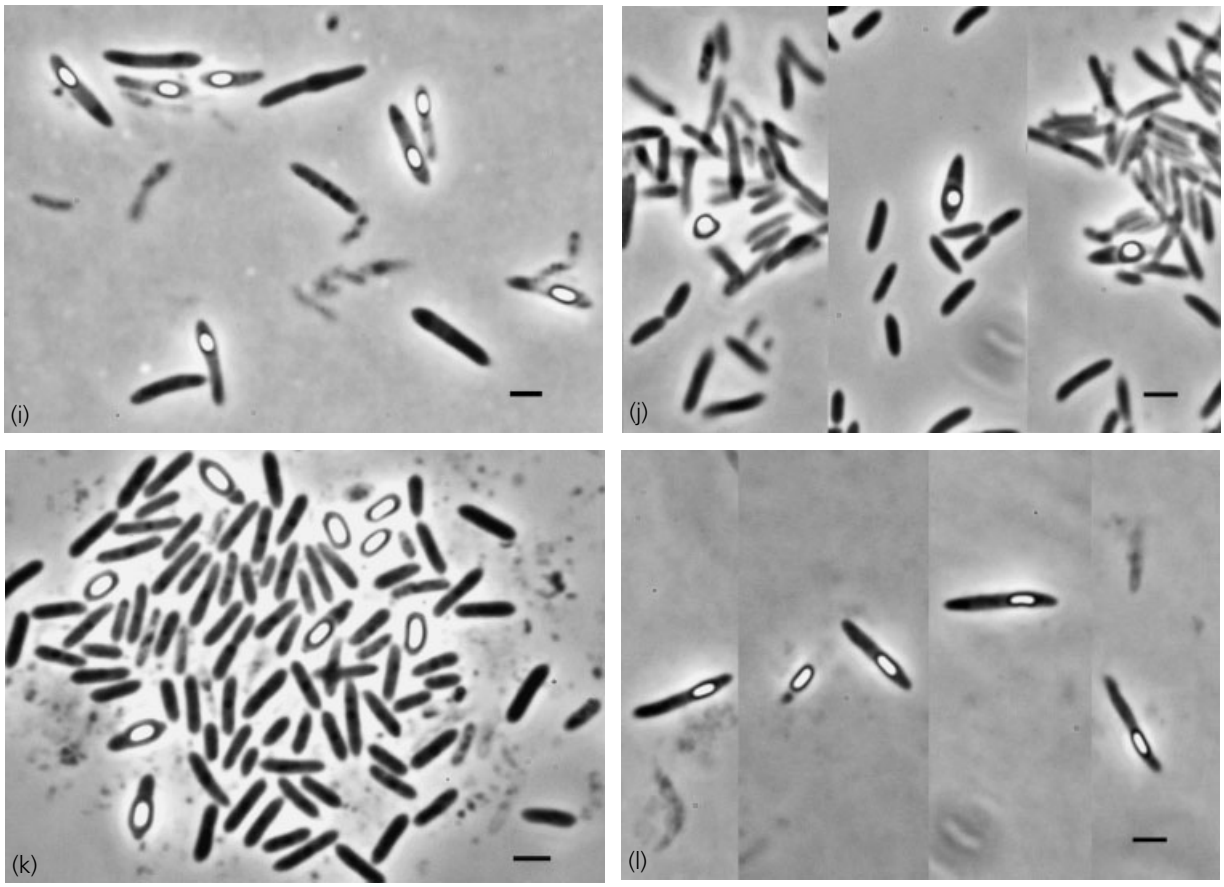

Figure 3 *Photomicrographs of endospore-forming bacteria viewed by phase contrast microscopy (Continued) Bar markers represent 2 μm.* **(i)** Brevibacillus brevis*, ellipsoidal, subterminal spores, swelling the sporangia;* **(j)** Brevibacillus laterosporus*, ellipsoidal, subterminal spores with thickened rims on one side, swelling the sporangia;* **(k)** Paenibacillus polymyxa*, ellipsoidal, subterminal spores, swelling the sporangia;* **(l)** Paenibacillus alvei*, cells with tapered ends, ellipsoidal to cylindrical subterminal spores, swelling the sporangium only slightly.*

(Berner et al. 1997); persistent bacteremias with strains showing resistance to vancomycin in two hemodialysis patients (A. von Gottberg and W. van Nierop, personal communication). Oral ciprofloxacin has been used successfully in the treatment of *B. cereus* wound infections. Clindamycin with gentamicin, given early, appears to be the best treatment for ophthalmic infections caused by *B. cereus*, and experiments with rabbits suggest that intravitreal corticosteroids and antibiotics may be effective in such cases (Liu et al. 2000).

Reports of infections with non-*B. cereus* group species are comparatively rare, but very diverse (Logan 1988; Berkeley and Logan 1997), and there have been several hospital pseudoepidemics associated with contaminated blood culture systems.

Bacillus licheniformis

This species has been reported from ventriculitis following the removal of a meningioma, cerebral abscess after penetrating orbital injury, septicemia following arteriography, bacteremia associated with indwelling central venous catheters (Blue et al. 1995), bacteremia during pregnancy with eclampsia and acute fibrinolysis, peritonitis in a continuous ambulatory peritoneal dialysis (CAPD) patient and in a patient with volvulus and small-bowel perforation, ophthalmitis, and corneal ulcer after trauma. There have also been reports of L-form organisms, phenotypically similar to *B. licheniformis*, occurring in blood and other body fluids of patients with arthritis, patients with neoplasms, clinically normal persons, and in association with infectious synovitis in birds (see Life cycle, above). Although some authors have claimed a relationship between these organisms and diseases with postulated immunological elements, and higher isolations from the synovial fluids and membranes of arthritic patients have been reported, Bartlett and Bisset (1981) were unable to confirm the latter association. *B. licheniformis* can cause food-borne diarrheal illness, and has been associated with an infant fatality (Mikkola et al. 2000). A toxin possibly associated with *B. licheniformis* food poisoning has been identified (Mikkola et al. 2000) but, in general, toxins or virulence factors widely accepted as responsible for symptoms periodically associated with *Bacillus* species

other than those in the *B. cereus* group have not been identified. *B. licheniformis* is frequently associated with bovine abortion and has been reproduced by experimental infection of cows, which demonstrated the tropism of the organism for the bovine placenta (Agerholm et al. 1997); this species has also been associated with abortion in water buffalo (Galiero and De Carlo 1998), and is occasionally associated with bovine mastitis (Blowey and Edmondson 1995). Many of these types of *B. licheniformis* and *B. cereus* infections are associated with wet and dirty conditions during winter housing, particularly when the animals lie in spilled silage (Blowey and Edmondson 1995); in one outbreak, a water tank contaminated with *B. licheniformis* was implicated (Parvanta 2000).

Bacillus subtilis

The name *B. subtilis* was often used to mean any aerobic, endospore-forming organism, but since 1970 there have been reports of infection in which identification of this species appears to have been made accurately. Cases associated with neoplastic disease include: fatal pneumonia and bacteremia, a septicemia, and an infection of a necrotic axillary tumor in breast cancer patients. Breast prosthesis and ventriculo-atrial shunt infections, endocarditis in a drug abuser, meningitis following a head injury, cholangitis associated with kidney and liver disease, and isolations from surgical wound-drainage sites have also been reported. A probiotic preparation of this species led to a fatal septicemia in an immunocompromised patient (Oggioni et al. 1998). *B. subtilis* has also been implicated in food-borne illness, and cases of bovine mastitis and of ovine abortion (Logan 1988; Berkeley and Logan 1997).

Other species

Organisms identified as *B. circulans* have been isolated from cases of meningitis, a cerebrospinal fluid shunt infection, endocarditis, endophthalmitis (Tandon et al. 2001), a wound infection in a cancer patient, and a bite wound (but see comments on this species in Identification, below), and *B. coagulans* has been isolated from corneal infection, bacteremia and bovine abortion. *B. pumilus* has been found in cases of pustule and rectal fistula infection, and in association with bovine mastitis. *B. sphaericus* has been implicated in a fatal lung pseudotumor, and meningitis (Logan 1988; Berkeley and Logan 1997).

Bacillus brevis has been isolated from corneal infection and has been implicated in several incidents of food poisoning; since these reports, the species was split (see Taxonomy, above) and transferred to the new genus *Brevibacillus*. Strains of the new species, *Brevibacillus agri*, have been isolated in association with an outbreak of waterborne illness in Sweden (Logan et al. 2002), and other *Brevibacillus* species have been found in human blood and bronchio-alveolar lavage specimens. *Br. laterosporus* has been reported in association with a severe case of endophthalmitis (Logan 1988; Berkeley and Logan 1997).

Paenibacillus alvei has been isolated from cases of meningitis, a prosthetic hip infection in a patient with sickle cell anemia, a wound infection, and, in association with *Clostridium perfringens*, a case of gas gangrene. *P. macerans* has been isolated from a wound infection following removal of a malignant melanoma, and from bovine abortion, and *P. polymyxa* from ovine abortion (Logan 1988).

Fish

There have been several reports of *Bacillus* infections among farmed fish. *Bacillus mycoides* was isolated from necrotic muscular lesions in channel catfish (*Ictalurus punctatus*) during an epizootic in a commercial pond in Alabama, USA, and similar lesions could be reproduced by subcutaneous injection of the isolate (Goodwin et al. 1994). An unidentified aerobic endospore-former was associated with a septicemic condition affecting a variety of widely cultivated freshwater fish in Nigeria, and its etiological role was confirmed by reinfection trials (Oladosu et al. 1994). Ferguson et al. (2001) isolated an unidentified *Bacillus* from a severe multi-focal, necrotizing and granulomatous infection of the intensively reared catfish *Pangasius hypophthalmus* in the Mekong delta; the condition was reproduced experimentally, but subsequently the pathogenicity of this organism was not demonstrable (Crumlish, M., personal communication).

Identification

Remember that these organisms do not always stain gram-positive. Before attempting to identify to species level it is important to establish that the isolate really is an aerobic endosporeformer, and that other inclusions are not being mistaken for spores. A Gram-stained smear showing cells with unstained areas suggestive of spores can be stripped of oil with acetone/alcohol, washed, and then stained for spores (see Appendix). Phase contrast (at 1000× magnification) should be used if available, as it is superior to spore-staining and more convenient. Spores are larger, more phase-bright, and more regular in shape, size, and position than other kinds of inclusion such as polyhydroxybutyrate (PHB) granules, and sporangial appearance is valuable in identification.

Members of the *B. cereus* group (see above) and *B. megaterium* (Figur 3a, c) will produce large amounts of storage material when grown on carbohydrate media,

but on routine media this vacuolate or foamy appearance is rarely sufficiently pronounced to cause confusion. Isolates of other organisms have often been submitted to reference laboratories as *Bacillus* species because they were large, aerobic gram-positive rods, even though sporulation had not been observed, or because PHB granules or other storage inclusions had been mistaken for spores.

Bacillus contains facultative anaerobes as well as strict aerobes, which can be a valuable characteristic in identification. For example, *B. licheniformis* and *B. subtilis*, which have very similar colonial (Figure 1j) and microscopic (Figure 3d, e) morphologies, are facultatively anaerobic and strictly aerobic, respectively; likewise, the two large-celled species *B. cereus* and *B. megaterium* are facultatively anaerobic and strictly aerobic, respectively.

The most widely used diagnostic schemes use traditional phenotypic tests (Gordon et al. 1973), or miniaturized tests in the API 20E and 50CHB Systems (bioMérieux, Marcy l'Etoile, France) (Logan, 2002; Logan and Berkeley, 1984). The API 20E/50CHB kits can be used for the presumptive distinction of *B. anthracis* from other members of the *B. cereus* group within 48 h. bioMérieux also offers a *Bacillus* card for the Vitek automated identification system. As many new species have been proposed since these schemes were established, updated API and Vitek databases are being prepared. Biolog (Biolog Inc., Hayward, California, USA) also offers a *Bacillus* database. The effectiveness of such kits can vary with the genera and species of aerobic endospore formers concerned, but they are improving with continuing development and increased databases (Logan 2002). It is stressed that their use should always be preceded by the basic characterization tests described below.

Other approaches include chemotaxonomic fingerprinting by fatty acid methyl ester (FAME) profiling, polyacrylamide gel electrophoresis (PAGE) analysis, pyrolysis mass spectrometry, and Fourier-transform infra-red spectroscopy. All these approaches have been successfully applied either across the genera or to small groups. As with genotypic profiling methods, large databases of authentic strains are necessary; some of these are commercially available, such as the Microbial Identification System software (Microbial ID. Inc., Newark, Del., USA) database for FAME analysis.

For diagnostic purposes, the aerobic endospore formers comprise two groups, the reactive ones that will give positive results in various routine biochemical tests, and which are therefore easier to identify, and the nonreactive ones which give few if any positive results in such tests. Nonreactive isolates, which are often members of the genus *Brevibacillus*, tend to dominate the identification requests sent to reference laboratories. When faced with aerobic endosporeformer contamination problems, bacteriologists in biotechnological industries frequently ask: 'Is it *Bacillus cereus* or *B. anthracis*, or is it another (and by implication, nonpathogenic) species?' It is probably fair to say that most routine laboratories that have the confidence to attempt the identification of an aerobic endosporeformer are well able to recognize members of the *B. subtilis* group and the *B. cereus* group. Once faced with a strain of *Brevibacillus* or *Paenibacillus*, however, it is a more difficult matter. Species of the former are usually inactive in routine biochemical tests – be they carbohydrate utilizations, or assimilations of carbohydrates, amino acids or organic acids as sole carbon sources – so that there are too few characters available to distinguish between the 11 species, most of which are very closely related. With *Paenibacillus*, the opposite problem arises; many of the species are highly active in the routine tests so that distinctions between them are confined to rather few variable characters. Table 1 shows reactions for some species belonging to both of these groups, and the phenotypic test schemes (Gordon et al. 1973; Logan and Berkeley 1984) outlined above may be used in conjunction with them. Characters for identifying other species may be found in Claus and Berkeley (1986), Heyndrickx et al. (1996, 1997, 1998) and Logan and Berkeley (1984).

The colonies of aerobic endosporeforming species outside the *B. cereus* group vary from moist and glossy (Figure 1d, f–i) through granular (Figure 1d) to wrinkled (Figure 1e, j); shapes vary from round to irregular, sometimes spreading (Figure 1k, l), with entire through undulate or crenate to fimbriate edges (Figure 1d–l). After 24–48 h incubation, colonial sizes of mesophilic strains typically range from 1–5 mm; color commonly ranges from buff or creamy-gray to off-white, but occasional strains may produce black, brown, orange, pink or yellow pigments; such pigmentation tends to be characteristic of species or subspecies. Elevations range from effuse through raised to convex. Consistency is usually butyrous, but mucoid and dry, adherent colonies are not uncommon. Hemolysis may be absent, slight (Figure 1h, i) or marked, partial or complete. *Bacillus subtilis* and *B. licheniformis* produce similar colonies which are exceptionally variable in appearance and often appear to be mixed cultures – the colonies are irregular in shape and of moderate (2–4 mm) diameter, and range in consistency from moist and butyrous or mucoid (with margins varying from undulate to fimbriate), through membranous with an underlying mucoid matrix (with or without mucoid beading at the surface), to a rough and crusty appearance as they dry (Figure 1j). The 'licheniform' colonies of *B. licheniformis* tend to be quite adherent. Rotating and migrating microcolonies (Figure 1k), which may show spreading growth, were observed macroscopically in approximately 13 percent of strains received as *B. circulans* (Logan et al. 1985) but this very heterogeneous species continues to undergo radical

taxonomic revision, with all spreading strains having been assigned to species within *Paenibacillus*. Motile, spreading microcolonies, which commonly have an unpleasant smell, are more typical of *Paenibacillus alvei* (Figure 1l).

The cells of aerobic endospore-formers may occur singly and in pairs, in chains (which may be of great length), and as filaments. Their rod-shaped cells are usually round-ended (the cells of members of the *B. cereus* group have often been described as squared), and *P. alvei* can produce frankly tapered ends (Figur 3l). Cell diameters range from 0.4 to 1.8 μm and lengths from 0.9 to 10.0 μm, but the cells of a particular strain are usually quite regular in size, and individual species normally have dimensions within fairly narrow limits. For example, cells of *B. pumilus* are typically 0.6–0.7 by 2.0–3.0 μm (Figure 3f), while those of *B. megaterium* are usually 1.2–1.5 by 2.0–5.0 μm (Figure 3c). Pleomorphism, showing as cells and filaments with swollen regions, and entirely swollen cells, may be observed in cultures grown in suboptimal conditions, and such stressed cultures sporulate poorly. Cytoplasm may stain uniformly or be vacuolate; vacuolation (the presence of inclusions is visible by phase-contrast microscopy as areas less refractive than spores, and in Gram stained preparations by unstained globules) is enhanced in some species (*B. cereus* and *B. megaterium*, for example; Figure 3a, c) by cultivation on an agar medium containing fermentable carbohydrate such as glucose, so that copious storage material is produced.

Sporangial morphologies (Figure 3) are characteristic of species, and so often valuable in identification, but an individual strain may show some variation and produce, for example, both oval and spherical spores (Figure 3c). The commonest spore shape is ellipsoidal or oval (Figure 3a, b, d, e, g, i, j, k), but shapes range from frankly cylindrical (Figure 3f, l) through ellipsoidal to spherical, and irregular forms such as kidney- or banana-shaped spores may be seen in some species. The position of the spore is also characteristic; the most frequently observed is a subterminal placement, and position can range from central through paracentral and subterminal to terminal. An individual strain may exhibit a range of spore positions. In small sporangia it is sometimes difficult to categorize spore positions with confidence. In just under half of the validly described *Bacillus* species the spores swell the sporangia slightly or appreciably, while in the remainder, sporangial swelling has not been observed, but both swollen and unswollen sporangia may be observed within a single strain. As already stated, sporangia of *B. thuringiensis* are characterized by their parasporal inclusions of crystalline protein (Figure 3b) known as δ-endotoxins, which are often toxic to insects and other invertebrates. Insecticidal strains of *B. sphaericus* also produce crystalline parasporal inclusions; these are less prominent than those of *B. thuringiensis*, but are generally visible with the aid of a good phase-contrast microscope (Priest 2002). *Brevibacillus laterosporus*, produces very distinctive ellipsoidal spores which have thickened rims on one side, so that they appear to be laterally displaced in the sporangia (Figur 3j).

Routine characters for distinguishing some of the aerobic endosporeformers most frequently encountered in clinical laboratories are given in Table 1.

Treatment

Information is sparse on treatment of infections with species outside the *B. cereus* group. Gentamicin was effective in treating a case of *B. licheniformis* ophthalmitis and cephalosporin was effective against *B. licheniformis* bacteremia/septicemia. Resistance to macrolides appears to occur naturally in *B. licheniformis* (Docherty et al. 1981). *B. subtilis* endocarditis in a drug abuser was successfully treated with cephalosporin, and gentamicin was successful against a *B. subtilis* septicemia. Penicillin, or its derivatives, or cephalosporins, probably form the best first choices for treatment of infections attributed to other *Bacillus* species. In the study by Weber et al. (1988), isolates of *B. megaterium* (13 strains), *B. pumilus* (4), *B. subtilis* (4), *B. circulans* (3), *B. amyloliquefaciens* (2) and *B. licheniformis* (1), along with five strains of *B. polymyxa* (now *Paenibacillus*) and three unidentified strains from blood cultures, over 95 percent of isolates were susceptible to imipenem, ciprofloxacin, and vancomycin; while between 75 and 90 percent were susceptible to penicillins, cephalosporins, and chloramphenicol. Isolates of *B. polymyxa* and *B. circulans* were more likely to be resistant to the penicillins and cephalosporins than strains of the other species – it is possible that some or all of the strains identified as *B. circulans* might now be accommodated in *Paenibacillus*, along with *B. polymyxa*. An infection of a human bite wound with an organism identified as *B. circulans* did not respond to treatment with amoxicillin and flucloxacillin, but was resolved with clindamycin (Goudswaard et al. 1995). A recurrent septicemia with *B. subtilis* in an immunocompromised patient yielded two isolates, both of which could be recovered from the probiotic preparation that the patient had been taking; one isolate was resistant to penicillin, erythromycin, rifampin, and novobiocin, while the other was sensitive to rifampin and novobiocin but resistant to chloramphenicol (Oggioni et al. 1998). The strains were later identified as *B. clausii* (Spinosa et al. 2000).

A strain of *B. circulans* showing vancomycin resistance has been isolated from an Italian clinical specimen (Ligozzi et al. 1998). Vancomycin resistance was reported for a strain of *B. popilliae* (now *Paenibacillus*) in 1965, and isolates of this species dating back to 1945 have been shown to carry a *vanA*- and *vanB*-like gene, that is to say a gene resembling those responsible for

high-level vancomycin resistance in enterococci. Vancomycin resistant enterococci (VRE) were first reported in 1986, and so it has been suggested that the resistance genes in *B. popilliae* and VRE may share a common ancestor, or even that the gene in *B. popilliae* itself may have been the precursor of those in VRE; *B. popilliae* has been used for over 50 years as a biopesticide, and no other potential source of *vanA* and *vanB* has been identified (Rippere et al. 1998). Of two South African vancomycin-resistant clinical isolates, one was identified as *P. thiaminolyticus* and the other was unidentified but considered to be related to *B. lentus* (Forsyth and Logan, unpublished); the latter was isolated from a case of neonatal sepsis, and has been shown to have inducible resistance to vancomycin and teicoplanin; this is in contrast to the *B. circulans* and *P. thiaminolyticus* isolates mentioned above, in which expression of resistance was found to be constitutive (A. von Gottberg and W. van Nierop, personal communication).

APPENDIX: STAINS

Spore stain

Prepare a heat-fixed smear as for Gram staining, and flood the slide with 10 percent aqueous malachite green. Leave for 40–45 min without heating, then rinse with tapwater and counterstain with 0.5 percent aqueous safranin for 30 s. View at 1000× magnification; spores are stained green, and cells and cellular debris pink-red.

M'Fadyean stain

As *Bacillus anthracis* is suspected, safety precautions must be taken throughout this procedure; all materials coming into contact with the specimen, including spent reagents and rinsings, must either be discarded into a disinfectant effective against endospores or autoclaved.

Make a thin smear from the specimen, and also from a positive control, on a clean slide and allow to dry. Fix by immersion in 95 percent or absolute alcohol for 30–60 s. Put a large drop (approximately 50 µl) of polychrome methylene blue (M'Fadyean stain) on the smear and ensure all the smear is covered by spreading the stain with an inoculating loop ('flooding' the slide is wasteful, unnecessary and environmentally undesirable). Leave for 1 min and wash the stain off with water (into 10 percent hypochlorite solution). Blot the slide and allow to dry. At 100–400× magnification, the organisms can be seen as fine short threads; at 1000× magnification (oil immersion), if virulent *B. anthracis* is present, the capsule should be seen as a clearly demarcated zone around the blue-black, often square-ended rods which lie in short chains of two to a few cells in number.

The positive control can be prepared by culturing a virulent strain in defibrinated horse blood as described in the text.

India ink negative stain for capsules

Place a large loopful of undiluted India ink on a clean slide, and mix in a small portion of the bacterial colony or a small loopful of the deposit from a centrifuged liquid culture. Drop a clean cover glass on, avoiding air bubbles, and press firmly between two sheets of blotting paper. When examined at 1000× under oil, the capsules appear as haloes around the highly refractive outlines of the bacterial cells. When capsules are absent, the ink particles directly abut the cell wall, and the cells are not easily seen. Phase-contrast is superior to bright-field microscopy, as the bacterial cells can be seen clearly in all cases.

APPENDIX: MEDIA

Bacillus cereus media (van Netten and Kramer 1992)

1. *Bacillus cereus* Medium (BCM), g/l: D-mannitol, 10.0; $(NH_4)_2PO_4$, 1.0; yeast extract, 0.2; KCl, 0.2; $MgSO_4.7H_2O$, 0.2; bromcresol purple, 0.04; agar, 20.0; adjust to pH 7.0 before autoclaving. Mix ingredients and heat to dissolve, then autoclave at 121°C for 15 min. Cool to 50°C and add 100 ml of 20 percent egg yolk emulsion. Mix gently then pour into Petri dishes.
2. Mannitol–yolk–polymyxin agar (MYP) g/l: D-mannitol, 10.0; peptone, 10.0; NaCl, 10.0; meat extract, 1.0; phenol red, 0.025; agar, 15.0; adjust to pH 7.1 before autoclaving. Mix ingredients and heat to dissolve, then autoclave at 121°C for 15 min. Cool to 50°C and add 10 mg/l polymyxin B sulphate (as filter sterilized solution) and 100 ml of 20 percent egg yolk emulsion per liter of base medium. Mix gently then pour into Petri dishes. This medium is commercially available from Difco.
3. Polymyxin–egg yolk–mannitol–bromthymol blue agar (PEMBA) g/l: D-mannitol, 10.0; peptone, 1.0; sodium pyruvate, 10.0; Na2HPO4, 2.5; NaCl, 2.0; KH_2PO_4, 0.25; $MgSO_4.7H_20$, 0.1; bromthymol blue, 0.1; agar, 18.0; adjust to pH 7.4 before autoclaving. Mix ingredients and heat to dissolve, then autoclave at 121°C for 15 min. Cool to 50°C and add 100 000 U polymyxin B sulphate (as filter sterilized solution) and 100 ml of 20 percent egg yolk emulsion per litre of base medium. Mix gently then pour into Petri dishes. The base medium, egg yolk emulsion, and polymyxin supplement are commercially available from Oxoid.

Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar, for isolation of *Bacillus anthracis* from environmental specimens (Knisely 1966)

Difco heart infusion agar (or Difco heart infusion broth with other agar base) is made up according to the manufacturer's instructions. EDTA (0.3 g/l) and thallous acetate (0.04 g/l) are added before autoclaving. (Note: thallous acetate is poisonous; avoid skin contact and inhalation by wearing gloves and weighing out the powder in a fume cupboard).

After autoclaving at 121°C for 15 min, the agar is cooled to 50°C and polymyxin (30 000 units/l) and lysozyme (300 000 units/l) are added in filter-sterilized solution. It is important that the agar is left at 50°C for long enough to ensure that this temperature has been reached throughout the medium before the polymyxin and lysozyme are added. After swirling to ensure even suspension of the ingredients, the agar is poured into Petri dishes.

Spread plates are prepared and incubated at 37°C for 36–48 h. Colonies of *B. anthracis* are smaller and smoother than on plain heart infusion agar, and *B. cereus* is generally inhibited by the medium.

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NOTES

1. Enquiries about gamma phage and indirect fluorescent antibody capsule staining should be addressed to the Diagnostics Systems Division, USAMRIID, Fort Detrick, Frederick, MD 21702-5011, USA.

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Listeria

JAMES McLAUCHLIN

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GENUS/SPECIES DEFINITION

Listeria are coccobacillary- to bacillus-shaped gram-positive organisms. They are nonsporing and motile by peritrichate flagella. They are aerobic and microaerophilic organisms that grow between <0 and 45°C. The organisms exhibit fermentative activities on carbohydrates, producing predominantly lactate and no gas from glucose. *Listeria* spp. are catalase-positive and oxidase-negative. The genus *Listeria* consists of six species: *monocytogenes*, *grayi*, *innocua*, *ivanovii*, *seeligeri*, and *welshimeri*. *L. monocytogenes* is the major pathogen for a wide variety of animals, including man, causing septic lesions in various organs. The G + C content of DNA is 36–42 mol% and the type species is *L. monocytogenes*. Sequences of the complete genomes of *L. monocytogenes* and *L. innocua* are available.

INTRODUCTION AND HISTORICAL PERSPECTIVE

In England in 1926, Murray, Webb, and Swann described a disease of rabbits and guinea pigs characterized by a marked mononuclear leukocytosis caused by a gram-positive bacillus, which they termed *Bacterium monocytogenes* (Murray et al. 1926). Pirie (1927) isolated the same bacterium from infected wild gerbils in South Africa and proposed the generic name *Listerella* in honor of the surgeon Lord Lister; later this was changed, for reasons of accurate nomenclature, to *Listeria* (Pirie 1940a, b).

In Denmark, Nyfeldt (1929) isolated *L. monocytogenes* from the blood cultures of patients with a mononucleosis-like infection (a rare manifestation of the disease); Burn (1936), in the USA, established listeriosis as a cause of both sepsis during the perinatal period and of meningitis in adults. Prior to 1926 there were published descriptions of disease likely to have been listeriosis; indeed, a 'diphtheroid' isolated from the cerebrospinal fluid of a soldier in Paris in 1919 was later identified as *L. monocytogenes* (Cotoni 1942). Listeriosis remained a relatively obscure disease, attracting limited attention until the 1980s, when a rise in the total numbers of human and animal cases in Europe and North America and a series of human food-borne outbreaks led to much renewed interest in the disease and the causative organism (Farber and Peterkin 1991). The number of reported human listeriosis cases has subsequently declined in Europe and North America (Tappero et al. 1995; McLauchlin 1996a, b; Smerdon et al. 2001; Goulet et al. 2001), although large food-borne outbreaks continue to occur (Anon 1999, 2000, 2001; Aureli et al. 2000; Maijala et al. 2001).

Listeriosis occurs in various animals, including humans, and most often affects the uterus at pregnancy, the central nervous system, or the bloodstream. During pregnancy, infection spreads to the fetus, which will either be born severely ill or die in utero. Listeriosis

usually presents as meningitis, encephalitis, or septicemia in non-pregnant individuals. In humans, infection occurs most often in the immunocompromised and elderly patients, pregnant women, and unborn or newly delivered babies. Infection can be treated successfully with antibiotics, but human infection has a mortality of 20–40 percent (Farber and Peterkin 1991). In domestic animals (especially in sheep and goats), listeriosis usually presents as encephalitis, abortion, or septicemia and is a cause of considerable economic loss (Low and Donachie 1997; McLauchlin and van der Mee-Marquet 1998). Consumption of contaminated food or feed is believed to be the principal route of infection (Farber and Peterkin 1991; Ryser and Marth 1998). However, in humans, infection can be transmitted, albeit rarely, by direct contact with the environment or infected animals (McLauchlin and Low 1994; O'Driscoll et al. 1999) or by cross-infection during the neonatal period (Schlech 1991).

L. monocytogenes is the major pathogenic species in both animals and humans (McLauchlin 1997a). However, in humans, occasional infections due to *L. ivanovii* (Cummins et al. 1994) and *L. seeligeri* (Rocourt et al. 1987a) have been reported. *L. ivanovii* infections may account for a significant proportion of cases of listeriosis in domestic animals, especially in sheep (Low and Donachie 1997). Rare infections due to *L. innocua* in domestic animals have occurred (Walker et al. 1994). *L. welshimeri* and *L. grayi* have not been shown to cause disease.

A more exhaustive account of the genus and the disease may be found in reviews by Gray and Killinger (1966), Seeliger and Jones (1986), Farber and Peterkin (1991), Bortolussi and Schlech (1995), McLauchlin (1996a, b), Low and Donachie (1997), Ryser and Marth (1998), Hof (2001), Vazquez-Boland et al. (2001), and Kathariou (2002).

HABITAT

Listeria are found ubiquitously in the environment and are distributed worldwide. Species of *Listeria* have been isolated from fresh water, wastewater, mud, and soil, especially when decaying vegetable material is present. Pirie (1927) first suggested that listeriosis may be transmitted through food, and since this route is now generally believed to be the principal route of infections for both humans and other animals (Farber and Peterkin 1991; Low and Donachie 1997), the distribution of *Listeria* in the environment is of importance in the transmission of the disease.

An extremely wide range of animals (mammals, birds, fish, and invertebrates) has been reported to carry *Listeria* spp. without apparent disease. In humans, carriage rates in feces of <3 percent for *Listeria* spp. and <1 percent for *L. monocytogenes* are most commonly reported (MacGowan et al. 1994; Oakley et al. 1992; Jensen 1993). Carriage in the gut is probably transitory (Cobb et al. 1996). Carriage of *L. monocytogenes* in the genital tract in the absence of disease is very rare (Oakley et al. 1992). *Listeria* spp. have been isolated from the upper respiratory tract and from seminal fluid of healthy animals (Gray and Killinger 1966). Because of the occurrence of listeria in the gut of animals and its survival in the environment, it is not surprising that *Listeria* spp. have been isolated from sites contaminated with human sewage, sewage sludge, or animal slurry.

The association of consumption of contaminated food with both outbreaks and sporadic human cases (Farber and Peterkin 1991) has resulted in much interest in food-borne listeriosis. *L. monocytogenes* has been isolated from a very wide range of foods for human consumption, including raw and processed meat, dairy products, vegetables, and seafood products (Farber and Peterkin 1991), and from food-production and storage environments (Cox et al. 1989). The ability to grow at refrigeration temperatures and tolerate preserving agents makes listeria of particular concern if present in refrigerated foods that are consumed without further cooking. Listeria in end-product foods results either from incomplete eradication during processing, or from contamination from sites within the food production environment. Contamination of food directly from infected animals is probably rare; however, *L. monocytogenes* can cause mastitis in cows, from which large numbers of the bacteria can then be shed in the milk (Bourry et al. 1995).

The relationship between the feeding of silage to domestic animals and the development of listeriosis has long been realized (Gray 1960). This is of particular importance when the pH is greater than 5.5 and the silage is of poor quality or has had prolonged exposure to aerobic conditions (Low and Donachie 1997). Modern practices of producing silage in large polythene-covered bales ('big bale') favors the growth of *L. monocytogenes* in comparison with production in the more traditional clamps and may, in part, explain the recent apparent increase in the incidence of listeriosis in domestic animals in Britain (Low and Donachie 1997).

Although found as gut commensals, *Listeria* spp. show many of the characteristics of bacteria adapted to a saprophytic existence, i.e. growth at low temperature, tolerance of sodium chloride and alkaline conditions, resistance to desiccation, motility at temperatures less than 25°C, presence in soil and decaying vegetable material, and, where pathogenic, a failure to produce host-specific clinical syndromes. However, there are specific adaptations that allow invasion of, and multiplication in, eukaryotic cells by *L. monocytogenes* (see Pathogenicity and virulence factors); these may have evolved for survival in unicellular and multicellular soil organisms. The 'true' habitat for the genus *Listeria* may be that of decaying plant material with occasional transitory residence in the gastrointestinal tract of animals;

additional properties may allow the utilization of the intracellular environments of free-living eukaryotic soil organisms by *L. monocytogenes*.

MORPHOLOGY

Young cultures consist predominantly of coccobacillary forms 1–3 μm long and 0.5 μm broad. As cultures age, short chains are generated. The poles of the cells are blunt. Smooth forms are almost always seen on fresh isolation; however, in older cultures (3–5 days) and with isolates subcultured on artificial media over many years, 'rough' forms with filamentous cells (6–20 μm long) may occur. Cultures are nonmotile at 37°C, but at temperatures <30°C, cells are motile by means of peritrichous flagella, with up to six flagella on each individual cell (Peel et al. 1988a, b). In hanging-drop preparations, characteristic tumbling movements are seen. Motility may also be demonstrated by the clouding of medium approximately 1–2 mm below the surface in soft agar in an 'umbrella-like' formation.

CULTURAL CHARACTERS

Listeria usually grow on most nonselective bacteriological culture media, especially when blood, serum, or a fermentable carbohydrate is added. For nonselective maintenance media, tryptose, tryptose soy, or brain–heart infusion agars are preferred. When cultured overnight on solid media containing blood, round, translucent colonies 1–2 mm in diameter with a 'ground-glass' crystalline appearance in the center are formed. The colonies are slightly raised, have an entire margin and a watery consistency, are easily emulsifiable, and are nonpigmented. The rough forms have larger, flatter colonies, with an irregular indented edge and are umbonate with a central crater, friable, and difficult to emulsify. All cultures have a characteristic sweet, buttermilk-like smell.

When growing on blood-containing agars, colonies of *L. monocytogenes* are surrounded by a narrow zone of β-hemolysis with an indistinct margin. *L. seeligeri* has a similar appearance to *L. monocytogenes* but exhibits a weaker zone of hemolysis. Colonies of *L. ivanovii* are surrounded by a wider and clearer β-hemolytic zone, which takes on a double appearance after 36–48 h.

Growth in broth is usually poor, but is greatly improved by the addition of 0.5–1 percent glucose. In this medium the smooth forms give rise within 24 h to a fairly dense suspension; after some days the organisms settle to the bottom in floccules. The organisms multiply rapidly in milk (Pine et al. 1989).

METABOLISM

A source of carbohydrate is essential for the growth of listeria and a number of fully chemically defined media to support growth have been described (Trivett and Meyer 1971; Phan-Thanh and Gormon 1997). Growth occurs at temperatures between <0 and 45°C (Walker et al. 1990); optimal growth occurs between 30 and 35°C. *L. monocytogenes* is one of the few pathogenic bacteria that can grow at 4°C, with a maximum doubling time of about 1–2 days. Growth does not generally occur outside the pH range 5.5–9.6.

Under anaerobic conditions the catabolism of glucose by all *Listeria* spp. is homofermentative, i.e. lactate is produced exclusively (Pine et al. 1989). Under aerobic conditions, cell yields are considerably increased, and all species produce lactic, acetic, isobutyric, and isovaleric acids plus acetoin and pyruvate; there are differences between strains in the relative amounts of the organic acids produced (Pine et al. 1989). *L. monocytogenes* possesses enzymes of the Embden–Meyerhof pathway (Miller and Silverman 1959) and a split noncyclic citric acid pathway, which has an oxidative and a reductive portion (Trivett and Meyer 1971). It has been suggested (Jones 1975) that this latter pathway is important in biosynthesis but not for the net gain of energy, which is supplied by glycolysis. *Listeria* spp. are catalase-positive; however, under some conditions this activity may be weak (Jones 1975). The catalase enzyme is of the 'heme' type. Cytochromes *b*, a_1, *o*, and *d* are produced (Seeliger and Jones 1986). Menaquinones are the sole isoprenoid quinones, and all species contain menaquinone-7 with minor amounts of menaquinone-6 and menaquinone-5 (Seeliger and Jones 1986).

GENETIC MECHANISMS

DNA base composition studies (Rocourt et al. 1982; Hartford and Sneath 1993) and 16S and 23S rRNA sequence studies (Collins et al. 1991; Thompson et al. 1992; Sallen et al. 1996; Graham et al. 1997) show that all species of the genus *Listeria* form a homogeneous group and, together with the nonpathogenic species *Brochothrix thermospacta* and *Brochothrix campestris*, justify family status as the Listeriaceae (Collins et al. 1991). 16S rRNA sequence analysis shows relationships with other gram-positive genera of low G + C ratio, including members of the genus *Bacillus* (Collins et al. 1991). Indeed, the completed genome sequences of *L. monocytogenes* and *L. innocua* show a close relationship to that from *Bacillus subtilis* and suggest a common origin (Glaser et al. 2001).

Complete genomes are available for one *L. monocytogenes* and *L. innocua* strain (Glaser et al. 2001). The *L. monocytogenes* genome contains 2 944 528 base pairs with 2 853 protein-coding genes, and *L. innocua* with 3 011 209 base pairs with 2 973 protein-coding genes (Glaser et al. 2001). The genes involved with pathogenesis are almost all clustered at a single locus (see section on Disease mechanisms for the invasion of mammalian

cells). Limited sequence information is available at some loci for the remaining four *Listeria* species.

Transposons Tn*1545*, Tn*916*, and Tn*917* (and their derivatives) introduced and expressed in *L. monocytogenes* have proved to be extremely useful tools in understanding the basis of virulence in the bacterium (Vazquez-Boland et al. 2001; Kathariou 2002). Transfer of Tn*1545* has been demonstrated between *Enterococcus faecalis* and *L. monocytogenes* in the digestive tract of gnotobiotic mice (Doucet-Populaire et al. 1991).

Plasmid DNA has been detected in all species of *Listeria*, most of which are larger than 20 MDa. Almost all the larger plasmids in *L. monocytogenes* encode resistance to cadmium (Lebrun et al. 1994a, b): the proportion of cultures with these large plasmids varies markedly depending upon the serovar of *L. monocytogenes* (McLauchlin et al. 1997). Plasmid DNA was detected in the complete sequence of the *L. innocua* genome and was shown to be 54 MDa in size with 79 genes (Glaser et al. 2001). Smaller plasmids encoding for resistance to tetracycline alone (3 MDa; Charpentier and Courvalin 1999) and for multiresistance to chloramphenicol, erythromycin, streptomycin, and tetracycline (25 MDa; Charpentier and Courvalin 1999) have been detected, although these are rare. Trimethoprim resistance due to the *dfrD* gene has been detected encoded on a 2.5 MDa plasmid of *L. innocua* (Charpentier and Courvalin 1999). Two bacteriocins have been detected encoded on a 1.9 kDa plasmid of *L. innocua* (Kalmokoff et al. 2001). Transfer of native listerial plasmids has been demonstrated in vitro between strains of *L. monocytogenes*, to other *Listeria* species, and to other species of bacteria including *Bacillus subtilis*, *Enterococcus faecalis*, *Streptococcus agalactiae*, and *Staphylococcus aureus* (Charpentier and Courvalin 1999). Cloning vectors based on the plasmid replicons pC194 (from *S. aureus*; Sullivan et al. 1984) and pE194 (from *Enterococcus*; Chakraborty et al. 1992) have been used successfully for gene-cloning experiments in listeria.

Lysogenic phage are commonly carried by listeria (Audurier et al. 1977; Loessner et al. 1994). They are generally morphologically similar, with isometric heads and long noncontractile tails and correspond to the Myoviridae or Syphoviridae families (Rocourt et al. 1986; Loessner et al. 1994). The complete genome sequence of one lysogenic phage, A118, has been reported (Loessner et al. 2000) and phage integration was shown to occur in a homolog of the bacillus *comK* gene, although no known function has yet been determined for this gene in listeria. To date, no phage conversion has been reported due to toxin genes associated with prophage sequences. A lytic phage (A511) of the Myoviridae family with a double-stranded DNA genome of approximately 116 kbp has also been described (Loessner et al. 1994). Information on the phage genomes indicate that these are composed of linear double-stranded DNA of 35–116 kbp in size (Rocourt et al. 1986; Loessner et al. 1994) with G + C base compositions of 37–39 mol% (Loessner et al. 1994). Lytic properties of sets of phages have been used for subtyping *L. monocytogenes* (Rocourt et al. 1985). Bacteriocin (monocin) production is common within the genus (Curtis and Mitchell 1992; Bannerman et al. 1996; Kalmokoff et al. 2001).

In addition to transposon mutagenesis, plasmid complementation experiments, techniques to generate in-frame point mutations by allelic exchange, and the introduction of genetic material by electroporation have been used to study the genetics of *L. monocytogenes*. These techniques, together with the expression of *L. monocytogenes* genes in *L. innocua* and bacteria from other genera, and the behavior of *L. monocytogenes* either in mammalian tissue culture or in experimentally infected mice, have led to a great increase in the understanding of the genes involved with virulence (Vazquez-Boland et al. 2001). For further information see section on Disease mechanisms for the invasion of mammalian cells.

CELL-WALL AND ENVELOPE COMPOSITION; ANTIGENIC STRUCTURE

The cell wall of listeria has the appearance of a thick multilayered structure typical of gram-positive bacteria (Ghosh and Murray 1967). The peptidoglycan of all species of *Listeria* is of the A1γ type of Schleifer and Kandler (1972), which contains L-alanine, D-glutamine, *meso*-diaminopimelic acid (as the diamino acid) and D-alanine (Fiedler et al. 1984).

Ribitol teichoic acids and lipoteichoic acid are present in all species (Fiedler and Ruhland 1987). In *L. monocytogenes* serovar 4b the teichoic acid consists of chains of ribitol, phosphate, and *N*-acetylglucosamine, the *N*-acetylglucosamine substituted with glucose and galactose residues (Uchikawa et al. 1986a). In *L. monocytogenes*, the lipoteichoic acid consists of 16–33 glycerol phosphate units substituted with a glycosyl side chain and attached to a galactose and glucose containing glycolipid (Uchikawa et al. 1986b).

The plasma membrane of *Listeria* spp. shows the typical trilamellar appearance with mesosomes (Ghosh and Murray 1967). The major phosholipids present in *L. monocytogenes* are phosphatidylglycerol, diphosphatidylglycerol, galactosylglucosyl diacylglycerol, and an unidentified phosphoglycolipid (Kosaric and Carroll 1971; Shaw 1974). In all *Listeria* spp., 14-methylhexadecanoic (17:0a) and 12-methyltetradecanoic acid (15:0a) predominate, with minor amounts of other saturated fatty acids (Ninet et al. 1992). Mycolic acids are not present.

Paterson (1939, 1940a) used bacterial agglutination and adsorption experiments with hyperimmune antisera to recognize O (somatic) and H (flagellar) antigens, and described four serological types. The scheme was

extended further by Seeliger and Donker-Voet (Seeliger and Höhne 1979) to include additional O antigen factors. In the Seeliger–Donker-Voet scheme, *L. monocytogenes* is subdivided into 13 serovars, and the remaining five species are similarly subdivided (Table 1). Fiedler et al. (1984) suggested that teichoic acids represent the immunochemical determinants in the serotyping scheme because there is a direct correlation between the ribitol teichoic acid structure and serotypes and species of *Listeria*. The flagella protein has been partially characterized and comprises a 29-kDa moiety (Peel et al. 1988a, b).

Cell-surface and secreted proteins occur, some of which are involved with the virulence of *L. monocytogenes* and *L. ivanovii*: i.e. the p60 protein (60 kDa) with murein hydrolase activity that is also secreted into culture supernates; the ActA protein (90 kDa) that is preferentially produced on the older pole of the cell; and the internalin family of proteins, located over all the cell surface and that is reminiscent of the M protein of *Streptococcus pyogenes*. The production of many of the surface-associated virulence genes is temperature-dependent and these are expressed above 30°C. Many, but not all, of the surface-associated virulence genes characterized to date are under the control of the central virulence regulator PrfA (Vazquez-Boland et al. 2001). For further information on the role of these in the pathogenicity of *L. monocytogenes*, see section on Disease mechanisms for the invasion of mammalian cells. L forms have been reported in *L. monocytogenes* (Gray and Killinger 1966) and may have a role in infection.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

All strains are sensitive to ampicillin, and this, in combination with an aminoglycoside, remains the treatment of choice in vivo (MacGowan 1990). Apart from the reported plasmid-encoded resistance to chloramphenicol, erythromycin, streptomycin, and tetracycline (Charpentier and Courvalin 1999), strains are almost universally sensitive to amikacin, amoxycillin, ampicillin, azlocillin, ciprofloxacin, chloramphenicol,

Table 1 *Somatic and flagellar antigens used for serotyping* Listeria

Serovar	Somatic (O factor) antigens	Flagellar (H factor) antigens
L. monocytogenes		
1/2a	I II III	AB
1/2b	I II III	ABC
1/2c	I II III	BD
3a	II III IV	AB
3b	II III IV (XII) (XIII)	ABC
3c	II III IV (XII) (XIII)	BD
4a	III (V) VII IX	ABC
4ab	III V VI VII IX X	ABC
4b	III V VI	ABC
4c	III V VII	ABC
4d	III (V) VI VIII	ABC
4e	III V VI (VIII) (IX)	ABC
7	III XII XIII	ABC
L. ivanovii		
5	III (V) VI (VIII) X	ABC
L. innocua[a]		
4ab	III V VI VII IX X	ABC
6a	III V (VI) (VII) (IX) XV	ABC
6b	III (V) (VI) (VII) IX X XI	ABC
L. welshimeri		
6a	III V (VI) (VII) (IX) XV	ABC
6b	III (V) (VI) (VII) IX X XI	ABC
L. seeligeri[a]		
1/2b	I II III	ABC
4c	III V VII	ABC
4d	III (V) VI VIII	ABC
6b	III (V) (VI) (VII) IX X XI	ABC
L. grayi	III XII XIV	E

a) *L. seeligeri* and *L. innocua* also contain undesignated combination of O antigens. Factor III is heat-labile. Factors in parentheses denotes that these are not always present.

clindamycin, coumermycin, doxycycline, enoxacin, erythromycin, gentamicin, imipenem, netilmicin, penicillin, rifampicin, trimethoprim, and vancomycin. Norfloxacin and ofloxacin have less activity, and *Listeria* spp. are resistant to the cephalosporins, phosphomycin, and polymyxin (MacGowan 1990; Charpentier and Courvalin 1999).

Tetracycline is less active against *L. monocytogenes*, and 2–5 percent of strains are highly resistant (Charpentier and Courvalin 1999): this rate of resistance has not changed over the past 30 years in the UK (Threlfall et al. 1998). Most of these highly resistant cultures contain a chromosomally encoded *tetM* gene (together with a transposon, int-Tn), which also confers resistance to minocycline. The *tetM* gene has also been detected in *L. innocua* and *L. welshimeri*. Plasmid-encoded tetracycline resistance in *L. monocytogenes* is rarer than that encoded chromosomally. The latter is encoded by a *tetL* (minocycline-sensitive; Charpentier and Courvalin 1999), or a *tetS* determinant (minocycline resistant). The *tetS* gene was first described in *L. monocytogenes*, but it has since been detected in *L. innocua*, *L. welshimeri*, and *Enterococcus faecalis*. Plasmid-encoded resistance to chloramphenicol (*cat221*), erythromycin (*ermAM*), streptomycin (*aad6*), and tetracycline (*tetS*) has been detected in *L. monocytogenes*. Another resistance gene to tetracycline (*tetK*) and a streptomycin-resistance gene (*aad6*) have been detected in *L. innocua*. A resistance gene to erythromycin (*ermC*) has been detected in *L. monocytogenes* and *L. innocua* (Charpentier and Courvalin 1999).

CLASSIFICATION

The genus was originally described as containing only *L. monocytogenes* (Pirie 1940a, b), however, six species are now recognized (Skerman et al. 1980; Moore et al. 1985). The group of bacteria originally named *L. monocytogenes* (*L. monocytogenes* sensu lato) was redefined on the basis of DNA/DNA hybridization studies (Rocourt et al. 1982) to comprise *L. innocua* (Seeliger 1981), *L. welshimeri* (Rocourt and Grimont 1983), *L. seeligeri* (Rocourt and Grimont 1983), *L. ivanovii* (Seeliger et al. 1984), and *L. monocytogenes* sensu stricto. The genus includes one other species, *L. grayi* (Errebo Larsen and Seeliger 1966). This classification was supported by the results of a second DNA/DNA homology study (Hartford and Sneath 1993), multilocus enzyme electrophoresis analysis (Boerlin et al. 1991), and 16 and 23S rDNA sequence analysis (Collins et al. 1991; Sallen et al. 1996). The DNA/DNA homology study by Hartford and Sneath (1993) suggested a very close relationship between *L. monocytogenes* and *L. innocua*, and there may be some overlap between these two species.

On the basis of both multilocus enzyme electrophoresis (Piffaretti et al. 1989; Bibb et al. 1990) and restriction fragment and sequence analysis of specific genes (Rasmussen et al. 1991; Gutekunst et al. 1992; Vines et al. 1992; Ericsson et al. 1995; Comi et al. 1997; Wiedmann et al. 1997; Vines and Swaminathan 1998), two major genetic 'sublines' of *L. monocytogenes* have been identified. These 'sublines' correspond to the serotypes with H antigens ABC (serovars 1/2b, 3b, and 4b), and those with H antigens AB or BD (serovars 1/2a, 1/2c, 3a, and 3c). These two groups may represent subspecies of *L. monocytogenes*, although phenotypic characters have not been found to distinguish the groups. A further lineage within serogroup four has also been identified (Rasmussen et al. 1995; Wiedmann et al. 1997).

On the basis of both phenotypic and genotypic characters of listeria, *L. grayi* shows a more distant relationship (Seeliger and Jones 1986; Boerlin et al. 1991; Collins et al. 1991; Kämpfer et al. 1991; Hartford and Sneath 1993), but there is clear justification for the retention of *L. grayi* within the genus (Rocourt et al. 1987b; Collins et al. 1991). There are two subspecies of *L. grayi* (Rocourt et al. 1992): *L. grayi* subsp. *grayi* and *L. grayi* subsp. *murrayi* (previously named '*L. murrayi*') (Welshimer and Meredith 1971).

The species previously known as '*Listeria denitrificans*' (Prévot 1961) is not a member of the genus *Listeria* and has been reclassified in a new genus as *Jonesia denitrificans* (Rocourt et al. 1987c).

Despite the genomic heterogeneity within the genus *Listeria* outlined above, there are surprisingly few phenotypic characters that distinguish between the different species (see section on Identification of *Listeria* species and subspecies).

Analysis of sequence data from the 16S rRNA confirms the close relationship within the genus *Listeria* and indicates a close phylogenetic relationship (93 percent sequence similarity) with the genus *Brochothrix*. This latter genus comprises two species (*Brochothrix thermosphacta* and *Brochothrix campestris*), which have many phenotypic properties in common with listeria (Seeliger and Jones 1986; Sneath and Jones 1986; Collins et al. 1991). Members of the genus *Brochothrix* have not been implicated in disease but are of economic importance as food-spoilage organisms, particularly of meat products (Sneath and Jones 1986). 16S rRNA sequence analysis shows relationships with other gram-positive genera of low G + C mol content, including members of the genera *Bacillus*, *Enterococcus*, *Aerococcus*, *Lactobacillus*, *Leuconostoc*, and *Streptococcus* (Collins et al. 1991).The completed genome sequences of *L. monocytogenes* and *L. innocua* show a close relationship to that from *Bacillus subtilis* and suggest a common origin (Glaser et al. 2001).

LABORATORY ISOLATION AND IDENTIFICATION

Culture from blood or cerebrospinal fluid does not require special media. Tissues should be homogenized,

suspended in broth, and subcultured on to blood agar. The incubation of medulla homogenates at refrigeration temperatures for some weeks has been reported as necessary to obtain cultures that grow on artificial media (Gray and Killinger 1966). For specimens such as feces, vaginal secretions, food, and environmental samples, special selective media are necessary.

Selective isolation techniques

Prior to the mid-1980s, 'cold enrichment,' utilizing the ability of listeria to outgrow competing organisms at refrigeration temperatures in nonselective broths, was the main method used for selective isolation (Gray and Killinger 1966). When growing on transparent media illuminated by oblique transmitted light and viewed at low magnification ('Henry' illumination technique) all listeria colonies have a characteristic blue color with a central 'ground-glass' appearance (Gray 1957). However, because of the degree of skill required in recognizing characteristic colonies, the lack of specificity and the slowness of these methods (some workers subcultured broths for up to 6 months), procedures have been much improved.

Media have been developed that rely on a number of selective agents; these include: acriflavin, lithium chloride, colistin, ceftazidime, cefotetan, fosfomycin, moxolactam, nalidixic acid, cycloheximide, and polymyxin. Such media have resulted in the widespread ability of microbiology laboratories (especially those involved with the examination of foods) to selectively isolate listeria. Numerous enrichment and selective isolation media have now been developed. Those mentioned here (or modifications of these) are used most frequently for the examination of foods. For selective broths, the US Food and Drug Administration (FDA) method (Lovett et al. 1987), the US Department of Agriculture (USDA) method (McClain and Lee 1988), or the Netherlands Government Food Inspection Service (NGFIS) method described by Van Netten et al. (1989) are most often used. Selective agars most frequently used are those of Curtis et al. (1989a; 'Oxford' formulation) or the PALCAM agar of Van Netten et al. (1989). These media are listed in internationally agreed standard methods (Anon 1996, 1998).

The FDA method is a single enrichment broth containing acriflavin, nalidixic acid, and cycloheximide. The USDA method consists of a double enrichment using first a University of Vermont primary enrichment broth 1 (UVM1; containing esculin nalidixic acid and acriflavin), which is subcultured into a Fraser broth (containing esculin, nalidixic acid, lithium chloride, and acriflavin). The NGFIS method uses a single L-PALCAM (or liquid-PALCAM) broth, the name of which is an acronym of the ingredients, polymyxin B, acriflavine, lithium chloride, ceftazidime, aesculin, and mannitol.

After incubation the selective broths are subcultured on to selective agars, most usually PALCAM agar (containing the same agents as L-PALCAM broth) or Oxford agar. The latter agar contains esculin, lithium chloride, cycloheximide, colistin, acrifalvin, cefotetan, and fosfomycin. On Oxford agar all *Listeria* spp. show a typical colonial appearance and hydrolyze esculin, to produce black zones around the colonies. On PALCAM agar the colonies have a similar appearance but with a cherry-red background. It is beyond the scope of this contribution to describe the preparation of these media; for further details see Baird et al. (1987). For comparisons of the efficiency of the different selective techniques for the isolation of listeria from foods see Warburton et al. (1991) and Hayes et al. (1992).

All *Listeria* species are isolated by these methods and are morphologically indistinguishable from each other. However Curtis et al. (1989b) reported differences in the minimal inhibitory concentrations of selective agents used in microbiological media and found that *L. ivanovii* and *L.seeligeri* were more sensitive to fosfomycin than *L. innocua*, *L. monocytogenes*, and *L. welshimeri*. Consequently, colonies of *L. ivanovii* and *L. seeligeri* grow more slowly and appear smaller on Oxford agar, which uses fosfomycin as a selective agent.

To differentiate *L. monocytogenes* from other *Listeria* species on selective agars, substrates have been added to selective media to detect phospholipase (Notermans et al. 1991) or β-glucosidase and enhanced hemolysis (Beumer et al. 1997). Selective media, based on lipase and β-glucosidase activity, which successfully differentiate *L. monocytogenes* from populations of other *Listeria* species, are commercially available (Vlaemynck et al. 2000).

Noncultural techniques such as those based upon immunoassays and the polymerase chain reaction (PCR) are used increasingly for the detection of listeria in enrichment broths for the examination of foods.

Identification of the genus *Listeria*

In addition to the properties outlined in Table 2, *Listeria* spp. produces acid without gas from a range of sugars. The choice of basal medium and pH indicator to demonstrate the production of acid is important; peptone water with phenol red or bromocresol purple as indicator and adjusted to pH 7 is recommended (Rocourt et al. 1983; Seeliger and Jones 1986). Almost all cultures of listeria produce acid from glucose, trehalose, salicin, amygdalin, cellobiose, aesculin, fructose, mannose, maltose, and glycerol. Acid is not produced from adonitol, dulcitol, erythritol, inulin, α-methyl-D-glucoside, raffinose, sorbose, arabinose, glycogen, or melibiose. Aesculin is hydrolysed. Litmus milk is acidified and slowly decolorized. The methyl red reac-

Table 2 *Differential characteristics of* Listeria *species*

	β-Hemolysis on blood sugar	Arylesterase activity	Acid produced from				CAMP test with	
			D-Mannitol	L-Rhamnose	D-Xylose	α-MM	*S. aureus*	*R. equi*
L. monocytogenes	+	–	–	+[a]	–	+	+	–
L. ivanovii	+	+	–	–	+	–	–	+
L. innocua	–	+	–	±[b]	–	+	–	–
L. welshimeri	–	+	–	±	+	+	–	–
L. seeligeri	(+)	+	–	–	+	±	(+)	–
L. grayi	–	+	+	±	–	NS	–	–

α-MM, α-methyl-D-mannoside (methyl α-D-mannopyranoside); ±, variable, not all strains able to perform this reaction; (+), weak reaction. NS, not stated;

a) Some strains do not ferment rhamnose, but these are rare.

b) Approximately 60 percent ferment rhamnose. Strains of *L. monocytogenes* occur that are nonhemolytic and do not produce a positive CAMP reaction. These are rare and probably avirulent.

tion and Voges–Proskauer (VP) test (O'Meara's or Barritt's method; Barrow and Feltham 1993) are positive. Catalase is produced, and the oxidase test is negative. H_2S, indole, and urease are not produced and there is no growth in citrate medium. Ornithine, lysine, glutamic acid, arginine decarboxylases, and arginine dihydrolase are not produced. Phosphatase is produced and methylene blue is decolorized. Tributyrinase activity is negative, and Tweens 20, 40, 60, and 80 are hydrolysed only slowly (Seeliger and Jones 1986).

From human clinical samples collected from normally sterile sites, almost all isolates will be *L. monocytogenes*. Hence, a simple presumptive identification scheme comprises of characteristic non-pigmented colonies with a ground-glass appearance; hemolytic on blood agar; gram-positive regular rods that stain evenly; catalase-positive; and characteristic tumbling motility at room temperature.

Identification of *Listeria* species and subspecies

Members of the genus *Listeria* are remarkably similar in their phenotypic characters (Seeliger and Jones 1986; Kämpfer et al. 1991). Rocourt et al. (1983) showed that the species of *Listeria* could be distinguished by the results of a small number of tests, and these, together with other tests to identify the members of this genus, are shown in Table 2.

Kämpfer and colleagues (Kämpfer et al. 1991; Kämpfer 1992) showed that *L. monocytogenes* could be distinguished from other members of the genus by the absence of an arylesterase active against alanine-substituted substrates. A simple 4-h protocol, based on the hydrolysis of DL-alanine-β-naphthylamide, which distinguishes *L. monocytogenes* from other *Listeria* spp., has been described (Clark and McLauchlin 1997; McLauchlin 1997b). Arylesterase activity on glycine-substituted substrates may be used for the identification of listeria (Monget 1992) and this reaction is used in a commercially available identification kit (Bille et al. 1992). The detection of phospholipase has been advocated for the discrimination of *L. monocytogenes* and *L. ivanovii* from the rest of the genus *Listeria* (Coffey et al. 1996).

On the basis of multilocus enzyme electrophoresis, DNA–DNA hybridization and rRNA restriction patterns, two subspecies of *L. ivanovii* have been described: *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Boerlin et al. 1992). These subspecies can be distinguished on the basis of ribose and *N*-acetyl-β-D-mannosamine fermentation.

There are two subspecies of *L. grayi* (Rocourt et al. 1992): *L. grayi* subs *grayi* and *L. grayi* subs *murrayi* (previously named '*L. murrayi*;' Welshimer and Meredith 1971). These subspecies can be distinguished on the basis of nitrate reduction, Tween 80 esterase activity, and fermentations of α-methyl-D-glucoside, D-lyxose, or D-turanose.

TYPING METHODS

The considerable recent interest in *L. monocytogenes* and in the epidemiology of listeriosis has resulted in the development of discriminatory subtyping methods. These methods utilize a range of phenotypic and genotypic characters. These methods have been evaluated in a multicenter international study (Bille and Rocourt 1996).

The combination of genetic and phenotypic methods has offered typing systems of the highest discrimination (Bille and Rocourt 1996). However, these systems require considerable investment in time, personnel, and equipment, and are usually only practicable in laboratories such as national reference centers with a particular interest in *L. monocytogenes*. Phage typing was the mainstay of larger studies but has been superseded by molecular biological methods, especially pulsed-field gel electrophoresis (PFGE). Simple methods based on resistance to heavy metals and plasmid analysis may be useful for screening large numbers of cultures by nonspecialist laboratories (McLauchlin et al. 1997).

Analysis by phage typing, multilocus enzyme electrophoresis, and pulsed-field gel electrophoresis of *L. monocytogenes* isolates from unrelated large human food-borne outbreaks in North America and Europe indicated that these strains show a high degree of similarity (Piffaretti et al. 1989; Buchrieser et al. 1993).

Serotyping

L. monocytogenes can be subdivided into 13 serovars by agglutination reactions with adsorbed rabbit antisera (see Cell-wall and envelope composition; antigenic structure, and Table 1). However, this offers only limited practical discrimination for epidemiological studies since the majority of strains causing disease (usually >90 percent) in humans and animals belong to serovars 1/2a, 1/2b, and 4b (Seeliger and Höhne 1979; McLauchlin 1997a; Low and Donachie 1997); the highest proportion of human isolates usually belongs to serovar 4b (Seeliger and Höhne 1979; McLauchlin 1997a) and those from animals to serovar 1/2a (Low and Donachie 1997). Almost all the large outbreaks of human listeriosis have been due to serovar 4b (McLauchlin 1997a).

The serovars of *L. monocytogenes* do not occur in the same proportions in different clinical groups of human patients (McLauchlin 1997a) or in different categories of disease in animals (Low and Donachie 1997). The reason for this is not known.

Other phenotypic methods

Lysogenic phages commonly occur in strains of *Listeria*. Patterns of lytic reactions with sets of phages offer systems of high discrimination, which are capable of application to relatively large numbers of cultures (Rocourt et al. 1985; Loessner 1991). Although a high proportion of the strains causing human infection are usually phage-typable, some groups of strains (particularly those belonging to serogroups 1/2 and 3) are often not phage-typable and additional typing schemes may be necessary.

Within the genus *Listeria*, bacteriocin (monocin) production is as common as the carriage of lysogenic phage (Curtis and Mitchell 1992). However, the use of monocins for subtyping has not provided good discrimination (Curtis and Mitchell 1992).

Analysis of the electrophoretic mobilities of constitutive enzymes (multilocus enzyme electrophoresis) has provided a method of good discrimination for *L. monocytogenes* (Piffaretti et al. 1989; Bibb et al. 1990).

Nucleic-acid-based methods

The extraction of total chromosomal DNA and the separation of restriction endonuclease digests by electrophoresis has been used to characterize *L. monocytogenes* strains (Nocera et al. 1993). Analysis of the large number of fragments generated and the complexity of resulting patterns present some considerable difficulty. The method can be improved by probing Southern blots of similar restriction endonuclease digests with probes for rRNA genes (Graves et al. 1991) or randomly cloned chromosomal DNA fragments (Ridley 1995).

The separation by pulsed-field gel electrophoresis of large DNA fragments obtained with low-frequency cleavage restriction endonucleases offers a method of characterizing whole listeria chromosomes (Brosh et al. 1991). This nucleic-acid-based method offers particularly high discrimination. Analysis of DNA fragments generated by the random amplification of polymorphic DNA (Mazurier and Wernars 1992) has also been described for subtyping *L. monocytogenes*. Results from methods based on random amplification of chromosomal sequences are still subject to laboratory variability; however, some of these molecular methods allow a greater degree of interlaboratory comparability, and successful networks of laboratories (Pulsenet) have been established using standardised methods for PFGE with automatic pattern comparison of strain databases over the internet (Swaminathan et al. 2001).

PCR-based methods have been described, including amplified fragment length polymorphism (AFLP) analysis (Aarts et al. 1999; Guerra et al. 2002). DNA sequenced-based approaches are likely to become widely used in the future (Cai et al. 2002).

ROLE IN THE NORMAL FLORA OF HUMANS

As outlined in Habitat, *L. monocytogenes* occurs in the gastrointestinal tract, which is presumably the initial site of invasion in most cases. Carriage in the gut is probably transitory. However, there is good evidence that some patients show long incubation periods (up to 90 days, with a median of 35 days) after the consumption of contaminated food (Linnan et al. 1988). Two episodes of infection have been reported in individual immunocompromised patients, some of these more than 1 year apart. On the basis that the same strain was shown to be responsible for the two episodes, it has been suggested that this represents long-term carriage and reactivation of the original infection (McLauchlin et al. 1991). The basis and site of the long-term carriage suggested here is not understood, although this may occur in hepatocytes (Vazquez-Boland et al. 2001). The recent outbreaks of listerial enteritis and fever (Aureli et al. 2000; Sim et al. 2002) have further highlighted the gastrointestinal tract as the initial site of invasion. Enteritis, however, is not always a feature of this disease (Linnan et al. 1988) and may be specific to certain *L. monocytogenes* strains. Although *L. monocytogenes* is a potential enteric pathogen, it is unclear whether routine bacteriological examination of stool samples from patients with diarrhoea will be clinically helpful (Hof 2001).

PATHOGENICITY AND VIRULENCE FACTORS

Advances using molecular biological techniques have further assisted in the understanding of listeriosis at the cellular level (Vazquez-Boland et al. 2001). However, much still remains obscure as to how this disease occurs in nature. It is characteristic of the natural disease that the attack rate in both humans and animals is usually low. Susceptibility to infection may be increased by external factors, but *L. monocytogenes* is a somewhat marginal pathogen. Hence, experimental models that attempt to reflect the natural infection may work poorly, and it may be necessary to challenge relatively large numbers of animals to produce clinical symptoms of disease in a small proportion.

The majority of isolates of *L. monocytogenes* are capable of causing disease; however, nonpathogenic variants have been described and there may be differences in virulence between strains (Conner et al. 1989; Brosch et al. 1993). Nevertheless, for considerations for public-health purposes, all *L. monocytogenes* should be regarded as potentially pathogenic (Hof and Rocourt 1992; McLauchlin 1997a).

Animal models and invasion of mammalian cells growing in vitro

A wide range of animals are susceptible to experimental listeric infection, but rabbits and mice are most

frequently used because both die following inoculation with live bacteria by the intravenous or interperitoneal route. Only strains of *L. monocytogenes* and *L. ivanovii* are virulent as measured by LD50 determination, the kinetics of bacterial growth in host tissue, survival in the liver and spleen, or the death of the experimental animal. All the nonhemolytic species of *Listeria* (*L. innocua*, *L. welshimeri*, and *L. grayi*) and the weakly hemolytic *L. seeligeri* are avirulent in mouse pathogenicity tests or by invasion and growth in mammalian cells in tissue culture (Kathariou 2002).

ANIMAL MODELS

The production of experimental keratoconjunctivitis (Anton's eye test) in guinea pigs or rabbits by instilling a live bacterial suspension into the conjunctiva (Anton 1934) historically has been used to demonstrate the virulence of *L. monocytogenes*. A purulent conjunctivitis (and occasionally a keratitis) develops within 24–48 h. This usually heals spontaneously and the animal rarely dies.

In mice, subcutaneous, intraperitoneal, intravenous, or oral inoculation with *L. monocytogenes* causes death after 1–7 days, the mortality rate depending upon the dose and route of inoculation. The LD50 for mice after intraperitoneal or intravenous inoculation ranges from 10^2 to 10^7 cfu (Conner et al. 1989; Brosch et al. 1993). At postmortem examination, listeric septicemia is characterized by multiple tiny focal necroses scattered most conspicuously throughout the liver, and also in the spleen, lungs, adrenal glands, tonsils, and intestinal tract (Gray and Killinger 1966). The organisms can be recovered from the spleen and heart blood. Marked differences in susceptibility to infection between different strains of inbred mice have been reported (Skamene 1983). Intraperitoneal carrageenan may be given to mice before inoculation with listeria to increase susceptibility to infection (Conner et al. 1989). Recent work has shown that the guinea pig is a better model of human infection than the mouse due to a single amino-acid difference between the murine and human E-cadherin molecules. This change reduces the affinity of the internalin A protein for the receptor and compromises invasion by *L. monocytogenes* in the mouse model (Lecuit et al. 1999). In guinea pigs and transgenic mice carrying the humanized E-cadherin gene, internalin was found to mediate invasion of enterocytes and crossing of the intestinal barrier (Lecuit et al. 2001). Virulence assays are usually performed using either intraperitoneal or tail-vein injection. Delivery directly into the animal overcomes the limitations of E-cadherin specificity when strains are administered by the oral route.

The inoculation of gerbils via the middle ear with a low infective dose produces a prolonged otitis media and persistent bacteremia and is followed by a rhombencephalitis in the brainstem (Blanot et al. 1997).

In rabbits (as in monogastric animals) a marked monocytosis is produced following parenteral inoculation, unless overwhelming numbers of bacteria are used, which results in very rapid death. The monocytosis reaches its maximum in 3–7 days (Gray and Killinger 1966).

Ovine encephalitis with characteristic histological features has been achieved experimentally by inoculating listeria into the dental pulp. Histological encephalitis is evident after 6 days, but the onset of clinical neurological disease varied from 20 to 40 days (Low and Donachie 1997).

Oral inoculation of mice, rats, and guinea pigs has been described (Kathariou 2002). Infection showed most consistency in gnotobiotic animals and interference with colonization by the natural microflora of the gastrointestinal tract has been suggested (Czuprynski and Balish 1981). An increase in the virulence of *L. monocytogenes* when grown at refrigeration temperatures has been reported in mice experimentally infected by the intravenous but not by the oral route (Czuprynski et al. 1989; Stephens et al. 1991). Similar effects have been described with chick-embryo infection (Kathariou 2002). Esophageal inoculation of juvenile rats with 10^6 cfu of *L. monocytogenes* showed an infection rate of approximately 50 percent in the liver or spleen (Schlech et al. 1993). A reduction in the acidity of the stomach by cimetidine treatment reduced the infective dose (Schlech et al. 1993). Infection of mice with *L. monocytogenes* via an aerosol has been reported (Bracegirdle et al. 1994). LD50 values after 4 days were 10^3–10^5 cfu. Feeding trials in cynomolgous monkeys showed that only those receiving 10^9 cfu developed fever, septicemia, loss of appetite, irritability, and occasional diarrhoea; those fed $\geqslant 10^7$ cfu, some of which were completely asymptomatic, shed the organism in the feces for up to 21 days (Farber et al. 1991).

Ligated ileal loops in rats have been used to investigate the initial intestinal stages of listeriosis (Pron et al. 1998) where the Peyer's patches were shown to be the preferential initial site of both invasion and replication. Dendritic cells beneath the epithelial lining of the Payer's patches are an early cellular target in invasion (Pron et al. 2001). In chick embryos inoculated by the intra-allantoic route, the LD50 is around 10^2 cfu. Lesions occur in the chorioallantoic membrane, liver, and heart, and the bacterium can be readily cultured from these sites (Vazquez-Boland et al. 2001; Kathariou 2002).

Chronic mastitis has been induced experimentally in cows by intramammary injection. Bacteremia was not detected and typically 10^3–10^4 *L. monocytogenes* cfu m/l were shed into milk for 9–12 months of the remaining lactation period (Bryner et al. 1989).

GROWTH IN TISSUE CULTURE

L. monocytogenes is able to infect a range of cell types growing in vitro, including enterocytes, macrophages,

and fibroblasts (Kathariou 2002). The use of such models has contributed much to the understanding of the factors involved in intracellular invasion and growth (see section on Disease mechanisms for the invasion of mammalian cells).

Disease mechanisms for the invasion of mammalian cells

L. monocytogenes has long been used as a low-grade intracellular pathogen to study cellular immunity (Kaufmann 1993), and more recent advances in molecular biology have further assisted the understanding of listeriosis at the cellular level (Vazquez-Boland et al. 2001). There has been considerable progress in understanding the mechanisms for invasion and multiplication, and cell-to-cell spreading of *L. monocytogenes* (and, to a lesser extent, *L. ivanovii*) is now better understood (Vazquez-Boland et al. 2001; Kathariou 2002).

The stages occurring during growth and cell-to-cell spread of *L. monocytogenes* are depicted in Figure 1. *L. monocytogenes* enters both phagocytic and non-phagocytic cells. A listerial surface protein, internalin A (which is reminiscent of the M protein of the group A streptococcus), has been shown to be involved with the initial stages of invasion of all cell types. A second cell-surface protein (internalin B) is required for entry into hepatocyte-like but not enterocyte-like cells. The receptor for internalin A is E-cadherin, which is a mammalian surface protein involved with cell-to-cell adhesion. The cell-wall teichoic acid is also required for adhesion of *L. monocytogenes* to mammalian cells (Abachin et al. 2002). The listerial cell-surface protein p60 (with murein hydrolase activity) may also be involved in the invasion of fibroblasts.

Within mammalian phagocytic cells, the majority of cells in the phagocytic vacuole are probably killed. However, those bacteria surviving in the vacuole and those in the membrane-bound compartment of the non-professional phagocyte (which probably confers some protection of the bacterium from host defenses in the extracellular environment), mediate the dissolution of the vacuole membrane by means of a thiol-activated hemolysin (listeriolysin O), in combination with the action of a phospholipase C (phosphatidyl inositol-specific). *L. monocytogenes* then enters the host cell cytoplasm, where growth occurs. In the cytoplasm, the organism becomes surrounded by polymerized host cell actin, which becomes preferentially polymerized at the older pole of the bacterium following cell division. The ability to polymerize actin confers intracellular mobility to the bacterium, is dependent on the Act A protein, and may be mediated by binding with host-cell profilactin and vasodilator-stimulated phosphoprotein, although the exact mode of action is not understood. The resulting 'comet-tail' like structure can push the bacterial cell into an adjacent mammalian cell, where it again becomes encapsulated in a vacuole. At this stage the vacuole is double-membrane-bound from the two host cells. The primary role of the second phospholipase enzyme (a broad-spectrum lecithinase enzyme that hydrolyzes phosphatidylcholine or lecithin) is believed to be involved in the subsequent dissolution of these membranes (although again the hemolysin and phospholipase C may also contribute in this process; a distinct role may not exist for each of the Hly, PlcBC, and PlcC proteins; rather, these enzymes work synergistically to achieve optimal levels of activity at different stages of the infection process). After release into the cytosol, intracellular growth and movement within the newly invaded cell are then repeated and the focus of infection continues to be enlarged by this process of cell-to-cell spread (Vazquez-Boland et al. 2001; Kathariou 2002).

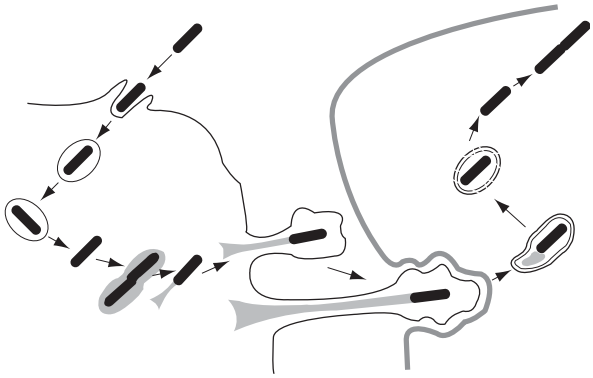

Figure 1 *Schematic representation for invasion of eukaryotic cells by* L. monocytogenes

The genes involved in the invasion and intracellular movement in mammalian cells are shown in Figure 2. These comprise the *plcA* gene encoding a phospholipase C, the *hly* gene encoding the listeriolysin, and the *actA* gene encoding a cell-surface protein involved with actin nucleation. The *plcB* gene encodes the lecithinase, which is activated by a metalloprotease (*mpl* gene). The genes *plcA*, *hly*, *mpl*, *actA*, and *plcB* are located in three adjacent operons. The operon containing the internalin gene family (*inlA*, *inlB*, *inlC*, *inlD*, *inlE*) is located quite closely on the bacterial chromosome, and a further internalin gene (*inlF*) is located elsewhere on the chromosome. The products of the *inlA* and *inlB* genes have been identified as associated with invasion of non-phagocytic cells. These genes are organized into a main virulence cluster containing adjacent operons, which are all regulated directly by the central virulence regulator PrfA. The internalin genes are located in a separate gene cluster and are regulated only partially by PrfA, there being a basal level of expression even in the absence PrfA-induction. Similar sets of virulence genes are also present in *L. ivanovii* and *L. seeligeri*. Virulence for *L. monocytogenes* is therefore a multifactorial property and at least nine genes and their products are required for infection, invasion, survival, motility, and

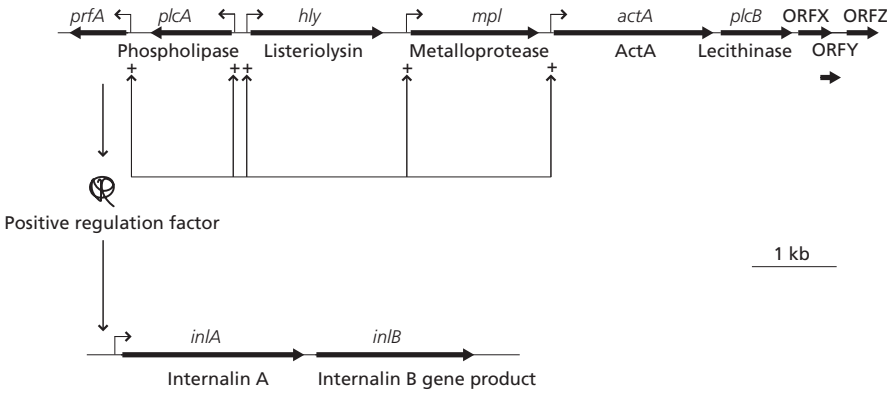

Figure 2 *Regulation of genes involved with the virulence of* L. monocytogenes

cell-to-cell spread (Vazquez-Boland et al. 2001), although additional genes (especially those for cell-surface components) are also likely to be involved with the infectious process (Autret et al. 2001; Engelbrecht et al. 1998; Raffelsbauer et al. 1998). It is possible that the genes for intracellular parasitism have evolved for *L. monocytogenes*, as well as *L. ivanovii* and *L. seeligeri*, to invade lower multicellular eukaryotic organisms resident in the environment. Internalin genes also occur in the nonpathogenic species *L. innocua* (Vazquez-Boland et al. 2001). Listeriolysin is one of the major extracellular proteins produced by *L. monocytogenes* under conditions of heat shock, and there is evidence to suggest that some of the above-mentioned genes are also regulated by the stage of the cell cycle and temperature. It has been shown that the disaccharide cellobiose represses the expression of the listeriolysin and phospholipase genes by an as yet uncharacterized mechanism (Park and Kroll 1993). It is tempting to speculate that the absence of this environmentally ubiquitous plant-derived molecule allows the induction of genes as a pathogenic response to the environment of eukaryotic cells.

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Erysipelothrix

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THE ORGANISM

Erysipelothrix are slender, rod-shaped bacteria with a tendency to form long filaments. They are nonmotile, nonsporing, and gram-positive but they decolorize easily and may appear gram-negative. The organisms are facultatively anaerobic and not acid-fast and growth is improved in the presence of 5–10 percent CO_2. The fermentative activity of *Erysipelothrix* spp. is very weak; acid but no gas is produced from glucose and certain other carbohydrates. *Erysipelothrix* spp. are catalase- and oxidase-negative. Typical strains form H_2S on triple sugar iron agar. There are at least 26 serotypes. The genus consists of two species: *Erysipelothrix rhusiopathiae* and *Erysipelothrix tonsillarum* (serovar 7) (Takahashi et al. 1987a). The G + C content of DNA of both species is 36–40 mol%. The type species is *E. rhusiopathiae*.

E. rhusiopathiae is widely distributed in the soil and surface waters of farms and in the sewage effluent of abattoirs. It is not normally found in soil and its presence reflects contamination by infected animals. Although killed by moist heat at 55°C for 15 min, *E. rhusiopathiae* can survive in the dried state for many years. In addition to its resistance to drying, *E. rhusiopathiae* is resistant to salting, pickling, and smoking. Meat and bacon may contain the organisms after pickling for 170 days or after 30 days in a mixture of salt and potassium nitrate (Reboli and Farrar 1991). In soil the organism is favored by an alkaline pH and high organic content (Ewald 1981). Soil that may retain viable organisms serves as a medium for infection of animals by the oral route.

THE DISEASE

Erysipelothrix infection in animals

Swine erysipelas is a common infection in animals caused by *E. rhusiopathiae* (Reboli and Farrar 1989). It is most commonly seen in pigs, where it has a major economic impact, but it has also been reported in sheep and poultry and can contribute to morbidity and mortality. The organism has also been isolated from a wide variety of other domestic and wild animal species. *Erysipelothrix* has been identified in up to 30 percent of chickens being processed through an abattoir in Japan (Nakazawa et al. 1998). In Japan, a survey of antibody to *Erysipelothrix* in cattle from nine prefectures demonstrated high rates of exposure (34 percent), with titers of 1:128 or above (Sawada et al. 2001). Low-serum titers were noted from areas where no swine industry was present, confirming the major ecological niche of the organism in swine. An Australian study using a polymerase chain reaction (PCR) gene-specific detection also demonstrated an infection rate of 32 percent in two abattoirs processing pigs, sheep, cattle, and chicken (Wang et al. 2002). The infection appears to be more common in summer and autumn months, and may have a periodic cycle between 4–5 years, possibly related to waxing and waning immunity in animal populations.

Four clinical types of erysipelas have been described in swine: acute septicemia, urticaria, endocarditis, and chronic arthritis. Cutaneous involvement may be seen in all forms of the disease, with the most characteristic being the quadrangular or rhombic patches in the mild

urticarial form ('the diamonds'). Acute septicemic erysipelas has a case fatality rate in swine of 80 percent within 3–4 days. Endocarditis in swine also ends fatally, but with a more prolonged course. The mild urticarial form spontaneously remits with minimal morbidity. The chronic arthritic form of erysipelas is characterized by crippling and growth retardation. In wild animals, autopsy evidence has suggested that the acute septicemic form of erysiplas is most common (Campbell et al. 1994). Septicemic infection appears to be more common in outbreaks or sporadic cases in poultry (Mutalib et al. 1993). A recent report of erysipelas septicemia in a flock of ring-necked pheasants in Connecticut, USA, noted a mortality of 6.2 percent over 6 days (Hennig et al. 2002). As the disease is rare and sporadic, there are no comparative data of incidence or prevalence in different geographic regions.

Human infection with *Erysipelothrix rhusiopathiae*

Human erysipeloid is an incidental zoonotic infection of man. Human infection is almost always acquired by direct handling of contaminated fomites. The disease is primarily an occupational hazard for individuals who handle meat, fish, or crustaceans or who work in any occupation that involves handling the byproducts of animal husbandry. In animals, the disease may be sporadic but can cause epidemic disease in swine, where it is carried in the upper respiratory tract and feces. It does not persist in soil or vegetation (Wood and Shuman 1974). Sporadic cases are also seen in human populations, but outbreaks of infection among fish handlers and abattoir workers have been described. Early descriptions of the disease include outbreaks in fish workers in Odessa (Stefansky and Grunfeld 1930) and in workers manufacturing bone buttons (Lawson and Stinnett 1933).

Erysipelothrix infections are not subject to mandatory reporting and no data exist on the incidence of disease or the prevalence of antibodies defining exposure in human populations, but the disease is rare. A serologic study (Molin et al. 1989) found antibodies to *E. rhusiopathiae* in 16 percent of 138 abattoir workers in Sweden. Although alcoholism has been described as a risk factor in some cases of *Erysipelothrix* bacteremia, the overall incidence of disease does not seem to be increased among immunocompromised patients. There are, however, case reports of *Erysipelothrix* septicemia in human immunodeficiency virus (HIV)-positive patients (Marne et al. 1996).

Rosenbach is credited with identifying the typical form of erysipeloid in 1884. He summarized his work in 1909 (Rosenbach 1909), and the disease in subsequent reviews has been referred to as the 'erysipeloid of Rosenbach.' The typical presentation is a localized cellulitis caused by direct inoculation of the organism into the skin. Rosenbach saw the infection most commonly in cooks, kitchen workers, butchers, and tanners, and described it as 'slowly progressing, sharply defined, and a slightly elevated, dark, violaceous, almost livid red zone, which develops around the site of inoculation' (Klauder et al. 1926). The incubation period is usually 1–2 weeks, and the lesion is usually painful. Constitutional symptoms are infrequent, but lymphangitis and lymphadenopathy occur in one-third of patients. Occasionally the local reaction can be more severe, with the formation of bullae, and fever can also develop.

In its most severe form, *Erysipelothrix* can cause sepsis and bacterial endocarditis (Gorby and Peacock 1988). However, only one-third of patients with the systemic form of infection have typical cutaneous disease. When sepsis is identified, it usually is a sign of coexisting bacterial endocarditis and is similar to the endocarditis seen in swine. Occupational or other exposure to infected fomites is usually obtained. The bacteremia may be complicated by abscess in other organs, glomerulonephritis, and central nervous system infection, including meningitis and brain abscess. The chronic arthritis syndrome seen in swine is not found in human infection, although localized osteomyelitis may occur at the time of the acute septicemic disease and infection postarthroscopy has been described (Allianatos et al. 2003).

Erysipelothrix infection has also been identified in a few cases of cat bite, although other pathogens such as *Pasteurella multocida* are much more common (Talan et al. 1999).

Laboratory diagnosis

Salient properties useful for the identification of *Erysipelothrix* spp. are listed in Table 1. *E. rhusiopathiae* grows well in blood and chocolate agar in 5–10 percent CO_2. For patients with bacteremia, isolation using automated systems works well. The best specimen for culture in cutaneous disease is usually a biopsy from the leading edge of the area of cellulitis. The biopsy should be transported and kept in 1 percent glucose prior to primary culture (Reboli and Farrar 1989). The organisms appear as elongated gram-positive rods in tissues using a Brown–Brenn stain. Identification of new isolates is based on examination of cell morphology, lack of hemolysis on blood agar, growth characteristics in nutrient gelatin, catalase and oxidase tests, production of acid from carbohydrates in a suitable medium, and hydrogen sulfide production.

In addition to the properties listed in Table 1, indole and acetoin (Voges–Proskauer (VP)) are not produced and the methyl red test is usually negative. Urea, aesculin, sodium hippurate, starch, cellulose, and casein are not hydrolyzed. Xanthine and tyrosine are not degraded. There is no discernible change in litmus milk and nitrate is not reduced (Reboli and Farrar 1991).

Table 1 *Characteristics of* Erysipelothrix *species*

Characteristic	*E. rhusiopathiae*	*E. tonsillarum*
Aerobic growth	+	+
Anaerobic growth	+	+
β-Hemolysis	–	–
α-Hemolysis	+	+
Motility	–	–
'Test-tube brush' growth in gelatin	+	+
H_2S production[a]	+	+
Aesculin hydrolysis	–	–
Acid from:		
Glucose	+	+
Mannose	+	±
Sucrose	–	+
Lactose	+	+
Maltose	+	+
Galactose	+	+
Rhamnose	–	–
Raffinose	–	–
Arabinose	–	–
Trehalose	–	–
Xylose	–	–
Glycerol	–	–
Inositol	–	–
Sorbitol	–	–
Mannitol	–	–
Dulcitol	–	–
Inulin	–	–
Salicin	–	±
Sensitivity in vitro to:		
Penicillin G (0.1 U/ml)	+	+
Ampicillin (0.1 μg/ml)	+	+
Kanamycin (100 μg/ml)	–	–
Pathogenic for swine	+	–[b]

±, variable, not all strains able to perform this reaction.
a) In triple sugar iron agar.
b) An occasional strain has been isolated from pig erysipelas (Takahashi et al. 1994).

Soto et al. (1994) reported the APl Coryne System to be suitable for the identification of *E. rhusiopathiae*. In specimens from suspected infections, bacteria with which *Erysipelothrix* is likely to be confused include *Actinomyces pyogenes*, *Arcanobacterium haemolyticum*, and *Listeria monocytogenes*. Unlike *Erysipelothrix*, these bacteria are all beta-hemolytic on blood, but care is required because colonies of *A. haemolyticum* display only very weak hemolysis after 24 h; none produces hydrogen sulfide in the base of TSl slants and *L. monocytogenes* is catalase-positive and motile. The resistance of *Erysipelothrix* to neomycin can also be used to distinguish it from *L. monocytogenes*.

The organism can be misidentified using standard laboratory algorithms as *Lactobacillus* or *Enterococcus* spp. in some instances (Dunbar and Clarridge 2000).

In veterinary laboratories, use of enrichment broth in combination with PCR may aid in the rapid diagnosis of swine erysipelas (Shimoji et al. 1998). While typing sera have been developed and used for many years in distinguishing *Erysipelothrix* serovars, serologic tests to identify human disease have not been employed. Serosurveys of animals using growth agglutination methodology have been supplemented by the development of enzyme immunoassays and latex agglutination tests, which detect exposure in animal populations but have not been useful diagnostically in human or animal disease where the organism is almost invariably identified by culture or histology.

Molecular methods for typing and identification of *E. rhusiopathiae* have undergone rapid development. Serotyping is being largely replaced by these molecular techniques. Multilocus enzyme electrophoresis was the first to be attempted for *Erysipelothrix* (Chooromoney et al. 1994), but DNA and RNA analyses have also been described. Ahrne et al. (1995) used restriction fragment length polymorphism (RFLP) of 16S ribosomal RNA and classified 32 type and reference strains. Organisms were placed in patterns A to I, and no clustering of previously determined serotypes was identified. Takeshi et al. (1999) developed a PCR methodology using four species-specific oligonucleotide primers after 16S RNA amplification. They were able to identify *E. rhusiopathiae* and *E. tonsillarum* in tissues of diseased animals within 5 h. Using 18 random primers, Okatani et al. (2000) identified a unique band in 64 of 66 strains of *E. rhusiopathiae* and a different band in 11 of 12 strains of *E. tonsillarum*. A combination of two primers (NK51 and NK6) appears to be most useful in strain analysis.

Okatani and his group have also reported the use of pulse field gel electrophoresis (PFGE) in the differentiation of *Erysipelothrix* strains (Okatani et al. 2001). They studied 70 strains of *Erysipelothrix* using three restriction enzymes. *Sma*I was the most effective and identified 63 distinct PFGE patterns in the 70 strains. This technology, widely used to study other epidemic infections, has not been used in investigations of outbreaks of swine erysipelas or human disease reported to date.

Metabolism

Erysipelothrix spp. are facultatively anaerobic. On primary isolation, growth occurs in the form of a band just below the surface of a soft agar culture. Whether this is due to a preference for CO_2 or for a reduced oxygen level is not clear. Growth is scant on the usual laboratory media. It is improved by glucose (0.2–0.5 percent) and by blood or serum (5–10 percent). The optimum temperature for growth is 30–37°C; growth occurs between 5 and 42°C. The optimum pH for growth is 7.2–7.6, range 6.8–8.2 (Karlson and Merchant 1941).

The exact growth requirements of *Erysipelothrix* spp. have not been determined; several amino acids, riboflavin, and small amounts of oleic acid are required (Hutner 1942). Growth is improved by tryptophan (Ewald 1981).

Glucose catabolism in *E. rhusiopathiae* is via the Embden–Meyerhof–Parnas pathway, although a small amount of glucose is used by the hexose monophosphate shunt (Robertson and McCullough 1968). Fermentation of glucose results in the production of lactic acid, with smaller amounts of acetic acid, formic acid, ethyl alcohol, and carbon dioxide. Exogenous citrate is not utilized. The organisms are catalase-negative and cytochromes and isoprenoid quinones are absent. (Jones 1986).

Morphology and cell-wall composition

Members of the genus exist in a rough and smooth form, each characterized by closely associated morphological and colonial appearances. In the smooth form (S-form), the cells appear as small, straight, or slightly curved, nonmotile, gram-positive rods 0.3–0.6 μm in diameter and 0.8–2.5 μm long with rounded ends. The cells occur singly, in pairs at an angle to give V forms, in clusters, and in short chains. In the rough form (R-form), long filaments more than 60 μm long predominate.

Both forms decolorize easily when gram-stained and both rods and filaments may appear as gram-negative containing bands of gram-positive staining that give a beaded effect. Metachromatic granules are absent. The cell morphology varies to some extent with the conditions of culture. It has been claimed that at pH 7.6–8.2, S-forms predominate, whereas pH values of 5.2–7.0 favor the production of R-forms (Zák et al. 1965). It has also been reported that S-forms grow better at 33°C and R-forms better at 37°C (Grieco and Sheldon 1970). In smears of blood and tissue taken from acute cases of infection, especially septicemia, the organisms are usually of the S-form of morphology; in chronic infections, arthritis, and endocarditis, R-forms are frequently detected together with S-forms (Ewald 1981). Capsules are considered to be absent (Ewald 1981; Jones 1986; Reboli and Farrar 1991), but Shimoji et al. (1994) noted the presence by electron microscopy of a capsule-like structure in virulent strains of *E. rhusiopathiae*. The cell wall of *Erysipelothrix* is of the gram-positive type. The cell-wall peptidoglycan diamino acid is lysine (Feist 1972) and the peptidoglycan type is the unusual B1δ (Schleifer and Kandler 1972). Mycolic acids are absent. Teichoic acids have not been detected (Feist 1972). Erler (1971) noted the presence of a large number of sugars in the cell wall of *E. rhusiopathiae*: galactose, glucose, arabinose, xylose, ribose, glucose-6-phosphate, and galactose-6-phosphate. A later study (Erler 1972a) indicated the presence of the amino sugars galactosamine, glucosamine, and fucosamine. From the monosaccharide and amino sugar patterns, Erler (1971, 1972a) recognized three chemotypes, but there appears to be no correlation between these and serotypes (Erler 1972b). L-forms of *E. rhusiopathiae* have been described (Pachas and Currid 1974; Todorov 1976).

The cellular fatty acids are of the 2- and 3-hydroxy and nonhydroxylated long-chain types. The hydroxylated fatty acids are primarily of the straight-chain series; the nonhydroxylated fatty acids are predominantly of the straight-chain saturated and monounsaturated series; small amounts of *iso*- and *anteiso*-methyl branched fatty acids are also present (Tadayon and Carroll 1971; Takahashi et al. 1994).

Pathogenicity and virulence factors

The basis for the virulence of *E. rhusiopathiae* is not fully understood. Most strains isolated from erysipelas in pigs belong to serotypes 1a, 1b, and 2. Serotype 1a is the most common in cases of septicemia (Reboli and Farrar 1991). Epitopes associated with serotype differences could play a role in virulence. Virulent strains of *E. rhusiopathiae* are reported to adhere more avidly to porcine kidney cell lines than do avirulent strains (Takahashi et al. 1987b). Similarly, strains of *E. rhusiopathiae* isolated from pig endocarditis or septicemia have been shown to adhere more strongly to pig heart-valve tissue in vitro than do other *E. rhusiopathiae* isolates (Bratberg 1981).

E. rhusiopathiae strains produce a hyaluronidase and a neuraminidase, which cleaves α-glycosidic linkages of sialic acid, a mucopolysaccharide on the surface of mammalian cells. Neuraminidase activity is higher in virulent than in avirulent strains (Krasemann and Müller 1975).

Virulence of *E. rhusiopathiae* may also be associated with the possession of a capsule-like structure that confers resistance to phagocytosis (Shimoji et al. 1994, 1996).

E. rhusiopathiae also possess a 65-kDa protein that shares a 56 percent identity with an *Escherichia coli* DnaK heat shock protein, which might contribute to virulence (Partridge et al. 1993).

Tamura et al. (1993), in a comparative study of protein profiles of *E. rhusiopathiae* and *E. tonsillarum*, noted that *E. rhusiopathiae* contained two major protein antigens with molecular masses of 32 and 56 kDa. By contrast, the nonvirulent *E. tonsillarum* contained only the 32-kDa protein. These authors speculated that the 56-kDa protein may play a role in the pathogenesis of *E. rhusiopathiae*.

Studies with a vaccine prepared from a NaOH-extracted antigen of a serovar 2 strain of *E. rhusiopathiae* led Kitajima et al. (1998) to suggest that the 67–64- and 62–60-kDa proteins in the NaOH extract play an

important role in protecting against *E. rhusiopathiae* infection. A 64-kDa cell-surface protein (SpaA) has recently been identified on the cell surface of most *E. rhusiopathiae*, but not *E. tonsillarum*, strains (Makino et al. 1998). The SpaA protein has eight repeated units, and mice vaccinated via the peritoneal route with *E. coli* expressing the complete protein (but not a deleted form without the repeats) were protected against subsequent subcutaneous challenge (1000 LD_{50} units) by *E. rhusiopathiae*.

TREATMENT

In the preantibiotic era, most cases of cutaneous erysipeloid resolved spontaneously. However, with the development of penicillin therapy in the 1940s, it was found that treatment would hasten recovery in mild disease (Stiles 1947) and may be life-saving in severe forms of infection, such as endocarditis. The organism remains susceptible to a wide variety of antimicrobial agents. A survey of 214 strains of *E. rhusiopathiae* isolated in Japan between 1988 and 1998 demonstrated that the β-lactam agents were the most active, and penicillin G remains the treatment of choice (Yamamoto et al. 2001). Cephalosporins, such as ceftriaxone and cefazolin, are also active, as are the quinolones, particularly enrofloxacin and danofloxacin. Aminoglycosides and sulfa drugs occasionally show resistance. A confirmatory study using 60 strains demonstrated MIC_{90} of 0.03 and 0.125 for penicillin and ceftriaxone. Quinolones were also active. Ciprofloxacin had the best activity in vitro (Fidalgo et al. 2002). Resistance to clindamycin or erythromycin has been reported, but these drugs remain alternative agents for the penicillin-allergic patient. Importantly, the organism is not susceptible to vancomycin, which is commonly used in this setting.

Patients with endocarditis present a special problem. Prosthetic valve endocarditis appears to be overrepresented in case reports of this disease, and valve replacement may be necessary for a cure in addition to antibiotic therapy. Despite medical and surgical therapy, the mortality rate of *Erysipelothrix* endocarditis approaches 50 percent (Gorby and Peacock 1988).

PREVENTION

Because of the rarity of infection in human populations, immunization against infection is not likely to be cost-effective. However, swine erysipelas remains a major veterinary problem, currently controlled with antibiotic treatment, prophylaxis, and immunization. A protective protein, SPA-A.1, has been characterized (Shimoji et al. 1999), which can induce protective antibody in mice. A 67-kDa protein from a serovar 2 *E. rhusiopathiae* strain has also been identified, which elicits protective antibody responses in mice using a formalin-killed vaccine encompassing several strains of the organism (Kitajima et al. 2000). The commercial swine vaccine contains inactivated serotype 2 strains of *E rhusiopathiae*, but provides cross-protection to other serovars. An erysipelas vaccine using live attenuated organisms has been studied, and use of commonly available antibiotics in feed does not appear to affect the immune response (Yamamoto et al. 2000). Other surface proteins of 64 kDa appear to be associated with protection against *E. rhusiopathiae* as well (Sato et al. 1999). Whether the SPA-A or other surface proteins will form the basis of other effective vaccines in veterinary medicine against this important pathogen remains to be determined. Interestingly, an attenuated strain of *E. rhusiopathiae* (YS-1) has also been used as a mucosal vaccine vector after substitution of the central region of SPA-A.1 with a gene coding for a P97 adhesion protein of *Mycoplasma hyopneumoniae*, a pathogen responsible for pneumonia in pigs (Shimoji et al. 2003). This could hold promise for immunizing proteins against other veterinary infections as *Erysipelothrix* is a common commensal microorganism in the respiratory and gastrointestinal tracts in animals and may be ideally situated as a vector.

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Corynebacteria and rare coryneforms

GUIDO FUNKE

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INTRODUCTION

Coryneform bacteria do not form a taxonomic entity, but are rather characterized by morphological traits, i.e. being asporogenous, irregularly shaped, nonpartially acid-fast, gram-positive rods which grow aerobically. The term 'coryneform' (Figure 1) is somewhat misleading since only true *Corynebacterium* spp. exhibit a typical club-shaped ('*coryne*' meaning 'club' in ancient Greek) morphology, whereas all the other bacteria outlined in this chapter show an irregular morphology. Nevertheless, the term 'coryneforms' is commonly used by many clinical microbiologists and will therefore be used in this chapter.

For didactical and clinical reasons some bacteria which may initially present as coryneforms are covered in different chapters in this textbook. These bacteria include members of the genus *Actinomyces* (see *Actinomyces* and related genera), *Propionibacterium* (see *Bifidobacterium*, *Eubacterium*, and *Propionibacterium*), partially acid-fast bacteria (see Chapter 45, *Nocardia* and other aerobic actinomycetes), and rapidly growing mycobacteria (see Rapidly growing mycobacteria). In addition, this chapter will focus on the medically relevant taxa only; therefore, species with importance for veterinary or environmental microbiologists will not be covered.

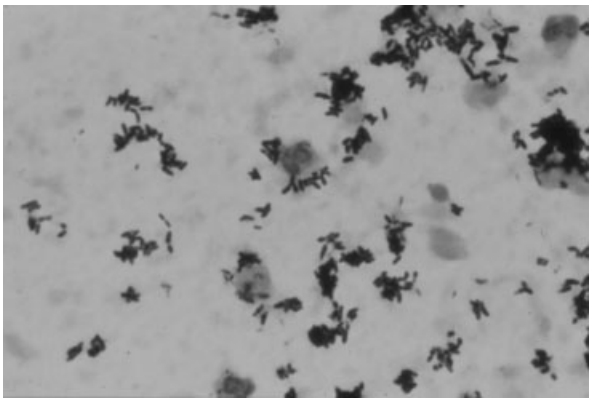

Figure 1 *Coryneform morphology*

The bacteria discussed in this chapter all belong to the class *Actinobacteria*, whose genera are characterized by specific 16S rDNA signature nucleotides (Stackebrandt et al. 1997). All the genera, except *Exiguobacterium* and *Gardnerella*, belong to the lineage of high guanine-plus-cytosine (G + C) content gram-positive bacteria. The group of coryneform bacteria is most diverse and genera can be differentiated by chemotaxonomic features (Table 1). In recent years, phylogenetic 16S rRNA gene sequencing investigations have confirmed the framework set by previous chemotaxonomic investigations.

NATURAL HABITAT

Many species of the medically relevant corynebacteria are part of the normal flora of the skin and mucous membranes in humans. It is interesting to note that not all corynebacteria are equally distributed over skin and mucous membranes, but rather occupy a specific niche.

Table 1 *Chemotaxonomic features of coryneform bacteria*

Genus	Major CFAs	Mycolic acids	Peptidoglycan diamino acid[a]	Acyl type
Corynebacterium	18:1ω9c, 16:0, 18:0	+[b]	*m*-DAP	Acetyl
Turicella	18:1ω9c, 16:0, 18:0	–	*m*-DAP	Glycolyl
Arthrobacter	15:0*ai*, 17:0*ai*, 15:0*i*	–	LYS	Acetyl
Brevibacterium	*15*:0*ai*, 17:0*ai*, 15:0*i*	–	*m*-DAP	Acetyl
Dermabacter	17:0*ai*, 15:0*ai*, 16:0*i*	–	*m*-DAP	ND[c]
Rothia	15:0*ai*, 17:0*ai*, 16:0*i*	–	LYS	ND
Exiguobacterium	17:0*ai*, 15:0*ai*, 16:0, 13:0*i*	–	LYS	ND
Oerskovia	15:0*ai*, 15:0*i*, 17:0*ai*	–	LYS	Acetyl
Cellulomonas	15:0*ai*, 16:0, 17:0*ai*	–	ORN	Acetyl
Cellulosimicrobium	15:0*ai*, 15:0*i*, 17:0*ai*	–	LYS	Acetyl
Microbacterium	15:0*ai*, 17:0*ai*, 16:0*i*	–	LYS, ORN	Glycolyl
Curtobacterium	15:0*ai*, 17:0*ai*, 16:0*i*	–	ORN	Acetyl
Leifsonia	17:0*ai*, 15:0*ai*, 16:0*i*	–	DAB	ND
Arcanobacterium	18:1ω9c, 16:0, 18:0	–	LYS	ND
Gardnerella	16:0, 18:1ω9c, 14:0	–	LYS	ND

ND, no data.
a) *m*-DAP, *meso*-diaminopimelic acid; LYS, lysine; ORN, ornithine; DAB, diaminobutyric acid.
b) Exceptions: *C. amycolatum*, *C. atypcium*, and *C. kroppenstedtii*.

C. diphtheriae can be isolated from the nasopharynx as well as from skin lesions, which represent a reservoir for the spread of diphtheria. Opportunistic pathogens such as *C. amycolatum*, *C. striatum*, and *Dermabacter hominis* are part of the normal human skin flora, but are not frequently found in throat swabs from healthy individuals. Coryneform bacteria prominent in the oropharynx include *C. durum* and *Rothia dentocariosa*. Other coryneform bacteria like *C. auris* and *T. otitidis* seem to have an almost exclusive preference for the external auditory canal. *C. macginleyi* has a tropism for the human conjunctiva. Another *Corynebacterium* with a distinctive niche is *C. glucuronolyticum*, which is almost exclusively isolated from genitourinary specimens. *C. urealyticum*, another genitourinary pathogen, has, like *C. jeikeium*, also been cultured from the inanimate hospital environment.

Arthrobacters are some of the most frequently isolated bacteria when soil samples are cultured, but *Arthrobacter cumminsii* seems to be present on human skin. Brevibacteria can be frequently found on dairy products (e.g. cheese), but are also inhabitants of the human skin. Members of the genera *Exiguobacterium*, *Oerskovia*, *Cellulomonas*, *Cellulosimicrobium*, *Microbacterium*, and *Leifsonia* have their habitats in the environment (e.g. soil and activated sludge). Microbacteria have also been isolated from hospital environment samples. Curtobacteria are primarily plant pathogens. The natural habitat of arcanobacteria is not fully understood, but *A. haemolyticum* is recovered from throat, as well as from wound swabs, whereas *A. bernardiae* is mainly found in abscesses adjacent to skin. It is unclear whether the two species are part of the normal skin and/or gastrointestinal flora. *A. pyogenes* is found on mucous membranes of cattle, sheep, and swine. *Gardnerella vaginalis* is part of the anorectal flora of healthy adults of both sexes, as well as in that of children. It is also part of the endogenous vaginal flora in women of reproductive age.

ISOLATION, IDENTIFICATION, AND TYPING SYSTEMS

In general, coryneform bacteria grow on nutrient agar at 37°C and on agar containing blood (e.g. sheep blood agar (SBA)), but not on enteric agar formulations; coryneform bacteria do not grow on MacConkey agar. However, if 'coryneforms' are recovered from this medium, they should be examined carefully in order to rule out rapidly growing mycobacteria. Selection of coryneform bacteria can be accomplished by inoculating media inhibitory to gram-negative bacteria such as those containing colistin sulfate and nalidixic acid (CNA). Another selective medium for coryneform bacteria contains 100 μg fosfomycin per ml (plus 12.5 μg of glucose-6-phosphate per ml) since nearly all coryneform bacteria (except *Arcanobacterium* spp. and *D. hominis*) are highly resistant to this compound. It is also possible to put disks containing 50 μg of fosfomycin (plus 50 μg of glucose-6-phosphate (already incorporated in the disk)) on the SBA plate and then examine the colonies which grow around the disk. If lipohilic corynebacteria like *C. jeikeium* or *C. urealyticum* are sought, then 0.1 to 1.0 percent Tween 80 should be added to an SBA plate (before pouring the medium). It is also possible to streak sterile filtered Tween 80 with a cotton swab onto SBA plates.

With very few exceptions (some arthrobacters, microbacteria, and curtobacteria, which have optimal growth temperatures between 30 and 35°C), the medically relevant coryneform bacteria all grow at 37°C. It is desirable to culture specimens for coryneform bacteria in a CO_2-enriched atmosphere since some taxa, e.g., *Arcanobacterium* spp. and *Rothia* spp., grow much better under those conditions. In almost every case all medically relevant coryneform bacteria grow within 48 h.

The primary plating medium for the cultivation of *C. diphtheriae* should be SBA plus one selective medium (e.g. cystine-tellurite blood agar (CTBA) or freshly prepared Tinsdale medium). The plates are read after 18 to 24 h of incubation at 37°C. Tellurite inhibits the growth of many noncoryneform bacteria, but even a few *C. diphtheriae* strains are sensitive to potassium tellurite and will therefore not grow on CTBA, but may grow on SBA. It is important to realize that growth on CTBA and tellurite reduction are not specific for *C. diphtheriae*, since many other coryneforms may also produce black colonies. The best medium for direct culturing of *C. diphtheriae* is probably Tinsdale medium; however, one of the limitations of this medium is its relatively short shelf life (<4 weeks). On Tinsdale plate, both tellurite reductase activity (as shown by black colonies) and cystinase activity (as shown by a brown halo around the colonies) can be observed. If neither CTBA nor Tinsdale medium is available, CNA plates are recommended for isolation of *C. diphtheriae*. However, in this case it is necessary to pick multiple colonies from CNA plates to rule out *C. diphtheriae* (first Gram staining, then subculturing, and biochemical testing).

The initial approach to identify coryneform bacteria comprises simple basic tests available in every microbiology laboratory. The Gram staining morphology of the cells can exclude the assignment to many genera (see under The genera and their species), e.g. *Corynebacterium*, *Turicella*, or *Dermabacter*. Colony morphology, size, pigment, odor, and hemolysis of colonies are also valuable characteristics in the differential diagnosis of coryneform bacteria.

The biochemical identification system outlined by von Graevenitz and Funke (1996) includes the following reactions: catalase; test for fermentation or oxidation (this is best observed in semisolid cystine Trypticase agar (CTA) medium); motility; nitrate reduction; urea hydrolysis; esculin hydrolysis; acid production from glucose, maltose, sucrose, mannitol, and xylose; CAMP reaction; and a test for lipophilia, which is performed only for catalase-positive colonies <0.5 mm in diameter. Lipophilic corynebacteria develop colonies up to 2 mm in diameter after 24 h on Tween-supplemented agar. For differentiation of arthrobacters and brevibacteria, utilization reactions of certain carbohydrates are recommended (Funke and Carlotti 1994; Funke et al. 1996a).

The presently available and widely used commercial identification systems include the API Coryne system (bioMerieux, Marcy lÉtoile, France) and the RapID CB Plus system (Remel, Lenexa, KS, USA). Both systems performed well in the published evaluations (Funke et al. 1997e, 1998f). However, it is emphasized to always question critically the identifications provided by any commercial identification system for coryneform bacteria and to correlate the results with simple basic characteristics such as macroscopic morphology and Gram stain results.

The presence of mycolic acids and their chain lengths can be detected by either thin-layer chromatography (TLC) or high-performance liquid chromatography (de Briel et al. 1992). These methods can be useful for the differentiation of *Corynebacterium* spp. (mycolic acids of 22 to 36 carbon atoms) from the partially acid-fast bacteria (mycolic acids of 30 to 78 carbon atoms), but may also provide evidence that a coryneform bacterium is not a *Corynebacterium* species (exceptions are *C. amycolatum*, *C. atypicum*, and *C. kroppenstedtii*) if mycolic acids are not detected. The detection of diamino acid of the petidoglycan by one-dimensional TLC is of value for determining the genus to which a particular strain may belong (see Table 1). The analysis of cellular fatty acids (CFA) by means of gas-liquid chromatography is a very useful method for the identification of coryneform bacteria on the genus level, but identification to the species level is impossible in most cases. The latter is because of closely related CFA profiles of coryneform bacteria belonging to the same genus (Bernard et al. 1991) and because the quantitative profiles observed depend strongly on the incubation conditions.

Molecular genetic-based identification systems for coryneform bacteria have been outlined in the 1990s. Restriction fragment length polymorphism analysis of the partly amplified and digested 16S rRNA gene has been demonstrated to be of use for identification of species within the genera *Corynebacterium* (Vaneechoutte et al. 1995) and *Brevibacterium* (Carlotti and Funke 1994). It is also possible to identify some corynebacteria on the species level by examining the length of the 16S–23S rRNA intergenic spacer region (Aubel et al. 1997). rRNA gene restriction fragment polymorphism analysis (ribotyping) has been demonstrated to allow the identification of corynebacteria if three different restriction enzymes (*Bst*EII, *Sma*I, and *Sph*II) are used (Björkroth et al. 1999). For pure taxonomic investigations of coryneform bacteria, full-length 16S rRNA gene sequencing and, on very rare occasions, quantitative DNA–DNA hybridizations might be necessary. In the routine clinical laboratory it is often sufficient to sequence the first 300 to 400 base pairs of the 16S rDNA gene in order to assign an isolate of a coryneform bacterium to a certain genus (Tang et al. 2000).

THE GENERA AND THEIR SPECIES

Genus *Corynebacterium*

The genus *Corynebacterium* presently consists of 62 species (and two taxon groups), 38 (and the two taxon groups) of which are medically relevant and which are covered in this chapter. The cell wall of corynebacteria contains *meso*-diaminopimelic acid (*m*-DAP) as diamino acid, as well as mycolic acids with 22 to 36 carbon atoms (Collins and Cummins 1986). *C. amycolatum*, *C. atypicum*, and *C. kroppenstedtii* are the only genuine *Corynebacterium* species which do not possess mycolic acids. Palmitic ($C_{16:0}$), oleic ($C_{18:1\omega9c}$), and stearic ($C_{18:0}$) acids are the main CFAs in all corynebacteria, and tuberculostearic acid can also be found in some species such as *C. urealyticum* (Bernard et al. 1991) and *C. confusum* (Funke et al. 1998c). The G + C content of *Corynebacterium* spp. varies from 46 mol% (in *C. kutscheri*) to 74 mol% (in *C. auris*) (Funke et al. 1995c), indicating the enormous diversity within the genus. The phylogenetic relationships within the genus *Corynebacterium* have been outlined (Pascual et al. 1995; Ruimy et al. 1995).

Gram staining of corynebacteria shows slightly curved, gram-positive rods with sides not parallel and sometimes slightly wider ends, giving some bacteria a typical club shape. It is emphasized that the club-shaped form of the rods is constantly observed only for true *Corynebacterium* spp., but not any other taxon of coryneform bacteria. True corynebacteria whose cellular morphology differ from the regular morphology include *C. durum*, *C. matruchotii*, and *C. sundsvallense* (see below under each species). If *Corynebacterium* cells are taken from fluid media, they are arranged as single cells, in pairs, in V forms, in palisades, or in clusters with a so-called Chinese letter appearance. Corynebacteria are always catalase-positive, and the medically relevant species are all nonmotile. The genus *Corynebacterium* contains both fermenting and nonfermenting species.

CORYNEBACTERIUM DIPHTHERIAE

Because of its clinical importance (see Table 2) this species is discussed in greater detail than the other species outlined in this chapter.

Colonial morphology

Traditionally, three types of *C. diphtheriae* have been described based on cultural and biochemical differences: biotype *gravis* (colonies $\geqslant$2 mm in diameter), *mitis* ($\geqslant$2 mm), and *intermedius* (<0.5 mm). The initial report that *gravis* strains caused the most severe cases of diphtheria (McLeod 1943) and that *mitis* strains were associated with milder clinical cases was not confirmed. On blood agar the tiny, transparent colonies of the *intermedius* biotype are typical of lipophilic corynebacteria and distinguish them from the larger, denser colonies of the nonlipophilic, slightly dry appearing *gravis* and *mitis* biotypes (Coyle et al. 1993). Most *mitis* and some *gravis* strains produce a narrow zone of weak β-hemolysis on blood agar, whereas *intermedius* strains are nonhemolytic. The ability to reduce tellurium salts to tellurium, which is precipitated in the cell to color the colonies gray, brown or black, is characteristic not only of *C. diphtheriae*, but also to a lesser extent of other corynebacteria. However, on a medium described by Tinsdale (1947) (containing L-cystine and potassium tellurite) only *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* produce a well delineated brown halo around the grayish-black colonies. A brown halo results when H_2S is produced by the action of cystinase on L-cystine and potassium tellurite is reduced to metallic tellurium.

Further characteristics

Colonies of *C. diphtheriae* from serum-based media (e.g. Loeffler slants) show pleomorphic rods some of which may be club-shaped (Figure 2). Cells may contain a few mostly terminal reddish-purple (metachromatic) polyphosphate granules which confer a beaded appearance. The biochemical characteristics of *C. diphtheriae* and its biotypes, including biotype *belfanti* as the nitrate reductase negative variant of biotype *mitis*, are given in Table 3. Of note is that all *C. diphtheriae* strains lack pyrazinamidase activity. Biotype *gravis* is distinguished from *mitis* by its ability to produce acid from glycogen. Propionic acid is one of the major end products of the glucose metabolism by *C. diphtheriae*. The cellular fatty acid profile of *C. diphtheriae* is particular in containing significant amounts of palmitoleic acid which is not observed in other corynebacteria (Bernard et al. 1991). *C. diphtheriae* is readily killed by heating at 58°C for 10 min. It is also susceptible to the usual disinfectants, but is relatively resistant to drying.

Toxin production

A comprehensive review on the biology and molecular epidemiology of diphtheria toxin has been written by Holmes (2000). Diphtheria toxin is synthesized by strains of *C. diphtheriae* that are lysogenic for one of a family of bacteriophages carrying *tox*, the gene for the exotoxin. It is a protein and the only presently recognized virulence factor of *C. diphtheriae*. Although the toxin inhibits protein synthesis in cell-free extracts of virtually all eukaryotic organisms, it is only toxic to the intact cells of certain mammals. Diphtheria toxin is a 535 amino acid, 58 kDa protein consisting of two fragments: an N-terminal catalytic fragment A (21 kDa amino acids 1–193) and fragment B (37 kDa) with two domains, an internal translocating one (205–378) and a C-terminal receptor-binding one (386–535). The proteo-

Table 2 *Frequent disease associations of coryneform bacteria*

Taxon	Disease or disease association
C. diphtheriae (toxigenic)	Throat diphtheria, cutaneous diphtheria
C. diphtheriae (nontoxigenic)	Endocarditis, foreign body infections, pharyngitis
Nonlipophilic corynebacteria:	
C. amycolatum	Wound infections, foreign body infections, bacteremia, urinary tract infections
C. glucuronolyticum	Genitourinary tract infections (mainly males)
C. minutissimum	Wound infections, urinary tract infections
C. 'nigricans'	Genitourinary tract infections (mainly females)
C. pseudodiphtheriticum	Respiratory tract infections, endocarditis
C. pseudotuberculosis	Lymphadenitis (occupational)
C. riegelii	Urinary tract infections (females)
C. striatum	Wound infections, respiratory tract infections, foreign body infections
C. ulcerans (toxigenic)	Respiratory diphtheria
Lipophilic corynebacteria:	
CDC goup F-1	Urinary tract infections
CDC group G	Catheter infections, bacteremia, endocarditis, wound infections
C. jeikeium	Endocarditis, bacteremia, foreign body infections, wound infections
C. macginleyi	Eye infections
C. urealyticum	Urinary tract infections, bacteremia, wound infections
Arthrobacter spp.	Urinary tract infections, bacteremia, foreign body infections
Brevibacterium spp.	Bacteremia, foreign body infections, malodorous feet
D. hominis	Wound infections, bacteremia
R. dentocariosa	Endocarditis, bacteremia, suppurative lesions
O. turbata	Foreign body infections, bacteremia
Cellulomonas spp.	Bacteremia, wound infections
C. cellulans	Foreign body infections, bacteremia
Microbacterium spp.	Bacteremia, foreign body infections, wound infections
A. bernardiae	Abscess formation (together with mixed anaerobic flora)
A. haemolyticum	Pharyngitis in older children, wound and tissue infections
A. pyogenes	Abscess formation, wound, and tissue infections
G. vaginalis	Bacterial vaginosis, preterm birth, endometritis, postpartum sepsis

lytic 'nicking' into fragments A and B occurs before or shortly after binding. Following binding to the receptor, the toxin enters the cell through receptor-mediated endocytosis. An acid pH triggers the translocation of fragment A across the endosomal membrane into the cytosol. The A fragment becomes enzymatically active following reduction of the disulfide bridge which connects it to the B fragment. Then it catalyses ADP ribosylation of the GTP-binding elongation factor 2 (EF-2) on diphthamide, a posttranslationally modified histidine. EF-2 normally effects the translocation of the growing polypeptide chain from the aminoacyl-tRNA to the peptidyl tRNA position on the ribosome. The energy for this reaction is provided by hydrolysis of GTP-ATP. Ribosylation of EF-2 inhibits this GTP-dependent translocation on the ribosome and thus the building of the polypeptide chain (Krueger and Barbieri 1995).

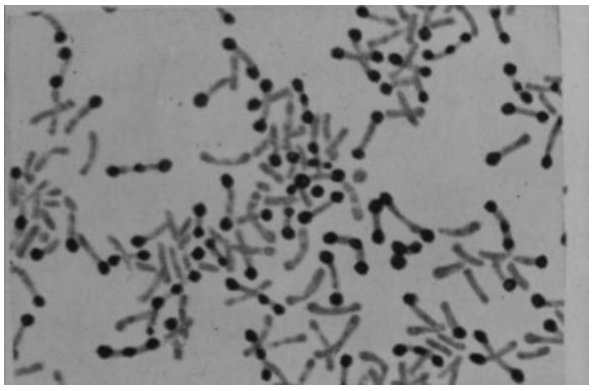

Figure 2 Corynebacterium diphtheriae *with metachromatic granules*

Addition of iron to the growth medium of *C. diphtheriae* inhibits production of diphtheria toxin. The regulation of diphtheria toxin expression by varying iron concentrations in the growth medium occurs at the level of transcription of the *tox* gene (Russell et al. 1984). The repressor molecule that regulates the *tox* gene is an iron-dependent protein that binds DNA by a sequence-specific mechanism and also regulates iron uptake by *C. diphtheriae* (Schmitt et al. 1992; Schmitt and Holmes 1994).

Table 3 *Characteristics of medically relevant* Corynebacterium *spp.*

Species	Fermentation/ oxidation	Nitrate reduction	Urease	Esculin hydrolysis	Pyrazinamidase	Alkaline phosphatase	Acid production from					CAMP reaction	Other features
							Glucose	Maltose	Sucrose	Mannitol	Xylose		
Nonlipophilic corynebacteria													
C. afermentans subsp. *afermentans*	O	–	–	–	+	+	–	–	–	–	–	V	
C. amycolatum	F	V	V	–	+	+	+	V	V	–	–	–	Most O/129 resistant, propionic acid detected
C. argentoratense	F	–	–	–	+	V	+	–	–	–	–	–	Chymotrypsin positive; propionic acid detected
C. atypicum	F	–	–	–	–	–	+	+	+	–	–	ND	Pinpoint colonies; lacking mycolics; β-glucuronidase +
C. aurimucosum	F	–	–	–	+	+	+	+	+	–	–	ND	Sticky colonies, slightly yellowish
C. auris	O	–	–	–	+	+	–	–	–	–	–	+	Slight adherence to agar; cleaved mycolics
C. confusum	F	+	–	–	+	+	(+)	–	–	–	–	–	Tyrosine negative; propionic acid detected
C. coyleae	F	–	–	–	+	+	(+)	–	–	–	–	+	
C. diphtheriae biotype *belfanti*	F	–	–	–	–	–	+	+	–	–	–	–	
C. diphtheriae biotype *gravis*	F	+	–	–	–	–	+	+	–	–	–	–	Glycogen positive
C. diphtheriae biotype *mitis*	F	+	–	–	–	–	+	+	–	–	–	–	Glycogen negative
C. durum	F	+	(V)	(V)	+	–	+	+	+	V	–	–	Adherence to agar; propionic acid detected
C. falsenii	F	–	(V)	–	(+)	+	(+)	V	–	–	–	–	Yellowish
C. freneyi	F	V	–	–	+	+	+	+	+	–	–	ND	α-Glucosidase positive; grows at 20°C and 42°C
C. glucoronolyticum	F	V	V	V	+	V	+	V	+	–	V	+	β-Glucuronidase positive; propionic acid detected
C. imitans	F	V	–	–	(+)	+	+	+	(+)	–	–	+	Tyrosine negative; O/129 resistant
C. matruchotii	F	+	–	V	+	–	+	+	+	–	–	–	'Whip handle' (Gram); propionic acid detected
C. minutissimum	F	–	–	–	+	+	+	+	V	V	–	–	Tyrosine positive
C. mucifaciens	O	–	–	–	+	+	+	–	V	–	–	–	Mucoid yellowish colonies
C. 'nigricans'	F	–	–	–	V	V	+	+	(+)	–	–	–	Black, adherent colonies
C. propinquum	O	+	–	–	V	V	–	–	–	–	–	–	Tyrosine positive

(*Continued over*)

Table 3 *Characteristics of medically relevant* Corynebacterium spp. (*Continued*)

Species	Fermentation/ oxidation	Nitrate reduction	Urease	Esculin hydrolysis	Pyrazinamidase	Alkaline phosphatase	Acid production from					CAMP reaction	Other features
							Glucose	Maltose	Sucrose	Mannitol	Xylose		
C. pseudodiphtheriticum	O	+	+	–	+	V	–	–	–	–	–	–	
C. pseudotuberculosis	F	V	+	–	–	V	+	+	V	–	–	REV	Propionic acid detected
C. riegelii	F	–	+	–	V	V	–	(+)	–	–	–	–	
C. singulare	F	–	+	–	+	+	+	+	+	–	–	–	Tyrosine positive
C. simulans	F	+	–	–	V	+	+	–	+	–	–	–	Reduces nitrite
C. striatum	F	+	–	–	+	+	+	–	V	–	–	V	Tyrosine positive
C. sundsvallense	F	–	+	–	V	V	+	+	+	–	–	–	Sticky colonies
C. thomssenii	F	–	+	–	+	+	+	+	+	–	–	–	*N*-Acetyl-β-glucusaminidase positive; sticky colonies
C. ulcerans	F	–	+	–	–	+	+	+	–	–	–	REV	Glycogen positive; propionic acid detected
C. xerosis	F	V	–	–	+	+	+	+	+	–	–	–	O/129 susceptible; propionic acid detected
Lipophilic corynebacteria													
C. accolens	F	+	–	–	V	–	+	–	V	V	–	–	
C. afermentans subsp. *lipophilum*	O	–	–	–	+	+	–	–	–	–	–	V	
C. appendicis	F	–	+	–	+	+	(+)	(+)	–	–	–	ND	TBSA positive
CDC group F-1	F	V	+	–	+	–	+	+	+	–	–	–	
CDC group G	F	V	–	–	+	+	+	V	V	–	–	–	Fructose positive; anaerobic growth positive
C. diphtheriae biotype *intermedius*	F	+	–	–	–	–	+	+	–	–	–	–	Propionic acid detected
C. jeikeium	O	–	–	–	+	+	+	V	–	–	–	–	Fructose negative; anaerobic growth negative
C. kroppenstedtii	F	–	–	+	+	–	+	V	+	–	–	–	Lacking mycolic acids; propionic acid detected
C. lipophiloflavum	O	–	+	–	+	+	–	–	–	–	–	–	Yellow
C. macginleyi	F	+	–	–	–	+	+	–	+	V	–	–	
C. urealyticum	O	–	+	–	+	V	–	–	–	–	–	–	

F, fermentation; O, oxidation; +, positive; –, negative; V, variable; ( ), delayed or weak reaction; ND, no data; REV, CAMP inhibition reaction; TBSA, tuberculostearic acid.

tox+ Bacteriophages

Murphy et al. (1974) demonstrated that the diphtheria toxin gene (*tox*) is located on the genome of the bacteriophage. Phages that carry *tox* integrate into the chromosome of *C. diphtheriae* by a process analogous to λ lysogenization of *Escherichia coli* (Buck and Groman 1981). Site-specific recombination occurs between an attachment site on the phage (*attP*) and one or two attachment sites on the bacterial chromosome (*attB1*, *attB2*) (Rappuoli and Ratti 1984). Although *attB*-related sequences also occur in a wide variety of *Corynebacterium* spp., the only species that are known either to be lysogenized by β phage or to produce diphtheria toxin are *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* (Cianciotto et al. 1986). Furthermore, *tox*-containing, nontoxigenic isolates of *C. diphtheriae* have been described (Cianciotto and Groman 1997).

Pathogenicity and toxin testing

Guinea pigs and rabbits are most susceptible to experimental infection with *C. diphtheriae*, whereas rats and mice are resistant (see review by Barksdale 1970). Subcutaneous inoculation with a toxigenic culture or filtrate into the flank of a guinea pig leads within 12–18 h to a local edema that gradually spreads. Depending on the dose, death usually occurs between 18 and 96 h. Hemorrhagic edema with a central necrotic area is present at the injection site. Regional lymph nodes and abdominal viscera are congested, but the most striking lesion is the swelling and congestion of the adrenal glands, with scattered hemorrhages in the medulla or cortex, or both.

For *in vitro* testing it is recommended that at least ten colonies of *C. diphtheriae* and related species be tested by the modified Elek method as described in a recent publication (Engler et al. 1997). A 3-h enzyme linked immunosorbent assay for the detection of diphtheria toxin from clinical isolates of *Corynebacterium* spp. has also been developed by the WHO Diphtheria Reference Unit (Engler and Efstratiou 2000). Another rapid and simple test for detecting diphtheria toxin, called the immunochromatographic strip (ICS) assay is also now available (Engler et al. 2002). In the latter test, pure cultures of toxigenic diphtheria strains may be identified within 10 to 15 min after 3-h incubation in an enrichment broth or detected in 4–6 h after clinical specimens containing toxigenic strains had been previously incubated in an enrichment broth for 16 h.

Polymerase chain reaction (PCR)-based methods for the detection of the diphtheria toxin gene (*tox*) in isolated bacteria have been developed and validated (Pallen 1991; Hauser et al. 1993). Recently, a real-time fluorescence PCR assay for rapid detection of the *tox* gene has also been described (Mothershed et al. 2002). The molecular based methods have advantages over the immunological methods as they may take as little as 4–5 h from the colonies to the final result, work from mixed cultures, and give clear-cut results.

Genome

During the writing of this chapter, researchers at the Sanger Institute have completed the sequencing of the genome of *C. diphtheriae* strain NCTC 13129. The genome is 2 488 635 bp long and has a G + C content of 53.5 percent. Further information can be obtained from www.sanger.ac.uk/projects/C_diphtheriae.

Typing methods

Outbreaks of *C. diphtheriae* in the states of the former Soviet Union and other locations have been studied by whole-cell peptide analysis, whole-genome restriction fragment length polymorphism analysis, ribotyping, pulsed-field gel electrophoresis, PCR single strand conformation polymorphism analysis of *tox* and *dtxR*, as well as of the 16s–23S rRNA gene spacer region, amplified fragment length polymorphisms, random amplification of polymorphic DNA, and multilocus enzyme electrophoresis (De Zoysa and Efstratiou 1999, 2000; Popovic et al. 2000). Sequencing studies with *C. diphtheriae* strains from the recent epidemic in the former Soviet Union have shown that point mutations within the *tox* gene were silent mutations, whereas multiple point mutations, which even led to amino acid substitutions, were observed in the *dtxR* gene (Nakao et al. 1997).

CLINICAL SIGNIFICANCE OF *C. DIPHTHERIAE*

Respiratory tract diphtheria and cutaneous diphtheria are the classical manifestations of diphtheria (Table 2), whereas cardiac and neurologic toxicity are the result of absorption and dissemination of diphtheria toxin.

Respiratory diphtheria

The initial differential diagnosis of respiratory diphtheria includes streptococcal tonsillopharyngitis, peritonsillar abscess, Vincent's angina, acute epiglottitis, infectious mononucleosis, and candidiasis. The diagnosis of *C. diphtheriae* becomes more likely if the patient is unimmunized or has not received booster immunizations, if the pretreatment antitoxin titer is below 0.01 IU/ml, and if the patient had a history of contact with a diphtheria case or has recently travelled to regions with endemic diphtheria.

In respiratory diphtheria, *C. diphtheriae* colonizes the mucosa of the nasopharynx and multiply locally without bloodstream invasion. After a short incubation period (usually 2–5 days) the patients start to complain about sore throat, malaise, and low grade fever (the latter as a result of substantial absorption of toxin). Within the

nasopharynx, the toxin induces a leukocyte response, tissue necrosis, and most significantly formation of a tough, adherent pseudomembrane which is composed of fibrin, dead cells, and bacteria. Initially, the membrane is white and glassy, but develops into a dirty grayish-green color. Characteristically, the membrane is strongly adherent and bleeding occurs when attempts are made to remove it. Membrane formation usually starts on the tonsils or in the posterior pharynx. However, the membrane may spread to the pharyngeal wall, fauces, soft palate, and even the bronchi. Laryngeal diphtheria can occur as a result of membrane extension, but may also be the only site of infection. In general, the extent of the membrane correlates with the severity of symptoms. As a result of severe pharyngeal diphtheria a significant edema of the submandibular areas and the anterior neck with lymphadenopathy may develop, resulting in the so-called patients' 'bullneck appearance'. Depending on the size of the respiratory infection due to *C. diphtheriae*, intubation or tracheostomy might be required. Untreated, severe forms of respiratory tract diphtheria can result in death (mortality rates vary from 3–15 percent) within a week of onset of symptoms. This may be due to cardiac toxicity of the diphtheria toxin (see below) or airway obstruction, or due to systemic toxin effects leading to severe prostration, stupor, and coma of the patient.

First evidence of cardiac toxicity occurs after 1–2 weeks of illness. Changes in ECG patterns can progress to more severe forms of arrhythmias. Myocarditis can present acutely with congestive failure and circulatory collapse. Patients with ECG changes due to diphtheria myocarditis have a mortality rate three to four times higher than those with normal tracings.

Neurologic toxicity is also proportional to the severity of the primary infection. Within the first few days of disease, local paralysis of the pharynx and palate regions may occur. Later, cranial neuropathies develop and peripheral neuritis (principally a motor defect) is seen from 2 weeks to 3 months after onset of disease. However, although slow, total resolution of all toxin-induced nerve damage is the rule.

Cutaneous diphtheria

C. diphtheriae can cause skin infections characterized by chronic nonhealing, punched-out ulcers, occasionally with a membrane and edematous rolled borders; group A streptococci and/or *Staphylococcus aureus* can also be often isolated from these ulcers. *C. diphtheriae*-associated skin ulcers are more frequently seen in subtropical countries and the tropics, whereas in industrialized countries this disease (frequently non-toxigenic strains) may be detected in people with low socioeconomic standard. Cutaneous diphtheria is rarely associated with signs of intoxication, but the infection itself induces high antitoxin levels. Patients with *C. diphtheriae* skin infections may shed the microorganism to the inanimate environment or induce respiratory diphtheria in other individuals. Transmission of *C. diphtheriae* from cutaneous lesions is often more efficient than from patients with throat diphtheria.

Invasive disease

Within the 1990s several reports indicated the emergence of invasive nontoxigenic *C. diphtheriae* strains mainly in drug and alcohol addicts causing endocarditis (associated with high mortality rates), osteomyelitis, or septic arthritis. Particular virulence factors have not been described for these nontoxigenic strains, many of which belonged to a clonal lineage (Funke et al. 1999). In parallel, nontoxigenic strains causing pharyngitis have also emerged in the last few years.

Treatment

The cornerstone of diphtheria therapy is the administration of diphtheria antitoxin, a hyperimmune antiserum produced in horses. It is of utmost importance to treat a patient with suspected respiratory diphtheria immediately, i.e. before laboratory results are available. Antitoxin neutralizes toxin which has not already bound to cells. The degree of protection due to antitoxin is inversely related to the duration of clinical illness prior to its administration. Between 20 000 and 120 000 intravenously administered IU are recommended depending on the stage and the severity of disease. Repeated injections have not been shown to be of additional benefit. Roughly 10 percent of the patients may show symptoms of hypersensitivity to horse proteins.

Antibacterial chemotherapy directed against *C. diphtheriae* is recommended to treat diphtheria by eliminating the organism, thereby terminating the toxin production and preventing the spread of the organism. Although several antibiotics including cefalothin, clindamycin, tetracycline, rifampin, chloramphenicol, and gentamicin have been shown in vitro to be effective against *C. diphtheriae*, penicillin or erythromycin are the drugs of choice. The recommended patient's total treatment period is 14 days. Patients should be maintained in strict isolation throughout therapy; after therapy, two consecutive negative cultures at 24-h intervals are required. Treatment of acute diphtheria with prednisone did not reduce the incidence of sequelae. If a person is a carrier of *C. diphtheriae* in the respiratory tract, a 7-day course with erythromycin is recommended for eradication. Cases of *C. diphtheriae* endocarditis or arthritis might be treated with a combination of penicillin or ampicillin together with gentamicin for 4–6 weeks.

Prevention

In general, the frequency of different symptoms and the severity of diphtheria as a disease are inversely

proportional to the patient's immunization history. Fatal outcomes are extremely rare in fully immunized individuals.

Immunization is done with a formalin-inactivated toxin vaccine. Children from 6 weeks to 7 years of age should receive three intramuscular injections of vaccine given at 4–8 weeks intervals, beginning at 6–8 weeks of age, followed by a fourth dose 6–12 months after the third. Children who have completed their primary immunization before the age of 4 years should receive a booster dose of diphtheria toxoid at the time of school entry. For epidemiological reasons it is desirable to reach a childhood vaccination coverage of at least 95 percent. Persons older than 7 years should receive booster immunization every 10 years. Antibody concentrations (measured by enzyme immunoassays or on Vero cell cultures) of 0.01–0.1 IU are thought to confer protection. Patients should receive toxoid immunization in the convalescent stage of their disease since an infection does not always induce adequate levels of antitoxin. Close contacts whose immunization status is unclear or incomplete should immediately receive the toxoid dose that is appropriate for their age and complete the proper series of immunizations. Prophylactic use of antitoxin in contact persons is not recommended.

Other nonlipophilic *Corynebacterium* spp.

C. AFERMENTANS SUBSP. *AFERMENTANS*

This species (Riegel et al. 1993) is part of the normal human skin flora and has up to now been isolated mainly from blood cultures. Colonies are, as for all the other nonlipophilic corynebacteria, 1–1.5 mm in diameter after 24 h of incubation. Colonies are whitish, convex with regular edges, and creamy. About 60 percent of all strains of this taxon are CAMP reaction positive. *C. afermentans* subsp. *afermentans* can be differentiated from *C. auris* and *T. otitidis* (both of which give the identical numerical code in the API Coryne system) by the consistency of its colonies (*C. auris* is slightly adherent to agar) and morphology on Gram staining (*T. otitidis* has longer cells). *C. afermentans* subsp. *afermentans* is generally susceptible to β-lactam antibiotics.

C. AMYCOLATUM

C. amycolatum is part of the normal human skin flora (Collins et al. 1988) and is the most frequently encountered *Corynebacterium* species in human clinical material (Funke et al. 1997f). This species does not contain mycolic acids, although it is a true *Corynebacterium*. Colonies are very typically dry, waxy, and grayish white with irregular edges. Isolates of this species are remarkable for their variability in basic biochemical reactions (Table 3) and, therefore, had been often misidentified in the past as *C. xerosis*, *C. striatum*, or *C. minutissimum* (Funke et al., 1996b, Zinkernagel et al. 1996). When tested on Mueller–Hinton agar supplemented with 5 percent sheep blood, nearly all *C. amycolatum* strains were resistant to the vibriocidal compound O/129 (150 μg disks). All *C. amycolatum* strains produce propionic acid as the major end product of glucose metabolism. *C. amycolatum* strains are very often multidrug resistant (Funke et al. 1996c).

C. ARGENTORATENSE

This species is mainly isolated from human throat cultures (Riegel et al. 1995b). Colonies are slightly rough. Glucose fermentation by *C. argentoratense* is quite rapid compared to the phenotypically similar but slowly fermenting *C. coyleae*. In addition, CAMP-negative *C. argentoratense* produces propionic acid, but CAMP-positive *C. coyleae* does not. *C. argentoratense* is the only medically relevant *Corynebacterium* expressing α-chymotrypsin activity. Although *C. argentoratense* is phylogenetically closely related to *C. diphtheriae*, it does not harbor the *tox* gene coding for the diphtheria toxin.

C. ATYPICUM

This recently defined species was isolated from an unknown human clinical source (Hall et al. 2003). Mycolic acids cannot be detected in cell wall hydrolysates of this true *Corynebacterium*. Although described as being nonlipophilic, colonies of *C. atypicum* are pinpoint after incubation at 37°C for 48 h. The biochemical screening profile of *C. atypicum* is given in Table 3. This species is unusual in lacking pyrazinamidase activity but expressing β-glucuronidase.

C. AURIMUCOSUM

It is important to note that the description of this recently established species was based on a single strain (Yassin et al. 2002a). On 5 percent SBA plates, colonies of this species are slightly yellow and sticky, but on Trypticase soy agar without blood, they appear colorless and slimy. The basic biochemical profile of this species is similar to that of *C. minutissimum* (Table 3).

C. AURIS

C. auris has been isolated almost exclusively from the ear region. Colonies are dryish and slightly adherent to, but do not penetrate agar (Funke et al. 1995c). All *C. auris* strains are strongly CAMP test positive. Abundant degradation products of mycolic acids are indirectly observed when CFA patterns are determined with the commercial Sherlock system. It is noteworthy that the minimum inhibitory concentrations (MIC) of β-lactam antibiotics for *C. auris* strains are elevated (Funke et al. 1996c).

C. CONFUSUM

This species has been isolated from patients with foot infections, a blood culture, and a breast abscess (Funke et al. 1998c). Acid from glucose is produced very weakly and growth under anaerobic conditions is weak. If glucose fermentation is judged to be negative, *C. confusum* strains can be misidentified as *C. propinquum*. However, in contrast to the latter species, *C. confusum* does not hydrolyze tyrosine and contains small amounts of TBSA (1–3 percent), whereas *C. propinquum* hydrolyzes tyrosine but does not contain TBSA. *C. confusum* is differentiated from *C. coyleae* and *C. argentoratense* by its ability to reduce nitrate.

C. COYLEAE

C. coyleae (Funke et al. 1997d) has been isolated mainly from cultures of blood and other normally sterile body fluids, but it may also be recovered from genitourinary specimens. The consistency of the colonies is either creamy or sticky. A slow fermentative acid production from glucose and a strongly positive CAMP reaction are the most significant phenotypic characteristics. *C. coyleae* is positive for cystine arylamidase, which is not observed for many other corynebacteria.

C. DURUM

This species was originally described as being isolated exclusively from respiratory tract specimens (Riegel et al. 1997a). *C. durum* is the most frequent *Corynebacterium* species isolated from throat swabs of healthy persons. It is a peculiar nonlipophilic organism that forms colonies of only 0.5 to 1 mm in diameter after aerobic incubation for 72 h. In the original description of this bacterium, beige and rough colonies with convolutions, and strong adherence to agar were mentioned. Gram stains of aerobic cultures may show long and filamentous rods with occasional 'bulges', but true *C. durum* strains do not have *C. matruchotii*-like 'whip handles'. Strains grow only weakly under anaerobic conditions. They always reduce nitrate, and some may exhibit weak and delayed urease and esculinase activities, whereas pyrrolidonyl arylamidase activity is not detected. *C. durum* usually ferments galactose and very often mannitol, whereas *C. matruchotii* is usually negative for those sugars.

C. FALSENII

Members of this species are mainly isolated from sterile body fluids (Sjöden et al. 1998). After 72 h, most strains described to date exhibit a yellowish pigment which is not observed in any other nonlipophilic *Corynebacterium* species encountered in clinical specimens (except the rarely found *C. aurimucosum* and the dryish *C. xerosis*). The most characteristic biochemical features of *C. falsenii* are a slow but fermentative acid production from glucose, a weak pyrazinamidase reaction, and quite weak urease activity.

C. FRENEYI

Strains of this species are usually isolated from skin-related material (Renaud et al. 2001). *C. freneyi* is phylogenetically and phenotypically closely related to *C. xerosis*. All *C. freneyi* strains exhibit α-glucosidase activity, which is not frequently observed in other nondiphtheria, nonlipophilic *Corynebacterium* species (very few *C. amycolatum* and all *C. xerosis* strains express this enzyme). *C. freneyi* can be differentiated from *C. xerosis* by glucose fermentation at 42°C and growth at 20°C, whereas *C. xerosis* is negative for these two reactions.

C. GLUCURONOLYTICUM

C. seminale is a junior synonym of *C. glucuronolyticum* (Funke et al. 1995a; Devriese et al. 2000). This species is probably part of the normal genitourinary flora of males, while its presence in females is uncertain. 16S rRNA gene sequences derived from fluids of patients with prostatitis were homologous to sequences derived for this species, indicating that *C. glucuronolyticum* might be involved in selected cases of prostatitis. This fermentative species is remarkable for its variability in basic biochemical reactions (Table 3). It is the only medically relevant *Corynebacterium* species exhibiting β-glucuronidase activity (except *C. atypicum*). When urea activity is present, it is very strong. All *C. glucuronolyticum* strains are CAMP reaction positive. Propionic acid is a major end product of glucose metabolism. *C. glucuronolyticum* strains are often tetracycline resistant and may also exhibit resistance to macrolides and clindamycin (Funke et al. 1996c).

C. IMITANS

This species was originally isolated from a nasopharyngeal specimen of a child suspected of having throat diphtheria, as well as from three adult contacts (Funke et al. 1997a). This was the first well-documented case of person-to-person transmission of a *Corynebacterium* other than *C. diphtheriae* in a nonhospital setting. Pyrazinamidase activity was only weak, as was the fermentation of sucrose, which may lead to the initial misidentification as an atypical *C. diphtheriae* strain (Table 3).
C. imitans is CAMP reaction positive and does not hydrolyze tyrosine, whereas the opposite reactions are observed for the phenotypically closely related *C. minutissimum*. *C. imitans* do not harbor the *tox* gene. In contrast to *C. diphtheriae*, *C. imitans* is resistant to O/129.

C. MATRUCHOTII

This species is a natural inhabitant of the oral cavity, but is a rare human pathogen. Microcolonies may appear flat or spider-like. *C. matruchotii* has a most unusual appearance in Gram stain, in that so-called 'whip handles' (i.e. filamentous bacteria with a single short

bacillus adjacent to the end of the filament, creating the illusion of a whip) are observed. *C. durum* strains (see above) have been misidentified as *C. matruchotii* in the past (Rassoulian Barrett et al. 2001). *C. matruchotii* are consistently negative for galactose, whereas *C. durum* strains can be positive.

C. MATRUCHOTII-LIKE STRAIN

This taxon is represented by a single strain, ATCC 43833 (Rassoulian Barrett et al. 2001). The strain has been deposited as *C. matruchotii*, but dot-blot hybridizations and 16S rDNA sequencing data revealed that it is distinct. Colonies are pinpoint to 0.1 mm in diameter, grayish-white, with a smooth, nonadherent texture. Biochemical screening reactions are similar to *C. minutissimum*, except that esculinase activity is detected for the *C. matruchotii*-like strain.

C. MINUTISSIMUM

This species is a member of the normal human skin flora. Most colonies are of a creamy texture, but some may also be sticky. *C. minutissimum* have a fermentative metabolism, produce acid from sucrose variably, and very few strains are able to produce acid from mannitol. *C. minutissimum* strains exhibit DNase activity and are also often pyrrolidonyl arylamidase positive. Nearly all strains hydrolyze tyrosine. Lactic and succinic acids are the major end products of glucose metabolism. Nearly all *C. minutissimum* strains are susceptible to O/129 (Früh et al. 1998).

C. MUCIFACIENS

This species is mainly isolated from blood cultures and other sterile body fluids (Funke et al. 1997c). Colonies are very distinct because they are slightly to overtly yellow and very mucoid. *C. mucifaciens* is the only presently known *Corynebacterium* species exhibiting such mucoid colonies. An extracellular substance (probably polysaccharides) has been demonstrated as the ultrastructural correlate of the mucoid colonies. *C. mucifaciens* has an oxidative metabolism; it consistently produces acid from glucose, but acid production from sucrose is variable (Table 3). TBSA can be detected in amounts of 1–2 percent of total CFAs. β-Lactams and aminoglycosides show very good activities against *C. mucifaciens* isolates.

C. 'NIGRICANS'

This taxon is an unusual black-pigmented *Corynebacterium* that seems to have its habitat in the genitourinary tract of mainly females (Shukla et al. 2001). The basic biochemical features are similar to *C. minutissimum*, but the black-pigmented *Corynebacterium* also pits the agar and has a strong adhesion to agar. Another unusual feature of this bacterium is that some strains can ferment glycogen (which is seen in very few true corynebacteria like some *C. diphtheriae* strains).

C. PROPINQUUM

This species is the closest phylogenetic relative of *C. pseudodiphtheriticum* and has the same niche (i.e. the oropharynx). Colonies are somewhat dryish. *C. propinquum* reduces nitrate and hydrolyzes tyrosine, but does not hydrolyze urea (Table 3). The species has an oxidative metabolism. Strains are usually susceptible to β-lactam antibiotics.

C. PSEUDODIPHTHERITICUM

This species is part of the normal oropharyngeal flora. As given in Table 2, this species has been well documented to cause respiratory tract infections in various populations. Colonies are slightly dry with entire edges. This nonfermenting species reduces nitrate and hydrolyzes urea, but does not produce acid from any of the commonly tested carbohydrates (Table 3). Some strains hydrolyze tyrosine. *C. pseudodiphtheriticum* strains are susceptible to β-lactam antibiotics, but resistance to macrolides and clindamycin has been observed.

C. PSEUDOTUBERCULOSIS

This species is phylogenetically closely related to *C. diphtheriae*, may harbor the diphtheria toxin gene, produces propionic acid as glucose fermentation product, and contains large amounts of the CFA $C_{16:1\ \omega7c}$. Like *C. ulcerans*, *C. pseudotuberculosis* is positive for urease and the CAMP inhibition test (i.e. complete inhibition of the effect of *S. aureus* β-hemolysin on sheep erythrocytes is achieved by streaking the presumed strain at a right angle toward *S. aureus* and incubating overnight; a β-hemolysin inhibition zone in the form of a triangle is observed). *C. pseudotuberculosis* is not susceptible to O/129, whereas *C. ulcerans* strains are (Hommez et al. 1999).

C. RIEGELII

This species is mainly isolated from females with urinary tract infections (Funke et al. 1998a). Some colonies are of creamy consistency, whereas others are sticky. *C. riegelii* strains exhibit extremely strong urease activity. A very peculiar feature of *C. riegelii* strains is the slow fermentation of maltose but not of glucose; no other defined *Corynebacterium* species has this characteristic (Table 3). The weak anaerobic growth corresponds to the weak fermentative metabolism.

C. SIMULANS

This species is isolated from skin-related specimens. *C. simulans* has been delineated from some *C. striatum*-like strains (Wattiau et al. 2000). Other close phylogenetic neighbors include *C. minutissimum* and *C. singulare*. *C. simulans* is the only valid *Corynebacterium* species

described to date which reduces nitrite. Further characteristics which separate *C. simulans* from the closely related fermentative corynebacteria are its inability to acidify ethylene glycol and to grow at 20°C (in contrast to *C. striatum*).

C. SINGULARE

This species (Riegel et al. 1997b) is infrequently found in clinical specimens. Key biochemical reactions are those for *C. minutissimum*, except that urease activity is observed (Table 3). The numerical API Coryne system profile is 6101125, indicating that pyrrolidonyl arylamidase activity is observed. *C. singulare* hydrolyzes tyrosine. This species does not produce propionic acid as fermentation product, differentiating it from *C. amycolatum*.

C. STRIATUM

C. striatum is part of the normal human skin flora. Nosocomial transmission of *C. striatum* has been documented. Some investigators described *C. striatum* colonies as somewhat like those of small coagulase-negative staphylococci. Some *C. striatum* strains have a positive CAMP reaction but this is usually not as strong as observed for other corynebacteria. Lactic and succinic acids are the major end products of glucose metabolism. All *C. striatum* strains are susceptible to O/129. *C. striatum* might be resistant to macrolides, clindamycin, tetracyclines, and quinolones.

C. SUNDSVALLENSE

This is a recently described species (Collins et al. 1999) which is infrequently encountered in clinical material. Colonies are buff or slightly yellowish and adherent to agar and have a sticky consistency. Gram stains show bulges or knobs at the ends of some rods, and these are not seen in any other corynebacteria. Fermentation of glucose, lactose, and sucrose is slow (Table 3). *C. sundsvallense* can be separated from *C. durum* by its positive α-glucosidase reaction and its inability to ferment galactose. It is further differentiated from *C. matruchotii* by expressing urease but not nitrate reductase activity.

C. THOMSSENII

This is a very rarely encountered *Corynebacterium* species. It is fastidious and slow-growing, resulting in colonies of <0.5 mm after 48 h, but it is not lipophilic (Zimmermann et al. 1998). After 96 h, colonies are molar tooth-like, very sticky, and slightly adherent to agar. This species is the only *Corynebacterium* species expressing *N*-acetyl-β-glucosaminidase activity.

C. ULCERANS

Phylogenetically, *C. ulcerans* is, together with *C. pseudotuberculosis*, the closest relative of *C. diphtheriae* (Riegel et al. 1995c), can harbor the diphtheria toxin gene, and contains significant amounts of $C_{16:1\omega7c}$. Colonies are somewhat dry and waxy and gray-white with light hemolysis. Strains of *C. ulcerans* are positive for glycogen, starch, and trehalose fermentation.

C. XEROSIS

The natural habitat of *C. xerosis* is unknown. Colonies are dry, granular, and yellowish with irregular edges. It is important to realize that nearly all '*C. xerosis*' strains which appeared in the literature before 1996 may have been misidentified *C. amycolatum* strains (Funke et al. 1996b). *C. xerosis* has a fermentative metabolism, is variable for the presence of nitrate reductase (Table 3), but always expresses α-glucosidase, as well as leucine arylamidase activities. Lactic acid is the major end product of glucose metabolism, and strains are, in contrast to *C. amycolatum*, susceptible to O/129.

Lipophilic *Corynebacterium* spp.

C. ACCOLENS

This species is found in specimens from eyes, ears, nose, and oropharynx (Neubauer et al. 1991). Colonies are, as for all other lipophilic corynebacteria, convex, smooth, and <0.5 mm in diameter on SBA. *C. accolens* has a variable pyrazinamidase reaction, but is negative for alkaline phosphatase, thus differentiating it from the morphologically and biochemically closely related CDC group G bacteria (Table 3). This species is susceptible to a broad spectrum of antibiotics.

C. AFERMENTANS SUBSP. *LIPOPHILUM*

Strains belonging to this species have been isolated mainly from blood cultures, as well as from superficial wounds. *C. afermentans* subsp. *lipophilum* has an oxidative metabolism and does not produce acid from any of the carbohydrates usually tested (Table 3). It is the only species of lipophilic corynebacteria which may exhibit a positive CAMP reaction. The numerical code of this species in the API Coryne system is 2100004, and so by that method it cannot be differentiated from the more robustly growing *C. afermentans* subsp. *afermentans*, *C. auris*, or *T. otitidis*. Strains are usually susceptible to β-lactam antibiotics.

C. APPENDICIS

The description of this recently defined species is based on a single strain isolated from a patient with appendicitis accompanied by abscess formation (Yassin et al. 2002b). *C. appendicis* is fermentative and its basic biochemical characteristics are given in Table 3. Unusually high amounts of TBSA (48 percent of total CFAs) have been described for this species whereas in other corynebacteria TBSA rarely accounts for more than 10 percent of total CFAs.

CDC GROUP F-1 BACTERIA

CDC group F-1 bacteria have not been given a species name. Although they are genetically distinct, no distinguishing phenotypic markers which clearly allow their separation from other defined *Corynebacterium* spp. have been found (Riegel et al. 1995a). Members of this taxon group are the only lipophilic fermentative corynebacteria able to hydrolyze urea. Of note is the negative alkaline phosphatase reaction (Table 3). CDC group F-1 strains are usually susceptible to penicillin, but are often resistant to macrolides.

CDC GROUP G BACTERIA

CDC group G bacteria possess chemotaxonomic features of true corynebacteria, but have not been given a species designation as it has so far been impossible to find phenotypic traits allowing for a unanimous definition (Riegel et al. 1995a). These bacteria can be separated from *C. jeikeium* by their anaerobic growth and fermentative acid production from fructose. Further biochemical features of CDC group G bacteria are given in Table 3. They might be multidrug resistant, but the most frequently observed resistance is to macrolides and clindamycin.

C. JEIKEIUM

This species is one of the most frequently encountered corynebacteria in clinical specimens. Nosocomial transmission has been described. It is often multiresistant, including β-lactams and aminoglycosides (Soriano et al. 1995). Quantitative DNA–DNA hybridizations has shown that *C. jeikeium* includes two genomospecies for which the penicillin and gentamicin MICs are low, but since they could otherwise not be differentiated phenotypically from the resistant *C. jeikeium* strains, they could not be proposed as independent species (Riegel et al. 1994). *C. jeikeium* is a strict aerobe which may oxidatively produce acid from glucose and sometimes from maltose (Table 3).

C. KROPPENSTEDTII

This is a rarely recovered species originally isolated from the sputum of a patient with pulmonary disease (Collins et al. 1998). Additional strains have been isolated from a breast abscess, open-lung biopsy specimen, and sputum (Bernard et al. 2002). Apart from *C. amycolatum* and *C. atypicum*, it is the only true *Corynebacterium* sp. lacking mycolic acids. It is also one of the few *Corynebacterium* spp. exhibiting esculinase activity (Table 3). This lipophilic species can be separated from *C. durum* and *C. matruchotii* by its colony morphology and from *C. glucuronolyticum* also by its negative CAMP reaction.

C. LIPOPHILOFLAVUM

This species is represented by only a single strain, which has been isolated from a patient with bacterial vaginosis (Funke et al. 1997b). It has the same biochemical screening pattern as *C. urealyticum*, except that it shows a strong yellow pigment and weaker urease activity.

C. MACGINLEYI

This species has been isolated almost exclusively from eye specimens (Riegel et al. 1995a; Funke et al. 1998d). When grown on Tween 80-SBA plates (better growth is usually observed on plates supplemented with 0.1 percent Tween 80 rather on those supplemented with 1.0 percent), some *C. macginleyi* strains exhibit a rose pigment, which is not seen for any other lipophilic *Corynebacterium* spp. This species is one of the very few corynebacteria not expressing pyrazinamidase activity (Table 3). Most *C. macginleyi* strains ferment mannitol, whereas the majority of other corynebacteria are unable to do so. Strains belonging to this species are susceptible to a broad range of antimicrobial agents.

C. UREALYTICUM

This species is one of the more frequently isolated clinically significant corynebacteria from clinical specimens. It is strongly associated with urinary tract infections. Recovery of this bacterium is often associated with alkaline urine, resulting in the production of struvite crystals. *C. urealyticum* is a strict aerobe and has a very strong urease activity. This species is almost always multidrug-resistant.

Genera other than *Corynebacterium*

GENUS *TURICELLA*

This genus is phylogenetically closely related to the genus *Corynebacterium*, but contains *T. otitidis* as the only species (Funke et al. 1994b). The cell wall contains *m*-DAP, but mycolic acids are not present (Table 1). The major CFAs for *T. otitidis* are the same as those detected in true corynebacteria, but all *T. otitidis* strains also contain significant amounts of TBSA. *T. otitidis* is the only coryneform bacterium that has a polar lipid profile without glycolipids. The G + C content varies between 65 and 72 mol%. Gram staining shows relatively long rods. *T. otitidis* is catalase positive, nonmotile, and an oxidizer. Colonies are whitish, convex, and creamy with entire edges and 1–1.5 mm in diameter after 48 h of incubation. Differentiation from the biochemically closely related *C. auris* and *C. afermentans* subsp. *afermentans* (see Table 3) is readily achieved on the basis of morphological features. Utilization reactions may also assist in the differentiation of these taxa. *T. otitidis* strains are uniformly strongly CAMP reaction positive. The MICs of β-lactam antibiotics for many

T. otitidis strains are very low; some strains may be resistant to macrolides and clindamycin (Funke et al. 1996c).

GENUS *ARTHROBACTER*

Presently, the genus *Arthrobacter* contains over 20 species, of which only a few have been recovered from human clinical specimens (Funke et al. 1996a). Lysine is the diamino acid of the cell wall (Table 1). $C_{15:0ai}$ represents more than 50 percent of total CFAs in most *Arthrobacter* spp. *A. cumminsii* has a distinctive CFA pattern with $C_{14:0i}$ and $C_{14:0}$ each representing 2–4 percent of all CFAs (Funke et al. 1998e). The G + C content range is from 59 to 70 mol%, indicating the diversity within the genus. In Gram stains of younger cultures jointed rods (i.e. rods in a rectangular form which contributed to the designation of this genus as '*arthros*', meaning joint in ancient Greek) might be observed but this is not possible for every species. Arthrobacters are catalase positive, motility is variable, and they are always oxidizers. *Arthrobacter* colonies are usually whitish-gray, slightly glistening, creamy, and 2 mm or more in diameter after 24 h. *A. cumminsii*, the most frequently isolated *Arthrobacter* in human clinical specimens, is slightly smaller than the other arthrobacters and may also exhibit a sticky consistency. Arthrobacters do not express a cheese-like smell, as often observed for the phenotypically closely related brevibacteria. Like brevibacteria, *Arthrobacter* spp. express DNase and gelatinase activity. The identification of arthrobacters on the species level might be achieved by carbohydrate utilization tests (Funke et al. 1996a). The penicillin MICs for most *Arthrobacter* strains are low. Aminoglycosides and quinolones show only weak activities against *A. cumminsii* strains.

GENUS *BREVIBACTERIUM*

meso-Diaminopimelic acid is the diamino acid of the cell wall. $C_{15:0ai}$ and $C_{17:0ai}$ usually represent more than 75 percent of all CFAs (Funke and Carlotti 1994). The G + C content varies between 60 and 67 mol%. Gram staining shows relatively short rods, which may develop into cocci when cultures are getting older. Brevibacteria are catalase positive, nonmotile, and oxidizers. Colonies are whitish-gray, convex, mostly creamy, and 2 mm or more in diameter after 24 h. *B. mcbrellneri* colonies tend to have a granular appearance. Many brevibacteria isolated from human clinical material give off a distinctive cheese-like odor. Brevibacteria are, in contrast to some arthrobacters, nonmotile and halotolerant. The species of this genus might be identified by carbohydrate utilization tests. More than 90 percent of all clinical *Brevibacterium* isolates are *B. casei*. The MICs of β-lactams for brevibacteria are often elevated (Troxler et al. 2001).

GENUS *DERMABACTER*

This genus presently comprises only one species, *D. hominis* (Jones and Collins 1988). *m*-DAP is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ usually account for 40–60 percent of all CFAs (Funke et al. 1994a). The G + C content varies between 60 and 62 mol%. Gram stains show very short rods which are often initially misinterpreted as cocci. *D. hominis* strains are catalase positive, nonmotile, and fermentative. Colonies are whitish, convex, of creamy or sticky consistency, and 1–1.5 mm in diameter after 48 h so that strains are sometimes mistaken for small-colony coagulase-negative staphylococci. *D. hominis* is one of the few coryneforms with the ability to ferment xylose. It is the only coryneform bacterium (covered in this chapter) to decarboxylate lysine and ornithine. *D. hominis* strains may be resistant to aminoglycosides.

GENUS *ROTHIA*

This genus belongs to the family *Micrococcaceae*. It is important to note that the previous *Stomatococcus mucilaginosus*, now *Rothia mucilaginosa* (Collins et al. 2000), exhibits cocci forms but also belongs to the genus *Rothia*. In contrast, *R. dentocariosa* clearly exhibits mainly rod forms and filamentous forms are normally not observed. *R. dentocariosa* genomovar II has been described but not formally given a species designation because it has not been possible to biochemically distinguish this taxon from the authentic *R. dentocariosa* sensu stricto (genomovar I) (Kronvall et al. 1998). Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ usually represent 40–60 percent of all CFAs. The G + C content ranges between 47 and 56 mol%. *R. dentocariosa* strains have a variable catalase reaction, are nonmotile, and exhibit a fermentative metabolism. Colonies of *R. dentocariosa* are whitish, raised, and smooth or rough or have a 'spoke-wheel' form, and can be up to 1.5 to 2 mm in diameter after 48 h. *Rothia* strains usually grow better in a CO_2-enriched atmosphere. The MICs of aminoglycosides for some *R. dentocariosa* strains are elevated, whereas penicillins usually show good *in vitro* activities against *Rothia* strains.

GENUS *EXIGUOBACTERIUM*

This genus is phylogenetically related to the so-called group 2 bacilli. Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ comprise only about 30–40 percent of total CFAs. *Exiguobacterium acetylicum*, the only medically relevant species within this genus, contains significant amounts of $C_{13:0i}$ and $C_{13:0ai}$, which are not found in any other coryneform taxon (Bernard et al. 1991). The G + C content is about 47 mol%. Exiguobacteria are found as relatively short rods in young cultures. Strains are catalase positive, motile, and fermentative. Colonies of *E. acetylicum* are plain, golden-yellow to orange, and up to 2 mm in diameter

after 24 h of incubation. Exiguobacteria might be confused with microbacteria, but CFA analysis provides a clear-cut distinction between the two genera (see Table 1). The pathogenic potential of *E. acetylicum* seems to be rather low; cases of pseudobacteramia have been observed.

GENUS *OERSKOVIA*

Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{15:0i}$ are the main CFAs in oerskoviae comprising about 40–60 percent of all CFAs. The G + C content is 70–75 mol%. Morphological features include extensive branching, vegetative hyphae, and penetration into agar, but no aerial hyphae. Gram stains show coccoid to rod-shaped bacteria originating from the breaking up of mycelia. *Oerskovia turbata*, the only presently described medically relevant species of this genus (apart from the recently reclassified *O. enterophila* (Stackebrandt et al. 2002)), is catalase positive, motile, and fermentative. Colonies are pale yellow to phosphorous yellow, convex, creamy, and about 1–2 mm in diameter after 24 h. *O. turbata* does not hydrolyze xanthine or hypoxanthine, whereas the related *Cellulosimicrobium cellulans* is able to do so.

GENUS *CELLULOMONAS*

Ornithine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{16:0}$ are the main CFAs. The G + C content is 71–76 mol%. Gram stains show small, thin rods. *Cellulomonas hominis*, the only species of this genus described as being isolated from human clinical material is catalase positive, and has a fermentative metabolism (Funke et al. 1995d). Colonies of *Cellulomonas* spp. are first whitish or pale or bright yellow, but after 7 days nearly all *Cellulomonas* strains are somewhat yellow. Colonies vary between 0.5 and 1.5 mm in diameter after 24 h, are convex and creamy, and have entire edges. *Cellulomonas* spp. are variable for the fermentation of mannitol. In contrast to all other members of the genus *Cellulomonas*, *C. hominis* did not hydrolyze cellulose in the test system used.

GENUS *CELLULOSIMICROBIUM*

This genus comprises at present only one medically relevant species, *C. cellulans*, which had been designated *Cellulomonas cellulans* and *Oerskovia xanthineolytica* in the past (Schumann et al. 2001). Lysine is the diamnino acid, $C_{15:0ai}$ and $C_{15:0i}$ are the predominant CFAs, and the G + C content is 74 mol%. It should be noted that *Cellulosimicrobium* is closely related to the genus *Oesrkovia*, but nevertheless distinct. Colonies of *C. cellulans* are similar to *O. turbata* and also pit agar. However, *C. cellulans* hydrolyzes either xanthine or hypoxanthine, whereas *O. turbata* does not and *O. turbata* strains might be motile, whereas *C. cellulans* strains are not.

GENUS *MICROBACTERIUM*

Since the mid 1990s it has been realized that the genera *Microbacterium* and *Aureobacterium* are phylogenetically intermixed and, therefore, it has been proposed to unify both genera in a redefined genus, *Microbacterium* (Takeuchi and Hatano 1998). Over 40 *Microbacterium* species have been validated but only a minority of them have been demonstrated to be of clinical importance. $C_{15:0ai}$ and $C_{17:0ai}$ are the two main CFAs, often representing up to 75 percent of the total CFAs (Funke et al. 1995b). The G + C content of *Microbacterium* spp. is 65–76 mol%, indicating the diversity within the genus. Gram stains show thin or short rods with no branching. Catalase activity as well as motility are variable. Microbacteria can be either fermenters or oxidizers. Microbacteria account for the majority of yellow-pigmented coryneform bacteria isolated from clinical specimens. All shades of yellow pigment are observed. Some microbacteria grow under anaerobic conditions, but only weakly. Some microbacteria are nitrate reductase negative which separates them from the phenotypically closely related genus *Cellulomonas* (Table 4). Final identification of *Microbacterium* species is best achieved by molecular genetic (e.g. 16S rRNA gene sequencing) investigations (Laffineur et al. 2003). Microbacteria are usually susceptible to vancomycin (except *M. resistens* (Funke et al. 1998b)), but susceptibility to other antimicrobial agents is unpredictable; in particular, resistance to aminoglycosides has been observed.

GENUS *CURTOBACTERIUM*

Curtobacteria belong to the peptidoglycan type B actinomycetes (i.e. cross-linkage between positions 2 and 4 of the two peptide subunits). Ornithine is the diamino acid and is the only amino acid composing the interpeptide bridge. Curtobacteria have an acyl-type acetyl peptidoglycan and menaquinone MK-9 as major MK, whereas the phenotypically closely related microbacteria possess a glycolyl type and MK-11,12 (Table 1). $C_{15:0ai}$ and $C_{17:0ai}$ represent more than 75 percent of all CFAs. The G + C contents range from 68 to 75 mol%. Gram stains show small and short rods with no branching. Catalase activity is positive, motility is observed in most strains, and all strains are slow oxidizers (in contrast to microbacteria which may oxidize any sugars more rapidly). Colonies are yellow or yellow-orange pigmented. Curtobacteria are usually nitrate reductase negative, but strongly hydrolyze esculin. The MICs of macrolides and rifampin for curtobacteria are very low.

GENUS *LEIFSONIA*

This genus (formerly known as *Corynebacterium aquaticum*) belongs to the peptidoglycan type B-type actinomycetes and, therefore cannot be true corynebacteria which actually possess an A type peptidoglycan (i.e. cross-linkage between positions 3 and 4 of the two

Table 4 *Biochemical characteritics of coryneform bacteria other than* Corynebacterium *spp.*

Taxon	Catalase	Fermentation/ oxidation	Motility	Nitrate reduction	Urease	Esculin hydrolysis	Acid production from:					Other features
							Glucose	Maltose	Sucrose	Mannitol	Xylose	
Turicella otitidis	+	O	–	–	–	–	–	–	–	–	–	CAMP reaction positive, long rods
Arthrobacter spp.	+	O	V	V	V	V	V	V	V	–	–	
Brevibacterium spp.	+	O	–	V	–	–	V	V	V	–	–	Odor cheese-like
Dermabacter hominis	+	F	–	–	–	+	+	+	+	–	V	Coccoid rods
Rothia dentocariosa	V	F	–	+	–	+	+	+	+	–	–	Adherence possible
Exiguobacterium acetylicum	+	F	+	V	–	+	+	+	+	+	–	
Oerskovia turbata	+	F	V	+	–	+	+	+	+	–	+	Xanthine not hydrolyzed
Cellulomonas spp.	+	F	V	+	–	+	+	+	+	V	+	
Cellulosimicrobium spp.	+	F	–	+	–	+	+	+	+	–	+	Hydrolysis of xanthine
Microbacterium spp.	V	F/O	V	V	V	V	+	+	+	V	V	
Curtobacterium spp.	+	O	V	–	–	+	+	V	V	V	+	Slow acid production
Leifsonia aquatica	+	O	+	V	–	V	+	V	V	+	+	
Arcanobacterium bernardiae	–	F	–	–	–	–	+	+	–	–	–	
Arcanobacterium haemolyticum	–	F	–	–	–	–	+	+	V	–	–	CAMP inhibition reaction
Arcanobacterium pyogenes	–	F	–	–	–	V	V	V	V	V	+	
Gardnerella vaginalis	–	F	–	–	–	–	+	+	V	–	–	Decolorized cells in Gram stain

+, positive reaction; –, negative reaction; V, variable reaction; O, oxidation; F, fermentation.

peptide subunits). *Leifsonia aquatica* (Evtushenko et al. 2000) is the only medically relevant species in this genus. Diaminobutyric acid is the diamino acid of the cell wall peptidoglycan, and $C_{15:0ai}$ and $C_{17:0ai}$ are the main CFAs, but represent <75 percent of all CFAs (Funke et al. 1994c). The G + C content is about 70 mol%. Gram stains show thin rods. The strains are catalase and oxidase positive (the latter is quite an atypical feature for coryneform bacteria), always motile, and oxidizers. The yellow pigment of *L. aquatica* colonies develops slowly within 3 to 4 days. *L. aquatica* does not hydrolyze either gelatine or casein, and has a stronger DNase activity than most microbacteria. The MICs of vancomycin for some *L. aquatica* strains were shown to be elevated (8 μg/ml).

GENUS *ARCANOBACTERIUM*

This genus presently contains six species, of which *A. haemolyticum*, *A. bernardiae*, and *A. pyogenes* are recovered from human clinical material. Lysine is the diamino acid of the cell wall, whereas lysine or ornithine is found in the phylogenetically closely related *Actinomyces* spp. The main CFAs in arcanobacteria are $C_{16:0}$, $C_{18:1\omega9c}$, and $C_{18:0}$ (as in *Corynebacterium* spp. and *T. otitidis*), but in contrast to corynebacteria, significant amounts of $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$ may also be detected (Bernard et al. 1991). The G + C content is 48–52 mol%. Gram stains show irregular gram-positive rods. All medical arcanobacteria are catalase negative, nonmotile, and fermentative. Succinic and lactic acids are the major end products of glucose metabolism. All arcanobacteria exhibit β-hemolysis on SBA; expression of hemolysis is best in a CO_2-enriched atmosphere. Colonies of the type species, *A. haemolyticum*, are 0.5 mm in diameter after 48 h of incubation. Of major value for the identification of *A. haemolyticum* is the so-called CAMP inhibition test. The protein responsible for this phenomenon is a phospholipase D excreted by *A. haemolyticum*, and this protein is genetically and functionally similar to the ones expressed by *C. ulcerans* and *C. pseudotuberculosis* (Cuevas and Songer 1993; McNamara et al. 1995).

A. pyogenes colonies are the largest of all arcanobacteria colonies, with diameters of up to 1 mm after 48 h of incubation. Of all the arcanobacteria, this species also shows the sharpest zone of β-hemolysis on SBA. The protein responsible for hemolysis, named pyolysin, is also an important virulence factor in vivo. Gram stains may show some branching rods. *A. pyogenes* is the only *Arcanobacterium* species of medical relevance that expresses β-glucuronidase activity and is capable of fermenting xylose.

A. bernardiae has glassy, whitish colonies of less than 0.5 mm in diameter after 48 h. Some colonies have a creamy consistency, whereas others are sticky. Most *A. bernardiae* strains belong to the very few coryneform bacteria that are able to ferment glycogen. Another peculiar feature of *A. bernardiae* strains is their ability to produce acid faster from maltose than from glucose.

The MICs of all β-lactams, rifampin, and tetracyclines for arcanobacteria are very low, whereas aminoglycosides and quinolones have reduced activities against arcanobacteria. Macrolides also exhibit excellent activities against arcanobacteria and are, therefore, an alternative to β-lactams for the treatment of infections, since treatment failures due to β-lactam antibiotics because of the inability of β-lactam antibiotics to act intercellulary have been reported.

GENUS *GARDNERELLA*

This genus does not have a particular phylogenetic relationship to any of the established genera described in this chapter. It is remotely related to the genus *Bifidobacterium*, and these two genera share some important characteristics, such as production of acetic and lactic acids as fermentation products. *G. vaginalis* is the only species belonging to the genus *Gardnerella*. Studies on the ultrastructure of its cell wall have demonstrated that it has a cell wall similar to but much thinner than the cell walls of other gram-positive bacteria (i.e. there is a smaller peptidoglycan layer). Lysine is the diamino acid of the cell wall, and $C_{16:0}$ and $C_{18:1\omega9c}$ are the predominating CFAs. The G + C content is 42 to 44 mol%. Gram stains show thin gram-variable rods or coccobacilli. Catalase is not produced, cells are nonmotile and have a slow fermentative metabolism. The optimal pH for growth of *G. vaginalis* is between 6 and 7.

G. vaginalis is associated with bacterial vaginosis (BV); its causative role in the syndrome is controversial (Spiegel 1991). In pregnant women, BV may lead to preterm birth, premature rupture of membranes, and chorioamnionitis. *G. vaginalis* may also be recovered from cultures of blood from patients with postpartum or postabortal fevers and may also cause infections in newborns. Although it might be recovered from the urethras of males, its disease association in males is questionable. Serious infections in sites other than those associated with the genital tract or obstetrics are very infrequently seen.

The 'gold standard' for the diagnosis of BV is direct examination of vaginal secretions and not the culture of *G. vaginalis*, since *G. vaginalis* can also be recovered from healthy women. However, isolation of *G. vaginalis* can support the diagnosis of BV. Cultures should be done on Vaginalis agar and incubated at 35–37°C in a 5 percent CO_2-enriched atmosphere or in a candle jar. β-Hemolyis is observed on human or rabbit blood-containing media, but not on SBA. Plates may be checked for growth of diffuse β-hemolytic colonies of less than 0.5 mm in diameter after 24 h, but very often *G. vaginalis* is best observed after 48 h only. Gram staining of the suspected colonies should confirm the diagnosis of *G. vaginalis*. Strains of *G. vaginalis* are

consistently α-glucosidase and starch hydrolysis positive, and 90 percent of strains hydrolyze hippurate. The identification of *G. vaginalis* can also be confirmed by antimicrobial agent disk inhibition tests with 50 μg metronidazole (inhibition present), 5 μg trimethoprim (inhibition present), and 1 mg of sulphonamide (inhibition absent). Metronidazole is also the drug of choice both for local therapy of BV and for systemic therapy of infections caused by BV-associated flora. Systemic infections due to *G. vaginalis* alone can be treated with ampicillin or amoxicillin, since β-lactamase-producing *G. vaginalis* strains have not been observed so far.

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Tropheryma

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INTRODUCTION

Tropheryma whipplei is the agent of Whipple's disease, a systemic condition of humans affecting the intestinal tract, as well as various other organs. The disease was described in 1907 by George H. Whipple; however its etiology remained unclear until 1961, when small, uniform bacteria were detected in affected tissues by electron microscopy. These bacteria resisted many attempts at cultivation in the laboratory, thus creating the perception that an enigmatic, culture-resistant bacterial pathogen is associated with Whipple's disease. Characterization of the Whipple's disease bacterium by phylogenetic analysis of 16S rDNA was achieved in the early 1990s, cocultivation of the bacterium with a human fibroblast cell line was achieved in 2000, and its complete genome was sequenced and analyzed in 2003. Whipple's disease is a relatively rare entity, with roughly 2 000 cases reported in the medical literature, but due to its varied manifestations the disease is part of the differential diagnosis of many clinical disorders across several medical specialties.

DEFINITION

T. whipplei is the only species of the genus. It belongs to the class *Actinobacteria* (Stackebrandt et al. 1997), which comprises phylogenetically related gram-positive bacteria with high genomic guanine and cytosine (G+C) content. The order and family designations of *T. whipplei* within the *Actinobacteria* have not been established. Currently, the most accurate markers for identification are the bacterium's 16S rRNA gene and 16S–23S rRNA intergenic spacer sequences (Maiwald et al. 1996). The type strain is *T. whipplei* Twist-Marseille CNCM I-2202^{T} (La Scola et al. 2001).

HISTORICAL PERSPECTIVE

In 1907, George H. Whipple performed an autopsy on a 36-year-old patient who had died after suffering for 5 years from an illness marked by arthritis, fever, weight loss, and cough (Whipple 1907). Whipple observed deposits of fat and fatty acids in the intestinal mucosa and mesenteric lymph nodes, and proposed the name 'intestinal lipodystrophy' for this disease. He also noted small, rod-shaped bacteria in silver-stained sections of a lymph node, but did not interpret this finding as causally related to the disease. The etiology of Whipple's disease remained unknown for more than five decades. Until 1947, the diagnosis was uniformly made at autopsy, and in 1949, the periodic acid-Schiff (PAS) stain was first utilized for the histopathologic diagnosis of Whipple's disease (Dobbins 1987). The first observation of successful antibiotic treatment was made in 1952, using chloramphenicol (Paulley 1952), and in 1961, two groups of investigators independently documented the presence of small, uniform bacteria in

affected tissues by electron microscopy (Chears and Ashworth 1961; Yardley and Hendrix 1961). Both the response to antibiotic treatment and the observation of uniform bacteria were confirmed by many investigators, but many subsequent attempts to cultivate the bacterium so implicated failed.

A monograph published in 1987 (Dobbins 1987), summarized the findings of 696 cases and characterized Whipple's disease as a rare, chronic, systemic disease with a predilection for middle-aged white men, as well as for outdoor professions. First information about the nature of the organism was obtained in the early 1990s, when broad-range 16S rDNA PCR was applied (Wilson et al. 1991; Relman et al. 1992). Analysis of the new 16S rRNA sequence revealed a phylogenetic position within the actinomycetes; the name *Tropheryma whippelii* was proposed. At the same time, the new sequence information provided the basis for a specific, PCR-based diagnostic test. A further advancement came in 2000, when stable cocultivation of the bacterium with a human fibroblast cell line was achieved (Raoult et al. 2000). This finding was reproduced (Raoult et al. 2001; Maiwald et al. 2003b), and the bacterium formally described as a new species, with modified latinization: *T. whipplei* (La Scola et al. 2001). Finally, the bacterium's complete genome sequence was determined, analyzed, and published in 2003 (Bentley et al. 2003).

CLINICAL MANIFESTATIONS

Whipple's disease is best described as a multisystem disease (Dobbins 1987). It is best known for its intestinal manifestations. These consist most commonly of malabsorption, diarrhea, abdominal pain, abdominal lymphadenopathy, and weight loss, which can be quite severe (Vital Durand et al. 1997; Maiwald et al. 2002). These intestinal manifestations are often preceded by years of arthralgias, and sometimes by low-grade intermittent fever (Vital Durand et al. 1997). Among the extraintestinal manifestations, those involving the central nervous system (CNS) and the heart are most prominent. Symptomatic CNS involvement occurs in approximately 10–40 percent of all cases and commonly presents with dementia (Louis et al. 1996). This can occur concurrently with intestinal disease at the time of initial presentation, but is more commonly seen when patients present with relapses during or after antibiotic treatment. Cardiac manifestations comprise endocarditis, myocarditis, and pericarditis (Vital Durand et al. 1997). *T. whipplei* is increasingly being recognized as one cause of 'culture-negative endocarditis' when PCR-based detection is used (Fenollar et al. 2001). Among various other manifestations, there are uveitis, skin hyperpigmentation, prosthetic joint infection, and spondylodiscitis (Maiwald et al. 2002).

EPIDEMIOLOGY AND PATHOGENICITY

According to a meta-analysis of 696 cases by Dobbins (1987), Whipple's disease occurs sporadically and affects men more commonly than women (male to female ratio ~7:1), with a peak of incidence around 50 years of age. Most cases are recorded in Europe and the USA, and white persons are most commonly affected. Among professional groups, there is a predilection for people in the farming and building trades (34 percent of all patients). Another epidemiologic study of 110 patients in Germany (von Herbay et al. 1997b) noted a relatively stable incidence of new cases over three decades, as well as a relatively even geographic distribution. The natural habitats of *T. whipplei* and the routes of disease transmission are still largely unknown. Several authors have reported positive PCR findings for *T. whipplei* in samples from asymptomatic persons (Ehrbar et al. 1999; Street et al. 1999), and suspected that the bacterium may be a normal commensal of humans, while others have not had similar findings, despite large numbers of tested specimens (Pron et al. 1999; Maiwald et al. 2001). One study detected *T. whipplei* sequences in the polymicrobial communities of municipal sewage (Maiwald et al. 1998). Immunologic abnormalities are thought to play a role as predisposing factors for Whipple's disease. Reduced numbers of monocytes expressing complement receptors (CD11b) and reduced cytokine (interleukin-12) production of macrophages have been found in some patients with Whipple's disease (Marth et al. 1994, 1997), among several other abnormalities. However, precisely defined immune defects, such as the absence of particular cell types, mediators, or receptors, have not been identified, and the disease is not over-represented in patients with known immune defects. Thus, the presumed immunologic disturbances in patients with Whipple's disease are likely to be subtle.

CLASSIFICATION AND PHYLOGENY

Analysis of the 16S rRNA gene, amplified by bacterial broad-range PCR, established a phylogenetic relationship of the Whipple's disease bacterium within the actinomycetes (Wilson et al. 1991; Relman et al. 1992). One analysis was based on a 645 bp sequence and reported *Rhodococcus equi* as the closest relative (Wilson et al. 1991), another utilized 1 321 bp of 16S rDNA and found the closest relatives to be representatives of the genera *Actinomyces*, *Rothia*, *Arthrobacter*, *Micrococcus*, *Terrabacter*, and *Dermatophilus* (Relman et al. 1992). A subsequent phylogenetic analysis (Maiwald et al. 1996) utilized a significantly increased actinomycete 16S rRNA database as compared with the initial studies, and found an intermediate position of the Whipple's disease bacterium between a cluster of actinomycetes with the rare group B peptidoglycan and members of the family

Cellulomonadaceae, with the common group A peptidoglycan. The highest 16S rDNA sequence similarities, both at 91.6 percent, were found to *Leifsonia aquatica* (previously '*Corynebacterium aquaticum*;' a group B peptidoglycan organism) and to *Cellulomonas cellasea* (group A). With these sequence similarities, *T. whipplei* is only distantly related to any other known cultivated bacteria. Additional phylogenetic information was obtained for the 23S rRNA and 5S rRNA genes (Maiwald et al. 2000), the *rpoB* gene (Drancourt et al. 2001) and a set of conserved genes comprising *atpD*, *tuf*, *groEL*, *rpoB*, and *rnpB* (Maiwald et al. 2003a), and all these analyses confirmed the position of *T. whipplei* within the actinomycetes. *T. whipplei* was formally described as a new species in 2001 (La Scola et al. 2001), and is now a member of the class *Actinobacteria* (Stackebrandt et al. 1997), although order and family affiliations are uncertain.

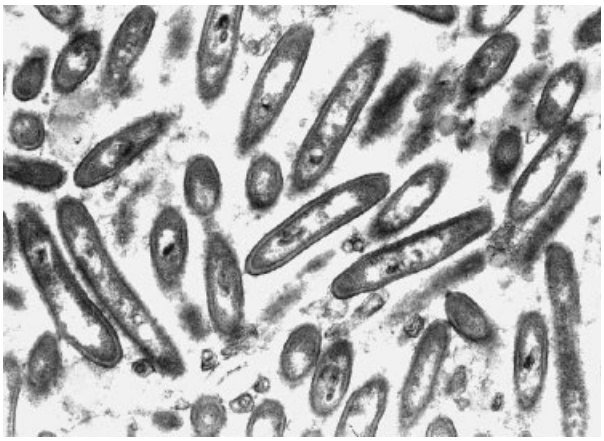

Figure 1 *Electron micrograph of a small intestinal biopsy of a patient with intestinal Whipple's disease. The bacteria are located in the extracellular spaces of the intestinal lamina propria. Courtesy of Axel von Herbay, University of Heidelberg, Germany*

MORPHOLOGY

After initial reports in the 1960s (Chears and Ashworth 1961; Yardley and Hendrix 1961), many other investigators detected small, uniform, rod-shaped bacteria in tissues and body fluids by electron microscopy (Dobbins 1987). These bacteria (Figure 1) measure 0.2–0.25 by 1.0–2.5 μm and have a distinctive, trilaminar appearance of the cell envelope (Silva et al. 1985; Dobbins 1987). The cell envelope consists – from inside to outside – of (1) a cytoplasmic membrane, (2) a ~20 nm thick cell wall with an inner polysaccharide and an outer peptidoglycan layer, and (3) a symmetric outer membrane (Silva et al. 1985). While the morphology of the peptidoglycan-containing cell wall is consistent with cell walls of other gram-positive bacteria, the outer membrane is not seen in other gram-positive organisms and unlike those observed in gram-negative bacteria. It was suspected that this membrane may be derived from the human host (Silva et al. 1985). The polysaccharide (glycopeptide) layer in the cell wall confers reactivity with the PAS-staining procedure, which is used for the histopathologic diagnosis of Whipple's disease. The organism is stained inconsistently gram-positive in tissues (Dobbins 1987), gram-negative in coculture with fibroblasts (La Scola et al. 2001), and is not acid-fast.

In tissue sections, morphologically intact and dividing bacteria are mostly seen in the extracellular spaces, whereas intracellular bacteria tend to undergo degradative changes, and after antibiotic therapy, only degraded cell wall remnants – still conferring PAS positivity – remain in macrophages (Silva et al. 1985; Dobbins 1987). These observations were confirmed by more recent in situ hybridization experiments, in which signals from a *T. whipplei*-specific rRNA probe – corresponding to metabolically active bacteria – were detected extracellularly in the intestinal lamina propria, but not in macrophages with PAS-positive material (Fredricks and Relman 2001). *T. whipplei* propagated in coculture with human fibroblasts has also been stained by immunofluorescence using mouse polyclonal antibodies (La Scola et al. 2001), as well as by YO-PRO nonspecific nucleic acids dye and by in situ hybridization, using a *T. whipplei*-specific oligonucleotide (Maiwald et al. 2003b). YO-PRO staining of culture material revealed distinctive small rods arranged in chains (Figure 2), and in situ hybridization was able to integrate bacterial morphology with the known 16S rRNA sequence of *T. whipplei*.

CULTURE

Many attempts were made to cultivate the Whipple's disease bacterium, but failed or yielded results that proved erroneous (Dobbins 1987). The use of nonsterile intestinal biopsy samples resulted in the isolation of contaminants by some investigators: *Streptococcus* spp., *Corynebacterium* spp., and *Haemophilus* spp. were among such organisms (Dobbins 1987). One spectacular attempt was published in 1997: based on the notion that PAS-positive macrophages are a prominent histopathologic feature, investigators inoculated interleukin-4-treated human macrophages with heart valve specimens from two infected patients (Schoedon et al. 1997). This treatment impairs microbicidal killing mechanisms that affect intracellular pathogens. The authors observed accumulation of PAS-positive intracellular material and the persistence of PCR-amplified products after cell passage, and interpreted this as indication of growth of *T. whipplei*. However, these findings were not reproduced by others (Zaaijer et al. 1998; Maiwald and Relman, unpublished results).

Cocultivation of *T. whipplei* from an infected heart valve with a human embryonic lung fibroblast cell line (HEL-299; ATCC CCL-137) was reported in 2000

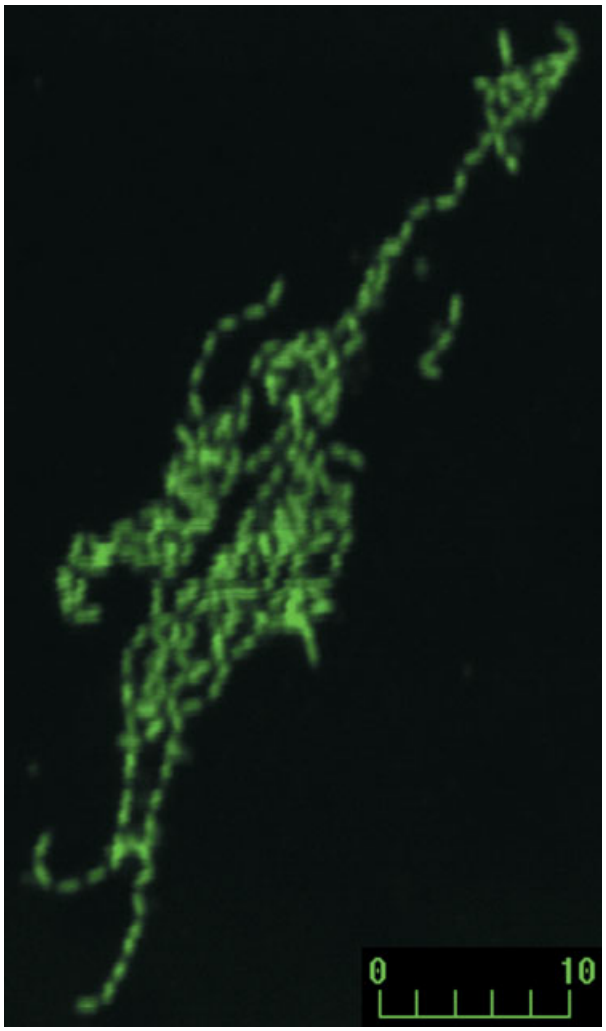

Figure 2 *Photomicrograph of supernatant from a culture of* T. whipplei *from CSF. Bacteria were stained with YO-PRO-1 nucleic acids dye. Note the distinctive appearance of small rods arranged in chains. Scale bar represents micrometers. Reproduced from Maiwald et al. (2003b) with permission from University of Chicago Press. Copyright 2003 by the Infectious Diseases Society of America*

(Raoult et al. 2000). The investigators used long-term culture conditions; a cytopathic effect was observed after 65 days of incubation, and microorganisms were seen by means of several staining procedures, including PAS staining. Seven culture passages were performed over 9 months; this involved expansion of cultures from 1 cm^2 of inoculated cell monolayers to 3 750 cm^2 of infected cells. The estimated bacterial doubling time was 18 days. Qualitative PCR was positive for *T. whipplei* after each passage, and microorganisms were stained positively by immunofluorescence using the patient's serum. The strain was designated *T. whipplei* Twist-Marseille CNCM I-2202^T (La Scola et al. 2001). A second strain was subsequently isolated from a duodenal biopsy specimen, using a decontamination protocol with antibiotics (Raoult et al. 2001).

These findings were reproduced and supplemented with additional observations by a second group of investigators, using infected cerebrospinal fluid (CSF) from two patients (Maiwald et al. 2003b). Two *T. whipplei* strains were propagated in co-culture with MRC-5 lung fibroblasts (ATCC CCL-171) and with human primary foreskin fibroblasts. Fifteen culture passages were completed over 17 months. The growth rate of the organisms was measured by quantitative competitive PCR, revealing a doubling time of 4 days. Fluorescent in situ hybridization with a *T. whipplei*-specific rRNA probe, a broad-range bacterial rRNA probe, and with nonspecific nucleic acid dye (YO-PRO) showed colocalized staining patterns within the same bacterial shapes of the cultivated strains. One of these strains from CSF was used for genome sequencing (Bentley et al. 2003). Taken together, all these data provide ample evidence that *T. whipplei* can be propagated in cell culture systems, but the long generation times and difficult culture conditions render this method unsuitable for routine laboratory use, at present.

DIAGNOSIS AND IDENTIFICATION

Histopathology of intestinal biopsies (Figure 3), taken by upper gastrointestinal endoscopy, is the standard

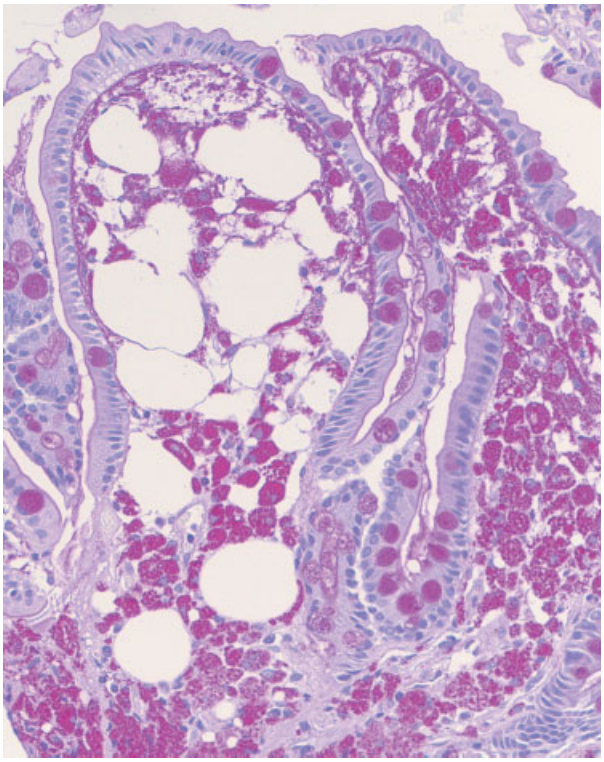

Figure 3 *Histology of a small intestinal biopsy of a patient with Whipple's disease, stained with the PAS reagent. The photo shows distended intestinal villi, deposits of lipid droplets (appearing as empty spaces), and the intestinal lamina propria infiltrated by macrophages with PAS-positive (red) inclusions. Courtesy of Axel von Herbay, University of Heidelberg, Germany*

first-line approach to the diagnosis of suspected Whipple's disease, since the overwhelming majority of patients have symptomatic or asymptomatic intestinal involvement (Maiwald et al. 2002). Accumulation of macrophages with PAS-positive inclusions in the intestinal lamina propria is the key diagnostic feature (von Herbay et al. 1996b). The PAS stain can also be applied to histopathology of various other tissues (e.g. lymph nodes) and to cytologic examination of body fluids (e.g. CSF). Since PAS-positive inclusions are not entirely specific, it has traditionally been recommended to confirm the diagnosis by electron microscopy, which shows bacteria of typical size and shape (Dobbins 1987). PCR is increasingly being used as a diagnostic tool for Whipple's disease (von Herbay et al. 1996a, 1997a; Ramzan et al. 1997). So far, the greatest body of information concerning clinical sensitivity and specificity has become available for PCR assays that were derived from the original one published by Relman and colleagues (1992). PCR has proven useful to confirm the histopathology of intestinal biopsies, and to establish the diagnosis with extraintestinal tissues and body fluids (Maiwald et al. 2002). Repeat intestinal biopsies and histology are recommended after the initiation of antibiotic therapy (von Herbay et al. 1996b), and it has been proposed that examination of CSF by PCR is useful, both for initial staging and for monitoring the central nervous system under therapy (von Herbay et al. 1997a).

The fastidious nature of *T. whipplei* prevents traditional bacterial identification tests from being feasible. As indicated, electron microscopy is useful to reveal the distinctive morphology of *T. whipplei*. In addition, monoclonal antibodies have been derived from a cultivated strain (Liang et al. 2002), and have shown promising results with *T. whipplei* from culture and with 22 bacterial controls, but have not yet been applied in diagnostic settings. As a consequence, identification is most accurate using DNA sequence data. Most comparative information concerning different strains of *T. whipplei* and other bacterial species has become available for 16S rDNA and the 16S–23S rDNA intergenic spacer (Maiwald et al. 1996, 2000; Hinrikson et al. 1999).

STRAIN DIFFERENTIATION

As a first step in exploring strain differences of *T. whipplei*, the 16S–23S rRNA intergenic spacer sequence (294 bp) was determined (Maiwald et al. 1996). Seven different spacer types have been described so far, most with exchange of a few nucleotides (Hinrikson et al. 1999; Maiwald et al. 2000; Geißdörfer et al. 2001). Types 1 and 2 are most common; one study with samples from 28 patients detected type 1 in 15 and type 2 in ten patients (Hinrikson et al. 1999), and another study with 43 patients detected type 1 in 14 and type 2 in 26 patients (Maiwald et al. 2000). In the latter study, types 1 and 2 were detected in a ratio of approximately 1:2 in patients from both Europe and the USA. Additional variability between strains was found in the 23S rRNA insertion sequence of *Actinobacteria* (one variable nucleotide; Hinrikson et al. 2000) and in the *groEL* heat shock protein gene (six nucleotides; Morgenegg et al. 2000). Both an analysis of conserved bacterial genes (Maiwald et al. 2003a) and genome sequence data (Bentley et al. 2003) further indicated the presence of variable number of tandem repeat (VNTR) loci in *T. whipplei*. VNTRs provide an important mechanism of bacterial sequence variation and have been used for strain typing in other species. A preliminary analysis of three VNTR loci plus the 16S–23S rRNA intergenic spacer in 11 strains yielded a unique pattern for each of the strains (Maiwald et al. 2003a), surpassing the discriminatory power of previous methods. However, since VNTRs can mutate easily, the stability of these patterns within individual strains needs to be explored.

ANTIMICROBIAL TREATMENT

Almost all data concerning treatment of Whipple's disease come from observations in case reports and case series (Keinath et al. 1985; Dobbins 1987; Feurle and Marth 1994; Maiwald et al. 2002). The recommended standard treatment consists of an initial 2-week intravenous course of penicillin G plus streptomycin or a third-generation cephalosporin, followed by long-term treatment ($\geqslant$1 year) with cotrimoxazole (Maiwald et al. 2002). It is important to use antibiotics that sufficiently cross the blood–brain barrier, since relapses affecting the CNS pose a major threat to patients during and after antibiotic treatment. Until the 1970s, tetracyclines were frequently used, with favorable effects on intestinal manifestations, but had an unacceptable rate of CNS relapses. Alternative drugs, albeit with less available clinical data, are imipenem or meropenem for intravenous induction (www.whipplesdisease.net), and cefixime, penicillin V, rifampin, and chloramphenicol for oral maintenance or salvage therapy (Maiwald et al. 2002). Initial data of in vitro antibiotic susceptibility testing have come from cultivated strains of *T. whipplei* (Masselot et al. 2003). Bacterial growth inhibition was measured by quantitative real-time PCR for the fluoroquinolones ciprofloxacin and levofloxacin. Relatively high minimal inhibitory concentrations (MIC) of 4–8 mg/l were found for ciprofloxacin, whereas low MICs of 0.25 mg/l were found for levofloxacin. At the same time, the fluoroquinolone target genes *gyrA* and *parC* were analyzed, and an amino acid exchange was found that is known to be associated with poor susceptibility in other bacteria.

GENETICS AND GENOMICS

Sequencing and analysis of the genome of *T. whipplei* strain TW08/27 (Maiwald et al. 2003b) revealed a size of

925 938 bp, a G+C content of 46.3 percent and 784 coding sequences (Bentley et al. 2003). This genome bears a number of interesting features, and three major themes can be recognized. First, it is the smallest genome sequenced for an actinomycete so far, and its G+C content is lower than that of other actinomycetes, which are typically high G+C organisms. *T. whipplei* has pronounced deficiencies in carbohydrate and energy metabolism, such as the absence of genes for the tricarboxylic acid cycle and the NADH dehydrogenase complex, and it has a lack of biosynthesis genes for several amino acids. Concordant with the reduced genome size, there is an increased proportion of genes devoted to 'housekeeping' functions. Comparison with other actinomycete genomes indicates that *T. whipplei* has been subject to gene loss, such as the loss of a *groES*-linked *groEL1* heat shock protein gene, while the *groEL2* gene is remaining.

Second, *T. whipplei* devotes a large amount of its coding capacity to the biosynthesis of molecules that are predicted to be located on the cell surface. This is the case with 15 percent of all predicted proteins, and 74 percent of the 116 proteins with no match in the database. A unique, heterogeneous family of proteins was found, consisting of 14 members and termed the WiSP proteins (for *T. whipplei* surface proteins). WiSPs range in size between 103 and 2 308 amino acids and are characterized by several common features, such as N-terminal signal sequences, C-terminal transmembrane domains, and conserved beta-strand motifs.

Third, the genome contains large regions of noncoding repetitive DNA and an array of mechanisms for creating genetic variability. The noncoding DNA is located in two regions, termed repeat clusters (RC) 1 and 2, which together comprise about 5 percent of the genome. This is considerably larger than any noncoding stretches observed in other bacteria. In addition, *T. whipplei* appears to use tandem repeat sequences, combined with a process termed slipped-strand mispairing, to vary the expression of surface molecules, and it exhibits unusual, localized sequence hypervariation in two of the WiSP proteins. Since these two WiSP genes are located within and share sequence similarity with the repeat clusters, it was suspected that this provides for a novel mechanism of sequence variation.

Taken together, *T. whipplei* bears the traits of a highly host-dependent organism, with reduced genome size and metabolic deficiencies. The extent of information devoted to surface molecules, together with 'built-in' mechanisms for antigenic variation, suggests that interaction with its host and evasion of the host's immune response play an important part in the organism's lifestyle, and are factors for sustaining a chronic infection. Thus, the determination of the genome sequence is a major step forward in the characterization of the organism, and has not only led to a better understanding of its biology, but also provides a wealth of new information, both at the DNA and the protein level, that can be used to design and improve diagnostic tests.

NOTE ADDED IN PROOF

At least three more significant publications have appeared recently: the sequencing of the genome of a second *T. whipplei* strain, Twist-Marseille CNCM I-2202^T (Raoult, D. et al. 2003. *Genome Res*, **13**, 1800–9), the culture of *T. whipplei* in a cell-free medium supplemented with amino acids (Renesto, P. et al. 2003. *Lancet*, **362**, 447–9), and the testing of antimicrobial susceptibility of *T. whipplei* in a cell culture system (Boulos, A. et al. 2004. *Antimicrob Agents Chemother*, **48**, 747–52).

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Actinomyces and related genera

GUIDO FUNKE

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DEFINITION

Actinomyces spp. are gram-positive rods, 0.4–1.0 μm wide, which may be straight, curved, or pleomorphic and occur singly, in pairs, clusters, or short chains. Filaments up to 50 μm in length are formed by members of some species. Some show true branching. They are nonacid-fast, nonmotile, and do not form endospores or conidia. The majority of species are facultative anaerobes; some are obligately anaerobic. Growth is stimulated by CO_2 and sodium carbonate in broth media. The optimum temperature for growth of all species is 35–37°C. Carbohydrates are fermented, generally with the production of formic, acetic, lactic, and succinic acids. There is little action on proteins, although organic nitrogen is required for growth. Species can be α-, β- or γ-hemolytic and hemolysis may depend on culture conditions. Some species show antigenic diversity and have more than one serotype. The cell wall peptidoglycan is, in most cases, of the Orn-Lys-Glu type, whereas *Actinomyces bovis* has Lys-Lys-D-Asp. In *Actinobaculum suis* the peptidoglycan includes Lys-Ala-Lys-D-Glu. Diaminopimelic acid and mycolic acids are absent. Cell wall polysaccharides include fucose, glucose, galactose, mannose, rhamnose, and 6-deoxytalose, and amino sugars; arabinose and xylose have not been detected. The major cellular fatty acids of *Actinomyces* comprise myristic (14:0), palmitic (16:0), stearic (18:0), and oleic acids (18:1ω9). The relative amounts of these acids vary among species. Smaller amounts of other acids (10:0, 16:1) may be found. The range of G + C content of the DNA is 55–71 mol% and the type species is *A. bovis*.

Related genera include *Arcanobacterium* (which is covered in Corynebacteria and rare coryneforms), *Actinobaculum* (Lawson et al. 1997), and *Varibaculum* (Hall et al. 2003d). Two former *Actinomyces* species, *A. bernardiae* and *A. pyogenes*, have been moved to the genus *Arcanobacterium* (Pascual Ramos et al. 1997b). *Actinobaculum* spp. (with *A. suis* as the type species) and *Varibaculum* sp. both merit genus status because 16S rDNA data showed long and deep phylogenetic lines with sequence divergences of 10–12 percent to the established *Actinomyces* spp. *Varibaculum cambriensis*, the type species of the genus *Varibaculum*, has a G + C content of only 51.7 mol%. Pascual Ramos et al. (1997b) demonstrated that the genus *Actinomyces* contains several clusters of species which may merit separate genus status. All genera covered in this chapter belong to the class of the *Actinobacteria* (Stackebrandt et al. 1997).

INTRODUCTION AND HISTORICAL PERSPECTIVE

The early history of the classification of *Actinomyces* was complicated by their histological resemblance to

fungi. As pointed out by Slack and Gerencser (1975), as early as 1827 (Meyen 1827), the name *Actinomyces* (ray fungus) had been given incorrectly to a previously named fungus. Slack and Gerencser (1975) list 16 generic names proposed from 1875 to 1934 to include organisms now considered to be *Actinomyces*. Harz (1879) initially provided a detailed histological description of an organism that formed granules in lesions in the jaws of cattle, which he named *Actinomyces bovis*. Harz could not cultivate the organism and it was not possible to compare it to those described subsequently by other workers. Apparently, Mosselman and Lienaux (1890) first isolated *A. bovis* as it is known today. However, a variety of organisms, including aerobes (Bostroem 1891) were also identified as *A. bovis*, further hindering the accurate definition of the genus.

Wolff and Israel (1891) described an anaerobic pathogen (*Actinomyces israelii*) isolated from two cases of actinomycosis in humans and demonstrated the pathogenicity and virulence of their isolates. However, new genera, often with the species names *bovis* or *israelii*, continued to be proposed to include branching pathogens of humans and other animals. It remained a problem to distinguish *Actinomyces* from other gram-positive genera with branching cells and to differentiate *A. bovis* and *A. israelii*. Wright (1905) defined *Actinomyces* as anaerobic and distinguished them from the aerobes identified by some workers as *A. bovis*, which he named *Nocardia*. Breed and Cohn (1919) recommended *Actinomyces* as a genus with the type species as *A. bovis* and this was accepted by Winslow et al. (1920).

Chemotaxonomic techniques made a dramatic impact on the classification of *Actinomyces*. Studies by Cummins and Harris (1958) and Cummins (1962) showed that *Actinomyces* spp. had cell walls typical of bacteria. Chemotaxonomy allowed better definition of the genus, resulting in reclassification of some species (Schaal 1986). In recent years, 16S rDNA sequence data (Pascual Ramos et al. 1997b) have been used extensively to delineate further species within the genus *Actinomyces* and to demonstrate the phylogenetic relationships between *Actinomyces* spp. and other actinomycetes (Embley and Stackebrandt 1994). Species currently placed into *Actinomyces* and related genera are shown in Table 1.

HABITAT

The natural habitats (Table 1) of most of the species of *Actinomyces* are known to be intestinal surfaces, as well as mucous membranes of humans and animals. *Actinomyces* spp. form a significant proportion of the normal flora of the oral cavities of humans and animals, where they occur as components of dental plaque on teeth and biofilms on mucosal surfaces (Bowden and Hardie 1973, 1978; Slack and Gerencser 1975; Bowden et al. 1979; Dent and Marsh 1981; Johnson et al. 1990). Generally, these organisms do not colonize other sites in the intestinal tract and, with certain exceptions, they can be described as uniquely oral organisms. However, some species can colonize tonsils (Slack and Gerencser 1975) and *A. israelii* is regularly present in the female genital tract (Persson and Holmberg 1984a). *A. turicensis* seems to be another *Actinomyces* species regularly present in the vaginal or gastrointestinal tract flora.

MORPHOLOGY

Cellular morphology

Cells are gram-positive, although occasionally they may appear beaded on Gram's stain. When grown on blood agar, the majority of species show diphtheroidal V and Y forms and may produce short coccobacilli. Several different cellular morphologies can occur together and often cells form clusters. Some species, including *A. israelii* (Figure 1a) and *A. bovis* produce long filamentous forms and show true nonseptate branching, which may also be seen with *Actinomyces viscosus*, *Actinomyces naeslundii* (Figure 1b), and *Actinomyces odontolyticus*. Cells of *Actinomyces* show a typical gram-positive wall structure when examined by electron microscopy (Slack and Gerencser 1975; Schaal 1986). Some species, in particular *A. naeslundii* and *A. viscosus*, have extensive fimbriae on their outer surfaces. Other species that have been examined (*A. israelii* and *A. odontolyticus*) have relatively smooth cell surfaces without fimbriae.

Colonial morphology and appearance in broth

Species such as *A. israelii*, which form long branching filaments, usually produce a granular deposit in broth with a completely clear supernate. The granules tend to be resistant to disruption. *A. naeslundii*, *A. viscosus*, and *Actinomyces gerencseriae*, which produce fewer, shorter filaments, also form a deposit in broth, but it is viscous and ropy and more easily suspended. Organisms that produce diphtheroidal or coccobacilliary cells usually grow as evenly dispersed suspensions.

On solid media, *Actinomyces* spp. produce a variety of colony forms. Typically, *A. israelii* produces a white-colored, rough, 'molar tooth' colony (Figure 1c), whereas *A. naeslundii*, *A. viscosus*, and *A. gerencseriae* usually produce convex, smooth, matt-surfaced cream-colored colonies (Figure 1d). *A. graevenitzii* colonies have been described as rough, dry, and adherent to blood agar (Sarkonen et al. 2001). Colonies of other species may be flat (*A. hyovaginalis*) or low convex (*Actinomyces neuii*). Species vary in their effect on

Table 1 Actinomyces *species and related genera*

Actinomyces species	Pathogenicity	Natural habitat	References
Acb. schaalii	Pathogen in humans	Unknown	Lawson et al. (1997)
Acb. suis	Pathogen in pigs	Recovered from urine of healthy boars	Ludwig et al. (1992); Lawson et al. (1997)
Acb. urinale	Pathogen in humans	Not known	Hall et al. (2003b)
A. bovis	Pathogen in animals	Not known	Schaal (1986)
A. bowdenii	Pathogen in dogs, cats	Not known	Pascual et al. (1999)
A. canis	Dogs	Not known	Hoyles et al. (2000)
A. cardiffensis	Pathogen in humans	Not known	Hall et al. (2002)
A. catuli	Pathogen in dogs	Not known	Hoyles et al. (2001b)
A. coleocanis	Pathogen in dogs	Not known	Hoyles et al. (2002)
A. denticolens	Non-pathogen	Oral cavity, cattle	Dent and Williams (1984a, 1984c)
A. europaeus	Pathogen in humans	Not known	Funke et al. (1997)
A. funkei	Pathogen in humans	Not known	Lawson et al. (2001)
A. georgiae	Associated with periodontal disease?	Oral cavity, humans	Johnson et al. (1990)
A. gerencseriae	Pathogen in humans and animals	Oral cavity, humans	Johnson et al. (1990)
A. graevenitzii	Pathogen in humans	Oral cavity ?	Pascual Ramos et al. (1997a)
A. hongkongensis	Pathogen in humans	Not known	Woo et al. (2003)
A. hordeivulneris	Pathogen in dogs	Oral cavity ?, dogs	Buchanan et al. (1984)
A. howellii	Nonpathogen	Oral cavity, cattle	Dent and Williams (1984b)
A. hyovaginalis	Pathogen in pigs	Porcine genital tract	Hommez et al. (1991); Collins et al. (1993)
A. israelii	Pathogen in humans and animals	Oral cavity, humans	Schaal (1986)
A. marimammalium	Pathogen in seals, porpoise	Not known	Hoyles et al. (2001c)
A. meyeri	Pathogen in humans Associated with periodontal disease?	Oral cavity, humans	Cato et al. (1984)
A. naeslundii	Pathogen in humans Associated with root caries and periodontal disease?	Oral cavity, humans	Johnson et al. (1990)
A. nasicola	Pathogen in humans	Not known	Hall et al. (2003e)
A. neuii	Pathogen in humans	Not known	Funke et al. (1993, 1994)
A. odontolyticus	Occasional pathogen in humans	Oral cavity, humans	Batty (1958); Schaal (1986)
A. oricola	Pathogen in humans	Not known	Hall et al. (2003c)
A. radicidentis	Pathogen in humans	Oral cavity?	Collins et al. (2000)
A. radingae	Pathogen in humans	Not known	Wüst et al. (1993, 1995); Vandamme et al. (1998)
A. slackii	Nonpathogen	Oral cavity, cattle	Dent and Williams (1986)
A. suimastitidis	Pathogen in pigs	Not known	Hoyles et al. (2001a)
A. turicensis	Pathogen in humans	Not known	Wüst et al. (1993, 1995); Vandamme et al. (1998)
A. urogenitalis	Pathogen in humans	Urogenital tract	Nikolaitchouk et al. (2000)
A. vaccimaxillae	Pathogen in cattle	Not known	Hall et al. (2003a)
A. viscosus	Nonpathogen	Oral cavity, animals	Johnson et al. (1990)
Vbc. cambriensis	Pathogen in humans	Not known	Hall et al. (2003a)

Acb., *Actinobaculum*; *Vbc.*, *Varibaculum*.

blood (see Tables 4 and 5) and growth conditions can affect the nature of the hemolysis. Colonies of some strains of *A. odontolyticus* turn brownish-red after incubation for 72–96 h on blood agar (Batty 1958; Johnson et al. 1990; Brander and Jousimies-Somer 1992).

CULTURAL CHARACTERISTICS

The optimum temperature for growth of all species is 35–37°C. The reported range of temperatures that support growth is 20–45°C depending on the species

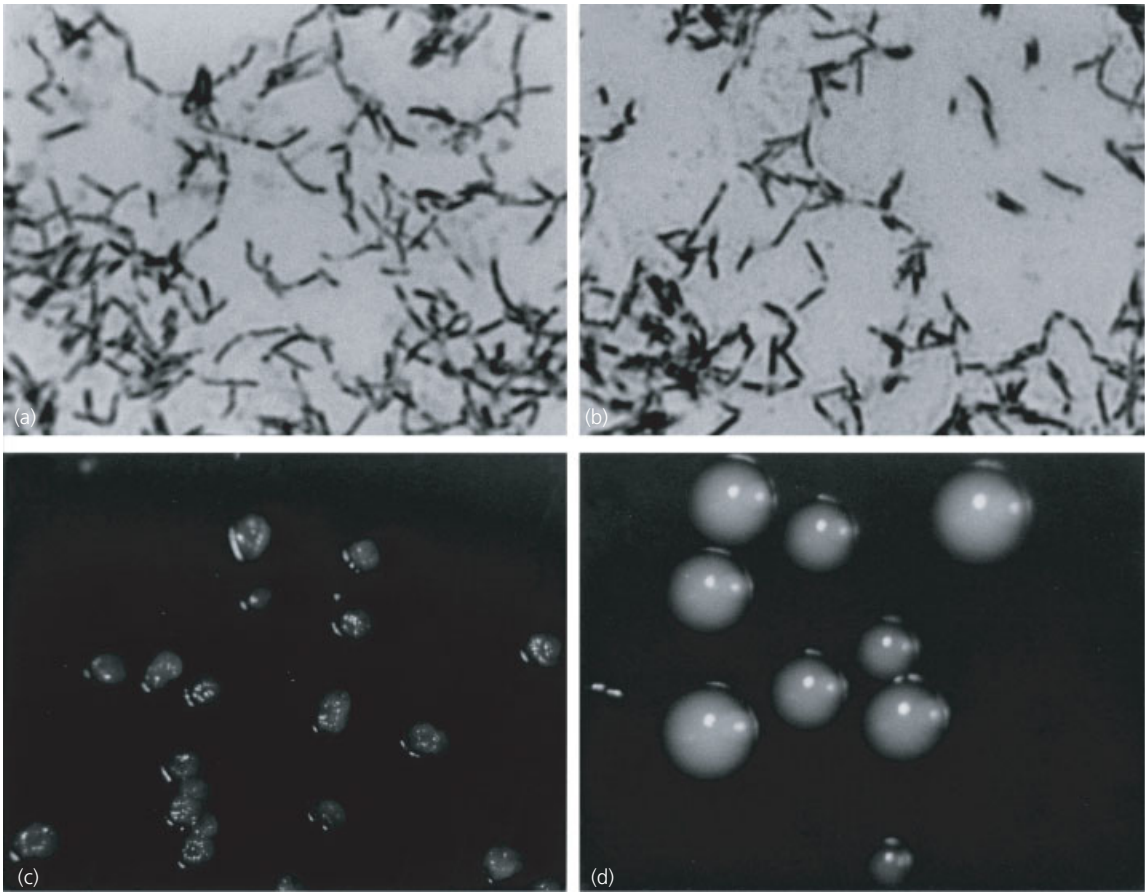

Figure 1 *Cellular and colonial morphology of* Actinomyces. **(a)** Actinomyces israelii. *Gram-stained smear from culture on blood agar (×810).* **(b)** Actinomyces naeslundii *genospecies 2. Gram-stained smear from culture on blood agar (×810).* **(c)** Actinomyces israelii *colonies on blood agar after incubation for 5 days (×3.4).* **(d)** Actinomyces naeslundii *genospecies 2 colonies on blood agar after incubation for 5 days (×3.4).*

(Schaal 1986). *A. israelii*, *A. gerencseriae*, *A. naeslundii*, *A. bovis*, *A. odontolyticus*, and *A. viscosus* grow on modern high quality nutrient agars in air with added CO_2, often without added enrichment. However, it is common practice to grow the organisms on solid media enriched with blood or, occasionally, serum. In some cases specific enrichments are recommended: *Actinomyces hordeivulneris* requires the addition of fetal calf serum (Buchanan et al. 1984), and *Actinomyces meyeri* requires vitamin K1 and Tween 80 (Cato et al. 1984). The nutrient requirements of the more recently described *Actinomyces* spp. are not known in detail.

Most species grow in high quality digest broths, often without added enrichment. The addition of serum to broth enhances growth. A feature of the growth of *Actinomyces* is their stimulation by CO_2, which can be provided in fluid media as a 1 percent v/v filter-sterilized solution of sodium carbonate (1 percent w/v) in distilled water. Reduced semisolid and cooked meat media can also be used.

The majority of the species of *Actinomyces* are facultative anaerobes, although strains of some species (*A. israelii*) may grow best under anaerobic conditions and *A. meyeri* and *Actinobaculum suis* are anaerobic. Some strains of *Actinomyces* may not grow under anaerobic conditions in the absence of CO_2 (Schofield and Schaal 1981). Therefore, strains of all species should be grown in 5–10 percent CO_2. Some *Actinomyces* spp. have the reputation of being slow-growing and, indeed, *A. israelii*, *A. bovis*, and *A. gerencseriae* may take 7–14 days to grow on initial isolation. However, once isolated and grown in pure culture, maximum growth usually occurs after incubation for 3–4 days.

METABOLISM

Examinations of the metabolism of glucose and CO_2 by *A. naeslundii* (*A. israelii*) were made by Buchanan and Pine (1965, 1967). Considerable interest has been shown by oral microbiologists in the biochemistry and physiology of *A. viscosus* and *A. naeslundii* (Brown et al. 1975; Kiel and Tanzer 1977; Miller and Somers 1978; Birkhed et al. 1979; de Jong et al. 1988; van der Hoeven and Gottschal, 1989; van der Hoeven and van den Kieboom 1990; Takahashi and Yamada 1992; Watson et al. 1993). Nothing is known in detail of the metabolic

activities of the more recently described species of *Actinomyces*.

Actinomyces spp. are fermentative, generally utilizing carbohydrates anaerobically in the presence of CO_2 or HCO_3^- to produce formic, acetic, lactic, and succinic acids. Separation of *Actinomyces* spp. and *Actinobaculum* spp. from related genera (e.g. *Rothia*) can be very difficult without the demonstration of succinic acid as endproduct (Sarkonen et al. 2001). Cultural conditions influence the amounts of these acids detected. The level of succinate in anaerobic cultures depends on the concentration of CO_2 or HCO_3^- available, whereas under aerobic conditions with CO_2, acetate is the major acid produced by *A. israelii*. Succinate is generated by *A. viscosus* via the succinate pathway (Brown and Breeding 1980). In *A. viscosus*, the stimulation of glycolysis by HCO_3^- has been related to enhanced activation of glyceraldehyde-3-phosphate dehydrogenase, resulting from the more efficient regeneration of NAD in the presence of bicarbonate (Takahashi and Yamada 1992). Different species can show variation in the pattern of acid products: *A. suis* may produce only trace amounts of succinate and strains of *A. meyeri* may not form lactic acid.

Sugar transport in *A. viscosus* is via a phosphoenolpyruvate phosphotransferase system and this organism also has an alternative system for sugar transport (Hamilton and Ellwood 1983). *Actinomyces* spp. ferment a wide range of carbohydrates, including mono-, di-, and trisaccharides, sugar alcohols, and some polysaccharides (see Tables 4 and 5). Some strains degrade starch (see Tables 4 and 5) and human oral strains produce enzymes that degrade levans (Miller and Somers 1978) and glucans (Johnson 1990). Genes for a neuraminidase (Henningsen et al. 1991; Yeung 1993) and a sucrase–fructanase (Norman et al. 1995) have been characterized from a strain of *A. viscosus*. Strains of *A. viscosus* from animals produce extracellular polysaccharides (van der Hoeven 1974; Birkhed et al. 1979).

Generally, *Actinomyces* spp. do not degrade proteins (see Tables 4 and 5). They are also limited in their abilities to decarboxylate or deaminate amino acids, except for the production of ammonia from arginine. Only few species hydrolyze urea.

CELL WALL COMPOSITION; ANTIGENIC STRUCTURE

Cell wall composition

Actinomyces spp. have cell walls typical of gram-positive bacteria. Species may have one of the types of mucopeptide based on the amino acid at position 3 of the stem peptide and the interpeptide bridge (Schleifer and Kandler 1972; Schleifer and Seidl 1985): Lys-Lys-D-Asp (*A. bovis*); Orn-Lys-D-Glu (*Actinomyces radingae*, *A. israelii*, *A. gerencseriae*, *A. viscosus*, *A. naeslundii*, *A. odontolyticus*, *A. hyovaginalis*); whereas arcanobacteria have Lys-Ala-Lys-D-Glu (*A. pyogenes*, *A. bernardiae*). The type strain of *A. radingae* has an unusual mucopeptide structure which has not been described previously (Wüst et al. 1995).

Neutral carbohydrate accounts for 25–45 percent by weight of the cell walls of *A. israelii*, *A. gerencseriae*, *A. naeslundii*, *A. viscosus*, and *A. bovis* and there is no evidence that teichoic acids are present. The wall polysaccharide component can be extracted with trichloracetic acid and yields of 5–24 percent by weight (Bowden et al. 1976; Bowden and Fillery 1978) are obtained. These polymers can contain one or more carbohydrates, with traces of amino sugars. The composition of the wall carbohydrate reflects, to some extent, the species of origin (Table 2).

Antigenic structure

Serological analysis has been used for decades to aid the identification and serotyping of *Actinomyces* spp. (Slack and Gerencser 1975; Gerencser 1979; Schaal and Gatzer 1985; Johnson et al. 1990). In general, the technique used has been fluorescent antibody (FA) staining, with polyclonal absorbed sera. However, FA staining gives no information on the nature of the antigens that have been demonstrated and absorbed sera do not reveal the numbers of antigens common to different species. Cross-reactions between *Actinomyces* spp. demonstrated predominantly by FA are shown in Table 3. Serological techniques that demonstrate the nature and extent of antigenic relationships between bacteria (Bowden 1993) have also been used to examine *Actinomyces* spp. These include immunodiffusion (Bowden et al. 1976; Schaal and Gatzer 1985); crossed immunoelectrophoresis (Holmberg et al. 1975a; Bowden and Fillery 1978); Western blotting (Putnins and Bowden 1993); and FA staining with monoclonal antibody (Firtel and Fillery 1988).

Actinomyces spp. have carbohydrate and protein antigens associated with the cell wall. In *Actinomyces denticolens*, *Actinomyces howellii*, *A. israelii*, *A. gerencseriae*, *A. naeslundii*, *A. odontolyticus*, and *A. viscosus* (Cummins 1962; Bowden et al. 1976; Bowden and Fillery 1978; Fillery et al. 1978; Putnins and Bowden 1993), the carbohydrate antigens are responsible for both species-specific and crossreactions. *A. israelii* and *A. gerencseriae* (*A. israelii* serotype II) share a common carbohydrate antigen, but *A. gerencseriae* has a second species-specific rhamnose-containing antigen (Bowden and Fillery 1978). *Actinomyces*, as typified by *A. naeslundii* and *A. viscosus*, have a complex mixture of surface antigens and epitopes (Firtel and Fillery 1988). Many of these are proteins, which can be extracted from isolated cell walls (Figure 2). In *A. naeslundii*, *A. viscosus*, *A. howellii*, *A. denticolens*, and *A. slackii*, the protein antigens are responsible for species-specific and crossreactions

Table 2 *Cell wall components and fermentation products of some* Actinomyces *species and other genera*

Genera and species	Significant cell wall components		Acid products from fermentation[c]
	Amino acids[a]	Carbohydrates[b]	
Actinobaculum			
A. suis	Ala, Lys, D-Glu	Rham	Form, Ac
Actinomyces			
A. bovis	Ala, Lys, D-Asp	Rham, 6-Deoxyt, (Gluc)[d]	Ac, Lac, Suc
A. denticolens	Ala, Lys, Orn, D-Glu	Rham	Ac, Lac, (Suc)
A. georgiae	Not known	Not known	Ac, Lact, Suc
A. gerencseriae	Ala, Lys, Orn, D-Glu	Gal, Rham	Ac, Lac, Suc
A. hordeivulneris	Ala, Lys, Orn, D-Glu	Gluc, Gal	Ac, Lact, Suc
A. howellii	Ala, Lys, Orn, D-Glu	Gluc, Rham	Ac, Lac, (Suc)
A. israelii	Ala, Lys, Orn, D-Glu	Gluc, Gal	Ac, Lac, Suc
A. meyeri	Not known	Not known	Ac, Lac, Suc
A. naeslundii	Ala, Lys, Orn, D-Glu	Rham, 6-Deoxyt,[e] (Gluc)	Ac, Lac, Suc
A. neuii	Not known	Not known	Ac, Lac, Suc
A. odontolyticus	Ala, Lys, Orn, D-Glu	Glu, Man, Rham, 6-Deoxyt	Ac, Lac, Suc
A. radingae	Ala, Lys, Orn,[f] D-Glu	Not known	Lac, Suc
A. slackii	Ala, Lys, Orn, D-Glu	Gluc, Rham, (Gal)	Ac, Lac, (Suc)
A. turicensis	Ala, Lys, Orn, D-Glu	Not known	Lac, Suc
A. viscosus	Ala, Lys, Orn, D-Glu	Glu, Rham, 6-Deoxyt, (Man, Gal)	Ac, Lac, Suc
Arcanobacterium	Ala, Lys, D-Glu	Not known	Ac, Lac, (Suc)
Propionibacterium	L-DAP, Gly (*meso*-DAP)	Glu, Gal, Man, Rham	Ac, Prop, Suc
Corynebacterium	*meso*-DAP	Gal, Arab	Ac, Prop, Lac, Suc
Rothia	Ala, Lys, D-Glu	Gal, Glu, Fruc	Ac, Lac, (Suc)

a) Amino acids: Ala, alanine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; Lys, lysine; L-DAP, L-diaminopimelic acid; *meso*-DAP, *meso*-diaminopimelic acid; Orn, ornithine.
b) Carbohydrates: Arab, arabinose; Fuc, fucose; Fruc, fructose; Gal, galactose; Gluc, glucose; Man, mannose; Rham, rhamnose; 6-Deoxyt, 6-deoxytalose.
c) Acids produced in presence of CO_2 for most species: Ac, acetic; Form, formic; Lac, lactic; Prop, propionic; Suc, succinic.
d) ( ), presence variable or present in trace amounts.
e) 6-Deoxytalose may not be present in all strains.
f) A new peptidoglycan type, ornithine may replace lysine in position 3 of the stem peptide.

Table 3 *Serological crossreactions among* Actinomyces *species*

Antiserum	Crossreacting species or serotype[a]
A. israelii	*A. gerencseriae*, *A. israelii* subtypes,[b] *A. naeslundii* genosp. 1, 2
A. gerencseriae	*A. israelii*, *A. israelii* subtypes, *A. naeslundii* genosp. 1, 2
A. naeslundii genospecies 1[c]	
A. naeslundii serotype I	*A. gerencseriae*, *A. israelii* subtype 1c, *A. naeslundii* genosp. 2
A. naeslundii genospecies 2[c]	
A. naeslundii serotype II	*A. naeslundii* serotypes I and III, '*A. viscosus* serotype II'
A. naeslundii serotype III	*A. naeslundii* serotypes I and II
'*A. viscosus* serotype II'	*A. gerencseriae*, *A. israelii*, *A. israelii* subtypes 1b, c, *A. naeslundii* serotypes I, II, and III, *A. bovis* serotype I, *A. meyeri*, *Actinomyces* serotype NV
A. viscosus	*A. naeslundii* genospecies 1, ' *A. viscosus* serotype II'
A. odontolyticus serotype I	*A. israelii*, ' *A. viscosus* serotype II'
A. odontolyticus serotype II	*A. israelii* subtype 1c, ' *A. viscosus* serotype II', *A. bovis* serotype II
A. bovis serotype I	*A. viscosus*, '*A. viscosus* serotype II', *A. meyeri*
A. bovis serotype II	*A. bovis* serotype I, *A. naeslundii* serotype II
A. hordeivulneris	*A. israelii*, *A. gerencseriae*, *A. viscosus*, '*A. viscosus* serotype II'

a) It is possible that protein antigens common to most of the species and serotypes exist and could be demonstrated by Western blotting (Putnins and Bowden 1993).
b) See Schofield and Schaal (1981) and Schaal and Gatzer (1985).
c) The genospecies as defined by Johnson et al. (1990). Data also taken from Gerencser (1979) and Schaal (1986).

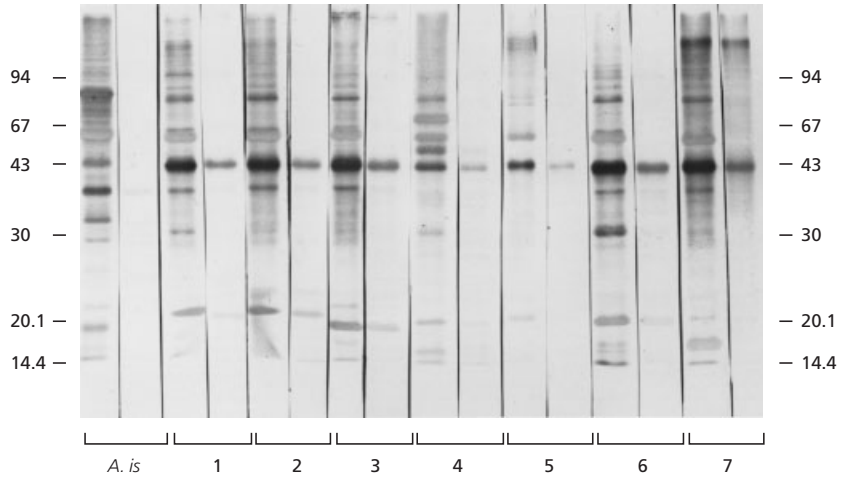

Figure 2 *Western blot of* Actinomyces *cell wall extracts. Cell wall protein extracts (Putnins and Bowden 1993) of* A. israelii, A. naeslundii *genospecies 1 and 2 and* A. viscosus*. The extracts are shown in pairs, the left lane of each pair was reacted with* A. viscosus *(ATCC 15978) antiserum and the right lane with the* A. viscosus *antiserum absorbed with* A. israelii *(NGB 17) cell walls. Lanes:* A. is. (A. israelii)*; 1, 2, 3, 4, and 6, representatives of* A. naeslundii *genospecies 2; 5,* A. naeslundii *genospecies 1 (ATCC 12104); and 7,* A. viscosus *(ATCC 15987). The results demonstrate wide crossreactivity among the strains and that many of the antigens are common between* A. israelii, A. naeslundii, *and* A. viscosus*. Molecular mass standards (kDa) left and right*

(Putnins and Bowden 1993). Western blotting has revealed crossreactions between *A. israelii* and other species (Figure 2). Perhaps the best defined antigens of *Actinomyces* are the surface fimbriae of *A. viscosus* and *A. naeslundii* (Masuda et al. 1983; Yeung and Cisar 1990; Yeung 1992). Little is known of the antigens of the more recently described *Actinomyces* spp.

Two types of fimbriae (types 1 and 2), which are antigenically distinct, can be detected on the cell surface of strains of *A. naeslundii* and *A. viscosus* (Cisar et al. 1991). Strains of *A. naeslundii* genospecies 2 isolates have both types 1 and 2 fimbriae, whereas those typical of *A. naeslundii* genospecies 1 only carry type 2. Testing strains using specific antibody against the fimbriae shows their wide distribution among strains of *A. viscosus* and *A. naeslundii* (Masuda et al. 1983; Cisar et al. 1991). Moreover, studies using a type 1 subunit-specific probe and specific antibody confirmed that the subunit was conserved among strains of *A. viscosus* and *A. naeslundii* from oral habitats in humans and animals and absent in *A. israelii* and *A. odontolyticus* (Yeung 1992).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Those *Actinomyces* spp. that have been tested are susceptible to a wide range of antimicrobial agents (Schaal 1986). *Actinomyces* spp. are highly sensitive to the β-lactam antibiotics and have a high to moderate sensitivity to tetracyclines, chloramphenicol, macrolides, lincomycins, fusidic acid, and vancomycin. Generally, the organisms are resistant to aminoglycosides and metronidazole. *Actinomyces* spp. are very infrequently reported to acquire or to develop antibiotic resistance. The antibiotic sensitivities of *A. meyeri* and *A. hordeivulneris* are similar, although they have not been examined in detail. Both these species are sensitive to penicillin G, tetracycline, and chloramphenicol; the former has also been shown to be sensitive to erythromycin and clindamycin. *Abc. suis* is sensitive to penicillin G, ampicillin, erythromycin, tetracycline, chloramphenicol, and clindamycin. Among the more recently described *Actinomyces* spp., *A. neuii* is susceptible to clindamycin, erythromycin, penicillin G, rifampicin, and vancomycin (Funke et al. 1993).

CLASSIFICATION

The gradual expansion and development of chemotaxonomy revolutionized the classification of actinomycetes. Traditional morphological characters had proved less than satisfactory in classification and chemotaxonomy was significant in defining these organisms at the level of the genus (Goodfellow and Cross 1984; Goodfellow and Minnikin 1985). Analysis of acid endproducts (Bowden and Hardie 1978; Schaal 1986), cell wall composition (Bowden and Hardie 1978), and peptidoglycan structure (Schleifer and Kandler 1972; Schleifer and Seidl 1985) of *Actinomyces* resulted in a more precise definition of the genus. Revisions to the genus were made, based on chemotaxonomic characters, e.g. *Actinomyces ericksonii* was shown to be a *Bifidobacterium* and *Actinomyces propionicus*, originally reclassified as *Arachnia propionica*, was subsequently named *Propionibacterium propionicus*. Methods to determine chemotaxonomic characters that aid the classification of *Actinomyces* are now well established (Goodfellow and Minnikin 1985;

Goodfellow and O'Donnell 1993) and include cell wall composition, acid endproducts, protein profiles of whole cells, and nucleic acid analysis.

In a numerical taxonomic study, Schofield and Schaal (1981) examined 134 strains of *Actinomyces*. *A. israelii* strains fell into a single, well-defined cluster, which could be subdivided into subclusters a–d, suggesting differences among the strains. Schaal and Gatzer (1985) confirmed the subdivisions among *A. israelii* and Schaal (1986) described them as 'subspecies,' the first two being represented by *A. israelii* serotypes I and II, the others being new groups. The majority (45 of 67) of strains of *A. naeslundii* and *A. viscosus* were recovered in two main clusters at 82.5 percent similarity. This similarity among strains of *A. naeslundii* and *A. viscosus* supported an earlier observation by Fillery et al. (1978) who had demonstrated an 88 percent similarity between 35 human isolates of *A. viscosus* serotype II and *A. naeslundii*. In this study, a separate cluster of three animal strains, including the type strain of *A. viscosus* serotype I, reacted only with *A. viscosus* serotype I antisera and the authors suggested that *A. viscosus* serotype I could represent a distinct group of animal organisms. By contrast, the human strains of *A. viscosus*, i.e. serotype II, and *A. naeslundii* could be seen as serotypes, rather than separate species. However, Schaal (1986) believed that *A. naeslundii* and *A. viscosus* should be retained as separate species. *A. odontolyticus* strains were recovered by Schofield and Schaal (1981) in a single cluster, as were three of four strains of *A. bovis*.

Buchanan et al. (1984) studied *Actinomyces* isolates from infections in dogs and found a group of strains (*A. hordeivulneris*) that were distinct from previously described species. Strains from healthy cattle were examined by Dent and Williams (1984a, b, c, 1986). Using chemotaxonomic and biochemical tests and DNA homology, they described three new species: *A. denticolens*, *A. howellii*, and *A. slackii*. *A. meyeri* (Cato et al. 1984) was the last species included by Schaal (1986) in his detailed review and definition of the genus. However, the two organisms '*Actinomyces suis*' and '*Actinomyces humiferus*' were considered by Schaal to be species *incertae sedis*. The latter species has been shown to belong to the genus *Cellulomonas* (Collins and Pascual 2000).

Johnson et al. (1990) studied human oral *Actinomyces* isolates, some of which did not conform to named species or react with standard antisera. Two new species were described: *A. gerencseriae*, to include isolates previously described as serotype II of *A. israelii*, and *Actinomyces georgiae*, to include organisms from the periodontal flora, previously called '*Actinomyces* D08'. These authors made a clear distinction between the animal organism *A. viscosus* serotype I and *A. naeslundii* and human strains of *A. viscosus* (serotype II). *A. viscosus* serotype I was retained as a distinct species, which was in accord with some numerical data (Fillery et al. 1978). They also showed a close relationship between *A. naeslundii* serotypes II and III, human strains of *A. viscosus* (serotype II) and serotype *Actinomyces* NV, which they considered to be examples of the same species. Because differentiation between strains in this latter group and *A. naeslundii* serotype I could be made only on the basis of serology and not on reliable phenotypic tests, they proposed two genospecies: *A. naeslundii* genospecies 1 to include *A. naeslundii* serotype I, and genospecies 2 to include the other group of human strains. *Actinomyces* WVA 963 was proposed as a third genospecies. The proposal by Johnson et al. (1990) for *A. viscosus* and *A. naeslundii* was supported by calculation of their antigenic similarities (Putnins and Bowden 1993).

The most recent method to be applied to *Actinomyces* classification has been the determination of phylogenetic relationships using data from sequence analysis of 16S rRNA (Embley and Stackebrandt 1994; Stackebrandt and Charfreitag 1990). The plethora of *Actinomyces* spp. described in recent years (see Tables 1 and 5) has been defined on extensive comparative 16S rDNA data. Undoubtedly, phylogenetic data have already made major contributions to the classification of *Actinomyces*. However, Embley and Stackebrandt (1994) emphasize the need for a polyphasic approach to bacterial classification, using genotypic and phenotypic features.

LABORATORY ISOLATION AND IDENTIFICATION

Isolation

Schaal (1984) discusses the laboratory diagnosis of diseases caused by actinomycetes, including *Actinomyces*. Detection of *Actinomyces* in clinical specimens by FA avoids the need for culture (Gerencser 1979) and has been particularly successful in demonstrating these organisms in samples from infections involving intrauterine devices (Leslie and Garland 1991). In general, isolation of *Actinomyces* from clinical and other specimens is relatively easy if certain criteria are met. These include: appropriate sample taking and transport; use of high quality media, or in certain cases selective media; incubation in an atmosphere with CO_2 or in broth media with HCO_3^-; incubation for a sufficient period of time; adequate spreading of sample on solid media; and careful examination of colonies. Usually *Actinomyces* will survive well in clinical samples, providing that oxygen is excluded and the samples do not dry. In the past, *Actinomyces* spp. were thought of as pathogens in pyogenic mixed anaerobic infections, with some emphasis placed on actinomycosis. Whereas the newly described species are often associated with mixed anaerobic infections, recent revisions in classification mean that *Actinomyces* may be encountered in samples from other sources.

Samples of pus or exudate from mixed infections can be examined for the presence of macroscopic masses of filaments, which appear as granules (sulfur granules or druzen) and are typical of actinomycosis (Slack and Gerencser 1975; Schaal 1984). Plating of a granule will often provide an enriched culture of *Actinomyces*. In the absence of obvious granules, samples may be plated directly, or after dilution in broth or reduced transport fluid (RTF) (Loesche et al. 1972). Isolation of individual colonies of *Actinomyces* from mixed samples is made easier if dilutions of the sample are plated. A simple spreading technique is usually effective. Samples of blood or swabs are dealt with in the normal way, taking care that the media, cultural conditions, and time of incubation are appropriate. Reliable recognition of *Actinomyces* colonies among those in mixed samples depends on experience. It is a good idea to grow known culture collection strains on the media that are being used for isolation, in order to become familiar with their colony forms.

Samples from the oral cavity can include dental plaque from different areas of the dentition, saliva, swabs of mucosal surfaces, and plaque taken from the gingival margin and subgingivally. Plaque can be removed by the use of sterile dental scalers, abrasive strips (Bowden et al. 1975), and dental floss. Various methods have been used to remove subgingival samples (Tanner and Goodson 1986). Oral samples are usually placed into RTF, suspended by mild sonication and diluted further in RTF, before plating.

Actinomyces spp. can be isolated on most good quality digest nutrient agar media that are enriched with serum or blood. Very often these standard media include hemin and vitamin K1 (Holdeman et al. 1977). Schaal (1984, 1986) recommended the CC medium of Heinrich and Kurth, and Tarozzi broth; details of the preparation of these media are given by Schaal (1986). Currently, although selective media are available for some *Actinomyces* spp., their use is restricted to oral samples and they are not commonly used in clinical laboratories.

Selective media have been designed to isolate actinomycetes (Beighton and Colman 1976) or, more specifically, *A. naeslundii* and *A. viscosus* (Ellen and Balcerzak-Raczkowski 1975; Kornman and Loesche 1978; Zylber and Jordan 1982) from oral samples. The medium of Beighton and Colman (1976) will support the growth of strains of *A. israelii*, *A. naeslundii*, *A. viscosus*, *A. odontolyticus*, and other oral pleomorphic gram-positive rods such as *Propionibacterium* (*Arachnia*), *Rothia*, and *Corynebacterium*. The media designed specifically for *A. viscosus* and *A. naeslundii* are not completely selective; they will all support some other oral organisms.

Identification

The identification of *Actinomyces* spp. has always presented a problem due to the lack of reliable, readily available tests to differentiate the species. Unfortunately, isolates are often identified on the basis of a few characters.

Facultative or obligatory anaerobic gram-positive pleomorphic rods can be assigned to a genus by analysis of acid endproducts (Holdeman et al. 1977; Bowden and Hardie 1978; Schaal 1986; von Graevenitz et al. 1994). This well accepted, relatively simple technique is routine in most laboratories. Typically, *Actinomyces* spp. produce significant amounts of succinic acid when they are grown anaerobically in the presence of CO_2 or HCO_3^-. There are exceptions (see Table 2) and gram-positive pleomorphic rods included in other genera can produce succinate (von Graevenitz et al. 1994). Despite these limitations, the analysis of acid endproducts remains an invaluable aid in assigning isolates to a genus. A second chemotaxonomic test that has not gained regular acceptance in routine or reference laboratories, perhaps because it is thought to be too complex, is determining cell wall composition. However, rapid methods for both preparation and analysis of *Actinomyces* walls (Boone and Pine 1968; Bousfield et al. 1985) and murein (mucopeptide) (Schleifer and Seidl 1985) have been described. Cell wall and endproduct analyses can be particularly valuable in differentiating *Actinomyces* from other gram-positive pleomorphic rods (see Table 2).

A valuable technique in the classification of bacteria, which is becoming more routine in identification, is electrophoresis of whole cell proteins and enzymes (Vauterin et al. 1993). The apparatus is simple. Adequate comparisons of protein profiles can be made visually initially and this technique has been used to characterize *Actinomyces* (Dent and Williams 1984a, b, 1986; McCormick et al. 1985). Although analysis of the cellular fatty acid composition is used to support identification of pleomorphic gram-positive rods (Bernard et al. 1991; von Graevenitz et al. 1994) and details are available on the fatty acids of *Actinomyces* spp., such analyses are not in general use due to the laboratory equipment required.

Very often, *Actinomyces* spp. have to be identified on the basis of phenotypic tests. However, species give variable results in many of the tests, which makes them of limited value. Johnson et al. (1990) noted 'that it continues to be exceedingly difficult to differentiate among the described species of *Actinomyces* by usual phenotypic tests, although a few tests are helpful.' Tables 4 and 5 list some selected tests that can be valuable in speciating isolates identified as *Actinomyces* on the basis of acid endproduct analysis. It is recommended that the original references (see Table 1) are consulted whenever possible, to obtain specific details and it must be borne in mind that test results may vary, depending on the media employed.

In many routine laboratories, commercial systems are used for identifying *Actinomyces* spp. on the species level. Santala et al. (2004) have recently demonstrated

Table 4 *Characteristics of* Actinomyces *species*

Character	*A. bovis*	*A. denticolens*	*A. georgiae*	*A. gerencseriae*	*A. hordeivulneris*	*A. howellii*	*A. hyovaginalis*	*A. israelii*	*A. meyeri*	*A. naeslundii*	*A. neuii*	*A. odontolyticus*	*A. radingae*	*A. slackii*	*A. turicensis*	*A. viscosus*
Growth																
O_2	–	(+)	–	–	nk	nk	nk	–	–	(+)	–	(+)	nk	+	nk	+
O + CO_2	(+)	+	+	(+)	+	+	+	(+)	(+)	+	+	+	+	+	+	+
AnO_2 + CO_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
catalase	–	–	–	–	+	+	–	–	–	(+)[a]	+	–	–	+	–	+
hemolysis	(β)	–	(α)	–	(β)	–	–	–	(α)	–	(α)/γ[b]	(α)	α	–	(β)w[c]	–
Hydrolysis of																
esculin	(+)	+	(+)	+	+	nk	+	+	(+)	+	–	(+)	+	–	–	+
gelatine	–	nk	(+)	–	nk	nk	–	–	–	–	–	–	–	nk	–	–
casein	–	nk	nk	–	nk	nk	nk	–	–	–	nk	–	–	nk	–	–
starch	AH	nk	A (H)	A (H)	–	nk	nk	–	–	–	nk	(AH)	H	nk	AH	–
Ammonia from																
arginine	–	nk	nk	+	nk	nk	–	+	–	(+)	nk	–	nk	nk	nk	(+)
urea	–	nk	nk	–	-	nk	–	–	(+)	(+)	–	–	–	nk	–	(+)
Acid from																
adonitol	–	nk	nk	nk	nk	nk	+	–	–	–	–/+[b]	(+)	nk	nk	nk	–
amygdalin	–	–	nk	+m	-	–	(+)	(+)	(+)	–	nk	–	–	–	–	–
arabinose	–	–	–	–	+	(+)	+	+m	(+)	–	(+)	(+)	+	–	(+)	–
cellobiose	–	–	nk	nk	+	–	(+)	+	–	(+)	(+)	–	+	–	(+)	–
erythritol	–	nk	nk	nk	nk	nk	nk	–	–	–	+	–	–	nk	+	–
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
glycerol	–	–	(+)	–	–	–	nk	–	(+)	(+)	+	(+)	nk	–	nk	(+)
glycogen	+	nk	+w	(+)	+	nk	(+)	(+)	(+)	(+)[d]	(+)	(+)	–	nk	–	–
maltose	(+)	+	(+)	+	+	(+)	+	+	+	+	+	(+)	+	nk	+	+

(*Continued over*)

Table 4 *Characteristics of* Actinomyces species (*Continued*)

Character	*A. bovis*	*A. denticolens*	*A. georgiae*	*A. gerencseriae*	*A. hordeivulneris*	*A. howellii*	*A. hyovaginalis*	*A. israelii*	*A. meyeri*	*A. naeslundii*	*A. neuii*	*A. odontolyticus*	*A. radingae*	*A. slackii*	*A. turicensis*	*A. viscosus*
Acid from (Continued)																
mannose	(+)	(+)	(+)	+	+w	(+)	+	+	–	+m	+	–	+	nk	(+)	+
mannitol	–	(+)	(+)	+m	–	–	nk	+m	–	–	+	–	–	–	–	–
raffinose	–	+	(+)	+m	+w	+w	–	+	–	+	+	–	+	+	(+)	+
rhamnose	–	–	+w	(+)	–	–	–	–	–	–	–	(+)	–	–	–	–
ribose	–	(+)	+	+m	–	–	nk	+m	+	(+)	+	(+)	+	nk	+	–
sucrose	+	+	+	+	+w	+	+	+	+	+	+	(+)	+	nk	+	+
trehalose	–	–	+	+	+	(+)w	–	(+)	–	(+)	+	–	–	nk	(+)	–
xylose	–	–	+m	+m	+	(+)w	+	+	+m	–	+	(+)	+	–	+	–
Enzymes[e]																
esterase-lipase	–	–	–	–	nk	nk	nk	–	–	–	nk	–	+w	nk	+	+
leucine-arylamidase	+	+	+	+	nk	nk	+	(+)	+	+	+	(+)	–	nk	+	+
valine-arylamidase	–	–	–	(+)	nk	nk	nk	–	(+)	–	–	–	–	nk	–	+w
acid phosphatase	–	(+)	–	–	nk	nk	nk	–	–	(+)	–	–	–	nk	–	–
β-galactosidase	–	+	–	+	nk	nk	+	+	–	(+)	+	(+)	+	nk	–	(+)
α-glucosidase	–	+	+	+	nk	nk	nk	(+)	+	(+)	–	–	+	nk	+	–
β-glucosidase	–	(+)	–	+	nk	nk	nk	+	–	+w	–	(+)	+	nk	–	+
N-Acetyl β-glucosaminidase	+	–	–	–	nk	nk	nk	–	–	–	–	–	+	nk	–	–
α-fucosidase	–	–	–	–	nk	nk	nk	–	–	–	–	(+)	–	nk	+w	–

+, positive; –, negative; ( ), variable reaction; A, acid; H, hydrolysis; m, most strains; nk, not known; w, weak.

a) *A. naeslundii* genospecies 1 is catalase negative.

b) *A. neuii* subsp. *neuii* / subsp. *anitratus*.

c) β-hemolysis O_2 + CO_2, γ-anaerobic.

d) *A. naeslundii* genospecies 1 seldom ferments glycogen.

e) APIZYM data.

Table 5 *Characteristics of recently established* Actinomyces *species and related genera*

Character	*A. europaeus*	*A. suis*	*A. schaalii*	*A. graevenitzii*	*A. bowdenii*	*A. canis*	*A. urogenitalis*	*A. radicidentis*	*A. marimammalium*	*A. catuli*	*A. funkei*	*A. suimastitidis*	*A. coleocanis*	*A. cardiffensis*	*V. Cambriensis*	*A. vaccimaxillae*	*A. urinale*	*A. nasicola*	*A. oricola*	*A. hongkongensis*
Growth																				
O + CO_2	+	–	+	+	+	+	+	+	+	+	+	+	+	(+)	(+)	+	(+)	(+)	(+)	–
AnO_2 + CO_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
catalase	–	–	–	–	+	+	–	+	–	V	–	–	–	–	–	–	–	–	–	–
hemolysis	α,β	–	–	nk	–	nk	nk	nk	–	nk	–	nk	nk	–	–	–	(β)	nk	–	–
Hydrolysis of																				
esculin	V	–	–	–	+	–	+	+	–	+	–	+	–	–	–	+	–	–	+	–
gelatine	nk	–	–	nk	–	–	–	W	–	–	–	–	–	–	–	–	–	–	–	nk
Ammonia from																				
arginine	nk	nk	–	nk	–	–	–	–	–	V	–	–	–	–	–	–	–	V	–	+
urea	–	+	–	–	–	–	–	V	–	–	–	–	–	–	–	–	V	–	–	–
Acid from																				
amygdalin	–	–	nk	–	nk	nk	nk	nk	nk	nk	nk	nk	nk	–	–	nk	nk	–	W	nk
arabinose	–	–	V	–	–	+	V	–	–	V	–	(+)	–	–	–	+	–	–	–	–
cellobiose	nk	–	nk	–	nk	nk	nk	nk	nk	nk	nk	nk	nk	–	–	nk	nk	+	+	nk
glucose	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–
glycogen	V	+	–	–	–	+	–	–	V	–	–	nk	+	–	–	V	–	–	–	nk
maltose	+	+	+	+	+	+	+	+	+	+	V	V	+	V	V	–	V	–	V	nk
mannose	V	–	V	nk	nk	nk	nk	nk	nk	nk	nk	nk	nk	V	–	–	–	–	nk	–
mannitol	–	–	–	–	–	–	V	+	–	–	-	–	–	–	–	V	–	–	–	nk
raffinose	nk	–	–	nk	+	V	+	nk	–	+	–	+	–	V	–	–	–	–	W	–
ribose	V	–	+	+	+	+	V	+	–	+	V	W	–	V	V	+	+	–	–	nk
sucrose	V	–	V	+	+	V	+	+	–	+	+	+	–	V	V	V	V	–	V	nk
trehalose	V	–	V	–	+	–	+	+	–	+	–	–	–	–	V	+	–	–	+	–
xylose	V	–	+	–	–	+	+	–	–	+	+	+	–	–	V	+	–	–	–	–
Enzymes																				
esterase-lipase	+	nk	nk	–	–	+	–	–	nk	+	–	nk	nk	nk	V	–	–	–	nk	nk
leucine-arylamidase	+	nk	nk	+	+	+	+	+	+	+	+	nk	nk	+	+	+	–	+	+	+
valine-arylamidase	+	nk	nk	–	–	–	+	–	nk	–	–	nk	nk	nk	–	–	–	+	nk	nk
acid phosphatase	nk	nk	nk	+	+	–	–	+	nk	+	+	nk	nk	nk	V	(+)	–	–	nk	nk
β-galactosidase	+	nk	nk	+	+	+	+	+	+	+	V	nk	+	–	V	–	–	+	V	–
α-glucosidase	+	nk	+	–	nk	+	+	+	–	+	+	+	+	+	+	+	–	+	+	–
β-glucosidase	-	nk	nk	–	+	+	+	+	–	+	–	+	–	–	–	+	–	V	+	–
N-Acetyl-β-glucosaminidase	–	nk	nk	+	–	+	+	–	nk	V	V	V	nk	–	–	–	–	+	–	–
α-fucosidase	nk	nk	nk	–	–	+	–	–	nk	–	–	nk	nk	–	–	+	–	–	–	–

+, positive; –, negative; V, variable; ( ), weak reaction; nk, not known.
Biochemical reactions were taken from the original publications. It is important to note that other authors came – in some cases – to different results using different commercial identification systems. *Actinomyces houstonensis*, as defined by Clarridge and Zhang (2002), is presently not a valid species.

that the most frequently used commercial identification systems all perform poorly, mainly due to not having included the recently described new *Actinomyces* spp. in their databases.

FA staining has provided one of the most useful methods for identifying isolates of *Actinomyces* and for direct demonstration of these organisms in clinical samples (Gerencser 1979; Schaal 1984; Leslie and Garland 1991). Techniques such as gel diffusion (Bowden et al. 1976; Schaal and Gatzer 1985) and crossed immunoelectrophoresis (Holmberg et al. 1975a, b) have also been used in identification. Simple whole cell slide agglutination (Putnins and Bowden 1993) has been shown to be useful for differentiating the two genospecies of *A. naeslundii*.

A few studies have explored the possibility of the serodiagnosis of actinomycosis caused by *A. israelii*. Early studies by Colebrook (1920) showed that patient's serum contained antibodies to *A. israelii* and, more recently, Holmberg et al. (1975b) have used crossed immunoelectrophoresis to diagnose *A. israelii* infections. Persson and Holmberg (1984b) evaluated crossed and countercurrent electrophoresis for the detection of genital actinomycosis and showed 98 percent specificity with 83 percent sensitivity. Weil and Schaal (1990) detected significant levels of antibodies to *A. viscosus* and *A. naeslundii* in 16 and 2 percent of healthy subjects, respectively. No significant responses were detected against *A. israelii* antigens.

Despite the ease of use and the specificity of serological methods in identification, there are drawbacks. A major one is the availability and standardization of antisera. Sera are not generally available. Also, sera can vary in their reactivity and specificity even when raised against a standard strain (Johnson et al. 1990). These authors also point out that *Actinomyces* isolates from the human gingival crevice may be phenotypically similar to some accepted species, but they fail to react or crossreact only weakly with the standard sera. Thus, the range of antisera available needs to be extended constantly to include new species.

TYPING METHODS

Historically, serology has been the most widely used method to type *Actinomyces* spp. and recently genetic typing methods are also being applied to *Actinomyces*.

Serotyping

Several *Actinomyces* spp. are known to include serotypes (see Table 3) (Gerencser 1979; Schaal and Gatzer 1985). Little is known of the antigens that define the serotypes. Perhaps the most complex group of *Actinomyces* with regard to serological subdivisions are those designated *A. naeslundii* genospecies 1 and 2 and phenotypically similar organisms (Johnson et al. 1990). It is apparent that *A. naeslundii* genospecies 2 (see Table 3) represents an antigenically heterologous group of organisms, with 33–79 percent antigenic similarity among strains (Putnins and Bowden 1993). *A. naeslundii* genospecies 1 could be more antigenically homogeneous, because to date it seems to include only strains of *A. naeslundii* serotype I. Little is known of the serological diversity among the more recently described *Actinomyces*, although *A. denticolens*, *A. howellii*, *A. slackii* (Putnins and Bowden 1993), and *A. hordeivulneris* (Buchanan et al. 1984) appear to be homogeneous. Little is known of any specific relationships between *Actinomyces* serotypes and pathogenicity. Details of the distribution of *Actinomyces* species, genospecies, and serotypes in humans with healthy or diseased gingivae are given by Johnson et al. (1990). *A. naeslundii* serotype I (genospecies 1) appears to be more closely associated with the tooth surface than genospecies 2.

Genetic typing

It is obvious that genetic typing and identification will largely replace serotyping for recognizing groups, types, and specific strains within *Actinomyces* spp. *Actinomyces* spp. have been typed by restriction fragment length polymorphism (RFLP) analysis of total DNA (Barsotti et al. 1993), and ribotyping (Bowden et al. 1993; Barsotti et al. 1994). With endonucleases *Bam*HI and *Pvu*II, over 130 ribotypes have been identified among 410 strains of *A. naeslundii* genospecies 1 and 2 (Johnson et al. unpublished). Barsotti et al. (1994) also ribotyped 64 strains of *Actinomyces* in a taxonomic study. Comparisons showed *A. gerencseriae*, *A. israelii*, *A. meyeri*, and *A. odontolyticus* to be distinct species. However, *A. naeslundii* and *A. viscosus* were not clearly differentiated. Hall et al. (1999, 2001) established an *Actinomyces* typing system based on amplified 16S ribosomal DNA restriction analysis (ARDRA) applying restriction enzymes *Hae*III and *Hpa*II.

ROLE IN THE NORMAL FLORA OF HUMANS

Actinomyces spp. are common in the mouths of humans and animals (Bowden et al. 1979; Dent and Marsh 1981). They form a significant component of dental plaque on the tooth surface and, together with the other components of the normal flora, aid in the protection of the host from colonization by exogenous pathogens. Samples from healthy sites on the gingivae harbor high proportions of several *Actinomyces* taxa, most of which can be considered to be associated with gingival health (Socransky et al. 1982; Dzink et al. 1988; Moore et al. 1991; Socransky and Haffajee 1992). *Actinomyces* play a significant role in the accumulation of dental plaque,

where their early colonization of the tooth surface and specific interactions with other oral organisms contribute to the composition of the community on the tooth surface (Kolenbrander and London 1992; Kolenbrander 1993). *A. naeslundii* genospecies 1 and 2 will coaggregate with a variety of other oral bacteria (Eifuku et al. 1990; Jenkinson et al. 1993; Hsu et al. 1994), oral epithelial cells, and salivary pellicles, via the type 2 fimbriae which can show subtle structural variations (Strömberg et al. 1992). The type 1 fimbriae on *A. naeslundii* genospecies 2 also promote attachment to tooth surfaces through interaction with acidic proline-rich proteins and statherin (Gibbons et al. 1988) and collagen (Liu et al. 1991). Data (Li and Bowden 1995) on in vitro biofilms of *A. naeslundii* suggest that biofilms of these organisms are significantly more resistant to removal by shear forces than streptococci, suggesting a role for *Actinomyces* in maintaining the integrity of oral biofilm communities.

PATHOGENICITY AND VIRULENCE

Traditionally, actinomycotic infections in humans have been viewed as mixed, involving predominantly *A. israelii* and *A. gerencseriae* with *A. naeslundii*, *A. viscosus* (*A. naeslundii* genospecies 2), and *A. odontolyticus* playing minor roles (Slack and Gerencser 1975; Bowden 1984; Schaal and Beaman 1984; Schaal 1986). The lesions of actinomycosis occur most commonly in the cervicofacial region, perhaps reflecting the oral habitat of *Actinomyces*, although infections may occur at any site in the body (Schaal 1986; Leslie and Garland 1991). *A. meyeri* and some of the more recently described *Actinomyces* spp. can also be isolated from abscesses in different areas of the body.

The pathogenesis of actinomycotic infections is based on the concept of an endogenous etiology with hematogenous spread of the organisms from the oral cavity (Bowden 1984). However, it now seems likely that *A. israelii* can persist in the genital tract of healthy females (Persson and Holmberg 1984a) and this could be the source of the organism for infections at this site. Although some of the more recently described *Actinomyces* spp. are isolated from mixed infections (e.g. *A. turicensis*, *A. radingae*), they can also occur alone (Sabbe et al. 1999).

Actinomyces spp. have also been proposed to have a role in two widespread human diseases, root surface caries, and periodontal disease. There is an extensive literature on the bacterial etiology of periodontal diseases (Holt and Bramanti 1991; Moore et al. 1991; Socransky and Haffajee 1992) and although some *Actinomyces* spp. may be found in higher numbers in association with lesions, the majority are not suggested to play a direct role (Dzink et al. 1988; Moore et al. 1991). In periodontal diseases, *Actinomyces* spp. are thought to contribute to plaque development and perhaps through the increase in plaque mass, to gingivitis. Also, through coaggregation they promote colonization of the gingival crevice by gram-negative anaerobic putative periodontal pathogens (Slots and Gibbons 1978; Ellen et al. 1988). In root surface caries, *Actinomyces* spp. are found in samples from lesions (Bowden 1990; van Houte et al. 1994) and one experimental model has shown that *Actinomyces* spp. can dominate the flora of lesions in root tissue (Nyvad and Kilian 1990).

Infections resembling actinomycosis occur in a variety of animals (Slack and Gerencser 1975; Schaal 1986), although the causative organisms have not always been isolated. The most well known is bovine actinomycosis or 'lumpy jaw'; the major pathogen is *A. bovis*, but *A. israelii* can cause bovine infections. *A. viscosus* has been reported to cause infections in cats and dogs, although *A. hordeivulneris* seems to be particularly associated with canine actinomycosis. Although *A. suis* (Ludwig et al. 1992), previously *E. suis*, is a well known pathogen in pigs, it has phenotypic characters distinct from the original description by Franke (1973). Another porcine pathogen, *A. hyovaginalis*, has been described from purulent vaginal discharges and aborted fetuses (Collins et al. 1993).

Experimental infections have been produced in a wide range of animals (Slack and Gerencser 1975), but mice seem to be the most reliable in terms of susceptibility (Behbehani and Jordan 1982). A guinea pig groin (Grenier and Mayrand 1983) and implanted chamber models (Figdor et al. 1992) have been used to study infections with *A. israelii*. This organism will usually produce lesions typical of actinomycosis, but the mice may not die and the infection usually resolves. *A. naeslundii*, *A. viscosus*, and *A. odontolyticus* may produce abscesses in mice, but they are less aggressive than *A. israelii*. *A. viscosus* and *A. naeslundii* have been associated with periodontal tissue destruction and root caries in experimental infections.

Those *Actinomyces* that have been tested in experimental animals cannot be regarded as particularly virulent pathogens. There has been considerable discussion on the role that ancillary organisms may play in enhancing the virulence of *Actinomyces*. Some studies have suggested nutritional interactions (Mayrand and McBride 1980), others that the granules of *Actinomyces* play a protective role (Jordan and Kelly 1983; Figdor et al. 1992) or that gram-negative organisms enhance the survival of *Actinomyces* (Jordan et al. 1984). In one study of experimental mixed infections, *A. israelii* was a component of mixtures of bacteria that produced abscesses, but it was not essential for abscess formation (Grenier and Mayrand 1983).

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Bifidobacterium, *Eubacterium*, and *Propionibacterium*

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BIFIDOBACTERIUM

GENUS DEFINITION

Bifidobacterium spp. are pleomorphic gram-positive rods showing true and false branching. They are nonmotile and nonsporing. Most species are strictly anaerobic; they grow between 20 and 45°C with optimum growth at 38°C. The organisms are aciduric, but killed by heat at 60°C in 5 min. They ferment various carbohydrates with production of acetic and lactic acids in the ratio 3:2. CO_2 is not produced. Glucose metabolism is characteristically and exclusively by the fructose-6-phosphate shunt. There is no proteolytic activity. They do not form oxidase, indole, or H_2S and are generally nonpathogenic to man and animals. *Bifidobacterium* spp. are normal flora of the mouth and intestine. The G + C content of the DNA is approximately 60 mol%. The type species is *Bifidobacterium bifidum*.

INTRODUCTION AND HISTORICAL PERSPECTIVE

The first member of this genus to be recognized was isolated from infants' stools by Tissier (1900), who called it *Bacillus bifidus*. The classification of the bifidobacteria has presented difficulties, but most workers consider that the bifidobacteria should be classed in a genus of their own, as suggested by Orla-Jensen (1924). This has been confirmed by phylogenetic studies that have demonstrated that the bifidobacteria are confined to a single deep cluster within the phylum *Actinobacteria* (Cole et al. 2003). Furthermore, all the bifidobacteria share the property peculiar to them among the nonsporing gram-positive anaerobes of degrading glucose by the fructose-6-phosphate shunt. Species definitions have been the subject of extensive revision. There are currently 28 species, ten of which have been isolated from humans. Bifidobacteria make up a high proportion of the bacteria in the gut flora of human infants and adults. The protective effects of bifidobacteria against enteric infection and cancer are now widely appreciated and the inclusion of bifidobacteria in probiotic products is widespread.

HABITAT

Bifidobacteria are found in the human mouth, the lower gut of humans and animals, and in sewage; they are occasionally isolated from clinical material.

MORPHOLOGY

A wide variety of cell morphologies are displayed and even a single strain may appear different under different cultural conditions. The classic bifid club-shaped protusions are shown by most species under conditions of nutrient limitation. Otherwise, morphologies range from the large, curved, irregular-shaped cells of *B. bifidum* and the palisade arrangement of *Bifidobacterium angulatum* to the characteristic star-like clusters of *Bifidobacterium asteroides*. Although it has been suggested that species identification is possible by examination of cell morphology (Scardovi 1986), this is not recommended without substantial experience of the genus.

ULTRASTRUCTURE

Electron microscopic examination of the ultrastructure of *B. bifidum* and *Bifidobacterium adolescentis* revealed some unusual features (Novik et al. 1994). Strains of both species exhibited an intracytoplasmic membrane complex containing lamellar, myelin-like, and vesicular structures. There were also numerous polysaccharide and polyphosphate inclusions. Ultrastructural reorganization was seen in ageing cultures with hypersynthesis of cell wall material, an increase in complexity and volume of the intracytoplasmic membrane complex together with structural changes to the nucleoid.

CULTURAL CHARACTERISTICS

Most species are strict anaerobes, but a few species, isolated from animals or bees, will tolerate O_2 in the presence of added CO_2, growing in 90 percent air + 10 percent CO_2.

METABOLISM

Biochemically, one of the more striking features of bifidobacteria is the formation of acetic acid in addition to lactic acid during the fermentation of glucose. This property, and the failure to form detectable gas, separate the bifidobacteria from the heterofermentative group of lactobacilli (Beerens et al. 1957). The absence of propionic acid from the products of fermentation separates *Bifidobacterium* from *Propionibacterium*. Glucose is utilized by the fructose-6-phosphate shunt, and detection of fructose-6-phosphate phosphoketolase is the most reliable test for assigning an organism to this genus (for method, see Scardovi 1986). Urease production is uncommon among human isolates, but ureolytic strains occur in all species. Animal strains of *Bifidobacterium longum* are most often strongly ureolytic. The catalase reaction is usually negative, but some oxygen-tolerant strains liberate O_2 from H_2O_2 when grown in air + 10 percent CO_2. Strains do not reduce nitrate. Many of the bifidobacteria isolated from the human gut are able to hydrolyze cholic acid and conjugated bile acids such as sodium glycocholate and sodium taurocholate (Drasar and Hill 1974; Ferrari et al. 1980).

CELL WALL COMPOSITION; ANTIGENIC STRUCTURE

Studies of cell wall murein of bifidobacteria have shown that the amino acid composition of the peptide side chains can be useful in species identification. However, other chemotaxonomic techniques, such as the analysis of cellular fatty acids and phosphoglycerides are not able to differentiate species (Exterkate et al. 1971; Veerkamp 1971).

The antigenic structure is complex and most workers have found a fairly high degree of strain specificity by agglutination reactions. The antigens taking part in these reactions are heat stable; the results are the same whether living or boiled organisms are used for the preparation of antisera. Unlike the lactobacilli, bifidobacteria do not seem to contain an extractable precipitinogen.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Bifidobacteria are uniformly susceptible to benzylpenicillin, the macrolides and lincosamines, chloramphenicol

and vancomycin, but are usually resistant to the aminoglycosides, nalidixic acid, and metronidazole.

CLASSIFICATION

Study of the DNA composition reveals a G + C content of 57–64 mol%, thus clearly differentiating the genus from *Lactobacillus* (33–53 percent G + C) and *Propionibacterium* (57–68 percent G + C). Substantial revision of the classification has taken place in recent years, based on phenotypic data augmented by genetic studies including phylogenetic analysis of 16S rRNA and other gene sequences and DNA:DNA relatedness. *B. bifidum* and *B. adolescentis* have been shown to form distinct genetic groups. The species, *B. dentium*, *B. angulatum*, *B. catenulatum*, and *B. pseudocatenulatum*, which are difficult to distinguish from *B. adolescentis* on the basis of fermentation patterns, are genetically distinct from each other and from *B. adolescentis*. The species *B. longum, B. infantis*, and *B. suis* have been unified as a single species, *B. longum,* divided into three biotypes (Sakata et al. 2002). At present, 28 species in all are recognized (Table 1). Of these, ten (*B. bifidum*, *B. breve*, *B. gallicum*, *B. longum*, *B. adolescentis*, *B. catenulatum*, *B. dentium*, *B. angulatum*, *B. pseudocatenulatum*, and *B. scardovii*) have been isolated from the human mouth, feces, urine, or vagina; 12 other species have been found in the intestines and feces of other vertebrate animals, including pigs, cattle, chickens, and rabbits. Three species are unique to the intestine of the honey bee, two species have been found only in sewage and one species has been isolated from an anaerobic digester.

Two species, *B. inopinatum* and *B. denticolens*, isolated from human dental plaque were proposed by Crociani et al. (1996) but were subsequently found, on the basis of HSP60 gene sequence relatedness, to each represent novel genera, and were therefore transferred to the genera *Scardovia* and *Parascardovia*, respectively (Jian and Dong 2002).

Table 1 *Current species of* 'Bifidobacterium'

Species	Typical sites of isolation
B. adolescentis	Human adult feces
B. angulatum	Human adult feces, sewage
B. catenulatum	Human adult and infant feces
B. gallicum	Human adult feces
B. longum	Human adult and infant feces, vagina
B. bifidum	Human adult and infant feces, calf feces
B. breve	Human infant feces, human vagina, calf feces
B. pseudocatenulatum	Human adult and infant feces, calf feces
B. dentium	Human dental caries, feces, vagina, miscellaneous human infections including abscesses, and wound infections
B. scardovii	Human adult clinical material
Parascardovia denticolens	Human dental caries
Scardovia inopinata	Human dental caries
B. merycicum	Bovine rumen
B. ruminantium	Bovine rumen
B. boum	Bovine rumen, pig feces
B. thermophilum	Bovine rumen, pig, and calf feces
B. pseudolongum	Bovine rumen, pig feces
B. animalis	Chicken, rat, rabbit feces
B. gallinarum	Chicken feces
B. pullorum	Chicken feces
B. asteroides	Bee intestine
B. indicum	Bee intestine
B. coryneforme	Bee intestine
B. choerinum	Pig feces
B. cuniculi	Rabbit feces
B. magnum	Rabbit feces
B. saeculare	Rabbit feces
B. minimum	Sewage
B. subtile	Sewage
B. thermacidophilum	Anaerobic digester

LABORATORY ISOLATION AND IDENTIFICATION

The nutritional requirements of bifidobacteria are still imperfectly known. Since the discovery that milk stimulated the growth of *B. bifidum*, considerable attention has been given to the study of 'bifidus factors' in human milk, but these studies have added little to our knowledge (Poupard et al. 1973). Bifidobacteria are very heterogeneous in their requirements for growth factors and vitamins; riboflavin and pantothenate are the most common requirements, but nicotinic acid, pyridoxine, thiamine, folic acid, and p-aminobenzoic acid may also be needed (Scardovi 1986). Most species are able to use ammonium salts as the sole source of nitrogen, but some species isolated from animals will not grow in the absence of organic nitrogen.

Many different media have been devised for isolating or enumerating the bifidobacteria in feces and sewage. The media contain a carbohydrate such as glucose, lactose, fructose, or maltose and complex mixtures of peptones, yeast extract, growth factors, and reducing agents. Antibiotics, particularly aminoglycosides, have been used to improve selectivity, but no universally satisfactory selective medium is at present available. Nonselective media that give good growth of the strains present in the habitat being studied are to be preferred (see Scardovi 1981).

Fermentation tests have been used traditionally to distinguish individual species. However, some species

such as *B. longum*, *B. dentium*, and *B. adolescentis* show similar patterns of sugar fermentation and this method cannot be used reliably for identification purposes. Biavati et al. (1982) showed that the comparison of protein profiles of soluble whole-cell proteins, using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), was a reliable method for the identification of species. Members of a single species have identical or nearly identical protein patterns. This method has been widely used subsequently both for identification and as a method for the preliminary characterization of new strains for classification purposes. Molecular methods have also been employed for this purpose. Oligonucleotide probes, designed from 16S rRNA sequence data, have been shown to be specific for *B. adolescentis*, *B. breve*, and *B. longum*; probes for *B. bifidum* showed specificity adequate for the identification of isolates from human material (Yamamoto et al. 1992). Extension and refinement of these approaches should permit rapid and even automated identification in the foreseeable future.

Although fermentation tests with simple sugars do not allow discrimination of species (Gavini et al. 1991), the use of complex carbohydrates has been shown by Crociani et al. (1994) to discriminate between species commonly isolated from human specimens (Table 2).

Isoenzyme patterns (of transaldolases and 6-phosphogluconate dehydrogenases) have proved useful for characterizing species of *Bifidobacterium*. Antisera against purified transaldolases have been used to distinguish groups of species from different habitats (Sgorbati and London 1982). Furthermore, β-galactosidase electrophoretic patterns successfully differentiated animal and human species of bifidobacteria and were more discriminatory than numerical analysis based on 45 tests in a comparative study (Roy et al. 1994).

Table 2 *Key for differentiation of* Bifidobacterium *species of human origin (Crociani et al. 1994)*

Substrate						
α-L-F	D-G	Ar	Ga	Gu	Am	Suggested species
+	+					*B. longum*
	–					*B. breve*
						(*B. pseudocatenulatum*)
–	–	+				*B. longum*
		–	+			*B. bifidum*
			–	+		*B. dentium*
				–	+	*B. adolescentis*
						(*B. pseudocatenulatum*)
					–	*B. catenulatum*

α-L-F, α-L-fucose; D-G, D-glucuronate; Am, amylopectin; Ar, arabinogalactan; Ga, gastric mucin; Gu, gum guar.
Species in parentheses is less likely. *B. angulatum, B. gallicum*, and *B. scardovii* are not included because of the low number of strains available.

ROLE IN NORMAL FLORA OF HUMANS

Bifidobacteria are reported to play an important regulatory role in the large intestine, controlling pH and protecting against infection by exogenous pathogens, particularly in infants (Modler et al. 1990; Saavedra et al. 1994). It has further been proposed that bifidobacteria may offer protection against cancer, not only of the lower gut but also at other body sites (Reddy and Rivenson 1993). These findings have led to considerable interest in the use of bifidobacteria in probiotics and a number of dairy products, which include bifidobacteria, are being developed (Isolauri et al. 2002).

The species most often found are: in babies, *B. longum* and *B. breve*, and in adults, *B. adolescentis*, *B. longum*, and *B. pseudocatenulatum* (Dehnert 1957, 1961; Reuter 1963; Beerens et al. 1980; Biavati et al. 1984; Mitsuoka 1984). *B. dentium* is part of the normal oral flora and can be isolated readily from dental plaque (Biavati et al. 1982).

PATHOGENICITY AND VIRULENCE FACTORS

B. dentium, *S. inopinata*, and *P. denticolens* are associated with dental caries and periodontal disease although whether they play a pathogenic role in these diseases is unknown (Scardovi and Crociani 1974; Moore et al. 1983; Crociani et al. 1996). *B. dentium* has also been isolated from clinical specimens obtained from a variety of sites. These are principally mixed infections, typically abscesses or wound infections around the head and neck area or the lungs. *B. dentium* can probably be classed as an opportunist pathogen like many other members of the oral microflora. Other bifidobacteria that have been isolated from clinical material, albeit rarely, include *B. longum*, *B. adolescentis*, *B. breve*, and *B. scardovii*, recovered from abscesses, urinary tract infections, and septicemia (Darbas et al. 1991; Hoyles et al. 2002).

BIFIDOBACTERIUM BIFIDUM

B. bifidum is common in the feces of breast-fed and bottle-fed infants and in the feces of adult humans and animals. It is nonpathogenic to man and laboratory animals. The G + C content of DNA is c. 60.1 mol% and DNA–DNA homology studies show *B. bifidum* to be a distinct species related to, but separate from, other bifidobacteria. It is a slender bacillus, approximately 4 μm long and 0.7 μm wide, with tapering, pointed ends. Arranged in pairs end to end, with the distal ends pointed and the proximal ends swollen, they generally lie parallel to one another, rarely intertwined. Two or three bacilli often radiate from a single point, forming a Y-shaped structure; clubbed forms and forms ending in knobs are not uncommon. They are often arranged

in palisades or Chinese letters. The general appearance is not unlike a diphtheroid bacillus. In culture, its morphology varies with the medium. Its characteristic appearance is that of a highly pleomorphic bacillus showing irregularly clubbed forms with branching often at both ends. Geniculate forms, forms ending in knobs, forms with lateral buds, bladder forms, and candle-flame forms may be seen. The absence of chain formation and long filaments is noteworthy. The species is nonmotile and nonsporing, stains uniformly in young cultures, but in older cultures irregular or granular staining is not uncommon. It is gram-positive in young cultures; later gram-negative forms appear. *B. bifidum* is non-acid fast. For a description of the morphology with illustrations, see Orla-Jensen (1943), Dehnert (1957, 1961), and Scardovi (1986).

Ammonium salts are utilized as sole source of nitrogen, but good growth is obtained on peptone–yeast extract–glucose agar with added cysteine and Tween 80 (Scardovi 1981). No growth occurs on nutrient agar. On glucose agar and human milk agar incubated anaerobically, colonies are usually low convex, grayish-brown, lenticular or ovoid, 0.5–2 mm in diameter, showing under the microscope a delicately granular structure, a brownish opaque center, a finer translucent periphery and a finely crenated edge. In glucose broth growth is moderate; in 3 or 4 days the organisms fall to the bottom of the tube, forming an abundant loose flocculogranular deposit, easily disintegrated on shaking. Very slight or no growth occurs at 20°C; optimum temperature is 38–39°C; upper limit is 45°C. Optimum pH is 6.5–7.0. The organism grows only in the absence of O_2; CO_2 is beneficial. Growth is improved by extracts of liver, yeast, and feces (Orla-Jensen 1943). Some strains are stimulated by 'bifidus factor' present in human milk. The organism requires riboflavin, nicotinic acid, folic acid, and p-aminobenzoic acid for growth; it is stimulated by Tween 80.

Glucose, lactose, and galactose are fermented; in addition, most strains ferment fructose and some attack sucrose, maltose, or melibiose. The addition of Tween 80 improves reliability of fermentation reactions. D-Lactic acid and acetic acid are the main end-products of metabolism. Oxidase, catalase, urease, indole, and H_2S are not produced, nitrate is not reduced, and arginine is not hydrolyzed.

In fluid cultures the organisms often die within 3 days. They are killed by heat at 55°C in 30 min and at 60°C in 5 min. They are resistant to acids; for preservation they should be subcultured weekly or freeze-dried.

The antigens are, as yet, incompletely studied. Cells are agglutinated by sera prepared against live or heat-killed organisms. The organism belongs to immunological specificity group B on the basis of its reaction with antitransaldolase sera (Sgorbati and London 1982).

EUBACTERIUM

GENUS DEFINITION

Eubacteria are uniform or pleomorphic gram-positive rods, motile or nonmotile, and nonsporing. They are strictly anaerobic and grow between 30 and 37°C. They may ferment various carbohydrates or peptone to produce mixtures of organic acids; gas is usually produced. They do not produce:

1. the large amounts of propionic acid characteristic of *Propionibacterium*
2. more acetic than lactic acid as does *Bifidobacterium*
3. lactic acid as the sole major product like *Lactobacillus*, or
4. the mixture of succinic and lactic acid formed by *Actinomyces*.

Some species are proteolytic; indole and H_2S may be produced. The G + C content of the DNA is 30–50 mol%. The type species is *Eubacterium limosum*.

INTRODUCTION AND HISTORICAL PERSPECTIVE

The first members of this genus to be described were isolated from the feces of man by Eggerth (1935), who assigned them to the genus *Bacteroides* as a temporary expedient. The genus *Eubacterium* was described by Prévot (1938). Since then, it has become a genus of convenience in that, as implied by its description above, it has become a repository for nonsporing gram-positive anaerobic bacilli that do not correspond to other, better defined, genera. *Eubacterium* therefore currently encompasses a wide variety of organisms which fall into a number of phylogenetic clusters. Consequently, extensive taxonomic revision of the genus is required.

HABITAT

Eubacterium species are found in the mouths and intestines of humans and animals, in the rumen, in abscesses and soft tissue infections, in the environment in soil and in extreme conditions.

MORPHOLOGY

The cell morphology is extremely variable, ranging from small coccobacilli to large filamentous bacilli. Cells often stain gram-variable.

CULTURAL CHARACTERISTICS

Growth is extremely variable but in the main eubacteria tend to be fastidious and many species are extremely

slow growing. *Eubacterium* spp. found in clinical specimens can be isolated on complex media suitable for anaerobes (Moore and Holdeman Moore 1986). Extended incubation of at least 7 days is required by the majority of species. All eubacteria are strict anaerobes; some are extremely sensitive to oxygen and require pre-reduced and anaerobically sterilized media for growth. The growth of a number of species found in the mouth and in clinical specimens is enhanced by the inclusion of arginine or lysine, or both, in culture media (Hill et al. 1987).

METABOLISM

As might be expected given such a diverse group, metabolism is extremely variable. Saccharolytic, nonfermentative, and proteolytic species have been described. A wide variety of patterns of production of volatile and nonvolatile fatty acids is seen. Some species reduce nitrate. *Eubacterium budayi* and *Eubacterium tenue* produce lecithinase (Moore and Holdeman Moore 1986). Some rumen strains such as *Eubacterium cellulosolvens*, *Eubacterium uniforme*, and *Eubacterium xylanophilum* can degrade fiber (van Gylswick and van der Toorn 1985, 1986). *E. limosum* is a versatile organism that can degrade amino acids, lactate, and methanol; conversely, this species can grow autotrophically on hydrogen and carbon dioxide or just carbon monoxide (Genthner et al. 1981; Genthner and Bryant 1982). For the majority of species, however, metabolic pathways are unknown.

CELL WALL COMPOSITION; ANTIGENIC STRUCTURE

The chemical composition of cell wall peptidoglycans shows wide variation between species and includes meso-diaminopimelic acid direct, ll-diaminopimelic acid–glycine types, and a type where peptide bridges are connected to the D-glutamic acid residue (Andreesen 1992), although relatively few species have been examined.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

In general, *Eubacterium* species are susceptible to all the major classes of antimicrobial agents. A detailed description of antimicrobial susceptibility by species is given by Moore and Holdeman Moore (1986).

CLASSIFICATION

The genus *Eubacterium* is extremely heterogeneous. Phylogenetic studies have revealed that *Eubacterium* species are widely distributed among the phyla *Firmicutes* (low G + C gram-positive) and *Actinobacteria* (high G + C) (Collins et al. 1994; Cheeseman et al. 1996; Wade et al. 1999).

Within the phylum *Firmicutes*, *Eubacterium* species are intimately associated with the clostridia, which themselves comprise a genus of considerable complexity. The only criterion for the placing of a species in *Eubacterium* or *Clostridium* is spore formation by the latter. However, individual lines of descent include both sporing and nonsporing species. The reason for this is unknown. Either spore formation is a feature that has emerged a number of times throughout evolution or nonsporing species carry sporulation genes that are either dormant or nonfunctional.

In recent years, a number of new species have been proposed for previously unnamed taxa. In addition, a number of new genera, including *Bulleidia*, *Mogibacterium*, and *Shuttleworthia*, have been proposed for groups of species formerly classified as *Eubacterium*. These changes are summarized in Table 3. Indeed, it has been suggested that *Eubacterium sensu stricto* should be restricted to *E. limosum*, *E. barkeri*, and *E. callanderi* (Willems and Collins 1996). At present, there are 43 species in the genus and, therefore, all but the three species mentioned above (and possibly *E. angustum* and *E. aggregans*) will be reclassified into either existing, or, more commonly, novel genera. A number of the present species are sufficiently different from their nearest relatives to warrant the proposal of monophyletic genera which will add considerably to the complexity of the classification of the *Firmicutes*.

Within the *Actinobacteria*, *Eubacterium lentum* has been renamed *Eggerthella lenta* within the family

Table 3 *Recently proposed species and taxonomic changes to* Eubacterium *and related genera*

Current nomenclature	Previous nomenclature
Actinobaculum suis	*Eubacterium suis*
Atopobium fosser	*Eubacterium fosser*
Bulleidia extructa	New species
Collinsella aerofaciens	*Eubacterium aerofaciens*
Cryptobacterium curtum	New species
Dorea formicigenerans	*Eubacterium formicigenerans*
Eggerthella lenta	*Eubacterium lentum*
Eubacterium aggregans	New species
Eubacterium infirmum	New species
Eubacterium minutum	*Eubacterium tardum*
Eubacterium sulci	*Fusobacterium sulci*
Filifactor alocis	*Fusobacterium alocis*
Holdemania filiformis	New species
Mogibacterium diversum	New species
Mogibacterium neglectum	New species
Mogibacterium pumilum	New species
Mogibacterium timidum	*Eubacterium timidum*
Mogibacterium vescum	New species
Pseudoramibacter alactolyticus	*Eubacterium alactolyticum*
Shuttleworthia satelles	New species
Slackia exigua	*Eubacterium exiguum*

Coriobacteriaceae (Wade et al. 1999). Also within this family are the new genera *Slackia*, *Collinsella*, and *Cryptobacterium*. These genera are closely related to *Atopobium*, a genus which includes organisms that produce lactic acid as their major metabolic end-product and which were formerly classified as *Lactobacillus* (see *Streptococcus* and *Lactobacillus*).

LABORATORY ISOLATION AND IDENTIFICATION

Careful specimen collection, and strict anaerobic transport, processing, and culture are undoubtedly important as is the use of a good nutritious culture medium. Incubation for 7 days is recommended as many species are slow growing. Identification of isolates can be difficult because most species tend to be unreactive in conventional biochemical tests. Schemes for the identification of commonly encountered species are shown in Tables 4 and 5.

A substantial proportion of fresh isolates do not correspond to named species; there remain a large number of, as yet, unnamed taxa. 16S rRNA gene sequence analysis is recommended for definitive identification of described species and phylogenetic placement of novel taxa.

ROLE IN NORMAL FLORA OF HUMANS

Eubacterium spp. comprise a substantial part of the normal flora of the mouth and large intestine (Finegold et al. 1983).

PATHOGENICITY AND VIRULENCE FACTORS

Twenty-two species of this group have been isolated from infections; these include *Collinsella aerofaciens*, *Eubacterium combesii*, *E. contortum*, *E. cylindroides*, *E. limosum*, *E. saburreum*, *E. tenue*, *E. rectale*, *Eggerthella lenta*, and *Pseudoramibacter alactolyticus*; *Eggerthella*

Table 4 *Differential characteristics of saccharolytic* Eubacterium *and related species encountered in human infections*

Species	Lactose	Maltose	Mannose	Sucrose	Indole	Nitrate reduction	Starch hydrolysis	Esculin hydrolysis	Metabolic end-products in PYG[a]
Bulleidia extructa	–	+	–	–	–	–	ND	–	a, L, (s)
Collinsella aerofaciens	V	+	+	V	–	–	–	V	A, F, L
Eubacterium contortum	V	+	V	+	–	–	–	+	A, f
E. cylindroides	–	–	+	V	–	–	V	+	a, B, L
E. limosum	–	V	–	–	–	–	V	+	A, B, L
E. moniliforme	–	V	+	–	–	V	V	–	a, B, L
E. multiforme	+	–	+	–	–	+	–	+	A, B, L
E. rectale	+	+	V	+	–	–	+	+	(a), B, L
E. saburreum	V	V	V	+	+	–	–	+	a, b, l
E. tenue	–	–	–	–	+	–	V	–	A, f
Pseudoramibacter alactolyticus	–	–	–	–	–	–	–	–	A, B, C

a, acetic acid; b, butyric acid; c, caproic acid; f, formic acid; l, lactic acid; ND, not determined; s succinic acid; V, variable reaction. Capital letters indicate major products.
a) PYG, peptone–yeast glucose broth.

Table 5 *Differential characteristics of asaccharolytic* Eubacterium *and related species encountered in human infections*

Species	Arginine hydrolysis	Nitrate reduction	Growth stimulated by arginine	Metabolic end products in PYG[a]	Rapid ID32A profile
Cryptobacterium curtum	+	–	+	None	2000 $012^{4}/_{0}00$
Eggerthella lenta	+	+	+	(a)	2000 0000 00
Eubacterium brachy	–	–	–	ib, iv, ic, (a, s)	0000 0000 00
E. combesii	–	–	ND	A, ib, B, iv, l	ND
E. minutum	–	–	–	B	0000 0120 01
E. nodatum	+	–	+	a, B	0000 0000 00
E. saphenum	–	–	+	a, b	0000 0000 00
E. sulci	–	–	–	a, B	0000 0400 00
Mogibacterium timidum	–	–	–	PAA	0000 0200 00
Slackia exigua	+	–	+	None	2000 $0^{2}/_{3}$37 05

a, acetic acid; b, butyric acid; ib, isobutyric acid; ic, isocaproic acid; iv, isovaleric acid; l, lactic acid; ND, not determined; paa, phenylacetic acid; s, succinic acid; V, variable detection. Capital letters indicate major products.
a) PYG, peptone-yeast glucose broth

lenta is the most frequently seen (Lewis and Sutter 1981; Sutter et al. 1986). *Eubacterium* spp. are typically recovered as part of a mixed flora from abscesses at various body sites. There have been several reports of the isolation of *Eubacterium* spp. from oral infections but because, in general, they are slow growing and have fastidious nutritional requirements, they have not been widely studied (Downes et al. 2001). Moore et al. (1982, 1983) found that *Eubacterium brachy*, *E. nodatum*, *E. saburreum*, *Mogibacterium timidum*, *Pseudoramibacter alactolyticus*, and 17 unnamed taxa made up a significant proportion of the subgingival microflora in chronic and severe advanced periodontitis. Higher isolation rates were reported by Uematsu and Hoshino (1992) who found that the genus made up 54 percent of the anaerobic flora of periodontal pockets. In addition, *Eubacterium* spp. are regularly isolated from carious dentine and endodontic and periapical infections (Hoshino 1985; Sundqvist 1992; Wasfy et al. 1992; Sato et al. 1993). Furthermore, oral asaccharolytic *Eubacterium* spp. are found in infections at a range of non-oral sites, albeit mainly in the head and neck region (Hill et al. 1987).

VIRULENCE FACTORS

The numerical associations between *Eubacterium* spp. and oral infections are strong. However, whether these bacteria play a causative role or merely colonize diseased sites is unknown. Because the majority of oral *Eubacterium* spp. have been described only recently, little work has been carried out in investigating these organisms for possible virulence factors. Since these organisms are always found in mixed infections, they may act in conjunction with other bacteria in the degradation of host tissues.

EUBACTERIUM LIMOSUM

This organism is isolated from the intestine of humans and vertebrate animals, including rats, poultry, and fish, and from sewage-sludge and mud. It may be isolated from wounds and abscesses, usually in mixed culture.

E. limosum is a gram-positive, nonmotile, irregular, club-shaped rod with occasional bifurcations; it may be gram-negative in old cultures. Ammonium salts are used as the main source of nitrogen. Good growth occurs on peptone–yeast extract agar or in peptone–yeast extract broth. Growth is often improved by the presence of fermentable carbohydrate. On blood agar incubated anaerobically, colonies are usually circular, entire, convex, up to 2 mm in diameter and translucent or slightly opaque. Growth in glucose broth is good, the broth becoming turbid with a smooth or viscous deposit. Slight or no growth occurs at 25°C; optimum temperature is 37°C; upper limit is 45°C. Glucose, fructose, mannitol, erythritol, and ribose are fermented; some strains attack maltose, mannose, and xylose. Acid and gas are produced. Major end-products of metabolism are acetic, butyric, and lactic acids together with CO_2 and H_2. Gelatin, but not meat, is digested by some strains. Indole is not produced. Nitrate is not reduced. The G + C content of DNA is 47 mol%.

For further information, see Moore and Cato (1965) and Moore and Holdeman Moore (1986).

EGGERTHELLA LENTA

Eggerthella lenta is isolated from human feces and from blood, postoperative wounds, and abscesses, commonly in mixed culture. It is a gram-positive, nonmotile coccobacillus. Growth is limited by the availability of arginine. On blood agar incubated anaerobically, colonies are circular, entire, 0.5–2 mm in diameter. Growth in glucose broth is moderate with a slight sediment. Carbohydrates are not fermented. Energy is obtained by the arginine dihydrolase pathway (Sperry and Wilkins 1976). Acetate is the major end-product of metabolism with trace amounts of lactate and succinate. Bile acids and corticosteroids are metabolized. The ability to metabolize digoxin may be clinically significant (Dobkin et al. 1983).

For more information, see Moore et al. (1971) and Moore and Holdeman Moore (1986).

PROPIONIBACTERIUM

GENUS DEFINITION

Propionibacterium spp. are pleomorphic gram-positive rods, 0.5–0.8 μm in diameter by 1–5 μm in length. Cells are club-shaped with one end rounded and the other tapered, and typically arranged in short chains or clumps. The species are anaerobic to aerotolerant and grow optimally in a temperature range of 30–37°C. Most isolates are nonmotile and catalase-positive. Carbohydrates are fermented with the production of large amounts of propionic and acetic acids and lesser amounts of isovaleric, formic, succinic, or lactic acids. The G + C content of the DNA ranges from 57 to 68 mol%. Eleven species and two subspecies are recognized (Table 6) with *P. freudenreichii* the genus type species.

INTRODUCTION AND HISTORICAL PERSPECTIVE

The original *Propionibacterium* species were described in cheese and dairy products by von Freudenreich and Orla-Jensen in 1906. Other species have been subsequently isolated in other fermented products and produce (e.g. silage, olives, orange juice). These species are referred to as dairy propionibacteria. A second group of *Propionibacterium* species, the cutaneous

Table 6 Propionibacterium *species*

Species	Source
P. acidipropionici	Isolated from dairy products
P. acnes	Isolated on skin
P. australiense	Isolated from granulomatous bovine lesions
P. avidum	Isolated on moist areas of skin
P. cyclohexanicum	Isolated from spoiled orange juice
P. freudenreichii subsp. *freudenreichii* subsp. *shermanii*	Isolated from dairy products
P. granulosum	Isolated from sebum-rich areas of skin
P. jensenii	Isolated from dairy products and silage
P. microaerophilum	Isolated from olive mill waste water
P. propionicum	Isolated from mouth, intestines, and vagina
P. thoenii	Isolated from dairy products

species, have been isolated on the surface of the skin and, less commonly, on mucous membranes. The four species in this group, *P. acnes*, *P. avidum*, *P. granulosum*, and *P. propionicum*, are associated with human colonization and disease.

HABITAT

P. acnes is one of the most common microbes on the surface of the human skin, appearing after puberty in the lipid-rich areas of the skin surface. The primary site of growth is the sebaceous gland (Leeming et al. 1984) and initiation of colonization is most probably the result of changes in the skin lipid composition, especially in the balance of the C_{18} saturated and unsaturated lipids (Nordstrom and Noble 1985; Patel and Nobel 1993). *P. granulosum* is also found in the sebaceous glands in post-pubescent individuals but generally in much lower concentrations compared with *P. acnes*. *P. avidum* prefers the moist areas of the skin (e.g. axillae, groin) and is much less commonly isolated in the lipid-rich skin areas. *P. propionicum* is a normal inhabitant of the mouth and vaginal surface.

MORPHOLOGY

P. acnes are 0.3–1.3 × 1.0–10 μm pleomorphic club-shaped rods when grown in broth (e.g. peptone yeast glucose (PYG) broth). The cells typically appear in irregular clumps commonly referred to as 'Chinese letter' arrangements. Colonies after growth for 2–3 days on blood agar plates are small (0.5 mm), circular, translucent to opaque, and typically white to gray, although pigmentation may appear after extended incubation. *P. avidum* grow slightly faster on blood agar plates and appear circular, white to light cream in color, and are generally β-hemolytic on sheep, human, and rabbit blood. *P. granulosum* grow better than *P. acnes* on blood agar plates with 1 mm or larger colonies observed after 2–3 days. Colonies appear white or grayish, smooth, and circular. *P. propionicum* are short rods, 0.2–0.8 × 1–5 μm with branching filamentous forms occasionally observed. Very small, filamentous, rough or smooth, white to gray–white colonies are observed on blood agar.

CULTURAL CHARACTERISTICS

P. acnes grows best on initial isolation in an anaerobic atmosphere although poor growth can be observed on media incubated in a CO_2-enriched aerobic atmosphere. Aerobic growth is improved following subculture. *P. avidum* is anaerobic or microaerophilic when initially isolated but can grow equally well in an aerobic or anaerobic atmosphere after a few subcultures. *P. granulosum* is anaerobic or microaerophilic, with poor or no growth aerobically. *P. propionicum* is a facultative anaerobe that grows best under anaerobic conditions with an optimum temperature of 35–37°C. Growth is not enhanced by carbon dioxide.

CELL WALL COMPOSITION

The cell wall peptidoglycan of *P. propionicum* contains either diaminopimelic acid (DAP) or lysine (Charfreitag et al. 1988). Most cutaneous *Propionibacterium* species possess cellular L-DAP, but a minority of *P. acnes* and *P. avidum* have *meso*-DAP. The G + C mol% of dairy propionibacteria is 65–68 mol%; however, the skin species tend to be lower: *P. acnes* 57–60 mol%, *P. avidum* 62–63 mol%, *P. granulosum* 61–63 mol%, and *P. propionicum* 63–65 mol%. The chief fatty acids are C_{15} unsaturated (Moss et al. 1967; Cummings and Moss 1990).

LABORATORY ISOLATION AND IDENTIFICATION

Propionibacterium species are generally slow-growing and require incubation for 2 or more days in an anaerobic atmosphere before growth is observed. Growth is best on blood supplemented agar media or enriched broth cultures. Differentiation of cutaneous species can be accomplished with a limited number of tests (Table 7). Demonstration of catalase production in *P. acnes* requires exposure of the colonies to oxygen for 30 min before testing.

Table 7 *Differential characteristics of cutaneous* Propionibacterium *species*

Species	Catalase	Acid from		Hydrolysis of		Indole production	Reduction of nitrate	Hemolysis
		Sucrose	Maltose	Esculin	Gelatin			
P. acnes	+	–	–	–	+	+	+	–
P. avidum	+	+	+	+	+	–	–	+
P. granulosum	+	+	+	–	–	–	–	–
P. propionicum	–	+	+	–	–	+	–	–

PATHOGENICITY, DISEASE, AND VIRULENCE FACTORS

The presence of *P. acnes* in sebaceous glands is important in the pathogenesis of acne vulgaris, although the mechanism has not been fully determined. *P. acnes* produces a variety of hydrolytic enzymes including proteases, neuraminidase, hyaluronidase, and lipases, which stimulate a localized inflammatory response. For example, lipase hydrolyzes triglycerides in sebum and frees fatty acids that can act as irritants to induce inflammation (Smith and Williams 1984). With the presence of inflammatory cells in the surrounding dermis, erythematous papules, pustules, and cysts characteristic of acne are produced (Greer 1985). Other factors that might be important in the inflammatory response involved in acne include the activation of complement, production of serum-independent chemotactic factors, and stimulation of lysosomal enzyme release from neutrophils (Brooks and Frazier 1991). The success in treatment of acne vulgaris has been correlated with decreasing the amount of sebum produced, decreasing the amount of hyperkeratosis within the follicular ducts and decreasing the numbers of *P. acnes* within the follicles.

P. acnes has also been associated with a variety of other clinical conditions including endocarditis involving both native and prosthetic valves, central nervous system shunt infections, chronic meningitis, brain abscess, subdural empyema, contact lens-associated conjunctivitis, endophthalmitis, dental and oral infections, pleuropulmonary infections, septic arthritis, osteomyelitis, and a number of other infections. The most common predisposing factors or conditions in patients with *P. acnes* infections other than acne vulgaris are the presence of foreign bodies, diabetes mellitus, and prior surgery or other trauma.

The other species of propionibacteria are primarily associated with opportunistic infections. *P. granulosum* has been found in lesions of the skin and deeper tissues and has been associated with septicemia in immunocompromised patients (Branger et al. 1987), although its role in disease is more difficult to define. *P. avidum* has been occasionally reported from deep lesions such as splenic abscess (Dunne et al. 1986), and *P. propionium* has been associated with infections of the lacrimal duct (Brazier and Hall 1993; Csukas et al. 1993).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Prior to the use of topical antibiotics for the treatment of *P. acnes* infections, most strains were susceptible to macrolides, tetracyclines, penicillins, and cephalosporins. Resistance to aminoglycosides was commonplace (Hoffler et al. 1980). Following the introduction of topical tetracyclines, macrolides, and clindamycin for treatment of *P. acnes* skin infections, there was a sharp rise in resistance to these antibiotics (Leyden et al. 1983; Eady et al. 1993). Currently, 30 percent of strains are resistant to erythromycin and more than 15 percent are resistant to tetracycline. The mechanism of antibiotic resistance has not yet been determined, but does not appear to reflect the acquisition of genes from other organisms. Nevertheless, erythromycin resistance is phenotypically indistinguishable from that of the majority of bacteria where resistance is due to methylation of the 23S ribosomal RNA, because most resistant *P. acnes* strains are inducibly or constitutively resistant to macrolide, lincosamide, and streptogramin B antibiotics (Eady et al. 1989a, b).

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Clostridium botulinum and *Clostridium tetani*

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GENUS AND SPECIES DEFINITIONS

The genus *Clostridium* has traditionally been classified as anaerobic or aerotolerant rod-shaped, fermentative bacteria that form endospores and obtain energy for growth by fermentation. The spores are usually wider than the vegetative organisms in which they are formed, imparting spindle shapes, the characteristic clostridium forms. The genus *Clostridium* is a large and diverse group of prokaryotes. A variety of taxonomic methods including mol% guanine and cytosine (G+C), DNA homology, and 16S rRNA studies (Cato et al. 1986; Cato and Stackebrandt 1989; Johnson and Francis 1975) indicates that the genus *Clostridium*, as currently classified, is an extremely heterogeneous group of bacteria characterized by their anaerobic metabolism and spore formation. The ninth edition of *Bergey's manual of systematic bacteriology* (Cato et al. 1986) listed 83 species in the genus *Clostridium*. The most recent tabulation of *Clostridium* species has brought the number to 177 (DSMZ 2003). Collins et al. (1994) suggested that five new genera of spore-forming rods be named: *Calormator*, *Filifactor*, *Moorella*, *Oxobacter*, and *Oxalophagus*. *Clostridium* has become one of the largest bacterial genera in numbers and diversity of species and is clearly in need of taxonomic refinement (Collins et al. 1994; Finegold et al. 2002b). A difficulty in resolving the heterogeneity within the genus is that 16S rRNA gene sequences do not appear to be adequate alone in distinguishing genera, and it is difficult to find genetic and phenotypic characters that enable rapid discrimination among genera and species.

In Collins et al.'s comprehensive paper (1994), 16S rRNA gene sequences of 129 clostridia and 69

representative species of other low G+C content gram-positive genera were used for delineation of relatedness. The analysis of the 16S rRNA gene sequences indicated that the 'clostridia' divided into 19 clusters. Most of the medically important species including neurotoxigenic *C. botulinum*, *C. baratii*, *C. butyricum*, and *C. tetani* reside in cluster I (*Clostridium* group I), which is very similar to the rRNA group I of Johnson and Francis (1975). Other clostridia causing intestinal or wound infections such as *C. difficile*, *C. histolyticum*, and *C. sordellii* reside in different clusters. Three 'clostridial' clusters make up 70 percent of the normal fecal flora of humans (Finegold et al. 2002b). These include the *C. coccoides* subgroup rRNA cluster XIVa, the *C. leptum* subgroup rRNA Cluster IV, and the *Clostridium* subgroup XIX (Finegold et al. 2002b; Collins et al. 1994). It is unknown if many of the species in these groups have the genes and capacity to produce clostridial neurotoxins. Classification based on genetic and protein relatedness may be good indicators of the evolutionary relationships of bacterial groups (Goodfellow and O'Donnell 1993), but it is often not practical to use these methods for routine and rapid identification of pathogenic organisms including clostridia that produce neurotoxins. Most investigators will continue to classify clostridia that produce neurotoxins into only a few species because of the characteristic mode of action and medical importance of these toxins. Recently, the genomic sequences of various clostridia have been published (Brüggemann et al. 2003; Nölling et al. 2001; Shimizu et al. 2002), and several other genome sequences are in progress, which should facilitate an understanding of the clostridia.

Overall, the genus *Clostridium* has a wide range of G+C mol% content of 22–55 mol%, whilst the toxigenic species have a much narrower range of G+C content of 24–29 mol% (Hatheway 1990, 1993; Hatheway and Johnson 1998). The clostridia have common phenotypic properties that have traditionally defined the genus. Designation of a bacterial isolate in the genus *Clostridium* has traditionally depended on the presence of four primary phenotypic characters (Hatheway and Johnson 1998; Hippe et al. 1992): (1) the presence of endospores; (2) an anaerobic energy metabolism not involving electron transport with oxygen as an acceptor; (3) inability to reduce sulfate to sulfide; and (4) a gram-positive cell-wall structure. They generally stain gram-positive, but some strains show a weak or negative Gram stain reaction particularly in later stages of growth. The peptidoglycans of neurotoxigenic clostridia that have been examined is linked by *meso*-diaminopimelic acid (Cummins and Johnson 1971; Schleifer and Kandler 1972; Weiss et al. 1981). *C. botulinum* and *C. tetani* are anaerobic heterotrophs, and many strains vigorously ferment proteins and carbohydrates. The do not perform a dissimilatory sulfate reduction, and this character separates them from *Desulfotomaculum*. The genus *Clostridium* was first proposed by Winslow et al. (Willis 1969, 1990) and adopted in the first edition of *Bergey's manual of determinative bacteriology* in 1923. The type species of the genus is *Clostridium butyricum* (Cato et al. 1986).

One of the most interesting features of the clostridia is their production of a wide diversity of biologically active proteins, many of which have roles in diseases of humans and animals. The clostridia produce more kinds of protein toxins than any other genus of microorganisms (van Heyningen 1950) and more than 20 toxins have been identified (Hatheway 1990). At least 15 species of *Clostridium* are known to produce protein toxins. These agents include neurotoxins, lipases, lecithinases, hemolysins, enterotoxins, cytotoxins, collagenases, permeases, necrotizing toxins, proteinases, hyaluronidases, DNases, ADP-ribosyltransferases, neuraminidases, and some others that are simply known as 'lethal toxins' (Hatheway 1990; Hatheway and Johnson 1998; Rappuoli and Montecucco 1997). *C. botulinum* and *C. tetani* produce characteristic neurotoxins (BoNT and TeNT) of extraordinary potency, and are the most poisonous toxins known with lethal doses of 0.2–1 ng/kg, or as little as 0.1–1 μg being required to kill an adult human (Gill 1982; Gunnison and Meyer 1930; Hatheway 1990; Lamana 1959; Morton 1961; Schantz and Johnson 1992; Schantz and Sugiyama 1974). The oral lethal dose may be much higher (Arnon et al. 2001). These neurotoxins are responsible for the characteristic disease syndromes of botulism and tetanus in humans and many animal species. In contrast to many other pathogens, *C. botulinum* and *C. tetani* cause true toxemias whereby the produced neurotoxin is solely responsible for the pathogenic effects. Botulinum and tetanus neurotoxins both affect the nervous system, causing either a flaccid paralysis (botulism) or a spastic paralysis (tetanus).

HISTORICAL BACKGROUND OF THE PATHOGENIC CLOSTRIDIA

The observation that microorganisms or 'animalcules' could exist in the absence of air was probably first made by Leeuwenhoek (Willis 1969; Dobell 1960). The development of the field of anaerobic bacteriology, however, is usually attributed to Pasteur, who in 1861 discovered anaerobiosis by observing that butyric acid fermentation occurred in the absence of oxygen (Pasteur 1861a, b, 1863a, b; see Willis 1969 for review). Pasteur noted that the anaerobic fermentation was due to a rod-shaped organism that he called '*Vibrion butyrique*,' which likely corresponds to the organism *Clostridium butyricum*, the type species of the genus *Clostridium*. Endospores were discovered independently by Ferdinand Cohn and Robert Koch in 1876, soon after Pasteur had made microbiology famous (Keynan and Sandler 1983). The clostridia were initially classified in the genus *Bacillus* because of their cylindrical shape and

formation of endospores, but in 1880 a new genus, *Clostridium*, was proposed by Prazmowski (1880) for the anaerobic spore-forming organisms (Cato and Stackebrandt 1989).

In the late 1800s, species of *Clostridium* pathogenic for animals and humans were discovered by several investigators (see Willis 1969). Although tetanus was known for thousands of years since the time of Hippocrates and the ancient Egyptians (reviewed in Bleck 1991), the demonstration that the causative agent was transmissible in tissue from an infected to an uninfected animal was reported in 1884 (Carle and Rattone 1884). *C. tetani* was subsequently isolated in 1889 (Bytchenko 1981; Kitasato 1889, 1891; Kobel and Marti 1985). Tetanus was demonstrated to be an intoxication caused by a protein neurotoxin (Tizzoni and Cattani 1890a, b). Similarly, the disease botulism was known for several hundred years as sausage poisoning (Kerner 1817, 1820, 1822; see Ergbuth (1998) and Kessler and Benecke (1997) for historical accounts) before the organism *C. botulinum* and properties of its neurotoxin were discovered in a remarkable bacteriological investigation of the disease that occurred during a funeral wake (van Ermengem 1897). The investigations by van Ermengem and the isolation of *Bacillus botulinus* (*Clostridium botulinum*) is a classic study in the field of clostridial pathogens and their toxins. During the late 1800s and early 1900s, seminal investigations were made on various aspects of the clostridia. These included the demonstration that tetanus, botulism, and certain other clostridial diseases were caused by exotoxins, investigations of the clinical diseases, and physiology of neurotoxin action (Adams et al. 1969; Bleck 1991; Burgen et al. 1949; Dickson 1918; Drachman 1971; Duchen 1971; Kao et al. 1976; Montecucco and Schiavo 1995; Tulloch, 1919–20) and the classic study on immunization to tetanus (Behring and Kitasato 1890). The association of botulism poisoning with various foods was also established (CDC 1979; Dewberry 1950; Kempner 1897; Leighton 1923; Meyer 1931, 1956, 1973; Meyer and Eddie, 1951; Meyer and Gunnison 1929), and that neurotoxigenic clostridia formed resistant endospores of considerable significance to the control of these diseases (Bauer and Meyer 1926; Bengston 1923, 1924; Bulloch 1938; Burke 1919; Fildes 1925, 1927). In the mid-to-late 1900s, it was established that botulism could also manifest from wound and intestinal infections (Chia et al. 1986; Cherington and Ginsburg 1975; Davis et al. 1951; Dolman 1964; Hall 1945; Henry 1916–17; Midura and Arnon 1976; Pickett et al. 1976; Robertson 1915–16; Smith 1977). Remarkably, botulinum toxin has recently gained notoriety as a pharmaceutical for treatment of a myriad of diseases (Borodic et al. 1996; Brin et al. 2002; Caya 2001; Drachman 1971; Ergbuth 1998; Jankovic and Brin 1991; Jankovic and Hallett 1994; Johnson 1999b, 1999c; Schantz and Johnson 1992; Scott 1989; Scott et al. 1973). The potentially evil use of botulinum toxin as a bioterrorism agent has also been considered recently (Arnon et al. 2001; Caya 2001).

Anaerobic bacteriology developed slowly during the early 1900s, partly due to the difficulties in isolation, purification, and identification of anaerobes (see Willis 1969, 1977). McIntosh and Fildes (1916) invented the anaerobic jar, which enabled agar plating and manipulation of anaerobes as well as the isolation of single colonies. In the wars of the early-to-mid 1900s, the frequent occurrence of serious anaerobic infections prompted the isolation, identification, and study of pathogenesis of many anaerobes, mainly using the anaerobic jar. The use of jars did not permit the study of certain very strict anaerobes, and Hungate in the late 1940s and 1950s developed the roll-tube technique for isolation of highly oxygen-sensitive anaerobes from the rumen (Hungate 1969). This technique works well for isolation of highly sensitive anaerobes, but is rather tedious and thus not practical for routine use in most clinical laboratories. Later, anaerobic cabinets and glove boxes were developed (Johnson 1999a; Levett 1991; Willis 1977), which enabled traditional bacteriological manipulations to be performed. The study of anaerobes still presents problems to contemporary microbiologists (Bittner 1980; Fernandez and Ciccarelli 1999; Hatheway 1988; Johnson 1999a; Poston 1995; Willis 1977). Isolation of many anaerobes in pure culture is often difficult, as anaerobes often exist in habitats involving mixtures of microbes, and cultures are difficult to purify to a single organism. Many anaerobic cultures tend to become contaminated and must be meticulously maintained. Modern molecular biology and genetic methods routinely applied to aerobes and facultative anaerobes have not been developed in most groups of strict anaerobes. Several texts and reviews provide useful descriptions of materials and methods for culture and study of clostridia involved in disease, including *C. botulinum* and *C. tetani* and their characteristic neurotoxins (Balows et al. 1988; Cato et al. 1986; CDC 1998a; Fernandez and Ciccarelli 1999; Habermann and Dreyer 1986; Hatheway and Johnson 1998; Holdeman et al. 1977; Johnson 1999a; Levett 1991; Moore et al. 1987; Rappuoli and Montecucco 1997; Willis 1969, 1977).

Early studies of clostridia were stimulated mainly by the association of the clostridia with human and animal infections and food poisoning. The genus was subsequently found to accommodate many organisms that have no obvious role in human or animal disease. Many of these organisms are of importance in nutrition and ecology as components of the intestinal tract, soils and muds, and other anaerobic environments. Several species are of biotechnological interest because of their ability to convert abundant substrates such as cellulose into industrially important organic solvents, acids, and other compounds (Bahl and Dürre 2001; Minton and Clarke 1989).

MILESTONES IN THE UNDERSTANDING OF BOTULISM AND TETANUS

Botulism

Although anecdotal evidence suggests that botulism occurred in ancient cultures (Smith 1977), the disease was first described in Germany by Muller (1735–93) and by the poet and noted physician Justinius Kerner (1786–1862) (Kerner 1817, 1820, 1822; see Dewberry 1950; Dickson 1918, Kessler and Benecke 1997, for reviews). In Germany, raw 'blood sausages' caused disease in 174 individuals and 71 deaths in a disease characterized by muscle paralysis and suffocation. The disease was referred to as sausage 'botulus' poisoning. During the period 1815–1822, Kerner showed experimentally that poison developed within the sausage and that exclusion of air was required for the poison formation. Kerner enriched a fatty substance from decomposed sausages that produced symptoms of botulism and death in animals, which he suggested as the toxic agent. Liebig (1843; cited in Dewberry 1950) suggested that the disease was due to a 'ferment', and Heller in 1953 suggested that a microscopic vegetative mold was responsible for the disease (Dickson 1918). Botulism was often referred to as Kerner's disease following these investigations (Meyer 1956).

A theory was also advanced that botulism was caused by putrefactive amines, but these substances failed to produce the disease in animals. Nauwerck in 1886 (cited in Dickson 1918) suggested that the toxic compounds were of bacterial origin and he isolated three bacilli from sausage that were proteolytic and putrefactive. Following the investigations in Germany, a disease with similar symptoms was recorded in Russia and Denmark from the consumption of fish, termed 'icythism.' Although numerous theories were proposed for the cause of botulism, its nature remained obscure until investigations by the Belgian microbiologist Emile Pierre van Ermengem.

In 1895, van Ermengem isolated an anaerobic bacillus from a ham implicated in a botulism outbreak that occurred at a funeral (van Ermengem 1897). He showed that on culturing the bacillus it produced a very potent toxin that was released into the medium. His success in isolating the anaerobe and demonstrating the extracellular nature of the toxin was a triumph and representative of the sophisticated nature of bacteriology in Europe at the time. Van Ermengem was uniquely qualified to elucidate the etiology of paralytic botulism: he had been a student of the eminent phyiologists Claude Bernard and Ranvier, and had also trained with Robert Koch (Bulloch 1938). Van Ermengem clearly established the etiology of botulism by isolation of '*Bacillus botulinus*' from the ham and the spleens and large intestines of persons who had died from the food poisoning. He also showed that a cell-free substance was solely responsible for the disease syndrome. Kempner (1897) subsequently showed that van Ermengem's cultures produced a substance that on injection in an inactive form gave rise to antitoxin in the blood of goats. This was the first evidence that antitoxin to botulinum toxin could neutralize toxicity and prevent death.

Van Ergengem's and Kempner's seminal investigations established several principles of botulism that remain valid today: (1) food-borne botulism is an intoxication and not an infection caused by *Clostridium botulinum*; (2) the toxin is produced in food by a specific organism '*Bacillus botulinus*;' (3) the toxin is not inactivated by digestive enzymes; (4) the toxin is labile to heat and alkali but is stable in acidic conditions; (5) the toxin is not produced in food with sufficient salt or acid; (6) *C. botulinum* produces heat-resistant endospores; (7) animals vary in their susceptibility to botulinal toxin; and (8) animals can develop immunity to the botulinal toxins by exposure to inactive toxin or toxoid.

Subsequent outbreaks of botulism indicated that there were at least two types of *C. botulinum*, designated A and B (Smith 1977). In the 1920s, Bengston (1923, 1924) isolated *C. botulinum* from fly larvae (*Lucilia caesar* and *L. sericata*) that produced a botulinum neurotoxin antigenically distinct from types A and B. She noted that chickens which ate these fly larvae died of a paralytic disease characterized by inability to keep their head erect – 'limber neck.' In the 1920s, a toxigenic organism was isolated from diseased cattle that resembled the fly larvae strains but which had antigenic differences (Seddon 1922). These strains were designated type C. An organism was isolated in 1927 in South Africa from cattle carcasses which produced a serologically distinct toxin from the previously described types. It was designated type D (Smith 1977). Type E was isolated from canned fish in Russia (Gunnison et al. 1936), and has been frequently isolated from marine and freshwater sediments (Dolman and Iida 1963). Type F *C. botulinum* was initially reported in Copenhagen, Denmark in 1958 from home-made liver paste implicated in an outbreak involving five persons, three of which had severe botulism and one died (Moeller and Scheibel 1960). Type F has rarely been reported to cause human botulism (Hoffman et al. 1982; Midura et al. 1972; Green et al. 1983; Hatheway 1995; Johnson and Goodnough 1998). The properties of *C. botulinum* type F was described by Dolman and colleagues (Dolman and Murakami 1961), and type F was suspected in an infant botulism case (Hoffman et al. 1982). Giménez and Ciccarelli (1970a, b) reported another distinct serotype from soil which was designated type G. *C. botulinum* type G was reported from infants that died from infant botulism in Switzerland (Sonnabend et al. 1981, 1985, 1987), but this finding has not been supported by subsequent studies. Type G is the only serotype not implicated in human or animal botulism. *C. botulinum* strains

that produce more than one serotype of toxin or that contain 'silent' or unexpressed genes have been isolated with increasing frequency (Franciosa et al. 1994; Giménez and Ciccarelli 1978; Hutson et al. 1996; reviewed in Franciosa et al. 2003; Giménez and Giménez 1993; Hatheway 1995; Johnson and Bradshaw 2001; Quinn and Minton 2001).

Studies in the 1960s and 1970s indicated that *C. botulinum* could be separated into different ecological and physiological groupings (Cato et al. 1986; Dowell and Dezfulian 1981; Gunnison and Meyer 1929a, b; Hatheway and Johnson 1998; Hauschild 1992; Holdeman 1970; Holdeman and Brooks 1970; Holdeman et al. 1977; Lynt et al. 1982; Moore et al. 1966; Popoff 1995), and this has remained as a major means of classification of *C. botulinum*. *C. botulinum* was initially categorized into three physiological groups on the basis of cultural, serological, and biochemical characteristics (Holdeman and Brooks 1970). The isolation of an organism that produced a botulinum-like toxin not neutralized by any of the known botulinum antisera and having distinct phenotypic characteristics (Giménez and Ciccarelli 1970a, b) prompted Smith and Hobbs (1974) to add a fourth phenotypic group within *C. botulinum* (Table 1).

Categorization of *C. botulinum* in four physiological groups (I–IV) has been widely accepted (Hatheway 1988; Quinn and Minton 2001; Popoff 1995; Smith 1977; Smith and Sugiyama 1988). The groups have been demonstrated to be distinct genetically by various methods including DNA hybridization (Cummins and Johnson 1971; Johnson 1973; Johnson and Francis 1975; Lee and Riemann 1970a, b; Nakamura et al. 1977, 1983; Suen et al. 1988a, b; Wu et al. 1972), pulsed field gel electrophoresis (PFGE) (Hielm et al., 1998a, b; Lin and Johnson 1995), multilocus enzyme electrophoresis (Altwegg and Hatheway 1988; Bories et al. 1993; Cato et al. 1982a), sequencing of genes encoding rRNA (Campbell et al. 1993; Collins et al. 1994; Collins and East 1998; East et al. 1996; Hutson et al. 1993, 1993–94; Willems et al. 1993), and gene arrangements within the toxin gene clusters (Dineen et al. 2003; Hauser et al. 1995; Johnson and Bradshaw 2001; Kubota et al. 1998; Quinn and Minton 2001). In a phylogenetic perspective, these groups would certainly form separate species, but the taxonomic description *Clostridium botulinum* has been retained because of its distinct ability to produce a characteristic neurotoxin of extraordinary potency.

This grouping is also supported by metabolic and structural properties including nutritional requirements (Belokopytov et al. 1982; Kindler et al. 1956; Mager et al. 1954; Malizio et al. 1993; Patterson-Curtis and Johnson 1992; Whitmer and Johnson 1988), resistance to salt and acidity and other environmental and food components (Anniballi et al. 2002; Glass and Johnson 2001; Hammer and Johnson 1988; Hutchinson 1992; Odlaug and Pflug 1978; Ohye and Scott 1957; Raatjes and Smelt 1979; Lynt et al. 1982; Sugiyama and Sofos 1988; Townsend et al. 1954), spore heat resistance and germination properties (Broussolle et al. 2002; Ito et al. 1968; Lund and Peck 2000; Lynt et al. 1975; Scott and Bernard 1982; Setlow and Johnson 2001), tolerance to air (Meyer 1924; Whiting and Naftulin 1992), minimum growth temperature (Eklund et al. 1976; Smith and Sugiyama 1988), endproduct formation (Mead 1971; Moore et al. 1966; Moss et al. 1970, 1980; Oguma et al. 1986; Smith and Sugiyama 1988), and toxin regulation. The four groups also have distinctive surface antigen relationships (Batty and Walker 1966; Glasby and Hatheway 1983, 1984a, b; Lynt et al. 1967; Solomon et al. 1969, 1971; Walker and Batty 1964) and by the morphology and host range of bacteriophages and antibacterial spectra of bacteriocins (boticins) (Dineen et al. 2000; Eklund et al. 1988; Graham 1978; Hall and Peterson 1922; Kautter et al. 1966; Kiritani et al. 1973; Lau et al. 1974; Mahony 1979; Smith 1975).

In recent years, organisms that produce more than one serotype of botulinal toxin or that carry genes for unexpressed toxin genes have been isolated (Hatheway 1995; Hutson et al. 1996; Johnson and Bradshaw 2001; Quinn and Minton 2001). Neurotoxin formation in *C. botulinum* has been reported to be unstable (Gunnison and Meyer 1929b; Lewis and Hill 1947; Schantz and Johnson 1992; Takumi et al. 1980; Townsend 1929), and the genes of the neurotoxin gene complex can be associated with mobile elements in certain serotypes (Eklund et al. 1987, 1989; Hauser et al. 1992, 1995; Quinn and Minton 2001; Zhou et al. 1993). Organisms

Table 1 Clostridia *producing botulinal neurotoxins*

Organism	Botulinal neurotoxin types	Related nontoxigenic clostridial species
Group I *C. botulinum*	A, B, F	*C. sporogenes*, *C. putrificum*
Group II *C. botulinum*	B, E, F	Nontoxigenic derivatives of type B, E (no species designation)
Group III *C. botulinum*	C_1, D	*C. novyi*, *C. oedematiens*
Group IV *C. botulinum*	G	*C. argentinense*, *C. subterminale*, *C. hastiforme*, some *Bacillus piliformis*
Toxigenic *C. baratii*	F	Typical *C. baratii* strains
Toxigenic *C. butyrium*	E	Typical *C. butyricum* strains

resembling *Clostridium baratii*, and *Clostridium butyricum* producing botulinum-like neurotoxins have been isolated from infants with botulism (Aureli et al. 1986; Hall et al. 1985; Hatheway 1995; McCroskey et al. 1986, 1991; Suen et al. 1988b). The isolation of neurotoxigenic *C. butyricum* and *C. baratii* have recently been obtained from wider geographical regions and have been associated with food-borne as well as infant botulism (Anniballi et al. 2002; Chaudry et al. 1998; Fenicia et al. 1999, 2002; Harvey et al. 2002; Meng et al. 1999; Paisley et al. 1995; Tsukamoto et al. 2002; Wang et al. 2000). Atypical toxin variants have been isolated, mainly from human cases of intestinal botulism (Hatheway et al. 1981; Hatheway and McCroskey 1989; Tabita et al. 1990, 1991; Griffin et al. 1997) but also from foods (Goodnough, Malizio, and Johnson, unpublished data). The species of clostridia producing botulinum neurotoxins are listed in Table 1 and the association of *C. botulinum* and *C. tetani* with disease in humans and animals is summarized in Table 2.

An important landmark in the understanding of botulism and tetanus were the isolation and characterization of the responsible neurotoxins. Botulinum neurotoxin was initially isolated as paracrystals by two groups working independently in the army biological laboratories at Fort Detrick (Abrams et al. 1946; Duff et al. 1957; Lamanna et al. 1946). Later, considerable evidence accumulated to show that the toxic substance was actually a protein complex comprised of several proteins, which could be separated into toxic and hemagglutinin fractions (DasGupta et al. 1966; DasGupta and Boroff 1967; DasGupta 1989; Sugiyama 1980). The type A neurotoxin was later isolated as a single, pure protein by ultracentrifugation under alkaline conditions (Schantz 1964; Schantz and Johnson 1992; Wagman and Bateman 1953; Wright 1955) and subsequently by column chromatography (DasGupta et al. 1966) and type E by Kitamura (see Simpson 1971). Methods for purification of the seven serotypes of botulinum neurotoxin have been reviewed (Malizio et al. 2000; Rappuoli and Montecucco 1997; Sugiyama 1980; Schantz and Johnson 1992). In a classic study, Burgen et al. (1949) showed that botulinum neurotoxin acts at the neuromuscular junction of motorneurons and prevents release of acetylcholine, thus causing deinnervation. Drachman and collaborators used this characteristic to conduct a fascinating series of experiments showing that botulinum neurotoxin was an excellent tool for research on the nervous system (Drachman 1971). Drachman obtained his neurotoxin from Edward J. Schantz at Fort Detrick and later at the University of Wisconsin for his studies (Drachman 1971; Schantz and Johnson 1997; Johnson 1999b, c). In turn, Schantz provided Alan B. Scott of the Smith Kettlewell Eye Research Institute in San Francisco with crystalline botulinum toxin complex for his pioneering studies on the use of botulinum neurotoxin for the treatment of strabismus and eventually a myriad of neuronal diseases (Brin et al. 2002; Scott et al. 1973; Scott 1989; Schantz and Johnson 1992, 1997). Its characteristics and utility as an ideal blocking agent were described by Drachman and Scott (Drachman 1971; Scott 1989; Johnson 1999b, c).

In the late 1980s and through the 1990s, the nucleotide and amino acid sequences of the genes encoding the various serotypes of botulinum neurotoxins and their associated nontoxic proteins comprising the toxin gene cluster as well as the nucleotide and amino acid sequence of tetanus neurotoxin were revealed and compared (reviewed in Collins and East 1998; Henderson et al. 1997; Lacy and Stevens 1999; Niemann 1991; Popoff and Marvaud 1999; Quinn and Minton 2001). These sequences provided valuable insights into the mechanisms of the toxins, which had remained enigmatic for many years. The analysis of the nucleotide and amino acid sequences revealed that the light chain contained a zinc-binding motif characteristic of a class of zinc-metalloproteases (Niemann 1991; Schiavo et al. 1992; Montecucco and Schiavo 1995; Wright et al. 1992). This discovery led to the remarkable finding that botulinum and tetanus neurotoxins selectively cleave core neuronal proteins, and this cleavage prevented release of neurotransmitters (reviewed in Humeau et al. 2000; Lalli et al. 2003; Montecucco 1995; Turton et al. 2002; Rossetto et al. 2003). Until this discovery that botulinum and tetanus neurotoxins were proteases, their mode of action had been a mystery. Recently three-dimensional structures have been obtained for botulinum neurotoxins types A and B and for the carboxy terminus region of tetanus neurotoxin, H_C (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000; reviewed in Lalli et al. 2003; Turton et al. 2002). These structures have been extremely useful in understanding the mechanisms of receptor binding, catalysis, trafficking into neurons, and for the rationale design and testing of inhibitors. Interestingly, the discovery that botulinum and tetanus neurotoxins were zinc-metalloproteases stimulated research into this catalytic mechanism for other toxins, including the discovery that anthrax lethal factor is also a zinc metalloprotease (Ascenzi et al. 2002; Duesbery et al. 1998; Kochi et al. 1994; Pannifer et al. 2001). Certain evolutionary families of zinc-metalloproteases (Rawlings and Barrett 1995) are well established to be involved in the virulence of various bacterial pathogens (Alouf and Freer 1999; Finkelstein et al. 1983; Häse and Finkelstein 1993; Rossetto et al. 2003; Schiavo et al. 2000).

Tetanus

In ancient times, physicians noted a relationship between wound infections and a vivid disease characterized by spasticity, violent movements, and death

Table 2 *Epidemiological features of botulism and tetanus in humans and animals*

Type	Discovered by	Animal species mainly affected	Common vehicles	Main geographical occurrence
1. Botulism				
A	Burke (1919)	Humans, occasionally chickens, mink, cattle	Vegetables, fruits, meat, fish	Worldwide, common in Western USA, Russia, China
B	van Ermengem (1897); Burke (1919)	Humans, horses, cattle	Vegetables, fruits, meats, fish	Worldwide, common in Europe (Poland, Germany, France, Italy, Spain, Norway, others), Russia, Canada
C_a	Bengston (1923)	Waterfowl, Chickens, turkeys, pheasants, other birds, occasionally dogs	Dead marsh vegetation	North and South America Africa, Australia
C_b	Seddon (1922)	Cattle, horses, sheep, mink, ferrets	Forage, carrion	UK, USA, Japan Australia
D	Theiler et al. (1927)	Cattle, horses	Carrion	South Africa, Australia
E	Gunnison et al. (1936)	Humans, fish, occasionally mink, waterfowl	Fish, marine mammals	Worldwide, Japan, Canada, USA, Russia, occasionally Europe
F	Dolman and Murakami (1961)	Humans (rare)	Meats	Denmark, California
G	Giménez and Ciccarelli (1970a, b)	None known	NA	Argentina, Switzerland
2. Neurotoxigenic *C. butyricum* and *C. baratii*				
C. butyricum (type E)		Humans	None known	Italy
C. baratii (type F)		Humans	None known	USA, China, India
3. Tetanus				
	Carle and Rattone 1884; Bytchenko 1981; Kitasato 1889	Humans, various		Worldwide, but mostly in developing countries

(reviewed in Bleck 1989, 1991; Bytchenko 1981; Cook et al. 2001). The disease syndrome is referred to as the seventh case in the Edwin Smith Surgical Papyrus, which described a patient with a penetrating skull wound who showed trismus and nuchal rigidity (Bleck 1991; Breasted 1930). Hippocrates and his contemporary Aretaues also described tetanus and its manifestations about 30 centuries ago. Galen implicated nerves in the disease and in motor function by showing that cutting a nerve in tetanus stopped the contractions but paralyzed the innervated muscles (Bleck 1991). In his 1824 textbook, Sir Charles Bell included a vivid illustration of a tetanus patient (Bell 1824). Sir William Gowers provided a concise description of the disease in 1988 'Tetanus is a disease of the nervous system characterized by persistent tonic spasm, with violent brief exacerbations. The spasm almost always commences in the muscles of the jaws and commences in the muscles of the neck and jaw, causing closure of the jaws (*trismus*, *lockjaw*) and involves the trunk more than those of the limbs. It is always acute in onset, and a large proportion of those who are attacked die' (Gowers 1888, cited in Bleck 1991).

The etiology of tetanus remained a mystery until 1884, when Carle and Rattone (1884, see Bytchenko 1981) demonstrated that infected tissue from a diseased animal could cause the disease in a healthy animal that was subsequently infected. Nicolaier (1884, 1886) showed that inoculation of soil into mice, guinea pigs or rabbits caused a syndrome that closely mimicked human tetanus. He noted the presence of long thin bacilli in the wounds and pus of the inoculated animals. He also noted that bacilli were occasionally observed in the sciatic nerve sheath and spinal cord. *C. tetani* was first reported in pure culture in 1889 by Kitasato in Berlin (Kitasato 1889). He showed that the heating of pus at 80°C for 45–60 min to destroy vegetative organisms facilitated the isolation of a pure culture by plating on gelatin and incubating in a hydrogen atmosphere. Faber (1890, cited in Finegold 1998) showed that cell-free extracts caused tetanus-like symptoms on injection into laboratory animals. These studies and those of Tizzoni and Cattoni (1890a, b) provided evidence that a cell-free toxin (tetanus neurotoxin; often referred to as tetanospasmin) was responsible for the disease. Behring and Kitasato (1890) discovered an immune response to inactive toxin, and in the 1920s Glenny and Ramon (Glenny et al. 1926, 1932; Ramon and Lemétayer 1934, 1935) independently discovered that the toxin treated with formalin caused inactivation, and on administration to animals, elicited an immune response. The prevention of tetanus intoxication by vaccination is one of the great discoveries of medicine. Vaccination has proved to be extremely effective in preventing tetanus intoxication in humans and animals. Recently, the genomic sequence of one strain of *C. tetani* has been determined, which has led to considerable insights into the pathogenicity and physiology of the pathogen (Brüggemann et al. 2003).

The primary habitat of *C. tetani* is soil, which serves as the reservoir for infection of wounds and in the intestinal tract of humans and animals. Although feces, particularly from horses, has been considered as a major habitat of *C. tetani*, studies have indicated that the stools are only a vehicle of passage (see Hatheway and Johnson 1998). Tetanus neurotoxin was isolated at about the same time as botulinum neurotoxin (Pillemer et al. 1948). Unlike botulinum neurotoxin, tetanus neurotoxin does not appear to occur naturally as a complex with nontoxic proteins (Robinson 1988; Robinson and Hash 1982). Since evidence suggests that the nontoxic proteins associated with botulinum neurotoxin protect the toxin in the gastric route (Kozaki et al. 1974; Oguma et al. 1995; Sugii and Sakaguchi 1975; Sugii et al. 1977a, b; Sakaguchi et al. 1981; Ohishi et al. 1977), their absence in tetanus toxin may explain partly why it is nontoxic by the oral route. Probably, other factors such as lack of intestinal receptors also contribute to the lack of tetanus oral toxicity. Methods for production of tetanus neurotoxin and the isolation of the high-producing 'Harvard' or 'Massachusetts' strain for production of toxoid were pioneered by Mueller, Miller and collaborators at Harvard (Latham et al. 1962; Mueller and Miller 1942, 1945, 1948, 1956). Formulation and testing as a vaccine and elicitation of cellular immunity were studied by various groups and was prompted by the need for protection of troops during war periods (Willis 1969; Middlebrook and Brown, 1995; Galazka and Gasse 1995).

Tetanus neurotoxin has a similar nucleotide and amino acid sequence, mechanism of action, and structural characteristics as botulinum neurotoxin (Lalli et al. 2003; Niemann 1991; Schiavo et al. 2000). However, tetanus neurotoxin has the unique ability of being retrogradely transported to the spinal cord, where it accumulates in the horn of the gray matter (Lalli et al. 2003). In the spinal cord it prevents the release of inhibitory neurotransmitters, leading to the harrowing spasticity characteristic of tetanus. Elegant research is currently being conducted on trafficking and its mechanism of action (reviewed in Lalli et al. 2003).

CLASSIFICATION OF NEUROTOXIGENIC CLOSTRIDIA

This section describes various taxonomic and physiological properties of pathogenic *Clostridium* with an emphasis on *C. botulinum* and *C. tetani*. The description is not restricted entirely to these species since pathogenic members of the genus *Clostridium* have common characteristics, and *C. botulinum* is actually an assemblage of different species of clostridia that form a characteristic neurotoxin. Therefore, *C. botulinum* is an 'artificial' species defined predominately by its ability to

produce the characteristic botulinum neurotoxin, and the taxonomic grouping does not have a phylogenetic basis. *C. botulinum* actually comprises at least four species. Defining the diverse array of botulinogenic species as *C. botulinum* is based on the medical significance of botulinum neurotoxin and its importance in disease. During the past 10–15 years, traditionally nonpathogenic clostridia have also been isolated that produce neurotoxins with high homology to botulinum neurotoxin (see section entitled Botulism). These include neurotoxigenic strains of *C. butyricum* that produce a neurotoxin very similar to that from *C. botulinum* type E and strains of *C. baratii* that produce a type F-similar neurotoxin. Neurotoxigenic *C. butyricum* and *C. baratii* strains have mostly been isolated from cases of infant botulism, but toxigenic *C. butyricum* and *C. baratii* have also been isolated from diverse geographic environments and associated with foodborne botulism (Anniballi et al. 2002; Fenicia et al. 1999; Harvey et al. 2002; Meng et al. 1999; Chaudry et al. 1998; Paisley et al. 1995; Tsukamoto et al. 2002; Wang et al. 2000). Cases of infant botulism due to neurotoxigenic *C. butyricum* were recently associated with *Clostridium difficile* colitis (Fenicia et al. 2002; Schechter et al. 1999). The isolation of neurotoxigenic *C. butyricum* and *C. baratii* suggest that the genes encoding the neurotoxin gene complex (Johnson and Bradshaw 2001; Dineen et al. 2003; Quinn and Minton 2001) can be transferred to various *Clostridium* species. In contrast to *C. botulinum*, *C. tetani* appears to be a fairly homogenous species. Thus, it is conceivable that the clostridial neurotoxin genes have been, and will be, transferred to additional species of *Clostridium* that await discovery. The neurotoxigenic clostridia isolated to date and producing botulinum or tetanus neurotoxin are listed in Table 2. Certain evidence suggests that neurotoxigenic clostridia in the intestinal tract of humans may be involved in neurological diseases such as autism (Finegold et al. 2002a), but these organisms await discovery.

HABITATS OF PATHOGENIC CLOSTRIDIA

The pathogenic clostridia are widely distributed in nature, but they have two principal habitats, the soil and the intestine of humans and animals (Beland and Rossier 1973; Smith 1978; Smith and Hobbs 1974; Smith and Williams 1984; Willis 1969). Several clostridia including pathogenic species appear to be common inhabitants of the intestinal tract of humans and animals, and 25–38 intestinal species have been reported depending on the survey (Finegold et al. 2002b; George and Finegold 1985). Clostridia have also been isolated from gastric and duodenal specimens (Finegold et al. 2002a, b). *C. tetani* is frequently found in human feces, but *C. botulinum* is rarely isolated from this source (Easton and Meyer 1924) except in infants with botulism (Arnon 1995, 2004; Hatheway 1995). *C. tetani* and *C. botulinum* are also found in most soils worldwide that have been surveyed (Dodds 1993; Hauschild 1992; Smith and Williams 1984; Willis 1969). It has been suggested that the main habitat of many clostridia is the intestines of animals and their presence in soil is due to fecal contamination. It is more likely that the primary habitat is soil; that they are digested together with plant foods harvested from the soil, and that some of them have adapted temporarily or permanently to the intestinal canal as a habitat. As a result of being predominant in soil, certain clostridia frequently occur in dust, milk, and sewage.

Interestingly, the worldwide distribution of *C. botulinum* differs in regions according to their toxin type and physiology. For example, proteolytic strains (Group I) of *C. botulinum* type A occur at a higher frequency in the western United States, whilst proteolytic type B occurs predominately in the eastern United States (Dodds 1993; Hauschild 1992; Popoff 1995; Smith and Sugiyama 1988). Nonproteolytic type B (Group II) predominate in western Europe. Type E is predominately associated with aquatic habitats including muds and brackish waters (Dolman and Iida 1963; Hatheway 1995; Johnson and Goodnough 1998). *C. botulinum*/*C. argentinense* producing type G neurotoxin has only been isolated from soils in Argentina. The ecological factors that determine the distribution of *C. botulinum* spores in nature are largely unknown. The distribution of different serotypes of *C. botulinum* worldwide has been reviewed (Hauschild 1992; Popoff 1995; Johnson and Goodnough 1998).

PHYSICAL AND METABOLIC PROPERTIES OF PATHOGENIC CLOSTRIDIA INCLUDING *C. BOTULINUM* AND *C. TETANI*

Staining reactions

C. botulinum and *C. tetani* stain readily with the usual dyes (see Hatheway and Johnson 1998; Paik 1980; Willis 1977). Considerable irregularity is noticeable in the depth of staining, especially in cultures that are more than 2 days old. Occasionally, metachromatic granules or points of more intense coloration are seen, especially when acetone is used for discoloration. In young cultures (<8 h), the bacilli usually stain uniformly gram-positive. On extended culture they may lose this property, and can be readily discolorized with ethanol. In the early stages of spore formation, the position of the spore is often marked by a region of intense staining; as it matures, however, the spore presents a colorless center surrounded by a peripherally stained ring. For initial staining of the organisms in the laboratory, Gram's stain is most commonly used. Clostridia are considered

gram-positive organisms, and have a gram-positive cell-wall structure, although some strains show the positive (violet) staining reaction only in young cultures (less than 12 h), and sometimes identification must be made without this observation. Often, Gram-stained smears of pure cultures show a mixture of positive and negative organisms.

Spores

The formation of resistant endospores is a common trait of *C. botulinum*, *C. tetani*, and other neurotoxigenic clostridia, although there is considerable variation in the propensity by which it occurs. The spores are wider than the vegetative bacilli and confer a distinctive appearance according to the position in which they arise. In *C. botulinum* and *C. tetani* the spores are subterminal to terminal giving the cells a club-shape or tennis-racket appearance. Spores are easily visualized by phase contrast microscopy as bright, refractile oval bodies in the mother cell. In a Gram stain, the cells typically stain but the spores do not acquire the stain and appear as refractile bodies. Sporulation of neurotoxigenic clostridia has been reviewed (Setlow and Johnson 2001; Woods and Jones 1986), and relatively little is known compared to other sporeformers, particularly *Bacillus* sp.

Spores of most species can be readily produced in chopped meat broth or agar (Dowell and Hawkins 1974; Hatheway and Johnson 1998; Holdeman et al. 1977), but spores produced in these media are difficult to purify for analyses. *C. botulinum* and *C. tetani* also sporulate in clear, complex media such as trypticase-peptone-glucose-yeast extract (TPGY) broth. Nutritional studies showed that sporulation in *C. botulinum* type B (group I) is stimulated by zinc (Kihm et al. 1988). Spores produced in clear media can be purified to over 99 percent purity by density gradient centrifugation from the clear media (Kihm et al. 1990). The clostridial spore released from the mother cell is structurally distinct from the vegetative cell in which they arise. Many clostridial spores contain an outermost layer, termed the exposporium, which often assumes a structurally complex morphology (Setlow and Johnson 2001). Underlying the exosporium is the spore coat, consisting of unusual proteins with a high degree of cross-linking. Beneath this layer is the spore cortex, consisting of peptidoglycan-like polymers. The central region of the spore, or the core, contains the spore's DNA, ribosomes, enzymes necessary for growth and germination, and deposits of divalent cations. The metal content of purified proteolytic *C. botulinum* type B spores is different from metals in spores of *Bacillus* that have been analyzed (Kihm et al. 1990). Overall, the spore structure and components is similar to that in the better studied *Bacillus*, but there are also clear differences that may be associated with different resistance mechanisms than those found in *Bacillus*. Proteolytic strains of *C. botulinum* can produce spores of high heat resistance and are the most important spore-former in the public health safety of thermally processed foods (Ito et al. 1968; Lund and Peck 2000; Setlow and Johnson 2001). Certain food regulations, such as the low-acid canned food laws, are based on a 'bot cook,' commonly considered as inactivation of 12 logs of the most resistant (type A) *C. botulinum* spores (Glass and Johnson 2001; Kim and Foegeding 1993; Lynt et al. 1975; Setlow and Johnson 2001). Spores from nonproteolytic *C. botulinum* strains (Group II), from types C and D (Group III), and type G (group IV) are less heat-resistant than those of proteolytic (Group I) strains. The non-neurotoxic species *C. sporogenes*, of which the spores have a higher heat resistance than *C. botulinum*, has been used as a surrogate to estimate the thermal process needed for a botulinum cook (Setlow and Johnson 2001).

In addition to heat, clostridial endospores are resistant to exposure to oxygen, chemical agents, irradiation, heating, and drying (Kim and Foegeding 1993; Lund and Peck 2000; Setlow and Johnson 2001). They can be inactivated with chlorine and with high concentrations of hydrogen peroxide (Kim and Foegeding 1993). The spores from Group I *C. botulinum* can survive boiling in aqueous media and in foods for more than 1 h. This is the reason that food-borne botulism can sometimes occur with foods that have been heat processed. The heat resistance of spores is often used to advantage in isolation of clostridia from mixed cultures, as heating kills vegetative flora, while the spores remain viable. Treatment of cultures with 50 percent ethanol will also inactivate most vegetative cells and will leave the spores viable (Koransky et al. 1978; Lilly et al. 1971). These methods are often useful in purifying clostridia from mixed or contaminated cultures.

The spore can remain in a dormant state for hundreds or perhaps thousands of years (Keynan and Sandler 1983), but dormancy can be broken by various treatments. The activation, germination, and outgrowth of spores depend on several factors. Often a heat treatment of 60–80°C for 10–20 min is applied for activation. The events that occur during heat activation are not known, but heating is a good method to break dormancy and facilitate subsequent germination. Cooked meat medium containing soluble starch (CMGS) is recommended for recovery of sporulated clostridia (Dowell et al. 1977a). The starch can bind certain lipid and fatty acid components of the medium and inoculum that can inhibit germination. The addition of lysozyme to media can also considerably increase the frequency of recovery, probably by substituting for germination enzymes that are destroyed by heat treatments (Lund and Peck 2000). The destruction of germination and other metabolic processes by heating or other treatments such as irradiation could be a cause of spores that are 'super'-dormant and do not germinate for many years until they are in

some manner shocked into a germinating vegetative state.

Flagella

C. botulinum and *C. tetani* are motile by peritrichous flagella, but motility can be difficult to demonstrate, especially in strains that have been laboratory subcultures for years and in cultures that have entered into a stationary phase. Young cultures in fluid medium, of 6–24 h old, are the most suitable for demonstration of motility. Motility may be observed by examining a drop of culture overlaid with a coverslip under the microscope using phase contrast or dark field illumination. Alternatively, growth in an anaerobic tube of motility medium (containing 0.5 percent agar) (Dowell et al. 1977a) will show diffuse growth throughout the medium. Flagella can be observed using Liefson's flagellar stain (Dowell and Hawkins 1974; Paik 1980).

Cell walls

Clostridium species are classified as gram-positive organisms and young cultures generally stain well, although sometimes the Gram reaction is difficult to demonstrate. The group I ('true clostridia') (Collins et al. 1994) have a typical gram-positive cell wall structure and cultures lack an outer membrane typical of gram-negative organisms. This remains an important taxonomic criterion, since members of the genus *Sporomusa*, also anaerobic sporeformers, possess a gram-negative cell wall (Andreesen et al. 1989). Certain other organisms grouped with the clostridia also have a gram-negative cell-wall structure (Finegold et al. 2002b). In gram-positive organisms, the peptidoglycan usually accounts for 80–90 percent of the cell-wall components forming a thick layer (Andreesen et al. 1989; Gottschalk 1986), while it accounts for only about 10 percent in gram-negative organisms and forms a relatively thin surrounding layer. DL diaminopimelic acid (*meso*-DAP) provides the crosslink in the *C. botulinum* and *C. tetani* strains for which it has been studied (Cato et al. 1986). The predominate cell-wall sugars in *C. tetani* are glucose and rhamnose (Cummins and Johnson 1971). The cell-wall peptidoglycan of a type A *C. botulinum* strain was found to contain glutamic acid, alanine, diaminopimelic acid, glucosamine, and muramic acid (Smith and Sugiyama 1988). The cell walls of other serotypes and groups would be expected to vary since it is a very heterogeneous species. The structure of the cell wall of *C. tetani* has not been elucidated.

Capsules

Most *Clostridium* sp., including *C. botulinum* and *C. tetani*, lack a capsule. A few clostridia have been shown to bear a capsule, especially *C. perfringens* and *C. butyricum*. Capsule formation is dependent on culture conditions, and may occur predominately in media containing serum or in the human or animal host. The serology of the capsular polysaccharide antigens is diverse (Klotz 1965), and this diversity has been used for establishing a common vehicle in outbreaks of food-borne disease caused by clostridia (Hatheway and Johnson 1998; Stringer et al. 1980).

Plasmids

Plasmids occur frequently in many *Clostridium* species, including strains of *C. botulinum* and *C. tetani* (Finn et al. 1984; Eklund et al. 1989). Most of the plasmids are cryptic, and *C. perfringens* is the only pathogenic clostridial species in which conjugative resistance plasmids (R plasmids) have been isolated and these generally carry a tetracycline resistance determinant (Rood 1998). A plasmid has been isolated that confers resistance to macrolide-lincosamide-streptogramin B antibiotics (Rood 1998). These plasmids have been very useful in developing genetic tools for the study of *C. perfringens*. It would be valuable to isolate conjugative plasmids from *C. botulinum* and *C. tetani* for the study of their physiology and pathogenicity, but such studies have not been pursued in depth and most plasmids detected in *C. botulinum* remain cryptic in function (Strom et al. 1984; Quinn and Minton 2001). Curing of the plasmids has not revealed an association of plasmids and toxin production in *C. botulinum* Group I and II, but loss of plasmids is associated with absence of toxicity in Group IV *C. botulinum*/*C. argentinense* producing type G neurotoxin (Eklund et al. 1988; Zhou et al. 1995) and in *C. tetani* (Finn et al. 1984; Laird et al. 1980). The association of plasmids with toxin production in *C. tetani* was initially suggested by Hara et al. (1977), and it was later shown that 52 toxin-producing strains of *C. tetani* harbored a single large plasmid of about 75 Mda (Laird et al. 1980). It was subsequently shown that the gene encoding tetanus neurotoxin resided on the plasmid (see Eklund et al. 1989 for review). Attempts to transfer the toxigenicity from a toxic strain to a nontoxic *C. tetani* strain were unsuccessful, suggesting that the plasmid does not have conjugal properties. Studies have also shown that the genes for type G neurotoxin in *C. botulinum* type G (*C. argentinense*) and associated nontoxic proteins in the neurotoxin complex are harbored on a plasmid (Eklund et al. 1988; Zhou et al. 1995). The type G botulinum neurotoxin gene complex was demonstrated to be harbored on an 81-Mda plasmid (Zhou et al. 1995). The function of such large plasmids in *C. tetani* and *C. botulinum* type G is not known. A gene encoding a bacteriocin has been shown to reside on a plasmid in strains of *C. botulinum* types A and B (Dineen et al. 2000). A related plasmid containing a boticin gene has also been demonstrated in *C. botulinum*

type A strain ATCC 2502 that is being used for genome sequencing. Plasmid profiles have been used for strain identification of *C. difficile* (Cornillot et al. 1995) and *C. perfringens* (Steinberg et al. 1987), but they have not been reported to be useful in strain identification of *C. botulinum* and *C. tetani*.

Bacteriophages

Many clostridia including neurotoxigenic species have been demonstrated to harbor bacteriophages (Dolman and Chang 1972; Eklund et al. 1976, 1987, 1989; Inoue and Iida 1968; Ogata and Hongo 1979). Research groups in the United States and in Japan demonstrated that bacteriophages mediated toxin production in *C. botulinum* types C and D (reviewed in Eklund et al. 1989; Johnson 1997). This relationship between neurotoxin and bacteriophages was established by curing of phages by treatment with ultraviolet light, mitomycin C, or acridine orange with associated loss of toxigenicity. These nontoxigenic isolates were subsequently found to be sensitive to bacteriophage infection, indicating that they had lost immunity and thus loss of bacteriophage infection. The direct demonstration of toxigenicity and bacteriophage was demonstrated by reinfection with phage and conversion to the toxigenic state with restoration of toxin formation. Phage interconversion of *C. botulinum* types C and D was also demonstrated by phage-loss and crossinfection. After loss of bacteriophages and toxicity from *C. botulinum* types C and D, the resulting nontoxigenic strains were indistinguishable, but both were demonstrated to belong to the heterogeneous group of clostridia comprising *Clostridium novyi* type A. Conversion of *C. novyi* to toxigenicity by phage infection was also demonstrated. The bacteriophage carrier state in *C. botulinum* types C and D has been termed pseudolysogeny, referring to the observations that the prophage–bacterium relationship is unstable and non-lysogens occur rather frequently. This was further demonstrated by incubating toxigenic cells in media containing antiserum raised against the bacteriophages. After several transfers, nearly the entire population had lost the ability to produce neurotoxin (see Eklund et al. 1989; Johnson 1997).

The relation of phages to toxin production has not been shown in other groups (including serotypes A, B, E, F, G) of *C. botulinum* (Eklund et al. 1989; Johnson et al. 1997). Although strains of *C. botulinum* in Group I contain prophages, curing of strains with mitomycin C and incubation in media containing acriflavine has not been correlated with loss of toxigenicity. However, introduction of the transposon Tn*916* into *C. botulinum* strain 62A (type A) led to strains that were tox^- and had a large 32-kb deletion that included all the genes for the neurotoxin complex (Lin and Johnson 1995; Johnson et al. 1997). These data suggest that the toxin locus can be unstable and is potentially indicative of phage-mediated loss of toxigenicity, particularly considering the large size ($\geqslant$12 kbp) of the toxin gene complex locus. Certain strains of *C. botulinum* in Group II lost their ability to produce neurotoxin using the mitomycin C/acriflavine treatment (Eklund et al. 1989). However, these cured strains did not appear to be phage sensitive and toxigenicity could not be restored by phage infection. This evidence for lack of correlation between bacteriophage infection and toxigenicity is indirect, and it is frustrating that phage-sensitive strains could not be isolated.

It is likely that the genes encoding the neurotoxin cluster can be transferred in nature because of the relatively recent isolation of *C. butyricum* strains and *C. baratii* strains that produce neurotoxins E and F, respectively (see previous citations in section entitled Botulism). However, the analysis of genome structure has not provided evidence for pathogenicity islands that occur frequently in several bacterial pathogens and have been associated with virulence gene transfer. Since *C. botulinum* and *C. tetani* contain plasmids and often numerous phages (Eklund et al. 1989; Johnson 1997), interspecies transfer may occur by mobilization of these potential moveable elements. The isolation of strains of *C. butyricum* and *C. baratii* that produced botulinum-like neurotoxins supports the fact that transfer occurs in nature, but the mechanism remains obscure. Indirect evidence has been presented that transfer of neurotoxigenicity can occur from *C. butyricum* to a nontoxigenic *C. botulinum* E-like strain and is probably mediated by either a plasmid or a defective bacteriophage (Hauser et al. 1992, 1995; Zhou et al. 1993). A gene encoding a bacteriophage-related lysozyme has been detected in the vicinity of a toxin gene cluster, lending indirect evidence that bacteriophages may reside in the vicinity of the toxin gene cluster on the chromosome (Quinn and Minton 2001). Thus, it may be fruitful to further pursue the possibility of phage transfer of botulinum neurotoxin genes in certain strains of *C. botulinum*.

Bacteriocins

Bacteriocins are antimicrobial peptides or proteins formed by bacteria which typically have inhibitory activity against closely related species (Jack et al. 1995). Bacteriocins and other uncharacterized antimicrobial substances are produced by a number of clostridia including *C. botulinum* (Dineen et al. 2000; Eklund et al. 1988; Graham 1978; Hall and Peterson 1922; Kautter et al. 1966; Kiritani et al. 1973; Lau et al. 1974; Mahony 1979; Smith 1975). Smith (1975) isolated strains of *B. cereus*, *C. sporogenes*, and *C. perfringens* that produced bacteriocins active against *C. botulinum*. The lantiobiotic nisin, which is produced by strains of *Lactococcus lactis* and is approved for certain food uses in the USA and UK,

has bactericidal activity in vitro against certain serotypes and strains of *C. botulinum* (Glass and Johnson 2001). Recently, a bacteriocin called boticin B was isolated and characterized from *C. botulinum* 213B (Dineen et al. 2000). DNA sequencing revealed the boticin B structural gene, *btcB*, to encode a protein of 50 amino acids with a calculated molecular mass of 5 138 Da. It had a narrow spectrum of activity against *C. botulinum* 62A and a *C. botulinum* strain 588 Ab, which produces type A neurotoxin as its major toxin and type B in considerably less quantities. The bacteriocin from 213B was shown to reside on an 18.8 kb plasmid. This is the first reported bacteriocin to be isolated from a group I *C. botulinum* strain, and it could have significance in the inhibition of related clostridia in the intestine or in foods. Recently, the strain *C. botulinum* ATCC 3502 whose genome is being sequenced, was shown to contain a ~20 kb plasmid that included a genes for a bacteriocin related to boticin B (www.sanger.ac.uk/Projects/C_botulinum).

Antigenic structure

Antigenic structure has been evaluated as a means of establishing a systematic method for identification and differentiation of strains of clostridia (reviewed in Hatheway and Johnson 1998). With the advance in molecular typing methods mainly involving DNA technologies such as PCR, PFGE, and nucleic acid hybridizations, interest in the serological approach has diminished. The main groups of clostridial antigens that have been studied are: (a) the antigens of the flagella and the bacterial bodies, (b) capsular polysaccharides, and (c) toxins or other factors found in culture filtrates. The use of fluorescent antibodies (FA) was evaluated for confirmation of infant botulism by identification of *C. botulinum* in patients' stools (Glasby and Hatheway 1983, 1984a). Since some strains of *C. sporogenes* also reacted with the antibodies, neurotoxin tests remained necessary and largely negated any potential advantage of FA identification. Molecular typing methods largely relying on nucleic acid methodologies and also specific immunoassays for clostridial toxins appear to offer more general applicable approaches.

GROWTH PROPERTIES OF *C. BOTULINUM* AND *C. TETANI*

General cultural methods for *C. botulinum* and *C. tetani*

The isolation and identification of neurotoxigenic clostridia presents certain practical difficulties (Bittner 1980; Johnson 1999a; Willis 1969, 1977). Clostridia are anaerobes and culturing requires reduced media to eliminate oxygen and toxic oxygen metabolites and an anaerobic environment for culture incubation such as an anaerobic chamber. Prompt collection of foods or clinical samples and rapid analysis is desired to prevent exposure to oxygen and reduction in viability of clostridial cultures. In general, *C. botulinum* and *C. tetani* are more sensitive to oxygen than many other clostridia such as *C. sporogenes* and *C. perfringens*. *C. botulinum* type C and the related nontoxigenic species *Clostridium novyi* are more oxygen sensitive than *C. botulinum* types A and B (Meyer 1924). Oxygen sensitivity restricts the habitat of vegetative cells to anaerobic environments, though endospores may survive in aerobic environments. In most environmental, food, and fecal samples, clostridial endospores are often present. In samples containing spores, competitor vegetative organisms can be eliminated by heat or by ethanol or phenethyl alcohol (Dowell et al. 1964, 1977a; Solomon et al. 1995). Incubation of the source materials at 60–80°C for 10–20 min is also usually sufficient to select for clostridial sporeformers. However, certain types of *C. botulinum* have heat-sensitive spores, and 50 percent alcohol treatment can facilitate the isolation of these cultures (Dowell et al. 1964; Johnston et al. 1964; Solomon et al. 1995). Mixed cultures are usually present in clinical specimens or foods, and oxygen-utilizing bacteria and fungi will utilize oxygen and provide an environment favorable for growth of clostridia. When clostridia are enriched in complex media from foods, fecal materials, or other complex sources, they may be outgrown by competitor organisms. Selective media have been useful for isolation of proteolytic clostridia from these sources (CDC 1998a; Dezfulian et al. 1981; Dowell et al. 1977a, 1981; Hatheway 1988; Mills et al. 1985; Willis 1977).

Difficulty may be encountered in obtaining and maintaining pure cultures of neurotoxigenic clostridia. Strains also tend to show genetic instability and variation in phenotypic properties including decrease in toxin production on repeated subculture in the laboratory (Gunnison and Meyer 1929a; Lewis and Hill 1947; Schantz and Johnson 1992). Newly isolated cultures should be stored as pure cultures in cooked meat medium preferably at −20 or −70°C. Toxin titers of *C. botulinum* have been found to decrease with storage under these conditions to levels that are unacceptable for toxin purification (Schantz and Johnson 1994; Lewis and Hill 1947). Nontoxigenic variants may exhibit enhanced sporulation and changes in lysogeny (Takumi et al. 1980). Subculturing in cooked meat medium can help maintain or restore toxin titers. Maintenance of high toxin producing cultures can require isolation of individual clones and testing for toxin production by assay in mice. An immunologic method was developed for qualitative assessment of toxin production within individual colonies (Goodnough et al. 1993).

C. tetani and *C. botulinum* generally grow optimally at 30–37°C, although certain strains of *C. botulinum* in group II (nonproteolytic strains of serotypes B, E and F)

can grow at temperatures as low as 3–4°C (reviewed in Hauschild 1992, ICMSF 1980, 1996, 1998; Glass and Johnson 2001). The optimum pH of growth for both species is about 7.0–7.4. *C. botulinum* and *C. tetani* will grow in liquid media from which oxygen has been removed such as by boiling or autoclaving, and which contain a reducing agent such as cysteine, thioglycollate, or metallic iron. For growth on solid media, an anaerobic environment is required such as an anaerobic chamber containing an atmosphere of 80–85 percent nitrogen, 5–10 percent carbon dioxide, and an initial concentration of hydrogen of 10 percent. Since concentrations of hydrogen near to 10 percent can be explosive if ignited, the initial level should not exceed 10 percent. In practice, the 10 percent level rapidly decreases as it reacts with oxygen to form water in the presence of a palladium catalyst (Johnson 1999a). Other methods of anaerobic culture on solid media can be employed such as anaerobic jars or Hungate tubes, but it is difficult to manipulate colonies in these systems. On solid media, growth can be relatively slow, and sometimes takes the form of a thin, effuse, often spreading film, which may be difficult to distinguish from the underlying medium, particularly with *C. tetani*. Film formation and swarming is promoted by moisture on the plates and low concentrations of agar. In particular, *C. tetani* tends to swarm or spread rapidly over a moist agar surface. *C. tetani* inoculated into the condensation of water of an agar slant, or laterally on a blood agar plate, in the course of a day spreads over the entire plate and the film is often so thin that were it not for its dentate spreading edge it might escape detection. Advantage may be taken of this property for isolation of the organism (Fildes 1925; Willis 1969, 1977). The spreading can be inhibited by increasing the concentration of the agar (e.g. 2.5–4 percent) (Dowell et al. 1977a), by certain chemicals, or by the addition of agglutinating antibody to the medium (Willis 1977; Williams and Willis 1970).

Routine isolation methods for pathogenic *C. botulinum* and *C. tetani* have been described (Dowell and Hawkins 1974; Dowell et al. 1977a; Hatheway 1988; Willis and Hobbs 1959; Willis 1969, 1977). Briefly, *C. botulinum* and *C. tetani* can be isolated from environmental samples, clinical specimens, and foods by heat treatment at 60–80°C for 10–20 min to select for spores, followed by incubation in a suitable rich broth such as cooked meat medium ±0.5 percent glucose, reinforced clostridium medium, or TPGY broth. For certain saccharolytic clostridia including Group I–III *C. botulinum*, it is often helpful to add 0.5 percent glucose. Individual colonies can be isolated on various solid media including TPGY, reinforced clostridium medium, cooked meat medium +0.5 percent glucose and starch, or blood agar plates. Selective media for toxigenic clostridia are based on the relative lack of inhibitory activity of certain antimicrobial agents against clostridia compared to other microbial groups. Inhibitors selective for clostridia include polymyxin B, neomycin, and crystal violet (Willis 1990), phenylethyl alcohol (Dowell et al. 1964), cycloserine (Hauschild and Hilsheimer 1974), and trimethoprim/sulfamethoxazole (Dezfulian et al. 1981; Mills et al. 1985). A selective agar medium (CBI) containing cycloserine, sulfamethoxazole, and trimethoprim was developed for isolation of proteolytic *C. botulinum* from human feces (Dezfulian et al. 1981). Modifications, especially the incorporation of thymidine phosphorylase, were recommended to suppress nonbotulinum fecal flora and facilitate more rapid isolation of *C. botulinum* (Mills et al. 1985). Initial presumptive identification of neurotoxigenic clostridia can include several of the following tests (CDC 1998a; Dowell and Hawkins 1974; Dowell et al. 1977a; Hatheway 1988; Holdeman et al. 1977; Willis 1977):

- cell morphology, Gram stain reaction, and size and the shape and position of mature endospores in the mother cell;
- biochemical reactions including lecithinase, lipase, proteolysis, indole formation;
- activity of metabolic pathways for utilization of specific carbohydrates as energy sources;
- volatile fatty acid endproducts determined by gas chromatography or high pressure liquid chromatography; and
- determined assay of neurotoxicity in mice with the use of proper controls.

For consistent identification, standardization of the growth conditions and the use of reference strains is recommended.

Many solid media are satisfactory for the study of colony morphology of clostridia including TPGY agar, blood agar, egg yolk agar, reinforced clostridial agar and other media that are rich in peptones, vitamins, and other nutrients. *C. botulinum* and *C. tetani* have nutrient requirements for numerous amino acids, and vitamins (Cato et al. 1986; Feeney et al. 1944; Whitmer and Johnson 1988). The growth of certain types of *C. botulinum* (e.g. type A) is stimulated by sugars, but *C. tetani* does not utilize sugars as carbon and energy sources and grows well on media lacking carbohydrate such as nutrient agar and blood agar. Most strains of *C. botulinum* Groups I–III form colonies after 24–48 h that are 2–8 mm in diameter with a translucent or semiopaque appearance and a rhizoid periphery (Cato et al. 1986; Holdeman et al. 1977). They sometimes have a raised colony center with a yellowish color. Colonies are generally gray in color with a matt to semiglossy surface (Batty and Walker 1966; Smith and Williams 1984; Willis 1969, 1977). As colonies of clostridia develop, the morphology changes depending on the conditions and the medium. Eventually, colonies of *C. botulinum* often take on an irregular shape. On blood agar, colonies are 2–6 mm in diameter, often irregular in shape with a rhizoid periphery, translucent to semiopaque, usually with a granular or ground glass occurrence, and are

beta-hemolytic (Cato et al. 1986; Willis 1969, 1977). *C. botulinum* type G generally form smaller and more translucent colonies, are more oxygen sensitive, and may form a spreading film (Hatheway and Johnson 1998).

Cooked meat medium (often supplemented with 0.5 percent glucose and/or starch) (CMGS), TPGY broth, and reinforced clostridial broth are good liquid media for vegetative growth and formation of spores (Ciccarelli et al. 1977; Hatheway and Johnson 1998). *C. botulinum* and *C. tetani* are generally observed microscopically as large, rod-shaped organisms, but the morphology of different strains can vary widely. Depending on the strain and culture conditions, the size varies and they can form pleomorphic shapes. The length of *C. botulinum* and *C. tetani* cells in liquid media is variable depending on media and conditions and can vary from about 3–8 μm in length and 0.6–1.4 μm in breadth (Cato et al. 1986; Willis 1969, 1977). Long filamentous forms up to 20 μm are often observed. The vegetative bacilli are straight to slightly curved, their sides parallel, and their ends rounded or somewhat truncated. Often they occur as single rods, but both species also frequently occur in pairs or in chains. As the cultures enter into stationary phase they can form their characteristic neurotoxins, and this process is associated with extensive autolysis in certain serotypes and strains. Depending on the nutritional and culture conditions, the organisms can form endospores that distend the sporangium, and this is followed by lysis and release of free, phase-bright endospores. When spores are present, *C. botulinum* and *C. tetani* show a distended subterminal sporangium, often described as a 'tennis racket' or 'drumstick' shape (Hatheway and Johnson 1998). Toxin production appears to be distinct or even antagonistic from sporulation, and strains used for toxin formation and for vaccine production generally have a nonsporulating phenotype (Schantz and Johnson 1992). Photomicrographs of smears of cells and colonies are presented in Figure 1.

Specific culture media are useful for determining phenotypic characteristics of *C. botulinum* (Cato et al. 1986; Hatheway 1988; Holdeman et al. 1977). Egg yolk agar is useful for detecting lecithinase and lipase enzymes secreted by *C. botulinum* and related organisms (Nagler 1939; McClung and Toabe 1947; Willis 1969, 1977). Lecithinase (phospholipase C) is indicated by the appearance of an insoluble, whitish-opaque pearly layer surrounding the colonies in the agar. Lipase reaction appears as an iridescent sheen on the surface of the colonies. Like other phenotypic tests, many other members of the genus *Clostridium* produce colonies with similar colony appearance. Many strains of *C. botulinum* and *C. tetani* are strongly proteolytic. Proteolysis is detected by digestion of chopped meat particles (CDC 1998a; Holdeman et al. 1977) or by digestion of casein in skim-milk plates (Hatheway 1988, 1995). In all biochemical tests, it is necessary to use positive controls to validate the method. Compilations of media and conditions for culture of clostridia are available (Cato et al. 1986; CDC 1998a; Dowell et al. 1977a; Holdeman et al. 1977; Johnson 1999a; Willis, 1977). For an earlier illustrated description of the morphological and colonial appearance of the pathogenic anaerobes see Batty and Walker (1966).

Identification of *C. botulinum* requires demonstration of the presence of botulinum neurotoxin (CDC 1998a; Hatheway 1988; Giménez and Giménez 1995; Johnson and Goodnough 1998), but this feature has not been required for *C. tetani*, as nontoxigenic strains of this species are allowed. Furthermore, from a clinical perspective, the diagnosis of tetanus generally relies on symptoms alone (Donadio et al. 1971; Bleck 1989, 1991; Cook et al. 2001; Hsu and Groleau 2001). Methods for mouse bioassay and typing of botulinum neurotoxin, including pitfalls in the techniques, have been described (Boroff and Fleck 1966; CDC 1998a, Hatheway 1988; Giménez and Giménez 1995; Schantz and Kautter 1978). International standards are extremely useful for typing of *C. botulinum* (Bowmer 1963; Schantz and Kautter 1978), but these are presently not available and efforts should be made to prepare new standard antitoxin (and toxin) preparations. Neurotoxin production in strains of *C. botulinum* and *C. tetani* can be lost or diminished in titer on repeated culture (Lewis and Hill 1947; Schantz and Johnson 1992; Goodnough et al. 1993). Gunnison and Meyer (1929b) conducted an extensive study of isolates of *C. botulinum* of types A, B, C, and D from various regions of the world. They emphasized that since the toxin-producing property of a strain may diminish, a classification based on physiological behavior possesses many advantages. They also emphasized that the final identification of *C. botulinum* should be made with toxin–antitoxin neutralization. They found that colony morphology was not characteristic of any specific type of *C. botulinum*. Gunnison and Meyer (1929a) also demonstrated that proteolysis and fermentation endproducts from sugars varied among the types. Moore et al. (1966) demonstrated that fermentation endproducts are useful in the identification of *Clostridium* species. Proteolysis, lipase reaction, fermentation endproducts, and toxin–antitoxin neutralization are primary tests used today for the identification of neurotoxigenic clostridia. In recent years, the classification of clostridia has increasingly relied on molecular techniques, particularly PFGE (Lin and Johnson 1995; Wang et al. 2000), and sequencing of genes encoding rRNA or interspatial regions (Finegold et al. 2002b; Lane et al. 1985; Olive and Bean 1999; Wilson 1995), but a more encompassing approach may be desired to define a bacterial lineage or species (Rosselló-Mora and Amann 2001).

PHYSIOLOGY OF *C. BOTULINUM* AND *C. TETANI*

Clostridia are anaerobic organisms that do not possess cytochrome oxidase or oxygenases (Andreesen et al.

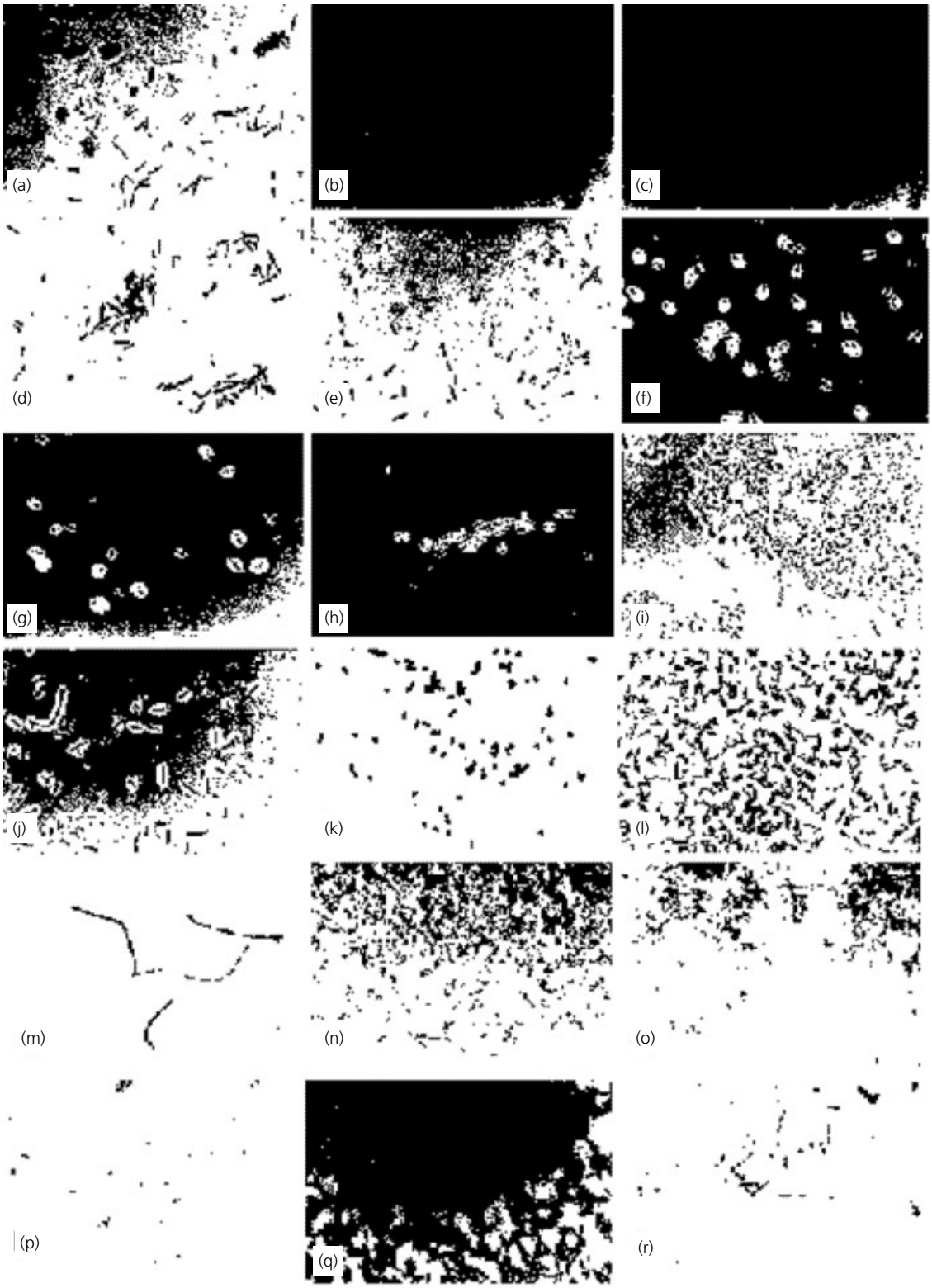

Figure 1 *Photomicrographs (× c. 1 500) of some representative species of* Clostridium*:* **(a)** C. botulinum *type A, 24 h CMGS, Gram stain;* **(b)** C. botulinum *type A, 72 h EYA, gram stain;* **(c)** C. botulinum *type B (nonproteolytic), 72 h EYA, Gram stain;* **(d)** C. botulinum *type E, 24 h CMGS, Gram stain;* **(e)** C. tetani*, 4 day EYA, phase contrast;* **(h)** C. botulinum *type B, 4 day EYA, phase contrast;* **(i)** C. botulinum *type E, 4 day EYA, phase contrast;* **(j)** C. tetani*, 4 day EY, phase contrast;* **(k)** C. perfringens*, 24 h CM, Gram stain;* **(l)** C. perfringens*, 24 h Schaedler agar, Gram stain;* **(p)** C. subterminale*, 28 h BAP, Gram stain;* **(q)** C. argentinese*, 72 h EYA, gram stain;* **(r)** C. innocuum*, 24 h thioglycollate broth, Gram stain. (Continued over).*

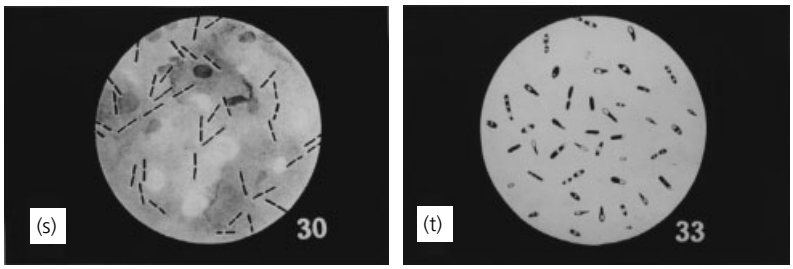

Figure 1 *Photomicrographs (× c. 1 500) of some representative species of* Clostridium. *(Continued)* **(s)** C. septicum*, 24 h CM methylene blue;* **(t)** C. chauvoei*, methylene blue) (from Hatheway and Johnson 1998).*

1989; Decker et al. 1970; Gottschalk 1986; Thauer et al. 1977). They derive their energy for growth by fermentation of organic compounds and by ion transport mechanisms rather than by oxidative processes involving oxygen (Andreesen et al. 1989). In classical clostridial fermentation processes, organic compounds serve as the terminal electron donor and also as the terminal electron acceptor (Andreesen et al. 1989; Morris 1975, 1976; Gottschalk 1986). This metabolism leads to the accumulation of reduced organic products. Traditionally, ATP was believed to be formed in clostridia solely by substrate level phosphorylation, and not by a respiratory process involving electron transport. Clostridia do not carry out dissimilatory sulfate reduction in which sulfate serves as an anaerobic electron acceptor, referred to as anaerobic respiration in certain bacteria such as *Desulfatomaculum* (Decker et al. 1970). Interestingly, the recent genome sequence of *C. tetani* shows the presence of the genes for a membrane-bound electron transport chain that were also identified previously in the genome of *C. perfringens* (Shimizu et al. 2002). It appears to have similar properties of the *Rhodobacter capsulatus*-specific nitrogen fixation system as well and to the NADH-ubiquinone oxidoreductase present in many aerobic pathogens such as *Vibrio cholerae*, *Salmonella typhyimurium*, *Yersinia pestis*, and *Haemophilus influenzae*, where it acts as a Na^+-translocating NADH dehydrogenase. However, since quinones apparently are not present in clostridia, it was postulated that the electron transport chain, if functional, could be involved in amino acid degradation (Brüggemann et al. 2003). It was also surprising to find abundant genes related to sodium ion transport and their extensive involvement in intermediary metabolism. *C. tetani* is unusual in that a vacuolar (V)-type ATPase appears to be the primary source for ion-motive force at the cytoplasmic membrane, and it did not possess the F_0F_1-type ATPase common in many other bacteria including *C. perfringens* and *C. acetobutylicum*. Thus, a mainstay of energy metabolism in *C. tetani* is the hydrolysis of ATP formed during fermentative metabolism by the V-type ATPase to generate a Na^+-motive force at the membrane. This sodium ion gradient would be used to drive various metabolic processes.

Since clostridia cannot use molecular oxygen and only thrive in its absence, they usually are unable to cope with oxygen or toxic oxygen derivatives (reactive oxygen species (ROS)). The clostridia, in general, lack certain protective enzymes such as catalase, although low levels of superoxide dismutase have been detected in certain strains (Morris 1975, 1976). In addition to being toxic to essential cellular components, oxygen may interfere with metabolism by oxidizing NADH through reactions catalyzed by NADH oxidases which many clostridia possess in substantial quantities (Andreesen et al. 1989). For many clostridia, oxygen seems to be bacteriostatic instead of bacteriocidal. Agar plate cultures of *C. butyricum* or certain types of *C. botulinum* can be removed from the anaerobic chamber for an hour or more, and growth of colonies will resume when they are returned to the chamber. Sometimes oxygen resistance is attributed to the recalcitrance of the spores, noting the normal presence of clostridia in soils and the viability of spores in dust carried by the wind. For germination and growth, spores or vegetative cells of most clostridia require a diminished oxygen level. Some attribute the inhibitory effect of oxygen to an increased oxygen reduction potential (E_h), because many anaerobic bacteria can grow in the presence of air provided a sufficiently low E_h is established in the medium. Such conditions are achieved by the use of reducing substrates, some of which act mainly by absorbing oxygen, others by establishing a low E_h after molecular oxygen has been nearly depleted or removed by physical means (Willis 1990). However, Morris (1975) pointed out the unreliability of E_h measurements in culture media and showed evidence that the inhibitory effect is possibly more dependent on oxygen than on E_h (Morris 1975). Media for culturing the clostridia usually contain reducing compounds such as cysteine, thioglycolate, glutathione, sulfites, or reduced iron compounds. Cooked meat medium, in addition to providing many essential nutrients and an environment conducive to sporulation and spore germination of vegetative clostridia, maintains anaerobic conditions through the binding or inactivation of oxygen by inherent compounds including hematin and sulfhydryl reducing compounds which bring about a negative oxidation reduction potential (Willis 1990). In many natural

environments such as foods, associated facultative or aerobic microflora will rapidly utilize oxygen, thus creating a reduced environment conducive to growth of clostridia in media and in foods (ICMSF 1980; Glass and Johnson 2001; Johnson 1999a; Larson and Johnson 1999).

Metabolism of carbohydrates, proteins, and nucleotide bases

Many clostridia have a strong capability to breakdown and catabolize many polymeric compounds including polysaccharides and proteins. Polysaccharides may be broken down to their sugar constituents, which are then utilized by the Embden–Meyerhof–Parnas pathway (Andreesen et al. 1989; Gottschalk 1986). Fermentation endproducts formed through the central intermediate of pyruvate are acetate, butyrate, lactate, and ethanol (Holdeman et al. 1977). Other alcohols such as isopropanol, butanol, and 1,2-propanediol may be formed by some species under certain conditions. Proteins are digested by proteolytic clostridia (including *C. tetani* and most serotypes of *C. botulinum*) and serve as a source of amino acids. The clostridia are diverse in their utilization of these as energy sources, as is evidenced by the variety of enzymes involved and the variety of metabolic endproducts (Holdeman et al. 1977). Certain amino acids are fermented singly, and can serve as sole carbon, nitrogen, and energy sources (Andreesen et al. 1989; Gottschalk 1986; Thauer et al. 1977). However, the fermentation of amino acids has traditionally been described as taking place in pairs with one serving as electron donor and the other as electron acceptor in what is known as the Stickland reaction (Stickland 1934; Gottschalk 1986). The electron donor loses one carbon while the electron acceptor maintains its original carbon chain length. In general, alanine, leucine, isoleucine, valine, histidine, tyrosine, phenyalanine, and tyrosine serve as electron donors; and glycine, proline, hydroxyproline, ornithine, and arginine as electron acceptors (Andreesen et al. 1989; Gottschalk 1986). However, some amino acids such as leucine, phenylalanine, and tryptophan can serve as either donor or acceptor for certain organisms. Straight chain amino acids are converted to isobutyrate, isovalerate, and isocaproate. Phenylalanine is converted to phenylpropionate, phenylacetate, and phenol, while tyrosine and tryptophan are metabolized to their hydroxyphenyl and indole equivalents, respectively (Andreesen et al. 1989; Gottschalk 1986). Although these schemes have traditionally been believed to be the main pathways for fermentation of amino acids, the recent elucidation of the genome sequence suggests a very different paradigm for the catabolism of amino acids in *C. tetani* and likely other clostridia. In this revised general pathway for the fermentation of amino acids, they are mainly transported into the cell by Na+ gradient mediated force, and most of the individual amino acids are then converted to pyruvate. The electrons are disposed of through oxidation of reduced ferredoxin and an electron transport system (Brüggemann et al. 2003). The roles of these different systems in amino acid catabolism and energy generation remains to be firmly established. Many clostridia can ferment purines and pyrimidines. The catabolism of these nucleotide bases follows pathways through conversion to glycine and b-alanine intermediates, respectively, and then to acetate and related endproducts.

DESCRIPTIONS OF THE SPECIES *C. BOTULINUM*, *C. TETANI*, AND OTHER NEUROTOXIGENIC CLOSTRIDIA

General description of *C. tetani*

C. tetani is one of the best known clostridia because it is the cause of the vivid disease referred to as tetanus (Adams et al. 1969; Bleck 1989, 1991; Faber 1890). It was probably first isolated by Kitasato from a patient with tetanus in 1889 (Kitasato 1889, 1891; Bytchenko 1981). Its physiological properties have been described (Smith 1975; Holdeman et al. 1977; Willis 1969, 1977). The mol% G+C content of the DNA is 25–26 percent (Cummins and Johnson 1971). It is a strict anaerobe, and will grow on the surface of agar media only in an anaerobic environment. On the basis of 16S rRNA gene homology, the organism is placed in the clostridial ribosomal RNA cluster 1 (Johnson and Francis 1975), and is closely related to *C. cochlearium* (Collins et al. 1994). The bacilli are 0.5–1.7 × 2.1–18.1 μm and often possess terminal endospores that give the sporing cells a drumstick appearance. Cells in cultures older than 24 h may appear gram-negative. *C. tetani* is motile by peritrichous flagella. Optimal growth occurs at 37°C, and little or no growth occurs at 25 or 42°C. Growth may not be apparent until after 48 h and may appear as a film rather than as discrete colonies because of the organism's vigorous motility and swarming. Media containing 3–4 percent agar are more conducive to formation of discrete colonies. On blood agar, the colonies are 4–6 mm in diameter, flat, translucent, gray with a matt surface, showing a narrow zone of clear (β-type) hemolysis. Colonies have irregular and rhizoid margins (Cato et al. 1986; Finegold 1998; Willis 1969, 1977). On clear media such as nutrient agar, colonies have a granular or ground glass appearance when viewed in transmitted light. Colonies do not form an opalescence or pearly layer indicative of lipase activity on egg yolk agar.

Most common biochemical tests for *C. tetani* are negative. Sugars are not fermented; milk and other protein complexes such as cooked meat are not digested. Neither lecithinase nor lipase is produced and nitrate is not reduced. Gelatin is slowly liquefied, requiring 7 or more days for complete liquefaction. Hydrogen sulfide and indole are produced. Cultures grown in

peptone-yeast extract broth cultures show endproducts of acetic, propionic, and butyric acids (Holdeman et al. 1977). Spores generally survive moderate heating (75–80°C for 10 min), but are usually destroyed after 100°C for 1 h (Adams et al. 1969; Eckmann 1967).

C. tetani is similar culturally and biochemically to *C. cochlearium* and organisms identified as *C. tetanomorphum*, but all three species could be clearly distinguished on the basis of DNA homology (Nakamura et al. 1979). Nontoxigenic strains of *C. tetani* have been found that correlate well with toxigenic strains by phenotypic and DNA comparisons, and may have lost the plasmid bearing the gene encoding tetanus toxin. Soluble proteins show different patterns (Cato and Stackebrandt 1989), but otherwise it is difficult to distinguish between the two species without DNA comparisons. Bacteriophages have been detected in *C. tetani* (Prescott and Altenbern 1967).

C. tetani is commonly found in soil samples in all parts of the world. Surveys in Japan, Canada, Brazil, and the USA have yielded 30–42 percent positive samples (Smith and Williams 1984). The organism is also commonly found in human and animal feces and different surveys have shown that *C. tetani* is present in 25–30 percent of human fecal specimens in China, England, and the USA (Bauer and Meyer 1926; Ten Broeck and Bauer 1922; Tulloch 1910–20). However, recent studies on human fecal flora failed to find *C. tetani* (George and Finegold 1985). Intestinal carriage may be responsible for naturally acquired immunity to the toxin (Veronesi et al. 1983; Griffin et al. 1997). Antitoxin, but not toxin, has been found in the serum of animals experimentally colonized with *C. tetani* (Wells and Balish 1983).

Since the clinical features of tetanus are so distinct and recognizable, laboratory investigation of cases is rarely done. However, studies have been performed to show the level of antibodies in the blood of humans immunized with tetanus toxoid (Gergen et al. 1995; Middlebrook and Brown 1995). Few organisms involved in tetanus have been thoroughly studied, and the strain in which the genome is being sequenced (Brüggemann et al. 2003) is a nonsporing strain that has been used for toxoid production. Our knowledge of *C. tetani* is based almost entirely on environmental isolates, or those obtained from normal humans or animals. It would be valuable to study strains from patients and animals with tetanus, as such studies of clinical isolates could reveal important and interesting bacteriological and pathological information.

Culture and isolation

Culture and isolation of *C. tetani* from wound specimens and environmental samples can be approached by the same methods. Samples of infected tissues taken from deep wounds of tetanus patients should be satisfactory, although wound swabs may also be suitable. Cooked meat medium to which starch has been added may be superior to cooked meat + glucose since *C. tetani* does not utilize glucose, and this might provide an unnecessary energy source that may be used by competing organisms. Heat treatment for spore selection could be used to advantage. Adams et al. (1969) recommended inoculating three broth cultures, heating the first at 80°C for 15 min, the second for 5 min, and no heating for the third. Beland and Rossier (1973) suggested inoculating soil samples after suspending in sterile saline; one portion is inoculated without treatment, and the second inoculated after heating at 60°C for 10 min. After incubating for 2 days at 37°C, the broth cultures are inoculated on to a small area on one side of a blood agar plate. The plates are incubated anaerobically at 37°C, and observed at 24 and 48 h for swarming growth. The bacteria at the leading edge of the swarming growth are picked and inoculated into chopped meat broth. After incubation, the cultures are observed microscopically, tested for tetanus neurotoxin, and examined for physiological characters.

C. tetani toxins

C. tetani produces two proven toxins, tetanus neurotoxin or tetanospasmin, and an oxygen-labile hemolysin known as tetanolysin (Adams et al. 1969; Bleck 1991; Cook et al. 2001; Finegold 1998; Mitsui et al. 1980). The structure, genetics, and cell biology of tetanus neurotoxin have been well studied and excellent reviews are available (Bizzini 1979; Habermann and Dreyer 1986; Lalli et al. 2003; Matsuda 1989; Montecucco 1995; Popoff and Marvaud 1999; Robinson and Hash 1982). In the analysis of the genome of *C. tetani*, Brüggemann et al. (2003) described other putative virulence-related genes including collagenase, fibronectin-binding protein, a putative PAF acetylhydrolase, a periplasmic immunogenic protein, myosin-cross-reactive antigen, virulence factor MviN, internalin A homologue, and surface layer proteins. Many of these genes were proposed as virulence factors on the basis of their homology with virulence factors from other bacterial pathogens. The gene for tetanus neurotoxin is carried on a large plasmid (Laird et al. 1980; Eklund et al. 1989; Brüggemann et al. 2003), and some strains identified as *C. tetani* lack the plasmid and are nontoxigenic. Although nontoxigenic strains are allowed in the species, unequivocal speciation of isolates as *C. tetani* requires demonstration of toxigenicity. Toxicity and neutralization tests can be performed by intramuscular injection of 0.1 ml of culture supernatant into the rear leg muscle of a mouse; a second mouse is injected with a mixture of 0.1 ml of supernatant and 0.1 ml of tetanus antitoxin (Gill 1982; Payling Wright 1955). A positive culture will cause the

development of a rigid paralysis in the injected muscle, with subsequent progression of the paralysis into the rest of the musculature of the body. No paralysis should be observed with antitoxin neutralized samples, unless the quantity of toxin in the sample is greater than the neutralizing capacity of the antitoxin. Test animals should be euthanized as soon as a conclusive judgment can be made of paralytic signs. It is estimated that the human lethal dose of tetanus and botulinum neurotoxins are 0.2–0.5 ng/kg (Gill 1982; Payling Wright 1955; Schantz and Johnson 1992), and extreme caution is needed in handling these toxins.

Tetanolysin is related functionally, serologically, and in nucleotide sequence to streptolysin-O and the oxygen-sensitive hemolysin of a variety of other organisms including *C. perfingens* (perfringolysin-O), and *Listeria monocytogenes* (Bernheimer 1976; Brüggemann et al. 2003). Purified tetanolysin-O has a molecular mass of 48 ± 3 kDa and appears to have several isoforms based on pI (Lucain and Piffaretti 1977; Mitsui et al. 1980). Some of the isoelectric point variation may be due to differences in charge between oxidized and reduced forms of the same molecule (Cowell et al. 1976). This group of hemolysins lyses a variety of cells such as erythrocytes, polymorphonuclear leukocytes, macrophages, fibroblasts, ascites, tumor cells, HeLa cells, and platelets (Bernheimer 1976). Lytic activity is reversibly inhibited by mild oxidizing conditions such as aeration or low concentrations of hydrogen peroxide, and reactivated by thiol reagents such as cysteine, reduced glutathione, or thioglycollate. The active toxin has an affinity for cholesterol and related sterols, and its lytic and lethal activities are inhibited by these compounds. Hardegree et al. (1971) showed in vivo effects of tetanolysin after intravenous injection into animals. In mice, pulmonary edema rapidly developed, followed by death. In rabbits and monkeys intravascular hemolysis was noted. Monkeys showed electocardiographic changes and a decreased heart rate indicating a cardiotoxic effect of tetanolysin. It is debatable if this toxin plays a role in *C. tetani* infections. The levels of tetanolysin produced by the organism in vivo is unknown, and serum that contains cholesterol is known to inhibit its activity in vitro.

General description of *C. botulinum*

C. botulinum and associated neurotoxigenic clostridia produce seven distinct serotypes of neurotoxins, A, B, C_1, D, E, F, and G. Although these clostridia differ widely in phenotypic and genetic relatedness, they all have the common property of producing a characteristic neurotoxic protein (DasGupta 1989; Smith and Sugiyama 1988; Sakaguchi 1983; Sugiyama 1980). The human lethal dose of botulinum neurotoxin is estimated to be as little as 0.1–0.5 ng/kg (Dolman 1964; Gill 1982; Meyer and Eddie 1951; Morton 1961; Schantz and Johnson 1992) and extreme caution is needed in working with the organism and its toxins (CDC 1998a; Malizio et al. 2000). The demonstration of botulinum toxicity by the mouse bioassay (Schantz and Kautter 1978) is essential to identify toxigenic strains (Hatheway 1988; Schantz and Johnson 1992; Solomon et al. 2001). In addition to demonstration of neurotoxicity and neutralization by serotype-specific antitoxins, the organsism has traditionally been identified and differentiated on the basis of the cultural and physiological characteristics. *C. botulinum* was originally divided into three metabolic groups by Holdeman and Brooks (1970), but with the discovery of *C. botulinum* type G (*C. argentinense*) (Giménez and Ciccarelli 1970a, b), a fourth metabolic group was added (Suen et al. 1988a). Organisms in groups I–III are motile, produce lipase as demonstrated on egg yolk agar, liquefy gelatin, and ferment glucose. *C. botulinum* type G (*C. argentinense*, Group IV) are nonsaccharolytic and proteolytic. Within the species *C. botulinum*, there exists variation from strain to strain and there is no characteristic morphology for distinguishing between physiological groups or toxin types. The basic properties of the Groups I–IV of *C. botulinum* and the neurotoxigenic organisms *C. butyricum* producing E-like neurotoxin and *C. baratii* producing F-like neurotoxin are presented in Table 1.

Groups of *C. botulinum*

GROUP I: *C. BOTULINUM* TYPE A, PROTEOLYTIC STRAINS OF TYPES B AND F

The GC content is 26–28 mol% (Cummins and Johnson 1971; Johnson and Francis 1975; Lee and Riemann 1970a, b). The genome size of *C. botulinum* type A (strain 62A) was initially estimated as $4\,039 \pm 40$ kbp as determined by pulsed field gel electrophoresis (Lin and Johnson 1995), whilst the genome size of Hall strain A (ATCC 3502), has been reported as 3 886 916 bp by genomic sequencing (www.sanger.ac.uk/Projects/C_botulinum). Although strains are commonly referred to by their source of isolation (e.g. Beans B, Beluga E) or by the person who isolated the strain (e.g. Hall A (Hall 1945)), different strains with this same acronym can show marked differences in genetic composition (Johnson and Bradshaw 2001; Lin and Johnson 1995) and caution must be exercised in assuming that strains described by the same acronym are identical. In fact, studies have shown considerable genetic variation in strains such as 'Hall A' (Lin and Johnson 1995). It would be valuable to have defined international reference strains for each serotype and group, preferably with their genome sequence known for comparative studies.

In TPGY broth cells are straight to slightly curved rods, $0.6\text{-}1.4 \times 3.0\text{–}20.2$ μm, and are motile by peritrichous flagella. Spores are oval and subterminal and

swell the rod. Sporulation is stimulated by zinc in the medium (Kihm et al. 1988) and the cells can accumulate high concentrations of transition metals compared to *Bacillus* sp. (Kihm et al. 1990). Colonies on blood agar are 2–6 mm in diameter, grayish, often irregular with a rhizoid perimeter, and are β-hemolytic. Group I *C. botulinum* have been isolated from soils, marine and freshwater sediments, foods, and the intestinal contents of humans, animals, birds, and fish. The incidence of spores in the environment shows an intriguing geographical distribution (Dodds 1993; Hauschild 1992; Johnson and Goodnough 1998; Popoff 1995).

Early studies suggested that *C. botulinum* and *C. sporogenes* could not be distinguished phenotypically. They were also demonstrated to share surface antigens (Gunnison and Meyer 1929a, b). *C. botulinum* type A and proteolytic strains showed high DNA homology with certain strains of *C. sporogenes* (Lee and Riemann 1970b; Wu et al. 1972). The two species also share many phenotypic properties. Later investigations showed that the DNA homology of two colony types of *C. sporogenes* differed in their DNA homology to *C. botulinum* (Nakamura et al. 1977). The DNA from strains forming circular or crenate colonies with matt to semi-glossy surface was 81 percent or more homologous to type A *C. botulinum* (strain A190), whilst those forming umbonate and rhizoidal colonies sporulated readily and the DNA differed from *C. botulinum* by 30–40 percent. *C. botulinum* and *C. sporogenes* also differ in cellular electrophoretic patterns (Cato et al. 1982a), cellular fatty acid content (Ghanem et al. 1991), chromatographic profiles of trimethylsilyl derivatives of whole cell hydrolysates, and sensitivity to lysins and mitomycin C (Kiritani et al. 1973).

Nutritional growth factors required for *C. botulinum* types A and B are arginine and phenyalanine (high concentrations) and biosynthesis concentrations of methionine, leucine, valine, isoleucine, glycine, histidine, tryptophan, tyrosine, and the vitamins pyridoxamine, p-aminobenzoic acid, biotin, nicotinic acid, and thiamine (Kindler et al. 1956; Whitmer and Johnson 1988). The ten required amino acids can be replaced with proteins by virtue of the organism's ability to synthesize proteases. Growth is stimulated by fermentable carbohydrates including D-fructose, D-glucose, glycerol, maltose, pyruvate, sorbitol, sucrose, and starch (Whitmer and Johnson 1988). The aromatic amino acids phenylalanine, tyrosine, and tryptophan are reduced to phenylpropionic, p-hydroxyphenylpropionic, and indolepropionic acids (Elsden and Hilton 1978, 1979). Arginine is the organic nutrient required in highest concentrations by group I *C. botulinum* and strongly represses toxin and protease formation in *C. botulinum* types A and B (Patterson-Curtis and Johnson 1989, 1992). Arginine promotes toxin formation by *C. botulinum* in moderately acidic foods (Malizio et al. 1993; Patterson-Curtis and Johnson 1992) and probably also protects the bacterium against environmental stresses including acidity and high osmolarity (Patterson-Curtis and Johnson 1992).

Fermentation products include acetic and butyric acids, hydrogen gas, and smaller quantities of propionic, isobutyric, isovaleric, and propyl, isobutyl, butyl, and isoamyl alcohols (Cato et al. 1986). The major acids are derived from carbohydrate fermentations and the minor components primarily from amino acid utilization. Ethanol, butanol, and deaminated amino acids including propionate, isobutyric, isovaleric, and hydroxycinnamic acids are produced in the appropriate culture conditions (Elsden et al. 1976; Elsden and Hilton 1978, 1979; Moss et al. 1970). *C. botulinum* type A and proteolytic type B possess a very active NAD+-glutamate dehydrogenase which is involved in amino acid utilization, release of ammonia, generation of a-ketoglutarate for biosynthesis, and protection against osmotic stress (Hammer and Johnson 1988).

The optimum temperature for growth of Group I *C. botulinum* is 35–40°C, and the minimum and maximum are 10 and 48°C, respectively (these and other physiological parameters are reviewed in Glass and Johnson (2001), Hobbs (1976), Kim and Foegeding (1993), Smith and Sugiyama (1988), and ICMSF (1996)). Most strains grow poorly below 15–20°C. The minimum pH for growth is 4.6–4.7 (Townsend et al. 1954) and the maximum is 8–9. Some reports indicate that *C. botulinum* type A can grow in laboratory media containing high levels of soya or milk protein at pH <4.6 (Raatjes and Smelt 1979). Growth or toxin formation has not been detected in commercial foods at pH <4.6. Fungi can catabolize organic acids in certain foods such as tomatoes (Huhtanen et al. 1976; Meyer and Gunnison 1929; Odlaug and Pflug 1978, 1979), raising the pH and enabling *C. botulinum* to grow in foods that had an initial pH less than 4.6. Acidification of a food can require several days for acid to equilibrate throughout the food (Sugiyama and Sofos 1988). The minimum A_w for growth of Group I *C. botulinum* is 0.94, or a brine content of 10 percent. As with the other environmental constraints, only rare strains will grow at A_w 0.94. Environmental constraints interact, and inhibitory conditions can be obtained in foods by combining subinhibitory levels of pH, salt, and other factors such as preservatives (Lynt et al. 1982; Glass and Johnson 2001; Rhodehamel et al. 1992; Sugiyama and Sofos 1988). The critical level of oxygen that will permit growth of group I *C. botulinum* is 1–2 percent (Meyer 1924; Whiting and Naftulin 1992), but this depends on other conditions such as A_w and pH.

Group I strains of *C. botulinum* associated with foodborne botulism and those associated with infant botulism in Japan have distinct genetic characteristics in their toxin complexes (Dineen 2003; Kozaki et al. 1974; Johnson and Bradshaw 2001; Sakaguchi et al. 1990; Tabita et al. 1990, 1991). The infant botulism strains initially characterized from Japan did not produce

hemagglutinin and were reported to have a toxin complex comprised only of neurotoxin and nontoxic nonhemagglutinating protein (NTNH). Surpisingly, they showed lower infective doses when fed orally to infant mice even though they lacked the protective hemagglutinin component. The infant strains more readily colonized infant mouse intestines and more rapidly produced toxin intraintestinally than food-borne strains. In contrast to the Japanese strains responsible for infant botulism, most *C. botulinum* strains from California causing infant botulism produced hemagglutinin (Tabita et al. 1990). Strain Kyoto-F, from infant botulism in Japan, was found to be closely related to *C. botulinum* from food-borne botulism, having 16S rRNA similarity of approximately 99.9 percent, but the amino acid sequences of the two neurotoxins had approximately 10 percent sequence divergence (Willems et al. 1993). However, the toxin complexes from strains that contain the classic type A complex including hemagglutinin (type I) (Johnson and Bradshaw 2001; Dineen 2003) differ in their structure from complexes derived from strains lacking hemagglutinin (type II), and the neurotoxins differ considerably in their amino acid sequence (~190 amino acids). Recent evidence shows that they also have structural differences that affect their neutralization by monoclonal antibodies produced against type I (unpublished data from E.A.J.'s laboratory).

C. botulinum has resistance and growth properties that require precautions to prevent it from growing in low acid foods (pH >4.6 and A_w >0.85) (Glass and Johnson 2001; Lund and Peck 2000; Setlow and Johnson 2001). Spores of group I *C. botulinum* have heat resistances ranging from D121°C = 0.03–0.23 min (ICMSF 1980) and D100°C ~30 min (Esty and Meyer 1922; Setlow and Johnson 2001; Smith and Sugiyama 1988). Certain strains of *C. sporogenes* produce spores with higher heat resistance (maximum D121°C ~1–2 min) than *C. botulinum*, and these strains can be used to determine the heat treatment required for obtaining a 12D inactivation or total lethality (F_0) as is recommended for shelf-stable low acid foods in cans, glass jars, or pouches. The required treatment for achieving F_0 of a food from *C. botulinum* spores is c. 3 min at 121°C or higher (Setlow and Johnson 2001). The commercial processing of certain foods is less than a F_0 of 3 min since other factors control their safety from *C. botulinum* (Rhodehamel et al. 1992; Sugiyama and Sofos 1988). Although used as a surrogate, caution must be exercised in concluding that growth of *C. sporogenes* in foods and its resistance properties will be identical to group I *C. botulinum*. In designing a process or food formulation to control *C. botulinum*, challenge studies using *C. botulinum* in the food should be performed to assure safety (Glass and Johnson 2001).

Organisms inhibiting *C. botulinum* or destroying its toxins in culture have been isolated from soil (Hall and Peterson 1922; Kautter et al. 1966; Smith 1975). *C. perfringens*, *C. sporogenes*, and *Bacillus* spp. have been shown to be inhibitory. Bacteriophages active on group I *C. botulinum* have also been isolated (Dolman and Chang 1972; Ogata and Hongo 1979; Smith 1977) but they have not been linked to toxin production or other phenotypic properties in group I *C. botulinum* (Eklund et al. 1989). Bacteriocins have been demonstrated in group I *C. botulinum* that inhibit other strains in this group (Kautter et al. 1966; Dineen et al. 2000).

GROUP II: *C. BOTULINUM* TYPE E AND NONPROTEOLYTIC TYPES E, B, AND F

The '*Bacillus botulinus*' originally isolated by van Ermengem (1897) was probably nonproteolytic *C. botulinum* type B (Group II) as indicated by van Ermengem's report and subsequent studies of Meyer and Gunnison (1929). The G+C content of group II *C. botulinum* is 27–29 mol%. The molecular size of genomic DNA does not appear to be available at this time. Cells in TPGY are straight rods, 0.8–1.6 × 1.7–15.7 μm, and are motile by peritrichous flagella (Cato et al. 1986). Spores are oval, subterminal, and generally swell the rod. Spores of type E have characteristic appendages and an exosporium, while spores of type F have an exosporium but lack appendages. Spores are readily obtained from *C. botulinum* type E, but are more difficult to obtain from nonproteolytic types B and F. Prolonged culture in cooked meat medium often yields spores in these strains. Colonies on blood agar are grayish, 1–5 mm in diameter, have mottled or mosaic internal appearance, and a rhizoid or irregular margin.

Cultures are saccharolytic and weakly or nonproteolytic. Gelatin is liquefied but casein or meat particles are not digested. Growth of Group II *C. botulinum* is stimulated by fermentable sugars. *C. botulinum* type E requires glucose or another carbohydrate energy source for growth and does not utilize arginine or intact protein as a source of energy (Whitmer and Johnson 1988). *C. botulinum* type E can use fructose, glucose, maltose, mannose, D-ribose, sorbitol, sucrose, and starch as energy sources (Whitmer and Johnson 1988). *C. botulinum* type E requires biosynthesis levels of seven amino acids (histidine, isoleucine, leucine, tryptophan, tyrosine, valine, and serine), adenine, and six vitamins (biotin, thimaine, pyridoxamine, folic acid, choline, and nicotamide). This organism utilizes ammonia as a nitrogen source, although growth is stimulated by organic nitrogenous nutrients, especially glutamate and asparagine. Neurotoxin formation was strongly repressed by tryptophan in a minimal medium (Leyer and Johnson 1990). The nutritional requirements of nonproteolytic *C. botulinum* type B unexpectedly differed from those required by *C. botulinum* type E. In contrast to type E, nonproteolytic type B required arginine and did not grow in a minimal medium that supported growth of type E

(Whitmer and Johnson 1988). Its nutritional requirements were more closely related to proteolytic strains of *C. botulinum* type A and B. The primary fermentation products of group II *C. botulinum* are butyric and acetic acids and hydrogen gas.

The optimum temperature for growth of Group II *C. botulinum* is 25–30°C, and the minimum temperature for growth of types B and E is 3–4°C (Dolman and Iida 1963; Eklund et al. 1967; Lynt et al. 1982; Ohye and Scott 1957). Most strains grow very poorly below ~7°C. The minimum pH for growth is 5.0 and the maximum ~8. The minimum A_w for growth of Group II *C. botulinum* is 0.97, and growth is inhibited by 5 percent brine. Growth is also inhibited by bile. Spores of Group II *C. botulinum* have relatively low heat resistance compared to Group I. Spores of *C. botulinum* 17B had a D82.2°C ranging from 1.49 to 32.2 min depending on the strain (Scott and Bernard 1982; Lynt et al. 1982). Strains of *C. botulinum* type E had D77°C ranging from 0.77 to 1.95 min (Ito et al. 1968) and D80°C ranging from 0.33 to 1.8 min. Scott and Bernard (1982) reported that they were able to obtain spores of a specific strain of *C. botulinum* type E with a D82.2°C greater than 4.9 min. Little is known of the factors contributing to heat resistance of *C. botulinum* spores, though their composition is probably different from spores of *Bacillus* (Kihm et al. 1990). Spore recovery can be enhanced by using lysozyme in the recovery medium which substitutes for lytic enzymes that are denatured during heating (Lund and Peck 2000).

Cultures in group II *C. botulinum* share somatic and spore antigens (Lynt et al. 1967, 1982), and agglutination to fluorescent antibody tests have been useful for identifying type E (Lynt et al. 1982; Nakamura et al. 1956; Walker and Batty 1964). Similarities in somatic antigens have been useful in identifying nontoxigenic 'botulinum-like' strains similar to types A, B, and E (Lynt et al. 1982). Antisera to somatic antigens of type E and nonproteolytic strains of types B and F cross-reacted with somatic antigens of these three types but not with type A or proteolytic strains of types B and F. Similarly, antisera to type A and the proteolytic strains of types B and F cross-reacted among each other but not with group II strains. The antigens from groups I and II also did not cross-react with other species of clostridia examined. Similar results were found for spore antigens (Lynt et al. 1982; Solomon et al. 1971), which differ from antigens on the surface of vegetative cells.

Strains of *C. botulinum* type E were reported to be inhibited by *C. perfringens*, while nontoxigenic strains were resistant (Smith 1975). Kautter et al. (1966) showed that type E-like organisms were antagonistic to *C. botulinum* type E and caused their lysis. Proteolytic strains of *C. botulinum* types A, B, and F were resistant. Two classes of inhibitory substances were partially purified and characterized (Kautter et al. 1966). One inhibitory factor was heat stable, dialyzable, inactivated by trypsin, and had a molecular weight less than 30 000 Da. Another inhibitory factor was less heat resistant and was reported to have a molecular weight of 4×10^7 Da. A bacteriocin-like substance was isolated from a nontoxigenic type E-like strain which was proposed to resemble a defective phage (Lau et al. 1974). Bacteriophages have been isolated from non-proteolytic *C. botulinum* (Sugiyama and King 1972). The isolation of nontoxigenic *C. botulinum* E-like strains has facilitated the study of neurotoxin gene transfer that probably occurs in nature. In one such study, the transfer of neurotoxigenicity from *C. butyricum* to an E-like strain was observed, and probably occurred through infection by a defective bacteriophage carrying the toxin genes (Zhou et al. 1993).

Clostridium butyricum strains that produce a neurotoxin neutralized by type E antitoxin were isolated from infant botulism cases in Rome, Italy (Suen et al. 1988b), and several isolates have subsequently been discovered in various geographical regions (Anniballi et al. 2002; Chaudry et al. 1998; Fenicia et al. 1999, 2002; Meng et al. 1997, 1999; Tsukamoto et al. 2002; Wang et al. 2000). DNA homology studies have shown that the isolated cultures are toxic variants of normally nontoxigenic *C. butyricum*. The isolation of toxigenic *C. butyricum* strains indicate that the genes encoding the botulinum neurotoxin complexes can be transferred to normally nontoxigenic species. Defective bacteriophages (Zhou et al. 1993) and/or plasmids (Hauser et al. 1992, 1995) may be involved in the interspecies transfer of the genes for botulinum neurotoxin and complexing proteins. Such interspecies transfers of toxin genes could have considerable significance for the food industry and medical microbiology. More research is needed to elucidate the mechanisms of toxin gene transfer and molecular mechanisms of loss of toxigenicity in groups I, II, and IV of *C. botulinum* and in *C. tetani*.

GROUP III: *CLOSTRIDIUM BOTULINUM* TYPES C AND D

The mol% G+C content of the DNA is 26–28. Cells in TPGY broth appear as straight rods, 0.5–2.4 × 3.0–22.0 μm and are motile by peritrichous flagella. Spores are oval, subterminal, and distend the cell. Surface colonies are circular, slightly raised and irregular, grayish white, with a mottled or mosaic internal appearance (Cato et al. 1986). On egg yolk agar certain strains of *C. botulinum* type C, and particularly the related organism *C. novyi*, produce a yellowish opacity surrounding the colonies due to lecithinase activity. This opacity is distinguished from lipase as it extends beyond the pearly zone located over and immediately around the colonies (Holdeman and Brooks 1970). This colonial pattern is similar but the lecithinase activity is less pronounced than observed in *C. novyi*.

C. botulinum types C and D and related nontoxigenic strains comprise a heterogenous group that are phylogenetically related according to DNA sequences encoding rRNA (Collins et al. 1994; Collins and East 1998; Hutson et al. 1993), whilst some strains have only 38 percent DNA relatedness (Lee and Riemann 1970a). Cultures of *C. botulinum* type C and D exhibit an interesting cross relation of bacteriophage sensitivity. Bacteriophages determining toxin production can cross-infect the two serotypes and strains cured of specific bacteriophages no longer producing toxin (Eklund et al. 1989). Holdeman and Brooks (1970) recognized that toxicity was unstable in *C. botulinum* type C and that nontoxigenic strains were indistinguishable from *C. novyi* type A. Two genetically different groups have been recognized in *C. botulinum* type C (Lee and Riemann 1970a; Nakamura et al. 1983). *C. botulinum* type C Stockholm had high genetic relatedness to *C. novyi* type B and *C. haemolyticum*. The four additional *C. botulinum* type C strains tested, all isolates from marine sediments, had low homology with *C. botulinum* type C Stockholm and *C. novyi* and *C. haemolyticum* but >91 percent homology with *C. botulinum* type C 85–8. The two *C. botulinum* type C groups also had different cultural properties (Nakamura et al. 1983; Oguma et al. 1986). The Stockholm group digested milk and produced indole and 85–8, but not the Stockholm group, fermented melibiose and galactose. Both groups produced lecithinase activity that was neutralized by antitoxin serum raised against *C. novyi*. *C. botulinum* type C could be distinguished from *C. novyi* by patterns of protein banding following gel electrophoretis (Cato et al. 1982a). Oguma et al. (1986) classified 11 toxigenic strains of *C. botulinum* types C and D and ten nontoxigenic derivatives into four groups based primarily on sugar fermentation profiles. The resulting groups were identical to the classification proposed for grouping by antigenicity of converting phages (Oguma et al. 1976b).

Cultures within group III are weakly nonproteolytic and they liquefy gelatin and some strains curdle casein but they do not digest meat (Holdeman and Brooks 1970). Growth is stimulated by fermentable carbohydrates with a reduction in pH to 5.2–5.7 in PYG broth. The group produces a unique pattern of endproducts including acetic, butyric, and propionic acids from glucose and lactate, acetic and butyric acids from pyruvate. *C. botulinum* type D and *C. novyi* have the distinct feature among tested anaerobes of producing propionate as a major endproduct from lactate (Holdeman and Brooks 1970). Strains of type C also catabolize glutamic acid, glycine, arginine, aspartic acid, and some strains convert threonine to propionate (Mead 1971). Lecithinase, indole formation, and production of hydrogen sulfide vary with the strains (Oguma et al. 1986).

Growth of group III *C. botulinum* can occur between 15 and 47°C, but optimum growth occurs at 35–40°C. Most strains do not grow below 25°C. The optimum pH ranges for germination of spores is 6.2–7.3 (Holdeman 1970), and like most neurotoxigenic clostridia, the optimum is from 6.6–7.2. Strains of *C. botulinum* type C are inhibited by soil isolates of *Bacillus*, probably by peptide antibiotics (Graham 1978).

Production of botulinal C_1 and D neurotoxins is dependent on bacteriophage infection (reviewed by Eklund et al. 1989). The genes for C_1 and D neurotoxins are carried by pseudolysogenic bacteriophages (Eklund et al. 1974, 1976, 1987, 1989; Inoue and Iida 1970; Oguma et al. 1976a, b). Other genes encoding proteins in type C and D botulinum toxin complexes (Inoue et al. 1996; Oguma et al. 1995; Sakaguchi 1983; Sugiyama 1980; Mutoh et al. 2003) including hemagglutinin (Oguma et al. 1976a) and nontoxin nonhemagglutinin (Hauser et al. 1993) are also associated with bacteriophage infection. For many years, *C. botulinum* type C was designated C alpha and C beta on the basis of complex toxin neutralization patterns found in early studies (Gunnison and Meyer 1929b). Owing to bacteriophage interconversion, strains of *C. botulinum* types C and D can produce both C_1 and D botulinal neurotoxins, and C_2 and C_3 toxins. Cultures that produce all three toxins were referred to as C alpha strains, while those producing only C_2 were C beta strains. Curing strains of their prophages yields cultures that make either C_2 or C_3 toxins. The genes for C_1 neurotoxin and C_3 exoenzyme are associated with bacteriophages (Eklund et al. 1989; Hauser et al. 1993). Because of the varied nature and variety of toxins produced, species and subspecies identification requires careful differentiation by toxin neutralization with antitoxins raised against highly purified toxins.

C. botulinum types C and D have frequently been isolated from marine and freshwater sediments, occasionally from birds and mammals not showing signs of botulism, and in large numbers from wild or domestic birds and their environment during outbreaks of botulism (Eklund and Dowell 1987). Examination of isolates suggests that marine and terrestrial groups of *C. botulinum* type C may differ in genetic and phenotypic properties (Segner et al. 1971). Animals vary in their susceptibility to type C and type D botulinal neurotoxins, to C_2 and C_3 toxins, and to intestinal infection. Monkeys, mice, and rabbits are very susceptible to type C botulinal toxin, and the lethal quantity in these animals ranges from 0.4 to 1 ng (Gill 1982; Prevot and Brygoo 1953; Payling Wright 1955). C_2 toxin has a unique structure as a binary toxin. It has cytotoxic activity in mice (Eklund et al. 1989) and also causes massive pulmonary edema in ducks. C_2 toxin causes vascular permeability, blood volume depletion, and fatal hypotension in mice (Ohishi 1983; Simpson 1984). C_3 toxin is an ADP-ribosyltranferase that acts on Rho protein, a member of the RAS superfamily of signaling proteins (Chardin et al. 1989). Like many clostridial toxins, C_1 and C_2 toxins can be activated by proteolysis (Sugiyama 1980).

C. BOTULINUM GROUP IV: *C. ARGENTINENSE* (SIC *C. BOTULINUM* TYPE G), CERTAIN STRAINS OF *C. SUBTERMINALE* AND *C. HASTIFORME* (GROUP IV)

Group IV comprises organisms producing botulinum neurotoxin serotype G. The organism originally isolated by Giménez and Ciccarelli in 1969 from Argentinian soils (Ciccarelli et al. 1977; Giménez and Ciccarelli 1970a, b) has phenotypic properties widely distinct from other neurotoxigenic clostridia. *C. botulinum* type G was later reported from soils in Switzerland (Sonnabend et al. 1981, 1987) and from necropsy specimens of four adults and one infant, but its involvement in human or animal botulism has not been confirmed. Glasby and Hatheway (1983) initially showed a serologic relationship between *C. botulinum* type G and some strains of *C. subterminale* and *C. hastiforme*. *C. subterminale* resembles *C. botulinum* type G genotypically and phenotypically except for neurotoxin formation. Later, *Clostridium argentinense* (species novum) was proposed for organisms related to type G *C. botulinum* (Suen et al. 1988a). All *C. botulinum* type G isolates examined were closely related to each other. Although toxigenic strains of *C. argentinense* have only been isolated from Argentina, nontoxigenic strains of the species have been obtained from clinical specimens including wound samples and blood cultures.

The G+C content of the DNA of *C. botulinum* type G is 28–30 mol% (Suen et al. 1988a). The size of its genomic DNA has not been determined. Cells in broth are straight to slightly curved rods, 0.5–2.0 μm wide and 1.6–9.4 μm long, motile by peritrichous flagella, and gram-positive (Giménez and Ciccarelli, 1970a, b; Suen et al. 1988a). Two colony types are observed on blood agar, one type showing small (1–4 mm), smooth, shiny, circular, or slightly irregular colonies. Another colony type is large and rough, resembling a fried egg, but this colony type is not phenotypically different from the smaller variant (Ciccarelli et al. 1977). On rabbit blood agar the colonies are β-hemolytic. The organism has unusual phenotypic properties compared to other neurotoxigenic clostridia and is weakly proteolytic and asaccharolytic (Giménez and Ciccarelli 1970a, b; Ciccarelli et al. 1977). The strain examined (strain 89) did not produce acid or gas from all sugars or carbohydrates examined. The primary volatile acid endproducts were acetic, isobutyric, butyric, and isovaleric. Pyruvate was assimilated and converted to ethyl alcohol and acetate. Valine is converted to isobutyric acid, leucine to isovaleric acid (Elsden and Hilton 1978), lysine is utilized, and indoleacetic acid is produced from tryptophan (Elsden and Hilton 1979). Abundant hydrogen gas, hydrogen sulfide, and hydroxyphenylacetic acid are formed. This unusual complement of endproducts reflects the proteolytic and asaccharolytic metabolism of *C. argentinense*. *C. botulinum* type G is also negative for lecithinase and lipase, which is unusual among the botulinal producing clostridia (Ciccarelli et al. 1977).

The organism produces very low titers of type G botulinal toxin in culture (Giménez and Ciccarelli 1970a, b; Nukina et al. 1988). The neurotoxin can be activated by proteolysis (Nukina et al. 1988), has a molecular size of 144 000 Da, and a specific toxicity of ca. 1×10^7 LD_{50} per mg in mice (Schiavo et al. 1994). The genes for *Clostridium botulinum* type G toxin complex and for a bacteriocin are on a plasmid (Eklund et al. 1988; Zhou et al. 1995). Type G botulinum neurotoxin was lethal by oral, intravenous, or subcutaneous routes to several animals including monkeys, guinea pigs, chickens, and mice (Ciccarelli et al. 1977). As with the other botulinum neurotoxin complexes, it was several hundred to thousand times less toxic by the oral route as compared to the intravenous route of administration (Ciccarelli et al. 1977).

NONPATHOGENIC CLOSTRIDIA DEMONSTRATED TO FORM NEUROTOXINS SIMILAR TO *C. BOTULINUM*

Clostridium butyricum

C. butyricum is the type species of the genus *Clostridium* (Prazmowski 1880) (Cato et al. 1986). The G+C content is 27–28 mol% and the optimum growth temperature is 25–37°C. The organism is mostly saccharolytic and uses a wide variety of carbon compounds including ribose, glycerol, mannitol, and lactate. It can also utilize certain polysaccharides including starch and pectin. Compared to many other clostridial species capable of producing botulinum neurotoxin, it is relatively nonfastidious and growth factors other than biotin are not required. Fermentation endproducts include butyrate, acetate, carbon dioxide, and hydrogen.

C. butyricum has traditionally been considered to be nonpathogenic. However, two neurotoxigenic strains were originally isolated from cases of infant botulism in Rome, Italy (Aureli et al. 1986; McCroskey et al. 1986). The detection and isolation of neurotoxigenic *C. butyricum* (and *C. baratii*) was difficult and required considerable persistence since the organisms could not be obtained by cultural methods commonly used for *C. botulinum*. Since the original isolation of neurotoxigenic *C. butyricum* from Rome, Italy, additional strains have been from infant botulism cases and from food-borne botulism outbreaks in China and India as described earlier. These data suggest that toxin gene transfer from *C. botulinum* type E to *C. butyricum* occurs at a higher frequency than originally suspected. Strains of type E-producing *C. butyricum* have been differentiated by sequencing of a 16S rDNA region (Pourshaban et al. 2002). Plasmids have been detected in *C. butyricum*, and the gene for the neurotoxin may be associated with a

plasmid (Hauser et al. 1992) or a bacteriophage (Zhou et al. 1993).

Clostridium baratii

Cells appear in broth as nonmotile straight rods, spores round to oval and swell the cell. The G+C content is 28 mol%, and the optimum growth temperature is 35–40°C. The organism has shown phenotypic similarities to *C. perfringens* and *C. absonum* (Cato et al. 1982b, 1986). The organism does not digest casein or gelatin. Most strains are nontoxigenic, although type F botulinal toxin has been detected in rare isolates. It is primarily saccharolytic and forms acetate, butyrate, and lactate as fermentation endproducts. *C. baratii* has been isolated from human wounds and infections, rat feces, and from soil. Strains of *C. baratii* that produce type F botulinum neurotoxin have been isolated from infants with botulism and also from food-borne botulism cases (Aureli et al. 1986; Hall et al. 1985; Harvey et al. 2002; McCroskey et al. 1991; Paisley et al. 1995). The type F neurotoxin produced by *C. baratii* has been partially characterized (Giménez et al. 1992).

Clostridium sporogenes

C. sporogenes was first described by Metchnikoff in 1908 (Metchnikoff 1908; Willis 1969). Although the organism is considered nonpathogenic, it is of importance because it shares many traits with group I *C. botulinum* and *C. putrificum* (Anonymous 1999; Hatheway and Johnson 1998; Olsen et al. 1995). It is widely distributed, has remarkable resistance properties, and has frequently been isolated from wounds (Willis 1969). It may be the most ubiquitous of all clostridia, present in soil, sewage, dust, and in the intestinal tract of vertebrates. Spores used within the food industry have a high heat resistance, with a D121°C exceeding 1 min, which is approximately five times that obtained from *C. botulinum*. It can serve as a model organism for studies of heat resistance in food systems. *C. sporogenes* shares common surface antigens with *C. botulinum* and *C. novyi* (Poxton 1984). Phages have been demonstrated in *C. sporogenes* (Betz and Anderson 1964), but it has not been reported if they are related to phages from *C. botulinum*.

The G+C content of *C. sporogenes* is 26 mol%, and the optimum growth temperature is 35–40°C. The range of carbohydrates used varies among strains, but all strains ferment glucose to butyrate, acetate, ethanol, carbon dioxide, and hydrogen. The organism requires several amino acids and vitamins for growth in defined media (Belokopytov et al. 1982; Whitmer and Johnson 1988). Many of the amino acids can be replaced by the analogous L-α-hydroxy-acids (Belokopytov et al. 1982). *C. sporogenes* has been important in understanding the mechanism by which amino acids supply carbon and energy to proteolytic clostridia. Like group I *C. botulinum*, *C. sporogenes* utilizes amino acids and sugars as its primary sources of carbon.

Clostridium subterminale

The G+C content is 28 mol%, and the optimum growth temperature is 37°C. It is often difficult to obtain spores from *C. subterminale* (and *C. botulinum* type G), but they can be produced in cooked meat medium, on anaerobic agar without egg yolk, or soil extract agar (Ciccarelli et al. 1977; Solomon et al. 1985). Spores are oval, subterminal, and distend the cell. Strains are generally negative for lecithinase and lipase which distinguishes them from other clostridia capable of producing botulinal neurotoxin.

C. subterminale does not ferment carbohydrates but it degrades proteins and amino acids. It oxidizes branched chain and aromatic amino acids as sources of energy (Elsden and Hilton 1978, 1979). Endproducts include those typically obtained from amino acid fermentations and include acetic, butyric, and isovaleric as major products, with minor quantities of isobutryic, formate, propionate, isocaproate, lactate, succinate, and phenylacetate (Cato et al. 1986). Abundant hydrogen gas is formed. A very active NAD+-dependent glutamate dehydrogenase has been characterized from the organism that is active in amino acid degradation (Winnacker and Barker 1970). *C. subterminale* can be distinguished from closely related *C. hastiforme* by spore location and formation of large quantities of hydrogen gas, and from *C. botulinum* type G by absence of a toxin lethal to mice in culture broth.

BOTULINUM NEUROTOXINS

The outstanding properties of *C. botulinum*, *C. tetani*, and neurotoxigenic *C. butyricum* and *C. barati* are their ability to synthesize highly-specific neurotoxins of extraordinary potency (estimated to be 0.1–0.5 ng/kg or 0.1–1 μg (approximately 40 U/kg) for a human by the IV or IM routes, see earlier references). In nonhuman primate studies, the oral lethal dose has been estimated to be 10–70 μg for a 70 kg human (Arnon et al. 2001; Maksymowych et al. 1999; Larson and Johnson unpublished data). The actual quantity of botulinum neurotoxin to cause intoxication is clearly debatable and depends on many factors including the presence of food or alcohol in the digestive tract, the nature of the toxin ingested, particularly if it is associated with protective nontoxin proteins, and the underlying health of the individual. Regardless, botulinum neurotoxin has the distinct feature of being extraordinarily toxic by the oral route compared to most other protein toxins. Botulinum neurotoxin can also be toxic by inhalation (van Holzer 1962; Johnson, personal communication; Park and Simpson 2003). These properties of botulinum neurotoxin mandate that rigorous safety precautions be employed in studying this group of

toxins (CDC 1998a; Malizio et al. 2000 and references therein).

Botulinum and tetanus neurotoxins comprise a family of pharmacologically similar toxins that bind to peripheral cholinergic synapses and block acetylcholine release at neuromuscular junctions (DasGupta 1989; Inoue et al. 1996; Lalli et al. 2003; Montecucco and Schiavo 1995; Sakaguchi 1983; Schiavo et al. 2000; Simpson 1971, 1981; Sugiyama 1980). Botulinum poisoning results in a characteristic flaccid paralysis that may affect all peripheral motoneurons (Hughes et al. 1981; Cherington 1990, 1998). Botulinum neurotoxins have also been reported to affect sensory and autonomic neuronal activity (e.g. Dressler and Benecke 2004; Jenzer et al. 1975). Botulinum neurotoxins are classified into seven serotypes (A, B, C_1, D, E, F, and G) on the basis of neutralization of toxicity by type-specific antibodies (Bowmer 1963; Hatheway 1988; Gimenez and Gimenez 1995). As previously discussed, these neurotoxins are the most poisonous substances known, and animals vary in susceptibility, with humans, horses, and guinea pigs among the most sensitive to botulinum toxin (Gill 1982; Morton 1961; Schantz and Johnson 1992; Whitlock and Buckley 1997). Rats, carrion-eating birds such as vultures (Kalmbach 1939), and certain other animals that feed on rotting carcasses (Ortiz and Smith 1994a, b; Reed and Rocke 1992) may be relatively resistant to poisoning by oral ingestion of botulinal toxins. However, these animals become poisoned when the toxin is injected intravenously, suggesting that the toxin is destroyed in the gastrointestinal (GI) tract or that receptors for internalization into the lymph are lacking in the gastric tract. Serotypes A, B, and E are mainly responsible for human botulism, while animals are affected by these and other serotypes (Table 2).

Tetanus neurotoxin and botulinum neurotoxins (serotypes A–G) comprise the clostridial neurotoxin family. The biochemistry, structure, pharmacology, and genetics of the neurotoxins and the genes encoding the toxins have been extensively reviewed (e.g. DasGupta 1989; Lalli et al. 2003; Montecucco and Schiavo 1995; Sakaguchi 1983; Sugiyama 1980; Schiavo et al. 2000; Simpson 1971, 1981, 1984; Turton et al. 2002) and only selected aspects are described here, particularly comparative aspects of botulinum and tetanus neurotoxins. Biologically active neurotoxins of *C. botulinum* and *C. tetani* are dichain molecules of approximately 150 kDa in molecular weight (Sugiyama 1980), which are produced as single polypeptide chains of about 150 kDa that undergo proteolytic cleavage during excretion or in the extracellular milieu to generate the fully active dichain molecule (DasGupta 1989; Sugiyama 1980). The fully active toxin molecules are composed of two chains that are termed the heavy chain (c. 100 kDa) and a light chain (c. 50 kDa) that are covalently connected by a disulfide bond. Although *C. botulinum* produces seven serologically distinct neurotoxins and *C. tetani* one known serotype, they all have similar nucleotide sequences and likely a common structure. The genes encoding all seven serotypes of tetanus and botulinum neurotoxins have been sequenced and the amino acid homology has been shown to be between 30–60 percent (reviewed in Niemann 1991; Lacy and Stevens 1999; Popoff and Marvaud 1999; Quinn and Minton 2001). The region of highest homology is in the middle of the light chain. This region has been shown in every instance to code for LxHExxH where the histidines in the sequence are coordinated with one Zn^{+2} atom. This zinc-binding motif is characteristic of metalloproteases (Rawlings and Barrett 1995). Bacterial extracellular zinc-containing metalloproteases are common in many genera of bacteria and are a major class of virulence factors and sometimes comprise or are associated with hemagglutinins (Häse and Finkelstein 1993). The bacterial zinc-containing metalloproteases were previously shown to be active on physiologically active substrates including fibronectin, lactoferrin, collagen, and mucin, and initially were discovered in the 1940s by Sir Macfarlane Burnet (Finkelstein et al. 1983). Although there was considerable precedent, the mechanism of the neurospecific clostridial toxins were more recently discovered in the 1980s, when it was found that they cleaved neuronal proteins involved in neurotransmitter release (Montecucco 1995; Schiavo et al. 1992; Wright et al. 1992; Montecucco and Schiavo 1995; Schiavo et al. 2000). Several other toxins and virulence factors including the lethal factor of the anthrax toxin complex are also zinc-metalloproteases (Ascenzi et al. 2002; Duesbery et al. 1998; Klimpel et al. 1994; Kochi et al. 1994; Pannifer et al. 2001).

Three functional domains have been identified in botulinum and tetanus neurotoxins (reviewed in Montecucco and Schiavo 1995; Lalli et al. 2003). The carboxyl terminus of botulinum heavy chain actually consists of two subdomains and is responsible for receptor binding to the neuronal cell surface (Lalli et al. 2003). The binding to nerve terminals occurs at extraordinary affinity contributing to their remarkable neurospecificity. The nature of the receptors are only beginning to be elucidated but probably include polygangliosides, specific neuronal proteins, and probably lipid rafts (Parton and Richards 2003; Lalli et al. 2003). The distinct trafficking of botulinum and tetanus neurotoxins implies that they bind to different receptor components and undergo distinct mechanisms of internalization and trafficking. After binding to the neuronal plasma membrane, botulinum and tetanus neurotoxins are directed towards different regions of motor neurons. Botulinum neurotoxins are probably endocytosed in lipid vesicles and the light chain is released into the cytosol of the cell via a translocation event through the phospholipid vesicle membrane. The light chain of botulinum neurotoxin probably remains in nerve terminus, while tetanus neurotoxin is directed to the fast axonal

retrograde transport system, and it is trafficked to the motor neuron soma located in the spinal cord (Lalli et al. 2003). Tetanus neurotoxin, but not botulinum neurotoxins, can also enter hippocampal neurons through synaptic vesicle recycling. In either location, tetanus and botulinum neurotoxins act by similar modes of action but with strikingly different physiological manifestations. The light chains of botulinum neurotoxin and tetanus toxin have been shown to be zinc endopeptidases (reviewed in Schiavo and Montecucco 1995; Schiavo et al. 2000; Turton et al. 2002; Lalli et al. 2003). In nerves, these zinc-endopeptidases cleave core proteins involved in trafficking and release of neurotransmitters including VAMP/synaptobrevin, SNAP-25, and syntaxin 1. These proteins comprise the synaptic members of proteins in the SNARE complex (soluble NSF (N-ethylmalemide-sensitive fusion protein) attachment protein) that have a central role in membrane fusion events in eurkaryotic cells (Lalli et al. 2003). The selective proteolysis of these SNARE proteins causes a block of neurotransmitter release from neurons. In the case of botulinum neurotoxin, cleavage is believed to be mainly responsible for inhibition of release of acetylcholine and the characteristic flaccid paralysis, whilst with tetanus neurotoxin, the release of inhibitory neurotransmitters glycine and gamma-aminobutryic acid is stopped and inhibitory interneurons are affected, resulting in the characteristic spastic paralysis. However, these neurotoxins probably have secondary effects such as inhibition of release of neuropeptides, stimulation of nerve growth, and other pharmacological actions. Despite elegant scientific investigations, much remains to be learned about tetanus and botulinum neurotoxins, such as secondary effects including relief of pain and inflammation by botulinum neurotoxins.

Botulinum neurotoxins are produced in culture media and in foods as aggregates of neurotoxin and nontoxic proteins that are noncovalently associated in protein complexes (Johnson and Bradshaw 2001; Inoue et al. 1996; Kitamura et al. 1969; Kozaki et al. 1974; Mutoh et al. 2003; Oguma et al. 1986; Ohishi 1983, 1984; Ohishi and Sakaguchi 1974; Sakaguchi 1983; Schantz 1964; Schantz and Johnson 1992; Sugii and Sakaguchi 1975; Sugiyama 1980). These complexes also contain RNA (Goodnough et al. 1993; Kitamura 2002). In contrast, tetanus neurotoxin is not known to be produced as a complex with other proteins. Botulinum neurotoxin complexes have been referred to as progenitor toxins and the purified neurotoxin obtained through chromatographic separation from the other associated proteins of the complex. Toxin complexes have been described as M for medium (235–400 kDa), L for large (450–500 kDa), and LL for very large (900 kDa). These toxin complexes vary in size from c. 900 000 daltons for type A LL toxin complex to c. 300 000 daltons for the type B M complex and type E complex, to 235 000 daltons for type F M complex. However, the botulinum toxin complexes vary according to the strain and method of purification and it is likely that in their natural state they are more complicated than often assumed in their composition, structure, and pharmacological actions depending on the serotype and evolutionary lineage. More research is needed to adequately characterize the botulinum toxin complexes that occur naturally in foods, the environment and clinical samples.

Relatively little is known of the environmental and nutritional factors that control the production of botulinum neurotoxins in *C. tetani*, *C. botulinum* (reviewed in Johnson and Bradshaw 2001), and in neurotoxigenic *C. butyricum* and *C. baratii* (Anniballi et al. 2002). It has been demonstrated that amino acids in the culture media are involved in the control of toxin formation and activation in *C. botulinum* Groups I and II. In Group I *C. botulinum* types A and B, arginine is important for control of production and activation of botulinum neurotoxin (Patterson-Curtis and Johnson 1989). In *C. botulinum* type E (Group II), tryptophan and other aromatic amino acids affect neurotoxin production (Leyer and Johnson 1990). Recently, conditions affecting production of type E neurotoxin in *C. butyricum* were studied (Anniballi et al. 2002). Histidine-containing peptides were shown by Mueller and colleagues (Latham et al. 1962; Mueller and Miller 1942, 1945, 1948, 1956) to be critical for production of tetanus neurotoxin (also see Johnson and Bradshaw 2001; Schantz and Johnson 1992). Much more research would be valuable in this area for a basic understanding of regulation of toxin synthesis, and also for the control of neurotoxigenic clostridia in the intestine and in foods. Studies on the molecular biology of neurotoxin production and regulation as well as comparative genomic studies could provide insight into the evolution of this important and fascinating group of neurotoxigenic clostridia and their characteristic neurotoxins.

DETECTION OF BOTULINUM AND TETANUS NEUROTOXINS

Following enrichment and isolation of pure cultures from foods or clinical specimens, identification of botulinogenic clostridia initially involves the demonstration of botulinum neurotoxin by bioassay in animals (Hatheway 1988; Schantz and Kautter 1978; Solomon et al. 1995, 2001). Foods, blood (serum), and stools should also be examined for the presence of botulinum toxin (Solomon et al. 1995). For toxin assay, centrifuged supernatatant from 5- to 7-day-old TPGY or cooked meat broths is injected intraperitoneally (IP) into mice and the animals observed for signs of botulism periodically for up to 4 days. Symptoms of botulism are usually observed within 24 h and manifest as reduced mobility, ruffling of fur, labored breathing, contraction of abdominal muscles, followed by convulsions and death. Animals showing these signs in 2–24 h of injection usually die within 48 h.

The presence of botulinum or tetanus neurotoxin is confirmed by neutralization with antitoxin and identified to serotype by neutralization with a type specific antitoxin (Giménez and Giménez 1995; Hatheway 1988; Solomon et al. 1995).

Complications are often encountered in the mouse assay for botulinum toxin, particularly deaths caused by nonbotulinal lethal agents. Fatalities occurring in less than 2 h or after 48 h should be considered suspect and the assay should be repeated. Fatalities from *C. tetani* or other clostridia may be encountered if soil or sediment samples are being examined. With foods or clinical specimens, nonbotulinum deaths from infection or endotoxins may occur. These nonspecific deaths often take place within a few hours of injection and mice do not exhibit the typical signs of botulism. Generally, it is possible to dilute out the nonspecific interfering agent using a gelatin-phosphate buffer to an endpoint where the nonspecific deaths are eliminated. Occasionally, a specimen or culture fluid will contain more than one serotype of botulinal toxin. Cultures which produce type C_1 neurotoxin may also produce type D neurotoxin in small amounts and can also produce C_2 toxin. There are also occasional reports of strains that produce two toxin types, in which one is at much higher titer than the other. When two toxin types are present in the sample being analyzed, it is necessary to neutralize the toxicity with a combination of monovalent antitoxins. Since botulinum neurotoxins are labile to heat (Woodburn et al. 1979; Siegel 1993), heating with loss of activity can also support that mice fatalities are caused by a heat-labile protein toxin. Nonproteolytic strains of *C. botulinum* produce botulinum toxins in a less active, unnicked form. These can be activated by a protease such as trypsin to achieve full toxicity. In certain foods, trypsinization can produce peptides exhibiting toxicity in mice, and the activation reaction should be stopped by adding soybean trypsin inhibitor after 30–60 min.

Botulinum neurotoxins can be quantitatively titered by determining the dose required to kill 50 percent of a population of test animals (Schantz and Kautter 1978). This quantity is defined as 1 LD_{50} and is a statistical measurement based on the number of animals in each test group and the susceptibility of the test animals to the toxin. Quantitation is usually done with 5–10 animals per dilution using 50–100 mice and the results plotted logarithmically with percent death on the vertical axis and dilution on the horizontal axis. The point at which the line connecting the percent death at each dilution crosses 50 percent is the dilution that contains 1 LD_{50} per injection volume (Schantz and Kautter 1978). From this curve the number of LD_{50} in the original sample is calculated. This method is both expensive due to the number of animals used, and time-consuming as well. For these reasons other methods of detecting botulinum toxin have been devised.

An alternative method of determining the quantity of active botulinum toxin in a sample is the intravenous time to death method (Boroff and Fleck 1966; Malizio et al. 2000). This method involves injection of a sample into the tail vein of an immobilized mouse. The time-to-death is noted and converted to the number of intraperitoneal LD_{50} using a standard curve prepared using the dilution to extinction method of Schantz and Kautter (1978). The method is fast and relatively accurate ($\pm$20 percent). Drawbacks to this method are the preparation of the standard curve and the need for a higher level of technical expertise. The method is only applicable when the quantity of toxin injected is within the linear portion of the standard curve (1×10^6 to 5×10^3 LD_{50} per ml of type A toxin). Standard curves are required for each serotype of toxin since the time to death differs for different serotypes (e.g. Malizio et al. 2000) and possibly composition and structure of the complexes.

Various immunological and DNA-based methods (primarily the polymerase chain reaction (PCR)) for detection of toxin-reactive proteins of toxin genes have been proposed as methods for detecting *C. botulinum* organisms or its neurotoxins (Doellgast et al. 1993; Ekong et al. 1995; Ferreira and Hamdy 1995; Ferreira et al. 2003; Hatheway and Ferreira 1996; Kozaki et al. 1989; Notermans and Nagel 1989; Szabo et al. 1994; Wu et al. 1972). These methods often appear promising in vitro and in culture media, but can result in inaccurate or false-positive detection in clinical samples and foods due to difficulties in sampling, extracting high quality DNA, inhibition of antibody binding or PCR inhibition by components in these complex matrices, and the presence of extraneous reactive DNA. Immunologic methods have the drawback of lack of sensitivity, expense, cross-reactions between different serotypes, and the detection of biologically inactive botulinum toxin. The immunologic method that appears most practical is the enzyme-linked immunosorbent assay (ELISA) (Ferreira et al. 2003). The various ELISA methods use an antibody or series of antibodies specific for a given toxin serotype to capture and label the toxin. PCR methods can have excellent sensitivity and potential feasibility (e.g. Fach et al. 1993; Franciosa et al. 1996; Wu et al. 2001), but they have drawbacks described above. Due to these drawbacks, it is feasible that ELISA or PCR can complement the mouse bioassay, but it is unlikely that these assays will completely replace the mouse assay. Various groups are developing automated biosensors for the rapid and sensitive detection of botulinum neurotoxins, but these need further development for demonstration of applicability in cultural, environmental, food, and clinical samples.

GENOMICS OF *CLOSTRIDIUM TETANI* AND *CLOSTRIDIUM BOTULINUM*

The genomic sequences of select *Clostridium* species have recently been determined including *C. tetani*,

C. perfringens, and *C. acetobutylicum* (Brüggemann et al. 2003; Shimizu et al. 2002; Nölling et al. 2001) and others are ongoing including *Clostridium difficile* and *C. botulinum*. The genome sequence for *C. botulinum* type A (Hall A strain) is in progress at the Sanger Institute. The genome sequence of a single strain of each *Clostridium* species has been determined, and it is not clear if the sequences will be representative of most strains, particularly in *C. botulinum* and *C. perfringens* in which several distinct groups of organisms occur within the species. The analysis of the genomes have revealed interesting features regarding the pathogenicity, spore formation, and metabolism of the clostridia.

The complete genome sequence of *C. tetani* E88, a variant of the famous Harvard or Massachusetts strain, a nonsporulating strain long used for production of toxoid, revealed several interesting features (Brüggemann et al. 2003). The genome was relatively small, consisting of a 2 799 250 bp chromosome encoding 2 372 orfs. The strain also harbors a 78 052 bp plasmid (pCL1), containing 61 orfs, including some involved in pathogenesis. The genes encoding tetanus neurotoxin, the positive regulatory protein *tetR*, and a collagenase that could destroy tissue are located on the plasmid. The chromosome of *C. tetani* had a mol% G+C content of 28.6 percent, and the plasmid pLC1 has an extraordinarily low G+C content of 24.5 percent. The genome of *C. tetani* had a low number of mobile elements, 16 transposases that were classified into four insertion sequence families, and most of these orfs appeared to be nonfunctional because of various classes of mutations. The G+C variation within the genome was very low and pathogenicity islands were not found. These data are intriguing as they suggest that the *C. tetani* genome is relatively stable compared to many other bacterial groups such as the Enterobacteriaceae. In line with the protein-based heterotrophic metabolism of *C. tetani*, the organism contained genes with strong similarity to protein/peptide-transporting systems, several genes for amino acid decomposition, and genes for more than 27 peptidases, including seven for predicted zinc-metallopeptidases. The presence of many genes for degradation of amino acids but the lack of genes for sugar utilization in *C. tetani* is in contrast to *C. perfringens* (Shimizu et al. 2002) and *C. acetobutylicum* (Nölling et al. 2001), which readily use sugars and other carbohydrates. Certain groups of *C. botulinum* are well-known to degrade various amino acids (reviewed in Barker 1981; Whitmer and Johnson 1988; Johnson and Bradshaw 2001), and groups I through III also ferment sugars, while this property is absent in group IV.

The complete genome sequence for a type A strains of *C. botulinum* (ATCC 3502) is being performed by the Sanger Institute in England (www.sanger.ac.uk/Projects/C_botulinum/). The analysis of the contigs containing the orfs has indicated several interesting properties of this *C. botulinum* type A strain. The genome appears similar to that of *C. tetani* in that there is no obvious evidence of foreign DNA acquisition. With regard to pathogenesis, in addition to containing the genes characteristic of the type A neurotoxin complex (Dineen et al. 2003), it contains two putative hemolysins. The toxin gene complex is flanked by IS elements, which are apparently nonfunctional since they contain several mutations. Interestingly, *C. botulinum* ATCC 3502 lacks many of the *spo/ger* genes found in *Bacillus* sp., and lacks many genes of the phosphorelay system involved in *Bacillus*. These results suggest that the mechanism of sporulation and spore germination is significantly different from that of *Bacillus*. Since *C. botulinum* is a very diverse species, it would be valuable to obtain the genome sequences of other strains and types, and to also elucidate the proteome or the total proteins expressed by the organism. The availability of the genomic sequences of *C. botulinum* will enable the development of microarrays for hybridization analysis of genes involved in pathogenesis, as well as genes in important processes including sporulation, stress responses, and genes essential for growth in the intestinal tract, wounds, and in foods.

SUSCEPTIBILITY OF NEUROTOXIGENIC CLOSTRIDIA TO CHEMICAL AND PHYSICAL AGENTS

Because of their ability to form endospores, all clostridia have a pronounced but variable resistance to heat, drying, chemicals, and disinfectants. In the vegetative state, they are about as resistant to heat and chemicals as nonsporing aerobes, but anaerobes may have a slightly greater susceptibility to various bactericides. The spores can exhibit marked resistance to physical and chemical treatments. Spores of Group I *C. botulinum* may withstand boiling for 3–4 h, and even survive at 105°C for ~100 min. Spores of *C. novyi* are slightly less resistant than *C. botulinum*. Among hospital disinfectants, the greatest sporocidal activity is shown by alcoholic hypochlorite and glutaraldehyde; iodophors, formalin, and phenolics are less effective (Willis 1990; Kim and Foegeding 1993). Spores of *C. botulinum* have a marked resistance to various types of irradiation including gamma rays. In dried earth or dust, spores of *C. tetani* can survive for years. Stock cultures of most clostridia remain viable for months to years. The susceptibility of *C. botulinum* to chemical and physical treatments was summarized by Kim and Foegeding (1993) and survival in foods has been reviewed (Glass and Johnson 2001; ICMSF 1996).

Susceptibility studies of clostridia to antibiotics have been summarized by Willis (1990). Swenson et al. (1980) and Dezfulian and Dowell (1980) established that *C. botulinum* is susceptible to many common antimicrobials but is resistant to nalidixic acid, trimethoprim/sulfomethoxazole, gentamicin, and cycloserine. Drugs of

choice for the prevention and treatment of clostridial wound infections are metronidazole and benzylpenicillin. Metronidazole is uniformly bactericidal for all anaerobic microorganisms, but is inactive against aerobes and facultative anaerobes. The aminoglycosides, on the other hand, show broad spectrum activity against aerobes and facultative anaerobes, but are inactive against anaerobes. Under highly reducing conditions such as occurs in the large bowel, the activity of aminoglycosides is greatly diminished or abolished. Vancomycin can also be used to treat clostridial infections, but it should be used sparingly.

EPIDEMIOLOGY AND CLINICAL ASPECTS

Tetanus

EPIDEMIOLOGY

Tetanus is a rare disease in the developed world including the USA and Western Europe (Bleck 1991; Cook et al. 2001; see also www.who.int/vaccines-diseases/diseases/NeonatalTetanus.html). However, it is a devastating disease worldwide, and although the true incidence is unknown it has been estimated that up to 1 million cases occur annually (Galazka and Gasse 1995; Cook et al. 2001; Dietz et al. 1996, who.int/vaccines-diseases/diseases/Neonatal tetanus.shtml). It remains endemic in many countries of the developing world (Cook et al. 2001). The global fatality rate of tetanus has been estimated as 30–50 percent (Hsu and Grolau 2001; Sanford 1995). At least 50 percent of these deaths tragically occur in neonates, who become infected with *C. tetani* shortly following birth and lack maternal antibodies for protection. Neonatal tetanus is the second leading cause of death from vaccine-preventable diseases in children worldwide (who.int/vaccines-diseases/diseases/Neonatal tetanus.shtml). The WHO set the goal in 1989 for eradication (<1 death per 100 000 live births) of neonatal tetanus by 1995, but it is still endemic in many developing countries. The two major groups at risk are the elderly and neonates. The WHO has described 'high risk' geographic regions including the African region, southeast Asia region, and western Pacific region. Tetanus, a tragic and regrettable disease, could be prevented by vaccination which is the most cost-effective public health intervention (Gergen et al. 1995). The primary strategies outlined for eradication of neonatal tetanus are vaccination of pregnant women with at least two doses of tetanus toxoid, and provision of sanitary delivery services to women. Fortunately considerable progress has been made in reducing the incidence of tetanus in most high risk regions (who.int/vaccines-diseases/diseases/Neonatal tetanus.shtml). Although vaccination has been successful in eradicating the incidence of tetanus in children, the levels of antibodies decline with age, and most cases of tetanus in developed countries such as the USA occur in people of 60 or more years of age, and it is recommended that booster immunizations be given every 10 years to all adults (Gergen et al. 1995). The incidence of tetanus in developed countries such as the USA is approximately 36–48 cases annually, or 0.15 cases per million population (CDC 1998b). Intravenous drug users and HIV-infected individuals are high risk groups for contracting tetanus.

CLINICAL ASPECTS

Tetanus generally follows infection in neonates or an acute injury in children and adults. The most common wounds are puncture wounds and lacerations, but can be associated with surgery, intravenous (IV) drug use, and complications of diabetes (CDC 1998b). Lack of immunization is the greatest risk factor for tetanus, which is associated with poorer segments of the population and in the elderly (Hsu and Groleau 2001). Once tetanus toxin is synthesized in the wound infection, it moves to the spinal cord, which generally takes 2–14 days (Bleck 1989, 1991; Sanford 1995). The closer the site of the wound (e.g. facial injuries) shortens the transit time. Shorter incubation times often result in a poorer prognosis. Within the central nervous system (CNS), it prevents the release of inhibitory neurotransmitters including gamma-aminobutyric acid (GABA) and glycine. When it reaches the spinal cord, localized or cephalic tetanus may occur initially, which is followed by generalized tetanus. Motor neurons as well as the autonomic nervous system are affected. Clinical tetanus has traditionally been classified into four types: generalized, local, cephalic, and neonatal (Bleck 1989, 1991). Generalized tetanus is most common and is associated with trismus, or inability to open the mouth (lockjaw) and with abdominal rigidity. Generalized tetanic spasms are the most vivid aspects of the disease, and has been described as a 'sudden burst of tonic contraction of muscle groups causing opisthotonos, flexion, and adduction of the arms, clenching of the fists, and extension of the lower extremities' (Weinstein 1973). Unlike epilepsy and certain other neurological disorders, generalized tetanus does not result in loss of consciousness and is extraordinarily painful.

Respiratory failure is the most serious early concern in generalized tetanus due to obstruction of the upper airway and the involvement of the diaphragmatic and abdominal musculature (Bleck 1989, 1991). Patients with severe tetanus formerly died rapidly from respiratory failure, and intensive respiratory support is necessary in treatment (Bleck 1991). For patients who survive the acute phase, death most frequently occurs by autonomic instability characterized by labile hypertension, tachycardia, pyrexia, dysrhythmias, and myocardial infarction are the common fatal events (Hsu and Groleau 2001). After initial observation, the disease usually progresses

for 10–14 days, and recovery typically requires 4 weeks. Recovery is due to formation of functional presynaptic neurotransmission that replaces the diseased neurons. In the absence of tetanus antitoxin, tetanus will continue as long as tetanus neurotoxin is being produced at the site of infection. Therefore, it is extremely important to immediately provide antitoxin and to immunize individuals who are suffering from tetanus to prevent continuing and recurrent disease.

Local tetanus is characterized by fixed rigidity of the muscles near the site of infection. The rigidity may be mild, somewhat painful, and can resolve spontaneously. However, it may be a signal of the onset of generalized tetanus, and should be treated with antitoxin and immunization. Cephalic tetanus is a type of local tetanus that affects the face, particularly the muscles innervated by the lower cranial nerves causing a facial paresis and characteristic grimace. Other neuromuscular conditions such as dysphagia can be seen. Many patients with cephalic tetanus have a poor prognosis, although mild cases also occur (Bleck 1989, 1991). Cephalic tetanus has been observed in individuals that are fully immunized as determined by the routine antibody assay, bringing into question the validity of the assay for measurement of neutralizing antibodies. Alternately, the toxin may reside in a region unavailable for antibody protection.

The majority of neonatal tetanus cases involves infection of the freshly cut umbilical cord, usually as a result of improper care following delivery, unsanitary delivery conditions, and absence of immunization of the mother. Passively transferred maternal antibodies can protect the neonate from tetanus, supporting that the disease occurs only in infants of mothers who lack adequate immunity (Bleck 1991). Passive immunization of the mother prior to delivery could also protect against neonatal tetanus. Common symptoms in the first two weeks of life in the neonate are irritability, poor feeding due to inability to suck, and generalized weakness, and these symptoms are followed by tetanic spasms and rigidity.

DIAGNOSIS AND TREATMENT OF TETANUS

The diagnosis of tetanus is made on clinical signs alone (Cook et al. 2001; Hsu and Groleau 2001; Sanford 1995). The clinical presentation of tetanus is usually so vivid that a presumptive diagnosis can be made in most cases (Bleck 1989, 1991; Finegold 1998; Hsu and Groleau 2001). The differential diagnosis from related presentations caused by infections, strychnine, or certain other poisonings, and myopathies or neuropathies have been described (Hsu and Groleau 2001). Treatment relies on neutralization of the neurotoxin activity, removing the source of toxin, and providing supportive care including ventilation and gastric control, and assistance for muscle spasms. Human tetanus immunoglobulin (HTIG) is administered to neutralize circulating tetanus neurotoxin, but in common with botulinum poisoning, HTIG cannot neutralize neurotoxin already present in neurons. Benzodiazepines are administered because of their GABA-agonist and relaxing properties, and antibiotics (particularly metronidazole) are given to control growth of *C. tetani* in wounds. Prevention of tetanus through vaccination is obviously the preferred means for control in human populations.

Botulism

EPIDEMIOLOGY

Foodborne botulism occurs in clustered geographic regions of the world (Hauschild 1992; Hatheway and Ferreira 1996; Johnson and Goodnough 1998; Glass and Johnson 2001). In most countries it is quite rare, although like other foodborne diseases, the actual incidence is undoubtedly greater than reported. Many mild cases without hospitalization are probably not diagnosed and reported, and botulism can also be misdiagnosed as other neurologic disorders. Even so, the characteristic and often severe symptoms of botulism and careful records of release of antitoxin for treatment probably make botulism one of the most accurately reported food-borne diseases.

Through his thorough ecological surveys and study of *C. botulinum*, K.F. Meyer concluded that the risk of botulism is greater in regions that have a high telluric incidence of type A, B, and E spores (Meyer 1956). The primary regions of the world with reports of human foodborne botulism are East Asia (China, Japan), North America, certain countries in Europe (Poland, Germany, France, Italy, Spain, Portugal, Denmark, and Norway), the Middle East (Iran), Latin America, Russia, and South Africa (Franciosa et al. 2003; Hauschild 1992). Human botulism is rare in the UK, although notable outbreaks, including the Loch Maree tragedy (Leighton 1923), the Birmingham incident (Ball et al. 1979), and the hazelnut yoghurt outbreak (Critchley et al. 1989; O'Mahony et al. 1990), have attracted much attention. Human botulism is also rare in Africa, Australia, Israel, Taiwan, Greece, New Zealand, India, Mexico, and several South American countries (Hauschild 1992).

Botulism is endemic in certain regions of the USA, particularly the Pacific coast states and in Alaska. Botulism outbreaks in the USA reached a peak during the 1930s and then declined due to effective preventive measures in the commercial canned food industry and the development of guidelines for home canning (Meyer 1956; Lynt et al. 1975; Gangarosa et al. 1971). From 1899 to 1949, there were 1 281 cases and 830 deaths, from 1950 to 1977 in the USA, 678 cases and 169 deaths, from 1978 to 1993, 423 cases and 31 deaths, and from 1990, 263 cases and 11 deaths (Rhodehamel et al. 1992, CDC 1979, 1991–1995, 1998a). Foods involved in mainland USA botulism are usually home-canned

fruits and vegetables. Of 182 outbreaks identified with foods during 1971–1989, 137 were caused by fruits and vegetables, 15 by meats, 13 by fish, and 17 by other foods including mixed vehicles. In the USA, approximately 60 percent of foodborne botulism cases have been caused by type A toxin, 18 percent by type B, and 22 percent by type E (Hatheway 1995). In Alaska, fermented meats and fish accounted for 98 percent of the outbreaks, most involving type E botulinal toxin (Eisenberg and Bender 1976a; Barrett 1991; CDC 2003; Wainwright et al. 1988; Barrett 1991). The incidence of botulism outbreaks in Alaska have increased due to the availability of plastic bags and heating stoves which provides a warm anaerobic environment for growth of *C. botulinum* (Eisenberg and Bender 1976b). Botulism often unexpectedly occurs with advances in food handling and changes in preservation practices. Poor process control and maintenance of chilled distribution of smoked whitefish from the Great Lakes resulted in a resurgence of botulism in the 1960s (Foster et al. 1965). In the 1970s, changes in packaging procedures and underprocessing led to botulinal toxin formation in canned mushrooms prepared by seven USA commercial producers (Lynt et al. 1975). Production of foods with minimal or no preservatives and relying primarily on refrigeration for controlling *C. botulinum* has led to recent outbreaks of botulism (Gangarosa et al. 1971; Hutchinson 1992; Rhodehamel et al. 1992). Increased consumption of foods in food service establishments in the USA have impacted on foodborne disease (Hedberg et al. 1994), including botulism (MacDonald et al. 1986; Shapiro et al. 1998). Several outbreaks of botulism involving commercially processed or restaurant-prepared foods have occurred in various regions of the world (Table 3).

Although foodborne botulism is rare, the consequences of botulism outbreaks are extremely serious and it is vital that the food industry process and formulate minimally processed foods to prevent growth and toxin formation by *C. botulinum*. Outbreaks of botulism involving commercial foods or foods prepared in restaurants have generated considerable publicity and expense. The average cost of a botulism case due to a commercially processed foods has been estimated to be as high as several million USA dollars when issues related to hospital costs, lost productivity, and legal expenses are considered (CAST 1994). Botulism is also associated with emotional impact on the patient and family (Cohen and Anderson 1986).

In Canada, during the period 1961–1989, 87 percent of botulism cases occurred in northern native Canadian communities. As in Alaska, type E botulinal toxin was involved and the prevalent foods included fermented meats from marine mammals or fish (Dolman and Iida 1963; Hauschild 1992; Hatheway 1995). The practice of holding meats at ambient temperatures until a desired flavor and texture is achieved allows *C. botulinum* type E to produce toxin. The largest outbreak in the history of Canada occurred in 1985 and the food vehicle was chopped garlic in oil (St. Louis et al. 1988). Canada and the USA have made efforts to reduce the incidence of botulism by encouraging strict adherence to temperature control by processors, distributors, retailers, and in restaurants (Rhodehamel 1992), acidifying or providing other *C. botulinum* inhibitory conditions in foods that receive minimal processing. Currently, there is concern of botulism from minimally processed refrigerated foods, restaurant prepared foods, vacuum packaged or modified atmosphere packaged foods, and sous-vide products (Rhodehamel 1992).

Botulism is relatively common on the Asian continent. Botulism was first documented in China in 1949, and *C. botulinum* was isolated from botulinogenic food in 1958 (Ying and Shuyan 1986). From 1958 through 1983, 986 outbreaks occurred in China affecting 4 377 individuals. The majority of cases have occurred in the northern provinces of Xinjiang (81.3 percent), Qinghai (3.4 percent), Xizang (4.5 percent), Shandong (5.6 percent), and Hebei (1.9 percent). Epidemiology of botulism is unique in China compared to other areas of the world: 824 outbreaks (86 percent of total diagnosed) have been caused by bean products, most (74 percent) by improperly fermented bean curd. Botulism occurs mainly in the winter and early spring when vegetables are scarce and homemade fermented foods are commonly prepared. During 1958–1983, type A botulism accounted for 93.4 percent, type B for 5.0 percent, mixtures of types A and B for 0.4 percent, and type E for 1.0 percent of the botulism cases in China. The incidence of botulism in China has decreased considerably since 1974 because of education of households, advice to food manufacturing plants, inclusion of 10 percent salt in bean curd fermentations, and heating of foods prior to consumption (Ying and Shuyan 1986).

The majority of botulism cases in Japan occur in the northern prefectures of Hokkaido, Akita, Aomora, and Iwate (Iida 1970). Nearly all cases are caused by fish or fermented fish products, particularly izushi, and involve type E toxin (Dolman and Iida 1963; Iida 1970; Hauschild 1992). Type E spores occur in high frequency in the soils and sediments of Hokkaido and Tohoku, and they readily contaminate fish. Prominent symptoms of type E botulism in Japan are dysfunction of salivary secretion and retention of urine. These symptoms have not been reported as frequently in types A and B human botulism. The case fatality rate has decreased over time since type E antitoxin was distributed and communities were advised about preparation of sushi. In 1984, an outbreak of type A botulism occurrred in Japan involving 36 cases with 11 deaths. The causative food was vacuum-packaged 'Karashi-Renkon' (deep-fried mustard-stuffed lotus root). Normally, this food is heated by residents, but it was eaten unheated by

Table 3 *Outbreaks of botulism associated with commercial and restaurant-prepared foods*

Food Product	Year	Location	Toxin type	No. cases	No. deaths	Reference
Brie	1973	Switzerland, France	B	75	0	Sebald et al. (1974); Collins-Thompson and Wood (1993)
Process cheese spread	1974	Argentina	A	6	3	Briozzo et al. (1983)
Commercial beef stew	1974	Georgia, USA	A	2	1	Blake et al. (1977)
Canned peppers	1977	USA	B	59	0	Terranova et al. (1978)
Potato salad	1978	USA	A	7	0	Seals et al. (1981)
Canned Alaskan salmon	1978	UK	E	4	2	Ball et al. (1979)
Kapchunka (salt-cured, uneviscerated white fish)	1981	USA	B	1	0	Rhodehamel et al. (1992)
Beef pot pie	1982	USA	A	1	0	Anonymous (1983)
Sauteed onions	1983	USA	A	28	1	MacDonald et al. (1985)
Karahi-renkon (vacuum-packed, deep-fried, mustard-stuffed, lotus root)	1984	Japan	A	36	11	Otofuji et al. (1987)
Chopped garlic in oil	1985	Canada	B	36	0	St. Louis et al. (1988)
Kapchunka	1987	USA/Israel	E	8	2	Slater et al. (1989); Telzak et al. (1990)
Chopped garlic in oil	1989	USA	A	3	0	Morse et al. (1990)
Hazelnut yoghurt	1989	UK	B	27	1	O'Mahony et al. (1990)
Faseikh (salted fish)	1991	Egypt	E	92	20	Weber et al. (1993b)
Cheese sauce	1993	USA	A	8	1	Townes et al. (1996)
Skordalia (Greek salad with baked potato)	1994	USA	A	30	0	Angulo et al. (1998)
Clam chowder	1994	USA	A	2	2	Food Chem News, 1994
Black bean dip	1994		A	1	0	Food Chem News, 1994
Marscapone cheese	1996		A	7	0	Aureli et al. (1996); Franciosa et al. (1999)

tourists who were presented with the product as a souvenir (Hayashi et al. 1986; Otofuji et al. 1987).

As with most countries, botulism is regionally distributed and is associated mostly with home-prepared foods. Outbreaks of fish-related botulism, 'ichthyism,' in Russia were reported in 1927 (Dewberry 1950), and were caused by eating sturgeon or improperly prepared caviar (Smith and Sugiyama 1988). Botulism in Russia and the Ukraine has mostly been caused by salted, dried, or smoked fish (Dolman 1964), though home-cured hams, preserved wild mushrooms, and stuffed eggplant have also been food vehicles involving type A and B botulinal toxins (Smith and Sugiyama 1988).

In Europe, most outbreaks of botulism are caused by improperly canned or cured meats such as hams or sausages. Nonproteolytic strains producing type B toxin are most commonly responsible for toxin production. Poland has experienced more botulism than any other nation, and most (92 percent) outbreaks occurred in rural areas by consumption of improperly canned or preserved meats, 7 percent by fish products. Botulism has occurred in Germany more often than other European countries except Poland (Smith and Sugiyama 1988). The usual vehicle is improperly preserved meats and type B botulinal toxin is usually involved. In France, botulism has occurred periodically, the usual vehicle being home-cured ham (Roblot et al. 1994). Type B toxin was responsible for 97 percent of the diagnosed cases. Botulism usually caused by type B botulinal toxin has sporadically been reported in Italy and Spain, usually from home-canned vegetables or home-preserved vegetables in oil. Botulism in Scandinavia is generally caused by improperly preserved fish and involves type E toxin.

Botulism is comparatively rare in the UK. In 1922, the famous Loch Maree tragedy caused eight illnesses and eight deaths from the consumption of sandwiches prepared with wild duck paste. As a result of this outbreak, the Ministry of Health made arrangements for a supply of botulinum antitoxin to be available at various locations. In 1989, the UK observed its largest botulism outbreak in its history (Critchley et al. 1989). Until this outbreak, there had only been 18 cases reported since the eight fatal ones in Loch Maree. The vehicle in the 1987 incident was hazelnut yoghurt. The hazelnut puree added to the yoghurt had been underprocessed and allowed *C. botulinum* type B to grow and produce toxin. Rapid diagnosis and action by the health authorities prevented additional cases by the commercial product.

Food-borne botulism has also been recorded sporadically in various other countries throughout the world (Hauschild 1992; Smith and Sugiyama 1988). Argentina and Mexico are the only countries in Central and South America that have reported significant numbers of botulism cases. Reports of human botulism are rare in African countries (Hauschild 1992). The first human outbreak in Africa was reported in 1979 and was caused by type A toxin in sour milk (Smith et al. 1979). A second outbreak was attributed to consumption of termites, a cultural cuisine. The termites had been kept in a sealed bag for 3 days which probably promoted growth of *C. botulinum* (Nightingale and Ayim 1980). From 1942 to 1984, only five outbreaks of botulism were reported in Australia, four associated with vegetables and one with canned tuna (Hauschild 1992).

Overall, the majority of foodborne botulism cases have been reported from the northern hemisphere (Hauschild 1992). Types A and B botulinum toxins are responsible for botulism in the more temperate regions, those from type E in colder regions. It seems paradoxical that botulism by proteolytic strains of *C. botulinum* types A and B occurs mostly in vegetables, while nonproteolytic type B mostly causes botulism in proteinaceous foods such as meats (Hatheway 1995). This pattern may be due to the heat resistance of spores from proteolytic strains, which survive the heating processes of home canning while the nonproteolytic spores are killed. Botulism by nonproteolytic type B is usually associated with minimally processed foods that are not heated prior to consumption. A final preventive measure that prevents botulism is the heating of foods before eating as all serotypes of botulinal toxin are labile to heat and are inactivated by treatment at 60–100°C for 10 min (Woodburn et al. 1979). Seasonal occurrence of outbreaks are associated with cultural customs of preserving foods. The low frequency of botulism in many countries is related to the density of *C. botulinum* spores in the environment and in foods, to hygienic practices in food production, and by cooking foods prior to consumption. Recently, certain foods not previously implicated have been associated with botulism. In Italy, botulism was associated with consumption of mascarpone cream cheese (Aureli et al. 1996, 2000; Franciosa et al. 1999), and in India, Gram (legume) flour was the source of neurotoxigenic *C. butyricum* implicated in a foodborne outbreak (Chaudry et al. 1998).

Approximately 2 000 hospitalized cases of infant botulism have been reported worldwide, with the large majority occurring in the USA (Arnon 2004; CDC 1998a), with about half the cases from California. Infant botulism has also been reported from various countries worldwide including Argentina, Australia, Japan, the UK, Canada (four cases), and rare cases in Chile, former Czechoslovakia, Denmark, France, the Netherlands, Spain, Sweden, Switzerland, and Taiwan (Dodds 1993, personal communications). Within the USA, infant botulism occurs in clustered geographic regions. The highest incidence has been reported from California, Colorado, Delaware, Hawaii, Utah, and Pennsylvania (Arnon 1995). The clustered geographic occurrence of infant botulism is not understood, but may be associated with the distribution of *C. botulinum* spore type and prevalence in the environment. This distribution has been

demonstrated in the USA, where the majority of type B cases are reported from the eastern states, and most type A cases from California. The environment, soils, dust, and surfaces of foods harvested from soils are probably the main sources of spores in infant botulism. The only known food source is honey (Arnon 1995; Arnon et al. 1979). In several infant botulism cases *C. botulinum* spores were isolated from honey fed to infants that contracted botulism, and in all cases examined the spore type has matched the toxin type found in the baby's feces (Arnon 1995; Arnon et al. 1982). The spore type contaminating honey depends on the geographical source and whether the honey is blended before marketing. While infant botulism is very rare in the UK, labeling of honey has been instigated. With education of physicians and parents to not feed honey to babies less than 1 year of age, the number of honey-related cases has diminished. Honey, but not corn syrup or dried infant cereals, is considered as a risk factor for infant botulism (Arnon 1995, 2004; Hauschild et al. 1988). Aminoglycosides have been found to potentiate symptoms of infant botulism and to prolong the illness (L'Hommedieu and Polin 1981; L'Hommedieu et al. 1979; Santos et al. 1981).

Proteolytic strains of *C. botulinum* serotypes A and B have been responsible for nearly all cases of reported infant botulism (Arnon 1995, 2004; Hatheway et al. 1981). However, in 1979, type F infant botulism caused by toxigenic *C. baratii* was reported from New Mexico (Arnon 1995, 2004). This case appeared to be an anomaly until 1987 when type F intestinal botulism was reported in an adult in Georgia, and the organism responsible was identified as *C. baratii* (McCroskey et al. 1991). Additional cases of *C. baratii* infant botulism have been reported (Harvey et al. 2002; Paisley et al. 1995). Like the first type F case, the onset time was very rapid, and the patients were quite young, 9 and 14 days of age, respectively. Type C infant botulism was reported in Japan (Oguma et al. 1990), type E cases from Italy caused by neurotoxigenic *C. butyricum* (Aureli et al. 1986), and three cases by type Bf in the USA (Arnon 1995). Additional cases of infant botulism caused by *C. butyricum* have occurred (Fenicia et al. 2002).

Infant botulism has been reported in babies up to 12 months of age, though c. 95 percent of cases have been reported from babies less than 6 months old. The youngest cases reported were 6 and 8 days of age, and the oldest 363 days (Arnon 1995). Infant botulism shows a peak in incidence in children 2–6 months of age, and the age-at-onset distribution shows a close match to the sudden infant death syndrome (SIDS). A proportion of SIDS cases may be the fatal result of a rapid onset, fulminant form of botulism, but this is debatable (Arnon 1995; Bohnel et al. 2001). Breast feeding appears to slow the development of the disease (Arnon et al. 1982), and has been considered a risk factor but in actuality may delay the disease for a sufficient period that the babies can be hospitalized and receive supportive care. Fulminant cases of infant botulism where the children died before hospitalization were formula fed. The case fatality rate of infant botulism in the USA is less than 2 percent, but infants are hospitalized for several weeks. Boys and girls are equally susceptible, and all major racial and ethnic groups are affected.

Intestinal infections by *C. botulinum* with accompanying in vivo botulinal toxin production has occurred in older children and adults, particularly in individuals who have undergone intestinal surgery (Chia et al. 1986; Freedman et al. 1986; McCroskey and Hatheway 1988; Griffin et al. 1997). Conditions associated with intestinal surgery such as lesions in the intestine and antibiotic treatment causing alterations in microflora could predispose humans to colonization by *C. botulinum* (Griffin et al. 1997). Use of intense immunosuppression and bowel sterilizing regimens in a 3-year-old girl with neuroblastoma were postulated to enable colonization by *C. botulinum* types A and B with ensuing intestinal botulism (Shen et al. 1994). In Japan, a botulism case in a 12-year-old girl associated with *C. botulinum* type Ab was recently reported (Kobayashi et al. 2003). Infections by *C. botulinum* in adults has been supported by the finding of botulinum toxin and organisms in the feces over a several month period (Hatheway 1995; McCroskey and Hatheway 1988; McCroskey et al. 1991; Griffin et al. 1997). Most patients with foodborne botulism in the USA are injected with trivalent antitoxin, and monitoring serum shows the absence of botulinum neurotoxin. However, in France, type B toxin was found in serum from individuals for up to 122 days after ingestion of toxic food. Relatively mild and progressive development of botulism occurred over several weeks in patients who had eaten chopped garlic in oil containing type B toxin and organisms (St. Louis et al. 1988). These observations suggest that *C. botulinum* type B could colonize the intestine of adults and continously produce toxin over several weeks to months. These examples involved type B botulinum toxin. *C. botulinum* type B may have a greater ability than type A to colonize the intestinal tract, which could help explain why more than 50 percent of the infant botulism cases in the USA involve type B, while type A foodborne botulism is three times more prevalent than type B.

Although botulism is extremely rare compared to many other microbial diseases, the disease has long fascinated physicians and scientists because of the peculiar symptoms and relatively high fatality rate of affected humans and animals. Human botulism outbreaks can have a dramatic impact on the intoxicated individuals, their families, and the communities in which they occur (Angulo et al. 1998; Cohen and Anderson 1986; Dolman 1964; Dolman and Murakami 1961). Outbreaks of animal botulism have periodically devastated populations of domestic and wild animals (Eklund and Dowell

1987; Smith 1977). Waterfowl poisonings are unique among most animal botulism outbreaks because toxin is produced within the rotting carcasses of the victims, which are consumed by secondary animals (Reed and Rocke 1992; Wobeser 1997). This results in enormous losses of birds in epizootic proportions. In North America, avian botulism leads to kills of hundreds to thousands of ducks and shorebirds annually.

CLINICAL ASPECTS OF BOTULISM

Botulism is one of the most daunting and terrifying diseases to affect humans and animals. Botulism is a true toxemia and results from the action of botulinum neurotoxin. The hallmark signs of botulism are a flaccid paralysis that usually descends bilaterally from the eye regions and subsequently paralyzes skeletal muscles. Muscle paralysis results from a presynpatic inhibition of acetylcholine at neuromuscular junctions. Since the discovery of *C. botulinum* and its characteristic neurotoxin more than a century ago (van Ermengem 1897), five clinical forms of botulism have described:

1. classic foodborne botulism
2. wound botulism
3. intestinal botulism, particularly in infants
4. inhalational botulism
5. botulism of unknown source.

Botulism caused by intentional poisoning such as in a bioterrorist event could be considered as a sixth class with potentially severe consequences (Arnon et al. 2001). Lastly, inadvertent botulism caused by inappropriate injection of botulinum neurotoxin during treatment of dystonic and other conditions can be considered as a sixth clinical class of botulism. All of the forms have the characteristic symptoms of skeletal muscle paralysis. Human botulism is usually caused by botulinum neurotoxin serotypes A, B, E, but rare cases have also been reported for types D and F (Hatheway 1995; Demarchi et al. 1958).

The symptomatology, diagnosis, and medical treatment of botulism have been periodically reviewed (Donadio et al. 1971; Cherington 1990, 1998; Hughes et al. 1981; Koenig 1971; Maselli and Bakshi 2000; Robinson and Nahata 2003; Tacket and Rogawski 1989). Botulism caused by food poisoning generally has an incubation period of 12–36 h following consumption of a toxic food. The incubation period is shorter when more toxin is consumed and the fatality rate of 'index' cases is higher (Woodruff et al. 1992). Type B foodborne botulism may have a much longer onset time of several days to a few weeks in mild cases (St. Louis et al. 1988; Koenig 1971; Woodruff et al. 1992). Patients with botulism may initially have gastrointestinal symptoms such as nausea, vomiting, abdominal cramps, and diarrhea (Hughes et al. 1981), but these signs may not be caused by botulinal toxin since they are not observed in infant or in wound botulism.

After absorption into the blood, the neurotoxin binds to motorneurons and causes flaccid muscle paralysis. Among the first indications of botulism are generalized fatigue and muscular weakness. These are soon followed by disturbances in ocular function including blurred and double vision, and the pupils become enlarged and unresponsive to light. As intoxication proceeds, weakness and drooping of the eye lids and facial muscles become noticeable, speech becomes slurred, and eventual loss of speech ensues. Paralysis of pharyngeal and laryngeal muscles causes difficulty in swallowing and breathing, giving a sensation of suffocation and throat constriction, and regurgitation of food through the nose may be observed. Sputum is often thick and tenacious and thickly coats the tongue. Paralysis of the muscles of the face and neck give the patient a dull appearance and they become unresponsive to stimuli. The inability of the patient to express their discomfort can cause hysterical attacks and mental depression. Humans with mild cases of botulism also exhibit poor coordination of the arms and legs. Autonomic symptoms of botulism may persist for months such as numbness, dizziness, dry mouth, and impairment of salivary and lacrimal secretions (Dressler and Benecke 2004; Goode and Shearn 1982; Jenzer et al. 1975). In severe cases, extreme muscular weakness leaves the patient unable to raise the head, arms, or legs. The patient becomes gradually weaker, fatigued, and finally, death occurs, generally by respiratory failure or possibly by cardiac dysfunction (Lamanna et al. 1988). Since botulinum neurotoxin primarily affects motor nerves, sensory responses, mental function and consciousness are usually present throughout the illness until the onset of respiratory failure and death. The requirement for intubation and ventilatory support is an indicator of the severity of the disease in patients. Woodruff et al. (1992) found that intubation was necessary for 67 percent of patients with type A botulism, 52 percent with type B, and 39 percent with type E. Of patients that were intubated, 72 percent involved single case illnesses, while only 54 percent were intubated from outbreak-associated illness. This suggests that patients with mild cases of botulism are often not hospitalized and these cases remain undiagnosed.

Convalescence and recovery from foodborne botulism is generally prolonged, requiring several weeks to months. A retrospective review of cases in the USA found a mean of 58 days of mechanical ventilation for type A, and 26 days for type B (Colice 1987). Recovery of speech and the ability to swallow recurs relatively early. Muscular weakness, vertigo, and constipation may persist for several months. The visual disturbances and ability to freely defecate are the last to regain function. Some patients continue to experience weakness, fatigue, and symptoms of impaired autonomic nervous system dysfunction such as dry mouth, constipation, and impotence even after 1–2 years following the onset of

botulism (Cherington 1990; Colebatch et al. 1989; Goode and Shearn 1982; Dressler and Benecke 2004).

Although the disease is largely understood, significant mortality (~5–7 percent) is still associated with foodborne intoxication in the USA. The lethal dose of botulinal toxins in humans is debatable and depends on the individual, the quantity of food and alcohol in the stomach, and the source and serotype of toxin (Schantz and Johnson 1992). Accidental cases of human botulism from ingestion of toxin contaminated foods showed symptoms of botulism and occasional death from as little as 0.1–1 μg of toxin (100–1 000 ng or 3 000–30 000 mouse LD_{50} doses) (Meyer and Eddie 1951; Schantz and Sugiyama 1974). The assumed lethality for humans is c. 0.1–0.5 ng/kg which is close to the lethal dose of various animals (Gill 1982). Toxins in culture supernatants or preformed in foods are toxic to humans, monkeys, mice, chickens, turkeys, and pheasants (Gill 1982; Smith 1977). Toxicity varies according to the serotype, species of animal, and the route of administration (oral, intraperitoneal, or intravenous routes).

Patients with botulism experience varying degrees of illness and are rendered helpless to different extents (Cherington 1990, 1998; Maselli and Bakshi 2000; Robinson and Nahata 2003). Many patients are unable to see because of diplopia and inability to raise their eyelids. Others exhibit difficulty in eating and drinking and autonomic signs (Dressler and Benecke 2004). Still others are admitted to intensive care units and experience difficulty in breathing and require intubation and respiratory assistance. Recently, cases of severe botulism with neuropathic features have been reported (Chang and Robinson 2000; Mackle et al. 2001). Disease sequelae including infectious diseases such as pneumonia are not uncommon. Stimulation of murine B cells to secrete immunoglobulins by *C. botulinum* 'lipoteichoic acid-like' molecules has been reported (Campos-Neto et al. 1995). Communication is disabled by inability to speak or to write due to ventilatory support and extreme weakness. Agitation, depression, and anxiety is common among patients with botulism (Cohen and Anderson 1986).

Infant botulism differs from foodborne and wound botulism in the ages of the affected individuals and in nearly always beginning with severe constipation of 3 days or more that precedes neurologic signs by one or more days (Arnon 1995). Infant botulism results from botulinum neurotoxin produced in the intestinal tract of infants and susceptible children and adults. Ingested spores of *C. botulinum* germinate and outgrow in the large intestine and produce botulinal toxin. Infant botulism was recognized in 1976 in California in two cases that occurred within 4 months of each other (Pickett et al. 1976). In the same year, Midura and Arnon (1976) isolated and identified *C. botulinum* and demonstrated botulinal toxin in the feces of infants with botulism. Infant botulism is now the most common cause of botulism in the USA and also has been demonstrated in certain other countries.

After initial constipation, the characteristic flaccid paralysis affects the baby's musculature in the head, neck, and upper regions of the body. Symptoms include dilated and sluggish response of pupils to light, ptosis of facial muscles, impaired sucking and poor feeding, inability to swallow, a feeble cry, lethargy, generalized weakness, and apathy. Paralysis may gradually become more severe with the baby unable to hold its head erect, increased weakness in extremities, and gradual or abrupt cessation in respiratory ability (Figure 2).

Figure 2 *Botulism syndromes:* **(a)** *boy with typical signs of foodborne botulism;* **(b)** *infant with botulism;* **(c)** *animal wound botulism.* **(a)** *and* **(c)** *reproduced coutesy of Charles Hatherway;* **(b)** *reproduced courtesy of Stephen Arnon.*

Infant botulism can be difficult to recognize because of the inability of babies to communicate their symptoms, and being uncommon, infant botulism is often initially misdiagnosed. Botulism can be misdiagnosed for other neurologic diseases such as myasthenia gravis, Guillain–Barré syndrome, tick paralysis, drug reactions, stroke, or viral and bacterial infections of the nervous system. A careful assessment of progression of clinical signs (L'Hommedieu and Polin 1981) and electrophysiologic examination (Gutierrez et al. 1994) can assist in recognizing infant and adult forms of botulism.

As with foodborne botulism, infant botulism varies in severity from mild ptosis and rapid recovery to severe paralysis requiring extended hospitalization. The current clinical picture is composed from those babies showing paralysis of sufficient severity to require hospitalization (Arnon 1995). At the most severe end of the clinical spectrum, babies manifest respiratory difficulties as soon as 5 h after the first neurologic effects are recognized (Thompson et al. 1980; Urdaneto-Carruyo et al. 2000). It is likely that a fulminant lethal infant botulism occurs in a limited proportion of the infant botulism cases, and this severe infant botulism and rapid death may be one cause of sudden infant death syndrome (Arnon 1995). Muscle strength generally returns in 4–8 weeks in their reverse order of occurrence (L'Hommedieu and Polin 1981). Of the classic symptoms, constipation is typically the last to disappear.

Wound botulism is the counterpart of tetanus and results from botulinal toxin produced from *C. botulinum* infecting a wound. Wound botulism is the rarest form of botulism, and only about 100 cases have been reported in the USA since its discovery in 1943 (Davis et al. 1951; Merson and Dowell 1973). Most cases involve type A botulinal toxin, due in part because about half of the USA cases have occurred in California, where type A spores are prevalent. Since wound botulism is so rare, misdiagnosis and delay in treatment have been reported (Birmingham et al. 1994). Symptoms of wound botulism are similar to those of foodborne botulism and manifests as a symmetrical descending paralysis starting in the ocular region. The case fatality rate has been c. 15 percent. Wound botulism is suspected following trauma or a superficial wound without obvious infection and no history of consumption of botulinogenic food. Wound botulism in the USA has recently been associated with intranasal use of cocaine and subcutaneous injection of heroin (Elston et al. 1991; MacDonald et al. 1986). Since 1991, c. 60 laboratory confirmed cases of wound botulism in California were injecting drug users (CDC 1991–95). An unfortunate case of wound botulism was also reported in a 5-year-old boy who died from toxin produced in a tooth abscess (Weber et al. 1993a). Wound botulism has been reported rarely (total of five cases) from France, Italy, Australia, and China (Hatheway 1995). *C. botulinum* has been demonstrated recently to infect intestinal lesions, particularly following surgery (Chia et al. 1986; McCroskey et al. 1991). Botulinum toxin is produced in these intestinal lesions and absorbed into the blood. Recently, colonization of wounds has been observed at higher than normal prevalence in drug users who inject narcotics, particularly heroin, in which the spores survive and grow in the wound with production of botulinum or tetanus neurotoxins (Werner et al. 2000; Passaro et al. 2000; Sandrock and Murin 2001; Chang and Ganguly 2003).

The rare occurrence of wound botulism in nonimmunized humans compared to tetanus indicates that *C. botulinum* is poorly infectious compared with *Clostridium tetani*. Spores of type A and B *C. botulinum* and *C. tetani* were isolated from 18.5 and 30 percent of soil samples in the USA (Smith 1978) and the incidence of spores does not explain the different infectious ability. The explanation apparently involves unidentified virulence factors present in *C. tetani* allowing the invasion and growth within human tissues. Tetanus spores will germinate in human and animal tissue of sufficiently low oxidation-reduction potential and produce a large population of vegetative cells in 6–24 h (Fildes 1927; Smith and MacIver 1974). Botulinum spores germinated but apparently failed to outgrow after subcutaneous injection or tissue implantation in mice (Suzuki et al. 1971; Suzuki and Grecz 1972). Injection of rat cecal contents together with *C. botulinum* type A spores (Joiner et al. 1980) allowed germination and outgrowth of *C. botulinum* spores, suggesting that nutritional requirements or host defenses may limit growth of *C. botulinum* in wound infections (Dezfulian 1989). *C. botulinum* can also infect wounds but toxin production is impaired or toxin is inactivated (Hall 1945). Although vast numbers of neonatal tetanus yearly fatalities occur worldwide (Stanfield and Galazka 1985), neonatal botulism is apparently unreported and of rare occurence.

Recovery from botulism depends on renewal of neural activity. Although not proved, sprouting from poisoned nerve trunks and formation of new myoneural junctions is required for recovery. This process of renewed neurotransmission requires several weeks to months for completion. Similar to foodborne botulism, infant botulism caused by type A botulinal neurotoxin is often the most severe in symptoms and time required for patient recovery. In California, the mean hospital stay for patients with type A infant botulism was 5.6 weeks, whereas the mean hospital stay for babies with type B infant botulism was 3.7 weeks. Fortunately death in hospitalized cases has been rare, and babies recover completely with no permanent weakness or neurologic abnormalities. When death or chronic morbidity has occurred, it has usually resulted from infections or other secondary complications.

Botulism has devasting effects on wild and domestic animal populations every year. Animal botulism is the

most prevalent type of botulism worldwide and has caused enormous losses of wild and domestic animals. Botulism has considerable economic importance in cattle, chickens, turkeys, geese, mink, and sheep (Eklund and Dowell 1987; Meyer 1956; Smith and Sugiyama 1988; Songer 1996). Vertebrates vary considerably in their susceptibility to botulism (Gill 1982; Gunnison and Meyer 1930). Humans, horses, and guinea pigs are among the most susceptible animals to oral exposure of botulinal toxins. Certain animals are relatively resistant to botulinal toxins including vultures (Kalmbach 1939), dogs, and rats. Many of these animals consume rotting vegetation or carrion. Resistance is probably due in most cases to the instability of botulinal toxin in the gut or its inability to enter into the blood, although vultures, and possibly other animals, possess substances in their blood that neutralize botulinum toxin (Kalmbach 1939). The oral dose for animals and humans varies considerably among individuals, as well as between species, and is difficult to quantitate since it is influenced by a number of factors including the amount of food present in the stomach. The ranges of susceptibility to botulinum toxins are smaller when the toxins are injected intraperitoneally, intravenously, or subcutaneously. Only twice the quantity of type B toxin was needed to kill guinea pigs orally as by subcutaneous injection, ten times as much for mice, and five times as much for rabbits (Gunnison and Meyer 1930). The signs and symptoms of animal botulism are similar to human disease and typically involve sudden weakness, loss of muscle control and tremors, recumbency, and eventually death. Clinical diagnosis of botulism in animals is confirmed by detection of botulinal toxin in the serum and/or feces.

Botulism in cattle, referred to as lamsietke in South Africa or forage poisoning in the USA, usually results from ingestion of preformed toxin (Smith and Sugiyama 1988). Growth of *C. botulinum* in the feed silage or supplementation of feeds with animal products such as ensiled poultry litter (Jean et al. 1995) can lead to intoxication. Cattle botulism is associated with phosphorous deficiency in the soil. Botulinal types B, C, and D have been responsible for intoxications. Type B poisoning is most common in the USA and Europe, while types C and D predominate in Australia and South Africa. Type A botulism was reported for the first time recently in cattle (zebu, *Bos indicus*) raised in Brazil for meat production (Schocken-Iturrino et al. 1990). Botulism in sheep resembles the disease of cattle, and is mostly a problem in Australia and South Africa (Smith and Sugiyama 1988).

Horses are among the most sensitive of animals to botulinal toxin types B, C, and D (Kinde et al. 1991, Swerczek 1980a, b; Johnston and Whitlock 1987). Botulism in horses manifests as sudden generalized weakness, diminished pupillary eye response, severe tremors of the fore and hind limbs, eventual recumbency, laborious breathing, and death. Generally toxin is produced by *C. botulinum* in the intestinal tract of horses, and thus resembles infant botulism (Swerczek, 1980a, b; Johnston and Whitlock 1987). Toxicoinfections in horses has been referred to as 'shaker foal syndrome.' Autopsy specimens have revealed intestinal lesions that may provide a suitable environment for multiplication of *C. botulinum*. Horse botulism can also be demonstrated by injection of preformed toxin. Feed containing animal carrion and water harboring cat or rodent carcasses has caused equine botulism (Kinde et al. 1991). Recently, outbreaks of botulism in dairy cows have occurred (Cobb et al. 2002; Galey et al. 2000), and cases of botulism have taken place in dogs (Barsanti et al. 1978) and particularly in horses, particularly in young colts (shaker foal syndrome) (Galey 2001; Wilkins and Palmer 2003; Whitlock and Buckley 1997). Botulism outbreaks have also occurred in various captive animals including monkeys (Smart et al. 1980).

Botulism is a considerable problem in the domestic farming of mink and foxes, primarily because farmers often feed their animals raw and partially decomposed meat or fish. Mink and foxes are voracious eaters, and can consume several hundred grams of food each day. Even low levels of toxin in food are suffcient to cause botulism because botulinum neurotoxin accumulates over a suitable period. Most mink botulism is usually caused by type *botulinum* toxin, although types A and E have been reported to cause intoxications. Type C toxoids are commercially available for immunizing minks. Botulism has also been observed in other domestic and zoo animals such as dogs, monkeys, turtles, and fish. The animals vary in their sensitivity to toxin, and may be quite resistant to certain serotypes and sensitive to others. Dogs seem to be most susceptible to type C toxin, monkeys to type A and B, turtles to type C, and fish to type E (Barsanti et al. 1978; Smith and Sugiyama 1988).

Among all phyla of animals, birds experience the largest outbreaks and botulism probably causes the most casualties of microbial diseases afflicting birds (Eklund and Dowell 1987). Nearly every family of birds seem susceptible to botulism (Jensen and Price 1987) except for carrion eating types such as vultures. Avian botulism has been reported from all continents except the Antarctic and is generally caused by type C botulinal toxin. Occasional outbreaks have involved types D and E botulinal toxins (Smith and Sugiyama 1988). Large outbreaks due to botulism have been reported in chickens, ducks, pheasants, pelicans, gulls, flamingoes, swans, and numerous other species of wild water fowl including endangered species, such as the bald eagle and peregrine falcon (Jensen and Price 1987). Most birds seem to acquire botulism through the consumption of preformed toxin often from consumption of invertebrates such as fly larvae (maggots) within decomposing dead carcasses.

DIAGNOSIS AND TREATMENT OF BOTULISM

The diagnosis of botulism relies primarily on assessment of clinical symptoms of the patients, and for foodborne incidents by the clustering of cases involving a small group of people, often a family or group of friends, who have eaten a common food. For many botulism cases, routine laboratory studies are of no aid in establishing a diagnosis (Koenig 1971). Detection of botulinal toxin in the blood, gastric aspirate, and food provides confirmation of botulism. Botulinal toxin has been detected in 60–70 percent of human patients with type E botulism in large outbreaks or endemic regions (Barrett 1991; Wainwright et al. 1988). Botulinal toxin is detected less often in type A cases, probably because type A toxin binds more rapidly to motorneurons. Isolation of *C. botulinum* from a suspect food, from feces of infants (Dowell et al. 1977b; Hatheway and McCroskey 1987), or from wounds provides supporting evidence for diagnosis of botulism but does not give confirmation since spores can be found in the feces of healthy individuals and from foods (Hatheway 1988).

Treatment of all forms of botulism requires rapid and meticulous supportive and respiratory care (Arnon 1995; Cherington 1998; Maselli and Bakshi 2000; May et al. 2002; Robinson and Nahata 2003). Admission to intensive care units and careful attention to airway sufficiency is of high priority. Intubation with mechanical ventilation, and nasogastric feeding are required in severe cases. Rapid administration of trivalent botulinal antitoxin is of value in decreasing morbidity and mortality from botulism (Tacket et al. 1984). In the USA, patients may be administered multivalent botulism equine antitoxin intravenously (1 000–10 000 International Units (IU) each of type A, B, and E antitoxins) as soon as a diagnosis is made. The product is available through government public health departments. One IU neutralizes 10 000 mouse LD_{50}s (types A and B) and 1 000 LD_{50}s (type E), which is sufficient to neutralize toxic doses encountered in human botulism. The antitoxin has a half-life in the serum of 5–7 days (Hatheway et al. 1984). It is necessary to administer antitoxin as soon as a diagnosis is confirmed since the antibodies probably do not neutralize neurotoxin that has bound to motorneurons, and certainly not toxin that has been internalized within the nerve by endocytosis. Antitoxin would thus not improve symptoms present at the time of administration, but may stop progression of the paralysis (Hatheway 1995). Over an 11-year period, equine antitoxin led to hypersensitivity reactions in about 9 percent of treated patients (Black and Gunn 1980). It would be useful to produce a supply of antitoxin from human donors or to produce 'humanized' animal antibodies by available molecular biology techniques. A clinical trial underway in California is designed to evaluate the use of botulism immune globulin (BIG) from human donors for infant botulism (Frankovich and Arnon 1991; Arnon 1995). Recently (October 2003) the Food and Drug Administration approved the use of 'Baby Botulism Immune Globulin' for the treatment of babies with botulism, which will considerably diminish morbidity and the length of hospital stays with accompanying emotional relief for the families (Arnon, 2003, personal communication).

Botulism in humans and animals is diagnosed by the characteristic flaccid paralysis and by the detection of botulinum neurotoxin in the blood, stool, or wound. The detection of *C. botulinum* in wounds, stools, or food products can also provide evidence for the occurrence of botulism. Electrophysiologic methods have also been useful in the presumptive diagnosis of botulism, particularly when neurotoxin detection is negative or inconclusive (Cherington 1998). As described (Cherington 1998), the most characteristic electrophysiologic abnormality is a small evoked muscle action potential in response to a single supramaximal nerve stimulus in an affected muscle. Single fiber EMG analyses can also show increased jitter and blocking, which decrease following activation.

Largely through meticulous supportive and respiratory care, the fatality rate in the USA from foodborne botulism decreased from 65 percent for the period 1899–1949 to 25 percent for the period 1950–1977. Good supportive and ventilatory care, and diagnosis of milder cases were probably mostly responsible for the drop in fatality rate during this period, but administration of antitoxin was also helpful. During the period 1978–1993, the fatality rate from foodborne botulism dropped to ~7 percent (Rhodehamel 1992; CDC 1979, 1991–1995). This encouraging trend in survival probably occurred by rapid diagnosis, improved supportive care in intensive care units, rapid administration of trivalent antitoxin, and prevention of secondary infections. The administration of trivalent antitoxin within 24 h of onset of type A botulism had a shorter course of illness, but the same fatality rate of those who received antitoxin later (Tacket et al. 1984). These results suggest that antitoxin treatment can be beneficial if administered rapidly. Aminoglycoside antibiotics are to be avoided in treatment of secondary infections since they can potentiate muscle paralysis (L'Hommedieu and Polin 1981).

Since botulism occurs rarely in humans, it is not practical to routinely immunize human populations. Natural immunity has rarely been observed in humans, even in individuals who have experienced repeated botulism or in infants continously exposed to toxin over several weeks in cases of infant botulism. The dose of toxin required to elicit an antibody response is not known, but is probably much higher than the lethal dose. Increasing numbers of humans have developed antibodies when injected periodically for treatment of neuromuscular disorders (Borodic et al. 1996). Humans who routinely handle botulinal toxins can be effectively immunized by pentavalent toxoid available from the CDC and other

international governmental agencies. Vaccines are commercially produced for immunization of domestic animals such as mink, horses, and cattle.

It is noteworthy that three cases of botulism occurred in laboratory workers in Germany who administered powdered type A toxin to laboratory animals (van Holzer 1962). This appears to be the only report of botulism from a laboratory accident and it emphasizes the care needed when working with botulinal toxins; aerosols can be formed in laboratory equipment such as centrifuges. This emphasizes the need for scrupulous laboratory practices in working with *C. botulinum* and their toxins. All materials suspected of containing botulinal toxin should be handled with maximum precaution and only by experienced personnel who have been immunized with pentavalent toxoid. Extreme caution should be taken to avoid contact of toxic materials with the eyes, mouth, or nasal mucosa. Centrifugation and other laboratory procedures that can generate aerosols should be performed only in sealed nonbreakable containers (stainless steel or plastic ware).

USE OF BOTULINUM TOXIN IN MEDICINE

Interest gradually waned in botulism after the 1930s as botulism in foods came under control. However, three important discoveries sparked a renewed interest in botulism and botulinum neurotoxin. The first was a series of outbreaks of type E botulism that occurrred in the USA in the 1960s due to the consumption of commercially packaged smoked whitefish and outbreaks in certain other commercial foods. The second was the discovery of infant botulism in the 1970s, and the involvement not only of recognized *C. botulinum* but also of newly recognized neurotoxigenic *C. butyricum* and *C. baratii* producing botulinum neurotoxin. The third, the development of botulinal toxin to treat humans who suffer from dystonias, hyperactive muscle disorders, cosmetic needs, and pain syndromes, had an even greater impact.

Botulinal toxin is increasingly being used to treat humans suffering from neurological diseases characterized by hyperactive muscle activity (Borodic et al. 1996; Brin et al. 2002; Jankovic and Brin 1991; Jankovic and Hallett 1994; Johnson 1999b, c; Moore 1995; Schantz and Johnson 1992, 1993, 1994, 1997). In December 1989, the USA Food and Drug Administration licensed type A botulinum neurotoxin complex as an orphan drug for the treatment of the human muscle disorders strabismus, hemifacial spasm, and blepharospasm in patients 12 years of age and older, by direct injection of the toxin into the hyperactive muscle. Botulinum toxin has seen a remarkable expansion in uses for the treatment of a number of dystonias, movement disorders, cosmetic problems, and pain disorders, all which have been notoriously difficult to treat by existing therapies. The use of the toxin for human treatment came about over 25 years ago through the collaborative research efforts of Alan B. Scott and Edward J. Schantz in the USA (Schantz and Johnson 1992, 1997). The key properties of botulinum toxin as a drug is its high specificity for motor neurons, its very high toxicity, which enables the injection of extremely low quantities, thereby avoiding side effects and an immunological response, and its long (several months) duration of action. The main drawbacks have been development of immunity in some patients, and diffusion of the toxin to neighboring tissues causing temporary ptosis (Hatheway and Dang 1994; Schantz and Johnson 1994, Borodic et al. 1996). The treatment of neurological disorders with botulinum toxin has opened a new field of investigation on the application of the toxin to nerve and muscle tissues in the human body.

SAFETY PRECAUTIONS FOR WORKING WITH *C. BOTULINUM*, *C. TETANI*, AND THEIR NEUROTOXINS

Botulinum and tetanus neurotoxins are considered the most poisonous substances known for humans and certain animals (Lamana 1959; Schantz and Johnson 1992). The toxicity of type botulinum neurotoxins and tetanus neurotoxins have been estimated to be 0.2–1 ng/kg of body weight, and as little as 0.1–1 μg could be lethal to humans (Schantz and Johnson 1992). Consequently, considerable care and safety precautions are necessary in working with *C. botulinum*, *C. tetani*, and their neurotoxins (CDC 1998a; Malizio et al. 2000). Only one known laboratory accident leading to severe botulism has been reported, which was caused by inhalational botulism caused by aerosols formed in the laboratory (van Holzer 1962). Because the consequences of an accidental intoxication with these neurotoxins are severe, safety must be a primary concern of scientists and clinicians interested in study of these toxins and the producer organisms. The Centers for Disease Control and Prevention recommends Biosafety Level 3 primary containment and personnel requirements for facilities producing and studying large (milligram) quantities of botulinum neurotoxins (CDC 1998a). All personnel that work with botulinum neurotoxins should be immunized with pentavalent toxoid available from the CDC. Although *C. tetani* and tetanus neurotoxin are not generally considered to be as hazardous in small quantities since most humans are immunized, it is advised that similar precautions be used for this toxin as are used for botulinum neurotoxins.

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Clostridium perfringens, *Clostridium difficile*, and other *Clostridium* species

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INTRODUCTION

Members of the genus *Clostridium*, including its pathogenic species, are found principally in the soil, and some species are present as a very small component of the bowel flora of humans and animals. The clostridia are not normally invasive, but they produce a number of toxins and enzymes that are responsible for their pathogenic effects. Pathogenic clostridia may cause two general types of infection: histotoxic infections, such as gas gangrene, after traumatic implantation into ischemic tissues; and toxin-induced disease in various target tissues, including the bowel and the nervous system. This chapter deals with the histotoxic and related infections, clostridial bacteremia, and clostridial infections of the gut, e.g., necrotizing jejunitis and *Clostridium difficile* infections. Food poisoning due to *Clostridium perfringens* is dealt with in Bacteriology of foods excluding dairy products. Tetanus and botulism, which are due to *Clostridium tetani* and *Clostridium botulinum*, respectively, are considered in *Clostridium botulinum* and *Clostridium tetani*.

HISTORICAL BACKGROUND TO PATHOGENIC CLOSTRIDIA

This section will concentrate on the pathogenic clostridia other than *C. botulinum* and *C. tetani*. In 1877, Pasteur discovered what we now know as *C. septicum*, which he called *Vibrion septique* (Pasteur and Joubert 1877). The organism, originating in the intestinal tract, appeared in the blood of animals which had died of anthrax. The blood samples had been taken at post-mortem intervals after which the anthrax bacillus had disappeared from the blood, and investigators were troubled by the unexpected symptoms in animals injected with the samples in an attempt to reproduce the anthrax. Pasteur explained that upon death of the animal, the aerobic anthrax bacillus died from lack of oxygen, but the anaerobic conditions favored the anaerobic *Vibrion*.

C. perfringens (*Bacillus aerogenes capsulatus*) was isolated from a patient who had died of tuberculosis in 1892 (Welch and Nuttall 1892). This organism, which has also commonly been known as *C. welchii*, is most closely related to *C. baratii* and *C. absonum*. *C. novyi*

was discovered in an extract of milk protein after it was injected into guinea-pigs and caused severe pathology and death (Novy 1894). The organism was recovered from the dead guinea-pigs and found to be distinct from the earlier pathogenic anaerobes.

In 1902, Tissier isolated *C. bifermentans* from putrefying meat (Tissier and Martelly 1902). The name applied at that time, *Bacillus bifermentans*, was chosen because this was the first anaerobe noted with the capacity to degrade both proteins and carbohydrates. This organism is nonpathogenic, but a similar, pathogenic organism was isolated from human gas gangrene cases 20 years later by Sordelli (1922); the organism is known as *C. sordellii*. Also first isolated from putrifying meat (beef infusion) was *C. carnis* (Klein 1904). This aerotolerant clostridium produces a soluble exotoxin and is pathogenic to mice, rats, and rabbits, with production of an edematous and congested local lesion.

C. histolyticum was first described by Weinberg and Seguin (1916) and *C. sporogenes* was first described by Metchnikoff (1908) and named *Bacillus sporogenes* at that time. Weinberg and Seguin (1915) were also the first to describe *C. fallax* following a study of war wounds. It has since rarely been isolated from such sites. Other clostridia recovered from war wounds include *C. cochlearium* and *C. sphenoides* (Douglas et al. 1920) and *C. tertium* (Henry 1916–17). *C. difficile* was discovered in 1935 as part of the fecal flora of healthy infants (Hall and O'Toole 1935). *C. cadaveris*, formerly known as *Bacillus capitovale*, was isolated by Snyder and Hall (1935) from a number of sources, including the pleural fluid of a sheep that had died of gas gangrene, the heart blood and peritoneal fluid at necropsy of cases of septic infection in humans, and the feces of normal infants.

CLASSIFICATION AND DESCRIPTION OF PATHOGENIC CLOSTRIDIA

Classification of bacteria has been carried out or proposed on the basis of morphology, disease association, pathogenicity, toxigenicity, source of isolation, physiological and metabolic characteristics, staining reactions, serologic properties, DNA relatedness, and ribosomal RNA gene sequence homology. Details are presented in *Clostridium botulinum* and *Clostridium tetani*, as is a description of the genus. Of particular interest is that of the 19 clusters generated by 16S rRNA sequence analysis (Collins et al. 1994), the clostridia of medical and veterinary importance are found in clusters I, II, and XI (Figure 1). Some toxigenic species are so similar in physiological characteristics to each other that they can be considered as toxin variants of a single species. For example, some consider *C. histolyticum* to be a toxin variant (type D) of *C. novyi*, especially as it is closely related to *C. novyi* type B at the DNA level (Nakamura et al. 1983a).

HABITAT

As indicated in *Clostridium botulinum* and *Clostridium tetani*, clostridia are almost ubiquitous. Within humans, pathogenic clostridia are found as a normal component of the gastrointestinal flora (George and Finegold 1985), in particular *C. perfringens*, and to a lesser extent *C. bifermentans* and *C. sporogenes*. These organisms can be a source of infection following exposure to tissues due to surgery or trauma.

PHYSICAL FEATURES

Morphology

Many of the clostridia are so pleomorphic that their identification on a morphological basis is difficult. The morphology of a strain varies widely, both within and between cultures.

Like the aerobic spore-bearing bacilli, they are large, rod-shaped organisms. They range in length from about 3 to 7–8 μm, but long filamentous forms are common. Their width ranges from about 0.4 to 1.2 μm. The vegetative bacilli are straight or curved, their sides parallel, and their ends rounded or somewhat truncated. Most are arranged singly, but some occur in pairs or in chains, others in bundles, the members of which are arranged parallel to each other. Irregular forms include navicular, or boat-shaped, organisms; citron forms shaped like a lemon with a small knob at each end; large swollen, nonsporing rods; snake-like filaments; deeply stained bulb-shaped types; and a great variety of so-called involution forms varying both in shape and in depth of staining. Autolysis frequently sets in when sporulation begins so that shadow forms are numerous, particularly in certain species. L-forms occur in clostridia, including *C. tetani* (Willis 1969). Key physical features of the pathogenic clostridia are shown in Table 1 and some examples of cellular morphology in *Clostridium botulinum* and *Clostridium tetani*.

Staining reactions

All members stain readily with the usual dyes. Great irregularity is noticeable in the depth of staining, especially in cultures more than a day or two old. Sometimes, metachromatic granules or points of more intense coloration are seen, especially when acetone is used for decolorization. For initial visualization of the organisms in the diagnostic laboratory, Gram's stain is most commonly used. In young cultures, the bacilli are all gram-positive. Some species rapidly lose this property, such as *C. novyi* and *C. haemolyticum*, and some are readily decolorized by the ethanol. Often, gram-stained smears of pure cultures reveal a mixture of positive and negative organisms. In the early stages of spore

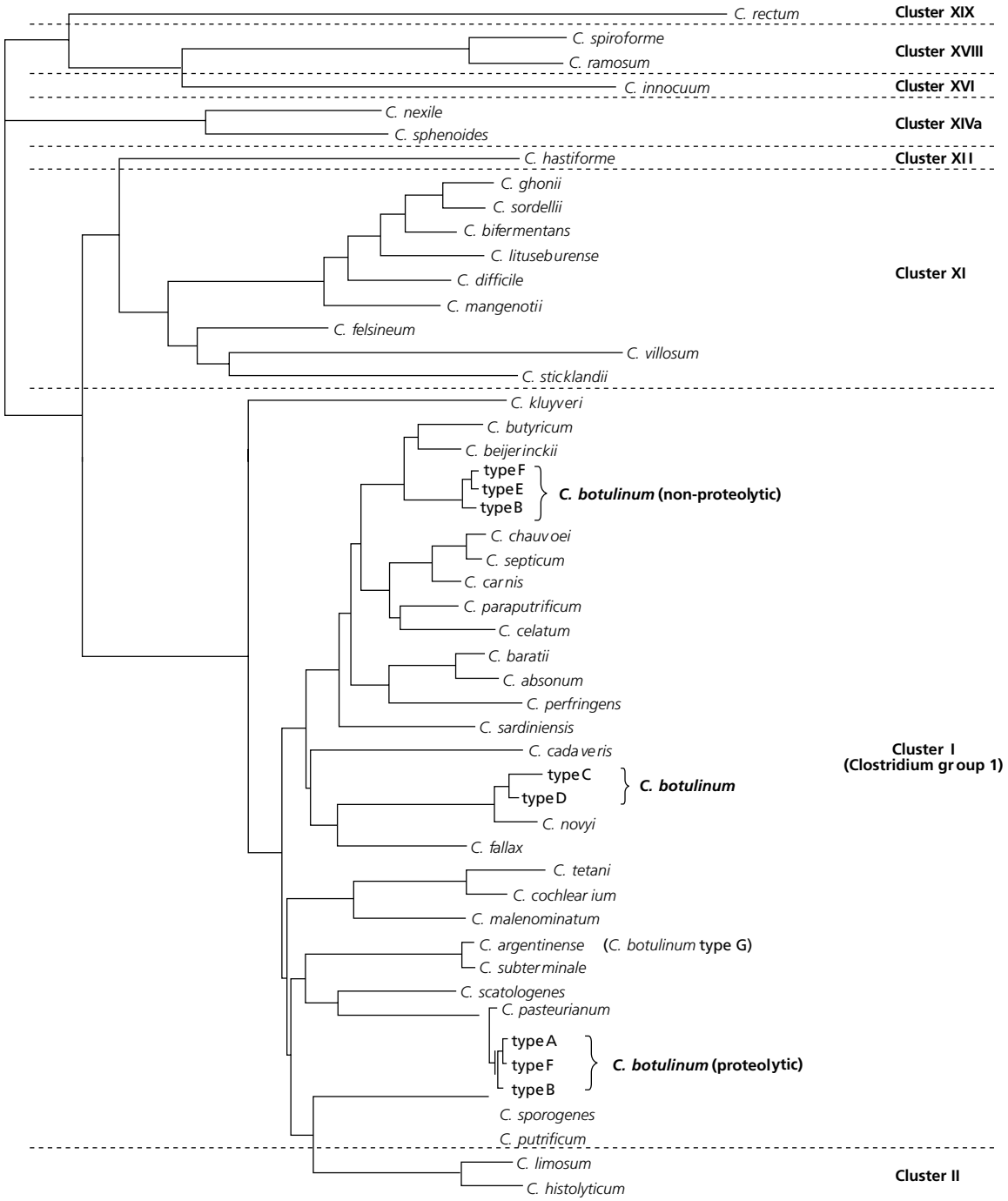

Figure 1 *Relationship of medically important* Clostridium *species based on 16S rRNA gene sequences. Data from Collins et al. (1994). Dendrogram for selected species provided by Paul A. Lawson, Institute for Food Research, Reading, UK.*

formation, the position of the spore is often marked by an area of intense staining; as it matures, however, the spore presents a colorless center surrounded by a peripherally stained ring.

Spores

Sporulation is common to all members, but there is considerable variation in the readiness with which it occurs. *C. sporogenes*, for example, spores readily on all media; *C. perfringens* only in particular media (Duncan and Strong 1968; Sacks and Thompson 1978). All the pathogenic members are able to form spores in the animal body, though *C. perfringens* does so rarely in the tissues or in culture media. However, extensive sporulation of ingested vegetative organisms in the intestine, concurrently with the release of enterotoxin, is involved in *C. perfringens* food-borne diarrhea (Hauschild et al.

Table 1 *Physical features of pathogenic clostridia*

Species	Size and shape	Spores	Motility
C. bifermentans	Straight rods. Single, pairs or short chains, 0.6–1.9 μm × 1.6–11 μm	Oval. Central to subterminal. Do not swell the cell	+
C. carnis	Straight rods. Single or short chains, 0.5–0.7 μm × 1.5–4.5 μm	Elongated. Subterminal to terminal. Swell the cell	+
C. chauvoei	Straight rods, single or pairs, 0.5–1.7 μm × 1.6–9.7 μm	Subterminal	+
C. difficile	Single straight rods, 0.5–1.9 μm × 3.0–16.9 μm	Oval. Subterminal. At 48 h bipolar spores may be seen	+
C. fallax	Straight rods. Single or pairs, 0.6 μm × 1.2-5 μm	Rare. Subterminal. Swell the cell	+
C. haemolyticum	Straight rods. Single, pairs or short chains, 0.5–1.6 μm × 1.6-18 μm	Oval. Subterminal. Swell the cell	+
C. histolyticum	Straight rods. Single, pairs or short chains, 0.5–0.9 μm × 1.3-9.3 μm	Subterminal	+
C. novyi	Straight rods. Single or pairs, 0.5–1.6 μm × 1.6–18 μm (Type B, 1.1–2.5 μm × 3.3–22.5 μm)	Oval. Subterminal. Swell the cell	+ (+, type B)
C. perfringens	Brick shaped straight rods. Single or pairs, 0.6–2.4 μm × 1.3–19 μm	Rarely seen	–
C. ramosum	V and Y form rods and straight rods in pairs or short chains	Spores uncommon and terminal	–
C. septicum	0.6–1.9 × 1.9–35	Oval. Subterminal. Swell the cell	+
C. sordellii	Straight rods. Single, pairs or short chains, 0.5–1.7 μm × 1.6–20.6 μm	Oval. Central to subterminal. Do not swell the cell	+
C. spiroforme	Curved rods. Single or arranged in coiled chains of varying length, 0.3–0.5 μm × 2–10 μm	Rarely seen. Terminal	–
C. sporogenes	0.3–1.4 μm × 1.3 × 16 μm	Oval. Sub-terminal. Swell the cell	+

1967). The spores of most members are wider than the vegetative bacilli. They therefore confer on the organism a distinctive appearance according to the position in which they arise. When they are formed at the equator, the clostridium is spindle-shaped; when subterminally, club-shaped; with an oval terminal spore the organism may look like a tennis racket; with a spherical terminal spore like a drumstick. Spores are easily visualized on a Gram-stained smear, or by phase-contrast illumination of a wet mount. Special spore stains are not necessary. General aspects of clostridial spores are covered in *Clostridium botulinum* and *Clostridium tetani*. Sporulation of clostridia has been reviewed by Woods and Jones (1986) and Setlow and Johnson (1997).

Resistance of spores to adverse conditions (e.g. heat) is of particular relevance to food safety and associated food-poisoning (see Bacteriology of foods excluding dairy products).

Flagella, cell walls, and capsules

Descriptions relating to clostridia in general are presented in *Clostridium botulinum* and *Clostridium tetani*. Particular points for the pathogenic clostridia which are the subject of this chapter are as follows. With the exception of *C. perfringens* and a few nonpathogenic species, all the members are motile by peritrichous flagella. Conversely, *C. perfringens* and *C. butyricum* are the only members with a classical capsule. The capsule of *C. perfringens* is noticeable in the animal body, and sometimes in cultures containing serum. The serology of the capsular polysaccharide antigens of *C. perfringens* is so diverse that it forms the basis of a typing scheme for food-poisoning outbreaks (see Bacteriology of foods excluding dairy products) and nosocomial antibiotic-associated diarrhea outbreaks (Borriello et al. 1984). Capsular material has also been shown to be present on the surface of *C. difficile* (Davies and Borriello 1990).

Diaminopimelic acid (DAP) provides the crosslink in the peptidoglycan for 53 of the 64 *Clostridium* species for which the information is available in *Bergey's Manual* (Cato et al. 1986). It is the *meso*-form for 47 species and the LL-form for six species (*C. carnis*, *C. fallax*, *C. perfringens*, *C. putrefaciens*, *C. thermoaceticum*, and *C. thermoautotrophicum*). Among the 11 species lacking DAP are *C. septicum*, *C. chauvoei*, and *C. tertium*. Lysine is usually the crosslinking component for the DAP-negative clostridia.

Plasmids

Rood and Cole (1991) have reviewed the incidence of plasmids in *C. perfringens*. Many cryptic plasmids have

been found. *C. perfringens* is the only *Clostridium* in which conjugative antibiotic resistance plasmids (R plasmids) have been identified. All of the conjugative R plasmids found in *C. perfringens* so far carry the same tetracycline resistance determinant. One nonconjugative R plasmid which confers resistance to the macrolide–lincosamide–streptogramin B antibiotics has been found. The gene for the enterotoxin of *C. perfringens* may be present on either the chromosome or a plasmid (Cornillot et al. 1995). Plasmid profiles have been used for strain identification of *C. difficile* (Steinberg et al. 1987) and *C. perfringens* (Mahony et al. 1987).

Bacteriophages

Bacteriophages have been described for many of the clostridia and their properties investigated (see Guelin et al. 1966; Prescott and Altenbern 1967; Sebald and Popovitch 1967; Mahony and Kalz 1968; Dolman and Chang 1972; Grant and Riemann 1976; Paquette and Fredette 1977; Mahony 1979; Ogata and Hongo 1979; Mahony et al. 1985). Phages are associated with neurotoxigenicity of *C. botulinum* types C and D, as well as production of α-toxin by *C. novyi*. Betz and Anderson, besides grouping the phages of *C. sporogenes*, found that many strains of this organism produced bacteriocin-like substances acting on other strains of the same species (Betz and Anderson 1964).

Bacteriocins

Bacteriocins are produced by a number of clostridia, for example *C. perfringens* (Mahony and Butler 1971; Mahony and Swantee 1978; Mahony 1979) and *C. difficile*. The technique of bacteriocin typing of *C. perfringens* has been used as a complement to serotyping in the laboratory confirmation of *C. perfringens* food poisoning outbreaks. It was helpful when the causative strain was serologically nontypable (Scott and Mahony 1982; Watson et al. 1982).

GROWTH AND APPEARANCE ON CULTURE MEDIA

Most of the clostridia with which we are concerned grow best at about 37°C, between pH 7.0–7.4 under anaerobic conditions, though many of them are capable of growing at temperatures of 20°C and even lower.

C. perfringens grows vigorously over the range of 20 to 50°C with an optimum of 45°C for most strains (Cato et al. 1986).

On solid media, growth is relatively slow, and sometimes takes the form of a thin, effuse, often spreading film, which may be difficult to distinguish from the underlying medium. Film formation is promoted by moisture. On first isolation *C. septicum* and *C. chauvoei* tend to spread rapidly over a moist surface. The spreading of clostridia is inhibited by certain chemicals. Most of them, however, are to some extent bacteriostatic. Inhibition of spreading without bacteriostasis may be effected by increasing the concentration of agar up to about 4 percent (Dowell et al. 1977), or by the addition of agglutinating antibody to the medium (Williams and Willis 1970).

Some clostridia produce colonies that fluoresce in longwave ultraviolet light (Willis 1990). *C. putrificum* produces red fluorescent colonies in the presence of blood and a fermentable carbohydrate due to the production of protoporphyrin. Colonies of *C. difficile* show yellow-green or chartreuse fluorescence on blood agar after incubation for 48 h; it is quite distinct from the nonspecific yellow fluorescence seen in cultures growing in the presence of neutral red, a property which *C. difficile* shares with a number of clostridia and is well recognized in cultures on MacConkey's agar.

For formulae of media for clostridia, see Dowell et al. (1977), Holdeman et al. (1977), and Willis (1977); for an illustrated description of the morphological and colonial appearance of the pathogenic anaerobes, see Batty and Walker (1966).

Culture media

AGAR PLATES

Single colonies are rounded, generally effuse, and have crenated, fimbriate, or rhizoid edges. They are generally translucent, not frankly opaque, grayish or exhibiting something of the color of the agar medium. *C. perfringens*, which is one of the less strict anaerobes, forms low convex colonies with an entire edge. *C. sporogenes* and *C. histolyticum* may form umbonate colonies with a raised center and a flat periphery. The colonial appearances are often characteristic, but some species give rise to variants which not only are unlike the typical colony, but which strongly suggest the occurrence of contamination. The colonies of some aerobic spore-bearing bacilli, growing anaerobically, simulate those of clostridia.

Several different types of colony may be formed, for example, by *C. sporogenes*. Two varieties of this species have been observed by a number of workers, beginning with Metchnikoff. Nakamura et al. (1977) designate a rhizoidal-colony variant as type I and a circular- or crenate-colony variant as type II. Type I strains showed much more abundant spores, but type II strains were generally more heat resistant, even when spores were not apparent on the stained slide. Type II strains were 81–91 percent related to a reference strain of *C. botulinum* type A by DNA hybridization, while type I strains were only 66–73 percent related.

BLOOD AGAR PLATES

On blood agar plates, both the colonial form and the degree and type of hemolysis may be characteristic.

Hemolysis is well developed after incubation for 24–48 h at 37°C; when the plates are stored in the dark at room temperature it often continues to increase. With a thick seeding the whole plate may be completely lysed. The nature and extent of hemolysis produced by different organisms may vary according to the species of erythrocyte used (see Willis 1969).

Many organisms give incomplete hemolysis after incubation for 24 h. After further incubation, this passes into the fully developed β-variety. In some cases, it is possible to specify the hemolytic factors concerned. For instance, the characteristic double-zone hemolysis produced by toxigenic strains of *C. perfringens* on routine horse-blood agar with an inner clear zone due to the θ-toxin, and an outer hazy zone due to α-toxin. On horse-blood agar containing added calcium chloride (0.5 percent) enhancement of α-toxin activity leads to the characteristic appearance of 'target' hemolysis (Evans 1945). *C. noyvi* produces a hot–cold hemolysin, so called because the hemolysis is more pronounced when the agar is cooled.

EGG YOLK AGAR MEDIA

Egg yolk agar media are very useful for the detection of the lecithinases and lipases that characterize certain pathogenic clostridia (Nagler 1939; MacFarlane and Knight 1941; McClung and Toabe 1947; Willis 1969). The intense diffuse opacity produced by some clostridia in egg yolk agar is due to lecithinases C, which are inhibited by specific antitoxins. The lecithinase producing organisms include *C. perfringens*, *C. bifermentans*, *C. sordellii*, and *C. novyi* types A, B, and D (*C. haemolyticum*). Some species produce an intense restricted opacity accompanied by a fine pearly layer overlying the colonies due to lipases. Lipolytic species include *C. novyi* type A. *C. sporogenes* and *C. botulinum* types A–F. These two effects, which are easily distinguished from one another, are also developed on the more complex lactose–egg yolk–milk agar (Willis 1990). This medium additionally shows lactose fermentation and proteinase effects, thus permitting an early presumptive identification of a number of different species.

MacCONKEY'S AGAR

Production of green fluorescent colonies by some clostridia on MacConkey's agar has been recognized for some time (Willis 1990). *C. perfringens* is particularly active in this respect. Fluorescence does not appear to be produced by facultative anaerobes. The fluorescence first becomes visible under ultraviolet light after incubation for 18–24 h, and is seen with ordinary illumination at about 24 h. It is due to some alteration in the neutral red that occurs under highly reduced conditions. The same effect is seen on other media containing neutral red, e.g. in cultures of *C. difficile* on cycloserine–cefoxitin fructose agar (George et al. 1979).

COOKED MEAT MEDIUM

Most clostridia grow well in cooked meat medium. All render the fluid turbid and most produce gas. The proteolytic species turn the meat black and may obviously digest it. The saccharolytic organisms do not digest the meat, and frequently turn it pink. The proteolytic organisms produce characteristic foul and pervasive odors, whereas with the saccharolytic organisms the odor is not foul or undetectable. This medium has the advantage of maintaining viability of many clostridia, sometimes for very prolonged periods.

SELECTIVE MEDIA

Selective media for the isolation of clostridia are based on the relative lack of inhibitory action of polymyxin B, neomycin, and crystal violet (see Willis 1990), phenylethyl alcohol (Dowell et al. 1964), cycloserine (Hauschild and Hilsheimer 1974), and trimethoprim/sulfamethoxazol (Dezfulian et al. 1981; Mills et al. 1985).

METABOLISM

Anaerobiosis

Clostridia are anaerobic organisms that possess neither cytochrome oxidase nor oxygenases (Decker et al. 1970). They derive their energy from fermentation of organic compounds rather than from oxidative processes involving oxygen (Andreesen et al. 1989). In the fermentation process, organic compounds serve as the primary electron donor and also as the terminal electron acceptor (Morris 1975). This leads to the accumulation of reduced organic products. ATP is formed by substrate level phosphorylation only, not by a respiratory process involving electron transport. The organisms do not carry out a dissimilatory sulfate reduction in which sulfate serves as an inorganic electron acceptor. This is referred to as anaerobic respiration (Decker et al. 1970).

Since anaerobic organisms cannot use molecular oxygen and can thrive in its absence, they often (or usually) are unable to cope with oxygen or the toxic products of oxygen consumption. In contrast, aerobes which are dependent on oxygen must be equipped to operate and survive under aerobic conditions. The clostridia in general lack catalase, although low levels of superoxide dismutase have been detected in some strains (Morris 1976). Oxygen may interfere with metabolism by oxidizing NADH through reactions catalyzed by NADH oxidases which many clostridia possess in substantial quantities (Andreesen et al. 1989). For many clostridia, oxygen seems to be not bactericidal, but bacteriostatic. Agar plate cultures of some pathogenic species, e.g. *C. perfringens*, can be removed from the anaerobic chamber for an hour or more, and growth of colonies will recommence after they are returned to the

chamber, whereas *C. haemolyticum* or *C. novyi* type B may survive for only a few minutes of exposure (Andreesen 1994). Sometimes oxygen resistance of the organisms is attributed to the spores, noting the normal presence of clostridia in soils and the viability of their spores in the dust carried by the wind. For growth, however, most clostridia require a greatly diminished oxygen level. Some attribute the inhibitory effect of oxygen to an increased oxidation–reduction potential (E_h), because many anaerobic bacteria can be grown in the presence of air provided a sufficiently low E_h is established in the medium. This can be done by reducing substances, some of which act mainly by absorbing oxygen, others establishing a low E_h after the molecular oxygen has been nearly used up or removed by mechanical means (Willis 1990). However, Morris points out the unreliability of E_h measurements in culture media and shows evidence that the inhibitory effect is possibly more dependent on oxygen than on E_h (Morris 1975). *C. histolyticum*, *C. tertium*, and *C. carnis* are exceptional in growing to a limited extent in the presence of air, though they do not form spores under these conditions (Willis 1990). Media for culturing the clostridia usually contain reducing compounds such as cysteine, thioglycollate, glutathione, sulfites, iron, or reduced iron compounds. Cooked meat medium, in addition to providing many essential nutrients and an environment conducive to germination of spores, as well as to sporulation of vegetative organisms, maintains anaerobic conditions through tying up the oxygen by unsaturated fatty acids catalyzed by hematin of the muscle, and the presence of sulfydryl compounds which bring about a negative oxidation–reduction potential (Willis 1990).

Metabolism of carbohydrates, proteins, and nucleotide bases

Polysaccharides may be broken down into their sugar components. Monosaccharides are utilized through the Embden–Meyerhof–Parnas pathway (Andreesen et al. 1989). Fermentation end-products formed through the central intermediate of pyruvate are acetate, butyrate lactate, and ethanol. Other alcohols such as isopropanol, butanol, and 1,2-propandiol may be formed by some species and under certain conditions.

Proteins are digested, obviously by the proteolytic clostridia (e.g. *C. sporogenes*), as well as other organisms in nature, and serve as the source of amino acids. The clostridia are diverse in their utilization of these as energy sources, as is evidenced by metabolic end-products (Holdeman et al. 1977; Lombard and Dowell 1982). Many amino acids may be fermented singly, and can serve as sole carbon, nitrogen, and energy sources (Andreesen et al. 1989). However, the fermentation of amino acids often takes place in pairs with one serving as electron donor and the other as electron acceptor in what is known as the Stickland reaction (Gottschalk 1986). The electron donor loses one carbon, while the electron acceptor maintains its original carbon chain length. In general, alanine, leucine, isoleucine, valine, histidine, tyrosine, phenylalanine, and tyrosine serve as electron donors; and glycine, proline, hydroxyproline, ornithine, and arginine as electron acceptors (Ljungdahl et al. 1989). However, some such as leucine, phenylalanine, and tryptophan can serve as either substrate for certain organisms (Gottschalk 1986). Straight chain amino acids yield acetate, propionate, butyrate, and valerate as end-products; branched chain amino acids are converted to isobutyrate, isovalerate, and isocaproate. Phenylalanine is converted to phenylpropionate, phenylacetate, and phenol, while tyrosine and tryptophan are metabolized to their hydroxyphenyl and indole equivalents, respectively (Andreesen et al. 1989).

The degradation of purines and pyrimidines follows pathways through conversions to glycine and β-alanine intermediates, respectively, then generally to acetate end-products.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

Because of their ability to sporulate, all clostridia have a pronounced but variable resistance to heat, drying, and disinfectants. In the vegetative state, they are about as resistant to heat and disinfectants as nonsporing aerobes, but anaerobes may have a slightly greater susceptibility to a large number of organic bactericides. The spores of *C. botulinum* may withstand boiling for 3–4 h, and even at 105°C may not be killed completely in less than 100 min. *C. novyi* is a little less resistant than *C. botulinum*. Spores of most strains of *C. perfringens* are destroyed by boiling in less than 5 min, but those of some food poisoning strains are often more heat resistant (Hobbs 1965). *C. sporogenes* can survive exposure for 8 days to a 5 percent phenol solution. Among hospital disinfectants, the greatest sporicidal activity is shown by alcoholic hypochlorite and glutaraldehyde. Iodophors, formalin, and phenolics are less effective (Willis 1990).

LABORATORY ISOLATION AND IDENTIFICATION

The conventional approach to diagnosis of a clostridial disease is usually to isolate the organism from, or to detect the toxin in, a suitable specimen. The organism is identified by performing appropriate differential tests on the isolated organism. Alternative methods, such as the use of fluorescent antibody reagents, have been attempted for identification of the organism without isolation, with variable success (Klotz 1965; Hunter and Rosen 1966; Glasby and Hatheway 1984). The poly-

merase chain reaction (PCR) for detection of toxin genes has been proposed as a means of detecting toxigenic organisms without culturing them (Szabo et al. 1994; Arzese et al. 1995; Baez and Juneja 1995; Buogo et al. 1995; Ferreira and Hamdy 1995; Franciosa et al. 1996). Staining of organisms with FA reagents (Batty and Walker 1963, 1966), determining electrophoretic patterns of soluble proteins (Cato et al. 1982), and cellular fatty acid analysis (Ghanem et al. 1991) have been used or proposed for use in identification of *Clostridium* species.

Attempts at isolation of clostridia will often be prompted by symptoms in a patient suggestive of a clostridial disease. Infections involving clostridia may be suggested by observing clostridial forms in Gram stains of smears from wounds or wound exudates. Isolation from agar cultures of materials containing a single or predominating organism is quite straightforward. For isolation from highly contaminated materials such as feces or soils, advantage is made of resistance of spores to heat and bactericidal agents, such as ethanol (Koransky et al. 1978). Sometimes cultures of the samples are tested for toxin, in particular neurotoxin (see *Clostridium botulinum* and *Clostridium tetani*) and toxin-positive samples are worked on further for recovery of the toxigenic organism. Selective and differential agar media are often very helpful (George et al. 1979; Mills et al. 1985). The lecithinase and lipase reactions detectable on egg yolk-based media are useful in selecting colonies for attempted isolation of *C. perfringens*, *C. botulinum*, *C. novyi*, *C. sordellii*, and *C. baratii*.

DIFFERENTIAL CHARACTERIZATION OF THE CLOSTRIDIA

The differential characteristics include morphological features such as spore position and shape, biochemical reactions in liquid and on solid media, metabolic products detected by gas–liquid chromatography (GLC), and the production of toxins and other biologically active antigenic agents.

Biochemical reactions

The biochemical reactions which reveal the physiological capabilities of the organisms in pure culture are determined using differential media and procedures described by others (Willis 1969; Dowell and Hawkins 1974; Dowell et al. 1977; Holdeman et al. 1977).

Metabolic products

An important adjunct to performing the tests on the differential media is the determination of the metabolic end-products by GLC (Holdeman et al. 1977; Lombard and Dowell 1982). In general, proteolytic organisms produce the 'isoacids' (isobutyric, isovaleric, isocaproic), however, *C. histolyticum* is one notable exception. It is evident that the phenylacetic and phenylpropionic acids produced from phenylalanine are detectable by this procedure, but the corresponding products from tyrosine (hydroxyphenyl derivatives) and tryptophan (indole derivatives) are not. Those products of amino acid metabolism have to be detected by alternative procedures (Moss et al. 1980). However, *C. difficile* converts tyrosine to p-cresol which can be detected by GLC.

Identifying the organism

On the basis of the characteristics determined by the above tests, the identity of the organism can be established with the dichotomous key devised by Holdeman et al. (1977).

Subtyping of strains of *Clostridium* species

Sometimes it is of interest to establish identity or non-identity between or among strains of a species with no distinguishing phenotypic characteristics, for epidemiological purposes, or to establish the source of contaminating or infecting organisms. This has been done with modest success in the serotyping (Hatheway et al. 1980; Stringer et al. 1980), bacteriocin typing (Mahony and Swantee 1978), and plasmid analysis (Mahony et al. 1987) of *C. perfringens*, and in investigation of outbreaks of antibiotic-associated pseudomembranous colitis caused by *C. difficile* (Clabots et al. 1993; Chachaty et al. 1994; Kato et al. 1994; Killgore and Kato 1994; Cartwright et al. 1995; Talon et al. 1995; Tang et al. 1995).

An example of serological approaches to distinguish between two different species is that applied to *C. septicum* and *C. chauvoei*. These two species are difficult to distinguish from each other on the basis of their physiological and toxigenic characteristics, and both organisms belong in 16S rRNA gene cluster I (Figure 1), and are closely related to each other on this basis. Proposed differential characteristics of sucrose fermentation and bacterial cell morphology on the liver surface in infected animals have been disputed (Al-Khatib 1969). It appears the most reliable means of differentiation is serological. Moussa studied 37 strains of *C. septicum* and 38 strains of *C. chauvoei* serologically and found eight different serologic groups (Moussa 1959). Five groups contained only *C. septicum* and two groups contained only *C. chauvoei*. The one mixed group contained only one strain of *C. septicum* and two of *C. chauvoei*. With the exception of the mixed group, the two species can be distinguished on the basis of their somatic antigens. *C. septicum* strains possess O-antigens 1 or 2, while *C. chauvoei* strains

possess O-antigen 3. Within each O-antigen group, strains may be differentiated on the basis of their H (flagellar) antigens. A common spore antigen was found for all strains regardless of species designation (Moussa 1959). The O-antigen differences provide the basis for distinguishing the two organisms by using specific fluorescent antibody reagents (Batty and Walker 1963). Vaccines for protection of animals against infection by *C. chauvoei* (Macheak et al. 1970) and *C. septicum* (Claus and Kolbe 1979) appear to be effective; but protection by *C. septicum* vaccines is somewhat strain specific and may be related to the antigenic diversity of those organisms as indicated by Moussa's studies (Moussa 1959).

TOXINS AND BIOLOGICALLY ACTIVE ANTIGENS

As stated in *Clostridium botulinum* and *Clostridium tetani*, the clostridia produce a wider variety of toxins than any other genus. These toxins and the lethality of some of them for mice are listed in Table 2 by organism. Greek letters have commonly been assigned as names of the toxins produced by many of the clostridia. The letter reflects the chronology of the discovery of each, rather than any indication of function or biological activity. There is no intended relationship between toxins with the same Greek letter designation produced by different species. This can be a source of confusion. A review of clostridial toxins published previously is the source of most of the information presented here (Hatheway 1990).

Pathogenic clostridia also have been shown to produce extracellular toxins or lipoteichoic acid-like molecules that act as superantigens, greatly stimulating the immune system (Bowness et al. 1992; Campos-Neto et al. 1995). For example, *C. perfringens* enterotoxin selectively stimulates peripheral blood lymphocytes bearing T-cell receptors. These superantigens probably contribute to pathogenesis by stimulating a hyperactive response of the immune system of mammalian hosts.

Genetics of toxigenesis

The genes encoding for several clostridial toxins are located on integrative lysogenic bacteriophages, while others are believed to be located on the chromosome and have not been shown to be associated with lysogenic bacteriophages (Johnson 1997; Rood et al. 1997). Genes encoding clostridial toxins that reside on the chromosome are the toxins of *C. difficile* and *C. perfringens* enterotoxin (human isolates). Genes encoding toxins that reside at least temporally on extrachromosomal virulence plasmids or bacteriophages are those of *C. botulinum* types C, D, and G, enterotoxigenic *C. perfringens* (animal isolates), and *C. tetani*. Analysis of toxin genes by gene probes, pulsed field gel electrophoresis, and other molecular approaches has demonstrated that these genes are frequently associated with unstable genetic elements such as plasmids, transposons, and bacteriophages. The presence of genes for virulence factors on extrachromosomal and moveable genetic elements often results in phenotypic properties, such as genetic instability and the capacity of toxigenicity to be dispersed by horizontal gene transfer to other microbial species.

The status of our knowledge of the molecular genetics of *C. perfringens* has been reviewed by Rood and Cole (1991). The chromosome maps for strains of toxin types A, B, D, and E show remarkably constant chromosome organization, and regardless of type, the strains represent a homogeneous group of closely related organisms. Type C could not be included in the comparison because the only available strain produced a very potent DNAase.

C. PERFRINGENS

The differences between toxin types seem to be restricted to which of the genes encoding the four major toxins are possessed. They cannot be differentiated on the basis of morphology, biochemical reactions, metabolic end-products (Cato et al. 1986), or electrophoretic analysis of soluble proteins (Cato et al. 1982). Types A–D were identical by 23S rRNA homology studies (Johnson and Francis 1975). The type strain is in *Clostridium* cluster I by 16S rRNA gene sequence analysis (Collins et al. 1994).

Toxins and toxin types of *C. perfringens*

The toxin types of *C. perfringens* A–D were initially established in 1931 on the basis of the studies of Wilsdon (cited by Glenny et al. 1933) and expanded to include type E after its discovery in 1943 (Bosworth 1943). The types are distinguished according to the production of the major toxins, α, β, ϵ, and ι, as indicated in Table 3. Toxin typing has generally been done by mouse lethality of culture fluids and neutralization with anti-A (anti-α), anti-B (anti-α + anti-β + anti-ϵ), anti-C (anti-α + anti-β), anti-D (anti-α + anti-ϵ), and anti-E (anti-α + anti-ι) antitoxin reagents (Dowell and Hawkins 1974). In recent years, commercial sources of typing reagents have been difficult to find (types A, C, D, and E reagents are currently available from TechLab, Blacksburg, VA, USA). Type B toxin type is determined by using the type C and D reagents. α-Toxin can be assumed by the lecithinase reaction on egg yolk agar. Immunoassays for β-, ϵ-, and ι-toxins have been proposed (Nagahama et al. 1991), but reagents for these tests are not available, and must be prepared by each prospective user. Since the nucleotide sequences for the genes for α-, β-, ϵ-, and ι-toxins have been published,

Table 2 *Toxins and biologically active antigens of clostridia (predominantly from Hatheway 1990)*

Species	Toxins	Size of molecule (kDa)	Activity/disease(Mouse LD_{50}; ng[a])[b]
C. argentinense (*C.botulinum* type G)	Neurotoxin	150	Botulism (experimental)
C. botulinum	Neurotoxin	150	Botulism (0.00625) ip
	C_2 (binary)		Permease/(45)ip
	component I	50	ADP-ribosylation
	component II	c. 80	Binding
	C_3	25	ADP-ribosylation
C. butyricum	Neurotoxin	145	Botulism, type E
C. baratii	Neurotoxin	141	Botulism, type F
C. bifermentans	β		Lethal
	HT	525	Equivalent to *C. difficile* toxin A
	LT	250	Equivalent to *C. difficile* toxin B
	Hemolysin	43	Oxygen-labile hemolysin
C. chauvoei/*C. septicum*	α	27	Lethal, necrotizing
	β	45	Deoxyribonuclease
	γ		Hyaluronidase
	δ		Oxygen-labile hemolysin
C. difficile (see also Table 4)	Toxin A	308	Enterotoxin/AAPMC[c] (26) ip
	Toxin B	270	Cytotoxin/AAPMC (1500)
	CDTa	43	ADP-ribosylation
	CDTb	80	Binding component
C. histolyticum	α		Necrotizing
	β		Collagenases
	Class I	68, 115, 79, 130	
	Class II	100, 110, 125	
	γ	50	Proteinase, thiol-activated
	δ	>10, <50	Proteinase
	ϵ		Oxygen-labile hemolysin
C. novyi/*C. haemolyticum*	α	250	Lethal
	β	32	Phospholipase C
	γ	30	Phospholipase C
	δ		Oxygen-labile haemolysin
	ϵ		Lipase
C. perfringens	Major		
	α	43	Phospholipase C/myonecrosis (50)
	β	40	Lethal, necrotic/enterotoxemia (8)
	ϵ	34	Lethal, permease/enterotoxemia (0.32)
	ι (binary)		Enterotoxemia (1560)
	Component a	40	ADP-ribosylation
	Component b	81	Binding
	Other		
	Enterotoxin	35	Food-borne diarrhea (1400)
	δ	42	Hemolysin (60)
	θ	51	Oxygen-labile hemolysin (167)
	κ	80	Collagenase (30 000)
	λ		Protease
	μ		Hyaluronidase
	ν		Deoxyribonuclease
	Neuraminidase	43, 64, 105, 310	*N* -acetylneuraminic acid glycohydrolase
C. sordelii (see also Table 4)	α	43	Phospholipase C (2500)
C. spiroforme	ι (binary)		Diarrhea in rabbits
	Component a	45	ADP-ribosylation
	Component b	92	Binding
C. tetani	Neurotoxin	150	Tetanus (0.015) ip
	Tetanolysin	48	Oxygen-labile hemolysin

a) Dose determined by intravenous injection unless indicated otherwise; i.p., intraperitoneal. Published values expressed in terms of N were converted to protein by multiplying by 6.25; those in MLD were converted to LD_{50} by multiplying by 0.5; and those expressed per kg of body weight were recalculated for 25 g mice.

b) For selected toxins.

c) AAPMC, antibiotic-associated pseudomembranous colitis.

Table 3 *Toxin types of* Clostridium perfringens *and major and minor toxins they produce (from Hatheway 1990)*

Toxins	Biological activity	Toxin type				
		A	B	C	D	E
Major(lethal)						
α	Lethal, lecithinase (phospholipase C)	+	+	+	+	+
β	Lethal, necrotizing, trypsin labile	−	+	+	−	−
ϵ	Lethal, permease, trypsin activatable	−	+	−	+	−
ι	Lethal, dermonecrotic, binary, ADP-ribosylating, trypsin activatable	−	−	−	−	+
Minor						
γ	Not defined, existence questionable	−	±	±	−	−
δ	Hemolysin	−	±	+	−	−
η	Not defined, existence questionable	±	−	−	−	−
θ	Hemolysin (O_2 -labile), cytolysin	±	+	+	+	+
κ	Collagenase, gelatinase	+	+	+	+	+
λ	Protease	−	+	−	+	+
μ	Hyaluronidase	±	+	±	±	±
ν	Deoxyribonuclease	±	+	+	±	±
Neuraminidase	*N* -acetylneuraminic acid glycohydrolase	+	+	+	+	+
Other						
Enterotoxin	Enterotoxic, cytotoxic	+	nt	+	+	nt

+, produced by most strains; ±, produced by some of the strains; −, no strain of the indicated toxin type has been shown to produce the toxin; nt, insufficient studies have been done with strains of the indicated toxin type.

gene probes or PCR could be used for determining toxin types (Songer and Meer 1996).

α-Toxin

Produced in large amounts by type A strains, α-toxin is a phospholipase C (EC 3.1.4.3) and appears to play a major role in gas gangrene. It hydrolyzes phosphatidylcholine and sphingomyelin, but not other phospholipids (Jolivet-Reynaud et al. 1988). It is a zinc metal protein and requires calcium ions for interaction with substrate. It is responsible for the lecithinase reaction on egg yolk agar and for the hazy zone of hemolysis on blood agar. The gene encoding α-toxin has been cloned and sequenced. The results show that the gene product is a 399-amino acid peptide with a molecular mass of 43 kDa (Okabe et al. 1989; Titball et al. 1989; Tso and Seibel 1989). Immunization of guinea-pigs with α-toxin protects them against gas gangrene when challenged with *C. perfringens* and toxin (Kameyama et al. 1972).

β-Toxin

β-Toxin is a major lethal toxin produced by type B and C toxin types. It is responsible for the lesions of necrotic enteritis of pig-bel (Murrell et al. 1966) and Darmbrand (Zeissler and Rassfeld-Sternberg 1949). The toxin purified by affinity chromatography has a molecular mass of 40 kDa and an isoelectric pH of 5.6 (Sakurai et al. 1981). Nucleotide sequencing of the gene encoding the β-toxin reveals an open reading frame of 1 008 nucleotides that encodes a protein of 336 amino acids with a molecular mass of 34.9 kDa (Hunter et al. 1993).

ϵ-Toxin

ϵ-Toxin is a prototoxin that is activated by proteolytic enzymes and is produced by both type B and type D strains. It increases the permeability of the intestine, thus enhancing its own uptake, and acts systemically as a lethal toxin (Buxton 1978). In the circulation, it causes swollen, hyperemic kidneys, edema in the lungs, and excess pericardial fluid (McDonel 1980a). The nucleotide sequence of the gene encoding ϵ-toxin shows that the mature prototoxin consists of 297 amino acids with a molecular mass of 33 kDa (Hunter et al. 1992). A 13 amino acid N-terminal peptide is cleaved during activation.

ι-Toxin

ι-Toxin is a binary toxin consisting of two subunits, ι-a and ι-b, which are immunologically and biochemically distinct (Stiles and Wilkins 1986a, b). The gene sequences encoding these subunits have been determined and the deduced amino acid sequences for the functionally active proteins correspond to peptides with molecular masses of about 40 and 81 kDa, respectively (Perelle et al. 1993). A mixture of both components is needed to demonstrate biological activity by mouse lethality or dermonecrosis. The light chain (ι-a) is an enzyme that ADP-ribosylates polyarginine (Simpson et al. 1987) and skeletal muscle and nonmuscle actin (Vandekerckhove et al. 1987; Schering et al. 1988). ι-a must gain entrance into the target cell before it can have any effect, but cannot do this of itself; ι-b recognizes a

binding site on the cell membrane, binds to the site, and interacts with ι-a to facilitate its entry. ι-Toxin of *C. perfringens* is similar to ι-toxin of *C. spiroforme* in serological, biological, and enzymatic activities (Popoff and Boquet 1988).

Minor toxins

Nine other factors produced by at least some strains of *C. perfringens* referred to as minor toxins have been identified and are listed in Table 3. At least some strains of all toxin types produce θ, κ, μ, ν, and neuraminidase. δ-Toxin is a hemolysin produced by type B and C strains, but not by type A, D, and E strains. λ-Toxin has not been detected in type A and C strains. It is a proteinase that digests gelatin, hemoglobin, and casein to some extent, but not collagen (Smith and Williams 1984). Strains of any toxin type may be encountered which fail to produce one or more of the indicated minor toxins. The minor toxins have been of primary interest in the early attempts at classifying members of the species. κ-Toxin (collagenase), μ-toxin (hyaluronidase), and λ-toxin (protease) appear to play roles in pathogenesis because of their ability to break down host tissues. γ- and η-Toxins have been proposed to account for discrepancies in neutralizing results obtained with specific antisera, but specific active substances related to those supposed entities have not yet been isolated (McDonel 1980a).

δ-Toxin has been recovered from *C. perfringens* type C and purified (Tixier and Alouf 1976; Alouf and Jolivet-Reynaud 1981). It has a molecular mass of 42 kDa and an isoelectric pH of 9.1. It has high hemolytic activity for erythrocytes from sheep, goats, and pigs, but is relatively inactive against those from humans, horses, rabbits, mice, and other mammals. The activity is inhibited by gangliosides (especially G_{M2}), but not by other lipid compounds such as sphingomyelin, lecithin, or cholesterol.

θ-Toxin, also known as perfringolysin-O, is responsible for the clear zone of hemolysis produced by at least some strains of all toxin types (McDonel 1980a; Smith and Williams 1984). It is an oxygen-labile, thiol-activated cytolysin, similar to but not identical with some hemolysins produced by other species (*Streptococcus pyogenes*, *Streptococcus pneumoniae*, *C. tetani*, and *C. novyi*) (Bernheimer 1976; Smith and Williams 1984). θ-Toxin has been purified by anion exchange and gel permeation chromatography (Yamakawa et al. 1977) and has a molecular size of 51 kDa by polyacrylamide gel electrophoretic (PAGE) analysis. This estimate agrees with amino acid analysis. θ-Toxin, activated by cysteine, is lethal for mice (intravenous injection) and the lethality is reportedly due to the cardiodepressant effect of toxin-induced release of endogenous mediators, such as platelet-activating factor (Stevens et al. 1988).

Tweten (1988a, b) has cloned and expressed the gene for θ-toxin in *E. coli*. The gene product was identical to the θ-toxin produced by the donor strain in amino terminal sequence and in SDS gel electrophoretic analysis. Both had an estimated molecular mass of 54 kDa and were of comparable hemolytic activity. The 1.8 kb chromosomal fragment generated a peptide of 499 amino acids that included a 27 residue signal peptide. The secreted form of the θ-toxin has a calculated molecular weight of 52.5 kDa. The toxin has 65 percent homology with streptolysin-O and 42 percent with pneumolysin. All three hemolysins share an identical region of 12 amino acid residues that include the single cysteine residue of the molecule, which is involved in the thiol activation.

Neuraminidase, or sialidase, serves many microorganisms as a pathogenicity factor in a variety of ways (Mueller 1976). Its action on erythrocytes may render them panagglutinable, resulting in increase of blood viscosity and promoting capillary thrombosis. Its modification of gangliosides on host cell surfaces may allow more direct contact of pathogens with the host, or it can provide suitable receptors for other toxins produced by the same or other microorganisms.

Nees et al. (1975) recovered and purified neuraminidase from *C. perfringens* strain ATCC 10543 and found it to be a single peptide with a molecular mass of about 64 kDa and exhibiting five components on isoelectric focusing, each with a different isoelectric pH, ranging between 4.7 and 5.4. After denaturation with SDS or 8 M urea, those differences disappeared and only a single component with an isoelectric pH of 4.3 was seen. Rood and Wilkinson (1976) recovered three different neuraminidases from strain CN 3870 with molecular masses of 310, 105, and 64 kDa. The two larger enzyme species were associated with hemagglutinin potency, but the 64 kDa species was devoid of that activity. Roggentin et al. (1988) cloned a 2.1 kb Sau3A DNA fragment from *C. perfringens* strain A99, whose product had neuraminidase activity. The active product was a peptide of 382 amino acids with a molecular mass of 42.8 kDa. Thus, different neuraminidase enzymes have been recovered from different *C. perfringens* strains.

Enterotoxin

Enterotoxin produced by *C. perfringens* is responsible for food-borne diarrhea which occurs after consumption of foods containing large numbers of the vegetative organism. It has been found in toxin types A, C, and D, but type B and E strains have not been sufficiently tested to establish whether they also produce it. Many, but not all, type A strains produce it. Enterotoxin studies have primarily involved toxin derived from type A strains. Enterotoxin is produced in the intestine during sporulation of vegetative organisms and it reacts with the surface of intestinal epithelial cells, causing

tissue destruction, and accumulation of fluids in the intestinal lumen (McClane 1992).

C. perfringens enterotoxin was isolated and purified (Hauschild and Hilsheimer 1971; Stark and Duncan 1972; Sakaguchi et al. 1973) and found to be a protein with a molecular mass of 35 kDa and an isoelectric pH of 4.3. The amino acid sequence has been determined (Richardson and Granum 1985; Granum 1986). The toxin consists of one peptide of 309 amino acids with a molecular weight of 34 262. The peptide has one free sulfydryl group. The activity of the enterotoxin is enhanced three-fold by treatment with trypsin (Granum et al. 1981). Trypsin cleaves at two sites, each involving a lysine residue, cleaving 15 or an additional 10 amino acids from the N-terminal end of the toxin (Richardson and Granum 1983). The trypsinized toxin then consists of 284 amino acids and two short peptides of 10 and 15 amino acids. Evidence shows that the short peptides do not separate from the main molecule in the absence of SDS or other denaturants. The nucleotide sequence of the enterotoxin gene has been determined. It encodes a 319 amino acid protein with a deduced molecular mass of 35.3 kDa (Czeczulin et al. 1993).

The mechanism of action of the enterotoxin seems to involve direct binding of the toxin to receptors on the surface of intestinal epithelial cells. Wnek and McClane (1983) isolated a 50 kDa protein from rabbit intestinal brush border membranes that specifically inhibits cytotoxicity for Vero cells. This molecule may be the specific receptor for the enterotoxin. Binding is followed by insertion of the entire molecule into the cell membrane, but no internalization into the cell (McClane et al. 1988). A sudden change of ion fluxes occurs, affecting cellular metabolism and macromolecular synthesis. As intracellular calcium ion levels increase, morphological damage occurs, resulting in greatly altered membrane permeability and loss of cellular fluid and ions and moderate-sized molecules (up to 3.5 kDa). Under some conditions, a loss of protein molecules may occur, but this may reflect cell death (McClane et al. 1988). Based on rabbit studies, the enterotoxin is most active in the ileum, moderately active in the jejunum, and essentially inactive in the duodenum (McDonel 1980b). It has been demonstrated that the enterotoxin acts as a superantigen, stimulating T-lymphocyte activity (Bowness et al. 1992). The observations suggest that superantigenic and enterotoxigenic properties are linked.

Several assays for detecting and measuring enterotoxin, including Western immunoblotting (Kokai-Kun et al. 1994), Vero cell toxicity, reversed passive latex agglutination (RPLA), and enzyme-linked immunosorbent assay (ELISA) (Berry et al. 1988) have been used. Kits for ELISA and RPLA are currently available from TechLab (Blacksburg, VA, USA) and Oxoid USA (Columbia, MD, USA), respectively. All of the tests for enterotoxigenicity require sporulation of cultures. Detecting the gene by use of a gene probe (Kokai-Kun et al. 1994) can show the potential of a strain for producing enterotoxin without requiring in vitro sporulation, but do not allow one to conclude that the strain can sporulate and produce enterotoxin in vivo.

C. DIFFICILE

Toxins A and B

C. difficile was not known to be pathogenic for man until it was implicated as the cause of antibiotic-associated pseudomembranous colitis. A toxin neutralized by *C. sordellii* antitoxin was found in the feces of patients (Larson et al. 1977; Rifkin et al. 1977), but the organism that produced it was actually *C. difficile* (Bartlett et al. 1978c; Larson et al. 1978). The toxin had both enterotoxic and cytotoxic activities, which were subsequently separated from each other and designated as toxin A and toxin B, respectively (Taylor et al. 1981). Purification of toxins A and B has been done by several researchers. Sullivan et al. (1982) separated the two toxins and purified them by DEAE ion-exchange chromatography and observed molecular masses of 440–500 kDa for toxin A and 360–470 kDa for toxin B. Banno et al. (1984) purified the corresponding toxins (designated D-l and D-2) to homogeneity as determined by PAGE analysis using gel filtration and ion-exchange chromatography. Their molecular masses were 550–600 kDa and 450–500 kDa, respectively. Each of the two toxins, D-1 and D-2, was converted to an apparent single, smaller molecular form, 190–200 kDa, determined by SDS-PAGE analysis after heating at 100°C for 5 min in the presence of SDS and 2-mercaptoethanol. The toxins were found to be immunologically differentiable by cross-neutralization studies in cyto toxicity and mouse lethal assays (Libby and Wilkins 1982; Banno et al. 1984). Cross-reactivity without neutralization between the toxins and corresponding heterologous monoclonal antibodies have, however, been observed (von Eichel-Streiber et al. 1987; Rothman et al. 1988), thus indicating common epitopes. However, it was shown that toxin A nonspecifically bound certain monoclonal antibodies (Lyerly et al. 1989) and that this nonspecific binding occurred through the carbohydrates on the Fab component (Cooke and Borriello 1998).

Structure–function analysis of large clostridial cytotoxins

Both *C. difficile* toxins have been cloned and sequenced and comparative sequence analysis undertaken (Barroso et al. 1990; Dove et al. 1990; Sauerborn and Von Eichel-Streiber 1990; von Eichel-Streiber et al. 1990, 1992a). Toxin A consists of 2 710 amino acid residues (308 kDa, pl 5.3) and toxin B of 2 366 residues (270 kDa, pl 4.1). Both toxins are about 48 percent identical and 63 percent homologous. *C. difficile* toxins A and B

appear to possess three major structural and functional domains: (1) the C-terminal domain; (2) a rather small hydrophobic region in the middle of the proteins; and (3) the N-terminal part harboring the enzyme activity of the toxins. This is similar to the structure of many other proteins, which act inside eukaryotic cells.

The C-terminus of the *C. difficile* toxins A and B is characterized by various oligopeptide repeats. The C-terminus of toxin A consists of 38 repetitive sequences containing seven groups of 30 amino acid residues and 31 groups of 20 amino acid residues. Toxin B possesses 23 repetitive oligopeptide groups with four groups of 30 amino acid residues and 19 groups of 20 amino acid residues (Dove et al. 1990; Lyerly and Wilkins 1995). These sequences exhibit significant homology with the repetitive sequences of glucosyltransferases of *Streptococcus mutans* and *Streptococcus sobrinus*. In the streptococcal glucosyltransferases, the sequences are involved in carbohydrate binding (von Eichel-Streiber and Sauerborn 1990; von Eichel-Streiber et al. 1992b). Therefore, it is suggested that the C-terminal oligopeptide repeats of *C. difficile* participate in binding of the toxins to the eukaryotic cell membrane. This hypothesis is supported by the findings that the monoclonal antibody PCG-4, which blocks the binding and enterotoxicity of toxin A, interacts with these repeating units (Frey and Wilkins 1992). Recently it was reported that the complete putative receptor-binding domain (residues 1 750–2 759) is necessary for receptor-binding and endocytosis (Frisch et al. 2003). The C-terminus of toxin A binds to the trisaccharide Gaα1–3Gaβ1–4GlucNAβ (Tucker and Wilkins 1991). This trisaccharide has been suggested to be part of the toxin receptor in brush border membranes of hamsters and in various cultured cells. However, because α-galactosyltransferase is absent in human tissue, this trisaccharide cannot be part of the receptor in man (Larsen et al. 1990). The same is true for the sucrase–isomaltase, which was identified as a toxin A receptor in rabbit ileal brush border, but is not present in all toxin-sensitive cells (Pothoulakis et al. 1996). Further, it has been reported that toxin A also binds to the glycophospholipid GalNAcβ3Galβ4GlucNAcβ3Galβ4Glcβ1Cer, which is found on human tissues (Teneberg et al. 1996). Thus the precise nature of the toxin A receptor(s) in the gut has still to be elucidated. Moreover, the receptor for toxin B is completely unknown so far.

By using electron microscopy and inhibitors of endocytosis and/or endosomal processing, evidence has been presented indicating receptor-mediated internalization of toxin A and B possibly via coated pits (Henriques et al. 1987; Kushnaryov and Sedmark 1989) and subsequent processing through a low pH compartment (Lyerly et al. 1986; Henriques et al. 1987; Fiorentini and Thelestam 1991). As deduced from other toxins, e.g. diphtheria toxin, the low pH compartment is likely to be important for refolding of the toxin, allowing exposure of hydrophobic toxin regions and subsequent membrane insertion (Montecucco et al. 1994; Collier 1995). This hydrophobic region appears to be located as a short stretch almost in the middle of the toxin's sequence, e.g. covering amino acid residues 956–1128 in toxin B. After internalization of the *C. difficile* toxins, this domain may be involved in the translocation of the toxins into the cytosol.

Recently, the low pH-induced pore-forming activity of toxin B was shown (Barth et al. 2001). Bafilomycin A1, which prevents acidification of endosomes, inhibits intoxication by toxin B. When the low pH of endosomes is mimicked in the cell culture medium, the toxin enters the cytosol and induces cytotoxic effects even in the presence of Bafilomycin. Under these conditions, toxin B facilitates the efflux of the radioactive $^{86}Rb^{+}$ isotope, indicating pore or channel formation. Increase in $^{86}Rb^{+}$ release is also observed with the toxin fragment 547–2 366, which covers the putative binding and translocation domains of toxin B (Barth et al. 2001). Also, in artificial membranes toxin B was shown to induce pores (Barth et al. 2001). Recent results give an answer to the question, whether only a part of the large toxin or the holotoxin is translocated into the cytosol to elicit its effects. When Vero cells are treated with toxin B, only the enzyme domain is recognized by antibody in the cytosol. The holotoxin remains associated with membranes. Treatment of culture medium with protease inhibitors prevent intoxication. These data indicate that toxin B is proteolytically cleaved during the uptake process (Pfeifer et al. 2003).

The N-terminal part of the *C. difficile* toxins carries the enzyme activity. Deletion analyses show that a fragment consisting of the N-terminal 546 amino acid residues of *C. difficile* toxins A and B possess full glucosyltransferase and glucohydrolase activities (Hofmann et al. 1997). However, further deletion of about 30 amino acid residues drastically reduces enzyme activity and a fragment of only 486 residues lacks enzyme activity. Similar structural requirements for enzyme activity were detected for the related *C. sordellii* lethal toxin which glucosylates Rac and additionally Ras subfamily proteins (e.g. Ras, Rap, Ral), but not RhoA. By constructing chimeras between *C. difficile* toxin B and *C. sordellii* lethal toxin, a sequence region was identified that defines the different substrate specificities of these toxins covering amino acid residues 368–468 (Hofmann et al. 1998). Further structure–function analysis of large clostridial cytotoxins (e.g. *C. difficile* toxins A and B) were obtained by sequence alignments with eukaryotic glycosyltransferases. Thereby, a DXD motif was identified that is conserved in all large clostridial cytotoxins, in many eukaryotic glycosyltransferases and also in various prokaryotic transferases not directly related to the cytotoxins (Figure 2) (Breton and Imberty 1999). Mutations (D286A and D288A) of the aspartate residues in this motif caused drastic

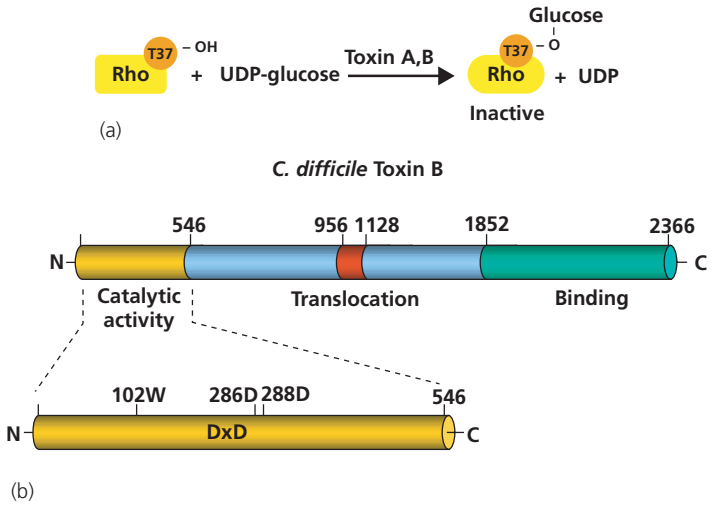

Figure 2 (a) *Inactivation of Rho proteins by* Clostridium difficile *toxins A and B. The toxins inactivate RhoA by glucosylation at threonine-37 (Rac and Cdc42 at Thr-35) using UDP glucose as a cosubstrate. Glucosylated Rho GTPases are inactive, as they can not interact with their effectors.* **(b)** *Localization of functional domains in the primary structure of* C. difficile *toxins A and B. Numbers indicate amino acid residues of toxin B. At the C-terminus, the toxins are characterized by oligopeptide repeats (amino acid residues 1 852–2 366) which are most likely responsible for carbohydrate binding. The hydrophobic region in the middle of the protein (residues 956–1 128) might be involved in translocation of the toxins into the cytosol. The N-terminus (residues 1–546) harbors the glucosyltransferase activity, the DXD motif and tryptophan-102 (W) are essential for enzyme activity and might be involved in binding of divalent cation and/or UDP-glucose.*

reduction (>5 000-fold) in enzyme activity and blocked photoaffinity-labeling with azido-UDP-glucose. Studies on the manganese dependency of DXD mutants suggested that this motif is involved in coordination of divalent cations and/or in UDP-glucose binding (Busch et al. 1998). The DXD-motif of large clostridial cytotoxins is positioned in a region ('extended' DXD-motif), which is significantly homologous with other glycosyltransferases. Interestingly, all these glycosyltransferases sharing the extended DXD-motif are α-retaining glycosyltransferases. Recent studies indicate that this might also be the case for clostridial cytotoxins. At least for *C. sordellii* lethal toxin, which is highly related to toxins A and B, it was shown that the glucose group, which is attached on to the target GTPase, is in the α-anomeric conformation (Vetter et al. 2000; Geyer et al. 2003). A conserved tryptophan residue (Trp-102) was identified, which is involved in the UDP-glucose binding by large clostridial cytotoxins (Busch et al. 2000). Replacement of Trp-102 by alanine decreased the glucosyltransferase activity by about 1 000-fold and blocked cytotoxic activity after microinjection. Recently, genes from pathogenic *Escherichia coli* strains (Burland et al. 1998), *Chlamydia trachomatis* (Stephens et al. 1998; Campbell and Richmond 1989), and *Pseudomonas fluorescens* were identified, which encode for large proteins, sharing sequence similarities with the enzyme domains of large clostridial cytotoxins, including the extended DXD-motif and the conserved tryptophan. All these genes are now grouped in family Nr 44 of glycosyltransferases, according to the system of Henrissat and coworkers (available on the internet at www.afmb.cnrs-mrs.fr/CAZY/GT_44.htlm (Campbell et al. 1997).

Toxin A is primarily responsible for the enterotoxic activity of *C. difficile* in animal models (Triadafilopoulos et al. 1987), but toxin B is much more potent as a cytotoxin than toxin A (Rothman et al. 1984; Pothoulakis et al. 1986a; Lima et al. 1988). Both toxins are lethal for mice, with toxin A having a potency 50–400 times that of toxin B (Taylor et al. 1981; Banno et al. 1984). However, recent studies suggest that both toxins are similarly enterotoxic or toxin B is even more potent than toxin A in humans (Hecht et al. 1988; Savidge et al. 2003). This is in agreement with the findings of toxin A–negative B-positive strains and their presence in clinical material (Torres 1991; Depitre et al. 1993; Borriello et al. 1992b; Lyerly et al. 1992; Rupnik et al. 1997). The physical characteristics and biological activities of the two toxins are compared in Table 4. The data for similar toxins produced by *C. sordellii* are also listed.

The enterotoxic effect of toxin A in the rabbit intestine appears to be due to tissue damage resulting from an inflammatory process induced by the toxin (Triadafilopoulos et al. 1987). Following injection of toxin A (but not toxin B) an infiltration of neutrophils into the ileum and a release of inflammatory mediators occur, causing fluid secretion, altered membrane permeability, and hemorrhagic necrosis. It has also been observed in vitro that toxin A acts as a powerful chemotactic agent for human neutrophils (Pothoulakis et al. 1986b).

The cytotoxic action of toxin B involves depolymerization of filamentous actin, causing a destructuring of the cell cytoskeleton and thus, a rounding of the cell

Table 4 *Physical characteristics and biological activities of toxins A and B of* C. difficile [a] *and HT*[b] *and LT*[c] *of* C. sordellii

Toxin	Molecular size (kDa)	Isoelectric pH	Dose (ng)			
			Tissue culture	Enterotoxic	Mouse lethality	Vascular permeability
C. difficile						
A	308[d]	5.3	10	1000[e]	50	1–10
B	270[d]	4.1	0.0002–0.001	Negative[e]	50	1–10
C. sordellii						
HT	c. 300	6.1	500	2000[e]	120	
LT	271	4.55	16.4	Positive[f]	2.94	

Note: Readers must refer to the original publications to determine concentration where dose is shown. Also, these figures will be dependent on the cell line used as their susceptibility differs.
a) Lyerly et al. (1988). (b) Mar tinez and Wilkins (1988). (c) Popoff (1987). (d) Wolfhagen et al. (1994). (e) Rabbit loop. (f) Guinea-pig loop.

(Pothoulakis et al. 1986a; Ottinger and Lin 1988). The high potency of toxin B is in line with an enzymatic action on proteins (low-molecular-mass GTPases, see below) involved in actin polymerization and regulation (Pothoulakis et al. 1986a).

The molecular mechanism of the action of *C. difficile* toxins has been elucidated. Both toxins are mono-*O*-glucosyltransferases that modify GTP-binding proteins of the Rho family. Members of this protein family, which comprise about 20 related proteins, are RhoA, B, C, Rac1, 2, 3, Cdc42, and others (Madaule and Axel 1985; Didsbury et al. 1989; Munemitsu et al. 1990; Vincent et al. 1992; Takai et al. 2001; Van Aelst and D'Souza-Schorey 1997; Bishop and Hall 2000; Ridley 2001; Matozaki et al. 2000; Kjoller and Hall 1999; Bokoch 2000). Rho proteins are GTPases, which are involved in the regulation of the actin cytoskeleton and act as molecular switches in a large array of signal processes (Collard 1996; Lim et al. 1996; Narumiya 1996; Hall 1998; Mackay and Hall 1998). The proteins belong to the 'Ras' superfamily (note Rho = Ras homologous) and are regulated by a GTPase cycle (Bourne et al. 1990, 1991). They are inactive in the GDP bound form and activated after GTP/GDP exchange. The active state of the proteins is terminated by hydrolysis of bound GTP. At least three different groups of proteins are involved in the regulation of Rho GTPases, including the activating guanine nucleotide exchange factors (GEF), the inactivating GTPase-activating proteins (GAP), and the guanine nucleotide dissociation inhibitors (GDI), which keep Rho GTPase in their inactive form in the cytosol. Like many small GTPases, Rho proteins are post-translationally modified by isoprenylation (farnesylation and/or geranylation) at the C-terminus. Isoprenylation is important for membrane binding and essential for activation of Rho GTPases.

Rho proteins regulate extracellular induced changes in motility, morphology, and cell polarity (Etienne-Manneville and Hall 2002). The various members of the Rho subfamily appear to regulate the actin cytoskeleton in a highly specific manner (Paterson et al. 1990; Ridley and Hall 1992; Ridley et al. 1992; Kozma et al. 1995; Nobes and Hall 1995). Whereas active Rho causes formation of stress fibers, Rac is involved in structuring of lamellipodia and membrane ruffling, and Cdc42 in the formation of filopodia or microspikes. Rho proteins are involved in a large array of signaling processes including phosphatidylinositol-4-phosphate-5-kinases (Chong et al. 1994), phospholipase β (Illenberger et al. 1998), and phospholipase D (Malcolm et al. 1994). They regulate proliferation (Olsen et al. 1995), and transcriptional activation (Hill et al. 1995), as well as cell tansformation and apoptosis (Hippenstiel et al. 2002). In respect to their role as targets of bacterial protein toxins, it is important to mention that Rho proteins have crucial functions in innate and aquired immunity. They are involved in migration of immune cells, in phagocytosis (Caron and Hall 1998), endocytosis (Schmalzing et al. 1995), secretion (Prepens et al. 1996), and NADPH oxidase regulation (Abo et al. 1991). Moreover, they are essential for the function of the so-called immunological synapse, which defines the interaction of cells of the immune system with target cells (Vicente-Manzanares et al. 2003; Reif and Cantrell 1998; Walmsley et al. 2003; Gu et al. 2003).

Molecular mechanisms of *C. difficile* toxins A and B

C. difficile toxins A and B glucosylate all Rho GTPases including RhoA, Rac, and Cdc42. RhoA is modified at threonine-37 (Rac and Cdc42 at the equivalent residue threonine-35) (Just et al. 1995a, b). This amino acid residue is significant for the functions of Rho proteins (Aktories and Just 1995; Wei et al. 1997; Ihara et al. 1998). Thr-37 is highly conserved and involved in the coordination of the magnesium cation, which is essential for the nucleotide binding. Moreover, Thr-37 is located in the so-called switch 1 region of the GTPase. This region undergoes major conformational changes upon activation and binding of GTP and is involved in the interaction of Rho proteins with their various effectors.

Accordingly, these interactions are dramatically changed after glucosylation. Immunoprecipitation studies show that RhoA glucosylated by toxin B is no longer capable of interacting with its effectors (e.g. protein kinase N) (Sehr et al. 1998). Thus, blocking of the interaction of Rho proteins with their effectors is most likely the central mechanism causing biological inactivation of glucosylated Rho (Figure 2). Additionally, Rho glucosylation causes a decrease in the affinity for nucleotides (GTP, GDP) and a reduction in Rho activation by the guanine nucleotide exchange factor LBC (Sehr et al. 1998). Surprisingly, glucosylation inhibits intrinsic and GTPase-activating protein-stimulated GTP hydrolysis (Sehr et al. 1998). Although the latter effects may prolong the active state of the Rho proteins, inhibition of the effector/GTPase interaction is dominant and defines the biological consequences of glucosylation. Similar functional consequences were obtained with Ras glucosylated by *C. sordellii* lethal toxin (Herrmann et al. 1998). It was shown that toxin B blocks the cytosol-membrane cycling of RhoA (Genth et al. 1999). In its GDP-bound inactive form, RhoA (and this is also true for the other Rho GTPases) is in the cytosol associated with GDI. Upon activation, the GTPase translocates to the membrane where it is released from GDI, nucleotide exchange is induced by GEFs and the interaction with effectors occurs. Termination of the active state of Rho by GTP hydrolysis increases the affinity for GDI, which extracts the GTPase from the membrane. This cycle is apparently blocked by glucosylation.

ADP-ribosyltransferase (CDT)

In addition to toxins A and B, a biologically active protein with ADP-ribosyltransferase activity (CDT) has been found in a strain (CD196) of *C. difficile* (Popoff et al. 1988). It was shown to modify cell actin in a manner similar to that of *C. botulinum* C_2 and *C. perfringens* ι toxin. This protein cross-reacts antigenically with the light chains of the ι binary toxins from *C. perfringens* type E and *C. spiroforme* (Popoff and Boquet 1988). CDT has a molecular mass of 43 kDa. Similar to the related binary toxins from *C. botulinum* (C2 toxin) and from *C. perfringens* (ι toxin), CDT consists of an enzyme component, which ADP-ribosylates actin and a binding component, which is involved in the transport of the enzyme component into the host cells (Stubbs et al. 2000). CDT belongs to the ι toxin-like subgroup of ADP-ribosyltransferases. Its binding component can be interchanged with the binding components of ι toxin and *C. spiroforme* toxin to transport the enzyme CDTa into the target cell. By contrast, the binding component of C2 toxin is unable to facilitate the uptake of CDTa. Similar to ι toxin-like enzyme, CDTa modifies nonmuscle and skeletal muscle actin (Gülke et al. 2001), whereas C2 toxin preferentially modifies nonmuscle actin (Aktories et al. 1986; Schering et al. 1988). Recent studies showed that about 6 percent of *C. difficile* isolates contain the genes for CDT (Stubbs et al. 2000). Interestingly, CDT is also found in *C. difficile* strains that do not produce large clostridial toxins (Rupnik et al. 2003). Therefore, it is of major importance to clarify the virulence potential of CDT, especially as toxin test kits at present available for *C. difficile* toxins do not cover CDT.

TOXIN VARIANTS

Recently, it was shown that a considerable toxin variability exists. The molecular basis are changes in the pathogenicity locus encoding the large clostridial cytotoxins. Toxin A and B are encoded by two genes *tcdA* and *tcdB*, which together with three additional genes (*tcdC*, *tcdD*, and *tcdE*) form a pathogenicity locus (Hundsberger et al. 1997; Moncrief et al. 1997a, b). Variant *C. difficile* strains with deletions, insertions or polymorphic restriction sites within this pathogenicity locus have been reported. So far, at least 20 different toxin types have been defined (Rupnik et al. 1998; Rupnik et al. 1997). The range of different cytopathic phenotypes induced in Vero cells by such strains has been described by Blake et al. (2004). Many of these, of several toxin B variants, have been described which differ in their substrate specificity. Of special interest are toxin B variants from strain 1470 (toxin type VIII) and from strain 8864 (toxin type X). Strain 1470 does not produce toxin A, but a toxin B subtype, which has a similar substrate specificity as *C. sordellii* lethal toxin. It glucosylates Rac and Cdc42, but not RhoA. In addition Ras proteins, including Ras, Rap, Ral, and R-Ras, are modified by toxin B from strain 1470. The toxin B variant from strain 8864 modifies these same substrate proteins. Another recently described toxin variant from strain C34 glucosylates Rho, Rac, and Cdc42 and also the above described Ras proteins.

C. SORDELLII AND *C. BIFERMENTANS*

Hall (1929) reported on the potent virulence of strains of *Bacillus sordellii* and demonstrated that pathogenicity was due to a toxin that caused severe edema that could be neutralized by a specific antiserum. For a while, *C. sordellii* was considered as a subgroup of *C. bifermentans*, distinguished by its ability to produce the potent lethal toxin responsible for the severe gelatinous edema. These organisms are related to *C. difficile* (see above), and belong to 16S rRNA gene cluster XI (Figure 1).

Toxins produced by *C. sordellii* and *C. bifermentans*

C. sordellii produces three toxins in common with the nonpathogenic *C. bifermentans*: (1) a lecithinase; (2) an oxygen-labile hemolysin; and (3) a fibrinolysin. The lecithinase is a phospholipase C, serologically related to the α-toxin of *C. perfringens* as evidenced by the positive

Nagler reaction (Willis 1969). It is relatively nontoxic for mice. The gene for the α-toxin of *C. bifermentans* has been cloned and is 64 percent homologous in coding sequence in comparison with the *C. perfringens* gene (Tso and Seibel 1989). It codes for a mature protein consisting of about 376 amino acids. This lecithinase from *C. bifermentans* has about 1/50 the activity of *C. perfringens* α-toxin in enzymic, hemolytic, and mouse lethality assays. The calculated molecular weight, although not given for the *C. bifermentans* lecithinase would be similar to the 43 kDa calculated for the *C. perfringens* α-toxin.

Major lethal toxins

A lethal factor referred to as β-toxin (Willis 1969) responsible for the pathogenicity of *C. sordellii* distinguishes it from *C. bifermentans*. Arsecularatne et al. (1969) found two different activities associated with the major lethal toxin, which were separable from each other in different preparations. Both toxic factors were dermonecrotic and hemorrhagic when injected into the skin of rats and guinea-pigs. The first activity (edematizing) caused massive edema and moderate areas of bright red hemorrhage when injected intradermally, subcutaneously, or intramuscularly into guinea-pigs, with death occurring within 24–36 h. The second factor (hemorrhagic) caused minimal edema, but confluent areas of brownish hemorrhage in the skin, and in the omentum, mesentery, and contiguous viscera when injected intravenously or intraperitoneally. The hemorrhagic factor appeared to be associated with sporulating cultures. Little lethality was noted for this toxin at doses with high hemorrhagic activity.

Nakamura et al. (1983b) examined 55 strains of *C. sordellii* for mouse lethality and cytotoxicity. They found a close correlation between the two activities. All 34 strains negative for mouse lethal toxin lacked cytotoxin. Lethal toxin titers paralleled cytotoxicity titers. Yamakawa et al. (1983) found that only two of 15 cytotoxigenic strains produced positive rabbit ileal loop responses (fluid accumulation). The lethal and cytotoxic effects, as well as the ileal loop response, were eliminated by preincubation of active culture fluid with either *C. sordellii* or *C. difficile* antitoxin. Two cytotoxins (I and II) were produced by one strain (3703), and were separated from each other by DEAE ion-exchange chromatography (Yamakawa et al. 1985). They could be distinguished from one another by specific antitoxins produced against each of them.

It appears that the lethal toxin classically designated as β-toxin (Willis 1969) actually consists of two components: lethal toxin (LT) and hemorrhagic toxin (HT). Popoff (1987) purified LT from a strain of *C. sordellii* and found it to be a protein with a molecular mass of 250 kDa and an isoelectric pH of 4.55. It was antigenically related to toxin B of *C. difficile*, but not to toxin A. Martinez and Wilkins (1988) purified HT and found a molecular mass of 525 kDa under nondenaturing conditions and an isoelectric pH of 6.l. HT is cytotoxic for cultured cells, lethal for mice, and causes an accumulation of hemorrhagic fluid in ligated rabbit ileal loops. It is immunologically related to toxin A of *C. difficile*. The properties and activities of HT and LT are listed and may be compared with the *C. difficile* toxins in Table 4. In summary, HT is equivalent to cytotoxin I (Yamakawa et al. 1985) and is related antigenically and in biological activity to toxin A of *C. difficile*. LT is equivalent to cytotoxin II and is related antigenically and in biological activity to toxin B of *C. difficile*.

C. NOVYI AND *C. HAEMOLYTICUM*

Diseases caused by pathogenic strains of *C. novyi* and *C. haemolyticum* appear to involve primarily the α-toxin of the former and the β-toxin of the latter. In gas gangrene in humans the α-toxin causes massive edema due to its effect on capillary permeability. In bacillary hemoglobinuria, the β-toxin destroys the circulating erythrocytes resulting in excretion of the hemoglobin in the urine. Simultaneously, blood is lost into the intestine because of destruction of the capillary endothelium. The grossly different pathological presentation of these two diseases has led to maintaining two separate species for the causative organisms (Smith and Williams 1984; Cato et al. 1986).

Toxins produced by *C. novyi*

Classification of three types of *C. novyi* is based on production of several toxic antigenic factors (Eklund 1993). *C. novyi* types A, B, and C, and *C. haemolyticum* are distinguished from each other by the toxins designated as α and β (Willis 1969; Smith and Williams 1984) (Table 5), based on the studies of Oakley and coworkers (Oakley et al. 1947; Oakley 1955; Oakley and Warrack 1959). Type A produces α only, *C. haemolyticum* produces β only, and type B produces both. Production of six other toxins by species and toxin type is also shown in Table 5. Type C strains produce none of the known toxins and are considered nonpathogenic. Nonpathogenic strains phenotypically similar to *C. novyi* type A, or to *C. botulinum* type C or D may be derived from pathogenic strains that have lost their infecting phages (Cato et al. 1986).

The lecithovitellin reaction has often been used to identify toxins of the *C. novyi* group. Rutter and Collee (1969) noted that positive lecithovitellin reactions could be due to lecithinase, lipase or both lecithinase and lipase activities. They pointed out that type A strains produce γ-toxin (a hemolytic lecithinase) and ϵ-toxin (a lipase). Type B and *C. haemolyticum* strains produce β-toxin (a hemolytic lecithinase), and also θ-toxin, which may be a lipase, though not demonstrable by a visible reaction on

Table 5 *Toxins of* C. novyi *and* C. haemolyticum[a]

Toxin	Activity	*C. novyi* type A	*C. novyi* type B	*C. novyi* type C	*C. haemolyticum*[b]
α	Necrotizing, lethal	+	+	–	–
β	Lecithinase; necrotizing lethal; 1hemolytic	–	+	–	+
γ	Lecithinase; necrotizing; hemolytic	+	–	–	–
δ	Oxygen-labile hemolysin	+	–	–	–
ϵ	Lipase (pearly layer)	+	–	–	–
ζ	Hemolysin	–	+	–	–
η	Tropomyosinase	–	+	–	–
θ	Opalescence in egg yolk	–	tr	–	+

a) From Hatheway (1990).
b) Sometimes referred to as *C. novyi* type D. tr, trace.

egg yolk agar. The β and γ lecithinases are serologically distinct. Rutter and Collee showed by thin-layer chromatography that *C. novyi* type B and *C. haemolyticum* strains break down lecithin into diglycerides, while *C. novyi* type A strains initially convert lecithin into diglycerides, but subsequently break down the diglycerides from the lecithinase reaction, as well as those present in the egg yolk suspension into free fatty acids and glycerol (Rutter and Collee 1969). Thus, *C. novyi* type A, *C. novyi* type B, and *C. haemolyticum* all possess lecithinase activity, but only type A possesses a readily demonstrable lipase. Those authors also showed a correlation between the mouse lethal activity of the α-toxin of types A and B strains and cytotoxicity of the cultures.

α-Toxin

Phillips et al. (1970) purified the α-toxin of *C. novyi* type B by gel filtration, adsorption on to alumina, and ultrafiltration, and reported it as a protein with a molecular mass of 132 kDa. Izumi et al. (1983) reported a molecular mass of 260–280 kDa for the toxin purified by a combination of ion-exchange and gel filtration chromatography. This was shown to be the more accurate figure from molecular studies (see below). The purified toxin had an isoelectric pH of 6.1. It had lethal, edematizing, and permeability activities but was free of the hemolytic and lecithinase activities that were present in starting culture fluid concentrate. The LD_{50} of the purified toxin for mice was 17 ng, and the OeD_{50} (50 percent edematizing dose) was 1.7 ng.

Eklund et al. (1976) found that α-toxin production in strains of *C. novyi* type A and type B was due to phages. Curing the organisms of their phages, NAI^{tox+} and NBI^{tox+}, respectively, rendered them nontoxigenic (with respect to α-toxin) and susceptible to reinfection by the homologous, but not the heterologous tox^+ phage. Reinfection resulted in their regaining toxigenicity. These phages affected only α-toxin production. Thus, the cured type A strain resembles nontoxigenic *C. botulinum* type C, and the cured type B strain resembles *C. haemolyticum*, since it continues to produce the β-toxin. Schallehn and Eklund (1980) infected a strain of *C. haemolyticum* with a tox^+ phage from type A strains, rendering it capable of producing α-toxin. It was thus indistinguishable from *C. novyi* type B on the basis of the major toxins it produced.

The α toxin belongs to the family of large clostridial cytotoxins. Other members of this toxin family are *C. difficile* toxins A and B (see detailed description under *C. difficile* toxins) and the lethal and hemorrhagic toxins from *C. sordellii* (Green et al. 1995; Just et al. 1996; Popoff et al. 1996). *C. novyii* α toxin consists of 2 178 amino acid residues with a molecular mass of 250 166 Da and shares about 33 and 32 percent identity and 48 percent homology with *C. difficile* toxins A and B, respectively (Hofmann et al. 1995). Like *C. difficile* toxin A and B, α toxin from *C. novyi* is a glycosyltransferase that modifies small GTPases of the Rho family. Rho proteins (Rho, Rac, and Cdc42) are regulators of the actin cytoskeleton and control signal transduction processes (see detailed description of Rho functions and actions of large clostridial cytotoxins under *C. difficile* toxins). However, the α toxin differs from *C. difficile* toxins A and B, because it uses UDP-GlcNAc instead of UDP-glucose as a cosubstrate and induces the *N*-acetylglucosaminylation of Rho proteins (Selzer et al. 1996). Protein substrates (Rho, Rac, and Cdc42) and their site of modification (threonine-37 of Rho, threonine-35 of Rac, and Cdc42) are the same for *C. novyi* α toxin as for *C. difficile* toxins (Selzer et al. 1996). Accordingly, the α toxin causes destruction of the actin cytoskeleton in cell culture in a manner indistinguishable from the effects of *C. difficile* toxin B (Bette et al. 1991). The *C. novyi* α toxin is more potent than *C. difficile* toxin B when compared in the mouse time to death assay. The LD_{50} after 100 h of treatment (intraperitoneal application) was about 300 ng/kg and 5 µg/kg for *C. novyi* α toxin and *C. difficile* toxin B, respectively (Bette et al. 1989, 1991). Both toxins exhibited the same potency in inducing rat paw edema (Bette et al. 1991).

β-Toxin

The β-toxin is lethal, hemolytic, necrotizing, and has lecithinase activity (Willis 1969). Darakhshan and Lauerman (1981) report a molecular weight of 32 kDa for β-toxin from *C. haemolyticum* purified by gel filtration chromatography. The toxin was labile at 60°C and rapidly inactivated by exposure to trypsin. The β-toxins of *C. novyi* type B and *C. haemolyticum* appear to be serologically identical (MacFarlane 1950). Although the enzymatic activity is that of phospholipase C, it is not related serologically to *C. perfringens* α-toxin or to the γ-toxin of *C. novyi* type A. *C. botulinum* type C produces a lecithinase that is comparable serologically to β-toxin of *C. novyi* type B and *C. haemolyticum* (Nakamura et al. 1983a). The lethality and pathogenicity of *C. haemolyticum* appear to be due to β-toxin that is produced in large amounts by this organism, more so than by *C. novyi* type B.

γ-Toxin

The γ-toxin produced by *C. novyi* type A is also a phospholipase C, but is serologically distinct from β-toxin, as well as from the α-toxin of *C. perfringens* (MacFarlane 1948). γ-Toxin was purified by Taguchi and Ikezawa (1975) by gel filtration chromatography, and the estimated molecular mass was 30 kDa. In addition to hydrolysis of lecithin, γ-toxin catalyzes the hydrolysis of phosphatidylinositol and phosphatidylglycerol, substrates not utilized by *C. perfringens* α-toxin. β- and γ-toxins may be determined and differentiated by hemolytic or lipovitellin assays using specific antitoxins (Willis 1969).

δ-Toxin

The δ-toxin is an oxygen-labile hemolysin that is serologically related to similar hemolysins such as the θ-toxin of *C. perfringens* and the tetanolysin of *C. tetani* (Willis 1969; Smith and Williams 1984). It is produced only by type A strains.

ϵ-Toxin

ϵ-Toxin is a lipase that is responsible for the 'pearly layer' associated with the bacterial colonies on egg yolk agar. This factor is seen only with type A strains, and is shared in common with *C. botulinum* types C and D. The ϵ-toxin decomposes triglycerides and diglycerides in the egg yolk medium, as well as diglycerides formed by hydrolysis of lecithin by γ-toxin (Rutter and Collee 1969).

ζ-Toxin

ζ-Toxin is a hemolysin produced only by type B strains. It is not related serologically to δ-toxin nor to any of the other oxygen-labile hemolysins, nor is it sensitive to oxygen (Willis 1969). There is no known role for these hemolysins in pathogenicity of the organisms that produce them.

η-Toxin

η-Toxin is a proteolytic enzyme produced by *C. novyi* type B and *C. haemolyticum*. It is active on the muscle proteins, myosin, and tropomyosin, and has been named tropomyosinase (MacFarlane 1955). Tropomyosin is hydrolyzed more completely than is myosin: 74 and 25 percent of the nitrogen of those respective substrates was converted to acid-soluble form. The enzyme is activated by sulfydryl reagents, such as cysteine, glutathione, and thiolactate. It is not inhibited by soybean trypsin inhibitor. It is neutralized by antisera prepared against *C. novyi* type B cultures. Its pathogenic action in muscle infection may be important, but this has not been studied (Smith and Williams 1984).

θ-Toxin

θ-Toxin, produced by *C. haemolyticum* (and possibly by *C. novyi* type B on the basis of antibodies produced against those strains), causes opalescence in egg yolk emulsions, but has not been characterized. It might be a lipase, serologically distinct from ϵ-toxin, and is nonhemolytic (Willis 1969).

GENETIC RELATEDNESS OF *C. NOVYI*, *C. HAEMOLYTICUM*, AND *C. BOTULINUM*

Nakamura et al. (1983a) examined four strains each of *C. novyi* type A, type B, and *C. haemolyticum* and five strains of *C. botulinum* type C. They found three groups of closely related organisms as indicated in Table 6. *C. novyi* type A is less related than either *C. novyi* type B or *C. haemolyticum* to *C. botulinum* type C. *C. novyi* type B and *C. haemolyticum* are very closely related to each other. One strain of *C. botulinum* type C (the Stockholm strain) is more closely related to the *C. novyi* type B/*C. haemolyticum* group than to the other four strains of *C. botulinum* type C.

C. SEPTICUM AND *C. CHAUVOEI*

C. septicum and *C. chauvoei* belong in 23S rRNA homology group I-D of Johnson and Francis (1975) and cluster I on the basis of 16S rRNA gene sequence homology (Figure 1).

C. HISTOLYTICUM

C. histolyticum is of interest because of its isolation from gangrenous and nongangrenous war wounds, its production of a lethal toxin that cross-reacts serologically with the α-toxin of *C. septicum*, and its production of a mixture of collagenases and other proteolytic enzymes that are unique in their efficiency of converting tissue proteins to amino acids and peptides. Although it is not

Table 6 *DNA relatedness of* C. novyi, C. haemolyticum *and* C. botulinum *type C*[a]

Species	No. of strains	Relation to reference strain (%)[b] from:		
		Group I	Group II	Group III
Group I				
C. novyi A	4	80–100	44–45	33–36
Group II				
C. novyi B	4	28–49	93–100	50–62
C. haemolyticum	4	26–38	93–100	50–62
C. botulinum C	1	42	85	54
Group III				
C. botulinum C	4	36–48	68–70	91–100

a) From Hatheway (1990); data condensed from Nakamura et al. (1983a).
b) Values indicate relatedness between DNA from the test strain and that of the reference strain.

a strict anaerobe (it may grow on agar media in the presence of air) and its nutritional requirements are not more stringent than many other clostridia, its growth may be inhibited by the presence of sugar in the medium, and contrary to some reports (Zeissler 1930; Prevot 1957), the heat resistance of spores may be rather low (Nishida and Imaizumi 1966).

Toxins of *C. histolyticum*

C. histolyticum produces five toxins, designated by the first five letters of the Greek alphabet, in the order of their recognition. It is pathogenic for laboratory animals (Willis 1969). Strains that produce appreciable amounts of α-(lethal) toxin cause death of a laboratory animal within 24 h of intramuscular injection of the organism. In the absence of α-toxin no toxemia ensues, but a progressive gangrenous infection may take place, with tissue destruction due largely to actions of the organism's collagenolytic and proteolytic toxins. The skin over the area of infected muscle is completely destroyed and the underlying tissues are grossly digested as are the soft parts of the bones (Aikat and Dible 1960). Ultimately the digestive process may terminate with autoamputation of the infected limb, but in some cases infection may spread into the trunk of the body, damaging vital organs and resulting in death (Weinberg and Seguin 1916; Willis 1969).

α-Toxin

Lethal toxicity of culture filtrates of *C. histolyticum* was reported by Weinberg and Seguin (1916). This lethal factor was designated α-toxin and noted to be necrotizing, but not hemolytic (Oakley and Warrack 1950). It has been noted that the α-toxin of *C. histolyticum* can be neutralized by antisera produced against toxic filtrates of *C. septicum* cultures. Similarly, the lethality of *C. septicum* cultures can be neutralized by *C. histolyticum* antisera (Sterne and Warrack 1964). Cross-neutralizations can be demonstrated both in mouse lethality and intradermal necrosis reactions.

Nishida and Imaizumi (1966) found that only six of 21 strains of *C. histolyticum* isolated from soil samples in Japan produced α-toxin in culture. The pharmacological and molecular properties of this toxin have not been studied in depth, probably because it is rather unstable. It is readily inactivated by proteolytic enzymes (Smith and Williams 1984). Bowen (1952) reported 85 LD_{50} per mg toxin per kg of body weight for mice for α-toxin partially purified by ethanol precipitation.

β-Toxin

β-Toxin has been identified as a collagenase (EC 3.4.24.3) or more recently as a group of seven collagenases (Bond and Van Wart 1984b; Van Wart and Steinbrink 1985). Collagenase is a zinc metalloprotease that cleaves native triple-helix collagen, as well as gelatin, into small fragments (Dixon and Webb 1979). Collagen is the most abundant protein in the animal body, constituting one-fourth to one-third of the total protein forming the insoluble fiber of connective tissue (White et al. 1978). Yoshida and Noda (1965) first separated *C. histolyticum* collagenase into two fractions designated as I and II. Both degraded collagen at similar rates, but collagenase II split more peptide bonds in the collagen molecule and was more efficient in degrading lower molecular weight polypeptides. Bond and Van Wart (1984a, b) and Van Wart and Steinbrink (1985) have identified seven distinguishable molecular forms of *C. histolyticum* collagenase, and designated each by a Greek letter; unfortunately five of these can be confused with the five toxins of this organism designated by the same Greek letters. Each collagenase is distinguished by its molecular mass, determined as 68, 115, 79, 100, 110, 125, and 130 kDa for collagenases α, β, γ, δ, ϵ, ζ, and η, respectively. Two subspecies of the α and β collagenases are noted on the basis of different isoelectric points. Class I collagenase activity is found with the α, β, γ, and η molecular species, and class II with the δ, ϵ, and ζ. The class I collagenases have extensive amino acid sequence homology with each other, and similar

homology is found among the class II enzymes, but the two classes have substantially different sequences (Bond and Van Wart 1984c). Evidence shows intragenic duplication in the β molecule, which may account for its higher molecular weight. This duplication may also be true for the other higher molecular weight collagenases. The collagenases are all related serologically, but some distinctions are evident by immunodiffusion analysis in agar gels. The two classes of collagenases have been shown to have similar but complementary substrate amino acid sequence specificities by which they synergistically digest collagen (Mookhtiar et al. 1985; Van Wart and Steinbrink 1985). β-Toxin no doubt plays a major role in the pathology of *C. histolyticum* infections in view of its ability to destroy collagen fibers. The toxin induces hemorrhage when placed on the surface of the lungs of animals, and causes hemorrhage and edema when injected into rat paws (Vargaftig et al. 1976). It causes a lethal intrapulmonary hemorrhage when injected intravenously (Smith and Williams 1984).

γ-Toxin

γ-Toxin is a thiol-activated proteinase that digests hide powder, azocoll, gelatin, and casein, but is inactive against collagen (Willis 1969). Its molecular weight is about 50 kDa (Smith and Williams 1984).

δ-Toxin

The δ-toxin is also a proteolytic enzyme. More specifically, it is an elastase (Willis 1969). It is reversibly inactivated by reducing agents. Takahashi et al. (1970) partially purified δ-toxin and found that it passed through a 50 kDa ultramembrane filter, but was retained by a 10 kDa membrane. The active fraction obtained by gel filtration and ion-exchange chromatography had high specific caseinolytic, as well as elastolytic, activity. It was not active on collagen. The elastolytic and caseinolytic activities were not affected by chelating agents.

ϵ-Toxin

The ϵ-toxin is an oxygen-labile hemolysin similar serologically to those produced by other clostridia such as *C. tetani*, *C. septicum*, and *C. novyi* (Willis 1969; Bernheimer 1976; Smith and Williams 1984).

C. SPIROFORME

Since 1964, there have been several reports of helically coiled, gram-positive anaerobic organisms isolated from animal feces and cecum contents (Bladen et al. 1964; Fitzgerald et al. 1965). Because spore formation was noted in some of these strains, they were recognized as clostridia (Koopman and Kennis 1977). Kaneuchi et al. (1979) compared 33 strains of coiled sporeforming organisms recovered from humans and animals. All of the organisms were nonproteolytic and nongelatinolytic, fermented glucose, and produced terminal to subterminal round spores. Two main groups were established on the basis of physiological characteristics and DNA relatedness. Twenty-one strains were designated as *C. cocleatum* and eight as *C. spiroforme*. Each of these two species has 46–60 percent relatedness with reference strains of the opposite species and 74–100 percent relatedness in comparison of strains within each species. The phenotypic differences noted (Kaneuchi et al. 1979) were fermentation of galactose by *C. cocleatum* and a more pronounced coiling by *C. spiroforme*. Both species were related to *C. ramosum*; 35–52 percent for *C. cocleatum*, and 32–53 percent for *C. spiroforme*. *C. ramosum* has a straight rod morphology and has α-methylmannosidase activities that are not found in the two coiled organisms. The relationship of *C. spiroforme* to *C. ramosum* on the basis of characteristics other than morphology is indicated further by their 16S rRNA similarity, as seen in Figure 1, with both organisms occupying cluster XVIII.

After *C. spiroforme* cultures are heated at 80°C for 10 min, uncoiled, straight cells are found that do not revert to the coiled structure upon subculture (Kaneuchi et al. 1979). No alterations of any other features of the organism are observed. The helical structure of the native organism consists of numerous single semicircular cells joined end to end (Borriello et al. 1986). The individual cells are nonmotile and gram-positive, with dimensions of 0.3–0.5 × 2.0–10 μm (Cato et al. 1986).

Much clinical interest in *C. spiroforme* arose when an organism with similar morphological and physiological characteristics which produced a toxin neutralizable with type E antitoxin of *C. perfringens* was consistently isolated from feces of scouring rabbits (Carman and Borriello 1982). Borriello and Carman (1983) reported complete agreement between the characteristics of toxigenic isolates from rabbits and the type strain of *C. spiroforme*, but the validity of the identity of the toxigenic strains has been questioned (Moore et al. 1987). ι-Toxin had been found previously in rabbit enterotoxemia, but investigators had attempted to recover ι-toxigenic *C. perfringens* (Patton et al. 1978; Baskerville et al. 1980). The toxigenic organism identified as *C. spiroforme* was subsequently implicated as the cause of both spontaneous and antibiotic-induced diarrhea and colitis in rabbits (Borriello and Carman 1983; Carman and Borriello 1984).

ι-Toxin

The ι-toxin of *C. spiroforme* is a binary toxin with components ι-a and ι-b (also called Sa and Sb) that correspond serologically and electrophoretically to the components of *C. perfringens* ι-toxin (Stiles and Wilkins 1986a). The electrophoretic mobilities of the latter are slightly faster. Although the *C. spiroforme* antiserum formed precipitates with both components of the toxin

from either species, *C. perfringens* antiserum formed visible precipitates only with the b-component of the *C. spiroforme* toxin on immunoelectrophoretic plates.

Popoff and Boquet (1988) purified the a-component of *C. spiroforme* ι-toxin by immunoaffinity and reported that it consists of a heterogeneous population of molecules with molecular masses ranging from 43 to 47 kDa. This is similar to the 47.5 kDa size of the ι-a component of *C. perfringens*. This component has ADP-ribosyltransferase activity towards actin. It was inactive in both cytotoxic and mouse lethality assays by itself, but was potentiated by the component ι-b from *C. spiroforme* or *C. perfringens*. ι-b from *C. spiroforme* potentiated ι-a from *C. perfringens* and also an ADP-ribosyltransferase (CDT) from *C. difficile* in those same assays. The components of the binary C_2 toxin of *C. botulinum* did not synergize with any of the binary components of the other three species.

C. FALLAX

Culture supernatants are considered nontoxic for mice, although there are reports that freshly isolated organisms produce a soluble toxin and are pathogenic for mice and guinea-pigs (Willis 1990).

GAS GANGRENE

History

Gas gangrene (clostridial myonecrosis) became prominent during the First World War. Its incidence was extremely high in battle wounds sustained during the campaigns in France and Belgium and the results were devastating. Since gas gangrene had been uncommon in earlier wars (Keen 1915; Bowlby 1919) and was uncommon in civilian injuries, it came to be regarded as a disease of modern war associated with extensive and heavily contaminated wounds.

The 'hospital gangrene' recorded before the introduction of antiseptic surgical techniques by Lister was anaerobic necrotizing fasciitis rather than clostridial myonecrosis (Bowlby 1919). Conditions recognizable as gas gangrene were rarely recorded in antiquity (Sussman 1958).

Incidence

INCIDENCE IN MILITARY DURING WAR

The incidence of gangrene in First World War wounds seems to have depended on the distribution of clostridia in the soil of the battlefield, being common in the agricultural fertile battlefields of northern Europe, but uncommon in Iraq, Egypt, and the Gallipoli peninsula. This is consistent with the observation that the clostridia responsible for gas gangrene reflect their presence in the soil (MacLennan 1943b, 1962).

In the first year of the First World War, the incidence of gas gangrene among casualties in the British expeditionary force was about 12 percent and the mortality rate some 25 percent. The incidence fell to 1 percent and less when the importance of early wound excision and other prophylactic measures were recognized (Report 1919; Bowlby 1919). During the Second World War the incidence of gas gangrene in British troops was 0.3–0.8 percent (MacLennan 1962) and in US forces was from 0 to 4.5 percent. During the Korean War, when early evacuation and treatment of casualties was practiced, the incidence of gas gangrene was 0.08 percent without mortality (Howard and Inui 1954). By the time of the Vietnam War, gas gangrene was rare and it was suggested that as a disease of war it was almost extinct (Unsworth 1973). Confirmation of this conclusion comes from the Falklands campaign and Operation Desert Storm during which gas gangrene was not observed in British troops or those under Western command. The availability of appropriate medical services appears to be crucial, because in these wars gangrene was observed in other troops.

INCIDENCE IN CIVILIAN PRACTICE

In the 1970s in the developed world, a civilian incidence of gas gangrene of 0.1–1.0 per 10^6 population per year was calculated (Roding et al. 1972; Hart et al. 1975). Wounds of the magnitude seen in war are uncommon in civilian practice and early appropriate surgical treatment and prophylaxis are usually readily available. This may not be the case in developing areas, where gas gangrene after accidental trauma is said to be common.

Though gas gangrene is characteristically associated with serious accidental trauma, it may follow clean elective surgery (Morton 1967; Parker 1967; Braithwaite et al. 1982; Gledhill 1982; Wells et al. 1985). It is a rare but serious complication that may follow the injection of adrenaline (Anon 1968; Teo and Balasubramanian 1983; Hallagan et al. 1992) or insulin (Chin et al. 1993), usually into the lower parts of the body. The causative organism is *C. perfringens*, which contaminates the skin of the perineum, buttocks, and thighs, and originates from the bowel. Clostridial infection of the myometrium, usually due to *C. perfringens*, was particularly related to criminal instrument-induced abortion but it is rare after normal childbirth (Butler 1945; Hill 1964). *C. perfringens* is not often present in the vagina, and then only in small numbers (Lindner et al. 1978; Thadepalli et al. 1978) and is derived from the bowel via the perineum. Spontaneous or metastatic gas gangrene is rare in humans (Willis 1969; Engeset et al. 1973; Gatt 1985; Narula and Khatib 1985; Mulier et al. 1993; Norgaard et al. 1993). It may be associated with diabetes mellitus (Gliemroth et al. 1996; Hengster and Pernthaler 1996).

Histotoxic clostridia may also be involved in many other types of infection. Some of these are considered

later in the present chapter. Useful discussions of clostridial soft tissue infections have been provided by Gorbach (1992) and Lorber (1995). Detailed reviews of gas gangrene and related infections have been published by MacLennan (1962), Willis (1991), and Finegold and George (1989).

Bacteriology

Clostridial wound infections may be of three types (MacLennan 1943b, 1962) with increasing severity. The first is simple contamination, when clostridia are present in injured tissues but without evidence of infection. Such contamination is common and the wounds heal by first intention without sequelae. The second is clostridial cellulitis, in which the infection is limited to local fascia, muscle is not involved and toxemia is minimal. Finally, in clostridial myonecrosis healthy muscle is affected and toxemia is severe. In the presence of tissue anoxia, simple contamination may rapidly progress to cellulitis and myonecrosis.

Anaerobic cellulitis is characterized by a foul, seropurulent infection of the depths of the wound without muscle involvement and toxemia is insignificant. The organisms responsible are proteolytic and nontoxigenic clostridia, and strains of *C. perfringens* of low toxigenicity. In gas gangrene, on the other hand, the important pathogens are predominantly toxigenic, including *C. perfringens*, *C. novyi*, *C. septicum*, *C. histolyticum*, *C. sordellii*, *C. bifermentans*, *C. sporogenes*, and less importantly, *C. fallax*, *C. carnis*, and *C. tertium*. Because of difficulties in growing *C. novyi* and *C. histolyticum*, they were not initially recognized as important (MacLennan 1962), but are now recognized as frequent pathogens with a high fatality, especially in combination with *C. sporogenes* (Smith and Williams 1984). Involvement of *C. difficile* in rare cases of gas gangrene has been suspected, but not clearly documented (MacLennan 1962). Since anaerobic infections are due to wound contamination, they are almost always polymicrobial. It is rare for gas gangrene to be monomicrobial. Surveys of the clostridial flora of gas gangrene in battle casualties have shown that *C. perfringens* and *C. novyi* are of major importance (MacLennan 1943b; Hamilton 1944–45; Cooke et al. 1945).

The bacterial flora present in gas gangrene commonly includes nonclostridial anaerobes and various facultative aerobes (Weinberg and Séguin 1918; MacLennan 1943a, b; Strawitz et al. 1955). Though this 'associated flora' plays no direct part in myonecrosis, it may facilitate tissue invasion by synergy with histotoxic clostridia.

C. botulinum may be present in wounds (Hall 1945) and may give rise to clinical botulism (wound botulism). When wound botulism occurs it is usually due to *C. botulinum* type A or B, and most cases have been reported from the USA (Davis et al. 1951; Thomas et al. 1951; Merson and Dowell 1973). Details are provided in *Clostridium botulinum* and *Clostridium tetani*.

Pathogenesis

Clostridia cannot multiply and produce disease in normal tissues because the high oxidation–reduction potential (E_h) of the circulating blood (+126–+246 mV) and of the tissues is above that necessary for the initiation of anaerobic bacterial growth (+74 mV for *C. perfringens*) (Oakley 1954; Futter and Richardson 1971). The central factor that allows anaerobes to grow in wounds is tissue anoxia. Trauma frequently reduces tissue perfusion and initiates the sequence of events that lead to tissue colonization by contaminating anaerobes. Facultative anaerobes, such as *Escherichia coli* and *Proteus* spp., that may also be present in the wound, contribute to the reduction of the local E_h by utilizing any remaining oxygen.

Anaerobic bacterial infections of humans are typically associated with locally damaged tissues and, commonly, also with a 'compromised' host. Once an anaerobic infection is established, toxinogenesis by the infecting organisms leads to a progressive weakening of host defenses. There is a spectrum of interaction between bacterial pathogenicity and host resistance. At one end of this spectrum the ubiquity of clostridia ensures that most accidental wounds are exposed to the risk of contamination at the time of injury, but contamination of the wound does not make gas gangrene inevitable. Anaerobic conditions must exist in the lesion if the organisms are to multiply. In the absence of an anaerobic environment, toxigenic clostridia remain dormant and are eliminated by the local defense mechanisms. Post-cholecystectomy bacteremia due to *C. perfringens* is an example of the other extreme of the spectrum. Before host defense mechanisms can react to the challenge, toxemia rapidly causes gross intravascular hemolysis and death. The predisposing factors have been discussed by Willis (1977, 1985, 1991) and Finegold and George (1989).

The course of events in *C. perfringens* infections that complicate clean elective surgery is similar to that associated with accidental trauma, but there are differences in the nature and degree of the initiating determinants. The infecting organisms are endogenous on the skin of the patient and are implanted by the surgical procedures. Mid-thigh amputation for obliterative arterial disease is associated with particular risk, as is lower-limb surgery in which foreign materials are implanted, such as pinning and plating of femoral fractures (Knutsdon 1983; Laszlow and Elo 1983). The application of tourniquets or use of bloodless field techniques during limb surgery, poor hemostasis, excessive use of diathermy or prolonged use of retractors may favor colonization by anaerobes. Even though surgical trauma is localized, the wound may be heavily contaminated with skin

commensals. Particularly in elderly patients, tissue perfusion may be greatly reduced by arterial disease or poor circulation. Foreign bodies, the effects of anesthesia, and surgical trauma are major contributory factors that add to post-surgical anaerobic sepsis.

Gas gangrene after injections into the buttock or thigh is due to the implantation of endogenous anaerobes into the muscle and the irritant or vasoconstrictor action of drugs, which reduce or abolish the local circulation in tissues that may already be subject to poor perfusion, with resulting profound reduction of tissue E_h. Adrenaline, with its vasoconstrictive effect, is particularly notorious in this connection (Evans et al. 1948; Bishop and Marshall 1960). A more recent risk is that associated with 'popping,' i.e. injection of heroin directly into muscle undertaken by injecting drug abusers. This is now a leading cause of wound botulisum (see *Clostridium botulinum* and *Clostridium tetani*), as well as other clostridial infections (Brazier et al. 2002).

A series of changes occurs in the damaged and anoxic tissues that lead to a rapidly falling E_h and establish an ideal environment for the growth of clostridia (see Oakley 1954; Willis 1969). The production of bacterial toxins and products of bacterial metabolism promotes the growth of the organisms so that gas gangrene becomes established. Defenses are further compromised, since neither phagocytes nor antibodies can enter the necrotic zone, and absence of perfusion prevents antimicrobial agents from reaching the affected tissues.

Culture filtrates of broth cultures of clostridia contain toxins, enzymes, and other substances that 'activate' the infection. Thus, young cultures of *C. perfringens* contain a nontoxic antigen that activates washed bacilli, and whose aggressive effect is neutralized by the corresponding antibody (Fredette and Frappier 1946). Older culture filtrates of *C. perfringens* type A contain several substances including α and θ toxins, collagenase (κ), hyaluronidase (μ), deoxyribonuclease (ν), and a fibrinolysin. Two other antigenic substances whose action is neutralized by antitoxic sera are recognized. The first sensitizes blood vessels to the action of adrenaline, and the other inhibits phagocytosis (Ganley et al. 1955). Injection of toxic filtrates into animals simulates the lesions in naturally infected human muscle (Kettle 1919; Govan 1946), which are characterized by edema, necrosis, and capillary and venous thrombosis. These are accompanied by proteolysis, lipolysis, release of lipid-derived phosphorus, and the tissues disintegrate as the result of the actions of bacterial collagenase, lecithinase, and hyaluronidase (Frazer et al. 1945; Robb-Smith 1945).

The role of clostridial toxins in gas gangrene

The onset of clinical gas gangrene coincides with toxin production, which initiates the extension of the syndrome, in which histotoxic necrosis is followed by bacterial invasion of necrosing tissue. Toxin also enters the circulation and contributes to the increasing toxemia and shock.

Three kinds of evidence indicate that biologically active clostridial products participate in gas gangrene:

1. The association of pathogenicity with ability to produce these substances
2. Mimicry of the disease by injection of these substances into susceptible tissue, and
3. The protective or curative effect of antibodies to the substances.

The pathogenicity of strains of *C. perfringens* type A is associated with production of α toxin, and hyaluronidase is produced by about half the toxigenic strains (Robertson and Keppie 1941; McClean et al. 1943; Keppie and Robertson 1944; Evans 1945; Kass et al. 1945). Production of collagenase (Evans 1947a) and θ toxin (Stevens et al. 1993a) are also associated with the virulence of *C. perfringens*. However, this simple association does not constitute proof of a causal link. Better proof is provided by the fact that the natural lesion can be reproduced by toxic filtrates that contain a number of active components. The more stringent immunological evidence that antibodies are protective is meagre. Thus, of the antibodies against α and θ toxin, hyaluronidase and collagenase, only α antitoxin protects guinea pigs (Evans 1943a, b, 1947b). The importance of α toxin is supported by the efficacy of active immunization with a toxoid, whereas the part played by hyaluronidase and collagenase in the genesis of gas gangrene appears to be less important.

The role of collagenase in causing proteolytic damage to uninfected muscle and in this way supplying amino acids and peptides for bacterial growth was postulated by Smith (1949). The spread of the infection may be facilitated by the hyaluronidase of *C. perfringens* or by hyaluronidase produced by other organisms present in the wound (McClean and Rogers 1944). Hyaluronidase itself cannot, to any significant extent, penetrate intercellularly (Hechter 1946), but with its assistance infected fluid under the pressure of edema may move more readily. One of the reasons for the success of early surgical treatment in gas gangrene may be the relief of such pressure. This is illustrated by the observation that simple incision of 4-hour-old *C. perfringens* lesions of the thigh muscles of guinea pigs allows their survival as compared with control animals not so treated (Hartley and Evans 1946).

The part the lecithinase (α toxin) plays in the myonecrosis is more readily understood than its role in the toxemia of gas gangrene. Filtrates of *C. perfringens* type A have an acute action on heart muscle and the isolated heart perfused with α toxin is killed and phosphoryl choline is released (Kellaway et al. 1940a, b; Wright 1950). The toxic action of lecithinase can be neutralized

by mixing it with lecithin before it is injected (Wright and Hopkins 1946). Similarly, dogs and mice can be protected against toxemia by the intravenous injection of lecithin isolated from human and animal tissue (Zamecnik et al. 1945; Gordon et al. 1954). However, toxin is rapidly fixed to muscle (Wright and Hopkins 1946) and little or no free toxin is detectable in the blood or tissues of humans or animals with toxemia (Balch and Ganley 1957; Linsey 1959). High concentrations of antitoxin in the blood do not protect against toxemia even if given early in the infection, and intravascular α toxin does not necessarily produce toxemia, though it induces hemolysis. This led MacLennan and Macfarlane (1945) and Macfarlane and MacLennan (1945) to question whether toxemia is due directly to α toxin. They suggested that, as in traumatic shock, the toxemia is due to the release of products of tissue injury secondary to the local effects of toxin (Zamecnik et al. 1947; Berg and Levinson 1955, 1957, 1959). Nevertheless, the absence of detectable α toxin in lesions or in the circulation in natural and experimental gas gangrene does not exclude its participation in local or general disease. In myonecrosis, disruption of sarcolemma and fragmentation of muscle fibers (Robb-Smith 1945) and edema are compatible with intoxication, and toxin may have a profound effect on the membranes of cells or cell particles (Macfarlane and Datta 1954). Hyaluronidase, deoxyribonuclease, collagenase, and fibrinolysin may play a role, but this has been difficult to prove. Thus, Aikat and Dible (1956) found no evidence that *C. perfringens* collagenase causes damage to connective tissue, which they suggested was due mainly to α toxin and hyaluronidase. They also concluded that in infections due to *C. novyi* the α toxin produces disruptive edema and muscle changes, and that ϵ toxin causes lipolysis. There is no evidence for an effect of the hyaluronidase of *C. novyi* or *C. septicum* in myositis (Aikat and Dible 1960).

The disease in rabbits infected experimentally with *C. perfringens* alone or together with *C. sporogenes* proceeds in two stages. First, capillaries are destroyed by α toxin and then the organisms give rise to a general bacteremia. The local lesion is the source of toxin and bacteria (Katich et al. 1964).

The toxins of *C. novyi* and *C. septicum* have not been separated to allow study of their effects in vivo. Immunological evidence for the part that the toxins of *C. perfringens*, *C. novyi*, and *C. septicum* plays in the genesis of gas gangrene comes from the protection afforded by toxoids prepared from the α toxins of these organisms, whereas passively administered antitoxin is ineffective in the presence of infected muscle, but this is true only for local myonecrosis. The toxemia associated with the natural disease remains unexplained. The systemic effects of *C. perfringens* α toxin are discussed by MacLennan (1962).

C. perfringens type A antitoxins are bactericidal for the bacteria in tissue fluids. Though the bactericidin does not agglutinate the organisms and is not a recognized antitoxin, it can be adsorbed by *C. perfringens* suspensions. It appears to be responsible for the protective effect of antitoxin in experimental infection in the chick embryo, guinea pig, and mouse. It is notable that the bactericidal effect of this factor is most evident at relatively high E_h, but it is known that antitoxin is generally ineffective in established experimental myonecrosis (Bullen and Cushnie 1962).

The pathology of gas gangrene has been described by Favata et al. (1944), Frazer et al. (1945), Govan (1946), and Aikat and Dible (1956, 1960).

Clinical presentation

The incubation period of gas gangrene after injury varies from 7 h to 7 days. Pain develops early in the region of the wound and increases in intensity with progressive swelling and edema. The pulse rate increases markedly, is feeble, and often impalpable and there is a mild to moderate pyrexia.

The wound is edematous, tender, and exudes a profuse serous or serosanguinous discharge. As the disease progresses, bubbles of gas appear in the discharge, crepitus may become evident in the tissues, and the skin becomes white and marbled. The patient is collapsed, profoundly toxemic, and shocked, but remains mentally alert and anxious. The blood pressure falls and peripheral venous collapse often makes venepuncture impossible. The syndrome usually terminates with sudden death due to circulatory failure. The presence of gas may not be obvious and is often a late development. Moreover, infections due to other organisms such as *Prevotella melaninogenica*, *E. coli* or even *Bacillus cereus* sometimes show copious gas formation (Bessman and Wagner 1975; Lewis et al. 1978; Fitzpatrick et al. 1979). Interstitial gas sometimes represents pockets of air that have been forced into the tissues during wounding. Radiological examination may identify gas in the muscle planes and helps to determine the extent of the infection (Kemp 1945).

Bacteremia is a rare, late, and usually agonal complication of clostridial myonecrosis. *C. perfringens* bacteremia is commonly associated with intravascular hemolysis and is more likely to be found in uterine gas gangrene that may follow abortion or childbirth, and as a rare complication of biliary tract surgery (Brown et al. 1948; Pyrtek and Bartus 1962; Hill 1964; Plimpton 1964; Turner 1964; Yudis and Zucker 1967).

It is essential to distinguish clinically between gas gangrene, clostridial cellulitis, and anaerobic streptococcal myositis, because of the important differences in their prognosis, and surgical and therapeutic manage-

ment (Lowry and Curtis 1947; Anderson et al. 1972). Apart from pseudomembranous colitis (PMC) due to *C. difficile*, and diarrhea due to *C. perfringens*, clostridial infections are not transmissible from person to person and do not pose problems of hospital cross-infection.

Accounts of gas gangrene have been published by MacLennan (1943b, 1962), Macfarlane and MacLennan (1945), and Weinstein and Barza (1973).

Diagnosis

Gas gangrene is essentially a clinical diagnosis. The presence of pathogenic clostridia in a wound, and particularly of *C. perfringens*, is of little pathognomonic significance. Anaerobic bacteriology cannot provide timely confirmatory evidence in a disease with an incubation period that may be as short as 4 h, and is on average 1–2 days. However, direct examination of the wound material must not be omitted because it may yield valuable information, especially for the diagnosis of anaerobic streptococcal myositis (see MacLennan 1943a; Memorandum 1943; Hayward 1945). In obstetric infection, the presence of capsulate *C. perfringens*-like bacilli in Gram-stained smears is almost diagnostic of pathogenic *C. perfringens* (Butler 1945).

Rapid enzymological tests have been proposed for the identification of clostridial products in wound exudates. A species-specific sialidase-inhibition test gives results in 2–6 h and agrees well with the results of bacteriological examination for *C. perfringens* infection, but not for *C. septicum* or *C. sordellii* (Roggentin et al. 1991). In an alternative approach, sialidases in exudates are bound in microtiter plates to immobilized polyclonal antibodies and the sialidase activity assayed against a fluorogenic substrate (Roggentin et al. 1993).

Apart from examination of smears, full bacteriological examination must include direct culture to assess the relative numbers of the various bacilli present and the inoculation of cooked meat broth for enrichment purposes and subsequent plating. The media used should include those that contain substrates, such as egg yolk and blood agar, to allow identification of various toxins. If suitable antitoxic sera are available, they may for convenience be prepared as half-antitoxin plates. Selective agar media facilitate early recognition and isolation of relevant organisms. Schemes for the bacteriological study of clostridial sepsis have been described by Hayward (1945) and Willis and Phillips (1988).

Prophylaxis and treatment

The most important prophylactic measure is early and adequate surgery (Latta 1951; Howard and Inui 1954; Lindberg et al. 1955; Strawitz et al. 1955; Wheatley 1967; Altemeier 1979; Anon 1984). Damaged muscle must be excised to leave only healthy, well vascularized tissue and the area irrigated to reduce numbers of contaminating microorganisms. Management is by delayed primary closure. The importance of this technique has repeatedly been emphasized by military experience (Ogilvie 1944; Shouler 1983; Anon 1984).

Incision of the gangrenous muscle along its length improves the survival rate of guinea pigs experimentally infected with *C. perfringens* (Hartley and Evans 1946), probably by reducing tension in the edematous muscle and improving the blood supply. In experimental *C. novyi* gas gangrene in sheep, wound incision alone fails to delay the onset of gas gangrene and has no effect on survival, but adequate excision greatly improves survival (Thoresby and Matheson 1967a, b). In humans, when formal wound excision for prophylactic purposes is not possible, the skin wound can be enlarged and the deep fascia incised to relieve tension and allow free drainage. Further excision can then be carried out at a later stage (Memorandum 1943).

Parenteral antimicrobial therapy must be started as soon as possible after wounding to ensure that therapeutic drug concentrations are present in the tissues at the time of surgery. Metronidazole is now regarded as the prophylactic antimicrobial compound of choice (Bartlett 1982). Metronidazole is given intravenously before surgery and then every 8 h for 24 h, followed by a 12-hourly schedule. In sheep with experimental high-velocity missile wounds contaminated with clostridia, penicillin alone given within 9 h of wounding and challenge is effective prophylaxis (Owen-Smith and Matheson 1968). In humans, antimicrobial prophylaxis alone is of no value, but its use together with surgery, particularly if this is carried out soon after wounding, reduces the risk of anaerobic infection (Altemeier et al. 1951; Garrod 1958; MacLennan 1962; Barber and Garrod 1963). Antitoxin is prophylactically and therapeutically ineffective and is no longer available.

Elective surgery in the hip and thigh region in elderly patients carries a significant risk of postoperative gas gangrene due to endogenous *C. perfringens* (Parker 1967). Prophylaxis in such patients consists of meticulous preoperative skin preparation (Ayliffe and Lowbury 1969) and peroperative prophylaxis with metronidazole or benzylpenicillin, continued for 48 h after surgery.

HYPERBARIC OXYGEN

Hyperbaric oxygen therapy has revolutionized the treatment of gas gangrene (Roding et al. 1972). Before its introduction in the early 1960s, successful management of gas gangrene required radical excision of the affected muscle. Muscles were excised from origin to insertion, or the limb amputated. Though controlled trials of hyperbaric oxygen have not been carried out, its effectiveness is supported by experimental (Boerema and Brummelkamp 1960) and clinical evidence (Brown et al. 1994). Over a period of 12 years in the Netherlands,

Roding et al. (1972) treated 130 patients, of whom 29 died (22.3 percent), only 14 from gas gangrene. The death rate in cases due to civilian trauma was only 12.2 percent, compared with 45.2 percent in postoperation cases. Often the use of hyperbaric oxygen led to the saving of limbs that would otherwise have been amputated. The disease was most effectively controlled in patients who survived for 48 h after the start of hyperbaric oxygen treatment. Hyperbaric oxygen treatment consists of exposure to oxygen at 2.5–3 atm pressure for 1.5–2 h as soon as possible after admission and is accompanied by resuscitation and intravenous metronidazole or penicillin. A single-patient chamber may be used, or a larger chamber that also accommodates medical staff. The larger chambers are pressurized with air and the patient is given oxygen by means of a face mask. Since hyperbaric oxygen does not revive necrotic tissue, surgical toilet, rather than radical excision, is carried out as soon as the patient's general condition has improved. Subsequent treatments in the chamber are given at intervals of about 8 h for the first 24 h and then at intervals of 12 h. Since many infections are mixed, a broad spectrum antibiotic may be given in addition to metronidazole (Hart et al. 1975; Darke et al. 1977; Skiles et al. 1978). An appropriate combination is metronidazole, gentamicin, and amoxycillin. Patients must be transferred early and speedily to a hyperbaric oxygen unit, because untreated gas gangrene runs a fulminating course and leads rapidly to fatal intoxication. Hyperbaric oxygen therapy has been reviewed by Rudge (1993).

Hyperbaric oxygen directly impairs the growth of clostridia (Hopkinson and Towers 1963; Bullen et al. 1966; Hill and Osterhout 1972), also prevents production of *C. perfringens* α toxin (van Unnik 1965; Kaye 1966), and kills vegetative bacilli. The part that hyperbaric oxygen, antimicrobial agents, and surgery play in the treatment of clostridial myositis has been examined in experimental animal models (Hirn et al. 1992; Stevens et al. 1993b). The effects of hyperbaric oxygen on microorganisms, and the factors that affect the response of clostridial infection to hyperbaric oxygen therapy have been reviewed by Gottlieb (1971). Hyperbaric oxygen may also promote the viability of tissues to which the blood supply is impaired.

ANTIMICROBIAL AGENTS

Histotoxic clostridia associated with wound infections are susceptible to several antimicrobial agents (Dornbusch et al. 1975). *C. septicum* and *C. perfringens* are highly susceptible to benzylpenicillin (Brazier et al. 1985), but some clostridia are resistant (Rosenblatt 1984; Brazier et al. 1985).

The activity of other penicillins against clostridia in decreasing order is amoxycillin, carbenicillin, flucloxacillin, and mecillinam. The combination of amoxycillin plus clavulanic acid (Augmentin) is active in vitro. The cephalosporins, cephamycins, and monobactams are poorly active against clostridia (Selwyn 1980; Brogden and Heel 1986), but imipenem (a carbapenem of the thienamycin class) has good activity in vitro (Wexler and Finegold 1985). The 4-quinolones are only moderately to poorly active against clostridia (Goldstein and Citron 1985; Delmee and Avesani 1986; King and Phillips 1986; Watt and Brown 1986). With some exceptions, metronidazole, chloramphenicol, benzylpenicillin, and erythromycin are the most effective anticlostridial drugs. Tetracyclines and clindamycin are less effective and the aminoglycosides much less so (Brazier et al. 1985; Robbins et al. 1987; Stevens et al. 1987a, b, c). The drugs of choice for the prevention and treatment of clostridial wound infections are metronidazole and benzylpenicillin.

OTHER CLOSTRIDIAL INFECTIONS OF HUMANS

The histotoxic clostridia may participate in infective conditions other than myonecrosis. Penetrating wounds may rarely result in brain abscess, acute purulent meningitis, panophthalmitis, endocarditis, septic arthritis, and intrapleural infections. *C. perfringens* is the organism most commonly responsible. These infections have been reviewed by Willis (1969, 1977, 1991) and Finegold and George (1989).

Bacteremia

C. perfringens is present on the skin over the antecubital fossa in 20 percent of subjects (Ahmad and Darrell 1976) and its presence in routine blood cultures is due to contamination from the skin. Contaminated skin may rarely be responsible for clinically significant bacteremia due to *C. perfringens* (Rose 1979).

Clostridia may invade the bloodstream late in gas gangrene. This is most commonly due to *C. perfringens* and *C. septicum* (Weinberg and Séguin 1918; Report 1919). *C. septicum* bacteremia may spontaneously complicate malignant disease of the colon and neutropenic enterocolitis (see below). *C. perfringens* bacteremia is rare after surgery on the gastrointestinal tract, or after the perforation of peptic ulcers. It is an uncommon complication of acute emphysematous cholecystitis (Willis 1969; Gorbach and Thadepalli 1975). Most idiopathic bacteremias are probably secondary to covert, often intraabdominal, pathological abnormalities.

Clinically significant bacteremia is dramatic in its onset and follows an overwhelmingly rapid course with intravascular hemolysis, shock, hemoglobinuria, renal failure, and rapidly deepening jaundice and cyanosis. The clostridium can be isolated by blood culture and is sometimes visible in Gram-stained blood films.

Endocarditis due to clostridia is rare (Case et al. 1972; Alvarez-Elcoro and Sifuentes-Osorio 1984). Clostridial bacteremia in patients with gas gangrene has a grave prognosis, but early or transient bacteremia, probably due to mechanical causes rather than invasion, is of little significance (Butler 1937; Gorbach and Thadepalli 1975).

C. septicum bacteremia has long been recognized, sometimes associated with spontaneous gas gangrene in which *C. perfringens* or *C. septicum* entered the circulation through a malignant lesion of the colon (Valentine 1957; Cabrera et al. 1965; Francois et al. 1994). Boggs et al. (1958) observed clostridial bacteremia in patients with leukemia and thought that it was due to invasion of infected leukemic infiltrates of the cecum and colon. The association of *C. septicum* bacteremia with ileocecal neoplasia is now well recognized.

The primary ileocecal syndrome has had a variety of names (typhlitis, ileocecal syndrome, necrotizing enteropathy) and is now usually known as neutropenic enterocolitis because of its association with severe neutropenia (see Borriello 1985a). The disease is frequently complicated by a severe and progressive sepsis due to invasion by *C. septicum* (Bignold and Harvey 1979; Rifkin 1980; King et al. 1984; Hiew et al. 1993). *C. septicum* probably plays the leading primary role in most cases of neutropenic colitis (Rifkin 1980; King et al. 1984). Most patients with *C. septicum* bacteremia have malignant disease including hematological malignancies (Koransky et al. 1979; Lorimer and Eidus 1994). Neutropenic patients who present with pyrexia, abdominal symptoms, and hypotension should raise the suspicion of endogenous clostridial infection (Warren and Mason 1970; Gorse et al. 1984; Pelfrey et al. 1984). The portal of entry for the organism is probably ulceration of the bowel. The organism proliferates locally to produce an enterocolitic lesion with submucosal edema, hemorrhage, and necrosis, which allows invasion of the bloodstream. *C. septicum* is relatively sparse in the human intestine and it is not known why it, rather than *C. perfringens*, behaves in this way.

Clostridial bacteremia has been reviewed by Spencer (1991) and Stevens et al. (1990).

Diseases caused by *C. sordellii*

MacLennan (1962) lists *C. sordellii* as one of six clostridia involved in histotoxic, gas gangrene-like infections. More recently, fatal *C. sordellii* infections have been noted in association with obstetrical surgical incisions (episiotomy) (Soper 1986; McGregor et al. 1989). Fatality in these cases was due to clinical shock, presumably toxin induced. The described cases were characterized by sudden onset of shock with severe hypotension, generalized tissue edema, accumulation of fluids in the peritoneum and other body cavities, increased hematocrit, marked neutrophilia, absence of rash or fever, limited or no myonecrosis and a rapid, lethal course. A case of spontaneous endometritis due to *C. sordellii*, having the same rapidly fatal clinical course has been described (Hogan and Ireland 1989). A fatality due to *C. sordellii* infection in a deep laceration of a thigh has also been reported (Browdie et al. 1975). Intramuscular injection of sterile fluid from cultures of *C. sordellii* isolated from the thigh wound caused edema and hemoconcentration in mice, and death within 12–48 h, the time until death depending on dose.

Pathogenicity of *C. bifermentans*

The involvement of *C. bifermentans* in infections has been indicated by its association with liver abscess (Nachman et al. 1989), endocarditis (Kolander et al. 1989), abdominal abscess (Rechtman and Nadler 1991), panophthalmitis (Rehany et al. 1994), and metastatic osteomyelitis (Scanlan et al. 1994), as well as gas-gangrene.

C. ramosum

The name *C. ramosum* was proposed in 1971 as a new combination to include organisms previously named *Bacillus ramosus*, *Nocardia ramosa*, *Actinomyces ramosus*, *Bacteroides ramosus*, and *Ramibacterium ramosum* (Holdeman et al. 1971). This organism is frequently isolated from clinical specimens, especially in intraabdominal sepsis (Smith and Williams 1984) and appendicitis. Culture supernatants are nontoxic for mice, but strains are pathogenic for guinea pigs. Pathogenicity may be lost in laboratory cultures (Cato et al. 1986).

Enteric infections

Clostridial diseases of the gut in man and animals are covered comprehensively elsewhere (Borriello 1985b). This section will deal primarily with the most important ones, namely those due to *C. perfringens* and *C. difficile*.

Food poisoning and other diarrhea

A mild diarrhea associated with the consumption of communal meals was for some time attributed to *C. perfringens* present in the food. In 1953 Hobbs and her colleagues (Hobbs et al. 1953) established that certain strains of *C. perfringens* type A were responsible for this foodborne disease, which is described in detail in Bacteriology of foods excluding dairy products.

It was not until 1984 that it was shown the enterotoxigenic strains of *C. perfringens* could cause diarrhea by a mechanism other than classical food poisoning (Borriello et al. 1984). The disease differs from food poisoning in a number of ways, the most important being that it is

more severe than food poisoning in most cases. The diarrhea is more profuse and of longer duration, and blood and mucus in the feces and abdominal pain occur more frequently (Larson and Borriello 1988). Histologic examination of rectal mucosa reveals mild edema, thus implying that there may be more severe lesions in proximal regions of the gut that account for fecal blood. The disease occurs almost exclusively in the elderly (Larson and Borriello 1988) which also differentiates it from food poisoning, and hospital-acquired cases are significantly associated with the use of antibiotics (Larson and Borriello 1988; Williams et al. 1985). It is difficult to explain why the disease predominantly affects the elderly, though it is possible that the elderly are more susceptible to colonization with *C. perfringens*, a proposal supported by the demonstration of persistent carriage of large numbers of organisms in healthy elderly patients who are institutionalized (Yamagishi et al. 1976).

A detailed epidemiological investigation of a cluster of cases of *C. perfringens* diarrhea (Borriello et al. 1985) showed widespread contamination of the hospital environment by the *C. perfringens* serotype, incriminated in 57 percent of areas sampled positive.

Although further studies have confirmed the importance of the observations reported in 1984 (Borriello et al. 1984) and indicate *C. perfringens* is a cause of between 2 and 15 percent of all cases of antibiotic-associated diarrhea (Hancock 1997; Modi and Wilcox 2001; Asha and Wilcox 2002), it is still underdiagnosed. The enterotoxin can be detected by its cytopathic effect in tissue culture for diagnostic purposes (Borriello et al. 1984). This approach has the advantage of also detecting *C. difficile* toxins, which are readily differentiated by the type of cytopathic effect produced. An enzyme immunoassay is also available for detection of *C. perfringens* enterotoxin which can be applied to analysis of fecal specimens. This has been evaluated and showed good concordance with tissue culture (Asha and Wilcox 2002).

Necrotizing jejunitis (enteritis necroticans, pig-bel)

Severe bowel disease due to *C. perfringens* type C was first described in 1946 in Germany as enteritis necroticans (Darmbrand). It was characterized by a diffuse sloughing enteritis of the jejunum, ileum, and colon. In 1963, a similar disease was identified in the native population of Papua New Guinea (Murrell and Roth 1963). This is a severe and often fatal disease due to *C. perfringens* type C. It was first recorded by Zeissler and Rassfeld-Sternberg (1949) in northwest Germany (Fick and Wolken 1949). Severe lower abdominal pain and diarrhea developed some hours after individuals had eaten rabbit, tinned meat, or fish paste. Blood and mucosal sloughs were present in the stools in some cases and there were deaths due to peripheral circulatory collapse or intestinal obstruction due to massive mucosal edema of the intestinal mucosa. The jejunum was principally affected.

Sporadic cases of necrotizing jejunitis have been recorded (Calnan 1950; Govan and MacIntosh 1951; Patterson and Rosenbaum 1952; Wright and Stanfield 1967; Severin et al. 1984). A second major outbreak was reported from New Guinea (Murrell and Roth 1963; Murrell 1967), in which *C. perfringens* type C was again identified as the probable pathogen (Egerton and Walker 1964). Subsequently, the syndrome was seen in other parts of the world (from East Africa: Foster 1966; Sekabunga 1966; Wright and Stanfield 1967; Gurovsky and Samuel 1972; from Thailand: Headington et al. 1967; Welch and Sumitswan 1975; Johnson et al. 1987; from India: Narayan and Rao 1970; Pujari and Deodhare 1980). It has been suggested that *C. perfringens* type A may occasionally also cause the disease (van Kessel et al. 1985).

In New Guinea, necrotizing jejunitis was referred to as the 'pig-bel' syndrome (New Guinea pidgin English, *pig-bel* = abdominal discomfort after a large pork meal), because of its relationship to the widespread practice of pork feasting in the locality. Murrell et al. (1966) defined pig-bel as an acute, patchy, necrotizing, and inflammatory disease of the small bowel in Highland Melanesians in New Guinea. The disease is predominantly one of children, males more commonly affected than females. It is characterized by anorexia, severe upper abdominal pain, bloody diarrhea, and vomiting and has been classified into four clinical types:

1. An acute form with fulminating toxemia and shock, with a mortality rate of around 85 percent
2. An acute surgical form in which there may be mechanical and paralytic ileus, acute strangulation and perforation, and peritonitis, with a mortality rate of about 40 percent
3. A subacute surgical form in which there may be acute and chronic small bowel obstruction, intestinal fistulae, ulcerative jejunitis, and peritoneal adhesions, with a mortality rate of about 40 percent, and
4. A mild form without complications and progressing to complete recovery.

The pathology of pig-bel has been described by Cooke (1979). Treatment includes resuscitation with blood transfusion, bowel decompression and resection, and antimicrobial therapy (Smith 1969; Pujari and Deodhare 1980; Millar 1981).

PATHOGENESIS

Pig-bel in New Guinea is due to *C. perfringens* and its β toxin. The organism is widely distributed in the village environment and is present in the feces of more than 70 percent of normal persons (Lawrence et al. 1979b). After ingestion, *C. perfringens* type C proliferates in the intestine and releases its β toxin, which under normal

circumstances is destroyed by intestinal proteinases. A number of factors may operate in Papua New Guinea to preserve the intraluminal activity of the β toxin, with resulting severe toxic intestinal damage (Lawrence and Walker 1976; Lawrence 1979; Walker 1985).

Though frank malnutrition is uncommon among native New Guineans, the diet is very low in protein, which markedly reduces production of intestinal proteinase enzymes. The staple diet is sweet potato, which provides up to 90 percent of dietary calories and contains heat-stable trypsin inhibitors. In addition, infestation with the roundworm *Ascaris lumbricoides*, which secretes a trypsin inhibitor, may contribute to reduction of proteinase activity. The occurrence of pig-bel, particularly in children, is due to their poor immunity to β toxin. When a high protein diet is eaten together with sweet potato, the conditions are ideal for the growth of *C. perfringens* type C and the production of β toxin, which is then less likely to be degraded by proteolytic enzymes.

Damage by the toxin occurs some time before symptoms are evident, by which time administration of antitoxin is ineffective (Rooney et al. 1979), but active immunization with *C. perfringens* type C β toxoid confers a high degree of lasting protection (Lawrence et al. 1979a; Walker et al. 1979; Davis et al. 1982). The pathogenesis of the disease and the effectiveness of immunization have been demonstrated by Lawrence and Cooke (1980).

An account of pig-bel is given by Davis (1984).

CLOSTRIDIUM DIFFICILE INFECTION

Clostridium difficile is most commonly associated with infections of the large bowel. It is the cause of many cases of antibiotic-associated diarrhea and nearly all cases of pseudomembranous colitis (PMC). As such, it is the most common identifiable bacterial cause of nosocomial diarrhea, and one of the most common anaerobic infections. However, infection is not restricted to the gut, and rare cases of extraintestinal infection have been reported (Feldman et al. 1995; Pron et al. 1995).

The importance of *C. difficile* as a pathogen is put in perspective by Willis (1988) who wrote: 'Among all of the many advances that have been made in our understanding of anaerobic bacteria and anaerobic bacterial disease during the last 40 years, elucidation of the syndrome of antibiotic-associated pseudomembranous colitis ranks as a major triumph.'

History

PMC was first described by Finney (1893) as a postoperative complication of gastroenterostomy. Over the years its etiology was variously ascribed to such causes as gastrointestinal surgery ('postoperative enterocolitis'), antibiotic therapy ('antibiotic-associated enterocolitis'), clindamycin therapy ('clindamycin-associated colitis'), and staphylococcal sepsis ('staphylococcal enterocolitis') (Gorbach and Bartlett 1977).

This, and other cases, preceded the use of antibiotics but interest in the disease was stimulated in the 1950s and 1960s because cases were associated with the administration of tetracycline and chloramphenicol (Reiner et al. 1952; Hummel et al. 1964) and again in the 1970s because of an association with the administration of clindamycin (Tedesco et al. 1974). These observations encouraged the development of an extremely active, though relatively short, period of research, which, together with a number of earlier, sometimes unrelated observations, culminated in the discovery of *C. difficile* as an etiological agent of antibiotic-associated PMC. In the course of a search for a possible viral cause of PMC, Larson et al. (1977), at the Clinical Research Centre, Harrow in the UK, reported the detection of a cytopathic agent in filtrates of feces from five patients with histologically proven disease. They concluded that the agent was probably a bacterial protein toxin. Animal model studies, largely in the USA, established that the toxin was clostridial in origin (Bartlett et al. 1977; Rifkin et al. 1977). These studies showed that disease in antibiotic pretreated hamsters could be transferred serially by intracecal injection of cecal contents from diseased animals, and that filtrates of these cecal contents were cytopathic for cells in tissue culture. More importantly, the cytopathic activity could be neutralized by *C. sordellii* antitoxin, though *C. sordellii* was not present in diseased animals. This dilemma was quickly resolved by the identification of *C. difficile* as the dominant *Clostridium* species in this material, that it produced disease in antibiotic pretreated hamsters, and further that it produced a cytopathic toxin that could be neutralized by *C. sordellii* antitoxin (Bartlett et al. 1977, 1978a, b; Rifkin et al. 1977). The final cornerstone of proof was put in place by the isolation of *C. difficile* from the feces of patients with PMC (Bartlett et al. 1978c; R.H. George et al. 1978; W.L. George et al. 1978; Larson et al. 1978).

Pathology

The disease is well defined histopathologically (Goulston and McGovern 1965; Price and Davies 1977; Price 1984) and almost invariably restricted to the colon. The mucosa becomes necrotic and, in fulminating cases, an exudative membrane forms, which resembles that seen in diphtheria. The pseudomembrane forms in multiple, friable yellow–white plaques that vary in size from a few centimeters to about 2 mm in diameter and are attached to the mucosal surface. They may become confluent. Between the mucosal plaques only congestion may be apparent. Microscopically, the membrane appears as a fibrinous exudate that contains leukocytes, epithelial cells, and mucin. The underlying intestinal submucosa shows a varying degree of necrosis and inflammation.

In the earliest stages of the disease 'summit-lesions,' tiny superficial intercryptal erosions, may be found (Price and Davies 1977). However, for accurate diagnosis the adjacent mucosa must show no more than edema and focal clusters of polymorphs. Where there is full thickness mucosal necrosis, the features are no longer diagnostic and may resemble other conditions that have a stage of complete mucosal necrosis, e.g. ischemia or radiation damage.

The association of infection with antibiotic use is due to the requirement for disruption of the normal stable gut microbiota (microflora) before *C. difficile* can establish and produce toxins. The barrier effect afforded by the normal microbiota is frequently referred to as colonization resistance. This barrier effect to infection with *C. difficile* and its disruption with antibiotics has been demonstrated in animals (Larson et al. 1980; Larson and Borriello 1990) and in vitro with human and animal fecal or cecal material (Borriello and Barclay, 1986; Borriello et al. 1988a). A variety of antibiotics can predispose to *C. difficile* infection (Borriello and Larson 1981; Tedesco 1984; George 1988), the frequency of association being related to the route of administration, frequency of use and the impact on the bacterial components of the gut microbiota. For example, McFarland et al. (1990) found that the short-term use of high-dose cephalosporins (<1 week; >10 g cumulative), or the use of broad spectrum penicillin for longer than 7 days, was associated with *C. difficile* infection. Although it is difficult to extrapolate the relative risk of infection associated with a given antibiotic from frequency of association of particular antibiotics, there is evidence from the hamster model of the disease that the major difference between oral antibiotics is the duration, rather than the degree, of susceptibility induced (Larson and Borriello 1990). It is possible that the higher incidence of infection in the elderly is due in part to their poorer colonization resistance (Borriello and Barclay 1986), so that a smaller effect by antibiotics is required to induce susceptibility to colonization with *C. difficile*. Neonates, who are in the process of developing a complex gut microbiota, are the other age group with a poor barrier effect. Indeed, *C. difficile* was first isolated from the feces of neonates (Hall and O'Toole 1935), in whom it is commonly present (Larson et al. 1982). *C. difficile* toxins are also commonly present in such feces (Libby et al. 1983; Borriello 1990). Why infants usually appear to be unaffected by the toxins remains unknown. Rarely, antibiotic-associated PMC occurs in older children (Viscidi and Bartlett 1981), and even more rarely in infancy (Scopes et al. 1980; Donta et al. 1981; Richardson et al. 1981; Price et al. 1990).

Pathogenesis

Once *C. difficile* is established in the bowel, disease is a consequence of the production of two large protein toxins, toxins A and B, both of which have been cloned and sequenced (Barroso et al. 1990; Dove et al. 1990). Nontoxigenic strains of *C. difficile* are known and may be present with toxigenic strains in patients with antibiotic-associated diarrhea (Borriello and Honour 1983). Earlier studies with toxigenic isolates indicated that toxins A and B are always present together (Lyerly et al. 1983). However, a cytotoxic isolate of *C. difficile* that lacks most of the gene for toxin A, but which is lethal in the hamster model of disease, has been described (Borriello et al. 1992b; Lyerly et al. 1992). The possibility that toxin A may be responsible for the enterocolitis is supported by the observations of Lyerly et al. (1985). In a series of animal feeding experiments they showed that the culture filtrates from a strain of *C. difficile* caused intestinal lesions and diarrhea, but filtrate from which toxin A had been removed was inactive. Moreover, although toxin B was inactive, the two toxins appeared to act synergistically. Various studies (Mitchell et al. 1986, 1987; Ketley et al. 1987; Triadafilopoulos et al. 1987) have shown that toxin A has both histotoxic and 'enterotoxic' activity and that the toxin is of major importance in the causation of tissue damage and luminal fluid accumulation in experimental animals. In rabbits with ileal and colonic loops, they showed that fluid secretion occurred when the toxin had penetrated to the deeper tissues of the bowel wall, and that this was probably the outcome of repeated cycles of toxin uptake and tissue damage. Gross hemorrhage occurred in the ileal tissues and villus architecture was severely damaged, giving rise to a bloody protein-rich luminal fluid. There was damage to the surface epithelium in the colon, with interstitial hemorrhages and a watery luminal fluid containing little protein. Besides the predominant effects of toxin A in animal models, there is evidence that in humans, toxin B may independently cause tissue damage (Riegler et al. 1995). Further evidence that toxin B causes inflammation of human intestinal tissue has been provided by use of immundeficient mice carrying human intestinal xenografs (Savidge et al. 2003). Some strains produce an ADP-ribosyltransferase toxin (see ADP-ribosyltransferase (CDT)). Any contributory role for this toxin in the disease process remains to be determined.

Although the cytotoxic and biochemical activities of *C. difficile* toxins A and B are very well understood at the molecular level (see *C. difficile*), the precise pathophysiological mechanisms leading to toxin-induced diarrhea or colitis are not clear. Studies with animal models show that injection of toxin A into colonic or ileal loop praparations causes increase in secretion, which is accompanied by an increase in mucosal permeability, epithelial cell destruction, and neutrophil infiltration. Thus the pathological feature of *C. difficile* toxin-induced alterations is clearly different from cholera toxin which increase fluid accumulation without overt signs of inflammation. Rho proteins are a major player

in the regulation of tight junctions of epithelial cells (Jou et al. 1998; Wittchen et al. 2003; Benais-Pont et al. 2003; Mitic and Anderson 1998) and inactivation of Rho disturbs the barrier functions of the epithelium (Nusrat et al. 1995; Nusrat et al. 2001). It was therefore suggested that inactivation of Rho proteins participates in increased fluid accumulation and diarrhea caused by *C. difficile*. Moreover, it was demonstrated that Rho proteins are required for endogenous and EGF-induced migration of small intestinal crypt cells, indicating that the GTPases are essential for a mechanism by which growth factors induce cell migration to reconstitute mucosal integrity (Santos et al. 1997). These mechanisms may be of relevance for the tissue damage of the intestinal epithelium observed during pseudomembranous colitis.

On the other hand, it was suggested that toxin-induced inflammation, involving activation of neurons and inflammatory cells in the intestine, are of major importance for the action of the toxins. Many por-inflammatory mediators are released subsequent to toxin application, including tumor necrosis factor-alpha, leukotrien B4 (Triadafilopoulos et al. 1989), and IL-8 (Branka et al. 1997; Mahida et al. 1996). Quite early it was proposed that the tissue damage is a consequence of toxin A-induced recruitment of neutrophils (Triadafilopoulos et al. 1987; Pothoulakis et al. 1988). A direct effect of toxin A on neutrophils has been described. However, extremely high concentrations of the toxin were necessary to elicit these effects (Pothoulakis et al. 1988). More likely is an activation of neutrophils by chemokines such as interleukin 8 (IL-8). This chemokine is released from monocytes after exposure to rather low concentrations of *C. difficile* toxins A and B (Linevsky et al. 1997). Similarly, release of IL-8 from toxin-affected intestinal epithelial cells has been described (Mahida et al. 1996). Further, it has been shown that toxin A stimulated macrophages produce an intestinal secretory factor which has many of the characteristics of IL-1β (Rocha et al. 1998).

Evidence has been presented indicating that substance P may be involved in the secretory and inflammatory effects of *C. difficile* toxins. Administration of a specific substance P-receptor antagonist effectively reduced toxin A induced intestinal fluid secretion and inhibited neutrophil infiltration (Pothoulakis et al. 1994; Wershil et al. 1998). Furthermore, it was shown that the neurokinin-1 (NK-1, substance P) receptor is required in *C. difficile*-induced enteritis, e.g. mice genetically deficient in the neurokinin-1 receptor were protected from secretory and inflammatory changes, as well as from epithelial cell damage induced by toxin A (Castagliuolo et al. 1998). Moreover, injection of toxin A into rat ileal loops causes increased substance P response in lumbar dorsal root ganglia and lamina propria macrophages, suggesting an increased release of substance P in the early phase of toxin A treatment (Castagliuolo et al. 1997). Similarly, antibodies against the calcitonin gene-related peptide (CGRP), which is a constituent of primary sensory neurons, reduced toxin A-induced responses of the gut (Keates et al. 1998). Also, neurotensin receptor antagonists significantly decreased toxin-A-induced colonic responses. Therefore, it was proposed that the neuropeptide, which is released in the intestine upon a large array of stimuli, is involved in toxin effects. So far it is unclear how the release of chemokines and/or the signaling by substance P are controlled or influenced by Rho GTPases, which are the direct targets of the *C. difficile* toxins A and B. Also, mast cells are suggested to play a crucial role in toxin A effects on the gut. Treatment with the mast stabilizer ketotifen inhibits intestinal responses to toxin A (Pothoulakis et al. 1993). In mast cell-deficient mice, toxin A application causes diminished neutrophil recruitment and decreased intestinal secretion (Wershil et al. 1998; Pothoulakis 2000). In monocytes exposed to toxin A, MAP kinase activation (p38) is required for IL-8 trancription and cell necrosis. Surprisingly, in these cells activation of p38 MAP kinase was observed after administration of toxin A within 1–2 min, whereas Rho glucosylation became evident not earlier than after about 15 min. These findings were interpreted to indicate that at least two independent effects are involved in toxin-induced effects on the gut. In addition it was reported that toxin causes early damage of mitochondria, depletion of ATP, and production of oxygen radicals (He et al. 2000).

In addition to the toxins, other factors produced by *C. difficile* may contribute to colonization of the gut and pathology. These have been reviewed (Borriello 1990; Borriello et al. 1990) and include secretion of proteases (Seddon et al. 1990), adhesion to mucus (Borriello et al. 1988c) and production of fimbriae (Borriello et al. 1988b). More recent work also indicated a role for cell surface proteins in adhesion. Of the two S-layer surface proteins implicated (Calabi and Fairweather 2002), one specifically interacts with epithelial cells (Calabi et al. 2002), as does the heat shock protein GroEL of *C. difficile* (Hennequin et al. 2001).

Clinical manifestations

C. difficile infection is almost always associated with recent or current administration of antibiotics and most commonly occurs in hospitalized elderly patients (Borriello and Larson 1981; McFarland et al. 1990). Disease is mainly restricted to the colon and may be associated with a spectrum of disease severity, ranging from trivial diarrhea, through moderately severe disease with watery diarrhea, abdominal pain and systemic upset, to life-threatening PMC. The latter may be accompanied by toxic megacolon (rare), electrolyte imbalance, and occasional bowel perforation. Frank blood in the stool is not common. The onset of

symptoms is frequently abrupt with explosive watery foul-smelling diarrhea (Bennett et al. 1984; Bartlett 1992) accompanied by abdominal pain, fever in some patients, and a raised blood leukocyte count.

Very rarely, reactive arthritis may occur as a consequence of gut infection (Cope et al. 1992). In total, 14 such cases have been reported, and six of 10 cases tested were HLA-B27-positive.

Diagnosis

Diagnosis of *C. difficile* infection is based on detection of toxin in the feces (Report 1994a). This can be achieved by application of fecal filtrates to cells such as African green monkey kidney (Vero) cells in tissue culture and observation of cytopathic effects that can be neutralized with specific antiserum (Chang et al. 1979; Borriello and Welch 1984), or by use of commercial enzyme immunoassays, the majority of which detect toxin A (Borriello et al. 1992a; Doern et al. 1992; Mattia et al. 1993). Kits which detect both toxins A and B are advocated because of their apparent improved sensitivity over toxin A alone kits, and particularly because of cases of diseases due to strains producing toxin B, but not toxin A (detailed in section on *C. difficile*) as reported by Johnson et al. (2001). Such kits make it possible for laboratories without tissue culture facilities to offer a same-day diagnosis. Stool samples that cannot be processed for toxin assay on the day of receipt should be refrigerated. An advantage of tissue culture for toxin detection is that other toxins can be detected, for example the enterotoxin of *C. perfringens*, another cause of nosocomial diarrhea (Borriello et al. 1984; Jackson et al. 1986; Larson and Borriello 1988).

Isolation of toxigenic *C. difficile* from samples of feces is not in itself diagnostic, but is essential for typing as part of the epidemiological investigations of outbreaks. The organism can usually be cultured by exploiting the selective action of cycloserine and cefoxitin (George et al. 1979; Willey and Bartlett 1979), by selection of its spores by alcohol treatment (Borriello and Honour 1981; Levett 1985), or by a combination of both (Borriello and Honour 1984). Recognition of *C. difficile* colonies on primary isolation is facilitated by their characteristic yellow–green fluorescence under long-wave ultraviolet light (George et al. 1979). Various methods have been used for typing or fingerprinting. Though no single standard method is at present accepted for this organism, many have been reviewed by Brazier (1995, 1998).

The presence of *C. difficile* toxin in feces is diagnostic of *C. difficile* infection, but this is not necessarily diagnostic of PMC, which can only be confirmed by detection of typical lesions on sigmoidoscopy and histological examination of tissue. Unfortunately, negative findings at sigmoidoscopy do not exclude the diagnosis, because the rectosigmoid may be spared (Seppälä et al. 1981).

Epidemiology and control

The incidence of the disease is uncertain. Published reports show a wide variation between studies and suggest that episodes of locally increased prevalence occur. These may be attributable to predisposing causes that affect particular groups, such as general surgical patients (Testore et al. 1988), oncology patients (Gerard et al. 1988), those with chronic renal disease (Bruce et al. 1982), or to hospital-acquired infection. Undoubtedly, the latter is often a factor together with other predisposing causes. The recognition that case clustering occurs, that the hospital environment may become contaminated with *C. difficile*, and that the organism can be detected in the feces of some in contact with infected patients, supports the view that *C. difficile* infection results from cross-infection in hospitals (Greenfield et al. 1981; Burdon 1982; Larson et al. 1982; Walters et al. 1982; Cumming et al. 1986; Heard et al. 1986; McFarland et al. 1989). It is recommended that infected patients should be isolated (Mulligan et al. 1980; Kim et al. 1981; Report 1994b), but efforts to limit dissemination of the organism in chronic-care and other long-stay wards may meet with little success (Bender et al. 1986). The 'carrier state' and its epidemiological associations have been reviewed by George (1986) and Mulligan (1988). Attempts to clear the organism from asymptomatic excreters are of no value, though various workers have attempted to achieve this (Johnson et al. 1992).

The spores of *C. difficile* are highly resistant and can survive on inanimate surfaces for many months (Fekety et al. 1981). Attempts to eradicate *C. difficile* from the hospital environment have not been very successful (Katz et al. 1988; Struelens et al. 1991). Most of the reduction achieved is more consistent with the physical removal of bacterial spores than with chemical disinfection. Where an acceptable level of reduction was achieved (Katz et al. 1988), the concentration and pH of the hypochlorite solution used would be unsuitable for long-term use. Fortunately, an exposure for 10 min to 2 percent alkaline buffered glutaraldehyde is suitable for the decontamination of medical equipment that cannot be steam sterilized (Rutala et al. 1993).

Community cases of *C. difficile* gastrointestinal infection are rare, even though the organism can be recovered from a wide variety of environmental sites, including soil (Hafiz and Oakley 1976), domestic animals (Princewell and Agba 1982), and household pets (Borriello et al. 1983; Riley et al. 1991).

There is little doubt that the most important factor in preventing infection with *C. difficile* is strict control of antibiotic use. This should be coupled with good enteric infection control practices, such as hand-washing, equipment sterilization, environmental cleaning, and isolation of patients with diarrhea (Report 1994b). The same approach is important for the control of outbreaks.

Further consideration of infection control practices to reduce cases of *C. difficile* infection are given in

Healthcare associated infections.

Treatment and management

When diarrhea develops during a course of antimicrobial therapy, withdrawal of the offending agent often leads to early resolution of symptoms, even in some cases of established PMC. Since fluid loss may be considerable, attention to fluid replacement and electrolyte balance is necessary. Antimicrobial therapy in the management of *C. difficile*-associated diarrhea and PMC should be reserved for patients with severe infections or for those in whom diarrhea fails to improve spontaneously within 48 h. A number of antibiotics, in particular vancomycin (Keighley et al. 1978; Tedesco et al. 1978) and metronidazole (Pashby et al. 1979; Teasley et al. 1983), have been used successfully and are reviewed by Wilcox and Spencer (1992). Vancomycin is poorly absorbed from the gastrointestinal tract and high concentrations are readily attained in the bowel after oral administration. This agent may thereby be looked upon as providing essentially topical therapy within the gut. Metronidazole, on the other hand, is rapidly absorbed from the upper gastrointestinal tract, but also produces therapeutic concentrations in diarrheal stools. These two agents appear to have similar efficacy and relapse rates and are both well tolerated.

The use of ion-exchange resins as binding agents for *C. difficile* toxin has not been attended with much benefit and antidiarrheal agents, such as loperamide and codeine, may prolong or exacerbate symptoms by delaying toxin elimination.

The optimum treatment for relapse or reinfection is less certain. In general, repeat courses of vancomycin or metronidazole are successful (Fekety et al. 1984; Bartlett 1985). A regimen of tapering doses, followed by pulsed treatment with vancomycin, has also proved effective (Tedesco et al. 1985). For very resistant cases, various bacteriotherapeutic approaches have been tried ranging from rectal infusion of normal feces to use of a nontoxigenic avirulent strain of *C. difficile* (Schwan et al. 1984; Gorbach et al. 1987; Seal et al. 1987; Surawicz et al. 1989; Schellenberg et al. 1994).

Intravenous immunoglobulin has been used successfully in two cases which failed to respond to standard antimicrobial therapy (Salcedo et al. 1997). The therapeutic role of passive immunotherapy awaits larger studies, and the possible use of vaccines in emerging but not yet available.

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Nocardia and other aerobic actinomycetes

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INTRODUCTION

The species usually considered to belong to the group of aerobic actinomycetes do not comprise a genetically closely related group. They form a loose assemblage of genera united by some similar features of colonial and/or Gram stain morphology. They are gram-positive rods that grow better aerobically than anaerobically, if indeed they grow anaerobically at all. As the designation 'actinomycete' suggests, many of the organisms form branching rods, but such branching may not occur at all in some species and genera, and is generally variable and dependent on growth conditions when it does occur. At least four of the genera (*Gordonia* [formerly *Gordona*], *Nocardia*, *Rhodococcus*, and *Tsukamurella*) include organisms that contain mycolic acids in their cell walls. Genera relatively commonly isolated in the clinical laboratory that are considered to belong to this group include *Actinomadura*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella*. Other genera that are sometimes also placed in this group include *Corynebacterium* and *Mycobacterium* (both of which include species with mycolic acids in their cell walls) (Brown and McNeil 2003), although in most discussions these two genera are dealt with separately. While all, or nearly all, the genera are included in the order Actinomycetales of the class Actinobacteria as proposed by Stackebrandt et al. (1997), they are distributed not only in different families but within different suborders. For example, the genera *Nocardia* and *Rhodococcus* are included in the family Nocardiaceae, which, along with families including Corynebacteriaceae and Mycobacteriaceae, are placed in the suborder Corynebacterineae. The genus *Streptomyces* is the only genus in the family Streptomycetacea, and is placed in the suborder Stretomycineae (Stackebrandt et al. 1997). Chun et al. (1996) have also recently presented a phylogeny of the mycolic acid-containing actinomycetes based

on 16S rRNA sequences. This entire group is undergoing considerable taxonomic flux not only at the species level but also at the genus level, with new genera being proposed, and merging of extant genera into others also being recommended. Only the following genera that contain species relatively frequently isolated in the diagnostic laboratory will be dealt with in detail here: *Actinomadura*, *Dermatophilus*, *Gordonia* (formerly *Gordona*), *Nocardia*, *Nocardiopsis*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella*. *Tropheryma*, containing only the species *Tropheryma whipplei*, considered the causative agent of Whipple's disease, will also be discussed, although it is not cultivable in the routine diagnostic laboratory. A few other genera containing species that have reportedly rarely been isolated from clinical specimens, but with what we think is incomplete documentation at best, or genera with organisms causing primarily allergic phenomena, will also be briefly mentioned; these are the genera *Amycolata*, *Amycolatopsis*, *Dietzia*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, and *Thermoactinomyces*. Some of the genera of 'thermophillic actinomycetes', implicated in hypersensitivity pneumonitis but virtually never isolated from clinical specimens, will also be briefly mentioned.

Generic placement of an isolate is facilitated by determination of such features as the types of diaminopimelic acid, mycolic acids, and sugars present in cells and cell walls (Brown and McNeil 2003; McNeil and Brown 1994; Goodfellow et al. 1998). Such determinations, however, are beyond the capacities of most diagnostic laboratories, which instead rely on such features as colonial morphology, presence or absence of aerial hyphae, degree of cellular acid-fastness, and reactions in a few key phenotypic tests (such as the ability to grow in the presence of lysozyme), for provisional generic placement. Several of these genera (particularly *Nocardia* and *Streptomyces*) contain numerous species, but only relatively few of the included species have thus far been demonstrated to be of clinical significance. Several investigators have used huge batteries of phenotypic tests to characterize species and to identify isolates, but the use of such batteries is far beyond the capacity of any diagnostic laboratory. The number of phenotypic tests that have been found useful for discriminating among these species and that are also feasible for use in the diagnostic laboratory is relatively small, and of these tests, few are regularly available in most laboratories. Hence, referral of most isolates of aerobic actinomycetes to a reference center, and the use of molecular methods including DNA sequencing and even DNA–DNA hybridization, are presently required for accurate species identification.

Little is known of the relative pathogenicity of the different species. There do appear to be differences in geographic distribution of, and types of infection most frequently produced by, even some closely related species. However, it is not known what the reasons for these differences are, and the problem is compounded by the fact that many species have only recently been recognized and were combined with others in the past, and that many isolates even today are identified either inaccurately or incompletely to the level of the species. Particularly when referring to the clinical literature, the ongoing taxonomic and nomenclatural problems posed by the aerobic actinomycetes should always be kept in mind, as adequate data to document species assignment are frequently lacking.

It is clear that there are antimicrobial susceptibility differences among some of these species; indeed, some *Nocardia* species were initially recognized on the basis of such differences. A recommended susceptibility testing procedure for the aerobic actinomycetes has been established by the NCCLS (2003). Recommended antimicrobial concentrations and interpretive breakpoints differ to some extent to those recommended for many other gram-positive organisms, and some experience with the technique is needed to assure both intra- and inter-laboratory reproducibility of results. Hence, for accurate susceptibility determinations, referral to a reference center may be advisable.

For many reasons, both the detection of the presence of an aerobic actinomycete, and determination of its clinical significance, are often difficult. Many of these organisms are widespread in the environment, particularly in soil, so that, when detected, the possibility that they may merely be contaminants needs to be considered. However, at least in our own setting, isolates of these organisms (other than *Streptomyces* species) are usually either documentable as, or presumed to be, significant pathogens. These organisms may not be visible, or may not be recognized, in Gram-stained direct smears of specimens, although the first clue to their presence may be the detection of beaded, branching, gram-positive rods by an astute technologist. Most of the species are relatively slow growing, and often will not be detected within the first 48 h of incubation. Even when present as pathogens, they may be present in cultures only as a few colonies or be overgrown by the normal flora in the specimen. Particularly in inexperienced hands, the colonies may not be recognized or they may be assumed to be a component of the inconsequential normal flora. For example, in the outbreak reported by Houang et al. (1980), the patient identified as the source of the outbreak had what were considered to be nonsignificant diphtheroids in her urine for 7 months before it was appreciated that the organisms were in fact a species of *Nocardia*. The modified acid-fast stain, critical for discrimination among these organisms, requires meticulous attention to details of technique, examination, and interpretation for accurate performance. Colonial and cellular morphology, and degree of acid-fastness of cells, are all subject to variation with species, medium and culture conditions, and

number and type of subcultures performed. Hence, even if an aerobic actinomycete is detected, its correct genus assignment, of considerable use in assessment of pathogenic significance, may not be possible in inexperienced hands.

TAXONOMIC RELATIONSHIPS

Given the rapidly increasing complexity in the taxonomy of the aerobic actinomycetes, we think that some explanation of the terminology we will be using is required. Given the widely accepted molecular-based definition of a species, based on DNA–DNA hybridization results and ΔT_m (Wayne et al. 1987; Stackebrandt et al. 2002), we do not believe that many species described without adequate molecular characterization will prove to be valid. This would seem to us an obvious point, but has hardly been honored by all authors and editors. On the other hand, given the current primarily DNA-based approach to delimiting individual species (Wayne et al. 1987; Stackebrandt et al. 2002), we do not think it is useful to insist on the presentation of phenotypic differences to distinguish a newly described species from other phenotypically similar ones. The aerobic actinomycetes present an excellent example of the problems inherent in the attempt to establish unique phenotypic patterns for any species. There may be phenotypic variability for any given characteristic within a species, requiring that an adequate number of strains be studied and introducing the additional problem of defining 'adequate.' A large array of phenotypic tests is available in only a small number of specialized laboratories. There are often subtle variations in such important factors as medium composition, inoculation method, incubation conditions, and result interpretation by different investigators, compounding the problems of intra- and interlaboratory reproducibility of results. Furthermore, there are differences in phenotypic attributes for the same species that can be found in different references, such as whether a certain carbohydrate can or cannot be used as a sole carbon source by a given species. Such differences frequently are unexplained, and the reader can only surmise as to whether the differences reflect taxonomic changes, differences in test methodology or interpretation, or – what we suspect is most likely – undetected typographical errors. To clarify another point, we think the term 'sensu stricto' should mean the restriction of a species epithet to the set of isolates that has been demonstrated to show (or that it is presumed could be demonstrated to show, if the appropriate tests were performed) sufficient molecular-based similarity to the type strain to be considered to belong to the same species as the type strain. We might note in this regard that we think there is a problem with the use of 'reference' strains in place of 'type' strains to establish conspecificity of a set of isolates. Currently only DNA–DNA hybridization and ΔT_m determination can unequivocally assign a 'reference' strain to the same species as the type strain, and more often than not, such conspecificity has been established by less rigorous procedures. It also sometimes seems to be not understood that at any given time there is only one strain that is the type strain for a species.

Somewhat different supraspecific and suprageneric arrangements of the aerobic actinomycetes have been proposed by different investigators, depending upon the set of properties analyzed (Chun et al. 1996; Stackebrandt et al. 1997). Such issues, while of phylogenetic and general biologic interest, are presently unsettled and of little relevance for the identification of unknown isolates, and hence will not be dealt with in detail here. Correct genus assignment is perhaps of greatest significance; such assignment can often be made by assessment of a few phenotypic features of the organism, although at times chemotaxonomic or molecular methods must be used. The modified acid-fast stain can rapidly provide useful information for distinguishing among the various aerobic actinomycete genera.

STAINING PROPERTIES

If visible at all in a direct Gram-stained smear, aerobic actinomycetes will appear as gram-positive rods that are often branched and beaded. Details of the Gram stain appearance may suggest particular genera; such features will be dealt with in the discussions of the individual genera below. A critical stain for both the detection of members of several genera in direct smears and for distinguishing among the genera is the modified acid-fast stain. The principal of this stain is the same as that of the regular acid-fast stain. Because of the cell-wall structure of members of some genera – more particularly, because of the mycolic acids present in the cell walls (such as in the genera *Nocardia* and *Mycobacterium*) – cells stained with a phenolic solution of carbol fuchsin resist decolorization with an acidic decolorizer. However, a weaker (modified) decolorization procedure must be used compared with that used for mycobacteria, or the cells will be decolorized. Hence, those aerobic actinomycetes that retain the carbol fuchsin dye when such a weaker decolorizing process is used are said to be modified acid-fast positive. Unfortunately, this stain requires considerable care and experience for its optimal performance and interpretation, and known positive and negative controls should always be simultaneously stained. The stain is most reliable when used directly on clinical specimens. Particularly when stained from cultures, only a fraction of the organisms will appear truly modified acid-fast positive. If an isolate appears acid-fast negative but the morphology of the cells and/or of the colonies are strongly suggestive of one of the modified acid-fast positive genera, the organism should be subcultured to a lipid-rich medium such as Lowenstein–Jensen or Middlebrook 7H10 agar and restained.

Isolates may recover or increase their acid-fast properties when grown on such lipid-rich media. Members of the genera *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* are generally modified acid-fast positive. Rarely, a *Corynebacterium* species may also appear modified acid-fast positive. Not surprisingly, virtually all isolates in the genus *Mycobacterium* will be uniformly and strongly positive with the modified acid-fast stain, as most such organisms are also positive with the regular acid-fast stain that uses a stronger decolonizing procedure.

Stain reagents

The staining reagents for the regular Kinyoun stain are commercially available, and can be used for performing the modified acid-fast stain. The carbolfuchsin is applied for 3 min, and then gently washed off. (If desired, the stain can be prepared by dissolving 0.3 g of basic fuchsin in 10 ml of 95 percent ethanol, and mixing this with a solution of 5 ml of phenol in 95 ml of distilled water; the solution should be filtered before use (Chapin and Lauderdale 2003).) We use the regular decolorizer (3 percent HCl in 95 percent ethanol) applied for 5–10 s (timed with a stopwatch), and then gently rinse off the decolorizer. A preferable decolorizing procedure, especially for those who do not perform the stain frequently, may be the use of a 1 percent sulfuric acid solution (1 ml of concentrated sulfuric acid added to 99 ml of distilled water), and decolorizing for 3 min before gentle rinsing of the slide. The slide is then counterstained with methylene blue (for example, 2.5 g of methylene blue chloride dissolved in 100 ml of 95 percent ethanol) for 30 s, gently rinsed, and allowed to air dry.

ISOLATION

If infection with a member of the aerobic actinomycetes is suspected, it is crucial that this information be communicated to the laboratory by the clinician to ensure that appropriate handling of the specimen is performed. Cultures for aerobic actinomycetes must be appropriately processed and plated and, because these organisms are relatively slow growing, inoculated media must be held for a minimum of 2 weeks (3 weeks for a tissue specimen) (McNeil and Brown 1994). Most species of aerobic actinomycetes will grow at 35°C, however, media should also be incubated at 28–30°C as some species show enhanced growth at these lower temperatures (Forbes et al. 1998). Certainly, any specimen which shows evidence of an aerobic actinomycete from observation of direct smears should be handled appropriately and held for extended incubation. Isolation of aerobic actinomycetes from normally sterile sites (blood, tissue, cerebrospinal fluid (CSF)) should be relatively uncomplicated, as most types of media which support the growth of aerobic bacteria, mycobacteria, and fungi will also allow isolation of aerobic actinomycetes (including sheep blood agar, chocolate agar, and Sabouraud dextrose agar) as long as an extended incubation is employed. Several types of commercially available blood culture media have been shown to support the growth of *Nocardia* spp., including biphasic fungal culture media (Roberts et al. 1974), Bactec NR-660 medium, and Bactec 12B medium (Vannier et al. 1992). Vannier et al. (1992) note that with a 5-day incubation time used for routine blood cultures with the Bactec NR-660 system, *Nocardia* spp. may not be automatically detected and terminal subcultures may be helpful to detect positive cultures. *Nocardia* spp. have also been recovered using a lysis-centrifugation system (Vannier et al. 1992).

The isolation of aerobic actinomycetes from respiratory specimens may be more difficult due to the presence of normal respiratory flora or other pathogens, especially if low numbers of the aerobic actinomycete are present in the sample. Decontamination techniques generally used to enhance isolation of mycobacteria from respiratory samples have been shown to be toxic to *Nocardia* spp. when exposure to the decontaminating agent extends for 30 min or more (Murray et al. 1987). In our hands a shorter decontamination step (15 min) has had little effect on the recovery of *Nocardia* spp. from contaminated specimens. With or without specimen decontamination, the use of appropriate selective or enhancement media is crucial for isolation of these organisms from potentially contaminated specimens.

The paraffin baiting technique has been used to selectively isolate *Nocardia* species. This technique, initially used for the isolation of actinomycetes from soil by Gordon and Hagan (1936), is based on the ability of *Nocardia* species, but not other bacteria, to utilize paraffin as a sole carbon source. For this technique, a glass rod coated with paraffin is immersed in a carbon-free broth into which the sample is inoculated. Mishra et al. (1973) report that after 1–2 weeks of incubation, *Nocardia* spp. from clinical specimens can be found growing on the paraffin at the surface of the broth; growth is then subcultured for further identification. Studies carried out in an attempt to isolate aerobic actinomycetes from soil have shown that the addition of inhibitory compounds to the broth media can decrease the numbers of contaminating species and enhance the recovery of aerobic actinomycetes. However, all techniques using this application of paraffin bait require subculture before identification procedures can begin.

Direct plating of sample onto selective solid media can allow more rapid detection of any aerobic actinomycete that may be isolated from the sample. Shawar et al. (1990) evaluated the use of carbon-free agar with added carbon sources, to which samples were directly plated. Media containing paraffin as a sole carbon source showed good recovery of *Nocardia* species with suppression of

normal respiratory flora and other contaminating species. Murray et al. (1988) found modified Thayer Martin agar to be a good selective medium for isolation of *Nocardia* spp., and several authors have reported successful isolation of *N. asteroides* from the selective buffered charcoal yeast extract agar (sBCYE) usually employed for isolation of *Legionella* spp. (Vickers et al. 1992; Kerr et al. 1992; Garrett et al. 1992). Vickers et al. (1992) reported that pretreatment of heavily contaminated sputum samples with a low pH KCl-HCl (pH 2.2 ± 0.1) solution and subsequent inoculation on to sBCYE effectively decreased the numbers of contaminating organisms and facilitated recovery of *N. asteroides* from these samples. A selective medium for recovery of *R. equi* (containing ceftazidime and novobiocin) has also been described (von Graevenitz and Pünter-Streit 1995).

For optimal recovery of aerobic actinomycetes, clinical specimens from sterile sites should be plated to an enriched medium (such as buffered charcoal yeast extract (BCYE) agar); specimens from respiratory sites should be plated to a selective enriched medium (such as sBCYE). Inoculated media can be incubated at 30 or 35°C and should be held for at least 2 weeks (3 weeks for tissue specimens). Cultures should be inspected on a regular basis, using a dissecting microscope, if possible, for earliest recognition of aerobic actinomycete-like colonies.

PHENOTYPIC IDENTIFICATION

The complete biochemical characterization of members of the aerobic actinomycetes requires extensive testing of numerous biochemical features (Tsukamura 1982; Kämpfer et al. 1990; Goodfellow 1971). Due to the infrequent isolation of these organisms, most of these biochemicals are impractical to perform or impossible to obtain in small quantities from commercial sources. This leaves a limited number of biochemical tests for characterization of patient isolates, and complicates the identification of many strains. Not all newly described species are biochemically characterized using the same set of parameters and some biochemical tests that are routinely used may be performed using methodologies which vary among laboratories. These methodologic inconsistencies include differences in medium formulation, inoculum preparation, incubation time and temperature, and interpretive criteria. The use of appropriate positive and negative controls is essential to eliminate the effects of these inconsistencies (Berd 1973). Schaal (1977) emphasizes that in order to obtain reliable and reproducible results with biochemical testing, careful attention to technique is important including the use of pure chemical ingredients and elimination of traces of nutrients from test inocula.

Variations in tests performed and the interlaboratory variation found using similar, but not identical, testing techniques may prevent correct species assignment for unknown isolates (Conville et al. 2003). In addition, some new species that are defined on the basis of an extremely limited number of isolates and minimal biochemical characteristics may not adequately reflect the actual variation within that species.

When using limited biochemical testing for identification of unknown isolates, the initial assignment of the isolate to the correct genus is often difficult (Berd 1973). Attention to microscopic and macroscopic morphology, as well as the use of discriminatory tests such as lysozyme resistance, may facilitate the choice of biochemicals and narrow the spectrum of possible species to which the organism may be assigned.

At least for the *Nocardia* species, minimal biochemical and susceptibility testing may give sufficient information to distinguish *N. brasiliensis*, *N. farcinica*, *N. otitidiscaviarum*, *N. nova*, and *N. pseudobrasiliensis* from the other *Nocardia* species. Kiska et al. (2002) propose an identification scheme for *Nocardia* species based on a limited number of biochemicals, assimilation tests from the API 20C Strip (Biomérieux, Durham, NC, USA) and antibiograms. While this scheme was tested using a minimal number of some species (*N. asteroides* drug pattern type IV, *N. transvalensis*, and *N. brevicatena*) good discrimination was obtained for most common, well-characterized species. However, for *N. nova* and similar species (*N. africana*, *N. veterana*) and the species belonging to 'complexes' (*N. asteroides*, *N. brevicatena*, *N. transvalensis*) molecular methodologies may be the only way to adequately discriminate among members of the genus. Indeed, molecular methods including the polymerase chain reaction (PCR), the restriction endonuclease assays (REA), and gene sequencing may be the most rapid, and probably the most accurate, methods for definitive identification of all of the aerobic actinomycetes.

Table 1 shows basic biochemical and susceptibility results for some commonly isolated species of *Nocardia*. Results are listed only for those tests that are helpful for species discrimination. Tests listed are commercially available and relatively easy to perform. It may be difficult to definitively identify members of *N. cyriacigeorgica* (presumed to be the same as *N. asteroides* drug pattern type VI); these organisms are best identified by molecular methods such as REA (see Molecular identification below). It is also important to note that isolates identified as *N. nova* may actually be members of *N. veterana*; REA is helpful to clearly identify isolates of *N. nova*.

We have included here some basic morphologic and biochemical characteristics which are frequently helpful for the identification of the aerobic actinomycetes.

Microscopic morphology

If carefully performed and interpreted, the modified acid-fast stain from colonial growth can be a helpful test

Table 1 *Biochemicals and susceptibility test results useful for discrimination of commonly-isolated* Nocardia *species* [a,b]

	N. abscessus	*N. cyriacigeorgica*[c]	*N. farcinica*	*N. nova*	*N. brasiliensis*	*N. pseudobrasiliensis*	*N. otitidiscaviarum*	*N. asteroides* IV/ *N. transvalensis* (sensu stricto)[d]
14-day arylsulfatase	–	–	NEG	POS[e]	NEG	NEG	–	–
acetimide utilization	NEG	VAR	POS	NEG	–	–	–	–
Hydrolysis of:								
casein	NEG	NEG	NEG	NEG	POS	POS	NEG	–
tyrosine	NEG	NEG	NEG	NEG	POS	POS	NEG	–
xanthine	NEG	NEG	NEG	NEG	NEG	NEG	POS	–
Opacification of Middlebrook	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG[f]
Susceptibility to:								
Amikacin	S	S	S	S	–	–	–	R
Cefotaxime	S	S	R[g]	S/I			–	–
Ceftriaxone	S	S	I/R	S/I			–	–
Ciprofloxacin	R	R	–	R	I/R	S	–	–
Erythromycin	R[h]	R[h]	R[h]	S[h]	–	–	–	–
Gentamicin	S	S/I	R	–	–	–	–	R[i]
Imipenem	R	S	–	S	–	–	–	–

a) Results from: Brown and McNeil (2003); Conville et al. (2003); Kiska et al. (2002); Wallace et al. (1988); Wallace et al. (1990); Wallace et al. (1991); Wallace et al. (1995); Wilson et al. (1997); Yassin et al. (2000b); Yassin et al. (2001).

b) Abbreviations: I, intermediate; NEG, negative; POS, positive; R, resistant; S, susceptible; VAR, variable; (–), not available or not important for species discrimination.

c) Presumed identical to *N. asteroides* drug pattern type VI.

d) *N. transvalensis* sensu stricto, isolates which resemble the *N. transvalensis* type strain

e) *N. nova* isolates are arylsulfatase positive, arylsulfatase negative isolates may be isolates of *N. veterana*; molecular identification needed for identification of *N. veterana*.

f) One isolate each of *N. asteroides* drug pattern type IV and *N. transvalensis* tested.

g) Clinical isolates resistant, type strain is susceptible or intermediate.

h) There are currently no NCCLS interpretive breakpoints for *Nocardia* species tested against erythromycin. The data for erythromycin susceptibilities are based on results reported by Wallace et al. (1988). A procedure for disk testing of erythromycin with *N. nova* and *N. farcinica* is reported by Wallace et al. (1991).

i) 80 percent of *N. transvalensis* sensu stricto clinical isolates are resistant, type and reference strains of *N. transvalensis* and reference strains of *N. asteroides* drug pattern type IV are resistant.

for initial genus assignment, as *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* are considered acid-fast or partially acid-fast (showing both acid-fast and non-acid-fast cells). Acid-fast properties can be influenced by a number of factors which include the lipid content of the media on which the organism is grown, and the age of the culture (Brown and McNeil 2003). Isolates suspected of belonging to the aerobic actinomycetes but which show ambiguous modified acid-fast staining reactions should be subcultured to a medium with a high lipid content (such as Lowenstein–Jensen medium or Middlebrook 7H10 agar) to enhance the acid-fast properties of the cells.

Characteristic morphologic structures of the aerobic actinomycetes can be visualized with the use of slide cultures on a minimal medium such as tap water agar. Using microscopic techniques, the size, structure, and degree of branching of substrate mycelium help to differentiate among the genera (Berd 1973; Brown and McNeil 2003; Gordon and Mihm 1962a; McNeil and Brown 1994). *Nocardia* species frequently show finely branched filaments that branch at right angles and may form secondary branches. The vegetative hyphae may fragment into bacillary or coccoid forms. *Rhodococcus* morphology is cyclic in nature, initially appearing bacillary, and then evolving into coccoid forms. The vegetative hyphae are branched and may be arranged in a zigzag fashion. *Gordonia* generally appear as short rods and also may display a cyclic growth cycle. *Tsukamurella* species usually present as long rods which fragment into three parts, while *Nocardiopsis* species show characteristic long, branched hyphae which fragment into zigzag chains of arthroconidia.

A complete microscopic evaluation will also include assessment of the presence or absence of aerial hyphae and conidia. Generally, species of *Actinomadura*, *Amycolatopsis*, *Nocardia*, *Nocardiopsis*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces*, and *Thermoactinomyces* will produce aerial hyphae while other genera of the aerobic actinomyces will not. *Nocardia* species may have short chains of conidia present on aerial hyphae, *Nocardiopsis* species produce chains of arthroconidia, members of *Thermoactinomyces* all produce spores which vary by species, and *Micromonospora* produce a characteristic single spore on the substrate hyphae (Brown and McNeil 2003).

Colonial morphology

Aerial hyphae can be frequently visualized macroscopically and will give a chalky appearance to the surface of the colonies. Of the acid-fast genera, only *Nocardia* produces aerial hyphae. The colonial morphology of members of the aerobic actinomycetes can vary widely, even within a genus, and should be used cautiously for tentative identification (Berd 1973).

BIOCHEMICALS

Acetamide hydrolysis

This biochemical tests for the presence of the enzyme acylamidase, which hydrolyzes acetamide with the production of ammonia after incubation for 7 days at 35°C. Care must be taken to note the appropriate color change in the medium as acetamide may be assimilated but not hydrolyzed (Remel Technical Information TI 60012-A). This biochemical is commercially available and is a distinguishing characteristic for *N. farcinica* (Kiska et al. 2002; Wallace et al. 1990).

Arylsulfatase

The enzyme arylsulfatase hydrolyzes the bond between the sulfate group and the aromatic ring of potassium phenolphthalein disulfate incorporated in the medium. With the addition of an alkali (sodium carbonate), free phenolphthalein is released, resulting in a red color change in the medium (Kent and Kubica 1985). The CDC Actinomycete Laboratory uses a modified methodology of the Kubica method, increasing the phenolphthalein concentration from 0.001 to 0.008 M (Brown and McNeil 2003). The test can be read at 3 days and 2 weeks, with most strains of *N. nova* showing positive results by 14 days (Brown and McNeil 2003). Arylsulfatase medium is available commercially, but the concentration of phenolphthalein in the product being used must be considered when comparing results obtained with results reported in the literature.

Carbohydrates

There are two methods used for determination of carbohydrate utilization for the differentiation of various species within most genera of the aerobic actinomyces, and either of these, or variations thereof, might be used in defining the carbohydrate reactions for newly described species. The 'Gordon' method utilizes basal medium with individual carbohydrates added; bromcresol purple is used as an indicator. The ability of organisms to oxidize carbohydrates with the production of acid results in a change in medium color from purple to yellow after 3 weeks incubation at 25 or 35°C (Berd 1973; Brown and McNeil 2003). The 'Goodfellow' method tests the ability of organisms to utilize various carbohydrates as a sole source of carbon. Basal medium with single carbon sources added are inoculated with an organism suspension; positive results are noted as heavier growth on medium containing the carbohydrate than is observed on basal medium after 3 weeks of incubation at 25 or 35°C (Brown and McNeil 2003). To our knowledge, neither of these carbohydrate media are available commercially; however, Brown and McNeil

(2003) give a detailed summary of media preparation, inoculation, incubation, and interpretation.

Gelatin hydrolysis

The ability of an organism to produce gelatinases (proteolytic enzymes) which liquefy or hydrolyze gelatin has been shown to be an important test to differentiate *N. brasiliensis* and *N. pseudobrasiliensis* from the other *Nocardia* species (Kiska et al. 2002; Brown and McNeil 2003). This medium is commercially available from several sources. After inoculation the test is incubated at 35°C for up to 7 days; tubes must be placed at 4°C for 15 min prior to checking for hydrolysis (Kiska et al. 2002).

Substrate decomposition

The differential ability of organisms to degrade or hydrolyze the amino acid tyrosine, the purine adenine, purine derivatives xanthine and hypoxanthine, and the milk protein casein have become important criteria for differentiation of members of *Nocardia* spp., *Rhodococcus* spp., and *Tsukamurella* spp. (Brown and McNeil 2003; Kiska et al. 2002). Typically, heavy inocula of the test organisms are placed on each of the substrate-containing agars; positive reactions are seen as clearing of the medium surrounding the colonial growth. All tests should be evaluated weekly; casein results should be recorded after 14 days total incubation, xanthine after 3 weeks incubation and adenine, hypoxanthine, and tyrosine after 4 weeks total incubation (Brown and McNeil 2003). Unfortunately, adenine and hypoxanthine are not readily available from commercial sources.

Lysozyme

Brown and McNeil (2003) describe growth in glycerol broth containing lysozyme as one of the most valuable tests for the discrimination of *Nocardia* from some other aerobic actinomycetes (*Nocardia* spp., *Thermoactinomyces* spp., and *Tsukamurella* spp. are lysozyme resistant; *Gordonia* spp. give variable reactions). Berd (1973) recommends the use of a very small inoculum into glycerol broth with lysozyme and a second similarly inoculated glycerol broth without lysozyme. After 4 weeks of incubation, results are interpreted by comparing the growth in broth containing lysozyme to the control without lysozyme. All *Nocardia* isolates tested by Brown and McNeil (2003) were resistant to lysozyme except for ten lysozyme-susceptible isolates which belonged to the *N. brevicatena* complex.

Middlebrook opacification

Flores and Desmond (1993) describe the ability of isolates of *N. farcinica* to opacify Middlebrook 7H10 or 7H11 agar within 2–10 days of incubation at 28 or 35°C. Opacification was not seen with isolates of '*N. asteroides*' (Carson and Hellyar 1994) although in a study of a limited number of organisms, rare isolates of *N. otitidiscaviarum* and *Rhodococcus* spp. were positive for this characteristic.

Temperature studies

The ability of members of the aerobic actinomycetes to grow at various temperatures can be an important differential characteristic, especially for members of *Tsukamurella* and the thermophilic actinomycetes. Replicates of the same medium (usually sheep blood agar or Sabouraud dextrose agar) are inoculated with a standardized inoculum and incubated at 35 and 45°C; after 3 days' incubation (Kiska et al. 2002) the amount of growth on the two tubes or plates is compared to determine temperature tolerances. If the presence of thermotolerant species is suspected, additional media should be incubated at 50°C.

Test systems and other identification methods

Several investigators have evaluated the usefulness of various identification systems for characterization of species of *Nocardia*, *Rhodococcus*, and *Gordonia*. These studies should be interpreted with the following caveat – most of these systems were evaluated using isolates of uncertain identity by today's standards. Re-evaluation of results based on molecular identifications of test isolates might affect the discriminatory abilities determined in these studies.

Using a combination of eight chromogenic test substrates from the MicroScan Rapid Anaerobe Identification Panels (Dade Behring, Deerfield, IL) and the *Haemophilus Neisseria* Identification Panels (Dade Behring), Biehle et al. (1996) were able to identify five species of *Nocardia* through the detection of preformed enzymes. Some heterogeneity of *N. asteroides* isolates was noted; identifications were obtained within 5 days. Boiron and Provost (1990) also evaluated the detection of preformed enzymes of *Nocardia* species and a limited number of other aerobic actinomycetes using the API ZYM System (Biomérieux, Durham, NC, USA). Identifications were obtained after a 5-h incubation; a difference of a minimal number of characteristics separated some species while others were unidentified by this system. Muir and Pritchard (1997) evaluated the use of the 32C Yeast Identification System (Biomérieux) for determining the assimilation profiles of numerous strains of *Nocardia* species and a limited number of other aerobic actinomycetes. After 7 days' incubation, a considerable amount of heterogeneity was found for most species tested, but predominant profiles for each

species were identified. Bizet et al. (1997) examined results of carbon utilization of isolates of *Rhodococcus*, *Gordonia*, and *Dietzia* species using carbon source utilization strips and found good identification to the species level using computer analysis of results.

Fiss and Brooks (1991) described a relatively simple procedure which tentatively separates *Nocardia* species and *Streptomyces* species from *Rhodococcus* species and the rapidly growing mycobacteria, *Mycobacterium chelonae* and *Mycobacterium fortuitum*. The procedure uses agar-based testing for siderophore detection, ethylene glycol degradation, and a disk test for β-galactosidase activity. The combination of these tests was not able to differentiate *Nocardia* spp. from *Streptomyces* spp. but was useful for distinguishing *Rhodococcus* spp. and the rapidly growing mycobacteria from the *Nocardia-Streptomyces* group. Provost et al. (1995) describe a novel procedure for the differentiation of *N. asteroides*, *N. farcinica*, and *N. nova* using a 'killer yeast' phenomenon. The two yeast strains, which produce extracellular glycoproteins ('killer toxins' which differentially inhibit *Nocardia* species), are streaked on to Sabouraud agar into which *Nocardia* species have been incorporated. After 5 days of incubation, areas of inhibition of the *Nocardia* species are visible surrounding the yeast inoculum, and each of the three *Nocardia* species tested show a different pattern of susceptibility to the toxins.

SUSCEPTIBILITY TESTING

The NCCLS has published an approved standard that includes susceptibility testing procedures for nocardiae and other aerobic actinomycetes (NCCLS 2003). In addition to isolates in the genus *Nocardia*, it is specifically recommended that isolates in the genera *Actinomadura*, *Gordonia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* be tested with the procedure for aerobic actinomycetes. Broth microdilution is the recommended method, with incubation in ambient air at $37 \pm 2°C$ for 3 days for most species, although a few may require incubation for up to 5 days. Drugs currently recommended for testing include amikacin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, sulfamethoxazole or trimethoprim-sulfamethoxazole, and tobramycin. Secondary drugs that could be considered for testing include cefepime, cefotaxime, doxycycline, gatifloxacin or moxifloxacin, and gentamicin. Interpretive breakpoints for most of these agents are also provided, and a recommended quality control procedure is specified. We think it advisable for a laboratory lacking extensive experience with the susceptibility testing of these organisms to assure that it can obtain results equivalent to those obtained by an experienced reference laboratory before reporting results for patient care purposes. Also, as the NCCLS updates its documents regularly, it is important for laboratories to assure that they are following the most currently recommended procedures.

CELL-WALL AND CELL-MEMBRANE COMPOSITION

DAP and sugars

The aerobic actinomycetes vary in their cell wall composition in regard to the isomeric form of diaminopimelic acid (DAP) and the presence of various cell-wall sugars. These components have been useful in discrimination among genera; based on DAP type and sugar content, the aerobic actinomycetes and related species can be classified into nine wall chemotypes (Lechevalier and Lechevalier 1970). Becker et al. (1964) and Lechevalier (1968), using whole cell hydrolysates and paper chromatography, demonstrated that the cell walls of most genera of aerobic actinomycetes contain *meso*-DAP with high concentrations of arabinose and galactose (wall chemotype IV), while *Streptomyces* contains L-DAP and no detectable cell-wall sugars. The genera *Actinomadura* and *Dermatophilus* possess cell walls with *meso*-DAP and madurose (Lechevalier and Gerber 1970); *Nocardiopsis* has *meso*-DAP and no detectable sugars. Staneck and Roberts (1974) describe a procedure using thin layer chromatography for detecting these components.

Isoprenoid quinones are found in the plasma membranes of most species of bacteria with menaquinones being the form of isoprenoid quinone found in the aerobic actinomycetes. The menaquinones can vary in the length and degree of saturation of their C_3 isoprenyl side chain. Using mass spectrometry, Collins et al. (1985) found that members of the aerobic actinomyces can be classified into a number of distinct groups based on the structure of their membrane menaquinones. The species of *Nocardia* studied showed similar menaquinone structures which differed from those obtained from *Mycobacteria* and *Rhodococcus* species. Several different patterns were observed among various species of *Rhodococcus*. Yassin et al. (1988) used thin layer chromatography and mass spectrometry to study the menaquinone composition of several genera of the aerobic actinomycetes. These investigators propose that menaquinone analysis, combined with DAP and carbohydrate analysis, would be useful methods for genus assignment among this group of organisms.

Lipids

Chromatographic analysis of cell wall-associated lipids has been used to differentiate genera and species of the aerobic actinomycetes. Species of *Dietzia*, *Gordonia*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* as well as members of the related genera *Corynebacterium* and

Mycobacterium are known to have high levels of mycolic acids as major constituents of their cell walls. The mycolic acids are high molecular weight α-branched, β-hydroxy fatty acids associated with the cell-wall peptidoglycan and contribute to the staining characteristics and pathogenicity of these organisms (Beaman and Beaman 1994). Mycolic acids of various organisms have been found to differ in the number of their constituent carbon atoms (Alshamaony and Goodfellow 1976). Corynemycolic acids (found in *Corynebacterium* spp.) are the shortest with an average of 32 carbon atoms, while nocardiomycolic acids have an average of 50 carbon atoms. Mycobacterial mycolic acids are the largest with more than 70 carbons in their long chains (Alshamaony and Goodfellow 1976). Mycolic acids also differ in the number of carbon–carbon double bonds and in the types of functional groups they contain.

Mordarska (1968) and Goodfellow et al. (1973) discussed Lipid A (a lipid characteristic of *Nocardia*, LCN-A), discovered on thin layer chromatography of cell-wall lipids which were present in *Nocardia* and *Rhodococcus* species but not in *Streptomyces* spp. These LCN-A molecules were found to be free nocardiomycolic acids. Using mass spectroscopy, Alshamaony and Goodfellow (1976) were able to characterize these free mycolic acids and showed 'rhodochrous' complex species to possess carbon numbers intermediate between those of *Corynebacterium* spp. and *Nocardia* spp.

Gas chromatography combined with mass spectrometry has been used to more completely analyze the mycolic acids of the aerobic actinomycetes. Using average carbon numbers and peak areas, Baba et al. (1997) found distinctive patterns for isolates of *N. nova* but were unable to definitively discriminate between isolates of *N. asteroides* and *N. farcinica*. In addition, significant carbon number variation was noted among isolates of *N. asteroides*, confirming the well-known heterogeneity of this species complex. Using a limited number of isolates, Nishiuchi et al. (1999) were able to differentiate isolates of *N. asteroides*, *N. farcinica*, and *N. nova* by the average number of carbon atoms, the relative degree of saturation in the mycolic acid molecules and the structure of the α-alkyl branch. Nishiuchi et al. (2000) used these same identifying criteria to discriminate among members of *Rhodococcus*, *Gordonia*, and *Dietzia* with species of *Rhodococcus* clearly dividing into two distinct groups.

Using high performance liquid chromatography (HPLC) of bromophenacyl esters of mycolic acids, Butler et al. (1986) showed the relative retention times of the carbon chains of *Corynebacterium*, *Rhodococcus*, *Nocardia*, and *Mycobacteria* species to be different enough to allow differentiation at the genus level. Further investigations by these authors (Butler et al. 1987) defined species-specific peak patterns for *Nocardia* and *Rhodococcus* species, with members of *Rhodococcus* splitting into short and long chain groups.

McNabb et al. (1997) used the Microbial Identification System and Library Identification software (MIDI, Inc. Newark, DE, USA) to identify a variety of species of aerobic actinomycetes. A custom reference library was assembled using data obtained from 39 type strains. After analysis of the pattern of mycolic acid peaks obtained for each test isolate, the software generates a similarity index to profiles stored in the library. The authors found 33.5 percent of isolates tested to give satisfactory similarity indices with library strains and recommend the system for grouping isolates into similar species or subspecies groups. One interesting finding was that none of the patterns of the *N. asteroides* strains tested matched the pattern of the *N. asteroides* type strain stored in the library.

MOLECULAR IDENTIFICATION

The aerobic actinomycetes are becoming increasingly difficult to differentiate on the basis of phenotypic characteristics alone due to the paucity of commercially available biochemical tests and the rapidly increasing number of recognized species (see Phenotypic identification section above). For these reasons, molecular methodologies have become the most reliable and rapid methods for precise species (and sometimes genus) assignment. The use of these methods has brought about the recognition of a number of new species, clarification of taxonomic relationships, and a new appreciation of the heterogeneous nature of this group of organisms.

Amplification of portions of the 16S rRNA gene and detection of the product by gel electrophoresis has been used to identify some members of the aerobic actinomycetes isolated in culture to the genus or species level. Laurent et al. (1999) describe genus-specific primers which allow amplification of the 16S rRNA gene of *Nocardia* species but not that of the other related aerobic actinomycetes. Bell et al. (1996, 1999) developed species-specific primers for the differential amplification of *R. equi* and several other *Rhodococcus* species grown in culture; cross-reactivity only occurred between *R. rhodochrous* and *R. ruber*.

Lungu et al. (1994) reported a PCR-REA which amplified a portion of the *groEL* gene (which codes for a heat shock protein) and subsequent digestion of the amplicons with the restriction endonuclease *Hae*III. Resulting restriction fragment length polymorphism (RFLP) patterns allowed differentiation of *Nocardia* species from *Mycobacterium* species and identification of many isolates of both of these genera to the species level. In 1995, Steingrube et al. reported a PCR-REA assay using amplification of the 65-kDa heat shock protein (HSP) gene and subsequent digestion with the restriction endonucleases *Msp*I and *Bsa*HI. This method allowed identification of 12 species and taxa of *Nocardia* and in addition, showed molecular differences among

the drug pattern types of *N. asteroides* described by Wallace et al. (1988). Enhancement of this method with the use of an additional endonuclease (Steingrube et al. 1997) allowed the identification of more species of aerobic actinomycetes and a decrease in the intraspecies variation seen among some species. Significant intraspecies variation was still observed with members of the genus *Streptomyces* and inconsistent amplification with *N. otitidiscaviarum* complicated identification of that species. In 2000, a PCR-REA method was described (Conville et al. 2000) using amplification of a 999-bp region of the 16S rRNA gene and subsequent REA which allowed the differentiation of nine species of *Nocardia*. When PCR-REA of portions of both the 16S rRNA gene and the 65-kDa HSP gene was employed, more accurate identifications could be obtained than was possible with the use of one gene region alone. In addition, discrepant results obtained from the two gene regions allowed the recognition of isolates which, as confirmed by 16S rRNA gene sequencing, were unusual (Conville et al. 2003) or undescribed species.

PCR-REA methodologies provide rapid (2–5 days) and accurate identification of many species of clinically relevant *Nocardia* (Wilson et al. 1998) and are reasonable alternatives to phenotypic testing for any laboratory with basic molecular biology capabilities. However, it is possible that as more *Nocardia* species are described and as species 'complexes' are more fully characterized, the number of restriction endonucleases needed to adequately identify these species will also increase, making the procedure more time consuming.

Specific information about identification of *Nocardia* isolates by REA can be found in the following references: HSP, Steingrube et al. (1997), Brown et al. (1997), Wilson et al. (1997); 16S rRNA, Conville et al. (2000, 2003).

While most molecular methods are used on growth from cultures, Qasem et al. (2001) describe a semi-nested PCR method with amplifies a portion of the *groEL* gene for detection of *Nocardia* spp. in blood and tissue samples of mice. This method was reported to be more sensitive than culture for those samples. This method was also able to detect *N. asteroides* and *N. farcinia* from spiked human blood samples.

Ribotyping is a somewhat labor-intensive method which has been shown to be useful for the identification of aerobic actinomycetes. Ribotyping involves extraction of DNA from bacterial cells, digestion with restriction endonucleases, and separation of the resulting fragments by gel electrophoresis. Fragments are transferred to a membrane using a Southern blot technique and hybridization is performed using labeled DNA probes to nucleotide sequences which code for rRNA. This method has been shown to be useful in the differentiation of *Rhodococcus* species and related strains (Jorks 1996; Lasker et al. 1992) and *Nocardia* species (Laurent et al. 1996; McNeil et al. 1997).

PCR-randomly amplified polymorphic DNA (RAPD) fingerprinting has also been used for the identification of *Nocardia* species. The procedure involves extraction of DNA from the organism and PCR using arbitrary primers and low stringency conditions. Gel electrophoresis of the amplification products shows patterns of banding which vary from strain to strain. Isik and Goodfellow (2002) studied the use of RAPD for identification of *Nocardia* spp. and obtained species-specific banding patterns for representatives of five species and unique patterns for single strains of eight other species. Multiple banding patterns were seen for representative isolates of *N. asteroides*, *N. nova*, *N. pseudobrasiliensis*, and *N. transvalensis*, possibly illustrating the heterogeneous nature of each of these species.

Sequencing of the 16S rRNA gene is becoming more widely used for more definitive species identifications of the aerobic actinomycetes. Using sequence analysis of *Streptomyces* sp., Stackebrandt et al. (1991) defined areas of the 16S and 23S rRNA genes which hold potential as targets for species-specific primers and probes. Cloud et al. (2002) evaluated a commercially available sequencing kit (MicroSeq 500, Perkin Elmer Applied Biosystems, Foster City, CA, USA) which provides sequence data for the first 500-bp region of the gene which, when used with an expanded database, seems to be sufficient for the identification of many clinically significant species of *Nocardia*. Sequence data from an extended region of the gene is necessary, however, for identification of a few species. Conville et al. (2000) described a hypervariable region of the 16S rRNA gene which provides species-specific base patterns for many *Nocardia* species. Due to the heterogeneous nature of the non-*Nocardia* aerobic actinomycetes, gene sequencing may be more reliable than phenotypic testing for the correct identification of these isolates. However, in using gene sequencing for identification, one must be careful to fully assess the sequence similarity of closely related species. It has been shown that some distinct *Nocardia* species show a high degree of sequence similarity (>99.0 percent of a 1350-bp region) (Conville et al. 2003) and that investigation of additional gene regions might more accurately determine species identity. Care must also be used when utilizing public databases as a basis for sequence comparison; errors inherent in the databases may result in an erroneous identification (Turenne et al. 2001). It may be that DNA–DNA hybridization may be the only definitive method for unequivocal species identification (Conville et al. 2003; Stackebrandt et al. 2002).

In the absence of adequate methods for the in vitro cultivation of *Tropheryma whipplei*, microscopy and molecular methods are the sole alternatives for the diagnosis of Whipple's disease. The use of broad spectrum 16S rRNA eubacterial primers, PCR and direct sequencing of the amplified product by Wilson et al. (1991) and Relman et al. (1992) resulted in extensive information

about the 16S rRNA sequence of the Whipple's disease-associated bacterium. From these sequences, specific primers were developed (Relman et al. 1992; von Herbay et al. 1996) and a probe sequence was identified and tested (Maiwald et al. 1996, von Herbay et al. 1996) which can be used as an adjunct to histiologic testing for the diagnosis. Using nested PCR, direct sequencing and single-strand conformation polymorphism, Hinrikson et al. (1999) detected three genetic variants in the 16S–23S ribosomal spacer region among 38 clinical isolates containing *Tropheryma whipplei*.

STRAIN TYPING

The aerobic actinomycetes are ubiquitous in nature and it is not surprising that some nosocomial infections with these organisms, especially with *Nocardia* spp., have occurred. In the hospital setting these species present especially serious complications for the immunocompromised patient, and several outbreaks have been documented in transplant units. Strain typing of isolates from these outbreaks is important for defining the scope of organism spread. Pseudoepidemics of species of aerobic actinomycetes caused by laboratory contamination have also been reported; typing of these isolates is necessary to pinpoint the source of contamination. Numerous methodologies have been applied to the epidemiologic study of *Nocardia* spp. (and occasionally to other members of the aerobic actinomycetes).

Prior to the widespread use of molecular techniques, immunologic or biologic methods were employed to characterize *Nocardia* strains involved in outbreaks. Pier and Fichtner (1971) describe an immunodiffusion method for strain typing isolates of *N. asteroides* which classifies isolates into four antigenic types. This method was used by Stevens et al. (1981) for strain typing of patient and environmental isolates involved in an outbreak in a renal transplant unit. All isolates in this outbreak were found to belong to antigen type III. Morace et al. (1988) applied a 'killer yeast' methodology to 13 strains of aerobic actinomycetes. All *N. asteroides* (six unrelated isolates) and *A. madurae* (five unrelated isolates) showed different patterns of sensitivity with a panel of nine yeast strains, and use of this system was proposed as a possible strain-typing methodology. Sahathevan et al. (1991) used a biotyping methodology to characterize three strains involved in an outbreak of *N. asteroides* in a liver disease unit. The method was based on detection of bacterial enzymes using fluorogenic substrates; the exact methodology and the enzymes examined were not described.

As with strain typing of other bacterial species, molecular methodologies have become important tools in evaluating strain relatedness of the aerobic actinomycetes. Plasmid profiling was one of the first molecular methods used for strain comparison; the method is based on the detection and comparison of plasmids of similar sizes from suspected outbreak strains. However, Provost et al. (1996) found plasmid analysis to be of little value in epidemiologic studies of *Nocardia* species as only 31 percent of 87 clinical isolates evaluated showed evidence of the presence of plasmids. Using the enzyme *Pvu*II and REA of total DNA, Patterson et al. (1992) investigated a pseudoepidemic of *N. asteroides* attributed to ineffective needle sterilization in the BACTEC TB System. Gel electrophoresis of the DNA digest of outbreak isolates showed RFLP patterns which were identical to one another but different from those of a reference strain. Auerbach et al. (1992) used biochemical and antibiogram data along with ribotyping with the restriction enzyme *Pst*I to investigate a pseudoepidemic of *T. paurometabolum* (see Molecular identification section for an overview of the ribotyping methodology). The source of this pseudoepidemic was traced to contamination in the laboratory at the time of specimen processing. Using ribotyping with the restriction enzyme *Pst*I, Lasker et al. (1992) found considerable heterogeneity with *R. equi* isolates and proposed that this methodology may be a useful epidemiologic tool in *R. equi*-related outbreaks.

Exmelin et al. (1996) compared ribotyping and RAPD analysis in an investigation of an outbreak of *N. farcinica* among heart transplant recipients (see Molecular identification section for an overview of the RAPD methodology). Ribotyping of outbreak strains with five enzymes showed no significant differences from unrelated reference strains. Using RAPD with two primers used alone or in combination, outbreak strains showed identical patterns while all unrelated reference strains showed different patterns.

Louie et al. (1997) investigated a pseudoepidemic of *N. asteroides* again linked to ineffective needle sterilization with the BACTEC 460 TB system. Patterns obtained with RAPD and pulse field gel electrophoresis (PFGE) showed outbreak strains to be identical to the index strain and also to the isolate cultured from the BACTEC needle heater. These isolates were significantly different from unrelated clinical isolates.

PFGE is also a popular method for studying the relatedness of many species of bacteria implicated in nosocomial infections. The technique involves preparation of genomic DNA and digestion of the DNA with restriction enzymes which have a limited number of recognition sites. The digests are then subjected to alternating-field electrophoresis; UV illumination of the ethidium bromide-stained gel reveals DNA banding patterns which vary depending on the DNA sequence of the organisms. Blümel et al. (1998) used PFGE with the enzyme *Ase*I to study the relatedness 18 isolates of *N. farcinica* recovered from postoperative infections over an 11-year period in a surgical ward. The authors found all patient isolates and two environmental *N. farcinica* isolates recovered from the operating suite to have identical PFGE patterns. The patterns of these isolates were

different from those of clinical isolates from other cities and other nearby environmental sites. The authors considered this strain to be endemic to the ward and thought it was most probably introduced during hospital renovation.

Strain typing may also prove useful in assessing the persistence of specific strains in patients with long-term infections. Provost et al. (1997) used RAPD to show that a single patient was infected with two strains of *N. asteroides*, while two additional patients remained infected with the same strains of *N. otitidiscaviarum*.

PATHOGENICITY

Through the use of animal models, a significant amount of information has been accumulated to define the disease-causing properties of *Nocardia* species and other aerobic actinomycetes. An early study by Gonzales-Ochoa and Sandoval-Cuellar (1976) found *N. brasiliensis* to be more virulent than *N. asteroides* and *N. caviae* when these organisms were inoculated into the plantar pad of mice. Also using mouse pathogenicity studies, Desmond and Flores (1993) found the LD_{50} for *N. farcinica* to be lower than that determined for *N. asteroides* and *N. nova*.

Based on numerous studies of nocardial virulence in murine models, Beaman and Beaman (1994) present an extensive review of the pathogenic capabilities of *Nocardia* species. Using strains of *N. asteroides* with varying degrees of virulence, these authors and their colleagues have defined specific properties of *Nocardia* which may contribute to their virulence in the human host. The complex nocardial cell wall is the site of many of these virulence factors. Some aspects of nocardial virulence are related to the phase of growth of the cell and the structure of the peptidoglycan-arabinogalactan-mycolate complex present in the cell wall. The chain length of mycolic acids and the relative level of unsaturation (number of double bonds) is also related to virulence. *Nocardia* species also possess a biologically active cord factor (trehalose-6, 6′-dimycolate) in the cell wall which is presumed to play a role in pathogenicity through its effects on leukocyte migration, and its immunomodulator properties. *Nocardia* species are known to be resistant to the 'oxidative burst' of the polymorphonuclear cells (PMN) responding to bacterial invasion; this is accomplished through the organism's ability to produce catalase (which breaks down peroxidase) and superoxide dismutase (SOD) (which inactivates superoxides, O_2^-) produced by the PMNs. Beaman and Beaman (1994) also discuss specific interactions between macrophages and invading nocardial cells. *Nocardia* species seem to be able to inhibit phagosome-lysosome fusion within the macrophage (possibly through the action of cord factor), which renders the macrophage incapable of destroying the nocardial cell. *Nocardia* also appear able to block acid production within the phagosome (the pH of which generally drops to 5 when organisms are engulfed), allowing the invading bacterium to survive and multiply within the vesicle intended for its destruction.

Black et al. (1983) determined that pathogenic strains of *Nocardia* that have been phagocytized by macrophages in vitro are able to affect the level of lysosomal enzyme activity, particularly that of acid phosphatase. Beaman et al. (1988) determined that *Nocardia* species use acid phosphatase as a carbon source, eliminating the killing activity of the enzyme. *N. otitidiscaviarum* has been shown to produce toxins in vitro, and other species produce hemolysins which play an uncertain role in pathogenesis.

The ability of nocardial species to form L-forms (cell-wall-deficient variants) has also been evaluated by Beaman and colleagues. These variants have been recovered from CSF from a patient with probable brain involvement, however the role of L-forms in the development of disease or their role in latent infections remains unclear (Beaman and Beaman 1994).

SEROLOGIC TESTING

Because *Nocardia* species are frequently difficult to isolate from clinical specimens, there have been attempts to diagnose *Nocardia* infections through detection of antibodies in serum to unspecified antigens recovered from culture filtrates or bacterial cells, or to specific nocardial proteins. The specificity of these tests must be adequately investigated, as most described assays are based on antigens derived from patient isolates identified as *N. asteroides* or *N. brasiliensis*. The usefulness of these serologic techniques requires assessment of the presence of these antigens from the numerous species of *Nocardia* now recognized as agents of human disease. In addition, false-positive results are likely to be obtained from patients infected with mycobacterial species or with other species of aerobic actinomycetes, especially with tests which use antigens prepared from disrupted bacterial cells (Sugar et al. 1985). Results from serologic tests may show low sensitivity, especially when testing sera from immunocompromised patients who have a decreased antibody response, but who are among those most likely to develop nocardial disease.

Tests have been developed based on the detection of serum antibodies to nocardial antigens present in culture filtrates (Blumer and Kaufman 1979; Shainhouse et al. 1978) or cell extracts (Humphreys et al. 1975), to specific proteins (Angeles 1987; Angeles and Sugar 1987; Salinas-Carmona et al. 1993; Sugar et al. 1985) or to a panel of these antigens (Kjelstrom and Beaman 1993). Various methodologies for antibody detection are employed by these tests including immunoprecipitation, immunoblot assays, complemen fixation tests and enzyme-linked immunosorbent assay (ELISA). Salinas-Carmona

et al. (1993) suggest that serologic tests may be useful for following the clinical course of infected patients as antibody titers have been shown to be related to the patient's clinical course. Serologic tests are not widely used and have been shown to be of limited practical utility; to our knowledge no commercial serologic test is available.

ACTINOMADURA

The most common infection caused by organisms in this genus is mycetoma. Such infections are usually initiated by trauma, are more common in tropical regions, and result in chronic, locally progressive infections producing considerable swelling and tissue destruction. Draining sinuses are frequent; 'grains' or 'granules,' consisting of clumps of organisms, may be found in the sinus discharge, and the color of the grains may assist in defining the etiology of the mycetoma (Brown and McNeil 2003). Mycetomas may be caused either by true fungi 'eumycotic mycetoma' or by aerobic actinomycetes 'actinomycotic mycetoma.' Worldwide, species in the genus *Actinomadura* evidently cause mycetomas less frequently than species in the genus *Nocardia* (Maiti et al. 2002; Lopez Martinez et al. 1992) (see below under *N. brasiliensis* for further discussion of mycetoma and its causative agents).

Clinically significant isolates of *Actinomadura* species do occur outside the tropics. In a report in 1990, McNeil et al., attributed 11.5 percent of the aerobic actinomycete isolates submitted to the CDC from October 1985 through February 1988 to the species *A. madurae*. Of the 42 isolates in that group, 57.1 percent were from sputum, 31.0 percent from wounds, and 2.4 percent each from blood and brain. By the susceptibility testing that they performed, all isolates were sensitive to amikacin, ceftriaxone, and imipenem. Over 80 percent of the isolates were sensitive to amoxicillin-clavulanic acid, cefotaxime, ciprofloxacin, doxycycline, minocycline, erythromycin, and trimethoprim-sulfamethoxazole; over half the isolates were resistant to ampicillin. Wüst et al. (1990) reported a patient on continuous ambulatory peritoneal dialysis who developed peritonitis attributed to *A. madurae*; the patient responded to treatment with amikacin. Both biochemical testing and cell-wall analysis was performed on the isolate. Interestingly, the patient, who had no history of foreign travel, worked in a flower shop, and the authors speculated that the plants or soils with which he came in contact may have been the source of the organism. McNeil et al. (1992b), reported a case of an IV drug abuser with acquired immune deficiency syndrome (AIDS) with fever and weight loss who had a blood culture positive for an isolate identified as *A. madurae*. Sputum cultures, as well as subsequent blood cultures, failed to grow the organism again. The patient expired despite treatment with a variety of antibiotics. At autopsy he was found to have a necrotizing pneumonia; no autopsy cultures were obtained, but pulmonary histopathology showed organisms morphologically consistent with *A. madurae*.

Species in this genus lack mycolic acids in their cell walls but their cells do contain the sugar madurose (McNeil and Brown 1994). Colonies may produce an aerial mycelium. These organisms appear as branching gram-positive rods.

Only three species in the genus are known to be human pathogens: *Actinomadura latina*, *Actinomadura madurae*, and *Actinomadura pelletieri* (Trujillo and Goodfellow 1997). *A. latina* is the most recently described; the species name refers to the fact that many pathogenic isolates in the genus have originated from Latin America (Trujillo and Goodfellow 1997). The three species can be discriminated in the laboratory by such features as color of substrate mycelium, and decomposition and sole carbon source utilization tests (Trujillo and Goodfellow 1997; Brown and McNeil 2003); note, however, that there are some differences in the positivity rates for the same tests in the latter two references.

AMYCOLATA AND AMYCOLATOPSIS

These two genera were proposed to accommodate two groups of species that lacked mycolic acids but differed in terms of their phospholipid patterns and menaquinones (Lechevalier et al. 1986). At least one species in each genus, *Amycolata autotrophica* and *Amycolatopsis orientalis*, has been reported to have been isolated from clinical specimens (McNeil and Brown 1994), but we have not been able to find any recent reports documenting their association with invasive disease. Some strains of the latter species produce vancomycin. Warwick et al. (1994), subsequently recommended that the genus *Amycolata* be merged into an emended genus *Pseudonocardia* on the basis of molecular studies.

DERMATOPHILUS

This genus currently contains two species, *Dermatophilus chelonae* and *Dermatophilus congolensis*; only *D. congolensis* is known to be a mammalian pathogen (and perhaps a pathogen of some non-mammalian species as well), but it only rarely causes human disease (McNeil and Brown 1994). The dermatitis produced by these organisms is known as dermatophilosis. Both members of the genus are unique among the aerobic actinomycetes in that they produce motile zoospores. Colonies do not produce an aerial mycelium, and the organisms do not contain mycolic acids and are not acid-fast (Brown and McNeil 2003; McNeil and Brown 1994). It is said that isolation of the organism may be difficult. It is recommended that material for culture be obtained from beneath skin scabs, and that incubation of some material be at 36°C in 5–10 percent CO_2. Contaminated speci-

mens may need to be inoculated into the skin of a rabbit in order to isolate the organism (Brown and McNeil 2003). The organisms have a characteristic morphology, with the formation of both transverse and longitudinal septa. While they are gram-positive, morphologic detail may be better seen in using a stain such as the Giemsa stain, which does not stain the organism so darkly. Organisms may be visualized directly in clinical material.

In a 1995 paper describing a new species pathogenic for chelonids, *D. chelonae*, the authors compared their isolates to a number of mammalian isolates of *D. congolensis*, and described a variety of phenotypic features, including colony morphology and biochemical reactions, for both species (Masters et al. 1995).

Disease in man is usually the consequence of contact with infected animals or materials from such animals (McNeil and Brown 1994). However, the organism may also cause the condition known as 'pitted keratolysis,' pitted lesions in the keratinized layer of the skin, evidently generally occurring on the feet. Gillum et al. (1988) reported a case of pitted keratolysis of the soles of both feet of a physician with no history of contact with infected animals; the organism was visualized histopathologically and isolated in culture. This condition is generally asymptomatic and self-limiting, and so may be more common than generally recognized; in these cases, it seems likely that infection results from soil contact. The organism (identified histopathologically and by immunofluorescence staining) was considered to be the causative agent of 'hairy' leukoplakia (a hyperkeratotic lesion of the tongue) in an HIV-positive animal handler (Bunker et al. 1988). Towersey et al. (1993) reported four cases of infection, including one patient who had nail as well as skin involvement; they also tabulated the clinical features of 26 other human cases for which they found reports in the literature.

DIETZIA

This genus was distinguished from the genus *Rhodococcus* on the basis of 16S rDNA sequence differences (Rainey et al. 1995). We are aware of only one report claiming the recovery of one species in the genus, *Dietzia maris*, from clinical specimens, both a blood culture and a catheter culture from an immunocompromised patient (Bemer-Melchior et al. 1999). The organism was identified on the basis of a variety of tests including HPLC, but it is not clear from the report which *Rhodococcus* species were included in the HPLC database, and molecular techniques were apparently not used.

GORDONIA

Many of the species currently placed in this genus (formerly *Gordona*) were previously placed in the genus *Rhodococcus*. An account of the history of the genus can be found in the discussion of rhodococcal systematics by Goodfellow et al. (1998). Stackebrandt et al. (1988), on the basis of phylogenetic heterogeneity demonstrated by 16S rRNA sequencing, proposed reviving the genus *Gordona* and emending the genus *Rhodococcus*, so that the genus *Gordona* would contain organisms with mycolic acids in the range of 48–66 carbon atoms and major amounts of dihydrogenated menaquinones with nine isoprene units, and the genus *Rhodococcus* would contain organisms with mycolic acids in the range of 34–52 carbon atoms and menaquinones with eight isoprene units. As is the case for species in the genus *Rhodococcus*, some phenotypic differences have been noted that may provide some discrimination among the different species in the genus, but it is not yet clear that phenotypic procedures can provide truly accurate identification of any of the species. The morphology of individual organisms and of bacterial colonies of the species in the genus is very similar to that of *Rhodococcus* species. Species-level identification is best made by using molecular methods. A brief account follows for those species for which we could find some account of their isolation from human clinical specimens.

Gordonia aichiensis

This species has been reported to have been obtained from human sputum (Klatte et al. 1994), but we were not able to find any case report regarding illness attributed to this species, nor any recent reports of its isolation from clinical specimens.

Gordonia bronchialis

An outbreak of seven cases of sternal wound infection following coronary artery bypass surgery was reported by Richet et al. (1991). (The report referred to the organism as *Rhodococcus* (*Gordona*) *bronchialis*.) All involved patients were men who had required multiple grafts during the surgery, but all incisions healed well postoperatively; the average time interval after surgery before appearance of infection was 53 days. Four patients required surgical debridement of the sternum, and two of these required sternectomy. Three patients did well with antibiotic treatment alone; no patient relapsed after treatment. Epidemiologic investigation traced the hospital reservoir of the infection to 'nurse A'. Cultures from nurse A's fingers after she wet them in a water bath in the course of performing an intraoperative clotting test, a nasal swab from nurse A, and air samples collected in an operating room in which nurse A had spent 5 min, all were positive for the same organism cultured from the patients. Further investigation resulted in growth of the organism from the scalp and vagina of the nurse and from the surfaces of her operating room locker, and also from the neck-scruff

skin of two of her three dogs, from a furnace vent in the floor where one of the dogs would often lie, and from the inside of the nurse's deck shoes. The nurse was assigned to duties not involving open-heart surgery, and there were no subsequent cases of wound infection caused by the organism. A regimen of vigorous hand washing, along with application of bacitracin to her hands and treatment with oral rifampin, failed to eliminate carriage from the hands and scalp of the nurse. Organisms were never cultured from the water bath, and it was surmised that transmission of the organism on her hands to the affected patients was facilitated by the droplets of water on her hands after the performance of the clotting tests. The organisms involved were identified by a variety of phenotypic tests, as well as mycolic acid analysis by HPLC. Molecular methods including plasmid analysis, restriction endonuclease analysis, and ribosome typing established that isolates from the nurse and the patients all belonged to the same strain. In vitro susceptibility testing showed the isolates to be susceptible to many agents, including amikacin, ceftriaxone, ciprofloxacin, erythromycin, imipenem, and trimethoprim-sulfamethoxazole. Antimicrobials used for patient treatment included ceftriaxone, ciprofloxacin, and trimethoprim-sulfamethoxazole. We are not aware of any subsequent reports of human infection attributed to this species.

Gordonia erythropolis

An isolate identified as this species (reported as *Rhodococcus erythropolis*) was reported from the sputum of one patient with a chest disorder (Osoagbaka 1989), but no further clinical details were provided, and, if any molecular methods were used to identify the isolate, these were not stated. This organism was also reported (again as *R. erythropolis*) as a cause of peritonitis in a patient receiving continuous ambulatory peritoneal dialysis (Brown and Hendler 1989); the patient responded to treatment with vancomycin. The organism was grown from culture of the dialysate and identified at a reference laboratory, but no other information about the organism is provided. In 1991, Vernazza et al. reported the case of an HIV-positive patient with multiple subcutaneous nodules, biopsy of one of which showed granulomatous inflammation with acid-fast rods. A culture grew out an organism identified at a reference laboratory as *R. erythropolis* (now *G. erythropolis*), but the procedures used to identify the isolate were not specified. An isolate of this species along with an isolate of *Rhodococcus luteus* (now *R. fascians*) was also reported from a patient with endophthalmitis (see below under *Rhodococcus*).

Gordonia rubropertincta

An isolate of this species (reported as *Rhodococcus rubropertinctus*) was considered to be the cause of lingular consolidation, accompanied by cough, fever, and weight loss in a young woman with no evident immunocompromise (Hart et al. 1988); the organism was visualized in and grown from pus obtained at bronchoscopy. The isolate was identified at a reference laboratory, and considerable phenotypic information concerning the organism was provided in the report.

Gordonia sputi

This species was initially described (as *Rhodococcus sputi*) on the basis of isolates obtained from human sputum specimens (Tsukamura and Yano 1985). Riegel et al. (1996) reported the case of a patient with metastatic melanoma who had several blood cultures positive for an isolate considered 'related' to this species. The isolate was carefully studied by a variety of techniques including DNA–DNA hybridization. The DNA hybridization results revealed only 60 percent similarity with the type strain of the species, suggesting that the isolate may have belonged to some other species. In 1999, Kuwabara et al. reported the case of a patient who developed mediastinitis attributed to this species after undergoing coronary artery bypass graft surgery. The isolate was identified on the basis of cell wall and phenotypic features, but no molecular studies such as DNA sequencing were performed. Lesens et al. (2000) reported a case of bacteremia and endocarditis considered to catheter-related that occurred in a patient with a hemoglobinapathy and multiple associated problems. This isolate was also extensively studied and reported as a *Gordonia* species; DNA–DNA hybridization studies indicated 55 percent relatedness to the *G. sputi* type strain.

Gordonia terrae

Martin et al. (1991) reported the case of a girl who developed a wrist lesion with accompanying axillary lymph node enlargement. Acid-fast bacilli were seen in a biopsy of the lesion, and an organism identified by the authors as a *Rhodococcus* species was isolated from a 7H9 broth culture of the specimen, but not from any of the other media planted. The isolate was noted by the authors to resemble *R. terrae* (now *G. terrae*) on the basis of its phenotypic features, but to be somewhat atypical. Nonetheless, the case is sometimes cited as one caused by *G. terrae*. Buchman et al. (1992) reported the cases of two patients who were receiving total parenteral nutrition and developed what was considered to be catheter-related sepsis. One isolate was considered related to *G. rubropertincta*, but could not be identified beyond the genus level even with the molecular methods employed. The other isolate was considered to be 'most likely *G. terrae* or "terrae-like"' on the basis of the studies performed, which included dot-blot DNA

hybridization. Drancourt et al. (1994) reported the case of a child with a primary cerebral rhabdoid tumor who developed a brain abscess attributed to this species following his third surgical ablation procedure. The isolate was biochemically somewhat atypical compared to the type strain, but was identified as *G. terrae* on the basis of ribotype analysis; DNA hybridization was not done. Drancourt et al. (1997) reported a case of central nervous system infection attributed to this species in an apparently immunocompetent patient; the organism was isolated from CSF, and radiographic studies indicated the presence of multiple brain abscesses. Sequencing of 1 481 positions of the 16S rRNA gene revealed 100 percent similarity with the sequence of *G. terrae*, but DNA–DNA hybridization was not done. Pham et al. (2003), reported on five patients with underlying malignancies who developed bacteremias which were considered catheter-related and were attributed to this species. Identification to the species level was based on the sequencing results for a 1 066 bp portion of the 16S rRNA gene, compared to the type strain. The percentage identity of the sequences of the five isolates with that of the type strain ranged from 98.5 to 99.9; for three of the isolates, it was 99.8–99.9. No DNA hybridization studies were performed. The patients all responded to treatment with a variety of antibiotics; in two cases, the catheter was removed.

NOCARDIA

General features

The nocardiae produce long, thin (0.5–1.2 μm diameter) hyphae that branch extensively. On Gram stain they generally appear as finely beaded gram-positive rods; the individual 'beads' may not be immediately juxtaposed to one another. When stained in direct clinical material, they are almost always weakly acid-fast positive. On solid media, colonies of most isolates form a fine, powdery aerial mycelium as the colony ages; the reverse is usually pigmented in a shade of yellow, orange, or salmon. When stained from cultures on a solid medium, the organisms easily break up into short rods and cocci, although a few longer, branching forms can often be found. However, the very long and branching forms typically seen in direct smears are rarely found in culture. Also, unless subcultured to lipid-rich media, the cells lose much of their acid-fastness with progressive subcultures. Nocardiae also typically produce an 'earthy' odor. (In fact, the odor attributed to soil is probably largely a consequence of aromatic compounds produced by nocardiae and other aerobic actinomycetes.) Conidia may be produced in culture, particularly on the aerial hyphae (Goodfellow and Lechevalier 1989). Nocardiae are generally recognized as such in the clinical laboratory by the combination of their typical cellular morphology (long, thin, branching rods), staining reaction (beaded on Gram stain, positive on modified acid-fast stain), and colonial morphology (colonies that may take approximately 3–7 days to become clearly visible to the naked eye, developing a whitish, powdery aerial mycelium and a more-or-less orange reverse).

Nocardiae also possess other important attributes, although these are rarely assessed in the clinical laboratory. They are aerobic, catalase-positive, and nonmotile, and have characteristic cell-wall components. In the previous edition of this work, Goodfellow (1998) detailed the chemotaxonomic features of the nocardiae and the other aerobic actinomycetes. Among the important features are *meso*-diaminopimelic acid in the peptidoglycan layer of the cell wall, both arabinose and galactose in the polysaccharide component of the cell wall, and cell wall mycolic acids containing an average of 50 carbon atoms (Alshamaony and Goodfellow 1976). A set of chemotaxonomic properties, including these and some others, has been recommended as the defining characteristics for organisms assigned to the genus *Nocardia* (Goodfellow 1998). At present, however, the decision to assign newly described species to the genus is based largely on degree of relatedness to other species of nocardiae as determined by molecular methods. On the basis of 16S rRNA sequence data, Chun and Goodfellow (1995) concluded that the genus *Nocardia* was most closely related to the genus *Rhodococcus*, and Roth et al. (2003) assessed the phylogeny within the genus, concluding that it contained at least ten taxa that warranted descriptions as new species.

Nocardiae will grow on a wide variety of media incubated in ambient air; buffered charcoal-yeast-extract agar supplemented with antibiotics (as used for the isolation of *Legionella* species) seems particularly useful for isolating nocardiae from specimens such as sputum that contain normal flora (Garrett et al. 1992). Nocardiae are typically found in a fungal culture in the clinical laboratory, and do grow at 30°C; however, particularly the pathogenic isolates will generally grow a little more rapidly at 35°C.

There are currently approximately 31 recognized species in the genus, not including several species for which transfer to another genus has been recommended (Euzeby 1997). From our review of the literature, largely confined to that in the English language, we are aware of data indicating isolation from human sources for the following species: *N. abscessus*, *N. africana*, *N. brasiliensis*, *N. brevicatena*, *N. carnea*, *N. cyriacigeorgica*, *N. farcinica*, *N. nova*, *N. otitidiscaviarium* (formerly known as *N. caviae*), *N. paucivorans*, *N. pseudobrasiliensis*, *N. transvalensis*, and *N. veterana*. Note that *N. asteroides* sensu stricto is not on the list; we are not aware of an isolate of that species having been documented as isolated from any human or other animal specimen. The organisms designated by Wallace et al. (1988) as

N. asteroides drug pattern types II and IV almost certainly belong to as yet unnamed species, and particularly the drug pattern II group probably consists of more than one species (Roth et al. 2003). (See below under *N. asteroides* for further discussion of these issues.) It can be anticipated that additional human pathogenic species will be described in the near future. Indeed, there is already some evidence available that the species *N. brevicatena* and *N. transvalensis* are in fact each species complexes. Other pathogenic species that will not be discussed in detail here include the fish pathogens *N. salmonicida* (Isik et al. 1999), *N. seriolae* (Kudo et al. 1988), the oyster pathogen *N. crassostreae* (Friedman et al. 1998), and the blueberry pathogen *N. vaccinii* (Goodfellow and Lechevalier 1989).

Disease presentations

The majority of infections caused by aerobic actinomycetes are produced by members of the genus *Nocardia*. The vast majority of infections result either from post-traumatic inoculation of the organism, or from inhalation and subsequent establishment of a respiratory focus. Most infections in the immunocompetent host are secondary to trauma and remain localized. Once a focus of infection is established in an immunocompromised host, metastatic foci may develop virtually anywhere. Disease caused by *Nocardia* species is often fairly indolent, even in an immunocompromised patient, and the infection may be widespread before the diagnosis is established. Often an invasive procedure such as a bronchoalveolar lavage must be performed to obtain adequate material containing the organism to establish the diagnosis of a respiratory infection. Diagnosis may be delayed even when an appropriate specimen has been obtained. The organisms may be undetectable or unrecognized in direct smears of clinical material. Cultures may not be held long enough for visible growth of the organism to occur, other contaminating organisms may prevent the detection of *Nocardia* colonies, or the detection of other potential pathogens may prematurely terminate the search for more slowly growing pathogens such as *Nocardia*. The data presently available suggest that no one species of *Nocardia* produces a unique form of infection. Perhaps the most that can be said at present is that post-traumatic infection in tropical regions, particularly mycetomas, if caused by a *Nocardia* species at all, are most frequently due to *Nocardia brasiliensis*. A recent report on a series of *Nocardia* infections in patients with chronic granulomatous disease (Dorman et al. 2002) gives some indication of the wide array of species involved in such infections. When molecular identification methods were applied to the 21 available isolates, the following identifications (with numbers of isolates in parentheses) were obtained: *N. asteroides* drug pattern type VI (now considered to be the same as *N. cyriacigeorgica*) (4), *N. farcinica* (4), *N. nova* (2), *N. otitidiscaviarum* (2), *N. pseudobrasiliensis* (1), *Nocardia* species (not further identifiable at the time of the study) (8). One of the then-unidentifiable isolates has since been found to be *N. veterana* (Conville et al. 2003). It is not surprising that in immunocompromised patients, infections with *Nocardia* species can occur along with other organisms such as fungi, or that some patients may be sequentially infected with different *Nocardia* species (Casale et al. 1984; Gaafar et al. 2001; Dorman et al. 2002). Stamm et al. (1983) reported the case of an immunocompromised patient who developed pulmonary infection attributed to *N. brasiliensis* and subsequently developed disseminated infection attributed to *N. asteroides*; the infections ultimately could not be controlled by sulfonamides and trimethoprim, and the patient succumbed, presumably from his disseminated infection. The isolation of three different *Nocardia* species at essentially the same time from a patient with sarcoidosis being treated with steroids has also been reported (Hidri et al. 2002); in that case, *N. asteroides* and *N. nova* were recovered from respiratory specimens and *N. otitidiscaviarum* was recovered from skin biopsies and urethral pus.

N. abscessus

This relatively recently described species (Yassin et al. 2000b) was named after the source of the initial isolates. It is the same organism as that formerly known as *N. asteroides* drug pattern I. Isolates of this drug pattern type were found to be susceptible to ampicillin, carbenicillin, and broad-spectrum cephalosporins; half the isolates had a high minimum inhibitory concentration (MIC) to imipenem (Wallace et al. 1988). DNA–DNA hybridization was used to demonstrate that this species was distinct from *N. asteroides* (in the strict sense). According to those who initially described the species, it differs phenotypically from the type strain of *N. asteroides* by its inability to hydrolyze esculin, to utilize gluconate as a sole carbon source, and to utilize proline as a sole source of nitrogen and carbon, and by its ability to utilize citrate and maltose as sole carbon sources.

N. africana

The description of this species was based on eight isolates obtained from the sputum of patients with pulmonary disease in Africa (hence the species name) (Hamid et al. 2001). On the basis of 16S rDNA sequencing the organism was found to be most closely related to *N. vaccinii*, to which it showed a maximum similarity of 98.7 percent. Among the phenotypic features of the organism found to be useful in separating it from other members of the genus were its inability to hydrolyze esculin or urea, its inability to use citrate or mannitol as sole sources of carbon, its ability to decompose casein,

and its ability to grow at 45°C. Some in vitro susceptibility test result differences between this and other species of *Nocardia* for some antibiotics were also reported, but the currently recommended NCCLS susceptibility testing procedure was not used. No clinical details pertaining to any of the patients from whom the organisms were obtained were provided. Hattori et al. (2003) subsequently reported isolating this species from a subcutaneous nodule of an 'American shorthaired cat'. The isolation of the organism occurred while the cat was in Japan, but no travel history for the cat was provided. Phenotypic testing of the isolate and of a 'reference strain' of *N. africana* produced identical results for both. The 16S rDNA portion of the isolate that was sequenced showed '99 percent homology' (according to the text of the manuscript, but '100 percent homology' according to the abstract) with the corresponding portion of the gene of the reference strain.

N. asteroides

The confusion regarding the strain designations and the nomenclature of early isolates placed in the '*Nocardia asteroides*' category results at least in part from the fact that the phenotypic properties initially used for the description and identification of aerobic actinomycetes did not allow clear discrimination among the various genera, let alone among the various species, involved (Gordon and Mihm 1962b). Consequently, many species considered distinct according to the modern molecular-based species definition were lumped together under the name '*N. asteroides*.' For a brief account of the early taxonomic history of this species, see below under *N. farcinica* (the name originally proposed for the first type strain of the genus *Nocardia*). It eventually became clear that groups of '*N. asteroides*' isolates with different in vitro susceptibility patterns were all, or nearly all, separate species (Wallace et al. 1988). Of the six drug pattern types distinguished by Wallace et al. (1988) drug pattern type I is now *N. abscessus*, drug pattern type III is *N. nova*, drug pattern type V is *N. farcinica*, and drug pattern type VI is most probably *N. cyriacigeorgica*. The other two drug pattern types (II and IV) have not been formally named as yet; drug pattern type II in particular may contain more than one species (Roth et al. 2003). A further complication is that the type strain of *N. asteroides* (ATCC 19247) is sufficiently different by 16S rDNA sequencing from both drug pattern types II and IV that it may be a different species from both. Also, it appears that the type strain does not fit into any of the drug pattern types described by Wallace et al. (1988) but would be included with the small number of 'miscellaneous' isolates that did not have any of the six patterns delineated (Wallace et al. 1988; Wallace and Steele 1988). Furthermore, it is not clear that any clinical isolates have been obtained that really belong to the same species (strictly defined) as the *N. asteroides* type strain. So even the phrase '*N. asteroides* sensu stricto' is ambiguous; it should refer to organisms belonging to the same species as the type strain, but in practice it essentially never does, because of failure to appreciate the extremely restrictive nature of 'sensu stricto' in this particular case. (Note in this regard the use of '*N. asteroides* sensu stricto' to refer to *N. asteroides* drug pattern type VI in one recent comprehensive reference (Brown and McNeil 2003). Sometimes these species were referred to together as the '*N. asteroides* complex' – but it is rarely clear which species are intended to be included in the 'complex.' More recently it has been found that susceptibility differences alone do not provide adequate discrimination among all the species actually involved, and yet additional species need to be distinguished (such as *N. veterana* from *N. nova*). As a consequence of all this taxonomic confusion, it is impossible to know to precisely which species most clinical reports – even quite recent ones – pertain, unless at least thorough molecular testing (such as RFLP analysis of more than one gene region and 16S rDNA sequencing) was performed. At present it seems to us an almost insurmountable problem to identify isolates in this group accurately to the species level by phenotypic testing. Even the most recent publications comparing the phenotypic features of these and other *Nocardia* species do not, for example, include *N. asteroides* drug pattern type II or the type strain of *N. asteroides* (which as indicated above may actually represent a species not yet reported to be a pathogen) (Brown and McNeil 2003; Kiska et al. 2002).

As the majority of *Nocardia* infections reported from nontropical regions have been attributed to this species, we will briefly review such infections here.

Infections attributed to this species have occurred in patients with a wide variety of immunocompromising conditions, including patients with cancer, AIDS, solid organ transplants, and chronic granulomatous disease. Infections in these patients most commonly begin with a pulmonary focus, and may subsequently disseminate to virtually any organ in the body, but one of the commonest sites of dissemination is the central nervous system. Occasionally infection is initially detected at a nonpulmonary site, with the (presumably initial) pulmonary focus only detected during the ensuing work-up.

Chouciño et al. (1996) presented six patients from their institution who developed *Nocardia* infection after bone marrow transplantation, and reviewed the cases of four other patients previously described in the literature. Eight of the patients had had leukemia, one had had non-Hodgkin's lymphoma, and one had had aplastic anemia. Six of the isolates were identified as *N. asteroides*, three as *N. nova*, and one as *N. otitidiscaviarum*. Four patients had soil or dust exposure prior to developing their infections. Four patients had a fungal infection diagnosed at the same time as, or shortly after, the

diagnosis of a *Nocardia* infection was made. Torres et al. (2002) reviewed episodes of nocardiosis occurring at their cancer center between 1988 and 2001. Of 39 isolates identified to the species level, 30 were called *N. asteroides* complex, seven, *N. brasiliensis*, and two, *N. otitidiscaviarum*. Forty-two infected patients were found in their retrospective review, giving an incidence of 60 cases per 100 000 admissions. Over half the patients (27, or 64 percent) had hematologic malignancies. Infection occurred after bone marrow transplantation in 13 patients. In the 30 days before the infections began, steroids were administered prior to 58 percent of the episodes, and chemotherapeutic agents, prior to 23 percent of the episodes. The infections were pulmonary in 70 percent, soft tissue in 16 percent, catheter-related in 7 percent, and disseminated (with lung involvement) in 2 percent. For the 15 patients for whom data regarding infection outcome were available, the infections were thought to have been contributory to the deaths of nine. *Nocardia* infections occurring from 1974 through 1986 at the same institution in patients with a variety of malignancies (and in one patient with no underlying disease) had been reviewed earlier by Berkey and Bodey (1989). They found 14 cases of infection; all were attributed to *N. asteroides*, except for one cutaneous infection attributed to *N. brasiliensis*. Ten patients had pulmonary involvement. Two patients with normal chest films had positive sputum cultures for *N. asteroides*; the organism was considered a colonizer, not a pathogen, in both.

Krick et al. (1975) discussed seven cases of pulmonary *N. asteroides* infection in heart transplant patients, six of which were diagnosed pre-mortem and five of which were confined to the lungs. In two patients the infection disseminated to the skin. All infected patients had been treated with prednisone and azathioprine. Percutaneous needle aspiration was done in all six patients who were diagnosed during life; diagnosis was established by the initial aspirate in four of the patients. The organism was eventually recovered from sputum cultures from three of the patients. Diagnosis and management in one patient were complicated by the prior and concurrent presence of *Aspergillus* pulmonary infection. One patient with pulmonary, calf, and shoulder lesions had a prolonged course and required several surgical interventions, including a mid-thigh amputation because of progressive leg infection. This patient eventually resolved his infection after a prolonged course of sulfisoxazole, along with multiple other antibiotics administered at various times during his infection.

There have been numerous reports of infection in renal transplant recipients. Houang et al. (1980) and Lovett et al. (1981) discussed an outbreak involving seven patients on a transplant unit in a London hospital. The index patient was eventually found to have had *N. asteroides* present in nephrostomy urine for some time, and the authors speculate that the manner in which her urine was disposed of in the unit may have resulted in environmental contamination, with 13 isolates being obtained from environmental samples. Five of the six subsequently infected patients had pulmonary involvement. The authors suggested that the outbreak was due to 'patient-to-patient transmission', but as the authors essentially indicate, the transmission more probably occurred through the intermediation of a contaminated environment. Isolate identity among the patients was presumed on the basis of phenotypic similarities; at that time molecular confirmatory testing had not been developed. Formaldehyde fumigation of the unit apparently resulted in termination of the outbreak. Wilson et al. (1989) discussed six cases of *N. asteroides* infection that had occurred between 1975 and 1987 in a total of 251 transplant patients; all involved a pulmonary focus and one also involved the skin. They also reviewed the literature regarding *Nocardia* infections in renal transplant patients up to that time; they found 88 additional patients for which sufficient data were available to be included in their review. Of these 88 patients, 88 percent had pulmonary involvement. Of the 81 cases for which organism identification was reported 78 were identified as *N. asteroides*, two as *N. brasiliensis*, and one as *N. caviae*. The most frequent symptoms were fever and cough. The most frequent radiographic chest abnormality was a nodular infiltrate, with some patients showing cavitation; a number of other radiographic abnormalities, including alveolar infiltrates and pleural effusions, were seen. Of the patients treated with a sulfa drug, 82 percent survived. Arduino et al. (1993) discussed nine cases of nocardiosis that had occurred in 1 255 renal transplant patients between 1980 and 1992, for an incidence of 0.7 percent. These patients had received a combination of cyclosporine and prednisone for immunosuppression, and had a lower incidence of *Nocardia* infection than an earlier group of 154 patients who had received azathioprine and prednisone for immunosuppression. In the earlier group there were four episodes of *Nocardia* infection, for an incidence of 2.6 percent. Of their nine patients, eight had pulmonary involvement, with one each of these also having brain and skin involvement. One patient had only skin involvement. Seven of the isolates were identified as *N. asteroides* and two as *N. brasiliensis*. Nearly all patients required a relatively invasive procedure such as a bronchoalveolar lavage or a biopsy to obtain diagnostic material. Eight patients were cured of their infections; trimethoprim-sulfamethoxazole and amoxicillin-clavulanic acid were the principal antimicrobials used. Unusual sites of infection in transplant patients in whom no pulmonary involvement was detected include a case of sphenoidal sinusitis attributed to *N. asteroides* in a renal transplant patient (Roberts et al. 1995), and a case of thyroid infection attributed to *N. asteroides* in a patient who had undergone a combined renal and liver transplant (Carriere et al. 1999).

Forbes et al. (1990) discussed seven liver transplant patients who developed *Nocardia* infection during a $3\frac{1}{2}$-year period during which 191 transplants had been performed. Four patients had pulmonary infection only; two had cerebral involvement, presented with grand mal seizures, and also had skin involvement, and one had an axillary mass and was subsequently found to have both pulmonary and cerebral involvement. One of the two patients who presented with seizures was also found to have pulmonary involvement at autopsy. Diagnostic material was obtained by bronchoscopy, fine needle aspiration of a pulmonary lesion, and aspiration of cutaneous lesions. A positive blood culture was obtained from one patient with disseminated disease. A diagnosis was made by direct examination of clinical material in several of the patients. The isolates from all the patients were identified as *Nocardia asteroides*. The patients were treated with a variety of antibiotics; three of the patients died, and in two of these, their *Nocardia* infections were considered to have played a major role in their demise. Susceptibility testing was done, although not using the procedure now recommended. The authors specifically note that one of the patients responded to trimethoprim-sulfamethoxazole despite an in vitro susceptibility result to the drug combination that suggested 'reduced' efficacy. Lopez et al. (1994) presented a case of testicular *Nocardia* infection in a liver transplant patient in whom no other focus of infection was found; they also reviewed four other reported cases of testicular involvement, all of which had occurred in immunocompromised patients, and in three of whom pulmonary involvement was also detected.

Hasain et al. (2002) reported on ten lung transplant patients who developed *Nocardia* infection over an approximately 10-year period at their institution, during which time 473 lung transplants had been performed. Isolates from three patients each were identified as *N. asteroides* complex, *N. farcinica*, and *N. nova*; the isolate from the remaining patient was identified as *N. brasiliensis*. There was pulmonary involvement in all ten patients; one patient also had brain and bone involvement. All three patients with *N. farcinica* infections died. In three of the four patients who died, death was attributed to their *Nocardia* infections.

Uttamchandani et al. (1994) discussed 30 patients with advanced HIV infection who were diagnosed with a *Nocardia* infection at their institution over a $4\frac{1}{2}$-year period; they also briefly mention several other case reports and series of such infections in HIV-infected patients published prior to their report. In 21 patients, infection was evidently confined to the lungs, and one patient was found to have involvement of lungs, skin, and CNS. In three patients, infection was first detected in a skin or soft tissue site; in five patients, it was first found in a variety of other sites. It is not entirely clear whether any of these eight patients with an initial nonpulmonary presentation had any subsequent evidence of pulmonary involvement. The authors note a significant association of intravenous drug abuse in the patients with a *Nocardia* infection; 53 percent were IV drug abusers, compared with an overall incidence of 30 percent in the 1 655 AIDS patients seen at their institution over the same time period. Isolates from all 30 patients were identified as *N. asteroides*. Of the patients with a pulmonary focus, 14 had radiographic evidence of upper lobe involvement, and 14 had an alveolar infiltrative pattern. Two patients had a reticulonodular pattern, and two, a mixed pattern. Cavitary lesions were eventually seen in 18 patients. Three patients had pleural effusions, and two of these required thoracostomy for empyema drainage. In most cases of pulmonary disease the diagnosis was made on a bronchoscopically obtained specimen. Of the 27 patients receiving antibiotics, 22 improved, but 14 of these relapsed in less than six weeks after antibiotics were stopped. Of the 25 patients who died, 12 were thought to have died from their nocardial infection, and the infection was thought to have been a contributing factor in the deaths of seven others. In 1994 Ray et al. described an HIV-positive, IV drug abuser who presented with septic knee arthritis and had concomitant pulmonary lesions; *N. asteroides* was isolated from cultures of both synovial fluid and sputum. Arabi et al. (1996) described an AIDS patient who presented with fever, nausea, and epigastric pain who had no discernible pulmonary involvement but did have widespread abscesses and positive blood cultures; in addition to being obtained from blood cultures, the organism, identified as *N. asteroides*, was also obtained from an aspirate of one of his markedly enlarged adrenal glands. This patient was initially treated with doxycycline because of a reported sulfonamide allergy; he initially improved, but subsequently relapsed, and ultimately responded well to trimethoprim-sulfamethoxazole.

Infection attributed to *N. asteroides* has also been reported in patients with systemic lupus erythematosus (SLE) (for example, by Gorevic et al. (1980), who reported three infections in men and noted that they had not seen any *Nocardia* infections in their female patients with SLE, despite that fact that they followed nearly seven-fold more women than men), in patients with chronic granulomatous disease (Dorman et al. 2002) (and see above under Disease presentations), in patients receiving steroids for rheumatoid arthritis (Narushima et al. 2002) and sarcoidosis (Jastrzembski et al. 2002), and very rarely in patients who are pregnant (Braun et al. 1991).

While the role of *Nocardia* species in brain abscesses is well known, *Nocardia* infection can cause, and, rarely, present as a subacute or chronic meningitis. In a review of 28 patients with meningitis caused by *Nocardia* species, Bross and Gordon (1991) reported that one was attributed to *N. caviae*, two to *N. brasiliensis*, and the

rest to *N. asteroides*; in 21 of these cases the organism was grown from a CSF culture, and in the remaining eight, from culture of some other site. Some predisposing condition was noted in 75 percent of the cases. A number of cases of endophthalmitis caused by this organism have also been reported (Knouse and Lorber 1990; Ng et al. 2002); virtually all have appeared to have resulted from hematogenous metastasis from another focus, usually pulmonary, and nearly all have resulted in blindness of the affected eye.

Nocardia species may occasionally be isolated from patients with some underlying pulmonary disease. For example, Lumb et al. (2002) reported isolating *N. asteroides* from three patients with cystic fibrosis. These authors noted that in these three patients, despite multiple isolations of the organism, it seemed more likely to be a colonizer than a pathogen.

In some of the reported cases of infection attributed to *N. asteroides*, the infection may have resulted from traumatic introduction of the organism. There have been a few reports of catheter-related bacteremia attributed to this organism in which no other tissue focus that could have served as the initial focus of infection was detected. Kontoyiannis et al. (2000) described two patients, one with Hodgkins' disease and the other with metastatic breast cancer, both of whom had positive blood and catheter-tip cultures. Lui et al. (2001) described a patient with thalassemia and diabetes who developed bacteremia; the bacteremia in this patient was cleared without removing the catheter. Torres et al. (2002) reported that in five of their 42 patients with cancer who developed *Nocardia* infections, the infections were catheter-related. Vanderstigel et al. (1986) reported the case of an IV heroin abuser who developed pulmonary infection but was apparently otherwise healthy. Although neither the heroin nor the injection paraphernalia used were available for culture, a history was obtained that the patient had hidden his syringes and needles in a flower-bed, and the authors speculated that his injection materials may have become contaminated with *N. asteroides* at that time.

Endocarditis caused by *Nocardia* species apparently may result either from traumatic introduction of the organism at surgery (presumably the most common cause), but may also result from intravascular dissemination from another traumatic focus, or from another established site of infection. Dhawan et al. (1998) described a patient who developed native mitral valve infection which was thought on the basis of its histopathological features to be most likely a *Nocardia* species, although no etiologic organism was cultured from any site in the patient. The patient had a history of recurrent skin infections, a Gram smear of the drainage from one of which revealed *Nocardia*-like organisms, but the organism was not isolated in culture; possibly one of these lesions was the source of her mitral valve involvement. These authors also reviewed six other cases of endocarditis in patients with prosthetic valves, five of which were attributed to *N. asteroides*, and one, to *N. farcinica*. Four of these six patients died. Watson and Wilson (2001) reported the case of an IV drug abuser who developed native aortic valve endocarditis attributed to *N. asteroides*.

N. asteroides has been associated with many other types of infection as well. Peritonitis associated with continuous ambulatory peritoneal dialysis has been reported (Arfania et al. 1981). A case of peritonsillar abscess attributed to *N. asteroides* was reported by Adair et al. (1987) in a patient with no known underlying disease and no evidence of pulmonary infection; however, the patient had a 5-year history of recurrent pharyngitis of unknown etiology. The authors hypothesized that the infection may have resulted from iatrogenic introduction of the organism during a tonsillar incision. Tsuboi et al. (1986) reported a patient with lymphocutaneous lesions (an ulcer and nodules on the right hand and forearm) mimicking sporotrichosis; cultures grew out organisms identified as *N. asteroides*. The patient, a fisheries student, had no history of trauma to the affected area; lesions had begun on the tip of his right index finger. The patient had no evidence of other disease at the time of presentation, but he did have a history of having had a lung abscess several years previously. Dinulos et al. (1999) presented the case of a previously healthy young girl who developed septic arthritis of her right knee; symptoms began some 6 weeks following puncture of the skin of the knee by a claw of a rooster that had attacked her. Multiple abscesses developed in the structures surrounding the knee; three drainage procedures and almost 5 months of antibiotic treatment were needed to eradicate the infection. Astudillo et al. (2001), reported a patient who developed a nodule on her arm at the site of a cat scratch 5 months previously; culture of a biopsy of the lesion grew out an organism reported to be this species, although no information was provided on how or where the identification was made.

There are a few reports of keratitis and corneal ulceration caused by *Nocardia* species; most are secondary to trauma. Tendolkar et al. (1998) presented a patient with a corneal ulcer who also had leprosy; there was no history of trauma, but the patient had no sensation in the affected eye, possibly secondary to her leprosy, and so would likely not have noticed any trauma that occurred. These authors also briefly reviewed the other cases of corneal ulceration attributed to *Nocardia* species that they found in the published literature. Also in 1998, Sridhar et al. retrospectively reviewed 16 cases of keratitis caused by *Nocardia* species (out of 908 culture-positive keratitis cases) that had been seen at their institution between early 1991 and the end of 1996. Four patients had a history of trauma. *N. asteroides* was identified in 15 patients, and *N. caviae* (now *N. otitidiscaviarum*) in one. A variety of

topical antibiotics were used as treatment. In 11 patients, there was healing with some residual corneal scarring, in one there was a persisting epithelial defect, one patient with concomitant *Streptococcus pneumoniae* infection required enucleation, and the other three were lost to follow up.

N. brasiliensis

Along with their detailed account of the infections attributed to this species in seven patients from their institution, and their review of 55 additional cases reported from the USA up to the time of preparation of their review, Smego and Gallis (1984) include a brief taxonomic history of the species. The organism that eventually became known as *N. brasiliensis* was first isolated in 1909 by Lindenberg from a Brazilian man with a mycetoma. It is relatively easy to differentiate from all other human pathogenic *Nocardia* species except *N. pseudobrasiliensis* because of its capacity to break down casein, hypoxanthine, and tyrosine, but not xanthine (Brown and McNeil 2003). Wallace et al. (1995) reported that within their collection of isolates that had all been considered to be *N. brasiliensis* there appeared to be another species, most isolates of which could be distinguished by several phenotypic features including decomposition of adenine and susceptibility to ciprofloxacin. Approximately 60 percent of their invasive isolates that had been considered to be *N. brasiliensis* appeared to belong to this new species; only about 3 percent of the *N. brasiliensis* isolates involved in localized cutaneous isolates appeared to be members of the new species. In the additional studies carried out for the subsequent description of this new species by Ruimy et al. (1996), as *N. pseudobrasiliensis*, molecular methods including DNA–DNA hybridization established that *N. pseudobrasiliensis* was indeed a species separate from *N. brasiliensis*. Given that *N. pseudobrasiliensis* was only recently recognized as a separate species, it is impossible to know how many infections attributed to *N. brasiliensis* reported in the older literature were in fact caused by *N. pseudobrasiliensis*. It seems particularly likely that *N. pseudobrasiliensis* was actually the etiologic agent in many of the cases of noncutaneous disease that had been attributed to *N. brasiliensis*. In their analysis of *N. brasiliensis* infections in 62 patients, Smego and Gallis (1984) noted a number of differences between such infections and those attributed to *N. asteroides*. *N. brasiliensis* caused disease primarily in immunocompetent patients (only 28 percent if the patients for whom adequate data were known to be available were immunocompromised) and infection often resulted from trauma. In one of the cases reviewed by Smego and Gallis the trauma involved was reportedly a cat scratch, and there have also been subsequent reports of lymphocutaneous infection following a cat scratch (Sachs 1992; Bottei et al. 1994). A case occurring at the site of an insect bite has also been reported (Paredes et al. 1988). As with disseminated infections attributed to *N. asteroides*, disseminated infections attributed to *N. brasiliensis* can involve virtually any body site. Koll et al. (1992) reported a case involving infection of the left knee, and briefly reviewed 13 additional reported cases of disseminated disease; among the sites involved were brain, liver, lung, and skin.

N. brasiliensis and other aerobic actinomycetes as causes of mycetoma

N. brasiliensis is probably the most common cause of actinomycotic mycetoma (see above under Actinomadura) in the western hemisphere, although, as most cases occur in relatively impoverished tropical regions, accurate figures are not available, and probably few if any isolates have been examined by modern molecular identification methods to assure that they belong to *N. brasiliensis* sensu stricto. The importance of trauma in relation to the development of infection was appreciated before the differences in the etiologic agents were understood (Pijper and Pullinger 1927). Indeed, the condition has been recognized for centuries. Khatri et al. (2002) cite a reference indicating that the first written reference to mycetoma occurs in 'the ancient Indian religious book, *Atharva Veda*, where it is described as ''Padavalmika'' (foot ant hill).' The condition, as it affects the foot, is often called 'Madura foot' the name it was evidently given by Gill in 1842, with its first recognition as a clinical entity occurring in Manurai, India, in 1842. Gonzáles Ochoa (1962) reported that *N. brasiliensis* was the etiologic agent of 94 percent of Mexican cases of mycetoma, while the organisms then called *N. asteroides* were responsible for less than 1 percent. He also noted that the prevalence of foot and back involvement was the consequence of the frequency of walking barefoot and of carrying contaminated fiber sacks on the back. Some 30 years later Castro et al. (1993), also noted the preponderance of *N. brasiliensis* among their cases of mycetoma from Sao Paulo, Brazil, with this species the etiologic agent in 13 of the 16 cases from which an actinomycete could be identified to the species level, and *N. asteroides* the agent in only one. In their report on mycetomas in Mali, Mahe et al. (1996) found that, of 30 actinomycotic mycetomas, 15 were caused by *Actinomadura pelletieri*, 12 by *Actinomadura madurae*, and three by *Streptomyces somaliensis*; identification of organisms was based on histopathologic examination of biopsies. In their report including 13 patients with actinomycotic mycetoma from Yemen, Khatri et al. (2002) attributed eight cases to *S. somaliensis*, one to *S. madurae*, and four to *Nocardia* species. In this study, identification was based on histopathological appearance of the grains. In their review of 264 cases of mycetoma seen at the Calcutta School of Tropical Medicine between 1981 and 2000, Maiti et al. (2002) report that 197 were actinomy-

cotic; of these, 75 were attributed to *N. brasiliensis*, 38 to *N. otitidiscaviarum*, 52 to *N. asteroides*, 9 to *Streptomyces* spp., and 23 to *Actinomadura* spp. At least some of their identifications were based on determining a few phenotypic properties of isolated organisms. There is a marked preponderance of mycetoma in males, presumably reflecting a greater likelihood of trauma to males than to females in settings such as agricultural work where exposure to actinomycetes may more likely occur. The importance of plant-related trauma in the etiology of mycetoma is highlighted by the report by Chávez et al. (2002) of 20 cases of perianal actinomycetoma. Of the 18 isolated and identified agents, 17 were *N. brasiliensis* and one was *A. madurae*; *Nocardia*-like granules were seen in the other two cases. Seventeen patients were male, and 18 were farmers. Of the 18 patients from rural areas, 14 reported that 'they defecated outdoors and sporadically wiped their rectums with different objects, such as herbs, leaves, grainless corn cobs, branches, etc.' – during the course of which they presumably sustained sufficient trauma to inoculate the etiologic agents. With regard to the case of a student who had always lived in Mexico City, the authors comment that 'the patient reported being homosexual and had the habit of introducing various vegetables into the anus, such as carrots, cucumbers, and radishes; we think that this is a possible explanation for the disease, as all of these vegetables are tubercles that grow underground and therefore must be rich in actinomycetes and fungi'. The patients with infections attributed to *Nocardia* species were treated with the combination of diaminodiphenylsulfone and trimethoprim-sulfamethoxazole, generally until clinical cure was achieved. The authors comment that treatment was more protracted than generally required for mycetomas involving the extremities, the average treatment period being 2.2 years, with a range of 6 months to 4.5 years.

N. brevicatena

Brown et al. (1997) recommended inclusion of three groups of *Nocardia* isolates into the '*Nocardia brevicatena* complex,' based on their similar pattern of aminoglycoside susceptibility, all the isolates being susceptible to amikacin, kanamycin, and tobramycin, and most being resistant to gentamicin. These groups included three ATCC 'reference strains' of *N. brevicatena*, 19 clinical isolates of '*N. asteroides* complex antibiogram type II' (see above under *N. asteroides* for a discussion of the drug pattern types), and ten lysozyme susceptible *Nocardia* isolates (presumably all from clinical sources) from Australia. By certain PCR-RFLP procedures applied to a region of the heat shock protein gene, this set of organisms could be distinguished from all other *Nocardia* species studied up to that time. Other PCR-RFLP procedures applied to the same gene region allowed discrimination among each of the three groups; therefore, it seems likely to us that further molecular study of this group will reveal that several different species are involved.

N. carnea

The organism now known as this species was initially described in 1891 by Rossi-Doria. The type strain has since been lost, and a neotype strain was selected by Gordon et al. (1978), in their study of several isolates which were thought to belong to this species. Of those isolates, a few had evidently been obtained from human clinical material. We are not aware of any recent reports of clinical isolates attributed to this species, nor of any molecular studies of the similarity of this to other currently accepted species within the genus.

N. corynebacterioides

This species was originally described as *Corynebacterium rubrum* by Crowle, and subsequently transferred to the genus *Nocardia* on the basis of its physiological, chemical, and ultrastructural features by Serrano et al. (1972). The specific epithet was changed to corynebacterioides because at that time '*Nocardia rubra*' was the valid name of another species. Serrano et al. (1972) describe the growth on solid media as 'orange–red pigmented colonies with a granular central region … surrounded by a smooth and shiny peripheral zone;' no mention is made of an aerial mycelium. In his taxonomic comments on the genus *Rhodococcus*, Goodfellow (1989) stated that *N. corynebacterioides* was a synonym of *Rhodococcus globerulus*, but *N. corynebacterioides* evidently remains the accepted name for this organism (Euzéby 1997). *Rhodococcus globerulis* has been reported at least once as a clinical isolate (see below under *Rhodococcus*). The appropriate generic placement, species epithet, and clinical significance of this species remain ambiguous.

N. cyriacigeorgica

This species was recently described by Yassin et al. (2001) as *N. cyriacigeorgici*; the name was corrected to its current spelling on notification (IJSEM 2001). It is presumed on the basis of 16S rDNA sequencing to be the same as the organisms grouped by Wallace et al. (1988), as *N. asteroides* drug pattern type VI (Roth et al. 2003; Conville et al. 2003). The type strain (the isolate to which the species name was first applied) was obtained from the 'bronchial secretions' of a patient with chronic bronchitis. The name refers 'to the origin of the name of the German town Gelsenkirchen where the type strain was isolated.' In a report in 2003 of which we have seen only the English abstract, Fux et al. (2003) stated that

theirs was the 'first report of invasive human infection' caused by this species; in fact, such evidence as is available thus far indicates that this species, although only recently formally named, is the most common human pathogen of all the species once lumped together as '*Nocardia asteroides*'. See the discussion above under *N. asteroides*.

N. farcinica

Ertl et al. (1987) reported a case of endocarditis attributed to this species that developed shortly after the patient's calcified aortic valve had been replaced with a prosthesis. No evidence of a pulmonary focus was detected, and the authors hypothesized that the organism may have been introduced intraoperatively, or that the operation may have reactivated a preoperatively acquired infection. These authors published their report before the work of Wallace et al. (1990), discussed immediately below, had been published. They do not report any of the specific phenotypic features of their isolate, but refer to another publication on classification of the aerobic actinomycetes wherein the procedures and tests for making the identification were presumably contained.

Wallace et al. (1990) reported that a significant percentage of isolates that they had studied, which at that time were considered to belong to the species *N. asteroides*, had the same antibiotic susceptibility testing profile, and the same results in a selected set of phenotypic tests, as the type strain and several reference strains of *N. farcinica*. This susceptibility test result profile was that of *N. asteroides* drug pattern type V (see above under *N. asteroides*). The group of isolates was noteworthy because of their significant degree of antibiotic resistance as compared with other isolates then contained in the species *N. asteroides*, and because of the role of many of the isolates in disseminated disease. These authors presented an illuminating account of the history pertaining to the designations of the species *N. asteroides* and *N. farcinica*. Briefly, the isolate obtained by Edmond Nocard in 1888 and thought to be the etiologic agent of bovine farcy was initially named *N. farcinica* by Trevisan in 1889, the genus name honoring the discoverer of the organism and the species epithet indicating the disease with which the organism was associated. However, the cultures of that isolate contained in two different collections were subsequently demonstrated to be very different, one a mycobacterium and the other a *Nocardia* species. When the *Nocardia* isolate was compared to isolates considered to be *N. asteroides* by Gordon and Mihm (1962b), it was considered indistinguishable from the *N. asteroides* strains. The recommendation of these authors that a new type species for the genus, with the name *N. asteroides*, be chosen was accepted in 1980. However, as work by other investigators (prior to that of Wallace et al. (1990)) had demonstrated that there were differences between the original *Nocardia* strain that had been designated *N. farcinica* on the one hand (along with some other strains as well), as compared with other *N. asteroides* isolates on the other hand, the International Judicial Committee allowed continued use of the name *N. farcinica*, although the nature of the entity to which the name referred was uncertain. Goodfellow and Lechevalier (1989) also discussed some of these problems, noting that the 'Gordon tests' could not distinguish between *N. asteroides* and *N. farcinica* but that the 'Goodfellow tests' could. Wallace et al. (1990) suggested the following set of phenotypic features for discriminating what they thought should be considered the separate species *N. farcinica* from the species *N. asteroides*: (1) Growth at 45°C within 3 days; (2) utilization of acetamide as a nitrogen and carbon source; (3) acid production from rhamnose; (4) resistance to cefamandole and tobramycin. At least 80 percent of the clinical isolates that they considered to belong to the species *N. farcinica* were positive for each of these features considered separately. In addition, all their *N. farcinica* isolates were susceptible to amikacin and sulfamethoxazole. Wallace et al. (1990) also referred to several earlier reports of infections attributed to *N. farcinica*. Subsequent molecular work has confirmed that *N. farcinica* is indeed a different species from *N. asteroides* and the other recently named species that have been separated out from the '*N. asteroides* complex' (Roth et al. 2003).

Miksits et al. (1991) reported a case of CNS infection in a renal transplant recipient attributed to this species. These authors were apparently unaware of the 1990 publication by Wallace et al. but they based their identification on criteria proposed by other investigators. They refer to publications by Schaal (1977) and Schaal et al. (1986), the latter of which we were unable to obtain, which described at least some of the phenotypic features by which *N. farcinica* could be distinguished from *N. asteroides* that were subsequently also reported by Wallace et al. (1990).

Schiff et al. (1993) reported the case of a previously healthy man who developed an infection attributed to this species after being accidentally hit in the face with a shovel. It took several months for symptoms to develop; when he presented some 6 months after the injury, he was found to have an abscess and osteomyelitis of the zygomatic arch. His infection resolved following surgical debridement and long-term treatment with amikacin. These authors also found and reviewed 13 other cases of infection attributed to this organism reported since 1966 (but not including the case reported by Miksits et al. 1991). Of the 14 cases considered by Schiff et al. (1993) 50 percent involved the lung, 29 percent, the brain, and 21 percent, skin and nearby tissues.

Torres et al. (2000) reported a case of disseminated infection attributed to this species in an elderly man

with lymphoma; the organism was isolated from a blood culture and from several urine cultures. These authors also reviewed 52 other cases reported in the literature for which there was adequate clinical information and from which the organism had been identified from a clinical specimen. They noted that the lung was the most commonly infected site (43 percent of the cases), with CNS infection occurring in one-third of the cases. Assuming that CNS infection always represents dissemination, 45 percent of the cases involved a disseminated infection. Included in their review were 14 cases of post-operative wound infection that occurred in two different hospitals; the source of the organism reportedly was dust from construction work that contaminated operating room air.

Some additional cases of interest include an elderly, apparently immunocompetent patient with no pulmonary findings who had an intracerebral abscess that was successfully treated with enucleation and both amikacin and imipenem, followed by oral minocycline (Krone et al. 1989); a patient with severe keratitis who presumably acquired the infection through cleaning her rigid contact lenses in nonchlorinated, presumptively contaminated rainwater stored in a basin (Eggink et al. 1997) (also included in the review by Torres et al. 2000); an AIDS patient with disseminated infection from whom the organism was cultured from both blood and CSF (Minamota and Sordillo 1998); and a case of laryngeal abscess and unilateral vocal cord paralysis in an immunocompromised man in whom no other focus of infection was identified (Cohen et al. 2000).

Wenger et al. (1998) reported the results of their investigation of an outbreak of sternotomy site infections which occurred in one hospital following open-heart surgery. Five patients developed such infections. A case–control study identified two risk factors, diabetes (four of the five patients), and exposure to one particular anesthesiologist (four of the five patients). Although the anesthesiologist had not participated in the surgical procedure performed on one of the infected patients, that anesthesiologist had participated in two surgical procedures on the day preceding the procedure on the patient who subsequently became infected. For strain identification both RFLP of chromosomal DNA and ribotyping were performed. Seven hand cultures from the anesthesiologist, as well as perineum and scalp cultures, were positive for *N. farcinica*. Of the ten colonies obtained from these hand cultures, RFLP analysis revealed seven distinct patterns. One of these patterns was identical to the pattern obtained from the isolates from four of the patients; another was identical to the pattern obtained from the patient with a pattern distinct from that of the other four patient isolates. No *Nocardia* isolates were obtained from body site cultures from 27 other hospital personnel. One of the infecting strains of *N. farcinica*, as well as several others, was also recovered from the anesthesiologist's house. Symptoms did not develop in the patients until 21–45 days post-operatively. All infections were serious, requiring some degree of debridement; three patients required sternum resection, and one required anterior mediastinal debridement. The infections in all patients ultimately resolved, but required long-term antimicrobial treatment. After the anesthesiologist began a program of vigorous hand washing, and some other barrier precautions were introduced as well, *N. farcinica* could no longer be cultured from his hands, and no additional infections developed.

N. nova

In a numerical analysis involving 107 characters reported in 1982, Tsukamura found that isolates considered to belong to the species *N. asteroides* could be divided into two subgroups; one of these he described as *N. nova* (nova meaning a new species). Seven characters were useful for distinguishing the isolates of *N. nova* from the isolates considered to be *N. asteroides*; these included 14-day arylsulfatase (positive in 71 percent of 14 *N. nova* isolates and negative in all of nine *N. asteroides* isolates), and utilization of citrate as a sole carbon source (negative for all *N. nova* isolates and positive 89 percent of *N. asteroides* isolates). Two of the 13 *N. nova* strains studied by Tsukamura (1982) were noted to have been isolated from the sputum of patients with pulmonary disease.

Wallace et al. (1991) presented their findings on the clinical significance of the organism and the characteristics by which it could be identified in the diagnostic laboratory. Of 223 isolates previously identified as *N. asteroides*, 17 percent were found to belong to this species. In all cases for which adequate data could be obtained, the isolate was considered to be clinically significant. Some 35 percent of the patients from whom an isolate was obtained were thought to have disseminated disease. Common single sites of involvement included skin, lungs, joints, and cornea. Infected patients had a spectrum of predisposing conditions similar to those noted for other *Nocardia* infections. Tests most useful in the diagnostic laboratory for identifying the species were 14-day arylsulfatase (positive in 75 percent of the isolates) and susceptibility to erythromycin (100 percent of the isolates). This species has the drug pattern type III susceptibility result profile previously described by Wallace et al. (1988) (see above under *N. asteroides*).

Monteforte and Wood (1993) described a cardiac transplant patient who developed pulmonary infection caused by both *N. nova* and *Aspergillus fumigatus*. Miron et al. (1994) described a child with acute lymphocytic leukemia who had a central venous catheter in place and developed pulmonary disease attributed to this species that was thought to be secondary to prior catheter-related infection. The child sustained some splinter injuries to his left palm while playing outside;

mild inflammation developed at one site but resolved with only local care. He presented one week later because of fever and diarrhea. Two blood cultures obtained through the left subclavian catheter were positive for what proved to be *N. nova*; peripheral blood cultures were negative and pulmonary examination was also negative. The patient was treated for one week with intravenous ampicillin, followed by four weeks of intravenous ceftriaxone and did well. The catheter was left in place. A few months later the child was found to have several nodular densities on a CT scan of the chest. Needle aspiration of lesions revealed no organisms, but cultures through the catheter were again positive for *N. nova*, while peripheral blood cultures were again negative. The catheter was removed, and the organism was also recovered from the catheter tip. The patient responded well to antibiotic treatment. The authors speculated that a local infection at the trauma site was followed by hematogenous dissemination to the catheter, with subsequent dissemination to the lungs from the catheter.

See above under *N. asteroides* for some additional reports of infections.

N. otitidiscaviarum

This species was originally described by Snijders; the name was chosen because the initial isolate was obtained from a guinea pig (genus *Cavia*) with ear inflammation (otitis) (Goodfellow and Lechevalier 1989; Clark et al. 1995). This is the same species as that known for a number of years as *N. caviae*. This species can usually be distinguished from other species in the genus by its ability to decompose xanthine (Brown and McNeil 2003).

This species has only rarely been reported as a human pathogen. Lee et al. (1994) reported a case of catheter-related sepsis attributed to this species in a bone marrow transplant patient with thalassemia; several blood cultures from one of the catheter lumens were positive, but not peripheral blood cultures or cultures from the other lumen. The organism was resistant to trimethoprim-sulfamethoxazole but susceptible to imipenem by in vitro testing; treatment with imipenem appeared to cure the infection. Clark et al. (1995) reported a previously healthy elderly man who developed an infection attributed to this species following a forehead laceration sustained in a motor vehicle accident. These authors briefly reviewed 27 other cases they were able to find in a review of the English language literature. Trauma was the most frequent predisposing event mentioned. Most of the patients for whom treatment results were reported improved with antibiotic treatment. References to a few reports of pulmonary and disseminated infections are also provided.

It is sometimes stated that sulfonamides with or without the combination of trimethoprim are ineffective against isolates of this species (Lerner 1996), but there are reports of the successful use of these agents for the treatment of such infections (Wallace et al. 1982; Clark et al. 1995).

N. paucivorans

This species was described by Yassin et al. (2000a), on the basis of isolates obtained from respiratory specimens from a patient with chronic lung disease. The species epithet means 'eating little', and was chosen because few compounds were found that the organism could utilize as a sole carbon source. By 16S rDNA sequencing and DNA–DNA hybridization the organism was found to be closely related to, but specifically distinct from, the type strain of *N. brevicatena*. A number of phenotypic features were found that were useful for distinguishing this organism from the other type strains in the genus that were studied by the authors. Eisenblätter et al. (2002) reported a case of CNS infection in an immunocompromised patient. The organism was eventually isolated from a CSF culture and initially identified by sequencing a portion of the 16S rDNA gene; the identification was confirmed by a national reference laboratory. The organism was determined to be susceptible by in vitro testing to virtually every antibiotic tested, although the recommended NCCLS procedure was not used. The patient showed sustained clinical improvement after a protracted course involving several different antibiotics.

N. pseudobrasiliensis

This species, phenotypically quite similar to *N. brasiliensis*, has been found to be the causative agent of a significant number of the pulmonary and invasive infections previously attributed to *N. brasiliensis*. See the discussion above under *N. brasiliensis*. Interestingly, disseminated disease in a Brazilian patient with AIDS caused by this species has been reported (Brown et al. 1999). As other cases have been reported from the USA and Australia, this species may well be distributed worldwide.

N. transvalensis

A description of some of the phenotypic properties of this species was provided by Gordon et al. (1978). From the title of the paper in which the species was originally described, which we have not been able to review, we presume the name of the species pertains to the region of South Africa from which the species was first isolated. Of the four apparently separate strains studied by Gordon et al. (1978) three were said to have been isolated from mycetomas.

McNeil et al. (1992a) reviewed the clinical features of 16 additional patients from whom *N. transvalensis* was

isolated. The phenotypic characteristics of the isolates were also described, as well as the antibiotic susceptibility results for 11 of the isolates. Of these 16 isolates, 15 were studied by the authors themselves; the results for the remaining isolate were obtained by the Queensland State Health Laboratory in Brisbane, Australia. Available information suggested that two of the isolates probably were colonizers; for four patients, the clinical significance could not be determined. Four patients had pulmonary infection, four had disseminated infection (with the brain involved in two patients), and two had localized, presumably trauma-related, infections (a mycetoma of the thumb in one patient and a corneal ulcer in the other). Many of these patients had some source of immunocompromise. Two patients had chronic granulomatous disease, and two had diabetes mellitus; one of these diabetic patients had also received a renal transplant. Of the three patients who were known to have died, all had disseminated disease. Almost two-thirds of the patients were male. Trimethoprim-sulfamethoxazole was used for treatment in nine of the ten patients considered to be infected, but a variety of other agents were used in many of these as well. For the 16 isolates, decomposition reactions were variable, with 25 percent of the isolates decomposing adenine, 31 percent, casein, 75 percent, hypoxanthine, 63 percent, tyrosine, and 56 percent, xanthine. The susceptibility testing procedure employed indicated a significant degree of resistance to many agents; 18 percent were resistant to amikacin and to trimethoprim-sulfamethoxazole, 70 percent were resistant to amoxicillin-clavulanate, 50 percent were resistant to cefotaxime and to ceftriaxone, 46 percent were resistant to minocycline, and 40 percent were resistant to ciprofloxacin.

Weinberger et al. (1995) described a patient who developed pulmonary and disseminated infection with *N. transvalensis* following a liver transplant. The patient's pulmonary manifestations were initially thought to be caused by a pulmonary embolism. The organism was eventually isolated from several respiratory specimens, an open lung biopsy, and an aspirate of a subcutaneous nodule in the temporal region. His infection appeared to clear with long-term trimethoprim-sulfamethoxazole therapy.

In an extensive study by Wilson et al. (1997) of 58 isolates that were either amikacin resistant, or in other respects similar to such isolates, it was found that four separate groups could be identified that could be recognized by certain phenotypic features and also by distinctive RFLP patterns obtained from amplification of a portion of the HSP gene. The 58 isolates studied could be separated into four subgroups; the authors proposed that the set of four subgroups be designated the *N. transvalensis* complex. One subgroup comprised what had been previously designated *N. asteroides* complex type IV (or *N. asteroides* drug pattern type IV; see above under *N. asteroides*), a second subgroup (which contained the *N. transvalensis* type strain) which was designated *N. transvalensis* sensu stricto, a third subgroup designated *N. transvalensis* new taxon 1, and a fourth subgroup designated *N. transvalensis* new taxon 2. According to the results presented in one of their tables, most isolates in all the subgroups were resistant not only to amikacin but also to gentamicin, kanamycin, and tobramycin. All their isolates in the subgroup *N. transvalensis* sensu stricto were resistant to amoxicillin-clavulanic acid, all their isolates in the subgroup *N. transvalensis* new taxon 2 were sensitive, and 13 percent of their isolates in the other two subgroups were resistant. The subgroups could also be distinguished by using a variety of substrates as sole carbon sources, but probably the most reliable procedure for distinguishing among the subgroups was RFLP. (Note that the phenotypic data for the four subgroups presented by Brown and McNeil (2003) differ significantly, without explanation, from those presented by Wilson et al. (1997). Also, the reason for the difference in frequency of amikacin susceptibility in the study of Wilson et al. 1997 and that of McNeil et al. (1992a) is not clear.) There was a clear difference in the geographic distribution of the four subgroups, based on the 58 isolates. All the isolates in *N. asteroides* complex type IV and in *N. transvalensis* new taxon 2 came from the USA, and none from Australia. Four of ten *N. transvalensis* sensu stricto isolates and six of eight *N. transvalensis* new taxon 1 isolates came from Australia, and the remainder from the USA. It is possible that each of the subgroups may prove to be a distinct species, but to the best of our knowledge, no DNA–DNA hybridization studies of isolates in the subgroups with one another have yet been done.

Yorke and Rouah (2003) described a patient with pulmonary and cerebral involvement with *N. transvalensis* who had initially been diagnosed as having lung cancer. The patient died despite antimicrobial treatment with several different agents and undergoing three craniotomies for resection of abscesses. It was not known whether or not the patient had some underlying immunocompromising condition.

Lopez et al. (2003), described the case of a heart transplant patient who developed a pulmonary infection with *Nocardia transvalensis* new taxon 2, isolated from the aspirate of a pulmonary nodule and identified at a reference laboratory using molecular methods. The patient was treated with trimethoprim-sulfamethoxazole and subsequently sulfamethoxazole alone, to which the organism had been found to be susceptible. He subsequently developed skin and brain lesions from which the same species was isolated; ribotyping was used to demonstrate that the initial and subsequent isolates were all the same strain. By in vitro testing, the subsequent isolates were found to be resistant to trimethoprim-sulfamethoxazole and sulfamethoxazole. The patient responded to treatment with ceftriaxone, ciprofloxacin,

and amoxicillin-clavulanic acid, and was subsequently maintained on oral ciprofloxacin without any recurrence of his infection. During his post-transplant course the patient also suffered from infections with *Aspergillus fumigatus* and *Cunninghamella bertholetiae*.

N. veterana

Gürtler et al. (2001) reported the first isolate of this species from a bronchoscopic lavage of an elderly man with a history of tuberculous pleurisy; although the patient had bilateral pulmonary lesions, the isolate was not thought to be clinically significant. No further clinical information regarding the patient was provided. The species name refers to the veteran's hospital at which the first isolate was obtained. By 16S rDNA sequencing, the organism appeared most closely related to *N. vaccinii*, but DNA–DNA hybridization demonstrated that it was a distinct species.

Kano et al. (2002) reported isolating this species from a culture of a biopsy of one of several cutaneous nodules on the right ankle of a middle-aged woman who had a history of systemic lupus eythematosus but was not being treated with steroids. No additional clinical information was provided. The 16S rDNA sequence of the clinical isolate showed 99.8 percent similarity to that of the type strain.

In 2003 there were three reports of infections caused by this species. Pottumarthy et al. (2003) reported the isolation of this species from a lung biopsy, a bronchoalveolar lavage, and sputum from a patient who had undergone a liver transplant, a patient with systemic lupus erythematosus, and a patient with bronchiectasis. The first two patients responded to trimethoprim-sulfamethoxazole treatment, and the third, to azithromycin. The isolates were identified by both phenotypic and molecular methods. The authors noted that by their RFLP analysis of the HSP gene, *N. nova* and *N. veterana* were indistinguishable. Godreuil et al. (2003) reported the isolation of this species from ascitic fluid of an AIDS patient with lymphoma who died before any treatment was begun. The isolate was identified by both phenotypic and molecular methods; the authors found that 16S rDNA gene of their isolate showed 100 percent identity to that of the type strain for the region sequenced, and they also noted that *N. veterana* appeared more closely related to *N. africana* than to *N. vaccinii*. Conville et al. (2003) reported a study of isolates obtained from fine needle aspirates of pulmonary lesions from two patients, one with chronic granulomatous disease and another who had undergone a single-lung transplant, as well as an isolate obtained from a bronchoalveolar lavage from a patient for whom no clinical history was available. The first patient responded well to a regimen involving a variety of different antimicrobial agents, and the second patient showed an initial response to trimethoprim-sulfamethoxazole treatment prior to the patient's decision to terminate immunosuppressive treatment and his subsequent demise. Both phenotypic and molecular methods were used to compare the clinical isolates to the type strains of *N. africana*, *N. nova*, *N. vaccinii*, and *N. veterana*. The routine performance of RFLP analysis of portions of both the 16S rDNA and HSP genes in their laboratory for *Nocardia* species identification made the authors suspect that they were dealing with an unusual species. The RFLP pattern obtained with the HSP gene was identical (resembling *N. nova*) for the patient isolates and all the type strains except that of *N. vaccinii*, while the pattern obtained for the 16S rDNA gene was identical for the patient isolates and the type strains of *N. africana* and *N. veterana*. Sequencing of the 16S rDNA gene revealed 100 percent similarity of the patient isolates to the sequence obtained from the *N. veterana* strain and 99.0 percent similarity to the *N. africana* type strain. DNA–DNA hybridization showed that one of the patient isolates was indeed *N. veterana* and not *N. africana* or *N. nova*. While some phenotypic differences were found among the isolates, too few isolates of *N. africana* and *N. veterana* were available to establish the reliability of the phenotypic tests for species discrimination. Susceptibility testing results were essentially the same for *N. africana*, *N. nova*, and *N. veterana*. The authors also noted that, as both *N. africana* and *N. veterana* have only recently been recognized and are phenotypically so similar to *N. nova*, published data on *N. nova* are likely to be confounded by inclusion of isolates belonging to one or both of the two more recently described species.

Treatment of *Nocardia* infections

Some treatment regimens described in various reports have been noted above. Probably the most commonly used treatment regimen for infection with *Nocardia* species in patients who can tolerate the agents is the use of a sulfonamide, usually in combination with trimethoprim; the efficacy of trimethoprim-sulfamethoxazole for both cutaneous and pulmonary nocardiosis was demonstrated by Wallace et al. (1982). A few isolates of several *Nocardia* species have been reported to be resistant to trimethoprim-sulfamethoxazole by in vitro testing at the time of initial isolation (Lee et al. 1994; Lopez et al. 2003; Cockerill et al. 1984; Garlando et al. 1992). Steingrube et al. (1991) reported the case of a cardiac transplant patient with *N. brasiliensis* infection whose initial isolate was sensitive in vitro to amoxicillin-clavulanic acid and who initially responded well to treatment with these agents, but who subsequently relapsed with a resistant isolate, that was determined to belong to the same strain by PFGE of genomic DNA restriction fragments. The resistant isolate was found to produce a beta-lactamase that differed from that of the initial isolate by a

small shift in the isoelectric point of its major band and to be 200-fold more resistant to clavulanic acid. Disseminated nocardiosis accompanied by a cerebral abscess attributed to *N. asteroides* has been successfully treated with the combination of amikacin and ceftriaxone (Garlando et al. 1992). Various drug and drug combinations, such as amikacin and imipenem, that can be used for the treatment of nocardiosis are discussed in the review by Lerner (1996). Linezolid has recently been reported to have been used successfully in the treatment of six patients with nocardiosis, four of whom had disseminated disease; the species involved were '*N. asteroides* sensu stricto,' *N. brasiliensis*, and *N. otitidiscaviarum* (Moylett et al. 2003). In vitro susceptibility testing results suggest that gatifloxacin and moxifloxacin may be superior to ciprofloxacin, and tigecycline, superior to minocycline, for the treatment of nocardiosis (Crist et al. 2003). In certain circumstances surgical intervention is required in addition to antimicrobial therapy for adequate treatment of a nocardial infection. Susceptibility testing of all clinically significant isolates should be performed by a laboratory with experience in such testing.

NOCARDIOPSIS

The transfer of the organism once known as *Actinomadura dassonvillei* to the proposed genus *Nocardiopsis* was made by Meyer (1976) on the basis of several features by which this species differed from other members of the genus *Actinomdura*, including the absence of the sugar madurose in whole cell hydrolysates, substrate hyphae that fragment into coccoid forms, and aerial hyphae that may have a 'zig-zag shape and divide into segments that then divide into smaller spores of irregular size.' Mycolic acids are absent. The genus name is derived from the word 'Nocardia' and the Greek word 'opsis,' which means 'appearance;' hence, the genus name means 'having the appearance of *Nocardia*' (Meyer 1976). Using molecular methods, Rainey et al. (1996), demonstrated that the genus was 'phylogenetically coherent' and that the species then recognized appeared to be quite closely related to one another.

Very few human infections have been attributed to *Nocardiopsis* species; most have been attributed to *N. dassonvillei* (Brown and McNeil 2003). Ajello et al. (1987) stated that in the 6-year period 1981 through 1986, 21 isolates of *N. dassonvillei* had been identified at the CDC. In one case presented by Beau et al. (1999), an isolate identified as *N. dassonvillei* was obtained from one blood culture of a patient with cholangitis; the organism was identified by a combination of phenotypic studies, cell-wall analysis, and molecular methods. An isolate identified as this species by Mordarska et al. (1998), on the basis of phenotypic features, glycolipid analysis, and chemotaxonomic and molecular studies was isolated from a 'pus sample' from a pulmonary abscess of a patient with chronic pneumonia.

Yassin et al. (1997b) described a new species, *N. synnemataformans*, on the basis of an isolate obtained from the sputum of a kidney transplant patient. The organism was shown to be a separate species from *N. dassonvillei* and other species in the genus by DNA–DNA hybridization. The authors also noted some phenotypic features by which the species in the genus might be distinguished. The aerial hyphae of the isolate were noted to form synnemata; hence the species name. No clinical details regarding the patient were provided, and we are not aware of any subsequent reports of isolation of this species.

The phenotypic, chemotaxonomic, and molecular aspects of the species in the genus were reviewed by Evtushenko et al. (2000). These authors also delineated a subspecies of *N. dassonvillei*, *N. dassonvillei* subsp. *albirubida*. Isolates of the species not in that subspecies would then automatically be in the subspecies *N. dassonvillei* subsp. *dassonvillei*.

Salazar et al. (2002) reported the development of genus-specific primers for use in the assignment of isolates to the genera *Nocardiopsis* and *Saccharothrix*.

PSEUDONOCARDIA

See above under *Amycolata* and *Amycolatopsis*.

RHODOCOCCUS

The genus *Rhodococcus* contains gram-positive to gram-variable, weakly acid-fast positive organisms that often appear coccal, but generally do also form short rods, which may show at least rudimentary branching. Colonies are often mucoid but may be smooth or rough; a more or less reddish pigment is often produced, but colony color may vary from buff to red, and some may be colorless. An aerial mycelium is generally absent, or at best rudimentary. A rod-coccus cycle continues during growth, as the longer rods fragment into cocci and short rods. The genus is definable by chemotaxonomic features such as the presence of *meso*-diaminopimelic acid, mycolic acids with carbon chains of 30–54 atoms in length, MK-8 as the predominant menoquinone, and cell walls rich in arabinose and galactose. The taxonomic history of the genus, and the features by which it can be distinguished from the other genera of aerobic actinomycetes, have been relatively recently recounted by Goodfellow et al. (1998). Members of the genus are widely distributed in the environment. Their abilities to synthesize or degrade a large variety of compounds make them of potential use for certain industrial procedures, or for environmental functions such as bioremediation (Bell et al. 1998). Several new species in the genus have recently been described from environmental sources (Euzéby 1997), and continuing recognition of

additional species is to be expected. Further study of the species currently in the genus may well result in delineation of additional genera (Goodfellow et al. 1998). A PCR-based procedure has been developed for the detection of several species in the genus in seeded artificial soil (Bell et al. 1999); some of the species detectable by the procedure, including *Rhodococcus erythropolis* and *Rhodococcus globerulus*, have been implicated in one or more reports of human infection. A PCR-based procedure for the identification of *Rhodococcus equi* isolates has also been described (Bell et al. 1996). Recently the species *R. equi* has been shown to be 'an heterogeneous taxon,' both on the basis of 16S rDNA sequence data and Curie-point mass spectrometric data (McMinn et al. 2000). It remains to be determined if the *R. equi* taxon contains several species within it, and if the PCR procedure of Bell et al. (1996) can recognize the molecular variants currently contained therein. Also, it is important to note the comment by Goodfellow et al. (1998), to the effect that species in the genus are difficult to identify phenotypically because of a lack of suitable tests, and that phenotypic tests can 'only give presumptive identifications.'

Rhodococcus equi

This species is currently the principal animal and human pathogen in the genus (Prescott 1991). The organism, initially isolated in 1923 by Magnusson from foals (hence the species epithet) and named *Corynebacterium equi*, is a significant cause of pneumonia and lung abscess in foals less than 4 months old, and is also commonly isolated from the cervical lymph nodes of pigs (Prescott 1991). The age range of particular susceptibility in horses is thought to be related to the gradual decline of maternally acquired antibody levels on the one hand, and the time required for the development of immunity secondary to organism exposure on the other. Disease appears most likely to develop in animals exposed to a large organism burden after the decline of maternally acquired antibody; most animals do not develop clinical disease if exposure is not too great. This species is a soil organism for which herbivore manure at the appropriate temperature apparently provides an ideal growth environment. The most common mode of infection is thought to be via inhalation of contaminated dust particles, but infection via the oral route and secondary to trauma may also occur. This species is evidently primarily an intracellular pathogen that eventually destroys the phagocytic cell within which organisms are contained.

Both the clinical and laboratory aspects of infection with *Rhodococcus equi* have been recently reviewed in depth by Cornish and Washington (1999). The first case of disease in man clearly attributable to this species (reported as *Corynebacterium equi*) was described by Golub et al. (1967). The patient had a progressive pulmonary infiltrate that eventually cavitated; he had a long history of immunosuppressive treatment, including prednisone, for 'plasma cell hepatitis.' The etiologic agent was not obtained until material was aspirated from the patient's lung via a catheter passed through a bronchoscope; thereafter, the organism could also be isolated from expectorated sputum. The isolate was identified in two separate reference laboratories. In addition to bronchoscopic drainage, treatment included administration of erythromycin for eight weeks; the cavity disappeared, and the organism could no longer be isolated from his sputum. However, six weeks after the erythromycin had been stopped, a subcutaneous abdominal nodule was noted, and the organism was again isolated from material obtained from the nodule. The patient was again treated with erythromycin, and had no further signs of infection. Interestingly, a history was eventually obtained from the patient that, although he had a clerical job, shortly before his infection developed he had spent a few weeks working in stockyards, cleaning pens that housed cattle, sheep, and pigs in a dusty environment contaminated with animal excrement.

Infection in humans remains relatively rare and has occurred primarily in individuals with some form of immunocompromise such as steroid treatment or HIV infection. The lung is the most common site of infection; particularly in the immunocompromised, dissemination via the blood may occur to virtually any body site. Occasionally, infection is first noted at nonpulmonary sites in patients with no apparent lung involvement; in the absence of a history of trauma, the route of infection in such cases generally cannot be determined. Capdevila et al. (1997) presented two cases of *R. equi* pneumonia in HIV-positive patients, and reviews 76 others that had been reported. In the 78 patients, the right upper lobe was involved in 41 percent, there was a single cavity in almost 60 percent, and positive sputum and blood cultures were obtained in approximately 60 percent and 50 percent, respectively. Weinstock and Brown (2002) also recently reviewed cases of *R. equi* infection. Only 10–15 percent of infections were found in immunocompetent hosts; approximately two-thirds occurred in HIV-infected patients, generally in those with low CD4 cell counts. Approximately half of the infected HIV-positive patients died as a result of the infection, compared to approximately 20 percent of infected HIV-negative patients. A nodular pulmonary infiltrate may be present; cavitation is fairly common (Scott et al. 1995). Histopathologically, microabscesses may be conspicuous, along with a marked histiocytic infiltrate. These macrophages may be periodic acid-Schiff stain positive, and resemble the cells seen in Whipple's disease. The presence of malakoplakia has been noted by several authors (Guerrero et al. 1999; Scott et al. 1995). Malakoplakia is a histopathologic lesion consisting of aggregates of histiocytes within some of

which are layered basophilic inclusions known as Michaelis–Gutmann bodies. These bodies are thought to be composed largely of undigested bacteria (Guerrero et al. 1999). In their multicenter review of infections in HIV-positive patients, Torres-Tortosa et al. (2003) noted that all the deaths in these patients due to *R. equi* infection occurred in patients who did not receive highly active antiretroviral therapy. Of their patients, 95.5 percent had pulmonary involvement, and 59.8 percent had positive blood cultures.

Infections also occur rarely in transplant patients; these have been recently reviewed by Perez et al. (2002). Cases have been reported in patients with bone marrow, heart, liver, lung, and kidney transplants.

Some patients, both immunocompromised and nonimmunocompromised, have a history of horse or pig exposure prior to the development of infection. Kedlaya et al. (2001) present an interesting case of an apparently immunocompetent man who developed pulmonary infection after having been stranded in a horse stable on a windy day when his car broke down, three weeks prior to hospital admission. These authors also review 19 other cases of infection in immunocompetent patients, including several trauma-related eye infections. Interesting case reports of infection in apparently immunocompetent hosts include a case of meningitis in a patient with no pulmonary findings (DeMarais and Kocka 1995), and a case of what was considered to be nosocomial meningitis following surgery in a patient with a prior history of head trauma (Scotton et al. 2000).

This species will grow well at 37°C on a variety of media. Colonies are generally smooth to mucoid, but may take approximately 48 h to develop their characteristic mucoid appearance. Pigmentation increases with time; a salmon color is often present after about 4 days. Individual organisms may be quite coccoid, but especially if stained early from liquid media, more bacillary forms may be seen. Organisms are modified acid-fast-positive, but often only a small percentage of the organisms retain the carbol fuchsin dye. Acid-fastness may be enhanced by culturing on media such as Lowenstein Jensen or Middlebrook 7H10 medium. This species is catalase- and urease-positive and can degrade adenine (Prescott 1991; Goodfellow et al. 1998). Molecular methods are probably the most definitive for identification of *Rhodococcus* species, particularly for those other than *R. equi*. Recently a PCR-based procedure for identifying *R. equi* that targets the cholesterol oxidase gene (*choE*) has been reported (Ladrón et al. 2003), but these authors examined only two isolates thought to belong to *Rhodococcus* species other than *R. equi*, so further study would seem to be required to establish the specificity of the assay. The enzyme in question is evidently responsible for the synergistic hemolytic reaction that occurs between isolates of *R. equi* and isolates of certain other species such as *Staphylococcus aureus*, and may be an important virulence factor. However, while considered characteristic for *R. equi*, such synergistic hemolysis is said not to be unique to the species (Prescott 1991).

There have been no treatment regimens for *R. equi* established by prospective clinical trials. In vitro, the organism has generally been found to be sensitive to aminoglycosides, erythromycin, fluoroquinolones, glycopeptides, imipenem, linezolid, and rifampin, although most in vitro studies probably have not used precisely the methodology now recommended by the NCCLS (Weinstock and Brown 2002; NCCLS 2003). Treatment recommendations for different circumstances are provided by Weinstock and Brown (2002). Mutations in the *rpoB* gene conferring rifampin resistance has recently been reported in a few isolates obtained from AIDS patients in northern Thailand (Asoh et al. 2003).

Rhodococcus species other than *R. equi*

Given the widespread occurrence of *Rhodococcus* species in the environment, at least occasional cases of infection caused by species other than *R. equi* would not be surprising. *Rhodococcus rhodochrous* was reported as isolated from a corneal ulcer of an elderly patient (Gopaul et al. 1988); no organisms were seen in a direct Gram smear. The organism was identified at a reference laboratory, and some phenotypic features of the organism are described. Both *Rhodococcus luteus* (now *Rhodococcus fascians*) and *Rhodococcus erythropolis* (now *Gordonia erythropolis*) were reported as the cause of endophthalmitis that occurred subsequent to extracapsular cataract extraction and implantation of a posterior chamber lens in an elderly, apparently immunocompetent woman (von Below et al. 1991). The organisms were identified at a reference laboratory, but other than noting that gram-positive bacilli were seen and cultured from material obtained subsequently from the eye, no information about the isolates is provided. *Rhodococcus globerulis* was recently reported as the cause of keratitis following laser in situ keratomileusis (Cuello et al. 2002). The isolate was identified in a reference laboratory, and phenotypic features of the isolate were reported. In none of these reports on isolation of *Rhodococcus* species were any molecular studies done, and given the problems with identifying *Rhodococcus* species isolates accurately by phenotypic procedures alone, we are not certain that the identity of these isolates has been adequately established. We have found a few other reports of human infection attributed either to a *Rhodococcus* either not identified to the species level, or to species other then *R. equi* but with no clinical or microbiologic data provided.

STREPTOMYCES

The characteristics by which the genus *Streptomyces* can be distinguished from other genera of aerobic actinomy-

cetes have been presented by McNeil and Brown (1994) and more recently by Anderson and Wellington (2001). Important features include the presence of both an aerial mycelium with conidia and a substrate mycelium, the presence of L-diaminopimelic acid in the cell wall, the absence of characteristic sugars in the cell wall, and the failure of organisms to stain with a modified acid-fast stain. A vast number of different species has been described, many in order to patent strains producing potentially useful compounds (Anderson and Wellington 2001). The genus is widespread in soil, and new species are continually being described. More study is warranted to assure that the currently described species have been appropriately distinguished based on current molecular criteria such as DNA–DNA hybridization. Given the large number of species in the genus, we think that an attempt to identify an isolate to the species level using phenotypic features alone is fraught with uncertainty. In their 1990 report on the species distribution and antibiotic susceptibility patterns of 366 aerobic actinomycete species evaluated from October 1985 through February 1988, McNeil et al. (1990) attributed 7.7 percent to the species *Streptomyces griseus* and an additional 7.7 percent to *Streptomyces* species. Of the 28 *S. griseus* isolates, 35.7 percent came from sputum, 32.1 percent from wounds, 25.0 percent from blood, and 7.1 percent from brain. However, no data were provided whereby the clinical significance of any of the isolates could be assessed.

Streptomyces somaliensis has been reported as a cause of actinomycotic mycetoma from many parts of the world (McNeil and Brown 1994); Hay et al. (1992) state that it is the main cause of actinomycotic mycetoma in Africa, but do not provide any substantiating data. Some phenotypic features of the species are given in a recent reference (Brown and McNeil 2003), but the extent to which these features will suffice to separate the species from other members of the genus is not clear. While most actinomycotic mycetomas involve a lower extremity site, cases of such mycetomas involving other parts of the body have also been reported, such as the craniofacial actinomycetoma in the patient described by Baril et al. (1999).

There are only a small number of invasive infections other than mycetoma attributed to *Streptomyces* species; many of these have been mentioned in recent reviews and case reports (McNeil and Brown 1994; Dunne et al. 1998). Reported infections have included endocarditis, pericarditis, peritonitis, brain abscess, and pneumonia.

Recent reports include the following: Dunne et al. (1998) reported the case of an HIV-positive patient with pneumonia; a sulfur granule was seen histopathologically, and a *Streptomyces* species was isolated from the biopsy culture. The patient responded well to initial treatment with ceftriaxone, followed by 6 months of clarithromycin.

Carey et al. (2001) reported the case of a woman with metastatic breast cancer with a central venous catheter in place, who developed fever after receiving an infusion of holistic preparations, including a compound called 'NeyTumorin.' The authors quoted a source describing this material as 'a combination of peptides and proteins of 15 different organs from fetal and young pigs and cows;' this mixture supposedly contained 'physiologic repair aids' that, via immunologic mechanisms, would enhance patients' responses to malignancies. Several blood cultures sets drawn both peripherally and through the catheter grew out a *Streptomyces* species. The catheter was removed and a culture of its tip was negative, but by the time of removal, the patient had been treated for several days with vancomycin. The patient's infection responded well to the combination of ceftriaxone and clarithromycin. The authors thought that the fact that the patient's fever resolved and that her blood cultures cleared after removal of the catheter made it likely that the infection was catheter related, with the source of the organism likely to be one of the holistic medications that the patient had received, although none of these preparations were available for culture.

Moss et al. (2003) reported the case of a girl with tibial osteosarcoma treated with a limb salvage procedure involving replacement of a portion of her tibia with a cadaveric bone graft. A central venous catheter had been in place for over a year. A brief episode of fever followed a methotrexate infusion; she had no other signs of infection on physical examination. Blood cultures obtained through the catheter at the time of the fever and several days later, as well as a subsequent peripheral blood culture, grew a *Streptomyces* species. The patient was treated with catheter removal and 6 weeks of vancomycin treatment and did well. The authors thought that the brief episode of fever was most likely attributable to the methotrexate itself, and considered that the bone graft was the potential source of an asymptomatic infection. The organism was identified as *Streptomyces bikiniensis* on the basis of 16S rDNA sequencing, but the authors did not state either the length of the portion of the gene sequenced or the degree of concordance of their sequence with published sequences for the *S. bikiniensis* 16S rDNA gene.

There have been no prospective, controlled studies of antibiotic regimens for treatment of *Streptomyces* infections. In their in vitro study, McNeil et al. (1990) reported that, of the isolates they identified as *S. griseus*, 10 percent were resistant to minocycline, 14 percent were resistant to erythromycin, 19 percent were resistant to imipenem, 20 percent were resistant to ceftriaxone, 29 percent were resistant to trimethoprim-sulfamethoxazole, and 43 percent were resistant to sulfamethoxazole. Clindamycin and vancomycin were not tested.

THERMOPHILIC ACTINOMYCETES: *SACCHAROMONOSPORA*, *SACCHAROPOLYSPORA*, AND *THERMOACTINOMYCES*

One or more species in each of these three genera have been implicated in the etiology of various forms of hypersensitivity pneumonitis (McNeil and Brown 1994), but we are not aware of any documentation of their involvement in invasive disease. These organisms will grow at temperatures of at least 50°C (McNeil and Brown 1994). Molecular methods appear to be the most useful for the identification of the species involved (Xu et al. 2002).

TROPHERYMA

Tropheryma whipplei, now generally considered to be the causative agent of Whipple's disease, was formally described and named in 2001 (La Scola et al. 2001). There have been three recent comprehensive reviews of the clinical, microbiological, and pathological features of Whipple's disease (Maiwald and Relman 2001; Marth and Raoult 2003; Fenollar and Raoult 2001). To briefly summarize a complex history, George Whipple is generally credited with the first description of the disease in 1907, although the condition had evidently been previously described 18 years earlier (Fenollar and Raoult 2001). Whipple's description was based on the autopsy findings of a physician who had suffered from fever, weight loss, diarrhea, and arthritis. The patient had aortic valve lesions, but the most prominent finding was the infiltration of mesenteric lymph nodes and the lamina propria of the small intestine with foamy macrophages, as a consequence of which Whipple named the condition 'intestinal lipodystrophy' (Fenollar and Raoult 2001). The disease, which primarily affects males, most commonly produces gastrointestinal symptoms but is in fact a systemic illness, and may in particular also involve the heart and the brain. In a study of relapse in relation to antibiotic treatment in 88 patients, Keinath et al. (1985), noted that, of 37 relapses in 31 patients, 13 primarily involved the CNS, and two, the heart. The heart may be affected without obvious signs of disease elsewhere, and Whipple's disease has been well documented to be a cause of culture-negative endocarditis (Gubler et al. 1999; Fenollar et al. 2001). An example of a most unusual presentation of the disease is the report by Rickman et al. (1995), of a patient whose presenting problem was bilateral uveitis, although she did have a long history of 'seronegative' arthritis. It has long been speculated that patients with Whipple's disease may have an underlying immune defect, and Marth et al. (1997) reported finding decreased production of interleukin-12 by monocytes and of interferon-γ by T cells, as well as decreases in serum immunoglobulin G2 and in transforming growth factor β. There is at least one report of infection in a patient with AIDS (Maiwald et al. 1995).

Whipple himself noted the presence of numerous rod-like organisms in some affected tissues, but apparently did not attribute any causal role to them (Relman et al. 1992; Fenollar and Raoult 2001). In the 1960s, bacteria-like structures were visualized in intestinal tissue by electron microscopy. Until recently diagnosis has depended largely on the detection of macrophages containing periodic acid-Schiff (PAS) staining inclusions, with electron microscopy recommended for confirmation, particularly if the diagnosis is being made on an extraintestinal specimen. These magenta-colored inclusions are comprised of both intact and degenerating organisms.

Despite numerous attempts, the organism has never been cultured in an axenic medium. There are two recent reports of culture of the organism by inoculating a human fibroblast cell line using a centrifugation shell-vial procedure; in the first report the organism was isolated from a mitral valve (Raoult et al. 2000), and in the second, from a duodenal biopsy (Raoult et al. 2001). In the first case a cytopathic effect was first detected 65 days after inoculation; in the second case organisms were detected by immunofluorescence staining 30 days after inoculation.

Despite the problems presented by the difficulties in culturing the organism, considerable progress has been made in elucidating genetic and phylogenetic features of the organism through the application of molecular biologic techniques directly to patient specimens. Wilson et al. (1991) were able to amplify and sequence an approximately 721-bp segment of bacterial 16S rDNA from a small bowel biopsy of a patient with Whipple's disease. The sequence obtained indicated that what the authors described as the Whipple's-associated bacterial organism (WABO) belonged to what was probably a hitherto undescribed genus, and was a member of 'the phylogenetic branch of gram-positive bacteria that possess a high G+C content.' Relman et al. (1992) sequenced approximately 90 percent of the 16S rDNA gene of the organism, working with frozen duodenal tissue from two patients with Whipple's disease, and formalin-fixed lymph node tissue from three others. The sequence obtained from the first patient differed by not more than 2 bp from the sequences obtained from the other five patients. No similar product was obtained from specimens from patients who did not have Whipple's disease. Only two differences were noted between the sequence obtained by Relman et al. (1992) and the corresponding portion of the sequence obtained by Wilson et al. (1991). The evolutionary tree constructed by Relman et al. (1992) indicated that the organism 'shares an ancestor with the actinobacteria that is more recent than the one it shares with the nocardioforms.' These authors proposed the name '*Tropheryma whip-*

pelii' for the organism. The genus name was derived from the Greek words *trophe*, meaning nourishment, and *eryma*, meaning barrier, referring to the malabsorption found in patients with Whipple's disease. The species name was chosen to honor George Whipple; the spelling was corrected to 'whipplei' in the formal description of the organism (La Scola et al. 2001). Maiwald et al. (1996), on the basis of still more extensive sequencing studies, concluded that the organism was not 'the deepest branching organism of the actinobacteria group,' finding that its phylogenetic position lay between the actinomycetes with group B peptidoglycan and the cellulomonads. Von Herbay et al. (1996) reported the development and evaluation of a system employing modified primers and a specific oligonucleotide probe, which they found to be a sensitive and specific procedure for making the diagnosis of Whipple's disease from formalin-fixed tissues. These authors also studied follow-up specimens obtained after treatment, and found that in most patients the PCR results had become negative by 18 months of treatment. Subsequent genetic studies indicated the existence of a few different sequence types of the organism (Hinrikson et al. 1999, 2000; Maiwald et al. 2000). Both the extent to which *T. whipplei* may be present in the environment and the frequency with which individuals may become colonized with the organism remain unknown. There have been some reports of positive PCR results for the organism that may have represented environmental or other contamination, or colonization, rather than true infection. Utilization of a quantitative real-time PCR, such as that reported by Fenollar et al. (2002), involving amplification of portions of two different genes, may prove helpful for distinguishing between infection and other circumstances in which small amounts of organism DNA may be present in a patient specimen.

There have been no randomized prospective studies of antibiotic therapy for Whipple's disease. The currently recommended regimen is the use of parenteral treatment for approximately 2 weeks with penicillin and streptomycin or with a third-generation cephalosporin, followed by a year or more of trimethoprim-sulfamethoxazole (Maiwald and Relman 2001; Marth and Raoult 2003). Effective treatment of CNS disease remains a significant problem.

TSUKAMURELLA

The genus *Tsukamurella* was created by Collins et al. (1988) to accommodate organisms that were similar to some then in the genera *Corynebacterium* and *Rhodococcus*, but that contained long-chain mycolic acids with 62–78 carbon atoms and 1–6 double bonds, which on pyrolysis yielded fatty acid esters with 20–22 carbon atoms. Also, the predominant menaquinone is MK-9. The organisms are gram-positive and modified acid-fast-positive but do not branch, and colonies do not produce an aerial mycelium. There are presently five species in the genus. Transfer of the species *Tsukamurella wratislaviensis* to the genus *Rhodococcus* has recently been proposed (Goodfellow et al. 2002); this species has evidently been isolated only from soil. Isolates assigned to all five species have been reported from human clinical specimens, but to date there have been only a small number of well-documented infections attributed to any of the five species. However, these organisms are easily mistaken for corynebacteria, and virtually certainly their incidence is higher than heretofore appreciated. Colonies of most species tend to be flat, dry, and velvety; color may vary from more or less nonpigmented to orange (Kattar et al. 2001). There do appear to be some phenotypic features useful for distinguishing among at least some of the species, but test results for some of these features are method-dependent (Kattar et al. 2001), and only a small number of isolates of each species have been studied, so these species are probably most reliably identified by molecular methods. Few data are available regarding antibiotic susceptibility, and there have been far too few cases for a controlled treatment trial. In one recent study in which five significant isolates, three of one species and one each of two others, were tested by E-test, all isolates were found to be susceptible to amikacin, ciprofloxacin, clarithromycin, imipenem, and sulfamethoxazole; all were resistant to *cefoxitin* (Schwartz et al. 2002).

Tsukamurella paurometabola

This species has a complex history, dealt with up to 1988 by Collins et al. (1988). Briefly, the organism first described as *Gordona aurantiaca* by Tsukamura and Mizuno (1971) was later transferred to the genus *Rhodococcus* and called *Rhodococcus aurantiacus*. Steinhaus (1941) isolated an organism from bed bugs that he named *Corynebacterium paurometabolum*. Collins et al. (1988) in their analysis of the 16S rRNA sequences of the two species, concluded that the 'exceptionally high level of sequence homology (>99 percent) exhibited by *Corynebacterium paurometabolum* and *R. aurantiacus* indicates that these taxa should be reduced to a single species.' DNA–DNA hybridization studies, however, were not done. Previous chemotaxonomic studies had shown that the two species were 'distinct from all other mycolic acid-containing taxa.' The type strain of *C. paurometabolum* was ATCC 8368; the type strain of *R. aurantiacus* was ATCC 25938. Collins et al. (1988) described *Tsukamurella paurometabolum* comb. nov. as the type strain of the genus, selecting the species epithet paurometabolum because it had priority over aurantiacus, and designating strain ATCC 8368 as the type strain of the species. The genus name was selected to honor the microbiologist Michio Tsukamura; the species name is from the Middle Latin word meaning 'little

changeable,' and presumably refers to the fact that the organism is relatively unreactive in most phenotypic tests. Subsequently, to be in accord with nomenclatural rules, the spelling of the species epithet was changed to 'paurometabola.' In a study of an outbreak of pseudoinfections caused by *T. paurometabola* reported in 1991, in which outbreak isolates were compared both with the new type strain ATCC 8368 and the strain that was the type strain for *R. aurantiacus* (ATCC 25938), Auerbach et al. (1992) noted that these two strains differed 'in terms of colonial morphology, biochemistry, antimicrobial susceptibility, and ribotyping,' and suggested that the two strains might in fact belong to separate species. The outbreak-related isolates were more similar to strain ATCC 25938 than to the type strain of *T. paurometabola*, ATCC 8368. In their study of various isolates of different species in the genus *Tsukamurella* published in 2001, in which the new species *Tsukamurella strandjordae* was described, Kattar et al. (2001) noted, 'We also found that the complete 16S rRNA gene sequence of *T. paurometabola* ATCC 25938 is 100 percent identical to that of *T. inchonensis* type strain ATCC 700082. These data, together with DNA–DNA hybridization and phenotypic data, provide strong evidence that *T. paurometabola* ATCC 25938 is a misnamed *T. inchonensis* strain.' Unfortunately, according to our understanding of the nomenclatural rules, the nomenclatural issues are more complex than Kattar et al. (2001) suggest. Had Collins et al. (1988) realized then that the two species they were synonomyzing were in fact different, they would assuredly have placed them both in the genus *Tsukamurella*, but retained the original species epithets, so there would have been two species placed simultaneously in the genus, *T. paurometabolum* (now corrected to *T. paurometabola*), and a species that they presumably would have designated '*Tsukamurella aurantiacus*' (which would have been corrected subsequently to '*T. aurantiaca*'). The species *T. inchonensis* (described in 1995 (Yassin et al. 1995)), if indeed the same as '*T. aurantiaca*' (initially described in 1971 as *Gordona aurantiaca*), would be merged into that species, and the name 'inchonensis' would be invalid. The environmental isolates studied by Auerbach et al. (1992) also would appear to belong to '*T. aurantiaca*.' Most, if not in fact all, other isolates attributed to the species *T. paurometabola* have not been sufficiently analyzed to be sure that they are not more appropriately placed in '*T. aurantiaca*' or elsewhere.

In the study of an outbreak of pseudoinfections attributed to *T. paurometabola* mentioned above, 12 isolates were obtained from ten patients at one hospital over a 17-month period. The phenotypic and molecular studies performed all indicated that the isolates belonged to the same strain, and were separable from two isolates obtained after the outbreak and from ATCC strains 8368 and 25938. Positive specimens tended to have certain features in common, such as being more likely to have been processed in the laboratory's TB/fungal room. All these features suggested a common-source outbreak, but a specific source could not be identified. A possibility was the use of saline obtained for a long period of time from the same bottle for processing specimens; *T. paurometabola* was not obtained from the one such bottle available for study, but several other microorganisms were. Once single-use vials were substituted, there was no recurrence of the outbreak for 2 years thereafter. The organism may be fairly widespread in the environment. In their review of the four cases of infection reported prior to their study that they found in the literature, Auerbach et al. (1992) suggested that at least three may have represented contamination rather than true infection. An example is the case of a woman with a relentless progressive process involving her right upper extremity, reported by Tsukamura et al. (1988). The patient underwent numerous surgical procedures, culminating in amputation of the arm without resolution of the process. A variety of different organisms were isolated from different specimens, but organisms identified phenotypically as *Rhodococcus aurantiacus* (now *T. paurometabola*) were isolated from several specimens in amounts ranging from 'several to 60 colonies.' No mention is made of direct visualization of any organisms in any of the specimens, and there was no response of the process to the administration of numerous antibiotics. However, the authors do note that 'the organism became resistant to 5 μg of ofloxacin per ml 2 months after the beginning of the administration,' although details of dates of organism isolation, drug administration, and isolate MICs are not provided.

Casella and Tommasi (1987) reported a case of peritonitis in a patient undergoing continuous peritoneal dialysis that was attributed to this species. The organism was reported as *Gordona aurantiaca* (*Rhodococcus aurantiacus*), and was identified at a reference laboratory, evidently on the basis of the numerous phenotypic features described by the authors.

Granel et al. (1996) reported a case of cutaneous infection attributed to this species in a man with a history of treated syphilis who presented with a three-month history of asymptomatic papular and pustular lesions and an indurated area on his left wrist. Noncaseating granulomas were seen on a skin biopsy, but apparently no organisms were visualized directly. On culture of a skin specimen a heavy growth of an organism was obtained that was identified as this species at a French national reference laboratory. The lesions resolved following local treatment with hexamidine and fusidic acid.

Lai (1993) reported an elderly immunocompromised woman with acute lymphoblastic lymphoma and a central venous catheter in place who became febrile. Several blood cultures, both peripheral and drawn

through the catheter, and the culture of the tip of the catheter after its removal, were all positive for an organism identified as this species at the CDC. The patient became afebrile 48 h after institution of vancomycin. The patient's treatment was later changed to erythromycin and gentamicin, and she did well. By the in vitro susceptibility testing procedures performed, the isolate was found to be sensitive to gentamicin but resistant to erythromycin. The procedures employed to identify the organism were not stated.

References to a few other case reports of infections attributed to this species can be found in the report and review by Schwartz et al. (2002).

Tsukamurella inchonensis

Yassin et al. (1995) described this species on the basis of isolates obtained from two patients; one had ingested hydrochloric acid and had four separate blood cultures positive for the organism, and the other had a necrotic lung tumor, the organism being isolated from a 'smear' of the lung. No further clinical information was provided. The organisms were found to be different from the *T. paurometabola* type strain by a variety of phenotypic and molecular procedures, including DNA–DNA hybridization. However, the strain ATCC 8368 was not compared to the patient isolates (see above under *Tsukamurella paurometabola*). The species name refers to the South Korean city of Inchon, where the type strain was isolated.

Tsukamurella pulmonis

Yassin et al. (1996) described this species on the basis of a sputum isolate from an elderly woman. The woman was described as having had cured pulmonary tuberculosis, and possibly an infection with another mycobacterium. She later appeared to have another pulmonary infection, at which time this organism was isolated. She was said to have recovered 'only after antibiotics that were active against the *Tsukamurella* strain in question had been administered;' no further clinical details were provided. The results of molecular studies, including DNA–DNA hybridization, comparing the isolate to the type strains of *T. paurometabola* and *T. inchonensis* indicated that the isolate was a new species of *Tsukamurella*; phenotypic differences between the isolate and the two type strains were also noted. The species epithet designates the site of origin (the lung) of the type strain. Of the six cases of catheter-related bacteremia in immunocompromised patients attributed to *Tsukamurella* species described by Schwartz et al. (2002), three were attributed to this species. These occurred in patients with metastatic breast cancer, severe combined immunodeficiency disease, and chronic renal failure. All patients did well following catheter removal and antibiotic treatment; different agents were used in different patients, and included trimethoprim-sulfamethoxazole, amikacin, clarithromycin, ciprofloxacin, and vancomycin. The isolates were identified by HPLC, dot-blot DNA–DNA hybridization, and 16S rRNA gene sequencing.

Tsukamurella tyrosinosolvens

Yassin et al. (1997a) described this species on the basis of four strains, two of which were each isolated from several different blood cultures from two patients with implanted cardiac pacemakers, and the other two of which were isolated from sputum specimens from two patients with chronic lung infections. The results of molecular studies, including DNA–DNA hybridization, comparing the isolates to the type strains of *T. paurometabola*, *T. inchonensis*, and *T. pulmonis* indicated that the isolates comprised a new species of *Tsukamurella*; phenotypic differences between the isolates and the three type strains were also noted. The species epithet refers to the ability of the isolates to hydrolyze tyrosine, which the authors thought was a characteristic feature of the species; the three type strains of the other species studied did not hydrolyze tyrosine. However, in their study of several isolates of different *Tsukamurella* isolates, Kattar et al. (2001) found that two of the four *T. inchonensis* isolates they evaluated did hydolyze tyrosine.

Of the six cases of catheter-related bacteremia in immunocompromised patients attributed to *Tsukamurella* species described in 2002 by Schwartz et al., one was attributed to this species. This occurred in a patient with chronic myelogenous leukemia who had recently received a bone marrow transplant. The organism was obtained from blood cultures drawn both peripherally and through the catheter. The catheter was removed accidentally, and the tip was not cultured. The patient was treated with a variety of antibiotics, including amikacin, clarithromycin, and ciprofloxacin, and did well. The isolate was identified by HPLC, dot-blot DNA–DNA hybridization, and 16S rRNA gene sequencing. Sheridan et al. (2003) reported the case of a patient with end-stage renal failure with a tunneled internal jugular hemodialysis line in place who developed a catheter-related infection attributed to this species. Blood cultures were positive on some six occasions over the course of approximately a year. The isolates were variously thought to be a *Corynebacterium* species, a *Bacillus* species, and *Nocardia otitidiscaviarum*. His presenting fevers responded to a variety of antibiotics. The catheter was removed during the last episode, and the same organism was cultured from the tip. The patient was treated with amikacin and trimethoprim-sulfamethoxazole and remained afebrile thereafter. The organism was ultimately identified by sequencing 16S rDNA from four of the isolates; all showed 'at least a

99 percent sequence identity with *Tsukamurella tyrosinosolvens* over 1290 base pairs.' However, with what degree of probability species-level identification can be made by such sequencing alone, perhaps particularly in this genus. In their initial description of the species, Yassin et al. (1997a) noted well over 99 percent sequence similarity for the 16S rDNA region of the organism they selected as the type strain for this species and the corresponding regions for several other *Tsukamurella* species type strains, including 99.9 percent similarity for the type strain of *T. pulmonis*.

Tsukamurella strandjordae

Kattar et al. (2001) described this species on the basis of several isolates obtained from the blood of a child with acute myelogenous leukemia who had a central venous catheter in place. The infection responded to catheter removal and treatment with trimethoprim-sulfamethoxazole (Schwartz et al. 2002). The organism was demonstrated to differ from other species in the genus by a variety of phenotypic and molecular procedures, including 16S rRNA gene sequencing and dot-blot DNA–DNA hybridization. The species name was selected to honor Paul Strandjord, the founder of the Department of Laboratory Medicine at the University of Washington. We are not aware of any other reports of isolation of this species.

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Mycobacterium tuberculosis complex, *Mycobacterium leprae*, and other slow-growing mycobacteria

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GENERAL CHARACTERISTICS

Definition

The genus *Mycobacterium* is currently the only genus in the family Mycobacteriaceae, order Actinomycetales, and related to other mycolic acid-containing genera. The minimal standards for including a species in this Genus are:

- acid-alcohol fastness (i.e. resist decolorization by acidified alcohol after being stained with a basic fuchsin dye);
- the presence of mycolic acids containing 60–90 carbon atoms, which are cleaved to C_{22}–C_{26} fatty acid methyl esters by pyrolysis; and
- a G + C content of the DNA of 61–71 mol% (Lévy-Frébault and Portaels 1992), with the only exception being *M. leprae* (>57 percent) (Good and Shinnick 1998).

Mycobacteria are nonmotile, nonspore-forming, weakly gram-positive, aerobic or microaerophilic, straight or slightly curved rod-shaped bacteria (0.2–0.6 × 1.0–10 μm) (Wayne and Kubica 1986). Some mycobacteria display coccobacillary or branched forms. Filamentous growth may occur, but on slight disturbance usually becomes fragmented into rods or coccoid elements. Currently, there are about 100 mycobacterial species that are usually grouped into two major divisions, 'slowly growing' and 'rapidly growing' mycobacteria. The slow growers require more than 7 days to produce visible colonies on solid media from a dilute inoculum under ideal culture conditions. Colony morphology varies among the species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigmented (yellow to orange, rarely pink). Some of the latter require light to form pigment (photochromogens), while others produce pigment in either the light or in the dark (scotochromogens) (Timpe and Runyon 1954; Runyon 1959). These divisions have, however, no formal taxonomic standing but are useful for a preliminary identification.

Slowly growing mycobacteria were among the first bacteria to be ascribed to specific diseases. In 1874, Armauer Hansen identified a rod-shaped bacillus (*Bacillus leprae*) in a tissue biopsy from a patient and suggested that it was the etiological agent of leprosy (Hansen 1880). Eight years later, Robert Koch identified another rod-shaped bacillus (*Bacterium tuberculosis*) as the causative agent of tuberculosis (TB) and formulated Koch's postulates for establishing a causal relationship between a suspected pathogen and a given disease

(Koch 1882). These two species were subsequently renamed *Mycobacterium leprae* and *Mycobacterium tuberculosis*, respectively, and placed in the genus *Mycobacterium* ('fungus bacterium,' named to reflect the mould-like pellicle formed by *M. tuberculosis* on liquid media) (Lehmann and Neumann 1896).

At the beginning of the twentieth century, *M. tuberculosis* was the only species of *Mycobacterium* routinely isolated from, and associated with, human disease. As time went on, more and more species of environmental mycobacteria, called nontuberculous mycobacteria (NTM) (formerly atypical mycobacteria or mycobacteria other than tubercle bacilli (MOTT)), were recognized as causes of human disease.

Habitat

Isolated from a wide variety of environmental sources, including water, soil, dust, and *Sphagnum* vegetation (reviewed by Kazda 1983; Collins et al. 1984; Hartmans and De Bont 1992), mycobacteria have been classified as obligate pathogens, facultative or opportunistic pathogens, or free-living saprophytes (Kazda 1983). Obligate pathogens are species that do not appear to multiply outside their hosts and include *M. tuberculosis* complex (i.e. *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. canettii*), *M. leprae*, *M. marinum*, and *M. paratuberculosis*. TB manifests both pulmonary and extrapulmonary in humans and warm-blooded animals and has become a new threat in developing countries in the most recent past. *M. leprae* causes chronic, granulomatous, and debilitating disease in humans. Leprosy-like infections have been documented in the nine-banded armadillo (*Dasypus novemcinctus*), chimpanzees (*Pan troglodytes*), mangabey monkeys (*Cercocebus torquatus atys*) (reviewed by Shinnick 1992), cats (Lawrence and Wickham 1963), rats (McCoy 1913), and buffalos (Lobel 1934). *M. paratuberculosis*, the etiological agent of Johne's disease, is shed in large numbers in the feces of infected cattle. Although this species can survive for long periods of time in the soil, there is no evidence that they actually multiply outside their animal hosts (Kazda 1983). Nevertheless, *M. paratuberculosis* has been suspected as a cause of Crohn's disease, an illness in humans with characteristic lesions resembling those of Johne's disease in cattle (reviewed by Chiodini 1989). Facultative pathogens are able to multiply outside the host as well and cause infections in humans or animals and include, among others, *M. avium* complex, *M. kansasii*, *M. malmoense*, *M. simiae*, *M. scrofulaceum*, *M. ulcerans*, and *M. xenopi*. Free-living saprophytes such as *M. gastri*, *M. gordonae*, and *M. nonchromogenicum* do not generally cause progressive infection or disease in immunocompetent individuals.

Nutritional requirements and metabolic activities

Given the wide spectrum of ecological niches occupied by these organisms, it is not surprising that the various species are metabolically very versatile and thus use a wide range of compounds as sources of essential nutrients. With the exception of *M. genavense*, *M. paratuberculosis*, and *M. haemophilum*, most mycobacteria are not fastidious, i.e. the basic nutritional requirements of mycobacteria growing in axenic media are similar to those of other bacteria. These include carbon, nitrogen, oxygen, phosphorus, sulfur, iron, sodium, potassium, magnesium, and trace elements (primarily zinc and manganese) (reviewed by Wheeler and Ratledge 1994). *M. leprae* represents the only mycobacterial species known so far where convincing evidence for growth in vitro is lacking.

CARBON AND NITROGEN SOURCES

Important carbon sources are carbohydrates (e.g. glucose, fructose, sucrose, mannose, trehalose, inositol, mannitol), organic acids, including short-chain (pyruvic, acetic, and citric) and long-chain (oleic and palmitic) acids, hydrocarbons (polycyclic aromatic compounds), and even CO_2, which is essential for optimal growth of many species (Ratledge 1982) and usually included in media as $NaHCO_3/Na_2CO_3$ or as CO_2 in the gas phase (5–10 percent). Laboratory culture medium traditionally contains glycerol because it is the only carbon source that can be utilized by all members of the genus, although it is not the optimal C-source for many species. For instance, pyruvate is a better C-source for *M. bovis*. Expressing a number of hydrolytic and degradative activities, mycobacteria may be able to utilize additional sources of carbon, such as Tween 80, which can be hydrolyzed to oleic acid.

In growth media, the preferred nitrogen source is asparagine or glutamine, although mycobacteria can obtain nitrogen from many inorganic (e.g. NH_4^+ salts) and organic sources (e.g. amides, amines, amino acids, nucleosides). A few species may be able to assimilate nitrate or nitrite via nitrate and nitrite reductases to generate NH_3.

ACQUISITION OF IRON

Iron is essential for the growth of mycobacteria both in vivo and in vitro. In secreting small water-soluble iron-binding siderophores called exochelins, mycobacteria have evolved a sophisticated system for scavenging iron from the environment and their eukaryotic hosts (reviewed by Wheeler and Ratledge 1994). The MS-type of exochelin (e.g. produced by *M. smegmatis*) remains water-soluble at all stages, whereas the MB-type (e.g. produced by *M. tuberculosis*) becomes chloroform extractable when complexed with Fe(III). Each type of

exochelin actually encompasses a family of small molecules whose masses range from approximately 0.7 to 0.8 kDa, which reflects varying numbers of CH_2 groups on an alkyl side chain (Gobin et al. 1995). Exochelins extract iron from various iron-containing molecules in their vicinity (e.g. ferritin) by virtue of their very high affinity for ferric ions (K_s of approximately 10^{25} M) which is used as a growth supplement for fastidious mycobacteria such as *M. genavense*. The ferriexochelin can then interact with a specific protein receptor on the mycobacterial cell surface and be transported to the interior of the cell. Alternatively, the ferric iron may be transferred to a related membrane-bound molecule called mycobactin. Mycobactins and exochelins share a common iron-binding core but differ in alkyl side chains, which accounts for the differences in polarity. Mycobactins can also bind free ferric ion and facilitate the transport of it across the membrane to the interior of the bacterium. Release of the iron from either ferriexochelin or ferrimycobactin is accomplished by reduction of the ferric ion to ferrous ion by an NADH-dependent reductase. Because of the efficient iron-scavenging systems, most mycobacteria require only small amounts of iron (1 μg Fe per ml) in culture medium for growth. *M. haemophilum* is unusual in that it requires high iron concentrations (2 percent ferric ammonium citrate or 40 μg hemin per ml) for growth.

Metabolic pathways in mycobacteria

In general, metabolic activities of mycobacteria are similar to those of other bacteria. General metabolic pathways and enzyme systems have been reviewed (Wheeler and Ratledge 1994). Of course, mycobacteria do have several unique biochemical activities related to the synthesis of mycobacteria-specific compounds, such as mycolic acids or the phenolic glycolipid I of *M. leprae*. Also, individual species express characteristic sets of enzymatic activities, probably related to their ecological niches, and these characteristic activities are exploited in species identification tests.

Relatively little is known about metabolic activities in two environments that are important in mycobacteria-caused diseases. First, the environment encountered by an intracellular pathogen is quite different from that encountered on an agar slant. So far, few studies have analyzed the metabolic activities of in vivo grown mycobacteria, although Barclay and Wheeler (1989) characterized some of the nutritional features of the intracellular environment and the corresponding mycobacterial metabolic activities. Second, both *M. tuberculosis* and *M. leprae* can persist in the human host for long periods in an apparently metabolically dormant state. An in vitro model of persisters has been developed by Wayne and Lin (1982) in which tubercle bacilli are allowed to settle to the bottom of a liquid culture. These bacilli cease to replicate, reduce their oxidative metabolism, and survive in anaerobic conditions much longer than actively metabolizing cells. Conversion of lipids into carbohydrates through the glyoxylate-shunt pathway may be essential for 'dormant' *M. tuberculosis* cells, allowing long-time survival in the host (McKinney et al. 2000; Bishai 2000).

Cell-wall structure and components

Mycobacteria have a complex outer envelope composed of several distinct layers (reviewed by Besra and Chatterjee 1994; Brennan and Draper 1994). The innermost layer is the plasma membrane, which displays an asymmetric bilayer structure. Inserted into it are proteins, phosphatidylinositol mannosides, and lipoarabinomannan. Next is the peptidoglycan layer, which determines the shape of the cell and is similar to peptidoglycans of other gram-positive bacteria. It contains repeating disaccharide units of *N*-acetylglucosamine-(β1–4)-*N*-glycolylmuramic acid cross-linked via L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine tetrapeptides, except in *M. leprae*, where the L-alanine is replaced by glycine (Draper 1986). About 10 percent of the *N*-glycolylmuramic acid residues are covalently attached to a branched-chain polysaccharide, arabinogalactan, via phosphodiester bonds. Distinct arabinose residues of the arabinogalactan molecules are esterified to high-molecular-weight mycolic acids. Finally, the outer surface of the mycobacterium is formed by the intercalation of medium-chain (e.g. mycocerosates) and short-chain (e.g. acylglycerols) lipids, glycolipids, and peptidoglycolipids into the uneven hydrophobic layer of mycolic acids. Proteins (e.g. porins, transport proteins) are found throughout the various layers.

Several components of the mycobacterial envelope are strongly immunologically active (Stewart-Tull 1983). For example, whole heat-killed mycobacteria are strong adjuvants and are the basis of Freund's complete adjuvant (heat-killed mycobacteria in oil). It is the peptidoglycan (murein) layer that contains this adjuvant activity. A water-soluble fragment of the peptidoglycan called muramyl dipeptide (MDP) (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) also acts as an adjuvant. MDP has the advantages over heat-killed mycobacteria in that it can be chemically synthesized, is commercially available, and is not immunogenic (i.e. it does not elicit an immune response to itself). Lipoarabinomannan (LAM) is also highly immunoreactive (see below).

LIPIDS

A prominent feature of the outer surface of mycobacteria is its high content of lipid, which accounts for up to 60 percent of the cell-wall weight and contributes to several biological features, including the hydrophobicity of mycobacteria, their tendency to form clumps and cords, their resistance to common lysis

procedures, and their ability to survive for a long period of time (reviewed by Brennan et al. 1990; Besra and Chatterjee 1994; McKinney et al. 2000; Bishai 2000). In general, the lipids are long-chain fatty acids (e.g. $CH_3(CH_2)_nCOOH$), which are often modified by the presence of unsaturated bonds, cyclopropane rings, or side groups such as methyl, methoxy, hydroxy, and keto groups.

Lipoarabinomannan

LAM is structurally and functionally related to the O-antigenic lipopolysaccharides of other bacteria (reviewed by Brennan et al. 1990; Besra and Chatterjee 1994). LAM is thought to be anchored in the plasma membrane and to extend through the cell wall to the surface of the mycobacterium. The membrane anchor is a diacylglycerol moiety, which in *M. tuberculosis* contains palmitic acid and tuberculostearic acid (TBSA) and which is attached to a phosphatidylinositol residue via a phosphodiester linkage. The polysaccharide backbone is also attached to the phosphatidylinositol and consists of α(1–6)-linked D-mannose residues to which short side chains of α(1–2)-linked D-mannose residues and α(1–5)-linked D-arabinose residues are attached. The termini of the LAM molecules may be branched hexa-arabinosides or linear tetra-arabinosides (Chatterjee et al. 1992a) and the arabinose termini may be capped with one to three mannose residues (Chatterjee et al. 1992b). Biological activities of LAM include strong seroreactivity, inhibition of interferon-γ-mediated activation of macrophages, induction of cytokine production and release by macrophages, scavenging reactive oxygen intermediates, and suppression of T-cell proliferation (Kaplan et al. 1987; Sibley et al. 1988; Barnes et al. 1992; Chatterjee et al. 1992c). Several parts of LAM are involved in these biological activities (reviewed by Besra and Chatterjee 1994). The terminal arabinose units are targets of circulating antibodies, and the degree of mannosylation of the terminal arabinose units influences the biological activities of LAM (e.g. ability to induce tumor necrosis factor alpha (TNF-α) production) (Chatterjee et al. 1992c). This difference may be related to virulence. The acyl groups are also required for some biological activities, such as the induction of cytokine production, because removal of the acyl groups by mild alkali treatment abolishes the activity. LAM has been identified as a key ligand of dendritic cell (DC)-specific intracellular adhesion molecule-3 grabbing nonintegrin (SIGN). DC-SIGN-mediated entry of *M. tuberculosis* in DCs in vivo is likely to influence bacterial persistence and host immunity (Tailleux et al. 2003). In addition, the cell-surface receptor DC-SIGN discriminates between *Mycobacterium* species through selective recognition of the mannose caps on LAM (Maeda et al. 2003).

Mycolic acids

Mycolic acids are a defining characteristic of members of the genus *Mycobacterium*, although related long-chain fatty acids also are found in the genera *Corynebacterium* (containing 28–40 carbon atoms) and *Nocardia* (containing 40–60 carbon atoms). The mycobacterial mycolic acids are α-branched, β-hydroxy fatty acids with 60–90 carbon atoms in the primary chain. The alkyl branches are attached to the α-position (the CH_2 group adjacent to the terminal carboxylic acid) and typically contain 22–26 carbon atoms. Mycolic acids are categorized according to oxygen-containing modifications found in the primary chain. If not containing any oxygen functions, in addition to the β-hydroxy group they are called α-mycolates. Those containing keto groups are ketomycolates, those with methoxy groups are methoxymycolates, and those with epoxy groups are epoxymycolates.

Each species of *Mycobacterium* appears to synthesize a unique set of mycolic acids, and this has been exploited for identifying mycobacteria (Minnikin et al. 1980; Daffé et al. 1983) (see below). The resulting pattern is compared with a library of reference patterns to identify the species.

ACETYLATED TREHALOSES

In 1950, Bloch isolated 6,6′-dimycolyl-α,α′-D-trehalose (trehalose dimycolate (TDM)) and suggested it was the cell-surface component responsible for the ability of virulent tubercle bacilli to form serpentine cords and to absorb the cationic phenazine dye neutral red. Subsequent studies have not borne out a role for TDM in either cord formation or virulence. Regardless, biological activities associated with cord factor include systemic toxicity, granulomagenic activity, and cytokine induction (Goren 1982).

Other trehalose-based lipids found in *M. tuberculosis* are sulfolipids, which are thought to play a role in the intracellular survival of tubercle bacilli by inhibiting phagosome activation (Goren et al. 1987). The sulfolipids are based on trehalose 2′-sulfate acetylated with hydroxyphthioceranic, phthioceranic, and saturated straight-chain fatty acids such as palmitate and stearate. Additional polar (Minnikin et al. 1985) and nonpolar (Besra et al. 1992) acetylated trehaloses have been described and suggested to be virulence factors, because these compounds have been found in virulent strains of *M. tuberculosis* only.

MYCOSIDES

The outer surface of a mycobacterium contains a heterogeneous group of biologically and immunologically active medium- and short-chain lipids, which are analogous structurally and functionally to the O antigens of gram-negative enteric bacteria (reviewed by Besra and

Chatterjee 1994). The mycosides fall into two major groups:

1 peptidoglycolipids, which contain mycoserosic acid, sugars and amino acids, and
2 phenol-phthiocerol glycosides.

Peptidoglycolipids often contain unusual sugar residues such as O-methyl rhamnose, fucose, or deoxytalose and play roles in determining serotype, colony morphology, and virulence. For example, peptidoglycolipids form the basis of an agglutination serotyping scheme for members of the *M. avium* complex. A peptidolipid also serves as the receptor for phage D4 attachment to mycobacterial cells.

Mycosides lacking amino acids are typically derivatives of phenol-phthiocerol and have been isolated and characterized from *M. kansasii*, *M. bovis*, *M. marinum*, and *M. leprae*. Phenolic glycolipid I (PGL-I) is found only in *M. leprae* and is synthesized and shed in large amounts (reviewed by Shinnick 1992). The immune response to the trisaccharide component of PGL-I has been used in immunodiagnostic tests to detect people infected with *M. leprae*. In addition, PGL-I can scavenge reactive oxygen intermediates and hence may play a role in the ability of *M. leprae* to survive within macrophages (Chan et al. 1989). PGL-I has also been implicated as playing a role in the specific immunosuppression seen in lepromatous leprosy patients (Mehra et al. 1984).

Antigens

Robert Koch's studies with antigens present in filtrates of cultures of *M. tuberculosis* were the beginning of a great deal of research, which still continues today, to define and characterize mycobacterial antigens.

TUBERCULINS

Tuberculin reactivity was described by Koch (1891) during a search for a remedy for TB. Koch inoculated tubercle bacilli into the flank of a guinea pig and observed a small nodule at the injection site a week or two later. This ulcerated and remained open and contained viable tubercle bacilli, until the animal died. About a month after inoculation, local lymph nodes were enlarged and lesions were present in many organs and tissues, leading to death of the animal about 3 months later. When the animal was reinoculated in the opposite flank 1 month after the original challenge, the lesion that developed was quite different. After a day or two, the skin at the injection site underwent necrosis and then sloughed off, leaving a shallow ulcer. Unlike the primary ulcer, this contained no viable bacilli and soon healed. A similar reaction, now known as the Koch phenomenon, was induced by injection of either killed tubercle bacilli or a heat-concentrated filtrate of the medium in which the bacilli had been grown, a preparation termed Old Tuberculin.

Old Tuberculin and its induction of a characteristic dermal reaction was utilized by von Pirquet in 1907 in epidemiological studies as an indicator of past infection by the tubercle bacillus. As Old Tuberculin contained some impurities from the medium, it induced small, nonspecific dermal reactions in uninfected people. This problem was resolved by Seibert in 1934, who purified the reagent by precipitating the proteins with acetone and ammonium sulfate. This reagent, purified protein derivative of tuberculin (PPD), remains the standard skin-testing reagent for human and animal use.

Skin-test reagents have also been developed for other species of the genus, including Burulin, which has been used in studies of *M. ulcerans* infections (Stanford 1983). Lepromin, a heat-sterilized homogenate of *M. leprae*-laden tissue, is a skin-test reagent used in the diagnosis of leprosy.

Tuberculin testing

The tuberculin test is used to determine the annual infection rate of TB in the community, to assess the effectiveness of control measures, and to indicate those requiring BCG vaccination or preventive therapy.

Weak or negative reactions do, however, not exclude a diagnosis of TB. Some people are intrinsically poor reactors and in others, reactivity is depressed due to advanced disease, old age, malnutrition, or immunosuppression, including human immunodeficiency syndrome (HIV) infection. Various skin-testing reagents have been developed from Koch's original Old Tuberculin. The most widely used is PPD produced by Seibert and her colleagues. The dosage of PPD is expressed in international units (IU) based on a biological standardization against an international standard preparation. In the Mantoux test, the only test that is internationally standardized, an exact amount (usually 0.1 ml) of tuberculin is injected intracutaneously and the diameter of the resulting induration is measured 72 h later. This test is the most satisfactory, as results can be quantified, but it requires some training and skill.

OTHER ANTIGENS

Mycobacterial antigens have generally been classified in terms of their chemical nature and distribution among species. Soluble antigens are the antigens found in the supernatant following high-speed centrifugation of a lysate of mycobacteria, which removes cell-wall fragments and other particulate matter. These antigens include most cytoplasmic and secreted proteins and soluble carbohydrates. Also, the sharing of antigens by the various species probably accounts for the lack of specificity of serological tests for tuberculosis.

Various chemical and immunochemical techniques have been used to characterize soluble antigens, including immunoelectrophoresis (Daniel and Janicki 1978), double diffusion in agar gel (Stanford and Grange

1974), and crossed immunoelectrophoresis (Closs et al. 1980). Although these tests identified up to 90 distinct lines of precipitation or antigens, until recently, only limited progress was made in characterizing a few of the precipitins because of difficulties in isolating and purifying the individual antigens. These difficulties were partially circumvented by the use of recombinant DNA technologies. Currently, mycobacterial antigens are being systematically characterized using a variety of sophisticated techniques including two-dimensional gel electrophoresis, mass spectroscopy, and high-performance liquid chromatography (HPLC). Overall, the combination of immunological studies, sophisticated biochemistry, recombinant DNA technologies, and the genome sequencing projects is producing a valuable catalogue of mycobacterial antigens (reviewed by Young et al. 1992; Anderson and Brennan 1994; Cole and Smith 1994; Cole et al. 1998).

One subset of soluble antigens contains proteins that are secreted during growth. The secreted proteins are the main antigenic constituents of culture filtrates of young, log-phase cultures of *M. tuberculosis*. Older cultures contain secreted proteins as well as many proteins released by autolysis of the tubercle bacilli. Secreted antigens have been suggested to be key targets of a protective immune response to *M. tuberculosis* (Horwitz et al. 1995; Roberts et al. 1995).

Soluble urokinase plasminogen activator receptor (suPAR) levels are elevated in TB patients and associated with mortality (Eugen-Olsen et al. 2002).

Insoluble antigens are those that are in the pellet following high-speed centrifugation of lysates and include cell-wall components, membrane-bound proteins, high-molecular-weight carbohydrates, lipids, mycolic acids, phenolic glycolipids, etc.

MYCOBACTERIAL GENOMES

The *M. tuberculosis* genome

The genomes of mycobacteria display a typical bacterial chromosomal structure (a single large circular DNA molecule), with a G + C content of 60–70 mol% (except for *M. leprae*, which is <57 mol%). Slow-growing mycobacteria have only one copy of the genes encoding the ribosomal RNAs, a feature that may contribute to their slow growth.

The complete genome sequence of *M. tuberculosis* H37Rv (Cole et al. 1998) consists of 4.41 Mb containing approximately 4 000 genes encoding proteins and 50 genes encoding RNAs. The 91 percent potential coding capacity and the gene density (1 gene for 1.1 kb) are typical for a prokaryote. The G + C content is constant throughout the genome, with an average of 65 percent. Unusually G + C-rich regions were detected, which correspond to sequences belonging to two large gene families, Pro-Glu (PE) and Pro-Pro-Glu (PPE). Genes are evenly distributed along the two DNA strands and 59 percent of the genes are transcribed with the same polarity as the replication fork. A high percentage of genes located on the leading strand, up to 75 percent in *Bacillus subtilis*, is thought to be associated with higher gene-expression levels, since replication and transcription act with the same polarity. The even distribution of genes in both DNA strands may explain the slow growth of *M. tuberculosis* and its 24-h generation time. In addition, the genome displays one single copy of the ribosomal RNA operon, located at unusually 1 500 kb from the origin of replication. This arrangement may also be related to the slow growth of *M. tuberculosis*, since most bacteria have several ribosomal RNA operons close to the origin of replication to exploit the gene-dosage effect obtained during replication.

Fifty-eight percent of the genes could be attributed a function, 27 percent presented similarity to putative proteins, and 15 percent resembled no known proteins, and may account for specific mycobacterial functions. The vast majority of the characterized genes of mycobacteria are structurally similar to those of other bacterial genera. By contrast, the *recA* genes of *M. tuberculosis* and *M. leprae* and some mycobacterial species are quite unusual (Fsihi et al. 1996; Colston et al. 2000). That is, the *recA* gene of *M. tuberculosis* encodes an 85-kDa protein, although the mature *M. tuberculosis* RecA protein has a molecular mass of only approximately 38 kDa. The primary translation product of the *M. tuberculosis recA* gene contains an internal amino acid sequence, called an intein, which is removed by a protein-splicing mechanism to produce an enzymatically active RecA protein. This is an interesting phenomenon because only a few examples of protein splicing are known and because it may be a novel way to regulate protein expression and function. The genome is characterized by the abundance of genes coding for enzymes involved in fatty-acid metabolism (more than 250, compared with 50 in *Escherichia coli*). All genes involved in aerobic metabolism as well as genes encoding anaerobic electron-transport chains are present. The ability of *M. tuberculosis* to adapt to environmental changes such as low oxygen tension may enable the bacilli to survive in granulomas or oxygen-poor tissues. About 9 percent of the genome is devoted to two multigene families of glycine-rich proteins, the PE and PPE families, which have a significantly higher genetic variability compared with the genome as a whole (Fleischmann et al. 2002). The PE and PPE repetitive structures may represent a source of antigenic variation or interfere with host immune responses by inhibiting antigen processing. More than 50 percent of the genes result from gene duplication, a similar percentage to that seen in other bacteria, except that the level of sequence conservation between copies is quite high, suggesting that duplication events occurred recently and that

M. tuberculosis is evolutionarily young (Sreevatsan et al. 1997).

M. tuberculosis is very rich in repeated DNA sequences, either insertion sequences (IS) or tandem repeats inserted in the intercistronic regions of genes within an operon (mycobacterial interspersed repetitive units (MIRU)). In H37Rv, 56 IS from eight different IS families have been identified. Some of these IS exhibit high similarities with those from other *Actinomycetes* such as *Rhodococcus* or *Nocardia*, suggesting that a horizontal transfer probably occurred in nature at the time the *M. tuberculosis* ancestor lived in soil and shared its ecological niche with other bacteria. IS may be the source of chromosomal rearrangements and deletions. In a population of *M. tuberculosis*, IS are present in one or two copies at the same loci, suggesting that most of these elements are inactive or exhibit a transposition immunity preventing the accumulation of additional copies. IS*6110* represents a notable exception with a wide range of copy numbers and a high polymorphism within the copy distribution. Whole-genome comparison of clinical isolates identified deletion regions (defined as sequences present in an isolate but missing from another one) and supports the hypothesis that genomic variation is largely caused by insertion sequence movement rather than point mutation (Brosch et al. 2000a; Ho et al. 2000). MIRUs, identified in 41 loci in H37Rv display polymorphism by changing the repeat copy number in most loci, which can be exploited for strain typing. Two prophages have been detected in H37Rv, which have been found stable through the analysis of various *M. tuberculosis* strains.

Although the spontaneous mutation rate of *M. tuberculosis* is similar to that of other bacteria (David 1973), sequence analysis of structural genes and of genes encoding host immune system protein targets showed a remarkably low polymorphism (Sreevatsan et al. 1997; Musser et al. 2000). By comparison, the level of allelic variation in *M. tuberculosis* is 2 000 times less than in *Borrelia burgdorferi* and 600 times less than in *Neisseria meningitidis*. The polymorphism within the *katG* gene, encoding a catalase-peroxidase, and the *gyrA* gene, encoding the subunit A of the gyrase, identifies three genetic groups. The first encompasses representatives of all tubercle bacilli, including *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*, whereas the two other groups include *M. tuberculosis* sensu stricto only.

All these observations indicate a clonal population structure of *M. tuberculosis* and a lack of interstrain genetic diversity with genome plasticity mainly generated by insertion sequences rather than point mutations and suggest that this pathogen is evolutionarily young (Sreevatsan et al. 1997; Brosch et al. 2000b; Musser et al. 2000; Kato-Maeda et al. 2001). The websites are www.genolist.pasteur.fr/TubercuList for *M. tuberculosis* H37Rv and www.tigr.org/tdb/CMR for *M. tuberculosis* CDC1551, respectively.

COMPARATIVE GENOMICS

DNA–DNA hybridization showed that all tubercle bacilli (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*) belong to a single species. However, the different members can be separated according to phenotypic tests as well as their host range and virulence. Recently, whole-genome comparisons showed insertion–deletion events that distinguished between the species. The different regions mainly correspond to metabolic functions and virulence factors. The distribution of these regions represents a useful tool for the identification of species within *M. tuberculosis* complex. Three of the deletions that contain genes for PE and PPE proteins, claimed to play a role in antigenic variation, may represent sites of hypervariability between tubercle bacilli (Gordon et al. 2001). Three regions affect genes coding for Esat6 protein family, potent T-cell antigens. Among these latter regions, RD1 is absent from all *M. bovis* BCG strains but is still present in *M. bovis* as well as in other *M. tuberculosis* complex members. This knowledge raised the development of diagnostic reagents for the tentative differentiation of vaccinated and infected individuals. The distribution of the deletion regions among the *M. tuberculosis* complex appears to be species-specific, providing promising new identification genetic tools for the differentiation of the *M. tuberculosis* complex species (Gordon et al. 1999). Comparative genomics of BCG vaccines using DNA microarrays allowed the drawing of the genealogy of BCG daughter strains and to date the year of the genetic events that prevailed to the differentiation of BCG strains (Behr et al. 1999).

The different regions correspond to sequences deleted either from *M. tuberculosis* or from *M. bovis*, suggesting that loss of genetic material occurs in both members of the complex and that *M. tuberculosis* cannot have evolved from *M. bovis*. The distribution of the genomic regions among the tubercle bacilli indicates that it is more likely that both species evolved from a common progenitor. Moreover, *M. tuberculosis* complex DNA detected in a North American Pleistocene extinct bison (dated 17 000 years ago) was identified closer to the current human bacilli *M. africanum* and *M. tuberculosis* than to *M. bovis*. Bovids were hence the vectors of the primordial organism that caused TB, and immigration of bovids over the Bering Strait introduced the disease to North America at least 20 000 years ago (Rothschild et al. 2001). A website is dedicated to the *M. bovis* genome sequence (www.sanger.ac.uk/Projects/M_bovis).

The *M. leprae* genome

M. leprae still remains noncultivable in synthetic media and represents the longest doubling time, estimated at around 14 days. The complete genome sequence shows a genome size of 3.27 megabases (Mb), much smaller than

that of *M. tuberculosis* (4.41 Mb) (Cole et al. 2001). The G + C content is also much lower, being 57.8 percent for *M. leprae* and 65.6 percent for *M. tuberculosis*, respectively. Comparative proteome analysis revealed an extreme reductive evolution, as only 391 soluble proteins could be detected (versus 1 800 in *M. tuberculosis*) instead of the 3 000 proteins predicted if all genes in the 3.27-Mb genome were active. Pseudogenes, with intact counterparts in *M. tuberculosis*, abound. The smaller genome size and the lower G + C value are partly due to the presence of only nine PE and PPE genes, also called RLEP, whereas *M. tuberculosis* contains more than 160 of these very (G + C)-rich genes. Important metabolic activities, such as siderophore production, part of the oxidative and most of the microaerophilic and anaerobic respiratory chains, as well as numerous catabolic systems have been deleted. By contrast, the anabolic pathway is relatively intact, except a possible auxotrophy for methionine. The *M. leprae recA* gene also contains an intein, but its structure and location are different from those of the *M. tuberculosis* intein (Davis et al. 1994).

Comparison of *M. tuberculosis* and *M. leprae* genomes shows mosaic structures, consisting of segments with conserved gene order and organization interspersed with different flanking regions. Extensive recombination events between dispersed repetitive sequences could explain this evolution (Philipp et al. 1996; Cole et al. 2001). Restriction fragment length polymorphism analysis of genomes of *M. leprae* isolates from diverse origins (patients from India, a monkey from West Africa, an armadillo from Louisiana) showed a high conservation of genomic sequences within the species, preventing further molecular epidemiological studies (Clark-Curtiss and Walsh 1989). The website for *M. leprae* is www.genolist.pasteur.fr/Leproma.

Genomes of other mycobacterial species

Repeated elements are also present in the other mycobacterial species. Genes from the PE and PPE families, also designated polymorphic GC-rich repetitive sequences (PGRS), major polymorphic tandem repeats (MPTR) have been detected in several species. Whereas the distribution of the MPTR (PPE genes) is very conserved in *M. tuberculosis*, these elements may be used as tools of molecular typing in *M. kansasii* and *M. gordonae*. According to the MPTR profile, *M. kansasii* strains can be divided into five groups or subspecies (Alcaide et al. 1997b; Picardeau et al. 1997a). The identification of subspecies is correlated to other markers, especially to the presence of IS*1652*, which is present in two subspecies only (Picardeau et al. 1997a). The PGRS sequences (coding for PE proteins) are also detected in *M. gordonae*, *M. kansasii*, *M. gastri*, *M. marinum*, and *M. szulgai* (Poulet and Cole 1995).

Insertion sequences have been identified in several mycobacterial species. Most IS have a narrow host range and may be used as targets for specific identification in polymerase chain reaction (PCR) tests. Some showed a high polymorphism of distribution and represent useful epidemiological markers. Although present in 15–20 copies in *M. paratuberculosis*, IS*900* does not display polymorphism and is useless for molecular typing of the strains (Green et al. 1989; Kunze et al. 1992; Pavlik et al. 1995). By contrast, IS*1245* and IS*1311* show a high polymorphism in *M. avium* (Guerrero et al. 1994; Picardeau and Vincent 1996; van Soolingen et al. 1998a).

Circular and linear plasmids

Unlike tubercle bacilli devoid of any plasmid, various mycobacterial species harbor plasmids. The first plasmids were described in 1979 in *M. avium* (Crawford and Bates 1979). Additional circular plasmids have been identified in the phenotypically related species *M. intracellulare* and *M. scrofulaceum* and later on in the rapidly growing species *M. chelonae*, *M. abscessus*, *M. fortuitum*, and *M. smegmatis* (Labidi et al. 1984; Meissner and Falkinham 1984; Beggs et al. 1995). Plasmids consist of elements of various sizes, from 5 kb to more than 100 kb. The prevalence of plasmids within strains varies from species to species. pAL5000, the smallest identified mycobacterial plasmid, has been entirely sequenced and led to the construction of shuttle vectors, allowing the expansion of the mycobacterial genetics (Rauzier et al. 1988). *E. coli*/mycobacteria shuttle vectors have been built from pAL5000 and several *E. coli* replicons for the transfer of DNA into mycobacteria.

In 1997, linear plasmids were described in several species, including *M. xenopi*, *M. celatum*, *M. branderi*, *M. avium*, and *M. fortuitum* (Picardeau and Vincent 1997, 1998). These elements range in size from 20 to 320 kb, and all of them exhibit an invertron-like structure with proteins covalently bound to their extremities. Plasmids from different species show cross-hybridizations, suggesting either possible interspecies horizontal transfer or a common origin. Analysis of the replication origin of a 23-kb plasmid, pCLP, shows that the origin of replication is identical to that of circular plasmids (Picardeau et al. 2000). Moreover, loci similar to those responsible for partition and postsegregational mechanisms in circular plasmids have been identified in pCLP (Le Dantec et al. 2001). All these observations underline the interrelation between circular and linear plasmids in mycobacteria.

Sequencing of pAL5000 did not allow the identification of functions of plasmidic genes other than those implied in replication and incompatibility determinants. For the other plasmids, few associated phenotypes have been identified (Pashley and Stoker 2000). Some plasmids are responsible for heavy-metal resistance, including resistance to mercury and copper (Meissner

and Falkinham 1984; Gavigan et al. 1997). Plasmids with genes responsible for morpholine or phenanthrene degradation have also been isolated (Waterhouse et al. 1991). The complete sequence of pCLP from *M. celatum* revealed genes similar to *M. tuberculosis* regions, especially those related to transposases. This observation suggests a possible horizontal transfer between the two mycobacterial species, as a possible trace from the time when *M. tuberculosis* and *M. celatum* shared the same ecological niche (Le Dantec et al. 2001).

Mycobacteriophages

To date, more than 250 bacteriophages have been described that infect various species of *Mycobacterium*. Mycobacteriophages are morphologically similar to phages of other bacteria and typically have hexagonal, icosahedral, or oval heads and long noncontractile tails. Phage I3 is the only mycobacteriophage with a contractile tail. The genomes of most mycobacteriophages are linear, double-stranded DNA molecules with a G + C content of 60–70 mol% and cohesive ends (Hatfull 2000).

Little is known about the mycobacterial surface receptors used by the phage (although mycoside C has been shown to be used by phage D4) or about the process by which phage DNA crosses the complex mycobacterial cell wall. Mycobacteriophages can produce bursts of 4–400 phage particles per infected cell or can establish a lysogenic state. A few phages establish true lysogeny (integration of a copy of the phage genome into the bacterial chromosome and immunity to superinfection), whereas many form pseudolysogens. In the pseudolysogenic state, phage DNA is maintained as an episomal plasmid instead of being integrated into the chromosome and is only transmitted to some of the daughter cells.

Mycobacteriophages have played important roles in the development of mycobacterial molecular biology. In a pioneering study, Tokunaga and Sellers (1964) demonstrated that mycobacteriophage D29 DNA could transfect *M. smegmatis* (Tokunaga and Sellers 1964). The most thoroughly analyzed mycobacteriophage is L5. The nucleotide sequence of this phage has been determined, open reading frames and potential protein products identified, and gene products and their functions characterized (Hatfull 2000). For example, the genes and gene products required for integration of this temperate mycobacteriophage into the mycobacterial chromosome have been identified and the mechanism of integration elucidated. This information led to the development of recombinant DNA vectors that can integrate foreign DNA into the mycobacterial genome. Other phage-derived vectors have been generated from Ms6 (Freitas-Vieira et al. 1998) and D29 (Ribeiro et al. 1997). All these vectors have the advantage of carrying a single copy of the gene at a defined chromosome site corresponding to the phage-attachment site.

One of the most promising uses of mycobacteriophages as clinical tools consists of luciferase reporter phages used for drug susceptibility testing of *M. tuberculosis* strains. *M. tuberculosis* strains are infected with a mycobacteriophage carrying the luciferase gene and exposed to antibiotics. The strain is reported as sensitive or resistant to the drug according to the light detection, i.e. the luciferase gene activity level related to the host strain viability (Jacobs et al. 1993; Carriere et al. 1997).

LABORATORY DIAGNOSIS OF MYCOBACTERIA

Because of the low infective dose of *M. tuberculosis* for humans (50 percent infective dose, fewer than ten bacilli), specimens from people with suspected or known TB must be considered potentially infectious and handled with appropriate precautions (CDC 1999). Control of aerosols and other forms of mycobacterial contamination is achieved in the laboratory by the use of properly functioning biosafety cabinets, centrifuges with safety carriers, meticulous processing techniques, and well-trained laboratory staff. BSC level 3 practices and facilities are required for laboratories assessed as being associated with a higher risk.

In recent years, there has been a growing number of new, rapid methods in clinical mycobacteriology, but remarkably, to date there is no single test for the detection of TB that can stand alone. Recent advances in this area include nonradiometric, fully automated systems for liquid culture and drug susceptibility testing (Pfyffer et al. 2003), chemical methods such as HPLC analysis of mycolic acids (Butler et al. 1992; Kellogg et al. 2001), and various molecular procedures for identification, as well as DNA fingerprinting techniques for molecular epidemiology (Vincent et al. 2003).

In the model diagnostic mycobacteriology laboratory described by Salfinger and Pfyffer (1994) and Hale et al. (2001), specimen decontamination and concentration, staining with fluorochrome dye, inoculation of primary isolation media, and direct detection of *M. tuberculosis* complex by nucleic acids amplification-based assays are carried out on the day the specimen is received. Depending on growth, identification tests by HPLC, DNA probes, or gene sequencing can be carried out a few days after inoculation of liquid media. If not identified by molecular methods (e.g. by a line probe assay) biochemical tests require 7–21 days for identification within *M. tuberculosis* complex after the primary culture is grown fully. Drug susceptibility testing is being completed in 7–10 days using modern growth-based systems (compared with at least 21 days using the conventional agar proportion method). Because of the need for rapid turnaround, authors have emphasized

procedures that streamline laboratory performance and identified subsets of priorities and activities for 'point-of-care,' 'fast track,' and 'specialty' laboratories. Taken together, such laboratories form a 'dynamic acid-fast network' to improve mycobacteriology laboratory service.

Collection of specimens and preparation for primary culture

Many different types of clinical specimens may be collected for mycobacterial analyses (Pfyffer et al. 2003). Mostly, they originate from the respiratory tract (sputum, tracheal, bronchial aspirates; bronchoalveloar lavage fluid specimens), but urine, gastric aspirates, tissue, biopsy specimens, and normally sterile body fluids (such as cerebrospinal fluid, pleural, and pericardial aspirates) are other commonly submitted specimens. There is seldom a need to examine blood, bone marrow, or feces in the routine diagnosis of TB, although they are usually analyzed for other mycobacterial infections in predominantly immunocompromised patients. Specimens should always be collected and submitted in leak-proof, disposable, appropriately labeled laboratory-approved containers without any fixatives. Generally, transport media or preservatives are not necessary, owing to the robust nature of mycobacteria. There are several guidelines that carefully describe how specimens have to be collected, submitted, and stored before processing in the laboratory (Kent and Kubica 1985; Pfyffer et al. 2003).

Patient specimens, especially sputum specimens, contain microorganisms other than mycobacteria. Treatment with a mucolytic agent frees the mycobacteria entrapped in mucus and makes the specimen easier to process. Treatment with a toxic agent kills contaminating microorganisms (Kent and Kubica 1985), but over-treatment will also kill many of the mycobacteria. The *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method is the procedure used in most clinical laboratories in the western hemisphere (Kent and Kubica 1985; Heifets and Good 1994; Smithwick 1994). Other methods are available for use when contamination is heavy. Overall, no single pretreatment method is ideal. The best yield of mycobacteria may be expected to result from the use of the mildest decontamination procedure that sufficiently controls contaminants.

Acid-fast smear microscopy

Mycobacteria are acid-alcohol fast, i.e. once stained with a specific dye, the cells resist decolorization with acidified ethanol. This staining characteristic is shared only by species of closely related genera that have mycolic acids as a constituent of their cell walls, e.g. *Dietzia*, *Gordonia*, *Tsukamurella*, *Skermania*, *Nocardia*, *Rhodococcus*, and *Corynebacterium* (Goodfellow and Minnikin 1984). In addition, *Legionella micdadei*, as well as the oocysts of *Cryptosporidium*, *Isospora*, and *Cyclospora*, may exhibit various degrees of acid-fastness. The basis of the acid-fast staining reaction is not fully understood, but it appears to be related to the presence of mycolic acids in the cell wall and to the integrity and viability of the cell (Kölbel 1984).

Microscopy provides a simple, sensitive, and rapid means of detecting open, infectious cases of pulmonary TB. Detection of acid-fast bacilli (AFB) by smear examination is, therefore, used in many developing countries as the only test to confirm the diagnosis of TB and to measure the relative infectiousness of a patient. To be detected microscopically, there must be between 5×10^3 and 5×10^4 AFB in 1 ml of sputum. While 10^6 AFB/ml of specimen usually results in a positive smear, only 60 percent of the smears are positive if 10^4 AFB/ml are present (European Society for Mycobacteriology 1991). The overall sensitivity of the smear has been reported to range from 22 to 80 percent (Lipsky et al. 1985). Normally, its predictive value for *M. tuberculosis* in expectorated sputum is over 90 percent. It is, however, not possible to distinguish members of the *M. tuberculosis* complex from other mycobacteria by their staining properties. Even though in liquid media *M. tuberculosis* shows serpentine cording, one has to be aware that cords are also seen with some NTM species (Morris and Reller 1993).

Smears can be taken directly from sputum specimens. However, it is advisable to perform it from a homogenized, decontaminated, and concentrated specimen, i.e. after pretreatment to enhance sensitivity. Standard methods are the Ziehl–Neelsen acid-fast stain (hot staining procedure), Kinyoun acid-fast stain (cold staining procedure), or auramine O fluorescence acid-fast stain (Collins et al. 1985; Kent and Kubica 1985). In the classical Ziehl–Neelsen procedure, stain is prepared by dissolving 0.3 g basic fuchsin in 10 ml 90–95 percent ethanol or methylated spirits and mixing with 90 ml of phenol solution (5 g phenol crystals in 95 ml distilled water). A volume of approximately 0.01 ml of specimen is smeared over a 2-cm^2 area of the slide and heat-fixed. Approximately five drops of fuchsin-phenol solution is added and the bottom of the slide heated with a Bunsen burner or electric heater until the stain begins to steam. After heating for 5 min, the smears are rinsed with tap water, and drained. Next, the smears are flooded with an acid-alcohol solution (3 ml concentrated hydrochloric acid plus 97 ml 90–95 percent ethanol or methylated spirits) for at least 2 min, rinsed in tap water, and drained. The smear is then counterstained with methylene blue for 1–2 min.

Fuchsin-stained smears should be examined with a light microscope by making three longitudinal sweeps of the stained area along the length of the slide. About 100 fields will be seen in a single sweep at a magnification of 1000-fold. Mycobacteria appear as red to pink rods on a blue background. Auramine-stained smears are examined with a fluorescence microscope at a lower power

(250×). Therefore, only 30 fields need to be examined. A smear can be examined in approximately 1.5 min with fluorescence microscopy (30 fields) as opposed to approximately 15 min by light microscopy (300 fields). If fluorescent particles are seen, they must be observed with higher magnification to confirm the typical morphology of AFB. Ideally, all fluorochrome-positive smears should be confirmed by a carbol-fuchsin staining method. A smear is considered negative if no AFB are seen. One or two bacilli per 300 fields indicate that another specimen should be analyzed.

Culture

Despite the advances in direct detection of *M. tuberculosis* complex in clinical specimens by molecular methods, culture is, at present, indispensable for a number of reasons. It (1) enhances diagnostic certainty, particularly when nucleic acid amplification (NAA)-based results are available; (2) provides biomass for further identification and antimicrobial susceptibility testing; and (3) detects NTM in addition to *M. tuberculosis* complex.

For detection of mycobacteria in clinical specimens, the current 'gold standard' consists of a combination of solid and liquid media. Solid and semisolid media have been known for a long time. Liquid media are more attractive as they offer significantly shorter turnaround times for detection of mycobacteria. In detecting as few as 10^1–10^2 viable organisms/ml of specimen in the optimal case, culture is more sensitive than smear. Also, it is the only reliable means to monitor effectiveness of therapy in TB patients (Vidal et al. 1996).

SOLID MEDIA

Egg-based media such as Löwenstein-Jensen (LJ), Ogawa, or Stonebrink have the capability to bind and neutralize toxic compounds encountered in clinical specimens. Usually, they contain malachite green, a dye that inhibits growth of contaminating organisms. Egg-based media have a long shelf life and support good growth of *M. tuberculosis* but need, on average, 18–24 days. Since they are nonsynthetic, the quality of the ingredients may vary considerably, which may affect the reproducibility of results. Also, when becoming contaminated, they may liquefy.

Agar-based media, e.g. Middlebrook 7H10 or 7H11, are chemically better defined and show growth of *M. tuberculosis* complex colonies within 10–12 days. Visual examination of the colonies is much easier. Negative aspects of agar-based media have limited shelf life and higher costs of preparation.

LIQUID MEDIA

Apart from traditional broths (e.g. Middlebrook 7H9 or Dubos Tween Albumin) and the biphasic Septi-Chek System (Becton Dickinson Microbiology Systems, Sparks, MD), the radiometric, semiautomated BACTEC 460TB System (Becton Dickinson; Siddiqi 1995) represented the most efficient and rapid technique to culture mycobacteria for more than two decades and is still used by many laboratories to date. By this method, ^{14}C-labeled palmitic acid as a carbon source in the medium is metabolized by microorganisms to $^{14}CO_2$, which is monitored by the instrument. The amount of $^{14}CO_2$ and the rate at which the gas is produced are directly proportional to the growth rate of the organisms. For *M. tuberculosis*, an average detection time of 8 days was found for smear-positive specimens, compared with 19 days on nonradioactive conventional solid medium (Roberts et al. 1983). For smear-negative specimens, an average recovery time of *M. tuberculosis* between 14 days in the BACTEC 460TB and 26 days on conventional medium has been reported (Morgan et al. 1983). Problems associated with the use of radioisotopes and the potential of needle punctures among technicians have implied, however, the search for nonradiolabeled and operationally safer alternatives.

Although utilizing different detection principles, the new culture concepts have in common that they are based on nonradiometric liquid media. Developments range from manual systems utilizing simple tubes (MB Redox, Heipha Diagnostica Biotest, Heidelberg, Germany; Mycobacteria Growth Indicator Tube (MGIT), Becton Dickinson) to fully automated systems (BACTEC MGIT 960, Becton Dickinson; MB Bac/T, Bio-Mérieux, Marcy-L'Etoile, France; ESP Culture System II, Trek Diagnostic Systems, Westlake, OH).

The nonautomated MB Redox technique is based on a modified Kirchner medium that contains a colorless tetrazolium salt as a redox indicator, which is reduced to colored formazan by actively growing mycobacteria. AFB can then be detected visually as pink to purple pinhead-size particles. Another manual culture method is represented by the MGIT (Becton Dickinson Microbiology Systems), which contains a modified 7H9 broth in conjunction with a fluorescence-quenching-based oxygen sensor. Growth of mycobacteria or other microorganisms in the broth depletes the oxygen, and the indicator fluoresces brightly when tubes are illuminated with ultraviolet (UV) light at 365 nm.

The fully automated systems that allow continuous monitoring of mycobacterial cultures are (1) the BACTEC MGIT 960 based on the MGIT technology; (2) the MB/BacT utilizing a colorimetric carbon dioxide sensor in each bottle to detect growth of mycobacteria; and (3) the ESP Culture System II, which is based on the detection of pressure changes in the headspace above the broth medium resulting from gas

production or consumption due to growth of microorganisms.

All these new systems have similar performance characteristics. In clinical evaluations, recovery rates and time to detection were similar to those of the BACTEC 460TB System and superior to those of conventional solid media (MB Redox (Somoskövi and Magyar 1999); manual MGIT (Somoskövi and Magyar 1999); BACTEC MGIT 960 (Hanna et al. 1999); MB/BacT (Hanna et al. 1999; Whyte et al. 2000); ESP Culture System II (Woods et al. 1997)). Fully automated systems are certainly less labor-intensive than conventional culture and address safety more appropriately. However, as a consequence of continuous monitoring, these systems are both instrument- and space-intensive. Also, some automated systems lack the versatility of the BACTEC 460TB System (e.g. no incubation temperature available other than 37°C; no medium available for the inoculation of blood).

GROWTH CONDITIONS

Inoculated Middlebrook agar media require a CO_2 atmosphere to ensure growth, but CO_2 is not essential for growth on egg-based media, although it stimulates early, more luxuriant growth. To ensure penetration of the gas into containers, tubes should be incubated in the slanted position with caps loose for at least 1–3 weeks before caps are tightened, and plates should be placed medium side down in CO_2-permeable plastic bags until the inoculum has been absorbed. The optimal temperature for recovery of most mycobacteria in the clinical laboratory is 35–37°C. *M. marinum*, *M. ulcerans*, and *M. haemophilum* require 25–33°C, while *M. avium* and *M. xenopi* should ideally be incubated at 40–42°C. The optimal pH for growth of mycobacteria is 6.0–6.5, but this may vary from species to species.

Many *Mycobacterium* spp. are obligate aerobes that will grow at or near the surface of semisolid medium (Collins et al. 1984). Microaerophilic species such as *M. bovis* and some *M. africanum* strains grow as a narrow band 5–20 mm below the surface of the medium.

Identification and typing methods of mycobacteria

The traditional methods of identification of mycobacteria, based on morphological and biochemical properties, are well established, standardized, reproducible, and relatively inexpensive, but they are limited in scope to the high number of species currently described. Mycobacteria are usually preliminarily identified by traits such as growth rate and pigmentation according to Runyon's criteria (Runyon 1959). Additional biochemical tests help for further characterization but do not always allow an accurate characterization at the species level. The current identification scheme of mycobacteria mainly relies on molecular methods along with some key phenotypic tests.

PHENOTYPIC TESTS

Biochemical tests

Determination of the growth rate remains a key test as mycobacteria are classified in rapidly and slowly growing species. Main tests also include determination of the preferential growth temperature, pigmentation, photoreactivity, and colony morphology. The most useful biochemical tests for the precise characterization of species within *M. tuberculosis* complex are the niacin accumulation test, two tests based on catalase, including a semiquantitative test at room temperature and thermotolerance, nitrate reductase, pyrazinamidase, and inhibition by thiophene-2-carboxylic acid hydrazide. A limited list of other tests useful for other mycobacteria includes arylsulfatase, iron uptake, sodium chloride tolerance, tellurite reduction, Tween 80 hydrolysis, and urease. Procedures and distinctive properties of most frequent mycobacteria in clinical practice have recently been revised (Vincent et al. 2003).

Mycolic acid analysis

Mycolic acids are present in all mycobacteria. Their composition is constant for all strains of a given species and varies from species to species (Minnikin et al. 1980; Daffé et al. 1983). An HPLC method for analysis of mycolic acid esters, has been standardized and demonstrated to be a rapid and reliable method for identification of many *Mycobacterium* species (Butler et al. 1991; Butler et al. 1992). In this procedure, mycolic acids are extracted from saponified mycobacteria, converted to *p*-bromophenacyl esters, and analyzed by HPLC. The mycolic acid esters are separated on a reversed-phase C18 column by a methanol-methylene chloride gradient elution and detected by UV or fluorescence detection spectrophotometry.

The standardized method recommends a visual comparison of a sample HPLC pattern to an atlas of reference strain patterns in combination with the use of peak height ratios. A fully integrated automated FL-HPLC system, SMIS (Sherlock Mycobacteria Identification System, MIDI, Inc., Newark, DE) was developed with a library containing entries for 26 mycobacterial species or groups. The SMIS has demonstrated an overall accuracy of 85–90.6 percent (Kellogg et al. 2001). Although initial equipment costs are high (approximately US $50 000), the method represents an interesting diagnostic tool. However, identification may be limited to assign a 'complex of species' and not individual species.

GENOTYPIC IDENTIFICATION OF MYCOBACTERIAL SPECIES

Commercially available tests

Several kits have been developed for the identification of mycobacterial strains. They consist of either separate probes or multiple probes dotted on a membrane strip.

Separate probes (Gen-Probe Inc., San Diego, CA) rely on the detection of rRNA and have been developed for *M. tuberculosis* complex, *M. avium* complex, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. gordonae*. Tests are very specific and sensitive for *M. tuberculosis* complex and *M. gordonae* (100 percent). Sensitivity may vary from 85 to 100 percent with the other species (Lebrun et al. 1992; Tortoli et al. 1996; Richter et al. 1999). Since probes consist of acridinium ester-labeled DNA, hybridization detection requires a luminometer. From culture, test time is within 2 h.

In the InnoLiPA multiplex probe assay (INNO-LiPA Mycobacteria, Innogenetics, Ghent, Belgium), probes consist of the internal transcribed spacer (ITS) region of approximately 280 bp, which separates the 16S and 23S rDNAs. The first version of the kit included probes for the *Mycobacterium* genus, *M. tuberculosis* complex, and seven specific probes for NTM (Viljanen et al. 1993). Several probes were included for *M. kansasii* and *M. chelonae*. Cross-reactions were expected to occur with *M. gastri* and *M. kansasii* as well as with *M. abscessus* and *M. chelonae*. A newly developed version includes additional probes and extends the identification to other species. Another multiplex probe assay is available from Hain Lifescience (Nehren, Germany). The kit is similar to the InnoLiPA, but probes consist of the specific alleles of the *gyrB* gene. The kit has no probe for the *Mycobacterium* genus but includes probes for *M. tuberculosis* complex and 14 other mycobacterial species.

Both assays run in 6 h and are more comfortably ensured with an automated machine, Auto-LiPA (Tecan, Switzerland), which performs washes and solution changes at the different steps of the procedure. According to various studies, specificity and sensitivity of the kits are high for most species but limited for the rapidly growing mycobacteria (Miller et al. 2000; Park et al. 2000; Tortoli et al. 2001; Mijs et al. 2002; Ruiz et al. 2002).

PCR/restriction enzyme analysis

The polymorphism of conserved genes can be determined using a simple PCR amplification of the gene followed by restriction enzyme digestion. Telenti et al. (1993) developed a method for mycobacteria based on the *hsp65* gene encoding for the 65-kDa heat-shock protein and proposed an algorithm of species identification based on the restriction profiles generated by *Hae*III and *Bst*EII. The method has been extensively applied and catalogs of profiles extended to various species (Devallois et al. 1997; Ringuet et al. 1999; Brunello et al. 2001). However, the method is hampered by the polymorphism of the gene within individual species (i.e. several alleles) on the one hand and by the high number of mycobacterial species, which both contribute to ambiguous profiles and complex databases, on the other hand. A website is dedicated to polymerase chain reaction/restriction enzyme analysis (PRA) profiles (www.hospvd.ch/prasite).

Other genes have been studied following the same procedure, PCR amplification followed by restriction enzyme analysis. These include the *rpoB* gene that mediates rifampin resistance, the *dnaJ* gene, and the 16S-23S rRNA spacer gene. None have been studied as extensively as *hsp65* (Takewaki et al. 1994; Kim et al. 1999; Park et al. 2000).

DNA sequencing

Because of the slow growth of mycobacteria, DNA sequencing for species identification is very attractive. Identification of species–specific signatures within variable regions of highly conserved genes such as 16S rDNA (*rrs* gene) or *hsp65* allowed the design of PCR protocols using genus-specific primers followed by the direct sequencing of the complete or full PCR products (Kirschner et al. 1993; Kapur et al. 1995; Springer et al. 1996). Other targets may also be used such as the gene encoding the 32-kD protein (Soini et al. 1994), the *dnaJ* gene (Takewaki et al. 1994), the *sod* gene encoding the superoxide dismutase (Zolg and Philippi-Schulz 1994), the *gyrB* gene coding for the gyrase subunit B (Kasai et al. 2000), the *rpoB* gene coding for the RNA polymerase (Kim et al. 1999), or the ITS 16S-23S sequence (Roth et al. 1998; Park et al. 2000; Roth et al. 2000). Catalogs of sequences of mycobacterial species may be retrieved from databases (websites at www.ncbi.nlm.nih.gov (GenBank/EMBL) and www.ridom.de/mycobacteria/).

Genes have different molecular clocks and the interspecies polymorphism varies from gene to gene. The molecular clock of the *rrs* gene (16S rRNA) is rather slow. In other words, species of recent divergence may have similar *rrs* gene sequences. As a matter of fact, *M. kansasii* and *M. gastri*, *M. ulcerans* and *M. marinum*, *M. shimoidei* and *M. triviale*, and *M. abscessus* and *M. chelonae* cannot be differentiated according to their *rrs* signatures (Kirschner et al. 1993). Moreover, no single 16S rDNA interstrain nucleotide sequence difference value that unequivocally defined species boundaries has been established for the *Mycobacterium* genus (Wayne et al. 1996). For instance, the *rrs* gene shows a two-nucleotide difference only in the 1 384-nucleotide sequence of the distinct species *M. szulgai* and *M. malmoense*, but one to seven different nucleotides in a 782-nucleotide segment of some *M. intracellulare* strains

(Wayne et al. 1996). By contrast, the molecular clock of the *hsp65* gene is much faster and several alleles are usually identified within a species. Species that cannot be differentiated with 16S rDNA sequencing such as *M. gastri* and *M. kansasii* or *M. abscessus* and *M. chelonae* show distinct *hsp65* sequences (Kapur et al. 1995; Ringuet et al. 1999).

GENOTYPIC MARKERS FOR SPECIES IDENTIFICATION WITHIN *M. TUBERCULOSIS* COMPLEX

All the *M. tuberculosis* complex members have the same *rrs* and *hsp65* alleles. The *gyrB* gene shows a polymorphism within the complex that allows a differential identification of the members of the *M. tuberculosis* complex (Niemann et al. 2000a). G*yrB* alleles have been included in a commercially available line probe assay for identification within *M. tuberculosis* complex (Hain Lifescience).

Several markers contribute to the differentiation of the different members of *M. tuberculosis* complex. The first one to be proposed to achieve this goal was the *mtp40* gene, coding for phospholipase C. The gene is usually present in *M. tuberculosis* and absent from *M. bovis*, but numerous exceptions have been described (Parra et al. 1991; Weil et al. 1996). *oxyR* and *pncA* genes show single mutations that allow the differentiation of *M. bovis* from the other members of *M. tuberculosis* complex. In *M. bovis*, the *oxyR* gene has an adenine at nucleotide 285 and the *pncA* gene has a guanine nucleotide at position 169, whereas guanine and cytosine are found at these positions, respectively, in *M. tuberculosis*, *M. africanum*, and *M. microti* (Espinosa de los Monteros et al. 1998). However, *M. tuberculosis* subsp. *caprae* is characterized by a polymorphism characteristic of *M. tuberculosis* in *pncA* and characteristic of *M. bovis* in *oxyR* (Aranaz et al. 1999). PCR tests developed with allele-specific primers allow an easy detection of the mutations and identification of *M. bovis* and *M. bovis* subsp. *caprae* (Espinosa de los Monteros et al. 1998). The comparative genomics of *M. tuberculosis* and *M. bovis* allowed the identification of regions of difference that contribute to the identification of the members of *M. tuberculosis* complex. Absence of RD1 is characteristic of *M. bovis* BCG, absence of RD4 of all *M. bovis*, and absence of RD9 of *M. africanum* (Gordon et al. 1999; Brosch et al. 2002). Moreover, spoligotyping, a strain-typing method described below, also contributes to the differential identification within *M. tuberculosis* complex.

DIRECT AMPLIFICATION TESTS

Because of the slow growth of the tubercle bacilli, great attempts were expected from direct amplification procedures to get a rapid, sensitive, and specific detection of *M. tuberculosis* complex directly from clinical specimens. Three commercial kits are currently available. Tests are directed against 16S rRNA (MTD, Gen-Probe Inc.) or against the 16S rRNA (*rrs*) gene (Amplicor, Roche Diagnostic Systems, Branchburg, NJ; Probetec, BD Biosciences, Sparks, MD). Manufacturers recommend the tests to be applied to respiratory specimens only. However, with specific modifications, detection of *M. tuberculosis* has been reported in nonrespiratory specimens (Vlaspolder et al. 1995; Pfyffer et al. 1996). Both tests are rapid and can be performed in a single working day. Although in vitro sensitivity is very high (as few as one cell equivalent), sensitivity in clinical specimens turned out to be less accurate and mainly depends on the number of bacilli per milliliter present in the specimen. The sensitivity of specimens containing 100 or fewer cells of *M. tuberculosis* per milliliter has been reported to be between 65 and 85 percent, whereas the sensitivity with samples containing more than 1 000 cells per milliliter was almost 100 percent (D'Amato et al. 1995; Moore et al. 1996; Pfyffer et al. 1996; Piersimoni et al. 1997; Alcala et al. 2001). The specificity is excellent for both tests. However, a discrepant positive test result was obtained with *M. celatum* containing specimens with the MTD kit (Tjhie et al. 2001). Since both tests rely on 16S rRNA or the *rrs* gene, it has to be stressed that detection corresponds to *M. tuberculosis* complex with no specific identification within the complex.

MOLECULAR TYPING *OF M. TUBERCULOSIS*

IS*6110* restriction fragment length polymorphism

The restriction fragment length polymorphism (RFLP) technique using the IS*6110* repetitive sequence as a probe is considered the gold standard for typing *M. tuberculosis* complex strains. IS*6110* is a 1 355-bp repetitive insertion sequence that displays variability both in the copy number, from 0 to approximately 25 in *M. tuberculosis* complex strains, and chromosomal positions. With a 3–4-year half-life, i.e. 50 percent of *M. tuberculosis* strains may display one band shift within a 3–4-year period, IS*6110* RFLP patterns are stable enough to ensure reliable epidemiological studies (van Soolingen 2001). A standardized protocol has been proposed for IS*6110* RFLP comparison (van Embden et al. 1993). Standardization of the procedure facilitates interlaboratory comparability of patterns, which still requires sophisticated software for image analysis and well-trained users (Salamon et al. 1998). A comparative study of several typing methods demonstrated that RFLP IS*6110* is the most discriminative method. PCR-derived methods targeting IS*6110* have been developed to shorten the delay of strain comparison (Haas et al. 1993; Prod'hom et al. 1997; Bonora et al. 1999).

Spoligotyping

The spoligotyping method (abbreviation for spacer oligotyping) relies on the polymorphism of the direct repeat (DR) locus, a unique locus specific for *M. tuberculosis* complex (Kamerbeek et al. 1997; van Soolingen 2001). The DR region contains multiple, conserved 36-bp repeats interspersed with nonrepetitive short spacer sequences of 34–41 bp length. The strains are tested by hybridizing their PCR-amplified DR regions to a membrane that consists of an array of 43 covalently bound oligonucleotides representing the polymorphic spacers identified in the *M. tuberculosis* H37Rv DR sequence (spacers 1–18, 21–32, 37–43) and in the sequence of *M. bovis* BCG (spacers 19–20 and 33–36). Strains vary in the numbers of 36-bp repeated elements and in the presence or absence of some spacers. Spoligotypes appear highly stable but less polymorphic than IS*6110* RFLP patterns (Kremer et al. 1999). On the other hand, spoligotyping allows the identification of prevalent genotypes, especially the Beijing genotype, frequently encountered in Asia and in the former USSR (Pfyffer et al. 2001) and responsible in the USA for the largest known epidemic of drug-resistant strains (Bifani et al. 1996; Agerton et al. 1999).

Spoligotypes contribute to the identification of the *M. tuberculosis* members. The *M. tuberculosis* spoligotypes are characterized by the absence of spacers 33–36 whereas *M. bovis* spoligotypes usually lack spacers 39–43 and *M. africanum* spoligotypes lack spacers 8, 9, and 39 (Table 1) (Kamerbeek et al. 1997; Viana-Niero et al. 2001). Moreover, *M. microti* as well as *M. canettii* and *M. tuberculosis* subsp. *caprae*, two recently described new taxons in the *M. tuberculosis* complex, display very characteristic spoligotypes (van Soolingen 2001; Vincent et al. 2003) (Table 1).

PCR strategies targeting loci other than IS*6110*

As indicated above, MIRUs are tandem repeats inserted in the intercistronic regions of genes within an operon and that display polymorphism by changing the repeat copy number in most loci. This variability can be exploited for strain typing, with the advantage that patterns are reduced to a simple numerical format, corresponding to the number of repeated units in each locus. A method based on 12 MIRU loci has been developed (Supply et al. 2000; Mazars et al. 2001).

Molecular typing of other mycobacterial species

Pulsed-field gel electrophoresis is the most widely used technique for mycobacteria other than members of *M. tuberculosis* complex. However, the description of insertion sequences (IS*1245*/IS*1311*) in *M. avium* allows discriminative analysis using either RFLP or a PCR-method targeting these elements (Guerrero et al. 1994; Picardeau and Vincent 1996; Picardeau et al. 1997b).

Achievements of molecular typing

Molecular typing allows the study of the population structure of the *M. tuberculosis* complex to define prevalent families and contributes to elucidating the intraspecies genetic microevolution. Molecular epidemiology considers that if strains show identical or highly similar patterns, then the respective patients belong to clusters of chains of transmission. Risk factors associated with clustering as well as places at risk for TB infection were identified in low- and high-incidence areas, in HIV-positive or -negative patients (Small et al. 1994; Bauer et al. 1998; Richner et al. 1999; Warren et al. 1999; van Soolingen 2001). However, these assumptions have to take into account the epidemiological situation of the region (level of incidence, trend of annual risk for infection) as well as the age of the patients, as it has been shown that, in some settings, clustering may be an unreliable indicator of recent transmission (Murray and Nardell 2002; Vynnycky et al. 2003). Moreover, DNA fingerprinting unexpectedly showed that transmission of TB can occur through very short contacts and outside the ring of contacts (van Soolingen 2001). Molecular studies indicated that strains resistant to isoniazid with different genetic mutations have different abilities to generate secondary cases (van Soolingen 2001). Molecular typing helps to differentiate relapses from exogenous reinfections and demonstrate false-positive cases due to laboratory cross-contamination of clinical samples during culture procedures (Small et al. 1993; van Rie et al. 1999).

Molecular typing of other mycobacteria was extensively used for *M. avium* strains isolated from acquired immunodeficiency syndrome (AIDS) patients. Studies showed the high diversity of the strains, mostly monoclonal infections and persistence of strains in tissues as well as in tap water over years (Arbeit et al. 1993; Picardeau et al. 1997b). Since strains from birds show a characteristic two-band pattern rarely found in humans, molecular studies demonstrated that birds represent an unusual source of *M. avium* infection in man (Ritacco et al. 1998). Molecular studies helped to elucidate nosocomial outbreaks due to rapidly growing mycobacteria, especially of respiratory tract colonization due to *M. fortuitum* (Hector et al. 1992) or postsurgical wound infections following a variety of surgical procedures (Brown-Elliott and Wallace 2002).

Susceptibility testing of *M. tuberculosis* complex

Drug susceptibility testing is mandatory on initial isolates of *M. tuberculosis* and related species from all patients. If culture remains positive over a longer period

Table 1 *Characteristic spoligotypes of some* Mycobacterium tuberculosis *complex members*

Strain	Spoligotype 1	9	19	29	39	43	Ref.
M. tuberculosis H37Rv	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ □ □ ■ ■ ■ ■ ■ ■ ■ ■ ■ □ □ □ □ ■ ■ ■ ■ ■ ■						Kamerbeek et al. 1997
M. bovis BCG Pasteur	■ ■ □ ■ ■ ■ ■ ■ □ ■ ■ ■ ■ ■ □ ■ □ □ □ □ □						Kamerbeek et al. 1997
M. tuberculosis Beijing or W	□ ■ ■ ■ ■ ■ ■ ■ ■ ■						van Soolingen et al. 2001
M. tuberculosis subsp. *caprae*	□ ■ □ □ □ □ □ □ □ □ □ □ □ □ □ □ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ □ ■ □ □ □ □ □ ■ ■ ■ ■ □ □ □ □ □						Aranaz et al. 1999
M. canettii	□ ■ □ □ □ □ □ ■ □ □ □ □ □ □						van Soolingen et al. 1997
M. microti	□ ■ ■ □ □ □ □ □						van Soolingen et al. 1998

■ positive hybridization; □ negative hybridization.

of time, susceptibility testing should be repeated to monitor a possible development of drug resistance. The new guidelines by the National Committee of Clinical Laboratory Standards (NCCLS) currently recommend repeating susceptibility testing at least every 3 months (NCCLS 2003).

Most experience is available on testing primary antibiotics, i.e. isoniazid (INH), rifampin (RMP), ethambutol (EMB), and pyrazinamide (PZA) by culture-based methods. Although there are three accepted methods for drug susceptibility testing of *M. tuberculosis* (i.e. the absolute concentration method, the resistance ratio method, and the proportion method) (Inderlied and Nash 1996), the latter is widely used in the western hemisphere. By this method, for most of the antituberculous drugs, resistance of *M. tuberculosis* is defined as presence of resistant organisms in >1 percent of a given population of *M. tuberculosis* cells. Therapy will ultimately be successful if the critical proportion of organisms and the critical concentration of a drug (i.e. the concentration that inhibits the wild type but not the resistant mutants) are strictly defined and, accordingly, the strain is fully susceptible to front-line drugs in vitro. Despite more rapid culture-based techniques for drug susceptibility testing, NCCLS, for instance, still considers the agar proportion method the gold standard against which all other, more rapid, culture-based methods have to be tested (NCCLS 2003).

Susceptibility results for the primary anti-TB drugs can be rapidly generated by the BACTEC 460TB. Once resistance to one or more drugs is being detected, it is good laboratory practice to confirm it either by a second method (e.g. the agar proportion method) or by another laboratory since the impact of resistant strains on patient management is serious (Hale et al. 2001). Although susceptibility testing on solid media (e.g. Middlebrook 7H10 agar or LJ) for confirmation of resistance takes some time, it is important since it can indicate false resistance due to a mixed population of *M. tuberculosis* and NTM. If resistance to one or several primary drug(s) is being detected, an extended spectrum of drugs may be tested, either by radiometric procedure (Pfyffer et al. 1999) or the agar proportion method (NCCLS 2003). Above all, susceptibility results should be reported without delay to the healthcare provider.

In the past decade, antimicrobial susceptibility testing has become a dynamic field spawning many new technologies that may one day prove successful in a clinical mycobacteriology laboratory. Among the novel strategies are, again, several of the new, non-radiometric culture methods. Preliminary studies, which include susceptibility testing of *M. tuberculosis* to INH, RMP, EMB, and streptomycin (SM) by the BACTEC MGIT 960 (Bémer et al. 2002), the MB Bac/T (Diaz-Infantes et al. 2000), and the ESP Culture System II (Bergman and Woods 1998), show excellent overall agreement with the proportion method on both solid and liquid media as well as promising turnaround times comparable to those by radiometry. Susceptibility testing of *M. tuberculosis* to PZA is more difficult due to the requirement of a more acidic pH value in the medium. The very first study based on the BACTEC MGIT 960 technique reports good agreement of the results with those generated by the BACTEC 460TB System (Pfyffer et al. 2002).

Apart from strictly growth-based strategies, an increasing number of approaches have assessed drug susceptibility by identifying alternative markers of drug-resistant metabolic activities. Among those are colorimetry, flow cytometry, bioluminescence (assay of mycobacterial adenosine triphosphate), and quantitation of mycobacterial antigens (for review, see Pfyffer 2000). Mycobacteriophage-based methods appear to be promising as well (Albert et al. 2001).

Although molecular approaches (cf. Ramaswamy and Musser 1998) would provide the clinician with susceptibility results within 1–2 days, most of these protocols are not yet ready for the clinical mycobacteriology laboratory. Resistance in *M. tuberculosis* complex organisms arises from mutations that are confined to chromosomal DNA and do not involve mobile genetic elements such as plasmids. Molecular research has shown that often more than a single mutation is responsible for drug resistance, making the whole issue very complex, particularly since different genes may be involved. Resistance to INH, for instance, appears to be the result of a single or multiple mutation(s) in the *katG*, *inhA*, *oxyR-ahpC*, and/or *kasA* gene(s). Worse, for some INH-resistant strains, the respective gene bearing the mutation has not yet been found. The only exception represents RMP resistance. Owing to the fact that more than 97 percent of all RMP-resistant strains carry a mutation in a specific core region of the *rpoB* gene (Telenti et al. 1993a), resistance to that drug can easily and reliably be detected by molecular methods. This may be achieved by gene sequencing or by a commercially available line probe assay (Rossau et al. 1997). The latter detects nucleotide changes in the relevant part of the *rpoB* gene by different oligonucleotide probes.

DESCRIPTION OF SPECIES AND CLINICAL RELEVANCE OF MYCOBACTERIA

Tuberculosis

HISTORY

TB is a disease of great antiquity and has almost certainly caused more suffering and death than any other infection. The clinical features of both respiratory and spinal TB were well described by Hippocrates in about 400 BC; accounts of the disease appeared in the

Vedas and other ancient Hindu texts, in which it was termed *rajayakshma* – the king of diseases – and it afflicted neolithic man and pre-Columbian Amerindians.

The transmissible nature of TB was established by Villemin in 1868 by inoculating rabbits with tuberculous material from humans and cattle. Villemin also established that scrofula (tuberculous lymphadenitis) and pulmonary TB were manifestations of the same disease process. In 1882, Robert Koch succeeded in cultivating the bacillus on inspissated serum and transmitted the disease to many animals of different species by inoculation with pure cultures of the bacillus. In addition to cultivating the causative organism, Koch succeeded in staining it by treatment with an alkaline solution of methylene blue for 24 h. Subsequently, Ehrlich improved the technique by using a hot solution of carbol fuchsin, and it is this technique, slightly modified by Ziehl and Neelsen whose names it bears, that is still widely used today.

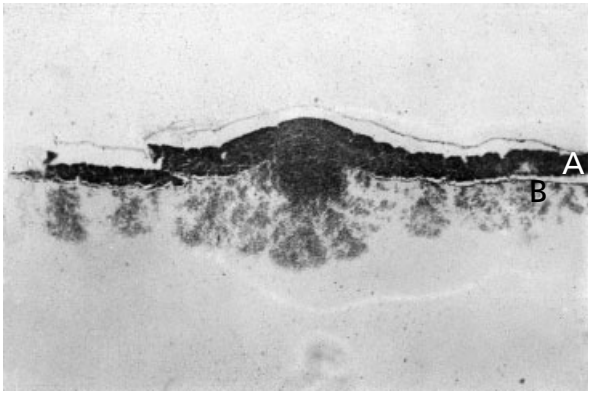

Figure 2 *Colony of* Mycobacterium bovis *(transversial section). Thin sections of colonies grown on Lowenstein–Jensen (LJ) medium were embedded in paraffin and stained with the Ziehl-Neelsen technique. Unlike* Mycobacterium tuberculosis*, most* Mycobacterium bovis *bacilli do not grow in the aerial part but grow microaerophilically in the medium. (A) Bacilli composing the aerial part of the colony. (B) Colony morphology on LJ medium.*

THE AGENTS

TB of man and animals is caused by a group of very closely related species forming *M. tuberculosis* complex. Although divided into species, on taxonomic grounds they are really variants of a single species. These are *M. tuberculosis* (Figure 1), the human tubercle bacillus, *Mycobacterium bovis* (Figure 2), the bovine tubercle bacillus, *M. africanum*, strains of human origin occurring principally in equatorial Africa, and *M. microti*, the vole tubercle bacillus. *M. microti* has been thought to be harmless for man and been used as a vaccine against TB in two large trials without any serious complications (Sula and Radkovsky 1976; Hart and Sutherland 1977). Recently however, molecular methods have identified genuine *M. microti* infections in both immunodeficient and immunocompetent individuals (van Soolingen et al. 1998b; Niemann et al. 2000b; Horstkotte et al. 2001).

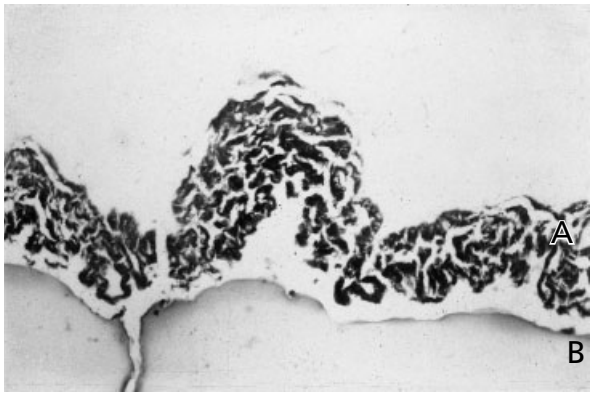

Figure 1 *Colony of* Mycobacterium tuberculosis *(transversal section). Thin sections of colonies grown on Löwenstein–Jensen (LJ) medium were embedded in paraffin and stained with the Ziehl–Neelsen technique. (A) Bacilli composing the aerial part of the colony. (B) Colony morphology on LJ medium.*

The complex also contains BCG vaccine, derived from a strain of *M. bovis*. Recently, a new taxon, *M. canettii*, was described for variants of *M. tuberculosis* characterized by smooth colonies. These exceptional strains have been isolated ony in man (van Soolingen et al. 1997; Pfyffer et al. 1998a; Miltgen et al. 2002).

EPIDEMIOLOGY

According to the World Health Organization's (WHO) estimates, more than one-third of the world's human population has been infected by the tubercle bacillus. Every year, more than eight million new cases occur among them, and more than two million die. Ninety-five percent of cases and 98 percent of deaths occur in low-income countries. TB is the cause of one in seven adult deaths and one in four preventable adult deaths, even though it is among the most cost-effective of all adult diseases to treat (Murray et al. 1990). It is a major cause of death of children, killing at least 500 000 annually. In 1993, the WHO took the unprecedented step of declaring TB a 'global emergency' (World Health Forum 1993).

In Europe, the average incidence was 168/100 000 people in 1950. After the introduction of antituberculous treatment, the incidence decreased to 89/100 000 in 1960 and to 17/100 000 in 1987. In 2002, 22 high-burden countries accounted for 80 percent of world cases. These countries are mainly in sub-Saharan Africa (Congo, Ethiopia, Kenya, Mozambique, Nigeria, South Africa, Tanzania, Uganda, Zimbabwe), East Asia (Afghanistan, Bangladesh, Cambodia, China, India, Indonesia, Myanmar, Pakistan, Philippines, Thailand, Vietnam), the eastern Mediterranean and Europe (Russia), and South America (Brazil).

Experience has shown that calculations of the incidence of TB on the basis of case-finding and notification

are very unreliable. In Great Britain, where there is a statutory requirement to notify TB, around 20 percent of cases are not notified (Sheldon et al. 1992). Determinations of the 'annual infection rate' or 'annual risk of infection' try to better define the epidemiological situation of TB. Infection for this purpose is defined as a conversion to tuberculin positivity, and the annual risk may be calculated from the results of tuberculin test surveys of children and young adults (Styblo 1984), provided that they have not had BCG vaccination. The annual infection rate has been used to make indirect estimates of the number of infectious cases of TB in communities.

In low-income countries, most infected people are in older age groups. Thus, in the USA and the UK, for example, only about 12 percent of people in the age range principally exposed to HIV, 15–45 years, are infected, whereas in sub-Saharan Africa, almost 50 percent of this age group are infected.

Impact of the HIV pandemic

Coexistence of HIV infection and TB has been hailed as one of the most serious threats to human health since the Black Death and called 'the Cursed Duet' (Chretien 1990). People infected by tubercle bacilli have about a 10 percent chance of developing TB during the remainder of their lives. By contrast, an HIV-positive person already infected by the tubercle bacillus has a 5–15 percent chance of developing overt disease annually, or up to 50 percent during the remainder of their relatively short lifespan (Selwyn et al. 1989; Dolin et al. 1994). The impact of the HIV/AIDS pandemic on the global toll of TB has been devastating. In 2001, there were an estimated 37.2 million adults and 2.7 million children infected by HIV worldwide, with 4.3 million adults and 0.8 million children newly infected during that year. In 2001, three million people died because of AIDS. In 2000, approximately 11 percent of worldwide new TB cases in adults, in the age range 15–49 years, were attributable to HIV infection, but this percentage reached 30 percent in some African countries. Of 1.9 million deaths due to TB worldwide, 18 percent were due to HIV. Conversely, TB was the immediate cause of 15 percent of all adult AIDS deaths. The prevalence of TB/HIV coinfection in adults was 0.41 percent worldwide but was over 5 percent in nine African countries. These figures stress that TB is a leading cause of HIV-related mortality and morbidity and that HIV is the most important factor fueling a TB epidemic in high HIV prevalence populations.

Although patients with HIV-related TB often respond to standard short-course chemotherapy, those in Africa are almost four times as likely to die of TB than HIV-negative patients within 13 months of diagnosis, mostly in the first month of therapy (Nunn et al. 1994). Even if therapy induces a bacteriological cure, the patient's subsequent life may be shortened. The reason for this is not fully understood, but there is evidence that immune responses in TB, and in other infections, induce cytokines that enhance the replication of the HIV and thus drive the patient into the full picture of AIDS with considerable shortening of life (Festenstein and Grange 1991). Although the details are not clear, there is evidence that TNF-α and other immunological mediators released in TB lead to transactivation of the HIV provirus and its subsequent replication (Osborn et al. 1989). In addition, TB causes a CD4+ T-cell lymphopenia, which may synergize with that induced by the HIV (Beck et al. 1985). Whatever the cause, the occurrence of active TB in the HIV-positive patient has very serious consequences, worsening the prognosis of HIV infection.

ENDOGENOUS REACTIVATION AND EXOGENOUS REINFECTION

For many decades, it was asserted that primary TB induced a degree of immunity sufficient to prevent exogenous reinfection. This, in turn, led to the belief that all post-primary TB is due to endogenous reactivation of a poorly understood 'latent' form of TB. The fallacy of this dogma became apparent during the conduct of an intensive TB control campaign in Canadian Inuit populations (Grzybowski et al. 1976). This campaign led, as was expected, to a large reduction in the incidence of primary TB and in the infection rate but also, and unexpectedly, to a reduction of incidence of the disease among older, previously infected people. This indicated that a significant proportion of cases of post-primary disease was due to exogenous reinfection.

DNA fingerprinting studies confirmed that exogenous reinfection occurs more often than previously believed (van Rie et al. 1999).

EPIDEMIOLOGY OF DRUG RESISTANCE

A pulmonary cavity contains from 10^6 to 10^8 bacilli, and a patient may have from 10^8 to 10^{10} bacilli. Considering the natural mutation rates of the bacilli towards antituberculous drugs (approximately 10^{-5} to 10^{-8}), some resistant mutants to any antibiotic are present in the bacillary population before any treatment. As there is no plasmid in tubercle bacilli, only a selection of resistant mutants leads to resistance in TB. The probability to find a mutant resistant to two drugs is 10^{-10} to 10^{-16}. This is the reason why standard antituberculous regimens associate three or four drugs to ensure elimination of resistant bacilli. There are two forms of drug resistance. Primary resistant disease occurs when an untreated person is infected by a strain that is already resistant. Secondary or acquired resistance is the result of a selection of mutants in patients receiving inadequate therapy. Acquired resistance may be due to factors related to the patient or to the clinician (e.g. poor compliance of the patient to therapy, misdiagnosis,

poor quality of drugs, poor therapy schemes) or factors related to medical structures (e.g. dysfunction of medical structures, difficult access to healthcare centers, lack of drug supply). By contrast, primary resistance reflects strains circulating in the community and is a good indicator for the quality of National Tuberculosis Programs.

Resistance to one of the drugs that were used in early regimens, SM or INH, is commonly encountered worldwide and does not pose a serious threat to TB control. Globally, approximately 10 percent of strains are resistant to a single drug (WHO 1997, 2000). Resistance to RMP is much more serious, especially as most strains resistant to this drug are also resistant to INH. Multidrug resistance (MDR) is usually defined as resistance to INH and RMP, with or without additional drug resistance. MDR TB fails to respond to standard short-course chemotherapy and it is necessary to use regimens containing drugs that are considerably less effective, more toxic, and more expensive.

In 1994, the WHO and the International Union Against Tuberculosis and Lung Disease (IUTLD) established a global drug-resistance surveillance project. The project aims were to develop a worldwide system of drug resistance surveillance, to determine the prevalence and assess trends of drug resistance in newly and previously treated patients, to determine the prevalence of mutidrug TB, and to monitor the effectiveness of TB control. The survey showed that drug- and multidrug resistance are ubiquitous but that the relative incidence varies greatly from region to region, with the occurrence of several 'hot spots' with a very high incidence. The latter include Argentina, the Dominican Republic, Estonia, Latvia, and Russia. Although there were limited data, a high incidence of resistance was also found in Asia. In some countries, the prevalence of drug resistance to any drug could be found in more than 60 percent of previously treated patients and MDR in more than 20 percent of these patients. In some countries from all continents (Estonia, Latvia, Russia, China, the Dominican Republic, Iran, the Ivory Coast), the prevalence of MDR was more than 5 percent in newly infected patients, jeopardizing the success of standard regimens. In 1997, more than 2 percent of all TB cases were multidrug resistant (WHO 2000).

The above survey stresses the importance of continued surveillance of the emergence and prevalence of antituberculosis drug resistance. To this end, the WHO has established a network of 22 Supranational Reference Laboratories to strengthen the local laboratories and validate the data of drug resistance collected all over the world.

EPIDEMIOLOGY OF HUMAN TB DUE TO *M. BOVIS*

Animal reservoirs of *M. bovis* pose a serious threat to human health. TB in cattle principally involves the lung and is spread from animal to animal by cough spray. Humans in direct contact with cattle may likewise be infected and develop primary pulmonary lesions. However, milk is the principal vector of transmission of *M. bovis*.

Well-executed bovine TB-eradication schemes including the slaughter of infected animals results in a rapid decline in the incidence of TB in cattle followed by a reduction in the incidence of primary manifestations of the disease, such as tuberculous lymphadenitis (scrofula), in the human population.

In countries where TB has been virtually eradicated in cattle, a few cases continue to occur in the human population. The great majority of such cases occur in people born before the disease was controlled in cattle and are thus assumed to be due to endogenous reactivation of old lesions. Evidence for human-to-human spread of disease due to *M. bovis* is limited. Whereas primary human TB of bovine origin is usually nonpulmonary, the lung is involved in about half the cases of post-primary disease (Grange and Yates 1994). A few cases of HIV-related TB due to *M. bovis*, and human-to-human spread, have been reported (Grange 1994). A large MDR epidemic due to *M. bovis* has been reported in Spain, involving AIDS and HIV-negative patients (Rivero et al. 2001).

Little is known concerning the zoonotic implications of *M. bovis* in the developing nations owing to a lack of laboratory facilities for the differentiation of this bacillus from *M. tuberculosis*. The very limited information has been reviewed by the WHO (Cosivi and Grange 1998).

THE DISEASE

M. tuberculosis is transmitted almost exclusively in cough spray from patients with open pulmonary TB and gains access to the body by inhalation of infective droplets usually less than 5 μm across. Patients with sputum that is positive on direct microscopical examination, and thus contains at least 5 000 bacilli in 1 ml, are the principal sources of infection (Rouillon et al. 1976). Not all patients infected by the tubercle bacillus develop overt disease. Only 10 percent of nonimmunocompromised infected people eventually develop active TB, 5 percent within the first 2 years following infection and 5 percent during their lifetime. The disease ratio in HIV-positive people is much higher, as indicated above. The interval between the initial infection and overt disease varies from a few weeks to several decades, and post-primary disease may be due to endogenous reactivation of the initial infection or to exogenous reinfection.

The initial lesion is usually in the lung (Figure 3), from which organisms reach other organs via the lymphatics and the bloodstream. Hematogenous spread occurs in primary TB with implantation of bacilli in many organs. In some people, particularly children under 3 years of age, these foci progress to serious, even

Figure 3 *Healed pulmonary tuberculosis with calcification. Anteroposterior chest radiograph showing many radiopaque healed lesions, particularly in the upper zones. (Courtesy of Dr P Ormerod.)*

fatal, disease principally involving the meninges, kidney, bones, and pleurae (Figures 4 and 5). Foci developing in the endothelium of major blood vessels may rupture and give rise to widespread small granulomata, a disease termed 'miliary TB' (Latin: *milium*, a millet seed). Disease occurring in a person never previously exposed to a tubercle bacillus is termed 'primary TB.'

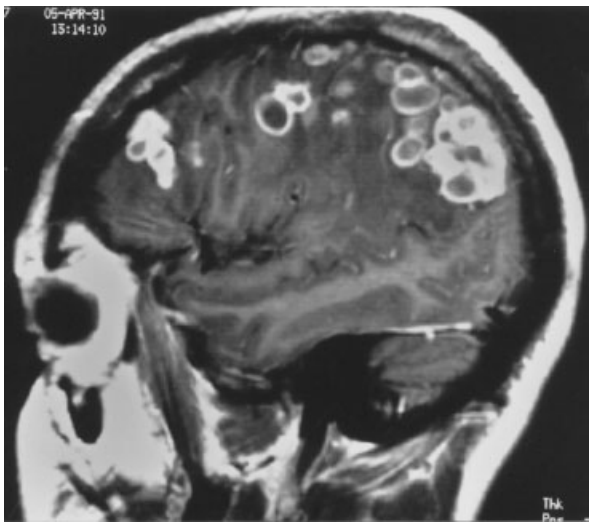

Figure 4 *Cerebral computerized tomography scan showing tuberculomas, principally in the left occipital region. (From Labhard et al. 1994.)*

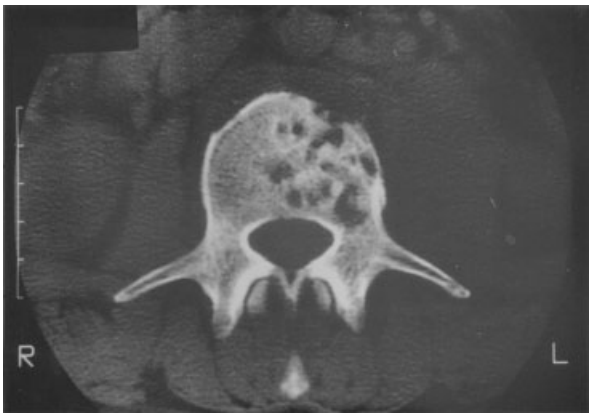

Figure 5 *Tuberculosis of the spine. Computerized tomography scan showing erosive lesions in the vertebral body. (Courtesy of Dr M. Humphries.)*

Infection results in a focus of disease (the Ghon focus) at the sites of implantation of the bacillus, usually the lung but occasionally other tissues. This focus, together with enlarged, infected regional lymph nodes, is termed the 'primary complex.'

The primary complex often resolves, but it may cause serious local complications. Most primary complexes resolve spontaneously, but a few tubercle bacilli may enter the poorly understood state of persistence or latency. Such infected but healthy people are usually, but not invariably, tuberculin-positive.

Post-primary TB

Post-primary TB develops in previously infected people either as a result of endogenous reactivation of latent disease or of exogenous reinfection. It usually occurs within 5 years after primary infection. The characteristic feature of such disease is extensive tissue necrosis. Very large tumor-like lesions termed tuberculomas may develop and, in common with primary lesions, the conditions within them do not favor mycobacterial growth. The necrotic tissue is softened and eventually liquefied by macrophage-derived proteases and, if the lesion erodes into a bronchus, the liquefied contents are discharged and a cavity is formed. In distinct contrast to closed lesions, the well-oxygenated cavities are ideal environments for bacillary replication, and their walls are lined by numerous bacilli.

Bacilli escaping from cavities may enter the sputum and be expectorated, thereby infecting other people. They may also spread through the respiratory tract and cause secondary lesions. On the other hand, spread of disease to lymph nodes and more distant organs is uncommon in post-primary disease, probably due to the obliteration of draining lymphatics and capillaries by the tissue necrosis and subsequent deposition of scar tissue. The formation of cavities and localization of the disease process characteristic of post-primary TB are dependent

on immune reactivity. Cavity formation is often limited or absent in immunocompromised people, and dissemination of disease to many organs frequently occurs (Festenstein and Grange 1991). Thus, TB in HIV-positive people with relatively limited immunosuppression resembles that in HIV-negative people but, in the more profoundly immunosuppressed, atypical forms of the disease are commonly seen (De Cock et al. 1992).

(For general reviews of the clinical features of TB, see Crofton et al. 1992; Grange 1996. For descriptions of childhood TB see Smith 2001).

Pathogenesis and immunology of TB

The clinical and histological features of TB are the result of the virulence of the tubercle bacillus and, more critically, the nature and effectiveness of the host's defense mechanisms.

Protective immune reactions in TB are principally cell-mediated, relying on macrophage activation and granuloma formation. Another important defense mechanism is the recognition and destruction of exhausted macrophages and other cells in which tubercle bacilli are replicating. Thus, regulated and directed cell destruction is an inevitable and essential part of the protective immune response in tuberculosis (Boom et al. 1991; Flynn et al. 1992). The outcome of infection by the tubercle bacillus depends critically on whether the host responds with a protective or tissue-necrotizing reaction.

Tubercle bacilli entering the tissues are taken up by macrophages. Entry of *M. tuberculosis* into human monocytes appears to be by a phagocytic mechanism mediated by complement receptor and the macrophage mannose receptors (Schlesinger et al. 1990; Schlesinger 1993). Recent results showed that heparin- or fibronectin-binding proteins, present on the bacterial surface, play a role to facilitate their binding to epithelial cells or macrophages (Pethe et al. 2001; Pasula et al. 2002). Once inside the macrophage, the intracellular mycobacteria employ a variety of survival strategies, which include: (1) prevention of an oxidative burst in phagocytosing cells and inhibition of phagosome-lysosome fusion; (2) resistance to lysosomal enzymes and reactive oxygen intermediates (by means of cell-wall lipids, including peptidoglycolipids (mycosides) and LAM and secretion of superoxyde dismutase; and (3) escape from the phagosome into the cytoplasm (Horwitz 1988; Sathish and Shinnick 1994; McDonough et al. 1993).

If the bacilli are not destroyed, they replicate and kill the cell. A local area of inflammation is thus established and more phagocytes are attracted to the site. Some bacilli are transported, probably within phagocytes, to the regional lymph nodes, where they are engulfed by antigen-presenting cells (APC). Other bacilli are transported further afield and may cause one of the extrapulmonary forms of primary disease such as tuberculous meningitis.

Epitopes from mycobacteria lying within phagosomes within the APC are presented on the cell surface by the major histocompatibility complex (MHC) class II (HLA-D) molecules to CD4+ helper T-cells, which undergo activation and clonal proliferation. These T-cells produce a range of cytokines, including interferon-γ (IFN-γ), which activates macrophages. Some T helper cells, however, also produce factors that lead to tissue-destroying hypersensitivity, as described below.

If the tubercle bacilli proliferate within the APC and escape from the phagosomes, their epitopes are presented to CD8+ T-cells by MHC class I (HLA-A and B) molecules. The CD8+ lymphocyte population contains cytotoxic T-cells that are able to lyse any cell presenting antigen in this manner.

Macrophage activation and granuloma formation

The evidence that a single human macrophage, even when fully activated in this manner, is able to kill tubercle bacilli is elusive. In disease, activated macrophages aggregate to form the characteristic lesion of TB and many other chronic infections, namely the granuloma. This consists of a compact palisade, many cells thick, of activated macrophages around the area of infection. Microscopically, these activated macrophages resemble epithelial cells and are thus termed epithelioid cells. Some of these fuse together to form multinucleate giant cells, which are characteristic of, but not exclusive to, granulomas of TB. The entire granuloma is much more capable of destroying tubercle bacilli than isolated macrophages (Dannenberg 1993). The palisade of metabolically active macrophages consumes oxygen diffusing into the granuloma, so that the center becomes anoxic and undergoes a type of necrosis, which, as it produces material similar in appearance to cottage cheese, is termed caseation. The anoxia and free fatty acids in the caseous center provide an environment that is highly unfavorable to the tubercle bacilli, many of which die. TNF-α plays a key role in protective immunity by maintaining the integrity of the granuloma (Flesch and Kaufmann 1990; Rook and Bloom 1994). It is, as described below, also a mediator of tissue-necrotizing immunopathology and it is responsible for the wasting (cachexia, consumption, or phthisis) of patients with advanced TB (Beutler et al. 1985).

In about 95 percent of primarily infected patients, these defense mechanisms render the disease quiescent, but some bacilli may survive for years or decades in a latent or persister state, the nature of which is still not well understood.

Protective immunity and immunopathology in TB

Though it is now both clear and obvious that tissue-necrotizing hypersensitivity and protective immune

reactions in tuberculosis are quite distinct, the main causes of difficulty were that protective and nonprotective responses were both associated with tuberculin positivity, that both were T-cell mediated, and that there was no place for a distinct tissue-necrotizing hypersensitivity reaction in the classical macrophage-based theory of mycobacterial immunity. Thus, there was a strong body of opinion that the various immune reactions were qualitatively identical and that their observed effects reflected the intensity of the reaction.

The dilemma was finally resolved by the demonstration that T helper (Th) cells mature into two distinct cells, Th1 and Th2, which secrete different, and mutually antagonistic, cytokines (Mosmann and Moore 1991). Necrosis occurs only in the presence of cytokines associated with the Th2 T-cell maturation pathway (Hernandez-Pando and Rook 1994). Th1-related cytokines, by contrast, do not induce this necrotizing reaction. Thus, the T-cell maturation pathway and the factors regulating it are of key importance to the nature of the immune response to infection by the tubercle bacillus.

Genetic factors in mycobacterial immunity

Because not all those exposed to the tubercle bacillus develop overt TB, some form of genetically determined innate resistance to disease has long been suspected. An allele, designated *bcg*, conferring natural macrophage-mediated resistance to several intracellular pathogens, including BCG, salmonellae and *Leishmania donovani*, has been described in the mouse (Skamene et al. 1989). It codes for a protein termed Nramp (natural-resistance-associated macrophage protein), which is involved in the generation of reactive nitrogen intermediates RNI from L-arginine (Vidal et al. 1993). A human homolog, *NRAMP1*, has been characterized (Cellier et al. 1994) and, in West Africa, polymorphisms in the gene coding for this protein have been shown to affect susceptibility to smear-positive pulmonary TB (Bellamy et al. 1998). In addition, naturally occurring mutation in the human genes for IFN-γ receptor and interleukin (IL)-12 receptor have been shown to result in increased susceptibility to mycobacterial infections (Manca et al. 2001).

Leprosy

HISTORY AND EPIDEMIOLOGY

Leprosy is caused by an intracellular acid-fast bacillus, *M. leprae*, one of the few human bacterial pathogens not yet cultured in vitro. In 1960, Shepard showed that its generation time is 10–11 days.

The disease is of great antiquity, being well described in written records from India (dated 600 BC) and from China (dated 150 BC). The first objective evidence of the disease, based on palaeopathology, was obtained from Egyptian mummies of the second century BC (Dzierzykray-Rogalski 1980).

From Egypt, it appears that leprosy spread to Europe; by AD 150, it had reached Greece, and a skeleton dated fourth century AD, excavated from a Romano–British cemetery in Dorset, UK, showed changes diagnostic of leprosy (Reader 1974). By the Middle Ages, it was widespread throughout Europe; it was introduced into Central and South America by the Spanish and Portuguese invaders, and subsequently into the southern states of the USA and the Caribbean isles by imported African slaves. It then declined rapidly in Europe, especially in the north, although a mini-epidemic occurred in Norway in the nineteenth century. From around AD 1850–1950, it spread widely in Oceania (see Browne (1985) for a detailed history of leprosy).

In 1983, the WHO estimated that the total number of cases of leprosy in the world was around 10.5 million, with over 5.3 million registered cases (Sansarriq 1983). At that time, treatment with dapsone alone was slow and often had to be continued for 20 years or for life in 'lepromatous' leprosy and for 5 years in 'tuberculoid' leprosy. The relapse rate was significant, especially in lepromatous leprosy. This form of treatment was replaced by multidrug therapy (MDT), consisting of dapsone, rifampin, and clofazimine (WHO Study Group 1982).

As a consequence, around 11 million leprosy patients have been cured over the past 15 years. The prevalence rate has dropped by 85 percent and leprosy has been eliminated in 98 countries (less than one case per 10 000 people). Furthermore, it has been estimated that early detection and adequate therapy has prevented three to four million people from being disabled. As of January 2000, the global leprosy prevalence rate was 1.25 cases per 10 000 people (WHO 2001). Between 2000 and 2005, approximately 2.5 million additional leprosy patients will have been detected. Special efforts will be needed to reach the leprosy elimination target in ten countries that account for 90 percent of the prevalence of the disease in the world (Brazil, Congo, Guinea, India, Indonesia, Madagascar, Mozambique, Myanmar, Nepal, and Tanzania).

The disease is more common among the lower economic classes and is associated with overcrowded, dimly lit living conditions (nasally excreted leprosy bacilli can survive for several days in poor light). Malnutrition appears to be much less important than with tuberculosis. After puberty, males are more often affected with 'spectrum' leprosy than females. In high-endemic areas, infection seems to occur mainly in children and young adults. Leprosy is extremely rare in infants (Brubaker et al. 1985). The incubation period is therefore assumed to be usually a matter of years (Feldman and Sturdivant 1976; Fine 1982). The infection rate in contacts appears to be high, but the attack rate for clinical disease is low, usually considerably less than the corresponding rate for TB.

Man is still regarded as the only significant reservoir of *M. leprae*, in spite of recent reports of natural infection with organisms resembling *M. leprae* in the nine-banded armadillo in Texas and Louisiana. The skin and nasal mucosa are the two most heavily infected tissues, but transmission via nasal secretions seems likely in view of the finding that they are responsible in lepromatous patients for a mean daily output of 10^7 viable bacilli (Davey and Rees 1974). Intact skin and the commonly occurring trophic ulcers are meager sources of bacilli (Weddell et al. 1963). Organisms may be shed from ulcerating lepromatous nodules, but these are rare. Infection by inhalation of droplets or droplet nuclei may be more common than infection via the skin. Although *M. leprae* is excreted in the breast milk of an untreated lepromatous mother (Pedley 1967), the numbers of leprosy bacilli inhaled by her baby are likely to greatly exceed the number ingested. Transplacental transmission remains uncertain, although there is evidence that mycobacterial antigen may cross the placenta in untreated lepromatous mothers (Melsom et al. 1982). Only 29 of the 51 leprous infants reported by Brubaker et al. (1985) had leprous mothers, of whom only 14 had lepromatous disease. There are additional factors, defined or undefined, that are thought to determine the clinical expression of the disease, such as ethnic, genetic, and environmental factors.

CLINICAL MANIFESTATION

Leprosy characteristically displays a great diversity of clinical manifestations (Sasaki et al. 2001; Ramos-e-Silva and Rebello 2001; Ishii 2003), the wide clinical 'spectrum' being related to host ability to develop and to maintain specific cell mediated immunity (CMI). In high resistant tuberculoid leprosy, the localized signs are restricted to skin and/or peripheral nerves; it is benign, chronic, and often self-healing, and the lepromin reaction is strongly positive. Low resistant lepromatous leprosy is a severe, generalized, progressive bacteremic disease, widely and symmetrically involving the skin, peripheral nerves, upper respiratory tract (from the nasal mucosa to the larynx), the reticuloendothelial system, especially the superficial lymph nodes, spleen and Kupffer cells of the liver, the anterior part of the eye, testes, and bone marrow; the lepromin reaction is negative.

Based on clear clinical, bacteriological (the concentration of bacilli in lesions), and immunohistopathological (the number and nature of lymphocytes in the leprous granuloma and the character of the bacterial host cells of the monocyte-macrophage series) evidence, Ridley and Jopling (1962) divided the spectrum into five groups, namely tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL), and lepromatous (LL). In addition, Ridley and Jopling noted a very early, prespectrum indeterminate (idt) form, usually consisting of a single hypopigmented, hypoaesthetic macule, difficult to diagnose, and self-curing in around 75 percent of cases, the remainder going on to develop some form of spectrum leprosy. While most tuberculoid patients are noninfectious, lepromatous (LL, BL) patients constitute the main source of infection.

In the TT form, there are only a few AFB, probably not more than 10^6 in total, but there is a strong CMI response with numerous epithelioid cells, giant cells, and lymphocytes, as in TB, although caseation only occurs, somewhat infrequently, in nerve trunks. In the LL form, there is a striking granulomatous response with many undifferentiated macrophages, often seen as large foamy cells (Virchow cells) packed with AFB. In advanced cases, there may be 10^9 *M. leprae* per gram of skin, with a patient total bacterial population of up to 10^{12} AFB. In each type of patient, the sensory, motor, and autonomic nerve fibers, are affected and AFB are present in Schwann and perineurial cells. No other bacterial species has the capacity to invade these nerve cells, even though mild histiocytic infiltrate may occur in nerves around blood vessels in advanced *M. lepraemurium* infection in mice. In tuberculoid infections, the asymmetrically affected nerves are infiltrated by epithelioid cells and lymphocytes, but there is only mild histiocytic infiltration in lepromatous cases. In all forms of leprosy, *M. leprae* organisms invade the nerves and eventually destroy nerve fibers. *M. leprae* invades both dermal nerves in skin lesions and nerve trunks, and occasionally, in TT and BT leprosy, one or more nerve trunks may be involved before the appearance of any skin lesions, so-called polyneuritic or neural leprosy. The most vulnerable sites are, for skin, those parts of the body that tend to remain cool and, for nerve trunks, those subject to trauma close to large joints. The Ridley–Jopling classification, which was originally devised for research purposes, has been used extensively because it contributes to our understanding of the clinical aspects of leprosy, giving guidance especially over prognosis and likely complications. For field use, however, a much simpler classification based on bacterial load has been devised.

Leprosy is an extremely chronic disease, which, if untreated, may be of lifelong duration. In TT and BT leprosy, after a variable length of time it may retrogress as the result of an increase in naturally acquired immunity, sometimes to relapse years later due to a decrease in CMI, perhaps associated with intercurrent infection, pregnancy, or old age. Even in lepromatous leprosy, disease activity used to vary spontaneously, although most patients died after perhaps 20 years, usually from renal failure due to secondary amyloidosis, intercurrent infection, or secondary pneumonia from laryngeal obstruction. Nowadays, the mortality from leprosy itself is negligible, with patients occasionally dying from secondary amyloidosis or from secondary infection of anaesthetic extremities and neuropathic ulcers.

During the course of the disease, acute immunologically mediated exacerbations, known as 'reactions,' commonly occur. Reversal or type 1 (Jopling 1978) reaction, which occurs in borderline leprosy and in a minority of the LL patients who have evolved from borderline, is associated with an increase in specific CMI. It is characterized by erythema and swelling of skin lesions, new skin lesions sometimes developing, and pain and tenderness of swollen nerves. Erythema nodosum leprosum (ENL) or type 2 reaction, which occurs only in LL and a small minority of BL patients, particularly during treatment, is due to an antibody–antigen reaction. Local formation of immune complexes in affected skin results in characteristic crops of small tender erythematous nodules resembling those in erythema nodosum, but more widespread, usually associated with malaise and pyrexia. Other systems may also be involved, leading to neuritis, iridocyclitis, orchitis, and immune complex nephritis. Severe chronic ENL may last for years, until the bulk of the dead AFB are removed after 8–10 years in LL leprosy; if such a reaction is not adequately suppressed, secondary amyloidosis may develop.

DIAGNOSIS

The diagnosis of leprosy is essentially a clinical one. Cardinal signs are anesthesia of the skin lesions, enlargement of nerves, and the presence of *M. leprae* in affected skin and nasal mucosa. Leprosy can, therefore, usually be correctly diagnosed by careful palpation of the peripheral nerves of predilection, with a pen, piece of cotton wool, or thermal tester to detect anesthesia, and a scalpel to remove material for microscopic examination.

The skin provides the most readily available tissue from which to obtain *M. leprae*, by means of the 'slit-skin smear' technique. *M. leprae* is present in the nasal mucosa and nasal secretions of untreated LL and many BL patients. A moistened swab is used for obtaining material from the surface of the inferior turbinate bones. Nasal secretion is collected, preferably early in the morning, by blowing the nose into a polyethylene handkerchief. All samples are examined as smears on glass microscope slides.

Smears are stained by the Ziehl–Neelsen technique (Figure 6), care being taken not to overdecolorize if acid-alcohol is used, since *M. leprae* is more acid-fast and less alcohol-fast than *M. tuberculosis*. Positive slit-skin smears are scored according to the density of the AFB, the bacterial index (BI) (Ridley 1958), which gives an indirect indication of the total bacterial load; serial smears will indicate the response of the patient to chemotherapy. Serial smears can also be used to assess the morphological index (MI) (Rees and Waters 1963), which gives a measure of the viability of *M. leprae* on the basis of morphology; uniformly staining bacilli are probably alive, whereas irregularly staining bacilli are dead (Rees 1985). In addition to microscopy, PCR-based assays have been established to conclusively diagnose the organism (Rastogi et al. 1999).

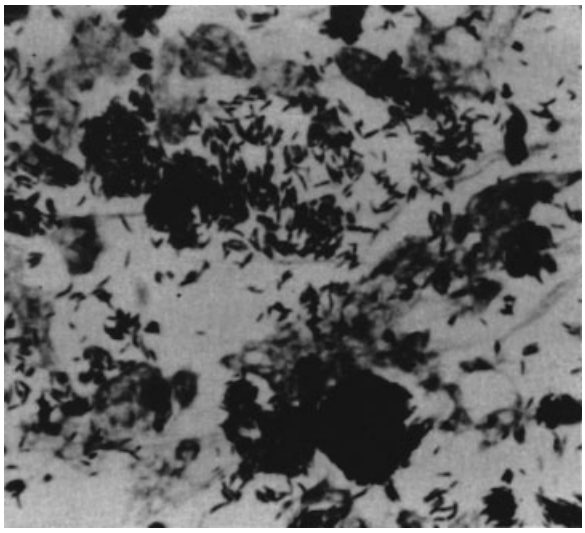

Figure 6 *Stained smear of nasal discharge from an untreated lepromatous patient showing* Mycobacterium leprae *singly and in clumps ('globi') (×1 000).*

Nontuberculous mycobacteria

NTM are frequently found in environmental habitats that may colonize and occasionally cause infection in humans and animals. Such infections are termed mycobacterioses. With the increasing prevalence of immunocompromised hosts, particularly in relation to the AIDS pandemic, NTM infections are becoming more prevalent (Brown-Elliott et al. 2002). The characteristics of these infections differ from those seen in immunocompetent human hosts, as do the organisms involved (Table 2).
Patients with depressed cellular immunity, such as those who have AIDS, lymphoproliferative disorders or transplants, and those on immunosuppressive therapy, are at particular risk.

AIDS patients differ from other immunocompromised patients in that high numbers of NTM can be recovered from blood. However, in these patients, NTM can also cause pulmonary infections (Rigsby and Curtis 1994). Although NTM are recoverable from sputum or bronchoalveolar lavage fluid, true invasion of the lung is difficult to demonstrate and pulmonary symptoms are often minimal. The radiographic appearance of NTM in AIDS differs from that in immunocompetent hosts. The chest X-ray may be normal or show nonspecific mediastinal and/or hilar adenopathy or, rarely, patchy alveolar infiltrates. The frequent detection of NTM in blood cultures reflects the disseminated nature of infection and the high bacterial burden. The mechanism by which organisms in the blood become recoverable from respiratory

Table 2 *Slow-growing nontuberculous mycobacteria (NTM) frequently encountered in clinical specimens and their pathogenic potential in humans and animals*

Human host				Animal host[a]
Immunocompetent		AIDS	Immunocompromised (non-AIDS)	
Pathogenic potential	**Most frequently nonpathogenic**	**Pathogenic potential**	**Pathogenic potential**	**Pathogenic potential**
MAC	*M. flavescens*	MAC	MAC	*M. farcinogenes*
M. celatum	*M. gastri*	*M. celatum*	*M. haemophilum*	*M. lepraemurium*
M. haemophilum	*M. gordonae*	*M. genavense*	*M. kansasii*	*M. paratuberculosis*
M. heckeshornense	*M. neoaurum*	*M. haemophilum*	*M. marinum*	
M. heidelbergense	*M. nonchromogenicum*	*M. kansasii*	*M. scrofulaceum*	
M. kansasii	*M. terrae*	*M. malmoense*	*M. tusciae*	
M. marinum	*M. triviale*	*M. marinum*	*M. xenopi*	
M. malmoense		*M. simiae*		
M. scrofulaceum		*M. szulgai*		
M. shimoidei		*M. xenopi*		
M. simiae				
M. szulgai				
M. ulcerans				
M. xenopi				

AIDS, acquired immunodeficiency syndrome; MAC, *Mycobacterium avium* complex.
a) Exclusively isolated from animals (in addition to other mycobacteria).

secretions is unknown. The increased incidence of disseminated *M. avium* complex (MAC) infection in AIDS patients is attributable to two principal factors: (1) greater surveillance since MAC bacteria were recognized as a potentially treatable cause of morbidity and (2) the increased survival of patients with AIDS. Rarely, there may be a genetic basis for disseminated NTM infection.

In transplant patients (Kesten and Chaparro 1999), NTM infections generally occur late in the post-transplantation period (range 10 days to 269 months; mean 48 months). Most are chronic infections of soft tissues and joints (cutaneous lesions on the extremities, tenosynovitis, arthritis) and osteomyelitis, often with multifocal involvement. Fever, leukocytosis, night sweats, weight loss, and lymphadenopathy are usually absent. Skin lesions are often painful erythematous subcutaneous nodules, which can progress to abscess formation. The most commonly involved joints include finger joints, wrists, elbows, ankles, and knees, and in these patients diagnosis is often delayed by several months. Pulmonary involvement occurs in approximately a quarter of patients, with radiographic evidence of pulmonary nodules or infiltrates, and over half the patients have concomitant extrapulmonary involvement. Pulmonary involvement is most frequently with *M. kansasii*. Risk factors for mycobacterioses in solid-organ-transplant recipients are poorly defined. For heart recipients, they include a history of open heart surgery and immunosuppressive therapy. The environment is the likely source in patients with transplant-related immunosuppression and other immunodeficiencies.

Patients with the uncommon 'hairy cell' leukemia appear to have a predilection for mycobacterial infection (approximately 5 percent of patients), mainly with *M. kansasii* and MAC (Castor et al. 1994). In contrast, infections by NTM in cystic fibrosis patients are common (Torrens et al. 1998).

In the immunocompetent host, NTM can cause infections in cutaneous, deep soft tissues, lymphatics, and other sites (e.g. skeletal, peritoneal catheter-related, ocular). These mycobacterioses are rare, indolent, and frequently misidentified. Active infections in immunocompetent hosts cause granulomatous inflammation of the lung.

However, very often, NTM lack clinical relevance and are, therefore, incorrectly labeled as colonizers or as the cause of infection. Discrimination between colonization and infection by NTM can be difficult. Some of the most important American Thoracic Society (ATS) criteria (American Thoracic Society 1997) for the diagnosis of pulmonary mycobacterioses are as follows: cavitary or noncavitary infiltrates (e.g. nodular infiltrates or bronchiectasis) should be present on a chest X-ray. Two or more respiratory specimens (sputum or bronchial washings) should demonstrate AFB on smear examination and/or moderate to heavy growth (2+ to 4+) on culture.

To date, human-to-human and animal-to-human transmission of NTM has not been documented. Inhalation of contaminated aerosols is the presumed mode of

inoculation of the lung. Geographical clustering of infections often matches the environmental distribution of NTM and supports the hypothesis that infections arise from environmental exposure. Host susceptibility is a factor in the development of infections, because in some areas exposure is universal. It is not known whether, as in the case of *M. tuberculosis*, latent infection is part of pathogenesis. Because of cross-reactivity between shared antigens, species-specific skin tests have not proved reliable to measure immune responses.

SPECIES FREQUENTLY INVOLVED IN HUMAN DISEASE

MAC

The bacteriological and clinical aspects of MAC infections have been reviewed by Inderlied et al. (1993) and Falkinham (1996).

MAC organisms show a characteristic heterogeneous colony morphology (Figure 7). Small translucent (smooth transparent (SMT)) colonies usually co-occur with glossy, whitish colonies (smooth domed (SMD)). There is even a third flat and dry morphology, which resembles *M. tuberculosis*. SMT are more frequently isolated from blood in AIDS patients (Belisle and Brennan 1994; Frothingham and Wilson 1994). Isolates from patients with disseminated MAC often contain mixtures of colony morphologies (Falkinham 1994; Slutsky et al. 1994; von Reyn et al. 1995). SMT bacteria have greater potential for intracellular multiplication in macrophages, have greater virulence in animal models, and are more resistant to antibiotics than SMD bacteria.

Traditionally, MAC has consisted of 28 serotypes ('serovars') comprising *M. avium* and *M. intracellulare*. Serovars 1–6, 8–11, and 21 are *M. avium*, while serovars 7, 12–20, and 22–28 are *M. intracellulare*. Inclusion of *M. scrofulaceum* (serovars 41–43) in the group *M. avium–M. intracellulare–M. scrofulaceum* (MAIS complex) is no longer appropriate. *M. avium* and *M. intracellulare* are easily distinguishable with molecular methods (Vincent et al. 2003). Based on phenotypic and genetic characteristics, three subspecies of *M. avium* have been proposed: subsp. *avium*, subsp. *paratuberculosis*, and subsp. *silvaticum* (Thorel et al. 1990).

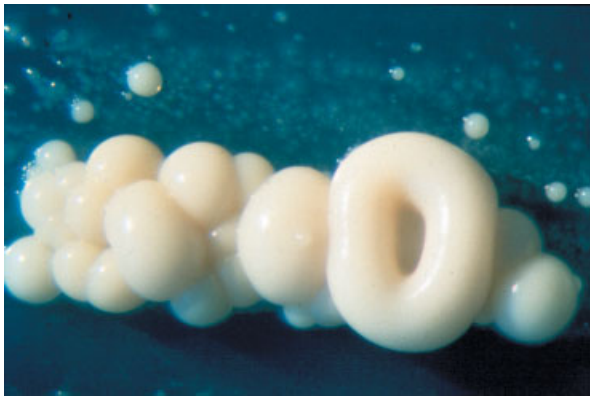

Figure 7 *Colony morphology of* Mycobacterium avium *complex (MAC) on Middlebrook 7H10 agar. Note the glossy, whitish (smooth domed) colonies, co-occurring with the small translucent (smooth transparent) colonies.*

MAC has been isolated from bedding material, house dust, soil, plants, swimming pools, hospital water, and natural bodies of water (Wolinsky and Rynearson 1968; Fry et al. 1986; du Moulin et al. 1988). The true prevalence of MAC infections are unknown because mycobacterioses are not reportable (Iseman 1989; Prince et al. 1989). Poultry, swine, and cattle may also be infected by MAC, which gets into the soil by fecal shedding from birds but not from cattle or swine (Inderlied et al. 1993). Serovars similar to those from birds have been isolated from humans living in their close proximity, but animal-to-human transmission of infection has not been documented. The predominant serovars in human and animal infections are different (Nel 1981). In spite of widespread environmental exposure to MAC in certain geographical regions, the incidence of clinical disease in immunocompetent hosts is extremely low. *M. avium* serovars account for more than 90 percent of typeable MAC that cause infection in AIDS, whereas in other patients almost half the isolates are *M. intracellulare* (Guthertz et al. 1989; Raszka et al. 1995). In the USA, for unknown reasons, bacteremia in AIDS is associated with a limited number of *M. avium* serovars (serovars 1, 4, and 8).

Before the advent of AIDS, the most common presentation of MAC infection was pulmonary disease occurring in middle-aged or older white male smokers (Figure 8a) and lung tissue shows either caseating or noncaseating granulomas. Pre-existing lung disease, including chronic obstructive pulmonary disease (COPD), previous TB, bronchiectasis (including cystic fibrosis), silicosis, chronic aspiration pneumonia, and bronchogenic carcinoma, is present in approximately 70 percent of patients (Hornick et al. 1988; Kilby et al. 1992; Wallace et al. 2002; McKlendin and Stark 2001). These lung diseases have in common substantial impairment of clearance mechanisms, which may allow colonizing bacteria to establish infection.

Another group of patients are predominantly nonsmoking females with nodular bronchiectasis (Figure 8b), but with otherwise no identifiable predisposing lung disease (Albelda et al. 1985; Prince et al. 1989). Many are elderly and, less commonly, younger women with pectus excavatum, scoliosis, or mitral valve prolapse (Iseman 1989; Reich and Johnson 1991; Kennedy and Weber 1994). Patients described a chronic productive cough, dyspnoea, malaise, and weakness. The disease was referred to as 'Lady Windermere's syndrome,' implying that the pathogenesis was linked to habitual cough suppression (Reich and Johnson 1992), but the true mechanism is unknown. Chest X-ray reveals

Figure 8 *Chest X-rays of patients with* Mycobacterium avium *complex (MAC) pulmonary infection.* **(a)** *Male smoker, age 50 years, with chronic obstructive pulmonary disease (COPD). Severe cavitary infiltrate and loss of volume of the left upper lobe are demonstrated.* **(b)** *Female nonsmoker, age 74 years, without history of lung disease, who has a persistent chronic cough. A reticular nodular infiltrate is seen in both the right middle and upper lobes.*

noncavitary infiltrates that frequently involves the right middle lobe and lingula. High-resolution computed tomography (CT) of the chest reveals characteristic small (<5 mm) nodules with associated bronchiectasis (Swensen et al. 1994). The disease process is indolent and slowly progressive, and patients may have symptoms for up to 10 years. This form of MAC infection may therefore be underdiagnosed. In a substantial proportion of patients, the disease is progressive, with a mortality as high as 20 percent (Prince et al. 1989; Teirstein et al. 1990; Wallace et al. 1998). Very infrequently, intrathoracic MAC infections are seen in immunocompetent children (Fergie et al. 1997).

MAC is also the leading cause of localized lymphadenitis in children, especially in those aged up to 5 years (Wolinsky 1995; Danielides et al. 2002). Cervical lymphadenitis due to MAC accounts for 63–80 percent of cases and *M. scrofulaceum* is the next most common (Schaad et al. 1979; Joshi et al. 1989).

In the early 1980s, before the epidemic of HIV infection and AIDS, disseminated MAC infection was very uncommon; fewer than 40 cases were reported between 1940 and 1984 (Falkinham 1994). More than half the patients were immunocompromised by malignancy or immunodeficient (Horsburgh et al. 1985). These infections remain uncommon other than in HIV-infected patients. Although disseminated disease is more common than focal disease in these patients (Johnson and Kiehn 1994), localized skin infection (Kakinuma and Suzuki 1994), arthritis (Disla et al. 1995), cholecystitis (Fantry and Sun 2002), and osteomyelitis (Aberg et al. 2002) (Figure 9) have been described. Disseminated MAC is associated with a high incidence of hematological abnormalities, such as leukemoid reactions, myelofibrotic changes, the hemophagocytic syndrome, polycythemia, pancytopenia, and myelodysplastic syndrome (Tsukada et al. 1994). In the latter syndrome, aggregates of macrophages filled with organisms are seen in the bone marrow without a granulomatous reaction and necrosis, similar to the overwhelming infections seen in late stage AIDS (Tsukada et al. 1994). Torriani et al. (1996) reported of highest concentrations of MAC in the reticuloendothelial organs.

In AIDS patients, the most common port of entry and site for dissemination is, in contrast to patients who are immunocompetent, believed to be the gastrointestinal tract followed by hematogenous dissemination (Torriani et al. 1996). The environmental source of MAC in AIDS patients is uncertain but may include potable water (Montecalvo et al. 1994; von Reyn et al. 2002), hard cheese (Horsburgh et al. 1994a), and soil (Yajko et al. 1995). Upon acquiring MAC, bacterial colonization and/or direct tissue invasion followed by intermittent bacteremia without dissemination is observed. Continuous

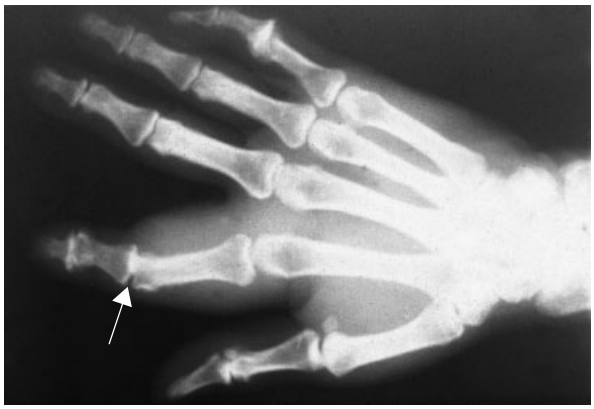

Figure 9 *Bone infection due to* Mycobacterium avium.

bacteremia with dissemination is usually accompanied by clinical symptoms and, finally, death (Torriani et al. 1994; Raszka et al. 1995). MAC organisms are frequently recovered from lymph nodes, liver, spleen, bone marrow, and the gastrointestinal tract. MAC infections in the eye, brain, meninges (Dwork et al. 1994; Gyure et al. 1995), CSF, skin, tongue, heart, lung, stomach, thyroid, breast, parathyroid, adrenal glands, kidney, pancreas, prostate, testes, skin and soft tissue, abdominal cavity, and, more recently, muscle (necrotizing myositis) (Miralles and Bregman 1994) have been documented.

Disseminated MAC infection develops in 60–65 percent of patients with antecedent respiratory colonization, but among all patients who develop disseminated disease, respiratory colonization is detected in only 21–33 percent before dissemination. Thus, culture is of limited value as a routine screening test. The strongest single risk factor for disseminated MAC in AIDS is the level of immunosuppression (Horsburgh et al. 1994b), and the risk of MAC infection increases as the CD4 count decreases (Henderson and Chapman 1994). It is most common with a CD4 T-lymphocyte count of less than 100 (Falkinham 1994). Symptomatic HIV-infected patients with CD4 lymphocyte counts of less than 50 are at substantial risk of MAC bacteremia. Disseminated MAC infection in HIV-infected people generally presents as a subacute but progressive syndrome of fever, chills, night sweats, cough, fatigue, diarrhea, and weight loss. Gastrointestinal complaints are particularly common and contribute greatly to morbidity. Diarrhea is due to small-intestinal or colonic involvement, and severe abdominal pain is due to enlarged retroperitoneal lymph nodes (Porrman and Katon 1994). Hepatosplenomegaly is detectable on physical examination in 10–50 percent of patients (Levin 1994). The most common laboratory abnormalities are worsening anemia and elevated alkaline phosphatase. Pulmonary MAC disease is rare in patients with disseminated MAC infection. Radiographic patterns are nonspecific and may include consolidating or nodular infiltrates and cavitation (Kalayjian et al. 1995). Epidemiological studies indicate that untreated disseminated MAC disease increases morbidity and shortens survival (Chin et al. 1994; Henderson and Chapman 1994; Horsburgh et al. 1994b). Median survival after the diagnosis of MAC is approximately 3–8 months versus approximately 10–16 months in CD4-matched HIV-infected patients without disseminated MAC (Hoover et al. 1995). Based on these data, treatment of MAC infection in AIDS patients, and other severely immunocompromised patients, appears to be justified because it improves symptoms and may increase survival (Horsburgh et al. 1991; Chin et al. 1994). Sahfran et al. (2002) have shown that therapy for MAC infection can safely be discontinued after highly active antiretroviral therapy (HAART).

Malabsorption complicates antibiotic regimens in AIDS patients. Placebo-controlled trials show a 50 percent reduction in MAC bacteremia in asymptomatic patients with rifabutin prophylaxis, and a US Public Health Service Task Force has recommended rifabutin for all AIDS patients with a CD4 cell count of less than 100 (Masur 1993; Nightingale et al. 1993), but *M. tuberculosis* must first be ruled out. A significant therapeutic advance in the treatment of symptomatic patients are extended-spectrum macrolide (ESM) agents with high-level activity against MAC. Primary therapy usually includes clarithromycin and ethambutol with or without rifampicin or rifabutin (Henderson and Chapman 1994). Though the current medical treatment for MAC is controversial, the ATS recommendations advocate empiric therapy with drug susceptibility testing for clarithromycin only (Iseman 2002; NCCLS 2003).

A partial response to treatment in terms of diminished symptoms, usually within 2–8 weeks of starting therapy, is seen in 72–95 percent of patients, but blood cultures may remain positive and the required duration of therapy appears to be indefinite (Porrman and Katon 1994; Sullam et al. 1994). Nonadherence to both MAC prophylaxis and antiretroviral therapy was associated with higher HIV type 1 RNA levels and a significant increase in the risk of developing an AIDS-defining complication or death (Cohn et al. 2002).

M. genavense

M. genavense has unusual fastidious growth requirements. Isolation has been most successful using the radiometric BACTEC 7H12 PZA test medium (Tortoli et al. 1994). Specimens stained for AFB must be observed carefully to detect small coccobacillary rods. Growth is inhibited in the *p*-nitro-α-acetylamino-β-propiophenone (NAP) test. Analysis of the 16S rRNA gene indicates that this species is closely related to *M. simiae*. It probably occurs frequently but is not detected because of failure to inoculate the proper medium, the requirement for a long incubation period, and the poor methods for identification. Nucleic acid-based techniques, such as gene amplification (16S rRNA) by PCR or in situ hybridization with highly specific probes are advisable for a definitive diagnosis.

The organism is probably widespread in the environment (Bessesen et al. 1993; Pechere et al. 1995). It is the major mycobacterial pathogen in birds, including parrots and parakeets, and has been isolated from pet birds with muscle wasting, hepatomegaly, and thickening of the small intestine (Hoop et al. 1993) as well as from a feline immunodeficiency virus (FIV)-positive cat (Hughes et al. 1999) and other mammals.

M. genavense has predominantly been associated with enteritis and genital and soft-tissue infections in HIV-positive and HIV-negative immunocompromised individuals. In tissues from humans infected with *M. genavense*, pathological changes are seen mainly in the small intestine, spleen, liver, and lymph nodes (Maschek et al.

1994; Leautez et al. 2000), while lungs, myocardium, and kidneys are usually not, or are only minimally, involved. Disseminated infections with this organism have been recognized in AIDS patients in the USA, Europe, and Australia (Coyle et al. 1992; Tortoli et al. 1998b; Thomsen et al. 1999) and manifest similar to those caused by MAC, with the important difference that stool specimens were more often smear-positive.

M. genavense infection is often associated with fever, anorexia, abdominal pain, chronic diarrhea, and massive weight loss, but splenomegaly, hepatomegaly, and lymphadenopathy are more prominent (Bessesen et al. 1993; Gaynor et al. 1994). A case–control study by Pechere et al. (1995) of 54 patients with disseminated *M. genavense* infection from Europe, North America, and Australia showed that most were extremely immunocompromised, 87 percent had fever and weight loss, 72 percent had anemia, 44 percent had diarrhea, 43 percent had splenomegaly, and 39 percent had hepatomegaly.

Investigation of patients with suspected *M. genavense* infection should include blood culture, bone-marrow biopsy, and, possibly, small-bowel biopsy.

M. haemophilum

M. haemophilum infections may be under-recognized because of the predilection of this species for a low incubation temperature (30°C) and its unique requirement for ferric ammonium citrate or hemin for growth. If *M. haemophilum* is suspected in a clinical specimen but the culture remains negative, the organism may be recovered by using a chocolate agar plate.

The natural habitat and means of acquisition are unknown. Potential routes of transmission include percutaneous inoculation, inhalation, and ingestion. Approximately 50 percent of infections have been in patients with AIDS, with a relatively large number reported from New York City (CDC 1991). The other cases have been in other immunosuppressed individuals (Saubolle et al. 1996; Gruschke et al. 2002; Paech et al. 2002) but also in immunocompetent pediatric patients with localized cervical lymphadenopathy (Samra et al. 1999) or with a pulmonary nodule (White et al. 1999). The classical clinical presentation is that of multiple skin nodules in clusters or without a definite pattern, commonly involving the extremities, and occasionally associated with fever, weight loss, tenosynovitis, septic arthritis, abscesses, draining fistulas, cellulitis, and osteomyelitis (Falkinham 1996). In addition to the human host, *M. haemophilum* infections have been reported from cats (Appleyard and Clark 2002) and from a royal python (Hernandez-Divers and Shearer 2002).

M. kansasii

M. kansasii forms visible niacin-negative, photochromogenic colonies on LJ slants at 2–3 weeks

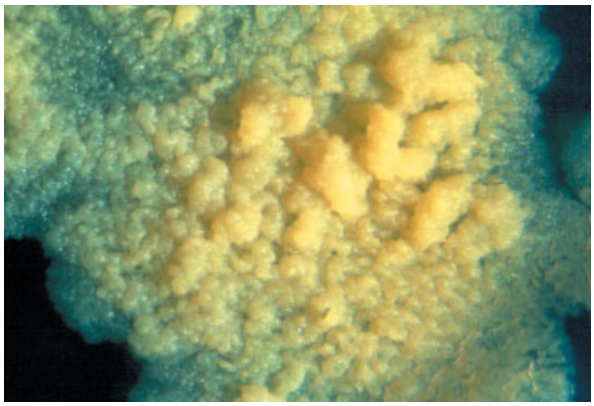

Figure 10 *Photochromogenic colonies of* Mycobacterium kansasii *on Middlebrook 7H10 agar.*

(Figure 10). After 2 weeks in ambient light, the colonies turn bright yellow or orange. The 'yellow bacillus,' as it was originally called, must be distinguished from other photochromogenic species, such as *Mycobacterium simiae* and *Mycobacterium szulgai*, which also may cause pulmonary mycobacterioses. In sputum smears, *M. kansasii* may appear as distinctive large, cross-barred AFB (Wolinsky 1979). Closely related to *M. gastri*, molecular studies have defined five genotypes of *M. kansasii*, all of which are able to cause human disease (Picardeau et al. 1997a). Subtyping *M. kansasii* may improve clinical management by distinguishing pathogenic from nonpathogenic subtypes, as demonstrated very recently in Switzerland (Taillard et al. 2003). The environmental reservoir of *M. kansasii* is unknown; it has occasionally been isolated from water sources but not from soil (Wolinsky 1979).

M. kansasii, first identified as a pulmonary pathogen by Buhler and Pollack (1953), is the second most common cause of pulmonary mycobacterioses in most parts of the world (O'Brien et al. 1987; Pang 1991; Debrunner et al. 1992; American Thoracic Society 1997). In the USA, Japan, and Switzerland, *M. kansasii* accounts for some 25 percent of pulmonary NTM isolates (O'Brien et al. 1987; Debrunner et al. 1992).

In contrast to most other pulmonary mycobacterioses, isolation of *M. kansasii* from sputum culture is less likely to represent colonization or contamination (Table 2). Colonization should be suspected in individuals without significant underlying lung disease (Ahn et al. 1982). It is hypothesized that colonization or infection results from inhalation of contaminated aerosols. Patients with active infection are typically male smokers (M:F ratio is 3:1) aged 50 years or older (Ahn et al. 1979; Pang 1991). They usually have coexisting COPD and often live in urban areas (O'Brien et al. 1987). *M. kansasii* infections may also coexist with lung cancer, previous TB, or pneumoconiosis (O'Brien et al. 1987; Pang 1991). It is common in mine workers in the UK and in South Africa (Corbett et al. 1999).

Symptoms and clinical characteristics of infection mimic tuberculosis, including cough, fever, night sweats, and weight loss. These are superimposed on the persistent symptoms of the coexisting lung disease. In 84–95 percent of cases, the chest X-ray shows apical cavitary infiltrates (O'Brien et al. 1987). *M. kansasii* may also mimic *M. tuberculosis* in that both rarely present as endobronchial lesions identified by bronchoscopy. However, unlike *M. tuberculosis*, the few *M. kansasii* endobronchial lesions that have been reported were in HIV patients (Connolly et al. 1993). Lymphadenitis and skin and soft-tissue infections due to *M. kansasii* and MAC have rarely been reported in immunocompetent individuals (Breathnach et al. 1995; Kotb et al. 2001). Such patients have cutaneous abscesses with ulceration and subcutaneous nodules. In most cases, skin trauma with exposure to contaminated water was thought to be the mode of inoculation. Subcutaneous nodules and sporotrichoid spread has been described for cutaneous *M. kansasii* infection (Breathnach et al. 1995).

Less commonly, *M. kansasii* (as well as *M. xenopi*) has been implicated in catheter-related infections (White et al. 1993). These infections are clinically indistinguishable from other bacterial causes but should be considered when routine bacterial cultures fail to grow. Mastoiditis that fails to respond to standard antibiotics is rarely due to NTM (Moerman et al. 1993). Presence of granulomatous inflammation on biopsy and culture confirm the diagnosis, and treatment is with surgery and antibiotic therapy.

Pulmonary and disseminated disease due to *M. kansasii* has been reported in patients infected with HIV (Parenti et al. 1995) and in patients with other immunocompromising conditions (Zvetina et al. 1992). *M. kansasii* is the second most common NTM to affect patients with AIDS in the USA (Bamberger et al. 1994). Horsburgh and Selik (1989) reported that *M. kansasii* accounts for 2.9 percent of the NTM that cause disseminated infections in AIDS patients.

The clinical presentation and prognosis of disease due to *M. kansasii* in patients with HIV was examined by Witzig et al. (1995). The patients had CD4 counts of less than 100 and a mean interval of 17 months between the diagnosis of AIDS and isolation of *M. kansasii*; 17 of the 49 patients had disseminated disease. Sputum smears were positive for AFB in 29 patients. It therefore appears that in HIV-infected patients, isolation of *M. kansasii* in sputum is usually associated with disease rather than colonization. Symptoms consisted of fever, weight loss, fatigue, night sweats, chills, cough, sputum production, dyspnea, chest pain, and occasionally hemoptysis. The physical findings were lymphadenopathy and occasionally splenomegaly, hepatomegaly, or skin lesions. In contrast to the study of Bamberger et al. (1994), most patients had abnormal chest X-rays, the majority with alveolar infiltrates and a smaller number with cavitation and interstitial infiltrates. Cavities have been reported as well (Patel and Wolf 2003). Disseminated disease was common and most often diagnosed by blood culture. Patients who received antimycobacterial treatment survived longer than those who did not.

Other types of *M. kansasii* infection in AIDS patients include pericarditis (Moreno et al. 1994), osteomyelitis (Weinroth et al. 1994), tenosynovitis (Blue et al. 2002), endobronchial lesions with obstruction (Connolly et al. 1993), sinusitis (Li et al. 1994), ocular infections (Gobels et al. 2002), and pericarditis (Pintado et al. 2001).

M. malmoense

The species has been recovered from soil and water (American Thoracic Society 1997). Lung infection due to this organism was first described by Schröder and Juhlin (1977) in Malmö, Sweden, where it is the second most common NTM isolate from the respiratory tract after MAC (Henriques et al. 1994). For reasons that remain unclear, reports of infection with this bacterium during the past decade have increased substantially in some countries (Henriques et al. 1994; Hoffner 1994; Tortoli et al. 1997; Doig et al. 2002).

The clinical features of pulmonary *M. malmoense* and MAC infection are similar. Some 70 percent of the patients have pre-existing pulmonary disease (e.g. TB, COPD, lung cancer) (Henriques et al. 1994). Radiographic features do not distinguish pulmonary *M. malmoense* infections from those due to other NTM or *M. tuberculosis* (Evans et al. 1993). *M. malmoense* is also the cause of cervical lymphadenitis in young children (Falkinham 1996), of infections of the skin (Schmoor et al. 2001), larynx (McEwan et al. 2001), and of tenosynovitis (Zenone et al. 1999), whereas other extrapulmonary or disseminated infections (Sarkar et al. 2001) have rarely been reported.

M. marinum

M. marinum is a photochromogenic species with an optimal growth temperature of 25–35°C. It is most commonly associated with a superficial cutaneous infection sometimes referred to as 'swimming-pool granuloma' or 'fish-tank granuloma'. Infections in snakes (Hernandez-Divers and Shearer 2002) and in aquatic animals are well known, e.g. in fish (Ucko et al. 2002; Dos Santos et al., 2002), sea urchins (De la Torre et al. 2001), and whales (Bowenkamp et al. 2001). In humans it causes superficial and deep infections in the extremities (Figure 11). Almost all infections occur after injuries during activity in contaminated water (e.g. fishing, handling fish, working in an aquarium) (Huminer et al. 1986; Johnston and Izumi 1987; Trampuz et al. 2002). After initial injury, the incubation period is generally 2–3 weeks and the typical presentation is a single papulonodular lesion that slowly enlarges and eventually begins to drain pus (Johnston and Izumi

Figure 11 Mycobacterium marinum *foot infection in a young boy who injured his fifth toe while swimming and developed a sporotrichoid spread of infection to the top of the foot. (Courtesy of M. Stone, MD, University of Iowa College of Medicine.)*

1987; Gluckman 1995). With time, it may become verrucous or ulcerated (Falkinham 1996). Superficial infections usually heal spontaneously, but the course is very prolonged and associated with discomfort. In approximately 20 percent of cases, local or lymphatic spread forms subcutaneous nodules along the regional draining lymphatics in a manner similar to sporotrichosis (Wolinsky et al. 1972; Gluckman 1995). Further extension to regional lymph nodes and systemic infection does usually not occur because of the low temperature required for optimal growth. Reports vary as to whether granulomas are seen in biopsies. Culture of biopsy specimens at 32°C increases the probability of diagnosis to 70–80 percent (Johnston and Izumi 1987; Gluckman 1995). Disseminated *M. marinum* infection has also been reported in patients with non-Hodgkin's lymphoma.

M. paratuberculosis

M. paratuberculosis is the etiological agent of Johne's disease, a chronic intestinal disease in ruminants. The species fails to synthesize mycobactin and growth media must be supplemented with iron. Mycobactin-dependent organisms resembling *M. paratuberculosis* have been isolated from wood pigeons; *M. paratuberculosis*, *M. avium*, and the isolates from wood pigeons are closely related by DNA–DNA homologies and may represent subspecies of a single species (Thorel et al. 1990). *M. paratuberculosis* has been suspected as a cause of Crohn's disease, a human intestinal illness with characteristic lesions resembling those of Johne's disease (Chiodini 1989; Ryan et al. 2002). There is evidence suggesting that the organisms may occur as spheroplasts in intestinal lesions, and recovery of this cell-wall free form with a requirement for mycobactin may be the reason why culture has been so unsuccessful.

M. simiae

M. simiae is one of the very few NTM synthesizing niacin. Unless tested for pigment production under the influence of light, it may be misidentified as *M. tuberculosis* by the inexperienced observer. The organism has been recovered from tap water and has been the agent of a nosocomial pseudo-outbreak (El Sahly et al. 2002). Clinical isolates have come from a few geographic areas, including the southwestern USA and the Caribbean (American Thoracic Society 1997) as well as Israel (Huminer et al. 1993). It is usually a respiratory colonizer and causes infection in only approximately 20 percent of cases (O'Brien et al. 1987; Falkinham 1996). Clinical manifestations such as osteomyelitis and chronic pulmonary and disseminated disease are rare (see Falkinham 1996; Alcala et al. 1999; Braun-Saro et al. 2002). Cases of *M. simiae* infection mimicking MAC in AIDS patients are known (Huminer et al. 1993; Legrand et al. 2000).

M. szulgai

Although *M. szulgai* is closely related to *M. malmoense*, based on its 16S rDNA sequence, phenotypic distinction between the two species is simple. *M. szulgai* is scotochromogenic at 37°C and photochromogenic at 25°C. Its distribution appears to be worldwide, but its natural reservoir is unknown.

M. szulgai is uncommonly isolated from pulmonary specimens, but when isolated it is generally pathogenic; one-half to two-thirds of infections are pulmonary (Maloney et al. 1987). Clinically, these resemble those due to *M. tuberculosis* and they generally occur in elderly males (Tortoli et al. 1998a). The remaining presentations include rare cases of bursitis, cervical lymphadenitis, tenosynovitis, cutaneous infections, and osteomyelitis (Falkinham 1996). Cases of *M. szulgai* infections in AIDS patients and disseminated disease in an immunocompetent patient have also been reported (Fang et al. 1999).

M. ulcerans

Failure to cultivate this organism in the past was due to its fastidious, heat-sensitive nature as well as to a long generation time compared with that of other NTM. Many conventional decontamination protocols used in mycobacteriology interfere with the viability of *M. ulcerans* (Palomino and Portaels 1998). Supplementation of media with egg yolk or reduced oxygen tension enhances the rate of recovery in the BACTEC 460TB System (Palomino et al. 1998). Molecular techniques have been developed that may help to lead to more rapid diagnosis (Portaels et al. 1997).

M. ulcerans was first described by MacCallum et al. (1948) in Bairnsdale in Australia and named for the

characteristic indolent necrotizing skin ulcer ('Bairnsdale ulcer') it causes. Cutaneous infections also occur in other tropical climates, including Central and South America, Southeast Asia, and West Africa, where the characteristic lesion is called the 'Buruli ulcer' (Muelder and Nourou 1990; Goutzamanis and Gilbert 1995). The frequency of *M. ulcerans* infection has long been underestimated due to the difficulties in isolating the pathogen. Today, *M. ulcerans* infection is the third most frequent mycobacterial disease in humans after tuberculosis and leprosy (Weir 2002).

M. ulcerans is considered an environmental saprophyte in tropical regions and has been isolated from swampy water in Uganda, but the natural reservoir has not been clearly identified (Muelder and Nourou 1990; Hayman 1993). The mode of transmission to humans is also uncertain, but traumatic inoculation into the skin is most likely. Disease probably occurs via direct contact with contaminated environment or via water-dwelling fauna such as insects. Most lesions appear on the extremities. At first, a hard, painless, and usually pruritic subcutaneous lump forms. After a few weeks, a shallow ulcer develops. *M. ulcerans* produces a toxin that causes necrosis (Van der Werf et al. 1999). The type of disease ranges from a localized nodule or ulcer to widespread ulcerative (Figure 12) or nonulcerative disease including osteomyelitis. If untreated, severe limb deformities with contractures and scarring are common. Disseminated *M. ulcerans* disease has been reported in an HIV-positive patient (Johnson et al. 2002). There is growing evidence that *M. ulcerans* also produces disease in wild animals such as lizards, oppossums, koala bears, armadillos, rats, mice, and cattle (Portaels et al. 2001).

M. xenopi

Strains of this species require 6–8 weeks to form visible, very fine, round colonies on primary isolation. With age, colonies may become pigmented. The optimum growth temperature for the species is 45°C.

Figure 12 *Buruli ulcer on the body of a young girl caused by* Mycobacterium ulcerans *(Courtesy of F. Portaels PhD, Institute of Tropical Medicine, Antwerp/Belgium.)*

The species name derives from the initial isolation from a dermal granuloma of the toad, *Xenopus laevis* (Schwabacher 1959). The usual habitat of *M. xenopi* is water. Case clusters have been observed along coastal regions of Europe and in Ontario, Canada. Pulmonary *M. xenopi* infections occur more frequently in the UK, Western Europe, and Canada (Smith and Citron 1983; Simor et al. 1984; Falkinham 1996) than in the USA, where *M. xenopi* accounts for less than 0.25 percent of mycobacteria isolated and reports of pulmonary infections have been more sporadic (O'Brien et al. 1987). In areas where *M. xenopi* is more endemic, it is most often a colonizer (60–70 percent of cases), so that the problem of distinguishing pathogens from colonizers of the respiratory tract is similar to that for MAC isolates. Increased isolation of the organism may, however, also be due to improved laboratory techniques (Donnabella et al. 2000).

Pulmonary disease is the most common manifestation of infection (American Thoracic Society 1997; Jiva et al. 1997; Bachmeyer et al. 2001), usually occurring in middle-aged and older males with underlying lung disease such as COPD, bonchiectasis, or old pulmonary scarring from previous pulmonary TB. The illness is subacute and characterized by cough, sputum production, weight loss, and malaise. Chest X-ray and histology of pulmonary lesions cannot be distinguished from TB and shows cavitary lesions of the upper lobes in 73–96 percent of patients; granulomas are seen on lung biopsy (Smith and Citron 1983; Simor et al. 1984). Extrapulmonary infections such as septic arthritis (Kelly et al. 1999), catheter-related infections, spondylitis (Danesh-Clough et al. 2000; Kulasegaram et al. 2001), and dissemination (Kesten and Chaparro 1999) have also been described both in immunocompetent and in immunocompromised individuals. Failure in hygiene practices has led to nosocomial outbreaks of *M. xenopi* in patients after discovertebral surgery (Astagneau et al. 2001).

NTM RARELY RECOVERED OR INFREQUENTLY CAUSING HUMAN DISEASE

Some NTM, including *M. gordonae*, *M. scrofulaceum*, and *M. terrae* complex, are frequently recovered from clinical specimens but are rarely associated with human disease. Some of the older case reports of infections attributable to these mycobacteria lack sufficient documentation of disease association or identification, the latter mainly due to unsatisfactory laboratory techniques. Other species, such as *M. asiaticum* or *M. shimoidei*, are globally so rarely recovered that most laboratories will never see them.

M. asiaticum

The first published report of human disease described five patients with pulmonary disease in Australia. There

were patients with respiratory disease in the USA and elsewhere as well (Taylor et al. 1990) and also with bursitis (Dawson et al. 1995) and tenosynovitis (Foulkes et al. 1998).

M. bohemicum

The organism is a bright yellow species that has been recovered from veterinary (goat) and environmental (freshwater) sources. Initially, it was isolated from a patient with Down's syndrome and tuberculosis (Reischl et al. 1998), but it was later also found in children with cervical lymphadenitis (Tortoli et al. 2000).

M. branderi

Isolated from respiratory specimens collected in Finland, this organism was characterized in 1995 and considered a new potential pathogen (Koukilä-Kähkölä et al. 1995). The nonpigmented species is closely related to *M. celatum*, *M. cookii*, and *M. xenopi* based on comparative 16S rRNA sequencing. Wolfe et al. (2000) reported a case in which *M. branderi* was the only organism isolated upon culture from a hand infection.

M. celatum

M. celatum shares morphological and biochemical characteristics with MAC, *M. malmoense*, and *M. shimoidei* and can, therefore, only be distinguished from them by molecular techniques. It has two copies of the rRNA gene. Because of the high similarity of the 16S rRNA gene sequence with that of *M. tuberculosis*, several strains have been misidentified in the past (Tjhie et al. 2001).

M. celatum has been isolated from diverse geographic regions, i.e. throughout the USA, Finland, and Somalia, mainly from respiratory tract, stool, and blood specimens. Many of the patients have been infected with HIV (Butler et al. 1993; Golizadeh et al. 1998). In addition, *M. celatum* has been isolated from an immunocompetent child suffering from cervical lymphadenitis and from an elderly female patient with a fatal pulmonary infection (Bux-Gewehr et al. 1998).

M. conspicuum

Growing at 22–31°C and producing pale yellow colonies on solid media, this organism is related to *M. asiaticum* and *M. gordonae*, as based on its 16S rRNA gene sequence. It is known to have been the etiological agent for disseminated disease in two AIDS patients (Springer et al. 1995).

M. gastri

This species was isolated from gastric washings and isolates have formed a clear and separate cluster in studies based on numerical taxonomy (Wayne 1984). Based on the 16S rRNA gene sequence *M. gastri* is indistinguishable from *M. kansasii*. Nevertheless, it is easily distinguishable from *M. kansasii* by its colony morphology and the inability to produce pigment and reduce nitrate. The species has not been associated with disease in man or animals and is encountered only rarely, even though it has been isolated from sputum and soil. Also, as judged by modern identification criteria, none of the published reports gave sufficient details to conclusively identify the organism as *M. gastri*.

M. gordonae

M. gordonae is the most commonly encountered 'nonpathogenic' species in clinical mycobacteriology laboratories owing to its wide distribution in water and soil. In only one of 38 published reports of *M. gordonae* infection was there convincing evidence that the organism played a relevant role in disease (Wayne and Sramek 1992). There are reports of peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (Harro et al. 1997). Importantly, Eckburg et al. (2000) have reviewed clinical and chest radiographic findings among people with sputum positive for *M. gordonae* and concluded that it is merely a nonpathogenic colonizing organism, even among people with local or general immune suppression and abnormal chest X-ray findings.

M. heckeshornense

Described in 2000, the scotochromogenic species was recovered from the lungs of an immunocompetent patient wih severe bilateral cavitary disease. On the basis of its unique 16S rRNA and 16S–23S spacer gene sequences, it appears to be a new species (Roth et al. 2000; Richter et al. 2001).

M. heidelbergense

Biochemically, *M. heidelbergense* is indistinguishable from *M. malmoense*, but it is unable to grow under microaerophilic conditions. Sequencing of the 16S rRNA gene positions the species on a branch separate from *M. malmoense* but within a group of slowly growing mycobacteria with a high degree of similarity to *M. simiae* (Haas et al. 1997). *M. heidelbergense* was first described in 1997 as an isolate from a child with recurrent lymphadenitis. The organism was also found to mimic a lung tumor in a previously healthy woman (Pfyffer et al. 1998b) (Figure 13).

M. interjectum

Closely related to *M. simiae*, colonies of this species are scotochromogenic. *M. interjectum* has been recovered from children with chronic lymphadenitis (Springer et al. 1993; De Baere et al. 2001) and from an AIDS patient with diarrhea (Green and Afessa 2000).

Figure 13 Mycobacterium heidelbergense *infection mimicking a lung tumor. Computed tomography scan at the level of the middle lung lobe showing a solid lesion located subpleurally, without calcification or necrosis.*

M. intermedium

The photochromogenic species can easily be confused with *M. asiaticum*, although differences exist in colony morphology. It has repeatedly been isolated from a patient with chronic pulmonary disease (Meier et al. 1993).

M. lacus

A novel nonchromogenic mycobacterium has been isolated from synovial tissue from an elderly female with bursitis of her elbow (Turenne et al. 2002). Its 16S rRNA gene sequence shows a close relationship with *M. malmoense*, *M. marinum*, *M. ulcerans*, and *M. tuberculosis* complex.

M. lentiflavum

First described in 1996, this species usually has a bright yellow pigment, a distinct pattern of mycolic and cellular fatty acids and does not grow at 45° C. The majority of the isolates were from respiratory specimens (Tortoli et al. 2002), but it was also the etiological agent of cervical lymphadenitis (Haase et al. 1997; Cabria et al. 2002) and of disseminated infection in HIV-positive patients (NgoNiobe et al. 2001; Ibanez et al. 2002).

M. palustre

Described in 2002, this organism has been the etiological agent of lymphadenitis in a child (Torkko et al. 2002).

M. scrofulaceum

Derived from 'scrofula,' the historical term was used to describe mycobacterial infections of the cervical lymph glands. Until the 1980s, the organism was the most common cause of mycobacterial cervical lymphadenitis in children. Nowadays, it has been replaced primarily by MAC (Wolinsky 1995). There are only a few reports of other human diseases, e.g. pulmonary disease, conjunctivitis, osteomyelitis, meningitis, granulomatous hepatitis, and disseminated disease (see Falkinham 1996).

M. shimoidei

First described in 1988 in a Japanese patient with cavitary lung disease, this is a thermophilic organism, growing well at 45°C. In general, *M. shimoidei* bacilli are unreactive in standard biochemical identification tests. Fewer than ten cases of *M. shimoidei* infection have been reported in the meantime (Mayall et al. 1999; Sundman et al. 2002). The disease resembles pulmonary tuberculosis and occurs in elderly males, in one case with pre-existing silicosis.

M. terrae complex

Unambiguous identification of the members of the complex (*M. terrae*, *M. triviale*, *M. nonchromogenicum*) requires molecular methods. Clinical disease due to *M. terrae* is generally limited to pulmonary disease and infections of the hand such as tenosynovitis or more severe manifestations (Figure 14) following local trauma (Smith et al. 2000). *M. nonchromogenicum*, which is ubiquitous in the aquatic environment, has been the cause of bacteremia in an AIDS patient (Mayo et al. 1998).

M. triplex

Most closely related to *M. simiae* and *M. genavense*, this differs from the former in not producing any pigment and from the latter in growing easily on solid media (Floyd et al. 1996). It was characterized in 1996 as an unusual cluster of slowly growing mycobacteria that strongly resembled MAC but were DNA gene-probe-negative. The majority of isolates were from either lymph nodes or respiratory specimens. *M. triplex* caused

Figure 14 *Tenosynovitis due to* Mycobacterium terrae.

a pulmonary infection in an immunocompetent patient (McMullan et al. 2002) and disseminated infections in an HIV-positive patient during antiretroviral therapy (Cingolani et al. 2000) and in a liver transplant patient (Hoff et al. 2001).

M. tusciae

The yellow-pigmented organism has been found in tap water and in a few clinical specimens in 1999, i.e. in a lymph node of an immunocompromized child and in a respiratory specimen of a patient with cystic fibrosis (Tortoli et al. 1999).

PSEUDOINFECTIONS

Pseudoinfection is the term used to describe a mistaken diagnosis because of clinical specimen contamination. With today's molecular techniques, pseudoinfections can easily be proven, e.g. whether cross-contamination has been linked to the laboratory procedures per se or to contaminated bronchoscopes (Bennett et al. 1994; Agerton et al. 1997; Gillespie et al. 2000). False-positive results may be generated at any step between specimen collection and reading of cultures (ASTPHLD 1997). Examples of such occurrences include the contamination associated with laboratory processing (Tokars et al. 1990; Bearman et al. 2002) and the contamination by *M. tuberculosis*, *M. xenopi*, and *M. gordonae* of hot water used to clean or rinse bronchoscopes after use (Steere et al. 1979; Nye et al. 1990; Gubler et al. 1992; Bennett et al. 1994). In each case, elimination of the contamination resulted in eradication of the pseudoinfection. Clusters of infections by these bacteria without supportive clinical evidence of infection are a clue that the cause may be contamination. Nosocomial pseudoinfection are associated with contamination of potable water and accumulated organic material at hot-water taps in hospitals (Costrini et al. 1981; Sniadack et al. 1993; El-Sahly et al. 2002).

TREATMENT OF MYCOBACTERIAL INFECTIONS, PREVENTION, AND CONTROL

Susceptibility to chemical and physical agents

Mycobacteria are as susceptible as other nonspore-forming bacteria to heat, and to some other physical and chemical agents, although some early work on the heat susceptibility of mycobacteria may have suggested otherwise (reviewed by Corper and Cohn 1937). For example, the conditions for pasteurization of milk were selected on the basis of the killing curves for tubercle bacilli (55°C for 1 h), because at that time they were considered the most heat-resistant organisms in milk. The discrepancies in the early studies were probably due to differences in methodology and selection of an end point. Materials containing mycobacteria can be readily sterilized by the standard conditions used (15 min at 121°C for clean materials and 30–60 min for infectious wastes) (Marsik and Denys 1995).

Heat susceptibility varies among the *Mycobacterium* spp. Tubercle bacteria are killed at >65°C for at least 30 min. The kill rate for a strain of *M. avium* isolated from human and porcine tissues was not significant at 60°C, whereas the decimal reduction values (D values) were 4 min or less at 65°C and 1.5 min or less at 70°C (Merkal and Crawford 1979). The temperature required to kill *M. bovis* was 6–7°C lower than that for *M. avium* in meat products (Merkal and Whipple 1980); in a study of nine species frequently isolated from domestic water supplies (Schulze-Röbbecke and Buchholtz 1992), D values at 55°C ranged from 6 min for an isolate of *M. kansasii* to approximately 6 h for an isolate of *M. xenopi*. Of the other isolates tested, *M. phlei* was rather heat-resistant, *M. marinum* was rather susceptible, and the five other species tested (*M. avium*, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, and *M. scrofulaceum*) were intermediate in their susceptibility to heat. In *M. thermoresistibile*, mycolic acid synthesis was considerably reduced at 55°C compared with 37°C and the production of fatty acids, presumably precursors of mycolic acids, was increased (Kremer et al. 2002).

Mycobacteria are generally resistant to acids and alkalis, and this feature is used to advantage in isolation procedures. As much as 2 percent sodium hydroxide, 2 percent sulfuric acid, or 2.5 percent oxalic acid can be used to kill contaminants in specimens prior to culture. However, the killing activity of acids and alkalis increases with increasing temperature of exposure, and resistance varies greatly among different species, so care must be taken in any decontamination procedure to avoid killing the mycobacteria of interest. Tubercle bacilli are also resistant to quaternary ammonium compounds and, indeed, cetylpyridinium chloride has been used for decontamination of clinical specimens prior to culture (Kent and Kubica 1985).

With respect to chemical disinfectants, mycobacteria are susceptible to a variety of chemical agents, including alcohols (70 percent ethyl alcohol; isopropyl is not as active as ethanol), chlorine, glutaraldehyde, iodophores, ethylene oxide, formaldehyde, hydrogen peroxide, and phenolic compounds (Marsik and Denys 1995). Derivatives of phenol in which a functional group (e.g. chloro, benzyl, phenyl, or amyl) replaces one of the hydrogen atoms on the aromatic ring are effective and safe to use (Marsik and Denys 1995). The three most commonly used derivatives are *ortho*-phenylphenol, *ortho*-benzyl-*para*-chlorophenol, and *para*-tertiary amylphenol. A ten-fold dilution of household bleach (5.25 percent sodium hypochlorite) provides a solution with approximately 5 000 ppm available chlorine, which may be used

for immersion of contaminated glassware, in discard pans in biological safety cabinets, and for general laboratory clean-up. However, the amount of chlorine needed to kill the various mycobacteria ranges from 1 000 to 10 000 ppm (Best et al. 1990; Marsik and Denys 1995). However, agents that are inactivated in the presence of organic matter (e.g. alcohols) cannot be relied upon to disinfect sputum and other protein-containing materials. With iodophors, the bactericidal effect depends on the content of available iodine as well as on the presence of organic matter (Best et al. 1990). Other agents such as malachite green, quaternary ammonium compounds, hexachlorophene, and chlorhexidine are bacteriostatic at best. Vaporization of *para*-formaldehyde crystals is used for the disinfection of large areas. The conditions for this procedure are very detailed since the area must be sealed to prevent the escape of toxic gas (CDC 1999).

Endoscopes are a potential source for the spread of mycobacteria and represent a challenge for disinfection. Alkaline 2 percent solutions of glutaraldehyde at pH 7.4–8.5 have been used, but claims for its activity against mycobacteria must be evaluated carefully and the exposure time must be adequate to assure tuberculocidal activity (Marsik and Denys 1995). Rutala and Weber (1995) reviewed studies relating to the tuberculocidal activity of glutaraldehyde and concluded that the number of bacilli on endoscopes could be reduced by four logs by a standardized cleaning protocol including immersion in a glutaraldehyde solution for 20 min at 20°C. Current requirements are that endoscopes be immersed in the glutaraldehyde solution at 25°C for 45 min.

Mycobacteria are resistant to freezing and desiccation and can survive for long periods on inanimate objects if protected from ultraviolet (UV) light, which is highly tuberculocidal. For example, 90 percent of a population of 10^6–10^7 cells of *M. tuberculosis* suspended in tap water at a depth of 2 cm can be killed by exposure to 5 000 μW s/cm^2 of UV light or by 99.99 percent with exposure to 20 000 μW s/cm^2 of UV light, doses that are similar to those required to kill other bacteria (Rubbo and Gardner 1965). Tubercle bacilli can remain suspended as a stable aerosol in air for many hours. The persistence of tubercle bacilli on surfaces and in air prompted development of methods for removal including treatment of the air and surfaces with UV light (Riley 1957, 1961), filtration of room air through high-efficiency particulate air filters (Rutala et al. 1995), and the use of respirators certified by the National Institute for Occupational Safety and Health (NIOSH 1995). Each procedure can clearly reduce the number of tubercle bacilli available to be inhaled. Given the low infective dose, strict adherence to the commonly accepted safety requirements in the mycobacteriology laboratory (e.g. CDC/NIH biosafety guide for laboratories; CDC 1999) is mandatory.

Susceptibility to antimicrobial agents

M. TUBERCULOSIS COMPLEX

Diseases caused by *Mycobacterium* spp. are difficult to treat, and the search for effective drugs has been active, the most intensive search being for drugs effective against TB, the mycobacterial disease of greatest public-health significance. Doub (1979) recounts some of the early events leading to the development of anti-TB drugs and concludes that 'of those that succeed [as anti-TB drugs], it is a story of serendipity and of chemical intermediates for planned drugs.' Synthetic drugs active against *M. tuberculosis* include *p*-aminosalicylic acid (PAS), PZA, INH, ethionamide, and EMB. Antibiotics are RMP, other rifamycins (e.g. rifabutin), SM, viomycin, kanamycin, capreomycin, and cycloserine. One of the newer active drugs is ofloxacin, a fluorinated carboxyquinolone, and linezolid, an oxazolidinone compound, has promising effects as well. The modes of action of the antimycobacterial drugs have been reviewed (Musser 1995; Inderlied and Pfyffer 2003).

The reliability and standardization of methods for testing the susceptibility of *M. tuberculosis* isolates to anti-TB drugs were discussed by Canetti et al. (1969) and the methods used routinely in most mycobacteriology laboratories have been reviewed (Kent and Kubica 1985; Heifets and Good 1994; Inderlied and Pfyffer 2003). A key to producing reliable results is the use of standardized drug concentrations to determine if a strain is resistant. As defined by Canetti et al. (1963): 'Resistance is clinically significant when at least 1 percent of the total bacterial population develops at the so-called critical concentrations.' The 'critical concentration' is the weakest concentration of drug that inhibits the growth of more than 95 percent of wild-type isolates of a given species using precisely defined experimental conditions. For *M. tuberculosis*, the critical concentration of INH in 7H10 agar medium is 0.2 μg/ml, RMP is 1.0 μg/ml, PZA is 25.0 μg/ml, and EMB is 5.0 μg/ml (Kent and Kubica 1985), while the critical concentrations in liquid media may be different (NCCLS 2003). Additional concentrations are often tested to provide information to physicians to assist them in designing a therapeutic regimen. However, the critical concentration is always included so that there is a historical basis to determine whether resistance increases in specific populations over time. Recognition of the emergence of resistance and the need to insert new drugs into a treatment regimen are dependent on collection of accurate data.

Drugs

Isoniazid INH (isonicotinic acid hydrazide) was first synthesized in the early part of the twentieth century, but its activity against tubercle bacilli was not discovered

until mid-century. The drug has been the mainstay of antituberculosis therapy for more than 40 years, but neither the target site in the mycobacterial cell nor the mode of action is well understood (Zhang and Young 1993; Musser 1995). The antibacterial spectrum of INH is limited largely to inhibition of strains of *M. tuberculosis*. The MIC for susceptible strains of *M. tuberculosis* is usually less than 0.05 μg/ml, and other species in the *M. tuberculosis* complex are also highly susceptible. However, MAC strains and strains of other species are not susceptible to INH. Middlebrook (1954) found that there was a link between INH resistance and reduced catalase activity. However, the exact relationship and molecular basis of INH resistance are still unknown. INH per se is not active and its activation is achieved by the catalase-peroxidase encoded by the *katG* gene. The KatG enzyme activates INH in a toxic metabolite that blocks the synthesis of mycolic acids (Zhang and Young 1993; Musser 1995). Numerous mutations, including point mutations and deletions, have been identified in the *katG* gene. Those located in part of the gene corresponding to the NH2 domain of the protein, i.e. the active site of heme binding, are correlated with a loss of the KatG activity and high resistance to INH whereas those located in part of the gene corresponding to the COOH domain, where INH binds, still have some KatG activity and are correlated with a low INH resistance. The low INH resistance may also be related to surexpression of two enzymes involved in the synthesis of mycolic acids, InhA and the InhB complex, composed of AcpM and KasA proteins. It has been proposed that activated INH binds to NADH, and that this complex once bound to InhA, an enoyl-acyl carrier protein reductase, inhibits mycolic acid synthesis. InhA surexpression correlated with mutations in the promoter region of *inhA* confers low resistance to INH and cross-resistance to the second-line drug ethionamide. The *kasA* gene encodes an enoyl-acyl carrier protein synthase involved in mero-mycolic acid synthesis. Mutations in *kasA* have been found in low INH-resistant strains and also in sensitive strains. Mutations in INH-resistant strains are associated with mutations in *katG* or *inhA*, and the significance of mutations in *kasA* is still unclear. Another gene, *ahpC*, has been involved. The AhpC protein contributes to detoxification of organic peroxydes into alcohols, according to a second pathway of defence against oxidative stress products. Mutations in the promoter region of *ahpC* yields an overexpression of AhpC, which compensates for the loss or diminished activity of KatG. Altogether, mutations in these genes may account for approximately 90 percent of INH resistance.

Rifampicin RMP was introduced in 1968 for the treatment of TB and is a first-line drug along with INH, PZA, and EMB. It is a broad-spectrum antibiotic that is active against *M. leprae*, *M. kansasii*, *M. haemophilum*, and *M. marinum*, as well as bacteria in other genera. The mechanism of action is similar to that in *E. coli* and involves inhibition of DNA-dependent RNA polymerase (Jin and Gross 1988; Musser 1995). Studies showed that more than 96 percent of RMP resistance could be attributed to mutations within an 81-bp region of the *rpoB* gene (Telenti et al. 1993a). The molecular mechanism of rifampin resistance in *M. leprae* is similar to that in *M. tuberculosis*.

Pyrazinamide PZA is a synthetic derivative of nicotinamide that is rapidly bactericidal for reproducing cells of *M. tuberculosis*; the average MIC is 20 μg/ml (Inderlied and Pfyffer 2003). The drug is inactive against nonreplicating tubercle bacilli and against all other species of mycobacteria, including *M. bovis*, MAC, and rapid growers. Optimal in vitro activity of PZA is observed at pH 5.5–6.0. Neither the mechanism of action nor the molecular basis for resistance is fully understood (Musser 1995) although there is evidence that the enzyme that hydrolyzes PZA to pyrazinoic acid, pyrazinamidase, is essential to susceptibility to the drug (Butler and Kilburn 1983). Lack of pyrazinamidase activity and its correlation with PZA resistance has been associated with mutations in the *pncA* gene that encodes the enzyme (Scorpio et al. 1997). Apparently, 72–97 percent of PZA resistance can be attributed to mutations in that gene.

Ethambutol EMB is a synthetic compound introduced in 1961 for the treatment of TB. Preliminary studies were carried out with the racemic mixture of the compound, but only the D-isomer is effective, which is less toxic than the racemic mixture. MICs against wild-type isolates of *M. tuberculosis* are 1–5 μg/ml, but the drug is rather inactive against other species (Inderlied and Pfyffer 2003). EMB inhibits cell-wall synthesis and is bacteriostatic. EMB resistance correlates with a specific mutation at codon 306 in the *embB* gene, which encodes arabinosyltransferase. Mutations in this codon were associated with MICs of 20–40 μg/ml for several EMB-resistant isolates of *M. tuberculosis* (Alcaide et al. 1997a).The mechanism of action probably involves inhibition of arabinogalactan synthesis (Takayama and Kilburn 1989) or synthesis of precursors of cell-wall components (Musser 1995). The drug has a synergistic effect in combination with ciprofloxacin, amikacin, and rifampicin against *M. malmoense* (Hoffner et al. 1993).

Streptomycin SM has been used extensively in the treatment of TB. Amikacin and kanamycin can be administered as well while gentamycin and tobramycin are inactive against mycobacteria at the usual concentrations attained in serum. SM is active in vitro against *M. tuberculosis*, *M. kansasii*, and *M. marinum*. The drugs must be given by injection and, over the years, SM in particular,

lost popularity and became difficult to obtain. Ototoxicity is the major side effect associated with aminoglycosides. Concerning SM resistance of *M. tuberculosis*, about 66 percent of strains display changes in the *rpsL* or 16S rRNA gene, as is seen in other bacteria (Musser 1995). This observation implies that there is at least one additional mechanism conferring resistance.

Drug regimens

Today's standard regimen (directly observed therapy, short-course (DOTS)) according to the WHO/IUATLD consists of a combination of three or four drugs (INH, RMP, EMB, PZA) for 2 months, followed by a dual combination of INH and RMP for 4 months. For multidrug-resistant TB, additional drugs have to be added and length of therapy adapted.

Prevention and control of human TB

The principles of TB surveillance and control have been reviewed in detail by Styblo (1986). As TB is almost always spread from person to person, chemotherapy, by rendering source cases uninfectious, is the main component of control programs. The impact of chemotherapy on the spread of infection depends on the effectiveness of the therapeutic regimens, the adherence (compliance) of the patients, and the percentage of cases that are diagnosed. The impact of chemotherapy on disease control also depends on the interval between onset of infectivity and commencement of therapy. The epidemiological impact of chemotherapy is considerably reduced if source cases have infected all their household contacts by the time they commence therapy. Patients receiving effective chemotherapy are rapidly rendered noninfectious even though they continue to excrete cultivable tubercle bacilli for several weeks. For practical purposes, patients are usually regarded as being noninfectious from the time that chemotherapy is started (Rouillon et al. 1976), though this is a risky assumption in regions and communities where multidrug resistance is common.

Cases may be detected passively, i.e. by waiting for those with symptoms to attend health centers, or actively, by searching for suspects, usually defined as people with a cough for 1 month or more in duration.

Contact tracing is a key component of tuberculosis control and up to 10 percent of cases of TB may be found in this way. Household contacts of patients with smear-positive pulmonary disease should be screened, as should the contacts of patients with nonrespiratory smear-negative pulmonary disease, as they may prove to be the source cases.

Vaccination strategies

A living, attenuated vaccine was produced by Calmette and Guérin from a tubercle bacillus isolated from a case of bovine mastitis by passaging it 230 times over a period of 11 years, by which time extensive animal studies showed that it had lost its virulence. This vaccine, bacille Calmette–Guérin (BCG), was given orally to neonates in 1921 but is now given by injection or multipoint inoculation.

The protective efficacy of BCG vaccine has been the subject of considerable controversy, as a number of major trials have shown its efficacy to vary from around 80 percent to none at all (Colditz et al. 1994). The various explanations of these discrepancies are outlined elsewhere. When given to an uninfected (tuberculin-negative) child, BCG offers protection against the serious forms of primary infection, especially those due to dissemination of bacilli from the primary complex. It is, however, much less effective in preventing tubercle bacilli from persisting in the tissues and becoming reactivated later in life, which greatly limits its use in the control of TB.

BCG protects equally well against leprosy as against tuberculosis (Brown et al. 1966) and protects children against cervical lymphadenitis caused by environmental mycobacteria (Romanus 1990). Thus, there is little doubt that the mycobacterial antigens that induce protective immunity are mostly or entirely among those that are common to all species in the genus.

LEPROSY

In view of the antiquity of leprosy, there are surprisingly few historical accounts of treatment. One substance, chaulmoogra oil, had been used to treat leprosy in India for 2 000 years and in China from the fifteenth century, and more purified preparations, given by mouth and by injection, were widely used during the period 1850–1900. Hansen and Looft (1895), in an excellent and nihilistic chapter, described how none of the then recommended remedies, including chaulmoogra oil and Koch's old tuberculin, helped their patients. From about 1900 onwards, derivatives of chaulmoogra oil, especially iodized ethyl esters, which could be administered parenterally, were developed, and Lara (1930) claimed that, since 1921 in Culion Leprosarium, 27.1 percent of 8 520 patients became skin-smear-negative for acid-fast bacilli following their intradermal use. By and large, it was only the tuberculoid and borderline cases that improved, and relapse rates were high.

In the sulfonamide era, it was found in 1941 that the disubstituted sulfone, sodium glucosulfone (Promin), protected guinea pigs from experimental tuberculosis. It proved inactive in human tuberculosis, but when given intravenously it was effective against lepromatous leprosy (Faget et al. 1943). Laryngitis was controlled, rhinitis improved, incipient blindness was prevented, and lepromatous ulcers often healed, even though the dermal infiltrate resolved only slowly and skin smears remained positive after 2 years. In 1948, the much

simpler, cheaper, and less toxic sulfone, dapsone (DDS) (Cochrane et al. 1949), which could be given orally as well as parenterally, was introduced, and by the early 1950s was recommended by WHO as the standard drug for the mass treatment of leprosy. However, relapses occurred in lepromatous patients unless treatment was maintained for many years, and from the early 1960s, relapse began to be reported in LL and BL patients who were still receiving treatment.

The mouse foot pad method provided evidence from more than 25 countries of an ever-increasing prevalence of relapsed lepromatous patients due to 'secondary' DDS resistance; it also demonstrated 'primary' DDS resistance in previously untreated lepromatous patients (Pearson et al. 1977). Against this background, the WHO convened a study group (WHO Study Group 1982) to advise on the catastrophic situation, which, if it continued, might destroy the value of dapsone – the least expensive antileprosy drug, with a proven record of efficacy and long-term safety. The Group recommended MDT for all leprosy cases (Table 3). It was anticipated that MDT might also shorten the length of treatment, just as the introduction of new and better drug combinations enabled the treatment of tuberculosis to be shortened. Two MDT regimens, designed for field use and based on the patient's bacterial load when untreated, were recommended. Patients were classified as either 'multibacillary,' which included all LL, BL, and BB patients plus those BT (and rare idt) patients who had a skin-smear BI reading of two or more at any site, or 'paucibacillary,' which included all smear-negative BT, TT, and idt cases plus those with no smear BI reading greater than one. Subsequently, for operational reasons (WHO 1988), it was agreed that all smear-positive patients should be classified as 'multibacillary.' A combination of only two of three drugs (rifampicin, dapsone, and clofazimine) was required for paucibacillary patients, as their bacterial load was too small to contain drug-resistant mutants. The choice of the three drugs was made on the basis of their high activity in mice; rifampicin alone was strongly bactericidal (see Rees 1985) and just as effective given monthly as daily (Waters et al. 1978). Monthly visits during the period of MDT ensured supervision for the administration of the most effective and costly drug (rifampicin) and of the 300-mg dose of clofazimine (Table 3). The results of MDT therapy have proved outstandingly good. In multibacillary disease, the cumulative risk of relapse on a 9-year follow-up has been 0.77 percent and in paucibacillary disease 1.07 percent (WHO 1994), although it is possible that many of the paucibacillary 'relapses' were in fact late type 1 reactions due to recognition of residual antigen rather than multiplication of surviving leprosy bacilli – the differential diagnosis is notoriously difficult. In view of these excellent results, a WHO Study Group (1994) recommended that the triple drug multibacillary regimen need only be given for 2 years in all cases; this appears very suitable for mass treatment campaigns, but as subsequently Jamet et al. (1995) reported a 20 percent relapse rate among a group of 35 previously untreated LL and BL patients who received this limited duration (2 years) chemotherapy, all the relapses occurring among patients with high bacterial loads, the original advice to continue treatment for 2 years and until skin smears become negative appears wiser for individual patients (see Waters 1995).

Since the introduction of MDT in 1982, three new groups of drugs have been found to be bactericidal for *M. leprae*, namely the quinolones pefloxacin and ofloxacin (but not ciprofloxacin), ofloxacin being given in the dosage of 400 mg daily, minocycline 100 mg daily, and clarithromycin 500 mg daily. All are less rapidly bactericidal than rifampicin, but more rapidly so than dapsone and clofazimine. The WHO Study Group (1994) has recommended that ofloxacin or minocycline should be used when patients refuse to accept clofazimine and has also suggested alternative regimens for those rare patients who suffer from rifampicin resistance or toxicity.

Prophylaxis

Measures for the control and prevention of leprosy include:

- early diagnosis, and treatment with MDT;
- health education to encourage early self-presentation;
- examination of domestic contacts associated with health education, so that any who subsequently develop symptoms of leprosy present rapidly, not waiting for annual surveillance; and
- vaccination of neonates with BCG, the vaccine used for tuberculosis.

The advent of successful MDT has increased public awareness and greatly encouraged early self-reporting.

Table 3 *The World Health Organization (WHO) Study Group's recommended treatments for leprosy*

Type of patient[a]	Rifampicin 600 mg once monthly	Dapsone 100 mg daily	Clofazimine 300 mg once monthly + 50 mg daily	Duration (months)
Multibacillary	+[b]	+	+	⩾24[c]
Paucibacillary	+	+	−[b]	6

a) For definitions, see text.
b) +, Treatment given; −, treatment not given.
c) Should be continued for at least 2 years and whenever possible until smears become negative.

WHO is aiming to 'eliminate leprosy as a significant public health problem' (defined as a prevalence of less than 1:10 000 population) by the year 2000; although some countries will not achieve this objective, the campaign, commenced in 1991, has helped to mobilize the medicopolitical will, resulting in widespread application of MDT.

There is still a need for a fully effective vaccine. Fernandez (1939) observed that over 90 percent of healthy lepromin-negative children became positive after receiving BCG. There followed a series of field trials of BCG against leprosy in three different countries (Myanmar, New Guinea, and Uganda) and two tuberculosis studies were analyzed for their leprosy protection (India and Malawi), all of which showed variable protection from 20 to 80 percent (see review by Fine 1988). Variable protection is also a feature of the use of BCG vaccine against tuberculosis. A second report from Malawi, based on a randomized controlled trial of single BCG, repeated BCG, or combined BCG, and killed *M. leprae* vaccine (Rees 1983) for the prevention of leprosy and tuberculosis (Karonga Prevention Trial Group, 1996), showed that in a population in which a single BCG vaccination affords 50 percent or more protection against leprosy, but none against tuberculosis, a second vaccination can add appreciably to the protection against leprosy. The addition of *M. leprae* to BCG did not add to the protection provided by BCG alone, although there was some suggestion that it might do so under the age of 15 years. Further long-term follow-up is required, especially as killed *M. leprae* but not killed BCG will protect mice against experimental foot-pad infection (Shepard et al. 1978). Second-generation vaccines using genetic engineering should quickly become feasible, if and when the protective antigens of *M. leprae* are identified.

Chemoprophylaxis is not currently recommended by WHO in leprosy endemic areas, partly because in the classical dapsone child study in India of Dharmendra and Noordeen (Noordeen 1969) more early cases of leprosy occurred in the dapsone group than in the control group during the first year of the study, although thereafter there was highly significant protection. However, in the UK and the USA, chemoprophylaxis is recommended for child household contacts of lepromatous cases, and BCG is also given as immunoprophylaxis in Britain. Kaur et al. (2002) reported quite satisfactory results from a combined 12-month WHO/MDT MB regimen and *Mycobacterium* w. vaccine in multibacillary leprosy.

Mitsuda reaction

This reaction, also called the 'lepromin reaction,' was described by Mitsuda in Japan in 1919. The test is carried out by the intradermal injection of 0.1 ml of an autoclaved extract of *M. leprae* from the skin lesions of lepromatous patients, the extract being referred to as 'integral lepromin.' The crude preparations of Mitsuda's era were standardized on the basis of content of tissue. Modern preparations are standardized according to their *M. leprae* content and prepared as described in a WHO Memorandum (1979). Standard Mitsuda lepromin contains 4.0×10^7 *M. leprae* per milliliter and has a shelf life of 2 years at 4°C. Mitsuda lepromin is increasingly being prepared from armadillo-derived *M. leprae*; such preparations, which are designated '(A),' are likely to replace eventually the lepromin, designated '(H),' derived from human lesions.

A positive reaction takes two forms, an early and a late one. The early reaction is characterized by an acute localized area of inflammation with congestion and oedema appearing usually within 24–48 h and tending to disappear within 3–4 days. The late reaction (Mitsuda reaction) is characterized by local infiltration of the skin starting at about 7 days and proceeding to the formation within 4 weeks of a nodule that may undergo central necrosis and ulceration and take several weeks to heal. The early or 'Fernandez' reaction (Fernandez 1940) is, like the tuberculin reaction, a delayed-type hypersensitivity (DTH). It is, however, usually poorly defined and of little practical use. In contrast, the Mitsuda reaction is not a measure of pre-existing DTH but is the manifestation of cell-mediated immunity, which the Mitsuda lepromin itself can induce. It thus discriminates between people who are capable of responding to *M. leprae* and those who cannot. It is positive in the majority of healthy adults, even in nonendemic areas, and cannot be used as a diagnostic test. On the other hand, it is of great value in classifying cases of leprosy (see above). Prognostically, the Mitsuda reaction is indicative of resistance to infection. Lepromatous patients are invariably Mitsuda-negative unless, or until, about 24 or more years have passed since they commenced effective chemotherapy without any relapse. Around this time, when presumably almost all the *M. leprae* antigens have been removed, some develop a weak positive reaction (Waters et al. 1990). Until then, they are not converted by repeated Mitsuda tests or by BCG, as healthy individuals frequently are. It is a remarkable fact that lepromatous patients, though failing to give a positive skin reaction to the leprosy bacillus, are able to do so in response to antigens of other mycobacteria, indicating that they are anergic both to *M. leprae*-specific antigens and to common mycobacterial antigens, but not to other species-specific antigens.

TREATMENT OF INFECTIONS BY NTM

Treatment of infections caused by NTM are, by far, less well established than for tuberculosis. Usually, a combination of several drugs is being administered for a long period of time (months up to 1–2 years,

depending on the species and the extent of disease). All NTM have in common that they are inherently resistant to PZA.

Pulmonary infections

With the advent of molecular testing in the laboratory, it is important to recognize the species, its potential to cause clinical disease, its susceptibility to antimicrobial drugs, and its response to drug/surgical therapy. In particular, with NTM present in respiratory specimens, it is not always easy to address these aspects, since not all individuals with sputum cultures positive for NTM require immediate treatment. In patients with severe lung problems or those with a single smear-negative, culture-positive sputum specimen, treatment should be delayed to determine whether the infection is invasive. The guidelines of the American Thoracic Society (1997) can be used to determine whether the infection is active. The need for treatment should be assessed critically in patients with medication intolerance or allergy, elderly patients with indolent disease, and patients in whom previous treatment has failed. Overall, prediction of treatment outcome from in vitro susceptibility tests continues to be a problem in infections with NTM (Heginbothom 2001; Research Committee of the British Thoracic Society 2002).

MAC. The MAC cell wall is a barrier to many antimicrobial agents (Rastogi et al. 1990). MAC isolates are generally resistant in vitro to most antituberculous drugs, and in vitro susceptibility correlates poorly with clinical response (Rynearson et al. 1971; Heifets 1988). In the past, long-term treatment failures had been commonplace and only 45–65 percent of patients responded long term to early multidrug treatment regimens (Etzkorn et al. 1986), but surgery combined with medical therapy has been effective in highly selected cases (Corpe 1981).

With the availability of newer drugs, better outcomes can be expected. The ESM drugs (e.g. clarithromycin, azithromycin) are the first with strong in vitro activity against MAC (Heifets et al. 1994). They are concentrated in lung tissue and macrophages, have a long half-life, have a postantibiotic effect, and are well tolerated orally. Availability of ESM antibiotics has led to a re-evaluation of in vitro susceptibility testing to predict the likely clinical response. Resistance to ESM in vitro may develop during monotherapy and thus predict treatment failure (Heifets et al. 1994; Wallace et al. 1994a).

Another advance is rifabutin, a derivative of rifamycin S, which is more active in vitro against MAC than rifampicin. Preliminary data suggest that clinical responses are improved only modestly when rifabutin is used in place of rifampicin (O'Brien et al. 1990). Side effects unique to rifabutin are also a drawback to its widespread clinical use. Drug combinations, such as ethambutol and rifampicin, are synergistic in vitro and effective in vivo, although MAC are resistant to these agents individually in vitro.

Usually, a regimen of an ESM, ethambutol, rifampicin, or rifabutin is recommended (ATS 1997). In severe disease the addition of streptomycin for the first 2–4 months of therapy is recommended. Treatment should be continued for 18 months to 2 years or at least 12 months after the last positive sputum culture.

M. kansasii. This is susceptible to most antituberculous agents except PZA. Some strains show intermediate resistance to INH (Wallace et al. 1990). A recommended regimen is INH, RMP, and EMB daily for 18 months. Strains isolated from patients treated with RMP are predictably susceptible to the drug at 1 μg/ml. As with most other pulmonary mycobacterioses, large controlled clinical treatment trials have not been reported. Medication regimens that include RMP have relapse rates of less than 8 percent (Ahn et al. 1981) and treatment can be shortened to 12 months if streptomycin is added for the first 3 months (Ahn et al. 1983). Resistance to RMP, which is caused by point mutations in the *rpoB* gene (Klein et al. 2001), predicts a poor outcome. RMP resistance has arisen as a result of inadequate initial therapy and has been a greater problem in HIV patients (Wallace et al. 1994b). Newer agents such as the ESMs and fluoroquinolones are active against *M. kansasii* in vitro and may be useful to treat patients infected with RMP-resistant bacteria (Jacobs 1999; Burman et al. 1998). A rifabutin-based regimen has been suggested for HIV-positive patients receiving antiretroviral therapy (Griffith 2002).

Other NTM. Treatment of pulmonary disease caused by other NTM remains controversial since it is uncertain whether in vitro testing predicts clinical response in the way it does for *M. tuberculosis* and *M. kansasii* and to a lesser extent for MAC. Agents to be primarily considered are clarithromycin, EMB, and RMP, while the utility for amikacin, INH, rifabutin, ciprofloxacin, and streptomycin are less certain (American Thoracic Society 1997). In a large randomized trial of treatments for pulmonary disease caused by MAC, *M. malmoense*, and *M. xenopi* in HIV-negative patients, the research committee of the British Thoracic Society (2001) missed a correlation of the in vitro susceptibility results with the patients' response to chemotherapy. In that study, treatment of *M. malmoense* with RMP and EMB for 2 years was preferable to RMP, EMB, and INH. Conversely, addition of INH reduced the failure of treatment/relapse rates for MAC and *M. xenopi*, but there were higher death rates overall. Lang-Lazdunski et al. (2001) pointed out that resection represented an important adjunct to chemotherapy for the treatment of *M. xenopi* pulmonary disease.

Extrapulmonary infections

Most *M. marinum* isolates are predictably susceptible to RMP and EMB. Alternative agents include

clarithromycin, amikacin, ciprofloxacin, tetracyclines, and sulfamethoxazole-trimethoprim. Even though treatment with adequate drugs is administered, surgical intervention (excision or debridement) is often necessary. Wild-type strains of *M. haemophilum* appear to be susceptible to quinolones, rifamycins, clarithromycin, and azithromycin, and usually resistant to EMB, INH, and SM. In vitro susceptibility test results may be helpful for the treatment of other NTM such as *M. xenopi*, *M. szulgai*, and *M. malmoense* because of the lack of information about the susceptibility patterns of these organisms. However, reports indicate that the correlation between susceptibility test results and therapeutic success was inconsistent (Wallace et al. 1988).

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Rapidly growing mycobacteria

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INTRODUCTION

The rapidly growing mycobacteria (RGM) were initially separated from the other nontuberculous mycobacteria by Runyon as members of his Group IV (Runyon 1965); as defined by him, these are 'Acid-fast bacilli which from small inocula mature at room temperature (25°C) within one week.' Unfortunately, not all isolates of all species of mycobacteria separate cleanly as either slow or rapid growers. For example, a significant percentage of *Mycobacterium marinum* and *Mycobacterium terrae* isolates – perhaps more than half of each – meet the technical definition of a RGM, although both species are traditionally grouped with the slow growers. It is also important to note that the definition of an RGM is not based on the time to detection of the organism on initial isolation; especially with modern liquid-based culture methods, some isolates of species generally considered to be slow growers, particularly when present in high concentration in a specimen, may occasionally be detected in less than 7 days. Conversely, some isolates of species that are generally considered to be RGMs may take more than 7 days to grow on initial isolation, especially if the culture is not incubated at the temperature optimum for the species in question. A further problem in the accurate initial categorization of an isolate is that different species of RGMs may be photochromogenic, scotochromogenic, or nonphotochromogenic (key features of the Runyon groups I, II, and III, respectively, of slowly growing nontuberculous mycobacteria). Therefore, not only may some mycobacterial isolates that belong to species considered to be slow growers initially be thought to be RGMs, but also some isolates that belong to species that are considered RGMs may initially be thought to belong to one of the groups of slowly growing nontuberculous mycobacteria.

Many case reports, even some published recently, make little or no mention of the procedures used for identifying isolates. Sometimes the only substantiation for an identification presented is that it was performed in a reference laboratory. Unfortunately, it then becomes impossible to know with certainty to what species the isolate or isolates actually belonged; this problem becomes magnified as new species are described and reference is made to the older literature. Deposition of unusual isolates, or of the sequences obtained from unusual isolates, in appropriate repositories would be helpful, but this also may not have been performed.

RGMs are widely distributed in the environment, particularly in soil and water (Wolinsky and Rynearson 1968; Wallace 1987; Carson et al. 1988; Covert et al. 1999); many outbreaks of infection are ultimately traceable to a contaminated water source. We are not aware of any reports documenting the transmission of an RGM directly from one patient to another. However, in one outbreak of otitis media, it appeared that patient-to-patient transfer via contaminated instruments played a significant role, although the ultimate source of the infecting strain may have been tap water (Lowry et al. 1988).

Different species of RGMs seem to have some propensity for causing infections at specific body sites, although it appears likely that any species could cause an infection at any site. We are aware of only a few studies of virulence factors of RGMs. Byrd and Lyons (1999) reported that rough colony variants of *M. abscessus* are more pathogenic than smooth colony variants, based on observations in murine pulmonary infection models and in human monocyte monolayers. They proposed that phenotypic changes occurred in the smooth colony variants, which affected phagosome development and function, cord formation, and monocyte–macrophage aggregation. Udou (1994) reported the presence of hemolytic activity by clinical isolates of *M. fortuitum*, *M. chelonae*, and *M. smegmatis*, which may be a virulence factor in cases of soft-tissue and cutaneous infection. However, it is clear that the precise immune status of the host is an important factor in the development of mycobacterial infections, and individuals suffering from a number of immune deficiencies in addition to acquired immunodeficiency syndrome (AIDS), hematologic malignancies, and iatrogenic immunosuppression have been found to be particularly susceptible to mycobacterial infections (Reichenbach et al. 2001).

Isolation of RGMs is generally not difficult, as they will grow on a wide variety of media. However, they may be missed if cultures are not held for at least 7 days, and their isolation may also be delayed (or possibly even prevented on occasion) if cultures are incubated only at higher temperatures such as the 35–37°C generally used for clinical specimens.

TAXONOMIC RELATIONSHIPS AND GROUPING

The phylogenetic relationships among members of the genus *Mycobacterium* have been studied using sequence data from the 16S rRNA gene (Stahl and Urbance 1990; Pitulle et al. 1992), the 23S rRNA gene (Stone et al. 1995), and the RNA polymerase (*rpoB*) gene (Kim et al. 1999). The analysis of these molecular data by these authors has supported the existence of separate evolutionary pathways for the RGMs and the slowly growing mycobacteria. However, the precise nature of the relationships observed among these two groups of organisms, as well as the extent to which the two groups are separated, seems to depend both on the phylogenetic algorithm and the specific genetic data employed. Please refer to the section on Molecular testing for more information on the phylogenetic relationships of the RGM.

As has occurred recently for many other groups of bacteria, the number of recognized species of RGMs has been increasing rapidly. Some of these new species have been defined largely or solely on the basis of their molecular features, and some descriptions are based on only one or a small number of isolates. This proliferation of species has made accurate identification of isolates of RGMs increasingly difficult. Adequate phenotypic data for identification are not available for many species, making identification based solely on phenotypic features increasingly uncertain for any isolate. To complicate matters further, any single molecular technique, such as sequencing the 16S rRNA, gene may not suffice for definitive identification, as some species that are considered distinct may have identical, or nearly identical, sequences. (For example, *M. goodii* and *M. smegmatis* reportedly differ in their 16S rRNA sequences by merely three base pairs, yet they show only 40 percent homology by DNA–DNA hybridization (Brown et al. 1999).) Therefore, not only for the convincing discrimination of new species, but even for the identification of isolates that may belong to poorly known species, DNA–DNA hybridization may be still be the only technique that provides unarguable results.

In their review of pathogenic nonpigmented or late-pigmenting RGMs, Brown-Elliott and Wallace (2002) proposed the following arrangement into groups, complexes, and species:

- *M. fortuitum* group
  - *M. fortuitum*
  - *M. peregrinum*
  - *M. fortuitum* third biovariant complex
    - Sorbitol-positive (in part, the proposed species *M. houstonense*)
    - Sorbitol-negative (in part, the proposed species *M. bonickei*)
    - *M. septicum*
    - *M. mageritense* (probably more appropriately placed in the *M. smegmatis* group)
  - *M. mucogenicum*
  - *M. senegalense*
- *M. chelonae-abscessus* group
  - *M. chelonae*
  - *M. abscessus*
  - *M. immunogenum*
- *M. smegmatis* group
  - *M. smegmatis*
  - *M. goodii*
  - *M. wolinskyi*

Other species not considered in the review by Brown-Elliott and Wallace, but mentioned here, include *M. aichiense*, *M. alvei*, *M. aurum*, *M. austroafricanum*, *M. brumae*, *M. confluentis*, *M. elephantis*, *M. fallax*, *M. farcinogenes*, *M. flavescens*, *M. gadium*, *M. gilvum*, *M. hassiacum*, *M. hodleri*, *M. holsaticum*, *M. marinum*, *M. moriokaense*, *M. neoaurum*, *M. novocastrense*, *M. obuense*, *M. parafortuitum*, *M. phlei*, *M. rhodesiae*, *M. terrae*, *M. thermoresistible*, and *M. vaccae*. Of these, *M. marinum* and *M. terrae* are generally considered with the slow-growing mycobacteria and have not been included in Table 1 or Figure 1. Some of these

Table 1 *Phenotypic characteristics of rapidly growing mycobacteria and availability of molecular and chromatographic characteristics* [a]

Mycobacterium sp.	Arylsulfatase, 3-day	Citrate	Inositol	Mannitol	Sorbitol	Iron uptake	MacConkey agar	Nitrate reduction	Pigment	NaCl tolerance	RFLP[b]	HPLC
abscessus	+	–	–	–	–	–	+	–	N	+	+	+
aichiense	+	–	+	+	–	NT	NT	–	S	NT	+	–
alvei	+	–	–	–	NT	–	–	+	N	–	+	+
aurum	V	–	+	+	–	NT	NT	+	S	NT	+	–
austroafricanum	+	+	+	+	–	NT	NT	+	S	NT	–	–
brumae	–	+	+	–	NT	+	–	+	N	–	+	TLC
chelonae	+	+	–	–	–	–	+	–	N	–	+	+
confluentis	–	NT	NT	NT	NT	–	NT	+	N	–	+	TLC
elephantis	–	–	NT	NT	NT	–	–	+	S	+	+	+
fallax	–	–	–	–	NT	–	–	+	N	–	–	GLC
farcinogenes	–	+	–	–	NT	–	NT	V	N	–	+	TLC
flavescens	V	–	–	V	V	–	–	+	S	V	+	–
fortuitum	+	–	–	–	–	+	+	+	N	+	+	+
fortuitum, third biovariant	+	–	+	+	Gp1:+Gp2:-	+	+	+	N	+	+	–
gadium	–	–	NT	NT	NT	NT	NT	+	S	NT	+	–
gilvum	–	–	V	+	–	NT	NT	+	S	NT	+	–
goodii	–	V	+	+	+	+	+	+	S	+	+	+
hassiacum	–	–	NT	NT	NT	–	–	–	S	+	-	+
hodleri	NT	–	+	+	+	NT	NT	–	S	NT	-	+
holsaticum	–	NT	NT	NT	NT	–	–	+	V	+	+	+
immunogenum	+	–	–	–	–	–	+	–	N	–	+	+
mageritense	+	V	+	+	+	+	+	+	N	+	+	+
moriokaense	+	–	V	+	+	NT	NT	+	N	V	-	TLC
mucogenicum	+	+	–	+	–	tan	+	V	N	–	+	+
neoaurum	+	+	+	+	–	+	–	V	S	+	+	GLC
novocastrense	–	NT	NT	NT	NT	–	+	+	P	+	-	TLC
obuense	+	–	V	+	V	NT	NT	–	S	NT	+	-
parafortuitum	V	V	–	V	–	NT	NT	+	V	V	-	-
peregrinum	+	–	–	+	–	+	+	+	N	V	+	+
phlei	–	+	–	+	+	+	–	+	S	+	+	TLC
rhodesiae	+	–	+	+	–	NT	NT	–	S	NT	+	-
senegalense	+	+	NT	NT	NT	NT	NT	+	N	+	+	TLC
septicum	V	–	NT	NT	NT	+	+	+	N	+	–	+
smegmatis	–	V	+	+	+	V	+	+	S	+	+	+
thermoresistibile	–	–	–	–	–	–	–	+	S	+	+	–
vaccae	V	V	V	+	–	+	–	V	V	V	+	–
wolinskyi	–	V	+	+	+	+	+	+	N	+	+	+

–, negative (approximately 20 percent or less positive); +, positive (approximately 80 percent or more positive); GLC, gas–liquid chromatography; N, nonphotochromogenic; NT, not tested; P, photochromogenic; S, scotochromogenic; TLC, thin-layer chromatography; V, variable, or approximately 20–80 percent positive.

a) For results of the polymyxin B inhibition test, see the section describing that test.

b) Heat-shock protein gene.

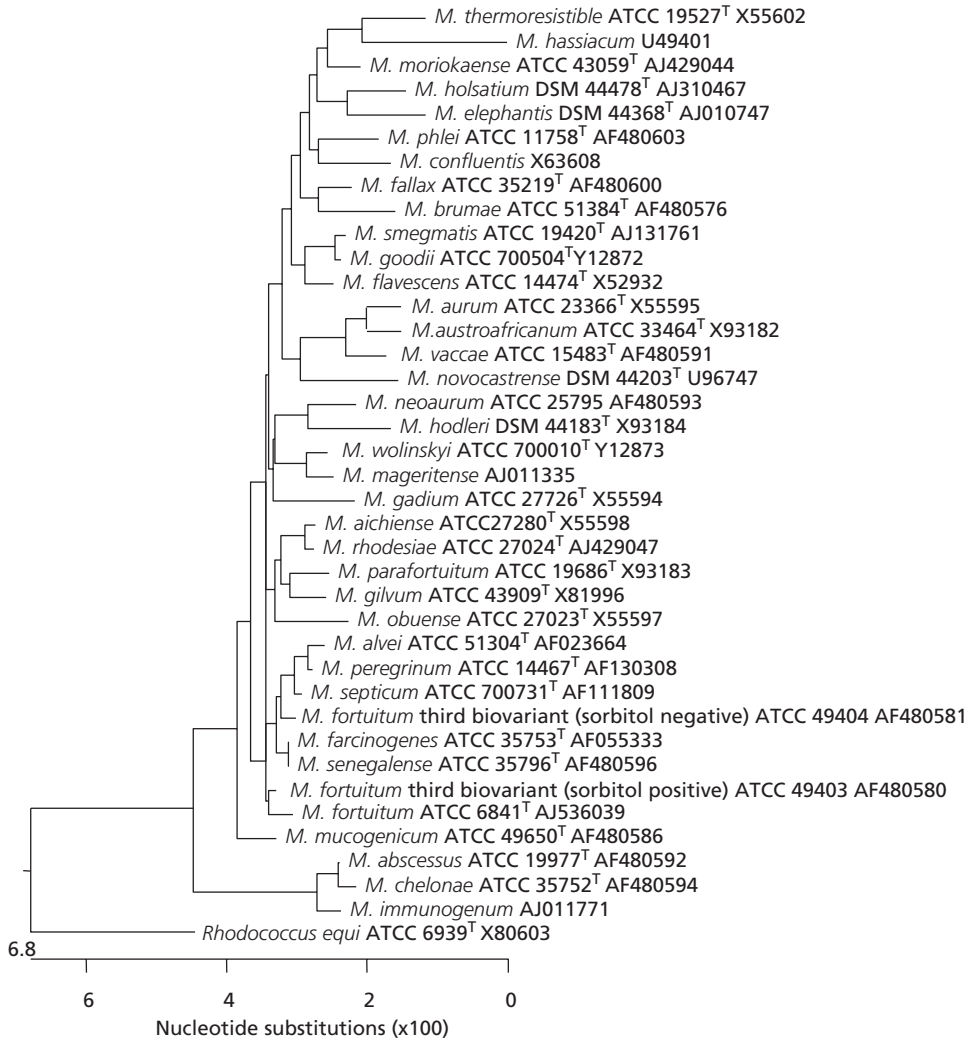

Figure 1 *Phylogenetic tree of type strains or representative strains of various species of rapidly growing mycobacteria prepared using the ClustalV algorithm of the Megalign software (DNAstar, Inc.).*

RGMs are discussed in another review (Brown-Elliott et al. 2002).

STAINING PROPERTIES

If mycobacteria, including RGMs, are visible at all in a direct Gram stain of a specimen, they may appear as negative images (as a result of failure to take up any components of the stain), as faint rods containing gram-positive beads, or rarely as gram-positive organisms suggestive of coryneforms. Hence, care is needed in the examination of gram-stained material so that RGMs are not dismissed as either staining artifacts or inconsequential contaminants. All mycobacteria, including RGMs, are considered to be acid-fast. However, there are several reports of RGMs staining less well with an auramine or auramine-rhodamine stain than with the Ziehl–Neelsen stain (Joseph et al. 1967; Flournoy 1993). Flournoy also found *M. fortuitum* to stain relatively less avidly than *M. chelonae* with the auramine stain. Our own experience (unpublished data) suggests that some isolates of RGMs may stain less well with the Kinyoun stain, the carbol fuchsin-based stain that we routinely use, than do most isolates of slowly growing mycobacteria. Nearly all are positive with the modified acid-fast stain used for Nocardia species, but it has been our personal observation that subculture to selected media may be necessary for a rare isolate to exhibit any acid-fastness whatever. The possibility that an organism that appears as a beaded gram-positive rod is a mycobacterium should not be dismissed too rapidly solely because it is negative by a regular acid-fast stain such as the Kinyoun stain. Although the individual cells of many species of RGMs appear relatively long, some may be coccobacillary, and there are no morphological features of individual cells of which we are aware that permit definite identification of an isolate as a RGM, let alone a particular species of RGM. See

Mycobacterium tuberculosis Complex, *Mycobacterium leprae*, and other slow-growing Mycobacteria for details on performing regular acid-fast stains, and Actinomyces and related genera for details on performing the modified acid-fast stain.

PHENOTYPIC IDENTIFICATION

A large number of biochemical tests have been used for the characterization of the RGMs, particularly in numerical taxonomic studies. However, clinical laboratories, for reasons of cost minimization, the need for rapid results, and the need for maintenance of expertise, must restrict themselves to the use of a relatively small battery of biochemical tests, if indeed they attempt identification of these organisms at all. It is our opinion that, given the large number of recognized species, only molecular methods can provide entirely accurate identification of RGMs. However, judicious use of a relatively small battery of biochemical tests can provide reasonably reliable identification of most of the more commonly encountered species. Such identification may be helpful for the initial selection of antimicrobial agents and for early recognition that an outbreak may be in progress. We describe here our procedures for performing selected biochemical and other phenotypic tests; detailed procedures for performing these and other tests are available in many standard references (Kent and Kubica 1985; Witebsky and Conville 1993; Pfyffer et al. 2003; Vincent et al. 2003). All laboratories do not perform these procedures in the same way, and full procedural details are often not provided in publications describing phenotypic features of RGMs. For most of these tests, we have indicated in Table 1 the expected reactions for the different species of RGMs when we have been able to find the relevant information. Data presented in the table have been abstracted from the references given in connection with the information presented under each species, and from some secondary sources as well (Kent and Kubica 1985; Witebsky and Conville 1993). For the most part, we have not repeated the data in the table in the accounts of the individual species. Some of the data are based on studies of only one or a very small number of isolates of a given species. Published data regarding phenotypic test results for all organisms need to be interpreted with caution; differences among laboratories with regard to testing materials such as media and reagents, and testing procedures and interpretive criteria, may result in different outcomes with a given isolate or species for what is nominally the same test. In some cases, data are not consistent from one publication to another, even when it is most likely that the same procedures have been used. We have provided results in the table obtained by the procedures we employ when possible, but more often than not, we have had to rely simply on results associated with a test procedure name. It should also be noted that, in any study, only a relatively small number of species are compared with one another with regard to results for a particular test performed at the same time in the same laboratory. Most media and reagents are now commercially available; recipes for preparation of such media will not be provided here. It is important that both positive and negative controls be performed with the majority of these phenotypic tests to ensure adequate medium, reagent, and technologist performance.

Arylsulfatase

This enzyme hydrolytically cleaves sulfate groups from aromatic rings. In the test, the production of free phenolphthalein from potassium phenolphthalein disulfate is detected by alkalinizing the medium with sodium carbonate. If free phenolphthalein has been produced, a pink to red color will be produced. Both 3- and 14-day tests are performed; the 3-day test is particularly useful for RGMs. In the 3-day test, a tube of 0.001-M substrate is inoculated with an appropriate volume (generally 0.1 ml) of a 1 McFarland density broth culture and incubated in ambient air for three days at 37°C. Thereafter, six drops of 2 N sodium carbonate are added, and the development of at least a pale pink color is considered positive.

Carbon utilization

The capabilities for using citrate, inositol, mannitol, and sorbitol as sole carbon sources are useful for identifying some species of RGMs. For this test, a 1:100 dilution in sterile normal saline of a barely visible broth culture of the organism is prepared; an additional dilution is made if turbidity is visible in the first dilution. Each slant is inoculated with 0.1 ml of the appropriate dilution, and incubated with the caps loose in ambient air at 27°C. Slants are examined for growth after 3, 7, and 14 days, and the results compared with growth on a base control. In our hands, this test did not prove reliable for the identification of *M. mucogenicum* (Conville and Witebsky 2001).

Colonial morphology

With experience, laboratorians find colonial morphology helpful in suggesting a particular species, but they should never make a definite identification on the basis of colonial morphology alone. With regard to some of the more commonly isolated species, the colonies of an *M. abscessus* isolate may be both rough and smooth, those of an *M. chelonae* isolate may be smooth with a thin surrounding fringe, and those of *M. mucogenicum* may be quite mucoid (Vincent et al. 2003).

Growth rate

A 1:2500 dilution in sterile normal saline of a 1 McFarland broth culture is prepared, and 0.1 ml of the dilution is spread evenly over the surface of a slant of a nonselective, enriched medium such as Lowenstein–Jensen (LJ). Slants are incubated with caps loosened and the slant surfaces kept horizontal at the optimum growth temperature for the organism (in ambient air, or, if at 37°C, in 8 percent CO_2). Slants are checked after 6 days for growth of isolated colonies clearly visible to the naked eye; if no growth is seen, the slants are checked again after 2 weeks of incubation, and weekly thereafter until growth is detected. By definition, RGMs show clearly visible growth within 7 days of incubation.

Iron uptake

Some RGMs can convert ferric ammonium citrate to iron oxide, thereby imparting a rust color to both the medium and the colonies. For this test, the same dilution is used as in the test for growth rate, and 0.1 ml is spread evenly over the surfaces of two LJ slants, one with and one without the substrate. Slants are incubated with caps loosened and the slant surfaces kept horizontal at 27°C in ambient air. Slants are checked weekly for up to 4 weeks for iron oxide production. Some isolates produce a tan rather than a rust color; while not a true positive reaction, such a result is useful for the identification of some species.

MacConkey agar without crystal violet

Many pathogenic species of RGMs can grow on this medium. A large loopful from a 1 McFarland broth culture is used to inoculate a plate of the medium, with streaking done to assure the production of isolated colonies. Plates are examined after 5 and 11 days of incubation at 27°C in ambient air. A positive result is indicated by good growth.

Nitrate reduction

This procedure tests the ability of an organism to reduce nitrate to nitrite. To perform this test, two loopfuls of growth from a culture on solid medium at least 2 weeks old are suspended in 0.2 ml sterile distilled water. 2 ml of sodium nitrate substrate solution is added, shaken manually, and incubated for 2 h in a 37°C water bath. The tube is removed from the water bath, and one drop of a solution of one part concentrated hydrochloric acid and one part distilled water, two drops of a 0.2 percent aqueous sulfanilamide solution, and two drops of a 0.1 percent aqueous solution of N-naphthyl-ethylenediamine dihydrochloride are added. A definite pink color, or anything darker, is considered positive. Negative tests are confirmed by adding zinc dust, which results in the production of a red color.

Pigment production

Most species of RGMs are nonphotochromogenic, their colonies being buff-colored or paler, regardless of whether they have been exposed to light. Some species are scotochromogenic, producing colonies that are pigmented (yellow to orange) from the time growth is visible, irrespective of exposure to light; isolates of some scotochromogenic species may become more pigmented after light exposure. Isolates of some species produce colonies that become pigmented with age; Brown-Elliott and Wallace state that most isolates of *M. smegmatis* and *M. goodii* become pigmented at 7–10 days (Brown-Elliott and Wallace 2002). See *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and other slow-growing mycobacteria for details regarding the performance of the light test.

Polymyxin B inhibition

This test can be helpful for distinguishing among some of the more commonly isolated RGMs. Most isolates of *M. fortuitum*, *M. fortuitum*, third biovariant, and *M. peregrinum* are inhibited by polymyxin B; most isolates of *M. abscessus*, *M. chelonae*, and *M. immunogenum* are not (Wallace et al. 1982; Wallace et al. 1993a; Wilson et al. 2001). Isolates of *M. mageritense* and *M. mucogenicum* give variable results (Wallace et al. 1993a; Wallace et al. 2002). For other species of RGMs, we have not been able to find adequate data pertaining to this test, and so we have not included this test in Table 1.

Careful attention to inoculum preparation is essential for accurate performance of this test. Four drops of oleic acid-albumin-dextrose-catalase solution are added to a Mueller–Hinton agar plate and spread evenly over the surface using a cotton swab. A tube of Middlebrook 7H9 medium that has been lightly inoculated and incubated overnight at 37°C in 8 percent CO_2 is standardized to a 0.5 McFarland concentration with sterile saline. A sterile cotton swab is dipped into the inoculum suspension, the excess is removed from the swab by pressing it against the side of the tube, and the Mueller–Hinton agar plate is inoculated as for a Kirby–Bauer susceptibility test. After allowing approximately 10 min for the plate to dry, a 300-unit polymyxin B susceptibility testing disk is placed on the plate, which is then incubated for 72 h in ambient air at 37°C. A clear zone of inhibition of any size is considered a positive result.

Sodium chloride tolerance

While isolates of many species of RGMs can grow on LJ medium containing 5 percent NaCl, isolates of

M. chelonae and *M. mucogenicum* generally cannot. We have found this test to be somewhat unreliable (Conville and Witebsky 1998), but others have found it more consistently useful (Yakrus et al. 2001). We have found it useful to: (1) inoculate slants with 0.1 ml of a suspension of organism grown in Tween albumin broth with glass beads at 27°C in ambient air to a concentration spectrophotometrically equivalent after vigorous vortexing to a 1 McFarland standard; (2) incubate slants at 35°C in ambient air, checking weekly for growth; (3) assure that more than 50 colonies are present on the control slant; and (4) interpret a result as positive only if at least 50 colonies of any size appear on the salt-containing slant.

Temperature studies

Many species of RGMs grow best at approximately room temperature, but determination of the optimum growth temperature, and the temperatures at which growth is possible at all, may be helpful for the identification of some species. Useful temperature ranges at which to test include 25–27, 30–32, 35–37, and 40–42°C; all incubations should be in ambient air. So far as we have been able to determine, only three of the species discussed here (*M. hassiacum*, *M. phlei*, and *M. thermoresistible*) will grow at 52°C; *M. hassiacum* is reported to be able to grow at 65°C (Schröder et al. 1997). Using a 1:2500 dilution in sterile normal saline of a 1 McFarland broth culture, LJ slants are inoculated with 0.1 ml spread evenly over the slant. Slants are incubated with the caps loose and the surfaces kept horizontal and examined weekly for 3 weeks. Relative amounts of growth at each temperature are noted. As the optimum and maximum growth temperatures for many species have not been determined unambiguously, we have not included temperature data in Table 1 but have noted both above and in the individual species accounts those few species known to be capable of growth at unusually high temperatures.

CHROMATOGRAPHY

Chromatographic techniques have become useful methods for the rapid identification of some species of RGM. These methodologies are based on the analysis of the mycolic acids associated with the peptidoglycan layer of the cell walls of mycobacteria and related organisms. The mycolic acids are long-chain, high-molecular-weight α-branched, β-hydroxy fatty acids that vary both in the number of their constituent carbon atoms and in the presence or absence of various structural groups that may be attached. Analytic procedures involve extraction of the mycolic acids from the cell walls and cleavage of these molecules to form fatty acid methyl ester derivatives and/or mycolic acid cleavage products, which can then be analyzed by a variety of chromatographic methods. The structures of mycolic acids have been shown to vary by genus and by species (Lechevalier et al. 1971; Minnikin et al. 1975); identification schemes take advantage of these dissimilarities and allow for the differentiation of species of mycobacteria from other aerobic antinomycetes (Minnikin et al. 1975) and from one another (Minnikin et al. 1984; Butler et al. 1986; Tisdall et al. 1979).

Thin-layer chromatography (TLC) has been used in the analysis of mycolic acids to discriminate among various species of mycobacteria and related actinomycetes (Minnikin et al. 1975). Using two-dimensional TLC, Minnikin et al. (Minnikin et al. 1984) separated numerous species of mycobacteria into 11 groups based on the structural types of mycolic acids; RGMs are included in seven of these groups. However, species-specific discriminations are not possible for a number of RGMs, and TLC data for recently described species are not available.

Gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) have been used to discriminate among the members of the genus *Mycobacterium*; species discrimination is made by analysis of the pattern of peaks obtained from chromatographic separation of mycolic acid derivatives, the retention times of these peaks, and, sometimes, the relative peak height. Tisdall et al. (1979, 1982) determined unique chromatographic patterns for *M. chelonae*, *M. fortuitum*, and several slowly growing mycobacteria using GLC and an identification system based on the presence of characteristic peaks. Utilizing HPLC, Steck et al. (1978) identified 24 different mycolic acids from four species of mycobacteria, which can aid in identification. Various other investigators (Thibert and Lapierre 1993; Guthertz et al. 1993; Butler and Kilburn 1990) have identified distinct chromatographic patterns of RGM species based on relative retention times and peak–height ratios. In 1996, the Centers for Disease Control and Prevention published a standardized method for HPLC identification of mycobacteria (Butler et al. 1996) that includes patterns for the identification of the *M. chelonae/abscessus* group, the *M. fortuitum*/*M. peregrinum* group, and *M. mucogenicum*, in addition to patterns for slowly growing mycobacteria. A computerized identification system has been developed that utilizes HPLC and pattern-recognition software to assist in the identification of mycobacterial isolates; an evaluation of this system found that it correctly identified between 83 and 100 percent of the species of RGMs (Kellogg et al. 2001). Enhancements of sample preparation may allow better discrimination of closely related mycobacterial isolates using this system (Müller et al. 1998).

Most described chromatographic methods are performed from growth on solid media; HPLC performed directly on clinical specimens has met with limited success (Jost et al. 1995). Methodologies

utilizing HPLC of mycobacterial isolates growing in liquid media may provide rapid identification of those isolates; however, a limited number of RGMs have been identified using these techniques (Cage 1994; Jost et al. 1995).

A number of variables may affect chromatographic results, complicating the comparison of results from different investigators. Methodologies used for the extraction and derivatization of mycolic acids may affect the types and sizes of patterns obtained; variations in column material and carrier gases, as well as the use of different detection methods, may also affect results. Users of these methods would be well advised to familiarize themselves with the patterns obtained for type strains with their particular system, and compare with results from unknown isolates with those obtained for the reference strains.

Table 1 gives information on the availability of mycolic acid chromatographic patterns for the RGMs mentioned in this chapter.

Other identification methods

Additional methods that have been utilized for the identification of RGM include pyrolysis mass spectrometry (Magee et al. 1997) and isoelectric focusing of β-lactamase enzymes (Sparks and Ross 1981). Multilocus enzyme electrophoresis has also been used to separate species of RGM from one another (Yakrus et al. 2001).

MOLECULAR TESTING

Molecular methodologies have added considerably to our understanding of the evolutionary relationships of all species of mycobacteria and of the RGMs in particular. Baess et al. (1982) used DNA–DNA hybridization to evaluate relationships among the RGMs and proposed a close phylogenetic relationship among *M. fortuitum*, *M. peregrinum*, *M. senegalense*, and *M. farcinogenes*. As stated previously, analysis of various gene sequences has shed light on the evolutionary relationships of the RGMs to one another and to other mycobacterial species. Figure 1 shows the phylogenetic relationships we have obtained for the species of RGMs discussed in this chapter, using sequences of type strains (when available) from the GenBank database, and aligning them with Megalign software (DNAStar, Inc., Madison, WI) using the ClustalV algorithm. The phylogenetic tree was constructed with Megalign using sequences of similar length (1305 bp).

The addition of various molecular methods to the procedures available for the identification of mycobacteria has allowed more accurate characterization of mycobacterial isolates and widened the spectrum of RGMs implicated in human disease. These techniques provide reliable, and often more rapid, identification alternatives to conventional (and generally less accurate) biochemical methods. While these methods may not be available in all laboratories, their use in regional or reference centers allows greater species discrimination for laboratories that can avail themselves of such service. Very few methods have been described for the detection and identification of RGMs directly from clinical specimens; the majority of the methods described here are applied most appropriately to growth on solid media.

Widespread use of automated methods for DNA sequencing of genes of mycobacterial species has dramatically increased the amount of molecular information available about these organisms. Several genes of interest have been studied for their ability to reliably discriminate among the growing number of recognized species. Several authors have shown that the hypervariable regions of the 16S rRNA gene may be useful for the discrimination of many of the species of RGMs (Rogall et al. 1990; Kirschner et al. 1992a). A commercially available sequencing system (MicroSeq 500 Bacterial Sequencing Kit, PE Applied Biosystems) has been evaluated for the identification of a number of mycobacterial species (Cloud et al. 2002; Patel et al. 2000). The usefulness of 16S rRNA sequencing is in considerable measure due to the large amount of 16S sequence information in molecular databases, with which the sequences of unidentified organisms can be compared. It is clear that the 16S rRNA gene is quite conserved within the genus *Mycobacterium*, and some species exhibit a high degree of similarity to each other, most notably the species pairs *M. abscessus* and *M. chelonae* (Patel et al. 2000), *M. goodii* and *M. smegmatis* (Brown et al. 1999), and *M. farcinogenes* and *M. senegalense* (Kirschner et al. 1992b). The use of an alternative gene region for restriction enzyme assays (REA) or sequencing or DNA–DNA hybridization may be essential in some cases for demonstrating that separate species are indeed involved (Domenech et al. 1994) (Baess 1982). Practically, some closely related species may, at present, actually be more reliably separated by phenotypic rather than by molecular methods.

The sequences of other genes have been investigated for their usefulness for reliably identifying various species of RGMs, including the 23S rRNA gene (Stone et al. 1995), the 16S-23S internal transcribed spacer (ITS) region (Park et al. 2000; Hamid et al. 2002; Roth et al. 2000), the *rpoB* gene (Kim et al. 1999), the *dnaJ* gene (Takewaki et al. 1994), and the 32-kDa protein gene (Soini and Viljanen 1997). Most of these genes are less highly conserved than the 16S rRNA gene and therefore show more variability among species. The usefulness and reliability of using sequences of these genes for the identification of clinical isolates is dependent on the number of species for which the sequence of the gene in question has been tested, the

analysis of the intraspecies variability in relation to the interspecies differences, and the quality of the sequence databases used for comparison.

In the absence of commercial DNA probe systems for the identification of RGMs, some investigators have evaluated the use of the polymerase chain reaction (PCR) and species-specific probes using the superoxide dismutase gene (Zolog and Philippi-Schulz 1994) and the 16S rRNA gene (Patel et al. 1997; de Beenhouwer et al. 1995). Successful use of these methodologies is complicated by the need for probes for all the various species of RGM.

Based on PCR methodologies initially developed by Telenti et al. (1993), Rinquet et al. (1999) evaluated methodologies employing sequences of a 441-base-pair (bp) fragment of the heat-shock protein (HSP) gene for multiple strains of ten species of RGM and found distinct sequence differences among the species. Intraspecies variability was common, but the degree of divergence within a species was less than that seen among different species (Ringuet et al. 1999). This gene region has proven to be particularly useful for the identification of many species of both slowly growing mycobacteria and RGMs, and now gene databases have considerable information about HSP sequences for this genus.

REAs have become popular rapid methodologies for the identification of mycobacteria; they are readily adapted to a basic molecular biology laboratory and are most frequently performed on growth from solid media. REAs involve PCR amplification of a specific region of a gene and subsequent restriction enzyme digestion of the amplified product. The differences in resulting restriction fragment length polymorphism (RFLP) patterns allow discrimination of a number of species and variants of those species. Vaneechoutte et al. (1993) initially described the use of this procedure for the identification of mycobacterial species utilizing a 1 500-bp region of the 16S rRNA gene.

Telenti et al. (1993) took advantage of the variability of the HSP gene in mycobacteria and developed a method that involves amplification of a 439-bp region of that gene and subsequent REA with two restriction endonucleases. With this method, Telenti was able to distinguish among 28 species of mycobacteria including both slowly and rapidly growing mycobacteria. Steingrube et al. (1995) focused the use of this procedure on the RGMs and created an algorithm for the identification of commonly isolated RGMs. Subsequent studies have increased the number of mycobacterial species identifiable by this method (Taylor et al. 1997; Devallois et al. 1997; Brunello et al. 2001) and have recognized species that show intraspecies variability in their RFLP patterns. In some cases, additional restriction enzymes must be used to adequately differentiate particular species (Brown et al. 1999). Enhancements such as the use of 10 percent polyacrylamide gel electrophoresis (Brunello et al. 2001) and computer-assisted software (Devallois et al. 1997) have improved the resolution of small bands and decreased the influence of gel to gel variation. Figure 2 shows RFLP patterns of several common strains of RGMs. Nonspecific amplification (see 240-bp band on BstEII digest for *M. peregrinum* in Figure 2) can sometimes occur, but bands resulting from this amplification should not be considered when analyzing RFLP results.

Other gene regions have been evaluated for the usefulness of REA, including the 16S–23S spacer region (Roth et al. 2000), the *dnaJ* gene (Takewaki et al. 1994), and the *rpoB* gene (Kim et al. 2001). These methodologies have been evaluated with a limited number of isolates, and their ultimate usefulness remains to be determined.

While most molecular methodologies have been applied to identification of isolated organisms grown on solid media, Kirschner et al. (1996) reported success with the identification of some species of mycobacteria directly from clinical samples using PCR amplification of the mycobacterial 16S rRNA gene and subsequent sequencing of that gene. Molecular methodologies have also been applied for the early identification of mycobacterial growth in liquid media. Kirschner et al. (1993) and Taylor et al. (1997) reported obtaining reliable results from PCR and sequencing of the 16S rRNA gene and PCR and REA of the HSP gene, respectively. In both cases, a limited number of samples containing RGMs were studied.

STRAIN TYPING

RGMs have frequently been implicated in nosocomial infections, and because of their propensity to thrive in water environments, they are often the cause of outbreaks or pseudo-outbreaks. In such cases, strain typing is useful for determining the relatedness of isolates and to assist in locating a common source. Historically, strains have been compared with one another using biochemical profiles or susceptibility to antibiotics or heavy metals (Wallace et al. 1989). While Lowry et al. (1988) used a high degree of aminoglycoside resistance to characterize an outbreak of *M. chelonae*, such phenotypic techniques are frequently not helpful in accurately characterizing outbreak strains, as their traits may not be significantly different from those of unrelated strains (Wallace et al. 1993b). The application of molecular techniques to outbreaks of infection and pseudo-infection caused by RGMs has considerably improved the reliability of epidemiologic studies. The study of plasmid profiles is based on the detection and comparison of plasmids of similar sizes from suspected outbreak strains (Wallace et al. 1989; Zhibang et al. 2002), but the technique requires considerable technical skill. Pulse field gel electrophoresis (PFGE) is the method of choice for the epidemiologic

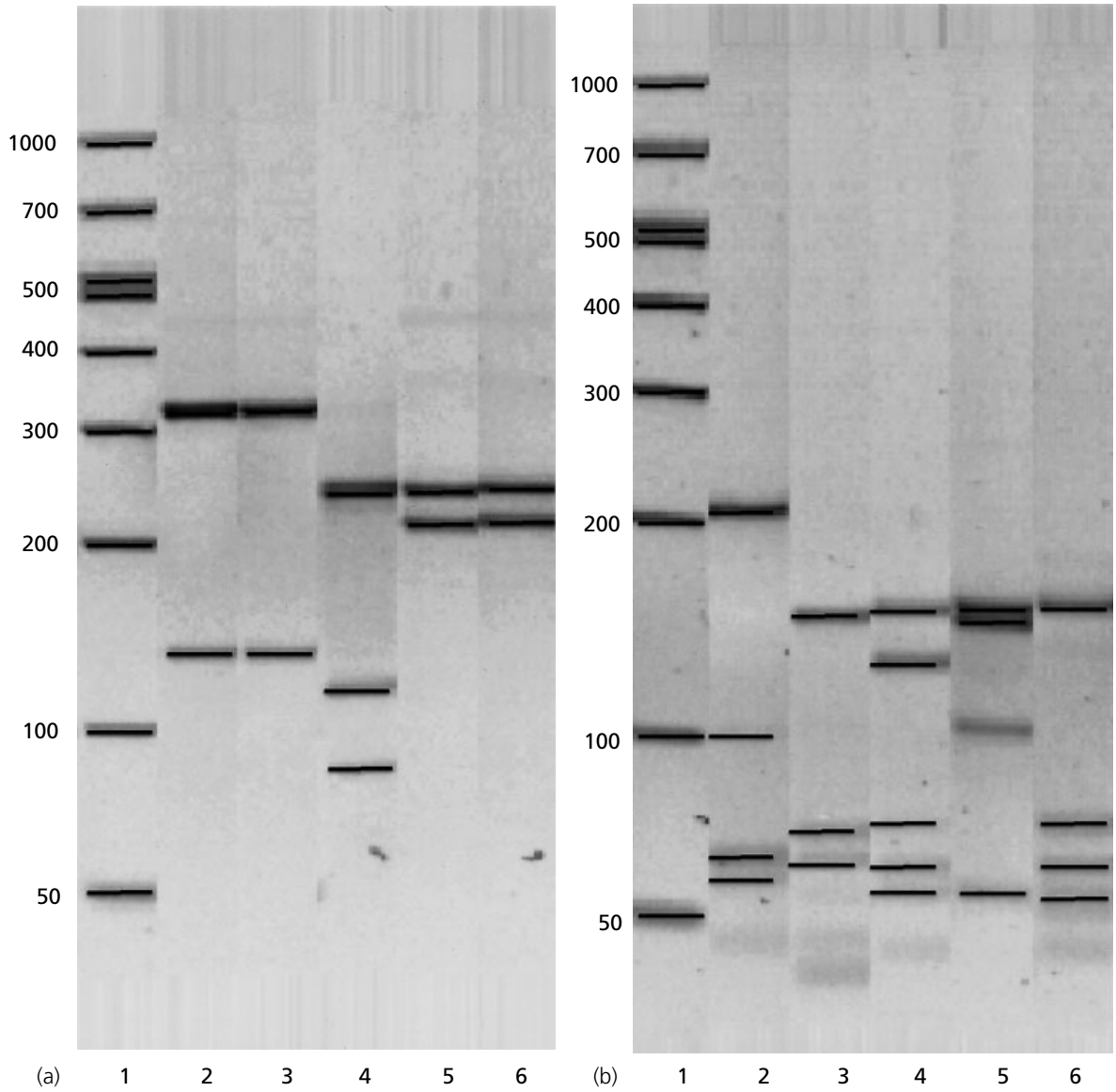

Figure 2 *Bio-Rad derived restriction fragment length polymorphism (RFLP) patterns of the 439 base-pair amplified region of the HSP gene for type strains of various rapidly growing mycobacteria (RGM). Digests were electrophoresed on 2.5 percent Metaphor agar gels. Bands smaller than 50 bp are not highlighted; only bands from the digestion of the 439 bp amplicon of the HSP gene are highlighted. Gel A,* BstE *II digests of HSP gene; Gel B,* Hae *II digests of HSP gene. Lane 1, bp marker; lane 2,* M. chelonae *ATCC 35752^T; lane 3,* M. mucogenicum, *ATCC 49650^T; Lane 4,* M. fortuitum, *ATCC 6841^T; lane 5,* M. peregrinum, *ATCC 14467^T; lane 6,* M. abscessus, *ATCC 19977^T.*

study of outbreak strains of many bacterial species, including RGMs. The technique of PFGE involves preparation of genomic DNA and digestion of the DNA with restriction endonucleases, which have a limited number of recognition sites. The digests are then subjected to alternating-field electrophoresis; ultraviolet (UV) illumination of the ethidium bromide-stained gel reveals DNA banding patterns, which vary depending on the DNA sequence of the organisms (Hector et al. 1992). This technique has been applied successfully to outbreaks of *M. fortuitum* (Hector et al. 1992) and *M. chelonae* (Wallace et al. 1993b). The use of PFGE is limited for the investigation of outbreaks involving *M. abscessus* because of spontaneous lysis or fragmentation of the *M. abscessus* DNA, which was been seen in nearly 40 percent of samples tested in one study (Wallace et al. 1993b). The use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been used successfully to compare strains of *M. abscessus* involved in outbreaks (Villanueva et al. 1997; Lai et al. 1998; Zhang et al. 1997). The procedure involves extraction of DNA and PCR using arbitrary primers and low-stringency PCR conditions. Gel electrophoresis of the amplification

products shows patterns of banding that vary from strain to strain. Zhang et al. (1977) proposed interpretive criteria that allow an objective comparison of results from outbreak strains; the technique is dependent on the use of freshly extracted DNA and the choice of appropriate primers for optimum differentiation. Multilocus enzyme electrophoresis has also been used occasionally for characterization of outbreak strains (Ashford et al. 1997).

ASSESSMENT OF CLINICAL SIGNIFICANCE

As RGMs are ubiquitous in the environment, care needs to be used in the assessment of the clinical significance of certain isolates, particularly in the case of a smear-negative specimen from a patient without a picture clearly compatible with an infection that could be caused by an RGM. The American Thoracic Society has provided some useful guidelines for assessing clinical significance, particularly of respiratory tract isolates (Wallace et al. 1997).

SUSCEPTIBILITY TESTING

A standardized susceptibility testing procedure recommended for use with RGMs has been published by the National Committee for Clinical Laboratory Standards (NCCLS) (2000). It may be difficult for laboratories that do not perform the procedure frequently to obtain reproducible results and results that are comparable to those from a reference laboratory. Because of the variety of techniques and interpretive criteria used in the past, caution is advised in assessing the interlaboratory comparability and the clinical relevance of published susceptibility testing results, even when what is described as the same methodology has been used by different investigators. The currently recommended susceptibility testing procedure is a broth microdilution procedure similar in principle to that recommended by the NCCLS for bacteria that grow aerobically. There are, however, differences in features such as recommended incubation periods, interpretive breakpoints for certain agents, and species-specific caveats. It is important to be aware that the majority of antituberculous agents are not effective for treatment of diseases caused by the RGMs. Those interested in performing susceptibility testing of RGMs are encouraged to consult the relevant NCCLS document, which will be updated periodically. Although susceptibility testing results are to some degree predictable from the species identification, there is sufficient intraspecies variability in susceptibility that testing of all clinically significant isolates is recommended.

SPECIES ACCOUNTS

In the accounts that follow, we will summarize the phenotypic and molecular features of the RGMs with approved names (information found at the 'List of Bacterial Names with Standing in Nomenclature' website by J. P. Euzéby (www.bacterio.cict.fr/)) that we have found through our literature search (almost entirely confined to that published in the English language) to have been reported to have been isolated from human sources, whether or not they have been clearly implicated as etiologic agents of disease. Clinical features of the diseases produced by RGMs will be summarized, as well as susceptibility test data when available. For the more common pathogens, a separate susceptibility section is included in the species account. Because of the economic impact of the disease, we will also briefly discuss the mycobacterial species responsible for African bovine farcy. In addition to cattle, other vertebrates for which RGMs have been found to be pathogenic include fish (Daoust et al. 1989; Bragg et al. 1990), cats (Malik et al. 2000), a horse (Booth and Wattret 2000), and others (Hines et al. 1995).

Wallace, Brown-Elliott, and their colleagues have studied the identification, disease association, susceptibility testing, and therapeutic management of human pathogenic RGMs for many years, and reference to their work is critical for an appreciation of the problems involved in the diagnosis and management of infections caused by these organisms. They have recently published a comprehensive overview of the pathogenic nonpigmented or late-pigmenting species (Brown-Elliott and Wallace 2002), and, with Pfyffer, Vincent, and Jost, have coauthored two chapters covering species pathogenic for man, providing details for performing many of the identification procedures feasible for diagnostic laboratories (Pfyffer et al. 2003; Vincent et al. 2003). In 1998, Wallace et al. reviewed the nosocomial outbreaks and pseudo-outbreaks caused by nontuberculous mycobacteria, and also summarized information pertaining to their occurrence in municipal and hospital water systems and their relative resistance to disinfecting agents such as chlorine and glutaraldehyde.

In the species accounts that follow, organisms are arranged alphabetically by species epithet. We have elected to review – as well as to critique – in some detail the cases and outbreaks that we think are of particular interest, rather than attempt a comprehensive list of infection sites and references. Hopefully, the references contained in the reports selected will contain sufficient information to enable those interested to access the larger body of published data. Reports are discussed under the currently recognized species most likely to have been involved, as best as we could determine on the basis of the data presented regarding the organisms discussed. When insufficient data were presented to

enable a presumptive assignment to a currently valid species, but the report is nonetheless considered sufficiently noteworthy, it is discussed under the species to which it was originally assigned, with a comment made regarding the uncertainty of the identification. The elevation to species of many taxa formerly considered subspecies or biovariants has further complicated the problem of reference searching and accurate species assignment. The organisms now assigned to *M. peregrinum* and the third biovariant complex of *M. fortuitum* were probably often reported as merely *M. fortuitum* in the past. *M. abscessus* was previously considered a subspecies of *M. chelonae* and not always clearly separated from it in infection reports. Indeed, it is probably the case that in the past, many laboratories reported any presumptively pathogenic rapid grower isolate as '*M. fortuitum-chelonae* complex,' or something equivalent.

M. abscessus

The initial description of this organism, by Moore and Frerichs in 1953, was based on an isolate from synovial tissue from the left knee of an 'unusually well preserved' 63-year-old woman who had injured and dislocated her left patella at the age of 14 while on a farm. The species name was presumably given to the organism by the original describers because it had been 'Observed in synovial lesions and in deep subcutaneous abscesses of the buttocks.' The authors also asserted, 'It is not fatal to man.' For the next 48 years the patient suffered some stiffness and pain, but developed increasing pain and swelling about 6 months prior to her presentation that resulted in a patellectomy and synovectomy. Many acid-fast bacilli were seen in sections of the synovium, and the patient was initially presumed to have tuberculosis. However, the organism grew well 'in a few days,' and on subculture incubated at room temperature, colonies were readily visible in seven days. Postoperatively, there was dissemination to both buttocks. Some of the lesions were initially treated with incision and drainage, but it took 18 months for the lesions to clear, apparently without any antimicrobial treatment. This case is noteworthy for the extremely long time period (some 48 years) between the time of presumed organism inoculation and the time of organism isolation. For many years, this organism was considered to be a subspecies of *M. chelonae*, but it was eventually shown by DNA–DNA hybridization to be a distinct species (Kusunoki and Ezaki 1992).

In 1976, an outbreak of sternal wound infections occurred that involved 19 patients who had surgery for valve replacement or coronary artery bypass grafts (Robicsek et al. 1978; Hoffman et al. 1981). Infections were noted 6–40 days postoperatively, when incisional pain or drainage developed. Five of the patients eventually died, probably all of complications related to their infections. Seven patients underwent total sternectomy in an attempt to control their infections; six of these did well, but one died with mycobacterial sepsis. Antibiotics used included erythromycin, kanamycin, and rifampin. The source of the organisms in this outbreak could not be identified. In 1983, Kuritsky et al. reported on an outbreak affecting six patients following cardiac surgery; five of the infections involved *M. abscessus*, and one involved *M. fortuitum*. Of the patients with *M. abscessus* infections, three had sternal wound infections, one had endocarditis, and one had a saphenous vein graft-site infection; the patient with *M. fortuitum* had a sternal wound infection. The infections were thought probably to have resulted from operative-field contamination stemming from the nonsterile ice used to cool the cardioplegia solution; no more infections occurred after sterile water was substituted for the nonsterile ice.

Bolan et al. (1985), reported their investigation of a 1982 outbreak of infections involving two cooperating hemodialysis centers; 27 of 140 patients had documented mycobacterial infections. One isolate was not available for specific identification, one was an *M. chelonae*-like organism (MCLO), and 25 isolates were *M. abscessus*. Of the 27 infected patients, nine had disseminated disease, 14 evidently had bacteremia alone, three had soft-tissue infections, and one had infection of the access graft. Of the 27 patients, 14 died over the course of a year, but as most had multiple medical problems and only one was autopsied, it was not clear to what extent the mycobacterial infections contributed to the deaths. All 140 patients had been dialyzed using processed hemodialyzers; both *M. abscessus* and MCLO were isolated from water used in the processing procedure. Part of the processing procedure involved filling the dialyzers with 2 percent aqueous formaldehyde and storing them for about 48 h before reuse. Several of the infecting isolates were unable to survive a 24-h exposure to 2 percent formaldehyde, but several dialyzers tested were found to contain formaldehyde concentrations of less than 2 percent. In 1990, Lowry et al. reported on five patients who had been dialyzed at a hemodialysis outpatient clinic and developed systemic infections. From four patients, *M. abscessus* was isolated from multiple sites; from another patient, *M. chelonae* and an MCLO were isolated in addition to *M. abscessus*. This outbreak was associated with the use of high-flux dialyzers; *M. abscessus* was isolated from the water used in the cleaning process of these dialyzers and was found to be able to survive to some extent in the hydrogen peroxide and peracetic acid-based disinfectant (the strength of which was found to deteriorate significantly over a few days) with which the dialyzers were filled after cleaning.

In 1987, Safranek, et al. reported postoperative wound infections in eight patients following facial and breast cosmetic plastic surgical procedures, all performed by the same physician in his office. The organism was found

in the gentian-violet skin-marking solution used by the surgeon, and was traced to the stock solution in the pharmacy that provided it. It was presumed that the distilled water used to make up the stock solution had been the source of the organism, but the water was not available at the time of the epidemiological investigation. Infections occurred following operations that were conducted over a period of 5.5 months. Incubation periods between date of surgery and date of symptom onset ranged from 9 to 55 days. During the same time period, 27 patients undergoing facial plastic surgery procedures and exposed to the contaminated skin-marking solution did not develop any clinically apparent infection. In 1991, Soto et al. reported an outbreak of cellulitis in 22 patients following rhinoplasty; the organism was also recovered from two of the involved hospital's water tanks and from surgical equipment.

In 1988, Lowry et al. reported on an outbreak of otitis media involving 18 patients, 17 of whom were seen at the same ear, nose, and throat (ENT) office; all cases appeared traceable to one index patient. The organism was cultured only from patients who had at least one tympanotomy tube in place or had at least one chronic tympanic membrane perforation. The ultimate source of the organism was thought to have been tap water; transfer among patients was considered probably to have resulted from inadequate instrument sterilization. In 1994, Franklin et al. reported on 20 sporadic cases of otitis media caused by this species. Of the 16 patients for whom the information was available, all had a tympanostomy tube in place. The majority of the patients lived in a southern coastal state, but the epidemiologic significance of this observation was not certain, in part because the reference laboratory that worked with the isolates was located in Texas. All six of the patients for whom a detailed history was available had been treated unsuccessfully with a variety of oral antibiotics and with otic drops containing steroids and aminoglycosides. The six patients for whom a detailed history was available all responded to the combination of surgical and antibiotic treatment, which included long-term oral erythromycin or clarithromycin in all six patients.

In 1990, Wenger et al. reported on an outbreak of foot infections in eight patients who had invasive procedures performed in a podiatry office. Two different risk factors were identified in this outbreak: patients who had procedures performed before 10:30a.m., and those having procedures performed on any inner toe (rather than the first and/or fifth toes), were more likely to develop infections. A handheld jet injector had been used to administer lidocaine to all affected patients. Fresh disinfectant solution, in which the injector tip was immersed between procedures, was prepared daily at approximately 8a.m. and discarded in the evening. The infecting organism was cultured from the jug in which the distilled water used to prepare the disinfectant solution was stored, and studies revealed that the number of organisms recoverable from the disinfectant solution decreased with time. After 4 h in the solution, the colony count had decreased by 98 percent, and no viable organisms were recovered after 24 h. It was presumed that the greater risk posed by procedures performed earlier in the day resulted from the injection of a higher number of colony-forming units present in the solution. Larger amounts of a bupivacaine–dexamethasone solution were injected in patients undergoing procedures on an inner toe than on an outer toe, and it was hypothesized that the larger steroid amount in such cases may have resulted in a decrease in the local inflammatory response and consequent increase in infection risk.

In 1996, Camargo et al. reported on an outbreak of skin lesions involving 232 of 298 patients studied, out of an exposed population numbering 667. The infections were traced to injections of contaminated lidocaine given by a so-called 'bioenergetic,' an alternative medicine practitioner, in Colombia, South America. Many patients had received numerous injections; there was a median of two lesions per patient, with a range of one to nine. The investigators of the outbreak noted that the lidocaine carpules used had rubber stoppers with several punctures and that the carpules contained differing volumes, suggesting that they had been refilled and reused repeatedly.

In 1997, Villanueva et al. presented a detailed analysis of a huge outbreak of postinjection infections involving 350 of approximately 2 000 patients who had all been treated by the same alternative medicine practioner in Barranquilla, Colombia. Other outbreaks of postinjection abscesses involving rapid growers were also briefly summarized. In most cases lesions developed within 30 days of an injection, but it was usually several months after injection before the etiologic agent was identified. Organisms were recovered from 205 of the 210 specimens cultured. Five of these were further studied in reference laboratories and identified as *M. abscessus*. The five isolates all shared the same typical susceptibility pattern for this species, and all were also resistant to inorganic mercury, cadmium, and arsenite. Two methods of DNA analysis were used to assess relatedness of the five isolates, PFGE and RAPD-PCR. PFGE could not be used, as the DNA broke or lysed spontaneously, a problem, the authors comment, that has been found previously with half the *M. abscessus* isolates investigated. Patterns obtained by RAPD-PCR, however, were identical for all five isolates. No specific environmental source for the outbreak could be identified, but it stopped when reusable injectors and related syringes were no longer used. Although assignment of patients to different treatment regimens was not randomized, it was nonetheless noteworthy that less than one-third of the patients who received no treatment or who were treated with either surgery or clarithromycin were cured, while 95 percent of the patients treated with surgery and clarithromycin were considered cured.

In 1998, Lai et al. used RAPD-PCR to determine that two outbreaks were actually pseudo-outbreaks, with the epidemic strain also found in contaminated autoclaved distilled water that had been used for specimen processing in the microbiology laboratory. The outbreak ceased when commercially prepared processing reagents were substituted for those prepared in house. Some unexplainable issues remained, however. While the epidemic strain was found in specimens from 14 different patients, three patients had at least two specimens, at least one of which was smear-positive, from which the epidemic strain was isolated, and four patients had positive specimens that apparently had not come into contact with the contaminated water.

In 1997, Ashford et al. reported the results of their investigation of a pseudo-outbreak of bacteremias involving 23 human immunodeficiency virus (HIV)-positive patients followed at the same hospital; nine isolates were available for study, seven of which were *M. abscessus* and two of which were *M. mucogenicum*. The blood culture procedure employed included the use of the Septi-Chek AFB system. That system involves resuspending a medium supplement in sterile water, and for this the hospital laboratory used water from a 1-l bottle stored in a refrigerator and used in conjunction with other media and reagents as well. *M. abscessus*, *M. fortuitum*, and other organisms were cultured from a supplement vial that was in use, but no organisms were obtained from three vials that had not been previously opened. Careful study of the laboratory blood culturing procedure revealed several lapses in technique whereby contaminants could have been introduced into the blood cultures. The seven *M. abscessus* patient isolates and the supplement isolate were found to be identical in their susceptibilities to 13 antimicrobial agents and in their multilocus enzyme electrophoresis patterns.

In 1998, Chadha et al. described an outbreak of postsurgical wound infections in 45 of 452 patients who had operations performed during a 5-month period at a children's hospital in New Delhi, India. The infections were traced to the water supply of the operating room. All the surgical instruments were cleaned in tap water in the operating room prior to sterilization, and major problems were discovered with the autoclave being used, which was not able to sterilize gauze impregnated with *M. abscessus*. Despite in vitro resistance of the organism, many patients were treated with an antituberculous regimen, coupled with incision and drainage in a few patients. After several months of therapy, all patients evidently cleared their lesions, but, given the prolonged course of some of the patients and the absence of any type of control group, it is not clear what role their treatment regimen played in their recovery.

In 1999, Galil et al. reported the largest outbreak of infections thus far recorded in the USA due to *M. abscessus*. At least 87 individuals developed postinjection abscesses following the use of an unlicensed material sold as adrenal cortex extract. Alternative medicine providers administered the material intramuscularly, intravenously, subcutaneously, or by several routes for indications such as chronic fatigue syndrome, immune system enhancement, infection, weight loss, and 'hypoadrenalism.' Cultures from vials of the material and from patients revealed identical organisms by both enzymatic and molecular methods. The material was apparently manufactured under nonsterile conditions; the handwritten formula for its preparation indicated the use of hydrocortisone in low concentrations combined with merthiolate as a preservative. After being placed in unlabeled vials, the material was shipped to another state, where labels with names of fictitious pharmaceutical firms were added. No evidence was found of the use of any animal products in the preparation of the material. Prior to its intravenous administration, some providers added vitamins, minerals, or crude liver extract. As is unfortunately typical for such outbreaks, it continued for a considerable period before being recognized – in this case, for over a year. Cultures of a patient's lesions for mycobacteria could be documented in only one-fourth of the cases. Less than a third of all patients received a drug with antimycobacterial activity and, of those who did, most received a course that was too short to be curative.

In 2000, Chetchotisakd et al. reported an unusual presentation of infections caused by rapid growers, many of which were assigned to this species, initially involving 16 adults who all presented with chronic bilateral cervical lymphadenopathy that ranged from 1 month to 5 years in duration. Other lymph node groups and organs were also involved in most of the patients. A full immunologic workup was not performed on these patients, but, as many of the patients had prior or concurrent infections with opportunistic pathogens, they may have had some underlying immunologic abnormality. Only five of the patients showed an initial complete response to antibiotic treatment, and three of these were known to have relapsed. None of the patients were related, and no clustering of cases was noted. All the patients presented to the same hospital in Thailand, but no source of the infections was identified. Most of the isolates were found by in vitro testing to be resistant to cefoxitin, an unusual feature of *M. abscessus* (Brown-Elliott and Wallace 2002).

A case of postoperative endocarditis involving a Bjork–Shiley aortic valve was reported (Altmann et al. 1975), with organisms isolated premortem from blood cultures and from valve vegetations obtained at autopsy. The organism was identified as *M. chelonei (abscessus)*, with the identification confirmed by agglutination testing; however, insufficient data are presented to be sure of which currently recognized species was involved. Also, a case of postoperative endocarditis involving Starr–Edwards mitral and aortic valves was reported

(Repath et al. 1976), with organisms isolated premortem from blood cultures and from valve vegetations obtained at autopsy.

A case of renal infection (most likely actually a disseminated infection, as the patient also had multiple nodular skin lesions) (Becker et al. 1980) was probably secondary to initial infection at an arteriovenous shunt site. No details regarding the biochemical reactions of the organism were presented, but the addition of cefoxitin to the patient's treatment regimen resulted in subsequent failure to isolate the organism from her urine, suggesting that the organism, which was identified by the Centers for Disease Control and Prevention (CDC) as *M. chelonei*, was in fact *M. abscessus* (Brown-Elliott and Wallace 2002).

In 1980, Sopko et al. reported on four patients with underlying emphysema who developed pulmonary infection; all had radiographically visible pulmonary infiltrates. A minimum of four positive sputum cultures was obtained from each patient; one patient had 21 positive cultures. In 1996, Olivier et al. discussed nontuberculous pulmonary disease in patients with cystic fibrosis and the problem of distinguishing colonization from infection. Cullen et al. (2000) described a cystic fibrosis patient who was thought to be colonized for 13 years but who was eventually considered to have had invasive disease for that entire period. Of the rapid growers that cause chronic pulmonary disease, this species is estimated to be the etiologic agent in approximately 80 percent of cases (Brown-Elliott and Wallace 2002).

In 1992, Fraser et al. reported on an outbreak of pseudoinfections traced to the use of an automated bronchoscope disinfector. Thirteen patients had positive cultures obtained during bronchoscopy with a flexible bronchoscope; the positive culture from another patient was obtained during esophageal endoscopy. Organisms with the same unusual high level of resistance to cefoxitin as the patient isolates were recovered from the rinse water from the automated disinfector. Attempts to disinfect the automated disinfector itself were unsuccessful; after institution of a rinse with 70 percent alcohol and forced-air drying of the bronchoscopes, no additional isolates of the epidemic strain were detected. It was postulated that the epidemic strain may have originally been introduced from tap water, although the investigators were not able to isolate it from 'unused' tap water. In 1994, Maloney et al. reported on another pseudoepidemic traced to the use of an automated washer. In this study, the relatedness of patient and environmental isolates was demonstrated by DNA analysis involving large restriction fragment patterns of genomic DNA. In neither of these studies was there any evidence of infection developing in any of the patients from whom the positive cultures had been obtained.

Other interesting case reports include the following: possible vascular catheter-associated septicemia in a premature infant (Speert et al. 1980), vascular catheter site infection (Engler et al. 1989), vascular catheter-associated bacteremia (Rodgers et al. 1996), disseminated disease in two renal transplant patients (Graybill et al. 1974), osteomyelitis following a foot puncture wound (Meredith and Sexton 1996), vertebral osteomyelitis in a steroid-dependent adolescent with systemic lupus erythematosus (Pruitt et al. 1993), infection of epicardial pacemaker wires (Cutay et al. 1998), tenosynovitis of a finger in a patient with anorexia nervosa (Zenone et al. 1998), pulmonary and pleural infection in a lung-transplant patient (Fairhurst et al. 2002), disseminated disease in immunocompromised patients (Pottage et al. 1982; Carpenter et al. 1984), disseminated skin infection in an elderly woman (Fitzgerald et al. 1995) and in a post-lung-transplant patient some time after pulmonary colonization (Swetter et al. 1993), fatal pulmonary infection post-lung-transplant in a patient with cystic fibrosis (Sanguinetti et al. 2001), keratitis following ocular trauma (Meisler et al. 1982), and mucocutaneous lesions of the glans penis and inguinal adenopathy following urethral dilatations (the organism was identified as *M. chelonei* but showed 'NaCl tolerance') (Zina et al. 1980).

SUSCEPTIBILITIES

Nearly all isolates of this species are either susceptible, or susceptible at an intermediate level, to amikacin, cefoxitin, and clarithromycin, but only about half the isolates have at least an intermediate level of susceptibility to imipenem and linezolid. Most isolates are resistant to ciprofloxacin, gatifloxacin, and doxycycline (Brown-Elliott and Wallace 2002). Note that the NCCLS currently recommends that imipenem not be tested against this species (NCCLS 2000). One study traced the genetic basis of clarithromycin resistance in this species to a point mutation in the 23S rRNA gene (Wallace et al. 1996). In 1999, Jongevos et al. reported the successful use of the combination of ofloxacin, ethambutol, and doxycycline for the treatment of subcutaneous nodules in a patient who could not tolerate either clindamycin or rifampin; however, no biochemical or susceptibility testing results were reported for the isolate.

Information concerning molecular identification of *M. abscessus* by REA of the HSP gene can be found in Telenti et al. (1993). Chromatographic identification is described by Butler et al. (1996).

M. aichiense

In the publication originally describing this species (Tsukamura et al. 1981), some isolates are said to have been obtained from patient sputum. No clinical details were provided, and we are not aware of subsequent reports of the isolation of isolates of this species from clinical material. The species name is derived from the

name of the Japanese prefecture, Aichi, from which the original isolates were obtained.

Information concerning molecular identification of *M. aichiense* by REA of the HSP gene can be found in Brunello et al. (2001).

M. alvei

The initial description of this species in 1992 involved the study of six isolates, four from environmental sources and two from human sputum specimens (Ausina et al. 1992). No assessment of the clinical significance of the sputum isolates was provided. The species name is from a Latin word meaning 'bed of a river;' the type strain was isolated from water from the Llobregat River in Spain. Some pathogenic human isolates belonging to this species have evidently been obtained recently (Brown-Elliott et al. 2002).

Information concerning molecular identification of *M. alvei* by REA of the HSP gene can be found in Brunello et al. (2001). Chromatographic identification is described by Ausina et al. (1992).

M. aurum

This species was originally described in 1966 (Tsukamura 1966a), and presumably was given its species name because of its orange-colored colonies. However, in a subsequent numerical taxonomic analysis of scotochromogenic RGMs, Tsukamura and Mizuno (1977) concluded that *M. aurum* could be considered either a subspecies of *M. parafortuitum* or a species that constituted part of the *M. parafortuitum* complex. They also distinguished two types within *M. aurum*, with somewhat different biochemical reactions. However, a subsequent numerical taxonomic study by Tsukamura et al. (1983), concluded that *M. aurum* could be considered a separate species. This organism has been reported as a cause of catheter-related bacteremia in a patient with lymphoma (Esteban et al. 1998). The isolate was identified at a national reference laboratory, but little information regarding the phenotypic characteristics of the organism was provided.

Information concerning molecular identification of *M. aurum* by REA of the HSP gene can be found in Telenti et al. (1993). Taylor et al. (1997) report that some strains of *M. aurum* may be indistinguishable from *M. neoaurum* by HSP REA.

M. austroafricanum

The original description of this species in 1983 by Tsukamura et al. (1983) was based on a numerical taxonomic study of water isolates from South Africa – hence the species name. This organism was reported from acid-fast, smear-negative, and mycobacterial culture-negative synovial tissue obtained from one patient with 'crystal arthritis.' The organism was detected by PCR amplification with genus-specific primers of DNA extracted from the tissue, followed by automated sequencing (van der Heijden et al. 1999). We are not aware of other reports of the organism from clinical specimens.

M. brumae

The initial description of this species in 1993 involved the study of 11 isolates, ten from environmental sources and one from a human sputum specimen (Luquin et al. 1993). The name is from a Latin word meaning 'of winter,' given to the organism because the first isolates were obtained in winter. No assessment of the clinical significance of the sputum isolate was provided.

Information concerning molecular identification of *M. brumae* by REA of the HSP gene can be found in Brunello et al. (2001). Chromatographic identification is described by Luquin et al. (1993).

M. chelonae

The initial species name for this organism was '*chelonei*,' derived from the Latin word for a tortoise, from which the organism was evidently originally isolated (Wayne and Kubica 1986). The spelling of the species name was subsequently changed to make it consistent with general usage. On the basis of a study of 40 different strains, Stanford et al. (1972) concluded that the organisms sometimes designated as the separate species *M. abscessus*, *M. borstelense*, *M. chelonei*, and *M. runyonii* all belonged to the same species, within which there were two geographic 'variants.' However, it was eventually shown by DNA–DNA hybridization studies that *M. abscessus* and *M. chelonae* were separate species (Lévy-Frébault et al. 1986; Kusunoki and Ezaki 1992). The names '*M. borstelense*' and '*M. runyonii*' are no longer considered valid.

Borghans and Stanford (1973) reported an outbreak of 47 cases of injection-related abscesses following administration of diphtheria–pertussis–tetanus–polio vaccine at a clinic in the Netherlands in 1969. Abscesses took up to 1 year to develop. Many patients required incision or excision of the abscesses. Evidently all lesions eventually resolved without specific antimicrobial therapy. No definite source for the outbreak was detected.

Gremillion et al. (1983) reported an outbreak of abscesses occurring in 13 of 42 student nurse assistants after practice injections. The authors reported that the material for the injections was taken from a hanging 1-l bottle of 0.9 percent sodium chloride, which was not changed over a 3-month period and which was noted to be cloudy and occasionally leaking. The bottle was not available for epidemiological investigation, having been

discarded prior to the recognition of the first case. The incubation period ranged from 6 to 16 weeks; all patients required some surgical intervention. Resolution of the infections took from 2 to 31 months.

There have also been a number of other case reports of infection subsequent to varying degrees of skin penetration, including two cases following acupuncture (Woo et al. 2002)

Laskowski et al. (1977) and Levy et al. (1977), reported on two patients with infections related to receipt of porcine heart valves that presumably had become contaminated during the course of the manufacturing process. By 1977, at least 25 contaminated porcine valve remnants had been detected by ten different medical centers (MMWR 1978), but only the two previously mentioned patients were known to have developed infections. The organism involved was identified by the CDC as *M. chelonei*, but no phenotypic features were mentioned in the reports. In 1984, Rumisek et al. (1985) reported the case of a young woman who had a mitral valve replacement with a porcine valve in 1976 and evidently did well for almost 3 years before developing symptoms; she eventually died more than 6 years after the initial valve replacement from complications related to *M. chelonae* endocarditis.

Grange (1992) reviewed mycobacterial infections occurring after heart valve replacement, including infections due to *M. chelonae* and *M. fortuitum* demonstrated or presumed to have resulted from implantation of contaminated valves, and postoperative sternal wound infections caused by each species, with cases tending to occur in clusters and probably due to contaminated water and perhaps to contaminated bone wax. Olalla et al. (2002) presented a case of *M. chelonae* endocarditis that occurred after aortic and mitral valve replacement, and reviewed the literature from 1966 to 2000 regarding *M. fortuitum* complex endocarditis.

Kuritsky et al. (1983) reported six patients with sternal wound infection, endocarditis, and saphenous vein graft-site infection caused by *M. chelonae* and *M. fortuitum*; the one *M. fortuitum* isolate caused a sternal wound infection. RGMs were cultured from several water sources in the hospital in which the infections occurred; the most likely source of the infecting organisms was thought to be nonsterile ice used to cool the cardioplegia solution before it was infused.

McCracken et al. (2000) reported a cluster of four immunocompromised patients with bacteremia, probably all related to procedures performed in the hospital radiology department, involving insertion of three Hickman lines and a biliary stent. Organism identification was performed by thin-layer chromatography.

Meyers et al. (2002) reported on an outbreak of cutaneous abscesses that developed in 34 of 82 patients who underwent liposuction procedures performed by a single physician over a period of approximately 6 months. An isolate of what was considered to be the same strain was obtained from an office faucet. The outbreak probably occurred as a consequence of inadequate infection control practices.

Boxerbaum (1980) reported the repetitive isolate of this organism from the sputum of an adolescent with cystic fibrosis; autopsy findings suggested that the organism may have contributed to the patient's demise. A review of hospital records revealed seven additional patients out of 430 with cystic fibrosis from whom a rapidly growing mycobacterium was isolated; five were *M. chelonae* and two were *M. fortuitum*. No phenotypic features of the organisms were provided, so, for example, it is not clear that the isolates reported to be *M. chelonae* were not in fact *M. abscessus*.

In 1983, Pappas et al. reported an outbreak resulting from contaminated flexible fiberoptic bronchoscopes involving 72 patients with positive cultures. Of these, nine patients became transiently colonized, and two with serious underlying diseases became infected. Bronchoscopes had initially been disinfected with glutaraldehyde; the problem was not resolved after attempting sterilization with ethylene oxide. The problem was eventually traced to puncture of the suction channel of the bronchoscopes, resulting in contamination of the bronchoscope interior with both *M. chelonae* and *Pseudomonas aeruginosa*. No biochemical data regarding the organism are presented, so the currently recognized species involved is not clear. Campagnaro et al. (1994) reported a pseudoepidemic that was traced to a single contaminated bronchoscope and a contaminated device for the cleaning of bronchoscopes. Cox et al. (1997) reported a pseudo-outbreak of 28 patients with positive bronchial washing specimens out of a total of 91 patients who had bronchial washings performed during a 3-month period. Lidocaine sprayers used during the bronchoscopy procedure were found to be contaminated with the organism; these sprayers were cleaned with a solution made with tap water, which was probably the ultimate source of the organism.

Gutman et al. (1974) report a case of a thyroid abscess in a 4-year-old child treated with surgical excision and one year of ethionamide, to which the organism was sensitive in vitro. Only minimal phenotypic data regarding the organism were presented in the report.

This species has been reported to be the cause of infections of the eye, particularly keratitis; most cases have followed ocular surgery or traumatic eye injuries (Khooshabeh et al. 1994; Bullington et al. 1992) (no biochemical results provided, and different susceptibility results obtained with different isolates) (Newman et al. 1984) (no biochemical results provided); apparently even such minor trauma as caused by the wearing of soft contact lenses can result in infection. Although in several of these reports the mycobacterial species causing the infection was not well characterized, in at least one report the organism was identified by molecular methods (Alvarenga et al. 2002).

A case of bilateral otits media and mastoiditis in a young child, symptoms of which began 6 months after insertion of myringotomy tubes, was reported by Avery et al. (1995). Interestingly, an initial isolate was considered by one reference laboratory to be resistant to all agents tested, with the exception of imipenem. (The cefoxitin minimum inhibitory concentration (MIC) was reported as >32, but note that current NCCLS guidelines (NCCLS 2000) would consider cefoxitin MICs of 32 and 64 to represent an intermediate level of susceptibility.) The patient was treated with imipenem and further surgery. A subsequent isolate was found at a second reference laboratory to be sensitive only to cefoxitin and ciprofloxacin but resistant to all other agents tested, including imipenem. Cefoxitin was not included in the patient's treatment regimen until receipt of the second report. The patient responded to the combination of cefoxitin and further surgical management. The cefoxitin susceptibility suggests that the organism may actually have been *M. abscessus* (Brown-Elliott and Wallace 2002). At the time of the report, the two organisms were considered conspecific, and no biochemical data for the organism are included in the report.

Infections have been associated with access devices such as intravascular (Flynn et al. 1988; Raad et al. 1991) and peritoneal catheters (Merlin and Tzamaloukas 1989; Hevia et al. 2000) (see also under *M. fortuitum*). Catheter-related infections may remain localized to the exit site or may result in disseminated infection; their course may become both chronic and complicated, with dissemination perhaps resulting in the seeding of other catheter sites (Hsueh et al. 1998).

In 1992, Singh and Yu reported the successful treatment of a case of pulmonary disease attributed to *M. chelonae* with the combination of cefoxitin and ciprofloxacin and reviewed the literature on pulmonary disease caused by this organism. However, they did not distinguish between cases caused by what were then considered to be the two subspecies, *M. chelonae* ssp. *chelonae* and *M. chelonae* ssp. *abscessus*.

In 1992, Wallace et al. reviewed 100 patients with skin, soft-tissue, or bone infections. Fifty-three patients had disseminated cutaneous disease; of these, three had positive blood cultures and two had osteomyelitis of the extremities. Thirty-five patients had cellulitis, a subcutaneous abscess, or osteomyelitis; 30 of these infections occurred after a penetrating injury or a medical procedure. Twelve infections were catheter-related. Four of the eight patients with intravenous catheters were bacteremic. Ninety-two percent of the patients with disseminated skin infection were receiving corticosteroids, either alone or in combination with other immunosuppressive agents. Of the antimicrobial agents tested in vitro, clarithromycin was the most active. Of the rapid growers, this species appears to be the one most commonly involved in disseminated cutaneous infections (Brown-Elliott and Wallace 2002).

SUSCEPTIBILITIES

Nearly all isolates of this species are either susceptible, or susceptible at an intermediate level, to amikacin, tobramycin, clarithromycin, gatifloxacin, and linezolid, but only about one-fifth have at least an intermediate level of susceptibility to ciprofloxacin, about one-fourth, to doxycycline, and two-fifths to imipenem (Brown-Elliott and Wallace 2002). Note that the NCCLS currently recommends that imipenem not be tested against this species; this is also the only species of the RGMs for which tobramycin results should be reported (NCCLS 2000). Wallace et al. (1993c) reported the successful use of clarithromycin as monotherapy in patients with skin disease. One patient who discontinued therapy early relapsed with a resistant isolate. Subsequently, Tebas et al. (1995) reported the development of clarithromycin resistance in a heart-transplant patient being treated for disseminated disease; the authors recommended the use of at least two agents, one of them clarithromycin, particularly in immunocompromised patients, for treating *M. chelonae* infections. Driscoll et al. (1997) reported a patient with recurrence of skin lesions with a resistant isolate after apparently successful treatment with clarithromycin monotherapy. One study traced the genetic basis of clarithromycin resistance in this species to a point mutation in the 23S rRNA gene (Wallace et al. 1996). Brown-Elliott et al. (2001) reported the successful use of linezolid in a patient with skin involvement of his right lower leg with an organism that had developed resistance to clarithromycin following treatment with that agent.

Information concerning molecular identification of *M. chelonae* by REA of the HSP gene can be found in Telenti et al. (1993). Chromatographic identification is described by Butler et al. (1996).

M. confluentis

The original description of this organism in 1992 was based on a single isolate obtained from the sputum of an apparently healthy male (Kirschner et al. 1992b). A brownish pigment, which subsequently turns black, is said to develop after incubation for 8–12 weeks; however, this pigment production was not found to be a stable property of the strain. In vitro, the type strain showed susceptibility to ethambutol, isoniazid, and streptomycin. The name is derived from the Latin word confluentis, which refers to the city of Koblenz, the source of the type strain.

Information concerning molecular identification of *M. confluentis* by REA of the HSP gene can be found in Brunello et al. (2001). Chromatographic identification is described by Kirschner et al. (1992b).

M. elephantis

The original description of this organism was based on as isolate obtained from a lung abscess in an elephant (Shojaei et al. 2000). A subsequent report in 2002 by Turenne et al., described 11 isolates obtained from patients in Ontario, Canada. Ten of these isolates were obtained from sputum specimens and were of uncertain clinical significance. One isolate was obtained from an axillary lymph node of a patient who presented with lymphadenopathy and had been tattooed recently. Histopathology showed caseating granulomas, and the isolate was presumed to be clinically significant. These authors provided a detailed comparison of the phenotypic and molecular features of their isolates as compared with the two colony types that they detected in the type strain. The sequence they obtained for the 16S rRNA gene from one of the two colony types differed by only one bp from the sequence that they obtained for the same region from their clinical isolates; the sequence obtained for the other colony type showed several additional bp differences. The *M. elephantis* sequence that we used in our tree diagram (Figure 1) is the one obtained for the clinical strains, and the phenotypic features in Table 1 for the species are those obtained for the clinical isolates. An isolate was also reported from the sputum of a patient in Belgium who had a history of both a respiratory tract infection and enteritis; the identification was made by molecular methods (Potters et al. 2003).

Information concerning chromatographic and molecular identification of *M. elephantis* by REA of the HSP gene can be found in Turrene et al. (2002).

M. fallax

Three of the 22 strains included in the study on which the original description of the species was based (Lévy-Frébault et al. 1983) were isolated from sputum specimens, but no mention was made of the possible clinical significance of the isolates; the other 19 isolates were from water and soil samples. The name fallax is a Latin word meaning deceptive, given to the organism as the colonies resemble those of *M. tuberculosis*. The organism has been found to be susceptible in vitro to cycloserine and ethambutol. We have not been able to find any subsequent accounts of isolation of the organism from other than environmental sources.

Information concerning chromatographic identification of *M. fallax* is described by Lévy-Frébault et al. (1983).

M. farcinogenes

This species and the closely related *M. senegalense* are considered to be the causative agents of bovine farcy, an infectious disease of cattle (Chamoiseau 1979; Hamid et al. 1991). The disease is apparently confined to Africa (Brown-Elliott and Wallace 2002). In a numerical phenetic study, Ridell and Goodfellow (1983) found several characters that proved useful for distinguishing between these species; most isolates of this species required more than 5 days for growth, whereas most isolates of *M. senegalense* grew within 5 days. Data presented by Chamoiseau (1979) suggest that most isolates of *M. farcinogenes* would be considered slow growers. These species are very closely related to each other, as well as to *M. fortuitum* (Pitulle et al. 1992; Kirschner et al. 1992a). The 16S rDNA sequences of both species are very similar, but there are differences in the 16S-23S rDNA internal transcribed spacer regions of the two species, and probes based on these differences have been developed that can be used for identification purposes (Hamid et al. 2002).

Information concerning molecular identification of *M. farcinogenes* by REA of the HSP gene can be found in Brunello et al. (2001). Chromatographic identification is described by Hamid et al. (1991).

M. flavescens

This organism is generally placed in the group of slowly growing scotochromogens; some isolates evidently grow rapidly enough that they could be classified as rapid growers. There are only a handful of reports of infection attributed to this organism (see brief summary as of 1997 by Fischer et al.). These infections have involved a variety of body sites.

Information concerning molecular identification of *M. flavescens* by REA of the HSP gene can be found in Telenti et al. (1993).

M. fortuitum

This species was considered a human pathogen when it was initially described in 1938 by da Costa Cruz. (See the discussion by Wayne and Kubica (1986) for a brief account of the history regarding the appropriate species name for this organism.) In 1981, Staneck et al. reported a patient with posttraumatic osteomyelitis in the ankle region after sustaining a compound fracture in a motorcycle accident. On the basis of the Gram stain appearance of the organism in pus from the lesion, described as an 'irregular-stained gram-positive filamentous branching organism,' and on the basis of the initial studies of the organism in culture, it was thought to be a *Nocardia* species. Further study of the organism after the patient failed to respond to therapy for a *Nocardia* infection revealed that the organism was in fact an RGM, identified as *M. fortuitum* in two reference laboratories, although specific biochemical data are not provided.

The lesion eventually responded after long-term administration of erythromycin and minocycline.

Nolan et al. (1991), reported on an outbreak involving six patients undergoing electromyography that was performed at the same facility; infections in all six patients began at a site at which a needle-electrode probe had been inserted. No source for the outbreak could be determined, but it was speculated that the source of the infection may have been contaminated tap water, which was used to rinse needles after they had been soaked in glutaraldehyde solution.

Plemmons et al. (1996) reported a case of otitis media and mastoiditis in an adolescent who had had multiple pressure equalization tubes placed on the same side for treatment of otitis media. Four other cases of otic infection due to *M. fortuitum* that had been reported to date were also reviewed. Susceptibility results of the patient's infecting organism were unusual, in that it was resistant to both amikacin and cefoxitin; the amikacin resistance was thought possibly to have resulted from previous topical treatment with aminoglycosides. The patient was treated with a combination of hyperbaric oxygen, limited debridement, and oral clarithromycin and trimethoprim-sulfamethoxazole, and ultimately did well.

Five patients undergoing open heart surgery at a community hospital between October 1975 and May 1976 developed infections at their median sternotomy sites from 6 to 44 days postoperatively; no source for the infections was found (Hoffman et al. 1981).

A few cases of endocarditis have been described, including a case affecting a native valve in an HIV-positive intravenous (IV) drug abuser (Spell et al. 2000) and cases affecting prosthetic valves (Narasimhan and Austin 1978). References to these and other cases can be found in the review by Olalla et al. (2002), although the review itself does not distinguish among the various species involved. A case of aortitis following sternal wound infection subsequent to aortic valve replacement has also been reported (Schlossberg and Aaron 1991).

In 1991, Raad et al. reported on 15 patients who had developed catheter-related infections caused by members of the *M. fortuitum* complex, and also reviewed the literature on catheter-related infections to date, finding an additional 14 cases. Nine of their own cases, and seven of those in their review, were attributed to *M. fortuitum*. The other cases were attributed to *M. chelonae*, but *M. abscessus* was not distinguished from *M. chelonae*. The authors noted that catheter removal was critical for successful management of such infections, with parenteral antibiotic administration also being of great importance. Rodgers et al. (1996), subsequently reported an additional case in an 18-month-old child who was evidently successfully treated by catheter removal alone, as it appeared that by in vitro testing the organism was resistant to the principal antibiotic used for treatment. In 2000, Hevia et al. presented five patients with peritoneal catheter exit-site infections, four of which were caused by *M. fortuitum* and one by *M. chelonae*; they also briefly refer to the few other reported cases that they could find. None of their four cases caused by *M. fortuitum* were associated with peritonitis, but there have been a few cases of peritonitis reported in patients undergoing chronic peritoneal dialysis (LaRocco et al. 1986; Woods et al. 1986). A case of ventriculoperitoneal shunt infection has also been reported (Midani and Rathmore 1999); the authors also briefly review a few other reported cases of central nervous system (CNS) infections attributed to *M. fortuitum*. The patient did well following shunt removal and antibiotic therapy.

In 1988, Varghese et al. reported a fatal pulmonary infection in a young woman with aspiration pneumonia secondary to esophageal achalasia; however, no biochemical data regarding the organism were provided Several fatal cases of pneumonia, generally in somewhat compromised hosts, have been described (e.g. Bevelaqua et al. 1981; Nussbaum and Heseltine 1990; Lessing and Walker 1993). Successful medical treatment of a child with Wilms' tumor, who had pulmonary disease with a vasculitic component, and positive cultures obtained from lung biopsy and blood, has been reported (al Shaalan et al. 1997). Successful medical treatment of a lung abscess in a patient who perhaps had underlying congenital cystic lung disease has also been reported (Pacht 1990).

Sack (1990) described an HIV-positive IV drug abuser who had disseminated cutaneous disease due to *M. fortuitum*; both *M. fortuitum* and *M. tuberculosis* were cultured from bronchial washings, and *Cryptococcus neoformans* was cultured from cerebrospinal fluid (CSF). Smith et al. (1996), described another patient with AIDS who had disseminated cutaneous disease; positive cultures for the organism were obtained from a skin biopsy and from bone marrow. At autopsy, the patient was noted to have basilar meningitis; a tissue specimen was histopathologically positive for acid-fast bacilli and grew *M. fortuitum*. Butt (1998) reported two HIV-positive patients with cervical lymphadenitis and reviewed the literature pertaining to lymphadenitis caused by *M. fortuitum*. One of their two patients had had *M. fortuitum* isolated from a sputum specimen several months before the development of lymphadenitis but had not been treated because of the absence of any pulmonary symptoms.

Clegg et al. (1983) discussed 17 patients who developed infection after augmentation mammoplasty over a 3.5-year period. Isolates from 12 of the patients were available for study; ten of these proved to be *M. fortuitum* and two *M. chelonae*. Infections became apparent from 1 week up to more than 2 years after surgery. The source of the organisms was not identified. Haiavy and Tobin (2002) reported two additional cases and briefly reviewed the literature.

In their 1996 review of meningitis caused by nontuberculous mycobacteria, Flor et al. (1996) mention six

patients who they found in their literature review with meningitis attributed to this species, but they mention no other species of RGM and provide no identification details.

Smith et al. (1984) reviewed their experience with finding mycobacteria in patients with cystic fibrosis. Mycobacteria were found in seven of 223 patients; three of the isolates were *M. tuberculosis*, two were unidentified, one was *M. chelonae*, and one was *M. fortuitum*. The patient from whom the *M. fortuitum* was isolated had multiple sputum specimens that were both smear- and culture-positive; this patient eventually died, evidently from overwhelming pulmonary mycobacterial infection.

In 1987, Hoy et al., reported on a pseudoepidemic involving bone-marrow specimens obtained from four patients. All four patients had underlying malignancies, and three had had bone-marrow transplantation. All specimens were obtained on the same ward, and three were obtained on the same day. Acid-fast smears of all four specimens were negative, and in none of the patients did a disseminated *M. fortuitum* infection seem very likely. The source of the organisms was finally determined when it was realized that all four patients had had viral bone-marrow cultures requested. When such cultures were requested, the syringe with needle and cap still attached was placed in ice obtained from the floor ice machines for transport to the laboratory. The same organism was grown from melted unconcentrated ice obtained from the machine on the floor that had housed the affected patients. Although the same organism was found in concentrated samples from other floors, organisms were not grown from unconcentrated samples from any other floor. After changing the method of specimen delivery to the laboratory, no additional positive cultures occurred.

In 1991, Burns et al. (1991) reported on an outbreak of respiratory tract colonization that was detected after 16 patients on an alcoholism rehabilitation ward had positive sputum cultures over a 6-month period; subsequent surveillance cultures detected several more colonized patients. None of the patients had an illness attributed to the organism. The outbreak was thought to have been caused by organisms present in water in the ward showers. By PFGE, an isolate obtained from a tap connected to the water line that supplied the showers, and all 16 patient isolates, were identical. Gebo et al. (2002) reported on an outbreak of respiratory tract colonization on a ward primarily for HIV-infected patients. The outbreak was recognized when it was noted that an unusually high number of *M. fortuitum* isolates had been obtained from patients on that ward; 60 specimens from 47 patients were culture-positive over a 13-month period, but none of the specimens was smear-positive. No clinical illness definitely attributable to the organism was found in any of the patients. Multiple isolates of the same species were obtained from an ice machine on the ward. PFGE was used to confirm strain identity of patient and ice machine isolates.

Winthrop et al. (2002) reported on their extensive analysis of an outbreak of furunculosis associated with footbaths at a California nail salon. One hundred and ten customers of the nail salon who developed furunculosis were identified. Of these, 34 had cultures that were positive for RGMs; 32 of the organisms were identified as *M. fortuitum*, and two were not identified. The outbreak was not detected until it had been ongoing for almost 6 months, when a physician reported to the local health department that she had seen four patients over the past 6 months with lower-extremity furunculosis of unknown etiology. Cultures from all ten of the footbaths in the salon grew *M. fortuitum*; other mycobacteria or nocardiae also were found in five of the footbaths. Isolates from 14 patients and six footbaths were compared by PFGE; the isolates from all the patients and three of the footbaths were found to be identical, while the isolates from the other three footbaths were not the same strain. Shaving of the legs prior to having a footbath was found to be a significant risk factor for infection. The median number of lesions per patient was two, with a range of one to 37. The authors noted that patients frequently delayed seeking medical attention because of the initial indolence of the infections, and that, once consulted, physicians generally did not consider the possibility of a mycobacterial infection, resulting in ineffective treatment and lack of clinical improvement. In those patients who eventually received appropriate oral antibiotic treatment, the lesions resolved, as they also did in some of the untreated patients. However, one untreated patient developed disseminated disease.

SUSCEPTIBILITIES

Nearly all isolates of this species are either susceptible, or susceptible at an intermediate level, to amikacin, cefoxitin, ciprofloxacin, gatifloxacin, imipenem, linezolid, and trimethoprim-sulfamethoxazole, but only about four-fifths have at least an intermediate level of susceptibility to clarithromycin, and about one-half to doxycycline (Brown-Elliott and Wallace 2002).

Information concerning molecular identification of *M. fortuitum* by REA of the HSP gene can be found in Telenti et al. (1993). Chromatographic identification is described by Butler et al. (1996).

M. fortuitum, third biovariant complex

Wallace et al. (1991) studied 85 isolates of this complex. By in vitro testing, all isolates were susceptible to amikacin, gentamicin, ciprofloxacin, imipenem, and sulfamethoxazole; one-quarter were resistant to doxycycline. Of the patients with skin and soft-tissue infections

for whom clinical histories were available, most had a history of trauma prior to the development of infection. Most patients responded well to drug treatment or a combination of drug and surgical management. Five patients were simultaneously infected with a second species of RGM. Twenty-six isolates were from a respiratory source; the clinical significance of these was not determined. Organisms in this group can be further subdivided on the basis of ability to use sorbitol as a sole carbon source; most sorbitol-positive isolates were found to be cefoxitin-resistant, while all sorbitol-negative isolates were susceptible. Recent studies have suggested that there may be six or more species within this complex (Brown-Elliott and Wallace 2002); the proposed name for most of the isolates within the sorbitol-positive group is *M. houstenense*, and the proposed name for most of those in the sorbitol-negative group is *M. bonickei*.

Isolates of this complex typically have at least an intermediate level of susceptibility to amikacin, cefoxitin (sorbitol-negative group only – see above), ciprofloxacin, gatifloxacin, imipenem, levofloxacin, linezolid, and sulfamethoxazole; some 80 percent of isolates have at least an intermediate level of susceptibility to clarithromycin (Brown-Elliott and Wallace 2002).

Information concerning molecular identification of *M. fortuitum* third biovariant by REA of the HSP gene can be found in Telenti et al. (1993).

M. gadium

The initial description of this organism was based on a sputum isolate from a patient with pulmonary tuberculosis from whom *M. tuberculosis* was isolated simultaneously (Casal and Calero 1974). The species name is a Latinized name for 'Cadiz,' the Spanish town where the patient from whom the organism was isolated lived. No pathogenic role could be attributed to the organism. We are not aware of any subsequent isolation from clinical specimens of organisms belonging to this species. A later comparison of this species with three other species of rapid growers, using molecular methods, suggested that this was indeed a distinct species (Garcia and Tabarés 1986).

Information concerning molecular identification of *M. gadium* by REA of the HSP gene can be found in Brunello et al. (2001).

M. gilvum

The initial description of this organism was based on a sputum isolate; no clinical information regarding the patient from whom the isolate was obtained was provided (Stanford and Gunthorpe 1971). The name of the organism is derived from a Latin word meaning 'pale yellow.' By in vitro testing, it was found to be susceptible to cycloserine and ethambutol.

Information concerning molecular identification of *M. gilvum* by REA of the HSP gene can be found in Brunello et al. (2001).

M. goodii

This organism was recently separated from *M. smegmatis* (Brown et al. 1999). Of 28 clinical isolates initially reported, six were responsible for pulmonary infections; and at least 18 of the remaining isolates were from posttraumatic or postsurgical infection sites. As with *M. smegmatis* and *M. wolinskyi*, the chronic lung disease that can be caused by this species tends to occur in patients with lipoid pneumonia. The organism was named after mycobacteriologist Robert Good. A case of olecranon bursitis caused by this organism was subsequently reported (Friedman and Sexton 2001). The patient's infection responded after the lesion had been aspirated several times and he had been treated with both doxycycline and ciprofloxacin. Typical antibiotic susceptibilities of this species are the same as those for *M. smegmatis* (Brown-Elliott and Wallace 2002). However, isolates of this particular species typically have an intermediate level of susceptibility to tobramycin (at an MIC of 2–8 μg/ml for taxonomic purposes) (Brown et al. 1999).

Information concerning chromatographic identification and molecular identification of *M. goodii* by REA of the HSP gene can be found in Brown et al. (1999).

M. hassiacum

The initial description of this organism was based on a single urine isolate that was not considered clinically significant (Schröder et al. 1997). The name refers to Hesse, the German province where the type strain was isolated. This species is remarkable for its ability to grow at 65°C. A second isolate from a single urine specimen of a patient with symptoms of cystitis has also been reported (Tortoli et al. 1998), but it was not clear that the organism played any role in the patient's illness. In vitro, both isolates were found to be susceptible to amikacin, clarithromycin, ethambutol, streptomycin, and trimethoprim-sulfamethoxazole. Although the organism grows rapidly, phylogenetic analysis indicated that it is most closely related to *M. xenopi* (Schröder et al. 1997).

Information concerning chromatographic identification of *M. hassiacum* can be found in Tortoli et al. (1998).

M. hodleri

The initial description of this species was based on an organism isolated from fluoranthene-contaminated soil (Kleespies et al. 1996). The organism was named in honor of Christian Hodler, a director of the Ministry of

Science and Culture in Lower Saxony, Germany, for his support of the natural sciences. On direct application of a PCR procedure for amplification of a portion of the 16S rRNA gene to synovial tissue obtained from a patient with rheumatoid arthritis, an amplicon was obtained that showed 97 percent sequence identity to the corresponding sequence for *M. hodleri* (van der Heijden et al. 1999); however, no mycobacteria were visualized in, or isolated from, the clinical material from this patient, and the significance of the positive PCR result was not clear.

Information concerning chromatographic identification of *M. hodleri* can be found in Kleespies et al. (1996).

M. holsaticum

The initial description of this species was based on organisms isolated from clinical specimens from nine patients in different regions of Germany (Richter et al. 2002). The species name refers to Holstein, the region in Germany in which the organism was first studied. Of the nine isolates, six were from sputum, and one each was from urine, gastric fluid, and an unknown source. No clinical information regarding any of the patients was provided. By in vitro testing, the isolates were said to be susceptible to ethambutol, ofoloxacin, and streptomycin.

Information concerning chromatographic identification and molecular identification of *M. holsaticum* by REA of the HSP gene can be found in Richter et al. (2002).

M. immunogenum

This is a newly described species phenotypically similar to *M. abscessus* and *M. chelonae* (Wilson et al. 2001). However, *M. immunogenum* has two ribosomal gene copies, a characteristic it shares with all other RGMs studied to date, except for *M. abscessus* and *M. chelonae*, each of which has only one copy. Many isolates of *M. immunogenum* have been recovered from metal-working fluids. The organism has been found in pseudo-outbreaks resulting from bronchoscopic contamination and has also been found to be the causative agent of human disease in a small number of cases to date. Patients from whom it has been isolated have had disseminated cutaneous disease, pneumonia, and foreign-body-related infections (Wilson et al. 2001). The organism is also likely to play an etiologic role in some cases of hypersensitivity pneumonitis, particularly in metal workers. The species name is a Latin word meaning 'eliciting an immune response.' In vitro isolates were found to be susceptible to amikacin and clarithromycin but resistant to cefoxitin, ciprofloxacin, doxycycline, imipenem, and sulfamethoxazole.

Information concerning chromatographic identification and molecular identification of *M. immunogenum* by REA of the HSP gene can be found in Wilson et al. (2001).

M. mageritense

The initial description of this species was based on five sputum isolates from different patients; none of the isolates was thought to be pathogenic (Domenech et al. 1997). The species name is derived from an Arabic name for Madrid (Magerit), where most of the initial isolates were obtained. Wallace et al. (2002) have presented data on six clinical isolates from North American patients. Isolates have come from sputum, a bronchoscopy specimen, an area of persisting cellulitis of the thigh postliposuction, blood, a sinus, and a wound. The isolates were noted to be very similar to isolates belonging to the sorbitol-positive group of the third biovariant but could be distinguished by carbohydrate utilization, relatively greater cefoxitin susceptibility, and molecular methods.

Information concerning chromatographic identification and molecular identification of *M. mageritense* by REA of the HSP gene can be found in Brown et al. (1999).

M. marinum

Some isolates of members of this species may grow rapidly enough to meet the technical definition of an RGM. However, this organism is traditionally grouped with the photochromogenic slowly growing mycobacteria and will not be discussed further here.

M. moriokaense

This initial description of this organism was based on a number of environmental isolates and a single sputum isolate from a patient with pulmonary tuberculosis (Tsukamura et al. 1986). The species name indicates the place (Morioka) where the species was initially isolated. On the basis of comparisons made with other RGMs at the time of its first description in 1986, the species could be distinguished from other rapid growers by the use of certain phenotypic tests, by its mycolic acid pattern, and by DNA relatedness studies. We could not find any subsequent reports of isolation of this species from clinical specimens.

Information concerning chromatographic identification of *M. moriokaense* can be found in Tsukamura et al. (1986).

M. mucogenicum

This organism was first recognized as a human pathogen in the late 1970s, when it was isolated from several

patients with peritonitis who were undergoing chronic peritoneal dialysis; two separate outbreaks, each at a different dialysis center, were involved, and seven sporadic cases were found as well (Band et al. 1982). Cross-infection from patient to patient was thought to have occurred as a result of both some design deficiencies in the dialysis machines and employment of inadequate disinfection procedures. The ultimate source of the organisms was not determined but was thought most likely to be tap water. One isolate belonging to this group (out of a total of 27) was also detected in the outbreak studied by Bolan et al. (1985). The organism was initially referred to as an MCLO. Even though the initial isolates were found to have phenotypic features that would differentiate them from what are now the separate species *M. abscessus* and *M. chelonae*, the species was not formally described and named until 1995 (Springer et al. 1995). The species name was selected because of the mucoid appearance of colonies of most isolates on solid media. In a study of 87 sporadic isolates of this organism from 84 patients, 54 were from the respiratory tract (Wallace et al. 1993a). Of those respiratory isolates, only two were thought to be clinically significant; one was from an open lung biopsy and the other was from the sputum of a patient who also had a positive blood culture. Both of these isolates were from AIDS patients. Isolates from skin, soft tissue, or blood were more likely to be considered clinically significant, but even in the case of blood isolates, a few that were obtained from only a single blood culture from patients who had other negative blood cultures were considered contaminants. Most of the skin and soft-tissue infections followed accidental trauma or surgery, and most of the patients with what were considered to be significant isolates had catheter-related sepsis. This organism has also been isolated from the blood of a patient with cirrhosis (Paterson et al. 1998); the source of the organism could not be determined, but the patient was on hemodialysis and did have an intravascular catheter in place. Additionally, it was isolated from liver tissue obtained at autopsy from a patient with granulomatous hepatitis, although the organism could not be visualized in sections of the liver (Goldblatt and Ribes 2002).

Zhibang et al. (2002) reported an outbreak of buttock infections in 86 patients following penicillin injection at a hospital in Chongqing, China. The infection spread in at least some patients from the injection sites to the inguinal lymph nodes. The source of the organisms was thought to be the soil in the basement in which the vials were stored on the ground; additionally, the vial lids were inadequately disinfected prior to entry of the vials. Susceptibility testing by an unspecified method revealed the organisms to be sensitive to amikacin, gentamicin, kanamycin, and vancomycin. The use of some or all the agents to which the organisms appeared susceptible, combined with surgery, evidently resulted in the cure of all patients within a year. The authors identified the organism involved as *M. abscessus*, but they stated that the isolates were mucoid, did not grow on 5 percent NaCl, utilized citrate, and were described as 'positive' for iron absorption. It seems most likely that these isolates actually belonged to the species *M. mucogenicum*. (*M. mucogenicum* is usually said to be negative for iron uptake, but some tan or rust color may develop) (Wallace et al. 1993a).

SUSCEPTIBILITIES

Unlike isolates of many other species of rapid growers, isolates of *M. mucogenicum* tend to be susceptible in vitro to a wide array of antimicrobials (Wallace et al. 1993a)

Information concerning molecular identification of *M. mucogenicum* by REA of the HSP gene can be found in Steingrube et al. (1995). Chromatographic identification is described by Butler et al. (1996).

M. neoaurum

This species was initially described by Tsukamura in 1972; the species name means 'a new gold-pigmented organism' (Wayne and Kubica 1986). Tsukamura subsequently published an additional study comparing this species with other scotochromogenic rapid growers (Tsukamura 1981). The first reported human infection attributed to this organism was a case of bacteremia occurring in an immunocompromised patient with a Hickman catheter in place; the patient was successfully treated with cefoxitin and gentamicin, without removing the catheter (Davison et al. 1988). Eight of nine blood cultures were positive. The organism was identified on the basis of its phenotypic and thin-layer chromatographic features; no molecular studies were carried out. Subsequently, there have been at least five case reports of infections attributed to this organism. Three of these have been cases of bacteremia in patients with Hickman catheters (Holland et al. 1994; George and Schlesinger 1999; Woo et al. 2000). One was a case of peritonitis in a patient receiving chronic ambulatory peritoneal dialysis (McNally and Mangino 2000), and another was from a patient with a urinary tract infection (Zanetti et al. 2001). The isolate from the patient with peritonitis was identified on the basis of results with thin-layer and high-performance liquid chromatography. One of the isolates was identified on the basis of its 16S rRNA sequence (Woo et al. 2000), and another on the basis of both restriction analysis of the PCR product from the *hsp65* gene and the 16S rRNA sequence (Zanetti et al. 2001). All these patients recovered from their infections.

Information concerning molecular identification of *M. neoaurum* by REA of the HSP gene can be found in Taylor et al. (1997); this species may be indistinguishable from *M. aurum*. Chromatographic identification is described by Woo et al. (2000).

M. novocastrense

The initial description of this species was based on an isolate obtained from a skin biopsy of a lesion on a child's hand (Shojaei et al. 1997). No additional clinical information regarding the patient from whom the organism was isolated was provided. The species name refers to the city of Newcastle in England. We are not aware of any subsequent reports of clinical isolates belonging to this species.

Information concerning chromatographic identification of *M. novocastrense* can be found in Shojaei et al. (1997).

M. obuense

In the publication initially describing this organism, isolates belonging to this species were said to have been isolated from patients with pulmonary disease (Tsukamura 1981). However, it is not clear whether the one isolate actually studied for that publication, which is indicated in one of the tables as having been 'received as' a member of this species and as having been obtained from the sputum of 'humans,' was actually considered to belong in this species. No specific clinical information regarding any isolate is provided. The species name refers to the place (Obu in Japan) where the species was initially isolated.

Information concerning molecular identification of *M. obuense* by REA of the HSP gene can be found in Brunello et al. (2001).

M. parafortuitum

The original description of this species was based on isolates obtained from soil; the species name is presumably intended to indicate its similarity to *M. fortuitum* (Tsukamura 1966b). In a table in a publication on the numerical taxonomy of organisms in the *M. parafortuitum* complex, several sputum isolates are listed that are labeled as *M. parafortutium* but are also said in a footnote to be 'outside the *M. parafortuitum* cluster' (Tsukamura et al. 1983). No additional clinical information is provided, and it is not clear to which species these organisms were actually considered by the authors to belong.

M. peregrinum

This species was initially described by Bojalil et al. (1962) on the basis of two isolates, one of which was from a child with 'bronchial aspiration.' The species name means 'strange' or 'foreign,' and it was applied because the initial isolates were the only nonpigmented organisms in the authors' 'branch I.' After some 30 years of debate regarding its taxonomic status, this organism was established as a valid species distinct from *M. fortuitum* by DNA–DNA hybridization criteria in 1992 (Kusunoki and Ezaki 1992). Although the occurrence of clinical isolates is noted in the publication establishing the species status of the organism, no specific clinical data are provided. Individual case reports of infections attributed to this organism have included a skin lesion (from which both this organism and *M. scrofulaceum* were isolated) in a fish fancier (Ishii et al. 1998), a thigh abscess at a site of continuous subcutaneous insulin infusion (Pagnoux et al. 1998), and catheter-related bacteremia in an immunocompromised patient (the organism in this case apparently having been identified on the basis of its glycopeptidolipids) (Rodríguez-Gancedo et al. 2001). Pipemidic-acid-susceptible and -resistant types (types 1 and 2, respectively, have been distinguished: type 1 is reported to have the same restriction pattern as the proposed species *M. bonickei*, and type 2 the same as the proposed species *M. houstonense*) (Brown-Elliott and Wallace 2002).

Information concerning molecular identification of *M. peregrinum* by REA of the HSP gene can be found in Telenti et al. (1993) and Steingrube et al. (1995). Chromatographic identification is described by Butler et al. (1996).

M. phlei

This species was described in 1899; the species name indicates a genus of grasses (*Phleum*) from which the organism was presumably initially isolated (Wayne and Kubica 1986). A noteworthy characteristic of this species is its ability to grow at 52°C (Saito et al. 1977). We have found three individual case reports of infection attributed to this species. One was a case of left-knee arthritis in a young boy; direct smear of synovial fluid was positive, and the organism cultured was identified as *M. phlei*, but only minimal biochemical data are presented (Aguilar et al. 1989). An abscess in the ankle region was also attributed to this organism, but direct smears of patient material were negative, fungal and mycobacterial cultures were negative, and the organism (regarding which no phenotypic features are presented other than that it was an RGM) was cultured only from the liquid medium that comprised a part of the routine bacteriologic culture (Spiegl and Feiner 1994). In a case of peritonitis in a patient on chronic ambulatory dialysis and who had several smear- and culture-positive specimens, the organism isolated was also identified as belonging to this species, but, other than susceptibility testing results and the fact that the organism was an RGM, no other phenotypic characteristics of the organism are mentioned (Paul and Devarajan 1998).

Information concerning molecular identification of *M. phlei* by REA of the HSP gene can be found in Devallois et al. (1997). Chromatographic identification is described by Kirschner et al. (1992b).

M. rhodesiae

Isolates belonging to this species were said to have been isolated from patients in Rhodesia with 'lung tuberculosis' (Tsukamura 1981). No specific clinical information regarding any isolate was provided; we are not aware of any subsequent reports of clinical isolates belonging to this species.

Information concerning molecular identification of *M. rhodesiae* by REA of the HSP gene can be found in Brunello et al. (2001).

M. senegalense

See the discussion under *M. farcinogenes*.

M. septicum

This organism was described on the basis of three blood culture isolates and a catheter-tip isolate obtained from a 2-year-old male with metastatic hepatoblastoma; both phenotypic and molecular tests supported the fact that these isolates were identical and represented a hitherto undescribed species (Hogg et al. 1999; Schinsky et al. 2000). The infection responded well to catheter removal and antimicrobial therapy. The species name refers to the clinical condition produced by the organism in the patient from whom it was first isolated.

Information concerning chromatographic identification of *M. septicum* can be found in Schinsky et al. (2000).

M. smegmatis

This species was described in 1899; the species name is derived from the Latin word for 'smegma,' from which the organism was presumably initially isolated (Wayne and Kubica 1986). In 1988, Wallace et al. reported on their experience with infections caused by this organism. Of 22 isolates from 21 patients, three were from respiratory tract sites, and the remainder were from infections involving skin, soft tissue, and/or bone. Seven infections occurred after cardiac surgery, including six sternal infections and one case of aortic valve endocarditis. By in vitro testing, all isolates showed what was considered to be at least some degree of susceptibility to amikacin, ciprofloxacin, doxycycline, ethambutol, and sulfamethoxazole. All of the 11 patients treated on the basis of the susceptibility testing results evidently responded well. These authors also studied some isolates obtained from cats and in addition noted that bovine mastitis had been attributed to this organism. In 1990, Plaus and Hermann reported two cases of injection site abscesses in weight lifters who had injected themselves with a veterinary preparation of an anabolic steroid; cultures of the injected material grew the same organism (Plaus and Hermann 1991). Newton et al. (1993) reported two cases of infection following trauma related to automobile accidents; no details were reported regarding the identification techniques employed. Cox et al. (1994) reported a case of *M. smegmatis* infection in a severely mentally disabled adolescent patient with concomitant lipoid pneumonia resulting from long-term mineral oil administration for chronic constipation; the organism was seen in, and cultured from, a lung biopsy specimen. The identification was confirmed in a reference laboratory; the patient appeared to resolve his infection with long-term antibiotic treatment. Gagnon (1994) reported the isolation of this organism from a foot puncture wound in a young girl; identification was performed in a reference laboratory and the patient responded well to debridement and trimethoprim-sulfamethoxazole. Infection following a facelift procedure has been reported by Pennekamp et al. (1997); the patient was treated with antibiotics and local wound care. A case of ultimately fatal disseminated infection due to *M. smegmatis* was reported in a child with interferon gamma receptor 1 deficiency by Pierre-Audigier et al. (1997). The organism, identified at a reference laboratory, was isolated both from a liver biopsy and from blood. The patient was apparently infected chronically with the organism for at least 5 years, until her death at 8 years of age. Skiest and Levi (1998) reported a case of catheter-related bacteremia caused by this species and *Enterococcus faecalis*; both organisms were recovered from blood and catheter-tip cultures and the patient responded well to catheter removal and antibiotic treatment. Typically, isolates of the *M. smegmatis* species group (*M. goodii*, *M. smegmatis*, and *M. wolinskyi*) are susceptible to amikacin, doxycycline, ethambutol, and sulfamethoxazole, have an intermediate level of susceptibility to ciprofloxacin, and have variable susceptibility to cefoxitin and clarithromycin (Brown-Elliott and Wallace 2002). However, isolates of this particular species have been found to be susceptible to tobramycin (at an MIC of $\leqslant 1$ μg/ml, for taxonomic purposes) (Brown et al. 1999).

Information concerning molecular identification of *M. smegmatis* by REA of the HSP gene can be found in Telenti et al. (1993). Chromatographic identification is described by Brown et al. (1999).

M. terrae

The *M. terrae* complex is composed of *M. terrae* and *M. nonchromogenicum*; *M. triviale* is also included by some authors. One text (Kent and Kubica 1985) indicates that 23 percent of the isolates of the complex (in the narrow sense) may meet the technical definition of a rapid grower. However, the members of this complex are generally considered to be slowly growing nonphotochromogens, and they will not be considered further here.

M. thermoresistible

This organism was presumably given its species name because of its ability to grow at temperatures of up to at least 52°C and to survive exposure to even higher temperatures (Saito et al. 1977). It was first reported as a pathogen following its isolation from a woman who presented with a 3-week history of weight loss, cough, and fever and who had several cavities on her chest X-ray (Weitzman et al. 1981). The organism was isolated from several pulmonary specimens. The patient responded to the combination of ethambutol, rifampin, and streptomycin. Subsequently reported human cases of infection caused by this organism have included a pulmonary granuloma in a man with common variable hypogammaglobulinemia (Liu et al. 1984), cutaneous infection at a surgical site in a diabetic heart-transplant recipient (Neeley and Denning 1989), abscesses following augmentation mammaplasty (Wolfe and Moore 1992), and localized cutaneous infection of a foot, also involving *M. fortuitum*, subsequent to stepping on gardening shears while spreading manure-containing mulch. In no case was there confirmation of the source of the infection by isolation of the organism, but the initial isolates of the species were obtained from soil (Tsukamura 1966a). Most patients responded slowly to treatment that generally included rifampin and ethambutol, in some cases with surgical drainage and foreign-body removal employed as well. The patient with the foot lesion apparently responded to the combination of levofloxacin and doxycycline.

Information concerning molecular identification of *M. thermoresistibile* by REA of the HSP gene can be found in Brunello et al. (2001).

M. vaccae

This species was described in 1964; the species name is derived from the Latin word for 'cow,' the source from which the organism was presumably initially isolated (Wayne and Kubica 1986). We have been able to find only one report of human infections caused by this organism (Hachem et al. 1996): three cases of pulmonary infection and one of cutaneous infection were reported. Two of the patients with pulmonary disease and the one with cutaneous disease had underlying malignancies. There was a history of exposure to cattle in two of the patients with pulmonary disease. The pulmonary infections appeared to respond to medical regimens including ciprofloxacin, and the patient with cutaneous disease appeared to respond to the combination of trimethoprim-sulfamethoxazole and minocycline. Organisms were isolated from only one specimen from each of the patients; apparently only one of the culture-positive specimens was also smear-positive for acid-fast bacilli on direct examination. No molecular studies were reported on the isolates.

Information concerning molecular identification of *M. vaccae* by REA of the HSP gene can be found in Telenti et al. (1993).

M. wolinskyi

This species was described on the basis of a study of eight human isolates (Brown et al. 1999). Of the seven patients for whom clinical histories were available, four had post-traumatic infections involving bone and/or soft tissue and three had postsurgical infections, including one arterial-venous shunt infection. They could not be distinguished from *M. smegmatis* or *M. goodii* by biochemical characteristics. There were some subtle differences among the three species noted by high-performance liquid chromatography (HPLC); molecular methods were the most reliable in clearly distinguishing among the three species. The organism was named after mycobacteriologist Emanuel Wolinsky. Typical antibiotic susceptibilities of this species are the same as those for *M. smegmatis* (Brown-Elliott and Wallace 2002), except that isolates of this species have been found to be resistant to tobramycin (at an MIC of $\geqslant$16 μg/ml, for taxonomic purposes) (Brown et al. 1999).

Information concerning chromatographic identification and molecular identification of *M. wolinskyi* by REA of the HSP gene can be found in Brown et al. (1999).

SUMMARY

Most, but perhaps not all, of the species generally considered to be RGMs are more closely related to one another than to the slow growers. The operational definition of an RGM is based on visible growth within 7 days on solid medium from a dilute suspension of organisms, not on the length of time for initial organism detection, although in most cases growth from the original specimen will be detectable in less than 7 days. Some isolates of species generally considered to be slow growers, however, may also meet the operational definition of an RGM.

On Gram stain, RGMs may appear as beaded gram-positive rods and may be presumed prematurely to be inconsequential contaminants because of their resemblance to coryneforms. RGMs usually stain adequately with regular acid-fast stains such as the Kinyoun stain, but some isolates may be relatively weakly acid-fast, and a modified acid-fast stain, such as that used for *Nocardia* species, may be useful to reveal their acid-fast properties.

RGMs are widely distributed in the environment and are relatively resistant to many disinfectants and so can relatively easily enter into, and persist in, materials used for diagnostic and therapeutic purposes.

Special media are not required for their isolation, but some isolates will grow better at room temperature,

and they do not grow as rapidly as most nonmycobacterial species, so they may be missed on routine bacterial culture of patient specimens. Also, it is possible that bacteremia is more frequent than currently reported, as in many cases of infection with these organisms, blood cultures may either not have been held for an adequate period of time or not have been obtained at all.

Many of the infections caused by these organisms are relatively indolent, and their possible etiologic role may not be considered early in the course of an epidemic, so their detection may be long delayed or perhaps missed altogether.

There have been many changes in the taxonomy of these organisms since the early reports on their occurrence were published. Unfortunately, both earlier as well as recent reports on these organisms often do not contain sufficient information about the isolates to be sure to which currently recognized species they belong. In some cases, species assignment has almost certainly been incorrect. Hence, care is needed in interpreting publications concerning these isolates. An increasing number of rapid-growing species is being recognized, and most laboratories have only a small array of phenotypic tests available for their identification. Furthermore, some of these tests may provide unreliable results, particularly in inexperienced hands. At the present time, molecular methods probably provide the most reliable for accurate identification of these organisms.

It is clear that isolates of certain species or species groups are much more commonly isolated from clinical materials than others, and it is also clear that some species are more commonly involved in certain types of infections than others. However, it is probably the case that virtually any species can potentially be involved in any type of infection. Also, mixed infections and episodes of contamination may occur.

Identification to the level of a specific species or species group is generally clinically relevant, not only to provide more information regarding the disease associations of particular species but, more importantly, also to provide an initial guide to selection of appropriate antimicrobial therapy. Most RGMs are resistant to the agents used for treating infections caused by members of the *M. tuberculosis* complex, although a few species are susceptible to ethambutol.

Many reports include in vitro susceptibility results, but as the methodology has frequently not been specified, and a variety of different procedures have been employed, the comparability and clinical relevance of much published data are uncertain. An NCCLS-recommended broth dilution susceptibility testing procedure has been established and should be used for all isolates considered to be clinically significant, as susceptibility status does not correlate perfectly with species assignment. However, susceptibility results, particularly for organisms obtained prior to the institution of treatment, which deviate markedly from expected results for the species, suggest the possibility of organism misidentification. Laboratories not experienced with the susceptibility testing procedure should ensure that they can obtain reproducible results that correlate with those obtained by a reference laboratory before reporting results on patient isolates.

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Neisseria

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INTRODUCTION

Definition

Members of the genus *Neisseria* are gram-negative diplococci that are normal inhabitants of the mucous membranes of mammals and reptiles; some species are primary pathogens of man. *Neisseria* are cocci 0.6–1.0 μm in diameter, occurring singly but more often in pairs with adjacent sides flattened; one species (*Neisseria elongata*) is an exception and consists of short rods 0.5 μm wide, often arranged as diplobacilli or in short chains. Division of the coccal species occurs in two planes at right angles to each other, occasionally resulting in tetrads. The species can largely be defined as either pathogenic or commensal, with *Neisseria gonorrhoeae* and *Neisseria meningitidis* being recognized as important human pathogens. The commensal Neisseriae are largely confined to the upper respiratory tract of humans (Smith et al. 1999).

All organisms belonging to the Neisseriaceae are aerobic chemoorganotrophs. They have a genomic DNA composition of 46.5–53.5 mole percentage of guanosine plus cytosine. They are oxidase- and catalase-positive (with one exception, *N. elongata*). Swarming motility does not occur and correspondingly flagella are absent. Members may express fimbriae (pili) that are associated with attachment, twitching motility, and genetic competence. They demonstrate natural transformation with homologous DNA at varying levels of efficiency. Most species are nutritionally fastidious, with an optimal growth temperature between 35 and 37°C. Some species are encapsulated, other species produce a greenish-yellow carotenoid pigment, and others are moderately α-hemolytic. Carbonic anhydrase is produced by all species, except the 'false Neisseriae' *Neisseria caviae*, *Neisseria ovis*, and *Neisseria cuniculi*.

Historical

Because of their association with disease, the pathogenic members of the Neisseriaceae were first described. *N. gonorrhoeae* (the gonococcus) was initially identified by Albert Neisser in 1879. Using microscopy, Neisser observed organisms within polymorphonuclear leukocytes (PMN) in gram-stained smears of urethral exudates from patients with gonorrhea, similar to those present in Figure 1. Shortly after this observation, this organism was successfully cultivated by Bumm (1885a, b) and by Lestikow and Loeffler (Lestikow 1882). The meningococcus (*N. meningitidis*) was first isolated in 1887 by Weichselbaum from the cerebrospinal fluid of patients with acute meningitis (Weichselbaum 1887). In 1906, von Lingelsheim described a number of commensal *Neisseria* species in nasopharyngeal cultures from healthy and diseased persons; these included *Micrococcus pharynges siccus* (*Neisseria sicca*), *Micrococus cinereus* (*Neisseria cinerea*), and *Micrococcus pharynges flavus* groups I, II, and III (*Neisseria subflava* biovars) (Von Lingelsheim 1906). Opportunistic members of the genus were subsequently identified. *Neisseria flavescens* was described in 1930 by Branham and *Neisseria lactamica* was described in 1969 by Hollis et al. Both these species were originally isolated from cererbrospinal fluid and thought to be responsible for

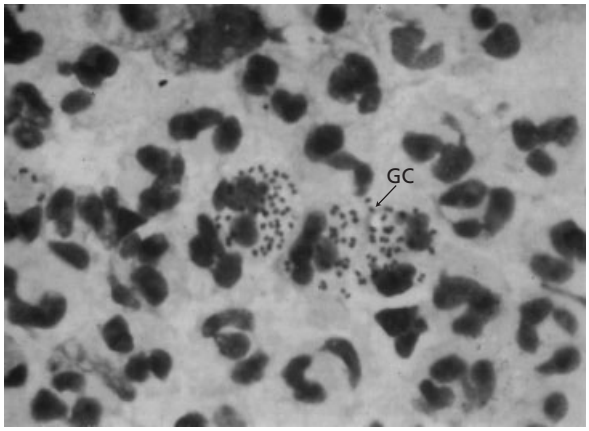

Figure 1 *Gram stain of the urethral exudates from a patient suffering from a local gonococcal infection.*

occasional cases of meningitis. *N. gonorrhoeae* subsp. *kochii* was isolated from conjunctival cultures from patients in rural Egypt and was first described in 1986 (Mazloum et al. 1986). *Neisseria* isolates formerly designated CDC group M-5 have been isolated from dog-bite infections and are now designated *Neisseria weaveri* (Andersen et al. 1993).

GENERAL FEATURES

Classification

The genus *Neisseria* is the type genus of the family Neisseriaceae (Bovre 1984). Strains belonging to the genus *Neisseria* may be identified by phenotypic characteristics including pigment expression, production of acid from various carbohydrates, production of polysaccharide from sucrose, and the reduction of nitrate and nitrite (Table 1). Using these criteria, 15 different *Neisseria* species, subspecies, and biovars isolated from humans and six species isolated from animals have been described (Knapp 1988; Barrett and Sneath 1994; Holmes et al. 1993). The majority of the human-associated species are placed into one of two major groups. Strains belonging to the first group (*N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. cinerea*, *N. polysaccharea*, *N. gonorrhoeae* subsp. *kochii*) grow as nonpigmented, translucent colonies on solid medium; strains belonging to the second group (*N. mucosa*, *N. sicca*, and *N. subflava*, including the biovars *subflava*, *flava,* and *perflava*) are referred to as the saccharolytic species and usually grow as opaque, yellow-pigmented colonies on solid medium.

Based on an analysis of ribosomal RNA genes by hybridization, *Neisseria* spp. can be divided into three main subgroups (Dewhirst et al. 1989; Rossau et al. 1989). The first subgroup is comprised of *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. polysaccharea*, and *N. cinerea*; the second subgroup is comprised of *N. flavescens*, *N. subflava*, and *N. sicca*; and the third subgroup is comprised of strains of *N. mucosa*. The results of 16S rRNA sequence analysis and DNA hybridization (Rossau et al. 1989; Dewhirst et al. 1989) suggest that the genera *Eikenella*, *Simonsiella*, and *Alysiella* and the CDC groups EF-4, M-5, and M-6 also be included in this family. Strains belonging to CDC group M-5 have been classified as *Neisseria weaverii* (Holmes et al. 1993), whereas strains belonging to CDC group M-6 have been classified as *Neisseria elongata* subspecies *nitroreducens* (Grant et al. 1990). Similar 16S rRNA analysis has also led to the recommendation that the genera *Acinetobacter*, *Moraxella,* and *Branhamella*, as well as the 'false Neisseriae' and *Kingella indologenes*, be removed from the family Neisseriaceae (Rossau et al. 1989; Dewhirst et al. 1989). Proposals have been made to assign *Branhamella catarrhalis* (formerly *Neisseria catarrhalis*) to the genus *Moraxella* in the family Moraxellaceae or to its own genus, *Branhamella*, in the family Branhamaceae (Catlin 1990).

As an alternative to phenotypic typing and 16S rRNA sequence homology, nucleic acid-based analysis has been useful in defining *Neisseria* spp. The three-subgroup organization defined by 16S rRNA organization has been further expanded to five phylogenetic groups based on the pattern of nucleotide sequence variation of the *recA*, *argF*, and *rho* genes, with the pathogenic *Neisseria* spp. associated with group 1 (Smith et al. 1999). Analysis of these data indicates that the human commensal *Neisseria* spp. can be separated into discrete groups of related species, but that their genetic relationships are distorted by interspecies recombination events facilitated by natural transformation.

Habitat

N. gonorrhoeae and *N. meningitidis* are obligate human pathogens. The majority of the human-associated species of *Neisseria* are nonpathogenic and are normal inhabitants of the upper respiratory tract (oro- and nasopharyngeal mucous membranes) (Knapp and Hook 1988). However, these commensal species may be infrequently associated with other mucosal sites and may occasionally be implicated as the etiologic agent of severe infections, including meningitis, osteomyelitis, endocarditis, cellulitis, arthritis, and pneumonia (Doern and Gantz 1982; Doern et al. 1982; Herbert and Ruskin 1981; Davis et al. 1983; Johnson 1983; Boyce et al. 1985; Gilrane et al. 1985; Wong and Janda 1992). Patients with various immune deficiencies, such as leukemia and acquired immunodeficiency syndrome (AIDS), are at increased risk for serious infections with the commensal *Neisseria* spp. (Schifman and Ryan 1983).

Children and adults may be colonized simultaneously by several *Neisseria* spp. or biovars. Colonization by nonpathogenic *Neisseria* spp., as well as *N. meningitidis*,

Table 1 *Differential characteristics of* Neisseria *species of human origin*

Species	Growth on:			Acid from:					Reduction of		Polysaccharide from sucrose	Catalase	DNAse	Pigment
	MTM, ML, and NYC media (35°C)	Chocolate or blood agar (22°C)	Nutrient agar (35°C)	Glucose	Maltose	Lactose	Sucrose	Fructose	NO_3^-	NO_2^-				
Human species														
N. gonorrhoeae	+	–	–	+	–	–	–	–	–	–	–	+	–	–
N. meningitidis	+	–	V	+	+	–	–	–	–	V	–	+	–	–
N. gonorrhoeae subsp. *kochii*	–	+	+	+	–	–	–	–	–	–	–	+	–	–
N. lactamica	+	V	+	+	+	+	–	–	–	V	–	+	–	–
N. cinerea[a]	V	–	+	–	–	–	–	–	–	+	–	+	–	–
N. polysaccharea	V	+	+	+	+	–	–	–	–	+	–	+	–	–
N. subflava	V	+	+	+	+	–	V	V	–	+	V	+	–	+
N. siccca	–	+	+	+	+	–	+	+	–	–	+	+	–	V
N. mucosa	–	+	+	+	+	–	+	+	+	+	+	+	–	+
N. flavescens	–	+	+	–	–	–	–	–	–	–	+	+	–	+
N. elongata subsp. *elongata*	–	+	+	–	–	–	–	–	–	+	–	–	–	+
N. elongata subsp. *glycolytica*	–	+	+	+	–	–	–	–	–	+	–	+	–	+
N. elongata subsp. *nitroreducens* (formerly CDC group M-6)	–	+	+	W+	–	–	–	–	+	+	–	–	–	+

+, Strains typically positive, but genetic mutants may be negative; –, most strains negative; MTM, modified Thayer–Martin medium; ML, Martin–Lewis medium; NYC, New York City medium; V, strain-dependent; W+, weakly positive. All species contain cytochrome oxidase.

a) Some strains grow on selective media even though they are colistin-susceptible.

may persist for several months (Knapp and Hook 1988). *N. lactamica* has been isolated more frequently from children than adults (Gold et al. 1978; Blakebrough et al. 1982), whereas colonization of the oropharynx by *N. polysaccharea* appears to occur infrequently and varies geographically (Riou et al. 1983; Boquete et al. 1986). *Neissera* spp. have been isolated from the oropharynx of animal species in addition to humans (Berger 1960, 1971; Andersen et al. 1993; Vedros et al. 1973, 1983; Dent 1982; Barrett et al. 1994).

Morphology, ultrastructure, and cell division

Neisseria spp. are gram-negative diplococci that vary in the ease with which they are decolorized by alcohol during Gram staining. Their cell morphology and arrangement are affected by the environmental conditions in which they are propagated and may vary between cells grown in vitro and those directly observed in specimens from an infected host. Observation of thin sections prepared from *N. gonorrhoeae* grown in vitro by electron microscopy exhibit cell structures that are similar to those of other gram-negative bacteria. Figure 2 summarizes the molecular organization of a typical *Neisseria* spp., which is largely a function of the biology by which these organisms divide. An undulating outer membrane, approximately 7.5–8.5 nm in thickness, appears as a bilayered structure (Fitz-James 1964; Swanson et al. 1971; Wegener et al. 1977) and contains lipo-oligosaccharide (LOS), proteins, and phospholipids (Morse and Hebeler 1978). The periplasmic space, the region between the cytoplasmic membrane and the outer membrane, comprises approximately 25 percent of the cell volume and contains proteins that are released upon osmotic shock (Chen et al. 1993) or by treatment with chloroform (Judd and Porcella 1993). Among the periplasmic proteins that have been identified are an iron-binding protein (Chen et al. 1993), a c-type cytochrome, and a homolog of elongation factor Tu (Judd and Porcella 1993). An electron-dense layer, approximately 6.0 nm in thickness, corresponding to the peptidoglycan layer of the bacterial cell envelope, is located within the periplasmic space (Swanson et al. 1971). Peptidoglycan isolated from *N. gonorrhoeae* grown at neutral pH comprises 1–2 percent of the cellular dry weight and has a composition similar to that of *Escherichia coli* (Hebeler and Young 1976). The peptidoglycan layer and outer membrane demonstrate adherent regions at regular intervals around the periphery of the cell (Fitz-James 1964; Wolf-Watz et al. 1975). Electron microscopy has also revealed the presence of membrane-bound vesicles in the cytoplasm of some cells located proximal to the site of septum formation, which resemble mesosomes (Fitz-James 1964; Murray et al. 1963).

It is likely that cell division is similar in all *Neisseria* spp., with the possible exception of *N. elongata* and *N. weaveri*. Cell division occurs by septation rather than by constriction (Westling-Haggstrom et al. 1977) and is initiated by an ingrowth of the cytoplasmic membrane enclosing a fold of peptidoglycan (Figure 3). Division planes are formed consecutively at right angles to each other, resulting in a transient tetrad formation (Murray et al. 1963; Catlin 1975; Westling-Haggstrom et al. 1977). Individual cells expand in only one dimension throughout most of the cell cycle (Westling-Haggstrom et al. 1977). Toward the end of the division period, initiation of growth in the second dimension typically begins before completion of the first septum. This change in growth direction enables these organisms to avoid a rod–sphere–rod transition and the large fluctuations in internal pressure that would occur if growth were unidirectional (Westling-Haggstrom et al. 1977). Subinhibitory concentrations of penicillin G, which induces filament formation in gram-negative rods and

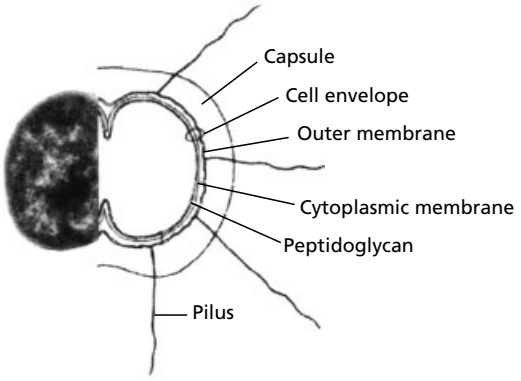

Figure 2 *Diagrammatic and electron-micrographic representation of* Neisseria *spp. To the left is a thin-section transmission electron micrograph showing the morphology of the cell membrane. The right-hand drawing is developed from the electron micrograph and other data.*

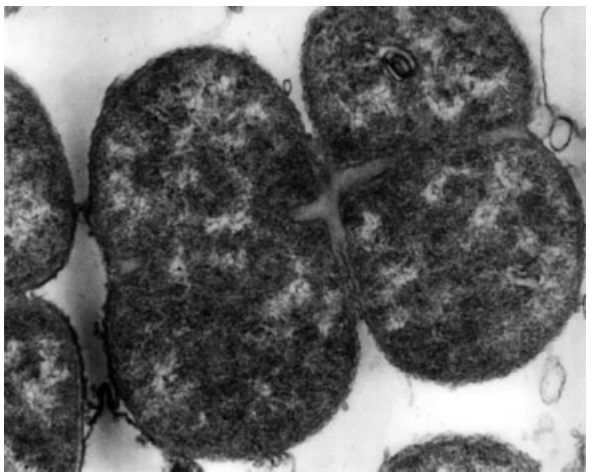

Figure 3 *Electron micrograph of* Neisseria *spp. undergoing cell division*

has been used to differentiate coccobacilli from cocci, does not induce filament formation in *N. gonorrhoeae* (Catlin 1975). However, subinhibitory concentrations of penicillin will induce filament formation in *N. elongata*, suggesting that cells of this species grow in a single dimension. A transmission electron micrograph of *Neisseria* spp. undergoing cell division is shown in Figure 3.

Genetic analysis of the gonococcal genome has identified a cluster of genes involved in cell division and cell-wall (dcw) biosynthesis. The gonococcal *dcw* gene cluster consists of 17 genes and is similar to that observed in gram-negative rods, such as *E. coli* and *Haemophilus influenzae*, and includes a protein encoded by *ftsZ*, which plays a central role in the cell division process by localizing to the division site, forming a contractile ring (Salimnia et al. 2000). Furthermore, it is known that division at the mid-cell in *E. coli* is maintained by a set of proteins designated MinC, MinD, and MinE, encoded by the *minB* genetic locus. It has been reported that *Neisseria* spp. possess MinC, MinD, and MinE homologs produced as part of a large 17-kb gene cluster (Ramirez-Arcos et al. 2001). Deletion of *minC* in *N. gonorrhoeae* led to abnormal cell division and cell lysis. The gonococcal *minC* gene can complement a *minC* mutant of *E. coli*; overexpression of gonococcal MinC causes filamentation in wild-type *E. coli* because it is a cell-division inhibitor (Ramirez-Arcos et al. 2001).

Cell-wall blebs produced by budding of the outer membrane have been observed both in lag-phase broth- or agar-grown cultures of *N. gonorrhoeae* and *N. meningitidis*. These blebs consist of outer-membrane components including LOS and proteins; they are produced by rapidly growing cells (Andersen et al. 1979; Andersen and Solberg 1978, 1988; Pettit and Judd 1992a,b). Blebs produced by *N. gonorrhoeae* contain both circular and linear DNA and may facilitate the intercellular transfer of plasmid or chromosomal DNA (Dorward et al. 1989).

Growth requirements

The natural habitat of the *Neisseria* spp. suggests that some species may have complex growth requirements. Many of these requirements are fulfilled by standard laboratory media. For example, *Neisseria* spp. other than *N. gonorrhoeae* and *N. meningitidis* will grow on nutrient agar at 35–37°C. Many of these nonpathogenic species will also grow in a chemically defined medium containing as few as five amino acids and two vitamins, although some require the addition of glucose and lactate, or both glucose and lactate for optimal growth (McDonald and Johnson 1975). Some species produce large amounts of ammonia during growth in these media, which will interfere with the detection of acid production from sugars like glucose. Consequently, the utilization of glucose by some *Neisseria* spp. may not correlate with results of tests that measure acid production (Table 1) (McDonald and Johnson 1975).

As a group, strains of *N. meningitidis* are relatively homogeneous and biosynthetically competent (Catlin 1973); most strains can grow on a relatively simple agar medium containing mineral salts, lactate and five amino acids. Cystine is required by <10 percent of strains (Catlin 1973). *N. gonorrhoeae* is more fastidious and will not grow in the absence of an energy source, such as glucose, pyruvate, or lactate (Morse 1978). All gonococcal strains require cystine for growth; glutamine and cocarboxylase are required by a significant proportion of strains upon primary isolation. For these reasons, a growth factor supplement containing these nutrients is added to media used for the primary isolation of *N. gonorrhoeae*. Many strains of *N. gonorrhoeae* have additional genetic defects in the pathways used for the biosynthesis of amino acids, purines, pyrimidines, and vitamins. This results in specific growth factor requirements and reinforces the need to use complex media for primary isolation of this microorganism. The use of growth factor requirements to differentiate between gonococcal strains, i.e. auxotyping, is a useful tool for epidemiological studies (Catlin 1973; Carifo and Catlin 1973). The development of defined media for growth of *N. gonorrhoeae* (Catlin 1973; Morse and Bartenstein 1980; La Scolea and Young 1974) has facilitated studies on gonococcal genetics and has helped to elucidate biosynthetic pathways.

Metabolism

N. gonorrhoeae and *N. meningitidis* dissimilate glucose via the Entner–Doudoroff (ED) pathway, with acetate and CO_2 as the primary end products (Morse et al. 1974; Holten 1975). With the exceptions of *N. cinerea* and *N. elongata* subsp. *elongata*, which lack 6-phosphogluconate dehydrase (Holten 1974), other *Neisseria* spp. also dissimilate glucose via the ED pathway (Holten 1975). Fermentative pathways like the Embden-Meyerhoff pathway do not appear to function in the dissimilation of glucose by these microorganisms (Morse et al. 1974). The lack of fermentative capability may explain why *Neisseria* spp. are aerobic and little or no growth occurs under strictly anaerobic conditions in the absence of an alternative terminal electron acceptor. Nevertheless, gonococci will remain viable for extended periods under anaerobic conditions (Short et al. 1982), and growth will occur when subtoxic concentrations of the alternative electron acceptor potassium nitrite are present (Knapp and Clark 1984). Nitrite, which is reduced by the constitutive enzyme nitrite reductase, may enable gonococci to survive within anaerobic environments in the host. Amino acids are utilized as an energy source via the tricarboxylic acid (TCA) cycle by *Neisseria* spp. capable of growth in a chemically defined medium without

glucose, pyruvate, or lactate. *N. gonorrhoeae*, which also has a functional TCA cycle (Hebeler and Morse 1976), is unable to grow in medium containing amino acids as the sole energy source. A high relative humidity (ca. 50 percent) is beneficial to the growth of all *Neisseria* spp. (Vedros 1984). All species of *Neisseria* (except the 'false *Neisseria*') contain carbonic anhydrase. However, increased concentrations of carbon dioxide (5–10 percent) are required for the growth of *N. gonorrhoeae* and will enhance the growth of *N. meningitidis* on solid medium (Vedros 1984). Bicarbonate can replace the requirement for carbon dioxide (Talley and Baugh 1975). The carbon dioxide (Platt 1976) and bicarbonate ions (Talley and Baugh 1975; Earl et al. 1976) stimulate gonococcal growth by reducing the lag phase. Incorporation studies using ^{14}C-labeled bicarbonate revealed that the majority of the bicarbonate was used for nucleic acid and protein biosynthesis during the lag phase (Morse 1976). This would be expected since the requirement for carbon dioxide can be met in part by the addition of uracil (Griffin and Racker 1956) and oxaloacetate (Griffin and Reider 1957) to the medium. The former is a direct precursor of RNA, whereas the latter can be used as a precursor of pyrimidines or proteins. Phosphoenolpyruvate carboxylase activity, which would be needed for the incorporation of bicarbonate, has been described in *N. gonorrhoeae* (Cox and Baugh 1977).

The optimum growth temperature of *Neisseria* spp. is 36–38.5°C, with a range of 22–40°C (Morse 1976; Vedros 1984). It has been reported that *N. gonorrhoeae* and *N. meningitidis* grow poorly or not at all below 30°C (Vedros 1984). However, several reports have suggested that some strains of *N. gonorrhoeae* may grow on solid or in liquid medium at temperatures as low as 25–28°C (Morse 1976; Annear and Grubb 1982). pH has a marked effect upon the metabolism and cell composition of *N. gonorrhoeae* (Morse and Hebeler 1978; Hebeler et al. 1979; Pettit et al. 1996, 1999). *N. gonorrhoeae* will grow in a glucose-containing medium over a pH range of 6.0–8.0 (Morse and Hebeler 1978), with the shortest generation time between pH 7.0 and 7.5. When gonococci are grown in continuous culture at constant pH values ranging from 6.3 to 7.2, the highest cell yields at all dilution rates are obtained at pH 6.75 (Brookes and Sikyta 1967).

GENOMICS

Organization of the neisserial chromosome

The genomes of *N. meningiditis* strains MC58 (group B) (Tettelin et al. 2000) and Z2491 (group A) (Parkhill et al. 2000) and the genome of the gonococcal strain FA1090 (unpublished) have been completely sequenced. As β-proteobacteria, *Neisseria* spp. are separated by over 100 million years of evolution from their next closest relatives, the γ-proteobacteria, of which *H. influenzae* and *E. coli* are examples. This is reflected by differences in size and chromosomal organization. *E. coli* has a genome size of greater than four megabases, whereas *N. meningitidis* and *N. gonorrhoeae* have genomes on the order of 2.2 megabases.

The repertoire of genes encoded by *N. gonorrhoeae* and *N. meningitidis* is highly homologous, identically expressing greater than 90 percent of their genes (Jordan et al. 2002). It is estimated that about 1 607 potential proteins are expressed by *N. meningitidis*. Of these, 281 are predicted to be important for information storage and processing, 306 for cellular processes, and 426 for metabolism; the remaining 615 are poorly characterized. The genomic and proteomic differences between the gonococcus and meningococcus are likely responsible for their differences in habitat and pathophysiology.

Genome analysis of *N. meningitidis* strain Z2491 indicates that this organism encodes a complete set of enzymes for glycolysis (apart from fruK and pfkA), gluconeogenesis, the pentose-phosphate and ED pathways, the pyruvate dehydrogenase complex, and the TCA cycle. In addition to the aerobic respiration genes, there are also both nitrite and nitrate reductases (Parkhill et al. 2000). *N. meningitidis* appears to be capable of de novo synthesis of most of the amino acids (with the exception of asparagine and methionine) and purine and pyrimidine nucleotides. However, the pathways for production of folic acid, molybdopterin, pantothenate, and pyridoxine are incomplete. This is in good agreement with the experimental data described under the sections on Growth requirements and Metabolism. A similar analysis of the gonococcal genome awaits completion.

In spite of their small genome size compared with that of other bacterial species, numerous repetitive elements are present in the *Neisseria* chromosome, ranging from short repeated sequences (10 bp to about 200 bp in length) to insertion elements (around 1 kb), to large gene duplications and prophage sequences (2.5–40 kb). Some of these repeats correspond to previously described sequences, such as the DNA uptake sequences important for the natural transformability of *Neisseria* spp. Most of these repeats are Correia sequences (discussed below) or inverted repeats flanking important virulence-associated genes. The relevance of these large repeated sequences is not understood at present, but they undoubtedly are involved in horizontal gene exchange.

The ability *N. gonorrhoeae* and *N. meningitidis* to exhibit antigenic diversity was well known prior to the sequencing of their genomes. One mechanism by which these organisms accomplish this is by phase variation, which can give rise to a large repertoire of phenotypes and facilitate survival in a changing environment (Saunders et al. 2000). Phase variation is generally associated

with reversible changes within DNA sequence motifs of fewer than ten base pairs. These repeat motifs are located either within open reading frames (ORF) or within promoter regions. Repeat sequences can be deleted or inserted by a process called 'slipped-strand synthesis.' Deletion or insertion of the repeat sequence located in the ORF alters the translational reading frame, often putting it out of frame and resulting in a nonsense protein (van Belkum et al. 1999; Belland et al. 1989; Stern and Meyer 1987). Genome sequence analysis has identified over 100 genes containing these repeats, suggesting that about 16 percent of neisserial genes are subject to phase variation. Alternatively, changes in the length of repeats located within promoters alters the relative position of promoter components and can thereby influence transcriptional levels of a given protein.

In addition to phase variation, horizontal gene transfer provides another mechanism for generating diversity in *N. gonorrhoeae* and *N. meningitidis*. Transfer of genetic information between *Neisseria* spp. occurs by transformation, conjugation, and, less frequently, transduction (Vogel and Frosch 2002). As a consequence of selective pressures in the human host, genetic exchange can confer advantages such as antibiotic resistance and antigenic mosaisism. This is particularly true for *N. gonorrhoeae* and *N. meningitidis*. The recognized mechanisms of genetic exchange are described below.

Transformation

Transformation in *Neisseria* was first described more than 40 years ago (Catlin and Cunningham 1961), when it was demonstrated that nearly all *Neisseria* species are transformable by chromosomal DNA from homologous (i.e. from the same species) and heterologous (i.e. from closely related species) sources. *Neisseria* spp. are unique among bacteria in being naturally competent for genetic transformation throughout their entire growth cycle (Biswas et al. 1977). The transformation frequency is much higher for piliated (Pil^+) gonococci than for nonpiliated (Pil^-) gonococci (Sparling 1966). Their autolytic nature facilitates the release of DNA into the local environment. An interesting hypothesis has been advanced that uptake of naked DNA may serve as a nutritional source (Finkel and Kolter 2001), suggesting that transformation and metabolism may be linked more closely than thought previously.

The specificity of transformation for homologous DNA is due to the presence of conserved ten-base-pair 'uptake sequences,' which are distributed throughout the neisserial genome (Kroll et al. 1998; Elkins et al. 1991). Exchange of DNA by transformation between different species of *Neisseria* occurs in vivo (Cannon and Sparling 1984), albeit at a much reduced frequency when compared with homologous transformation, most likely because this heterologous DNA lacks the specific uptake sequence. Homologous transformation involves binding of double-stranded DNA to the bacterial cell surface followed by entry into a compartment, where it becomes resistant to exogenous nuclease. By a mechanism that is not completely understood, pilin (encoded for by *pil*E), and two loci referred to as *pil*T and *com*P are critical for the transport of DNA across the outer and cytoplasmic membrane (Koomey 1998; Aas et al. 2002). One strand of the DNA enters the cytoplasm, while the other strand is degraded (Finkel and Kolter 2001). Finally, after a recombination event, the new DNA is integrated into the chromosome, requiring RecA activity and homologous sequences in the recipient DNA for incorporation of the DNA into the chromosome.

Conjugation

Conjugal transfer of DNA, which occurs in *N. gonorrhoeae* and *N. meningitidis*, is different from that in *E. coli* because a plasmid-encoded pilus does not appear to be required. Measurable transfer of episomal DNA occurs only when cells are on a solid surface. However, one report suggests that certain strains of gonococci may contain a genetic island containing genes that confer serum resistance, the production of a cytotoxin, and transfer factor genes homologous to the *E. coli* F-factor (Dillard and Seifert 2001). Two gonococcal conjugal plasmids of 24.5 and 25.2 mDa mediate the transfer of resistance (R)-plasmids to both pathogenic and nonpathogenic *Neisseria* spp., *E. coli*, and *H. influenzae* (Biswas et al. 1980). Although these conjugal plasmids have been found to function transiently in *E. coli*, they are not stably maintained in any species other than *N. gonorrhoeae*, *N. meningitidis*, or *N. cinerea* (Genco et al. 1984). The mobilization of R-plasmids is often accompanied by transfer of the conjugal plasmids. However, no evidence of stable cointegrate formation has been found. Several of the R-plasmids can be mobilized by *E. coli* conjugal plasmids and transferred to other *E. coli* strains, *Salmonella minnesota*, and *H. influenzae* (Guiney and Ito 1982), suggesting that *E. coli* and related species may play a role in the dissemination of the R-plasmids to pathogenic *Neisseria* spp.

Small repetitive elements and transposons

Small repetitive elements (SRE), often called 'Correia elements,' are sequences of unknown function, and are characteristic of neisserial genomes (Correia et al. 1986, 1988). The 156-bp Correia elements fall into four major classes distinguished by a 50-bp internal deletion and five point mutations (Buisine et al. 2002). The elements resemble a transposon with 25-bp inverted repeats and a TA duplication at the target site. Within the element

there is a functional integration host factor binding site. The genomic distribution of Correia elements is essentially random except for some small 'Correia-less' regions apparently derived by horizontal exchange. Correia elements have a high potential for creating functional promoters because there is a perfect TATA box at each end of the element. The distribution and number of Correia elements in the genomes of *N. gonorrhoeae* and *N. meningitidis* suggests that these elements were acquired before the divergence of these species.

While transposable elements have become valuable tools for molecular genetic analysis and manipulation, no naturally occurring transposons have been described for *Neisseria* spp. Investigators have exploited several exogenous transposons for use in *N. gonorrhoeae* and *N. meningitidis*, including Tn916 and derivatives of Tn1545 (Kathariou et al. 1990; Nassif et al. 1991). However, direct transposon mutagenesis with Tn916 is not highly efficient in *N. meningitidis*; nor has it been developed for use in *N. gonorrhoeae*. Although several interesting *N. meningitidis* mutants have been isolated, including strains altered in capsular polysaccharide and LPS and auxotrophic mutants (Stephens et al. 1994), mutagenesis with Tn916 has been difficult. Several shuttle mutagenesis systems have also been described for targeting and modification of distinct chromosomal genes (Seifert and So 1991; Haas et al. 1993). A mini-Tn system based on Tnl721 transposition functions appears to be particularly useful for genetic studies in the pathogenic *Neisseria* spp.

Plasmid-mediated antimicrobial resistance

The increased proportion of antimicrobial-resistant bacterial strains is a challenge to the management of infectious diseases. The inappropriate use of broad spectrum antibiotics, combined with self-administration, has provided the selective pressure for the emergence of antimicrobial-resistant bacteria and has contributed to the appearance and dissemination of antimicrobial-resistance encoding plasmids.

The discovery of penicillinase-producing *N. gonorrhoeae* (PPNG) in 1976 (Ashford et al. 1976) foreshadowed the demise of penicillin as effective therapy for the treatment of gonorrhea and ensured that future control of this infection would require the use of more costly treatment regimens. To date, six gonococcal β-lactamase plasmids have been described (Table 2).

The origin of these β-lactamase plasmids is uncertain, but the β-lactamase gene itself and some of its surrounding DNA are homologous with the ampicillin transposon TnA (Roberts and Falkow 1978). The β-lactamase encoded by TnA is a TEM1-type enzyme. The left-hand 60 percent of the transposon, including the structural gene for the transposase, has been lost. It is thought that the right-hand 40 percent of TnA found in *N. gonorrhoeae* may have originated in the Enterobacteriaceae. The non-TnA sequences of the gonococcal β-lactamase plasmids appear to be deletion derivatives of *Haemophilus* spp. plasmids with which they share an identical origin of transfer (OriT). β-Lactamase plasmids of *N. gonorrhoeae* are not autotransmissible but may be mobilized between strains by either the 24.5-mDa conjugative plasmid or the 25.2-mDa TetM-containing conjugative plasmid (Genco and Clark 1988). A majority of *N. gonorrhoeae* isolates also contain a 2.6-mDa cryptic plasmid, the function of which is not understood.

Table 2 *Plasmids that have been described for* Neisseria gonorrhoeae

Designation	Plasmid size (mDa)	Conjugative	Resistance determinant
African	3.2	–	β-Lactamase
Asian	4.4	–	β-Lactamase
Rio	2.9	–	β-Lactamase
Toronto	3.1	–	β-Lactamase
Nimes	4.0	–	β-Lactamase
New Zealand	6.0	–	β-Lactamase
Dutch	25.2	+	TetM
American	25.2	+	TetM
Conjugative	24.5	+	None
Cryptic	2.6	–	None

N. gonorrhoeae strains have been isolated in the USA with plasmid-mediated high-level resistance to tetracycline (minimum inhibitory concentration (MIC) 16–64 μg/ml) (Knapp et al. 1988). Tetracycline resistance in these strains is associated with the presence of the 25.2-mDa conjugative TetM cointegrate plasmid. The *tetM* gene is a determinant that was described originally as part of the streptococcal conjugative transposons Tn916 and Tnl545 (Burdett et al. 1982). How the *tetM* gene became associated with the gonococcal conjugal plasmid is unknown. Tetracycline-resistant *N. gonorrhoeae* isolates can transfer the 25.2-mDa plasmid to *N. meningitidis*, commensal *Neisseria* spp., and *Haemophilus* spp. Studies have also indicated that in the presence of the 25.2-mDa and β-lactamase plasmids, *N. meningitidis* and commensal *Neisseria* spp. have the potential to act as donors to further disseminate both plasmids. This may increase the acquisition and spread of the β-lactamase plasmids in *N. meningitidis*, which could have serious consequences, since penicillins are often used to treat these infections. To date, plasmids are found at various frequencies in different strain collections of *N. meningitidis*, and their presence is sometimes correlated with β-lactamase-mediated resistance to penicillin. Sequence analysis has shown that meningococcal plasmids encoding β-lactamase activity are virtually identical to those described for the gonococcus (Backman et al. 2000).

Transduction

Transduction is not considered a major genetic mechanism among *Neisseria* spp. Bacteriophages active against nonpathogenic *Neisseria* spp. have been isolated (Stone et al. 1956; Phelps 1967; Steinberg et al. 1976). These bacteriophages exhibit a narrow host range as measured by plaque formation. Lysogenic strains of *N. perflava*, *N. sicca*, and *N. flavescens* have also been reported. Genome analysis has revealed regions of similarities to Mu-like phages. These phage DNA regions are clearly mosaic with obvious sequence similarity to phage Mu interspersed among DNA segments that are unrelated to Mu (Morgan et al. 2002). Some genes located within phage DNA regions encode for surface-exposed proteins capable of eliciting bactericidal antibodies (Masignani et al. 2001). These findings support the possibility that transduction may be a rare means of genetic exchange.

ANTIGENIC STRUCTURE

The cell envelope of *N. gonorrhoeae* and *N. meningitidis*, like that of other gram-negative bacterial species, consists of a cytoplasmic membrane separated from the outer membrane by the periplasmic space. The external leaflet of the outer membrane is strategically arrayed at the interface between the host and microbe, such that molecules associated with this surface (1) implement the host pathology or (2) represent targets for a host immune response. This external leaflet is comprised of a complex mixture of phospholipids, lipooligosaccharides, and proteins, as shown for the gonococcus in Figure 4.

However, the neisserial outer membrane differs from that of *E. coli* in at least two important ways. First, *Neisseria* spp. appear to lack a functional analog of Braun lipoprotein, which serves to anchor the *E. coli* outer-membrane to the relatively rigid middle layer of peptidoglycan. This may explain the spontaneous release of outer-membrane fragments or 'blebs' from viable meningococci (DeVoe and Gilchrist 1973). Since blebs can display surface-associated antigens in their native orientation, they may be capable of binding antibodies to outer-membrane antigens, thereby serving as antigenic decoys for the organism.

Pili

Pili are hair-like filamentous proteins composed of repeating peptide subunits (pilin) that extend several micrometers from the bacterial cell surface. Pili have been observed on *N. gonorrhoeae* (Jephcott et al. 1971; Swanson et al. 1971), *N. meningitidis* (DeVoe and Gilchrist 1974; McGee et al. 1977), and the nonpathogenic *Neisseria* spp. (Wistreich and Baker 1971; McGee et al. 1977). The length of each pilus filament varies;

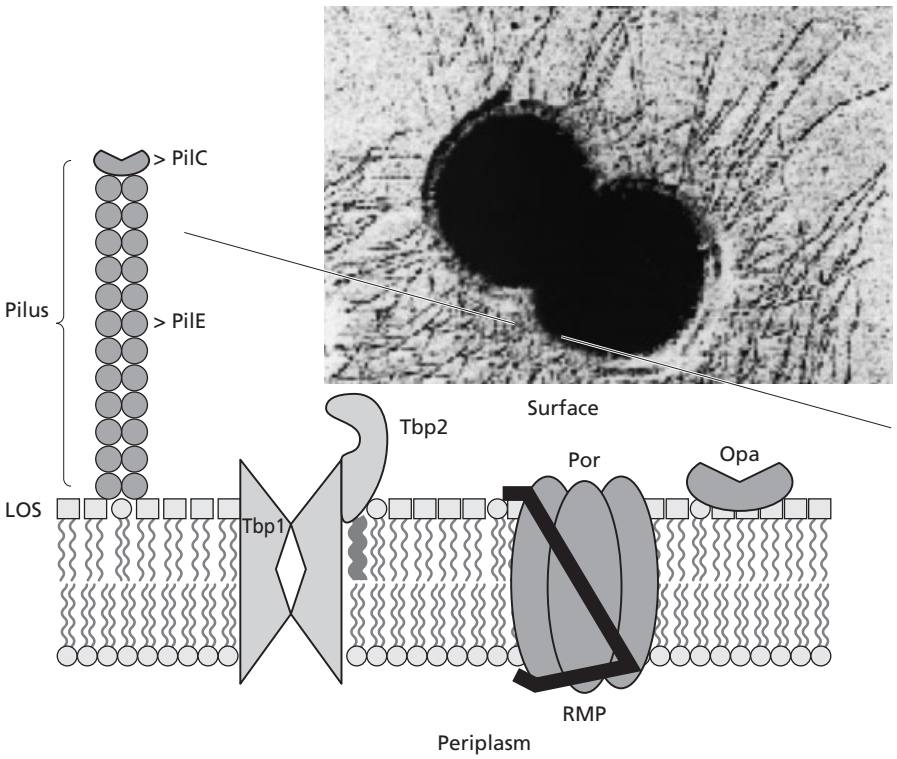

Figure 4 *Electron micrograph of a gonococcal cell producing multiple pili. The corresponding diagram depicts several of the surface-associated gonococcal antigens described in the text.*

short pili (175–210 nm in length) have been observed only on nonpathogenic *Neisseria* spp. (Wistreich and Baker 1971), whereas long pili (up to 4 300 nm in length) have been observed on both pathogenic and nonpathogenic *Neisseria* spp. (McGee et al. 1977). Piliation is associated with a characteristic colonial morphology in *N. gonorrhoeae* (Jephcott et al. 1971); pili are observed only on cells from small domed highlighted colonies (types 1 and 2) and not on cells from large flat colonies (types 3 and 4) (Kellogg et al. 1963). However, there does not appear to be a similar association with *N. meningitidis* or the nonpathogenic *Neisseria* spp. (Jephcott et al. 1971). Because of the association of colonial morphology with piliation status of the gonococcus, much is known about the biochemistry and pathobiology of this organism pili.

In the gonococcus, and presumably other *Neisseria* spp., pili participate in a number of different functions including DNA transfer, bacterial attachment to host cells, intrabacterial adhesion, and bacterial movement. As with many bacterial pathogens, gonococcal pili are important in pathogenesis. For example, the natural transformation of gonococci is significantly enhanced by the expression of pili (Rudel et al. 1992, 1995a, c; Cannon and Sparling 1984; Fussenegger et al. 1997). The attachment properties of gonococcal pili confer tissue tropism as well as the ability to overcome the bacterial–host cell charge repulsion (Long et al. 1998, 2001). Finally, gonococcal pili mediate twitching motility, a mode of migration along liquid–solid or air–liquid interfaces (Wall and Kaiser 1999; Merz et al. 2000; Merz and So 2000).

Evidence for the involvement of pili in gonococcal pathogenesis began with the seminal observations by Kellogg and colleagues in 1963, who reported that clinical material freshly propagated on solid media gave rise to gonococcal colonies of two morphotypes, termed T_1 and T_2 (now designated as piliated or P^+) (Kellogg et al. 1963). Upon nonspecific passage in vitro, two additional colonial forms appeared, termed T_3 and T_4 (now designated as nonpiliated or P^-). Piliated colonial morphotypes were subsequently found to be substantially more infectious for male volunteers following intraurethral challenge than nonpiliated colonial morphotypes (Kellogg et al. 1963).

Amongst bacteria, several structural classes of pili exist (e.g. type 1, Gal-Gal, etc.), which have either unique structural or receptor binding characteristics. Gonococci express a type 4 pilus defined by the ordered assembly of a principal repeating polypeptide subunit termed pilin, a polypeptide of 18–22 kDa (ca. 150 residues) encoded by the *pil*E locus (Parge et al. 1995). The pilin protein of type 4 pili is synthesized as a precursor protein with a unique secretory signal sequence shared by many type II secretion systems (Sandkvist et al. 1997). During processing, the pilin precursor is modified by the PilD prepilin peptidase/transmethylase (Lory and Strom 1997). The result is a mature pilin subunit with a methylated phenylalanine residue at its N-terminus (N-met-Phe). This maturation is coupled with transport and assembly. A protein designated PilQ has been described as one of many protein products associated with pilus biogenesis and function (Collins et al. 2001; Tonjum and Koomey 1997). This protein was previously described as a high-molecular-weight common outer-membrane antigen and designated as OMP-MC (Tsai et al. 1989). PilQ is surface-exposed and common to diverse gonococcal isolates. It is thought to function by forming a multimeric channel in the outer membrane through which the assembled pilus fiber is presented. This process results in the aggregation of thousands of pilin molecules in an extended linear structure, which can be readily viewed in negatively stained transmission electron micrographs of gonococci propagated on agar medium (Figure 4).

The atomic structure of gonococcal pilin can explain much of the serologic and biologic data previously reported in the literature (Parge et al. 1995; Forest et al. 1996). The pilin monomer is organized as an N-terminal hydrophobic tail of approximately 60 residues that folds into an unusually long α-helix followed by a C-terminal globular head region of approximately 90 residues. The amino terminal helical region packs as a 'stalk' presenting an antigenically variable C-terminal 'flower' on the surface of this oligomer. The C-terminal pilin domain can be phosphorylated and glycosylated, further increasing the antigenic diversity of this oligomeric structure. The *pilE* gene of *N. gonorrhoeae* and *N. meningitidis* exhibits 'phase variation.' Switching from the P^+ to the P^- state occurs at rates as high as 10^{-3} per cell generation. Reversion to the piliated state also occurs, although at slower rates, varying from 10^{-6} to 10^{-8} reversions per generation (Cannon and Sparling 1984). The control of phase variation is complex and appears to involve several mechanisms. Deletions and frameshift mutations result in the production of truncated pilin molecules and a stable nonpiliated phenotype.

Gonococcal pili were originally thought to exist as a homopolymer. However, it is now accepted that a 110-kDa protein (PilC) is associated with the pilus structure (Rudel et al. 1992, 1995a, b, c). The gene for this protein, designated *pilC*, can exist within a single gonococcal strain as two distinct loci (*pilC1* and *pilC2*) (Jonsson et al. 1995; Mellies et al. 1997). It is unclear whether PilC functions as an adhesin oriented at the pili terminus, distal to the gonococcal cell surface, or whether PilC functions in the assembly, retraction (twitching motility), or genetic competence (transformation) associated with piliated gonococci (Merz and So 2000; Merz et al. 2000). Reversible phase variation can result from changes in the signal peptide-coding region of *pilC* (Jonsson et al. 1992, 1994, 1995).

Another mechanism of diversity is antigenic variation. *N. gonorrhoeae* and *N. meningitidis* contain at least one

copy of the expressed major pilus subunit gene, *pilE*, and multiple copies of *pilS*, which are transcriptionally silent, incomplete pilin loci carrying variant sequences encoding the C-terminal 90 residue 'flower' of mature pilin (Meyer et al. 1984; Haas and Meyer 1986). These *pilS* loci consist of the semi- and hypervariable regions that give rise to the antigenic diversity of pili. Antigenic variation results from recombination events between silent and expressed loci, which result in the formation of diverse pilins either by a transformation-mediated recombination with DNA released from lysed gonococci or by recombination between a silent locus and an expressed locus on the same chromosome (Seifert et al. 1988). The entire *pil*S locus, or only a portion of it may recombine with the *pil*E locus. As a result of this process, it has been proposed that the diversity of pilin variants approaches that of the B-cell repertoire of antigen-combining sites.

In contrast to gonococci, meningococci express two quite distinct classes of pili, which have been termed class 1 and class 2 pili (Table 3) (Heckels 1989). Class 1 pili closely resemble gonococcal pili, whereas class 2 pili are composed of smaller pilin subunits that share conformational determinants with class 1 and gonococcal pili (Heckels 1989). The biochemistry and pathophysiology of the meningococcal class 2 pili are not well understood.

Porins

Gonococcal and meningococcal outer membranes contain integral membrane proteins that form pores and are correspondingly referred to as porins (Leith and Morse 1980; Newhall et al. 1980; Douglas et al. 1981). Porin function is necessary for survival, allowing nutrients to pass through the outer membrane and waste products to exit. The genomes of the *N. gonorrhoeae* and *N. meningitidis* contain two porin genes, *porA* and *porB*. Unlike meningococci, which express both PorA and PorB proteins, gonococci contain mutations in the promoter region and frameshift mutations in the coding region of *porA* that effectively silence this locus (Feavers and Maiden 1998). Hence, *N. gonorrhoeae* express a single porin designated PorB. Deletion of PorB is lethal for *N. gonorrhoeae* propagated in vitro, underscoring its critical presence for gonococcal survival even in the absence of host defenses.

PorB of *N. gonorrhoeae* forms a trimeric complex in the outer membrane with the monomeric reduction-modifiable protein (RMP) (Newhall et al. 1980; Leith and Morse 1980). By one estimate, PorB comprises approximately 60 percent by weight of the total repertoire of gonococcal outer-membrane proteins (Johnston and Gotschlich 1974). The apparent molecular mass of the gonococcal PorB is between 32 and 39 kDa, depending upon the gonococcal isolate. PorB has a pore size estimated to be 2.5 nm and has been demonstrated to be an anion-selective channel essential for the diffusion of solutes with molecular masses of less than 1 kDa into the periplasmic space and diffusion of small hydrophilic molecules out of the periplasm (Douglas et al. 1981).

An examination of geographically distinct isolates of *N. gonorrhoeae* reveals that PorB can be separated into two immunologically distinct classes, referred to as PIA and PIB (Carbonetti and Sparling 1987; Gotschlich et al. 1983, 1987a). The differences in antigenicity between PIA and PIB are associated with substantial variation of these loops. More subtle variations within these loop sequences account for over 40 distinguishable serovars, defined by reactivity with PorB-specific monoclonal antibodies (Mee et al. 1993). PorB expression is stable upon passage in vitro. Unlike other gonococcal surface antigens, it is not subject to high-frequency phase or antigenic variation within a strain. Nevertheless, gonococcal PorB protein

Table 3 *Outer-membrane proteins produced by* Neisseria gonorrhoeae *and* Neisseria meningitidis

N. gonorrhoeae	*N. meningitidis*	Function
Pilin	Class I pilin	Adherence to epithelial cells and erythrocytes
–	Class II pilin	
Protein I (PIA or PIB)	Class 2 and 3	Function as porins; anion-selective
–	Class 1	Function as porins; cation-selective
Protein III (Rmp)	Class 4	Bind blocking bactericidal Ab; homology to OmpA
Protein II (Opa)	Class 5	Adherence to epithelial and phagocytic cells
PilC	PilC	Epithelial cell-specific pilus-associated adherence
Pan I	Pan 1	Anaerobically induced lipoprotein; may function in nitrate reductase system
Oxi A	–	Oxygen-induced protein; function unknown
Lip (H.8)	–	Lipoprotein; function unknown
Laz	–	Lipid-associated azurin
36 kDa adhesin	–	Glycolipid-binding adhesin

–, no gonococcal or meningococcal equivalent has been identified.

undergoes antigenic variation in vivo over time, as shown by the diversity of serovars.

PorB has been implicated in specific responses by host epithelial and phagocytic cells that are relevant to gonococcal pathogenesis (Lorenzen et al. 2000). Purified and detergent solubilized PorB can be incorporated into natural or synthetic lipid bilayers, conferring the property of voltage gating to these bilayers (Young et al. 1983). PorB can translocate from the outer membrane of viable gonococci to the plasma and mitocondrial membranes of eukaryotic cells or to artificial lipid bilayers, where voltage-dependent changes can be detected (Mauro et al. 1988). The gating properties of certain porins are modulated by nucleotide triphosphates (Rudel et al. 1996). Purified PorB interferes with degranulation by neutrophils (Haines et al. 1991), suggesting that it may play a role in 'managing' the host inflammatory response to gonococcal infection. The addition of purified PorB to human monocytes triggers a Ca^{2+} influx (Muller et al. 1999). The significance of this property is related to the fact that activation of macrophages and granulocytes is initiated by hyperpolarization of their plasma membranes (Young et al. 1983). Thus, disruption of this polarization may limit the ability of host cells to respond to and control gonococcal infection.

The ability of the gonococcus to transcytose through the epithelial cell barrier is recognized as an important property that contributes to infection (Figure 5). The contribution of the gonococcal PorB to transcytosis is suggested by experiments using isogenic gonococcal strains carrying different *por* alleles (van Putten et al. 1998a). Constructs expressing PorB associated with strains isolated from disseminated gonococcal infections conferred an increased ability to invade epithelial cells. Further, this invasive phenotype was influenced by nucleotide triphosphates or polyphosphate (van Putten et al. 1998a). The current view is that PorB functions to promote invasion in combination with other surface-associated adhesions (Merz and So 2000).

The PorB phenotype contributes to complement-mediated killing of gonococci by normal human serum. Nearly all gonococcal strains expressing a PIA PorB are serum-resistant, apparently because serum proteins that downregulate complement binding proteins (C4pb and factor H) are bound to loop 5 sequences found in PIA porins, but not in PIB porins (Ram et al. 2001a, b). PIB serum-resistant variants also bind C4pb via a conformational epitope between loops 5 and 7 (Ram et al. 2001b). Strains sensitive to serum killing fail to bind these factors but may acquire serum resistance if sialylated LOS binds factor H (Ram et al. 1999).

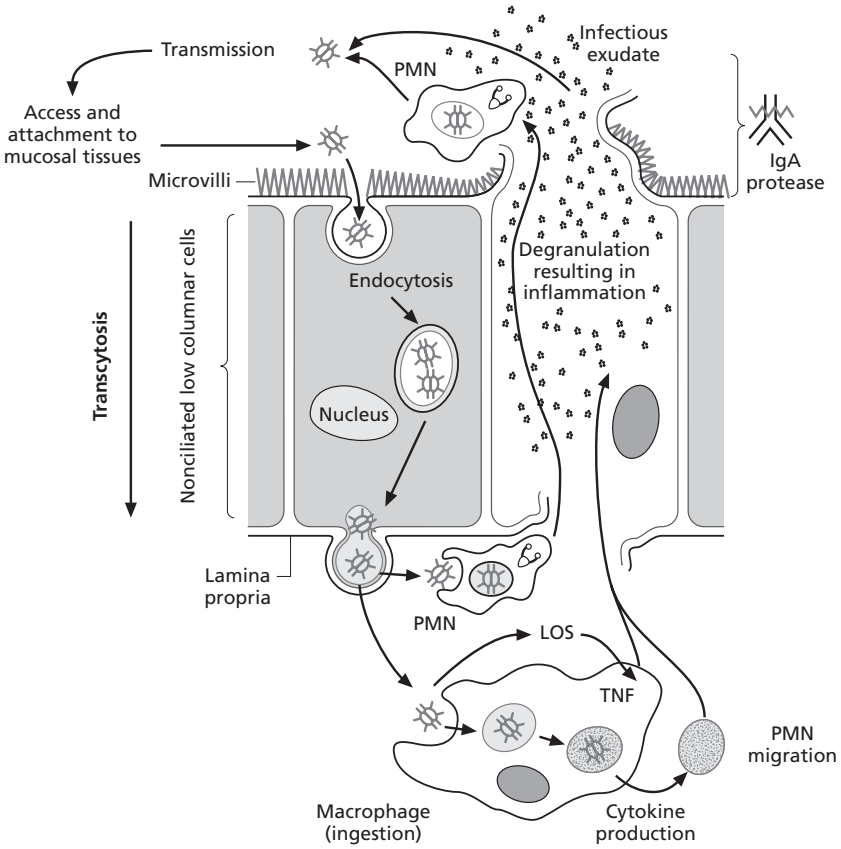

Figure 5 *Summary of the mechanism by which the gonococcus transcytoses through the epithelial cell and cause infection.*

In contrast to gonococci, *N. meningitidis* simultaneously produce two porins. Expression of a given porin is a stable trait for a given strain. However, differences that occur between strains are responsible for antigenic diversity. One porin is the class 1 protein (also known as PorA), produced by almost all meningococcal isolates, although there is considerable variation in the amount produced for any given strain (Poolman et al. 1980). PorA has been demonstrated to be cation-selective. In addition, all meningococcal isolates posses a gene that encodes for either the class 2 or class 3 proteins, which are homologs of gonococcal PIA and PIB porins (Table 3) (Blake and Gotschlich 1987; Judd 1989). Like gonococcal PIA and PIB, the meningococcal class 2 and 3 proteins form anion-selective pores (Tommassen et al. 1990) and are likely to play a similar role in pathogenesis. Antigenic differences in class 2/3 proteins are used to serotype strains of *N. meningitidis*, whereas antigenic differences in the class 1 protein are used for subserotyping (Verheul et al. 1993).

Rmp

Rmp is a highly conserved surface-exposed outer-membrane protein expressed by all pathogenic *Neisseria* spp. Its descriptive name derives from its migration properties in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Rmp has an apparent molecular mass of approximately 31 kDa under nonreducing conditions but migrates more slowly in the presence of reducing agents (Blake 1985). Historically, gonococcal Rmp was termed protein III, while the meningococcal homolog was designated as the class 4 protein (Table 3). The gonococcal and meningococcal genomes contain a single copy of this gene. Gonococcal and meningococcal mutants that do not produce Rmp are viable when cultured in vitro (Wetzler et al. 1992), suggesting that this protein is not essential for cell growth. Surface-labeling studies, susceptibility to cleavage by cathepsin G, and immunoprecipitation with a monoclonal antibody provide evidence for surface-exposed domains (Shafer and Morse 1987; Swanson et al. 1982). However, Rmp does not exhibit phase or antigen variation (Blake 1985).

Examination of the Rmp sequence reveals a sequence that is partially homologous with the carboxy-terminal portion of the OmpA proteins of *Shigella dysenteriae*, *E. coli* and *Enterobacter aerogenes* (Gotschlich et al. 1987b). Some antibodies, termed blocking antibodies, bind to surface-exposed regions of Rmp and interfere with the bactericidal activity of antibodies directed to other surface antigens such as PorB and LOS (Joiner et al. 1985; Rice et al. 1986; Rice 1989). It has been proposed that 'natural' antibodies to homologous regions of the enterobacterial OmpA proteins crossreact with Rmp and function as blocking antibodies (Plummer et al. 1993). Antibodies to Rmp correlate with an increase in susceptibility to gonococcal infection. Antibody to Rmp can be found in the serum and vaginal fluid of patients convalescing from gonorrhea, although in general Rmp is not as immunogenic as other surface-exposed gonococcal antigens (Lammel et al. 1985). Immunization of human volunteers with protein I preparations containing trace amounts of Rmp induce a similar blocking antibody response.

Opacity-associated proteins

A group of related surface-exposed proteins, historically referred to as proteins II for *N. gonorrhoeae* and class 5 proteins for *N. meningitidis* (see Table 3), are now refered to as Opas for opacity associated. The term Opa is derived from the observation that gonococcal colonies, propagated on clear typing media and viewed through a stereomicroscope using light reflected from a mirror below the stage, exhibit an opacity phenotype ranging from transparent to opaque (Bhat et al. 1992; Swanson 1978; Swanson et al. 1992). Colonies appearing transparent generally, but not always, lack Opas. However, there appears to be no relationship between colony opacity and expressions of these proteins in *N. meningitidis* (Hagman and Danielsson 1989); rather, these have been designated as Opas based on sequence homology to the gonococcal Opas. A gene that encodes a similar protein has been identified in *N. lactamica* and other nonpathogenic *Neisseria* spp. (Aho et al. 1987; Wolff and Stern 1995). The potential repertoire of Opa proteins is greater in *N. gonorrhoeae* than in *N. meningitidis*, with 12 or more genes found in the gonococcal genome and four or fewer genes found in the mengococcal genome (Meyer and van Putten 1989). No more than two loci are present in the chromosomal DNA of *N. lactamica* and *N. flava*, and only a single copy exists in the genomes of *N. mucosa*, *N. subflava* and *N. sicca* (Wolff and Stern 1995).

Opas comprise a family of surface-exposed outer-membrane proteins with apparent subunit molecular weights between 24 and 30 kDa. As a group, they are basic proteins with isoelectric points above 9.0 (Blake 1985; Blake et al. 1995) and are heat-modifiable (i.e. when heated at 37°C prior to SDS-PAGE analysis, they migrate with a higher relative mobility than when heated at 60°C). Organisms forming opaque colonies usually express one or more Opas that affect intergonococcal adhesion and give rise to the opacity properties of these colonies (Swanson 1978, 1980; King and Swanson 1978; Swanson and Barrera 1983; Swanson et al. 1988). These proteins are not presented as part of an extended appendage such as a pilus; rather, they are intimately associated with the outer membrane. Whereas pili appear to be crucial for the first contact of bacteria with host cells from rather long range, Opas are thought

to promote more intimate contact with host cells resulting in subsequent cell signaling.

The predicted amino acid sequences of Opas contain a relatively conserved 'semivariable' region near the N-terminus of the molecule and two hypervariable regions that differ markedly in amino acid sequence and length (Dempsey et al. 1991). Both hypervariable regions appear to contribute to the antigenic and functional properties of individual Opa family members.

Opa expression is variable. Different colonies isolated from a single strain may express none, one or several serologically distinct Opas simultaneously (Swanson 1978). Each of the Opa genes is complete with a functional promoter (Stern et al. 1986; Stern and Meyer 1987). All of the Opa protein genes are transcribed, although not all are translated. Opa expression is controlled at the level of translation by the presence of up to 28 identical tandem pentameric repeats (CTCTT) encoding the signal peptide (Stern et al. 1984; Stern et al. 1986). Intact Opas are produced only when the number of the repeat units places the initiator AUG codon in-frame with the mature protein coding sequence. A change in the number of repeats results from slipped-strand mispairing of the pentameric units during DNA replication and is independent of RecA activity. Fluctuation between different Opa expression patterns in vitro occurs at rates as high as one per 1 000 cell divisions (Mayer 1982). When fully expressed, the Opa content of the outer membrane nearly equals that of PorB.

Translational regulation accounts for the expression of serologically distinct Opas within a single strain. This antigenic diversity is further enhanced through recombination between *opa* genes within and among *Neisseria* spp. resulting in chimeric structural genes with different combinations of hypervariable regions (Swanson et al. 1992). Sequence analysis of gonococcal and meningococcal *opa* genes suggests that horizontal genetic exchange and recombination between *N. gonorrhoeae* and *N. meningitidis* DNA occurs in nature (Hobbs et al. 1994), as shown by the presence of at least one identical *opa* hypervariable locus in isolates of the two species.

Opa proteins also function as adhesins to facilitate gonococcal interactions with human epithelial and phagocytic cells (Blake et al. 1995; Chen et al. 1995; Belland et al. 1992; Dehio et al. 2000). Expression of certain Opa proteins favors binding of gonococci to human polymorphonuclear cells but not to human epithelial cells (Kupsch et al. 1993). A subset of Opa proteins can induce oxidative killing by human polymorphonuclear cells, whereas a different subset induces oxidative killing by human peripheral blood monocytes (Belland et al. 1992). Variability in Opa-mediated host cell binding and activation is likely a reflection of variation in host cell receptors and the intracellular pathways stimulated by specific Opa binding to these receptors.

Early studies of naturally infected patients suggested that Opa expression was strongly correlated with the clinical manifestations of gonorrhea. In women with endocervicitis, recovery of opaque colonial variants was maximum at midcycle. In men, opaque colonial variants were associated with uncomplicated urethritis, pharyngitis, and proctitis. By contrast, recovery of the transparent colonial morphology was associated with disseminated gonococcal infection, pelvic inflammatory disease, and endocervicitis during menstruation (James and Swanson 1978). Antibodies to Opa proteins can be detected in convalescent serum and vaginal fluid of patients treated for gonorrhea, indicating that these proteins are expressed and immunogenic in vivo (Zak et al. 1984). Variable Opa expression was demonstrated in an analysis of 200 variants of a single strain isolated during an outbreak of penicillin-resistant gonorrhea (Schwalbe et al. 1985). Swanson and colleagues demonstrated a transition to the opaque phenotype in vivo in males challenged by urethral inoculation with a transparent colonial variant (Swanson et al. 1988, 1992). Meningococci isolated from different anatomical sites may also differ in the expression of Opa proteins (Poolman et al. 1980; Tinsley and Heckels 1986).

In more recent studies employing the male urethral challenge model, volunteers were challenged with transparent gonococci followed by characterization of the Opa protein expression of gonococci recovered from the urine, urethra, and semen (Jerse et al. 1994). The results confirmed the strong selection for Opa proteins in vivo, which are expressed more rapidly, in greater number, and with more variation than in the inoculum variant passed in vitro. However, no single Opa protein type was preferentially expressed during early infection of the male urethra, and different subjects inoculated with the same bacterial suspension developed infections with gonococci that varied dramatically in the Opas expressed.

By using isogenic gonococci differing only in Opa expression and well-defined cell lines, two candidate host receptors have been identified. One receptor appears to be the heparan sulfate proteoglycans (HSPG) (Chen et al. 1995; Grant et al. 1999). Certain Opa proteins that contain highly cationic surface-exposed loops bind strongly to HSPG, resulting in invasion of certain epithelial cells such as conjunctival cells. This binding is markedly reduced by competition with soluble heparin, heparan sulfate, or HSPGs. In addition, the serum proteins vitronectin and fibronectin in conjuction with their integrin receptors (and other cell proteins) encourage HSPG-mediated binding (Duensing and van Putten 1997; van Putten et al. 1998b; Freissler et al. 2000). Opa-mediated binding to HSPG receptors results in stimulation of intracellular signaling pathways involving acid sphingomyelinases (Makino et al. 1991) and cytoskeletal rearrangement (Grassme et al. 1996), events that may facilitate invasion by gonococci.

A second target for many Opas is the CEACAM (formerly called CD66) family of host cell receptors. These host cell surface antigens are represented by multiple isoforms, each with a different expression pattern on various epithelial and immune cells. This differential expression and subsequent Opa recognition may account for the tissue tropism exhibited by the isogenic gonococcal Opa variants. As in the case of binding to HSPG receptors, binding to CEACAM results in activation of intracellular signaling cascades, including acid sphingomyelinases (Hauck et al. 2000), protein-tyrosine kinases (Hauck et al. 1998), and cytoskeletal rearrangement (Dehio et al. 1998; Meyer 1998, 1999). Recent studies suggest that Opa^+ gonococci binding to CEACAM1 (also called CD66a or BGP) on $CD4^+$ T cells and to CEACAM3 (CD66d or CGM1a) on B cells and neutrophils may result in inhibition of host immune responses (Boulton and Gray-Owen 2002).

Opa expression is strongly correlated with the most basic aspects of host–parasite interaction. One interpretation of studies of Opa biology is that this family of proteins and the genetic mechanisms that underlie its phase and antigenic variation are essential to the ecology of the organism, if not for virulence per se. Expression of these antigenic and phase-variable surface proteins during infection appears to be arbitrary, placing an apparent premium on antigenic and attachment diversity. Opa expression may also play a role in active immune suppression by gonococci, consistent with the familiar picture of reinfection, even by strains of *N. gonorrhoeae* with the same porin serotype (Hobbs et al. 1999).

Surface proteins involved in iron acquisition

Analysis of the cellular composition of gonococci and meningococci grown in vitro on rich complex medium may not accurately reflect the composition of these organisms in vivo. The natural environment of the human host is defined by many parameters. Notable examples include extremes in temperature, pH (Pettit et al. 1995, 1996, 1996), and growth-essential nutrients (Mietzner et al. 1998). The latter is of particular significance, since natural environments are frequently limited in one or more essential nutrients due to depletion by the host and endogenous microbial populations. Hence, nutrient insufficiency may be the most common environmental extreme to which a pathogen is exposed. Investigations have focused on cellular components expressed when these organisms are propagated under conditions that simulate those of a less-than-hospitable host. For example, using continuous culture, Morse et al. (1983) were able to modulate the structural and antigenic composition of gonococcal LOS using glucose as a rate-limiting nutrient. Other reports suggest that oxygen tension may influence the synthesis of novel proteins (Lissenden et al. 2000; Clark et al. 1988). However, most studies have been focused upon the influence of iron on the molecular composition of gonococci and meningococci (Sparling 1998; Mietzner et al. 1998; Schryvers and Stojiljkovic 1999).

Within the human host there is little free iron, most being complexed to human transferrin in serum and human lactoferrin in secretions (Welch 1992). Iron in the form of heme may be bound to proteins, such as hemoglobin, haptoglobin, or hemopexin (Mietzner et al. 1998; Schryvers and Stojiljkovic 1999). These host proteins represent the principal reservoir of growth-essential iron for pathogenic bacteria. In turn, the ability of pathogenic microorganisms to scavenge iron from their host environment and incorporate this element into proteins is a crucial requirement for the production of disease (Weinberg 1984). Many pathogenic bacteria employ soluble low-molecular-weight siderophores that compete for this iron source (Neilands et al. 1987). The inability to detect the secretion of a soluble siderophore by the gonococcus (Mietzner et al. 1998) and by the meningococcus (Archibald and DeVoe 1979) was the first indication that these organisms might employ a fundamentally different mechanism for iron acquisition. Ultimately this was resolved by Archibald and DeVoe (Archibald and DeVoe 1979), who demonstrated that iron-saturated transferrin sequestered within a dialysis bag and immersed in iron-limiting media was able to support the growth of a siderophore-producing strain of *E. coli*, but not *N. meningitidis*, suggesting that direct contact with transferrin was required for meningococcal growth but not for that of *E. coli*. Almost three decades after these original experiments were performed, it is well accepted that a human transferrin receptor-binding complex is present on the surface of pathogenic *Neisseria* (Clarke et al. 2001). The specificity of this receptor for human transferrin has been postulated to be a factor in restricting these organisms to the human host (Schryvers and Stojiljkovic 1999).

All strains of *N. gonorrhoeae* and *N. meningitidis* isolated from infected patients are able to utilize iron from human transferrin (McKenna et al. 1988). However, only subsets of gonococcal strains are able to utilize iron from human lactoferrin and hemoglobin (Mickelsen et al. 1982). Key to this process is the expression of specific receptors that bind human transferrin, human lactoferrin, or hemoglobin at the bacterial surface. One further source of iron is an outer-membrane receptor formerly referred to as FrpB (Beucher and Sparling 1995) but now designated FetA for ferric enterobactin transport (Carson et al. 1999). This receptor recognizes phenolate siderophores produced by other microorganisms. A common property of pathogenic *Neisseria* is the exploitation of endogenous host iron sources such as transferrin, lactoferrin,

hemoglobin, or phenolate siderophores, without expending metabolic energy to make a scavenging (e.g. siderophore) compound.

The neisserial lactoferrin, hemoglobin, and enterobactin receptors all demonstrate some capacity for phase variation (Schryvers and Stojiljkovic 1999), allowing organisms expressing these surface proteins to potentially escape immune responses directed at these proteins, making these poor targets for vaccine development. There may be a selective advantage to expression of the gonococcal hemoglobin receptor during infection of women in the early phases of their menstrual cycles (Anderson et al. 2001). In contrast, the transferrin receptor is expressed by all gonococci, does not demonstrate phase variation, and is required for human infection (Cornelissen et al. 1998). Expression of a functional transferrin receptor is critical to growth of gonococci in vivo, as an engineered mutation inactivating the transferrin receptor in a strain of *N. gonorrhoeae* that naturally lacks the lactoferrin receptor rendered the bacteria unable to cause experimental urethral infection in humans (Cornelissen et al. 1998). Interestingly, expression of the transferrin receptor is not required for establishing an infection in a murine vaginal model (Jerse et al. 2002).

The transferrin receptor of *N. gonorrhoeae* and *N. meningitidis* is comprised of a heterodimer transcribed from a bicistronic operon whose promoter is regulated by the iron-dependent transcriptional repressor 'Fur' (for ferric uptake repressor) (Ronpirin et al. 2001). The first gene in this operon encodes a lipoprotein (TbpB) that is tethered to the neisserial cell surface and imparts binding and recognition properties to the transferrin receptor oligomeric complex (Anderson et al. 1994; Boulton et al. 1999). The second gene in this operon (TbpA) is an integral outer-membrane protein that is thought to function as a gated porin (Masri and Cornelissen 2002). Sequence analysis of TbpA reveals homology with the TonB-dependent outer-membrane siderophore receptors FepA, FecA, IutA, FhuA, and FhuE (Clarke et al. 2001). By SDS-PAGE, the molecular masses of the gonococcal TbpA and TbpB are estimated to be approximately 100 and 85 kDa, respectively (Schryvers and Lee 1989). Similar homologues of TbpA and TbpB exist in *N. meningitidis*. Structural and antigenic diversity has been reported for TbpA (Cornelissen et al. 2000), although the variability appears to be greater for the more surface-accessible TbpB (Rokbi et al. 2000; Legrain et al. 1998).

The stoichiometry of TbpA to TbpB in the transferrin receptor complex does not appear to be fixed and may vary as a function of the degree of iron starvation of gonococci (Masri and Cornelissen 2002). Under extreme conditions, there may be as many as 20 copies of TbpB to one copy of TbpA. The gonococcal transferrin receptor preferentially binds iron-saturated human transferrin over apo-transferrin (Retzer et al. 1998). TbpA or TbpB can bind human transferrin independent of the other. However, both are required for the efficient transport of the growth-essential iron (Anderson et al. 1994; Cornelissen et al. 1993).

To accomplish its function, the neisserial transferrin receptor must be surface-exposed. It has been proposed that human transferrin bound to the gonococcal cell surface may also act as a bridging ligand to facilitate attachment and invasion of organisms (Heine et al. 1996). More recent studies of human epithelial cell cultures infected with *N. gonorrhoeae* indicate that the former have reduced levels of transferrin receptor messenger RNA and cycling of human transferrin receptors, suggesting that infection alters epithelial cell transferrin-iron homeostasis at multiple levels (Bonnah et al. 2000).

Other surface and secreted protein antigens

Pathogenic *Neisseria* spp. display a uniform stress response irrespective of the nature of the environment. A number of stress (or heat-shock) proteins have been identified. The most abundant neisserial stress protein is genetically conserved among the *Neisseria* spp. and has considerable homology to the Hsp60 heat-shock protein family (Pannekoek et al. 1992). Analysis of patient sera indicates that this antigen is expressed and immunogenic in vivo.

Another environmental factor influencing the expression of gonococcal genes is oxygen. The growth of *N. gonorrhoeae* can be accomplished in the absence of oxygen with nitrite as a terminal electron acceptor. Under these growth conditions, gonococci express several additional proteins and repress the expression of others (Keevil et al. 1986; Clark et al. 1987). The major anaerobically induced protein is a surface-exposed 54-kDa lipoprotein that is designated Panl (Hoehn and Clark 1992). Coisolation of gonococci and obligate anaerobic microorganisms from the site of infection, and the presence of antibodies against the anaerobically induced proteins in the sera of patients with neisserial disease (Clark et al. 1988), suggest that anaerobic growth of *Neisseria* spp. may occur in vivo. *N. meningitidis* also has the gene for the Panl protein. However, it is expressed at significantly lower levels when cultivated under the same conditions as the *N. gonorrhoeae*.

Recent studies suggest that ribosomal protein L12 is exposed on the surface of *N. gonorrhoeae* and is involved in invasion of cells in the female genital tract that express the lutropin receptor (Spence and Clark 2000). This surprising observation further emphasizes the complexity and redundancy of mechanisms for attachment and invasion of host cells in these remarkably versatile pathogens. Genome scanning of *N. menin-*

gitidis has identified a region with homology to the Mu phage of *E. coli*. Three of these phage-encoded genes have been reported to be surface-exposed (Masignani et al. 2001).

NadA, a novel surface antigen of *N. meningitidis*, has been described in 52 of 53 strains of hypervirulent lineages and is absent in other *Neisseria* spp. This protein, which has a predicted molecular structure strikingly similar to other bacterial adhesins such as YadA from *Yersinia*, may be important for meningococcal–host cell interactions (Comanducci et al. 2002). In addition, a number of other outer-membrane proteins have been described in both *N. gonorrhoeae* and *N. meningitidis*. These include the H.8 antigen (a lipoprotein of unknown function) (Cannon 1989), Laz (a lipid-associated azurin) (Woods et al. 1989), and a 36-kDa glycolipid-binding adhesin (Paruchuri et al. 1990).

The neisserial IgA1 protease is actively secreted by viable *N. gonorrhoeae* and *N. meningitidis* during in vitro and in vivo growth. IgA1 protease attacks the hinge region of IgA1, thus creating Fab and Fc fragments (Plaut et al. 1975). These fragments are immunologically inactive for all Fc-mediated antibody functions and for functions that require a divalent immunoglobulin molecule. The IgA1 hinge region is rich in proline, serine, and threonine residues. This region is deleted from the hinge region of the IgA2 subclass, thus accounting for the resistance of IgA2 antibodies to digestion by this protease. Analysis of the substrate specificity within the IgA1 hinge region has led to the identification of two types of gonococcal IgA1 proteases. Type 1 recognizes a proline–serine sequence, whereas type 2 recognizes a proline–threonine sequence. Although all gonococcal strains produce an IgA1 protease, individual strains produce either the type 1 or the type 2 variant, but never both. The type 1 and type 2 gonococcal IgA1 protease structural genes have been cloned and when expressed in *E. coli* are secreted into the growth media (Koomey et al. 1982). Thus, in both *E. coli* and *N. gonorrhoeae*, the protease must traverse the cytoplasmic membrane, the periplasmic space, and the outer membrane. The enzyme is synthesized as a 169-kDa precursor. The first 27 amino acids, which comprise a typical leader sequence, are cleaved as the enzyme is translocated across the cytoplasmic membrane. A C-terminal β-domain of approximately 45 kDa mediates the translocation of the protease across the outer membrane. During this process, the β-domain, which remains embedded in the outer membrane, is cleaved from the secreted 121-kDa proenzyme. Finally, a C-terminal α-domain of approximately 12 kDa is autoproteolytically cleaved from the mature enzyme, leaving the secreted IgA-1 protease with a final molecular mass of approximately of 106 000-kDa (Pohlner et al. 1995). It has been proposed that IgA1 protease may play a role in the pathogenesis of *N. gonorrhoeae* and *N. meningitidis* by cleaving the lysosomal glycoprotein LAMP1. This in turn allows for the transcytosis of gonococci or meningococci through the epithelial cell layer (Lin et al. 1996).

Polysaccharides

Several *Neisseria* spp. synthesize polysaccharide capsules and exopolysaccharides (Table 4). Among the pathogenic *Neisseria* species, *N. gonorrhoeae* lacks a conventional polysaccharide capsule, whereas capsule production by the meningococcus is closely associated with virulence. Morphological evidence suggests that gonococci may express a nonconventional capsule composed of polyphosphate (Hendley et al. 1977; James and Swanson 1977; Reimann et al. 1988). Whether this proposed polyphosphate surface structure contributes to gonococcal infection in vivo has yet to be unambiguously demonstrated.

N. meningitidis expresses a polysaccharide capsule. Antigenic differences in the capsular polysaccharides provide the basis for serogrouping *N. meningitidis*. Presently A, B, C, H, I, K, L, W-135, X, Y, Z, and 29E are recognized serogroups among meningococci (Frasch 1989) (Table 4). Structural characterization of meningococcal capsular polysaccharides indicates that they are either homopolymers (serogroups A, B, C, and X), or heteropolymers comprised of a monosaccharide-glycerol (serogroups H and Z), disaccharide (serogroups I, K, W-135, Y, and 29E), or trisaccharide (serogroup L) repeating units. The capsular polysaccharides are linked to a hydrophobic moiety, 1,2-diacylglycerol, which is thought to help anchor one end of the polysaccharide chain in the meningococcal outer membrane (Gotschlich et al. 1981). Both the biochemistry and the genetics of group B capsular polysaccharide synthesis have been elucidated (Masson and Holbein 1983; Frosch et al. 1989). Genome analysis indicates that the genes encoding enzymes for the synthesis of the meningococcal capsule are segregated on pathogenicity islands. They are organized as an operon that encodes the biosynthetic machinery and a separate operon that encodes transport machinery (Tzeng et al. 2001, 2002). The capsule of *N. meningitidis* functions as a virulence factor because of its antiphagocytic activity. In general, meningococcal capsular polysaccharides are immunogenic in humans. Under certain growth conditions, some nonpathogenic *Neisseria* spp. synthesize extracellular polysaccharides that may also be considered capsular polysaccharide. For example, *N. perflava* possesses the enzymes that form a glycogen-like polysaccharide (Okada and Hehre 1974). Other *Neisseria* spp. (e.g. *N. polysacchareae*, *N. denitrificans*, *N. canis*, *N. cinerea*, and *N. subflava*) produce a similar polysaccharide during growth on a medium containing 1–5 percent sucrose (MacKenzie et al. 1977, 1978a, b, c).

Table 4 *Chemical composition of capsular polysaccharides and exopolysaccharides of* Neisseria *spp.* [a]

Species	Structural repeating unit[b]
N. meningitidis	
Serogroup A[c] (homopolymer)	ManNac-(1-P$\xrightarrow{\alpha}$6)- 3 | OAc
Serogroup B (homopolymer)	NeuNAc-(2$\xrightarrow{\alpha}$8)-
Serogroup C[d] (homopolymer)	NeuNAc-(2$\xrightarrow{\alpha}$9)- 7 8 | | OAc OAc
Serogroup H (monosaccharide–glycerol repeating unit)	$\rightarrow$ 4)α-D-Gal-(1$\rightarrow$2)-Gro-(3-P $\rightarrow$
Serogroup I (disaccharide repeating unit)	$\rightarrow$ 4)α-L-GulNAcA(1$\rightarrow$3)β-D-ManNAcA(1$\rightarrow$ | 4OAc
Serogroup K (disaccharide repeating unit)	$\rightarrow$ 3)-β-D-ManNAcA-(1$\rightarrow$4)-β-D-ManNAcA-(1$\rightarrow$ | 4-OAc
Serogroup L (trisaccharide repeating unit)	$\rightarrow$ 3)-β-D-GlcNAc-(1$\rightarrow$3)β-D-GlcNAc-(1$\rightarrow$3)α- D-GlcNAc-(1-P-
Serogroup W-135 (disaccharide repeating unit)	6-D-Gal(1$\xrightarrow{\alpha}$4)-NeuNAc(2$\xrightarrow{\alpha}$6)
Serogroup X (homopolymer)	GlcNAc(1-P$\rightarrow$4)-
Serogroup Y (BO)[e] (disaccharide repeating unit)	6-D-Glc(1$\xrightarrow{\alpha}$4)-NeuNAc(2$\xrightarrow{\alpha}$6) | OAc
Serogroup Z (monosaccharide–glycerol repeating unit)	D-GalNAc(1$\xrightarrow{\alpha}$1)-Gro-(3'-P$\xrightarrow{\alpha}$4)
Serogroup 29E (disaccharide repeating unit)	D-GalNAc(1$\xrightarrow{\beta}$7)-KDO(2$\xrightarrow{\alpha}$3)- 4 5 | OAc
N. lactamica	Antigenically similar to capsular polysaccharide of *N. meningitidis* serogroup B
N. polysaccharea	Glycogen-like structure containing mainly 1,4-linked α-D-glucopyranosyl residues; ca. 6% 4,6-di-*O*-substituted α-D-glucopyranosyl branch points[f]
N. sicca	Polymer of GalNAc
N. subflava biovar *perflava*	Similar to *N. polysaccharea*[f]

a) From Morse and Knapp 1991.
b) Gal, galactose; Glc, glucose; ManNAc, *N*-acetylmannosamine (2-acetamido-2-deoxy-D-mannose; NeuNAc, *N*-acetylneuraminic acid (sialic acid); OAc, *O*-acetylated; Gro, glycerol; P, phosphate; GulNAc, *N*-acetylgalactosamine (2-acetamido-2-deoxy-D-galactose).
c) Group A is substituted with *O*-acetyl at C3 on about 70 percent of the ManNAc-P residues.
d) Group C is substituted on C7 or C8 with 1 mol of *O*-acetyl per mole of sialic acid. One-quarter of the sialyl residues are not acetylated. Some di-*O*-acetylated (C7 and C8) may exist.
e) The Y polysaccaride contains 1.3 mol of *O*-acetyl per NeuNAc residue. The most probable site for acetylation is C3, C4, or C7. ^{13}C nuclear magnetic resonance studies have shown that the serogroup BO polysaccharide is identical to serogroup Y, although BO contains 1.8 mol of *O*-acetyl per mole of NeuNAc.
f) Produced in medium containing 1 to 5 percent sucrose.

LOS

Lipopolysaccharide (LPS) is the major glycolipid component of the outer membrane of gram-negative bacteria. High-molecular-weight LPS from enteric bacteria such as *E. coli* is comprised of a lipid A moiety, a core oligosaccharide, and a repeating O-antigen polysaccharide. Gonococcal and meningococcal LPS homologs are smaller glycolipid molecules that lack the repeating O-antigen polysaccharides (Mintz et al. 1984), and are consequently referred to as LOS. They are similar in size to the LPS from rough mutants of *Salmonella*, except that they exhibit considerable antigenic diversity. The oligosaccharide component of meningococcal and gonococcal LOS consists of relatively short, multiantennary glycans (Kim et al. 1994; Phillips et al. 1990) consisting of two regions: (1) a basal oligosaccharide linked to keto-deoxyoctulonate (KDO) and (2) a middle linear segment containing a varying number of residues that determine the length of the LOS. The nonreducing terminal sugar is similar in monosaccharide content and anomeric linkages to human cell-surface glycosphingolipids (Mandrell and Apicella 1993; Mandrell et al. 1993). Similar LOSs have been identified in other bacteria (e.g. *H. influenzae*, *Haemophilus ducreyi*, and *Bordetella pertussis*) that colonize or infect the mucosal surfaces of the respiratory and genital tracts.

Most gonococcal strains produce from one to six LOS species, which are antigenically and structurally distinct. This structural heterogeneity can be experimentally demonstrated as differences in migration when analyzed by SDS-PAGE (Griffiss et al. 1987a, b, 1988). Like the LOS of *H. influenzae*, the structural heterogeneity of the LOS oligosaccharide is modulated by expression of different glycosyltransferases as a result of high-frequency genetic events such as slipped-strand DNA synthesis (Danaher et al. 1995). The development of a typing system based on LOS is not practical because the antigenic variability of gonococcal LOS within a single strain is so marked. In contrast, meningococci exhibit a much more stable LOS upon which a serological typing system has been developed that separates strains into 12 immunotypes.

Variations in neisserial surface oligosaccharide composition can lead to further modification by host factors. For example, Smith et al. (1992) noted that gonococci acquired resistance to killing activity associated with normal human serum after brief exposure to human blood or secretions and ascribed this to the sialylation of a 4.2-kDa LOS species. Sialylation of LOS requires a gonococcal sialyltransferase and the host-derived substrate CMP-NANA, which is found in saturating concentrations in all human tissues. Sialylation of LOS is more pronounced during growth with lactate as compared with growth with glucose (Smith et al. 1992). This may be facilitated by lactate generation from granulocytes that form the exudates in gonorrhea (Frangipane and Rest 1993) and may explain why organisms harvested directly from a urethral exudate are highly sialylated (Parsons et al. 1992).

Antibodies specific for gonococcal LOS can be detected in sera and vaginal fluids of infected patients (Lammel et al. 1985). In addition, LOS contains a principal antigenic determinant recognized by 'natural' bactericidal antibody of the IgM class that, in combination with complement, mediates the killing of serum-sensitive strains. LOS also serves as the target antigen for much of the bactericidal antibody present in normal or convalescent sera (Rice and Kasper 1977). Gonococci expressing full-length LOS (containing the lacto-*N*-neotetraose moiety) are serum-sensitive, whereas sialylation of LOS renders them serum-resistant. Decreased uptake and killing of sialylated gonococci by PMNs has been reported (Rest et al. 1994). The modulation of LOS may have a significant role in invasion of cells (van Putten et al. 1995). Sialylated gonococci are less invasive for a variety of host cells when compared with nonsialylated gonococci (van Putten 1993). However, other factors also influence this property. For example, the invasiveness of some gonococcal strains depends upon expression of a terminal glucose (Minor et al. 2000) or other terminal sugars (e.g. lactosamine) on the LOS (Harvey et al. 2001a, b).

LOS promotes injury to the mucosa and may be responsible for the loss of ciliary activity and the sloughing of ciliated epithelial cells (Greg et al. 1981) by inducing the proinflammatory cytokine, tumor necrosis factor alpha (TNF-α) (McGee et al. 1999). It has been proposed that this is an important contributor to gonococcal salpingitis and may mediate postsalpingitis infertility and ectopic pregnancy (McGee et al. 1999). LOS possesses potent endotoxic activity and is believed to be the toxin responsible for adrenal cortical necrosis seen in severe meningococcal disease.

The structural and antigenic similarity noted above between the terminal oligosaccharides of LOS and human cell-surface glycosphingolipids has been examined (Mandrell and Apicella 1993; Harvey et al. 2001b). One terminal LOS trisaccharide was found to resemble a precursor of human blood group antigens and another appears to be related to the globoseries glycolipids, which constitutes the *E. coli* P-pilus receptor on human uroepithelial surfaces. The 'molecular mimicry' of neisserial LOS with host cells may confer several advantages for infection: (1) LOS may resemble 'self' antigens of the host, reducing their immunogenicity; (2) human cell-surface lectin-like proteins, which normally recognize specific glycosphingolipids, could bind LOS and thus mediate epithelial cell adherence and invasion by gonococci; and (3) host-derived glycosyltransferases, which normally modify the oligosaccharide moieties of glycosphingolipids, might also modify gonococcal LOS, creating neoantigens on the gonococcal surface.

CLINICAL DISEASE

Epidemiology

Of the pathogenic members of the Neisseriaceae, the gonococcus is responsible for infections with the highest morbidity, whereas the meningococcus causes infections with the greatest mortality. *N. gonorrhoeae*, the causative agent of the sexually transmitted disease (STD) (also referred to as sexually acquired infection (SAI)), gonorrhea, was responsible for approximately 360 000 cases of gonococcal infection in the USA in 2000 (about 132 cases per 100 000 population) and an estimated 62 million cases worldwide (Agacfidan and Kohl 1999). This statistic is even more staggering when one considers that like all acute STDs, gonorrhea is a disease of young people; 65 percent of new infections involve individuals under 25 years of age (Figure 6). The majority of these infections are confined to the genital mucosa of males and females. However, any site lined by columnar cells may be infected.

In males, urethral infection may be asymptomatic or present as a painful, but ultimately self-limited, urethral inflammation. Acute gonococcal urethritis is characterized by an exuberant granulocytic inflammatory discharge. Gonococcal orchitis and epidymitis are infrequent complications of urethral infection. Pharyngeal disease may be more common than previously appreciated, and anal intercourse may lead to gonococcal proctitis. Organisms that are increasingly resistant to conventional antibiotics have been isolated from rectal cultures (McFarland et al. 1983). In women, gonococci may cause asymptomatic or symptomatic endocervical infections or upper-genital-tract disease. Gonococcal infections of women are closely associated with infertility and, as a result, females disproportionately suffer the consequences of infection. For both men and women, but more commonly in women, local gonococcal infection can progress to bacteremia with attendant septic arthritis. In fact, gonococcal arthritis is the most common etiology of arthritis in young people. Finally, gonococcal infection facilitates transmission of HIV-1. In HIV-infected individuals, gonococcal urethritis greatly increases shedding of HIV in mucosal secretions and semen (Kaul et al. 2002). In HIV-negative individuals, gonococcal infection recruits cells and cytokines capable of enhancing acquisition of HIV and facilitates breaks in mucosal integrity, leading to increased virus infectivity (Cohen 1997).

In contrast, meningococcal infections exist in a largely asymptomatic carrier state within healthy individuals. Meningococci may enter the bloodstream of susceptible individuals, causing a bacteremia. If organisms cross the blood–brain barrier and reach the meninges, meningitis is likely to ensue. The incidence of meningococcal meningitis is approximately 0.5–5 cases per 100 000 population, and these rates vary geographically. For example, there are regions defined by latitude referred to as 'meningococcal belts' that extend throughout areas of Africa where rates of meningococcal disease are extremely high. Unlike gonorrhea, meningococcal disease does not discriminate by age or gender – it is among the leading causes of meningitis in infants, adults, and the elderly, where approximately 10 percent of cases are fatal; and among survivors, approximately 10 percent suffer permanent hearing loss or other serious sequelae.

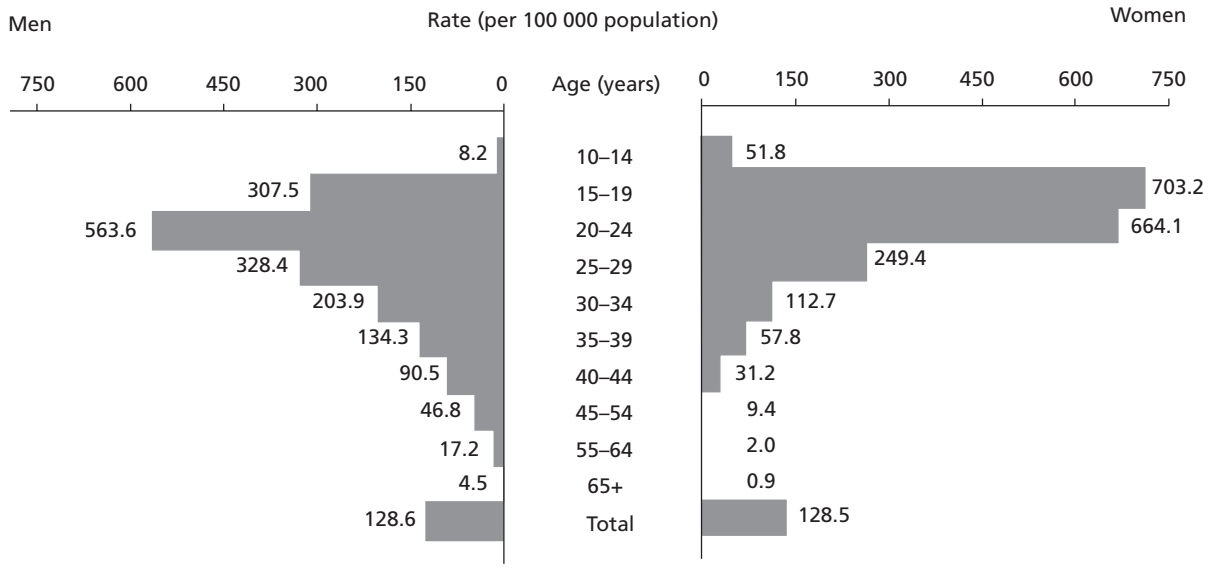

Figure 6 *Age- and gender-specific rates of gonococcal infection in the USA, 2000. Figure courtesy of the Centers for Disease Control and Prevention, Atlanta, Georgia.*

Pathology

Pathogenic *Neisseria* spp. do not produce a specific product or 'toxin' that in and of itself accounts for the symptoms of gonorrhea or meningitis. Rather, it is the ability of these organisms to multiply on, and invade, mucosal surfaces while avoiding host defense mechanisms. Ultimately, it is their ability to incite a vigorous host immune response that causes disease pathology. The capacity to multiply in vivo is a function of their ability to access growth-essential nutrients from the host, including efficiently scavenging growth-essential iron from human transferrin, lactoferrin, or hemoglobin using protein systems described above. These organisms evade host defenses by avoiding antigenic detection through the production of a capsule or antigenic variation of surface proteins. They also evade host defenses by invading and transcytosing through columnar epithelial cells (e.g. endocervix, urethra, rectum, oropharynx, nasopharynx, and conjunctiva). Host factors can also contribute the pathogenesis of infections caused by *Neisseria*, e.g. individuals with complement deficiencies are predisposed to infection by pathogenic *Neisseria* spp.

Diagnosis

The laboratory diagnosis of gonorrhea is made presumptively and then confirmed. This process involves identifying characteristics that distinguish *N. gonorrhoeae* from other *Neisseria* spp. that may be present in the specimen (Table 1). Nonpathogenic *Neisseria* spp. are normal inhabitants of the orpharynx and nasopharynx and are occasionally isolated from sites that are infected by *N. gonorrhoeae*. The presence of multiple pairs of gram-negative diplococci within a neutrophil is highly characteristic of gonorrhea when the specimen is obtained from a genital site. The Gram stain of a urethral exudate is about 95 percent sensitive in symptomatic men but only 50–70 percent sensitive in asymptomatic men. In women, the specificity of the Gram stain is complicated by the presence of other bacteria in the female genital tract flora that may have a similar morphology. The sensitivity and specificity of the Gram stain for diagnosing rectal infections is lower than for cervical infection.

N. gonorrhoeae can be cultured on selective media. However, attention to detail is necessary for optimal isolation. In men, the best specimens are a urethral exudate or a urethral scraping. In women, cervical swabs are preferred over urethral or vaginal specimens. Swabs may be transported to the laboratory in a suitable transport medium, although, ideally, specimens should be inoculated on to appropriate media and incubated immediately after collection. Urethral, cervical, and pharyngeal specimens are inoculated on to selective media, such as Thayer–Martin or Martin–Lewis. These are complex media that contain antimicrobial (vancomycin, colistin) and antifungal (nystatin) agents to inhibit the growth of normal flora. Specimens collected from normally sterile sites such as blood, synovial fluid, and conjunctiva may be inoculated on to a nonselective medium, such as chocolate agar. Direct detection methods for *N. gonorrhoeae*, such as amplified and nonamplified DNA-based assays, are also available. These assays have an advantage where culture is impractical because specimen viability is not required.

N. gonorrhoeae is distinguished from other *Neisseria* spp. by acid production from carbohydrates. The gonococcus is only capable of producing acid from glucose (Table 1). Gonococcal strains can be characterized according to their nutritional requirements (auxotyping) (Table 5). A panel of monoclonal antibodies specific for epitopes on the outer membrane protein I has also been used to subtype strains. Strains exhibiting specific reaction patterns are termed serovars. A combined serovar–auxotype classification provides

Table 5 *Phenotypic classification systems of* Neisseria gonorrhoeae *and* Neisseria meningitidis

Organism	System	Basis	No. of groups or types	Names
N. gonorrhoeae	Serovars	Protein I	24 PIA, 31 PIB	PIA, . . . PIA-24 PIB-1, . . . PIB-31
	Auxotypes	Nutritional requirement	35[a]	ARG^-, PRO^-, etc.
N. meningitidis[b]	Serogroups	Capsular polysaccharide	12	A, B, C, X, Y, Z, 29E W-135, H, I, K, L[c]
	Serotypes	Class 2/3 proteins	20	1, 2a, 2b, 2c, . . . 21[d]
	Subserotypes	Class 1 protein	10	P1.1, P1.2, . . . P1.6[d]
	Immunotypes	LOS	12	L1–L12

a) Auxotypes determined by growth response patterns on a standard set of defined media (Catlin 1978).
b) A strain can express more than one serosubtype or immunotype-specific epitope, e.g. L3,7,9.
c) See Table 4 for structure of the capsula polysaccharides.
d) Not all the numbers between 1 and 21 and between PI.1 and PI.16 are used.

resolution among gonococcal isolates and is useful in epidemiological investigations (Sparling et al. 1990).

In cases of suspected meningococcal disease, specimens of blood, cerebrospinal fluid, and nasopharyngeal secretions are typically collected and examined for the presence of *N. meningitidis*. Cerebrospinal fluid should be concentrated by centrifugation and a portion inoculated on to chocolate or blood agar. Nasopharyngeal specimens require plating on a selective medium such as Thayer–Martin media, while blood cultures are typically plated on rich nonselective media, such as chocolate or blood agar. Growth of these organisms requires incubation in an aerobic atmosphere containing 5 percent CO_2. The presence of oxidase-positive, gram-negative diplococci provides a presumptive identification of *N. meningitidis*. For confirmation, production of acid from glucose and maltose, but not sucrose, lactose, or fructose, may be used (Table 1). Gram-stained smears of cerebrospinal fluid may also be diagnostic.

The presence of a polysaccharide capsule is the major distinguishing structural feature separating *N. meningitidis* (and many nonpathogenic *Neisseria* spp.) apart from *N. gonorrhoeae*. This capsule is believed to play a role in the resistance to PMN-mediated phagocytosis. It also provides the basis for grouping of these organisms. To date, 12 different meningococcal serogroups are identified based on the chemical composition of the polysaccharide (Table 5). The serological group is determined by a slide agglutination test, using polyvalent and monovalent antisera. In addition to serotyping by capsular polysaccharide, *N. meningitidis* strains can be divided into certain serotypes and subserotypes based on antigenic determinants of certain outer-membrane proteins and LOS (Table 5). A typing scheme based on isoenzyme electrophoresis has also been used to study the epidemiology and geographical distribution of *N. meningitidis* strains (Crowe et al. 1987).

Treatment; antibiotic susceptibility and resistance

Pathogenic *Neisseria* spp. are generally susceptible to treatment with antibiotics. The key is delivering the correct antibiotic in a timely fashion. This is particulary true for meningitis caused by *N. meningitidis*, where the time from symptomology to death can be less than a day. Penicillin is still the drug of choice to treat meningococcal meningitis and meningococcaemia. Penicillin does not readily penetrate the blood–brain barrier. However, when the meninges are acutely inflamed it can traverse this barrier. Recently, penicillin-resistant *N. meningitidis* strains have been described (Brett et al. 1992), indicating that continued use of this drug may be in jeopardy. The *penA* gene from penicillin-resistant *N. meningitidis* is very diverse and 30 mosaic genes have been found in only 70 different isolates. In contrast, only three different mosaic *penA* genes have been identified in *N. gonorrhoeae*. It appears that the opportunity for acquiring DNA from commensal *Neisseria* spp. is greater for *N. meningitidis*, which normally inhabits the nasopharynx. Tetracycline resistance in *N. meningitidis* due to the acquisition of the 25.2-mDa TetM plasmid has also been described (Roberts and Knapp 1988). Sulfonamide resistance in *N. meningitidis* is due to chromosomally mediated determinants (Fermer et al. 1995). Recent evidence indicates that the origin of the sulfonamide resistance determinants genes may have been from other *Neisseria* spp.

Gonococcal infection is complicated by the high incidence of coinfection with other STDs such as chlamydia, the high mobidity of the disease, and the rapid evolution of resistance to conventional and new-generation antibiotics as described below. Due to the development of resistance involving multiple mechanisms, penicillin is no longer the antimicrobial of choice for the primary treatment of gonorrhea. Resistance to β-lactam antibiotics results from the destruction of the antibiotics by β-lactamases or the presence of mutated penicillin-binding proteins (PBP) with decreased binding affinity for penicillin (Dowson et al. 1989). Resistance to penicillin in non-β-lactamase producing *N. gonorrhoeae* is chromosomally mediated and due to the production of low-affinity forms of PBPs combined with reductions in the permeability of the cell envelope to penicillin. Alterations in PBPs have also occurred in *N. meningitidis*, resulting in some strains with decreased susceptibility to penicillin. In both *N. gonorrhoeae* and *N. meningitidis*, chromosomal resistance to penicillin results from low-affinity mosaic forms of PBP-2, a bifunctional enzyme with transpeptidase and transglycosylase activities. These have arisen by the replacement of parts of the PBP-2 gene (*penA*) with the corresponding regions of genes from closely related commensal species, such as *N. flavescens* and *N. cinerea* (Dowson et al. 1989).

The increase in the prevalence of β-lactamase-producing strains of *N. gonorrhoeae* in the 1970s and 1980s has resulted in a shift to other antibiotics for the treatment of gonorrhea, including tetracycline, sulfonamides, macrolides, third-generation cephalosporins, and fluoroquinolones. Concomitantly, chromosomal- and plasmid-mediated resistance of *N. gonorrhoeae* and *N. meningitidis* to agents such as tetracycline, sulfonamides, and erythromycin has arisen (Table 6). The widespread distribution of the 25.2-mDa conjugative TetM plasmid in strains of *N. gonorrhoeae* has rendered tetracycline as an ineffective alternative for penicillin. In addition, strains with multiple chromosomal resistance to penicillin, tetracycline, erythromycin, and cefoxitin have been identified in the USA and many other parts of the world (Ng et al. 2002; Marquez et al. 2002; Anonymous 2002; Rouquette-Loughlin et al. 2003; Chaudhry et al. 2002; Trees et al.

Table 6 *Antibiotic resistance in* Neisseria[a]

	N. gonorrhoeae		*N. meningitidis*		Commensal *Neisseria*	
	Plasmid	Chromosomal	Plasmid	Chromosomal	Plasmid	Chromosomal
Pencillin	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+
Sulfonamides	–	+	–	+	+	+
Trimethoprim	–	+	–	?	–	+
Spectinomycin	–	+	–	?	?	?
Streptomycin	–	+	–	+	+	+
Quinolones (ciprofloxacin)	–	+	–	?	?	?
Erythromycin	–	+	–	+	?	?
Rifampicin	–	+	–	+	?	?
Pen, Su, Sm[b]	–	–	–	–	+	–

a) +, Antibiotic resistance gene has been reported on either a plasmid or the chromosome; –, antibiotic resistance has not been reported; ?, not known.
b) Multiple antibiotic resistance to penicillin, sulfonamides, and streptomycin is encoded on a single plasmid.

2000). Indeed, even fluoroquinolones can no longer be reliably used to treat gonorrhea in patients from Asia (McGee and Grewal 2002; Knapp et al. 1997) and this trend is increasing in the USA (Figure 7). Plasmid and chromosomally mediated antibiotic resistance has also been described in a number of commensal *Neisseria* spp. (Qvarnstrom and Swedberg 2002). Of interest is the reported plasmid-mediated multiple drug resistance to sulphonamides, streptomycin and penicillin (Table 6), which could potentially spread to *N. meningitidis*.

Prevention

Like most infectious diseases, hygienic considerations are useful in the control of neisserial infections. However, the asymptomatic nature of meningococcal infection makes it difficult to control its spread, particularly in crowded environments such as military barracks and college dormitories.

The severity of meningococcal disease warrants a vaccinologic approach for the prevention of this life-threatening disease. However, the limited morbidity of the disease does not dictate mass vaccination. Vaccine formulations based on *N. meningitidis* serogroups A, C, W-135, and Y capsular polysaccharides are available for use in humans and elicit bactericidal antibodies directed against the capsule. These vaccines have been proven to be effective and are administered to high-risk groups, such as military personnel confined to close quarters. The group B capsular polysaccharide is poorly immunogenic because of its similarity to sialic acid moieties found in human tissues, and no effective vaccine has been developed.

As with other STDs, effective control of gonorrhea can be achieved with barrier protection, education, diagnosis, and contact tracing. In principle, the high morbidity of gonococcal infection would ideally be controlled through a vaccinologic approach. In practice, the concept of mass vaccination for the prevention of an

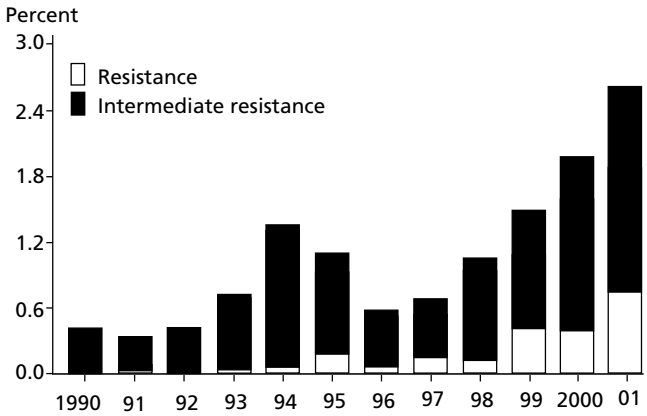

Figure 7 *Increase in the proportion of intermediately resistant and resistant gonococcal isolates to ciprofloxacin from 1990 to 2001. This demonstrates the rapid emergence of resistances of gonococci to this widely used antibiotic. Figure courtesy of the Centers for Disease Control and Prevention, Atlanta, Georgia.*

STD is one that is undergoing social evolution and is probably not acceptable by today's standards. A strong argument has been made for vaccination of high-risk populations for the prevention of gonorrhea. However, the antigenic variation exhibited by the gonococcus makes the development of a vaccine very challenging, if not impossible. Currently, no vaccine exists for the prevention of gonococcal infection.

SUMMARY

The *Neisseria* are a group of closely related species that range from commensal organisms that exist on healthy mucosal surfaces in mammals to those that cause significant human disease. In many respects, such as iron acquisition and polysaccharide production, the biology of these organisms exhibit remarkable similarity to the biology of eukaryotic cells as we know it. Other aspects, such as antigenic variation and horizontal gene exchange, provide mechanisms whereby these organisms can survive in a hostile environment by evading host defenses and the inhibitory activity of some antimicrobials. Regardless, their evolution has placed them into a discrete family within the microbial kingdom.

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Acinetobacter and *Moraxella*

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TAXONOMIC REVIEW: FAMILY MORAXELLACEAE

The taxonomic status of this family has a long history of debate and change. Certainly the newer molecular techniques have greatly facilitated the current definitions. Following is a brief historical perspective for the genera discussed in this chapter. Supportive data and arguments can be made for each position, as definitive taxonomic placement for each the genera has not reached consensus.

Acinetobacter

Beijerinck in 1911 is credited with isolating a microbe representative of the genera from the soil and naming it *Micrococcus calcoaceticus* (Baumann et al. 1968b). The genera expanded and underwent numerous defining changes. Important to note is that most of the changes were based on the individual isolate's saccharolytic ability. Oddly, this characteristic is perhaps the most clinically relevant as the genus undergoes definition. The saccharolytic strains resembling Beijerinck's initial isolate were successively called *Herellea vaginicola*, 'B5W,' *Bacterium anitratum*, and *Achromobacter anitratus*. Likewise, the asaccharolytic strains were penned *Mima polymorpha*, *Moraxella lwoffii*, and *Achromobacter lwoffii* (Henriksen 1973). Brisou and Prévot proposed the generic designation of Acinetobacter in 1954. Their 'genus' was a heterogenous group inclusive of nonmotile, gram-negative, oxidase-positive, and oxidase-negative saprophytes distinguishable from other bacteria by their lack of pigmentation (Ingram and Shewan 1960). Studies of Baumann et al. (1968b) moved the oxidase-positive strains to the genus *Moraxella*. In 1971 the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria suggested that the genus *Acinetobacter* should include only the oxidase-negative strains (Lessel 1971). A transformation test was described (Juni 1972; Weyant et al. 1996) and is still used for the genus-level identification. This research technique involves the conversion of auxotrophs to prototrophy. Since the organisms classed within *Acinetobacter* were similar, a monotypic genus was proposed, *Acinetobacter calcoaceticus*, comprising two varieties, var. *anitratus* (formerly *Herellea vaginicola*) and var. *lwoffii* (formerly *Mima polymorpha*) (Juni 1984).

DNA homology studies have recognized at least 25 genomospecies (Bouvet and Grimont 1986; Bouvet and Jeanjean 1989; Gerner-Smidt and Tjernberg 1993; Ibrahim et al. 1997; Nemec et al. 2001; Nishimura et al. 1988; Tjernberg and Ursing 1989), of which to date ten are named (Bouvet and Grimont 1986; Nemec et al. 2001; Nishimura et al. 1988). Of note, genomic species 1, 2, 3, and 13 are quite difficult to distinguish in the clinical laboratory using the standard biochemicals and instrumentation; therefore, these species are referred to as the *Acinetobacter calcoaceticus–baumannii* complex. Of the unnamed species, very little is known about the clinical significance (Table 1).

Table 1 *Frequently isolated saccharolytic and asaccharolytic* Acinetobacter *species*

Named species	Associated genomic species[a]	Associations with nosocomial outbreaks[b]	Laboratory observations
A. calcoaceticus	1		Saccarolytic, typically nonhemolytic; difficult to separate in clinical laboratory; considered as *A. calcoaceticus-baumanii* complex[c]
A. baumannii	2	++	
	3	+	
	13		
A. haemolyticus	4		Typically hemolytic
A. johnsonii	7	+	Asaccharolytic; typically nonhemolytic
A. lwoffi	8	+	
A. radioresistens	12		

a) Data from Bouvet and Grimont (1986), Bouvet and Jeanjean (1989), and Tjernberg and Ursing (1989).
b) Bouvet and Grimont (1987).
c) Gerner-Smidt et al. (1991).

During this period the genus was considered a member of the family Neisseriaceae. However, recent molecular methods, rRNA–DNA hybridization assays and 16S rRNA typing, have moved *Acinetobacter*, along with *Moraxella*, *Psychrobacter*, and related organisms, to the family Moraxellaceae (van Landschoot et al. 1986; Rossau et al. 1989; Rossau et al. 1991).

Moraxella

The genus *Moraxella* has also undergone several taxonomic revisions within the past 20 years. Initially, Bovre (1979, 1984) argued that the genus consisted of two subgenera, *Moraxella* and *Branhamella*, within the family Neisseriaceae. Subsequent DNA–rRNA hybridization studies and 16S rRNA analysis suggested that *Moraxella*, *Branhamella*, and the 'false *Neisseria*' (*Neisseria caviae*, *Neisseria ovis*, and *Neisseria cuniculi*) all belong in the same family, but not within the family Neisseriaceae (Rossau et al. 1986; Dewhirst et al. 1989). Rossau et al. (1991) proposed the creation of a new taxonomic family to include the subgenera *Moraxella* and the genera *Acinetobacter* and *Psychrobacter*. Opposing, Catlin (1991) suggested that only the *Moraxella* and *Branhamella* be placed within a new family called Branhamaceae. Data from Veron et al. (1993) and Enright et al. (1994) support the exclusion of *Moraxella* and *Branhamella* from the family Neisseriaceae. Not unexpectedly, the taxonomic status of the inclusive species continues to evolve. This has been reviewed by Pettersson et al. (1998).

Respecting historical conventions, *Branhamella* will be discussed separately from *Moraxella*.

ACINETOBACTER

Microbiology

Members of the genus *Acinetobacter* are gram-negative, catalase-positive, and oxidase-negative. During periods of rapid growth (exponential phase), the organisms typically appear bacillary to coccobacillary 1–1.5 by 1.5–2.5 microns in size. Notably they become more coccoid or diplococcal as the cultures age (stationary phase). The colonies are 1–2 mm in diameter (smaller than typical Enterobacteriaceae) and are typically domed, smooth to slightly mucoid, and opaque. Pigmentation is usually grayish-white, although some strains may appear pale yellow. Hemolytic activity on blood agar is variable, although a diffusible brown pigment has been observed (Pagel and Seyfried 1976) when glucose has been added to the medium (Siau et al. 1998). Acinetobacters are nonlactose fermenters but may produce a slight pinkish hue that could be mistaken for lactose fermentation. The acinetobacters are nonmotile, but occasionally an odd twitching motility can be demonstrated (Lautrop 1961; Lautrop 1974; Barker and Maxted 1975). Older cultures frequently capsulate, occasionally causing problems with destaining the crystal violet. For the most part, the species are not fastidious and capably grow on the standard nutritional medium used within the laboratory. Occasionally, strains may be encountered that are fastidious, failing to grow in nutrient broth, and forming smaller colonies on blood agar (Weyant et al. 1996). Utilization of carbohydrates varies considerably among the species. Nitrates are not reduced to nitrites. The ability to hydrolyze gelatin and urease is variable. They are strictly aerobic and are capable of growing at a wide range of temperatures. However, it is observed that most clinical strains grow optimally at 37°C, while the environmental isolates prefer lower temperatures. For clinical isolates, growth on MacConkey is variable and presenting as either colorless or light pink colonies (Table 2).

Identification of the acinetobacters is often problematic for all but the reference laboratories. Unfortunately, the databases of most of the commercially available identification systems are variable in their ability to identify all isolates. Gerner-Smidt et al. (1991) and Kämpfer et al. (1993) have proposed various phenotypic

Table 2 *Characteristics of the* Moraxellaceae *and similar genera*

	Actinetobacter	*Moraxella*	*M. catarrhalis*	*Neisseria*	*Kingella*
Cell morphology	B, CB, DC	B	DC	DC/B	CB
Oxidase	−	+	+	+	+
Catalase	+	+	+	+[a]	−
Glucose (acid)	±	−	−	±	+
Dnase	−	−[c]	+	−	−
NO_3 reduction	−	±	+	−[b]	+
Butyrate esterase			+	−	

B, bacillus; DC, diplococcus; CB, coccobacillus.
a) *Neisseria elongata* is variable for catalase.
b) *N. mucosa* reduces NO_3.
c) *M. canis* is Dnase positive.

schemes for identification. Review of these schema shows that they are not practical for the typical clinical laboratory owing to their requirement for a large number of biochemicals.

Epidemiology

The acinetobacters are saprophytic and ubiquitous. They can be found throughout the natural and hospital environment on a wide range of dry and moist inanimate surfaces (Getchell-White et al. 1989; Towner et al. 1991). Recent studies have documented that hospital environments, such as intensive care units (ICU), can harbor carbapenem-resistant *A. baumannii*. Only after closing the affected ICU and thorough cleaning of the environment and equipment with hyochlorite and terminal disinfection were the resistant strains eliminated (Aygun et al. 2002). Additionally, a study by Brooks et al. (2002) documented that pan-resistant acinetobacters could be recovered from the surfaces of dispensers of hand soap containing 2.0 percent chlorhexidine.

Perhaps most importantly, they are considered as part of the commensal flora of man and other animals, where they occasionally present as opportunistic pathogens. As commensals, they have been isolated from axillae, groin, digit webs, and other external fossa of about 25 percent of the population (Taplin et al. 1963; Somerville and Noble 1970).

Notably, several studies have demonstrated sustained carriage on the hands of healthcare workers (Adams and Marrie 1982; Larson et al. 1986). In his study, Larson (1981) determined that acinetobacters were the most common gram-negative organisms carried on the skin of hospital personnel. Members of the genus have been isolated from practically every type of patient specimen. This ubiquity often presents problems for the clinician. Therefore, careful evaluation of the role of an isolate to an infectious process is required.

Interestingly, it appears that the genomic distribution of the various acinetobacters differs greatly throughout the diverse hospital environments. *A. baumannii* is the species isolated most frequently. Assimilation tests have identified 19 biotypes (Bouvet and Grimont 1987). Traub and Leonhard (1994) have described 32 serovars. The other named genomospecies, *A. lwoffii*, *A. haemolyticus*, and *A. johnsonii* follow in frequency (Table 1).

As with many other genera, many different typing systems have been devised to segregate the various members of this genus. Refer to Bouvet (1991) for a review of early attempts at typing schema. Conventional methods have presented problems that have precluded their adaptation. These methods have included biotyping (Bouvet et al. 1990), phage-typing (Vieu et al. 1979), serotyping (Traub and Leonhard 1994), and bacteriocin typing (Andrews 1986).

Currently, several molecular methodologies have been developed and used within the clinical setting for the investigation of nosocomial outbreaks, although it is important to note that no particular system has been accepted as the 'gold standard.' These techniques have included plasmid profile analysis (Gerner-Smidt and Tjernberg 1993; Seifert et al. 1994a, b), restriction endonuclease digestion and pulsed-field gel electrophoresis of total chromosomal DNA (Gouby et al. 1992; Struelens et al. 1993; Liu and Wu 1997; Grundmann et al. 1997), random amplified polymorphic DNA profiles (Gräser et al. 1993; Struelens et al. 1993), ribotyping (Gerner-Smidt 1992; Bernards et al. 1997), cell-envelope and outer-membrane protein profiles (Dijkshoorn et al. 1987a, 1987b), and multilocus enzyme electrophoretic typing (Thurm and Ritter 1993).

Interestingly, strain specificity has been demonstrated for various acinetobacters against numerous bacteriophages (Ackermann et al. 1994). Typically, most of these bacteriophages are lytic and are range-restricted to the original strain of *Acinetobacter* from which they were isolated.

Virulence and pathogenic factors

Usually acinetobacters are not pathogenic, but they do have components that are capable of enhancing their virulence in debilitated individuals. Perhaps the most important and similar to other gram-negative organisms,

is the presence of endotoxin. Endotoxin is a lipopolysaccharide (LPS) moiety of the outer membrane and in which the toxic lipid component, lipid A, is embedded. It has been demonstrated that the LPS is responsible for toxicity in mice and pyrogenicity in rabbits. Endotoxin is considered partially responsible for the febrile response during septic episodes. For acinetobacter pneumonia, Obana (1986) demonstrated an enhanced virulence in the presence of mixed infections with other bacteria species in the mouse model. Obana's study could not identify conclusively the definitive nature of this enhancement.

The research by Borneleit and Kleber (1991) on the matrix of the cell wall and outer membrane demonstrates special properties capable of influencing the permeability properties of the cells. Fimbriae have been documented that are potentially capable of facilitating adhesion to human epithelial cells. A polysaccharide capsule has been described that perhaps limits phagocytosis (Anstey et al. 1992). This capsule certainly aids the bacterium to survive under dry conditions (Wendt et al. 1997). These properties in conjunction with protein S layers and slime (Obana et al. 1985; Borneleit and Kleber 1991) also potentially enhance the virulence of the organism.

Interestingly, it has been noted that certain strains have been shown to produce siderophores (e.g. aerobactin) and iron-repressible outer-membrane receptor proteins (Smith et al. 1990; Echenique et al. 1992; Actis et al. 1993). These abilities are also potentially important virulence determinants. For further information about *Acinetobacter*, see Juni (1978), Towner et al. (1991), and Bergogne-Bérézin et al. (1996).

Clinical significance

Generally acinetobacters are not fastidious and are capable of surviving in various environmental niches. Most importantly, they are adept at acquiring resistance to multiple antibiotics. Fortunately, most isolates are simply colonizers in healthy individuals (Bergogne-Berezin and Towner 1996; Cisneros et al. 1996). However, determining the clinical significance of a particular isolate can be problematic for the clinician, particularly for intensive care patients and others with extensive surgical or antibiotic intervention. The opportunistic potential of this genus for causing hospital-acquired infections is well documented. Studies by Corbella et al. (1996) have shown that the digestive tracts of patients within ICUs often serve as reservoirs for multiresistant *A. baumannii* strains involved in hospital outbreaks.

The respiratory tract is the prominent site for hospital-acquired infections, with endotrachael tubes frequently implicated. Additionally, isolates found from the urinary tract, wounds, and catheter sites may eventually lead to septicemia (Bergogne-Berezin and Towner 1996; Bernards et al. 1997; Cisneros et al. 1996; Seifert et al. 1995). Less frequently, members of the genus have been associated with arthritis, endocarditis, meningitis, osteomyelitis, peritonitis related to continuous ambulatory peritoneal dialysis, and ventilator-related pneumonias (Bergogne-Berezin and Towner 1996; Jimenez-Mejias et al. 1997). As surmised, risk factors for acquiring acinetobacter infections can be attributed to antibiotic exposure, surgery, instrumentation, ventilation, and ICU admission (Bergogne-Berezin and Towner 1996; Cisneros et al. 1996; Wisplinghoff et al. 2000).

Management, therapeutic options, and susceptibility testing

Guidelines providing minimum inhibitory concentration (MIC) and disk diffusion interpretative criteria for suggested groups of antibiotics have been provided by the National Committee for Clinical Laboratory Standards (NCCLS). Regardless, the clinical management of opportunistic and coinfections with the acinetobacters is perhaps one of the biggest challenges facing clinicians today, particularly in medical/surgical, rehabilitation, and intensive care units. Resistance among clinical isolates has developed rapidly. This is perhaps the most striking feature of the genus. Clinical isolates are predictably resistant to penicillin, ampicillin, first-generation cephalosporins, and chloramphenicol. Activity is variable against carbenicillin, tetracyclines, aminoglycosides, second- and third-generation cephalosporins, quinolones, trimethoprim-sulfamethoxazole, and carbapenems (Bergogne-Bérézin and Towner 1996; Cisneros et al. 1996; Seifert et al. 1993; Visalli et al. 1997). Recent reports have documented the successful use of intravenous colistin as a safe alternative to the carbapenems (Garnacho-Montero et al. 2003). Several investigators have demonstrated a potential role for sulbactam in patient management (Corbella et al., 1998; Levin 2002).

Due to the unpredictable multiresistant patterns of nosocomial strains, consideration must be given to the prevalent susceptibility profiles within the institution (Jones 2001). Strains of *A. calcoaceticus–baumanii* complex typically are more antimicrobial-resistant than *A. lwoffii*, probably due to their higher prevalence within the hospital environment and possibly their ability to acquire antibiotic resistance. Given the overall unpredictable nature of resistance, susceptibility testing is warranted for clinically significant isolates. Additionally, studies have demonstrated the value of cohorting and aseptic techniques as important factors in the containment of resistant strains (Podnos et al. 2001).

Genetic resistance

Transformation is probably not a significant event among the *Acinetobacters* (Juni 1978). The process was

first demonstrated by Juni and Janik (1969) with *A. calcoaceticus* strain BD4 and its microencapsulated *trpE27* mutant derivative BD413. Juni (1972) demonstrated that the transformation of strain BD413 *trpE27* to the wild-type phenotype could be used as the basis of the genetic test for identifying members of the genus. The ease with which strain BD413 can be transformed has been used with a wide range of advanced molecular procedures, allowing the detailed fine-structure genetic investigations of the chromosomal organization and metabolic regulation of this strain (Ornston and Neidle 1991).

Strain specificity appears to exist with regards to transduction. Using P78, a temperate phage, Herman and Juni (1974) demonstrated a low-frequency generalized transduction with its specific host, while failing to lysogenize 389 other strains of *Acinetobacter*. This process has not been studied extensively.

Towner and Vivian (1976) and Hinchliffe and Vivian (1980) demonstrated that conjugation could occur with a broad host-range of plasmids using *Acinetobacter* strain EBF 65/65. Although very little is known of the mobilization events, the system has been important for the mapping of mutations on the circular chromosomal linkage group in strain EBF 65/65 (Towner 1978; Vivian 1991).

Plasmids and transposons are perhaps the most interesting to researchers and clinicians and probably serve an important role in the biology of *Acinetobacter* spp. (Towner 1991). It has been observed that more than 80 percent of *Acinetobacter* isolates appear to carry multiple indigenous plasmids of variable molecular size (Gerner-Smidt 1989; Seifert et al. 1994a). Unfortunately, studies involving plasmids and transposons have been riddled with problems most likely due to difficulties in lysing organism's cell wall. Transposons, as opposed to plasmids, may be more important to the development of antibiotic resistance in clinical isolates (Towner 1991). This observation may be due to the lack of a suitable test system for studying plasmid-mediated transfer.

MORAXELLA

Microbiology

Species belonging to genus *Moraxella* are gram-negative, although they may appear to resist decoloration. Those species originally classed within the genus are usually coccobacillary, while *Moraxella catarrhalis*, which were originally placed in the genus '*Branhamella*,' tend to appear as diplococci. Occasionally they may form short chains. Cellular pleomorphism is typical as the culture ages or with growth at increased temperature. All moraxellas are strict aerobes, oxidase-positive, and catalase-positive. On blood agar, moraxellas form circular, smooth, domed colonies after 24 h of incubation. They have a translucent gray color. They are nonmotile, although Piechaud observed a sluggish motility on nutrient agar in a special oil chamber (Jones and Jephcott 1990). All species have fimbriae and, with the exception of *M. bovis*, are encapsulated. They are asaccharolytic and negative for DNase, urease, indole, and hydrogen sulfide. Citrate utilization, nitrate reduction, and gelatin liquefaction are variable. Their optimal growth temperature is 33–35°C. Several of the species are weakly hemolytic on blood agar. Growth is variable on MacConkey agar. Given their relative inertness, speciation often presents problems in the clinical laboratory. The commercially available identification systems generally have limited ability to speciate this genus accurately. A reference laboratory may be useful if this is required (Table 2).

As mentioned previously, studies suggest that those species historically associated with the genus '*Branhamella*' should be moved to the genus *Moraxella*. However, for convenience, the discussion of *Moraxella* (*Branhamella*) *catarrhalis* will follow that of *Moraxella*.

MORAXELLA NONLIQUEFACIENS

M. nonliquefaciens is the most commonly isolated species of the genus. Kaffka first described this species in 1955, noting that it was unable to liquefy gelatin. This species is most commonly isolated from the upper respiratory tract. Graham et al. (1990) reviewed 933 isolates of moraxella submitted to the Centers for Disease Control and Prevention and found that *M. nonliquefaciens* was the most common isolate from nose, throat, mouth, or eye.

The species is quite variable (Bovre 1967; Bovre and Henriksen 1967). Typically colonies are smooth and translucent to semiopaque. On blood agar, colonial growth may reach 1 mm in diameter after 48 h. Pitting and spreading have been observed. Using data from nutritional studies, DNA content, and genetic transformation, species typical for *M. osloensis* and *[Moraxella] phenylpyruvica* were removed. Of particular note the status of *[M.] phenylpyruvica* is questionable. Studies by Rossau et al. (1991) and Enright et al. (1994) suggest that this group of organisms is most closely related to *Psychrobacter immobilis*.

MORAXELLA LACUNATA

Members of this species are part of the typical flora found on the mucous membranes of humans and animals. Morax and Axenfeld (Jones and Jephcott 1990) first described this group in a case of conjunctivitis. Smears of secretions often show the organism associated with polymorphonuclear leukocytes as well as desquamated epithelial cells.

They are more fastidious than other members of the genus. Colonial growth on blood or chocolate agar can

be poor. Colonies usually pit the agar and form dark halos in the media surrounding the colonies. The literature describes *M. liquefaciens* as a variant *M. lacunata*. This variant is capable of liquefying gelatin rapidly and growing at room temperature. Clinically, it has been implicated in serious invasive disease. These differences, being minimal, Henriksen and Bovre (1968a) reject separate species classification.

MORAXELLA OSLOENSIS

Being less fastidious, *M. osloensis* was split from *M. nonliquefaciens* by Bovre and Henriksen (1967). The species is a common resident of the human genital tract and other mucous membranes. To the inexperienced, it can be confused with *N. gonorrhoeae*. Unlike the gonococcus, it does grow well on blood agar. Questions remain regarding the taxonomic status of this species (Rossau et al. 1991; Vandamme et al. 1993). Case studies have associated *M. osloensis* with septic arthritis, osteomyelitis and septicemia.

MINOR SPECIES

There are several minor species of questionable human clinical significance but worthy of mention. *M. atlantae* is fastidious, forming <0.5-mm colonies on blood agar only after 48 h of incubation. The colonies may pit blood agar (Bovre et al. 1976). *M. lincolnii* was described by Vandamme et al. (1993). The species is not particularly fastidious. On blood agar it forms smooth, whitish, nonhemolytic, convex colonies (1–3 mm) within 48 h. Optimal growth is at 28–33°C, but it can also grow at 36–37°C. Both species are part of the normal flora of the human respiratory tract. Little is known about their pathogenic potential.

ANIMAL-ASSOCIATED SPECIES

M. caviae was first isolated from the throat of guinea pigs (Pelczar et al. 1949). Pelczar (1953) named it *Neisseria caviae*. Colonies are caramel to dusty brown. It is asaccharolytic but is capable of reducing nitrate and nitrite. It is DNase-positive and will hydrolyze tributyrin.

M. bovis was first isolated by Jones and Little (1923) and described as being the causative agent of infectious bovine keratoconjunctivitis. The organism grows poorly on nutrient agar and broth (Watt 1951). It fails to reduce nitrate but it does liquefy gelatin.

Lindqvist (1960) isolated *M. ovis* from sheep with keratoconjunctivitis. Colonies tend to be nonpigmented, glossy, and translucent. It is asaccharolytic and capable of reducing nitrate to nitrite and hydrolyzing tributyrin. It is DNase-negative.

Jannes et al. (1993) described *M. canis*, an isolate from dogs that had been confused previously with *M. catarrhalis*. *M. canis* produces large colonies of which some strains may be mucoid similar to some of the Enterobacteriaceae. Interestingly, microscopically the cells resemble *M. catarrhalis* (Jannes et al. 1993). Additionally, like *M. catarrhalis*, it is asaccharolytic, reducing nitrate and producing a DNase, and is capable of hydrolyzing tributyrin. Unlike other species, it produces gamma-glutamyl aminopeptidase and a brown pigment on Mueller–Hinton agar.

Other named species commonly found in animals include *M. caprae* (goats), *M. cuniculi* (rabbits), and *M. equi* (horses).

Clinical significance

Apart from *M. catarrhalis*, the moraxellas have little pathogenic potential. When they are implicated as pathogens, their clinical presentations are similar among all the species. There are case reports suggesting their occasional implication in arthritis, conjunctivitis, endocarditis, keratitis, meningitis, septicemia, and otolaryngologic infections (Graham et al. 1990; Johnson et al. 1995; Shah et al. 2000).

Therapeutic options and susceptibility testing

Susceptibility testing is seldom warranted, as most moraxellas are susceptible to the β-lactams, tetracyclines, quinolones, and aminoglycosides (Fass and Barnishan 1980; Rosenthal et al. 1978; Spangler et al. 1996), as well as the conventional topical ocular antimicrobials. β-Lactamases are not significant in moraxella species other than *M. catarrhalis* (Rosenthal et al. 1978).

MORAXELLA (BRANHAMELLA) CATARRHALIS

Microbiology

M. catarrhalis are gram-negative diplococci. They tend to resist decolorization. Cellular division occurs along two planes at right angles, hence the occasional formation of tetrads. Their similarity to the *Neisseria* species on Gram stains is striking and necessitates their identification. However, studies by Doern and Morse (1980) suggested that isolates from sources (e.g. middle-ear fluid or sinus aspirate) unlikely to yield neisseria are pathognomonic for *M. catarrhalis*.

They are strict aerobes and, like other *Moraxella*, they are oxidase- and catalase-positive. They are asaccharolytic and negative for indole and hydrogen sulfide. They reduce nitrate and nitrite. Importantly, they produce DNase and butyric acid esterase, enabling their differentiation from *Neisseria* species. The organism is not fastidious. It produces 1–3-mm whitish-gray colonies on blood and chocolate agars within 24 h of incubation.

Although their optimum growth temperature is 35–37°C, they are capable of growing well at 28–30°C. The colonies usually remain intact when manipulated with a bacteriological loop, hence the similarity to a hockey puck on a flat surface. Some strains of *M. catarrhalis* are capable of growing on Thayer–Martin agar and other colistin-containing agars (Blackwell et al. 1978; Doern et al. 1981; Durussel and Siegrist 1988). A selective medium containing acetazolamide and antibiotics has been described (Vaneechoutte et al. 1988) (Table 2).

The species has had an interesting taxonomic and clinical history. Initially, Seifert, who described a case of purulent tracheobronchitis and penned the putative agent as *Micrococcus catarrhalis*, defined the species as a *Micrococcus* in 1882. In the following years, the species was subsequently transferred to the *Neisseria* and then to the *Branhamella*, before being placed within the *Moraxella*. Ghon and Pfeiffer (1902) recognized the species as a pathogen. Shortly thereafter, investigators in both Europe and the USA (Benzancon 1905; Jordan 1908; Hiss and Zinsser 1910) confirmed its pathogenic potential. In 1921, Gordon attempted to define a relationship between the organism and the common cold. Failing to find any association, he submitted that *M. catarrhalis* should be considered as a commensal. Interestingly, Berger (1963) and later Knapp et al. (1984) realized that part of the problem in defining the pathogenic nature of *M. catarrhalis* was the organism's frequent misidentification with the respiratory commensal *Neisseria cinerea*. Detailed reviews of this species have been published (Catlin 1990; Verghese and Berk 1991; Enright and McKenzie 1997; Verduin et al. 2002).

Epidemiology and clinical significance

M. catarrhalis is seldom recovered from the oropharynx of healthy adults (approximately 5 percent): however, higher carriage rates in children and the elderly (approximating 50 and 25 percent, respectively) have been demonstrated (Knapp and Hook 1988; Vaneechoutte et al. 1990; Ejlertsen et al. 1994). In infants, it appears that seasonal nasopharyngeal colonization, peaking in winter, is common. Infections are thought to represent an extension of a colonized area. Studies by Murphy (1996) using various molecular techniques discuss the dynamics of this process. It has been shown to be a frequent cause of lower respiratory tract infections (e.g. pneumonia and tracheobronchitis) in adults and sinusitis and otitis media in children.

Giebink (1989) estimated that approximately 10–15 percent of all cases of otitis media were caused by *M. catarrhalis*. Similar rates have been attributed for cases of sinusitis (Jousimies-Somer et al. 1989). Significantly, it appears that patients with underlying chronic pulmonary disease are most affected (Klingman et al. 1995). Doern (1986) suggested that it is the third-ranking pathogen in this particular patient population following *Streptococcus pneumoniae* and *Haemophilus influenzae*. It is suggested that respiratory infections due to *M. catarrhalis* occur primarily in the winter months (DiGiovanni et al. 1987; Mbaki et al. 1987; Davies and Maesen 1988). *M. catarrhalis* has also been reported to cause keratitis, suppurative arthritis, bacteremia, meningitis, endocarditis, and urogenital infections. Additionally, it has been established as a nosocomial respiratory pathogen (Patterson et al., 1988; Richards et al. 1993).

Virulence and pathogenic factors

Numerous cell components have been studied that are putative virulence factors. Hellio et al. (1988) demonstrated the presence of a polysaccharide capsule. LPS have been studied in detail (Fomsgaard et al. 1991; Masoud et al. 1994). This cell-wall component was shown to have marked similarities to that of *N. gonorrhoeae* in that the LPS has no long repeating units and that no blebs or vesicles are released from the outer membrane (Murphy 1989, 1990; Murphy and Bartos 1989; Murphy and Loeb 1989).

There also appears to be a homogeneity among the strains for both LPS and the outer-membrane proteins (OMP). Antigenic characterization has determined that the antigenic nature of the OMPs is somewhat conserved among the various strains. This has been an active area for research into potential vaccines (Bartos and Murphy 1988; Murphy and Loeb 1989).

It appears that properties pertaining to serum resistance, fimbriation, and hemagglutination vary among strains. Wistreich and Baker (1971), using several ATCC strains, reported that *M. catarrhalis* lacked fimbriae and failed to agglutinate human erythrocytes. Murphy (1989) disputed the absence of fimbriae with his studies using clinical isolates, and Soto-Hernandez et al. (1989) observed their ability to agglutinate human erythrocytes. Jordan et al. (1990) found that 43 percent of disease-producing isolates were serum-resistant, while only 13 percent of colonizing strains were serum-resistant.

Investigators have found that the bacteria contain pili. As with other bacteria, the pili are presumed to facilitate the adherence of the bacterium to the epithelial cells of the host (Ahmad et al. 1996).

Therapeutic options and susceptibility testing

Before 1970, all strains of *M. catarrhalis* were susceptible to penicillin and were β-lactamase negative (Barber and Waterworth 1962; Catlin and Cunningham 1964; Baumann et al. 1968a; Ahmad et al. 1987). In 1977, the presence of a β-lactamase was first reported in *M. catarrhalis* in Sweden, followed in France and England

(Malmvall et al. 1977; Percival et al. 1977; Philippon et al. 1986). In the subsequent years, the ability to produce β-lactamase developed very rapidly and simultaneously around the world. In 1990, Wallace et al. after reviewing strains stored at the Centers for Disease Control Prevention reported an approximate increase in the ability to produce β-lactamase from 40 to 75 percent during the latter 1970s. During the LIBRA Surveillance initiative, in the 1249 isolates that were collected from 290 participating laboratories β-lactamase production was demonstrated in 94.2 percent of these strains. Three types of β-lactamase have been defined, BRO-1, BRO-2, and BRO-3. These were shown to be inducible and cell-membrane-associated. Approximately 90 percent of the β-lactamase-producing strains *M. catarrhalis* possess the BRO-1 enzyme (Wallace et al. 1989, 1990; McGregor et al. 1998). This organism is susceptible to β-lactamase-stable penicillins, cephalosporins, cephamycins, tetracyclines, trimethoprim-sulfamethoxazole, fluoroquinolones, aminoglycosides, and macrolides. It should be considered uniformly resistant to penicillin, ampicillin, methicillin, vancomycin, and clindamycin. Approved standards and antimicrobial susceptibility are unavailable; therefore, treatment for *M. catarrhalis* infections is usually empiric.

A significant area of recent research involves the production of a viable multivalent vaccine for the prevention of otitis media. Logical target components would include not only *M. catarrhalis* but also *S. pneumoniae* and the nontypable strains of *H. influenzae*. This progress has been reviewed by McMichael (2000) and Snow (2002).

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Veillonella and other anaerobic gram-negative cocci

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CLASSIFICATION

The Veillonellaceae are a family of gram-negative obligate anaerobic cocci that are divided into three recognized genera: *Acidaminococcus*, *Megasphaera*, and *Veillonella*. All three genera are nonmotile, nonsporing and do not produce oxidase. These genera can be differentiated by their mol% guanine and cytosine (G+C) of 56.6, 53.1–54.1, and 40.3–44.4 percent, respectively. Analysis of their end-products of metabolism can also discriminate between these genera, with *Veillonella* species producing acetic and propionic acids, *Acidaminococcus* producing acetic and butyric acids, and *Megasphaera* producing acetic, propionic, isobutyric, butyric, isovaleric, valeric, and caproic acids. *Acidaminococcus* is distinct in its ability to ferment amino acids as its major energy source and is asaccharolytic, as mainly are *Veillonella* spp., while *Megasphaera* can ferment a few simple carbohydrates.

The genus *Veillonella* was first described by Veillon and Zuber (1898) under the epithet *Staphylococcus parvulus*. It was subsequently renamed by Prevot (1933), in honor of the senior author of the original description. *Peptostreptococcus elsdenii* was described by Gutierrez et al. in 1959, but Rogosa (1971) subsequently suggested that it should be reclassified as a new genus *Megaspheara*. Rogosa had also previously described the genus *Acidaminococcus* in 1969. *Acidaminococcus* consists of only one species, *A. fermentans*, while *Megaspheara* consists of three species and *Veillonella* currently contains seven species although eight serogroups have been described (Mays et al. 1982).

Recently, a new member of the family has been proposed by Carlier et al. (2002) who described *Anaeroglobus germinatus* gen. nov., sp. nov. This gram-negative coccus was isolated from a post-operative fluid collection, and analysis by both phylogenetic and phenotypic methods led the authors to suggest that this organism represented a novel lineage within the family Veillonellaceae. Table 1 summarizes a few simple tests that can differentiate between the three recognized genera comprising the Veillonellaceae.

HABITAT

Veillonella spp. are essentially residents of the oral cavities of man and other mammals, especially rodents. They may reside in several niches within the oral cavity including the buccal mucosa, subgingival plaque, and in particular, the dorsum of the tongue, to which Gibbons and van Houte (1975) found they adhered particularly well. Sutter (1984) found they were the most numerous anaerobic bacterium present in a study of adult human saliva. They are also present on the oral mucosal surfaces and in the saliva of 2-month-old edentulous infants, and at 1 year of age 100 percent of infants harbored *Veillonella* spp. (Kononen et al. (1999). As veillonellae are frequently swallowed, they may be

Table 1 *Simple differential characteristics of* Acidaminococcus, Megasphaera, *and* Veillonella

Characteristic	*Acidaminococcus*	*Megaspheara*	*Veillonella*
Cell morphology (diameter in microns)	0.6–1.0	2.4–2.6	0.3–0.5
Fermentation of carbohydrates	–	+	– (w)
UV red fluorescent colonies	–	–	+
Nitrate reduction	–	–	+

UV, ultraviolet.

present in the upper respiratory tract and form part of the intestinal microflora. They may also be found in low numbers in urogenital sites. In man, three species have been described, namely *V. parvula*, *V. dispar*, and *V. atypica*. Other mammals harbor different species that are sometimes species-specific in their host–parasite relationship. For example, *V. criceti* are found only in hamsters (*Cricetus* spp.), *V. caviae* only in guinea pigs (*Cavia* spp.), and *V. ratti* only in rats (*Rattus* spp.). However, other species such as *V. rodentium* may be found in the oral cavities of either rats, rabbits, or hamsters.

Acidaminococcus fermentans is found in the intestinal tracts of a variety of mammals including man. *Megasphaera* contains three species: *M. elsdenii*, *M. cerevisiae*, and a recently proposed new species, *M. micronuciformis*. *M. elsdenii* is a commensal of pigs' cecae and may be found in the rumen of cattle and sheep. It has been found in relatively large numbers in over 10 percent of human feces sampled by Werner (1973) and Sugihara et al. (1974). *M. cerevisiae* was first described in 1985 by Engelmann and Weiss as an isolate from spoiled beer. Two isolates of *M. micronuciformis* sp. nov. were described by Marchandin et al. (2003) from human clinical material. These isolates both had 99.8 percent 16S rDNA sequence homology with a *Megasphaera* sp. (oral clone BU057) but had only 94.5 and 93.8 percent sequence homology with *M. elsdenii*, and *M. cerevisiae*, respectively. They also differed enough in terms of biochemical reactions and metabolic end-products to suggest they belonged to a novel species.

ROLE IN NORMAL FLORA

There is much interest in the role of nitrate and nitrite in the human diet, in which evidence is emerging that *Veillonella* spp. play an integral role. A recent review by Archer (2002) reported that 93 percent of ingested nitrite originates from saliva. He explained that nitrate, which we ingest predominantly from green-leafed vegetables in our diet, is absorbed from the gut into blood plasma. It then concentrates in the salivary glands from where it is secreted into the oral cavity and converted to nitrite by the action of nitrate-reducing bacteria. As *Veillonella* spp. are found in very high numbers in saliva (Sutter 1984) and on the surface of the tongue, and are strong reducers of nitrate (Atsumi and Ueha 1977, 1978), it is believed they play a key role in this process. Nitrite is then either swallowed where it is reduced by gastric acidity to nitric oxide (NO) which has known antibacterial properties, or acidified in situ to NO by acid-producing oral bacteria. The effect of amoxicillin on salivary nitrate concentration was investigated by Dougall et al. (1995) who noted a significant drop in salivary nitrite compared to controls indicating removal of nitrate-reducing bacteria by the antibiotic. They suggested that removal of nitrate reducers and hence a reduction in NO may explain the overgrowth of oral *Candida* spp. sometimes seen in these patients. Allaker et al. (2001) demonstrated a nitrite-related in vitro effect on the growth and survival of bacteria implicated in periodontal disease, namely: *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Eikinella corrodens*. Nitrite is also excreted in sweat on the surface of the skin and Weller et al. (2001) performed experiments on acidified nitrite from sweat that showed enhanced antimicrobial activity on bacterial skin pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes*, and fungi such as *Trichophyton mentagrophytes*. If subsequent studies prove that nitrite acts in synergy with gastric acidity as a barrier to gastrointestinal pathogens, and plays a role in the defense of our teeth and skin, it would highlight the potential importance of *Veillonella* spp. in playing a key role in human innate immunity.

Little is known about the specific roles of *Acidaminococcus* and *Megasphaera* in the human gut ecosystem.

ROLE OF VEILLONELLACEAE AS OPPORTUNIST PATHOGENS

Infrequently, *Veillonella* spp. may be isolated from various sites of human infection but are often in mixed culture. Brook (1996) reviewed infections in children due to *Veillonella* and reported an overall incidence of 4 percent. They were isolated most frequently in mixed culture from abscesses, cases of aspiration pneumonia, burns, bites, and sinus infections. In 5 percent of cases however, they were in pure culture. Tan et al. (2001) also reported *Veillonella* spp. in mixed culture in children's neck infections. Rare examples of *Veillonella* infections in pure culture are occasionally found in the literature, such as a prosthetic joint infection due to *V. dispar* (Marchandin et al. 2001), endocarditis due to *V. dispar* (Loughrey and Chew 1990), and osteomyelitis

due to a *Veillonella* sp. (Barnhart et al. 1983). Meningitis due to *Veillonella* spp. is also extremely rare and the most recent case was described by Bhatti and Frank (2000) in which *V. parvula* was recovered in pure culture from the cerebrospinal fluid (CSF) of a 47-year-old woman secondary to chronic sinusitis.

Veillonella spp. may be found in association with the formation of dental caries although they are not believed to be causative. They form co-aggregates with cariogenic streptococci, and are believed to ameliorate the effect of lactic acid which is known to be cariogenic, by converting it to less harmful acids such as acetic and propionic acid.

Acidaminococcus and *Megasphaera* are very rarely associated with human infection. Isolated case histories of infections with these organisms can be found in the literature, such as Brancaccio and Legendre's description of a case of endocarditis due to *M. elsdenii* (1979), and Sugihara et al.'s (1974) description of *Acidaminococcus* isolated as part of a mixed flora from a closed abdominal abscess and a putrid lung abscess.

VIRULENCE FACTORS

In common with the gram-positive anaerobic cocci, there has been relatively little study of the virulence mechanisms of the Veillonellae and even less on *Acidaminococcus* and *Megasphaera*. The cell walls of *Veillonella* spp. are known to contain a highly endotoxic lipopolysaccharide (LPS). This LPS has an unusually high lipid content of 60 percent, but in agglutinin titration experiments on rabbits was found not to be highly antigenic (Delwiche et al. 1985). However, Heath et al. (1987) reported that LPS and lipoteichoic acid from *Veillonella* induced human monocytes to release collagenase thus inducing cytokine production. In a monkey model, Warfvinge et al. (1985) showed that *Veillonella* LPS could induce migration of polymorphonuclear leukocytes, and Nagashima (1990) reported a similar affect on monocytes in vitro.

CULTURAL CHARACTERISTICS

All Veillonellaceae are obligately anaerobic, and in common with most other anaerobic genera are susceptible to the anti-anaerobic agent metronidazole. The optimum growth temperature is 30–37°C. As chemoorganotrophs, they require complex and nutritious media for growth in vitro and some species of *Veillonella* have specific growth requirements for the amino acids cadaverine and/or putrescine.

Veillonella spp.

Colonies of all *Veillonella* species are morphologically similar. They are small, 0.5–1.0 mm in diameter, round, entire with a raised center, and are transparent or opaque with a gray–greenish appearance on blood-containing media. Chow et al. (1975) demonstrated that while *Acidaminococcus* and *Megaspheara* colonies did not fluoresce under illumination with long wave (365 nm) ultraviolet light, colonies of *Veillonella* species produced a brick-red fluorescence. Brazier and Riley (1988) later demonstrated that this pigment was a porphyrin whose fluorescence was best seen on brain–heart infusion blood agar examined directly on removal from the anaerobic atmosphere. This property was shared by seven different *Veillonella* species, but one in particular, *V. criceti*, produced a strong fluorescence on all the media tested.

On Gram staining, cells of *Veillonella* are very small (<0.5 microns) cocci in clumps, pairs or short chains. Differentiation between *Veillonella* species and a closely related anaerobe, *Dialister pneumosintes*, is difficult on microscopical features alone, but the latter do not reduce nitrate and differ in their metabolic end-products.

Certain strains produce catalase, and *V. alcalescens* was a synonym used for catalase producing variants of *V. parvula*.

Acidaminococcus fermentans

After 48-h incubation, surface colonies of *A. fermentans* are very small, 0.1–0.2 mm in diameter, round, smooth, entire, and gray to translucent in color. In broth cultures, turbidity is uniform but may be restricted to the deeper layers away from the interface with the air.

Megaspheara elsdenii

Colonies after 4 days incubation are 0.5–2.0 mm in diameter, circular, with a glistening or rough surface. They may be butyrous in consistency or adhere to the medium. In agar-deep cultures the colonies are larger (4 mm) disk-shaped and honey-yellow in color.

Laboratory maintenance

Short-term storage of *Veillonella* spp. and other Veillonellaceae can be achieved by inoculation into good quality cooked meat medium and incubation at 37°C until visible growth appears, followed by storage of the medium at ambient temperature away from strong sunlight. For long-term storage, cryopreservation is preferable and freeze-drying is the best method. However, for laboratories not equipped with freeze-drying apparatus the use of commercially available cryobeads in vials stored at −80°C are perfectly adequate.

LABORATORY IDENTIFICATION

The identification of *Veillonella* to species level can be a problem for clinical or veterinary diagnostic micro-

biology laboratories. Presumptive identification to genus level can be made by Gram stain, (small gram-negative cocci <0.5 μm), absence of growth in air or 5 percent carbon dioxide in air, and a simple disk test for reduction of nitrate to nitrite. Differentiation to species level is best performed by a reference laboratory, involving detection of acetic and propionic acid metabolites by gas–liquid chromatography to confirm the genus, and further phenotypic or genotypic tests to speciate the isolate. Although veillonellae are generally nonfermentative, some strains of *V. criceti* may weakly ferment fructose enough to give a positive result (acid end-product) in sugar fermentation tests. Although some species produce catalase, *Veillonella* spp. are generally phenotypically unreactive and commercial kits designed for the identification of anaerobes may not yield an identification to species level, and may not even include those veillonellae of animal origin in their databases. However, with supplementation of a few simple tests and the use of a specialist anaerobe identification manual, reasonable results can be obtained for the identification of the common human isolates of *V. atypica*, *V. dispar*, and *V. parvula*. Due to their rarity, it is unlikely any isolates of *Megaspheara* or *Acidaminococcus* would be recognized by commercially available identification kits and should be referred to a reference laboratory for confirmation.

Genomic methods such as restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S ribosomal DNA (16SrDNA PCR RFLP) have been shown to differentiate between all the described species. Sato et al. (1997a) used universal 16S primers and PCR amplification on ATCC type strains of *V. atypica*, *V. caviae*, *V. criceti*, *V. dispar*, *V. parvula*, *V. ratti*, and *V. rodentium*, in combination with *Mnl*I and *Sau3*AI restriction endonucleases to produce species-specific RFLP profiles. A second paper by the same group used the restriction enzyme *Mnl*I alone and this proved efficacious in differentiating between the human oral isolates, *V. parvula*, *V. dispar*, and *V. atypica* (Sato et al. 1997b). This method has been evaluated and adopted for the identification of *Veillonella* spp. in the National Public Health Service Anaerobe Reference Laboratory for England and Wales (NPHS-ARL).

ANTIBIOTIC SUSCEPTIBILITIES

There are few specific data in the literature on the antibiotic susceptibilities of the Veillonellacea. Reig et al. (1997) reported high levels of resistance to penicillin amongst 40 clinical isolates of *Veillonella* spp. from Spain. Eighty-five percent had a minimum inhibitory concentration (MIC) above the NCCLS recommended breakpoint of 2 mg/l. They could not detect β-lactamase production however, and concluded that altered penicillin-binding proteins were responsible for its poor activity. The first description of a β-lactamase produced by an anaerobic coccus was that of Galan et al. (2000) who investigated a penicillin-resistant strain of *A. fermentans* isolated from a perianal abscess. By nucleotide sequence analysis they identified a new class A β-lactamase called ACL-1. This enzyme had less than 50 percent amino acid homology with any other previously described class A β-lactamases from gram-negative organisms, and the authors suggested it may have been obtained from a gram-positive organism by horizontal gene transfer.

The NPHS-ARL has a database of the antibiotic susceptibilities by disk testing of all the isolates referred for identification including *Veillonella* spp. Of 49 isolates currently in its database, there were 31 (63 percent) resistant to penicillin, slightly lower than the level reported from Spain. There were 12/49 (24 percent) resistant to amoxiclavulanic acid and surprisingly 2/49 (4 percent) resistant to metronidazole, but no resistance to clindamycin or chloramphenicol. On the basis of this and several published susceptibility studies, chloramphenicol, clindamycin, and metronidazole appear the most active agents against *Veillonella* spp. (Rolfe and Finegold 1981, 1982; Sutter and Finegold 1976).

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The Enterobacteriaceae: general characteristics

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INTRODUCTION

The family Enterobacteriaceae is one of the most important bacterial families. It includes the plague bacillus *Yersinia pestis* and the typhoid bacillus *Salmonella* serotype Typhi (*Salmonella typhi*) which are two of the most important bacterial pathogens in human history. It also includes two genera of intrinsic enteric pathogens *Shigella* and *Salmonella*; essentially all strains in these two genera can cause diarrhea or intestinal infections (Centers for Disease Control and Prevention (Anonymous) 2003). Two other genera, *Escherichia* (Gamage et al. 2003; Misselwitz et al. 2003; Naimi et al. 2003; Riley et al. 1983; Vallance et al. 2002) and *Yersinia*, also include enteric pathogens; however, only a few of the many serotypes (strains) have the virulence factors that enable them to infect the intestinal tract or allow them to colonize it and produce enterotoxins. Many other species have an association with diarrhea (Murata et al. 2001), but their causal role is uncertain. Several other species of Enterobacteriaceae frequently cause extraintestinal human infections (Diekema et al. 1999; Edmond et al. 1999; O'Hara et al. 2000a) and some have an association with chronic diseases such as arthritis (Yu and Kuipers 2003). Many other species occasionally cause infections or occur in human clinical specimens, and often it is difficult to determine their clinical significance. Other genera and species do not cause disease or occur in human clinical specimens, but can be confused with those species that do. The family also includes plant and animal pathogens and a number of species that have been favorite subjects for studies on genetics, physiology, metabolism, antibiotic resistance, ecology, epidemiology, pathogenicity, virulence, and vaccines.

In this tenth edition of *Topley and Wilson*, the material on Enterobacteriaceae is divided in a manner similar to the ninth edition. One difference is that the chapter *Escherichia and Shigella* has been divided; each genus now has its own chapter. A second difference is that *Plesiomonas* is now classified in the family (see section entitled Inclusion of the genus *Plesiomonas*); in previous editions, this oxidase-positive species was included in chapters with two oxidase-positive relatives, *Vibrio* and *Aeromonas*.

The introductory chapter on Enterobacteriaceae has changed only slightly over the last several editions of this book; however, our revision is a major one. We have updated some of the material covered in the ninth edition, but felt that it was equally important to add new sections and recent references. Because of space limitations and because they are fully discussed in the following six chapters (*Escherichia*; *Shigella*; *Salmonella*; *Proteus*, *Morganella*, and *Providencia*;

Yersinia; and *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia*, and other *Enterobacteriaceae*), many topics in the present chapter are only briefly mentioned. The statement 'see ... in the chapter(s) that follows for more details and references' is implied in every instance. Many other chapters in the tenth edition have useful information on the Enterobacteriaceae. Those chapters of particular interest are the ones that cover: history; cell structure and function; taxonomy and nomenclature; diversity; genetics; bacteriophages; antibiotics; water; milk and foods; normal flora; pathogenicity and virulence; typhoid and paratyphoid; bacillary dysentery; *E. coli* diarrhea; acute enteritis; food-borne illnesses; food poisoning; plague and other *Yersinia* infections; infections of the urinary tract; and hospital-acquired infections.

For a historical approach to the family and for invaluable basic information on isolation, identification, and serotyping, the classic books by Edwards and Ewing (1972), Ewing (1986), and Kauffmann (1969) are essential reading. The more recent book *The Enterobacteria* by Janda and Abbott (1998) covers the whole family, and is also highly recommended. Other books and encyclopedias (Lederberg 2000) cover many specific topics such as basic biology, nomenclature, and classification (Boone et al. 2001; Brenner et al. 2005), laboratory media and methods (Atlas and Parks 1997; Balows et al. 1988; Murray et al. 2003), infectious diseases (Farthing and Keusch 1988; Mandell et al. 2000), and hospital (nosocomial) infections (Mayhall 2000). Also, try searching www.booksinprint.com (not free, but available in many libraries).

Several reviews and chapters also cover the entire family and are recommended (Balows et al. 1992; Brenner 1992a, b, 1984; Brenner and Farmer 2005; Farmer et al. 1985; Farmer 2003; Krieg 1984). Many articles, books, chapters, reviews, and internet sites cover the individual genera and species in the family, and are discussed in the following six chapters (Chapters 52–57).

Phenotypic definition

Most genera and species in the family Enterobacteriaceae share the following properties: they are small gram-negative rods; do not have unusual cell structures or produce spores; are motile by peritrichous flagella; grow on ordinary laboratory media formulated with peptones or meat extracts; grow both aerobically and anaerobically; grow without the addition of sodium chloride or other supplements; grow well on MacConkey agar; are active biochemically; ferment D-glucose and other sugars, often with gas production; are catalase positive and oxidase negative; reduce nitrate to nitrite; contain the enterobacterial common antigen (eca); and have a 39–59 percent guanine-plus-cytosine (G + C) content of DNA (Balows et al. 1992; Brenner et al. 2005), with most of the genera in the range 49–59 mol%. Table 1 expands on this definition, and gives quantitative data and the exceptions. It also lists the species most frequently isolated from human and animal diseases and those that occur in clinical specimens. Table 2 (p. 14) compares Enterobacteriaceae with three genera that are close relatives.

Host-adapted species that are uncultivable, difficult to culture, or slow growing have evolved in some genera (Table 3, p. 14). A number of new or unusual species that have not yet been isolated from human clinical specimens have been reported (Table 3).

Molecular and genetic definition, relatives, population structure, and evolution

When techniques such as DNA–DNA hybridization and 16S r-RNA sequencing (Fox et al. 1992; Stackebrandt and Goebel 1994) that measure evolutionary distance are used, genera and species in the family are more closely related to *Escherichia coli*, the type species of the type genus of the family, than they are to organisms in other families. The phylogenetic position of the family Enterobacteriaceae is in the gamma subclass of *Proteobacteria*, a subclass also known as the 'purple bacteria' (Figures 1 and 2, pp. 15 and pp. 16).

The figures show that the closest relatives of Enterobacteriaceae are the families Alteromonadaceae, Vibrionaceae, Aeromonadaceae, and Pasteurellaceae.

The use of DNA–DNA hybridization (Brenner and Farmer 2005; Brenner 1984; Farmer et al. 1985) has greatly expanded the number of species in the family and has led to a basic understanding of the phylogenic relationship of the genera and species (Boyd et al. 1996; Brunder and Karch 2000; Ochman and Groisman 1995; Oelschlaeger et al. 2002; Oyston et al. 2003; Szakal et al. 2003; Vallance et al. 2002; Vulic et al. 1997). 16S rRNA sequencing data have been very helpful in defining phylogenetic relationships at the level of genus and family. Multilocus enzyme electrophoresis (MEE) has been used to define population structure and evolutionary relationships in *Escherichia coli–Shigella* (Dobrindt et al. 2003; Neidhardt 1996), *Salmonella* (Neidhardt 1996), *Yersinia*, and in several other genera, species, and serotypes. Several evolutionary pathways are now understood: the evolution of *Shigella* from *Escherichia coli*; the evolution of *Escherichia coli* O157 from *Escherichia coli* O55:H7 (Whittam et al. 1993) and the evolution of *Yersinia pestis* from *Yersinia pseudotuberculosis* which happened relatively recently.

History

An excellent way to follow the history of the family Enterobacteriaceae is to start with the first edition of *Topley & Wilson* and follow the evolution of genus and species names and individual topics through its ten

Table 1 *Biochemical reactions [a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b]*

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation
*Budvicia aquatica**[b]	0	93	0	0	80	33	0	0	0	0	27	0	0	0	100	53	87	0	60	0	0	0	0	0
Buttiauxella agrestis	0	100	0	100	0	0	0	0	0	100	100	0	80	60	100	100	100	0	100	0	100	0	0	0
Buttiauxella brennerae	0	100	0	0	0	0	0	0	0	33	100	0	100	100	100	100	0	0	100	0	100	67	0	0
Buttiauxella ferragutiae	0	100	0	0	0	0	0	100	0	80	60	0	40	0	100	100	0	0	100	0	100	0	0	100
Buttiauxella gaviniae	0	100	0	20	0	0	0	0	20	0	80	0	60	100	100	40	60	0	100	0	100	100	0	0
Buttiauxella izardii	0	100	0	0	0	0	0	0	0	100	100	0	67	100	100	100	100	0	100	0	100	0	0	0
*Buttiauxella noackiae**	33	100	0	33	0	0	100	0	67	0	100	0	100	100	100	100	0	0	100	0	100	0	0	0
Buttiauxella warmboldiae	0	100	0	33	0	0	100	0	0	0	100	0	33	100	100	100	0	0	100	0	100	0	67	0
*Cedecea davisae**	0	100	50	95	0	0	0	0	50	95	95	0	86	91	100	70	19	100	100	0	99	0	0	0
*Cedecea lapagei**	0	40	80	99	0	0	0	0	80	0	80	0	100	99	100	100	60	0	100	0	100	0	0	0
*Cedecea neteri**	0	100	50	100	0	0	0	0	100	0	100	0	65	100	100	100	35	100	100	0	100	0	0	100
Cedecea species 3*	0	100	50	100	0	0	0	0	100	0	100	0	100	0	100	100	0	50	100	0	100	0	0	0
Cedecea species 5*	0	100	50	100	0	0	0	0	50	50	100	0	100	0	100	100	0	100	100	0	100	0	0	100
*Citrobacter freundii**	33	100	0	78	78	44	0	0	67	0	89	0	89	11	100	89	78	89	100	11	0	0	0	100
*Citrobacter diversus (C. koseri)**	99	100	0	99	0	75	0	0	80	99	95	0	0	95	100	98	50	40	99	40	15	99	0	99
*Citrobacter amalonaticus**	100	100	0	95	5	85	0	0	85	95	95	0	99	1	100	97	35	9	100	1	30	0	0	99
*Citrobacter farmeri**	100	100	0	10	0	59	0	0	85	100	97	0	93	0	100	96	15	100	100	2	9	0	0	98
*Citrobacter youngae**	15	100	0	75	65	80	0	0	50	5	95	0	95	5	100	75	25	20	100	85	10	0	5	100
*Citrobacter braakii**	33	100	0	87	60	47	0	0	67	93	87	0	100	0	100	93	80	7	100	33	0	0	0	100
*Citrobacter werkmanii**	0	100	0	100	100	100	0	0	100	0	100	0	100	100	100	100	17	0	100	0	0	0	0	100
*Citrobacter sedlakii**	83	100	0	83	0	100	0	0	100	100	100	0	100	100	100	100	100	0	100	100	17	0	0	100
*Citrobacter rodentium**	0	100	0	0	0	100	0	0	0	100	0	0	0	100	100	100	100	0	100	0	0	0	0	100
*Citrobacter gillenii**	0	100	0	33	67	0	0	0	33	0	67	0	100	100	100	100	67	33	100	0	0	0	0	100
*Citrobacter murliniae**	100	100	0	100	67	67	0	0	67	0	100	0	100	0	100	100	67	33	100	100	33	0	0	100
*Edwardsiella tarda**	99	100	0	1	100	0	0	100	0	100	98	0	0	0	100	100	0	0	0	0	0	0	0	0
Edwardsiella tarda biogroup 1*	100	100	0	0	0	0	0	100	0	100	100	0	0	0	100	50	0	100	100	0	0	0	0	0
*Edwardsiella hoshinae**	50	100	0	0	0	0	0	100	0	95	100	0	0	100	100	35	0	100	100	0	50	0	0	0

(*Continued over*)

Table 1 *Biochemical reactions [a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation
Edwardsiella ictaluri	0	0	0	0	0	0	0	100	0	65	0	0	0	0	100	50	0	0	0	0	0	0	0	0
*Enterobacter aerogenes**	0	5	98	95	0	2	0	98	0	98	97	0	98	95	100	100	95	100	100	5	100	98	95	100
Enterobacter cloacae complex*	0	5	100	100	0	65	0	0	97	96	95	0	98	75	100	100	93	97	100	15	75	25	15	95
'Enterobacter agglomerans complex'*	20	50	70	50	0	20	20	0	0	0	85	2	35	65	100	20	40	75	100	15	65	7	15	30
*Enterobacter gergoviae**	0	5	100	99	0	93	0	90	0	100	90	0	0	96	100	98	55	98	99	0	99	0	0	0
*Enterobacter sakazakii**	11	5	100	99	0	1	50	0	99	91	96	0	99	18	100	98	99	100	100	5	99	0	75	0
*Enterobacter taylorae (E. cancerogenus)**	0	5	100	100	0	1	0	0	94	99	99	0	98	100	100	100	10	0	100	0	92	0	0	1
Enterobacter amnigenus biogroup 1*	0	7	100	70	0	0	0	0	9	55	92	0	100	91	100	100	70	100	100	0	91	0	0	9
Enterobacter amnigenus biogroup 2*	0	65	100	100	0	0	0	0	35	100	100	0	100	100	100	100	35	0	100	0	100	0	0	100
*Enterobacter asburiae**	0	100	2	100	0	60	0	0	21	95	0	0	97	3	100	95	75	100	100	0	100	0	0	100
*Enterobacter hormaechei**	0	57	100	96	0	87	4	0	78	91	52	0	100	100	100	83	9	100	100	87	44	0	0	0
*Enterobacter intermedius**	0	100	100	65	0	0	0	0	0	89	89	0	65	100	100	100	100	65	100	100	100	0	0	100
Enterobacter cancerogenus	0	0	100	100	0	0	0	0	100	100	100	0	100	100	100	100	0	0	100	0	100	0	0	0
Enterobacter dissolvens	0	0	100	100	0	100	0	0	100	100	0	0	100	100	100	100	0	100	100	0	100	0	0	100
Enterobacter nimipressuralis	0	100	100	0	0	0	0	0	0	100	0	0	100	100	100	100	0	0	100	0	100	0	0	100
Enterobacter pyrinus	0	29	86	0	0	86	0	100	0	100	43	0	0	86	100	100	14	100	100	0	100	0	100	0
*Escherichia coli**	98	99	0	1	1	1	0	90	17	65	95	0	3	0	100	95	95	50	98	60	40	5	1	94
Escherichia coli, inactive*	80	95	0	1	1	1	0	40	3	20	5	0	1	0	100	5	25	15	93	40	10	3	1	75
*Escherichia fergusonii**	98	100	0	17	0	0	0	95	5	100	93	0	0	35	100	95	0	0	98	60	65	98	0	0
*Escherichia hermannii**	99	100	0	1	0	0	0	6	0	100	99	0	94	0	100	97	45	45	100	19	40	0	0	0
*Escherichia vulneris**	0	100	0	0	0	0	0	85	30	0	100	0	15	85	100	97	15	8	100	0	30	0	0	1
Escherichia blattae	0	100	0	50	0	0	0	100	0	100	0	0	0	100	100	100	0	0	0	0	0	0	0	0
Shigella dysenteriae (Group A)*	45	99	0	0	0	0	0	0	2	0	0	0	0	0	100	0	0	0	0	5	0	0	0	30
Shigella flexneri (Group B)*	50	100	0	0	0	0	0	0	5	0	0	0	0	0	100	3	1	1	95	1	0	0	0	29

(*Continued over*)

Table 1 *Biochemical reactions [a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation
Shigella boydii (Group C)*	25	100	0	0	0	0	0	0	18	2	0	0	0	0	100	0	1	0	97	5	0	0	0	43
Shigella sonnei (Group D)*	0	100	0	0	0	0	0	0	2	98	0	0	0	0	100	0	2	1	99	0	0	0	0	2
*Ewingella americana**	0	84	95	95	0	0	0	0	0	0	60	0	5	0	100	0	70	0	100	0	80	0	0	0
*Hafnia alvei**	0	40	85	10	0	4	0	100	6	98	85	0	95	50	100	98	5	10	99	0	13	0	0	0
Hafnia alvei biogroup 1	0	85	70	0	0	0	0	100	0	45	0	0	0	45	100	0	0	0	55	0	55	0	0	0
*Klebsiella pneumoniae**	0	10	98	98	0	95	0	98	0	0	0	0	98	93	100	97	98	99	99	30	99	90	95	99
*Klebsiella oxytoca**	99	20	95	95	0	90	1	99	0	0	0	0	97	98	100	97	100	100	99	55	100	99	98	99
*Klebsiella ornithinolytica**	100	96	70	100	0	100	0	100	0	100	0	0	100	100	100	100	100	100	100	10	100	100	95	100
*Klebsiella planticola**	20	100	98	100	0	98	0	100	0	0	0	0	100	100	100	100	100	100	100	15	100	100	100	92
*Klebsiella ozaenae**	0	98	0	30	0	10	0	40	6	3	0	0	88	3	100	50	30	20	100	2	97	97	55	65
*Klebsiella rhinoscleromatis**	0	100	0	0	0	0	0	0	0	0	0	0	80	95	100	0	0	75	100	0	98	100	95	100
Klebsiella terrigena	0	60	100	40	0	0	0	100	0	20	0	0	100	100	100	80	100	100	100	20	100	100	80	100
*Kluyvera ascorbata**	92	100	0	96	0	0	0	97	0	100	98	0	92	96	100	93	98	98	100	25	100	0	0	40
*Kluyvera cryocrescens**	90	100	0	80	0	0	0	23	0	100	90	0	86	86	100	95	95	81	95	0	100	0	0	45
*Kluyvera georgiana**	100	100	0	100	0	0	0	100	0	100	100	0	83	50	100	17	83	100	100	33	100	0	0	0
*Leclercia adecarboxylata**	100	100	0	0	0	48	0	0	0	0	79	0	97	93	100	97	93	66	100	86	100	93	0	0
*Leminorella grimontii**	0	100	0	100	100	0	0	0	0	0	0	0	0	0	100	33	0	0	0	83	0	0	0	0
*Leminorella richardii**	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
*Moellerella wisconsensis**	0	100	0	80	0	0	0	0	0	0	0	0	70	0	100	0	100	100	60	0	0	100	0	0
Morganella morganii subsp. *morganii**	95	95	0	0	20	95	95	1	0	95	95	0	98	1	99	90	1	0	0	0	0	0	0	0
Morganella morganii biogroup 1*	100	95	0	0	15	100	100	100	0	80	0	0	90	5	100	93	0	0	0	0	0	0	0	0
Morganella morganii subsp. *sibonii**	50	86	0	0	7	100	93	29	0	64	79	0	79	0	100	86	0	7	0	0	0	0	0	0
Obesumbacterium proteus biogroup 2	0	15	0	0	0	0	0	100	0	100	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Pantoea dispersa	0	82	64	100	0	0	9	0	0	0	100	0	82	9	100	0	0	1	100	0	0	0	0	0

(*Continued over*)

Table 1 *Biochemical reactions* [a] *of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae*[a,b] *(Continued)*

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation
Photorhabdus luminescens (25°C)	50	0	0	50	0	25	0	0	0	0	100	50	0	0	75	0	0	0	0	0	0	0	0	0
*Photorhabdus asymbiotica**	0	0	0	20	0	60	0	0	0	0	100	80	20	0	100	0	0	0	0	0	0	0	0	0
*Plesiomonas shigelloides**	100	90	0	0	0	0	3	100	98	99	95	0	1	0	100	0	80	0	0	0	0	0	95	0
Pragia fontium	0	100	0	89	89	0	22	0	0	0	100	0	0	0	100	0	0	0	0	0	78	0	0	0
*Proteus mirabilis**	2	97	50	65	98	98	98	0	0	99	95	90	98	2	100	96	2	15	0	0	0	0	0	0
Proteus vulgaris complex*	98	95	0	15	95	95	99	0	0	0	95	91	99	0	100	85	2	97	0	0	50	0	0	0
*Proteus penneri**	0	100	0	0	30	100	99	0	0	0	85	50	99	0	100	45	1	100	0	0	0	0	0	0
Proteus myxofaciens	0	100	100	50	0	100	100	0	0	0	100	100	100	0	100	100	0	100	0	0	0	0	0	0
*Providencia rettgeri**	99	93	0	95	0	98	98	0	0	0	94	0	97	0	100	10	5	15	100	0	50	100	90	1
*Providencia stuartii**	98	100	0	93	0	30	95	0	0	0	85	0	100	0	100	0	2	50	10	0	2	5	95	1
*Providencia alcalifaciens**	99	99	0	98	0	0	98	0	0	1	96	0	100	0	100	85	0	15	2	0	1	98	1	1
*Providencia rustigianii**	98	65	0	15	0	0	100	0	0	0	30	0	100	0	100	35	0	35	0	0	0	0	0	0
Providencia heimbachae	0	85	0	0	0	0	100	0	0	0	46	0	8	0	100	0	0	0	0	0	0	92	46	0
*Rahnella aquatilis**	0	88	100	94	0	0	95	0	0	0	6	0	0	100	100	98	100	100	100	88	100	0	0	94
Salmonella Group I[c] strains*	1	100	0	95	95	1	0	98	70	97	95	0	0	0	100	96	1	1	100	96	0	0	35	95
Salmonella serotype Typhi*	0	100	0	0	97	0	0	98	3	0	97	0	0	0	100	0	1	0	100	0	0	0	0	99
Salmonella serotype Choleraesuis*	0	100	0	25	50	0	0	95	55	100	95	0	0	0	100	95	0	0	98	5	0	0	0	90
Salmonella serotype Paratyphi A*	0	100	0	0	10	0	0	0	15	95	95	0	0	0	100	99	0	0	100	90	0	0	0	95
Salmonella serotype Gallinarum*	0	100	0	0	100	0	0	90	10	1	0	0	0	0	100	0	0	0	100	90	0	0	0	1
Salmonella serotype Pullorum*	0	90	0	0	90	0	0	100	10	95	0	0	0	0	100	90	0	0	100	0	0	0	0	10
Salmonella Group II strains*	2	100	0	100	100	0	0	100	90	100	98	2	0	95	100	100	1	1	100	90	5	0	5	100
Salmonella Group IIIa strains*	1	100	0	99	99	0	0	99	70	99	99	0	1	95	100	99	15	1	100	0	0	0	0	99
Salmonella Group IIIb strains*	2	100	0	98	99	0	0	99	70	99	99	0	1	95	100	99	85	5	100	1	0	0	0	99
Salmonella Group IV strains*	0	100	0	98	100	2	0	100	70	100	98	0	95	0	100	100	0	0	98	0	60	5	0	100
Salmonella bongori (Group V)*	0	100	0	94	100	0	0	100	94	100	100	0	100	0	100	94	0	0	100	94	0	0	0	100

(*Continued over*)

Table 1 *Biochemical reactions [a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation
Salmonella Group VI strains*	0	100	0	89	100	0	0	100	67	100	100	0	0	0	100	100	22	0	100	67	0	0	0	0
*Serratia marcescens**	1	20	98	98	0	15	0	99	0	99	97	90	95	3	100	55	2	99	99	0	95	40	75	99
Serratia marcescens biogroup 1*	0	100	60	30	0	0	0	55	4	65	17	30	70	0	100	0	4	100	96	0	92	30	30	92
Serratia liquefaciens complex*	1	93	93	90	0	3	0	95	0	95	95	90	90	2	100	75	10	98	100	0	97	5	60	95
*Serratia rubidaea**	0	20	100	95	0	2	0	55	0	0	85	90	25	94	100	30	100	99	100	0	99	99	20	1
Serratia odorifera biogroup 1*	60	100	50	100	0	5	0	100	0	100	100	95	60	0	100	0	70	100	100	0	98	50	100	100
Serratia odorifera biogroup 2*	50	60	100	97	0	0	0	94	0	0	100	94	19	0	100	13	97	0	97	0	45	55	100	100
*Serratia plymuthica**	0	94	80	75	0	0	0	0	0	0	50	60	30	0	100	40	80	100	100	0	94	0	50	65
*Serratia ficaria**	0	75	75	100	0	0	0	0	0	0	100	100	55	0	100	0	15	100	100	0	100	0	55	100
Serratia entomophila	0	20	100	100	0	0	0	0	0	0	100	100	100	0	100	0	0	100	100	0	100	0	0	0
*'Serratia' fonticola**	0	100	9	91	0	13	0	100	0	97	91	0	70	88	100	79	97	21	100	91	100	100	30	100
*Tatumella ptyseos**	0	0	5	2	0	0	90	0	0	0	0	0	0	0	100	0	0	98	0	0	55	0	0	0
*Trabulsiella guamensis**	40	100	0	88	100	0	0	100	50	100	100	0	100	0	100	100	0	0	100	0	13	0	0	100
Xenorhabdus nematophilus	40	0	0	0	0	0	0	0	0	0	100	80	0	0	80	0	0	0	0	0	0	0	0	0
*Yersinia enterocolitica**	50	97	2	0	0	75	0	0	0	95	2	0	2	0	100	5	5	95	98	0	20	0	30	99
*Yersinia frederiksenii**	100	100	0	15	0	70	0	0	0	95	5	0	0	0	100	40	40	100	100	0	92	0	20	100
*Yersinia intermedia**	100	100	5	5	0	80	0	0	0	100	5	0	10	5	100	18	35	100	100	0	100	0	15	100
*Yersinia kristensenii**	30	92	0	0	0	77	0	0	0	92	5	0	0	0	100	23	8	0	100	0	15	0	15	100
*Yerrsinia rhodei**	0	62	0	0	0	62	0	0	0	25	0	0	0	0	100	0	0	100	100	0	0	0	0	100
Yersinia aldovae	0	80	0	0	0	60	0	0	0	40	0	0	0	0	100	0	0	20	80	0	0	0	0	60
*Yersinia bercovieri**	0	100	0	0	0	60	0	0	0	80	0	0	0	0	100	0	20	100	100	0	20	0	0	100
*Yersinia mollaretii**	0	100	0	0	0	20	0	0	0	80	0	0	0	0	100	0	40	100	100	0	20	0	0	100
*Yersinia pestis**	0	80	0	0	0	5	0	0	0	0	0	0	0	0	100	0	0	0	97	0	70	0	0	50
*Yersinia pseudotuberculosis**	0	100	0	0	0	95	0	0	0	0	0	0	0	0	100	0	0	0	100	0	25	0	0	0
*'Yersinia' ruckeri**	0	97	10	0	0	0	0	50	5	100	0	30	15	0	100	5	0	0	100	0	0	0	0	50
*Yokenella regensburgei** *(K. trabulsii)*	0	100	0	92	0	0	0	100	8	100	100	0	92	0	100	100	0	0	100	0	8	0	0	0

(*Continued over*)

Table 1 *Biochemical reactions [a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	Indole production	Methyl red	Voges–Proskauer	Citrate (Simmons)	Hydrogen sulfide (TSI)	Urea hydrolysis	Phenylalanine deaminase	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Motility	Gelatin hydrolysis (22°C)	Growth in KCN	Malonate utilization	D-Glucose, acid	D-Glucose, gas	Lactose fermentation	Sucrose fermentation	D-Mannitol fermentation	Dulcitol fermentation	Salicin fermentation	Adonitol fermentation	*myo*-Inositol fermentation	D-Sorbitol fermentation
Enteric Group 58*	0	100	0	85	0	70	0	100	0	85	100	0	100	85	100	85	30	0	100	85	100	0	0	100
Enteric Group 59*	10	100	0	100	0	0	30	0	60	0	100	0	80	90	100	100	80	0	100	0	100	0	0	0
Enteric Group 60*	0	100	0	0	0	50	0	0	0	100	75	0	0	100	100	100	0	0	50	0	0	0	0	0
Enteric Group 63	0	100	0	0	0	0	0	100	0	100	65	0	0	0	100	100	0	0	100	0	100	0	0	100
Enteric Group 64	0	100	0	50	0	0	0	0	50	0	100	0	100	100	100	50	100	0	100	0	100	100	0	0
Enteric Group 68*	0	100	50	0	0	0	0	0	0	0	0	0	100	0	100	0	0	100	100	0	50	0	0	0
Enteric Group 69	0	0	100	100	0	0	0	0	100	100	100	0	100	100	100	100	100	25	100	100	100	0	0	100
Enteric Group 137*	100	100	0	0	0	70	0	0	20	100	100	0	100	0	100	0	100	100	100	0	100	0	0	100

Organism	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	Alpha-methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
*Budvicia aquatica**[b]	80	0	100	0	93	0	0	0	0	0	0	27	0	20	27	0	0	0	100	0	93	0	0	0
Buttiauxella agrestis	100	100	100	100	100	100	100	0	0	100	100	0	60	100	60	0	0	0	100	0	100	0	100	0
Buttiauxella brennerae	100	100	33	100	100	100	100	0	0	100	100	67	67	67	0	0	0	0	100	0	100	0	100	0
Buttiauxella ferragutiae	100	0	100	100	100	100	100	40	0	100	0	0	0	60	0	0	0	0	100	0	100	0	100	0
Buttiauxella gaviniae	100	0	100	60	100	100	100	0	0	100	0	80	0	80	40	0	0	0	100	0	100	0	100	0
Buttiauxella izardii	100	33	100	100	100	100	100	0	0	100	67	0	33	100	67	0	0	0	100	0	100	0	100	0
*Buttiauxella noackiae**	100	0	100	100	100	100	100	33	0	100	0	0	0	100	100	0	0	0	100	0	100	0	100	0
Buttiauxella warmboldiae	100	0	100	100	100	100	100	0	0	100	0	0	0	0	0	0	0	0	100	0	100	0	100	0
*Cedecea davisae**	0	10	0	100	100	100	100	5	0	45	0	100	0	0	0	0	91	0	100	0	90	0	100	0

(*Continued over*)

Table 1 *Biochemical reactions* [a] *of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae*[a,b] *(Continued)*

Organism	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	Alpha-methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
*Cedecea lapagei**	0	0	0	100	0	100	100	0	0	100	0	100	0	0	0	60	100	0	100	0	99	0	100	0
*Cedecea neteri**	0	0	0	100	100	100	100	0	0	100	0	100	0	0	0	0	100	0	100	0	100	0	100	0
Cedecea species 3*	0	100	0	100	100	100	100	50	0	100	100	100	0	0	0	50	100	0	100	0	100	0	100	0
Cedecea species 5*	0	100	0	100	100	100	100	0	0	100	100	100	0	0	0	50	50	0	100	0	100	0	100	0
*Citrobacter freundii**	100	44	100	100	89	100	44	11	0	0	100	0	100	100	100	44	0	0	100	0	89	0	100	0
Citrobacter diversus (*C. koseri*)*	99	0	99	100	100	100	99	40	0	1	0	98	99	95	90	75	0	0	100	0	99	0	100	0
*Citrobacter amalonaticus**	99	5	100	99	99	100	100	2	0	5	0	0	60	96	96	86	0	0	99	0	97	0	100	0
*Citrobacter farmeri**	100	100	100	100	100	100	100	75	0	0	100	0	65	100	93	80	0	0	100	0	100	0	100	0
*Citrobacter youngae**	100	10	100	95	100	100	45	0	0	5	10	5	90	100	100	65	0	0	85	0	90	0	100	0
*Citrobacter braakii**	100	7	100	100	100	100	73	33	0	0	80	0	87	100	93	53	0	0	100	0	80	0	100	0
*Citrobacter werkmanii**	100	0	100	100	100	100	0	0	0	0	0	0	100	100	100	100	0	0	100	0	100	0	100	0
*Citrobacter sedlakii**	100	0	100	100	100	100	100	0	0	17	100	0	83	100	100	83	0	0	100	0	100	0	100	0
*Citrobacter rodentium**	100	0	100	100	100	100	100	0	0	0	0	0	0	100	100	0	0	0	100	0	100	0	100	0
*Citrobacter gillenii**	100	0	100	100	100	100	67	0	0	0	67	0	67	67	100	0	0	0	100	0	67	0	100	0
*Citrobacter murliniae**	100	33	100	100	100	100	100	0	0	0	33	0	100	100	100	33	0	0	100	0	100	0	100	0
*Edwardsiella tarda**	9	0	0	100	0	0	0	0	0	0	0	0	30	0	25	0	0	0	100	0	0	0	100	0
Edwardsiella tarda biogroup 1*	100	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
*Edwardsiella hoshinae**	13	0	0	100	0	100	0	0	0	0	0	0	65	0	0	0	0	0	100	0	0	0	100	0
Edwardsiella ictaluri	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
*Enterobacter aerogenes**	100	96	99	99	100	100	100	95	0	98	99	100	98	90	95	50	0	0	100	0	100	0	95	0
Enterobacter cloacae complex*	100	97	92	100	99	100	99	85	0	30	90	15	40	75	30	75	0	0	99	0	99	0	100	0
'Enterobacter agglomerans complex'*	95	30	85	89	93	97	55	7	0	60	50	50	30	40	25	30	0	0	85	0	90	75	98	0
*Enterobacter gergoviae**	99	97	99	100	99	100	99	2	0	97	97	97	100	2	97	93	0	0	99	0	97	0	100	0
*Enterobacter sakazakii**	100	99	100	100	100	100	100	96	0	100	100	0	15	1	1	96	0	0	99	0	100	98	100	0
Enterobacter taylorae (*E. cancerogenus*)*	100	0	100	99	100	100	100	1	0	90	0	0	1	75	0	35	0	0	100	0	100	0	100	0

(*Continued over*)

Table 1 *Biochemical reactions[a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	Alpha-methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
Enterobacter amnigenus biogroup 1*	100	100	100	100	100	100	100	55	0	91	100	0	0	35	9	0	0	0	100	0	91	0	100	0
Enterobacter amnigenus biogroup 2*	100	0	100	100	100	100	100	100	0	100	100	0	0	100	0	0	0	0	100	0	100	0	100	0
*Enterobacter asburiae**	100	70	5	100	97	100	100	95	0	95	0	0	11	21	30	87	0	0	100	0	100	0	100	0
*Enterobacter hormaechei**	100	0	100	100	96	100	100	83	0	0	0	0	4	96	13	74	0	0	100	0	95	0	100	0
*Enterobacter intermedius**	100	100	100	100	100	100	100	100	0	100	100	0	100	100	100	0	0	0	100	0	100	0	100	0
Enterobacter cancerogenus	100	0	100	100	100	100	100	0	0	100	0	0	0	100	0	33	0	0	100	0	100	0	100	0
Enterobacter dissolvens	100	100	100	100	100	100	100	100	0	100	100	0	0	100	0	100	0	0	100	0	100	0	100	0
Enterobacter nimipressuralis	100	0	100	100	100	100	100	100	0	100	100	0	0	100	0	0	0	0	100	0	100	0	100	0
Enterobacter pyrinus	100	0	100	100	0	100	100	0	0	100	0	0	0	0	0	0	0	0	100	0	100	0	100	0
*Escherichia coli**	99	50	80	95	95	98	2	0	0	35	75	5	75	95	95	90	0	0	100	0	95	0	98	0
Escherichia coli, inactive*	85	15	65	80	70	90	2	0	0	5	40	5	65	30	85	40	0	0	98	0	45	0	97	0
*Escherichia fergusonii**	98	0	92	96	96	96	96	0	0	46	0	100	20	0	96	96	0	0	100	0	83	0	100	0
*Escherichia hermannii**	100	40	97	100	100	100	97	0	0	40	0	8	3	97	35	78	0	0	100	0	98	98	100	0
*Escherichia vulneris**	100	99	93	100	100	100	100	25	0	20	100	0	25	78	2	30	0	0	100	0	100	50	100	0
Escherichia blattae	100	0	100	100	100	75	0	0	0	0	0	0	100	50	50	0	0	0	100	0	0	0	100	0
Shigella dysenteriae (Group A)*	45	0	30	15	4	90	0	0	0	0	0	0	10	0	75	0	0	0	99	0	30	0	100	0
Shigella flexneri (Group B)*	60	40	5	30	2	65	0	0	0	0	55	1	10	0	30	8	0	0	99	0	1	0	100	0
Shigella boydii (Group C)*	94	0	1	20	11	85	0	0	0	0	15	0	50	0	50	0	0	0	100	0	10	0	100	0
Shigella sonnei (Group D)*	95	3	75	90	2	100	5	0	0	0	25	0	15	10	90	0	0	0	100	0	90	0	100	0
*Ewingella americana**	0	0	23	16	13	99	10	0	0	50	0	99	24	0	35	10	0	0	97	0	85	0	99	0
*Hafnia alvei**	95	2	97	100	98	95	15	0	0	7	0	0	95	0	70	15	0	0	100	0	90	0	100	0
Hafnia alvei biogroup 1	0	0	0	0	0	70	0	0	0	0	0	0	0	0	30	0	0	0	100	0	30	0	100	0
*Klebsiella pneumoniae**	99	99	99	98	99	99	98	90	0	99	99	98	97	90	95	75	0	0	99	0	99	0	99	0
*Klebsiella oxytoca**	98	100	100	100	100	100	100	98	2	100	99	98	99	93	98	90	0	0	100	0	100	1	100	0
*Klebsiella ornithinolytica**	100	100	100	100	100	100	100	100	0	100	100	100	100	96	100	95	0	0	100	0	100	0	100	0
*Klebsiella planticola**	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	62	0	0	100	0	100	1	100	0

(*Continued over*)

Table 1 *Biochemical reactions[a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	Alpha-methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
*Klebsiella ozaenae**	98	90	55	95	95	98	92	70	0	80	97	95	65	25	50	2	0	0	80	0	80	0	100	0
*Klebsiella rhinoscleromatis**	100	90	96	100	100	100	100	0	0	30	100	100	50	0	50	0	0	0	100	0	0	0	100	0
Klebsiella terrigena	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	20	0	0	100	0	100	0	100	0
*Kluyvera ascorbata**	100	98	100	100	99	100	100	98	0	99	99	0	40	90	35	50	0	0	100	0	100	0	100	0
*Kluyvera cryocrescens**	100	100	100	100	91	100	100	95	0	100	100	0	5	81	19	86	0	0	100	0	100	0	100	0
*Kluyvera georgiana**	100	100	83	100	100	100	100	100	0	100	100	0	33	83	50	83	0	0	100	0	100	0	100	0
*Leclercia adecarboxylata**	100	66	100	100	100	100	100	0	0	100	100	96	3	93	83	28	0	0	100	0	100	37	100	0
*Leminorella grimontii**	100	0	0	0	83	0	0	0	0	0	0	0	17	100	100	0	0	0	100	0	0	0	0	0
*Leminorella richardii**	100	0	0	0	100	0	0	0	0	0	0	0	0	50	100	0	0	0	100	0	0	0	0	0
*Moellerella wisconsensis**	0	100	0	30	0	0	0	0	0	0	100	75	10	0	30	10	0	0	90	0	90	0	100	0
Morganella morganii subsp. *morganii**	0	0	0	0	0	0	0	0	0	0	0	0	5	0	95	0	0	0	90	0	10	0	98	99x
Morganella morganii biogroup 1*	0	0	0	0	0	0	0	0	0	0	0	0	100	0	100	0	0	0	90	0	20	0	100	99x
Morganella morganii subsp. *sibonii**	0	0	0	0	0	100	0	0	0	0	0	0	7	7	100	0	0	0	100	0	0	0	100	99x
Obesumbacterium proteus biogroup 2	0	0	15	50	15	85	0	0	0	0	0	0	0	0	15	0	0	0	100	0	0	0	85	0
Pantoea dispersa	100	0	91	82	100	100	55	0	0	0	0	100	27	0	9	100	0	0	91	0	91	27	100	0
Photorhabdus luminescens (25°C)	0	0	0	25	0	0	0	0	0	0	0	0	0	0	50	0	0	0	0	0	0	50	100	0
*Photorhabdus asymbiotica**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	20	0	0	0	0	0	60	100	0
*Plesiomonas shigelloides**	0	0	0	95	0	100	0	0	0	0	70	0	35	0	50	8	0	0	100	99	90	0	10	0
Pragia fontium	0	0	0	0	0	0	0	0	0	78	0	0	0	0	0	0	0	0	100	0	0	0	0	0
*Proteus mirabilis**	0	1	1	0	98	98	1	0	0	0	0	0	70	0	87	20	92	50	95	0	0	0	0	99x
Proteus vulgaris complex*	0	1	5	97	95	30	0	60	1	50	0	0	60	0	80	25	80	80	98	0	1	0	0	99x
*Proteus penneri**	0	1	0	100	100	55	0	80	0	0	0	0	55	0	85	5	45	40	90	0	1	0	0	99x
Proteus myxofaciens	0	0	0	100	0	100	0	100	0	0	0	0	100	0	100	0	100	50	100	0	0	0	0	99x
*Providencia rettgeri**	0	5	70	2	10	0	3	2	75	35	5	100	60	0	95	60	0	0	100	0	5	0	100	99
*Providencia stuartii**	1	7	0	1	7	98	5	0	0	0	0	0	50	0	90	75	0	10	100	0	10	0	100	99

(*Continued over*)

Table 1 *Biochemical reactions[a] of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae[a,b] (Continued)*

Organism	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	Alpha-methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
*Providencia alcalifaciens**	1	1	0	1	1	2	0	0	0	0	0	0	15	0	90	40	0	0	100	0	1	0	100	99
*Providencia rustigianii**	0	0	0	0	0	0	0	0	0	0	0	0	5	0	50	25	0	0	100	0	0	0	100	99
Providencia heimbachae	0	0	100	54	8	0	0	0	0	0	0	92	0	0	69	0	0	0	100	0	0	0	100	99
*Rahnella aquatilis**	100	94	94	94	94	100	100	0	0	100	100	0	13	30	6	6	0	0	100	0	100	0	100	0
Salmonella Group I[c] strains*	99	2	95	97	97	99	5	2	0	5	95	0	5	90	90	90	0	2	100	0	2	0	100	0
Salmonella serotype Typhi*	2	0	0	97	82	100	0	0	0	0	100	0	20	0	100	0	0	0	100	0	0	0	100	0
Salmonella serotype Choleraesuis*	0	1	100	95	98	0	0	0	1	0	45	1	0	0	85	1	0	0	98	0	0	0	95	0
Salmonella serotype Paratyphi A*	100	0	100	95	0	100	5	0	0	0	95	0	10	0	0	0	0	0	100	0	0	0	100	0
Salmonella serotype Gallinarum*	80	10	10	90	70	50	10	0	1	0	0	0	0	50	100	0	0	10	100	0	0	0	100	0
Salmonella serotype Pullorum*	100	1	100	5	90	90	5	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100	0
Salmonella Group II strains*	100	0	100	100	100	100	0	8	0	15	8	0	25	96	50	95	0	0	100	0	15	0	95	0
Salmonella Group IIIa strains*	99	1	99	98	100	99	1	1	0	1	95	1	10	90	5	90	0	2	100	0	100	0	100	0
Salmonella Group IIIb strains*	99	1	99	98	100	99	1	1	0	1	95	1	10	30	20	75	0	2	100	0	92	0	100	0
Salmonella Group IV strains*	100	0	98	100	100	100	50	0	0	0	100	5	0	0	65	70	0	0	100	0	0	0	100	0
Salmonella bongori (Group V)*	94	0	88	100	100	100	0	0	0	0	94	0	0	88	0	100	0	0	100	0	94	0	100	0
Salmonella Group VI strains*	100	0	100	100	100	100	0	0	0	0	89	0	33	89	100	89	0	0	100	0	44	0	100	0
*Serratia marcescens**	0	2	0	96	7	99	5	0	1	95	0	0	95	0	75	50	98	98	98	0	95	0	99	0
Serratia marcescens biogroup 1*	0	0	0	70	0	100	4	0	0	96	0	0	92	0	50	4	75	82	83	0	75	0	100	0
Serratia liquefaciens complex*	98	85	15	98	100	100	5	5	0	97	75	0	95	0	75	40	85	85	100	0	93	0	100	0
*Serratia rubidaea**	100	99	1	99	99	100	94	1	0	94	99	85	20	0	70	80	99	99	100	0	100	0	100	0
Serratia odorifera biogroup 1*	100	100	95	100	100	100	100	0	0	95	100	0	40	5	100	60	35	100	100	0	100	0	100	0
Serratia odorifera biogroup 2*	100	7	94	100	100	100	100	0	7	40	96	0	50	0	100	65	65	100	100	0	100	0	100	0
*Serratia plymuthica**	100	94	0	94	94	100	88	70	0	81	93	0	50	0	100	55	70	100	100	0	70	0	100	0
*Serratia ficaria**	100	70	35	100	100	100	100	8	0	100	40	100	0	0	17	40	77	100	92	8	100	0	100	0
Serratia entomophila	0	0	0	100	40	100	0	0	0	100	0	60	0	0	100	80	20	100	100	0	100	0	100	0
*'Serratia' fonticola**	100	100	76	97	85	100	6	91	0	100	98	100	88	0	58	15	0	0	100	0	100	0	100	0

(*Continued over*)

Table 1 *Biochemical reactions* [a] *of the named species, subspecies, biogroups, and enteric groups of the family Enterobacteriaceae*[a,b] *(Continued)*

Organism	L-Arabinose fermentation	Raffinose fermentation	L-Rhamnose fermentation	Maltose fermentation	D-Xylose fermentation	Trehalose fermentation	Cellobiose fermentation	Alpha-methyl-D-glucoside fermentation	Erythritol fermentation	Esculin hydrolysis	Melibiose fermentation	D-Arabitol fermentation	Glycerol fermentation	Mucate fermentation	Tartrate, Jordan's	Acetate utilization	Lipase (corn oil)	DNase (25°C)	Nitrate → nitrite	Oxidase, Kovacs	ONPG test	Yellow pigment (25°C)	D-Mannose fermentation	Tyrosine hydrolysis
*Tatumella ptyseos**	0	11	0	0	9	93	0	0	0	0	25	0	7	0	0	0	0	0	98	0	0	0	100	0
*Trabulsiella guamensis**	100	0	100	100	100	100	100	0	0	40	0	0	0	100	50	88	0	0	100	0	100	0	100	0
Xenorhabdus nematophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	0	0	20	20	0	0	60	80	0
*Yersinia enterocolitica**	98	5	1	75	70	98	75	0	0	25	1	40	90	0	85	15	55	5	98	0	95	0	100	0
*Yersinia frederiksenii**	100	30	99	100	100	100	100	0	0	85	0	100	85	5	55	15	55	0	100	0	100	0	100	0
*Yersinia intermedia**	100	45	100	100	100	100	96	77	0	100	80	45	60	6	88	18	12	0	94	0	90	0	100	0
*Yersinia kristensenii**	77	0	0	100	85	100	100	0	0	0	0	45	70	0	40	8	0	0	100	0	70	0	100	0
*Yerrsinia rohdei**	100	62	0	0	38	100	25	0	0	0	50	0	38	0	100	0	0	0	88	0	50	0	100	0
Yersinia aldovae	60	0	0	0	40	80	0	0	0	0	0	0	0	0	100	0	0	0	100	0	0	0	100	0
*Yersinia bercovieri**	100	0	0	100	100	100	100	0	0	20	0	0	0	0	100	0	0	0	100	0	80	0	100	0
*Yersinia mollaretii**	100	0	0	60	60	100	100	0	0	0	0	0	20	0	100	0	0	0	100	0	20	0	100	0
*Yersinia pestis**	100	0	1	80	90	100	0	0	0	50	20	0	50	0	0	0	0	0	85	0	50	0	100	0
*Yersinia pseudotuberculosis**	50	15	70	95	100	100	0	0	0	95	70	0	50	0	50	0	0	0	95	0	70	0	100	0
*'Yersinia' ruckeri**	5	5	0	95	0	95	5	0	0	0	0	0	30	0	30	0	30	0	75	0	50	0	100	0
*Yokesnella regensburgei** (*K. trabulsii*)	100	25	100	100	100	100	100	0	0	67	92	0	0	0	0	25	0	0	100	0	100	0	100	0
Enteric Group 58*	100	0	100	100	100	100	100	55	0	0	0	0	30	0	60	45	0	0	100	0	100	0	100	0
Enteric Group 59*	100	0	100	100	100	100	100	10	0	100	0	10	10	60	50	50	0	0	100	0	100	25	100	0
Enteric Group 60*	25	0	75	0	0	100	0	0	0	0	0	0	75	0	75	0	0	0	100	0	100	0	100	0
Enteric Group 63	100	0	100	100	100	100	100	65	0	100	0	0	0	65	0	0	0	0	100	0	100	0	100	0
Enteric Group 64	100	0	100	100	100	100	100	0	0	100	0	100	0	100	50	0	0	0	100	0	100	0	100	0
Enteric Group 68*	0	0	0	50	0	100	0	0	0	0	0	0	50	0	0	0	0	100	100	0	0	0	100	0
Enteric Group 69	100	100	100	100	100	100	100	100	0	100	100	0	0	100	0	25	0	0	100	0	100	0	100	0
Enteric Group 137*	100	100	100	100	100	100	100	80	0	100	100	0	100	100	50	100	0	0	100	0	100	0	100	0

a) Each number is the percentage of positive reactions after 2 days of incubation at 36°C (unless a different temperature is indicated). The vast majority of these positive reactions occur within 24 h. Reactions that become positive after 2 days are not considered. ONPG, *o*-nitrophenyl-β-D-galactopyranoside; TSI, triple sugar iron agar. Several 'species' in the table are composed of two or more DNA–DNA hybridization groups, so the term 'complex' should be understood to follow the species name (see text).
b) An '*' beside the name of an organism indicates that it occurs in human clinical specimens.
c) The Roman numerals refer to the seven *Salmonella* subgroups that are biochemically and genetically distinct.

Table 2 *Properties of Enterobacteriaceae*, Plesiomonas *(the family's only oxidase-positive genus), and three oxidase-positive genera that are relatives*

Test or property	Enterobacteriaceae	*Plesiomonas*	*Aeromonas*	*Photobacterium*	*Vibrio*
Genetic relationship to *Escherichia coli*	Higher	Higher	Lower	Lower	Lower
Enterobacterial common antigen (eca)	+	+	–	–	–
Oxidase reaction	–	+	+	+	+
Peritrichous flagella when grown in liquid media	+	V	–	–	V
Sheathed polar flagellum	–	–	–	–	+
Guanine + cytosine content of DNA (mol percent)	38–60	51	57–63	40–44	38–51
Associated with diarrhea and extra-intestinal infections in humans	+	+	+	–	+
Na^+ is required for growth or stimulates growth	–	–	–	+	+
Sensitive to the vibriostatic compound O129	–	+	–	+	+
Lipase production	V	–	+	V	+
D-Mannitol fermentation	+	–	+	–	+

+, most genera, species and strains are positive; –, most genera, species and strains are negative; V, genera or species are variable.

Table 3 *New, unusual, fastidious, or unculturable genera and species that have been classified* [a] *in the family Enterobacteriaceae, and which do not normally occur*[b] *in human clinical specimens. The unculturable and extremely fastidious organisms have been classified in the family Enterobacteriaceae based mainly on 16S ribosomal RNA sequencing data*

Classifications in the family Enterobacteriaceae

Human pathogen:

Calymmatobacterium granulomatis (*Klebsiella granulomatis) – causes donovanosis (granuloma inguinale)*

Associated with plants, but occasionally with human clinical infections:

Genus *Pantoea*, seven species[b], two subspecies

Pathogenic for, or associated with plants:

Genus *Brenneria*, six species; causes a variety of diseases of deciduous trees and walnut trees

Genus *Erwinia*, nine to 28 species, several named subspecies; major plant pathogen, also exists as a saprophyte and epiphyte

Genus *Pectobacterium*, four to six species, several named subspecies; causes soft rot and a variety of other plant diseases including blights, cankers, die back, leaf spot, and wilts

Phlomobacter fragariae – unculturable species that occurs in the sieve tubes of phloem tissue of plants

Associated with extreme environments:

Alterococcus agarolyticus – habitat is costal hot springs

Pathogenic for, or associated with insects:

Genus *Arsenophonus* – two species, *A. nasoniae*, which is culturable and was originally described as causing 'son-killer' trait in a parasitic wasp, and *A. tiatominarum* which has not been cultured

Buchnera aphidicola – Cannot be cultivated outside the aphid host; essential for the survival of the aphid

Genus *Photorhabdus* – three species, three subspecies. Two species are found only in nematodes and the insects that are infected by the nematode. One species *P. asymbiotica* causes human wound infections

Wigglesworthia glossinidia – Obligate intracellular endosymbiont of tsetse flies (Aksoy 1995)

Genus *Xenorhabdus* – five species; found only in nematodes and the insects that the nematodes infect

Other organisms:

Aranicola proteolyticus

a) The table includes some organisms whose eventual classification may as 'relatives' of Enterobacteriaceae. For more information about these organisms, refer to the internet site of J.P. Euzéby (www.bacterio.cict.fr). b) There have been clinical isolates for some of the species.

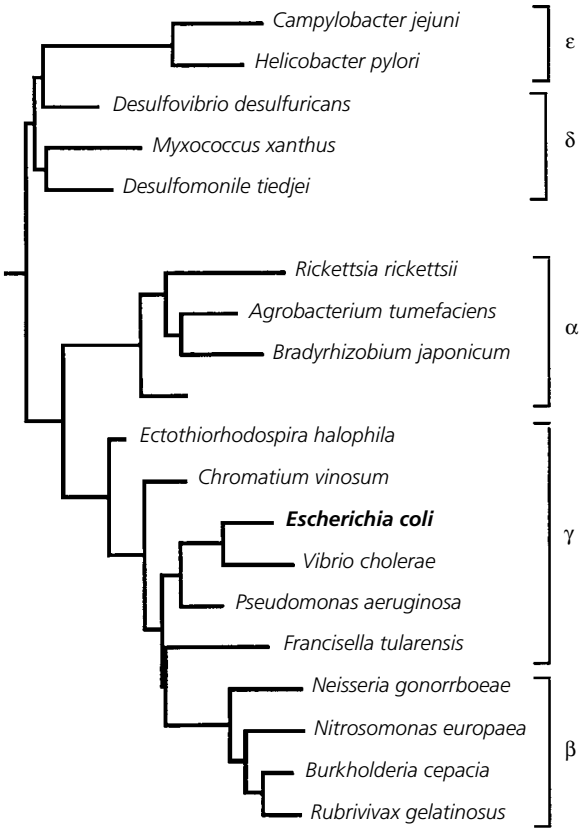

Figure 1 *The Proteobacteria. This illustrates the closest evolutionary relatives, based or 16S r-RNA sequencing data, of Enterobacteriaceae represented by* Escherichia coli *(the type species of the type genus of the family).*

editions (1929, 1936, 1946, 1955, 1964, 1975, 1983, 1990, 1998, and the current one). This is an invaluable way to rapidly gain a historical perspective for an unfamiliar subject. The family name Enterobacteriaceae was first proposed by Rahn in 1937, but many of its species are much older. *Serratia marcescens* was first described and named by Bizio in 1823. Because it produces a distinct red pigment, *Serratia marcescens* can be traced in written history to long before the bacteriological era. Gaughran (1969) argues that the first recorded account of *Serratia* dates back to 332BC. This organism typically grows on grains such as polenta (corn), and produces its characteristic red pigment. This led the superstitious to believe that a supernatural force caused 'bright red blood' to spontaneously appear; 'a miracle!'. The microbiological explanation for the 'bleeding polenta' miracle came over 2 000 years later with the description of the red pigment prodigiosin that is produced by *S. marcescens* (and a few other bacteria) as it grows on bacteriological media and substrates such as polenta and other grains. Other species were described in the late 1880s and 90s; *Yersinia pseudotuberculosis* in 1883, *Salmonella typhi* in 1884, *Salmonella cholerae-suis* in 1885, and *Yersinia pestis* in 1894. These organisms were originally given different names, and their nomenclature and classifications have changed considerably through the ten editions of this book.

A few bacterial diseases have unique clinical symptoms or epidemiology and can be identified with certainty in the historical record. Two classical examples are plague (*Yersinia pestis*) and cholera. Typhoid fever (*Salmonella typhi*) was also defined as a distinct enteric disease before the bacteriological era (when bacteria were first cultivated on artificial media and seriously studied in the late 1800s). This was accomplished in the 1850s by William Budd who carried out classical investigations on the epidemiology and pathology of typhoid. This was 30 years before the isolation of the typhoid bacillus.

The Enterobacteriaceae were instrumental in our understanding of the way that living organisms work at the cellular and genetic level (Watson et al. 1987, 2004). Most of this pioneering work was carried out using strains K12 and B of *Escherichia coli*, and seven virulent 'T coliphages' (bacteriophages) and the temperate bacteriophage λ of this species. Conjugation or 'bacterial sexuality' was discovered in 1946 by Lederberg and Tatum in *E. coli* K12, and transduction was discovered in 1952 by Zinder and Lederberg in *Salmonella typhimurium* using phage P22. A partial list of molecular and genetic breakthroughs that were done in, or utilized species of Enterobacteriaceae includes:

- discovery of the bacteriophage
- lactose metabolism and the lactose operon
- deciphering the genetic code
- definition of genes in terms of biochemical function
- biosynthetic pathways for DNA, RNA, and proteins
- the histidine operon of *Salmonella typhimurium*
- entry of molecules into cells
- comparative analysis of the genes and metabolism of *Escherichia coli* and *Salmonella typhimurium* (see www.EcoSal.org)
- structure, biosynthesis, and genetic regulation of cell membranes, walls, capsules, flagella, and other appendages
- plasmids that carry virulence factors and genes for catabolic pathways such as lactose fermentation
- the resistance transfer factor (RTF) and plasmid-mediated antibiotic resistance
- the synthesis of insulin, other hormones, and many other useful products in bacteria rather than by mammalian cells

NOMENCLATURE AND CLASSIFICATION

As time passed and scientific evidence accumulated, older medical terms such as 'agues' and 'fevers' were replaced with more precise and operationally defined

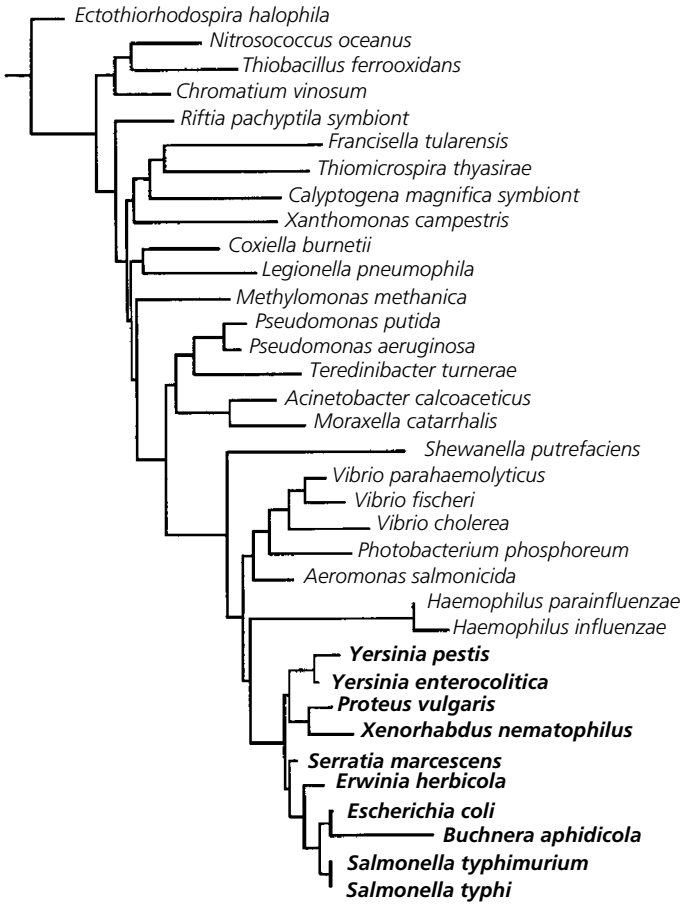

Figure 2 *The γ subdivision of the Proteobacteria. This illustrates the evolutionary relatitionships of 10 species of Enterobacteriaceae and their closest relatives, and is based or 16S r-RNA sequencing data.*

terms such as typhoid, typhus, influenza, and rhinovirus infection. A similar progression occurred with the nomenclature and classification of Enterobacteriaceae. Many critics complain that this has been a torturous process, but we would argue that this is merely progress that takes place in all scientific and medical fields as knowledge accumulates (Farmer et al. 1985). Unfortunately, there are some specialists in bacterial taxonomy who give instability rather than stability to nomenclature and classification.

The nomenclature and classification of the genera, species, subspecies, biogroups, and serotypes of Enterobacteriaceae are constantly being reevaluated, and the literature contains many differing opinions (Brenner and Farmer 2005; Farmer 2003; Euzeby 1999; Ezaki et al. 2000a, b; Farmer et al. 1985; Krieg 1984). Genera and species were once defined by biochemical and antigenic analysis. More recently, newer techniques such as nucleic acid hybridization, nucleic acid sequencing, and comparisons of enzymes, proteins, and other cellular structures (see the related chapters in this volume), have made it possible to determine the evolutionary relationships of organisms in the family (Brenner and Farmer 2005; Carter et al. 1999; Farmer et al. 1985; Janda and Abbott 1998) (Figure 3, p. 17). The use of a single technique, DNA–DNA hybridization, has led to the discovery of many new genera and species and has resulted in the proposed reclassification of some of the older ones (Brenner and Farmer 2005; Farmer 2003) (Figure 4, p. 18).

This chapter will, at least, mention the different names and classifications that the reader is likely to encounter in the scientific literature and in material accompanying commercial products. The nomenclature and classification given in Tables 1 and 3 (p. 3 and 14), is a compromise based on all available evidence, and includes most of the genera, species, subspecies, biogroups, and unnamed enteric groups included in the family (Brenner and Farmer 2005; Farmer 2003; Farmer et al. 1985). If two names are widely used for the same organism, both are mentioned in this chapter with one in parentheses. Most of the 'nonclinical' organisms in the family are also included because there is a possibility that they will be isolated from a human clinical specimen some day (Brenner and Farmer 2005; Farmer et al. 1985).

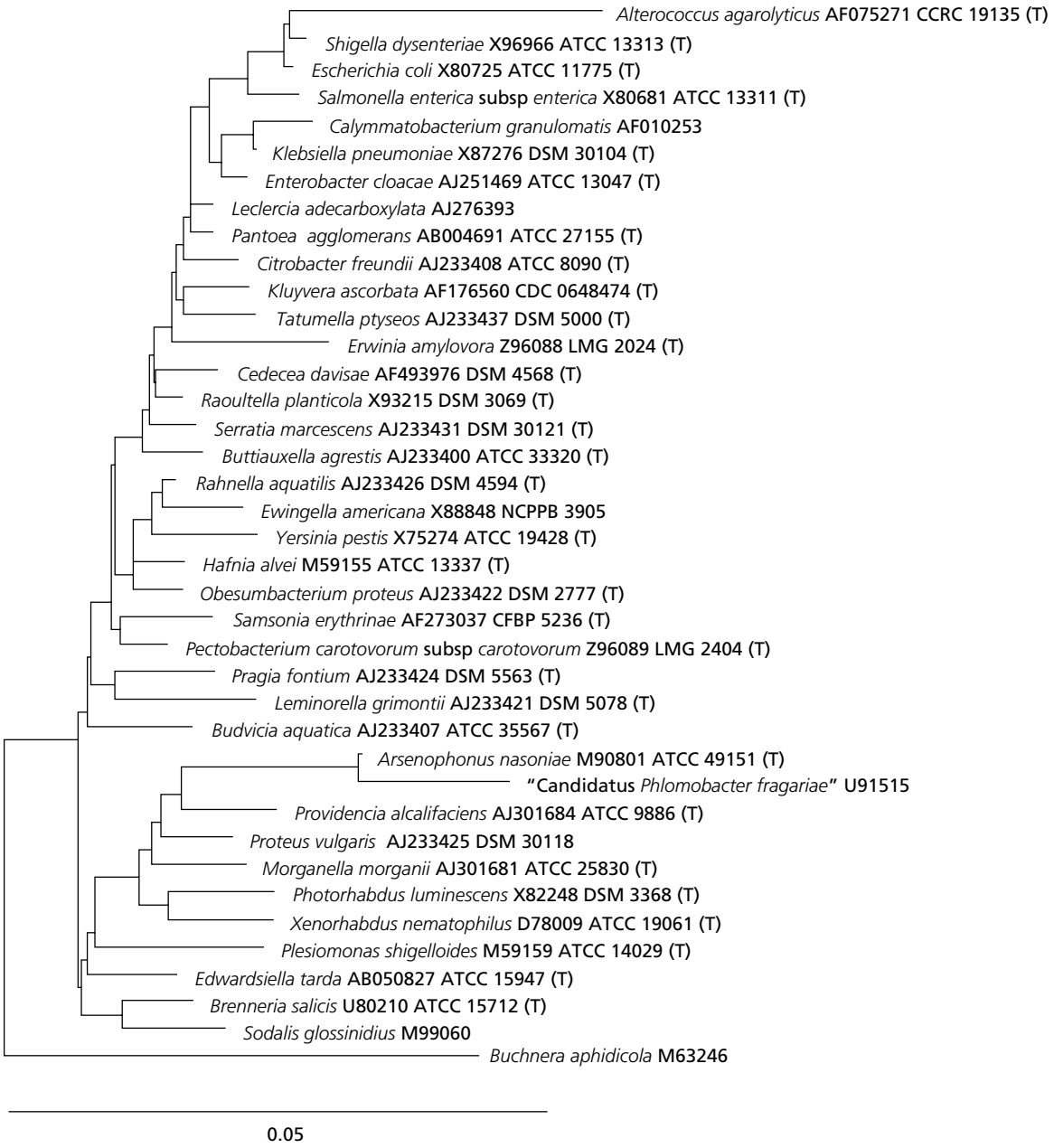

Figure 3 *The evolutionary relationships of the genera and species of Enterobacteriaceae based on 16S r-RNA sequencing data. (From Farmer 2005; used with permission)*

Historical perspective for understanding the evolving nomenclature and classification of the family

The bacteriological era started in the mid decades of the 1800s with the pioneering studies of Pasteur on the microbial origin of fermentation and those of Koch on the bacterial agent that causes anthrax. However, it was not until the 1880s and 1890s that many of the important bacterial pathogens were described. Bacteriological methods were extremely crude when the first bacterial species were described. It was almost impossible to compare descriptions of bacteria that were written by different authors and determine if they were describing the same, or a different species. This led to great confusion in the early literature.

Many of the early species now classified in Enterobacteriaceae were originally described as belonging to the genera *Bacillus* and *Bacterium*. Examples include *Escherichia coli* (first named *Bacterium coli commune* in 1885), *Shigella dysenteriae* (*Bacillus dysenteriae* in 1898), *Salmonella cloleraesuis* (*Bacillus clolerae-suis* [sic] in 1894), *Salmonella typhi* (*Bacillus typhi* in 1886), *Yersinia*

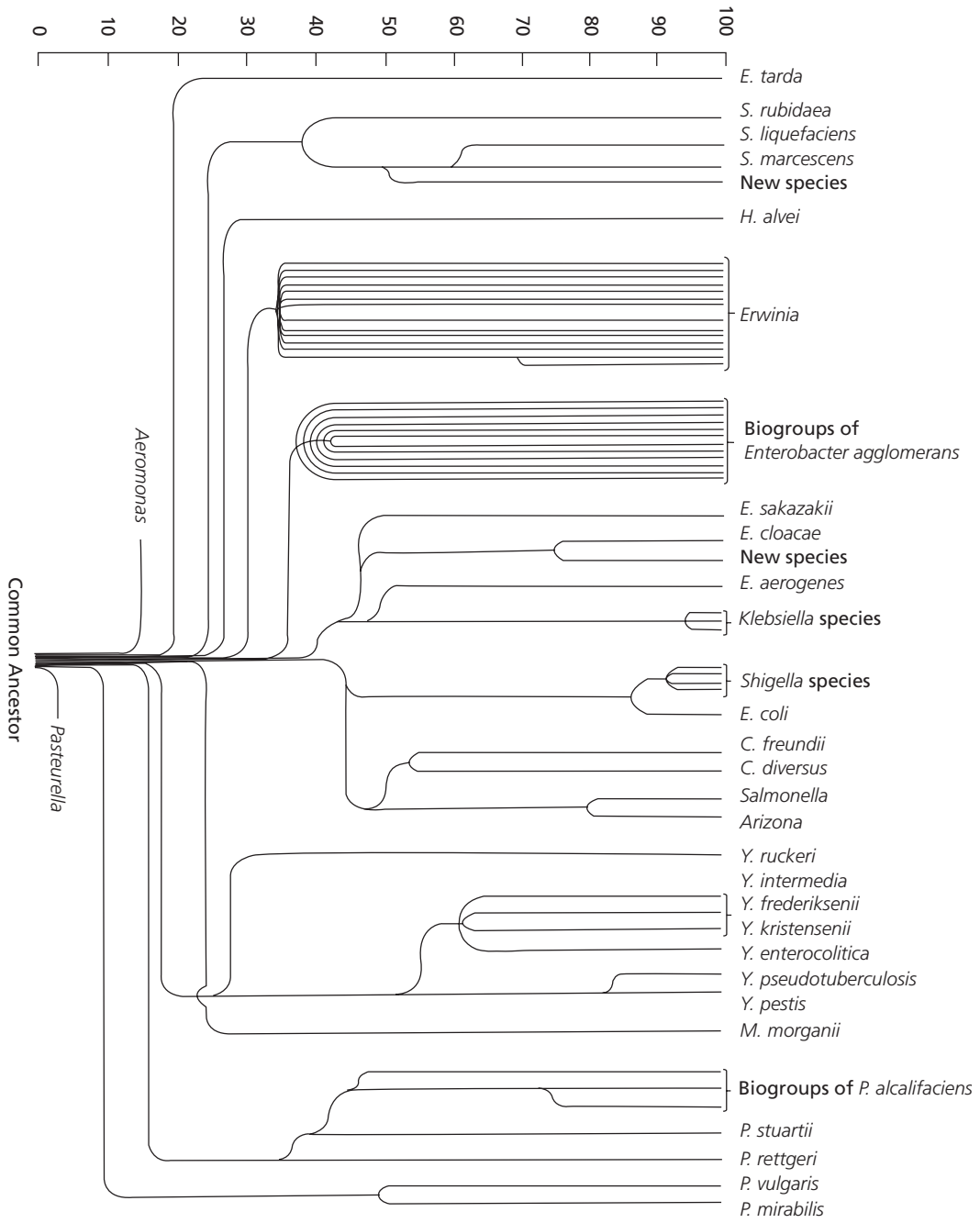

Figure 4 *The evolutionary relationships of Enterobacteriaceae based on DNA–DNA hybridization (from Brenner 1984; used with permission). The ordinate is the percentage relatedness by DNA–DNA hybridization.This figure is a simplified attempt to depict relatedness of each species of enterobacteria to all other species. It assumes a common ancestor from which all of the organisms have diverged. The horizontal branch depicts relatedness of an organism to all organisms that have not branched. For example,* E. trada *is about 20 percent related to all organisms except those that are below its branch point (*Aeromonas*,* Proteus*,* Providencia*, and* Pasteurella*. Similarly, the two* Citrobacter *species* C. freundii *and* C. diversus *are about 45 percent related to the species above them, and diverged at a point in time such they are now 50 percent related.*

pestis (*Bacterium pestis* in 1896), *Yersinia pseudotuberculosis* (*Bacillus pseudotuberkulosis* [sic] in 1889), and the plant pathogen *Erwinia tracheiphila* (*Bacillus tracheiphilus* [sic] in 1895). Some genus names were used briefly and then disappeared, such as *Hyalococcus pneumoniae* (1886, now *Klebsiella pneumoniae*). Other genera such as the genus *Micrococcus* were redefined and now exclude Enterobacteriaceae; in 1892 *Micrococcus amylovorus* (now *Erwinia amylovora*) was described. A few of the early species were classified in genera that have stood the test of time such as *Serratia marcescens* (1823) and *Proteus mirabilis* (1885).

The genus *Bacillus* was eventually redefined to include only gram-positive bacteria that produce endospores. Thus many of the species of Enterobacteriaceae had to be reclassified in the genus *Bacterium*. This genus name was applied to a broad group of gram-negative, nonsporing rods occurring in the intestinal tract of humans and animals, on plants and in the soil, and leading a saprophytic, commensal, or pathogenic existence. These organisms have been studied for many years by groups of workers with widely differing objectives. The medical and veterinary bacteriologists concentrated their attention on those organisms that caused illness in humans and animals. The plant pathologists were most interested in those that possessed enzymes capable of digesting vegetable tissues. Industrial bacteriologists studied in detail a few strains that carried out useful fermentations or interfered with manufacturing processes. Sanitary bacteriologists were concerned with distinguishing the organisms found in the intestine from those living saprophytically outside the human or animal body. This diversity of approach led to much confusion, and the same organism was often studied independently, and under different names, by several groups of workers who used methods that were not comparable. The species of Enterobacteriaceae were classified in the genus name *Bacterium* through several editions of *Topley & Wilson*.

A classification of the Enterobacteriaceae that resembles the one used today emerged largely under the stimulus of the Enterobacteriaceae Subcommittee of the International Committee on Systematic Bacteriology (see references under 'Enterobacteriaceae Subcommittee'). Attempts were made to coordinate the experiences of workers in the different fields, and there is now general agreement on the definition of the family Enterobacteriaceae and the main lines on which it should be subdivided.

As long ago as the early 1890s, it was pointed out by Theobald Smith that certain important groups of organisms pathogenic to humans and animals differed from most of the nonpathogenic forms in failing to ferment lactose, and the genera *Salmonella* and *Shigella* were defined for these non-lactose fermenters. The lactose fermenters were, for the most part, thought to be normal inhabitants of the intestinal tract of humans and the higher animals, or to exist on plants or in the soil. However, it is now known that some genera contain members that ferment lactose rapidly, others that do so late and irregularly, and yet others that are lactose non-fermenting. Furthermore, it is now known that *Escherichia coli*, usually a rapid lactose fermenter, is an important cause of acute enteritis in persons of all age groups, and in animals. For many years the term 'coliform bacteria' has been used for those members of the Enterobacteriaceae that generally, though by no means invariably, ferment lactose.

Current classification of the family

The number of genera and species in the family has increased dramatically during the last four decades. A useful way to follow this increase is to compare this chapter with previous editions of *Topley and Wilson* or to a bacteriology reference book such as *Bergey's Manual of Determinative (Systematic) Bacteriology*. For example, there were 12 genera and 36 species in 1974 when the eighth edition of *Bergey's Manual of Determinative Bacteriology* was published (Buchanan and Gibbons 1974). By 1984, when the first edition of *Bergey's Manual of Systematic Bacteriology* was published, the family contained 20 genera and 76 species (Krieg 1984). The total rose to 30 genera and 107 species by the ninth edition of *Bergey's Manual of Determinative Bacteriology* in 1994. The family presently contains over 40 genera and 150 named species and subspecies, along with a growing number of named biogroups, 'enteric groups', DNA–DNA hybridization groups (genomospecies) and unnamed organisms (Brenner and Farmer 2005; Farmer 2003). Most of these organisms are discussed in the following six chapters (Chapters 52–57).

How do proposed changes in classification become established?

Contrary to popular opinion, there is no designated international body that considers every proposed change in classification and then issues an 'official classification' that becomes established as a matter of law. However, for many years the Subcommittee on Enterobacteriaceae of the International Committee on Systematic Bacteriology has been charged with the task of monitoring and discussing the nomenclature and classification of Enterobacteriaceae. This subcommittee can make recommendations on matters of classification but has rarely done so in the last few years. Even if this subcommittee studies a specific 'proposed reclassification,' it can only make a recommendation, which can then be accepted or rejected by the scientific community. *Changes in classification are decided by usage not by a judicial decision or action* (see the pertinent chapter in this volume for further discussion). Sometimes several classifications are widely used, and all can be 'correct.' Six names that represent the same organism are: the typhoid bacillus, *Salmonella typhi*, *Salmonella* serotype Typhi, *Salmonella* serovar Typhi, *Salmonella choleraesuis* subspecies *choleraesuis* serovar Typhi, and *Salmonella enterica* subspecies *enterica* serovar Typhi. Classifications are nomenclaturally correct if they conform to all the rules in the *Bacteriological Code* (*International Code of Nomenclature of Bacteria*). However, classifications can be useful or not useful; practical or impractical; based on sound data or erroneous data; can be frequently used in

the literature, or rarely used; and can give stability or instability to the literature.

Proposed changes in classification

Recently, several 'alternative classifications' have been proposed in the literature. Some of these appear to be good ones because they are totally in agreement with all scientific data. These have been incorporated in Table 1 (p. 3). However, others have been based on data from a single laboratory and have not been fully discussed or widely accepted by the scientific community. Table 1 (p. 3) gives the nomenclature and classification used by the reference laboratories at the Foodborne and Diarrheal Diseases Branch, at the Centers for Disease Control and Prevention. It will, no doubt, differ from other nomenclatures and classifications.

INCLUSION OF THE GENUS *PLESIOMONAS*

In the last edition, the genus *Plesiomonas* was classified in the family Vibrionaceae along with *Vibrio* and *Aeromonas*, and the oxidase-positive genera were covered in separate chapters. Because *Plesiomonas* is closer to Enterobacteriaceae than Vibrionaceae based on 16S rRNA sequencing, and because it contains the enterobacterial common antigen, it has been included in the family Enterobacteriaceae (Table 1, p. 3) in this edition. However, *Plesiomonas* is oxidase positive, a characteristic not shared with other species of Enterobacteriaceae, and it should be understood that *Plesiomonas* is a distant relative of *Escherichia coli* (Figure 3), the type species of the type genus of Enterobacteriaceae. Thus, the classification of *Plesiomonas* in the family Enterobacteriaceae might best be viewed as tentative.

RECLASSIFICATION OF *XENORHABDUS LUMINESCENS* DNA HYBRIDIZATION GROUP 5 AS *PHOTORHABDUS ASYMBIOTICA*

In Table 1 (p. 3), the organism originally classified (Boemare et al. 1993; Farmer et al. 1989) as *Xenorhabdus luminescens* DNA hybridization group 5 is now classified as *Photorhabdus asymbiotica* (Fischer-Le Saux et al. 1999). It has caused rare cases of bacteremia and wound infection in the United States (Farmer et al. 1989) and Australia (Peel et al. 1999).

KLUYVERA COCHLEAE IS A SYNONYM OF *ENTEROBACTER INTERMEDIUM*

Phenotypic and 16S rRNA sequencing data indicate that *Kluyvera cochleae* is essentially identical to *Enterobacter intermedium*. All evidence indicates that *Kluyvera cochleae* is a junior subjective synonym, thus *Enterobacter intermedium* is the name used in Table 1 (p. 3).

Other proposed changes in nomenclature and classification; problems and controversies

Several other proposals are controversial, more recent, or require further evaluation by the scientific community.

PROPOSED CLASSIFICATION OF THREE *KLEBSIELLA* SPECIES IN *RAOULTELLA*

In 2001, Drancourt et al. proposed that *Klebsiella planticola*, *K. ornithinolytica*, and *K. terrigena* be reclassified in a new genus *Raoultella* as *R. planticola*, *R. ornithinolytica*, and *R. terrigena*. These three species are extremely similar to *Klebsiella pneumoniae* in their phenotypic properties, making differentiation very difficult (Farmer et al. 1985). This alternative classification needs further evaluation.

PROPOSED RECLASSIFICATION OF *CALYMMATOBACTERIUM GRANULOMATIS* AS *KLEBSIELLA GRANULOMATIS*

Calymmatobacterium granulomatis is an organism that has received little attention in industrialized countries. In the seventh edition of the *Manual of Clinical Microbiology*, *Calymmatobacterium* was mentioned only twice (pp. 25 and 50). It was listed as an aerobic bacterium that can be found in the genital area, and under the topic 'specimen management' it was mentioned under the diseases granuloma inguinale or ulcerative donovanosis, with the notes 'mostly a tropical disease' and 'culture is nonproductive.' *Calymmatobacterium granulomatis* has been described as a highly pleomorphic gram-negative rod that does not grow on laboratory media. Diagnosis of granuloma inguinale has been traditionally carried out by showing the presence of 'Donovan bodies' in Giemsa-stained smears of mononuclear cells or in histiocytes from the patient's genital ulcers.

It had been assumed for almost a century that *Calymmatobacterium granulomatis* has no relationship to the 'easy to culture' organisms of the family Enterobacteriaceae. Carter et al. (1999) recently proposed that *Calymmatobacterium granulomatis* be reclassified in the genus *Klebsiella* as *K. granulomatis*. This proposal was based both on nucleotide sequence relatedness, and on disease similarity. Granuloma inguinale is a disease similar in some ways to rhinoscleroma, also a tropical disease (nose infection) caused by *K. rhinoscleromatis*.

This alternative classification was used in the eighth edition of the *Manual of Clinical Microbiology*, but while it is being evaluated and discussed, we would urge that both scientific names be used, with writer's preference listed first: '*Calymmatobacterium granulomatis* (*Klebsiella granulomatis*)' or '*Klebsiella granulomatis* (*Calymmatobacterium granulomatis*).'

'UNCULTURABLE ENTEROBACTERIACEAE' AS POSSIBLE CAUSES OF HUMAN DISEASES OF UNKNOWN ETIOLOGY

That *Calymmatobacterium granulomatis* is probably an unculturable species of *Klebsiella* is a stunning finding. This suggests that other diseases of unknown etiology may be caused by unculturable Enterobacteriaceae. It is possible that strains of Enterobacteriaceae have evolved in a host–parasite relationship in such a way that they become unculturable or difficult to culture. Examples of this host adaptation have already been demonstrated in insects. The unculturable Enterobacteriaceae species *Buchnera aphidicola* and the slow growing species *Arsenophonus nasoniae* are well documented examples from insect pathology. Unculturable bacteria including Enterobacteriaceae should be looked for in diseases of unknown etiology including Crohn's diseases, ulcerative colitis, tropical sprue, 'Brainerd diarrhea' (see: www.cdc.gov/ncidod/dpd/parasites/diarrhea/brainerd_diarrhea.htm), ankylosing spondylitis, and many others.

NOMENCLATURE, CLASSIFICATION, AND REPORTING OF THE GENUS *SALMONELLA*

After much study and discussion, there is now good agreement on several topics in the controversy over the nomenclature and classification of the genus *Salmonella* (Ezaki et al. 2000a, b; Farmer et al. 1984; Reeves et al. 1989). However, there are still several problem areas where different names and classifications are being used. These include the names *Salmonella choleraesuis* versus *Salmonella enterica* (Euzeby 1999); the use of the terms 'serotype' (Brenner et al. 2000) versus 'serovar' (Popoff 2001; a simple solution is to abbreviate both terms as 'ser.', which nicely avoids this problem); the best way to write the names of the serotypes (serovars); the use of names versus antigenic formulas for some of the serotypes; and the argument whether some well-known serotype names should be eliminated and combined with other serotypes (F.W. Brenner et al. 2000; Popoff 2001). These issues are discussed in *Salmonella*; for a different opinion see the recent analysis by Farmer (2003).

THE '*ENTEROBACTER AGGLOMERANS* COMPLEX' – *PANTOEA*

This is a confusing subject (Kwon et al. 1997), and writers continue to make errors in the definition and circumscription (boundaries) of *Pantoea agglomerans*. In 1972, Ewing and Fife redefined the name *Enterobacter agglomerans* to include a wide variety of organisms previously known under many different names. These investigators also defined 11 different biogroups to recognize the phenotypic diversity of the many strains included in *Enterobacter agglomerans*. This name has become a useful one for clinical microbiologists, and it has been used extensively in the literature. Systematic analysis by Brenner and coworkers using DNA–DNA hybridization indicated that *Enterobacter agglomerans* is very heterogeneous, with at least 14 DNA hybridization groups (Brenner 1992a). For this reason, the names '*Enterobacter agglomerans* complex' (Farmer 2003) or '*Enterobacter agglomerans* group' (Farmer et al. 1985) have been used to better indicate the heterogeneity of this 'species' (Table 1, p. 3). However, it has been very difficult to find simple tests to differentiate and identify all of the DNA hybridization groups (Farmer et al. 1985). For this reason, workers have been reluctant to subdivide the *Enterobacter agglomerans* complex until a definitive classification could be proposed (Farmer et al. 1985). Gavini et al. (1989) took the first step in a more logical classification for this complex group, by proposing that the group of six strains that Brenner et al. (1984) had defined as 'DNA hybridization group 13 of *Enterobacter agglomerans*' be classified in a new genus, *Pantoea*. In the same paper they also defined a new species in the genus, *Pantoea dispersa* (Gavini et al. 1989) that corresponded to *Enterobacter agglomerans* DNA hybridization group 3 of Brenner (Brenner et al. 1984).

However, their new classification has caused serious problems. Some authors have broadened the original definition of Gavini et al. for *Pantoea agglomerans* to include most or all of the dozens of organisms included in the '*Enterobacter agglomerans* complex.' Since DNA–DNA hybridization is not routinely carried out, and since simple tests are not available to identify strains definitively to the level of DNA hybridization group, it seems prudent to retain the vernacular name '*Enterobacter agglomerans* complex' as a convenient name for clinical microbiologists for routine identification. This term is defined biochemically in Table 1 (p. 3), and it should be emphasized that it is used merely for convenience and because the name *Enterobacter agglomerans* is well understood and widely used in the literature. Eventually, this term will be replaced with a better classification. When definitive testing in a reference laboratory (usually including DNA hybridization) is carried out, more precise names can be used in reporting. Examples could include: *Pantoea agglomerans* (DNA hybridization group 13), *Pantoea dispersa* (DNA hybridization group 3), *Enterobacter agglomerans* DNA hybridization group 1, etc. Table 1 gives the phenotypic definition of the vernacular name *Enterobacter agglomerans* complex. We hope this term will prove useful for reporting isolates in most microbiology laboratories, since few if any can carry out DNA–DNA hybridization. We would strongly argue against using a classification in which *Pantoea agglomerans* would be synonymous with the '*Enterobacter agglomerans* complex'; this would only restate an old problem in terms of a new name.

THE NAMES *ENTEROBACTER TAYLORAE* AND *ENTEROBACTER CANCEROGENUS*

Enterobacter taylorae and *Enterobacter cancerogenus* may be two names for the same organism (Grimont and

Ageron 1989). However, they have different type strains; therefore, they are not 'objective synonyms' under the rules of the *Bacteriological Code*. Until the identity of these two organisms is confirmed by other laboratories, both names will be used (Table 1, p. 3).

THE NAMES *CITROBACTER DIVERSUS* AND *C. KOSERI*

The names *Citrobacter diversus* and *C. koseri* have both been used in the literature for some time, but the name *C. diversus* has been used much more frequently. Many workers recognized the phenotypic similarity of these two organisms and thought that they might be the same. They have different type strains, so considering them as the same will always be a subjective matter. They can be considered 'subjective synonyms,' but not 'objective synonyms' (which must have the same type strain). The name *Citrobacter diversus* became the correct name for this organism on 1 January 1980, when the *Approved Lists of Bacterial Names* was issued, because under the laws of priority it was the older name. However, in 1993 the Judicial Commission of the International Committee on Systematic Bacteriology issued an Opinion (Judicial Commission of the International Committee on Systematic Bacteriology 1993) that the name *Citrobacter koseri* should be conserved over the name *Citrobacter diversus*, even though the name *Citrobacter diversus* was the older name, was on the *Approved Lists of Bacterial Names*, was the correct name under the rules of the *Bacteriological Code*, and was the name used most frequently in the literature. This 'opinion' seems to contradict the logic of issuing the *Approved Lists of Bacterial Names* and starting the priority of names on 1 January, 1980. This topic needs much more discussion by the scientific community and is beyond the scope of this chapter. Both names are listed in Table 1, p. 3.

How many species of Enterobacteriaceae are there?

The current count is now well over a hundred, but the actual number could be in the thousands. We conclude that well-documented processes of evolution are at work. Others would argue for the alternative explanation, 'divine creation.' The strengths and weaknesses of this explanation are beyond the scope of this chapter. We would only observe that if a supernatural being is involved in establishing the population structure of the family Enterobacteriaceae, she is definitely a 'splitter' rather than a 'lumper.'

This is becoming apparent as methods such as DNA–DNA hybridization and 16S rRNA sequencing are being used to study strains isolated from human clinical specimens, plants, animals, and the environment. Grimont et al. (1981) found five *Cedecea* species in a small collection of clinical strains that had been reported 'unidentified,' but were phenotypically similar. Similarly, Müller et al. (1996), found six new species of *Buttiauxella* and two new species of *Kluyvera* (actually one, see *Kluyvera cochleae* above) in a large collection of strains isolated from snails. Additional DNA–DNA hybridization subgroups, which are probably new species (sometimes called 'genomospecies'), have been found in systematic studies of *Enterobacter cloacae* (Brenner 1992a, b), *Proteus vulgaris* (O'Hara et al. 2000b), *Rahnella aquatilis* (Brenner et al. 1998), *Citrobacter* (Brenner et al. 1993) *Yersinia* (Brenner 1992a; Brenner et al. 1999b), and many of the enteric groups (Farmer et al. 1985). This 'complexity of nature' causes a tremendous problem for the routine identification and reporting of isolates from human clinical specimens. As a compromise solution, Farmer (2003) argued for using the term 'complex' as a more precise and descriptive term for those 'species' that are actually composed of several DNA–DNA hybridization groups. For example, the '*Enterobacter cloacae* complex' is defined as a vernacular name and is composed of five (or more) DNA hybridization groups that are phenotypically very similar and also very closely related to the type strain of *Enterobacter cloacae*. This is in contrast to the precise scientific name *Enterobacter cloacae* which should be strictly limited to those strains related at the species level to the type strain. Several other species 'complexes' in the family have been defined and discussed (Farmer 2003).

Most of the Enterobacteriaceae that medical personnel and clinical microbiologists encounter every day belong to just a few of the many species described (Ewing 1986). However, the expanding number of Enterobacteriaceae species is becoming a serious problem for reference laboratories and for commercial identification systems. Identification methods are becoming inadequate for complete and accurate identification. When a commercial identification system gives an unusual organism for a final identification, there are several possibilities to consider. The first is that the identification is correct, just unusual. Another possibility is that the identification is incorrect because another aerobic or anaerobic organism is present as a contaminant, and the biochemical profile is the result of the metabolic activities of the mixture. Another possibility is that a handling or coding error was made somewhere along the way. Before a final report of an unusual organism is issued, it is advisable to carry out several checks. This could include repeating the biochemical tests in the same commercial system after confirming the absence of a contaminating aerobic or anaerobic organism; testing the isolate in another commercial identification system; and comparing the strain's antibiogram with known patterns reported for this organism. If these steps do not resolve the problem, a reference laboratory can be contacted for advice, and often, the culture will be accepted for further study. Different commercial systems often give different identifications for the same strain. The 'gold standard' for identification is

DNA–DNA hybridization; 16S rRNA sequencing is a less accurate, but more readily available alternative. However, a reference laboratory's identification based on phenotypic characteristics will be the final step for most strains.

Keeping up with new genera and species

Two internet sites are very useful for following the nomenclature and classification of the family and for learning of new species that are being proposed. The National Library of Medicine 'PubMed' site contains an alphabetical list of the genera that is particularly useful. The internet site of J.P. Euzéby (www.bacterio.cict.fr) gives nomenclature, classifications, original literature citations, and detailed information for all of the genera and species in the family Enterobacteriaceae and its relatives. Unfortunately, it does not have an alphabetical list of the genera and species currently classified in the family.

It can not be overemphasized that most of the newly described organisms in Tables 1 and 3 (p. 3 and 14) are only very rarely found in clinical specimens (Farmer et al. 1985). Most clinically significant isolates belong to 20–25 species that have been well known for many years (Ewing 1986).

Another controversy – nomenclature for Shiga toxins/verotoxins produced by *E. coli* and *Shigella*

In a similar vein to the problems described for *Salmonella*, several different names are being used in the literature for the cytotoxins produced by *E. coli* and *Shigella*. This topic is critical because of the importance of *E. coli* O157 and other strains that produce these toxins (see *Escherichia*). Several different commercial assays for these toxins are being marketed; thus it is essential to read the package insert carefully to determine exactly which toxin(s) the kit is detecting, and to word laboratory reports accordingly.

For almost 100 years, it has been known that *Shigella dysenteriae* serogroup O1 produces a potent cytotoxin known as Shiga toxin. More recently, it has been shown that certain strains of *E. coli* that cause intestinal infections produced a similar toxin (Alexandre and Prado 2003) that was first detected because it was cytotoxic for Vero cells in tissue culture. A number of recent studies have defined these proteins from *Shigella dysenteriae* O1 and *E. coli*, and there is agreement that they comprise a family of toxins. They are being referred to in the literature as Shiga toxin (ST), Shiga-like toxins (SLT), and verotoxins (VT); and at least five different toxins are involved (Calderwood et al. 1996). The *E. coli* (EC) strains that produce these toxins are often referred to as 'STEC' and 'VTEC.' Recently, Calderwood et al. (1996) summarized the data available and proposed a new nomenclature for the toxins and for their corresponding genes. They recommended that strains of *E. coli* that produce these toxins be called 'Shiga toxin-producing' *E. coli*, which would replace the previous term, 'Shiga-like toxin producing.' They also recommended that the new toxin name be crossreferenced with the corresponding verotoxin name. With this nomenclature, a laboratory report for a stool culture might be worded, 'positive for *E. coli* O157:H7, which produces Shiga toxins Stx1 (VT1) and Stx2 (VT2).' Hopefully, the differences between those using the two different nomenclatures will be resolved, resulting in a single nomenclature.

CELL STRUCTURE AND CELLULAR ANTIGENS

Cell morphology

Unlike many other bacteria, the Enterobacteriaceae are essentially devoid of cell organization or internal structure when stained and examined under the light microscope. Most are small rods about 2–3 μm in length by 0.6 μm in width, with parallel sides and rounded ends. They stain evenly (no granules are seen; however, a few stains, such as the plague bacillus, stain as as 'bipolar') and are gram-negative, non-acid-fast, and do not form endospores.

However, there are exceptions to this normal cell morphology. Some strains are almost coccal in form; others are long, sometimes filamentous rods. There is a tendency for the coccobacillary, or the elongated form, to predominate in any single strain, but some cultures show a wide diversity in this respect.

Some important structures and antigens found in strains of Enterobacteriaceae include the cytoplasm, cell membrane, cell wall, enterobacterial common antigen, flagella, capsules, and fimbriae. Many important enteric pathogens synthesize attachment and colonization factors which are important in virulence.

Major cellular antigens and antigenic notation

The most important cellular structures for practical antigenic analysis are the cell wall (O antigens), flagella (H antigens), and capsules (K antigens). A fourth antigen F (for fimbriae) is occasionally used. Complete antigenic analysis is usually carried out in very specialized reference laboratories and utilizes sera produced in rabbits or monoclonal antibodies. For most Enterobacteriaceae, the antigenic formula is written after the genus and species names, and some general guidelines and examples follow:

- Antigens are given a number in the order in which they are discovered beginning with number 1. Examples: O1, O2, O3; H1, H2, H3; and K1, K2, K3, etc.
- The major antigens (O, H, and K) are separated by colons.
- In many species there is a schema only for the O antigen. Typical reports would be *Shigella dysenteriae* O9 and *Providencia alcalifaciens* O26.
- Subfactors of an antigen (defined for a few antigens) are separated by commas. A few O antigens have been given subfactor designations that are indicated by lower case letters. Example: the two different subfactors of *E. coli* O111 – O111a,b and O111a,c. In some species the O antigens are indicated by a series of factors and subfactors. Examples in *Yersinia enterocolitica* include: O1, O1,2,3, O2a,2b, O5, O5,27.

Slight differences in the antigenic composition can be very important. Strains of *Y. enterocolitica* O5,27 typically cause diarrhea and intestinal infection and contain virulence factors, but strains of *Y. enterocolitica* O5 typically do not cause diarrhea and lack virulence factors.

- In the genus *Klebsiella* the schema for K antigens is usually the only one used. A typical report would be *Klebsiella pneumoniae* K3.
- In other species there is a schema for O and H antigens. Typical reports would be *Escherichia coli* O157:H7 and *Serratia marcescens* O14:H12.
- A few reference laboratories have antisera for all four antigen types. A report from the International *Escherichia* Center might read *Escherichia coli* O6:K2:H1:F7.
- A major exception to these guidelines is the genus *Salmonella* in which actual names are given to 'species,' i.e. serotypes, instead of formulas. The name *Salmonella* serotype Enteritidis is used to report strains with O antigen factors 9, and 12 and H (phase 1) antigen factors g and m (see *Salmonella*).

Each of these cellular structures and antigens is discussed in the sections and chapters that follow. Also see the section on 'Typing methods.'

Cell envelope, cell wall, and O antigen

Most strains of Enterobacteriaceae are 'smooth' and synthesize a cell envelope and wall (Figure 1 in *Escherichia*) that are typical of other gram-negative bacteria (see the following six chapters, 52–57). The main structural components of the cell envelope are a thin inner layer of mucopeptide and a thicker outer layer. This outer layer is composed of a polymolecular complex of lipopolysaccharide with protein and lipid and probably also a lipoprotein element linked to the mucopeptide (Freer and Salton 1971).

The lipopolysaccharide (LPS) complex is a characteristic and integral component of the outer membrane of all gram-negative bacteria. It is now recognized that LPS consists of two parts with contrasting properties:

1. the hydrophilic polysaccharide component consisting of the O-specific polysaccharide together with the core oligosaccharide, and
2. the hydrophobic lipid A component that is responsible for the numerous pathophysiological effects of 'endotoxin' in humans and animals.

In the Enterobacteriaceae the link between the polysaccharide and lipid A components is provided by 2-keto-3-deoxyoctonic acid (KDO). Because of the acid liability of the ketosidic bond, lipid A can be obtained as a precipitate by mild acid treatment of LPS.

Lipid A is a complex phosphoglycolipid and its complete structure was established only in the comparatively recent past (Rietschel 1984). A general structure found in a number of taxonomically distinct families is characterized by a central biphosphorylated β(1,6)-linked D-glucosamine disaccharide which is substituted by up to 4 mol of ester- and amide-bound (*R*)-3-acyloxyacyl or (*R*)-3-hydroxyacyl residues. Lipid A from different bacteria may vary in the chain length of its 3-hydroxy fatty acids, the substitution pattern of its 3-hydroxyl group, and that of its backbone phosphoryl groups. In *E. coli*, *Proteus*, and *Salmonella*, the disaccharide backbone carries 4 (*R*)-3-hydroxytetradecanoyl groups in positions 2, 3, 2′ and 3′ and two phosphoryl residues in positions 1 and 4′. The 3-hydroxyl groups of up to three of these hydroxy fatty acids (positions 2, 2′, and 3′) are acylated by nonhydroxylated acyl residues whereas nonacylated, nitrogen-containing residues such as 4-amino-4-deoxy-l-arabinopyranose and phosphorylethanolamine may be bound to phosphoryl groups. The hydroxyl group in position 4 of the glucosamine disaccharide is free and that in position 6′ serves as the attachment site for KDO and hence for the polysaccharide components of LPS.

All LPSs of gram-negative bacteria, whether from smooth strains or rough mutants and from pathogenic and nonpathogenic strains, are potent endotoxins, and the endotoxic principle is lipid A. The effects of endotoxin on the animal body are numerous; when administered parenterally to a suitable animal it gives rise to fever, leukopenia followed by leukocytosis, hyperglycemia with a subsequent fall in the blood sugar far below the normal level, and lethal shock after a latent period; it provokes the localized and the generalized Shwartzman reaction and causes hemorrhagic necrosis in tumors and at the site of an intradermal injection of adrenaline; it gives rise to nonspecific resistance to the intraperitoneal injection of a variety of gram-negative bacteria and to damage by irradiation; and it has a powerful adjuvant effect on the antibody response to unrelated protein antigens but not to polysaccharide

antigens. No other biological material gives rise to such a wide variety of reactions, though some other bacterial products, for example, staphylococcal enterotoxin and the pyrogenic exotoxin of *Streptococcus pyogenes*, cause a number of similar effects. The mode of action of endotoxin is not fully understood. There is reason to believe that its action may be responsible for many of the consequences of established infections with gram-negative rods. For a review of the pathophysiology of endotoxin see Hinshaw (1985); for a review of its chemistry see Rietschel (1984).

Synthetic analogs of lipid A have been prepared and tested in a number of test systems, including lethal toxicity, B lymphocyte mitogenicity, macrophage activation, induction of crossed tolerance, and expression of lipid-A antigenicity, these synthetic part-structures were of activity comparable to that of free bacterial lipid A. However, in two test systems, pyrogenicity and local Shwartzman reaction, the synthetic analogs exhibited weak or no endotoxic activity. It is likely that distinct determinants are necessary for the expression of different biological activities of lipid A; the studies with synthetic analogs suggest that the presence of (*R*)-3-acyloxyacyl groups may be necessary for the full expression of pyrogenicity and the local Shwartzman reaction (Rietschel et al. 1985).

O ANTIGEN

Many Enterobacteriaceae can be subdivided by means of serological or molecular tests into a number of O-antigenic groups. Most O antigens are characterized by a single antigen, but others are a combination of antigenic 'factors,' which may appear in different combinations in other members of the family. There is also extensive sharing of O antigens between otherwise quite unrelated organisms. Nearly all the *Shigella* serotypes are antigenically related to one or more O groups of *E. coli*; the relation is sometimes unilateral, sometimes reciprocal, and sometimes one of complete identity. For example, the O antigen of *S. dysenteriae* 3 is identical to that of *E. coli* O124. Such a relation between *Salmonella* and *E. coli* serotypes is less common. There is also considerable sharing between the O antigens of *E. coli* and *Providencia*; and relationships, including some of identity, occur between *Y. enterocolitica* and certain serotypes of *E. coli* and *Salmonella* (Thomas et al. 1983).

Chemical characterizations and structural analyses have demonstrated that all *Salmonella* strains have a core oligosaccharide with the same structure (Lüderitz et al. 1971). A further five closely related structures have been found in *E. coli* (Jansson et al. 1981). By contrast, a great number of O-specific polysaccharides exist and differ in their composition or structure or in both. They are all made up of repeating oligosaccharide units so that each can be defined according to the structure of one of its repeating oligosaccharides and its joining linkage. Over 100 enterobacterial O antigens have been defined in this way and the details are summarized in the review of Jann and Jann (1984). For example, O-specific polysaccharides with a repeating sequence of mannose-rhamnose-galactose are found in *Salmonella* of groups A, B, D, and E of the Kauffmann–White scheme (see *Salmonella*). The chemical characterization of the epitopes ('immunodominant sugars') within these polysaccharides has also been described. Such polysaccharides also represent the receptors for bacteriophages that are responsible for antigenic conversions that depend on the effect of bacteriophage-induced endoglycosidases or a deacetylase. For example, groups E1 and E2 of *Salmonella* are interrelated by conversion with bacteriophage ϵ_{15}. The O-specific polysaccharides of some *E. coli* strains (but not *Klebsiella* or *Salmonella*) contain acidic components such as hexuronic acids, *N*-acetylneuraminic acid or phosphate. Thus, LPS with neutral and with acidic O-specific polysaccharides exist and can be distinguished by immunoelectrophoresis (Ørskov et al. 1977). The LPS prepared from any bacterial strain is always heterogeneous with respect to chain length of the O-specific polysaccharide and this can be demonstrated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the complete LPS (Jann et al. 1975). This phenomenon is generally interpreted as indicating the presence of individual LPS molecules with O-specific polysaccharide chains differing in size by multiple increments of oligosaccharide repeating units.

ROUGH VARIANTS

Rough variants of Enterobacteriaceae form LPS, but it does not contain any of the repeating O-specific oligosaccharides of the corresponding smooth form (Lüderitz et al. 1960). Rough variants are of two major classes or serogroups: RII (chemotype Ra), which contains all the core sugars, and RI (chemotype Rb), which together with a further series of mutants (chemotypes Rc to Re) have various defects in core structure (Lüderitz et al. 1966). A class of 'semi-rough' mutants (Naide et al. 1965), intermediate in characters between smooth and RII organisms, has core sugars but may form only part of the O-specific polysaccharide. The so-called T forms of *Salmonella* strains (see *Salmonella*) have serological characters resembling those of rough mutants and have a normal core structure, but form O-specific polysaccharide composed of ribose and additional galactose in place of the sugars of the corresponding smooth form (Wheat et al. 1967).

There is uncertainty about the part played by the O antigen in the virulence of the Enterobacteriaceae. If virulence is defined as the ability to multiply in the tissues of experimental animals after intravenous or intraperitoneal injection, and to cause a fatal infection, there is little doubt that most smooth enterobacteria are

more virulent than their rough variants. Semi-rough variants are usually of intermediate virulence. In studies with a strain of *E. coli* O18a,c:K1:H7 that had caused meningitis in an infant, Smith and Huggins (1980) showed that mutants lacking either the O18a,c or the K1 antigens were much less virulent for mice (by intraperitoneal injection) and chickens (by a variety of routes). Mäkelä et al. (1973) have shown that even quite subtle changes in the O antigens of otherwise isogenic *Salmonella* serotype Typhimurium derivatives can affect their virulence for mice when injected intraperitoneally. Heat-killed vaccines made from smooth organisms, or LPS extracted from them, give protection against injections of the homologous organisms, and also sometimes against related organisms sharing the same O antigen; for example, vaccines of smooth *Salmonella* serotype Paratyphi B (a serological group B serotype) will protect against infection with serotype Typhimurium (also a serological group B serotype) (Schütze 1930). Vaccines made from rough variants, or 'rough' polysaccharide, usually do not have this effect. On the other hand, protection by living vaccines, whether of smooth or rough strains, is often superior to that obtained with killed vaccines of smooth strains, and may be effective against organisms with unrelated O antigens. Very little is known about the significance of the polysaccharide portion of the O antigen in infections in the natural host. For further information about the virulence of enterobacteria and the part played in it by O antigens, see Molecular basis of bacterial adaptation to a pathogenic lifestyle, Roantree (1967) and Hinshaw (1985).

Flagella, motility, and H antigens

Most Enterobacteriaceae synthesize flagella and are motile. The main exceptions are the genera *Klebsiella*, *Leminorella*, *Moellerella*, and *Shigella*. *Y. pestis* is nonmotile, in contrast to the other species in the genus which are motile at lower temperatures, but nonmotile at 37°C. Most strains of *E. coli* are motile. In the genus *Salmonella*, the only constantly nonmotile serotypes are Gallinarum and Pullorum. In the other genera, nonmotile mutants occasionally occur. Flagella are always peritrichous except in *Tatumella*, whose flagella tend to be polar, subpolar, or lateral.

Leifson (1960), who made measurements on stained films, found the normal flagellar wavelengths to vary between 2.13 and 3.02 μm. He also described several variations in the shape of the flagella, of which the 'curly' was the most frequent. 'Curly' flagella have about half the normal wavelength, and function inefficiently. They occur as genetically stable mutants, and can also be induced phenotypically, in *Proteus* but not in other members of the Enterobacteriaceae, by growth at pH 5.5 (Hoeniger 1965). Nonmotile variants may be either nonflagellate or flagellate but 'paralysed.' In the salmonellae there is an unusual type of variation – phase variation (Chapter 54) – in which the production of flagella of different antigenic constitution is rapidly alternated. A similar phenomenon may occur occasionally in *E. coli* (Ratiner 1985). For reviews of the genetic control of flagellation, see Joys (1968) and Iino (1969). A study of the ultrastructure of the flagella surface in *E. coli* of different H serotypes showed that they could be grouped into a number of 'morphotypes,' each containing up to 15 different H serotypes (Lawn et al. 1977).

The upper limit of temperature for motility is often considerably lower than that for growth. Raising the temperature from 37 to 44°C inhibits the motility of *Salmonella* strains, and the production of flagella, while having no effect on the growth rate (Quadling and Stocker 1962). Most of the Enterobacteriaceae are motile at 37°C but some, notably *Enterobacter*, *Hafnia*, *Tatumella*, and *Yersinia*, should be tested at a lower temperature. 'Swarming' over the surface of solid media is a character only of certain strains of *Proteus* (see *Proteus*, *Morganella*, and *Providencia*).

Fimbriae

Many Enterobacteriaceae produce fimbriae (Constable 1956; Duguid et al. 1955; Duguid and Gillies 1957, 1958; Duguid 1959). Duguid distinguished between common fimbriae, which are usually present in large numbers (50–100) per cell, often influence the physicochemical characters of the organism, are chromosomally determined, and do not have an essential role in bacterial conjugation, and sex pili, which are present in smaller numbers, are determined by conjugative plasmids, and appear to be organs of conjugation (Duguid et al. 1966; Duguid 1968). Strains may carry both common fimbriae and sex pili, and more than one type of common fimbriae. Within the strain, there are individual cells with many common fimbriae and others with none, and there is reversible variation between the fimbriate and the non-fimbriate phase.

By means of electron microscopy, common fimbriae may be classified according to their number and their dimensions. In this way Duguid et al. (1966) found six types of common fimbriae, of which five were found among the Enterobacteriaceae, and all are peritrichate. Type 1 fimbriae are strongly hydrophobic and render the organism adhesive to plant, fungus, and animal cells, cause agglutination of untreated erythrocytes, and lead to the formation of a pellicle on the surface of liquid media. Adhesiveness and the ability to cause hemagglutination are reversed by D-mannose; fimbriae of this type are described as mannose-sensitive (MS). Type 2 fimbriae do not confer adhesiveness. Type 3 fimbriae facilitate adhesiveness to plant and fungal cells and to glass surfaces, but not to animal tissue cells or erythrocytes unless these are first treated with tannic acid or

heated to 70°C; these properties are uninfluenced by mannose and are termed mannose-resistant (MR). Type 4 fimbriae also cause MR adhesiveness, but this is active against untreated red cells. Type 5 fimbriae were not found in the Enterobacteriaceae. Type 6 fimbriae are rather scanty in number (4–40 per cell), longer than fimbriae of other types and do not cause adhesiveness. Fimbriae of type 1 are found in *Enterobacter*, *Escherichia*, *Klebsiella*, most *Salmonella* strains, *Shigella flexneri*, and *Serratia*; of type 2 in a few of the *Salmonella* strains that do not form type 1 fimbriae; of type 3 in *Klebsiella* and *Serratia* strains together with fimbriae of type 1; of type 4 only in *Proteus* strains; and of type 6 only in otherwise non-fimbriate strains of *K. ozaenae*.

Little is known about the function of the common fimbriae. Type 1 fimbriae are able to mediate adhesion to a wide range of human and animal cells that contain mannose residues. Such adhesion might favor pathogenicity and there are some examples of this (Duguid 1968; Duguid and Old 1980). However, the possible role of type 1 fimbriae in urinary tract infection remains controversial (see the reviews of Harber 1985 and Reid and Sobel 1987 for further details). On the other hand, the ability of bacteria with type 1 fimbriae to form a pellicle on the surface of static fluids increases their access to oxygen and leads to additional multiplication of the organism (Old et al. 1968; Old and Duguid 1970). This might be an advantage to saprophytic enterobacteria in their natural environment. Adhesiveness of type 3 fimbriae to plant root hairs and fungal mycelia might serve a similar function (Duguid 1968).

More recently, a number of filamentous protein structures resembling fimbriae have been described in *E. coli*. These cause MR hemagglutination, and there is good evidence that they play an important part in the pathogenesis of diarrheal disease and in urinary tract infection. They include the K88 antigen found in strains causing enteritis of pigs, the K99 antigen found in strains causing enteritis of calves and lambs, and the colonization factor antigens (CFA) found in enterotoxigenic *E. coli* of human origin (see *Escherichia*). Fimbriae that are important in urinary tract infection and cause MR hemagglutination are distinguished according to their receptor specificities. These include the P fimbriae that bind specifically to receptors present on the P blood group antigens of human erythrocytes and uroepithelial cells (see Bacterial infections of the urinary tract).

The sex pili or conjugative pili also vary morphologically (Bradley 1980a, b). Conjugative plasmids have been classified according to their inability to coexist in the same bacterial strain (incompatibility typing), and electron microscopy reveals that three morphologically distinct groups of pili are determined by plasmids of different incompatibility groups. Thin flexible pili are determined by plasmids of incompatibility groups I, B, and K; thick flexible pili are determined by plasmids of groups C, D, the F complex, H1, H2, J, T, V, and X; and rigid pili are determined by plasmids of groups M, N, P, and W.

Capsules and K antigens

Some species synthesize capsules and form mucoid colonies when first isolated; however, the capacity to produce capsules may be lost on prolonged storage or subculture on artificial media. The amount of capsular material varies enormously from strain to strain. Sometimes it forms a well circumscribed capsule around the organisms, but at others it becomes free in the surrounding medium as 'loose slime' (Duguid 1951). Capsulation is the rule in *Klebsiella*, and also occurs in some strains of *Enterobacter* and *E. coli*. *Klebsiella* capsules are composed mainly of acid polysaccharides and many antigenically distinct capsular polysaccharides have been recognized. The capsules of *E. coli* appear to contain one of two substances: type-specific polysaccharides (K antigens) and a more widely distributed mucoid (M) substance formed by many different strains (Kauffmann 1944). Rare strains that produce extracellular slime of the latter type occur in *Salmonella*, *Shigella*, and most other genera. Slime production is the rule among forms of *Salmonella* Paratyphi B that do not utilize D-tartrate, and is favored by continued incubation at low temperature. When grown on medium with high osmolarity, most *Salmonella* and *E. coli* strains produce extracellular slime (Anderson 1961; Anderson and Rogers 1963). This is a colanic acid (Goebel 1963; Grant et al. 1969) which appears to be identical with Kauffmann's M substance (see *Escherichia*; and 54, *Salmonella*).

The enterobacterial common antigen

The enterobacterial common antigen (Böttger et al. 1987; Erbel et al. 2003; Kunin et al. 1962; Whang and Neter 1962) is one of the defining characteristics for the family. It was first observed in indirect hemagglutination tests between antisera against *E. coli* of O-group 14 and erythrocytes coated with supernatants or extracts of cultures of other enterobacteria. The antigen–antibody reaction can also be detected by hemolysis in the presence of complement and by immunofluorescence (Aoki et al. 1966).

The enterobacterial common antigen has been detected in strains of, essentially, all the genera of Enterobacteriaceae tested. The major exception is absence in *Erwinia chrysanthemi* (Böttger et al. 1987; Mäkelä and Mayer 1976). Its detection in *Plesiomonas* was one of the strongest arguments for classifying this genus in the family. The latter finding might once have been surprising but several studies confirm that *Plesiomonas* and the genera *Aeromonas*, *Photobacterium*, and

Vibrio are phylogenetically linked with the Enterobacteriaceae and Pasteurellaceae to form a monophyletic unit within the γ subgroup of the Proteobacteria; in particular, *Plesiomonas shigelloides* is related to *Proteus* to the extent that it is generally agreed that this oxidase-positive organism should be classified in 'extreme boundaries' of the family Enterobacteriaceae (see Ruimy et al. 1994).

When vaccines of intact cells were injected into animals, only *E. coli* O14 and O56 gave rise to antibody against common antigen, but ethanol-soluble fractions of LPSs from other members of the Enterobacteriaceae also did so (Suzuki et al. 1964). Hammarström and colleagues (1971) were unable to separate the common antigen of *E. coli* O14 from the O polysaccharide and found only core constituents in their preparations. They concluded that *E. coli* O14 and O56 were lacking in O-specific polysaccharides; some rough strains formed much antibody to common antigen, and members of O groups with complicated O-specific polysaccharides formed little. Antigenicity was thus related to the accessibility of core constituents. Chemical analysis has shown that the common antigen is an amino sugar polymer consisting of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannoseaminuronic acid, partly esterified by acetic and palmitic acids (Mayer and Schmidt 1979). The common antigen usually occurs as a free glycolipid in the outer membrane but in certain strains, including those that are immunogenic for the common antigen, it is linked to the LPS core.

For further information about the chemistry of the O polysaccharides, see Lüderitz et al. (1966, 1971), Stocker et al. (1966), Ørskov et al. (1977), and Rietschel (1984); for general reviews of the structure and function of O antigens see the monograph by Weinbaum et al. (1971), Jann and Jann (1987), and Chapters 52–57.

Genetic organization – the chromosome, plasmids, and phages

The DNA of Enterobacteriaceae species is presumably organized as a single chromosome, in contrast to *Vibrio cholerae* which apparently has two. Recently, the complete genetic sequence of two strains of *E. coli* has been published (Hayashi et al. 2001). The chromosome of a pathogenic strain of *E. coli* O157 is 859 kb larger than that of of *E. coli* K12. There is a 4.1 Mb sequence highly conserved between the two strains, which may represent the fundamental backbone of the *E. coli* chromosome. There are 1.4 Mb of sequences that are O157:H7-specific, and most are probably foreign DNAs that were acquired by horizontal transfer. Twenty four prophages and prophage-like elements explain more than half of the O157:H7-specific sequences. Differences in the DNA of strains of the same species with a different evolutionary history form the basis of most of the molecular typing methods being used routinely in clinical microbiology and research laboratories.

Many strains of Enterobacteriaceae contain additional DNA in the form of plasmids and bacteriophages (phages), which code for important proteins such as toxins and colonization factors that are involved in disease production. Plasmids can also carry genes that code for a variety of enzymes involved in carbohydrate metabolism and cell wall synthesis. The chapters on *Escherichia*, *Shigella*, *Salmonella*, and *Yersinia* provide excellent examples of the practical importance of these extra-chromosomal elements.

NUTRITION AND GROWTH

Organisms in the family Enterobacteriaceae vary in their nutritional requirements, but almost all grow readily on peptone and meat extract nutrient media commonly used in the laboratory. Unlike most vibrios they do not require added NaCl for growth, but some strains are salt tolerant and will grow in NaCl concentrations above 6 percent. They grow under both aerobic and anaerobic conditions, although the growth is better in air which contains approximately 20 percent oxygen. The optimum temperature for most species of medical importance is 35–37°C. The range over which growth occurs is fairly wide, extending from a high of about 45°C to a low around the freezing point of water. Many *Enterobacter* and *Serratia* strains grow poorly, or not at all, at 44°C, and differ in this respect from *E. coli*. Moreover, many coliform bacteria (Leclerc et al. 2001) have a temperature optimum lower than 37°C, and others, which appear to grow as well at 37°C as at 30°C, are less active biochemically at the higher temperature. These organisms frequently grow slowly at temperatures as low as −1.5°C. They form an important part of the coliform flora of water and soil, and are commonly found in chilled meat (Eddy and Kitchell 1959). Many of the coliform bacteria of water that can grow at refrigerator temperatures are *Hafnia alvei* or *Serratia* strains. *Yersinia enterocolitica* is able to multiply rapidly at 4°C (Hurvell 1981) which may be important for its role in foodborne diseases and in infections acquired from the infusion of refrigerated blood and blood products.

Growth on solid media

On agar media, colonies of Enterobacteriaceae are relatively large, with an average diameter of 2–3 mm, but vary considerably in size (Table 4). They may be circular, raised, and low convex, with an entire edge and smooth surface; they may be flatter with a more irregular surface, and a more effuse and irregular edge; or they may assume the well known vine-leaf form that is commonly described as characteristic of *Salmonella*

Table 4 *Colonial appearance of the most common Enterobacteriaceae on MacConkey agar and sheep blood agar* [a]

Genus or species	Appearance and typical colony diameter on	
	MacConkey agar	Sheep blood agar[b]
Salmonella and *Shigella*	Colorless, flat, 2–3 mm	Smooth, 2–3 mm
Yersinia enterocolitica	Colorless, less than 1 mm	Smooth, less than 1 mm
Escherichia coli (lactose positive)	Red, usually surrounded by precipitated bile, 2–3 mm	Smooth, 2–3 mm
Escherichia coli (lactose negative)	Colorless, 2–3 mm	Smooth, 2–3 mm
Klebsiella pneumoniae	Pink, mucoid, 3–4 mm	Mucoid, 3–4 mm
Enterobacter	Pink, not as mucoid as *Klebsiella*, 2–4 mm	Smooth, 3–4 mm
Proteus vulgaris and *Proteus mirabilis*	Colorless, flat, often swarm slightly, 2–3 mm	Swarm in waves to cover plate
Other *Proteus*, *Providencia*, and *Morganella*	Colorless, flat, no swarming, 2–3 mm	Flat, 2–3 mm, no swarming

a) Most strains appear this way, but there are exceptions.
b) Most strains of Enterobacteriaceae are nonhemolytic, which is in contrast to many strains of *Aeromonas* and *Vibrio* which are hemolytic. However, a few strains of *E. coli*, and occasionally other species of Enterobacteriaceae, are strongly hemolytic.

typhi. Even with freshly isolated strains the range of variation is wide and the most varied colonial forms may be seen when old laboratory strains are examined. Apart from the possible appearance of rough variants, a single strain may show several different types of colony if successive subcultures in broth are interspersed with platings and subculture of individual colonies. A few strains produce dwarf colonies on nutrient agar but form colonies of normal size when provided with a source of reduced sulfur, such as sulfide or cysteine (Gillespie 1952).

A few genera and species have very distinct colonial morphologies that lead to instant recognition. This includes the mucoid colonies of *Klebsiella* and the swarming growth of *Proteus* strains. The differential value of the mucoid colony type is diminished only slightly by the fact that certain other organisms, including a few strains of *E. coli*, may produce mucoid colonies.

All members of the Enterobacteriaceae, with the exception of a few *Klebsiella* strains from the respiratory tract, grow in the presence of bile salts and form colonies on MacConkey agar nearly as large as those on nutrient agar. Occasional strains produce yellow or orange pigments (Parr 1937; Thomas and Elson 1957); and strains of the following more recently described species also produce yellow pigment: *Enterobacter sakazakii*, *Leclercia adecarboxylata*, and the *Enterobacter agglomerans* complex. In addition, many *Serratia* strains synthesize pigments of the prodigiosin family of chemical compounds and can appear light pink to bright red or orange.

Growth in liquid media

Typically, Enterobacteriaceae are 'smooth' and grown in broth with uniform turbidity. A slight deposit may form as growth increases, but this is easily dispersed by shaking the tube. Growth and turbidity increases rapidly during the first few hours, but then slows as toxic metabolic end products begin to accumulate. Pellicle formation is rare except when cells produce fimbriae, whose production can be enhanced by serial subculture in liquid medium. *Y. pestis* and *Y. pseudotuberculosis* are exceptions; they produce little turbidity in the center of the broth tube but a form of deposit on the sides and bottom of the tube. 'Autoagglutination in broth' is a useful method of detecting pathogenic strains of *Yersinia*.

REQUIREMENTS FOR VITAMINS AND AMINO ACIDS

Many strains of Enterobacteriaceae grow on Simmons citrate agar, an indication that they do not require vitamins or amino acids. Other genera and species have complex growth requirements, which is discussed in Chapters 52–57.

Susceptibility to physical and chemical agents

Most members of Enterobacteriaceae are killed by exposure to a temperature of 55°C for approximately 1 h, or to 60°C for 15–20 min. On the whole, *E. coli* strains tend to be slightly more resistant to heat than most other coliform bacteria. A small proportion of them are not completely destroyed by exposure to 60°C for 30 min in broth, or to pasteurization at 62.8°C for the same time in milk (Wilson et al. 1935). The 'aerogenes type' tends to be slightly more resistant to chlorine in water than the 'coli' or 'intermediate types' (Bardsley 1938). In raw water stored under atmospheric conditions, coliform bacteria may remain alive for weeks or months. '*Klebsiella aerogenes*' (*K. pneumoniae*) tends

to survive rather longer than *E. coli*, but the results are influenced by, among other things, the temperature (Platt 1935; Raghavachari and Iyer 1939b). In feces stored at 0°C, coliform organisms may persist for a year or more, the coli types often being gradually supplanted by the intermediate and aerogenes types (Parr 1938).

Most members of the Enterobacteriaceae are susceptible to all classes of chemical disinfectants (see Microbial susceptibility and resistance to chemical and physical agents) and, like other gram-negative rods, to sodium azide (Snyder and Lichstein 1940). Differences in susceptibility to dyes and other chemicals that are of use in enrichment and selective media for the isolation of pathogenic members of the family are mentioned in Microbial susceptibility and resistance to chemical and physical agents. The susceptibility of individual organisms to antibiotics and other antimicrobial agents varies greatly and is described at appropriate points in the following chapters.

Permanent preservation of cultures and working cultures

Compared to many other organisms, strains of Enterobacteriaceae are easy to grow and maintain unless they are allowed to dry on plating media, agar slants or in 'deeps.' Their die off is mainly due to drying. Freezing and storage in liquid nitrogen is the method of choice for long-term preservation. If liquid nitrogen storage is not available, cultures can be stored in a laboratory freezer at −70°C, or even in a 'home freezer' at –10 to −20°C. The lowest temperature available should be used (see Farmer (2003) for technical details). If a home freezer is used, a simple method is available to insure that freezing and thawing is not taking place, which could cause death of the stored culture. An aqueous solution of a dye in a closed tube is frozen, then inverted in the freezer. If thawing is taking place, the liquid will flow from the top of the tube to the bottom.

An alternative method is freeze drying (lyophilization) and storage at 4°C, the method used with good results by the national type culture collections in England, the United States, and many other countries.

It is often convenient to prepare 'working cultures' for daily use. Growth from an agar plate is touched and stabbed to the bottom of 13 × 100 mm screw cap tube of a peptone-meat extract type medium such as 'working stock medium' (Farmer 2003). The culture is grown overnight, and checked to insure that there is good growth. The tube can be sealed by closing the screw cap tightly. This procedure does not give an air tight fit, and water from the medium with begin to evaporate (at a rate dependent on the particular tube) and eventually strains will begin to die. To avoid this problem, a small volume (approximately 0.3 ml) of sterile mineral oil can be added to cover the surface (approximately 3 mm above the interface) which prevents evaporation of the medium. A second method to seal the culture is to insert a number '000' white rubber stopper into the neck of the screw-cap tube to seal it. Many species of Enterobacteriaceae survive for decades with this method, but some species will die. Before working cultures are made, important cultures should be frozen or lyophilized as explained above.

HABITATS

Enterobacteriaceae are distributed worldwide and have been isolated from many different sources. One of their most common ecological niches is the intestine of humans and other animals (Balows et al. 1992; Brenner and Farmer 2005; Brenner et al. 2005). Other common sources include plants, soil, water, fruits, vegetables, grains, flowering plants, trees, and a wide variety of foods. Some species occupy very limited ecological niches. For example, *Salmonella typhi* causes typhoid fever in humans. In contrast, strains of *Klebsiella pneumoniae* cause human infections, ranging from asymptomatic colonization of the intestinal, urinary, and respiratory tracts to fatal pneumonia, septicemia, and meningitis. *K. pneumoniae* also occurs in the environment and contributes to biochemical and geochemical processes (Krieg 1984).

HUMAN INFECTIONS, PATHOGENICITY, AND VIRULENCE

The family includes several of the most important bacterial pathogens. Enterobacteriaceae may account for 80 percent of clinically significant isolates of gram-negative bacilli and 50 percent of clinically significant bacteria in clinical microbiology laboratories. They account for nearly 50 percent of septicemia cases, more than 70 percent of urinary tract infections, and a significant percentage of intestinal infections. Common human diseases include diarrhea, other intestinal infections, meningitis, septicemia, bacteremia, urinary tract infection, pneumonia, wound infections, and abscesses. They are a major component of the normal intestinal flora of humans, but are relatively uncommon as normal flora of other body sites. Several species of Enterobacteriaceae are very important causes of nosocomial infections (Mayhall 2000).

Extraintestinal infections

Except for the species of *Shigella*, which rarely cause infections outside the gastrointestinal tract, many species of Enterobacteriaceae commonly cause extraintestinal infections. However, a small number of species, i.e. *E. coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, *Enterobacter aerogenes*, the *Enterobacter cloacae* complex, and *Serratia marcescens*, account for most of these infections. Urinary tract

infections, primarily cystitis, are the most common, followed by respiratory, wound, bloodstream, and central nervous system infections. Many of these infections, especially sepsis and meningitis, are life-threatening and are often hospital acquired. Because of the severity of these infections, prompt isolation, identification, and susceptibility testing of Enterobacteriaceae isolates are essential.

Intestinal Infections

Several organisms in the family Enterobacteriaceae are also important causes of intestinal infections of humans and animals worldwide. Although other species in the family have been associated with diarrhea (Wadström et al. 1976) or even implicated as causes of diarrhea, only organisms in four genera, *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia*, have been clearly documented as enteric pathogens. These four genera are discussed in the following chapters (*Escherichia*; *Shigella*; *Salmonella*; and *Yersinia*). Other Enterobacteriaceae such as *Citrobacter*, *Edwardsiella*, *Hafnia*, *Morganella*, *Proteus*, *Klebsiella*, *Enterobacter*, and *Serratia*, have an 'association with diarrhea,' and have occasionally been implicated (Balows et al. 1992; Wadström et al. 1976). Strains that produce biologically active compounds (these are often overstated as being 'enterotoxin-producing strains') of these Enterobacteriaceae have been isolated from people with diarrhea (Wadström et al. 1976), but the etiological role of these strains is uncertain. Some laboratories issue reports for stool cultures, such as '*Klebsiella pneumoniae* isolated in essentially pure culture (ten of ten colonies tested); please consult the laboratory to discuss possible significance,' to reflect this drastic change in the stool flora. There is no evidence that strains of these other genera are important causes of diarrhea.

In contrast, the evidence for the etiological role of *Plesiomonas shigelloides* in diarrhea is somewhat stronger. A safe generalization would be that 'certain strains of *P. shigelloides* may cause diarrhea in certain people under certain conditions, but it is probably not an intrinsic pathogen.' For an intrinsic pathogen, most strains would cause diarrhea in most people, under most conditions.

ISOLATION

Microscopic examination

Before stool specimens are cultured they should be examined visually for the presence of blood or mucus, but microscopic examination should not be done routinely because of its lack of specificity. Although identification by fluorescent-antibody staining is theoretically possible for all enteric pathogens, it has been of limited success because the method is difficult and there are many serological cross-reactions among the species of Enterobacteriaceae (Ewing 1986). This technique has been limited to detection of *Salmonella* strains (primarily by the food industry) and certain serogroups of *E. coli* and to outbreak investigations.

Extraintestinal specimens

Most strains of Enterobacteriaceae grow readily on the plating media commonly used in clinical microbiology laboratories. MacConkey agar, generally interchangeable with eosin methylene blue (EMB) agar, is usually used, because it allows a preliminary grouping of enteric and other gram-negative bacteria. The most common isolates of Enterobacteriaceae have a characteristic appearance on blood agar and MacConkey agar that is useful for preliminary identification (Table 4, p. 29). Broth enrichment can increase the isolation rate if small numbers of Enterobacteriaceae are present, but this step is not normally required.

Intestinal specimens

Media that should be used routinely for intestinal specimens include a nonselective medium such as blood agar, a differential medium of low to moderate selectivity such as MacConkey agar, and a more selective differential medium such as xylose-lysine-deoxycholate (XLD) agar or Hektoen enteric (HE) agar. A broth enrichment such as selenite (or GN or tetrathionate) can be included, particularly if the specimen is not optimal. A highly selective medium such as brilliant green agar or bismuth sulfite agar can also be included for isolating strains of *Salmonella*. A special plate, such as sorbitol-MacConkey (SMAC) agar (or one of its modifications) can be added to enhance the isolation of Shiga toxin-producing strains of *E. coli* O157:H7. This medium should be used if the stool is frankly bloody or if the patient has a diagnosis of hemolytic-uremic syndrome (HUS), and it can be used for all fecal specimens if resources permit. When *Y. enterocolitica* is suspected, a selective-differential medium, such as cefsulodin-irgasan-novobiocin (CIN) agar (also called *Yersinia* selective agar), can be added. A complete stool culture procedure should also include media for isolation of *Campylobacter* and possibly *Vibrio* strains in areas where cholera and other *Vibrio* infections are common.

IDENTIFICATION

There are many different approaches to identifying strains of Enterobacteriaceae (Farmer et al. 1985; Farmer 2003; and Chapters 52–57).

Conventional biochemical tests in tubes

Tube testing was once used by all clinical microbiology laboratories, and it is still widely used in reference and public health laboratories (Ewing 1986; Farmer 2003). Although some laboratories prepare their own media from commercial dehydrated powders, most of the common media are also available commercially in glass tubes that are ready to inoculate. Growth from a single colony is inoculated into each tube, and the tests are read at 24 h and, usually, also at 48 h. In many reference laboratories, most tests are often kept for 7 days to detect delayed reactions. Unfortunately, the media and tests are not completely standardized, and few laboratories use exactly the same formulations or procedures. Even with these variables, this approach usually results in correct identifications of the common species of Enterobacteriaceae. Table 1 (p. 3) gives the results for Enterobacteriaceae in 47 tests (for the media and methods used to generate the data in this table, see Hickman and Farmer 1978; Farmer et al. 1985; Farmer 2003).

Computer analysis to assist in identification

Two microcomputer programs have been developed in the CODC's Enteric Reference Laboratory to assist with the identification of Enterobacteriaceae cultures. 'George' and 'Strain Matcher' were described in the 1985 review of the family (Farmer et al. 1985). A detailed description and information for obtaining them have traditionally been available. Hopefully these programs will be modified to run on current operating systems, and will be made available via the Internet.

Screening tests, using all information available

Over the years, CDC's enteric reference laboratories have found that many genera, species, and serotypes can be tentatively identified with a minimum number of screening tests (Table 5). More precise identification can be made by using a complete set of tests or commercial identification systems. Because of limited availability of certain reagents (bacteriophage O1, *Yersinia* sera, etc.) these screening tests may be more useful in a reference or research laboratory.

Example 1. A blood isolate has the following properties: colonies on MacConkey agar are 2–3 mm in diameter, are bright red and nonmucoid, and have precipitated bile around them; it is indole positive and 4-methylumbelliferyl-β-D-glucuronidase (MUG) positive; it grows at 44.5°C; and is resistant to five of the 12 antibiotics tested. These results are completely consistent with *Escherichia coli*.

Example 2. An isolate from the feces of a diarrhea patient has the following properties: colonies on MacConkey agar are 2–3 mm in diameter and colorless; colonies on XLD agar are 2–3 mm and black; the isolate agglutinates in *Salmonella* polyvalent O serum and in *Salmonella* O-group B serum; the MUCAP test (hydrolysis of 4-methylumbelliferyl caprylate (Biolife, Milano, Italy); and lysis by the *Salmonella*-specific bacteriophage O1 are positive; and it is resistant to seven of the 12 antibiotics tested. All these results are consistent with *Salmonella* serogroup B.

'Kits' for identification

A kit is defined as a panel of miniaturized or standardized tests that are available commercially. The approach for using kits is similar to the conventional tube method, with the main differences being in the miniaturization, number of tests available, suspending medium, and method of reading and interpreting results (sometimes by machine). Kits are now used by most laboratories (Murray et al. 2003). Kits usually give the correct identification for the most common species of Enterobacteriaceae, but they may not be as accurate for some of the new species. It is important to check the instruction manual to determine which organisms are included in the database and the number of strains that were used to define each organism. The main problem with kit-based identification is that the tests used (usually about 20 tests) are becoming inadequate to differentiate all of the current species of Enterobacteriaceae given in Tables 1 and 3 (pp. 3 and 14).

This is also becoming a problem with conventional tube tests, even when the 47 tests given in Table 1 (p. 3) are carried out. Unusual identifications or 'no identification' obtained with a kit could be verified by other methods or approaches, but referral to a reference laboratory may be the best alternative. Other methods might include trying a different kit (which will have similar limitations), or using more expensive research techniques such as molecular tests, or 16S rRNA sequencing (Murray et al. 2003).

Problem strains

Most strains of Enterobacteriaceae grow rapidly on plating media and on media used for biochemical identification, but occasionally a slow-growing or fastidious strain is encountered. Some strains grow poorly on blood agar but much better on chocolate agar incubated in a candle jar. This characteristic suggests a possible nutritional requirement or a mutation involving respiration. There are slow-growing strains of *E. coli*, *K. pneumoniae*, and *Serratia marcescens*, and typical biochemical

Table 5 *Screening tests for genera and species of Enterobacteriaceae often isolated from human clinical specimens*[a]

Organism (genus, species or serotype)	Test or property[b]
Salmonella	Lactose$^-$, sucrose$^-$, H_2S^+, O1 phage$^{+,c}$ MUCAP$^{+,d}$ agglutinates in polyvalent serum[b], typical colonies on media selective/differential for *Salmonella* (brilliant green agar, SS agar, Rambach agar, etc.), lyzed by the *Salmonella* specific bacteriophage,[c] often antibiotic resistant
Salmonella typhi	H_2S^+ (trace amount only), agglutinates in group D serum
Shigella	Nonmotile, lysine$^-$, gas$^-$, agglutinates in polyvalent serum, biochemically inactive, often antibiotic resistant, molecular test: PhoE^{+d}
Shigella dysenteriae	Agglutinates in group A serum, D-mannitol$^-$
Shigella dysenteriae O1	Catalase$^-$, agglutinates in O1 serum, Shiga toxin$^+$
Shigella flexneri	Agglutinates in group B serum, D-mannitol$^+$
Shigella boydii	Agglutinates in group C serum, D-mannitol$^+$
Shigella sonnei	Agglutinates in group D serum, D-mannitol$^+$, ornithine decarboxylase$^+$, lactose$^+$ (delayed), colony variation: smooth to rough
Escherichia coli	Extremely variable biochemically, indole$^+$, MUG$^+$, grows at 44.5°C, sometimes antibiotic resistant, PhoE$^+$ molecular test[d]
Escherichia coli O157:H7	Colorless colonies on sorbitol-MacConkey agar, MUG$^-$, D-sorbitol$^-$ (or delayed), agglutinates in O157 serum, and H7 serum
Yersinia	Grows on CIN agar, often more active biochemically at 25°C than 36°C (motile at 25°C, nonmotile at 36°C), urea$^+$
Yersinia enterocolitica, pathogenic serotypes	CR-MOX$^+$, pyrazinamidase$^-$, salicin$^-$, esculin$^-$, pathogenic serotypes agglutinate in O typing sera: 3; 4,32; 5,27; 8; 9; 13a,13b; 18; 20; or 21
Yersinia enterocolitica O3 (a pathogenic serotype)	D-Xylose$^-$, agglunitates in O3 serum, tiny colonies at 24 h on plating media
Yersinia enterocolitica, nonpathogenic serotypes	CR-MOX$^-$, pyrazinamidase$^+$, salicin$^+$, esculin$^+$, do not agglutinate in typing O sera: 3; 4,32; 5,27; 8; 9; 13a,13b; 18; 20; or 21
Citrobacter	Citrate$^+$, lysine decarboxylase$^-$, often grows on CIN agar, strong characteristic odor
Hafnia	Lysed by *Hafnia* specific bacteriophage[c], often more active biochemically at 25°C than 36°C
Klebsiella	Mucoid colonies, encapsulated cells, nonmotile, lysine$^+$, very active biochemically, ferment most sugars, VP$^+$, malonate$^+$, resistant to carbenicillin and ampicillin
Enterobacter	Variable biochemically, citrate$^+$, VP$^+$, resistant to cephalothin
Serratia	DNase$^+$, gelatinase$^+$, lipase$^+$, resistant to colistin and cephalothin
Serratia marcescens	L-arabinose$^-$
Serratia, other species	L-arabinose$^+$
Proteus-Providencia-Morganella	Phenylalanine$^+$, tyrosine hydrolysis$^+$, often urea$^+$, resistant to colistin
Proteus	Swarms on blood agar, pungent odor, H_2S^+, gelatin$^+$, lipase$^+$
Proteus mirabilis	Urea$^+$, indole$^-$, ornithine$^+$, maltose$^-$
Proteus vulgaris	Urea$^+$, indole$^+$, ornithine$^-$, maltose$^+$
Providencia	No swarming, H_2S^-, ornithine$^-$, gelatin$^-$, lipase$^-$
Morganella	Very inactive biochemically, no swarming, citrate$^-$, H_2S^-, ornithine$^+$, gelatin$^-$, lipase$^-$, urea$^+$
Plesiomonas shigelloides	Oxidase$^+$, lysine$^+$, arginine$^+$, ornithine$^+$, myo-inositol$^+$

a) This table gives only the general properties of the genera, species, and serogroups, so there will be exceptions. See Table 1 in this chapter and Chapters 52–57 for more details and more precise data. The properties listed for a genus or group of genera generally apply for each of its species, and the properties listed for a species generally apply for each of its serotypes.
b) Biochemical test results are given as percentages in Table 1. The serological tests refer to slide agglutination in group or individual antisera (O1, O3, etc.) for *Salmonella*, *Shigella*, *Yersinia*, or *Escherichia coli*, respectively.
c) These are two bacteriophage tests useful for identification.
d) CIN, cefsulodin-irgasan-novobiocin agar (a plating medium selective for *Yersinia*); CR-MOX, Congo red, magnesium oxalate agar (a differential medium useful for distinguishing pathogenic from nonpathogenic strains of *Yersinia*); MUCAP, 4-methylumbelliferyl caprylate (a genus specific test for *Salmonella*); MUG, 4-methylumbelliferyl-β-D-glucuronidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PhoE, a test done by PCR that is sensitive and specific for *E. coli*/*Shigella* (see text and Sprierings et al. 1993); VP, Voges–Proskauer.

reactions of these strains usually require extended incubation. Another type of problem organism is sometimes isolated from patients who are being treated with antimicrobial agents. Li et al. (1974) described such 'pleiotropic' (having multiple phenotypic expression) mutants of *S. marcescens* and *Salmonella* after exposure

to gentamicin. These strains react atypically in many of the standard biochemical tests and were difficult to identify. A different type of pleiotropic mutant induced by chemical exposure was reported by Lannigan and Hussian (1993). A *Salmonella* strain lost the ability to produce hydrogen sulfide, reduce nitrate to nitrite, and produce gas from glucose because of chlorate resistance acquired after exposure to Dakin's solution (a solution that contains chlorate and is found in hospitals). Some atypical and slow-growing strains become more typical and grow better when they are transferred several times. Laboratories occasionally isolate strains that grow rapidly but have a biochemical reaction profile that does not fit any of the described species, biogroups, or enteric groups of Enterobacteriaceae. At present, this type of culture can only be reported as 'unidentified.' It may be an atypical strain of one of the organisms listed in Table 1 (p. 3), or it may belong to a new species that has not been described (Farmer et al. 1985; Warren et al. 2000). Additional testing at a state, national, or international reference laboratory can often answer this question, and has in the past led to the discovery of new causes of human infections (Brenner and Farmer 2005; Farmer et al. 1985).

Identification and differentiation – a historical perspective

The tests listed in Tables 1 and 5 were developed over a period of over 100 years, a fact that the current generation of laboratory scientists does not seem to appreciate.

It was recognized early that the fermentation of sugars provided a useful means of distinguishing certain pathogenic enterobacteria from the coliform bacteria. Theobald Smith, for instance, observed that *E. coli* fermented lactose, whereas *Salmonella* serotype Typhi did not (Smith 1890); and the production of acid and gas from glucose by *E. coli*, but of acid only by *Salmonella* serotype Typhi, was pointed out by Chantemesse and Widal in 1891. Shigellae were also distinguished from the coliform bacteria and divided into a few broad groups by similar methods (see *Shigella*). For many years, interest was concentrated on the serological identification of *Salmonella* and *Shigella*, and the biochemical classification of the remaining enterobacteria was left mainly in the hands of the water bacteriologists, who had a profound influence on it between 1900 and 1950.

In his original description of the coliform bacteria, Escherich (1885) noted the occurrence of two types: one, '*Bacterium coli*,' formed fairly large rods, was motile, and clotted milk slowly; the other, '*Bacterium lactis aerogenes*,' formed shorter, plumper rods, was nonmotile, and clotted milk more actively. In the next few years, the introduction of suitable indicators of acidity (Wurtz 1892) and of means of detecting gas production (Smith 1890; Durham 1898) added to the ease with which fermentation tests could be carried out. Various elaborate classifications of the coliform bacteria were made according to the range of their ability to attack sugars, but none of these proved satisfactory (Bergey and Deehan 1908; MacConkey 1905, 1909; Jackson 1911).

An important correlation between biochemical activity and natural habitat was observed at about this time; it was found that '*B. lactis aerogenes*' (later called '*Bacterium aerogenes*') was an infrequent inhabitant of the intestine, but was often found on vegetation and in the soil, whereas '*B. coli*' was a constant intestinal parasite (Winslow and Walker 1907). This was of practical as well as theoretical importance, and the presence or absence of '*B. coli*' in water supplies came to be regarded as a valuable indication of fecal pollution. The ability to distinguish between those that were of intestinal origin and those that might occur in unpolluted waters was aided by the discovery that there were differences between the results of fermentation of one and the same sugar by different coliform bacteria. Thus, there were differences in the amount of gas produced from glucose and the ratio of CO_2 to H_2 in the gas (Smith 1895; Harden and Walpole 1906), in whether or not acetylmethylcarbinol was produced, and in the final pH (see Bacterial growth and metabolism). Levine (1916a, b) established that there was a high negative correlation between the results of the VP test for acetylmethylcarbinol and the MR test for final acidity in glucose-containing media. In a series of intensive studies, he placed on a firm foundation the conclusion that the lactose-fermenting coliform bacteria could be divided into two primary divisions; the first comprised strains that produced a moderate amount of gas with a CO_2:H_2 ratio of about 1:1, were VP^- and MR^+, included most of the strains isolated from the feces of humans and animals, and corresponded to '*B. coli*'; the second comprised strains that produced a greater amount of gas with a CO_2:H_2 ratio of about 2:1, were VP^+ and MR^- and were often found on plants and in unpolluted water. These could be further subdivided into '*B. aerogenes*' which did not liquefy gelatin and '*Bacterium cloacae*' which did.

Later, Brown (1921) drew attention to the usefulness of a medium containing citrate for distinguishing '*B. aerogenes*' from '*B. coli*,' and Koser (1923, 1924, 1926) devised a synthetic medium in which citrate was the sole source of carbon. He was able to subdivide coliform bacteria into an MR^+, VP^-, $citrate^-$ 'coli type,' an MR^-, VP^+, $citrate^+$ aerogenes type, and an MR^+, VP^-, $citrate^+$ 'intermediate type'; only the first of these was commonly of fecal origin.

Considerable help was also afforded by the test introduced in 1904 by Eijkman who found that 'coli' but not 'aerogenes' strains were able to form gas in a glucose broth medium incubated at 46°C. After a chequered career, this test was improved and standardized (Levine

et al. 1934; Wilson et al. 1935) by the institution of accurate temperature control at 44°C and the replacement of glucose broth by MacConkey's lactose bile salt broth. It was then shown to be a highly specific test for fecal coliform bacteria in the natural waters of many countries. However, as shown by Raghavachari and Iyer (1939a) and by Boizot (1941), organisms that were MR^-, VP^+, $citrate^+$, but produced gas from lactose at 44°C were common in the surface waters in some tropical countries. These organisms, referred to by British water bacteriologists as 'Irregular VI', can be distinguished from fecal coliform bacteria by their inability to form indole at 44°C (Mackenzie et al. 1948). These tests, with the addition of indole production and gelatin liquefaction, for many years formed the basis of the classification of coliform bacteria used by British water bacteriologists. In addition to their usefulness in practice, they had the advantage of conforming in broad outline with the biochemical classification of the Enterobacteriaceae as a whole that gained general acceptance in the 1950s (Enterobacteriaceae Subcommittee Report 1954a, b, Kauffmann 1956c, 1959; Kauffmann et al. 1956).

For the detailed biochemical classification of the Enterobacteriaceae, however, many additional tests are required. The fermentation of sugars is of little value for the establishment of broad groupings, but action on individual substrates is often useful to distinguish between closely related groups. For example, the production of acid, with or without gas, from adonitol, cellobiose, glycerol, inositol, and starch is useful in making subdivisions within *Klebsiella* and *Enterobacter* (Kauffmann 1956a, b). In several genera, lactose fermentation is late or irregular. The introduction of the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) test for β-galactosidase (Bacterial growth and metabolism), which quickly reveals potential ability to attack this sugar, has added uniformity to the characters of *Citrobacter* and some biochemical varieties of *Enterobacter*, *Klebsiella*, and *Shigella* (Bülow 1964; Lapage and Jayaraman 1964). Other useful tests, also described in Chapter 3, include those for the utilization of various organic acids as sole carbon sources or their fermentation in peptone media, for the oxidation of gluconate, the decarboxylation or deamination of amino acids and for the hydrolysis of hippurate. It must be pointed out that virtually all members of the Enterobacteriaceae produce some hydrogen sulfide under optimal conditions, and that the term 'H_2S production' is used in this group to indicate the ability to produce blackening in triple sugar iron agar, in Kligler's medium or in ferrous chloride gelatin. The test for urea hydrolysis was originally introduced for the recognition of *Proteus*, but many other members of the Enterobacteriaceae give positive reactions that are of value in classification when a suitable medium is used (see Bacterial growth and metabolism). Tetrathionate reductase is produced by nearly all strains of *Citrobacter*, *Proteus*, *Salmonella*, and *Serratia* and by a few strains of *Enterobacter*, *E. coli*, and *Klebsiella*, but is not produced by strains of *Edwardsiella* and *Shigella* (Le Minor 1967).

In general, the Enterobacteriaceae produce few exoenzymes. Rapid liquefaction of gelatin (in 1–2 days) occurs only in some *Proteus* strains and in *Serratia*, slower liquefaction (in 3–7 days) in salmonellae of subgroups II, IIIa, IIIb, and IV, and very slow liquefaction (in 15–30 days or more) in *Enterobacter* spp. and *Klebsiella oxytoca*. Deoxyribonuclease production and lipolysis are confined to *Serratia* and some *Proteus* strains (Davis and Ewing 1964; Martin and Ewing 1967), whereas strains of *Cedecea* are also lipolytic. According to Mulczyk and Szewczuk (1970), nearly all *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Serratia* strains, but no other enterobacteria, produce pyrrolidonyl peptidase. For further information about the biochemical characters of enterobacteria, see Kauffmann (1969), Lapage et al. (1979), Farmer et al. (1985), and Ewing (1986).

Table 1 (p. 3) summarizes the usual behavior in some commonly used biochemical tests of the Enterobacteriaceae that are of clinical interest. Details of the biochemical characters that differentiate between the species within these genera are given in the following chapters. In some genera, exceptions occur so frequently that it is difficult to adequately describe the biochemical reactions in a single table. In other genera there are distinct differences between different species, subspecies or biochemical varieties (biotypes or 'biovars').

Molecular methods for identification

Molecular methods have proved useful for identification to the level of family, genus, species, serotype, clone, and strain and for differentiating pathogenic from nonpathogenic strains (see Molecular basis of bacterial adaptation to a pathogenic lifestyle). For example, a polymerase chain reaction (PCR) test for the *phoE* gene appears to be a sensitive and specific test for determining if a strain belongs to the *Escherichia-Shigella* group (Sprierings et al. 1993). However, few if any of these molecular methods are commercially available. In the USA, commercial tests must also be approved by the Food and Drug Administration if they are used on human clinical specimens. Each county will probably have its own regulatory requirements. These problems have greatly restricted the use of molecular methods in clinical microbiology laboratories. However, they have proved extremely useful in a research setting.

Commercial products

A wide variety of commercial products and reagents are available for the Enterobacteriaceae, but availability is constantly changing. The best approach is to go to suppliers' internet sites to check availability, technical

information, and price. Some of the main companies and names useful in internet searches include: Difco, BBL, Becton Dickinson, BD Biologicals, Oxoid Inc., Denka Seiken, Eiken, Institut Pasteur Production, SA Scientific, Murex Biotec Ltd, ProLab Diagnostics, Remel, and Meridian Diagnostics Inc.

The US Food and Drug Administration's *BAM Manual* (www.fda.gov) lists many additional resources that are useful.

TYPING METHODS

Traditional (conventional) typing methods include serotyping, biotyping, antibiotic resistance patterns (antibiograms), bacteriophage (phage) typing, bacteriocin production and sensitivity, outer membrane protein (OMP) pattern typing, and plasmid profile analysis. Serotyping is still one of the most useful techniques because of its stability and application at the international level. For example, a culture of *E. coli* O157 would be typed as O157 by most laboratories in the world that are using adequate sera. With other typing methods it is extremely difficult to assign the same type name when different laboratories are doing the typing.

In recent years, molecular typing methods have replaced many of the traditional methods. A wide variety of these methods are described in the following chapters. Their main advantages include intralaboratory reproducibility and the application of the same method to many different organisms. Other advantages include their excellent discriminatory power, speed, and use of commercial equipment and reagents. A spectacular success has been the use of pulsed field gel electrophoresis (PFGE) patterns in the epidemiological analysis of the enteric pathogens. A molecular subtyping network named PulseNet (www.cdc.gov/pulsenet) is allowing pathogen tracking at the national and international level for *E. coli* O157, *Salmonella*, and other organisms. The following six chapters (Chapters 52–57) also discuss typing methods and their applications.

ANTIBIOTIC SENSITIVITY AND RESISTANCE

When antibiotics were first introduced, there was only slight resistance among the species of Enterobacteriaceae. Today, antibiotic resistance is much more common among strains isolated from humans and animals (Bradford 2001; Mammeri et al. 2001; Palucha et al. 1999). Antibiotic resistance patterns vary depending on the organism and its origin.

There are two basic types of resistance. Intrinsic resistance is a genetic property of most strains of a species and evolved long before the clinical use of antibiotics. For example, essentially all strains of *Serratia marcescens* have intrinsic resistance to penicillin G, colistin, and cephalothin. Table 6 (p. 36) lists some of the common species of Enterobacteriaceae and their intrinsic resistance patterns. The second type of antibiotic resistance is acquired resistance. Bacteria become resistant when they are exposed to the evolutionary selective pressure of antibiotics (or other agents that result in crossresistance). A second mechanisms for acquired resistance is through horizontal transfer of resistant genes which is usually through plasmid acquisition (see Bacterial genetics and Microbial susceptibility and resistance to chemical and physical agents which discuss antibiotics and antibiotic resistance).

Antibiotic susceptibility testing is usually carried out on isolates in clinical laboratories, and provides an 'antibiogram' that is useful for determining clinical

Table 6 *Intrinsic antimicrobial resistance in some common Enterobacteriaceae*

Genus/species	Most strains are resistant to
Buttiauxella species	Cephalothin
Cedecea species	Polymyxins, ampicillin, cephalothin
Citrobacter amalonaticus	Ampicillin
Citrobacter freundii	Cephalothin
Citrobacter diversus (*C. koseri*)	Cephalothin, carbenicillin
Edwardsiella tarda	Colistin
Enterobacter cloacae	Cephalothin
Enterobacter aerogenes	Cephalothin
Many other *Enterobacter* species	Cephalothin
Escherichia hermannii	Ampicillin, carbenicillin
Ewingella americana	Cephalothin
Hafnia alvei	Cephalothin
Klebsiella pneumoniae	Ampicillin, carbenicillin
Kluyvera ascorbata	Ampicillin
Kluyvera cryocrescens	Ampicillin
Proteus mirabilis	Polymyxins, tetracycline, nitrofurantoin
Proteus vulgaris	Polymyxins, ampicillin, nitrofurantoin, tetracycline
Morganella morganii	Polymyxins, ampicillin, cephalothin
Providencia rettgeri	Polymyxins, cephalothin, nitrofurantoin, tetracycline
Other *Providencia*[a] species	Polymyxins, nitrofurantoin
Serratia marcescens[b]	Polymyxins, cephalothin, nitrofurantoin
Other *Serratia* species	Polymyxinsc, cephalothin
'*Serratia fonticola*'	Ampicillin, carbenicillin, cephalothin

a) Most strains of *Providencia stuartii* are also resistant to cephalothin and tetracycline.
b) *Serratia marcescens* can also be resistant to ampicillin, carbenicillin, streptomycin, and tetracycline.
c) Most *Serratia* species are resistance to polymyxins, but some strains have unusual zones of inhibition, 10–12 mm or larger even though they are resistant when tested by other methods.

Table 7 *Synonyms of some Enterobacteriaceae names and terms*

Synonym	Name used in this chapter
Arizona hinshawii	*Salmonella* subgroups IIIa and IIIb
Calymmatobacterium granulomatis	*Klebsiella granulomatis*
Citrobacter amalonaticus biogroup 1	*Citrobacter farmeri*
Citrobacter intermedius biotype a	*Citrobacter amalonaticus*
Citrobacter intermedius biotype b	*Citrobacter diversus*
Citrobacter koseri	*Citrobacter diversus*
Citrobacter species 9	*Citrobacter rodentium*
Citrobacter species 10	*Citrobacter gillenii*
Citrobacter species 11	*Citrobacter murlinae*
Donovania granulomatis	*Klebsiella granulomatis*
Edwardsiella Hawke's Group GA 7752	*Edwardsiella ictaluri*
Edwardsiella anguillimortifera	*Edwardsiella tarda*
Enteric Group 1	*Escherichia vulneris*
Enteric Group 8	*Kluyvera*
Enteric Group 9	*Enterobacter sakazakii*
Enteric Group 10	*Escherichia fergusonii*
Enteric Group 11	*Escherichia hermannii*
Enteric Group 15	*Cedecea*
Enteric Group 17	*Enterobacter asburiae*
Enteric Group 19	*Enterobacter taylorae*
Enteric Group 40	*Ewingella americana*
Enteric Group 41	*Leclercia adecarboxylata*
Enteric Group 45	*Koserella trabulsii*
Enteric Group 46	*Moellerella wisconsensis*
Enteric Group 47	*Klebsiella* Group 47
Enteric Group 57	*Leminorella*
Enteric Group 59	*Buttiauxella noackiae*
Enteric Group 63	*Buttiauxella ferragutiae*
Enteric Group 64	*Buttiauxella gaviniae*
Enteric Group 75	*Enterobacter hormaechei*
Enterobacter agglomerans DNA 3	*Pantoea dispersa*
Enterobacter agglomerans DNA 6	*Pantoea ananas*
Enterobacter agglomerans DNA 13	*Pantoea agglomerans*
Enterobacter tayloreae	*Enterobacter cancerogenus*
Erwinia cancerogena	*Enterobacter cancerogenus*
Erwinia dissolvens	*Enterobacter dissolvens*
Erwinia nimipressuralis	*Enterobacter nimipressuralis*
Escherichia adecarboxylata	*Leclercia adecarboxylata*
Hawke's group GA 7752	*Edwardsiella ictaluri*
Klebsiella aerogenes	*Klebsiella pneumoniae*
Klebsiella edwardsi	*Klebsiella pneumoniae*
Klebsiella edwardsi var. edwardsi	*Klebsiella pneumoniae*
Klebsiella edwardsi var. atlantae	*Klebsiella pneumoniae*
Klebsiella mobilis	*Enterobacter aerogenes*
Klebsiella pneumoniae subsp. *pneumoniae*	*Klebsiella pneumoniae*
Klebsiella pneumoniae subsp. *ozaenae*	*Klebsiella ozaenae*
Klebsiella pneumoniae subsp. *rhinoscleromatis*	*Klebsiella rhinoscleromatis*
Kluyvera species 3	*Kluyvera georgiana*
Kluyvera cochleae	*Enterobacter intermedium*
Koserella trabulsei	*Yokenella regensburgei*
Levinea amalonatica	*Citrobacter amalonaticus*
Levinea malonatica	*Citrobacter diversus*
0157 ('zero' 157)	O157 ('capital oh' 157)
Paracolobactrum anguillimortiferum	*Edwardsiella tarda*

(*Continued over*)

Table 7 *Synonyms of some Enterobacteriaceae names and terms (Continued)*

Synonym	Name used in this chapter
phagovar	phage type
Photorhabdus luminescens DNA hybridization group 5	*Photorhabdus asymbiotica*
Providencia alcalifaciens biogroup 3	*Providencia rustigianii*
Providencia friedericiana	*Providencia rustigianii*
Proteus inconstans A	*Providencia alcalifaciens* and *Providencia stuartii*
Proteus inconstans B	*Providencia stuartii*
Proteus morganii	*Morganella morganii*
Proteus rettgeri	*Providencia rettgeri*
Salmonella serotype (or serovar) Typhi	*Salmonella typhi*
Salmonella cloleraesuis subspecies *choleraesuis* Serotype (or serovar) Typhi	*Salmonella typhi*
Salmonella enteritidis	*Salmonella* serotype *Enteritidis*
Salmonell cloleraesuis subspecies *choleraesuis* serotype *Enteritidis*	*Salmonella* serotype *Enteritidis*
Salmonell cloleraesuis subspecies *bongori*	*Salmonella bongori*
serovar	serotype
Shiga-like toxin	Shiga toxin
Shigella serogroup A	*Shigella dysenteriae*
Shigella serogroup B	*Shigella flexneri*
Shigella serogroup C	*Shigella boydii*
Shigella serogroup D	*Shigella sonnei*
Xenorhabdus luminescens	*Photorhabdus*
Xenorhabdus luminescens DNA hybridization group 5	*Photorhabdus asymbiotica*
Vero toxin	Shiga toxin
Yersinia pseudotuberculosis subspecies *pestis*	*Yersinia pestis*
Yersinia biogroup X2	*Yersinia aldovae*
Yersinia biogroup 3A	*Yersinia mollaretii*
Yersinia biogroup 3B	*Yersinia bercovieri*

susceptibility to the antibiotics tested. Two other uses of antibiograms are the comparison of isolates in epidemiologic studies and as an aid to identification.

The antibiogram as a marker in epidemiological studies

When the selective ecological pressures of antibiotics are changed, the resistance patterns of epidemic and endemic strains in an institution or geographical area may also change. These changes have been documented in institutional outbreaks that have lasted for several months or longer. Even though it has some limitations in stability, the antibiogram is probably the most useful and practical laboratory marker for comparing strains. It can be extremely helpful in recognizing and analyzing infection problems.

Use of the antibiogram for identification

The antibiogram of a culture can be compared with those of known isolates (Table 6, p. 36) to provide a different approach to identification. Sometimes, the antibiogram and laboratory's identification are incompatible; for example a culture of *Klebsiella* that is susceptible to ampicillin and carbenicillin, or a culture of *Enterobacter* that is susceptible to cephalothin. In these instances, the culture should be checked for purity, and both the identification and the antibiogram may need to be repeated.

LITERATURE AND THE INTERNET

The literature on Enterobacteriaceae is voluminous; this is an understatement! Two free internet search mechanisms are highly recommended: PubMed (www.ncbi.nlm.nih.gov/entrez/query.fcgi) for published articles and Google (www.google.com) for internet citations. Some search topics yield thousands of hits; others yield only a few. For example, a recent PubMed search yielded 218 605 hits for Enterobacteriaceae, 196 000 for *Escherichia coli*, 1 006 for *Citrobacter freundii*, one for *Photorhabdus asymbiotica* and one for Enteric Group 137. The number of hits for these five subjects on Google was 231 000, 254 000, 4 560, 33, and 10, respectively.

In doing searches it is important to include synonyms and alternative names (Table 7, p. 37) for genera, species, serotypes, and scientific terms. On a recent

PubMed search, *Citrobacter koseri* gave 44 citations, but *Citrobacter diversus* gave 203. Table 7 lists some of the more common and important synonyms to use in searches. Euzyby's internet site gives synonyms for most of the genera and species of Enterobacteriaceae. For example, in searching for the 'Shiga-vero' class of toxins produced by *E. coli* O157, all these terms should be tried: 'Shiga-like toxin', 'Shiga toxin', and 'vero toxin' (searches are not case sensitive; 'Shiga' gives the same hits as 'shiga').

It is important to consider subtle differences and common errors. O antigens are written with a 'capital O' as in O157 ('oh' 157). However, a common error is to write this as 0157 ('zero' 157). A Google search for the error '0157' yielded a surprising 22 500 hits compared to 41 600 hits for the correct 'O157'.

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Escherichia

TOM CHEASTY AND HENRY R. SMITH

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GENUS AND SPECIES DEFINITION

Members of this genus are oxidase-negative, gram-negative, rod-shaped bacteria, usually motile by peritrichous flagella and do not produce spores. They are facultatively anaerobic; gas is usually produced from fermentable carbohydrates. They are methyl red-positive and Voges–Proskauer-negative. Many strains produce polysaccharide capsules or microcapsules.

The type species is *Escherichia coli*. Most strains of this species promptly ferment lactose or give a positive *o*-nitrophenyl-β-D-galactopyranoside (ONPG) reaction. They produce indole, fail to hydrolyze urea or to grow in Møller's KCN broth. H_2S production is not detectable on triple sugar iron (TSI) agar or Kligler's iron agar (KIA), phenylalanine is not deaminated, gelatin is not liquefied, and gluconate is not oxidized. Most strains decarboxylate lysine and utilize sodium acetate, but they do not grow on Simmons' citrate agar. Other species included in the genus *Escherichia* are *Escherichia blattae* (Burgess et al. 1973), *Escherichia fergusonii* (Farmer et al. 1985), *Escherichia hermannii* (Brenner et al. 1982a), and *Escherichia vulneris* (Brenner et al. 1982b) (see Table 1). A sixth species *Escherichia albertii* (associated with cases of diarrhea in Bangladeshi children) has recently been proposed (Huys et al. 2003).

INTRODUCTION AND BRIEF HISTORICAL PERSPECTIVE

E. coli was first identified by the German pediatrician Theodore Escherich during his studies of the intestinal flora of infants. He described the organism in 1885 as *Bacterium coli commune* (Escherich 1885) and established its pathogenic properties in extraintestinal infections (Escherich 1894). The name *Bacterium coli* was widely used until 1919, when Castellani and Chalmers defined the genus *Escherichia* and established the type species *E. coli* (Castellani and Chalmers 1919).

E. coli is an organism that has been intensively studied under various aspects. In general bacteriology, it has been used as a model organism for studies of cell structure, growth, and metabolism. Later, it became an important vehicle for the cloning of genes from prokaryotic and eukaryotic cells, and for the expression of gene products. *E. coli* is used as a control organism for the testing of the efficacy of antimicrobial agents and disinfectants, and is considered an indicator organism of fecal contamination in food, water, and the environment. It plays an important role as part of the intestinal microbial ecosystem in humans and warm-blooded animals. Last but not least, some strains are important pathogens for these hosts.

Table 1 Escherichia *differentiation of species*

Test or property	*E. coli* Normal	*E. coli* Inactive	*E. fergusonii*	*E. hermannii*	*E. vulneris*	*E. blattae*	*E. albertii*
Motility	+	–	+	+	+	–	–
Indole production	+	v	+	+	–	–	–
Lysine decarboxylase	+	v	+	–	v	+	+
Ornithine decarboxylase	v	v	+	+	–	+	+
Acetate utilization	+	v	+	v	v	–	+
Growth in KCN	–	–	–	+	v	–	–
Yellow pigment	–	–	–	+	v	–	–
Gas produced during fermentation	+	–	+	+	+	+	+
Fermentation of:							
adonitol	–	–	+	–	–	–	–
D-arabitol	–	–	+	–	–	–	–
cellobiose	–	–	+	+	+	–	–
lactose	+	v	–	v	v	–	–
D-mannitol	+	+	+	+	+	–	+
mucate	+	v	–	+	v	v	v
D-sorbitol	+	v	–	–	–	–	–
sucrose	v	v	–	v	–	–	–

Normal, typical biochemical reactions; inactive, reactions previously associated with '*Escherichia dispar*'; KCN–, failure to grow in presence of potassium cyanide.

HABITAT

E. coli is a component of the normal intestinal flora of both humans and warm-blooded animals (mammals and birds). The organism is excreted with the feces and may survive in the environment. However, it appears that there is no independent existence outside the body. Accordingly, *E. coli* is considered an indicator organism for fecal contamination and is an important parameter in food and water hygiene. In organs outside the intestinal tract, *E. coli* may cause a variety of diseases, but the responsible strains as well as strains causing enteritis in humans and mammals, are characterized by the presence of specific virulence factors. Infections with such strains develop either by the endogenous route (e.g. urinary tract or gall bladder infections, septicemia), or they are spread in the hospital via contaminated equipment and by the hands of the nursing staff (urinary and respiratory tract infections, wound infections, septicemia, meningitis). The natural reservoirs of enterovirulent strains are the intestines of humans, enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC) or domestic animals (ETEC) and Vero cytotoxin-producing (VTEC). The organisms are transmitted by direct contact or via contaminated food and water.

E. fergusonii, *E. hermannii*, and *E. vulneris* have been isolated from clinical specimens and from the intestinal contents of humans and warm-blooded animals. They are considered to be opportunistic pathogens and have occasionally been associated with wound infections in humans. The atypical *Escherichia* species *E. blattae* is found only as part of the intestinal flora of cockroaches.

MORPHOLOGY

Escherichia form rod-shaped cells of between 2.0 and 6.0 μm in length and 1.1 and 1.5 μm in width with rounded ends (Ørskov 1984). The shape may vary from coccoid to long filamentous rods. In some strains one of these forms may prevail, but others may show a wide diversity (Gross and Holmes 1990). By the usual staining methods the cells appear homogeneous without granules; spores are not formed.

Escherichia strains, with the exception of the 'inactive' biovar of *E. coli*, are usually motile by a set of peritrichous flagella. These proteinaceous structures form long, slender appendages of 19–24 nm diameter which extend about 15–20 μm from the cell surface (Lawn et al. 1977; Silverman and Simon 1977). As shown by Lawn et al. (1977), all H antigens of *E. coli* have a morphologically distinct surface pattern that is identical within each strain and among H antigens associated with different O groups. The different morphological types could be grouped into six structurally related morphotypes.

Capsules or microcapsules made of acidic polysaccharides are common in *E. coli*. They may vary in their size and thus may be detected by light microscopy or, as microcapsules, only by serological or chemical techniques (Ørskov and Ørskov 1984). Mucoid strains sometimes produce extracellular slime which is either a polysaccharide of certain K antigen specificities, or a

common acid polysaccharide formed in many *E. coli* strains, as well as other Enterobacteriaceae. The latter is described as M antigen and composed of colanic acid (Ørskov and Ørskov 1984).

E. coli produce different kinds of fimbriae (or pili) that vary in structure and antigenic specificity. These are filamentous, proteinaceous, hair-like appendages surrounding the cell in varying numbers. If present in large numbers they may influence the physicochemical properties of the organisms and render the strains O-inagglutinable. Fimbriae are either chromosomally or plasmid-encoded, and more than one type of fimbriae may be carried by one individual cell (Ørskov and Ørskov 1984). Within a fimbriate strain, there are individual cells with many fimbriae and others with none and there is reversible variation between the fimbriate and nonfimbriate phase (Gross and Holmes 1990). Fimbriae are hydrophobic and exert host- or organ-specific adhesion properties.

METABOLISM, CULTURAL CHARACTERISTICS, AND GROWTH REQUIREMENTS

Escherichia are facultatively anaerobic organisms. They are chemo-organotrophic, having both a respiratory and a fermentative type of metabolism, but growth is less plentiful under anaerobic conditions. The optimal growth temperature is 37°C at which they grow well on ordinary media containing 1 percent peptone as carbon and nitrogen source (Holt and Krieg 1994). According to the state of the lipopolysaccharide of the outer membrane, the growth on solid media is characterized by glistening, smooth (S), or dry, wrinkled, rough (R) colonies, respectively. In liquid media, S forms show homogeneous turbid growth within 12–18 h, whereas R forms agglutinate spontaneously, forming a sediment on the bottom of the test tubes (Ørskov 1984). Pellicle formation on the surface of liquid media can be seen in heavily fimbriate strains after prolonged incubation (>72 h) at 37°C, or can be induced by serial subcultures under these conditions.

E. coli exerts pronounced metabolic activity between 15 and 45°C (Holmes and Gross 1990); under optimal conditions the generation time is 20 min. Exotoxins, such as enterotoxins and hemolysins, are best produced at about 37°C, are translocated into the periplasmic space and therefore may be liberated in higher amounts by exposure to 2 000 IU/ml of polymyxin B for 30 min (Bockemühl 1992). In contrast to most other coliform bacteria, *E. coli* ferments lactose and produces indole at 44°C. This combination has been used for identification in food and water bacteriology. After 18–24 h of incubation at 37°C, *E. coli* forms large (2–3 mm), circular, convex, and nonpigmented colonies on nutrient and blood agar; hemolysin is produced by a number of strains. *E. coli* strains are resistant to low concentrations of bile salts (e.g. 0.05 percent sodium deoxycholate) and grow as large red colonies on MacConkey agar. *E. coli* is more heat resistant than most other species of Enterobacteriaceae and survives at 60°C for 15 min or at 55°C for 60 min (Holmes and Gross 1990).

Escherichia strains can be maintained for years in tightly closed nutrient agar stabs or, better, on Dorset egg medium kept in the dark at room temperature. However, even without further subcultures such strains tend to mutate to the 'rough' form and to lose virulence plasmids. Important cultures should be preserved in tryptic soy broth containing $\geqslant$10 percent glycerol at −70°C, in liquid nitrogen or as lyophilized cultures (Ørskov 1984; Rowe and Gross 1984).

IMPORTANT GENETIC MECHANISMS

For decades *E. coli* has been used as a model organism to study bacterial genetics for various reasons: no special nutritional or growth requirements, rapid proliferation with doubling times as short as 20 min, easy availability and nonpathogenicity.

Consequently, a variety of very basic genetic mechanisms has been discovered or have at least been extensively studied in this organism. These include our basic understanding of DNA replication, transcription, and translation processes; DNA restriction and modification systems where modification by methylases protects the cell's own genome from being restricted; F factor as a conjugational donor of chromosomal markers at high frequency (Hfr strains); recombination; DNA repair mechanisms; organization and expression of sets of genes that determine the metabolism of various compounds, e.g. the lactose operon. It is, therefore, not surprising that the genome of *E. coli* K12 was the one that was first analyzed. It consists of a single circular DNA molecule of about 4.6×10^6 base pairs with a molecular weight of 4×10^9 and a total length of about 1.4 mm. Many of the genes have been mapped and this knowledge has increased recently with the publication of the sequence of the entire genome of two strains of *E. coli* O157 which has a significantly larger genome of approximately 5.5×10^6 base pairs, (Perna et al. 2001; Hayashi et al. 2001). In addition, *E. coli* has been widely used as a cloning vehicle. It is beyond the scope of this chapter to go into detail regarding genetic mechanisms. The reader is referred to an excellent collection of reviews which cover most aspects of the cellular and molecular biology of this organism (Neidhart et al. 1996).

CELL WALL COMPOSITION AND ANTIGENIC STRUCTURE

Cell wall structure

First attempts to characterize the nature of the *E. coli* cell surface structures were made by Kauffmann in the

1940s using serological methods (Kauffmann 1943, 1944). During his investigations, he observed that antisera prepared from boiled cultures (O antisera) did not agglutinate many freshly isolated strains, but these cultures became agglutinable after heating at different temperatures. Kauffmann distinguished several surface antigens with different physical and serological properties and named them A, L, and B antigens (Kauffmann 1965).

The elucidation of the chemical structure of the gram-negative cell wall and its outer layers (Lüderitz et al. 1966; Lüderitz et al. 1973; Jann and Westphal 1975; Ørskov et al. 1977; Jann and Jann 1987; Rietschel et al. 1987) not only explained the serological differences and cross-reactions between antigens, but also recognized the pathophysiological importance of certain compounds now known as endotoxins.

As shown in Figure 1, the outer layers of *E. coli* consist of the outer membrane with phospholipids, lipid A and proteins, from which protrude the polysaccharide (LPS) chains, overlayed by capsular polysaccharides (CP) (Jann and Jann 1987). Both LPS and CP are the chemical basis of O and K antigens, respectively, and contribute to the pathogenicity of the organisms.

As reviewed by Jann and Jann (1987, 1992), LPS and CP are synthesized at the cytoplasmatic site of the cytoplasmic membrane of the bacteria from where they are transported to the outer membrane. Whereas CP are further secreted to form the bacterial capsule, LPS reside and are integrated into the cell wall through hydrophobic interaction. They consist of lipid A as the hydrophobic moiety linked to the O-specific polysaccharides through an oligosaccharide linkage region, called core region (Figure 1).

Lipid A, of which only one chemical structure is known in *E. coli* (Jann and Jann 1987), mediates as endotoxin many different in vivo activities including pyrogenicity, local Shwartzman reactivity, lethal toxicity in mice, adjuvant activity, induction of interferon and tumor necrosis factor. In humans, these biological properties contribute to a variety of pathophysiological effects, such as fever, hypotension, disseminated intravascular coagulation, and septic shock (Rietschel et al. 1987).

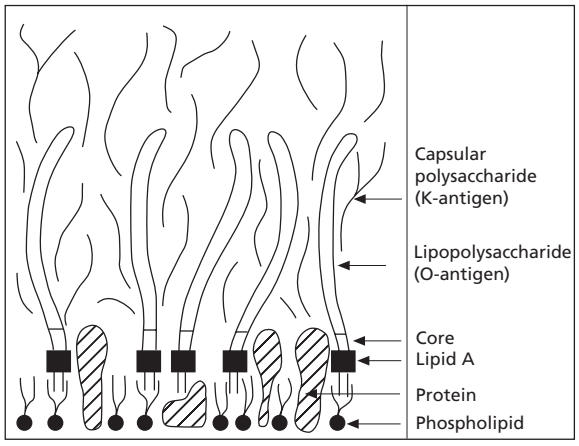

Figure 1 *Cell wall structure of* Escherichia coli *(modified from Jann and Jann 1987).*

Five different core structures have been identified in *E. coli* (Jann and Jann 1987) and 174 serological specificities of the polysaccharide chains have been defined. Loss of the O-specific polysaccharide moiety results in a mutation from smooth (S) to the rough (R) form of the bacteria; R mutants express R-LPS which consists only of lipid A and the core oligosaccharide. The polysaccharide chains consist of oligosaccharide repeating units that generally are neutral, but may also have acidic components in *E. coli* (Jann and Jann 1987).

The capsular (K) antigens of *E. coli* are acidic polysaccharides that, in view of their chemical, physical, and microbiological characteristics, have been divided into two groups. Group I CPs are of a high molecular weight (>100 000), are related to the *Klebsiella* capsular antigens and are coexpressed with O groups 8, 9, 20, and 101 of *E. coli*. They are stable at pH 6 and when heated to 100°C, correspond to the capsular antigen type A of Kauffmann (1965). Group II capsular antigens are of lower molecular weight (<50 000), are related to the K antigens in *Neisseria meningitidis* and *Haemophilus influenzae*, and are coexpressed with many *E. coli* O groups. They are heat-labile (B- and L-type capsular antigens, Kauffmann 1965) and do not resist pH 6. The chemical identity or relatedness between certain K antigens is associated with similar pathogenic traits. Thus, *E. coli* K1 antigen is identical to the K antigen of *N. meningitidis* type b, and both are related to the capsules of *H. influenzae* types a and b, all of which are characterized by an invasive capacity of the respective strains. In these organisms the capsules are responsible for both evasion of the unspecific host defense through interruption of the complement cascade and evasion of immune recognition due to molecular mimicry of substances found in the host's tissues. For more details on this fascinating topic, the reader is referred to the reviews of Jann and Jann (1987, 1992).

Antigenic structure of *E. coli*

Complete serotyping of *E. coli* includes the determination of the O, K, and H antigens, i.e. O:K:H, and if an additional fimbrial virulence factor is present, it is O:K:H:F. Examples given by Ørskov and Ørskov (1984) are O6:K2:H1:F7 for a typical strain from a patient with pyelonephritis, O18ac:K1:H7 for a meningitis isolate, or O111:H2 (no K antigen) for a strain from a case of infantile enteritis. Since a large number of single antigens has been described in *E. coli* and since most of these can be found in various combinations, a complete typing scheme comparable to the Kauffmann–White

scheme for *Salmonella* would be too extensive and of no practical applicability. Furthermore, as shown below, K-typing is restricted to a few specialists and, therefore, complete typing has been performed only on a rather small number of strains. Certain O groups can be associated with a number of different K and H antigens and, furthermore, a number of O groups are antigenically complex and would require further subdivisions. Therefore, only standard strains for the established O, K, and H antigens have been defined and listed (Ørskov et al. 1977, 1991; Ørskov and Ørskov 1984; Ewing 1986).

O ANTIGENS

Of the numbered O groups 1–181 (Scheutz et al. 2004), seven have been excluded for different reasons. Two of them (O31 and O93) proved to be identical with previously defined antigens (O1 and O8, respectively), four grouped strains were later identified as *Citrobacter* (O67, O72, O94, O122) and one reference strain was lost (O47) (Ørskov and Ørskov 1984; Ewing 1986). *E. coli* O antigens are not type- or species-specific, but numerous cross-reactions are known to occur between individual *E. coli* O antigens and also with the O antigens of *Shigella*, *Citrobacter*, *Salmonella*, *Providencia*, and *Yersinia* (Ørskov and Ørskov 1984; Ewing 1986; Holmes and Gross 1990). Therefore, determination of the O antigen must always be associated with a proper biochemical identification of the isolate. This is especially important for shigelae that share partial or identical O antigens with *E. coli* (Table 2).

O antigens are heat-stable and are not inactivated by heating at 100°C for 2.5 h (Kauffmann 1965). Such preparations are used for antiserum production in rabbits, but cultures carrying A-type capsular antigens need to be heated to 120°C for 2 h. Likewise, determination of the O antigens of *E. coli* requires, as a rule, heat treatment of the strain suspensions for 1/2 h at 100°C. Whereas typing of all defined O antigens is very laborious, requires extensive experience and is, therefore, restricted to reference laboratories, typing of a narrow scope of O groups may be helpful for the screening of certain pathotypes, such as EPEC, EIEC, or VTEC. Serogroups frequently associated with these enteropathogenic *E. coli* types are listed in Table 3. However, most of the virulence factors are encoded by mobile genetic elements, such as plasmids or phages, and therefore may be absent in such isolates, or may be present in other O groups. The problems encountered with the determination of *E. coli* O groups are discussed in the Laboratory methods section.

K ANTIGENS

Based on different sensitivities to heat treatment, Kauffmann (1965) differentiated three serological groups of *E. coli* envelope antigens. Strains with type A capsular antigens mostly grew with more or less mucoid colonies, were thermostable (100°C) and became O-agglutinable only after heating at 120°C for 2 h. These antigens are now known to belong to the group of class I capsular polysaccharides according to Jann and Jann (1987). The heat-labile antigens L and B were defined by their different agglutinin-binding power following inactivation at 100°C. However, results obtained for L- and K-type antigens were often inconsistent upon repeated examination of the same culture. Finally, Ørskov et al. (Ørskov et al. 1977; Ørskov and Ørskov 1984) showed that these K antigens included a variety of surface structures such as fimbriae, flagella, outer-membrane proteins, and capsular polysaccharides that vary quantitatively according to the culture conditions. These authors also showed that the 'heat-labile' polysaccharide K antigens in principle are thermostable and eluted from the bacterial surface during boiling, thus making K agglutination of boiled suspensions of bacteria impossible. Using immunoelectrophoresis with cellular extracts, these authors demonstrated the presence of capsular antigens in all the former A- and L-containing strains, but only in a few strains in which B antigens had been described. This method distinguished five reaction patterns to which all described K antigens could be allocated. Therefore, the term K antigen was suggested as the common name for all types of capsular polysaccharide antigens.

Table 2 *Examples of identical O antigens in* Shigella *and* E. coli

Shigella	Serovar	Corresponding *E. coli* O group	*Shigella*	Serovar	Corresponding *E. coli* O group
S. dysenteriae	2	112ac	*S. boydii*	1	149
	3	124		2	87
	5	58		4	53
	12	152		5	79
				8	143
S. flexneri	2b	147		11	105
	4b	135		14	32
	5	129		15	112ab

Modified from Ewing (1986).

Table 3 *Serogroups of* E. coli *frequently associated with intestinal infections in humans*

Pathotype	Predominant O Serogroups
Enteropathogenic (EPEC) infantile diarrhea	O26, O55, O86, O111, O114, O119, O125ac, O126, O127, O128ab, O142, O158
Enteroinvasive (EIEC) dysenteric disease in all age groups	O28ac, O112ac, O124, O136, O143, O144, O152, O164, O167, O173
Enterotoxigenic (ETEC). Common cause of travelers' diarrhea. Cause of diarrheal disease in both humans and animals.	O6, O8, O15, O20, O25, O27, O63, O78, O128ac, O148, O153, O159, O167
Vero cytotoxigenic (VTEC) or Shiga toxin-producing (STEC). Diarrhea frequently bloody, in all age groups, in young children leading to hemolytic–uremic syndrome (HUS)	O5, O26, O91, O103, O111, O113, O128ab, O145, O157
Enteroaggregative (EAggEC) associated with persistent diarrhea and travelers' diarrhea	O3, O44, O51, O77, O86, O92, O111ac, O125, O126
Diffusely adherent (DAEC) diarrhea in children	O1, O2, O21, O75

Adapted from Ørskov and Ørskov (1984), Ewing (1986), Bockemühl (1992), Willshaw et al. (2000).

For the reasons described above, K typing of *E. coli* is too complicated for routine purposes and is restricted to a few specialist reference laboratories.

H ANTIGENS

Flagellar antigens are heat-labile proteins. The described 53 H antigens are numbered H1 to H56. Of these, H50 has been withdrawn and H13 and H22 were deleted after identification of the type strains as *Citrobacter freundii* (Ørskov and Ørskov 1984). Although usually one antigenic type of flagellum is produced by motile strains of *E. coli*, phase variation (as found in motile *Salmonella* strains) has been described between flagellar antigens H3 and H16, and H4 and H16 (Ratiner 1982).

H antigens are determined by slide or tube agglutination using broth cultures or growth on semisolid media from actively motile strains. As most *E. coli* strains are poorly motile when they are first isolated, one or several passages through semisolid media (e.g. in U-shaped tubes) is usually necessary to obtain highly flagellate bacteria. Sometimes motility is better at 30°C than at 37°C. Most H antigens are type-specific and there are only a few antigenic relationships of practical importance (Ewing 1986). The methods applied for the determination of *E. coli* H antigens have been described by Ørskov and Ørskov (1984) and Ewing (1986). Although H typing is of importance for a precise phenotypic characterization of *E. coli* strains, its performance is usually limited to reference laboratories.

F ANTIGENS

Certain fimbrial antigens are involved in the adhesion process and, therefore, are important virulence factors. They are expressed at 37°C, but not at 18°C; chemically they are heat-labile proteins. Fimbrial antigens agglutinate a variety of erythrocytes that can be used for their characterization. Most *E. coli* strains produce a type of fimbria whose hemagglutinating capacity is inhibited in the presence of mannose, mannose-sensitive hemagglutination (type 1 fimbriae). In addition, strains associated with diarrheal or extraintestinal disease may produce fimbriae that still hemagglutinate in the presence of mannose, mannose-resistant hemagglutination; their formation is usually encoded by plasmid genes. Ørskov and Ørskov (1984) have developed a scheme for the serological typing of fimbrial antigens associated with different disease conditions (Table 4).

LABORATORY ISOLATION AND IDENTIFICATION

Isolation

Escherichia spp. may be recovered from various sites of the body either as normal flora or as causative agents of a variety of infections (gastrointestinal tract, urinary tract and wound infections, meningitis, septicemia, etc.). Transport and culture of extraintestinal specimens follow general practices as escherichiae are less fastidious than many of the other species that may be present in such materials. Specimens from normally sterile body sites are plated on the usual nonselective media such as blood, chocolate, or nutrient agar. If materials are likely to be contaminated or contain different organisms (e.g. specimens from the respiratory or the urinary tract, or from wound infections), media of low selectivity such as MacConkey, CLED (cysteine, lysine electrolyte deficient), or eosin–methylene blue

Table 4 *Ørskov and Ørksov typing scheme for fimbrial antigens of* E. coli

Fimbrial antigens	Previous designation	Disease association
F: 1	Type 1	
F: 2	CFA /I	ETEC, human
F: 3	CFA /II	ETEC, human
F: 4	K 88	ETEC, pig
F: 5	K 99	ETEC, bovine
F: 6	987	ETEC, pig
F: 7	C 1212	UTI, human
F: 8	C 1254–79	UTI, human
F: 9	3669	UTI, human
F: 10	C 1960–79	UTI, human
F: 11	C 1976–79	UTI, human
F: 12	C 1979–79	UTI, human

F: 1, Common fimbrial antigen.
UTI, urinary tract infection. Modified from Ørksov and Ørskov (1984).

(EMB) agar should be included in the set of plating media.

Direct culture on MacConkey or EMB agar of freshly passed stool specimens is the usual method for isolation of enterovirulent *E. coli* from patients with enteric infections. Rectal swabs are less desirable, but may be taken from acutely ill patients, e.g. in hospital.

The chance of detecting a pathogen can be increased if specimens are collected in the acute stage of the disease. Repeat examinations are of value, especially in convalescents or patients with previous antibiotic treatment. As *E. coli* is part of the normal intestinal flora, at least ten, but preferably more, colonies should be picked from the isolation media and tested for the presence of virulence markers. Cases of ETEC and VTEC infection have been identified in which one colony of pathogenic *E. coli* was detected among 200–300 'apathogenic' isolates (Altwegg and Bockemühl, 1998). Alternatively, polymerase chain reaction (PCR) targeting virulence-associated gene sequences can be performed on colony sweeps from MacConkey or other moderately selective agar plates. For the detection of toxin-producing strains, tests can be performed on colony sweeps or extracts of fecal specimens. Ideally such a procedure should be followed by subculture and identification of the responsible pathogenic strain.

Identification

Escherichia are identified phenotypically by use of a variety of biochemical tests, useful tests to differentiate the species are summarized in Table 1. Problems may arise in separating certain metabolically inactive *E. coli* strains from *Shigella* as intermediate forms may occur. Shigellae, by definition, are nonmotile and do not decarboxylate lysine or hydrolyse arginine.

A wide variety of biological, immunological, and molecular methods are now available to identify the different groups of enterovirulent strains of *E. coli*. Unfortunately, many of these methods are not widely used in routine diagnostic laboratories. Simple screening methods such as agglutination with specific antisera, which only identify such strains with a certain probability, have been employed in many laboratories. Thus, collaboration with specialized or reference laboratories is usually necessary to substantiate infections with enterovirulent *E. coli*.

EPEC

Although this pathotype is rather heterogeneous and different virulence factors have been identified, such strains are frequently associated with certain *E. coli* O groups that can be tested by use of agglutinating antisera. Screening for '*E. coli* serogroups related to infantile diarrhea' may be applied in outbreaks, but is controversial for sporadic diarrheal cases of infants below 1 year of age and rarely used now. Ten or more lactose-positive colonies are subcultured on blood or nutrient agar and agglutinated with a set of polyvalent and monovalent OK antisera. A subculture yielding a clear agglutination with a monovalent OK antiserum is heated at 100°C for 30–60 min and titrated in parallel with a reference culture of the same O group. The agglutination is read after overnight incubation at 50°C.

Enteropathogenic *E. coli* (EPEC) produce attaching and effacing lesions of intestinal epithelial cells. Definite identification of EPEC strains includes the fluorescence actin staining method (Knutton et al. 1989) (Figure 2) and cell culture tests with HEp-2 or HeLa cells to demonstrate localized adherence (Scaletsky et al. 1984; Cravioto et al. 1991). An ELISA for the detection of EPEC that show localized adherence has been described (Albert et al. 1991), this ELISA detected all strains that showed good localized adherence, whereas EPEC strains that did not show adhesion were negative. Molecular diagnosis by PCR (Figure 3) or colony hybridization to detect genes encoding for intimin (*eae*), bundle-forming pili (*bfpA*) or the EPEC adherence factor (EAF) plasmid (Schmidt et al. 1993a; Franke et al. 1994; Gunzburg et al. 1995). Alternatively *eae*, EAF, and *bfp* probes can be used to test strains belonging to EPEC serogroups, as well as other *E. coli*. Classical EPEC outbreak strains are positive with all three probes, whereas most EPEC serogroup strains isolated during the last decade in the UK are *eae*-positive, but negative with the EAF probe (Smith et al. 1990; Scotland et al. 1991, 1993). The nomenclature of 'typical,' that is *eae*+ and EAF+ and 'atypical,' *eae*+ and EAF-EPEC strains, has been introduced. Other differences between 'typical' and atypical' EPEC include patterns of adhesion in tissue culture and reservoirs of infection (Trabulsi et al. 2002). The importance of the *eae*+ EAF-strains in human disease has yet to be fully established, though outbreaks have been reported in Finland, Japan, USA,

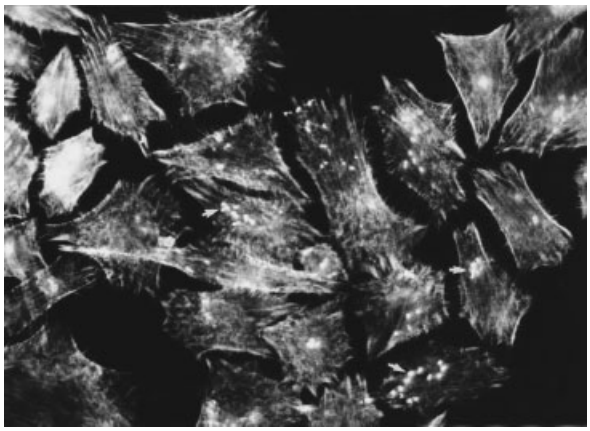

Figure 2 *Fluorescent actin staining (FAS) test of VTEC and enteropathogenic* E. coli*. Intimate adherence is characterized by actin polymerization (arrows).*

and the United Kingdom (Viljanen et al. 1990; Yatsuyanagi et al. 2003; Hedberg et al. 1997; Jenkins et al. 2003).

It is likely that EPEC do not represent a homogenous group, but rather consist of pathogenic strains with different virulence mechanisms.

EIEC

Enterinvasive *E. coli* (EIEC) do not decarboxylate lysine and are usually non- or late lactose fermenters. They belong to a limited number of serotypes, most of which are nonmotile and their somatic (O) antigens are identical with, or related to, certain *Shigella* antigens (see Table 2). EIEC strains are confirmed by demonstration of invasiveness (Serny or cell culture tests, see Figure 4), immunologically by an ELISA (Pál et al. 1985), or by detection of the *ipaH* gene present on the chromosome and on the virulence plasmid, which is also present in *Shigella* strains (Sethabutr et al. 1993; Lüscher and Altwegg 1994). Thus, if the invasive capacity has been established, subsequent species confirmation by biochemical tests is essential.

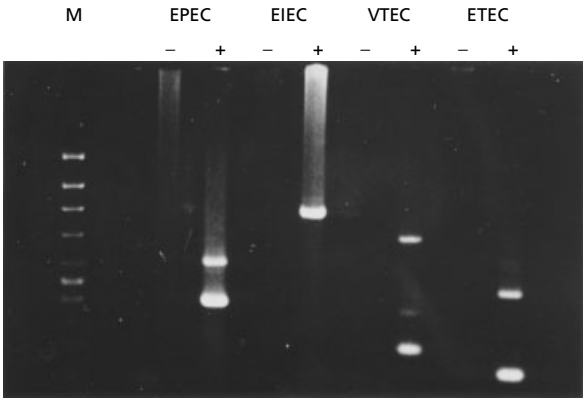

Figure 3 *Amplification of virulence genes of different* E. coli *pathotypes by the polymerase chain reactions: M, molecular weight marker, EPEC,* eaf *(top band),* eae *(lower band); EIEC,* ipaH*; VTEC,* vtx1B *(top band), VTEC-specific* eae *(middle band),* vtx2B *(lower band); ETEC,* lt *(top band),* st *(lower band).*

ETEC

Following earlier methods of enterotoxin detection by cell culture techniques (heat-labile enterotoxin (LT)) (Figure 5) or in animal models (the suckling mouse assay for the heat-stable enterotoxin (ST), and the rabbit ileal loop for LT and ST) (Figures 6 and 52.7), immunological tests are now commercially available. Furthermore, the genes encoding for LT and ST production can be detected by PCR or DNA probes (Abe et al. 1992; Lüscher and Altwegg 1994; Schultsz et al. 1994; Wray and Woodward 1994; Stacy-Phipps et al. 1995).

VTEC/STEC

Over 200 serotypes associated with the production of Vero cytotoxin (or Shiga toxin) have been identified in *E. coli* strains of human origin (Flemming Scheutz, personal communication). Of these, serotypes O157:H7 and O157:H-, plus a few others (see Table 5), are predominant; VT-producing strains of O group 157 usually do not ferment sorbitol and do not produce β-glucuronidase. For their detection, sorbitol MacConkey (SMAC) agar is of great value. This medium is commercially available. However, caution is needed as some strains of *E. coli* O157:H- first isolated in Europe, but recently isolated in Australia and Scotland, are able to ferment sorbitol rapidly and are β-glucuronidase positive (Gunzer et al. 1992; Bettelheim et al. 2002; Allison 2002); these and >90 percent of the other VT-producing serovars of *E. coli* will be missed if SMAC alone is used for their detection. By supplementing SMAC with cefixime or cefixime–tellurite, the selectivity for sorbitol-negative O157 VTEC can be improved (Chapman et al.

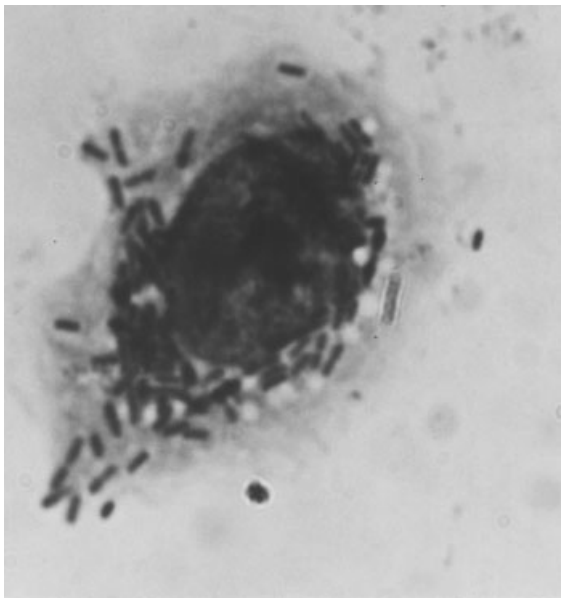

Figure 4 *Intracellular location of enteroinvasive* E. coli *(EIEC) in HeLa cells*

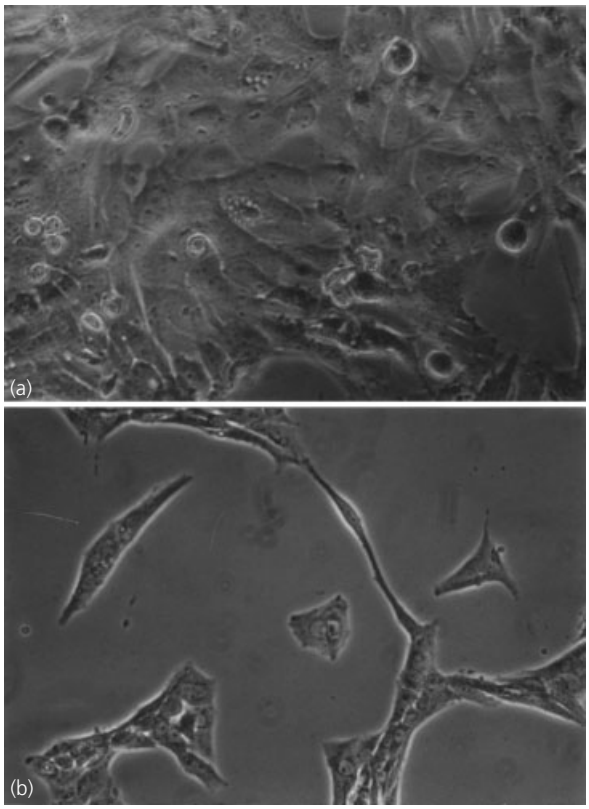

Figure 5 *Action of leat-labile (LT) of* E. coli *on cultured cells in vitro (courtesy of Virion, Switzerland)*

1991; Zadik et al. 1993). Detection rates of O157 VTEC from fecal samples can be improved by the use of a culture enrichment (liquid) stage before plating on to agar (Hindle et al. 1995).

Agglutinating sera and latex coagglutination tests for O group 157 are commercially available. Due to the antigenic relationship with a number of O antigens of *E. coli* and other enterobacteria (Aleksic et al. 1992), a positive slide agglutination should be confirmed by titration of heated antigens, as described for EPEC.

Figure 6 *Assay of* E. coli *enterotoxin (LT or ST) in the rabbit ileal loop test showing several negative and two positive loops with significant fluid accumulation*

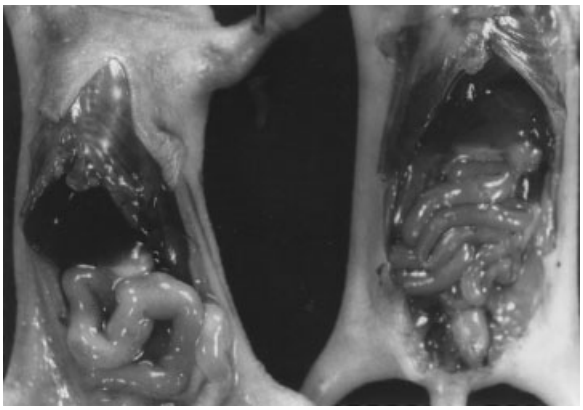

Figure 7 *Fluid accumulation due to* E. coli *heat-stable enterotoxin in the suckling mouse test: left, positive reaction; right, negative reaction*

Most strains of *E. coli* O157:H7 and many VTEC strains of other serovars produce a plasmid-encoded hemolysin that can be detected by subculture on a special blood agar plate (Schmidt et al. 1994) or for the presence of the *ehlyA* gene using PCR (Schmidt et al. 1995a).

Detection of cytotoxin production in the Vero cell culture test, confirmed by a neutralization assay, is the standard method for identification of VT-producing *E. coli*. This test is laborious and requires extensive experience and special laboratory facilities. Commercially available enzyme immunoassays (Scheutz et al. 2001) can be used for cytotoxin detection in both stool specimens and from *E. coli* isolates. Alternatively, a latex agglutination test (Unipath/Oxoid) can be used to demonstrate separately VT1 and VT2 production in culture filtrates.

Karch et al. (1996) showed that the sensitivity of VTEC O157 detection varies considerably with the diagnostic methods applied. Stool culture on SMAC, as well as direct PCR from stool specimens can detect about 10^5 *E. coli* O157 per gram of feces in a background flora of 10^7 coliform bacteria. The sensitivity can be increased to 10^2–10^3/g feces by pre-enrichment with, and subsequent separation of, paramagnetic particles coated with O157 antibodies (immunomagnetic separation (IMS)). IMS beads for detection of serogroups O26, O103, O111, and O145 are also available.

In view of the high proportion of concomitant *E. coli* and the comparatively low number of excreted VTEC in stool specimens, especially in patients with hemolytic–uremic syndrome (HUS), it is necessary to test using methods of increased sensitivity compared with direct plating. Therefore, screening of a large number of isolates for VT genes using molecular methods such as PCR or colony hybridization (Newland and Neill 1988; Rüssmann et al. 1995), or use of IMS for VTEC O157, have become the methods of choice in adequately equipped laboratories. Alternatively, PCR can be

Table 5 *Infective doses of enterovirulent* E. coli

Pathogen	Infective dose (cfu)	Reference	Type of study
EPEC	High (10^8–10^{10})	Donnenberg et al. (1993)	v
		DuPont et al. (1971)	v
		Savarino (1993)	v
		Ferguson and June (1952)	v
ETEC	High (10^6)	Levine et al. (1979)	v
EIEC	High (10^6–10^{10})	DuPont et al. (1971)	v
VTEC	Low (<100)	Griffin and Tauxe (1991)	e
		Wachsmuth (1994)	e
		Burnens et al. (1993), Willshaw et al (1994) c15 org/gram	e , also microbiological
EAggEC	High 10^{10}	Nataro et al. (1995)	v
DAEC	High (?)	Previously classified as EPEC	

e, epidemiological studies; cfu, colony forming units; v, volunteer studies.

performed on colony sweeps from MacConkey plates (Lüscher et al. 1992), giving an excellent sensitivity as illustrated by the fact that up to 200 colonies would have to be screened from positive plates to allow the isolation of the VTEC strain. After identification of a VT-producing colony, biochemical determination of the species must follow (World Health Organization 1994). Identified cultures should immediately be suspended in Luria–Bertani or tryptic soy broth supplemented with 20–50 percent glycerol and stored at −70°C to prevent loss of the VT encoding genes. The same procedure is recommended for the preservation of ETEC and other enterovirulent *E. coli* cultures.

For a detailed description of all the media and the established test procedures, the reader is referred to one of the manuals of microbiological laboratory methods (Ewing 1986; Balows and Hausler 1991; Burkhardt 1992; Gilligan et al. 1992; Murray and Baron 1995; Willshaw et al. 2000; Scheutz et al. 2001). Patients with *E. coli* O157 infection develop antibodies against O157 LPS which can be determined by ELISA or immunoblot, differentiating IgA, IgM, and IgG antibody classes (Chart et al. 1989; Bitzan et al. 1991, 1993). It is now possible to detect these antibody classes in saliva (Chart et al. 2003). For tables relating to commercial availability of reagents for the serological detection of *E. coli* O157 and other VTEC-associated O groups, IMS kits, media for phenotypic markers, and EIA/ELISA tests for toxin detection, the reader is referred to the excellent chapter by Scheutz et al. (2001).

EAggEC

Enteroaggregative *E. coli* (EAggEC) are characterized by a unique, 'stacked brick-like' adherence pattern which can be demonstrated in HEp-2 cells (Nataro et al. 1987; Scotland et al. 1989b) and a 6 h test is recommended. Some strains show the ability of bacterial clump formation at the surface of liquid cultures (Albert et al. 1993). These and other properties are associated with the presence of large plasmids from which a DNA probe (Baudry et al. 1990) and PCR primer pairs have been derived (Schmidt et al. 1995b). Such strains have been designated 'typical' EAggEC. HEp-2-positive isolates that are either probe-positive or probe-negative (designated 'atypical' EAggEC) have been found. Therefore the HEp-2 assay should remain the gold standard for the detection of EAggEC (Nataro and Kaper 1998; Okeke and Nataro 2001).

DAEC

Strains of the group are identified by testing for attachment to HEp-2 cells in a diffuse pattern using a 6 h assay (Scotland et al. 1989a). Two DNA probes for diffuse adhesion that do not cross hybridize have been developed from two different *E. coli* strains. The first probe derived from genes encoding the F1845 fimbrial adhesin (Bilge et al. 1989) has high specificity but low sensitivity indicating the possibility of several adhesins for the diffuse phenotype. With the availability of the sequence of the F1845 pilin subunit (Genbank Accession Number M27725) increased specificity in the detection of F1845 could enhance accuracy in detection of DAEC. The second probe for DAEC detects an outer membrane autotransporter protein AIDA-I (Benz and Schmidt 1992), but these appear to be rare among DAEC. This suggests that different gene sequences may determine the same phenotype of diffuse adhesion.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Before the era of antibiotics, there was only a low incidence of resistance to antimicrobials among the species of Enterobacteriaceae. Intrinsic resistance, i.e. resistance of most strains of a given group against a particular antibiotic, is rare in the genus *Escherichia* and essentially includes ampicillin and carbenicillin resistance in *E. hermannii* (Farmer 1995). The susceptibility of

individual isolates now varies greatly and, therefore, antibiograms are often used as epidemiological markers that can easily be determined. This nonintrinsic resistance is usually plasmid mediated.

E. coli strains with transferable resistance to one or multiple drugs can be isolated readily from the feces of the general population and from animals (Holmes and Gross 1990). Between 1971 and 1982, *E. coli* isolated from urinary tract infections with resistance to ampicillin, sulphonamides, tetracyclines, and trimethoprim increased considerably in general practice and in hospitals. This indicated that susceptibility testing to antimicrobials is essential for diagnostic laboratories. Resistance to ciprofloxacin was observed in 6 percent and resistance to four or more drugs in 28 percent of extraintestinal *E. coli* isolates including isolates from blood and cerebrospinal fluid in England and Wales in two studies (Threlfall et al. 1997, 1999).

In a recently published study, approximately 20 percent of O157 VTEC isolates were found to be resistant to antimicrobial agents with resistance to streptomycin, sulphonamides, and tetracycline most common and resistance to fluoroquinolones was not observed (Willshaw et al. 2001). Amongst isolates of EAggEC obtained during the infectious intestinal disease study in England from 1993 to 1996, 54 percent of strains isolated from diarrheal cases were resistant to antimicrobials and 19 percent were multiresistant (resistant to four or more antimicrobials (Wilson et al. 2001).

TYPING METHODS

There are numerous methods available for subtyping *Escherichia* spp. Each method has advantages and disadvantages when applied to a specific situation. 'Conventional' typing methods such as biotyping, phage and colicin typing, resistotyping, and serotyping have been replaced by or used in combination with molecular techniques that are applicable to a wide variety of organisms with only minimal adaptation in the procedures. These newer methods are often more discriminatory, but are also technically more demanding, often more expensive, and require specialized equipment.

Conventional typing methods

Biotyping, the most widely used typing method, includes a wide range of biochemical identification tests and characterizes an organism with regard to the presence or absence of these phenotypic markers. In some situations, biotyping has proved adequate for strain discrimination in *E. coli* (Crichton and Old 1985).

Typing based on differences in susceptibilities to antimicrobials (resistance or R typing) is sometimes useful in distinguishing between strains. However, resistance is most often plasmid mediated and, therefore, tends to be an unstable character as resistance plasmids are easily lost or newly acquired. Because antibiograms are usually done on a routine basis for clinically significant isolates, they may provide the first indication for a common origin of two or more strains. Nevertheless, under selective ecological pressure by antimicrobials, resistance patterns may change rapidly.

Bacteriophages have been used since 1945 to subdivide *E. coli*. For *E. coli* serogroups O111, O55, and O26, a phage typing system was established in the early 1950s and was subsequently shown to provide a great deal of information about the distribution of such strains throughout the world. For further information on the phage typing of *E. coli* see the review by Milch (1978). The problem with phage typing lies in the fact that suitable phage suspensions are not readily available and are usually restricted to reference laboratories. For example, Gershman et al. (1984) used a reduced set of 32 phages and found 178 different phage types among 866 *E. coli* isolates from cases of bovine mastitis; only 37 strains were not typable. A few years later, a phage typing scheme was introduced for isolates of *E. coli* O157:H7 that are of paramount importance in diarrheal disease and the hemolytic–uremic syndrome (see below) (Ahmed et al. 1987). From the UK it was reported that the predominant phage types (PT) of *E. coli* O157 have remained unchanged between 1982 and 1991, although the proportion of strains belonging to types 2 and 49 has increased (Frost et al. 1993). Since then there has been a change in the predominant phage types within the United Kingdom with the emergence of PT 21/28 as the dominant PT (CDR June 2003). The number of human isolates of O157 VTEC in England and Wales increased each year from 1 in 1982 to a high of 1087 in 1997 (Willshaw et al. 2001) and since 1999 have steadily fallen to just under 600 in 2002 (CDR June 2003). An upturn was observed in 2003 with the numbers rising to 675 (CDR 2004). The subdivision of O groups, or of phage types within an O group, by colicin typing has proved to be practicable (Holmes and Gross 1990). Typing antisera are not readily available with the possible exception of those used for identifying O serogroups of *E. coli* that are associated with certain virulence properties. Full serotyping (the identification of both the somatic O-antigen and the flagella H-antigen) is usually restricted to reference centers, but may be extremely helpful in identifying new pathogenic strains. This was demonstrated with the emergence of O157:H7 strains associated with outbreaks originating from undercooked hamburgers and other sources.

Molecular typing methods

Recent advances in molecular methods are having a direct effect upon strain typing and can be seen to complement and enhance conventional culture-based methods for the detection, identification, typing, and epidemiological analysis of the wide variety of *E. coli*

strains associated with causing disease. Genomic molecular methods can offer more discrimination by 'fingerprinting' than phenotypic methods. Such methods include detection of specific genes by oligonucleotide probes and PCR and the use of PCR for amplification of chromosomal sequences. Examples of these techniques include restriction fragment length polymorphism (RFLP) typing, restriction endonuclease analysis of chromosomal DNA by pulsed-field gel electrophoresis, fluorescence-based amplified fragment length polymorphism (FAFLP), and multilocus sequence typing (MLST).

Multilocus enzyme electrophoresis (MLEE), which allows quantitative analysis of strain relationships, has revealed that the *E. coli* O157:H7 clone is most closely related to a clone of O55:H7 strains that has been associated with worldwide outbreaks of infantile diarrhea (Whittam et al. 1993). Based on these results, the authors hypothesized that the 'new' pathogen emerged when an O55:H7-like progenitor which was already adherent to intestinal cells acquired secondary virulence factors (toxins, additional adhesins) via horizontal gene transfer and recombination. Enzyme electrophoresis has also been used to analyze clonal relationships among bloodstream isolates of *E. coli* (Maslow et al. 1995). One cluster contained the majority of isolates and was indistinguishable from the largest cluster of each of two other collections of *E. coli* that caused pyelonephritis and neonatal meningitis, respectively, and thus defined a group with increased virulence. MLEE offers only moderate discrimination as many unrelated isolates appear to share common traits.

Plasmid profiles (resistance plasmids and small cryptic plasmids) have been widely used to investigate the epidemiology of diseases associated with escherichia and have proved to be more discriminatory than conventional typing methods on several occasions. For isolates with similar plasmid profiles, further discrimination can be achieved by digestion with restriction endonucleases. Results are obtained within 1 or 2 days, instead of several days for the more sophisticated typing methods. However, plasmid profiles are usually less sensitive when investigating disease outbreaks than methods that include digestion of high molecular weight chromosomal DNA with restriction enzymes followed by analysis of the resulting fragments by pulsed-field gel electrophoresis (PFGE) or by a combination of agarose gel electrophoresis, Southern blotting, and hybridization with different kinds of probes (e.g. ribotyping). When analyzing 61 isolates of *E. coli* O157:H7 isolated from patients, food samples, and calf fecal samples, 21 distinct genomic profiles were generated by PFGE after digestion with endonuclease *Xba*I, whereas only five different plasmid profiles were generated (Meng et al. 1995).

Ribotyping has also been used extensively and with excellent results for subtyping *E. coli*. The sensitivity is dependent on the selection of restriction endonucleases used. Another approach uses randomly amplified polymorphic DNA (RAPD) for strain separation. This method is not necessarily dependent on purified genomic DNA and thus is both simple and rapid. Poor reproducibility is a significant disadvantage especially when comparing results obtained in different laboratories, because pattern variations may depend on the brand of *Taq* polymerase or the type of thermocycler used (Meunier and Grimont 1993). RAPD seems to be more discriminatory than other molecular methods in some situations, but may be less suitable in others. In common with all typing methods, combinations are likely to give the best separation of unrelated isolates. For example, 60 *E. coli* strains previously delineated in 36 ribotypes by *Eco*RI and *Hin*dIII digests exhibited 28 different RAPD fingerprints (Cave et al. 1994). However, some strains of identical ribotype had clearly different RAPD patterns.

FAFLP is an assay based on the fluorescent analysis of an amplified subset of restriction fragments using polyacrylamide gel electrophoresis to provide accurate band sizes necessary for accurate homology assessment. Zhao et al. (2000) showed that FAFLP analysis of *E. coli* O157 strains provided greater genetic resolution than PFGE. Guan et al. (2002) examined isolates of *E. coli* from livestock, wild animals, and man and found AFLP analysis of the isolates to be more discriminatory than the combination of antibiotic resistance and analysis of the sequences of the 16S rRNA genes of the *E. coli* strains.

Maiden et al. (1998) proposed multilocus sequence typing (MLST), which is based on MLEE, but assigns alleles at each locus directly by nucleotide sequencing, rather than indirectly from the electrophoretic mobilities of the gene product on a starch gel. An advantage of MLST is that there is no need to culture the strain as fragments for sequencing can be amplified directly from the clinical sample. Sequence data are easy to standardize making the creation of a 'typing' database relatively straightforward and readily available. The use of MLST for typing isolates of *E. coli* O157 was reported by Noller et al. (2003), but it is unsuitable as the strains had a striking lack of genetic diversity in all the housekeeping genes examined. In contrast, for studying *E. coli* O78 isolates, Adiri et al. (2003) found MLST showed a positive correlation between virulence and clonal origin.

ARRAY TECHNOLOGY

DNA microarrays are a means by which the detection capabilities of PCR can be enhanced. They permit rapid detection of sequence variation within a defined locus and also detection of multiple products from multiplex PCR. Microarrays can also be used to fingerprint bacterial isolates. For an overview of microarrays in

microbiology, see Ye et al. (2001). Dobrindt et al. (2003) compared pathogenic and commensal *E. coli* using DNA arrays and concluded that DNA array technology allowed them to assess the genetic diversity and genome content amongst the different types of *E. coli* studied.

ROLE IN NORMAL FLORA OF HUMANS

The only site of the human body where *E. coli* is regularly found as a colonizer is the intestinal tract where it represents the most prevalent (cultivable) facultatively anaerobic bacterial species (Williams Smith 1965). However, about 99 percent of the normal bacterial intestinal flora are strict anaerobes. Because of the extraordinary complexity of the intestinal microflora, it is very difficult to identify specific functions for individual species.

E. coli and other Enterobacteriaceae are able to synthesize a wide range of vitamins in vitro. It has been shown by using gnotobiotic rats, that these animals can rely completely on their intestinal microflora for their requirement of vitamin K, folic acid, the vitamin B complex, biotin, and vitamin E (Drasar and Barrow 1985). There is no doubt that intestinal bacteria contribute to the vitamin requirements of many animals, but no evidence has yet been presented that this is also true for man. In contrast, the fact that gastrointestinal disturbances are relatively rare in patients who receive drugs that have no or minimal activity against anaerobes, but good activity against facultative anaerobes (e.g. quinolones), but are quite common in patients treated with antimicrobials (e.g. ampicillin) that eliminate anaerobic, as well as facultatively anaerobic bacteria (Wollschlager et al. 1987), suggests that facultative anaerobes are not a very important component for maintaining the gastrointestinal equilibrium. On the other hand, colicinogenic *E. coli* have been suggested as one of the significant factors of gastrointestinal tract protection in the course of shigellosis (Bures et al. 1979). The period of *Shigella* excretion was significantly reduced if an appropriate colicinogenic *E. coli* strain was present in the intestinal flora of patients.

PATHOGENICITY AND VIRULENCE FACTORS

E. coli strains can cause an impressive variety of different types of disease, including septicemia, pneumonia, meningitis, bladder and kidney infections, hemolytic–uremic syndrome (HUS), diarrhea, and dysentery. However, different strains that have acquired distinct sets of virulence genes cause different clinical syndromes. Despite this extremely broad spectrum of pathogenicity, the majority of *E. coli* strains have to be considered avirulent in the intestine.

Enterovirulent *E. coli*

E. coli strains capable of causing any type of enteric disease have often been referred to as 'enteropathogenic *E. coli* or EPEC' and for many years EPEC were only looked for in children <3 years of age. This term is now used very specifically for one of the six different classes of enterovirulent (or diarrheagenic) *E. coli* that have been associated with gastrointestinal disease by case-control studies (Kaper 1994; Nataro and Kaper 1998).

For many years, some of these strains were identified by serotyping based on the association of serogroup and virulence type (see Table 3). However, the reliability of serotyping is limited because not all isolates of serogroups associated with EPEC are enteropathogenic and pathogenicity is not restricted to particular serogroups or serovars (Valentini et al. 1992; Law 1994). With the molecular characterization of the various virulence genes involved in the pathogenesis of enterovirulent *E. coli* (Table 6), diagnostic tests evolved that detected these genes by hybridization, PCR or a combination of both. These tests provided a much more coherent identification of these groups of diarrheal pathogens.

Adherence is one of the important virulence mechanisms of enterovirulent *E. coli*. Three main patterns of adherence have been described (Nataro et al. 1987): localized, diffuse, and aggregative (Figure 8). Classification and identification of adherence patterns is confusing for several reasons (Law 1994): different adhesion assay methods may give different adhesion patterns and some authors do not separate EPEC and diffusely adhering *E. coli* (DAEC), but rather consider them as class I EPEC (strains with localized adherence belonging to serogroups that are most commonly associated with outbreaks of infantile diarrhea) and class II EPEC (diffusely adherent strains belonging to serogroups less often incriminated in outbreaks of diarrhea), respectively. Very little is known as yet about some of the mechanisms involved in adhesion of DAEC and EAggEC and, therefore, strains cannot yet be easily identified using molecular markers instead of cell culture assays.

Enteropathogenic *E. coli*

EPIDEMIOLOGY AND DISEASE

During the 1940s, outbreaks of infantile enteritis occurred with increased frequency in hospitals and nurseries and they were more common in the winter months. These outbreaks were often severe, with high attack and fatality rates. Bray (1945) investigated such an outbreak in a London hospital and showed that the epidemic strain of *E. coli* belonged to a serogroup that was subsequently recognized as *E. coli* O111. Similar studies in Aberdeen in 1947 and 1948 implicated two

Table 6 *Some known virulence factors of enterovirulent* E. coli

Virulence factor/gene	Location	Putative role of gene product in disease
Enteropathogenic *E. coli* (EPEC)		
bfpA	p	Bundle-forming pilus (BFP) mediates nonintimate localized adherence
dsbA	c	Disulfide isomerase which catalyzes disulfide bond formation in the periplasmic space; essential for active BFP
eae	c	Intimin; mediates intimate adherence to epithelial cells
		Induction of tyrosine phosphorylation and release of inositol phosphate from cells
per	p	'Plasmid encoded regulator': transcriptional activator of *eaeA*
sepA/sepB	c	Secretion of (virulence) proteins
Verotoxigenic *E. coli* (VTEC)		
eae	c	Mediates intimate adherence to epithelial cells; high similarity to *eae* of EPEC
vtx gene family	ph	A1B5 toxin (similar to Shiga toxin). A subunit specifically removes one base from eukaryotic 28S rRNA and results in inhibition of protein synthesis. B subunit is responsible for binding to receptor on host cell
EHEC-hlyA	p	Enterohemolysin, ±60 percent homology to α-hemolysin genes (*hlyA*, *hlyC*) of *E. coli*
EHEC-hlyC		
Enterotoxigenic *E. coli* (ETEC)		
LT	p	Heat-labile A1B5 toxin (similar to cholera toxin). A subunit induces an increase in intracellular cAMP via ADP-ribosylation of a G protein. B subunit mediates binding to the ganglioside GM1 receptor
ST	p	Small, heat-stable and non-immunogenic peptide toxin that induces an increase in intracellular cGMP via activation of guanylate cyclase
CFAs, CSs	(p)	Various types of colonization factor antigens or coli surface associated antigens that mediate adherence to the small bowel epithelium

c, chromosome; p, plasmid; (p), usually but not always located on plasmids; ph, lysogenic phage. Some *vtx* genes are chromosomally encoded.

serogroups of *E. coli*, O55 and O111, as the causative organisms of epidemics (Giles and Sangster 1948; Smith 1949). In these studies there were two peaks in the seasonal incidence; this was highest in March and April, was mainly due to cases in hospital and was accompanied by high mortality. A smaller peak in July was predominantly due to cases in the community. It appeared that an outbreak in the community had occurred before or at the same time as the hospital outbreaks and that cross-infection in the hospital had followed the introduction of the infection by babies admitted while still excreting the causative organism.

During the 1950s, many epidemics due to enteropathogenic *E. coli* (EPEC) were reported in Europe and North America. In Britain, these outbreaks continued during the 1960s and early 1970s. In late 1967, there was an epidemic in several hospitals in Teeside in which the mortality rate was high (Annotation 1968). Two *E. coli* strains were responsible for these infections, serotypes O119:H6 and O128:H2. A similar outbreak occurred in hospitals in the Manchester area, but the causative organism in these cases was O114:H2 (Jacobs et al. 1970). In 1970 and 1971 outbreaks due to O142:H6 occurred in several hospitals in the Glasgow area and also in a Dublin hospital (Love et al. 1972; Hone et al. 1973; Kennedy et al. 1973). In Dublin the infection appeared to be spread by crossinfection after the admission to hospital of infected infants from the community. Retrospective studies of *E. coli* O142 strains isolated from outbreaks in Scotland, England, Northern Ireland, Eire, Indonesia, Canada, and the USA suggested that a single enteropathogenic clone may have spread to all those countries (Gross et al. 1985). Since the early 1970s, few outbreaks of infantile enteritis caused by EPEC have been reported in Britain or the USA. Sporadic cases continue to occur with a peak in the summer months.

In contrast to the situation in Britain and the USA, EPEC strains are among the most frequent causes of diarrhea in infants in developing countries (Levine and Edelman, 1984; Cravioto et al. 1988; Gomes et al. 1989; Kain et al. 1991; Trabulsi et al. 2002). EPEC infections show a marked seasonality and are associated with warm

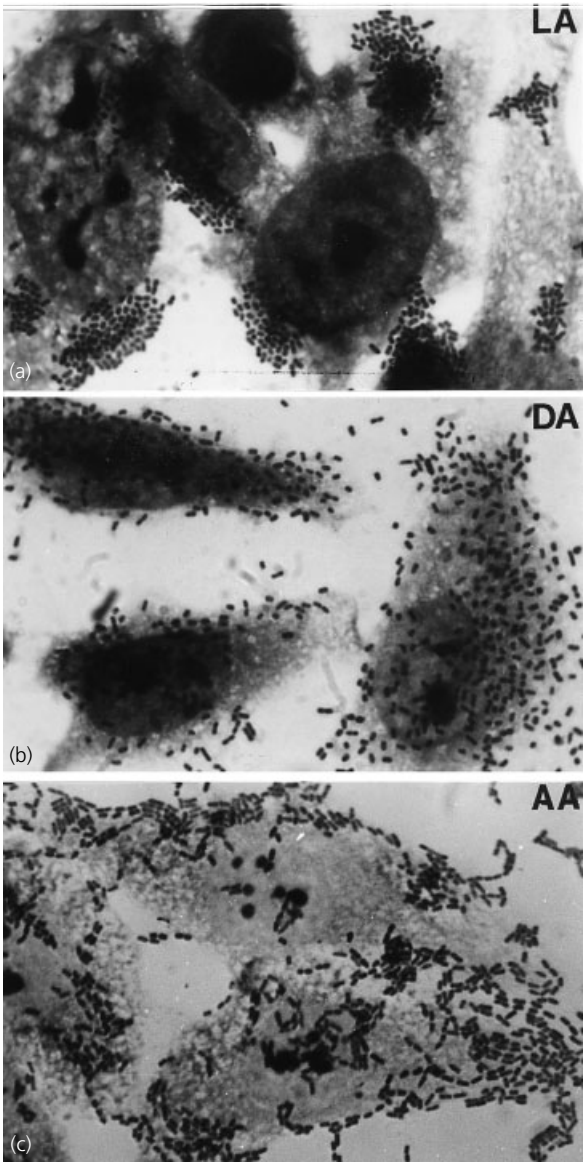

Figure 8 *Light microscopy of Giemsa-stained HeLa cells showing three types of adherence in enterovirulent* E. coli*: localized (LA, top), diffuse (DA, middle) and aggregative (AA, bottom) (photographs courtesy of Dr S. Knutton)*

season peaks. Outbreaks in institutions are often reported, but sporadic cases and outbreaks occur very frequently in the general community. Several studies have shown that the peak incidence of enteritis was always in the few months after the beginning of the weaning period. Because weaning is often late in developing countries, the age distribution differs from that observed in Europe and North America. Contamination of weaning foods appears to contribute to the transmission of EPEC in developing countries. Most EPEC infections occur in the first 3 years of life, but the importance of EPEC as a cause of adult enteritis is difficult to evaluate because few laboratories test for EPEC in patients over 3 years. Outbreaks in the adult population have been reported in the USA and Britain (Schroeder et al. 1968; Vernon 1969).

Although the pathogenicity of EPEC strains involved in outbreaks was confirmed by volunteer studies (Ferguson and June 1952; June et al. 1953), for a long time there was no suitable animal model or reliable in vitro test available to demonstrate the pathogenicity of putative EPEC isolates. Therefore, serogrouping remained the only means of identifying such isolates.

Virulence factors

EPEC pathogenesis is not yet understood in all details and several steps seem to be multifactorial. In 1992, Donnenberg and Kaper proposed a three-stage model and in 1998 Knutton et al. proposed a four-stage model.

In the first stage, nonintimate adherence between the bacterium and the epithelial cell is mediated by the bundle-forming pilus (BFP) and intimin along with EspA filaments (short, surface-associated filaments) (Girón et al. 1991a). Formation of active BFP is dependent on two genes, *bfpA* and *dsbA*, located on the 55–77 MDa EPEC adherence factor (EAF) plasmid and on the chromosome, respectively. The adherence pattern is called 'localized' because organisms attach to one or two small areas of the cell surface in microcolonies and it is clearly different from the diffuse or aggregative adherence patterns seen in other classes of enterovirulent *E. coli* (Scaletsky et al. 1984; Nataro et al. 1987).

In the second stage, the initial attachment of EPEC using BFP and EspA filaments results in the injection of the translocated intimin receptor (Tir) via a type III secretion system directly into the host cell, resulting in tyrosine protein kinase activation and formation of the intimin receptor (tyrosine phosphorylated Tir) and to actin rearrangements. During the third stage, EspA filaments and other surface structures are eliminated from the area of intimate attachment, intimin binds to phosphorylated Tir and polymerized actin and other cytoskeletal elements accumulate beneath the intimately attached bacteria.

Finally, in the fourth stage, further actin polymerization and accumulation of cytoskeletal elements at the site of bacterial attachment results in the production of the mature A/E lesion with the characteristic pedestal structure. Host cell processes are disrupted by the translocated effector molecules allowing a loss of tight-junction integrity and mitochondrial function, resulting in electrolyte loss and cell death.

Enteroinvasive *E. coli*

EPIDEMIOLOGY AND DISEASE

Enteroinvasive *E. coli* (EIEC) are very similar to *Shigella* spp. in every way except for their lower acid

resistance which results in a higher infectious inoculum (DuPont et al. 1971) and their inability to produce Shiga toxin (produced only by *Sh. dysenteriae* 1) which may be related to the fact that so far no complications, such as HUS, have been observed in EIEC infections.

Virulence

EIEC strains are invasive pathogens and the first step in this process is the attachment to the host cell. This induces rearrangements in the cytoskeleton of the cells and eventually leads to phagocytosis of the bacteria. The invasion plasmid antigen (Ipa) D is believed to be involved in adhesion. Other proteins, IpaB and IpaC, which are also encoded by genes located on the virulence plasmid, can be found at the bacterial surface and in extracellular fluid. Their excretion requires the presence of membrane expression of invasion (*mxi*) genes. IpaB and IpaC seem to be essential for phagocytosis and for rupture of the phagocytic vesicle which precede rapid multiplication in the cytoplasm. Intercellular spread, which again involves continuous reorganization of the host cell actin filaments, is mediated by two proteins, IcsA (also called VirG) and IcsB, and ultimately leads to cell death. Although relatively little work has been done to characterize EIEC virulence, it is assumed that they are virtually identical to those of *Shigella* spp. For a fuller account on the mechanism of the invasion process see *Shigella*.

Enterotoxigenic *E. coli*

EPIDEMIOLOGY AND DISEASE

Enterotoxigenic *E. coli* (ETEC), first recognized in the late 1960s as a cause of cholera-like diarrhea in India, resemble *Vibrio cholerae* in that they induce profuse, watery diarrhea by elaboration of toxins that act on the mucosal cells. They adhere to the small intestinal mucosa, but do not invade. There are no apparent histological changes and little inflammation.

ETEC are a common cause of dehydrating diarrhea in children in developing countries, particularly when they are weaned. The disease is usually less severe than cholera (Eisenstein 1990), but can be fatal, especially in infants, and results in some kind of immunity which is directed against the colonization factor antigens. ETEC may also affect adults from industrialized countries (where these pathogens are not endemic) who visit developing nations. They are considered to be the leading cause of travelers' diarrhea, accounting for up to 75 percent of these cases (Guerrant 1990) and also in soldiers deployed to developing countries.

VIRULENCE FACTORS

There are two classes of virulence factors in ETEC, both of which are essential for their pathogenicity: enterotoxins (LT, STa, STb) (see Table 7) and various adhesive factors termed colonization factor antigens (CFA) or coli surface (CS) associated antigens.

Enterotoxins can be classified according to their heat stability. Whereas heat-labile toxin (LT) is inactivated by incubation at 100°C for 30 min, heat-stable toxin (ST) retains toxin activity after such treatment (Smith and Halls 1967). LT is very similar to cholera toxin in structure (about 75 percent identity at the amino acid level). The mechanism of action of the two toxins seems to be identical: binding to the GM1 ganglioside receptor located at the apical surface of mucosal cells mediated by the B subunits, entry of subunit A into the cell followed by ADP-ribosylation of a G protein (second messenger) and activation of adenylate cyclase. The concomitant increase in intracellular cAMP concentration leads to the massive secretory diarrhea seen with ETEC and *V. cholerae*. There is a second type of heat-labile toxin (LT II) which has the same basic structure and mechanism of action as LT I (Holmes et al. 1985). However, the two toxins do not cross react immunologically. In addition, LT II has a different receptor (Hewlett 1990), has been found primarily in strains of animal origin, and has not been associated with disease either in animals or humans. STs are a family of small (17–19 amino acids) heat-stable and nonimmunogenic toxins that are either soluble (STa or ST I) or insoluble (STb or ST II) in methanol (Burgess et al. 1978; Lee et al. 1983). The Sta toxins produced by human and animal strains are remarkably homogeneous, yet two types have been described that are referred to as STh (produced by human strains only) and STp (produced by human and animal strains), respectively. They cause an increase in cGMP levels (in contrast to cAMP by LT) in the host cells which leads to fluid loss similar to that induced by LT (Guerrant et al. 1980). STb acts by a different mechanism from that of STa and produces diarrhea in piglets. There is no evidence that STb-positive ETEC strains are diarrheagenic in humans. The frequency with which LT and ST are found in ETEC isolated from persons with travelers' diarrhea varies between studies with usually less than 50 percent of the strains producing both toxins (Guerrant 1990; Wolf et al. 1993). This is important when selecting laboratory tests for the detection of ETEC because at least 25 percent of the cases would be missed if only tests for LT (such as the immunodiffusion test, Figure 9) were used.

In order to cause disease, ETEC must not only produce an enterotoxin, but must also be able to colonize the small bowel of animals and humans. There are several types of pili (fimbriae) that mediate adhesive properties: type 1 pili are usually found in *E. coli* strains from the resident microflora and do not seem to contribute to the pathogenic potential of ETEC strains (Salyers and Whitt 1994). Pili that are important for ETEC include K88, 987P, F41, F107, and 2134P

Table 7 *Characteristics of toxins produced by enterovirulent* E. coli

	Subunit structure	Eukaryotic cell surface receptor	Location of genes	Function	Associated disease
Shiga toxin	A1B5	Gb3[a]	Chromosome	B subunit: binds to host cell receptor. A subunit: specific removal of one base in the 28S rRNA of eukaryotic cells; inactivates the ribosome	Human HUS, hemorrhagic colitis, diarrhea
VT1	A1B5	Gb3	Temperate bacteriophage		
VT2	A1B5	Gb3			
VT2c	A1B5	Gb3			
VT2d	A1B5	Gb3			
VT2e	A1B5	Gb4[b]	Temperate bacteriophage Chromosome		Pig edema disease
VT2f	A1B5		Temperate bacteriophage		Found only in pigeon feces
LT-I	A1B5	GD1	Plasmid	B subunit: binds to host cell receptor A subunit: ADP-ribosylation of host cell proteins	Diarrhea
LT-Ip (pig)					
LT-Ih (human)					
LT-II	A1B5	GM1	Chromosome		Diarrhea
LT-IIa					
LT-IIb					
ST I (STa)	Single molecule	Glycoprotein receptors	Plasmid	Activation of guanyl cyclase: elevation of cGMP levels	
ST Ip					Diarrhea in animals and man
ST Ih					Diarrhea in man
ST II (STb)	Single molecule		Plasmid	Prostaglandin E_2 activation	Diarrhea in pigs

a) Globotriosylceramide
b) Globotetraosylceramide

(in strains infecting pigs), K99 (strains infecting pigs, lambs, and calves) and at least 21 colonization factor antigens and coli surface antigens specific for humans have been defined (Elsinghorst 2002). CFA/I and CFA/II are the most commonly found adhesins in human ETEC (but many strains do not produce either of these antigens, and there are additional ETEC adhesins). Another distinct pilus has been detected and termed 'longus' because it can extend for over 20 μm from the cell surface (Girón et al. 1994); the structural gene (*lngA*) is encoded on a large plasmid. In contrast to other ETEC pili, *lngA* is widely distributed among ETEC strains independent of their geographical origin, serovar, toxin production, or other antigens expressed. Its suitability as a vaccine component remains to be shown.

Other virulence factors include Tia, an outer membrane protein that directs adherence and invasion by ETEC strain H10407, LeoA part of an outer membrane secretion apparatus (Fleckenstein et al. 1996, 2000) and TibA, an outer membrane glycoprotein associated with adherence and invasion by ETEC strain H10407 (Elsinghorst and Weitz 1994).

Vero cytotoxin-producing *E. coli*

EPIDEMIOLOGY AND DISEASE

Vero cytotoxin-producing *E. coli* (VTEC) were described first by Konowalchuk in 1977 and *E. coli* of several different O serogroups were reported to produce a cytotoxin active on Vero cells (African green monkey kidney cells). In 1982, one particular serotype of *E. coli* (O157:H7) was identified as the causative agent involved in two outbreaks of a distinctive bloody diarrheal syndrome (Riley et al. 1983). Since then, these organisms have received much attention, not only in the USA, but also in many other developed countries as a cause of epidemic or sporadic bloody and non-bloody diarrhea, HUS, and thrombotic thrombocytopenic purpura (TTP). These sometimes fatal complications roughly occur at a rate of 5–10 percent of all cases (Griffin and Tauxe 1991; Wachsmuth 1994; Griffin 1998; Smith et al. 1998).

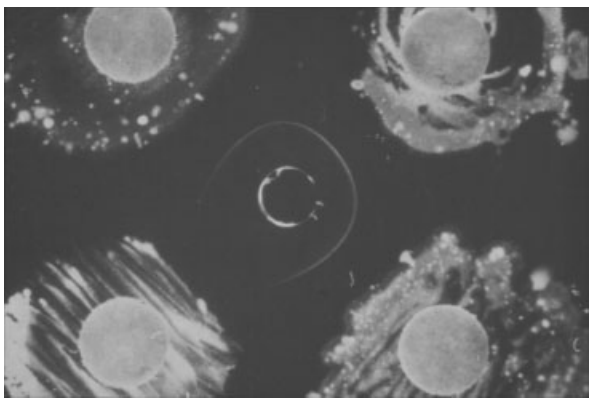

Figure 9 *Immunodiffusion (BIKEN) test for the demonstration of heat-labile toxin of* E. coli

Soon after the discovery of the special significance of *E. coli* O157:H7 it was recognized that some strains of other *E. coli* serovars may exhibit a similar virulence potential. Because one of their major virulence factors (see section on Virulence factors, below) is the production of toxins that are closely related to Shiga toxin and that have a cytotoxic effect on Vero cells, these organisms have also been called Shiga toxin producing *E. coli* (STEC) (O'Brien et al. 1982). The term enterohemorrhagic *Escherichia coli* (EHEC) was introduced for those strains producing a bloody diarrhea and therefore EHEC represent a subset of VTEC or STEC (Griffin and Tauxe 1991). Analogous to other virulence types of diarrheagenic *E. coli*, there seems to be a limited number of serogroups that are of major importance (see Table 5). However, at least 200 different VTEC serotypes have so far been isolated from humans, animals, and foodstuffs (World Health Organization 1994, 1998). In addition to O157 and O26, serogroup O111 strains may be of special epidemiological importance: the first outbreak of non-O157 associated HUS in Italy was due to serotype O111:H$^-$ (Caprioli et al. 1994) and the same or a very closely related clone (as determined by PFGE) has been reported in Germany (Karch 1994). Furthermore, a large community outbreak of HUS associated with *E. coli* O111:H$^-$ was reported from Australia (Centers for Disease Control 1995). At least in Europe, the majority of sporadic cases of VTEC-associated diarrhea are caused by *E. coli* serovars other than O157:H7 (Lüscher et al. 1992; Huppertz et al. 1996; Tozzi et al. 2003). The UK appears to differ from mainland Europe in that infections associated with non-O157 VTEC are relatively rare, but very few laboratories screen for these organisms.

An unusual outbreak of severe disease (including cases of HUS) caused by Verocytotoxin-producing *Citrobacter freundii* occurred in Germany (Tschäpe et al. 1995). The overall epidemiological significance of this finding is not clear, but it adds weight to the earlier finding (Schmidt et al. 1993b) that the genes coding for Vero cytotoxins, which are encoded by temperate phages, can be found in other species of the Enterobacteriaceae (*Sh. sonnei*) and might be horizontally transferred from one species to another.

The main reservoirs for VTEC strains are the gastrointestinal tract of cattle, other farm animals, and humans (Griffin and Tauxe, 1991; Hancock et al. 2001; Wasteson 2001). Consequently, the majority of outbreaks have been the result of transmission via foods of bovine origin, such as beef and raw milk, but apple cider, potatoes, and other vehicles for transmission have also been identified. In addition, direct person-to-person transmission is important, especially in families and child care

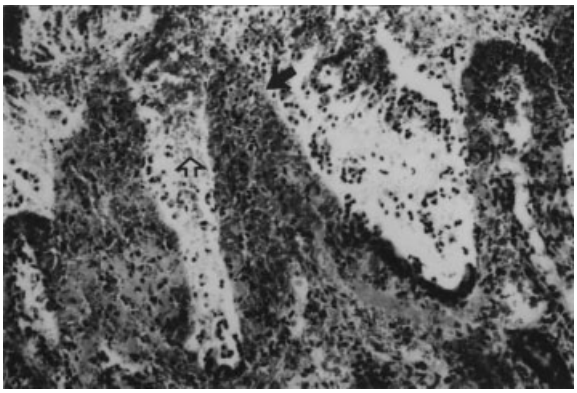

Figure 10 *Enteritis due to VTEC O139 in a piglet. Characteristic necrosis of villi (arrow) and presence of blood (open arrow) (reproduced with permission, from Dedié et al. 1993: 73)*

facilities (Belongia and Osterholm 1993; Reida et al. 1994), and is facilitated by the low infectious dose of these organisms (see Table 5). Long-term shedding of *E. coli* O157 despite clinical cure has been found in 13 percent of patients, particularly in children (Karch et al. 1995). In recent years the importance of direct or indirect contact with animals and their feces, particularly cattle, has been increasingly recognized as an important transmission route.

VIRULENCE FACTORS

VTEC are characterized by the production of cytotoxins that are similar to Shiga toxin. It is now generally agreed that the terms VTEC and STEC may be used and that they are interchangeable. There are two major types of Vero cytotoxin, VT1 and VT2, and both can occur in a single strain. Variants of VT2 exist and are associated with human disease (VT2c, VT2d), with pig edema disease (VT2e), and with feral pigeons (VT2f), respectively. VTs are A1/B5-type toxins and are usually determined by lysogenic phage (Scotland et al. 1983; Williams Smith et al. 1983). Subunit A is the active toxic component which specifically removes the adenine base at position 4324 of eukaryotic 28S rRNA, resulting in ribosome inactivation (Endo et al. 1988), whereas subunit B mediates binding to the eukaryotic host cell receptors Gb3 and Gb4, respectively.

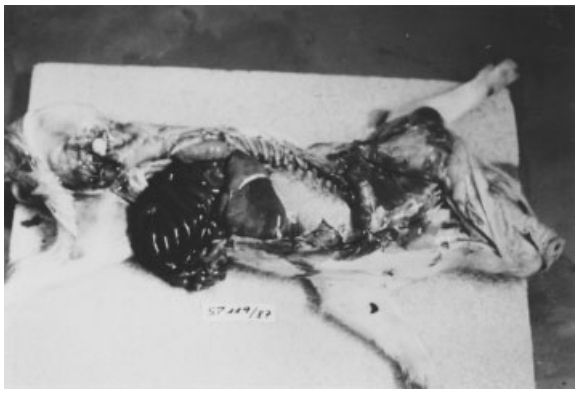

Figure 11 *Lethal hemorrhagic enterocolitis due to VTEC infection in a piglet (reproduced with permission, from Dedié et al. 1993: 72)*

In newborn piglets, VTEC produce attaching and effacing lesions (Figures 10 and 11) that are very similar, if not identical, to those produced by EPEC isolates (Tzipori et al. 1989). One of the genes, *eae*, involved in mediating this intimate adherence to epithelial cells, shares 86 percent homology at the nucleotide level and 83 percent homology at the amino acid level with the *eae* gene of EPEC strains (Yu and Kaper 1992). This extremely high homology is not evenly distributed over the entire gene: with 94 percent homology in the N-terminal (comprising 75 percent of the gene) but only 49 percent in the C-terminal: (comprising 25 percent of the gene) which represents the putative receptor-binding site. The variability in the C-terminal region may reflect the fact that EPEC are primarily found in the small intestine, whereas the major site for VTEC is the distal ileum and the colon (Kaper 1994). As with EPEC, it is the close association between Tir and intimin that facilitates in part the formation of the A/E lesion. At least 10 subtypes of intimin (starting with α) have been reported in EPEC and VTEC and the subtype associated with VTEC O157 is γ (Jenkins et al. 2003).

A plasmid-encoded hemolysin of *E. coli* O157:H7 has been described (Schmidt et al. 1995a). The two open reading frames found on a 5.4 kb *Sal*I restriction fragment share approximately 60 percent homology with the *hlyC* and *hlyA* genes of the *E. coli* α-hemolysin operon. This EHEC hemolysin ('enterohemolysin') may be of clinical importance because it was found in all O157 strains tested (but only in 12 of 25 non-O157 VTEC) and was reactive with 19 of 20 sera of patients with HUS (but with only one of 20 control serum samples). However, the role of enterohemolysin in disease has not yet been demonstrated.

Enteroaggregative *E. coli*

EPIDEMIOLOGY AND DISEASE

Although initially isolated from adults with travelers' diarrhea (Mathewson et al. 1985), enteroaggregative *E. coli* (EAggEC) are now mainly considered a cause of bloody or protracted infantile diarrhea as shown by studies in various nonindustrialized countries and also a cause of sporadic cases of diarrhea in developed countries (Law 1994; Nataro and Kaper 1998; Okeke and Nataro 2001). Some EAggEC strains belong to some of the classical EPEC serogroups (e.g. O44, O111, O86, O126) (Scotland et al. 1991, 1996; Knutton et al. 1992; Wilson et al. 2001). This is another indication that

serotyping is of limited value for the identification of the various *E. coli* virulence types.

VIRULENCE FACTORS

EAggEC bind to small intestinal cells without any obvious histological changes and are not invasive, but knowledge of their virulence factors is very limited. Both toxin production and adhesion probably play an important role in pathogenesis. EAggEC produce a toxin (called enteroaggregative heat-stable toxin (EAST)) that induces fluid secretion and is antigenically related to the heat-stable toxin of ETEC (Savarino et al. 1991). However, the genes encoding EAST have also been detected in other diarrheagenic *E. coli* and nonpathogenic *E. coli*. EAggEC also produce a toxin similar to the α-hemolysin of *E. coli* associated with urinary tract infection (Baldwin et al. 1992). Other enterotoxins linked to virulence in EAggEC are Pet, a high molecular weight enterotoxin belonging to the autotransporter family of secreted proteins (Eslava et al. 1998) and the *Shigella* enterotoxin ShET1, found predominantly in *Shigella flexneri* 2a strains (Fasano et al. 1995). Four morphologically distinct types of fimbriae have been identified by Knutton et al. (1992), including so-called aggregative adherence fimbriae I (AAF/I; Nataro et al. 1992) that are very similar to the bundle forming pili of EPEC. AAF/I were present in 43 of 44 isolates (Knutton et al. 1992) and their presence correlated well with a positive hybridization signal using a DNA probe that had been derived from the 60 MDa plasmid of EAggEC which confers aggregative adherence when introduced into a nonadherent *E. coli* strain and which also encodes the EAST toxin (Baudry et al. 1990; Savarino et al. 1991; Nataro et al. 1992). Based on the sequence of a fragment of the EAggEC probe a PCR assay was developed that correlated well, but not perfectly, with aggregative adherence to HEp-2 cells (Schmidt et al. 1995b). Other aggregative adherence fimbriae, AAF/II and AAF/III, have now been reported (Czeczulin et al. 1997; Bernier et al. 2002).

Diffusely adherent *E. coli*

EPIDEMIOLOGY AND DISEASE

Diffusely adherent *E. coli* (DEAC) belonging to classical EPEC serogroups were named class II EPEC because they did not hybridize with the EAF probe (Nataro et al. 1985). Their significance as a diarrheagenic agent is controversial. In some reports the isolation rates of strains that show diffuse adherence in the HeLa cell assay and/or that hybridize with the F1845 DNA probe (see Virulence factors below) were similar in infants with diarrhea and in controls (Chatkaeomorakot et al. 1987; Gomes et al. 1989; Cravioto et al. 1991). Two other studies, however, reported a strong association between DAEC and diarrhea in Mayan and Bangladeshi children (Girón et al. 1991b; Baqui et al. 1992). DAEC are classified as one of the six classes of diarrheagenic *E. coli* and yet little is known about the virulence properties of this group. Studies have been hampered by the lack of a specific DA probe or PCR test for this group of *E. coli* (Nataro and Kaper 1998), However, Kyaw et al. (2003) have published their findings of the presence of a type III secretion system in DAEC isolated from cases of diarrhea in Brazilian children.

VIRULENCE FACTORS

DAEC are clearly different from EPEC in that they are always negative in the FAS test, which correlates with attaching and effacing lesions (Knutton et al. 1989) and for hybridization with the EAF probe (Gomes et al. 1989). Their mode of adherence is different from EPEC strains and two adhesins have been identified: a 100 kDa, plasmid-encoded protein from an O126:H27 isolate (Benz and Schmidt 1992) and a chromosomal fimbrial adhesin from a serogroup O75 isolate (Bilge et al. 1989). A DNA probe (F1845) derived from the chromosomal adhesin gene has been used in epidemiological studies, but the results of probe tests do not correlate 100 percent with HEp-2 assays (Levine et al. 1988; Girón et al. 1991b), suggesting that a number of different genetic determinants may encode diffuse adherence. In addition, Smith et al. (1994) showed that the F1845 probe cross-hybridized with 80 of 86 *E. coli* O44:H18 strains that exhibited aggregative adherence and that were positive with the EAggEC probe. A study from France found that children carrying F1845 DNA probe-positive DAEC had significantly longer hospital stays than those with probe-negative DAEC (Poitrineau et al. 1995).

Uropathogenic *E. coli*

E. coli is the most common organism found in community-acquired urinary tract infection (UTI), causing more than 80 percent of the cases. The bacteria follow an ascending route of infection in over 90 percent of all UTIs, with infection starting with colonization of the periurethral area (Bacheller and Bernstein 1997). These *E. coli* are primarily derived from the fecal flora of the host (Plos et al. 1995), but hematogenic infections do occur (Ward and Jones 1991). In addition to host factors (sex, age, sexual activity, use of spermicides, and also genetic factors like the presence of receptors for Pap fimbriated bacteria), which affects the susceptibility of patients to UTIs, there are various possible colonization and virulence factors, such as adhesins, toxins, LPS, iron acquisition, presence of capsules, or serum resistance, that determine the uropathogenicity of *E. coli* strains (Salyers and Whitt 1994).

Adhesion to epithelial cells of the urogenital tract is generally accepted as being essential for uropathogenic

bacteria because otherwise the bacteria would be washed out rapidly. This view has been questioned by Gordon and Riley (1992) who calculated that with a doubling time of about 50 min bacteria could maintain themselves without adhering in the bladder. However, in 100 percent of *E. coli* isolates from the urine of patients with pyelonephritis, but only in 17 percent of *E. coli* isolates from patients with asymptomatic bacteriuria, P or Pap (pili associated with pyelonephritis) were found (O'Hanley et al. 1985). This suggests that these pili are significant virulence factors. There are many different antigenic variants of Pap pili. They all recognize the same type of receptors which are glycolipids containing the disaccharide α-D-galactopyranosyl-(1,4)-β-D-galactopyranose (gal-gal) (Leffler and Svanborg-Eden 1980). Gal-gal is sufficient for inhibiting hemagglutination (O'Hanley et al. 1983) and for blocking attachment (Svanborg-Eden et al. 1982) of Pap fimbriated bacteria to uroepithelial cells. Biosynthesis, assembly, and regulation are encoded by a single operon located on the *E. coli* chromosome (Hultgren and Normark 1991), resulting in a single polycistronic mRNA transcript and, consequently, coordinated expression of all genes essential for fimbrial production. In addition, uropathogenic *E. coli* (UPEC) can also express afimbrial adhesins like AFA I, AFA III, or Dr adhesins which are also encoded by gene clusters (Garcia et al. 1994). These adhesins recognize the Dr blood group antigen as receptor and mediate mannose-resistant hemagglutination (Swanson et al. 1991). When women with chronic UTI were inoculated with a mixture of an *E. coli* strain isolated from a patient with stable bacteriuria and with a strain transformed with adhesin gene sequences, the wild-type strain persisted and caused stable bacteriuria, whereas the transformant strain was eliminated within 48 h (Andersson et al. 1991). These results suggest that acquisition of adherence factors may be a prerequisite to, but is not sufficient for, survival in the urinary tract of humans. More recent reports indicate that UPEC can invade and colonize the bladder epithelium establishing a urinary tract reservoir, allowing an explanation of recurrent UTI that does not necessarily involve reinfection via the gastrointestinal (GI) tract (Mulvey et al. 2000).

UPEC are a heterogeneous group of strains belonging to a limited number of O-serogroups. Specific adhesins aid in colonization and invasion. Several toxins are produced, these include cytotoxic necrotizing factor 1 (CNF1), hemolysin, and the secreted autotransporter toxin (Sat). They also possess pathogenicity islands containing large blocks of genes not found in fecal isolates. There are, therefore, a number of virulence factors which may enhance the ability of UPEC strains to cause UTI, but, in this process, how many of these factors are necessary or expressed by an individual infecting strain is still not clear.

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Shigella

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A BRIEF HISTORICAL PERSPECTIVE

Although Chantemesse and Widal (1888) in France and Ogata (1892) in Japan were the first to recognize the etiological agent of bacillary dysentery, culture problems associated with isolation of *Shigella* from patients' stools probably hampered final identification, thus the discovery and identification of these microorganisms is generally attributed to the Japanese microbiologist Kiyoshi Shiga who successfully isolated and identified in 1898 what is currently known as *Shigella dysenteriae* 1 (the Shiga bacillus). The genus name *Shigella* was therefore attributed in 1919 by Castellani and Chalmers in the honor of its discoverer, as part of an attempt to differentiate the agents of bacillary dysentery from the newly created genus *Escherichia* and species *Escherichia coli*. It took half a century, however, for microbiologists to agree on the current concept of four *Shigella* species/subgroups (Enterobacteriaceae Subcommittee 1954).

GENUS AND SPECIES DEFINITION

Shigella are nonspore-forming, gram-negative bacteria. In contrast to the classical characters described for *E. coli*, they are nonmotile, do not produce gas from carbohydrates, decarboxylate lysine, or hydrolyze arginine. Sodium acetate is used only occasionally and indole is produced only by certain serovars.

Based on DNA–DNA hybridization studies, *E. coli* and *Shigella* are so closely related that they could be considered as a unique species. However, for historical and practical, as well as pathophysiological, reasons they are considered as separate species. For similar reasons, *Shigella* themselves even split in four 'species', *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*. These four 'species' actually simply consist of four subgroups (i.e. A, B, C, and D, respectively) that differ by key biochemical and serological characters. The G + C content of the *Shigella* chromosome is 48–53 mol%, similar to the *E. coli* G + C percentage. The genome of *Shigella flexneri* serotype 2a has recently been sequenced. The chromosome is 4.16 Mbases in size, similar to the chromosome of *E. coli* K12. Among its 3 235 ORFs, a backbone of 3 030 is shared with *E. coli* K12, and 2 911 with *E. coli* O157:H7. Compared to these two *E. coli* strains 175 ORFs therefore constitute the genomic identity of *Shigella flexneri* (Jin et al. 2002; Wei et al. 2003). This is clearly insufficient to define a sensu stricto species. The genetic identity of *Shigella* compared to *E. coli*, in addition to some specific chromosomal genes, is conferred by the presence of a large 213 kb virulence plasmid that encodes the invasive phenotype, and by the lack or alteration of a certain number of chromosomal genes (black holes), among which those encoding lysine decarboxylase (LDC), β-galactosidase, and flagella. These

genetic differences are reflected in the positivity or negativity of a certain number of metabolic markers that are routinely used for diagnostic purposes in clinical laboratories.

HABITAT OF *SHIGELLA* AND CONSEQUENCES ON TRANSMISSION

Humans represent the only significant reservoir of *Shigella*. Monkeys can be carriers and even develop the disease under the stress conditions of capture, but this does not play a significant role in the epidemiological characteristics of the disease. Excretion of the bacteria is highest at the peak of disease symptoms, thus the feco–oral transmission makes human to human spread the most efficient mean of transmission. *Shigella*, however, may survive in food for several days, thus making shigellosis a significant cause of food-borne infections. It can also be transported by flies and protection of food against insects has proved to be an efficient means of prevention in endemic areas.

CLINICAL MANIFESTATIONS

Shigellosis is essentially an acute rectocolitis caused by invasion of the rectal and colonic epithelia by *Shigella*. The damage is mostly in the recto-sigmoid area, with the mucosa appearing swollen, with mucus, hemorrhages, and ulcers. Histopathological analysis (Mathan and Mathan 1991) shows continuous and diffuse lesions that are characterized by mucosal edema, focal hemorrhages reflecting rupture of mucosal capillaries, and an epithelial infiltrate consisting of polymorphonuclear leukocytes, lymphocytes, and mast cells. Crypt abscesses are also observed. Bacteria can be seen in the epithelium of the surface and upper third of the colonic crypts. They are often associated with abscesses and mucosal ulcers releasing a luminal exudate consisting of a meshwork of fibrin, individual colonocytes or detached epithelial fragments, erythrocytes and polymorphonuclear leukocytes forming a white mucopurulent layer adhering to the epithelial lining. Later in disease evolution, mononuclear cells may massively infiltrate the lamina propria. These lesions clearly account for the clinical symptoms that characterize shigellosis.

Although dysentery is the classical syndrome associated with *Shigella* infection, its severity varies according to a spectrum encompassing severe dysentery, watery diarrhea or asymptomatic infection (Keusch and Bennisch 1989). Often, a diarrheal episode precedes the appearance of dysentery. In experimentally infected volunteers, the classical symptomatic triad comprising fever, intestinal cramps, and bloody-muco-purulent diarrhea is observed in approximately 50 percent of the individuals (DuPont et al. 1969), thus emphasizing the heterogeneity of the host response to the pathogen. The incubation period is 1–4 days and although the stool frequency is high, often reaching 100 movements/day, severe dehydration is rare because the loss of fluid remains limited. The clinical pattern may however appear more serious, especially in malnourished children in the most impoverished areas, with dehydration and septicemia giving dysentery a profile of increased gravity and possible lethality.

S. dysenteriae 1 is the *Shigella* species that is most often associated with these severe forms, although all *Shigella* species have the potential to cause fatal disease that reflect severe complications such as hypoglycemia (Bennish and Wojtyniak 1991), toxic megacolon, intestinal perforation with peritonitis and hemolytic uremic syndrome (HUS) causing acute kidney failure (Butler et al. 1984), and seizures (Bennish and Wojtyniak 1991). In a retrospective study based on hospital admissions carried out in Bangladesh, lethal infection occurred in 7 percent of the patients (Bennish et al. 1990). The risk factors for fatal outcome were a young age (<1 year), lethargy, abnormally low level of serum proteins, and thrombocytopenia. In addition, severe, prolonged cases of shigellosis in young children led to malnutrition and stunted growth. In the most impoverished areas, shigellosis may be the first cause of growth retardation in infants and young children (Henry 1991).

Last but not least, reactive arthritis may be observed as a sequellae of *S. flexneri* infection, expression of the HLA-B27 histocompatibility antigen being a strong predisposing factor (Kapasi et al. 1992).

EPIDEMIOLOGY

Shigella has a narrow distribution in nature, essentially inhabiting the intestinal tract of humans and captive primates. Although shigellae are impossible to grow from environmental samples, they are consistently present, particularly in sewage, since accidental contamination of water supplies by their effluents is regularly followed by an outbreak of shigellosis. Most cases of shigellosis follow person-to-person transmission, although some outbreaks may reflect food or water contamination. Throughout history, epidemics of shigellosis have always occurred among human gatherings in poor hygiene conditions: armies in campaign, besieged populations, pilgrimages, and refugee camps.

Shigellosis is endemic throughout the world, although 99 percent of infections occur in the developing world. According to a review (Kotloff et al. 1999), the total number of yearly cases is 164.7 million, 1.1 percent of which result in death. Children less than 5 years old account for 69 percent of the clinical episodes and 62 percent of the deaths. In spite of excellent epidemiological reviews, shigellosis is a rapidly evolving disease that requires permanent updates with regard to the general incidence and evolution of the respective species

and serotypes. This will become even more important when good vaccine candidates become available.

Shigella is a highly infectious microorganism, as few as 100 shigellae given orally have been shown to cause the disease in 25–50 percent of North-American volunteers (DuPont et al. 1989). Relative resistance of *Shigella* to gastric acidity may partly account for this high infectivity.

The transmission of *Shigella* occurs essentially through the fecal–oral route. It is spread by direct contact with infected individuals because the bacteria show significant survival on the skin (Islam et al. 1997). The high infectivity of *Shigella* is practically reflected in the high attack rate of infection observed during outbreaks in day care centers (33–73 percent) and the rate of secondary cases observed in family members of the sick children (26–33 percent) (Weissmann 1975; Weissmann et al. 1974). *Shigella* can also be transmitted by contaminated food and many examples of outbreaks of food-borne infections have been reported with contaminated products as diverse as lettuce, onions, coconut milk, hamburgers, and pasta dishes (Boyce et al. 1982; Rennel and Levine 1986). Shigellosis is also a sexually transmitted disease transmitted by oro-genital sexual practices (Dritz and Black 1974).

It must be emphasized, again, that shigellosis is, overwhelmingly, a third world disease which is mainly associated with the conditions prevailing in underdeveloped regions: overcrowding, poverty, poor personal hygiene, absence of food control, inadequate water supplies, and sewage. In these conditions, where children often suffer from malnutrition, the rate of serious complications may be high. In Bangladesh, an epidemic of *S. dysenteriae* 1 infection has been associated with a 42 percent increase in children mortality between 1 and 4 years (Bennish and Wojtyniak 1991). Similarly, in the refugee camp of Goma in 1994, the Shiga bacillus has been shown to cause a 30 percent attack rate and a 38 percent mortality rate. The high level of multiresistance of the strains isolated in these circumstances concurs in the severity of the disease.

DISTRIBUTION OF SEROGROUPS AND SEROTYPES

S. flexneri and *S. sonnei* are essentially responsible for the endemic form of shigellosis, whereas *S. dysenteriae* 1 is responsible for rapid and possibly deadly epidemics. *S. boydii* is rarely found, it belongs to the endemic form of the disease. In developing areas, the most common species is *S. flexneri*, mainly the serotypes 1b, 2a, 3a, 4a, and 6. The prevalence of these dominant serotypes varies considerably depending on the area under study and in a given area the pattern may be very unstable, thus requiring permanent surveillance and update. Regarding geographical distribution, *S. dysenteriae* 1 is more prevalent in South Asia and in sub-Saharan Africa. In industrialized regions, *S. sonnei* largely predominates, actually, occurrence of a switch between *S. flexneri* and *S. sonnei* is a sign of economical development (Kotloff et al. 1999).

THE GENOME OF *SHIGELLA*

The genome sequences of two different isolates of the same *S. flexneri* 2a serotype have been published (Jin et al. 2002; Wei et al. 2003). The whole chromosome is composed of 4 607 203 bp to which the 221 618 bp virulence plasmid must be added. The *Shigella* chromosome, although very similar in size and gene composition to the genome of *E. coli* K12, is characterized by several original features such as the presence of an extraordinary number of insertion sequences (314 IS elements, a 7-fold higher number compared to *E. coli* K12); 13 events of translocation and inversion involving a segment larger than 5 kb, most of them being related to a deletion or an acquisition of DNA sequences encompassing several pathogenicity islands, probably introduced by bacteriophages. *S. flexneri* is characterized by the presence of hundreds of pseudogenes, their inactivation being linked to various events such as introduction of stop codons, frame shift mutations, truncations, and integration of insertion sequences. This massive loss of gene integrity accounts for the massive loss of metabolic functions that is so characteristic of *Shigella.* It also illustrates the fact that the evolutionary process that has led *E. coli* to become *Shigella* has been characterized not only by a gain of genes and pathogenicity islands, but also by a loss of genes.

PATHOGENESIS OF SHIGELLOSIS

Following oral contamination, shigellae transit through the gastric barrier and the small intestine before reaching their site of invasion, the colonic and rectal mucosae. The disease process is characterized, in the classical form of the disease, by the occurrence of an acute rectocolitis with epithelial abscesses and ulcerations that cause the dysenteric symptoms. In severe forms, the terminal ileum may also be affected. The reasons for the rather specific tropism of *Shigella* to the colonic and rectal mucosae remains largely unexplained.

Virulent *Shigella* isolates express an invasive phenotype which is essentially reflected in the capacity of the bacteria to penetrate into nonphagocytic epithelial cells and to spread from cell to cell. The spreading process is correlated with the development of a strong inflammatory reaction causing the rupture and destruction of the epithelial barrier (Sansonetti 2001). *Shigella* causes rupture, invasion, and inflammatory destruction of the colonic epithelial barrier through a complex process. *Shigella* cannot invade epithelial cells apically, thus it exploits M cells to cross the colonic epithelial barrier. M cells of the follicle-associated epithelium (FAE) that

covers solitary lymph nodes within the colonic mucosa carry out the sampling of luminal antigens and their delivery to the local immune system. M cells show a poorly organized brush border, lack apical glycocalix and express high endocytic activity. They form a typical pocket filled by mononuclear cells. Several invasive microorganisms use them to cross the epithelial barrier (Raupach et al. 1999). Following crossing of the FAE, bacteria reach the dome area of the lymphoid follicle, where they are phagocytosed by macrophages and dendritic cells. *Shigella* kills macrophages by apoptosis (Zychlinsky et al. 1992), then escapes to the subepithelial tissue and invades the epithelial lining basolaterally. Apoptosis of the macrophages allows *Shigella* to escape phagocytes and to initiate inflammation because apoptosis is caused by the activation of Caspase-1, a cysteine protease that cleaves the procytokines pro-IL-1β and pro-IL-18, thus releasing their active products (Chen et al. 1996). As a consequence, polymorphonuclear cells (PMN) infiltrate the infected site and destabilize the epithelium (Sansonetti et al. 1999a). Rupture of the coherence of the epithelial barrier allows more bacteria to translocate, thereby providing access to the permissive basolateral side of epithelial cells. Bacteria then disseminate throughout the epithelium by spreading from cell to cell. Intracellular shigellae reprogram epithelial cells to express pro-inflammatory cytokines and chemokines such as IL-8, thus exacerbating inflammation (Sansonetti et al. 1999b).

Bacterial determinants of pathogenicity

The 213 kb virulence plasmid contains the genes required to express the *Shigella* invasive phenotype (Sansonetti et al. 1982). Sequencing of the virulence plasmid of a *S. flexneri* 5a isolate (Buchrieser et al. 2000) has confirmed the presence of a 31 kb pathogenicity island (PAI) encoding a type III secretion system (TTSS) (*mxi spa* genes) and its cognate effector proteins that are injected into the membrane or the cytoplasm of target cells (*ipaA-D*, *ipgB1*, and *ipgD* genes). The TTSS of *Shigella* is composed of approximately 20 proteins that assemble into a structure spanning both the inner and outer membranes, and extending outside by approximately 60 nm (Blocker et al. 1999). Following contact between bacteria and host cell, IpaB and IpaC are inserted as a complex into the host membrane and form a 25 Å pore transporting the other invasins into the cell cytoplasm.

This PAI is necessary and sufficient for entry of bacteria into epithelial cells, macrophage apoptotic death, and activation of polymorphonuclear cells (Maurelli et al. 1985; McCormick et al. 1998; Sasakawa et al. 1988; Zychlinsky et al. 1992). It also contains the encoding proteins secreted by the TTSS, and the genes encoding the cytoplasmic chaperones, IpgC and IpgE. The *virB* gene codes for the activator required for transcription of these operons (Adler et al. 1989). The virulence plasmid carries other genes outside the PAI encoding proteins that are also secreted through the TTSS; these comprise five *ipaH* genes, specific functions of which have not yet been identified. IpaH7.8 has been implicated in the escape of *Shigella* of the phagosome in macrophages (Fernandez-Prada et al. 2000) and IpaH9.8 can be transported into the host cell nucleus and stimulates the protein secretion from *Shigella* (Toyotome et al. 2001) and the multigene family, *osp* with unknown function. In addition, other genes have been identified in pWR100, such as *sepA* encoding a protein with proteolytic activity whose inactivation leads to an attenuation of virulence (Benjelloun-Touimi et al. 1995; Benjelloun-Touimi et al. 1998). Last but not least, *icsA* (*virG*) encodes the outer membrane protein directly responsible for the motility of bacteria within the host cytoplasm (Bernardini et al. 1989; Egile et al. 1999; Goldberg and Theriot 1995; Lett et al. 1989; Makino et al. 1986). Gene *virK* encodes a protein required for a proper production or localization of IcsA (VirG) (Nakata et al. 1992) and *sopA* (*icsP*) encodes an outer membrane protease implicated in the cleavage of IcsA (Egile et al. 1997; Steinhauer et al. 1999).

Bacteria–host cell cross talks

Upon contact with nonpolarized, cultured epithelial cells, *Shigella* induce local formation of actin-dependent cell membrane extensions, thus causing a macropinocytic pocket engulfing the microorganism. Ipa invasins orchestrate the cytoskeletal rearrangements necessary for bacterial invasion (Ménard et al. 1993; Parsot and Sansonetti 1996; Tran Van Nhieu et al. 1997). In contrast, *Shigella* can only invade the basolateral side of confluent, polarized epithelial cells (Mounier et al. 1992).

In contrast to other invasive pathogens, *Shigella* shows little cell binding ability. The IpaB/C complex secreted by *Shigella* has been shown to bind the $\alpha_5\beta_1$ integrin, the receptor of fibronectin (Watarai et al. 1996); however integrins are not exclusive receptors for the IpaB/C complex. During *Shigella* entry, a functional role for CD44, the receptor for hyaluronic acid, has been observed. IpaB can bind to this receptor in epithelial cells (Skoudy et al. 1999) leading to local recruitment and possibly activation of ezrin.

Both Cdc42 and Rac, two small GTPases of the Rho family induce actin polymerization in their activated GTP-bound form through the activation of the Arp2/3 complex. They are recruited to *Shigella* entry foci and are essential for *Shigella* uptake by cultured epithelial cells (Mounier et al. 1999). The proto-oncogene *c-src* is also involved in the extension of cellular projections, thereby facilitating the entry process (Dumenil et al. 1998). Actin polymerization at the point of bacterial

contact with the epithelial cell is dependent upon IpaB and C forming a pore. IpaC translocated into the host cell cytosol acts as a direct effector for *Shigella*-induced actin polymerization, induces formation of filopodial and lamillipodial extensions dependent on Cdc42 and Rac (Tran Van Nhieu et al. 1999). IpaA binding to vinculin promotes the association of vinculin with F-actin and induces F-actin depolymerization. As a consequence, the formation of new projections that could repel the bacterium away from the host cell surface is stopped.

Following entry, the bacteria disrupt their vacuole and enter the cell cytoplasm, multiply, and express an actin-based motility phenotype (Bernardini et al. 1989; Lett et al. 1989). The actin-based motility of *Shigella* is dependent on IcsA(VirG), an outer membrane protein which causes actin nucleation and polymerization at the bacterial surface (Bernardini et al. 1989) through the binding and activation of N-WASP, a member of the WASP family of proteins, which itself binds and activates the Arp2/3 complex which induces actin nucleation (Egile et al. 1999). When bacteria reach the inner face of the host cell membrane, a protrusion is formed that is phagocytosed by the adjacent cell, thus allowing cell-to-cell spread. E-cadherin, an adhesion protein localized in intermediate junctions, and its partners regulating the junction, are involved in intercellular spread (Sansonetti et al. 1994). Lysis of the cellular membranes allows bacteria to reach the cytoplasm of newly infected cells without extracellular step.

Chromosomally encoded virulence factors

The virulence of *S. flexneri* and most probably other *Shigella* species and serotypes is also due to chromosomally encoded factors which include the *wba* and *wbb* genes encoding biosynthesis of the major surface antigen, the lipopolysaccharide (LPS), the *mra* and *mrb* genes encoding the synthesis of the peptidoglycan (PGN), and other less characterized pathogenicity islands such as SHI-1, SHI-2, and *Shigella* resistance locus (SRL) (Ingersol et al. 2002; Turner et al. 2001).

LPS comprises three structural components: the lipid A (endotoxin), the core oligosaccharide, and the variable O-side chains that constitute the somatic antigen carrying the serogroup/serotype specificities. The lipid A accounts for a significant part of the intestinal inflammation observed in the course of experimental shigellosis (d'Hauteville et al. 2002). A large portion of the humoral response is directed against this somatic antigen and the protection that it mediates is largely serotype-specific. SHI-O contains the O-antigen serotype conversion genes carried by lysogenic bacteriophages. Two conserved genes, *gtrA* and *gtrB*, and one gene specific of the particular modification of the O-antigen have been recognized (Allison 2000). The PGN, even limited as in other gram-negative microorganisms is a potent inducer of inflammation, particularly through its recognition by the Nod system, as described below. PGN is a heteropolymer composed of two aminosugars: N-acetylglucosamine and N-acetyl-muramic acid and a limited number of amino acids, some of which never occur in proteins. Short peptide chains are attached to the carboxyl group of the muramic acid, the most common sequence being L-alanine-D-glutamic acid-*meso*-diaminopimelic acid-D-alanine-COOH. Presence of the *meso*-DAP in position three of the peptide is rather characteristic of gram-negative microorganisms. SHI-1, a 46 kb pathogenicity island encodes the ShET1 enterotoxin (*set1B* and *set1A*) and two autotransporters (SigA and Pic) which may participate to *Shigella* virulence. The ShET1 enterotoxin has an A1B5 structure similar to cholera toxin and is found essentially in *S. flexneri* 2a (Fasano et al. 1995). SHI-2 is present in all *S. flexneri* isolates. It encodes the aerobactin scavenging system that is essential for *Shigella* acquisition of ferric iron in the iron-limiting conditions that prevail at the intestinal surface and in tissues; it also encodes colicinV immunity which seems to be important for luminal survival (Moss et al. 1999). The SRL is an island encoding resistance to streptomycin, ampicillin, chloramphenicol, and tetracycline (Turner et al. 2001).

In addition to the core of virulence determinants that have been described essentially in *S. flexneri*, the serotype 1 of *S. dysenteriae* is characterized by the expression of Shiga toxin, a potent cytotoxin belonging to the A1B5 model which is encoded by a chromosomal cistron: *stxAB* at centisome 28 (Sekizaki et al. 1987; O'Brien and Holmes 1987). Shiga toxin is potently cytotoxic through its capacity to block protein biosynthesis, although alternative causes of cell death may exist. StxA carries the enzymatic activity of Shiga toxin, whereas StxB mediates its binding to its ganglioside receptor, Gb3 (Keusch and Jacevicz 1977). Once internalized by receptor-mediated endocytosis, Shiga toxin undergoes retrograde transport, reaches the Golgi compartment from which StxA crosses the membrane, and reaches the cytosol (Sandvig et al. 1991). StxA is a glycosidase that removes adenine from one particular residue in the 28S RNA of the 60S ribosomal subunit of eukaryotic cells, in a way similar to the plant toxin Ricin (Endo et al. 1988). It has not yet been convincingly demonstrated that Shiga toxin affects intestinal epithelial cells and actually accounts for diarrhea. Gb3 is barely expressed by intestinal epithelial cells, but rather by other cells, particularly endothelial cells. Evidence indicates that Shiga toxin is primarily a toxin for the vascular endothelium and that, in the course of shigellosis, it may superpose a component of severe intestinal ischemia to the invasive process, thus accounting for the increased severity of infections caused by the Shiga bacillus (Fontaine et al. 1988).

Invasion and inflammatory destruction of the epithelial barrier

The acute inflammatory response generated by *Shigella* infection is characterized by diffuse erythema and swelling of the mucosa, focal hemorrhages and a mucopurulent exudate. At the later stages of shigellosis, the histopathological lesions cannot be differentiated from those observed in acute ulcerative colitis. Cytokines and chemokines are mediators of tissue damage. In patients at the acute and convalescent stages of *S. flexneri* and *S. dysenteriae* 1 infection, immunohistochemistry of rectal samples shows a pattern of IL-1, IL-4, IL-6, IL-8, TNFα, and IFNγ production (Raqib et al. 1995a, b). Severe disease is associated with increased IL-1β, IL-6, TNFα, and IFNγ expression. IL-1 is essentially produced by monocytes/macrophages, whereas IL-6 and IL-8 are produced by epithelial cells. The initial step of tissue invasion of *Shigella* through the follicle-associated epithelium leads to bacterial phagocytosis by macrophages, followed by apoptotic death of the phagocytes (Zychlinsky et al. 1992, 1996). Being caused by the activation of Caspase-1, macrophage apoptosis also causes the release of the pro-inflammatory cytokines IL-1β and IL-18, thereby initiating the inflammatory cascade characteristic of shigellosis (Sansonetti et al. 2000).

In response to bacterial invasion, intestinal epithelial cells provide important signals for the initiation and amplification of an acute mucosal inflammatory response. They express an array of pro-inflammatory molecules, particularly IL-8, a potent chemoattractant for PMN (Jung et al. 1995), although not responsible for their transepithelial migration (McCormick et al. 1995). IL-8 causes PMN-mediated arrest of *Shigella* translocation through the intestinal epithelium into the lamina propria at the cost of massive epithelial destruction (Sansonetti et al. 1999b). Presence of intracellular shigellae leads to NF-κB activation (Philpott et al. 2000). NF-κB is a major transcriptional activator for the main pro-inflammatory genes, including IL-8. Nuclear translocation of NF-κB and activation of the pro-inflammatory genes occur in epithelial cells in response to engagement of the cytosolic microbial sensor Nod1 by the peptidoglycan released by intracellular shigellae (Girardin et al. 2001, 2003). Largely in response to the early events of interaction occuring at the level of infected mucosal lymph nodes and to the stimulation of Nod1 in invaded epithelial cells, a massive infiltration of mononuclear and polymorphonuclear cells occurs that transmigrate and disrupt the epithelium, opening a way for bacterial invasion at the basolateral membrane domain (Perdomo et al. 1994a, b). *Shigella* are efficiently killed by PMN. If PMN contribute initially to the development of invasion, they are ultimately involved in the resolution of infection, particularly through the degradation of the bacterial virulence factors by elastase (Weinrauch et al. 2002) and the killing of invasive shigellae (Mandic-Mulec et al. 1997).

LABORATORY DIAGNOSIS

Fecal leukocytes

Patients presenting the acute stage of a dysenteric syndrome are naturally suspected of shigellosis, although a similar clinical picture can be observed in the course of acute amoebic colitis due to *Entamoeba histolytica*, and in the course of infections by *Campylobacter*, *Salmonella*, and *Yersinia*. Microscopic examination of stool smears reveals a striking contrast between amoebiasis where erythrophagocytic trophozoites are observed with very few PMN, whereas, in the case of shigellosis, PMN are massively present (50 PMN per high power microscopic field (Speelman et al. 1987)).

Optimizing chances of *Shigella* isolation

Diagnosis of shigellosis depends largely on microbiological examination. In the course of epidemiological studies, immunological assays for anti-*Shigella* LPS antibodies may substitute, in retrospect, as a method to diagnose *Shigella* infection. Molecular methods, such as polymerase chain reaction (PCR) directly performed on stool samples, still require extensive validation. Multiple methods are available to isolate *Shigella* from feces samples. Shigellae are present in the stools of acutely ill patients in concentrations that may vary between 10^3 and 10^9 cfu per gram of stool. These viable microorganisms are, however, extremely fragile and they quickly die off if stool samples are not properly processed. Rectal swab specimens yield the highest rate of successful isolation in the acute phase of the disease, compared to fresh stools and anal swabs. Streaks of blood and/or muco-purulent material that contain large amounts of bacteria should always be cultured as they contain an almost pure culture of *Shigella*. Ideally, once collected, specimens should be immediately inoculated into cultured media since *Shigella* is unable to survive a brisk drop in temperature and the competitive conditions, such as drop in pH and lack of nutrients, created by overgrowth of other bacteria. If a delay in transport to the laboratory is anticipated, direct inoculation of the specimens should be performed into media such as buffered glycerol saline (BGS), with a pH indicator. Refrigerated BGS preserves *Shigella* viability better than the Cary–Blair transport medium. From the inoculated transport medium, plating on isolation medium should ideally be carried out within 6 h.

Isolation of *Shigella*

There is no reliable enrichment procedure for *Shigella* from any type of sample (i.e. stools, food or environmental samples); therefore, isolation should be attempted as quickly as possible following collection of

the samples, on different media in order to increase the chances of successful isolation. The following media are advised: (1) a low-selectivity medium such as MacConkey or eosin-methylene blue (EMB) agar, (2) a high-selectivity medium such as Hektoen, *Salmonella-Shigella* (SS), or xylose-lysine-deoxycholate (XLD) agar, (3) and a nonselective medium such as bromocresol-purple agar lactose (BCP). SS medium appears more convenient for field studies but is not adapted to isolation of *S. dysenteriae* serotype 1 and *S. sonnei*.

Identification of *Shigella*

Shigella are nonlactose-fermenting Enterobacteriaceae that yield colonies after 12–18 h incubation at 37°C. Suspect colonies may be subcultured on a more selective medium before identification is started. On MacConkey, Hektoen, BCP and SS agar, typical colonies have a small diameter of 0.5–1 mm; they are convex and translucent with a smooth surface. Those suspect colonies can be directly identified by several commercial systems. The major identification criteria that will identify *Shigella* from other members of the Enterobacteriaceae are: (1) glucose positive, usually without production of gas, (2) lactose negative, H_2S negative, non motile. In addition, the four groups of *Shigella* (i.e. A = *S. dysenteriae*; B = *S. flexneri*; C = *S. boydii*; and D = *S. sonnei*) can be differentiated on the basis of further biochemical properties. *Shigella* typically does not produce gas in the presence of glucose with the exception of *S. flexneri* 6, *S. boydii* 13 and 14, and *S. dysenteriae* 3. The four groups are LDC−, acetate−, and mucate−. Only *S. sonnei* ferments lactose slowly, some isolates being mucate positive, or capable of decarboxylating ornithine. Strains of *S. dysenteriae* are characteristically negative for mannitol fermentation and production of catalase, and they are occasionally o-nitrophenyl-β-D-galactopyranoside (ONPG)+. Subgrouping is extremely difficult based upon biochemical differences, thus the quick serotyping when sera are available is recommended. However, serological methods using group- and type-specific antisera must be performed only following biochemical presumptive identification.

A simplified algorithm of identification can be proposed:

- Isolate is nonmotile and LDC−
- With the few exceptions mentioned above, isolate does not produce gas during glucose fermentation
- Isolate grows on synthetic medium plus acetate (Trabulsi and Ewing medium) and is Christensen's citrate–.

Serotyping should be performed at this stage.

Serogrouping of *Shigella* isolates

Somatic antigenic specificities allow *Shigella* to be divided into four serogroups (species). With the exception of *S. sonnei* which comprises a single serotype, *S. flexneri* comprises of 6 serotypes, *S. dysenteriae* 8, and *S. boydii* 18.

Although *Shigella* can be presumptively identified at the group (genus) level with biochemical tests, their identity (serogroup/species) must be confirmed by slide agglutination with polyvalent (serogroups A–D) antisera. Below species level, serotyping is usually carried out in reference laboratories. When biochemically identified isolates of *Shigella* do not agglutinate in *Shigella* antisera, a bacterial suspension should be heated (100°C for 15 min) and tested again for agglutination. In case of a persistent lack of agglutination, isolates should be sent to a reference center for further study, as such isolates could correspond to enteroinvasive *E. coli* (EIEC). Recently, a method of 'molecular serotyping' has been developed that makes use of amplification of O-antigen gene clusters (Coimbra et al. 1999).

The major characteristics of *Shigella* that are relevant to their diagnostic identification are summarized in Table 1.

Molecular diagnosis

Several sequences related to the pathogenicity of *Shigella* have been used to diagnose *Shigella* and EIEC and differentiate them from *E. coli* isolates belonging to the resident flora. PCR techniques performed on diarrheal stools (Lüscher and Altwegg 1994) and food (Lindqvist 1999) have revealed a strong potential for sensitive and specific diagnosis. The use of PCR to amplify a specific *virA* gene fragment from the *Shigella* virulence plasmid (Villalobo and Torres 1998) or an *ipaH* gene fragment also from the plasmid (Sethabur et al. 2000) have proven useful to detect *Shigella* and EIEC. These techniques have the potential to be used in clinical centers (Islam et al. 1998).

Routine diagnosis of the *Shigella* invasive phenotype

These aspects of diagnosis are restricted to reference laboratories. The reference test that confirms the invasion phenotype of a bacteriologically identified gram-negative bacillus is the capacity of the isolate to induce an acute keratoconjunctivitis in the guinea pig's eye, following inoculation in the conjunctival sac of a droplet at a concentration of 10^8 cfu/ml. This test is also known as the Sereny test (Sereny 1957). This reference test tends to be abandoned because it causes a long, painful disease in infected animals, to the profit of in vitro assays using cultured epithelial cells such as HeLa cells.

TREATMENT OF SHIGELLOSIS

The early clinical phase of shigellosis is often characterized by an episode of watery diarrhea that may persist

Table 1 *Characteristics of* Shigella

	Mannitol	Indole	Serotypes	Antigens	Comments
S. dysenteriae (subgroup A)	–	– +	1,3,4,5,6,10,11,12,13 2, 7, 8	Each serotype possesses a distinct antigen.	ONPG+, catalase-, arabinose-
S. flexneri (subgroup B)	+	[–] [+] –	1, 2 3, 4, 5 6	Several antigenic determinants are shared by the species members. Coagglutination is possible and highly absorbed antisera are needed.	*S. flexneri* 6 var. Manchester and var. Newcastle are gas+[a]
S. boydii (subgroup C)	+	–	1,2,3,4,6,8,10,12,14,18	The 18 serotypes are antigenically distinct, except serotypes 10 and 11 sharing a common antigen other than their specific antigen.	*S. boydii* 10 is ADH+, arabinose+, treahalose+ *S. boydii* 14 is gas+, mannitol-, maltose+, sorbitol-, arabinose+, trehalose+, ADH+
		+	5,7,9,11,13,15,16,17		*S. boydii* 17 is ADH+, arabinose+, trehalose+, *S. boydii* 9 is ONPG+ (African strains may be lactose+)
S. sonnei (subgroup D)	+	–	only one serotype	It undergoes irreversible antigenic variation from phase I (smooth and virulent) to II (rough, non virulent). A plasmid encodes the form I antigen and the ability to invade intestinal epithelial cells. The phase I is not antigenically related to other *Shigella* groups but identical with *Plesiomonas shigelloides* serotype C27.	*S. sonnei* usually ferments lactose or saccharose after 3, 4 days at 37°C. 4 major biotypes (99 percent of the strains): a: ONPG+, xylose–, rhamnose+ g: ONPG+, xylose-, rhamnose+ d: ONPG–, xylose+, rhamnose+ f: ONPG–, xylose–, rhamnose+

a) Var. Manchester is glucose+, mannitol+, and var. Newcastle is glucose–, mannitol–.

and eventually represent most of the observed symptomatology. When such symptomatology occurs in infants and young children, it may require oral rehydration therapy. Intravenous fluid replacement may be required in the most severe cases. The mild diarrheal forms of shigellosis are not generally recognized as such and consequently are not given antibiotics. In the case of dysentery, antibiotics should be administered. Bacillary dysentery is actually one of the few instances among enteric infections for which antibiotics are indicated (Levine 1986). Orally-administered absorbable antibiotics such as ampicillin and sulfamethoxazole-trimethoprim are the molecules of choice, as well as nalidixic acid. There is, however, increasing resistance to these 'first-line' antibiotics, particularly in *S. dysenteriae* 1. This makes fluoroquinolone ciprofloxacine the drug of choice in areas of multiresistance, although its cost makes its use difficult in the most impoverished regions. Multiresistance in *Shigella* is currently causing a major crisis in highly endemic areas and in the course of *S. dysenteriae* 1 epidemics, particularly in the context of humanitarian disasters such as refugee camps. This critical situation legitimates renewed efforts for the development of a vaccine.

PREVENTION OF SHIGELLOSIS

Interruption of disease transmission

Transmission of shigellosis is efficiently interrupted by public health measures avoiding contact with contaminated feces. When clean water and good sanitary conditions are available, safe disposal of excreta, food protection, and person-to-person protection by hand washing, and elimination/protection against flies are efficient preventive measures. In impoverished, endemic areas, effective prevention is more difficult and requires a more global approach that includes both the construction of infrastructures (i.e. construction of latrines) and social interventions. Considering the technical difficulties and costs inherent to this approach, education remains the priority in prevention: encouraging avoidance of fecal contamination and promoting breast feeding are cost-effective measures.

Both epidemiological and experimental evidence indicate that *Shigella* infection elicits subsequent protective immunity against a similar serotype (Ferreccio et al. 1991). In addition, the experimental challenge of North-American volunteers initially infected by *S. sonnei* or *S. flexneri* 2a showed 75 percent protection when this challenge was carried out with the homologous serotype (Herrington et al. 1990; Kotloff et al. 1995). Additional experimental evidence in macaque monkeys (Formal et al. 1991) and epidemiological observations in human populations (Cohen et al. 1992) emphasize the fact that protection against *Shigella* infection is serotype-specific. Passive protection of subsequently challenged volunteers with bovine colostrum containing high titers of antibodies (essentially secretory IgA) directed against the *Shigella* O-antigen (Tacket et al. 1992), and experimental evidence that passively administered monoclonal IgA also directed against the somatic antigen, protect against murine lung infection following an intranasal challenge (Phalipon et al. 1995), strongly suggest that protection against *Shigella* infection is largely achieved at the mucosal surface by secretory IgA directed against the somatic antigen, thus confirming the sero-dependence of protection. Recently, O-antigen specific secretory IgA have been shown to achieve mucosal protection through their capacity to carry out immune exclusion of shigellae from the epithelial surface via their trapping and elimination in the mucus (Phalipon et al. 2002).

It remains, however, to evaluate the possible role of serotype-specific serum IgG in immune protection. These antibodies may transudate at the site of colonic invasion/inflammation and become bactericidal by activating the complement and/or acting as opsonins (Robbins et al. 1992). This issue of secretory IgA mediating immune exclusion versus serum IgG mediating bactericidal activity at the sites of mucosal invasion is of primary importance because it supports the rational for vaccine development that is dominated by one alternative: live attenuated strains administered orally versus polysaccharide conjugate administered parenterally.

Initial attempts at protecting against shigellosis were based on whole-cell killed preparations administered parenterally. These attempts were unsuccessful, thus leading to the concept that parenteral immunization could not provide efficient protection against shigellosis, and that for a disease that remained essentially local, mucosal immunization with live-attenuated, orally administered vaccine candidates represented a better option. In the mid-1960s, initial attempts using multiple oral doses of a streptomycin dependent (SmD) strain showed encouraging results, thus providing the proof of concept for this approach. In addition, these studies confirmed the sero-dependence of protection (Mel et al. 1971). According to this principle, over recent years, attenuated strains have been genetically engineered on rational bases by knocking out bacterial genes encoding either metabolic pathways that are essential for the survival of bacteria in the tissues, such as *aro*, *gua* or *thy* mutants, or factors that are essential for the pathogenicity/virulence of the microorganism, such as *icsA*, or a combination of both (Phalipon and Sansonetti 1995). Several of these live vaccine candidates are currently under clinical trial. They do show protection in North-American volunteers (Coster et al. 1999), although these encouraging data need to be assessed in endemic areas, particularly in the pediatric population.

Alternatively, a sub-unit parenteral vaccine composed of purified-detoxified *S. sonnei* LPS conjugated to a

protein toxoid has shown 70 percent homologous protection in the course of a *S. sonnei* outbreak occuring among immunized Israeli military recruits (Cohen et al. 1996).

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Salmonella

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DEFINITION AND BACKGROUND

Salmonellae are organisms that conform to the definition of the Enterobacteriaceae. Most strains are motile. Apart from a few exceptions that form acid only, they produce acid and gas from glucose and mannitol, and usually also from sorbitol; they ferment sucrose or adonitol rarely, and rarely form indole. Acetylmethylcarbinol is not formed. They do not hydrolyze urea or deaminate phenylalanine, usually form H_2S on triple sugar iron agar, and use citrate as sole carbon source. They form lysine and ornithine decarboxylases. The many serotypes in the group are closely related to each other by somatic and flagellar antigens and most strains show diphasic variation of the flagellar antigens. Salmonellae are primarily intestinal parasites of vertebrates; they are pathogenic for many species of animals, giving rise to enteritis and to typhoid-like diseases. The G + C content of their DNA is 50–53 mol% and the type species is *Salmonella enterica*.

The genus *Salmonella* was originally created by medical bacteriologists to include organisms that gave rise to a certain type of illness in humans and animals and were related to one another antigenically. Later it became clear that salmonellae had many common biochemical characters. Now more emphasis is placed on biochemical activity than antigenic structure in their definition. Individual strains atypical in one or more biochemical tests usually have so many other biochemical characters in common with other salmonellae that there is little difficulty in classifying them. An organism which, on biochemical grounds, appears to be a salmonella will usually possess one or more antigens in common with other salmonella types but the possession of salmonella antigens is itself insufficient for an organism's inclusion in the group.

The genus *Salmonella* includes not only the familiar serotypes that are pathogens of mammals but also other serotypes formerly considered to be biochemically aberrant types. It is now considered to comprise two species: *S. enterica* and *Salmonella bongori*. There are six subspecies of *S. enterica*, the most important of which is *S. enterica* subsp. *enterica* (subspecies I) which includes the typhoid and paratyphoid bacilli and most of the other serotypes responsible for widespread disease in mammals. Members of the other five subspecies (II–VI) are, in the main, parasites of cold-blooded animals or are found in the natural environment.

NOMENCLATURE

The terminology introduced by early workers accorded specific rank to each antigenically distinguishable salmonella type. The species names given were generally descriptive of the disease or the host with which the serotype was associated, and were sometimes incorrect. Thereafter, the convention was established that each new type should be named after the place in which it was first isolated. Whereas the first published

table contained some 20 serotypes, each considered to be a species, the current number is 2 399 (Popoff et al. 1995).

However, they are no longer accorded specific status because more modern taxonomic techniques (Le Minor et al. 1982a, b) suggested that all serotypes of *Salmonella* probably belonged to one DNA-hybridization group within which seven subgroups were identified; and, following the strict rules of the Bacteriological Code, Le Minor et al. (1986) correctly proposed the name *Salmonella choleraesuis* for the only genospecies of *Salmonella*. That same name, however, had long been used to designate a salmonella serotype and its use was likely to lead to errors of interpretation by clinicians and others unfamiliar with the complexities of bacterial nomenclature. To avoid such confusion, Le Minor and Popoff (1987) requested that the name of this species be changed from *S. choleraesuis* to *S. enterica*, an epithet never before used to designate a salmonella serotype, and that proposal has been generally adopted (Ewing 1986; Old 1992; Popoff and Le Minor 1992). The seven DNA-hybridization subgroups were designated as subspecies: *S. enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori*, and *indica* (Le Minor and Popoff 1987). However, the DNA–DNA hybridization studies of Le Minor and colleagues (1982a, 1986) suggested that DNA subgroup V (*S. enterica* subsp. *bongori*) had evolved significantly from the other six subspecies. Results from multilocus enzyme electrophoresis (MLEE) studies supported that observation and the elevation of *S. enterica* subsp. *bongori* to the level of species as *S. bongori* was proposed (Reeves et al. 1989).

Because names of serotypes escape the strictures of the Bacteriological Code, they have no more nomenclatural ranking than phage type or biotype designations and are no longer given specific status (Popoff and Le Minor 1992). Serotype names should be given in roman (not italic) type as, for example, serotype (ser.) Typhimurium. Furthermore, only serotypes of subsp. *enterica* are still named; serotypes of other subspecies of *S. enterica* and of *S. bongori* are no longer named but designated by their antigenic formulae. Designations such as *Salmonella* ser. Typhimurium, *Salmonella* Typhimurium or even simply Typhimurium are convenient for use in clinical situations and indicate, furthermore, that the named serotype is a member of subsp. *enterica*. This practice will be followed in this chapter.

Subtypes of serotypes recognized by phage typing or biotyping should not be named as if they were serotypes. Thus, the names of salmonellae (e.g. '*S. java*') distinguishable from established serotypes by biotype characters only should not be perpetuated and most have been deleted from the Kauffmann–White scheme of serotypes (Popoff and Le Minor 1992).

PHENOTYPE

Morphology

The morphology of salmonellae is similar to that of other enterobacteria. With the exception of Gallinarum-Pullorum, they are motile by peritrichous flagella. Nonmotile strains may be isolated from clinical cases.

Most salmonellae form common fimbriae and most fimbriate strains (80 percent) possess type-1 fimbriae associated with mannose-sensitive adhesive properties (Duguid et al. 1966; Duguid and Old 1980). These fimbriae are composed of fimbrillin subunits (MW c. 21 kDa) containing a high proportion (40 percent) of hydrophobic amino acids. Failure to form type-1 fimbriae is the rule in some serotypes, e.g. Paratyphi A, Gallinarum-Pullorum and is unusual in others, e.g. Paratyphi B, Typhi and Typhimurium (Duguid et al. 1975; Duguid and Old 1980, 1994). Strains of Gallinarum-Pullorum form type-2 fimbriae which are morphologically and antigenically like type-1 fimbriae but nonadhesive (Duguid et al. 1966; Crichton et al. 1989; Duguid and Old 1994).

Some strains of serotypes such as Enteritidis and Typhimurium that are usually type-1 fimbriate produce thin fimbriae (<3 nm diameter) the presence of which renders them O-inagglutinable (Rohde et al. 1975). Originally described as nonadhesive, they agglutinate tannic acid-treated red blood cells (Adegbola et al. 1983) and behave like type-3 fimbriae commonly present in Klebsielleae and Proteeae (Old and Adegbola 1985). Other kinds of thin fimbriae produced by strains of Enteritidis include 'SEF 14KDα' fimbriae which, unlike type-3 fimbriae, are nonhemagglutinating; thin aggregative fimbriae may also be produced by Enteritidis strains (see Clegg and Swenson 1994). About one-third of strains of subsp. *arizonae* and *diarizonae* and a few strains of subsp. *salamae* also produce thin fimbriae which, though they agglutinate tanned red blood cells, are probably not type-3 fimbriae (see Adegbola et al. 1983).

Strains of serotypes Salinatis and Sendai produce mannose-resistant and eluting hemagglutinins (MREHA) which, though common in *Escherichia coli* (Duguid et al. 1955), are rare in salmonellae. MREHA production by Salinatis and Sendai is associated with thin (3.6 nm diameter) and thick (13.6 nm diameter) fimbriae, respectively (Old et al. 1989; Yakubu et al. 1989).

Cultural characters and growth requirements

Salmonellae grow over a wide temperature range from 7 to 48°C, at pH 4–8 and at water activities above 0.93 (Baird-Parker 1991). Under special conditions, they may

proliferate at <4°C (d'Aoust 1991) and withstand extremes of pH <4 (Foster 1992). In aerobic and anaerobic conditions, salmonellae grow readily on ordinary media, producing colonies indistinguishable from those of other Enterobacteriaceae. Simple carbon compounds can be used as a source of carbon and energy and a wide range of nitrogenous compounds as nitrogen source on first isolation or after adaptation. Most salmonellae are prototrophic and grow in minimal salts medium with a suitable carbon source. Auxotrophic serotypes adapted to a single host species (see above) will grow on a minimal medium supplemented with appropriate growth factor(s). Auxotrophic strains of prototrophic serotypes are not uncommon, e.g. c. 7 percent of Typhimurium strains (Duguid et al. 1975).

Some serotypes of salmonellae, particularly Paratyphi B, produce mucoid colonies best developed at low temperature, low humidity, and high osmolarity (Anderson and Rogers 1963). The presence of a mucoid surface layer, the M antigen of Kauffmann, inhibits O and H agglutinability. Because the M antigen is identical in all salmonella serotypes and is like the colanic acid materials of other Enterobacteriaceae, it is unimportant diagnostically. The 'mucoid-wall test' is positive with most strains of Paratyphi B that cause enteric fever (Kauffmann 1966).

Susceptibility to chemical agents

Many observations on the resistance of salmonellae to different chemical substances have been derived from attempts to devise enrichment and selective media for the isolation of salmonellae from samples containing other coliform bacilli. The use of dyes, particularly those of the triphenylmethane group, as inhibitors has been well defined since the early 1900s when malachite green and later brilliant green were used. Brilliant green-containing media are useful for the isolation of most serotypes other than Typhi. RV medium, a modification by Vassiliadis et al. (1981) of the original Rappaport medium, in which the concentration of malachite green was reduced to 0.004 percent w/v, is considered superior to other enrichment media. Tetrathionate broth, especially the modification of Kauffmann–Müller containing brilliant green and 0.018 M tetrathionate, is useful for a wide range of serotypes including Dublin, but not for Typhi which is best recovered through selenite broth.

Many salmonellae multiply at 43°C and incubation of enrichment media such as selenite or RV broths at that temperature is recommended for the isolation of salmonellae. Solid media containing bile salts or pure sodium deoxycholate, often in the presence of other inhibitory substances, are generally used as selective plating media. Bismuth sulfite agar, often recommended for Typhi, is perhaps even more useful for the detection of lactose-fermenting salmonellae. Many other media based on the action of yet other inhibitory substances have been devised though few are in use today. For a discussion of the use and choice of these media and modifications of those described above, the authoritative review of Fricker (1987) is invaluable.

Biochemical activities

The common biochemical characters of the salmonellae are shown in Table 1. Although most strains conform closely to this pattern, there are several exceptions; no organism should, therefore, be excluded on the result of a single test. The usual reactions include:

- fermentation of glucose, maltose, mannitol, and sorbitol with the production of acid and gas
- absence of fermentation of sucrose, salicin, and adonitol

Table 1 *Biochemical differentiation of species and subspecies of* Salmonella

	Subspecies of *S. enterica*						
Reaction	***enterica***	***salamae***	***arizonae***	***diarizonae***	***houtenae***	***indica***	***S. bongori***
ONPG	–	–	+	+	–	d	+
Gelatinase	–	+	+	+	+	+	–
Galacturonate	–	+	–	+	+	+	+
Growth in KCN	–	–	–	–	+	–	+
Malonate	–	+	+	+	–	–	–
Dulcitol	+	+	–	–	–	d	+
Mucate	+	+	+	d	–	+	+
d-Tartrate	+	–	–	–	–	–	–
γ-Glutamyltransferase	+	+	–	+	+	+	+
β-Glucuronidase	d	d	–	+	–	d	–
Salicin	–	–	–	–	+	–	–
Sorbitol	+	+	+	+	+	–	+

+, >90 percent of strains positive; –, >90 percent of strains negative; d, some strains positive, others negative.

- failure to produce indole, to hydrolyze urea or to deaminate phenylalanine and
- a positive methyl red reaction and a negative Voges–Proskauer reaction.

It should be noted, however, that members of subsp. *houtenae* acidify salicin and that strains of subsp. *indica* do not acidify sorbitol (Table 1). Gas is not formed by Typhi, biotype Gallinarum or by occasional strains of other serotypes; maltose is usually not fermented by biotype Pullorum strains; and Typhisuis does not ferment mannitol. Occasional strains have been recorded that form acid from lactose, sucrose or raffinose, produce indole, hydrolyze malonate or split urea. Some of these atypical properties are plasmid mediated (Le Minor et al. 1974; Buissière et al. 1977). The positive reaction to the methyl red test, and the negative reactions to the phenylalanine and Voges–Proskauer tests appear to be constant. Other biochemical characters are variable, and some of them are the means by which *S. enterica* is subdivided into subspecies (Table 1). Within subsp. *enterica* there are several types that depart from the usual pattern; Table 2 shows the reactions of five of them, and other examples will be found in the description of individual types at the end of this chapter.

Most salmonellae grow with citrate as the sole carbon source though important exceptions include Typhi, Paratyphi A, Typhisuis and Sendai and biotype Pullorum strains of serotype Gallinarum. Most salmonellae also give a positive reaction for H_2S in triple sugar iron agar, but exceptions include strains of Paratyphi A, Choleraesuis (sensu stricto), Typhisuis, Sendai, Abortusequi, and Berta, as well as a few strains of Typhi. Liquefaction of gelatin, which may be slow or rapid, is characteristic of members from all subspecies other than *enterica* and *S. bongori* (Table 1). Nearly all salmonellae produce arginine dihydrolase and lysine and ornithine decarboxylases but not glutamic acid decarboxylase. Notable exceptions are Typhi and biotype Gallinarum strains which fail to decarboxylate ornithine, and Paratyphi A, which does not attack lysine. Fermentation of organic acids is common; most salmonellae of subsp. *enterica* rapidly attack *d*-tartrate, citrate, and mucate though there are exceptions. Several types, notably Paratyphi A, Sendai and most enteric fever-causing strains of Paratyphi B are *d*-tartrate negative.

Screening media for the simultaneous detection of several biochemical reactions are widely used in the routine identification of salmonellae. On triple sugar iron agar they show H_2S production and absence of fermentation of lactose, sucrose, and salicin; on lysine iron agar they show the production of H_2S and lysine decarboxylase. The use of screening media is time-saving when appropriately controlled. For further information about the biochemical reactions of salmonellae, see Ewing (1986).

Antigenic structure

The antigens used to define the serological types of salmonellae include:

- the O antigens, heat-stable polysaccharides that form part of the cell wall lipopolysaccharide (LPS)
- the H antigens, heat-labile proteins of the flagella that in salmonellae have the almost unique character of diphasic variation and
- surface polysaccharides that inhibit the agglutinability of the organisms by homologous O antisera, of which the Vi antigen of Typhi is the most important example.

The O polysaccharides have a core structure that is common to all enterobacteria and side chains of sugars attached to the core determine O specificity. Salmonellae may be classified into 'chemotypes' each of which contains organisms with the same sugars in their side chains. Chemotypes may include salmonellae with different O antigens and in many cases also *E. coli* strains of several O groups and other enterobacteria, but the organisms are serologically distinct because of differences in the sequence of sugars in the side chains, particularly at the distal end. Minor cross-reactions between salmonella O serogroups, and between the O antigens of salmonellae and other members of the Enterobacteriaceae, are quite common. The traditional method of detecting salmonella O antigens is by the agglutination of heated suspensions of organisms by rabbit antisera

Table 2 *Biochemical reactions of some* Salmonella *serotypes of subsp.* enterica *(subsp. I)*

Reaction[a]	Most serotypes	Typhi	Paratyphi A	Choleraesuis	Gallinarum[b]	Pullorum[b]
Gas from sugars	+	–	+	+	–	+
Utilization of citrate	+	–	–	d	+	–
H_2S	+	w	–	–	–	–
Lysine decarboxylase	+	+	–	+	+	+
Ornithine decarboxylase	+	–	+	+	–	+
Motility	+	+	+	+	–	–

–, Negative reaction; +, positive reaction; d, delayed positive reaction; w, weakly positive in triple sugar iron medium.
a) For aberrant reactions of other serotypes, see text.
b) Gallinarum and Pullorum are biotypes of serotype Gallinarum.

raised against boiled organisms; known cross-reactions are removed by suitable absorption of the sera.

Some of the H antigens of *Salmonella* are also found in *Citrobacter*; they comprise a large number of factors arranged in different combinations in the different serotypes. In most salmonellae the flagellar antigens exist in two alternative phases; to identify a serotype completely it is necessary to determine the antigenic factors present in both phases. For information about the practical identification of serotypes, the reader should consult reference publications (Kauffmann 1966; Ewing 1986; Popoff and Le Minor 1992). An up-to-date list of salmonella serotypes can be obtained from most national salmonella centers. Kelterborn (1967, 1987) gives a comprehensive account of the first isolation and geographical distribution of nearly 2 000 serotypes. Additions to the list of officially recognized serotypes are published annually (see Popoff et al. 1995).

ANTIGENIC NOTATION

The antigenic formula consists of three parts, describing the somatic O antigen, the phase-1 H antigen and the phase-2 H antigen, in this order. The three parts are separated by colons, and the components of each part by commas. The somatic O antigens are given Arabic numerals. The phase-1 H antigens are designated a to z, a series that is complete except for j. H1 antigens described after z are designated as z with subscripts, e.g. z_1 to z_{83}. The phase-2 H antigens are designated by Arabic numerals (1–12), but phase 2 may also contain antigenic components of the e series or one of the z series. Some H antigens are phase-specific, others appear in both phases.

Certain somatic factors are present in some members only of a particular serotype. For example, the O factor 5 is absent from many strains of Paratyphi B; this is indicated by placing the factor within a square bracket in the antigenic formula (see Table 4, p. 18). Some somatic factors are underlined; this is because they are determined by the presence of a phage (see below), e.g. O factor 1 in Typhimurium and the factors 15 and 15,34 in Anatum (Table 3).

The Kauffmann–White diagnostic scheme

Primary subdivision is into O serogroups, each of which shares a common somatic antigen. Where more than one O antigen is present, one of them is the major O antigen and is regarded as determining the group to which the strain shall be allocated. O serogroups were formerly designated by letters of the alphabet and some groups were subdivided into subgroups. In this chapter, however, each O serogroup is designated by its characteristic O factor(s) with the letters formerly used to designate the O serogroups shown in parentheses (see Table 4). The series of O antigens numbered O1–67 is not continuous because some O antigens were originally assigned to bacteria that proved subsequently not to be salmonellae. Again, some of the 67 O antigens are minor antigens (e.g. O12), which have no discriminatory value; others (e.g. O5) arise by chemical modification of a known major O antigen or are determined by phage conversion. Thus, only 46 O serogroups are defined by the 67 O antigens described (see Lindberg and Le Minor 1984; Popoff and Le Minor 1992).

The serotypes classified in the scheme include members of all subspecies and of *S. bongori*. An antigenic scheme was developed independently from the Kauffmann–White scheme for the characterization of salmonellae of subspp. *arizonae* and *diarizonae* when they were considered to belong to a separate genus ('*Arizona*') distinct from *Salmonella*. It is convenient and sensible to designate the serotypes of all subspecies of *Salmonella* by one set of symbols. Hence, the '*Arizona*' formulae have been deleted from the scheme (Popoff and Le Minor 1992). In the Kauffmann–White scheme, only antigens of diagnostic value are cited. For most purposes a simplified version of the scheme, based on only 12 O, 18 H sera, and the Vi serum, gives sufficient information for routine diagnostic identifications (see Ewing 1986).

The distribution of 2 399 known salmonella serotypes among the different subspecies is: *enterica* (1 416), *salamae* (477), *arizonae* (94), *diarizonae* (317), *houtenae* (66), *indica* (10), and *S. bongori* (19) (Popoff et al. 1995). The majority (96.5 percent) of the serotypes of subsp. *enterica* belong to 6 O serogroups (O4–O1,3,19) and the rest are scattered throughout the remaining serogroups. Most of the serotypes from subspecies other than *enterica* belong to higher O serogroups O11–67, some of which also contain serotypes of *enterica*. Serotypes in subspp. *arizonae* and *houtenae* and *S. bongori* are monophasic.

VARIATION IN THE O ANTIGENS

Rough mutant strains can be classified in a series from Ra, which lacks the O-specific side chains only, through Rb to Re which show a progressive loss of sugar constituents from the core. Agglutinability in the presence of 0.3 percent w/v auramine and heat-stability tests are considered particularly suitable for the differentiation of smooth from rough forms. Although the different rough forms behave similarly in these tests, rough strains of chemotypes Ra–e show steady gradation in their sensitivity to antibiotics, detergents, and dyes. Rough variation occurs rarely in nature but is common in strains maintained through many generations on ordinary laboratory media.

Lysogenic conversion

The specificities of O antigens may be modified in the process of lysogenic conversion by phages, which genetically determine subtle changes in the chemistry of the

Table 3 *Illustration of the Kauffmann–White scheme of serological classification*

O serogroup[a]	Serotype[b]	O antigens[c]	H antigens	
			Phase 1	Phase 2
2 (A)	Paratyphi A	1,2,12	A	[1,5]
4 (B)	Paratyphi B	1,4,[5],12	B	1,2
	Stanley	1,4,[5],12,27	D	1,2
	Schwarzengrund	1,4,12,27	D	1,7
	Saintpaul	1,4,[5],12	e,h	1,2
	Derby	1,4,[5],12	f,g	[1,2]
	Agona	1,4,12	f,g,s	[1,2]
	Typhimurium	1,4,[5],12	I	1,2
	Bredeney	1,4,12,27	l,v	1,7
	Brandenburg	1,4,[5],12,27	l,v	e,n,z_{15}
	Heidelberg	1,4,[5],12	R	1,2
7 (C_1)	Choleraesuis	6,7	C	1,5
	Paratyphi C	6,7[Vi]	C	1,5
	Livingstone	6,7,14	D	l,w
	Montevideo	6,7,14	g,m,[p],s	[1,2,7]
	Thompson	6,7,14	K	1,5
	Virchow	6,7	R	1,2
	Infantis	6,7,14	R	1,5
	Mbandaka	6,7,14	z_{10}	e,n,z_{15}
8 (C_2–C_3)	Muenchen	6,8	D	1,2
	Newport	6,8,20	e,h	1,2
	Hadar	6,8	z_{10}	e,n,x
9 (D_1)	Typhi	9,12[Vi]	D	-
	Enteritidis	1,9,12	g,m	[1,7]
	Dublin	1,9,12[Vi]	g,p	-
	Panama	1,9,12	l,v	1,5
	Gallinarum	1,9,12	-	-
3, 10 (E_1)	Anatum	3,10,[15],[15,34]	e,h	1,6
1, 3, 19 (E_4)	Senftenberg	1,3,19	g,[s],t	-
11 (F)	Rubislaw	11	R	e,n,x
13 (G)	Kedougou	1,13,23	I	l,w
6, 14 (H)	VI	1,6,14,25	A	e,n,x
16 (I)	II	16	g,[m],[s],t	[e,n,x]
18 (K)	IIIa	18	z_4,z_{32}	-
21 (L)	IIIb	21	I	1,5,7
43 (U)	IV	43	z_4,z_{23}	-
48 (Y)	V	48	z_{35}	-
60–	IIIb	60	R	e,n,x,z_{15}
61–	IIIb	61	K	1,5,[7]

a) Some serogroups were formerly designated by letters indicated in parentheses.
b) Serotypes of *S. enterica* subsp. *enterica* are named; others are designated by antigenic formula only in other subspecies of *S. enterica* (II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae*; and VI, *indica*) and in *S. bongori* (V).
c) Somatic factors associated with phage conversion are underlined, e.g. 14.['x'] Antigen 'x' not present in all strains of serotype.

repeating unit of the polysaccharide (see Lindberg and Le Minor 1984). The new specificities appear in salmonellae within minutes of their infection with phage. The presence of factor O1 in serotypes of groups O2, 4, and 9 is associated with lysogenization by phages such as P22 whereas other phages determine O1 in higher serogroups such as O13, 40, 42, and 51; not all O1 factors, however, e.g. that in group O1,3,19 (Table 3), are phage determined.

The sequential conversions by phages ϵ15 and ϵ34 of serotypes of serogroup O3,10 (formerly E_1) resulted in the appearance of new specificities designated, respectively, 3,15 and 3,15,34. On the basis of what were minor changes in the structure of the repeating unit of the antigen O3,10, special serogroups (formerly E_2 and E_3) were established and the sequentially lysogenized variants of serotypes of serogroup E_1 were given new serotype names. Thus, when Anatum (3,10:e,h:1.6) was

lysogenized with phage ϵ15 it became Newington (3, 15:e,h:1,6), which in turn, when lysogenized by ϵ34 became Minneapolis (3, 15, 34:e,h:1,6). The serotype Anatum is now designated as 3,10[15],[15, 34]:e,h:1,6 and its variants are not named. Although serotype names for the lysogenized variants of extant serotypes have been deleted from the current list of *Salmonella* serotypes (Popoff and Le Minor 1992), not all *Salmonella* reference laboratories have adopted that protocol. Phage conversions responsible for many other O antigen modifications are of little or no diagnostic significance.

Plasmid conversion

The first observation of changes in the specificities of O antigens of *Salmonella* resulting from plasmid conversion was made with regard to O54. The serotype Tonev (21,54:b:e,n,x) contains a small plasmid, loss of which results in failure to express factor O54; thus, the plasmidless variant becomes (21:b:e,n,x), i.e. Minnesota of group O21 (Popoff and Le Minor 1985). Although strains of the other 12 serotypes of serogroup O54 contain plasmids similar to that of Tonev, plasmidless variants not expressing O54 could be derived from only eight of them; the latter O54$^-$ variants were indistinguishable from serotypes already classified in other O serogroups. Some serotypes of serogroup O54, therefore, should be deleted from the scheme; thus, if Minnesota could be redefined as 21, 54:b:e,n,x in a style analogous to that used for lysogenized variants, the serotype Tonev could be suppressed. But some problems remain. With the loss of expression of O54 by Winnipeg (54:e,h:1,5), a new factor (O8) not normally expressed by that serotype appears and it is thereby converted to Ferruch (8:e,h:1,5). Neither of these serotypes, however, produces the other's O-determining antigen, i.e. strains of serotype (8,54:e,h:1,5) have not been described in nature. Thus, Winnipeg should be retained as well as the names of four other serotypes from which O54$^-$ variants have not yet been derived. The heterogeneous group O54 has been retained provisionally in the current scheme (Popoff and Le Minor 1992).

Form variants may show complete or partial loss of O antigen, e.g. somatic antigen O5 is not present in all cultures of Paratyphi B or Typhimurium and, hence, is designated as [5] (Table 3).

VARIATION IN THE H ANTIGENS

Most serotypes of *Salmonella* (of subspecies other than *arizonae* and *houtenae* and *S. bongori*) show the phenomenon of diphasic variation, i.e. they express alternately two kinds of flagella with different antigenic specificities. Typhimurium, for example, produces flagella of antigenic specificity i in phase 1 and of specificity 1,2 in phase 2. Salmonellae switch phases at characteristic frequencies and the transition can be induced in the laboratory by their cultivation in semi-solid agar containing antiserum against phase-1 or phase-2 flagellar antigens. Thus, growth of phase-2$^+$ Typhimurium in the presence of H-antiserum 1,2 induces transition to phase 1 and selects bacteria expressing flagella of i specificity.

The major components of the flagellar filaments are highly antigenic proteins called flagellins which are encoded by twi independent, chromosomally located genes (*fliC*) (phase 1) and *fljB* (phase 2). Salmonellae in phase 1 express flagellin of the *fliC* gene and in phase 2 express flagellin of the *fljB* gene along with an associated repressor of the phase-1 gene (coded by the *fljA* gene). The regulation of the phase switching is complex but depends on a recombinational event involving a specific invertible sequence of DNA (970 bp long) that contains the promoter region of the phase-2 operon. With this invertible sequence in one orientation, the promoter is correctly positioned to allow reading of the *fljB* gene along with the repressor (from *fljA*) that switches off the phase-1 gene. In its other orientation, the phase-2 gene cannot be read, the repressor of phase-1 gene is not formed and so flagella with phase-1 antigenicity are formed. The invertible region also determines the frequency of inversion and, hence, of phase variation and functions essentially as a 'flip-flop' switch; it is related, therefore, to similar sequences involved in other phase-variable systems (see Silverman and Simon 1980).

Some serotypes of *Salmonella* are usually monophasic, expressing in nature flagella of only one phase, e.g. Paratyphi A (1,2,12:a: -). The growth of Paratyphi A in the presence of H antiserum induces flagella with antigens 1,5 characteristic of phase 2. Thus, in the Kauffmann–White scheme Paratyphi A is written 1,2,12:a:[1,5] (Table 4). On the other hand, when Abortusequi (4,12: - :e,n,x), a natural serotype monophasic in phase 2, is cultivated in the presence of H antiserum, e,n,x, variants with phase-1 flagella of specificity a are selected that are monophasic in phase 1, and as stable as the naturally occurring phase-2 monophasic strain; it requires culture in H antiserum a to induce reversion to the common phase-2 only type. Natural serotypes fixed in one H phase are known to be associated with mutations linked to regulatory genes.

Other serotypes, such as Typhi (9,12:d: -), appear to be monophasic and most populations encode flagellin of phase 1 with d antigenic specificity. Culture of Typhi in the presence of H antiserum d selects variants with a different phase-1 specificity of the j type. These uncommon phase-1 (R-phase) variants occasionally arise in nature, e.g. some Typhi populations from Indonesia form a variant phase-1 j allele which arises as a result of a deletion of a portion of 261 bp from the central region of the phase-1 flagellin gene (Frankel et al. 1989). An interesting example of R-phase H antigens arising in nature (Guinée et al. 1981) comes from a series of isolates in Indonesia in 1979–80 from patients who had authentic typhoid fever, were biochemically typical of

Typhi, but were unreactive in the H antisera d and j (R) previously associated with Typhi. These isolates possessed a new flagellar antigen z_{66}, thought to be encoded by a phase-2 locus (Selander et al. 1990b), and when they were grown in the presence of H antiserum z_{66}, gave rise to variants with the d or j specificities usually associated with the phase-1 flagella of Typhi. This is important diagnostically because that strain of Typhi, which is restricted to Indonesia, was Vi unreactive and gave negative reactions in the Widal test with standard d suspensions.

New flagellar antigens

Comparison of the nucleotide sequences of the phase-1 flagellin locus (*fliC*) of strains of five serotypes – Paratyphi A (specificity a), Choleraesuis (c), Muenchen (d), Typhimurium (i), and Rubislaw (r) – showed that the terminal parts of the genes are highly conserved (Wei and Joys 1985, 1986) probably because of their importance in polymerization and secretion of the flagellin proteins. The central region of the gene is highly variable with marked differences in length and sequence and yet without impairment of flagellar motility; the central region of the gene, which determines the major antigenic epitopes of the flagellin exposed in the flagellar surface, is subject to little or no functional constraint. Do new flagellar antigens arise, therefore, by random mutational drift in these flagellin genes? If so, then variations in their nucleotide sequences might be expected even among strains within a serotype.

When the central regions of the genes coding for phase-1 flagellin of i specificity were sequenced for six strains of Typhimurium of different genetic backgrounds (i.e. of different electrophoretic types (ET) as judged by MLEE), they were invariant (Smith and Selander 1990). Similarly, the sequences of the central parts of the phase-1 flagellin of strains of serotype Heidelberg with antigenic specificity of the r type were also invariant (Smith et al. 1990). Thus, the *fliC* genes are not evolving by sequence drift at unusually high rates.

The serotypes Heidelberg (1,4,[5]12:r:1,2) and Typhimurium (1,4,[5]12:i:1,2), which are indistinguishable other than by their phase-1 flagellins, are genetically close (genetic distance 0.2, as determined by MLEE), yet the *fliC* alleles (r and i) show considerable divergence with a 19 percent nucleotide sequence difference (Selander and Smith 1990). Likewise, strains of Typhimurium and Muenchen (which have a genetic distance of only 0.22 despite belonging to different O serogroups) showed a 50 percent difference in the nucleotide sequence in the *fliC* genes coding for phase-1 flagellins of antigenic types i and d.

By contrast, the sequences of the *fliC* genes coding for phase-1 flagella of antigenic specificity i in serotypes from different subspecies (Typhimurium in *enterica* and an $O48^+$ serotype in *arizonae*) that are distantly related (genetic distance of 0.72) showed, at the chromosomal level, a small difference of 2.2 percent in their phase-1 (i) alleles; likewise strains of serotypes Muenchen and Typhi (with a genetic distance of 0.47) had phase-1 (d) alleles that differed in nucleotide sequence by only 1.3 percent (Smith et al. 1990). The existence of flagellin genes that are almost identical in serotypes that are genetically unrelated is best explained by the horizontal transfer of these genes; their recombination after transfer provides an explanation of new flagellar antigen combinations. Phage mediated transfer of genetic material, demonstrated for salmonellae by Zinder and Lederberg (1952) seems the most likely method.

Triphasic strains

Occasional strains of *Salmonella* are found in nature that express three or more antigenically distinct kinds of flagella (Ewing 1986). It has long been known that when triphasic serotypes, such as Montgomery or Salinatis (now deleted from the Kauffmann–White scheme), were grown in H antiserum d, normal diphasic variants (11:a:e,n,z_{15}, and 1,4,12, 27:e,h:e,n,z_{15}, respectively) were obtained from which the third flagellar antigen type d was irreversibly lost (Edwards et al. 1957). Genetic analysis has shown that a triphasic variant of serotype Rubislaw has, in addition to the phase-1 (*fliC*) and phase-2 (*fljB*) genes (r and e,n,x, respectively) of Rubislaw, a third flagellin gene (*flpA*) carried on a large plasmid (Smith and Selander 1991). The gene *flpA* contained a central region coding for a phase-1 antigen (d) coupled with a phase-2 promoter. In phase 2, the triphasic variant expressed its real phase-2 flagellin (e,n,x) and one (d) normally associated with phase-1 flagella. Growth of the triphasic isolate in the presence of H antiserum d gave a diphasic variant indistinguishable from Rubislaw expressing the normal phase-1 and 2 flagellar antigens (r:e,n,x) of that serotype; the *flpA* gene had been completely deleted from the plasmid (Smith and Selander 1991). When the deletion of the *flpA* gene from the plasmid was incomplete, an antigenic variant (j) of the d allele was formed in variants that were still triphasic. The existence of similar large plasmids carrying phase-1 flagellin (d) genes in distantly related triphasic serotypes (including Salinatis) in different *Salmonella* subspecies is strong evidence that horizontal transfer of flagellar genes has occurred. With their subsequent recombination new flagellar antigens are formed (Selander and Smith 1990; Smith and Selander 1991).

Nonmotile strains of salmonellae may occasionally be found with flagella that are antigenically and morphologically like those of the motile parent strains. The failure of Mot-strains to rotate the flagella is thought to be associated with defects in the mechanism of energy transduction (MacNab 1987). Flagellate salmonellae may

give rise to non-flagellate H^- strains to which it is difficult to assign serotype names.

Strains of Gallinarum-Pullorum (1,9,12: - : -) are permanently nonflagellate salmonellae from which spontaneous reversions to motility have not been reported. However, in transduction experiments they have been shown to carry factors determining phase-1 flagellar antigens of the g,m type, like serotype Enteritidis (1,9,12:g,m: -). Sequencing studies have shown that the phase-1 (*fliC*) flagellin gene in most strains of Gallinarum is complete and identical to that of Enteritidis (g,m), and that in strains from three other Gallinarum ETs (Gal,2,2a) there was a stop codon (position 495) towards the distal part of the gene (Li et al. 1993). On the other hand, the sequence of the *fliC* gene of strains of Pullorum from different ETs differed from that of Enteritidis by having 2–3 nonsynonymous substitutions in the central part of the gene and a synonymous substitution at its end; thus, if ever expressed, the flagellar specificities of Pullorum might well be other than g,m (Li et al. 1993). Similarly, the few strains of Gallinarum-Pullorum analyzed seem to have complete genes encoding the flagellar-hook protein (*flgK*) that are similar to that of Enteritidis. Thus, the genetic basis of the nonmotility of these strains is as yet unclear but it has been speculated that it is due to a mutation in a gene (e.g. *fliA*) of the flagellar regulon (Li et al. 1993).

Vi antigen

Vi is a capsular polysaccharide of α-(1→4)-linked *N*-acetyl-D-galactosaminouronic acid, variably acetylated at C2/C3 positions (Daniels et al. 1989); it prevents immune serum mediated killing, is antiphagocytic (by inhibiting binding of C3b and antibodies to LPS), and increases resistance to peroxide. The rate of synthesis and cell-association properties of Vi are determined by structural (*viaB*) and functional (*viaA*) elements at distinct chromosomal sites (Makela and Stocker 1969).

Felix and Pitt (1934) demonstrated that Typhi strains cultured from the blood of patients with typhoid fever were inagglutinable in O9 serum, showed that the virulence of O-inagglutinable strains of Typhi was greater for mice than O-agglutinable strains and proposed the name Vi (virulence) antigen for the implicated surface structure. The virulence and protective roles of Vi were established by the following observations:

- Vi^+ strains of Typhi (and Paratyphi C) have a lower LD50 for mice than Vi^- strains
- active immunization with Vi from Typhi, or other serotypes, protects mice against subsequent challenge with Typhi (but not Paratyphi C)
- a higher incidence of disease, including bacteremia and fever, is seen in volunteers challenged with Vi^+ Typhi strains than with Vi^- strains
- isolates of Typhi and Paratyphi C from the blood of patients with typhoid or enteric fever are invariably Vi^+ and
- Vi vaccine prepared under nondegrading conditions gives excellent protection in controlled trials in areas with high attack rates of typhoid fever (see Felix and Pitt 1934; Robbins and Robbins 1984; Tacket et al. 1986; Klugman et al. 1987; Daniels et al. 1989).

Vi is produced by strains of three salmonella serotypes – Typhi, Paratyphi C, and Dublin – that are genetically distant. The presence of *via* genes in all but a few (c. 1 percent) strains of Typhi and Paratyphi C suggests that Vi is a property long established in these serotypes; by contrast, production of Vi by Dublin strains is restricted to Du1, a clone of limited geographical distribution, that may have acquired it recently by horizontal transfer of *via* genes (Selander et al. 1990b). Strains rich in Vi form opaque colonies but the amount of Vi present in different strains and serotypes varies considerably; thus, continued laboratory culture of Vi^+ strains may lead to loss of Vi production. Again, isolates of Paratyphi C, 50 percent of which are phenotypically Vi^-, produce less Vi per cell than Typhi, release it more rapidly into the medium and show a higher frequency of reversion from Vi^+ to Vi^- than Typhi (Daniels et al. 1989). Rare strains of *Citrobacter freundii* produce large amounts of cell-associated Vi identical to that of Typhi and Paratyphi C (Daniels et al. 1989) and so are ideal sources of Vi antigen and useful for the preparation of Vi antiserum (Ewing 1986). An invertible insertion sequence located within *viaB* is responsible for variable expression of Vi by *C. freundii* strains (Snellings et al. 1981).

In the absence of positive cultures from patients with typhoid fever, other methods are needed to confirm the diagnosis. The detection of serum antibodies to LPS, flagella, and Vi antigens forms the basis of the long-established Widal test. A rapid immunoblotting procedure with Enteritidis LPS and Muenchen flagella has been developed that provides same-day diagnostic results (Chart et al. 1995). The performance of this and other established tests for Vi has been reviewed (Chart 1995). Specific DNA probes to detect the Vi genes have also been developed (Rubin et al. 1989) and a nested polymerase chain reaction (PCR)-based technique specific for the *viaB* gene of the Vi pathogenicity island has also been developed (Hashimoto et al. 1995). However, this technique has never been applied in the clinical setting and in any event would be only of limited use in detecting infections caused by Vi-negative strains. More recently, a second nested PCR based on the *fliC* gene has been evaluated (Kumar et al. 2002). Although this PCR test has shown an increased sensitivity in identifying the presence of *S.* Typhi in previously negative blood cultures, its applicability in the field setting needs to be evaluated.

FIMBRIAL ANTIGENS

Only one of the five type-1 fimbrial antigens described in *Salmonella* is common to the type-1 fimbriae of all *Salmonella* serotypes (Duguid and Campbell 1969; Duguid 1985); that common antigen is also present on the type-1 fimbriae of *C. freundii* but not on those of other Enterobacteriaceae (Duguid 1985; Adegbola and Old 1987). Type-1 fimbrial antibodies, which may be produced by a majority of typhoid patients and vaccinees (Brodie 1977), are responsible for many of the false positive reactions obtained in Widal tests (Duguid 1985).

Type-3 fimbriae are commonly formed by many Enterobacteriaceae but not by *E. coli* and by only a few serotypes of *Salmonella* (Adegbola and Old 1983; Old and Adegbola 1985; Clegg and Swenson 1994); the type-3 fimbriae of *Salmonella*, *Enterobacter*, *Klebsiella*, and *Yersinia* belong to a single antigenic group, distinct from those of other Enterobacteriaceae (Old and Adegbola 1985). Type-3 fimbriae, when present, render salmonellae O-inagglutinable but may be removed by heating at 100°C for 2.5 h (Rohde et al. 1975).

The ability of many of the novel types of fimbriae described in *Salmonella* (see Clegg and Swenson 1994) to mask other antigens and to give false positive results in serotyping tests is unknown. Nevertheless, consideration of the fimbriation status of salmonellae is important and much of the early work in this area needs reassessment.

Molecular aspects

Both the O-antigen and H antigen gene clusters of *S. enterica* have been subjected to extensive molecular characterization over the last decade (Jiang et al. 1991; Liu et al. 1991; Curd et al. 1998; Wang et al. 2002). These differences have been exploited for the development of a serogroup-specific PCR assay for serogroup O:6, 14 (Fitzgerald et al. 2003), and it is understood that further PCR assays for other serogroups are under development (Fields et al. 2002).

POPULATION STRUCTURE AND TYPING

Population structure

Phenotypic methods such as serotyping, phage typing, biotyping, and antibiogram typing (R-typing) are convenient for the primary discrimination of *Salmonella* strains and have yielded useful markers for highlighting epidemic strains in epidemiological studies. They do not, however, provide a ready means for the genetic analysis of population structure in *Salmonella*.

The work of Selander and colleagues, who pioneered the study of clonal relationships and evolutionary structure of many species of medically important bacteria, used MLEE analysis to estimate the genetic distances among *Salmonella* serotypes (Selander et al. 1986; Selander and Musser 1990). Their studies confirmed that the population structure of *Salmonella* is clonal and that many common serotypes, e.g. Typhimurium, are monophyletic, i.e. represented by one, or a few, genetically similar clones probably derived from a common ancestor, that are distributed world wide and are resident on the same branch of the salmonella phylogenetic tree. That finding explains why serotyping is so useful in the epidemiological analysis of monophyletic serotypes. Other (c. 25 percent) serotypes are polyphyletic and comprise clones that are genetically distant and found on different branches of the phylogenetic tree; clones of these polyphyletic serotypes, e.g. Derby, often show distinct geographical distributions and/or host associations.

Other aspects of population structure of *Salmonella* that emerged from these genetic-based MLEE studies include the following:

- strains of the same serotype, e.g. Paratyphi B, may belong to genetically distant groups
- strains of different serotypes, e.g. Heidelberg and Typhimurium, may be genetically indistinguishable
- salmonellae causing typhoid and enteric fevers in humans are phylogenetically distant, implying that convergent evolution of the properties of host adaptation and invasiveness occurred recently in these human-adapted serotypes and
- the lineages of serotypes adapted to avians and other animals may be close.

Furthermore, used in conjunction with comparative nucleotide sequencing, particularly of the *fliC* gene, MLEE has shown that the H1 flagellins of genetically similar serotypes may be extensively divergent, whereas serotypes from diverse chromosomal backgrounds, even of different subspecies, may have closely similar nucleotide sequences for the highly polymorphic central region of the H1 gene. These observations suggest that recombinational events and horizontal gene exchange of both O and H genes among different lineages of *Salmonella* are likely to have been important mechanisms for the generation of new serotypes.

Multi locus sequence typing (MLST), which is targeted at comparing sequence diversity at multiple, conserved housekeeping genes (Maiden et al. 1998), has recently been applied to seven housekeeping genes in a collection of 26 isolates of *S.* Typhi collected from diverse geographical locations (Kidgell 2003). The results confirmed earlier findings from MLEE typing (see above) and conclusively demonstrated the existence of four sequence types of *S.* Typhi from distinct geographical areas as well as providing an estimate of the age of the organism (ca. 50 000 years old) (Kidgell et al. 2002). The study also demonstrated that MLST may form the basis for a molecular typing scheme for *S.* Typhi.

Serotyping

BACTERIOPHAGE TYPING METHODS

The underlying principle of phage typing is the host specificity of bacteriophages and on this basis several phage-typing schemes have been developed for serotypes of clinical or epidemiological importance.

Typhi

The first phage typing scheme based on the principle of phage adaptation was that developed for the differentiation of Typhi (Craigie and Yen 1938); in this scheme progressive adaptations were made of Vi phage II, which is specific for Vi (capsular) antigen of Typhi (Felix and Pitt 1934), is highly adaptable and shows a high degree of specificity for the last strain on which it has been propagated. The extraordinary adaptation of Vi phage II is due in part to the selection of spontaneously occurring host-range phage mutants by the bacterium and in part to a nonmutational phenotypic modification of phage by the host strain. The mechanisms underlying the Vi type specificity of Typhi have been discussed fully elsewhere (Anderson and Williams 1956).

The method of Vi phage typing was standardized in 1947 (Craigie and Felix 1947) and with further adaptations of Vi phage II, a further 95 types were defined and internationally recognized bringing the total number of Vi phage types to 106 (Edelman and Levine 1986). Vi phage typing is now the internationally accepted method for differentiation of Typhi and the scheme is used in specialized WHO-approved reference centers world wide. The types most widespread and abundant throughout the world are E1 and A, followed by B2, C1, D1, and F1; other less common types are found that are prevalent temporarily or permanently in some countries only, or even within certain geographical areas within a country (Jegathesan 1983). The value of the Vi phage typing method was demonstrated, for example, by investigations of the sudden appearance in the UK in 1990 of multiresistant strains belonging to Vi phage type M1 and associated with patients recently returned from Pakistan (Rowe et al. 1990). Subsequently, multiresistant strains of Vi phage type E1 have been associated with patients returning from India (Rowe et al. 1995), where strains of type E1 have caused numerous outbreaks since 1990 (Prakash and Pillai 1992). Again Vi phage typing has linked outbreaks in several countries in the Arabian Gulf to immigrant workers from the Indian subcontinent (Wallace et al. 1993; Mirza et al. 1996).

A serious limitation of the usefulness of Vi phage typing is that one type, usually A or E1, may be so common in a country as to limit the usefulness of epidemiological information. Several attempts have been made to overcome this problem; members of common phage types have been further discriminated by biochemical tests, by the use of a battery of unadapted O phages (Nicolle et al. 1954) and, more recently, by various molecular methods, e.g. insertion sequence (IS) *200* for Vi phage types E1 and M1 (Threlfall et al. 1993c) and pulsed field gel electrophoresis (PFGE) (Thong et al. 1994; Connerton et al. 2000).

Other serotypes

In contrast to the phage typing scheme for Typhi, which is based primarily on adaptations of bacteriophage Vi II, phage typing schemes for other serotypes depend to a limited extent on phage adaptability and, for the most part, are based on patterns of lysis produced by serologically distinct phages isolated from a variety of sources. In addition to Typhi, other serotypes for which published phage typing schemes are in routine use in the UK and include: Paratyphi B (Felix and Callow 1943, 1951), Typhimurium (Callow 1959; Anderson 1964), Hadar (de Sa et al. 1980), Enteritidis (Ward et al. 1987) and Virchow (Chambers et al. 1987). More than 50 phage types are now recognized in the Enteritidis scheme, the value of which was realized on an international scale following the global pandemic of Enteritidis from the late 1980s to the early 1990s. For the Typhimurium scheme, 232 phage types were designated (Anderson et al. 1977b) and a further 40 types have been recognized subsequently. Supplementary schemes have been developed in several countries to discriminate Typhimurium strains untypable by conventional typing schemes. Again, phage typing schemes have been developed, as required, for several other serotypes which became predominant in particular areas, e.g. a phage typing scheme for Agona was developed in the UK in the 1980s when isolations of Agona were at a high level (Ward L.R., unpublished), but the scheme was not put into effect until 1995 following an international outbreak associated with a contaminated kosher snack imported into the UK and the USA from Israel (Anonymous 1995). Phage typing schemes for Enteritidis and Typhimurium used in different countries of the EU have now been rationalized as part of an international collaborative venture for salmonella surveillance (Fisher et al. 1994; Fisher 1999). More recently, attempts have been made to develop DNA-based phage typing for Typhimurium, utilizing 84 phage-type specific markers revealed by fluorescent amplified fragment length polymorphism fingerprinting (FAFLP) of 46 Typhimurium isolates of Australian origin (Hu et al. 2002). It is possible that this method may be used for the development of a microarray-based molecular phage-typing scheme for this and other serotypes.

It is important to realize that phage types cannot always be regarded as indicative of clonality because phage type conversions may result from the acquisition of both plasmids (Anderson and Lewis 1965; Anderson

et al. 1973; Threlfall et al. 1978; Barker 1986; Frost et al. 1989) and bacteriophages (Threlfall et al. 1980; Rankin and Platt 1995).

Biotyping

Subdividing common salmonella serotypes according to their biochemical characters is sometimes of value in epidemiological investigations. In many serotypes there are few biochemical tests in which significant numbers of strains behave differently and so the number of identifiable biotypes is small. But, when available, biotyping is a useful adjunct to phage typing for it can subdivide a large group of untypable strains or members of common phage types (as in Typhi).

Its usefulness has been greatest in studying the epidemiology of infections with Typhimurium for which Duguid et al. (1975) developed a biotyping scheme based on the use of 15 biochemical characters. Thirty-two potential primary biotypes were defined by the possible combinations of positive and negative reactions in the five tests most discriminating for this serotype: D-xylose, *m*-inositol, L-rhamnose, *d*- and *m*-tartrates; primary biotypes were designated by numbers (1–32) and full biotypes by appending to these numbers letters which indicated results in ten secondary tests. To date, representatives of 24 primary and 184 full biotypes have been identified. In different studies the system has afforded a high degree of discrimination and the biotype characters have proved remarkably stable (Duguid et al. 1975; Anderson et al. 1978; Barker and Old 1979, 1980, 1989; Barker et al. 1980; Old and Barker 1989; Wray et al. 1987).

When biotyping was used in conjunction with phage typing, each system complemented the other and combined data allowed characterization of 574 different 'phage biotypes' among >2 000 strains of Typhimurium (Anderson et al. 1978). Strains of several phage types could be subdivided by biotype; thus, strains of phage type 141 included representatives of three distinct, yet stable, biotypes 1f, 9f, and 31bd (Barker and Old 1979), probably reflecting the acquisition of the same phage type-determining character by different biotype lines of the serotype. Furthermore, strains of several biotypes were found that had diversified in phage type by acquisition of different phage type-determining characters. Combined phage type-biotype studies help:

- to determine with greater confidence the fine relationships among strains
- to characterize variants that arise from a strain in the course of its epidemic spread and
- to indicate likely phage type interconversions (Anderson et al. 1978; Barker et al. 1980; Barker 1986).

Modified versions of the Typhimurium biotyping scheme have been successfully applied to the epidemiology of other salmonella serotypes: Agona (Barker et al. 1982), Livingstone (Old et al. 1994; Crichton et al. 1996), Montevideo (Reilly et al. 1985) and Paratyphi B (Barker et al. 1988). Regrettably, despite its undoubted discriminatory ability, biotyping is now rarely used and in many laboratories has been superseded by a range of DNA-based molecular methods.

Molecular typing methods

A range of molecular typing methods based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA has now been developed either to supplement the more traditional phenotypic methods of typing (serotyping, phage typing, biotyping) or, in some cases, as methods of discrimination in their own right. Molecular typing methods based on the characterization of plasmid DNA and which have been used for the differentiation of *Salmonella* include plasmid profile typing, plasmid fingerprinting and the identification of plasmid mediated virulence genes. By contrast, more recent methods have sought to identify small regions of heterogeneity within the bacterial chromosome. Five chromosomally based methods have been used for *Salmonella*: ribotyping; random cloned chromosomal sequence (RCCS) typing; insertion sequence (IS) *200* typing; PFGE; and PCR-based methods such as random amplified polymorphic DNA typing (RAPD), enterobacterial repetitive intergenic consensus typing (ERIC-PCR), repetitive extragenic palindromic element typing (REP-PCR), amplified fragment length polymorphism fingerprinting (AFLP), and variable number of tandem repeats fingerprinting (VNTR). The first three methods use DNA–DNA hybridization to identify restriction fragment length polymorphisms (RFLP) following detection of single or multicopy gene sequences across the bacterial genome; the fourth method (PFGE) is a modification of conventional agarose gel electrophoresis (AGE) that permits analysis of the whole bacterial genome on a single gel. The PCR-based methods are based on amplification of specific DNA sequences by PCR to produce characteristic groups of fragments dependent upon the origin of the template DNA. A detailed review describing these methods and their applicability for salmonella epidemiology is available (Threlfall et al. 1994a).

The methods used most extensively in support of epidemiological investigations of *Salmonella* are plasmid typing, ribotyping, RCCS typing, IS*200* fingerprinting and, more recently, PFGE and AFLP fingerprinting.

PLASMID TYPING

Many wild-type strains of salmonellae carry plasmids differing in both molecular mass and number. Plasmid typing, based on the numbers and molecular weight of plasmids after extraction of partially purified plasmid

DNA and AGE, has been used for differentiation within serotypes, e.g. Muenchen (Taylor et al. 1982b), Goldcoast (Threlfall et al. 1986a), and Berta (Threlfall et al. 1992c), and phage types (Wray et al. 1987; Hampton et al. 1995; Lawson et al. 2004), but is restricted to strains of serotypes possessing plasmids and is of limited use in serotypes in which the majority of isolates contain only one plasmid. Thus, until recently, plasmid profile typing has been of only limited value in Enteritidis (Erdem et al. 1994), in which the majority of isolates carry a single plasmid of 38 000 kDa (Helmuth et al. 1985). However, since the year 2000, an increasing number of *S.* Enteritidis phage types have carried one or more plasmids in addition to the 3 800 kDa serotype-specific plasmid, and together with PFGE, this property has been exploited in investing numerous outbreaks in the UK in 2002 and 2003. The sensitivity of the plasmid profile typing may be increased by cleaving plasmid DNA with a limited number of restriction endonucleases and the resultant plasmid 'fingerprint' may be used to discriminate between plasmids of similar molecular mass (Platt et al. 1986). On this basis a hierarchical plasmid fingerprinting scheme has been proposed (Platt et al. 1986). As with plasmid profile typing, the use of plasmid fingerprinting is restricted to plasmid-carrying strains; furthermore, carriage of multiple plasmids may make the interpretation of results extremely difficult.

CHROMOSOMALLY BASED METHODS

For salmonellae that do not carry plasmids or possess only serotype-specific plasmids (SSP), it may be necessary to use chromosomally based methods such as ribotyping which has been used for differentiation within serotypes, e.g. Dublin (Chowdry et al. 1993) and Typhi (Altwegg et al. 1989), and within phage types, e.g. Typhi (Grimont and Grimont 1986). However, ribotyping alone is not highly discriminatory and, in general, it is used in combination with other genotypic methods such as IS*200* fingerprinting.

Observations of heterogeneity in chromosomal restriction enzyme-generated fragment patterns may be enhanced by the use of randomly cloned sequences of chromosomal DNA as single-stranded gene probes and this method of typing, originally developed in the USA by Tompkins and colleagues (1986), provided a method for the differentiation of strains of Typhimurium but was of limited use for Dublin and Enteritidis.

A third RFLP-based method is that of insertion sequence typing. Insertion sequences belong to a class of mobile genetic elements which contain only those genes necessary for their own transposition. In particular, the *Salmonella*-specific 708 bp insertion sequence IS*200* (Lam and Roth 1983) has been shown to be distributed on conserved loci on the chromosome of many *Salmonella* serotypes with copy numbers ranging from one to 25 (Gibert et al. 1990). In some serotypes, identification of the number and distribution of IS*200* elements in the genome has provided a method of discrimination suitable for epidemiological investigations. In particular, IS*200* fingerprinting has been used for discrimination within serotypes such as Brandenburg (Baqúar et al. 1994), Infantis (Pelkonen et al. 1994), and Heidelberg (Stanley et al. 1992) for which phage typing schemes are not available and which do not contain extrachromosomal plasmid DNA. However, when applied to serotypes that can be subdivided by phage typing, IS*200* fingerprinting appears to be of more limited value; thus, in Enteritidis the method defined only three clonal lineages among 27 phage types and did not differentiate within phage types (Stanley et al. 1991). For both Typhimurium and Paratyphi B biotype Java, preliminary studies have suggested that IS*200* fingerprinting may be as discriminatory as phage typing for some investigations (Ezquerra et al. 1993; Stanley et al. 1993), a finding recently exploited in the typing of Paratyphi B strains of biotype Java from a large outbreak of human infection in France, caused by contaminated goats' milk cheese (Desenclos et al. 1996).

Although the RFLP-based methods described above provide fingerprints that are easy to interpret and reproduce, ribotyping, RCCS typing, and IS*200* fingerprinting may be nondiscriminatory. Furthermore, in the case of IS*200* fingerprinting, not all serotypes possess these elements. By contrast, PFGE, a variation of conventional AGE that uses alternation between two field orientations to separate linear DNA fragments of a magnitude ranging from >6 000 to <10 kb, can provide a fingerprint of the whole genome. This method is being used increasingly for the fingerprinting of *Salmonella*, and has provided subdivision within both serotype, e.g. Typhi (Thong et al. 1994), Newport (Ward et al. 2002), Stanley (Kirk et al. 2004) and phage type, e.g. Enteritidis PT 4 (Powell et al. 1994, 1995), *S.* Typhimurium DT 104 (Lawson et al. 2004). Since the mid-1990s PFGE has been used for characterization of a strain of Agona PT 15 responsible for an international outbreak of salmonellosis in at least four countries (Threlfall et al. 1996b) and for the discrimination of a strain of *S.* Newport responsible for an outbreak of salmonellosis in England and Wales in 2001 associated with the consumption of contaminated salad vegetables originating in Spain (Ward et al. 2002). When combined with ribotyping and IS*200* fingerprinting, a genotypic method was also provided for the subdivision of strains of Panama isolated in several countries over an extended period of time (Stanley et al. 1995).

The use of PFGE for the subtyping of *S. enterica* (and several other enteric pathogens) has now been standardized in the USA and has been developed into a national network – PulseNet – for the molecular subtyping of various *S. enterica* serotypes and also *Escherichia coli* O157:H7 (Swaminathan et al. 2001). In Europe, a nine country network (Salm-gene) has been

established targeted at harmonizing PFGE and other molecular methods for the subtyping of *S. enterica* within human Salmonella reference laboratories within the European Union (Peters et al. 2003). The harmonized PFGE method is compatible with those used in PulseNet and the long term objective will be to develop a major international network on a global scale, involving both human and veterinary laboratories, for the standardized molecular subtyping of *S. enterica*. The power of this method, coupled with its potential for international standardization, has been exploited in the identification of an outbreak of multiresistant *S.* Typhimurium, DT 104b epidemiologically associated with contaminated lettuce in five European countries and Iceland in the summer of 2000 (Lindsay et al. 2002; Crook et al. 2003), and more recently, in an outbreak of *S.* Stanley and *S.* Newport in three continents associated with peanuts originating from China (Kirk et al. 2004). In both outbreaks the electronic exchange of gel profiles obviated the necessity for the exchange of strains through the postal system, and resulted in the rapid implementation of effective intervention strategies.

The three PCR-based methods RAPD, ERIC-PCR, and REP-PCR have all been used experimentally for differentiation within both serotypes and phage types. However, results are not yet sufficiently reproducible for widespread use in large-scale outbreak investigations. In contrast, AFLP appears to have considerably more potential, both for outbreak investigations and for exploring phylogenetic relationships within *S. enterica*. Fluorescent amplified fragment length polymorphism fingerprinting (FAFLP), which utilizes digestion of genomic DNA with two restriction enzymes followed by ligation of specific oligonucleotide adapters and PCR amplification of specific subsets of fragments using one fluorescently-labeled primer and an unlabeled primer designed to prime from the ligated adapter sits, has been critically evaluated in comparison to PFGE (Lindstedt et al. 2000). The method has subsequently been exploited both for outbreak investigations (Lindsay et al. 2002; Scott et al. 2001; Lawson et al. 2004), and for exploring the genetic structure of the SARA, SARB, and SARC *Salmonella* reference collections (Scott et al. 2002) and various *S*. Typhimurium phage types (Lan et al. 2003). Single-enzyme amplified fragment length polymorphism fingerprinting (SAFLP), which follows the same principles as FAFLP but utilizes only one enzyme and does not involve the incorporation of a fluorescin label (Peters and Threlfall 2001) is a more simple method possibly more suited for day-to-day use in the reference laboratory setting, and has also been used both independently of, and also in conjunction with PFGE, in outbreak investigations (Sood et al. 2002).

VNTR is based on the presence and subsequent identification of units of repeated DNA elements in the genome. Such elements, known as VNTRs, range from about 10 to 100 base pairs (bp). Following the publication of the *S.* Typhimurium LT2 genome (McClelland et al. 2001) VNTR fingerprinting has been experimentally applied to the subtyping of *S.* Typhimurium DT 104 (Lindstedt et al. 2003). Further evaluation of the method in a range of different serotypes is required before this method can be used for outbreak investigations.

Outer-membrane protein and lipopolysaccharide profiles

Analysis of outer-membrane protein (OMP) patterns has demonstrated considerable homogeneity among strains within each of the serotypes Choleraesuis, Heidelberg, Infantis, Panama, and Typhimurium; by contrast, a limited degree of heterogeneity was observed within Dublin and Enteritidis, with a few strains lacking one major OMP (Helmuth et al. 1985). Because of the limited heterogeneity observed, it is unlikely that OMP typing can be used in epidemiological investigations. However, it has demonstrated that some strains of Typhimurium are deficient in OMP F and, because of this deficiency, show low-level resistance to a range of antibiotics, including ciprofloxacin, resulting from decreased uptake of the respective antibiotics through cell wall porins.

Analysis of LPS content, though not a useful tool for subdivision within a serotype, has proved useful in investigating changes of phage type within certain serotypes; thus, irreversible loss of the ability to synthesize LPS accounts for the conversion of PT 4 to PT 7 in Enteritidis strains (Chart et al. 1989).

EPIDEMIOLOGY

Habitat

Salmonellae are primarily intestinal parasites of humans and many animals, including wild birds, domestic pets, and rodents; they may also be isolated from their blood and internal organs. They are found frequently in sewage, rivers, and other waters and soil in which they do not multiply significantly. Under suitable environmental conditions, they may survive for weeks in waters and for years in soil. They have been isolated from many foods, including vegetables and fruit, used by humans (Rampling 1990; Report 1993) and are important contaminants of animal protein-feed supplements.

Some serotypes are adapted to specific hosts, e.g. Abortusovis, Gallinarum, Typhi, and Typhisuis are confined respectively to sheep, fowl, humans, and swine (or closely related species). The majority, however, are ubiquitous serotypes inhabiting a wide range of hosts. Members of subsp. *enterica* predominate among mammals; those from other subspecies are isolated infrequently from, and are rarely pathogenic for, humans and

other mammals. Serotypes from subspp. *salamae*, *arizonae*, *diarizonae*, and *houtenae* are commonly found in the intestinal tract of cold-blooded animals.

Susceptibility to antimicrobial agents

Until about 1960, nearly all salmonellae were sensitive to a wide range of antimicrobial agents but since 1962 resistance, frequently plasmid mediated, has appeared in salmonellae world wide. The relative importance of antibiotic resistance, and the serotypes in which it occurs, differs from country to country. For example, in developed countries in Western Europe such as the UK, resistance is common in serotypes associated with bovine animals, e.g. Typhimurium, but is relatively uncommon in serotypes associated with poultry, e.g. Enteritidis. It must also be realized that within Typhimurium, resistance may be concentrated in a relatively few phage types, often associated with bovine animals, e.g. definitive phage types (DT) 29, 204c, and 104, whilst remaining uncommon in phage types associated with poultry, e.g. DTs 9, 10, and 12 (Palmer and Rowe 1986; Frost et al. 1995). By contrast, in many developing countries resistance is found in several serotypes that are almost invariably linked to severe outbreaks of salmonellosis in humans without obvious animal involvement (for review, see Rowe and Threlfall 1984).

DEVELOPED COUNTRIES

The question of whether antibiotic resistance in non-typhoidal salmonellas associated with food animals is a human public health problem in developed countries has been a contentious issue since the late 1960s. In the UK, following concern about the increasing incidence of multiresistance (to four or more antimicrobial agents) in strains of salmonellae, particularly Typhimurium DT 29 in humans and bovine animals (Anderson 1968), the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine (the Swann Committee) recommended in 1969 that certain therapeutic antibiotics, at that time widely used in food animals without prescription, should be available only on prescription (Anonymous 1969). A further recommendation of the Swann Committee was that certain antibiotics should be reserved specifically for prophylaxis and therapy and should not be used for growth promotion. By 1970, DT 29 had disappeared from bovine animals in Britain and for the next 6 years <8 percent of isolations of Typhimurium from cattle and 3 percent of isolations from humans were multiresistant (Rowe and Threlfall 1984). However, from 1975 to the mid-1980s there was a substantial upsurge in the occurrence of multiresistant Typhimurium strains in humans and food animals, particularly calves. The phage types involved were different from those that caused outbreaks in the 1960s, with the related DTs 204, 193, and 204c predominating (Threlfall et al. 1978, 1980, 1985). A feature of this outbreak was the sequential acquisition of plasmids and transposons coding for a wide range of antimicrobial agents, e.g. ampicillin (A), chloramphenicol (C), gentamicin (G), kanamycin (K), streptomycin (S), sulfonamides (Su), tetracyclines (T), and trimethoprim (Tm). The acquisition of resistance by strains of these phage types seemed to coincide with the introduction and use of at least some of the antimicrobial agents in attempts to combat infections in calves by Typhimurium strains resistant to an increasing range of antibiotics (reviewed by Rowe and Threlfall 1984). This epidemic also provided the first conclusive evidence of the introduction and use, in calf husbandry, of a veterinary aminoglycoside antibiotic (apramycin) giving rise to resistance to gentamicin, a related antibiotic which is used for treating severe systemic infections in humans (Threlfall et al. 1985, 1986). Over the next 5 years the incidence of drug resistance in both human and bovine isolates of Typhimurium continued to rise (Ward et al. 1990; Threlfall et al. 1993a), and the Expert Group on Animal Feedingstuffs (The Lamming Committee) recommended not only that antibiotics giving cross-resistance to those used in human medicine should not be used as growth promoters, but also that their prophylactic use in animals should be reconsidered (Anonymous 1992a).

Since 1991 there has been a further substantial increase in the incidence of both resistance and multiple resistance in Typhimurium strains in England and Wales. In 1994, 78 percent of isolations from humans were resistant to at least one antimicrobial agent and 62 percent were multiresistant (Frost et al. 1995). Of particular importance in this increase in the incidence of multiresistance in Typhimurium since 1991 has been an epidemic in cattle and humans in England and Wales of multiresistant (MR) strains of DT 104 of R-type ACSSuT. In contrast to DTs 29, 204c, and 193, in *S.* Typhimurium DT 104 the ACSSuT resistance genes are inserted in the chromosome (Threlfall et al. 1994a). MR *S.* Typhimurium DT 104 of R-type ACSSuT was first identified in the UK in the early 1980s from gulls and exotic birds. With the exception of a small outbreak in Scotland in the mid-1980s there were no isolations from humans until 1989, by which time MR *S.* Typhimurium DT 104 had also been isolated from cattle. Over the next 5 years this strain became epidemic in bovine animals throughout the UK (Anonymous 1998). However, in contrast to previous epidemic MR phage types such as DTs 29, 204, 193, and 204c, which were for the most part confined to cattle, MR DT 104 has also become common in poultry, particularly turkeys, pigs, and sheep (Anonymous 1998). Human infection with MR *S.* Typhimurium DT 104 has been associated with the consumption of chicken, beef, pork sausages, and meat paste (Wall et al. 1994), and to a lesser extent with

occupational contact with infected cattle (Wall et al. 1995).

Although some variants have been identified, when studied by PFGE the majority of MR *S.* Typhimurium DT 104 are characterized by a distinctive *Xba*I-generated macrorestriction fingerprint (Ridley and Threlfall 1998; Prager et al. 1999; Lawson et al. 2004). Over the last 4 years this particular clone has caused outbreaks of infection in food, animals, and humans in numerous European countries and as far afield as South Africa, the United Arab Emirates, and the Philippines. In 1996, infections with MR DT 104 were recognized in cattle and humans in North America, both in Canada and the USA (for reviews, see Threlfall 2000, 2002).

Of particular concern has been the resistance of the organism to a wide range of therapeutic antimicrobials. Furthermore, in some countries there have been reports of an apparent predilection of the organism to cause serious disease (Wall et al. 1994; Miller 1997; Mølbak et al. 1999; Helms et al. 2002). However, a study in the UK of over 70 000 salmonella infections in the 3 year period 1994–96 demonstrated that MR *S.* Typhimurium DT 104 appears no more invasive than other common serotypes and phage types in terms of isolations from blood culture in comparison to fecal isolations (Threlfall et al. 1998).

Genetic analysis has demonstrated that in MR *S.* Typhimurium DT 104 of R-type ACSSuT, the complete spectrum of antibiotic resistance is chromosomally encoded (Threlfall et al. 1994a). Investigation of a selection of resistant strains by PCR demonstrated integron hot spots in two copies as two discrete bands of approximately 1.0 and 1.2 kilobases (kb). Direct nucleotide sequencing of the individual amplicons indicated that the 1.0 kb gene product was the enzyme ANT (3′)-1a, responsible for resistance to streptomycin (and also to spectinomycin); the 1.2 kb amplicon contained the gene *bla* P1, encoding the β-lactamase CARB-2 (PSE-1) and the *sul1* gene coding for resistance to sulfonamides (Ridley and Threlfall 1998; Sandvang et al. 1998). It is particularly noteworthy that all isolates of MR DT 104 of the R-type ACSSuT phenotype have contained the same gene cassettes irrespective of source (food, animal, or human), or country of origin. Subsequent studies have demonstrated that the genes for chloramphenicol and tetracycline resistance are grouped between the ampicillin/sulfonamide and streptomycin/spectinomycin (SSp) integrons described above (Briggs and Fratamico 1999; Casin et al. 1999). Antibiotic resistance in MR DT 104 of R-type ACSSuT is therefore comprised of a sequence of approximately 1.4 kb, containing the A/Su and SSp integrons, and intervening plasmid-derived genes coding for resistance to chloramphenicol and tetracyclines (Briggs and Fratamico 1999).

Since 1992, there has been an increasing number of isolates of *S.* Typhimurium DT 104 which, as well as possessing chromosomally encoded resistance to ACSSuT, have also acquired plasmid mediated resistance to sulphonamides and trimethoprim (R-type ACSSuTTm) (Threlfall et al. 1996a, 1999b; Threlfall 2000). A further development in 1995 was the substantial increase in multiresistant DT 104 exhibiting decreased susceptibility to ciprofloxacin (MIC 0.250–0.500 mg/l). This is of particular concern because ciprofloxacin is regarded as the drug of choice for the treatment of invasive salmonellosis in humans. In isolates of multiresistant *S.* Typhimurium DT 104 with decreased susceptibility to ciprofloxacin, this property is chromosomally encoded. Amplification and sequencing of the 120 bp quinolone resistance determining region (QRDR) in a panel of isolates of MR DT 104 of R-type ACSSuTCp has identified two discrete base substitutions at codon aspartate (Asp)-87 and further point mutations at codons Ser-83 and Ala-119 (just outside the QRDR), all giving rise to decreased susceptibility to ciprofloxacin (Ridley and Threlfall 1998). The most common mutation in Asp-87 involved change from **G**AC (aspartate) to **A**AC (asparagine). An identical mutation giving rise to decreased susceptibility to ciprofloxacin has recently been identified in a strain of MR DT 104 responsible for an outbreak in Denmark in the summer of 1998, associated with pork of Danish origin (Mølbak et al. 1999). More recently the mutation in codon 87 from G**A**C to G**G**C has been identified in isolations of MR *S.* Typhimurium DT 104 from humans, cattle, milk, and a milk filter in a substantial outbreak in northwest Lancashire in the summer of 1998 (Walker et al. 2000). This outbreak was associated with a breakdown in on-farm pasteurization. It is particularly noteworthy that the fluoroquinolone antibiotic marbofloxacin had been in use on the farm in the months preceding the outbreak, and this may have contributed to the persistence and dissemination of the multiresistant strain in the farm environment.

The emergence and spread of MR DT 104 with decreased susceptibility to ciprofloxacin in the UK followed the licensing for veterinary use of the related fluoroquinolone antibiotic enrofloxacin in November 1993 (Threlfall et al. 1998, 1999a). This antimicrobial has subsequently been used extensively in both cattle and poultry for treatment and prophylaxis. A consequence of this has been the rapid development of resistance to quinolones in strains of MR DT 104 from food-producing animals (Piddock and de Jong 1999). Such resistance has been particularly apparent in strains of MR *S.* Typhimurium DT 104 from turkeys but also in strains from chickens and cattle (Davies et al. 1999). For humans, the clinical significance of decreased susceptibility to ciprofloxacin is controversial. However, in the outbreak in Denmark in the summer of 1998, four of 11 hospitalized patients did not respond to treatment with ciprofloxacin and there were two deaths (Mølbak et al. 1999). This outbreak clearly demonstrates the potential of MR DT 104 for causing serious disease and the clin-

ical consequences of decreased susceptibility to ciprofloxacin in this epidemic strain.

Decreased susceptibility to ciprofloxacin is not just confined to MR *S*. Typhimurium DT 104 in the UK. In a study of over 27 000 of *Salmonella* isolates from cases of human salmonellosis in ten European countries in the year 2000, over 14 percent of isolates exhibited such resistance. Furthermore, such decreased susceptibility was observed in numerous serotypes and phage types (Threlfall et al. 2003a). Although the use of fluoroquinolone antibiotics in humans cannot be totally excluded, there is little doubt that such resistance is a direct consequence of the use of such antibiotics in food-producing animals in many countries in Europe. It should be similarly realized that multiple resistance in salmonellas in developed countries is not confined to *S.* Typhimurium DT 104 and related strains. In Spain, emergent multiresistant strains of *S. enterica* serotype [4,5,12:i:-] have been associated with an increasing number of human infections since the mid-1990s (Guerra et al. 2000). Such strains have also caused infections in humans in the UK (Walker et al. 2001) and Denmark. In these strains resistance has been mediated by an unusual plasmid containing resistance genes located within a class 1 integron and also the *spvA*, *spvB*, and *spvC S*. Typhimurium plasmid virulence genes (Guerra et al. 2002). Similarly, in Greece, multiresistant strains of *S.* Blockley have caused numerous infections in humans since 1996 (Tassios et al. 2000). It should be emphasized that although the most common presentation has been gastroenteritis, in infections caused by both *S. enterica* serotype [4,5,12:i:-] and *S.* Blockley, in some cases patients have not responded to antimicrobial treatment, possibly as a consequence of the wide resistance spectrum of the organisms concerned.

In other developed countries, multiple antibiotic resistance has been reported in a number of serotypes from food animals. For example, in the USA, multiple resistance has been reported in Saintpaul, Heidelberg, Newport, and Typhimurium (Neu et al. 1975; Holmberg et al. 1984) and such resistance has often been attributed to the use of antibiotics in animal feeds. In 2000 the first reported case of domestically-acquired ceftriaxone-resistant *Salmonella* in a case of human salmonellosis in the USA involved the transmission of a resistant strain from cattle to the child of a veterinarian, following treatment of the infected herd with this antimicrobial (Fey et al. 2000). More recently closely-related strains of multidrug-resistant *S.* Newport with plasmid-encoded resistance to ceftriaxone have been associated with numerous infections in both cattle and humans in North America (Rankin et al. 2002), to become the third most common serotype causing salmonellosis in man in the USA in from 2000 to 2002. This organism commonly shows resistance to ACSSuT with additional resistance to third-generation cephalosporins mediated by the CMY-2 beta-lactamase gene (Rankin et al. 2002; Carattoli et al. 2002; Carattoli 2003). Spread of this strain has been linked to the use of related third-generation cephalosporins in cattle in the USA (Rankin et al. 2002).

As antibiotics are not recommended for the treatment of mild to moderate salmonella-induced enteritis in humans, it may be argued that in developed countries drug resistance in nontyphoidal salmonellas is of little consequence to human public health. However, antibiotics are used for the treatment of salmonellosis in immunocompromised patients and sometimes for treating particularly vulnerable patients; in such cases treatment with an appropriate antibiotic is often essential and may be life-saving. Thus, it would appear that in developed countries, and particularly in the UK, the injudicious use of antibiotics in food animals has encouraged the spread and persistence of multiresistant salmonellas in these animals. This has undoubtedly contributed to the overall occurrence of such strains in humans and has also resulted in the appearance and spread of strains resistant to antibiotics used for the treatment of invasive disease in humans.

To combat the development of resistance in zoonotic pathogens to such important drugs as the fluoroquinolones, it is hoped that such antimicrobials will only be used in food animals when absolutely necessary. In the UK, recommendations targeted at the development of a coherent strategy aimed at reducing the veterinary use of antibiotics have recently been published by the Advisory Committee on the Microbiological Safety of Food (ACMSF), in their recent report on Microbial Antibiotic Resistance in Relation to Food Safety (Anonymous 1999).

DEVELOPING COUNTRIES

In developed countries, the most common presentation in patients with salmonellosis is mild to moderate gastroenteritis. The disease is normally self-limiting with <2 percent of infections being extraintestinal (Threlfall et al. 1992b) and mortality is <0.5 percent. Nosocomial outbreaks are uncommon and the majority of infections are related to the consumption of infected food. The majority of strains have food-animal reservoirs and the most common serotypes involved are Enteritidis, Typhimurium, Hadar, and Virchow. By contrast, in developing countries, particularly in South America, Africa, the Indian subcontinent, and South East Asia, the most common presentation is that of severe gastroenteritis often accompanied by septicemia (up to 40 percent in some outbreaks) and with up to 30 percent mortality. The common pattern has been for several hospitals, often situated many miles apart, to be involved. The majority of outbreaks have occurred in neonatal and pediatric wards, but community outbreaks in villages and small towns have also been reported. The clinical disease has been severe with enteritis frequently accompanied by septicemia. The main method of transmission

Table 4 *Multiresistant salmonellae in developing countries*

Serotype and phage type	Years	Country/area	Resistance patterns[a]
Wien[b]	1970–84	Northern Africa	ACSSuT
		Southern Europe, India	ACSSuTTm
Typhimurium PT 208[b]	1974–84	Middle East	ACSSuT, ACGKSSuT
Johannesburg[b]	1974–78	South Africa	ACKSSuT
Oranienburg[c]	1974–86	Indonesia	ACKSSuT
Typhimurium PT Nc[b]	1976–80	Kenya	ACSSuT
Typhimurium PT Un[b]	1976–80	Liberia	ACKSSuT
Typhimurium PT 66/122[b]	1978–84	India	ACKSSuT, ACGKSSuT, ACGKSSuTTmNx
Bareilly[b,c]	1986–92	India	ACKSSuT
Typhimurium PT Un[b]	1978–94	India	ACKSSuT, ACKSSuTFu, ACGKSSuT, ACGKSSuTTmNx
Typhimurium PT Un	1986–2002	Turkey	ACKSSuT, ACGKSSuTTm, ACGKSSuTTmNx
Senftenberg	1996–2002	India	ACKSSuT, ACGKSSuTTm, ACGKSSuTTmNx, ACCtGSSuTTmNx

Nc, not-conforming; Un, untypable.
a) Resistance to: A, ampicillin; C, chloramphenicol; Ct, ceftriaxone; Fu, furazolidone; G, gentamicin; K, kanamycin; Nx, nalidixic acid/decreased susceptibility to ciprofloxacin; S, streptomycin; Su, sulfonamides; T, tetracycline; Tm, trimethoprim
b) Outbreak.
c) Sporadic.

is by person-to-person spread either in hospitals or in the community. The strains involved are invariably multiresistant, often with resistance to up to nine antimicrobial agents. A disturbing development in recent years has been an increase in the incidence of multiresistant strains with high-level resistance to nalidixic acid (MIC: >100 mg/l) and with reduced susceptibility to ciprofloxacin (MIC: 0.1 mg/l cp 0.0075 mg/l for nalidixic acid-sensitive strains). This has been particularly evident in countries such as India (Lewin et al. 1991) particularly for *S.* Typhimurium (Lewin et al. 1991), but also for *S.* Senftenberg (Gupta et al. 1999). Examples of outbreaks caused by such multiresistant strains are shown in Table 4.

With the exception of resistance to furazolidone and, since 1990, to nalidixic acid coupled with decreased susceptibility to ciprofloxacin, resistances are invariably plasmid encoded. A particular property of the majority of these multiresistant strains is the possession of a plasmid of the F_I*me* incompatibility group coding not only for multiple resistance but also for production of the hydroxamate siderophore aerobactin, which is a known virulence factor for some enteric and urinary tract pathogens. These plasmids, first identified in Typhimurium DT 208 which caused numerous epidemics in many Middle Eastern countries in the 1970s (Anderson et al. 1977a), were subsequently identified in a strain of Wien responsible for a massive epidemic that began in Algiers in 1969 but spread rapidly thereafter through pediatric and nursery populations in many countries throughout North Africa, Western Europe, the Middle East, and eventually the Indian subcontinent over the next 10 years (Le Minor 1972; McConnell et al. 1979; Casalino et al. 1984). F_I*me* plasmids have also been identified in several unrelated phage types of Typhimurium that have caused substantial outbreaks in Africa (Kenya, Liberia), India, and Turkey (Frost et al. 1982) (Table 4). A 30 year retrospective molecular study of this group of plasmids, from strains of *S.* Typhimurium from several countries, has demonstrated that the plasmids have evolved through sequential acquisition of integrons carrying different arrays of antibiotic resistance genes (Carattoli et al. 2001; Carattoli 2003). Although not clinically proven, the epidemiological evidence strongly suggests that possession of the F_I*me* plasmid has contributed to the exceptional virulence of these epidemic clones.

SALMONELLA TYPHI

An appropriate antibiotic is essential for the treatment of patients with typhoid fever; until the mid-1970s, chloramphenicol was the undisputed drug of choice. Although a few sporadic isolations of chloramphenicol-resistant Typhi had been reported before 1970 (see Anderson and Smith 1972), the first epidemic caused by a chloramphenicol-resistant strain occurred in Mexico in 1972 (Anonymous 1972; Olarte and Galindo 1973). At about that same time, a second substantial outbreak occurred in India (Paniker and Vilma 1972) and in the succeeding 5 years outbreaks were reported in several other countries, notably Vietnam, Indonesia, Korea, Chile, and Bangladesh (Anderson 1975). A feature of all chloramphenicol-resistant strains from outbreaks in Mexico, the Indian subcontinent, and South East Asia was that, although the strains belonged to different Vi phage types, resistance to chloramphenicol was encoded by a plasmid of the H_1 incompatibility group, and was often found in combination with resistance to streptomycin, sulfonamides, and tetracyclines (R-type CSSuT) (Anderson 1975; Threlfall et al. 1991). Since 1989,

outbreaks caused by Typhi strains resistant to chloramphenicol, ampicillin, and trimethoprim, and with additional resistances to streptomycin, sulphonamides, and tetracyclines (R-type ACSSuTTm), have been reported in many developing countries, especially Pakistan and India (Threlfall et al. 1992a; reviewed by Rowe et al. 1997). Multiresistant strains have also caused outbreaks in Bangladesh (Albert et al. 1991), several countries in South East Asia (Tinya-Superable et al. 1995) and in both north and South Africa (Coovadia et al. 1992; Mourad et al. 1993). The recent emergence in developing countries of Typhi strains with resistance to trimethoprim and ampicillin has caused many problems because, since 1980, these antibiotics had been used extensively for the treatment of patients infected with chloramphenicol-resistant Typhi strains (Mandal 1991).

Without exception, in all outbreaks involving multiresistant Typhi so far studied, the complete spectrum of multiple resistance has been encoded by plasmids of the H_1 incompatibility (Inc) group (Rowe et al. 1991; Threlfall et al. 1992a). However, although resistances in outbreak and endemic strains of *S.* Typhi are encoded by Inc H_1 plasmids, evolutionary diversity within this compatibility group has been observed in such plasmids from multiresistant strains from Vietnam over a 10 year time period in the 1990s (Wain et al. 2003).

In the UK, resistance to chloramphenicol was rarely encountered until 1990 (Rowe et al. 1987, 1990), but since then Typhi strains resistant not only to chloramphenicol but also to trimethoprim and ampicillin have been isolated with increasing frequency. The majority of these strains have been isolated from travelers with a history of recent return from countries where multiresistant strains have either caused outbreaks or have become endemic. In all multiresistant Typhi strains isolated in the UK since 1990, resistance to chloramphenicol, ampicillin, and trimethoprim, together with resistance to streptomycin and sulphonamides (ACSSuTTm) has been encoded by plasmids of the H_1 incompatibility group.

Because multiresistant *S.* Typhi is now endemic in many developing countries and is also common in travelers returning to developed countries, ciprofloxacin is generally used for the treatment of typhoid in both developing and developed countries (Anand et al. 1990; Mandal 1991). It is therefore a concern that multiresistant Typhi strains with plasmid-encoded resistance to chloramphenicol, ampicillin, and trimethoprim and additional chromosomal resistance to ciprofloxacin have been reported both in the UK (Rowe et al. 1995) and in outbreaks of infection in several developing countries, including Tajikistan (Murdoch et al. 1998) and Vietnam (Parry et al. 1998). In the Tajikistan outbreak, which occurred in 1996–97, 50 000–60 000 cases of typhoid fever were reported (Mermin et al. 1999). In the UK, 23 percent of over 200 isolates in 1999 exhibited such resistance (Threlfall and Ward 2001) and in a recent study of over 400 strains isolated from patients in 10 European countries in the 3 year period 1999–2001, between 20 and 26 percent exhibited decreased susceptibility to ciprofloxacin, often in combination with resistance to chloramphenicol, ampicillin, and trimethoprim (Threlfall et al. 2003b). Although the MIC of these strains to ciprofloxacin was below the anticipated serum level for this antimicrobial following administration at recommended doses, treatment failures have been increasingly noted (Threlfall et al. 1999c). In such cases, third-generation cephalosporin antibiotics such as ceftriaxone or cefotaxime have been suggested as possible alternatives (Parry et al. 1998; Threlfall et al. 1999c). In strains with decreased susceptibility to ciprofloxacin, four different mutations have been identified in *gyrA* in a study of 17 different strains from five countries (Walker et al. 2003), confirming that such strains were not clonal either in respect of phage type or of ciprofloxacin susceptibility.

Azithromycin, a macrolide antibiotic, has also been evaluated for the treatment of infections caused by multiresistant typhoid, with encouraging results (Wallace et al. 1994). However, more clinical trials are required before this antibiotic can be recommended for first-line treatment ahead of such antimicrobials as ciprofloxacin and ceftriaxone.

SALMONELLA PARATYPHI A

Infections caused by *S.* Paratyphi A, although in general not as severe as typhoid fever, may also require antimicrobial intervention before the results of susceptibility tests are available. A substantive increase in *S.* Paratyphi A with decreased susceptibility to ciprofloxacin has also been reported in India since the late 1990s (Chandel et al. 2000). As with *S.* Typhi, a similar increase in strains of *S.* Paratyphi A with decreased susceptibility to ciprofloxacin isolated from patients from ten European countries between 1999 and 2001 has also been observed (Threlfall et al. 2003b).

NOTES ON SELECTED SEROTYPES OF SALMONELLAE

Subspecies I

AGONA (1,4,12:F,G,S:[1,2])

Rarely isolated before 1969, this serotype was imported into the USA and many European countries in contaminated Peruvian fishmeal, which was used in animal feed stuffs. Agona soon became entrenched in pigs and poultry (Lee 1974; Turnbull 1979) and by 1971 an animal-food cycle independent of imported products was established, e.g. in the UK (Palmer and Rowe 1986). Thereafter, Agona was soon present in the top ten sero-

types isolated from humans in the UK and elsewhere; its subsequent decline remains unexplained (Le Minor et al. 1985; Palmer and Rowe 1986). The combined use of phage typing and biotyping revealed little type divergence (Barker et al. 1982). A different phage typing scheme recognizing 38 phage types (LR Ward, unpublished) was used to investigate an outbreak of Agona that occurred in the UK from late 1994 to early 1995, associated with a kosher, savory peanut-based snack imported from Israel. All isolates from cases in the UK and from others in Israel, Canada, and the USA and from the snacks, belonged to PT 15 (Anonymous 1995). The clonal identity of the outbreak strain was confirmed by PFGE (Threlfall et al. 1996b).

MLEE has shown that the serotype is monophyletic; all isolates of Agona examined belonged to one clone (Reeves et al. 1989; Boyd et al. 1993). Of the three distinct lineages of serotype Derby (1,4,[5],12:f,g:[1,2]) (Beltran et al. 1988), one (De31) is more closely related in ET to that of Agona (Ag1) (from which it differs in only four of 26 enzyme loci) than to those of the other Derby clones (De1, De13) (Boyd et al. 1993).

CHOLERAESUIS (6,7:C:1,5)

This swine-adapted serotype commonly causes enteritis in pigs but may be isolated occasionally from other animals including humans, e.g. in the UK from 1981 to 1990 there were only 27 isolations from humans, 74 percent of which were invasive (Threlfall et al. 1992b). About 50 percent of recorded human infections are associated with prolonged pyrexia; over one-third result in localized pus formation and 20 percent are fatal (Saphra and Winter 1957). Common pyemic manifestations include pneumonia, septic arthritis and osteomyelitis, meningitis, and endocarditis. Choleraesuis grows better in brilliant green-containing media than in selenite broth or on MacConkey or deoxycholate agars. Traditionally three biotypes (Choleraesuis, Decatur, and Kunzendorf) have been recognized (see Table 5), each containing different components of the H1 antigen factors c_{1-3}. Two other salmonella serotypes with the same antigenic formula as Choleraesuis are still retained in the Kauffmann–White scheme (Popoff and Le Minor 1992): Typhisuis and Paratyphi C. Typhisuis too is swine adapted but may occasionally be isolated from other animals including humans in whom the clinical presentation is like that of Choleraesuis (Saphra and Wassermann 1954). Typhisuis grows poorly on ordinary media, produces gas sparsely, and is distinguishable biochemically (Table 5); among salmonellae it is unusual in being mannitol nonfermenting and lysine decarboxylase-negative. Most strains of the human-adapted Paratyphi C are difficult to distinguish from Choleraesuis and Typhisuis but produce Vi antigen, which, however, is rapidly lost on laboratory culture; Paratyphi C strains (Vi^+ or Vi^-) give a positive result with a *viaB*-specific probe (Selander et al. 1990b).

Analysis by MLEE indicates a very close genetic relationship among most clones of Choleraesuis, Typhisuis, and Paratyphi C. Most (82 percent) strains of Choleraesuis belong to one dominant clone (Cs1) and all but two clones of Choleraesuis form a tight genetic cluster to which Typhisuis clones (Ts1, Ts2) also belong (Beltran et al. 1988; Selander et al. 1990b). About 90 percent of Paratyphi C strains belong to clones Pc1 and Pc2 which, with most other Pc clones, also cluster in this same lineage; it has been suggested that the major clones of Choleraesuis, Typhisuis, and Paratyphi C shared a common, invasive ancestor from which they evolved with a shift to human hosts by Paratyphi C (Selander and Smith 1990). However, some clones of Choleraesuis (Cs6,13), Typhisuis (Ts3), and Paratyphi C (Pc4) are distantly related to the above major genetic group (Selander et al. 1990b). Furthermore, the three clones representative of biotype Decatur (Dt1-3) are distantly related to those of serotype Choleraesuis (Selander et al. 1990b); yet Decatur has been subsumed into Choleraesuis (Popoff and Le Minor 1992).

DUBLIN (1,9,12 [Vi]:G,P: -)

Dublin is highly host-adapted to cattle but other animals including humans are sporadically infected. In Britain, it is the second most common serotype in cattle (Anonymous 1994a, 2002). In infected calves, it causes severe diarrhea, whereas in adult cattle it is responsible for septic abortion associated with a 70 percent mortality and prolonged carriage by surviving animals. Human infections are relatively uncommon in the UK, e.g. only

Table 5 *Biochemical reactions of salmonellae of antigenic formula 6,7:c:1,5*

		Fermentation of				
Serotype	**Biotype**	**Arabinose**	**Trehalose**	**Dulcitol**	**Mucate**	**Production of H_2S**
Choleraesuis	Choleraesuis	–	–	–	–	–
Choleraesuis	Kunzendorf	–	–	–	–	+
Choleraesuis	Decatur	+	+	+	+	+
Paratyphi C[a]		+/–	+/–	+	–	+
Typhisuis		+	+	–	–	–

–, Negative reaction (in 5 days); +, positive reaction (in 2 days); +/–, some strains are positive, others negative.
a) Most strains are Vi^+ but production of Vi is rapidly lost on subculture.

430 human isolations in England and Wales between 1981 and 1990 (Threlfall et al. 1992b), but are more common in other parts of Western Europe such as France (Le Minor et al. 1985). Numbers of human infections, particularly in western states of the USA, increased steadily after 1964, especially among elderly, debilitated male patients in whom mortality (35 percent) was high (Taylor et al. 1982a). Human infection often follows the consumption of raw milk and large milk-borne outbreaks have been described (Small and Sharp 1979). There is a high incidence of bloodstream invasion; thus, 25 percent of isolates from humans in the UK were from blood (Threlfall et al. 1992b); abscess formation in bones and joints has also been noted.

Of three closely related clones (Du1, Du3, Du4) recognized by MLEE, 82 percent of strains belong to Du1 which is globally distributed (Beltran et al. 1988; Selander et al. 1992). Gene probing reveals that *viaB* genes are confined to clone Du3, restricted in its distribution to France and the UK; whether clones Du1 and Du3 differ in type or severity of infection in humans or animals is unknown (Selander et al. 1992). Both MLEE and sequencing of *fliC* genes show that Dublin is closely related to Enteritidis, (1,9,12:g,m:[1,7]), particularly to clonal lineage SECLIII, and that Rostock (1,9,12:g,p,u: -) is identical by MLEE to Du1 (Selander et al. 1992; Li et al. 1993; Chowdry et al. 1993). Most wild-type strains carry a large (52 000 kDa) serotype-specific plasmid (SSP) implicated in virulence for BALB/c mice, not required for blood invasion in humans and of uncertain status in bovine infections (Manning et al. 1986; Heffernan et al. 1987). Most strains (96 percent) require nicotinic acid for growth. Some biotype diversification is present (Walton 1972; Fierer and Fleming 1983), but molecular techniques have been necessary for the typing of Dublin strains (see Liebisch and Schwarz 1996). Among human isolations in the UK, two clones (*Sd*RI, *Sd*RII) of identical IS*200* profile were identified on the basis of ribotyping; it has been suggested that strains of ribotype I may be associated with invasive disease in humans whereas those of ribotype II are associated only with noninvasive disease (Chowdry et al. 1993). Strains formerly assigned to clone Du2 belong to a new serotype (1,9,12:g,m,p: -) distantly related to Dublin (Selander et al. 1992). Non-motile strains of Dublin (1,9,12: - : -), indistinguishable by MLEE from Du1, carry a plasmid (52 000 kDa) the restriction pattern of which is the same as that of wild-type Dublin strains (Selander et al. 1992).

ENTERITIDIS (1,9,12:G,M:[1,7])

This serotype usually causes food poisoning in humans, but known complications include severe septicemia and pyemic conditions such as spinal meningitis and subcutaneous abscesses, as described, for example, among Liberian infants among whom very high mortality rates (45 percent) were noted in children <1 year old (Hadfield et al. 1985). Enteritidis is commonly among the top three serotypes from humans in many countries world wide (Rodrigue et al. 1990; Schroeter et al. 1994); in the UK, for example, reported isolations increased 16-fold in the period 1981–94 (Threlfall et al. 1994c; Frost et al. 1995), Enteritidis accounting for 54 percent of all salmonellae isolated from humans in 1995 (Anonymous 1996). Its main reservoir is in poultry flocks, poultry meat, and contaminated eggs being important vehicles of human infection (Coyle et al. 1988; Cowden et al. 1989a, b; de Louvois 1993, 1994). Primary differentiation of strains is best achieved by phage typing; the scheme of Ward et al. (1987) with 11 phages originally recognized 32 types and has now been extended to include 16 phages recognizing >80 distinct types (Ward L.R., unpublished). The unprecedented epidemic of Enteritidis in the UK and several other western European countries in the late 1980s and early 1990s was caused by a strain of PT 4 which spread in poultry flocks, selected by environmental pressures associated with poultry husbandry. An unusual feature of the epidemic was the transovarian spread of the strain in the avian host (Humphrey et al. 1989; Timoney et al. 1989). In other countries epidemics were caused by strains of other phage types: PTs 8 and 13 in the USA (Rodrigue et al. 1990), PT 6a in Greece (Vatopoulos et al. 1994), and PT 1 in several eastern European countries; other PTs associated with poultry and poultry products are 7, 7a, 23, 24, and 30 (Threlfall et al. 1993b).

The largest number of isolations of PT 4 was recorded in 1993 in the UK, when 17 371 infections were identified (Wall and Ward 1999). There has been a dramatic decline since 1997, with around 6 700 isolations of PT 4 identified in 1999 (Anonymous 1999). The reasons for this decline are multifactoral. Several codes of practice for the control of salmonellas in chickens have been in operation in the UK since 1993. There have also been many improvements in the poultry industry in infection control and hygiene at breeding sites, and vaccination against *S*. Enteritidis started in breeder flocks in the UK in 1994 and in layer flocks in 1998 (Ward et al. 2000; Davies and Breslin 2003). Nevertheless, Enteritidis still remains the most common serotype from humans in the UK and in 2002 and 2003 there have been an unprecedented number of outbreaks of infection caused by phage types other than PT 4 (Anonymous 2003). The reasons for this are not fully understood but it is possible that the importation of infected eggs into the UK from other countries in the EU may be a contributory factor (O'Brien et al. 2004).

Despite its predominance, the overall level of antimicrobial resistance in Enteritidis in the UK is relatively low in comparison to *S*. Typhimurium (15–19 percent in 1981–94), and multiple-drug resistance in isolates from food animals or humans is rare (Threlfall et al. 1993a; Threlfall et al. 2003c). Further discrimination within

major PTs has been obtained by the use of molecular techniques.

Enteritidis is polyphyletic and clone En2 is distantly related to other ETs of this serotype, except in its *fliC* gene sequence (Beltran et al. 1988; Li et al. 1993). The dominant clone En1 is closely related to clones of the cattle-adapted serotype Dublin (Du1,3) and to the avian-adapted types Gallinarum and Pullorum (Boyd et al. 1993; Li et al. 1993).

GALLINARUM (1,9,12: - : -)

Two distinct entities, distinguished by biotyping and by causing different pathologies in their avian hosts (Blaxland et al. 1956), are recognized within this nonmotile serotype. Bioserotype Gallinarum is the agent of fowl typhoid, a septicemic disease of young and adult birds, usually spread by contaminated food or water. In natural infections, mortality is high. Bioserotype Pullorum causes an acute diarrheal illness in chicks; adult birds may be chronically infected but remain healthy. Spread occurs by transovarian transmission to eggs in carrier hens. Now uncommon in the UK and USA, these bioserotypes have been prevalent since the 1970s in Africa, Asia, and South America (da Silva 1985). Sporadic cases occur in humans and other animals. The virulence plasmids of both types are similar in size and structure (Barrow and Lovell 1989), but the mechanisms responsible for different diseases in avian hosts are unknown. Whereas Gallinarum strains grow well on ordinary enteric media, brilliant green-containing media are recommended for Pullorum strains, some of which, however, are very sensitive to brilliant green and bile salts. Pullorum, but not Gallinarum, strains exhibit phase-variable expression of antigen $O12_2$ subfactor. Most Gallinarum strains belong to PT 2 of Lilleengen (1952); the phage-typing scheme of Anderson (1964) recognizes 13 phage types of Pullorum. Gallinarum strains are remarkably homogeneous in biotype: they are anaerogenic, ferment dulcitol and maltose promptly and are ornithine decarboxylase-negative (Crichton and Old 1990). At least five biotypes of Pullorum are recognized (Crichton and Old 1990).

MLEE analysis has shown that both Gallinarum and Pullorum are monophyletic and closely related to Enteritidis (Li et al. 1993). Only five ETs were identified for Gallinarum strains, most (93 percent) of which belonged to the globally distributed clone Ga2. By contrast, Pullorum strains showed greater evolutionary divergence with seven ETs identified. The dominant global Pullorum clone (Pu3 and its variants) contained 61 percent of strains; clones Pu2 (26 percent strains) and Pu4 (13 percent) were apparently restricted to the UK and Germany, respectively (Li et al. 1993). These groupings are in agreement with findings from ribotyping, IS*200* profiles and PFGE (Olsen et al. 1996). The existence of a hybrid type, dulcitol-positive, and rhamnose-negative like Gallinarum but gas-producing and ornithine decarboxylase-positive like Pullorum (Crichton and Old 1990), was confirmed by the presence of a unique ET (Ga/Pu1) closer in overall genetic structure to Gallinarum than to Pullorum (Li et al. 1993). The kind of disease produced by strains of Ga/Pu1 is not known. All three lineages are thought to have been derived by loss of motility and independent host adaptations to birds, from a motile Enteritidis-like ancestor of broad host range.

HADAR (6,8:Z_{10}:E,N,X)

Rarely isolated before 1971, Hadar became one of the prevalent serotypes isolated from humans in many countries in the 1970–80s (Le Minor et al. 1985; Palmer and Rowe 1986; Reilly et al. 1988; Farmer 1995), its sudden epidemic spread being attributed to its introduction via contaminated feed supplements to turkey flocks; its major reservoir, in the UK, for example, is turkeys (Anonymous 1992b) with ducks and geese increasingly affected (Anonymous 1994a). Hadar remains a common serotype in the UK, accounting for c. 2 percent of salmonella isolations from humans in 1994–95 (Frost et al. 1995). The phage-typing scheme of de Sa et al. (1980) has been expanded and currently 62 PTs are recognized, using nine typing phages; PTs from humans and turkeys are similar (Palmer and Rowe 1986). Resistance to antimicrobial agents has increased dramatically in Hadar isolates in the UK from 2 to 90 percent in the period 1981–94 and 13 percent of strains were multidrug-resistant (pattern, ACST); since 1991, the incidence of decreased susceptibility to ciprofloxacin increased from 0 to 48 percent (Threlfall et al. 2003c). This increase is probably linked to the licensing of enrofloxacin for use in food production animals in the UK in the early 1990s. MLEE indicates that Hadar is a monophyletic serotype (Reeves et al. 1989).

HEIDELBERG (1,4,[5],12:R:1,5)

Consistently ranked among the top ten serotypes isolated from humans in different countries in Europe and in the USA (Le Minor et al. 1985; Reilly et al. 1988; Threlfall et al. 1992b; Farmer 1995), Heidelberg usually causes gastroenteritis but is occasionally associated (3.3 percent of cases) with bloodstream invasion. It is well entrenched in poultry, particularly turkeys, in the UK (Anonymous 1992b).

Epidemiological and phylogenetic groups of Heidelberg have been distinguished by IS*200* profiling; the copy number of IS*200* varied from four to six with at least one insertion site being serotype-specific (Stanley et al. 1992). Seven unique, stable IS*200* fingerprints identified clones: one was associated with human infections, a second comprised strains from chicken sources and a third was derived from that chicken clone; in addition, there were four minor clones. Clones could be further

subdivided by plasmid analysis showing, for example, that all chicken isolates carried a unique (23 000 kDa) plasmid of the Inc X group (Stanley et al. 1992). Populations of Heidelberg have been shown by MLEE to comprise eight ETs which form a single cluster of closely related genotypes; most (87 percent) strains belong to the globally dominant clone He1 (Beltran et al. 1988). This monophyletic serotype is a member of a large phylogenetic complex to which Typhimurium, Saintpaul, (some clones of) Paratyphi B/Java, and Muenchen also belong (Beltran et al. 1988, 1991).

INFANTIS (6,7,14:R:1,5)

The primary reservoir of this serotype is poultry but it has spilled over to cattle in some countries (Anonymous 1992b; Pelkonen et al. 1994). Infantis features regularly in the top ten prevalent serotypes isolated from humans in many developed countries (Le Minor et al. 1985; Reilly et al. 1988; Pelkonen et al. 1994), and there is some evidence of an enhanced propensity to cause invasive disease with 4.3 percent of strains isolated from extraintestinal sites (Wilkins and Roberts 1988). A phage-typing scheme for Infantis (Kasatiya et al. 1979) has not been widely applied. Again, resistance to antimicrobial agents is rare and few (18 percent) strains have plasmids (Pelkonen et al. 1994).

Thus, primary strain discrimination of Infantis has been molecular based. A study in Finland, where Infantis is the most common serotype identified from cases of food poisoning in humans, showed that most Infantis isolates had 3 (97 percent of strains) or >4 (71 percent) copies of IS*200*. Used together with ribotyping, IS*200* profiling identified 15 different genotypes and clearly differentiated between isolates from humans and other animal sources of infection; all strains from domestic, and most (96 percent) imported, cases of Infantis infection in humans belonged to genotypes distinct from those strains entrenched in domestic broiler chickens and cattle (Pelkonen et al. 1994). MLEE analysis has identified four clones and showed that 96 percent of isolates belong to a predominant, globally distributed clone (In1). The serotype may be polyphyletic; only one isolate of an aberrant clone (In3) of uncertain status did not cluster with the other (99 percent) strains (Beltran et al. 1988; Boyd et al. 1993).

MONTEVIDEO (6,7,14:G,M [P],S:[1,2,7])

Serotype Montevideo is recognized as a cause of serious illness in sheep (Sharp et al. 1983) in which it is the second most commonly isolated serotype in the UK; it is also common in poultry but less so in cattle (Anonymous 1994a). Montevideo is less commonly isolated from human cases in Scotland than in England and Wales; it is also regularly among the top ten serotypes from humans in the USA (Farmer 1995).

Biotyping recognized two major biogroups, the distribution of which was different in parts of the UK. Biogroup 10di was predominant in all animal species in Scotland but only in sheep in England and Wales; biogroup 2d, on the other hand, was responsible for almost all infections in humans, poultry, and cattle in England and Wales but for only one-quarter of human infections in Scotland (Reilly et al. 1985). MLEE recognized two genotypically distinct ETs in this monophyletic serotype (Boyd et al. 1993).

PANAMA (1,9,12:L,V:1,5)

This serotype was rarely isolated in the UK before 1938. Imported dried-egg products were responsible for its establishment in pigs which, with pig-meat products, remain a major source of human infection; nevertheless, Panama is now isolated from a wide variety of animals in the UK (Anonymous 1992b). For many years it has ranked in the top ten serotypes isolated from humans in the UK (Lee 1974; Turnbull 1979; Threlfall et al. 1992b) and elsewhere (Le Minor et al. 1985; Stanley et al. 1995). In humans, there is evidence of an above-average ability to cause infections in blood and other extraintestinal sites (Wilkins and Roberts 1988; Threlfall et al. 1992b).

The phage typing scheme of Guinée (1969) differentiates only eight PTs and is of limited value epidemiologically. IS*200* profiling, which shows that four (of 7–9) loci are conserved among all strains, is considered to be the most appropriate genotyping method (Stanley et al. 1995). Although a high degree of discrimination has been achieved and many individual genotypes identified when IS*200* profiling is used with plasmid analysis, these techniques have not yet been applied to epidemiological investigations. MLEE analysis recognized 13 clones, the predominant one of which (Pn1) contained 75 percent of strains (Selander et al. 1990b). Clones of the monophyletic Panama are closely related to those of the polyphyletic serotype Miami (1,9,12:a:1,5) (Selander and Smith 1990; Selander et al. 1990b).

PARATYPHI A (1,2,12:A:[1,5])

This serotype, host-adapted to humans, is an important cause of enteric fever in Asia, the Middle East, Africa, and South America (Report 1982); it is infrequently isolated from animals and occurs naturally in the H1 phase. Among salmonellae of subsp. I, it is unusual in being H_2S-negative, xylose nonfermenting and often anaerogenic (Ewing 1986). It is auxotrophic and, hence, unable to utilize citrate as sole carbon source in Simmons's medium, and is unreactive with other organic acids (see Table 3).

Of four clones recognized by MLEE, 2 (Pa1, Pa3) are widely distributed (Selander and Smith 1990; Selander et al. 1990b). Serotype Sendai (1,9,12:a:1,5), a rare cause of enteric fever, resembles Paratyphi A in its biochem-

ical properties, auxotrophy and in its host adaptation to humans; it is, however, aerogenic, ferments xylose and possesses O factor 9 rather than O2. The immunodominant sugars of factors O2 and 9, the O antigens of which are otherwise identical, are paratose and tyvelose, respectively; gene *rfbJ*, encoding cytidine 5′-diphosphate (CDP)-tyvelose epimerase which converts CDP-paratose to CDP-tyvelose, is active in strains of serogroup O9 but is present in a mutant form in strains of serogroup O2 (Verma and Reeves 1989). Four clones of Sendai (Se1-4) identified by MLEE are closely related to those of Paratyphi A (Selander et al. 1990b). Serotype Miami (1,9,12:a:1,5), though antigenically like Sendai, differs from it in being prototrophic, in not forming a fimbrial MREHA and in causing gastroenteritis rather than enteric fever. Strains of Miami are genetically diverse and are more closely related to serotype Panama (1,9,12:1,v:1,5) which also causes gastroenteritis, than to Paratyphi A or Sendai. Two clones of Miami (Mi1 and Mi2) are adapted to, but not invasive for, humans. Serotype Miami is not a biotype variant of Sendai and is correctly distinguished from Sendai in the Kauffmann–White scheme (Popoff and Le Minor 1992). The possible derivation of Paratyphi and Sendai from a Panama-like ancestor would have involved both a restriction in host range to humans and acquisition of invasiveness potential (Selander and Smith 1990).

Within *S.* Paratyphi A the increasing occurrence of strains with decreased susceptibility to ciprofloxacin, often associated with infections not only in patients in the Indian subcontinent but also with patients returning to developed countries, is becoming a major problem for the first-line treatment of infections (Chandel et al. 2000; Threlfall et al. 2003b).

PARATYPHI B (1,4 [5],12:B:1,2)

Paratyphi B is also known as Schottmuelleri, but this synonym is rarely used now. The phage typing method of Anderson (1964) differentiates 55 phage types and the biotyping scheme of Duguid et al. (1975) 56 full biotypes (Barker et al. 1988); used together, they provide excellent strain discrimination for epidemiological studies (Old and Barker 1989). Two groups of strains exist:

1. some cause enteric fever in humans, are rarely isolated from animals, usually form a mucoid (slime) wall at room temperature and 90 percent are *d*-tartrate nonfermenting (dT^-)
2. others cause gastroenteritis in humans, are also isolated from animals and food, do not form a slime wall and are usually *d*-tartrate fermenting (dT^+), i.e. are of biotype Java.

Cases of gastroenteritis occur sporadically but a few large, food-borne outbreaks have been reported, e.g. in the UK in 1988 (Anonymous 1994b) and in France in 1993 (Desenclos et al. 1996). In this latter outbreak, which was associated with contaminated goats' milk cheese, the symptoms were those of gastroenteritis and the implicated strain was of PT var. 3, which in the UK is normally associated with Java. Thus, it seems likely that the French outbreak was caused by strains of biotype Java, and not of Paratyphi B which is associated with paratyphoid illness in humans. Since 2002, there has been a massive increase in dT^+ Paratyphi B (biotype Java) in the Netherlands, with concomitant human infections. The majority of strains have been drug-resistant (van Pelt et al. 2003). Infections with similar strains possibly associated with imported poultry from the Netherlands have also been recorded in Scotland (Brown et al. 2003). The above findings clearly indicate the present confusion in nomenclature, which is important to resolve international comparisons of salmonella-associated disease. Discrimination between strains of dT^- (paratyphoid-like) and dT^+ (biotype Java, non paratyphoid-like) strains of *S.* Paratyphi B can be facilitated by MLEE, PFGE, IS*200* fingerprinting, and more recently, by the presence of the *sopE*, *sopD*, and *sopE1* virulence genes (Prager et al. 2003).

Of 23 ETs recognized by MLEE (Selander et al. 1990a, b), only three are significantly represented in natural populations: 89 percent of dT^- strains belong to clone Pb1, and 84 percent of dT^+ strains (Java) belong to clones Pb3 and Pb4. The traits of adaptation to humans and ability to cause enteric fever have evolved, probably recently, in only one clone (Pb1) which is globally distributed but otherwise phenotypically similar to the clones that cause gastroenteritis and are of broad host range (Selander et al. 1990a, b). The serotype is polyphyletic. Thus, clones Pb1, 2, and 3 are genetically close; but the four variant ETs of clone Pb5 (comprising monophasic strains) and clones Pb4, 6, and 7 are genetically closer to strains of Heidelberg, Saintpaul, and Typhimurium, from which they differ antigenically in H1 only, than to clones Pb1, 2, and 3 (Selander and Smith 1990).

TYPHI (9,12 [Vi]:d: -)

This human-adapted serotype is estimated to cause annually 12–21 million cases of typhoid fever in humans and up to 700 000 deaths (Thong et al. 1994; Ivanoff 1995; Mirza et al. 1996; Pang et al. 1998). With appropriate antimicrobial therapy, mortality is reduced from 30 to <1 percent. Multidrug resistance in Typhi strains has become a problem in many countries but is less so in Africa and South and Central America (Mirza et al. 1996). Ciprofloxacin is now the antibiotic of choice for multidrug-resistant strains, although the efficacy of this antimicrobial is rapidly becoming eroded. Children infected with drug-resistant strains are iller for longer periods and have higher mortality than those infected with drug-sensitive strains (Bhutta et al. 1991). Strains are homogeneous in biotype.

Primary discrimination of Vi^+ strains is best achieved by phage typing (Craigie and Yen 1938; Anderson and Williams 1956) which recognizes 106 PTs; Vi^- strains may be discriminated by PFGE. The number of IS*200* loci varies from ten to 25 (Gibert et al. 1990); whilst IS*200* profiling may distinguish between drug-resistant and sensitive strains within common PTs, such as E1 or M1, it is less discriminating than phage typing, e.g. the type strains of the commonest PTs (E1, M1, and A) have identical IS*200* profiles (Threlfall et al. 1993c, 1994e). Among Typhi strains from Indonesia, some (16 percent) express an H1 flagellar antigen of type j resulting from a deletion in the central antigen-determining part of the H1-d gene; others express a z_{66} flagellar antigen, thought to be an H2 locus (Guinée et al. 1981; Frankel et al. 1989; Selander et al. 1990b). Typhi strains of type 9,12,[Vi]:j:- are associated with milder clinical illness in older patients and are less motile and less invasive for HEp-2 cells in vitro than Typhi strains of type 9,12,[Vi]:d:- (Grossman et al. 1995).

Two clones were identified by MLEE: Tp1 (83 percent) was dominant worldwide and Tp2 (16 percent) was found in Africa, particularly in Senegal; dual infections with Tp1, Tp2 were observed in some patients in Senegal (Selander et al. 1990b). Indonesian strains, whether of H type d, j or z_{66}, belonged to Tp1. A very few (<1 percent) Typhi strains that do not have vi antigen genes are otherwise of similar MLEE profiles to Vi^+ strains of Tp1, Tp2. As measured by MLEE, Typhi strains are genetically distant from other salmonellae, including other serotypes that are host-adapted and/or invasive (Selander et al. 1990b).

Studies by Thong et al. (1997), have demonstrated that the genome size of *S.* Typhi isolates can differ by as 20 percent, which is suggestive of a large number of insertions and deletions. The gene sequence is also variable due to rearrangements caused by homologous recombination between rRNA genes (Liu and Sanderson 1995; Echeita and Usera 1998; Ng et al. 1999). The recent sequencing of the genome of a multiple drug-resistant strain of *S.* Typhi, isolated from a patient in Vietnam (Parkhill et al. 2001), has thrown new light on the genome structure of this important pathogen and will undoubtedly reveal much more about the fundamental structure of this important organism as well as resulting in significant advances in the development of molecular-based techniques for the diagnosis of typhoid fever.

TYPHIMURIUM (1,4,[5],12:I:1,2)

Typhimurium is one of the top three serotypes isolated world wide over many decades from humans, domestic and wild animals, foodstuffs, and the environment, with cattle and poultry important sources of infection for humans (Palmer and Rowe 1986). In the UK, multiple-drug resistance (MDR) to >4 antimicrobial agents has increased 12-fold in isolates from humans in the period 1981–94 (Frost et al. 1995) and in 2000, 82 percent of isolates were drug-resistant of which > 70 percent were multiresistant (Threlfall et al. 2003c); since 1980 the majority of isolates from bovine hosts showed MDR which also increased in pigs and, to a lesser extent, in poultry (Threlfall et al. 1993a). In strains of types prevalent from 1981 to 1990, e.g. DTs 204, 204c, and 193, MDR was plasmid mediated (Threlfall et al. 1986, 1994b). Since 1992, a particular cause for concern in the UK has been Typhimurium of DT 104, which is currently endemic in cattle and has caused many human infections. Broad spectrum MDR in strains of DT 104 is chromosomally located (Threlfall et al. 1994b, 1996a), and additional chromosomal resistance to quinolones has been found in poultry isolates (Frost et al. 1995). In 2001, a major outbreak of MDR DT 104 in England and Wales was epidemiologically associated with contaminated lettuce (Horby et al. 2003). Additional discrimination within Typhimurium phage types can be achieved by biotyping (Duguid et al. 1975; Anderson et al. 1978; Barker and Old 1979, 1980); their combined use is highly discriminatory and provides excellent strain characterization for epidemiological purposes (Old and Barker 1989). However, these methods have now been to all intents replaced by molecular methods, of which PFGE has become the 'gold standard' for discrimination within phage types (see above).

Detailed genetic studies of the main biotype markers identified major clones, e.g. the FIRN (fimbriation-, inositol- and rhamnose-negative) group (Old and Duguid 1979; Barker and Old 1989). Additional strain discrimination within the serotype or phage types can be achieved, where needed, by molecular methods (see Threlfall and Frost 1990; Threlfall et al. 1994a). MLEE analysis of natural populations has identified 17 closely related clones in this monophyletic serotype that cluster with clones of Heidelberg, Saintpaul, and Paratyphi B/Java (Beltran et al. 1988, 1991; Selander and Smith 1990; Selander et al. 1990a, b).

Sequencing of the genome of *S.* Typhimurium strain LT2 (McClelland et al. 2001) has increased our understanding of the fundamental structure of this organism and should lead to major advances both in diagnosis and typing as well as contributing to an understanding of the factors contributing to the pathogenicity of the organism in humans and food production animals.

VIRCHOW (6,7:R:1,2)

Before 1975, this serotype accounted for <1 percent of isolations from humans in the UK but during 1981–94 it became the third commonest serotype, accounting for 9 percent of human isolations (Frost et al. 1995). A phage-typing scheme (Chambers et al. 1987), based on 13 phages and distinguishing 57 phage types (Torre et al. 1993), is the method of choice for primary strain discri-

mination; however, six phage types (PTs 8, 31, 26, 21, 19, and 2, in ranking order) accounted for 62 percent of all isolates in 1992 (Threlfall et al. 1993a). This phage-typing scheme has now been extended and 68 types are recognized using 15 phages (Ward L.R., unpublished). For further discrimination within major phage types, plasmid profiling is better than IS*200* sequence distribution (Torre et al. 1993), although as with Typhimurium, PFGE has become the method of choice for subdivision within phage type. Poultry, particularly chicken, is a major source of infection for humans (Reilly et al. 1988; Threlfall et al. 1993a).

There has been a significant increase (16–71 percent) in drug resistance in Virchow between 1981 and 1999 with many (9–19 percent) strains showing multiple resistance to >4 antimicrobial agents (Threlfall et al. 1993a, 2000; Frost et al. 1995). Phage types in which resistance was common included PT 19 associated with poultry imported from France; in contrast, multiple-drug resistance was less common in home-produced poultry (Torre et al. 1993; Threlfall et al. 2003c). Quinolone resistance has emerged in different phage types, possibly associated with the use of enrofloxacin. Diverse studies have shown that among nontyphoidal salmonellae, Virchow has a high potential for invasion in humans with high incidences of isolations from blood (Mani et al. 1974; Todd and Murdoch 1983; Wilkins and Roberts 1988; Threlfall et al. 1992b). Hence, the emergence of resistance to the likely therapeutic drug of choice in invasive Virchow infection causes much concern.

Although the vast majority of salmonellae recovered from humans in developed countries are serotypes from subsp. I, e.g. 99.73 percent of 67 767 isolates in France in a period of 4 years (Le Minor et al. 1985), evidence increasingly suggests that some serotypes from other subspecies may be pathogens of humans and animals and these will now be considered.

Subspecies II

The biochemical characters for identification of *S. enterica* subsp. *salamae* are given in Table 1. The O and phase-1 H antigens of most of the 477 serotypes described are similar to those of serotypes from subsp. *enterica* (Rohde 1965; Popoff and Le Minor 1992). Most retrospective studies have confirmed their rare occurrence in humans; thus, only 27 isolates of serotypes of subsp. II were found among 81 936 isolates, mostly from humans, reported in different surveys, e.g. Central Africa before 1960 (see Schrire et al. 1987), Malaysia 1973–82 (Jegathesan 1984) and France 1980–83 (Le Minor et al. 1985). The main reservoirs of this subspecies are healthy or diseased reptiles and diverse cold-blooded animals (Rohde 1965).

The first indication that strains of subsp. II might be found more often in human populations closely associated with the indigenous fauna of remote areas in some countries came from Iveson et al. (1969) in Western Australia. More recently, a relatively high frequency (6.4 percent) of isolation of subsp. II strains from humans was evident in southern Africa in the period 1979–84 (Schrire et al. 1987). Many isolates were from patients with symptoms of gastroenteritis or dysentery and, whilst most of them occurred sporadically, some clustering of cases was noted, e.g. of the former serotype Mobeni from children in an institutional outbreak. Isolates belonged to 203 serotypes, 45 of them newly described types present in both Namibia and South Africa. Schrire et al. (1987) speculated that a combination of poor conditions of hygiene and contamination of food and water supplies by wild animals contributed to the greater frequency of human infections with strains of subsp. II and also to the widespread presence of so many exotic serotypes in humans in that region.

Subspecies IIIa and IIIb

The members of these two subspecies (previously designated as *Salmonella* subgenus III or '*Arizona hinshawii*') are now named *S. enterica* subsp. *arizonae* (monophasic strains) and *S. enterica* subsp. *diarizonae* (diphasic strains); 94 and 317 serotypes, respectively, have been identified (Popoff et al. 1995). The biochemical properties helpful in their identification are presented in Table 2. Soon after their first isolation from reptiles in western states of the USA, it became clear that they were an important cause of diarrheal illness in turkey poults, with spread traced from farm to farm and infection transmitted by hatchery eggs (see Edwards et al. 1959). As avian pathogens, particularly of chickens and turkeys, they are still of worldwide economic importance. The major serotype of subsp. *arizonae* in turkeys for 30 years has been 18:z_4,z_{32}:-; but other serotypes (18:z_4,z_{23}:- and 53:z_4,z_{23}:-), formerly important in turkeys, are now rarely isolated from them (Edwards et al. 1956; Weiss et al. 1986). The serotypes present in other avian species are not necessarily those prevalent in turkeys. For example, in earlier surveys, serotype 40:z_4,z_{23}:- of subsp. IIIa was responsible for 50 percent of chicken infections but was rare in turkeys (Edwards et al. 1956).

Epidemiological surveys in Australia have indicated that the distribution of serotypes of these two subspecies in reptiles, both captive and wild, is similar and not influenced by their proximity to humans (Iveson et al. 1969). Again, there are subtle differences in the serotypes carried by different reptilian species (Weiss et al. 1986) and wild snakes are known to yield more commonly strains of serotypes from *arizonae* than *diari-*

zonae (Edwards et al. 1956; Weiss et al. 1986). Reptiles remain an important reservoir of these subspecies (Weiss et al. 1986). Consumption of rattlesnake meat or powder is an unusual source of extraintestinal infections in patients with chronic medical conditions including AIDS (Noskin and Clarke 1990).

Members of these subspecies are often considered uncommon human pathogens, but reports of their involvement in serious human illness are not new (Edwards et al. 1959). In a survey in the USA over 30 years (Weiss et al. 1986), 374 isolates of human origin included representatives of 71 serotypes; the four most common were one from subsp. *arizonae* (18:z_4,z_{32}:-) and three from *diarizonae* (61:l,v:1,5,7; 61:k:1.5,7; and 60:r:e,n,x,z_{15}); together they accounted for 55 percent of all human isolates. Serotypes 18:z_4,z_{23}:- and 21:g,z_{51}:- from *arizonae* and 61:k:1,5,7 from *diarizonae* were isolated more commonly from extraintestinal than intestinal sites and accounted for 50 percent of all extraintestinal isolates. Weiss et al. (1986) suggested that strains of these three serotypes are more invasive than other serotypes of these two subspecies and that they are associated with higher mortality in, and have enhanced virulence for, humans. The proportion of these isolates giving rise to serious septicemic and pyemic illnesses is greater than for most other salmonella types (see Johnson et al. 1976). In a survey in the UK over 25 years (Hall and Rowe 1992), 66 isolates of human origin included representatives of 29 serotypes; the most common was 61:l,v:1,5,7 which was also the commonest from terrapins. Many (35 percent) patients had a history of foreign travel. However, 41 percent of UK patients were children of <4 years of age, many of whom had not been abroad and for whom contact with terrapins or other reptiles, especially pet snakes, was a potential source of infection (Hall and Rowe 1992). In the UK series, only two patients had extraintestinal infections.

Differences in the frequency with which individual serotypes occurred in humans over the years probably reflect corresponding adaptations of individual serotypes in different animal hosts. Thus, serotype 18:z_4,z_{32}:-, the prevalent one in turkeys for several decades, is now the most common type found in humans (Weiss et al. 1986) from whom it was absent 30 years ago (Edwards et al. 1956). Outbreaks of human infection are predominantly food-borne (Edwards et al. 1959), and important serotypes prevalent in domestic animals like cattle (61:l,v:1,5,7) and sheep (18:z_4,z_{32}:-; 61:l,v,:1,5,7; 61:k:1,5,7) are also frequently isolated from humans.

Subspecies IV–VI

Most of the 66 serotypes of *S. enterica* subsp. *houtenae* (subsp. IV) belong to high O serogroups and are usually isolated from cold-blooded animals, particularly in the tropics, and from the inanimate environment. Available evidence suggests that they are isolated infrequently from humans in developed countries (Le Minor et al. 1985). Ten serotypes of *S. enterica* subsp. *indica* (subsp. VI) and 19 serotypes of *S. bongori* (Reeves et al. 1989), formerly designated as *S. enterica* subsp. *bongori* (subsp. V), have been defined (Popoff et al. 1995). The biochemical properties of these three groups are detailed (see Table 2); however, information about their epidemiology is sparse.

PATHOGENICITY AND DISEASE

Natural infections

Salmonellae cause disease in a wide range of species of vertebrates; most of the serotypes pathogenic in mammals and birds belong to subsp. I. Broadly speaking, the disease may present in three ways. First, a few host-adapted serotypes habitually cause systemic disease in their hosts. When the manifestations of systemic disease are mainly septicemic, as is usually the case with typhoid and paratyphoid bacilli in humans, the clinical picture is one of enteric fever with an incubation period of 10–20 days, but with outside limits of 3 and 56 days depending on the infecting dose (Mandal 1979). Diarrhea, starting 3–4 days after onset of fever and lasting, on average, 6 days, may occur in 50 percent of cases of typhoid fever and is more common in younger, than in older, children or adults (Roy et al. 1985); intestinal symptoms, however, may be absent or insignificant. Second, certain other serotypes – Blegdam, Bredeney, Choleraesuis, Dublin, Enteritidis, Panama, and Virchow in humans and Gallinarum in adult fowl – are also invasive but tend to cause pyemic infections and to localize in the viscera, meninges, bones, joints, and serous cavities. Third, most other salmonellae, the ubiquitous serotypes found in a number of animal species, tend to cause an acute, but mild, enteritis with a short incubation period of 12–48 h, occasionally as long as 4 days.

Nevertheless, these distinctions are not clear-cut. Local abscesses and even pyemia may develop occasionally as a late complication of a diarrheal disease. Serotypes that usually cause enteritis in healthy adults may cause septicemia or pyemic infections in young or elderly patients. The incidence of laboratory-confirmed salmonellosis in AIDS patients is 20–100 times that in the general population (Angulo and Swerdlow 1995). In American states with a high incidence of AIDS, the proportion of salmonella isolations from blood among men 25–49 years of age increased from 2.8 percent before the HIV epidemic (1978–82) to 14.2 percent in 1983–87, with Enteritidis, Typhimurium, and Dublin being more opportunistically invasive than other serotypes (Levine et al. 1991). Recurrent nontyphoidal salmonella septicemia (RSS) has been included as an AIDS-defining illness since 1987. Typhimurium,

generally associated with enteritis in humans, may give rise to more severe disease in other hosts. Again, the typhoid bacillus, often considered to cause mild and atypical infections in children, will give rise to severe infections with high mortality in children whose previous health and nutrition have been poor (Scragg et al. 1969).

Convalescent patients may continue to excrete salmonellae, usually in the feces or urine but from other sites after pyemic infections, long after clinical cure. Asymptomatic persons may also harbor salmonellae unknowingly. Both remain potentially infectious for weeks or months and some become life-long excreters.

Experimental studies and virulence determinants

Extremely large doses of typhoid bacilli given orally or intravenously to chimpanzees produce an illness closely resembling typhoid fever in humans (Gaines et al. 1968) though the incubation period is shorter and ulceration of the lymphoid tissue of the small intestine does not occur. In mice and other small laboratory animals, oral administration of Typhi does not give rise to typhoid fever; when injected in small doses, there is no evidence that Typhi multiplies freely in their tissues. With some virulent strains of Typhi, it is necessary to inject $>10^7$ bacilli into the mouse peritoneum to cause death; but similar doses of killed bacilli will cause a purely toxemic death. Fatal infections may, however, be produced in mice when smaller doses (10^2–10^7 bacteria) are given intraperitoneally with agents such as gastric mucin that depress host resistance. Typhi, therefore, is not an invasive pathogen for small laboratory animals; its failure to cause disease in these species may result from its inability to compete successfully for iron essential for its growth (O'Brien 1982).

Typhimurium infection in the mouse is, however, a useful model for typhoid fever in humans. Orally administered organisms pass rapidly through the mouse intestine with only a small proportion found in the large intestine after the first few hours. Bacteria infect the ileal mucosa and Peyer's patches and spread thereafter to the draining lymph nodes. Typhimurium is resistant to killing by phagocytic cells and spreads through the lymphatics to form infective foci in the spleen and liver (Carter and Collins 1974).

INVASIVE INFECTION

The factors responsible for the virulence of salmonellae are still ill-defined, although factors contributing to the virulence of different serotypes for food production animals are becoming more clear. The following sections summarize our understanding of the complex interactions between different salmonella serotypes, their hosts and host cells. Molecular techniques have helped to elucidate some of the critical steps in these host–pathogen interactions and to identify genes involved. The ability of salmonellae to invade, survive, and replicate within eukaryotic cells is essential for successful infection. Salmonellae possess several factors which contribute to the disease process; these virulence-promoting factors are generally conserved among species, as are the mechanisms by which bacteria interact with the host cell. Such factors facilitate entry into (invasion of) nonphagocytic cells, survival in the intracellular environment and replication within host cells (for reviews, see Finlay et al. 1992; Guiney et al. 1995).

After oral ingestion of organisms in sufficient numbers to promote onset of disease, the next stage in the disease process is penetration of the intestinal epithelium. Transmission electron microscopy has revealed that invasive salmonellae interact with the well-defined apical microvilli of epithelial cells, thereby disrupting the brush border. Once these cells have been penetrated, the bacteria are enclosed in membrane-bound vacuoles within the host cytoplasm. Virulent salmonellae survive and multiply within these vacuoles, eventually laying their host cells and disseminating to other parts of the body. Salmonellae also depolarize epithelial barriers by affecting the integrity of tight junctions between cells, which has the result of promoting significant cytotoxic damage to epithelial cells.

The induction of protein synthesis de novo is necessary for invasion and is regulated by the microenvironment (low oxygen), growth phase or the epithelial cell surface. Only viable, metabolically active salmonellae can adhere to host cell surfaces. Adherence is followed almost immediately by internalization into host cells. The bacterial genes necessary for salmonellae to enter eukaryotic cells have yet to be fully characterized but at least six different genetic loci appear to be involved and an additional hyperinvading locus has been identified (Finlay et al. 1992; Guiney et al. 1995).

Internalization of salmonellae through the apical surface of epithelial cells is associated with disruption of the eukaryotic brush border. After a lag period of about 4 h, invading organisms begin to multiply within the vacuoles of host epithelial cells. For Typhimurium, it has been demonstrated that this intracellular replication is essential for pathogenicity. Salmonellae remain within membrane-bound inclusions and their survival appears to involve blockage of phagosome-lysosome fusion events.

The involvement of *Salmonella* pathogenicity island (SPI)2 in the translocation of secreted effector proteins into target epithelial cells in mice has been elucidated (Wallis and Galyov 2000). More recently, the use of signature-tagged mutagenesis has helped identify genes in the SPI-2 in *S.* Dublin, responsible for the induction of both systemic and enteric salmonellosis in calves (Bispham et al. 2001). It is possible that such studies will contribute to the development of effective vaccines for the prevention of *S.* Dublin infection in cattle.

SEROTYPE-SPECIFIC PLASMIDS

Analysis of the plasmid content of salmonella strains belonging to a range of serotypes of epidemiological importance in humans and food animals has demonstrated that some (but not all) serotypes possess plasmids of high molecular mass that may be regarded as SSPs. Serotypes harboring such plasmids include Enteritidis, Typhimurium, Dublin, Choleraesuis, Pullorum, and Gallinarum. In contrast, SSPs have not been identified in serotypes such as Virchow and Hadar, both of which cause disease in humans, nor in Typhi. These plasmids, which range in molecular mass from about 30 000 kDa (Choleraesuis) and 38 000 kDa (Enteritidis) to 62 000 kDa (Typhimurium) (Helmuth et al. 1985), are generally nonconjugative and do not carry easily identifiable phenotypic markers such as drug resistance.

The results of animal challenge experiments have indicated that SSPs are involved in the virulence in their host strains for certain strains of inbred mice, e.g. BALB/c. Studies by Pardon et al. (1986) and Gulig and Curtis (1987) have demonstrated that the 62 000 kDa SSP of Typhimurium genetically determined its spread beyond the small intestine to deeper tissues such as the mesenteric lymph nodes or the spleen. They also observed the attenuation of virulence of plasmid-cured strains in parenterally inoculated mice.

Although SSPs from different serotypes possess substantial regions of nonhomologous DNA, all possess a highly conserved common region which carries salmonella plasmid virulence (*spv*) genes (Gulig et al. 1993) responsible for their virulence in mice (Williamson et al. 1988a). In Dublin, *spv* genes have been shown to be clustered within an 8 kb *Sal*I-*Xho*I restriction endonuclease fragment (Williamson et al. 1988b) and a homologous region has been identified in the SSPs of Typhimurium, Enteritidis, Choleraesuis, and several other serotypes (Williamson et al. 1988a). Within this region, a highly conserved 3.5 kb *Hin*dIII fragment has been shown to have homology with the virulence regions of the SSPs of Dublin and Enteritidis (Woodward et al. 1989).

Six *spv* genes within the common 8 kb *Sal*I-*Xho*I fragment have now been sequenced, namely *spvR*, *spvA*, *spvB*, *spvC*, *spvD*, and *spvE*. The *spvE* gene encodes a 13 kDa protein, the *spvD* gene a 25 kDa protein and the *spvC* gene a 28 kDa protein, which is located both in the outer membrane and the cytoplasm. The latter protein has a demonstrable role in virulence, as it has been shown that transposon insertion mutants in the *spvC* gene of SSP result in a significant drop in the number of Typhimurium isolated from the spleen of orally infected mice. Moreover, only one-fifth of these mice died after infection with an SSP transposon-attenuated strain as compared to those infected with wild-type bacteria (Norel et al. 1989). The *spvB* gene encodes a 65 kDa protein of unknown function which is located both in the outer membrane and the cytoplasm, and the *spvA* gene a 28 kDa protein also of unknown function.

Although essential for the virulence of most strains for certain strains of mice, the role of SSP virulence plasmids in the pathogenesis of disease in humans and other animal species is not clear. Results with cattle have suggested that these plasmids are required for bacteremia (Pardon et al. 1986) but not necessarily for gastroenteritis. SSP-like plasmids have been shown to be necessary for the intracellular survival of their host organisms in mouse phagocytes (Buchmeier and Heffron 1991) and it has been suggested that they may enhance survival of bacteria in extraintestinal infections in humans (Fierer et al. 1992). However, studies have demonstrated that for Enteritidis, the possession of an SSP is not necessary for the induction of enteritis in humans (Threlfall et al. 1994d). Likewise, similar SSPs have not been identified in certain phage types of Typhimurium isolated from cases of diarrhea in humans (Brown et al. 1986).

TOXINS

Early evidence suggesting that food-poisoning salmonellae of diverse serotypes produced 'enterotoxins' with biological and antigenic properties of cholera-like (CT) toxins (Sandefur and Peterson 1977; Jiwa 1981; Finkelstein et al. 1983) could not be confirmed by other workers (Wallis et al. 1986a; Clarke et al. 1988).

Other possible explanations for salmonella-induced diarrhea in gastroenteritis invoke mechanisms involving some kind of immune response in the intestine or salmonella entry into enterocytes. Support for these latter hypotheses comes from the following observations:

- interaction between salmonellae and inflammatory cells leads to release of prostaglandins mediating fluid secretion, which is reversible in monkeys by inhibitors of prostaglandin synthesis (Gianella et al. 1977; Gianella 1979)
- microscopy studies of experimental Typhimurium infection show that fluid secretion is preceded by extensive infiltration of inflammatory cells and that cell damage, when it occurs, is limited to the tips of the villi and shortened villi may lead to enhanced secretion (Wallis et al. 1986b)
- salmonellae induce epithelial cells to secrete IL-8, a potent chemoattractant of neutrophils which might contribute to inflammation, cell damage and fluid secretion and
- salmonella entry into epithelial cells in monolayers brings about many biochemical changes including the phosphorylation of epidermal growth factor (EGF) receptor and activation via phospholipase A2 of leukotriene synthesis which may result in actin rearrangements and disruption of the integrity of the tight junctions on epithelial cells, with resultant cytotoxic damage and fluid loss (Finlay et al. 1992; Pace et al. 1993).

There is, therefore, a range of mechanisms whereby salmonellae might cause gastroenteritis and diarrhea production may be multifactoral.

In both experimental and natural conditions, salmonella infections may occur via the conjunctivae, by inhalation and by insect bites as well as by the oral route. No attempt will be made here to describe in detail the infections in humans and other animals by other serotypes, but some information will be found in the descriptions of individual serotypes and also in earlier editions of this book.

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Proteus, *Morganella*, and *Providencia*

BERNARD W. SENIOR

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The bacteria of the genera *Proteus*, *Morganella*, and *Providencia* belong to the tribe Proteeae in the family Enterobacteriaceae. They have one biochemical characteristic that distinguishes them from all other members of the Enterobacteriaceae, with the exception of the rare genera *Tatumella* and *Rahnella*: the ability to deaminate oxidatively certain amino acids to the corresponding keto acid and ammonia. Tests for phenylalanine or tryptophan deaminase are the ones commonly made and a rapid method for this reaction has been devised (Giammanco et al. 1985). Members of the tribe are also readily recognized by the red-brown, melanin-like pigment they form when cultured under aerobic conditions on media containing iron and an aromatic L-amino acid, such as phenylalanine, tryptophan, tyrosine or histidine (Polster and Svobodova 1964; Müller 1985).

In addition, most strains of species of *Proteus*, *Morganella*, and *Providencia* share the following common but not unique characteristics: motility, resistance to KCN (except *Prov. heimbachae*), the ability to degrade tyrosine (except *P. myxofaciens*) (Sheth and Kurup 1975) and an inability to acidify lactose, dulcitol, and malonate or form arginine dihydrolase or β-galactosidase or decarboxylate lysine (except for some *M. morganii* strains). All are methyl-red positive and, except for some *Proteus* spp., Voges–Proskauer negative.

PROTEUS

DEFINITION

Proteus spp. give a characteristic spreading growth ('swarming') on appropriate solid media, oxidatively deaminate amino acids, hydrolyze urea but fail to acidify mannose. The G + C content of the DNA is 38–40 mol%.

The genus, named after the Greek deity Proteus who had the ability to assume different shapes, presently has five named species: *Proteus mirabilis* and *Proteus vulgaris* (formerly known together as *Proteus hauseri*), *Proteus myxofaciens* described by Cosenza and Podgwaite (1966), *Proteus penneri* (formerly *P. vulgaris* biotype 1) described by Hickman et al. (1982), and *Proteus hauseri* (formerly *P. vulgaris* biogroup 3) described by O'Hara et al. (2000) in honor of Gustav Hauser who first described the genus (Hauser 1885). The

genus also contains three unnamed genomospecies (O'Hara et al. 2000).

The type strain of *P. mirabilis* is ATCC 29906, NCTC 11938, CDC PR14; 9165-79. The type strain of *P. vulgaris* is ATCC 29905, NCTC 13145, CDC 9166-79. The type strain of *P. penneri* is ATCC 33519, NCTC 12737, CDC 1808-73. The type strain of *P. hauseri* is ATCC 700826, CDC 1732-80. The type strain of *P. myxofaciens* is ATCC 19692, CDC 9338-76.

MORPHOLOGY

After growth for 24–48 h on solid media, most cells are rods, 1–3 μm long by 0.4–0.6 μm wide, though short, fat, coccobacillary forms are not uncommon. In young cultures that are swarming (see section on Swarming) on solid media, many of the cells are long, curved, and filamentous, reaching 10, 20 and even up to 80 μm in length. In older cultures, the organisms have no characteristic arrangement: they may be distributed singly, in pairs or in short chains. However, in young swarming cultures, the filamentous cells tend to be arranged concentrically like the isobars in a diagram of a cyclone.

Except for nonflagellate variants and those with paralyzed flagella, all strains in young cultures are actively motile by peritrichate flagella. The flagella are more variable in shape than those of most other enterobacteria, normal and 'curly' forms sometimes being found together on the same organism and even in the same flagellum. The form of the flagellum is also influenced by the pH of the medium. *P. mirabilis* has several genes coding for flagella, but normally only one type of flagellin, FlaA, a protein of 40 kDa is formed. However, it may undergo antigenic variation as a result of spontaneous mutation and this may be an important survival mechanism in vivo (Belas 1994).

Under appropriate cultural conditions, strains of *Proteus* species form one or more of at least six different types of peritrichous fimbriae. Some are associated with virulence. Many have been purified and their genes cloned. Some of the fimbriae can be distinguished morphologically and by their hemagglutinating characteristics. Among these, mannose-resistant *Proteus*-like (MR/P), channelled, thick (7–8 nm in diameter) fimbriae and mannose-resistant *Klebsiella*-like (MR/K), nonchannelled, thin (4–5 nm in diameter) fimbriae are frequently found in *Proteus* strains. By contrast, mannose-sensitive (MS), channelled, thick (8–9 nm in diameter) fimbriae are found only infrequently (Old and Adegbola 1982; Adegbola et al. 1983; Yakubu et al. 1989). Other fimbriae such as ambient temperature fimbriae (ATF) (Massad et al. 1994a), *Proteus mirabilis* fimbriae (PMF) (Massad et al. 1994b) and uroepithelial cell adhesin (UCA) (Wray et al. 1986) are unable to agglutinate erythrocytes. A given cell of most isolates will often bear more than one type of fimbria and it is unusual to find a strain bearing only MS, or MR/P fimbriae.

CULTURAL CHARACTERISTICS

Proteus strains grow well on ordinary media, but most strains, particularly if grown at 22–30°C, do not form discrete colonies on nutrient agar. Instead they exhibit spreading growth (see Swarming) even on the surface of appropriate well-dried media. In nutrient broth, *Proteus* gives rise to a uniform turbidity with a slight to moderate powdery deposit and a faint ammoniacal odor. A thin, fragile pellicle may develop in old cultures. In minimal salts media, *Proteus* shows a requirement for nicotinic acid.

A number of selective and indicator media have been designed for *Proteus* and the other genera of the Proteeae. That of Malinowski (1966) incorporates alanine, potassium nitrate, and dyes so that the alanine decarboxylating strains of the tribe give rise to blue-gray colonies distinct in color from both other lactose non-fermenters including *Salmonella* and *Shigella* spp. (white-gray) and also *Escherichia coli* (red) which cannot decarboxylate this amino acid. An infusion agar containing bile salts, lithium chloride, sodium thiosulfate, and sodium citrate inhibits the growth of almost all enterobacteria, except Proteeae (Xilinas et al. 1975). The medium of Hawkey et al. (1986) contains tryptophan and tyrosine and, to inhibit gram-positive bacteria, clindamycin. Through oxidative deamination of tryptophan, and tyrosine degradation, strains of the Proteeae form dark brown colonies surrounded by a clear area of degraded tyrosine crystals.

Swarming

This is a characteristic feature of most isolates of *Proteus* spp. in which a group of cells at the edge of a developing microcolony migrate to an uninoculated area of the medium. It is a good example of 'quorum sensing' in which the initiation and regulation of transcription of particular genes occurs only when a critical cell density is reached. However, swarming is not unique to *Proteus* and similar behavior can be found in some other bacteria outwith the tribe, such as *Serratia marcescens* and *Vibrio parahaemolyticus*. The process of swarming growth in *Proteus* is exceedingly complex and probably involves more than 50 genes. A simplified scheme of one cycle of swarming in *Proteus* is presented in Figure 1.
During an incubation period of about 2–4 h at 37°C, the bacterial cell, which is in the form of a sparsely flagellate, short (2–4 μm) bacillus, referred to here as a vegetative cell, receives a number of signals. The signals from the environment include, for example, its viscosity, temperature, the presence of a solid surface, and the composition and concentration of nutrients. The signals

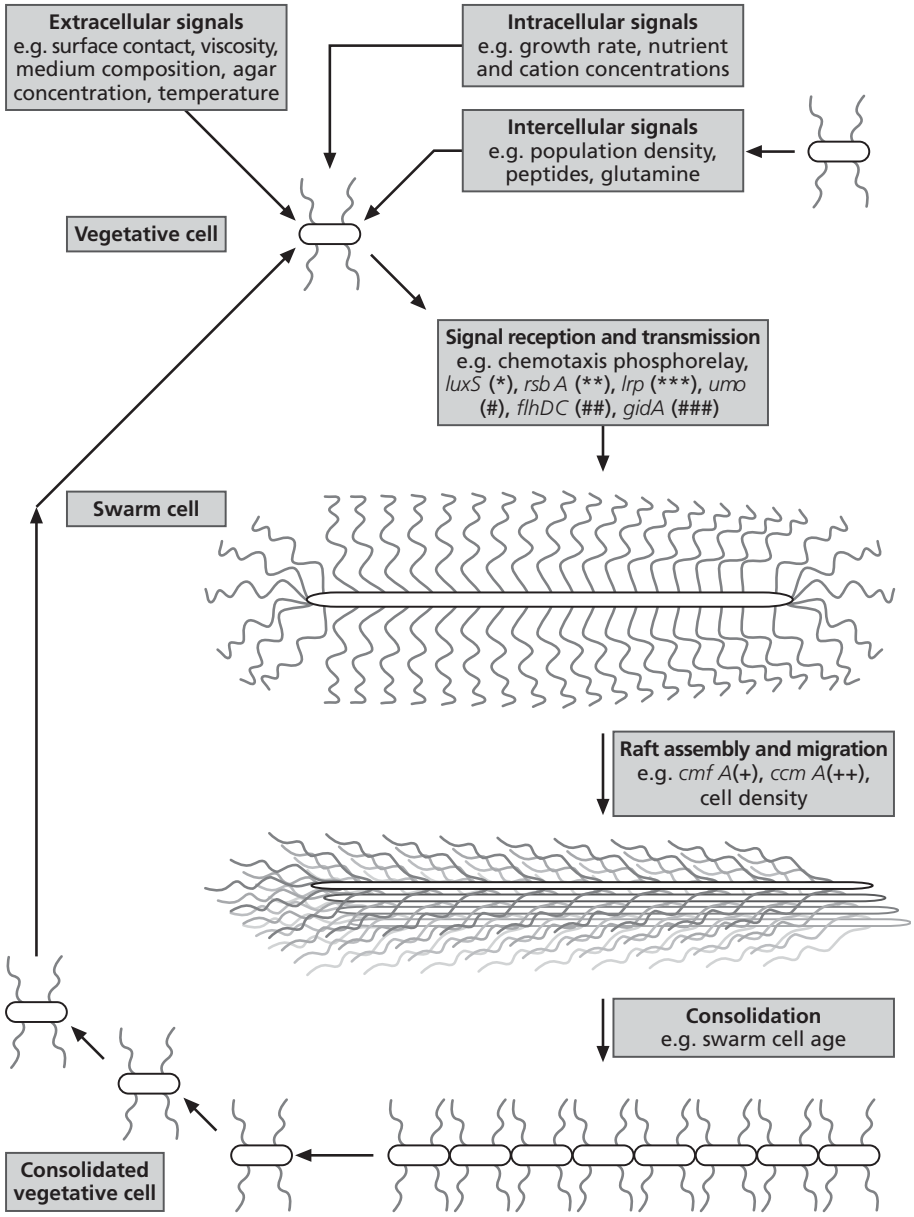

Figure 1 *Simplified scheme of some of the factors and genes involved in the cycle of swarming growth in* Proteus. *For further information on particular genes see: *Schneider et al. 2002; **Belas et al. 1998; ***Hay et al. 1997; #Dufour et al. 1998; ##Furness et al. 1997; ###Belas et al., 1995; +Gygi and Hughes 1995; ++Hay et al. 1999.*

from within and between the vegetative cells include, among other things, growth rate, population density, and the presence and concentration of particular peptides and amino acids such as glutamine (Allison et al. 1993) and other key substances such as the quorum sensing autoinducer 2 (AI-2), a derivative of furanone, produced by *luxS* (Schneider et al. 2002). The signals are received and transmitted between the vegetative cells by a variety of mechanisms which may include a chemotaxis two-component phosphorelay regulatory system, *rsbA*, a two-component membrane sensor kinase that coordinates the initiation of swarming (Belas et al. 1998), *lrp* a global transcriptional regulator linking physiological signals to swarmer differentiation (Hay et al. 1997), *flhDC* the flagellar master operon that regulates the synthesis of flagella and cell septation (Furness et al. 1997), *umo* genes which upregulate *flhDC* (Dufour et al. 1998), and *gidA* which is involved in the initiation of chromosome replication (Belas et al. 1995).

The consequence of the reception and transmission of the many signals is the coordinate transcription of many genes and the inhibition of transcription of others. This results in the synthesis, assembly, and movement of flagella, the de novo synthesis of qualitatively different lipopolysaccharides and exopolysaccharides, the synthesis of an acidic capsular polysaccharide (Rahman et al.

1999), alterations in membrane fatty acid composition and fluidity, peptidoglycan synthesis, cell division, and proteolysis of peptides (Belas et al. 1995; Fraser and Hughes 1999; Gue et al. 2001). The cell now synthesizes several hundreds of flagella and continues to grow in length, but not in width, because septum formation and cell division are inhibited, as are the synthesis of non-agglutinating fimbriae (NAF) and mannose-resistant/*Proteus* like (MR/P) pili (Latta et al. 1999). Eventually a multinucleate, densely flagellate, nonseptate, elongated cell 20–80 μm in length, known as a swarm cell, is formed. The enzymic activity, antibiotic sensitivity, cell wall permeability, lipopolysaccharide composition, and response to amino acid attractants of the swarm cell differ from those of the vegetative cell. The swarm cell is also different from the vegetative cell in that it now secretes virulence factors such as urease, hemolysin, and protease (Allison et al. 1992a).

After a further lag period of about 3 h at 37°C, a series of events causes the swarm cells to migrate. The process is not haphazard and it requires close contact between swarm cells. This ultimately may facilitate signalling between cells and coordinate flagellar action. The swarm cells first align themselves in small groups along their long axes. Mutant swarm cells which lack the gene *ccmA*, for an integral membrane protein, develop as curved cells and are unable to migrate, possibly because they are not aligned along their long axes (Hay et al. 1999). The swarm cells through *cmfA* secrete a capsular polysaccharide (Gygi and Hughes 1995) which may help to stabilize the group of swarm cells. Then by multicellular interaction and signaling, probably influenced by swarm cell density and age, there is the simultaneous and co-ordinated migration in unison with swarm cells. They emerge from the edge of the colony in small groups as rafts and, facilitated by the acidic polysaccharide which possibly creates a fluid environment by extracting water from the agar beneath the swarm cells, swarm a short distance before returning to the colony. As the swarming period proceeds, the cells move out in larger groups and travel further before returning. The duration and extent of this swarming period is highly variable according to the strain and the cultural conditions. The swarming growth on a plate may eventually appear as a uniform film of growth extending over the whole plate (continuous swarming) or as a series of concentric circles of growth around the point of inoculation (discontinuous swarming; Figure 2). In discontinuous swarming, migration usually ceases after about 2 h and the cells enter a period of consolidation during which the swarm cells differentiate back to the vegetative short bacillary form by division at several positions along the length of the swarm cell. The vegetative cells then grow and multiply until such a time as swarm cell formation is initiated again and the process is repeated. This cyclical swarming and consolidation, which may occur every 4 h or so, gives rise to the series of alternating concentric circles of thick and thin growth over a plate (Figure 2).

The cause of swarming has given rise to much speculation. Lominski and Lendrum (1942, 1947) proposed that swarming was the outcome of a negative chemotactic response to metabolic products that accumulated in agar in areas of high population density. They implied that swarm cell formation was stimulated by the toxic products. This theory may not be correct, for although *Proteus* cells are believed to form toxic products (indeed the antiswarming effect of charcoal has been attributed

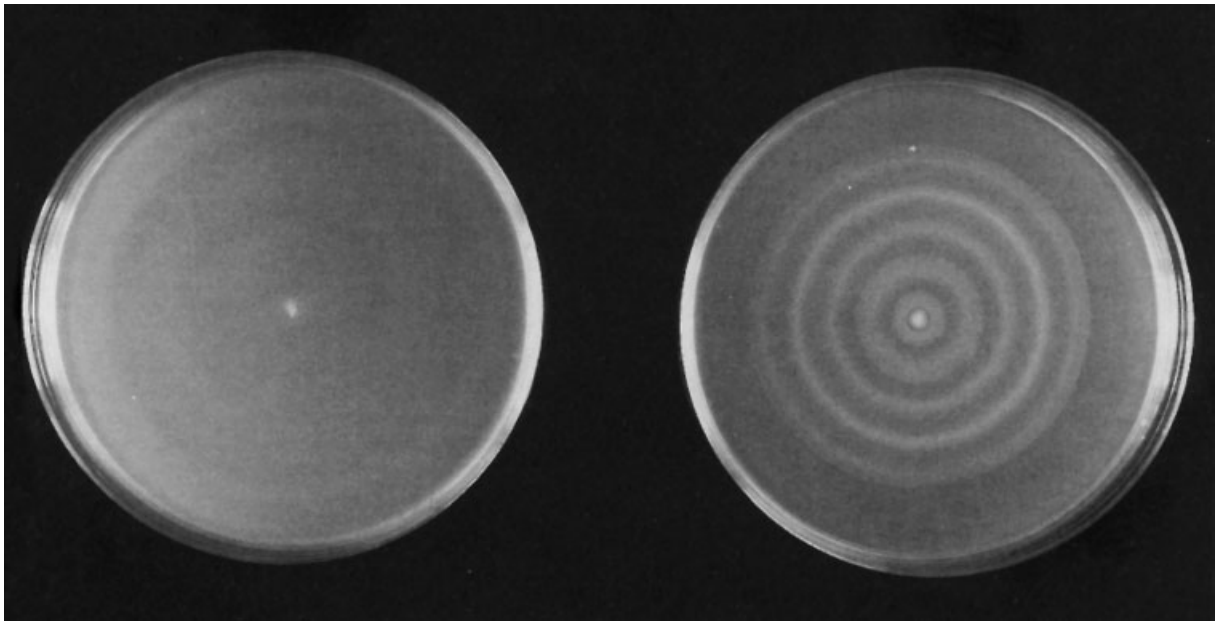

Figure 2 *Swarming growth given by* Proteus *strains: left, continuous swarming; right, discontinuous swarming (×0.75).*

to the adsorption of such compounds) that may lead to the formation of filamentous cells, there is no evidence that such filaments are true swarm cells capable of swarming. By contrast, Williams and Schwarzhoff (1978) believed swarm cell formation was not the result of the effect of toxic compounds and that the movement of swarm cells was a nonchemotactic event (Williams et al. 1976). Others disagree with both these views and believe swarming to be a positive chemotactic response to deteriorating nutritional conditions. Swarm cells have been found to be chemotactic and they respond to fewer attractants than vegetative cells. Some attractants are mutually exclusive and attract either swarm cells (glutamine) or vegetative cells (glutamate). This may indicate the presence of separate sensory components in swarm and vegetative cells that are coupled to different forms of motility in *Proteus* (Allison et al. 1993). Today, most regard swarming to be neither a response to starvation nor an obligatory development stage, but rather a means of maximizing biomass production and substrate utilization.

Irrespective of the reasons for swarming, swarming is an important, though not obligatory, development stage which has a major bearing on *Proteus* pathogenicity. The ability of *Proteus* to differentiate into a population of swarm cells results in upregulation of their virulence factors (Allison et al. 1992a). They are now capable of rapid migration over the mucosae of the bladder and the ureter, which they ascend against a flow of urine and mucus, and are enabled to infect and damage the kidney as has been shown in vivo in mice (Allison et al. 1994).

In spite of these things, however, it is the nonswarming cellular form which is predominantly seen in the bladder, ureter, and kidney during ascending urinary tract infection (Jansen et al. 2003). Antigenic variation in the flagella of hyperflagellate swarm cells which has been found in vitro, may permit resistance to detection by the host immune system and aid survival (Murphy and Belas 1999). *Proteus* swarming is also associated with biofilm formation and the encrustation and blocking of urinary catheters (Stickler et al. 1998).

Anti-swarming agents

The swarming of *Proteus* may make it difficult to isolate in pure culture other bacterial pathogens with which it is present in clinical specimens. Many ways of inhibiting swarming have been described. These include:

- physically restricting the movement of *Proteus* cells by means of agar overlays or poured plates, or by increasing the agar concentration to 3–4 percent w/v
- preventing the formation of or interfering with the structure or activity of flagella through the incorporation into media of polyvalent H antisera, ethanol 5.5 percent, boric acid 0.1 percent, detergents, bile salts, or other surface-active agents (Lominski and Lendrum 1942), or β-phenethyl alcohol (Kopp et al. 1966)
- retarding the cell growth rate by incorporating into media growth inhibitors such as sulfonamide, neomycin, chloral hydrate, barbiturates, sodium azide, or purine bases.

Some anti-swarming methods act by mechanisms which are as yet unexplained. Activated charcoal has been found by Smith and Alwen (1966) to inhibit swarming without affecting motility, but some components of media, notably blood, make the charcoal ineffective. Swarming is inhibited in MacConkey agar by the bile salts and in Wilson and Blair's medium by the bismuth sulfite. Swarming rarely occurs in the absence of electrolytes. Useful alternative anti-swarming media are Lab-Lemco without added salt, and cysteine lactose electrolyte-deficient (CLED) agar (Mackey and Sandys 1966). Regrettably, many of these anti-swarming agents are unsuitable for use when isolating certain pathogenic bacteria. Some agents are toxic and prevent the growth of delicate pathogens. Others make their recognition difficult by altering the colonial morphology of the organism or, by lysing red blood cells, preventing the recognition and detection of hemolytic organisms. One exceptional anti-swarming agent is *p*-nitrophenylglycerol, which at low concentrations inhibits the formation of swarm cells without affecting the formation or motility of flagella (Kopp et al. 1966). It is also without effect on red blood cells, the colonial morphology of other organisms and their antibiotic sensitivity and is nontoxic for a wide range of delicate bacterial pathogens such as *Neisseria gonorrhoeae* (Senior 1978).

Colonial appearances

Proteus strains form several sorts of discrete colony on MacConkey agar; Belyavin (1951) described three of these, which he designated phases. Phase A colonies are smooth, 3–4 mm in diameter, with a beaten-copper surface; morphologically they consist of regular bacillary forms 5–6 × 0.5 μm which on other media give rise to discontinuous swarming. Phase B colonies are smaller, smoother, and more conical; they consist of highly pleomorphic cells (coccobacilli, filaments, and giant cells) that do not swarm on other media. Phase C colonies are flat, rough, and larger than phase A colonies. They consist of long filaments that give rise to continuous swarming on other media. The dominant type-specific O antigen is not found in phase B cells. Phase variations A→B and A→C are reversible. Coetzee and Sacks (1960) described five colonial variants, three of which, Y, W, and Z, correspond to Belyavin's phases A, B, and C, respectively. Cultures arising from a single cell of one particular variant eventually gave rise to all the others.

METABOLIC ACTIVITIES

The characteristic metabolic activity of Proteeae of the oxidative deamination of certain amino acids results in the formation of α keto acids. These have strong iron-binding properties and they may function as siderophores in the tribe because the Proteeae, unlike others in the family Enterobacteriaceae, are unable to form hydroxamate and catecholate siderophores (Drechsel et al. 1993). All species within the tribe also have the ability to degrade indole on prolonged incubation. It is important therefore, when indole formation is to be used as an identifying characteristic, to use a standard medium such as tryptone and to read the reaction after incubation for only 1 day (Müller 1986).

Early reports on the hemolytic activity of *Proteus* are discrepant, probably because of variation in the culture media, conditions of assay, and type of red blood cells used. Sheep red blood cells are more sensitive than human red blood cells, and mouse red blood cells are relatively resistant, to hemolysins from *P. mirabilis* (Peerbooms et al. 1983). Two distinct hemolysins, HpmA (Welch 1987) and HlyA (Koronakis et al. 1987) are found in *Proteus*. HpmA is a 166 kDa calcium-independent hemolysin and is produced by virtually all strains of *P. mirabilis*, most strains of *P. vulgaris*, and some strains of *P. penneri* (Senior and Hughes 1988; Swihart and Welch 1990). HlyA, a 110 kDa calcium-dependent hemolysin, is less common in *Proteus*. It is found in some isolates of *P. vulgaris* and *P. penneri* (Koronakis et al. 1987; Senior and Hughes 1988; Senior 1993) and is very similar to the HlyA hemolysin of *Escherichia coli* and *Morganella morganii*. Some strains of *P. vulgaris* and *P. penneri* form both hemolysins. Both HpmA and HlyA cause the lysis of a wide variety of cell types in addition to erythrocytes, by forming cation-selective pores in cell membranes. In *P. mirabilis*, HpmA usually remains cell-associated. As a result, strains of this species do not appear to be hemolytic on blood agar when swarming is prevented. The calcium-dependent hemolysin HlyA forms more obvious hemolytic zones on blood agar (Senior and Hughes 1988).

EXTRACELLULAR PRODUCTS

Most strains of *P. mirabilis*, *P. vulgaris*, *P. penneri*, and *P. hauseri* produce a unique EDTA-sensitive metalloproteinase. It is thought to be an important virulence factor because it can cleave at unique sites the heavy chain of human IgA1, IgA2, IgG, and both free and IgA-bound secretory component (Senior et al. 1987, 1988; Loomes et al. 1990, 1992; Almogren et al. 2003). Furthermore, *P. mirabilis* protease causes degradation of the antibacterial peptide LL-37 of the innate immune system (Schmidtchen et al. 2002). Strains of *P. mirabilis* associated with urinary tract infections also form hyaluronidase.

All strains of *Proteus* spp., with rare exceptions, form potent urease enzymes whose genes have been cloned. The nickel-containing cytoplasmic enzyme is chromosomally determined and is inducible in *Proteus*. The enzyme comprises a number of subunits (α, β, and γ) and in *P. mirabilis* is of the form α_2, β_4, γ_4 with a mass of 212–250 kDa. The enzyme is determined by three structural genes, *ureA*, *ureB*, and *ureC* and accessory genes *ureD*, *ureE*, *ureF*, and *ureG*. Transcription is controlled by a repressor protein UreR which is specific for urea. The urease of *P. mirabilis* hydrolyzes urea between six and 30 times faster than that of *Morganella* or *Providencia* spp. and this feature undoubtedly contributes to the greater virulence of this organism than the others for the urinary tract.

HABITAT

P. myxofaciens has been isolated only from gypsy-moth larvae (*Porthetria dispar*), but the other *Proteus* spp. are widely distributed in nature and constitute an important part of the flora of decomposing matter of animal origin. They are constantly present in rotten meat and sewage, and very frequently in the feces of man and animals. They are commonly found in garden soil and on vegetables. In addition to their wide saprophytic existence, strains of *Proteus* cause septic infections in man and animals.

LABORATORY ISOLATION AND IDENTIFICATION

Proteus strains grow readily on a wide variety of media in air over a wide temperature range below 42°C, but optimally at 34–37°C. For the isolation and identification of *Proteus*, the specimen should be plated directly for single colonies on either a medium that does not permit swarming, such as MacConkey or CLED agar, or one of the indicator and selective media referred to above. Enrichment culture in tetrathionate broth before subculture to solid media may be helpful. After overnight incubation at 37°C, a single colony of gram-negative pleomorphic bacilli that is oxidase-negative and unable to ferment lactose should be suspended in a small amount of nutrient broth to provide an inoculum for mannose peptone water and for either a nutrient agar medium enriched with L-tryptophan 1 percent and an antiswarming agent (e.g bile salts), such as that described by Müller (1985) or Senior and Leslie (1986), or a similar medium enriched with L-phenylalanine 1 percent instead of tryptophan. After overnight incubation at 37°C, isolates belonging to the Proteeae form colonies surrounded by a red-brown color on the medium containing tryptophan and form a transient green color after the addition of aqueous ferric chloride 10 percent to the phenylalanine medium. Those that also acidify mannose belong to the genera *Morganella* or

Providencia and can be identified further by biochemical tests according to Table 1 and the scheme in Figure 3. Those that do not acidify mannose are strains of *Proteus*. Most of them swarm on nutrient agar or blood agar at 30°C and degrade urea rapidly. They are identified further according to the scheme in Figure 3 by determining their ability to form indole and ornithine decarboxylase, acidify maltose and xylose, and degrade aesculin. Most isolates from human clinical specimens are *P. mirabilis*. They are more readily identified from swarming growth by being indole-negative and forming ornithine decarboxylase and H_2S. Media to examine these reactions in combinations have been devised (Senior 1997a).

BIOCHEMICAL REACTIONS

All *Proteus* strains form acid from glucose and most also form small amounts of gas. Unlike strains of *Morganella* and *Providencia*, no strain of *Proteus* forms acid from mannose, mannitol, adonitol, or inositol. Lactose fermentation is rare and when present is encoded by a plasmid acquired from outside the genus. All species produce an inducible urease; this enzyme is distinct from the urease of *M. morganii* and that of *P. penneri* is unique from those of others of the tribe (Senior et al. 1980; Rosenstein et al. 1981; Mobley et al. 1987). All strains are catalase-positive and oxidase-negative. All species except *P. myxofaciens* form acid from xylose and all except *P. mirabilis* form acid from maltose. *P. mirabilis* is the only species in the genus to produce ornithine decarboxylase, and together with *P. penneri* (and *P. myxofaciens*), are the only indole-negative species in the genus. Sucrose is acidified by most strains of all species except *P. mirabilis* in which only about 15–20 percent strains are sucrose-positive. *P. myxofaciens* characteristically forms copious amounts of slime in broth at 25°C and is the only species in the tribe that fails to degrade tyrosine. Among the indole-positive strains of *Proteus*, those that also acidify salicin and degrade aesculin are strains of *P. vulgaris*, whereas those that do not acidify salicin or degrade aesculin are either strains of *P. hauseri* or strains of genomospecies 4, 5, and 6 (O'Hara et al. 2000). *P. hauseri* strains can be distinguished from the latter in being unable to acidify rhamnose, utilize acetate or Jordan's tartrate or form lipase or DNase, whereas the genomospecies have the ability to carry out one or more of these activities (O'Hara et al. 2000). Growth on Simmons's citrate medium is variable in some species but does not occur with *P. penneri* and *P. hauseri* strains. Most strains of *Proteus* are methyl-red-positive, but only *P. myxofaciens* and some *P. mirabilis* strains give a positive Voges–Proskauer reaction when tested at 37°C.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

Proteus cells are readily destroyed by moist heat at 55°C for 1 h and by common disinfectants such as phenolics or halogens. However, some strains isolated in hospitals, particularly of *P. mirabilis*, have a significant resistance to chlorhexidine, of which as much as 800 mg/l may be required to prevent multiplication (Stickler 2002).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

The susceptibility of members of the Proteeae to concentrations of antibiotic attainable in the body is highly variable. Some strains of *Prov. stuartii*, for example, have resistance patterns as wide as any encountered in the clinical laboratory but those of others, for example *P. mirabilis*, may well be very sensitive to a broad range of antibiotics. Moreover, because the individual species of a genus often differ widely in antibiotic susceptibility, accuracy in speciation of isolates is of great importance. With this in mind, antibiotic sensitivities recorded in the literature should be read with caution. 'Indole negative *Proteus*' usually refers only to *P. mirabilis* strains and excludes *P. penneri* strains: 'indole positive *Proteus*' usually refers to collections of strains which may be either *P. vulgaris* or *P. hauseri* or both and '*Providencia* spp.' often refers only to *Prov. stuartii*.

All species of the tribe are resistant to nitrofurantoin. This antibiotic is most active under acid conditions and it has been thought that its inactivity on the Proteeae was a consequence of the alkaline conditions which form during growth of many of the members of the tribe. However, this may not be the complete explanation for it does not explain the resistance to nitrofurantoin of urease-negative species of the tribe like some *Prov. stuartii* strains. Different strains of the Proteeae show varying responses to tetracycline and this antibiotic is useful diagnostically, for example in biotyping *M. morganii* isolates. However, apart from this, tetracycline has little clinically useful activity in treating infections with members of the Proteeae.

Strains of *Proteus* spp. have some unusual antibiotic sensitivity characteristics. Thus, whereas most enterobacteria are susceptible to polymyxin B and colistin but resistant to novobiocin, all *Proteus* spp. are resistant to polymyxin and colistin and many, particularly strains of *P. vulgaris*, are sensitive to novobiocin. No information is available about the antibiotic susceptibility of *P. myxofaciens*. However, this organism has never been associated with infection in man. In general, *P. mirabilis* is the most sensitive species of the genus.

The sensitivity of *Proteus* spp. to penicillins and cephalosporins is complicated (Garrod et al. 1981) and is

Table 1 *Usual biochemical reactions of species of* Proteus, Morganella, *and* Providencia

							Providencia						
										stuartii			
	Proteus									Biogroup			
Property	***mirabilis***	***vulgaris***	***penneri***	***hauseri***	***myxofaciens***	***Morganella morganii***	***rettgeri***	***alcalifaciens***	***rustigianii***	**4**	**5**	**6**	***heimbachae***
Gas from glucose	+	(+)	V	–	+	(+)	(–)	(+)	V	–	–	–	–
Acid from													
Mannose	–	–	–	–	–	+	+	+	+	+	+	+	+
Maltose	–	+	+	+	+	–	–	–	–	–	–	–	V
Xylose	+	+	+	+	–	–	(–)	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	+	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	+	+	–	–	–	+	+
Inositol	–	–	–	–	–	–	+	–	–	–	+	+	V
Trehalose	+	–	V	–	+	V**	–	–	–	+	+	+	–
Salicin	–	+	–	–	–	–	V	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	V	–	–	–	–	–	+
Urease	+	(+)	+	+	+	+	+	–	–	+	–	–	–
Indole	–	+	–	+	–	(+)	+	+	+	+	+	+	–
Ornithine decarboxylase	+	–	–	–	–	(+)	–	–	–	–	–	–	–
Esculin hydrolysis	–	+	–	–	–	–	V	–	–	–	–	–	–
H_2S (TSI medium)	+	V	(–)	V	+*	(–)	–	–	–	–	–	–	–
Gelatin liquefaction	+	(+)	V	+	+	–	–	–	–	–	–	–	–
Lipase production	+	(–)	V	–	+	–	–	–	–	–	–	–	–
Methyl red	+	(+)	+	+	+	(+)	+	+	V	+	+	+	(+)
Voges–Proskauer	V	–	–	–	+	–	–	–	–	–	–	–	–

The following properties are common to virtually all: motile; acid in glucose; no acid in dulcitol, lactose, sorbitol, raffinose and arabinose; nitrate reduced to nitrite; phenylalanine deaminated; arginine dihydrolase-; malonate-; mucate-; +, positive reaction within 48 h; –, negative reaction; ( ) reaction of most strains; V, some strains positive, others negative; *, reaction after 3–4 days; **, subsp. *morganii* –ve, subsp. *sibonii* +ve.

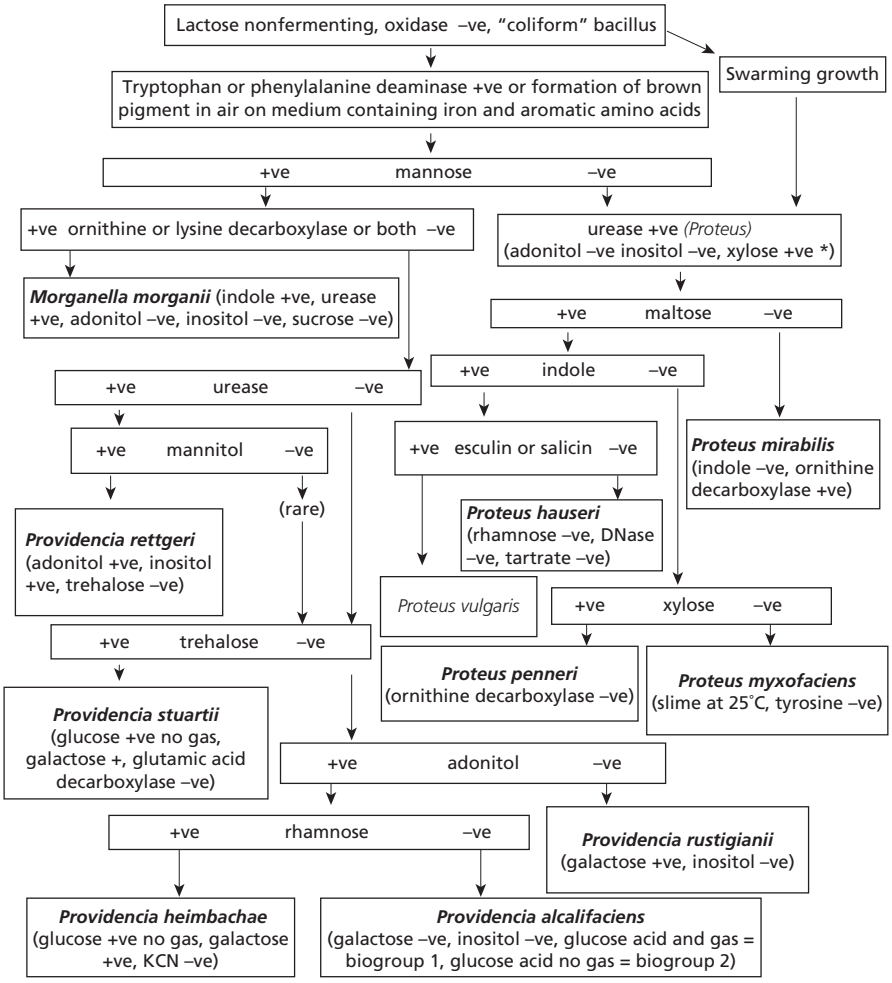

Figure 3 *Scheme for identification of species in the tribe Proteeae: ( ), confirmatory reactions;* *P. myxofaciens *is xylose −ve.*

determined partly by mechanisms of intrinsic resistance and partly by whether they produce β-lactamases. Some *P. mirabilis* strains are unique among the Proteeae in being sensitive to amounts of benzylpenicillin that are attainable in urine. These strains are also fully sensitive to the extended spectrum penicillins, such as ampicillin, and cephalosporins like cephaloridine, cephalothin, cephalexin, and loracarbef. By contrast, *P. vulgaris* and *P. penneri* strains are resistant to these antibiotics, but are often sensitive to other cephalosporins such as cefetamet, cefoxitin, and latamoxef (Penner et al. 1982; Fuksa et al. 1984) as are strains of *P. mirabilis*. *P. vulgaris* strains are characteristically more resistant to cefamandole than other species in the Proteeae. They are also exceptional among the Enterobacteriaceae in having an inducible β-lactamase (cefuroximase) that has high activity against β-lactamase-stable cephalosporins such as cefuroxime and cefotaxime. Most strains of *Proteus* spp. are sensitive to cefixime, cefpodoxime, cefdinir, and ceftazidime, but are resistant to cefsulodin. It is important when interpreting the sensitivity of 'indole positive *Proteus*' to some of the newer oral cephalosporins by the disk diffusion method, to exercise caution (Biedenbach and Jones 1994). Most *P. mirabilis* strains are sensitive to cefepime and cefpirome.

Most strains of *P. mirabilis* and *P. vulgaris* are very susceptible to the acylureidopenicillins, such as azlocillin, mezlocillin, and piperacillin, and the carboxypenicillins, such as carbenicillin and ticarcillin, whereas *P. penneri* strains may be somewhat more resistant to them (Hawkey et al. 1983). Most β-lactamase-producing strains of *Proteus* spp. are sensitive to the carbapenems imipenem and aztreonam. The susceptibility of strains of *Proteus* to tetracyclines is variable, but that to chloramphenicol is mainly species-dependent: most strains of *P. mirabilis* and *P. vulgaris* are sensitive, but *P. penneri* strains are chromosomally resistant (Hickman et al. 1982; Fuksa et al. 1984). Most strains of *Proteus* spp. are susceptible to nalidixic acid and other quinolones (Hawkey and Hawkey 1984) and also to the aminoglycosides gentamicin, tobramycin, netilmicin, and amikacin. However, from time to time strains may acquire plasmids encoding for resistance to some of these or to trimethoprim and to other antibiotics to which they are

normally sensitive. These resistance characters may be transferred to other *Proteus* strains, to enterobacteria and to *Pseudomonas aeruginosa*.

CELL ENVELOPE COMPOSITION AND ANTIGENIC STRUCTURE

Proteus spp. are not normally considered to be capsulate organisms. However, *P. mirabilis* and *P. vulgaris* strains have been found to be able to form a capsule or slime layer of acidic polysaccharides on the outer surface of the cell identical to the O-specific chain of their lipopolysaccharide (LPS). It plays an important role in virulence by assisting swarming and growth of struvite crystals leading to stone formation in the urinary tract (Beynon et al. 1992; Dumanski et al. 1994; Gygi and Hughes 1995; Rahman et al. 1999). Beneath is the outer membrane containing outer membrane proteins (OMP), antigenically related to those of other members of the Enterobacteriaceae, and LPS. In *P. mirabilis* there are three major OMPs of 39 kDa (OmpA), 36 and 17 kDa. *P. mirabilis* OmpA enhances the production of IgG to LPS, is a strong adjuvant and a mitogen in vitro for mouse B lymphocytes. Furthermore, it inhibits the production of oxygen radicals and interleukin-1, but increases the secretion of TNFα by LPS-stimulated macrophages (Weber et al. 1993). In *P. mirabilis*, LPS exists in two forms: approximately two-thirds of it is in the form LPS I which has a relatively short O polysaccharide chain, whereas the remainder is in the form LPS II, which has very long O polysaccharide chains (Gmeiner 1975). Some of these may be so long that they have been considered as capsules (Beynon et al. 1992). The variation in structure and composition of the O-specific polysaccharide part of *Proteus* LPS accounts for the O antigenic heterogeneity in the genus. The structure and composition of many of these polysaccharides are known (Knirel et al. 1993; Rozalski et al. 1997). In addition to the sugars commonly found in enterobacterial LPS, a frequent feature of *Proteus* O polysaccharides is the presence of amino acids like L- and D-alanine, L-lysine, L-serine, and L-threonine linked to uronic acids, and the presence of lactic acid ethers, and hydroxybutyryl, pyruvic, and phosphate groups. However, these unusual components are often not a part of the immunodominant epitopes. The O polysaccharides of some *P. mirabilis* and some *P. penneri* strains are very unusual as they resemble the ribitol teichoic acids of gram-positive bacteria in having ribitol phosphates (Toukach et al. 2002). Analysis of the oligosaccharides in the core of *Proteus* LPS shows that it can be of six different types. The presence in it of D-galacturonic acid, as is also found in the core of the LPS of *M. morganii* and *Prov. rettgeri*, distinguishes it from that of *E. coli* and *Salmonella* (Radziejewska-Lebrecht et al. 1990). The resistance of *Proteus* spp. and others of the Proteeae to polymyxins is because of the presence of L-arabinoso-4-amine in their LPS (Basu et al. 1986).

The LPS of some strains of *Proteus* has epitopes shared with some *Rickettsia* spp. (Amano et al. 1993). During particular (but not all) rickettsial infections, antibody, usually of the IgM class, is formed, causing the agglutination of one or more *Proteus* strains designated X19, X2, and XK. The LPS of each of these strains has been purified and characterized (Mizushiri et al. 1990). These are, respectively, strains of *P. vulgaris* serotype O1 and serotype O2 and *P. mirabilis* serotype O3. Thus patients with epidemic louse-borne typhus infected with *Rickettsia prowazekii*, or Rocky Mountain spotted fever (*Rickettsia rickettsii*) develop serum antibodies that agglutinate *Proteus* OX19 cells but not OXK cells, whereas those with scrub typhus (*Rickettsia tsutsugamushi*) develop serum antibodies that agglutinate only OXK cells. The detection of antibodies to these bacteria has been used in the past to indicate rickettsial infection (the Weil–Felix reaction). The test is being replaced by more specific serological and polymerase chain reaction (PCR) methods. The LPS of *P. mirabilis* O29 can affect the cytoskeleton of erythrocytes causing alteration in their membrane lipids and proteins and rendering them osmotically fragile (Gwozdzinski et al. 2003).

The peptidoglycan component of the cell wall of *Proteus* species, and indeed all members of the Proteeae, is unusual among the enterobacteria in that the C6 hydroxyl group of many (29–57 percent) of the *N*-acetylmuramyl residues is O-acetylated. This modification renders the cell wall resistant to lysozyme and possibly to many muramidases and autolysins (Clarke 1993).

There is much antigenic heterogeneity among strains of *P. mirabilis*, *P. vulgaris*, and *P. penneri* and many different O and H antigens have been described. The simplified scheme devised by Kauffmann and Perch (Kauffmann 1966) for serotyping *P. mirabilis* and *P. vulgaris* distinguished 49 O antigens and 19 H antigens. Since then, the O antigenic composition of many *P. penneri* strains has been investigated and now more than 70 O-serotypes of *Proteus* have been defined. Although some O antigens are species specific, many are not and may be found not only shared between strains of several of the other species of *Proteus* (Penner and Hennessy 1980; Kondakova et al. 2003), but also on occasions, with bacteria outwith the genus.

The O antigens of *Proteus* are resistant to heating at 100°C, to ethanol and to dilute HCl, whereas the H antigens are destroyed by these treatments. Agglutination tests for the determination of O antigens should be made with cultures that have been boiled for 1 h because some living and some formalized cultures are O-inagglutinable. O agglutination tests are best read after incubation for 20 h and H agglutination tests after 4 h in a water bath at 50°C. Cross-reactions between H antigens are numerous and complex. The most frequently encountered strain in clinical infections is *P. mirabilis* serotype

O3. Other frequently encountered serotypes are O6, O10, O26, and O30. The most commonly encountered flagellar antigens are H1, H2, and H3.

TYPING METHODS

Phage typing

Phages have been isolated from lysogenic strains and from sewage, and several groups of workers have developed phage typing schemes. Lysis is generally restricted to members of the species of origin of the phage, but occasionally it is seen in members of other species in the tribe and, rarely, in other enterobacteria. The schemes of Pavlatou et al. (1965) and Schmidt and Jeffries (1974) are useful for typing strains of *Proteus* and other members of the Proteeae. Those of Vieu and Capponi (1965) and France and Markham (1968) are applicable to typing strains only of *Proteus* and allocate over 80 percent of strains to a type. The systems devised by Izdebska-Szymona et al. (1971) and Hickman and Farmer (1976) permit the typing only of *P. mirabilis* strains. The last of these is highly discriminatory and reproducible, permitting 94 percent of 200 strains to be differentiated into 113 types.

Bacteriocin typing

Cradock-Watson (1965) distinguished strains of *Proteus* by determining the range of activity of their bacteriocins on a set of indicator strains. Most strains of *P. mirabilis* have the genetic potential to produce one of 13 different bacteriocins (proticins) which resemble contractile phage tails (Senior 1983a) and to be sensitive to a different one, or more, of them. The scheme devised by Senior (1977a) in which *Proteus* strains were examined for both proticin production and proticin sensitivity (P/S typing) has given the greatest discrimination: 250 strains were differentiated into 90 P/S types. The production of proticin 3 is associated with strains that are virulent for the urinary tract (Senior 1979). The P/S type of an isolate is independent of its O serotype (Senior and Larsson 1983).

Other typing methods

The Dienes phenomenon forms the basis of another typing method (Dienes 1946). When different strains of *Proteus* spp. are allowed to swarm towards each other, a line of completely or partially inhibited growth forms where the spreading growths of incompatible strains meet. Such a line does not form between identical strains (Figure 4). The determinant of Dienes compatibility was thought to be the H antigen until others found serologically identical strains to be incompatible and strains with the same O antigens and different H antigens were sometimes compatible. Senior (1977b) showed that 204 swarming strains of *Proteus* could be differentiated into 98 compatibility groups and that the determinants of compatibility were the proticin production and sensitivity characters of the swarming strains. Strains of the same P/S type, irrespective of their species or O and H antigenic structure, were compatible, whereas strains of different P/S types were incompatible (Senior and Larsson 1983). Kashbur et al. (1974) devised a scheme for resistotyping *P. mirabilis* strains. They found it discriminated between strains that appeared to be identical by other typing methods.

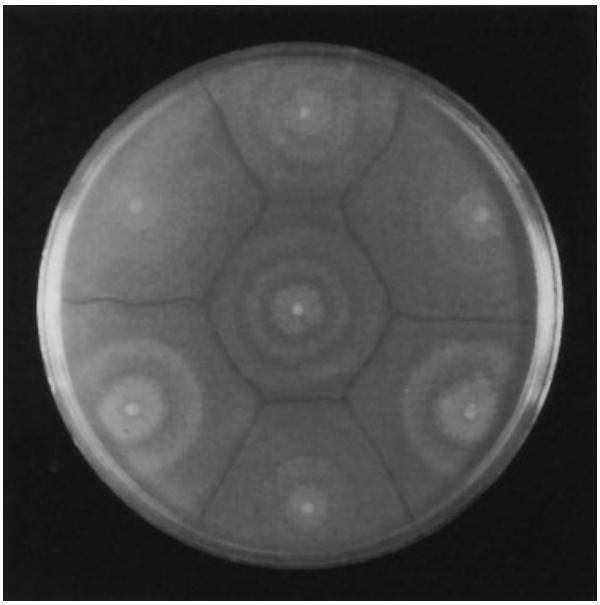

Figure 4 *Dienes typing of* Proteus *strains. A line of inhibited growth is formed only where the swarming growths of different strains meet. The strain at the top of the plate is the same as the one in the center. Each of the remaining strains is different from its immediate neighbor (×0.85).*

Some workers have compared the electrophoretic profiles of outer-membrane or total cell proteins of strains and used this as a typing method (Kappos et al. 1992; Costas et al. 1993). The method is relatively simple and reproducible, but not very discriminatory. It can be useful in typing strains of *P. vulgaris* and *P. hauseri*. The discriminating power of the method is greatly increased when combined with multilocus enzyme electrophoresis (Kappos et al. 1992), but this method is labor intensive and time consuming.

Latterly, typing schemes based on polymerase chain reaction methods and the analysis of *Proteus* DNA have been devised. Among these are randomly amplified polymorphic DNA (RAPD), repetitive sequenced based PCR, tandem tetramer-based microsatellite fingerprinting (Cieslikowski et al. 2003), ribotyping (Pignato et al. 1999) and pulsed field gel electrophoresis (PFGE) methods.

As *P. mirabilis* is the species in the genus most frequently encountered in a clinical situation, typing schemes which give the greatest discrimination between strains of this organism by methods that are repeatable, rapid and simple to perform, are those of greatest value in studies of cross-infection. Bingen and colleagues (1993) reported that PCR typing with the use of a single arbitrary primer was as reproducible and as discriminating as ribotyping, but much simpler and quicker to perform. Comparisons between the discriminating ability of the Dienes test with ribotyping and PFGE have shown all three methods to be comparable and all to be highly discriminating (Pfaller et al. 2000). Of these methods, the Dienes test is by far the simplest to perform.

Thus, if only a few strains of *P. mirabilis* are to be compared, the rapid and simple Dienes typing method is usually the most suitable one to use. When larger numbers of strains are to be examined, and possibly on a routine basis, consideration should be given to the use of P/S typing or serotyping methods. Since these methods are independent of each other, the best discrimination is given by a combination of P/S typing and O serotyping.

PATHOGENICITY AND VIRULENCE FACTORS

Nothing is known about the pathogenicity of *P. myxofaciens* for it has never been isolated from man. However, members of all the other species of *Proteus* are pathogenic for man. *P. mirabilis* is the most frequently encountered species of the tribe in human infections and is responsible for 70–90 percent of all *Proteus* infections in man. *P. vulgaris*, *P. hauseri*, and *P. penneri* cause similar types of infection to *P. mirabilis* because their habitat and virulence factors are similar. However, they are isolated less frequently and they may be less virulent.

The most common site of *Proteus* infection is the urinary tract and *P. mirabilis*, the species most frequently implicated, is possibly the organism after *Escherichia coli* most frequently associated with urinary tract infections. *Proteus* urinary tract infections are common in young boys and elderly patients (Senior 1979). In the latter, they are often associated in domiciliary patients with diabetes or structural abnormalities of the urinary tract, but in hospital patients with various forms of urological instrumentation or manipulation. *Proteus* urinary tract infections tend to be more serious than those caused by *E. coli* and other coliforms, for these are usually confined to the bladder whereas *Proteus* spp. have a predilection for the upper urinary tract (Fairley et al. 1971; Svanborg Eden et al. 1980).

The virulence of *P. mirabilis* for the urinary tract arises through the interplay of several virulence factors of which the most important is urease. This nickel-containing cytoplasmic enzyme is inducible solely by urea. The concentration of urea in urine is sufficiently high for the ureases of strains of all *Proteus* spp. to be able to work at the maximum rate, that of *P. mirabilis* in particular giving the greatest rate of urea hydrolysis. The enzyme hydrolyzes the urea in urine to ammonia and carbon dioxide. This reaction may be important to the bacteria by providing them with a source of usable nitrogen for growth in urine. However, the formation of ammonia also leads to alkalinization of the urine and at pH values above 8, calcium and magnesium ions are precipitated in the form of struvite ($MgNH_4PO_4.6H_2O$) and carbonate-apatite ($Ca_{10}(PO_4)CO_3$) crystals. The formation of these crystals is also influenced by the chemical structure of the polysaccharide of the LPS and its ability to bind calcium and magnesium ions (Torzewska et al. 2003). Ultimately the crystals are bound by the polysaccharide slime or glycocalyx formed by the cell and thereby create bladder and kidney stones. Experiments in mice infected transurethrally with either uropathogenic or isogenic urease-negative strains of *P. mirabilis* have confirmed that urease is a critical virulence determinant for colonization of the urinary tract, stone formation and the development of acute pyelonephritis (Johnson et al. 1993). Urease activity together with hemolysin (see below) also cause the death of human renal proximal tubular epithelial cells (Mobley et al. 1991). Furthermore, urease-induced formation of ammonia protects the bacterial cell from the effects of complement by inactivating it.

Fimbriae of *Proteus* spp. also play a significant but more subtle role in virulence for the urinary tract as shown by in vivo experiments with genetically designed strains. MR/P fimbriae are expressed in vivo, and though not essential for infection, appear to play a significant role in the colonization of the upper part of the urinary tract. Their presence correlates with the development of acute pyelonephritis (Bahrani et al. 1994). The expression of MR/P fimbriae is phase variable. When 'switched on', colonization of the bladder occurs. The later colonization of the kidney is thought to be dependent upon MR/P fimbrial expression being 'switched off' (Li et al. 2002). MR/P fimbriae are thought to be a promising candidate for a vaccine to prevent *P. mirabilis* urinary tract infection. PMF appear to be important for colonization of the bladder rather than the kidney (Massad et al. 1994b). Both UCA and MR/K fimbriae bind to uroepithelial cells and the latter also bind to Bowman's capsule of the glomeruli and tubular basement membranes of the kidney (Sareneva et al. 1990). MR/K fimbriae also play a major role in the adhesion of *Proteus* to catheters and the persistence of infection in catheter-associated *Proteus* bacteriuria. It is not yet known whether ATF fimbriae and *P. mirabilis* P-like fimbriae have a role in *Proteus* virulence for the urinary tract. However the PmpA protein of the latter fimbriae has a close similarity with the pyelonephritis-associated (Pap) fimbrial protein of uropathogenic *E. coli*.

Hemolysins are also known to be important virulence factors of *Proteus* spp. Hemolytic strains are much more lethal for mice than nonhemolytic ones (Peerbooms et al. 1983, 1985). Both HpmA and HlyA are cytotoxic for a wide variety of cell types and together with urease they play an important role in cell invasion and internalization – which appears to take place by a mechanism not dependent on actin polymerization – and ultimately cell death (Peerbooms et al. 1984; Allison et al. 1992b; Chippendale et al. 1994). Despite having similar urease and hemolysin genes, hemolysin and urease are expressed at higher levels in *P. mirabilis* than in *P. vulgaris*. This may be the explanation for the greater virulence of *P. mirabilis*.

Motility and swarming are additional properties of *Proteus* spp. which are important, though possibly not absolutely essential, in virulence. Pazin and Braude (1974) showed that antibodies to flagella prevented *P. mirabilis* infecting the kidney. Motility is also critical for the successful infection of burns by *P. mirabilis* (Harmon et al. 1989). The development of flagella is important in swarm cell formation in which other virulence determinants, urease, hemolysin, and protease, are co-ordinately expressed (Allison et al. 1992a). *P. mirabilis* mutants lacking flagella are noninvasive to uroepithelial cells and motile but nonswarming cells are 25 times less invasive than wild-type motile, swarming cells (Allison et al. 1992b).

Proteus proteinase, which cleaves a variety of immunoglobulin types including IgA and other nonimmunoglobulin substrates, is thought to be another important virulence factor. Active enzyme and immunoglobulin fragments can be detected in the urine of patients with a *Proteus* urinary tract infection (Senior et al. 1991), indicating it is synthesized and active in vivo. The cleaved antibody fragments have defective immune effector functions and thereby the effectiveness of the immune response to the organism is limited (Loomes et al. 1993). The proteinase may also play a role in degrading antibacterial peptides of the innate defense system (Schmidtchen et al. 2002) and in generating products like glutamine to induce invasive swarm cell formation. In vivo experiments in mice have shown that proteinase-negative mutants can infect the bladder, but have severely reduced ability to infect the kidney and form abscesses.

Infection of the urinary tract with *Proteus* spp. may also give rise to hyperammonemic encephalopathy and coma. Urinary tract infection often also leads to bacteremia. *Proteus* bacteremias can be difficult to treat. In extreme situations they may progress to septicemia with septic shock and disseminated intravascular coagulation. The polysaccharide component of *P. mirabilis* LPS has been shown to stimulate the adhesion of blood platelets to collagen (Zielinski et al. 2002). *Proteus* bacteremias have a mortality rate of 15–88 percent according to the severity of the underlying disease (Lewis and Feckety 1969).

Proteus spp. are frequently isolated both in pure culture and also with other organisms from various superficial septic lesions. Their presence in mixed culture favors the multiplication of pathogenic anaerobes. Occasionally, in addition to their isolation from blood, they are isolated in pure culture from abscesses and cerebrospinal fluid (Chang et al. 2002). Both *P. mirabilis* and *P. vulgaris* may cause osteomyelitis. In neonates, infection of the umbilical stump often leads to a highly fatal proteus bacteremia and meningitis.

P. mirabilis may also be a microbial trigger for the development of rheumatoid arthritis (RA). The urine of RA patients is more frequently found to carry small numbers of *P. mirabilis* than is that of healthy controls (Wilson et al. 1990; Senior et al. 1999). This frequent and prolonged antigenic stimulus may explain the raised levels of IgG (Ebringer et al. 1985, 1988) or IgM (Senior et al. 1995) specific for *P. mirabilis* seen specifically in RA patients. It has been suggested that RA then develops because of molecular mimicry between the peptide ESRRAL found in an outer membrane protein of *P. mirabilis* necessary for the secretion of HpmA hemolysin and the peptide sequence EQRRAA found in the β chain of HLA types associated with RA (Ebringer et al. 1992).

The pathogenicity of *Proteus* spp. for laboratory animals is variable. Virulent strains on introduction into tissue are able to proliferate and invade the bloodstream. Strains of lower virulence cause chronic inflammation, either of the suppurative or of the infective granuloma type.

The inoculation intraperitoneally of 0.5–1.0 ml of a 24-h broth culture of a virulent strain generally proves fatal to rats and mice in 18–48 h and to guinea pigs and rabbits in 1–7 days. The LD_{50} for mice of 21 strains ranges from 2 million to 600 million living organisms (Miles 1951). Large doses given intravenously to mice result in a fatal septicemic illness; smaller doses of some strains produce necrotic lesions in the kidneys of survivors. Studies on the virulence of *P. mirabilis* and *P. vulgaris* for mice by determinations of the LD_{50} and KID_{50} (50 percent kidney infecting dose) have shown that although there can be wide variation in virulence between different strains of *P. mirabilis* (Peerbooms et al. 1982), on the basis of LD_{50}:KID_{50} ratios, *P. mirabilis* strains are generally more virulent than *P. vulgaris* strains.

MORGANELLA

DEFINITION

Morganella spp. do not show true swarming on solid nutrient media. They acidify glucose, usually with gas formation, and mannose but not lactose, sucrose, salicin, mannitol, xylose, adonitol, or inositol. They oxidatively deaminate amino acids and produce urease,

but do not utilize citrate. Most strains form ornithine decarboxylase and indole but do not produce H_2S nor liquefy gelatin nor form lipase. G + C content of the DNA is 50 mol%.

The genus has one species, *M. morganii*, with two subspecies, *morganii* and *sibonii*. The type strain of *M. morganii* subsp. *morganii* is ATCC 25830, NCTC 00235, CDC 4567-84. The type strain of *M. morganii* subsp. *sibonii* is ATCC 49948, NCTC 12357, CDC 8103-85.

The organism is named after Morgan (1906) who isolated it from the feces of infants with diarrhea. Rauss (1936) suggested it was closely related to *Proteus* because, although it failed to liquefy gelatin, it formed indole, degraded urea, and exhibited spreading growth on media with reduced agar concentrations at 20–28°C. The observation by Henriksen and Closs (1938) that both *Proteus* and Morgan's bacillus degraded phenylalanine to phenylpyruvic acid gave additional support to the suggestion and for many years the organism was known as *Proteus morganii*. The proposal of Kauffmann (1956) that it should be placed in a separate genus, *Morganella*, did not at first receive general acceptance, but the evidence in its favor is now overwhelming: the G + C content of the DNA of *Morganella* is considerably higher than that of *Proteus*; DNA–DNA hybridization studies show less than 20 percent genetic homology between the two genera and other members of the Proteeae (Brenner et al. 1978); there are differences in the properties of enzymes common to *Morganella* and other members of the tribe. More recent extensive DNA–DNA hybridization studies have shown the existence of two subspecies: *M. morganii* subsp. *morganii* with four biogroups and *M. morganii* subsp. *sibonii* with three biogroups (Jensen et al. 1992).

GENERAL PROPERTIES

Most isolates of *M. morganii* are motile because of peritrichous flagella. These may differ in wavelength and are not formed in some strains, particularly if cultured above 30°C. Strains also form thin and thick peritrichous fimbriae (Old and Adegbola 1982). On prolonged incubation on soft (1 percent) agar at 22°C, growth may spread to form a film (Rauss 1936) but true swarming, which is associated with characteristic changes in cellular morphology, migration, and consolidation on 1.5 percent agar, is not seen with the possible exception of a few isolates of subspecies *sibonii* biogroup F strains (Jensen et al. 1992).

Morganella strains, unlike *Proteus* strains, require both niacin and pantothenic acid for growth. They grow readily on most culture media as convex colonies 2–3 mm in diameter after overnight incubation at 37°C. Nutrient broth cultures have an unpleasant smell. Approximately 30 percent of strains are hemolytic on blood agar through the production of a calcium-dependent hemolysin (Senior and Hughes 1988). Because of an inability to acidify sucrose and lactose, *M. morganii* strains form colorless colonies on MacConkey, eosin-methylene blue, xylose-lysine-deoxycholate, and *Salmonella-Shigella* agars. *M. morganii* strains form a number of phosphatases including extremely high levels of phosphate irrepressible acid phosphatase, a feature rare among enterobacteria but shared with *Providencia stuartii*. Modified MacConkey media have been devised that exploit this feature and readily and specifically reveal the presence of these organisms as red colonies (Thaller et al. 1992). Liquid cultures of mixed gram-negative bacteria can be enriched for *M. morganii* through the incorporation of Irgasan – a chlorophenoxyphenol (Toora et al. 1994). The biochemical characteristics of *M. morganii* are fairly uniform (see Table 1). Mannose fermentation readily distinguishes it from isolates of *Proteus*. Urease production and the ability of most strains to decarboxylate ornithine or lysine or both, together with an inability to utilize citrate, readily distinguish them from strains of *Providencia* spp. Lactose fermenting strains of *M. morganii* have been encountered but are rare and, like some of those which form lysine decarboxylase, arise through carriage of stable plasmids (Tierno and Steinberg 1975; Cornelis et al. 1981).

M. morganii strains do not ferment many sugars, glucose and mannose being the ones characteristically acidified. The reaction of strains to trehalose is a means of distinguishing the two subspecies. Trehalose fermenters are members of the subspecies *sibonii* (named after the Danish bacteriologist Knud Siboni who first recognized them), whereas trehalose nonfermenters, are members of the subspecies *morganii*. Subdivision of subspecies *sibonii* into biogroups E, F, and G is based on the ability of strains to produce lysine or ornithine decarboxylase or both, and indole and grow in the presence of KCN. Subdivision of subspecies *morganii* into biogroups A, B, C, and D is based largely on the ability of strains to form lysine or ornithine decarboxylase, or both, or neither (Jensen et al. 1992). Other useful differentiating features include motility, glycerol fermentation, and tetracycline sensitivity (Siboni 1976; Senior and Vörös 1990; Jensen et al. 1992). Most clinical isolates of *M. morganii* and the ones responsible for most of the more serious infections in man are of subsp. *morganii* biogroup A (ornithine decarboxylase (ODC) +ve and lysine decarboxylase (LDC) −ve). Biogroup B strains (ODC +ve, LDC +ve) are usually nonmotile and rapid (24 h) fermenters of glycerol. The most common biogroup of subsp. *sibonii* is that of G (ODC +ve, LDC −ve) which is encountered in 10–14 percent of clinical specimens (Farmer et al. 1985; Janda et al. 1996). *Morganella* strains, unlike those of *Proteus*, produce urease constitutively. The enzyme is structurally and antigenically different from that produced by strains of species of *Proteus* and *Providencia* and at 590 kDa is one of the largest enzymes known (Guo and Liu 1965;

Smit and Coetzee 1967; Senior et al. 1980; Rosenstein et al. 1981; Hu et al. 1990). The fatty acid component of the lipopolysaccharide of *Morganella* strains is similar to that of *Escherichia* and *Shigella* and different from that of *Proteus* and *Providencia* strains in having higher levels of dodecanoic ($C_{12:0}$) acids (Vasyurenko and Chernyavskaya 1990).

M. morganii strains can be typed by various methods. They can be separated into a small number of types by biotyping (Rauss and Vörös 1959; Jensen et al. 1992). Phages that lyse *M. morganii* generally do not attack strains of *Proteus* and *Providencia*. They have been used for phage typing (Schmidt and Jeffries 1974) which defines more types. *M. morganii* strains have ribotype profiles which are unique to them among the Proteeae, but it is not clear whether ribotyping can distinguish between strains of the different subspecies (Pignato et al. 1999). Greater discrimination is given by protein profile typing, 21 different types being distinguished on the basis of differences in profile of major outer membrane proteins of 35–40 kDa (Senior and Vörös 1990; Costas et al. 1990a). Serological typing is most useful as *M. morganii* is antigenically heterogeneous, 55 O, 4 K, and 38 H antigens having been identified and more than 77 different serotypes are recognized (Rauss et al. 1975; Vörös and Senior 1990). Typing by bacteriocin production and sensitivity (Senior 1987) is also highly discriminating. However, because the last three typing methods are independent of each other, the greatest discrimination is given by a combination of serotyping, protein profile, and bacteriocin typing methods (Senior and Vörös 1989).

Possible virulence factors of *M. morganii* strains for man include the calcium-dependent hemolysin and the constitutively produced urease (Senior 1983b). The hemolysin is serologically and functionally identical to *E. coli* hemolysin HlyA (Koronakis et al. 1987) and is associated with the high virulence of strains for mice and chick embryos (Emódy et al. 1982). It is more commonly produced by non-intestinal isolates of particular O serotypes (Vörös and Senior 1990) and it acts by forming cation-selective channels of about 1 nm diameter in membranes. It may aid invasion of host cells and lead to their death.

Although the urease of *M. morganii* strains has high affinity for urea and the potential to cause alkalinization of urine and stone formation, this rarely happens in vivo in man. Indeed, only rarely is *M. morganii* associated with urinary tract infections in the uncatheterized. This may be because of its inability to grow rapidly in urine and make it very alkaline (Senior 1983b). Even in those patients with catheters, *M. morganii* has difficulty in raising urinary pH and forming crystalline biofilms (Stickler et al. 1998). The reason for these things may be a consequence of *M. morganii* being unusual in the tribe in having a bifunctional urease which is activated at acid pH (Young et al. 1996). Thus the role of the urease may be to promote colonization of the intestinal tract by protecting the organism against the acidity of the stomach. Possibly as a consequence of this, *M. morganii* is frequently found in human feces and has also been isolated from the feces of other animals and reptiles. It is often found in diarrhetic stools in the absence of other known bacterial enteropathogens. However, firm evidence for its etiological role in enteritis has not yet been presented. Experiments with germ-free mice have shown *M. morganii* does not cause inflammatory bowel disease (Jiang et al. 2002).

From time to time *M. morganii* is associated with a variety of infections, particularly in neonates, pregnant women, the immunocompromised, and the diabetic. It has been isolated from blood, usually after surgery, cerebrospinal fluid, sputum, wounds, and abscesses – including those from snake bites (Jorge et al. 1994). It can cause an ecthyma gangrenosum-like eruption on the skin and can be a cause of septic arthritis. Outbreaks of hospital-acquired infections – some of them serious and even fatal – have been reported (Tucci and Isenberg 1981; Williams et al. 1983). *M. morganii* has been associated with cases of pneumonia in some animals. Because it has potent histidine decarboxylase activity, *M. morganii* has the potential to cause histamine food poisoning through the ingestion of contaminated unrefrigerated fish and particularly scombroid fish, such as tuna, sardines, pilchards, and anchovies.

With the exception of tetracycline which has been used to distinguish the subspecies of *M. morganii*, there is no evidence that strains of different biogroups differ in their sensitivity to antibiotics (Stock and Wiedemann 1998a). *M. morganii* strains are usually sensitive to quinolones, such as nalidixic acid and ciprofloxacin, to aminoglycosides such as gentamicin and tobramycin, to trimethoprim, cotrimoxazole, chloramphenicol, and aztreonam, and the carbapenems imipenem and meropenem. Care should be taken in interpreting results with the latter two antibiotics when measuring sensitivity by turbidity because of cell elongation prior to lysis (Biedenbach and Jones 1995). Although most isolates of *M. morganii* are sensitive to piperacillin with or without tazobactam, ticarcillin, and mezlocillin, they are resistant to other penicillins and many of the newer cephalosporins including cefuroxime, loracarbef, and cefazolin usually through the formation of inducible chromosomal ampC β lactamases. Some third generation cephalosporins, such as cefoxitin, when used to treat serious infections, are good inducers of these enzymes in *M. morganii* and give rise to strains resistant to third generation cephalosporins. *M. morganii* isolates with resistance to fourth generation cephalosporins such as cefotaxime and ceftriaxone through plasmid-mediated SHV- and TEM-derived extended-spectrum β lactamase production, have been reported (Coudron et al. 1997; Tessier et al. 1998; Power et al. 1999). Such resistant strains usually remain sensitive to cefpirome and

imipenem. The sensitivity of *M. morganii* strains to some of the newer oral cephalosporins such as cefixime, cefpodoxime, and cefetamet, when determined by disk diffusion as opposed to dilution methods, should be interpreted with care (Biedenbach et al. 1993). Most *M. morganii* strains are sensitive to latamoxef, ceftazidime, cefotaxime, cefepime, and cefpirome, the latter antibiotic also being active against ceftazidime-resistant isolates (Sader and Jones 1994). Strains show variation in their susceptibility to tetracyclines and sulphonamide.

PROVIDENCIA

DEFINITION

Strains of *Providencia* spp. do not swarm on solid nutrient media. All form acid from glucose and mannose, but gas formation from glucose is variable. They do not form acid from lactose or, except for some strains of *P. heimbachae*, maltose; they oxidatively deaminate amino acids. Most strains of species other than *P. rustigianii* form acid from one or more of the sugar alcohols adonitol, arabitol, erythritol, inositol, or mannitol. They do not produce ornithine decarboxylase or H_2S or form lipase or liquefy gelatin. Urease is formed only by *P. rettgeri* and some strains of *P. stuartii*. G + C content of the DNA is 39–43 mol% (Owen et al. 1987).

According to DNA hybridization studies and extensive phenotypic analyses, the genus has five species: *Providencia alcalifaciens* type strain ATCC 9886, NCTC 10286, CDC 9014-82, *Providencia stuartii* type strain ATCC 29914, NCTC 11800, CDC 2896-68, *Providencia rettgeri* type strain ATCC 29944, NCTC 11801, CDC 9167-79, *Providencia rustigianii* type strain ATCC 33673, NCTC 11802, CDC 132-68, and *Providencia heimbachae* type strain ATCC 35613, NCTC 12003, CDC 8025-83.

Stuart and colleagues (1943) described organisms they considered intermediate in character between *Proteus* and *Shigella*. Kauffmann (1951) placed them in a new genus, *Providencia*. Ewing et al. (1954) found that *Providencia* strains could be separated into two biochemical groups: one formed gas from glucose and fermented adonitol but not inositol, the other formed no gas from glucose and fermented inositol, but not adonitol. The organisms of these groups were given the specific names *P. alcalifaciens* and *P. stuartii*, respectively (Ewing 1962).

Providencia rettgeri is the name now given to the organism isolated by Rettger (1909) from a cholera-like epidemic in chickens and found by others to be anaerogenic and capable of fermenting mannitol. Rustigian and Stuart (1943) later assigned it to the genus *Proteus* as *Proteus rettgeri* when they found it hydrolyzed urea. However, Kauffmann (1953) believed the organism should be separated from the genus *Proteus* and proposed the name *Rettgerella*. The results of other workers, which indicated *Proteus rettgeri* had biochemical properties more similar to *Providencia* than to *Proteus* or *Morganella*, supported the view that the organism was classified in the wrong genus. Smit and Coetzee (1967) also showed that *Proteus rettgeri* and *Providencia* strains had similar phenylalanine deaminases. Penner and colleagues described a number of biotypes of *Proteus rettgeri*, one of which, apart from forming urease, resembled *P. stuartii* and had *Providencia* O antigens (Penner et al. 1975, 1976). Subsequent DNA hybridization studies by Brenner and colleagues (1978) showed that the latter were in fact urease-forming strains of *P. stuartii* and that all *Proteus rettgeri* strains were more related to *Providencia* than to *Proteus*. They proposed that the organism be known as *Providencia rettgeri*.

The name *P. rustigianii* was proposed by Hickman-Brenner et al. (1983) for strains previously called *P. alcalifaciens* biogroup 3 (Ewing et al. 1972) in honor of an earlier worker Robert Rustigian. DNA hybridization studies (Brenner et al. 1978) had shown this biogroup was not closely related to any of the other biogroups of *Providencia* or to the species of other genera in the tribe. The organism isolated by Müller (1983) from the feces of five different types of penguin and called *P. friedericiana* was later shown by DNA–DNA hybridization studies to be identical to *P. rustigianii*. The latter name became the approved one because it had been validated earlier (Hickman-Brenner et al. 1986). *P. heimbachae* is the name given to organisms also isolated from penguin feces by Friederike Heimbach. Initially they were thought to be a biogroup of *P. friedericiana*, but have been shown subsequently by DNA–DNA hybridization to form a distinct species (Müller et al. 1986).

GENERAL PROPERTIES

In general, strains of *Providencia* spp. are motile at 20–25°C by means of peritrichate flagella, but may be less active at 37°C. Fimbriae of five different morphological types have been found in members of the genus (Old and Adegbola 1982). *Providencia* strains do not swarm. They can often be recognized by their 'fruity' smell and as yellow or orange-centered colonies on deoxycholate citrate agar because the ferric hydroxide in the medium is precipitated at the alkaline pH which forms during growth. *Providencia* strains, unlike those of *Proteus* and *Morganella*, require neither niacin nor pantothenic acid for growth. The important biochemical reactions characteristic of the different species and biogroups of the genus are presented in Table 1.

Providencia strains differ from others of the Proteeae through their ability to form acid from mannose and their inability to decarboxylate ornithine or lysine. Except for strains of *P. rustigianii*, those of other *Providencia* spp. form acid from one or more of the

polyhydric alcohols adonitol, arabitol, erythritol, inositol, or mannitol.

P. rettgeri strains are genetically diverse, but are readily distinguished in the genus by their ability to ferment mannitol and degrade urea. Biogroups of *P. rettgeri* can be distinguished by their reactions in rhamnose, salicin, and erythritol (Penner et al. 1975). Some 75 percent of isolates of *P. rettgeri* also have the ability to acidify erythritol. This reaction is unique to this species among members of the Proteeae.

P. stuartii is an organism of great clinical importance because of its tendency to be resistant to many antibiotics and to cause nosocomial infections. However, it is often misidentified, particularly with commercial kits, because strains frequently carry plasmids that may bear genes for the fermentation of sucrose or lactose or the hydrolysis of urea. Thus the organism may show a variable phenotype. It forms, along with *M. morganii*, large amounts of phosphatase, and media have been devised to simplify its recognition (Thaller et al. 1992). *P. stuartii* strains are readily distinguished in the genus by their ability to form acid from trehalose. Unusual isolates, with some properties shared with *P. rettgeri*, can also be distinguished in that *P. stuartii* strains do not decarboxylate L-glutamic acid (Fischer et al. 1989).

P. rustigianii is distinguished from other species in the genus by its inability to form acid from any of the polyhydric alcohols inositol, mannitol, adonitol, arabitol, or erythritol. It forms acid from galactose, but not trehalose.

Strains that have the properties of the genus, but do not degrade urea or form acid from trehalose but do form acid from adonitol, are either *P. alcalifaciens* or *P. heimbachae*. *P. alcalifaciens* strains form indole, but do not form acid from either rhamnose or galactose. Information from ribotyping and polymorphisms of DNA and enzymes suggests there may be more than one species among strains presently identified as *P. alcalifaciens* (Picard et al. 1991).

P. heimbachae strains form acid from both rhamnose and galactose. They differ from other members of the Proteeae in not growing in the presence of KCN and are unusual among *Providencia* in being citrate-negative and indole-negative in certain media (but indole-positive in others). Indole reactions need to be carefully controlled with regard to medium and time of incubation when identifying strains of *Providencia* spp., for some strains may subsequently degrade the indole that they had formed (Müller 1986).

The genus *Providencia* is antigenically heterogeneous. Strains have thermostable O antigens, thermolabile H antigens, and some also have capsular (K) antigens. In general, each species has its own distinctive O antigens, 46 O-antigenic groups having been defined in *P. alcalifaciens* (Penner et al. 1979a), 17 in *P. stuartii* (Penner et al. 1979b), and 93 in *P. rettgeri* (Penner and Hennessy 1979). In addition to serotyping, strains of *Providencia* can be differentiated by phage typing (Chouteau et al. 1974), bacteriocin typing (Stickler and Thomas 1976; McHale et al. 1981), and protein profile typing on SDS polyacrylamide gels (Costas et al. 1987; Holmes et al. 1988; Costas et al. 1989, 1990b). *P. stuartii* has also been typed on the basis of restriction fragment length polymorphisms of rRNA cistrons (Owen et al. 1988).

Although *P. alcalifaciens* is occasionally found in normal human feces, it is found more often in the stools of patients with diarrhea and gastroenteritis because some strains are thought to be the causative agent of these conditions both in man and animals (Haynes and Hawkey 1989; Albert et al. 1992, 1998; Tribe and Rood 2002; Chlibek et al. 2002). A medium which readily detects *P. alcalifaciens* in fecal samples has been devised (Senior 1997b). *P. alcalifaciens* strains which have been found on salad vegetables and fruits (Visnawathan and Kaur 2001) and can be carried by flies (Urban and Broce 1998), have been associated with large outbreaks of foodborne infection (Murata et al. 2001). Among the isolates of *P. alcalifaciens* associated with diarrhea are some which are able to invade a variety of tissue culture cell lines (Janda et al. 1998) and these belong to only a few ribotypes, whereas others are noninvasive and comprise many different ribotypes (Guth and Perrella 1996). The invasive isolates appear to be of a clonal lineage (Guth et al. 1999) and, facilitated by a secreted 28 kDa protein (Rahman et al. 2002), they enter host cells either by endocytosis and polymerization of actin – a process inhibited by cytochalasin D which inhibits microfilament formation, or by disruption of tight junctions and multiplication in intercellular spaces (Mathan et al. 1993; Albert et al. 1995; Guth et al. 1999). Although the condensation of actin filaments by invasive strains is in a manner similar to that caused by *Shigella flexneri* and different from that caused by enteropathogenic *E. coli* (Albert et al. 1992, 1995), DNA sequences homologous to the virulences genes of these and other invasive enteric bacteria have not been detected in *P. alcalifaciens* nor does invasiveness appear to be plasmid determined (Sobreira et al. 2001).

P. stuartii strains, though only occasionally isolated from infected wounds and burns, are among the most common isolates from urine of long-term catheterized elderly patients, particularly women, in old people's homes and geriatric wards in hospitals (Warren 1986; Rahav et al. 1994). In these situations, strains can persist in patients for several months and serve as the reservoir of infection for others. Strains are particularly difficult to eradicate because of their formation of MR/K fimbriae which bind to uroepithelial cells and the catheter, and also because they are frequently resistant to many antibiotics. Such strains may give rise to a fatal bacteremia (Warren 1986). In animal models, isolates vary in their uropathogenicity, which is associated with motility and the ability to form urease and fimbriae.

P. rettgeri has been isolated from poultry and is part of the normal fecal flora of a number of reptiles and amphibians. It has been isolated from surface waters and is probably widespread in nature. It is not commonly found in human feces but has been associated with nosocomial infections of the urinary tract (Edwards et al. 1974; Lindsey et al. 1976), its active urease causing alkalinization of urine and encrustation of catheters (Stickler et al. 1998). Urinary tract infections with *P. rettgeri* are usually of less importance than those due to *P. stuartii* because of the greater sensitivity of the former to antibiotics. From time to time *P. rettgeri* is also the cause of infections of wounds, burns, and blood.

Most isolates of *P. rustigianii* have come from human feces and those of penguins and pigs. *P. heimbachae* strains have also been isolated from penguin feces and an aborted bovine fetus, but until recently this organism had not been isolated in man. However, an isolate from human feces which was phenylalanine deaminase negative and had other biochemical characteristics different from the type strain was confirmed by DNA–DNA hybridization to be *P. heimbachae* (O'Hara et al. 1999). At the present time it is very unusual to find isolates of *P. rustigianii* and *P. heimbachae* in clinical specimens from man.

Providencia strains, like other Proteeae, are resistant to polymyxin B and colistin. In general, *P. alcalifaciens* and *P. rustigianii* strains are naturally sensitive to a wide range of antibiotics, including aminoglycosides and quinolones, and antibiotic resistance is not a common problem. This is also probably true for *P. heimbachae* although only a few isolates have been tested (Müller et al. 1986; O'Hara et al. 1999). The opposite is true for *P. stuartii*. *P. rettgeri* strains have a position in between these extremes (Stock and Wiedemann 1998b). *P. stuartii* is the most antibiotic-resistant species of the Proteeae and one of the most resistant organisms known (Hawkey 1984). Most strains are resistant to tetracyclines, chloramphenicol, sulfonamides, nitrofurantoin, and many penicillins, cephalosporins, and the aminoglycosides gentamicin, tobramycin, and netilmicin. Resistance to these aminoglycosides is a consequence of a chromosomal gene in *P. stuartii* coding for the antibiotic modifying enzyme 2'-N-acetyltransferase (Rather et al. 1993). This enzyme also acetylates peptidoglycan in *P. stuartii*, affecting the morphology of the cells (Payie et al. 1996). *P. stuartii* is much more resistant than *P. rettgeri* and *P. alcalifaciens* to the substituted penicillins mezlocillin, azlocillin, piperacillin, carbenicillin, and ticarcillin (Penner et al. 1982; Hawkey et al. 1983). Among the antibiotics often found useful for treating *P. stuartii* urinary tract infections are the oral cephalosporins cefuroxime, cefetamet, and cefpodoxime or ciprofloxacin (Cornaglia et al. 1995). *Providencia* spp. are also sensitive to ceftazidime, cefotaxime, imipenem, and the monobactam aztreonam. The aminoglycoside amikacin is often useful for treating gentamicin-resistant strains. Among the quinolone antibiotics, ciprofloxacin is one of those most active against *P. stuartii* (Hawkey and Hawkey 1984). Clinical isolates of *P. stuartii* are often resistant to disinfectants such as chlorhexidine and cationic detergents such as cetrimide and benzalkonium chloride, but are sensitive to phenols and glutaraldehyde.

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Yersinia

AUDREY WANGER

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GENUS/SPECIES DEFINITION

The genus *Yersinia* belongs to the family Enterobacteriaceae. They are gram-negative rods (coccobacilli) with rounded ends. *Yersinia* are facultative anaerobes that ferment glucose, in addition to other sugars, without the production of gas, are oxidase-negative and catalase-positive, reduce nitrate, and grow on nutrient agar. *Yersinia* are motile at 22–30°C, but not at 37°C. Their cell-wall and antigenic composition also resemble those of other members of the family Enterobacteriaceae and some are identical. Many of the members of the genus *Yersinia* possess the ability to grow under extreme ranges of temperature and are well adapted to survival in the environment. As a genus, these organisms are distinct from most other members of the family Enterobacteriaceae in that they are smaller in size and stain in a bipolar fashion, resembling a 'safety pin' (not seen with the Gram stain or from isolates grown in culture). *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* are human pathogens, and *Yersinia ruckeri* is a fish pathogen, whereas the other species are commensals. The G + C content of the DNA of the genus is 46–50 mol%. Eighty percent homology exists between *Y. pestis* and *Y. pseudotuberculosis*, while only 20–55 percent homology exists between *Y. enterocolitica* and *Y. pseudotuberculosis* and *Y. ruckeri*. *Y. pestis* is the type species for the genus.

Y. pestis is nearly genetically identical to *Y. pseudotuberculosis* and is thought to have evolved from *Y. pseudotuberculosis* 1 500–20 000 years ago, before the first plague pandemic. Consideration was given to combining the species; however, they remain distinct due to the differences in clinical disease and public-health implications of *Y. pestis* (Oyston and Isherwood 2002; Achtman et al. 1999).

INTRODUCTION AND BRIEF HISTORICAL PERSPECTIVE

Members of the genus *Yersinia* were formerly included in the genus *Pasteurella* and finally removed in the 1970s, although this transfer was suggested by Thal in 1954 (Bercovier and Mollaret 1984) due to their dissimilarity with other members of this genus. The name *Yersinia* was derived from the French bacteriologist Alexander Yersin, who first isolated the plague bacillus in Hong Kong in 1894 (Gyles and Thoen 1993; Solomon 1995). Included in this new genus, in addition to

Y. pestis, was *Y. pseudotuberculosis*, first isolated by Malassez and Vignal in 1883 after inoculation of guinea pigs with material isolated from a skin lesion of a child who died of meningitis. Also moved from the genus *Pasteurella* to the newly formed genus *Yersinia* was the species *Y. enterocolitica*, described by Schleifstein and Coleman in 1939, who first called it *Bacterium entercoliticum* (Cover and Aber 1989) and later *Pasteurella pseudotuberculosis rodentium* and *Pasteurella* X. It was given its current designation of *Y. enterocolitica* in 1964 by Frederiksen (Bottone 1977).

Y. ruckeri, the causative agent of enteric redmouth disease of fish, was first described in 1955 and added to the genus *Yersinia* in 1978 on the basis of DNA analysis (Furones et al. 1993). Three additional species, *Yersinia frederiksenii*, *Yersinia intermedia*, and *Yersinia kristensenii*, were designated in 1980 and *Yersinia aldovae* in 1984 (Bercovier and Mollaret 1984). *Y. aldovae*, *Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii* are often referred to as *Y. enterocolitica*-like group as a matter of convenience. Strains of *Yersinia*, isolated in the USA and Germany, that agglutinated with *Y. enterocolitica* H antisera but were untypable with O antisera and differed biochemically from other *Yersinia* by being negative for indole, Voges–Proskauer, and citrate were classified as a new species, *Yersinia rohdei* (Aleksic et al. 1987). *Y. enterocolitica* biogroup 3 was divided into 3A, which fermented L-sorbose and either *i-* or *myo*-inositol, and 3B, which did not ferment either; these were proposed as a new biogroup, number 6 (Wauters et al. 1987). However, DNA studies revealed that they were distinct enough for each to be a separate species and they were subsequently named *Yersinia mollaretii* and *Yersinia bercovieri*, respectively (Wauters et al. 1988).

The Philomiragia bacterium, isolated from a muskrat in 1959, was included in the genus *Yersinia* as proposed by Jensen in 1969 due to similar morphology and its DNA relatedness to *Y. pestis* (Hollis et al. 1989). In 1980 this categorization was reconsidered and the organism was renamed *Francisella philomiragia* and will not be discussed further in this chapter.

HABITAT

Y. pestis survives in nature in the stomach and proventriculus of the flea and in the soil of animal burrows. *Y. pestis* is maintained in the environment by enzootic spread of the organism among rodents (natural reservoir host), which usually have an unapparent infection and low risk for transmission to humans. Epizootic plague spreads rapidly among susceptible rodents, which die off rapidly (Gage 1998). *Y. pestis* is transmitted to humans either through the bite of the flea or by regurgitation during attempts at taking a blood meal; the organism causes sylvatic plague, the most common form of plague found in the USA (Gutman 1992). *Y. pestis* is also known to infect more than 200 species of rodents and small animals (Gutman 1992), which can also be a source of human infection. Endemic areas for plague include areas of Southeast Asia (predominantly Vietnam), Africa (predominantly Madagascar and Tanzania), South America (predominantly Brazil and Peru), and the western USA (Gage 1998).

Y. pseudotuberculosis can also be transmitted by rodents or other infected animal species, including wild birds (Fantasia et al. 1993), although, like *Y. enterocolitica*, the most common mode of transmission is by food or water contaminated with infected fecal material (Gyles and Thoen 1993). Both *Y. enterocolitica* and *Y. pseudotuberculosis* can survive for long periods in the environment due to their ability to grow with minimal nutritional requirements and their ability to remain metabolically active at extremes of temperature (Brubaker 1991; Tashiro et al. 1991). Although *Yersinia* as a group is sensitive to inactivation by heat (56°C) or chemical disinfectants (phenol reagents), *Y. pestis* can remain viable in dried blood for at least 3 weeks and for months in the soil. *Y. enterocolitica* is also found in the intestines of a variety of animal species; although in most cases the serotypes identified in animals are not pathogenic for humans, pathogenic serotypes have been isolated from healthy pigs (Doyle 1990). In parts of Europe and Japan, the most common animal reservoir of *Y. enterocolitica* is pigs. Contamination of food such as chocolate milk and tofu has also been implicated as an outbreak source for *Y. enterocolitica*.

Many of the *Yersinia*-like species are normally found in the environment, especially in water, and some have been isolated from animals or humans with gastrointestinal disease. Interestingly, a major reservoir for these organisms is wild mice. *Y. frederiksenii* is found in water and soil and is considered a human commensal of the gastrointestinal tract. Colonization of the gastrointestinal tract has been demonstrated in hospital staff as well as infection in medical residents who drank unpasteurized milk (Cafferkey et al. 1993). Both *Y. mollaretii* and *Y. bercovieri* are found in the environment and thought to be saprophytes (Wauters et al. 1988). *Y. rhodei* has been isolated from surface water, stools from two patients with diarrhea, and several healthy dogs (Aleksic et al. 1987). Its significance as a human or animal pathogen is unknown at this time. *Y. ruckeri* is a fish pathogen that causes an inflammation of the head region affecting trout as well as salmon (Furones et al. 1993). It is common throughout Europe, North America, South Africa, and Australia. *Y. intermedia* is also found in the environment as well as in fresh-water fish, oysters, shrimp, snails, wild and domestic animals, foods, and humans with gastritis (Sulakvelidze 2000). *Y. aldovae* is also found in water and soil but has not been found to be associated with disease in either animals or humans.

MORPHOLOGY

Members of the genus *Yersinia* are gram-negative coccobacilli that exhibit bipolar staining, particularly when stained from clinical material. Wayson's dye mixture (methylene blue with carbolfuchsin) or Giemsa's stain is recommended for staining *Y. pestis* (Gutman 1992). *Yersinia*, especially *Y. pestis*, can also appear quite pleomorphic, particularly when colonies growing on nutrient agar medium are stained. *Yersinia* spp. produce no endospores and have no envelope, except for *Y. pestis*, which has been shown to have an envelope in vivo and in vitro when grown at 37°C on a rich medium such as serum agar (Burrows 1963). The envelope is thought to represent the F1 glycoprotein antigen complex (Chen and Elberg 1975). The organisms are smaller in size then other members of the family Enterobacteriaceae (0.5–0.8 μm in diameter and 1–3 μm in length). *Y. pestis* is nonmotile, whereas both *Y. enterocolitica* and *Y. pseudotuberculosis* are motile, with peritrichous or paripolar flagella, when grown at 22°C, but not at 37°C (Gutman 1992). When cultivated below 30°C, all species of *Yersinia* except *Y. pestis* produce flagella and are motile. Pease (1979) demonstrated biochemically and electron-microscopically that, at room temperature, strains of *Y. enterocolitica* could go through a transition to a spheroplast type L-form, where they could revert back to an irregular shaped structure with an intact cell wall. It has also been hypothesized by Zykin (1994) that conversion of *Y. pestis* into L-forms allows them to persist in fleas or ticks for prolonged periods before conversion back to a bacillary form and transmission to a susceptible host.

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

Yersinia are able to grow at temperatures ranging from 4 to 43°C and over a pH range of 4–10 (*Y. pestis* and *Y. pseudotuberculosis*, pH 5.0–9.6), although the optimum is pH 7.2–7.4 (Bercovier and Mollaret 1984). A combination of low pH and low temperature is used to prevent *Y. enterocolitica* from growing in foods at refrigeration temperature (Brocklehurst and Lund 1990). *Yersinia* are also salt-tolerant. *Y. ruckeri* will grow in salt at a concentration of 3 percent, whereas *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* will grow in up to 5 percent salt. All species can grow on nutrient agar, although colony size is smaller and growth is slower for *Y. pseudotuberculosis* and *Y. pestis* (Bercovier and Mollaret 1984). Colonies of *Y. pestis* can be as small as 0.1–0.2 mm after 24 h of growth on nutrient agar at 37°C. With increased incubation, the colonies reach a size of 1–1.5 mm in diameter. All other species of *Yersinia* will reach a size of 1–1.5 mm after 24 h and 2–3 mm after 48 h of incubation at 37°C, although small colony variants often remain. The colony morphology also changes to become opaque with a gray to yellow color in the center; they remain transparent and gray to white in color on the periphery ('Chinese hat' shape). Growth and size of the colonies may be slightly improved by the addition of blood to the medium. Hemolysis is not observed when grown on blood agar. Most *Yersinia* will grow on MacConkey medium, although growth of *Y. pseudotuberculosis* and *Y. pestis* is variable. *Y. pseudotuberculosis* is the only species of the genus that will grow as small gray to black colonies on tellurite medium (Brzin 1963).

Growth of *Yersinia* is moderate in liquid media. After 48 h of incubation, some turbidity is evident, whereas other Enterobacteriaceae will become turbid in less than 18 h of growth in liquid media. *Y. pestis* and *Y. pseudotuberculosis* do not show uniform turbidity but settle to the bottom of the tube while the supernatant remains clear. A pellicle forms, which disintegrates to form flocculent masses and larger deposits at the bottom of the tube (more evident in peptone water than in nutrient broth).

When grown at 25°C on minimal medium, *Y. pestis* requires L-methionine and L-phenylalanine, whereas other species do not. At 37°C, all species require supplements to grow on minimal medium (thiamine, biotin, and other amino acids) (Burrows 1963). *Y. pestis* has the particular requirements of isoleucine, glycine, threonine, valine, a reducing agent, and supplementary CO_2 when grown under these conditions (Brubaker 1991).

All species of *Yersinia* have a requirement of iron for growth. Virulent strains of *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* also have a requirement for a low concentration of calcium in order to express some of the antigens involved in the virulence of these species. The concentration of calcium in the medium is critical for the expression of virulence factors of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. The mechanism by which calcium affects virulence is discussed further in Bacteriocins and bacteriophages.

METABOLISM

Yersinia are aerobic and facultatively anaerobic and, therefore, both oxidative and fermentative. As with other members of the family Enterobacteriaceae, *Yersinia* ferment glucose with the production of acid and little or no gas. Fructose, galactose, maltose, mannitol, mannose, N-acetylglucosamine, and trehalose are fermented. Rhamnose, melibiose, raffinose, and α-methyl-D-glucoside are fermented soley, by *Y. frederiksenii* (Sulakvelidze 2000). *Y. enterocolitica*, *Y. frederiksenii*, and *Y. intermedia* produce acetoin when incubated at 28°C but not at 37°C.

GENETIC MECHANISMS

Genetic mechanisms of *Yersinia* are thought to be similar to those of *Escherichia coli*, although no known

intrinsic factors of conjugation or transduction have been specifically described. Sex factors or bacteriophages of *E. coli* have been shown to allow transfer of DNA between *E. coli* and *Yersinia* (Brubaker 1991). Although rare in clinical isolates, plasmids have been described in *Yersinia*, particularly in *Y. enterocolitica*. Plasmids involved in fermentation of both lactose and raffinose have been described. Plasmids that code for various virulence factors and antibiotic resistance genes have also been described (Ferber and Brubaker 1981). All pathogenic species of *Yersinia* possess a 70-kb plasmid, a low-calcium-response plasmid that contains the gene necessary for virulence of these organisms. *Y. pestis* has two additional plasmids, a 10-kb plasmid (pPst), which encodes pesticin I, and pFra, which encodes the envelope antigen fraction 1 and murine toxin (Filippov et al. 1990).

In vitro transfer of DNA in *Yersinia* species has rarely been successful. Conchas and Carniel (1990) described the parameters for high-efficiency transformation of *Yersinia* by electroporation, a method used extensively for *E. coli*. They were successful at transferring *E. coli* plasmid DNA into *Yersinia* at a frequency of up to 10^5–10^7 transformants per microgram of DNA. Cutrin et al. (1994) also used electroporation on a strain of *Y. ruckeri* and achieved a transformation efficiency of 6×10^5 (Cutrin et al. 1994).

Hertwig et al. (1999), described transduction of small plasmids of *Yersinia* species using phages found in nature; however, transduction of the large virulence plasmid was not successful.

CELL-WALL AND ENVELOPE COMPOSITION; ANTIGENIC STRUCTURE

The cell-wall and antigenic structure of the genus *Yersinia* is, in general, very similar to that of other members of the family Enterobacteriaceae. The cell walls of these organisms are made up of lipopolysaccharide (LPS) consisting of a lipid A, core region, and an O-specific side chain, as seen with other Enterobacteriaceae, with minor genus and species differences. The nature of the LPS of *Y. enterocolitica* does vary for certain serogroups. The pathogenic serogroups 3, 8, and 9 are composed of galactosamine, glucosamine, 3-deoxy-D-mannoctulosonic acid (KDO), 2 heptoses, glucose, and galactose (Cornelis et al. 1987). *Y. pestis* lacks the extended O-group structure present in the other species. When *Y. pseudotuberculosis* is grown at 26°C instead of 37°C, short O-group side chains are formed.

Y. pestis has more than 20 antigens, many of which are shared with *Y. pseudotuberculosis* and *Y. enterocolitica* and some of which are essential for virulence of the organisms. The fraction 1 envelope antigen (F1) is a heat-labile, water-soluble antigen that stimulates immunity in both mice and humans and is produced only when the organism is grown at 37°C on protein-rich media (see Bacteriocins and bacteriophages, for further discussion of the role of this protein in virulence). Other antigens associated with *Yersinia* species are the V and W antigens. This complex is composed of two proteins, both with a molecular weight of 90 kDa. The V protein is cell-bound and the W protein is an excreted protein. The VW antigens, as well as outer-membrane proteins, Yops, are associated with virulence of the organisms and are described in detail in Bacteriocins and bacteriophages. Two of these Yops in *Y. enterocolitica*, YopC and YopF, function as porins based on permeability studies performed with nitrocefin and cephaloridine (Brzostek and Nichols 1990). Other antigens produced by *Y. pestis* include an intracellular toxin called murine toxin, composed of two high-molecular-weight proteins (toxin A and toxin B) and a lipopolysaccharide endotoxin (Gyles and Thoen 1993).

Y. enterocolitica has more than 70 serotypes based on O and H antigens. O:3 and O:9 are the most common serotypes associated with human disease in Europe, Japan, and Canada, and serotypes O:8 and O:5,25 are the most common in the USA (Currie 1998). Since 1981, there has been a trend in California and New York in the increase in the prevalence of serotype O:3 as the predominant type in cases of gastroenteritis (Bissett et al. 1990). Serotypes O:1, O:2, O:3, O:5, O:6, O:7, and O:8 are responsible for most cases of severe disease in animals. There is little antigenic relationship between *Y. enterocolitica* and other *Yersinia* species; however, cross-reaction has been noted between *Y. enterocolitica* serotype O:9 and *Brucella*. There is some similarity between *Y. enterocolitica* and *Vibrio cholerae* (Isshiki et al. 1992). Flagellar or H antigens are present but are complex, highly species-specific, and not useful for serotyping (Cornelis et al. 1987). Although no true capsule has been described, some strains do have a K antigen (Cornelis et al. 1987). Pathogenic strains also produce an enterotoxin similar in nature to *E. coli* ST. A detailed discussion of the antigens of *Y. enterocolitica* can be found in Wauters (1981).

There are six serotypes of *Y. pseudotuberculosis* and eight subtypes based on the presence of 15 O antigens. Serotype O:1 is associated with 90 percent of all cases of human disease. Some cross-reactivity has been noted between the O antigens of *Y. pseudotuberculosis* and some serotypes of *Salmonella* (Gutman 1992). At least five distinct flagellar (H) antigens are produced at temperatures below 30°C in *Y. pseudotuberculosis*. O and H serotypes of *Y. pseudotuberculosis* cross-react to some extent with other members of the family Enterobacteriaceae and have some distinct geographical distributions. For a description of the LPS O antigens of *Y. ruckeri* serotype 01 and *Y. kristenseii*, see Beynon et al. (1994) and L'vov et al. (1992), respectively.

CLASSIFICATION

Members of the genus *Yersinia* are classified into 11 species, based on biochemical analysis. The species of

most concern to humans is *Y. pestis*, the agent of plague. *Y. enterocolitica* and *Y. pseudotuberculosis* are also human pathogens, whereas the other species, with the exception of *Y. ruckeri*, which is a fish pathogen, are environmental, nonpathogenic organisms (Romalde 1993). *Y. pestis* and *Y. pseudotuberculosis* are biochemically homogeneous. *Y. enterocolitica* as a species is very heterogeneous and was initially divided into five biogroups based on the following biochemical reactions: indole production, hydrolysis of esculin and salicin, lactose oxidation, acid from xylose, trehalose, sucrose, sorbose, and sorbitol, o-nitrophenyl-β-D-galactopyranoside (ONPG), ornithine decarboxylase, Voges–Proskauer reaction, and nitrate reduction (Wauters 1981). The scheme was later modified by Wauters to include six biotypes. Biogroup 1 was divided into two groups. Group 1A includes environmental strains that lack virulence plasmids and are pyrazinamidase-, esculin-, and β-D-glucosidase positive. The strains that compose biogroup 1B are esculin-, pyrazinamidase-, and β-D-glucosidase-negative and belong to one of the following serogroups: O:4, O:8, O:13, O:18, O:20, or O:21. Biogroup 1B strains are reportedly pathogenic to humans in North America (Cornelis et al. 1987).

Y. pestis can also be divided into three biogroups (antigua, medievalis, and orientalis), based on their ability to reduce nitrate and to ferment glycerol and melibiose. These biogroups were also found to correlate with the geographical distribution and strain-specific rodent species.

CLINICAL DISEASE AND IMMUNE RESPONSE

Fleas acquire *Y. pestis* by ingestion of blood from an infected rodent. Infection in the flea occurs predominantly in the alimentary tract. Approximately 3–9 days after the infected meal, a mass of organisms, hemin, and fibrin-like material enlarges to block the stomach so that further blood meals cannot be swallowed and are regurgitated into the host (Perry and Fetherston 1997).

Once in a human host, *Y. pestis* has a predilection for lymphoid tissue, although the organism can infect the lungs and can spread via respiratory secretions to other humans. Humans infected with *Y. pestis* develop lesions in the blood vessels; these lesions discolor the skin, giving the disease the name 'black plague.'

There are three clinical forms of plague in humans. The most common is bubonic plague, in which a swollen painful lymph node (bubo) develops at the site of infection. Usually only a single bubo develops; however, the organisms can spread to other nodes via the bloodstream. The second most common form is septicemic plague, which usually arises due to dissemination of organisms into the blood from an infected bubo (cases of inadequately treated bubonic plague). Complications may include pneumonia, meningitis, and endophthalmitis. The rarest, but most serious, form of the disease is pneumonic plague. Primary pneumonic plague occurs following close contact and inhalation of *Y. pestis* organisms from another human or animal (cat) with plague pneumonia (Gage 1998).

Y. pseudotuberculosis causes a mesenteric lymphadenitis in infected humans, which mimics appendicitis (Fukushima 1987). Severe septicemia can occur in immunocompromised patients (Salyers and Whitt 1994). For the most part, it is a self-limited disease; however, in patients with liver damage, the organism can disseminate (Naktin and Beavis 1999). Complications can include erythema nodosum, Reiter's syndrome, and nephritis with a duration of 1 week to 6 months (Quan 1998).

Children infected with *Y. enterocolitica* most often present with enterocolitis; however, older children can develop severe disease, often mistaken for appendicitis (Cover and Aber 1989). Adults more often present with reactive arthritis (Reiter's syndrome) or erythema nodosum (Viitanen et al. 1991). Adults may also develop enteric disease followed in some cases by immune-mediated arthritis. The HLA type B27 is associated with increased risk of arthritis. The only reported cases of human-to-human transmission of this organism were due to transfusion of contaminated blood (Cover and Aber 1989). Again, the ability of *Y. enterocolitica* to survive for extended periods at refrigeration temperatures facilitates the growth of the bacteria in contaminated blood products (Feng et al. 1992; Baert et al. 1994).

The immune response to *Y. pestis* is both humoral and cellular. In plague, antibody to F1 capsular antigen gives protection in animals against cutaneous challenge with *Y. pestis* but may not protect against air-borne challenge (Gage 1998). Mice were also found to be protected from challenge with *Y. pestis* after development of both an antibody response and cell-mediated response to recombinant V antigen. Recombinant F1 + V antigens have been evaluated as a possible vaccine candidate (Williamson 2001). In *Y. enterocolitica* the major protective portion is the T-cell response (CD4 and CD8). A 66-kDa heat-shock protein might induce cytokine production in macrophages contributing to induction of an immune response (Noll and Auterwith 1996). Protection of mice against *Y. enterocolitica* can be transferred by HSP60 reactive T cells (Noll and Autenrieth 1996).

LABORATORY ISOLATION AND IDENTIFICATION

Because of the potential hazards to laboratory personnel, the microbiological isolation of *Y. pestis* is not usually performed in routine clinical laboratories without biosafety isolation hoods and equipment.

Specimens such as aspirates from lymph nodes or other body fluids collected from a patient suspected of having plague should be sent to a laboratory with the proper facilities to culture the organism. Care in transport is essential.

Y. pestis is inactive in the majority of biochemical tests and therefore is difficult to identify; many commercially available identification systems do not include this species in their database. Suspected isolates should be sent to a national reference laboratory for identification and epidemiological investigation. Serological diagnosis using microhemagglutination, complement fixation, or enzyme immunoassay can be used to detect antibodies to *Y. pestis* in the patient's serum. *Y. pestis* antigens can also be detected directly in patient samples. A rapid test described by Chanteau et al. (2003) uses a monoclonal antibody to detect F1 antigen present in large amounts in the blood and bubo fluid from patients infected with *Y. pestis*. Amplification of two plasmid-encoded genes, plasminogen activator and antigen fraction 1, previously sequenced by Sodeinde and Goguen in 1989 (Sodeinde et al. 1992), successfully identified *Y. pestis* in as little as 4 μl of blood from infected mice in experiments simulating the quantity of organisms found in the blood of infected humans (Norkina et al. 1994). Although some homology exists between portions of these genes of *Salmonella typhimurium* and *E. coli*, primers used were selected to avoid homologous regions. Bardarov et al. (1990) cloned and characterized a chromosomal fragment of DNA that was specific for *Y. pestis* and has the potential as a diagnostic tool for the direct identification of *Y. pestis* in clinical samples.

Culture of *Yersinia* other than *Y. pestis* can be readily performed in established clinical microbiology laboratories. *Yersinia* will grow on nonselective media and can be isolated from specimens obtained from usually sterile sites by inoculating blood agar medium. *Yersinia* grow well at 35°C; however, a lower isolation temperature is recommended for specimens that may be contaminated with other bacteria, e.g. incubation at 25°C allows a selective advantage for the isolation of *Yersinia* from stool specimens. Cold enrichment of the stool (incubation of the sample in saline at 4°C for 1–3 weeks) has been shown to increase the frequency of isolation of the organism (Davis et al. 1980). Isolation of *Yersinia* from contaminated specimens is further enhanced by the use of a selective medium, such as cefsulodin–Irgasan–novobiocin (CIN) medium. This medium inhibits the majority of other enteric bacteria. On CIN medium, *Y. enterocolitica* may be identified by its characteristic colony morphology (red pigmented colonies surrounded by a clear zone). In a review of various antimicrobials as a supplement to a broth medium for selective recovery of *Y. enterocolitica*, Toora et al. (1994) found Irgasan to be the most effective. Addition of other antimicrobials either did not inhibit all enteric bacteria or inhibited some *Yersinia* strains as well (Toora et al. 1994). Selective enrichment for *Y. enterocolitica* from food was best achieved using Irgasan ticarcillin broth (de Boer 1992).

All species of *Yersinia* produce catalase but not oxidase. Specific identification of *Yersinia* requires the use of individual biochemical utilization tests. Sharma et al. (1990) successfully used the API 20E (a commercially available system containing more than 20 biochemical reactions in microtubes inoculated simultaneously) for species-level identification of *Y. enterocolitica*, *Y. frederiksenii*, and *Y. kristensenii* using 175 stool isolates collected in Canada. Neubauer et al. (1998) compared the API 20E, API Rapid32 IDE, Micronaut E, and polymerase chain reaction (PCR) for identification of ten different species of yersinia using a set of 118 well-characterized strains. API 20E had the highest sensitivity and specificity and was the most cost-effective method for identification. Identification of *Yersinia* species other than *Y. enterocolitica* and *Y. pseudotuberculosis* was problematic with all of the systems studied.

De Ryck et al. (1994) proposed a screen method for the rapid identification of enteric pathogens, including *Y. enterocolitica*, from stools. Colonies resembling *Y. enterocolitica* on CIN medium were transferred to rhamnose broth. Strains that were rhamnose-negative and motility-negative, as determined by a wet mount from rhamnose broth, were identified presumptively as *Yersinia* species (De Ryck et al. 1994). Kitch et al. (1994) evaluated the RapID onE (Innovative Diagnostics, Inc.) for the identification of 379 enteric organisms including *Yersinia* species. The system correctly identified eight out of eight isolates of *Yersinia* species. The Vitek enteric pathogens card also correctly identified five isolates of *Y. enterocolitica* (Imperatrice and Nachamkin 1993). Identification of *Yersinia* species can be misinterpreted due to the slow growth of the organism at 35°C. *Y. enterocolitica* and *Y. frederiksenii* differ by only one biochemical fermentation of rhamnose, which occurs slowly at 35°C (Cafferkey et al. 1993). Therefore, biochemical analysis of *Y. enterocolitica* and the related species should be performed at temperatures of less than 30°C (see Table 1 for biochemical tests helpful in the identification of *Yersinia* spp.).

PCR assays have also been shown to be useful in the identification of *Y. enterocolitica* and *Y. pseudotuberculosis* directly from either food or clinical specimens, alleviating the need for the cumbersome and time-consuming biochemical identification of these isolates. Since plasmids of *Y. enterocolitica* are shared with other species of *Yersinia*, PCR assays for detection of virulence lack species-specificity (Ibrahim et al. 1992). Nakajima et al. (1992) described a PCR assay based on the amplification of a region of the *inv* gene of *Y. pseudotuberculosis* and the *ail* gene from pathogenic

Table 1 *Biochemical reactivity of* Yersinia *spp.*

	Y. pestis	*Y. pseudo-tuberculosis*	*Y. entero-colitica*	*Y. frederiksenii*	*Y. kristensenii*	*Y. ruckeri*	*Y. mollaretii*	*Y. bercovieri*	*Y. rohdei*	*Y. aldovae*	*Y. intermedia*
Motility (25°C)	–	+	+	+	+	v	+	+	+	+	+
Ornithine decarboxylase	–	–	+	+	+	+	+	+	v	+	+
Urease	–	+	+	+	+	–	+	+	v	+	+
VP (25°C)	–	–	+	+	–	v	–	–	–	+	+
Citrate (25°C)	–	–	–	v	–	+	–	–	+	+	+
Indole	–	–	v	+	v	–	–	–	–	–	+
Rhamnose	–	+	–	+	–	–	–	–	–	+	+
Sucrose	–	–	+	+	–	–	+	+	+	–	+
Cellobiose	–	–	+	+	+	–	+	+	+	–	+
Sorbose	–	–	+	+	+	–	+	–	ND	ND	ND
Sorbitol	–	–	+	+	+	–	+	+	+	+	+
Melibiose	v	+	–	–	–	–	–	–	v	–	+
Raffinose	–	v	–	–	–	–	–	–	v	–	+
Fucose	ND	–	v	+	v	ND	–	+	ND	v	v

ND, not done; v, variable; –, negative; +, positive.
References: Bercovier and Mollaret 1984; Aleksic et al. 1987; Wauters et al. 1988; Gutman 1992; Holt and Krieg 1994.

Y. enterocolitica. They found that the limit of detection of their assay was 10^3–10^4 cfu of bacteria in a suspension of river water. Detection of a pure culture of *Y. enterocolitica* using a duplex PCR with primers for *ail* and 16S rRNA genes was found to be very specific for species identification and invasive strains (Wannet et al. 2001). Rasmussen et al. (1994) used primers synthesized from the *inv* region of *Y. enterocolitica* and demonstrated specific detection of pathogenic *Y. enterocolitica*. Feng et al. (1992) developed a PCR assay for the direct detection of *Y. enterocolitica* in blood, based on the detection of a portion of the *virF* gene on the pYV plasmid and a portion of the *ail* gene located on the chromosome. The assay was able to detect 500 cfu in 100 μl of blood

In addition to speciation, analysis of the serotype is warranted since there is an association between serotype and pathogenicity (Farmer et al. 1992); however, O antisera for serotyping are not readily available to routine laboratories. Other methods have been described to differentiate virulent from avirulent strains of *Y. enterocolitica*, such as presence of a 40–48-kDa plasmid, autoagglutination, tissue culture cell assay, serum resistance, hydrophobicity, and virulence in animals. The majority of these methods are impractical for most laboratories, although autoagglutination has been shown to be the best in vitro predictor of virulence in *Y. enterocolitica* (Kapperud et al. 1987). Other more practical methods include the use of biochemicals such as aesculin, salicin, and pyrazinamide. Cimolai et al. (1994) defined a pathogenic biotype as strains that were negative for all three assays, and a nonpathogenic biotype as positive for all three. Strains belonging to the so-called pathogenic biotypes were more likely than the nonpathogenic biotypes to be isolated from young children with bloody diarrhea. Selective media such as Congo red or Congo red magnesium oxalate (CRMOX) agar will identify strains that possess two factors associated with the presence of a virulence plasmid, Congo red dye uptake, and calcium-dependent growth at 35°C, have also been used (Farmer et al. 1992). Other authors have proposed the use of a medium containing crystal violet in which only the virulent, plasmid-containing colonies will retain the dye. Farmer et al. (1992) described the use of reactivity in several assay systems together to increase the sensitivity and specificity: pyrazinamidase, CRMOX agar, salicin fermentation–esculin hydrolysis, and D-xylose fermentation (see Table 2).

PCR assays that specifically target regions of the genome responsible for virulence have also been developed, due in part to the inconsistent nature of the above-described assays (Kwaga et al. 1992). Wren and Tabaqchali (1990) used a PCR assay based on detection of a portion of a plasmid essential for virulence. However, plasmids can be lost on subculture, causing a false-negative result (Ibrahim et al. 1992). Fenwick and Murray (1991) amplified a portion of the attachment invasion locus on the chromosome of pathogenic *Y. enterocolitica*. Robins-Browne et al. (1989) confirmed the use of a probe directed at all regions specific to pathogenic strains. Other more specific targets for amplification included the chromosomally encoded *yst* gene (enterotoxin) (Ibrahim et al. 1992). A more sensitive PCR assay was developed, which specifically detects pathogenic strains of *Y. enterocolitica* by amplification of the *yst* gene. Using a TaqMan assay on spiked samples of pork, the investigators were able to detect as few as 10^2 CFU/ml of organisms (Vishnubhatla et al. 2000).

Table 2 *Association of biochemical reactivity with pathogenicity and the presence of the virulence plasmid*

Biochemical reactivity	Pathogenicity	Presence plasmid
Red colonies CRMOX	+	+
Pyrazinamidase production	–	–
Salicin fermentation	–	–
Aesculin hydrolysis	–	–
Retains crystal violet dye	+	+

CRMOX, Congo red magnesium oxalate.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Alzugaray et al. (1995) studied the antimicrobial profile of clinical isolates of *Y. enterocolitica* serotype O:3. All were susceptible to amoxycillin–clavulanic acid, cefoxitin, fosfomycin, gentamicin, kanamycin, neomycin, tetracycline, naladixic acid, norfloxacin, ciprofloxacin, and trimethoprim. All were resistant to ampicillin (Alzugaray et al. 1995). In a study of 375 strains of *Yersinia* species isolated from food in France, only four were resistant to non-β-lactam antibiotics (Ahmedy et al. 1985). According to a study by Pham et al. (1991) of 100 clinical isolates of *Y. enterocolitica* in Australia, all isolates were susceptible to chloramphenicol, ciprofloxacin, gentamicin, tetracycline, trimethoprim, sulpha drugs, ticarcillin–clavulanate, and imipenem. All isolates were resistant to ampicillin (Pham et al. 1991). Biotype-related differences were observed to other β-lactam antibiotics. Biotypes 1A and 3 were resistant to cefoxitin and amoxycillin–clavulanate and biotype 3 was susceptible to ticarcillin. Biotype 4 strains were susceptible to cefoxitin and amoxycillin–clavulanate and resistant to ticarcillin. These differences are thought to be attributed to differences in types of β-lactamases produced (Pham et al. 1991). Two chromosomally mediated β-lactamases have been described in *Y. enterocolitica* (Ahmedy et al. 1985). Enzyme A is constitutive and is a broad-spectrum enzyme; enzyme B is inducible and is a cephalosporinase (Pham and Bell 1993). Inducible β-lactamase was found in all isolates of *Y. enterocolitica* biotypes 1A and 3 but in none of the isolates of biotype 4 (Pham and Bell

1992). Induction of β-lactamase production can be detected using a modified disk diffusion technique, using an imipenem disk as the inducer and a cefotaxime disk as the substrate (Pham and Bell 1993).

A streptogramin A acetyltransferase gene (sat), previously found only in gram-positive organisms, was found on the chromosome of all isolates of *Y. enterocolitica* tested. The function of this plasmid-encoded gene in strains of staphylococci and streptococci is to confer resistance to streptogramins. Gram-negative organisms are naturally resistant to streptogramins due to the impermeability of their outer membrane. The role of this gene in *Y. entercolitica* is unknown; however, it may be responsible for acetylation of an unidentified substrate (Seone and Garcia Lobo, 2000).

A study of antibiotic susceptibility of 54 isolates of *Yersinia bercovieri*, *Y. mollaretii*, *Y. aldovae*, and *Y. ruckeri* to 69 antibiotics found most of the strains to be susceptible to carbapenems, aminoglycosides, aztreonam, quinolones, tetracyclines, chloramphenicol, nitrofurantoin, and trimethoprim sulfamethoxazole (Stock et al. 2002). Differences were found among the penicillins based on expression of two different chromosomal β-lactamases (class A and class C enzymes). The presence of one or both of these β-lactamases was found to be correlated with the serotype in *Y. enterocolitica* (Pham et al. 2000). An AmpC type enzyme is assumed to be responsible for resistance of strains of *Y. mollaretti* to cefoxitin. No β-lactamases have been detected in strains of *Y. pseudotuberculosis* or *Y. pestis, Y. aldovae*, and *Y. ruckeri*; however, regardless of in vitro susceptibility to β-lactam antibiotics, in a mouse model using a pathogenic strain of *Y. pseudotuberculosis*, the only antibiotic found to be effective was ofloxacin (Lemaitre et al. 1991). Bonacorsi et al. (1994) found that 18 strains of *Y. pestis* tested were susceptible to amoxycillin, cefotaxime, ceftriaxone, streptomycin, gentamicin, ofloxacin, and doxycycline.

In a study of antimicrobial susceptibility of 78 strains of *Y. pestis* (57 from patients in Vietnam with plague between 1985 and 1993), the most active antimicrobials were ciprofloxacin, ofloxacin, and ceftriaxone; streptomycin, tetracycline, and chloramphenicol (agents most commonly used to treat patients with plague) were less active (Smith et al. 1995).

Based on serotype, *Y. ruckeri*, the fish pathogen, was found to vary in susceptibility to antimicrobial agents. Of 50 isolates tested by De Grandis and Stevenson (1985), two were resistant to tetracycline and sulphonamide, two antimicrobials commonly used for therapy of disease caused by this organism. Resistance was associated with the presence of a 3.6×10^4-kDa plasmid that was transferable to both another strain of *Y. ruckeri* and a strain of *E. coli*.

Nonpathogenic isolates of *Y. entercolitica* belonging to biotype 1A were tested for their susceptibility to heavy metals. Minimum inhibitory concentration (MIC) to cadmium and arsenic ranged from 2.5 to 5 mM and from 5 to 10 mM, respectively.

TREATMENT AND PREVENTION

First-line therapy for treatment of plague (particularly the pneumonic form) is a 10-day course of streptomycin. Chloramphenicol is an alternative option for treatment of bubonic or septicemic plague, particularly in cases of meningitis, and can be used in combination with streptomycin. Fluoroquinolones, although active in vitro and in animal models of disease, have not been tested in humans (plague manual). Seven days' treatment of doxycycline or trimethoprim/sulfamethoxazole is recommended for prophylaxis. Implementation of surveillance programs, public education in avoidance of sick animals in endemic areas, personal protection including insect repellent, and aggressive flea control all contribute to prevention of plague (Dennis et al. 1999).

Uncomplicated disease in a normal host due to *Y. enterocolitica* is most often untreated. Antibiotics have not been shown to reduce the severity or duration of entercolitis or appendicitis-like syndrome (Naktin and Beavis 1999). Disseminated disease is treated with trimethoprim/sulfamethoxazole or a fluoroquinolone, or, alternatively, a third-generation cephalosporin.

Antibiotics are not usually indicated for treatment of most infections due to *Y. pseudotuberculosis*; however, with septicemia, patients are usually treated with ampicillin or amoxicillin and an aminoglycoside.

TYPING METHODS

As with other organisms, many methods exist for epidemiological comparison of strains of *Yersinia*, including phenotypic markers such as biotyping, serotyping, bacteriophage typing, and antibiotic susceptibility patterns; genotypic differences such as restriction endonuclease digestion of plasmid (REAP) or restriction endonuclease digestion of chromosomal (REAC) DNA; digestion of genomic DNA and separation by pulsed field gel electrophoresis (PFGE); and ribotyping and analysis of PCR-generated fragments. Results from multilocus enzyme electrophoresis (MLEE) studies found *Y. enterocolitica* species to be clonal, but distinct groups, correlating with virulence of the strains, were identified (Caugant et al. 1989). Fukushima et al. (1993) compared biotyping, serotyping, phage typing, REAP, REAC, and antibiotic susceptibility patterns for 138 strains of *Y. enterocolitica* serotype O:5,27, isolated from both humans and animals in the USA, Japan, Canada, Australia, the Netherlands, and Germany. For the epidemiological investigation of relatedness of other genera, the best method for the analysis of *Yersinia* strains was a combination of several techniques used in conjunction with the epidemiological information about the sources

of the isolates. Fukushima et al. concluded that the combination of REAP, biotyping, and H-antigen typing would yield the most information about the relatedness of strains of this group of organisms. Kapperud et al. (1990) also determined that multiple methods would be useful; however, in general, genotypic rather than phenotypic methods were more discriminatory. Since most isolates of *Y. enterocolitica* contain only a single virulence plasmid, typing using plasmid DNA analysis is not useful in strain differentiation (Blumberg et al. 1991). Although REAP or REAC seem to be useful techniques, inter- and even intralaboratory comparisons can be difficult due to the multitude of bands on the gel. The use of gel electrophoresis of restriction digests in combination with a specific probe can facilitate the use of this method by many laboratories. Picard-Pasquier et al. (1990) compared ribotyping with MLEE and found the methods to be equivalent for the differentiation of strains of *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. intermedia*, *Y. aldovae*, *Y. frederiksenii*, and *Y. kristensenii*. Andersen and Saunders (1990) confirmed the usefulness of ribotyping to subtype serogroups of *Y. enterocolitica*. Strains of *Y. enterocolitica* can be typed more specifically using PCR ribotyping of the discriminatory spacer regions of the rRNA (Lobato et al. 1998). Saken et al. (1994) found PFGE following *Not*I digestion of genomic DNA of *Y. enterocolitica* strains to be more discriminatory than other genotypic methods. PFGE is the method of choice for differentiating *Y. enterocolitica*-like species, although a new technique, multilocus sequence typing, which compares nucleotide sequences among strains at several select 'housekeeping genes,' may provide more specific information regarding strain relatedness (Sulakvelidze 2000). PFGE was also found to be a useful epidemiological tool for typing of *Y. pestis* (Lucier and Brubaker 1992).

Different typing methods have been applied to the analysis of the clonal relationship of isolates of *Y. pseudotuberculosis*. Although most isolates of *Y. pseudotuberculosis* have a single plasmid associated with virulence, analysis by REAP differentiated isolates into their serotype as well as their geographical origin (Fukushima et al. 1994). The relatedness of strains of *Y. pseudotuberculosis* has been evaluated using an advanced PCR-based genotypic technique, random amplified polymorphic DNA (RAPD) (Makino et al. 1994). This technique was found to be useful in the study of outbreak strains in Japan.

Filippov et al. (1990) analyzed 242 isolates of *Y. pestis* from various areas of the former USSR, Mongolia, China, Vietnam, India, Indonesia, Brazil, and Africa for the presence of plasmid DNA. The majority of the isolates possessed the same three plasmids; however, 20 strains from different areas contained an additional cryptic plasmid of approximately 6 000 kDa. They also found changes in the size of pCad and pFra (plasmids found in all isolates of *Y. pestis*) in strains from particular regions.

An insertion sequence (IS100) found in all isolates of *Y. pestis*, located on the pesticin plasmid, was also found to hybridize to strains of *Y. pseudotuberculosis* that were susceptible to pesticin (McDonough and Hare 1997) and may be helpful in identifying those strains of *Y. pseudotuberculosis* that are more closely related to *Y. pestis* on an evolutionary basis and tracking them in the environment. Even though *Y. pestis* has many more copies of the insertion sequence than other *Yersinia* sp., PFGE seems to be a more specific method for epidemiological analysis of strains of *Y. pestis* (Huang et al. 2002).

BACTERIOCINS AND BACTERIOPHAGES

Y. pestis produces two bacteriocins, pesticin I and pesticin II. Pesticin I is plasmid-mediated and active against *Y. pseudotuberculosis* and some strains of *E. coli* and *Y. enterocolitica* (Staggs and Perry 1992). The compound is a polypeptide with a molecular weight of 63 kDa. Its mode of action is to degrade the cell-wall peptidoglycan of sensitive strains (Brubaker and Surgalla 1962; Ferber and Brubaker 1979). Pesticin II, produced by both *Y. pestis* and *Y. pseudotuberculosis*, is active only against select strains of *Y. pestis* (Fetherston et al. 1995). Csiszar and Toth (1992) demonstrated production of bacteriocins by clinical isolates of *Y. enterocolitica* belonging to various serogroups except O:3. The majority of the O:3 strains were sensitive to the bacteriocins produced by strains of other serogroups and are not well characterized (Rakin et al. 1994). Sensitivity of *Y. enterocolitica* strains to pesticin is associated with the production of a 65-kDa outer-membrane protein (FyuA), also thought to act as a receptor for yersiniabactin (Haag et al. 1993). Bacteriocins have also been described in *Y. intermedia* that are produced at 25°C but not at 37°C and are active against strains of *Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii* (Bercovier and Mollaret 1984). Toora (1995) has described a bacteriocin in *Y. kristensenii*. The bacteriocin was found to be extracellular with activity specific for strains of *Y. enterocolitica*.

Bacteriophages active against *Yersinia* species have been described (Nilehn and Ericson 1969; Nicolle et al. 1973). Typing schemes based on bacteriophage activity have been described for *Y. enterocolitica*; however, other epidemiological methods have been found to be more useful (see Laboratory isolation and identification).

Temperate bacteriophages that undergo numerous mutations may result in defective bacteriophages of which the tail-like particles act as bacteriocins. A bacteriocin of this nature has been found in a strain of *Y. enterocolitica* associated with a food-borne outbreak further designated entercoliticin. The bacteriocin had activity against the pathogenic serogroups of *Y. enterocolitica* (Strauch et al. 2001).

The complete sequence of a T3-like lytic bacteriophage, specific to *Y. enterocolitica* serotype O:3, was reported by Pajunen et al. (2001).

ROLE IN NORMAL FLORA OF HUMANS

Although *Yersinia* species are not considered part of the normal flora in humans, *Y. enterocolitica* can occasionally be found in the intestinal tract without any associated disease (Jawetz et al. 1978). Other *Yersinia* spp. may be found as normal fecal flora from healthy humans and animals (Quan 1998).

PATHOGENICITY AND VIRULENCE FACTORS

Although the three species of pathogenic *Yersinia* infect their hosts through different routes, all have a predilection for invasion and survival within the lymphoid tissue of their host. *Y. pestis*, which is transmitted by the bite of the flea, are carried into the bloodstream and rapidly disseminate and localize in the lymph nodes, where they cause plague.

Gastroenteritis, the most common disease manifestation of *Y. enterocolitica*, occurs following ingestion of contaminated food or water. The bacteria enter the intestines, are taken in by M cells, and subsequently enter lymphoid follicles within the intestines (Peyer's patches). *Y. pseudotuberculosis* is the etiologic agent of mesenteric adenitis and septicemia and is contracted as described above for *Y. enterocolitica* (Bleves and Cornelis 2000; Natkin and Beavis 1999).

Pathogenicity of *Yersinia* species is mediated by a 70–75-kb plasmid (pYV) and two additional plasmids in *Y. pestis* (Straley et al. 1993). These plasmids control the four major virulence factors in this genus: adhesin/invasin proteins, excreted antiphagocytic proteins (Yops), proteins involved in processing and excretion of the Yops (Ysc), and regulatory proteins (Lcr). The role of each of these factors in the pathogenicity of *Yersinia* species will be discussed separately. Chromosomal factors, including the production of an enterotoxin, are also thought to play a role in virulence, since strains lacking the plasmid have been found to survive within macrophages (Charnetzky and Shuford 1985) and cause disease in an animal model as well as in humans (Fukushima 1987).

Invasion of *Y. enterocolitica* and *Y. pseudotuberculosis* through mucosal surfaces and colonization of these surfaces is based, at least in part, on the production of a toxin (Yst), similar to the heat-stable toxin produced by *E. coli* (Iriarte and Cornelis 1995). The role of the toxin in the production of diarrhea has not been determined; however, it is only produced at temperatures below 30°C, which lends evidence against it having a role in vivo. It has also been found in both pathogenic and nonpathogenic strains of *Y. enterocolitica*. The *yst* gene has been cloned and sequenced in an attempt to learn more about its role in vivo. Hybridization studies using a portion of the cloned sequence found the *yst* gene to be present in pathogenic but not in nonpathogenic serotypes of *Yersinia*. Homologous DNA was also found in some strains of *Y. kristensenii* (Delor et al. 1990). An exotoxin has also been identified, cloned, and sequenced from *Y. pseudotuberculosis* (Ito et al. 1995). This toxin, *Y. pseudotuberculosis*-derived mitogen (YPM), acts as a superantigen by activation of specific human T cells in the presence of immune cells bearing MHC class II molecules. Little homology was demonstrated with superantigens from other bacterial species (Miyoshi-Akiyama et al. 1995). It has been suggested that YPM is involved in mediating systemic symptoms associated with human disease due to *Y. pseudotuberculosis* (Abe et al. 1993). In a mouse model of infection, the animals infected with YPM-deficient mutants survived for a significantly longer period of time (Carnoy et al. 2000).

Other invasion factors include invasin and Ail, two proteins under the control of chromosomal genes, and the plasmid-encoded protein YadA. Invasin is produced maximally at room temperature and only at body temperature in the presence of low pH, whereas YadA and Ail are produced in higher concentrations at 37°C. Ail, the product of the *ail* gene, plays a role in adherence, invasion, and serum resistance in virulent isolates of *Y. enterocolitica* (Wachtel and Miller 1995). Both invasin and YadA have been demonstrated to penetrate tissue culture cells and to bind to integrins in intestinal tissue (Pepe and Miller 1993; Skurnik et al. 1994). YadA has also been shown to bind to fibronectin (Tertti et al. 1992). YadA and *ail* are also responsible for the ability of *Yersinia* species to resist the activity of serum, a trait only expressed at 37°C in *Y. enterocolitica* and *Y. pseudotuberculosis*. The *inv* gene, encoding invasin, has been described in all strains and various species of *Yersinia*, whereas *ail* has only been found in pathogenic strains of *Yersinia* spp. (Pierson and Falkow 1993). Revell and Miller (2001) noted that the inv gene in *Y. pestis* is a disrupted form of the gene.

Following colonization of mucosal surfaces, avoidance of phagocytosis is the next step in the pathogenicity of *Yersinia*. The antiphagocytic ability of this genus is under the control of at least 11 Yops, at least three of which have been directly associated with virulence: YopE, YopH, and YopM (Straley et al. 1993). Yops function in two ways, interfering directly with the signal transduction of the phagocytes or directly attacking the host cells, and thereby inhibiting phagocytosis by macrophages (Straley et al. 1993; Visser et al. 1995). YopM is an example of an excreted protein that interferes with signal transduction by inhibiting platelet aggregation. YopH possesses protein tyrosine phosphatase activity, acting on host proteins specifically, inhibiting respiratory burst in macrophages (Galyov et al. 1993, Green et al. 1995). YopE acts directly on the host cell by destroying

actin filaments (Rosqvist et al. 1991). YopP (YopJ in *Y. pestis*), a 33-kDa protein, has been associated with inhibition of signaling pathways that lead to apoptosis in macrophages by decreasing the production of tumor necrosis factor alpha (TNFα) (Orth 2002).

LcrV, previously designated V antigen in *Y. pestis*, is found in all species of *Yersinia* and has a role in regulation and expression of Yops as well as an unknown role in the virulence of *Yersinia* species (Salyers and Whitt 1994). Genes *yopB* and *yopD* also have a regulatory role in the control of expression of Yops. Proteins YopB and YopD are excreted under conditions of low concentrations of calcium (Hakansson et al. 1993). YopB has been demonstrated to suppress the production of TNFα by phagocytes (Beuscher et al. 1995). Virulence genes on *Yersinia* are controlled by two independent regulatory systems, temperature and cellular concentration of calcium. Secretion of Yops occurs only at 37°C and in the absence of calcium (Cornelis 1994). A chromosomal gene, *ymoA*, functions as the temperature controller (Cornelis et al. 1991), turning on the *virF* gene, which allows expression of *yop* and *ysc* genes (Michiels et al. 1991; Salyers and Whitt 1994). Calcium concentrations are regulated by *lcr* genes (LcrE (YopN) and LcrH) (Forsberg et al. 1991).

Pathogenic strains of *Yersinia* can be divided into those with low virulence and those with high virulence based at least in part on their ability to obtain and store iron. In fact, severe disease in humans due to *Y. enterocolitica*, even those of low pathogenicity, are usually associated with an underlying disease, particularly disorders involving iron metabolism (Chambers and Sokol 1994). Addition of iron to an animal infected with a strain of low pathogenicity will allow the organism to disseminate (Carniel 2001). Highly pathogenic strains of *Yersinia* possess a group of genes responsible for iron acquisition and storage, termed the high-pathogenicity island (HPI). This island has been found in other pathogenic species of enterobacteriaceae as well. The HPI is a large fragment of chromosomal DNA (35–43 kb) that contains the yersiniabactin genes, several insertion sequences, and a mobility gene (bacteriophage P4-like integrase gene). The majority of the HPI is highly conserved among the three pathogenic species of *Yersinia*. *Yersinia* produce both a siderophore, which is active at low temperature, and an iron storage system induced at 37°C. Two high-molecular-weight iron-regulated proteins (HMWP) (1 and 2) were found to be encoded on the chromosome of virulent strains of *Yersinia* (Gyles and Thoen 1993; Guilvout et al. 1995). The gene *irp2* encodes HMWP2, which is only found in virulent strains of *Y. enterocolitica* (Haag et al. 1993; Rakin et al. 1994). Haag et al. identified a siderophore from the supernatant of actively growing *Y. enterocolitica*, yersiniabactin (yersinio-phore), and by creating mutants associated the protein with virulence. The iron transport genes are thought to be regulated by Fur protein, as found in other species of Enterobacteriaceae (Staggs and Perry 1992).

Yersinia species also possess a chromosomal gene that encodes a fibrillar protein in pathogenic serotypes of *Y. enterocolitica* known as Myf (Cornelis 1994). Myf is composed of MyfA (a 21-kDa protein), MyfB (a chaperone), and MyfC (an outer-membrane protein) (Iriarte and Cornelis 1995). As with other virulence factors, the fibrillar protein has homology with proteins with similar function in enterotoxigenic *E. coli*. These proteins are hypothesized to work in conjunction with Yst to aid in the colonization of mucous membranes. *Y. pseudotuberculosis* and *Y. pestis* have a protein with similar function, called pH6 antigen or pilus adhesin (PsaA), not found in *Y. enterocolitica*, which may be involved in defense once the organisms are within the phagocyte (Cornelis 1992; Salyers and Whitt 1994).

Stress proteins found in many bacterial genera are also produced by *Yersinia* species. These proteins may aid the bacteria in survival within phagocytes by neutralizing toxic products of these host cells (Yamamoto et al. 1994).

Y. pestis has two additional plasmids not present in other species of *Yersinia*. The 100-kb plasmid contains the genes responsible for antiphagocytic protein capsule (Fra) and a toxin that is lethal in mice and acts by preventing the action of adrenaline (epinephrine) (Gyles and Thoen 1993). The capsular protein F1 is encoded by caf1 (capsular antigen fraction 1 gene). The role of F1 in pathogenesis in humans is unclear; however, antibodies are protective in an animal model. The plasmid also contains the *ymt* (Yersinia murine toxin) gene. Ymt protein has no role in pathogenesis in humans but is important for colonization in the flea gut (Salyers and Whitt 1994). The second *Y. pestis*-specific plasmid is 9.5 kb and contains the gene(s) responsible for plasminogen activator protease (Pla), which has a role in vivo in the ability of the organism to resist the host's ability to contain the infection locally, possibly by resistance to killing by complement (Sodeinde et al. 1992; Straley et al. 1993; Friedlander et al. 1995). Pla is also capable of regulating the presence of Yops, which would allow the organisms to be ingested by monocytes when, unlike other *Yersinia* species, they would be protected from killing. Podladchikova et al. (1994) sequenced an insertion sequence (IS 100) found on the 9.5-kb plasmid of *Y. pestis*, the function of which is currently unknown. *Y. pestis* also has the ability to store hemin, which occurs maximally at 26°C. Storage of iron is manifested as pigment production in virulent strains of *Y. pestis* and is regulated by the chromosomal locus *pgm* (Gyles and Thoen 1993). The F1 antigen or protein capsule is antiphagocytic and is also immunogenic (Friedlander et al. 1995).

Furones et al. (1990) compared the cell extracts of serotype I strains of *Y. ruckeri* with those of other

nonpathogenic serotypes. They found the presence of a heat-sensitive lipid only in the serotype I strains. In vivo studies confirmed this substance to be a virulence factor. The addition of sodium dodecyl sulfate (SDS) to culture medium enhances selective growth of pathogenic strains of *Y. ruckeri*.

A 7.0×10^4-kDa plasmid thought to be involved in the virulence of *Y. ruckeri* has been described. This plasmid has been demonstrated to be unique from similar size plasmids in other species of *Yersinia* (Guilvout et al. 1988). Virulent isolates of *Y. ruckeri* have also been found to possess a heat-sensitive factor (Furones et al. 1990). This factor, designated extracellular products (ECP) by Romalde and Toranzo, was found to be involved in the pathogenesis of ERM disease in fish. Romalde et al. (1991) also hypothesized that *Y. ruckeri* produce a siderophore when grown under conditions of low iron concentrations, which may have a role in the virulence of the organism.

The sequencing of two strains of *Y. pestis*, one from the subtype or biovar Mediaevalis and one from the biovar Orientalis as well as the genome of *Y. enterocolitica* and *Y. pseuodotuberculosis* (data not yet published), will likely add to our knowledge and to the elucidation of more precise information regarding the genetic mechanisms of these species in the near future (Deng et al. 2002; Parkhill et al. 2001).

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Citrobacter, *Enterobacter*, *Klebsiella*, *Plesiomonas*, *Serratia*, and other members of the Enterobacteriaceae

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INTRODUCTION

This chapter describes important members of the Enterobacteriaceae that are not discussed in the preceding chapters (Enterobacteriaceae: general characteristics, *Escherichia*, *Shigella*, *Salmonella*, *Proteus*, *Morganella*, and *Providencia*, *Yersinia*). Some of the genera discussed in this chapter are commonly associated with human disease (e.g. *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia*) while other genera are uncommon pathogens. With a few exceptions, the genera described in this chapter conform to the general definition of the Enterobacteriaceae given in Enterobacteriaceae: general characteristics: they are aerobic and facultatively anaerobic organisms that ferment glucose and give a positive catalase and a negative oxidase reaction (*Plesiomonas* is oxidase positive); all of them reduce nitrate to nitrite; and only in *Tatumella* are there motile strains that do not exhibit peritrichous flagella.

CITROBACTER

Definition

Citrobacter species are motile organisms that ferment glucose usually with gas production, mannitol, rhamnose, maltose, mannose, trehalose, and xylose. They may or may not ferment lactose, but nearly always produce β-galactosidase. They give a positive methyl red (MR) test and a negative Voges–Proskauer (VP) test. They do not deaminate phenylalanine or decarboxylate lysine. They do not hydrolyze gelatin or produce lipase or DNase. These organisms are found in human clinical specimens, soil and water, and in the intestinal tract of animals. The G + C content of the DNA is 52–54 mol% and the type species is *Citrobacter freundii*.

Classification

Werkman and Gillen (1932) proposed the establishment of the genus *Citrobacter* to include certain 'intermediate'

coliform bacteria that produced trimethylene glycol from glycerol. The name did not find immediate acceptance and these organisms were described as '*Escherichia freundii*' by Yale (1939). A number of lactose-negative or late lactose-fermenting organisms described some years later shared certain somatic antigens with salmonellae and many possessed the Vi antigen of *Salmonella* serotype Typhi. These organisms were known as the Ballerup–Bethesda group until West and Edwards (1954) showed their similarity to strains of '*E. freundii*.' Kauffmann (1956) subsequently revived the name *Citrobacter* for them and the species became known as *Citrobacter freundii*.

An outstanding feature of *C. freundii* is its ability to blacken triple sugar iron agar through the production of H_2S. In 1971, Young and colleagues described a group of organisms that were H_2S-negative in this medium and placed them in a new genus *Levinea*, in which there were two species, *Levinea amalonatica* and *Levinea malonatica*. Ewing and Davis (1972) suggested that *L. malonatica* was a later synonym of *Citrobacter diversum* (see Werkman and Gillen 1932) and proposed the revival of the name *C. diversus*, with the change of spelling for grammatical reasons. However, in 1970, Frederiksen had also described a group of organisms for which he proposed the name *Citrobacter koseri*. Subsequent studies (Crosa et al. 1974; Sakazaki et al. 1976) showed that *C. diversus* and *C. koseri* are synonyms. The authors of this chapter preferred the name *C. koseri* because the description of *C. diversus* given by Ewing and Davis (1972) differs in several respects from the original description of the species (Holmes et al. 1974). The Judicial Commission of the International Committee on Systematic Bacteriology no longer recognizes *C. diversus* and has, instead, validated the name *C. koseri* in its place. Brenner and coworkers (1977) re-examined strains of *L. amalonatica* and suggested that these might also be included in the genus *Citrobacter* as a third species, *Citrobacter amalonaticus*.

The genus now consists of 11 genomospecies separable by their biochemical characters (Brenner et al. 1993). *C. amalonaticus* and *C. koseri* proved to be homogeneous species, as previously described. *C. amalonaticus* biogroup 1, as described by Farmer et al. (1985a), was shown to be a separate homogeneous species, which was named *Citrobacter farmeri*. The *C. freundii* complex was quite heterogeneous; *C. freundii* sensu stricto, as represented by the type strain, contained only nine of 66 strains in this complex, whereas the remaining 57 strains were members of seven genomospecies. Genomospecies 5, containing 21 strains, was named *Citrobacter youngae*; genomospecies 6, containing 15 strains, was named *Citrobacter braakii*; genomospecies 7 and 8, each containing six strains, were named *Citrobacter werkmanii* and *Citrobacter sedlakii*, respectively. Genomospecies 9, 10, and 11, each containing three strains, were named *Citrobacter gillenii*, *Citrobacter murliniae*, and *Citrobacter rodentium*.

Morphological and cultural characteristics

In these respects, strains of *Citrobacter* resemble most other members of the Enterobacteriaceae. They grow well on ordinary media producing smooth, convex colonies 2–4 mm in diameter on nutrient agar. They are not pigmented. Rough or mucoid forms sometimes occur.

Biochemical activities

The chief biochemical reactions of the 11 *Citrobacter* species are shown in Enterobacteriaceae: general characteristics, Table 1. The key tests used to differentiate the *Citrobacter* species are listed in Table 1. The expansion in number of species necessitates a wider range of, and more specialized, tests than

Table 1 *Differentiation of species of* Citrobacter

Property	*C. amalonaticus*	*C. braakii*	*C. farmeri*	*C. freundii*	*C. gillenii*	*C. murliniae*	*C. koseri*	*C. rodentium*	*C. sedlakii*	*C. werkmanii*	*C. youngae*
Indole	+	V	+	V	–	+	+	–	V	–	V
Ornithine decarboxylase	+	+	+	–	–	–	+	+	+	–	–
Malonate utilization	–	–	–	–	+	–	+	+	+	+	–
Acid from:											
Adonitol	–	–	–	–	–	–	+	–	–	–	–
Dulcitol	–	V	–	–	–	+	V	–	+	–	+
Melibiose	–	V	+	+	V	V	–	–	+	–	–
Sucrose	–	–	+	+	V	V	V	–	–	–	V

+, Most strains positive; –, most strains negative; V, some strains positive, others negative

previously required. Janda et al. (1994) attempted identification of 235 *Citrobacter* strains to the species level on the basis of 15 conventional tests. 100 percent of the *C. amalonaticus* group strains and 81 percent of the *C. freundii* complex strains could be definitively assigned to one of the previously established or more recently designated species or hybridization groups of the genus *Citrobacter*. Within the *C. freundii* complex, *C. freundii* predominated (37 percent), followed by *C. youngae* (24 percent), *C. braakii* (13 percent), and *C. werkmanii* (6 percent). The ability of commercial identification systems to identify the more recently recognized species of *Citrobacter* has been evaluated by O'Hara et al. (1995). Strains of *C. freundii* able to produce ornithine decarboxylase but unable to produce β-galactosidase have been shown by various techniques (including DNA–DNA hybridization) to represent only a separate biotype and not a separate species (Popoff and Stoleru 1980).

Susceptibility to antimicrobial agents

C. freundii is usually susceptible to aminoglycosides and chloramphenicol and susceptibility to ampicillin, cephalosporins, and tetracycline varies. Holmes and colleagues (1974) showed that 79 percent of *C. freundii* strains were resistant to cephaloridine and susceptible to carbenicillin, whereas 96 percent of *C. koseri* strains were susceptible to cephaloridine and resistant to carbenicillin. Lund et al. (1974) found that both *C. amalonaticus* and *C. koseri* were generally resistant to ampicillin and carbenicillin. Gross and Rowe (1983) reported that resistance to cephaloridine, gentamicin, neomycin, streptomycin, and sulfonamides also occurred frequently among *C. koseri* strains.

Typing methods

Serotyping schemes have been proposed for *C. amalonaticus* (Sourek and Aldová 1976), *C. freundii* (West and Edwards 1954; Sedlák and Slajsová 1966), and *C. koseri* (Gross and Rowe 1975; Gross et al. 1981). Determination of outer-membrane protein profiles has been found potentially useful in epidemiological investigations of disease caused by *C. koseri* (Kline et al. 1988, 1989). Harvey et al. (1995) evaluated the epidemiological usefulness of an automated procedure for analysis of polymerase chain reaction (PCR)-generated DNA fingerprints to confirm the vertical transmission of *C. koseri* from a mother to her infant. A monoclonal antibody has been developed against *C. freundii* O36 (Shearman et al. 1984).

Habitat and pathogenicity

Members of the genus *Citrobacter* are often found in the feces of humans and may be isolated from a variety of clinical specimens. They do not often give rise to serious infections, except *C. koseri*, which has been responsible for several outbreaks of neonatal meningitis (Gross et al. 1973; Gwynn and George 1973; Duhamel et al. 1975; Parry et al. 1980; Graham and Band 1981; Lin et al. 1987). Neonatal meningitis is usually accompanied by the development of brain abscesses and tends to have high rates of morbidity and mortality. Morgan et al. (1992) described brain abscesses occurring in two infants; both survived, albeit with some minor degree of brain damage. Eppes et al. (1993) reported a case of a neonate with *C. koseri* meningitis and brain abscesses who relapsed after initial antibiotic therapy and from whom the same organism (but a genetically different strain) was recovered from cerebrospinal fluid (CSF) 4 years later during a neurosurgical procedure. A case of brain abscesses following urinary infection in an adult was described by Booth et al. (1993). The patient was treated with surgical drainage, cefotaxime, and netilmicin. Strains from CSF possess a minor outer-membrane protein of 32 kDa, which may serve as a marker for strains that are likely to cause meningitis or brain abscess in human neonates.

Clonally identical isolates of verotoxinogenic *C. freundii* proved responsible for a summer outbreak of severe gastroenteritis followed by hemolytic–uremic syndrome (HUS) and thrombotic thrombocytopenic purpura in a nursery school and kindergarten (Tschape et al. 1995). Infant food has been identified as a vehicle in a nosocomial outbreak of *C. freundii* in a neonatal intensive care unit of a large hospital where colonization and clinical diseases due to the agent had been observed (Thurm and Gericke 1994). Occasionally, like *C. koseri*, this organism can also cause neonatal meningitis (Joaquin et al. 1991–2).

KLEBSIELLA

Definition

Klebsiella organisms are nonmotile and usually capsulate. They ferment adonitol, inositol, mannitol, salicin, and sorbitol, and often also lactose and sucrose. Most strains form gas from sugars; gas production from starch is an important diagnostic feature. Characteristically they give a negative MR and a positive VP reaction, but strains from the respiratory tract often give the opposite reactions and may have other atypical biochemical reactions. Nearly all grow on Simmons' citrate medium and in Møller's KCN medium. They are nonpigmented, do not deaminate phenylalanine and, with a few exceptions, fail to liquefy gelatin. They are found in the bowel and respiratory tract of humans and animals and in soil and water and cause a variety of septic infections in humans and animals. The G + C content of the DNA is 52–58 mol% and the type species is *Klebsiella pneumoniae*.

Classification

In earlier editions of this book, the name '*Klebsiella aerogenes*' was used for the nonmotile, capsulate, gas-producing, acetylmethylcarbinol-forming strains commonly found in human feces and in water. They were thought to correspond to the nonmotile, gas-producing organism from feces described by Escherich (1885) as '*Bakterium lactis aerogenes*,' referred to by later workers as '*Bacterium aerogenes*,' and subsequently transferred to a reconstituted genus *Klebsiella* (Report 1954). Unfortunately, the term '*Bacterium*' (later '*Aerobacter*') *aerogenes* was also used by water bacteriologists to refer to organisms that were later shown to be motile and are now placed in the genus *Enterobacter*. In an attempt to resolve the resultant confusion, some taxonomists adopted *pneumoniae* as the specific epithet for the nonmotile *aerogenes*-like organisms, although it had earlier been used to designate certain biochemically atypical strains of *Klebsiella* from the respiratory tract. The view of these workers prevailed, and the name *K. pneumoniae* appeared in the Approved Lists of Bacterial Names (Skerman et al. 1980) whereas '*K. aerogenes*' was omitted.

A number of other nonmotile and capsulate organisms occur in the respiratory tract of humans and other animals. In 1882, von Frisch cultivated a capsulate organism from patients with rhinoscleroma; in 1883, Friedländer isolated a similar organism – generally known as Friedländer's bacillus – from the lungs of patients who had died of pneumonia; and Abel (1896) described the ozaena bacillus. These three organisms, and others from the respiratory tract, form a biochemically heterogeneous group but are antigenically related to some of the more biochemically typical strains now included in *K. pneumoniae*. Various specific names, including '*Klebsiella atlantae*,' '*Klebsiella edwardsii*,' *Klebsiella ozaenae*, *Klebsiella pneumoniae* (as the name was initially used), and *Klebsiella rhinoscleromatis*, have been attached to biochemical varieties in this group. Both American and British authors, in the past, used the name *K. pneumoniae* to apply only to respiratory strains believed to correspond to Friedländer's bacillus. Studies of DNA–DNA hybridization (Brenner et al. 1972) indicated, however, that these respiratory strains are so closely related genetically to the more biochemically typical strains now included in *K. pneumoniae* that they should all form part of the single species *K. pneumoniae*. The epithets *ozaenae* and *rhinoscleromatis*, together with *pneumoniae*, have been proposed as subspecies of *K. pneumoniae* but in the last edition of this text, *K. ozaenae* and *K. rhinoscleromatis* were retained as species in their own right.

Four other members of the genus *Klebsiella*, though biochemically similar to *K. pneumoniae*, are accorded separate specific status. An organism that formed indole and liquefied gelatin slowly was described by Flügge (1886) as '*Bacterium oxytocum*' (Lautrop 1956). The decision to consider it a separate species – *K. oxytoca* – is supported by evidence from DNA–DNA hybridization tests, which suggest that assignment to a separate genus may even be appropriate (Jain et al. 1974). From *K. oxytoca* must be differentiated another indole-producing species, formerly known as enteric group 47, but later designated as *Klebsiella ornithinolytica* (Sakazaki et al. 1989). This species occurs only rarely in clinical specimens and was the only *Klebsiella* species to decarboxylate ornithine. *Klebsiella planticola* (Bagley et al. 1981) and *Klebsiella trevisanii* (Ferragut et al. 1983) have been shown to be synonyms (Gavini et al. 1986), the former name taking priority. Strains of the species also occur rarely in clinical material (Freney et al. 1986). Another organism, named *Klebsiella terrigena*, also showed genetic differences from *K. pneumoniae* but was largely indistinguishable from it in routinely used tests (Izard et al. 1981a). It has been found mainly in soil and water.

Since the last edition of this text was published, the genus *Klebsiella* has undergone extensive revisions. *K. pneumoniae*, *K. ozaenae*, and *K. rhinoscleromatis* are now accepted as subspecies within the species *K. pneumoniae*. For the sake of simplicity, all references to *K. pneumoniae* in this chapter are to the specific subsp. *pneumoniae*. *K. ornithinolytica*, *K. planticola*, and *K. terrigena* have been moved from the genus *Klebsiella* into the new genus *Raoutella* (Drancourt et al. 2001). *Klebsiella variicloa* is a newly described species found associated with bananas, rice, sugar cane, and corn (Rosenblueth et al. 2004). This species will not be discussed further in this chapter. Finally, *Calymmatobacterium granulomatis* has been moved into the genus *Klebsiella* (Carter et al. 1999; Kharsany et al. 1999). *Klebsiella granulomatis* produces granulomatous lesions in humans. The disease was first recognized in India by McLeod in 1882. The lesions have been referred to in the literature by a variety of names: granuloma inguinale, granuloma venereum, and donovanosis. The cellular inclusions that are the specific hallmark of the disease were first described by Donovan (1905) and came to be known as Donovan bodies. Originally named *Calymmatobacterium granulomatis* by Aragao and Vianna (1913), it was isolated by Anderson et al. (1945) by inoculation into chick-embryo yolk sacs and named *Donovania granulomatis*; however, until recently, *C. granulomatis* maintained priority until genetic analysis conclusively showed the species more properly belongs in the genus *Klebsiella*.

Morphological and cultural characteristics

Members of the genus *Klebsiella* tend to be somewhat shorter and thicker than the other enterobacteria and

are straight rods about 1–2 μm long and 0.5–0.8 μm wide, with parallel or bulging sides and rounded or slightly pointed ends. The cells are either in pairs end to end or are arranged singly. In the body, diplobacilli, very like pneumococci, are commonly seen. They are nonmotile. When the capsule is pronounced, it can be demonstrated even by Gram stain. Capsular material is produced in greater amount in media containing a relative excess of carbohydrate (Duguid and Wilkinson 1953) and is a nitrogen-free polysaccharide (Toenniessen 1921; Heidelberger et al. 1925). Wilkinson et al. (1954a) purified the capsular polysaccharides of a number of strains belonging to capsular type 54, and showed that they contained about 50 percent D-glucose, 10 percent L-fucose, and 29 percent glucuronic acid. Mucoid strains produced the largest amount of polysaccharide, and most of it was present as capsule or slime; smooth strains produced less and nearly all of it was intracellular. Rough strains produced least (Wilkinson et al. 1954b). Wherever the polysaccharide material was situated, its chemical composition was the same (Dudman and Wilkinson 1956). There were, however, differences in physical properties and in molecular weight between capsular material and loose slime (Wilkinson 1958). The polysaccharides of the different capsular types are all complex acid polysaccharides (Dudman and Wilkinson 1956), which contain a uronic acid – usually glucuronic acid but occasionally galacturonic acid – as well as a considerable amount of pyruvic acid (Wheat et al. 1965). They resemble the group I K antigens of *Escherichia coli* (Jann and Jann 1983).

Most strains of *Klebsiella* are fimbriate but some of the respiratory strains form an exception (Duguid 1959, 1968). Biochemically typical strains of any capsular type usually form fimbriae of both type 1 and type 3. The respiratory strains, which originally were the sole constituents of *K. pneumoniae*, form only type 1 fimbriae. *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* are invariably nonfimbriate, as are certain biochemically aberrant respiratory strains belonging to capsular types 1 and 2. The type 1 fimbriae of different species of *Klebsiella* are antigenically similar and related to those of *Enterobacter aerogenes* (Adegbola and Old 1987). In vitro, the *Klebsiella* type 3 fimbrial adhesin appears to mediate adherence to human respiratory tissue.

When much capsular material is produced, the growth on agar is luxuriant, grayish-white, and mucoid. This is due, no doubt, to the high proportion of water, 92 percent (Toenniessen 1921), in the capsular material. The condensation water on an agar slope is converted into a white mucoid mass. In broth, the organism grows freely, giving rise after a few days to a pronounced viscosity, so that the medium takes on the consistency of melted gelatin. Usually there is no liquefaction of gelatin, but often a large bubble of gas accumulates just beneath the surface, giving, on first sight, the appearance of liquefaction.

The cultural appearances of capsulate strains are subject to considerable variation. Nonmucoid variants appear on serial subculture on solid medium, particularly when the plates are incubated for several days (Toenniessen 1913), either as translucent peripheral outgrowths or as secondary colonies in the substance or on the surface of the original colonies. Sometimes the whole colony may dry up and wither away, leaving an effuse translucent layer looking like ground glass – aptly called by Collins (1924) 'suicide colonies.' Nonmucoid variants form colonies closely resembling those of *E. coli*. Reversion to the mucoid form may occur, often suddenly. For further information about colonial variation, see Toenniessen (1914), Hadley (1927), Goslings (1935), and Read et al. (1957). Some of the respiratory strains grow poorly or not at all on MacConkey agar.

The organisms are killed by moist heat at 55°C in 30 min. They may survive drying for months (Loewenberg 1894). When kept at room temperature, cultures remain viable, as a rule, for weeks or months. They are facultatively anaerobic, but growth under strictly anaerobic conditions is poor. There is no hemolysis of horse or sheep red cells. The optimum temperature for growth is 37°C, the limits are 4 and 43°C, depending on the species. Some strains form a slightly brownish pigment.

Cysteine-requiring strains that are auxotrophic for this amino acid because of defects in the sulfur assimilatory pathway account for about 1.5 percent of urinary tract isolates of *Klebsiella* spp. (and *E. coli*); such strains may be seen as small (c. 1 mm) colonies on MacConkey agar. Problems with the isolation, identification, and sensitivity testing of cysteine-requiring *Klebsiella* strains have been studied by McIver and Tapsall (1988) who suggested methods by which these may be minimized. McIver and Tapsall (1990) examined cysteine-requiring strains of *Klebsiella* to test the ease with which various test systems identified clinical isolates of cysteine auxotrophs. A significant proportion of the cysteine-requiring strains were not adequately identified by growth-dependent tests, which used various peptones as a nutrient source. Problems were encountered with all test systems examined: conventional methods and the commercial API 20E, MicroScan, and Vitek systems. The performance of the test systems was only partly improved when inocula were derived from appropriately supplemented media. However, the problems of the growth-dependent tests were resolved when a cysteine-supplemented suspension was used to inoculate each test system.

K. granulomatis is identified in infected tissue by the presence of Donovan bodies, which consist of organisms contained within the cytoplasmic vacuoles (phagosomes) of large macrophages, formerly known as Pund or Greenblatt cells. Bodies are sometimes found inside polymorphonuclear leukocytes or lying free outside cells. The organisms themselves are coccobacilli or short rods, 1–1.5 μm in length and 1 μm in width; they may be curved or dumb-bell shaped. They are nonmotile and

gram-negative, best stained in tissues by the Wright–Giemsa method (which stains the body of the bacterium blue or purple and the capsule pink), by Papanicolau's method or by various silver strains. A slow-Giemsa (overnight) tissue section staining technique has been described as being much superior than tissue smears and recommended for ultimate confirmation of *K. granulomatis* (Sehgal and Jain 1987). Bipolar staining produces a 'closed safety-pin' appearance. The organism usually has a prominent capsule within the phagosome, but capsules may be absent in rapidly multiplying organisms in fresh lesions. Infected macrophages may contain as many as 25 organisms. The cell membrane and cell wall have structures typical of gram-negative organisms.

Fine structure analysis of *K. granulomatis* in human tissue has revealed the presence of a complex cell envelope (Chandra and Jain 1991). The cytoplasm of these organisms showed presence of electron-dense polar material, in addition to regular bacterial structures such as mesosome, ribosomes, and other nuclear material. The origin of fimbriae and blebs (surface appendages) was clearly endogenous to the cell wall. The morphology of fibrium at the site of its attachment of the cell membrane has been described along with a distinct layer of homogenous material of varying density surrounding the organism, suggesting the possibility of it being a capsule.

K. granulomatis can be isolated by inoculating infected material into the yolk sac of embryonated hens' eggs (Anderson 1943). It can be cultured in vitro only with great difficulty. For example, it has been successfully grown on media containing fresh egg-yolk material (Dienst et al. 1948), in a semisynthetic medium containing lactalbumin hydrolysate (Goldberg 1959) and has been maintained in tissue culture. Moreover, *K. granulamatis* has been successfully isolated in pure culture by the monocyte co-culture system from both biopsy specimens and genital ulcer scrapings (Kharsany et al. 1997). The optimal temperature for growth is said to be 37°C.

In the absence of culture methods, an indirect immunofluorescence technique may prove to be valuable for the diagnosis of individual cases of granuloma inguinale and as an epidemiological tool in studies of the disease (Freinkel et al. 1992).

Biochemical activities

The chief biochemical reactions of *Klebsiella* species and subspecies grown in culture are presented in Enterobacteriaceae: general characteristics. The key biochemical tests used to differentiate these *Klebsiella* species are listed in Table 2. Members of the genus ferment a wide range of sugars, but their behavior in this and other respects is far from uniform. *K. pneumoniae* is the most active fermenter of the genus, producing acid and gas from all, or nearly all, the sugars usually employed, with the possible exception of dulcitol. Attention should be drawn to their ability to form gas from starch. Hormaeche and Munilla (1957) advocated a medium containing nonsoluble starch on which practically all strains of *K. pneumoniae*, and practically no other members of the Enterobacteriaceae, are able to form gas within 4 days. The other biochemical reactions of *K. pneumoniae* are fairly uniform. They are indole-negative, do not liquefy gelatin, and grow on Simmons' citrate medium. They are urease-positive, utilize malonate and have a lysine decarboxylase. *K. oxytoca* closely resembles the biochemically typical *K. pneumoniae* strains, except that it liquefies gelatin slowly, forms indole, and nearly always acidifies dulcitol (Malcolm 1938; Ørskov 1955c; Lautrop 1956). *R. planticola* also closely resembles the biochemically typical members of *K. pneumoniae* and indole-producing strains may easily be confused with *K. oxytoca*. Conventional methods usually fail to differentiate *R. planticola* and *R. terrigena* from *Klebsiella*. Monnet and Freney (1994) combined four carbon substrate assimilation tests and two conventional tests; their scheme identified these species with a specificity and a sensitivity of 100 percent, although this was based on only few strains.

K. pneumoniae subsp. *rhinoscleromatis* is anaerogenic, does not acidify lactose, gives a positive MR reaction and a negative VP reaction, does not grow on Simmons'

Table 2 *Differentiation of* Klebsiella *species*

	K. pneumoniae			
Properties	**Subsp. *pneumoniae***	**Subsp. *ozaenae***	**Subsp. *rhinoscleromatis***	***K. oxytoca***
Indole	–	–	–	+
Voges–Proskauer	+	–	–	+
Urea hydrolysis	+	–	–	+
Lysine decarboxylase	+	V	–	+
ONPG	+	+	–	+
Growth at:				
4°C	–	–	–	–
10°C	–	–	–	+
44°C	+	V	UK	+

+, Most strains positive; –, most strains negative; V, some strains positive, others negative; UK, unknown; ONPG, o-nitrophenyl-β-galactopyranoside test

citrate medium, does not form lysine decarboxylase, and is urease- and gluconate-negative. Some strains fail to grow in Møller's KCN medium. *K. pneumoniae* subsp. *ozaenae* forms a less clear-cut taxon, including strains that are late lactose and sucrose fermenters, are sometimes anaerogenic, and also give a positive MR and a negative VP reaction. They are malonate- and gluconate-negative. Few strains form lysine decarboxylase, but some attack arginine slowly.

Susceptibility to antimicrobial agents

Biochemically typical strains of *K. pneumoniae* are resistant to a wider range of antibiotics than are most *E. coli* strains. They are nearly always naturally resistant to ampicillin; they were at one time usually susceptible to cephalosporins, thus differing from strains of *Enterobacter*, which are almost invariably cephalosporin-resistant (Benner et al. 1965). Their resistance to chloramphenicol, streptomycin, and tetracycline varied from strain to strain but they were usually susceptible to gentamicin and the polymyxins. Since that time, resistance to a number of antibiotics, notably various cephalosporins and gentamicin, have become common in strains found in hospitals. Transferable enzymatic resistance to third-generation cephalosporins during a nosocomial outbreak of infection with multiresistant strains has been described by Brun-Buisson et al. (1987). Other authors have also noted such transferable resistance, for example to cefotaxime and ceftazidime, in nosocomial strains due to their production of plasmid-borne extended-spectrum β-lactamases; in such circumstances, active agents are likely to include carbapenems (imipenem, meropenem), cefotetan, and the β-lactam–β-lactamase inhibitor combinations such as amoxycillin–clavulanic acid, ampicillin–sulbactam, and piperacillin–tazobactam. Cefoxitin is said to be more active than ampicillin–sulbactam. French et al. (1996), however, reported a hospital outbreak caused by a strain of *K. pneumoniae* K2 producing the extended-spectrum β-lactamase SHV-5. Some of the isolates produced up to five times as much enzyme as the other isolates and were resistant to β-lactam–β-lactamase inhibitor combinations. The outbreak was controlled by patient isolation and attention to hand-washing.

Subminimal inhibitory concentrations of β-lactam drugs such as cephems, monobactams, and penicillins may cause thinning of the capsule of *K. pneumoniae* with increases in the hydrophobicity and decreases in the negative charge of the cell surface. This renders *K. pneumoniae* more susceptible to phagocytic activity by reducing the physical repulsion between the bacteria and phagocytes, thereby enhancing the susceptibility of the bacteria to phagocytic killing activity (Nomura et al. 1995).

Many of the respiratory strains, on the other hand, are more susceptible to antibiotics. A few of them are susceptible to as little as 2 μg/ml benzylpenicillin, and most of them are susceptible to ampicillin, chloramphenicol, streptomycin, and tetracycline. Some *K. pneumoniae* subsp. *oxytoca* strains have been found resistant to aztreonam and cefuroxime, but susceptible to cefotaxime, ceftazidime, and imipenem. *K. pneumoniae* subsp. *rhinoscleromatis* isolates are inhibited by clinically achievable concentrations of amoxycillin–clavulanate, cefpodoxime, cefuroxime, cephalexin, chloramphenicol, ciprofloxacin, and trimethoprim–sulfamethoxazole (Perkins et al. 1992). In Canada, *Klebsiella* spp. are highly susceptible to most antimicrobial agents with only one of 656 isolates tested showing resistance to third-generation cephalosporins (Toye et al. 1993). Similarly, in Finland, resistance to cefuroxime has remained rare and cefuroxime remains an alternative to third-generation cephalosporins in the treatment of *Klebsiella* septicemia. In neonates, ampicillin-based regimens are more likely than cefuroxime to produce drug-resistant strains (Tullus and Burman 1989).

For hand-washing, 0.5 percent chlorhexidine in isopropyl alcohol yielded sterile post-disinfection finger washings more often than 4 percent chlorhexidine digluconate; both preparations were more effective than soap and water, and with each residual action was demonstrable.

Because *K. granulomatis* cannot be grown in vitro, the activity of antimicrobial agents is predicted by clinical experience. Spontaneous resolution has been reported (Pradinaud et al. 1981) but active treatment should be undertaken. The use of antimony has given way to antibiotics. Penicillin, ampicillin, and cephalosporins are ineffective against *K. granulomatis* but can eliminate significant secondary infection. Tetracyclines are probably the most widely favored drugs (World Health Organization 1981; Velasco et al. 1972), but chloramphenicol and thiamphenicol are also very effective. Erythromycin (Robinson and Cohen 1953), gentamicin, and streptomycin (Greenblatt 1953) have been used successfully. More recently, co-trimoxazole (Latif et al. 1988) and norfloxacin (Ramanan et al. 1990) have been found to be very effective.

Antigenic structure

In studies of the antigenic structure of this group, most attention has been paid to the capsular (K) antigens. A great advance was made when American workers showed the presence in the group of capsular polysaccharides (Avery et al. 1925; Heidelberger et al. 1925; Julianelle 1926a, b, c). Julianelle (1926a) recognized three serological types (A, B, and C) among the respiratory klebsiellae, distinguishable by agglutination, adsorption, precipitation, and protection tests, and an undifferentiated group. The type specificity depended on a polysaccharide in the capsule. Goslings and Snijders (1936) described three further capsular antigens (D, E, and F) among *K. pneumoniae* subsp. *ozaenae* strains,

and Kauffmann (1949) described eight more. Kauffmann proposed that the capsular types should be referred to by arabic numerals in place of the capital letters used previously. The former types A, B, C, and D became types 1, 2, 3, and 4, and the new types were numbered consecutively. Since then, many further types have been recognized (Brooke 1951; Edwards and Fife 1952; Edmunds 1954; Ørskov 1955a; Durlakowa et al. 1967) and the present total is about 80. Unfortunately, the prevalence of nontypable strains, which can be up to 25 percent, limits the usefulness of capsular serology as an epidemiological tool.

In addition to the capsular antigens, there are smooth somatic antigens that occur in various combinations with the capsular antigens (Goslings 1933; Goslings and Snijders 1936). Kauffmann (1949) studied spontaneous noncapsulate variants of capsulate strains and recognized three of these O antigens among members of the first 14 capsular types. Ørskov (1954) examined a much larger selection of strains and was able to add only two more O antigens. Of these five *Klebsiella* O antigens, four were identical with or related to *E. coli* O antigens. So far, 12 O antigens have been identified but two of these, O1 and O6, are identical (Ørskov and Ørskov 1984). It is thus possible to divide *Klebsiella* strains into a small number of O groups, which may be further subdivided into capsular types. Organisms with the same capsular antigen may, however, occur in several O groups. It is unlikely, therefore, that the identification of the O antigen, which in any case presents considerable technical difficulties, is of much practical value in the classification of the capsulate members of the genus *Klebsiella*. In addition to the type-specific acid polysaccharide, a neutral polysaccharide, which is serologically active but not type-specific, has also been described (Gormus and Wheat 1971).

There is some association between antigenic structure, biochemical activities, and habitat. Thus, members of the first six capsular types occur most frequently in the human respiratory tract, though occasionally they may be found in other parts of the body or even in other animals (Edwards and Fife 1955). They are not, however, by any means the only types found in the respiratory tract. Ørskov (1955b), for example, found that only 16 percent of strains from sputum belonged to capsular types 1–6. Nearly all the biochemically aberrant respiratory klebsiellae are to be found in capsular types 1–6, though a number of strains in types 1–3 are biochemically typical strains of *K. pneumoniae*. Capsular type 3 contains nearly all the *K. pneumoniae* subsp. *rhinoscleromatis* strains, as well as most of the respiratory strains now included in *K. pneumoniae*. Members of *K. pneumoniae* subsp. *ozaenae* belong to capsular types 4–6, and make up all, or nearly all, of these types. Capsule type 66 proved common in *K. oxytoca*, but not in *K. pneumoniae*, whereas K types 2, 7, and 33 were frequently found in *K. pneumoniae*, but not in *K. oxytoca* (Podschun 1990).

There is considerable overlapping between the antigens of the genus *Klebsiella* and certain quite unrelated organisms. Capsular type 2, for example, is similar immunologically to the type 2 *Streptococcus pneumoniae* (Avery et al. 1925). Capsular antigens are usually detected by means of the capsular 'swelling' reaction, but agglutination, complement fixation, indirect immunofluorescence (Riser et al. 1976), and countercurrent immunoelectrophoresis (Palfreyman 1978) have also been employed.

Other typing methods

Many *Klebsiella* strains produce bacteriocins (klebocins), which appear to be distinct from colicins because they have no action on *E. coli* (Stouthamer and Tieze 1966). Many of the so-called pneumocins from *K. pneumoniae* have a narrow range of activity on other *Klebsiella* strains, often mainly on members of the same biochemical variety. Bacteriocin typing can be carried out by a traditional cross-streak method (Hall 1971) or by means of liquid preparations of bacteriocins after induction with mitomycin C (Edmondson and Cooke 1979). Epidemiological analysis may be improved by the use of bacteriocins as an adjunct to capsular serotyping (Bauernfeind et al. 1981). Phage typing by means of a set of temperate phages derived from other *Klebsiella* strains has also been advocated (Slopek et al. 1967). Bacteriophage typing can be an effective adjunct to serotyping in distinguishing serologically cross-reactive isolates (Gaston et al. 1987). Humphries (1948) described an enzyme, present in lysates of phage-infected organisms, capable of dissolving the capsule of type 1 organism. It did not inhibit growth itself nor was it toxic to the cells. Sikka et al. (1989) advocated a combination of bacteriocin and resistogram typing. Biotyping has been advocated in place of serotyping in less well equipped laboratories (Simoons-Smit et al. 1985).

The advent of molecular typing methods has seen the successful application of various approaches to the typing of isolates of *Klebsiella*. These include β-lactamase typing, multilocus enzyme electrophoresis (MEE) (Nouvellon et al. 1994) and one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins (Costas et al. 1990). Twelve protein types were recognized; comparison with established typing methods indicated that the level of discrimination of SDS-PAGE was similar to that achieved with conventional typing methods but the strains were grouped differently. Random amplification of polymorphic DNA (RAPD) analysis (Wong et al. 1994; Eisen et al. 1995) of *K. pneumoniae* has been considered as discriminatory as restriction fragment length polymorphism (RFLP) analysis using pulse field gel electrophoresis (PFGE), yet quicker and less costly. Restriction enzyme analysis by PFGE was found useful by Poh et al. (1993) as was ribotyping by Thompson et al. (1993).

Pathogenicity

Strains of *K. pneumoniae* occasionally give rise to community-acquired cases of severe bronchopneumonia, and also to more chronic destructive lesions with multiple abscess formation in the lungs (Limson et al. 1956). Such lesions are usually but not always associated with members of capsular types 1–5, which are frequently biochemically atypical. However, the main importance of *Klebsiella* as a pathogen for humans is in causing infections in hospital patients; the strains responsible are nearly always biochemically typical members of *K. pneumoniae*, most of which belong to higher-numbered capsular types. These strains cause widespread colonization of hospital patients. Clinical sepsis develops in surgical wounds and in the urinary tract; a number of patients have bacteremic infections and some of them die. Colonization of the respiratory tract is very common, but its clinical significance is often difficult to assess in patients with serious underlying diseases. Some of them develop bronchopneumonia in which the klebsiellae appear to be the primary infecting agent. Biochemically typical strains of *K. pneumoniae* are also responsible for various septic diseases in animals.

Experimentally, the virulence of *K. pneumoniae* is subject to considerable variation. Apart from the fact that smooth variants tend to be pathogenic for laboratory animals and rough variants nonpathogenic, there is a great difference in the virulence of individual smooth strains. Some strains will kill mice in a dose of 0.2 ml of a 24 h broth culture diluted 10^6 times; others fail to kill even with 0.2 ml of the undiluted culture. Capsular types 1 and 2 are usually very virulent to mice when injected intraperitoneally; other types are generally nonvirulent or of only low virulence to mice. Virulence does not appear to depend on capsule formation. The types just quoted as being of low virulence possess capsules in the same way as the highly virulent types 1 and 2. Moreover, noncapsulate, highly virulent variants have been described (Toenniessen 1914).

After subcutaneous injection of a very small dose (e.g. about 10 µl of a 24 h broth culture of a virulent strain) into mice, the animals die in 12–72 h. At postmortem examination, there is a local exudate, the associated lymph glands are swollen, and the spleen is enlarged. Encapsulated rods are found in the blood and viscera. Guinea pigs are refractory to subcutaneous but succumb to intraperitoneal injection, death occurring in 12–72 h. The fatal dose is about 0.01 ml of a 24 h broth culture. At postmortem examination, there is a viscous exudate in the peritoneum; the spleen may be enlarged, and the adrenals hemorrhagic. The rods are found in large numbers in the blood and viscera. Rabbits appear to be more resistant, but they succumb after intravenous or intraperitoneal injection with a dose of about 0.1 ml of a broth culture. Intraperitoneal inoculation is likewise fatal to pigeons.

Bacterial pathogens must multiply in order to establish an infection and in order to multiply they must acquire iron. *Klebsiella* strains make use of two high affinity iron uptake systems, one employing aerobactin, the other enterochelin (Williams et al. 1984). Production of aerobactin can be correlated with virulence and the genes encoding aerobactin have been found located on a plasmid (Nassif and Sansonetti 1986). In addition to aerobactin production, another phenotype can be correlated with the presence of this virulence plasmid: the mucoid phenotype of the bacterial colonies. This mucoid phenotype is considered an important virulence factor of *K. pneumoniae*; it is due to the plasmid-encoded production of a substance that is different from colanic acid and the capsular polysaccharide. *Klebsiella* strains show resistance to complement mediated serum killing and phagocytosis if both K and O antigens are present; resistance to the former seems related to the O antigens and the latter to the K antigens (Williams et al. 1983; Tomás et al. 1986). Immunization directed against the capsular polysaccharides has been successful in rats (Cryz et al. 1986a) and humans (Cryz et al. 1986b). It has been postulated that production of a capsule-like extracellular material may mediate aggregative adhesion of *K. pneumoniae*, which might explain the persistence of this organism inside the host gastrointestinal tract. Agents known to reduce capsule expression in *K. pneumoniae*, such as bismuth compounds or salicylate, also enhance phagocytic uptake of bacteria. Effecting a decrease in the production of capsular polysaccharide, the primary *K. pneumoniae* virulence factor, with salicylate may therefore have therapeutic potential (however, sodium salicylate can affect both antibiotic susceptibility and synergy). Salicylate accentuates phagocytosis of *K. pneumoniae* by making subcapsular antigens and components accessible to immune and nonimmune host defenses, and vaccination with subcapsular antigens may exhibit optimal protection against lethal infection when combined with salicylate therapy.

Although Thomison (1951) produced miliary granulomata in the brain of chicks by intracerebral inoculation of *K. granulomatis*, the bacterium is considered nonpathogenic for laboratory animals. In humans, it appears to have a low propensity to cause infection and requires direct inoculation through a break in the skin or mucous membranes to establish an infection. The incubation period is ill-defined and probably varies between a few days and 2–3 years, but most infections manifest themselves within 3–40 days of exposure (Clarke 1947). Granuloma inguinale is a chronic, destructive, granulomatous condition of the superficial tissue of the genital region. The initial subcutaneous nodules break down to give ulcers that, unlike those of chancroid, are painless; they exhibit beefy red granulations. The ulcers slowly enlarge and may produce daughter lesions and result in much local tissue loss and scarring. Spread to the inguinal region

can mimic lymphadenitis by producing a periadenitis (pseudobubo), but true bubo formation does not occur. Extensive scarring and pelvic fibrosis can result. Primary oral infection can occur (Garg et al. 1975) and distant spread to the face and involvement of the liver, thorax, and bones (Kirkpatrick 1970) have been reported. Although observed predominantly in adults, granuloma inguinale has been reported in two infants (8 months and 5 months of age) causing mastoiditis and otitis media, respectively. A temporal lobe abscess also developed in the 8-month-old child whereas the second child had a polypoid mass of the middle ear that on biopsy showed the features of granuloma inguinale. The mother of this child had biopsy-proven granuloma inguinale of the uterine cervix. The authors indicated that this agent can be transmitted during vaginal delivery and that careful cleaning of neonates to infected mothers be recommended (Govender et al. 1997).

ENTEROBACTER

Definition

Enterobacter organisms are metabolically active organisms with most strains able to ferment glucose, usually with gas production, as well as arabinose, cellobiose, maltose, mannitol, mannose, rhamnose (except *E. asburiae*), trahalose, and xylose (except *E. pyrinus*). All species reduce nitrate to nitrite, but have negative reactions for indole production, H_2S production, hydrolysis of gelatin, or production of lipase, DNase, or oxidase. All species are nonpigmented with the exception of *E. sakazakii* which is yellow-pigmented. *E. aerogenes*, a commonly isolated species, is distinguished from the other species by production of arginine dihydrolase and fermentation of adonitol, D-arabitol, and myo-inositol. The habitat of *Enterobacter* is soil and water, but they are occasionally found in the human bowel flora. They occasionally cause septic infection in humans. The G + C content of the DNA is 52–60 mol% and the type species is *Enterobacter cloacae*.

Classification

The organism that Escherich (1885) described as '*Bacterium lactis aerogenes*' was nonmotile; it was subsequently renamed '*Bacterium aerogenes*,' and in 1900 was transferred to a separate genus '*Aerobacter*,' by Beijerinck. The terms '*Bacterium*,' '*Aerobacter*' or '*Klebsiella aerogenes*' have been applied by successive generations of water bacteriologists to all VP-positive coliform bacteria, whether motile or nonmotile, except for those that liquefy gelatin rapidly. Later, some bacteriologists defined '*Aerobacter aerogenes*' as a motile organism and so distinguished it from *Klebsiella*. Motile, gelatin-liquefying '*Aerobacter aerogenes*'-like organisms were usually described by water bacteriologists as '*Bacterium cloacae*.' When it was discovered that many of the motile organisms from water and feces liquefied gelatin slowly they were transferred to '*cloaca*,' taking the specific epithet *aerogenes* with them. Hormaeche and Munilla (1957) and Hormaeche and Edwards (1958) recognized as 'Cloaca A' and 'Cloaca B' the organisms now called *E. cloacae* and *Enterobacter aerogenes*, respectively (Report 1963). The genus has been renamed successively '*Aerobacter*' (Hormaeche and Edwards 1958) and *Enterobacter* (Hormaeche and Edwards 1960). The latter name gained general acceptance (Report 1963), and the genus *Enterobacter* is considered to include all the motile '*Aerobacter aerogenes*'-like organisms, except those that form a red pigment and strains related to them, which are placed in the genus *Serratia*. *E. cloacae* is heterogeneous and comprises at least six genomic groups; however, the groups could not be differentiated phenotypically so there seemed to be no reason for an attempt to split *E. cloacae* into two or more species (Lindh and Ursing 1991).

In addition to *E. aerogenes* and *E. cloacae*, other species have been subsequently recognized in the genus. In 1976, Richard and colleagues described a new group of organisms that was most similar to *E. aerogenes*. Subsequently the name *Enterobacter gergoviae* was proposed for this group, strains of which are found in various clinical specimens and in the natural environment (Brenner et al. 1980). Strains previously regarded as yellow-pigmented, non-sorbitol-fermenting variants of *E. cloacae* were shown by DNA–DNA hybridization to warrant recognition as a separate species for which the name *Enterobacter sakazakii* was proposed (Farmer et al. 1980). Also described from clinical isolates are *Enterobacter asburiae* (Brenner et al. 1986), *Enterobacter hormaechei* (O'Hara et al. 1989), and *Enterobacter taylorae* (Farmer et al. 1985b). The latter was subsequently shown to be a later synonym of *Enterobacter cancerogenus* (Schonheyder et al. 1994) so the latter has priority. *Enterobacter amnigenus* with two biogroups (Izard et al. 1981b) has been isolated along with *Pseudomonas aeruginosa* from an intravenous catheter from the blood of a heart transplant patient (Bollet et al. 1991a), and *Enterobacter intermedius* (Izard et al. 1980) has been recovered in wound, bile, and fecal specimens, as well as blood. *Enterobacter dissolvens*, *Enterobacter nimipressuralis*, and *Enterobacter pyrinus* are mentioned purely for completeness because these species have not been encountered in human clinical specimens.

An anaerogenic, yellow-pigmented organism, which was described as '*Bacterium typhiflavum*' by Dresel and Strickl (1928), and as '*Chromobacterium typhiflavum*' in some of the earlier editions of this book, deserves description here. This organism is not uncommon in the human upper respiratory tract and in swabs from superficial lesions (Slotnick and Tulman 1967; Gilardi et al. 1970; Bottone and Schneierson 1972; Meyers et al. 1972); it has also been isolated from various animals

(Muraschi et al. 1965; Lev et al. 1969). The '*typhiflavum*' strains are indistinguishable from *Erwinia herbicola*, which is anaerogenic and does not liquefy pectin (Graham and Hodgkiss 1967). Although *E. herbicola* is the name accepted by many microbiologists, the name *Enterobacter agglomerans* has been commonly used in the American literature. Ewing and Fife (1972) recognized seven anaerogenic and four aerogenic biochemical varieties within the species. However, these 11 biogroups showed little correlation with the results of DNA–DNA hybridization, by which at least 14 DNA-relatedness groups can be identified within the species. One of these DNA-relatedness groups, DNA hybridization group 13, has been renamed *Pantoea agglomerans* (Gavini et al. 1989). Unfortunately, many microbiologists incorrectly refer to the complex of species that forms *Enterobacter agglomerans* as synonymous with *P. agglomerans*. The correct classification of the organisms in the *E. agglomerans* complex remains to be resolved (Brenner et al. 1984).

Morphological and cultural characteristics

Although motility has historically been an important distinguishing character between *Enterobacter* and *Klebsiella*, some species of *Enterobacter* are nonmotile (*E. asburiae*, *E. dissolvens*, *E. nimipressuralis*) or have variable motility (*E. gergoviae*, *E. hormaechei*, *E. intermedius*, *E. pyrinus*). In fluid media, sausage-shaped aggregations or symplasmata may occur in *P. agglomerans*; these appear to be chains of spherical bodies, 7–13 μm in diameter, consisting of organisms within a matrix (Lev et al. 1969). The colonies of *Enterobacter* strains may be somewhat mucoid, but the amount of extracellular material formed is usually not great. In *P. agglomerans* there is pigment of an ochre or rusty yellow color; on further incubation, granular structures corresponding to the symplasmata appear, as well as biconvex bodies with a clear-cut margin. The latter probably represent downgrowths into the medium (Cruickshank 1935; Graham and Hodgkiss 1967).

P. agglomerans grows poorly on MacConkey agar and is not hemolytic on blood agar. In a gelatin stab, liquefaction usually begins in 6–10 days. Growth occurs between 20 and 37°C, but the optimum is near 37°C.

Biochemical activities

In general, the fermentative activity of *Enterobacter* strains is more limited than that of typical strains of *Klebsiella*, but most *Enterobacter* strains do form gas from cellobiose and ferment rhamnose, characters which distinguish them from most species of *Serratia*. Some *Enterobacter* strains give a weak urease reaction in Christensen's medium. In the modified medium advocated by Kauffmann (1954), however, *Klebsiella* strains are urease-positive and *Enterobacter* strains usually negative (but *E. gergoviae* and *E. hormaechei* are exceptions to this rule). Although growth at 37°C is usually good, many *Enterobacter* strains give atypical biochemical reactions unless tested at a lower temperature. When strains of *P. agglomerans* are tested at 37°C, the MR reaction is positive and the VP reaction negative, though the latter is often positive at 30°C. The main cultural and biochemical characteristics of *Pantoea* and of *Enterobacter* are given in Enterobacteriaceae: general characteristics. The key biochemical tests used to differentiate human strains of *Enterobacter* are listed in Table 3.

E. aerogenes resembles *Klebsiella* in its wide range of fermentative activity and in its ability to form gas from cellobiose, glycerol, and inositol, but it does not form gas from starch. It also differs from *Klebsiella* in being motile and in forming lysine and ornithine decarboxylase. Liquefaction of gelatin in 7–60 days distinguishes it from all the subspecies of *K. pneumoniae* but not from *K. oxytoca*. Nevertheless, Bascomb and colleagues (1971) believed that it should be transferred to a reconstituted *Klebsiella* genus as *Klebsiella mobilis* and this is supported by DNA–DNA hybridization data. The proposal has, however, not gained wide acceptance. *E. cloacae* differs from *E. aerogenes* in attacking arginine but not lysine and in its inability to form gas from glycerol and inositol. It sometimes fails to liquefy gelatin. *E. gergoviae* differs from *E. aerogenes* in producing urease, and in giving negative results in the following tests: KCN tolerance, and fermentation of adonitol, *i*-inositol, D-sorbitol, and mucate. *E. sakazakii* differs from *E. cloacae* in being yellow-pigmented, producing extracellular deoxyribonuclease, and failing to ferment D-sorbitol. Only strains of *E. asburiae* fail to ferment malonate, give a positive result in the MR test and fail to ferment L-rhamnose; only strains of this species and of *E. hormaechei* and *E. taylorae* fail to ferment melibiose.

Clinical, animal, and plant isolates of *P. agglomerans*, representing different geographical areas, have been characterized phenotypically (Lindh et al. 1991). No strain decarboxylated ornithine and 22 strains, mainly plant isolates, showed delayed acid production from α-methyl-glycoside. In these two characters the results differed from the description of *P. agglomerans* given by Gavini et al. (1989).

Susceptibility to antimicrobial agents

Strains of *Enterobacter* are nearly always highly resistant to first-generation cephalosporins (Benner et al. 1965) by virtue of producing a β-lactamase with predominantly cephalosporinase activity (Jack and Richmond 1970). Many are also resistant to chloramphenicol, streptomycin, and tetracycline. At one time, most were suscep-

Table 3 *Differentiation of human species of* Enterobacter

Properties	*E. aerogenes*	*E. amnigenus*	*E. asburiae*	*E. cancerogenus*	*E. cloacae*	*E. gergoviae*	*E. hormaechei*	*E. intermedius*	*E. sakazakii*
Methyl red	–	–	+	–	–	–	V	+	–
Voges–Proskauer	+	+	–	+	+	+	+	+	+
Urea hydrolysis	–	–	V	–	V	+	+	–	–
Arginine dihydrolase	–	–	V	+	+	–	V	–	+
Lysine decarboxylase	+	–	–	–	–	+	–	–	–
Ornithine decarboxylase	+	V	+	+	+	+	+	+	+
Growth in KCN	+	+	+	+	+	–	+	V	+
Acid from:									
Adonitol	+	–	–	–	V	–	–	–	–
Melibiose	+	+	–	–	+	+	–	+	+
Rhamnose	+	+	–	–	+	+	–	+	+
Sorbitol	+	–	+	–	+	–	–	+	–
Sucrose	+	+	+	–	+	+	+	V	+
Yellow pigment	–	–	–	–	–	_	–	–	+

+, Most strains positive; –, most strains negative; V, some strains positive, others negative

tible to kanamycin and nearly all to gentamicin (Toala et al. 1970). Most British strains of *E. cloacae* remain susceptible to the third-generation cephalosporins, amikacin, gentamicin, and nalidixic acid but more than 10 percent show resistance to ampicillin, carbenicillin, cefuroxime, and sulfamethoxazole. Following treatment with ampicillin, isolates can develop markedly elevated minimum inhibitory concentrations (MIC) to several agents including cefotaxime, ceftazidime, cefuroxime, and piperacillin. Administration of amdinocillin in combination with ceftazidime is said to confer a protective effect against emergence of resistance to ceftazidime. In Canada, many isolates of *Enterobacter* spp. (>16 percent) are resistant to the third-generation cephalosporins with marked cross-resistance to aztreonam (76.6 percent), piperacillin (63.1 percent), and piperacillin–tazobactam (44.9 percent) (Toye et al. 1993). Ciprofloxacin, cefepime, cefpirome, gentamicin, imipenem, and tobramycin were consistently active against most isolates of *Enterobacter* spp. including those resistant to the third-generation cephalosporins. Elsewhere, resistance to imipenem has been observed, the proportion being 1.3 percent of isolates in the USA; the resistance appears to be related to mutants deficient in the production of nonspecific porins.

Ehrhardt and Sanders (1993) reviewed the effects of the introduction of extended-spectrum cephalosporins into clinical use, which caused the prevalence of *Enterobacter* spp. to increase because of their natural resistance to earlier cephalosporins and their ability to develop resistance rapidly to the newer drugs. β-Lactam resistance in this genus is due, for the most part, to the presence of a Bush group 1 chromosomal cephalosporinase. This enzyme is normally inducible and resistance to older cephalosporins, aminopenicillins, and cephamycins results from either the extreme lability of the drugs to the enzyme or from their inducer activities. Resistance to newer cephalosporins, monobactams, and penicillins is attributable to the selection of mutants that express large amounts of the enzyme. Such mutants arise as the result of a spontaneous mutation in one of the regulatory genes responsible for suppressing enzyme expression. Since the enzyme has very high affinity for the newer cephalosporins, this, coupled with the slow penetration of the drugs into the cell, provides a very efficient mechanism of resistance. Surveys in the USA and elsewhere have shown that the increased prevalence of multi-β-lactam-resistant *Enterobacter* strains is due to the increased use of the newer cephalosporins. Attempts to prevent these problems include the more judicious use of newer β-lactam antibiotics and the development of enhanced-potency cephalosporins, which are able to evade the resistance mechanism because they have lower enzyme affinity and permeate more rapidly into the cell. Monotherapy with an extended-spectrum cephalosporin should be avoided because this increases the selection of resistant strains. Plasmids also play a role in resistance, with up to five aminoglycoside-modifying enzymes being detected on the same R plasmid; *E. cloacae* is thus also important as a gene pool for the spread of resistance to other bacteria of clinical relevance.

Fussle et al. (1994) traced the development of resistance by *E. cloacae* during the treatment of pulmonary infections in intensive care patients. During therapy with

cefotaxime and tobramycin, the strains from 47 percent of the patients became resistant to cefotaxime within 6 days. In all cases, resistance encompassed all other broad-spectrum penicillins and cephalosporins tested, as well as aztreonam. Development of resistance regularly led to persistence of bacteria. Resistance to ciprofloxacin, imipenem, or tobramycin was not observed. Treatment of 25 patients with persisting infections was successful in six of seven patients receiving ciprofloxacin and in 17 of 18 patients treated with imipenem.

Huber and Thomas (1994) described how strains of *E. aerogenes* and *E. cloacae* with inducible β-lactamase developed resistance when cefoxitin was added to cefuroxime disks; this approach can be used in routine clinical laboratories to detect latent resistance due to chromosomally mediated inducible β-lactamase in enterobacters. Yu et al. (1996) proposed use of the spiral plater in identifying those antimicrobial agents that induce few or no spontaneous mutants and, therefore, may be more likely to be successful in treating infections due to *Enterobacter* spp. Higashitani et al. (1995) found that when *E. cloacae* strains were treated with piperacillin in combination with tazobactam, the in vitro frequency of emergence of resistant strains (mutants producing β-lactamase) was lower than with bacteria treated with piperacillin or ceftazidime. *Enterobacter* strains differ from *Serratia* strains in being susceptible to the polymyxins (Greenup and Blazevic 1971).

Outbreaks of infection may need to be controlled by interrupting the transmission of the epidemic strains, by measures such as cohort nursing, diligent hand-washing before and after procedures (*E. cloacae* has been found still present on the fingertips after 15 repeated courses of hand-washing and applications of disinfectants), and thorough environmental cleaning including decontamination of any equipment believed to have acted as a persistent reservoir. Pharmacy intervention may also be necessary. Increasing resistance to ceftazidime and some other β-lactam antibiotics developed among *Enterobacter* spp. after increased use of ceftazidime at a 500-bed community teaching hospital (Schentag 1993). Severe restrictions on the use of ceftazidime were imposed by pharmacy intervention. For empiric therapy of severe infections, a combination of piperacillin plus tobramycin was most frequently substituted for ceftazidime. Susceptibility patterns of *Enterobacter* returned to baseline within 3 months after active intervention reduced the use of ceftazidime by 98 percent of its peak. There was no development of enhanced resistance to piperacillin or tobramycin or to other antibiotics on the formulary. It was concluded that active pharmacy intervention resulted in reversion of susceptibility of *Enterobacter* spp. to baseline for ceftazidime and the other β-lactam antibiotics (i.e. aztreonam, cefotaxime, ceftriaxone, mezlocillin, and piperacillin) for which covariance (i.e. parallel decline of the organisms' sensitivities) or cross-resistance had developed.

Antigenic structure

An O-antigen typing scheme has been described for *E. cloacae* and currently comprises 30 O types (Gaston 1988). According to Sakazaki and Namioka (1960), there is no significant relation between the capsular antigens of *Klebsiella* and strains with the characteristics of *E. cloacae*. However, the capsular antigens of *E. aerogenes* are similar to those of *Klebsiella* and isolates can frequently be typed with *Klebsiella* capsular antisera (Gaston et al. 1989).

Other typing methods

Schemes for biotyping and bacteriocin typing of *E. aerogenes* have been described; for biotyping there is available the PhenePlate-*Klebsiella*/*Enterobacter* (KE) system automated biochemical fingerprinting system, which is performed in microtitration plates (Kuhn et al. 1991). Georghiou et al. (1995) applied several molecular techniques to study the epidemiology of infections due to *E. aerogenes*: restriction endonuclease analysis of chromosomal DNA and repetitive-element polymerase chain reaction (rep-PCR) with primers based on repetitive extragenic palindromic (REP), and enterobacterial repetitive intergenic consensus (ERIC) bacterial DNA sequences; genomic fingerprinting with rep-PCR appeared most advantageous, especially when REP primers (rather than ERIC primers) were used.

Most interest lies in typing *E. cloacae* and the most successful approach appears to be O serology as the primary method of strain discrimination with strains of the same O serotype then being subdivided by phage typing (Gaston 1988). Costas et al. (1989) used high-resolution SDS-PAGE of whole cell proteins and found discrimination similar to that achieved with conventional typing methods; all strain groups recognized by combined sero–phage typing were also found by SDS-PAGE. Ribotyping and arbitrarily primed PCR (Grattard et al. 1995) and RAPD (Davin-Regli et al. 1996) appeared to be reliable methods for typing *E. aerogenes* strains implicated in nosocomial infection. RFLP of total DNA and rDNA regions (Bingen et al. 1992), ribotyping (Garaizar et al. 1991; Poilane et al. 1993), small-fragment restriction endonuclease analysis of *Eco*RI DNA digests, PFGE of genomic restriction fragments (Haertl and Bandlow 1993), and RAPD (Riain et al. 1994) have all similarly proved useful in the investigation of outbreaks due to *E. cloacae*.

Habitat and pathogenicity

The normal habitat of *Enterobacter* is believed to be soil and water, but the organism is occasionally found in the feces and the respiratory tract of humans. In recent years, infection of hospital patients with *E. aerogenes*

and *E. cloacae* has been reported more often than formerly, but *Enterobacter* is a much less important cause of hospital infection than is *Klebsiella*. Most infections are of the urinary tract (Eickhoff et al. 1966), although members of the genus are an important cause of bacteremia in some hospitals (Gaston 1988). Shlaes (1993) described a prospective study of *Enterobacter* bacteremia conducted in several patients. The results indicated that (1) emergence of resistance was more frequent during treatment with third-generation cephalosporins than with other antibiotics; (2) the addition of an aminoglycoside to the cephalosporin did not prevent emergence of resistance; (3) previous treatment with cephalosporins was associated with bacteremia caused by multiresistant *Enterobacter* spp.; and (4) infection with multiresistant *Enterobacter* spp. was associated with a higher mortality than infection with susceptible strains.

The author concluded that avoidance of third-generation cephalosporins for surgical prophylaxis and therapy in patients in whom *Enterobacter* infections are suspected or proven should lower the prevalence of *Enterobacter* bacteremias and mortality and prevent the emergence of multiresistance. The organism has been shown to have serious implications following eye surgery; in the episode described by Mirza et al. (1994), the use of contaminated swabs led to infections resulting in useful vision being retained in only one of ten eyes. Colonization with *Enterobacter* spp. has been found to increase in cardiac surgery patients given cefazolin prophylaxis (Flynn et al. 1987).

The clinical significance of the more recently described *Enterobacter* spp. remains to be determined but *E. sakazakii* is known to have caused neonatal meningitis and sepsis (Muytjens et al. 1983; Gallagher and Ball 1991). Two unrelated hospital outbreaks, involving bacteremia, meningitis, and colonization of neonates, were investigated by a combination of typing methods: antibiograms, chromosomal restriction endonuclease analysis, MEE, plasmid analysis, and ribotyping. All but the antibiograms proved effective as epidemiological typing methods, especially when used in combination (Clark et al. 1990). *E. taylorae* is known to have caused osteomyelitis (Westblom and Coggins 1987). The latter organism was also reported as the cause of severe nosocomial infections in four patients; the isolates were not susceptible to cephalosporins or penicillins (unlike most members of the genus (Rubinstien et al. 1993). As well as being an occasional pathogen in predisposed human patients, *P. agglomerans* has caused bacteremia when administered in contaminated intravenous fluids (Lapage et al. 1973).

In mice, lipopolysaccharide (LPS) from *P. agglomerans* has demonstrated relief of morphine dependence (Okutomi et al. 1992) and a protective effect on gastric ulcers (Inagawa et al. 1992). When administered intradermally, LPS was less toxic and elicited a tumor response in combination with cyclophosphamide; Goto et al. (1996) therefore foresaw an application to cancer treatment even in humans.

SERRATIA

Definition

Serratia organisms are small, motile, gram-negative rods that ferment glucose, mannitol, mannose, salicin, and trehalose with the production of acid and sometimes of a small bubble of gas. They are indole-negative (except some strains of *Serratia odorifera*), urease-negative (except for some strains of *Serratia fonticola* and *Serratia marcescens*), and generally grow on Simmons' citrate medium. They do not deaminate phenylalanine but the most common species (*S. liquefaciens*, *S. marcescens*) decarboxylate lysine and ornithine. Most species liquefy gelatin rapidly, usually within 2–3 days, and produce lecithinase, lipase, and deoxyribonuclease. Some strains produce a red nondiffusible pigment. *Serratia* species are typically found in soil and water, but some strains occur in the animal body. They may cause septic infection in humans. The G + C content of the DNA is 53–59 mol% and the type species is *Serratia marcescens*.

Classification

S. marcescens was first described by Bartolomeo Bizio in 1823 as a cause of 'bleeding polenta' (Breed and Breed 1924). Similar organisms have been isolated at various times from water, soil, and sewage, from contaminated foodstuffs and, less frequently, from the animal body. Although the pigmented strains were the first described, they form only a small part of a group of mainly nonpigmented organisms (Davis et al. 1957).

S. marcescens was the first species recognized in this genus. Today, eight species are generally recognized, with all but one (*S. entomophila*) isolated from humans. *Serratia liquefaciens* was formerly known as '*Enterobacter liquefaciens*,' and was transferred to *Serratia* as a result of a numerical taxonomy study (Bascomb et al. 1971). *S. odorifera* (Grimont et al. 1978) comprises two distinct biochemical varieties (referred to as biogroups 1 and 2), but DNA–DNA hybridization tests indicate that it forms a single species. *Serratia plymuthica* (Grimont et al. 1977) and *Serratia grimesii* resemble *S. liquefaciens* in biochemical characters and have been referred to as '*S. liquefaciens*-like' (Grimont et al. 1982). *Serratia rubidaea* is the name used by Ewing and colleagues (Ewing et al. 1972, 1973), but their description of this organism differs in several respects from that given originally for '*Bacterium rubidaeum*.' Despite this discrepancy, the name *S. rubidaea* has seen most frequent use, particularly in the American literature, and will be used here in the interests of uniformity. *Serratia ficaria* is associated with figs and a species of fig wasp (Grimont et al. 1979)

but it has also been isolated from human sources (Gill et al. 1981; Pien and Farmer 1983; Brouillard et al. 1984). *Serratia fonticola* (Gavini et al. 1979), a species from water, is possibly misplaced in *Serratia*. *Serratia entomophila* has been isolated from water sources but not human specimens.

Morphological and cultural characteristics

S. marcescens is described in detail because it is the species most commonly encountered in clinical specimens. It is, on the whole, smaller than the average coliform bacterium. However, its size is subject to considerable variation; even on the same type of medium a single strain may at one time give rise to coccobacilli and at another to rods indistinguishable from other coliform bacteria. Capsules are not visible around cells grown on nutrient agar, but Bunting et al. (1949) found that capsular material was formed on a well-aerated medium poor in nitrogen and phosphate. Aucken et al. (1997) showed that capsules could be demonstrated around cells of most of the O-serotype reference strains when grown on suitable agar media. Most *Serratia* strains are motile with peritrichous flagella. The flagella are usually best seen in cultures grown at temperatures below 37°C (Fulton et al. 1959).

Colonies of *S. marcescens* on agar are usually undifferentiated for the first day or two, and then may develop a convex, pigmented, and relatively opaque center and an effuse, colorless, almost transparent periphery with an irregular crenated edge. However, the colonial characters of a strain are liable to considerable variation; colonies may vary in size, shape, opacity, surface, and consistency. Similarly, there is great variation in their ability to produce the red pigment, which is formed only in the presence of oxygen and at a suitable temperature. The optimum temperature for pigment formation is not necessarily the same as that for growth. Thus many strains grow best at 30–37°C but form little or no pigment, whereas at lower temperatures growth is poorer and pigment formation is abundant. The ability to form pigment is commonly diminished and often irreversibly lost after repeated subculture. *S. liquefaciens* and *S. odorifera* are nonpigmented but red pigment is formed by many strains of *S. plymuthica* and *S. rubidaea*.

The red pigment, prodigiosin, is soluble in absolute alcohol, acetone, benzol, carbon disulfide, chloroform, and ether, but is insoluble in water. Wrede and Rothhaas (1934) purified it and assigned to it a tripyrrylmethene structure. This was confirmed by Hubbard and Rimington (1949, 1950). Williams et al. (1956a, b) (see also Green et al. 1956) consider that prodigiosin is not a single substance. They separated it into three red fractions and one blue fraction with different absorption spectra. Prodigiosin is also formed by some organisms unrelated to *Serratia*, including an actinomycete (Perry 1961) and certain gram-negative rods from seawater that attack sugars oxidatively are oxidase-positive and have polar flagella (Lewis and Corpe 1964).

Except for the possibly misplaced species *S. fonticola*, all *Serratia* strains are actively proteolytic. Liquefaction of gelatin is usually obvious within 2–3 days. On blood agar, many produce a narrow zone of hemolysis in 24 or 48 h. Most strains produce opacity in egg-yolk media, due to the formation of a lecithinase C (Monsour and Colmer 1952; Klinge 1957); except for *S. fonticola*, they also produce deoxyribonuclease (Schreier 1969).

The optimum temperature for growth varies; some strains grow as well at 37°C as at 30°C, many have an optimum between 30 and 37°C, and a few fail to grow at all at 37°C. Many grow at 1–5°C.

Biochemical activities

The biochemical reactions of the eight species of *Serratia* are given in Enterobacteriaceae: general characteristics, Table 1. The key biochemical tests used to differentiate *Serratia* species are listed in Table 4. Acid is usually formed promptly in glucose, glycerol, maltose, mannitol, sorbitol, sucrose, and trehalose. Cellobiose and inositol may be acidified in a few days. Lactose is not acidified, except by strains of *S. fonticola* and *S. rubidaea*, or is acidified late, but β-galactosidase is always formed. Dulcitol is fermented only by *S. fonticola* and rhamnose only by strains of *S. ficaria*, *S. fonticola*, and *S. odorifera*. The strains are either completely anaerogenic or form small bubbles of gas filling no more than 5 percent of a Durham's tube. Gas is not formed in cellobiose, glycerol, inositol, or starch.

MacConkey agar with sorbitol (1 percent) and colistin (200 IU/ml) has been proposed for better isolation of *S. marcescens* from clinical samples.

Susceptibility to antimicrobial agents

Serratia strains, like *Enterobacter* strains, are nearly always highly resistant to cephalosporins. Such resistance is frequently plasmid mediated and readily transferable to other members of the Enterobacteriaceae. As with other organisms described in this chapter, prior antimicrobial therapy with an extended-spectrum cephalosporin is an important risk factor for subsequent acquisition of an organism resistant to third-generation cephalosporins. Resistance to ampicillin and gentamicin varies from strain to strain, but many strains destroy these antibiotics enzymatically. *Serratia* strains, unlike *Enterobacter* strains, are resistant in vitro to polymyxins (Ramirez 1968). In an experimental model of rabbit endocarditis, Potel et al. (1992) found that intermittent amikacin dosing gave excellent bactericidal activity

Table 4 *Differentiation of species of* Serratia

Properties	*S. ficaria*	*S. fonticola*	*S. liquefaciens*	*S. marcescens*	*S. odorifera*	*S. plymuthica*	*S. rubidaea*
Lysine decarboxylase	–	+	+	+	+	–	V
Ornithine decarboxylase	–	+	+	+	V	–	–
Malonate utilization	–	V	–	–	–	–	+
Acid from:							
Adonitol	–	+	–	V	V	–	+
Arabinose	+	+	+	–	+	+	+
Cellobiose	+	–	–	–	+	V	+
Rhamnose	V	V	V	–	+	–	–
Sorbitol	+	+	+	+	+	V	–
Sucrose	+	V	+	+	+	+	+
Xylose	+	V	+	–	+	–	+
Red pigment	–	–	–	V	–	+	+

+, Most strains positive; –, most strains negative; V, some strains positive, others negative

within the first 24 h in severe *S. marcescens* infections. Aztreonam is also effective. *S. liquefaciens* is said to be more resistant than *S. marcescens* to amikacin, aztreonam, and ceftazidime.

Some strains of *S. marcescens* may grow in chlorhexidine-based disinfecting solutions; such solutions may therefore be a potential source of infection, for example, where used for contact lenses. Cells adapted to chlorhexidine may persist or grow in solutions with different antimicrobial agents, including benzalkonium chloride. The organism has also persisted in a dialyzer in spite of the implementation of recommended sterilization procedures.

Antigenic structure

Davis and Woodward (1957) studied the O antigens of 16 cultures of *S. marcescens* and identified six O groups. There was no apparent relation between serological group and pigment production. Ewing et al. (1959) examined 115 *Serratia* cultures and established an antigenic typing scheme with nine O groups, 13 separate H antigens, and 46 serotypes. This scheme was then extended to 15 O groups (Ewing et al. 1962) and the present total is 28 (Pitt and Erdman 1984; Traub 1985, 1991c; Aucken et al. 1996). Serogroup O14 predominates in clinical specimens. Another seven H antigens have also been described by Traub and Kleber (1977) and Le Minor and Pigache (1977); these may conveniently be detected by motility-inhibition tests (Traub and Kleber 1977). Between 16 and 50 percent of all strains may belong to the combined serotype O14:H12. Gaston and Pitt (1989) developed a dot immunoassay that appeared to offer greater accuracy than agglutination tests for serotype identification in *S. marcescens*. Subsequently, 14 chemically and serologically distinct K types have been identified among the 28 O-serotype strains (Aucken et al. 1997).

Traub (1991a) has compared bacteriocin typing, biotyping, and serogrouping (O antigens) of *S. liquefaciens*. Biotyping afforded least discrimination because 85.5 percent of the isolates comprised two biotypes; bacteriocin typing differentiated 84.3 percent of the isolates into 20 types; polyclonal rabbit anti-O immune sera identified 16 O antigens, and all 83 isolates could be serogrouped. Traub (1991b) differentiated 12 flagellar (H) antigens among 79 motile isolates with polyclonal anti-H rabbit immune sera.

Other typing methods

Serratia strains produce two different groups of bacteriocins or 'marcescins' (Prinsloo 1966; Eichenlaub and Winkler 1974). Group A bacteriocins are resistant to chloroform, heat, and proteolytic enzymes; they are active against other *Serratia* strains and sometimes also against less closely related enterobacteria. Group B bacteriocins are susceptible to these agents; they are active against other enterobacteria but not against *Serratia* strains. Bacteriocin-susceptibility tests (Traub and Raymond 1971) have been used as a means of typing *S. marcescens*. According to Traub (1980), 73 patterns of susceptibility can be detected; unfortunately, however, small differences in pattern may occur on replicate typing. Nevertheless, the method may be useful to subdivide strains of the same O serogroup from an outbreak of infection. Several phage typing systems have been reviewed by Traub (1980). Biotyping has also been described and the scheme of Grimont and Grimont (1978) has been improved by Sifuentes-Osornio and Groschel (1987). Alonso et al. (1993) compared bacteriocin type, biotype, and serotype with rDNA RFLP patterns (ribotypes) for the type identification of *S. marcescens*. They concluded that, due to the accessibility of biotyping and the lack of commercially available antisera for *S. marcescens*, the biotype and ribotype together

provide reliable markers of strain identity. Bale et al. (1993) reached similar conclusions from a combination of biotyping and DNA restriction fragment analysis. One-dimensional SDS-PAGE of whole cell proteins has also been used (Holmes et al. 1990). Comparison with O serotyping indicated that the level of discrimination by SDS-PAGE was similar; however, as with O serotyping, a secondary scheme, such as phage typing, was necessary to differentiate strains of the same protein type. PCR amplification of enterobacterial repetitive intergenic consensus sequences has also been shown to provide a rapid and simple means of typing isolates for epidemiological studies (Liu et al. 1994).

Although most interest lies in typing isolates of *S. marcescens*, typing has been performed on strains of other species. Sader et al. (1994) found macrorestriction analysis of chromosomal DNA by PFGE useful for typing isolates of *S. odorifera* (biogroup 2).

Habitat and pathogenicity

S. marcescens is widely distributed in nature. Pigmented strains have at times caused alarm by giving rise to red colors in various foods (e.g. Klein 1894), by simulating the appearance of blood in the sputum (Woodward and Clarke 1913), or by producing stains on babies' napkins (Waisman and Stone 1958). Pigmented and nonpigmented strains are found from time to time in the human respiratory tract and in feces. Accounts of human disease due to *S. marcescens* have become increasingly frequent in recent years. Most infections occur in hospital patients; they include sepsis of the meninges, of the respiratory and urinary tracts, and of wounds. Septicemia is common, and endotoxic shock and endocarditis have been reported; some strains become established endemically in hospitals (see, for example, Wilfert et al. 1970). Outbreaks of infection are frequently reported, such as epidemic septic arthritis associated with an antiseptic (Nakashima et al. 1987), one involving a special care baby unit (Gransden et al. 1986) and one in a cardiac surgery unit (Wilhelmi et al. 1987). Only a small proportion, usually <10 percent, of the strains responsible for infection are pigmented. Some strains produce a cell-bound hemolysin and have a higher pathogenic potential than strains failing to produce the hemolysin. For a review of infections caused by *S. marcescens*, see Acar (1986).

On experimental inoculation into laboratory animals, organisms of this group are harmless except in very large doses. Culture filtrates of *S. marcescens* have been used at times in the treatment of cancer, but there is no reason to believe that the substance producing hemorrhage and necrosis in the tumor is other than the endotoxin (Wharton and Creech 1949). The inhalation of aerosols of *S. marcescens* is said to cause an acute, short-lived illness with respiratory and general constitutional symptoms (Paine 1946).

The other *Serratia* spp. also occur commonly in the natural environment, especially in water. *S. liquefaciens* and *S. odorifera* occur with some frequency in human clinical specimens, whereas only occasional strains of *S. rubidaea* (Parment et al. 1984) and *S. plymuthica* (Clark and Janda 1985; Horowitz et al. 1987) are seen. Chmel (1988) isolated a strain of *S. odorifera* (biotype 1) from the blood and urine of an alcoholic man with cirrhosis and signs of septic shock. Mermel and Spiegel (1992) described a case of nosocomial sepsis due to *S. odorifera* (biotype 1). *S. rubidaea* was isolated from the bile and blood of a patient with a biliary tract carcinoma obstructing the common bile duct and who underwent invasive procedures (Ursua et al. 1996). *S. plymuthica* has been documented from a case of chronic osteomyelitis and two cases of sepsis secondary to central venous catheter infection. Reina et al. (1992) presented a case of a woman aged 50 years who was admitted to hospital as a result of a process affecting the lower respiratory tract, which was associated with a community-acquired bacteremia due to this organism; the patient was treated with erythromycin and gentamicin and was discharged home 7 days after admission with complete clinical cure. Carrero et al. (1995) reported the isolation of *S. plymuthica* from six patients: three from blood cultures (in which the patients had lymphoblastic leukemia, lymphoma, or stroke), two from exudates (following knee and abdominal surgery), and one from the peritoneal fluid of a patient with cholecystitis. The infection was considered nosocomial in five cases and community acquired in the other. The pathogenicity of *S. ficaria* is questionable but it has been reported as a cause of septicemia in an elderly cancer patient (Darbas et al. 1994). *S. fonticola* has been reported from a very large leg abscess in a patient following an accident (Bollet et al. 1991b) and from pus from a severe infection of the hand following a road traffic accident (Pfyffer 1992).

CEDECEA

Definition

Cedecea are usually motile and usually produce gas from glucose. Acid is produced from arabinose, cellobiose, maltose, mannose, D-mannitol, salicin, and trehalose. Indole is not produced. Most strains utilize citrate. They do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gelatin is not liquefied. Most strains grow in Møller's KCN medium. Lysine is not decarboxylated. The organisms are isolated from the human respiratory tract but their clinical significance is uncertain. Three species have been named (*C. davisae*, *C. lapagei*, and *C. neteri*) (Grimont et al. 1981; Farmer et al. 1982) and two species remain unnamed (*Cedecea* species 3 and species

5). The G + C content of the DNA is 48–52 mol% and the type species is *Cedecea davisae*.

Cultural characteristics and biochemical activities

Members of the genus grow well on ordinary media, producing convex colonies about 1.5 mm in diameter on nutrient agar at 37°C. The usual biochemical characters of *Cedecea* are summarized in Enterobacteriaceae: general characteristics, Table 1. The key tests to differentiate *Cedecea* species are listed in Table 5. Properties not mentioned in the definition of the genus include failure to form acid from adonitol, L-arabinose, dulcitol, *i*-inositol, or L-rhamnose; most strains ferment malonate and form arginine dihydrolase. The differential characters of *C. davisae*, *C. lapagei*, and *C. neteri* are shown in Table 5.

Susceptibility to antimicrobial agents

Cedecea strains are susceptible to amikacin, carbenicillin, chloramphenicol, gentamicin, kanamycin, minocycline, nalidixic acid, nitrofurantoin, streptomycin, sulfonamides, tetracycline, tobramycin, and trimethoprim. Strains are resistant to ampicillin, cephalothin, colistin, and polymyxin B. Resistance to third-generation cephalosporins has also been reported (Berkowitz and Metchock 1995).

Habitat and pathogenicity

All *Cedecea* strains so far described have been isolated from human clinical specimens, predominantly from the respiratory tract. There is, as yet, little clear evidence that they play a pathogenic role, although *C. davisae* and *C. neteri* have been reported to cause bacteremia (Farmer et al. 1982; Perkins et al. 1986) and a *Cedecea* strain has been reported from a cutaneous ulcer (Hansen and Glupczynski 1984).

Table 5 *Differentiation of species of* Cedecea

Property	*C. davisae*	*C. lapagei*	*C. neteri*
Methyl red test	+	V	+
Voges–Proskauer	–	+	–
Ornithine decarboxylase	+	–	–
Acid from:			
Lactose	–	+	+
Sorbitol	–	–	+
Sucrose	+	–	+
Xylose	+	–	+

+, Most strains positive; –, most strains negative; V, some strains positive, others negative

EDWARDSIELLA

Definition

Edwardsiella species are motile organisms that ferment glucose, often with the production of some gas. Maltose and D-mannose are fermented, but few other sugars. Lysine and ornithine are decarboxylated but arginine dihydrolase is not produced. The genus is pathogenic for catfish, eels, and other animals; occasional human infections have been reported. The G + C content of the DNA is 55–59 mol% and the type species is *Edwardsiella tarda*.

Classification

In 1962, a group of enterobacteria that appeared to be quite distinct from all others were described under the name 'Asakusa' (Sakazaki 1965). The main habitat of this organism appears to be the gastrointestinal tract of lower animals but a number of isolations have been made from human feces, and a few cases of febrile diarrhea and systemic infection in humans have been reported (Okubadejo and Alausa 1968; Sonnenwirth and Kallus 1968; Jordan and Hadley 1969; Bockemühl et al. 1971).

The organism has been named *Edwardsiella tarda* (Ewing et al. 1965). Grimont and coworkers (1980) described a distinct biochemical variety of *E. tarda* differing from typical members of the species in fermenting L-arabinose, mannitol, and sucrose, but these atypical strains are closely related to *E. tarda* by DNA–DNA homology and are generally referred to as '*E. tarda* biogroup 1'. Walton et al. (1993) encountered an unusual strain (H_2S-negative, fermenting sucrose) that mimicked a biogroup 1 isolate (it was isolated from a mixed infection from a woman suffering from cholelithiasis). Two other species have been described: *Edwardsiella hoshinae* (Grimont et al. 1980) has been isolated only from animals, and *Edwardsiella ictaluri* (Hawke et al. 1981) has caused several outbreaks of enteric septicemia in catfish and was earlier called *Edwardsiella* group GA7752 (Hawke 1979).

Cultural characters and biochemical activities

E. tarda grows well on ordinary media but produces only small colonies of 0.5–1 mm diameter after 24 h. Biochemical activities are described in Enterobacteriaceae: general characteristics; also see Sakazaki (1967), Ewing et al. (1969), and Bascomb et al. (1971).

Susceptibility to antimicrobial agents

Studies indicate that all three *Edwardsiella* spp. are susceptible to most commonly prescribed antibiotics

(including penicillin, which is unusual in the Enterobacteriaceae, and other β-lactam antibiotics). All strains were susceptible to fluoroquinolones, doxycycline, and gentamicin (Reger et al. 1993). Resistance to colistin and sulfonamides has been reported (Reger et al. 1993).

Antigenic structure

The H and O antigens of *E. tarda* appear to be unrelated to those of other enterobacteria (Sakazaki 1965). Two different serotyping schemes were originally described: Sakazaki (1967) recognized 17 O antigens, 11 H antigens, and 18 O–H combinations; and Ewing (1986) recognized 49 O antigens, 37 H antigens, and 148 O–H combinations. The two systems have now been combined to form a single scheme comprising 61 O groups and 45 H antigens (Tamura et al. 1988).

Other typing methods

Some *E. tarda* strains produce bacteriocins, which are active against other *E. tarda* strains and against *Yersinia*, but not against *Proteus* (Hamon et al. 1969). *E. tarda* strains are not sensitive to colicins.

Habitat and pathogenicity

Edwardsiella strains are frequently isolated from feces or other specimens from healthy cold-blooded animals and their environment, particularly fresh water. The natural reservoir appears to be the gastrointestinal tract of cold-blooded animals. *Edwardsiella* strains are pathogenic for catfish, eels, and other animals, sometimes causing economic loss. Of the three recognized species, only *E. tarda* has been demonstrated to be pathogenic for humans. Principal infections associated with this species include gastroenteritis, wound infections (Jordan and Hadley 1969) such as cellulitis or gas gangrene associated with trauma to mucosal surfaces, and systemic disease such as cholecystitis, meningitis, osteomyelitis, and septicemia. Zighelboim et al. (1992) noted only three previous cases of patients who had documented hepatic abscess due to *E. tarda*; two patients died, and the third required a laparotomy and drainage. They reported a fourth case that was successfully managed with antibiotic therapy alone. Risk factors associated with *E. tarda* infections include exposure to aquatic environments or exotic animals (e.g. amphibia or reptiles), pre-existing liver disease, conditions leading to iron overload, and dietary habits (e.g. raw fish ingestion). *E. tarda* is rarely found in the feces of healthy people. Although a higher isolation rate has been found in patients with diarrhea (Ewing et al. 1965), the role of the species in the etiology of diarrhea requires further study.

The organism does have invasive capabilities. Janda et al. (1991) investigated the pathogenic characteristics of 35 *Edwardsiella* strains from clinical and environmental sources. Most *E. tarda* strains were invasive in HEp-2 cell monolayers, produced a cell-associated hemolysin and siderophores, and bound Congo red; many strains also expressed mannose-resistant hemagglutination against guinea pig erythrocytes. *E. hoshinae* strains bound Congo red and differed in their invasive and hemolytic capabilities whereas *E. ictaluri* strains did not produce either factor; neither *E. hoshinae* nor *E. ictaluri* expressed mannose-resistant hemagglutination nor elaborated siderophores. Selected strains of each species tested for mouse lethality indicated strain variability in pathogenic potential, with *E. tarda* strains the most virulent. Their findings indicate some potentially important differences in pathogenic properties that may help explain the environmental distribution of the three species and their differing abilities to cause disease in humans. Janda and Abbott (1993) found that *E. tarda* produces a hemolysin that is at least partially regulated by the relative availability of iron and may play a role in human disease.

EWINGELLA

Definition

The genus *Ewingella* is represented by one species, *E. americana*. *Ewingella* organisms are usually motile and do not produce gas from glucose. Acid is produced from arabitol, mannitol, mannose, salicin, and trehalose. Indole is not produced. All strains grow on Simmons' citrate medium. They do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and there is no liquefaction in nutrient gelatin stab culture within 5 days but weak gelatinase production detectable by the plate test. Growth does not occur in Møller's KCN medium. Lysine and ornithine are not decarboxylated and arginine dihydrolase is not produced. The organisms are isolated predominantly from the human respiratory tract but their clinical significance is uncertain. The G + C content of the DNA is 53–55 mol% and the type species is *Ewingella americana*. This organism was described by Grimont et al. (1983) as strains resembling *Cedecea*.

Cultural characteristics and biochemical activities

E. americana grows well on nutrient agar, producing convex colonies about 1.5 mm in diameter.

Biochemical activities are given in Enterobacteriaceae: general characteristics, Table 1. In addition to the properties mentioned in the definition, most strains produce β-galactosidase and give a positive result

in the VP and methyl red tests; they do not ferment adonitol, arabinose, dulcitol, inositol, raffinose, sorbitol, or sucrose. Although thought to resemble *Cedecea*, several characters distinguish *E. americana* from this genus. For example, *E. americana* has negative reactions and *Cedecea* positive reactions for growth in KCN, lipase production, gas production with glucose, and fermentation of cellobiose, maltose, and xylose. More difficult, and not discussed by Grimont et al. (1983), is the differentiation of *E. americana* from *P. agglomerans*. Typically, *E. americana* does not ferment malonate, sucrose, or xylose but strains atypical in these characters may not be easily assigned to one or other species.

Susceptibility to antimicrobial agents

E. americana strains are susceptible to carbenicillin, chloramphenicol, colistin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfadiazine, tetracycline, and tobramycin; they are resistant to cephalothin and penicillin G.

Habitat and pathogenicity

Although rare in occurrence, the organism is most commonly associated with the human respiratory tract. Whereas the pathogenicity of the species remains to be determined, *E. americana* has been reported from polymicrobial bacteremia (Pien et al. 1983), from two blood cultures from a 75-year-old man after cholecystectomy (Devreese et al. 1992), and from wound colonization (Bear et al. 1986). The organism was isolated separately from both eyes of a woman aged 30 years (Heizmann and Michel 1991). Her clinical signs and symptoms included adhesive eyelids, itching, and edematous upper and lower lids. Relief of symptoms was effected with amoxycillin–clavulanate therapy. Plasmid profiling was used to investigate an episode of pseudobacteremia due to the contamination of blood coagulation tubes with *E. americana* (McNeil et al. 1987a, b).

HAFNIA

Definition

Hafnia organisms are usually motile and usually produce gas from glucose. Acid is formed from arabinose, glycerol, maltose, mannitol, mannose, rhamnose, trehalose, and xylose. Indole is not produced. Most strains utilize citrate (if tested at 30°C) but do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gelatin is not liquefied. Most strains grow in Møller's KCN medium. Arginine dihydrolase is not produced but both lysine and ornithine are decarboxylated. The organisms occur in the feces of humans and other animals and are also widespread in the natural environment. The G + C content of the DNA is 52–57 mol% and the type species is *Hafnia alvei*.

Classification

This organism has at times been placed in the genus *Enterobacter* under the names '*E. alvei*' and '*E. hafniae*.' However, DNA–DNA hybridization studies reveal that the organism deserves separate generic status (Brenner 1978). Three DNA-relatedness groups have been recognized in *H. alvei* and these may be elevated to separate species at some future date.

Morphological and cultural characteristics

Strains grow well on ordinary media, producing grayish convex colonies 1–2 mm in diameter on nutrient agar at 37°C after incubation for 24 h.

Biochemical activities

H. alvei ferments a much narrower range of sugars than do members of the genus *Enterobacter*: it acidifies salicin and sucrose late, or not at all, and lactose and sorbitol only rarely, and does not attack adonitol, dulcitol, or inositol. It is not encapsulated and does not liquefy gelatin. Møller (1954) greatly advanced the knowledge of this species when he showed that, although strains were able to grow at 37°C, many of their biochemical activities at this temperature were irregular. The effect of temperature on motility was also critical: many were nonmotile at 37°C, but nearly all were motile at 30°C. At 22°C they all gave a positive VP reaction, grew on Simmons' citrate medium, and formed gas in sugars, though any one or all of these reactions might be inhibited at 37°C.

Antigenic structure

Forty-nine serotypes were recognized by Sakazaki (1961) and the total was extended to 197 by Matsumoto (1963, 1964).

Susceptibility to antimicrobial agents

Isolates that display resistance to conventional antibiotics, including cephalosporins and penicillins, have been reported (Klapholz et al. 1994).

Habitat and pathogenicity

Hafnia organisms are isolated from the feces of humans and other animals; they are also found in sewage, soil, water, and dairy products. They were rarely considered

pathogenic, but Albert et al. (1992) described seven strains from diarrheal stools of children that resembled enteropathogenic *Escherichia coli* (EPEC). These strains produced attaching and effacing (AE) lesions in rabbit ileal loops and fluorescent actin staining in infected HEp-2 cells. In addition, a DNA probe, from a chromosomal gene required by EPEC to produce AE lesions, hybridized to chromosomal DNA from all seven *H. alvei* strains. These findings indicated a sharing of virulence-associated properties at the phenotypic and genetic levels by *H. alvei* and EPEC. *H. alvei* strains with these properties were considered diarrheagenic. Ridell et al. (1994) found an epidemiological association of *H. alvei* with diarrhea, because the organism was isolated from 12 (16 percent) of 77 adult Finnish tourists to Morocco who developed diarrhea and from none of 321 tourists without diarrhea ($p < 0.001$). The strains were negative for the AE lesion by an in vitro fluorescent actin staining test and also did not show homology to the *E. coli* AE gene (*eaeA*) by PCR. The results suggested that a mechanism or mechanisms other than the AE lesion may also be involved in the association of *H. alvei* with diarrhea. It has also been noted that *eaeA*-positive strains have characteristic biochemical properties, including the ability to assimilate 3-hydroxybenzoate and the inability to assimilate histidine and 2-ketogluconate (Ridell et al. 1995). Ginsberg and Goldsmith (1988) reported a neonate with necrotizing enterocolitis and subsequent ileal perforation from whom the organism was isolated from both blood and stool. *H. alvei* may also be a potential pathogen in patients with chronic underlying illness (Klapholz et al. 1994).

KLUYVERA

Definition

Kluyvera organisms are motile and usually form gas from glucose. Acid is produced from arabinose, cellobiose, glycerol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sucrose, trehalose, and xylose. Indole is produced by most strains. Most strains grow on Simmons' citrate medium. They do not hydrolyze urea or form H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gelatin is not liquefied. Most strains grow in Møller's KCN medium. Arginine dihydrolase is not produced; most strains decarboxylate lysine and all decarboxylate ornithine. They occur in human clinical specimens and the natural environment. The G + C content of the DNA is 55–57 mol% and the type species is *Kluyvera ascorbata*.

Classification

The name *Kluyvera* was originally proposed in 1956 by Japanese workers; they recognized two species: '*Kluyvera citrophila*,' in which citrate was utilized, and '*Kluyvera noncitrophila*,' in which it was not. These names were little used in succeeding years and do not appear in the Approved Lists of Bacterial Names (Skerman et al. 1980). In 1981, Farmer and coworkers reproposed the name *Kluyvera*; their studies of DNA–DNA hybridization led them to recognize three genomic groups, two of which they named *K. ascorbata* and *Kluyvera cryocrescens*. The third genomic group was subsequently named *Kluyvera georgiana* (Müller et al. 1996).

Cultural characteristics and biochemical activities

Strains grow well on ordinary media and form colonies 1–2 mm in diameter on nutrient agar after 24 h at 37°C.

The characteristic biochemical reactions of the genus are given in Enterobacteriaceae: general characteristics, Table 1). Most workers will attempt to identify strains only to the genus level. In addition to the reactions mentioned previously, neither adonitol nor *i*-(*myo*)inositol is acidified, the MR test is positive, and the VP test negative. Most strains ferment malonate.

Susceptibility to antimicrobial agents

Kluyvera strains are generally susceptible to antimicrobial agents (see Farmer et al. 1981). Strains of *K. cryocrescens* often display large zones of inhibition around disks containing carbenicillin and cephalothin whereas strains of *K. ascorbata* do not.

Habitat and pathogenicity

Strains of Kluyvera have been isolated from milk, sewage, soil, and water, but most of those so far identified have come from human sources, especially sputum and, less commonly, urine; a number were from feces (some from patients with severe diarrhea) (Fainstein et al. 1982, Aevaliotis et al. 1985) and a few from blood cultures (one associated with a catheter-related infection) (Wong 1987). Thaller et al. (1988) recorded the isolation of a *K. cryocrescens* strain in pure culture from a gall-bladder pus specimen from a 76-year-old woman with acute cholecystitis. Yogev and Kozlowski (1990) treated a child who developed peritonitis due to *K. ascorbata*. The repeated isolation of the organism in pure culture from the peritoneal fluid and its isolation from postmortem subdiaphragmatic microabscesses suggested it was clinically significant. There is, however, as yet, little definite evidence that the organisms are of clinical significance in humans. Luttrell et al. (1988) described a soft tissue infection caused by *Kluyvera* spp. in a previously healthy woman. Successful treatment required incision and drainage of

the wound in addition to the administration of antibiotics. Sierra-Madero et al. (1990) described a case of bacteremia and mediastinitis in a man following open-heart surgery. Dollberg et al. (1990) described a *Kluyvera* sp. recovered from the urine of a previously healthy 5-year-old child with clinical and laboratory evidence of acute pyelonephritis. Their findings suggest that this organism can cause severe disease even in previously healthy, immunocompetent children.

LECLERCIA

Definition

Leclercia organisms are usually motile and usually produce gas from glucose. They often form a yellow pigment. Acid is produced from adonitol, arabinose, cellobiose, dulcitol, lactose, maltose, mannitol, rhamnose, salicin, trehalose, and xylose. Indole is formed. They do not grow on Simmons' citrate medium, seldom hydrolyze urea, and do not produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and there is no liquefaction in nutrient gelatin stab culture within 5 days, but weak gelatinase production is detectable by the plate test. Strains grow in Møller's KCN medium but show no decarboxylase activity. They are isolated from various human clinical specimens, food, water, and the environment; their clinical significance remains uncertain. The G + C content of the DNA is 52–55 mol% and the type species is *Leclercia adecarboxylata*.

Classification

This organism was originally described as *Escherichia adecarboxylata* by Leclerc (1962) from a collection of strains isolated from water. Strains of the species are, however, easily distinguished from *E. coli* as they acidify adonitol, ferment malonate, and are KCN tolerant, as well as failing to form lysine decarboxylase or produce alkali when grown on Christensen's citrate medium. Ewing and Fife (1972) included *E. adecarboxylata* in *Enterobacter* (now *Pantoea*) *agglomerans* (syn. *Erwinia herbicola*), but the former yields gas from glucose, produces indole, and acidifies dulcitol and inositol, all characters that distinguish it from typical strains of *P. agglomerans*. More recent DNA–DNA hybridization studies show that *E. adecarboxylata* should be placed in a separate genus for which the name *Leclercia* was assigned (Tamura et al. 1986).

Cultural characteristics and biochemical activities

Strains grow well on ordinary media. The biochemical characteristics of the organism are given in Enterobacteriaceae: general characteristics, Table 1).

In addition to the characters mentioned above, strains fail to produce acid from glycerol, inositol, and sorbitol. The MR reaction is positive and VP reaction negative. Malonate is fermented and β-galactosidase produced.

Susceptibility to antimicrobial agents

All strains are susceptible to ampicillin, carbenicillin, cephaloridine, colistin, gentamicin, kanamycin, and nalidixic acid, but resistant to penicillin. Most strains are also susceptible to chloramphenicol, streptomycin, sulfadiazine, and tetracycline.

Habitat and pathogenicity

Isolations have been made from various human clinical specimens including blood, feces, sputum, urine, and wounds. There are no definite indications of pathogenicity. Strains also occur in the environment, food, and water.

LEMINORELLA

Definition

Leminorella organisms are nonmotile, and they may or may not produce gas from glucose. Acid is produced from arabinose and xylose and generally also from glycerol. Indole is not formed. Strains may or may not grow on Simmons' citrate medium. They do not hydrolyze urea but produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gelatin is not liquefied. Growth does not occur in Møller's KCN medium. They do not show any decarboxylase activity. They are isolated predominantly from human fecal specimens but their clinical significance remains to be determined. The G + C content of the DNA has not been determined and the type species is *Leminorella grimontii*.

Classification

This genus, with its two species *L. grimontii* and *L. richardii*, was described by Hickman-Brenner et al. (1985). DNA–DNA hybridization studies indicated the existence of a third species but it was represented by only a single strain so no name was proposed for it.

Cultural characteristics and biochemical activities

In general, colonies are not as large as those of other species of Enterobacteriaceae, but growth occurs on MacConkey agar. *Leminorella* strains are unique amongst the Enterobacteriaceae in that although they produce H_2S and ferment arabinose and xylose, they are otherwise

relatively inactive. The biochemical characteristics of the genus and tests useful in differentiating the two named species are shown in Enterobacteriaceae: general characteristics, Table 1. If strains grow only weakly, they may fail to give appropriate results in these tests making species assignment difficult or incorrect. Strains of *L. richardii* may fail to produce acid from glucose even though they acidify arabinose, glycerol, and xylose. Some strains of *L. grimontii* are anaerogenic. Strains fail to produce acid from adonitol, cellobiose, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose, and trehalose. The MR reaction is positive in most strains of *L. grimontii* and negative in strains of *L. richardii*; the VP reaction is negative in both species. Malonate is not fermented and β-galactosidase is not produced.

Susceptibility to antimicrobial agents

Leminorella strains are susceptible to colistin, but resistant to ampicillin, carbenicillin, cephalothin, and penicillin.

Habitat and pathogenicity

Two strains of *L. grimontii* were isolated from urine and all other *Leminorella* strains have come from fecal specimens. Their clinical significance remains to be determined.

MOELLERELLA

Definition

Moellerella organisms are nonmotile and do not produce gas from glucose. Acid is produced from adonitol, glycerol, lactose, mannitol, raffinose, and sucrose. Indole is not formed. Strains may or may not grow on Simmons' citrate medium. They do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gelatin is not liquefied. Strains may or may not grow in Møller's KCN medium and they show no decarboxylase activity. They are isolated predominantly from human feces but their clinical significance remains to be determined. The G + C content of the DNA has not been determined and the type species is *Moellerella wisconsensis*. This genus and its single species were described by Hickman-Brenner et al. (1984).

Cultural characteristics and biochemical activities

Strains grow well on ordinary media. On MacConkey agar, colonies are bright red with precipitated bile around them, thus resembling *E. coli* colonies. The characteristic biochemical reactions of *M. wisconsensis* are given in Enterobacteriaceae: general characteristics, Table 1. In the original description of the species, strains were reported to grow on Simmons' citrate medium and not to ferment arabinose; strains do not form acid from dulcitol, inositol, maltose, rhamnose, salicin, sorbitol, trehalose, or xylose. The MR reaction is positive and the VP reaction negative. Malonate is not fermented and β-galactosidase is produced.

Susceptibility to antimicrobial agents

Strains are resistant to colistin but susceptible to cephalothin, chloramphenicol, gentamicin, and nalidixic acid.

Habitat and pathogenicity

The species is predominantly isolated from human feces and, although in some cases the patient was reported to have had diarrhea, there is no evidence at present that *M. wisconsensis* is the cause. The organism has also been reported from a bronchial aspirate.

RAHNELLA

Definition

Rahnella organisms are motile and produce gas from glucose. Acid is produced from arabinose, dulcitol, lactose, raffinose, rhamnose, and sorbitol. Indole is not formed. Strains grow on Simmons' citrate medium. They do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated but gelatin is slowly liquefied. Strains do not grow in Møller's KCN medium and show no activity in lysine or ornithine decarboxylase media, but some strains produce a dihydrolase for arginine. The organisms are isolated predominantly from water and nonpolluted soil, but their clinical significance remains to be determined. The G + C content of the DNA is 53 mol% and the type species is *Rahnella aquatilis*. This genus and its single species were described by Izard et al. (1979).

Cultural characteristics and biochemical activities

Strains grow well on ordinary media at 30 and 37°C; they also grow at 4°C, but not at 41°C. The biochemical characteristics of the organism are given in Enterobacteriaceae: general characteristics, Table 1).
In addition to the characteristics mentioned above, strains acidify glycerol, maltose, mannitol, salicin, sucrose, trehalose, and xylose but fail to produce acid from adonitol and inositol. The MR reaction is negative and the VP reaction is positive. Malonate is fermented and β-galactosidase is produced.

Susceptibility to antimicrobial agents

Funke and Rosner (1995), who isolated the organism from blood cultures of a human immunodeficiency virus (HIV)-infected intravenous drug abuser, obtained a favorable outcome after a 14-day course of ciprofloxacin.

Habitat and pathogenicity

Goubau et al. (1988) described a case of septicemia in a patient with acute lymphoblastic leukemia. Harrell et al. (1989) reported the isolation of *R. aquatilis* from the bronchial washing of a patient with acquired immunodeficiency syndrome (AIDS). Alballaa et al. (1992) reported a case of urinary tract infection in a renal transplant patient. Hoppe et al. (1993) described repeated isolations of the organism from the blood of a pediatric bone marrow transplant recipient, probably related to inappropriate handling of a Hickman catheter. A case of a surgical wound infection in a patient who underwent a prosthetic surgical intervention was reported by Maraki et al. (1994). Oh and Tay (1995) reported isolation of *R. aquatilis* from the blood of a diabetic patient and a patient with laryngeal carcinoma; both patients recovered from their infection after treatment with parenteral antibiotics including gentamicin.

TATUMELLA

Definition

Tatumella organisms differ from other members of the Enterobacteriaceae in that, when grown at 25°C, over 50 percent of strains are motile by means of polar, subpolar, or lateral flagella (versus peritrichous flagella). Gas is not formed from glucose; acid is produced from glycerol, salicin, sucrose, and trehalose. Indole is not formed. Most strains grow on Simmons' citrate medium at 25°C. The organisms do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is deaminated and gelatin is not liquefied. Growth does not occur in Møller's KCN medium. Lysine and ornithine are not decarboxylated, but some strains produce a dihydrolase for arginine. The organisms are isolated predominantly from the human respiratory tract but their clinical significance is as yet uncertain. The G + C content of the DNA is 53–54 mol% and the type species is *Tatumella ptyseos*.

Classification

This genus and its single species were described by Hollis and coworkers (1981). Studies of DNA–DNA hybridization show that, despite the absence of peritrichous flagellation, the species is correctly placed in the Enterobacteriaceae. Its members also differ from others in this family in growing less well and in surviving less readily on laboratory media.

Cultural characteristics and biochemical activities

Colonies are 0.5–1.0 mm in diameter, low convex with entire edges, semitranslucent, smooth, and glossy after incubation for 24 h on blood agar. In general, colonies are not as large as those of other species of Enterobacteriaceae, but growth occurs on MacConkey agar. The characteristic biochemical reactions of the species are given in Enterobacteriaceae: general characteristics, Table 1). In addition to the properties mentioned above, strains fail to form acid from adonitol, L-arabinose, dulcitol, *i*-inositol, lactose, maltose, D-mannitol, raffinose, L-rhamnose, D-sorbitol, and starch; the MR and VP reactions are negative, and malonate is not fermented. A few strains form arginine dihydrolase, particularly at 25°C.

Susceptibility to antimicrobial agents

Unlike most of the other members of the Enterobacteriaceae, strains of *Tatumella* are susceptible to cephalosporins and penicillins, and to aminoglycosides, chloramphenicol, and tetracycline.

Habitat and pathogenicity

The organism is commonly associated with the human respiratory tract. Most isolates have been recovered in sputum, but *Tatumella* has also been found in feces and urine, and in blood cultures. Its pathogenicity is at present uncertain.

YOKENELLA

Definition

Yokenella organisms are usually motile and usually produce gas from glucose. Acid is formed from arabinose, cellobiose, maltose, mannitol, rhamnose, trehalose, and xylose. Indole is not produced. Most strains utilize citrate, but do not hydrolyze urea or produce H_2S detectable in triple sugar iron agar. Phenylalanine is not deaminated and gelatin is not liquefied. Generally, they grow in Møller's KCN medium. Lysine and ornithine are decarboxylated and most strains produce arginine dihydrolase. They are isolated predominantly from human wounds but their clinical significance is as yet uncertain. The G + C content of the DNA is 58–60 mol% and the type species, *Yokenella regensburgei*, was described by Kosako et al. (1984).

Cultural characteristics and biochemical activities

Strains of *Y. regensburgei* grow well on ordinary media. The biochemical characteristics of *Y. regensburgei* are given in Enterobacteriaceae: general characteristics, Table 1). Additional characteristics not given in the definition of the genus include failure to form acid from adonitol, dulcitol, lactose, sorbitol, and sucrose; β-galactosidase is produced. *Y. regensburgei* differs from the closely related species, *H. alvei*, in its failure to ferment glycerol, ability to produce acid from melibiose, resistance to both colistin and the *Hafnia*-specific bacteriophage, and having a very weak catalase reaction.

Susceptibility to antimicrobial agents

Strains of *Y. regensburgei* are resistant to colistin and penicillin and susceptible to chloramphenicol, gentamicin, kanamycin, nalidixic acid, and sulfadiazine.

Habitat and pathogenicity

Y. regensburgei is infrequently encountered but a number of isolations have been made from human wounds. The organism has been isolated from a 74-year-old man with a septic knee and from a 35-year-old immunocompromised woman who had a transient bacteremia without overt signs of sepsis (Abbott and Janda 1994).

OTHER GENERA

The following genera have been proposed, but will not be discussed in detail: *Erwinia* from plants; *Budvicia* (Bouvet et al. 1985) and *Pragia* (Aldová et al. 1988; Schindler et al. 1992) from water; *Buttiauxella* (Ferragut et al. 1981; Müller et al. 1996) from slugs, snails, and water; *Obesumbacterium* (Shimwell 1963; Priest et al. 1973) from breweries; and *Xenorhabdus* (see Thomas and Poinar 1979) associated with certain nematodes and with insect larvae parasitized by them. The former *Xenorhabdus luminescens* now resides in a separate genus *Photorhabdus*; it has been reported in clinical material (Farmer et al. 1989), but it is rare. *Trabulsiella guamensis* and enteric groups 58, 59, 60, 68, and 69 are also not discussed.

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Vibrio

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INTRODUCTION

The taxonomy of the Vibrionaceae is controversial and rapidly evolving. The family was originally proposed by Veron in 1965. The last edition of *Bergey's Manual for systematic bacteriology* included four genera in the family: *Vibrio*, *Aeromonas*, *Photobacterium*, and *Plesiomonas* (Baumann et al. 1984). *Aeromonas* has now been given its own family, the Aeromonadaceae. *Plesiomonas* remains controversial, but is more closely related to enterics, particularly *Proteus*, than to *Vibrio*. This leaves *Vibrio* and *Photobacterium* as the only remaining genera in Vibrionaceae that appear on the Approved Lists of Bacterial Names (www.bacterio.cict.fr). Several other genera have been proposed for addition to Vibrionaceae: *Allomonas*, *Listonella*, *Enhydrobacter*, *Salinivibrio*, and *Enterovibrio* (www.the-icsp.org/taxa/vibrio). These genera, as proposed, would not include the recognized human pathogens or the most well-known environmental strains. The exception is that it has been proposed that *V. anguillarum*, an important fish pathogen, be moved to *Listonella anguillarum*. The human pathogenic member of *Photobacterium*, *Photobacterium damselae*, was formerly *V. damsela* and will be discussed in this chapter.

There are currently more than 40 proposed species of *Vibrio* (www.the-icsp.org/taxa/vibrio), of which only about a dozen are human pathogens (Holmberg 1992; Tison 1999). A number are also important pathogens of aquatic animals (Baumann et al. 1984). The most famous member of the genus is *Vibrio cholerae*, the causative agent of cholera. It is not clear when cholera first arose; descriptions of disease suggestive of cholera appear in Sanskrit writings more than 1 000 years ago (Longmate 1966; Politzer 1959). Cholera is one of the classic plagues and in the nineteenth century was almost unequaled in its ability to engender fear and panic. Seven cholera pandemics have been recorded since 1817 (Barua 1992; Kaper et al. 1995). Cholera was one of the first diseases recognized, by John Snow, to be waterborne (Holmberg 1992; Longmate 1966). With the advent of modern water purification and sewage treatment, cholera has become a rarity in the developed world. However, it continues to be a significant cause of morbidity and mortality worldwide, particularly in developing countries with poor sanitation. The current or seventh pandemic initially appeared in the Celebes islands in Indonesia in 1961 and has since spread through Asia, the Middle East, Africa, parts of Europe, and Latin America (Barua 1992; Politzer 1959). The appearance of cholera in Latin America, in the 1990s, was the first time cholerae had spread to the Americas in over a century. Between 1991 and 1993, there were more than 820 000 cases and 7 000 deaths in South America (Tauxe et al. 1994). Also within the past decade, a new serogroup, O139, has emerged as an

agent of epidemic cholera. From October 1992, *V. cholerae* O139 Bengal was recovered from cholera patients in India and Bangladesh and this new serogroup spread rapidly through Asia. It has been proposed that this may be the beginning of the eighth pandemic, but only time will reveal whether this strain will spread globally (Kaper et al. 1995; Morris et al. 1994; Chiang and Mekalanos 1999).

Cholera has become rare in the developed world, but an appreciation of the importance of other *Vibrio* spp. as human and animal pathogens is growing. These noncholera vibrios cause serious wound infections wherever people are exposed to warm seawater. People with underlying liver or immune disorders can develop highly fatal septicemia following consumption of raw or undercooked seafood (Strom and Paranjpye 2000; www.cdc.gov/ncidod/dbmd/diseaseinfo/vibriovulnificus_g.htm). *V. vulnificus* is the species most commonly associated with septicemia and is responsible for >90 percent of the deaths due to vibrios in the USA (www.cdc.gov/ncidod/dbmd/diseaseinfo/files/VibCSTE99web.pdf; www.cdc.gov/ncidod/dbmd/diseaseinfo/files/CSTE_Vibrio_2000.pdf). Noncholera vibrios also cause sporadic gastroenteritis associated with the consumption of seafood. Of these, *V. parahaemolyticus* is the most frequently isolated. It is one of the most important causes of gastroenteritis in Asia and is the *Vibrio* species most frequently isolated from clinical samples in the USA (Morris and Nair 2001; Tison 1999). A pandemic due to *V. parahaemolyticus* O3/K6 has been documented (Arakawa et al. 1999; Chiou et al. 2000; Chowdhury et al. 2000).

DEFINITION

Genus

Vibrios are gram-negative, asporogenous, straight, curved, or comma-shaped rods. They are motile by means of sheathed monotrichous or multitrichous polar flagella (McCarter 2001). Vibrios are halophilic and predominately oxidase-positive (Baumann et al. 1984). Sodium chloride stimulates the growth of all vibrios and is required by most. The minimal concentration for optimum growth varies from 0.029 to 4.1 percent NaCl. Vibrios are facultatively anaerobic. They can ferment many sugars with mixed acid production. Respiration uses oxygen as the electron acceptor. Vibrios are ubiquitous in aquatic environments. They are found in the water column, in sediments, and associated with plants, invertebrates, and vertebrate animals within those ecosystems. The interaction of vibrios with animals may be commensal, mutualistic, or pathogenic. The guanine and cytosine (G+C) content of DNA is 38–51 mol%. The type species is *V. cholerae*.

Species

Many new species have been proposed; some are defined only by DNA–DNA hybridization studies or rDNA sequences. Currently there are 48 proposed *Vibrio* species listed by the Vibrio Working Group of the International Committee on Systematics of Prokaryotes (ICSP) (www.the-icsp.org/taxa/vibrio). Twelve species are considered human pathogens: *V. alginolyticus*, *V. cholerae*, *V. cincinnatiensis*, *V. damsela* (*P. damselae*), *V. fluvialis*, *V. furnissii*, *V. harveyi* (synonym *V. carchariae*), *V. hollisae*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus* (Morris and Nair 2001; Tison 1999). These species are the most frequently isolated by clinical laboratories. However, the importance of some of these species as pathogens remains unclear.

HABITAT

Vibrios are ubiquitous aquatic organisms whose range is determined by salinity, temperature, and nutrient availability (Heidelberg et al. 2002). In temperate climates, vibrios peak in the water column during the summer months and may be very abundant. The seasonal distribution of human infections generally correlates with this increase. When water temperature drops, so does the concentration of vibrios and they may become limited to sediments (Barbieri et al. 1999; Baumann et al. 1984; DePaola et al. 2003; Morris and Nair 2001; Motes et al. 1998). Vibrios are normal inhabitants of marine, estuarine, and, to a more limited extent, fresh-water environments (Pinhassi and Berman 2003; Strom and Paranjpye 2000). Vibrios that require lower concentrations of salt, such as *V. cholerae* and *V. mimicus*, can be found in fresh-water lakes and rivers as well as estuarine and marine environments. Although fecal contamination of drinking water is important in the epidemiology of cholera, it should be stressed that *V. cholerae* is a normal inhabitant of even pristine aquatic habitats (Colwell and Huq 1994; Holmberg 1992). Infection of humans does act as an amplification cycle that is important in the epidemic spread of *V. cholerae* (Morris and Nair 2001; Merrell et al. 2002). In developing countries, shedding of *V. cholerae* in diarrheal stools contaminates local water supplies, resulting in higher counts of *V. cholerae* in the water and increased rates of human infection. However, increased concentrations in rivers may predate and predict human outbreaks (Franco et al. 1997). Halophilic vibrios are abundant in estuarine and marine environments. They can form biofilms and may be intimately associated with plants, fish, and zooplankton, shellfish, and other invertebrates (Castro et al. 2002; Chiavelli et al. 2001; Heidelberg et al. 2002; Ostling et al. 1993; Pedersen et al. 1997a; Watnick and Kolter

1999). Their relationship with higher organisms can range from mutualistic to pathogenic. Strains of *V. fischeri* are symbionts of sepiolid squid, colonizing their light organs and supplying bioluminescence (Nishiguchi 2002). *V. parahaemolyticus* and *V. vulnificus* are concentrated by filter feeders such as oysters (DePaola et al. 1998; Morris and Nair 2001; Strom and Paranjpye 2000; Wright et al. 1996). *V. anguillarum*, *P. damselae*, and *V. vulnificus* are important pathogens of fish (Biosca et al. 1996; Love et al. 1981; Pedersen et al. 1997a).

Vibrios can exist in the environment in several forms. The most easily studied is planktonic, when they occur in the water column as free-living individual cells. This occurs most frequently when nutrients are plentiful and water temperatures are warm. When nutrients are limited, the cells become less metabolically active, lose their flagella, and take on a small round morphology called a spherical ultramicrocell (Oliver 1993; Ostling et al. 1993). Cytoplasmic granules disappear and the nuclear region becomes condensed (Hood et al. 1986). During cold periods, vibrios may be found primarily in sediments or attached to surfaces. Attachment to specific substrates such as shellfish and copepods may require the production of specific enzymes or lectins (Chiavelli et al. 2001; Pruzzo et al. 1996). Cells may also respond to starvation and/or cold by entering a viable but nonculturable (VBNC) state. It is not clear whether ultramicrocells and VBNC forms are related. In the VBNC state, the cells are physiologically active but will not grow under routine laboratory conditions (Colwell and Huq 1994; Xu et al. 1982). This state is accompanied by ultrastructural changes including a reduction in cell size and changes in the outer membrane (Kondo et al. 1994; Oliver 1993; Strom and Paranjpye 2000). *V. cholerae* can also excrete an exopolysaccharide that leads to formation of clumps in liquid culture and a wrinkled or rugose colony morphology on laboratory media (White 1938). These rugose forms show increased resistance to chlorine and other biocides (Rice et al. 1993). This ability of vibrios to respond to changes in their environment may be what allows them to persist in so many environmental niches.

MORPHOLOGY

Microscopic

Vibrios are straight to curved or comma-shaped and 0.5–0.8 μm wide (Baumann et al. 1984). Rapidly growing cultures often show straight forms that are indistinguishable from other gram-negative rods. Curved and comma shapes are most common in the early stationary phase. Old or stressed cultures may form coccoid shapes, long filaments, and S forms. In liquid media, vibrios show vigorous darting motility.

Colony morphology

Vibrios can produce a large variety of colony morphologies on nonselective media. Most produce smooth, convex, creamy colonies. Acapsular isolates tend to produce translucent colonies, while encapsulated strains are more opaque and may even be somewhat muccoid for heavily encapsulated *V. vulnificus* strains. Capsule production can be reversibly switched off resulting in a mix of translucent and opaque colonies, and single colonies may produce translucent and opaque sectors (Johnson et al. 1992; Simpson et al. 1987; Wright et al. 1990). Rough mutants, which have truncated lipopolysaccharides (LPS), produce a more granular appearance and cannot be serotyped. Vibrios can also switch between the common smooth morphology and a wrinkled, or rugose, colony morphology that is adherent to the agar and difficult to emulsify (White 1938). In *V. cholerae*, this phenotype is associated with the production of an exopolysaccharide and increased resistance to chlorine, ultraviolet (UV) light, and other environmental stresses (Rice et al. 1993). *V. parahaemolyticus*, *V. harveyi*, and *V. alginolyticus* may swarm on solid media due to the production of lateral flagella (McCarter 2001). Swarming occurs most frequently at room temperature.

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

Culture

Samples for vibrios should be processed expeditiously and never refrigerated. Fecal specimens should be placed in Cary–Blair transport media. Enrichment broths such as alkaline peptone water, incubated for 6–8 h, can be useful for increasing recovery of *V. cholerae* from formed stools, food samples, and water samples. A second enrichment step is often used for food and water samples. Vibrios grow rapidly on most nonselective media containing 0.5–1.0 percent NaCl. Most vibrios will not grow in nutrient agar without added salt and the range of salt concentrations that support growth is an important test for identifying the many species of vibrios (Tison 1999; Kaper et al. 1995).

Several selective media have been designed to isolate *V. cholerae* but their sensitivity and specificity for other species of vibrios is not clear. The most commonly used selective media is thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Some species, such as *P. damsela*, *V. hollisae*, and *V. cincinnatiensis*, do not grow or grow poorly on TCBS. There is some loss of plating efficiency even for *V. cholerae* on TCBS and this varies from manufacturer to manufacturer and from lot to lot (Franco et al. 1997; West et al. 1982; Tison 1999). In addition to selecting for vibrios, TCBS differentiates

strains by their ability to ferment sucrose. *V. cholerae*, *V. alginolyticus*, and *V. fluvialis* ferment sucrose, producing yellow colonies. Green colonies are nonsucrose-fermenting species, such as *V. vulnificus*, *V. mimicus*, *V. parahaemolyticus*, *P. damselae*, *V. hollisae*, and, *V. metschnikovii*. Other selective media include taurocholate tellurite gelatin agar (TTGA) (or Monsur's agar) (Kay et al. 1994), polymyxin-mannose-tellurite agar (Shimada et al. 1990), and thiosulfate-chloride-iodide agar (Pfeffer and Oliver 2003). Vibrios will also grow well on blood agar and usually as clear lactose-negative colonies on MacConkey agar after overnight incubation. Blood agar plates from stool cultures should be examined for oxidase positive colonies to increase recovery of vibrios and *Aeromonas* spp. (Tison 1999)

Blood agar is particularly useful for isolation, recognition, and identification of vibrios as it allows one to test individual colonies for oxidase production. *V. cholerae* and *V. vulnificus* tend to form smooth, creamy, buff-colored colonies. Others, such as *V. metschnikovii*, and *V. alginolyticus*, appear as rather flat grayish glistening colonies and *V. alginolyticus* usually swarms. *V. mimicus*, *V. hollisae*, *V. fluvialis*, *P. damsela*, *V. metschnikovii*, and some *V. cholerae* are β-hemolytic on T-soy agar with five percent sheep blood after overnight incubation at 35–37°C. Classical biotype *V. cholerae* O1 are nonhemolytic, but most strains of eltor biotype, serogroup O139, and non-O1, non-O139 *V. cholerae* are β-hemolytic. *V. metschnikovii* produces a double zone of hemolysis. Alpha-hemolytic or nonhemolytic species include *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus*. Although not β-hemolytic on sheep-blood agar, *V. parahaemolyticus* is β-hemolytic on Wagatsuma agar, which contains human blood. This is known as the Kanagawa phenomenon and is associated with virulence (Tison 1999; Joseph et al. 1982).

Growth requirements

All vibrios are halophilic, and most will not grow in media lacking sodium chloride. The range of salt that supports growth is a useful characteristic for species identification. *V. cholerae* and *V. mimicus* have the lowest requirement for salt and will grow on media without added salt (e.g. nutrient agar). Marine species such as *V. alginolyticus* and *V. aerogenes* have the highest salt requirements (Baumann et al. 1984). Because most common laboratory media contain only 0.5 percent salt, it is important to add salt to one percent to decarboxylase broths, sugar fermentation broths, and other biochemicals when identifying vibrios.

Vibrios generally grow well between 14 and 40°C. *V. metschnikovii* and *V. anguillarum* will grow slowly at 4°C, but most strains rapidly become nonviable or nonculturable below 10°C. Vibrios also tolerate a range of pH from 6.5 to 9.0. The gastric acid barrier is an important protection against vibrio infection and patients with reduced stomach acidity are at increased risk for infection. Food can act as a buffer, reducing the infectious dose required to produce cholera (Kaper et al. 1995).

METABOLISM

D-Glucose is fermented with the production of acid but rarely gas, except in *V. furnissii*, *V. aerogenes*, and, rarely, *P. damselae* (Baumann et al. 1984). The main products of fermentation are formic, acetic, lactic, succinic, and pyruvic acids and ethanol. Some species produce acetoin and acetyl methyl carbanol (Voges Proskauer positive). Vibrios are highly degradative, excreting many enzymes, including cytolysins, proteases, elastases, amylase, gelatinases, chitinases, nucleases, lipases, sulfatases, and lecithinases, into the substrate (Baumann et al. 1984; Ostling et al. 1993; Pedersen et al. 1997a; Janda et al. 1999). Most species are oxidase-positive and reduce nitrate to nitrite. All are chemo-organotrophs; most can grow in minimal medium with D-glucose and NH+ as sole carbon and nitrogen sources, respectively.

CELLULAR ORGANIZATION

Flagella

Flagella allow the bacteria to move through liquids and respond to food sources through chemotaxis and are involved in biofilm formation. Most vibrios, when grown in liquid media, produce a single polar flagellum with a sheath that is an extension of the outer membrane (McCarter 2001). Vibrios with monotrichous flagella include *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae*, *V. harveyi*, *V. vulnificus*, and *L. anguillarum*. Some species (*V. harveyi*, *V. fischeri*, *V. logei*, *V. salmonicida*) produce tufts of 3 to 12 sheathed polar flagella (Baumann et al. 1984). In *V. parahaemolyticus*, expression of polar flagella requires about 60 genes that are mostly contained within two regions. Flagella are required for full virulence of *V. cholerae* and *V. parahaemolyticus* (Kaper et al. 1995; McCarter 2001).

On solid or highly viscous media, some vibrios produce elongated swarmer cells that produce both polar and peritrichous flagella. The large numbers of peritrichous flagella are not sheathed, have a different wavelength than the polar flagella, and allow the organism to swarm across the surface of the agar. *V. parahaemolyticus*, *V. alginolyticus*, *V. diabolicus*, and *V. pectenicida* show this swarming behavior (Baumann et al. 1984; McCarter 2001). The lateral flagella are encoded by a separate region of the chromosome from the polar flagella and controlled by the *scr*ABC genes in *V. parahaemolyticus* (Boles and McCarter 2002).

Pili

Vibrios also produce a variety of pili or fimbriae (Baumann et al. 1984). One of the best studied is the toxin coregulated pilus (TCP), produced by epidemic strains of *V. cholerae*. TCP is a type IV pilus encoded on a pathogenicity island and is critical for virulence for two reasons. Studies with isogenic mutants have shown that TCP is required for attachment to human intestinal mucosa and that this is a necessary step in infection (Kaper et al. 1995; Klose 2001). TCP is also the attachment site of CTXϕ, the lysogenic phage carrying cholera toxin (CT) genes (Chiang and Mekalanos 1999).

V. cholerae O1 of the eltor biotype and *V. cholerae* O139 produce a second type IV pilus, the mannose-sensitive hemagglutinin (MSHA) (Chiang and Mekalanos 1999; Tacket et al. 1998). MSHA, thin flexible pili, are rarely produced by classical strains (Kaper et al. 1995). The role of MSHA in human virulence is somewhat unclear. Antibodies against MSHA are protective against infection with eltor strains (Osek et al. 1992), but a deletion mutant in MSHA still produced diarrhea and an immune response in volunteers (Tacket et al. 1998). MSHA may also play a role in adherence to zooplankton (Chiavelli et al. 2001).

A third colonization factor is produced by CT-producing *V. cholerae*. The *cep* gene is located in the core virulence region near the CT genes. *Cep* has a predicted amino-acid sequence that shows homology with a flexible pilus of *Aeromonas hydrophila* and mutations show reduced colonization in infant mice (Kaper et al. 1995). However, the role in human pathogenesis, if any, is unclear.

Nonepidemic strains of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* also have pili that may be involved in colonization (Honda et al. 1988; Nakasone and Iwanaga 1991; Nakasone and Iwanaga 1990a; Nakasone and Iwanaga 1990b; Paranjpye et al. 1998). Further work is needed to determine the role of any of these pili in virulence.

Cell wall

The LPS of vibrios is an important component of the cell wall. It is also the major determinant of serogroup. As in other gram-negative bacilli, vibrio LPS consists of lipid A, core polysaccharide, and an O polysaccharide side chain that determines the serogroup. In *V. cholerae* O1, the lipid A consists of a β-1,6-linked glucosamine disaccharide backbone with two phosphoryl groups (Janda et al. 1988). The core polysaccharide contains KDO (keto-3-deoxy-D-mannose-octulosonic acid), D-glucose, L-glycero-D-*manno*-heptose, D-fructose, and ethanolamine phosphate (Manning et al. 1994). There is variation in core polysaccharide among *V. cholerae* serogroups (Nesper et al. 2002). The O-polysaccharide of *V. cholerae* O1 is a perosamine (4,6-dideoxy-D-mannose) homopolymer acetylated with 3-deoxy-L-glycero-tetronic acid with approximately 17–18 repeating subunits (Manning et al. 1994). The O polysaccharide of O139 strains has a hexasaccharide repeating subunit identical to the repeating subunit of the capsular polysaccharide (CPS). However, the LPS is truncated with only one or two subunits of the O139 antigen (Cox et al. 1996; Knirel et al. 1997). There is a great deal of heterogeneity in the O-side chains of vibrios (Iguchi et al. 1989; Iguchi et al. 1993; Kondo and Hisatsune 1989). Like *V. cholerae* O139, the LPS O-side chains of *V. parahaemolyticus* and *V. vulnificus* are very short and are termed lipooligosaccharides (LOS) (Bahrani and Oliver 1991; Iguchi et al. 1995).

Capsules

V. vulnificus and non-O1 *V. cholerae* produce acidic polysaccharide capsules that are important to their ability to cause extraintestinal infections (Johnson et al. 1992; Simpson et al. 1987; Wright et al. 1990). The *V. vulnificus* capsule shows a high degree of heterogeneity and is present on pathogenic and environmental strains (Bush et al. 1997; Hayat et al. 1993). *V. parahaemolyticus* produces a capsule (Chatterjee and Neogy 1972), but its role in virulence is not known. *V. cholerae* O1 do not produce capsules, but capsules are commonly produced by *V. cholerae* of serogroups other than O1 (Morris and Nair 2001). An acapsular mutant of a non-O1, non-O139 *V. cholerae* strain was less virulent in a mouse model (Johnson et al. 1992). *V. cholerae* O139 is a special case. These strains produce a polysaccharide capsule with a hexasaccharide repeating subunit (Preston et al. 1995). The O139 LPS has one or two of this identical repeating subunit as its O-side chain (Cox et al. 1996). The genes encoding the capsule and O antigen are found in the same chromosomal location as the O1 antigen genes in eltor strains (Comstock et al. 1996; Stroeher et al. 1998). The presence of a capsule in these strains may be the result of an error in LPS synthesis rather than by synthesis of a separate polysaccharide as occurs in other *V. cholerae*. However, the presence of a capsule still leads to the possibility that O139 strains have the potential to cause extraintestinal infections in susceptible hosts.

GENETIC MECHANISMS

Chromosomes

The entire genome of *V. cholerae* has recently been sequenced and it was discovered that it has two circular chromosomes (Trucksis et al. 1998; Heidelberg et al. 2000). *V. parahaemolyticus* also has two chromosomes (Makino et al. 2003; Tagomori et al. 2002). One of the chromosomes may have started as a megaplasmid, but

both now contain essential genes. Recombination between the chromosomes can occur. It is not known whether all vibrios share this unique genetic organization.

Plasmids and integrons

Plasmids are common in vibrios, but their functions are largely unknown (Biosca et al. 1996; Pedersen et al. 1997b; Wiik et al. 1989). Recently, a number of transmissible integrons encoding antibiotic-resistance genes have been found on plasmids or on the chromosome of vibrios The SXT element, a conjugative integrating element, has been isolated from *V. cholerae* O139 strains and encodes resistance to streptomycin, trimethoprim, and sulfamethoxizole. This 62-Kb region is integrated into a specific site on the chromosome and is self-transmissible by conjugation (Chiang and Mekalanos 1999; Waldor et al. 1996). In another study of 94 non-O1, non-O139 *V. cholerae* strains examined, 22 strains were found to have class I integrons on plasmids or in the chromosome. These integrons encoded resistance to trimethoprim, amikacin, tobramycin, streptomycin, spectinomycin, and erythromycin (Thungapathra et al. 2002).

Phage

There are over 180 tailed and at least ten filamentous phage, both lytic and lysogenic, that infect vibrios (Kapfhammer et al. 2002). These can be used for phage typing schemes and may be responsible for horizontal gene transfer. One of the more exciting new discoveries about *V. cholerae* pathogenicity is that the genes for cholera toxin are carried on the lysogenic bacteriophage CTXϕ and that the receptor for CTXϕ is the TCP (Waldor and Mekalanos 1996; Mekalanos et al. 1997). CTXϕ is a filamentous phage that is usually integrated into the *V. cholerae* genome but can also replicate as a plasmid (Waldor et al. 1996; Mekalanos et al. 1997). Both cholera toxin and TCP are largely conserved within clonal serogroup O1 and O139 strains, but these findings suggest that horizontal gene transfer may allow new epidemic strains to arise in the future (Chiang and Mekalanos 1999). This possibility is supported by the recent observation that CT and TCP were found in a clonal group of serogroup O141 strains that causes severe diarrhea (Dalsgaard et al. 2001).

A general transducing phage, CP-T1, has been identified from *V. cholerae*. This temperate phage is capable of mediating horizontal gene transfer. Transfer of cholera toxin genes and the TCP pathogenicity island into nontoxigenic strains can also be mediated by CP-T1 (O'Shea and Boyd 2002; Boyd and Waldor 1999). General transducing phage have also been described for *V. parahaemolyticus* and *V. alginolyticus* (Muramatsu and Matsumoto 1991).

The temperate bacteriophage K139, isolated from O139 and O1 strains, has recently been sequenced (Kapfhammer et al. 2002). This phage is a member of the kappa family of phage, which have previously been described in *V. cholerae* O1. These phage encode a protein, Glo, that may be involved in virulence (Chiang and Mekalanos 1999). This phage uses the O1 antigen as its receptor, suggesting that phage infection predated the conversion of an O1 eltor to serogroup O139.

Bacteriophage have also been found in *V. parahaemolyticus* (Chan et al. 2002), *V. vulnificus* (DePaola et al. 1998), and *V. harveyi* (Oakey and Owens 2000). The role of these phage in the ecology and virulence of vibrios remains to be determined. However, all of these recent developments in the genetics of vibrios suggest that horizontal gene transfer and recombination are relatively frequent events and the genome of vibrios is plastic and continually evolving. It is likely that we will see the emergence of new pathogenic clades of *V. cholerae* and other vibrios in the future.

LABORATORY ISOLATION AND IDENTIFICATION

Genus identification

The first step in laboratory identification of vibrios is to assign the isolate to the correct genus. This is becoming more challenging because of changes in phenotype, discovery of more *Vibrio* spp. with unusual characteristics, and the fact that many laboratories no longer have access to conventional biochemicals. Many of the miniature biochemical test panels used in today's clinical laboratories are not reliable in differentiating *Vibrio* spp. from *Aeromonas* spp. or in correctly identifying the species of either of these genera (Carnahan et al. 1996). These systems may have a limited number of vibrios in the database and the panels can give false negative tests due to the low salt concentration. It is important for the laboratory to be aware of these difficulties and to proactively look for vibrios in clinical specimens, particularly when there is a history of consumption of raw or undercooked shellfish, exposure of wounds to seawater, or foreign travel. Vibrios, on nonselective media are similar in appearance to enterics, *Aeromonas* spp., and *Plesiomonas shigelloides*. Vibrios grow well on commonly used nonselective media but may be overgrown by other bacteria, particularly in stool cultures. Addition of a selective agar such as TCBS can increase recovery of vibrios. Growth and colony morphology on TCBS agar is helpful in identifying vibrios but is not sufficient even as a screening tool. Not all vibrios will grow on TCBS and there is significant lot-to-lot variation in selectivity. The oxidase test is readily available in clinical laboratories and particularly critical for separating vibrios, *Aeromonas* spp., and *Plesiomonas shigelloides* from

enteric gram-negative rods. Vibrios (except *V. metschnikovii* and *V. gazogenes*), aeromonads, and *P. shigelloides* are oxidase-positive (Baumann et al. 1984; Tison 1999). In the Kovac's oxidase test, fresh growth is exposed to a 1 percent aqueous solution of n,n,n′,n′-tetramethyl-p-phenylenediamine. Oxidase-positive colonies produce a purple color. It is important to use fresh growth from media that does not contain carbohydrate and to use simultaneous positive and negative controls. Tests such as triple sugar iron (agar) (TSI) or Kligler's iron agar (KIA) can be useful to separate vibrios from Enterobacteriaceae and oxidase-positive nonfermentors. Vibrios ferment sugars but do not produce gas from glucose (except some strains of *V. furnissii* and *P. damsela*). Susceptibility to the vibriostatic compound 2,4-diamino-6,7-diisopropyl-pteridine phosphate (O/129) used to be a key test for identifying vibrios. In the past, all vibrios were sensitive to 150 μg O/129 and all aeromonads and enterics were resistant. Unfortunately, in recent years, resistance to O/129 has become widespread in *V. cholerae* (Huq et al. 1992; Nath and Sanyal 1992) and has also appeared in other species (Pedersen et al. 1997a; Dalsgaard et al. 1997). The string test and growth in 6 percent NaCl remains valuable for identifying vibrios. The string test is performed by mixing a large loop of growth from a noninhibitory agar with a drop of 0.5 percent sodium deoxycholate on a glass microscope slide (Baumann et al. 1984; Tison 1999). The deoxycholate lyses vibrios, releasing the cellular contents, which causes the solution to become viscous. When the loop is slowly lifted off the slide, a viscous 'string' is visible between the slide and the loop-like cheese on a particularly gooey pizza. Some strains of *V. parahaemolyticus*, *V. cincinnatiensis*, and *P. damsela* give variable results. Most *Aeromonas* spp., *P. shigelloides*, and enterics are string-test-negative. Salt requirements are a valuable test for separating these groups of organisms and can be helpful for species identification. The salt requirement is tested by growth in nutrient broth with 0 percent or 6 percent NaCl added. Vibrios can grow at 6 percent NaCl, while aeromonads, *P. shigelloides*, and enterics cannot. The halophilic vibrios cannot grow at 0 percent NaCl. Salt broths must be inoculated lightly so that they are not visibly turbid before incubation, and the inoculum should be fresh from a nonselective medium. The tubes should be examined at 24 h, but negative tubes may be held for up to 7 days (Morris and Nair 2001; Tison 1999). Key tests are summarized in Table 1. Several tests should be considered before definitively assigning a strain to a specific genus. Clinical laboratories may wish to involve a reference laboratory in identification of these organisms. However, due to the significance of vibrios or aeromonads isolated from blood, sterile body sites, or wounds, a preliminary report should be communicated to the physician as rapidly as possible and not be delayed for definitive identification.

Species identification

For many years, laboratory identification of vibrios was relatively simplistic. Vibrios need only to be divided into two groups *V. cholerae* O1 and nonagglutinable (NAG) or noncholera vibrios (NCV). Identification of *V. cholerae* O1 meant not only a diagnosis of cholera but also a public-health emergency. NAG or NCV vibrios were regarded as essentially similar. A relatively few biochemical tests and a slide agglutination test were sufficient to distinguish these two groups. We now recognize that not all *V. cholerae* O1 produce CT, that other serogroups may produce CT, that there are many species of NCV, and that identification of other *Vibrio* species is important for understanding their pathogenesis and epidemiology. Identification of vibrios is no longer simple but requires a battery of biochemical tests. Testing for toxins and other virulence factors may also be required.

To begin identification of the pathogenic vibrios, fermentation of sucrose, as seen on TCBS agar, is helpful. *V. cholerae*, *V. alginolyticus*, *V. fluvialis*,

Table 1 *Differential characteristics of* Plesiomonas shigelloides, *Vibrionaceae, Aeromonidaceae, and Enterobacteriaceae*

Test	*V. cholerae*/ *V. mimicus*	Halophilic vibrios	*Aeromonas*	*Plesiomonas shigelloides*	Enterobacteriaceae
Oxidase	+	+[a]	+	+	−
String test	+	+[b]	−	−	−
Gas from glucose	−	−[c]	+	−	V
10 μ O/129	S[d]	S/R	R	S/R	R
150 μ O/129	S[d]	S	R	S/R	R
0% NaCl[e]	+	−	+	+	+
6% NaCl	+	+	−	−	−

+, 90 percent positive reaction; −, 90 percent negative reaction; V, variable or ±; S, susceptible; R, resistant.
a) *V. metshcnikovii* and *V. gazogenes* are oxidase-negative.
b) *V. parahaemolyticus*, *V. cincinnatiensis*, and *P. damsela* are variable.
c) *V. furnissii* and *P. damsela* are variable.
d) Many strains of *V. cholerae* in India and Bangladesh have become resistant.
e) Performed in nutrient broth.

V. furnissii, and *V. cincinnatiensis* ferment sucrose and have yellow colonies. *V. parahaemolyticus*, *V. mimicus*, *V. vulnificus*, *P. damselae*, *V. hollisae*, and *V. metschnikovii* do not ferment sucrose, so their colonies are green. Occasional strains of *V. vulnificus* that ferment sucrose have been reported. For definitive, reliable identification, classical tube biochemicals should be used and supplemented to a final concentration of 1 percent NaCl. Tests should be incubated in ambient air at 35–37°C and read daily for up to 3 days. *V. cholerae* and *V. mimicus* can be differentiated from other vibrios by their ability to grow in nutrient broth without added salt. *V. cholerae* is sucrose-positive and *V. mimicus* is sucrose-negative. Different concentrations of salt from one to eight percent can help differentiate species (Baumann et al. 1984). Lysine decarboxylase (LDC), ornithine decarboxylase (ODC), and arginine dihydrolase (ADH) are key tests for dividing vibrios into groups. *V. hollisae* is the only clinical species that is negative for all three tests. *P. damselae*, *V. fluvialis*, and *V. furnissii* are ADH-positive. Other critical tests are indole, Voges–Proskauer, Simmon's citrate, and fermentation of arabinose (Tison 1999; Baumann et al. 1984). Fermentation of myo-inositol is key for identification of *V. cincinnatiensis*. Key tests for separating the pathogenic vibrios are listed in Table 2.

Biotypes

V. cholerae O1 can be broken into classical and eltor biotypes. Classical strains are nonhemolytic, do not agglutinate chicken erythrocytes, are Voges–Proskauer-negative, and are sensitive to polymyxin B. Eltor isolates have the opposite reactions, although nonhemolytic strains are becoming more common. The biotypes can also be separated by susceptibility to a panel of lytic bacteriophage. Isolates from the sixth pandemic were all of the classical biotype. The seventh pandemic is characterized by eltor biotype strains, although occasional classical isolates are still seen (Kaper et al. 1995).

V. vulnificus also has two biotypes. Biotype 1 strains colonize shellfish and are associated with human infections. They are indole- and ornithine decarboxylase-positive. Biotype 2 strains are associated with infections of culture eels, have the same serotype, and are indole- and ornithine decarboxylase-negative (Biosca et al. 1996; Strom and Paranjpye 2000).

ANTIMICROBIAL SUSCEPTIBILITY

Treatment of cholera and other severe diarrhea with antibiotics can reduce the duration of the disease and length of time that vibrios are shed, but it is less important than appropriate fluid and electrolyte replacement (Bhattacharya 2003; Kaper et al. 1995). Cholera is often treated with tetracycline (Holmberg 1992). Before the advent of *V. cholerae* O139, trimethoprim-sulfamethoxazole and furazolidone were also used to treat cholera, but O139 strains are generally resistant to these drugs (Waldor and Mekalanos 1994). On the other hand, prompt, appropriate, and aggressive antibiotic treatment of extraintestinal vibrio infections is critical for survival. There are limited studies on the susceptibility of the halophilic vibrios. Most vibrios tested are susceptible to tetracyclines, aminoglycosides, chloramphenicol, monobactams, carbapenems, and fluoroquinolones (Morris et al. 1985; Tang et al. 2002; Khan et al. 2002; Wong et al. 2000). A combination of minocycline and a fluoroquinolone or cefotaxime may be most effective (Chuang et al. 1998; Tang et al. 2002). Antibiotic resistance in *V. cholerae* has increased dramatically in recent years, making susceptibility testing a necessity (Morris and Nair 2001; Dromigny et al. 2002; Thungapathra et al. 2002; Ranjit and Nurahan 2000; Garg et al. 2000).

TYPING

Serotyping

The main vibrio antigenic structures important for serotyping are similar to those in enterics. The O-side chain of the LPS constitutes the somatic (O) antigen and the flagellum constitutes the H antigen. The O antigen forms the basis for most vibrio typing schemes, as the H antigens are common to all vibrio species. Most serotyping schemes for vibrios were developed before it was known that vibrios have capsules and therefore capsular (K) antigens have not been extensively studied.

There are serotyping schemes for several species of vibrios. These are of great importance in tracking outbreaks. However, only typing sera for *V. cholerae* serogroups O1 and O139 and serotypes Inaba and Ogawa are readily available. The use of the extended serotyping schemes is generally limited to a few reference laboratories. Several serotyping schemes have been developed for typing *V. cholerae* based on the O antigen side chain of the LPS (Kaper et al. 1995). They all identify strains of the sixth and seventh pandemics as serogroup O1, but differ for other serogroups. The Sakazaki typing scheme is currently most widely used, recognizing more than 140 serogroups for *V. cholerae* (Shimada et al. 1994). Serogroup O1 can be further divided into Inaba, Ogawa, and Hikojima serotypes. Hikojima represents an unstable transitional state where both Inaba and Ogawa antigens are expressed (Manning et al. 1994; Tison 1999). During epidemics, strains can shift between serotypes with a change of Ogawa to Inaba being more common than the converse. This change is due to mutations in *rfbT* (Kaper et al. 1995).

V. parahaemolyticus has 13 O antigens and more than 60 K antigens (Wong et al. 1996; Janda et al. 1988). *V. vulnificus* has seven O antigen groups, but all flagellar

Table 2 *Differential characteristics of* Vibrio *spp. of clinical importance*

	V. cholerae	*V. mimicus*	*V. parahaemo-lyticus*	*V. algino-lyticus*	*V. vulnificus*	*P. damselae*	*V. fluvialis*	*V. furnissii*	*V. hollisae*	*V. metschni-kovii*	*V. cinncinati-ensis*	*V. harveyi*
TCBS	Yellow	Green	Green	Yellow	Green	Green/poor growth	Yellow	Yellow	Poor growth/ green	Green/poor growth	Poor growth	Variable
0% NaCl[a]	+	+	–	–	–	–	–	–	–	–	–	–
1% NaCl[a]	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	–	–	–	–	–	+	+	+	–	V	–	–
Lysine decarboxylase	+	+	+	+	+	V	–	–	–	V	V	+
Ornithine decarboxylase	+	+	+	V	V	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+	–	+	+
Nitrate nitrite	+	+	+	+	+	+	+	+	+	–	+	+
Indole	+	+	+	V	+	–	V	V	+	V	–	+
Voges–Proskauer	V[b]	–	–	+	–	+	–	–	–	+	–	V
Simmon's citrate	+	+	–	–	V	–	+	+	–	V	V	–
Urea	–	–	–[c]	–	–	–	–	–	–	–	–	–
Gelatin	+	V	+	+	V	–	V	V	–	V	–	–
Arabinose	–	–	V	–	–	–	+	+	+	–	+	–
Myo-Inositol	–	–	–	–	–	–	–	–	–	–	+	–

+, 90 percent positive reaction; –, 90 percent negative reaction; V, variable or ±. Key reactions are in pink.

a) Growth in nutrient broth.

b) Classical *V. cholerae* are negative and El Tor are positive.

c) There is an uncommon, pathogenic clade of *V. parahaemolyticus* that is urease-positive.

antigens are the same (Shimada and Sakazaki 1984). At least 23 serotypes of *V. anguillarum* have been identified (Pedersen et al. 1999). *V. fluvialis* and *V. furnissii* can also be serotyped (Shimada et al. 1999).

Phage typing

The sensitivity pattern of different strains of *V. cholerae* to lytic bacteriophage is another useful tool for tracking the spread of cholera. Phage can be used for differentiating classical from eltor biotypes of *V. cholerae* O1 (Mukerjee 1963; Takeya et al. 1981) and now phage specific for O139 Bengal strains have been identified (Albert et al. 1996). Additional phage have allowed discrimination between O1 strains of the same serotype and biotype (Basu and Mukerjee 1968). An updated typing scheme has been reported (Chattopadhyay et al. 1993), and typing has been expanded to include O139 Bengal strains (Chakrabarti et al. 2000). A phage typing scheme has also been reported for *V. fluvialis* (Suthienkul 1993). The use of phage typing is limited to a few reference labs by the availability of typing phage and a lack of consensus in the typing schemes (Kaper et al. 1995).

Molecular typing

The difficulties inherent to phage typing have led to a search for new, easier, and more sensitive typing methods. Modern molecular typing methods are widely available, highly discriminatory, and robust (Arbeit 1999). Standard molecular typing methods work well with vibrios. These include multilocus enzyme electrophoresis (MLEE or MEE), amplified fragment length polymorphisms (AFLP), pulsed-field gel electrophoresis (PFGE), ribotyping, and sequencing (Kaper et al. 1995; Popovic et al. 1995; Romalde et al. 2002; Thungapathra et al. 2002; Wong et al. 2000). MLEE or zymovar analysis uses variations in the electrophoretic mobility of several enzymes to divide bacteria into electrophoretic types. This method has been used to track the spread of cholera in South America and Southeast Asia (Beltran et al. 1999; Wachsmuth et al. 1993) and can be expanded to other vibrios (Momen and Salles 1981). AFLP has been used to differentiate strains of *V. cholerae* (Lan and Reeves 2002), *P. damselae* (Botella et al. 2002), and other vibrios (Janssen et al. 1996). PFGE may the most commonly currently used method for molecular typing due to its high degree of discrimination and applicability to many organisms (Arbeit 1999). It has been used for a variety of vibrios (Osawa et al. 1996; Pedersen et al. 1997a; Romalde et al. 2002; Ryang et al. 1999; Wong et al. 2000; Yeung et al. 2002). PFGE is easy to perform, very versatile, reproducible, and discriminatory. However, gel patterns can be somewhat difficult to interpret and lab-to-lab comparison of gels is particularly problematic. Ribotyping analyzes restriction fragment length polymorphisms of the genes encoding rRNA. Its advantages are that it can be used on any bacterium and an automated instrument that allows excellent lab-to-lab comparison of data has been developed (Gendel et al. 2001; Yeung et al. 2002). Sequencing of single genes such as *ctxA*, *ctxB*, *hsp60*, and *recA* shows promise as a molecular typing method (Kaper et al. 1995; Kwok et al. 2002; Stine et al. 2000). It is currently limited by the expense of sequencing, but it has the advantages of excellent lab-to-lab reproducibility and ease of analysis. Most importantly, sequence data provide information about the phylogeny and evolution of pathogens that is not available from the other typing methods. Recent evidence that recombination is common in bacteria, means that the use of multiple genes, or multilocus sequence typing (MLST), is preferred over sequencing of a single gene (Byun et al. 1999; Smith et al. 2000).

NORMAL FLORA

Vibrios are not part of the usual flora of humans. They should always be reported by the clinical microbiology laboratory when recovered. Isolation of a vibrio from a sterile body site or a wound is highly significant. However, not every individual carrying vibrios in the gastrointestinal tract shows symptoms. This is seen during cholera epidemics, where up to 60–75 percent of patients may be asymptomatic (Kaper et al. 1995). Chronic carriers of vibrios appear to be rare, but short-term shedding in asymptomatic patients has been reported for *V. cholerae* during cholera epidemics and in pilgrims returning from Mecca, and for other vibrios in visitors to the US Gulf Coast (Morris and Nair 2001).

PATHOGENICITY

Vibrios cause a range of infections from diarrhea, to localized wound, eye, and ear infections, to necrotizing fasciitis, to highly fatal septicemia. The most important and well-known pathogen in the genus is *V. cholerae*, the etiologic agent of epidemic cholerae. Cholera ranges in severity from inapparent infections to cholera gravis with massive efflux of fluids and electrolytes. In its most severe form, cholera is characterized by prolific secretory diarrhea, resulting in 'rice-water' stools. Fluid loss can reach 200 ml/kg body weight/day (Chiang and Mekalanos 1999). Untreated, cholera leads to hypovolemic shock, acidosis, circulatory collapse, and death in adults as well as children (Kaper et al. 1995). Epidemic strains of *V. cholerae* (serogroups O1 and O139) produce cholerae toxin. Until a decade ago it was believed that only serogroup O1 strains could cause epidemic cholera. In 1992, a new serogroup, O139, appeared in India and Bangladesh. This O139 Bengal strain caused an epidemic that spread rapidly through

Asia. The clinical picture was identical to that of cholera, except that adults were more frequently affected, showing that previous infection with O1 cholerae was not protective. (Morris et al. 1994; Waldor and Mekalanos 1994). It has since been shown that O139 Bengal strains are very closely related to O1 eltor strains (Bik et al. 1995; Johnson et al. 1994; Stine et al. 2000; Waldor and Mekalanos 1994). The major difference is that the genes responsible for O-antigen and capsule biosynthesis in O139 have replaced the O1 antigen gene of O1. O1 and O139 strains produce the same virulence factors: cholera toxin, zonnula occludens toxin (ZOT), accessory cholera enterotoxin (ACE), and TCP. *V. cholerae* is a classic model of a toxigenic enteric pathogen; the bacterium uses a pilus, TCP, to colonize the gut and CT causes the diarrhea (Holmberg 1992). The importance of CT and TCP have been unequivocally demonstrated by volunteer, animal, and epidemiologic studies. Cholera toxin has been intensively studied in terms of mode of action, site of action, genetic regulation and molecular structure. Rarely, strains of non-O1, non-O139 *V. cholerae*, and *V. mimicus*, particularly in Asia, carry CT. Fluid replacement by oral rehydration therapy and/or intravenous fluids is the key to treating cholera (Bhattacharya 2003; Kaper et al. 1995).

Although *V. cholerae* is the only vibrio that causes cholera, several other species cause diarrhea. *V. parahaemolyticus* is a leading cause of food poisoning in Japan and Southeast Asia (Joseph et al. 1982; Wong et al. 2000). According to Centers for Disease Control and Prevention (CDC) surveillance, *V. parahaemolyticus* is also the most common vibrio isolated and the leading cause of vibrio gastroenteritis in the USA (www.cdc.gov/ncidod/dbmd/diseaseinfo/files/VibCSTE99web.pdf; www.cdc.gov/ncidod/dbmd/diseaseinfo/files/CSTE_Vibrio_2000.pdf). A newly identified serotype, O3:K6, has recently been responsible for pandemic outbreaks (Arakawa et al. 1999; Chiou et al. 2000; Yeung et al. 2002). Other species associated with diarrhea are *V. alginolyticus*, non-CT-producing *V. cholerae*, *V. fluvialis*, *V. hollisae*, and *V. mimicus* (Morris and Nair 2001; Janda et al. 1988). A variety of enterotoxins are associated with these organisms, but are not as well studied as CT. Several other species, such as *V. furnissii*, *V. mimicus*, *V. fluvialis*, *V. hollisae*, and *V. vulnificus*, have been isolated from stools of patients with diarrhea, but causality is not clearly demonstrated (Dalsgaard et al. 1997; Holmberg 1992).

In addition to causing enteric disease, vibrios are important causes of wound infections and septicemia. Wound infections are often associated with injuries exposed to estuarine or marine environments and can be associated with significant tissue destruction (Barber and Swygert 2000; Penland et al. 2000). *V. vulnificus* generally produce the most severe infections, often requiring surgical debridement. The wound infection can advance to cellulitis with extensive skin necrosis, myositis, and necrotizing fasciitis that may mimic gas gangrene. Wound infections may progress to septicemia, and the fatality rate ranges from 20 to 30 percent (Morris and Nair 2001; Strom and Paranjpye 2000). Noncholera strains of *V. cholerae*, *V. alginolyticus*, and *P. damselae* also cause wound infections (Tison 1999).

Septicemia occurs secondary to wound infections or primarily following consumption of raw or undercooked seafood. *V. vulnificus* is the most important cause of primary septicemia due to vibrios. In the USA, infection typically follows consumption of raw oysters. Data from the CDC showed that more than 95 percent of patients had consumed raw oysters within the past 7 days (Strom and Paranjpye 2000). *V. vulnificus* is responsible for more than 90 percent of deaths due to vibrios. Fatalities were predominantly in people older than 50 and most occur in men. Estrogen may play a protective role, explaining the gender difference in disease (Merkel et al. 2001). Septicemia occurs primarily in people with predisposing conditions such as liver disorders, immunosuppression, increased serum iron, or other chronic diseases. Cases have been reported from patients with cirrhosis, a history of alcohol abuse, cancer, liver transplantation, acquired immune deficiency syndrome (AIDS), hemochromatosis, and thalasemia major (Strom and Paranjpye 2000). Patients present with a sudden onset of fever and chills, vomiting, diarrhea, and abdominal pain. Secondary skin lesions often appear, progressing to bullae formation and necrosis. Endotoxic shock often occurs and can rapidly lead to death. Primary septicemia is fatal in 60–75 percent of patients. Rapid treatment of septicemia and wound infections with antibiotics is critical (Morris and Nair 2001; Strom and Paranjpye 2000). Tetracyclines or fluoroquinolones appear to be effective and may be used in combination with gentamicin or chloramphenicol (Chuang et al. 1998; Morris et al. 1985).

Vibrios are also important pathogens of animals. *V. vulnificus* of biogroup 2 are pathogens of eels (Biosca et al. 1996). *P. damselae* was originally described as a cause of ulcers in damselfish (Love et al. 1981) and has been associated with wound infections of a variety of fish and other marine vertebrates (Pedersen et al. 1997a). *V. anguillarum* is the causative agent of disease in marine fish and shellfish (Pedersen et al. 1997b). *V. tapetis* causes brown ring disease in clams (Castro et al. 2002). These diseases can cause losses to aquaculture; their prevalence in the wild is unknown.

VIRULENCE FACTORS

V. cholerae

The most well-known virulence factor of vibrios is CT, a heat-labile enterotoxin produced by *V. cholerae* of serogroups O1 and O139. This toxin is responsible for the fulminate, dehydrating diarrhea characteristic of cholera gravis. CT is a member of the family of heat-

labile enterotoxins that includes the *Escherichia coli* LT. This multimeric toxin has one A subunit (holotoxin, MW 27.2 kDa) that is active intracellularly and five identical B subunits (MW 11.6 kDa) that form a ring. The A subunit is activated by cleaving the holotoxin to form A_1 and A_2 peptides joined by a disulfide bond. The B subunit ring interacts with G_{M1} receptors on human cells, allowing the toxin to bind. The G_{M1} receptor is a ganglioside [galactose β1→3 *N*-acetylgalactosamine β1 (*N*-acetylneuraminic acid α2→3) →glucose-ceramide]. Neuraminidase, also secreted by *V. cholerae*, aids in the binding of CT by cleaving other gangliosides to G_{M1}. Once binding occurs, the A subunit translocates into the cell and interacts with G proteins that control adenylate cyclase. Adenylate cyclase catalyzes the conversion of adenosine 5′ triphosphate (ATP) to cyclic adenosine 5′-monophosphate (cAMP). The increased levels of intracellular cAMP causes increased secretion of chloride ions by intestinal crypt cells and decreased NaCl-linked absorption by villus cells, changing the osmotic gradient. This change in the transepithelial osmotic gradient causes fluids to be excreted into the lumen of the bowel, resulting in diarrhea (Kaper et al. 1994; Klose 2001). CT may also increase the intensity of diarrhea by interacting with prostaglandins and the autonomic nervous system. (Kaper et al. 1995; Peterson and Ochoa 1989).

The secretory effect of CT can be detected in animal models such as the removable intestinal tie adult rabbit diarrhea (RITARD) model, rabbit ileal loop (RIL) model, or the infant mouse assay (Pierce et al. 1988; Spira et al. 1981; Spira et al. 1983). CT can also be detected in vitro by enzyme-linked immunosorbent assay (ELISA) and gene probes or in tissue culture (Nair and Takeda 1994; Franco et al. 1997).

The structural genes, *ctxAB*, encoding CT lie within core region encoded on a lysogenic bacteriophage, CTXϕ (Waldor and Mekalanos 1996). The core element also contains additional toxin genes, *ace* and *zot*, and a colonization factor gene, *cep*, and is flanked by RS1 elements. The RS1 elements allow for amplification and deletion of the core region (Waldor and Mekalanos 1994). There are multiple copies of the core region in most strains; classical strains generally have two widely separated copies, while the core region of eltor and O139 strains is found at single chromosomal locations, although there may be tandem repeats (Kaper et al. 1995; Trucksis et al. 1998).

Before CT can exert its effect, *V. cholerae* must be able to colonize the intestine. The major colonization factor for epidemic strains of *V. cholerae* is the toxin-coregulated pilus. Mutations that lack TCP have reduced colonization and diarrhea in animal and volunteer models (Kaper et al. 1995). TCP is composed of long filaments 7 nm in diameter that form bundles (Taylor et al. 1987). The major pilin subunit, TcpA, shares homology with type IV pili of other bacteria. Eltor and classical biotypes both express TCP, although there are some variations. The predicted amino-acid sequences of the two biotypes share 82–83 percent homology; there are some epitope differences, and the environmental conditions required for expression are different. Expression of TCP requires genes in the *tcp* operon and *tcp*G at a separate location (Chiang and Mekalanos 1999). The *tcp* operon lies within a pathogenicity island, called the vibrio pathogenicity island (VPI) or the TCP pathogenicity island (Karaolis et al. 1998; Faruque et al. 1998). TCP serves as the receptor for CTXϕ.

Expression of CT and TCP is regulated by the ToxR-ToxT regulatory cascade. This system allows the bacterium to respond to a variety of environmental signals. The ToxR system controls expression of at least 17 genes including CT, TCP, Ace, and the outer-membrane proteins OmpT and OmpU. ToxR is a 32-kDa transmembrane protein with homology to two-component response regulators. ToxR binds directly upstream of *ctxAB*, *ompU*, and *toxT*, increasing their expression, possibly with the aid of ToxS. ToxT, an AraC-like protein, can also directly upregulate CT expression. ToxT, in turn, activates other genes, including those for production of TCP. This system allows multiple genes at different locations on the chromosome to be coordinately regulated so that they respond to the same environmental conditions (Bina et al. 2003; Chiang and Mekalanos 1999; Mekalanos et al. 1997; Klose 2001). ToxR homologs have been found in many other *Vibrio* spp. (Lin et al. 1993; Vuddhakul et al. 2000). ToxR has been shown to directly regulate genes in *V. anguillarum* (Okuda et al. 2001) and to affect regulation of outer-membrane proteins and bile resistance in *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus* (Provenzano et al. 2000).

V. cholerae O1, with a deletion of *ctxA*, can still produce significant diarrhea and abdominal discomfort in volunteers. This may be due to the presence of ZOT and ACE (Morris and Nair 2001). The *zot* and *ace* genes are located within the CTXϕ element containing CT genes. ZOT acts by loosening the tight junctions (zonnula occludens) of small-intestinal mucosa, thereby increasing intestinal permeability (Fasano et al. 1991; Fasano et al. 1997; Kaper et al. 1995). ACE increases the short-circuit current in Ussing chambers and causes fluid accumulation in RIL (Trucksis et al. 1993). Both of these toxins may add to the loss of fluids into the lumen of the bowel. Other studies have shown that *zot* and *ace* are involved in CTXϕ morphogenesis (Chiang and Mekalanos 1999). Further work is needed to clarify the relative importance of these dual roles of ZOT and ACE, and how they evolved.

Additional toxins can occur in *V. cholerae* but are not well characterized. These can occur in both epidemic and nonepidemic strains of *V. cholerae*. These include a shiga-like toxin that has not been cloned and several

hemolysins/cytolysins (Kaper et al. 1995). Some non-O1, non-O139 *V. cholerae* produce a heat-stable enterotoxin that has been shown to cause diarrhea by epidemiologic and volunteer studies (Arita et al. 1986; Bagchi et al. 1993; Morris et al. 1990). This toxin has 50 percent protein sequence homology to the heat-stable toxin (ST) of enterotoxigenic *Escherichia coli* (ETEC) (Ogawa et al. 1990). To differentiate the heat-stable toxin of *E. coli* (ST) from that of *V. cholerae*, the *V. cholerae* toxin is called non-agglutinating (vibrio) stable toxin (NAG-ST), because non-O1 *V. cholerae* are referred to as non-agglutinating vibrios in the older literature. There is an association of NAG-ST with Sakazaki O14 serogroup strains suggesting a clonal origin (Sarkar et al. 2002). It is possible that *V. cholerae*, like *E. coli*, has nonpathogenic members as well as pathogenic clades characterized by specific virulence genes or pathogenicity islands.

V. vulnificus

V. vulnificus is primarily an agent of septicemia and wound infections and its virulence is not well understood. Host factors are clearly of great importance in *V. vulnificus* infections; septicemia is rarely seen in people without underlying conditions. Strains also show considerable variation in virulence in mouse models and no one has yet been able to clearly link specific factors with increased virulence. The one feature that has conclusively demonstrated to play a role in pathogenesis is the polysaccharide capsule (Simpson et al. 1987). Acapsular mutants showed a significant increase in the LD_{50} in mouse models (Wright et al. 1990). However, most strains of *V. vulnificus* produce capsules, regardless of virulence (Bush et al. 1997; Hayat et al. 1993; Stelma et al. 1992). As with other pathogens, the CPS protects the bacteria from complement and phagocytosis (Johnson et al. 1984; Musher et al. 1986; Strom and Paranjpye 2000; Tamplin et al. 1985). The capsule may also contribute directly to septic shock by activating inflammatory cytokines (Powell et al. 1997).

Other virulence factors that have been proposed are LPSs, cytolysin, protease/elastase, collagenase, pili, and the ability of the organism to acquire iron from the host. The LPSs of many gram-negative pathogens are known to be potent inducers of septic shock (Alexander and Rietschel 2001). Studies with *V. vulnificus* LPS suggest that it is less active than that from *E. coli*, although it does elicit inflammatory cytokines (Powell et al. 1997; Strom and Paranjpye 2000). Further work is needed to understand the role of LPS and CPS in the induction of shock.

Iron utilization is clearly important in the pathogenicity of *V. vulnificus*. Diseases leading to increased serum iron are a risk factor for *V. vulnificus* susceptibility, and iron loading of mice decreases the LD_{50} (Wright et al. 1981; Wright et al. 1985). *V. vulnificus* produces siderophores and can utilize a number of iron-containing compounds (Simpson and Oliver 1983). Shifting from a high to a low iron environment may be a signal for expression of virulence factors (Strom and Paranjpye 2000).

A number of extracellular enzymes have been shown to have activity in animal and cell culture modes, but their importance in virulence is not clear (Morris and Nair 2001; Strom and Paranjpye 2000). Mutations in cytolysin, protease, or elastase resulted in little change in the LD_{50} in mouse models, suggesting that these toxins are not necessary for virulence (Shao and Hor 2000; Wright and Morris 1991).

Pili are important colonization factors for other vibrios and may contribute to *V. vulnificus* pathogenicity. A recent report that inactivation of *vvpD*, with resulting loss of pili, caused a significant increase in the LD_{50} in mouse models is intriguing. However, this mutation also had defects in protein export that could be responsible for the loss of virulence (Paranjpye et al. 1998). At this time, it is clear that CPS and iron-acquisition systems are important for survival in the blood stream of the host; what is not clear is how *V. vulnificus* produces the extensive tissue destruction and shock characteristic of infections with this organism.

V. parahaemolyticus

Our knowledge about the factors responsible for virulence of *V. parahaemolyticus* and, even more so, other *Vibrio* spp. is very limited. *V. parahaemolyticus* pathogenesis has traditionally been associated with production of a thermostable direct hemolysin (Vp-TDH) that can be detected by β-hemolysis on Wagatsuma agar, designated the Kanagawa phenomenon (Tison 1999). Deletion of the gene encoding TDH reduces, but does not eliminate, the enterotoxic effect in Ussing chambers and rabbit ileal loops (Xu et al. 1994). In recent years, a variety of TDH-related toxins (Vp-TRH) have been found in Kanagawa-negative clinical strains. TRH and TDH share about 70 percent sequence homology. Recently, urease-positive strains have been associated with outbreaks (Osawa et al. 1996; Wong et al. 2000). The genes for TDH and urease (*tdh* and *ure*) are linked in a region that looks like a pathogenicity island (Park et al. 2000). Pili have also been proposed as necessary virulence factors.

Research on the virulence factors of the other *Vibrio* species has only just started. Indeed, many of these species were not accurately identified until the past decade or so, and we are still trying to understand their significance in human and animal disease. Many vibrios produce pili, toxins, enzymes, capsules, and other products that could be involved in virulence. Many years of research will be needed to clarify these issues. Although *V. cholerae* is one of the most intensively studied bacterial pathogens; it still has the power to

surprise us. Vibrios will continue to infect us and intrigue us for the foreseeable future.

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Aeromonas

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DEFINITION

Aeromonads are gram-negative, oxidase- and catalase-positive, facultatively anaerobic rods. Most species and subspecies are motile by means of a single polar flagellum. Some strains also produce numerous lateral flagella. Nitrates are reduced and anaerogenic fermentation of many common monosaccharides, disaccharides, glycosides, and some alcoholic sugars (D-mannitol, glycerol) occurs. Acid is rarely produced from pentoses (except L-arabinose), methyl pentose, trisaccharides, and noncarbohydrate compounds (*m*-inositol). Aeromonads are resistant to both 10 and 150 μg of the vibriostatic agent O/129. Nutritional requirements are simple. Salt supplementation is not required for growth; high salt concentrations (6–7 percent) are inhibitory. Many extracellular enzymes are produced including cytolytic toxins, proteases, nucleases, lipases, sulfatases, lecithinase, chitinase, amylase, and stapholysin. Aeromonads are commonly found in aquatic environments, soil, and agricultural produce and in the gastrointestinal contents of fish, reptiles, amphibia, and higher vertebrates. Some species are pathogenic for humans and fish. The G + C content of DNA is 57–63 mol%.

There are presently 17 species in the genus *Aeromonas* (Table 1). At least ten of these have been implicated in human disease, but *A. hydrophila*, *A. caviae*, and *A. veronii* biovar sobria have been most commonly isolated from human infections. The type species is *A. hydrophila*.

HABITAT

Aeromonads are common inhabitants of the microbial biosphere. They can be isolated from virtually any freshwater source excluding hot springs (Hazen et al. 1978). During colder seasons they are often recovered from poikilothermic animals and freshwater environments although usually in low numbers. However, during the warmer months of the year their relative numbers increase due to elevated water temperatures (Chauret et al. 2001). Aeromonads can be isolated from potable water sources (wells, reservoirs, drinking fountains) (Borrell et al. 1998; Chauret et al. 2001; Villari et al. 2003) and numerous foods including dairy products, meats, fish, shellfish, and fresh produce (Krovacek et al. 1992; Kirov 2001; Isonhood and Drake 2002). Recovery from such diverse sources hinders epidemiological investigations attempting to link cases of *Aeromonas*-associated gastroenteritis with particular food vehicles. *Aeromonas* species can also be recovered from the marine environment, particularly from estuaries, bays, and inlets where salinity values are lower. *A. caviae* is prevalent in marine samples, while *A. hydrophila* hybridization groups (HG) (HG1, HG2, and HG3), *A. veronii* biovar sobria and *A. popoffii* predominate in freshwater (Borrell et al. 1998; Soler et al. 2002). Although *Aeromonas* is not generally considered to be part of the normal human flora, fecal carriage rate in asymptomatic individuals can approach 3 percent in temperate regions, and reach over 30 percent in developing countries

Table 1 *Current members of the genus* Aeromonas

Species	HG[a]	Former names/synonyms	Comment
A. allosaccharophila	–		
A. bestiarum	2	*A. hydrophila* genomospecies 2	
A. caviae	4	*A. punctata*	
A. culicicola	–		
A. encheleia	11	*A. eucrenophila* subgroup II	
A. eucrenophila	6		
A. hydrophila [5][b]	1		
A. jandaei	9		
A. media	5		
A. molluscorum	–		
A. popoffii	–		
A. salmonicida [5]	3		Human strains contained in this HG are motile and grow at 35°C
A. schubertii	12	Enteric group 501	
A. simiae	–		
A. sobria	7		Not the *A. sobria* isolated from clinical samples
A. trota	–	*A. enteropelogenes*	
A. veronii	10		Biovar veronii [ODC^+]
A. veronii	8	*A. ichthiosmia*	Biovar sobria [ODC^-]; this is the species isolated from clinical samples previously referred to as '*A. sobria*'

ODC, ornithine decarboxylase
a) CDC hybridization group (Janda 2001)
b) Number in parenthesis indicates number of subspecies.

(Kirov 2001). Aeromonads have been isolated from many other species and higher vertebrates including birds, dolphins, seals, alligators, opossums, dogs, pigs, cattle, sheep, horses, water buffaloes, tamarins, and monkeys. A notable habitat is the digestive tract of *Hirudo medicinalis*, the medicinal leech, in which a pure culture of *A. veronii* biovar sobria is normally found (Graf 1999a).

MORPHOLOGY AND CELL STRUCTURES

Aeromonas bacilli are (0.3–1.0) mm × (1.0–3.5) mm in size. They are morphologically indistinguishable from other enteric organisms such as *E. coli*. Curved forms are not present. Cellular morphology is independent of culture conditions.

S-Layers

The outermost surface of some strains of *Aeromonas* species including *A. salmonicida*, *A. hydrophila*, *A. schubertii*, and *A. veronii* is the S-layer, or paracrystalline surface layer (formerly termed A-layer). The S-layer can be detected by electron microscopic analysis (thin section) of uranyl acetate stained cells (Figure 1a, b) (Kay and Trust 1991; Kokka et al. 1991). A single 50–53 kDa protein forms the S-layer through an entropy-driven self-assembly process: the subunits are arranged in a tetragonal array. The structural genes for the S-layers of *A. salmonicida* (*vapA*), *A. hydrophila* (*ahsA*) and atypical *A. salmonicida* ($vapA_{atyp}$) have been cloned and sequenced (Belland and Trust 1987; Chu et al. 1991; Thomas and Trust 1995a; Maurice et al. 1999). The *vapA* gene encodes a 502 amino acid protein, containing a 21-residue amino-terminal signal peptide that is cleaved following translocation of VapA across the cytoplasmic membrane. The *ahsA* gene encodes a protein that has a predicted M_r of 45 000, however the observed molecular mass of the purified protein is 52 000. This is due to post-translational modification of AhsA, as evidenced by the presence of phosphotyrosine in AhsA and also in S-layers of *A. veronii* biotype *sobria* strains (Thomas and Trust 1995a). Production of VapA is regulated by multiple promoters, and by the product of the *abcA* gene, a bifunctional protein also required for lipopolysaccharide (LPS) side-chain biosynthesis (Noonan and Trust 1997). S-layer subunits are secreted across the outer membrane by specific proteins with homology to proteins of the general secretory pathways: in *A. hydrophila* to the PulD family of type II secretion proteins and in *A. salmonicida* to a family of ATP-binding proteins (Thomas and Trust 1995b; Noonan and Trust 1997). Following assembly, the array is held in position by hydrophobic and polar interactions, and it has been proposed that the LPS O-polysaccharide side chains help to anchor the S-layer to the outer membrane, with the O-chains protruding through the S-layer array (Noonan and Trust 1997).

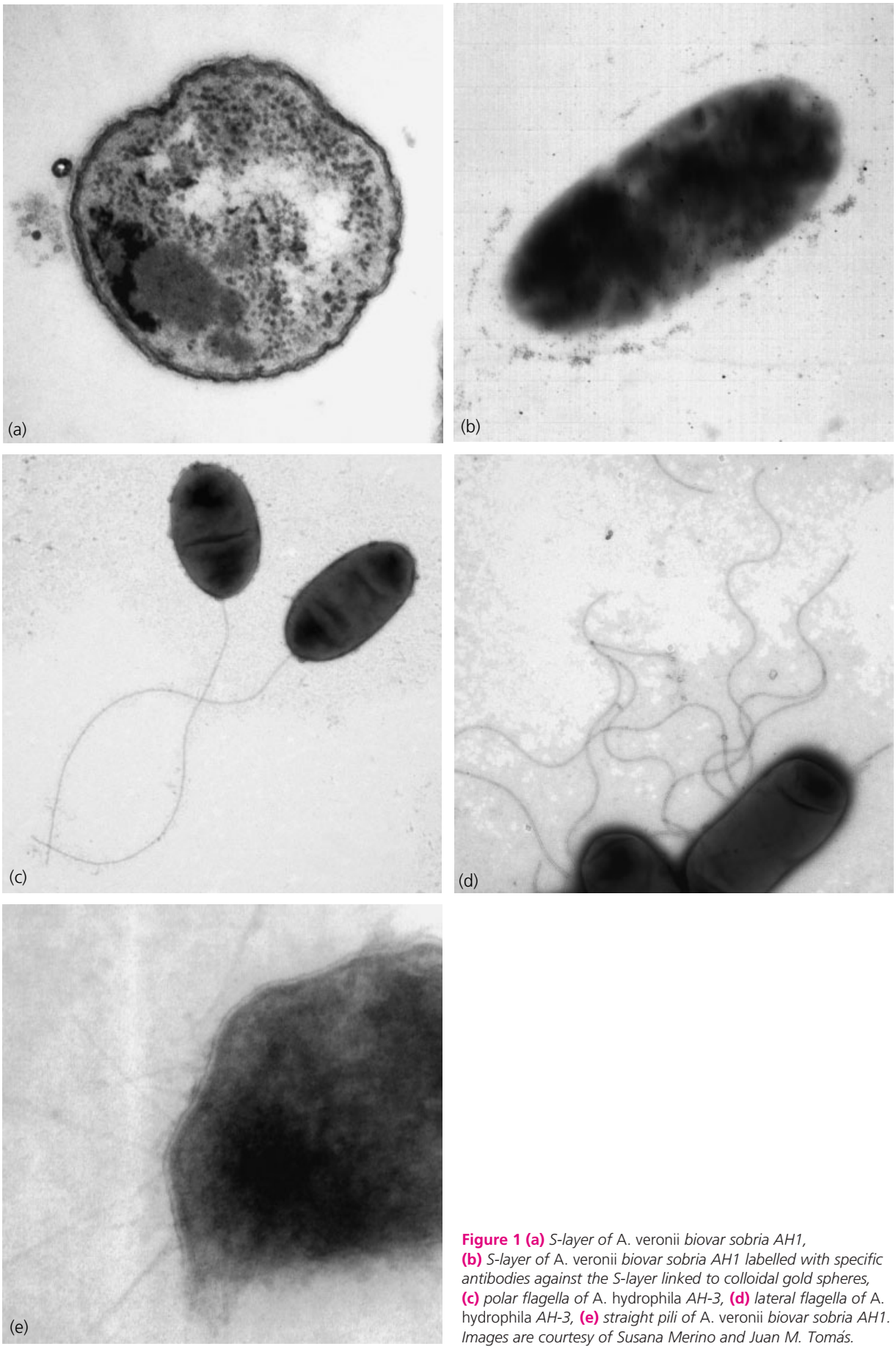

Figure 1 (a) *S-layer of* A. veronii *biovar sobria AH1,* **(b)** *S-layer of* A. veronii *biovar sobria AH1 labelled with specific antibodies against the S-layer linked to colloidal gold spheres,* **(c)** *polar flagella of* A. hydrophila *AH-3,* **(d)** *lateral flagella of* A. hydrophila *AH-3,* **(e)** *straight pili of* A. veronii *biovar sobria AH1. Images are courtesy of Susana Merino and Juan M. Tomás.*

Capsules

Capsules have been detected in several *Aeromonas* species using light microscopy of India ink preparations and electron microscopy of ruthenium red-stained thin sections (Kuijper et al. 1989; Martinez et al. 1995). The capsule of *A. salmonicida* has been shown to be composed of glucose, mannose, rhamnose, mannuronic acid, and *N*-acetylmannosamine (Garrote et al. 1992), while capsules of *A. hydrophila* strains belonging to serotypes O:11 and O:34 contained D-glucose, D-mannose, L-rhamnose, D-mannuronic acid, and acetic acid (Martinez et al. 1995). Zhang and colleagues have recently cloned the capsule gene cluster of an *A. hydrophila* O:18 isolate from a diseased fish: the 17 562 base pair sequence includes 13 genes, which are arranged into three regions similar to those found in group II capsule gene clusters of other gram-negative bacteria (Zhang et al. 2002). Region II contained five genes with homologies to capsule biosynthesis genes found in other bacteria, and regions I and III contained capsule transport genes. In a further study, two types of group II capsules were identified on the basis of their region II structure (Zhang et al. 2003). Ten out of 33 *A. hydrophila* isolates were found to produce group II capsules, with group IIA mainly found in the O:18 and O:34 serogroups, and IIB in the O:21 and O:27 serogroups.

Lipopolysaccharide

The LPS of aeromonads consists of typical lipid A–core oligosaccharide–O polysaccharide components. Electrophoretic analysis of the LPS of mesophilic strains reveals three major patterns: heterogeneous ladder-like bands typical of smooth LPS, homogeneous bands of medium length uniquely associated with the O:11 serogroup, and a fast-migrating band suggestive of a rough chemotype, seen in O:22 strains (Janda 2001). Early investigations by Shaw and Hodder (1978) identified three major core oligosaccharide chemotypes based upon the presence or absence of D-glucose, D-galactose, D-glycero-D-manno-heptose and L-glycero-D-mannoheptose in each form. Further studies have revealed the presence of some additional sugars as part of the core component, including 3-acetamido-3,6-dideoxy-L-glucose in chemotype III (Banoub and Shaw 1981) and D-glucosamine in chemotype I (Michon et al. 1984). The basic structure for each of these three chemotypes has been published (Banoub and Shaw 1981; Banoub et al. 1983; Michon et al. 1984). The O-polysaccharide structure has been determined for a few selected isolates. Chemical analysis of two serologically related strains of *A. salmonicida* and *A. hydrophila* revealed a very similar repeating unit structure with →4)-L-Rha linked (1→3) to either *N*-acetylmannosamine (*A. salmonicida*) or *N*-acetylglucosamine for *A. hydrophila* (Shaw et al. 1983; Shaw and Squires 1984). Both strains showed partial O-acetylation of either *N*-acetylmannosamine at position 4 or L-rhamnose at position 2. An *A. caviae* isolate also contained L-rhamnose linked to *N*-acetylmannosamine, but its pentasaccharide repeating unit also contained D-galactose, *N*-acetylgalactosamine and D-glucuronic acid (Linnerborg et al. 1996). The O-polysaccharide of an *A. hydrophila* O:34 strain contained *N*-acetylgalactosamine, D-mannose and 6-deoxy-L-talose (6dTal) arranged in tetrasaccharide repeating units (Knirel et al. 2002). The 6dTal exhibited an unusual random acetylation, carrying no, one or two 0-acetyl groups at any positions. Finally, *A. trota* O-polysaccharide was found to consist of hexasaccharide units containing sugars including 3,6-dideoxy-L-xylo-hexose or colitose (Knirel et al. 1996). The serological cross-reactivity of the *A. trota* strain and *Vibrio cholerae* O139 Bengal is explained by the presence of a similar colitose-containing epitope in the capsular polysaccharide of the latter.

Genetic analysis of LPS biosynthesis genes in aeromonads is limited as yet. The O-antigen gene cluster from a virulent *A. hydrophila* strain has been cloned and sequenced (Zhang et al. 2002). The cluster is 17 296 base pairs long and comprises 17 open reading frames (ORF). Genes for the synthesis of rhamnose and mannose, transferase genes and saccharide-processing genes were identified. The *abcA* gene of *A. salmonicida* encodes an ATP-binding cassette transport protein required in O-polysaccharide biosynthesis and in energizing O-polysaccharide transport to the cell surface (Chu et al. 1995).

Flagella

Mesophilic aeromonads usually possess a single, polar flagellum, but can also express multiple lateral flagella (Figure 1c, d). The constitutively expressed polar flagellum is unsheathed, and allows motility in liquid environments (Rabaan et al. 2001). Genes and loci involved in the production of polar pili have been identified in several species. A polar flagellin locus of *A. caviae* contains five genes: two flagellins are encoded by *flaA* and *flaB* (Rabaan et al. 2001). Each is a glycosylated protein with predicted size of 31.7 kDa. The two flagellins share 92 percent amino acid homology, but both are required for optimal flagellum function (Rabaan et al. 2001). A polar flagellar locus (*flg*) of *A. hydrophila* contains 16 genes, including two chemotaxis genes, but no flagellin structural genes (Altarriba et al. 2003). The region is conserved in *A. caviae* and *A. veronii* biovar sobria. The *flmA* and *flmB* genes are found in *A. caviae* and other mesophilic aeromonads, and are involved in polar flagellum assembly (Gryllos et al. 2001). An early report described peritrichous flagella on about 18 percent of mesophilic *Aeromonas* strains grown on solid medium at 22°C (Shimada et al.

1985). Optimization of growth conditions revealed that at least 50 percent of mesophilic aeromonads produce unsheathed lateral flagella (Kirov et al. 2002). Lateral flagellins were recently purified from *A. caviae* and *A. hydrophila* (Gavin et al. 2002). They are glycosylated proteins that differ from polar flagellin, and share highest amino acid homology with the lateral flagellin LafA of *V. parahaemolyticus*. Lateral flagellin genes *lafA-U* (*A. hydrophila*) and *lafA1*, *lafA2*, and *fliU* were cloned and sequenced (Gavin et al. 2002). As with the tandem polar flagellin genes, *A. caviae* has two lateral flagellin genes *lafA1* and *lafA2*, while *A. hydrophila* has only one (*lafA*). Mutagenesis studies showed that both *lafA1* and *lafA2* are required for motility in semi-solid agar, and for swarming motility on Eiken agar (Gavin et al. 2002; Kirov et al. 2002) suggesting that the *A. caviae* flagellum has a complex filament composed of both lateral flagellins. Mutations that abolished polar flagellum production apparently inhibited lateral flagellum expression, although lateral flagellin was still detectable by Western blot (Gavin et al. 2002; Kirov 2003). The *flgN* gene in the polar flagella operon of *A. hydrophila* is required for lateral flagella formation and swarming (Altarriba et al. 2003) demonstrating another link between the two flagellar types.

Despite *A. salmonicida* being defined as a nonflagellated, nonmotile species of the genus, there have been sporadic reports suggesting that some strains may exhibit motility (McIntosh and Austin 1991). Umelo and Trust (1997) presented the first genetic evidence that *A. salmonicida* is capable of producing flagella: the tandemly located *flaA* and *flaB* genes are 79 percent homologous to each other and share high homology to the polar flagellin genes of *A. caviae* (Umelo and Trust 1997; Rabaan et al. 2001). Electron microscopy showed that *A. salmonicida* can indeed express unsheathed polar flagella, albeit on less than 1 percent of cells grown under normal laboratory conditions (Umelo and Trust 1997). *A. salmonicida* also possesses a full complement of lateral flagellum *laf* genes, which are functional with the exception of the flagellin gene *lafA* (Merino et al. 2003). As *lafA* is inactivated by insertion of transposase 8, of the IS3 family, this may explain the lack of production of lateral flagella in *A. salmonicida*.

Pili

Aeromonas pili fall into two major morphological types: short, straight or rigid pili that are numerous on the cell surface, and long, flexible pili present in lower numbers (Figure 1e) (Carrello et al. 1988; Kirov et al. 1995).
Straight pili have diameters of 7–9 nm, are composed of subunits of 17–18 kDa, and are common in environmental isolates (Ho et al. 1990; Honma and Nakasone 1990; Kirov et al. 1995). The N-terminal amino acid sequence of pilin from a clinical *A. hydrophila* isolate showed homology with *E. coli* type 1 and Pap pilins (Ho et al. 1990). Unlike these *E. coli* pili, however, the straight pili do not mediate hemagglutination.

The flexible pili have diameters of 4–7 nm and are the predominant type on fecal isolates (Carrello et al. 1988; Kirov et al. 1995; Kirov and Sanderson 1996). They are type IV pili, whose pilin subunits share significant N-terminal amino acid homology. Two families of type IV pili have been identified. The Bfp (for bundle-forming pili) have pilins of 19–23 kDa (Hokama et al. 1990; Nakasone et al. 1996; Kirov et al. 1998). Their N-terminal amino acid sequences have closer homology to the type IVA pilins such as the mannose-sensitive hemagglutinin of *V. cholerae*, than to the type IVB pilin of *E. coli* Bfp (Kirov and Sanderson 1996). Unlike other type IV pili, *Aeromonas* Bfp do not mediate twitching motility (Kirov et al. 1999). A second family of type IV pili was identified following the cloning of the gene cluster *tap*ABCD from a strain of '*A. hydrophila*' (*A. bestiarum* HG2) (Pepe et al. 1996). The cluster has also been cloned from *A. veronii* biovar sobria (Barnett et al. 1997) and *A. salmonicida* (Masada et al. 2002) and has been identified in other Bfp-positive strains and in all *Aeromonas* species (Barnett and Kirov 1999). Tap pilins have a predicted size of 17 kDa and are more closely related to type IVA pilins of *P. aeruginosa* and *N. gonorrhoeae* than to *Aeromonas* Bfp (Barnett et al. 1997). A third flexible pilus is not of type IV: it is produced by an *A. hydrophila* strain at 22°C under iron-depleted conditions and is composed of a novel 46 amino acid polypeptide termed mini-pilin (Ho et al. 1990). The mini-pilin structural gene *fxp* was not detected in over 160 other *Aeromonas* strains (Ho et al. 1992; Kirov 2001). The *fxp* gene is homologous to *cep*, which encodes the virion capsid protein of CTX Phi, a filamentous phage encoding cholera toxin. It has thus been suggested that this flexible pilus may actually be a filamentous phage (Waldor and Mekalanos 1996).

Outer membrane proteins

Four outer membrane proteins of *A. hydrophila* have been purified and shown to be channel-forming proteins or porins (Jeanteur et al. 1992). Porin I, a 47 kDa OMP, was identified as a maltose-inducible maltoporin. It was subsequently cloned from *A. salmonicida*, where the size predicted from the sequence was 46 kDa (Dodsworth et al. 1993), and from *A. veronii*, where the predicted size of the 48 kDa 'Omp48' was 44 kDa (Vazquez-Juarez et al. 2003). This discrepancy may be due to association of porins with LPS, which could affect size determination on SDS-PAGE. Porin II of *A. hydrophila*, a 39 kDa protein (Jeanteur et al. 1992), exhibits highest sequence similarity to porins OmpN, PhoE, and OmpF from *E. coli* (Nogueras et al. 2000). A 43 kDa porin of *A. salmonicida* has an N-terminal amino acid sequence

identical to that of porin II (Lutwyche et al. 1995). Porin III (36 kDa) is inducible in low-phosphate medium, and resembles the general anion porin PhoE. Porin IV (27 kDa) has a unique N-terminal sequence and forms small cation-specific channels. Proteins V (30 kDa) and VI (25 kDa) were only partially characterized (Jeanteur et al. 1992). A 28 kDa OMP of *A. salmonicida*, with relatively nonselective pore-forming ability, was reported to have amino-terminal sequence homology with protein V (Lutwyche et al. 1995). This was subsequently identified as one of two *A. salmonicida* OmpA homologs which migrate as a doublet on SDS-PAGE with apparent M_r of 27/28 kDa prior to extraction with hot phenol, or 32/33 kDa following phenol extraction (Costello et al. 1996). Numerous functions have been attributed to the OmpA family of proteins, which are major OMPs of gram-negative bacteria, including maintenance of cell structure and morphology. The possession of two tandem *ompA* genes was shown to be widespread among *Aeromonas* spp. (Costello et al. 1996).

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

On nonselective agar media *Aeromonas* isolates typically appear as tan to buff-colored, smooth, convex colonies 3–5 mm in diameter after overnight incubation at 35–37°C. Most *A. salmonicida* strains involved in fish infections produce a brown water-soluble pigment on tyrosine-containing agar (Donlon et al. 1983) and rare strains belonging to other groups, in particular some biotypes of *A. media*, *A. bestiarum*, and *A. eucrenophila* elaborate a similar pigment on media such as trypticase soy agar (Janda et al. 2002; Abbott et al. 2003). Most aeromonads can grow in media containing up to 4 percent NaCl and over a pH range of 5–9 (Austin et al. 1989). Alkaline peptone water (pH 8.6), commonly used for the isolation of vibrios, can also be used to enrich for the presence of *Aeromonas* species. Because growth requirements are simple, aeromonads can be routinely cultured on many nonselective, differential, and selective agars. Many *Aeromonas* strains produce β-haemolysis on sheep blood agar, a phenotypic characteristic useful in separating aeromonads from other enteric bacilli. Some strains produce a double zone hemolysis, with an outer incomplete zone of clearing and an inner zone of complete β-hemolysis.

Classically, aeromonads have been divided into two large groups based upon differential temperature optima for growth. Psychrophilic strains, most commonly exemplified by the fish pathogens *A. salmonicida* ssp. *salmonicida*, *A. salmonicida* ssp. *masoucida*, and *A. salmonicida* ssp. *smithia*, grow best at temperatures ranging between 22 and 28°C (Austin et al. 1989). Mesophilic strains, primarily associated with human infections, grow optimally between 35 and 37°C. However, many mesophilic strains can grow at temperatures ranging between <5 and 45°C (Kirov 2001; Isonhood and Drake 2002).

METABOLISM AND PHYSIOLOGY

Aeromonads are chemo-organotrophs using a wide variety of sugars and carbon sources for energy (Popoff 1984). Glucose is metabolized both aerobically and fermentatively with or without the production of gas (CO_2, H_2). Transport systems have been characterized for sugars including maltose (Honer zu Bentrop et al. 1994), fructose and mannose (Binet et al. 1998). When grown in the presence of glucose, certain strains of *A. hydrophila*, *A. veronii* biovar sobria and *A. caviae* become nonviable after 24 h, a phenomenon termed suicide or acid toxicity. This has been attributed to catabolite repression by glucose of tricarboxylic acid (TCA) cycle enzymes, an overproduction of acetic acid and decrease in pH below 5.2 (Namdari and Cabelli 1990). Both acid toxicity and amylase production by *A. hydrophila* require the transcriptional regulator cAMP receptor protein (CRP) and are linked to cAMP levels (Kidd and Pemberton 2002). *A. hydrophila* and *A. salmonicida* also regulate gene expression through an *N*-acylhomoserine lactone-dependent quorum sensing system, which controls production of proteases and biofilm development (Lynch et al. 2002).

A number of studies have documented the existence of a viable but nonculturable (VBNC) state in *A. salmonicida* and *A. hydrophila*. In one case, storage of *A. hydrophila* in sterilized distilled water at 4°C resulted in a VBNC state after 7 weeks, while storage at 25°C caused the bacteria to enter the classical starvation survival state with little decline in viability, forming a mixture of rods and cocci (Mary et al. 2002). The extent of resuscitation from the VBNC state is still controversial (Wai et al. 2000; Mary et al. 2002).

GENETIC MECHANISMS

Physical and preliminary genetic maps of *A. hydrophila* and *A. salmonicida* have been constructed, revealing single circular chromosomes and genome sizes of 4.5 and 4.6 Mb, respectively (Dodd and Pemberton 1998; Umelo and Trust 1998). The location of six loci for degradative enzymes and ten sets of ribosomal genes were defined in the *A. hydrophila* genome, while the *A. salmonicida* map showed that some known virulence-associated genes are not clustered on the chromosome. Further details await completion of the *A. salmonicida* subsp. *salmonicida* genome project which is currently in progress (see http://wit.integratedgenomics.com/GOLD/).

In *A. salmonicida*, close to 100 percent of the strains analyzed carry multiple plasmids: small, multi-copy plasmids of 1–6 kb and large 11–150 kb plasmids (Toranzo et al. 1983; Belland and Trust 1989; Boyd et al. 2003).

Most *A. salmonicida* subsp. *salmonicida* strains carry a highly conserved group of cryptic plasmids of 5.0, 5.2, and 5.4 kb, which have now been sequenced, revealing them to be ColE1 and ColE2-type plasmids (Boyd et al. 2003). For other *Aeromonas* species the frequency of plasmid carriage is much lower. Chang and Bolton (1987) screened 75 clinical isolates and found only 20 (27 percent) to carry plasmids. Only one plasmid (pSOB1) was conjugative upon mating experiments with *E. coli*. In later studies, 39 percent of human fecal aeromonads carried plasmids (Morita et al. 1990) and transferable plasmids were detected in 33 percent of motile *Aeromonas* species from Indian clinical and environmental sources (Chaudhury et al. 1996), but plasmid carriage rate was only 16 percent in (mainly) Australian isolates (Brown et al. 1997). Some plasmids detected in *A. hydrophila* have been assigned to the IncC or IncU incompatibility groups. IncU plasmids are rarely found in genera other than *Aeromonas* (Hedges et al. 1985; Aoki and Takahashi 1987).

Plasmid-mediated antibiotic resistance in select *Aeromonas* strains includes cephalexin, cefoxitin, erythromycin, and furazolidone (Chaudhury et al. 1996), novobiocin and carbenicillin (Hanes and Chandler 1993), streptomycin and sulfonamides (Hedges et al. 1985) and kanamycin, streptomycin, tobramycin, and ticarcillin (Chang and Bolton 1987). Several classes of tetracycline resistance determinants have been described on R plasmids including *tet*A, *tet*C and *tet*D (Aoki and Takahashi 1987; Schmidt et al. 2001; L'Abée-Lund and Sørum, 2002). In addition to antibiotic-resistance markers, several other properties have been found to be plasmid-mediated in individual *Aeromonas* strains including production of a mini pilin (Ho et al. 1992), precipitation after boiling (Toranzo et al. 1983), regulation of attachment and hemolytic activities (Hanes and Chandler 1993), and production of a Shiga-like toxin (Haque et al. 1996), but none of these plasmids are widely disseminated among motile aeromonads (Brown et al. 1997). Virulence plasmids may play a more significant role in *A. salmonicida*: Type III secretion genes in *A. salmonicida* subsp. *salmonicida* are located on a 140 kb plasmid, loss of which correlates with loss of virulence for cultured fish cells (Stuber et al. 2003).

CLASSIFICATION AND TAXONOMY

The classification and nomenclature of aeromonads over the past 30 years has led to much confusion and misunderstanding in the literature regarding the role various species play in disease processes of both clinical and veterinary importance. This confusion has been exacerbated by the development of multiple molecular tools to assess the phylogenetic depth and relatedness of aeromonads and aeromonad-like organisms, leading to an expanding number of species and subspecies within the genus. This process will undoubtedly continue for the foreseeable future as microbiologists investigate aeromonads recovered from various environmental and animal sources including aquatic ecosystems, soils, insects, and fish.

Phylogenetic studies

It is now well established that aeromonads represent a unique line in the γ-subclass of the phylum *Proteobacteria*. Early studies by Colwell et al. (1986) employing 5S and 16S rRNA cataloging indicated aeromonads possessed enough phylogenetic depth and evolutionary distance from other members of the family *Vibrionaceae* (*Vibrio*, *Photobacterium*, *Plesiomonas*) to warrant their transfer to a discrete new eubacterial family, the *Aeromonadaceae*. These pioneering studies have been confirmed in subsequent phylogenetic relatedness investigations using molecular chronometers such as small-subunit (16S) rDNA and β-subunit of DNA gyrase (*gyrB*) gene sequencing (Martinez-Murcia et al. 1992; Ruimy et al. 1994; Yãnez et al. 2003). Thus, consensus agreement from a number of phylogenetic studies indicates that *Aeromonas* species represent a distinct line of bacteria in the γ-subclass from those of *Vibrio*, *Plesiomonas*, and other enterobacteria. At present the family *Aeromonadaceae* consists of two genera, *Aeromonas* and *Tolumonas*.

Classification

The classification and naming (nomenclature) of *Aeromonas* species, subspecies, and groups has a checkered history. The early history (prior to 1960) recognized two main groups or species based upon biochemical characteristics, growth temperatures, and disease syndromes. These two groups were *A. hydrophila* (motile, growth at 35°C, principally associated with human/animal diseases) and *A. salmonicida* (nonmotile, best growth at 25–28°C, fish pathogen). By the mid-1970s, at least two distinct groups within *A. hydrophila* had been detected based upon numerical taxonomy analysis of over 200 independent characters (Popoff and Véron 1976). These two groups were designated *A. hydrophila* and *A. sobria*.

By the late 1970s and early 1980s a systematic evaluation of the DNA relatedness of all aeromonads was independently undertaken by Canadian, French, and United States groups using DNA–DNA hybridization. The end result of these studies was that of the four recognized species at that time, three of these (*A. hydrophila*, *A. sobria*, *A. caviae*) were genetically heterogeneous at the DNA level, that is, multiple DNA groups (taxa) existed in each species although they could not be phenotypically separated from one another (Popoff et al. 1981). Thus these three heterogeneous nomenspecies actually contained multiple DNA groups within them and were often referred to as phenospecies.

Based on DNA pairing studies principally conducted by the Centers for Disease Control (CDC) and the Institute of Pasteur, an unofficial consensus system of naming each DNA group, or HG, was proposed to avoid confusion. The type strains of previously named species, such as *A. hydrophila*, *A. salmonicida*, *A. caviae*, and *A. sobria* constituted HGs 1, 3, 4, and 7 respectively; the remaining HGs remained unnamed since biochemical traits to clearly separate these groups from existing species were not available. Initially 12 separate DNA groups could be discerned based upon DNA–DNA reassociation kinetics. By the late 1980s, phenotypic characteristics that could separate several of these groups were discovered and a number of these taxa were named such as *A. veronii* (HG 10) and *A. schubertii* (HG 12). By the early 1990s most of these original HGs were named and the use of formal HGs became outdated as 16S rDNA sequencing and other molecular tools took center stage in the naming of species since DNA hybridization studies were more expensive, technically demanding, and more time consuming to generate results.

Table 1 lists the 16 currently named *Aeromonas* species using the nomenclature that is most familiar to microbiologists worldwide, although some of these proposed species names may be in violation of rules of the *Bacteriological Code 1990*, and therefore their taxonomic standing may be questionable. The most recently named species of the genus *Aeromonas* are *A. culicicola*, isolated from the midgut of female *Culex quinquefasciatus* mosquitoes (Pidiyar et al. 2002), *A. simiae*, isolated from monkey feces (Harf-Monteil et al. 2004), and *A. molluscorum* isolated from bivalve mollusks (Miñana-Galbis et al. 2004).

Classification issues and controversies

There are currently a large number of issues with many of the named species. This includes priority of publication (*A. enteropelogenes* = *A. trota*), deposition of type strain in two readily accessible international culture collections (*A. culicicola*), and the legitimacy of other proposed species. In addition, it does not appear feasible for many of the more recently described species to be identified using simple conventional biochemical tests. Examples would include *A. allosaccharophila*, *A. encheleia*, and *A. culicicola*. Even with older established species, such as *A. media* and *A. eucrenophila*, individual isolates can present a diagnostic challenge to the best laboratories. Finally, there is still taxonomic confusion in the medical literature regarding '*A. sobria*' and its use in commercial identification databases. The correct name for isolates with the '*A. sobria*' phenotype that are isolated from clinical material is '*A. veronii* biovar/biotype sobria'. An excellent resource for more information on such issues is the List of Bacterial names with Standing in Nomenclature (LBSN) of Euzéby (www.bacterio.cict.fr/).

LABORATORY ISOLATION AND IDENTIFICATION

Isolation

Aeromonads grow well on most media routinely used for the isolation of gram-negative pathogens. From sterile body sites such as blood or cerebrospinal fluid (CSF), or from contaminated sites where all species are typically identified (wounds), their isolation is straightforward. However, when recovery from the gastrointestinal tract is attempted, their isolation can sometimes be problematic. Many *Aeromonas* isolates (species) are sucrose-positive and/or lactose-positive and almost all strains fail to ferment D-xylose. Thus, moderate selective and differential media such as MacConkey, Hektoen enteric, and xylose lysine desoxycholate agars are unsatisfactory for the routine isolation of all fecal isolates. Many other enteric media including selective agars (brilliant green, bismuth sulfite) and eosin methylene blue are highly inhibitory to aeromonads and are therefore of little practical value either (Desmond and Janda 1986).

The approach to the recovery of aeromonads from gastrointestinal samples is dependent upon both the relative frequency of their isolation from stool samples plus the isolation rate of several other enteric pathogens such as *Yersinia enterocolitica*. If the incidence of *Aeromonas*-associated gastroenteritis is not high then the most practical approach is probably to include a blood agar plate (with or without ampicillin), as a safeguard to recover strains not picked up on moderately selective and differential media (e.g. MacConkey). Blood agar has the advantage of detection of β-hemolytic colonies, which covers most *Aeromonas* species and strains (Abbott et al. 2003). Many strains of *Aeromonas* produce characteristic 'double-zones' of hemolysis on blood agar consisting of an outer zone of partial (incomplete) hemolysis and an inner zone of complete hemolysis. Colonies growing on blood agar can be spot tested for oxidase and indole positivity, further confirming the presence of aeromonads. The addition of 10–20 μg/ml of ampicillin to blood agar helps to reduce the amount of competing microflora; however, it should be noted that 5–10 percent of all aeromonads and all strains of *A. trota* are ampicillin-susceptible and thus will be missed on media to which this antibiotic has been added.

A couple of other approaches to the isolation of aeromonads from feces are possible. If the incidence of *Y. enterocolitica* is high enough, cefsulodin-irgasan-novobiocin (CIN) agar can be used for a dual purpose as studies have shown that both pathogens can be successfully isolated off this medium (Altorfer et al.

1985). On CIN agar, aeromonads produce 'bulls eye' colonies. If the incidence of *Aeromonas* gastroenteritis is high (>1 percent), a selective medium specifically designed for the recovery of aeromonads may be warranted and Jeppesen (1995) has reviewed a number of these media.

Identification

SCREENING REACTIONS

While it is anticipated that most oxidase- and indol-positive strains recovered from the gastrointestinal tract will most likely belong to the genus *Aeromonas*, such isolates can be confused with less frequently encountered species belonging to the genera *Vibrio* and *Plesiomonas*. Because of this phenotypic similarity between these groups in rapid spot tests and the fact that commercial identification systems are notoriously poor in correctly identifying these species (Park et al. 2003), it is important to perform screening tests prior to complete biochemical characterization of isolates in a conventional or miniaturized format. There are a number of tests that are useful in placing such isolates into the appropriate genus. These tests include growth on nutrient agar with 0 percent NaCl and on thiosulphate-citrate-bile salts-sucrose (TCBS) agar, susceptibility to the vibriostatic agent O/129, gas from glucose fermentation, acid from the fermentation of sucrose and *m*-inositol, and Møeller's decarboxylase and dihydrolase tests (Abbott 2003). Once results are determined to the genus level, complete biochemical characterization can be undertaken with the appropriate choice of biochemical tests.

COMMERCIAL

There have not been any comprehensive studies published on the ability of commercial systems to correctly identify aeromonads, vibrios, and plesiomonads to species since the initial investigations of Overman et al. (1985). However, a number of case reports suggest that the accuracy of these systems is less than what would be desirable (>90 percent accuracy). Major problems include the inability of kit systems to identify aeromonads to species, distinguishing *A. caviae* from *V. fluvialis*, and the misidentification of the *Aeromonas* species (in particular the ornithine decarboxylase-positive *A. veronii* biovar veronii) as *Vibrio* species (Overman et al. 1985; Abbott et al. 1998; Park et al. 2003).

CONVENTIONAL

Conventional identification is, at present, the method of choice for identifying *Aeromonas* isolates to species in the clinical laboratory. For the most common species associated with human disease (*A. hydrophila*, *A. caviae*, *A. veronii* biovar sobria) a few simple tests that are readily available in most laboratories can accurately identify these genomospecies. These tests include Voges–Proskauer, Møeller's decarboxylase and dihydrolase reactions, gas from glucose, esculin hydrolysis, and fermentation of L-arabinose. To identify the less frequently encountered species a larger collection of tests needs to be employed, including some assays (DL-lactate, stapholysin, elastase) which are not readily available in many diagnostic laboratories. The presence of some rare species can be suspected, however, by unusual biochemical test results such as the inability to ferment D-mannitol (*A. schubertii*), susceptibility to ampicillin (*A. trota*), and acid production from D-sorbitol (*A. salmonicida*). Biochemical tests useful in the identification of *Aeromonas* species that can be isolated in the clinical laboratory are listed in Table 2. Flow charts and special tests to separate *Aeromonas* groups or complexes can be found in Abbott et al. (2003).

MOLECULAR

Over the past few years a number of probes have been developed to detect isolates belonging to the genus *Aeromonas* or specific genomospecies. These molecular assays could be used to identify isolates where conventional or commercial biochemical test results fail to provide a satisfactory answer with a high level of confidence. A digoxigenin-generic DNA probe to detect all *Aeromonas* species has been published. The probe targets a 237 bp region of the glycerophospholipid-cholesterol acetyltransferase gene (Chacón et al. 2002); similar organisms including vibrios and a number of enteric species were nonreactive with this probe. Species-specific probes or polymerase chain reaction (PCR) assays have also been developed for *A. hydrophila* (Cascón et al. 1996; Oakey et al. 1999), *A. caviae* (Khan and Cerniglia 1997), *A. jandaei* and *A. schubertii* (Ash et al. 1993), and *A. trota* (Khan and Cerniglia 1997; Khan et al. 1999). While most of these probes/assays look very promising, few have undergone rigorous analysis including testing with wild type aeromonad isolates and against nonrelated gram-negative species including vibrios, plesiomonads, and enteric bacteria.

ANTIMICROBIAL SUSCEPTIBILITY

Aeromonads are generally ampicillin resistant and susceptible to the third generation cephalosporins, aminoglycosides, tetracycline, trimethoprim–sulfamethoxazole, chloramphenicol, and newer groups or classes of agents including the quinolones, carbapenems, and monobactams (Burgos et al. 1990; Koehler and Ashdown 1993; Vila et al. 2002). Variable resistance is reported to agents including the penicillins ticarcillin and piperacillin, and to some of the first and second generation cephalosporins (Janda 2001). In some cases the variability may be due to species specificity of anti-

Table 2 *Distinguishing biochemical properties of medically relevant* Aeromonas *species*

Character	*A. hydrophila*	*A. bestiarum*	*A. salmonicida*[a]	*A. caviae*	*A. media*	*A. eucrenophila*	*A. veronii* biovar sobria	*A. veronii* biovar veronii	*A. jandaei*	*A. schubertii*	*A. trota*
Indole	+	+	+	V	+	+	+	+	+	–	+
Voges–Proskauer	+	V	V	–	–	–	+	V	+	V	–
Møeller's tests											
lysine	+	V	V	–	–	–	+	+	+	V	+
ornithine	–	–	–	–	–	–	–	+	–	–	–
arginine	+	+	V	+	V	V	+	–	+	+	+
Lipase (corn oil)	+	+	+	V	V	+	+	+	+	+	–
Glucose (gas)	+	V	V	–	–	V	+	+	+	–	V
Acid from:											
L-arabinose	V	+	+	+	+	V	–	–	–	–	–
mannose	+	+	+	V	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	–	V
D-sorbitol	–	–	+	–	–	–	–	–	–	–	–
sucrose	+	+	+	+	+	V	+	+	–	–	V
Esculin	+	V	+	V	V	V	–	+	–	–	–
DL-lactate	V	–	–	+	V	–	–	–	–	V	+
Urocanic	–	+	+	+	+	–	–	–	–	–	V
Stapholysin	V	V	V	–	–	–	–	–	–	–	–
Elastase	V	–	V	–	–	–	–	–	–	–	–

Data from Abbott et al. (2003). +, ⩾85 percent positive; –, ⩾85 percent negative; V, variable (16–84 percent positive)
a) Mesophilic strains only

microbial profiles. In vitro susceptibility to first generation cephalosporins such as cephalothin and cefazolin is species associated as most strains of *A. veronii* biovar sobria are susceptible while the majority of *A. hydrophila* and *A. caviae* isolates are resistant (Koehler and Ashdown 1993; Vila et al. 2002). As yet there are few reports that examine large numbers of the more recently recognized species. In a study of the antimicrobial susceptibility patterns of four species implicated in human disease, the majority of *A. jandaei* isolates were resistant to ticarcillin, piperacillin, and imipenem, while *A. veronii* biovar veronii isolates were 100 percent susceptible to piperacillin and 100 percent resistant to ticarcillin (Overman and Janda 1999). *A. trota* is unique among the aeromonads in being susceptible to ampicillin (Carnahan et al. 1991) and 80 percent of strains were also susceptible to cefazolin (Overman and Janda 1999). Resistance to colistin has been identified as differentiating *A. jandaei* and *A. hydrophila* (resistant or variably resistant) from other aeromonads (Fosse et al. 2003a). In contrast, Kampfer and colleagues (1999) found little evidence of species specificity in a study of strains representing 14 of the currently described species.

Variable levels of resistance may also reflect acquired resistance in isolates from particular geographical regions. Although clinical *Aeromonas* isolates have been reported to contain R plasmids these do not appear to play a major role. Ko and colleagues (1996) have reported emerging antibiotic resistance in clinical isolates in Taiwan, to agents including tobramycin, cefixime, trimethoprim-sulfamethoxazole, and tetracycline, but the origin of this resistance is unknown. Class 1 integrons mediate antibiotic resistance in environmental strains of *A. salmonicida* (L'Abée-Lund and Sørum 2001) and motile aeromonads (Schmidt et al. 2001), with gene cassettes conferring resistance to oxytetracycline, sulfonamide/trimethoprim, and aminoglycosides. These integrons are in some cases associated with conjugative R plasmids, and perhaps may provide an environmental pool of resistance genes capable of transfer to human pathogenic aeromonads.

Resistance to β-lactam antibiotics in *Aeromonas* species is often caused by inducible β-lactamases, with some strains producing multiple enzymes. Such resistance can be induced by a wide range of antibiotics including penicillin, cephalosporins (including cefoperazone), and carbapenems (Bakken et al. 1988). At least three different, chromosomally mediated β-lactamases can be produced by mesophilic aeromonads: group 1/class C cephalosporinase, group 2d/class D penicillinase and group 3/class B metallo-β-lactamase (Rossolini et al. 1996). The genes for the three β-lactamases of *A. veronii* biovar sobria have been cloned and sequenced (Walsh et al. 1998): *cepS* encodes a group 1 cephalosporinase, *AmpS* is a group 2d enzyme hydrolyzing mainly penicillins, and *imiS* encodes a class 3 metallo-β-lactamase active mainly against carbapenems. Equivalent genes have been sequenced from *A. hydrophila* and *A. jandaei*. Sequence analysis of chromosomal cephalosporinase of *A. caviae*, Cav-1, suggested it to be the putative ancestor of plasmid-mediated group1/class C β-lactamases of the FOX family found in *E. coli* and *Klebsiella* (Fosse et al. 2003b).

TYPING

A number of different methodologies have been developed to type *Aeromonas* isolates. Typing methods inherently are not used to identify isolates to species (such as a DNA probe), but rather to determine strain relatedness (identity, nonidentity) between two or more isolates. There are a number of reasons why typing methods may be employed. These include epidemiologic investigations related to potential outbreaks or pseudo-outbreaks of disease caused by aeromonads, to identify biotypes within established genomospecies, to detect biochemically unique biovars within a species which may represent a new taxon (species), and the association of specific subtypes within a species with ecologic niches, disease settings, or pathogenic potential.

Conventional

By far the simplest and least expensive method for determining strain relatedness is biotyping. This technique is underutilized today due to the advent of molecular typing procedures, yet is highly accurate if performed correctly and can be done in most laboratories. *Aeromonas* species are a very metabolically active group and can attack a wide range of substrates including carbohydrates and digest or hydrolyze numerous protein, lipid, and complex carbohydrate-like compounds including casein, elastin, starch, and esters (Abbott et al. 2003). At least two, and preferably three, biochemical test reactions should be different between two or more strains to ensure that they are not related if one uses biotyping methods to fingerprint strains. These test results should be reproducible under defined conditions. For determining new biotypes, the same rules apply except that the biochemical differences must always be to the same tests.

There are some inherent problems with biotyping. First, biochemical reactions are both temperature- and media-dependent so these factors are important to control (Altwegg et al. 1987). A second issue is that some biochemical reactions with some *Aeromonas* species appear to be linked as recently reported for the fermentation of melibiose and raffinose (Abbott et al. 2003). Finally, some laboratories may not have a number of substrates or tests that show considerable variability within the genus such as gluconate oxidation, utilization of DL-lactate, and production of the enzymes elastase and stapholysin just to name a few.

Serotyping

Another useful typing system is based upon the presence or absence of unique lipopolysaccharide antigens (somatic, O) on the cell surface. Serogrouping of aeromonads is an established typing technique since isolates possess many different combinations of somatic antigens. The most widely used serogrouping system has been that developed by the National Institute of Health (Tokyo, Japan), which consisted of 44 O-serogroups (Sakazaki and Shimada 1984). The system has subsequently been expanded to now include 96 distinct O serogroups (Korbsrisate et al. 2002).

Serotyping is a useful technique but does have a number of limitations. Few laboratories today still perform serogrouping of aeromonads. The National Institute of Health (Tokyo, Japan) has discontinued serogrouping although the O-serogroup 1 to O-serogroup 44 and R strains are available from the Japan Collection of Microorganisms, RIKEN (Wako, Saitama, Japan). Several serogroups (O:11, O:16, O:18, O:34, O:83) are also fairly common, so that existence of isolates that possess these identical antigens does not necessarily imply that they are the same strain. Finally, a fairly large percentage of strains are 'untypable' or 'rough' which makes the technique useless for determining strain relatedness for these isolates.

Molecular

Molecular methods have certainly come into vogue as the most common method used to type or fingerprint *Aeromonas* strains despite the cost, technical equipment and expertise required, and the limited association of aeromonads with outbreak disease. Common molecular methods currently utilized include restriction fragment length polymorphism (RFLP) of 16S rRNA genes and 16S–23S rDNA spacers (Borrell et al. 1997; Martinez-Murcia et al. 2000), ribotyping (Moyer et al. 1992), random amplification of polymorphic DNA (RAPD) and PCR amplification of enterobacterial repetitive intergenic consensus (ERIC) sequences (Davin-Regli et al. 1998). Pulsed-field gel electrophoresis (PFGE) has also been used in several studies to fingerprint *Aeromonas* strains (Talon et al. 1996; Borchardt et al. 2003). Satisfactory resolution has been obtained using restriction endonucleases *Xba*I, *Spe*I, and *Swa*I.

As with other techniques, there are certain limitations to molecular typing methods. Co-migration of RAPD-PCR amplicons has been noted in one study (Oakey et al. 1998) and another investigation has indicated that RFLP analysis of PCR-amplified 16S rRNA genes can produce misidentifications (Graf 1999b). Thus, all interpretations of strain relatedness based upon a single technique should be interpreted cautiously, and the ideal situation would be to use two or more distinct typing methods.

BACTERIOPHAGES AND BACTERIOCINS

Aeromonas bacteriophages belonging to the families *Myoviridae*, *Siphoviridae*, and *Podoviridae* have been identified and partially characterized (Ackermann et al. 1985; Ackermann 2001). These bacteriophages exhibit different specificities for components located on the cell surface. The receptor for some phages (LPS-core oligosaccharides, O-antigen polysaccharide, flagellum) has been identified (Merino et al. 1992). Two *Aeromonas* phages belonging to the *Myoviridae* are distantly related to coliphage T4 (Matsuzaki et al. 1999). There are no reports of well-characterized bacteriocins in *Aeromonas*, but bacteriocin-like activity has been observed, including a recent report of antibacterial activity by *A. hydrophila* against a number of gram-positive genera (Messi et al. 2003). No recognized phage or bacteriocin typing system for aeromonads has been established.

PATHOGENICITY AND VIRULENCE FACTORS

Aeromonads are involved in both intestinal and extra-intestinal human infections (Janda 1991). A large number of putative virulence factors have been identified, but in most cases definitive evidence linking these to human disease is lacking.

In gastroenteritis, the chief *Aeromonas* virulence factor in the disease process is the enterotoxin (Janda 1991). A number of distinctly different molecules produced by aeromonads have been shown to possess enterotoxigenic activity. The most common enterotoxin is a cytotoxic/cytolytic β-hemolysin, or aerolysin, produced by species including *A. hydrophila*, *A. veronii*, *A. caviae*, and *A. trota*. The structural gene *aerA* was first cloned from an *A. bestiarum* fish isolate (Howard and Buckley 1986); its homolog *actA* from a diarrheal *A. hydrophila* strain shares 89 and 93 percent DNA and amino acid homologies, respectively (Chopra and Houston 1999). Aerolysins have molecular weights ranging from 49 to 54 kDa, cause fluid accumulation in infant mice and rabbit ligated ileal loops and are rapidly lethal for mice. They are also cytotoxic for a wide variety of eukaryotic cells (Janda 2001). Recent findings provide possible mechanisms for the intestinal fluid secretion and pathology associated with *Aeromonas* infection: ActA and an aerolysin-like hemolysin of *A. veronii* biovar sobria stimulate the production of cyclic AMP (Chopra et al. 2000; Fujii et al. 2003). ActA also stimulates the production of tumor necrosis factor alpha and other proinflammatory cytokines and activates arachidonic acid metabolism in macrophages, due to initiation of several early cell signaling pathways (Chopra et al. 2000; Ribardo et al. 2002). The pro-aerolysin has been crystallized and shown to be a pore-forming toxin that oligomerizes into a ring-like

heptameric structure: this inserts into membranes and forms channels, destroying cell integrity and causing apoptosis in certain cell types (Fivaz et al. 2001). The inactive precurser binds to a variety of receptors, by recognizing the glycan cores of glycosylphosphatidyl inositol-anchored membrane proteins (Abrami et al. 2003). A second family of beta-hemolysins exhibits 45–51 percent amino acid sequence homology to the *V. cholerae* HlyA hemolysin. Aeromonad HlyA toxins have less than 20 percent amino acid sequence identity with Act/aerolysin and are larger (64–69 kDa) (Wong et al. 1998).

A number of cytotonic enterotoxins have also been described. They are nonhemolytic, and most cause either elongation of CHO cells or rounding of Y1 adrenal cells and an increase in intracellular cAMP levels, but sizes and reactivity with cholera antitoxin (CT) are variable. Non-CT-reactive cytotonic enterotoxins include heat-stable proteins of 15–20 kDa and the 71 kDa Ast protein (Chopra and Houston 1999; Sha et al. 2002). A 44 kDa heat-labile protein (Alt) also studied by Chopra and colleagues (1996) possesses some amino acid homology to phospholipase C of *A. hydrophila*. Other enterotoxins have exhibited DNA homology and/or immunological cross-reactivity with CT (Schultz and McCardell 1988; Chopra and Houston 1999) and with Shiga-like toxin 1 (Haque et al. 1996).

Aeromonads secrete a wide variety of enzymes and other molecules, the role of which in human infection is generally not yet established. They include several metalloproteases and serine proteases and a number of DNases that have been cloned and sequenced. *Aeromonas* secretes multiple lipases, including glycerophospholipid:cholesterol acetyltransferase (GCAT) that is implicated in fish pathogenicity (Janda 2001). Lecithinase (phospholipase C) was shown to be required for virulence of *A. hydrophila* O:34 strains in mice (Merino et al. 1999). *A. hydrophila* was recently reported to express enolase activity under in vivo growth conditions; enolase is a glycolytic enzyme implicated as a virulence factor in gram-positive bacteria (Sha et al. 2003). Acquisition of iron from the host is essential for virulence, and many bacterial pathogens secrete siderophores that are compounds with high affinity for iron. Aeromonads secrete at least two catechol siderophores: a novel phenolate termed amonabactin is the predominant siderophore in *A. hydrophila* and *A. caviae*, while enterobactin is present in *A. veronii* biovar sobria and *A. jandaei* (Byers and Arceneaux 1993).

Adhesion to the intestinal epithelium is likely to be an essential virulence factor for aeromonads causing enteric infections. Filamentous structures implicated as adhesins include the flexible Bfp pili and both polar and lateral flagella (Kirov et al. 1999; Gryllos et al. 2001; Gavin et al. 2002; Kirov 2003). Other potential adhesins include LPS, a 43 kDa outer membrane protein (OMP), carbohydrate-reactive porins, capsule and the S layer (Francki and Chang 1994; Merino et al. 1996; Noonan and Trust 1997; Kirov 2001; Rocha-De-Souza et al. 2003). Some strains of *A. hydrophila*, *A. veronii* biovar sobria, *A. caviae* and *A. schubertii* have been demonstrated to invade cultured epithelial cells such as HEp-2 and intestinal Caco-2 and Henle 407 (Lawson et al. 1985; Krovacek et al. 1995; Shaw et al. 1997; Granum et al. 1998; Rocha-De-Souza et al. 2003). A suggested link with dysentery or hemorrhagic colitis is not yet confirmed; inability to establish an animal model that faithfully reproduces the gastrointestinal syndrome associated with *Aeromonas* infection has hindered such investigations. One study using the removable intestinal tie adult rabbit diarrhea model has been partially able to link histological and pathological changes in the intestine with the probable site (ileum) of infection and invasion (Pazzaglia et al. 1990).

For systemic disease, results of cumulative LD50 studies in mice support clinical data indicating that strains belonging to the phenospecies *A. hydrophila* and *A. sobria* are inherently more pathogenic than *A. caviae* (Janda et al. 1994) while *A. jandaei* and *A. schubertii* are also documented causes of bacteremia (Janda and Abbott 1998). Virulence factors thought to play a role in invasive disease include S layers (Kokka et al. 1992), capsule (Aguilar et al. 1999), LPS (Merino et al. 1998), β-hemolysin activity (Chopra and Houston 1999), and resistance to complement-mediated lysis (Janda et al. 1994) which in mesophilic aeromonads is mediated by both capsular and LPS layers (Merino et al. 1998; Aguilar et al. 1999). *Aeromonas* virulence is clearly multifactorial, and it is likely that new virulence factors, such as the recently-discovered *cagA*-like gene in environmental isolates (Datta et al. 2003), await discovery.

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Campylobacter and *Arcobacter*

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CAMPYLOBACTER

DEFINITION

Campylobacter organisms are slender, spirally curved, nonspore-forming, gram-negative rods, 0.2–0.5 μm wide by 0.5–5 μm long. Cells may form coccoid bodies in old cultures or cultures exposed to air. Except for *C. gracilis* and *C. hominis*, cells are motile with a characteristic corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell (*C. showae* has multiple unipolar flagella). Campylobacters are microaerophilic to anaerobic and have a respiratory type of metabolism. They grow at 37°C but not at 15°C. Hydrogen may be required by some species for microaerobic growth. They are oxidase-positive (except *C. gracilis*). They are nonsaccharolytic; energy is obtained from amino acids or tricarboxylic-acid-cycle intermediaries. Menaquinone-6 and a methyl-substituted menaquinone-6 are the major respiratory quinones. Guanine plus cytosine (G+C) content of DNA 29–46 mol%. The type species is *Campylobacter fetus*.

HISTORICAL PERSPECTIVE AND CLASSIFICATION

The generic name *Campylobacter* (Greek, curved rod) was given by Sebald and Véron (1963) to the group of bacteria formerly known as the microaerophilic vibrios. These organisms cause two major groups of disease: fetal infections of cattle and sheep and acute enterocolitis of humans. The former was for many years the only known pathogenic activity of campylobacters; it was only during the 1970s that their role as enteropathogens came to light (Butzler et al. 1973; Skirrow 1977). It is likely, however, that the first recorded encounter with campylobacters was in 1886 when Theodor Escherich described small vibrios in the large-intestinal mucus of infants who had died of cholera infantum. His drawings leave little doubt that he was looking at *Campylobacter*

jejuni (or *Campylobacter coli*), but he was unable to cultivate them and he did not think they were pathogenic (Escherich 1886).

The first isolation of campylobacters was made in 1906 by McFadyean and Stockman (1913) from the uterine exudate of aborting sheep. A few years later Smith isolated a similar organism from fetuses of aborting cows and named it *Vibrio fetus* (Smith and Taylor 1919). The disease came to be known as vibrionic abortion. Much later, Florent (1959) showed that a form of the infection known as bovine infectious infertility was due to a variety of *V. fetus* transmitted from carrier bulls to cows during coitus. He named the organism *Vibrio foetus* var. *venerialis* (now *Campylobacter fetus* subsp. *venerealis*).

In 1927, another microaerophilic vibrio was isolated from the jejunum of calves with diarrhea and later named *Vibrio jejuni* (Jones et al. 1931). In 1944, Doyle isolated a similar vibrio from pigs suffering from swine dysentery; he named it *Vibrio coli* (Doyle 1948). These became *C. jejuni* and *C. coli*, respectively, with the formation of the genus *Campylobacter* by Sebald and Véron in 1963, although no examples of the original *V. jejuni* or *V. coli* strains had at that time survived. King (1962) carried out the first systematic examination of the few microaerophilic vibrios that had then been isolated from humans and in so doing she gave the first definitive description of the group now represented by *C. jejuni* and *C. coli* (her 'related vibrios'). These species are now known to be among the leading causes of acute bacterial diarrhea worldwide.

In the 1970s there was much confusion over campylobacter nomenclature (Skirrow 1990), but the classification of Véron and Chatelain (1973) forms the basis of currently approved nomenclature. The application of molecular methods, notably 16S rRNA sequencing, shows that the genera *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Wolinella* form a group, rRNA superfamily VI, which Vandamme et al. (1991) regarded as a distinct phylum far removed from other eubacteria. Trust et al. (1994) make a case for placing the group in the epsilon subdivision of the class Proteobacteria.

Campylobacter and *Arcobacter* are now included in the family Campylobacteraceae as proposed by Vandamme and De Ley (1991). *Campylobacter* currently contains 18 species, of which three, *C. fetus*, *C. hyointestinalis*, and *C. jejuni*, are divided into subspecies (Table 1). The taxonomy of campylobacters is reviewed by On (2001).

HABITAT

Campylobacters enjoy a widespread reservoir in nature. *C. jejuni* and *C. coli* are present in a wide variety of wild and domestic animals, especially birds. Their host adaptation is reflected by their high optimum growth temperature of around 42°C. Domestic poultry are especially prone to colonization and are a major source of human infection (Vellinga and Van Loock 2002). Pigs are the main hosts for *C. coli*. Another species belonging to this 'thermophilic' group, *C. lari*, is regularly found in seagulls. As a result of this extensive animal reservoir, virtually all natural waters are contaminated with *C. jejuni*, *C. coli*, and *C. lari*.

Figure 1 Campylobacter fetus *from a 48-h blood agar culture (×1 300)*

C. fetus subsp. *venerealis* is more strictly parasitic in that it is adapted to the bovine genital tract and not to the intestine. *C. sputorum* biovar sputorum, *C. concisus*, *C. rectus*, *C. curvus*, *C. showae*, and *C. gracilis* form part of the natural gingival flora of humans.

Figure 2 Campylobacter jejuni *from a 24-h blood agar culture (×1 300)*

Table 1 *Cultural and biochemical characteristics of* Campylobacter *and* Arcobacter

	C. fetus subsp. *fetus*	*C. fetus* subsp. *venerealis*	*C. hyointestinalis*	*C. lanienae*	*C. mucosalis*	*C. sputorum* biovar sputorum	*C. sputorum* biovar bubulus	*C. sputorum* biovar fecalis	*C. concisus*	*C. rectus*/ *C. curvus*	*C. showae*	*C. hominis*	*C. gracilis*	*C. jejuni* subsp. *jejuni*	*C. jejuni* subsp. *doylei*	*C. coli*	*C. hyoilei*	*C. lari*	*C. upsaliensis*	*C. helveticus*	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*
Wavelength of cell	M	L	L	L	L	L	L	L	--	--/L	--	--	--	S	S	S	--	S	S	M	L	M	--
Flagellar arrangement	m	m	m	a	m	m	m	m	m	m	l	--	--	a	a	a	a	a	a	a	m/a	a	m/a
Growth at: 25°C	+	+	w	–	+	–	–	–	–	–	–	–	--	–	–	–	--	–	–	–	+[a]	+[a]	+[a]
42°C	V	–	+	+	+	+	+	+	+	+	+	–	--	+	w	+	+	+	+	+	V	–[b]	V
Growth requires formate or H_2	–	–	–	--	+	–	–	–	+	+	+	--	+	–	–	–	--	–	–	–	–	–	–
Anaerobic growth with 0.1% TMAO	–	–	+	--	–	–	–	+	–	--	--	+	+	–	–	–	--	+	–	–	--	--	--
Catalase	+	+	+	+	–	–	–	+	–	–	+	–	–[c]	+	V	+	+	+	–/w	–	w	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	V	--	+	–	+	+	+	+	+	+	V	+
Nitrite reduction	–	–	–	+	V	V	V	V	+	+	+	--	+	–	–	–	+	–	–	--	–	–	–
Hippurate hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
Indoxyl acetate hydrolysis	–	–	–	–	–	–	–	–	–	+	+	–	--	+	+	+	--	–	+	+	+	+	+
H_2S production in:																							
TSI medium	–	–	+[d]	–	+	+	+	+	+	+	+	–	+	–	–	+[e]	+	+[e]	–	–	–	–	–
FPB medium	–	–	--	--	–	+	+	V	–	--	--	--	--	V	–	–	--	w	–	–	–	–	–
Growth on 0.04% TTC agar	–	–	–	–	–	–	–	–	–	--	--	–	--	+	–	+	--	V	–	--	V	V	–
Growth in the presence of																							
1.5% NaCl	V	–	–	–	–	V	+	+	+	--	--	--	--	–	–	–	+	+	–	--	+	–	–
3.5% NaCl	–	–	–	–	–	–	+	+	–	--	–	--	--	–	–	–	–	–	–	--	V	–	V
1% glycine	+	–	+	–	V	+	+	+	+	+	V	+	--	+	+	+	+	+	V	--	V	V	+
Sensitivity to:																							
nalidixic acid	R	R	R	R	R	R	R	R	R	R	R	V	S	V	S	S	S	R[f]	S	S	S	S	S
cephalothin	S	S	S	R	S	S	S	S	V	S	S	S	R	R	R	R[g]	R	R	S	S	R	V	S

Based on Table 1 in Nachamkin and Skirrow (1998) The methods used to perform these tests are stipulated by Ursing et al. (1994).
a, amphitrichate; l, lophotrichate; L, long (>1.9 μm); m, monotrichate; M, medium (1.3–1.8 μm); S, short (0.8–1.3 μm) (fixed preparations); TMAO, trimethylamine *N*-oxide; TTC, 2,3,5-triphenyltetrazolium chloride; V, some positive/sensitive, some negative/resistant; w, weak reaction; +, positive reaction; –, negative reaction; -, unknown or not applicable.

a) Also grow at 15°C.
b) Not all strains grow at 37°C.
c) Also oxidase-negative.
d) Positive only in the presence of H_2 or formate.
e) Blackening where there is water of syneresis after 7 days in the presence of H_2.
f) Urease-positive subgroup (UPTC) sensitive to nalidixic acid.
g) Occasional strains sensitive.

MORPHOLOGY

The most striking feature of campylobacters is their spirillar conformation (Figures 1 and 2). This reflects adaptation to the mucous environment of the intestinal mucosa, as spiral bacteria are able to move easily through viscous fluids (Ferrero and Lee 1988; Shigematsu et al. 1998). Most campylobacters have a single unsheathed flagellum inserted at one or both poles of the cell (monotrichate or amphitrichate). Exceptions are *C. showae*, which has up to five unipolar flagella, and *C. gracilis*, which has none. At each pole of the bacterial cell there is a thickening of the inner cytoplasmic membrane in the form of a truncated cone, tapering in *C. jejuni* and blunt in *C. fetus*. At the apex of this cone there is a cup-shaped depression occupied by a large (80–120 nm) disk pierced at its center by the hook of the flagellum. This arrangement is similar to that in *Spirillum* spp., except that in *C. fetus* and *C. jejuni* the disk has a 'cartwheel' structure consisting of 11 spoke-like arms (Curry et al. 1984). This structure is apparently unique to campylobacters.

Motility is rapid and darting, with the bacteria spinning around their long axes in a corkscrew fashion. *C. jejuni* swim at an average speed of nearly 40 μm/s compared with about 13 μm/s for *Escherichia coli* (Shigematsu et al. 1998; Karim et al. 1998). They are able to pass through a 0.6-μm membrane filter, a property used for isolating *Campylobacter* spp. from clinical samples (Bolton 2001). In some species, notably *C. jejuni* and *C. lari*, cultures that are postmature or exposed to atmospheric oxygen undergo coccal transformation (Figure 3). This seems to be a degenerative process in response to toxic oxygen derivatives and low osmolality (Harvey and Leach 1998; Reezal et al. 1998). Several studies have shown that these coccal forms do not grow on subculture, but the possibility that they can revert to spiral forms in the gut remains unanswered. The existence of viable but nonculturable (VBNC) cells is somewhat contentious, but Tholozan et al. (1999) claim to have shown that such cells have a much increased cell volume with low potassium content and membrane potential.

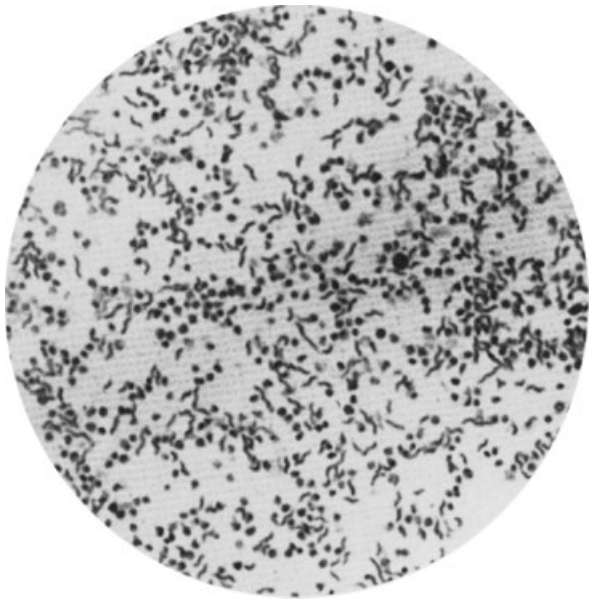

Figure 3 Campylobacter jejuni. *Predominantly coccal forms from a blood agar culture exposed to air for 24-h (×1 300)*

CULTURAL CHARACTERISTICS, METABOLISM, AND GROWTH REQUIREMENTS

Campylobacters are strictly microaerophilic and do not grow in air on primary isolation, yet oxygen (5–10 percent) is normally required for growth. Plate cultures of *C. jejuni* exposed to air have been shown to adapt to aerobic conditions (Jones et al. 1993). The oxygen sensitivity of campylobacters is due to the vulnerability of their strongly electronegative dehydrogenases to superoxides and free radicals, especially when they are in a resting state. The key to successful cultivation of campylobacters is to add supplements that scavenge these offending compounds. A simple and widely used supplement consists of ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP), each to a final concentration of 0.05 percent. The effect of media composition on the aerotolerance of campylobacters is explored and reviewed by Hodge and Krieg (1994). Most campylobacters require CO_2 (1–10 percent) for growth (Bolton and Coates 1983).

Some species, notably *C. sputorum* and *C. fetus*, can grow anaerobically in the presence of certain electron acceptors such as fumarate, aspartate, or nitrate (Véron et al. 1981); indeed, *C. sputorum* has been described as an aerotolerant anaerobe, and growth is not enhanced by adding FBP. *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, and *C. hyointestinalis* usually require hydrogen for primary growth. Hydrogen also enhances the growth of *C. jejuni* and *C. coli*, which use it as a major energy source through the action of powerful dehydrogenases (Hoffman and Goodman 1982). *C. fetus* possesses cytochromes *b* and *c*, and *C. jejuni* cytochromes *b*, *c*, and *d*. Cytochrome *c* is membrane-bound.

The fatty-acid composition of campylobacter cells is distinctive. The major fatty acids are tetradecanoic (14:0), hexadecanoic (16:0), hexadecenoic (16:1), octadecanoic (18:0), octadecenoic (18:1), and, in the case of *C. jejuni* and *C. coli*, 19-carbon cyclopropane (19:0 cyc) acids (Lambert et al. 1987; Brondz and Olsen 1991). The major isoprenoid quinones of campylobacters are menaquinone-6 and a methyl-substituted menaquinone-6 (Moss et al. 1984). The latter appears to be unique to campylobacters.

Under ideal conditions, *C. fetus* and most other campylobacters produce visible growth after 24 h at

37°C, but colonies are not well formed until 48 h. *C. jejuni*, *C. coli*, and *C. lari* grow optimally at 42–43°C and for this reason are sometimes referred to as the 'thermophilic' group, even though they are not thermophilic in the strict sense. Colonies of most species are circular and convex, but those of *C. jejuni*, *C. coli*, and *C. lari* are flat and droplet-like and tend to spread on moist agar (Figure 4). A light, even turbidity is produced in broth cultures, but if a little agar is added growth this is restricted to a band about 2 mm below the surface.

Susceptibility to physical and chemical agents

In general, campylobacters are more susceptible to adverse environmental conditions than most bacteria.

HEAT AND COLD

C. fetus and *C. jejuni* give D-values of 1 min or less at 60°C in raw, pasteurized, or skimmed cows' milk (Gill et al. 1981). Thus, conventional pasteurization procedures should kill large inocula. Exposure to temperatures of 50°C, or below 4°C, causes sublethal injury to *C. jejuni* and makes organisms unduly susceptible to rifampicin and 0.1 percent sodium deoxycholate (Humphrey and Cruikshank 1985). A similar effect is produced by exposure to ethylenediaminetetraacetic acid (EDTA), so the nature of injury is probably cell-membrane damage. Freezing and thawing causes a 1–2 $\log_{10}$ fall in viable numbers, but bacteria remain alive for many months at –20°C. *C. jejuni* can survive for several weeks in natural water at 4°C, but for much shorter periods at temperature above 15°C.

DRYING

Heavy saline suspensions of *C. jejuni*, *C. coli*, and *C. laridis* exposed to drying survive for 2–10 h at 37°C (Doyle and Roman 1982a), whereas suspensions in skimmed milk or Brucella broth dried and held at 4°C survive for several weeks (Fernandez et al. 1985).

GAMMA AND ULTRAVIOLET RADIATION

C. jejuni is at least as sensitive to γ-radiation as salmonellae (Lambert and Maxcy 1984) and more sensitive to ultraviolet (UV) irradiation than *E. coli* (Butler et al. 1987).

DISINFECTANTS

Hypochlorites, phenols, iodophors, and quaternary ammonium compounds have all been shown to kill *C. jejuni* within 1 min at standard working dilutions. Chloramine at a concentration of 1 mg/l kills *C. jejuni* at least as rapidly as it does *E. coli* (Blaser et al. 1986a).

PH

C. fetus, *C. jejuni*, and *C. coli* have a pH grow range of 5.5–8.0. At pH values below 5.0 or above 9.0 there is a progressive inactivation although some strains of *C. fetus* can grow slowly at pH 9.0.

SALT

Most campylobacters do not grow in broth or on semi-solid agar containing more than 1.5 percent NaCl. *C. sputorum* subsp. *bubulus* and biotype fecalis are the main exceptions in that they grow in the presence of 3.5 percent NaCl. *C. jejuni* and *C. coli* are progressively inactivated by concentrations higher than 1.5 percent, but the rate is temperature-dependent (Doyle and Roman 1982b). For example, at room temperature *C. jejuni* can survive for 3–5 days in the presence of 4.5 percent NaCl, whereas at 4°C it can survive for 3 weeks in 6.5 percent NaCl, albeit with a fall in viable numbers of several $\log_{10}$ values. Therefore it is possible that *C. jejuni* and *C. coli* could survive in salted uncooked meats if initial contamination was heavy (Abram and Potter 1984).

Figure 4 Campylobacter jejuni. *Surface colonies on blood agar, 24 h at 37 C °*

ASCORBIC ACID

Ascorbic acid is bactericidal to *C. jejuni* at 5 mmol/l, a concentration that is present in a wide range of foods (Juven and Kanner 1986).

GENETICS

C. fetus, *C. jejuni*, and *C. coli* have small genomes ranging in size from 1100 to 1700 kb, which is only about one-third the size of the *E. coli* genome. This is consistent with the limited biochemical repertoire of campylobacters. The genome of *C. jejuni* strain (NCTC 11168) has been sequenced (1 641 481 bp) and found to contain several hypervariable regions encoding surface structures. These probably reflect a survival strategy whereby the organism is able to live for long periods outside its animal hosts. The study of campylobacters at the molecular level has posed problems. Taylor (1992) suggested that difficulties in cloning and expressing genes from *C. jejuni* might be caused by the presence of unusual campylobacter promoter sequences not utilized effectively in *E. coli*. The latter may lack accessory genes that are needed to process campylobacter gene products, and DNA may be unstable due to differences in methylation. In order to facilitate the cloning and expression of campylobacter genes, a number of shuttle and suicide plasmid vectors have been developed (Taylor 1992). Housekeeping and antibiotic-resistance genes have been cloned, but the only virulence factors that have been studied in any detail are the flagellar genes (see section on Flagella and motility).

Plasmids ranging in size from 2.0 to 162 kb have been detected in 19–32 percent of isolates of *C. jejuni* and *C. coli*, but apart from tetracycline resistance, no function has been attributed to them.

The subject of campylobacter genetics is dealt with fully by Taylor (1992) and also by Bereswill and Kist (2002).

CELL-WALL COMPOSITION AND ANTIGENIC PROPERTIES

The historical development of this subject is described in Skirrow 1990. The outer membrane of campylobacters includes specific immunogenic proteins and lipopolysaccharide (LPS) with a lipid A portion similar to that of other gram-negative bacteria.

Surface antigens

C. fetus and *C. rectus* are unique among campylobacters in possessing a microcapsule, or S layer, consisting of high-molecular-weight proteins (90–160 kDa) arrayed in the form of a lattice. Unlike most protein antigens, it is able to resist boiling for several minutes. In the case of *C. fetus*, this S layer confers resistance to serum-mediated killing and phagocytosis, which may explain why *C. fetus* is able to cause systemic infection (Blaser and Pei 1993). It is an immunodominant antigen and it carries at least four antigenic specificities. Significantly, antigenic variation may arise in vivo in the bovine genital tract.

Nothing equivalent to this S layer has been found in *C. jejuni* or *C. coli*. These species have major outer-membrane proteins (MOMP) of MW 43–44 kDa in single isometric form (porins), which are only weakly immunogenic but cross-reactive with antisera raised against other *Campylobacter* spp. *C. fetus* strains possess two MOMPs of MW 45–47 kDa, each consisting of a single or predominant isomer. Other (heat-labile) protein antigens are expressed on the cell surface and flagella. In the case of *C. jejuni*, *C. coli*, and *C. lari* they are the dominant immunogens during infection, and they are the serodeterminants for the Lior serogrouping scheme, which recognizes some 160 serogroups. Sialylation of these surface proteins and glycolipids strongly influences serum resistance and virulence (Guerry et al. 2000a).

Peb1 is a conserved protein antigen among *C. jejuni* and *C. coli* strains to which most infected patients seroconvert (Pei et al. 1991). It would appear to be a promising vaccine candidate.

LPS (heat-stable) antigens

C. fetus has two heat-stable LPS antigens, A and B, with high-molecular-mass components. Strains of *C. fetus* subsp. *venerealis* possess the A antigen, whereas strains of subsp. *fetus* possess either the A or the B antigen.

By contrast, the LPS and lipooligosaccharide (LOS) components of *C. jejuni*, *C. coli*, and *C. lari* show great antigenic diversity. These antigens were originally thought to form the basis of the Penner serogrouping scheme of Penner and Hennessy (1980) (see below), but it now appears that the main determinants are high-molecular-weight capsular polysaccharides (CPS), which are possessed by most strains (Karlyshev and Wren 2001). Campylobacter CPSs are unusual in being heat-stable, unlike the CPSs of other gram-negative bacteria. LPS antigens are immunogenic in patients naturally infected with campylobacters, but the nature of the antibody response has received only limited study (Blaser and Perez-Perez 1992).

LABORATORY ISOLATION AND IDENTIFICATION

In a clinical context the main role of the laboratory is to detect campylobacters in the feces of patients with diarrhea. For same-day deliveries to the laboratory, feces can be transported in conventional containers, but if longer delays are anticipated, feces should be put in transport medium such as Cary–Blair medium and kept

cool. Transport medium should also be used for rectal swabs.

Direct detection in feces

In the case of liquid feces, a direct smear or wet preparation may reveal the presence of curved rods characteristic of campylobacters. For Gram staining, it is essential to counterstain with carbol fuchsin or basic fuchsin, since campylobacters do not stain well with safranin.

Commercial kits are available for the direct detection of *C. jejuni* and *C. coli* antigens in feces, for example by latex agglutination (Hindiyeh et al. 2000; Dediste et al. 2003). Molecular identification methods based on the polymerase chain reaction (PCR) can detect several species of campylobacter, including uncommon species that are difficult to culture. An advantage of PCR methods over culture is the detection and speciation of the organism on the same day, but they are expensive and labor-intensive and do not provide an isolate for typing or sensitivity testing (Kulkarni et al. 2002).

Culture and isolation

Most *Campylobacter* spp. require a microaerobic atmosphere containing about 5 percent O_2, 10 percent CO_2; and 85 percent N_2. Some species of *Campylobacter*, such as *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, and *C. hyointestinalis*, may require hydrogen for primary isolation. Commercial gas packs may not yield enough H_2 for the isolation of these species.

The conventional method for isolating the common enteric *Campylobacter* species from feces is primary plating on selective media and incubation at 42°C in a microaerobic atmosphere. There is a distinct advantage in incubation at 42°C rather than 37°C for the isolation of *C. jejuni* and *C. coli* (Gee et al. 2002). Selective media are blood-based, or blood-free- charcoal-based, and contain one or more antibiotics, mainly cefoperazone, that suppress the bacterial enteric flora. Some of the less common *Campylobacter* species are inhibited by conventional selective agents and incubation at 42°C. Therefore, where the presence of such organisms is likely, such as in patients with autoimmune deficiency syndrome (AIDS), immunocompromised patients, and deprived children in developing countries, stool filtration and culture on a nonselective medium should also be used (Lastovica and le Roux 2000; Bolton 2001).

Enrichment cultures may be beneficial in instances where few organisms may be expected, such as in feces delayed before delivery to the laboratory or in formed feces during convalescence from disease (Bolton 2001).

The isolation of campylobacters from blood and other normally sterile sites is usually fortuitous. Most blood-culture systems are capable of detecting campylobacters, usually in the anaerobic medium. The vital step is to incubate subcultures under microaerobic conditions. The same goes for specimens such as joint fluids, where cultures are likely to be negative unless microaerobic conditions are provided.

Food and environmental specimens

The isolation of campylobacters from food and environmental samples requires more complex methods. Enrichment cultures are essential and are often preceded by preincubation at 37°C to repair bacteria sublethally damaged by exposure to heat or cold (Bolton 2001).

Biochemical activities and identification

The most widely adopted approach for the identification of campylobacters is based on classical phenotypic characters that include morphological appearances, growth temperatures, biochemical reactions, and tolerances (Procop 2001). These are summarized in Table 1, p. 3.

Campylobacters are biochemically inactive compared with many other bacteria. They do not utilize sugars or produce indole, but all except *C. gracilis* produce oxidase. Most species produce catalase and all reduce nitrate to nitrite apart from *C. jejuni* subsp. *doylei*. Hippurate hydrolysis is a unique feature of *C. jejuni*, although Totten et al. (1987) found that 1.6 percent of *C. jejuni* strains were hippurate-negative. Both *C. jejuni* and *C. coli* grow at 42°C and are resistant to cephalothin and cefoperazone, which are valuable agents for inclusion in selective media. The phenotypic identification of other *Campylobacter* species can be difficult and unreliable. Consequently, molecular methods, such as DNA–DNA hybridization, PCR–DNA fingerprinting, and PCR–restriction fragment length polymorphism analysis, have been developed (Englen et al. 2003), although the complexity and the cost of these procedures preclude their use in most diagnostic laboratories.

A pragmatic approach to identification is taken in diagnostic laboratories, where virtually all isolates are *C. jejuni* or *C. coli* and their differentiation has little or no clinical significance. Only if there are epidemiological or other special reasons for speciation or typing is there a need for cultures to be referred.

Immune response and serodiagnosis

Patients infected with enteric campylobacters develop both serum and intestinal antibodies. Serum antibodies (mainly IgG and IgM) peak within 2–4 weeks after infection and then rapidly decline. In populations repeatedly exposed to infection, such as those in developing countries, IgA antibody concentrations increase progressively and this is accompanied by immunity (Blaser et al. 1986b).

In culture-negative cases of suspected *C. jejuni* infection, for example reactive arthritis, erythema nodosum, and Guillain–Barré syndrome (GBS), a serological diag-

nosis is possible. Immunodiagnostic assays are not standardized, but they are necessary for the study of postinfective sequelae and for the estimation of protective immunity in epidemiological studies. A complement-fixation test proved to be the most practicable test to detect *C. jejuni* and *C. coli* group antigens in a comparative trial by Van Duin et al. (1993). Strain-specific tests, such as the bactericidal assay, are useful for investigating outbreaks (Jones et al. 1981); enzyme-linked immunosorbent assay (ELISA) may also be adapted for this purpose. Cawthraw et al. (2002) identified several potential marker antigens indicating long-term immunity, including flagellin, the MOMPs, and a 40-kDa antigen. These antigens may be suitable for assessing immune status, although their protective status is unknown. Assays that can be carried out on saliva are awaited.

A curious antigenic coincidence is shown by about a quarter of patients with campylobacter infection, whose sera give positive reactions in the rapid microagglutination and indirect florescent antibody tests for legionellosis (Boswell 1996).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Campylobacters are normally susceptible to a variety of antimicrobial agents including macrolides, aminoglycosides, chloramphenicol, and tetracycline, but all are highly resistant to trimethoprim. *C. fetus* and several other *Campylobacter* species are sensitive to β-lactam antibiotics, but *C. jejuni*, *C. coli*, and *C. lari* are resistant to most β-lactams, including cephalosporins; some strains produce β-lactamase. Sensitivity to nitroimidazoles is a feature of several species, which is curious for nonanaerobic bacteria, but there is considerable variation between strains. *C. fetus* strains are universally resistant.

C. jejuni and *C. coli* readily acquire resistance to fluoroquinolones, which limits their use in the treatment of campylobacter enteritis. Resistance to the new quinolones is secondary to a mutation causing a change in the A subunit of DNA gyrase (Cooper et al. 2002). Erythromycin resistance is acquired less readily; resistance rates of less than 5 percent are usual for *C. jejuni* but they may rise to 80 percent in *C. coli*. Resistance to tetracycline is plasmid mediated.

Campylobacter enteritis is a self-limiting disease for which antimicrobial therapy is not usually required. However, infants and immunocompromised individuals are at higher risk of developing severe infection. In those cases, early antibiotic therapy can decrease the severity and the duration of illness. Until recently, if antibiotics were indicated for the treatment of campylobacter infection, macrolides or fluoroquinolones were considered the drugs of choice (Skirrow and Blaser 2002). However, in the past 5 years, a rapidly increasing number of *Campylobacter* strains all over the world have been found to be resistant to fluoroquinolones or macrolides (Fernandez 2001). Fluoroquinolone resistance has risen sharply since the late 1980s in Asia and Europe following the introduction of these antimicrobial agents (notably enrofloxacin) in veterinary practice (Endtz et al. 1991). The approval of fluoroquinolone use in poultry in 1995 was associated with an abrupt increase in fluoroquinolone resistance of *C. jejuni* from zero in 1990 to 13 percent in 1997 to 18 percent in 1999. The use of fluoroquinolones in the treatment of human campylobacteriosis was also found to give rise to resistance in *C. jejuni* (Fernandez 2001).

Despite decades of use, the rate of resistance of campylobacters to erythromycin remains low in industrialized countries, but resistant strains are common in developing countries, notably Thailand (50 percent) and Zimbabwe (14 percent) (Coker et al. 2002).

Susceptibility tests for *Campylobacter* spp. are not standardized (there is no recommendation by the National Committee for Clinical Laboratory Standards (NCCLS)), so there is variability in the susceptibility data reported. The agar dilution method, using Mueller–Hinton agar supplemented with 5 percent horse blood and incubated for 16–18 h under microaerobic conditions, provides a standard. As there is good correlation between disk diffusion and agar dilution methods, disk diffusion provides a reliable, easy, and inexpensive method for susceptibility testing of *C. jejuni* and *C. coli* to many antibiotics (Gaudreau and Gilbert 1997). The E test has been found to compare favorably with agar dilution methods, but it tends to produce lower minimum inhibitory concentration (MIC) values for nalidixic acid and erythromycin (Engberg et al. 1999).

TYPING METHODS

Many typing systems have been devised to study the epidemiology of campylobacter infections and they vary in complexity and ability to discriminate between strains. The most frequently used systems are biotyping and serotyping, sometimes in conjunction with phage typing. Auxotyping, protein profiling, lectin binding, and multilocus enzyme electrophoresis have been tried but without much success. Genotypic methods, such as restriction endonuclease analysis, ribotyping, and restriction analysis of PCR products can be highly discriminatory, but there is no standardization. An evaluation of phenotypic and genotypic methods for subtyping *C. jejuni* is given by Nielsen et al. (2000).

Biotyping and serotyping

Biotyping schemes based on limited biochemical tests can group *C. jejuni*, *C. coli*, and *C. lari* into broad categories. Although discrimination is low, biotyping is useful as a first step for epidemiological investigation,

and finer discrimination can be obtained by combining biochemical and resistance tests to give a numerical biotype code (Bolton et al. 1984).

Two major serotyping schemes are used worldwide: the Penner scheme (Penner and Hennessy 1980) based on heat-stable O (LPS, LOS and CPS) antigens, and the Lior scheme (Lior et al. 1982) based on heat-labile surface protein antigens using a bacterial agglutination method (160 serogroups for *C. jejuni*, *C. coli*, and *C. lari*). The two schemes are complementary and when used together they can give good discrimination even with restricted panels of antisera.

The Penner system classically uses passive hemagglutination to detect antigen–antibody reactions, which are predominantly governed by CPS antigens. Others have used direct agglutination of boiled whole cells, which detects LPS and LOS as well as CPS antigens. Comparative tests show that some strains share the same capsular and LPS or LOS antigens, whereas others appear to share a common capsular antigen but differ in their LPS or LOS antigens and vice versa (Oza et al. 2002). The general subject of serotyping based on heat-stable antigens is reviewed by Moran and Penner (1999).

The major drawback of all serotyping systems is their limited availability owing to the time and expense needed to maintain quality antisera.

Phage typing

All of the published campylobacter phage typing schemes are closely related and based on all or part of the original phage set of Grajewski et al. (1985). The phages that comprise the Salama schemes (Salama et al. 1990) are a combination of six of those from the Grajewski scheme and ten phages isolated in the UK. Frost et al. (1999) found that the use of this phage-typing scheme in conjunction with serotyping enabled them to screen large numbers of *C. jejuni* and *C. coli* isolates with a high level of discrimination.

Genotyping

Some genotyping methods, such as ribotyping, pulsed-field gel electrophoresis (PFGE), and flagellin typing (*fla* typing), are in use, but because of genetic instability (gene transfer, intramolecular recombination, nonclonality), most genotyping methods are only useful for the investigation of outbreaks. They are of little use for source tracing or global epidemiology (Wassenaar 2003). Flagellin typing and PGFE, for which standardized procedures have been developed, can be used for short-term epidemiological studies. As both methods are affected by instability of the genome, it is recommended that such typing data should be used in combination with another method, especially for strains from samples of different time and place. Internationally agreed typing schemes are urgently needed to facilitate a greater understanding of the global epidemiology of *Campylobacter* infections (Frost et al. 1999).

PATHOGENICITY AND VIRULENCE FACTORS

The main significance of campylobacters in terms of human disease is their role as enteric pathogens, but other campylobacter-associated diseases must first be considered. Expanded accounts of campylobacter-associated diseases, including those of animals, can be found in Skirrow 1998.

Reproductive disease

C. fetus subsp. *fetus* causes epizootic abortion in sheep and sporadic abortion in cattle; *C. fetus* subsp. *venerealis* is a major cause of venereally transmitted infertility in cattle (Skirrow 1994). *C. fetus* subsp. *fetus* infections originate in the gut and cause acute placentitis that is fatal to the fetus. *C. fetus* subsp. *fetus*, *C. jejuni*, and *C. coli* are rare causes of human abortion with similar pathology (Skirrow and Blaser 2002; Lastovica et al. 2002). *C. jejuni*-associated abortion has also been reported in goats, mink, dogs, and cynomolgus monkeys (Skirrow 1994).

Systemic campylobacteriosis

C. fetus is an uncommon cause of bacteremia or septicemia, sometimes with focal infection, in patients with immune deficiency or serious underlying disease. This is distinct from the transient bacteremia sometimes seen in campylobacter enteritis due to *C. jejuni* or *C. coli*, although these species can also cause septicemia and focal infection in compromised patients. *C. fetus* infection probably arises from intestinal colonization, but intestinal symptoms occur in only about one in four instances. A high incidence of thrombophlebitis and endocarditis indicates that *C. fetus* has an affinity for endovascular surfaces. An important virulence factor in *C. fetus* is its microcapsule, or S layer (see section on Surface antigens), which protects the bacteria from serum killing and phagocytosis (Blaser and Pei 1993).

Periodontal disease

C. sputorum, *C. concisus*, *C. rectus*, *C. curvus*, *C. showae*, and *C. gracilis* are often found in abundance, with other bacteria, in septic periodontal pockets, but their role in the pathogenesis of periodontal disease is unclear. *C. rectus* appears to be the most important of the group (Rams et al. 1993); it secretes a 104-kDa cytotoxin that

cross-reacts with another known dental pathogen, *Actinobacillus actinomycetemcomitans* (Gillespie et al. 1993). *C. rectus* LPS may promote periodontal tissue inflammation by stimulating the plasminogen activator–plasmin system (Ogura et al. 1995).

The human disease associations of campylobacters other than *C. jejuni* and *C. coli* are described by Lastovica et al. (2002).

Miscellaneous diseases of animals

C. jejuni, *C. coli*, and possibly other species, can cause diarrhea in some animals, notably monkeys, dogs, and probably cats. Owing to the ubiquity of campylobacters, animals develop immunity from an early age, which makes it difficult to assess the significance of infection. A similar pattern is found in children in developing countries. *C. jejuni* mastitis in cows is well documented. Campylobacters are excreted in profusion in milk from infected udders, often before abnormalities have become apparent, a feature that has far-reaching public-health consequences (Orr et al. 1995)

C. mucosalis, *C. hyointestinalis*, and *C. hyoilei* have all been found in association with the puzzling group of pig diseases known as the proliferative enteropathies. *C. hyoilei* seems to be the one most likely to be involved in its pathogenesis, but many other factors confuse the issue (Alderton et al. 1995).

Campylobacter enteritis

C. jejuni and *C. coli* are a major cause of acute enterocolitis in humans. In most industrialized countries they are, together, the most commonly identified cause of acute diarrheal illness. Other *Campylobacter* species are associated with diarrheal disease, but much less frequently. *C. upsaliensis* is the next most important (reviewed by Bourke et al. 1998). The proportion of *C. upsaliensis* among fecal campylobacter isolations ranges from 0.8 percent (UK) through 12 percent (Belgium) to 18 percent (South African children). The equivalent figures for other species are: *C. lari*, 0.1 percent (UK); *C. hyointestinalis*, 1.3 percent (South African children); and *C. jejuni* subsp. *doylei*, 0.5 percent (Belgium), 1.1 percent (Thai children), and 9.1 percent (South African children). These campylobacters are occasionally isolated from blood or internal organs, usually from patients with immune deficiency or underlying disease (Lastovica et al. 2002).

C. jejuni and *C. coli* cause an enterotoxigenic-like illness with watery diarrhea, or an inflammatory enterocolitis characterized by fever, the presence of blood and white cells in feces, and occasionally bacteremia (Skirrow and Blaser 2002). At least three mechanisms by which campylobacter may induce illness can be postulated in the basis of clinical syndromes: (1) intestinal adherence of ingested organisms and production of bacterial enterotoxins; (2) bacterial invasion and proliferation within the intestinal mucosa; and (3) extraintestinal translocation.

Cell association and invasion

C. jejuni strains have been shown to invade in vitro cell lines and translocate polarized intestinal epithelial cell lines such as Caco-2 cells (Konkel et al. 1992). They can also interact with M cells in the Peyer's patches of rabbits, which may indicate an additional pathway for entry into the submucosa. Ketley (1995) identified four distinct phenotypes among clinical strains: noninvasive, invasive, invasive with transcytosis, and transcytosis without invasion. Carvalho et al. (2001) found a 1.6-kb DNA band more frequently among *C. jejuni* and *C. coli* isolates from children with diarrhea (63 percent) than in isolates from symptom-free children (16 percent), and a fragment of 518 bp of the iam locus was amplified in 85 percent and 20 percent of isolates, respectively.

C. jejuni 81–176, which has been studied by numerous investigators, invades an intestinal derived cell line (Caco-2) and appears within membrane-bound vacuoles (Russell and Blake 1994). The mechanism of internalization is not known, but uptake of 81–176 into another intestinal cell line, INT407, was blocked by microtubule depolymerization and inhibitors of coated-pit formation, but not by inhibitors of microfilament depolymerization (Oelschlaeger et al. 1993). Uptake into the cell appears to be dependent upon bacterial protein synthesis. After uptake, there is evidence of intracellular survival of the organism (Konkel et al. 1992). Superoxide dismutase (sodB) might have some role in intracellular survival (Pesci et al. 1994). *C. jejuni* appears to produce proteins when cultured in association with eukaryotic cells, which may be important for internalization of the organism (Konkel et al. 1993). Peb1 is a conserved surface protein in *C. jejuni* and *C. coli* that may be involved in adherence (Pei and Blaser 1992) and it has homology with Enterobacteriaceae glutamine-binding protein (*glnH*), lysine–arginine–ornithine (LAO) binding protein, and histidine-binding protein (*hisJ*).

The subject of cell invasion and gut colonization is reviewed by Hu and Kopecko (2000).

Flagella and motility

Flagella and motility are important determinants for attachment and invasion. Campylobacter colonization and infection in a variety of animal models is dependent upon intact full-length flagella and active motility, although other colonization factors may be involved (Nachamkin et al. 1993).

Two genes, *flaA* and *flaB*, are involved in the expression of the flagellar filament and are arranged in tandem in both *C. jejuni* and *C. coli* (Guerry et al. 2000b). Components of intestinal mucin, particularly l-fucose, are chemotactic for *C. jejuni*, and motility towards these components may be important in the pathogenesis of infection (Hugdahl et al. 1988). Another gene, *flbA*, is involved in the synthesis of *Campylobacter* flagella and shows homology with virulence-related proteins of *Yersinia pestis* LcrD and *Salmonella typhimurium* InvA (Miller et al. 1993).

Toxins

Although several toxin activities have been described (cholera-like enterotoxin, Shiga-like toxin, hepatotoxin, cytodistending toxin) genome sequencing identified only one gene locus (cdt) with homologies to known toxins (Parkhill et al. 2000). The cytolethal distending toxin (CDT) affects epithelial morphology, causing distension and eventual cell death in in vitro models (Hassane et al. 2003). If the toxin is similarly active on rapidly dividing and differentiating cells within the crypts of the intestine, it could lead to loss of function and erosion of the epithelial layer and thereby cause diarrhea. CDT expression varies between strains (Park 2002). The subject of toxin production by campylobacters is reviewed by Wassenaar (1997).

Other factors

Several investigators have examined the ability of *C. jejuni* to acquire iron from exogenous sources. *C. jejuni* does not appear to produce its own siderophores but can utilize exogenous siderophores from other bacteria as iron carriers (Baig et al. 1986). *C. jejuni* can obtain iron from hemin and hemoglobin. Hemolytic activity described in *C. jejuni* does not appear to be iron regulated (Pickett et al. 1992). One of the iron-responsive regulatory genes, ferric uptake regulator (for), may have some role in the regulation of undetermined virulence factors (Wooldridge et al. 1994).

Postinfection sequelae

Noninfective reactive arthritis is observed in about 1 percent of patients recovering from campylobacter enteritis (Pasternack 2002). The risk of this complication is increased among people bearing the HLA-B27 allele.

During the past decade, evidence has appeared showing an association of *Campylobacter* spp. infection with acute inflammatory demyelinating polyneuropathy, otherwise known as GBS, including the Miller–Fisher variant (Mishu Allos 1998). Studies have also shown an association between campylobacter infection and an illness, clinically similar to GBS, known as acute motor axonal neuropathy (AMAN) that occurs primarily in northern China and may occur in other parts of the world (McKhann et al. 1993). GBS affects one to two people per 100 000 population in the USA each year. Although *C. jejuni* infections are a common trigger of GBS, the risk of developing GBS is less than 1 case per 1 000 campylobacter infections. Patients with campylobacter-associated GBS are, on average, more severely affected than those without such an association; they often require intensive hospital treatment and end up with long-term disability. Several studies show that the disease is particularly associated with campylobacter serotypes Penner type O:19 in Japan and the USA and Penner type O:41 in South Africa. Specific glycopeptide moieties on the campylobacter lipopolysaccharide closely resemble normal human ganglioside, such as GM1, present in peripheral nerve myelin and it is postulated that enteric infection triggers the production of cross-reactive antibodies, which then attack normal structures (Asbury 2000; Nachamkin et al. 2002).

SUMMARIZED DESCRIPTIONS OF SPECIES OF CAMPYLOBACTER

Campylobacter fetus: *C. fetus* (Smith and Taylor 1919; Véron and Chatelain 1973) is the type species of the genus. There are two subspecies: subsp. *fetus* and subsp. *venerealis* (Florent 1959), which are difficult to distinguish. The latter fails to grow with 1 percent glycine or produce H_2S from cysteine with lead acetate paper indicator. Cells are 0.3–0.5 μm wide by 2–3 μm long, with occasional longer filamentous forms. It is microaerophilic but can grow anaerobically in the presence of fumarate or nitrate; it grows at 25°C and some strains grow at 42°C. Colonies are circular, 1–1.5 mm, convex, translucent, and nonspreading. The species is sensitive to penicillins, including benzylpenicillin, and to most cephalosporins, but resistant to nalidixic acid and metronidazole. It possesses a group-specific glycoprotein microcapsule or S layer with antiphagocytic properties. *C. fetus* causes reproductive disease in cattle and sheep, and occasionally systemic infection in immunodeficient or otherwise diseased humans. Average genome MW 1.56×10^9. G+C content of DNA 35.5 ± 1.0 mol%. Type strain CIP 5396.

Campylobacter hyointestinalis: *C. hyointestinalis* (Gebhart et al. 1985) is phenotypically similar to *C. fetus* but produces H_2S in freshly prepared triple sugar iron (TSI) or sulfide–indole–motility (SIM) media in the presence of H_2 or formate. There are two subspecies: subsp. *hyointestinalis* and subsp. *lawsonii* (On et al. 1995a), which differs genetically and in its failure to grow in the presence of 1.5 percent bile. Colonies scraped from agar cultures appear yellow. Subsp. *hyointestinalis* is found in pigs with proliferative enteropathy and in various other animals; also in humans with diarrhea and proctitis,

but its pathogenicity is uncertain. Subsp. *lawsonii* has been isolated only from pig stomachs. G + C content of DNA 32–36 mol%. Type strains ATCC 35217 (subsp. *hyointestinalis*) and NCTC 12901 (subsp. *lawsonii*).

C. lanienae: *C. lanienae* (Logan et al. 2000) is closely related to *C. hyointestinalis* subsp. *lawsonii* and phenotypically the two cannot be distinguished from one another. It was first isolated from the feces of healthy abattoir workers (Logan et al. 2000) and has since been isolated from the feces of healthy pigs (Sasaki et al. 2003). G + C content of DNA 36 mol%. Type strain NCTC 13004.

Catalase-negative group

Campylobacter mucosalis: *C. mucosalis* (Roop et al. 1985a) was first described by Lawson and Rowland (1974) and formerly regarded as a subspecies of *C. sputorum*. It is catalase-negative and similar to *C. sputorum* but requires hydrogen or formate for growth. Colonies are a dirty yellow color. Three serogroups (A, B, and C) have been described. It is found in large numbers in the intestinal mucosa of pigs with porcine intestinal adenomatosis and related diseases, but disease is not reproduced by inoculation of pure cultures. G + C content of DNA 38 mol%. Type strain NCTC 11000.

Campylobacter sputorum: *C. sputorum* (Prévot 1940; Roop et al. 1985b) was first isolated by Tunnicliff (1914) from a patient with acute bronchitis. It was originally regarded as an anaerobe but it is really a facultative microaerophile. Cells are curved rods 0.3–0.6 μm wide by 2–4 μm long, occasionally filamentous. It is monotrichate. Colonies are 1–2 mm in diameter, circular, gray, and convex; they may produce greening on horse-blood agar. Most strains reduce nitrite as well as nitrate and produce H_2S from TSI medium. G + C content of DNA 29–31 mol%. There are three biotypes:

1. Biotype sputorum – catalase-negative, accounting for about 5 percent of cultivable organisms isolated from human gingival crevices and can be isolated from the feces of about 2 percent of healthy people. It is essentially a commensal, though occasionally it has been isolated from blood cultures and once from a leg abscess. Type strain ATCC 35980.
2. Biotype bubulus – differs by producing abundant H_2S from TSI and SIM media and growing in the presence of 3.5 percent NaCl. It was first mentioned by Prévot (1940) who called it '*V. sputorum* var. *bubulum*.' It can be isolated from the genital tract of healthy cattle and sheep of both sexes. It has not been implicated in disease but it may be confused with the pathogen *C. fetus* subsp. *venerealis*. Type strain CIP 53103.
3. Biotype fecalis – resembles biotype bubulus except that it is catalase-positive. Originally described by Firehammer (1965) as '*V. fecalis*,' it can be isolated from sheep feces and the genital tract of cattle. It probably causes enteritis in sheep and cattle. Enteritis has been produced experimentally in calves. Type strain NCTC 11415.

Campylobacter concisus: *C. concisus* (Tanner et al. 1981) is difficult to distinguish from *C. mucosalis*, with which it has been confused. Strains formerly known as EF group 22 are of this species (Vandamme et al. 1989). Cells are 0.5–1.0 μm wide by 4 μm long and gently curved. Colonies are small, convex, and translucent. It is isolated from gingival cervices and periodontal lesions and is commonly found in human feces, but rarely in blood. Its pathogenicity is unknown. G + C content of DNA 34–38 mol%. Type strain ATCC 33237.

Campylobacter rectus* and *Campylobacter curvus: *C. rectus* (Tanner et al. 1981) and *C. curvus* (Tanner et al. 1984) were originally placed in the genus *Wolinella*; these species are now classed as campylobacters (Vandamme et al. 1991). Like *C. sputorum* and *C. concisus* they are catalase-negative and found in gingival crevices and periodontal lesions, but they are oxidase-positive only in the modified test of Tarrand and Gröschel (1982). They require formate or fumarate. The two species may be separated by their sensitivity to various dyes and by their morphology. Cells of *C. rectus* are usually straight, 0.5–1.0 μm wide, and less than 4 μm long, and they possess a high-molecular-weight S layer. Both species produce three types of colony according to the medium: convex, flat spreading, and agar corroding. Corroding colonies may be mistaken for those of [*Bacteroides*] *ureolyticus* and *C. gracilis*. Their pathogenicity is unknown. G + C content of DNA 42–46 mol%. Type strain for *C. rectus* NCTC 11489; *C. curvus* NCTC 11649.

Campylobacter showae: *C. showae* (Etoh et al. 1993) is similar to *C. rectus* but it has two to five unipolar flagella and is catalase-positive. It is microaerophilic in the presence of fumarate with formate or hydrogen, but grows best anaerobically. Previously described as *Wolinella*-like strains from human subgingival dental plaque and infected root canals, its pathogenicity is unknown. G + C content of DNA 44–46 mol%. Type strain ATCC 51146.

Campylobacter hominis: *C. hominis* (Lawson et al. 2001) is a fastidious, slow-growing, essentially anaerobic species that shares with *C. gracilis* the unusual feature (for campylobacters) of being nonmotile. Cells are short straight aflagellate rods. It can be detected in the feces of about 30 percent of healthy people. G + C content of DNA 32–33 mol%. Type strain NCTC 13146.

Campylobacter gracilis: *C. gracilis* (Tanner et al. 1981) was previously called *Bacteroides gracilis* but it was transfered to *Campylobacter* by Vandamme et al. (1995). Atypically for campylobacters it is oxidase-negative, but like other campylobacters it possesses

cytochromes b and c and CO-binding cytochrome c; it does not possess detectable type a and d cytochromes. Cells are straight aflagellate nonmotile rods. It is most closely related to *C. concisus*, *C. rectus*, *C. curvus*, and *C. showae*, based on 16 rRNA sequencing, and shares many of their characters. Found in gingival crevices and has been isolated from visceral, head and neck infections in humans. G + C content of DNA 44–46 mol%. Type strain ATCC 33236.

[Bacteroides] ureolyticus: According to Vandamme et al. (1995) this bacterium (Jackson and Goodman 1978) is a member of the family Campylobacteraceae. It differs from campylobacters in its fatty-acid composition, proteolytic metabolism, and its ability to hydrolyse urea. Isolated from patients with superficial ulcers, soft-tissue infections, and urethritis. G + C content of DNA 28–29.5 mol%. Type strain NCTC 10941, ATCC 33387.

'Thermophilic' campylobacters

Principal species have an optimum growth temperature higher than 37°C and are able to grow at 43°C, but not at 25°C. Most are small spiral rods, 0.3–0.5 μm wide by 2–3 μm long, with short wavelength and single bipolar flagella (amphitrichate). They readily undergo coccal transformation.

Campylobacter jejuni: *C. jejuni* (Jones et al. 1931; Véron and Chatelain 1973) is the only campylobacter to hydrolyse hippurate. There are two subspecies. *C. jejuni* subsp. *jejuni* causes campylobacter enteritis in humans and some animals, and also abortion in sheep. Colonies have a strong tendency to spread on moist agar; postmature growth develops a 'metallic' sheen. The average genome MW is 2.16×10^9. G + C content of DNA 31.2 ± 1.1 mol%. Type strain CIP 702. *C. jejuni* subsp. *doylei* (Steele and Owen 1988) is a less common and more fastidious organism mainly found in the feces, and occasionally blood, of socially deprived children with diarrhea. Filtration methods and incubation at 37°C are necessary for primary isolation. It does not reduce nitrate. G + C content of DNA 29 ± 1 mol%. Type strain NCTC 11951.

Campylobacter coli: *C. coli* (Véron and Chatelain 1973) was putatively first isolated by Doyle (1948) from piglets with swine dysentery. Pigs are the main host, but *C. coli* is also commonly found in avian and other animal species. It is closely related to *C. jejuni*, but it does not hydrolyse hippurate. Colonies are like those of *C. jejuni* but there is less tendency to swarm, or form a 'metallic' sheen on postmature growth. Its pathogenicity is similar to that of *C. jejuni*. G + C content of DNA 32.6 ± 1.0 mol%. Type strain CIP 7080.

Campylobacter hyoilei: *C. hyoilei* (Alderton et al. 1995) is closely related to *C. coli* and is distinguished by its ability to reduce nitrite and produce H_2S in TSI medium. Isolated from intestinal lesions of pigs with proliferative enteritis. G + C content of DNA 35 ± 1.5 mol%. Type strain CCUG 33450.

Campylobacter lari: *C. lari* (Benjamin et al. 1983) was first isolated from the cloacal contents of seagulls of the genus *Larus*. Originally named *C. laridis* and later changed to *C. lari*, it is commonly found in birds and a wide variety of other animals, notably dogs, and in natural water. It is common in seawater and shellfish. Some of the latter strains belong to a urease-positive subgroup (UPTC) described by Bolton et al. (1985). *C. lari* accounts for only about 0.1 percent of human campylobacter infections, but it was the apparent cause of an outbreak of water-borne enteritis in Canada. *C. lari* and UPTC strains have been isolated from the blood of patients with underlying diseases. G + C content of DNA 32.1 ± 0.5 mol%. Type strain NCTC 11352.

Campylobacter upsaliensis: *C. upsaliensis* (Sandstedt and Ursing 1991) was initially referred to as 'CNW' (catalase-negative or weakly reacting) campylobacter. Like *C. jejuni* subsp. *doylei*, these organisms are best isolated from feces by membrane filtration at 37°C. Seven serogroups are recognized. Isolated from the feces of dogs with or without diarrhea, the feces of human patients with diarrhea, and occasionally from the blood of patients with underlying disease. G + C content of DNA 35–36 mol%. Type strain NCTC 11541.

Campylobacter helveticus: *C. helveticus* (Stanley et al. 1992) is closely related to *C. upsaliensis* and is distinguished by its inability to grow on potato-starch medium or to reduce selenite. The flat translucent colonies are unusual in being adherent on blood agar, and they may have a blue-green hue. Some O antigens are shared with *C. upsaliensis*. This species is commonly present in the feces of domestic cats, and less commonly in dogs. Pathogenicity unknown. G + C content of DNA 34 mol%. Type strain NCTC 12470.

ARCOBACTER

DEFINITION

Gram-negative, motile, nonspore-forming, spirally curved rods, 0.2–0.9 μm wide by 1–3 μm long. Cells in old cultures form coccal bodies. A single unsheathed polar flagellum (monotrichate) imparts a rapid corkscrew-like motility. Growth range usually 15–37°C but some strains grow within the range 5–42°C; optimal growth at 30°C under microaerobic conditions. Grow aerobically and anaerobically after primary isolation. Respiratory metabolism; hydrogen not required. Non-saccharolytic. Oxidase- and catalase-positive. G + C content of DNA 27–31 mol%. Type species *Arcobacter nitrofigilis*.

HISTORY AND CLASSIFICATION

Arcobacters were first described in the 1970s by Ellis and colleagues, who isolated them from aborted fetal tissues from pigs and cattle (Ellis et al. 1977; Ellis et al. 1978). Because of their similarity to campylobacters and their

ability to grow aerobically at 30°C, they were originally considered to be aerotolerant campylobacters (Neill et al. 1979) and a new species, *Campylobacter cryaerophila* (Greek *cruos*, cold; *aer*, air; *philos*, friend), was proposed to include them (Neill et al. 1985). Later, Thompson et al. (1988) showed, by partial 16S rRNA sequence analysis, that *C. cryaerophila* and *C. nitrofigilis* (a nitrogen-fixing organism isolated from roots of a salt-marsh plant) exhibited 87 percent homology with each other and 68 percent homology with other campylobacters. This called for the formation of a new genus, which was subsequently defined and named *Arcobacter* (bow-shaped rod) by Vandamme et al. (1991), and the two species became *Arcobacter cryaerophilus* and *A. nitrofigilis*, respectively (*A. nitrofigilis* designated the type strain).

At the same time, Kiehlbauch et al. (1991) independently described two distinct genetic groups among 78 aerotolerant campylobacters isolated from humans, primarily with diarrheal illness, and animals on several continents. Group 1 contained the type strain of *C. cryaerophila*, but most strains belonged to group 2, which was named *C. butzleri*. The *C. cryaerophila* group was divided into two DNA hybridization subgroups designated 1A and 1B. Later, *C. butzleri* was reclassified as *Arcobacter butzleri*, and a novel species, *A. skirrowii*, was proposed for another group of animal-associated *Arcobacter* strains (Vandamme et al. 1992b; Vandamme and Goossens 1992). Thus, three species have been isolated from humans and animals: *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*; of these, *A. butzleri* seems to be the main human pathogen.

HABITAT

Arcobacters are widely distributed in nature. Natural waters are frequently contaminated with arcobacters and are a potential source of infection. They have been isolated from raw and effluent sewage and drinking-water reservoirs in Germany. They have also been isolated from mussels (Houf 2001).

Arcobacters have also been found in the feces of various animals, notably cattle, sheep, pigs, and poultry (Mansfield and Forsythe 2000). Domestic poultry are especially prone to colonization. Houf et al. (2001a) isolated them from 100 percent of neck-skin samples from laying hens, and they have been found in 0–97 percent of poultry carcasses. *A. butzleri* was the predominant species, but *A. cryaerophilus* was also represented. Molecular studies show a great genetic diversity among such strains and their origin remains unclear (Houf et al. 2002). Arcobacters have also been found in animal products such as ground pork (89 percent positive in a study by Collins et al. 1996). The *Arcobacter* type strain, *A. nitrofigilis*, has been found only on the roots of a small marsh plant, *Spartina alterniflora*, in Nova Scotia (McClung et al. 1983), but it is conceivable that it may have an animal host.

Morphology

Arcobacters are gram-negative, nonspore-forming, S-shaped, or spirally curved rods 0.2–0.9 μm wide by 1–3 μm long (Figure 5); occasional cells as long as

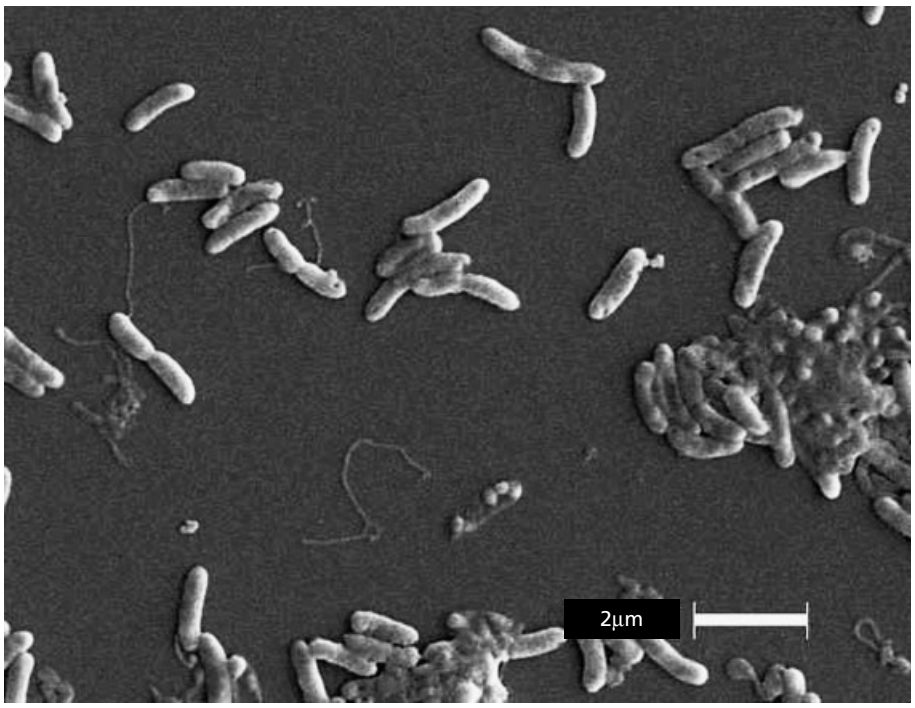

Figure 5 *Scanning electron micrograph of* Arcobacter butzleri. *Courtesy of Professor Heriberto Fernandez, Institute of Clinical Microbiology, Universidad Austral de Chile, Valdivia, Chile*

20 μm have been described. Cells may form spherical coccoid bodies in old cultures or cultures exposed to air. Arcobacters possess a single unsheathed polar flagellum (monotrichate configuration); dividing cells may appear amphitrichate (Figure 5). The ultrastructure is probably like that of campylobacters, but specific studies have not been done. The small size and active motility of arcobacters, like campylobacters, enables them to pass through membrane filters that retain almost all other bacteria, a feature that is used to advantage for their isolation.

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

Optimal growth occurs at 30°C under microaerobic conditions (3–10 percent oxygen). Arcobacters have a respiratory type of metabolism and use organic and amino acids as carbon sources. Carbohydrates are not fermented or oxidized. They are oxidase-positive and hydrogen is not required for microaerobic growth. After primary isolation in a microaerobic environment, growth is possible in aerobic or anaerobic atmospheres. Growth temperature ranges are difficult to define owing to variation in cultural conditions and individual laboratories. There is also variation between species. All grow between 15 and 36°C, although *A. nitrofigilis* strains are struggling at 36°C (Kiehlbauch et al. 1991). *A. butzleri* and *A. skirrowii* are the most thermotolerant species, all strains growing at 40°C and some at 42°C (On et al. 1996). Almost 40 percent of *C. cryaerophila* strains grow at 4°C (Boudreau et al. 1991) or 5°C (Neill et al. 1985). Biochemical activity is modest.

Colonies of *A. butzleri* and *A. cryaerophilus* grown for 48 h under optimal conditions are, respectively, 2–4 mm and 1–3 mm in diameter and are convex with an entire edge; swarming may occur on moist agar. Colonies of *A. butzleri* are whitish, whereas those of *A. cryaerophilus* tend to be dirty yellow. Colonies of *A. skirrowii* grown for 48 h under optimal conditions are 1–3 mm in diameter, with a flat irregular edge and grayish appearance; growth is usually sparse. Most strains produce greening ('alpha-hemolysis') on sheep- and horse-blood agar, as do some strains of *A. butzleri*.

Susceptibility to physical and chemical agents

Although arcobacters, like campylobacters, appear more sensitive to adverse conditions such as heat, acidity, drying, disinfectants, and irradiation than enterobacteria, evidence is sparse. Arcobacters are slighly more tolerant of alkaline conditions than campylobacters; almost all strains tolerate pH values of 6.8–8.0 and some can survive 5.5–9.5. Otth et al. (2001) found *A. butzleri* to be more resistant to desiccation than the classical enteropathogenic *Campylobacter* species. Phillips (1999) found that the growth of *A. butzleri* was inhibited by 0.5 percent, 1.0 percent, and 2.0 percent lactic and citric acids. Sodium citrate (2 percent), and to a lesser degree sodium lactate, inhibited growth for 8 h, but not for longer periods.

GENETICS

The arcobacter genome has not yet been studied.

CELL-WALL COMPOSITION AND ANTIGENIC PROPERTIES

Little is known. Tsang et al. (1996) identified, by Western immunoblot, a hemagglutinin in the form of an immunogenic protein of about 20 kDa. The hemagglutinating activity is sensitive to proteolytic enzyme digestion and heat treatment of 80°C and above. This arcobacter hemagglutinin is possibly a lectin-like molecule that binds the erythocytes via a glycan receptor containing D-galactose as part of its structure.

LABORATORY ISOLATION AND IDENTIFICATION

Fecal specimens destined for the laboratory should be handled and treated in the same way as for campylobacters. The primary culture of arcobacters from clinical material is a more complex and lengthy procedure than it is for *C. jejuni* and *C. coli*. Arcobacters are sensitive to polymyxins and rifampicin, which are used in several conventional campylobacter-selective media, and they require lower incubation temperatures. Selection may be attained by inoculating media containing alternative antimicrobials, or by membrane filtration on nonselective agar, or by a combination of both. A recommended protocol is to use selective enrichment followed by plating on selective agar (Vandenberg et al. 2002). Stool samples are inoculated into an enrichment broth (Brucella broth + antibiotic supplement consisting of piperacillin 75 mg/l, cefoperazone 25 mg/l, amphotericin B 10 mg/l, trimethoprim 20 mg/l, cycloheximide 100 mg/l + 5 percent laked horse blood) and incubated for 24 h at 25°C in a microaerobic atmosphere (5 percent O_2; 6 percent H_2; 10 percent CO_2; 79 percent N_2). After incubation, 40 μl of enriched broth is inoculated on arcobacter selective medium (De Boer et al. 1996). The plates are incubated for 3 days at 25°C in a microaerobic atmosphere and the plates are examined daily.

Food and environmental specimens

Several protocols have been described for the isolation of arcobacters from food and the environment since they were first isolated from bovine and porcine fetal tissues

on leptospira (EMJH) medium (Ellis et al. 1977). Enrichment cultures are an essential part of each. Various selective antimicrobial cocktails have been used. Most contain cefoperazone with one or more other agents, such as vancomycin, piperacillin, and teicoplanin (Houf 2001). Houf et al. (2001a) described a new fast and reliable method for the isolation of arcobacters from poultry using amphotericin B, cefoperazone, 5-fluorouracil, novobiocin, and trimethoprim as selective agents in a new enrichment procedure. This method was validated for the isolation of all three *Arcobacter* species and showed the value of enrichment, which increased the isolation rate from 50 to 73 percent.

Identification

The identification of arcobacters, like campylobacters, remains difficult because of cross-specific phenotypic similarities and relative biochemical inertia. Tolerance and sensitivity tests are of some help, but are inherently prone to error. The most reliable feature that differentiates arcobacters from campylobacters is the ability of arcobacters to grow in air and at 15°C, but it is not always possible to distinguish between arcobacter species by phenotypic characters alone. Most *A. butzleri* strains are only weakly catalase-positive, grow in 1 percent glycine and 1.5 percent NaCl, and are resistant to cadmium chloride (2.5 μg disk), whereas *A. cryaerophilus* strains show strong catalase activity and sensitivity to cadmium chloride (Schroeder-Tucker et al. 1996). Other differentiating characters are shown in Table 1, p. 3.

The definitive identification of *Arcobacter* spp. must rely on recently developed molecular methods, which have considerably facilitated and speeded up the process, although they usually require the skills of a specialist laboratory (Marshall et al. 1999; Houf et al. 2002). The use of 16S rRNA-based DNA probes, PCR assays, ribotyping, and restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA or 23S rRNA all differentiated *A. butzleri* from other arcobacters but did not differentiate *A. cryaerophilus* from *A. skirrowii* (Mansfield and Forsythe 2000). These three organisms were easily identified by a multiplex PCR assay (Houf et al., 2000).

TYPING METHODS

A coordinated serological typing scheme for arcobacters has yet to be developed. Boudreau et al. (1991) recognized 18 antigenic types of *C. cryaerophila* based on formalinized whole-cell (FWC) suspensions. Lior and Woodward (1993) recognized 65 serogroups among 427 *A. butzleri* isolates also based on FWC suspensions. No cross-reactivity was observed against *C. jejuni*, *C. coli*, and *C. lari*. Strains of serogroups 1 and 5, followed by 2, 6, and 12 were found to be the most common among human isolates.

Genotyping methods, such as PCR-mediated DNA fingerprinting (Vandamme et al. 1993), are more successful, but they are varied and specialized and lack standardization. Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis is useful for discriminating between arcobacter strains isolated from poultry carcasses (Houf et al. 2002).

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Susceptibility tests for *Arcobacter* spp. are not standardized; consequently, the literature contains some variation in the susceptibility data reported. Recommendations for the agar dilution method include using Mueller–Hinton agar supplemented with 5 percent horse blood, incubated for 16–18 h under microaerobic conditions. Like campylobacters, arcobacters are inherently resistant to trimethoprim and vancomycin, both agents used in campylobacter selective media. However, they are sensitive to polymyxins (e.g. colistin) and rifampin at concentrations used in several such media (Houf et al. 2001b). *A. skirrowii* is the most susceptible species. 5-Fluorouracil, novobiocin, and teicoplanin are without any inhibitory effect on arcobacters at concentrations effective for their selective isolation.

Kiehlbauch et al. (1992) evaluated the in vitro activities of 22 antimicrobial agents against 78 human and animal isolates belonging to two aerotolerant *Arcobacter* species, *A. cryaerophilus* and *A. butzleri*, using a broth microdilution technique. Most isolates were resistant to cephalosporin antibiotics, with the exception of cefotaxime. *C. cryaerophila* DNA hybridization group 1A isolates were generally susceptible to the tetracyclines, chloramphenicol, nalidixic acid, azythromycin, erythromycin, and roxythromycin and moderately susceptible to clindamycin and ampicillin. The MICs of tetracyclines were higher for *A. butzleri* and *A. cryaerophila* DNA hybridization group 1B isolates than for *C. cryaerophila* DNA hybridization group 1A isolates, but most strains were still susceptible to doxycycline and tetracycline; all isolates were susceptible to minocycline. Surprisingly in this study, *A. butzleri* and *A. cryaerophila* DNA hybridization group 1B isolates were generally resistant to macrolide antibiotics (including erythromycin).

Using a disk diffusion method, Atabay and Aydin (2001) tested 39 *A. butzleri* strains isolated from broiler chickens for their susceptibility to 23 antimicrobial agents. All isolates were resistant to aztreonam, cefuroxime, cephalothin, oxacillin, penicillin G, and trimethoprim-sulphamethoxazole. Of the 39 isolates tested, 26 were also resistant to ampicillin, amoxycillin, and amoxycillin/clavulanic acid. One isolate was resistant to and four showed intermediate resistance to erythromycin.

All isolates were susceptible to aminoglycosides (gentamicin, amikacin, tobramycin), nalidixic acid, enrofloxacin, chloramphenicol, tetracyclines, and nitrofurantoin. More studies are needed before concluding that there are differences in antimicrobial susceptibility between *Campylobacter* spp. and *Arcobacter* spp.

PATHOGENICITY

The extent of animal and human disease caused by arcobacters is largely unknown. Their abundance in healthy animals, particularly poultry, indicates that they are only occasionally pathogenic. The areas in which pathogenicity is suspected are twofold: reproductive disease in pigs, cattle, and sheep, and diarrhea in humans and possibly some animals.

Reproductive disease

All three animal-associated *Arcobacter* species (*A. cryaerophila*, *A. butzleri*, *A. skirrowii*) have been isolated from the internal organs of aborted cattle, sheep, and pig fetuses, but their significance is in doubt as they can also be isolated from the internal organs of healthy fetuses. In Denmark, arcobacters were isolated from 23 of 55 aborted pig fetuses that tested negative for established abortifacient pathogens (On et al. 2002). Arcobacters have also been isolated from infertile sows with vaginal discharge and experimental infection lowered conception rates in sows artificially inseminated with arcobacter-containing semen (Houf 2001). The fact that *A. skirrowii* has been isolated from the preputial sheath washings of bulls (Vandamme et al. 1992b) takes on fresh significance here, as the circumstances begin to parallel those of infectious infertility caused by *C. fetus*. There is a single report of *A. butzleri* bacteremia in a preterm human infant that probably became infected in utero (On et al. 1995b).

Diarrheal disease

Arcobacters have been isolated from the feces of both humans and animals with diarrhea, including lambs, pigs, cattle, horses, and monkeys. *A. butzleri* is the species most often encountered in humans, usually in patients with diarrhea (Dediste et al. 1998; Lastovica et al. 2002; Vandenberg et al. 2002). The diarrhea tends to be watery and less intense but more persistent than in campylobacter enteritis. An outbreak in school children infected with a single strain of *A. butzleri* was characterized by recurrent abdominal cramps without diarrhea (Vandamme et al. 1992a). *A. butzleri* has occasionally been isolated from blood cultures and from peritoneal fluid from patients with acute appendictis (Mansfield and Forsythe 2000; Lastovica et al. 2002). *A. cryaerophilus* is also associated with enteritis and bacteremia, but much more rarely. The contribution of arcobacters to human diarrheal disease is not known because specific cultures for them are not routinely carried out in cases of enteritis (Engberg et al. 2000; Houf 2001).

The role of arcobacters as a cause of diarrhea in animals is less clear. *A. butzleri* is probably enteropathogenic for macaque monkeys, as it was isolated from colonic specimens from three macaques with active colitis (Anderson et al. 1993). *A. butzleri* in particular can colonize the intestine and other organs of neonatal pigs (Wesley et al. 1996). Pathogenic mechanisms are unknown. Toxin production and the ability to invade tissues may be mechanisms by which arcobacters cause disease (Fernandez et al. 1995; Johnson and Murano 2002). Arcobacters produce CDT-like activity on cell cultures but in a different manner to that of campylobacter CDT, because no CDT amplimers have been found in any of the arcobacter isolates. Cytotoxins are unlikely to be a major factor in pathogenesis, as they are associated with bloody diarrhea, and this symptom is rarely described in patients with arcobacter diarrhea.

Bovine mastitis

An outbreak of bovine mastitis was apparently caused by an *Arcobacter* strain that was subsequently used to reproduce the disease in four young cows (Neill et al. 1982).

SUMMARIZED DESCRIPTIONS OF *ARCOBACTER* SPECIES

Arcobacter nitrofigilis: *A. nitrofigilis* (McClung et al. 1983) was formerly assigned to *Campylobacter*, but transfered to *Arcobacter* as the type species of the genus by Vandamme et al. (1992b). It is a nitrogen-fixing bacterium found on the roots of a small marsh plant in Nova Scotia. Not found in animals or humans. G + C content of DNA 27.9–28.8 mol%. Type strain ATCC 33309.

Arcobacter butzleri: *A. butzleri* (Kiehlbauch et al. 1991) was formerly included in *Campylobacter* but transfered to *Arcobacter* by Vandamme et al. (1992b). The curved to S-shaped rods, 0.2–0.4 μm wide by 1–3 μm long, are catalase-negative or weakly positive. Colonies are entire, white, and opaque; 16 biotypes and 65 serogroups are recognized. This species is associated with diarrhea in humans and animals, occasional systemic infection in humans, and abortion in cattle and pigs; it is found in water, sewage, and poultry and other meats. G + C content of DNA 28–29 mol%. Type strain ATCC 49616.

Arcobacter cryaerophilus: *A. cryaerophilus* (Neill et al. 1985), formerly in the genus *Campylobacter*, is

now classified as *Arcobacter*; there are two subgroups (Vandamme et al. 1992b). Cells are 0.4 μm wide by 1.8 μm long but may attain a length of over 20 μm. Colonies on blood agar are domed, entire, and yellowish in the case of subgroup 2 strains. It is isolated from normal and aborted ovine and equine fetuses and was the apparent cause of an outbreak of bovine mastitis. It is also found in the feces of domestic animals and occasionally from humans, although some strains originally identified as this species have since been identified as *A. butzleri*. G + C content of DNA 31 ± 1 mol%. Type strain NCTC 11885.

Arcobacter skirrowii: *A. skirrowii* (Vandamme et al. 1992b) cells are 0.2–0.4 μm wide by 1–3 μm long. Colonies are flat, greyish, and α-hemolytic on blood agar. It is isolated mainly from the preputial fluid of bulls, but also from aborted bovine, porcine, and ovine fetuses, poultry, and the feces of animals with diarrhea. The organism has also been isolated from an elderly man with chronic diarrhea (Wybo et al. 2004). G + C content of DNA 29–30 mol%. Type strain CCUG 10474.

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Helicobacter

ROBERT J. OWEN

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Diagnosis	8		

DEFINITION

Helicobacters are helical, S-shaped, or curved gram-negative rods, 0.3–1.0 μm wide by 1.5–10.5 μm long. They are motile by means of single or multiple unipolar, bipolar, or lateral flagella. In most species the flagella are sheathed. Periplasmic fibrils or an electron dense glycocalyx/capsule-like layer have been observed on the cellular surface of several species. Cells exposed to air and in older cultures may form coccoid bodies. They are nonsporing and most are microaerophilic with respiratory metabolism. Hydrogen stimulates growth and may be required by some species. They are nonsaccharolytic, and oxidase-positive and most are catalase-positive. Helicobacters do not grow at 25°C. For convenience, two groups of species can be defined: those that colonize the gastric mucosa of humans and animals (gastric group); and those that colonize the intestines and possibly hepatic tissue of humans and animals (enterohepatic group). Strains within these groups can be further categorized on the basis of cellular morphology. The gastric species typically produce abundant urease. The G + C content of their genomic DNA is in the range 30–48 mol% with most having values of 36 ± 2 mol%. The type species *Helicobacter pylori* is one of the most common human gastric infections worldwide and plays a major role in the pathogenesis of peptic ulceration and gastric cancer. *H. hepaticus* is currently the best studied of the enterohepatic *Helicobacter* species because of its ability to cause liver cancer in mice, whereas *H. cinaedi* is the most commonly encountered species in human clinical samples, although isolation rates remain low compared to *H. pylori*.

HISTORICAL PERSPECTIVE AND CLASSIFICATION

The existence of gastric spiral organisms colonizing the mucus and glands of the stomach of healthy dogs was first reported by Bizzozero in 1893. Salomon (1896) confirmed their presence in dogs and also found them in cats and rats, but not in man. It is unlikely that these organisms were *Helicobacter pylori*, particularly as they were associated with parietal rather than mucus-secreting cells. The first observation of spiral-shaped organisms in the human stomach may have been by Krienitz (1906) who described three different types of 'spirochetes' in the stomach of a patient with carcinoma of the lesser curve, one of which may have been *H. pylori*. In 1938, Doenges observed spiral bacteria that he also reported as 'spirochetes' in the gastric mucosa of about 50 percent of human stomachs examined at routine necropsies from accident victims, but he was unable to correlate their presence with gastric diseases.

Freedberg and Barron (1940) reported similar organisms in 37 percent of gastrectomy specimens from patients with peptic ulcer or carcinoma. Following the introduction of flexible fibreoptic gastroscopy during the 1970s, Steer and Colin-Jones (1975) came near to identifying *H. pylori* and its association with gastritis in a large study of biopsy specimens, but misinterpretation of culture results led to the wrong conclusion that the organisms seen were *Pseudomonas aeruginosa*.

In 1982, Warren and Marshall were the first to culture and identify what is now known as *H. pylori* and to establish the association between the presence of this organism in the human gastric mucosa and the occurrence of histological gastritis (Warren and Marshall 1983, 1984). Within a few years of the publication of Warren and Marshall's work many reports appeared worldwide, confirming the close association between *H. pylori* and chronic active gastritis, as well as with gastric and duodenal ulcers. The historical associations between bacteria and peptic ulcer disease are described by Rathbone and Heatley (1992). The organism was named *Campylobacter pyloridis* by Marshall et al. (1984), but this was later changed to the more grammatically correct *C. pylori*. Further studies showed that the organism differed sufficiently from true campylobacters to justify the formation of a new genus, *Helicobacter* (Goodwin et al. 1989a). It soon became apparent that similar organisms colonized the stomach of a wide variety of animals other than humans, and that certain spiral bacteria colonizing the intestines of rodents and other animals also belonged to *Helicobacter*.

Helicobacter, together with *Campylobacter*, *Arcobacter*, and *Wolinella*, was shown to belong to a distinct bacterial grouping, rRNA superfamily VI, based on 16S RNA–DNA hybridization analysis (Vandamme et al. 1991); and the classification was in keeping with lineages defined by 16S rDNA phylogenetic analysis which placed *Helicobacter* and these other genera in the epsilon subclass of the class *Proteobacteria*. Although specific fatty acid profile, specific ultrastructure, respiratory quinones, growth requirements, and enzyme characteristics were originally described to distinguish helicobacters, the use of 16S rDNA analysis is crucial for their classification as there are no other consistent taxonomic markers to facilitate separation of *Helicobacter* from allied genera such as *Campylobacter* and *Arcobacter*.

Helicobacter pylori is the type species of the genus *Helicobacter* that was proposed by Goodwin et al. (1989a). The genus originally contained two species: *H. pylori*, the human gastric pathogen, and *H. mustelae*, a similar bacterium found in the stomach of ferrets. Although these two species had several campylobacter-like characters, they were separated from the genus *Campylobacter* principally on the basis of their unique and very different rRNA sequence. Since then the number of species in *Helicobacter* has expanded and currently, the genus contains 18 validly named species, two *Candidatus* species and three putative new species (names effectively described, but not formally validated). Species, their hosts, and habitat are detailed in Table 1. The gastric group is represented by about 12 species and the enterohepatic group by a further 21 species, the majority of the latter being isolated from small rodents. The mouse represents a convenient and widely used animal model for *H. pylori* infection and as a consequence its gastrointestinal tract flora has been thoroughly investigated in order to screen for natural infections with other *Helicobacter* species in laboratory animals. Enormous diversity is indicated by 16S rDNA sequences with at least 30 additional potential species candidates awaiting more precise definition, and recommended minimal standards for describing new species of the genus have been proposed (Dewhirst et al. 2000). *H. pylori* and *H. heilmanii* are the principal species which have been associated with human gastric disease. Amongst the enterohepatic group, human infections are mainly associated with *H. cinaedi* and *H. fennelliae* as causes of enteritis and proctocolitis, especially in homosexual men, and they may sometimes also cause bacteremia in immunocompromised patients. Interestingly, additional species *H. pullorum*, *H. canadensis*, and *H. winghamensis* have also been associated with human gastroenteritis. Distinguishing features of those members of the *Helicobacter* genus associated with human disease are compared in Table 2. Characteristics for distinguishing the other species are described elsewhere (Dewhirst et al. 2000) and in the new species descriptions.

HABITAT

Helicobacters colonize the gastric or intestinal mucosa of humans, and a wide variety of other mammalian hosts, notably dogs, cats, sheep, pigs, cattle, as well as many small rodents, and birds. Isolation of organisms in extragastric sites is rare, although there are reports of *H. pylori* in dental plaque, saliva, and feces as well as *H. pylori*, *H. pullorum*, and *H. cholecystus* in liver tissue and the biliary tract. Presence of such organisms at these locations and in the natural environment (soil, river water, and sea water) is rarely based on direct culture and is mainly reliant on polymerase chain reaction (PCR) detection assays so it is unclear if the organisms are viable.

The surface of the stomach mucosa is the major habitat of *H. pylori* and other gastric species. The bacteria live in and beneath the layer of mucus that covers the gastric mucosa; in this situation the pH is near neutrality. The vast majority of *H. pylori* are located adjacent to surface and pit gastric epithelial cells (Figure 1). Any part of the stomach may become colonized, but the mucus-secreting epithelium of the antrum is the favored site. Areas of gastric metaplasia in the duodenum or elsewhere in the digestive tract may

Table 1 *Gastric and enterohepatic members of the* Helicobacter *genus*

Gastric *Helicobacter* taxa		Enterohepatic *Helicobacter* taxa	
Species	**Natural host**	**Species**	**Natural host**
Simple cell morphology		**Simple cell morphology**	
H. pylori[a]	Humans	*H. cinaedi*[b]	Humans, hamsters
H. mustelae	Ferrets	*H. fennelliae*	Humans, dogs, macaques
H. suncus	House musk shrews	*H. pullorum*	Humans, chickens
H. acinonychis	Cheetahs	*H. canis*	Humans, dogs, cats
		H. canadensis	Humans
Complex cell morphology		*H. winghamensis*	Humans
H. felis	Cats, dogs, cheetahs	*H. pametensis*	Wild birds, pigs
H. salomonis	Cats, dogs	*H. muricola*	Korean wild mouse
H. bizzozeronii	Cats, dogs	*H. marmota*	Woodchucks, cats
		H. hepaticus	Mice
		H. rodentium	Mice
		H. typhlonius	Mice
		H. ganmanii	Mice
Non-culturable with complex cell morphology			
H. heilmannii	Humans, pigs, cats, dogs, monkeys, cheetahs	*H. mesocricetorum*	Hamsters
Candidatus H. bovis	Cattle		
Candidatus H. suis	Pigs	*H. cholecystus*	Hamsters
		Complex cell morphology	
		H. bilis	Mice, dogs, cats
		H. trogontum	Rats
		H. muridarum	Mice, rats
		H. aurati	Syrian hamsters

A proposal has been made to place the organism previously known as *Flexispira rappini* within the genus *Helicobacter* as *H. rappini* but the taxonomy has not yet been fully resolved.

a) This species includes *H. nemestrinae*.

b) This species includes *H.* sp. strain mainz and *H. westmeadii*.

also become colonized, but *H. pylori* does not colonize areas of intestinal metaplasia in the stomach. The electron microscope shows adherence of *H. pylori* to human gastric mucus cells and in some cases involves the formation of pedestal attachments. At such sites there is effacement of microvilli, flattening of cells, with loss of mucin granules, disruption of cytoskeletal elements, and alteration of intercellular complexes (Goodwin et al. 1986). Colonization of the mucosa by *H. pylori* is almost invariably associated with the histological pattern of chronic active gastritis or antral type-B gastritis (Price 1991), which is clearly distinct from type-A autoimmune inflammation of the acid-secreting mucosa of the body of the stomach, found in pernicious anemia. This pattern is usually characterized by mononuclear cell infiltration associated with superficial infiltration of polymorphonuclear leukocytes of the epithelium (Robert and Weinstein 1993). The amount of inflammation may be highly variable, ranging from minimal inflammatory infiltration of the lamina propria with intact glandular architecture, to severe dense inflammation with microabscess formation, reactive epithelial atypia, and the presence of intraepithelial neutrophils. In general, there is a correlation between the number of *H. pylori* organisms present and the degree of polymorph infiltration. On average, bacteria are present in less than 5 percent of biopsy specimens that are histologically normal and, when present, are located most commonly in the gastric body. Since the distribution of bacteria may be patchy, occasional cases of chronic superficial gastritis with neutrophil activity may fail to show *H. pylori* organisms due to sampling errors (Bayerdörffer et al. 1989). If left untreated the infection does not usually resolve spontaneously, but persists for life. Over the course of years or decades, there is extension of the inflammatory process into the deeper portions of the mucosa, partial loss of the pyloric glands and endocrine cells (i.e. atrophy) of the antrum, and a progressive replacement of the mucosa by intestinal metaplasia (Sipponen et al. 1991). Some gastric species only colonize the nonacid-secreting mucosa, whereas others, such as *Helicobacter felis*, can colonize the canaliculi of acid-secreting oxyntic cells.

H. cinaedi and other enterohepatic species of *Helicobacter* colonize the intestines of humans and a variety of other mammals, in particular small rodents where their presence has been studied extensively, and in birds.

Table 2 *Cultural and biochemical characteristics of* Helicobacter *spp. associated with human infection*

	H. pylori	*H. cinaedi*	*H. fennelliae*	*H. canis*	*H. pullorum*	*H. canadensis*	*H. wiinghamensis*	*H. hepaticus*
No. flagella/cell	4–8	1–2	1–2	1–2	1	1-2	1-2	2
Distribution of flagella	Bp	Bp	Bp	Bp	Mp	Bp	Bp	Bp
Sheathed flagella	+	+	+	+	–	–	–	+
Periplasmic fibers	–	–	–	–	–	–	–	–
Growth at 42°C	(–)	V	(–)	+	+	+	–	–
Catalase	+	+	(+)	–	+	+	–	+
Nitrate reduction	–	+	–	–	+	+	–	+
Urease	+	–	–	–	–	–	–	+
Alkaline phosphatase	+	(–)	(–)	+	–	–	–	–
γ-Glutamyl transpeptidase	+	–	–	+	ND	–	ND	–
Indoxyl acetate hydrolysis	(–)	(–)	+	+	–	+	+	+
Growth in presence of 1% glycine	–	–	–	–	–	+	ND	+
Susceptibility to:								
Nalidixic acid	R	R	S	S	R	R	R	R
Cephalothin	S	I	S	I	S	R	R	R

+, 100% of strains positive; –, 0% strains positive; (+) 80–94% strains positive; (–) 7–33% strains positive; V, 42–66% strains positive; S, sensitive; R, resistant, I, intermediate; ND, not determined; Bp, bipolar; Mp, monopolar.

Typically, organisms are recovered from colonic crypts and adjacent mucous layers, as well as from the feces. Some intestinal species are capable of colonizing the stomach when acid secretion is defective, for example, there is molecular evidence of organisms resembling *H. cinaedi* in human antral biopsy specimens although their roles in chronic gastritis and peptic ulcer disease remain unclear (Peña et al. 2002). Since the isolation of *H. hepaticus* from the livers of laboratory mice with chronic active hepatitis and associated liver tumors (Ward et al. 1994), there has been a growing interest in the ability of helicobacters to infect such tissue. The species of *Helicobacter* associated with hepatic disease in animals include *H. canis*, *H. bilis*, *H. cholecystis*, and *H. pullorum* (Table 1). A number of studies also suggest that helicobacter DNA is sometimes present in the human liver, bile, or biliary tree tissue, but the interpretation of these findings remain controversial (Fox et al. 1998; Avenaud et al. 2000; Nilsson et al. 2000a, b). In most cases efforts to cultivate helicobacters direct from such tissue was unsuccessful apart from the isolation of *H. pylori* from a patient in Brazil with hepatic abnormalities but it was unclear if the helicobacter was the cause of the disease (de Magalaes Queiroz and Santos 2001).

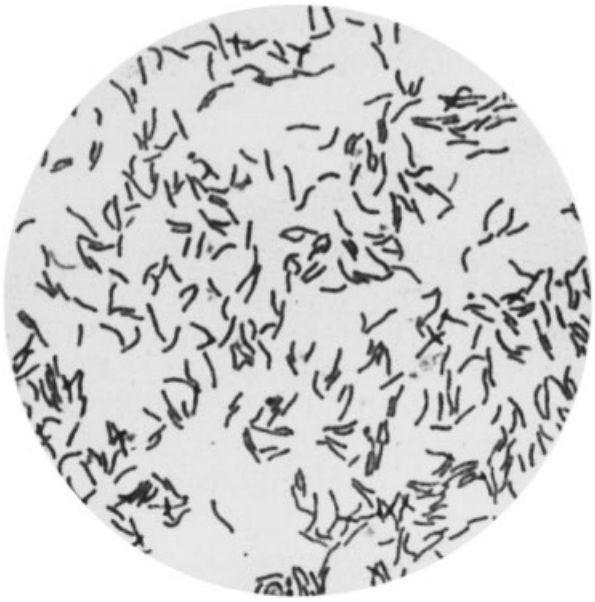

Figure 1 Helicobacter pylori *from a 3-day chocolate agar culture (×1 300)*

BACTERIOLOGY

Morphology

There is much morphological diversity among *Helicobacter* species. Cells may vary from simple rod-like forms with unsheathed bipolar flagella that resemble *Campylobacter* to forms with increased complexity – long spiral cells with periplasmic fibers and bundles of sheathed multiple flagella. Within the gastric species, *H. felis* is unique in that its cells possess periplasmic fibers that are independent of the flagella (Lee and O'Rourke 1993), whereas other culturable species such as *H. bizzeroni* and *H. salomonis* and nonculturable species such as '*H. heilmannii*' have tufts of up to 23 multiple flagella. By contrast, the species with simpler cell morphology (between two and eight bipolar sheathed flagella and no periplasmic fibers) such as *H. pylori* cells take the form of curved, or S-shaped gram-negative

rods, 0.5–0.9 μm wide by about 3 μm long with a wavelength of about 2.6 μm. By electron microscopy, the organism is spiral with bluntly rounded ends and with between four and eight sheathed, unipolar, flagella; the sheath is continuous with the outer membrane of the cell wall. Some flagella have a terminal bulb. A glycocalyx-like material surrounding the cell is also apparent. In agar cultures spiral forms are less obvious and cells appear more as singly curved rods (Figure 1). *H. pylori* undergoes coccal transformation on exposure to air within 1–2 h at room temperature, and in this state it fails to grow on subculture. Such coccoid forms do not appear to be virulent (Eaton et al. 1995). The enterohepatic species typically have a simple cell morphology with some species, such as *H. pullorum* and *H. rodentium* exhibiting unsheathed flagella.

Cultural characteristics, metabolism, and growth requirements

No species of *Helicobacter* grow aerobically but all grow under microaerobic conditions with the exception of *H. ganmani* which is strictly anaerobic. *H. pylori* is strictly microaerophilic and CO_2 (5–20 percent) and high humidity are required for growth. *H. pylori* requires media containing supplements similar to those used for campylobacters: blood, hemin, serum, starch, or charcoal. However, *H. pylori* is inhibited by the bisulfite in the ferrous sulfate–sodium metabisulfite–sodium pyruvate (FBP) campylobacter 'aerotolerance' supplement. Growth is best on media such as moist blood agar, or brain–heart infusion agar with 5–10 percent horse blood. Strains grow in various liquid media supplemented with fetal calf or horse serum (Shahamat et al. 1991). Some strains grow in serum-free media, notably bisulfite-free brucella broth (Hawrylik et al. 1994). Sheep blood, either whole or laked (lyzed), when used as a broth supplement, has been shown to inhibit the growth of *H. pylori* (Coudron and Stratton 1995). All strains grow at 37°C, some grow poorly at 30 and 42°C, but none grows at 25°C. Colonies from primary cultures at 37°C usually take 3–5 days to appear and are circular, convex, and translucent (Figure 2). They seldom grow bigger than 2 mm in diameter even if incubation is extended beyond 1 week. They are weakly hemolytic on 5 percent horse blood agar. Motility is weak or absent when grown on agar.

Species of *Helicobacter* are inactive in most conventional biochemical tests. Over the past 20 years a panel of ten phenotypic tests used in combination with morphotype based on presence of periplasmic fibers, number and distribution of flagella and presence of flagella sheath, have been established as the standard phenotypic criteria for species differentiation, and form the basis of the recommended minimal standards for describing new species of *Helicobacter* (Dewhurst et al. 2000). All species produce oxidase and catalase with the notable exception

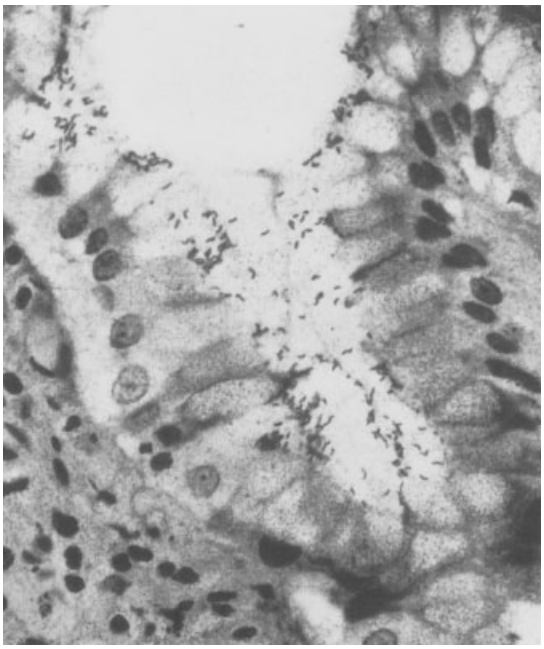

Figure 2 *Section of antral mucosa showing gastric pit colonized with* Helicobacter pylori. *Warthin–Starry silver stain (×560)*

of *H. canis* and *H. winghamensis*. Other key characteristics such as nitrate reduction to nitrite, alkaline phosphatase hydrolysis, urease hydrolysis, indoxylacetate hydrolysis, gamma-glutamyl transpeptidase activity, growth at 42°C, and susceptibility to nalidixic acid and cephalothin vary between species and sometimes amongst strains within species. Key tests for differentiating species within the gastric and enterohepatic groups associated with human infections are shown in Table 2: the important enterohepatic animal species, *H. hepaticus*, is included for comparison. For instance, *H. pylori* is notable for the strong production of urease, catalase, and alkaline phosphatase. Furthermore, all strains produce DNAase, leucine aminopeptidase, and γ-glutamylaminopeptidase. The urease enzyme of *H. pylori* is one of the most widely studied in helicobacters and is a nickel containing high molecular weight protein (approximately 600 kDa) with maximum activity at 45°C and pH 8.2. Remarkably, it forms 6 percent of protein produced by the organism. An almost identical urease is produced by *Helicobacter nemestrinae*, now reclassified as *H. pylori* (Suerbaum et al. 2002), *Helicobacter mustelae*, and *H. felis* (Turbett et al. 1992). The presence of urea (5 M) enables *H. pylori* to tolerate pH values down to 2.6, because the production of ammonia raises the pH in the immediate vicinity of the bacterial cell. The importance of this action for colonization of the stomach is obvious.

GENOMICS

The genetics of *Helicobacter* was transformed in 1997 by the publication of the complete annotated genome

sequence of *H. pylori* strain 26695 isolated in the UK in the early 1980s from a patient with gastritis (Tomb et al. 1997). Prior to then, strategies used to clone genes from *Campylobacter*, such as shuttle mutagenesis, have been used to express *H. pylori* urease genes in *C. jejuni*, and *E. coli* to study flagellin and *cagA* genes (Tummuru et al. 1994). Other genes that have been successfully cloned and expressed include the gene for vacuolating cytotoxin (Schmitt and Haas 1994) and *recA* (Thompson and Blaser 1995). Many analyses in the pregenomic era reviewed by Taylor (1992) addressed genomic structure and diversity using techniques such as pulse field gel electrophoresis to generate physical maps, as well as ribotyping, randomly amplified polymorphic DNA, and PCR-RFLP that showed extensive nucleotide diversity between strains and have provided a valuable basis for strain fingerprinting (Owen et al. 2001a). Plasmid transfer has not been reported in *H. pylori*, but plasmids have been detected in 48–58 percent of wild strains (Stanley et al. 1992).

In 1999, the genome sequence of a second strain of *H. pylori* was reported. This strain was isolated in the USA in 1994 from a patient suffering from a duodenal ulcer and duodenitis (Alm et al. 1999). The two sequences have been the focus of extensive comparative analyses (Doig et al. 1999; Marais et al. 1999) which highlight a number of key similarities as well as differences in relation to physical arrangement of the genome, allelic diversity within orthologous genes, and strain specific differences within the hypervariable region (plasticity zone; Table 3). The majority of genes in both strains lie at the same relative positions, apart from ten regions containing some large rearrangements that needed to be artificially transposed and/or inverted to enable the genes to be located on a single genome map. In contrast to conserved gene order, there was significant nucleotide diversity between orthologous genes. Due to redundancy in the genetic code, that diversity was not reflected at such a high level in the respective encoded proteins. Comparative analyses between the sequenced genomes have identified up to 7 percent of the coding capacity (117 genes) was strain specific and over 70 percent of these had no predicted function. Almost half the strain-specific genes are contained in a plasticity zone that has a lower G + C mol% content than the remainder of the genome – 35 percent compared to 39 percent. The subsequent availability of the *C. jejuni* NCTC11168 genome has enabled comparison of the two closely related genera (Parkhill et al. 2000). There were significant differences between the genomes, notably in energy metabolism and chemotaxis systems, and 17 percent of the *H. pylori* predicted genes were found to be *H. pylori*-specific, but with an unknown function. Further study of these genes is essential as they may encode many of the phenotypic characteristics that have enabled *H. pylori* to adapt to the gastric environment.

Further new perspectives on the *H. pylori* genome were provided by the publication of the complete genome sequence of *H. hepaticus* (Suerbaum et al. 2003). With a size of 1.799 Mb and a base composition of 35.9 mol% G + C, the genome was slightly larger than those of *H. pylori* and *C. jejuni* (Table 3). *H. hepaticus* lacked orthologs of most known *H. pylori* virulence factors including adhesins, the VacA toxin, and almost all cag pathogenicity island proteins. The genome contains a 71-kb genomic island and several genomic islets whose G + C content differs from the rest of the genome. Comparisons of other isolates of *H. hepaticus* by microarray hybridization showed extensive variation within the species. Extensive physiological similarities with *C. jejuni* reflect enteric colonization, but the reasons for its carcinogenic potential are not immediately apparent from the genome sequence.

CELL WALL COMPOSITION AND ANTIGENIC STRUCTURE

Helicobacters have the typical cell wall structure of gram-negative bacteria comprised of lipopolysaccharide (LPS) attached to a semipermeable outer membrane, a peptidoglycan layer, and an inner cell membrane. The fatty acid composition of *H. pylori* is distinctive and one of the main criteria for exclusion from *Campylobacter*. It is characterized by long-chain fatty acids composed

Table 3 *General features of the genomes of* Helicobacter *strains*[a]

	H. pylori 26695	J 99	*H. hepaticus* ATTC 51449
Size (base pairs)	1 667 867	1 643 631	1 799 146
(G + C) %	39	39	35.9
Gene copies:			
23S–5S rRNA	2	2	1
16S rRNA	2	2	1
Predicted coding sequences	1 590	1 496	1 825
Proteins with assigned functions	899	877	1 022
Proteins with no assigned functions	325	347	487

a) By comparison, the *C. jejuni* NCTC 11168 genome was 1 641 481 base pairs (30.6% G + C) and was predicted to encode 1 654 proteins. Only 55.4% of *C. jejuni* genes had orthologs in *H. pylori* (Parkhill et al. 2000).

predominantly of tetradecanoic (14:0) and 19-carbon cyclopropane (19:0 cyc) acids with lower amounts of hexadecanoic (16:0), octadecanoic (18:0), octadecenoic (18:1), and linoleic (18:2) acids (Moran 1995). Phosphatidylethanolamine (79.1 percent), lysophosphatidylethanolamine (16 percent), and phosphatidylcholine (1.9 percent) constitute the polar head groups of neutral phospholipids. Acidic phospholipids contain phosphatidic acid (52.7 percent) and phosphatidylserine (47.3 percent). *H. pylori* does not possess the methyl-substituted menaquinones (thermoplasma quinones) present in *Campylobacter* species. Cholesterol glucosides are a unique characteristic of the lipid content of various *Helicobacter* spp. (Haque et al. 1996).

Lipopolysaccharides

The lipopolysaccharides of *H. pylori* have been intensively investigated and appear to have unique biological properties – notably in inducing a low immunological response that may be an adaptation to aid both persistence and infection in its ecological niche in the gastric mucosa (Moran 1995). Fresh isolates of *H. pylori* possess LPS with a ladder-like polysaccharide side chain like those of 'smooth' strains of Enterobacteriaceae, but are different in producing O-chains of relatively constant chain length. Antigenic and structural differences detected in electrophoretic, lectin binding and immunoblotting, and hemagglutinin analysis indicate considerable strain diversity and have been suggested as a possible basis of strain specific typing schemes (Mills et al. 1992). However, in vitro growth condition may induce changes in LPS expression as strains may change from smooth to rough LPS when grown on conventional solid media and may be reversible when grown in liquid medium (Moran 1995).

An interesting feature of *H. pylori* LPS is that several structural studies have shown mimicry of Lewis x and/or Lewis y blood group determinants. Serological screening has detected those antigens in up to 90 percent of strains. It has been speculated that bacterial expression of Lewis antigens identical to those present in the human gastric mucosa may camouflage *H. pylori* in its ecological niche particularly in the early phase of infection (Appelmelk et al. 1997). However, Lewis x is not a stable trait and LPS can display a high frequency of phase variation (Appelmelk et al. 1998). This phenomenon has been attributed to a slip-strand mechanism that changes the length of nucleotide repeats that introduce translational frameshifts leading to the production of inactive truncated gene products. For instance, the poly C tracts of the alpha (1,3)-fucosyltransferase genes may directly contribute to changes in Le expression, although the length of the C repeats does not correlate with Lewis x and Lewis y expression in all clinical isolates (Appelmelk et al. 1999).

LIPID A

The lipid A component of *H. pylori* LPS has been characterized in a number of detailed structural studies and like the LPS appears to have evolved a number of modifications associated with a long-term chronic infection. Unlike lipid A of many bacterial species that of *H. pylori* appears to lack 3-hydroxydecanoic acid, as well as unusual phosphorylation and fatty acid substitutions which could in part explain the lower biological activities (Moran et al. 1997). For instance, *H. pylori* LPS binds more slowly than *E. coli* LPS to lipid-binding protein and also exhibits poorer binding to CD14.

OUTER MEMBRANE PROTEINS

A large number of integral outer membrane proteins in five paralogous gene families comprising three to 33 members have been inferred from genomic analysis of *H. pylori*. These genes represent approximately 4 percent of a strain's coding capacity, but functional analysis has been reported on only a few (Alm et al. 2000). The porins are one of the best characterized families of proteins localized in the outer membrane of *H. pylori*. Five members of the Hop family of proteins labelled A, B, C, D, and E with molecular weights of 29 to 75 kDa have been identified as porins containing nonspecific water-filled channels that allow small hydrophilic molecules, nutrients, and even small antibiotics to diffuse passively across the outer membrane (Exner et al. 1995; Alm et al. 2000). Furthermore, a member of the Hop protein family, BabA (HopS), have been implicated as an adhesin mediating binding to the blood group antigen Lewis B on human host cell surfaces and could be an important factor in virulence (Ilver et al. 1998). The wide range of outer membrane protein identified may reflect adaptation of the species to the unique gastric environment.

TYPING METHODS

As *H. pylori* is the most widely encountered and clinically most important species in the genus *Helicobacter*, it is the species most thoroughly investigated with respect to potential typing methods. Nevertheless, at present there is no generally agreed system for defining types or subtypes within the species, even though information on strain type is recognized to be of key importance in:

- Monitoring the effects of therapy and intervention procedures
- Establishing association of disease severity with a particular strain type and identification of markers that might contribute in assessing benefit of eradication in patients with nonulcer dyspepsia
- Investigation of routes of transmission
- Finding clues to the common ancestry of strains worldwide that might have relevance to design of vaccines.

Phenotyping methods

H. pylori is remarkably homogenous in its phenotypic expression. The only biochemical differences found between strains of widely different origin are in the activities of a few enzymes, which divide strains into four biotypes, one of which accounts for 85 percent of strains (Owen 1995). Characterization of the protein profiles, lipopolysaccharides or surface antigens have been proposed as typing criteria in order to differentiate *H. pylori* strains isolated in different parts of the world. Unfortunately, most of the strains display little heterogeneity and fall into few different groups so that these techniques are not useful for strain differentiation. For example, hemagglutination and lectin typing have shown recognizable patterns, but the systems have not been developed. The LPS antigens of *H. pylori* are sufficiently diverse for their potential use in typing (Mills et al. 1992), but the approach has not been developed further. Lewis blood group determinants are present in about 80 percent of strains and it was proposed they could form the basis of an O-antigen serotyping scheme using specific monoclonal antibodies: the scheme was compromised by the frequent occurrence of phase variation (Simoons-Smit et al. 1996).

Genotyping methods

Most successes have been with molecular methods that exploit the considerable genetic diversity of *H. pylori*. Methods of molecular fingerprinting applied include ribotyping, restriction fragment length polymorphism (RFLP) of known loci such as the urease encoding genes, and *flaA* and *flaB* the genes for flagellins, pulse field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). The subject is reviewed by Owen et al. (2001a). The molecular fingerprint methods have proved useful mainly for tracing the intrafamilial spread of *H. pylori* (Bamford et al. 1993) or for differentiating between recrudescence and reinfection after attempts to eradicate *H. pylori*. The PCR methods in particular have brought to light the extreme heterogeneity of *H. pylori*, with each infecting strain displaying an almost unique fingerprint. This degree of diversity, attributed to a nonclonal population structure, is confirmed by the use of sequence typing applied to housekeeping genes, as well as to pathogenicity associated genes (Owen and Xerry 2003).

While it has not yet been possible to assign a particular genovar to specific gastric diseases (e.g. chronic gastritis versus peptic ulcer or gastric cancer) using high resolution fingerprinting methods; there is some evidence that certain broader genotypes can be recognized based on presence of *cagA*, the cytotoxin associated gene, which provides a marker for the presence of a genomic pathogenicity island (Censini et al. 1996). There is contradictory evidence that *cagA*+ strains are associated with more severe clinical outcomes in western populations, whereas in East Asian populations virtually all strains are *cagA*+ and the association with disease severity is much weaker (Yamaoka et al. 1999; Owen et al. 2001b). Interestingly there is evidence that the 3′ end of *cagA* contains repeat motifs that may be associated with greater disease severity in East Asians (Yamaoka et al. 1998). Another widely investigated marker of potential virulence is the *vacA* genotype – this gene encodes a toxin that is secreted by the bacterium and causes vacuoles in epithelial cells (Telford et al. 1994). The gene has a mozaic structure and two parts show particular sequence heterogeneity (Atherton et al. 1995). The s-region encoding the signal polypeptide occurs as an s1 type with three subtypes a, b, and c, or an s2 type. Within the m-region (middle), m1 or m2 types exist and within m2 subtypes m2a and m2b can be recognized. Like *cagA*, the *vacA* genotypes have a particular geographical distribution. For instance, in Europe most strains from northern, central, and eastern Europe are subtype *vacA* s1a, whereas East Asian patients often contain subtype s1c (van Doorne et al. 1998). The *cagA* and *vacA* types provide the most widely recognized means of characterizing strains, e.g. genome sequenced strains 26695 and J99 have genotypes *cagA*+/*vacA*s1a/m1, and *cagA*+/*vacA*s1b/m1, respectively.

There is a growing interest in the use of multilocus sequence typing (MLST) defined by alleles present at each of seven core genes, as a method for molecular typing and large datasets have been generated for important gram-negative and gram-positive bacterial pathogens (Maiden et al. 1998). The technique has limited value for defining epidemiological types within *H. pylori* as recombination occurs so frequently that alleles within isolates from a defined geographic area are all different and unique and discrete clones or clonal complexes cannot be readily discerned (Suerbaum et al. 1998; Achtman et al. 1999; Falush et al. 2001). There is evidence for geographic populations and subpopulations of *H. pylori* around the world from population-based analysis of multilocus sequences (Falush et al. 2003).

DIAGNOSIS

Various tests are available to assist in the diagnosis of *H. pylori* infection. These tests can be categorized into those that are based on direct assessment of gastric biopsies (invasive) and indirect tests (noninvasive) that detect an immunological response (i.e. antibodies against *H. pylori*) or metabolic products (i.e. urease activity) of *H. pylori*.

The ‘gold standard’ for the detection of *H. pylori* has been defined as culture, or histopathological examination of gastric biopsies, depending on the experience of the investigator. In order to optimize the diagnosis of *H.*

pylori, it is usually recommended that several tests be used together. The choice of particular tests will depend on locally available facilities, cost considerations, and clinical circumstances in which the diagnosis of *H. pylori* is to be made. The different tests for the detection of *H. pylori* and where relevant other species are briefly described in the following section and are summarized in Table 4.

Histology

The morphology of *H. pylori* is sufficiently distinctive for diagnosis to be made by light microscopy. The conventional histological hematoxylin–eosin (HE) stain at high magnification is able to detect most cases of *H. pylori* infection unless the number of organisms is very low. The organisms are best seen in or near adherent mucus on the luminal side of the gastric surface and pit epithelial cells. There are more sensitive staining methods for the diagnosis of *H. pylori*, including the modified Giemsa or Warthin–Starry silver stain (Madan et al. 1988). The Warthin–Starry stain as originally used by Warren (Warren and Marshall 1983) gives excellent results but it is quite labor-intensive, costly, and requires experienced technicians for reliable performance. The modified Giemsa stain is simpler and less labor-intensive. Other stains that have been recommended for the diagnosis of *H. pylori* include acridine orange (fluorescence), cresyl-fast violet, Gimenez, or Brown–Hopps. Phase-contrast microscopy of a wet preparation of crushed tissue is convenient and quick. Gram staining of mucus smears or of biopsy imprints can also be used, but is generally considered to be less sensitive than the other stains. The sensitivity and specificity of histology for the diagnosis of *H. pylori* are over 95 percent in experienced hands and when multiple gastric biopsies are obtained. An advantage of histology is that it allows the degree of inflammation and any other associated pathologies to be assessed. A novel but as yet not widely evaluated development for the detection of *H. pylori* is by means of an in situ immunofluorescent hybridization (FISH) assay (Trebesius et al. 2000).

Culture

The most specific method to diagnose infection is culture of *H. pylori* from gastric biopsies, but its sensitivity depends on both the biopsy transport conditions and the expertise available in the individual laboratory (Han et al. 1995). However, *H. pylori* is a fastidious slow growing organism that requires special culture conditions, so relying on culture alone can mean missing a proportion of infected cases for technical reasons, including overgrowth of other bacteria and low bacterial

Table 4 *Summary of tests for detection of* H. pylori

Test	Sensitivity[a] (%)	Specificity[a] (%)	Cost[b]	Endoscopy required	Comments
Histology	90–>95	95–99	++	Yes	Multiple antral biopsies recommended because of the irregular distribution of the organisms, allows evaluation of the degree of inflammation
Culture	75–90	100	+++	Yes	Variable results depending on the expertise of the laboratory; required for susceptibility testing and epidemiological studies
Rapid urease test (e.g. CLO test)	85–95	90–>95	+	Yes	Can be performed rapidly; decreased sensitivity immediately after antimicrobial treatment
Gastric biopsy PCR	95	100	+++	Yes	Technically very demanding; not yet available in clinical practice
^{13}C breath test (non-radioactive)	95	98–100	++	No	Preferred in pregnant women, childen; not widely available; well suited to early follow-up of antimicrobial treatment
^{14}C breath test (radioactive)	95	90–100	++	No	Small radiation exposure; well suited to follow-up of antimicrobial therapy
Serology	80–95	85–>95	+	No	Widely available; useful tool for screening patients before endoscopy and also for seroepidemiological studies; not appropriate for short-term treatment monitoring
Stool antigen test	94	97	++	No	Relatively new noninvasive test; useful for pediatric patients; applicable for detection of active infection before and after therapy

a) Average estimates from data reported in the literature.
b) +, least expensive; ++, moderately expensive; +++, most expensive.

load. Attention to transport conditions of the biopsies is particularly crucial for an adequate diagnostic yield. Biopsy specimens should be transported in a moist state and when suspended in saline solution or distilled water, must be cultured within 4 to 6 h of being taken. Storage beyond this time should be at 4°C, or at −20°C if the period is more than 2 days. Specimens can be kept in Stuart semisolid transport medium for 24 h if stored at 4°C (Soltesz et al. 1992). No recommended transport medium is used universally, but the various suggested transport media for biopsy samples, include normal saline, 20 percent glucose, and Stuart's medium (Vaira et al. 2002), as well as commercially available medium (e.g. Portagerm pylori, Bio-Merieux). An in-house helicobacter transport medium used successfully for several years for both biopsies and cultures comprises brain heart infusion broth (3.5 percent), yeast extract (2.4 percent), sterile horse serum (5 percent), and Dents antibiotic supplement (vancomycin/trimethaprim/cephsoludin/amphotericin) (Dent and McNulty 1988).

Selective media for culture that suppress nasopharyngeal flora and other potential contaminants are available commercially and should be used together with a nonselective fresh blood-based agar, such as Columbia agar containing 10 percent horse blood, in order to ensure optimal recovery of *H. pylori*. Samples should be prepared for direct examination and plating on nonselective and selective media, and plates should be incubated in a microaerobic environment at 37°C under humid conditions for at least 5–7 days before being discarded. Colonies may be visible after 3–5 days of incubation, but may take longer to appear on primary isolation. Colonies are small, domed, translucent, and sometimes weakly hemolytic. The sensitivity of culture ranges from 75 to 90 percent, even in expert hands. Because of the unique characteristics of *H. pylori*, only a few simple tests are needed for identification, including Gram-stain appearance, catalase, oxidase, and urease tests. A more detailed account of the laboratory isolation of *H. pylori* is given by Jerris (1995). The disadvantage of culture as a detection method is that it relies on availability of a gastric biopsy, culture takes at least 7 days, and availability may be restricted to specialist laboratories all of which lead to increased cost. In specialized laboratories, sensitivity of culture can be over 90 percent, but this can be reduced in patients that have had prior antibiotic therapy, while lowering gastric pH with proton pump inhibitors (PPI) can lead to overgrowth of contaminating oral bacteria. The main value of culture is in providing additional information that might be useful in patient management – notably for determination of antibiotic resistance, which is becoming an increasing problem. Furthermore, in some cases it may be helpful to have information on the strain type to establish if persistence of symptoms may be due to failure to eradicate or to reinfection, or if the strain has features indicative of virulence potential, such as presence of the cag pathogenicity island.

Intestinal helicobacters, such as *H. cinaedi*, *H. fennelliae*, and *H. pullorum*, can be isolated on some campylobacter-selective agars, or by filtration on nonselective agar (Kiehlbauch et al. 1995). However, for these species there is no alternative to culture as no other diagnostic tests are available at present.

Rapid urease test

H. pylori produces such abundant urease that the enzyme can be detected in specimens of infected gastric mucosa placed directly in a solution of urea with phenol red as an indicator. In the original test, Christensen's urea broth was used but unbuffered urea solutions, if freshly prepared, and the use of gels instead of broth give quicker results (Marshall et al. 1987; Arvind et al. 1988). A number of modifications have been described and kits are now widely available from commercial sources (e.g. Campylobacter-like organism (CLO) test). The test can have a sensitivity of at least 80 percent and a specificity approaching 100 percent when the reaction is positive within 1 h. False positives may occur by contamination with urease-producing bacteria from the oral cavity. In clinical practice, biopsy urease test results should be read within 24 h. It has the advantage of diagnosing *H. pylori* status in most cases soon after endoscopy, which is helpful for the clinician planning therapeutic interventions. Test kits are accurate and reliable and are much less expensive than histology or culture of gastric biopsies (Leodolter et al. 2001).

Urea breath test

The urea breath test (UBT) is currently the standard noninvasive test for detecting *H. pylori* with the highest accuracy in pre- and posttreatment conditions. Urea breath tests are based on the principle that urea that has been labeled with a carbon isotope is administered orally and is hydrolyzed by urease produced by viable *H. pylori* in the stomach, if present. As a result, ammonia is produced and bicarbonate is excreted in the breath as CO_2. The amount of labeled CO_2 in breath is indicative of the amount of urease activity and can be used as a marker of *H. pylori* infection. Two types of isotopically labeled urea have been developed: the [^{13}C]urea (Graham et al. 1987) and [^{14}C]urea (Marshall and Surveyor 1988). Both tests measure active infection, have the great advantage of being noninvasive, and are useful in making a primary diagnosis as well as monitoring response to therapy. The [^{13}C]urea breath test, although widely used in clinical practice, is more costly because it requires mass spectrometry equipment for measuring the excretion of the ^{13}C nonradioactive isotope. On the other hand, the [^{14}C]urea breath test, which is simpler to perform than the ^{13}C test, only requires a scintillation

counter (available at many hospitals) for measuring the excreted labeled CO_2. The ^{14}C test is thus less expensive, but it involves exposure to a long-lived radioactive isotope, albeit at a very low dose; therefore, testing with ^{13}C is preferred for use in children and in pregnant women. A recent review summarizing numerous studies showed that the UBT was sensitive (90–100 percent) and specific (86–100 percent) (Vaira and Vakil 2001). The [^{13}C] UBT has an excellent sensitivity regardless of age, but the specificity decreases in infants and young children. Breath tests have also proved very reliable in indicating cure or therapeutic failure 1 month after antibiotic therapy (Logan et al. 1994). However, false-negative results can occur in patients that have had prior antibiotic, bismuth salts, or PPI therapy, so treatment with a drug influencing the UBT should be stopped as soon as possible according to guidelines before performing the UBT.

Serology

H. pylori infection results in a systemic as well as local immune response characterized by an elevation of specific IgG and IgA in serum, elevated secretory IgA, and low-level gastric IgM (Rathbone et al. 1986), phenomena that have permitted development of a multitude of serological tests for the detection of *H. pylori*. Several serological techniques have been used for the study of *H. pylori* infection (e.g. complement fixation, hemagglutination, immunoblot, fluoroimmunoassay), but conventional enzyme-linked immunosorbent assay (ELISA) is considered the optimal serological method. Various antigens have been employed for detection of *H. pylori*-specific antibodies, including whole cells and whole-cell sonicates, acid-glycine extracts, and heat-stable antigens, or more purified enriched antigen fractions such as urease and a 120 kDa protein. Tests using a mixture of the more purified antigen preparations have generally been more sensitive and specific than tests using whole-cell or sonicated extracts (Hirschl et al. 1990). Many kits using the enriched antigens for detecting specific *H. pylori* IgG are available commercially. The best of these serum ELISA tests generally yield sensitivities and specificities around 85 and 79 percent, respectively, compared to histology or urease testing (Goossens et al. 1992; Loy et al. 1996; Leodolter et al. 2001). Since elevated IgG antibody levels may persist for years in untreated individuals, serology has been very useful for epidemiological studies and also for screening individual dyspeptic patients before endoscopy (Megraud et al. 1989; Sobala et al. 1991b). After successful bacterial eradication, antibody titers tend to decrease to approximately 50 percent of pretreatment levels at about 6 months (Kosunen et al. 1992). There are, however, large person-to-person variations in changes in antibody titers after successful therapy and in some individuals low levels of IgG may persist for years after *H. pylori* eradication (Glupczynski et al. 1992). As serological assays have a lower diagnostic accuracy they should only be used for screening of *H. pylori* infection after careful local validation. They are of limited value for short-term monitoring of antimicrobial therapy and use of the urea breath tests (^{13}C or ^{14}C tests) may be more appropriate in this setting.

Stool antigen test

The stool antigen test is a more recently developed immunoassay for detection of *H. pylori* antigens in stool samples. The ELISA-based kit test for detection of specific antigen in stool specimens has been met with considerable interest as it is noninvasive, does not require highly specialized equipment and, unlike serology, is more likely to provide evidence of active, rather than past infection. Furthermore, it may be more appropriate for use in pediatric patients than either serology or other invasive methods. Numerous evaluative studies have been conducted to determine the performance of stool antigen kits as a means of primary diagnosis, as well as post-eradication follow-up. A recent review of 43 studies comprising a total of 4 769 patients reported that overall the Premier Platinum HpSA (Meridian Diagnostics) kit was a highly sensitive (92.4 percent) and specific (91.9 percent) means of diagnosing infection in untreated patients (Gisbert and Pajares 2001). Analysis of 25 studies examining a total of 2 078 post-treatment patients demonstrated that the HpSA test could also be used as a sensitive (88.3 percent) and specific (92.0 percent) follow-up test at $\geqslant$4 weeks to assess eradication outcome, although some studies gave contradictory results (Gisbert and Pajares 2001). Results reported from one of the few studies that have compared the HpSA kit with the newer IDEIA HpStAR (Dayko) kit suggested that the latter is marginally more sensitive (93.8 versus 98.0 percent) for primary diagnosis of pediatric infection (Makristathis et al. 2000). Similarly, the IDEIA HpStAR kit was reportedly more specific than the HpSA kit for post-treatment follow-up of pediatric patients (Makristathis et al. 2000). A different study of 148 adult dyspeptic patients demonstrated that the sensitivity of the IDEIA HpStAR kit for follow-up of patients after eradication therapy was higher (88.6 percent) than that of the HpSA kit (80.0 percent) (Leodolter et al. 2003). Overall indications are that these stool tests have been shown to be highly sensitive and specific when applied to adult (Odaka et al. 2002; Vaira et al. 2002) and to pediatric populations (Konstantopoulos et al. 2001; Oderda et al. 2000; Koletzko et al. 2003) for testing pretreatment (Gisbert and Pajares 2001; Konstantopoulos et al. 2001; Makristathis et al. 2000; Oderda et al. 2000) and post-therapy (Gisbert and Pajares 2001; Konstantopoulos et al. 2001; Leodolter et al. 2003; Makristathis et al. 2000; Vaira et al. 2002).

Near patient tests using whole blood

Rapid office-based tests using whole blood have been developed as they are quick and easy to perform, but sensitivity and specificity vary widely. Their inaccuracy therefore precludes their use as diagnostic tests in a general practice setting (Leodolter et al. 2003).

Salivary and urine antibody assays

The use of commercial assays to detect anti-*H. pylori* IgG antibodies in saliva or urine are attractive alternatives as they are completely noninvasive and easy to perform. However, a large trial of a salivary assay showed the accuracy was unacceptable (sensitivity 81 percent and specificity 73 percent) (Luzza et al. 2000). However, data on urine antibody assays indicate an accuracy comparable to that of the serum ELISA in Japanese (Kato et al. 2000) and European populations (Leodolter et al. 2003).

Molecular tests

There are no commercially available assays for the detection of *H. pylori* in gastric biopsies even though several validated genus and species-specific probe hybridization and PCR assays have been described and have recently been reviewed (Kabir 2001). The method with the potential for greatest sensitivity is detection of *H. pylori*-specific DNA by PCR. Gastric biopsy material and gastric juice aspirates have been used for PCR diagnosis targeting various DNA sequences, including regions of the 16S rRNA gene (Bell et al. 2003), urease subunit genes (Romero-Lopez et al. 1993), the *vacA* gene (Chisholm and Owen, 2003), and a portion of a gene encoding a species-specific 26 kDa protein (Hammar et al. 1992). Results with these PCR tests have yielded ranges of sensitivities and specificities depending on the material analyzed and the assays (Kabir 2001). Molecular techniques may become more relevant in the future for the diagnosis of *H. pylori* infection. As they are technically more complex than standard kit assays, they are essentially a specialist laboratory tool at present, but can be useful to clarify unusual results – for instance, UBT or CLO test positive but culture negative specimens.

String test

The swallowed string test (Enterotest) is a minimal invasive approach as an alternative to endoscopy for direct sampling of gastric fluid, although there is no information on its use in clinical practice. Sampling of gastric juice provides a good measure of the infecting strain population shed from the gastric mucosa (Williams et al. 2000) so analysis of the string sample by culture or PCR could be informative when its accuracy has been established.

GENERAL TREATMENT APPROACHES

Who to treat

The favorable impact of *H. pylori* eradication on the long-term clinical course of peptic ulcer disease is now well established. Cure of the infection heals duodenal and gastric ulcers refractory to antisecretory drugs, speeds up ulcer healing, heals ulcers without concomitant gastric acid suppression and, most importantly, virtually prevents recurrences and ulcer complications. There is substantial reduction in the risk of gastric and duodenal ulcer recurrence to <10 percent within a year when *H. pylori* has been successfully eradicated (Tytgat 1994). In 1994 the US National Institutes of Health Consensus Conference (NIH Consensus Conference 1994) and other groups, including the *H. pylori* European Study Group and the European Society of Primary Care Gastroenterology, have since recommended that all ulcer patients infected with *H. pylori* should be treated with antimicrobial agents – now well established as a cost-effective strategy. However, considerable confusion has continued over management of *H. pylori* infection, particularly among primary care physicians. The fact that many countries lacked national guidelines lead to the first Maastricht Consensus Report in which recommendations were made enabling the development of European guidelines (EHPSG 1997). The guidelines were subsequently updated at the Maastricht 2-2000 Consensus Meeting – a central element of these guidelines was to define who to treat and how to treat them (Malfertheiner et al. 2002). For patient management, the key recommended strategy was that a 'test and treat' approach should be offered to adult patients under the age of 45 years (the age cut-off may vary locally according to the mean age of gastric cancer onset) with persistent dyspepsia, having excluded those with predominantly gastroesophageal disease (GERD) symptoms, nonsteroidal antiinflammatory drug (NSAID) users, and patients with alarm symptoms. However, the role of *H. pylori* in functional dyspepsia and the evidence to support its treatment continue to be controversial.

How to treat – first-line therapies

Because of its unique location in the gastric mucosa and mucus, the treatment of *H. pylori* infection initially proved to be difficult. Many antimicrobial agents are poorly secreted in the gastric mucosa or are inactivated in the acid environment of the stomach lumen. This means that in vitro activity of various agents usually does not translate into success in vivo. Numerous clinical

trials of different combinations of antibiotics, proton pump inhibitors, and bismuth-containing compounds have been undertaken over the past 15 years. The ideal treatment should be easy to comply with, cheap, have minimal side effects, and attain high eradication rates. On these criteria, the most effective treatment options presently available as recommended by the Maastricht 2-2000 Consensus Report (Malfertheiner et al. 2002) use antibiotics combined with either proton pump inhibitors (PPI) or ranitidine bismuth citrate (RBS). Potential side effects are nausea, diarrhea, and taste disturbances. One week triple therapies combining a PPI such as omeprazole and a low dose of two antibiotics (metronidazole or amoxicillin plus clarithromycin) are the overall most effective eradication regimens with eradication rates of about 90 percent (Harris and Misiewicz 2001). Omeprazole, pantoprazole, and lansoprazole remain the reference PPIs in the first-line regimens for *H. pylori* eradication (Ulmer et al. 2003). Ranitidine-bismuth-citrate may be used as an alternative to a PPI in combination with clarithromycin and either metronidazole or amoxicillin for a week. In areas of high metronidazole resistance, amoxicillin should be used as an alternative antibiotic. A comprehensive review of treatment options is given elsewhere (Harris and Misiewicz 2001; Suerbaum and Michetti 2002).

How to treat – second-line therapies

Even with the current most effective treatment regimens, about 10–20 percent of the patients will fail to eradicate the infection and will remain *H. pylori* positive after 4 weeks or longer. Failures are due mainly to bacterial resistance or poor compliance. Widespread resistance to antibiotics used in eradication therapy, in particular metronidazole and clarithromycin, is a source of growing concern because of its impact on eradication. The clinical relevance of antibiotic resistance is increasingly accepted as an issue and its importance in therapy has been highlighted in several recent meta-analyses of over 60 studies (Dore et al. 2000; van der Wooden et al. 1999). For instance, an analysis of studies published between 1983 and 1997 found that metronidazole resistance reduced effectiveness by 37.7 percent, but most striking was the finding that clarithromycin resistance reduced effectiveness by an average of 55 percent (Dore et al. 2000).

An optimal strategy for retreatment after initial failure of eradication has not yet been established, but the recommended second-line therapy according to the Maastricht 2-2000 report is use of quadruple therapy – a PPI with bismuth, metronidazole, and tetracycline (an antibiotic unlikely to have been used previously in front-line therapy). It is advisable that the antibiotic susceptibility of the organism is determined before retreating because of high resistance rates in some populations to metronidazole. Resistance to both clarithromycin and metronidazole is a major concern in retreatment – for instance, in a German study, 46 percent of 554 isolates from patients in whom one or more therapies had failed were resistant to both antibiotics (Heep et al. 2000). The use of ranitidine-bismuth-citrate-based triple therapy is another possibility for retreatment (Suerbaum and Michetti 2002). Subsequent failures should be referred and handled on a case-by-case basis. Interestingly a number of studies have shown rifabutin-based rescue therapies might offer an encouraging alternative strategy for eradication.

It should be noted that probiotics have been investigated in a number of trials and although the results appear promising there is insufficient evidence to date to support their use as an alternative to standard anti-*H. pylori* therapies (Cremonini et al. 2001).

Antibiotic resistance

There is a clear difference between developing countries where a high proportion of strains may be resistant and the developed countries where resistances range from 10 to 50 percent (Megraud 2001). In a European multicenter study, the mean rate of resistance to metronidazole was 33.1 percent, to clarithromycin 9.9 percent, and to amoxicillin 0.8 percent (Glupczynski et al. 2001). Resistance to metronidazole was significantly higher in females, while resistance to clarithromycin was significantly higher in children and teens. Similarly, metronidazole resistance rates of between 29 and 40 percent have been reported in England (Teare et al. 1999; Glupczynski et al. 2001; Parsons et al. 2001), although rates may be significantly higher in some communities (Banatvala et al. 1993).

A number of phenotypic methods have been used to detect in vitro antimicrobial resistance in *H. pylori*, of which the most important are agar dilution, disk diffusion, and Epsilometer test (E-test) methods (Megraud 2001). The E-test appears to be an easy method for determining minimal inhibitory concentrations (MIC) as an alternative to the more labor-intensive agar dilution method. However, the European Study Group multicenter analysis highlighted discrepancies between E-test and agar dilution for metronidazole and lack of reproducibility in some isolates (Glupczynski et al. 1991). The National Committee for Clinical Laboratory Standards in the United States has published guidance on susceptibility testing by agar dilution (NCCLS 1999) and the British Society of Antmicrobial Chemotherapy on susceptibility testing by the E-test (King 2001). However, as there are no published internationally agreed susceptibility methods by disk diffusion specifically for *H. pylori*, a method based on review of the relevant literature comparing E-test or agar dilution with disk diffusion was recommended by the PHLS

Helicobacter Working Group (McNulty et al. 2002). Key factors considered that might contribute to the discrepancies seen with metronidazole susceptibility testing, but rarely with clarithromycin were: culture medium, age of cultures, size of inoculum, anaerobic preincubation, disk content, zone size breakpoints, and mixed cultures. In addition to the phenotypic methods, a number of molecular assays have been developed for the detection of mutations in the 23S rRNA gene associated with clarithromycin resistance (Versalovic et al. 1996; Owen 2002). Use of a real-time PCR format, for instance, enables assays to be applied directly to analysis of DNA extracted from gastric biopsies without the need for culture, thus enabling resistance results to be available on the same day, compared to 7–12 days by conventional methods (Chisholm et al. 2001). At present there are no molecular assays for resistance to metronidazole as the mechanism of resistance to that antibiotic is not fully understood.

Prevention

With our lack of knowledge concerning the exact sources, reservoirs, and modes of transmission of *H. pylori*, there is currently no possibility of preventing this infection. Primary prevention of infection in childhood may be, in the future, the most effective way to eliminate gastric cancer in high-risk populations, but this must await the development of an effective vaccine. Prophylactic and therapeutic vaccination with *H. pylori* antigens, such as urease, as a candidate vaccine component have been successful in animal models, but the translation to a human vaccine remains difficult, in part because the immunology of the stomach remains poorly understood (Ruggiero et al. 2003).

PATHOGENICITY AND VIRULENCE FACTORS

H. pylori is highly adapted to its role as a gastric pathogen and its success appears dependent on two major groups of determinants, most of which are currently viewed as 'putative' (Dunn et al. 1997). First, maintenance factors which allow the organisms to colonize and to persist long term, and second, virulence factors that contribute to three major pathogenic effects that are either direct or as part of the modulation of the host response – gastric inflammation, disruption of the gastric mucosal barrier, and altered gastric physiology.

Infected individuals are typically characterized by chronic gastric inflammation, but most do not progress to develop peptic ulcer disease or gastric cancer. The reasons that some individuals develop more serious disease is not understood, but the most likely explanation appears to lie in a complex combination of host, environment, and strain-related factors. Putative pathogenicity factors including virulence mechanisms of *H. pylori* have been the focus of intense investigation over the past 15 years. Efforts are now aided by the availability of two complete genome sequences that have enabled developments, in areas such as gene expression and proteomics, to provide new insights. A number of key bacterial factors that may contribute to pathogenicity and/or virulence have been described and characterized, and are highlighted below.

Urease

H. pylori urease is an important adaptive enzyme that is essential for the establishment of gastric mucosal colonization according to animal experimental studies with urease-negative mutants and urease inhibitors (Mobley 2001). Urease may serve to protect the organism from gastric acid by converting urea to ammonia and carbon dioxide, thus surrounding the bacterium with an alkaline layer that neutralizes the acid. Urease may also function as a virulence factor as it appears to be an important cause of the inflammation seen in the lamina propria that is the hallmark of chronic *H. pylori* infections. Furthermore, in vivo urease activity is toxic to human gastric epithelial cells (Mai et al. 1992; Segal et al. 1992).

Catalase and superoxide dismutase

Other adaptative enzymes that form part of the antioxidant defense mechanism facilitating the survival of *H. pylori* in the gastric mucosa include catalase and superoxide dismutase – both of which protect bacteria against the toxic effects of reactive oxygen species, such as hydrogen peroxide and associated hydroxyl radicals released by activated gastric neutrophils infiltrating the lamina propria and epithelium (Hazell et al. 2001).

Flagella

The spiral shape and up to six polar sheathed flagella of *H. pylori* provide its motility in gastric juice and gastric mucus. Urease enzyme, by breaking down the urea in gastric juice, appears to generate enough bicarbonate and ammonia around *H. pylori* to allow its safe migration through the gastric acid barrier and its arrival at the protective mucus layer. Motility and urease have been shown to be essential factors for colonization in an animal model using gnotobiotic piglets (Eaton et al. 1989). *H. pylori* also possess a flagella hook which is essential for the attachment of flagella. Mutants with mutations in the *flgE* gene, which encodes the flagellar hook protein, are nonmotile and aflagellate but continue to synthesize flagellins (Luke and Penn 1995).

CagA pathogenicity island

The cytotoxin associated gene (*cagA*) is present in approximately 70 percent of strains (type 1) and is a marker for the presence of a distinctive genomic pathogenicity island (designated *cag*PAI) – a 40 kb region consisting of about 30 putative genes encoding a functional type IV secretion system capable of direct translocation of complex proteins into eukaryotic cells. Most *cagA*+ strains contain a complete and contiguous *cag*PAI and the gene order is relatively well conserved, although several possible structures of the *cag*PAI have been proposed (Censini et al. 1996). CagA, the highly immunodominant 128-kDa protein product of *cagA*, is produced by all type 1 strains (Covacci et al. 1993). It has been shown to be translocated into cultured gastric epithelial cells, where it induces host cell kinases that phosphorylate tyrosine residues in CagA adjacent to the site of bacterial adhesion on the host gastric epithelial cells (Asahi et al. 2000; Backert et al. 2000) This in turn activates eukaryotic signal transduction pathways and cytoskeletal plasticity (Odenbreit et al. 2000; Segal et al. 1999; Stein et al. 2000). Several putative tyrosine phosphorylation motifs such as EPIYA have been identified in CagA (Stein and Bagnoli 2002). The precise link among *cagA*, the production of the CagA protein, and strain virulence is controversial. For instance, there are marked regional differences in frequencies of type 1 strains as *cag*PAI carriage is higher in East Asian strains compared to European strains. It appears currently that *cagA* status alone is insufficient to reliably predict either virulence or association with gastric ulcers (Graham et al. 1996; Mitchell et al. 1996; Jenks et al. 1998).

Vacuolating cytotoxin

Another interesting aspect of *H. pylori* pathogenicity is the so-called vacuolating cytotoxin. This secreted exotoxin is expressed in about 50 percent of *H. pylori* strains and is responsible for inducing vacuoles in a variety of eukaryotic cell lines (Leunk et al. 1988). Oral administration of purified cytotoxin to mice causes ulceration and gastric lesions bearing some similarities with the pathology observed in humans (Telford et al. 1994). The cytotoxin has been characterized and purified as an 87 kDa protein which shows limited homology with other known bacterial toxins (Cover and Blaser 1992). The gene encoding this cytotoxin is called *vacA* and has been cloned by Cover et al. (1994). The *vacA* gene is present in all *H. pylori* strains, regardless of whether they express toxin activity in vitro and is often used as a marker of enhanced virulence potential. However, analysis of the role of VacA in disease is complicated by the extensive variability in the *vacA* sequences. It has been established that *vacA* has a mosaic structure and two parts of the gene show particular sequence heterogeneity (Atherton et al. 1999). The s-region encoding the signal peptide located at the 5′ end of the gene occurs as an s1 and s2 type. Within type s1, several subtypes (s1a, s1b, and s1c) can be distinguished. Within the m-region (middle), there are m1 (m1a and m1b subtypes) and m2 types. Despite numerous studies, evidence for reproducible associations between *vacA* genotype and disease severity remains controversial – for instance the link between the s1 allele and peptic ulcer disease (Atherton et al. 1997). By contrast, it is better established that strains with the s2 form of *vacA* are nontoxigenic, cause little inflammation, and are rarely associated with peptic ulceration (Letley et al. 1999). The toxin-positive and toxin-negative strains present large dissimilarities (Cover et al. 1994) reflecting the possible combinations of the distinct s- and m-region types determining the production and cell-specific activity of the cytotoxin. Furthermore, there is evidence that the Vac A molecules exhibit different cell specificities according to the presence of specific receptors on host cells and this was found to be dependent on the type of allele present in the m-region (Pagliaccia et al. 1998).

Adhesins

Adherence of *H. pylori* to specific host-cell macromolecular receptors is essential to stabilize it against mucosal shedding into the gastric lumen and to ensure good access to nutrients from gastric epithelium damaged by infection. The best defined *H. pylori* adhesin–receptor interaction found to date is that between the Lewis b blood group antigen binding adhesin, BabA (an outer membrane protein) and Lewis b antigens – terminal fucose, containing blood group antigens found in the gastrointestinal mucosa as well as on red blood cells (Ilver et al. 1998). The adhesin binding to blood group O-specific antigens may help explain the predisposition of people with blood group O to peptic ulcer disease and gastric adenocarcinoma. The *babA* gene exists in two distinct forms – *babA1* comprises an almost complete open reading frame (ORF), but lacks the necessary initiation site and cannot be translated. The second form, *babA2* contains a 10-bp insert at its 5′ end with an in-frame ATG start codon permitting expression of the ORF which correlates with adhesion. The presence of *babA* in *H. pylori* also correlates with duodenal ulcer and adenocarcinoma (Gerhard et al. 1999). However, the spectrum of *H. pylori*-adhesin receptors is complex and adaptive. There is evidence that BabA is not an absolute requirement and that other adhesins independent of BabA may interact with Lewis b antigens (Testerman et al. 2001). For example, a sialyldimeric Lewis x glycosphingolipid was identified as the sialic acid binding adhesin (SabA) (Mahdavi et al. 2002). It was suggested that the ability of strains to adhere to

sialylated glycoconjugates during chronic inflammation might contribute to virulence and the chronicity of infection.

Lewis antigens

The cell envelope of *H. pylori* contains LPS, the O-polysaccharide chain of which expresses blood group antigens similar to those of the host – predominantly type 2 blood group determinants Lewis x and Lewis y, although type 1 determinants Lewis a and Lewis b have also been described. Bacterial expression of these antigens has been suggested as providing a mechanism by which the bacterium can evade the immune system by mimicking the Lewis antigens expressed on the gastric epithelium, and so aid in colonization (Appelmelk et al. 1997).

EPIDEMIOLOGICAL ASPECTS OF *H. PYLORI*

Prevalence

H. pylori is responsible for one of the world's most common bacterial infections. It is usually acquired without knowledge of the time of exposure, the source or the magnitude of the infecting dose. In adult infections, it is likely from volunteer experiments that the infectious dose is high, possibly in the order of 10^5 to 10^9 organisms depending on the strains (Feldman et al. 1998). *H. pylori* infection persists silently in most infected individuals and causes superficial gastritis that persists for years, leading to chronic inflammation. In developing countries the organism is acquired early in childhood, with infection rates of 50–60 percent by the age of 10 and up to 90 percent in adults (Megraud et al. 1989). By contrast, in developed countries few infections occur during childhood and a gradual increase in prevalence is observed with age (at a rate of about 0.5–1 percent per year), leading to infection rates of 20–30 percent by the age of 20, and of about 50 percent at 50–60 years. Differences within age groups in adulthood within a population seem to reflect a birth cohort affect, indicating a higher rate of infection in earlier decades caused mainly by poor living conditions in earlier birth cohorts (Banatvala et al. 1994). A study of the prevalence of *H. pylori* in the general population of England and Wales showed that an estimated total of 7.5 million people had an active infection (Vyse et al. 2002). Estimated prevalence varied by region and was highest in London. Prevalence also varies between populations of different ethnic origin. In the USA, healthy adult Hispanic and black populations have seropositivity rates several-fold higher than non-Hispanic white populations, independently of age, gender, geographical location (urban versus rural), educational level, and current income (Graham et al. 1991b). The reasons for these highly significant differences in seroprevalence rates among ethnic groups are not well understood, but socioeconomic conditions during childhood, such as crowding, poor sanitation, and close contact with infected persons, appear to be important risk factors. A twin study from Scandinavia comparing the rates of infection in monozygotic and dizygotic twin pairs reared apart or together has suggested a large inherited susceptibility to infection, in addition to environment-related factors (Malaty et al. 1994). The prevalence of *H. pylori* has been investigated in selected populations in a variety of countries around the world, and is reviewed elsewhere (Pounder and Ng 1995; Brown 2000).

Sources and transmission of infection

The natural hosts for *H. pylori* are humans. Closely similar, but distinguishable organisms have been isolated in primates (Goodwin et al. 1989b) – these include *H. nemestrinae* which is now classified as *H. pylori* (Suerbaum et al. 2002), and *H. acinonychis* isolated from captive cheetahs (Eaton et al. 1993). *H. pylori* has been isolated from the stomachs of domestic cats, raising the possibility that the organism may be a zoonotic pathogen with transmission occurring from cats to humans (Handt et al. 1994, 1995). There is no primary environmental reservoir and person-to-person transmission is probably the dominant mode of transmission, although controversy exists over whether fecal–oral or oral–oral spread predominates. From its protected niche in the gastric mucosa *H. pylori* could either be transmitted by the oral–oral route with episodes of gastroesophageal reflux permitting access to the mouth, or be excreted in the feces. *H. pylori* has been isolated from the feces (Thomas et al. 1992; Kelly et al. 1994), from dental plaque (Shames et al. 1989), and from saliva (Ferguson et al. 1993), albeit on few occasions, but it has been detected by polymerase chain reaction from such specimens in a greater proportion of infected subjects suggesting that transmission by both routes can indeed occur (Mapstone et al. 1993). The increased rates of infection among children of West African mothers who premasticate their infants' food (Albenque et al. 1990) and among Chinese who share eating utensils favors oral–oral transmission. An increased rate of infection has also been reported among endoscopists not wearing gloves (Mitchell et al. 1989). However, rates of infection among dental workers were not elevated compared to age-matched control groups (Malaty et al. 1992).

The main epidemiological evidence supporting fecal–oral transmission, which may occur particularly in developing countries, is the similarity of the seroepidemiology with that of hepatitis A (Graham et al. 1991a). Whether by oral–oral route or by fecal–oral transmission, intra-

familial spread appears to occur in western populations (Drumm et al. 1990; Rowland 2000). When a parent or a child is infected, the other family members are usually more likely to be infected than family members from uninfected index cases (parent or children). Cross-sectional studies have shown that both a high density of living and limited access to sanitary facilities are significantly associated with higher infection rates, especially when promiscuous living conditions prevailed during childhood (Mendall et al. 1992). Likewise, *H. pylori* seropositivity rates are also notoriously higher in persons living in institutions. For example, at a Bangkok orphanage *H. pylori* seropositivity was 74 percent in children 2–4 years old, well above the rates in children living in rural Thailand (Perez Perez et al. 1990). In another study from Australia, Berkowicz and Lee (1987) found three times the expected prevalence of antibody among inmates of an institution for the mentally retarded. Transmission of *H. pylori* in the endoscopy suite can occasionally occur. Patient-to-patient transmission by an endoscope is a potential threat which has been reported at a frequency of three infections for every 1 000 gastroduodenoscopies in one study from the Netherlands (Langenberg et al. 1990). Contact with secretions from infected patients may also subject endoscopists to risk of infection. There have been detailed reports of accidental infections, as well as epidemics of gastritis transmitted by infected gastric secretions (Gledhill et al. 1985; Sobala et al. 1991a). The prevalence of *H. pylori* infection was significantly higher in a group of Australian gastroenterologists than in an age-matched group of control subjects (Lin et al. 1994). There is little evidence for transmission between couples (Perez Perez et al. 1991). Nor is there any correlation with sexual preference, number of sexual partners, history of sexually transmitted diseases, or infection with human immunodeficiency virus among those infected with *H. pylori* compared with those who were not infected (Polish et al. 1991).

Waterborne transmission may be important, though not yet proven, especially in areas of the world that have high rates of *H. pylori* infection and less than adequate water quality (Brown 2000). Consumption of vegetables irrigated or washed with untreated or contaminated water may also be a source of infection. A study performed on raw municipal wastewater from an open wastewater canal on the US–Mexico border reported for the first time culture of *H. pylori* from an environmental source (Lu et al. 2002). There is growing evidence from PCR for *Helicobacter* DNA in drinking water and that biofilms may act as a reservoir in water distribution systems in developing (Bunn et al. 2002) and in developed countries (Park et al. 2001). Furthermore, the presence of *H. pylori* has been detected using fluorescent in situ hybridization in wastewater and river water samples in Spain (Moreno et al. 2003).

CLINICAL CONSEQUENCES OF *H. PYLORI* INFECTION

The clinical consequences of *H. pylori* infection are highly variable and appear to be influenced by both microbial and host factors, as well as possible environmental factors. Acute infection, usually in childhood, causes transient hypochlorhydria, after which chronic gastritis will develop in virtually all persistently colonized persons – most of whom will never have symptoms.

Epidemic hypochlorhydria and acute gastritis

Two outbreaks of mild gastrointestinal illness with abdominal pain, nausea, and vomiting were reported in previously healthy people who, in a study on stomach secretions underwent gastric instrumentation (Ramsey et al. 1979; Gledhill et al. 1985). All suffered profound hypochlorhydria which lasted for several months. Retrospective histological examination of material from these outbreaks showed *H. pylori*-like bacteria and active gastritis; moreover, analysis of stored sera from one study demonstrated rising titers of *H. pylori* antibodies. It was presumed that the two outbreaks resulted from cross-infection by instruments passed into the stomach. In two volunteer experiments similar acute manifestations occurred after the ingestion of pure cultures of *H. pylori* (Marshall et al. 1985; Morris and Nicholson 1987).

Chronic gastritis

Most frequently *H. pylori* infection presents as a chronic infection and results in an asymptomatic superficial gastritis. There is now much evidence for a causal relationship between *H. pylori* and chronic superficial gastritis:

- Accidental and voluntary ingestion of *H. pylori* has resulted in sporadic cases or in epidemics of gastritis (see above).
- Natural infection and experimental challenges in various animals simulate human infection with resulting chronic gastritis (Fox and Lee 1993).
- Antimicrobial therapy to eradicate infection results in resolution of gastritis (Glupczynski et al. 1988).
- *H. pylori* only overlies the gastric epithelium and its infection is associated with only certain types of gastroduodenal inflammation (Ormand et al. 1991).
- There is a universal local and systemic immune response to *H. pylori* that can be detected serologically (mainly IgG and IgA antibodies).
- The levels of *H. pylori* specific antibodies decrease with therapy, concomitant with diminution in inflammation (Rauws et al. 1988).

Peptic ulceration

The life-time risk of peptic ulcer in a person infected with *H. pylori* ranges from 3 percent in the United States to 25 percent in Japan (Suerbaum and Michetti 2002). A causal relationship between *H. pylori* and peptic ulceration has been more difficult to establish because only a small proportion of infected individuals develops peptic ulcer and until recently there has been no satisfactory animal model. Longitudinal case–control studies over a 3–10-year period have shown that patients with *H. pylori* infection have a four- to 13-fold increase in the risk of developing duodenal or gastric ulcers (Sipponen et al. 1990; Nomura et al. 1994). Strongest evidence for the pathogenic role of *H. pylori* in peptic ulcer was provided by the marked decrease in recurrence rates of ulcers following *H. pylori* eradication (Hentschel et al. 1993). However, in a landmark study (Ohkusa et al. 2003), it was shown that *H. pylori* could induce duodenal ulceration in the Mongolian gerbil model, finally fulfilling Koch's postulates for duodenal ulceration. The close association between *H. pylori* and peptic ulceration is now generally accepted. Infection with *H. pylori* is a prerequisite for the occurrence of almost all duodenal ulcers in the absence of other precipitating factors such as nonsteroidal anti-inflammatory drug use or Zollinger–Ellison syndrome (Nensey et al. 1991). Over 95 percent of patients with duodenal ulcer have *H. pylori* gastritis. Patients with higher acid output are likely to develop a diffuse antral predominant gastritis which predisposes them to duodenal ulcers.

The association between *H. pylori* and gastric ulcer is slightly less strong, about 80 percent of patients with non-NSAID-induced gastric ulcers being infected. Patients with lower acid output are more likely to have corpus predominant gastritis and multifocal atrophy which predisposes them to gastric ulcer. Our knowledge of the exact role of *H. pylori* in the pathogenesis of this disease is still incomplete and certain host or organism characteristics remain to be characterized.

Gastric malignancy

Descriptive epidemiological studies have shown a higher frequency of gastric cancer in populations with higher prevalences of *H. pylori* infection and a geographical correlation has been found between *H. pylori* infection and gastric cancer death rate (Eurogast Study Group 1993). More importantly, several prospective case–control serological studies have detected a three- to six-fold increased gastric cancer risk in individuals infected with *H. pylori* and an attributable risk of at least 50 percent of all gastric cancers (Forman et al. 1991; Nomura et al. 1991; Parsonnet et al. 1991). One prospective study has shown that *H. pylori* chronic diffuse gastritis did significantly increase the risk of developing intestinal metaplasia and atrophic gastritis over a mean follow-up of 10 years (Kuipers et al. 1995); these pathological lesions are widely accepted as precursors of gastric carcinoma. Thus in patients with low acid output and chronic *H. pylori* infection, corpus predominant atrophic gastritis can trigger a sequence of pathological events involving intestinal metaplasia and dysplasia leading to distal gastric carcinoma. Since gastric cancer develops only in a small proportion of infected subjects and because it may also occur without evidence of past *H. pylori* infection, the relationship between *H. pylori* and carcinogenesis is not straightforward. Most of the evidence is epidemiological and thus open to different interpretations. A number of studies have described the development of gastric cancer in *H. pylori*-infected Mongolian gerbils, but this model has a number of limitations. The role of strain diversity in determining virulence factors important in disease progression needs further investigation, as does the role of human genetic diversity in determining predisposition to gastric cancer risk and their interaction with environmental factors especially diet.

H. pylori infection significantly increases the risk of gastric mucosa-associated lymphoid tissue (MALT) lymphoma and up to 98 percent of patients with this disease are infected with *H. pylori* (Wotherspoon et al. 1991; Parsonnet et al. 1994; Wotherspoon 2000). Successful eradication of *H. pylori* by specific antimicrobial treatment has resulted in complete or partial tumor regression in 27 out of 33 (82 percent) patients with low-grade MALT gastric lymphoma (Bayerdörffer et al. 1995) with a yearly recurrence rate of approximately 5 percent (Stolte et al. 2002) However, long-term follow-up will be needed to clarify whether the remission is lasting with high grade lymphomas.

Nonulcer dyspepsia

The association between *H. pylori* and nonulcer dyspepsia (NUD) is controversial. Dyspepsia is common and imposes a substantial clinical and financial burden on health services. Cross-sectional population studies have suggested that up to 23 percent of functional dyspepsia in the community is attributable to *H. pylori* infection. However, in a randomized controlled trial, it was found that community screening and treatment for *H. pylori* produced only a 5 percent reduction in dyspepsia and that the small benefit had no impact on quality of life (Moayyedi et al. 2000).

Gastroesophageal reflux disease

The role of *H. pylori* in the pathogenesis of gastroesophageal reflux disease (GERD) is complex and remains incompletely understood. The incidences of esophageal diseases, including reflux disease and esopha-

geal carcinomas, have increased in recent years, whereas the incidences of *H. pylori*-related ulcer disease and distal gastric adenocarcinoma have declined. These epidemiological trends prompted a debate that presence of *H. pylori*, particularly *cagA*-positive strains, might have a protective role for GERD (Richter 2001). Despite the conflicting data, it would appear that *H. pylori* does not have a major causal role in pathogenesis of GERD and in the development of Barrett's esophagus, dysplasia, and adenocarcinoma of the esophagus and esophogastric junction (Sharma 2001).

Extragastric diseases

H. pylori has been weakly linked, mainly by PCR evidence, to numerous extragastric diseases that include ischemic heart disease, Parkinson's disease, inflammatory bowel disease, skin disease, and liver and biliary tract disease. However, for most of these conditions bacteria have rarely been isolated to provide convincing evidence that *H. pylori* or other helicobacters might have a causal role (Fox 2002).

SUMMARY OF DESCRIPTIONS OF *HELICOBACTER* SPECIES

Gastric helicobacters (in alphabetical order)

HELICOBACTER ACINONYCHIS

H. acinonychis (acinonyx) (Eaton et al. 1993) is closely related to *H. pylori* by 16S rRNA phylogenetic analysis. The species consists of short, spiral rods 0.3 μm wide by 1.5–2.0 μm long with between two and five sheathed unipolar flagella. It is able to hydrolyze urea and is isolated from the gastric mucosa of captive cheetahs (*Acinonyx jubilatus*) with gastritis. The G + C content of DNA of the type strain is 30 mol% and the type strain is CCUG 29263.

HELICOBACTER BIZZOZERONI

H. bizzozeroni is a long spiral organism isolated from the gastric mucosa of dogs (Hänninen et al. 1996). The bacteria are 5–10 μm long by 0.3 μm wide and each cell has 10 to 20 sheathed flagella at both ends of the cell, but lack periplasmic fibrils. The organisms are microaerophilic and grow at 37 and 42°C, but not at 25°C. It hydrolyzes urea and reduces nitrate and, in contrast to *H. felis*, hydrolyzed indoxylacetate. The type strain is ATCC 700030.

HELICOBACTER FELIS

H. felis is a morphological distinct organism first isolated from the gastric mucosa of a cat (Paster et al. 1991). Cells are rigid, tightly spiral, 0.4 μm wide by 5–7.5 μm long, with between five and seven spirals per cell surrounded by periplasmic fibrils appearing as concentric helical ridges in pairs, threes, or singly. Tufts of 14–20 bipolar sheathed flagella are positioned slightly off center at the end of the cell. It is microaerophilic, but can grow anaerobically, growing at 42°C. It is commonly present in the gastric mucosa of cats and dogs, sometimes associated with gastritis. The pathogenicity for humans is unknown although a gastric infection has been detected by PCR (Germani et al., 1997). The species has highest similarity by 16S rDNA sequencing to *H. bizzozeroni* and *H. salomonis*. The species can be propagated in mice, so is useful in screening trials for anti-*H. pylori* chemotherapeutic agents, studies of pathogenic mechanisms, and for development of a vaccine model. The G + C content of DNA is 42.5 mol% and the type strain is ATCC 49179.

HELICOBACTER HEILMANNII

H. heilmannii was first described and originally named *Gastrospirillum hominis* by McNulty et al. (1989). It is the only helicobacter, other than *H. pylori*, to colonize the human stomach and be associated with gastritis. A survey of gastric biopsies in south east England using PCR showed that *H. heilmannii*-like organisms had a prevalence rate of 2.3 percent (Chisholm and Owen 2003). Although the nomenclature implies a defined species, 16S rDNA phylogenetic analysis demonstrated that *H. heilmannii* comprises two related but distinct types. Type 1 is closely related to *Candidatus H. suis*, an organism found in pigs (Mendes et al. 1990), whereas type 2 is more closely related to the *H. bizzozeroni/H. felis/H. salomonis* subgroup that commonly infect cats and dogs. It has not been cultured, but electron micrographs reveal tightly spiral, bipolar flagellated cells that lack periplasmic fibrils.

HELICOBACTER MUSTELAE

H. mustelae (Fox et al. 1989) was first described as a subspecies of *C. pylori*, but was later elevated to specific status and transferred to *Helicobacter* by Goodwin et al. (1989a). It is similar to *H. pylori*, but has multiple lateral as well as polar flagella. Growth occurs at 42°C and anaerobically with CO_2. It reduces nitrate and is isolated from the normal or inflamed gastric mucosa of the ferret (*Mustela putorius furo*). The G + C content of DNA is 36–41 mol% and the type strain is ATCC 43772.

HELICOBACTER NEMESTRINAE

H. nemestrinae (Bronsdon et al. 1991) was originally described as phenotypically similar and closely related genetically to *H. pylori*. It is isolated from the gastric mucosa of the pigtailed monkey (*Macaca nemestrina*); there is no associated pathology. The species has been proposed as a junior synonym of *H. pylori* (Suerbaum et al. 2002).

HELICOBACTER PYLORI

H. pylori (Marshall et al. 1984) was originally named *Campylobacter pyloridis*, but changed to *C. pylori* and later moved by Goodwin et al. (1989) to *Helicobacter* as the type species of the genus. Cells are curved or spiral with between four and eight unipolar sheathed flagella, some with terminal bulbs. Colonies are nonpigmented, translucent, 1–2 mm in diameter. There is variable growth at 42°C. The species may grow in air enriched with 10 percent CO_2, but not anaerobically. It is nitrate-negative. *H. pylori* is the major cause of gastritis in humans and is strongly associated with peptic ulceration and gastric cancer. It does not naturally infect animals, but has been found in domestic cats. Complete genomes have been sequenced for two strains (26695 and J99). The G + C content of DNA is 39 mol% and the type strain is NCTC 11637 (ATCC 43504).

HELICOBACTER SALOMONIS

H. salomonis is a canine gastric helicobacter closely related to *H. felis* and *H. bizzozeroni* (Jalava et al. 1997). Cells are only slightly spiral, 5–7 μm long and 0.8–1.2 μm wide and show an unusual slow wavelike motion. Each cell has tufts of sheathed flagella at one or both ends. All strains are urease and nitrate positive. Isolates were from healthy pet dogs and adult experimental dogs – clinical significance is not known. The type strain is CCUG 37845.

HELICOBACTER SUNCUS

H. suncus is the name proposed for a helicobacter with bipolar sheathed flagella (one at each end) isolated from the stomach of house musk shrews (*Suncus murinus*) with chronic gastritis (Goto et al. 1998). The organism is able to rapidly hydrolyze urea and to reduce nitrate to nitrite. Phylogenetic analysis indicates the organism is closely related to *H. mustellae*. The name has not been validated and no type strain has been designated.

CANDIDATUS H. BOVIS

The name *Candidatus Helicobacter bovis* was proposed by De Groote et al. (1999a) for a new helicobacter-like organism detected in the pyloric part of the abomasa of calves and adult cattle. The organisms were described on the basis of biochemical, immunohistochemical, and electron microscopical data as cultivation was not successful. Phylogenetic analysis placed the organism, corresponding to the reference sequence R2XA, within *Helicobacter* with *H. bilis* as its closest validly named neighbor.

CANDIDATUS H. SUIS

The name *Candidatus Helicobacter suis* was proposed by De Groote et al. (1999b) for a new helicobacter associated with ulcer disease in the stomachs of pigs (Mendes et al. 1990). The tightly spiral microorganism was uncultured and described on the basis of biochemical, immunohistochemical, and electron microscopical data. Phylogenetic analysis placed the organisms within *Helicobacter* and closely related to the *H. felis*, *H. bizzozeroni*, and *H. salomonis* with the latter species as its closest validly named neighbor. In situ hybridization linked the reference sequence to '*Gastrospirillum suis*'-like bacteria which are thought to resemble *H. heilmannii* type I.

Enterohepatic helicobacters

HELICOBACTER AURATI

H. aurati is a urease-positive species cultured from the gastrointestinal tissues (inflamed stomachs and ceca) of adult Syrian hamsters (Patterson et al. 2000). The species is fusiform with multiple bipolar sheathed flagella and periplasmic fibers. It is most closely related to *H. muridarum* and *H. hepaticus*. A type strain was proposed (MIT 97-5075c), but the name has not been validated by publication in the *International Journal Systematic and Evolutionary Microbiology*.

HELICOBACTER BILIS

H. bilis (Fox et al. 1995) cells are fusiform to slightly spiral, 0.5 μm wide by 4–5 μm long, entwined with periplasmic fibers. There are tufts of sheathed flagella numbering three to 14 at each end. Coccoid cells form in old cultures. Colonies appear as a thin spreading layer on agar medium. It is microaerophilic and grows at 42°C and in 20 percent bile and 0.4 percent triphenyltetrazolium chloride. It is urease-positive and can be isolated from the colons and ceca of mice, and the bile and livers of mice with hepatitis. The type strain is ATCC 51630.

HELICOBACTER CANADENSIS

H. canadensis is a novel species cultured from the feces of diarrheic humans, although its pathogenic role is not established (Fox et al. 2000). Cells are slender, curved to spiral rods and motile by means of nonsheathed, single unipolar, or bipolar flagella. Growth occurs at 37 and 42°C. The bacteria are urease, alkaline phosphatase, and gamma glutamyl transpeptidase negative, but catalase and oxidase positive. The species is closely related to *H. pullorum*, but is distinguishable by the ability to hydrolyze indoxyl acetate. The type strain is NLEP-16143 (ATCC 700968).

HELICOBACTER CINAEDI AND HELICOBACTER FENNELLIAE

H. cinaedi and *H. fennelliae* (Totten et al. 1985), first isolated from rectal swabs of homosexual men with symptoms of proctitis, were placed in the genus *Campylobacter*, but were subsequently reclassified in *Helicobacter* by Vandamme et al. (1991). Superficially they

resemble *C. coli*, but grow more slowly and require blood. They possess single, sheathed, predominantly bipolar flagella. Pinpoint translucent colonies are formed after 48 h and develop into flat, watery, spreading colonies like young *C. jejuni* cultures. The main phenotypic test distinguishing the two species is nitrate reduction – *H. fennelliae* cultures do not reduce nitrate – although the two species are clearly distinguishable from each other by 16S rDNA phylogenetic analysis. Both species are associated with gastroenteritis and proctitis in homosexual men and occasional systemic infection in patients with underlying immunodeficiency or serious disease (Kiehlbauch et al. 1994, 1995). *H. cinaedi* apparently forms part of the normal intestinal flora of hamsters (Gebhart et al. 1989) and there is molecular evidence of the species in human gastric biopsies (Peña et al. 2002). The G + C content of DNA of *H. fennelliae* is 35 mol% and the species type strain is ATCC 35684. The G + C content of *H. cinaedi* is 37–38 mol% and the type strain is ATCC 3568. Extensive phylogenetic diversity has been documented within *H. cinaedi* and it should be noted that strains previously referred to as *H. westmeadiae* and *Helicobacter* sp. strain Mainz represent additional strains of *H. cinaedi* (Vandamme and On 2001).

HELICOBACTER CANIS

H. canis (Stanley et al. 1993) consists of slender helical rods 0.25 μm wide by 4 μm long with between one and two spiral turns and single, bipolar sheathed flagella. Pinpoint, nonpigmented, alpha-hemolytic colonies form after 48 h on blood agar, growing at 42°C. The species is catalase-, urease-, and nitrate-negative. It is isolated from feces of diarrheic or healthy dogs; one strain has been isolated from human feces. The pathogenicity is unknown. The G + C content of DNA is 48 mol% and the type strain is NCTC 12739.

HELICOBACTER CHOLECYSTUS

H. cholecystus is a filamentous (3–5 μm long) motile helicobacter with a single polar sheathed flagellum isolated from the gallbladders of hamsters with cholagiofibrosis and centrilobular pancreatitis (Franklin et al. 1996). Cells grow under micoaerobic and anaerobic conditions. There is growth at 37 and 42°C, but not at 25°C. The species is positive for catalase and nitrate activity, but does not hydrolyze urea. The type strain is Hkb-1.

HELICOBACTER GANMANI

H. ganmani is a urease-negative species isolated from the intestines (mucous scrapings) of laboratory mice (Robertson et al. 2001). Isolates possess single, bipolar, unsheathed flagella, but are unusual in that they only grow anaerobically at 37°C and are incapable of growth under microaerobic conditions. They are positive for oxidase and reduce nitrate to nitrite, whereas most are catalase negative, and do not hydrolyze hippurate or indoxylacetate. In a phylogenetic analysis, the species is most closely related to *H. rodentium* which also has unsheathed flagella. The type strain is CCUG 43526.

HELICOBACTER HEPATICUS

H. hepaticus (Fox et al. 1994) consists of curved to spiral rods, 0.2–0.3 μm wide by 1.5–5.0 μm with between one and three spiral turns with single, sheathed, bipolar flagella. It grows as pinpoint colonies, but may spread on agar. It does not grow at 42°C. Urease-positive, it is isolated from the colons and ceca of mice, and the livers of mice with active, chronic hepatitis and liver cancer. It is the prototype enterohepatic *Helicobacter* species. The complete genome sequence of strain ATCC 51449 has been determined with a size of 1.8 M base pairs and predicted to encode 1 875 proteins (Suerbaum et al. 2003). The type strain is ATCC 51448.

HELICOBACTER MESOCRICETORUM

This organism is a spiral-shaped bacterium with bipolar, single, nonsheathed flagella isolated from the feces of asymptomatic Syrian hamsters (Simmons et al. 2000).

HELICOBACTER MARMOTAE

Members of this species are slender curved to spiral rods that are motile by means of sheathed single unipolar or bipoar flagella. Isolates are urease positive but nitrate negative. Bacteria were isolated from the liver of woodchucks and feces of clinically normal cats. The type strain is MIT 98-6070 (ATCC BAA-546) (Fox et al. 2002).

HELICOBACTER MURICOLA

This species has a pair of nonsheathed, bipolar flagella, is positive for urease, catalase, and oxidase, and reduces nitrate to nitrite. It is phylogenetically most closely related to *H. muridarum* and to *H. pullorum*. The bacteria (two strains) were isolated from the ceca and fecal pellets of Korean wild mice. The proposed type strain is w-06 (Won et al. 2002).

HELICOBACTER MURIDARUM

H. muridarum (Lee et al. 1992) is morphologically distinct. Cells are helical, 0.5–0.6 μm wide by 3.5–5.0 μm long with between two and three spirals per cell. Spherical forms occur in older cultures. There are bipolar tufts of between ten and 14 sheathed flagella. Each cell is entwined by nine to 11 criss-crossing periplasmic fibrils. It is microaerobic and grows only at 37°C. Urease-positive, it can be isolated from the intestinal mucosa of rats and mice. Its pathogenicity is unknown. The G + C content of DNA is 34 mol% and the type strain is ATCC 49282.

HELICOBACTER PAMETENSIS

H. pametensis (Dewhirst et al. 1994) cells are curved, 0.4 μm wide by 1.5 μm long with single, bipolar, sheathed flagella; occasionally there is a third flagellum. The species grows at 42°C, but does not hydrolyze urea. It is isolated from tern, gull, and swine feces; its pathogenicity is unknown. The G + C content of DNA is 38 mol% and the type strain is ATCC 51478 (CCUG 29255).

HELICOBACTER PULLORUM

H. pullorum (Stanley et al. 1994) is a campylobacter-like organism with single, unipolar, unsheathed flagellum (atypical for a helicobacter). Cells are gently curved slender rods 3–4 μm long and it grows at 42°C. It forms pinpoint, nonpigmented, alpha-hemolytic colonies on 5 percent horse blood agar. Urease-negative, it is isolated from asymptomatic broiler chickens, the livers and intestinal contents of laying hens with features of vibrionic hepatitis, and feces from humans with gastroenteritis. The G + C content of DNA is 34–35 mol% and the type strain is NCTC 12824.

HELICOBACTER RODENTIUM

H. rodentium is a spiral-shaped bacterium (1–3 turns) motile with bipolar, single, nonsheathed flagella isolated from the intestines (colon and cecum) and feces of laboratory mice (Shen et al. 1997). Growth occurs at 37 and 42°C, but not at 25°C. The bacteria are urease negative and weakly catalase and oxidase positive, reduce nitate to nitrite, but do not hydrolyze indoxylacetate or hydrolyze hippurate. Pathogenic potential is unclear. The closest named relative of the species is *H. ganmanii*. The type strain is ATCC 700285.

HELICOBACTER TROGONTUM

H. trogontum is a fusiform to slightly spiral rod-shaped bacterium (4–6 μm in length) with periplasmic fibers that has been cultured from the colonic mucosa of laboratory rats (Mendes et al. 1996). Cells are motile by means of tufts consisting of between three and seven sheathed flagella. The organisms are able to grow at 42°C, have catalase, oxidase, and urease activity, but do not reduce nitrate. Closest taxonomic neighbors are murine species *H. hepaticus* and *H. muridarum*. The type strain is ATCC 700114.

HELICOBACTER TYPHLONIUS

H. typhlonius is a curved to spiral, rod-shaped bacterium with no periplasmic fibers which was cultured from feces of naturally infected laboratory mice (Franklin et al. 2001). Cells are motile by single, sheathed, bipolar flagella. The organism is oxidase and catalase positive and reduces nitrate to nitrite, but does not hydrolyze urease. It can induce enteric disease in various strains of immunodeficient mice. The species is most closely related to *H. hepaticus*, but is unique in containing a large (166-bp) intervening sequence in its 16S rDNA, is urease negative, and does not hydrolyze indoxyl acetate.

HELICOBACTER WINGHAMENSIS

This species was cultured from the stool samples of patients with symptoms of gastroenteritis, although a pathogenic role was unclear (Melito et al. 2001). The cells were about 2 μm in length and motile by one or two nonsheathed flagella. The organisms grow in a microaerobic atmosphere at 37°C, but not at 42°C in either aerobic or anaerobic atmospheres. The species is oxidase positive but catalase, urease, and nitrate negative. The closest relatives are organisms designated as *Flexispira* taxon 1. The type strain is NLEP 97-1090.

HELICOBACTER RAPPINI

Flexispira rappini was originally described by Kirkbride et al. (1985) and is now recognized to be part of a complex group of similar organisms. It has never been formally named, but has been shown to be closely related to *H. muridarum* and is probably a helicobacter (corresponding to *Flexispira* taxon 5 or taxon 8) (Solnick and Schauer 2001; Vandamme and On 2001). Its cells are straight and fusiform, but with criss-crossing periplasmic fibrils, like those of *H. muridarum*. It shows serpentine flexibility with changes in cell length and shape and grows as a thin spreading film on agar. It is microaerophilic and urease-positive and is isolated from aborted sheep fetuses, the feces of humans with chronic diarrhea, and the intestinal mucosa of laboratory mice. The G + C content of DNA is 33–34 mol%.

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Pseudomonas

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INTRODUCTION

The aerobic pseudomonads are rod-shaped, gram-negative bacteria, motile by means of one or more polar flagella. They are not acid-fast and do not form spores, prosthecae, or cysts. They have an absolute aerobic metabolism and the catalase reaction is positive. The guanine and cytosine (G+C) content of the DNA ranges from 57 to almost 70 mol%. The pseudomonads are nonexacting and can grow normally using simple sources of carbon and nitrogen. Many of them are saprophytic, but some are plant pathogens and others are opportunistic pathogens of humans and animals. A detailed taxonomic study of this group of organisms (see below) has demonstrated that actually it includes several genera of bacteria, but now the name *Pseudomonas* is reserved only for species closely related to the most outstanding species, *Pseudomonas aeruginosa*. The importance of this species derives from the widespread distribution of its strains in nature, their resistance to many antibacterial compounds, and the number of pathogenicity factors that they can produce.

P. aeruginosa is one of the so-called 'fluorescent' species because they produce pigments that fluoresce under ultraviolet (UV). Other fluorescent pseudomonads (*P. putida* and *P. fluorescens*, and many others that have been described recently) are less common human opportunistic pathogens. One of the reasons may be because their optimal temperature of growth is lower than that of *P. aeruginosa*, but they may also be poor producers of virulence factors. Many others are plant pathogenic. There are also nonfluorescent *Pseudomonas* species (*P. stutzeri*, *P. alcaligenes*, *P. pseudoalcaligenes*). Precise methodological procedures have allowed taxonomists to recently describe a number of new species; most of them produce fluorescent pigments.

BRIEF HISTORICAL PERSPECTIVE

Up to the first half of the twentieth century, many of the descriptions of species of *Pseudomonas* in successive editions of *Bergey's Manual of determinative bacteriology* were decidedly poor when viewed from present-day standards, and the interpretation of revisions and improvements was difficult since many of the original cultures were no longer available. This situation was most unfortunate because these organisms were very common in nature and, in case one became interested in their study, it was practically impossible to do much work with them because the original descriptions presented awkward problems in establishing priorities. Many of them were so inadequate that one description could very well be applied to many newly isolated strains.

Towards the middle of the twentieth century, several workers in various countries manifested a desire to attempt a satisfactory classification of species since the genus had become a dumping ground for phylogenetically unrelated organisms. In fact, the number of species catalogued in Index Bergeyana (Buchanan et al. 1966) and its supplement (Gibbons et al. 1981), and those subsequently described in journal publications,

amounted to more than 800 names. Of these, many had been been applied to plant pathogenic species. Unfortunately, the lack of proper criteria for species description added considerably to the confusion created by this nomenclatural hypertrophy and, with few exceptions, the identification of strains isolated from nature and their assignment to known species became an almost impossible task. Fortunately, in 1926, den Dooren de Jong, working in Delft under the supervision of the famous microbiologist Beijerink, published his doctoral thesis, a very significant piece of work centered on the microflora able to convert a large variety of organic carbon compounds to carbon dioxide. This work very soon led him to discover the extraordinary mineralizing ability of the pseudomonads (den Dooren de Jong 1926).

In spite of their similarity in basic phenotypic characteristics, these organisms are remarkably diverse in their capacity to live at the expense of many different organic compounds as sole sources of carbon and energy. This aspect of their diversity helps in their taxonomy (den Dooren de Jong 1926; Palleroni 1984; Stanier et al. 1966). Based on their remarkable metabolic diversity, den Dooren de Jong devised a method of classification of the organisms of his collection, in which he tested their capacity of using each of a long list of carbon compounds as a single source of carbon and energy. This was described eloquently by Skerman in 1949, who pointed out that in spite of the similarity of the organisms in their basic phenotypic characteristics, their classification could perhaps be attempted on the basis of nutritional properties. Although this methodology failed to attract the attention of bacteriologists, den Dooren de Jong's data were indeed attractive to the biochemists. In her classic book, Marjorie Stephenson (1930) transcribes several of the pages with the data of den Dooren de Jong obtained with *Pseudomonas* species, but it took about 40 years to see any positive reaction among the bacteriologists.

Work performed on many strains of *Pseudomonas* at the Department of Bacteriology of the University of California at Berkeley, mainly along the lines suggested by den Dooren de Jong, resulted in the proposal of a system of classification of *Pseudomonas* species based on phenotypic characteristics with emphasis on the utilization of various organic compounds as sole carbon and energy sources (Stanier et al. 1966). Later this was followed by DNA–DNA hybridization studies, which confirmed the proposed system of phenotypic classification. However, in spite of the progress, the results of these studies suggested that the species assigned to the genus *Pseudomonas*, a number considerably smaller than years before, constituted a group that was not phylogenetically uniform. A clear demonstration of the phylogenetic heterogeneity of the genus was eventually achieved by means of an estimation of the ribosomal RNA (rRNA) similarities, using rRNA–DNA hybridization as an analytical criterion (Palleroni et al. 1973).

Subdivisions of the genus *Pseudomonas* into species

The rRNA/DNA hybridization studies demonstrated that *Pseudomonas* could be subdivided in rRNA homology groups, each of which represented at least a separate genus. The name *Pseudomonas* was reserved for rRNA group I, which included *P. aeruginosa*. This group will be the subject of this chapter, and the rest of the genera will be treated elsewhere in this book. The species of *Pseudomonas* can be subdivided into two groups, the fluorescent and the nonfluorescent species. Much of the description that follows refers to the type species of the genus, *P. aeruginosa*, which has not only all the general properties assigned to members of the genus but also properties that are rather uncommon.

The number of *Pseudomonas* species to be included in the new edition of *Bergey's Manual of systematic bacteriology* will be about 65 because it has been decided that the list of species should not go beyond January 2000. Therefore, this number can only be taken as an approximation. There are, however, a number of new species, mostly fluorescent, which perhaps is the result of defining more sharply some of the groups related to the biovars described originally under the collective names of *P. putida* and *P. fluorescens*.

An interesting question pertaining to the nomenclature is the fact that at the beginning, the first fluorescent species carried names that referred to some striking phenotypic character of the strain or of its host (*aeruginosa*, *fluorescens*, *putida*, *aureofaciens*, *chlororaphis*, *syringae*, etc.). After some time, the names of scientists who collaborated in *Pseudomonas* research or the places of collection of the samples were used for naming the new species. As a consequence, these new names had less informative value for the noninitiated scientist

One of the most surprising results of the rRNA/DNA hybridization experiments was the finding that *E. coli* appeared to be closer to some *Pseudomonas* species than these were to others of the sensu lato genus. Before these experiments were done, no bacteriologist would have accepted the idea that this level of phylogenetic relationship existed between a peritrochously, fermentative organism (such as an enteric bacterium) and an oxidative, polarly flagellated one. After the finding in my own laboratory, many pieces of confirmation came from different laboratories. More recently, Sugawara and Nikaido (1992), working with the properties of the OmpF channel, came to the following conclusions: 'These results are gratifying because they suggest the evolutionary relationship between the enteric porins of high flux rate, such as OmpF and OmpC, and *P. aeruginosa*, whose outer membrane could be considered basically as a modified enteric outer membrane except that it lacks these classical porins. Thus, in the later organism, the very ineffective porin function of the

OmpA/OprF-type protein, which is hidden behind the much higher fluxes through the classical porins in *E. coli*, now becomes predominant.'

The best determinative procedure for the identification of purposes is the study of rRNA, but hybridization of this macromolecule with DNA is no longer the method of choice. It has been replaced by the direct determination of rRNA sequences, because this is very reliable and informative for the allocation of new isolates to the proper rRNA homology group.

It is important to emphasize here the fact that the rRNA similarity studies performed with the aerobic pseudomonads have been extended to many other groups of prokaryotes in other laboratories, demonstrating their usefulness in phylogenetic studies of these organisms. The development of a taxonomic system for *Pseudomonas*, therefore, has had a more general significance than simply providing bases for species classification within a single bacterial genus.

HABITATS

The aerobic pseudomonads can be found in many different materials; therefore, most bacteriologists are familiar with *Pseudomonas* and the related genera. Pseudomonads have been found in many materials used in clinical laboratories and medical practice. In addition to their nutritional versatility, which explains their ubiquity and their importance in the recycling of organic matter, they can be dangerous opportunistic pathogens because of their tolerance to physical, chemical, and antibacterial compounds.

However, there are exceptions. Acid media (pH below 5.5) and hot habitats in general are free of them. Actually, at one time the incapacity for growth at pH below 5.5 was included among the negative group characteristics of aerobic pseudomonads. In fact, with minor exceptions, there are no true acidophilic or thermophilic aerobic pseudomonads. Recently, a thermotolerant species was described, with the name of *P. thermotolerans* (Manaia and Moore 2002). Even so, the list of materials where the presence of pseudomonads can be demonstrated is impressive. It includes water (even samples labeled 'sterile') (Favero et al. 1971; Lantos et al. 1969), irrigating fluid (Mitchell and Hayward 1966), solutions purported to have antiseptic activity (Plotkin and Austrian 1958; Mitchell and Hayward 1966; Cragg and Andrews 1969; Kedzia 1977; Jin et al. 1994), medications, utensils, and medical instruments (Bruch 1971; Jessen 1965; Moffett et al. 1967; Moffett and Williams 1967; Shooter et al. 1969).

Pseudomonads are found in natural or processed foods (Farmer 1976) and occasionally these materials are sources of infections of patients in hospitals (Kominos et al. 1972; Shooter et al. 1971; Shooter et al. 1995). External sources of infection can be various materials, such as plants and flowers, brought by visitors or health personnel (Bergan 1975; Cho et al. 1975; Green et al. 1974). This is to be expected, because pseudomonads can be isolated from healthy and diseased plants (Palleroni and Doudoroff 1972; Schroth et al. 1977; Trust and Bartlett 1976). Since pseudomonads can also colonize animals and humans, they can spread among individuals (Hoadley and McCoy 1968; Matthews and Fitzsimmons 1964; Shooter et al. 1966). Together with the genera *Burkholderia* and *Stenotrophomonas*, the species of *Pseudomonas* are the most formidable opportunistic pathogens causing infections in hospitalized patients.

From all the above, it is evident that prevention of contamination with pseudomonads is practically impossible when you are dedicated to medical practice, to social relations, or to agriculture. In hospitals, among the species, the most common is a formidable opportunistic pathogen, *P. aeruginosa*, and therefore, the medical concern with infection of immunologically compromised patients in burns and neonatal units, and in acquired immune deficiency syndrome (AIDS) and cancer wards, is well justified indeed.

MORPHOLOGY AND CELLULAR STRUCTURE

In the following sections, much of the information will refer to *P. aeruginosa* because this is the most important species from the medical point of view, but occasional reference will also be made with respect to the other species.

P. aeruginosa cells are rod-shaped and have a single flagellum inserted at the tip of the cell. Other species of the genus appear to be different, but this is primarily in the number of flagella, which is normally higher than one. A discussion of the morphology of various aerobic pseudomonads of different species can be found elsewhere (Palleroni 1984). Although the general definition refers to the shape of rods, in some species the cells tend quite often to be more oval than cylindrical. A property that is fairly common in all instances is the arrangement of relatively short cells in pairs, but since the width/length relation can vary within wide limits, strains of some species (*P. putida* and fluorescent plant pathogens) can have long cells that resemble filaments. As in other bacterial groups, the cells are short in the stationary phase of growth. Under certain growth conditions and oxygen limitation, filamentation can be induced in cells of strains of *P. putida* and *P. fluorescens*, but not in *P. aeruginosa* (Jensen and Woolfolk 1985).

As mentioned before, *P. aeruginosa* can be differentiated from the rest of the fluorescent species in having a single polar flagellum. However, careful observation of flagella-stained preparations shows that there are exceptions (see Sarin et al. 2002, Figure 1). As can be seen in that figure, some cells deviate from the prototype both in the number of flagella and

in their insertion. The significance of these deviations may be quite limited, since many microscopic fields had to be examined to detect them. The normal flagellar number has always been taken to be one per cell, but now it is known to be controlled by FleN, a putative adenosine 5′-triphosphate/guanosine 5′-phosphate (ATP/GTP) binding protein. Disruption of *fleN* results in multiflagellation and this is also associated with a chemotactic defect (Dasgupta and Ramphal 2001). On the other hand, the capacity of *P. aeruginosa* of swarming on the surface of solid media, as in the case of *P. stutzeri* (see below), may be due to these lateral flagella but this point deserves a comparative study with other species.

Two genes were identified and named *fleS* and *fleR* and found to be homologous to members of the subclass of two-component systems involved in transcriptional regulation of a diverse array of genes from sigma 54 promoters. They are arranged in a putative SR operon, located upstream from a flagellar *fliE* gene.

P. aeruginosa displays multiple phenotypes when a biofilm develops. The changes have been monitored using microscopy and in situ reporter gene analysis to follow biofilm physiology. Five stages of physiology development were identified (Sarin et al. 2002): (1) reversible attachment; (2) irreversible attachment; (3) maturation-1; (4) maturation-2; and (5) dispersion. Cells at the state of biofilm do not differ very much from those in a planktonic type of population as far as the sensitivity to antibacterial compounds is concerned (Spoering and Lewis 2001).

In a report from a different group of workers, strain 57RP formed a phenotypically heterogeneous population by a mechanism called phase variation. In this population there were variants that formed small, rough colonies that spontaneously emerged when the strain was cultivated as a biofilm or in static liquid cultures. These variants produced abundant type IV fimbriae, displayed defective swimming, swarming, and twitching motilities, and were impaired in chemotaxis (Déziel et al. 2001).

Motility was not studied systematically in any of the species of *Pseudomonas*, with the exception of *P. aeruginosa*. Out of 354 strains, Jessen (1965) found that 20 strains (5 percent) were nonmotile. Five of these 20 strains subsequently acquired the characteristic of motility. Lateral flagella of shorter wavelength, in addition to the polar one, may be observed in *P. stutzeri*. Perhaps they are involved in propelling the cell over the surface of solid media (Palleroni et al. 1970; Shinoda and Okamoto 1977).

Fimbriae or pili are present in most species (Fuerst and Hayward 1969), and they cause aggregation and attachment to inert or living surfaces, or may have receptors for the attachment of bacteriophages (Bradley 1972; Bradley 1974). They can even participate in causing twitching motility on the surface of solid media (Bradley 1980). The determinants of pili in *P. aeruginosa* may have been received by horizontal transmission from organisms of lower G+C content in their DNA, as suggested by the sequence homology with pilins of unrelated organisms such as *Neisseria* and *Moraxella* (Buchanan and Pearce 1979), and by differences in codon usage when compared with the genes of these organisms (West and Iglewski 1988). Unfortunately, however, the taxonomic implications of our knowledge on the pilin markers at present are rather limited.

Aside from movement due to swimming under the action of flagella, and twitching of the cells, which obeys the propulsion of the type IV pili, there is also a more purposeful displacement of the cells due to attraction or repellent action of chemical or biological compounds, and this falls under the category of chemotaxis. The propeller here is also the fimbriae or pili, and the translocation is related to the composition of fatty acid of the solid medium. Synthetic dilauroyl- and dioleoyl-phosphatidylethanolamine were capable of directing the *P. aeruginosa* motility, while many others were not (Kearns et al. 2001).

Salicylate depresses the motility of *Burkholderia cepacia* and of several other species of different genera, but it has no effect on *P. aeruginosa* at the same concentration. Its effect on many of the organisms could be related to osmoregulation (Kunin et al. 1995).

The composition of the cell envelopes of aerobic pseudomonads has been the subject of much research, which initially was performed mainly on well-known species, such as *P. aeruginosa*, but was later extended to other species in search of correlations with their taxonomic allocations. An adequate summary of the research up to the 1980s was presented in *Bergey's Manual of Systematic Bacteriology* (Palleroni 1984), and only some supplementary information will be given here.

Poly-3-hydroxybutyrate (PHB) is accumulated in the form of refractile granules inside of the cells of species of genera that are very similar to *Pseudomonas*. This is a good negative character, because, with very few exceptions, none of the species of *Pseudomonas* accumulate the polymer intracellularly. Some accumulation was observed in one of the strains of *P. pseudoalcaligenes* (Stanier et al. 1966). Some weak synthesis may occur in other species of the genus *Pseudomonas* but is never sufficiently active to result in the formation of granules visible under the light microscope. In spite of all this, *P. aeruginosa* is considered to produce substantial amounts of alginate. A comprehensive discussion of this problem is given by Gacesa (1998).

Synthesis of other poly-3-hydroalkanoates has been reported for the fluorescent pseudomonads (Hori et al. 1994; Huijberts et al. 1994), and a gene from *P. aeruginosa* has been cloned (Timm and Steinbüchel 1990). Membranous structures such as mesosomes (Brown et al. 1990; Carric and Berk 1971) and rhapidosomes (Baechler and Berk 1972; Yamamoto 1967) can be identified by electron microscopy in some of the species.

The cell envelopes constitute a complex system that can be resolved into nine components by freeze-etching (Gilleland and Stinnett 1973; Lickfield et al. 1972). Some of these compounds have structures that are removed by ethylenediaminetetraacetic acid (EDTA) treatment and are reconstituted by addition of magnesium ions. Different species vary in their sensitivity to EDTA, which correlates with a high phosphorous content of the cell walls (Wilkinson and Galbraith 1975). Descriptions of *P. aeruginosa* outer-membrane properties and of protein components involved in permeability are found in excellent review articles (Bellido et al. 1992; Nikaido 1992; Nikaido and Hancock 1986).

Monoclonal antibodies for outer-membrane proteins have been isolated and characterized by Hancock and collaborators (Hancock et al. 1982). One of the antibodies specific for an antigenic epitope on an outer-membrane protein of *P. aeruginosa*, H2, was recommended as an identification tool for use in clinical laboratories (Mutharia and Hancock 1985). This epitope was detected in all 17 serotype strains of the species and, in addition, in other *Pseudomonas* species that were tested, but not in any species of other genera of aerobic pseudomonads tested.

Culture characteristics

The phenotypic studies on the pseudomonads by Stanier et al. (1966) included the determination of ability to grow at two temperatures, 4 and 41°C. *P. aeruginosa* was unable to grow at the lower temperature but it grew very well at the higher. As a matter of fact, we could grow all our strains at 44°C, although very slowly.

Cultures on solid media have a characteristic, rather agreeable, aromatic odor. In comparison, *P. putida* receives its name from the latin adjective *putida*, meaning stinking and fetid.

The colonies of *P. aeruginosa* are of two types on most solid media. One is large and smooth, with flat edges and elevated centers ('fried-egg' appearance), and the other is small, rough, and convex. Clinical materials are, in general, good sources of the large colony type, while the small type is commonly obtained from natural sources (Véron and Berche 1976). Variation of the large type shifting to the small colony type is observed frequently, but the reverse variation is extremely rare. A third colony type (mucoid) can often be obtained from respiratory and urinary tract secretions, and was first observed by Sonnenshein (1927). Mucoid mutants of *P. aeruginosa* can be divided into two groups according to whether the mucus (alginate) is produced in chemically defined media (Vasil et al. 1977). Additional colony types can be observed, but, as pointed out by Jessen (1965), the type of colony cannot be correlated with other properties of the strain or the medium, so the detailed description is of doubtful value. Jessen (1965) states that at least five colony types may be distinguished. The following descriptions are taken directly from his thesis.

1. Circular, smooth colonies. The circumference is almost circular and the edge entire. The colonies are convex, dome-shaped, with a smooth, shining surface; translucent, homogeneous, and a whitish-gray colour. The consistency is soft.
2. Irregular, contoured colonies. The circumference is irregularly lobate and the edge entire or fimbriate. The colonies are convex, with a contoured, beaten-copper appearance, and a smooth, shining surface. They are translucent, slightly granular, and of a whitish-gray color. The consistency is soft.
3. Dry, flat colonies. The circumference is almost circular, the edge lobular. The colonies are low, raised, with the irregular surface seen in rough colony types. They are almost opaque, granular, and whitish-gray. The consistency is friable.
4. Mucoid colonies. Most often somewhat larger than other colony types; flowing out, convex, with entire edges. The surface is smooth, moist, and shining. The colonies are almost opaque, homogeneous, whitish or grayish-green. The consistency is soft, more or less viscid.
5. Rugose colonies. Most often somewhat smaller than other colony types; round, with lobular margins. The surface is wrinkled, with irregular or radiant crests, and usually dry. The colonies are opaque, granular, gray or whitish. The consistency is membranaceous.

The first three types have flat, restricted swarming outgrowths. A combination of features characteristic of the majority of *P. aeruginosa* cultures on solid media was (1) a tendency to localized swarming from the edge of the colony; (2) the green and fluorescing pigments; (3) the fruity odor; and (4) the occurrence of shining metallic patches on top of the culture.

The colony structure of *P. aeruginosa* shows a variety that should be interesting to know for identification and epidemiological purposes. *P. fluorescens*, on the other hand, is a very complex species with many natural variants, and their study assures an interesting and large harvest of information in the future. Classification of the colony types of fluorescent pseudomonads, however, is in its infancy, and we can refer almost exclusively to the pioneering observations of Rainey and Travisano (1998), who classified the types of colonies obtained in different habitats. From the ancestral 'smooth' (SM morph), they obtained the three principle morph classes, (SM), wrinkly spreader (WS), and fuzzy spreader (FS), names that succinctly describe the colony types (Rainey and Travisano 1998).

We do not have sufficient information about the different biovars of *P. fluorescens* that occasionally are isolated as opportunistic pathogens,. Aside from the fluorescent organisms, the nonfluorescent species that

has the most interesting colonies is *P. stutzeri*, because they can go from the smooth, round, and creamy type to leathery, folded units that are almost impossible to suspend in water (van Niel and Allen 1952).

PIGMENTATION

Many strains of *Pseudomonas* produce pigments, some of which are water-soluble and diffuse into the culture media, while others remain associated with the cell mass. Pigments are very striking and useful for determinative purposes, and their visual impact is so convincing that additional tests usually become subordinate tools when it comes to the identification of some species. However, they are quite unreliable as taxonomic characteristics, because their production depends on the composition of the medium and on the general environmental conditions surrounding the cells. Pigment formation may be erratic, it may be lost by mutation, or several pigments may be produced making it difficult to decide whether the diagnostically important component is part of the mixture. The following pigments are of taxonomic interest.

Fluorescent pigments

The so-called fluorescent pseudomonads are characterized by the production of pigments that are water soluble, diffuse freely into the medium and fluoresce brightly under UV light. Production of these pigments is a property of, among others, *P. aeruginosa*, *P. putida*, *P. fluorescens*, *P. chlororaphis*, *P. syringae*, *P. cichorii*, and *P. flavescens*, and is manifested in media of low iron content. The medium universally used for stimulating the production of fluorescent pigments is the 'King B' medium (King et al. 1954). Another effective method for fluorescent pigment stimulation is the aseptic addition of 10 percent of egg white to a common complex medium (Garibaldi 1967).

Little progress was made about the chemical nature of fluorescent pigments (pyoverdines) until relatively recently (Demange et al. 1986; Meyer et al. 1990; Wendenbaum et al. 1983). Today we know that they are chromopeptides consisting of three parts: a chromophore of quinolinic nature, a peptide chain, and a side chain in various combinations. Since there are different chromophores, peptides, and side chains, the possible number of different pyoverdines is quite large. Differences observed within the third element of the siderophores, that is, the peptide chain, may be expressed in the presence of unusual aminoacids, some of which are D-aminoacids. It is thought that the presence of these 'unnatural' components may prevent the destruction of the siderophores by proteolytic enzymes. Interesting variations in the composition and activity of the siderophores may be found in the literature (Fuchs 2001).

A re-evaluation of the taxonomic value of fluorescent pigment as well as of the siderophores produced by nonfluorescent pseudomonads places these compounds in a more prominent level than before as taxonomic tools (Meyer et al. 2002). I shall also be mentioning them when dealing with pathogenicity.

Phenazine pigments

Pyocyanin, a blue phenazine derivative characteristic of *P. aeruginosa*, is diffusible and its production can be enhanced by growth in the 'King A' medium (King et al. 1954). Other phenazine pigments are characteristic of *P. chlororaphis* (chlororaphin, green; frequently crystallizes in the medium) and of *P. chlororaphis* (*P. aureofaciens*) (phenazine-α-carboxylate, orange, soluble). Some strains of *P. aeruginosa* produce phenazine-α-carboxylate (Chang and Blackwood 1969), and others produce a variety of phenazine pigments of the chlororaphin family, aside from phenazine-α-carboxylate (for references, see Palleroni and Doudoroff 1972).

The most characteristic of all the pigments produced by *P. aeruginosa* is pyocyanin. This pigment, together with a sheen of the colonies and a characteristic odor are useful characters for a tentative identification. Pyocyanin is soluble in water, and the color depends on the pH. Changing the pH of an aqueous solution, this passes from colorless (at alkaline pH), to yellow, and then to red (at acid pH). Pyocyanin can be extracted with chloroform, where it gives a blue-green color. In water solution, the pigment changes to reddish upon acidification.

Of 354 strains of *P. aeruginosa* studied by Jessen (1965), 92 percent gave pyocyanin and fluorescein. One of the strains dissociated into pigmented and nonpigmented variants. In 2 percent no fluorescein was evident, and in the remaining 6 percent pyocyanin was never seen. About 1 percent of the strains were totally unpigmented. Some strains of *P. aeruginosa* are capable of producing additional pigments that may even obscure the characteristic pyocyanin and fluorescein. One, pyorubrin, is of reddish color, and it was described many years ago (Meader et al. 1925); another pigment is brownish and belongs to the chemically undefined group of melanins (pyomelanin). This pigment, in common with other melanins, is produced from aromatic amino acids such as tyrosine or phenylalanine (Yabuuchi and Ohyama 1972), while pyorubrin production is enhanced by the addition of glutamate to the medium.

Two red pigments produced by some strains were described by Herbert and Holliman (Herbert and Holliman 1964; Holliman 1957) and given the names 'aeruginosin A' and 'aeruginosin B' (this choice of names was unfortunate, since the name aeruginosin is also applied to the bacteriocins produced by this species). Finally, some strains have been reported to produce phenazine-1-carboxylate (Chang and Black-

wood 1969; Takeda 1958), dihydroxyphenazine-1-carboxylic acid (Korth 1962), chlororaphin (Takeda 1958), and oxychlororaphin (Chang and Blackwood 1969; Sierra 1957). Thus, the capacity of *P. aeruginosa* for the production of different pigments is quite extensive, and even though the production of pyocyanin and, to a lesser degree, the fluorescent pigments, has a diagnostic value, in some strains these pigments may be absent or masked by others. Extraction of pyocyanin into chloroform (in which most of the other pigments are insoluble) is recommended in such cases.

Lemonnierin

Lemonnierin, an intracellular, insoluble blue pigment characteristic of *P. fluorescens* biotype IV (*P. lemonnieri*), is a derivative of 3,3-bipyridyl (Kuhn et al. 1965), whose structure has been determined (Ferguson et al. 1980; Jain and Whalley 1980).

FATTY-ACID COMPOSITION

In what follows, the fatty-acid compositions are indicated by a carbon, C, with a subscript with two numbers. The first is the number of carbon atoms of the acid and the second is the number of double bonds; the carbon where the hydroxyl group is located is also indicated; the iso in an odd-number acid indicates that a methyl is located in the second to the last carbon in the chain. The straight-chain saturated fatty acid $C_{16:0}$ and the straight-chain unsaturated fatty acid $C_{18:1}$ are present in all the *Pseudomonas* species that have been analyzed, and therefore their presence lacks taxonomic value. In contrast, the distribution of hydroxy acids, cyclopropane acids, and branched-chain acids provides a very useful identification tool. For *Pseudomonas*, 10:0 3-OH and 12:0 3-OH. For other rRNA homology groups: group 2, 14:0 3-OH, 16:0 3-OH, and 18:1 2-OH; group 3, 10:0 3-OH; group 4, 14:0 2-OH; group 5, 12:0 2-OH, 14-0 2-OH, and 14:0 3-OH; and group 6, 12:0 3-OH, 11:0 iso 3-OH, and 13:0 iso 3-OH. The ubiquinone Q-9 is present in *Pseudomonas* and not in the other groups. This summary of the differences between *Pseudomonas* and other rRNA homology groups has been taken from the summary of the work by Stead (1992), in which a comparison is made with the contribution of Oyaizu and Komagata (1983). A modern taxonomic evaluation of fatty acids in the taxonomy of *Pseudomonas* has been published by Vancanneyt et al. (1996). It can be seen that groups 2–6 can be very well differentiated from *Pseudomonas* (group 1), and therefore, fatty-acid analysis approaches the resolving power of rRNA sequence determinations for taxonomic purposes (Vancanneyt et al. 1996).

PLASMIDS

The pseudomonads are a very rich source of plasmids carrying genes for a wide variety of functions, such as resistance to antibiotics and miscellaneous antibacterial agents (including simple chemicals, such as metals and inorganic anions), resistance to bacteriophages, bacteriocins, or physical agents, and inductions of fertility or of particular metabolic properties. In this last category are plasmids with genes coding for enzymes for degradative pathways of aromatic compounds and their halogenated derivatives. Since many of these compounds are toxic to higher organisms and constitute a serious threat to human health, much work on environmental microbiology is now directed to a rational use of the properties that these plasmids confer to the bacterial cells, either in their natural state or after modification by recombinant DNA techniques.

An important point here is the stability of the plasmid at the temperature of the human body, which may not be tolerated by some of the plasmids.

The best known of the catabolic plasmids is toluene (TOL), found in some *P. putida* strains, which confers the ability of growth on toluene, xylenes, and related compounds (Assinder and Williams 1990). A plasmid similar to TOL is NAH, with genes coding for the enzymes required to degrade naphthalene (Dunn and Gunsalus 2003). Plasmids like TOL and naphthalene (NAH) are genetically complex, because the biochemical pathways for the corresponding pathways are biochemically complicated. Other plasmids, however, can even facilitate the assimilation of simple organic compounds, like malonate (Kim and Kim 1994). It is obvious that the catabolic plasmids contribute to the nutritional diversity of the aerobic pseudomonads.

As in the case of the plasmids of the enteric bacteria, plasmids of the pseudomonads can be classified in incompatibility groups. Thirteen groups have been identified (Jacoby 1986) and in addition there are some plasmids not yet allocated to any of these groups.

Boronin (1992a, b) has addressed the important questions concerning the genetic composition, ecology, and diversity of plasmids in the widespread species of fluorescent pseudomonads, as well as the practical consideration of determining the most appropriate combination of plasmids and hosts to take maximum advantage of their metabolic catabolic properties towards chemical contaminants in the environment. Of the 13 *Pseudomonas* incompatibility groups, most of the isolates in the survey group carried plasmids belonging to groups P1–P5 (Boronin 1992a).

About three-quarters of the catabolic plasmids isolated in a survey come from *Pseudomonas* and half of them have been found in *P. putida* (Sayler et al. 1990). Unfortunately, many of the strains labeled *Pseudomonas* in the survey have not been precisely identified, which suggests the necessity of following precisely formulated procedures now available for precise taxonomic analysis (Palleroni 1993).

METABOLISM

The well-deserved reputation of the aerobic pseudomonads for their important role in the degradation of low-molecular-weight organic compounds has its roots in the work of den Dooren de Jong (1926). The remarkable catabolic activities of the aerobic pseudomonads, and among them the *Pseudomonas* species, have made them choice subjects for the search of novel catabolic pathways and their regulatory mechanisms. Our knowledge of this ability has been expanded by the discovery of the participation of these organisms in the degradation, under aerobic conditions, of xenobiotic compounds that are found in many environments as a consequence of human activities.

Strain-specific gene islands have been found integrated into conserved $tRNA^{Gly}$. This type of island derives from mobile elements that, upon integration, would endow the recipient with strain-specific metabolic properties, perhaps giving advantage over others in a given habitat. This is one way of conceiving the remarkable capacity of these organisms (Larbig et al. 2002). Indeed, aside from the fact that these organisms are dangerous opportunistic human pathogens, they have a respectable capacity for degrading many toxic compounds and their intermediates under aerobic conditions or conditions of denitrification.

A large amount of literature describes the biochemical transformations of many organic compounds by pseudomonads, among them species of *Pseudomonas*, and they do this in media of very simple composition, which has facilitated research considerably. A book on the genetics and biochemistry of species of the genus (Clarke and Richmond 1975) is a good source of references up to the mid-1970s. More recent highly recommended sources are available (Galli et al. 1992; Sokatch 1986). Many descriptions of biochemical activities have been included in books on bacterial metabolism and in recent years in publications dealing with the degradation of environmental contaminants and biotechnological applications. Research performed with *Pseudomonas* species on carbohydrates has resulted in the discovery of the Entner–Doudoroff pathway, of basic importance in the aerobic degradation of monosaccharides. One of the details of particular importance in *P. aeruginosa* is the fact that, among the compounds tested, only monosaccharides are utilized, while other fluorescent species may use di- and even trisaccharides. This peculiarity has been discussed by workers interested in the permeability of the bacterial outer membrane (Bellido et al. 1992; Nikaido and Hancock 1986; Yoshimura and Nikaido 1982). Similarly, the participation of *Pseudomonas* species on aromatic compounds has been very important for an understanding of the mechanisms of fission of the benzene ring.

An interesting detail in the taxonomy of species of the genus is the capacity of degradation of arginine under anaerobic conditions. This property was discovered in relation to the capacity of arginine for maintaining the motility of the cells in an anaerobic medium. It is related to the production of ATP in the conversion of arginine to citrulline and citrulline to ornithine, and it is called the arginine dihydrolase system. The system is positive for some species that use this amino acid aerobically, but in other groups there are species capable of growth at the expense of arginine but negative for the arginine dihydrolase. Within the genus *Pseudomonas*, the species that are positive are the fluorescent ones (the property is variable for the fluorescent plant pathogens). *P. mendocina* and *P. alcaligenes* are both positive, and *P. pseudoalcaligenes* is variable. *P. stutzeri* is negative. Aside from having the arginine dihydrolase system, *P. aeruginosa* is capable of very limited growth with arginine under conditions of anaerobiosis (Komatsu et al. 1994).

Pseudomonas species still attract the attention of bacteriologists, molecular biologists, and molecular geneticists. Recently, an issue of the journal *Microbiology* (vol. 146, no. 10, October 2000) was dedicated to the biology and diversity of *Pseudomonas*. A mini review by Spiers, Buckling, and Rainey discusses the causes of *Pseudomonas* diversity, and its main conclusion is that there is nothing special at the basis of the remarkable diversity of this very interesting genus, aside from 'a long evolutionary history (and concomitant opportunities for lateral gene transfer) and continual exposure to a range of spatially and temporally complex environments' (Spiers et al. 2000). In a laboratory experiment, a single week was sufficient to create a range of niche-specialist genotypes (Rainey and Travisano 1998). A surface polymer might be involved in this phenomenon, although this is at the hypothesis level.

P. aeruginosa as an opportunistic pathogen

P. aeruginosa is the type species of the genus. Its history is long and interesting, but it will be summarized to a minimum here. Even when this organism is extremely common in a vast number of natural materials, it was first recognized as the cause of 'blue pus' in wounds and, for this property, named *Bacillus pyocyaneus* by Gessard. The name was changed by Sédillot in 1850 to *Pseudomonas pyocyanea*, and later called *Pseudomonas aeruginosa* by Schroeter in 1872 when it was realized that the specific designation *aeruginosa* has priority over *pyocyanea*.

The cells are monotrichous, which is a useful differential character separating *P. aeruginosa* from other fluorescent pseudomonads. Some strains of the species are nonflagellated. Of Jessen's strains, 20 were thus permanently nonmotile because of the lack of flagella

(Knirel et al. 1996). On the other hand, a small proportion of the cells may have more than one flagellum per cell or have flagella that are not polarly inserted in the cell (Figure 1).

In *P. aeruginosa* there are divalent ions bridging the many phosphate groups present in the outer membrane. The low permeability, together with the efflux mechanism are very effective at keeping antibacterial compounds out of contact with the cytoplasm of the cells, but, if any molecules manage to get in, they may be subjected to the action of beta lactamases or of enzymes that modify them by acetylation, phosphorylation, or adenylylation.

RESISTANCE TO ANTIMICROBIAL COMPOUNDS

Several properties of this species help the cells resist antibacterial compounds. One is the composition of the outer membrane, where most prominent is the low permeability to those compounds. This most interesting subject has been studied by Nikaido, Hancock, and collaborators. Some aspects of *P. aeruginosa* outer membrane properties have been covered by these two authors (Nikaido and Hancock 1986). Among the mechanisms that contribute to the protection of the cells is an active efflux by which there is an active transport of the antibiotic back to the outside of the cell. The

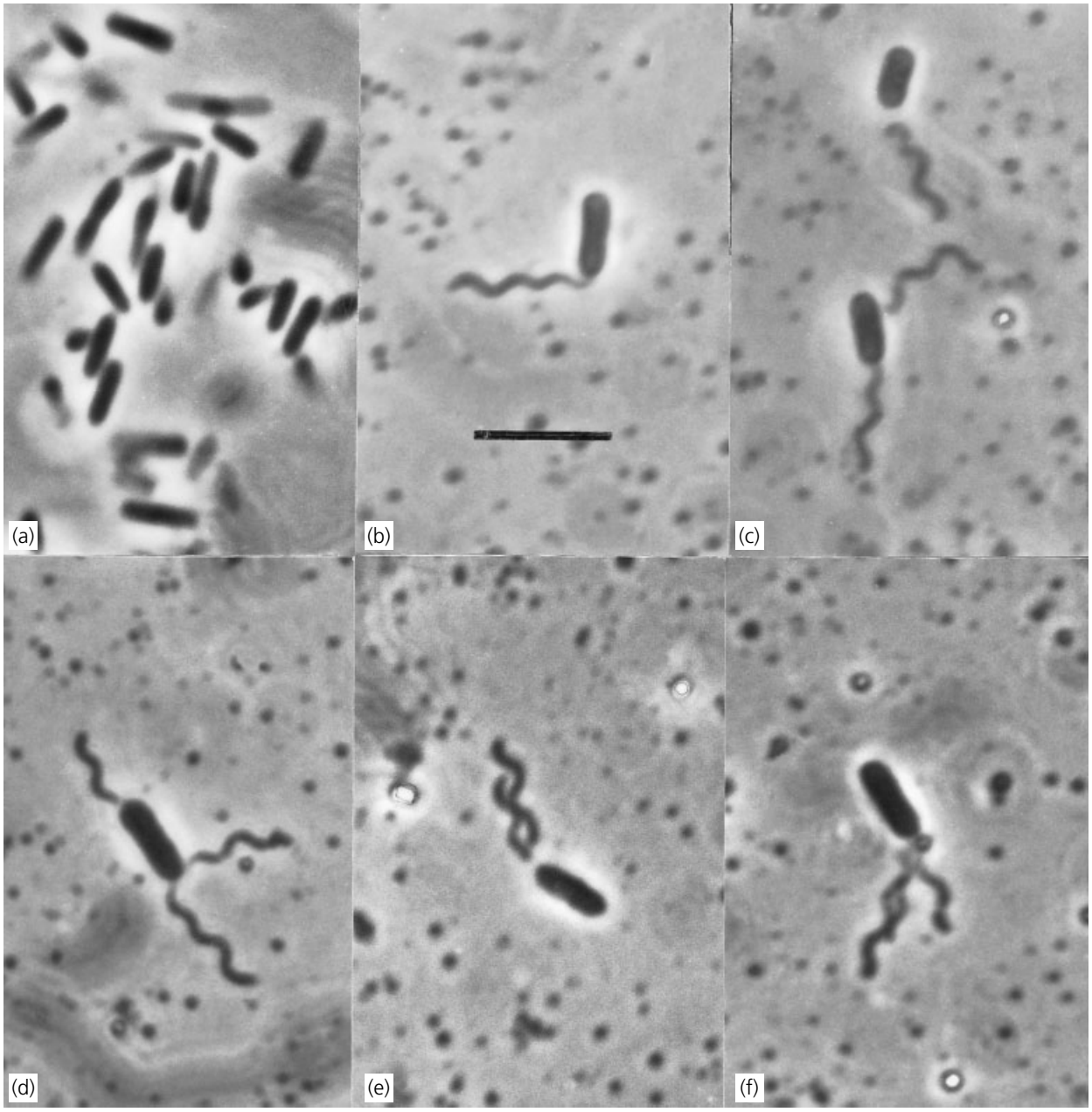

Figure 1 P. aeruginosa *cells stained by the Leifson procedure:* **(a)** *'normal' monotrichous flagellation;* **(b–f)** *multitrichous flagellated cells with at least one subpolar flagellum* **(b and f)**, *and one lateral flagellum* **(e)**. *Bar = 4 μm*

precise details of this mechanism are not clearly known, since the work with mutants suggests that there might also be changes in the composition of the cell envelope composition by which the cell architecture and stability may be changed.

P. aeruginosa does not have the trimeric porins (proteins that produce nonspecific channels) characteristic of the enteric bacteria, and it is left with a monomeric porin (Opr F), allowing a very slow rate of solute diffusion. In *E. coli*, on the contrary, most solutes encounter a wide road of penetration. This accounts in part for differences in antibiotic sensitivities of the two species (Nikaido 1992).

The membrane composition of the outer membrane of *P. aeruginosa* has been studied in detail. All strains have the proteins Opr E, F, H1, H2, and I, of molecular weights 44, 39, 21, 20.5, and 9-12 kDa, respectively. Proteins G (25 kDa) and D2 (45 kDa) are not in all strains, and protein D2 is involved in the penetration of basic amino acids.

The reaction to antibiotics is very variable. For instance, the aminoglycosides may be subjected to various types of modifications, such as acetylation, adenylylation, or phosphorylation, and beta lactams may be destroyed by beta lactamases. This means that a dynamic situation ensues, with participation of the cell porins. For other antibiotics, such as chloramphenicol, there may be a gene regulating the cell permeability. Tetracyclines penetrate through porins and porin mutants are not very resistant. The resistance due to efflux is rather common and it can be due to either chromosomal or plasmid genes. A tetracycline-inducible gene product, Tet, is membrane-bound and is required for efflux. A convenient discussion of the different resistance mechanisms has been presented by Quinn in a chapter in the book *Pseudomonas* by Galli et al. (1992).

PRODUCTION OF VIRULENCE FACTORS

P. aeruginosa is a common etiologic agent of corneal infections, and a murine corneal scratch model has been developed to study this problem extensively (Preston et al. 1995). The phospholipids phospatidyl inositol and phosphtidyl serine present in the mucus of the cell surfaces may function as the *P. aeruginosa* receptors and contribute to selective bacterial-host interactions responsible of the interactions that initiate the infection (Panjwani et al. 1996).

P. aeruginosa is the most common opportunistic pathogen of all *Pseudomonas* species. In addition to its resistance to antimicrobial compounds, *P. aeruginosa* is able to take advantage of its capacity of excreting various virulence factors in the form of enzymes and toxins. The arsenal of this organism has several extracellular proteases, among which the most prominent are an alkaline protease and an elastase, a cell-associated protein cytotoxin (leukocidin), two extracellular proteins that are adenosine 5′ (ADP)-ribosyl-transferases (toxin A and exoenzyme S), two hemolysins (phospholipase C and a rhamnolipid hemolysin), an adenylate kinase, and hydrogen cyanide, with which *P. aeruginosa* is capable of killing *Drosophila* or *Caenorhabditis* larvae (Gallagher and Manoil 2001). In fact, D'Argenio et al. (2001) proposed the use of *Drosophila* as a model host for *P. aeruginosa* infection studies. A recent study has included a strain (PA14) that causes disease in humans and also in nonvertebrate hosts, including plants, *Caenorhabditis elegans*, and the greater wax moth, *Galleria mellonella*. A subtractive hybridation technique allows the recovery of relevant genomic differences. Using the technique of representational difference analysis, the study has shown that strain PA14 differs substantially from an avirulent mutant strain in several genes. In addition, a gene homologous to the *ybtQ* gene of *Yersinia mellonella* is present in PA14 and absent in the mutant strain. This suggests that the virulence differences between two strains may be detected by the representational difference analysis (Choi et al. 2002).

Of all the extracellular enzymes produced by *P. aeruginosa*, exotoxin A (ETA) is the most toxic. It is a single polypeptide with two fragments and four S-S bridges, and it functions to transfer ADP-ribose from nicotinamide dinucleotide (NAD) to the elongation factor 2, an enzymatic reaction that shuts off peptide-chain elongation and protein synthesis. This action is identical to that of the diphtheria toxin, although the enzymes are unrelated. Its action leads to death (Vasil et al. 1993; Vasil et al. 1977). The pathogenicity of *P. aeruginosa* does not depend solely on the expression of the toxin A gene because negative mutants still are pathogenic, albeit less so (Woods et al. 1980).

Another extracellular toxic enzyme, exotoxin S, is also an ADP-ribosyltransferase (Radke et al. 1999), but the adenosine 5-diphosphate-ribosyl fragment taken from NAD is transferred not to elongation factor 2 (EF-2) but to vimentin, a filamentous protein. This enzyme is capable of stimulating the in vitro proliferation of murine lymphocytes (Barclay et al. 1999).

Among the important virulence factors are several proteases. Their presence is tested en bloc by growth on an agar medium containing gelatin, casein, elastin, or other proteins. After growth, the plate is flooded with a reagent capable of precipitating the proteins (for instance, a solution of mercuric chloride in HCl), and the proteolytic activity is noted by absence of a white precipitate under and around the bacterial patch. The proteases are differentiated by their physicochemical properties (pI, pH optimum, substrates). An analysis of excreted proteases shows that at least three proteases may be present: an alkaline protease, a general protease, and an elastase (Morihara 1964). The enzymes can be distinguished by their physicochemical characteristics (optimal pH, isoelectric point, substrate specificity). The three enzymes isolated by Morihara (1964), whose early

work was carried out with a strain of doubtful identity, were called neutral, semialkaline, and alkaline protease. Elastase corresponded to the semialkaline, which is active on elastin, casein, hemoglobin, egg albumin, and fibrin. Elastase and alkaline protease cause vascular lesions and necrosis and descematocele formation in the cornea of animals (Twining et al. 1986), as well as detachment of respiratory epithelial cells and interference with ciliary beating (Amitani et al. 1991). These two enzymes are metalloproteases and are inhibited by chelating agents. Strains low in protease synthesis are less pathogenic in burned tissues. On the other hand, strains that are high producers of proteases in many cases are less virulent because of ETA, and this is destroyed by excess of protease.

Experimentally, lipase production by *P. aeruginosa* strains can be conveniently tested on plates of common media that have been supplemented with Tween 80 (Sierra 1957). The medium contains calcium ions, and the bacterial patches will be surrounded by a precipitate of the calcium salts of the fatty acids liberated from Tween 80 in case of positive reactions. Most *P. aeruginosa* strains produce lipase, particularly during the late log phase. Oils or fats can be used instead of Tween 80.

The lipase is released in a form tightly bound to the lipopolysaccharide (LPS). From the genetical point of view, the gene lipA of *P. aeruginosa* has homology with that of *P. alcaligenes*, adding to other similarities between these two species found in the early studies of Stanier et al. (1966). The purification of the extracellular enzyme and the separation from the LPS has been described (Stuer et al. 1986). The enzyme seems to be synthesized in small amounts (about 200 molecules per cell), each molecule having an exceptionally high molecular activity (about 2.2×10^4/s).

At least two hemolysins are produced by *P. aeruginosa*: phospholipase C, which is heat-labile, and a rhamnolipid that is heat-stable (Esselmann and Liu 1961; Sierra 1957). *P. aeruginosa* gives a positive egg-yolk reaction, but the interpretation of the results is not always clear. To this reaction, phospholipase C contributes with the formation of an opacity in the medium around the growth region.

Early observations showed that *P. aeruginosa* infections in man and animals did not cause death because of sepsis in the classical sense (no invasion of the bloodstream by the organisms). Instead, the effect of the infection is death due to exotoxin excreted by the bacteria that inhabit external tissues. For many years, these early observations were forgotten and the pathogenesis was attributed to endotoxin as in the case of other gram-negatives.

Aside from the major virulence factors mentioned above, there are several miscellaneous factors. One of these has to do with iron metabolism. As is true with other pathogenic organisms, *P. aeruginosa* has to compete for iron with the tissues of the host (Britigan et al. 2000). Two siderophores have been identified, pyoverdine and pyochelin. In the literature a third one is mentioned, ferribactine, but this is considered to be a side product of pyoverdine. As a matter of fact, ferribactine has been described for *P. fluorescens*, not for *P. aeruginosa*. In this latter species, there is no evidence for the cyclization process that normally allows the formation of the chromophore. There are three types of pyoverdine and only one of the three is present in each strain. The loss of this pigment is correlated with the loss of virulence. Its specificity is practically used for the purpose of typing (siderotyping) (Prof. J. Meyer, personal communication; for citations, see Meyer et al. 1997).

Flagella and fimbriae also contribute to the lung infection. The flagellar cap protein FliD participates in the adhesion of *P. aeruginosa* cells to mucin, and the normal phenotype can be restored in cap negative mutants by providing the gene *fliD* alone (Arora et al. 1998, 2000). In fact, the adherence of *P. aeruginosa* to the mucous surfaces of the lungs occurs through the action of nonpilus adhesins binding to mucin. The mutants that lost this property are also nonmotile (Arora et al. 1996). Comolli et al. (1999) have demonstrated that mutants of the genes *pilT* and *pilU* of type IV are responsible for decreased colonization in the liver but not in the lungs, relative to the parent strains.

P. aeruginosa exhibits direct twitching motility up phosphatidylethanolamine gradients, while many other species were inactive under the same conditions (Kearns et al. 2001). A system for control of flagellin synthesis by FlgM exists in *P. aeruginosa* that resembles more that in *Vibrio cholera* than the one in *E. coli* because the first two were sigma 28-dependent (Frisk et al. 2002). In any case, *P. aeruginosa* flagella are important in establishing a respiratory tract infection, and may act to tether the cells in their initial interaction with the epithelial membranes (Feldman et al. 1998). *P. aeruginosa* flagella are important to allow the cells to move to different liquid habitats and to select the best conditions. Perhaps as a consequence of nutritional requirements, the cells also move to establish respiratory tract infections and may act as tethers in initial interactions with epithelial membranes. This function is offset by the contribution of flagella to host clearance mechanisms facilitating phagocytic clearance and the role of flagellar genes in mucin binding and clearance (Feldman et al. 1998).

Pyocyanin, a pigment that is a redox-active compound, increases the release of interleukin-8 (IL-8) by both normal and cystic fibrosis airway epithelial cell lines and by primary airway epithelial cells. This release increases the intracellular oxidant stress (Denning et al. 1998). A novel virulence factor property was demonstrated for azurin, the redox protein by Yamada et al. (2002). The role of pulmonary alveolar macrophages in defense of the lung against the pseudomonads was esti-

mated, and the results suggest that these macrophages do not play an important role in defense against *P. aeruginosa* (Cheung et al. 2000).

Quorum sensing in *P. aeruginosa*. In several species of gram-negative bacteria, a system of communication from cell to cell has been detected by means of the use of diffusible acyl homoserine lactones and in this way it is possible a coordination of the gene expression with the density of the cell population. It is possible that these compounds may function as virulence determinants by themselves, as has been postulated in the case of N-(3-oxododecanoyl)-L-homoserine lactone, and the results suggest that the quorum-sensing systems are active in cases of cystic fibrosis and that they may control the expression of virulence of *P. aeruginosa* in the lungs of the patients (Erickson et al. 2002).

P. aeruginosa synthesizes two acyl-homoserine lactones (AHL), N-(butyryl)-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL). These are required for quorum-sensing. The invariant lactone ring comes from S-adenosylmethionine, while the variable acyl chains come from the cellular acyl-acyl carrier protein pool, a reaction that is carried by specific AHL synthases that exhibit acyl-chain specificity (Hoang et al. 2002). Modulations in the activity of this enzyme may explain the variations in the chain lengths of the lactones.

The molecules of *P. aeruginosa* that provoke the quorum-sensing (*N*-(3-oxododecanoyl)-homoserine lactone) are capable of inducing inflammation by themselves in vivo (Smith et al. 2002) and they may have immunomodulatory activity (Telford et al. 1998), but with time they undergo a lactonolysis in a manner that depends on the temperature, the pH, and the length of the acyl chain (Yates et al. 2002). The *P. aeruginosa* quorum-sensing system regulates the expression of several virulence factors, including elastase and rhamnolid. A strain of *P. aeruginosa*, however, can produce these factors despite a nonfunctional quorum-sensing system. When supplied in *trans*, *E. coli dnak* mutation suppressor gene completely suppresses those two virulence factors (Branny et al. 2001).

Typing methods

Typing procedures are useful for epidemiology, that is to establish the origin of strains causative of infections, and are very important to guide treatment in environments of limited dimensions.

BACTERIOCIN TYPING

Bacteriocins are proteins produced by one strain that are lethal against the cells of other strains of the same species. Pyocins, bacteriocins produced by *P. aeruginosa*, can be used to classify *P. aeruginosa*. There are three morphological types of pyocins. The R type resembles the tails of T-even coliphages (Lee et al. 1999), and the F types are flexuous filaments. The third type, the S type, has low molecular weight, diffuses through the agar, and has no discernible details under the electron microscope. The pyocin produced by an unknown strain is tested against a series of indicator strains. This is the most common method. In a variation, the pyocin sensitivity of the unknown is measured, instead of its production. Nonfluorescent pseudomonads are not sensitive to pyocins, but fluorescent members may be so.

SEROLOGIC TYPING

The serological typing is based on the reaction of a heat stable O-antigen with a set of antisera initially proposed by Habs in 1957. Habs worked with 70 strains of bacteria and came up with 12 somatic groups, forming the basis of the International Antigenic Typing Scheme (IATS). Later there were several proposals to extend this number to reach 17 by addition of a few new small groups, but they were never accepted internationally. The antisera are prepared against live or boiled cell suspensions, and in the agglutination reaction, boiled or auclaved bacteria can be used.

DNA RESTRICTION ANALYSIS

The endonucleases that are used in this type of analysis can be those that cut the DNA frequently or those that do not do so, a property that depends on the composition of the DNA and on the enzyme specificity. *Pseudomonas* is a taxon whose DNA is relatively rich in G+C pairs, and will give more fragments after digestion with an enzyme having a high affinity for this configuration than with adenine and thymidine (A+T). For macrorestriction genome analysis, few bands are obtained by using enzymes that attack infrequently the DNA (that is, by using enzymes with high affinity for A+T pairs) and, in doing so, the fragments will be of large size and fewer in number. Their separation by electrophoresis will require special instrumentation.

Other species of the genus *Pseudomonas*

There are many other species of the genus *Pseudomonas* that could be mentioned here, although in their majority, as far as we know, they lack medical interest. Within the fluorescent subgroup of *Pseudomonas* species are *P. fluorescent*, *P. putida*, and *P. syringae*. The latter is of much interest to plant pathologists, because its species are specifically pathogenic for plants, and although they seldom kill the plants, they deteriorate the quality of the fruits, and, with that, their economical value.

P. fluorescens is the most complex species of the whole genus. Stanier et al. (1966) subdivided it into biotypes (a name changed later to 'biovars'), but all the

subdivisions are far from convincing. The exceptions may be biovar D and E, characterized by different colors (orange for D due to a soluble pigment phenazine-α-carboxylate and green for E due to the insoluble pigment chlororaphin). These two biovars probably deserve the category of separate species.

Other properties that have been used for the internal subdivision of *P. fluorescens* are the denitrification or formation the gas from nitrate in an anaerobic medium, and the property of forming a slime (a levan) in a medium of high sucrose concentration. There are *P. fluorescens* biovars that denitrify as vigorously as *P. aeruginosa*. The property of denitrification separates very neatly those biovars of *P. fluorescens* from *P. putida*, which never denitrifies, but, as said before, not all *P. fluorescens* possess this capacity. A universal property useful for the separation of *P. fluorescens* from *P. putida* is the liquefaction of gelatin, a positive property for the former species and negative for the latter.

Both species contain less phosphorous in the outer membrane, and therefore are not as sensitive to EDTA as *P. aeruginosa* (Wilkinson et al. 1973). This compound removes the divalent cations that link the phosphate groups and which are required for the outer membrane stability.

Roles of *P. fluorescens* in human Crohn's disease and murine inflammatory bowel disease have been suggested (Wei et al. 2002). These authors have found an I2 sequence associated with the disease. The sequence was isolated and identified as an open reading frame in the *P. aeruginosa* genome. The originating species was *P. fluorescens*, but homologies were detected in 13 other *Pseudomonas* species.

Other species of *Pseudomonas* are *P. stutzeri* and a related one, *P. mendocina*, that I isolated from soils in Mendoza, Argentina. Both are occasionally isolated from clinical materials. Many other species form a long list, but in their majority are of no medical interest.

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Burkholderia spp. and related genera

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The genus Burkholderia	1	Melioidosis	20
Burkholderia cepacia complex	1	*Burkholderia mallei*	26
Burkholderia gladioli	11	Glanders	27
The pseudomallei group	12	Miscellaneous opportunist 'pseudomonads'	29
Burkholderia pseudomallei	12	References	32
B. thailandensis	14		

THE GENUS BURKHOLDERIA

The genus *Burkholderia* was proposed by Yabuuchi et al. (1992) to encompass the species formerly grouped within the rRNA group II of the genus *Pseudomonas*. Seven species were transferred with *B. cepacia* as the type species of the genus. *Pseudomonas pickettii* and *Pseudomonas solanacearum* were subsequently moved to the genus *Ralstonia* (Yabuuchi et al. 1995). Today the genus *Burkholderia* comprises a complex group of microorganisms exhibiting extreme diversity not only in their host specificity but also in their biochemical and physiological activity. *Burkholderia* are key microbial constituents of the rhizosphere and have significant roles including the provision of nutrients to the growing plant nodule, fixation of atmospheric nitrogen, inhibition of plant pathogens including fungi, and degradation of complex compounds. They are versatile and inhabit ecological niches as varied as rice paddy water, acid water holes, and sea water. This versatility has led to the use of a number of *Burkholderia* species, particularly the *B. cepacia* complex, for biocontrol, bioremediation, and plant growth promotion. The introduction of a biocontrol strain with the planting material results in the colonization of the rhizosphere and the suppression of pathogenic competing bacteria and fungi with proven economic benefits (Bowers and Parke 1993). Other *Burkholderia* strains have been widely used for bioremediation of soils and agricultural environments due to their ability to degrade complex hydrocarbons and herbicides. A universally accessible database of such strains has been established (Urbance et al. 2003) (www.bsd.cme.msu.edu/bsd/index.html).

Currently, approximately 30 species have been incorporated into the genus and they range from obligate plant and animal pathogens through symbiotic organisms to plant associated species that cause opportunist infections in man. Table 1 lists the species so far recognized and their habitat or biological property. The medically significant species are the members of the *B. cepacia* complex and *B. gladioli*, *B. mallei*, and *B. pseudomallei*. Closely related genera such as *Ralstonia* and *Pandorea* are also occasionally recovered from human clinical specimens. Identification of species and their differentiation from others is difficult by cultural and biochemical test methods. Classification is best achieved by application of polyphasic taxonomic methods (Vandamme et al. 1997) and by indexing polymorphisms in specific gene targets (rRNA, *recA*) amplified by polymerase chain reaction (PCR) assays (Segonds et al. 1999; Mahenthiralingam et al. 2000a).

BURKHOLDERIA CEPACIA COMPLEX

Taxonomy

This species complex owes its present epithet to the description by Burkholder (1950) of a bacterium

Table 1 *Origins of selected* Burkholderia *spp.*

Species	Source/property	Reference
B. andropogonis[a]	Stripe disease of sorghum, leaf spot of velvet bean	Palleroni (1984); Coenye et al. (2001b)
B. anthina	Genomovar VIII of *B. cepacia* complex	Vandamme et al. (2002a)
B. ambifaria	Genomovar VII of *B. cepacia* complex	Coenye et al. (2001f)
B. caledonica	Rhizosphere of plants in Scotland	Coenye et al. (2001c)
B. caribensis	Soil, exopolysaccharide forming	Achouak et al. (1999)
B. caryophylli	Rot of carnations and onions; induces root nodule formation	Palleroni (1984)
B. cepacia	Genomovar I of *B. cepacia* complex	Vandamme et al. (1997)
B. cenocepacia	Genomovar III of *B. cepacia* complex	Vandamme et al. (2003)
B. cocovenenans[b]	Produces toxoflavin, bongkrekic acid, food poisoning associated with fermented corn flour	Zhao et al. (1995); Coenye et al. (1999)
B. dolosa	Genomovar VI of *B. cepacia* complex; induces root nodule formation	Coenye et al. (2001d); Vermis et al. (2003)
B. fungorum	Roots of plants, fungi, and clinical specimens	Coenye et al. (2001c)
B. gladioli[c]	Rot of onions, rice, flowers	Ura et al. (1996); Coenye et al. (2001a)
B. glathei	Fossil lateritic soil in Germany	Zolg and Ottow (1975)
B. glumae	Rot of rice grains and seedlings	Urakami et al. (1994)
B. graminis	Wheat rhizosphere, corn, and grasses	Viallard et al. (1998)
B. hospita	Transconjugants acquiring plasmids pJP4 or pEMT1[d]	Goris et al. (2002)
B. kururiensis	Acquifer polluted with trichloroethylene	Zhang et al. (2000a)
B. mallei	Causative agent of glanders, no known environmental source; BW agent	Palleroni (1984)
B. multivorans[e]	Genomovar II of *B. cepacia* complex	Stanier et al. (1966)
B. phenazinium	Soil, produces iodinin	Viallard et al. (1998)
B. phymatum	Induces root nodule formation in tropical legumes	Vandamme et al. (2002b)
B. pseudomallei	Causative agent of melioidiosis; rural	Whitmore (1913)
B. pyrrocinia	Genomovar IX of *B. cepacia* complex	Vandamme et al. (2002a)
B. sacchari	Soil in sugar cane plantation, produces polyhydroxyalkanoates	Brämer et al. (2001)
B. stabilis	Genomovar IV of *B. cepacia* complex	Vandamme et al. (1997)
B. terricola	Transconjugants acquiring plasmids pJP4 or pEMT1[d]	Goris et al. (2002)
B. thailandensis	Environment, Thailand	Brett et al. (1998)
B. ubonensis	Soil, Thailand; grouped with *B. cepacia* complex	Yabuuchi et al. (2000)
B. vietnamiensis	Genomovar V of *B. cepacia* complex; rice seedlings, Vietnam	Gillis et al. (1995)
'*B. tropicalis*' and '*B. brasiliensis*'	Tropical fruit, nitrogen fixers. Tentatively named species	Estrada-De Los Santos et al. (2001)

a) Two pathovars, *B. andropogonis* pv. *andropogonis* and pv. *stizolobii*.
b) Also known as '*Pseudomonas farinofermentans*,' junior synonym of *B. gladioli*.
c) Two pathovars, *B. gladioli* pv. *gladioli* and pv. *allicola*.
d) Plasmids containing genes for degradation of 2,4-dichlorophenoxyacetic acid.
e) Previously 'eugonic oxidizers group I,' *Pseudomonas kingii*.

responsible for rot in onion bulbs. The organism was later referred to as '*Pseudomonas multivorans*' by Stanier et al. (1966) and as *Pseudomonas kingii* by Jonsson (1970). Snell et al. (1972) showed that *P. kingii* was synonymous with *P. cepacia*, the name originally proposed by Burkholder, and confirmed the proposition of Ballard et al. (1970) that the latter name be preferred. *B. cepacia* was named as the type species (type strain ATCC 25416) following the transfer of the species from the genus *Pseudomonas* to the new genus *Burkholderia* (Yabuuchi et al. 1992).

B. cepacia has long been thought not to be a homogeneous species. Using numerical analysis techniques, Yohalem and Lorbeer (1994) distinguished four phenons within a large collection of isolates of *B. cepacia* from diverse environmental and clinical sources and concluded that three of these groups were more different from each other than would be expected of a subspecies, but the groups were joined in a cluster at approximately 80 percent similarity. Furthermore, an exploration of the genetic structure of the species by Wise et al. (1995) by multilocus enzyme electrophoresis,

showed that the mean genetic diversity was low (0.57) and the limited association between the alleles investigated suggested frequent recombination and relatively little clonality. However, it was the pioneering work of Vandamme et al. (1997) using polyphasic taxonomic methods including biochemical test reactions, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins, DNA–DNA hybridization, and gas liquid chromatography of fatty acid methyl esters that revealed a complex of five genomovars (genetically distinct but phenotypically similar) among strains isolated from cystic fibrosis (CF) patients.

To date, the *B. cepacia* complex comprises nine genomovars, *B. cepacia* (I), *B. multivorans* (II), *B. cenocepacia* (III; Vandamme et al. 2003); *B. stabilis* (IV), *B. vietnamiensis* (V), *B. dolosa*, (VI), *B. ambifaria*, (VII), *B. anthina* (VIII), and *B. pyrrocinia* (IX) (Table 1) (Coenye et al. 2001g). *B. ubonensis*, originally named *B. uboniae* by Yabuuchi et al. (2000), has close similarities to the complex but so far it has not been named as a member. A panel of experimental strains representative of the first five genomovars (Mahenthiralingam et al. 2000b) has been widely used by investigators and this was recently extended to include the later genomovars VI–IX (Coenye et al. 2003c).

Representatives of the first five genomovars were classified into four restriction fragment length polymorphism (RFLP) groups on analysis of PCR amplified 16S rDNA. *B. cepacia* genomovar I and III were grouped together but the others fell in different RFLP groups (Segonds et al. 1999). Bauernfeind and colleagues (1999) utilized 16S and 23S rDNA sequences to identify *B. vietnamiensis* and *B. multivorans* specifically, but were also unable to discriminate between genomovars I, III, and IV. The 16S rDNA RFLP approach was extended to all nine members of the complex by Vermis et al. (2002b) using *Alu*I, *Cfo*I, and *Dde*I restriction enzymes. Only genomovar VI was identified unambiguously and approximately half of the *B. vietnamiensis* strains gave the same pattern as genomovars I and III. Genomovars II and IV gave unique restriction profiles but two strains of each had divergent profiles which were also observed in other genomovars. Automated ribotyping was applied to the discrimination of the genomovars by Brisse et al. (2000), but although broadly they could be distinguished from each other, several ribotype patterns were evident within individual genomovars. Ribotyping, however, clearly distinguished the closely related species *B. gladioli* from the *B. cepacia* complex. In general, ribotyping was concordant with the results obtained by 16S rDNA RFLP analysis. Taken together these studies suggest that there is insufficient variation within the rRNA operon to specifically identify all members of the complex.

tRNA intergenic length polymorphism analysis (tDNA-PCR) was used for the classification of *B. cepacia* complex isolates and the product was sequenced by capillary electrophoresis. Genomovar V isolates gave identical tDNA profiles to genomovar IX, and genomovars I and VIII could not be distinguished from each other (Storms et al. 2002). DNA–DNA hybridization using genomic DNA macroarrays for the differentiation of *Burkholderia* spp. was proposed by Ramisse et al. (2003) who constructed a data matrix based on the relative DNA–DNA relatedness values of selected member species. This allowed the differentiation of *B. mallei* from *B. pseudomallei* and also discriminated between genomovars I, II, III, IV, V, and IX.

The *recA* gene was targeted by Mahenthiralingam et al. (2000a) and RFLP analysis of PCR amplified *recA* from reference and clinical isolates showed sufficient sequence variation to discriminate all five genomovars then known. Genomovar III was subdivided into two *recA* groups III-A and III-B and specific PCR assays for each of the genomovars were proposed following alignment of *recA* sequences for the design of PCR primers. Subsequently Vermis et al. (2002a) evaluated the *recA*-PCR tests on a large collection of *B. cepacia* complex isolates and found that the assays for genomovars II, III, and VII were highly sensitive and invariably specific but genomovar I strains cross-reacted with genomovar IX. New *recA* RFLP types were found within the collection which suggests that novel genomovars await definition.

Isolation and identification

B. cepacia complex organisms are slender rods with multitrichous flagella that may stain irregularly owing to the accumulation of poly-β-hydroxybutyrate. They are aerobic and grow well on nutrient agar but often prefer temperatures of 25–35°C for 48 h for optimal growth. Most strains grow at 41°C but not at 42°C, and none grows at 4°C. Cultures on blood agar often become nonviable after 3–4 days and survival on refrigerated agar slopes is poor; some workers favor maintaining cultures as suspensions in sterile tap water. Colonies on nutrient agar are opaque; those of some strains are grayish-white, but others are at first yellowish and later take on an intense reddish-purple color due to the formation of a nondiffusible phenazine pigment; the yellow pigment is best demonstrated on iron-containing media. Members of the *B. cepacia* complex resemble *B. pseudomallei* in not forming ammonia from arginine and in being lysine- and ornithine decarboxylase-positive; acid may be formed from dulcitol but not from ethanol in ammonium-salts medium. The oxidase reaction is characteristically slow and nitrate is not reduced; some strains produce a melanin-like pigment on tyrosine agar.

Selective media are necessary to optimize isolation of the species from specimens with mixed flora and several media, some of them also differential, have been described that exploit the biochemical diversity and intrinsic resistance of the complex to antibacterial

agents. They include: an oxidation–fermentation agar base supplemented with lactose, polymyxin B, and bacitracin (Welch et al. 1987); a commercially available agar containing crystal violet, bile salts, ticarcillin, and polymyxin B (Gilligan et al. 1985); a medium containing the selective agents 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan and polymyxin B (Wu and Thompson 1984). Henry et al. (1999) reported that a medium containing lactose and sucrose in an enriched casein and yeast extract with polymyxin, gentamicin, and vancomycin was superior to oxidation-fermentation agar and the commercially available selective medium for the recovery of *B. cepacia* from CF sputum. For the optimal isolation of *B. cepacia*, sputum should be liquefied, diluted in saline, and selective agar plates incubated at 37°C for 48 h and then at room temperature for up to 5 days. A liquid enrichment medium (Malka broth) supplemented with polymyxin B is recommended for the isolation of the organism from environmental swabs and soils (Butler et al. 1995). A number of pseudomonads and other polymyxin-resistant gram-negative species are able to grow on *B. cepacia* selective media and the identity of isolates should be confirmed by additional tests. Some strains of *B. cepacia* from CF sputum are auxotrophic for specific single or multiple amino acids. These mutants arise from prototrophic parent strains and are maintained by the nutritionally rich environment of the CF airway (Barth and Pitt 1995).

Van Pelt et al. (1999) found that automated biochemical identification assays, such as Vitek and Microscan, gave only 'fair' identification of a panel of molecularly defined *B. cepacia* complex isolates, but API 20NE microgalleries misidentified only a single strain of the complex. This was supported by Henry et al. (2001), who concluded that the API 20NE strip was reasonably reliable for identifying all members of the complex, except *B. stabilis*. Classification of *B. cepacia* complex clinical isolates to genomovar level is best achieved by amplification of the *recA* gene to assign the isolate to the complex. RFLP analysis with *Hae*III restriction enzyme is then used to generate genomovar specific patterns (Mahenthiralingam et al. 2000a).

Genomics

B. cepacia possesses one of the largest genomes so far sequenced among gram-negative bacteria. The DNA sequence of *B. cenocepacia* strain J2315 is now complete, and the sequences are available on the Internet (www.sanger.ac.uk/Projects/B.cepacia). The genome totals 8.056 Mb in three chromosomes of 3.870, 3.217, and 0.876 Mb, and a plasmid of 92.7 kb, with a G + C content of approximately 66.9 percent. The multiple replicon structure was investigated by Wigley and Burton (2000) in clinical and environmental strains by pulsed-field gel electrophoresis (PFGE). Three or four large replicons were found more frequently in clinical than in environmental strains. The genome is also rich in insertion sequences and these two features may have played a key role in the evolution of novel degradative functions and the unusual adaptability of the bacterium (Lessie et al. 1996).

Susceptibility to antimicrobials

B. cepacia can contaminate various pharmaceutical products, especially antiseptic solutions such as chlorhexidine and cetrimide, and a number of outbreaks have arisen in hospitals as a direct consequence of the proliferation of the organism in such solutions (Martone et al. 1987). The species is resistant to most antibiotics. Nearly all strains are resistant to the aminoglycosides, polymyxin, ticarcillin, azlocillin, and imipenem, whereas variable susceptibility is shown to temocillin, aztreonam, ciprofloxacin, and tetracycline. A proportion of strains remain relatively susceptible to trimethoprim–sulfamethoxazole and chloramphenicol. A study of *B. cepacia* from CF patients in Britain found that about three-quarters of isolates were susceptible to ceftazidime, piperacillin (+ tazobactam), and meropenem but usually at only 'high' breakpoint levels (Pitt et al. 1996).

A survey of *B. cepacia* complex isolates, of known genomovar status, showed that the minimum inhibitory concentrations of individual antimicrobials varied widely within genomovars, although clinical and environmental strains of *B. vietnamiensis* were marginally more susceptible to ceftazidime and chloramphenicol than strains from other genomovars (Nzula et al. 2002). Various combinations of two, three, or even four antibiotics may exhibit synergy in vitro against *B. cepacia*. Manno et al. (2003) found that less than half of *B. cepacia* complex isolates tested were susceptible to combinations of ciprofloxacin-piperacillin and rifampin-ceftazidime. They also showed that rates of antagonism for cotrimoxazole and chloramphenicol in combination with β-lactams were higher than those observed for ciprofloxacin plus β-lactams.

Resistance to β-lactams is a consequence of a combination of low permeability of the outer membrane and inducible β-lactamases. Decreased expression of the major porins leads to elevated resistance to β-lactams (Parr et al. 1987) and the insensitivity to cationic antibiotics results from the sparse degree of phosphorylation of its lipopolysacchharide (LPS) coupled with the presence of protonated 4-amino-4-deoxy-l-arabinose in lipid A or core oligosaccharide (Cox and Wilkinson 1991). β-Lactam resistance may also be mediated by a class A β-lactamase very similar to the chromosomal β-lactamase of *Klebsiella oxytoca* (Trepanier et al. 1997). A multidrug resistance efflux pump called BcrA may contribute to high level resistance to tetracycline and nalidixic acid (Wigfield et al. 2002) and another

multidrug efflux protein has been reported in *B. vietnamiensis* (Fehlner-Gardiner and Valvano 2002). Plasmids can be detected in *B. cepacia* but their contribution to overall resistance is unknown (Sabaté et al. 1994); however, antibiotic resistance is mostly associated with large plasmids (146–222 kb). Conjugative transfer of plasmid DNA from *P. aeruginosa* to *B. cepacia*, and from *B. cepacia* transconjugants to other strains of the same species, has been demonstrated (Lennon and DeCicco 1991).

Outbreaks in hospitals

A common feature of early reports of outbreaks of *B. cepacia* infections in hospitals was contamination of water supplies, often in pharmacies, antiseptic/disinfectant solutions, tubing for irrigation, and monitoring lines (Martone et al. 1987). Today, outbreaks are less common owing to the use of prepackaged proprietary preparations, which decreases the risk of common source contamination. Nevertheless, extrinsic contamination of nebulized medications, such as albuterol, has been implicated in outbreaks in intensive care units (Pegues et al. 1996) and cardiac wards (van Laer et al. 1998). Patients tend to be colonized rather than infected and patient-to-patient spread is rare; prolonged carriage by non-CF patients has not been documented. There are conflicting reports concerning an association between *B. cepacia* and chronic granulomatous disease. Respiratory infection in these patients appears to be independent of the degree of lung damage (O'Neil et al. 1986), and the organism is found in only a minority of cases (Nakhleh et al. 1992). Phagocytic cells from these patients do not produce peroxides necessary for the intracellular killing of bacteria.

Cystic fibrosis

The major impact of *B. cepacia* infection has undoubtedly been in CF patients in whom it was first recognized in the mid-1970s. However, it did not become a clinically significant problem until 10 years later when the prevalence of the organism rose to nearly 40 percent of patients attending a major treatment center in Toronto, Canada (Isles et al. 1984) and elsewhere in the USA (Thomassen et al. 1985). The clinical outcome of *B. cepacia* infection in CF patients may vary from asymptomatic carriage through a gradual but accelerated decline to a rapidly fatal fulminant septicemia in a minority of cases, the latter condition being termed 'cepacia syndrome.' The contribution of *B. cepacia* to clinical deterioration and lung damage in the majority of patients is unclear. Peckham et al. (1994) found no significant difference in inflammatory markers and lung function between *B. cepacia* colonized and noncolonized patients, but more recent studies show a clear excess of mortality in patients colonized with an epidemic strain of *B. cepacia* particularly where lung function was poor (Ledson et al. 2002). Moreover, the severity of pulmonary exacerbation was no greater in patients with multiple organisms compared with those harboring *B. cepacia* alone (McManus et al. 2003).

The frequency of *B. cepacia* complex organisms among all CF patients is generally low (approximately 5 percent of patients in UK centers), but this may increase dramatically if transmissible strains are implicated. Infections are more common in adult (>15 years) than pediatric patients. It is a widely accepted practice to segregate *B. cepacia*-positive CF patients from others not harboring the organism and this, among other infection-control measures, has been shown to interrupt spread and significantly reduce acquisition of the organism by new patients (Whiteford et al. 1995). However, a 6 year review of molecular types of isolates of *B. cepacia* from 56 patients referred to a North American transplantation center showed that patient-to-patient spread was rare and no evidence was found that they represented a major source of *B. cepacia* strains found in the resident CF clinic population (Heath et al. 2002). This may be explained by the fact that the great majority of the strains examined lacked the *cblA* gene which is almost unique to strains of the ET12 clonal lineage (Johnson et al. 1994). This highly transmissible lineage originated in Ontario and spread through north-eastern centers in the USA to the UK, apparently carried by CF patients returning from summer camp holidays (Anon 1994). This strain was subsequently identified in approximately one-third of UK and Ireland patients colonized with *B. cepacia* (Pitt et al. 1996).

It has also been repeatedly demonstrated that *B. cepacia* can be transmitted between CF patients both in and out of hospitals (Govan et al. 1993; D.L. Smith et al. 1993). Guidelines for patients and their carers have been put forward by CF associations in Europe and North America in an attempt to reduce cross-infection. They stress the need for good personal hand hygiene, the avoidance of intimate contact between individuals and sharing of rooms for physiotherapy, and nebulizer care to minimize bacterial contamination, etc. They also highlight the importance of regular sensitive and specific bacteriological screening to establish the colonization status of patients and inform segregation policies. It is noteworthy that transmission of strains can occur between non-CF and CF patients (Holmes et al. 1999).

GENOMOVARS

More than half of *B. cepacia* complex isolates from CF patients are of genomovar III; in the USA approximately 75 percent are *recA* lineage IIIb, whereas in Canada IIIa strains prevail. The second most common is

B. multivorans (genomovar II) and reports of its frequency range from approximately 5 to 37 percent. The other genomovars are uncommon (Mahenthiralingam et al. 2002). Accordingly, most clustering of outbreak isolates are associated with genomovar III strains but genomovar II may occasionally be implicated in outbreaks. Nevertheless, most genomovar II strains from CF appear to be genotypically unique (Turton et al. 2003). The finding that some virulent genomovar III strains can superinfect patients already colonized with genomovar II strains is of clinical importance as the former are universally associated with clinical decline and a higher mortality (Mahenthiralingam et al. 2001). Owing to the shortage of donors, heart-lung, or lung transplantation is available as an end-stage treatment to only a minority of CF patients. Patients infected with *B. cepacia* complex organisms present an additional problem in their selection for transplant lists as long-term survival has been closely correlated with genomovar status. Although outcomes are variable with some 1 year mortality rates of between 50 and 100 percent, survival rates greater than 75 percent have also been reported (Mariencheck et al. 2001). However, the survival rates of genomovar III and non-genomovar III colonized patients differed significantly and mortality was almost exclusively associated with the former in both Great Britain and North America (De Soyza et al. 2001; Aris et al. 2001).

TRANSMISSIBILITY FACTORS

Three transmissibility genetic factors have so far been described in *B. cepacia*; *cblA* which encodes for thick bundles of peritrichous (cable) fimbriae (Sun et al. 1995), an insertion sequence hybrid IS*1356*/IS*402* (Tyler et al. 1996), and the *B. cepacia* epidemic strain marker (BCESM), a 1.4 kb sequence containing an open reading frame with homology to transcriptional regulatory genes (Mahenthiralingam et al. 1997). The strong association of these markers with epidemic strains led Clode et al. (2000) in the UK to suggest that strict segregation of patients might only be appropriate for those harboring transmissibility marker-positive genomovar III strains. This was contested by LiPuma et al. (2001) in the USA who found a relatively low frequency of BSCEM (23 percent) and *cblA*-positive (1.6 percent) strains in their patient population and attributed the difference to the spread of the ET12 clone in the UK. Recent work by Baldwin et al. (2004) has demonstrated that BCESM is part of a novel genomic island encoding genes linked to both virulence and metabolism.

Parsons et al. (2003) reported the results of a subtractive hybridization technique to identify genomic regions within the ET12 lineage of *recA* type IIIA that were absent from a nontransmissible genomovar IIIB strain. Fifteen sequences were identified, five of which showed significant similarity to known sequences. Many of the regions identified had low guanine and cytosine (G + C) content, a feature indicative of pathogenicity islands, but one region shared sequence similarity with capsule/polysaccharide-production or export genes of *B. mallei* and *B. pseudomallei*. This finding, taken together with the observations of Reckseidler et al. (2001) who performed similar experiments with *B. pseudomallei* polysaccharide gene clusters, suggests that the *B. cepacia* complex contains a polysaccharide gene cluster that is related but different from that of *B. mallei*, *B. pseudomallei*, or *B. stabilis*.

ACQUISITION FROM THE ENVIRONMENT

B. cepacia has been detected in air samples taken after physiotherapy and can persist in the air after the room is vacated (Ensor et al. 1996). It is able to survive for long periods in respiratory droplets on environmental surfaces but this property varies with the strain (Drabick et al. 1996). It is also a common contaminant of dental water systems (Pankhurst and Philpott-Howard 1996). The species complex is relatively common in the domestic environment (Mortensen et al. 1995) but is infrequent in salads and fresh foods.

The question arises as to whether the natural environment represents a significant risk for CF patients. Butler et al. (1995) concluded that the environment represented a low risk but the pathogenic potential of environmental strains remained unclear. This contrasted with the view of Bevivino et al. (1994), who found that environmental strains lacked the abilities to produce gelatinase and an hydroxamate siderophore, and they failed to attach to human uroepithelial cells. A more recent study by this group compared traits related to virulence and transmissibility in large numbers of isolates from Italian CF patients with isolates from the maize rhizosphere (Bevivino et al. 2002). Genomovar III isolates comprised the majority of the strains examined in both the environmental and clinical collection with *B. ambifaria* also being quite frequent in the former. The clinical group generally contained a higher proportion of antibiotic resistant strains and were equally as able as environmental strains to macerate onion tissue and produce proteases. There were, however, marked differences between the groups in hemolytic activity with more environmental than clinical strains expressing a hemolysin. The *esmR* gene was found exclusively among genomovar III isolates with a marked prevalence in clinical isolates, but only one of the 143 isolates tested positive for *cblA* underlining the rarity of clone ET12 in Italy.

Holmes et al. (1998) warned about the potential danger of the widespread application of strains to the agricultural environment for biodegradation and biocontrol purposes. There is evidence of evolutionary divergence in 16S rDNA sequences of rhizosphere isolates from their clinical counterparts (Tabacchioni et al.

1995), but diversity in this locus among clinical strains also occurs as evidenced by the wide range of ribotyping patterns found among CF isolates (Pitt et al. 1996). A survey by Richardson et al. (2002) showed that most rhizosphere isolates had closest affinity with *Burkholderia* spp. with known bioremediation and biocontrol capabilities and were unrelated to taxa comprising plant or human pathogenic strains. Nevertheless, the finding of a genomovar III strain from agricultural soil in the USA by LiPuma et al. (2002) suggests that human pathogenic strains are not necessarily distinct from environmental strains and patients may, on occasion, acquire these strains direct from the environment.

Cell surface structures

CELLULAR LIPIDS

Like other gram-negative bacteria, *B. cepacia* possesses a complex cell envelope in which a thin layer of peptidoglycan is sandwiched between the cytoplasmic membrane and the outer membrane which is composed of lipids, outer membrane proteins (OMP), and LPS. The polar lipids contain two forms each of phosphatidylethanolamine (PE) and an ornithine amine lipid (OL), the forms differing only in the presence or absence of a 2-hydroxy fatty acid; diphosphatidylglycerol (DPG) is a third component. The average distribution of lipid phosphorus in cell wall extracts is DPG (23 percent), PE1 (50 percent), PE2 (27 percent). Ornithine-containing lipids contribute 10–15 percent to the total cellular lipids with a preponderance of OL2 over OL1 (Cox and Wilkinson 1989). The distinctive range of 2-hydroxy acids in *B. cepacia* conferred by PE2 and OL2 is also characteristic of other *Burkholderia* spp. (Galbraith and Wilkinson 1991). Lipid composition, particularly an increase in 2-hydroxy acids in PE1, is strongly influenced by growth temperature and change in the growth rate also affects the PE:OL ratio (Taylor et al. 1998).

OUTER MEMBRANE PROTEINS

Two types of OMP profiles are characteristic of clinical isolates of *B. cepacia*. The first type is characterized by proteins of 39, 27, and 18 kDa and, in addition, the second contains proteins in the size range 80–90 kDa in growth limiting conditions (Livesley et al. 1998). The 39 and 27 kDa proteins function as porins for small hydrophilic solutes, constitute antigens, and contribute to antibiotic resistance (Parr et al. 1987). The major porin protein, coded OpcP1, has been cloned and the predicted *opcP* gene encoded a mature protein of 35.7 kDa (Tsujimoto et al. 1997). An enzyme-linked immunosorbent assay (ELISA) test using the 80 kDa OMP has been shown to be of value for distinguishing between *B. cepacia* derived from colonized compared to noncolonized CF patients (Lacy et al. 1997). Expression of a 97 kDa putative heme-binding protein was linked to genomovar III strains by Smalley et al. (2001). This protein is located in the outer membrane and binds iron (III) protoporphyrin IX in both the oligomeric and monomeric forms. It may serve as a receptor for the uptake of iron or facilitate cell-surface deposition of ferrihemes which protect the cell against neutrophil-derived hydrogen peroxide attack in the inflamed lung. Two other heme-binding OMPs of 77 and 147 kDa were identified when genomovar IIIa strains were exposed to increasing concentrations of iron (III) protoporphyrin IX, these proteins were not found in genomovar I strains. They may therefore be pivotal to the virulence of the organism and given their prominence on the cell surface may prove to be suitable candidates for vaccine development (Smalley et al. 2003).

LIPOPOLYSACCHARIDES

LPS forms the immunodominant structure of the outer membrane. It has a general architecture similar to the enterobacteria comprised of lipid A, core oligosaccharide, and O-specific type chain. There are, however, some unusual features including: (1) a low degree of phosphorylation in the core oligosaccharide (Cox and Wilkinson 1991); (2) extensive substitution by 4-amino-4-deoxy-L-arabinose in the lipid A; (3) a low content of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo); (4) the substitution of Kdo by D-glycero-α-D-talo-2-octulosonic acid (Ko); this disaccharide is also present in *B. pseudomallei*; and (5) the presence of two distinct polymeric fractions in several reference strains which creates ambiguity about the identity of individual O antigens and in some cases detracts from serospecificity (Wilkinson and Pitt 1995a).

Compared with many bacteria, *B. cepacia* produces remarkably simple O-antigen polymers. They are linear with di- or tri-saccharide repeating units although some contain monosaccharides. Many of the polymers also occur in other gram-negative species and identical structures have been reported in *B. vietnamiensis* and *B. cepacia* (Gaur and Wilkinson 1997). The chemical structure of the O-antigen of an environmental strain of *B. cepacia* was reported by Cimino et al. (1998) which was apparently unique to *Burkholderia* spp. and for O-antigens in general.

At least three serological typing systems have been described independently for the grouping of *B. cepacia* isolates (see Wilkinson and Pitt 1995a), but Werneburg and Monteil's scheme (1989) has probably been the most widely used. This differentiates the species into nine O and five H types with a typability of 98 percent and 43 percent, respectively, for isolates from the environment and nosocomial infection; typability rates fall significantly with CF isolates. O-serotype and susceptibility to lipopolysaccharide-binding bacteriophages was not correlated with genomovar status (Kenna et al.

2003). Evans et al. (1999) used SDS-PAGE and immunoblotting to define the chemotype and antigenic cross-reactivity of 16 strains of four genomovars of *B. cepacia*. All strains of genomovars I and II had smooth LPS but genomovar III strains of the ET12 lineage were of either rough or smooth LPS chemotype. A rabbit serum raised to the index ET12 strain (J2315) revealed antigenic relatedness between LPS core antigens in different genomovars; variation in LPS chemotype was also observed with broth and agar grown cells of the same strain.

EXOPOLYSACCHARIDES

B. cepacia produces two distinct acidic exopolysaccharides, PSI and PSII. PSI is characterized by the repetition of a disaccharidyl unit while PSII has a more complex heptasaccharidyl repeat unit. PSI was found associated with PSII in two environmental strains, but PSII alone was produced by most mucoid strains examined (Cérantola and Montrozier 2001). *B. cepacia* rarely grows as a mucoid form on agar media but production of the exopolysaccharide is encouraged by growth in glycerol-based media with high salt content (Cérantola et al. 2000).

FLAGELLA AND PILI

In common with *P. aeruginosa*, *B. cepacia* expresses two protein flagellin antigenic types which are distinguishable by molecular size. The type II flagellins have an approximate size of 45 kDa and are readily differentiated by SDS-PAGE from the 55 kDa type I flagellins; however, a flagellin of 70 kDa has also been reported for a single strain (Hales et al. 1998). A third flagellar protein of 31 kDa was found by Montie and Stover (1983) but this may have been an artifact of the isolation procedure rather than complete flagellin. The diameter of the flagella of type I strains is wider than that of type II strains (approximately 18.0 vs. 14.5 nm). The N-terminal sequence of the type II flagellin shows close identity with *B. pseudomallei* flagellin. In common with other flagellins, the *B. cepacia* sequences exhibit high levels of homology in the conserved terminal regions but differ considerably in the central region. No evidence of phase variation has been reported but Hales et al. (1998) found extensive flagellin sequence variation and separated the 16 strains examined into ten RFLP groups using two restriction enzyme digests. Flagella contribute to the virulence of many bacterial species and *B. cepacia* is no exception. Tomich et al. (2002) attributed the ability of a clinical strain of *B. cepacia* (genomovar III) to invade cultured respiratory epithelial cells to be linked to its flagellar status. Loss of motility in transposon generated mutants was strongly correlated with reduced invasiveness due to disruption of the *fliG* locus and was not due to defective adherence of the mutant to the test cells.

At least five different structural classes of pilus structures (cable, mesh, filamentous, spine, and spike) have been identified in *B. cepacia*. The cable pilus was strongly implicated in the spread of the ET12 clone in the USA and UK (Goldstein et al. 1995), whereas the mesh pili were the most widely expressed being found in both epidemic and nonepidemic CF isolates; the spike pili were commonly associated with environmental isolates. A 22 kDa protein located on the cable pilus mediates cell binding with high affinity for respiratory mucins and lung epithelial cells (Sajjan and Forstner 1993) through the receptor, cytokeratin 13, the expression of which is increased in CF lungs (Sajjan et al. 2000, 2002). Chiu et al. (2001) showed that strains expressing cable pili form large clumps on the surface of the target cell whereas non-cable piliated strains predominantly attach as single cells. They used dextrans (10 000 Da) to inhibit the adherence of strains and this was more effective against non-cable-piliated strains than those expressing this pilus; the inhibitory effect was readily reversible.

Typing of *B. cepacia*

BACTERIOCINS AND BACTERIOPHAGES

A scheme utilizing production and sensitivity to bacteriocins was developed by Govan and Harris (1985) and identified 14 producer and 23 susceptibility types in 44 combinations among 95 percent of *B. cepacia* isolates. It is a simple agar overlay technique and results can be obtained within 24 h but there may be substantial variation among outbreak-related strains (Rabkin et al. 1989).

A phage-typing scheme for *B. cepacia* has not been described, but the contribution of phages to genomic plasticity, adaptability, and virulence of the complex was explored by Langley et al. (2003). Temperate and lytic phages (T-even like and lambda-like) were isolated from soils and river sediments but proved not to be species specific within the complex. Lysogeny was relatively common and the shared host range of two temperate phages from *B. cenocepacia* and *B. stabilis* suggests that integration of the same phage can occur in different species of the complex.

CHROMOSOMAL DNA ANALYSIS

Ribotyping has been widely applied to epidemiological studies of *B. cepacia*. LiPuma et al. (1988) found 14 different rRNA patterns based on the positions of 4–8 hybridization bands in *Eco*RI digests of DNA from CF strains and at least 50 patterns were identified by Pitt et al. (1996) among strains from UK CF patients. PCR has been used to detect polymorphisms in the intergenic spacer regions of rRNA (Kostman et al. 1992), and arbitrarily primed PCR revealed strain lineages similar to those obtained by PFGE of *Xba*I

digests, or ribotyping (Bingen et al. 1993). Insertion sequence primers originally intended for the identification of *Bordetella pertussis* were found to differentiate between strains of *B. cepacia* from unrelated sources and show homology among epidemiologically related isolates (Cimolai and Trombley 1995). PCR amplification of the flagellin gene, *fliC*, of an extensively characterized panel of *B. cepacia* complex strains (Mahenthiralingam et al. 2000b) discriminated *B. multivorans* from other genomovars on the basis of amplicon size with the exception of a genomovar I strain; four *B. vietnamiensis* strains failed to yield an amplicon (Winstanley et al. 2001).

Multilocus restriction typing (MLRT), which indexes variation at five chromosomal loci by restriction analysis of PCR-amplified genes, was described by Coenye and LiPuma (2002). The genes targeted were *recA*, *gyrB*, *fliC*, *cepIR*, and *dsbA*, and most clusters delineated by MLRT corresponded closely to those defined by PFGE. The method was used to investigate the population structure of a large collection of clinical and environmental isolates of genomovar III from widespread geographical sources. The majority of isolates fell into just three major clonal lineages. Varying degrees of genetic recombination were found and the authors speculated that the differences in population structure stem from differences in evolutionary biology and ecology (Coenye and LiPuma 2003).

PLASMIDS

McKevitt and Woods (1984) found plasmids in only 23 percent of CF isolates and seven had the same plasmid. However, enzyme digest lysates and salt–ethanol precipitated DNA revealed plasmids in all but one of 16 isolates examined by Gonzalez and Vidaver (1979). Furthermore, 31 of 37 diverse strains proved positive for plasmids when a rapid alkaline lysis method was used (Lennon and DeCicco 1991). Reported plasmid sizes range from 1.7 to 224 kbp with up to eight plasmids per strain (Wigley and Burton 1999).

OTHER TYPING METHODS

Multilocus enzyme electrophoresis (MLEE) has proved of value for outbreak investigation and studies of the genetic structure of different strain populations. In particular, Wise et al. (1995) showed that there was only limited association between enzyme alleles and this was suggestive of frequent recombination within the species. MLEE in combination with ribotyping allowed the definition of various strain lineages and determination of linkage between strains from different geographical locations, which confirmed that the Toronto clone was genetically indistinguishable from the epidemic strain in the UK (Johnson et al. 1994). Quantitative differences in fatty acid profiles can also be epidemiologically significant (Mukwaya and Welch 1989), and pyrolysis mass spectrometry has been reported to be more discriminating for strain identification than SDS-PAGE (Corkill et al. 1994).

Pathogenicity of *B. cepacia*

Strains of *B. cepacia* from animal or plant sources are relatively avirulent (LD_{50} 1×10^8 colony-forming units (CFU)) for normal mice but they persist in high numbers in a burned mouse model (Stover et al. 1983). The species produces a variety of extracellular putative virulence factors that may contribute to its pathogenicity for plant and animal hosts. These include lipases, hemolysins, and proteases, among others (Wilkinson and Pitt 1995b).

Lipases of different types including esterases and lecithinases have been described; a 25 kDa protein which attacked Tweens but lacked phospholipase C activity was purified from a CF isolate that was not cytotoxic (Lonon et al. 1988). *B. cepacia*, unlike *P. aeruginosa*, has distinct phospholipase C and hemolysin activities and about 40 percent of isolates apparently express hemolytic activity (Vasil et al. 1990). A hemolysin that induces apoptosis (programmed cell death) and degranulation of mammalian phagocytes was described by Hutchinson et al. (1998). This compound was identified as a lipopeptide and expressed surface tension lowering activity; a functional role of suppression of the host's immune response was suggested.

An extracellular 36 kDa zinc metalloprotease was purified from *B. cepacia* which induced bronchopneumonia when instilled into the trachea of rats. It also cleaved gelatin, hide powder, and human collagen types I, IV, and V (McKevitt et al. 1989). Immunization of an animal with a peptide corresponding to the conserved metalloprotease significantly decreased the severity of experimental lung infections (Sokol et al. 2000b). Genetic analysis subsequently showed that the predicted amino acid sequences of the protease from *B. cepacia* and *B. pseudomallei* were similar to the thermolysin-like family of metalloproteases and was likely to be secreted via general secretory pathways (Corbett et al. 2003). *B. cepacia* may actively degrade respiratory mucins by the activity of a mucin-sulfatase. Desulfation increases the susceptibility of mucins to degradation by bacterial enzymes and the released carbohydrates and amino acids probably support bacterial growth. Mucin-sulfatase activity was detected in all nine strains of *B. cepacia* and four of six *P. aeruginosa* strains tested; aryl-sulfatase activity was also found in *B. cepacia*, but at levels approximately 20-fold lower than in *P. aeruginosa* (Jansen et al. 1999).

ENTRY AND SURVIVAL IN CELLS

It is widely held that the invasion of host cells and intracellular survival of *B. cepacia* is fundamental to its establishment in the host and a necessary prerequisite

for disease. Using the gentamicin protection assay, Burns et al. (1996) were the first to show that *B. cepacia* invaded cultured respiratory epithelial cells and progressed into the cytoplasm where they were enclosed by membrane-bound vacuoles. Live bacteria were necessary for invasion of the cell layer and this was inhibited by cytochalasin D, an inhibitor of eukaryotic microfilament formation. Intracellular survival and growth of *B. cepacia* complex isolates in *Acanthamoeba* was demonstrated by Marolda et al. (1999) and extracellular bacteria grew saprophytically on byproducts released by the amoebae. These results suggest a symbiotic relationship between the species complex and free-living amoebae in which the latter acts as a reservoir for the organism and even possibly serves as a vehicle for its transmission among CF patients. A macrophage model of Martin and Mohr (2000) confirmed these findings with strain J2315, but also showed that an environmental strain had similar invasive properties for macrophages but not for epithelial cells and was unable to survive or replicate intracellularly. They concluded that intracellular survival allowed *B. cepacia* to evade the host immune response. A different view on the interaction of *B. cepacia* with macrophages was taken by Saini et al. (1999). Using fluorescence microscopy and a gentamicin protection assay, they showed that *B. dolosa* (genomovar VI) and *B. vietnamiensis* (genomovar V) survived in murine macrophages for at least 5 days without significant bacterial replication. Entry of the bacteria into the macrophages stimulated production of tumor necrosis factor (TNF) and primed them to release toxic oxygen radicals which was attributed to LPS as they were blocked by polymyxin B. On the strength of these results, they proposed a model where repeated cycles of phagocytosis and cellular activation without bacterial killing promotes a deleterious inflammatory response causing tissue destruction and deterioration of lung infection. This scenario fits with the course of chronic respiratory infection in CF patients due to *P. aeruginosa*.

B. cepacia has been localized by immunohistology in CF patients with 'cepacia syndrome' in the exudates within bronchial lumens and between airway epithelial cells (Sajjan et al. 2001). In patients with chronic infections the organisms were diffusely distributed but in acute infections the distribution was more focal with *B. cepacia* located on injured airway surfaces and in abscesses. In the progression from the chronic state to invasive infection, *B. cepacia* migrates across the epithelial barrier to invade the lung parenchyma and capillaries thereby initiating septicemia. Schwab et al. (2002) explored the universality of *B. cepacia* complex invasion pathways in well differentiated human airway epithelial cell cultures. They found that all three strains tested (genomovars II, III, and IV) circumvented the mechanical barriers of mucus and ciliary transport to penetrate epithelial cells but they used different routes. The genomovar III strain formed biofilms at the apical cell surface and invaded and destroyed the cells by disruption of the glycocalyx and rearrangements of the actin cytoskeleton. In contrast, the genomovar IV strain did not form biofilms and the majority of bacteria that penetrated the epithelium were located between the cells, suggesting paracytosis. The genomovar II strain penetrated the epithelium both by cell destruction and paracytosis. Cieri et al. (2002) assessed the relative invasiveness of different genomovars of *B. cepacia* by their capacity to invade cultured respiratory epithelial cells and in a murine model of lung infection. They found that invasion of the cell line was relatively low compared to other pathogens and was often variable from assay to assay. Invasion was most evident with genomovar III strains (75 percent invasive) and was lowest in genomovars I and IV (25 percent). There was good correlation between the in vitro and in vivo assays. In contrast, BALB/c mice rendered leukopenic by administration of cyclophosphamide prior to intranasal challenge with different genomovars cleared all of nine genomovar III strains tested, but with variable toxicity, compared with five of six genomovar II strains which persisted at stable numbers in the lungs for several days with minimal toxicity (Chu et al. 2002). They speculated that genomovar III strains provoked higher levels of toxicity, thereby inducing a more effective immune response and clearance.

Many gram-negative bacteria use a conserved secretory system, type III, to deliver virulence proteins directly into host cells. Parsons et al. (2001) identified a gene cluster which was found in all of *B. cepacia* complex genomovars, except genomovar I. They also found a putative gene homologous to the *virB1* gene of *Brucella suis*. One of the genes identified by Tomich et al. (2003) in their examination of type III secretory gene homologs in *B. cepacia*, designated *bscN*, encodes an ATP-binding protein believed to generate energy driving virulence factor secretion. An isogenic *bcsN* mutant was significantly attenuated in comparison to the parent in terms of recovery from mouse lungs and spleens and induction of an inflammatory cell infiltrate.

SIDEROPHORES

At least four different siderophore compounds are produced by *B. cepacia* and include: ornibactins, pyochelin, salicylic acid (formerly azurechelin), and cepabactin. The ornibactins and salicylic acid compounds are produced by the majority of clinical isolates (Darling et al. 1998). Two genes with significant homology to pyoverdine biosynthetic genes of *P. aeruginosa*, *pvdA* and *pvdD*, are required for ornibactin synthesis, and an outer membrane receptor protein with a predicted molecular mass of 81.7 kDa was shown to be necessary for utilization of ornibactin for iron transport or growth; a mutant lacking the receptor was also significantly less virulent than its parent strain (Sokol et al.

1999, 2000a). Homologs of the universal ferric uptake regulator (fur) protein, which functions as a transcription repressor of many genes in bacteria in response to iron, has been demonstrated in *B. cepacia* with 90 percent sequence homology to the *fur* gene of *B. pseudomallei*. In contrast to the *fur* gene in *Escherichia coli*, transcription of which is activated by oxidative stress, hydrogen peroxide and superoxide generators had no effect on *fur* transcription in *B. cepacia*. The *fur* promoter was also not responsive to iron availability (Lowe et al. 2001).

ENDOTOXIN

The LPS of *B. cepacia* is probably the most significant of the virulence factors in mediating the augmented inflammatory response of CF patients infected with the species. Isolated LPS has relatively low toxicity; LD_{50} values in mice of approximately 1 mg have been reported but compared with *P. aeruginosa*, it has good endotoxic activity and induces approximately nine times as much TNF-α (Shaw et al. 1995). This was confirmed by Zughaier et al. (1999a) who, in contrast to the earlier study, found that induction of TNF-α by LPS was almost completely CD14 mediated. Monocytes primed with *B. cepacia* LPS produced more superoxide anion than cells primed with *P. aeruginosa* or *Stenotrophomonas maltophilia* LPS. However, the melanin-like pigment elaborated by some strains on tyrosine-enriched agar appears to attenuate the monocyte respiratory burst by acting as a scavenger of the superoxide anion thus protecting the organism from attack by free radicals (Zughaier et al. 1999b). The proinflammatory cytokines interleukin (IL)-6 and IL-8 are upregulated by LPS from strain J2315 and it also increases the surface expression of the β_2 complement receptor 3 on neutrophils and to prime the respiratory burst activity of these cells. Genomovar I strains are also able to prime the neutrophil respiratory burst response (Hutchinson et al. 2000; Hughes et al. 1997). The lack of nitric oxide (NO) activity in the lungs of CF patients is thought to contribute to the survival of *B. cepacia* in these patients. Smith et al. (1999) reported that superoxide, hydrogen peroxide, or NO alone failed to kill J2315 but when combined they reduced the bacterial count by more than 1 000-fold. Superoxide dismutase did not inhibit the process but catalase did, suggesting that NO and hydrogen peroxide mediated bacterial killing.

An unusual interaction of polymyxin B with *B. cepacia* LPS was recently described by Shimomura et al. (2003). They found that although *B. cepacia* contains 4-amino-4-deoxy-L-arabinose in its inner core region which should prevent binding of polymyxin B and thus confer constitutive colistin resistance, polymyxin B-immobilized beads were able to bind to *B. cepacia* LPS with high affinity. Remarkably, polymyxin B augmented the IL-1β-inducing activity of *B. cepacia* LPS to levels similar to other gram-negative LPS despite the fact that the LPS had previously been shown to be a weak IL-1β inducer (Shimomura et al. 2001).

QUORUM SENSING

Co-colonization of CF patients with *P. aeruginosa* and *B. cepacia* is infrequent but when it occurs it is associated with increased morbidity. Communication between the two species occurs via the *N*-acyl homoserine lactone (AHL) quorum-sensing mechanism with the possible effect of coordinately regulating their virulence factor expression (Lewenza et al. 2002). In the *B. cepacia* complex, the CepIR quorum sensing system regulates protease expression, biosynthesis of ornibactin, swarming motility, and biofilm formation. A second system, BviIR, is operative in *B. vietnamiensis* (Lutter et al. 2001). The stationary-phase sigma factor (RpoS), a regulatory system involved in the response to high cell density, is also an alternative sigma factor which directs transcription of a large number of genes involved in adaptation to nutrient-limiting conditions and induction of virulence factors. Aguilar et al. (2003) found that the quorum-sensing system of the onion pathogenic strain (ATCC 25416) had a negative effect on *rpoS* gene expression. They also confirmed that the *cep* locus regulates protease production and onion pathogenicity. Cell signaling genes, specifically an AHL synthase gene and its corresponding transcriptional regulator, were located on a 31.7 kb pathogenicity island in *B. cenocepacia* by Baldwin et al. (2004).

BURKHOLDERIA GLADIOLI

B. gladioli was previously known as '*Pseudomonas marginata*' but is closely related by DNA hybridization to *B. cepacia* (Ballard et al. 1970). Strains grow readily on polymyxin-containing selective media but they can be differentiated from *B. cepacia* by their negative reactions for oxidase enzyme and utilization of maltose and lactose. *B. gladioli* may also be confused with *Oligella ureolytica* due to its variable urea-hydrolyzing properties. Several reports attest to the clinical significance of *B. gladioli*, not only as an opportunist in the CF lung but also as a cause of primary invasive infection in these patients and patients with chronic granulomatous disease (Khan et al. 1996; Hoare and Cant 1996). Multiresistant isolates with shared phenotypic properties of *B. cepacia* and *B. gladioli* were reported from CF patients by Simpson et al. (1994) who found the 'Edinburgh epidemic strain' of *B. cepacia* to give equivocal reactions in biochemical tests and fatty acid analysis. Nosocomial acquisition of '*B. gladioli*' by CF patients has been reported in the literature (Wilsher et al. 1997), but the isolates were subsequently identified as *B. cepacia* (Clode et al. 1999). A PCR assay for the species based on 23S rDNA sequences was shown by Whitby et al. (2000) to have a sensitivity of 96 percent and to be 100 percent specific.

THE PSEUDOMALLEI GROUP

The glanders bacillus was isolated by Loeffler and Schutz in 1882 from a horse dying of glanders and was named '*Bacillus mallei*' by Zopf in 1885 (see Buchanan et al. 1966). An organism, in many respects resembling it, was isolated by Whitmore and Krishnaswami (1912) from a glanders-like disease of humans in Rangoon; it was designated '*Bacillus pseudomallei*' by Whitmore (1913). Subsequently, Stanton and Fletcher (1921, 1925) gave the name melioidosis to the disease and '*Bacillus whitmori*' to the causative organism. Because the two organisms cause similar diseases they were classified together but confusion over their taxonomic position led to them being placed in a variety of bacterial genera (for early history, see the seventh edition of this book). Palleroni (1984) classified the species as members of the genus *Pseudomonas* of rRNA homology group II. However, Yabuuchi et al. (1992) proposed that all members of that rRNA group be reclassified within the genus *Burkholderia* and the new names have been validly published.

BURKHOLDERIA PSEUDOMALLEI

Habitat

B. pseudomallei is an environmental saprophyte present in soil and water in a limited geographical area – South East Asia, the Philippines, Northern Australia, parts of Africa, and South and Central America – but may also be isolated in temperate climates (Dance 1991; Yabuuchi and Arakawa 1993). A damp climate and a terrain of flooded low-lying plains appear to favor the organism. Counts of *B. pseudomallei* in soil vary with region in countries such as Thailand and this may be a significant determinant of the frequency of melioidosis in the population (Smith et al. 1995b).

Isolation and identification

The culture and manipulation of *B. pseudomallei* (and *B. mallei*) presents a risk to laboratory personnel and so all procedures involving live cultures must be performed within a category level 3 containment facility.

B. pseudomallei are slender rods 0.3–0.5 μm in width and 1–2 μm long with two to four polar flagella per cell. The smooth colonial form of *B. pseudomallei* often appears as long parallel bundles of 'filaments' – really chains of closely associated rods (Miller et al. 1948a) embedded in interstitial substance. In the rugose form, the shorter, oval cells are more irregularly arranged and there is no interstitial substance. Staining is irregular and bipolar staining (safety-pin appearance) is seen, particularly of *B. pseudomallei* in films of pus; accumulation of poly-β-hydroxybutyrate may be demonstrated by staining with Sudan black.

B. pseudomallei grows well on simple media, including nutrient, blood, and MacConkey agars, but it does not grow on deoxycholate citrate or Salmonella–Shigella agars. After overnight incubation on nutrient agar at 37°C, the colonies are 1–2 mm in diameter. The growth may be smooth and mucoid or rough, with a dull wrinkled, corrugated, or honeycombed surface. In the smooth form, colonies are round, low convex, amorphous, translucent, and grayish-yellow, with a smooth, glistening surface and an entire edge; although mucoid in consistency, they are easily emulsified. After several days they become opaque, yellowish-brown, uneven, and often umbonate. Chambon and Fournier (1956) described numerous different colonial forms and Stanton et al. (1927) illustrated an ultra-rugose form, with an extremely corrugated surface and a tenacious consistency. Frankly mucoid forms are uncommon. No extracellular pigment is formed. The smooth colonial form of *B. pseudomallei* tends not only to die out rapidly in culture but also to kill other pseudomonads, including rough variants, in its vicinity; Rogul and Carr (1972) attributed this to the production of ammonia. The growth has a characteristic earthy or musty odor although sniffing of agar cultures is discouraged on safety grounds. α-Hemolysis is observed on sheep blood agar; older cultures produce β-hemolysis. Colonial morphology is best observed on Ashdown's medium which is a simple agar containing crystal violet, glycerol, and gentamicin (Ashdown 1979b), colonies concentrate the dye from the medium and grow rugose or smooth. This medium was recently modified by Howard and Inglis (2003) and contained less glycerol than the original; crystal violet, which is apparently inhibitory for some mucoid isolates, was replaced by Nile Blue. Typical colonies of *B. pseudomallei* are shown in Figure 1. Many strains grow poorly below 25 C°but all grow at 41°C. The species forms acid from various sugars in Hugh and Leifson's or ammonium-salts media, produce ammonia from arginine, and has extracellular hydrolytic enzymes that degrade poly-β-hydroxybutyrate

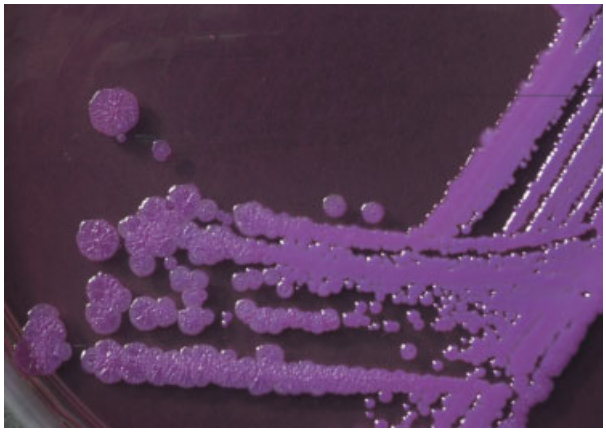

Figure 1 *Growth of* B. pseudomallei *on Ashdown's agar medium after 48 h incubation*

and starch. Gelatin and Tween 80 are hydrolyzed, it is usually potassium cyanide (KCN) resistant and does not attack malonate. The following characteristics of *B. pseudomallei* help to distinguish it from *B. cepacia*: production of ammonia from arginine, hydrolysis of starch, formation of heat-stable phosphatase, and negative lysine decarboxylase reaction. The patterns of acidification of sugars are rather similar. *B. cepacia* generally attacks salicin and adonitol, but *B. pseudomallei* does not. Atypical strains occur in both species and further confirmatory tests may occasionally be necessary.

Dance et al. (1989c) recommend a simple screening procedure for the confirmation of identity of *B. pseudomallei* for an oxidase positive, gram-negative rod showing bipolar or irregular staining. Organisms are tested for resistance to disks containing colistin 10 μg and gentamicin 10 μg and subcultured on Ashdown's medium and incubated for 48–72 h. The API 2ONE kit proved to be highly efficient for the confirmation of the identity of *B. pseudomallei* (Dance et al. 1989c), but false identification of some other pseudomonads as *B. pseudomallei* sometimes occurs. Wuthiekanun et al. (1990) evaluated Ashdown's medium with and without pre-enrichment in selective broth (containing colistin and crystal violet) for the isolation of *B. pseudomallei* from clinical specimens and concluded that enrichment improved the chances of recovery of the organism from sites with an extensive normal flora (see also Walsh and Wuthiekanun 1996). The inclusion of colistin (50 mg/l) to broth enrichments markedly improves the yield of *B. pseudomallei* from clinical specimens (Walsh et al. 1995c); the antibiotic should also be added to the basal salt medium containing L-threonine (Gallimand's medium) to optimize the recovery of the organism from soil (Wuthiekanun et al. 1995a).

Nonculture methods

Three rapid serological assays have been described for the identification of *B. pseudomallei* from agar culture. A specific and sensitive latex agglutination test with a rabbit polyclonal antibody was used by M.D. Smith et al. (1993). This was strongly reactive with the LPS antigen which is remarkably conserved within the species but which also exhibits extensive crossreaction with *B. mallei* (Pitt et al. 1992). Another latex agglutination test with an exopolysaccharide specific monoclonal antibody also proved to be highly specific and sensitive for the species (Steinmetz et al. 1999). Lastly, a monoclonal antibody bound to latex towards a 39 kDa *B. pseudomallei* outer membrane protein was able to detect approximately 5×10^6 CFU/ml in a direct agglutination assay with a sensitivity and specificity of 94 and 98 percent, respectively. This reagent also proved valuable for confirmation of the organism in blood cultures and potentially could speed up the diagnosis of melioidosis by 2 days (Pongsunk et al. 1999).

An oligonucleotide probe following partial sequencing of the 23S rRNA was constructed and used for the identification and detection of *B. pseudomallei* by hybridization or by direct PCR (Lew and Desmarchelier 1994). The probe hybridized with DNA from *B. mallei* but did not react with other *Pseudomonas* spp. A combination of the two methods allowed the detection of approximately 10^4 CFU/ml in spiked blood samples and sensitivity was increased 100-fold by concentration of bacteria from 0.5 ml of blood. Kunakorn and Markham (1995) described a seminested PCR which was used to generate products that were quantitatively detected by enzyme immunoassay. The target DNA sequence was the internal transcribed spacers between 16S and 23S rRNA. The primers did not amplify DNA from *B. mallei* and a sensitivity of 75 bacteria per ml was claimed. The 16S rRNA gene was targeted by Dharakul et al. (1996) for the design of a nested PCR system for the detection of *B. pseudomallei* in septicemic melioidosis. *B. mallei* was not included in specificity tests but no crossreactions were evident with other gram-negative species tested. As few as two bacteria were reported to be detectable by the assay and it could be used with equal efficiency for buffy coat or pus specimens. A *B. pseudomallei*-specific DNA probe sequence was utilized for the design of two 22-mer primers to amplify a 178 bp product from several clinical isolates. This PCR system detected as little as 0.5 fg of DNA which equated to a sensitivity in spiked whole blood of one bacterial cell per ml (Rattanathongkom et al. 1997). Sura et al. (1997) designed primer sets from random sequencing of *B. pseudomallei* DNA which amplified a specific 124 bp product from clinical strains. Using a combination of PCR and hybridization, the lower limit of detection was 35 CFU/ml in bacterial suspensions and approximately 400 CFU in liver tissue which was below the positive threshold for culture. There is some dispute nevertheless, regarding the performance of various PCR assays for diagnosis of melioidosis. Haase et al. (1998) found that the intergenic primer set (Kunakorn and Markham 1995), failed to detect *B. pseudomallei* in their collection of Australian isolates and the 23S rRNA primer set (Lew and Desmarchelier 1994) was insufficiently sensitive to detect the organism in clinical specimens that gave positive results by culture. In this group's laboratory, the 16S rRNA-derived primer set had a sensitivity approaching 100 percent for clinical samples and identified three culture-negative cases. However, samples from a third of patients with other diseases were also positive by this PCR. These findings are in marked contrast to those of Kunakorn et al. (2000) who reported the sensitivity and specificity of the 16S rRNA PCR to be only 41 and 47 percent with actual blood samples from patients diagnosed by blood culture. Some patients had been treated with antimicrobial drugs active against *B. pseudomallei* before the specimens for PCR were collected which would have reduced the number of

viable bacteria. An optimized protocol for rapid and specific detection of *B. pseudomallei* was described by Hagen et al. (2002). They used a seminested PCR assay targeting a genus-specific sequence of the ribosomal protein subunit (rpsU) 21 and a nested PCR to amplify the filament forming flagellin gene (*fliC*). The combination of PCR and sequencing of the amplicons resulted in high sensitivity and specificity in fixed tissue sections and avoided the need for culture of the organism.

Susceptibility to physical and chemical agents

B. pseudomallei is able to grow on 0.1 percent cetrimide agar and is resistant to various dyes. It survives for up to 30 months in moist clay soil (Thomas and Forbes-Faulkner 1981), but for a much shorter time in dry sandy soil. In experimental conditions, a water content in soil of <10 percent rendered the bacteria nonviable within 70 days, but at >40 percent they survived for 726 days (Tong et al. 1996). These studies also showed that the organism is killed within 18 days on nutrient medium at 0°C, at pH values below 5 and above 8, and is more susceptible to ultraviolet rays than permanent soil bacteria. It also has a remarkable capacity to survive in nutrient-free medium and can be recovered from triple-distilled water without any additional nutrient after more than 3 years (Wuthiekanun et al. 1995b).

Most strains of *B. pseudomallei* are susceptible to imipenem, piperacillin, doxycycline, amoxycillin–clavulanic acid, azlocillin, ceftazidime, and ticarcillin–clavulanic acid, with a minimum inhibitory concentration (MIC) of <2 mg/l; approximately 10 percent of strains exhibit resistance to chloramphenicol (Dance et al. 1989b). Of four carbapenems tested by Smith et al. (1996), biapenem was the most active but all carbapenems had bactericidal activity against strains resistant to ceftazidime, co-amoxiclav or both. Resistance to aminoglycosides, the polymyxins, and the penicillins is uniform.

B. pseudomallei is unusual among 'pseudomonads' in that intrinsic ampicillin resistance is mediated by a clavulanate-sensitive β-lactamase (Livermore et al. 1987); three different mechanisms of acquired β-lactam resistance have been described (Godfrey et al. 1991). Sequencing of the genome of *B. pseudomallei* strain K96243 has identified class A, C, and D β-lactamase genes but not a class B (metallo-)β-lactamase gene (www.sanger.ac.uk/Projects/B_pseudomallei/). The *B. pseudomallei* class A β-lactamase, which is susceptible to clavulanic acid inhibition, confers selective resistance against ceftazidime and is encoded by the *penA* gene. This gene is identical to that found in *B. mallei* (Tribuddharat et al. 2003). A class D β-lactamase gene from *B. pseudomallei* was cloned and sequenced and the enzyme shown to be an oxacillinase with homology to oxacillinases from *Ralstonia pickettii* and *Aeromonas* spp. (Niumsup and Wuthiekanun 2002). Interestingly, an environmental strain from a rice field possessed the class D β-lactamase gene suggesting that the enzyme may play an alternative role in cell physiology in addition to inactivating β-lactams.

Small colony variants (diameter of 1 mm or less) can be isolated following exposure of an isolate to ceftazidime, ciprofloxacin, or gentamicin, and these show a significant increase in the MICs of various unrelated classes of antimicrobials (Haußler et al. 1999). The significance of these variants in treatment failure and relapse of melioidosis remains to be determined.

A multidrug efflux system, AmrAB-OprA, which mediates resistance to aminoglycoside and macrolide antibiotics has been shown to be unique to *B. pseudomallei* (Moore et al. 1999). Using a well-defined transposon mutagenesis system two genes were identified, *amrA* and *amrB*, and inactivation of either of these resulted in enhanced susceptibility to these classes of antibiotics; the *amr* locus did not confer resistance to tetracyclines, chloramphenicol, fluoroquinolones, or β-lactams. *B. pseudomallei* also exhibits high levels of resistance to cationic antimicrobial peptides such as polylysine, protamine sulfate, human neutrophil peptides, and polymyxins (Eickhoff et al. 1970; Jones et al. 1996). Burtnick and Woods (1999) concluded that the architecture of the *B. pseudomallei* cell prevents binding of polymyxin with lipid A molecules in the LPS of the organism owing to the protective barrier of O-antigen and outer core LPS components.

B. THAILANDENSIS

Wuthiekanun et al. (1996) compared the biochemical characteristics of a large number of '*B. pseudomallei*' isolates from clinical and environmental sources. The clinical isolates were, in the main, uniform in their reactions but a number of the soil isolates were of a different phenotype. All of the latter had the ability to assimilate arabinose but failed to assimilate dulcitol or erythritol. This biotype, termed ara$^+$, was shown by Smith et al. (1997) to be nonvirulent compared with the highly virulent ara$^-$ clinical strains. Further evidence that the ara$^+$ phenotype was distinct from clinical *B. pseudomallei* was provided by ribotyping. Two groups of organisms were distinguished by their ribotype patterns. Ribotype group I consisted of both clinical and environmental strains and was ara$^-$, whereas ribotype group II strains were exclusively from the environment and were all ara$^+$ (Trakulsomboon et al. 1997). Sufficient differences in 16S rRNA encoding genes were found (Dharakul et al. 1999) to allow the development of a multiplex PCR assay to differentiate between the two biotypes but the differences were, however, not sufficient for classification into a new species. Nevertheless, further rRNA

sequence data resulted in the proposal of the new species *B. thailandensis* by Brett et al. (1998).

Genomics

Like *B. cepacia*, the genome of *B. pseudomallei* K96243 is relatively large compared with other bacteria at 7.24 Mb. It consists of two chromosomes of 4.07 and 3.17 Mb with a G + C content of 68 percent (Holden et al. 2004). It is rich in insertion sequences, some of which are found in *B. cepacia* (Mack and Titball 1998). Brown and Beacham (2000) used the technique of subtractive hybridization to compare the genomes of *B. pseudomallei* with *B. thailandensis* and found that of 43 subtracted clones only six were present in both genomes and four of these appeared to represent transposable genetic elements. Many of the sequences unique to *B. pseudomallei* were of unknown function and a proportion showed similarity to known virulence genes. The widely used transposon for insertional mutagenesis studies, Tn*5*-OT182, contains *bla* and *tetAR* genes encoding ampicillin resistance and tetracycline, respectively, and as the MIC of the latter for most isolates seldom exceeds 12.5 μg/ml, the use of 50 μg/ml tetracycline allows the selection of cells into which the transposon has inserted. Tn5-OT182 is described as a self-cloning, promoter probe transposon that integrates randomly into the *B. pseudomallei* chromosome (De Shazer et al. 1997; Woods et al. 1999). The transposon also has a ColE1 origin of replication allowing rapid cloning of flanking DNA and a promoterless *lacZ* at one end that facilitates the formation of transcriptional fusions when the transposon integrates downstream of a promoter. For optimal transformation by electroporation, kanamycin should be added to the growth medium; frequencies of 8×10^3 transformants are achievable compared with 10^2 transformants by chemical methods (Mack and Titball 1996).

Cell surface structures

CELLULAR LIPIDS

The cell wall lipids and fatty acid composition of *B. pseudomallei* were investigated by Phung et al. (1995a). They found, in a panel of environmental and clinical strains, that the major lipids are phosphatidylglycerol, two forms of phosphatidylethanolamine and two forms of ornithine-containing lipid. The total cellular fatty acids were characterized by the presence of 2-hydroxy-palmitic, hexadecanoic, octadecanoic, myristic, and 3-hydroxy palmitic acids. There was no significant difference between strains of human and environmental origins. A polar glycolipid containing glucitol and other unknown sugars with no hydroxy and 2-hydroxy fatty acids was used as an antigen in an ELISA for the diagnosis of melioidosis by the detection of antibody in patients' serum samples. Patients with melioidosis formed specific IgG antibodies to the antigen but a small number of controls were also positive with this antigen (Phung et al. 1995b).

OUTER MEMBRANE PROTEINS

Five major OMPs have been identified in *B. pseudomallei* of 70, 38, 31, 24, and 17 kDa and they were common to all strains tested by Gotoh et al. (1994). An oligomeric protein of 110 kDa migrates as a 38 kDa protein after heating at 95°C and probably acts as a porin. A 19.5 kDa antigen, which probably equates with the 17 kDa protein above, was shown to be specific for *B. pseudomallei* by Western blotting and potentially useful for the serodiagnosis of melioidosis (Anuntagool et al. 1993).

LIPOPOLYSACCHARIDES

B. pseudomallei is antigenically homogeneous due to conservation of the O-antigenic LPS (Pitt et al. 1992). Thus, polyclonal or monoclonal antibodies raised against whole cells or purified LPS of a strain will react with all strains, with rare exceptions. This common LPS antigen is largely specific to *B. pseudomallei* but some cross-reactions occur with *B. mallei* and, less so, with *B. cepacia*; nevertheless, the wide distribution of the LPS antigen makes this an ideal candidate for a vaccine and studies have demonstrated significant passive protection with anti-LPS antibodies (Bryan et al. 1994). Independent studies have shown that a single strain of *B. pseudomallei* produces two distinct partially *O*-acetylated S-type LPS molecules (Knirel et al. 1992; Perry et al. 1995). Type I O-PS is an unbranched homopolymer with the structure 3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1- and type II O-PS is an unbranched heteropolymer with repeating D-glucose and L-talose units with the structure →3)-β-D-glucopyranose-(1→3-6-deoxy-α-L-talopyranose-(1-2→. Part of the 6-deoxy-L-talose (6dTal*p*) is 2-*O*-methylated and 4-*O*-acetylated and part only 2-*O*-acetylated. These two O-antigens are present in approximately equal proportions in the LPS.

Isshiki et al. (2001) reported that the LPS of *B. pseudomallei* partitions preferentially into the phenol phase following hot phenol–water extraction. Using gel-permeation column chromatography they recovered three major fractions (I–III) from the phenol phase purified LPS; only fraction II gave a typical O-repeating unit ladder pattern in SDS-PAGE while the fraction III corresponded to LPS core elements. In keeping with this, the latter fraction induced the highest level of TNF in tissue cell lines, fraction II less so, and no activity was detected in fraction I. The authors concluded that fraction I was not an LPS constituent but a capsular polysaccharide of 1,3-linked 2-*O*-acetyl-6-deoxy-β-heptane.

The biological activity of *B. pseudomallei* LPS is similar to enterobacterial LPS, but comparative tests showed that the former exhibits weaker pyrogenic

activity in rabbits, is less toxic for galactosamine sensitized mice, and is a weaker activator of murine macrophages than enterobacterial LPS. However, *B. pseudomallei* LPS exhibited stronger mitogenic activity to murine splenocytes even of LPS resistant mice (Matsuura et al. 1996).

A cluster of 15 genes appears to be linked to O-PSII production and the *wbiA* locus is required for the *O*-acetylation of the L-6dTal*p* residues which constitutes an antibody recognition site (DeShazer et al. 1998; Brett et al. 2003). Serum-sensitive mutants generated by transposon mutagenesis lacked the O-PSII polysaccharide and this moiety was therefore necessary for serum resistance and virulence of the organism (DeShazer et al. 1998). IgG antibody responses of patients with melioidosis to the two polysaccharides confirmed that only the level of antibody to O-PSII was significantly higher in patients who survived than in those who died, indicating that this response was protective and that O-PSII would be a potentially useful component of a vaccine against the infection (Charuchaimontri et al. 1999). Ho et al. (1997) examined the O-PS specificity and functional activity of a protective polyclonal antiserum, recognizing O-PSI and O-PSII, and an IgM monoclonal antibody specific for O-PSII. Both antibodies mediated phagocytic killing by polymorphonuclear leukocytes. Patients with acute melioidosis formed antibodies to both O-PS, but asymptomatic patients who were seropositive by the indirect hemagglutination test using sonicated whole cells as antigen were negative for these anti O-PS antibodies. O-PSII is also expressed by *B. thailandensis* and by *B. mallei*, but in the latter species it lacks acetyl modifications at the *O*-4 position of the L-6dTal*p* residues (Burtnick et al. 2002).

EXOPOLYSACCHARIDES

Steinmetz et al. (1995) described the characterization of a species-specific exopolysaccharide (EPS) of *B. pseudomallei*. They isolated a monoclonal antibody, following immunization of a mouse with a mucoid strain, which showed specificity in immunoblots for a carbohydrate antigen (molecular weight (MW) >150 kDa) and was poorly resolved by SDS-PAGE. The antigen had a capsule-like appearance by electron microscopy and all mucoid and nonmucoid strains tested reacted with the antibody in ELISA. Crossreactivity was found only with *B. mallei* but no LPS was detected in the purified exopolysaccharide. Structurally, the polysaccharide was a linear tetrasaccharide consisting of three galactose residues and one Kdo residue (Nimtz et al. 1997); this composition was confirmed with a different strain of *B. pseudomallei* by Masoud et al. (1997). Kawahara et al. (1998) found that when the species was grown in the absence of glycerol, two polysaccharides were produced, one of which was mainly 1,4-linked glucan and the other was composed of galactose and Kdo. However, when the organism was grown in glycerol, a different and novel polysaccharide compound containing galactose, rhamnose, glucose, and a uronic acid was synthesized. The capsule is readily observed by transmission electron microscopy of ruthenium red-stained cells. Three distinct forms were detected by Puthucheary et al. (1996) in cultures taken from human blood agar; a macrocapsular form seen as a fibrogranular structure of medium density on the outside of the cell wall approximately 0.1–0.25 μm; a microcapsulate form with a compact granular layer of <0.1 μm and a noncapsulate form lacking electron dense material.

B. thailandensis lacks the tetrasaccharide EPS and this difference has been exploited as the basis for a rapid test to differentiate it from *B. pseudomallei*. Anti-LPS antibodies react with both species while the anti-EPS antibody reacts only with *B. pseudomallei* (Wuthiekanun et al. 2002).

The role of EPS as a virulence factor was elegantly confirmed by the work of Reckseidler et al. (2001) who used the technique of subtractive hybridization to identify virulence determinants in the genome of *B. pseudomallei*. One of the subtractive clones encoded a protein with homology to a glycosyltransferase from *P. aeruginosa* and insertional inactivation of this gene in the wildtype strain resulted in an acapsular mutant with an LD_{50} of 3.5×10^5 CFU compared with an LD_{50} of <10 CFU for the wildtype. A similar finding was reported by Atkins et al. (2002a) who isolated a mutant by signature tagged mutagenesis which was attenuated in a mouse model of melioidosis. A plasmid that had been modified to incorporate one of 96 tags was transformed into a wild-type strain by electroporation. The transposon inserted into the same gene cluster identified by Reckseidler et al. (2001) and the resulting mutant demonstrated a 10^5-fold increase in median lethal dose and did not react with an antibody to EPS. Monoclonal antibodies to EPS were shown to be significantly more protective in passive immunization studies than antibodies to surface proteins and LPS (Jones et al. 2002).

FLAGELLA

The flagellar antigens of *B. pseudomallei* are serologically homogeneous; a polyclonal serum prepared against purified flagella of a reference strain reacted with all but one of 65 strains tested by Brett et al. (1994). Purified monomer flagellin proteins were of MW 43.4 kDa and NH_2-terminal amino acid sequence analysis of the protein showed significant homology with flagellins from *Proteus mirabilis*, *Bordetella bronchiseptica*, and *P. aeruginosa*. A flagellin-specific antiserum also conferred protection to infection with live organisms in an experimental model. Using motility mutants selected by transposon mutagenesis, DeShazer et al. (1997) concluded that the genetic basis of flagellar synthesis and operation in *B. pseudomallei* was similar to that found in other

organisms as many of the genes showed homology to flagellar and chemotaxis proteins in *E. coli* and *Salmonella typhimurium*. No significant difference was found in the animal virulence of a flagellar mutant and the wild-type strain suggesting that flagella and/or motility are probably not virulence determinants in the model used. However, an isogenic mutant with a deletion in *fliC* (aflagellate and nonmotile) was not impaired in its ability to invade and replicate in cultured human lung cells compared with the wildtype, but it was avirulent for intranasal infection of BALB/c mice (Chua et al. 2003). Further evidence of a virulence role for the flagella of *B. pseudomallei* was provided by the demonstration that the organism tethers itself to the external surface of *Acanthamoeba*; a non-flagellate isogenic mutant did not demonstrate flagellum-mediated endocytosis in contrast to the wildtype strain (Inglis et al. 2003).

A conjugate of purified flagellin and O-polysaccharide from the same strain was prepared by Brett and Woods (1996) to exploit the antigenic conservation of both antigens in the species in order to maximize production of protective antibody in the vaccine recipient. Adipic acid dihydrazide was used as the linker molecule and this elicited high titer IgG responses to both components in rabbits; these antibodies were also protective of mice challenged with both the vaccine strain and a heterologous strain.

Typing of *B. pseudomallei*

PHAGES

Thirty bacteriophages active on *B. pseudomallei* were isolated from water samples around Hanoi, Vietnam, by Leclerc and Sureau (1956) and their lytic reactions indicated the presence of two different strains in the area. Several temperate phages from *B. pseudomallei* will lyse *B. mallei* but not other pseudomonads (Smith and Cherry 1957; Manzenyuk et al. 1994). Other cross-reacting phages described include a temperate phage from *B. thailandensis* that formed plaques on *B. mallei* but not on *B. pseudomallei* or other *B. thailandensis* strains (Woods et al. 2002), and a transducing phage from *B. cepacia* which lysed 13 of 40 *B. pseudomallei* strains tested (Langley et al. 2003).

CHROMOSOMAL DNA ANALYSIS

Ribotyping was first used by Lew and Desmarchelier (1993) to group strains of *B. pseudomallei*. Using the restriction enzyme *Bam*HI, they classified 22 different groups among 100 isolates from various animal, human, and environmental sources. Twenty-nine isolates from different sources had the same ribotype pattern and thus the system defined groups rather than distinct strains. This was confirmed by Currie et al. (1994) who found strains of the same ribotype isolated over a period of 25 years in temperate south-western Australia. These strains were subsequently shown to be heterogeneous by random amplified polymorphic DNA (RAPD) analysis (Haase et al. 1995). RAPD was combined with multilocus enzyme electrophoresis to examine isolates of *B. pseudomallei* from different Australian communities (Norton et al. 1998) and revealed two broad groups that appeared to be linked to disease presentation. One group consisted mainly of patients with respiratory infection, whereas the second group was more varied in presentation. Ribotyping was also utilized to determine the relationship of primary and relapse isolates of *B. pseudomallei* from patients with recurring infection after clinical resolution of their primary illness and showed that relapse was most often due to infection from an endogenous source with the same strain as that isolated from the original infection (Desmarchelier et al. 1993). *Eco*RI was preferred by Sexton et al. (1993) to distinguish a single ribotype group of strains of *B. pseudomallei* which were then further divided by hybridization patterns following *Sal*I, *Hin*dIII, and *Pst*I restriction endonuclease digestion. A survey of 350 isolates of *B. pseudomallei* recovered over 71 years from 23 countries revealed 44 *Bam*HI patterns comprised of three to eight hybridization bands accounting for 15 band positions in a size range of 3–28 kb (Pitt et al. 2000). Two ribotypes together accounted for almost half of the collection and one of these was statistically associated with an Asian origin. A combination of ribotyping with *Xba*I DNA macrorestriction digests resolved by PFGE revealed a number of different strains within a predominant ribotype among Malaysian strains of *B. pseudomallei* suggesting that infection was acquired from multiple sources (Vadivelu et al. 1997).

A DNA probe which could be used to detect and type *B. pseudomallei* was advocated by Sermswan et al. (1994). This consisted of a 1.5 kb cloned chromosomal DNA fragment, a radioactively labeled insert which could detect 4×10^4 bacterial cells. Southern blots following *Hin*dIII restriction revealed at least eight groups among 60 clinical isolates.

The DNA pattern methods above are not well suited to interlaboratory comparisons and so multilocus sequence typing (MLST), which indexes variation within fragments of seven housekeeping genes, was applied to a large collection of human, animal, and environmental geographically disparate strains (Godoy et al. 2003). They identified 71 sequence types among 128 *B. pseudomallei* strains and the number of alleles per locus varied from four to 15 (average 8.6) projecting a potential 3.4 million different allelic profiles within the species. All *B. pseudomallei* isolates were grouped together and were clearly resolved from *B. thailandensis*. The five isolates of *B. mallei* tested were identical and clustered with the *B. pseudomallei* isolates; alleles of six of the seven loci in *B. mallei* were present in *B. pseudomallei* indicating that the former is a clone of the latter. The study

concluded that on population genetic grounds, *B. mallei* should not be given separate species status.

Pathogenicity

TOXIC PRODUCTS

The role of toxic products in the pathogenesis of melioidosis is unclear. Liu (1957) showed that *B. pseudomallei* produced a lethal and dermonecrotic toxin and Heckly and Nigg (1958) separated two heat-labile toxic constituents, both of which were lethal for mice when injected intraperitoneally but only one of which was dermonecrotic. Proteolytic and lecithinase activity has been demonstrated in toxic fractions. Ismail et al. (1987) developed an ELISA for the detection of antibody to *B. pseudomallei* exotoxin in mice. They isolated a protein of approximately 31 kDa from a broth culture that had been incubated for 7 days and raised a specific monoclonal antibody to it in mice. The protein was a potent inhibitor of DNA and protein synthesis in cultured macrophages (Mohamed et al. 1989). A low molecular weight, ~3 kDa, cytolethal toxin was described by Haase et al. (1997) from culture filtrates of isolates of *B. pseudomallei* from different sources. The purified toxin lost approximately 60 percent of activity after pronase treatment and was relatively heat stable. It was toxic for McCoy cells and cytopathic effects were visible after exposure for 1 h. There was some correlation of cytotoxic activity with source of isolates with soil isolates being the least toxic and those from patients with melioidosis encephalitis the most active. It is unclear whether these toxins (31 and 3 kDa) represent those described earlier by Heckly and Nigg (1958). A highly purified glycolipid (acidic rhamnolipid) of 762 Da was identified in heated culture filtrates of *B. pseudomallei*. The rhamnolipid is composed of two molecules of rhamnose and two molecules of β-hydroxytetradecanoic acid (Häußler et al. 1998). It exhibited toxicity on various cell lines and lysed mouse, sheep, and human erythrocytes.

EXTRACELLULAR ENZYMES AND SIDEROPHORES

An extracellular protease of approximately 36 kDa was shown to be a metalloenzyme, requiring iron for maximal activity, which was inhibited by ethylenediaminetetraacetic acid (EDTA). The protease was active against various substrates, including immunoglobulins, but was weakly elastolytic. Antibodies to *P. aeruginosa* alkaline protease crossreacted with the *B. pseudomallei* protease (Sexton et al. 1994). A type II secretion gene cluster which controls the secretion of protease, lipase, and phospholipase C was identified by DeShazer et al. (1999) and two genes were found to be necessary for the maximal secretion of these enzymes. There was no correlation between the level of protease activity and the virulence of *B. pseudomallei* for mice injected intraperitoneally (Gauthier et al. 2000). Winstanley et al. (1999) identified a cluster of genes in *B. pseudomallei* homologous to, and in the same order as, a group of genes in *Ralstonia solanacearum* which in the latter are located in a type III secretion-associated pathogenicity island. These genes were present only in the ara^- phenotype and absent from ara^+ strains suggesting they have a role in virulence (Winstanley and Hart 2000).

A survey of 100 clinical isolates of *B. pseudomallei* showed that 91 percent produced lecithinase, lipase, and protease, but none was positive for elastase; 93 percent formed a hemolysin (Ashdown and Koehler 1990). *B. pseudomallei* also produces an acid phosphatase enzyme that may play a role in intracellular survival (Dejsirilert et al. 1989). The acid phosphatase can be readily isolated from both whole cells and culture supernatant and the cell-associated enzyme appears to serve as a receptor for insulin (Kondo et al. 1996). The gene encoding acid phosphatase, *acpA*, was sequenced in *B. pseudomallei* and *B. thailandensis*. PCR primers designed on the *B. pseudomallei acpA* gene were used to amplify an *acpA* homolog from *B. mallei* and differences of 15 and three amino acids in predicted amino acid sequences of the AcpA protein in *B. thailandensis* and *B. mallei* were found (Burtnick et al. 2001).

B. pseudomallei obtains iron in serum and cells through a hydroxamate siderophore, malleobactin, which is optimally produced under iron-deficient conditions (Yang et al. 1991). The siderophore sequesters iron from transferrin and lactoferrin at neutral and acid pH and appears to acquire iron more effectively from these sources than from the host cell (Yang et al. 1993).

ENTRY AND SURVIVAL IN CELLS

The adherence of *B. pseudomallei* to mammalian cells has not been extensively explored but attachment of the organism to fresh pharyngeal epithelial cells has been documented (Ahmed et al. 1999). Attachment appeared from electron microscopic studies to be mediated by a thin electron dense layer which was thought to be capsular polysaccharide; fimbriae were not observed. A specific receptor–ligand system was reported by Kanai et al. (1997). Using thin layer chromatography they demonstrated specific binding of *B. pseudomallei* acid phosphatase to the tissue glycolipids gangliotetraosylceramide (asialo GM1) and gangliotriaosylceramide (asialo GM2). Furthermore, they showed that the acid phosphatase is most probably a protein tyrosine phosphatase enzyme which plays a significant role in a two component signal transfer mechanism.

Several features of melioidosis suggest that *B. pseudomallei* may occupy an intracellular niche. These include the observation of structurally intact bacteria within macrophages in experimental melioidosis (Narita et al. 1982), an augmented cellular immune response (Brown

et al. 1990), and the demonstration of intracellular survival and growth (Pruksachartvuthi et al. 1990; Egan and Gordon 1996). A three-fold $\log_{10}$ increase in bacterial cell numbers over a period of 21 h was observed by Pruksachartvuthi et al. (1990) and the phagocytes remained viable and retained their capacity to produce an oxidative burst for the first hour of incubation. The occurrence of latency and the tendency for melioidosis patients to relapse are also consistent with the organism surviving intracellularly. Jones et al. (1996) showed that *B. pseudomallei* has the capacity to invade HeLa, CHO, A549, and Vero cultured cells. The organisms survived in rat alveolar macrophages and were localized inside membrane-bound vacuoles in human monocyte-like cells. They were also resistant to host antimicrobial peptides. In a subsequent study, mutants deficient in the ability to invade A459 cells were generated by transposon mutagensis and two open reading frames were identified. The predicted proteins shared considerable homology with a two-component regulatory system involved in the regulation of heavy metal resistance in other organisms and these were linked to Cd^{2+} and Zn^{2+} resistance in the wild-type strain (Jones et al. 1997). Both the mutant and wild-type exhibited similar virulence for animals suggesting that this property was not linked to virulence. Harley et al. (1998) explored intracellular survival of the species further by studying the uptake of a test strain by fresh mouse peritoneal macrophages and cultured cells by electron microscopy. They found that *B. pseudomallei* appeared initially in phagosomes with intact membranes, but fusion with lysosomes was not observed. Loss of the membrane occurred rapidly and the organisms escaped into the cytoplasm.

The presence of multinucleated giant cells has been reported in the tissues of patients with melioidosis (Wong et al. 1995); these cells are apparently formed by fusion of single cells. This was investigated by Kespichayawattana et al. (2000) who showed that when phagocytic and nonphagocytic cells were labeled with two different markers, mixed, and then infected with *B. pseudomallei*, direct cell-to-cell fusion occurred leading to giant cell formation. Staining of the infected cells with rhodamine-conjugated phalloidin revealed actin rearrangement into a comet tail appearance and this led to the formation of actin associated membrane protrusions containing bacteria which led to the death of the cell through apoptosis. Subsequently, Breitbach et al. (2003) showed that the mechanism of induction of actin polymerization by *B. pseudomallei* differs substantially from those evolved in other intracellular pathogens such as those in the genera *Listeria* and *Shigella*.

The novel approach of co-culture of *B. pseudomallei* with *Acanthamoeba* spp. has given clearer insights into the mechanisms of entry of the organism into a host cell (Inglis et al. 2000a). Bacterial adhesion to the trophozoite surface and rapid bacillary rotation were followed by endocytosis and the appearance of vacuoles containing single bacilli, then multibacillary vacuoles, and finally formation of external bacillary tufts; intracellular bacteria remained motile for up to 72 h, a feature which might be indicative of a symbiotic relationship in the natural environment.

INDUCTION OF CYTOKINES AND EXPERIMENTAL INFECTION

B. pseudomallei is a potent activator of complement. Activation occurs rapidly regardless of the presence of specific antibody, and in the absence of the latter the alternative pathway of complement activation is predominant (Egan and Gordon 1996). These workers further showed that phagocytosis was significantly greater if the bacteria were opsonized with fresh rather than inactivated human serum indicating that complement is involved in the process. Oxidative metabolites were produced by phagocytes only when challenged with opsonized bacteria. A strong dose-dependent inhibition of intracellular growth of *B. pseudomallei* was observed with interferon (IFN)-γ-activated macrophages (Miyagi et al. 1997). The induction of bactericidal activity correlated well with the production of nitric oxide (NO) by these cells and NO was suppressed in the presence of a specific competitive inhibitor of NO synthesis. Superoxide dismutase and catalase significantly inhibited macrophage activity and the bacteria were susceptible to the killing effects of chemically generated NO and superoxide anion in a macrophage-free system. These results suggest that IFN-γ-induced macrophage activity against *B. pseudomallei* is mediated to a large extent by reactive nitrogen intermediates and less so by reactive oxygen-dependent killing mechanisms.

In an experimental mouse model, resistance to acute infection with *B. pseudomallei* was completely dependent on the production of IFN-γ in vivo as administration of neutralizing antibody towards IFN-γ lowered the 50 percent lethal dose from $>5 \times 10^5$ to approximately 2 CFU in a mouse model (Santanirand et al. 1999). This was associated with a marked increase in bacterial numbers in the liver and spleen of these animals. Neutralization of either TNF-α or IL-12, but not granulocyte–macrophage colony-stimulating factor, also increased susceptibility to infection.

Independent studies by Leakey et al. (1998) and Hoppe et al. (1999) both showed that C57BL/6 mice (C57BI/6 in Leakey's paper) were more resistant to infection by *B. pseudomallei* than BALB/c mice. The latter exhibited an acute course of melioidosis while the former appeared to mimic the chronic form of the disease. Following intravenous injection, BALB/c mice had a progressive bacteremia leading to death within 96 h while C57BL/6 mice typically remained asymptomatic for up to 6 weeks. However, the course of infection was highly dependent on the infective dose and the bacterial strain used (Hoppe et al. 1999). In comparison with BALB/c mice, the bacterial levels in C57BL/6

mice were decreased 12 h after infection, which suggests that an innate immune mechanism towards *B. pseudomallei* early in the infection contributed to the resistance of these mice. They also had a significantly higher IgG2a/IgG1 ratio that might be indicative of a T helper type 1 immune response. Attenuation of the virulence of *B. pseudomallei* for BALB/c mice was achieved through the selection of a mutant auxotrophic for branched chain amino acids by transposon mutagenesis causing disruption of the *ilvI* gene encoding the large subunit of the acetolactate synthase enzyme (Atkins et al. 2002b). Mice inoculated intraperitoneally with the auxotroph were protected against challenge with the wild-type strain.

A diabetic rat model, induced by injection of the animal with streptozotocin, was used by Woods et al. (1993) on the basis that melioidosis in humans is significantly associated with pre-existing diabetes mellitus. They showed that insulin inhibited the growth of *B. pseudomallei*, in vivo and in vitro, and the organism grew at an increased rate in sera from diabetic rats; the addition of human recombinant insulin, recovered the growth rate to levels similar to controls. Isotope studies showed that *B. pseudomallei* possessed a specific high-affinity binding site for human insulin. Diabetic rats were also significantly more susceptible to septicemia following challenge with *B. pseudomallei* than controls. However, doubt was cast on the validity of these results when it was demonstrated by Simpson and Wuthiekanun (2000) that growth of *B. pseudomallei* was inhibited by m-cresol at concentrations found in pharmaceutical insulin preparations; they concluded that the previous findings may have been confounded by the presence of the preservative m-cresol.

B. pseudomallei and *B. thailandensis*, but not *B. mallei*, kills the nematode *Caenorhabditis elegans*, apparently by an active process. Pathogenicity for the nematode did not always correlate with animal pathogenicity and killing did not involve LPS, aminoglycoside/macrolide efflux pumping, type II pathway secreted exoenzymes or motility (O'Quinn et al. 2001).

MELIOIDOSIS

Clinical manifestations

The clinical spectrum of *B. pseudomallei* infection is extremely broad, analogous to the range of disease caused by *Staphylococcus aureus*, for example. Melioidosis has been referred to as 'the remarkable imitator' (Poe et al. 1971). The majority of infections appear to be subclinical, although melioidosis may run a fulminant, rapidly fatal course, particularly in the immunocompromised. Several attempts have been made to develop a clinical classification of melioidosis, but none is entirely satisfactory. Infections may be acute or chronic, localized or disseminated, but one form of the disease may progress to another and individual patients are often difficult to categorize. Several reviews have summarized the clinical manifestations of melioidosis (Howe et al. 1971; Leelarasamee and Bovornkitti 1989; Punyagupta 1989; Dance 1990; White 2003).

MILD AND SUBCLINICAL INFECTIONS

In endemic areas such as north east Thailand, antibodies to *B. pseudomallei* are found in approximately 80 percent of children by the time they are 4 years old (Kanaphun et al. 1993), so the majority of infections are presumably mild or asymptomatic. A flu-like illness associated with seroconversion has been reported from Australia (Ashdown et al. 1989).

LATENT INFECTIONS

In people who have resided in endemic areas for a limited time, the infection may remain latent for over 20 years before becoming clinically apparent (Chodimella et al. 1997), giving rise to the nickname 'Vietnamese time bomb' (Goshorn 1987). Relapses usually occur at times of intercurrent stress (e.g. other acute infections, burns or trauma, malignancies, diabetes mellitus). The sites and mechanisms of persistence are unknown, although clinically silent, chronic, localized foci of melioidosis in the lung, liver, or spleen have been reported in animals, and intracellular survival may play a part. The proportion of seropositive individuals who harbor latent infection, and are therefore at risk of relapse, is also uncertain, although Currie et al. (2000a) found evidence of recrudescence in only 3 percent of prospectively studied cases.

CLINICAL DISEASE

Only a small proportion of *B. pseudomallei* infections are sufficiently severe to come to medical attention. The literature is full of individual case reports and case series, of which several may be of interest to readers (Whitmore 1913; Stanton and Fletcher 1932; Everett and Nelson 1975; Rode and Webling 1981; Guard et al. 1984; Chaowagul et al. 1989; Punyagupta 1989; Vatcharapreechasakul et al. 1992; Puthucheary et al. 1992; Kosuwon et al. 1993; Lumbiganon and Viengnondha 1995; Currie et al. 2000b). Common clinical pictures are summarized below.

SEPTICEMIC MELIOIDOSIS

Forty-five to sixty percent of cases of culture-positive melioidosis have positive blood cultures (Suputtamongkol et al. 1994a; Currie et al. 2000b). The majority of these present clinically as community-acquired 'sepsis syndrome,' with a short history (median 6 days; range 1 day to 2 months) of high fever and rigors (Chaowagul et al. 1989). Only half have evidence of a primary focus of infection, usually in the lung or skin and subcutaneous tissues. Confusion and stupor, jaundice, and diarrhea may also be prominent features, as may acute respiratory distress syndrome (Puthucheary et al. 2001).

Initial investigations usually reveal anemia, a neutrophil leukocytosis, coagulopathy, and evidence of renal and hepatic impairment. Such patients often deteriorate rapidly, developing widespread metastatic abscesses, particularly in the lungs, liver, and spleen, and metabolic acidosis with Kussmaul's breathing. Once septic shock has supervened, the mortality approaches 95 percent, many patients dying within 48 h of hospital admission. Other poor prognostic features include absence of fever, leukopenia, azotemia, and abnormal liver function tests (Chaowagul et al. 1989).

If the patient survives this acute phase, the manifestations of the multiple septic foci which result from bacteremic dissemination become prominent. Any site or tissue may be involved, but the most common foci are in the lungs, liver and spleen, skin and soft tissues, and prostate. An abnormal chest X-ray is found in 60–80 percent of patients, the most common pattern being widespread nodular shadowing (Figure 2) (Dhiensiri et al. 1988; Chaowagul et al. 1989; Puthucheary et al. 1992). Liver and splenic abscesses are usually multiple (Figure 3) (Vatcharapreechasakul et al. 1992). Cutaneous pustules or subcutaneous abscesses occur in 10–20 percent of cases (Chaowagul et al. 1989). Other common sites for secondary lesions include the kidneys, bones, and joints. Involvement of the central nervous system may comprise cerebral abscesses, or 'neurological melioidosis,' characterized by peripheral motor weakness, brain-stem encephalitis, aseptic meningitis, and respiratory failure, which probably reflects direct invasion of the central nervous system rather than being toxin mediated (Woods et al. 1992; Currie et al. 2000b).

B. pseudomallei bacteremia may also cause a less fulminant disease. Such patients usually have a swinging fever, often associated with profound weight loss. This presumably reflects overspill from occult or apparent localized foci of infection. Patients with multiple, noncontiguous foci of infection, which probably reflect bacteremia at some stage, behave similarly.

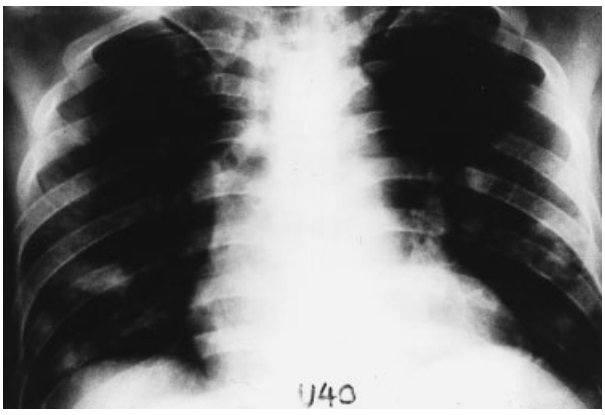

Figure 2 *Chest X-ray showing widespread nodular opacities ('blood-borne pneumonia') in septicemic melioidosis. Reproduced with permission of W.B. Saunders*

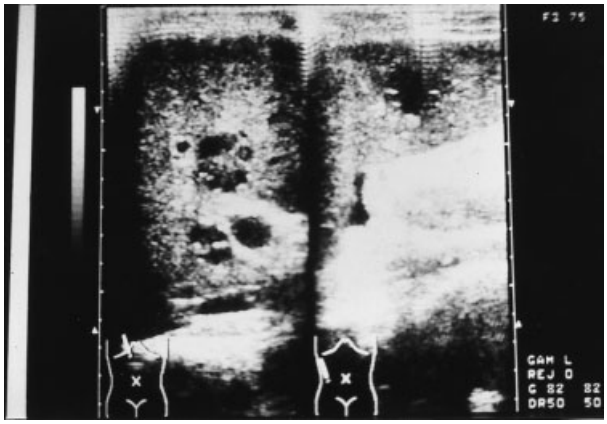

Figure 3 *Abdominal ultrasound showing multiple echo-lucent areas (abscesses) in the liver in septicemic melioidosis. Reproduced with permission of The Medicine Group (Journals) Ltd*

LOCALIZED MELIOIDOSIS

Localized melioidosis occurs most frequently in the lung. This may manifest as acute bronchitis or pneumonia, but most often as a subacute cavitating pneumonia accompanied by profound weight loss, which is often confused with tuberculosis or lung abscess (Everett and Nelson 1975) (Figure 4). Relative sparing of the apices and the infrequency of hilar adenopathy may help to distinguish melioidosis from tuberculosis (Dhiensiri et al. 1988). Any lung zone may be affected, although there is

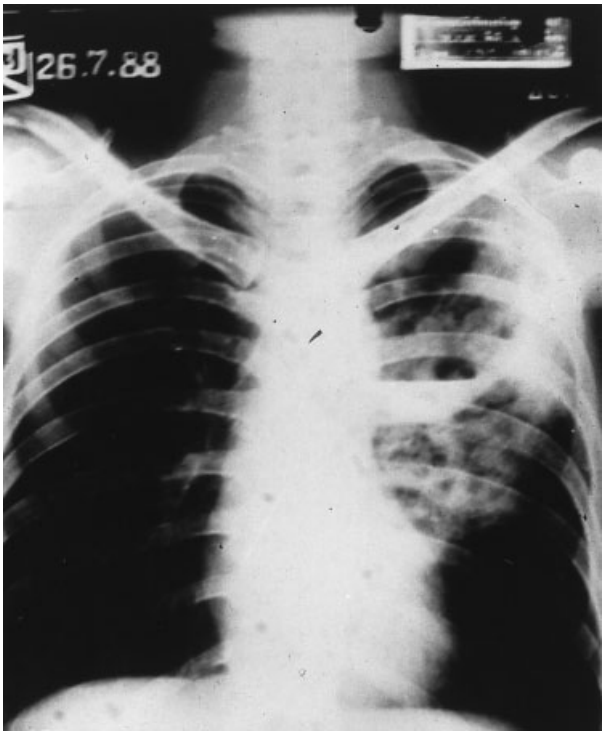

Figure 4 *Chest X-ray in pulmonary melioidosis showing a large area of consolidation with cavitation and an air/fluid level. Reproduced with the permission of the editor, PHLS Microbiology Digest*

Figure 5 *Acute suppurative parotitis with abscess formation, a common manifestation of melioidosis in children. Reproduced with permission of The Medicine Group (Journals) Ltd*

a predilection for the upper lobes. Complications include pneumothorax, empyema, and purulent pericarditis, and ultimately progression to septicemia.

Acute suppurative parotitis is a characteristic manifestation of melioidosis in children, accounting for approximately one third of pediatric cases in north east Thailand (Figure 5) (Dance et al. 1989a). The reason for this strong age–site association is unclear. Most cases are unilateral and result in parotid abscesses which require surgical drainage, although they may rupture spontaneously into the auditory canal. Facial nerve palsy and septicemia are rare complications.

Localized *P. pseudomallei* infection without bacteremia may affect any other organ. Well-described examples include cutaneous and subcutaneous abscesses, lymphadenitis, osteomyelitis, septic arthritis, liver and/or splenic abscesses, cystitis, pyelonephritis, prostatic abscesses, epididymo-orchitis, keratitis, and brain abscesses.

Epidemiology of melioidosis

GEOGRAPHICAL DISTRIBUTION

The main areas of melioidosis endemicity are South East Asia and northern Australia. Following the early work by Whitmore in Burma and Stanton and Fletcher in the Federated Malay States (now Malaysia), further cases were observed in the French and Dutch colonies in South East Asia (now Vietnam, Cambodia, and Indonesia) prior to the Second World War. More cases, possibly 2 000–5 000/year, occur in Thailand than any other country in the region. Since 1949, melioidosis has been recognized as being endemic in northern Australia (Rode and Webling 1981; Guard et al. 1984). It has been proposed that the organism was only introduced to Australia by troops returning from endemic areas at the end of the war (Fournier 1965), although this remains controversial. In recent years it has become clear that the disease is also prevalent elsewhere in south and east Asia, including China and Taiwan, Singapore, and the Indian subcontinent (Dance 1991, 2000a), which has been the source of infection for several recent cases diagnosed in Britain (Dance et al. 1999). Sporadic cases have also been reported from the Pacific Islands, central Africa, and latterly Central and South America and the Caribbean (Dance 1991, 2000a). The disease is probably under-diagnosed in many of these regions, since relatively sophisticated laboratory facilities are necessary to confirm the diagnosis. Furthermore, reports of cases in Iran and an epizootic which occurred in France during the 1970s (Mollaret 1988) have indicated that the disease is not confined to the tropics.

RESERVOIRS AND TRANSMISSION

Stanton and Fletcher (1932) were the first to identify melioidosis in animals. They concluded that the disease was a zoonosis with a reservoir in rodents. However, workers in French Indochina, noticing that the disease was sometimes acquired after contact with muddy water and that wild rats were rarely infected, later proved that *B. pseudomallei* was really an environmental saprophyte, readily isolated from soil and surface water in endemic areas (Chambon 1955). The organism is particularly associated with rice paddy (Strauss et al. 1969), and using selective media it may be recovered from 68–78 percent of rice fields in Thailand (Nachiangmai et al. 1985; Wuthiekanun et al. 1995a). The ecology of *B. pseudomallei* is poorly understood. It is thought to persist in deeper clay layers during the dry season, rising to the surface after the annual rains (Thomas et al. 1979). Its presence in greater numbers in the soil of some areas (Smith et al. 1995b), which presumably depends on a range of chemical (e.g. pH), biological, and environmental factors (Dance 2000b; Inglis et al. 2001), may affect the incidence of melioidosis in these areas.

Man and animals are thought usually to become infected by inoculation or contamination of wounds or mucosae with soil or surface water, although a specific episode of exposure is only identified in 6–25 percent of cases (Suputtamongkol et al. 1994a; Currie et al. 2000b). Aerosols of dust were thought to be a source of infection for helicopter crews during the Vietnam war (Howe et al. 1971), and two recent outbreaks in Australia have been associated with contamination of potable water supplies, although the precise mode of infection in these clusters was unclear (Inglis et al. 2000b; Currie et al. 2001). There is increasing evidence that inhalation of aerosols during heavy monsoons has previously been

underestimated as a source of infection (Currie and Jacups 2003). The role of ingestion and insect vectors in natural transmission is doubtful. Although melioidosis has been observed in a wide range of animal species (including rodents, primates, sheep and goats, pigs, cattle, horses, deer, dogs and cats, dolphins, koalas, kangaroos, camels, crocodiles, and birds), transmission from animals to humans has rarely been reported, and person-to-person spread is very uncommon (Kunakorn et al. 1991a; Abbink et al. 2001). Iatrogenic infection from contaminated injections has also been reported occasionally, as have laboratory-acquired infections, resulting in the classification of *B. pseudomallei* as a containment level 3 pathogen.

DESCRIPTIVE EPIDEMIOLOGY

Melioidosis is a disease of people in regular contact with soil and water, such as rice farmers in south east Asia and aboriginals in Australia. Twenty to 50 human cases are diagnosed annually in Australia, whilst several thousand are thought to occur each year in Thailand. Suputtamongkol and colleagues (1994a) reviewed the epidemiology of culture-positive melioidosis seen over a 5-year period in Ubon Ratchatani, north-east Thailand, where melioidosis accounts for 18 percent of community-acquired septicemia (Chaowagul et al. 1989). The average annual incidence was 4.4/100 000 population, with evidence of space–time clustering. All age groups were affected, with the peak incidence occurring from 40–60 years. The male:female ratio was 3:2, and in males aged 60–69 years, the annual incidence approached 30/100 000. As observed elsewhere, the disease was markedly seasonal, 75 percent of cases presenting during the rainy season. Although this pattern suggests that most cases are recently acquired, the incubation period before the onset of clinically apparent melioidosis has varied from 2 days to 26 years, and it is possible that some other seasonal factor precipitates relapses of latent infections. Other risk factors confirmed in this study were occupational exposure to soil and water (81 percent of cases were rice farmers or their children) and the presence of underlying disease (identified in 53 percent of cases), particularly diabetes mellitus, which increased the relative risk by up to 100-fold in some population groups.

The epidemiology of melioidosis in other countries, such as Australia and Singapore, is broadly similar to that in Thailand, although the male:female ratio is generally higher (up to 5:1) (Heng et al. 1998; Currie et al. 2000b), and the reported incidence is up to ten-fold higher (Faa and Holt 2002), which probably reflects better ascertainment. The incidence is generally highest during particularly heavy monsoons (Merianos et al. 1993).

Pathology and pathogenesis

The pathogenesis of melioidosis is poorly understood and until recently had been relatively little studied. *B. pseudomallei* is a pyogenic organism, causing localized abscesses or granulomata at the site of primary infection, depending on the duration of the lesion, and septicemia when the bloodstream is invaded, which may in turn lead to seeding and localized foci of infection in other tissues. A high level of bacteremia (>50 CFU/ml) is associated with a fatal outcome (Walsh et al. 1995a). The outcome of contact with *B. pseudomallei* presumably depends on the host's immune system, the virulence of the infecting strain, and the size and route of the initial inoculum.

HISTOPATHOLOGY

Lesions of melioidosis are extremely variable in nature, from an acute, necrotizing inflammatory process with abscess formation, to chronic granulomatous inflammation, and sometimes a mixed picture is seen (Piggott and Hockholzer 1970). Although the process may be difficult to distinguish from other inflammatory conditions, features which may be helpful include the presence of intracellular 'globi' of gram-negative bacilli combined with giant cells in a background of acute necrotizing inflammation (Wong et al. 1995).

HOST DEFENSE

Melioidosis which is severe enough to be seen in hospital is an opportunistic disease, as 50–70 percent of patients have underlying predisposition to infection. Diabetes mellitus is particularly important in this regard, and other underlying conditions reported have included chronic renal disease, malignancy, immunosuppressive treatment (particularly steroids), liver disease, alcohol or drug abuse, thalassemia, pregnancy, and, in Australia, kava consumption (Chaowagul et al. 1989; Suputtamongkol et al. 1994a, 1999; Currie et al. 2000b). More recently, cystic fibrosis has also emerged as a predisposing factor for pulmonary melioidosis (O'Carroll et al. 2003).

Little is known about the specific immunological mechanisms responsible for protection, although cell-mediated immunity is probably of particular importance. Relapse of latent infection also usually occurs at times when cellular immunity is likely to be suppressed (see above). In this context, it is perhaps surprising that infection with the human immunodeficiency virus does not appear to predispose to melioidosis.

HOST RESPONSE

Once infected, the majority of patients mount a vigorous humoral and cellular immune response. This is presumably usually effective in eliminating infection in view of the frequency of mild or asymptomatic infection. Most studies show that 90 percent or more of patients with culture-positive melioidosis have detectable IgG and IgM antibodies at some stage in their illness. Evidence from studies in experimental animals suggests that

antibodies to the exotoxin, LPS, flagellin, flagellin–LPS conjugates, and capsular polysaccharides, may all have some protective effect (Smith et al. 1987; Bryan et al. 1994; Brett et al. 1994; Brett and Woods 1996; Ho et al. 1997; Jones et al. 2002). In human infections, Charuchaimontri et al. (1999) found that the level of antibody to O-PSII, but not O-PSI, was significantly higher in patients who survived melioidosis than in those who died.

There is also evidence of massive activation of the cellular immune system. Evidence from animal models suggests that this may be partially protective. However, an over-vigorous response may be damaging. The serum levels of TNF-α, IFN-γ, and soluble IL-2 receptors as well as IL-6, IL-8, IL-10, IL-12p40, IL-15, IL-18, granzymes A and B, procalcitonin, and CXC chemokines have all been found to be elevated in patients with severe melioidosis, and some of these correlate with disease severity and outcome (Suputtamongkol et al. 1992; Friedland et al. 1992; Smith et al. 1995a; Lauw et al. 1999, 2000a, b; Simpson et al. 2000).

Diagnosis

The protean manifestations of melioidosis make it difficult to diagnose on clinical grounds alone. The diagnosis should be considered in any patient who has ever visited an endemic area who presents with septicemia, abscesses, or chronic suppuration, particularly if there is evidence of an underlying disease such as diabetes mellitus. Specific diagnosis depends on the detection of *B. pseudomallei* or of corresponding antibodies. The laboratory should always be warned if melioidosis is suspected, both to enable appropriate methods and media to be employed and to ensure that appropriate containment measures are taken.

MICROSCOPY AND CULTURE

The organism should be sought in blood, pus, sputum, urine, or any other appropriate specimen. Direct immunofluorescent microscopy may be worthwhile in endemic areas, and has a specificity of 73 percent and a sensitivity of 99 percent compared with culture (Walsh et al. 1994). Definitive diagnosis depends on isolation and identification of *B. pseudomallei*, of which asymptomatic carriage has never been reported. The organism grows readily on most laboratory media, although it may take 48 h or more to develop characteristic colonial morphology. The sensitivity of culture of sites with a normal flora may be increased by the use of selective media such as Ashdown's agar (Wuthiekanun et al. 1990), and selective broth pre-enrichment increases the yield still further (Wuthiekanun et al. 1990; Walsh et al. 1995c). Culture of a throat swab alone using these selective techniques has an overall sensitivity of 36 percent for the diagnosis of melioidosis (79 percent in sputum-positive patients), which is particularly useful in children or others who cannot produce sputum (Wuthiekanun et al. 2001). Identification of cultures may be conducted using conventional biochemical tests or commercial kits such as the API 20E (Ashdown 1979a), Microbact 24E (Thomas 1983), API 20NE (Dance et al. 1989c), Minitek discs (Ashdown 1992a), and Vitek 1 (Lowe et al. 2002), although none is more accurate than a few simple tests (colonial appearance on Ashdown's medium, resistance to gentamicin and colistin) in experienced hands (Dance et al. 1989c). Identification is often delayed in nonendemic areas, however, because microbiologists are not familiar with the appearance and characteristics of the organism. More rapid results may be obtained using immunofluorescence (Naigowit et al. 1993), direct or latex agglutination (M.D. Smith et al. 1993; Rugdech et al. 1995; Anuntagool et al. 2000), or a molecular approach (Gee et al. 2003). Overall, latex agglutination with a monoclonal antibody to the extracellular polysaccharide of *B. pseudomallei* is probably the most useful approach, being simple, rapid, specific, and applicable both to isolates and broth cultures (Wuthiekanun et al. 2002). Unfortunately these reagents are not commercially available.

DETECTION OF ANTIBODIES

Several tests have been described for the serodiagnosis of melioidosis, although the nature of the immune response to infection and the antigens involved have been poorly characterized. The test most widely used in endemic areas is an indirect hemagglutination (IHA) test, which detects IgM antibodies to crude heat-stable antigens, probably predominantly lipopolysaccharide, (Ashdown 1987). The test is poorly standardized, and there is considerable interlaboratory variation between titers which are regarded as positive. Crossreactions occur with some other organisms such as *Legionella pneumophila* (Klein 1980), and the background seropositivity rate is high in endemic areas, leading to a low specificity (Chaowagul et al. 1989). Nonetheless, a single high titer (>1:40) in someone from a nonendemic area, or a rising titer, may be diagnostically useful. ELISA, which detect IgG antibodies, appear to give similar results (Ashdown et al. 1989). Assays which detect specific IgM (indirect immunofluorescence, ELISA) correlate better with disease activity (Ashdown 1981; Khupulsup and Petchclai 1986; Ashdown et al. 1989; Kunakorn et al. 1990, 1991b) and are thus more useful in endemic areas and in follow-up of patients on treatment. There is little doubt that some of the newer assays employing more purified antigens are an improvement on the IHA test (Sermswan et al. 2000; Wongratanacheewin et al. 2001), but none of these tests has ever been subjected to a large scale, multicenter analysis.

Control

Since *B. pseudomallei* is ubiquitous in the environment in endemic areas, it is almost impossible for those whose occupations involve soil and water contact to avoid exposure. It might be worthwhile for those particularly at risk (e.g. diabetics) to avoid rice farming, or use protective footwear and gloves, although the effectiveness of this has not been evaluated. Elimination of the organism from soil using disinfectants was attempted in the French outbreak (Mollaret 1988). No *B. pseudomallei* vaccine has been licensed for human use, although several groups are working on vaccine development (Warawa and Woods 2002) and experimental vaccines have been used in animals. Patients should ideally be nursed in standard isolation, although person-to-person spread is very rare.

Treatment

Patients with septicemic melioidosis usually require aggressive supportive treatment and should ideally be managed in an intensive care unit. Particular attention should be paid to correction of volume depletion and septic shock, respiratory and renal failure, and hyperglycemia or ketoacidosis. Abscesses should be drained whenever possible.

B. pseudomallei is intrinsically resistant to many antibiotics, including aminoglycosides and early beta-lactams. Indeed, a complete lack of response to penicillin and gentamicin, a combination often used for the empirical treatment of septicemia in the tropics, is characteristic of melioidosis (Chaowagul et al. 1989).

Until the mid-1980s, various empirical combination regimens were used to treat melioidosis, usually including a tetracycline, chloramphenicol, and co-trimoxazole, but mortality remained unacceptably high. Several recent studies, summarized by White (2003) and Samuel and Ti (2003), have shown that the mortality of acute severe melioidosis can be substantially reduced by the use of newer beta-lactam agents. Treatment is divided into two phases – an initial parenteral phase, during which the objective is to prevent death, and a subsequent eradicative phase, when the aim is to reduce relapse.

Suitable agents for use in the acute phase include ceftazidime, imipenem, and meropenem, and cefoperazone-sulbactam, with or without co-trimoxazole (White et al. 1989; Sookpranee et al. 1992; Simpson et al. 1999; Chetchotisakd et al. 2001b). Appropriate doses are given in Table 2. Co-amoxiclav is associated with a higher failure rate (Suputtamongkol et al. 1994b), and ceftriaxone and cefotaxime should not be used (Chaowagul et al. 1999b). Acute phase treatment should be given for at least 2 weeks and sometimes longer, depending on clinical response. The carbapenem antibiotics have some theoretical advantages over ceftazidime (Smith et al. 1994, 1996; Walsh et al. 1995b) and particularly encouraging results have been obtained in Darwin, Australia, using meropenem plus co-trimoxazole, with adjunctive granulocyte colony-stimulating factor, with mortality in melioidosis septic shock falling from 95 percent to 10 percent (Cheng et al. 2004), although these findings need further verification in prospective, comparative studies.

Table 2 *Doses of antimicrobials for the treatment of melioidosis in the acute phase (first 2–4 weeks)*

Drug	Dose (mg/kg/day)	Reference
Ceftazidime	120	White et al. (1989)
Ceftazidime plus	100	Sookpranee et al. (1992)
Co-trimoxazole	8/40	
Imipenem[a]	50	Simpson et al. (1999)
Co-amoxiclav	160	Suputtamongkol et al. (1994b)
Cefoperazone-sulbactam plus	25	Chetchotisakd et al. (2001b)
Co-trimoxazole	8/40	

a) Meropenem in equivalent doses, with or without co-trimoxazole, is probably equally effective.

Following parenteral treatment, prolonged oral antibiotics are needed to prevent relapse, which occurs in up to 23 percent of patients (Chaowagul et al. 1993), and is more common in patients with more severe disease. Recurrent disease is almost always due to relapse, although reinfection appears to occur occasionally (Desmarchelier et al. 1993). The risk can be reduced to less than 10 percent if antibiotics are given for a total of 20 weeks (Rajchanuvong et al. 1995). The combination of chloramphenicol (40 mg/kg/day), doxycycline (4 mg/kg/day) and co-trimoxazole (10 mg + 50 mg sulphamethoxazole/kg/day) was associated with a lower relapse rate than co-amoxiclav (60 mg amoxycillin + 15 mg clavulanic acid/kg/day) in the latter study, although this may have been accounted for by differences in compliance, so either regimen is probably adequate. Fluoroquinolones and doxycycline alone are inadequate for eradication treatment (relapse rates 18–25 percent) (Chaowagul et al. 1997, 1999a), as is the combination of ciprofloxacin plus azithromycin (relapse rate 22 percent) (Chetchotisakd et al. 2001a). Co-amoxiclav is preferable in children and pregnant or lactating women due to its lower toxicity. It is possible that the chloramphenicol may be omitted (Chetchotisakd et al. 2001a), and co-trimoxazole alone has been used in Australia (Currie et al. 2000b; Cheng et al. 2004), but this too needs further evaluation in prospective comparative studies. In patients who have mild localized disease, any of the oral regimens described above may be used, although the optimum duration is uncertain.

Outcome and follow-up

Even with optimal treatment, the overall mortality rate is 40–50 percent in Thailand (range 14–74 percent) (White 2003), but lower in Australia (19 percent) (Currie et al. 2000b). The relapse rate ranges from 1 to 26 percent (White 2003). In patients who survive, there is often chronic morbidity resulting both from the disease itself and the underlying conditions. Patients require long-term follow-up to detect relapse. Serial measurements of IgM (Ashdown 1981) or C reactive protein (Ashdown 1992b) may give an early clue to relapse. Susceptibility tests should be carried out on isolates obtained during or after treatment, since antibiotic resistance may emerge in 5–10 percent of cases (Dance et al. 1989b).

BURKHOLDERIA MALLEI

Cultural characteristics

B. mallei is an obligate animal parasite (particularly of equine species) that was at one time widespread in the world. Isolation of the organism is exceedingly rare today and restricted to a few foci in Asia, Africa, and the Middle East. Cells of *B. mallei* are straight or slightly curved (2–5 μm long and 0.3–0.8 μm wide), with rounded ends and irregularly parallel or wavy sides; the cells are arranged singly, in pairs end to end, in parallel bundles or in Chinese-letter form. Granular inclusions may be observed and cells may stain irregularly. A capsule is not evident by light microscopy and flagella are absent. *B. mallei* is aerobic but like other pseudomonads can utilize nitrate as an alternative electron acceptor for anaerobic growth. It grows less well than *B. pseudomallei* on nutrient agar and forms smooth, gray translucent colonies 0.5–1 mm in diameter in 18 h at 37°C (see Miller et al. 1948a).

According to Holmes et al. (1986), *B. mallei* is nonmotile, does not grow on McConkey or cetrimide agar, does not form pigments, is catalase positive, and few are oxidase positive. All strains reduce nitrate but not nitrite, and grow and form inclusions on poly hydroxy butyrate agar. Gelatin hydrolysis, urease activity, and production of lecithinase is variable; almost all hydrolyze tyrosine but without formation of pigment. All strains break down arginine but not lysine or ornithine. The following sugars in ammonium salts medium are converted: arabinose (89 percent), glucose (99 percent), glycerol (89 percent), inositol (99 percent), mannitol (67 percent), sorbitol (78 percent), and trehalose (89 percent).

Antimicrobial susceptibility

Isolation of the organism from contaminated samples is best achieved by culture on media supplemented with agents that inhibit the growth of gram-positive bacteria (e.g. bacitracin, crystal violet). Data on the antibiotic susceptibility of *B. mallei* are scanty. The few strains reported appear to be sensitive to sulfonamides and usually also to streptomycin, tetracycline, and novobiocin; some were sensitive to chloramphenicol (Miller et al. 1948a; Al Izzi and Al Bassam 1989). More recently, Kenny et al. (1999) examined 17 strains obtained from culture collections and reported the following susceptibilities in broth dilution (MIC μg/ml) for 90 percent of the strains tested: ampicillin >64, piperacillin 16, amoxicillin-clavulanate 8, cefuroxime >64, ceftazidime 8, imipenem 0.25, chloramphenicol >64, doxycycline 2, ofloxacin 8, ciprofloxacin 8, gentamicin 0.5, azithromycin 4, rifampin 16, sulfamethoxazole >64, trimethoprim 32, co-trimoxazole >64. They concluded that the superior tissue penetration of azithromycin over gentamicin made this a possible candidate antibiotic for treating glanders.

Genomics

B. mallei and *B. pseudomallei* are very closely related genetically by DNA–DNA hybridization (Rogul et al. 1970) and relatively minor differences exist between the two genomes. A PCR based on the nucleotide difference thymidine versus cytosine at nucleotide 2143 in the 23S rDNA sequences was used to differentiate *B. mallei* from *B. pseudomallei*. A 526 bp product was obtained with a primer of the following sequence 5′-CAC CGA AAC TAG CA-3′ at an annealing temperature of 47°C (Bauernfeind et al. 1998). A single nucleotide difference was also found in the flagellin *fliC* gene between the two species despite the fact that *B. mallei* is nonmotile; there is a shift from guanine to cytosine at position 798. However, Sprague et al. (2002) showed that this mutation was not *B. mallei* specific and was also found in some strains of *B. pseudomallei*.

The complete genome sequence has recently been published (Nierman et al. 2004).

Antigenic structure

B. mallei strains are antigenically heterogeneous and form two or three groups, only one of which is related to *B. pseudomallei* (Stanton et al. 1927; Dodin and Fournier 1970). The species shares LPS antigens with *B. pseudomallei* and a polyclonal antiserum to *B. pseudomallei* LPS was shown to bind in Western blots with eight of ten *B. mallei* strains examined by Burtnick et al. (2002); the negative strains lacked O-repeating units. An 18.7 kbp region of the *B. mallei* DNA sequence was identified that contained 16 open reading frames identical to those previously defined as the O-PS gene cluster in *B. pseudomallei* (DeShazer et al. 1998). *B. mallei* LPS has a similar chemical

structure but it is devoid of an *O*-acetyl group at the 4′ position of the L-talose residue. Loss of the O-PS moiety in two strains resulted in loss of serum resistance and an IS*407*-like element was identified in the O-PS biosynthetic gene cluster in these serum sensitive strains suggesting that the presence of this element is responsible for the loss of expression of O-PS (Burtnick et al. 2002).

A complement fixation test is prescribed for international trade of horses. It is reported to be 90–95 percent accurate, a positive result is obtained within 1 week of infection and remains positive in chronic cases. For the test antigen, the overnight growth from glycerol-agar slopes is harvested in saline and heated at 65°C for 1 h. For details of this and other diagnostic tests and procedures for glanders, see van Zijderveld and Bongers (2000).

GLANDERS

Glanders is a zoonotic disease of equines which may occasionally be transmitted to man or other animals (sheep, goats, dogs, and cats). Traditionally, 'glanders,' a systemic disease involving the nasal mucosa and lower respiratory tract, has been distinguished from 'farcy,' a cutaneous infection with lymphangitis also caused by *B. mallei*. In contrast with melioidosis, there has been remarkably little recent work on this disease. The history of glanders was reviewed by Wilkinson (1981).

Clinical manifestations

Glanders has a similar range of clinical presentations to that of melioidosis, as originally observed by Whitmore (1913). Occult infections are probably under-reported. At the other end of the spectrum, glanders may present as overwhelming sepsis resulting in severe toxemia and widespread abscesses, culminating in delirium, shock, and death. The disease may be fatal within days or may persist for many years with remissions and relapses. In reality, the distinction between glanders and farcy is artificial since, as with melioidosis, an acute phase may precede or follow chronic disease and localized cutaneous infection may disseminate or may result from metastatic infection.

Characteristic local manifestations include wound infection and ulceration, lymphangitis with abscesses along the course of lymphatic drainage ('farcy buds'), ulceration of the respiratory (especially nasal) mucosa, polyarthritis, pneumonia, and lung abscesses, and nodular abscesses in any site, particularly muscle and subcutaneous tissue. A widespread necrotizing skin eruption has been described as a terminal feature of disseminated glanders. Readers requiring a more detailed description of the manifestations of glanders are referred to the excellent review by Howe (1950). A recent laboratory-acquired case in a military researcher who had failed to wear gloves when working with *B. mallei*, despite the fact that he was diabetic, was reported by Srinivasan et al. (2001). His illness closely resembled melioidosis, with initial axillary lymphadenopathy followed by a systemic illness with liver and splenic abscesses. Despite the history of working with *B. mallei*, both the clinical and laboratory diagnosis of this case were delayed, which highlights the potential difficulties of making a diagnosis of these rare infections.

Epidemiology

GEOGRAPHICAL DISTRIBUTION

In the early part of the twentieth century, equine glanders was present worldwide and was still relatively common in Europe and the United States. Over 200 000 horses were destroyed as a result of glanders during the First World War (Howe 1950). No naturally acquired cases have been reported in the USA or the UK since 1938, although occasional laboratory-acquired infections have occurred (Howe and Miller 1947; Srinivasan et al. 2001). Currently, equine glanders is still known to occur in parts of the Middle East, Africa, and Asia, although the true incidence is uncertain (Arun et al. 1999). Occasional cases have arisen amongst carnivora in nonendemic areas, probably through the ingestion of contaminated imported meat (Alibasoglu et al. 1986).

RESERVOIRS AND TRANSMISSION

Human glanders, which was rare even when equine glanders was common, is primarily an occupational disease of those in close contact with infected animals. Natural infection is thought to usually result from contamination of wounds, abrasions or mucous membranes. The infectivity by these routes is probably low, although the organism is particularly hazardous to laboratory workers who may acquire infection by inhalation (Howe and Miller 1947). This route may also account for some naturally acquired cases. Person-to-person spread has occurred, so patients require isolation.

B. mallei is more delicate than *B. pseudomallei* and is usually considered to be an obligate parasite. It may, however, survive for up to 4 weeks in water (Howe 1950), although the role of ingestion in human infections is uncertain.

Pathology and pathogenesis

Following inoculation, *B. mallei* establishes local infection, often with ulceration, and may subsequently disseminate via the lymphatics or bloodstream.

HISTOPATHOLOGY

As with melioidosis, the nature of glanders lesions varies according to the duration of infection. Nodular foci of infection range from abscesses with acute inflammation to chronic granulomata with central caseous necrosis (Howe 1950). These lesions are said to be distinguishable from tuberculous granulomata (Zubaidy and Al-Ani 1978). Involvement of small blood vessels and lymphatics with inflammation and endothelial proliferation progressing to subintimal and medial degeneration and thrombosis is characteristic (Howe 1950).

HOST DEFENSE

Several animal species are susceptible to experimental glanders, but species vary in their susceptibility to infection, with cattle, pigs, rats, and fowl being intrinsically resistant. It is not clear whether underlying diseases render humans susceptible to glanders.

HOST RESPONSE

Both humoral and cellular immune responses occur in natural and experimental infections with *B. mallei*, but these have not been well characterized. However, neither natural infection nor immunization induces protection in horses, despite some success with the latter in guinea pigs (Mohler and Eichhorn 1914).

Diagnosis

CULTURE

As with melioidosis, the clinical manifestations of glanders are rarely pathognomonic. The diagnosis thus hinges on an appropriate history (contact with animals in an endemic area or laboratory exposure) and on isolation and identification of *B. mallei* from an appropriate clinical specimen (e.g. blood, wound swab or pus, sputum) or the detection of specific antibodies. Although *B. mallei* will grow on orthodox media, the laboratory should be alerted if the diagnosis is suspected, both to increase the chances of detection and because, like *B. pseudomallei*, *B. mallei* requires containment level 3 accommodation. Its rarity means that most microbiologists are unfamiliar with its characteristics, and it might easily be overlooked unless sought specifically. Selective techniques have been described (Howe 1950), but recent experience of these is limited.

SERODIAGNOSIS

Serological tests (complement fixation, bacterial agglutination or hemagglutination, or ELISA), may be available through reference or veterinary laboratories (Verma et al. 1990), but are of little use for diagnosis of acute cases and may give crossreactions in patients exposed to *B. pseudomallei*. Skin testing with a crude extract of *B. mallei* (mallein) has been used for screening animals and has been reported to be useful in human glanders, but cannot be recommended because of its unreliability, the risk of adverse reactions, and problems obtaining mallein. As yet no rapid diagnostic tests have been developed.

Control

The remarkable decline of glanders has been attributed variously to the compulsory slaughter of infected or seropositive animals, the decreasing use of the horse, and the removal of communal water troughs (Blancou 1994). There is no effective vaccine for either human or animal use, and prevention in those countries where glanders persists will depend on veterinary public health measures and the continued reduction of reliance on the horse as a means of transport.

Treatment

SPECIFIC TREATMENT

Since most human infections occurred in the pre-antibiotic era, data on treatment are scarce. Unlike *B. pseudomallei*, *B. mallei* is sensitive in vitro to aminoglycosides and azithromycin, although it is unknown whether these agents would be effective in vivo. Otherwise, their susceptibility profiles are similar (Al Izzi and Al Bassam 1989; Kenny et al. 1999; Heine et al. 2001). Sulfonamides in full doses for at least 20 days were effective in curing experimental infections (Miller et al. 1948b) and have been used successfully to treat human cases (Howe and Miller 1947). The recent American case (Srinivasan et al. 2001) was effectively cured by treatment with imipenem plus doxycycline followed by azithromycin plus doxycycline for a total of 6 months (despite relative in vitro resistance to azithromycin). Until further data are available, treatment for severe glanders should comprise one of the regimens validated for the treatment of melioidosis.

OUTCOME AND FOLLOW-UP

The mortality of reported cases, many of whom have not been effectively treated, has been high (over 90 percent), but this should be reduced if appropriate antibiotics are given. Long-term follow-up to detect relapse should be arranged.

Bioterrorist agents

Both *B. mallei* and *B. pseudomallei* appear on the category B list of critical agents published by the US Centers for Disease Control and Prevention (Rotz et al. 2002). The organisms have long been considered to be of relevance to military medicine due to the deployment of troops within endemic areas, particularly during monsoon periods. They also share the characteristics

that they are inexpensive to produce in low technological facilities, they have high infectivity, long environmental survival, can be disseminated into the environment, and have the capacity to cause severe disease and high mortality. *B. mallei* and *Bacillus anthracis* were released by the Germans during the First World War to infect shipments to the Allies of horses, mules, cattle, and sheep (Wheelis 1998). The first reported case of human glanders since 1949 occurred in a microbiologist with insulin-dependent diabetes working in the US Army Medical Research Institute for Infectious Diseases. The infection was presumably contracted via skin puncture with infected material (Srinivasan et al. 2001). Animals do not appear to represent a significant reservoir for the transmission of *B. pseudomallei*. Nevertheless, the demonstration that some human cases of melioidosis in Hong Kong were caused by strains with the same DNA macrorestriction pattern and MLST sequence type as strains from captive sea mammals and birds in an aquatic park raises the possibility that animal to man transmission may occur (Godoy et al. 2003).

MISCELLANEOUS OPPORTUNIST 'PSEUDOMONADS'

Pandoraea spp.

The genus *Pandoraea* is a close relative of *Burkholderia* and was first described by Coenye et al. (2000) as nonfermentative gram-negative rods isolated predominantly from soil and the sputum of CF patients. They identified four species, *P. apista*, *P. pulmonicola*, *P. pnomenusa*, and *P. sputorum* and also reclassified *Burkholderia norimbergensis* as a new *Pandoraea* species. The taxonomy was developed further by Daneshvar et al. (2001) who assigned nine isolates of CDC weak oxidizer group 2 to the new genus and described four unnamed genomospecies. PCR assays based on 16S rDNA were developed by Coenye et al. (2001e) and are recommended for the differentiation of *Pandoraea* from the *B. cepacia* complex. The few *Pandoraea* strains tested by Daneshvar et al. (2001) were resistant to ampicillin, extended spectrum cephalosporins and aminoglycosides, but varied in their susceptibility to ciprofloxacin.

Ralstonia spp.

Formerly known as CDC group IV c-2, the genus *Ralstonia* was defined by Yabuuchi et al. (1995). It contains several gram-negative rods of diverse ecological function including, for example, the plant pathogen *R. solanacearum*, the biomediation agent *R. eutropha*, metal resistant species such as *R. metallidurans*, and the opportunistic human pathogen, *R. pickettii* (formerly *Burkholderia pickettii*).

16S rDNA sequence analysis suggests that there are two distinct sublineages within the genus; the *R. eutropha* lineage which comprises nine species and the *R. pickettii* lineage represented by a further five species. Members of the *R. eutropha* lineage have peritrichous flagella, do not produce acid from glucose, and are susceptible to colistin. On the other hand, the *R. pickettii* lineage have one or more polar flagella, form acid from several sugars, and are resistant to colistin. On the basis of these features and other phylogenetic data, Vaneechoutte et al. (2004) proposed that the *R. eutropha* lineage should be classified as a novel genus *Wautersia* gen. nov. Both lineages contain species of human clinical significance. Identification of *Ralstonia* species in the diagnostic context is aided by the use of amplified ribosomal DNA restriction analysis which allows differentiation of 12 species (Segonds et al. 2003). However, Coeyne et al. (2003a) found that some clinical and environmental isolates of *R. pickettii* represented a new species for which the name *R. insidiosa* was proposed. Another novel species, *R. respiraculi* sp. nov. has also recently been recovered from the respiratory tract of CF patients (Coenye et al. 2003b).

R. pickettii is the species most commonly implicated in outbreaks of infection in hospitals owing to its capacity to contaminate solutions used for patient care, such as respiratory therapy solutions and wound irrigation systems (Yoneyama et al. 2000), and blood culture bottles (Boutros et al. 2002). Although less frequent, *R. paucula* can cause clusters of nosocomial infections some of which may be serious and even life threatening (Ramos et al. 1993).

Delftia (*Comamonas*) *acidovorans*

The family Comamonadaceae comprises a number of soil bacteria, but *Comamonas acidovorans* has been isolated on a number of occasions from hospital patients and their environment. Wen et al. (1999) proposed, on the phylogenetic basis of 16S rRNA genes, that *C. acidovorans* be removed from the genus *Comamonas* and renamed as *Delftia acidovorans* with ATCC 15668T as the type strain. The organism is able to degrade polyurethane, a widely used industrial polymer and utilize certain herbicides (Nakajima-Kambe et al. 1999; Muller et al. 2001). *D. acidovorans* is a common contaminant of dental unit water lines (Stampi et al. 1999) and has been occasionally reported as a cause of eye infections (Stonecipher et al. 1991). Some strains are able to grow on polymyxin-containing agar and may be misidentified as *B. cepacia* in sputum specimens from CF patients (Henry et al. 1997). It acidifies mannitol but not ethanol, glucose, or maltose in ammonium-salts media and is generally asaccharolytic. Strains are variable in their antimicrobial susceptibility but usually are resistant to

gentamicin and susceptible to the ureidopenicillins, tetracyclines, quinolones, and co-trimoxazole (von Graevenitz 1985). Resistance to β-lactams is the product of synergy between β-lactamase and outer membrane protein alterations (Ravaorinoro and Therrien 1999).

Stenotrophomonas maltophilia

CLASSIFICATION

This organism was originally classified as '*P. maltophilia*' but was transferred to the genus *Xanthomonas* in 1983 and subsequently as the sole member to the genus *Stenotrophomonas* (Palleroni and Bradbury 1993). The latter species was clearly distinguished from the former by 16S rRNA gene homology studies (Nesme et al. 1995). Cells of *S. maltophilia* stain evenly and do not accumulate poly-β-hydroxybutyrate. They usually have several polar flagella and are actively motile particularly at 18–20°C. On nutrient agar at 37°C they form opaque grayish colonies with a yellowish tinge; on blood agar the growth can have a faint lavender hue. A brownish pigment is formed by some strains on tyrosine-containing agar and because of its requirement for methionine it will not grow in Koser's citrate medium. *S. maltophilia* is an obligate aerobe that will not grow below 5°C or above 40°C. The oxidase reaction is negative or equivocal and they usually acidify maltose and less commonly glucose, but do not attack other sugars in ammonium-salts media. They decarboxylate lysine but not ornithine, and split esculin, gelatin, Tween 20, and DNA (Denton and Kerr 1998).

HABITAT

The species is ubiquitous and has been isolated from a range of water sources including rivers, wells, bottled water, and sewage. It can also be recovered from soil and the plant rhizosphere and in the latter environment its bioremediation and antifungal properties have been explored for commercial crops (Berg et al. 1996). A strain of *S. maltophilia* has also been shown to grow in a medium containing benzene, toluene, or ethylbenzene as the carbon source (Lee et al. 2002). Members of the species are common food contaminants and have been isolated from frozen fish, milk, and meat carcasses. A recent report highlighted *S. maltophilia* as the most common contaminant identified from embryos and semen samples stored in liquid nitrogen and the organism was further shown to significantly suppress fertilization and embryonic development in vitro (Bielanski et al. 2003). Human carriage is variable; fecal carriage rates have been reported to be as high as 33 percent in patients with hematological malignancy but only 3 percent in a control patient group (Kerr et al. 1991). Carriage on the hands of hospital workers in outbreaks is uncommon.

OPPORTUNIST INFECTION

The species is occasionally associated with outbreaks of infection in hospitals, particularly in intensive care units, being recovered from bypass equipment, defrost water baths in operating theaters, and ice-making machines. *S. maltophilia* has been isolated from patient specimens as the causative organism of a wide variety of infections but sepsis is unusual unless certain risk factors are prevalent in the patient population, i.e. prior surgery, shock, mechanical ventilation, and broad spectrum antimicrobial prophylaxis, particularly imipenem, to which it is intrinsically resistant (Elting et al. 1990; Laing et al. 1995). Friedman et al. (2002) found that the most common characteristics in cases of bacteremia were the presence of an indwelling central venous catheter and previous antimicrobial therapy; 18 percent of patients died within 7 days and a significant correlation was found between deaths and a failure to remove the indwelling catheter or treat with appropriate antimicrobials. A survey of opportunist infection of human immunodeficiency virus (HIV) patients revealed 61 episodes of *S. maltophilia* infection in 59 HIV-infected patients. Approximately 78 percent of cases were clinically septic with bacteremia while lower respiratory tract and urinary tract infection were relatively uncommon leading to the conclusion that compared with non-typhoid *Salmonella* spp. bacteremia, HIV patients were at significantly higher risk of developing *S. maltophilia* disseminated infection (Calza et al. 2003). However, the attributable mortality rate (26.7 percent) for *S. maltophilia* bacteremia was found to be similar to the rate for other nosocomial bacteremias, but there was an eightfold increase in the risk of death (Senol et al. 2002). Despite its incidence in bloodstream infections, the respiratory tract is the most common site of infection in hospitalized patients and many patients have preexisting lung conditions. Kollef et al. (1995) considered that the presence of the organism in the sputum was the most important predictor of mortality in late-onset ventilator-associated pneumonia, but the distinction between colonization and true infection is not easily made.

ENDOCARDITIS

Most cases of endocarditis due to *S. maltophilia* occur in intravenous drug users or as a complication of prosthetic valve surgery, and endocarditis outside these situations is uncommon. Khan and Mehta (2002) reviewed 23 cases of *S. maltophilia* endocarditis and found that all patients were pyrexial and prosthetic valves were involved in half of the cases. The underlying risk factors were prior valvular or congenital heart disease surgery (60 percent), intravenous drug abuse (32 percent), and infected intravascular lines (18 percent). The most frequently used antibiotics for therapy were aminoglycosides, trimethoprim-sulfamethoxazole, and penicilllins. Mortality was 39 percent and was equally distributed between patients

with prosthetic and native valve endocarditis. *S. maltophilia* may occasionally cause infections of the central nervous system, the eyes, urinary tract, skin and soft tissues, bone and joints, and the gastrointestinal tract (see Denton and Kerr 1998).

INFECTION IN CYSTIC FIBROSIS

S. maltophilia has increasingly been isolated from the sputum of CF patients since 1979 and evidence suggests that in some large centers its incidence among CF patients has risen year on year. Talmaciu et al. (2000) reported that since 1993 the incidence of *S. maltophilia* positive patients increased from 2.8 to 6.2 percent in 1997. These patients had worse growth parameters, clinical scores, and spirometric values than *S. maltophilia*-negative patients and there appeared to be a higher incidence of colonization with *Staphylococcus aureus* among those patients who grew *S. maltophilia*. Nevertheless, they concluded that acquisition of *S. maltophilia* had little repercussion on the clinical status of the patients. Considerable variation has been reported in the prevalence of the species in CF centers worldwide (19 percent in a UK center and over 30 percent in a Spanish center, see Denton and Kerr 1998) and this may be attributable to, among other factors, the wider use of carbapenems and longer duration of hospitalization of patients. CF patients may harbor strains for long periods and, to date, crossinfection between patients is not widespread. *S. maltophilia* may be mistaken for *B. cepacia* as it grows reasonably well on selective media containing polymyxin B (Burdge et al. 1995).

TYPING

Strains of *S. maltophilia* can be divided into a number of types by heat-stable antigens (Schable et al. 1992), and further subdivided by ribotyping with *Eco*RI (Bingen et al. 1991), PFGE (Laing et al. 1995), and arbitrary primer-PCR (Chatelut et al. 1995). Marty (1997) reviewed the typing methods available at that time and concluded that PFGE of *Xba*I or *Spe*I total chromosomal digests offered the highest discrimination and defined populations at the strain level. Ribotyping with restriction endonucleases (*Bam*HI, *Cla*I, *Eco*RI) was less discriminatory but PCR fingerprinting with primers targeting repeat motifs (enterobacterial repetitive intergenic consensus (ERIC), repetitive extrapalindromic (REP)) was recommended for the study of outbreaks. Denton et al. (1998) used PFGE and ERIC-PCR to investigate the molecular epidemiology of the organism in a CF center and concluded that there was no evidence of person-to-person transmission although some patients apparently acquired their colonizing strain from the hospital environment. A common feature of typing studies of *S. maltophilia* is the high diversity of strain types recovered and the low incidence of cross-infection between patients.

ANTIMICROBIAL SUSCEPTIBILITY

The determination of the antimicrobial susceptibility of *S. maltophilia* is problematic. The species exhibits a temperature-dependent variation in susceptibility to several antibiotics including aminoglycosides and polymyxin B (Wheat et al. 1985), and this variation has been linked with alterations in outer membrane fluidity and LPS composition (Rahmati-Bahram et al. 1996). Disk diffusion tests are considered inaccurate and lack reproducibility particularly for quinolone agents (Pankuch et al. 1994; Denton and Kerr 1998). The concentration of Zn^{2+} in the medium has a profound effect on the observed susceptibility of strains to imipenem, but this does not appear to be so for meropenem. There are three basic mechanisms of resistance to β-lactam agents; production of the β-lactamase L1 which confers resistance to the penicillins, β-lactamase L2 (specific for the cephalosporins), and low permeability of the outer membrane due to efflux pumps (Mett et al. 1998; Zhang et al. 2000b). A 50 kDa outer membrane protein was expressed by many multiple drug resistant strains and this protein showed antigenic homology with a protein secreted by *P. aeruginosa* strains hyperexpressing drug efflux systems. Moreover, antibody to OprM, the outer membrane component of the MexAB-OprM multiple drug resistance efflux system of *P. aeruginosa*, reacted strongly with the *S. maltophilia* protein (Zhang et al. 2000b).

A comprehensive review of the antimicrobial susceptibility of *S. maltophilia* is given by Denton and Kerr (1998). The tetracyclines doxycycline and minocycline, co-trimoxazole, and the third-generation cephalosporins have the highest activity against the organism, whereas the antipseudomonal penicillins are only moderately active. Most strains are resistant to aminoglycosides. Gabriel et al. (2004) studied 673 initial CF patient isolates for their susceptibility to ten antimicrobial agents and also conducted checkerboard dilution studies of eight agents against them. The majority (78 percent) of isolates were resistant to all agents in two or more antimicrobial classes, but 91 percent of these were inhibited by at least one antimicrobial combination. Doxycycline was active against 80 percent of isolates but co-trimoxazole inhibited only 16 percent of isolates. However, synergistic or additive activity was demonstrated for cotrimoxazole with ticarcillin-clavulanate (65 percent of isolates), ciprofloxacin with ticarcillin-clavulanate (64 percent), among others.

Brevundimonas diminuta and *Brevundimonas vesicularis*

These are closely related species of monotrichate organisms with flagella of very short wavelength. They were previously classified as rRNA homology group IV within the genus *Pseudomonas*, but Segers et al. (1994) proposed the name *Brevundimonas* following a

comprehensive study of phenotypic and genotypic characters. They require pantothenate, biotin, and cyanocobalamin for growth; *B. diminuta* also requires cysteine or methionine. They grow rather slowly on ordinary nutrient media. Features other than growth requirements that distinguish *B. vesicularis* from *B. diminuta* are that *B. vesicularis* gives only a weak oxidase reaction, forms a carotenoid pigment, and thus has yellow or orange colonies, and acidifies glucose and maltose in ammonium-salts media. Both species are rare in pathological specimens and of doubtful clinical significance, although occasional serious infections may occur (Gilad et al. 2000; Chi et al. 2004).

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Pasteurella, *Mannheimia*, *Actinobacillus*, *Eikenella*, *Kingella*, *Capnocytophaga*, and other miscellaneous gram-negative rods

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The organisms included in this chapter are a diverse group that encompasses pathogens of humans, animals, or both. Members of the genus *Pasteurella*, *Mannheimia*, and *Actinobacillus* are found predominantly in animals. Except for those due to *Actinobacillus actinomycetemcomitans* and a few other species, most human infections with organisms belonging to these genera result from animal bites or scratches. *Eikenella*, *Kingella*, *Cardio-*

bacterium, and certain *Capnocytophaga* species are members of the human oropharyngeal flora that can behave as pathogens primarily in immunocompromised hosts and those with underlying disease. Canine *Capnocytophaga* species and CDC group EF-4 organisms are also associated with animal exposures, with the former species being capable of causing severe, life-threatening disease. *Dysgonomonas* species, and *D. capnocytophagoides* in particular, are also opportunistic agents. *Chromobacterium* species are environmental bacteria that are capable of causing rare systemic infections. In this chapter, the taxonomy, virulence factors, clinical significance, cultural characteristics, and antimicrobial susceptibility of these organisms will be addressed.

PASTEURELLA AND *MANNHEIMIA* SPECIES

TAXONOMY AND CHARACTERISTICS OF THE GENUS *PASTEURELLA* AND THE GENUS *MANNHEIMIA*

Pasteurella species are members of the family Pasteurellaceae. This family also includes the genera *Haemophilus*, *Actinobacillus*, *Mannheimia*, *Lonepinella*, *Phocoenobacter*, and *Gallibacterium* (Christensen et al. 2003; Dewhirst et al. 1992; Foster et al. 2000; Osawa et al. 1995). At present, the taxonomic structure of the family Pasteurellaceae is complicated and unclear, and taxonomists are currently attempting to unravel the relationships among the members of the described genera and the proper assignment of various species to these and other genera (Angen et al. 1999; De Ley et al. 1990; Dewhirst et al. 1992, 1993a). The delineation of these genera was previously based on DNA hybridization studies, with species showing greater than 50 percent homology being included in the same genus with little regard for phylogenetic origin and phenotypic characterization (Mutters et al., 1985a, b). Since 1985, new species within the family Pasteurellaceae have been assigned to genus *Actinobacillus* or genus *Pasteurella* in a more or less arbitrary fashion. Examination of 16S rRNA sequences has shown that the older species of *Pasteurella* – *P. pneumotropica*, *P. aerogenes*, *P. ureae*, and the *P. haemolytica* group – are not closely related to the type species of *Pasteurella* (*P. multocida* sensu stricto) or to each other and probably belong in either the genus *Actinobacillus* or in new genera (Dewhirst et al. 1992; Mutters et al. 1985a; Sneath and Stevens 1990). As a first step in the dissection of this complex group of organisms, *P. ureae* was formally reassigned to the genus *Actinobacillus* as *Actinobacillus ureae* in 1986 (Mutters et al. 1986).

In 1985, several new *Pasteurella* species were described based on DNA–DNA hybridization. These new species included *P. dagmatis*, *P. canis*, *P. stomatis*, *P. anatis*, and *P. langaa* (Mutters et al. 1985a). These strains encompassed the former biotype Henriksen *P. pneumotropica* strains (the so-called *Pasteurella* gas) as *P. dagmatis*, and the former *P. multocida* biotype 6 as *P. canis* and *P. stomatis*. These studies also resulted in the transfer of the avian strains *Haemophilus avium* and *Haemophilus paragallinarum* to the genus *Pasteurella* as *P. avium* and *P. volantium*, respectively (Mutters et al. 1985a; Mutters et al. 1985b). Both *P. avium* and *P. langaa* represent V-factor-independent strains of avian origin. Recently *P. anatis* was reclassified in the new genus *Gallibacterium* as *Gallibacterium anatis* (Christensen et al. 2003). Sequencing of 16S rRNA from the type strains of the various *Pasteurella* species demonstrated a single genetic cluster containing the type strains of *P. multocida*, *P. avium*, *P. canis*, *P. dagmatis*, *P. gallinarum*, *P. langaa*, *P. stomatis*, and *P. volantium* as members of the genus *Pasteurella* sensu stricto (Dewhirst et al. 1992, 1993b; Frederiksen 1993) Two additional species – designated *Pasteurella* species A and B – were considered to be members of the genus but remained unnamed. Using both genomic fingerprinting techniques and quinone analysis, Kainz and coworkers in Austria suggested that *Pasteurella* sensu stricto should be restricted to *P. multocida*, *P. canis*, *P. dagmatis*, *P. stomatis*, and *Pasteurella* species B, and that *P. multocida* subsp. *septica* may constitute a separate species within the genus (Kainz et al. 2001). Despite efforts to restructure the genus, several isolates have been described and assigned to the genus *Pasteurella* that are very likely misnamed strains that belong to other or to new genera. These *Pasteurella* species included *P. mairi*, *P. bettyae*, *P. caballi*, *P. testudinis*, *P. seminis*, *P. lymphangitidis*, *P. granulomatis*, and *P. trehalosi* (Schlater et al. 1989; Sneath and Stevens 1990). *P. lymphangitidis* has now been excluded from the genus *Pasteurella* based on DNA–DNA hybridization and phenotypic characteristics, and in 1999 members of the *P. haemolytica* group (including *P. haemolytica* sensu stricto and *P. granulomatis*) were placed in the new genus *Mannheimia* or reclassified as the previously described species *P. trehalosi* (see below) (Angen et al. 1999; Sneath and Stevens 1990). The biochemical characteristics of *Pasteurella* species sensu stricto are found in Table 1, while those of the missclassified *Pasteurella* species are found in Table 2.

New *Pasteurella* species have been described on the basis of mostly single isolates. Although the taxonomic status of these newer isolates is unclear at present, the provisional assignment of these organisms to the genus *Pasteurella* underscores the heterogeneous nature of the genus and the need for additional studies on the taxonomic structure of this group. *Pasteurella skyensis* has been isolated from Atlantic salmon and exhibited similarities in 16S rRNA sequences with a strain from a porpoise that was designated, but not validly published, as *Pasteurella phocoenarum* (Birkbeck et al. 2002). Kainz et al. (2001) mentioned a strain called *Pasteurella*

Table 1 *Phenotypic characteristics for Identification of* Pasteurella *species*

Characteristic	*P. multocida* ssp. *multocida*	*P. multocida* ssp. *septica*	*P. multocida* ssp. *gallicida*	*P. canis*	*P. stomatis*	*P. dagmatis*	*P. gallinarum*	*P. volantium*	*P. avium*	*P. langaa*	*Pasteurella* sp. A	*Pasteurella* sp. B
Hemolysis	–	–	–	–	–	–	–	–	–	–	–	–
NAD requirement	–	–	–	–	–	–	–	+	+	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	–	+	+
Alkaline phosphatase	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate red	+	+	+	+	+	+	+	+	+	+	+	+
Urease	–	–	–	–	–	+	–	–	–	–	–	–
Indole	+	+	+	+	+	+	–	–	–	–	–	V
Lysine decarboxylase	–	–	–	–	–	–	–	–	–	–	–	–
Ornithine decarboxylase	+	+	+	+	–	–	–	d	–	–	–	+
Glucose, gas	–	–	–	–	–	$+^{w}$	–	–	–	–	–	–
Acid from:												
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	–	–	–	–	–	–	–	–	–	V	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	–	–	–	–	–	–	–	–
Esculin	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	+	+	+	–	–	–	–	+	–	+	V	+
Sorbitol	+	–	+	–	–	–	V	–	–	–	–	–
Dulcitol	–	–	+	–	–	–	–	–	–	–	–	–

+, 90 percent or more of the strains positive within 1–2 days; –, less than 10 percent of the strains positive within 14 days; d, delayed reaction; V, 11–89 percent of the strains positive; $+^{w}$, weak positive.

Table 2 *Phenotypical characteristics of misclassified* Pasteurella *species and Gallibacterium anatis*

Characteristic	*Gallibacterium anatis*	*P. aerogenes*	*P. bettyae*	*P. caballi*	*P. mairii*	*P. pneumotropica*	*P. testudinis*	*P. lymphangitidis*	*P. trehalosi*
Hemolysis	V	–	–	–	–	–	+	–	+
Oxidase	+	+	–	–		+	+	+	+
Catalase	+	+	V	–	+	+	+	+	–
Alkaline phosphatase	+	–	+	NA	+	+	+	–	+
Urease	–	+	–	–	+	+	–	+	–
Indole	–	–	–	NA	–	+/+^{w}	+	–	–
Nitrate red	+	+	+	NA	+	+	+	–	+
Lysine decarboxylase	–	–	–	NA	+	V	–	–	–
Ornithine decarboxylase	–	+	–	+	+	+	–	–	–
Glucose, gas	–	+	V, +^{w}	NA	–	–	–	–	–
Acid from:									
Galactose	+	+	–	+	+	+	+	+	–
Sucrose	+	+	–	NA	+	+	+	+	+
L-Arabinose	V	+	–	–	+	V	V	+	–
Inositol	V	–	–	–	V	(+)	+	–	(+)
Salicin	–	–	–	–	–	–	–	–	+
Esculin	–	–	–	–	–	–	+	–	+
Mannitol	+	–	–	V	+	–	+	+	+
Sorbitol	V	–	–	–	V	–	+	–	+
Dulcitol	–	–	–	NA	–	–	–	–	–

+, 90 percent or more of the strains positive within 1–2 days; (+), 90 percent or more of the strains positive within 3–14 days; –, less than 10 percent of the strains positive within 14 days; V, 11–89 percent of the strains positive; +^{w}, weak positive; NA, data not available.

leonis that may be a valid species according to 23S rRNA sequencing and quinone analysis. Finally, a new subspecies of *P. multocida*, designated subspecies *tigris*, has been described as an isolate from a tiger-bite wound in a child, but this finding has not been validated by further investigations (Capitini et al. 2002). Among the established *Pasteurella* species, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *P. canis*, *P. dagmatis*, and *P. stomatis* are the most frequently isolated species in human infections (Escande and Lion 1993).

P. haemolytica strains are recognized as prominent pathogens in domesticated animals, causing severe diseases and major economic losses in the cattle, sheep, swine, and poultry industries. Historically, *P. haemolytica* strains were divided into biotypes designated A (cattle-associated) and T (sheep-associated) (Carter 1984). Biotype A strains produced acid from arabinose but not trehalose while biotype T strains produced acid from trehalose but not arabinose. These biotypes were each divided into 17 serotypes, with serotypes 1, 2, 5–9, 11–14, 16, and 17 belonging to biotype A, and serotypes 3, 4, 10, and 15 belonging to biotype T. In 1985, *P. haemolytica* sensu stricto was excluded from the genus *Pasteurella* on the basis of DNA–DNA hybridization studies, and DNA–rRNA hybridization confirmed its exclusion from the *Pasteurella*, *Haemophilus*, and *Actinobacillus* genera (Mutters et al. 1985a). Genotypic investigations of trehalose-positive *P. haemolytica* (biotype T) strains has resulted in their reclassification as *Pasteurella trehalosi*, even though they show marginal genetic affinity with the genus *Pasteurella* (Sneath and Stevens 1990). Examinations of many diverse trehalose-negative (biotype A) *P. haemolytica* strains by phenotypic criteria, multilocus enzyme electrophoresis, ribotyping, and 16S rRNA sequencing has resulted in the proposal of a new genus, *Mannheimia*, to accommodate the trehalose-negative isolates of the *P. haemolytica* complex and *P. granulomatis* (Davies et al. 1996; Ribeiro et al. 1989). Based on 16S rRNA sequencing, four distinct related clusters corresponding to five new species have been delineated in the new genus *Mannheimia*: *M. haemolytica* and *M. glucosida* (cluster I), *M. ruminalis* (cluster II), *M. granulomatis* (cluster III), and *M. varigena* (Angen et al. 1999). These new species are most closely related to genus *Actinobacillus* (particularly *A. lignieresii*) and *P. trehalosi*. *Mannheimia* species can be separated from genus *Pasteurella* by failure of the former species to produce acid from mannose and from most animal-associated *Actinobacillus* species by being urease-negative. *Mannheimia* species are mannitol-positive, which separates them from members of the genus *Haemophilus*, which are mannitol-negative.

Members of the genus *Pasteurella* have certain phenotypic characteristics in common (Carter 1984; Mutters et al. 1984, 1985b). *Pasteurella* species are nonmotile, gram-negative, facultatively anaerobic coccobacilli or rods. Most species are oxidase-positive, catalase-positive, and alkaline phosphatase-positive, and reduce nitrate to nitrite. Most species also produce acid from glucose, fructose, mannose, and sucrose, and none of them hydrolyze starch or salicin. Members of the genus *Pasteurella* and animal-associated *Actinobacillus* species are difficult to distinguish phenotypically (Mutters et al. 1984). Like the pasteurellae, *Actinobacillus* species are characteristically oxidase- and catalase-positive gram-negative coccobacilli that reduce nitrate to nitrite. Several members of both genera also produce urease.

VIRULENCE FACTORS OF *PASTEURELLA MULTOCIDA* AND *MANNHEIMIA HAEMOLYTICA*

Virulence factors have been investigated primarily with *P. multocida* strains that are associated with economically important diseases in animals, such as fowl cholera, atrophic rhinitis in swine, and hemorrhagic septicemia and bronchopneumonia in cattle (Christensen and Bisgaard 1997; Horadagoda et al. 2002). Infections in animals may involve a variety of anatomic sites. Some *P. multocida* strains are encapsulated, and four capsular groups – A, B, D, and E – have been described. In cattle, hemorrhagic septicemia is associated with *P. multocida* capsular type B strains, while capsular type A strains cause respiratory distress syndromes and bronchopneumonia (Frank 1989; Horadagoda et al. 2002). Most human isolates of *P. multocida* belong to capsular group A and, to a lesser extent, group D. These capsular groups can be divided further by somatic O antigen typing. Strains of *P. multocida* that cause atrophic rhinitis in swine produce a 145-kDa dermonecrotic toxin that is an essential virulence factor for the development of this progressive respiratory tract infection (Lichtensteiger et al. 1996). This molecule also activates several cellular signal-transduction pathways (Orth et al. 2003). Toxin production is primarily associated with capsular types A and D, and the toxin gene, *toxA*, is located in a conserved region of the *P. multocida* chromosome. Toxigenic capsular type A strains associated with atrophic rhinitis and nontoxigenic capsular type A strains associated with pneumonia represent distinct subpopulations of *P. multocida* (Chen et al. 2002; Davies et al. 2002). Phenotypic and genotypic analysis of toxigenic strains have shown that these strains are not clonal, and no significant differences could be found between toxigenic strains of porcine or human origin when investigated by biochemical, serological, or genomic typing methods (Donnio et al. 1999). Molecular methods have been developed for the direct detection of the *toxA* gene in *P. multocida* (Lichtensteiger et al. 1996). The role of the dermonecrotic toxin, if any, in human disease is not known. Clinical isolates of *P. multocida* also produce

lipases, which may also be potential virulence factors for this organism (Pratt et al. 2000).

Among the pasteurellae, only *P. multocida* exhibits the broad host range that is reflected in various types of infections in a wide variety of hosts. Among the other species, *P. avium*, *P. volantium*, *G. (P.) anatis*, *P. langaa*, and *P. gallinarum* are commensals of various avian species but may also cause respiratory tract and systemic infections in these same animals (Christensen and Bisgaard 1997; Lin et al. 2001). *P. gallinarum*, in particular, is associated with various types of pathological lesions in poultry. *P. testudinis* has been isolated from tortoises living in the Mojave and Sonora deserts of the USA. This species can be recovered from the respiratory tracts of clinically healthy animals as well as from tortoises with upper respiratory tract disease (Snipes et al. 1995).

M. haemolytica strains are well recognized as the etiologic agent of bovine pneumonic pasteurellosis or shipping fever. In cattle, this infection is triggered by overcrowding, stress, immunosuppression, and concomitant viral respiratory tract infection. These species also cause diseases in pigs, sheep, and hares, with *M. haemolytica* being the most frequently isolated pathogen (Angen et al. 2002). *M. haemolytica* strains isolated from cattle with pneumonic pasteurellosis produce a leukotoxin that is species-specific and has cytolytic activity against ruminant lymphoid cells (Highlander 2001; Jeyaseelan et al. 2002). The leukotoxin is a 105–108-kDa protein that belongs to the RTX toxin family, which also includes the *E. coli* hemolysin, the *B. pertussis* adenylate cyclase/hemolysin, and the *A. pleuropneumoniae* Apx toxin (Saadati et al. 1997). Repeats in toxin (RTX) refers to the presence of characteristic, glycine-rich repeat units at the C-terminus of the peptide molecule. At low concentrations, *M. haemolytica* leukotoxin activates neutrophils, induces formation of inflammatory cytokines, and causes cytoskeletal changes that lead to apoptosis (Stevens and Czuprynski 1996). At higher concentrations, the toxin causes pore formation in the cell membranes, which results in cell swelling and lysis. In vitro, the leukotoxin causes cytopathic changes in the bovine alveoli, where it is associated with the membranes of degenerating macrophages and neutrophils. Leukotoxin-negative mutants of *M. haemolytica* show a significant reduction in virulence in animal models in comparison with leukotoxin-producing strains (Tatum et al. 1998). In addition, differences in the incidence and severity of ovine pneumonic pasteurellosis and lipopolysaccharide (LPS) chemotypes suggest an important role for the LPS in determining host-species susceptibility to lung infection (Hodgson et al. 2003). Other virulence factors associated with iron acquisition and surface adhesins homologous with those found in nontypable strains of *Haemophilus influenzae* (i.e. Hia, Hsf, HMW1, and HMW2) have also been found in *M. haemolytica* (Lo 2001). Fibrinogen-binding proteins have been described that may be important virulence factors in the pathogenesis of fibrinous pneumonia caused by *M. haemolytica* and *P. trehalosi* (McNeil et al. 2002).

PASTEURELLA MULTOCIDA

Clinical significance

P. multocida is the most frequently recovered *Pasteurella* species from human specimens and is also recovered from a wide variety of animals. The organism can commonly be cultured from the oral cavities of healthy domesticated cats (70–90 percent) and dogs (40–66 percent) and is also found in a wide variety of other animals, including cattle, horses, swine, sheep, fowl, rodents, rabbits, monkeys, lions, panthers, lynx, birds, reindeer, and buffalo (Talan et al. 1999). In some animals, this organism causes serious infections, including shipping fever and hemorrhagic septicemia in cattle, cholera in fowl, atrophic rhinitis in swine, and pleuritis, pneumonia, abscess formation, chronic rhinitis, otitis media, and septicemia in laboratory rabbits (Christensen and Bisgaard 1997; Horadagoda et al. 2002).

The majority of human infections are wound infections associated with bites and scratches from cats and dogs. These infections are characterized by rapid development of pain, erythema, swelling, cellulitis with or without abscess formation, and purulent or serosanguinous drainage at the wound site (Westling et al. 2000). Bone and joint infections may result from implantation of *P. multocida* into the bone and joint spaces by a traumatic bite, by hematogenous seeding of the joint spaces, or by contiguous spread from an adjacent cellulitis. Bacteremia occurs predominantly in the settings of preexisting liver disease (e.g. cirrhosis) or other underlying conditions, such as solid neoplasms, hematologic malignancies, systemic lupus erythematosus, and human immunodeficiency virus (HIV) infection (Raffi et al. 1987). However, profound sepsis due to *P. multocida* has been seen following animal bites in individuals with no underlying disease (Ruiz-Irastorza et al. 1995). In patients with bacteremia, hematogenous dissemination may result in intraabdominal infection, meningitis, pneumonia, septic arthritis and bursitis, and vertebral osteomyelitis (Raffi et al. 1987). *P. multocida* can also be recovered from the upper and lower respiratory tracts, where it may exist as a commensal or as a cause of epiglottitis, pneumonia, empyema, lung abscess, adult respiratory distress syndrome, bronchitis, sinusitis, tonsillitis, and otitis media (Ory et al. 1998; Wine et al. 1997). *P. multocida* is an infrequent cause of intraabdominal infections, including postsurgical wound infections, spontaneous bacterial peritonitis, appendicitis, and intraabdominal and splenic/hepatic abscesses (Chun et al. 2003; Raffi et al. 1987). Patients who develop these infections invariably have underlying diseases such as cirrhosis, solid-organ tumors, and/or hematologic malignancies.

P. multocida may be a rare cause of central nervous system infections (e.g. meningitis, brain abscess), ocular infections (e.g. keratitis, endophthalmitis), and both native and prosthetic valve endocarditis (Genne et al. 1996; Hoffman et al. 1987; Layton 1999). With this wide clinical spectrum, *P. multocida* may be recovered from a variety of clinical specimens, particularly from immunocompromised hosts.

Cultural characteristics and identification

P. multocida grows well on chocolate and sheep blood agar, forming smooth gray colonies that are 0.5–2.0 mm in diameter after 24-h incubation at 35–37°C in 5–7 percent CO_2. The organism is nonhemolytic and does not grow on MacConkey agar or other types of selective or differential enteric media. Isolates from respiratory tract specimens may be mucoid. A characteristic odor (like an *E. coli*, but more pungent) is frequently noted, perhaps due to the formation of large amounts of indole by the organism. *P. multocida* is oxidase-positive, catalase-positive, ornithine decarboxylase-positive, indole-positive, and urease-negative. The spot indole test using the *p*-aminocinnamaldehyde reagent is usually strongly positive. *P. multocida* produces acid, but no gas, from glucose, sucrose, and mannitol, but not from maltose or lactose. For typical isolates recovered from likely sources, such as a cat bite or scratch, the demonstration of oxidase-positive, gram-negative bacilli that are strongly spot indole-positive and fail to grow on MacConkey agar is usually sufficient to presumptively identify *P. multocida*. *P. multocida* strains may be further broken down into three subspecies (subspecies *multocida*, *septica*, and *gallicida*) based on acid production from sorbitol and dulcitol. Identification to subspecies is of interest in the study of veterinary isolates but is not particularly relevant or important for human clinical isolates.

Antimicrobial susceptibility

Antimicrobial susceptibility testing indicates that *P. multocida* isolates are broadly susceptible to a variety of antimicrobial agents in vitro. The organism is susceptible to penicillin, ampicillin, amoxicillin-clavulanate, broad-spectrum penicillins (i.e. ticarcillin, piperacillin, mezlocillin), expanded-spectrum cephalosporins (i.e. cefotaxime, cefoperazone, cefuroxime, ceftazidime, ceftizoxime), tetracyclines, and chloramphenicol (Cormican and Jones 1995; Goldstein and Citron 1988; Noele and Teele 1986). Less activity has been noted with the first-generation cephalosporins, such as cephalothin and cefazolin, and with the semisynthetic penicillins (Goldstein and Citron 1988; Goldstein et al. 1988; Noele and Teele 1986). Aminoglycosides have moderate to poor activity against *P. multocida* strains in vitro, and the organism is resistant to vancomycin and clindamycin (Goldstein and Citron 1988). Among the macrolide antibiotics, azithromycin, clarithromycin, roxithromycin, and dirithromycin have greater activity against *P. multocida* than erythromycin (Goldstein and Citron 1993; Goldstein et al. 1999a). *P. multocida* strains are also very susceptible to the fluoroquinolones and oxazolidinones, but clinical data supporting their efficacy as chemotherapeutic agents are limited (Goldstein and Citron 1988, 1993; Goldstein et al. 1999a, b). Animal isolates of *P. multocida* are often resistant to tetracycline due to the presence of a transposon-associated *tet*(*H*) tetracycline-resistance determinant (Hansen et al. 1996). For therapy of *P. multocida* infections in cattle and pigs, aspoxicillin, ceftiofur, and amoxicillin have minimal inhibitory concentration (MIC) values well within the therapeutic range (Chang and Carter 1976; Yoshimura et al. 2001). Despite the in vitro susceptibility of *P. multocida* to many agents, clinical experience with many of them is limited and, therefore, precludes their being recommended for empiric therapy. At the present time, penicillin and the expanded-spectrum parenteral cephalosporins remain the agents of choice for treatment of infections caused by *P. multocida*. Trimethoprim-sulfamethoxazole and ciprofloxacin may be acceptable therapeutic alternatives to parenteral penicillin and cephalosporins when oral therapy is preferred in cases following animal bites (Lion et al. 1996b).

Resistance to penicillin via plasmid-mediated β-lactamase production is fairly uncommon in *P. multocida* and was reported first among animal isolates. In 1991, the first β-lactamase-producing *P. multocida* isolate from a human was recovered from a respiratory tract specimen (Rosenau et al. 1991). This isolate was resistant to penicillin, ticarcillin, cephalothin, sulfonamides, and tetracyclines; clavulanate and sulbactam reduced the MIC of the isolate to β-lactam drugs by 64-fold. This β-lactamase was characterized as a ROB-1-type β-lactamase according to substrate and inhibition specificities and isoelectric point determinations.

OTHER *PASTEURELLA* SPECIES ISOLATED FROM HUMAN INFECTIONS

Pasteurella pneumotropica

P. pneumotropica is part of the respiratory tract flora of dogs, cats, rats, and mice. In laboratory rodents, the organism causes lower respiratory tract infections and abscesses (Artwohl et al. 2001). Man acquires the organism via traumatic exposure to animals, such as dog and cat bites. Human infections attributed to *P. pneumotropica*include meningitis, bacteremia with shock, bone and joint infections, wound infection, cellulitis, and pneumonia (Cuadrado-Gomez et al. 1995; Minton 1990). *P. pneumotropica* peritonitis in a dialysis patient has also

been described as a consequence of contamination of the dialysis tubing by a pet hamster (Campos et al. 2001). Recently, molecular methods (i.e. 16S rRNA sequencing) have been used to confirm a bacteriological diagnosis of *P. pneumotropica* septicemia in a previously healthy woman who was frequently scratched by her cats (Frebourg et al. 2002). Specimens yielding positive cultures have included wound drainage, bone fragments, joint fluid, throat swabs, urine, pleural fluid, and blood. It is likely that most descriptions of *P. pneumotropica* infections in humans are due to *P. dagmatis*, which is the former Henriksen biovar of *P. pneumotropica*.

After 24-h incubation, colonies of *P. pneumotropica* on blood agar are variable in size (0.5–1 mm in diameter), smooth, convex, and nonhemolytic. They are oxidase-positive, catalase-positive, urease-positive, indole-positive, and ornithine decarboxylase-positive (Table 2). The latter two identification tests help to differentiate *P. pneumotropica* from *A. ureae*, which is negative for these two reactions. Some strains of *P. pneumotropica* will grow on MacConkey agar. The positive urease test and differential reactions for maltose and mannitol help to differentiate this organism from *P. multocida*. Classically, strains of *P. pneumotropica* could be differentiated into two distinct biotypes – Jawetz and Heyl – on the basis of biochemical tests. A molecular method based on 16S rDNA fragment amplification random amplified polymorphic DNA analysis has been described that is able to separate these biotypes more readily than conventional phenotypic approaches (Kodjo et al. 1999).

Another species that is closely related to *P. pneumotropica* was assigned the provisional name *Pasteurella* sp. new species 1 or *Pasteurella* gas (Carter 1984). It has also been isolated from animal bites, miscellaneous specimens from humans, and oropharyngeal specimens from dogs and cats. This organism resembles *P. pneumotropica* in cultural characteristics and biochemical reactions. Colonies tend to be slightly larger than those of *P. pneumotropica* after 24-h incubation. Like *P. pneumotropica*, it is indole-, urease-, and maltose-positive, but it can be differentiated by lack of ornithine decarboxylase activity and failure to produce acid from xylose. Urease, ornithine decarboxylase, and acid production from maltose are helpful for differentiating this species from *P. multocida*. Urease-negative *P. pneumotropica*-like isolates have also been referred to as *Pasteurella* species taxon 16 (Bisgaard and Mutters 1986). Some isolates of *Pasteurella* gas produce small amounts of gas from glucose and other carbohydrates. Some members of this taxon, plus some other *P. pneumotropica*-like organisms have been reclassified as *P. dagmatis*. *P. pneumotropica* isolates are usually susceptible to penicillin, ampicillin, first-, second-, and third-generation cephalosporins, piperacillin, tetracycline, erythromycin, chloramphenicol, and ciprofloxacin, and most strains are susceptible to the aminoglycosides and trimethoprim-sulfamethoxazole (Cuadrado-Gomez et al. 1995).

Pasteurella aerogenes

P. aerogenes is a part of the oropharyngeal and intestinal flora of swine. Rare human infections have resulted from bites, tusk wounds, and other occupational exposures to pigs and boars (Lester et al. 1993; Ejlertsen et al. 1996). This organism is also associated with abortion and stillbirth in animals, including pigs, dogs, and rabbits (Fodor et al. 1992). *P. aerogenes* was isolated from the ears and throat of a stillborn child delivered by a women in the thirty-first week of pregnancy (Thorsen et al. 1994). The same organism was isolated from vaginal cultures following the delivery. On investigation, the authors learned that the woman worked as an assistant on a pig farm in Denmark. It was not known whether the organism infected the fetus hematogenously or by ascending genital tract infection. *P. aerogenes* was also reported as a cause of vertebral osteomyelitis in a previously healthy 62-year-old male without a history of animal contact, pre-existing disease, or immunosuppression (Quiles et al. 2000).

P. aerogenes produces smooth, convex, circular, nonhemolytic colonies on blood agar and also grows on MacConkey agar. Most strains are catalase-positive, oxidase-variable, indole-negative, and urease-positive; most isolates also produce ornithine decarboxylase (Table 2). As the name implies, this species is aerogenic, meaning that gas is produced from glucose during fermentation. *P. aerogenes* isolates recovered from human wounds have been susceptible to ampicillin, cephalothin, cefotaxime, cefuroxime, aminoglycosides, ciprofloxacin, and tetracycline (Ejlertsen et al. 1996). Strains recovered from animals may be resistant to ampicillin and cephalothin due to β-lactamase production and are also frequently resistant to tetracyclines.

Pasteurella canis and *Pasteurella stomatis*

Both *P. stomatis* and *P. canis* have also been isolated from wound infections resulting from dog bites (Holst et al. 1992; Pouedras et al. 1993; Talan et al. 1999). *P. canis* strains are divided into two biotypes; biotype 1 is found in the oral cavity of dogs, whereas biotype 2 has been recovered from calves (Mutters et al. 1985a). In the study conducted by Holst and colleagues, 28 of 159 strains examined were identified as *P. canis*; all of these were from dog-bite wounds and belonged to biotype 1 (Holst et al. 1992). Ten of the 159 strains were identified as *P. stomatis*; eight were from dog-bite wounds and two were from abscesses. In the eight *P. stomatis* wound infections, *P. multocida* or *P. canis* were coisolated with *P. stomatis*.

P. canis and *P. stomatis* isolates are oxidase- and catalase-positive. Most strains of *P. canis* are indole-positive, ornithine decarboxylase-positive, and urease-negative. *P. stomatis* strains are also usually indole-positive (reac-

tions may be weak) and urease-negative, but they are ornithine decarboxylase-negative (Table 1). *P. canis* and *P. stomatis* are susceptible to ampicillin, amoxicillin-clavulanate, cephalothin, cefotaxime, linezolid, the aminoglycosides, tetracycline, piperacillin, and the fluoroquinolones (Goldstein et al. 1998, 1999a, b; Holst et al. 1992; Pouedras et al. 1993).

Pasteurella dagmatis

P. dagmatis is a part of the oral flora of dogs and cats and is associated with bites and scratches from these animals. Of 159 *Pasteurella* strains submitted to a veterinary laboratory in Denmark, five isolates were identified as *P. dagmatis* (Holst et al. 1992). Human infections with this organism included single isolates from a cellulitis, a groin abscess, a throat abscess, and two isolates from dog-bite wounds. *P. dagmatis* has also been recovered from cat-bite wounds (Zbinden et al. 1988). This organism is a rare cause of pneumonia, bacteremia, and endocarditis (Fajfar-Whetstone et al. 1995; Rosenbach et al. 2001; Sorbello et al. 1994).

After 24 h, colonies of *P. dagmatis* are 1–3 mm in diameter and are nonhemolytic on sheep blood agar. This organism is oxidase- and catalase-positive, indole-positive, urease-positive, and ornithine decarboxylase-negative, and produces small amounts of gas from glucose (some isolates of this species were previously referred to a *Pasteurella* gas) (Table 1). No growth is seen on MacConkey agar. *P. dagmatis* isolates from human infections have been susceptible to penicillin, ampicillin, ticarcillin, cephalosporins and cephamycins, aminoglycosides, tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole (Fajfar-Whetstone et al. 1995; Sorbello et al. 1994).

Pasteurella bettyae

P. bettyae (formerly known as CDC group HB-5 and then as *P. bettii*) has been isolated primarily from the genitourinary tract and related specimens (i.e. vagina, cervix, urethra, and amniotic fluid). In 1989, a cluster of five patients with urethritis, pelvic inflammatory disease, or Bartholin's gland abscesses was identified in Tennessee, USA, in which *P. bettyae* was isolated as the etiologic agent. This outbreak suggested that the organism may be a sexually transmitted pathogen (Baddour et al. 1989). *P. bettyae* may also be associated with genital ulcer disease. In a study performed in Rwanda, the organism was isolated from 25 (3.6 percent) of 675 patients (204 women and 471 men) with genital ulcer disease, but from only one of 983 patients without genital ulcer disease (Bogaerts et al. 1990). Of 145 men with a urethal discharge but without genital ulcers, *P. bettyae* was isolated from only one patient. In 1996, *P. bettyae* was isolated from blood cultures of a 25-year-old woman with peripartum bacteremia and as a cause of fatal pneumonia and pleural effusion in a 40-year-old man with HIV infection (Moritz et al. 1996; Shapiro et al. 1996).

P. bettyae is a gram-negative coccobacillus that grows on both blood agar and chocolate agar; ability to grow on MacConkey agar is variable. After 24 h of incubation in a CO_2 environment, colonies are 0.5–1 mm in diameter, nonhemolytic, smooth, and white. The organism is oxidase-variable or delayed, although positive results are usually obtained with the tetramethylphenylene diamine reagent. The catalase reaction is usually negative. Nitrate is reduced to nitrite, but not to gas, and ornithine decarboxylase and urease are not produced. Acid is formed from glucose (along with gas) and sometimes mannose, but not from maltose, sucrose, lactose, mannitol, sorbitol, trehalose, or xylose (Table 2). Indole production after overnight incubation in tryptone broth is a variable reaction. The amount of indole formed may be small, so xylene extraction of the broth and use of Ehrlich's indole reagent are often required for its detection.

P. bettyae isolates are usually susceptible to ampicillin, cephalosporins, aztreonam, imipenem, fluoroquinolones, aminoglycosides, trimethoprim, and trimethoprim-sulfamethoxazole, and are resistant to erythromycin, clindamycin, vancomycin, and tetracycline. All five isolates recovered in the Tennessee cluster were susceptible to ampicillin, whereas seven of the 24 isolates tested from the Rwanda study were ampicillin-resistant and produced a β-lactamase (Baddour et al. 1989; Bogaerts et al. 1990).

Pasteurella caballi

P. caballi is an inhabitant of the upper respiratory tract of horses (Blackall et al. 1997; Schlater et al. 1989). This organism has also been isolated in pure and/or mixed culture from equine infections, including endocarditis, pneumonia, peritonitis, wounds, abscesses, and genital tract infections (Church et al. 1998; Schlater et al. 1989). Documented cases of human *P. caballi* infection include an inflammatory, fluctuant finger lesion that occurred in a veterinarian who worked with horses and ponies, and from a horse-bite wound (Bisgaard et al. 1991; Escande et al. 1997). Isolates of *P. caballi* are usually oxidase-positive, catalase-negative, and nonhemolytic on sheep blood agar, and do not grow on MacConkey agar. Indole and urease are not produced, although some strains are ornithine decarboxylase-positive. *P. caballi* strains are broadly susceptible to antimicrobial agents; some strains, however, are resistant to lincomycin, streptomycin, and sulfonamides and are intermediate in susceptibility to penicillin G (Schlater et al. 1989). The isolate obtained from the veterinarian was susceptible to all antimicrobials tested, except penicillin and the sulfonamides (Bisgaard et al. 1991).

Pasteurella gallinarum

P. gallinarum is a gram-negative coccobacillus that primarily inhabits the respiratory tract of domestic fowl, although isolates have also been obtained from pigs and rats (Christensen et al. 2002c). The first report of a human infection with *P. gallinarum* appeared in 1995, when the organism was recovered from blood cultures of a 12-year-old Saudi male who developed endocarditis 10 years after surgical repair of a ventricular septal defect (Al Fadel Saleh et al. 1995). The isolate in this report was susceptible to penicillin, ampicillin, and cefotaxime, and the patient responded to 6 weeks of therapy with the latter two agents. Interestingly, the patient did not report any bites, scratches, or contact with any animals. A second report of a human infection appeared in 1999 from Japan, when *P. gallinarum* was isolated from blood cultures of a 34-year-old man with acute gastroenteritis (Arashima et al. 1999).

Isolates of *P. gallinarum* are oxidase- and catalase-positive and nonhemolytic on sheep blood agar and do not grow on MacConkey agar. Indole, urease, and ornithine decarboxylase are not produced. This isolate was susceptible to ampicillin, cefazolin, cefotaxime, azithromycin, imipenem, norfloxacin, and the aminoglycosides, and the patient responded to cefazolin therapy.

MANNHEIMIA SPECIES

Members of the former *P. haemolytica* complex are rarely isolated from humans, and infections have usually resulted from occupational or recreational exposure to animals. Rivera and colleagues reported a case of aortic graft infection due to both *P. haemolytica* biotype A (*M. haemolytica*) and group C β-hemolytic streptococci in a 50-year-old man (Rivera et al. 1994). Yaneza and colleagues reported a case of endocarditis due to this organism (Yaneza et al. 1991). This patient had no contact with cattle or other farm animals. *M. haemolytica* isolates are susceptible to penicillin, ampicillin, amoxicillin-clavulanate, macrolides (i.e. azithromycin, clarithromycin, erythromycin), chloramphenicol, the aminoglycosides, and the fluoroquinolones, but are resistant to linezolid (Goldstein et al. 1999a, b). Biochemical characteristics for the identification of *Mannheimia* species are found in Table 3.

GALLIBACTERIUM SPECIES

G. anatis and *Gallibacterium* genomospecies 1 are causes of salpingitis, peritonitis, and respiratory tract infections in chickens (Christensen et al. 2003; Lin et al. 2001). These organisms have not been isolated from infections in humans.

ACTINOBACILLUS SPECIES

TAXONOMY

Actinobacillus species are small, gram-negative, nonmotile, nonsporeforming bacilli that are predominantly found in animals. Besides the association with animals, these organisms are similar to members of the genus

Table 3 *Phenotypic characteristics for identification of* Mannheimia *species*

Characteristic	*M.haemolytica*	*M. granulomatis*	*M. glucosida*	*M. ruminalis*	*M. varigena*
Hemolysis	+	–	+	–	+
Oxidase	+	d	+	V	+
Catalase	+	d	+	+	+
Alkaline phosphatase	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Urease	–	–	–	–	–
Indole	+	–	–	–	–
Lysine decarboxylase	–	–	–	–	–
Ornithine decarboxylase	–	–	V	–	V
Glucose, gas	–	–	–	–	–
Acid from:					
Galactose	+	+	+	+	+
Sucrose	+	+	+	+	+
L-Arabinose	–	–	V	–	+
Inositol	V	V	(+)	–	V
Salicin	–	–	V	–	V
Esculin	–	V	V	–	–
Mannitol	+	+	+	+	+
Sorbitol	+	+	+	V	–
Dulcitol	–	–	–	–	–

+, 90 percent or more of the strains positive within 1–2 days; (+), 90 percent or more of the strains positive within 3–14 days; –, less than ten percent of the strains positive within 14 days; d, delayed reaction; V, 11–89 percent of the strains positive; $+^{w}$, weak positive.

Pasteurella in many of their cultural and phenotypic characteristics. The genus *Actinobacillus* is heterogeneous and contains *Actinobacillus* species defined on the basis of hybridization and sequencing data as species sensu stricto, as well as species located outside the genus either in prospective genera of Pasteurellaceae or other families. The established species *A. lignieresii*, *A. suis*, *A. capsulatus*, *A. equuli*, *A. pleuropneumoniae* (formerly *Haemophilus pleuropneumonaie*), and *A. hominis* represent the core group of true actinobacilli. In 1986, the former *Pasteurella* species *P. ureae* was formally transferred to the genus *Actinobacillus* as *Actinobacillus ureae*; this species is also a member of the genus sensu stricto (Dewhirst et al. 1992; Mutters et al. 1986). Other *Actinobacillus* species that have been described, such as *A. capsulatus*, *A. muris*, and *A. seminis*, clearly belong to other genera based on DNA–rRNA hybridization and 16S rRNA sequencing (Dewhirst et al. 1992; Mutters et al. 1986). The genus also includes some of Bisgaard́s taxa; Bisgaard taxon 9 is now classified as *A. arthritidis* and Bisgaard taxon 11 is now classified as *A. equuli* subsp. *haemolyticus* (Blackall et al. 1997; Christensen et al. 2002a, b). *Actinobacillus* genomospecies 2 represent *A. lignieresii*-like strains isolated from horses (Christensen et al. 2002a). Bisgaard taxon 5 remains unnamed at this time. The former Bisgaard taxon 12 has been named as *A. muris*. *A. seminis* and *A. rossii* are located outside the genus *Actinobacillus* (Foster et al. 1999). *A. scotiae*, *A. succinogenes*, *A. delphinicola*, *A. minor*, *A. indolicus*, and *A. porcinus* have also been classified as additional *Actinobacillus* (Foster et al. 1996, 1998; Guettler et al. 1999; Maller et al. 1996). *A. salpingitidis*, which includes biovars 5 and 8 of the avian *P. haemolytica/P. anatis/A. salpingitidis* complex, has been reclassified as genomospecies 1 of the newly described Pasteurellaceae genus *Gallibacterium* (which also includes the former *Pasteurella anatis* as *Gallibacterium anatis*) on the basis of a polyphasic approach using 16S rRNA sequencing, DNA–DNA hybridization, pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and other techniques (Christensen et al. 2003). The biochemical characteristics for identification of *Actinobacillus* species sensu stricto are found in Table 4, while those species of provisional taxonomic status are found in Table 5.

As mentioned, most *Actinobacillus* species are associated with animals. Members of the genus and their animal associations include *A. lignieresii* (horses), *A. equuli* (primarily horses), *A. capsulatus* (rabbits), *A. muris* (mice), *A. seminis* (sheep, rams), *A. delphinicola* (sea mammals), *A. scotiae* (porpoises), and *A. succinogenes* (bovine rumen). *A. pleuropneumoniae*, *A. suis*, *A. rossii*, *A. minor*, *A. porcinus*, and *A. indolicus* are found in the respiratory tract of swine. Genetically confimed *A. suis* has also been isolated from a cat (Daignault et al. 1999). Three species – *A. actinomycetemcomitans*, *A. ureae*, and *A. hominis* – are found in humans.

Members of the genus *Actinobacillus* are small coccobacillary, nonsporeforming, gram-negative bacteria. On blood and chocolate agar, they form small, translucent

Table 4 *Phenotypic charcteristics for identification of* Actinobacillus *species sensu stricto*

Characteristic	*A. lignieresii*	*A. equuli*	*A. pleuro-pneumoniae*	*A. suis*	*A. arthritidis*	*A. hominis*	*A. ureae*
Oxidase	+	+	+	+	+	+	+
Catalase	+	V	+	+	–	+	
Alkaline phosphatase	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+
NAD dependence	–	–	+	–	–	–	–
Ornithine decarboxylase	–	–	+	–	–	–	–
Esculin hydrolysis	–	–	–	+	–	d	–
Acid from:							
Arabinose	V	V	–	+	–	–	–
Galactose	V	V	V	V	(+)	(+)	–
Inositol	–	–	–	–	–	–	–
Lactose	d	+	(+)	(+)	(+)	V	–
Maltose	+	+	d	+	+	+	+
Mannitol	+	+	+	–	+	+	(+)
Mannose	+	+	V	+	+	V	V
Salicin	–	–	–	+	–	V	–
Sorbitol	–	–	–	–	+	–	–
Sucrose	+	+	+	+	+	+	+
Trehalose	–	(+)	–	+	–	+	–
Xylose	(+)	+	+	+	+	+	–

+, 90 percent or more of the strains positive within 1–2 days; (+), 90 percent or more of the strains positive within 3–14 days; –, less than ten percent of the strains positive within 14 days; d, delayed reaction; V, variable; 11–89 percent of the strains positive.

Table 5 *Phenotypic charcteristics of selected* Actinobacillus *species with pending taxonomic status*

Characteristic	*A. actinomycetem-comitans*	*A. capsulatus*	*A. muris*	*A. seminis*	*A. rossii*	*A. minor*	*A. delphinicola*	*A. succinogenes*	*A. scotiae*	*A. porcinus*	*A. indolicus*
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	NA	–	+	–	–	+
Alkaline phosphatase	+	+	+	–	V	NA	NA	+	NA	NA	NA
Urease	–	+	+	–	+	+	–	–	+	–	–
NAD dependence	–	–	–	–	–	+	–	–	–	+	+
Ornithine decarboxylase	–	–	–	V	–	–	V	NA	V	–	–
Esculin hydrolysis	–	+	+	–	–	–	NA	NA	NA	NA	NA
Acid from:											
Arabinose	+	+	–	V	+	–	NA	+	–	V	–
Galactose	(+)	+	–	V	(+)	NA	–	+	V	NA	+
Inositol	–	–	V	–	+	NA	–	–	–	V	–
Lactose	–	+	–	–	(+)	+	–	+	+	V	V
Maltose	+	+	+	+	–	+	–	+	–	V	+
Mannitol	+	+	+	V	+	–	–	+	–	V	–
Mannose	+	+	+	–	–	+	+	+	–	V	+
Salicin	–	+	+	–	–	NA	–	+	–	NA	NA
Sorbitol	–	+	–	–	–	–	–	+	–	V	–
Sucrose	–	+	+	–	–	+	–	+	–	V	+
Trehalose	–	+	–	–	–	NA	–	–	–	NA	NA
Xylose	V	+	–	–	+	NA	–	+	–	NA	NA

+, 90 percent or more of the strains positive within 1–2 days; (+), 90 percent or more of the strains positive within 3–14 days; –, less than 10 percent of the strains positive within 14 days; V, 11–89 percent of the strains positive; NA, data not available.

colonies of 1–2 mm diameter. In comparison with *Pasteurella*, colonies of *Actinobacillus* species are smaller. All members of the genus *Actinobacillus* are positive for either oxidase or alkaline phosphatase. As with other members of the family Pasteurellaceae, these organisms reduce nitrate to nitrite, do not grow in Simmons's citrate medium, and do not acidify adonitol or sorbose. Acid is produced from glucose, fructose, sucrose, and xylose without gas formation, and no acid is produced from inositol and dulcitol. True *Actinobacillus* species are usually urease-positive and acidify maltose and mannitol, but not trehalose. Tests for ornithine decarboxylase, arginine dihydrolase, and indole are usually negative. The G + C content of the DNA is 40–43 mol%. The type species is *A. lignieresii*.

A. actinomycetemcomitans, the principal human isolate, shares many cultural and biochemical characteristics with the haemophili. Transformation, DNA hybridization, 16S rRNA sequence data, and immunodiffusion studies have demonstrated that *A. actinomycetemcomitans* is more closely related to *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* than to the other *Actinobacillus* species (Olsen et al. 1999; Tanner et al. 1982). In 1985, a proposal was made to transfer *A. actinomycetemcomitans* to the genus *Haemophilus* as *Haemophilus actinomycetemcomitans* (Potts et al. 1985). However, this proposal was rejected due to a lack of similarity to the type species of genus *Haemophilus* (i.e. *H. influenzae*). Others have argued that *A. actinomycetemcomitans* and *H. aphrophilus* should not be included in the genus *Haemophilus* because they do not require X or V factors. However, recently described species from swine – *A. minor*, *A. porcinus*, and *A. indolicus* – are all V-factor-dependent, as is *A. pleuropneumoniae*. At the present time *Actinobacillus* species, including *A. actinomycetemcomitans*, remain in the family Pasteurellaceae, which is now included among the γ-Proteobacteria (Garrity and Holt 2000).

VIRULENCE AND PATHOGENICITY OF *ACTINOBACILLUS* SPECIES

Actinobacillus actinomycetemcomitans

The most important *Actinobacillus* in human infections, *A. actinomycetemcomians* is implicated in periodontal infections (see below), but the exact role of the organism in these infectious processes is not clear. Several potential virulence factors have been identified in strains of *A. actinomycetemcomitans*. Isolates produce a leukotoxin, which is a 116-kDa protein that binds to cell membranes of neutrophils, monocytes, and T-lymphocytes (Taichman et al. 1980). In contrast to other RTX toxins, the leukotoxin of *A. actinomycetemcomitans* exists in both cell-associated and extracellular forms (Kachlany et al. 2000). Highly leukotoxic forms appear to exhibit increased pathogenic potential (Guthmiller et al. 2001). The toxin induces pore formation in cell membranes, causes degranulation of lysosomal contents to the polymorphonuclear cell surface, induces cleavage and fragmentation of chromosomal DNA, possibly by activation of endogenous nucleases, and protects the organism from phagocytosis (Johansson et al. 2000; Mangan et al. 1991). Production of leukotoxin in the supragingival area may result in localized degranulation of polymorphonuclear leukocytes (PMN) and release of lysosomal contents that may enhance inflammation and cause the localized immunosuppression central to the development of periodontal lesions. This toxin is encoded by an operon consisting of four genes (*ltxC*, *ltxA*, *ltxB*, and *ltxD*), of which *ltxA* is the structural gene (Kolodrubetz et al. 1996). The ltxC protein is required for activation of the leukotoxin protein, while the *ltxB* and *ltxD* gene products are involved in secretion of the toxin polypeptide (He et al. 1999). Essentially all *A. actinomycetemcomitans* strains contain the leukotoxin operon, but the level of leukotoxin produced by different strains varies (Hritz et al. 1996). Some highly leukotoxic strains of *A. actinomycetemcomitans* produce ten to 20 times more leukotoxin than minimally leukotoxic strains due to the presence of a 530-bp deletion within the toxin promoter region or to the presence of an insertion sequence element adjacent to the leukotoxin promoter region (Brogan et al. 1994; Haubek et al. 1995; He et al. 1999). These highly toxic clones are associated with localized juvenile periodontitis and other severe periodontal infections (Zambon et al. 1996).

In addition to leukotoxin, several other putative virulence factors have been described in strains of *A. actinomycetemcomitans*. Fresh isolates possess long fimbriae that are maximally produced under anaerobic conditions and are lost on serial subculture (Scannapieco et al. 1987). These fimbriae are composed of a 52–54-kDa subunit protein. Fimbriated cells aggregate spontaneously, demonstrate increased adherence to hydroxyapatite beads, and promote adherence to and colonization of oral mucosal surfaces and tissues, respectively (Mintz and Fives-Taylor 1994a). A gene locus was recently identified in *A. actinomycetemcomitans* strains that had greater than 90 percent sequence identity with the cytotoxin distending gene (*cdt*) of some pathogenic *E. coli* strains and *H. ducreyi* (Mayer et al. 1999). Strains possessing this gene produced toxin in vitro, while naturally occurring toxin-negative strains had large deletions within the *cdt* gene locus itself. In vitro, cytolethal distending toxin causes distention and death of Chinese hamster ovary and other cell lines and is able to cause cell cycle arrest of lymphocytes (Tan et al. 2002). Among the periodontopathogenic bacterial species found in the oral cavity, *A. actinomycetemcomitans* is the only one that has been found to produce this toxin (Yamano et al. 2003). The cytotoxic and immunosuppressive properties of this toxin may function to cripple the host immunity and contribute to the pathogenesis of aggressive periodontitis.

A. actinomycetemcomitans is able to actively invade human cells and cell lines (Brissette and Fives-Taylor 1999). This ability may protect the organism from humoral and cellular immune factors and from the effects of some antimicrobial agents. Cellular invasion by *A. actinomycetemcomitans* involves both actin-dependent and actin-independent pathways. Invasiveness is not associated with any particular strain type as defined by restriction fragment length polymorphism analysis, and leukotoxin production is seen more frequently in noninvasive strains of *A. actinomycetemcomitans* (Lepine et al. 1998). A surface polysaccharide antigen of *A. actinomycetemcomitans* has also been shown to stimulate bone resorption in a mouse bone culture system and and to induce osteoclast-like cells in mouse bone-marrow tissue cultures (Ueda et al. 1995). Alveolar bone loss during the pathogenesis of periodontal infections such as localized juvenile periodontitis may reflect the activity of this antigen in vivo. Ochiai and colleagues isolated and purified an immunosuppressive fraction (ISF) from *A. actinomycetemcomitans*, immunized animals with ISF, and performed adoptive transfer experiments to provide sensitized T-cells to unimmunized mice (Ochiai et al. 1994) These ISF-induced T-cells exerted an immunosuppressive effect on B-cell function and on the production of antibodies against *A. actinomycetemcomitans*. ISF may act as a virulence factor by suppressing localized antibody production, thereby preventing opsonization and microbial clearance. Lastly, the cell wall LPS of *A. actinomycetemcomitans* possesses endotoxic properties similar to those of other bacteria. In the gingival crevices, LPS stimulates osteoclasts to initiate bone resorption, activates complement, and induces lysosomal release from polymorphonuclear leukocytes.

A. actinomycetemcomitans strains also produce several enzymes and other factors that may contribute to virulence. Enzymes that are able to degrade immunoglobulins G, M, and A are produced by *A. actinomycetemcomitans* and may prevent opsonization by antibody (Gregory et al. 1992). Cell-associated and extracellular components are also produced that are able to bind the Fc region of immunoglobulin molecules (Tollo and Helgeland 1991). Release of these molecules from the organism may contribute to periodontitis by interfering with bacterial opsonization, phagocytic activity of polymorphonuclear cells, and fixation of complement by antigen–antibody complexes. This binding component is apparently a 25–32-kDa outer-membrane protein (OMP) related to the OmpA of *E. coli* (Mintz and Fives-Taylor 1994b). *A. actinomycetemcomitans* strains also produce collagenases and proteases that may act on connective tissue and basement-membrane collagens (Gazi et al. 1997; Robertson et al. 1982). These enzymes may function to facilitate spread of the organism in the soft tissue and to generate small peptides that have biological activity as mediators of inflammation.

Actinobacillus pleuropneumoniae

In animal production facilities *A. pleuropneumoniae* is a significant pathogen of swine. The species causes a severe and often fatal fibrinous hemorrhagic bronchopneumonia as well as acute infections. *A. pleuropneumoniae* produces three different RTX toxins, the so-called Apx toxins. Similar toxins are produced by porcine *A. suis* and *A. rossii* (Kamp et al. 1994; Kuhnert et al. 2003; Schaller et al. 2000). In addition to RTX toxins, *A. pleuropneumoniae* strains express transferrin-binding proteins and urease during infection. It could be shown that urease activity may cause sufficient impairment of the local immune response. The ability to utilize transferrin-bound iron is presumably required for multiplication and persistence of *A. pleuropneumoniae* in the porcine respiratory tract. Construction of bacterial mutants with deletions in both transferrin-binding protein genes resulted in strains that were unable to cause symptomatic infections and to engender an immune response. These transferrin-binding proteins could be regarded as virulence factors of *A. pleuropneumoniae* (Baltes et al. 2001, 2002).

ACTINOBACILLUS ACTINOMYCETEMCOMITANS

Clinical significance

A. actinomycetemcomitans is a part of the normal flora of the oral cavity, particularly in the gingival and supragingival crevices. Based on reactions of specific monoclonal antibodies with exposed, cell-surface carbohydrate moieties of the LPS, this species can be divided into six serotypes, designated a to f (Gmur et al. 1993). Serotypes a, b, and c are the most prevalent, comprising more than 80 percent of oral strains with equal frequencies (Saarela et al. 1999). While it has been suggested that certain serotypes (e.g. serotype b) may be associated with specific forms of periodontal disease, biotyping and rRNA gene probe studies have demonstrated significant phenotypic and genomic heterogeneity among strains belonging to the same serotype (Asikainen et al. 1995; Dogan et al. 1999b; Saarela et al. 1993). Different strains within the same serotype may also be detected with arbitrarily primed polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism analyses (Dogan et al. 1999a). Additional studies using sequence analysis of 16S rRNA genes, and PCR assays for leukotoxin, cytolethal distending toxin, major fimbrial subunit antigen, and serotype-specific O-polysaccharide genes have shown that *A. actinomycetemcomitans* strains can be divided into three lineages consisting of all serotype b strains, all serotype c strains, and strains belonging to serotypes a, d, e, and f (Kaplan et al. 2002). All of these serotypes and lineages

have been isolated from patients with periodontal disease, indicating that all of them have pathogenic potential.

A. actinomycetemcomitans is associated with actinomycotic infections, endocarditis, bacteremia, wound infections, and dental infections. The organism's name is derived from its recognized concomitant isolation with *Actinomyces* species from abscesses and other infections (Amrikachi et al. 2000; Kuijper et al. 1992; Zijlstra et al. 1992) *A. actinomycetemcomitans* is also a cause of both native valve and prosthetic valve endocarditis (Collazos et al. 1994; Kaplan et al. 1989; Steitz et al. 1998). Native valve endocarditis usually occurs in individuals with antecedent valvular damage due to congenital heart diseases (e.g. congenital aortic stenosis, bicuspid aortic valve disease, atrial-venticular septal defect, or mitral valve insufficiency due to rheumatic heart disease). Prosthetic mitral and aortic valve endocarditis have also been reported in individuals with porcine and mechanical valves and pacemakers, and localized complications have included pericarditis and paravalvular abscess (Van Winkelhoff et al. 1993; Wilson 1989). Predisposing factors in the development of *A. actinomycetemcomitans* endocarditis include poor dentition or recent dental manipulations. *A. actinomycetemcomitans* endocarditis usually follows an indolent course. Fever, weight loss, chills, cough, and night sweats are common, along with a heart murmur. Hepatosplenomegaly and conjunctival or splinter hemorrhages are variably present. Complications include septic emboli, coronary arteritis, vasculitis, congestive heart failure, valvular damage requiring prosthetic valve placement or replacement, infectious complications of hematogenous dissemination (e.g. endophthalmitis, glomerulonephritis, septic arthritis), and death (Binder et al. 2003; Kaplan et al. 1989; Lass et al. 1984; Steitz et al. 1998; Viallard et al. 2002).

A. actinomycetemcomitans is a recognized pathogen in the development of periodontitis, particularly in younger individuals. This organism is causally related related to a type of early-onset periodontitis called localized juvenile periodontitis (LJP) (Gustke 1998a). LJP is a disease of older children and young adults (aged 12–26 years) that is characterized by rapid degeneration and destruction of the alveolar bone supporting the first permanent molars and incisors. Subgingival bone loss in these areas results in the development of deep gingival pockets that bleed readily with probing. During the development of this condition, there is ordinarily minimal plaque and calculus accumulation and little apparent gingival inflammation (Gustke 1998a). Progression of bone loss to involve adjacent teeth leads to generalized juvenile periodontitis or rapidly progressive periodontitis. In the USA, periodontal disease associated with *A. actinomycetemcomitans* is more common among African-American, Asian, and Hispanic individuals than among Caucasians (Slots 1999).

Periodontal disease associated with *A. actinomycetemcomitans* evokes both systemic and localized immune responses. LJP patients produce high titers of serum IgG to *A. actinomycetemcomitans* serotype antigens, which are O-side chain oligosaccharides in the cell-wall LPS (Gu et al. 1998). The LPS of the organism also induces localized gingival inflammation and allows the organism to establish itself in subgingival plaque. In addition to a potent leukotoxin and an LPS, *A. actinomycetemcomitans* produces several other properties that contribute to its virulence as a periodontal pathogen (see above). Some of these factors also stimulate the production of antibodies in serum and crevicular fluid (Gu et al. 1998). The immune response to microbial antigens may help to limit the periodontal process and failure to stimulate humoral or cell-mediated responses against the organism and/or its products may be partially responsible for progression to more severe, generalized periodontal disease. In addition to LJP and other generalized forms of periodontal disease, *A. actinomycetemcomitans* is also implicated in the pathogenesis of Papillon–Lefevre syndrome, which is an inherited disease characterized by hyperkeratosis of the palms and soles and extensive periodontal destruction, resulting in the loss of both primary and permanent teeth (Velazco et al. 1999).

Other infections caused by *A. actinomycetemcomitans* result from contiguous spread of the organism from its habitat in the oral cavity or from hematogenous dissemination during bacteremia. These infections include brain abscess, cervical lymphadenitis, cellulitis, septic arthritis, osteomyelitis, vertebral discitis, and ostreomyelitis, subcutaneous and intra-abdominal abscesses, and urinary tract infections (Hammerberg et al. 1998; Kaplan et al. 1989; Molina et al. 1994; Nashi et al. 1998; Zijlstra et al. 1992). Pulmonary infections with *A. actinomycetemcomitans* in the absence of *Actinomyces* species are rare, with only four cases reported before 1990 in the English literature. In a case reported by Yuan and coworkers, the infection involved the lung, the soft tissue of the chest wall, and the overlying ribs and sternum (Yuan et al. 1992). The portals of entry for these various types of infections have included oral lesions, prior pulmonary infections, skin abrasions, thoracotomy sites, and urinary tract instrumentation.

Cultural characteristics and identification

A. actinomycetemcomitans grows slowly on chocolate and blood agars, with visible colonies appearing after 48–72 h. Colonies are small, smooth, translucent, and nonhemolytic, and have slightly irregular edges. Fresh clinical isolates are adherent to the agar and are difficult to emulsify. With prolonged incubation (i.e. 5–7 days), colonies may develop a central density that appears as a four- or six-pointed star. This characteristic is lost on repeated subculture and the colonies become less adherent. As with *H. aphrophilus*, growth in broth is scant and adherent to the sides of the tube. On Gram stain, the organisms

appear as pale staining, gram-negative coccobacilli. Longer cells may be noted after several subcultures.

Characteristics for identification of *A. actinomycetemcomitans* include lack of growth on MacConkey and other enteric agars and positive reactions for catalase production and nitrate reduction (Table 5). The organism is oxidase-negative (occasional strains may be weakly positive), is urease-negative, does not produce indole, and does not require X or V factors. Lysine and ornithine decarboxylase and arginine dihydrolase reactions are negative. Most strains strongly ferment glucose, fructose, and mannose; acid production from maltose, mannitol, and xylose varies. *A. actinomycetemcomitans* can be differentiated from *H. aphrophilus* in that the former is catalase-positive and *o*-nitrophenyl-β-D-galactopyranoside (ONPG)-negative, and acid is not produced from lactose, sucrose, or trehalose.

Molecular methods for detection and identification of *A. actinomycetemcomitans* have also been described (Dogan et al. 1999a; Riggio and Lennon 1997). Genetic studies have shown that the gene for the 23S ribosomal RNA is split into two smaller forms in *A. actinomycetemcomitans*, while the transcript is continuous in *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, and *H. influenzae* (Preus et al. 1992). Recognition of this atypical RNA pattern on polyacrylamide gel electrophoresis can provide accurate identification and strain differentiation. Direct probe- and amplified probe-based methods have also been developed for detection of *A. actinomycetemcomitans* and other periodontal pathogens directly in subgingival plaque samples (Komiya et al. 2000; Tran and Rudney 1999). Amplification of the *lktA* gene, the structural gene for the *A. actinomycetemcomitans* leukotoxin, has also been used for identification and direct detection of the organism in gingival fluid and subgingival plaque (Goncharoff et al. 1993). While typing and characterization of *A. actinomycetemcomitans* strains may be accomplished by serology, other traditional and molecular-based methods that have been used include antibiogram typing, biotyping based on fermentation of mannose, mannitol, and xylose, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer-membrane proteins, restriction endonuclease analysis of whole chromosomal DNA, restriction fragment length polymorphisms, and ribotyping (Asikainen et al. 1995; Han et al. 1991; Suzuki et al. 2001; Van Steenbergan et al. 1994). Molecular-based assays may be of particular value in differentiating atypical strains of *A. actinomycetemcomitans* (e.g. catalase-negative strains) from hemophili and for epidemiological studies (Riggio and Lennon 1997).

Antimicrobial susceptibility

Strains of *A. actinomycetemcomitans* are susceptible to third-generation cephalosporins (e.g. ceftriaxone, cefuroxime, cefixime, cefotaxime, ceftazidime, cefipime), fluoroquinolones (e.g. ciprofloxacin, ofloxacin, levofloxacin, clinafloxaccin), tetracycline, doxycycline, azithromycin, trimethoprim-sulfamethoxazole, rifampin, and the aminoglycosides (Kugler et al. 1999; Madinier et al. 1999; Muller et al. 2002; Pajukanta et al. 1992a, b, 1993). While amoxicillin shows good activity against *A. actinomycetemcomitans*, between 30 and 40 percent of strains are resistant to benzylpenicillin (Kugler et al. 1999; Madinier et al. 1999; Paju et al. 2000). Penicillin-resistant strains do not produce β-lactamase enzymes, so resistance is presumably due to altered penicillin-binding proteins (Paju et al. 2000). Amoxicillin-clavulanate does not demonstrate any greater activity than amoxicillin alone, which further supports the lack of β-lactamase activity (Madinier et al. 1999). In vitro susceptibility to amoxicillin does not necessarily predict a favorable clinical outcome, and because of this, the third-generation cephalosporins (i.e. ceftriaxone) are considered the drugs of choice for empiric therapy of serious infections, such as endocarditis (Wilson et al. 1995). In vitro studies indicate that combinations of antimicrobial agents for the treatment of *A. actinomycetemcomitans* endocarditis may be synergistic, additive, or antagonistic (Pavicic et al. 1992). Hence, the efficacy of combination therapy cannot be predicted and must be determined for individual strains by either an agar dilution technique or an E-test (Pajukanta et al. 1992b).

Treatment of *A. actinomycetemcomitans*-associated periodontal disease involves subgingival debridement, scaling, root planing, and antimicrobial therapy directed against periodontitis-associated bacteria. Successful treatment partially depends on the eradication and suppression of *A. actinomycetemcomitans* and other bacteria from deep periodontal pockets. The currently recommended regimen includes tetracycline, or doxycycline, or combination therapy with amoxicillin and metronidazole (Gustke 1998b). While most strains have remained susceptible to tetracyclines, the agents of choice for many years, plasmid-associated, tetb-mediated tetracycline resistance in *A. actinomycetemcomitans* has been reported (Roe et al. 1995; Walker and Karpinia 2002). The amoxicillin–metronidazole combination is associated with fewer treatment failures and effectively eradicates and suppresses subgingival growth of *A. actinomycetemcomitans* (Van Winkelhoff et al. 1992). Since most strains of *A. actinomycetemcomitans* are resistant to metronidazole in vitro, success of the combination therapy may be partially due to the activity of amoxicillin on *A. actinomycetemcomitans* and the broad activity of metronidazole against coinfecting anaerobic periodontal organisms (i.e. *Prevotella* and *Porphyromonas* species). Fluoroquinolones (e.g. ciprofloxacin, moxifloxacin) may also be effective agents for the treatment of *A. actinomycetemcomitans*-associated periodontal infections (Kleinfelder et al. 2000; Muller et al. 2002).

ACTINOBACILLUS UREAE

Genetic and phenotypic studies have shown that several *Pasteurella* species (i.e. *P. ureae*, *P. pneumotropica*, and *P. aerogenes*) are closely related to animal species in the genus *Actinobacillus* (Bia et al. 1978; Dewhirst et al. 1992; Escande et al. 1984; Mutters et al. 1984). Phenotypic similarities include similar oxidase and catalase reactions, reduction of nitrate to nitrite, growth on MacConkey agar, production of urease, and fermentation of several carbohydrates (Table 4). At present, *P. ureae* is the only species that has formally been transferred to the genus *Actinobacillus* as *Actinobacillus ureae* (Mutters et al. 1986).

A. ureae is an uncommon commensal of the human respiratory tract and a rare isolate from several types of human infections, including bacteremia, endocarditis, meningitis, bone-marrow infection, atrophic rhinitis, bronchitis, pneumonia, conjunctivitis, otitis media, and peritonitis (Avlami et al. 1997; Bigel et al. 1988; Bogaerts et al. 1985; Brass et al. 1983; Kingsland and Guss 1995; Noble et al. 1987). In most cases, an underlying or predisposing condition is present, such as postsurgical infection, diabetes, periodontal disease, emphysema, or alcohol-associated cirrhosis of the liver. Cases of meningitis have been associated with previous skull trauma (assault, cranial surgery) and with underlying disease, including HIV infection (Kaka et al. 1994; Kingsland and Guss 1995).

A. ureae is a pleomorphic gram-negative bacillus; on staining, some strains form distinct filaments. After 24-h growth on blood agar in a CO_2-enriched atmosphere, colonies are smooth, 1 mm in diameter, and nonhemolytic or weakly α-hemolytic. Some isolates may appear mucoid. The organism is usually oxidase- and catalase-positive, reduces nitrate to nitrite, does not ordinarily grow on MacConkey agar, and hydrolyzes urea rapidly (Table 4). No indole is produced, and lysine and ornithine decarboxylase and arginine dihydrolase reactions are negative. Acid is produced from glucose, maltose, sucrose, and mannitol; no acid is produced from lactose, xylose, or trehalose. The other biochemically similar *Actinobacillus* species may be differentiated from *A. ureae* by acid production from additional carbohydrates (e.g. lactose and xylose) and by the abilities of the former species to grow on MacConkey agar. Molecular methods have also been used for the diagnosis of *A. ureae* meningitis (Whitelaw et al. 2002). *A. ureae* strains are susceptible to most antimicrobial agents, including penicillin, ampicillin, cephalothin, cefoxitin, tetracycline, trimethoprim-sulfamethoxazole, and the aminoglycosides.

ACTINOBACILLUS HOMINIS

A. hominis is a rare human isolate that has been recovered from sputum specimens of individuals with chronic lung disease and from empyema fluid (Friis Møller 1981). Wust and colleagues reported on two patients with hepatic failure due to chronic hepatitis B infection and alcoholic cirrhosis, respectively, in which *A. hominis* septicemia occurred as the terminal event in their illness (Wüst et al. 1991). In a study conducted in Copenhagen on 36 patients with *A. hominis* lower respiratory tract infection and/or bacteremia, chronic alcoholism, cardiovascular disease, drug addiction, chronic psychiatric disorders, and chronic obstructive pulmonary disease were frequent predisposing factors (Friis-Møller et al. 2001). *A. hominis* strains are usually oxidase-positive and catalase-negative and do not grow on MacConkey agar. The organism is strongly urease-positive but does not produce indole or ornithine decarboxylase (Table 4).

ANIMAL SPECIES IN THE GENUS *ACTINOBACILLUS*

Actinobacillus species found in animals include *A. lignieresii*, *A. equuli* subsp. *equuli*, *A. equuli* subsp. *haemolyticus*, *A. suis*, *A. pleuropneumoniae*, *A. seminis*, *A. muris*, *A. capsulatus*, *A. rossii*, *A. delphinicola*, *A. scotiae*, *A. succinogenes*, *A. arthriditis*, *A. minor*, *A. porcinus*, and *A. indolicus* (Christensen et al. 2002a, b; Foster et al. 1996, 1998, 1999; Guettler et al. 1999; Maller et al. 1996; Phillips 1984; Sneath and Stevens 1990). Also included in this group are other unnamed taxa (i.e. Bisgaard taxa 5, and 8, and *Actinobacillus* genomospecies 2 (formerly certain strains of Bisgaard taxon 9)). These species comprise parts of the normal flora of certain animals and may occasionally cause disease in them.

A. lignieresii is associated with granulomatous infections in the oral cavity (wooden tongue disease) and gastrointestinal tracts of cattle, and pneumonic and cutaneous infections in sheep and other ungulates. Under normal conditions, *A. suis* and *A. equuli* colonize the upper respiratory tract and the oral cavity, and the genital tract of horses and pigs, but they can cause a variety of diseases in these animals. *A. equuli* can be isolated from the respiratory tracts of diseased animals and from newborn foals dying within the first week after birth with septicemia (Mair et al. 1974; Rycroft and Garside 2000). *A. equuli* may also cause bacteremia, peritonitis, nephritis, osteomyelitis, and arthritis in horses and swine and both localized and systemic infections in monkeys, calves, dogs, and rabbits. *Actinobacillus suis* has been recovered from septicemic pigs and has been associated with pneumonia, pleuritis, pericarditis, and polyarthritis in swine and horses (Peel et al. 1991). *A. pleuropneumoniae* is an important pig pathogen causing porcine pleuropneumonia, a highly contagious respiratory infection. This severe and often fatal fibrinous hemorrhagic bronchopneumonia leads to significant economic losses (Bossa et al. 2002). *A. seminis* is restricted to sheep and is a causative agent of abortion, epididymitis, orchitis, and sterility (De la Puente-Redondo et al. 2000; Foster et al.

1999). *A. muris* resembles Bisgaard taxon 12, a group of bacteria isolated from laboratory white mice, and is found as a normal component of the oropharyngeal and genital tract flora of these animals (Bisgaard 1986). *A. capsulatus* causes arthritis in rabbits, and *A. rossii* is found in the vaginas of postparturient sows and aborted piglets (Blackall et al. 1997). *A. delphinicola* and *A. scotiae* have been isolated postmortem from different pathological lesions of the lung, cervix, and uterus or from intestinal contents of stranded porpoises, dolphins, and a whale (Foster et al. 1996). Their role in infectious diseases in these animals is unknown. *A. succinogenes* is found in the bovine rumen (Guettler et al. 1999). *A. arthriditis* has been isolated from diseased and healthy horses, as has *Actinobacillus* genomospecies 2, which represents equine strains of *A. lignieresii* (Christensen et al. 2002a). Biochemical characteristics for identification of these organisms are found in Tables 4 and 5.

The pathogenicity of recently described *Actinobacillus* species such as *A. minor*, *A. indolicus*, and *A. porcinus* is still unclear. Investigations have demonstrated that these newer species have a low potential for colonization of the upper respiratory tract of gnotobiotic piglets and show no pathogenicity in these animals (Chiers et al. 2001). On the other hand, a considerable number of isolates of these three groups were recovered in pure cultures from pneumonic lungs of pigs. Consequently, it will be necessary to critically review the opinion that these NAD-dependent Pasteurellaceae are only agents colonizing the mucosa (Kielstein et al. 2001).

Human infections with animal species of *Actinobacillus* usually occur as a result of animal-related trauma. *A. lignieresii*, *A. equuli*, and *A. suis* have been recovered from human clinical specimens, including horse- and sheep-bite wounds, joint fluid, blood, and sputum (Ashhurst-Smith et al. 1998; Benaoudia et al. 1994; Escande et al. 1996; Peel et al. 1991). All three species have also been isolated from the human upper respiratory tract (Sakazaki et al. 1984). Ashhurst-Smith and colleagues reported a case of *A. equuli* bacteremia and sepsis in a butcher after sustaining a knife cut at work (Ashhurst-Smith et al. 1998). Although this patient had a prosthetic mitral valve in place, endocarditis was not a complication. The remaining *Actinobacillus* species have not been isolated from human clinical specimens.

EIKENELLA CORRODENS

TAXONOMY

Holme (1950) first applied the name corroding bacillus to an anaerobic *Haemophilus*-like organism that was isolated from actinomycotic lesions. Similar organisms were described by Henriksen (1948) and by Eiken (1958) and were originally thought to be facultative strains of the obligately anaerobic species *Bacteroides corrodens*. This classification was disputed when the facultative nature of these strains was determined (Henriksen 1969). Similar organisms were also described by King (1964) and were given the name CDC group HB-1. Phenotypic and genotypic studies demonstrated that these facultative corroding bacilli were distinct from the anaerobic species. The obligately anaerobic species was reclassified in the genus Bacteroides and named *Bacteroides ureolyticus*. The facultative strains were placed in the monospecific genus *Eikenella* as *Eikenella corrodens* in honor of Eiken's earlier studies on this organism (Jackson and Goodman 1972). The genus *Eikenella* was originally classfied in the family Brucellaceae (Henriksen 1969). Using 16S ribosomal RNA sequencing and rRNA–DNA hybridization, Dewhirst et al. (1990a), Rossau et al. (1989), and others demonstrated a close taxonomic relationship between *E. corrodens* and *Neisseria* species. *E. corrodens* is the only species in the genus and is now included in the emended family Neisseriaceae in the β-subgroup of the Proteobacteria (Dewhirst et al. 1990a).

VIRULENCE AND PATHOGENICITY

Several workers have examined *E. corrodens* strains in efforts to define potential virulence factors. A lectin-like protein found on the surface of *E. corrodens* strains appears to function in adherence to human crevicular epithelial cells and interacts with a galactose-bearing receptor on epithelial cell surfaces (Yamazaki et al. 1988). This molecule also functions as a hemagglutinin. *E. corrodens* strains express type 4 pili and exhibit phase variation, resulting in an irreversible transition from the piliated to the nonpiliated state (Villar et al. 1999, 2001). On agar media, piliated cells form small colonies that pit the agar; nonpiliated cells produce larger, nonpitting colonies. The genetic loci responsible for pilus structural proteins, pilus synthesis, and export have been described (Villar et al. 1999, 2001). Piliated cells synthesize and export the pilus proteins to the cell surface, while nonpiliated variants synthesize pili but do not export them. Piliated cells also display twitching motility and are competent to undergo transformation, while nonpiliated cells lack these features. A 33–42-kDa principal outer-membrane protein has also been described that can trigger the release of lysosomal enzymes by macrophages and also can stimulate or depress macrophage activity in a dose-dependent manner (Maliszewski et al. 1983). Like other gram-negative bacteria, *E. corrodens* has an outer-membrane LPS that has similar biological activities to the LPS found in enteric bacteria (Progulske et al. 1984). Structurally, the LPS of *E. corrodens* differs from that of enteric bacteria in that it lacks 2-keto-3-deoxyoctulosonic acid, heptose sugars, and β-hydroxymyristic acid. Several antigens on the surface of *E. corrodens* broadly cross-react with those of other organisms, including *Kingella kingae*, *Kingella denitrificans*, and *Moraxella bovis* (Chen et al. 1990a).

CLINICAL SIGNIFICANCE

E. corrodens is a member of the normal oropharyngeal and upper respiratory tract flora (Marsden and Hyde 1971). It is also found in the gastrointestinal and genitourinary tracts. In the oral cavity, *E. corrodens* plays a role in periodontal disease, gingivitis, periapical infections, and root-canal infections (Chen and Wilson 1992). Young patients with periodontal disease have the highest concentrations of *E. corrodens* in subgingival plaque sample, while very low numbers of organisms are found in similar specimens from individuals with no periodontal disease (Suda et al. 2002). *E. corrodens* may also be isolated from other head and neck infections, including ocular infections, sinusitis, otitis media, mastoiditis, submandibiular abscesses, subdural and extradural empyemas, and brain abscess (Arana et al. 1998; Asensi et al. 2002; Assefa et al. 1994; Hemady et al. 1992; Heymann and Drezner 1997; Joseph et al. 1998; Klein et al. 1990; Paul and Patel 2001; Sane et al. 1999). By extension from periodontal, middle ear, or sinus infections, the organism may enter the central nervous system, leading to meningitis, brain or paraspinal abscesses, and subdural empyema (Asensi et al., 2002; Brill et al. 1982). Aspiration of oropharyngeal contents may lead to mixed pleuropulmonary infections involving *E. corrodens* (Hoyler and Antony 2001; Joshi et al. 1991; Killen et al. 1996). These patients are usually immunocompromised or have underlying lung disease associated with compromised local defense mechanisms.

E. corrodens is uncommonly associated with other serious infections. *E. corrodens* bacteremia and endocarditis occur in immunocompromised hosts, intravenous drug abusers, and individuals with previous valvular damage following dental procedures or oral surgery, although endocarditis has also been seen in healthy individuals with no recognized risk factors (Fainstein et al. 1981; Olopoenia et al. 1994; Watkin et al. 2002). Infections of prosthetic valves and other indwelling vascular prostheses associated with *E. corrodens* have also been reported (Decker et al. 1986; Heiro et al. 2000). Bacteremia with or without endocarditis may occur in individuals with diseases such as rheumatoid arthritis and hematological malignancies (Fainstein et al. 1981; Monkemuller and Bronze 1998). In recent years, *E. corrodens* has been isolated from the blood, subcutaneous abscesses, and soft-tissue infections of individuals who are users of injectable drugs (Angus et al. 1994; Gonzalez et al. 1993; Olopoenia et al. 1994). The use of saliva for skin cleansing prior to drug injection is the likely pathogenesis of these infections. *E. corrodens* may also be recovered from cellulitis, osteomyelitis, and joint-space infections as a result of human bites, clenched-fist injuries (i.e. fist fights), and wounds contaminated with oropharyngeal contents (Bonnet et al. 1997; Sagerman and Lourie 1995). In these settings, the organism is isolated in mixed culture with other facultative and obligately anaerobic organisms found in the oral cavity. Bacteremic *E. corrodens* infections resulting in hematogenous cellulitis, septic arthritis, vertebral osteomyelitis, intervertebral diskitis, and paraspinal abscess have also been reported (Emmett and Allman 2000; Flesher and Bottone 1989; Lehman et al. 2000; Raab et al. 1993). *E. corrodens* infections involving the vertebral column may occur as a complication of spinal surgery (Ang and Ngan 2002). *E. corrodens* is able to survive in the gastrointestinal tract and may be recovered from abdominal, splenic, hepatic, and pancreatic abscesses and from peritoneal infections along with facultative and anaerobic gastrointestinal flora (Arnon et al. 1999; Joseph et al. 1998; Ramos et al. 1994; Stein et al. 1993). Although uncommon, gynecological infections, including chorioamnionitis, endometritis, and cervicitis, have been described and may result from colonization of intrauterine devices (Andres et al. 2002; Jeppson and Reimer 1991).

CULTURAL CHARACTERISTICS AND IDENTIFICATION

E. corrodens will grow on both blood and chocolate agar but does not grow on MacConkey agar, eosin methylene blue (EMB) agar, or other selective or differential media. Colonies are small (0.5–1.0 mm) after 48 h. About 50 percent of isolates may pit the agar as they grow, and both pitting and nonpitting variants may be observed in the same culture. A pale yellow pigment (observed best on a white swab swept through growth on a chocolate agar plate) is usually produced, and most strains have an odor suggestive of sodium hypochlorite (i.e. Chlorox bleach). On Gram stain, the organisms appear as regular, slender, gram-negative bacilli or coccobacilli measuring 1.5–4 microns, with rounded ends. The biochemical characteristics for identification of *E. corrodens* strains are fairly uniform. The organisms are oxidase-positive and catalase-negative, although rare strains may be weakly catalase-positive (Table 6). The organism reduces nitrate to nitrite and does not require X or V factors, although hemin is necessary for aerobic growth. Nonpitting isolates may be mistaken for *Haemophilus* species and, when tested with X and V factor disks, display growth around the X disk but not the V disk. Indole and urease are not produced, but most strains are lysine decarboxylase- and ornithine decarboxylase-positive. *E. corrodens* is asaccharolytic and does not ferment or oxidize carbohydrates. The organism is not encapsulated, is nonmotile, and does not form spores.

Molecular methods for detection, identification, and typing of *E. corrodens* have been investigated as well. *E. corrodens*-specific DNA probes have been designed by analysis of 16S rRNA, identification of unique nucleotide sequences, and chemical synthesis of probe molecules that

Table 6 *Biochemical characteristics for identification of* Eikenella corrodens, Cardiobacterium hominis, Kingella *species, and* Suttonella indologenes

Characteristic	*E. corrodens*	*C. hominis*	*K. kingae*	*K. denitrificans*	*K. oralis*	*S. indologenes*
Hemolysis, SBA	–	–	β[a]	–	–	–
Oxidase	+	+	+	+	+	+
Catalase	–[b]	–	–	–	–	–
Reduction of nitrate to nitrite	+	–	–	+	–	–
Reduction of nitrite to N_2 gas	–	+	+	+	–	+
Indole	–	+	–	–	–	+
Urease	–	–	–	–	–	–
Ornithine decarboxylase	+	–	–	–	–	–
Esculin hydrolysis	–	–	–	–	–	–
ONPG	–	–	–	–	–	–
Gas from glucose	–	–	–	–	–	–
Acid from:						
Glucose	–	+	+	+	+w	+
Maltose	–	+[c]	+	–	–	+
Fructose	–	+	–	–	–	+
Sucrose	–	+	–	–	–	+
Lactose	–	–	–	–	–	–
Xylose	–	–	–	–	–	–
Mannitol	–	+[c]	–	–	–	–
Mannose	–	+	–	–	–	+
Galactose	–	–	–	–	–	–
Trehalose	–	–	–	–	–	–
Raffinose	–	–	–	–	–	–
Sorbitol	–	+	–	–	–	–

+, positive reaction; –, negative reaction; +w , weak positive reaction; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; SBA, sheep blood agar.
a) Most strains produce a soft β-hemolysis on sheep blood agar.
b) Rare strains may be weakly catalase-positive.
c) Rare strains may be maltose- and/or mannitol-negative.

are complementary to these species-specific sequences (Lippke et al. 1991). These probes enable direct detection of the organism in subgingival plaque samples of periodontitis patients. Molecular strain typing methods, including SDS-PAGE, pulsed-field gel electrophoresis, restriction endonuclease analysis, and arbitrarily primed PCR have been developed to determine relatedness of *E. corrodens* to other bacteria and to delineate strain differences among clinical *E. corrodens* isolates (Chen and Ashimoto 1996; Chen et al. 1990b; Steffens et al. 1994).

ANTIMICROBIAL SUSCEPTIBILITY

Antimicrobial susceptibility testing of *E. corrodens* is difficult because of its fastidious nature and slow growth. Most studies have used agar dilution susceptibility testing because the organism grows poorly in broth used for routine susceptibility testing. In 1998, Alcala and colleagues reported a broth microdilution procedure for *E. corrodens* using *Haemophilus* test medium (Alcala et al. 1998). Depending on the antimicrobial agent, between 75 and 97 percent agreement (± 1 $\log_2$ dilution) was noted between the microbroth and agar dilution procedures. Most *E. corrodens* strains are susceptible to ampicillin, amoxicillin, and tetracyclines and are resistant to the penicillinase-resistant penicillins, clindamycin, vancomycin, erythromycin, metronidazole, and the aminoglycosides (Chen and Wilson 1992; Goldstein et al. 1978; Luong et al. 2001). Penicillin susceptibility may vary from strain to strain. Most isolates are variably susceptible to the first-generation cephalosporins, with cefazolin being the most active (Goldstein et al. 1978). *E. corrodens* is also susceptible to cefoxitin, ceftriaxone, cefotaxime, cefepime, ciprofloxacin, and the carbopenems (imipenem, meropenem) (Alcala et al. 1998; Cormican and Jones 1995; Goldstein et al. 1978; Kugler et al. 1999). Rare β-lactamase-positive strains have been reported (Goldstein et al. 1978; Lacroix and Walker 1991). The *E. corrodens* β-lactamase enzyme is highly cell-associated, shows little activity against cephalosporins, and is strongly inhibited by clavulanate and sulbactam (Lacroix and Walker 1991).

KINGELLA SPECIES AND *SUTTONELLA INDOLOGENES*

TAXONOMY

The type species of the genus *Kingella*, *K. kingae*, was originally described in the 1960s and at that time was

called CDC group M-1. Early descriptions of this organism suggested a relationship with the moraxellae, except that these strains were catalase-negative (Snell 1984). CDC group M-1 was renamed *Moraxella kingii*, and subsequently this name was taxonomically amended to *Moraxella kingae* (Bovre et al. 1974). Because of significant genetic, chemotaxonomic, and phenotypic differences, Henriksen and Bovre recommended that *M. kingae* be removed from the genus *Moraxella* and renamed as the new genus and species *Kingella kingae* in the family Neisseriaceae in 1976 (Henriksen and Bovre 1976). In that same year, Snell and LaPage proposed the transfer of two other moraxella-like, oxidase-positive, catalase-negative, saccharolytic species to the genus *Kingella* (Snell and LaPage 1976). These new species included the organism previously called TM-1 described by Hollis et al. (*Kingella denitrificans*), and indole-positive strains that had originally been isolated from cases of conjunctivitis (*Kingella indologenes*) (Hollis et al. 1972; Snell and LaPage 1976). Based on 16S rRNA sequence comparisons, Dewhirst and colleagues showed that *K. indologenes* was more closely related to *C. hominis* than to the other *Kingella* species or to the other members of the family Neisseriaceae (Dewhirst et al. 1990b). Also, despite the similar phenotypic characteristics of *C. hominis* and *K. indologenes*, the G + C content of their DNA differed enough to warrant separate genus and species status. Consequently, *K. indologenes* was removed from the family Neisseriaceae and placed as the only species in the new genus *Suttonella* as *Suttonella indologenes* (after the Australian microbiologist R.G.A. Sutton) in the family Cardiobacteriaceae in the γ-subgroup of the Proteobacteria (Dewhirst et al. 1990b; Garrity and Holt 2000). *K. kingae* and *K denitrificans* remain in the emended family Neisseriaceae, which is also in the γ-subgroup of the Proteobacteria (Dewhirst et al. 1990a; Garrity and Holt 2000; Rossau et al. 1989). In 1993, Dewhirst and colleagues described a new *Kingella* species that was recovered from human dental plaque of a patient with adult periodontitis (Dewhirst et al. 1993b). This new species was named *Kingella orale* and subsequently was emended to *Kingella oralis*. This organism constitutes 0.4 percent of the total microbiota in dental plaque from periodontally healthy individuals and about 4.6 percent of the microbiota in dental plaque from adult and juvenile periodontitis patients (Chen 1996).

CLINICAL SIGNIFICANCE

Although they are part of the normal upper respiratory and genitourinary tract flora of humans, *Kingella* species, and *K. kingae* in particular, are increasingly being recognized as important human pathogens. Since its initial description, *K. kingae* was considered to be a rare cause of infection in patients with endocarditis. However, during the past decade, *K. kingae* has emerged as a significant pathogen in pediatric patients, causing primarily bacteremia and skeletal infections (Douglas et al. 1998). This organism can be isolated from the upper respiratory tract of infants and children; almost 75 percent of children over 6 months of age have been found to carry the organism in their oropharynx at some time (Yagupsky and Dagan 2000; Yagupsky et al. 2002). The prevalence of the organism in the respiratory tract is highest among children aged 6 months to 4 years, which parallels the ages having the highest attack rates for invasive disease (Yagupsky et al. 2002). Carriage rates are lower among children under 6 months and over 4 years of age. Studies in daycare-center populations have demonstrated that *K. kingae* can be transmitted from child to child by the respiratory route in daycare settings (Slonim et al. 1998). The portal of entry for the organism into the blood stream is probably via breaches in the oropharyngeal mucosa.

Bone and joint infections are the most common clinical manifestations of *K. kingae* infection in children; these infections present as bacteremia, septic arthritis, osteomyelitis, discitis, tenosynovitis, and dactylitis (Birgisson et al. 1997; Dodman et al. 2000; Moylett et al. 2000; Yagupsky and Dagan 2000). In fact, with the availability of effective vaccines against *H. influenzae* type b, *K. kingae* has essentially replaced *H. influenzae* as the most common gram-negative bacterium causing osteoarticular infections in children under 3 years of age (Dodman et al. 2000). Most infants with systemic *K. kingae* infections present with low-grade fevers and often have concurrent viral upper respiratory tract infections (Birgisson et al. 1997; Dodman et al. 2000). In most cases, *K. kingae* septic arthritis is an acute infection, with patients usually presenting within 3 days of the appearance of signs and symptoms. These children are usually febrile, and the involved joints are swollen and tender and display decreased ranges of motion. Blood cultures from these patients are frequently negative (Dodman et al. 2000). *K. kingae* septic arthritis is usually monoarthritic, with the most frequently involved joints being the knee, hip, and ankle. Osteomyelitis due to *K. kingae* is usually an indolent infection that follows a subacute course, with patients usually presenting 7–10 days after ther appearance of symptoms. Patients with osteomyelitis of the long bones usually are unable to bear weight on the affected limb, and there is local tenderness over the involved bone (Moylett et al. 2000). Hematogenous seeding of the intervertebral disk leads to spondylitis and intervertebral discitis (Dodman et al. 2000; LaScola et al. 1998). Disk-space infections may involve the lumbar, thoracic, lumbosacral, or thoracolumbar spaces (Yagupsky and Dagan 2000). Children with invasive *K. kingae* infections often have significant underlying conditions, such as acute lymphocytic leukemia or congenital heart disease. Although rare, bone and joint infections due to *K. kingae*, including intervertebral

discitis and septic arthritis, have been diagnosed in adults (Esteve et al. 2001; Meis et al. 1992).

As with several other fastidious gram-negative bacilli, *K. kingae* and the other *Kingella* species are rare causes of bacteremia and endocarditis. *K. kingae* endocarditis, as with the other fastidious bacteria discussed thus far, primarily occurs in people with underlying heart disease or cardiac prostheses, although cases in people with no previous heart disease have been reported (Chakraborty et al. 1999; Ferber et al. 1997; Wells et al. 2001; Wolak et al. 2000). Unlike bone and joint infections, *K. kingae* endocarditis is seen more in adults and school-age children, although cases in children may occur as well (Wells et al. 2001; Yagupsky and Dagan 2000). Complications, including pericarditis, pericardial abscess, embolic phenomena, mycotic aneurysm, cerebral infarction, and congestive heart failure, also may occur in the clinical course of *K. kingae* disease. *K. kingae* bacteremia, endocarditis, and meningitis have been reported in patients with severe underlying diseases, including systemic lupus erythematosis and acquired immunodeficiency syndrome (AIDS) (Urs et al. 1994; Wolak et al. 2000). Many patients are noted to have poor oral hygiene, pharyngitis, or mucosal ulcerations due to treatment for other conditions (e.g. radiation therapy). *K. kingae* bacteremia without endocarditis has also been reported in immunocompetent adults following dental manipulations (Roiz et al. 1997). Additional complications of *K. kingae* bacteremia and bone infections include meningitis, hematogenous endophthalmitis, soft-tissue infection, and corneal abscess (Carden et al. 1991; Mollee et al. 1992; Rolle et al. 2001; Van Erps et al. 1992). *K. kingae* has been isolated from blood and sputum specimens of patients with pneumonia, epiglottitis, and tracheobronchitis (Kennedy and Rosen 1988). Septicemia with *K. kingae* in immunocompromised children and adults may also mimic systemic neisserial infections (i.e. meningococcemia or disseminated gonococcal infection) in its clinical presentation, with the presence of characteristic skin lesions and joint involvement (Redfield et al. 1980; Shanson and Gazzard 1984; Toshniwal et al. 1986).

K. denitrificans and *S. indologenes* have rarely been isolated from clinically significant infections. *K. denitrificans* has been cultured from the upper respiratory and genitourinary tracts, where it constitutes a part of the normal flora, and has been documented as a rare cause of septicemia and both native and prosthetic valve endocarditis (Hassan and Hayek 1993). Patients with endocarditis ranged from 22 to 66 years of age and all but one either had pre-existing valvular disease or had prosthetic valves in place. Many of the patients had undergone dental procedures without prophylaxis or had concomitant respiratory tract infections. *K. denitrificans* has also been recovered from empyema fluid in a patient with bronchogenic carcinoma, from bone-marrow cultures of an AIDS patient, and from amniotic fluid of a 23-year-old woman with chorioamnionitis (Maccato et al. 1991; Minamoto and Sordillo 1992; Molina et al. 1988). *S. indologenes* has been isolated from human eye infections and was first reported in association with a case of prosthetic valve endocarditis in 1987 (Jenny et al. 1987; Snell and LaPage 1976). *K. oralis* has not been associated with any infectious process.

CULTURAL CHARACTERISTICS AND IDENTIFICATION

Kingella species are plump gram-negative bacilli or coccobacilli that sometimes occur in pairs or short chains. Individual cells measure 1–3 microns in length They are oxidase-positive and, unlike *Neisseria* and *Moraxella* species, catalase-negative. All species grow on chocolate and blood agars but do not grow on MacConkey agar or other enteric media. A selective medium for recovery of *K. kingae* from respiratory tract specimens has been described that is composed of trypticase soy agar containing 5–7 percent sheep blood and vancomycin (2 μg/ml) (Yagupsky et al. 1995). The vancomycin inhibits the gram-positive oropharyngeal bacterial flora and facilitates detection of the β-hemolysis produced by the organism. All *Kingella* and *Suttonella* species do not produce lysine or ornithine decarboxylases, arginine dihydrolase, indole, acetoin, or urease (Table 6).

K. kingae is β-hemolytic on sheep blood agar; the hemolytic reaction is soft, resembling that of group B streptococci, and may only be noted in areas of confluent growth or after the removal of the colony from the agar surface. On prolonged incubation, some strains of *K. kingae* (and *K. denitrificans*) may pit the agar like *E. corrodens*. *K. kingae* produces acid from glucose and maltose only. *K. denitrificans* was originally called TM-1 and was first recognized by its ability to grow on Thayer–Martin medium (Hollis et al. 1972). *K. denitrificans* produces acid only from glucose in supplemented media, rapid carbohydrate degradation tests, and various kit systems, and it is also prolyl-aminopeptidase-positive. Because of these characteristics, *K. denitrificans* may be misidentified as *N. gonorrhoeae*, particularly in specimens from the oropharyngeal and genitourinary tracts. This organism, unlike the other species in the genus, reduces nitrate to nitrite, and most strains will also reduce nitrite to nitrogen gas on prolonged incubation. *S. indologenes* produces acid from glucose, maltose, and sucrose, but not from lactose, and is distinguished by its ability to produce indole. Like *C. hominis*, this characteristic should be determined in tryptone broth with xylene extraction and addition of Ehrlich's indole reagent. *S. indologenes* may be differentiated from *C. hominis*, which it closely resembles, by appearance on Gram stain, positive alkaline phosphatase activity, and failure to produce acid from mannitol and sorbitol (Bruun et al. 1984; Dewhirst et al. 1990b). *K. oralis* produces acid from glucose only, but it may be

differentiated from *K. denitrificans* by its failure to reduce nitrate and nitrite and the absence of prolyl-aminopeptidase activity (Dewhirst et al. 1993a). *E. corrodens* may be differentiated from both *C. hominis* and *Kingella* species by its Gram stain morphology, failure to ferment carbohydrates and its positive ornithine and lysine decarboxylase reactions. Biochemical reactions for identification of *Kingella* species and *S. indologenes* are presented in Table 6.

While identification of these organisms is straightforward once they are isolated, recovery of *K. kingae* from clinical specimens, particularly joint-fluid specimens, is sometimes difficult. Yagupsky and coworkers found that direct plating of aspirates was much less sensitive for recovering *K. kingae* than inoculation of BACTEC blood culture media (Yagupsky et al. 1992). Among 100 specimens cultured by both methods, 34 grew significant organisms; ten of the 11 *K. kingae* isolates that were obtained grew only in the BACTEC medium and were not recovered on direct plating. The discrepancy in the isolation rate of *K. kingae* was not observed for other organisms causing bone and joint infections. *K. kingae* has also been detected in blood and synovial fluids using the BacT/Alert system (Organon Teknika) and the pediatric Isolator 1.5 microbial tube (Wampole Laboratories, Cranbury, NJ, USA) (Lejbkowicz et al. 1999; Yagupsky and Press 1997). Using pooled synovial fluids seeded with small inocula of 24 different *K. kingae* isolates, Host and coworkers detected 100 percent of the 24 isolates using the BacT/Alert Aerobic and BacT/Alert Pedi-BacT aerobic bottles, 88 percent using the BacT/Alert FAN aerobic bottle, and 63 percent using the Bactec Plus Aerobic F bottle (Host et al. 2000). Improved recovery of *K. kingae* from joint-fluid specimens inoculated into blood culture media may be due to the dilution of inhibitory factors in synovial fluid by the culture medium (e.g. broth-based blood culture systems) or by removal of the organisms from the synovial fluid milieu (e.g. Isolator cultures). Inoculation of osteoarticular fluids into blood culture bottles is now considered the standard of practice for processing of these specimens in the clinical laboratory (Moylett et al. 2000; Yagupsky et al. 1992). Molecular methods (e.g. PCR) have also been used for the direct detection of *K. kingae* in joint fluid (Stahelin et al. 1998).

ANTIMICROBIAL SUSCEPTIBILITY

K. kingae strains are susceptible to penicillin, ampicillin, oxacillin, cephalosporins of all generations, chloramphenicol, the aminoglycosides, trimethoprim-sulfamethoxazole, and ciprofloxacin. Some strains may be relatively resistant to erythromycin, and most strains are resistant to clindamycin, lincomycin, and vancomycin (Birgisson et al. 1997; Jensen et al. 1994; Kugler et al. 1999). Isolates of *K. denitrificans* have antimicrobial susceptibilities similar to those of *K. kingae*; growth of *K. denitrificans* on Thayer–Martin agar indicates that they are resistant to vancomycin and colistin (Hollis et al. 1972). The *K. indologenes* endocarditis isolate reported by Jenny et al. (1987) was susceptible to ampicillin, cefazolin, and tobramycin. In 1993, Sordillo and colleagues recovered a *K. kingae* strain from the blood of a 29-year-old AIDS patient that produced β-lactamase and was highly resistant to ampicillin, cefazolin, and ticarcillin, but was susceptible to β-lactamase-resistant agents and combination drugs containing β-lactamase inhibitors (clavulanate and sulbactam) (Sordillo et al. 1993). Among five cases of *K. kingae* infection reported from Iceland in 1997, three of the five isolates produced β-lactamase enzymes and were resistant to pencillin and ampicillin (Birgisson et al. 1997).

CARDIOBACTERIUM HOMINIS

TAXONOMY

C. hominis is the only species in the genus *Cardiobacterium*. This organism, originally called CDC group IID, was described and named *Cardiobacterium hominis* by Slotnick and Dougherty in 1964 (Slotnick and Dougherty 1964). These workers demonstrated its unique biochemical features and antigenic unrelatedness to other fastidious gram-negative and gram-positive bacteria. The organism possesses a typical gram-negative cell wall with an unusually dense outer layer and terminal, tufted polar caps that are adherent to the cell membrane (Reyn et al. 1971). These properties may explain the atypical appearance of the organism on gram stains (see below). In 1990, Dewhirst and coworkers examined *Cardiobacterium* strains by 16S ribosomal RNA sequence analysis and found that this organism and *Kingella indologenes* were closely related to one another and differed significantly from reference strains of other fastidious organisms (Dewhirst et al. 1990b). The family Cardiobacteriaceae was proposed to include *C. hominis*, *Kingella indologenes*, and the related soil organism *Dichelobacter nodosus*. Phenotypic similarities also supported the genetic relatedness of *C. hominis* and *K. indologenes* (Bruun et al. 1984). *K. indologenes* was removed from the family Neisseriaceae, renamed *Suttonella indologenes*, and placed in the family Cardiobacteriaceae along with *Cardiobacterium* and *Dichelobacter* species (Dewhirst et al. 1990b). The family Cardiobacteriaceae belongs to the proposed order Cardiobacteriales in the γ subdivision of the Proteobacteria (Garrity and Holt 2000).

CLINICAL SIGNIFICANCE

C. hominis is an organism of very low virulence and has the unique characteristic of causing endocarditis almost exclusively; about 50 cases of endocarditis have been reported in the literature (Currie et al. 2000). The

organism is a part of the normal respiratory tract flora, but because of its slow growth it is rarely noted in respiratory tract specimens. *C. hominis* enters the bloodstream and usually infects previously diseased or damaged heart valves (e.g. rheumatic heart disease, ventricular septal defects). However, *C. hominis* endocarditis may also develop in individuals with no evidence of previous heart disease. About 20 percent of reported *C. hominis* infections have involved various types of prosthetic cardiac valves, including aortic homograft tissue valves, porcine xenograft aortic and mitral valves, mechanical aortic and mitral valves, and pacemaker leads (Apisarnthanarak et al. 2002; Nurnberger et al. 1998; Taveras et al. 1993). Patients are usually middle-aged to elderly and have poor dentition and/or a history of recent dental procedures. The infection follows a very subacute course, with an insidious onset, low-grade fevers, and vague symptoms that may linger for several months before a diagnosis is made. *C. hominis* tends to form large, friable vegetations on the heart valves, so the most serious complications include septic embolization, mycotic aneurysms, and congestive heart failure (Currie et al. 2000; Lin and Vieco 1995). *C. hominis* bacteremia and sepsis without endocarditis may also occur in immunocompromised patients (Ronnevik and Neess 1981). Isolation of *C. hominis* from specimens other than blood cultures is rare. Francioli and colleagues diagnosed cardiobacterial meningitis in a patient as a complication of endocarditis, and Rechtman and Nadler reported the recovery of *C. hominis* along with *Clostridium bifermentans* from an abdominal abscess and the blood of a diabetic male patient with adenocarcinoma of the kidney and caecum (Francioli et al. 1983; Rechtman and Nadler 1991). In 1998, Nurnberger et al. reported a *C. hominis* infection of a pacemaker atrial lead without valvular involvement that was complicated by hematogenous vertebral osteomyelitis.

CULTURAL CHARACTERISTICS AND IDENTIFICATION

C. hominis is principally isolated from blood cultures. The organism grows slowly, producing no visible change in the blood culture medium, such as turbidity or hemolysis. In a recent case report, the organism was recovered only after blind subculture of blood culture bottles on the tenth day of incubation (Lu et al. 2000). Gram stains of positive blood bottles may not reveal the organism; low-speed centrifugation of an aliquot of the medium to remove red blood cells with subsequent high-speed centrifugation to pellet small numbers of organisms may aid in their visualization. On Gram stains, the organisms may appear gram-variable, with a tendency for the cells to retain the crystal violet dye at the poles. Individual cells may appear swollen at one or both ends, resulting in tear-drop, dumbbell, and lollypop-shaped organisms. Individual cells are 1.5–3.0 microns in length. The cells may appear in rosette-like clusters or lined up in picket-fence arrangements in their orientation with one another. The morphology of the individual cells is dependent on the type of culture medium used; in media containing yeast extract, cells appear as uniform, gram-negative bacilli with rounded ends.

C. hominis grows as very small, glistening, opaque colonies on enriched blood or chocolate agar generally after 48–72 h at 35°C in 5–7 percent CO_2. Some strains may also pit the agar on further incubation. No growth is observed on MacConkey agar or other enteric selective and/or differential agars. *C. hominis* is oxidase-positive, catalase-negative, nitrate-negative, and urease-negative (Table 6). The positive oxidase and negative nitrate reduction reactions help to differentiate this organism from *H. aphrophilus/paraphrophilus* and *A. actinomycetemcomitans*, but are similar to reactions seen for *Eikenella*, *Kingella*, and *Suttonella*. Indole production can be detected in heart infusion or typtone broth. These media are heavily inoculated (with a swab) and incubated for 48 h. Extraction with xylene or chloroform and use of Ehrlich's reagent rather than Kovac's reagent allows detection of the small amounts of indole produced by this organism. Hydrogen sulfide production can be detected using lead acetate strips. *C. hominis* produces acid fermentatively from glucose, fructose, sucrose, mannose, and sorbitol; acid production from maltose and mannitol is variable, with most strains producing positive reactions. Lactose, xylose, galactose, trehalose, and raffinose are not fermented. Phenotypically, *C. hominis* is very similar to *S. indologenes* (Bruun et al. 1984). *C. hominis* can also be differentiated from *S. indologenes*, *E. corrodens*, and *Kingella* species by cellular fatty acid analysis (Wallace et al. 1988). As with other agents of bacterial endocarditis, molecular methods have been used successfully for detection of *C. hominis* directly in clinical specimens (Mueller et al. 1999; Nikkari et al. 2002).

ANTIMICROBIAL SUSCEPTIBILITY

Antimicrobial susceptibility testing of *C. hominis* is difficult because of its fastidious requirements and slow growth rate. In addition, breakpoints for susceptibility testing, optimal media, and incubation requirements have not been established for testing *C. hominis* or other fastidious organisms. Strains of *C. hominis* are generally susceptible to most antimicrobial agents, including penicillin, ampicillin, cephalothin, third-generation cephalosporins (i.e. cefixime, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, and cefepime), imipenem, meropenem, the fluoroquinolones, aminoglycosides, chloramphenicol, rifampin, and tetracycline (Kugler et al. 1999). Patients with *C. hominis* endocarditis have usually been treated with penicillin alone or penicillin combined with an aminoglycoside. Cures have also been achieved with cefazolin and ciprofloxacin monotherapy (Christen

1990; Vogt et al. 1994). At the present time, the recommended treatment includes the third-generation cephalosporins (Wilson et al. 1995). β-Lactamase-producing, penicillin-resistant strains of *C. hominis* are rare. In 1994, a β-lactamase-producing strain of *C. hominis* was isolated from a case of endocarditis in a 76-year-old man (Le Quellec et al. 1994). This strain was susceptible to tetracycline, rifampin, vancomycin, and imipenem and was resistant to erythromycin, trimethoprim-sulfamethoxazole, gentamicin, amoxicillin, ticarcillin, cefotaxime, and piperacillin. The patient responded to vancomycin/rifampin followed by amoxicillin-clavulanate. The second β-lactamase-producing isolate was recovered from a 66-year-old woman (Lu et al. 2000). This isolate was resistant to penicillin and ampicillin, and was susceptible to amoxicillin-clavulanate, cefotaxime, ceftriaxone, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Most patients with native valve endocarditis due to *C. hominis* are successfully treated with antibiotics alone, although some may require partial valve resection or valve replacement due to hemodynamic compromise, embolic phenomena, or progressive cardiac failure (Christen 1990; Taveras et al. 1993).

CAPNOCYTOPHAGA SPECIES

TAXONOMY

Capnocytophaga species were first described in 1956 by Prevot at the Pasteur Institute in Paris. At first, these organisms were thought to be indole-negative, fermentative variants of *Fusobacterium nucleatum* and, consequently, they were christened *F. nucleatus* var. *ochraceus*. Examination of the G + C content of these strains revealed a closer relationship to bacteria called *Ristella* species than to the fusobacteria, so then these strains were renamed *Ristella ochracea*. At the same time, Loesche was examining isolates of *Bacteroides oralis* from the human oral cavity and noted that some of these strains were long and thin with pointed ends, again suggestive of fusobacteria. Loesche suggested the name *Bacteroides oralis* var. *elongatus* for these strains. While the original isolates of Prevot have been lost, further examination of *Ristella ochracea* and *Bacteroides oralis* var. *elongatus* confirmed that these names described the same organism. The organism's name was then changed to *Bacteroides ochraceus*. Elizabeth O. King at the Centers for Disease Control and Prevention (CDC) was also studying several thin, fusiform gram-negative isolates recovered from human clinical specimens during the early 1960s (King 1964). These strains were designated CDC group DF-1. DF means dysgonic fermenter, referring to the organism's poor fermentative capabilities in media lacking serum supplementation. These strains were facultative in their metabolism and showed a distinct requirement for CO_2 for growth on agar media. During the late 1970s, workers at the Forsyth Dental Center in Boston published several studies on a group of fusiform, gram-negative bacilli that required CO_2 for growth and demonstrated gliding motility (Holt et al. 1979; Leadbetter et al. 1979; Socransky et al. 1979; Williams and Hammond 1979). These organisms appeared to have a role in the pathogenesis of human periodontal diseases and were named *Capnocytophaga* species, reflecting the organism's CO_2 requirement for growth. Laboratory groups at University of California, Los Angeles (UCLA) and the Wadsworth VA Hospital compared strains and concluded that the *Capnocytophaga* species of the Boston investigators, CDC group DF-1, and *Bacteroides ochraceus* described the same organisms (Newman et al. 1979; Williams et al. 1979). The type species was named *Capnocytophaga ochracea* and related isolates studied at the same time were named *Capnocytophaga sputigena* and *Capnocytophaga gingivalis*. Subsequent research supported the species status of *Capnocytophaga*, the synonomy of the various species described by US and European scientists, and the inclusion of three separate species within the genus (Dees et al. 1982). In 1994, two new human *Capnocytophaga* species – *C. granulosa* and *C. haemolytica* – were identified in human dental plaque specimens (Ciantar et al. 2001; Yamamoto et al. 1994). Based on 16S rRNA analysis, these five *Capnocytphaga* species are classified in the family Flavobacteriaceae along with *Flavobacterium*, *Chryseobacterium*, and *Weeksella* species, in the proposed order Flavobacteriales in the phylum Bacteroidetes (Garrity and Holt 2000; Vandamme et al. 1986).

CDC group DF-2 was originally isolated in 1976 from blood and spinal fluid cultures of a patient following a dog bite (Bobo and Newton 1976). Subsequently, several reports describing similar organisms were published, and from 1976 to 1987, over 150 isolates of this organism, designated CDC group DF-2 (for dysgonic fermenter) were sent to the Special Bacteriology Branch of the CDC (Hicklin et al. 1987). In 1989, DF-2 and several DF-2-like organisms were classified as *Capnocytophaga* species based on phenotypic characteristics and DNA relatedness studies (Brenner et al. 1989). CDC group DF-2 is now called *Capnocytophaga canimorsus* (Latin for dog bite) and the DF-2-like strains are called *Capnocytophaga cynodegmi* (Greek for dog bite). Of these two species, *C. canimorsus* is isolated far more frequently and appears to be more virulent. Studies in cultured mouse macrophages have shown that *C. canimorsus* is phagocytized, multiplies intracellularly, and causes cell death within 48 h, whereas *C. cynodegmi* demonstrated no cytotoxic effects (Fischer et al. 1995). While these organisms differ from human *Capnocytophaga* species in several phenotypic characteristics, they are similar to them with respect to Gram stain morphology, cellular fatty acids, gliding-type motility, and cultural conditions for growth. *C. canimorsus* and *C. cynodegmi* are classified with the human *Capnocytophaga* species in the family

Flavobacteriaceae in the phylum Bacteroidetes (Garrity and Holt 2000).

CLINICAL SIGNIFICANCE

Capnocytophaga species found in humans are gliding, gram-negative bacteria that are part of the normal oropharyngeal flora. These organisms likely play a role in the pathogenesis of localized juvenile periodontitis and other periodontal diseases. *Capnoctyophaga* species may also be agents of sepsis in patients with malignancies, granulocytopenia, and other severe underlying illnesses, including myeloblastic leukemia, acute lymphocytic leukemia, adenocarcinoma, multiple myeloma, Hodgkin's disease, and endometrial carcinoma (Bilgrami et al. 1992; Geisler et al. 2001; Gomez-Garces et al. 1994; Kristensen et al. 1995; Mantadakis et al. 2003). Bacteremic episodes with these bacteria often coincide with periods of profound granulocytopenia due to the hematological malignancies and to administration of cytotoxic chemotherapy. Mucositis and oral ulcerations characteristically found in profoundly immunosuppressed patients receiving radiation or cytotoxic chemotherapy establish an efficient entry route for the organism into the blood stream (Baquero et al. 1990; Kristensen et al. 1995). *Capnocytophaga* species are mostly isolated from respiratory sources (i.e. gingival crevices, periodontal pockets) and are occasionally isolated from blood and cerebrospinal fluid (Geisler et al. 2001; Kim et al. 1996; Mantadakis et al. 2003). Rarely, isolates may be recovered from the lower respiratory tract, lung abscesses, wound infections, pleural/peritoneal fluids, joint fluid, bone, conjunctival, corneal, and vitreal specimens (Elster et al. 1983; Heidemann et al. 1988; Parenti and Snydman 1985; Rubsamen et al. 1993; Testillano Tarrero et al. 1989; Wasserman et al. 1995; Winn et al. 1983). *Capnocytophaga* species have been isolated from female genital tract infections as causes of serious intrauterine, intra-amniotic, and perinatal infections (e.g. endometritis, amnionitis, chorioamnionitis), preterm abortion, congenital bacteremia, and neonatal sepsis (Alanen and Laurikanien 1999; Douvier et al. 1999; Feldman et al. 1985; Mayatepek et al. 1991; Mercer 1985). Endocarditis, cervical lymphadenitis, empyema, lung abscess, sinusitis, vertebral osteomyelitis, subphrenic abscess, pyonephrosis, liver abscess, osteomyelitis, and clenched-fist injuries involving *Capnocytophaga* species have been reported in both immunocompromised and nonimmunocompromised patients (Buuhoi et al. 1988; Duong et al. 1996; Elster et al. 1983; Parenti and Snydman 1985; Seger et al. 1982; Tay et al. 1985; Weber et al. 1997). In 1995, the first case of *Capnocytophaga* species (*C. sputigena*) as a cause of continuous ambulatory peritoneal dialysis-related peritonitis was documented in a 73-year-old man with end-stage renal disease (Esteban et al. 1995). In 2000, the newly described species *C. granulosa* was isolated from an abscess in an immunocompetent patient (Ebinger et al. 2000).

Studies of virulence factors produced by *Capnocytophaga* have focused on their recognized role in periodontal disease. Isolates from oral lesions produce various aminopeptidases, proteases, elastases, and chymotrypsin-like enzymes (Gazi et al. 1997; Spratt et al. 1995). These enzymes may function as virulence factors directly by causing degradation of periodontal tissue and indirectly by their action on proteins in dental plaque that generate small molecules having known inflammatory potential in periodontal disease (e.g. bradykinin). These molecules cause increases in vascular permeability, polymorphonuclear cell accumulation, and pain. *C. ochracea* and *C. sputigena* produce neuraminidase, an enzyme produced by many other microorganisms and considered to contribute to virulence (Moncla et al. 1990). *Capnocytophaga* species also produce a dialyzable substance that alters the microscopic appearance of neutrophils and markedly inhibits chemotaxis in vitro (Shurin et al. 1979; Van Dyke et al. 1982). This substance may act locally in the oropharyngeal tissue to abrogate neutrophil cell function in those patients who are already immunocompromised. Extracts prepared from sonicated cells of *Capnocytophaga* species contain a 14-kDa protein substance that causes dose-dependent inhibition of lymphocyte proliferation in response to mitogens (Ochiai et al. 1998). *Capnocytophaga* species also secrete IgA-proteases, allowing the disabling of the mucosal immune response (Frandsen et al. 1995). Extracellular polysaccharide production may also contribute to virulence by inhibiting the T-lymphocyte response to mitogens and antigens (Bolton et al. 1985). Finally, the LPS of *Capnocytophaga* species may render some strains resistant to the bactericidal effects of normal human serum (Wilson et al. 1987).

C. canimorsus infections are associated with dog bites or close contact with dogs (Hovenga et al. 1997; Lion et al. 1996a; Pers et al. 1996; Saab et al. 1998). The most serious clinical manifestations occur in individuals with underlying diseases or conditions that predispose them to severe infection with the organism, including hepatic disease secondary to alcoholism, previous splenectomy related to other medical circumstances, Hodgkin's disease, hairy cell leukemia, pulmonary fibrosis, malabsorption syndrome, renal disease, chronic obstructive pulmonary disease, peptic ulcer disease, Waldenstrom's macroglobulinemia, and the use of systemic or topical corticosteroids (Hartley et al. 1994; Kullberg et al. 1991; Martone et al. 1980; Ruddock et al. 1997; Yu et al. 2000). The frequent association of systemic *C. canimorsus* infection with asplenia suggests that the reticuloendothelial system plays an important role in containing the spread of the organism. Major clinical features of *C. canimorsus* infection include wound infection with cellulitis, meningitis, bacteremia with septic shock, renal failure, hemorrhagic skin lesions reminiscent of menin-

gococcal disease, pneumonia with empyema, and both native and prosthetic valve endocarditis (Andersen and Pedersen 1992; Blanche et al. 1994; Chambers and Westblom 1992; Kooter et al. 1999; Le Moal et al. 2003; Ngaage et al. 1999; Rosenman et al. 2003). Fulminant *C. canimorsus* sepsis may clinically resemble overwhelming meningococcal disease, with the development of disseminated intravascular coagulation, purpura fulminans, rapidly evolving hemorrhagic skin lesions, symmetrical peripheral gangrene, and the Waterhouse–Friderikson syndrome (Bryson et al. 2003; Hartley et al. 1994; Kullberg et al. 1991; Mirza et al. 2000). Unusual presentations and complications of *C. canimorsus* sepsis have included precipitous hypotension complicated by adult respiratory distress syndrome, myocardial infarction, acute abdominal symptoms, the hemolytic-uremic syndrome, musculocutaneous mononeuropathy, thrombotic thrombocytopenic purpura without disseminated intravascular coagulation, and dialysis-related peritonitis (Bannerjee et al. 1993; Chadha and Warady 1999; Depres-Brummer et al. 2001; Ehrbar et al. 1996; Finn et al. 1996; Howell and Woodward 1990; Kok et al. 1999; Mulder et al. 2001; Sawmiller et al. 1998; Tobe et al. 1999). *C. canimorsus* has been isolated from the oropharynx and saliva of dogs and, in at least one case of sepsis, the strain recovered from the patient's blood was also recovered from gingival swab specimens from the patient's dog (Martone et al. 1980). Eye infections, including angular blepharitis, chronic corneal ulcers, corneal ulcer with perforation, and endophthalmitis caused by *C. canimorsus*, have also been reported (De Smet et al. 1990; Glasser 1986; Kiel et al. 1987; Phipps et al. 2002; Zimmer-Galler and Pach 1996). Interestingly, a few cases of *C. canimorsus* infection, including keratitis and systemic infection, have occurred in individuals who sustained bites and/or scratches from domestic cats (Carpenter et al. 1987; Chodosh 2001; Mahrer and Raik 1992; Valtonen et al. 1995). In 2001, the first case of cellulitis, pneumonia, and bacteremia due to *C. cynodegmi* was reported in a diabetic man following a dog bite (Sarma and Mohanty 2001).

CULTURAL CHARACTERISTICS AND IDENTIFICATION

Capnocytophaga species found in humans are slow-growing, with colonies becoming visible generally after 48 h incubation and developing a characteristic morphology after this time. All species require a CO_2-enriched environment for growth; this may be provided in a CO_2 incubator or a candle jar. The colonies of the organism are yellow, tan, or slightly pinkish and have marginal finger-like projections (gliding motility) appearing as a film surrounding the central area of the colony. The central parts of the colonies also have a moist, mottled appearance. *Capnocytohaga* species grow on blood and chocolate agar, but not on MacConkey agar. Good growth may also be observed on modified Thayer–Martin agar because of the organism's resistance to vancomycin, colistin, and trimethoprim. The organisms are gram-negative, fusiform in shape, and appear straight or slightly curved. Pleomorphism, with swollen or large coccal cells, is characteristic in older cultures. All human species are catalase-negative, are oxidase-negative, and produce acid from glucose, maltose, sucrose, and mannose, but not from ribose, xylose, mannitol, or sorbitol (Socransky et al. 1979). Indole and urease are not produced, and both lysine and ornithine decarboxylase and arginine dihydrolase reactions are negative. *Capnocytophaga* species produce characteristic uniform profiles on cellular fatty acid analysis that allow genus-level but not species-level identification (Dees et al. 1982). Species are identified by an expanded battery of tests (e.g. nitrate and nitrite reduction, starch and dextran hydrolysis, etc.). The newly described species *C. haemolytica* is β-hemolytic on sheep blood and is the only human hemolytic species (Yamamoto et al. 1994). *C. granulosa* strains form intracellular granular inclusions that stain with carbol fuchsin when the organisms are grown anaerobically in peptone-yeast glucose broth under anaerobic conditions (Yamamoto et al. 1994). These two newly described species can also be differentiated from the three previously described human *Capnocytophaga* species by determination of various aminopeptidase activities. Biochemical characteristics of the five species of *Capnocytophaga* are shown in Table 7.

C. canimorsus is usually recovered from blood cultures, although other specimens (wound cultures, aspirates from cellulitis, cerebrospinal fluid (CSF)) may also yield the organism. *C. cynodegmi* has been recovered from dogs' mouths and from localized dog-bite wounds. In cases of high grade bacteremia, the bacteria may actually be observed on smears of perpheral blood (Mirza et al. 2000). The organism has been recovered in several types of blood culture media, and growth is generally slow. The lysis-centrifugation method (Isolator) has also been used successfully for recovery of *C. canimorsus* (Tison and Latimer 1986). In most reports, blood cultures usually become positive 3–7 days after collection. The organism grows on both blood and chocolate agar that is incubated at 35°C in a CO_2 incubator or a candle jar with increased humidity (Heltberg et al. 1984). Poor growth on routine sheep blood agar has been attributed to the use of a trypicase-soy base; better growth is seen when a heart infusion base is employed. Heltberg and coworkers found that media supplemented with cysteine supported the best growth (Heltberg et al. 1984). Commercial supplemented chocolate agars containing IsoVitalex (which contains cysteine) or other similar enrichments are satisfactory. Pinpoint colonies appear after 3 or 4 days' incubation. After a few more days, the colonies appear circular, smooth, and convex. On Gram stain, the bacteria appear

Table 7 *Biochemical characteristics for identification of* Capnocytophaga *species*

Characteristic	*C. ochracea*	*C. gingivalis*	*C. sputigena*	*C. haemolytica*	*C. granulosa*	*C. canimorsus*	*C. cynodegmi*
Hemolysis, SBA	–	–	–	(a	–	–	–
Oxidase	–	–	–	–	–	+	+
Catalase	–	–	–	–	–	+	+
Gliding motility	+	+	+	+	+	+	+
Growth on MacConkey agar	–	–	–	–	–	–	–
Reduction of nitrate to nitrite	–	V	–	+	–	–	V
Reduction of nitrite to N_2 gas	–	–	–	–	–	V	V
Indole	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	+	+
Ornithine decarboxylase	–	–	–	–	–	–	–
Esculin hydrolysis	V	–	–	+	–	V	+
Glycogen hydrolysis	V	–	–	+	–	+	V
Starch hydrolysis	+	–	–	+	+	+	+
Acid production from:							
Glucose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	–	+
Lactose	+	V	–	+	+	–	+
Xylose	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–
Mannose	+	+	+	+	+	V	+
Hydrolysis of:							
L-Alanyl-aminopeptidase	2+	2+	3+	–	1+	NA	NA
L-Arginine aminopeptidase	2+	2+	3+	–	2+	NA	NA
L-γ-Glutamyl aminopeptidase	1+	1+	1+	–	1+	NA	NA
L-Leucyl aminopeptidase	2+	2+	3+	–	2+	NA	NA
L-Lysyl aminopeptidase	2+	2+	3+	–	2+	NA	NA
N-α-benzoyl-DL-arginyl-aminopeptidase	–	1+	–	–	–	NA	NA

+, positve reaction; –, negative reaction; NA, data not available; SBA, sheep blood agar; V, variable reaction.
a) β-Hemolytic characteristic lost on repeated subculture.

as thin fusiform bacilli that are 2–4 microns in length. Some cells may appear slightly curved. Like the other fastidious bacteria discussed thus far, no growth is observed on MacConkey agar. *C. canimorsus* and *C. cynodegmi* are catalase-positive and oxidase-positive. These two reactions differentiate them from *Capnocytophaga* species found in humans, which are both oxidase- and catalase-negative. The canine species are also arginine dihydrolase-positive and ONPG-positive. Lysine and ornithine decarboxylase tests are negative. *C. canimorsus* and *C. cynodegmi* are differentiated by carbohydrate utilization tests, with the latter organism producing acid from a wider variety of sugars. Table 7 shows the biochemical reactions of *C. canimorsus* and *C. cynodegmi* along with the biochemical characteristics of the human *Capnocytophaga* species discussed previously. The term 'dysgonic' means that biochemical test media for identification, including the basal medium for carbohydrate fermentation, should be supplemented with serum (3–5 drops/5 ml broth) to obtain reliable and consistent reactions.

ANTIMICROBIAL SUSCEPTIBILITY

Capnocytophaga species found in humans are generally susceptible to erythromycin, clindamycin, tetracycline, and the fluoroquinolones and are variably susceptible to penicillin, aztreonam, colistin, and metronidazole (Arlet et al. 1987; Roscoe et al. 1992; Rummens et al. 1986). *Capnocytophaga* strains that are resistant to penicillin produce novel β-lactamase enzymes that confer resistance to expanded-spectrum cephalosporins and penicillins (Foweraker et al. 1990; Rosenau et al. 2000). These β-lactamase-producing strains are highly resistant to penicillin, amoxicillin, and cefazolin; addition of clavulanate results in a 64-fold decrease in the amoxicillin MIC for over 90 percent of the β-lactamase-producing strains (Roscoe et al. 1992). These strains are also less susceptible to cefuroxime, cefotaxime, and ceftazidime than β-lactamase-negative strains. Imipenem appears to be active against both β-lactamase-positive and β-lactamase-negative *Capnocytophaga* isolates (Roscoe et al. 1992). *Capnocytophaga* species are usually resistant to aminoglycosides (i.e. gentamicin, tobramycin, amikacin, netilmicin), trimethoprim, colistin, and vancomycin, although some strains may be susceptible (Roscoe et al. 1992; Rummens et al. 1986). Isolates have been reported that are resistant to the fluoroquinolones (Geisler et al. 2001). Gomez-Garces et al. reported a case of fatal bacteremia caused by a ciprofloxacin-resistant β-lactamase-producing strain of *C. sputigena* that was resistant to all β-lactams (except cefoxitin) and aminoglycosides, but was susceptible to tetracycline, erythromycin, clindamycin, aztreonam, and imipenem (Gomez-Garces et al. 1994). Often, immunocompromised and granulocytopenic patients may be treated empirically with a combination of a β-lactam antibiotic and an aminoglycoside, and cidal levels of antimicrobials are usually required for an optimal therapeutic response. Therefore, rapid recognition of the salient features of this organism by the microbiologist is important. The intrinsic resistance of *Capnocytophaga* species to aminoglycosides and the production of β-lactamase enzymes by some clinical isolates (detected by the nitrocefin test) should be reported to the physician as soon as possible.

Slow growth of *C. canimorsus* on agar medium and failure of some strains to grow in certain types of broth media have generally precluded adequate studies of the antimicrobial susceptibility of this organism. Unstandardized studies using disk-diffusion procedures designed for more rapidly growing bacteria had previously reported that *C. canimorsus* strains were susceptible to most antimicrobials but were resistant to aminoglycosides. In 1988, Verghese and associates reported on a broth-dilution technique using Schaedler broth as the growth medium (Verghese et al. 1988). All eight *C. canimorsus* strains tested were susceptible to all antibiotics, including penicillin, erythromycin, ticarcillin, piperacillin, cefazolin, cefaperazone, cefotaxime, ceftazidime, chloramphenicol, trimethoprim-sulfamethoxazole, and fluoroquinolones. Susceptibility of *C. canimorsus* to sulfonamides and aminoglycosides is unclear at present, since in vitro results with these agents appear to be method-dependent. Antibiotics that are generally more active against gram-positive organisms, such as vancomycin, clindamycin, erythromycin, and rifampin, were also active against *C. canimorsus*. These data support the clinical efficacy observed with penicillin (mean penicillin MIC = 0.04 μg/ml ± 0.01 μg/ml for all strains tested).

DYSGONOMONAS SPECIES

Dysgonomonas is a new genus of fastidious gram-negative bacilli that includes the former CDC group DF-3 as *Dysgonomonas capnocytophagoides* (Hofstad et al. 2000). Biochemically, this organism resembles *Capnocytophaga* species, but 16S rRNA sequencing indicates genetic relatedness to the anaerobic gram-negative species *Bacteroides distasonis*, *Bacteroides uniformis*, and *Prevotella melaninogenica* (Vandamme et al. 1986). The first two reports of this organism in association with human disease were published in 1988. In one case, *D. capnocytophagoides* was isolated in pure culture from multiple stool specimens of an elderly woman with common variable hypogammaglobulinemia (Wagner et al. 1988). In the second case, *D. capnocytophagoides* was recovered from the blood of a granulocytopenic male with acute lymphocytic leukemia following intensive radiation and chemotherapy (Aronson and Zbick 1988). Gill and coworkers (1991), at the National Cancer Institute, screened stool specimens from 690 patients and found 11 patients with diarrhea and with moderate to heavy growth of *D. capnocytophagoides*. Four of these 11

patients responded to treatment, while the organisms were cleared spontaneously in the remaining seven patients. Blum and colleagues isolated *D. capnocytophagoides* from stool specimens of eight patients over a 1-year period (Blum et al. 1992). These eight patients were immunocompromised or had severe underlying disease. While some patients who harbor this organism in the gut are symptomatic, with chronic diarrheal illness, others are asymptomatic (Blum et al. 1992). The association of enteric infection with *D. capnocytophagoides* and immunosuppression and common variable hyogammaglobulinemia has been noted by other investigators (Heiner et al. 1992; Melhus 1997). *D. capnocytophagoides* is also a rare isolate from soft-tissue infections, decubitus ulcers, and the urinary tract of compromised patients (Bangsborg et al. 1990; Melhus 1997; Schonheyder et al. 1991)

D. capnocytophagoides can be isolated from stool specimens using media for isolation of *Campylobacter* species, including cefoperazone–vancomycin–amphotericin (CVA) blood agar. Inoculated media are incubated at 35°C in 5–7 percent CO_2. Nonselective blood agar should also be inoculated, since some strains fail to grow on CVA blood agar (Gill et al. 1991). The organism has also been recovered on blood agar containing kanamycin and vancomycin following anaerobic incubation (Melhus 1997). *D. capnocytophagoides* grows slowly, with pinpoint colonies being visible on agar medium after 24 h incubation. Colonies are gray-white, smooth, and nonhemolytic after 48–72 h of incubation, and some strains produce a sweet odor during growth on agar media (Aronson and Zbick 1988; Blum et al. 1992; Gill et al. 1991; Wagner et al. 1988). On Gram-stained smears, the organisms appear as small gram-negative coccobacilli. Both oxidase and catalase reactions are negative, nitrate is not reduced, and urea is not hydrolyzed. Indole is produced in tryptone broth and esculin is hydrolyzed. Lysine and ornithine decarboxylases and arginine dihydrolase are not produced. The organism ferments glucose, xylose, and maltose; most strains also produce acid from sucrose and lactose but do not produce acid from mannitol. DF-3-like strains have also been described that are consistently sucrose-negative and slightly hemolytic on blood agar (Daneshvar et al. 1991). Chromatography can also be used for identifying this organism, since all strains consistently demonstrate 12- and 13-methyltetradecanoate in their cell walls, with minor amounts of tetradecanoate and hexadecanoate being present as well (Bernard et al. 1991; Daneshvar et al. 1991; Wallace et al. 1989). In addition to *D. capnocytophagoides*, two other *Dysgonomonas* species have been described. A single isolate of an organism designated *Dysgonomonas gadei* was isolated from a human gall bladder, and *Dysgonomonas mossii* was recovered from human clinical specimens (Hofstad et al. 2000; Lawson et al. 2002). The biochemical characteristics of *Dysgonomonas* species are summarized in Table 8.

Antimicrobial susceptibility of *D. capnocytophagoides* isolates has been determined by disk diffusion, broth dilution, and E-test methods (Aronson and Zbick 1988; Blum et al. 1992; Gill et al. 1991; Heiner et al. 1992; Melhus 1997; Wagner et al. 1988). By in vitro testing, *D. capnocytophagoides* strains are resistant to several antimicrobial agents, including penicillin, ampicillin, ampicillin-sulbactam, aztreonam, aminoglycosides, first-, second-, and third-generation cephalosporins, erythromycin, ciprofloxacin, and vancomycin. Most isolates are susceptible to trimethoprim-sulfamethoxazole and chloramphenicol and variably susceptible to piperacillin, clindamycin, tetracycline, and imipenem. Patients usually respond to therapy with trimethoprim-sulfamethoxazole, clindamycin, or tetracycline (Blum et al. 1992; Gill et al. 1991).

CDC GROUPS EF-4A AND EF-4B

Formerly a group of uncertain taxonomic affiliation, these bacteria have recently been classified as unnamed members of the family Neisseriaceae (Rossau et al. 1989). CDC group EF-4 (eugonic fermenter 4) is part of the normal oral flora of cats and dogs and may cause purulent cutaneous and pulmonary infections in these animals (Corboz et al. 1993; Weyant et al. 1994). EF-4 has been isolated from human wounds resulting from scratches and bites from cats and dogs (Talan et al. 1999). More severe infections have included bacteremia in a 65-year-old woman with metastatic small-cell carcinoid of the liver, endophthalmitis following a corneal laceration from a cat scratch, and chronic external otitis media resulting from a dog licking the ears of its owner (Dul et al. 1983; Roebuck and Morris 1999; Vartian and Septimus 1989).

CDC group EF-4 is a gram-negative coccobacillus that grows on both blood and chocolate agars; some strains may also grow on MacConkey agar. After 24 h of incubation, the colonies are about 1 mm in diameter, opaque, smooth, and yellowish, have an entire edge, and are weakly β-hemolytic or nonhemolytic. Some strains have a distinct popcorn-like odor. In the single bacteremic case, EF-4 was detected radiometrically by the BACTEC system after 2 days' incubation. The organism is nonmotile, oxidase-positive and catalase-positive and reduces nitrate to nitrite. Some strains may also reduce nitrite to nitrogen gas (Bruun et al. 1984). The organism does not produce urease or indole. EF-4 strains are divided into subgroups EF-4a and EF-4b based on carbohydrate utilization (see Table 8). EF-4a strains ferment glucose, do not produce acids from other carbohydrates, and are arginine dihydrolase-positive (Hanner et al. 1991; Weyant et al. 1994). EF-4b strains similarly produce acid oxidatively rather than fermentatively from glucose only. EF-4b strains also do not reduce nitrate all the way to gas and are arginine dihydrolase-negative (Hanner et al.

Table 8 *Biochemical characteristics for the identification of* Dysgonomonas *species and CDC groups EF-4A and EF-4B*

Characteristic	*D. capnocytophagoides*	*D. gadei*	*D. rossii*	Group EF-4A	Group EF-4B
Hemolysis, SBA	–	–/α	–	–/αw	–/αw
Oxidase	–	–	–	+	+
Catalase	–	+	V	+	+
Motility	–	–	–	–	–
Growth, MacConkey agar	–	–	–	V	V
NO_3 to NO_2	–	–	–	+	+
NO_2 to N_2 gas	NA	NA	NA	V	V
Indole	V	V	+	–	–
Acetoin	–	–	–	NA	NA
Urease	–	–	–	–	–
Gelatin hydrolysis	NA	–	–	V	–
Alkaline phosphatase	+	+	+	NA	NA
Arginine dihydrolase	–	–	–	+	–
Ornithine decarboxylase	NA	NA	NA	NA	NA
Esculin hydrolysis	+	+	+	–	–
Gas from glucose	–	–	–	–	–
Acid produced from:					
Glucose	+	+	+	+	+
Adonitol	–	–	NA	NA	NA
L-Arabinose	+	+	NA	NA	NA
Cellobiose	NA	+	NA	NA	NA
Dulcitol	–	–	NA	NA	NA
Erythritol	NA	–	NA	NA	NA
Fructose	NA	+	+	NA	NA
Glycogen	NA	–	NA	NA	NA
Inositol	–	–	+w	NA	NA
Lactose	+	+	+	–	–
Maltose	+	NA	+	–	–
Mannitol	–	–	+w	–	–
Mannose	+	+	+	NA	NA
Melezitose	NA	+	NA	NA	NA
Melibiose	+	+	NA	NA	NA
Raffinose	+	+	V	NA	NA
Rhamnose	NA	+	+w	NA	NA
Ribose	NA	+w	NA	NA	NA
Salicin	NA	+	+	NA	NA
Sorbitol	–	–	NA	NA	NA
Starch	NA	+	NA	NA	NA
Sucrose	+	+	+	–	–
Trehalose	–	+	+	NA	NA
Xylose	+	+	+	–	–
β-Glucuronidase	–	+	–	NA	NA
N-acetyl-β-glucosaminidase	–	+	+	NA	NA
α-Fucosidase	–	+	+	NA	NA

+, positive reaction; –, negative reaction; +w, weak positive reaction; NA, data not available; SBA, sheep blood agar.

1991). EF-4b strains may be subdivided into two subgroups based on acid production from gluconate (Ganiere et al. 1995). Most EF-4 isolates are susceptible to penicillin, ampicillin, cefazolin, chloramphenicol, tetracycline, erythromycin, clarithromycin, trimethoprim-sulfamethoxazole, the quinolones, and the aminoglycosides (Goldstein and Citron 1993).

CHROMOBACTERIUM VIOLACEUM

Chromobacterium violaceum is an infrequently encountered gram-negative bacillus that is normally found in the soil of tropical and subtropical regions, including Southeast Asia, South Africa, South America (Argentina, Brazil), Guyana, India, Malaysia, Trinidad, and Australia

(Kaufman et al. 1986; Martinez et al. 2000). The organism is geographically restricted between latitudes 35°N and 35°S (Dromigny et al. 2002). The organism is also found in soil in the southeastern regions of the USA, and human infections have been reported from Florida, Louisiana, and South Carolina (Midani and Rathore 1998). Most strains of *C. violaceum* synthesize a nondiffusible violet pigment called violacein. This pigment is ethanol- and acetone-soluble and insoluble in water, chloroform, ether, and benzene. Genes encoding the enzymes responsible for violacein synthesis reside on a 14.5-kb fragment of the *C. violaceum* genome (Pemberton et al. 1991). Nonpigmented strains do occur, and both pigmented and nonpigmented colonies may be observed together on blood agar plates. *C. violaceum* is the only member of the genus and is related to members of the emended family Neisseriaceae (Rossau et al. 1989).

C. violaceum is a rare human pathogen that is associated with sepsis and bloodstream dissemination. Infections are often fatal unless they are treated promptly, and relapses of disease occur frequently. The organism is usually acquired by contact with soil or stagnant water and can cause localized cellulitis and abscesses (Chattopadhyay et al. 2002; Lee et al. 1999; Petrillo et al. 1984). Entry of the organism into the bloodstream is associated with the development of septic shock, pneumonia, and abscesses in the lungs, brain, liver, and spleen (Moore et al. 2001). Clinically, its presentation is reminiscent of the septicemic form of melioidosis caused by *Burkholderia pseudomallei* (Chong and Lam 1997; Ti et al. 1993). Complications of bacteremia with *C. violaceum* include meningitis, osteomyelitis, vertebral abscess, urinary tract infection, otitis media, purulent conjunctivitis, and orbital cellulitis (Feldman et al. 1984; Shao et al. 2002; Shenoy et al. 2002; Shetty et al. 1987; Simo et al. 1984; Tucker et al. 1979). The organism may also cause severe gastrointestinal disease, particularly in children (Ballal et al. 2000; Dromigny et al. 2002). More severe clinical presentations occur in individuals with chronic granulomatous disease and conditions that affect neutrophil function (Macher et al. 1982; Sorenson et al. 1985). Other predisposing factors for severe illness with *C. violaceum* include malnutrition and alcoholism (Martin and Brimacombe 1992). The mortality rate for *C. violaceum* may exceed 60 percent. Infections also occur in animals, including gibbons, pigs, and buffaloes.

C. violaceum is a facultatively anaerobic, nonfastidious, motile rod that is catalase-positive and oxidase-variable. Individual cells are 1.5–3 microns long, 0.5–1 micron wide, and sometimes slightly curved. Since the oxidase test may be difficult to interpret because of pigmentation, anaerobic incubation of cultures will allow this characteristic to be determined, since violacein is not produced in the absence of oxygen. The organism posesses a single short polar flagellum and one or more longer subpolar or lateral flagella (Sneath 1956). *C. violaceum* grows optimally at 25°C, although most strains do grow at 35–37°C. Colonies on blood agar are smooth and circular, with an entire edge. Pigmentation can vary from pale violet to almost black and becomes more pronounced on prolonged incubation. If violacein is produced, further biochemical testing confirms the identification. However, nonpigmented, oxidase-positive strains may be confused with *Aeromonas* or *Vibrio* species because they are often also indole-positive (Lee et al. 1999). Unlike most *Aeromonas* species, *C. violaceum* is lysine decarboxylase-negative. The lysine-decarboxylase-negative aeromonads (the *A. caviae* group) are mannitol-positive, while *C. violaceum* is mannitol-negative. *C. violaceum* reduces nitrate to nitrite, does not hydrolyze esculin or ONPG, and is arginine dihydrolase-positive (see Table 9). Lysine and ornithine decarboxylase reactions are negative. Casein and gelatin are hydrolyzed, but starch is not hydrolyzed. The organism is a carbohydrate fermenter. Acid is produced from glucose, fructose, mannose, and trehalose; some strains also acidify maltose and sucrose. Lactose, mannitol, and xylose are not fermented. Pigmented and nonpigmented strains give the same biochemical reactions for identification. Growth occurs on MacConkey agar and other selective media for coliforms and some *C. violaceum* isolates may be β-hemolytic on blood agar.

C. violaceum is susceptible to a variety of antimicrobial agents, including aminoglycosides, tetra-

Table 9 *Biochemical characteristics of* Chromobacterium violaceum

Characteristic	Reaction	Characteristic	Reaction
Catalase	+	Acid from:	
Oxidase	V	Glucose	+
Ornithine decarboxylase	–	Sucrose	V
Arginine dihydrolase	+	Lactose	–
Lysine decarboxylase	–	Xylose	–
Arginine dihydrolase	+	Mannitol	–
Esculin hydrolysis	–	Mannose	+
Nitrate reduction	+	Trehalose	+
Indole	V	Maltose	V
ONPG hydrolysis	–	Growth on MacConkey agar	+

cyclines, chloramphenicol, trimethoprim-sulfamethoxazole, the fluoroquinolones (especially ciprofloxacin), piperacillin, imipenem, and aztreonam (Aldridge et al. 1988). Most strains are resistant to cephalosporins, susceptible to the cephamycins (i.e. cefotetan), and resistant to rifampin and vancomycin. Treatment of infections consists of surgical drainage of abscesses and purulent collections, debridement, and appropriate antimicrobial therapy (Midani and Rathore 1998). Although the optimal therapeutic approach to this organism is not known given the rarity of infections, the drug of choice is intravenous gentamicin administered for at least 14 days. Patients treated with shorter courses of therapy frequently relapse.

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Haemophilus

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DEFINITION

Members of this genus are small- to medium-sized coccobacilli or rods, often markedly pleomorphic, sometimes filamentous. The organisms are gram-negative, nonmotile, nonsporeforming and nonacid-fast. They are aerobic and facultatively anaerobic, and require one or both of two accessory growth factors – factor X (hemin or other porphyrins) and factor V (coenzyme I; nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP+)). They are chemo-organotrophic with both a respiratory and a fermentative type of metabolism. Sugars are attacked fermentatively. Oxidase and catalase reactions vary between species and strains; many are positive. Nitrate is reduced to nitrite. The species are obligate parasites of humans and animals, inhabiting particularly mucous membranes. The guanine and cytosine (G+C) content of DNA is 37–44 mol%. The type species is *H. influenzae*.

INTRODUCTION AND HISTORICAL PERSPECTIVE

In 1892, Pfeiffer claimed that the small gram-negative hemophilic coccobacillus that he had isolated in large numbers from the sputum of patients suffering from epidemic influenza was the causative agent of that disease. The specific name *Haemophilus influenzae* (Winslow et al. 1917), given to the organism, is a permanent reminder of this erroneous association. In fact, although *H. influenzae* was named as the type species, it was not the first member of the genus to have been described. In 1883, Koch described a bacillus causing conjunctivitis in Egypt, now named *H. aegyptius*.

Several other small gram-negative bacteria that share many of the characteristics of *H. influenzae* have since been described. All of these organisms require one or both of two accessory growth factors – factor X and factor V. The use of growth-factor requirement as the major criterion for defining the genus *Haemophilus* has been questioned in the light of DNA–DNA hybridization and 16S rRNA sequence comparison (Mutters et al. 1984; Dewhirst et al. 1992). Occasional strains in other genera within the family Pasteurellaceae and other unrelated genera exhibit growth-factor requirements similar to those of *Haemophilus* spp. However, many workers still accept that a requirement for X-factor or V-factor, or both, is a prerequisite for inclusion of a small gram-negative bacterium in the genus *Haemophilus* (Kilian and Biberstein 1984; Kilian personal communication).

SPECIES IN THE GENUS

The organisms we have included in the genus are listed in Table 1. Opinions differ on the validity of some of the species and it may be difficult to make clear-cut distinctions between others. DNA–DNA hybridization studies suggest the genus should be limited to *H. influenzae*, *H. aegyptius*, *H. haemolyticus*, *H. parainfluenzae*, and '*H. intermedius*' (Mutters et al. 1984). Comparison of partial *infB* sequences supports these findings (Hedegaard et al. 2001). Phylogenetic analysis by 16S rRNA sequence comparison suggests that the genus comprises *H. influenzae*, *H. aegyptius*, *H. haemolyticus*, *H. segnis*, *H. aphrophilus*, *H. paraphrophilus*, and perhaps *Actinobacillus actinomycetemcomitans* (Dewhirst et al. 1992). DNA–rRNA hybridization indicates that *H. aphrophilus* and *A. actinomycetemcomitans* are located on a separate branch of the family Pasteurellaceae distinct from the genus *Haemophilus* (De Ley et al. 1990). Clearly the exact delineation of the genus *Haemophilus* awaits further clarification.

The term *H. influenzae* biogroup aegyptius (Brenner et al. 1988) has been adopted for strains associated with Brazilian purpuric fever (BPF) – a severe pediatric infection characterized by conjunctivitis – proceeding to a frequently fulminant bacteremic infection. This term is often used synonymously with *H. aegyptius* but there is as yet no clear genetic evidence that they are identical (Kilian et al. 2002). Genomic analysis using polymerase chain reaction (PCR)-based subtractive hybridization suggests that strains of *H. influenzae* biogroup *aegyptius* causing invasive disease, harbor unique chromosomal regions that may have been acquired from unrelated microbial sources (Smoot et al. 2002). *H. aegyptius*, *H. influenzae* biogroup *aegyptius* and *H. influenzae* share over 90 percent genomic nucleotide sequence homology (Brenner et al. 1988) and are indistinguishable in routine laboratory tests.

H. aphrophilus is considered to belong to the genus because of its apparent requirement for X-factor on primary isolation and is so named because it needs carbon dioxide for growth (*aphrophilus* = foam loving). The X-factor requirement is readily lost on subculture. DNA–DNA hybridization studies indicate that *H. aphrophilus* and *H. paraphrophilus* are closely related organisms (Potts et al. 1986) and, possibly, do not constitute separate species. Potts and Berry (1983) have suggested it may be more appropriate to designate one of these two organisms as a subspecies, a view endorsed by Sedlacek et al. (1993). Since *H. aphrophilus* is X-factor-dependent and *H. paraphrophilus* is V-factor-dependent, we have regarded them as two species. DNA–DNA hybridization analyses of *Actinobacillus actinomycetemcomitans*, *H. segnis*, *H. aphrophilus*, and *H. paraphrophilus* have shown these four species are at least 28 percent related (Potts et al. 1985, 1986), consistent with their placement in a single genus. Accordingly, it has been proposed that *A. actinomycetemcomitans* should be reassigned to the genus *Haemophilus* as *H. actinomycetemcomitans* despite being X- and V-factor-independent. However, this chapter is restricted only to X- and/or V-factor requiring organisms. Casin et al. (1985) found the level of DNA hybridization between *H. ducreyi* and other *Haemophilus* spp. was very low

Table 1 *Species of* Haemophilus*: growth requirements, haemolytic activity and host origin*

Species	Requirement for			Hemolysis on horse blood agar	Host
	X	V	CO_2		
H. influenzae	+	+	–	–	Human
H. aegyptius	+	+	–	–	Human
H. haemolyticus	+	+	–	+[b]	Human
H. haemoglobinophilus	+	–	–	–	Dog
H. ducreyi	+	–	v	V	Human
H. aphrophilus	+[a]	–	+	–	Human
H. parainfluenzae	–	+	–	–	Human, monkey
H. parahaemolyticus	–	+	–	+[b]	Human
H. paraphrohaemolyticus	–	+	+	+[b]	Human
H. paraphrophilus	–	+	+	–	Human
H. segnis	–	+	–	–	Human
H. parasuis	–	+	–	–	Pig
H. paragallinarum	–	+	+	–	Poultry
H. paracuniculus	–	+	+	–	Rabbit
H. felis	–	+	+[c]	+/–	Cat

+, positive; –, negative; v, variable.
a) Requires hemin-containing media on primary isolation.
b) May lose hemolytic activity on subculture.
c) CO_2 requirement lost on subculture.

(0.6 percent relatedness) and they suggested that *H. ducreyi*, which is also phenotypically distinct from all other *Haemophilus* spp., is misplaced in this genus.

Hemophili isolated from swine and fowl have posed considerable problems of nomenclature and taxonomy (see Biberstein and White (1969); Blackall (1989)). Biberstein and White (1969) proposed the name *H. parasuis* for those V-factor-dependent strains, which are frequently isolated from the respiratory tract of pigs. An X- and V-factor dependent analog, *H. suis*, described by Lewis and Shope (1931) is of uncertain validity. The organism formerly classified as *H. pleuropneumoniae* has been transferred to the genus *Actinobacillus* (Pohl et al. 1983) on the basis of its phenotypic and DNA relatedness to *A. lignieresii*. The emended species *A. pleuropneumoniae* is composed of V-factor requiring and V-factor-independent biovars. A V-factor requiring *Haemophilus* sp., *H. paragallinarum*, causes infectious coryza in poultry. The existence of an X- and V-factor-requiring organism, '*H. gallinarum*' (Schalm and Beach 1936) has been questioned (Page 1962; Roberts et al. 1964) and it appears likely that these strains were erroneously described as being dependent on X and V factors owing to technical limitations. Unfortunately these strains are no longer available. *H. paragallinarum* appears to be more closely related to the genus *Actinobacillus* than to *Haemophilus* on the basis of DNA hybridization studies (Mutters et al. 1984; Piechulla et al. 1985) and its complement of respiratory quinones (Höllander and Mannheim 1975). *H. paracuniculus* has been isolated from the small intestine of rabbits with mucoid enteritis (Targowski and Targowski 1979). An organism isolated from the respiratory tract of cats has been named *H. felis* (Inzana et al. 1992) Other species of *Haemophilus* have been described, but have not been validated. These include '*Haemophilus somnus*' (Baillie et al. 1973) and '*Haemophilus agni*' (Kennedy et al. 1958).

HABITAT

Haemophilus spp. are obligate parasites of the mucous membranes of humans and a wide variety of animal species (Table 1). They demonstrate a marked degree of host specificity; each species being associated exclusively with one host species. *Haemophilus* spp. commonly inhabit the human upper respiratory tract (Kilian et al. 1972) and mouth (Kilian and Schiøtt 1975) and may also be isolated from the intestinal tract (Palmer 1981) and vagina (Kilian 1976). A similar habitat is seen in pigs (Harris et al. 1969), monkeys (Rayan et al. 1987), poultry (Hinz and Kunjara 1977), and other birds (Grebe and Hinz 1975). Some species are regular members of the normal flora of their host while others seem only to be associated with disease (see review by Kilian and Frederiksen (1981)).

CELL MORPHOLOGY

H. influenzae is a slender, short, poorly staining gram-negative rod or coccobacillus, 0.3–0.5 μm × 0.5–1.0 μm with rounded ends. The organisms are relatively difficult to stain by Gram's method. Prolonged counterstaining for 5–15 min with very dilute carbol fuchsin often gives satisfactory results. Some cultures are uniformly coccobacillary. At other times they are markedly pleomorphic, with many long filamentous forms. Either form may be observed in Gram-stained smears of the cerebrospinal fluid in cases of hemophilus meningitis (Figure 1).

Capsules are found in some species, notably *H. influenzae*, *H. parasuis*, and *H. paragallinarum*. Occasional capsulate strains of *H. parainfluenzae* have been described (Sims 1970). The capsules of *H. influenzae* and *H. paragallinarum* are important in pathogenicity.

CULTURAL CHARACTERISTICS

Growth factor requirements

Specific growth factor requirements are major criteria by which the genus *Haemophilus* is defined. These species will not grow in the absence of certain factors that are present in blood, hence the generic name *Haemophilus* or 'blood-loving'. *H. influenzae* requires two accessory growth factors, a heat-stable growth promoting substance present in red blood cells (X factor), and a heat-labile vitamin-like substance (V factor). Neither of these factors is a single substance.

X FACTOR

Fildes (1924) named hemin as X factor and suggested it plays a role as an enzyme in respiration. Various iron-containing compounds, such as hemes or protoporphyrin IX, can satisfy a requirement for X factor. It was thought that *H. influenzae* had an absolute requirement

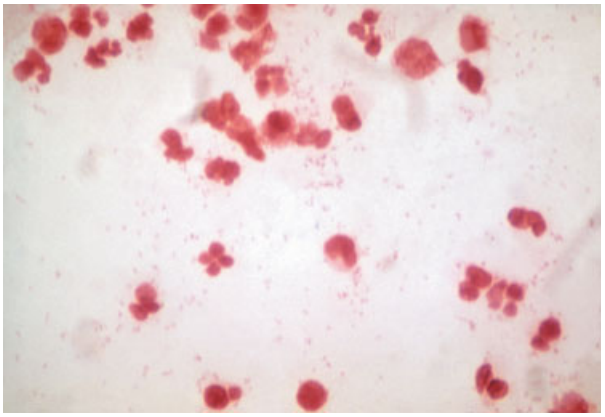

Figure 1 *Gram-stained smear of cerebrospinal fluid from a case of* H. influenzae *type b meningitis (Bar = 5 μm)*

for heme to supply both the iron requirement of the bacterium and essential porphyrin (White and Granick 1963; Coulton and Pang 1983). It now appears that, in most instances, only protoporphyrin IX is required (Pidcock et al. 1988) provided iron can be obtained from another source, because *H. influenzae* has a ferrochelatase that catalyses the insertion of iron into the protoporphyrin ring (White and Granick 1963). *H. influenzae* does not produce a hemolysin (Kilian 1976) and therefore cannot acquire its iron from the hemolysis of red blood cells. Herrington and Sparling (1985) have shown that *H. influenzae*, but not *H. parainfluenzae*, can use human transferrin as a sole source of iron. *H. influenzae* can acquire hemin from various sources, including hemoglobin–haptoglobin complexes, heme–hemopexin, and heme–albumin (Pidcock et al. 1988). The requirement for X-factor is substantially reduced, but not eliminated, during anaerobic growth (Gilder and Granick 1947) and strains might thus be erroneously regarded as X-factor independent. When grown in complex media, *H. influenzae* requires NAD 0.2–1.0 mg/l and hemin up to 10 mg/l (Evans et al. 1974).

Hemin-dependent strains of *Haemophilus* spp. are unable to convert δ-aminolaevulinic acid (ALA) to protoporphyrin (White and Granick 1963; Biberstein et al. 1963), a process involving five enzyme-mediated steps (Figure 2). X-dependent strains lack some or all of these enzymes. The ability to synthesize porphobilinogen and porphyrins from ALA is the basis of the porphyrin test, which was first described by Biberstein et al. (1963) and subsequently developed by Kilian (1974). This test is convenient and, if properly performed, is a reliable method for demonstrating a hemin requirement. However, it does not exclude the possibility of a requirement for hemin because of a lack of enzymes later in the biosynthetic pathway. Some *Haemophilus* strains apparently require hemin itself since they lack the ferrochelatase responsible for incorporating iron into the hemin molecule (White and Granick 1963). *H. aphrophilus* may lose its requirement for X-factor following subculture and thus appear to be X- and V-factor independent.

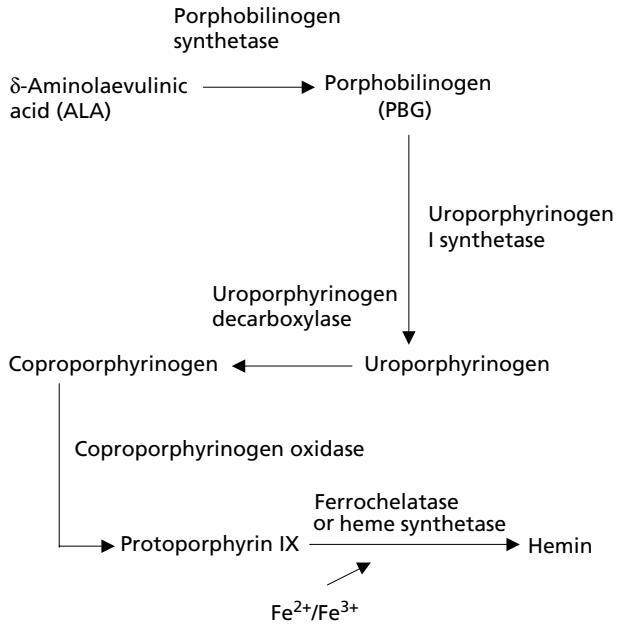

Figure 2 *Main steps in porphyrin and hemin biosynthesis*

V FACTOR

This second factor is present in the tissues of plants and animals and is synthesized by most bacterial species other than *H. influenzae*. It was originally thought that this thermolabile substance was a vitamin, hence the name V-factor (Thjøtta and Avery 1921). Pittman (1935) showed that V-factor is involved in oxidation-reduction processes in the growing bacterial cell. V-factor requirement can be satisfied by NAD^+ or by precursors of this coenzyme. (See reviews of aspects of V-factor dependency by Cyanomon et al. (1988) and Niven and O'Reilly (1990).) Although V-factor is present in blood it is not available to hemophili because it is largely intracellular. Additionally the blood of many animal species contains NADase activity (Krumwiede and Kuttner 1938). Heating blood agar to about 75°C, until it acquires a chocolate color, releases V-factor from red cells and also inactivates NADase activity.

Staphylococcus aureus, and some other organisms, release V-factor during growth. This diffuses into the surrounding medium, enhancing the growth of any organisms requiring V-factor. Any V-factor-requiring species of *Haemophilus* will show satellitism, as will occasional strains of other bacterial species of unrelated genera, for example, some strains of streptococci, neisseria, and diphtheroids. V-factor-independent isolates of *H. parainfluenzae* have been described (Windsor et al. 1991). This finding is associated with the acquisition of a 5.25-kb plasmid from *H. ducreyi* (Martin et al. 2001). Plasmid-encoded V-factor-independent strains of *H. paragallinarum* (Bragg et al. 1993) have been reported from South Africa.

Haemophilus spp. are fastidious not only in their requirements for X- and V-factor but also in their need for other nutritional factors, which vary among the species. *H. influenzae* requires pantothenic acid, thiamine, and uracil and some strains also need purine and cysteine (Holt 1962). *H. parasuis* and *H. ducreyi* require serum. *H. paragallinarum* requires serum and sodium chloride (Rimler et al. 1977), though it is not halophilic in the usual sense.

Physical requirements for growth

The optimum temperature for growth is 35–37°C. The minimum temperature for growth is 20–25°C. Most

Haemophilus spp. are killed by heating at 55°C for 30 min. *H. influenzae* grows better in aerobic than in anaerobic conditions. Raised carbon dioxide tension improves the growth and may be required for a number of species, including *H. aphrophilus*, *H. paraphrophilus*, *H. paraphrohaemolyticus*, and *H. paragallinarum*. Under the usual conditions found in a bench incubator or incubator room, some recently isolated strains of *Haemophilus* spp. may grow only feebly or not at all. Therefore, carbon dioxide should be added to the atmosphere for the primary isolation and may be beneficial for subsequent cultures of *Haemophilus* spp. Many strains that have an apparent carbon dioxide requirement on primary isolation rapidly become adapted to growing in air.

Type of growth

COLONIAL MORPHOLOGY

The colonies produced by *Haemophilus* spp. on solid media vary considerably with the individual strains and with the medium used. On blood agar, *H. influenzae* forms translucent low, convex, or flat pinpoint colonies. On a more favorable medium, such as chocolate agar, Levinthal's agar, or Fildes' agar, colonies of noncapsulate strains are typically grayish, transparent, smooth, and low convex or flat with a slightly splayed-out entire edge (Figure 3). They attain a size of 0.5–0.8 mm after incubation for 24 h at 37°C, enlarging to 1–1.5 mm by 48 h. Morphologically atypical strains produce more granular colonies. Capsulate strains of *H. influenzae* form larger, more opaque, smooth, mucoid colonies 3–4 mm in diameter, which may appear to coalesce. Colonies of capsulate strains grown on clear media, such as Levinthal's agar, are iridescent when examined by obliquely transmitted light. The iridescence is due to dispersion of light by the arrangement of organisms in a grid-like pattern like a diffraction grating (Engbaek 1950). This phenomenon is best observed in 18-h cultures, gradually fading after prolonged incubation.

Colonies of *H. influenzae* grow to a larger size in the vicinity of certain other bacterial colonies, e.g. staphylococci, which secrete V factor (satellitism) (Figure 4). Strains from cases of meningitis and epiglottitis usually produce indole, which gives the cultures a characteristic pungent smell.

The various colonial forms of the other *Haemophilus* spp. tend to resemble noncapsulate *H. influenzae* with a few exceptions. *H. haemoglobinophilus* colonies at first resemble *H. influenzae* but become more opaque and larger, reaching 1–2 mm diameter at 24 h. They grow well on blood agar and growth is not enhanced around staphylococci. *H. aphrophilus* and *H. paraphrophilus* form small, highly convex, granular colonies. Colonies of *H. paraphrophilus* and *H. aphrophilus* may pit the agar, particularly on primary isolation on blood agar (Chadwick et al. 1995) leading to possible misidentification as *Eikenella corrodens*. Colonies of *H. parainfluenzae* can be flat and smooth or rough and wrinkled, reaching a size of 1–2 mm at 24 h. *H. segnis* is slow growing, the colonies being smooth or granular, odor-free and about 0.5 mm after incubation for 48 h. *H. parasuis* is also slow growing, the colonies reaching a diameter of 0.5 mm after 48–72 h. *H. paragallinarum* is usually capsulate, producing iridescent colonies on transparent media.

In liquid media, such as Levinthal's broth or Filde's broth, most strains of *H. influenzae* produce a uniform turbidity. Occasional strains that produce a flocculent deposit with a slight turbidity of the supernatant suspension are found to be filamentous on microscopic examination.

HEMOLYSIS

Pritchett and Stillman (1919) observed that gram-negative rods requiring X and V factors could be hemolytic. Pittman (1953) distinguished *H. haemolyticus* and

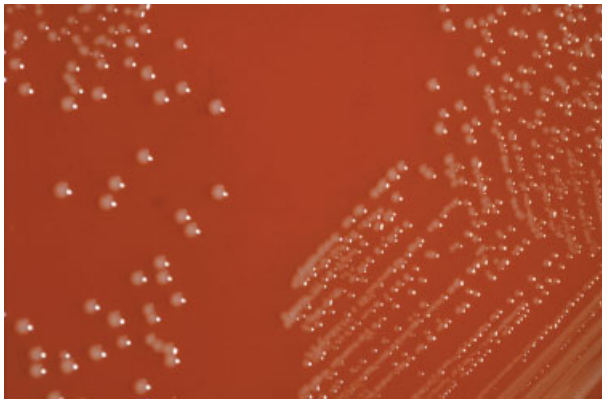

Figure 3 *Culture of* H. influenzae *on chocolate agar*

Figure 4 *Culture of* H. influenzae *on whole horse blood agar showing satellitism of colonies adjacent to growth of* Staphylococcus aureus

H. parahaemolyticus from the nonhemolytic species *H. influenzae* and *H. parainfluenzae*. Zinnemann et al. (1971) described the hemolytic species *H. parahaemolyticus*, which requires V factor and carbon dioxide. Kilian (1976) noted that, in a proportion of strains of *H. parahaemolyticus* and *H. parahaemolyticus*, hemolysis is an unstable property.

METABOLISM

All of the strains possess functional enzymes of the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways, the hexose monophosphate shunt, tricarboxylic acid cycle, and gluconeogenesis, with a high activity of malate dehydrogenase (see Tatusov et al. (1996) for a review of *Haemophilus* metabolism).

GENETIC MECHANISMS

Transformation and transduction

The genome of *H. influenzae* has been shown to be a 1.9 Mb circle with a G+C content of c. 37 percent and genetic maps for *H. influenzae* strains Rd (capsule type d) and Eagan (capsule type b) and *H. parainfluenzae* have been constructed by use of pulsed field gel electrophoresis (Barcak et al. 1991; Butler and Moxon 1990; Kauc and Goodgal 1989). *H. influenzae* strain Rd was the first bacterium for which the entire DNA sequence was determined (Fleischmann et al. 1995). Several restriction-modification systems have been identified and characterized from hemophili. *H. influenzae* is naturally transformable; under growth-limiting conditions it will become competent to bind and take up double-stranded DNA and recombine these fragments into any homologous sites on the chromosome. Natural transformation from *H. influenzae* into *Neisseria meningitidis* appears to have occurred at some time in the past (Kroll et al. 1998). Temperate bacteriophages, but no virulent or transducing phage, have been identified in *H. influenzae* (Barcak et al. 1991).

Plasmids and transposons

Plasmids have been identified in *H. influenzae* and other species of *Haemophilus*. Large (c. 60 kb) conjugative plasmids are associated with multiple antibiotic resistance and are generally integrated into the chromosome (Stuy 1980; Levy et al. 1993). They encode a wide range of resistances, including ampicillin, chloramphenicol, tetracycline, erythromycin, and aminoglycoside resistance. These plasmids share sequence homology and can be detected in fully susceptible strains. It has been suggested that there is a core 'cryptic' plasmid, which can acquire antibiotic resistance genes (Leaves et al. 2000). TnA or Tn*10*-like transposons, carrying ampicillin or tetracycline resistance genes respectively, are often carried on these plasmids and are thought to have been acquired from Enterobacteriaceae by a cryptic plasmid in *H. parainfluenzae* (Brunton et al. 1986). Chloramphenicol resistance genes have been detected as an insertion into one of the inverted repeat sequences flanking the tetracycline resistance transposon (Brunton et al. 1986). Indistinguishable plasmids have been identified from epidemiologically unrelated *H. influenzae*, suggesting conjugative spread in nature (Dimopoulou et al. 1992). Small (<10 kb) cytoplasmic, nonconjugative, plasmids encoding Tem-1 β-lactamase mediated ampicillin resistance have been identified in various *Haemophilus* species; these share homology with similar plasmids from *Neisseria* spp. (Brunton et al. 1986). A 4.4-kb cytoplasmic plasmid has been associated with the production of ROB-1 β-lactamase in *H. influenzae* (Daum et al. 1988). These plasmids are similar to those found in *H. pleuropneumoniae* (now classified as *Actinobacillus*) (Daum et al. 1988) and an animal source for this resistance has been postulated (Medeiros et al. 1986). A 24-kb plasmid p3031 has been associated with the majority of *H. influenzae* biogroup *aegyptius* strains from BPF although not all strains possess the plasmid (Tondella et al. 1995).

Insertion sequences are associated with *H. influenzae* type b (Hib) and have been detected in some noncapsulate *H. influenzae* (NCHI) (St. Geme et al. 1994). Many virulence-associated genes have been investigated; in particular, the genetics of encapsulation has been investigated in detail. A cluster of genes, termed the *cap* locus (18-kb), controls capsule production. The *cap* locus can be divided into three regions. Region 1 contains the four conserved *bex* genes (*bex* A–D), which control export of capsular polysaccharide to the cell surface of the organism. Region 2 contains four genes involved in polysaccharide biosynthesis. These genes are capsular-type specific. Region 3 contains two open reading frames involved in polysaccharide export (Kroll et al. 1991b). Most strains of types a, c–f strains possess a single copy of the *cap* locus, but the majority of clinical isolates of type b strains possess two copies in tandem, one copy having a deletion of *bex* A. This duplication renders this region unstable, with a marked propensity for recombination. In some cases recombination results in mutants possessing only a single copy of the *cap* locus, and lacking *bex* A. These strains do not express capsule and are termed b-strains. A similar deletion has been reported in clinical isolates of *H. influenzae* type a (Kroll et al. 1994) Sometimes recombination results in a mutant with up to five copies of the *cap* locus. The amount of capsule produced increases in proportion to the number of copies of *cap* genes, and these strains show increased virulence in animal models (Roche and Moxon 1995).

Population genetics

Pennington (1993) reviewed the population genetics structure of *H. influenzae*. A multilocus enzyme electrophoresis (MLEE) study of 2 209 capsulate strains from 30 countries showed the population structure to be clonal with nine clones responsible for most invasive Hib disease (Musser et al. 1990). In contrast, studies of noncapsulate *H. influenzae* showed a panmictic structure with innumerable clones (Porras et al. 1986; Musser et al. 1986). Noncapsulate strains are thought to have arisen by convergent evolutionary loss of the ability to synthesize or express a polysaccharide capsule from a capsulate ancestor (Musser et al. 1986). Preliminary studies using multilocus sequence typing (MLST) show a similar clustering of isolates to that obtained by MLEE (Meats et al. 2003).

CELL ENVELOPE COMPOSITION: ANTIGENIC STRUCTURE

Capsule

There are six antigenic types of capsule in *H. influenzae*, designated a–f (Pittman 1931), each one composed of a linear polymer of disaccharide units. The capsules are negatively charged, acidic, and hydrophobic. The capsules of types a, b, c, and f contain phosphodiester linkages and are, therefore, teichoic acids, whereas types d and e contain glycosidic linkages with no phosphate, and unusual sugars, and are true polysaccharides (Moxon and Kroll 1990). Types a and b differ from the others in containing the 5-carbon component ribitol, and type b is unique as both sugars are pentoses. The type b capsule has been the most extensively characterized and consists of a phosphodiester-linked polymer of polyribosyl ribitol phosphate (PRP). The type b capsule antigen (PRP) cross-reacts with a wide variety of bacteria including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, and *Escherichia coli* K100 (reviewed by Slack 1990).

Hib disease is associated with a lack of antibodies to polyribosyl ribitol phosphate. Infants less than about 2 years old have little or no anticapsular antibodies once maternal antibodies decline and are especially susceptible to infection with Hib. Immunization of infants with PRP fails to elicit protective levels of antibody in this age group (Peltola et al. 1977). This is due to the T-cell-independent nature of the immune response to polysaccharide antigens. The conjugation of PRP to a protein carrier stimulates anti-PRP antibodies in young infants and is the basis of current Hib vaccines (Jones 1993). The PRP capsule also stimulates complement-mediated bacteriolysis (Moxon and Kroll 1990; Tunkel and Scheld 1993).

Pili

Pili have been demonstrated on *H. influenzae* (reviewed by Gilsdorf et al. 1997). Hib from the nasopharynx usually express pili, whereas isolates from blood and cerebrospinal fluid do not; this has been shown to be due to reversible phase variation, which occurs at a frequency of about 10^{-4} to 10^{-3} per generation. Following serial subculture, the majority of isolates do not express pili. Strains of *H. aegyptius* and *H. influenzae* biogroup *aegyptius* are more stably piliated (Gilsdorf et al. 1997). Piliated *H. influenzae* bind to erythrocytes, which contain the Anton (AnWj) antigen (van Alphen et al. 1986). A cluster of five genes (*hifA*, *B*, *C*, *D*, *E*) governs pilus expression. Most strains possess a single copy of this cluster, but two copies are found in *H. aegyptius* and *H. influenzae* biogroup *aegyptius* (Read et al. 1998), which may explain the greater stability of piliation in these species. Nonhemagglutinating pili that adhere to HeLa cells are found in strains of *H. influenzae* biotype IV (Rosenau et al. 1993).

Lipo-oligosaccharide

The major constituent of the outer membrane is lipo-oligosaccharide (LOS). In *H. influenzae*, this comprises lipid A linked to outer core oligosaccharides, via a single phosphorylated 2-keto-3-deoxyoctulosonic acid moiety and three heptoses. The lipid A backbone is a glucosamine disaccharide substituted by two phosphate groups, as in enterobacterial lipid A, but with a simpler fatty acid composition of only tetradecanoic and its 3-hydroxylated derivative (reviewed by Moxon and Maskell 1992). At least two species-specific antigenically distinct components of lipid A have been detected among capsulate and noncapsulate strains. The outer core oligosaccharides contain mainly glucose and galactose, with the molar ratios and linkages varying between capsular types a–f, and a sialylated terminal lactosamine (reviewed by Roche and Moxon 1995). LOS biosynthesis is encoded by the *lic* genes located in three multigene chromosomal loci (Hood et al. 1996). Several LOS epitopes are similar to those in pathogenic *Neisseria* species and are cross-reactive with human glycosphingolipid epitopes. Extensive antigenic variation in LOS, between strains and within strains, has been documented for capsulate strains and NCHI. Variation in colonial morphology is also associated with variation in LOS (Roche and Moxon 1995). Antibodies to LOS have been demonstrated after infection but are not protective.

Outer-membrane proteins

The outer membrane of *H. influenzae* contains 10–20 different proteins with a few major polypeptides accounting for 80 percent of the total outer-membrane

protein (OMP) (Loeb and Smith 1982). A numerical and alphabetical classification system for OMPs, which are sarkosyl insoluble, are in use (reviewed by van Alphen 1993). The major OMPs are P1 (or protein a, MW 46–50-kDa), P2 (or protein b/c, a doublet, MW 36–42 kDa with porin activity), P4 (or protein e, a lipoprotein, MW 30-kDa, involved in heme uptake), P5 (or protein d/f; two conformers of the same protein) and P6 (or protein g, a lipoprotein, MW16 kDa). The OMP originally designated P3 consists of both the non-heat-modified form of P1 and the heat-modified form of P5. OMPs P1, P2, and P5 are heterogeneous in molecular weight and form the basis for typing systems. Monoclonal antibody studies have shown OMP P1 epitopes are not conserved between Hib or NCHI strains (Panezutti et al. 1993). P2 is the most abundant OMP and appears to contribute to virulence (Cope et al. 1990). P2 is conserved in type b strains but extremely diverse and shows antigenic drift in NCHI; antibodies are strain-specific (Duim et al. 1994; Murphy 1994). OMP P6 has been shown to be conserved in Hib and NCHI and bactericidal antibodies have been detected in human sera (Nelson et al. 1988; Barenkamp 1992).

Conserved epitopes, which elicit protective antibody responses against all *H. influenzae* have been sought as candidates for inclusion in component vaccines. Antibodies to OMP P1 were shown to be partially protective against Hib in an infant rat bacteremic model (Granoff and Munson 1986; Gonzales et al. 1987; Loeb 1987) and antibodies to P2 and P6 were shown to be protective in this model (Granoff and Munson 1986; Green et al. 1987). OMP P4 is reported to be a conserved nonimmunogenic protein and anti-P4 serum was not protective against Hib meningitis in infant rats (Granoff and Munson 1986; van Alphen 1993). In contrast, antibodies raised against P4 were shown to be bactericidal for Hib strain Eagan and six NCHI strains (Green et al. 1991). Rabbit antibodies to OMP P5 did not protect infant rats from infection with Hib in the infant rat bacteremic model (Granoff and Munson 1986). Human cellular immunity to the P6 OMP of NCHI has been detected (Kodama and Faden 1995).

There are a number of other minor outer membrane proteins, including PCP, OMP 26, D15, protein D, and several heme- and transferrin-binding proteins. Antiserum raised against a purified outer-membrane lipoprotein PCP (MW 16-kDa, which co-migrates with OMP P6) was bactericidal for nine of 11 NCHI strains but not for Hib strain Eagan (Deich et al. 1990). Synergistic killing of NCHI by anti-e and anti-PCP (Green et al. 1991) and anti-PCP and anti-P6 sera (Deich et al. 1990) has been reported. D15 is a high molecular weight protein (MW 103-kDa) which is highly conserved. Antibodies against D15 have been detected in convalescent serum from the majority of infants with invasive Hib disease and shown to reduce the level of bacteremia in infant rats (Kimura et al. 1985; Thomas et al. 1990).

CLASSIFICATION

Tables 1 and 2 show that members of this genus can be classified on the basis of their requirement for X- and V-factors, hemolytic activity, and biochemical activities; requirement for an increased partial pressure of carbon dioxide in the atmosphere for primary isolation is also of some help. Tests of carbohydrate utilization and acid production can be of considerable value in the classification of *Haemophilus* spp. The results of these

Table 2 *Biochemical characteristics of* Haemophilus *spp.*

	Production of				Acid from							
Species	**Cat**	**Ox**	**Ind**	**Ure**	**Glu**	**Fru**	**Gal**	**Lac**	**Man**	**Suc**	**Tre**	**Xyl**
H. influenzae	+	+	D	D	+	−	+	−	−	−	−	+
H. haemolyticus	+	+	±	+	+	W	+	−	−	−	−	V
H. haemoglobinophilus	+	+	+	−	+	−	−	−	+	+	−	+
H. ducreyi	−	+	−	−	−	−	−	−	−	−	−	−
H. aphrophilus	−	−	−	−	+	+	+	+	−	+	+	−
H. parainfluenzae	±	+	D	D	+	+	+	−	−	+	−	−
H. parahaemolyticus	±	+	−	+	+	+	±	−	−	+	−	−
H. paraphrohaemolyticus	+	+	−	+	+	+	V	−	−	+	−	−
H. paraphrophilus	−	+	−	−	+	+	−	+	−	+	+	−
H. segnis	V^{w}	−	−	−	W	W	$\pm^{w}$	−	−	W	−	−
H. parasuis	+	−	−	−	+	+	+	V	−	+	−	−
H. paragallinarum	−	−	−	−	+	+	−	−	+	+	−	V
H. paracuniculus	+	+	+	+	+	+	−	−	−	+	−	−

Cat, catalase; D, different reaction given by biotypes; Fru, fructose; Gal, galactose; Glu, glucose; Ind, indole; Ox, oxidase; Lac, lactose; Man, mannitol; Suc, sucrose; Tre, trehalose; Ure, urease; Xyl, xylose; V, variable; W, weak reaction +, positive; −, negative; ±, variable, most strains negative. Data compiled from the work of Kristensen (1922); Lewis and Shope (1931); Tunevall (1951); Biberstein and White (1969); Sneath and Johnson (1973); Kilian (1976); Hinz and Kunjara (1977); Kilian and Theilade (1978); Targowski and Targowski (1979); Zinnemann (1980); Broom and Sneath (1981); Sturm and Zanen (1984); Kilian and Biberstein (1984).

tests are, to some extent, affected by the media used, the period of observation, and other conditions of the test, and thus they should be interpreted with caution. The degree of acidity may be slight and hemophili can produce acid in media devoid of sugar (Tunevall 1951). All hemophili, with the exception of *H. ducreyi*, 'ferment' glucose anaerobically (Sneath and Johnson 1973). The exact mode of carbohydrate breakdown in these tests is not known and the term 'fermentation' is being applied in a somewhat loose sense to refer to metabolism with acid production. Most strains of *H. parainfluenzae*, *H. haemolyticus*, *H. aphrophilus*, and *H. paraphrophilus* produce small amounts of hydrogen and carbon dioxide from glucose (Kilian 1976). The remaining species do not produce any gas, indicating differences in the pathways of carbohydrate metabolism within the genus.

Almost all strains of *Haemophilus* reduce nitrate to nitrite (Kilian 1976). Catalase and oxidase reactions can be used to differentiate species of *Haemophilus*. Biberstein and Gills (1961) noted the catalase activity of hemophili can vary with the conditions of the test; some catalase-negative strains become catalase-positive when provided with sufficient hemin. *H. influenzae* is invariably oxidase positive, *H. aphrophilus* and *H. ducreyi* are oxidase negative, the oxidase reaction of other species is variable. Urease activity is found in many strains of *H. influenzae*, *H. parainfluenzae*, *H. parahaemolyticus*, and *H. paraphrohaemolyticus* (Kilian 1976).

LABORATORY ISOLATION AND IDENTIFICATION

Isolation

The isolation of *Haemophilus* spp. from clinical material requires the use of media that fulfil the fastidious growth requirements of these microorganisms. Ordinary blood agar is not suitable because growth of strains requiring V factor is limited by the V-factor deficiency of this medium. Most species grow well on chocolate agar. Rennie et al. (1992) found that GC agar base supplemented with 1 percent yeast autolysate and 5 percent sheep blood (chocolated) was superior to other medium bases and growth supplements for the primary isolation of *Haemophilus* spp. (except *H. ducreyi*). Levinthal (1918) devised a transparent and nearly colorless medium, which is capable of supporting the growth of *H. influenzae*. The use of such a medium is essential for observing the iridescence of capsulate strains of *H. influenzae*. A transparent medium may also be prepared from Fildes' peptic digest of blood (Fildes 1920). A number of completely synthetic media have been devised, but Tebbutt (1984) found none of these defined media was adequate for growth of a wide range of strains of *H. influenzae*. The addition of 1 percent IsoVitalex (BBL) to chocolate agar may increase the recovery of *H. aegyptius*.

For primary isolation of hemophili from specimens from the respiratory tract, a medium that supports good growth of hemophili can be made somewhat selective by the addition of substances that inhibit other members of the normal respiratory tract flora, e.g. media containing bacitracin (Barber 1969) and vancomycin, bacitracin, and clindamycin (Chapin and Doern 1983) have been described. Möller et al. (1993) described NAG medium, (blood agar base supplemented with *N*-acetyl glucosamine, hemin and NAD) which improves the isolation of *H. influenzae* from the sputum of cystic fibrosis patients. Overgrowth of *Pseudomonas aeruginosa* can lead to difficulties in recovering *H. influenzae* in sputum samples from patients with cystic fibrosis. To some extent this can be resolved by incubating cultures anaerobically. Vastine et al. (1974) found IsoVitalex supplemented chocolate agar was the most useful medium for the isolation of *Haemophilus* spp. from the conjunctiva. Sturm (1986) described a selective culture medium containing vancomycin, bacitracin, clindamycin, and amphotericin B to facilitate the isolation of *H. influenzae* and *H. parainfluenzae* from genital tract specimens. Michaels et al. (1975) described a Levinthal agar supplemented with high titer *H. influenzae* type b (Hib) antiserum. Barbour et al. (1993a) devised an improved antiserum agar consisting of Columbia agar supplemented with yeast extract, hemin, NAD, bacitracin, and type b antiserum. On this medium, colonies of *H. influenzae* type b can be readily discerned in mixed cultures from respiratory material since they have an obvious halo of antigen–antibody precipitate (Figure 5).

Cultures should be incubated at 37°C in an aerobic atmosphere enhanced with 5–10 percent carbon dioxide.

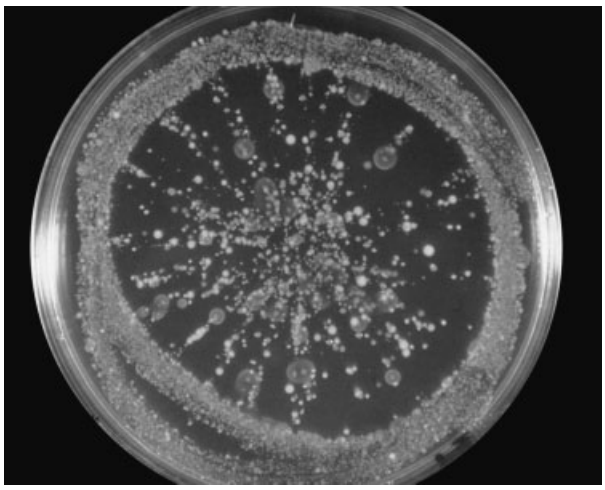

Figure 5 *Culture of a throat swab on antiserum agar showing halos around colonies of Hib (by courtesy of Dr M Barbour)*

Identification

A presumptive *Haemophilus* spp. isolate should first be tested for its X- and V-factor requirements. These requirements are usually demonstrated by the absence of growth on media deficient in X and V factor but otherwise nutritionally adequate, except near paper disks or strips impregnated with X, V, or X plus V factors (Figure 6). Consistent results in these tests for growth factor requirements will be obtained only if a small inoculum is used, to avoid carry over of the factors. V-factor requirement of strains can be inferred by demonstrating satellitism near a streak of *S. aureus*. As V factor is heat-labile, media in which all the constituents have been autoclaved will reliably be free of it. Most of the basic fluid and solid media contain traces of X factor, as do some brands and batches of peptone. Doern and Chapin (1984) found trypticase soy agar to be more reliable than either brain heart infusion agar or Mueller–Hinton agar for testing the X-factor requirement of *Haemophilus* spp. Columbia agar is also generally satisfactory for this purpose. Inzana et al. (1987) devised a broth system for the rapid determination of the X- and V-factor requirements of *Haemophilus* spp., which gives results in 4–5 h. Kilian (1974) suggested that probably no complex medium which otherwise satisfies the growth requirements of *Haemophilus* spp. is completely free of X factor. He found that 18 percent of *H. influenzae* strains were misidentified as *H. parainfluenzae*, and claimed more reliable results by testing the ability of strains of *Haemophilus* spp. to synthesize porphyrins from ALA.

The porphyrin test (Kilian 1974) provides an accurate and reliable method for determining X-factor requirement (Figure 2). A substrate of 2-mm ALA and 0.08 mm $MgSO_4$ in 0.1 M phosphate buffer pH 6.9 is used. A loopful of growth from a 24 h chocolate agar culture is added to 0.5 ml of substrate and the mixture is incubated for 4 h at 37°C. Porphyrin production is demonstrated by the development of a red fluorescence when the mixture is exposed to a Wood's lamp (maximum emission 360 nm). Alternatively, the presence of porphobilinogen can be demonstrated after incubation for 24 h by adding 0.5 ml of Kovac's reagent. A red color in the aqueous phase indicates the presence of porphobilinogen. *H. parainfluenzae* can be used as a positive control. A disk method for the porphyrin test, (Lund and Blazevic 1977) and a porphyrin test agar (Gadberry and Amos 1986) have also been described.

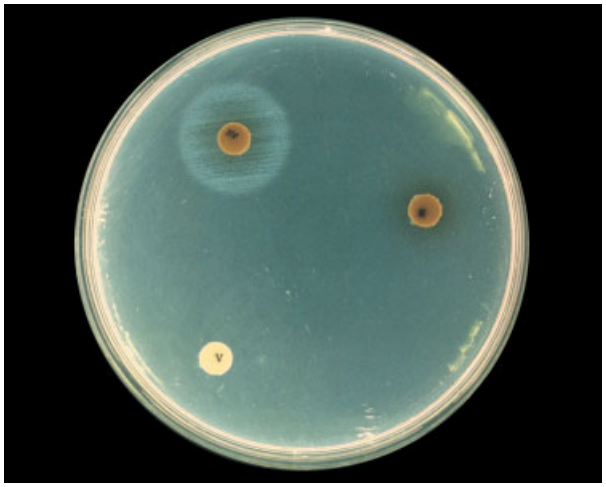

Figure 6 *Growth factor requirement of* H. influenzae. *Putative* Haemophilus *strain sown on Columbia agar plate. Disks containing X factor, V factor, and X + V factors placed on surface of inoculated plate. Colonies of* H. influenzae *grow in vicinity of disk containing X + V factor, but not near the disks containing either factor alone.*

BIOCHEMICAL TESTS

Hemolysis of horse blood agar is of value in differentiating *H. influenzae* from *H. haemolyticus*, and *H. parainfluenzae* from *H. parahaemolyticus*. Carbohydrate utilization can be studied in phenol red broth base supplemented with 1 percent of the carbohydrate and 10 mg/l each of NAD^+ and hemin (Kilian 1976). The results can be read after incubation for 24 h. Some species, notably *H. segnis*, show weak reactions and *H. ducreyi* does not ferment any carbohydrate in conventional tests (see Table 2).

Three biochemical tests – for indole, urease, and ornithine decarboxylase – can be used to biotype *H. influenzae* and *H. parainfluenzae* (Kilian 1976). Biotyping can be conveniently performed in media that do not support growth since the tests detect the presence of preformed enzymes or enzyme systems (Kilian 1976). Several commercial micromethod kits have been used successfully to identify *Haemophilus* spp. and biotype *H. influenzae* and *H. parainfluenzae*. These include API 10E, API 20E, and HNID (Quentin et al. 1992), API NH (Barbé et al. 1994), Rapid NH and RIM-Haemophilus (Murphy et al. 1990). Porphyrin-based testing methods are the most reliable way of identifying *Haemophilus spp* (Munson et al. 2002)

It is impossible to reliably distinguish *H. influenzae*, *H. influenzae* biogroup *aegyptius* and *H. aegyptius* using standard means of identification. A number of tests have been suggested to differentiate *Haemophilus aegyptius* from *H. influenzae*, including lack of xylose fermentation, troleandomycin susceptibility, and the agglutination of human red cells, but none of these tests give an unequivocal identification.

Molecular techniques are now being applied to the detection and identification of *Haemophilus* spp. in clinical specimens and for confirmatory tests on isolates. Species-specific DNA probes and PCR-based techniques can be used to detect *H. influenzae* in clinical material (Daly et al. 1991; Rådström et al. 1994; Ueyama et al. 1995; Corless et al. 2001) and to determine the capsular

genotype of isolates of *H. influenzae* (Falla et al. 1994). A PCR technique has also been employed to identify *H. parainfluenzae* (Hamed et al. 1994).

Partial 16S rRNA sequencing can be used to identify many species of *Haemophilus*, although it will not differentiate *H. influenzae* from *H. aegyptius* or *H. aphrophilus* from *H. paraphrophilus* (Dewhirst et al. 1992). The sequences obtained by this technique can be compared with those in the BLAST database (www.ncbi.nlm.nih.gov/BLAST/). Quentin et al. (1996) described a PCR-based method, using 16S rRNA sequences, to identify strains of the genital *Haemophius* genospecies, which can differentiate such strains from strains of *H. influenzae* biotype IV sensu stricto. The commercial MicroSeq 500 16S rDNA identification system has been shown to be of use in identifying bacterial isolates that have ambiguous biochemical profiles (Woo et al. 2003).

PRESERVATION AND STORAGE

Cultures of *Haemophilus* spp. can be stored for a short time on chocolate agar slopes in screw-capped bottles or tubes. The slopes should be heavily inoculated with *H. influenzae*, incubated overnight at 37°C, and subsequently stored at room temperature. Provided the cap is air-tight and is not unscrewed, the culture will remain viable for up to 4–6 weeks. For long-term storage, cultures can be maintained at –70°C in tryptic soy broth supplemented with 15–20 percent glycerol. Aulet de Saab et al. (2001) found that good results were obtained if a heavy inoculum was suspended in one of the following; skim milk supplemented with 1 percent glucose, 0.5 percent yeast extract, and 10 percent glycerol, trypticase soy broth plus 25 percent glycerol or brain heart infusion broth plus 25 percent glycerol. 100 percent of the stored strains were recovered from any of these cryopreservative broths after 2 months' storage at −20°C. Votava and Střitecká (2001) concluded that trypticase soy broth with 10 percent glycerol and 40 percent horse serum was a suitable medium for storing strains of *Haemophilus* spp. at –70°C. Lyophilized cultures can be stored for many years.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

H. influenzae is commonly susceptible in vitro to therapeutically achievable concentrations of ampicillin, cephalosporins, chloramphenicol, tetracycline, aminoglycosides, rifampicin, meropenem, ciprofloxacin, and oral sulphonamides. Representative minimum inhibitory concentrations (MIC) of a range of antimicrobial agents for *H. influenzae* are shown in Table 3.

For many years, ampicillin was the treatment of choice for *H. influenzae* infections, but the increasing prevalence of resistance to ampicillin and several other antibiotics has prompted changes in the way these infections are treated.

Table 3 *Susceptibility of* Haemophilus influenzae *to antimicrobial agents*

Agent	MIC[a] (mg/l)
Ampicillin/amoxycillin[b]	0.5
Amoxycillin: clavulanic acid (2:1)	0.5/0.25
Cefuroxime	1.0
Cefotaxime	0.06
Ceftriaxone	0.03
Cefaclor	4
Imipenem	2–4
Meropenem	0.03–0.5
Aztreonam	0.12
Chloramphenicol	0.5
Tetracycline	0.5
Rifampicin	1
Erythromycin	2–32
Clarithromycin	8
Azithromycin	0.25–2
Ciprofloxacin	0.06
Levofloxacin	0.03
Trimethoprim	0.5
Co-trimoxazole	2–4
Gentamicin	0.5

MIC determined by an agar-dilution technique; inoculum c. 10^4 colony forming units (cfu) Data compiled from Slack (unpublished).
a) 90 percent of strains inhibited by concentration less than value stated.
b) β-lactamase negative strains

The first reports of clinical resistance to ampicillin in cases of *H. influenzae* meningitis appeared in 1974 (Gunn et al. 1974). The ampicillin resistance in these strains was mediated by a TEM-1 β-lactamase (Williams et al. 1974). Ampicillin-resistant strains of *H. influenzae* are now distributed throughout the world. Markedly differing rates of resistance to ampicillin have been reported in various parts of the world (Table 4). Jones et al. (2002) noted that, in nationwide surveys in the United States, the rate of β-lactamase production in *H. influenzae* remained virtually unchanged from 1997 to 1999. However, in the same studies, the rate of resistance to trimethoprim-sulfamethoxazole increased from 2 percent in 1997–1998, to 15.5 percent in 1999. A second β-lactamase, designated ROB-1, has been described in *H. influenzae* (Rubin et al. 1981) and has been associated with failure of ampicillin therapy. A third, designated VAT-1, has been described in *H. influenzae* (Vali et al. 1994; Shanahan et al. 1996). Production of a β-lactamase is the most common mechanism of ampicillin resistance in *H. influenzae*, but a small number of strains exhibit reduced susceptibility to a range of β-lactam agents without β-lactamase activity being detectable (Bell and Plowman 1980; Markowitz 1980). The mechanism of ampicillin resistance in these β-lactamase-negative, ampicillin resistant (BLNAR) strains is thought to be decreased binding to penicillin binding proteins (PBP)

Table 4 *Prevalence of antimicrobial resistance among clinical isolates of* Haemophilus influenzae *from different parts of the world*

	Percentage (%) of strains resistant in							
Agent	**Australia**	**Brazil**	**Canada**	**China**	**Pakistan**	**Spain**	**UK**	**USA**
Ampicillin	23.4	11	24	5.8	16.6	32	17.3	33
Co-amoxiclav	<1	<1	<1	<1	<1	0.5	<1	0.3
Cefuroxime	1.5	0	1.7	<1	4	<1	<1	0.6
Azithromycin	0	0	2.7	0	0	0.3	0	0.5
Clarithromycin	4.0	8	1.7	2.6	0	8.7	4.6	2.1
Ciprofloxacin	0	<1	<1	<1	0			<1
Co-trimoxazole	17.2	53	13.7	51.3	53	52.1	20.9	14.6

Data compiled from Critchley et al. (2000), Hoban et al. (2001), Sahm et al. (2000), Thornsberry et al. (1999), Turnak et al. (2001), and Zhanel et al. (2000).

3A and 3B (Mendelman et al. 1984, 1990; Clairoux et al. 1992). Garcia-de-Lomas et al. (2002) found 9 percent of *H. influenzae* isolated from cases of community-acquired respiratory tract infection in Spain were BLNAR. It has been suggested that strains with altered PBPs may be less virulent (Clairoux et al. 1992). Tolerance to β-lactam antibiotics (MBC/MIC ratio >32) has also been described in *H. influenzae* (Bergeron and Lavoie 1985), but its clinical significance is unclear.

Chloramphenicol resistance in *H. influenzae* was first noted in 1976 (Manten et al. 1976). The resistance is usually of a high level and is almost always due to the production of a plasmid-mediated chloramphenicol acetyl transferase (CAT) type II (Roberts et al. 1980). Non-CAT-mediated resistance has been observed. This form of resistance is chromosomally encoded, and is due to a permeability barrier to the uptake of chloramphenicol by the cell, associated with the loss of an outer-membrane protein (Burns et al. 1985). Almost all CAT-mediated chloramphenicol-resistant strains are also resistant to tetracycline (Roberts et al. 1980). Resistance to both ampicillin and chloramphenicol in *H. influenzae* type b was first reported in 1980 from Bangkok (Simasthien et al. 1980) and has since been recognized in many parts of the world, including the USA and the UK. Strains resistant to chloramphenicol or chloramphenicol and ampicillin are still relatively uncommon, accounting for 1–2 percent of all isolates in most parts of the world.

Resistance to co-trimoxazole varies from region to region, but in some countries now exceeds 50 percent (Table 4). Resistance to β-lactams, macrolides, and co-trimoxazole is more frequently observed in respiratory isolates than in blood culture isolates. Resistance rates also tend to be higher in NCHI than in *H. influenzae* type b. The emergence of rifampicin-resistant *H. influenzae* following chemoprophylaxis with oral rifampicin has been reported (Murphy et al. 1981; Nicolle et al. 1982). The combination of rifampicin and trimethoprim (1:1) is synergic and bactericidal and may prevent the selection of rifampicin-resistant mutants (Yogev et al. 1982) (see Jones and Pfaller (1998), Hoban et al. (2001), Turnak et al. (2001) for reviews of antimicrobial resistance in *H. influenzae*).

Antimicrobial resistance has been reported in a number of other *Haemophilus* spp. β-lactamase-mediated ampicillin resistance has been found more frequently in *H. parainfluenzae* than in *H. influenzae* (Green et al. 1979; Scheifele and Fussell 1981). β-Lactamase activity has also been seen in *H. parahaemolyticus*, *H. paraphrophilus* (Green et al. 1979; Jones et al. 1976), and *H. ducreyi* (Brunton et al. 1979). Non-β-lactamase-mediated ampicillin resistance has been noted in *H. parainfluenzae* (Walker and Smith 1980). Plasmid-mediated chloramphenicol resistance (Cavanagh et al. 1975) and aminoglycoside resistance (Le Goffic et al. 1977) have also been described in this species.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *H. influenzae* by disk-diffusion procedures is significantly influenced by technical variations in inoculum size, culture medium, and antimicrobial content of the disks. Jorgensen et al. (1987) have described a simple transparent susceptibility testing medium for *H. influenzae*: Mueller–Hinton medium supplemented with hematin 15 mg/l, NAD 15 mg, and yeast extract 5 g, known as haemophilus test medium (HTM). The National Committee for Clinical Laboratory Standards (NCCLS) specifies the use of HTM for susceptibility testing of *H. influenzae*. The British Society for Antimicrobial Chemotherapy (BSAC) working party on susceptibility testing stipulates the use of Iso-Sensitest agar supplemented with 5 percent defibrinated horse blood and 20 mg/l NAD (Andrews 2001; King 2001). β-Lactamase-producing strains of *Haemophilus* spp. may appear susceptible to ampicillin in conventional disk susceptibility tests. Disk tests can also fail to detect non-β-lactamase-mediated ampicillin resistance if the content of the ampicillin disk is too high (Philpott-Howard et al. 1983; Mendelman et al. 1986). More accurate results can be obtained if a low content (2 μg) ampicillin disk is used and a test for β-lactamase is employed routinely, e.g. chromogenic cephalosporin test (O'Callaghan et al. 1972), or acidimetric paper strip test (Slack et al. 1977a). Tests for β-lactamase are especially important since there have been cases of invasive *H. influenzae* type-b infection from

which mixed populations of β-lactamase-positive and β-lactamase-negative strains have been isolated (Jubelrier and Yeager 1979; Stewardson-Krieger and Naidu 1981). The chromogenic cephalosporin, Nitrocefin, is only weakly labile to the ROB-1 enzyme (Rubin et al. 1981) which therefore may not be detected by this method. The presence of ROB-1 β-lactamase activity in isolates of *H. influenzae* can be inferred from resistance to cefaclor (Karlowsky et al. 2000).

For chloramphenicol susceptibility tests a low content disk (2.5, 5, or 10 μg) should be used. A test for chloramphenicol inactivation (e.g. Slack et al. 1977b; Azemun et al. 1981; Satish et al. 2001) can also be applied. Doern et al. (1987) found that a 1-h tube assay for CAT activity (Azemun et al. 1981) was significantly more accurate than a 30 min commercially available impregnated disc test. Tests for trimethoprim and erythromycin susceptibility are prone to considerable laboratory errors and the results should be interpreted with caution. The E-test is an antimicrobial agent gradient-coated plastic test strip which allows MIC determinations on solid media, which can be conveniently used for antimicrobial susceptibility tests of *Haemophilus* spp. (Jorgensen et al. 1991) (see review on in vitro susceptibility testing of *H. influenzae* by Doern (1992)).

The detection of antimicrobial resistance genes by the use of DNA probes or PCR is now possible for several antimicrobial agents (see reviews by Courvalin (1991), Tenover et al. (1993)).

TYPING METHODS

The limited diversity of Hib from disease has been demonstrated by many techniques, including OMP subtyping, ribotyping, pulsed field gel electrophoresis (PFGE), MLEE (Musser et al. 1990), and MLST (Meats et al. 2003), and has been explained by clonal theory (Pennington 1993). Subtyping techniques therefore only prove useful in epidemiological studies if a strain with an infrequent subtype is involved. Defined subtyping schemes enable global comparisons. In contrast, NCHI exhibit a much wider variety of phenotypes and genotypes and can be readily characterized in local epidemiological studies by many techniques, but the consequential difficulty in defining subtypes makes interlaboratory comparisons of large numbers of strains impossible. For phylogenetic studies, MLST is now the favored technique since the results are more precise and less ambiguous than MLEE. The *H. influenzae* MLST scheme is available at www.mlst.net.

Capsule typing

H. influenzae strains are differentiated on the basis of capsule expression into capsulate and noncapsulate strains. Six antigenically distinct types of capsule were detected by agglutination (Pittman 1931) and designated a–f. Type e strains have been shown to possess two

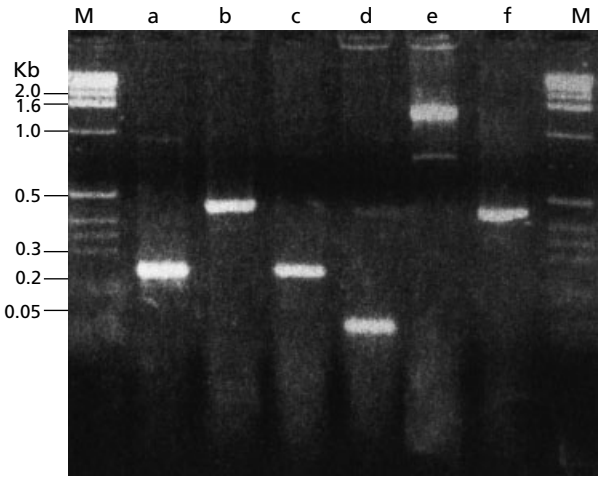

Figure 7 *PCR amplification products from* H. influenzae *capsule types a–f. M is a size marker*

capsular antigenic components, e1 and e2; a minority have only the e2 component (Williamson and Zinneman 1951). Capsule type has long been determined by agglutination-based methods (serotyping), but autoagglutination by some strains and cross-reactions between antisera have been noted (Falla et al. 1993). DNA-based methods of determining capsular type are now available. DNA hybridization patterns obtained with a probe (pUO38) containing *cap* genes have been shown to be capsule-type specific and give a characteristic pattern for capsule-deficient mutants of type b (Hib$^-$) strains (Kroll et al. 1991a). Fourteen capsular genotypes were detected among 222 isolates of capsulate *H. influenzae* from all over the world (Musser et al. 1990) with four types (b [S], b[G], b[V], and b[O]) detected among 85 type b strains. A study of 425 Hib from England and Wales identified three rare variants (Leaves et al. 1995). Fragments of capsule-associated DNA, specifically IS*1016* sequences, have been detected in noncapsulate strains (St. Geme et al. 1994). Primers designed to amplify products of different sizes from each of the six capsular types in PCR enable the unequivocal determination of capsular genotype (Falla et al. 1994) (Figure 7). The detection of Hib$^-$ and other unusual genetic arrangements of capsule-associated genes is also possible with PCR (Leaves et al. 1995).

Biotyping

H. influenzae and *H. parainfluenzae* can each be subtyped on the basis of three biochemical tests (for indole, urease, and ornithine decarboxylase) into eight biotypes (Table 5) (Kilian 1976; Oberhofer and Back 1979; Gratten 1983; Sottnek and Albritton 1984).

Most clinical isolates of *H. influenzae* are distributed between four biotypes (I, II, III, and IV); Hib isolates from invasive disease are mainly biotype I, and the majority of NCHI are biotype II or III. *H. influenzae* biogroup *aegyptius* is contained within biotype III and a

subset of NCHI biotype IV strains have been implicated as an obstetric pathogen, causing maternal, neonatal, and urogenital infections (reviewed by van Alphen 1993). These strains are now regarded as a cryptic genospecies of *Haemophilus*.

OMP subtyping

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs has formed the basis for subtyping Hib. Two OMP subtyping schemes, with different nomenclatures, have been advocated. With a normal SDS–PAGE system, nine OMP subtypes have been defined in Hib (van Alphen 1994). Gradient SDS–PAGE gels classified 587 Hib isolates from all over the world into 33 subtypes (Musser et al. 1990). Five of the OMP subtypes are common to both schemes (van Alphen 1994). A few subtypes account for the majority of infections worldwide but the predominant subtypes vary geographically, in different host populations and at different times. Three OMP subtypes accounted for c. 75 percent of Hib in the USA (Granoff et al. 1982) whereas OMP subtype 1 (3L in the scheme of Granoff et al. 1982) accounted for 93 percent of Hib in the Netherlands and western Europe (van Alphen et al. 1987). OMP-enriched preparations have been used to distinguish between strains of NCHI. The enormous heterogeneity of NCHI precludes the definition of useful subtypes but OMP profiles have been used successfully to characterize strains in local outbreaks (Jordens et al. 1993). OMP subtyping has been shown to distinguish *H. influenzae* biogroup *aegyptius* from other biotype III *H. influenzae* (Carlone et al. 1985). Traditional methods for subtyping *H. influenzae* have been reviewed by van Alphen (1993). OMP electrophoretic patterns of *H. parainfluenzae* have been shown to be extremely heterogeneous (Quentin et al. 1989).

DNA-based typing and characterization

DNA-based methods for subtyping or characterizing strains of *H. influenzae* include restriction endonuclease analysis (REA) of chromosomal DNA, hybridization with probes for ribosomal RNA encoding genes (rDNA) and PCR-based methods. A subtyping scheme for Hib based on the pattern of *Eco*RI fragments of chromosomal DNA hybridizing with a probe for 16S and 23S rDNA (ribotyping) defined 22 ribotypes (Leaves and Jordens 1994). REA with *Bam*HI and ribotyping have been shown to concur with OMP analysis for characterizing NCHI (Bruce and Jordens 1991). Ribotyping has been used to discriminate NCHI strains (Stull et al. 1988; Bruce and Jordens 1991; Jordens et al. 1993; Smith-Vaughan et al. 1995) and *H. influenzae* biogroup *aegyptius* strains (Irino et al. 1988). REA with *Bam*HI has been used to characterize *H. parainfluenzae* (Kerr et al. 1993) and ribotyping to characterize *H. aphrophilus* and *H. paraphrophilus* (Sedlácek et al. 1993).

PCR-based amplification of randomly amplified polymorphic DNA (RAPD) (Figure 8), inter-

Table 5 *Biotypes of* H. influenzae *and* H. parainfluenzae

Species	Biotype	Production of		
		Indole	Urease	Ornithine decarboxylase
H. influenzae	I	+	+	+
	II	+	+	–
	III[a]	–	+	–
	IV	–	+	+
	V	+	–	+
	VI	–	–	+
	VII	+	–	–
	VIII	–	–	–
H. parainfluenzae	I	–	–	+
	II	–	+	+
	III	–	+	–
	IV	+	+	+
	V[b]	–	–	–
	VI	+	–	+
	VII	+	+	–
	VIII	+	–	–

a) *H. influenzae* biotype III and *H. influenzae* biogroup *aegyptius* are indistinguishable biochemically but may be differentiated on the basis of OMP profiles.

b) V-factor dependent strains negative in all three tests have been designated '*H. parainfluenzae* biotype V'. Similar negative reactions are given by *H. paraphrophilus* (but this is lactose positive) and *H. segnis* (poor growth and ferments slowly). (Kilian, personal communication)

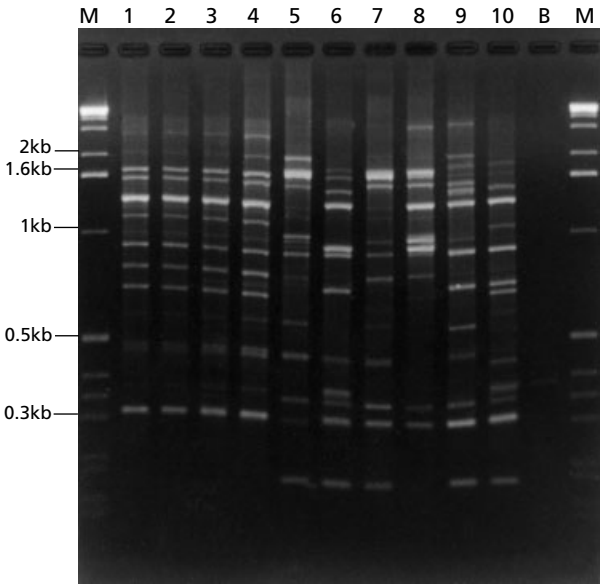

Figure 8 *Randomly amplified polymorphic DNA (RAPD) generated fingerprints of NCHI. B, reagent blank; M, size marker. Note: epidemiologically unrelated strains are distinct whereas isolates 1–4 are indistinguishable and probably represent spread of a single strain.*

enterobacterial repetitive intergenic consensus (ERIC) sequences, *omp*P2 and rDNA have been used to characterize NCHI. In outbreak investigations, the results from RAPD, inter-ERIC amplification and OMP profiling were shown to correlate well (Jordens et al. 1993; van Belkum et al. 1994). Restriction analysis of the *omp*P2 amplification product was used to monitor the spread of *H. influenzae* (probably NCHI) between individuals in a closed community in Antarctica (Hobson et al. 1995). Amplification of c. 6 kb of rDNA, followed by restriction analysis with *Hae*III, was shown to correlate well with probe-based ribotyping (of *Xba*I digests) for NCHI from Aboriginal children in Australia (Smith-Vaughan et al. 1995).

Typing methods for animal strains

A serotyping scheme (not based on capsules) has been reported for *H. paragallinarum* and a biotyping scheme based on the combination of serotyping, carbohydrate fermentation patterns, and antimicrobial drug resistance patterns, together with REA, has been applied successfully to epidemiological studies of this species (Blackall et al. 1990). REA and ribotyping have shown that NAD-independent *H. paragallinarum* from chickens suffering from infectious coryza are indistinguishable and hence clonal (Miflin et al. 1995). These strains are now widespread in South Africa (Horner et al. 1995). HPG-2 PCR, based on a randomly cloned fragment of *H. paragallinarum* (Chen et al. 1996) can be used to identify cultures of both NAD-dependent and NAD-independent *H. paragallinarum* directly from the colonies and to distinguish these organisms from other avian respiratory tract pathogens, such as *Pasteurella* spp. and *Ornithobacterium rhinotracheale* (Miflin et al. 1999). A serotyping system has been described for *H. parasuis* (Kielstein et al. 1991).

BACTERIOCINS

Most *H. influenzae* type b produce a bacteriocin 'hemocin' (LiPuma et al. 1992), which is active against capsulate *H. influenzae* strains of other types, NCHI, other *Haemophilus* spp. and certain enterobacteria, but not against gram-positive bacteria. *Haemophilus* spp. vary in their susceptibility; nontypable *H. influenzae*, *H. haemolyticus*, and *H. parainfluenzae* have been shown to be the most susceptible and *H. parahaemolyticus* the least susceptible (Venezia et al. 1977). A large proportion of strains of *E. coli* and related genera were susceptible to hemocin. Less closely related genera, including *Klebsiella*, *Proteus*, and *Enterobacter* were uniformly resistant. This bacteriocin appears to act by inhibiting DNA synthesis (Streker et al. 1978).

ROLE IN NORMAL HUMAN FLORA

Humans are the hosts of *H. influenzae*, *H. haemolyticus*, *H. aphrophilus*, *H. parainfluenzae*, *H. parahaemolyticus*, and *H. segnis*. Hemophili can be found almost universally in the human pharynx by use of selective culture media. In the mouth, hemophili are found on the tongue, palate, cheeks, and teeth, in dental plaque and in saliva (Sims 1970). *H. influenzae* constitutes only about 10 percent of the hemophili found in the pharynx and is virtually absent from the extensive hemophilus flora of the oral cavity (Kilian and Frederiksen 1981).

The upper respiratory tract of the neonate is sterile, but rapidly becomes colonized by a wide variety of organisms, including NCHI. Faden et al. (1995) demonstrated that the rate of nasopharyngeal colonization increases during infancy, reaching 34 percent at 1 year and 44 percent at 2 years. By the age of 5–6 years, over 50 percent of children are colonized, often with multiple strains. Carriage rates remain high thereafter, but adults usually only carry a single strain (Kuklinska and Kilian 1984). The age of primary acquisition varies between different populations. For example, almost all Australian Aboriginal children are colonized with *H. influenzae* by 1 month of age in contrast to 1.5 percent of non-Aboriginal children (Leach et al. 1994) The carriage of *H. influenzae* including Hib is a dynamic process with intermittent colonization with one or more strains (Trottier et al. 1989) (see Garcia-Rodriguez and Martinez (2002) for a review of the dynamics of nasopharyngeal colonization). Several studies have measured the nasopharyngeal and oropharyngeal carriage rates of Hib prior to the introduction of routine

Hib immunization of infants. Oropharyngeal carriage of Hib is uncommon during the first 6 months of life, rises to an average 3–5 percent among children aged 3–5 years, and then gradually declines with increasing age. Many factors influence reported colonization rates, including age (Sell et al. 1973), ethnic origin (Turk 1963), day-care attendance (Ginsburg et al. 1977), close family contact with a case of invasive Hib disease (Turk 1975; Michaels and Norden 1977), season of the year (Henderson et al. 1982), recent antibiotic therapy (Howard et al. 1988), sampling (Michaels et al. 1976), and cultural techniques (Chapin and Doern 1983). The use of standardized swabbing techniques and Hib antiserum agar (Barbour et al. 1993a, b) is recommended for these studies. *H. influenzae* types e and f are also carried in the pharynx at similar rates to Hib (Barbour et al. 1995) (see review of Hib carriage by Moxon (1986)). Day care attendance is one of the major risk factors for increased carriage of *H. influenzae* and other respiratory pathogens (Peerbooms et al. 2002). Hib immunization of infants results in reduced pharyngeal carriage of Hib (Takala et al. 1991; Takala et al. 1993; Mohle-Boetani et al. 1993). This appears to be the result of reduced or delayed acquisition of Hib in the first year of life (Barbour et al. 1995). Giving Hib vaccine to a child does not rapidly terminate carriage (Barbour et al. 1995). To date, carriage rates of *H. influenzae* type e and f appear to be unaffected by Hib immunization.

The hemolytic species *H. parahaemolyticus* and *H. paraphrohaemolyticus* have been isolated from the pharynx (Kilian et al. 1972; Kawakami et al. 1982) and oral cavity (Sims 1970). *H. haemolyticus* is found in the nasopharyngeal flora of c. 1 percent of the population (Kilian et al. 1972). *H. parainfluenzae* is a ubiquitous member of the flora of the nasopharynx and mouth, accounting for c. 75 percent of the hemophili isolated from these sites (Kilian and Schiøtt 1975). It may also be found in the normal vaginal flora (Kilian 1976) and has been reported in 26 percent of fecal specimens (Palmer 1981). *H. segnis* forms a significant proportion of the flora of dental plaque (Liljemark et al. 1984) and is also found to a lesser extent in the oropharynx. *H. paraphrophilus* and *H. aphrophilus* show a predilection for teeth (Kilian and Schiøtt 1975; Kraut et al. 1972). *H. aphrophilus* has been found in dental plaque in about one quarter of the adult population (Kraut et al. 1972).

PATHOGENICITY AND VIRULENCE FACTORS

Pathogenicity

CAPSULATE *H. INFLUENZAE*

Within the genus *Haemophilus*, *H. influenzae* is the most important species causing human disease. *H. influenzae* is strictly a parasite of humans and is associated with the normal commensal flora of the respiratory tract. Infections caused by *H. influenzae* generally result from invasion of the bloodstream or contiguous spread from the respiratory tract. Hib causes serious invasive disease in children less than 5 years of age. This is predominantly meningitis, but also includes epiglottitis, pneumonia, cellulitis, osteomyelitis, and septic arthritis (reviewed by Jordens and Slack 1995). Up to 20 percent of survivors of Hib meningitis have long-term neurological sequelae. Risk factors for Hib disease are mainly socioeconomic and include overcrowding, attendance at day-care centers, chronic illness, and lack of access to good health care facilities. Other capsular types may also cause invasive disease, notably type a, especially in nonindustrialized countries. The introduction of vaccines effective against Hib in infants has dramatically reduced the incidence of Hib disease in children (Morbid Mortal Weekly Rep 1995) and consequently the epidemiology of Hib disease is changing (Heath and McVernon 2002). An eight-fold increase in *H. influenzae* type a meningitis has been reported in Brazil, following the introduction of routine infant immunization with conjugate Hib vaccine (Ribeiro et al. 2003). Adderson et al. (2001) described a cluster of invasive infections due to *H. influenzae* type a in previously healthy children and Perdue et al. (2000) reported the incidence of non-type-b capsulate *H. influenzae* infections in Alaskan residents increased by 120 percent between 1980 and 1990 and between 1991 and 1996. Restriction digest pattern analysis of 48 recent invasive isolates of type a, e, and f *H. influenzae* showed them to be of very limited genetic diversity, possibly reflecting the emergence of hypervirulent clones of these serotypes (Omikunle et al. 2002).

NCHI

NCHI are often associated with infection of previously damaged tissues, causing respiratory infections in patients with chronic bronchitis, bronchiectasis or cystic fibrosis. They account for approximately one-third of cases of acute otitis media and acute and chronic sinusitis (Foxwell et al. 1998). They can also cause community-acquired pneumonia and conjunctivitis. Recent reports show that NCHI are also an important cause of invasive disease in both adults and children (reviewed by Jordens and Slack 1995). In most cases of NCHI meningitis, there are predisposing factors such as head injury, with or without a cerebrospinal fluid leak, otitis media or sinusitis, and the infection is presumed to result from direct extension (Bol et al. 1987). In some cases of NCHI bacteremia, the patient has an underlying humoral immune deficit (Farley et al. 1992). Several candidate NCHI vaccines, based on major (P1, P2, P4, P5) and minor (P6, D15, TbA/B) OMPs, LOS, and adhesins (HMW, Hia, pili, P5) are currently under investigation (Poolman et al. 2001). Strains of NCHI biotype

IV are a significant cause of urogenital and neonatal infections (Wallace et al. 1983). A subset of these strains is genetically distinct and has been described as a cryptic genospecies related to *H. influenzae* and *H. haemolyticus* (Quentin et al. 1993). *H. influenzae* biogroup *aegyptius* is associated with an acute form of seasonal conjunctivitis seen mainly in hot climates and in children. It has been implicated as the cause of BPF (Morbid Mortal Weekly Rep 1986).

OTHER *HAEMOPHILUS* SPECIES

H. parainfluenzae, *H. parahaemolyticus*, *H. paraphrohaemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* are of relatively low pathogenicity but occasionally cause disease including endocarditis, meningitis, epiglottitis, septic arthritis, osteomyelitis, brain abscess, acute pharyngitis, acute cholecystitis, empyema of the gallbladder, jaw infections, appendicitis, and pancreatic abscess (see Albritton (1982) for a review of infections caused in humans by *Haemophilus* spp. other than *H. influenzae*). Fastidious gram-negative bacilli causing endocarditis are collectively known as the HACEK group, which includes *H. influenzae*, *H. parainfluenzae*, *H. aphrophilus*, *H. paraphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp. (see Brouqui and Raoult (2001) for a review of HACEK endocarditis). *H. haemolyticus* is not thought to be pathogenic. Riggio and Lennon (1997) described the use of restriction enzyme analysis of PCR-amplified 16S rRNA genes to distinguish *A. actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus*. Doğan et al. (1999) used an arbitrarily primed PCR method to differentiate these three species.

H. parasuis is an important opportunist pathogen of swine, particularly animals under stress, causing respiratory-tract infections and Glasser's syndrome (polyserositis) (see Nicolet (1992) for description). *H. haemoglobinophilus* has occasionally been implicated in urogenital inflammation in dogs (Osbaldiston 1971) but is probably of low pathogenicity. *H. paragallinarum* causes infectious coryza in chickens (Hinz and Kunjara 1977), and *H. paracuniculus* is associated with mucoid enteritis in rabbits (Targowski and Targowski 1979). For pathogenicity of *H. ducreyi*, see below.

Virulence factors

Primates are the only species that naturally suffer from invasive Hib disease and infant primates provide a good model (Tunkel and Scheld 1993). Most animals do not develop meningitis after bacteremia unless the permeability of the blood–brain barrier is increased. However, infant rats develop bacteremia and meningitis after intranasal challenge with Hib (Moxon et al. 1974). This has been a valuable model for studying the early pathogenic events of meningitis. Tunkel and Scheld (1993) reviewed the pathogenesis and pathophysiology of bacterial meningitis. NCHI have been studied in a chinchilla model of otitis media (Leake et al. 1994).

CAPSULATE *H. INFLUENZAE*

The possession of the type b capsule has been shown to be a major virulence factor. Animal studies of isogenic strains transformed with capsule-associated DNA confirmed earlier observations that type b strains were more virulent than type a strains, which were more virulent than the other capsular types (Zwahlen et al. 1989). The bacterial capsule aids colonization and is probably the most important virulence factor for invasive disease as it protects against phagocytosis and complement-mediated lysis (Moxon and Kroll 1990; Tunkel and Scheld 1993). Other virulence factors which may be involved in adherence and colonization of Hib include lipopolysaccharide (Moxon and Maskell 1992), which is expressed differently in the nasopharynx and the bloodstream, and fimbriae, which have been shown to be important for nasopharyngeal colonization but not for invasion. The role of pili and other structures in adherence and invasion have been reviewed by van Alphen and van Ham (1994). OMP P2 has been shown to affect the virulence of Hib (Sanders et al. 1993) and purified peptidoglycan was shown to cause brain edema and inflammation in rabbit and rat models (Burroughs et al. 1993; Roord et al. 1994). Hemocin, a bacteriocin, is strongly associated with type b strains (LiPuma et al. 1992). IgA proteases are produced by all Hib and have been shown to be inhibited by human milk (Plaut et al. 1992). *H. influenzae* requires iron for growth in vivo and can acquire this from host iron-binding proteins, such as transferrin, by a siderophore-independent mechanism involving two transferrin-binding proteins, Tbp1 and Tbp2 (Williams and Griffiths 1992; Gray-Owen et al. 1995). Transferrin-binding ability is strongly associated with Hib from invasive infection (Hardie et al. 1993) and may be important for pathogenicity. The iron-binding protein Tbp1 is a member of the TonB family and has been shown to be essential for invasion of Hib in an animal model (Jarosik et al. 1994).

NCHI

Studies suggest that pili (Sirakova et al. 1994) and OMP P2 (Sanders et al. 1993) may be important virulent factors for NCHI. The secretory products IgA1 protease and ciliotoxin may contribute to the pathogenesis of pneumonia. Peptidoglycan from NCHI caused inflammation of the middle ear in the chinchilla otitis media model (Leake et al. 1994). The lipooligosaccharide biosynthesis gene *lic2B* has been identified as a putative virulence factor in strains of NCHI causing otitis media (Pettigrew et al. 2002) (see St Geme (2002) for a review of NCHI pathogenesis). Isolates of the cryptic genospecies of *Haemophilus*, associated with urogenital,

maternal, and neonatal infections, contain the enzyme, copper- and zinc-superoxide dismutase, which is not found in *H. influenzae* sensu stricto (Langford et al. 2002). This periplasmic enzyme may contribute to the virulence of these strains by protecting the organisms from phagocytic superoxide (Wilks et al. 1998). Invasive isolates of *H. influenzae* biogroup *aegyptius* recovered from cases of BPF harbor a 24 Mda cryptic plasmid and a chromosomally integrated bacteriophage HP1 not found in non-BPF strains (Li et al. 2003). The plasmid does not encode any recognizable virulence factors (Kroll et al. 2002). In addition at least 16 unique chromosomal loci have been identified in BPF-causing strains (Li et al. 2003). It has been suggested that at least some of these may have been acquired by horizontal gene transfer from unrelated microbial sources (Smoot et al. 2002). The gene *Bpf001* encodes a protein homologous to the *Legionella pneumophila* epithelial cell entry-enhancing protein EnhC and to the product of the meningococcal gene *NMB0419*, which have been implicated in invasion of respiratory epithelial cells (see Marrs et al. (2001), for a review of virulence factors of *H. influenzae*).

HAEMOPHILUS DUCREYI

Introduction and historical perspective

Ducrey (1889) first reported the presence of characteristic bacilli in stained smears prepared from the exudates of soft genital sores (chancroid). Bezançon et al. (1900) succeeded in isolating the organisms on nutrient agar plus 30 percent whole fresh rabbit blood. Tomasczewski (1903) successfully reproduced soft chancre in humans with pure cultures of *H. ducreyi* (see Albritton (1989) for historical review). *H. ducreyi* differs in many respects from other members of the genus and there has been doubt over its taxonomic position for many years. *H. ducreyi* was originally assigned to the genus *Haemophilus* because of its requirement for X factor.

The techniques of genetic transformation (Albritton et al. 1986), DNA hybridization, and SI nuclease treatment (Casin et al. 1985), DNA–RNA hybridization (De Ley et al. 1990) and 16S RNA sequencing (Rousseau et al. 1991; Dewhirst et al. 1992) have all been used to clarify the taxonomic position of *H. ducreyi*. The results suggest that *H. ducreyi* is a member of the Pasteurellaceae but clusters differently to the true hemophili. However, no formal renaming has been proposed.

Morphology

In the purulent discharge from the ulcerated surface of chancroid lesions, several morphological forms may be seen. The organisms may lie intracellularly or more commonly extracellularly, and appear as pleomorphic coccobacilli or short rods. The characteristic arrangement of organisms as 'shoals of fish' or 'railroad tracks' are rarely evident in clinical material, and the ulcers generally harbor a polymicrobial flora. Sturm et al. (1987) found direct Gram staining of ulcer exudate identified only 10 percent of culture-positive cases. Additionally, they found only 5 percent of patients with a clinical diagnosis of chancroid had a positive Gram-stained smear. These studies underline the difficulties in making the diagnosis of chancroid. Accurate diagnosis of chancroid depends on the ability to identify *H. ducreyi* as the causative agent.

In smears prepared from growth on solid media, the organisms appear as isolated individuals, in groups or as short chains; growth in liquid media is difficult to achieve, but in such cultures long tangled chains may be seen.

Cultural characteristics and growth requirements

H. ducreyi is a fastidious organism which grows only slowly on chocolate agar and culturing *H. ducreyi* is difficult. Isolation of *H. ducreyi* has a sensitivity of about 70 percent compared to PCR in an unselected population of men with genital ulcers (Johnson et al. 1995). Several media have been devised to improve the isolation of *H. ducreyi*. It is generally accepted that the use of two media is optimal for the recovery of *H. ducreyi*, a gonococcal agar-based and a Mueller–Hinton-based medium to each of which is added 1 percent IsoVitalex together with either 5 percent chocolatized horse blood or 1 percent hemoglobin and 5 percent fetal calf serum. Activated charcoal (0.2 percent) can be used instead of fetal calf serum (Lockett et al. 1991), which has the advantage of being a much cheaper medium. To isolate *H. ducreyi* from clinical specimens, media can be made selective by the addition of vancomycin 3 mg/l. However, Jones et al. (1990) described strains of *H. ducreyi* that are inhibited by 3 mg/l vancomycin. Fresh plates with a moist surface should be used and they should be incubated for 3–4 days at 33–36°C with added moisture and carbon dioxide. For many laboratories that isolate *H. ducreyi* infrequently, the availability of fresh selective media will be a problem. Dangor et al. (1993) described a thioglycollate–hemin-based transport medium from which viable *H. ducreyi* can be isolated after 4 days at 4°C.

The colonial appearance of *H. ducreyi* varies with the medium used. Typically, after incubation for 24 h, the colonies are small, yellow-gray, translucent or semi-opaque. Cultures often appear 'mixed' with a variety of colonial sizes and appearance. The colonies are very cohesive and can be pushed intact across the agar surface. It is extremely difficult to emulsify the colonies. *H. ducreyi* does not grow well in liquid media. Strains

may be preserved on moist slopes of a medium such as Sheffield medium for about 4 weeks at 4°C or in liquid nitrogen for up to 2 years (Hafiz et al. 1984) (see Trees and Morse (1995) and Lewis (2000a) for discussion of media and diagnostic techniques).

Identification

H. ducreyi requires X factor but not V factor for growth. Hammond et al. (1978) found the optimal hemin requirement for *H. ducreyi* was 200–500 mg/l.

The biochemical activities of *H. ducreyi* are shown in Table 2. Kilian and Biberstein (1984) considered *H. ducreyi* to be apparently asaccharolytic, but commented that some strains show a late positive reaction with glucose. *H. ducreyi* has a characteristic enzyme profile (Casin et al. 1982) and a number of rapid kit systems have been used to identify it by its enzymic activity. These include API-ZYM (Casin et al. 1982), Minitek System (Oberhofer and Back 1982), and RapDNH (Hannah and Greenwood 1982).

A number of noncultural diagnostic tests have been developed for *H. ducreyi*, including indirect immunoflorescence microscopy of clinical material with polyclonal or monoclonal antisera (Hansen and Loftus 1984; Schalla et al. 1986; Finn et al. 1990), a blot-radio-immunoassay (Hansen and Loftus 1984) and enzyme-linked immunosorbent assay (ELISA)-based antigen detection (Roggen et al. 1993). Hansen and Loftus (1984) have produced two monoclonal antibodies which recognize all strains of *H. ducreyi* and which are capable of detecting *H. ducreyi* in skin lesions in experimental animals. Parsons et al. (1989) described ^{32}P-labeled DNA probes for *H. ducreyi*. The probes will detect 10^4 CFU of *H. ducreyi*. They could be used to confirm the identity of *H. ducreyi* isolates but lack the sensitivity required to detect *H. ducreyi* in clinical material.

PCR methods have been developed for the diagnosis of chancroid (Chui et al. 1993; Johnson et al. 1994; Orle et al. 1994; West et al. 1995). A multiplex PCR assay has been described for the simultaneous amplification of DNA from *H. ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 (Orle et al. 1996) which appears to be more sensitive than culture for the detection of these agents in genital ulcers. Although such techniques are valuable for the confirmation of *H. ducreyi* isolates, their value in the clinical diagnosis of chancroid is not yet established. Johnson et al. (1994) demonstrated that PCR assays performed on genital ulcer specimens are less sensitive than assays based on *H. ducreyi* cultures because of the presence of *Taq* polymerase inhibitors in DNA preparations from ulcer material. Tekle-Michael et al. (2001) described a heminested PCR assay for the detection of an *H. ducreyi*-specific protein *p*27, which appears to be sufficiently sensitive and specific to be applied to clinical specimens.

Susceptibility to antimicrobial agents

The susceptibility of *H. ducreyi* to antimicrobial agents varies between geographical areas (see review by Dangor et al. (1990)). Resistance is more prevalent in areas where chancroid is common. In countries where *H. ducreyi* infection is endemic, most isolates are β-lactamase producers and are also resistant to tetracycline. Resistance to trimethoprim-sulfamethoxazole is being seen increasingly, especially in the Far East. Ison et al. (1998) discussed the epidemiology of global antibiotic resistance in *H. ducreyi*. Most isolates of *H. ducreyi* are susceptible to erythromycin and the newer macrolides clarithromycin, azithromycin, and roxithromycin. Other agents that show excellent activity against *H. ducreyi* in vitro include the quinolones such as ciprofloxacin, perfloxacin, and fleroxacin, rifampicin, spectinomycin, ceftriaxone, and co-amoxiclav. Azithromycin and ceftriaxone offer the advantage of single-dose therapy. Ceftriaxone therapy has been associated with treatment failures in Kenya (Tyndall et al. 1993) (see www.bashh.org/guidelines/ceguidelines.htm, and Schmid (1999) for guidelines on the management of chancroid).

Antimicrobial susceptibility testing of *H. ducreyi* is technically difficult, requiring specially supplemented media. In addition the slow and variable growth of isolates and the difficulties of emulsifying the cohesive colonies mean that it is hard to standardize inocula.

Typing methods

Sturm (1981) differentiated *H. ducreyi* into two types: electron microscopic preparations reveal an outer membrane in type 1, which is lacking in type 2. Both types can produce chancroid-like lesions in monkeys. Odumeru et al. (1983) have identified seven subtypes of *H. ducreyi* by OMP subtyping. Slootmans et al. (1985) described an indirect immunoflorescent technique for typing *H. ducreyi*. The techniques of ribotyping (Sarafian et al. 1991) and plasmid analysis (Sarafian and Knapp 1992) have been applied to *H. ducreyi*. Lectin typing of *H. ducreyi* (Korting et al. 1988) appears to be of dubious value (see review by Trees and Morse (1995) for discussion of typing methods).

Pathogenicity

H. ducreyi causes the sexually transmitted disease chancroid, or soft chancre (soft sore). Asymptomatic carriage of the organism has been reported in men (Kinghorn et al. 1983) and women (Khoo et al. 1977) but it is unclear whether this is persistent or transient carriage or recovery of the organism during the incubation period. *H. ducreyi* has not been isolated from nonhuman sources. Avirulent strains, as defined in an animal model, can be produced by repeated passage in vitro,

but have not been reported on primary isolation. These avirulent strains produce no cutaneous lesion when inoculated intradermally into a rabbit; virulent strains produce necrotic lesions. Several in vitro models have been used to study the pathogenesis of *H. ducreyi*. Animal models have also been developed (reviewed by Trees and Morse (1995)). Spinola et al. (1994) studied experimental infections in humans.

The potential virulence factors of *H. ducreyi* include a novel pilus (Brentjens et al. 1996), LOS (Campagnari et al. 1991), a cytolethal distending toxin (Purven and Lagergård 1992), a hemoglobin-binding OMP (Stevens et al. 1996), a hemolysin (Palmer et al. 1996), a filamentous hemagglutinin-like protein (Ward et al. 1998), and copper-zinc superoxide dismutase (San Mateo et al. 1998) (see Spinola et al. (2002) for a review of *H. ducreyi* pathogenesis). Several candidate vaccines are under investigation, based on an outer membrane protein or a toxoid prepared from *H. ducreyi* hemolysin. Trees and Morse (1995), Lewis (2000b), Al-Tawfiq and Spinola (2002), and Bong et al. (2002) provide excellent comprehensive reviews of *H. ducreyi* and chancroid.

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Brucella

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INTRODUCTION AND HISTORY

A type of fever characterized by fairly regular remissions or intermissions has been recognized along the Mediterranean littoral since the time of Hippocrates. Many names have been applied to it, often relating to localities in which it was particularly prevalent; Malta fever, Mediterranean fever, Gibraltar or Rock fever, and undulant fever are probably the best known. The cause of this disease was obscure until 1887 when Bruce reported numerous small coccal organisms in stained sections of spleen from a fatally infected soldier and isolated an identical organism in culture from spleen tissue of four other soldiers. This bacterium, which he named *Micrococcus melitensis*, produced a remittent fever in inoculated monkeys; one animal died from the infection and the organism was recovered in pure culture from the liver and spleen. Later, Wright and Smith (1897) described an agglutination test for detecting antibodies to *M. melitensis* in the serum of humans and animals. With this test, Zammit (1905) detected infection in Maltese goats and, with others, showed the goat to be the natural host of *M. melitensis*. Humans became infected by the consumption of raw milk or cheese (Report 1905–7; Horrocks and Kennedy 1906).

Alice C. Evans (1918a) drew attention to the similarity between *M. melitensis* and the organism described by Bang (1897) as the cause of contagious abortion of cattle. Her observations were confirmed by others, and Meyer and Shaw (1920) proposed the generic name *Brucella* for the two organisms, in honor of Sir David Bruce. The possible pathogenicity of the second organism, *Brucella abortus*, for humans was suggested by Evans (1918b) and confirmed by others, including Bevan (1921–22).

Sheep and goats are the preferred natural hosts of *B. melitensis*, but other animals may also be infected. As with the other members of the genus, *B. melitensis* tends to localize in the reticuloendothelial system and the genital tract; genital infection of the pregnant female typically results in abortion. Humans are susceptible to infection, often manifest initially as an acute fever. Chronic complications may succeed the acute phase and the term 'brucellosis' is used as a convenient description for all phases of the disease. *B. melitensis* is fairly widely distributed throughout the world but is particularly common in countries around the Mediterranean littoral, the Arabian peninsula, central Asia, and parts of Latin America. It probably accounts for the majority of cases of brucellosis in humans. This species is not found in northern Europe, Australia, or New Zealand.

Cattle are the preferred natural hosts of *B. abortus*, but it can also infect other animals. Although usually less virulent than *B. melitensis*, it can also cause brucellosis in humans. *B. abortus* is even more widespread

than *B. melitensis* and has, at one time or another, been isolated in virtually all cattle-raising countries of the world. Over the past few decades, control campaigns have dramatically reduced the prevalence of *B. abortus* infection in most developed countries and eliminated it altogether from some. Countries which have reported the eradication of bovine brucellosis include Australia, Canada, the Czech Republic, Germany, Holland, Japan, the Scandinavian countries, and New Zealand. It is now uncommon in the USA and most countries of western Europe. There are indications of a resurgence in some parts of eastern Europe which formerly reported a low incidence, including Russia and Ukraine.

The third member, *B. suis*, was first recorded in 1914 by Traum, who reported its isolation from the fetus of a sow. In 1943, Huddleson implicated *B. suis* as an agent of brucellosis (Huddleson 1943). It is a natural parasite of pigs, frequently producing a generalized infection but with a tendency to localize in the genital tract. Infection can also be transmitted to other animals, although the host range tends to be narrower than that of *B. abortus* and *B. melitensis*, possibly for geographical rather than biological reasons. Infection is now rare in the USA, but is prevalent in parts of Latin America, southern China, and South East Asia. In Australia, it occurs in feral swine. In contrast with the *B. suis* strains typified by those described by Traum, other types have been found which show a different host range and pathogenicity. Thus, Thomsen (1934) isolated strains from pigs in Denmark which differed in certain cultural properties from those found in the USA and were less pathogenic for humans. These Danish or European porcine strains have hares and swine as their natural hosts, and have been classified as *B. suis* biogroup ('biovar') 2; the American strains have been assigned to *B. suis* biogroups 1 and 3. Two additional *B. suis* biogroups have also been defined, neither of which causes natural infections in swine. *B. suis* biogroup 4 was originally described as a separate species, *B. rangiferi tarandi* (Davydov 1961), and infects reindeer in Alaska, Canada, and northern Russia, causing reproductive failure. It is transmissible to humans and causes an undulant-fever syndrome. *B. suis* biogroup 5 has only been found in mouse-like rodents in the Caucasus (Vershilova et al. 1983). However, it is known to be pathogenic for humans and causes a disease similar to that produced by the other *B. suis* biogroups. Generally, *B. suis* strains are less widely distributed geographically than *B. abortus* or *B. melitensis*.

The fourth member of the genus, *B. canis*, was reported by Carmichael and Bruner (1968) as the cause of abortion among beagle dogs in the USA. It has since been found in dogs of various breeds in many countries including Argentina, Brazil, China, the Czech Republic, Germany, Japan, Madagascar, Mexico, Papua New Guinea, Peru, and the Philippines. Occasional cases of infection have also been reported in humans, usually presenting as a mild pyrexial illness (Carmichael 1990; Schoenemann et al. 1986; Weber 1982).

The fifth member of the *Brucella* group, *B. ovis*, was first observed at about the same time in Australia and New Zealand (Buddle and Boyes 1953; Simmons and Hall 1953) and identified as the cause of sexually transmitted epididymitis in rams. It has since been found in most other sheep-raising countries including Argentina, Chile, France, Germany, South Africa, USA, Spain, and countries of the former Soviet Union. Although serological evidence suggests that humans can be infected by this organism (Gavrilov et al. 1972), it has not been confirmed to be a cause of overt disease.

Unlike *B. abortus*, *B. melitensis*, and *B. suis*, in which virulence is associated with the smooth colonial form, *B. canis* and *B. ovis* have been found only in the nonsmooth phase. This has implications for the serological diagnosis of infections caused by these organisms because smooth and non-smooth *Brucella* strains are antigenically distinct.

The last member, *B. neotomae*, was isolated by Stoenner and Lackman (1957) from desert wood rats in a remote area of Utah, USA. It has not been associated with disease in humans or other species and further isolates have not been recorded.

Regardless of the infecting species, cases of brucellosis in humans have many features in common, but the severity of disease and the occurrence of complications vary to some extent with the organism responsible. In general, *B. melitensis* produces the most severe and acute human infections. Biogroups 1 and 3 of *B. suis* may produce severe acute infections but have a particular tendency to cause chronic suppurative lesions affecting the skeletal system. *B. abortus* and *B. canis* tend to produce a milder disease, with a higher proportion of subacute and subclinical infections. Indeed, human disease caused by *B. canis* has been diagnosed only infrequently, but serological studies suggest that exposure to infection is common in some populations (Monroe et al. 1975).

DEFINITION

Brucellae are small nonmotile, nonsporing, gram-negative coccobacilli, or short rods. They grow rather slowly on ordinary nutrient media; growth is improved by serum or blood. Metabolism is aerobic; there is no growth under strictly anaerobic conditions. Growth is often improved by carbon dioxide, which is essential for some strains. The organisms have little or no fermentative action on carbohydrates in conventional media. They oxidize certain amino acids, urea cycle intermediates, and carbohydrates; oxidative patterns are useful for identification. They are catalase positive and usually oxidase positive, reduce nitrates, and hydrolyze urea to a variable extent. They are susceptible to genus-specific phages, with the lytic pattern dependent on

species and colonial phase. The smooth phase expresses a distinctive outer-membrane lipopolysaccharide devoid of heptose; the O chain is a homopolymer of 4-formamido-4,6-dideoxymannose. The genus is a member of the family Brucellaceae in the α2 subdivision of the Proteobacteria. The species are intracellular parasites of humans and animals producing characteristic infections, particularly of the reticuloendothelial and reproductive systems. They contain two chromosomes, and the G + C content of the DNA is 58–59 mol%. The type species is *B. melitensis*.

On the basis of DNA–DNA homology, the genus has been defined as monospecific and a system of nomenclature proposed which identifies each type as a biogroup of *B. melitensis* (Verger et al. 1985). Although this is in accordance with taxonomic practice, this nomenclature has not been widely accepted because of the confusion that could be caused to nonspecialists; therefore, the current terminology should be retained for practical purposes. Subdivision into nomen species depends on principal natural host, oxidative metabolic pattern, and phage sensitivity. Restriction fragment polymorphism with selected enzymes may also be useful. The polymerase chain reaction (PCR) with appropriate primers is effective for identification at genus and possibly subgenus level (Fekete et al. 1992). Classification into 'biovars' (biogroups) is based on carbon dioxide requirement, hydrogen sulfide production, dye sensitivity, and agglutination by monospecific sera to A, M, and R antigens. In practice, presumptive identification of isolates is made on the basis of cultural characteristics, cellular morphology, and serological reactivity. Association with particular disease patterns may give an indication of identity (see Robertson et al. 1980; Corbel 1993).

HABITAT

Epidemiology

In the past, *B. abortus* accounted for most cases in northern Europe. However, because of the successful eradication or control of bovine brucellosis, the human cases now seen are increasingly due to *B. melitensis* infections acquired in other countries. Most cases of human brucellosis are caused by *B. melitensis* and their geographical distribution very closely follows the distribution of ovine and caprine brucellosis. In Europe, the highest prevalence of human brucellosis occurs in the countries of the Iberian peninsula and the Mediterranean littoral. France, Greece, Italy, Portugal, Spain, and Turkey account for most cases but the infection is also present in Albania and is occasionally reported from countries of the former Yugoslavia (Report 1986a).

Human brucellosis, mainly caused by *B. melitensis*, is also prevalent on the southern and eastern edges of the Mediterranean basin, particularly in Tunisia, Libya, Egypt, Israel, Lebanon, and Syria, and in the Arabian Peninsula and Iran. In Kuwait and Saudi Arabia, the prevalence in the human population has increased as a result of attempts to develop intensive livestock production units against a background of infection in the indigenous animal population (Report 1986a; Mousa et al. 1988). The introduction of measures for the control of the disease in animals has been followed by a decline in the number of human cases in recent years.

Brucellosis is also common in many parts of North, Central, and South America, particularly in Mexico, Argentina, Brazil, Colombia, Ecuador, and Peru. Most reported cases are caused by *B. melitensis*, but *B. abortus* accounts for some. Infections caused by *B. suis*, mainly of biogroup 1, are common in Argentina, Brazil, and Colombia (Lopez-Merino 1989). Brucellosis is seen infrequently in Canada and the USA, but is common in Mexico in humans, livestock, and other animals.

B. abortus and *B. melitensis* infections in humans are widespread in many African countries, but the true prevalence is unknown. *B. melitensis* infection is common in some areas of the Indian subcontinent and was formerly prevalent in Mongolia and northern China, but has declined as a result of the intensive vaccination of sheep and goats. *B. abortus* and *B. suis* infections are widespread in many Asian countries but the true prevalence of human brucellosis in this region is unknown.

SOURCES AND TRANSMISSION

Brucellosis is a zoonosis and, with few exceptions, infections in humans result from direct or indirect contact with animal sources. Rare instances of person-to-person transmission have been recorded, either in circumstances implicating sexual contact (Goossens et al. 1983; Stantic-Pavlinic et al. 1983; Mantur et al. 1996), or by the transfer of tissue, including blood and bone marrow (Naparstek et al. 1982). Laboratory-acquired brucellosis, a much greater problem, has been caused by *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* as a result of accidental ingestion, inhalation, injection, and mucosal and skin contamination. Circumstantial evidence implicates exposure to infectious aerosols generated during the manipulation of cultures as one of the most common sources of laboratory infection. Several studies have suggested that in the past, brucellosis was acquired in the laboratory more frequently than any other bacterial disease (Pike 1978; Collins 1983). In Britain, the Advisory Group on Dangerous Pathogens has classified *Brucella* as a pathogen requiring containment at the level of category 3 (Report 1994). Recommendations for appropriate precautions to be taken when handling *Brucella* cultures have been made (Corbel et al. 1979b; Report 1986b).

Most cases of infection caused by *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* arise from occupational or

domestic contact with infected animals or with an environment contaminated by their discharges. Farmers and their families, abattoir workers, butchers, and veterinarians are particularly at risk. Infected animals that have recently aborted or given birth present the greatest hazard; infection may occur through ingestion or inhalation, or by contamination of the conjunctiva or skin with discharges. *B. abortus* 'strain 19' and *B. melitensis* 'Rev 1' – two live vaccine strains – retain virulence for humans, and infections have resulted from accidental exposure to them (Sadusk et al. 1957; Olle Goig and Canela-Soler 1987).

The main source of infection for the general population is dairy produce prepared from infected milk. *B. melitensis* presents the greatest hazard. The milk of infected sheep and goats may contain large numbers of viable organisms, which become concentrated in products such as soft cheeses. Indeed, soft cheese has been recognized as a major vehicle of infection in the Mediterranean region, the Middle East, and Latin America (Eckman 1975; Sabbaghian 1975; Al-Dubooni et al. 1986; Report 1986a).

B. melitensis may be transmitted from infected sheep and goats to cattle kept in contact with them. Camels, which are susceptible to *B. abortus* and *B. melitensis*, can be infected in the same way; their milk is an important source of infection for humans in Middle Eastern countries and in Mongolia (Report 1971).

Cattle infected with *B. abortus* probably present the greatest hazard to humans through direct contact after calving or abortion, or at slaughter. This is reflected in the occupational nature of the disease in Britain and other developed countries (Buchanan et al. 1974b; Report 1980). Of the 494 cases reported to the Public Health Laboratory Service in England and Wales in the period 1956–61, 69 percent were in males and 71 percent in the 10–50 year age group. Farm and dairy workers, abattoir workers, butchers, and veterinary surgeons accounted for a disproportionate number of male cases, whereas housewives and schoolchildren predominated among the female cases. Dalrymple-Champneys (1960) attributed about 80 percent of nonoccupational cases to the consumption of raw milk and cream. At that time, at least 5 percent of herd samples of raw milk were infected with *B. abortus* (Report 1961). Cases among the nonoccupationally exposed population were confined to those who drank tuberculin-tested or other types of raw milk. They were virtually nonexistent in urban populations supplied with pasteurized milk. After the introduction of eradication measures for bovine brucellosis, the disease in the general population declined rapidly. Of 419 cases reported in the period 1975–79, only 13 were attributed to the consumption of raw milk, the remainder being occupational in origin (Report 1980). By 1984 the decline was greater still, and even occupationally acquired cases were rare. Serological surveys have indicated that even in populations consuming raw milk, the number of cases of clinical brucellosis is small in proportion to the number of people with antibodies to *B. abortus*. This indicates that *B. abortus* has a high infectivity for humans but is less virulent than some other types.

Infection caused by *B. suis* biogroups 1 and 3 has a much more limited distribution than that caused by *B. abortus* and *B. melitensis*. It is essentially an occupational disease of slaughterers and meat packers, who handle carcasses of infected pigs, and hence affects mainly adult males (Hendricks 1955). It was formerly common in the USA, especially in the middle-western states, but has declined dramatically as a result of the virtual eradication of the disease from the pig population. Epidemiological studies made when the infection was common showed that only a minority of those exposed developed overt, as opposed to a subclinical, infection (Hardy et al. 1931, 1932; Huddleson et al. 1933; Heathman 1934; Buchanan et al. 1974a). The organism gains entry via the conjunctiva, abraded skin, respiratory tract, or by ingestion (Hendricks et al. 1962; Buchanan et al. 1974a). The disease is often prolonged and debilitating, with a tendency to produce severe complications. Occasionally, *B. suis* infects cattle, causing milk-borne outbreaks in the general population (Borts et al. 1943). This is not uncommon in Argentina, Brazil, and Colombia, where *B. suis* infection of humans is prevalent. The disease also occurs in the South Pacific region and in southern China. Occasional cases contracted from feral pigs occur in the southern USA and in Australia (Crichton and Medveczky 1987).

Worldwide, *B. abortus* biogroup 1 is usually the most common cause of brucellosis in cattle in developed countries and in others in which intensive systems of management are used. *B. abortus* biogroup 3 tends to predominate in indigenous cattle populations in Africa and Asia but is now the most common type in western Europe. *B. melitensis* biogroup 1 is probably the most common type worldwide but biogroup 2 predominates in Italy and is at least as common as biogroup 1 in countries surrounding the Arabian Gulf. *B. suis* biogroup 2 is now uncommon but occurs sporadically in hare populations throughout Europe from the Baltic to the Adriatic Sea. *B. suis* biogroups 1 and 3 are now uncommon in the USA but frequent in Mexico, Colombia, Argentina, southern China, and Indonesia. *B. suis* biogroup 4 is confined to reindeer populations in the Arctic regions of Alaska, Canada, and Russia. *B. suis* biogroup 5 is found only in rodents in the Caucasus. *B. canis* has been implicated as a cause of brucellosis in humans, the manifestations being similar to those resulting from infection with *B. abortus*, *B. melitensis*, and *B. suis*, but the disease is usually mild (Polt et al. 1982). Serological studies suggest that subclinical infection is common in some populations (Monroe et al. 1975).

MORPHOLOGY

Typically, *Brucella* spp. occur as small gram-negative coccobacilli, but coccal and bacillary forms also occur. The cells are short and slender; the axis is straight; the ends are rounded; the sides may be parallel or convex outwards. In length, they vary from about 0.6 to 1.5 μm, and in breadth from 0.5 to 0.7 μm. The short forms may appear as oval cocci, or, if they are arranged singly, in pairs end to end, or in small groups; sometimes short chains of four to six members may be seen, especially in liquid media. Because of their frequently coccoid appearance, their bacillary nature may be in doubt, but it may be noted that they are smaller than any of the gram-negative cocci. Moreover, when arranged in pairs, their long diameter is in the same axis as that in which they are lying, as distinct from the gram-negative diplococci, whose long axis is generally at right angles to that in which they are lying (Figure 1).

B. melitensis tends to be more coccal in form than *B. abortus*, but this is not consistent enough to be of value for identification. The bacillary forms of *B. abortus* and *B. suis* are most readily apparent when grown on a rich medium, when individual cells may reach 2–3 μm in length. *B. melitensis* usually remains coccoid and rarely exceeds 1 μm in length. The organisms stain fairly well with the ordinary dyes. They are gram-negative, non-acid-fast, nonmotile, and nonsporing. Bipolar staining can occur and irregularity in the depth of color may be seen, especially in old cultures in which irregular forms appear. L-forms have been isolated from natural sources (Nelson and Pickett 1951; Corbel et al. 1980) and from cultures treated with penicillin, glycine, or hormones (Hines et al. 1964; Roux and Sassine 1971; Meyer 1976). They have the morphology and structure typical of bacteria that are cell wall defective. Although sometimes isolated from infected individuals, it has not been shown that these organisms retain their pathogenicity.

Figure 1 *Scanning electron micrograph of* B. abortus *strain 19 (bar = 2 μm)*

Brucella cells resist decolorization by dilute solutions of acids and alkalis and advantage has been taken of this in differential staining procedures for the examination of infected tissues. Modified Ziehl–Neelsen, Macchiavello, or Hansen and Köster's methods may be used (Christofferson and Ottosen 1941; Stamp et al. 1950). These procedures are not absolutely specific for *Brucella* and false-positive reactions may be given by chlamydiae, *Coxiella burnetii*, and several species of gram-negative bacilli. Immunospecific staining with labeled immunoglobulins will differentiate *Brucella* from most other organisms but not from *Yersinia enterocolitica* O9. False-positive staining reactions may also be given by gram-positive cocci containing protein A or protein G (Corbel 1973a; Brewer and Corbel 1980).

Brucella strains have many structural features in common with other gram-negative bacteria. Nevertheless, they show a number of important differences from the gram-negative bacilli typified by *Escherichia coli*. In electron micrographs from negatively stained preparations, the cell surface appears rugose, probably because of the presence of structures formed from the lipopolysaccharide (LPS)–protein complex which comprises the outer membrane. This layer, which is about 9 nm thick, encloses a muramic acid-containing peptidoglycan layer 3–5 nm thick which is much more prominent in *Brucella* cells than in *E. coli* (Dubray and Plommet 1976). Internal to this layer is a zone of low electron density, the periplasmic region. Closely associated with the inner cell membrane are polyribosome complexes. The cytoplasm contains small vacuoles and polysaccharide-containing granules (Dubray 1972). The chromosomal material has been located as an osmiophobic mass interspersed with delicate osmiophilic filaments (De Petris et al. 1964; Peschkov and Feodorov 1978) (Figure 2).

CELLULAR COMPOSITION

The gross cellular composition is similar to that of other gram-negative bacteria. Thus, the dry weight of the cell comprises an average 40–50 percent protein, 20–25 percent carbohydrate, c. 7 percent total lipid (2.5 percent bound lipid, 2 percent phospholipid, and 2.5 percent neutral lipids), 10–15 percent RNA, and 1.5–3 percent DNA. The precise proportions of these constituents are very dependent on conditions of growth.

In relation to detailed chemical composition, brucellae strains show some distinctive features. *Brucella* cells are more resistant to the action of lysozyme, even in the presence of chelating agents and nonionic detergents, than are enterobacterial cells which suggests some unusual features in peptidoglycan structure. The latter comprises a glycan skeleton of D-glucosamine and muramic acid linked by short chains of alanine, glutamic acid, and α,ε-diaminopimelic acid. However,

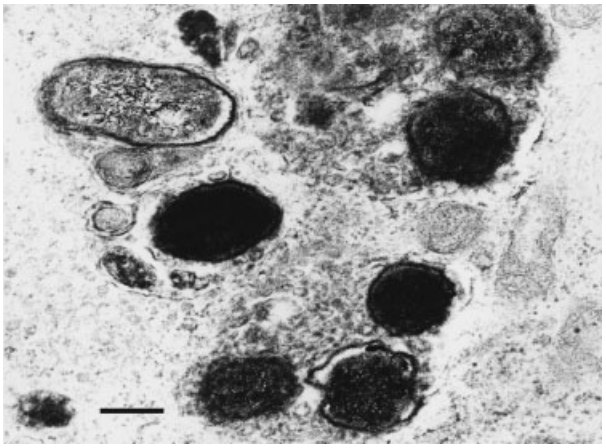

Figure 2 *Negatively stained thin section of bovine macrophage infected with* B. abortus *biogroup 1. The bacteria are enclosed in vacuoles and their outer membranes and internal structure are visible (bar = 0.25 μm)*

it also contains covalently linked proteins and lipids (Cherwonogrodzky et al. 1990).

In *B. abortus*, the outer-membrane proteins include the 88–94 kDa high molecular weight group 1, the 43 kDa and 36–38 kDa porin proteins of group 2 (Douglas et al. 1984) and the 25–27 kDa proteins of group 3, as well as minor proteins of 15–31 kDa. A lipoprotein of 8 kDa is also covalently linked to the peptidoglycan skeleton. These components are also present in the other nomen species but with quantitative differences (Santos et al. 1984; Verstreate and Winter 1984). In addition to the above, *B. melitensis* contains a 31 kDa outer-membrane protein and another of 39–40 kDa.

In all *Brucella* strains, the group 2 proteins are by far the most abundant. The genes for the 36 kDa porins (*omp2a* and *omp2b*) have been cloned and the peptide sequences determined (Ficht et al. 1989). The group 3 proteins were assumed to be analogs of OMP A but sequence data have not confirmed this.

The 8 kDa lipoprotein resembles the Braun lipoprotein of *E. coli* in molecular weight, isoelectric point, and amino acid composition, but differs from it in being surface exposed (Gomez Miguel et al. 1987). *Brucella* is also distinguished by being particularly rich in myristic, palmitic, and stearic acids, in containing moderate quantities of cis-vaccenic and arachidonic acids, low quantities of C17 and C19 cyclopropane fatty acids, and no hydroxy fatty acids. It is believed that this unusual fatty acid composition contributes to hydrophobic interactions in the *Brucella* outer membrane and enhances its stability (Cherwonogrodzky et al. 1990).

Another distinctive polysaccharide component is the material identified as polysaccharide B, also known as component 1 or second component. This was thought to be an antigen related to the O polysaccharide (Diaz et al. 1979). However, it was subsequently shown to be a cyclic polymer of 17–24 β-1,2 linked D-glucose residues (Bundle et al. 1988). It is apparently nonantigenic and similar to the cyclic β-D-glucans synthesized by many other bacteria. It readily forms noncovalently linked complexes with *Brucella* LPS and O chain.

The lipid composition of *Brucella* cells has a number of distinctive features which reinforce the taxonomic affiliation of the genus to the α2 subdivision of the Proteobacteria. The free lipid fraction comprises mainly phospholipids and neutral lipids. Phosphatidylcholine is the principal phospholipid, in contrast to most bacterial species (Thiele and Schwinn 1973), although it is also present in *Agrobacterium tumefaciens*. Its presence confers distinctive properties on the outer membrane and may account for its reduced susceptibility to phospholipases and lysozyme. Phosphatidylglycerol and diphosphatidylglycerol are also present as major lipids. Phosphatidyl ethanolamine, phosphatidyl serine, and candiolipin are represented as minor components. The fatty acids associated with the phospholipid fraction are unusual in that lactobacillic (C19: cyclic) acid, typical of gram-positive but not gram-negative species, is usually the major cyclopropane fatty acid, together with its metabolic precursor cis-vaccenic (C18:1 cyclic) acid. It should be noted that *B. canis* is an exception to this and has cis-vaccenic acid as the major fatty acid with lactobacillic acid in only trace amounts. This largely accounts for the distinct position of *B. canis* vis-à-vis the other nomen species when fatty acid composition is used for taxonomic analysis (Tanaka et al. 1977).

The neutral lipids include unusual wax-like esters containing large quantities of palmitic (C16:0) acid and moderate amounts of myristic (C14:0), palmitoleic (C16:1), stearic (C18:0), and cis-vaccenic (C18:1) acids. Lactobacillic acid is absent from these compounds.

Other distinctive free lipid components of *Brucella* include ubiquinone Q10 and ornithine-containing lipids (Thiele and Schwinn 1973). The latter make up 32 percent of the total neutral lipid. They contain lactobacillic, cis-vaccenic, and palmitoleic acids in ester linkage and palmitic and stearic acids in amide linkage but no hydroxy fatty acid. The function of the ornithine lipids is unknown but they are structural components of the outer membrane and it has been suggested that they are implicated in the attachment of *Brucella* cells to the surface of macrophages and lymphocytes (Cherwonogrodzky et al. 1990). Minor free lipid components include 1,3 and 1,2 diesters of glycerol and monoesters of ethylene glycol.

The bound lipids are mainly associated with the LPS, lipoprotein, and glycolipid fractions of the outer membrane. The composition of the LPS is unusual in that the lipid A backbone is formed of 2,3-diamino-2,3-dideoxy-D-glucose as well as D-glucosamine. This feature is present in some other members of the Proteobacteria (Weckesser and Mayer 1988). Both amide- and ester-linked fatty acids are attached to the aminoglycose skeleton. The amide-linked acids include 3-O-(C16:0)

12:0 (25 percent), 3-O-(C16:0) 13:0 (4 percent), 3-O-(C16:0) 14:(64 percent), and 3-O-(C18:0) 14:(7 percent) as diesters with 3-OH(C16:0) as the unsubstituted fatty acid. The ester-linked acids comprise C:16:0, 3-OH-C16:0, 3– -C18:0, and C18:0 acids which account for 37, 12.5, 3.5, and 4.5 percent, respectively, of the total fatty acids. Lactobacillic and unsaturated fatty acids are absent from the lipid A of *Brucella* although represented in many other lipid components (Cherwonogrodzky et al. 1990).

ANTIGENIC STRUCTURE

Surface antigens

LPS ANTIGENS

The antigenic composition of the *Brucella* cell surface varies with the colonial phase of the organism. All smooth *Brucella* strains crossreact extensively in agglutination tests with antisera prepared against smooth cultures. Similarly, all rough strains show extensive crossreactions in tests with antisera to nonsmooth cultures. However, there is little or no crossreactivity between the major surface antigens of smooth and completely nonsmooth cultures, with varying degrees between strains in transition from smooth to rough phases. Failure to appreciate these differences led to much confusion in earlier studies on the antigenic structure of *Brucella* (Wilson and Miles 1964). The studies of Wilson and Miles (1932) represented a major advance in the understanding of the antigenic structure of the *Brucella* cell surface. By careful crossadsorption of antiserum to typical *B. abortus* strains with cells of *B. melitensis* and vice versa, they produced antisera that were monospecific for the A determinant of *B. abortus* or the M determinant of *B. melitensis*. They attributed the crossreactivity of smooth strains to the presence of a small proportion of the heterospecific antigen in the homologous strain. Subsequently, Miles and Pirie (1939a, b) identified the serological activity with a formylated phospholipid-aminopoly-hydroxy compound isolated from culture supernates. Later studies showed that the A and M epitopes were associated with the LPS complexes that partitioned into the phenol phase on extraction by the Westphal method (Diaz et al. 1968).

Initial attempts at chemical characterization of the structures responsible for the crossreactivity of smooth strains and the A and M specificity were unsuccessful. Subsequently Perry and colleagues concluded that the O chain of smooth LPS was degraded by conventional hydrolysis procedures. By carbon-13 and proton nuclear magnetic resonance spectroscopy in combination with polarimetry and methylation analysis, they demonstrated that the O chains from smooth *B. abortus* and smooth *B. melitensis* were both homopolymers of 4,6 dideoxy-4-formamido-D-mannose (N-formyl-D-perosamine) (Caroff et al. 1984a, b; Bundle et al. 1987). In the case of *B. abortus* LPS, the O chain consists of about 100 glycose residues nearly all linked α-1,2 but with a very small proportion linked α-1,3.

In the case of *B. melitensis* LPS, the O chains consist of unbranched linear polymers of pentasaccharide units comprising four residues linked α-1,2 and one residue linked α-1,3. The difference in linkage produces penta- or hexasaccharide units with different preferred conformations (Figure 3). This hypothesis has been confirmed by crystallographic studies with monoclonal antibodies which differentiate the rod-like A epitope from the kinked M structure (Rose et al. 1993). The common presence of nonterminal tetrasaccharide units of α-1,2 linked N-formyl-D-perosamine explains the crossreactivity observed between the LPS of all smooth *Brucella* strains. The A- and M-specific epitopes are actually present as minority structures in both types of LPS. In the case of strains typified by *B. abortus* biogroup 1, the O chains contain predominantly A epitopes with a very small proportion of M epitope attributable to the few α-1,3 linked residues. In strains typified by *B. melitensis* biogroup 1, the reverse situation applies. This confirms the hypothesis of Wilson and Miles (1932) that A and M epitopes resided on a single molecule, the observed specificity being determined by the relative proportions of each.

By means of slide agglutination, agglutinin absorption, or enzyme immunoassays with polyclonal antisera or monoclonal antibodies specific for the A and M epitopes, the A or M antigen predominance of *Brucella* strains can be determined (Wilson and Miles 1932; Olin 1935; Meikle et al. 1989; Bundle et al. 1989). This information is useful in classifying the smooth *Brucella* nomen species into biogroups. It should be noted that strains of *B. abortus*, *B. melitensis*, or *B. suis* can be A, M, or A and M antigen positive (Table 1) (Dubray and Limet 1987). Strains that are both A and M antigen positive synthesize LPS with O chains that contain both A and M structural features in relatively high proportions (Perry and Bundle 1990).

N-Acylated-D-perosamine also occurs in the O chains of the LPS complexes of *E. coli* 0157, *Salmonella* Kauffmann–White group N (Bundle et al. 1987), *Stenotrophomonas maltophilia* strain 555, *Vibrio cholerae* (Redmond 1979; Kenne et al. 1982), and *Yersinia enterocolitica* 09 (Caroff et al. 1984b), all of which crossreact serologically with smooth *Brucella* (for references, see Corbel 1985). Crossreactions also occur between *Brucella* and *Francisella tularensis* (Francis and Evans 1926; Ohara et al. 1974), but it is not known if the latter organism contains D-perosamine.

The structure of the LPS of rough *Brucella* strains is basically similar to that of smooth strains. Both contain the same lipid A in which both 2,3-diamino-2,3-dideoxyglucose and 2-amino-2-dioxyglucose occur in the glycose

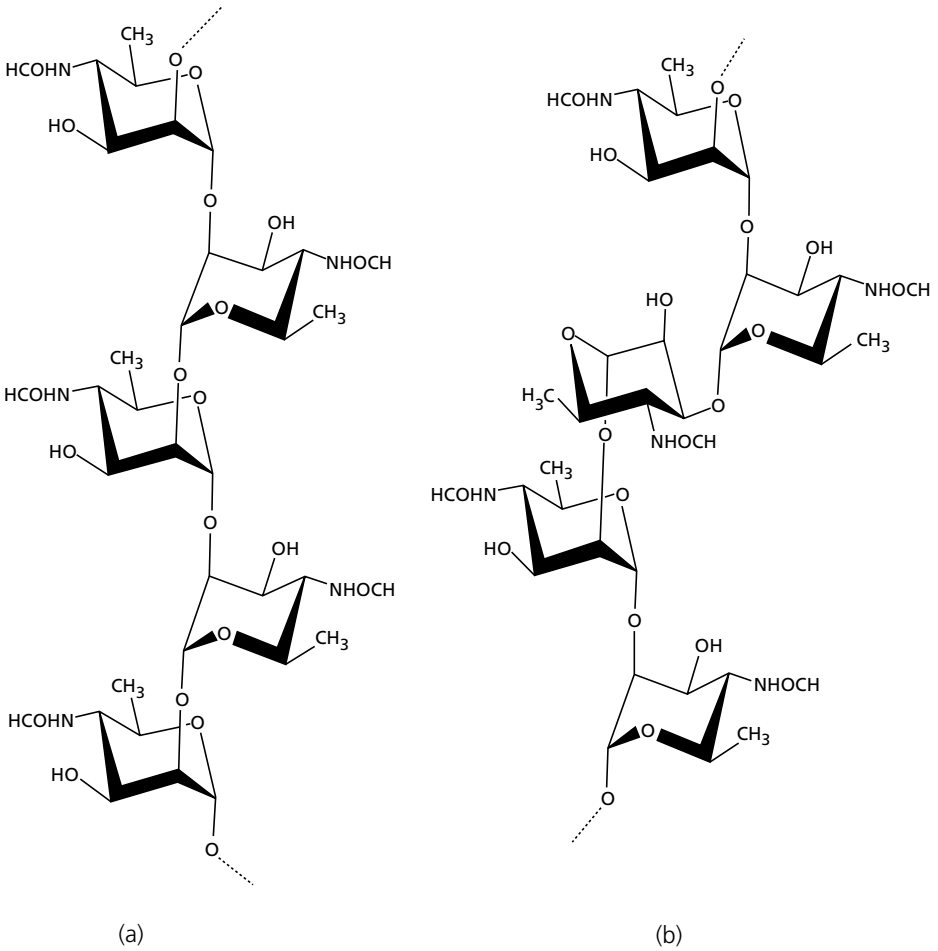

Figure 3 *Structures of* **(a)** *A type LPS of* B. abortus *biogroup 1 and* **(b)** *M type LPS of* B. melitensis *biogroup 1 (after Bundle et al. 1989)*

backbone. This is linked through 2-keto-3-deoxyoctulosonic acid (KDO) to the core polysaccharide composed of glucose, mannose, and 6-amino-6-deoxglucose (quinovosamine). Heptose is notably absent from *Brucella* LPS. A short homopolymer of N-formyl perosamine has been reported in the LPS of some rough *B. abortus* strains and *B. canis* (Perry and Bundle 1990). However, some rough LPS preparations do not contain quinovosamine suggesting that this is the terminal glycose of the core oligosaccharide and that the glucose, mannose residues are the main determinants of R specificity (Moreno et al. 1984). If nonsmooth *Brucella* strains are to be examined in agglutination procedures, the suspensions need to be stabilized by special buffers (Corbel et al. 1979a). Serological crossreactions have been reported between rough *Brucella* strains and *Actinobacillus equuli*, *Pasteurella multocida*, and mucoid strains of *Pseudomonas aeruginosa* (Weber 1976; Carmichael et al. 1980).

The smooth LPS is the immunodominant antigen of the *Brucella* cell surface and a major virulence factor. It has endotoxic activity but shows some major differences in activity from enteric endotoxins typified by *E. coli* LPS. For example, it is much less toxic for rabbits, chick embryos, and endotoxin-sensitive mouse strains. It is much more toxic than *E. coli* endotoxin for endotoxin-resistant mouse strains and is effective in stimulating interleukin-1 and tumor necrosis factor (TNF)-α production in these. It is also toxic for macrophages and is antigenic for spleen cells and B-, but not T-lymphocytes. Its lipid A does not bind polymyxin B and this does not inhibit its mitogenic or toxic activity. *Brucella* LPS has an unusual adjuvant activity in that it stimulates high levels of IgM and IgG3 antibodies in mice. It is a major protective antigen in mice and other species, including cattle.

OTHER POLYSACCHARIDE ANTIGENS

Various polysaccharides structurally related to *Brucella* LPS O chain have been described. These include 'native antigen' or 'native hapten.' This can be extracted from LPS or whole cells by mild methods (Moreno et al. 1987, 1979). Its association with lipid and protein is controversial. It is believed to be O chain which has not been incorporated into LPS. The rough strain *B. melitensis*

Table 1 *Characters of the species and biogroups ('biovars') of* Brucella[a]

Species	Biogroup	CO_2 requirement	H_2S production	Growth in presence of 1 in 50 000 (20 μg/ml)		Agglutination by monospecific serum[b]			Preferred natural host	Remarks
				Thionin	Basic fuchsin	A	M	R		
B. melitensis	1	–	–	+	+	–	+	–	Sheep, goat	Typical melitensis
	2	–	–	+	+	+	–	–	Sheep, goat	
	3	–	–	+	+	+	+	–	Sheep, goat	
B. abortus	1	(+)	+	–	+	+	–	–	Cattle	Typical abortus
	2	(+)	+	–	–	+	–	–	Cattle	Wilson II type
	3	(+)	+	+[c]	+	+	–	–	Cattle	Rhodesian type
	4	(+)	+	–	(+)	–	+	–	Cattle	
	5	–	–	+	+	–	+	–	Cattle	British 'melitensis'
	6	–	–	+[c]	+	+	–	–	Cattle	
	9	–	+	+	+	–	+	–	Cattle	
B. suis	1	–	+	+	(–)	+	–	–	Pig	American suis
	2	–	–	+	–	+	–	–	Pig, hare	Danish suis
	3	–	–	+	+	+	–	–	Pig	American 'melitensis'
	4	–	–	+	(–)	+	+	–	Reindeer	'*B. rangiferi tarandi*'
	5	–	–	+	–	–	+	–	Rodents	Caucasus only
B. neotomae		–	+	–	–	+	–	–	Desert wood rat	USA only
B. canis		–	–	+	(–)	–	–	+	Dog	Canine abortion organism
B. ovis		+	–	+	(–)	–	–	+	Sheep	Ram epididymitis organism
B. 'maris'		+ or –	–	+	+	+	+ or –	–	Marine mammals	Pathogenicity unknown

+, positive; –, negative; (+), usually positive; (–), usually negative.

a) For oxidative metabolism, see Table 2; for phage susceptibility, see Table 4.

b) A, abortus; M, melitensis; R, rough.

c) *B. abortus*, biogroup 3 grows in the presence of 1 in 25 000 thionin; biogroup 6 does not. Former *B. abortus* biogroups 7 and 8 are no longer regarded as valid.

B115 has been shown to synthesize smooth O chain which accumulates in the cytoplasm but is not assembled into complete LPS nor exported to the cell surface (Cloeckaert et al. 1992b).

The component identified as polysaccharide B, and formerly advocated as a diagnostic reagent (Diaz et al. 1979), is now known to be a nonantigenic cyclic D-glucose which forms complexes with smooth LPS or O chain (Perry and Bundle 1990).

OUTER-MEMBRANE PROTEINS

The outer-membrane proteins include the outer-membrane proteins of groups 1, 2, and 3, the most quantitatively important of which are the group 2 porins. These, and other proteins associated with the peptidoglycan fraction of the outer membrane, have been investigated as candidate protective antigens. In general, attempts to demonstrate protection with monoclonal or polyclonal antibodies to these have indicated low activity in the absence of antibody to smooth LPS (Jacques et al. 1992). However, this does not disprove their role in protective immunity as they could still be implicated in relevant cell-mediated responses.

The role of outer-membrane proteins in the response of cattle to *Brucella* infection has been investigated. Proteins with molecular masses of 10, 16.5, 19, 25–27, and 36–38 kDa elicited antibody responses in cattle infected with *B. abortus*, although the pattern of response to each varied between individual animals. Antibodies to the 89 kDa protein were not specific for *Brucella* (Cloeckaert et al. 1992a, b). The 8 kDa lipoprotein covalently linked to peptidoglycan is antigenically related to the Braun lipoprotein of *E. coli* and is thus a potential source of crossreaction (Gomez Miguel et al. 1987).

Intracellular antigens

The internal antigens released on breakage of *Brucella* cells are, for the most part, proteins, glycoproteins, and peptides common to all strains irrespective of colonial phase and biogroup. With polyclonal antisera raised against homogenates of disrupted cells, 30–40 individual precipitating components have been detected by Western blotting or two-dimensional immunoelectrophoresis procedures. Most of these are still detected by antisera adsorbed with washed whole cells indicating that they are not exposed on the cell surface. Few have been identified with structural components of the cell but attempts have been made to characterize those most relevant to serological responses in infected animals or humans (Stemshorn and Nielsen 1977, 1981; Schurig et al. 1978; Stemshorn et al. 1981).

Some internal antigens are associated with the ribosomal fraction. They may be involved in both antibody and cell-mediated responses to infection (Corbel 1976b). The L7/L12 ribosomal protein has been identified as an important component of brucellin INRA and shown to elicit delayed hypersensitivity reactions in sensitized animals (Bachrach et al. 1994).

The antigens designated A1, A2, A3, A4, B1, B2, and C by Schurig et al. (1978) have been examined for their role as indicators of infection status. The A2 antigen is identical with the heat-stable glycoprotein described by Stemshorn and Nielsen (1981) and Stemshorn et al. (1981), and the 20 kDa protein described by Zygmunt et al. (1992). An 18 kDa cytoplasmic protein has been described as an indicator of active infection in cattle and humans (Goldbaum et al. 1993). Hitherto, experience of protein antigens has indicated a high specificity but relatively low sensitivity for detection of infected individuals. In brucellosis, the antibody response is dominated by the LPS antigen.

In vivo antigens

Evidence that *Brucella* cells grown in vivo exhibited differences from cells grown in culture media has been presented (Smith and Fitzgeorge 1964; Fitzgeorge and Smith 1966). They reported that virulent *B. abortus* extracted from infected bovine placenta was more resistant to intracellular killing by bovine buffy coat phagocytes than the same strain grown in vitro. Furthermore, the effect was neutralized by antiserum from infected animals. The protective factor was associated with the cell envelope, preparations of which from organisms grown in vivo promoted the intracellular survival of attenuated strains. Similar effects were observed if the virulent strain was grown in culture medium supplemented with bovine allantoic fluid. These important observations have not been followed up and the composition of the protective factor produced in vivo has not been determined.

It is now well established for other bacteria that growth under conditions analogous to those found in vivo induces environmentally regulated antigens. The most extensively studied have been the iron-regulated proteins and it has been clearly demonstrated for a number of bacteria, including the pathogenic *Neisseria*, *Pasteurella*, and *Yersinia* species, that novel proteins associated with iron acquisition are produced under such 'in vivo' conditions. These proteins are essential for growth of the organisms and for the establishment of infection (for further information, see Griffiths 1993). Little information is available on the effect of iron restriction on the growth of *Brucella* in vivo. It is known, however, that *Brucella* responds to adverse environmental conditions by synthesis of chaperonins or heat shock proteins analogous to the GroEL and Htr proteins of *Escherichia coli* (Tatum et al. 1994).

Brucella also responds to an acidic environment by synthesis of stress-related proteins. These are produced

in at least two phases; between pH 6.5 and 4.5 the proteins synthesized are probably involved in pre-acid shock inducible pH homeostasis, whereas those induced at pH 3.8 are probably involved in acid tolerance (Lin and Ficht 1995). During intracellular growth in a macrophage cell line, production of novel proteins of 62, 28, 24, and 17 kDa was observed. The 62 kDa protein was identified as Hsp 62, a GroEL homolog. The 24 kDa protein was probably equivalent to the Asp 24 acid-induced protein, whereas the 17 and 28 kDa proteins were apparently specific to macrophage-induced stress. These observations are clearly relevant to understanding the mechanisms of intracellular survival of *Brucella*.

GENETICS

In recent years, there have been considerable advances in knowledge of the molecular genetics of *Brucella*. These have provided an indication of the phylogenetic relationships of the genus and its possible origins, and have pointed the way towards a system of classification based on genetic rather than phenotypic characteristics.

Genome composition

The average genome complexity for *Brucella* and the closely related *Ochrobactrum* and *Phyllobacterium* genera falls within the range 2.37–2.82 $\times$ 10^6 kDa (de Ley et al. 1987; Allardet-Servent et al. 1988). The DNA of all members of the genus contains approximately 58–59 mol% G + C. This is quite different from the values for *Bordetella bronchiseptica* and *Francisella tularensis* which were formerly classified as *Brucella* species on the basis of superficial phenotypic similarity. It is identical with the value for *Ochrobactrum anthropi* (formerly CDC group Vd, see Cieslak et al. (1992)) and very close to those for *Agrobacterium*, *Rhizobium*, and *Phyllobacterium* (Table 2).

A partial physical map of the *Brucella* genome was produced by Allardet-Servent et al. (1991). Extension of this study led to the conclusion that the genome comprised two chromosomes of 2.1 and 1.5 mbp, respectively. This has been confirmed by insertion of unique restriction sites (Jumas-Bilak et al. 1995). Two rRNA operons and the gene for the DnaK heat shock protein are located on the larger replicon, and another rRNA operon and the gene for the GroEL chaperonin are located on the smaller one. This indicates that both replicons encode functions essential for survival and replication and hence are chromosomes and not plasmids (Michaux et al. 1993). The presence of multiple replicons is also typical of the *Agrobacterium* and *Rhizobium* genera, now known to have genetic affinity with *Brucella*.

DNA–DNA hybridization studies have indicated a high degree of homogeneity within the genus (Hoyer and McCullough 1968a, b; Verger et al. 1985; de Ley et al. 1987). The DNA relatedness of all the nomen species is 96 $\pm$ 4 percent in the most recent studies. The apparent deletion of 6 percent of sequences in *B. ovis* relative to the other species reported by Hoyer and McCullough (1968b) has not been confirmed but is close to the limits of error of the methodology used. It is notable that *B. ovis* has been reported to show slight differences from the others in restriction endonuclease digestion pattern (O'Hara et al. 1985).

In general, restriction endonuclease analysis of whole DNA with frequently cutting enzymes has been of

Table 2 *Comparison of DNA and rRNA composition of* Brucella *and other bacteria*

Species	DNA G + C (mol%)	DNA homology (%)	16S rRNA/DNA		5S rRNA homology (%)
			Binding	T_m(e) (°C)	
B. melitensis	57.9–59.2	96±4	0.19	80.2	100
B. abortus	58.2–58.9	96±4	0.18	80.0	100
B. suis	57.9–58.2	96±4	0.18	80.1	100
B. neotomae	57.9	96±4	0.18	80.3	100
B. ovis	58.1	96±4	0.18	80.5	100
B. canis	57.9	96±4	0.18	80.3	100
Agrobacterium tumefaciens	60.2–60.6	⩾6	0.10	72.6	95.1
Bartonella bacilliformis	39	<5	–	–	95.4
Bartonella quintana	38.3–39	<5	–	–	95.8
Bordetella bronchiseptica	68.9	<5	0.06	57.4	–
Francisella tularensis	32.8	<5	0.08	56.7	–
Mycoplana dimorpha	63.8	~6	0.14	74.2	–
Ochrobactrum anthropi	57.6–59	23±7	0.15	77.7	~98
Rhizobium meliloti	62.3	~6	0.07	72.0	–

–, not available.

limited value in differentiating the members of the group. The use of infrequently cutting enzymes combined with pulsed field gel electrophoresis has differentiated *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, and *B. canis* along conventional lines, although it has not distinguished the biogroups (Allardet-Servent et al. 1988).

Restriction endonuclease analysis combined with probes for specific genes has demonstrated a number of polymorphisms. Of most taxonomic significance are polymorphisms in the *omp2* porin gene encoding the 36 kDa outer-membrane protein responsible for determining sensitivity to dyes (Ficht et al. 1988). Polymorphism has also been demonstrated in the gene encoding the BCSP 31 surface protein. This is associated with a 0.9 kb insertion sequence which is present at higher frequency in *B. ovis* than in the other nomen species (Halling and Zehr 1990).

RNA analysis

Both the 5S and 16S rRNA have been sequenced and have disclosed previously unexpected relationships to other genera. The 16S rRNA sequence is typical of the α-2 subdivision of the Proteobacteria. A high degree of sequence homology (over 95 percent) has been reported with the rRNA of *Agrobacterium*, *Ochrobactrum*, and the *Bartonella/Rochalimaea* group (Dorsch et al. 1989; Relman et al. 1992). Comparison of 16S rRNA/DNA binding patterns and sequence homology has confirmed the relationship of *Brucella* to *O. anthropi* and, less closely, to *Agrobacterium*, *Mycoplana*, *Phyllobacterium*, and *Rhizobium*. It has also indicated an affiliation to other intracellular pathogens including *Bartonella bacilliformis*, *Bartonella henselae*, and *Bartonella quintana*. Observations on the 5S rRNA sequences have indicated that *Brucella* is distinct from the *Bartonella* group, with a sequence homology of 86.6 percent compared with 92 percent. DNA–DNA hybridization studies have confirmed the close relationship between *Brucella* and *Ochrobactrum*. The similarity to *Agrobacterium* and *Rhizobium* is less evident and no relatedness to *Bartonella* has been found by this method (Table 2).

Plasmids and genetic exchange

Conjugative transfer of the broad host-range plasmid R751 from *E. coli* to *Brucella* and thence between *Brucella* species has been demonstrated (Verger et al. 1993). This was associated with transfer of trimethoprim resistance. Transfer of tetracycline resistance to *Brucella* by an *E. coli* plasmid had been reported previously by Rigby and Fraser (1989). These observations suggest that *Brucella* strains could acquire multiple antibiotic resistance by plasmid transfer.

The transduction of antibiotic resistance by phages isolated from atypical *Brucella* strains has also been described (Rigby 1990).

Molecular cloning

Numerous *Brucella* genes have now been cloned and expressed in heterologous systems. These have included the genes for the 36 kDa porin protein (Ficht et al. 1988), the copper–zinc superoxide dismutase (Bricker et al. 1990), the BCSP 31 protein (Mayfield et al. 1988), various outer-envelope proteins (De Wergifosse et al. 1995), heat shock proteins (Cellier et al. 1992), and many others. The regulatory proteins for some of these genes have also been identified and resemble those of *E. coli* (Mayfield et al. 1988).

Deletion mutants of *Brucella* have also been produced by genetic modification, mainly with the objective of determining the role of specific genes such as *htrA*, *recA*, and the copper–zinc superoxide dismutase (*sodC*) gene in pathogenesis (Tatum et al. 1993).

Phages

Although the occurrence of bacteriocins specific for *Brucella* has not been confirmed, numerous phages lytic for *Brucella* have been isolated. These phages are all closely related DNA phages of the same morphological group (Pedoviridae), with a base composition of 45–47 mol% G + C and a genome size of 25×10^3 kDa. They are stable to anionic and nonionic detergents, and to common organic solvents with the exception of chloroform, but are inactivated by heat, cationic detergents, and oxidizing agents. Their stability to proteolytic enzymes varies between strains. Restriction endonuclease and serological studies have indicated that all are very similar and can be regarded as host-range variants of a single type of phage. For convenience, they have been classified into six groups on the basis of host range (Corbel and Thomas 1983).

The phages of group 1, typified by the Tbilisi (Tb) strains, replicate only in smooth *B. abortus* and to a much lesser extent in *B. neotomae* cultures. They can produce 'lysis-from-without' on smooth *B. suis* cultures when applied at high concentration. Tb phage alone can be used for identification of smooth *Brucella* species if preparations standardized at routine test dilution (RTD) and 10 000 RTD are used. It is of no value for the identification of nonsmooth cultures and, in practice, identification is facilitated by using a battery of phages selected from groups 1, 3, 5, and 6.

The phages of group 2, typified by the Firenze (Fi75/13) strain, replicate in smooth *B. abortus* and *B. neotomae* and, to a much smaller extent, in *B. suis* cultures. Those of group 3, typified by the Weybridge (Wb) strain, replicate with similar efficiency in smooth

cultures of *B. abortus*, *B. neotomae*, and *B. suis*, but not usually in *B. melitensis* cultures although this varies with strains from different geographical locations (Corbel 1987). Phages of group 4, typified by the Berkeley (BK2) strain, replicate in smooth cultures of all *Brucella* species, although *B. melitensis* strains from some localities may be lyzed with low efficiency (Corbel 1987). The group 5 phages R (rough), R/O, and R/C are lytic for rough strains of *B. abortus*, *B. ovis*, or *B. canis* but not for smooth cultures (Corbel and Thomas 1985). The group 6 phages, represented by the Izatnagar (Iz1) strain, are lytic for smooth cultures of all *Brucella* species and for rough cultures of *B. melitensis* and *B. suis* and, to a much smaller extent, *B. ovis* and *B. canis* (Corbel et al. 1988). Another set of phages, the Nepean (Np) strains, resemble those of Group 1 in host range but do not lyze *B. suis* strains at 10^4 RTD. They have been proposed as a seventh group (Rigby 1990). The lytic patterns produced by representatives of these groups are shown in Table 3. For further information on *Brucella* phages and phage typing, see Corbel and Thomas (1983), Corbel (1984, 1987), and Young and Corbel (1989). The earlier studies on *Brucella* phages were summarized by Drozevkina (1963).

METABOLISM AND BIOCHEMICAL PROPERTIES

All *Brucella* strains are aerobic and require oxygen for growth. No growth occurs under strictly anaerobic conditions. Some strains require supplementary carbon dioxide. Although acid production from sugars has been demonstrated under special conditions (Pickett and Nelson 1955), metabolism is essentially oxidative. *Brucella* strains are catalase positive and most are oxidase positive. They also have superoxide dismutase activity mediated by two distinct enzymes, a manganese superoxide dismutase of typical prokaryote type (Sriranganathan et al. 1991) and a copper-zinc enzyme (Beck et al. 1990). In the case of *B. abortus*, the respiratory system has been shown to make use of cytochromes *a* and a_3, cytochrome *b*, cytochrome *c*, cytochrome *o*, flavoproteins, and ubiquinone (Rest and Robertson 1975). Energy-yielding processes involve the monophosphate pathway and the tricarboxylic acid cycle (Robertson and McCullough 1968). Glucose is metabolized by most strains but isoerythritol is used preferentially by *B. abortus*, *B. melitensis*, and *B. suis*. *B. ovis* shows little activity towards either substrate; its preferred energy source is unknown, but it has been shown to oxidize water-soluble components of various ovine tissues very actively (Redwood and Corbel 1983).

Most *Brucella* strains produce nitrate reductase and will reduce nitrate to nitrite and may also reduce nitrite, especially *B. suis* biogroup 1. *B. ovis* and, occasionally, strains of other species, do not reduce nitrate. Hydrogen sulfide is produced from sulfur-containing amino acids. Strains of *B. abortus*, *B. neotomae*, and *B. suis* biogroup 1 produce it in sufficient quantity to assist in their identification; the other species produce it in small quantities or not at all. *Brucella* strains do not produce indole from tryptophan. They have the capacity to synthesize most, if not all, amino acids essential to growth. The genes for biosynthesis of proline and leucine have been cloned (Essenberg and Sharma 1993).

Brucellae do not form acetylmethylcarbinol from glucose and hence give negative results in the Voges–Proskauer test. They also give negative reactions in the methyl red test although acid production from glucose can be demonstrated by sensitive techniques (Pickett 1994). The reaction for *o*-nitro-phenol β-D-galactosidase is usually negative. Urease is produced by most strains but the activity varies considerably between species and even between strains within species. *B. canis* and *B. suis* consistently give strong urease reactions, producing a magenta color on Christensen's medium within 5 min. At the other extreme, *B. ovis* is a weak producer of urease and some strains may give negative reactions even after incubation for 7 days. Strains of *B. abortus*, *B. melitensis*, and *B. neotomae* fall between these extremes and usually give positive urease reactions after 1 h or more. Nevertheless, variation occurs between strains and the test is of limited value for species identification (Corbel and Hendry 1985).

The oxidative activity of *Brucella* strains towards selected amino acid and carbohydrate substrates varies in a manner which correlates closely with other properties used to define the species. Originally, manometric methods were used to detect oxidation (McCullough and Beal 1951; Meyer and Cameron 1957, 1961a, b) and, although hazardous and time-consuming to perform, they are still useful when quantitative studies are to be performed. Nevertheless, quantitative variations in activity between strains within a species can cause confusion. To overcome this problem, Verger and Grayon (1977) established three levels of oxidative activity, corresponding to low, medium, and high oxygen uptake, which they used to produce a metabolic profile for each of the main species. The metabolic activity towards selected substrates can also be determined qualitatively by thin layer chromatography (Balke et al. 1977; Corbel and Morgan 1984). The patterns of oxidative activity characteristic of the species are shown in Table 4.

There is some variation in oxidative pattern between the biogroups of *B. suis*, but in our experience this is not sufficiently reliable for identification. With an extended range of substrates, further differentiation of *B. melitensis* strains beyond the three currently recognized biogroups has been reported (Arnaud-Bosq et al. 1987). Caution should be exercised, however, in attributing significance to minor differences in oxidative activity as this can be influenced by many factors including method of preservation of the strain and cultural conditions.

Table 3 *Lytic activity of phages for smooth (S) and rough (R)* Brucella *species*

			B. abortus		*B. suis*		*B. melitensis*		*B. neotomae*		*B. canis*	*B. ovis*
Phage group	**Phage strain**	**Titer**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**R**	**R**
1	Tb	RTD	L	NL	NL	NL	NL	NL	NL or PL	NL	NL	NL
		RTD $\times 10^4$	L	NL	L	NL	NL	NL	L	NL	NL	NL
2	Fi 75/13	RTD	L	NL	PL	NL	NL	NL	L	NL	NL	NL
3	Wb	RTD	L	NL	L	NL	V	NL	L	NL	NL	NL
4	BK_2	RTD	L	NL	L	NL	L or PL	NL	L	NL	NL	NL
5	R	RTD	V	L	NL	NL	NL	NL	NL	NL	NL	NL
	R/O	RTD	V	L	V	NL	NL	NL	V	NL	NL	L
	R/C	RTD	NL	L	NL	NL	NL	NL	NL	NL	L	L
6	Iz_1	RTD	L	NL	L[a]	V[a]	L or PL	V	L	PL	NL	NL
					PL[b]	NL[b]						
7	Np	RTD	L	NL	NL	NL	NL	NL	L	NL	NL	NL
		RTD $\times 10^4$	L	NL	NL	NL	NL	NL	L	NL	NL	NL

L, confluent lysis; PL, partial lysis; single plaques or growth inhibition; NL, no lysis; V, variable, some strains lysed; RTD, routine test dilution.

a) Biogroups 1 and 4.

b) Biogroups 2, 3 and 5.

Table 4 *Oxidative metabolism of species of* Brucella

	B. melitensis	*B. abortus*	*B. suis*	*B. neotomae*	*B. canis*	*B. ovis*
Amino acids						
L-Alanine	+	+	v*	v	v	v
L-Asparagine	+	+	v*	+	–	+
L-Glutamate	+	+	v*	+	+	+
L-Arginine	–	–	+	–	+	–
DL-Citrulline	–	–	+	–	+	–
L-Lysine	–	–	v*	–	+	–
DL-Ornithine	–	–	+	–	+	–
Carbohydrates						
L-Arabinose	–	+	v*	+	v	–
D-Galactose	–	+	v*	+	v	–
D-Ribose	–	+	+	v	+	–
D-Xylose	–	v	–	–	–	–
D-Glucose	+	+	+	+	+	–
Isoerythritol	+	+	+	+	v	–

+, positive; –, negative; v, variation between strains; v*, variation between biogroups of some assistance in classification.

SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

The members of this group exhibit the usual susceptibility of vegetative bacteria to heat and disinfectants. In aqueous suspensions of moderate density they are destroyed by heating for about 10 min at 60°C, but in very dense suspensions they may survive much higher temperatures (Swann et al. 1981). In agar cultures kept sealed at 4°C, they generally survive for at least 1 month, and often longer. If lyophilized, especially in the presence of a protecting agent, they will survive for decades.

Considerable attention has been paid to their resistance under natural conditions and this subject is discussed in the report of the Mediterranean Fever Commission (1905–7). So many factors determine the exact outcome of any given observation under natural conditions that it is dangerous to draw general conclusions. In favorable circumstances, however, *B. melitensis* may remain viable for 6 days in urine, 6 weeks in dust, and 10 weeks in water or soil. In pickled hams from naturally infected pigs, *B. suis* can live for as long as 3 weeks, but it is apparently destroyed by smoking (Hutchings et al. 1951). *B. suis* may live on sacking for 4 weeks and in sterile feces for 100 days in the dark. *B. abortus* may survive for 7 months in infected uterine exudate kept at about freezing point (Bang 1897). It can also survive in bovine urine for 4 days, feces for 120 days, aborted fetuses for 75 days, and liquid manure for up to 2.5 years. In raw milk at room temperature it seems to die out fairly rapidly with the production of acid. Acid production also seems to be the cause of its rapid death in butter, cheese, and yogurt, although variable reports exist on its survival in these materials. In general, survival in soft, nonacid cheeses is much longer than in hard, lactic- or propionic-acid-fermented cheeses. Pasteurization, whether by holder or high-temperature short-time cycles, kills *Brucella* in milk. Ultraviolet and γ-radiation at normal sterilizing dosage are rapidly lethal to *Brucella* provided that the organisms are not protected by the suspending medium. Ethanol, isopropanol, iodophors, phenols, hypochlorite, ethylene oxide, and formaldehyde (either gaseous or as aqueous formalin) are effective disinfectants for *Brucella* under appropriate conditions; they are killed by 1.0 percent phenol in 15 min. Xylene and calcium cyanamide have been recommended for killing *Brucella* in manure but prolonged contact times are required (FAO/WHO 1986).

CULTURAL ASPECTS

Biohazard aspects

Brucella strains have frequently been implicated in laboratory-acquired infections and are appropriately classified as dangerous pathogens. In Britain, they are assigned as Class 3 pathogens in the hazard classification of the Advisory Committee on Dangerous Pathogens (1994). Cultures and other materials potentially containing *Brucella* should only be handled under adequate containment conditions. Clinical specimens and isolates from patients with febrile disease should always be regarded with suspicion until positively identified. Accidental infections have occurred among unsuspecting laboratory staff. Misidentification of cultures through the use of inadequate methods can present a particular hazard. The incorrect identification

of *B. melitensis* as *Moraxella phenylpyruvica* through the use of API20NE rapid identification tests has resulted in several cases of laboratory-acquired brucellosis (Batchelor et al. 1992; Luzzi et al. 1993).

Cultural properties

Apart from different carbon dioxide requirements, all members of the genus resemble each other closely in cultural properties. Although laboratory-adapted strains can often be induced to grow in simple minimal media containing ammonium ion as sole nitrogen source (Gerhardt 1958), growth tends to be slow and colony size small. Most *Brucella* strains have much more exacting nutritional requirements, especially on primary isolation, and growth is much improved by the addition of blood, serum, and tissue extracts. In general, several amino acids, thiamin, biotin, nicotinamide, magnesium, iron, and manganese are required for growth. The growth of many strains is improved by pantothenate and isoerythritol. Indeed, the latter substance is used by *B. abortus*, *B. melitensis*, and *B. suis* as an energy source in preference to glucose. Liver-infusion agar was formerly recommended for the culture of *Brucella* spp. but has been superseded by more consistent media based on high quality peptone preparations, usually tryptic digests of soybean protein. These will support the growth of all but the most fastidious strains. *B. ovis*, *B. abortus* biogroup 2, and some strains of biogroups 3 and 4, grow very poorly in the absence of blood or serum, and media enriched with these are essential for their isolation.

On serum dextrose agar or similar medium, most *Brucella* strains produce raised, convex, circular, translucent colonies, 0.5–1 mm in diameter, after incubation for 48 h at 37°C. In direct light, the colonies of smooth strains appear a clear honey color, but in reflected light they have a distinctive bluish-gray translucency. Nonsmooth strains produce colonies of similar size but of much more variable color and consistency. They are usually more opaque than smooth colonies, with a granular dull surface, and range in color from off-white or yellowish to buff or even brown. Unlike smooth colonies, which are soft and easily emulsifiable, nonsmooth colonies can be friable, tacky, or obviously slimy.

Strains of the less fastidious types, especially *B. melitensis* and *B. suis*, will grow on bile-salt media producing small lactose-negative colonies. Growth in gelatin is poor and liquefaction is not produced. On blood agar, the colonial appearance is not distinctive; true hemolysis is not produced but a greenish brown discoloration develops around the colonies. In static liquid culture, maximum turbidity is produced after 7 days or longer. Most strains produce a uniform turbidity with a variable deposit. A surface pellicle may also be produced, especially by carbon dioxide-independent strains. Dissociation from smooth to nonsmooth colonial forms occurs readily in static liquid media and may be marked by a change in the deposit from light and powdery to extremely viscous.

Dissociation is best detected by examination of 4-day-old cultures on glycerol dextrose agar under a stereoscopic plate-microscope with obliquely reflected light (Henry 1933; FAO/WHO 1958). Smooth colonies appear small, circular, convex, translucent, and grayish-blue with a smooth glistening surface. The undivided cells are uniformly short rods or coccobacilli arranged singly. Colonies of rough strains are of much the same size, but are less convex, more opaque, and have a dull dry yellowish-white granular appearance. The individual cells are rather larger than those of the smooth form, and occasional long slender rods are present. Mucoid colonies are transparent, grayish or slightly orange, and slimy. The distinction between smooth and rough colonies can be increased by flooding plates with a 0.05 percent aqueous solution of crystal violet and pouring off the excess dye 15 s later. Smooth colonies then appear a light bluish-green, rough colonies are red to purple (White and Wilson 1951) (Figure 4). Smooth colonies form uniform suspensions in saline or aqueous acriflavine. Nonsmooth colonies are autoagglutinable in unbuffered saline solutions and in the presence of acriflavine. They can also be agglutinated by heating and by antiserum to the R antigenic determinant, or by the lectin concanavalin A (Corbel et al. 1979a).

For the isolation of *Brucella* from contaminated material, the use of selective media is generally required. Various formulations have been developed, but the most generally useful are based on that of Farrell (1974). This contains bacitracin, cycloheximide, nalidixic acid, nystatin, polymyxin B, and vancomycin in a serum dextrose agar base. A liquid medium containing a similar formulation with the addition of amphotericin B and D-cycloserine was devised by Brodie and Sinton

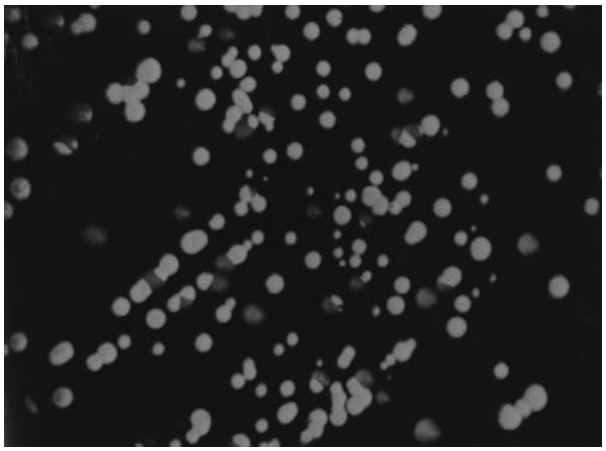

Figure 4 *Colonies of* B. melitensis *biogroup 1 grown on glycerol-dextose agar. Addition of 1 percent crystal violet solution to the plate has produced dark staining of the nonsmooth (R) colonies but left the smooth (S) colonies unstained.*

host specificity, and, with the exception of occasional *B. canis* infections in other carnivores and in humans, seem to have little capacity to spread beyond their usual hosts.

Typically, in all host species *Brucella* grows intracellularly, producing a variable bacteremic phase followed by localization in the reticuloendothelial system. In the preferred host, localization also occurs in the tissues of the genital tract and in the mammary glands. In the case of *B. abortus*, *B. melitensis*, and *B. suis*, this has been attributed to the presence of isoerythritol in the target tissues of cattle, sheep, goats, and pigs (Smith et al. 1961, 1962). Abortion is a frequent consequence of infection in the pregnant female, and orchitis and epididymitis can result in the male. Sexually immature animals are often less susceptible to the disease.

All species of *Brucella* can establish infection in guinea pigs and mice, but the severity and duration varies considerably. In general, *B. melitensis* and *B. suis* strains produce the most severe effect, *B. abortus* produces less severe but more obviously granulomatous lesions, and the remaining species produce a more transient infection accompanied by few, if any, gross lesions (Braude 1951; Isayama et al. 1977). There is some evidence that *B. melitensis* and *B. abortus* induce different cellular responses (Young et al. 1979).

The basis of the virulence of *Brucella* strains is incompletely understood. Nonsmooth variants of the normally smooth species are usually of greatly reduced virulence. This suggests a role for the O chain of the smooth LPS in determining virulence; supportive evidence for this is provided by the protective effect of monoclonal antibodies directed towards A or M determinants (Montaraz et al. 1986).

LPS, and possibly another cell-envelope component, have also been implicated in the blocking of the bactericidal action of normal serum and of phagocytes, permitting the survival of *Brucella* in polymorphonuclear and mononuclear phagocytes (Smith and Fitzgeorge 1964; Kreutzer and Robertson 1979). However, other factors are also involved in this process. The organisms are able to suppress the oxidative burst at least in polymorphonuclear phagocytes. *B. abortus* releases a low molecular weight nucleotide fraction containing guanine monophosphate and adenine which inhibits the intracellular bactericidal mechanisms of bovine phagocytes (Canning et al. 1986). The organisms also release low molecular weight RNA which may block the action of cationic proteins including lysozyme and defensins (Corbel and Brewer 1980).

The copper–zinc superoxide dismutase also plays a part in facilitating intracellular survival of *Brucella* by blocking the formation of activated oxygen and probably also hypohalogen acids. Mutants unable to synthesize the enzyme produce ten-fold lower infection levels in mice than wild-type strains (Tatum et al. 1992).

Survival within macrophages is associated with the synthesis of proteins of molecular weight 17, 24, 28, 60, and 62 kDa. The 62 kDa protein corresponds to the Gro EL homolog Hsp 62 and the 60 kDa protein is an acid-induced variant of this. The 24 kDa protein is also acid-induced and its production correlates with bacterial survival under acidic conditions (pH <4). The 17 and 28 kDa proteins are apparently specifically induced by macrophages and correlated with intracellular survival (Lin and Ficht 1995).

Another stress-induced protein, HtrA, was shown to be involved in the induction of an early granulomatous response to *B. abortus* in mice. This was associated with a reduction in the levels of infection during the early phase. However, it did not prevent a subsequent increase in bacterial numbers and *htrA*-deficient mutants ultimately produced levels of splenic infection similar to those given by wild-type *B. abortus* (Tatum et al. 1994). Similarly, *recA*-deleted mutants produced a lower initial spleen viable count than *recA*-positive strains but still established persistent infection (Tatum et al. 1993). Clearly a number of still unknown factors are involved in promoting protective immune responses to *Brucella*.

Antibodies to LPS provide some protection against infection but this is incomplete. Even opsonized bacteria are not effectively killed by macrophages (Caron et al. 1994). Antibodies to cell-surface proteins are even less effective than LPS in eliminating *Brucella* (Dubray 1987). However, protein antigens may play a significant role in triggering cell-mediated responses. The clearance of infection is dependent upon Th1-type responses; the stimulation of γ-interferon producing $CD4^+$ cells is crucial. These are most effectively induced by live *Brucella* cells (Zhan et al. 1995). TNF-α is also important for intracellular killing of *Brucella* and its production is suppressed by live but not by killed cells (Caron et al. 1994). Interestingly, purines or nucleosides appear to be involved in the suppression (Caron et al. 1994). The mechanisms whereby infection with *Brucella* results in tissue damage and disease are still unknown. No exotoxins have been detected and not all of the effects observed can be explained by the presence of endotoxin. Indeed, although *Brucella* LPS shares many of the properties of the endotoxins of other gram-negative bacteria, it is intrinsically less toxic than enterobacterial LPS, possibly because of a lower efficiency in releasing inflammatory cytokines and particularly TNF-α (Berman and Kurtz 1987; Caron et al. 1994).

Pathogenesis

Brucellae enter the body via the alimentary tract, conjunctival mucosa, respiratory tract, or skin. At or near the site of entry, they are likely to be ingested by either mononuclear or polymorphonuclear leukocytes. However, virulent brucellae can depress chemotaxis and phagocytosis by polymorphonuclear leukocytes (Ocon et al. 1994). Phagocytosis is facilitated by pre-existing antibodies but is not dependent upon them (Canning

et al. 1988). A lectin which binds to receptors on the cell membrane of the B lymphocyte has been identified on the surface of brucella cells (Bratescu et al. 1981). This, or a similar component, may also promote endocytosis of brucellae by other cell types.

Virulent brucellae have the capacity to survive in both polymorphonuclear and mononuclear phagocytes. In both types, the organisms release soluble factors distinct from LPS that inhibit phagolysosome fusion (Frenchick et al. 1985). They also inactivate the myeloperoxidase–hydrogen peroxide–halide systems by releasing adenine and guanosine monophosphate which prevent degranulation (Bertram et al. 1986; Canning et al. 1986). These, or similar factors, are probably responsible for inhibiting the oxidative burst (Kreutzer et al. 1979). Purines are also implicated in the suppression of production of TNF-α by monocytes (Caron et al. 1994). This is a major factor in determining the capacity of virulent brucellae to survive in monocytes.

It is evident that the O chain of smooth-type LPS plays a role in intracellular survival as smooth strains generally survive much more effectively than rough variants (Kreutzer and Robertson 1979). However, its precise role is unclear. Other factors that contribute to intracellular survival by protecting the bacteria from hydrogen peroxide and superoxide include catalase (Fitzgeorge et al. 1965) and copper–zinc superoxide dismutase. Strains deficient in the latter produced ten-fold lower splenic counts in experimental infections in mice (Tatum et al. 1992).

Macrophage activation plays a key role in determining the outcome of brucella infection and interferon-γ (IFN-γ) is essential for this process (Cheers and Pagram 1979; Stevens et al. 1992; Zhan and Cheers 1993). However, activation alone is not effective in eliminating the infection (Jones and Winter 1992). In contrast to some other bacteria, in which iron depletion inhibited in vivo growth, iron-saturated transferrin is reported to promote the elimination of brucellae from infected macrophages by catalyzing the formation of hydroxyl radical (Jiang and Baldwin 1993).

After phagocytosis, brucellae probably multiply in the lymph nodes draining the site of entry. Subsequently, as the bacteria are released from the dying cells that they have parasitized, they enter the blood and produce the bacteremia that normally accompanies the acute febrile phase of the disease. Bacteremia varies in degree with the severity of the disease but is likely to be sustained during the early phase of the acute disease, becoming intermittent as this passes. From the blood, the organisms are distributed throughout the reticuloendothelial system and may be present in large numbers in the liver and spleen. They may also localize in many other sites including the joints, heart, kidneys, central nervous system (CNS), and genital tract. The nature of the lesions produced depends to some extent upon the type of infecting organism. Thus, *B. abortus* strains more typically produce a granulomatous reaction (Young 1979). *B. suis* strains are often associated with chronic suppurative lesions, especially in the joints and spleen (Spink 1956).

Antibodies to the brucella LPS develop within a few days of the onset of the acute phase or, in subacute cases, may precede the onset of symptoms. Cell-mediated responses to brucella antigens may develop in parallel with or independently of the antibody response. Some patients remain anergic throughout the disease, whereas others develop cell-mediated responses but do not produce antibodies; both antibodies and cell-mediated immune responses develop in most patients. The cell-mediated response, particularly the production of activated mononuclear phagocytes, is probably largely instrumental in promoting recovery from the disease (Serre et al. 1987; Baldwin and Winter 1994).

The mechanisms whereby the clinical effects of the disease are produced, particularly the fever, asthenia, and depression of the CNS, are not clearly understood. Endotoxin and hypersensitivity to brucella antigens have been invoked to explain them; similar effects have been produced by partly purified fractions containing these antigens (Abernathy and Spink 1958). Such preparations are capable of releasing cytokines, including interferons and TNF-α (cachectin) from mononuclear cells (Berman and Kurtz 1987). The physiological effects of these mediators are remarkably similar to many of the clinical manifestations of brucellosis.

Clinical manifestations

Acute or subacute disease follows an incubation period which can vary from 1 week to 6 or more months. In most patients for whom the time of exposure can be identified, the incubation period is between 2 and 6 weeks. The length of the incubation period may be influenced by many factors, including the virulence of the infecting strain, the size of the inoculum, the route of infection, and the resistance of the host. *B. melitensis* infections are likely to be manifest as acute disease of sudden onset; infections caused by the other species are often subacute, with an insidious onset and prolonged and indeterminate incubation period. Acute brucellosis is characterized by an intermittent fever with a maximum temperature that usually lies within the range 38–41°C but is sometimes substantially higher; hyperpyrexia, which occurs in some cases, is a significant cause of death. Typically, the body temperature is often near normal during the early part of the day but rises sharply during the late afternoon or evening; this is accompanied by chills, shivering, malaise, nausea, and extreme fatigue. After reaching its peak, the temperature shows a rapid fall, accompanied by profuse sweating. The temperature rises may follow a regular pattern, especially early in the disease, but increasingly long intervals between them may occur later. The

characteristic undulant fever is most likely to be seen in untreated cases in which the disease has persisted for some time. For a description of the clinical manifestations of undulant fever, see Dalrymple-Champneys (1960) and Hughes (1897).

The febrile phase is associated with anorexia, weakness, severe fatigue, and loss of weight which may be rapid and substantial. Headache is a frequent symptom as are muscular and joint pains. Objective physical signs other than fever are few in uncomplicated cases. About two-thirds of patients with *B. melitensis* infection have enlargement of the liver, spleen, and superficial lymph nodes, sometimes accompanied by transient jaundice, whereas only about one-third of patients with *B. abortus* or *B. suis* infection have splenomegaly, and fewer have perceptible enlargement of the liver. A cough sometimes accompanies the acute disease and gastrointestinal disturbance is not uncommon in brucellosis resulting from ingestion of infected foods or milk. Most patients with acute brucellosis will recover from the physical effects of the disease in 1–3 months, but subjective symptoms may persist for much longer. The untreated disease is, however, associated with significant mortality. In *B. melitensis* infections, the case-fatality rate has been estimated as between 2 and 5 percent, but in infections caused by other species it is substantially lower. Death may result from hyperpyrexia, severe toxemia, endocarditis, meningoencephalitis, or other serious complications. In some patients, especially those untreated, the acute or subacute disease may persist or recur for 6 months or more, the disease then being regarded as 'chronic.' Complications, which may develop during the acute or chronic stages, most frequently affect the bones and joints, cardiovascular system, CNS, genitourinary tract, and reticuloendothelial system (Spink 1956; Dalrymple-Champneys 1960; Young 1983; Young and Corbel 1989; Madkour 1989).

About 10 percent of patients develop bone or joint complications, usually spondylitis or arthritis of the hip, knee, ankle, or sacroiliac joints. Paravertebral abscess is fairly frequently associated with spondylitis; osteomyelitis of the cranium, ribs, or limb bones has also been reported. Reactive arthritis may follow acute or chronic brucellosis; in patients of European origin, an association with HLA-B27 antigen has been observed (Hodinka et al. 1978), but no such association has been found in patients of ethnic groups in which this antigen is rare (Alarcon et al. 1981).

Nearly all patients with brucellosis experience nonspecific symptoms referable to the CNS, including headache, irritability, loss of concentration, insomnia, anxiety, and depression. In some 3–5 percent of patients with *B. melitensis* infection, but fewer with infection caused by other types, neurobrucellosis may develop as a result of invasion of the CNS. Meningitis and meningoencephalitis are the usual manifestations of this, but polyradiculoneuritis and meningomyelitis may also occur (Bashir et al. 1985; Shakir 1986). Neurobrucellosis requires prompt and vigorous treatment with tetracycline, rifampicin, and streptomycin if death or permanent disability is to be avoided (Shakir et al. 1987; Al-Deeb and Madkour 1989).

Thrombophlebitis and endocarditis are the most frequent cardiovascular complications of brucellosis. Endocarditis causes a high proportion of the fatalities in brucellosis, especially that caused by *B. abortus* and *B. suis*, and requires vigorous and sustained antibiotic treatment and cardiac surgery.

Epididymo-orchitis, which is the most common genitourinary complication in the male, may be acute or chronic. Brucellosis in pregnancy may result in abortion; the bacteria have been isolated from the placenta and fetal membranes (Poole et al. 1972; Young 1983). There is little indication, however, that *Brucella* spp. have a specific tropism for the human placenta, such as that seen in ungulate species. Many pregnant patients with acute brucellosis have proceeded to term even without antibiotic treatment (Williams 1973).

Other complications include a diffuse or focal hepatitis, which may be accompanied by acute inflammatory changes and parenchymal degeneration, or may be granulomatous. The former are said to be frequent in *B. melitensis* infection whereas *B. abortus* and *B. suis* are more commonly associated with granuloma formation (Young 1995). Hepatic and splenic abscesses may also develop, especially in patients with *B. suis* infection.

There is some confusion over the nature of chronic brucellosis. This term should be reserved for disease that has persisted for 6 months or longer, with or without complications, but with objective evidence of brucella infection as the cause. In practice, such evidence may be difficult to obtain. Blood culture is usually unsuccessful in the chronic phase. Culture of bone marrow or material removed surgically from localized lesions may be more successful. Serological tests are frequently unhelpful, but radioimmunoassay and enzyme immunoassay are reported to be useful (Parratt et al. 1977; Magee 1980; Araj et al. 1986a, b). The use of PCR may offer a means of detecting infection in some of these cases (Fekete et al. 1990; Matar et al. 1996). However, the selection of appropriate primers is crucial and this procedure is not without its pitfalls. It has yet to be fully evaluated in chronic brucellosis. Illness associated with nonspecific symptoms but no physical signs, and a history of occupational or other exposure to *Brucella*, is often diagnosed as chronic brucellosis. Not surprisingly, it usually responds poorly to specific treatment.

Diagnosis

The variable symptoms, the paucity of distinctive physical signs, and the occurrence of subclinical and atypical infections, make the clinical diagnosis of brucellosis in humans particularly difficult. Bacteriological and serological examination are usually essential for confirmation of

the diagnosis, but even these tend to give unsatisfactory results in the chronic phase of the disease. Brucellosis should always be considered as a possible cause of pyrexia of undetermined origin, and of any acute or chronic inflammatory disease that presents diagnostic difficulty. A history of occupational or environmental exposure to possible sources of infection may assist diagnosis. No specific biochemical changes are associated with the disease. Mild leukopenia with a relative lymphocytosis, sometimes accompanied by a secondary anemia and thrombocytopenia, is a common finding in acute or subacute brucellosis. The erythrocyte sedimentation rate is usually in the normal range except in severe cases.

CULTURE

Isolation of a *Brucella* sp. is the most certain means of reaching a diagnosis. It is most likely to be successful during the acute phase of infection caused by *B. melitensis* or *B. suis*, but is less successful with *B. abortus* infections. Samples of any body fluid, or of tissue collected surgically or at necropsy, may be cultured. Blood is the material most commonly examined, but bone marrow may be more likely to give positive results (Gotuzzo et al. 1986). Blood cultures are best performed on samples collected during the pyrexial phase, preferably while the temperature is rising. The isolation rate is improved if several samples are taken over a period of 24 h. At least 3–5 ml should be inoculated into 20–100 ml of serum dextrose broth, tryptone soya broth, or trypticase–soy broth and incubated at 37°C in air enriched with CO_2 5–10 percent. Larger volumes of blood have been cultured successfully in automated blood culture systems. If desired, a replicate sample may be incubated in air or anaerobically. If an anticoagulant is needed during transport of the specimen, trisodium citrate or preservative-free heparin should be used. Blood clot may also be cultured but should be homogenized first. There is some evidence that buffy coat samples are more likely than whole blood to give positive results.

Selective media are not necessary for the culture of human blood samples taken with aseptic precautions but should be used if samples of excreta or contaminated tissues are to be examined. The liquid media recommended above can be made selective by the addition of an antibiotic mixture containing: amphotericin B 1 mg/l, bacitracin 25 mg/l, cycloheximide 100 mg/l, D-cycloserine 100 mg/l, nalidixic acid 5 mg/l, polymyxin B 6 mg/l, and vancomycin 20 mg/l. This antibiotic mixture is suitable for most strains of *B. abortus*, *B. melitensis*, and *B. suis*. It may be inhibitory, however, for strains of *B. abortus* biogroups 2, 3, and 4 and some strains of *B. melitensis*, *B. canis*, and *B. ovis*. For these organisms, media may be made selective by adding an antibiotic mixture of vancomycin 3 mg/l, sodium colistimethate 7.5 mg/l, nitrofurantoin 10 mg/l, and nystatin 12 500 units (Brown et al. 1971).

When liquid media are used for enrichment or primary isolation, subcultures should be made every 3–5 days on to a suitable solid medium such as heated blood agar or serum dextrose agar, which is then incubated at 37°C in air enriched with CO_2 5–10 percent. The need for frequent subculture can be avoided by use of a two-phase system (Castañeda 1947) in which both solid and liquid media are contained within the same bottle. Even with this system, subcultures to fresh solid medium should be made immediately when growth appears, to minimize the dissociation of smooth cultures to the nonsmooth colonial phase. This procedure is capable of achieving isolation rates of up to 85 percent from patients with acute *B. melitensis* infection. This rate may be improved if the blood, bone marrow, or other tissue suspensions are treated by the lysis-concentration method of Finegold et al. (1969) combined with centrifugation rather than filtration (Kolman et al. 1991). Because many patients may have received prior treatment with inappropriate antibiotics that inhibit but do not kill brucellae, all cultures should be incubated for at least 6 weeks, with frequent subculture, before they are discarded as negative. Rapid growth detection methods such as the Bactec or BACT/ALERT systems may also increase the sensitivity of isolation when combined with the lysis-concentration method (Kolman et al. 1991; Solomon and Jackson 1992), but samples should not be discarded as negative without subculture.

The PCR using a 223 bp region of the 31 kDa surface protein of *B. abortus* has given promising results in the diagnosis of both acute and chronic relapsing brucellosis (Matar et al. 1996). The procedure merits further evaluation, particularly for assessment of patients who have received antibiotics or have suspected chronic brucellosis.

SEROLOGICAL TESTS

Most patients with acute brucellosis produce antibodies of the IgM isotype within a few days of onset of the disease. These antibodies are rapidly followed and superseded by IgG, and to a lesser extent IgA, antibodies. Maximum titers are reached in the third or fourth week of disease and then slowly decline; however, antibodies usually persist throughout the active phase of the disease and in some cases for long after. In subacute or chronic brucellosis this pattern is generally not seen, the serological response consisting of a sustained production of IgG and sometimes IgA antibodies (Araj et al. 1986a; Serre et al. 1987; Ariza et al. 1992). IgE antibodies to *Brucella* spp. have also been observed in some brucellosis patients, but their clinical significance is uncertain (Escande and Serre 1982). These patterns are of diagnostic relevance because antibodies of the IgA, IgG, and IgM isotypes vary considerably in their activity in different serological tests.

Numerous serological procedures have been tried in the diagnosis of human brucellosis but few have

achieved lasting application. Until recently, the most widely used was the standard tube-agglutination test (SAT). In many laboratories this has now been superseded by the rose bengal plate test and enzyme-linked immunosorbent assay (ELISA). Other useful tests include the 2-mercaptoethanol agglutination test, the complement fixation test, and the Coombs antiglobulin test and radioimmunoassay (Buchanan et al. 1974b; Diaz et al. 1976; Magee 1980; Araj et al. 1986a; Report 1986b; Diaz and Moriyon 1989). The SAT should be performed with a standardized suspension of heat-killed smooth brucella cells in saline containing phenol 0.5 percent. Incubation may be at 37 or 50°C for 18 h. There is some advantage in performing parallel tests with suspensions of smooth biogroup 1 strains of *B. abortus* and *B. melitensis*, but these must be properly standardized. The use of both detects infection caused by M antigen-dominant strains more readily than *B. abortus* antigen alone. It does not, as commonly supposed, identify the infecting strain, because both *B. abortus* and *B. melitensis* biogroups share similar antigenic structures. In many countries the agglutinating suspensions are standardized against the international standard for *Brucella abortus* antiserum, a practice to be recommended as it permits comparison of tests performed by different laboratories and on different occasions within a single laboratory. Microagglutination procedures are also available (Baum et al. 1995). If infection by *B. canis* or another nonsmooth brucella is suspected, a stable suspension of the appropriate type must be used. Recommendations have been made for the preparation of such suspensions (Report 1986b).

Most patients with acute brucellosis develop agglutinin titers of 640 or more by the end of the third or fourth week of illness. These tend to decline fairly rapidly once the acute phase is over, and may become low within 3 months. Rising titers, or titers that decline after appropriate antibiotic treatment, indicate recent active infection.

The interpretation of stable titers in the low or intermediate range (20–160) is difficult. In symptomless patients they probably indicate past or latent infection. In patients with symptoms suggestive of brucellosis they may indicate active infection, especially if there is no history of occupational or other previous exposure to infection. In patients with a history of prolonged exposure, titers of this magnitude are common even in the absence of disease and reliance cannot be placed upon agglutination tests alone. In such patients, low agglutinin titers should be investigated by testing with additional procedures such as ELISA and Western blotting. PCR may also be useful in such cases but has received limited evaluation (Matar et al. 1996). The absence of agglutinins does not exclude brucellosis, as many cases have been recorded in which a positive blood culture was obtained despite a negative agglutination reaction (Gilbert and Dacey 1932; Heathman 1934).

Prozones sometimes occur in the agglutination test. They have been attributed to antibody excess (Serre et al. 1970) or to blocking of agglutinins by non-agglutinating IgG or IgA isotypes (Heremans et al. 1963; Wilkinson 1966). This emphasizes the need to use more than one serological test for diagnosis. Of the various rapid slide or plate agglutination tests for brucella antibodies, the most effective is the rose bengal plate or card test. This uses, as antigen, a dense suspension of smooth brucella cells stained with rose bengal and suspended in an acid buffer. The test discriminates against agglutinins of low avidity and is not subject to prozones. It compares favorably with the SAT in specificity and for detecting antibodies in human sera (Buchanan et al. 1974b; Diaz et al. 1976; Cernyševa et al. 1977) and is useful for detecting antibodies in cerebrospinal fluid in cases of neurobrucellosis (Diaz et al. 1978).

Buchanan et al. (1974b) concluded that the complement fixation and 2-mercaptoethanol tests were the most accurate indicators of active disease. On the grounds of technical convenience the latter was preferred; it has also been used to assess the response to treatment (Buchanan and Faber 1980). The Coombs antiglobulin test and the complement fixation test tend to become positive later in the course of the disease than the agglutination test and are likely to be still reactive in the chronic phase (Kerr et al. 1967, 1968; Robertson et al. 1980). Unfortunately, the titers observed at this stage are often no greater than those of symptomless patients with a history of either occupational exposure to *Brucella* or of acute infection with recovery. For this reason, Farrell et al. (1975) concluded that the diagnosis of chronic brucellosis had to be based on clinical symptoms. Because these are largely subjective, however, it is desirable to obtain objective evidence of infection, and some progress has been made through the use of enzyme-labeled antibody assays.

Magee (1980) concluded that enzyme-labeled antibody assays were as sensitive as radioimmunoassay and could as easily be made specific for immunoglobulin isotypes. He identified four classes of sera:

1. those from acute cases with very high concentrations of IgG antibody and moderate ones of IgM and IgA
2. those from chronic cases having high concentrations of IgG but not IgM or IgA antibodies
3. those with low residual titers of IgG, probably representing past infection, and
4. those with little or no brucella-specific antibody.

These results are broadly consistent with those of Lindberg and colleagues (1982) who found that patients in the acute stage had high IgM and rising titers of IgG antibodies to *B. abortus* LPS antigen, whereas those in the chronic stage had raised titers of IgG antibody alone. Araj et al. (1986a) also concluded that enzyme immunoassay was an effective method for diagnosing acute and chronic brucellosis; and it was useful for

detecting antibodies in the cerebrospinal fluid of patients with neurological complications (Araj et al. 1986b).

Immunoblotting against extracts of *Brucella* cells or purified protein antigens has been reported to differentiate active disease from past exposure to infection (Goldbaum et al. 1993).

All serological tests for brucellosis that are based on detection of antibodies to the LPS antigen of smooth strains are subject to interference from antibodies produced in response to bacteria with structurally related antigens. These include *E. coli* O:157 (Stuart and Corbel 1982), *Francisella tularensis* (Francis and Evans 1926), *Stenotrophomonas maltophilia* (Corbel et al. 1984a), *Salmonella* serotypes of Kauffmann–White group N (Corbel 1975), *Vibrio cholerae* (Wong and Chow 1937; Feeley 1969), and *Yersinia enterocolitica* O:9 (Ahvonen et al. 1969). With the exception of *F. tularensis*, in which the crossreacting antigen has not been fully characterized, these crossreactions result from antibodies evoked by epitopes containing 4-amino-4,6-dideoxymannose (perosamine) (Redmond 1979; Bundle et al. 1987). That produced in response to *Y. enterocolitica* O:9 is the most complete as its polysaccharide O chain is almost identical with that of strains of *Brucella* in which the A antigen dominates (Caroff et al. 1984a, b). Antibodies produced in response to *Y. enterocolitica* O:9 react in agglutination, antiglobulin, immunofluorescence, complement fixation, and immunoprecipitation tests with brucella antigens (Corbel and Cullen 1970). Various attempts have been made to devise tests that distinguish brucellosis and yersiniosis, but none is entirely satisfactory (Corbel and Cullen 1970; Diaz and Dorronsoro 1971; Mittal and Tizard 1981; Corbel et al. 1984b). The crossreactions produced by other bacteria are usually less troublesome than those produced by *Y. enterocolitica*, but diagnostic problems have been caused by *E. coli* O:157 (Notenboom et al. 1987), *F. tularensis* (Foshay 1950; Behan and Klein 1982), and by vaccination with *V. cholerae* (Eisele et al. 1946). For further discussion, see Corbel (1985).

TESTS FOR CELL-MEDIATED IMMUNITY

Cell-mediated immunity is believed to play a major role in recovery from infection with *Brucella*. Most patients show evidence of a cell-mediated immune response to brucella antigens as the disease progresses from the acute phase to either recovery or the chronic disease (Serre 1989). There is usually a delayed-hypersensitivity type reaction to the intradermal injection of 0.05–0.1 ml of a suitable preparation of antigen. Such a reaction usually manifests itself within 6–24 h as a slightly raised, sometimes tender, erythematous plaque 2–6 cm or more in diameter. Occasionally, much more severe local reactions accompanied by slight fever and malaise develop. False-positive reactions generally appear rapidly and disappear within 24 h. Delayed reactions of the type sometimes encountered in tuberculosis have been reported; in these, an inflammatory response develops in recently infected patients at the site of an intradermal test performed weeks previously.

Numerous antigen preparations, referred to collectively as 'brucellins,' by analogy with tuberculin, have been employed in such tests. They range from culture supernates such as 'abortin' (M'Fadyean and Stockman 1909) or 'melitin' (Burnet 1922) to extracts prepared by physical disruption of cells or by chemical fractionation, including acid or alkaline hydrolysis (Alton and Jones 1967). Such preparations are poorly defined, of variable composition, and often contain LPS antigen which stimulates an antibody response in the recipient and interferes with the interpretation of subsequent serological tests. No reference preparations exist and there are no agreed standard doses; consequently, the interpretation of results is difficult. Because of these problems there have been moves towards the development of a defined nonantigenic preparation based on the extraction procedure of Bhonghibhat et al. (1970). Such a preparation ('Brucellin INRA') has been widely used in France in a screening test for animal brucellosis (Report 1986b).

In general, the intradermal test with brucellin is analogous to the tuberculin test in that it demonstrates previous exposure to infection without clearly indicating its significance. Some patients with high antibody titers react weakly or negatively to brucellin, whereas those without antibodies sometimes react strongly. Provided that an LPS-free preparation is available, the test may be of value in distinguishing between brucellosis and infections caused by bacteria that share antigenic determinants with the brucella O chain. However, it is not recommended as a routine procedure for the diagnosis of active brucellosis.

In vitro tests, based on cell-mediated immunity, for the diagnosis of brucellosis in humans, include the macrophage migration-inhibition test, the leukocyte-lysis test, and the lymphocyte-stimulation test (Peraldi et al. 1976; Report 1986b). All require freshly collected cells, are difficult to standardize, and have yet to be evaluated with well-defined antigenic preparations. They cannot be recommended for diagnostic purposes.

Prophylaxis

The ideal method of prevention, namely the avoidance of direct or indirect contact with infected animals or their products, is usually unattainable for those at risk through occupational exposure. The hazards can be reduced, however, by working practices that minimize the risk of skin or mucosal contamination or the inhalation of infectious aerosols. Adequate protective clothing, including impermeable gloves, should be worn by people handling animals that have recently aborted or given birth; particular care should be taken when disposing of placental and fetal

material. The latter should be incinerated or buried in quicklime and any part of the premises contaminated by discharges should be thoroughly decontaminated with an approved agricultural disinfectant used at the recommended concentration. Specific recommendations have been made for hygienic measures to be adopted by those concerned with the husbandry and slaughter of animals (Report 1986b), and by laboratory workers (Alton et al. 1975; Corbel et al. 1979b; Report 1986b).

Most cases of nonoccupational brucellosis, which result from the consumption of dairy or other animal products, could be eliminated by adequate heat treatment (pasteurization, boiling, or sterilization) of milk, and the thorough cooking of meat products.

No satisfactory vaccine exists for the prevention of human brucellosis. Two attenuated vaccines used in animals, *B. abortus* strain 19 and *B. melitensis* Rev 1, retain virulence for humans and are therefore unacceptable. *B. abortus* strain 19-BA, a more attenuated variant of strain 19, has been used in the former USSR and China to vaccinate populations at high risk of infection from *B. melitensis* (Vershilova 1961). The vaccine is given by the intradermal route, usually by scarification, and is said to produce severe reactions only rarely. The immunity produced is, however, of short duration and where there is a high risk of infection, annual booster doses are required. Repeated doses of these vaccines are apparently associated with an increasing risk of hypersensitivity reactions. Other attenuated strains of *B. abortus* or *B. melitensis* have been used in China, but precise information on their safety and efficacy is lacking. Attempts have been made to produce vaccines from purified fractions of *Brucella*. A peptidoglycan fraction (Lopez-Merino et al. 1976), used in France (Bentejac et al. 1984), is apparently intended to give a minimal degree of protection which will be reinforced by subclinical infection. Its protective value is probably limited, and cases of acute brucellosis have occurred in recipients. Epidemiological evidence and the histories of cases of laboratory-acquired infection indicate that even attacks of clinical brucellosis give only incomplete protection against reinfection, particularly by strains of high virulence. This suggests that a safe and effective vaccine against human brucellosis will be difficult to produce.

Treatment

Brucella strains are susceptible to a wide range of antibiotics in vitro, but few have proved effective in the treatment of brucellosis. Good intracellular penetration is essential for in vivo activity against *Brucella* and thus there is limited correlation between in vitro performance and therapeutic efficacy. β-Lactam antibiotics show limited activity against *Brucella* (Hall and Manion 1970). Some strains are inhibited by benzylpenicillin, ampicillin, and amoxycillin. Most strains are resistant to methicillin, nafcillin, ticarcillin, and piperacillin. Similarly, first- and second-generation cephalosporins show limited activity against *Brucella*, but some third generation cephalosporins such as cefotaxime, ceftizoxime, and ceftriaxone have minimum inhibitory concentrations (MIC) in the range 0.25–2 mg/l (Palenque et al. 1986). The MIC for chloramphenicol is in the range 2–3 mg/l for most strains. Sensitivity to macrolides is variable. With the exception of *B. abortus* biogroup 2 and *B. ovis*, most strains are resistant to erythromycin, with $MIC_{90} \geqslant 16$ mg/l. Sensitivity to dirithromycin and roxithromycin is similar but clarithromycin and azithromycin show 2–8-fold greater activity (Garcia-Rodriguez et al. 1993).

Most *Brucella* strains are inhibited by streptomycin, gentamicin, kanamycin, tobramycin, and amikacin at concentrations of 1–4 mg/l (Mortenson et al. 1986). Streptomycin augments the activity of tetracycline in infected cell cultures and this is borne out by therapeutic experience (Richardson and Holt 1962; Colmenero et al. 1989). Sensitivity to tetracyclines is universal, with MICs in the region of 0.1 mg/l (Hall and Manion 1970). *Brucella* strains are also highly sensitive to rifamycins; the MICs for rifampicin are in the range 0.1–2 mg/l with MICs at approximately four times this concentration (Hall and Manion 1970; Corbel 1976a). Single-step resistance develops rapidly in vitro (Corbel 1976a) and has also been observed during the course of therapy (Rautlin de la Roy et al. 1986). Rifapentine has similar activity to rifampicin but has superior pharmacokinetic properties (Garcia-Rodriguez et al. 1993).

Brucella strains are generally resistant to nalidixic acid but show in vitro sensitivity to the fluoroquinolones. The MICs for ciprofloxacin are in the range 0.5–1 mg/l, but therapeutic results have been disappointing (Bosch et al. 1986). Sensitivity to co-trimoxazole is borderline with the MIC_{90} being just within the breakpoint (Garcia-Rodriguez et al. 1993). This is consistent with the high relapse rate observed with this drug.

Of agents subjected to clinical evaluation, the tetracyclines have emerged as the most effective group. In the past, the recommended treatment for all forms of brucellosis in adults was tetracycline given orally to a total daily dose of 1–2 g for a minimum of 6 weeks. In severe infections, or in those accompanied by localized lesions, this was supplemented by streptomycin in intramuscular doses of 1 g daily for the first 2–3 weeks. Rifampicin has been shown to exert strong inhibitory and bactericidal activity against *Brucella* strains in vitro (Corbel 1976a) and was effective in both experimental infections (Philippon et al. 1977) and clinical studies (Bertrand et al. 1979). It has proved useful against brucellosis in young children (Llorens-Terol and Busquets 1980) and is the antibiotic of choice for brucellosis in pregnancy.

The regimen recommended by the World Health Organization consists of rifampicin 600–900 mg and doxycycline 200 mg, both taken as a single daily dose for

a minimum of 6 weeks (Report 1986b). The superiority of this over tetracycline in combination with streptomycin has been questioned (Ariza et al. 1985). Strains of *Brucella* resistant to rifampicin have been reported to emerge during treatment (Rautlin de la Roy et al. 1986). Mousa and colleagues (1988) concluded that a combination of tetracycline, streptomycin, and rifampicin gave the best results for severe infections. Later studies suggest that combinations of streptomycin and tetracycline, rifampicin and doxycycline, or rifampicin and ofloxacin give comparable results provided that treatment is sufficiently prolonged (Ariza et al. 1985; Akova et al. 1993).

Alternatives to these regimens include co-trimoxazole 2–3 g daily for at least 6 weeks. However, relapses after this treatment are common and it is not recommended except when rifampicin and tetracyclines cannot be used. Moxalactam together with chloramphenicol has been used successfully in the treatment of human infection caused by *B. canis* (Tosi and Nelson 1982) and, together with rifampicin and gentamicin, for brucella endocarditis. Gentamicin alone has been recommended as an alternative treatment for uncomplicated brucellosis (Wundt 1982).

For the treatment of brucellosis with complications, modifications of the standard regimens have been used. A combination of rifampicin 600–900 mg daily and tetracycline 2 g daily orally for 8–12 weeks, supplemented with intramuscular streptomycin 1 g per day for the first 6 weeks, has been recommended for patients with meningoencephalitis (Shakir 1986). Similar schedules have been used for brucella endocarditis, usually in conjunction with surgical treatment of the heart valves.

The response of chronic brucellosis to treatment is much less satisfactory than that of the acute disease. This is attributable in part to the vague symptoms, difficulty in diagnosis, and irreversible changes in musculoskeletal tissues. Repeated courses of antibiotic therapy may alleviate symptoms in some cases, but in the majority they do not. Immunostimulant therapy with levamisole has been claimed to benefit such patients (Thornes 1977), but objective evidence from properly controlled trials is lacking. Treatment with immunosuppressive agents has also been tried (Thornes et al. 1982), but is difficult to justify in a nonlethal disease; the administration of anti-inflammatory agents may produce symptomatic relief in some patients although the effects are likely to be temporary. Antigen therapy is potentially hazardous and is not now recommended (Report 1986b).

SUMMARIZED DESCRIPTIONS OF SPECIES

Brucella abortus

The organisms are gram-negative coccobacilli or short rods, 0.8–1.5 μm long by 0.6–0.8 μm wide. The species is catalase and oxidase positive and usually requires supplementary carbon dioxide for growth, especially on primary isolation. It usually produces hydrogen sulfide from sulfur-containing amino acids or proteins. Most isolates hydrolyze urea but some may not. They generally grow in the presence of basic fuchsin, methyl violet, pyronin, and safranin O but not thionin, at standard concentrations. They reduce nitrate to nitrite and may also reduce nitrite. Isolates are normally smooth on primary isolation. Smooth strains may have A or M surface antigens reactive in tests with monospecific antisera, depending upon biogroup. They oxidize L-alanine, D-alanine, L-asparagine, L-glutamic acid, D-galactose, D-glucose, D-ribose, and isoerythritol but do not oxidize D-xylose, L-arginine, DL-citrulline, DL-ornithine, or L-lysine. Cultures in the smooth or smooth-intermediate phase are lyzed by Brucella phages of groups 1, 2, 3, 4, and 6; nonsmooth strains are lyzed by phages of group 5 at RTD and 10^4 RTD. Nonsmooth cultures are lyzed by Brucella-phage R at RTD. The species is usually pathogenic for cattle, causing abortion, but may also infect other species including sheep, goats, dogs, horses, and humans. Guinea-pigs, rabbits, and mice are susceptible to experimental infection. Seven biogroups are recognized: former biogroups 7 and 8 are no longer considered valid. See Table 5 for reference strains.

Brucella melitensis

The organisms are gram-negative cocci or coccobacilli, rarely rods; 0.6–1.2 μm long by 0.5–0.7 μm wide. They are catalase and oxidase positive, and do not require supplementary carbon dioxide for growth. They do not produce hydrogen sulfide, or no more than a trace, when grown on recommended media. They usually hydrolyze urea but some strains may not. They usually grow in the presence of basic fuchsin, thionin, methyl violet, pyronin, and thionine blue at the standard concentrations. They reduce nitrate to nitrite and may also reduce nitrite. Smooth cultures may have the A, M, or both A and M surface antigens reactive in tests with monospecific sera. They oxidize D-glucose, isoerythritol, L-alanine, D-alanine, L-asparagine, and L-glutamic acid, but do not oxidize L-arabinose, D-galactose, D-ribose, D-xylose, L-arginine, DL-citrulline, DL-ornithine, or L-lysine. Cultures in the smooth phase are usually susceptible to lysis by phages of groups 4 and 6 at RTD and 10^4 RTD. The species is usually pathogenic for sheep and goats but may also infect other species, including humans. Guinea-pigs, rabbits, and mice are susceptible to experimental infection. See Table 5 for reference strains.

Brucella suis

The organisms are gram-negative coccobacilli or rods, 0.8–1.5 μm long by 0.6–0.8 μm wide. They are catalase and

Table 5 Brucella *reference strains*

Species	Biogroup	Strain	NCTC no.	ATCC no.
B. abortus	1	544	10093	23448
	2	86/8/59	10501	23449
	3	Tulya	10502	23450
	4	292	10503	23451
	5	B3196	10504	23452
	6	870	10505	23453
	9	C68	10507	23455
B. melitensis	1	16M	10094	23456
	2	63/9	10508	23457
	3	Ether	10509	23458
B. suis	1	1330	10316	23444
	2	Thomsen	10510	23445
	3	686	10511	23446
	4	40	11364	23447
	5	513	11996	
B. neotomae		5K33	10084	23459
B. ovis		63/290	10512	25840
B. canis		RM6/66	10854	23365

oxidase positive, and supplementary carbon dioxide is not required for growth. They produce large amounts of hydrogen sulfide (biogroup 1), or none at all (other biogroups), and hydrolyze urea rapidly. They usually grow in the presence of thionin but most strains are inhibited by basic fuchsin, methyl violet, pyronin, and safranin O at standard concentrations. Isolates are normally in the smooth phase on primary isolation. In smooth cultures, the A surface antigen is predominant except for cultures of biogroup 4 which react equally with antisera monospecific for the A and M surface antigens, and biogroup 5 which reacts only with M monospecific serum. They usually oxidize D-ribose, D-glucose, isoerythritol, D-xylose, L-arginine, DL-citrulline, DL-ornithine, and L-lysine. Oxidation of L-asparagine, L-glutamic acid, L-arabinose, and D-galactose varies with the biogroups, and they do not oxidize L-alanine or D-alanine. Smooth or smooth-intermediate cultures are lyzed by *Brucella* phage of groups 3, 4, and 6 at RTD and 10^4 RTD. Phages of group 2 produce partial lysis at RTD; those of group 1 do not produce lysis at RTD, but are lytic at 10^4 RTD. The species is usually pathogenic for pigs, except biogroups 4 and 5 which are usually pathogenic for reindeer and rodents, respectively. Biogroup 2 also naturally infects hares. Many other species are susceptible to the other biogroups, including humans. Guinea pigs, rabbits, and mice are susceptible to infection but the severity varies with the biogroup. See Table 5 for reference strains.

Brucella neotomae

The organisms are gram-negative coccobacilli 0.6–1.4 μg long by 0.6 μm wide. They are catalase positive and oxidase negative and do not require supplementary carbon dioxide for growth. They usually produce hydrogen sulfide profusely and hydrolyze urea rapidly. They do not grow in the presence of basic fuchsin even at 7.5 mg/l, nor in the presence of safranin O at 100 mg/l or thionine blue at 2 mg/l, but will grow in the presence of thionin at 7.5 mg/l. They reduce nitrate to nitrite. Cultures are usually smooth on primary isolation. In smooth cultures, the A surface antigen is predominant. They oxidize L-asparagine, L-glutamic acid, L-arabinose, D-galactose, D-glucose, isoerythritol, D-xylose, but do not oxidize L-alanine, D-alanine, L-arginine, DL-citrulline, DL-ornithine, or L-lysine. Oxidation of D-ribose may be variable. Smooth or smooth-intermediate cultures are lyzed by *Brucella* phages of groups 2, 3, 4, and 6 at RTD. Phages of group 1 produce partial lysis with few very small plaques at RTD, complete lysis at 10^4 RTD. Rough or mucoid cultures are not lyzed by these phages at RTD or 10^4 RTD. The species is pathogenic for the desert wood rat (*Neotoma lepida* Thomas). Authenticated natural infections of other species have not been reported. Mice are more susceptible than guinea pigs to experimental infection. No biogroups are recognized. For reference strain see Table 5.

Brucella ovis

The organisms are gram-negative cocci, coccobacilli, or short rods, 0.7–1.2 μm long by 0.5–0.7 μm wide. They are catalase positive and oxidase negative. They require supplementary carbon dioxide for growth, do not produce hydrogen sulfide and hydrolyze urea weakly or not at all. They usually grow in the presence of basic fuchsin and thionin at standard concentrations. Production of nitrite from nitrate is variable. A true smooth

phase on primary isolation has not been described. Rough (R)-specific surface antigens crossreacting with other nonsmooth *Brucella* are predominant. They oxidize L-alanine, D-alanine, L-asparagine, D-asparagine, L-glutamic acid, DL-serine, and adonitol, but do not oxidize L-arabinose, D-galactose, D-glucose, D-ribose, isoerythritol, D-xylose, L-arginine, DL-citrulline, DL-ornithine, or L-lysine. The cultures are not lyzed by *Brucella* phages of groups 1, 2, 3, 4, and 6 at RTD or 10^4 RTD but are lyzed by pages R/O and R/C of group 5 at RTD. The species is pathogenic for sheep, causing epididymitis in rams and sometimes abortion in ewes; it produces low-grade splenic infection in guinea pigs and mice. No biogroups are recognized. See Table 5 for reference strain.

Brucella canis

The organisms are gram-negative coccobacilli or rods, 0.5–2 μm long by 0.5–0.6 μm wide. They are catalase positive and oxidase positive. They do not require supplementary carbon dioxide for growth, do not produce hydrogen sulfide and hydrolyze urea very rapidly. Most strains reduce nitrate to nitrite. Growth is usually inhibited by basic fuchsin but not by thionin at the standard concentrations. A smooth phase is not known; cultures are always in the rough or mucoid phase on primary isolation. Cultures form a mucoid sediment in liquid media. Rough-specific surface antigens crossreact with other nonsmooth strains of *Brucella*. They oxidize D-ribose, D-glucose, L-arginine, DL-citrulline, DL-ornithine, and L-lysine. Oxidation of isoerythritol is variable, and they do not oxidize L-alanine, D-alanine, L-asparagine, L-glutamic acid, L-arabinose, and D-galactose. They are not lyzed by *Brucella* phages of groups 1, 2, 3, 4, or 6 but are lyzed by phages of group 5 at RTD and 10^4 RTD. The species is pathogenic for dogs, causing abortion in pregnant females and epididymo-orchitis in males. It is occasionally transmitted to humans. It produces splenic infection, sometimes accompanied by 'tapioca-grain' nodules, in guinea pigs; it also produces low-grade infection in mice. No biogroups are recognized. See Table 5 for reference strain.

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Francisella

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INTRODUCTION AND HISTORY

Francisella tularensis is the etiologic agent of tularemia, an acute, febrile zoonotic disease (Bell 1981; Eigelsbach and McGann 1984; Ellis et al. 2002; Foshay 1950; Francis 1925). *F. tularensis* was first isolated and characterized in 1912 during an outbreak of a 'plague-like' disease in Tulare County, California (McCoy and Chapin 1912). During the following two decades *F. tularensis* was isolated from several locations in North America, Japan, and the Soviet Union. Microbiologists recognized the common antigenicity of the strains isolated in the different countries, but noted the differences in the virulence between the North American strains and strains isolated elsewhere. The genus designation honors an American pathologist, Edward Francis, who studied tularemia during the 1920s and 1930s, and the species name and the name of the disease derive from Tulare County.

Interest in tularemia in Western countries declined during the latter part of the twentieth century as the number of cases steadily declined. However, at the beginning of the twenty-first century, there has been a resurgence of interest in tularemia as *F. tularensis* was recognized as a potential agent of bioterrorism.

FRANCISELLA SPECIES AND STRAINS

Francisella forms separated on the basis of human clinical disease

The application of new tools for phylogenetic classifications has led to the discovery of new bacteria that belong in the genus *Francisella*, and for proposals for reorganizing the names of species and subspecies of members in the genus. However, these important molecular epidemiology studies have not altered the two major clinical categories that have long been recognized for *F. tularenis*, namely the North America-specific, highly virulent *F. tularensis* type A (or *F. tularensis* subspecies *tularensis*, or subsp. *nearctica*) and the less virulent *F. tularensis* type B (or *F. tularensis* subspecies *holarctica*). The type A biovar of *F. tularensis* causes human clinical illness that is quite distinct from the disease caused by all other *Francisella* species or subspecies. This biovar of *F. tularensis* probably has a human ID_{50} of less than ten organisms, and causes death in untreated individuals at a rate of 5–20 percent. In individuals treated with antibiotics the death rate is still more than 2 percent.

All cases of tularemia outside of North America, and an undefined percentage of cases in North America, are caused by strains of *F. tualarensis* type B. The type B biovar probably has a similar ID_{50} to the type A biovar but causes a much less severe disease. Death due to type B biovars of *F. tularensis* is rare, even in the absence of antibiotic treatment.

Within the broad grouping of the type B biovar are two recognized subspecies: *F. tularensis* subsp. *holarctica* (previously referred to as *F. tularensis* var. *palaearctica*) and *F. tularensis* subsp. *mediasiatica* (Olsufjev and Meshcheryakova 1983). The latter subspecies is found in Central Asia and may be less virulent than the *holarctica* subspecies.

F. novicida has traditionally been considered a separate *Francisella* species. However, recently, many authors have begun to refer to *F. novicida* as '*F. tularensis* subsp. *novicida*.' While this may or may not be justified from a bacterial systematics point of view, it should not confuse those concerned with clinical tularemia. Since the identification of *F. novicida* in the early 1950s, there have been only three confirmed cases of human infections, and no deaths, due to this microbe. During the same time period there have been tens of thousands of cases of tularemia caused by *F. tularensis* type A and type B. Laboratory infections are quite common with *F. tularensis* but unknown with *F. novicida*, further supporting the notion that *F. novicida* is not normally a pathogen of humans

F. philomiragia has been isolated from several human patients (Hollis et al. 1989; Sicherer et al. 1997). Most, but not all, of these infections were initiated after exposure to ocean water, and many of the patients had been immunosuppressed by treatment with corticosteroids following a near-drowning episode (Wenger et al. 1989). Other patients have had chronic granulomatous disease (Sicherer et al. 1997), rendering their polymorphonuclear leukocytes (PMN) incapable of producing normal amounts of bactericidal oxidative metabolites. However, it appears that immunocompetent individuals can also be infected with *F. philomiragia*, and it is likely that many infections in the past have gone unrecognized as resulting from this microbe.

Phylogenic relatedness of *Francisella* species

DNA hybridization studies (Hollis et al. 1989) and 16S RNA analysis (Forsman et al. 1994) have shown that all of the *F. tularensis* isolates tested as well as *F. novicida* strains are highly similar. Using the hybridization or sequence criteria *F. tularensis* and *F. novicida* appear as one species. *F. philomiragia* strains appear as close but distinct relatives of *F. tularensis* and *F. novicida*.

Members of the genus *Francisella* lie in the γ-subdivision of the *Proteobacteria* group solely with *Wolbachia persica*, an obligate intracellular bacterium frequently found in ticks. Two other endosymbiont of the ticks, *Ornithodoros moubata* and *Dermacantor andersoni* were found to be highly related to *Francisella* species (Niebylski et al. 1997; Noda et al. 1997). The endosymbiont of *Dermacantor andersoni* was found to have transovarial transmission to female progeny but no experimental transmission to rodents. This endosymbiont caused a mild infection in experimentally infected guinea pigs, and could multiply in Vero cells

Although researchers have tried to show the relatedness of *F. tularensis* to other gram-negative invasive pathogens, such as *Brucella* and *Yersinia*, using antiserum cross reactivity and DNA homology, these relationships have not been verified by 16S rRNA sequence analysis. However, 16S rRNA phylogeny studies do show that *Francisella* species are distantly related to *Legionella* and *Coxiella*.

DISTRIBUTION

The type B biotype of *F. tularensis* is found throughout Europe, Asia, and North America, and it infects a number of small animals, especially rodents. The type A strain has been found only in North America where it is thought to have a reservoir in rabbits and their ectoparasites. However, both the type A and the type B biotypes can infect a large number of animals and their true reservoir is not clearly established. *F. philomiragia* has been associated with waters along both coasts of North America but has also been isolated from patients living at a distance from any ocean. Although both *F. novicida* and *F. philomiragia* have been associated with dead muskrats (Jensen et al. 1969; Larson et al. 1955) it is likely that other animals can act as hosts or reservoirs.

MORPHOLOGY AND GROWTH

F. tularensis appears as a bipolar-staining, coccobacillus under observation by light microscopy. When viewed by electron microscopy, logarithmic phase *F. tularensis* appears as a rod of approximately 0.3×1.0 μm. Smaller cocci forms and larger pleomorphic forms can be found, especially when cells are collected from the stationary phase of growth or when infected tissue if observed. Although accumulated evidence provides strong support for the existence of a capsule, it is nevertheless very difficult to visualize the capsule either by light or electron microscopy, since the capsule is dislodged very easily from cells.

F. tularensis is an obligate aerobe that has an optimum growth temperature of 37°C. *F. tularensis* can grow on several types of rich, solid media. The addition of cysteine is required for the growth of many *F. tularensis* strains but not for the growth of *F. novicida* and *F. hilomiragia*. Commercially available cysteine heart agar (Difco) supplemented with 5 percent defribrinated blood from rabbit, sheep, or horse provides a good growth medium for all types of *Francisella*. A peptone-cysteine agar has been used to differentiate colony morphotypes of *F. tularensis* (Eigelsbach et al. 1951). Trypticase soy broth, supplemented with 0.1 percent cysteine, a modified Mueller–Hinton broth (Baker et al. 1985), or Chamberlain's defined medium (Chamberlain 1965) can be used for the liquid cultivation of *Francisella*. *F. philomiragia* may require the addition of NaCl for growth in some media.

LIPID AND CARBOHYDRATE SURFACE MOLECULES

All of the *Francisella* species have unusual fatty-acid composition that is specific to the genus, and, as such,

serves as a useful taxonomic or diagnostic tool (Jantzen et al. 1979). All strains have long chain C_{18}–C_{26} saturated and unsaturated fatty acids as well as two long-chain hydroxy fatty acids (3-OH $C_{12:0}$ and 3-OH $C_{18:0}$). Since the hydroxy fatty acids are not associated with phospholipids it is possible that they are part of the lipid A structure of the lipopolysaccharide (LPS).

The surface of *F. tularensis* is covered by a carbohydrate-rich capsule. Removal of the capsule, either through biochemical treatment or through mutation, abolishes virulence of *F. tularensis* (Hood 1977; Sandström et al. 1988). The capsule apparently serves to protect *F. tularensis* from complement-mediated killing, and may act as an antiphagocytic factor. Capsules have not been demonstrated in *F. novicida* or *F. philomiragia*, but exhaustive studies have not been carried out.

F. tularensis has a smooth form of LPS, the O-side chain of which may have the same structure as the carbohydrate portion of the capsule. The chemical structure of the O-antigen of two type B strains of *F. tularensis* has been determined, and appears to be conserved among different *F. tularensis* strains (Conlan et al. 2002; Vinogradov et al. 1991). The LPS of *F. tularensis* has very little endotoxic activity as measured both by the *Limulus* amoebocyte lysate assay and the galactosamine-sensitized mouse test (Sandström et al., 1992). Since macrophages can be stimulated for control of intracellular bacterial growth with a combination of endotoxin from typical gram-negative bacteria and interferon-γ, *F. tularensis* may have a survival benefit because of the low endotoxic properties of its LPS.

In the 1950s Eigelsbach reported the appearance of colony opacity mutants of *F. tularensis* (Eigelsbach et al. 1951), and it is very likely that the variants represented changes in surface carbohydrates. More recently, Cowley and coworkers demonstrated that colonial phase variation in the live vaccine strain of *F. tularensis* was due to variation in LPS and perhaps capsule (Cowley et al. 1996). These changes involved alterations in both the O-antigen and the endotoxin. In wild-type strains the endotoxin fails to stimulate the production of tumor necrosis factor (TNF) in cultured rat macrophages. In strains expressing the variant form of LPS, the endotoxin induces TNF and nitric oxide, which inhibits bacterial growth.

The change in endotoxin form also affects the growth of *F. tularensis* in animals. When strains separately expressing the two forms of LPS were coinjected into rats, it was found that the presence of the strain with the variant form of LPS suppressed the growth of the strain expressing the wild-type form by as much as 170-fold (Cowley et al. 1997).

F. novicida expresses only the 'variant' form of LPS, and, hence, may represent a *F. tularensis* strain that is locked into one LPS form. This LPS phenotype may explain why *F. novicida* does not cause disease in humans while still being highly virulent in mice. If human macrophages respond in a similar fashion as rat macrophages this would lead to TNF and nitric oxide production, and suppression of *F. novicida* growth, but this point has not yet been studied directly.

CLINICAL ASPECTS

Diagnosis

Traditionally, diagnosis of tularemia is made through clinical assessment which is confirmed by serology using a micragglutination technique with a commercially available *Francisella*-specific antisera. However, serum agglutinating antibodies are usually not detectable until at least the second week of disease, and thus diagnosis by conventional serology may not be timely. Further, since the antibody response to *Francisella* infection may persist for several decades, a single positive titer does not discriminate between recent and past infection; a four-fold rise in titer between acute and convalescent sera samples is generally accepted as evidence of recent infection. Because *Francisella* is a notorious laboratory-acquired infection, clinicians usually only attempt identification by culture when a reference laboratory with appropriate biosafety containment facilities is available. Culture from sputum, pharyngeal secretions, and ulcers has been successful, but *F. tularensis* is rarely isolated from blood except in the later stages of disease.

Other rapid diagnostic tests for tularemia have been described in the research literature, including enzyme-linked immunosorbent assay (ELISA) (Bevanger et al. 1988; Viljanen et al. 1983), a handheld biosensor based on binding of the *Francisella* LPS, (Grunow et al. 2000), and a variety of polymerase chain reaction (PCR)-based assays, but none of them are yet available for routine use. PCR assays have been designed using sequence information for a *F. tularensis* lipoprotein (Junhui et al. 1996; Long et al. 1993) and an outer-membrane protein (Fulop et al. 1996). To date, these appear specific for *Francisella* and are not positive using clinical isolates of other related bacteria such as *Yersinia*. However, it remains to be determined whether these assays will be specific for *F. tularensis* and/or able to detect *F. novicida* or *F. philomiragia*.

Disease

F. tularensis can enter the body through a number of routes, and the route of infection has a significant impact on the resulting disease. Most commonly, infection results from vector-borne transmission or from a cut while dressing wild meat. The resulting infection is usually limited to an ulceroglandular form, but occasionally it progresses to a typhoidal or pneumonic form. Inhalation or ingestion of *F. tularensis* can lead

directly to the pneumonic or typhoidal forms of tularemia.

Before the availability of antibiotics, the mortality rate from tularemia in North America was between five and ten percent (Evans et al. 1985). Since the introduction of antibiotics the mortality rate has been about 2.5 percent. As important, the introduction of antibiotics has reduced the course of disease from months to days. However, when pneumonic tularemia occurs the prognosis is poor, even with antibiotic treatment. There has seldom been any differentiation between the type A and type B biovars when studying clinical infections in North America, so it is difficult to accurately know the mortality of the type A strain. Deaths from type B biovars of *F. tularensis* in Europe and Asia are extremely rare. The mechanisms involved in causing the symptoms of tularemia are poorly understood.

Treatment

Historically, streptomycin has been the drug of choice for treatment of tularemia. However, with the potential side effects of extended streptomycin treatment and its limited availability, some clinicians have used a variety of other antibiotics to treat tularemia (Enderlin et al. 1994; Johansson et al. 2000; Risi and Pombo 1995). Most commonly, gentamicin, tetracycline, and ciprofloxacin have been used, with a treatment regime of 2–4 weeks. All of these antibiotics have a cure rate approaching 90 percent, with relapse rates of about 5–10 percent. Although chloramphenicol has a comparable cure rate to these other drugs, it should not be considered a drug of choice, given the danger of chloramphenicol inducing aplastic anemia in patients. β-Lactams have proven to be ineffective for treatment of tularemia. Erythromycin can be effective against some strains of *F. tularensis*, but its use should be avoided, since many strains are resistant to it.

GENETIC AND BIOCHEMICAL ANALYSIS OF VIRULENCE

Intracellular growth

F. tularensis is clearly a facultative intracellular bacterium, although the relative amount of intracellular growth versus extracellular growth in vivo has not been extensively examined. In the murine model of infection with the live vaccine strain (LVS) of *F. tularensis*, only intracellular growth has been observed. *F. tularensis* has been found inside macrophages and hepatocytes (Conlan and North 1992) but not in PMNs. Apparently, *F. tularensis* can enter macrophages via a microfilament-independent pathway (Fortier et al. 1994).

Following entry into macrophages, *F. tularensis* lives inside vacuoles that do not fuse with secondary lysosomes (Anthony et al. 1991; Fortier et al. 1995). Acidification of the vacuole is apparently necessary for growth of *F. tularensis*, probably as a means of releasing iron from transferrin or another host iron-binding protein. Treatment of murine macrophages with interferon-γ (IFN) results in the production of nitric oxide and cessation of *F. tularensis* growth (Anthony et al. 1992; Fortier et al. 1992). Since this growth arrest can be reversed by the addition of excess iron-pyrophosphate, it is likely that NO production directly or indirectly limits access to iron that would normally be available to *F. tularensis*. Recent work has demonstrated that *F. tularensis* resides in a membrane-bound vesicle for several hours but gains access to the host-cell cytoplasm by 8 h after cell entry (Clemens et al. 2004).

F. tularensis grows vigorously in several types of macrophages harvested from mice, guinea pigs or rats (Anthony et al. 1991). *F. novicida* is unable to grow in rat macrophages since its endotoxin stimulates the production of nitric oxide. One study found that the contents of lysosomes were excluded from early *F. tularensis*-laden phagosomes. However, much more work is needed to define how *F. tularensis* induces modification in the maturation of the phagosome.

Mutants with decreased virulence

Although there are limited tools for genetically manipulating *F. tularensis*, there has been some progress identifying genes needed for full virulence or growth in macrophages. Two separate transposon mutagenesis schemes have been used in *F. novicida* in efforts to generate LPS or intramacrophage growth mutants (Cowley et al. 2000; Gray et al. 2002; Mdluli et al. 1994). Inactivation of MinD, a protein involved in cell division, leads to avirulence, as do mutations in the ValAB operon, proteins needed for transport and modification of LPS. Insertions in *purF* (needed for purine biosynthesis), *clpA* (encoding a heat shock protease), or a locus that shows similarity to several hypothetical proteins in gram-negative pathogens all result in strains with reduced intramacrophage growth. One mutant resulted from an insertion into a gene that encodes a 23-kDa protein (Gray et al. 2002). This protein was identified by Golovliov et al. (1997) as the major protein induced after introduction of *F. tularensis* into a culture of macrophages. An insertion that creates a mutant with defective LPS and sensitivity to serum and deoxycholate also results in poor growth of *F. novicida* in macrophages (Cowley et al. 2000)

A genetic locus encoding putative regulator genes, *mglAB*, was isolated by virtue of the reduced production of acid phosphatase in the mutant (Baron and Nano 1998). The original mutant suffered a point mutation that led to a nonconservative amino-acid substitution (T54K) in MglA. This mutant, as well as insertion

mutants in either *mglA* or *mglB*, had about 10 000-fold less growth in macrophage cultures than wild-type *F. novicida*. MglAB mutants are avirulent in mice. Complementation of the mutant with the wild-type *mglAB* locus restored wild-type growth in macrophages.

MglAB shows high similarity to SspAB of *Escherichia coli*. These proteins are known to regulate other genes during nutritional stress, and can associate with RNA polymerase. Mutants in *mglA* show the loss of expression of several other proteins, including a putative secreted 70-kDa protein.

It also appears that all *Francisella* strains possess a respiratory burst-inhibiting acid phosphatase (Reilly et al. 1996). However, insertion mutations that cause the loss of the phosphatase do not impair intracellular growth (Baron et al. 1999).

Genomics and proteomics

Essentially all of the *F. tularensis* Shu4 (highly virulent) genome has been sequenced. However, to date, no type III secretion system has been found in the genome (Karlsson et al. 2000; Prior et al. 2001); such secretion systems are an essential part of several bacterial virulence loci and pathogenicity islands. Further, genes that encode toxins or adherence factors have not been identified.

Three studies of the *Franicsella* proteome have been carried out. (Havlasova et al. 2002; Hernychova et al. 2001; Kovarova et al. 2002). A comparison of proteins from the live vaccine strain to a clinical isolate of *F. tularensis* revealed 50 differentially expressed proteins. Eight of these proteins were identified, and they included the acid phosphatase and an isoform of the 23-kDa protein described previously (Golovliov et al. 1997). The 23-kDa protein was also highly expressed in cell-line macrophages ($NRAMP^s/BCG^s$) that are relatively resistant to *F. tularensis* growth, when compared with expression levels in congenic cell-line macrophages ($NRAM^r/BCG^r$) that are relatively susceptible to *F. tularensis* growth.

IMMUNE RESPONSE TO *F. TULARENSIS* INFECTION

Animal models

The immune response to *F. tularensis* has been studied over many years in a variety of animal models, although the pathogenesis of infection in animals is less well characterized (in part because of the difficulty in deriving informative bacterial mutants). *Francisella* infects a wide variety of mammals, many of which serve as reservoirs and vectors in nature. Animals used as experimental models include the mouse, rat, guinea pig, rabbit, and monkey. The most information is available from studies using mice, which are obviously convenient but also have been considered reasonable models since the course and nature of *Francisella* infection appears to be similar in mice and humans (Fortier et al. 1991; Tärnvik 1989). Outbreaks of *Francisella* infection have been reported in a variety of nonhuman primates, including those in laboratory colonies and zoos. Experimental infection of rhesus macaques and cynomolgus monkeys has been described, with marked similarities between monkey and human disease, although monkeys may be somewhat more susceptible than humans (Saslaw and Carlisle 1969). Disease progression and pathology have been described in rhesus-monkey models of aerosol infection with both type A (White et al. 1964) and type B (Schricker et al. 1972) *F. tularensis*. Infected monkeys had an acute febrile illness with pneumonia. Bacteria multiplied readily on the lungs, and disseminated to regional lymph nodes, spleen, and liver; surviving animals cleared bacteria within 2 months. Monkeys experimentally vaccinated with a *Francisella* vaccine strain appear to have protection against secondary aerosol challenge as well (Saslaw and Carlisle 1969).

Immunity to *Francisella* in humans and animals

Importantly, documented cases of *Francisella* reinfection are rare (Burke 1977), indicating that live infection stimulates protective immunity. It is fairly clear from studies in both humans and animals to date that, as for many intracellular pathogens, specific T-cell responses and activation of macrophages for intracellular killing are probably of most importance in protective immunity against *Francisella*. Although there are some indications that specific antibodies may have a minor contribution in some circumstances, in general, strategies that engender production of large amounts of specific anti-*Francisella* antibodies, such as the use of killed or subunit vaccines, provide at best poor protection against less virulent strains or lower challenge doses.

Much of what is currently believed about the specific mechanisms of immunity to *Francisella* derives from study of the LVS in a murine model of infection, and must be extrapolated to human infection with caution. Nonetheless, many of the features uncovered using the murine model appear to reflect what is known about human infection. In mice, a very strong innate immune response follows within hours of primary infection with LVS. This response includes production of IFN and TNFα, both of which are required for initial survival of infection. While the specific cell type responsible for the production of each remains to be determined, it is clear that neither is initially derived from either T or B cells, as *scid* mice lacking both lymphocyte populations produce both IFN and TNF and survive for as long as 3 weeks after infection (Elkins et al. 1996). Neutrophils

recruited to the site of infection are also required for initial survival of LVS infection (Elkins et al. 1996; Sjöstedt et al. 1994); their exact effector function remains to be determined, but in the liver may involve lysis of infected hepatocytes (Conlan and North 1992). Production of IFN and TNF during innate responses activates macrophages for control of intracellular bacterial growth, in mice via the production of nitric oxide (Anthony et al. 1992; Fortier et al. 1992). Surprisingly, interleukin (IL)12, while made in abundance very quickly after LVS infection of mice, is not required for initial survival. Instead, the p40 chain of IL12, but not IL12 p70, is only required much later in infection for final clearance of bacteria (Elkins et al. 2002).

In general, this strong innate immune response functions to provide time for activation and expansion of specific effector T cells. The exact effector functions of T cells is the subject of current study, but they almost certainly involve further production of IFN. However, the action of IFN may only be bacteriostatic, not bactericidal; indeed, the accumulated evidence to date indicates that IFN is necessary, but not sufficient, for protection and final clearance of bacteria (Elkins et al. 2003). Studies in various knockout mice, as well as those depleted of T-cell subpopulations, indicate that either $CD4^+$ or $CD8^+$ T cells can provide protection during both primary and secondary infections (Yee et al. 1996), although the specific effector mechanisms used by each likely differ (Cowley and Elkins, 2003). It does not appear that cytotoxic T cells or perforin-secreting cells are involved, however (Cowley and Elkins, unpublished data). Further, although $CD4^+$ or $CD8^+$ T cells were sufficient for protection, another $Thy1.2^+CD4^-CD8^-$ population of cells may also provide some protection (Conlan et al. 1994). To date, γ/δ^+ T cells appear to have only a minor role following LVS infection or challenge, although this may reflect a difference between LVS and fully virulent *Francisella*. In humans, there is a large polyclonal expansion of $V\gamma9V\delta2$ T cells in the peripheral blood of tularemia patients within the first 7 days after the onset of symptoms, and these levels remain high for over a year (Kroca et al. 2000; Poquet et al. 1998; Sumida et al. 1992). The $V\gamma9V\delta2$ T cells appear to respond to a phosphoantigen found in virulent tularemia, and a comparable response was not observed in LVS vaccine recipients (Poquet et al. 1998). The role of B cells, both in terms of antibody production as well as other effector functions (e.g. antigen presentation, cytokine production, and chemokine production), also remains a subject of ongoing study. In the mouse models, specific anti-*Francisella* antibodies appear to provide little benefit. CD4 knockout mice have very poor LVS specific antibody responses but readily survive maximal lethal LVS challenge (Yee et al. 1996), and transfer of LVS specific IgG provides very limited protection that is highly route-dependent (Rhinehart-Jones et al. 1994).

Similar to murine studies, long-lasting human T-cell responses to both *Francisella* infection and LVS vaccination are readily detectable in in vitro assays. Peripheral blood lymphocytes (PBL) from vaccinated people proliferated in response to whole formalin-killed LVS within 1–2 weeks after vaccination (Karttunen et al. 1991; Koskela and Herva 1982) and as late as 9 years later (Tarnvik et al. 1985). Similarly, PBLs from tularemia patients responded to LVS antigens as much as 30 years after the onset of disease (Ericsson et al. 2001; Ericsson et al. 1994). $CD4^+$ T-cell proliferative responses in particular were readily detected following stimulation of PBLs from both LVS-vaccinated and naturally infected people (Ericsson et al. 2001; Sjöstedt et al. 1992; Sjöstedt et al. 1990). In contrast, it has been more difficult to detect $CD8^+$ T-cell proliferation or IFN production in vitro (Sjöstedt et al. 1990; Surcel 1990), although this may be due to culture conditions and/or the need for $CD4^+$ T-cell help (Sjöstedt et al. 1992). The *Francisella* antigens recognized by human T cells are not well characterized, but include membrane proteins, heat-shock proteins, and lipoproteins (Ericsson et al. 2001; Ericsson et al. 1994; Sjöstedt et al. 1990; Sjöstedt et al. 1989; Surcel et al. 1991). To date no obvious immunodominant antigens have been identified. In addition to readily detectable T-cell responses, specific anti-*Francisella* IgM, IgG, and IgA antibodies are found in sera within 2 weeks after vaccination or infection (Koskela 1985). While useful for diagnosis, there is no correlation between levels of antibodies in humans and protection (Tärnvik 1989). For example, vaccinated laboratory workers who had high titers of agglutinating antibodies at the time of infection nonetheless suffered severe tularemia following laboratory exposure (Overholt et al. 1961).

Vaccines

Following identification of tularemia as a bacterial disease, interest in development of a vaccine soon followed. In the USA, these efforts began along traditional lines with development of killed whole-cell vaccines. It soon became clear, however, that killed vaccine or antigen preparations were relatively ineffective (Foshay 1950), although some amelioration of symptoms following systemic challenge (but not aerosol challenge) was suggested (Saslaw et al. 1961a; Saslaw et al. 1961b). In the Soviet Union, live attenuated strains were developed from *F. tularensis* type B clinical isolates (Tigertt 1962), and mass vaccination campaigns were undertaken in the USSR in the late 1940s with a clear reduction in overall disease burdens (Sandström 1994). Indeed, attenuated strains, including one designated Strain 15, are still produced and in use today in Russia and other former Soviet Union republics, although only in areas where tularemia is considered endemic.

In the 1950s, an ampoule of mixed tularemia vaccine from the USSR was transferred to the USA. This culture was expanded, then passaged through mice with an accompanying increase in virulence, and finally isolated from moribund animals. The resulting strain was designated LVS (Eigelsbach and Downs 1961). LVS is thus significantly more virulent for mice than strains used in Soviet mass-vaccination programs, and the genetic basis of attenuation of LVS remains unknown. The success of LVS as a vaccine in humans remains a subject of study as an investigational vaccine under IND (Investigational New Drug) sponsored by the Department of Defense, and it is not licensed for use in the USA. Several somewhat limited studies have been offered in support of the efficacy of LVS as a vaccine against either type A or type B tularemia. An analysis of laboratory-acquired cases at one research facility before and after LVS was introduced for vaccination was interpreted to indicate that LVS prevented typhoidal or pneumonic tularemia fairly well, and at least ameliorated symptoms of ulceroglandular tularemia (Burke 1977). However, this analysis is likely limited by the changes in biosafety, containment, and laboratory procedures that progressed over the same time period. Aerosol challenge studies in vaccinated human volunteers were performed in the late 1950s, in which volunteers were given LVS by scarification and subsequently challenged by inhalation with virulent type A Schu S4 (McCrumb 1961). Results in this limited population suggested that some protection against development of symptoms was afforded, at least at lower challenge doses; similar observations in a monkey model have also been published (Eigelsbach et al. 1962).

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Legionella

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DEFINITION

Legionellae are nutritionally fastidious, pleomorphic gram-negative organisms. They do not form endospores or microcysts and are not encapsulated. In vivo they grow as short rods or coccobacilli measuring 0.3–0.9 μm in width and 2.0–6.0 μm in length, on artificial media they may be seen as rods or occasionally as filamentous forms 6.0–20.0 μm or more in length. They are motile with a single monopolar flagellum although nonmotile strains do occur. Branched chain fatty-acids predominate in the cell wall which also contains major amounts of ubiquinones with more than ten isoprene units in the side chain. L-Cysteine and iron salts are required for growth in vitro, carbohydrates are not fermented or oxidized, and nitrate is not reduced to nitrite. Legionella are chemoorganotrophic using amino acids as carbon and energy sources. They are urease-negative, catalase-positive, and give variable results in the oxidase test. The legionellae have a genome size of approximately 2.5×10^9 daltons (~3.9 Mb) and a guanine-plus-cytosine content of 33–52 mol%.

The family Legionellaceae comprises a single genus *Legionella*. There are some 50 formally described species and many more await full characterization (Table 1). They are members of the gamma-subgroup of the class Proteobacteria. The type species is *Legionella pneumophila* subsp. *pneumophila* (Brenner et al. 1979, 1988).

HISTORICAL PERSPECTIVE

A dramatic outbreak of an acute, febrile respiratory illness occurred in July 1976 among Legionnaires (veterans of the US army) who had gathered at the 58th convention of the Pennsylvania Branch of the American Legion in Philadelphia, USA. Among the 4 400 attendees there was a total of 182 convention-associated cases, 29 of whom died (Fraser et al. 1977). The outbreak aroused considerable interest and lurid names were devised by the newspapers to describe the illness: 'Legion Fever,' 'Philly Killer,' 'Legion Malady,' and 'Legionnaires' disease.' Surprisingly, the name 'Legionnaires' disease' was taken up by the scientific community and this has, at least in part, helped to perpetuate media interest in this otherwise unremarkable form of pneumonia.

The search for the cause of the Philadelphia outbreak was extensive and only after more than 6 months of investigation was the isolation of the causative organism reported (McDade et al. 1977). In order to identify the organism, investigators from the Centers for Disease Control (CDC) had used rickettsial isolation techniques. Guinea-pigs were inoculated with autopsy material from

Table 1 *Summary of biochemical and phenotypic properties of Legionellaceae* [a]

Species	Multiple serogroups	Isolated from humans	Auto-fluorescence	Hippurate hydrolysis	Brown pigment on TBYE	β-Lactamase production	Gelatinase production	Flagellum
L. pneumophila[b]	15	Y	–	+	+	+	+	+
L. adelaidensis		N	–	–	–	–	+	+
L. anisa		Y	BW	–	+	+	+	+
L.beliardensis		N	–	–	–	+	+	+
L. birminghamensis		N	–	–	–	+	+	+
L. bozemanii	2	Y	BW	–	+	+	+	+
L. brunensis		N	–	–	+	+	+	+
L.busanensis		N	–	+	–	NR	+	+
L. cherrii		N	BW	–	+	–	+	+
L. cincinnatiensis		Y	–	–	+	+	+	+
L. drozanskii[c]		N	–	NR	NG	NR	NR	NR
L. dumoffii		Y	BW	–	+	+	+	+
L. erythra	2	N	R	–	+	+	+	+
L. fairfieldensis		N	–	–	–	–	–	+
L.fallonii[c]		N	–	NR	NG	NR	NR	NR
L. feeleii	2	Y	–	–	+	V	–	+
L. geestiana		N	–	(+)	(+)	–	+	+
L. gormanii		Y	BW	–	+	+	+	+
L. gratiana		N	–	–	–	+	+	+
L. gresilensis		N	–	(+)	–	+	+	+
L. hackeliae	2	Y	–	–	+	+	+	+
L. israelensis		N	–	–	–	–	+	–
L. jamestowniensis		N	–	–	+	+	+	+
L. jordanis		Y	–	–	+	+	+	+
L. lansingensis		Y	–	–	–	–	–	+
L. londiniensis		N	–	–	+	+	+	–
L. longbeachae	2	Y	–	–	+	+	+	+
L. lytica[c]		Y	BW	NR	NG	NR	NR	+

(*Continued over*)

Table 1 *Summary of biochemical and phenotypic properties of Legionellaceae*[a] *(Continued)*

Species	Multiple serogroups	Isolated from humans	Auto-fluorescence	Hippurate hydrolysis	Brown pigment on TBYE	β-Lactamase production	Gelatinase production	Flagellum
L. maceachernii		Y	–	–	–	–	–	+
L. micdadei		Y	–	–	–	–	–	+
L. moravica		N	–	–	+	+	+	+
L. nautarum		N	–	–	–	+	–	+
L. oakridgensis		Y	–	–	+	+	+	–
L. parisiensis		Y	BW	–	+	+	+	+
L. quateirensis		N	–	–	+	+	+	+
L. quinlivanii	2	N	–	–	+	–	+	+
L. rowbothamii[c]		N	BW	NR	NG	NR	NR	NR
L. rubrilucens		N	R	–	+	+	+	+
L. sainthelensi	2	Y	–	–	+	+	+	+
L. santicrusis		N	–	–	+	+	+	?–
L. shakespearei		N	–	–	–	+	+	+
L. spiritensis	2	N	–	(+)	+	+	+	+
L. steigerwaltii		N	BW	–	NG	+	+	?–
L. taurinensis		N	R	–	–	+	+	+
L. tucsonensis		Y	BW	–	–	+	+	+
L. wadsworthii		Y	–	–	–	+	+	+
L.waltersii		N	–	+	–	+	+	+
L. worsleiensis		N	–	–	+	+	+	+
L. genomospecies 1		N	–	–	+	–	+	+

Table adapted from Harrison and Taylor (1988b).
BW, blue/white autofluorescence; N, no; NG, no growth on this medium; NR, not reported; R, Red autofluorescence; Y, yes; V, variable results; +, positive result; (+), weakly positive result; –, negative result.
a) All legionellae grow on primary isolation on BCYE agar, but fail to grow on BCYE agar from which L-cycteine has been omitted; they are Gram stain negative and catalase positive, all fail to reduce nitrate. Results for the oxidase test are often reported as weakly-positive or variable but this test is unreliable due to contamination of the test by the BCYE growth medium.
b) *L. pneumophila* comprises three subspecies: *L. pneumophila* subsp. *pneumophila* (the type species of the genus), subsp. *pascullei*, and subsp. *fraseri*.
c) These species were formally called *Legionella*-like amoeabal pathogens. They grow only weakly on BCYE at 30°C.

a Legionnaires' disease (LD) patient and tissue from febrile guinea-pigs was then inoculated into embryonated hens' eggs. In this way McDade and colleagues (1977) were able to demonstrate the presence of gram-negative bacilli in preparations of the infected eggs. Convalescent sera from most patients identified in the outbreak reacted in an indirect immunoflourescent antibody test (IFAT) with antigens prepared from the yolk sacs of infected eggs, supporting the view that the bacterium was the etiological agent of LD.

CDC investigators also recognized epidemiological similarities between the Philadelphia outbreak, where an air-conditioning system was implicated as the source of infection, and several earlier outbreaks of previously unexplained illness. These included one outbreak in Washington DC during 1965, and one in the same Philadelphia hotel in 1974 which occurred amongst delegates to a convention of the Independent Order of Odd Fellows (Terranova et al. 1978). Examination of stored sera collected from patients during these outbreaks demonstrated that the Legionnaires' disease bacterium, as it was called, had also caused these outbreaks (McDade et al. 1977). Thus, although newly recognized, LD was clearly not a new illness. During these early investigations an outbreak of unexplained flu-like illness in Pontiac, Mitchigan during 1968 was also re-examined. Serological analysis of stored sera showed that, in addition to causing pneumonia, legionellae could give rise to a quite distinct clinical syndrome. This syndrome, subsequently called Pontiac fever was characterized by a very high attack rate but in contrast to LD was a relatively mild and self-limiting flu-like illness (Glick et al. 1978).

In the summer of 1973, a group of Scottish tourists returned to Glasgow, UK from a 10-day holiday in Benidorm, Spain. One of the party was dead on arrival at the airport, two others were seriously ill and they died shortly after. Investigations revealed that a further 86 people, who had stayed in the same hotel, had also suffered a respiratory illness. The cause of the illness was not determined at the time but in 1977, in the wake of the Philadelphia outbreak, stored sera from these patients were re-examined and a diagnosis of LD was established in eight cases, including the three men who had died (Grist et al. 1979). In August and September 1977, a further cluster of cases was identified in the Nottingham area and subsequently that year 25 sporadic cases of LD were reported in the UK establishing the global nature of LD (Macrae et al. 1979).

CLINICAL ILLNESS AND EPIDEMIOLOGICAL FINDINGS

Legionella infection of man (legionellosis) can vary from a mild flu-like illness to severe life-threatening pneumonia. Classically, legionellosis presents as pneumonia of sufficient severity to require admission to hospital and such infections, referred to as Legionnaires' disease (LD), have been extensively characterized. However, investigations into large outbreaks of LD almost always identify patients who have milder symptoms often with little respiratory involvement (Fraser et al. 1977; Westminster Action Committee 1988; Garcia-Fulgueiras et al. 2003). The spectrum of this nonpneumonic legionellosis, which encompasses any condition that is not LD, but where there is some microbiological evidence of legionella infection, is much less well studied. The term 'Pontiac fever' is sometimes used interchangably with nonpneumonic legionellosis, however, it should only be applied for those cases characterized by a self-limiting, flu-like illness with a high attack rate and short incubation period (Glick et al. 1978).

Legionnaires' disease

While overall LD is a relatively uncommon form of pneumonia, in the context of severe pneumonia it is much more significant, probably being the second commonest form accounting for 14–37 percent of cases (Hubbard et al. 1993). LD has no special features that clearly distinguish it from other pneumonias but the clinical picture may be suggestive of the diagnosis (British Thoracic Society 2001; Mayaud and Dournon 1988). Compared to pneumonia of other etiologies, patients with LD are reported to be more likely to smoke, to have diarrhea, to have neurological symptoms, and evidence of multisystem involvement. They are less likely to have comorbidity.

LD presents as an acute lobar pneumonia eventually progressing to extrapulmonary symptoms and multisystem failure. The incubation period is typically 2–10 days, although it can be significantly longer. Early symptoms include headache, generalized weakness, fever, myalgia, rigors, and a nonproductive cough. Dyspnea, fatigue, râles, bradycardia, hemoptysis, bacteremia, and extrapulmonary abnormalities are common. Central nervous system dysfunction, involvement of the heart, spleen, bone marrow, brain, lymph nodes, and renal system have all been documented, and symptoms of disorientation, confusion, nausea, vomiting, renal insufficiency, and diarrhea are common. Routine laboratory findings are not particularly helpful. Liver abnormalities are frequently found with elevated serum levels of transaminases and/or alkaline phosphatase or, in some cases, of bilirubin. Proteinuria, hematuria, and renal insufficiency are infrequent in patients receiving early treatment. Elevation of creatinine phosphokinase and aldolase blood levels, suggestive of muscle damage, is seen in some patients (Sopena et al. 1998; Mayaud and Dournon 1988).

The prognosis for patients promptly treated with appropriate antibiotics is good and they usually recover even if they are immunocompromised (Mayaud et al. 1984; Lim et al. 2001). In contrast, the prognosis is poor

in patients not treated before acute respiratory failure and shock develop (Heath et al. 1996; El-Ebiary et al. 1997). This is usually late in the course of the illness. In such cases rhabdomyolysis, acute renal failure, pancytopenia, disseminated intravascular coagulation, icterus, or coma are not infrequent (Mayaud and Dournon 1988). However, in most instances, patients die from the respiratory distress syndrome and not from the extrapulmonary manifestations of the disease.

Pontiac fever

Pontiac fever is an acute, nonpneumonic, flu-like illness that is usually self-limiting (Glick et al. 1978). The attack rate is very high (>90 percent) and the disease affects previously healthy, and often young, individuals. The incubation period is short, varying from a few hours to 2–3 days and the illness resolves spontaneously, usually within 2–6 days. Outbreaks of Pontiac fever have been reported to be caused by *L. pneumophila*, *L. feeleii*, *L. micdadei*, and *L. anisa* (Fenstersheib et al. 1990; Herwaldt et al. 1984; Glick et al. 1978; Goldberg et al. 1989). Mixed outbreaks have been reported where cases of both LD and Pontiac fever have been seen (Goldberg et al. 1989; Benin et al. 2002a; Girod et al. 1982). Interestingly, although *Legionella* species have been isolated from LD patients in such mixed outbreaks, the organism has never been isolated from a patient with Pontiac fever. While this may be due to the differing severity of the respective illnesses, some authors have speculated that it may be because Pontiac fever is caused by inhalation of dead legionellae or by a hypersensitivity reaction to their amebal host (Rowbotham 1986). Although the subject of considerable speculation, the explanation for the differences between LD and Pontiac fever remains unclear.

Epidemiology

INCIDENCE

There is no evidence of person-to-person spread of LD, infection being acquired primarily by inhalation of aerosol containing viable organisms or, particularly in immunocompromised patients, by aspiration of bacteria in water. Cases of LD have been reported from countries throughout the world but the reported incidence varies considerably. Surveillance data for 2002 from 31 countries across Europe identified almost 5 000 cases with an average annual rate of 10.1 cases per million population. However, the incidence varied markedly between countries, ranging from 0.0 to 34.1 cases per million (Joseph 2004). This variation may in part be a reflection of genuine differences in incidence but is due mainly to considerable under-ascertainment in many countries. As an example, the reported incidence of LD in England and Wales is ~200 cases per year (a rate of ~4.0 cases per million). However, prospective studies in the UK and elsewhere indicate that approximately 2.0–5.0 percent of cases of all pneumonia cases requiring admission to hospital are LD (Woodhead et al. 1986; Lim et al. 2001; Marston et al. 1997). This equates to between 2 000 and 5 000 cases per annum in England and Wales suggesting that 10 percent or less of LD cases are recognized and reported each year. Estimates from the US suggest that less than 5 percent of LD cases are reported to their national surveillance scheme (Benin et al. 2002b).

Legionnaires' disease is seen in patients of all ages but is most frequent in adults over 40 years old and is very rare in children. There are many more cases among males than among females (typically >2:1) although this may be a reflection of other coincident risk factors, such as smoking, rather than a function of gender per se. Significant risk factors include a compromised immune system, recent surgery, congestive heart failure, chronic bronchitis, liver cirrhosis, renal insufficiency, and heavy smoking. In certain patient groups and settings therefore, such as hospitalized organ transplant patients, the incidence is much higher than 5 percent. Reported mortality varies considerably but is significantly higher in nosocomially acquired cases than in community-acquired cases. Recent data suggest that overall the rate is falling. In the US the average mortality was 20 percent between 1980 and 1998 falling from a high of 34 percent in 1985 to 11.5 percent in 1998 (Benin et al. 2002b). Similarly in Europe mortality fell from 13 percent in 1998 to 6 percent in 2002 (Joseph 2004). While there is some evidence that the decline is due to improvements in speed of diagnosis and hence the initiation of appropriate therapy (Heath et al. 1996; Formica et al. 2000), improved ascertainment of less severe cases probably accounts for most of the fall (Benin et al. 2002b). In support of this view, mortality rates appear to be low in outbreaks of LD that are investigated using urinary antigen assays compared with those seen in outbreaks where this sensitive and rapid technology was not used. For example, in the Central London outbreak of 1988 the mortality was 3/79 (4.3 percent) (Westminster Action Committee 1988) and in the Murcia outbreak of 2001, the largest to date, the mortality was only 5/449 (1.1 percent) (Garcia-Fulgueiras et al. 2003). In contrast, in the Stafford Hospital outbreak which occurred in 1985 before urine assays were available, 22/68 (32 percent) patients died (O'Mahony et al. 1990) and in the 1999 'Flower show' outbreak in the Netherlands 21/188 (11 percent) of patients died. In this latter case although urinary antigen testing was available, it was rarely used in the Netherlands prior to the outbreak (Den Boer et al. 2002).

A seasonal variation in the incidence of LD has been recognized in many parts of the world with a peak incidence in late summer and early autumn in both the

northern and southern hemispheres (Benin et al. 2002b; Li et al. 2002; Joseph et al. 1999). However, there have been several major outbreaks during the winter and early spring following periods of unseasonably warm weather (Westminster Action Committee 1988; Watson et al. 1994).

Although there is a good understanding of the ecology of legionellae and a substantial body of legislation regarding their control, large outbreaks of LD continue to occur (Den Boer et al. 2002; Garcia-Fulgueiras et al. 2003). However, although highly publicized, these outbreaks account for less than 25 percent of cases (Joseph et al. 1999). There is some evidence to suggest that many of the remaining 75 percent of cases are associated with a common source of infection although this is not recognized at the time (Bhopal et al. 1991). Cooling towers have been identified as the source in most of the major outbreaks of LD but, overall, water distribution systems are the most frequently implicated sources (Joseph 2004). In recent years, whirlpool spas, either in use or on display, have been identified as a major vehicle for transmission of LD (Den Boer et al. 2002; De Schrijver et al. 2003; McEvoy et al. 2000)

NOSOCOMIALLY ACQUIRED LD

Hospitals often have large and complex water distribution systems and a population of susceptible patients. It is not surprising therefore that nosocomially acquired LD is a significant problem, accounting for 888 (9 percent) of cases in Europe in 2000–2002 (Joseph 2004). Although overall this represents only a small number of cases annually, the mortality in this group of patients is disproportionately high at ~30 percent (Benin et al. 2002b). A second feature of nosocomial LD is that a very much larger proportion of infections is caused by non-*L. pneumophila* serogroup 1 strains than is reported overall. Both these observations probably reflect the immunocompromised status of most patients in the nosocomial group.

LEGIONELLA ABLE TO CAUSE HUMAN INFECTION

To date, 19 species have been isolated from clinical material (Table 1), however infections caused by species other than *L. pneumophila* are rare and are almost always seen in patients who are immunocompromised (Benin et al. 2002b; Muder and Yu 2002; Dournon 1988). One exception to this is *L. longbeachae* serogroup 1 which is now recognized to be a major cause of community-acquired pneumonia in Australia: between 1996 and 2000 42 percent of notified cases of LD in Australia were caused by *L. longbeachae* (Li et al. 2002). The reservoir for this organism appears to be potting mixes prepared from composted indigenous wood where it is found growing in association with amebae (Hughes and Steele 1994). Cases of *L. longbeachae* infection associated with potting composts have recently been reported outside the Antipodes (Anonymous 2000)

The majority of sporadic, and almost all outbreak-associated, cases of LD are caused by a subset of so-called 'virulent' strains of *L. pneumophila* serogroup 1. These strains are characterized by their reaction with a particular monoclonal antibody and are referred to as MAB2+ve (Dournon et al. 1988) or MAb3/1+ve (Helbig et al. 2002) depending on the origin of the antibodies being used. These antibodies recognize a virulence-associated epitope on the lipopolysaccharide of *L. pneumophila* serogroup 1 strains (see section Somatic 'O' antigens). Strains of *L. pneumophila* serogroup 1 that are MAB2-ve or 3/1-ve, together with strains of other *L. pneumophila* serogroups, account for most remaining cases (Joseph 2004). With a few exceptions, *Legionella* species other than *L. pneumophila* are only seen as the cause of rare sporadic cases of LD or persistent endemic problems in large hospitals (Muder and Yu 2002). It does, however, seem probable that under the appropriate circumstances, any *Legionella* species could cause infection as no consistent differences have been observed between the virulence traits of the various species examined to date (Alli et al. 2003; O'Connell et al. 1996a).

HABITAT

Extensive environmental studies indicate that legionellae are ubiquitous organisms occurring worldwide in natural freshwater habitats including streams, lakes, rivers, natural spas, water in hydrothermal areas, and subterrestrial groundwater sediments (Fliermans et al. 1981; Ortiz-Roque and Hazen 1987; Bornstein et al. 1989; Verissimo et al. 1991; Fliermans and Tyndall 1993). Isolates have also been obtained from moist potting soils, mud, riverbanks, and rainwater run-off (Hughes and Steele 1994; Koide et al. 1999). Legionellae have been isolated from a diverse range of man-made habitats including air-conditioning systems, potable water supplies, ornamental fountains, and plumbing fixtures and fittings in hospitals, shops, and homes (Tobin et al. 1981; Bartlett and Bibby 1983; Hlady et al. 1993; Memish et al. 1992; Stout et al. 1992; Redd et al. 1990; Colbourne and Trew 1986). It is these man-made environments that are the source of almost all cases of LD, and control of legionellae in them is the key to the prevention of LD.

Early studies found that legionellae could be detected by immunofluorescence (IF) in most natural bodies of water examined but could only be isolated and cultured from a few of these (Fliermans et al. 1981). Because of the questionable specificity of the IF reagents that were used at that time, the isolation rate was considered to be the more accurate indication of the likely prevalence of legionellae. However, the isolation rate is, in large part, dependent on the temperature of the body of water

examined, legionellae being readily isolated from water between 20 and 40°C, but rarely isolated from colder sources. It is now known that at low temperatures, in common with many environmental organisms, legionellae enter a 'dormant state' where they remain viable but cannot easily be cultured (Steinert et al. 1997; Colbourne et al. 1988; Ohno et al. 2003) . Hence, isolation rates probably give a poor estimate of the true prevalence of legionellae.

The widespread distribution of legionellae, even in low nutrient environments, appears to contrast with fastidious growth requirements for culture and isolation of the organism in the laboratory. The explanation is that naturally legionellae grow in close association with other microorganisms. Tison and colleagues (1980) first demonstrated that *L. pneumophila* could grow in mineral salts medium at 45°C in association with cyanobacteria (*Fischerella* sp.). In the same year Rowbotham (1980) observed that when legionellae were phagocytosed by *Acanthamoeba* trophozoites in vitro, they could survive and multiply within the amebal vacuole. It has subsequently been shown that other amebae and ciliates such as *Hartmanella*, *Naegleria*, *Valkampfia*, *Echinamoeba*, *Tetrahymena*, and *Cyclidium* can also be parasitized (Barbaree et al. 1986; Breiman et al. 1990) and it is recognized that these host–parasite relationships are fundamental to the propagation and distribution of legionellae within the environment. This close association has several potential benefits for legionellae, such as the provision of adequate concentrations of nutrients in otherwise nutrient poor conditions; protection from harsh environmental extremes of pH, temperature, osmolarity, and chemical disinfectants, and possibly by providing a vehicle of dissemination within amebal cysts (Barker and Brown 1994; Kilvington and Price 1990).

In common with most other aquatic bacteria, legionellae preferentially colonize surfaces at the aqueous/solid interface (Wright et al. 1989). Here, together with their protozoal hosts, other bacteria, and algae, they form a complex consortium of organisms termed 'biofilm.' Biofilm formation provides its constituent organisms with nutritional advantages and protection from adverse environmental influences compared to their planktonic counterparts. Although it is now generally accepted that legionella do not replicate in the extracellular environment, they have been shown to form microcolonies within the biofilm (Rogers and Keevil 1992).

MORPHOLOGY

By electron microscopy, *Legionella* organisms appear as short rods approximately 0.3–0.9 μm wide and 1–3 μm long (Rodgers et al. 1978; Chandler et al. 1979; Rodgers 1979). From human or experimentally infected tissues, the pathogen appears coccobacillary in shape but filamentous forms occur after extended cultivation and on poor quality bacteriological media. Typically, the bacteria have nonparallel sides with tapering ends and, like many other gram-negative bacteria they possess wrinkled or rugose surfaces and poly-β-hydroxybutyrate (PHB) granules. Division is by a pinching, septate binary fission (Figure 1). The organism possesses a single monopolar flagellum (Heuner and Steinert 2003). Flagella are unsheathed, 14–20 nm wide and up to 8 μm long (Figure 2). Motile organisms have been observed in wet mounts as well as within phagosomes of infected cells. Under conditions of exponential growth in enriched media, the legionellae also possess long and short fimbriae or pili (Figure 3) (Rodgers et al. 1980; Stone and Abu Kwaik 1998).

Negative-staining, thin-sectioning, scanning, and freeze-fracture electron microscopy of legionellae organisms show a cellular architecture typical for other gram-negative prokaryotes and have been reviewed by Rodgers (1985). The presence of PHB granules in the organism has been confirmed by Sudan black B staining, freeze-fracture electron microscopy, and Nile red spectrofluorometry (James et al. 1999). During active growth, the cytoplasm of the bacterium is rich in dispersed ribosomes each 25 nm in diameter and intermixed with a fine skein of nuclear elements. The limiting cell envelope

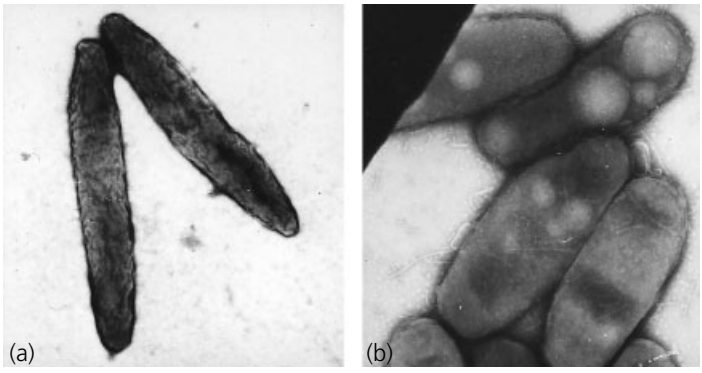

Figure 1 L. pneumophila *organisms negatively stained with 1 percent phosphotungstic acid.* **(a)** *Organisms showing tapered ends and rugose surfaces. Magnification ×12 600.* **(b)** *organisms showing poly-β-hydroxybutyrate granules; fine fimbriae are also evident. Magnification ×21 000 (reproduced with permission from Rodgers and Davey 1982)*

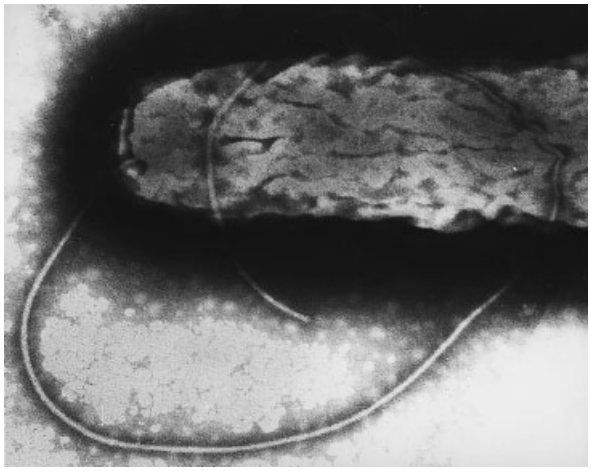

Figure 2 *Electron micrograph of* L. pneumophila *stained with 1 percent phosphotungstic acid and showing the rugose surface of the organism and the polar attachment of a flagellum. Magnification ×66 000 (reproduced with permission from Rodgers et al. 1980)*

of the organism is approximately 25 nm in thickness and comprises the periplasm located between the outer and cytoplasmic membranes, each measuring 7–10 nm. Like many of the aquatic pseudomonads, the legionellae possess a peptidoglycan cell wall that is best visualized after partial plasmolysis. A well-defined capsule or glycocalyx has not been consistently reported. It should be noted, however, that individual bacteria located within the cytoplasm of infected cells are frequently surrounded by an electron-lucent zone of variable thickness suggestive of a microcapsule or slime layer (Rodgers 1985).

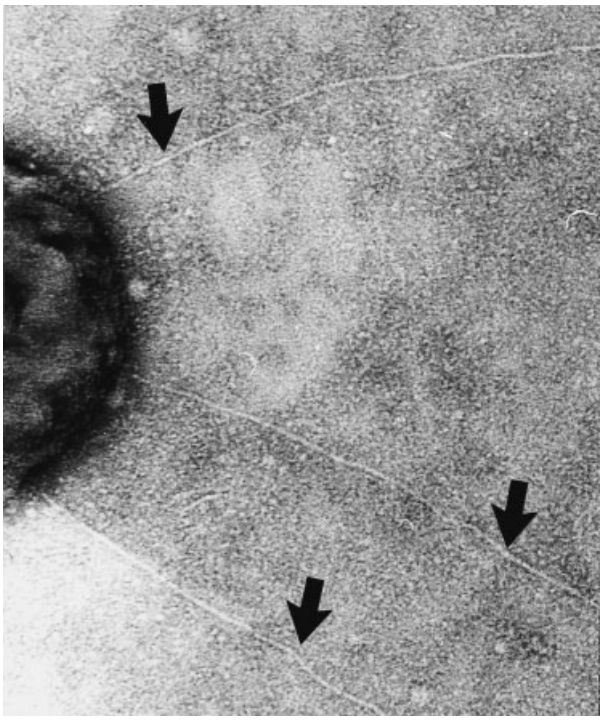

Figure 3 *Part of an* L. pneumophila *organism showing fimbriae (arrows). Stained with 1 percent phosphotungstic acid. Magnification ×126 000 (reproduced with permission from Rodgers et al. 1979)*

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

Buffered charcoal yeast extract (BCYE) agar with added L-cysteine, ferric pyrophosphate, α-ketoglutarate and *N*-2-acetamido-2-amino-ethanesulfonic acid (ACES) buffer is the medium most widely used for organism isolation (Feeley et al. 1979; Edelstein 1982). On primary isolation, colonies usually first appear after 3–5 days of incubation but they may be visible in about 36 h from specimens where large numbers of legionellae are present with few contaminating organisms. Growth tends to be delayed by about 12–24 h where selective agars are used (Dournon 1988). When first visible, the colonies have a granular or speckled opalescence resembling ground glass. They are round with an entire edge, glistening, convex (Figure 4). Initially, colonies show a blue–green or pink iridescence (Figure 5) that becomes less marked with prolonged incubation: colonies that are more than a week old are much less characteristic, being flat and whitish (Harrison and Taylor 1988a).

For culture, the organism shows a marked requirement for L-cysteine and iron salts whereas α-ketoglutarate augments growth (Edelstein 1981), and charcoal detoxifies free fatty acids and depletes inhibitory oxygen radicals (Hoffman et al. 1983). The presence of amino acids, trace metals, and a humid atmosphere containing CO_2 stimulates growth and media should be buffered to pH 6.85–6.95. Incorporation of 1 percent albumin into isolation and growth media enhances the isolation rate for some *Legionella* spp. including *L. micdadei* (Morrill et al. 1990), whereas glycine is useful for the cultivation of environmental isolates (Wadowsky and Yee 1981), and bromocresol purple and bromothymol blue may assist in differentiating species (Vickers et al. 1981).

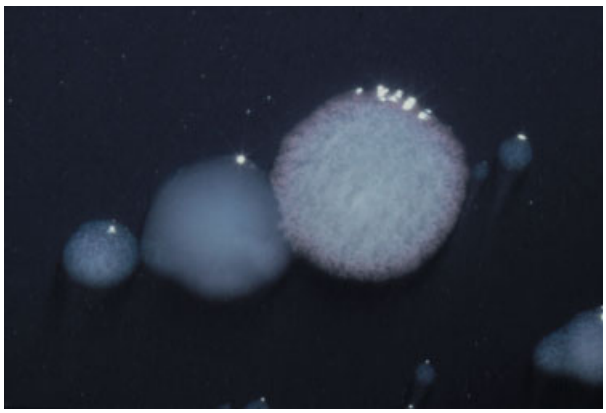

Figure 4 *Primary isolation plate showing* L. pneumophila *colonies 48 h after they first become visible (3–5 days after inoculation). A pink and blue iridescence can be seen at the periphery of colonies (original magnification ×3).*

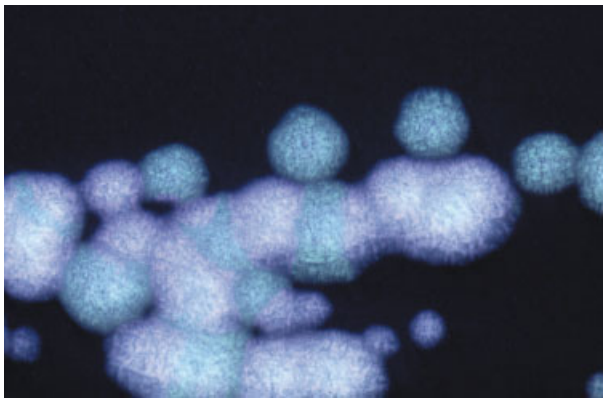

Figure 5 *Typical appearance of* Legionella *colonies showing the distinctive 'ground glass' appearance of and the variation in color that can be seen in young colonies (original magnification ×3)*

METABOLISM AND GROWTH REQUIREMENTS

Legionellae will grow in the temperature range of 29–40°C with an optimum of ~35°C. However, they will withstand higher temperatures, of 50°C and above, for a considerable time (>30 min), a characteristic which is exploited in their culture and isolation from clinical and environmental specimens (Dennis 1988). At temperatures below 20°C, particularly in nutrient poor environments, legionellae will enter a metabolically inert 'viable but nonculturable (VBNC)' state and can no longer be cultured on laboratory growth media. In this state the organisms retain viability and virulence for long periods, becoming metabolically active on entry into amebal or mammalian cells (Steinert et al. 1997; Ohno et al. 2003).

Legionellae are oligotrophs and utilize amino acids rather than carbohydrates as energy sources (Tesh et al. 1983) with sugars neither oxidized nor fermented. Glucose catabolism is achieved through the Entner–Doudoroff and pentose phosphate pathways, whereas the Krebs cycle facilitates carbon assimilation, and the gluconeogenic anabolic enzymes of the Embden–Meyerhof pathway are responsible for sugar synthesis (Hoffman and Pine 1982). Although there are some differences in amino acid requirements between species, subspecies, and strains, the amino acids arginine, cysteine, methionine, serine, threonine, and valine are required as essential nutrients by all isolates, whereas other strains also need isoleucine, leucine, phenylalanine, and tyrosine for growth (George et al. 1980; Tesh and Miller 1981). Proline, glutamate, and glutathione have also been found to stimulate the growth of some *Legionella* strains. In keeping with the use of the Krebs cycle as the major energy-producing pathway through amino acid catabolism, the activity of NADH in the organism is high (Hoffman 1984). The presence of calcium, cobalt, copper, magnesium, manganese, molybdenum, nickel, vanadium, and zinc in trace amounts all enhance bacterial growth (Reeves et al. 1981), but sodium chloride is inhibitory.

Iron is essential for all intracellular organisms and *L. pneumophila* has evolved multiple iron acquisition mechanisms. Factors involved include heme utilization (O'Connell et al. 1996b), a novel siderophore (legiobactin) that appears to be unique in its structure and regulation (Liles et al. 2000) and two internal ferric reductases (Poch and Johnson 1993). Genetic data indicate that *L. pneumophila* also produces a hydroxymate-like siderophore that promotes macrophage infection and a pyoverdin-like siderophore (Hickey and Cianciotto 1997; Viswanathan et al. 2000). L-Cysteine is needed for incorporation into proteins by the organism, but the lack of two key biosynthetic enzymes, serine transacetylase and *O*-acetylserine sulfydrylase, responsible for the conversion of serine to cysteine, results in the need for added amounts of this amino acid (Hoffman 1984). These enzymes are present in *L. oakridgensis*, which consequently does not require added cysteine after primary isolation (Orrison et al. 1983).

The organisms are obligate aerobes and are catalase- and superoxide dismutase-positive, weakly peroxidase-positive, oxidase-variable and possess the cytochromes *a–d*. Nitrates are not reduced and urea is not hydrolyzed. With few exceptions, the various species of *Legionella* liquefy gelatin. The positive hippurate hydrolysis of *L. pneumophila* forms a useful means to differentiate this species from many of the other members of the genus (Table 1).

Studies by Rowbotham (1983, 1993) have shown that some legionellae originally described as *Legionella*-like amebal pathogens (LLAP) can be obtained from environmental and clinical samples by cocultivation with amebae, but cannot be isolated using standard legionella culture media such as BCYE. Subsequent work has shown that these LLAPs are *Legionella* spp. and most can be grown, albeit poorly, on BCYE provided a reduced incubation temperature of 30°C is used (Adeleke et al. 2001).

GENETIC ANALYSIS

DNA homology

The genomes of the various species of *Legionella* are approximately 2.5×10^9 daltons (~3.9 Mb) (Bender et al. 1990) with a guanine and cytosine (G+C) content ranging from 38 to 52 mol% (average 42 mol%). The exception to this is *L. gresilensis*, which has a reported G+C content of 33 mol%. Based on DNA homology techniques, the legionellae are distinguishable from, and unrelated to, other bacterial genera. Indeed, following the initial isolation of *L. pneumophila*, DNA homology studies were used to confirm the phenotypic evidence that the organism constituted not only a new genus, but also a new bacterial family (Brenner et al. 1979). DNA relatedness is generally high (>90 percent) within

species, but ranges from 1 to 67 percent between different species within the genus (Brenner 1986). Many cloned genes of *L. pneumophila* possess homologies in other *Legionella* species, as shown for the *fur*, *mip*, *flaA*, *pilB*, *htpB*, *ompS*, and *sodB* genes.

Plasmids

The first report of a plasmid in *Legionella* was a 30 MDa cryptic plasmid found in *L. pneumophila* (Knudson and Mikesell 1980). Since then, plasmids of various sizes (12–>120 kb) have been found in many other species of *Legionella* (Brown et al. 1982; Maher et al. 1983; Doyle and Heuzenroeder 2002). Plasmid bearing isolates of *L. pneumophila* are frequently obtained from environmental sources but less commonly from clinical specimens. Brown and colleagues (1992) reported that strains with plasmids persisted in the environment longer than did plasmidless strains, and in support of this view, one 36 MDa plasmid has been shown to mediate resistance to ultraviolet (UV) radiation (Tully 1991). Legionella plasmids seem to be generally stable upon passage on laboratory media or in animals (Brown et al. 1982; Edelstein et al. 1986; Harrison and Saunders 1994) and they are mostly self-transmissible by conjugation (Mintz et al. 1992). The existence of an unstable 30 Kb genetic element, probably of phage origin, has been shown to be responsible for the phase-variable expression of virulence-associated lipopolysaccharide (LPS) in a strain of *L. pneumophila* (Luneberg et al. 2001). In the fully virulent wild-type strain the element is located on the bacterial chromosome, but in an avirulent phase-variation mutant the element is excised from the chromosome and replicates as a high copy number plasmid. The regulatory mechanism of this phase-variation has not yet been determined. Similarly, a large plasmid in a strain of *L. longbeachae* has been reported to encode a transcriptional regulator (LrpR). A *lrp*R mutant was found to have a reduced ability for intra-amebal replication and was attenuated for virulence in a guinea-pig model of infection (Doyle and Heuzenroeder 2002).

CELL-WALL COMPOSITION AND ANTIGENIC STRUCTURES

Cell-wall composition

The cell walls of *Legionella* species are typical of gram-negative organisms being trilaminar with an outer membrane, peptidoglycan layer, and cytoplasmic membrane; however, the composition is unusual. The subunits of peptidoglycan are extensively cross-linked producing a highly stable layer that confers some resistance to degradation by lysozyme (Amano and Williams 1983). The LPS composition of *Legionella* species has also been shown to be unusual, with the monosaccharide and the fatty acid composition being particularly complex in structure (Sonesson et al. 1994b). The LPS comprises a Lipid A part with 16–24 amide-linked 3-hydroxy fatty acids, 5–11 ester linked nonhydroxy fatty acids, and long-chain ester linked (ω-1)-oxo fatty acids and (ω-1)-hydroxy fatty acids. These features appear to be unique and characteristic for *Legionella* species (Sonesson et al. 1994a, b). It is thought that these unusually long branched-chain fatty acids may inhibit binding of the CD14 LPS receptor on macrophages thus reducing the endotoxic activity of *Legionella* LPS by ~1 000 fold compared to that of salmonella LPS (Wong et al. 1979; Neumeister et al. 1998). The LPS core oligosaccharide lacks the heptose and phospahate of classic LPS, but contains abundant 6-deoxy sugars and is highly O-acetylated. The O-chain polysaccharide of the *L. pneumophila* serogroup 1 LPS is a α(2–4)-linked homopolymer of the 5-*N*-acetimidoyl-7-*N*-acetyl derivative of 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid, termed legionaminic acid.

Although qualitatively similar, the whole-cell fatty-acid profiles of some *Legionella* species show marked and consistent quantitative differences and have consequently been used as chemotaxonomic markers. In addition, all species contain unusual members of the ubiquinone group of respiratory isoprenoid-quinones that contain 9–15 isoprenyl unit side-chains. The combination of whole-cell fatty-acid profiling and ubiquinone analysis has proved a valuable, although not routine, tool for the identification of legionellae (Diogo et al. 1999; Wait 1988).

Somatic 'O' antigens

LPS is the immunodominant antigen and *Legionella* are divided into serogroups by their reaction with hyperimmune antisera containing antibodies directed against the LPS, or somatic 'O', antigens. To date, 70 serogroups have been recognized among the 49 named species although in the cases of *L. sainthelensi* serogroup 2 and *L. erythra* serogroup 2 these are indistinguishable from *L. rubrilucens* and *L. santicrucis*, respectively (Table 1) (Harrison and Saunders 1994). There is also considerable antigenic overlap between some of the serogroups, particularly within the blue–white autofluorescent species complex, and therefore, hyperimmune antisera require extensive adsorbtion to render them serogroup specific. Conversely, there is significant antigenic heterogeneity within some serogroups such as *L. pneumophila* serogroup 1 (Thomason and Bibb 1984). Panels of monoclonally derived antibodies (mAb) directed against LPS epitopes have been used extensively to subgroup strains of *L. pneumophila* serogroup 1 for epidemiological purposes (Joly et al. 1986a; Helbig et al. 2002; Saunders and Harrison 1988).

Dournon and colleagues (1988) were the first to notice that strains (designated mAb2+ve) that reacted with one

such mAb were more likely to be isolated from patients than were strains that did not react (mAb2−ve strains). This led to the widely held view that mAb2+ve strains are more virulent than mAb2− strains. Some evidence that mAb2−ve strains infect human monocytes less well than do mAb2+ve strains was reported (Dournon and Rajagopalan 1987); however, more recent studies have not confirmed this (Edelstein and Edelstein 1993; Luck et al. 2001). An alternative suggestion is that the LPS of mAb2+ve strains is more hydrophobic than mAb2−ve LPS resulting in better survival of mAb2+ve strains in aerosols which, in turn, makes it more likely that they will be inhaled in a viable state by a susceptible individual than will be mAb2−ve strains. Two sets of data support this hypothesis. Dennis and Lee (1988) showed that mAb2+ve strains survived significantly better than did mAb2−ve strains in a laboratory generated aerosol held at a a suboptimal relative humidity of 60 percent. Secondly, Luck and colleagues (2001) isolated a mAb2−ve mutant from a mAb2+ve wild-type parent strain. Analysis showed that mAb2−ve strain had a point mutation in the active site of O-acetyltransferase and consequently its LPS contained legionaminic acid that lacked 8-O-acetyl groups: earlier work by the same group had shown that increased 8-O-acetylation of legionella LPS resulted in increased hydrophobicity of the organism (Zahringer et al. 1995)

Flagella

In addition to 'O' antigens legionella have 'H' or flagellar antigens (Figure 2). The single monopolar flagellum is composed of one major subunit, FlaA, of approximately 48 kDa. The flagellin is encoded by the *flaA* gene, which is found in almost all *Legionella* species so far described (Heuner et al. 1995). Expression of flagellin is associated with virulence and is regulated by growth phase and a range of environmental factors including temperature, osmolarity, and viscosity of growth medium (Heuner et al. 1999; Bosshardt et al. 1997). Flagella from all species share common antigens, a property which can be useful in confirming the identity of new or unknown strains of legionella (Harrison and Taylor 1988a; Bornstein et al. 1991). It has also been shown that flagella can be subtyped using panels of mAbs (Saunders and Harrison 1988).

TAXONOMY AND CLASSIFICATION

The causative agent of LD was initially designated the Legionnaires' disease bacterium (McDade et al. 1977). DNA–DNA homology studies on the few clinical and environmental isolates then available demonstrated that they were members of a distinct species and the family Legionellaceae and the genus *Legionella* were subsequently defined in 1979 for a single species *Legionella pneumophila* (Brenner et al. 1979). The rational for the creation of a new family was based largely on phenotypic grounds, as *L. pneumophila* was distinct from other gram-negative bacteria in both growth requirements and cell wall composition. Since this time, 48 other species of *Legionella* have been identified and formally described (Park et al. 2003) and the validity of the family Legionellaceae has been substantiated both phenotypically and phylogenetically (Harrison and Saunders 1994; Ratcliff et al. 1997).

Analysis of *L. pneumophila* strains by multilocus enzyme electrophoresis (MLEE) showed significant differences in enzyme profiles and suggested that the species should be split into three (Selander et al. 1985). Subsequent DNA–DNA hybridization studies essentially confirmed this, but as these DNA groups showed no consistent phenotypic differences the groups were designated as three subspecies: *L. pneumophila* subsp. *pneumophila*, *L. pneumophila* subsp. *pascullei*, and *L. pneumophila* subsp. *fraseri* (Brenner et al. 1988).

Classification at the genus level was initially controversial with some workers maintaining that the family should be divided into three genera: *Legionella*, *Fluoribacter*, and *Tatlockia* on the basis of some limited phenotypic differences (Garrity et al. 1980). Alternative names have been formally proposed and consequently *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. micdadei*, and *L. maceachernii* may be found cited in the literature as *F. bozemanae*, *F. dumoffii*, *F. gormanii*, *T. micdadei*, and *T. maceachernii*, respectively, (Fox and Brown 1993; Garrity et al. 1980). More recent phylogenetic studies have again demonstrated that the species of the family Legionellaceae are monophyletic showing no discrete divisions (Figure 6) (Harrison and Saunders 1994; Adeleke et al. 2001; Ratcliff et al. 1997; Fry et al. 1991). The only possible excepton to this is *L. geestiana* which appears to be an outlier of the genus in terms of both G+C content and *mip* and 16SrDNA gene sequences.

LABORATORY DETECTION AND IDENTIFICATION

As discussed above, the clinical picture of LD is not sufficiently distinct to establish a diagnosis with certainty. Laboratory tests are therefore essential for a definitive diagnosis. The main approaches to the laboratory diagnosis of legionella infections are culture of the causative organism, demonstration of the organism, its components or products in clinical specimens, and demonstration of significant levels of antibody directed against the organism in patients' sera.

Culture

Although frequently found in aquatic environments not associated with LD, legionellae do not occur in humans

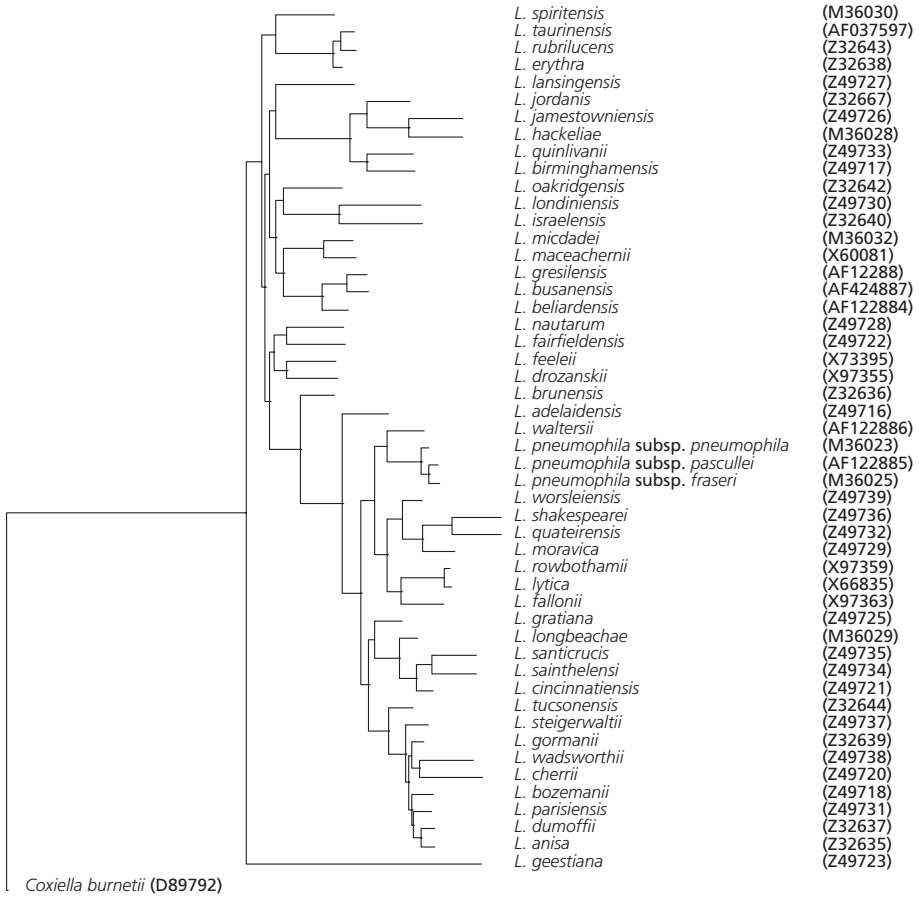

Figure 6 *Phlylogenetic tree showing the relationships between the small subunit RNA/DNA sequences from 48 of the 49 described species of* Legionella *(no sequence was available for* Legionella *genomospecies 1). The tree was constructed by using multiple aligned distances and neighbor joining analyses and rooted by reference to* Coxiella burnetii. *GenBank/EMBL accession numbers are shown in parentheses (kindly provided by Dr NK Fry).*

in the absence of disease (Bridge and Edelstein 1983). Hence, culture and isolation of legionellae from clinical specimens equates with infection and constitutes the 'gold standard' for diagnosis. Furthermore, culture and isolation may be the only way to establish a diagnosis if there are no reagents available to detect the infecting species/serogroup, or where a patient does not produce antibodies. Importantly, isolation of the infecting strain also allows subtyping to be undertaken providing valuable epidemiological data for the control and prevention of further cases.

The sensitivity of culture varies widely depending upon the laboratory attempting isolation but in situations where clinical awareness is high is in the order of 50–80 percent (Winn 1993; Dournon 1988). Specimens for culture should be taken as early as possible and ideally before antibiotic treatment is initiated. Lower respiratory tract specimens are most likely to yield positive results but sputa are quite satisfactory provided the specimens are pretreated. Severe forms of disease are also associated with bacteremia, and legionellae have been found in a wide range of nonrespiratory samples, such as blood, liver, spleen, and wounds, especially from samples taken at postmortem (Dournon 1988).

Selective media have been developed for the cultivation of legionellae from clinical and environmental specimens that are likely to contain other microbes. The two most widely used are based on BCYE agar and contain cephamandole, polymyxin B, and anisomycin (Edelstein 1981) or glycine, vancomycin, polymyxin B, and anisomycin (Wadowsky and Yee 1981). As these antibiotics are somewhat inhibitory to legionellae, growth is slower following inoculation on to these media. Pleural and lavage fluids should be concentrated by centrifugation prior to processing. Specimens can be heat treated at 50°C for 30 min should the presence of contaminating organisms be suspected or prove problematic. Additionally, an acid treatment similar to that used in environmental isolation work can be useful in severely contaminated samples. Sputum and lung tissue should be examined neat and diluted (10^{-1} and 10^{-2}) in distilled water or Page's saline to reduce the impact of inhibitors and contaminants (Dournon 1988).

Demonstration of the organism or its components

Despite the many advantages of culture, the time taken to obtain results by this method is measured in days. In contrast the direct demonstration of legionella antigen or nucleic acids in clinical specimens can be achieved within a few hours of specimen collection. Furthermore, a diagnosis may be established by visualization or detection of the organism in tissue even when they are no longer viable, after antibiotic therapy or retrospectively in fixed tissues.

Bacteriological stains such as Gimenez and modified Warthin–Starry have been used successfully to detect legionellae in clinical material (Greer et al. 1980; Pounder 1983) but these stains may reveal any bacterial species and so a specific diagnosis cannot be established. IF using rabbit hyperimmune antisera has been used to diagnose LD since legionellae were first recognized, but the poor sensitivity and the questionable specificity of these reagents have severely limited its use. Using reagents derived from monoclonal antibodies, usually in the form of fluorescein-isothiocyanate (FITC)-conjugates, can circumvent the problem of specificity, and one of these directed against the *L. pneumophila* major outer-membrane protein (MOMP) (Gosting et al. 1984) is now widely used for direct immunofluorescent antibody (DFA) detection of legionellae in both clinical and environmental samples (Figure 7). This reagent reacts with all the serogroups of *L. pneumophila* and so eliminates the need for repeated testing of a specimen with many different antisera. The specificity has been found to be excellent allowing a diagnosis to be confidentially established very rapidly (Edelstein et al. 1985). However, the sensitivities of both IF and DFA are still considerably lower than that of culture.

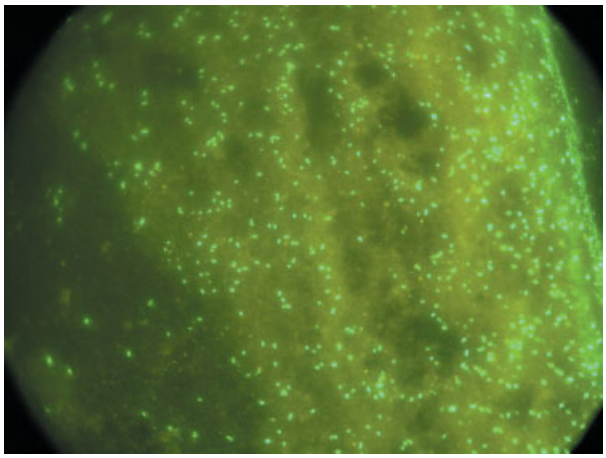

Figure 7 *DFA staining using an* L. pneumophila *specific FITC-conjugated monoclonal antibody of a water concentrate taken from a cooling tower associated with a large outbreak of LD*

Urinary antigen EIAs

It is now well established that the use of enzyme immunoassays (EIA) for the detection of *L. pneumophila* antigen in urine, allow diagnoses of LD to be established early in the course of infection (Hackman et al. 1996; Birtles et al. 1990; Harrison et al. 1998). Until the early 1990s, the use of such assays was restricted to those few reference laboratories able to develop and maintain in-house EIAs (Sathapatayavongs et al. 1982; Birtles et al. 1990) but commercially produced kits are now widely available. Currently available kits have been shown to be highly specific and reliable in routine use (Harrison and Doshi 2001; Dominguez et al. 1999) and legionella urinary antigen detection has become the single most widely used diagnostic methodology. The limitation to this method is the poor sensitivity that the kits have for LD caused by any legionellae other than the 'virulent' (mAb2+ve) *L. pneumophila* serogroup 1 strains. Practically this means that while the sensitivity is ~90 percent for the diagnosis of community-acquired LD, it is <50 percent for nosocomially acquired LD (Helbig et al. 2003).

Attempts to overcome this limitation led to the development of a 'broad spectrum' EIA that uses hyperimmune serum raised against a pool of *Legionella* species and serogroups to capture the soluble antigen (Tang and Toma 1986). Unfortunately, this method of producing the capture antiserum is not amenable to standardization and this methodology has not been translated into a commercial assay. An alternative approach is to use reagents prepared against defined *L. pneumophila*, or preferably *Legionella* genus, common antigens. While the successful detection of common protein antigens such as momp or mip in urine has not been reported, preliminary studies suggest that a *Legionella* genus common 19 kDa peptidoglycan-associated lipoprotein (PAL) target is detectable in the urine of experimentally infected guinea-pigs (Kim et al. 2003). Whether or not this is also true for humans with a natural infection remains to be seen.

Molecular methods

Methods for the detection of legionella nucleic acid have been available since the early 1980s when Kohne and colleagues (1984) developed a radiolabeled DNA probe complementary to regions of the ribosomal RNAs of all 20 *Legionella* species known at the time. A commercial nucleic acid hybridization test based on this work, and intended for the detection of legionellae in clinical samples, was introduced soon after. This kit was shown to be reasonably sensitive performing similarly to IF, but was less sensitive than culture (Finkelstein et al. 1993; Pasculle et al. 1989). Furthermore, questions concerning the kit's specificity (Laussucq et al. 1988) and its use of radioisotopes limited the kit's utility and it was subsequently withdrawn from the market.

The theoretical sensitivity of polymerase chain reaction (PCR) (i.e. detection of one copy of target sequence) quickly led to widespread use as an alternative to direct probing, which typically requires large numbers of organisms. Furthermore, as alternatives to radioisotope labeling are now available which have at least equivalent sensitivity and none of the inherent disadvantages, PCR is now seen as the molecular method of choice, promising advantages of both specificity and sensitivity. Development of PCR-based assays for legionellae have focused on three main targets. Assays designed to detect *L. pneumophila* typically use primers directed against the *mip* gene while those targeting *Legionella* species use primers for either the 5SrRNA or 16SrRNA genes (Starnbach et al. 1989; Mahbubani et al. 1990; Lisby and Dessau 1994; Jonas et al. 1995; Ballard et al. 2000; Fry and Harrison 1998). Where lower respiratory tract (LRT) samples are used, PCR appears to be at least as sensitive as culture (Jaulhac et al. 1992; Cloud et al. 2000; Jonas et al. 1995). However, this does not seem to be the case for detection of legionellae in environmental samples, where the sensitivity is consistently lower than culture (Ballard et al. 2000; Riffard et al. 1903). The difference is probably due to the concentration steps, typically 100-fold, which are integral to the processing of environmental samples. *Legionella* DNA has been detected in nonrespiratory samples such as serum and urine and leukocytes samples, but with a lower sensitivity than culture (Lindsay et al. 1994; Murdoch et al. 1996; Helbig et al. 1999). At present the status of PCR for the diagnosis for LD is much the same as applied to urinary antigen detection in the 1980s, that is, confined to a few specialist and reference laboratories able to use it in conjunction with the full range of other diagnostic approaches. Until validated commercial kits are available, the real impact of PCR will not be known.

Diagnosis by estimation of antibody levels

For more than 20 years the primary method for the diagnosis of LD was by serology and although now displaced by urinary antigen testing, estimation of antibody levels against killed preparations of legionellae is still a very commonly employed method for the diagnosis of LD. It is an important tool for case finding during outbreak investigations and for late or retrospective diagnosis. However, this approach does not often allow a diagnosis to be established during acute illness, and so has little value in patient management or in the early detection of outbreaks.

Historically, the IFAT was the method used in 1977 to establish that *L. pneumophila* was the causative agent of LD (McDade et al. 1977). Since this time, several variants of this test have been comprehensively evaluated for the diagnosis of infection caused by *L. pneumophila* serogroup 1, and the IFAT became the reference test for LD diagnosis (Wilkinson et al. 1981; Harrison and Taylor 1988b). The test used in the UK and parts of Europe until the late 1990s employed a formalized yolk-sac antigen (FYSA) and FITC labeled anti-human conjugate capable of detecting IgG, IgM, and IgA. This test was both sensitive (~80 percent) and highly specific (>99 percent) (Bornstein et al. 1987; Harrison and Taylor 1988b), the latter being particularly important in a disease of low prevalence such as LD, if a positive result is to have any real significance. Reagents for this IFAT are no longer available but various commercially produced IFAT reagents, typically using agar-grown antigens, can be obtained. Reagents for IFATs to detect infection caused by species and serogroups other than *L. pneumophila* serogroup 1 are also commercially available, however it should be emphasized that the sensitivity and specificity of IFAT using these reagents is largely unknown.

In addition to the IFAT, several other methods for antibody detection such as microagglutination (Harrison and Taylor 1982; Farshy et al. 1978) and indirect hemagglutination (Edson et al. 1979; Lennette et al. 1979) have been devised. As EIAs are widely used in microbiology, several have been developed for *L. pneumophila* (Wreghitt et al. 1982; Barka et al. 1986; Herbrink et al. 1983). Reagent manufacturers market diagnostic kits using this methodology but these usually use antigens prepared from pools of *L. pneumophila* serogroups 1–6. None of these assays use purified antigens and so the EIA plates are coated with a complex mixture of bacterial proteins and LPS. The use of such crude pooled antigens may in part be responsible for the poor performance of these assays (Elder et al. 1983; Malan et al. 2003; Harrison and Taylor 1988b).

Identification of species

A bacterium that grows on BCYE but not on the same medium lacking supplemental L-cysteine, is Gram's stain negative and catalase-positive can be presumptively identified as a *Legionella* spp. (Harrison and Taylor 1988a). Very few organisms that meet these criteria will not be subsequently confirmed as *Legionella*, however the same does not apply if blood agar is used as the nutritional control medium. Some heterotrophic non-*Legionella* bacteria, particularly of environmental origin, can show L-cysteine dependence (Thacker et al. 1981) and so will grow on BCYE but not on blood agar. Also, it should be noted that while all legionellae, with the exception of *L. oakridgensis*, require L-cysteine for primary isolation, some species and many laboratory adapted strains will grow in its absence thereafter (Orrison et al. 1983; Harrison and Taylor 1988a).

For most clinicians, establishing the identity of the organism at the genus level is quite sufficient as there is

no significant difference between the illnesses caused by the various *Legionella* species or their treatment. However for epidemiological, prevention, and control or research purposes it is frequently essential to be able to precisely characterize the species. There are a few simply demonstrated phenotypic characteristics that allow the Legionellaceae to be subdivided into groups of species, or in some cases into individual species (Table 1). For example, when exposed to long-wave UV light at 366 nm, the colonies of some species autofluoresce brilliant blue–white (the blue–white autofluorescent clade of ten species) or autofluoresce red (*L. erythra*, *L. rubrilucens*, and *L. taurinensis*) (Figure 8). Hippurate hydrolysis can be useful for the identification of *L. pneumophila* if more specific reagents are not available and some species produce characteristic colored colonies when grown on dye-containing media. Additionally, many species produce a brown pigment (a polymer of homogentisic acid) from D-phenylalanine or tyrosine, and most will liquefy gelatin. Unfortunately, most species of *Legionella* cannot be differentiated using these tests. As members of the Legionellaceae are asaccharolytic, the various biochemical tests commonly used to identify non-*Legionella* bacteria in microbiology laboratories either give weak or variable results or are absent altogether. Commercial identification systems are of little use. Mauchline and Keevil (1991) used the BIOLOG substrate utilization system to successfully distinguish some species, but the method was expensive and needed modification for use. Commercial reagents for the serological identification of the various *L. pneumophila* serogroups are widely available and usually reliable, but serological identification of the other species and serogroups is much more complex and usually can only be undertaken with confidence by specialist laboratories.

Genetic analysis by DNA hybridization is probably still the definitive way to identify individual species. However, a number of other molecular approaches are also useful and in some cases may be better. Most *Legionella* species can be differentiated by their rRNA gene restriction digest patterns (Grimont et al. 1989; Saunders et al. 1988) and this method (ribotyping) has recently been evaluated using a commercially available platform. Results indicate that when a sufficiently comprehensive database of profiles has been established, it may be an effective, albeit expensive, methodology (Cordevant et al. 2003). The increasing introduction of DNA sequencing technologies to routine microbiology laboratories suggests that DNA sequence-based identification of organisms will become commonplace. An identification scheme based on DNA sequence differences in the legionella macrophage infectivity potentiator (*mip*) gene has been proposed (Ratcliff et al. 1998) and is likely to become the method of choice. With the exception of *L. geestiana* this approach allows the unambiguous identification of all species so far described.

Epidemiolgical typing

Legionellosis is acquired by inhalation or aspiration of legionellae from a contaminated environmental source. Thus, when a case of legionellosis is recognized, others may become infected from the same source if appropriate control action is not taken to reduce the risk of transmission. The source of the outbreak or incident can be determined by epidemiological investigation together

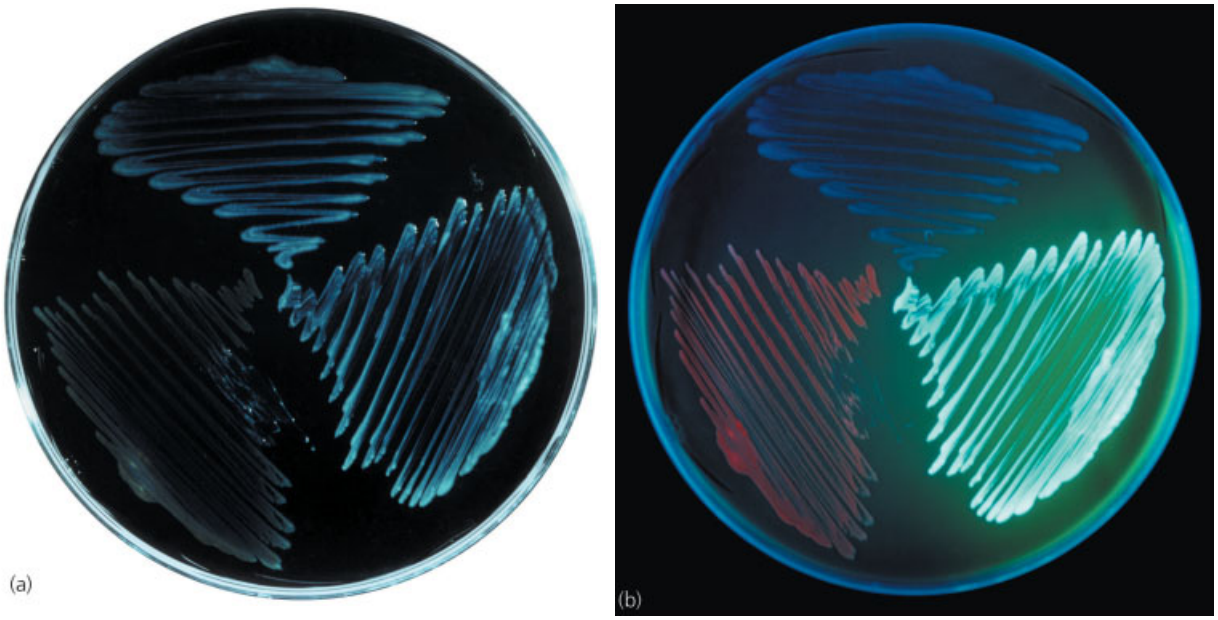

Figure 8 *Demonstration of autofluorescence of* L. pneumophila, L. anisa, *and* L. erythra *grown on BCYE when* **(a)** *examined under natural light or* **(b)** *illuminated with long-wave UV light at 366 nm.* L. bozemanii *colonies autofluoresce brilliant blue–white,* L. erythra *colonies autofluoresce red. Colonies of* L. pneumophila *do not autofluoresce (kindly provided by N. Doshi).*

with characterization of legionellae isolated from patients and putative sources (Harrison and Saunders 1994; Fry et al. 1999). Occasionally, the species causing infection is so rarely encountered that simply isolating the organism from an associated environmental site is sufficient evidence that it was probably the source of infection. A cluster of nosocomial cases of LD caused by *L. dumoffii* in nonsterile distilled water used for respiratory equipment is one such example (Joly et al. 1986b). However, the vast majority of cases of legionellosis are caused by *L. pneumophila* and most of these are serogroup 1 strains. These strains are also frequently encountered in the environment so isolates of this serogroup must be further differentiated if a convincing epidemiological link is to be established.

Isolates of *L. pneumophila* serogroup 1 can be rapidly subgrouped using mAb subgrouping with panels based on the international mAb subgrouping panel (Joly et al. 1986a; Helbig et al. 1997). However, mAb subgrouping lacks discrimination, dividing *L. pneumophila* serogroup 1 into only 8–10 phenons depending on which mAb panels are used (Fry et al. 1999). Consequently, in addition to this phenotypic typing method, a large number of molecular methods have been successfully employed for epidemiological purposes. These include MLEE, ribotyping, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (FRLP), restriction endonuclease analysis (REA), and arbitrarily primed-PCR (Edelstein et al. 1986; Harrison and Saunders 1994; Fry et al. 1999; Van Belkum 2002). One of these methods, AFLP analysis, has been adopted as an international standard and is now widely used by members of the European Working Group for Legionella Infections (EWGLI) (Fry et al. 2002; Garcia-Fulgueiras et al. 2003) (Figure 9). Two interlaboratory studies have assessed the reliability of this AFLP method. The method was robust, rapid, and allowed the exchange of data between laboratories, however proficiency testing showed that a significant proportion of laboratories could not correctly identify isolates 100 percent of the time. Incorrect identification was typically a result of data analysis issues (e.g. difficulties comparing the AFLP banding patterns) rather than the method itself (Fry et al. 1999, 2002). One way to simplify analysis and data exchange is to use DNA-sequence-based approaches to type the organism of interest. The advantages of this approach are now well rehearsed and include portability between laboratories and the consequent ease with which global databases can be established (Maiden et al. 1998). Recent work has shown that this approach is applicable to *L. pneumophila*. Using only three gene loci (*flaA*, *mompS*, *proA*) to identify allelic variation, Gaia and colleagues (2003) were able to achieve a level of discrimination equivalent to that obtained by AFLP. Adding further gene targets is likely to improve the discrimination still further.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Antibiotics

Erythromycin is the classic drug of choice for the treatment of LD, however its selection as the standard treatment was based primarily on a review of limited treatment data from the 1976 Philadelphia outbreak and

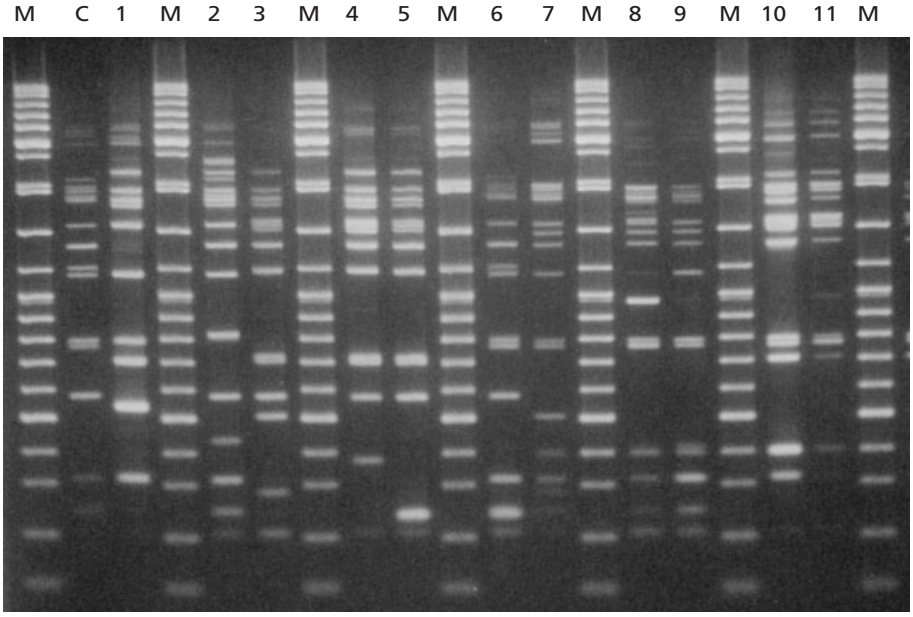

Figure 9 *Example of the profiles obtained using the internationally agreed EWGLI protocol for AFLP typing (Fry et al. 2002). C, control strain EUL137; M, marker (GeneRuler, MBI Fermentas); tracks 1–12, unrelated strains of* L. pneumophila *serogroup 1*

early experiences with nosocomially acquired LD rather than from prospective clinical trials (Fraser et al. 1977; Kirby et al. 1978). No substantial prospective clinical trial has been published and, given the comparative scarcity of LD cases, it is unlikely that an adequate comparative clinical trial of the disease will ever be completed (Edelstein 1998). Consequently, decisions about new therapies have to be based on laboratory experiments and limited clinical data.

In vitro, legionellae are susceptible to a very wide range of antibiotics (Schulin et al. 1998), but for several reasons these data are not necessarily predictive of their in vivo efficacy (Gibson and Fitzgeorge 1983). Firstly, the media used to culture legionellae inhibit the activity of some antibiotics making valid determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) difficult (Edelstein and Meyer 1980; Marques and Piedade 1997). Secondly, and more importantly, some antibiotics that are active in vitro (e.g. β-lactam antibiotics) are not active in vivo because their cell permeability is poor and they do not reach the intracellular legionellae (Vilde et al. 1986). To overcome these obstacles, both animal and intracellular infection models have been developed and these appear to provide clinically relevant results (Gibson and Fitzgeorge 1983; Edelstein 1998).

The antimicrobial agents that show the best activity against legionellae are those that are concentrated within the phagocytic cells such as macrolides and quinolones and these antibiotics form the basis of recent treatment guidelines (British Thoracic Society 2001; Bartlett et al. 2000). Although erythromycin is the traditional drug of choice, studies reveal that it is only inhibitory, and reversibly so, in its activity against intracellular legionellae (Horwitz and Silverstein 1983). In addition, it is often poorly tolerated by many patients and treatment failures are not infrequent (Edelstein 1998). In contrast, azithromycin is bactericidal even at quite low concentrations (Edelstein and Edelstein 1991), is highly effective in preventing disease in an aerosol-infected guinea pig model (Fitzgeorge et al. 1993), and is well tolerated by patients. Azithromycin is only licensed for oral use in some countries and is not suitable for treatment of severe legionellosis in this form. Although clarithromycin is significantly less active than azithromycin, it is more active than erythromycin and so is recommended in some guidelines (British Thoracic Society 2001). Most fluoroquinolones are more active than erythromycin in vitro and some have been shown to be highly effective in both animal and cell-culture models of infection (Dournon et al. 1986; Rajagopalan-Levasseur et al. 1990; Edelstein et al. 1996).

Other agents and control measures

Under laboratory conditions, legionellae are quite susceptible to a wide variety of inactivation protocols. The organism is inactivated readily by drying, and optimal survival in aerosols occurs in atmospheres of relatively high humidity (Berendt 1980; Dennis and Lee 1988). The thermal killing of legionellae varies widely depending upon the test conditions but it was reported in one study that *L. pneumophila* could be recovered from a model plumbing system at temperatures between 20 and 50°C, but did not survive at 60°C or above (Rogers et al. 1994). In addition, *L. pneumophila* organisms were readily inactivated by exposure to 1.25 ppm of chlorine in 15 min and by 5 ppm in 1 min (Wang et al. 1979), and at these concentrations the electron transport of the organism was impaired and cell lysis induced. A 99 percent reduction in *L. pneumophila* bacterial cell numbers was effected in 5 min using 0.1 μg/ml ozone and in 30 min with 1 mg/ml H_2O_2 (Domingue et al. 1988), whereas UV radiation produced a 5 $\log_{10}$ kill in less than 1 h (Muraca et al. 1987). However, as is the case with the in vivo and in vitro evaluation of antibiotics, the disinfecting regimens determined in the laboratory are not necessarily reflected in field trials.

Control measures depend on the eradication or suppression of legionella in contaminated water supplies, especially those from which an aerosol can be generated. The control regime used will be largely dictated by a range of local environmental factors such as the complexity of the water system, the degree of pre-existing contamination (e.g. biofilm) and the usage of the system (Lin et al. 1998). Disinfectants that are suitable for legionella inactivation are metal ions (copper and silver), oxidizing agents, nonoxidizing agents, and UV light. Oxidizing agents include halogen-containing compounds (chlorine, bromine), ozone, and hydrogen peroxide. Nonoxidizing reagents include heterocyclic ketones, guanidines, thiocyanates, and halogenated glycols. Among the oxidizing agents, chlorine is the most widely used for potable water, and for cooling towers the halogen releasing organic, bromo-chloro-dimethyldantoin (BCDMH), has been found to be quite effective (Bentham and Broadbent 1995). Among the nonoxidizing agents, 2,2-dibromo-3-nitropropionamide (DBNPA) is probably the most effective (Kim et al. 2002). Whichever regime is selected, it must be noted that the biological load in these systems profoundly influences inactivation rates. In addition to disinfection, remedial cleaning and flushing of water systems, as well as the removal of sediment from hot water tanks, coupled with water treatment and constant water monitoring, are crucial to prevent the explosive outbreaks recorded to date.

PATHOGENICITY AND VIRULENCE FACTORS

Legionella species are not per se adapted to the human host. That they cause disease in humans is essentially an accident of nature; the pathogenic mechanisms have

evolved to enable legionellae to survive in their protozoal host also enabling them to multiply in alevolar macrophages.

Intracellular replication

Infection in humans occurs following inhalation of fine aerosol (<5 μm particle size) containing organisms that are both viable and virulent. Once within the alveoli, the legionellae are taken up by alveolar macrophages where they multiply. As a result of this multiplication in polymorphonuclear leukocytes (PMN), more macrophages and erythrocytes infiltrate the alveoli causing capillary leakage and so edema (Baskerville et al. 1983). Two modes of entry into macrophages have been observed. The first termed 'coiling phagocytosis,' in which long phagocytic pseudopods coil round the organism, was extensively studied by Horwitz and colleagues (Horwitz and Silverstein 1980; Horwitz 1983; Horwitz and Maxfield 1984; Shuman and Horwitz 1996). Although initially thought to be highly significant, workers have since reported that other strains of *L. pneumophila* are taken up by conventional phagocytosis (Rechnitzer and Blom 1989). Phagocytosis is mediated by a three component, receptor–ligand–acceptor molecule, system comprising complement receptors on phagocytes (CR1 and CR3), fragments of the complement component C3 fixed on the bacterial surface and the MOMP, which acts as the C3 acceptor molecule, in the bacterial outer membrane (Payne and Horwitz 1987). During phagocytosis, the plasma membrane is remodeled with components of the phagocyte being sorted into, or out of, the nascent phagosome: the CR3 receptors are concentrated within the phagosome while class-I and -II major histocompatibility complex (MHC) molecules are excluded from it. This mode of entry appears to limit the oxidative burst of the phagocyte and so enhances intracellular survival of the legionellae. A less well-characterized opsonin-independent mode of entry has also been reported (Stone and Abu Kwaik 1998). Once within the macrophage, legionellae are able to reprogram the phagocytes endocytic pathway to escape killing by inhibiting acidification of the nascent phagosome and preventing phagosome/lysosome fusion. Subsequently, the phagosome recruits smooth vesicles, mitochondria, and rough endoplasmic reticulum and within 4–8 h a ribosome-lined replicative vacuole is formed. Multiplication of the legionellae proceeds by binary fission with a doubling time of approximately 2 h. During the late replicative phase, the legionella-containing phagosome merges with lysosomes but replication continues until the host cell is packed with organisms and finally disrupts releasing bacteria to infect further host cells. The actual killing and lysis of the phagocytes appears to occur in a biphasic manner. In the early stages of infection legionellae induce apoptosis, a process that is mediated by the activation of caspase-3 (Gao and Abu Kwaik 1999), but once bacterial replication ceases the legionellae cause necrosis of their host cell probably by inducing a pore-forming toxin (Alli et al. 2000).

Growth phase

A striking feature of *L. pneumophila* is the significant phenotypic plasticity that it exhibits in different growth phases. In planktonic, nutrient poor conditions, they can assume a VBNC state while two distinct phenotypes have been described during their intracellular life-cycle. From careful microscopic observations, Rowbotham (1980) first reported that once within the ambeal host, legionellae first went through a 'replicative phase' forming large bacteria-filled vacuoles. They then entered an 'active infective phase' marked by their synchronous conversion from nonmotile rods to highly motile coccobacilli that were seen to escape from the lysed host cells and disperse (Rowbotham 1980). This observational model has subsequently been confirmed by phenotypic and molecular studies of *L. pneumophila* infected macrophages. The replicative legionellae are sodium-resistant, nonmotile bacilli and grow exponentially, while the postexponential infective phase organisms express a number of traits associated with virulence including, sodium-sensitivity, cytotoxicity, osmotic resistance, enhanced resistance to biocides, the capacity to evade phagosome-lysosome fusion, and motility. One of the triggers for the switch from the replicative to the infective phase seems to be amino acid depletion and involves the stringent response and the sigma factor RpoS (Bachman and Swanson 2001; Heuner et al. 2002). It is postulated that while intracellular conditions are suitable, the legionellae replicate in their specialist phagosome, but when nutrients become limiting, the bacteria coordinately express several traits that bring about their escape from the depleted cell and their transmission to a new host (Swanson and Hammer 2000).

Identification of genetic determinants of virulence

In recent years, a number of genetic loci have been identified in *L. pneumophila* that are required for its intracellular survival. Many, but not all, of these loci are necessary for the infection process in both mammalian and protozoan cells.

THE *MIP* GENE

The first virulence associated gene of *L. pneumophila* to be identified was the macrophage infectivity potentiator (*mip*) gene which encodes a 24 kDa protein surface protein (Mip). Mip is a prokaryotic homolog of the FK506-binding proteins and exhibits peptidyl-prolyl *cis/trans* isomerase (PPIase) activity. *mip*-like genes have

been detected in all other species of legionella and some other bacteria. Cianciotto and colleagues (1990), using a pair of isogenic strains (one of which was deficient in Mip), demonstrated that Mip was required for the full expression of virulence by *L. pneumophila*. It appears that Mip is required either for optimal internalization of the legionellae by macrophages or for resistance to the bactericidal mechanisms active shortly after phagocytosis. It is, however, noteworthy that although much less efficiently, the Mip-deficient mutant did multiply in macrophages and did produce illness in guinea-pigs. Clearly Mip can only contribute in part to the virulence of a strain.

THE *DOT/ICM* GENE

The Dot/Icm system of *L. pneumophila* is encoded by genes located in two 20 kb regions of the chromosome which may, at least in some strains, constitute a single pathogenicity island (Vogel and Isberg 1999; Brassinga et al. 2003). The *dot* (defective in organelle trafficking) and *icm* (intracellular multiplication) loci were independently identified by two different laboratories (Segal and Shuman 1997; Vogel et al. 1998) so the majority of the 24 genes so far identified have two designations**.** The Dot/Icm system shares homology with type IV transporters of other pathogenic bacteria such as *Bordetella pertussis*, *Agrobacterium tumefaciens*, *Helicobacter pylori*, and *Brucella* species. Type IV systems, which are thought to have evolved from systems involved in conjugative transfer of DNA, are critical for the pathogenic process as they export important virulence factors across bacterial membranes. Hilbi and colleagues (2001) showed that the Dot/Icm system is required for stimulation of uptake of the bacteria upon contact with the host cell. Two proteins have been identified that are secreted by Dot/Icm: DotA and RalF. DotA, previously thought to be an inner membrane protein, may actually be a pore-forming protein that is inserted into the host membrane (Nagai and Roy 2001). RalF is required for the localization of the ADP-ribosylation factor on phagosomes containing *L. pneumophila* (Nagai et al. 2002).

OTHER IMPLICATED GENES

L. pneumophila contains a type II secretion system that is required for the expression of type IV pili (Liles et al. 1998). The system is known to transport several molecules including the 38–42 kDa Zn-metalloprotease. This enzyme, variously called tissue destructive protease, zinc metalloprotease, and major secretory protein, has been studied in detail and has been shown to be cytotoxic and hemolytic. Experimentally, it causes pulmonary lesions which closely resemble those seen in infection (Quinn et al. 1989; Conlan et al. 1988; Szeto and Shuman 1990; Williams et al. 1993). The type II secretion system is dependent on the *pilBCD* genes that are involved in the biogenesis of a temperature-regulated type IV pilus (Stone and Abu Kwaik 1998). Two genes have been studied in detail: *pilE* (pilin protein) and *pilD* (prepilin peptidase). The *pilE* gene/pilin is not required for intracellular growth but may be involved in attachment to the host cells. However, the prepilin peptidase is essential for pilus production and type II secretion of proteins. Other genes that promote various stages of intracellular infection, but which are less well studied, included the *pmi* (protozoan macrophage infectivity) gene and *mil* (macrophage-specific infectivity) gene (Gao et al. 1998).

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Bordetella

ROGER PARTON

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DEFINITION

Bordetella spp. are minute, gram-negative, nonacid-fast, nonsporing coccobacilli. They do not ferment carbohydrates. There are eight named species, four motile and four nonmotile. Seven species appear to be obligatory parasites, mostly inhabiting the surface of the respiratory tract of man and other warm-blooded animals, including birds. These are aerobic and their optimum temperature is 35–37°C. Some species appear to be capable of survival within mammalian cells. One species is an environmental organism capable of anaerobic growth. Four species are known to produce a range of virulence factors and expression of these factors can be modulated by environmental signals. The G + C content of the DNA is 60–70 percent and the type species is *B. pertussis*.

INTRODUCTION

Until recently, there were four recognized species in the genus. *B. pertussis* was described by Bordet and Gengou (1906) as the cause of whooping cough. *Bordetella bronchiseptica* was first obtained by Ferry (1910) from the respiratory tract of dogs and is now known to be an important respiratory pathogen in wild and domesticated animals, and occasionally affects man. *Bordetella parapertussis* was isolated by Bradford and Slavin (1937) from cases of mild whooping cough and recognized as being different from *B. pertussis*. More recently, ovine strains of *B. parapertussis*, distinct from human strains, have been identified. *Bordetella avium* was first proposed as a species by Kersters et al. (1984) to include agents of respiratory disease of turkey poults originally isolated by Filion et al. (1967). Four more species are now recognized. The species *Bordetella holmesii* was proposed for a group of strains, isolated from 1983 onwards, from blood cultures of patients, some of whom were immunocompromised (Weyant et al. 1995). *Bordetella hinzii* was the name proposed by Vandamme et al. (1995) for a *B. avium*-like group of organisms found mainly in the respiratory tracts of chickens and turkeys and thought to be nonpathogenic. Possibly the first such isolate, however, was from human sputum in 1957 (Cookson et al. 1994). *Bordetella trematum* contains isolates from human wound and ear infections (Vandamme et al. 1996). The most recently named species, *Bordetella petrii*, consists of a single isolate obtained from an anaerobic, dechlorinating microbial consortium from a fluidized bed bioreactor that had been inoculated with river sediment (von Wintzingerode et al. 2001).

For a more detailed, historical perspective, and access to the earlier literature on *Bordetella*, the chapter by Wardlaw (1990), in a previous edition of this volume, is recommended. The main aim of this chapter is to summarize the vast amount of recent information on the genus. The whole-genome sequences of *B. pertussis*,

B. parapertussis, and *B. bronchiseptica* have now been completed (Parkhill et al. 2003) and sequencing of the *B. avium* genome is in progress (www.sanger.ac.uk/Projects/B_avium/). This information is already being used to further our understanding of these organisms, how they grow and respond to changing environmental conditions, and how they interact with their respective hosts to cause disease.

CLASSIFICATION

The genus *Bordetella*

The first three recognized species were included in the genus *Haemophilus* before the introduction of the genus *Bordetella* by Moreno-Lopez (1952) and named in honor of the pioneering work of Jules Bordet. *B. bronchiseptica* had previously been included in the genera *Alcaligenes* and *Brucella*. The original specific name *bronchicanis* was changed to *bronchiseptica* after isolation of similar organisms from animal species other than dogs. *B. avium* was identified originally as *Alcaligenes faecalis*. The *B. hinzii* group had been referred to previously as *A. faecalis* type II, turkey coryza (TC) bacterium type II, *Alcaligenes* spp. strain C_2T_2 as well as *B. avium*-like. Their present name was proposed to honor K.-H. Hinz, in recognition of his work on avian isolates of *Bordetella*. *B. holmesii* was formerly known as CDC nonoxidizer group 2 (NO-2) and was named in honor of B. Holmes for his contribution to the study of unusual and opportunist bacteria. The isolates now classified as *B. trematum*, to reflect their association with wounds and other exposed parts of the human body, were collected originally as atypical bordetellae or unidentified organisms. *B. petrii* was named in honor of R.J. Petri, the inventor of the Petri dish.

On the basis of a numerical taxonomic study covering a wide range of tests and properties, Johnson and Sneath (1973) suggested that the genus *Bordetella* should be grouped in the family Brucellaceae. The three species then known, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, were grouped with *A. faecalis* and *Brucella*. This group had affinities with *Acinetobacter*, *Moraxella*, and *Neisseria* strains, but was distinct from the *Haemophilus*, *Actinobacillus*, *Pasteurella* group. Subsequently, their taxonomic position became uncertain until De Ley et al. (1986), after extensive DNA–rRNA hybridization and phenotypic analyses, proposed that *Bordetella* should be included in a new family, Alcaligenaceae. The four species known at the time, now including *B. avium*, were found to belong to the third rRNA superfamily but their only close neighbor was the genus *Alcaligenes* and these two genera had a common separate position within the gram-negative bacteria. On the basis of the T_m parameter (melting temperature of the rRNA–DNA hybrids), the nearest neighbors to the bordetellae (T_m 79.0–81.9°C) were *Alcaligenes* (now *Achromobacter*) *xylosoxidans* (T_m 79°C) followed by *Alcaligenes faecalis* (T_m 75.5°C). *Brucella* strains were quite unrelated (T_m 58°C). More recent evidence, from 16S rRNA gene sequence analysis of all eight *Bordetella* spp. has confirmed a common phylogeny with *Achromobacter* and *Alcaligenes* (Weyant et al. 1995; Gerlach et al. 2001). These three genera are now assigned to the β-Proteobacteria, Class *Neisseriae*, Order *Burkholderiales*, Family Alcaligenaceae (Garrity 2001). They also have similar cellular fatty acid profiles, with $C_{16:0}$ and $C_{17:0cyc}$ (except in *B. pertussis*) as their main constituents, and contain ubiquinone 8 as the major respiratory quinone. *Bordetella*, *Achromobacter*, and *Alcaligenes* are clearly closely related and phenotypically similar. Some differential characteristics of these and other phenotypically similar genera are described elsewhere (Johnson and Sneath 1973; Kersters et al. 1984; Pittman 1984b; Vandamme et al. 1995, 1996; von Wintzingerode et al. 2001).

Species designation

Various studies have suggested that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are very closely related: DNA–DNA hybridization analyses; T_m values of their rRNA–DNA hybrids; mean G + C contents of their genomic DNA; similarity in their 23S rRNA gene sequences and, most recently, the comparison of whole genome sequences. Another estimate of genetic relatedness was obtained by Musser et al. (1986) in a survey by multi-locus enzyme electrophoresis of 15 metabolic enzymes in 60 strains of mammalian bordetellae obtained from world wide sources at different times. They found such limited genetic diversity, compared with most other pathogenic bacteria, to suggest that continued recognition of separate species was justified only on clinical and historical grounds. This, and other evidence, has led to the conclusion that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are subtypes of a single genomic species (strains whose DNAs are 70 percent or more related, and with 5°C or less divergence in T_m values). By the same criteria, *B. avium*, *B. holmesii*, *B. hinzii*, *B. trematum*, and *B. petrii* each form distinct species (Vandamme et al. 1995, 1996; Weyant et al. 1995; von Wintzingerode et al. 2001). For the sake of convenience in this chapter, in discussing the distinctive phenotypic and pathogenic properties of the bordetellae, the traditional specific names are appropriate. Some of the differential features of these species are shown in Table 1.

Evolutionary considerations

A number of lines of evidence have been used to write a possible evolutionary history of the *Bordetella* spp. (Rappuoli 1994; Gerlach et al. 2001). Their close

Table 1 *Differential characteristics of* Bordetella *species*

Characteristic	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	*B. hinzii*	*B. holmesii*	*B. trematum*	*B. petrii*
Growth: colonies visible in (days)	3	1–2	1	1	2	2–3	1	1–2
Peptone agar:								
growth	–	+	+	+	+	+	+	+
browning	–	+	–	–	–	+	–	–
Growth on MacConkey agar	–	+	+	+	+	v	+	+
Growth on Simmons citrate agar	–	v	+	v	+	–	–	?
Motile, peritrichous flagella	–	–	+	+	+	–	+	–
β-like hemolysis	+	+	v	v	–	–	–	–
Nitrite formation[b]	–	–	+	–	–	–	v	–
Denitrification[b]	–	–	–	–	–	–	v	+
Oxidase	+	–	+	+	+	–	–	–
Urease	–	+	+	–	v	–	–	–
Major cellular fatty acids[a]	$C_{16:1w7c}$ $C_{16:0}$	$C_{16:0}$ $C_{17:0cyc}$	$C_{16:0}$ $C_{16:1w7c}C_{17:0cyc}$	$C_{16:0}$ $C_{17:0cyc}$	$C_{16:0}$ $C_{17:0cyc}$	$C_{16:0}$ $C_{17:0cyc}$	$C_{16:0}C_{17:0cyc}$	?
G + C content of DNA (mol%)	66.7–68.9	66.4–69.0	68.1–69.5	60.2–62.6	65–67	61.5–62.3	64–65	63.8

+, positive; –, negative; v, some strains positive, others negative; ?, unknown.
Data from: Pittman (1984b), Kersters et al. (1984), Weyant et al. (1995), Vandamme et al. (1995, 1996), von Wintzingerode et al. (2001).
a) The number before the colon is the number of carbon atoms and the number after the colon is the number of double bonds; c, *cis* isomer; cyc, cyclopropane ring; w, double bond position.
b) API 20 NE test.

phylogenetic relationship with *Achromobacter* and *Alcaligenes*, which are widespread in the environment, suggests that the ancestral bordetellae were free-living and some evolved to infect warm-blooded animals. Certain properties exhibited by the pathogenic species still reflect this environmental origin: temperature sensing and regulation of virulence factor expression accordingly; the presence of flagella in some species and greater expression of motility at low temperature; the ability of *B. bronchiseptica* and *B. avium* to grow and survive for long periods in low nutrient conditions such as natural waters. The newly described *B. petrii* may be a close relative of these ancestral bordetellae.

A phylogenetic tree for the first four species to be described was based on the nucleotide sequence of the pertussis toxin (PT) gene and the presence or absence in the chromosome of two insertion sequences (Gross et al. 1989). During evolution, the first to diverge, to infect birds, was *B. avium*. Its distinctiveness is shown by its lower G + C content and lack of genes for PT. More recent genotyping studies based on multi-locus enzyme electrophoresis and the distribution of insertion sequences have suggested that *B. pertussis* and human and sheep strains of *B. parapertussis* evolved at different times and from distinct clones of *B. bronchiseptica* (van der Zee et al. 1997). *B. pertussis* strains and human strains of *B. parapertussis* are both still very homogeneous, clonal populations (Mastrantonio et al. 1998; Yuk et al. 1998b) indicating their relatively recent adaptation to humans**.** Indeed, the first descriptions of the highly characteristic disease caused by *B. pertussis* date from Paris in the mid-16th century and it has been suggested that the development of the human population of Europe at that time, to a sufficient size, density, and mobility, could have provided a suitable niche for the emergence of the organism (Wardlaw 1988). Evidence from whole genome sequencing, based on pairwise synonymous substitution rates for all 3 000 genes common to the three species, provides much longer estimates for the divergence of the species: 0.7–3.5 million years for *B. bronchiseptica* and *B. pertussis* and 0.27-1.4 million years for *B. bronchiseptica* and *B. parapertussis* (human strain). However, these estimates are based on the genome data from a single representative of each species and it is recognized that *B. bronchiseptica* is sufficiently genetically diverse that isolates more closely related to the last common ancestors could exist (Parkhill et al. 2003).

MORPHOLOGY AND ULTRASTRUCTURE

The bordetellae are minute rods or coccobacilli (0.2–0.5) × (0.5–2) μm, arranged singly or in pairs. Occasionally, filamentous forms several μm in length may be seen. *B. avium*, *B. bronchiseptica*, *B. hinzii*, and *B. trematum* are motile by peritrichous flagella; the other four species are nonflagellate. In *B. avium*, the proportion of motile rods is greater at room temperature than at 35°C and in *B. bronchiseptica* motility is expressed in the Bvg^- phase. *B. trematum* is equally motile at 25 and 37°C. *B. pertussis* and other bordetellae are reported to be capsulated when freshly isolated but the significance of a capsule for virulence is unknown. A carbohydrate 'slime-layer' has been demonstrated when *B. pertussis* is grown in vitro as a biofilm (Bosch et al. 2000). Genes for the biosynthesis of a polysaccharide capsule have been identified in the bordetella genome sequences but whether they are expressed, and under what conditions, is uncertain (Antoine et al. 2000; Parkhill et al. 2003).

Early ultrastructural studies showed fine filamentous appendages on the surface of the bordetellae and, with *B. pertussis*, similar filaments and membranous vesicles were found in the culture supernate. Subsequently, appendages identified as fimbriae, 3–5 nm in width and 100–250 nm in length, have been demonstrated on the surface of at least five species. The cellular location of some of the surface-related components of *B. pertussis* (Table 2) has been determined by immunoelectron microscopy with gold-labeled monoclonal antibodies (Blom et al. 1994). Antibodies to agglutinogens (AGG) 2 and 3 labeled, respectively, long and short fimbriae protruding from the cell surface. The presence of pertactin (PRN) was revealed by intense and evenly distributed labeling of the surface of the cell. Antibody to filamentous hemagglutinin (FHA) stained aggregates of material between or adherent to the cells, which suggests that this component is readily shed. Previous work had shown that purified FHA consists of fine filaments 4 × 50 nm and is distinct from the fimbriae. Antibodies to PT and adenylate cyclase toxin (ACT) labeled amorphous material adherent to or between cells, again indicating that these products are readily dispersed after export. In a study of antigen production for the manufacture of pertussis vaccines, the levels of cell-associated and released antigens were found to depend on growth phase and producer strain (Westdijk et al. 1997). Large amounts of PT and PRN were released during growth and the amount of cell-associated FHA declined rapidly. Intracellular globules of poly-hydroxybutyrate (PHB) may be seen during the early stages of growth of *B. pertussis* (Thalen et al. 1999) and other bordetellae. Based on the above information and that given later in this chapter, a diagrammatic representation of the disposition of the important cellular components of the bordetellae is shown in Figure 1.

CULTURAL CHARACTERISTICS

Growth of *B. pertussis* was first achieved by Bordet and Gengou (1906) with a glycerol, potato-extract medium without peptone but containing 50 percent blood. This medium, with minor modifications, is still in general use. Sheep or horse blood is routinely used and the amount is usually reduced to 15–20 percent, thereby making

Table 2 *Virulence factors of* Bordetella *species*

Virulence factor	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	Probable role in pathogenicity
Tracheal cytotoxin (TCT)	+	+	+	+	Ciliostasis, epithelial cell cytotoxicity
Dermonecrotic toxin (DNT)	+	+	+	+	Local inflammatory effects
Endotoxin[a]	+ LOS	+ LPS	+ LPS	+ LPS	Pyrogenicity
Adenylate cyclase toxin (ACT)	+	+	+	–	Interference with immune effector cells
Pertussis toxin (PT)	+	–	–	–	Interference with immune effector cells
Fimbriae	+	+	+	+	Adhesins/invasins
Filamentous hemagglutinin (FHA)	+	+	+	+	Adhesin/invasin
Pertactin (PRN)	+	+	+	–	Adhesin/invasin
BrkA	+	?	+	–	Adhesin/invasin, serum resistance
Tracheal colonization factor (TCF)	+	–	–	–	Adhesin
Type III secretion system	–	v	+	?	Interference with immune effector cells

+, present; –, absent; v, variable, reported in ovine strains; ?, unknown.
a) LOS, lipo-oligosaccharide; LPS, lipopolysaccharide.

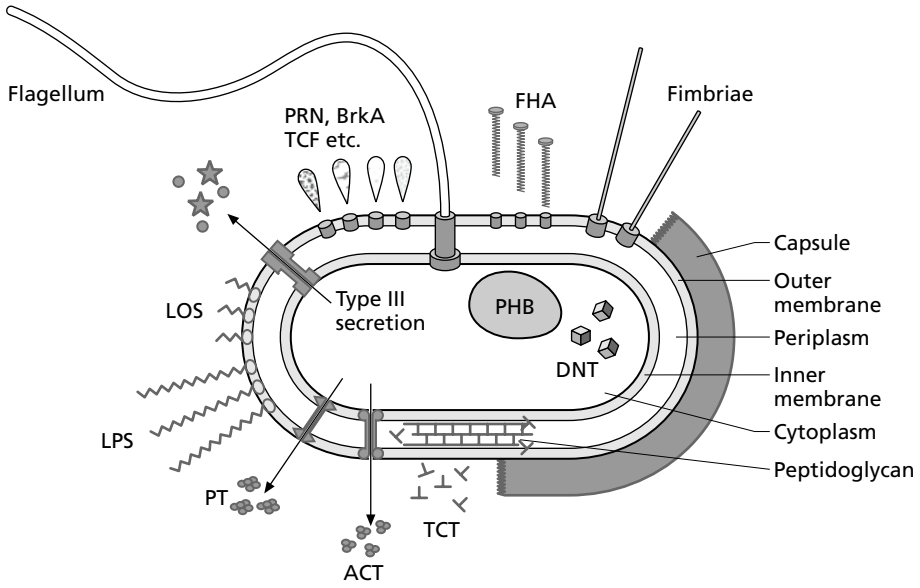

Figure 1 *Diagrammatic representation of the disposition of the important cellular components of the bordetellae. Not all components are expressed in all species.*

hemolysis around the colonies easier to see. Various selective media have been developed for clinical specimens and solid and liquid media without blood are available, for example, for vaccine production. *B. pertussis* is the most fastidious of the bordetellae and media suitable for *B. pertussis* are also suitable for the other species, although the latter can also be grown on peptone-containing media such as trypticase soy agar and MacConkey agar. Some of the cultural characteristics of the bordetellae are shown in Table 1. The time of development and size of the colonies differ between the species.

On Bordet–Gengou (BG) medium, *B. pertussis* forms tiny (0.5 mm diameter), smooth, convex, pearl-like, glistening colonies with entire edges and surrounded by a narrow zone of hemolysis. After 5–6 days, the diameter of the colonies has increased to 2–3 mm. Colonies of *B. parapertussis* develop more quickly and become slightly larger whereas those of *B. bronchiseptica* are larger still and appear within 24 h. On peptone- or tyrosine-containing agar, *B. parapertussis* and *B. holmesii* produce a brown discoloration of the medium. *B. avium* on Columbia agar containing 7 percent ox blood or veal infusion agar may show two colony types. Some strains produce small, pearl-like colonies with entire edges and glistening surfaces 1 mm in diameter after 24 h. Others are larger with smooth surfaces. *B. holmesii* colonies on heart infusion agar with rabbit blood are described as punctate, semiopaque, convex, and round with complete edges. *B. hinzii* isolates have one of two distinct colony types. When grown on trypticase soy agar, some strains produce round, raised, glistening, grayish colonies about 2 mm in diameter after 48 h at 37°C in air containing 5 percent CO_2. Under the same conditions, other strains produce flat, dry, crinkled colonies up to 5 mm in diameter. *B. trematum* colonies on blood agar are grayish-cream, convex, and circular with entire edges. *B. petrii* forms creamy-white colonies of different sizes when grown aerobically on Luria–Bertani agar at 30°C and small, irregular, slightly red colonies when grown anaerobically on selenate-containing medium.

Variation during growth is a notable feature of the bordetellae, both in vitro and in vivo. Cultures frequently exhibit different colony types on the same agar surface, or after a few transfers. Changes in colony morphology on repeated subculture, with concomitant loss of antigenic and virulence attributes and alterations in growth requirements, have long been known for *B. pertussis*. Peppler and Schrumpf (1984) described phenotypic variants of *B. pertussis* that had flat, nonhemolytic colonies on BG agar, unlike the domed, hemolytic wild-type colonies; some of the variants could be selected for growth on nutrient agar. Colonial variation in *B. pertussis* is probably caused by the combined processes of serotype variation, phase variation and antigenic modulation and similar phenomena occur in other bordetellae.

GROWTH REQUIREMENTS

Although complex media containing blood are often used for their initial isolation, *Bordetella* spp. have relatively simple nutritional requirements (Rowatt 1957; Thalen et al. 1999). These include amino acids as carbon and nitrogen sources, nicotinamide or nicotinic acid, and an organic source of sulfur. *B. bronchiseptica* and *B. avium* can also grow on carbon sources such as lactate or citrate plus ammonium ions in place of amino acids. Despite these simple nutritional requirements, *B. pertussis* is a fastidious organism and growth may be

inhibited by various components, such as unsaturated fatty acids, present in normal media, or by contaminating detergents from the glassware. *B. parapertussis*, *B. bronchiseptica*, *B. avium* and, presumably, the other species, are less susceptible to these inhibitors. Surprisingly, *B. bronchiseptica* and *B. avium* are able to grow in low nutrient conditions, such as in natural waters and even in unsupplemented phosphate-buffered saline, but this property is not shared by *B. pertussis* or *B. parapertussis* (Porter et al. 1991; Porter and Wardlaw 1993; Cotter and Miller 1994).

Early liquid media developed for bulk growth of *B. pertussis* for vaccine production and free from blood or tissue extracts, contained salts, amino acid mixtures, growth factors, and soluble starch. Despite the inclusion of starch, which helps to reduce the effects of the above-mentioned inhibitors, these media do not allow growth from small inocula and, when solidified with agar, do not permit single cells to form separated colonies. In place of starch, albumin, charcoal or ion-exchange resins have been used. Ammonia is formed during growth of the bordetellae and consequently media must be adequately buffered. A simple chemically defined medium for *B. pertussis* was developed by Stainer and Scholte (1971). Stainer–Scholte (SS) medium contains glutamic acid, proline, and cystine, together with ascorbic acid, nicotinic acid, glutathione, salts, and Tris buffer; again, it supports the growth of *B. pertussis* only from a heavy inoculum. This difficulty was overcome by Imaizumi et al. (1983) who supplemented SS medium with heptakis (2,6-*O*-dimethyl)β-cyclodextrin. Cyclodextrins form water-soluble complexes with hydrophobic molecules such as fatty acids. The resulting medium, solidified with agarose, was the first defined medium for *B. pertussis* to allow reliable growth from dilute inocula, a property only previously associated with blood-containing media such as BG agar. Supplementation of the medium with casamino acids further improved growth. Cyclodextrin not only improves the growth of *B. pertussis* but also greatly enhances the yield of PT, FHA, and ACT in the culture supernate (Hozbor et al. 1994). Unlike *B. pertussis*, *B. bronchiseptica* is able to grow on a variety of conventional media but these can affect production of virulence factors. For example, growth on nutrient agar and brain heart infusion broth induced the avirulent Bvg^- phase and growth on tryptic soy agar and in tryptone phosphate broth induced an intermediate, Bvg^i phase (Passerini de Rossi et al. 2001).

LABORATORY ISOLATION AND IDENTIFICATION

Successful isolation of *B. pertussis* from patients depends on various factors (Onorato and Wassilak 1987; Friedman 1988; Hoppe 1988; Gilchrist 1991; Guiso 1997; Müller et al. 1997; Hallander 1999; Kerr and Matthews 2000). Cultivation of the organism is more likely to be successful early in the disease and less so during the paroxysmal stage. Age and immunization status of the patient and any preceding antimicrobial therapy are also important. Factors that can be controlled by the bacteriologist include the methods of specimen collection, transport, and culture.

Nasopharyngeal swabs or nasopharyngeal aspirates give better isolation rates than cough plates, throat swabs or nasal swabs. Calcium alginate swabs have been found to be superior to those made from other materials. Although direct plating of specimens is the method of choice, transport media can be used. For swabs, Hoppe (1988) recommended the transport medium of Regan and Lowe (1977), that contains half-strength charcoal agar, horse blood, and cephalexin, the latter to suppress the normal nasopharyngeal flora. For transporting nasopharyngeal secretions, casamino acids broth held at 4°C is suitable and, for longer-term preservation, there is no significant loss of viability on storage at −20°C in casamino acids or in serum inositol suspending medium (Cassiday et al. 1994). For primary isolation of *B. pertussis*, the medium developed by Regan and Lowe (1977) containing charcoal agar, with defibrinated horse blood 10 percent and cephalexin (40 mg/l) is recommended. Amphotericin B may be added if problems with fungal contaminants occur. A synthetic cyclodextrin-containing medium with cephalexin has a much longer shelf-life than BG agar. With simulated nasopharyngeal swab specimens, however, recovery on BG agar was 80 percent, and on cyclodextrin medium was <60 percent, of the numbers obtained on Regan and Lowe medium (Morrill et al. 1988). Plates should be incubated aerobically in a humid atmosphere at 35 rather than 37°C and for up to 7 days. The characteristic tiny, smooth, convex, pearl-like colonies of *B. pertussis* usually become visible after 3–4 days, whereas those of *B. parapertussis* appear after 2–3 days. The latter organism is readily isolated on culture media used for *B. pertussis* but Moredun Bordetella medium, a charcoal agar containing cycloheximide and spectinomycin, is highly specific for *B. parapertussis* but inhibits *B. pertussis* (Connor et al. 1996).

B. bronchiseptica is readily isolated by nasal or tracheal swabbing of animals with bordetellosis. Blood agar or MacConkey agar, with or without selective agents such as penicillin, streptomycin, and nystatin may be used and other selective media have been described (Goodnow 1980; Rutter 1985). Tracheal specimens from infected dogs and cats often contain high numbers of bordetellae and in pure culture (Bemis 1992). MacConkey agar is also suitable for *B. avium*. For isolation of *B. holmesii*, BG medium containing methicillin or oxacillin was found to be suitable, whereas cephalexin, commonly used for other bordetellae, was inhibitory (Mazengia et al. 2000).

Identity can usually be confirmed by Gram staining, urease, oxidase, and nitrate reduction reactions

(Table 1) and, for *B. pertussis*, by typing with fluorescent antibody or by slide agglutination with specific antisera. There are species-associated differences in other biochemical tests. For example, *B. hinzii* can be distinguished from *B. avium* by its ability to cause alkalinization of a wider range of substrates, to assimilate caprate and to decarboxylate histidine (Vandamme et al. 1995). Some investigators have resorted to determination of cellular fatty acid composition and 16S rRNA sequencing for confirmation of identity of 'unusual' isolates of bordetellae, such as *B. bronchiseptica*, *B. hinzii*, and *B. holmesii* from humans (Kattar et al. 2000; Gadea et al. 2000; Wallet et al. 2002).

Various serological tests such as agglutination, complement fixation, and enzyme-linked immunosorbent assay (ELISA) have been used to confirm the diagnosis of pertussis and parapertussis and are valuable in epidemiological and vaccine efficacy studies. They are less suitable for rapid diagnosis and generally require seroconversion to distinguish active disease from high titers due to vaccination or maternal antibodies. ELISA tests for IgG antibodies to PT and FHA are widely used and sensitive diagnostic methods and criteria for a positive diagnosis have been suggested by Hallander (1999). Antibody responses to PT are considered to be specific for pertussis whereas seroconversion for FHA may also occur after *B. parapertussis* infection. The direct detection of *B. pertussis* or its components in clinical specimens is also possible (Onorato and Wassilak 1987; Friedman 1988; Gilchrist 1991; Hallander 1999). Such methods have the advantages of speed and ability to detect organisms that are no longer viable. The direct fluorescent antibody test uses fluorescein-labeled antibodies to *B. pertussis* and *B. parapertussis* to detect the bacteria in nasopharyngeal secretions. The test is technically demanding, of low sensitivity, and may give false negative results if the number of organisms in the specimen is low, or false positive results, due to lack of technical expertise, poor quality slides or antigenic cross-reactions with other organisms. It is recommended as an adjunct to, rather than a replacement of, culture. Tests for the detection of *B. pertussis* components such as adenylate cyclase, by its enzymic activity, and pertussis toxin, by immunoassay or by cytotoxicity assay, either directly in respiratory secretions or after enrichment, appear to be sensitive and specific but have not found routine use.

The most rapid and sensitive detection methods for *B. pertussis* and other bordetellae are those involving the polymerase chain reaction (PCR). Such techniques are particularly suitable for fastidious, slow growing pathogens such as *B. pertussis*. With primers directed to the pertussis toxin gene promoter, discrimination between *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* is possible (Reizenstein et al. 1993). A PCR assay targeting multicopy species-specific insertion sequences allows the simultaneous detection and discrimination of *B. pertussis* and *B. parapertussis* and is sensitive enough to detect a single cell of either pathogen (van der Zee et al. 1993). With knowledge of the *Bordetella* genomes, new species-specific and genus-specific targets could be identified. With the advent of real-time PCR technology, identification can be completed within 1 h after extraction of the DNA from a swab sample (Sloan et al. 2002). A real-time PCR method targeting the IS*481* insertion sequence was able to detect and discriminate between *B. pertussis* and *B. holmesii* (Poddar 2003). PCR assays allow a much greater detection rate for *B. pertussis* infection than culture, particularly in vaccinated individuals, in patients given antibiotics, and in patients with mild or atypical symptoms (Schmidt-Schläpfer et al. 1997). As with culture, the methods of sample collection and processing are crucial as is the choice of primers, PCR conditions, and detection system (Meade and Bollen 1994; Müller et al. 1997). Hewlett (2000) has cautioned that efforts to culture *B. pertussis* should continue, to detect strain variation, antibiotic resistance, and other properties of the organism that could be missed by a PCR-based system. Others have highlighted the dangers of false positives with PCR methods and the need for fully validated methods (Lievano et al. 2002).

TYPING METHODS

Serotyping has long been used for epidemiological investigations of *B. pertussis* isolates and depends on the presence or absence of specific AGGs on the bacterial cell surface. These are detected by slide agglutination tests traditionally with antisera raised in rabbits and made monospecific by absorption with heterologous strains. In an early definitive study, Eldering et al. (1957) described 14 heat-labile AGGs amongst *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. AGGs 1–6 were specific for *B. pertussis*. AGG 1 was common to all strains, and AGGs 2–6 were found in various combinations on different isolates. Currently, only AGGs 1, 2 and 3 are widely recognized. Thus, the four serotypes of *B. pertussis* are 1,2,3; 1,2; 1,3; and 1. AGG 1 is common to all fresh isolates and is thought to be located on the lipo-oligosaccharide component of the cell envelope. The antigenic determinants of AGGs 2 and 3 are located on fimbriae. Monoclonal antibodies to the serotype antigens are now available and have been suggested for adoption as standard serotyping reagents (Guiso et al. 2001; Xing et al. 2001).

A variety of much more discriminating DNA fingerprinting methods has now been applied to the various bordetellae. For example, pulsed-field gel electrophoresis (PFGE) has proved to be useful for epidemiological studies and numerous DNA types have been identified amongst *B. pertussis* isolates even in individual outbreaks (de Moissac et al. 1994; Hardwick et al. 2002). PFGE has also been used to discriminate between isolates of *B. bronchiseptica* (Binns et al. 1998), *B. parapertussis* (Mastrantonio et al. 1998), and *B. holmesii*

(Mazengia et al. 2000). Ribotyping and restriction endonuclease analyses have been used to differentiate between *B. avium* and *B. hinzii*, and to distinguish numerous types within each species (Register et al. 2003). Multilocus sequence typing (MLST) methods based on virulence genes encoding surface proteins have detected potentially important polymorphisms in the *B. pertussis* population (van Loo et al. 2002). With advances in sequencing technology, such information is readily obtainable and more easily transferable between laboratories and databases than results of other methods based on whole-genome comparisons. To facilitate the comparison of *B. pertussis* populations between different countries and for investigation of the effectiveness of different vaccination regimes used, a standardized methodology for epidemiological typing of *B. pertussis* has been proposed, based on serology, PFGE, and gene typing (Mooi et al. 2000).

BIOCHEMICAL ACTIVITIES AND METABOLISM

Studies on the metabolic activities of the bordetellae have been concerned mainly with defining nutritional requirements or with biochemical tests for identification, and for taxonomic and phylogenetic purposes. Biochemical activity, as usually determined in the laboratory, is generally regarded as weak in that none ferments sugars. Other metabolic studies have been concerned with vaccine development and virulence mechanisms. As a step towards constructing live attenuated bordetellae as vaccine strains for mucosal delivery, the *aroA* gene of *B. pertussis* and *B. bronchiseptica* has been targeted. This gene encodes an enzyme essential for the biosynthesis of aromatic amino acids and other aromatic compounds and *aroA* mutants are highly attenuated in mice (Roberts et al. 1990; Stevenson and Roberts 2002). Leucine and methionine auxotrophs of *B. pertussis* generated by transposon mutagenesis were also attenuated in a mouse model (Weiss and Goodwin 1989). The enzymes catalase, superoxide dismutase, and peroxidase, which are important in oxygen metabolism and for survival of some bacteria within phagocytes, are present in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, and the other five species are known to produce catalase. A catalase-deficient construct of *B. pertussis* was more sensitive to killing by hydrogen peroxide than the wild-type strain but its ability to survive within human polymorphs was unchanged (DeShazer et al. 1994). *B. bronchiseptica* has potent urease activity and, with some other pathogens, this enzyme is thought to be important in host colonization and disease production. Surprisingly, Monack and Falkow et al. (1993) found that a urease deletion mutant of *B. bronchiseptica* was better able to colonize the respiratory tract of guinea pigs than the parent strain. They suggested that urease may be more important to *B. bronchiseptica* in a possible environmental niche rather than in pathogenicity.

The whole-genome sequences of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* have revealed that all three appear to encode complete pathways for the biosynthesis of nucleotides, co-factors, and amino acids (except cysteine), consistent with their ability to grow on defined media. Their inability to utilize sugars as carbon sources is explained by the absence of genes encoding glycolytic enzymes such as glucokinase, phosphofructokinase, and fructose 1-6 bisphosphatase (Parkhill et al. 2003). With the availability of the genome sequences, it should be possible to elucidate every metabolic pathway of these organisms and, with the aid of transcriptome analysis and proteomics, to determine how they respond to different environmental conditions (Preston and Maskell 2002).

Iron is an essential nutrient for bacteria and successful pathogens must be able to cope with the iron-restricted environment found in host tissues. Here, the level of free iron is kept low by the iron-binding proteins lactoferrin and transferrin. Some pathogens produce high-affinity iron chelators (siderophores) to remove iron from these proteins and others have surface receptors that bind the host proteins directly to facilitate iron transport into the cell. Yet others can utilize iron from other sources, such as heme. There is evidence, supported by the genome sequences, that *B. pertussis* and other bordetellae possess all of these types of iron uptake mechanism and their expression appears to be controlled, as in other gram-negative bacteria, by the repressor protein Fur with ferrous iron as the co-repressor (Vanderpool and Armstrong 2001; Murphy et al. 2002; Parkhill et al. 2003). Hydroxamate siderophores have been purified from *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, and shown to be identical to alcaligin, the iron chelator of *Alcaligenes* (now *Achromobacter*) *denitrificans*. They also have receptors, such as the ferric-enterobactin receptor BfeA, for siderophores that are produced by other microorganisms. A mutant of *B. pertussis*, defective in the inner membrane proteins required for the transport of the various iron complexes into the bacterium had a reduced ability to colonize the lungs of mice, indicating the importance of such iron-uptake systems for growth in vivo (Pradel et al. 2000)

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

The effectiveness of antibacterial agents against bordetellae in vitro does not necessarily reflect their usefulness against the bacteria localized on the ciliated epithelium of the respiratory tract due to the difficulty in maintaining suitable concentrations of the drugs at this site (Hoppe 1998). Antibiotic treatment in the early (catarrhal or early paroxysmal) stages can ameliorate the symptoms of pertussis and eliminate the infection.

Later treatment may have no clinical benefit for the patient but may help to limit the spread to susceptible contacts (Cherry et al. 1988). Several antibiotics including erythromycin, tetracycline, choramphenicol, and trimethoprim-sulfamethoxazole are effective in vivo, but erythromycin is generally the drug of choice for treatment of pertussis patients and for post-exposure prophylaxis of close contacts; erythromycin estolate is reported to reach higher concentrations than other formulations. Short-term treatment with clarithromycin or azithromycin, because of their improved absorption and longer half-life, has been shown to be at least as effective as standard erythromycin treatment (Aoyama et al. 1996; Bace et al. 1999; Lebel and Mehra 2001). These agents, or trimethoprim-sulfamethoxazole, may be appropriate for treatment of adult pertussis patients who cannot tolerate erythromycin (Hoppe 1996). Routine antibiotic susceptibility testing of *B. pertussis*, a slow growing and nutritionally fastidious organism, is cumbersome and is usually unnecessary. However, erythromycin-resistant isolates have been reported and have prompted the development of standardized testing methods. An agar-dilution method has been recommended, using Mueller–Hinton agar plates supplemented with 5 percent horse blood and containing different concentrations of antibiotic. A dense inoculum (c. 10^4 cfu) of each isolate is applied with a multipoint inoculator to a small spot on the plates. Minimum inhibitory concentration (MIC) values are determined after incubation for 72 h at 36°C in a humid atmosphere. The E-test method, based on the diffusion of a concentration gradient of the antibiotic from a plastic strip into a spread-inoculated agar plate, is simpler but poor growth of some isolates from a low inoculum may cause problems (Hoppe 1998; Hill et al. 2000).

Early attempts to modify the course of pertussis illness by passive immunization were of doubtful value but newer preparations of anti-pertussis immunoglobulin have shown some promise. High-titer immunoglobulins prepared from adults immunized with either pertussis toxoid (PTd) or PTd plus FHA gave a significant reduction in the number of whoops, but not cough or vomiting, when administered intramuscularly to pertussis patients in a double-blind, placebo controlled trial (Granstrom et al. 1991). Similarly, a high-titer serum from donors immunized with PTd reduced the paroxysmal coughing in children with severe pertussis (Bruss et al. 1999). Further studies on pertussis immunotherapy are clearly warranted.

There is little information on the most appropriate clinical treatment for parapertussis patients. *B. parapertussis* is reported to be generally more resistant to most antimicrobials than *B. pertussis* but they are equally susceptible to trimethoprim-sulfamethoxazole and fluoroquinolones in vitro and these agents are likely to be effective in vivo (Hoppe 1999). In the treatment of animal bordetellosis, delivery of standard amounts of antimicrobial agents by the oral or parenteral routes may be insufficient to reduce bacterial numbers in the respiratory tract and to alleviate symptoms (Bemis 1992). Increased dosage or local administration of the agent by aerosol or intratracheal injection may be beneficial. Another difficulty is the frequent development of resistant strains of *B. bronchiseptica*. In surveys on the in vitro susceptibility of *B. bronchiseptica* (Woolfrey and Moody 1991; Bemis 1992), isolates were generally susceptible to aminoglycosides, anti-pseudomonal penicillins, chloramphenicol, tetracyclines, imipenem, quinolones, and polymyxins. Those that were generally ineffective were erythromycin, some cephalosporins, streptomycin, clindamycin, and nitrofurans. In some cases, treatment failure has been shown to be due to R factors. Plasmids encoding multiple drug resistance have been described and some are capable of conjugal transfer to other organisms. Antibiotic resistance plasmids are also commonly encountered in *B. avium* but have not been described in *B. pertussis*. In a survey of Australian isolates of *B. avium*, all were resistant to erythromycin and most were resistant to sulfonamides and streptomycin (Blackall et al. 1995). *B. hinzii* has been described as a multidrug resistant organism with MICs of different antimicrobials resembling those for strains of *B. bronchiseptica* (Funke et al. 1996; Kattar et al. 2000).

VIRULENCE FACTORS

Bordetella spp. produce an array of toxins, aggressins, and adhesins that are presumed to be important in colonization of their respective hosts and ensuring their survival and propagation. The best characterized species is the human pathogen *B. pertussis* and many of its products have been rigorously purified and assessed for their protective activity in experimental animals. Following the pioneering work of Weiss, Falkow and coworkers (Weiss et al. 1983, 1984; Weiss and Falkow 1984), genes encoding the putative virulence factors have been identified by transposon mutagenesis and many have been cloned and sequenced and expressed in other bacteria to investigate their properties. Strains engineered with precise mutations have been used to determine the effect of the loss of individual factors on virulence-related properties. With *B. pertussis*, however, the role of these factors in pathogenesis has been difficult to define since reliance has to be placed on in vitro assays or animal models of the human infections, particularly in the mouse, in which different conditions may prevail. Nevertheless, further information has been provided by studies of *B. bronchiseptica* and *B. avium* in their natural hosts. With this in mind, the putative virulence factors of the bordetellae will now be described. A summary of the expression of these factors in four species is shown in Table 2. The more recently described species, *B. hinzii*, *B. holmesii*, *B. trematum*, and *B. petrii* have been investigated for production of PT, ACT, dermonecrotic toxin (DNT), FHA or PRN by

genetic and immunological analysis but results suggest that they do not possess these factors (Gerlach et al. 2001; von Wintzingerode et al. 2001).

Tracheal cytotoxin

Tracheal cytotoxin (TCT) is produced by at least four species of bordetellae. It was discovered by Goldman and colleagues from its ability to cause ciliostasis and ciliated cell extrusion in hamster tracheal organ cultures and inhibition of DNA synthesis in hamster trachea epithelial (HTE) cell cultures. The destruction of ciliated cells is similar to that seen in necropsy material from human pertussis and in turkey coryza (Goldman 1988). Such effects could well account for some of the pathological events of *Bordetella* respiratory infections such as accumulation of mucus, coughing, and predisposition to secondary infections. TCT is an unusual toxin, with a low molecular weight (MW) of 921 Da. It is a disaccharide tetrapeptide (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-(L)-alanyl-γ-(D)-glutamyl-*meso*-diaminopimelyl-(D)-alanine), equivalent to a monomeric subunit of the bacterial peptidoglycan derived from the normal turnover of peptidoglycan during growth of the cell envelope. Unusually in bacteria, this subunit is released by the bacterium rather than recycled (Cookson et al. 1989). Another bacterium known to release such a toxin is *Neisseria gonorrhoeae,* which causes a similar cytopathology in fallopian tubes. These toxins belong to a family of muramyl peptides with diverse biological activities such as pyrogenicity, adjuvanticity, arthritogenicity, induction of slow-wave sleep, and stimulation of IL-1 production. Nitric oxide, synthesized in response to IL-1, appears to be the actual cytotoxic factor (Heiss et al. 1994). In a study of hamster tracheal organ cultures infected with *B. pertussis*, Flak and Goldman (1999) showed that the production of cytokine-inducible NO synthase (iNOS) was induced in nonciliated cells and mucus-secreting cells but not in ciliated cells. Moreover, the effect was reproduced by a combination of TCT and *B. pertussis* lipo-oligosaccharide (LOS), but not by either agent alone (Flak et al. 2000). A similar synergistic response, resulting in induction of IL-1, iNOS, NO production, and inhibition of DNA synthesis was shown in HTE cells. It has been suggested that the production of inflammatory mediators by mucosal epithelial cells in response to molecules such as muramyl peptides and lipopolysaccharide (LPS) or LOS is part of a normal innate response to infecting bacteria. The abnormal release of large amounts of TCT by *Bordetella* spp., in combination with LOS, causes an exaggerated host response that results in respiratory tract pathology (Flak et al. 2000). Although the effects of purified TCT and LOS can be demonstrated on ciliated epithelia, Bvg$^-$ strains of *B. pertussis*, which produce both of the toxins but none of the usual adhesins, cause little damage when incubated with respiratory epithelia. It may be that during infection, adhesins are required to bring *B. pertussis* into close contact with target cells for their toxic effect to be induced.

DNT

DNT activity is found in the four species listed in Table 2. Genetic studies have supported earlier findings that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* produce DNTs which are essentially indistinguishable in their physicochemical, serological, and biological properties whereas that of *B. avium* is similar (Walker and Weiss 1994). The products of the *dnt* genes of *B. pertussis* and *B. bronchiseptica* are 1 464 amino acids in length with a MW of 160.6 kD and there are only 11 residues different between the two polypeptides (Pullinger et al. 1996). DNT is a cytoplasmic component of the bacteria and not normally released by actively growing cells.

DNT has also been known as heat-labile toxin because its activity is destroyed by heating to 60°C for c. 10 min. It was the first bordetella toxin to be described and yet its role in disease has not been clearly defined. DNT is largely responsible for the mouse-lethal toxicity of freshly harvested *B. pertussis* cells and the mouse toxicity test for pertussis vaccine was introduced primarily to ensure that DNT is destroyed during vaccine manufacture. It has potent vasoconstrictive activity and in experimental animals causes death or loss of normal weight gain, spleen atrophy, and ischemic lesions or necrosis of skin. The dermonecrotizing activity appears to be due to a specific constrictive effect on vascular smooth muscle. Such an effect on the highly vascularized tissues in the respiratory tract could induce a local inflammatory reaction and thus account for some of the pathology of pertussis (Nakase and Endoh 1988).

Despite its potency in various bioassays, *B. pertussis* mutants deficient in DNT production were unaltered in their ability to cause a lethal infection in mice. However, from studies of other *Bordetella* species in their natural hosts, DNT appears to have a role both in disease and in colonization. DNT in *B. bronchiseptica* seems to have a role in producing the turbinate atrophy associated with atrophic rhinitis in pigs by causing degenerative changes in osteoblasts and impairing bone formation (Horiguchi et al. 1994). A *B. bronchiseptica* strain deficient in DNT production was associated with considerably less turbinate atrophy and pneumonia in pigs compared to the wild-type parent and also had reduced ability to colonize the nasal cavity (Brockmeier et al. 2002). Similarly, a DNT-deficient strain of *B. avium* was less virulent and persisted for a shorter time than the parent strain in young turkeys, and had reduced ability to adhere to turkey ciliated tracheal cells in vitro (Temple et al. 1998).

The various effects of DNT on target cells include stimulation of DNA and protein synthesis and formation of actin stress fibers and focal adhesions. These effects are thought to be mediated by its activity as a transglutaminase catalyzing the deamidation or polyamination of the guanosine triphosphate (GTP)-binding protein Rho and other members of the Rho family involved in regulation of the cytoskeleton. This results in constitutive activation of the Rho proteins (Masuda et al. 2002). In this respect, it resembles cytotoxic necrotizing factor 1 of *Escherichia coli*, with which it shares some structural homology. The receptor-binding or cell-internalization domain of DNT resides at the N-terminus of the molecule whereas the catalytically active domain lies at the C-terminus (Matsuzawa et al. 2002).

Endotoxin

Despite the close genetic relatedness of the bordetellae, their endotoxins are different. The endotoxin of *B. pertussis* (LOS) can be resolved electrophoretically into a slow-moving band, band A, and a fast-migrating band B (Peppler 1984). Band B (LPS-B) is composed of lipid A linked via a single ketodeoxyoctulosonic acid residue to a branched core consisting of glucose, heptose, glucuronic acid, glucosamine, and galactosaminuronic acid. Addition of a trisaccharide (2-*N*-acetyl-4-*N*-methylfucosamine, 3-di-*N*-acetylmannosaminuronic acid and *N*-acetylglucosamine) to LPS-B produces LPS-A (Band A) (Chaby and Caroff 1988; Caroff et al. 2000). *B. pertussis* endotoxin, like that of some other bacterial pathogens, is generally referred to as a LOS (Brodeur et al. 1993). By contrast, *B. parapertussis* and *B. bronchiseptica* endotoxins usually consist of a smooth-type LPS with high MW O-polysaccharide components, usually with the O-antigen comprising a homopolymer of 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid, although differences in O antigen structure between *B. bronchiseptica* strains have been noted (Vinogradov et al. 2000). *B. pertussis* lacks the *wbm* genetic locus for O-antigen biosynthesis in *B. bronchiseptica* and *B. parapertussis*. *B. avium*, *B. holmesii*, and *B. hinzii* have different O-antigen repeat structures whereas *B. trematum* expresses only a single O antigen unit. The lipid As of the bordetellae have some unusual structural features. For example, *B. pertussis* and *B. parapertussis* lipid A is hypo-acylated (penta-acylated) compared to enterobacterial lipid As whereas *B. bronchiseptica* and *B. hinzii* are fully hexa-acylated. There are also species-specific differences in the fatty acyl components of the lipid As of the bordetellae (Zarrouk et al. 1997; Preston and Maskell 2001).

There is no clear understanding of the role of endotoxin in *Bordetella* infections although it may act in concert with other bordetella toxins, as described above for TCT. *B. pertussis* LOS has the usual properties of endotoxins such as general toxicity, pyrogenicity, and adjuvanticity as well as some unusual properties such as the ability to induce antiviral activity, B cell mitogenicity, and polyclonal B cell activation (Chaby and Caroff 1988). LOS may be responsible for the mild fever in early pertussis. In addition, it probably causes much of the reactogenicity of whole-cell vaccine and a main aim in pertussis acellular vaccine development has been to eliminate this toxin. Nevertheless, LOS may have a role in immunity to pertussis infection. Pertussis vaccine that has been boiled to destroy all but the heat-stable LOS antigens still protects mice against lethal intranasal challenge with *B. pertussis*. Anti-LOS antibodies may be bactericidal for *B. pertussis* in the presence of complement and some anti-LOS monoclonals were able to passively protect mice from lethal (Shahin et al. 1994) and sublethal (Mountzouros et al. 1992) challenge with *B. pertussis* irrespective of their bactericidal activity in vitro. The presence of smooth LPS in *B. bronchiseptica* correlated with resistance to killing in vitro by antibody and complement (Byrd et al. 1991) and antimicrobial peptides (Banemann et al. 1998) whereas *B. pertussis*, lacking the O-polysaccharide side chains, was more sensitive to these agents.

Mutagenesis of the LPS/LOS biosynthesis genes has been done to obtain mutants expressing a range of defined LPS molecules. For example, when the *wlb* locus, which is required for addition of the trisaccharide to the core region to form LPS-A, was deleted from *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*, mutants of each were defective in their ability to colonize different sites of the mouse respiratory tract (Harvill et al. 2000). Mutants in the *wbm* locus of *B. bronchiseptica* and *B. parapertussis* that were deficient in O-antigen biosynthesis both lacked the complement resistance associated with the wild type, but only the *B. parapertussis* mutant had reduced ability to colonize the mouse respiratory tract (Burns et al. 2003). It thus appears that LPS structures play roles in infection that are specific to each organism. There is evidence that the main regulator of virulence gene expression, the *bvg* locus, influences LPS structure in *B. bronchiseptica* but not in *B. pertussis* (van den Akker 1998). In the Bvg^+ mode, the lipid A is modified by palmitoylation and this modification was shown to be important for long-term persistence of *B. bronchiseptica* in the mouse respiratory tract (Preston et al. 2003).

ACT

The adenylate cyclase (AC) of *B. pertussis* was first shown to be associated with toxic activity by its ability to attenuate neutrophil superoxide production and bactericidal capabilities. Subsequently, ACT was shown to penetrate and intoxicate a wide range of cell types. However, immune effector cells such as neutrophils, monocytes, macrophages, dendritic cells, and natural

killer cells, necessary for combating infection, are thought to be the primary targets (Hewlett and Gordon 1988; Guermonperez et al. 2001). *B. parapertussis* and *B. bronchiseptica* have ACT activity similar to that of *B. pertussis* but the toxins are antigenically distinct. ACT or CyaA, the product of the *cyaA* gene, is a bifunctional protein of 1 706 amino acids with both AC and hemolytic activities. It is a member of the repeats in toxin (RTX) family of calcium-dependent, pore-forming cytolysins, of which the prototype is *E. coli* hemolysin (Coote 1992). The AC domain is located in the N-terminal 400 amino acids and the C-terminal portion, of 1 306 amino acids, has homology to the other RTX cytolysins, confers weak hemolytic activity and provides a means of entry into the target cell for the N-terminal AC moiety (Ladant and Ullmann 1999). It is this moiety that is responsible for the weak β-hemolysis seen around colonies of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* on Bordet–Gengou agar plates. ACT is exported by a type I secretion pathway that requires a nonprocessed signal sequence at the C-terminus of the toxin and also the products of the accessory genes *cyaB*, *cyaD*, and *cyaE*. It is synthesized as an inactive protoxin and is converted to the active form by palmitoylation of Lys983 catalyzed by the product of another accessory gene *cyaC*.

Although at high concentrations ACT can bind to and enter a variety of cell types including red blood cells, only cells expressing CD11b are intoxicated by low, presumably more physiological, levels of the toxin. ACT binds with high affinity to the CD11b moiety of the CR3 receptor (CD11b/CD18) and such binding may explain its primary action on immune effector cells. Both ACT and FHA bind to the integrin CD11b/CD18 and it has been suggested that they may act cooperatively to deplete CD11b/CD18-expressing leukocytes (Guermonperez et al. 2001). A direct physical association between ACT and FHA has also been demonstrated and may play a role in the efficient delivery of the toxin to target cells (Zaretsky et al. 2002). ACT monomers are sufficient to deliver the AC domain to the target cell interior and to form cation-selective channels in the target cell membrane. With red blood cells however, hemolysis requires oligomerization of the toxin monomers in the cell membrane (Gray et al. 1998). Although the AC domain can gain access to the cytosol of target cells by direct translocation across the plasma membrane, processes involving vesicular trafficking appear to contribute to cytotoxicity for macrophages (Khelef et al. 2001).

Once inside the cell, the AC moiety is activated by the endogenous eukaryotic protein calmodulin, a property it shares with the edema factor of *Bacillus anthracis*. It then causes unregulated synthesis of cyclic AMP from adenosine triphosphate (ATP), thus interfering with normal cell functioning, before it is degraded (Hanski 1989). Purified ACT is also able to induce apoptosis in target cells, a property shared with some other RTX toxins. For example, ACT is able to induce apoptosis in mouse alveolar macrophages in vitro and in vivo (Khelef and Guiso 1995; Gueirard et al. 1998). Studies with *Bordetella* mutants deficient in ACT activity in mouse models have confirmed its importance as a virulence factor and that phagocytic cells are the primary target. Such *B. pertussis* mutants have reduced ability to cause lethal infection in infant mice and to colonize the lungs of older mice (Weiss et al. 1984; Weiss and Goodwin 1989; Khelef et al. 1994). After comparing the effects of wild-type and ACT^- *B. bronchiseptica* strains in normal and immunodeficient mice, it was concluded that neutrophils were critical for early defense against infection and that ACT was responsible for overcoming this arm of the innate immune system (Harvill et al. 1999b). Other work has shown that expression of ACT inhibits uptake of *B. pertussis* by human respiratory epithelial cells (Bassinet et al. 2000) and Fc receptor-mediated attachment and opsonophagocytosis by human neutrophils (Weingart et al. 2000).

ACT has been shown to be a protective antigen for mice against *B. pertussis* challenge. Immunization with native ACT or recombinant active ACT promoted more rapid clearance of *B. pertussis* from the respiratory tract than in controls or in mice immunized with inactive (nonacylated) recombinant ACT (Betsou et al. 1993; Hormozi et al. 1999). Apart from any potential use in new pertussis vaccines, ACT may have other important applications in vaccinology (Ladant and Ullmann 1999). Genetically detoxified, recombinant ACT has been shown to be an effective vehicle for delivery of heterologous epitopes and induction of cell-mediated immunity. A variety of $CD8^+$ T cell epitopes has been inserted into the catalytic domain of AC. The resulting AC toxoids are still cell-invasive and deliver these epitopes into the cytosol of MHC class I antigen-presenting cells with subsequent priming of cytotoxic T lymphocytes. Both protective antiviral and therapeutic anti-tumor responses have been stimulated in this way (Saron et al. 1997; Fayolle et al. 1999).

PT

PT is generally considered to be expressed only by *B. pertussis* and is actively secreted. Structural genes for toxin subunits and toxin export are present in *B. parapertussis* and *B. bronchiseptica*, but they appear to be transcriptionally silent due to defective promoters or activator (BvgA) binding sites (Arico and Rappuoli 1987; Parkhill et al. 2003). However, two atypical strains of *B. bronchiseptica* have been described that appeared to have produced the toxin during infection (Stefanelli et al. 1997). PT has attracted wide interest not only because of an assumed central role in the pathogenesis of whooping cough and as a proven vaccine antigen but

also because of its remarkable range of biological activities and its use as a probe of eukaryotic cell signaling processes (Wardlaw and Parton 1988b; Ui 1990; Krueger and Barbieri 1995; Ryan et al. 1998). Some of the in vitro and in vivo activities of PT are listed in Table 3.

PT is a complex protein of 105 kDa with an enzymic moiety, the A or S1 subunit, and a heteropentameric B subunit responsible for binding to target cells and inserting the S1 subunit into the cytoplasm. The B oligomer is made up of four different polypeptides S2–S5, in the molar ratio 1:1:2:1, in the form of two dimers S2–S4 and S3–S4 held together by S5. Carbohydrate-recognition domains on S2 and S3 target PT to eukaryotic cells by binding preferentially to glycoproteins and glycolipids. The hemagglutinating, T cell mitogenic, and at least some of the adjuvant activities of PT are due solely to the B subunit binding to cell surfaces (Ui 1988; Roberts et al. 1995; Ryan et al. 1997). The 3D crystal structure of PT has been resolved (Stein et al. 1994; Hazes et al. 1996).

PT is actively secreted by *B. pertussis*. Each polypeptide is synthesized with an N-terminal signal sequence and translocated to the periplasm through the inner membrane by a *sec*-mediated process. The holotoxin is assembled here and transported across the outer membrane via a mechanism involving nine pertussis toxin liberation (Ptl) proteins. These are believed to form a complex spanning the inner and outer membranes and passing through the peptidoglycan layer (Burns 1999). This process is known as type IV secretion and is similar to systems used by other pathogens to export effector proteins. Although secreted, some PT remains surface-associated and may participate in the adhesion of *B. pertussis* to cells such as human ciliated cells and macrophages. Thus, PT may be unusual as both a toxin and adhesin (Tuomanen 1988). Others have questioned the role of PT as an adhesin and have suggested that it is the enzymic activity and resulting pharmacological effects of PT that aid the bacterium in colonizing the mouse respiratory tract, even in the absence of FHA (Alonso et al. 2001). Nevertheless, both PT and FHA are required for efficient binding to target cells such as human ciliated cells and they also act cooperatively in the uptake of *B. pertussis* by macrophages. PT upregulates the function of the macrophage integrin CR3 and thereby promotes attachment and uptake of *B. pertussis* via FHA (Tuomanen 1993). Activation of CR3 and augmentation of FHA binding has also been described for fimbriae and the Arg-Gly-Asp (RGD) domain of FHA.

Table 3 *Some activities of pertussis toxin*

Activity
Demonstrated at the cellular level in vitro:
ADP-ribosylation of G proteins
Hemagglutination
Adhesion/invasion
Inhibition of neutrophil oxidative burst
Inhibition of monocyte migration
Inhibition of histamine release from mast cells
T cell mitogenicity
Demonstrated in experimental animals and *in humans:
Protective antigen (in toxoided form)*
Induction of leukocytosis*
Enhancement of insulin secretion*
Inhibition of adrenaline hyperglycemia*
Sensitization to histamine
Sensitization to anaphylaxis, anoxia, endotoxin
Adjuvanticity
Enhancement of vascular permeability
Acute toxicity/lethality

Upon receptor binding, PT is taken into the eukaryotic cell by receptor-mediated endocytosis and undergoes vesicular trafficking and membrane translocation to reach its target proteins on the inner surface of the plasma membrane. PT needs to be activated to exert its toxic effects. This requires ATP and the reduction of a disulfide bond in the S1 subunit to liberate it from the B pentamer (Xu and Barbieri 1995; Hazes et al. 1996). Many of the activities of PT depend on the enzymatic activity of the S1 subunit which is an adenosine diphosphate (ADP)-ribosyltransferase that catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to target proteins. In this way, PT resembles cholera toxin and *E. coli* heat-labile toxin. PT ADP-ribosylates the α subunits of eukaryotic GTP-binding regulatory (G) proteins (Krueger and Barbieri 1995). The modified G proteins are unable to function in normal signal transduction processes across the membrane and, because different cell types and signaling systems are affected, the ensuing biological effects are numerous (Table 3). One such effect, the leukocytosis induced by PT, may be explained by its inhibition of L-selectin expression by leukocytes, preventing their migration to areas of infection and homing of blood lymphocytes to peripheral lymphoid tissues (Hudnall and Molina 2000; Hodge et al. 2003).

PT is important for virulence of *B. pertussis* in mouse models of infection. One probable target is the phagocytic cells, in limiting their migration to the site of infection in the respiratory tract and in reducing their phagocytic potential and killing mechanisms (Weiss and Hewlett 1986; Schaeffer and Weiss 2001). Some of the systemic effects of pertussis infection or pertussis vaccination in man, such as leukocytosis, enhancement of insulin secretion, and inhibition of adrenaline hyperglycemia (Table 3), can be reproduced in animals by injection of PT and some of the effects are long lasting. Because of this, PT has been proposed as having a central role in the pathogenesis of pertussis and being in some way responsible for the prolonged course of the disease and for the symptoms which persist after the infection has been cleared (Pittman 1984a). Its actual

role, however, and its main site of action in the host, whether locally in the respiratory tract or systemically, perhaps in the central nervous system, remain obscure (Hewlett 1997). In view of the many common features of bordetella diseases and the fact that PT is unique to *B. pertussis*, it is difficult to assign a preeminent role to PT in either pathogenesis or host specificity. Nevertheless, immunization with PT, in toxoided form (PTd), protects mice against both intracerebral and respiratory challenges with *B. pertussis* (Sato and Sato 1988). Monocomponent acellular vaccines containing PTd have been shown to be somewhat efficacious in a number of field trials in children and chemically detoxified PTd has been a major component of acellular pertussis vaccines in use since 1981 (Sato et al. 1984). Such chemical toxoiding however, is known to reduce immunogenicity and fully immunogenic, recombinant PTd, has been developed (Rappuoli et al. 1992). One such molecule (PT-9K/129G) produced by site-directed mutagenesis of the active site of the enzymic moiety, has been included in acellular vaccines tested in large scale field trials and shown to be efficacious.

Fimbriae

B. pertussis produces two types of fimbriae (pili) which carry the antigenic determinants of the serotype 2 and 3 agglutinogens and individual strains may express one, both or neither. Serotype 2 and 3 fimbriae are composed predominantly of protein subunits of Fim2, 22.5 kDa, and Fim3, 22 kDa, respectively (Mooi 1994). Serologically cross-reactive fimbrial subunits of different molecular weights are found in other bordetellae. A gene *fimA*, which is homologous to the genes for Fim2 and Fim3, is not expressed in *B. pertussis* because of a deletion at its 5′ end. In contrast, intact *fimA* genes are present in *B. bronchiseptica* and *B. parapertussis*, and *fimA* is expressed in *B. bronchiseptica*. It has been proposed that *fimA* represents the primordial *Bordetella* fimbrial gene. Other gene products, FimB and FimC, are required for the secretion and assembly of these fimbriae. Additional fimbrial genes have now been identified in the whole-genome sequences, including an operon for type IV pilus biosynthesis that is present in *B. bronchiseptica* but not in *B. pertussis* or *B. parapertussis* (Parkhill et al. 2003).

Bacterial fimbriae are usually involved in adhesion but the exact role of bordetella fimbriae has not been clearly defined. They do not appear to play a role in attachment of *B. pertussis* in its usual location, on human respiratory ciliated cells (Tuomanen 1988). However, binding to other cell types, including monocytes and a human laryngeal epithelial cell line, but not a human bronchial epithelial cell line, has been reported (Van den Berg et al. 1999). Type 2 and 3 fimbriae of *B. pertussis*, in addition to the major fimbrial subunits, also contain FimD, a protein of 40 kDa, as a minor component located at the tip of the fimbrial structures. This protein enables the bacterium to bind to cells via their cell-surface integrin VLA-5. Binding of FimD to VLA-5 activates the monocyte CR3 receptor and significantly enhances FHA-mediated attachment of *B. pertussis* to these cells (Hazenbos et al. 1995). Both the major and minor fimbrial subunits can also mediate binding to glycosaminoglycans, structures ubiquitous on many mammalian cell surfaces and in the respiratory tract (Geuijen et al. 1998). This property is shared with FHA.

Other evidence suggests that fimbriae are important in both pathogenesis and immunity. Of the four recognized serotypes (1,2,3; 1,2; 1,3; and 1) of *B. pertussis*, only the first three (possessing fimbriae) infect man. Mutants of *B. pertussis* and *B. bronchiseptica*, unable to produce fimbriae, had reduced ability to colonize the tracheas of mice and rats, respectively (Geuijen et al., 1997; Mattoo et al. 2000). Purified fimbriae have been shown to protect mice against respiratory infection with *B. pertussis* and, with challenge strains of different serotypes, protection was found to be serospecific. Moreover, infection of mice that were immune to one fimbrial type led to selection of *B. pertussis* strains that produced the other type (Robinson et al. 1989). These findings are consistent with suggestions that protection of children with whole-cell pertussis vaccine is to some extent serospecific (Preston 1988). For these reasons, it is recommended by WHO that strains expressing both types of fimbriae are included in whole-cell pertussis vaccines (WHO 1979; Fry et al. 2001). Purified type 2 and 3 fimbriae are also included in some multicomponent acellular pertussis vaccines.

FHA

FHA appears to be the major adhesin of *B. pertussis* and one of the two hemagglutinins (the other being PT), and its primary role appears to be in colonization of the ciliated respiratory epithelium. Similar, but not identical, proteins are present in *B. parapertussis* and *B. bronchiseptica* and *B. avium* also produces an FHA-like protein (Spears et al. 2003). Genes encoding other FHA-like proteins have been identified in the genome sequences of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* and await further characterization for their possible role in host–parasite interactions (Parkhill et al. 2003).

FHA is synthesized as a large precursor protein of 367 kDa. The precursor protein is exported to the bacterial surface where it forms filamentous (4 × 50 nm) appendages and is thought to be anchored by its C-terminal moiety. Transport across the inner membrane is directed by a 71 amino acid signal sequence which is then removed. Transport across the outer membrane requires an accessory outer membrane protein FhaC (Jacob-Dubuisson et al. 2000). Eventually, the C-terminal

portion is cleaved by the surface-bound serine protease SphB1 (Coutte et al. 2001) and the N-terminal 220 kDa 'mature' FHA is released. Release of FHA from the surface of *B. pertussis* may facilitate transmission of the organism within the respiratory tract or from host to host (Arico et al. 1993). A *B. pertussis* mutant defective in the SphB1 protease had a reduced ability to colonize the mouse respiratory tract, suggesting that release of FHA allows the bacteria to disperse and colonize new sites (Coutte et al. 2003). It has also been proposed that FHA (and PT) released from *B. pertussis* in the respiratory tract could bind to other bacteria by a process which has been called 'piracy of adhesins', allowing them to efficiently adhere to cilia and promoting secondary bacterial infections (Tuomanen 1988).

As an adhesin, FHA is unusual in having multiple binding activities. These allow *B. pertussis* to adhere to different cell types, including ciliated epithelial cells and macrophages, possibly at different stages of infection and in conjunction with other adhesins (Locht et al. 1993; Mooi 1994). FHA has a CR3 recognition domain that allows adhesion to macrophages and other cell types. Another of its binding activities is due to an RGD domain that binds to leukocyte response integrin in concert with integrin-associated protein and causes upregulation of CR3 (Ishibashi et al. 1994). In the absence of opsonizing antibodies, such FHA-mediated binding of *B. pertussis* to macrophages via CR3 leads to phagocytosis that does not result in subsequent killing and may be critical in the intracellular survival of this organism. FHA has also been reported to mediate attachment of *B. pertussis* to neutrophils but in a way that fails to promote phagocytosis (Weingart and Weiss 2000). FHA also has a carbohydrate recognition domain specific for binding lactosylceramides (Prasad et al. 1993). This is responsible for attachment of FHA to respiratory cilia and to some extent to macrophages, although it is not important for uptake of *B. pertussis* into macrophages. Recognition of lacotsylceramides is also a property of the S2 subunit of PT. In addition, FHA, has a binding activity for glycosaminoglycans, substances ubiquitous in the respiratory tract, with similar, but not identical, specificity to that of the fimbrial subunits (van den Berg et al. 1999). With FHA, this specificity is responsible for its hemagglutinating activity (Hannah et al. 1994). By electron microscopy, FHA filaments can been seen to have a globular head, a long shaft of c. 35 nm and a small flexible tail. Molecular modeling suggests that the shaft is composed of three parallel β sheets with the CR3, RGD, and carbohydrate recognition domains located near the middle. The hemagglutinating/glycosaminoglycan binding domain is located at the head (Kajava et al. 2001).

Infection experiments have shown that FHA mutants of *B. pertussis* (Kimura et al. 1990) and *B. bronchiseptica* (Cotter et al. 1998) have a reduced ability to colonize and persist in the upper respiratory tract of mice and rats, respectively. Immunization with FHA alone will protect mice against aerosol challenge with *B. pertussis* and FHA enhances the protective activity of PTd in mice against a lethal intracerebral (i.c.) challenge. This i.c. challenge test is a standard test of pertussis vaccine potency. In view of its adhesive and mouse-protective activities, FHA is one of the prime antigens in acellular pertussis vaccines. FHA also has immunomodulatory properties. It can inhibit the production of IL-12 by macrophages in response to LPS and γ-IFN, by stimulating the production of IL-10 (McGuirk and Mills 2000).

PRN

PRNs are surface-associated and secreted proteins produced by the three closely related *Bordetella* spp. (Table 2). PRN from *B. pertussis* (P.69) has an apparent MW of 69 kDa. Homologous proteins, of slightly different size, are found in *B. parapertussis* (P.70) and *B. bronchiseptica* (P.68). They are encoded as large precursor proteins. The precursor of *B. pertussis* PRN is 93.5 kDa, from which a 3 kDa N-terminal signal sequence and a 30 kDa C-terminal portion are cleaved proteolytically to yield mature PRN of 60 kDa. This molecule migrates anomalously in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with an apparent MW of 69 kDa, hence its descriptor P.69. The signal sequence is responsible for *sec*-mediated transport of the pertactin precursor across the inner membrane. The C-terminal 30 kD of the precursor is necessary for translocation of pertactin from the periplasm through the outer membrane to the cell surface (Charles et al. 1989, 1994).

P.69 has been shown to mediate attachment of *B. pertussis* to, and invasion of, some tissue culture cell lines (Roberts et al. 1991; Leininger et al. 1991, 1992) although other evidence suggests that expression of pertactin by *B. pertussis* may actually impede its invasion of human tracheal epithelial cells (Bassinet et al. 2000). Like FHA, PRN has an RGD, integrin-binding domain but there is conflicting evidence on the role of this domain in attachment and invasion (Arico et al. 1993; Everest et al. 1996). The position of the RGD site in the tertiary structure of pertactin indicates that it is unlikely to function in eukaryotic cell binding (Emsley et al. 1996). Nevertheless, PRN and FHA may act in concert for efficient cell binding. Studies with a *B. bronchiseptica prn* mutant show that PRN is involved in attachment of the organism to porcine ciliated respiratory cells and alveolar macrophages (Forde et al. 1999). *B. pertussis prn* mutants, however, were unaffected in their ability to colonize the lungs of mice (Roberts et al. 1991; Khelef et al. 1994).

Despite the uncertainty on the exact role of PRN in pathogenicity, it is an important protective immunogen of *Bordetella* spp. and, when given parenterally or

intransally, P.69 is protective in mice against aerosol challenge with *B. pertussis*. The related molecule P.68 has been reported to protect mice and pigs from *B. bronchiseptica* infection (Roberts et al. 1992, 1993). There has been much interest, therefore, in PRN as a vaccine component. It was shown to be present in a significant amount in one of the early Japanese acellular vaccines and it is now included as a main constituent in some of the newer acellular formulations**.** A B-cell epitope, recognized by both humans and mice, has been identified in a particular region, region 1, of the PRN molecule and shown to be important in immunity to *B. pertussis*. Region 1 is composed of amino acid repeats (GGxxP) and is adjacent to the RGD motif. Variation in region 1 (giving types prn1, 2, 3 etc.) has been detected in circulating strains of *B. pertussis*. In an intranasal challenge model in mice, a whole-cell vaccine prepared from strains expressing prn1 afforded less protection against prn2 and prn3 strains than against prn1 strains (King et al. 2001).

Other virulence-associated factors

PRN and a number of other related, Bvg-regulated proteins in bordetellae are now recognized as members of the family of autotransporter proteins found in gram-negative pathogens. They consist of a secreted, N-terminal (passenger) domain and a C-terminal domain that is thought to oligomerize in the outer membrane to form a β-barrel pore to allow secretion of the passenger protein. At the cell surface, the passenger domain may be proteolytically cleaved and released or it may remain associated with the cell (Henderson and Nataro 2001; Veiga et al. 2002). In addition to PRN, four such proteins, SphB1, BrkA, TCF, and Vag8 have been characterized. The genome sequences of the bordetellae have revealed the presence of a number of genes encoding other autotransporters but each of the three species contains a different complement of such proteins (Parkhill et al. 2003). Thus, the autotransporter mechanism appears to be common in the bordetellae for transport of functionally different proteins to the cell surface.

Bordetella resistance to killing (BrkA) is synthesized as a large precursor, of 103 kDa, and is processed into the mature 73 kDa surface protein and a 30 kDa outer membrane protein (Fernandez and Weiss 1994). A *B. pertussis* mutant deficient in BrkA was less virulent for mice than the parent strain, less adherent to HeLa cells, less invasive for these cells and at least 10-fold more susceptible to killing by normal human serum. Possession of BrkA by *B. pertussis* inhibits the antibody-dependent classical pathway of complement activation by preventing the deposition of C4 on the bacterial surface. *B. pertussis* does not activate the antibody-independent branches of the classical pathway. BrkA, therefore, protects the bacterium against all of the effects of complement activation such as upregulation of the immune system and opsonization, as well as against direct killing via the membrane attack complex. Such a resistance mechanism is useful even for pathogens of the respiratory tract where significant levels of complement do occur and increase during inflammation (Barnes and Weiss 2003). Antibodies to BrkA can neutralize this resistance (Oliver and Fernandez 2002). Sequences homologous to the *brkA* gene are present in *B. parapertussis* and *B. bronchiseptica* and the protein has been detected in some strains of *B. bronchiseptica* by immunoblotting with a monoclonal antibody to BrkA. However, loss of BrkA from *B. bronchiseptica* does not render the organism sensitive to complement killing (Rambow et al. 1998) presumably due to the resistance conferred by its smooth LPS. BrkA also confers resistance to killing by antimicrobial peptides (Fernandez and Weiss 1996)

Tracheal colonization factor (TCF) is expressed only in *B. pertussis* and a mutant lacking this protein had reduced ability to colonize mouse trachea compared with the parent strain (Finn and Stevens 1995). It is expressed as a precursor of apparent MW 90 kDa and cleavage releases the mature protein of apparent MW 60 kDa, leaving the 30 kDa C-terminal fragment in the outer membrane. Intranasal immunization of mice with an attenuated *Vibrio cholerae* strain expressing TCF reduced the ability of *B. pertussis* to colonize the trachea (Chen et al. 1998).

An autotransporter protein, Vag8, first described in *B. pertussis* (Finn and Amsbaugh 1998), appears to be involved in Type III secretion in *B. bronchiseptica*. Genes encoding components of Type III secretion systems and potential translocated proteins have been identified in *B. bronchiseptica*, ovine *B. parapertussis*, human *B. parapertussis*, and *B. pertussis*, but appear to be expressed only in the first two (Yuk et al. 1998a, 2000). Such systems are responsible for translocating effector proteins directly from the bacterial cytoplasm to the cytoplasm of eukaryotic cells. In *B. bronchiseptica*, its expression is Bvg-regulated and it appears to be involved in secretion of at least three polypeptides. These products enable long-term persistence of *B. bronchiseptica* in the tracheas of rodents. They appear to interfere in various ways with the action of both the innate and acquired immune systems and are cytotoxic for macrophages and epithelial cells (Harvill et al. 1999a; Heininger et al. 2002; Stockbauer et al. 2003).

VIRULENCE REGULATION

Serotype variation

The major serotype antigens or AGGs 1, 2, and 3 occur in various combinations on different strains of *B.*

pertussis and thereby differentiate the four widely recognized serotypes 1,2,3; 1,2; 1,3; 1. The AGGs are all lost during phase variation (see below), but AGGs 2 and 3 can also be lost or regained independently in vitro and in vivo. Such changes occur at about one in every 10^3–10^4 cell divisions (Willems et al. 1990). AGGs 2 and 3 are located on fimbriae and serotype variation appears to be due to spontaneous frameshift mutations in the promoter region of the fimbrial subunit genes.

Phase variation and phenotypic modulation

Phase variation in *B. pertussis* was first described by Leslie and Gardner (1931) as a stepwise degradative process from phase I, with the characteristics of fresh isolates, to phase IV, with the different phases being distinguished by colony morphology, hemolysis, and serology. Phases I and II were toxic for guinea pigs whereas phases III and IV were not. It is now clear that degradation of *B. pertussis* strains is even more complicated than the above four stages but the spontaneous occurrence of phase IV or 'avirulent-phase' mutants is well known. Such strains no longer express the above-mentioned virulence factors associated with fresh isolates (Wardlaw and Parton 1988b; Stibitz and Miller 1994), with the exception of TCT and endotoxin. Phase variation occurs at a frequency of one per 10^3–10^6 organisms and is occasionally reversible. Intermediate phase variants of *B. pertussis*, expressing different combinations of virulence factors, have also been reported (Cotter and DiRita 2000).

Expression of virulence factors in bordetellae is also regulated in response to growth conditions in vitro. The term 'antigenic modulation' was introduced by Lacey (1960) to describe the freely reversible phenotypic changes that occur when *B. pertussis* is grown with high levels of certain salts, organic acids or at low temperatures. The so-called C (cyanic) mode or avirulent phenotype was originally distinguished from the X (xanthic) mode or virulent phenotype by serology and cultural characteristics. A serologically distinct intermediate or I mode, produced under submodulating conditions, was also identified. Subsequent work has shown that many of the virulence factors are lost during the X to C transition, as are certain outer-membrane proteins, adhesiveness, and hydrophobicity (Wardlaw and Parton 1988a).

The similar loss of virulence factors during phenotypic modulation and phase variation suggested that a common regulatory mechanism was involved and that phase variation was the genotypic equivalent of phenotypic modulation. Thus, phase I and X mode are essentially the same, as are phase IV and C mode, although the ability of phase IV strains to grow on nutrient agar (Leslie and Gardner 1931; Peppler and Schrumpf 1984) suggests that additional changes may occur upon repeated subculture. The difference is that the X to C change is environmentally regulated, affects all cells in the population and is freely reversible, whereas the phase I to IV change is an infrequent, mutational event that requires subsequent clonal selection for recognition, and reversion is equally infrequent or impossible. Weiss and colleagues (Weiss et al. 1983; Weiss and Falkow 1984) demonstrated that a single Tn*5* insertion in the *B. pertussis* chromosome abolished the expression of the virulence factors such as PT, ACT, FHA, and DNT. They had thus identified a virulence regulatory locus which they designated *vir*, and which was later renamed *bvg* (bordetella virulence gene). Thus the organism may undergo phenotypic modulation between the virulent (Bvg^+) or avirulent (Bvg^-) phase or mode according to growth conditions in vitro but the actual signals that modulate the Bvg system in vivo/ex vivo are unknown. Phase variation is caused by spontaneous mutations in the *bvg* locus and both reversible, frameshift mutations and irreversible deletions have been found in avirulent phase strains. Sequences homologous to *bvg* are present in *B. bronchiseptica*, *B. parapertussis*, and *B. avium* and they are related to so-called two-component signal transducing systems in other bacteria.

A number of virulence-repressed genes (*vrg*) have now been identified but, in most cases their functions are unknown. They are regulated by the *bvg*-locus in a reciprocal fashion to the virulence-activated genes (*vag*) encoding the virulence factors described above. Some *vrg* gene products in *B. bronchiseptica* have metabolic functions and are additionally regulated by growth phase, with maximal expression in the late log or stationary phase (Schneider et al. 2002). The regulation of virulence expression via the *bvg* locus (Cotter and DiRita 2000; Coote 2001; Bock and Gross 2001; Cotter and Jones 2003) is summarized in Figure 2. Transcription analysis has revealed that the *vag* genes are turned on sequentially. Activation of the genes encoding the adhesins FHA and fimbriae (early *vag*s) occurs immediately after the shift to Bvg^+ growth conditions whereas expression of those encoding PT and ACT (late *vag*s) begins after 2 h. Expression of PRN occurs at an intermediate time. The timing of activation of the *vag* genes appears to depend on the affinity, the number and orientation of BvgA binding sites upstream of *vag* genes and the concentration of BvgA within the cell.

Significance of virulence regulation

The chameleon-like potential for change in *B. pertussis* and other bordetellae is thus apparent (Figure 3) but its significance in the life-style of these organisms is less clear. There is evidence that fimbrial (serotype) variation in *B. pertussis* occurs in animal models of infection and in human infections. This type of variation and variation in other surface components that arise

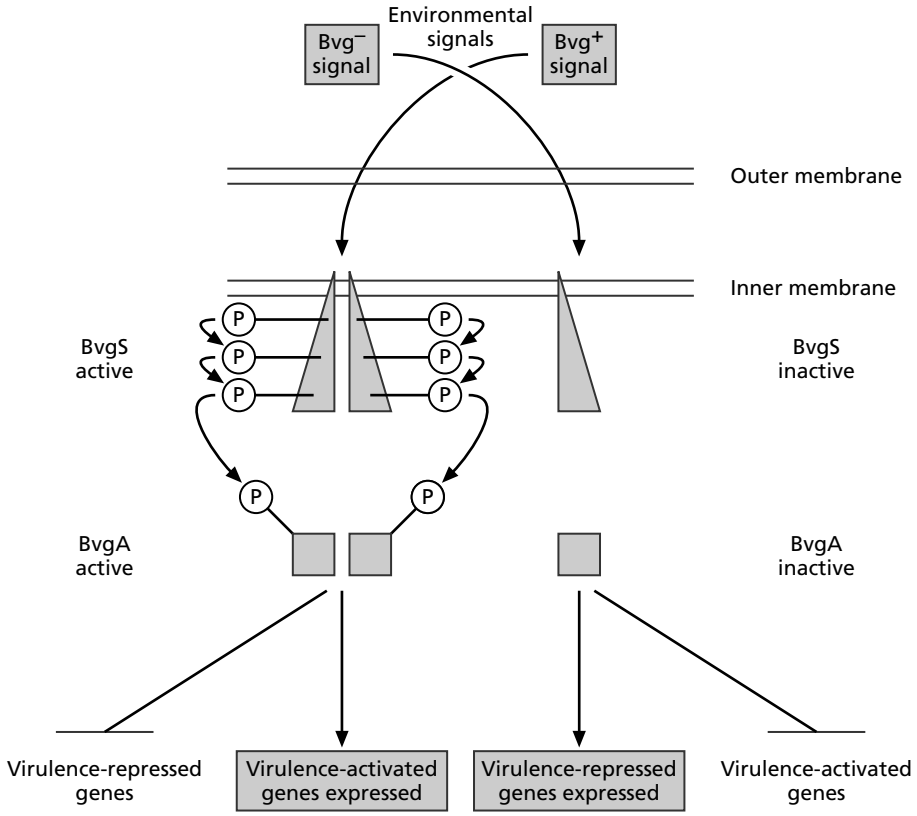

Figure 2 *Bvg-mediated virulence regulation. The* bvg *locus encodes two proteins BvgS and BvgA with homology to 'two-component' signal-transducing proteins in other bacteria and which function as dimers. BvgS is a sensor protein spanning the inner membrane and BvgA is a cytoplasmic response regulator protein able to bind to the DNA. Incoming (Bvg⁺, virulent mode) signals are transmitted to the cytoplasmic portion of BvgS which autophosphorylates and then, via a phosphorelay system, activates BvgA. Phosphorylated BvgA allows transcription of the virulence-activated genes (*vags*) and causes repression of other, virulence-repressed genes (*vrgs*). Under modulating (Bvg⁻, avirulent mode) growth conditions, BvgS and BvgA are inactive and only the* vrgs *are expressed.*

spontaneously could be useful in evading the host immune response and could be selected for in vivo due to natural or vaccine-induced immunity. With regard to phenotypic modulation, it may be that the ability of a *B. pertussis* population to respond rapidly to environmental influences is more than just a laboratory artifact or an evolutionary remnant from the ancestral, environmental bordetellae. There has been speculation that it may undergo modulation, not only to evade the host immune response, but to aid transmission, to prolong survival between hosts or to colonize some site other than the ciliated surface of the human respiratory tract. The idea of a significant role for the coordinate regulation of virulence is strengthened by the knowledge that this process also occurs in *B. bronchiseptica*, *B. parapertussis*, and *B. avium*. Rappuoli (1994) has suggested that the *bvg*AS system evolved to enable the bordetellae to distinguish between the external environment, at a low temperature, from the host environment at a higher temperature. Temporal control of virulence factors, so that adhesins are expressed first and toxins later, would be appropriate if a Bvg⁻ phase organism was transferred to a new host or to a new site within the host where it had to combat host defenses.

However, it is now clear that different sets of genes are controlled by *bvg*AS in these species. For example, in *B. bronchiseptica*, but not in *B. pertussis*, Bvg-activated factors include a type III protein secretion system, possibly involved in combating the host immune system, and LPS modification. *Bvg*-activated factors unique to *B. pertussis* include PT and TCF. Some factors that are expressed only in the Bvg⁻ phase of *B. bronchiseptica* and not in that of *B. pertussis* include an adhesin (Register and Ackermann 1997), flagella (Akerley and Miller 1993), and urease (McMillan et al. 1996). Thus, *bvg*-regulation appears to control different functions in these two species and this may be related to their different life-styles. *B. bronchiseptica*, but not *B. pertussis*, is capable of environmental survival and growth in nutrient-limited conditions. The Bvg⁻ phase of *B. bronchiseptica* appears to be necessary for this. However, studies with Bvg⁺ and Bvg⁻ phase-locked mutants in animal models have shown that the Bvg⁺ phase of both species is necessary and sufficient for respiratory infection and that expression of *bvg*⁻-repressed factors in the Bvg⁺ phase hinders the infection process (Martinez de Tejada et al. 1998; Merkel et al. 1998). In *B. bronchiseptica*, *bvg* regulation could allow

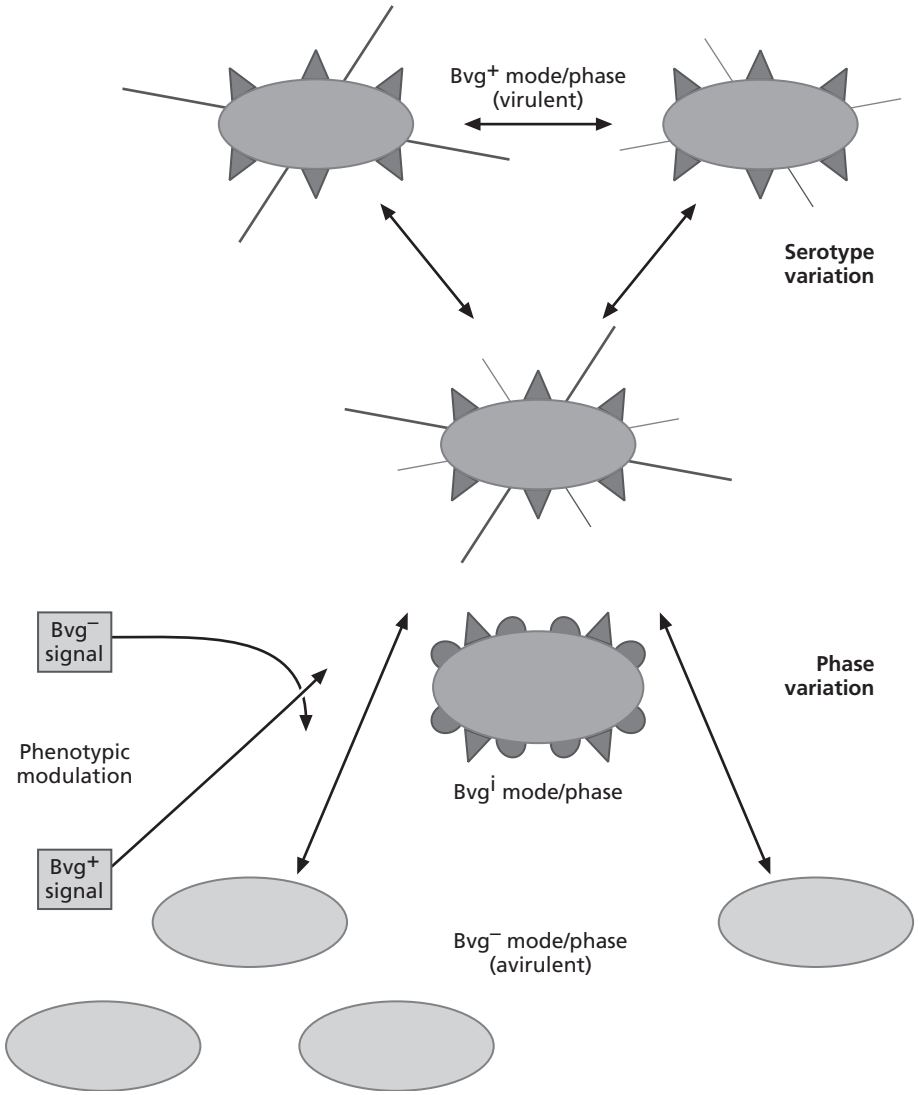

Figure 3 *Variation in* B. pertussis. *The combined processes of serotype variation, phenotypic modulation, and phase variation make* B. pertussis *and other bordetellae highly variable and, potentially, highly adaptable organisms. These processes may be involved in evasion of host immune responses, transmission or survival at different sites in vivo or ex vivo. Phenotypic modulation affects all cells in the population and is freely reversible whereas serotype and phase variation are infrequent, mutational events.*

the organism to adapt to the very different conditions in vivo (perhaps in different hosts) and ex vivo between hosts (Cotter and Miller 1994, 1997).

The role of the Bvg^- phase of *B. pertussis* may not be clear but it has been suggested that an intermediate phase (Bvg^i), between Bvg^+ and Bvg^-, is important for sensing location within the respiratory tract and perhaps for transmission to new hosts. Such a phase has been reported for *B. bronchiseptica* and *B. pertussis* and is characterized by the absence of *vrg* products, the presence of some but not all *vag* products, and some products unique to the Bvg^i phase (Cotter and Miller 1997; Cotter and DiRita 2000). Organisms in the nasal cavity, at a significantly lower temperature than those deeper in the respiratory tract, may be in the Bvg^i phase and primed for transmission to a new host. A Bvg^i phase-specific protein BipA, that may be involved in the transmission process, has been identified in *B. bronchiseptica* and the *bipA* gene is present in *B. pertussis* (Antoine et al. 2000; Cotter and DiRita 2000). BipA is a large outer-membrane protein that has a region of homology with the intimin protein of enteropathogenic *E. coli* and invasin of *Yersinia* spp. and therefore may be involved in adherence (Stockbauer et al. 2001). For further speculation on the role of *bvg* regulation in *Bordetella* species, see Cotter and DiRita (2000), Coote (2001), Bock and Gross (2001), Cotter and Jones (2003).

A second two-component system named regulator of intracellular stress (Ris) has been described in *B. bronchiseptica* and *ris* genes are present in all eight *Bordetella* species (Jungnitz et al. 1998; von

Wintzingerode et al. 2001). The *ris*AS locus is regulated by environmental conditions but independently of *bvg*AS and appears to be optimally expressed at 37°C and in the absence of magnesium. Ris-regulated products include an acid phosphatase and they appear to play a role in persistence in mouse lungs and also in intracellular survival, by conferring resistance to phagosomal acidification and reactive oxygen intermediates. Thus, Ris may be important in the chronic progress of *B. bronchiseptica* infections (Jungnitz et al. 1998; Zimma et al. 2001).

GENOME ORGANIZATION

The whole-genome sequences of representative strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are now available (Parkhill et al. 2003). The strains chosen for sequencing were *B. pertussis* Tohama I, a strain widely used for laboratory studies, *B. parapertussis* strain 12822, a human isolate, and *B. bronchiseptica* RB50, a rabbit isolate. The final assembly of the *B. pertussis* sequence was verified by comparison with a physical map of its genome constructed by Stibitz and Garletts (1992). These workers had used PFGE to locate the positions of the virulence genes and some 'housekeeping' genes**.** Apart from the close proximity of *fha*, *bvg*, and of some genes involved in fimbrial assembly, they had found no significant linkage of the virulence genes. Comparison of the genome sequences has supported earlier suggestions that *B. pertussis* and *B. parapertussis* have evolved independently from a *B. bronchiseptica*-like ancestor. The *B. bronchiseptica* RB50 genome is 5 338 400 bp with 5 007 predicted genes, whereas that of *B. parapertussis* 12 822 is 4 773 551 bp with 4 404 genes and that of *B. pertussis* Tohama I is 4 086 186 bp with 3 816 genes. Relatively few genes are unique to either the *B. pertussis* or *B. parapertussis* strains and few, if any, of these are thought to be involved in pathogenicity. In contrast, *B. bronchiseptica* RB50 has over 600 genes not present in the either of the other two species. *B. pertussis* has 358 pseudogenes, *B. parapertussis* 220, and *B. bronchiseptica* 19. Many of the genes that are missing or inactivated in *B. pertussis* and *B. parapertussis* are thought to be involved in transport and metabolism, regulation of gene expression, and synthesis of surface structures. It thus appears that the characteristics of *B. pertussis* and *B. parapertussis* that distinguish them from each other and from *B. bronchiseptica*, i.e. biochemical properties, virulence factor expression, and host range, are due predominantly to large scale loss of genes and gene function rather than to acquisition of new genes.

There is a high degree of colinearity between the genomes of *B. bronchiseptica* and *B. parapertussis*, whereas that of *B. pertussis* shows major differences in gene arrangement that is unusual with closely related species (Parkhill et al. 2003). Insertion sequence (IS) elements are abundant in the *B. pertussis* Tohama I genome, with 238 copies of IS*481*, 17 copies of IS*1663* and six copies of IS*1002*. *B parapertussis* contains 22 copies of IS*1001* and nine copies of IS*1002* whereas *B. bronchiseptica* has none of these particular IS elements. The genome rearrangements in *B. parapertussis* and especially in *B. pertussis* appear to have been caused mainly by recombination between IS elements. Marked differences in genome organization between different strains of *B. pertussis* noted by Stibitz and Yang (1997) were presumed to be due to such IS-mediated events. Pseudogene formation has also resulted from this process of rearrangement and from in-frame stop codons and frameshifts. Weiss (1992) had speculated that the growth deficiencies and fastidious nature of *B. pertussis* was caused by the presence of such IS elements in the genome. IS*481* is also present in 8–10 copies, in *B. holmesii* (Reischl et al. 2002)

Antoine et al. (2000) have searched the genome sequence of *B. pertussis* for new potential virulence factors by screening for genes that were regulated positively or negatively by the *bvg* locus using a transcriptional fusion reporter plasmid. The genes identified include more putative toxins, adhesins, and iron-uptake systems. Further investigation of the bordetella genome sequences will no doubt help to identify other virulence factors and protective antigens, and provide a better understanding of their mechanisms of virulence regulation and pathogenic potential.

BACTERIOPHAGES

Bacteriophages have been described in various *Bordetella* species and bacteriophage genes have been identified in the genome sequences (Parkhill et al. 2003). *B. avium*-specific temperate phages have been characterized and phage resistance was associated with changes in LPS composition of the bacterium (Shelton et al. 2002). Rauch and Pickett (1961) isolated a number of bacteriophages from *B. bronchiseptica* strains and each strain showed a unique phage sensitivity pattern. Some of the phages were lytic for *B. parapertussis*, but not for *B. pertussis*. An unusual group of temperate bacteriophages was recently identified in *B. bronchiseptica* (Liu et al. 2002). The viral particles had icosahedral heads and short necks with six tail fibers ending in unusual globular structures. Their genomes consisted of 42.5 kb of double-stranded DNA. The original isolates had a marked tropism for Bvg^+ rather than Bvg^- bordetellae and PRN was identified as one of the phage receptors. From these bacteriophages, variants could be derived that had switched tropism to Bvg^--phase or could form plaques on either Bvg^+ or Bvg^- strains. The three types of variants were capable of converting between the different tropisms. A reverse transcriptase-mediated mechanism was shown to be responsible and capable of generating a vast repertoire of phage specificities.

HABITAT AND PATHOGENICITY

Until recently, the bordetellae were considered to be obligate, noninvasive parasites of the respiratory tracts of warm-blooded animals, including birds, with a predilection for the respiratory ciliated epithelium (Wardlaw 1990). This definition has been confounded by more recent observations. First, most strains of the newly described species *B. holmesii* and *B. trematum* were isolated from human blood or wounds, respectively, and were not associated with respiratory infection. There are also reports of the isolation of *B. pertussis*, *B. bronchiseptica*, and *B. hinzii* from human blood. These isolations appear to have been mainly from immunocompromised patients and so an increasing number of such findings is perhaps inevitable. Second, it has become apparent that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* have mechanisms for attachment to, invasion of, and survival in, both epithelial cells and phagocytes. The significance of this habitat is not yet clear as none appears to replicate intracellularly. *B. pertussis* may be capable of only short-term survival whereas *B. bronchiseptica* is better able to survive in the acidic environment of the mature phagolysosome (Schneider et al. 2000). However, an intracellular phase may be significant in the protracted course of bordetella infections or in establishment of a quiescent or chronic stage. The importance of cell-mediated immunity to *B. pertussis* infection, demonstrated in animal models, may be due in part to destruction of such infected cells (Mills et al. 1998). Third, although the bordetellae were generally regarded as having little capability for growth and survival outside their hosts, *B. petrii*, an environmental isolate, has now been described. Moreover, it has been shown that *B. bronchiseptica* and *B. avium* are able to survive and grow in low nutrient conditions such as natural waters (Porter and Wardlaw 1993; Porter et al. 1994; Cotter and Miller 1994). Phenotypic modulation appears to be required for this survival. Finally, the ability of at least four *Bordetella* species to regulate virulence factor expression in response to external signals via the *bvg* and *ris* loci strongly suggests that they could have alternative habitats, either in vivo or ex vivo, where such regulation would be an advantage.

In considering the pathogenicity of members of the genus, the range of virulence factors produced by each species (Table 2) should be examined. This may provide clues as to which factors are responsible for the unique or common features of the diseases. Such evidence has been used by Goldman (1988) to propose that TCT, produced by at least the first four described species, has a central role in the pathogenesis of bordetella infections. The common features of the diseases in their natural hosts caused by these species include age-related susceptibility to infection, adherence of organisms to ciliated epithelium and loss of ciliated cells from the respiratory tract, reduced weight gain of the host, excessive mucus production, and some form of cough. Secondary infections also appear to be common and increase the mortality rate in untreated cases (Wardlaw and Parton 1988a).

Despite their common features and close genetic relationship, *B. pertussis* and *B. bronchiseptica* have clearly different pathogenic mechanisms. *B. pertussis* causes a severe, acute, potentially life-threatening disease in humans, whereas *B. bronchiseptica* establishes a persistent, often asymptomatic infection in its animal hosts. In a direct comparison of the strains of *B. pertussis* and *B. bronchiseptica* chosen for the genome sequencing project, Harvill et al. (1999a) showed that *B. bronchiseptica* was more persistent in colonizing the respiratory tract of immunocompetent mice, grew to greater numbers, caused greater lung pathology, and stimulated a greater antibody response than *B. pertussis*. It was also more aggressive in immunodeficient mice and more cytotoxic for a mouse macrophage cell line. In a subsequent study which included the *B. parapertussis* genome strain, this organism resembled *B. bronchiseptica* in some of these properties and *B. pertussis* in others (Heininger et al. 2002).

There is no completely satisfactory explanation for the different host specificities of the bordetellae (Table 4), although adhesion mechanisms to host ciliated cells seem to be influential. *B. pertussis* has been shown to adhere best to human cilia, *B. bronchiseptica* and *B. parapertussis* adhered best to non-human mammalian, but not avian, cilia and *B. avium* adhered specifically to avian ciliated cells (Tuomanen 1988). To determine whether host specificity of the bordetellae might be related to different nutrient conditions in their respective hosts, Porter and Wardlaw (1994) collected tracheobronchial washings (TBW) from seven vertebrate species. *B. bronchiseptica* and *B. avium* grew well in most of the TBW samples, *B. parapertussis* grew in TBW from man, sheep, and certain other species but, surprisingly, *B. pertussis* did not grow in any sample,

Table 4 *Host specificities of* Bordetella *species*

Species	Host	Source of human isolates
B. pertussis	Man	Respiratory tract
B. parapertussis	Man, sheep	Respiratory tract
B. bronchiseptica	Mammals, including man	Respiratory tract
B. avium	Birds	–
B. hinzii	Birds, man	Respiratory tract, blood
B. holmesii	Man	Blood, respiratory tract
B. trematum	Man	Wounds, ear

including that from man. Considering the genomic differences between three species, Parkhill et al. (2003) have suggested that the limited host range of *B. pertussis* and *B. parapertussis* compared to their *B. bronchiseptica*-like ancestor can be explained by their loss of host interaction mechanisms. The virulence of *B. pertussis* for humans could be due to overexpression or constitutive expression of virulence factors that are subject to regulatory processes in the other species. With the high rate of transmission that occurs in the human population, the loss of ability to cause only limited damage to the host and for environmental survival is no longer a selective disadvantage for *B. pertussis*.

Bordetella pertussis

The pathogenicity of *B. pertussis* has been studied in various models in vivo and in vitro, in cell and organ cultures. In vivo studies with *B. pertussis* have been, of necessity, in various unnatural animal models of infection and the relative merits of each of these models have been reviewed (Sato and Sato 1988; Shahin and Cowell 1994). Mouse models are used most often but a coughing rat model has the potential for investigation of the mechanisms of cough production and immunity in pertussis (Parton et al. 1994; Hall et al. 1998). The results of such studies, particularly with mutants deficient in individual virulence factors, have clearly shown that pertussis pathogenesis is complex and multifactorial. Loss of any one of a number of toxins and adhesins has been shown to affect the organism's ability to colonize the host or cause pathological changes. Based on the information in the preceding and following sections, a possible sequence of events in pertussis is shown in Figure 4.

In a model using human nasal turbinate tissue in an air–interface organ culture, bacteria bound initially to mucus, cilia, and areas of epithelial damage. Bacterial replication led to a great increase in the number of *B. pertussis* attached to the cilia and was associated with the loss of ciliated and non-ciliated cells and increased secretion of mucus (Soane et al. 2000). New insights into the host response to *B. pertussis* infection are now being obtained by using the human genome sequence information and DNA microarrays to measure gene transcription from human cells exposed to the organism. For example, when a bronchial epithelial cell line was infected with *B. pertussis*, expression of a very large number of genes was altered (Belcher et al. 2000). Some were up-regulated but many more were down-regulated. Those up-regulated included genes for proinflammatory cytokines and chemokines and anti-apoptotic factors. This was in spite of the fact that *B. pertussis* components are known to inhibit chemotaxis of leukocytes and to induce their apoptosis. This study also revealed increased mucin gene expression and the ability of *B. pertussis* to bind to mucin. With human peripheral blood monocytes, exposure to *B. pertussis* appeared to cause an active suppression of the induction of TNFα that normally is induced in response to bacterial stimulation (Boldrick et al. 2002).

Before widespread vaccination, pertussis was primarily a disease of young children in that the majority of typical cases occurred in the age group 1–5 years with the most severe disease and most fatalities in early infancy. Passive protection is only poorly passed on from the mother to the newborn. All ages are susceptible, however. Pertussis is highly communicable with attack rates ranging from 50 to 100 percent, depending on the intensity of exposure. Transmission can mostly be attributed to droplet infection from active cases. Asymptomatic, culture-positive carriers have been detected (De Schutter et al. 2003), but their role in transmission of the disease, in the absence of coughing, is unknown. There is no known non-human host, vector or environmental reservoir of *B. pertussis*.

The clinical features of pertussis tend to vary with age, general health, and immune status, and adults and older children often show mild or atypical symptoms. Typical pertussis can be divided into the catarrhal, paroxysmal, and convalescent stages. After an incubation period of 7–14 days, the catarrhal stage resembles a non-distinctive, upper respiratory tract infection with a mild cough, excessive mucus production, and sometimes mild fever. The cough becomes increasingly severe and, after 7–10 days, the patient enters the paroxysmal stage. This is characterized by bouts of uncontrollable coughing, up to 20 or more in 24 h, when the patient is attempting to clear the tenacious mucus from the airways. The paroxysms are often followed by the distinctive, inspiratory whoop. Vomiting and hypoxia may also ensue. A typical feature of the late catarrhal and early paroxysmal phases of pertussis is a pronounced leukocytosis with a predominant lymphocytosis. The paroxysmal stage may last for 1–6 weeks or more, then the cough gradually subsides over a prolonged convalescent period. A high percentage of all reported cases of pertussis require hospitalization, especially those in infants <6 months of age. Secondary infections, such as otitis media and pneumonia, are relatively frequent, especially in infants, and may result from impairment of clearance mechanisms by the bacterium. Complications resulting from pressure effects of the coughing paroxysms include subconjunctival hemorrhage, pneumothorax, epistaxis, subdural hematomas, hernias, and rectal prolapse. Central nervous system disturbances, in the form of seizures and encephalopathy, may also occur and are thought to be due to the hypoxia or, possibly, to bacterial toxins. Further information on clinical aspects of pertussis may be found elsewhere (Cherry et al. 1988; Hodder and Mortimer 1992; Cherry 1996; Hewlett 2000).

Pertussis or whooping cough is responsible for severe morbidity and mortality world wide, mainly in

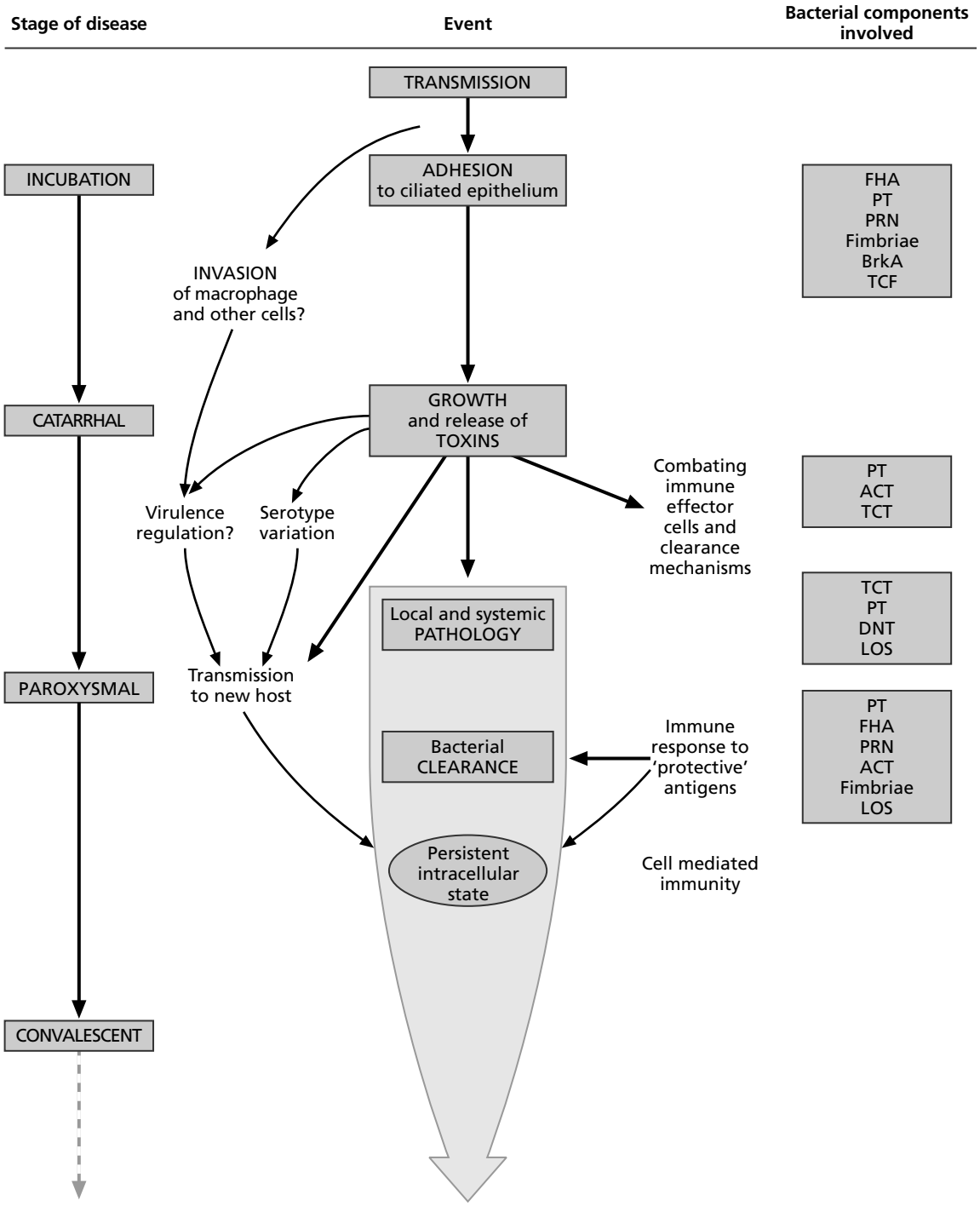

Figure 4 *The sequence of events in pertussis. The postulated roles for the various bacterial components at the different clinical stages of disease are based on evidence from in vitro and in vivo models of infection.*

unvaccinated communities. Each year, there are some 20–40 million cases, 90 percent of which occur in developing countries, and an estimated 200 000–300 000 fatalities (WHO: www.who.int/vaccines-diseases/diseases/pertussisvaccine.htm). Fortunately, global immunization schemes are steadily reducing these figures. In some vaccinated communities, the problems have been rather different. In the mid-1970s, concern about the possible harmful effects of whole-cell vaccines in some countries such as the UK, and the associated decline in vaccine uptake, resulted in recurrences of pertussis epidemics. As vaccine uptake recovered, control was once again established. There is no doubt that vaccination, where practiced, has greatly reduced the incidence and severity of pertussis but it also appears to have changed the age distribution of the disease. Most cases in vaccinated communities occur in adolescents and adults with waning immunity and in infants too young to have been

vaccinated (Edwards et al., 1999; Van Buynder et al. 1999). It is now recognized that adults may constitute an important reservoir of infection and that pertussis is the cause of a high percentage of cases of persistent coughs in adults (Black 1997; Cherry 1999). Reports of re-infection of adults, diagnosed with pertussis as children, have also been documented, suggesting that immunity induced by infection, like that induced by vaccination, is not solid. When most adults had pertussis as children and then were regularly re-exposed to infection, there was a high level of adult immunity. In well-immunized populations, the opportunity for this boosting of vaccine-induced immunity is limited. Serological studies on vaccinated and unvaccinated populations have suggested that, whether immunization is practiced or not, the disease is endemic (Cherry 1999). Also, in populations vaccinated or not, epidemics occur every 3–4 years, presumably due to the number of susceptible individuals in the population. A lack of effect on this frequency by introduction of vaccination or changes in vaccine uptake rates seems to indicate that whole-cell vaccines provide better protection against disease than against infection and that vaccination has little effect on the prevalence of *B. pertussis* in the population (Fine 1988). This may be partly because the vaccines are administered parenterally and as such are unlikely to stimulate mucosal immunity in the respiratory tract.

Despite the proven efficacy of whole-cell vaccines, the real and perceived problems associated with their use prompted the development of acellular pertussis vaccines, fully characterized in terms of their antigenic components and in which the toxic components of *B. pertussis* are absent or inactivated (Hewlett and Cherry 1997; Edwards et al. 1999; Decker and Edwards 2000; Roberts and Parton 2001). Two such vaccines, a monocomponent PTd and a two-component PTd + FHA vaccine, were used in a large-scale, double-blind, placebo-controlled field trial in 1986–87 in Sweden where pertussis was prevalent (Ad Hoc Group 1988). Their reactogenicity was low, as expected, but efficacy values against culture- or serologically-confirmed pertussis (54 percent for the monocomponent PTd and 69 percent for the two-component vaccine) were lower than would have been expected for a whole-cell vaccine, although such a vaccine was not included in this trial. Both vaccines, however, gave better protection (c. 80 percent) against severe pertussis.

As a result of the outcome of the Swedish trials, plans were made for further field trials of other acellular pertussis vaccine formulations and, where possible, to compare them with whole-cell vaccines and placebo controls. A number of vaccines were selected, containing from one (PTd alone) to five components: PTd (chemically or genetically toxoided), FHA, pertactin and fimbrial antigens 2 and 3. Large-scale trials of these acellular preparations were carried out in the early-mid 1990s in various countries. The design and outcomes of these trials, and their limitations, have been reviewed extensively (Decker and Edwards 2000; Edwards et al. 1999; Halperin 1999; Hewlett and Cherry 1997; Plotkin and Cadoz 1997). All of the acellular preparations were efficacious, with estimates of efficacy against severe pertussis ranging from 59–93 percent. In general, the multicomponent vaccines (containing three to five antigens) were better than the one- and two-component products. However, the best whole-cell vaccines were as good or better than the acellular vaccines with efficacy estimates from 94–98 percent. In all trials where whole-cell vaccines were included, the acellular vaccines were significantly less reactogenic in terms of both local and systemic reactions. On the basis of the above results, all of the acellular vaccines used in the trials have now been licensed and are in use in some countries, but the less expensive whole-cell vaccines will remain in use in many countries for some time to come. Whole-cell pertussis vaccines are considered to be too reactogenic for use in adults so, in view of the lower reactogenicity of acellular preparations, it has been recommended that they are used to boost immunity in older children and adults (Olin 1997; Plotkin and Cadoz 1997; Campins-Marti et al. 2002). Despite the proven efficacy of acellular and whole-cell vaccines, the mechanisms by which they produce immunity in humans remains uncertain. It is still not clear which are the most important components or what the optimal composition might be, although serological data from the most recent vaccine trials indicated that antibodies to PRN, Fim2 and Fim3, and to a lesser extent PT, were associated with clinical protection (Cherry et al. 1998; Storsaeter et al. 1998; Hewlett and Halperin 1998). Future pertussis vaccine developments may involve the creation of stable, genetically defined, attenuated *B. pertussis* strains suitable for use as live vaccines and possibly for delivery of heterologous antigens (Stevenson and Roberts 2003). Intranasal delivery of these or other, non-living antigen formulations would avoid the need for injection and would help to stimulate effective mucosal immunity.

In recent years, a resurgence of pertussis has been reported in some countries, including US, Australia, Canada, and the Netherlands, even though they have maintained high vaccination rates (Crowcroft and Britto 2002). The reasons for this are unclear but the increasing number of susceptible adults, as mentioned above, improved diagnosis and reporting in this age group, as well as lower vaccine efficacy, have been suggested. The recent discovery of structural variants of PRN and the S1 subunit of PT in circulating *B. pertussis* strains may also be a factor. In the Netherlands, when the *prn* and *ptx* genotypes of contemporary, historical, and vaccine strains were compared, non-vaccine types were found to have gradually replaced the types included in the whole-cell vaccine. This led to the suggestion that a process of 'vaccine-driven evolution' may have selected for strains that are antigenically distinct from the vaccine strains

(Mooi et al. 1998, 2000; van Loo and Mooi 2002). Similar, but not identical, antigenic shifts have been reported in other countries (Mooi et al. 1999; Mastrantonio et al. 1999; Cassiday et al. 2000), perhaps reflecting the use of vaccines of different composition, but not all are associated with a resurgence of pertussis (Fry et al. 2001). However, such findings may have implications for the efficacy of both whole-cell and especially acellular vaccines with their more limited antigenic repertoire. There is therefore a need for continued surveillance and typing of circulating strains and there may be a future need to adjust the composition of these vaccines to contain several pertactin and PT types (Mooi et al. 1998).

Bordetella parapertussis

B. parapertussis is generally considered to cause a milder form of disease than *B. pertussis*. Although it may be true that it is isolated less frequently than *B. pertussis* and that many parapertussis infections are asymptomatic, this organism is capable of causing typical whooping cough (Hoppe 1999). In two studies of coughing illness in Germany, paroxysmal coughing, whooping, and vomiting were all features of parapertussis infections, with frequencies approaching those in pertussis cases (Heininger et al. 1994; Wirsing von König and Finger 1994). The main difference was that lymphocytosis, due to PT which is produced only by *B. pertussis*, was not a feature of parapertussis. These findings suggest that PT may play a role in exacerbating the typical symptoms of whooping cough but that other toxins are primarily responsible. In both studies, the percentage of patients who had received pertussis vaccine was greater in the parapertussis group than in the pertussis group. This observation supports previous evidence that there is little cross-immunity between the two infections. For example, Lautrop (1971) showed that, over a 20-year period in Denmark, the epidemics of pertussis and parapertussis both occurred in 3–4 year cycles but were out of phase with each other. In view of the close relationship between *B. pertussis* and *B. parapertussis*, and their similar range of virulence factors, the lack of cross-immunity is remarkable. Khelef et al. (1993) showed that FHA, PRN, and ACT from *B. pertussis* given parenterally, were protective antigens for mice against intranasal challenge with *B. pertussis* but not against *B. parapertussis* suggesting that their virulence factors have important antigenic differences. However, broncho-alveolar washes from mice convalescing from respiratory infection with one of these organisms gave some reciprocal protective immunity to subsequent challenge with the other. It was suggested that mucosal immunity, including FHA-specific secreted IgA, were important for this reciprocal protection (Watanabe and Nagai 2001).

Until recently, *B. parapertussis* was considered to be a purely human parasite, like *B. pertussis*, but its isolation from healthy and pneumonic lambs in New Zealand and Scotland (Porter et al. 1994) has changed this view. Human and ovine strains, although closely related, are phenotypically and genetically distinct (van der Zee et al. 1997). Thus, there is no evidence for transmission of *B. parapertussis* between humans and sheep. Ovine isolates have been shown to cause pneumonia in experimentally infected lambs and, when administered with *Pasteurella haemolytica*, to cause more severe disease than either agent alone. This situation parallels that with *B. bronchiseptica* and *Pasteurella multocida* in atrophic rhinitis in pigs.

Bordetella bronchiseptica

This organism is an important pathogen of domestic and laboratory animals and it is often carried asymptomatically. Its broad host range includes dogs, cats, pigs, guinea pigs, rats, mice, rabbits, horses, turkeys, monkeys, humans, and numerous wild animals. *B. bronchiseptica* is one of the causes of atrophic rhinitis, an economically important disease of swine, characterized by inflammation of the nasal turbinates, turbinate atrophy, snout deformation, and retarded growth (Rutter 1985; Woolfrey and Moody 1991). Although *B. bronchiseptica* alone is capable of causing mild to moderate turbinate atrophy, the severe, progressive disease appears to be caused by a subsequent colonization by toxigenic *P. multocida* and an interaction between the two species. Both produce a heat-labile, dermonecrotizing toxin that, on repeated local or parenteral administration, causes progressive turbinate atrophy. It is thought that TCT released by *B. bronchiseptica* enhances colonization by *P. multocida* by inducing ciliostasis and mucus accumulation. Another less common disease of pigs due to *B. bronchiseptica* is bronchopneumonia in piglets. Clinical signs include coughing, sometimes with whooping and dyspnea. *B. bronchiseptica* also causes infectious tracheobronchitis, or kennel cough, in dogs (Goodnow 1980; Bemis 1992). This is a highly contagious disease characterized by various degrees of coughing, sometimes paroxysmal, and purulent nasal discharge. Some pneumonia-related deaths occur but infection is generally limited to the tracheobronchial tree and the resulting pathological changes are similar to those described for whooping cough. The role of *B. bronchiseptica* in humans has been reviewed by Woolfrey and Moody (1991). They concluded that, despite the considerable exposure to animal sources of the organism, it is isolated only rarely from humans. It may be found as a respiratory tract commensal and as an opportunist, sometimes associated with septicemia in compromised patients.

Bordetella avium

This is the causative agent of turkey coryza, or turkey rhinotracheitis, although other birds are affected

(Kersters et al. 1984). Turkey coryza is a highly contagious disease of turkey poults and symptoms include oculonasal discharge, tracheal mucus production, coughing, loss of appetite, and decreased weight gain. It is sometimes a mild disease but conditions of stress and secondary infections, for example by *P. multocida*, can result in a high mortality rate. Histopathological examination shows attachment of the bacteria to the ciliated epithelium resulting in inflammation, loss of ciliated cells, and distortion of the mucosa and tracheal rings. The clinical features similar to those of other bordetella diseases suggest that the virulence factors, such as TCT and DNT, common to these species, have a central role in bordetellosis (Spears et al. 2003). Alternatively, *B. avium* may possess other virulence factors. For example, a protein toxin, osteotoxin, has been described that is cytotoxic for mammalian cells, including osteogenic and tracheal cells. The toxin is distinct from DNT and has β-cystathionase activity (Gentry-Weeks et al. 1993).

Bordetella hinzii, Bordetella holmesii, Bordetella trematum

There is little information on the pathogenic potential of these recently described species and they do not appear to contain the range of virulence factors in the above-mentioned species. The first strains of *B. holmesii* to be described were isolated from human blood cultures and several of these patients were known to have had an underlying condition that may have enhanced their susceptibility to infection (Weyant et al. 1995). More recently, *B. holmesii* has been isolated from sputum (Tang et al. 1998) and, with increasing frequency, from nasopharyngeal secretions of patients, mainly young adults, with pertussis-like symptoms (Mazengia et al. 2000). With strains of *B. hinzii* isolated from the respiratory tracts of turkeys and chickens, attempts to produce disease experimentally were unsuccessful (Vandamme et al. 1995). However, strains have been obtained from humans including from the blood of an AIDS patient (Cookson et al. 1994) and from the sputum of a patient with cystic fibrosis. In this latter patient, the isolations were made after periods of pulmonary exarcerbations of the disease (Funke et al. 1996). *B. hinzii* has also been identified as the cause of a case of fatal septicemia (Kattar et al. 2000). *B. trematum* has been associated only with human wound and ear infections and not with respiratory tract disease (Vandamme et al. 1996). Like *B. bronchiseptica*, these three strains appear to be opportunists in man. No hosts other than man have been reported for *B. holmesii* and *B. trematum*.

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Borrelia

DANIÈLE POSTIC

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INTRODUCTION

The genus *Borrelia* is associated with two distinct diseases, Lyme borreliosis (LB) and relapsing fevers. As they belong to the same genus, the bacteria responsible for each kind of disease share various phenotypic and genetic properties. However, the mechanisms of transmission, geographic distribution, physiopathology of infection, and the course of the diseases are very different. They have transmission by arthropods in common. Whereas relapsing fevers and some of their etiologic agents were known as early as the ninetenth century, *Borrelia* species related to LB have only been known since the 1980s. This chapter will focus mainly on *Borrelia* species associated with LB.

HISTORY

A review of the historical perspective of arthropod-borne spirochetoses has been given by W. Burgdorfer (Burgdorfer 2001).

The term 'relapsing fever' was used for the first time at Edinburgh in 1843. However, it was during an epidemic of recurring fevers in 1867–1868 that Obermeier observed the agent of the louse-borne relapsing fever. First it was named *Spirochaeta recurrentis* by Lebert in 1874, and then *Spirochaeta obermeieri* in 1875. In 1906, in East Africa, Novy and Knapp (1906) described *Spirochaeta duttonii* as the agent responsible for the human tick disease described by Livingstone in 1857, and they showed that the disease was transmitted by the tick *Ornithodoros moubata*. *S. duttonii* was named after Dutton, who observed the disease some years earlier, together with Todd in Kenya (Dutton and Todd 1907). For these spirochetes, Swellengrebel created the genus *Borrelia* in 1907, in honor of the French physician, A. Borrel. At the beginning of the twentieth century, Sergent and Foley first established the role of lice in the transmission of *Spirochaeta recurrentis*. Whereas the role of different species of *Ornithodoros* as vectors of tick-borne relapsing fevers was first demonstrated by C. Nicolle (Rodhain 1989) and then by Balthazard, major advances in the field of *Borrelia* would not have been possible without the development of an artificial culture medium by Kelly in 1971, further modified by Stoenner and Barbour and finally named BSK medium (Barbour 1984).

In 1982, W. Burgdorfer, who studied *Rickettsia* in *Ixodes scapularis*, formerly *I. dammini* ticks, found spirochetes in the midgut of these ticks which had been collected on Shelter Island, New York (Burgdorfer et al. 1982). The responsibility of these spirochetes was demonstrated (Steere et al. 1983) in the epidemic of arthritis that occurred in children and adults some years earlier in the county of Lyme in Connecticut (Steere et al. 1977). The spirochete was identified as a new species of *Borrelia* and named *Borrelia burgdorferi* (Johnson et al. 1984) in honor of W. Burgdorfer. With *B. burgdorferi*, many clinical manifestations, described as early as the 1880s in Europe, found an etiologic

agent. The association was established with the dermatologic manifestations of erythema migrans (Afzelius 1910; Lipschütz 1913) and acrodermatitis chronica atrophicans (Buchwald 1883; Herxheimer and Hartmann 1902), as well as with the neurologic manifestations described by Garin and Bujadoux (1922) and Bannwarth (1941). *Erythema migrans* was first described in the USA in 1970 (Scrimenti 1970). The multisystem illness named Lyme disease and now Lyme borreliosis, was then described by Steere (Steere et al. 1986; Steere 1989). *Borrelia burgdorferi* became the subject of extensive research, and the genome of *B. burgdorferi* sensu stricto was sequenced in 1997 (Fraser et al. 1997).

MORPHOLOGY

Borrelia are spirochetes of 0.2–0.5 μm in diameter and 10–30 μm long (Figure 1). Their size, as well as the number of spires, varies depending on the physiological state of the bacteria and the age of the culture. They are irregularly twisted and their spires are easily visible. *Borrelia* stain poorly, if at all, with Gram stain. Therefore, they are usually observed using dark-field microscopy.

They share the same basic ultrastructural features as other members of the Spirochaetaceae. An outer membrane encloses the protoplasmic cylinder, which consists of a peptidoglycan layer and an inner membrane enclosing the internal components of the cell. Seven to 11 periplasmic flagella are subterminally attached at each end of the cell cylinder and overlap at the center. They lie between the outer membrane sheath and the cell cylinder. These flagella propel the spirochetes by rotation (Goldstein et al. 1994). They are involved both in the motility of spirochetes and in their shape. Mutants lacking flagella become rod-shaped without wave-like structure (Motaleb et al. 2000). Flagella are crucial for spirochetes surviving in ticks as well as in mammalian hosts.

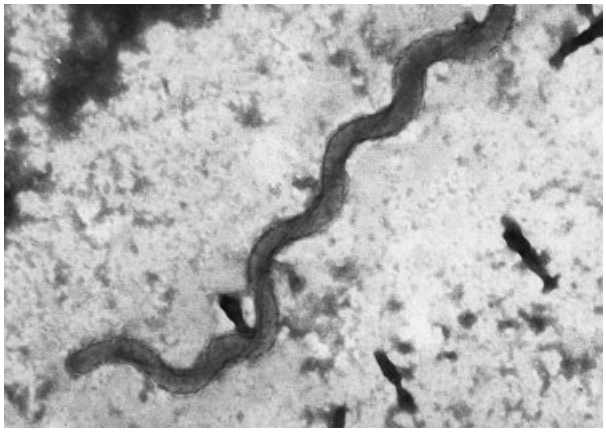

Figure 1 *Electron micrograph of a* Borrelia burgdorferi *cell negatively stained with ammonium molybdate*

TAXONOMY AND GENETIC DIVERSITY

Borrelia burgdorferi sensu lato

The etiological agent of LB was recognized in 1982 by Willy Burgdorfer. Two years later, this spirochete was named *Borrelia burgdorferi* (Johnson et al. 1984). Since that time, 11 related species have been identified on the basis of DNA relatedness, which are gathered together under the term *B. burgdorferi* sl. However, many related strains from both North America and Europe (Postic et al. 1999; G.Q. Wang et al. 1999b) are still not assigned to any established species. These species are not equally distributed throughout the world, and only three of them are known to cause LB in humans. *B. burgdorferi* sensu stricto (*B. burgdorferi* ss) is the sole species to cause LB in the USA, whereas *B. burgdorferi* ss, *B. garinii* (Baranton et al. 1992), and *B. afzelii* (Canica et al. 1993) are the causative agents of LB in Europe. Finally, *B. garinii* and *B. afzelii* are the two pathogenic species present in Asia, where *B. burgdorferi* ss is absent (Table 1).

With regard to the nonpathogenic species, some of them are restricted to a specific tick vector and host, and have a limited geographic spread. This is the case with *B. andersonii* specifically isolated on the western coast of the USA from the cottontail rabbit and its tick, *I. dentatus* (Anderson et al. 1989; Assous et al. 1994; Marconi et al. 1995). It is also the case with the Japanese species *B. tanukii* and *B. turdi* isolated from their respective ticks, *I. tanuki* and *I. turdus* (Fukunaga et al. 1996a). *B. japonica* (Kawabata et al. 1993) is the only *Borrelia* species isolated in Japan from *I. ovatus*, a tick common and widely distributed in this country. *B. sinica* was isolated from *Niviventer confucianus* and *I. ovatus* in Sichuan and Anhui provinces and seems to be restricted to China (Masuzawa et al. 2001). *B. lusitaniae* is restricted to Europe (De Michelis et al. 2000; Gern et al. 1999; Le Flèche et al. 1997) and, surprisingly, to North Africa (Younsi et al. 2001), where it is by far the predominant species also transmitted by *I. ricinus*.

In contrast, *B. valaisiana* (Wang et al. 1997) is a species widely distributed in Europe and Asia (but not in America) and offers a huge genetic diversity (Masuzawa et al. 2000; Wang et al. 2000; Derdakova et al. 2003). There is no association between this species and a tick species, since *B. valaisiana* has been isolated from *I. ricinus* in Europe, *I. columnae* in Japan, *I. nipponensis* in Korea, and *I. granulatus* in China and Korea (Miyamoto and Masuzawa 2002).

In the USA, *B. bissettii* (Postic et al. 1998) has been isolated from *I. pacificus* and *I. spinipalpis* in California and Colorado (Norris et al. 1997; Schwan et al. 1993; Schneider et al. 2000). The usual host is the woodrat *Neotoma fuscipes*. Only one strain has been isolated from *I. scapularis* in upstate New York (Anderson et al.

Table 1 *Characteristics of* Borrelia *species*

Species	Disease	Vector	Geographical distribution
American relapsing fever species			
B. hermsii	American tick-borne relapsing fever	*Ornithodoros hermsi*	Western USA, Canada
B. turicatae	American tick-borne relapsing fever	*O. turicata*	Southwestern USA
B. parkeri	American tick-borne relapsing fever	*O. parkeri*	Western USA
B. mazzottii	American tick-borne relapsing fever	*O. talaje*	Southern USA, Mexico, Central and South America
B. venezuelensis	American tick-borne relapsing fever	*O. rudis*	Central and South America
African relapsing fever species			
B. recurrentis	Louse-borne relapsing fever	*Pediculus humanus humanus*	Worldwide. Endemic in Sudan, Ethiopia, Somalia
B. duttonii	East African tick-borne relapsing fever	*O. moubata*	Central, eastern, and southern Africa
B. hispanica	Hispano-African tick-borne relapsing fever	*O. erraticus*	Spain, Portugal, Morocco, Algeria, Tunisia, Greece
B. crocidurae, *B. merionesi*, *B. microti*, *B. dipodilli*	North African tick-borne relapsing fever	*O. erraticus*	Morocco, Libya, Egypt, Iran, Turkey, Senegal, Kenya
Asian relapsing fever species			
B. persica	Asiatic-African tick-borne relapsing fever	*O. tholozani*	Middle East, Central Asia
B. caucasica	Caucasian tick-borne relapsing fever	*O. verrucosus*	Iraq, southwestern former USSR
B. latyschewii	Caucasian tick-borne relapsing fever	*O. tartakovskyi*	Southcentral, southwestern former USSR, Iraq, Iran, Afghanistan
Relapsing fever species of veterinary significance			
B. anserina	Avian borreliosis	*Argas persicus* and other *Argas* spp.	Worldwide
B. theileri	Bovine borreliosis	*Boophilus microplus*, *B. annulatus*, *B. decoloratus*, *Rhipicephalus evertsi*	South Africa, Australia, Brazil, Mexico
B. coriaceae	Epizootic bovine abortion (?)	*Ornithodoros coriaceus*	California
***B. burgdorferi* sensu lato**			
B. burgdorferi sensu stricto	Lyme borreliosis	*Ixodes scapularis*	Midwestern and eastern USA
		I. pacificus	Western USA
		I. ricinus	Europe
B. garinii	Lyme borreliosis	*I. ricinus*	Europe
		I. persulcatus	Russia, China, Japan
B. afzelii	Lyme borreliosis	*I. ricinus*	Europe
		I. persulcatus	Russia, China, Japan
B. valaisiana	Lyme borreliosis?	*I. ricinus*	Europe
		I. persulcatus	Japan
B. lusitaniae	Non pathogenic	*I. ricinus*	Europe, North Africa
B. andersonii	Non pathogenic	*I. dentatus*	Western USA
B. bissettii	Non pathogenic?	*I. spinipalpis*	Western USA
		I. pacificus	Southeastern USA
B. japonica	Non pathogenic	*I. ovatus*	Japan
B. turdi	Non pathogenic	*I. turdi*	Japan
B. tanukii	Non pathogenic	*I. tanuki*	Japan
B. sinica	Non pathogenic	*I. ovatus*	China
Intermediate *Borrelia* species			
B. miyamotoi	Non pathogenic?	*I. persulcatus*	Japan
B. lonestari	Mild Lyme borreliosis?	*A. americanum*	Southern USA

1990). *B. bissettii* also occurs in southeastern USA (Lin et al. 2001).

Genetic diversity proceeds from molecular mechanisms such as mutations, deletions, insertions, substitutions, plasmid gene rearrangements, as well as natural selection that controls the rate of accumulation of genetic variability. Either the whole genome or limited targets on the genome have been used to evidence the overall inter- or intraspecies diversity. Pulsed-field gel electrophoresis (PFGE) (Iyer et al. 2001), ribotyping (Fukunaga et al. 1993; Postic et al. 1990), random amplified polymorphic DNA (RAPD) (Wang et al. 1998), and multilocus enzyme electrophoresis (MLEE) (Boerlin et al. 1992) are current detection tools to differentiate species and also genotypes within each species of the *B. burgdorferi* sl complex. Polymerase chain reaction (PCR)-based methods, species-specific PCR, PCR-RFLP analysis (Postic et al. 1994; Postic et al. 1999), SSCP-PCR (Guttman et al. 1996; Norris et al. 1997), and AP-PCR (Welsh et al. 1992; Foretz et al. 1997) have been successfully used for nucleic acid sequence analysis. The most extensive targets studied to access the genetic diversity of *B. burgdorferi* sl are plasmidic genes coding for outer surface proteins such as *ospA* (Qiu et al. 1997; Wilske et al. 1993; Masuzawa et al. 1996), *ospC* (Livey et al. 1995; Theisen et al. 1995, Baranton et al. 2001; Dykhuizen and Baranton 2001; Marti Ras et al. 1997; Seinost et al. 1999; I.N. Wang et al. 1999c), *erp* (Akins et al. 1999; Stevenson et al. 2002; Sung et al. 1998), and *p66* (Bunikis et al. 1998; Norris et al. 1997). However, chromosomic genes such as the *rrs* gene (Le Flèche et al. 1997; Schneider et al. 2000; Ciceroni et al. 2001), the *fla* gene (Fukunaga et al. 1996b), the *hbb* gene (Valsangiacomo et al. 1997), as well as noncoding intergenic spacers (Liveris et al. 1995; Postic et al. 1994; Postic et al. 1998), are other widely used targets for classifying *Borrelia* strains. Plasmid content has been used as a tool for demonstrating phenotypic diversity (Barbour 1988; Schwan et al. 1988; Simpson et al. 1990).

LB is a multisystemic disorder characterized by skin, joint, cardiac and neurological involvement (Steere 2001). Evidence suggests that each species of pathogenic *B. burgdorferi* sl is preferentially associated with a distinct clinical form of the disease (Assous et al. 1993; Van Dam et al. 1993). *B. burgdorferi* ss is the agent of Lyme arthritis, and it is the reason why this clinical manifestation is the most frequently encountered in the USA. *B. garinii* and *B. afzelii* are mainly, but not exclusively, involved in the neurologic manifestations of the disease and in the late cutaneous manifestation of acrodermatitis chronica atrophicans (ACA), respectively. The three species cause the primary lesion of erythema migrans (EM). It is not known why the majority of untreated patients with EM recover, whereas only a few patients will develop late neurologic or arthritic manifestations. Moreover, some patients acquire the disease but show these secondary manifestations only, without initial EM. It is likely that differences in the pathogenic potential of strains could be an explanation. Factors leading to the pathogenicity are multiple and the fact that they belong to a pathogenic *Borrelia* species is not sufficient to predict the course of the disease. Indeed, no ACA has ever been reported in Japan, although *B. afzelii* is one of the most abundant pathogenic species there. In addition, some *Borrelia* strains are responsible for clinical manifestations other than the expected ones (Busch et al. 1996; Rijpkema et al. 1997). *B. valaisiana* DNA has been detected in the skin of patients with EM, but bacteria belonging to this species have never been isolated from any patients' material (Rijpkema et al. 1997). Whether this species is pathogenic for man remains questionable.

There is strong evidence that the genetic diversity of *B. burgdorferi* sl extends beyond the species level. Heterogeneous ecological and clinical data might be explained by species differences, but also by strain variations within a species. Therefore, the main interest of improved molecular tools to evaluate the genetic diversity of *B. burgdorferi* sl lies in the interrelationships that are apparent between some genotypes and clinical and epidemiological features. Interestingly, mixed populations of genetically and antigenically distinct *B. burgdorferi* clones may infect mice and infections can be acquired either by coinfection or by sequential infection. The lack of crossimmunization may play a role in the maintenance of the genetic heterogeneity of *B. burgdorferi* sl in nature (Hofmeister et al. 1999). It has been shown that some different genotypic characteristics were not independent, resulting in predominant genotypes (Iyer et al. 2001). Moreover, a positive correlation has been found between some genotypes and the ability of corresponding strains to invade and disseminate into the host leading to late manifestations of LB (Baranton et al. 2001; Seinost et al. 1999; Wormser et al. 1999). Although the mechanisms of pathogenicity of *B. burgdorferi* sl are not yet known, easy and rapid methods should be used as markers to facilitate further study of determinants of virulence.

B. miyamotoi and *B. lonestari*

While they are associated with hard ticks, *B. miyamotoi* and *B. lonestari* appear to be more closely related to relapsing fever species than to the *B. burgdorferi* sl complex (Marti Ras et al. 1996).

B. miyamotoi has been isolated from *I. persulcatus* and a rodent *Apodemus argenteus* in Japan and individualized as a new species on the basis of DNA relatedness studies (Fukunaga et al. 1995). The pathogenic potential of this species is unknown.

The evidence of an erythema migrans-like rash in the southern USA led Barbour et al. (1996) to further study the lone star tick *Amblyomma americanum*, a hard tick

that frequently bites humans. The geographic range of this tick coincides with that of the atypical disorder. A spirochete was identified in approximately 2 percent of ticks by immunofluorescence and PCR methods. However, this organism could not be cultivated in BSK medium used for *B. burgdorferi* and relapsing fever *Borrelia*. This new *Borrelia* species was named *B. lonestari*, on the basis of phylogenetic analysis of genes encoding the flagellin and the 16S rRNA. *B. lonestari* DNA has been amplified from the skin of a patient with erythema migrans (James et al. 2001), but whether *B. lonestari* is the actual etiologic agent of Lyme disease-like illness remains to be elucidated.

Recently, a new *Borrelia* species was identified for the first time from *I. scapularis* ticks in the northeast of the USA. This novel *Borrelia* clustered with relapsing fever group and was much more related to *B. miyamotoi* than to the North American *B. lonestari* or *B. theileri* (Scoles et al. 2001). Similarly, two DNAs closely related to *B. miyamotoi* were identified from *I. ricinus* ticks in Sweden (Fraenkel et al. 2002). Ecological and biological characteristics of these *B. miyamotoi*-like organism are not yet known.

Relapsing fever *Borrelia*

Taxonomy of relapsing fever *Borrelia* was initially based on the concept of cospeciation, each *Borrelia* species being associated with its specific vector (Felsenfeld 1965). Despite some advances in cultivation methods and molecular analysis of the diversity, the classification of this group of species has not undergone significant changes. *B. recurrentis*, the first spirochetal agent observed in the blood of a patient suffering a louse-borne relapsing fever by Obermeier in 1868, is now easily cultivable (Cutler et al. 1994). Successful isolation of *B. duttonii* was also achieved (Cutler et al. 1999).

Most of the phylogenetic relationships between these related spirochetes were based on analysis of the 16S rRNA sequences, despite its weak resolving power due to its high level of conservation (Marti Ras et al. 1996). Phylogenetic trees derived from the analysis of alignments of flagellin sequences also provide useful data for delineating species, consistent with those inferred from 16S rRNA gene analysis (Cutler et al. 1997; Scoles et al. 2001).

The North American species *B. hermsii*, *B. parkeri*, and *B. turicatae* usually cluster together, although *B. hermsii* is located on a separate branch within this cluster. According to DNA relatedness data, these three species belong to only one (Hyde and Johnson 1984).

B. crocidurae, *B. duttonii*, *B. recurrentis*, and *B. hispanica*, the tick-borne group transmitted by argasid ticks of the species *Ornithodoros*, cluster together, distantly related to the 'Spain' relapsing fever strain (Anda et al. 1996).

A third group consists of *B. miyamotoi* and related species, *B. lonestari* and *B. theileri*. *B. coriaceae* is located closely but separate to this group. *B. anserina* is deeply separated from other *Borrelia* species. The species status of *B. anserina* and *B. coriaceae*, the etiological agents of avian borreliosis, and epizootic bovine abortion respectively, have been established by DNA–DNA homology (Hyde and Johnson 1984; Johnson et al. 1987).

EPIDEMIOLOGY

LB is one of the most common tick-borne diseases in temperate regions of the northern hemisphere. *B. burgdorferi* sl is an obligatory parasite, maintained in nature within a complex enzootic cycle involving arthropod (*Ixodes* ticks) and vertebrate hosts. *B. burgdorferi* sl is transmitted to humans by ticks of the complex *Ixodes ricinus*. The main species responsible for transmission of LB are *I. ricinus* in Europe, *I. persulcatus* in Asia, *I. scapularis* in the central and eastern United States, and *I. pacificus* in the west part of the USA. Large variations in the life cycle of ticks occur according to climatic conditions and host availability. After egg-laying, larvae become abundant, usually at the beginning of summer. Larvae feed on small mammals (essentially rodents) and then molt into nymphs. After nymphs find an appropriate host, small mammals, birds, or even humans, they molt into adults. Lastly, adult females take a blood meal on larger animals and then will lay eggs that will enter a new cycle. This cycle may take 1, 2, or sometimes more years to complete, depending on environmental conditions. Apart from the roles of vector and reservoir, the role of amplifier has been also suggested for *I. scapularis* (Hodzic et al. 2001). Considerable variation in the infection rates among *Ixodes* ticks was observed between different geographic regions. Mixed infections have been documented in ticks (Misonne et al. 1998; Postic et al. 1997) and in reservoirs.

However, the geographic range of LB and even that of *B. burgdorferi* sl never coincide with the range of *Ixodes* ticks. Actually, the behavior of ticks towards humans and the presence and diversity of vertebrate hosts also play a critical role in the maintenance of *B. burgdorferi* sl and in the prevalence of the disease. Hence, if a particular tick species harbors a particular *Borrelia* species, but never or seldom bites humans, the prevalence of the disease will be low. Besides, it has been demonstrated that the infection prevalence of nymphs drastically declines with the increase in host diversity (LoGiudice et al. 2003). This fact is particularly important since the infection of ticks is directly correlated with the disease risk to humans.

A large range of animals act as reservoirs for *B. burgdorferi* sl species. However, a strong association has been described in Europe between specific hosts and *B. burgdorferi* sl species, such as *B. afzelii* and small

rodents (Hu et al. 1997; Humair et al. 1999), as well as *B. garinii* and *B. valaisiana* and birds (Humair et al. 1998; Olsen et al. 1995). However, *Apodemus* mice have been identified as reservoirs of *B. garinii* OspA serotype 4 in Switzerland (Huegli et al. 2002). In contrast, *B. burgdorferi* ss seems to be less specialized. It has been hypothesized that a complement-mediated selection in the midgut of the feeding tick could be a component of the host specificity of *B. burgdorferi* sl (Kurtenbach et al. 2002a, b). The Erp protein family could be involved in the binding to host-derived complement proteins which confers resistance to the complement of the host species (Hellwage et al. 2001; Alitalo et al. 2002; Stevenson et al. 2002). Those *Borrelia* that are sensitive to the complement of a given species are immediately killed in the tick midgut before transmission (Kurtenbach et al. 2002a). Thus, surface-exposed lipoproteins upregulated in mammalian hosts would be involved in a strategy for evading the host–immune response. However, coinfections with different *Borrelia* species are frequently observed in ticks and particularly in nymphs (Christova et al. 2001; Liebisch et al. 1998; Misonne et al. 1998; Pichon et al. 1995; Jenkins et al. 2001). As nymphs have taken only one blood meal, it is likely that these mixed infections have been acquired on the same animal. This is in contradiction with the previous hypothesis. It is likely that several mechanisms contribute to the persistence of *B. burgdorferi* sl in nature.

Clearly, climate influences the LB incidence. A significant correlation has been reported between LB incidence and moisture levels occurring during the 2 preceding years (Subak 2003). The recent climate change scenarios could contribute to a different repartition of the disease throughout the globe in the future.

Tick-borne relapsing fever *Borrelia* are transmitted by soft bodied ticks belonging to *Ornithodoros* species. *B. duttonii*, transmitted by *O. moubata*, is responsible for an endemic relapsing fever in sub-Saharan East Africa. *B. crocidurae* transmitted by *O. erraticus sonrai* is the cause of endemic relapsing fever in western Africa (Trape et al. 1991). The mode of transmission, either by the way of saliva, or by the coxal fluid, varies according to the stage of the tick. Interestingly, argasid ticks take numerous blood meals lasting some minutes compared to several days for *Ixodes* ticks. In North America, endemic relapsing fever occurs mainly in western regions. *O. hermsii* specifically transmits *B. hermsii*, as *O. turicata* and *O. parkeri* specifically transmit *B. turicatae* and *B. parkeri*, respectively.

The louse-borne species *B. recurrentis* caused widespread severe epidemics in the nineteenth century with a high mortality rate. Epidemic relapsing fever is currently associated with poor living conditions and social disruption. Now, the disease persists in a few isolated foci in Ethiopia (Sundnes and Haimanot 1993), Somalia, and Sudan (de Jong et al. 1995). The vector is the human body louse, which harbors *B. recurrentis* in the hemolymph. Contamination of man occurs through crushing of the infected louse on abraded skin.

Man is considered as the sole reservoir for *B. duttonii* and *B. recurrentis*. Some species only have veterinary significance. *B. theileri*, transmitted by *Boophilus microplus*, the cattle tick, and *B. coriaceae* transmitted by *O. coriaceus* cause borreliosis in livestock.

GENETICS

The entire sequence of the genome of *B. burgdorferi* ss has been available since 1997 (Fraser et al. 1997). The genome of *B. burgdorferi* ss strain B31 consists of a 910 kb linear chromosome, and 21 extrachromosomal elements comprising 12 linear plasmids and nine circular plasmids (Casjens et al. 2000). Genetic maps of the three pathogenic species are highly conserved, contrasting with physical maps which show significant differences (Ojaimi et al. 1994) (Figure 2). An unusual organization of ribosomal genes occur in *B. burgdorferi* sl, with only one copy of the 16S rRNA gene (*rrs*) and two copies tandemly repeated of 23S rRNA (*rrl*) and 5S rRNA (*rrf*) genes (Fukunaga and Sohnaka 1992; J.J. Schwartz et al. 1992b).

Coding sequences represent 93 percent of the chromosome, 59 percent code for biological functions, 12 percent matched with sequences coding for hypothetical functions, and 29 percent are new genes. The G + C content of the chromosome is 28.6 percent. The chromosomes of several isolates are very similar except in the right-end DNA which could be plasmid derived (Casjens et al. 2000). Authors have speculated that analysis of *B. burgdorferi* ss was consistent with a single copy of the chromosome per bacterium, in bacteria colonizing the mouse (Morrison et al. 1999). This contrasts with *B. hermsii* which has multiple copies of both chromosome and plasmids (Hinnebusch and Barbour 1992).

The plasmids comprise 610 kb and thus represent >40 percent of the total genome. The overall G + C content of plasmids ranges from 20.7 to 31.6 percent, but large variations in the G + C content occur according to their positions, mostly in lp17, lp28-1, and lp56. The linear elements have covalently closed termini or telomeres, characterized by short hairpin loops of DNA (Barbour and Garon 1987; Hinnebusch and Barbour 1991).

No less that 161 paralogous gene families have been identified, among which 107 contain at least one plasmid gene (Casjens et al. 2000). Casjens et al. identified 670 potentially functional genes and 167 pseudogenes on the B31 strain plasmids. Of the 670 plasmid genes, 5.8 percent are similar to previously sequenced genes of known function and 2.1 percent are similar to genes of unknown function. Most of the pseudogenes appear to be mutationally damaged genes, and their actual significance remains unknown. A very high degree of genetic redundancy occurs in *B. burgdorferi* plasmids, which is not yet completely understood. Multiple similar, but

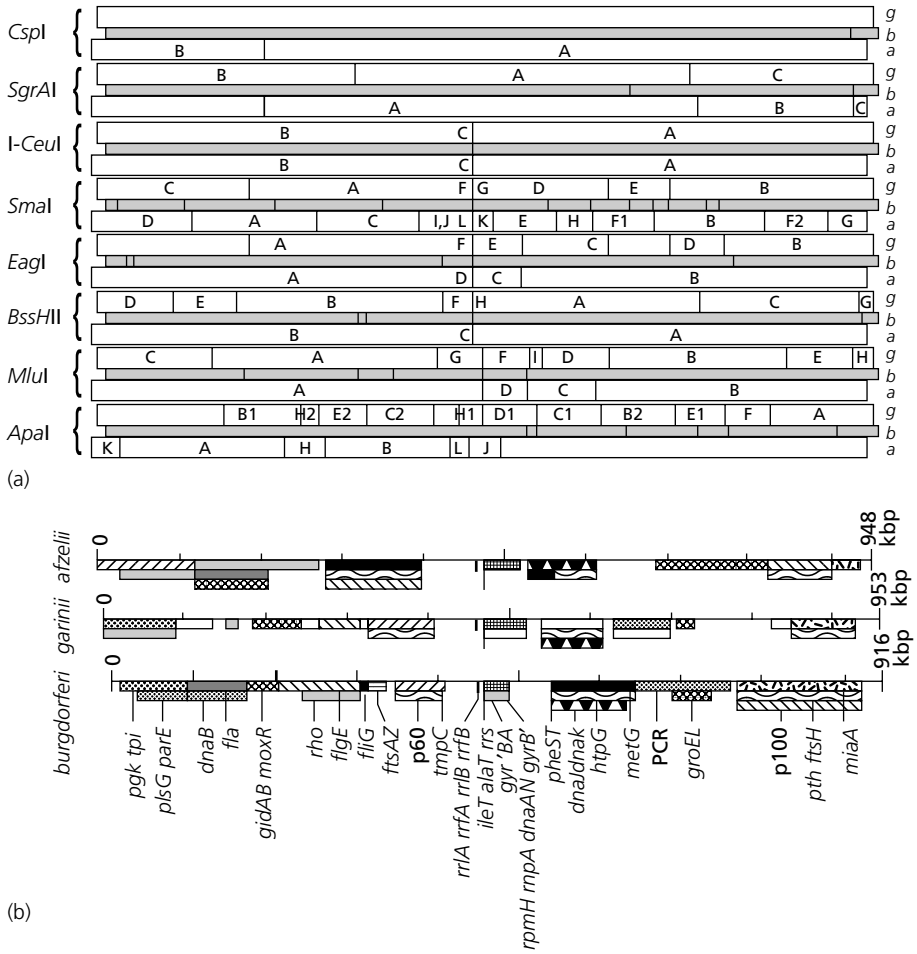

Figure 2 *Maps of the chromosomes of* B. garinii *20047,* B. burgdorferi*, 212 and* B. afzelii *VS461.* **(a)** *Physical maps. For each enzyme, the* B. garinii *map is represented at the top by open boxes, the* B. burgdorferi *map (Davidson et al. 1992; Csp1 and 1-Ceu1 sites mapped in this study) is represented in the middle by shaded boxes, and the* B. afzelii *map is represented at the bottom by open boxes. The three maps have been aligned by the centrally located* MluI *site in rrs. Fragments are designated A, B, etc. Only some of the* ApaI *sites and fragments are included in each map.* **(b)** *Genetic maps. Fill patterns for individual genes or gene clusters are the same in all three maps, with the* B. burgdorferi *map providing the key for the correspondence between specific fill pattern and gene/gene cluster. The genes* alaT *and* ileT *were not mapped for* B. garinii *and* B. afzelii*, while* pgk, tpi, tmpC, metG, PCR*, and the* p60 *gene were not mapped for* B. afzelii*. Redrawn from Ojaimi et al. 1994, with permission.*

slightly different, copies of various sequences lie on the plasmids. For example, up to nine very homologous cp32 plasmids can be maintained in a single *B. burgdorferi* isolate (Casjens et al. 2000). Interestingly, Eggers et al. speculated that cp32 plasmids are packaged by a lysogenic bacteriophage, suggesting a role in lateral gene transfer within a strain, as well as between strains (Eggers and Samuels 1999; Eggers et al. 2001).

The large number of plasmids raises issues concerning the replication and segregation mechanisms of the various replicons. It has been demonstrated that the central region of the chromosome was the site of a switch in polarity of GC skew, from which the replication could proceed bidirectionally (Picardeau et al. 1999). The same mechanism seems to occur in some plasmids (Picardeau et al. 2000). The sequence of the candidate replication origin is unique for each plasmid as experimentally demonstrated for cp32 and cp9 (Eggers et al. 2002; Stewart et al. 2001). A gene cluster containing *soj/parA* homolog is closely linked to the candidate origin of replication and suggests that these genes and other paralogous gene families could be involved in partitioning of both circular and linear plasmids (Casjens et al. 2000; Picardeau et al. 2000).

Compared to the chromosome, plasmids contain a few coding sequences. The larger fraction (14.5 percent) of plasmid genes code for lipoproteins or putative lipoproteins potentially involved in interactions between bacteria and host, whereas only approximately 5 percent of chromosome genes code for such putative lipoproteins. In contrast, only a few, if any, putative virulence genes have been identified (Fraser et al. 1997). Genes encoding major outer surface proteins are located on plasmids: *ospC* gene on the cp26 plasmid. *ospA* and *ospB* organized in a single operon and thus cotranscribed, are located on the

largest linear plasmid. They play an important role in bacteria–host interactions.

Most of the plasmids can be lost during culture or mouse passage procedures. The loss of specific plasmids has often been correlated with the attenuation or loss of infectivity (Schwan et al. 1988; Xu et al. 1996). Purser et al. determined the association between individual plasmids and infectivity. In particular, linear plasmids lp25 and lp28-1 are associated with infectivity for mice. The clones that lacked lp28-1 but contained lp25 expressed an intermediate-infectivity phenotype (Purser and Norris 2000). It has been speculated that these two plasmids encode proteins required for colonization and short-term maintenance in the skin and bladder, whereas lp28-1 is not required for colonization of the joint (Labandeira-Rey and Skare 2001). In contrast, circular and linear plasmids cp9, cp32-3, lp21, lp28-2, lp28-4, and lp56 were not required for infectivity in the mouse model used (Purser and Norris 2000).

Up until recently, no efficient system was available to study the function of genes in pathogenic *Borrelia*. Major advances have been made by P. Rosa and her team (Rosa et al. 1996). They first developed a method of gene inactivation by allelic exchange, using a mutated allele of the endogenous *gyrB* that confers coumermycin resistance (Rosa et al. 1996), and then a kanamycine cassette (Bono et al. 2000) as selectable markers. Alternatively, Sartakova et al. (2000) introduced a wide host range plasmid pGK12 into a noninfectious B31 strain, using the erythromycin resistance as a selective marker. This plasmid was propagated in B31 without recombination or integration to the chromosome. Recently Stewart et al. (2001) constructed a shuttle vector, pBSV2, derived from a 3.3 kb fragment of the circular plasmid cp9 containing three ORFs, which was able to replicate autonomously in the high passage *B. burgdorferi* B31 strain. The three ORFs are required to confer autonomous replication. The transformation efficiency of the noninfectious strain is high 1.8×10^{-6} and pBSV2 is extremely stable with 100 percent colonies retaining the plasmid after 25 generations of *B. burgdorferi*. However, a lower transformation efficiency was obtained with the infectious strain N40, which was more refractory to genetic manipulations. This may be due to an incompatibility of the shuttle vector with the resident cp9, which is lost in the high passage strain. In order to analyze phenotypes of mutant *B. burgdorferi* ss in an infectious cycle, Elias et al. (2002) derived several wild clones from the B31 strain infectious for mice. By using carefully optimized electrotransformation conditions, they demonstrated transformation with a shuttle vector and gene inactivation by allelic exchange. However, gene inactivation was successful in only one infectious clone. A similar approach was used by Eggers et al. (2002) to construct a cp32-based shuttle vector. Complementation studies are now feasible and, despite potential caveats in in vivo studies, open the way to access the role of numerous putative virulence genes in *B. burgdorferi* sl.

PATHOGENICITY AND RELATIONS HOST–VECTOR BACTERIA

The causative agents of LB and relapsing fevers have to undergo considerable physiological adaptations during the life cycle between their two niches, arthropods and mammals. Differential gene expression, up- or down-regulation of outer surface proteins, and starting of regulatory pathways are some adaptive changes developed in response to various environmental factors.

B. burgdorferi sl adapt to the environment during the different steps of their life cycle, within unfed ticks, during the blood meal, and then in the reservoir host or in humans. The pathogenesis of LB is multifactorial. Mechanisms that may contribute to dissemination and pathogenicity are poorly understood. Positive correlations have been reported between some *B. burgdorferi* sl genotypes and pathogenicity (Seinost et al. 1999) and severity of the disease in a murine model (Wang et al. 2002). Moreover, the success of tick transmission and therefore the pathogenicity for the host, appears to be more isolate-dependent than species-dependent (Crippa et al. 2002).

The sequential events leading to human infection involve first the acquisition of *Borrelia* by the vector and then the transmission to the host (Humair and Gern 2000).

First, the acquisition of *B. burgdorferi* sl by ticks at the tick attachment site is an active phenomenon, which involves tick saliva and requires an active participation of *B. burgdorferi* sl (Gern and Rais 1996; Hodzic et al. 2001). Transmission to the host is linked to the frequency of contacts between the vector and the host (Humair and Gern 2000). When the tick attaches to the host, spirochetes migrate from the midgut of the tick to the salivary glands, and are inoculated with the tick saliva. Transmission generally occurs from 17 to 48 h after *I. ricinus* attachment (Kahl et al. 1998) and is extremely efficient during the third day of *I. scapularis* nymphal feeding (Piesman 1993). The efficiency of cofeeding transmission increases the probability for ticks to acquire infection from the host (Randolph et al. 1996), facilitating the transmission cycle. The nymphal developmental stage is the most efficient for transmitting most human infections.

During tick feeding and transmission to the host, several changes take place in *B. burgdorferi* sl. First, there is a pronounced multiplication of spirochetes in *I. scapularis* ticks (de Silva and Fikrig 1995), although this phenomenon has never been evidenced in *I. ricinus* ticks. Simultaneously, whereas spirochetes in unfed ticks express the protein OspA at their surface, as ticks start to feed on the host, the repression of OspA synthesis is concomitant with the upregulation of OspC (Pal et al.

2000; Schwan and Piesman 2000). It has been demonstrated that the protein OspA specifically binds to proteins of the gut of *I. scapularis* and therefore plays a determinant role in colonization of the vector (Pal et al. 2000). The downregulation of this protein during tick feeding permits *Borrelia* to migrate towards salivary glands. Diverse factors such as temperature, cell density, and pH (Schwan and Piesman 2000; Carroll et al. 1999) are involved in the switch that allows spirochetes to migrate from the midgut to the salivary glands. OspC is involved in the transmission from tick to mammal, but not from mammal to tick (Schwan and Piesman 2000).

Invasion of host tissues is due to the expression of a number of surface proteins and associated with changes in several genes, mostly plasmid-encoded genes including *ospEF* homologues, *ospC*, *dbpAB*, *p21*, and *bbk32* (de Silva and Fikrig 1997; Suk et al. 1995; Anguita et al. 2000; Probert et al. 1995), which are preferentially expressed in vivo. The processes of adaptation and immune evasion involve modifications in gene expression and recombination events. Crystal structure of proteins OspA, OspC, and VlsE involved in these mechanisms have been outlined (Li et al. 1997; Eicken et al. 2001, 2002).

DNA microarrays have been developed to identify the adaptive responses to different experimental conditions of temperature, pH or environment that mimic the different steps of the *B. burgdorferi* sl life cycle (Liang et al. 2002b; Ojaimi et al. 2003; Revel et al. 2002). Among 215 ORFs differentially expressed at 23 and 35°C, 134 have unknown function, 63 percent of these genes are plasmid encoded. Genes encoding proteins of known function are mostly cell envelope genes upregulated at 35°C. Genes involved in intermediary metabolism are more highly expressed at 23°C than at 35°C. Genes encoding for putative hemolysins are significantly more expressed at 35°C. These data emphasized the role of several cp32 lipoprotein-encoded genes in the enzootic cycle of *B. burgdorferi* (Ojaimi et al. 2003).

A gene conversion mechanism has also been evidenced in VlsE antigenic variation (Zhang and Norris 1998a). The high level of conservation of the *vls* locus, the *vmp*-like sequence locus, among various *B. burgdorferi* sl, but not present in an in vitro-passaged *B. burgdorferi* ss isolate, suggests a role in the physiology and pathogenesis of Lyme disease spirochetes (Anguita et al. 2000; Wang et al. 2001). A *vlsE* recombination would be induced by mammalian host factors, independently of adaptive immune response (Zhang and Norris 1998b).

ospEF-related genes, also designated *erp* locus, result in antigenic variants (Nguyen et al. 1994; Sung et al. 2000), which may contribute, together with VlsE antigenic variation, to the establishment of mammalian infection. Infected mice produce antibodies that also recognize Erp proteins within 4 weeks of infection, sera from LB patients also recognize several of these proteins. Not all the Erp proteins are essential for mammalian infection, as spirochetes lacking the plasmid cp32-2, encoding ErpC and ErpD proteins, remain infectious and can be transmitted from ticks to mammals (Stevenson et al. 1998).

OspC expression is upregulated in the feeding tick, while the DbpA expression is only detected in the mammalian host early after infection. The expression of these two proteins is regulated by the sigma factors *RpoS* and *RpoN* (Hübner et al. 2001). OspC is a very antigenically variable molecule (Fung et al. 1994; Wilske et al. 1996) and the *ospC* gene offers huge diversity (Livey et al. 1995; Seinost et al. 1999; Theisen et al. 1995), with a comparable variation within a local population (I.N. Wang et al. 1999). The immunodominant OspC antigen elicits protective antibodies (de Silva and Fikrig 1997). Liang et al. (2002a) demonstrated that anti-OspC antibodies are both necessary and sufficient to select spirochetes not expressing OspC during infection. However, no variation was observed in the sequence of *ospC* gene 6 months after infection of OspC-immunized mice with clonal *Borrelia* strains. The authors concluded that variation in *ospC* gene does not seem to be a crucial mechanism for immune evasion during chronic infection of a given patient (Hodzic et al. 2000). However, a strong diversifying selection is applied to this gene as revealed by inter- and intraspecies transfers (Baranton et al. 2001; Jauris-Heipke et al. 1995; Livey et al. 1995; G.Q. Wang et al. 1999). Recently, Grimm et al. (2004) demonstrated that *B. burgdorferi* ss strictly required OspC to infect mice.

Anguita et al. (2000) characterized the pathogenicity of derivatives of a clonal population of *B. burgdorferi* ss N40. They showed that spirochetes passaged 75 times in vitro remained infectious, but did not cause arthritis or carditis. This was not due to a loss of genes since sequences could be readily amplified. In fact, the high-passage strain was defective in the upregulation of genes preferentially expressed during mammalian infection, including *dbpAB*, *bba64*, and genes that map the cp32 family of plasmids. This defect in differential gene expression resulted in the inability to colonize and disseminate within the host.

Pathogenicity is often facilitated by the expression of surface protein that adhere to extracellular ligands. From the *B. burgdorferi* ss B31 strain, Probert et al. identified a 47 kDa peptide encoded by the *bbk32* gene and which specifically binds fibronectin. This may be a mechanism for establishing persistent infection. However, other *B. burgdorferi* ss or *B. garinii* strains do not express any fibronectin-binding activity, whereas some *B. afzelii* express a protein 45 kDa in size (Probert and Johnson 1998). Similarly, *B. burgdorferi* ss binds plasminogen at its surface, and the OspA protein has been identified as a binding site (Fuchs et al. 1994; Coleman et al. 1995).

B. burgdorferi sl does not produce lipopolysaccharide (LPS) and no gene codes for LPS synthesis (Fraser et al.

1997). Lipoproteins activate inflammatory cells through Toll-like receptor 2 (TLR2). These TLR2-dependent signaling pathways are facilitated by CD14 (Hirschfeld et al. 1999). They play a critical role in the innate host defense. However, they are not necessary for inflammatory and acquired humoral responses (Lien et al. 1999; Wooten et al. 2002).

Relapsing fevers may range from subclinical, with mild spirochetemia, to severe forms leading to a high mortality level. The pathogenesis is diverse and depends on the *Borrelia* species and the host. The epidemic louse-borne fever caused by *B. recurrentis* is the most severe. It is characterized by one to five acute recurrences of fever with febrile episodes for 2 to 9 days and an afebrile interval of about 9 days. Recurrences are sometimes complicated by cardiac, neurologic, ocular manifestations, or bleeding associated with thrombocytopenia. Mortality is high in untreated cases. Tick-borne relapsing fever due to *B. duttonii* and *B. crocidurae* are usually less severe, although they can cause neurologic or meningoencephalitic, hepatic or cardiac complications.

Relapses depend on the ability of *Borrelia* to undergo an antigenic variation. Similarly to *B. burgdorferi* sl, relapsing fever *Borrelia* carry genes homologous to the *ospC* gene, encoding the variable surface antigens of Vmp families. These proteins are the target of the immune host response leading to the mechanism of antigenic variation, now well understood in *B. hermsii* and *B. turicatae*. Each of the 40 *vmp* related genes in *B. hermsii*, now reclassified in *vsp/vlp* for genes encoding small (Vsp) and large proteins (Vlp), respectively (Cadavid et al. 1997), can be found either in a silent or an expressed version. Only one gene is expressed at a time. Antigenic variation occurs by the switch of a gene previously located in a silent site towards an expression site that contains the promoter element allowing the transcription (Kitten and Barbour 1990; Plasterk et al. 1985). This switch involves interplasmidic recombination between two linear plasmids and occurs at the frequency of 10^{-3}–10^{-4} per generation. Therefore, the Vmp proteins are the determinants of serotype (Barbour 1990; Marti Ras et al. 2000) and undergo antigenic variation. After infection, *Borrelia* actively multiply in the bloodstream and the fever occurs. When the immune response against a prominent outer surface lipoprotein develops, spirochetes disappear from the blood. A new population coated with a new lipoprotein appears in the blood causing the febrile recurrence. Each change in Vmp synthesis allows a new serotype to establish in the host, leading to bacteremia and a relapse of fever. This mechanism allows the infection to be prolonged in mammals and increases the likelihood of transmission.

Moreover, the Vsp proteins are also associated with tissue tropism (Zuckert et al. 2001; Pennington et al. 1999). It has been shown that different serotypes of *B. turicatae* were associated with different severity and different clinical manifestations. For example, infection with the serotype B is associated with a greater spirochete burden in the joints of infected mice, a greater inflammatory response, and finally, more severe arthritis. Conversely, infection with the serotype A is predominantly associated with invasion of the central nervous system (Cadavid et al. 1994; Pennington et al. 1997). In the same way, a tissue tropism directed to the brain of infected mice was observed for the serotype 2 of *B. crocidurae*, causing more severe neurologic manifestations than did serotype 1 (Shamaei-Tousi et al. 1999).

A role in the stimulation of human monocytes to produce tumor necrosis factor has been attributed to the Vmp of *B. recurrentis* (Vidal et al. 1998). Therefore, another consequence of the antigenic variation could be the modulation of the host inflammatory response.

Another mode of immune evasion has been proposed for *B. crocidurae*, consisting of an aggregation of erythrocytes by spirochetes (Burman et al. 1998). This erythrocyte-rosetting mechanism allows the erythrocyte-covered spirochetes to avoid the cells of the host's immune system. Therefore, the onset of the specific immune response is delayed. Moreover, histopathologic changes in the lungs, kidneys, and brain of infected mice, with inflammatory response and cell death, and microemboli in vessels, explain the clinical symptoms observed in relapsing fever in humans (Shamaei-Tousi et al. 1999).

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

Borrelia are microaerophilic, slow-growing spirochetes. They grow best in liquid media at 30–35°C with cell yields of 10^7–10^8 per ml. The generation time at 35°C varies from 12 to 24 h. Growth occurs in soft solid media (1 percent agar) with a 50–100 percent plating efficiency. The colonies are subsurface and do not have distinguishing characteristics. The nutritional requirements have not been fully defined, but are complex. *Borrelia* spp. have the unusual requirement for *N*-acetylglucosamine (Kelly 1971). They are similar to *Treponema*, requiring long-chain saturated and unsaturated fatty acids. Other nutritional requirements include glucose and amino acids. Serum proteins, such as bovine serum albumin, are a component of the medium and probably function primarily as a nontoxic source of fatty acids.

METABOLISM

Carbohydrates, such as glucose, serve as a major energy source and the predominant metabolic endproduct is lactic acid (Livermore et al. 1978; Pickett and Kelly 1974). The majority of the outer proteins of *B. burgdorferi* are covalently modified with lipids (Brandt et al. 1990). These lipoproteins are synthesized by the

pathway common for bacterial lipoproteins and contain the tricyl-*S*-glycerylcysteine structural moiety. *B. burgdorferi* is catalase-negative (Kelly 1976).

LABORATORY DIAGNOSIS

Samples

Borrelia can be isolated from diverse sources, human specimens, but mostly vector ticks and animal hosts.

The best suitable human specimens are cutaneous tissues obtained by punch biopsies from early erythema migrans and late acrodermatitis lesions. Biopsy fragments are placed into BSK medium supplemented with antibiotics. After 7 days of incubation at 34°C, the skin biopsy specimen is transferred to BSK medium without antimicrobial agents. Both the tube of isolation medium from which the skin biopsy was removed and the tube of antibiotic-free BSK containing the skin biopsy are further incubated and examined for spirochetes over 2 months.

Ticks collected by dragging vegetation can be maintained alive over several weeks in optimal conditions of relative humidity and saturation deficit (Perret et al. 2003). Before cultivation, ticks are dropped in 70 percent ethanol for 1 min and rinsed with sterile water. Ticks are cut into two pieces, triturated, and placed in a tube containing 4 ml of medium.

Ear skin biopsies (approximately 2×2 mm by 0.4 mm thick) are aseptically collected from anesthetized rodents or birds with a sterile dermal punch, and placed directly into BSK medium. Rodents are sacrificed with pentobarbital and dissected under sterile conditions. Blood and tissue samples from urinary bladder, heart, and spleen are aseptically removed and each organ placed into BSK medium.

Borrelia isolation and identification

The culture medium is derived from Kelly medium (Kelly 1971) which was the first medium developed for isolation and growth of *B. hermsii*. The medium was then modified, basically by adding a component for cell culture, CMRL, which contains amino acids, vitamins, various growth factors, and yeast extract (Barbour 1984). *N*-acetylglucosamine, a constituent of peptidoglycan, is essential for growth. The medium is now known by the acronym BSK for Barbour, Stoenner and Kelly, and commercialized as BSK-H (Pollack et al. 1993). Some authors have proposed minor modifications to the medium (Preac-Mursic et al. 1986; Sinsky and Piesman 1989).

The medium can be made selective by adding antibiotics such as kanamycin (8 μg/ml), neomycin (30 μg/ml), nalidixic acid (100 μg/ml), rifampicin (50 μg/ml), 5-fluorouracil (100–250 μg/ml), fosfomycin (20–100 μg/ml), or amphotericin B (2.5 μg/ml). These modifications may be suitable for cultivation of infected skin samples or ticks.

Cultures are incubated at 34°C. The optimal growth temperature lies between 30 and 37°C. Cultures must be examined weekly using a dark field microscope. The generation time varies from 12 to 24 h at 35°C. Therefore, cultures are examined weekly over 2 months.

Genus identification is based on the analysis of phenotypic characteristics such as morphology, size, and motility. Species identification is based on molecular PCR-based methods performed on culture thermolysates. Among these methods, the restriction polymorphism of the amplification product of the intergenic *rrf-rrl* region is simple and efficient (Postic et al. 1994). This is a method allowing amplification of DNA from all *Borrelia* species within the complex *B. burgdorferi* sl, the identification lies on the species-specific pattern after enzymatic restriction. Alternative methods consist of amplification of conserved genes followed by hybridization with probes specific for each species (Assous et al. 1994; Jaulhac et al. 1996; Luft et al. 1992a; Picken 1992; Demaerschalck et al. 1995; Kawabata et al. 1994; Nocton et al. 1996).

Results

The early cutaneous manifestations of LB are often typical, the diagnosis is easy and does not need any biological confirmation. In contrast, due to the large spectrum of late, nonspecific manifestations, there is a need for complementary diagnosis laboratory tests. However, the cell density of *B. burgdorferi* sl in tissues is extremely low, particularly in the bloodstream. This is the reason for the low rates of cultures usually reported from clinical samples.

The best success rates are obtained from skin biopsies with a sensitivity ranging from 56 percent (Robertson et al. 1999), or 60 percent (Wormser et al. 1992; Nadelman et al. 1996) to 80 percent (Berger et al. 1992). Culture from other sites are not usually successful and the sensitivity varies considerably between studies. Successful cultivation from blood has rarely been reported. The rate of recovery is significantly greater from plasma cultures than from serum cultures (Wormser et al. 2000, 2001). Karlsson et al. (1990) isolated *B. burgdorferi* sl from CSF of 11 percent of patients who fulfilled the criteria for neuroborreliosis. This rate is in the range generally reported.

In contrast, *B. burgdorferi* sl can be easily isolated from ticks and host animals. The urinary bladder and the ear are the major source sample of *B. burgdorferi* sl isolation from mammals.

In contrast, a high level of bacteremia in relapsing fevers allows easy recognition of spirochetes on blood smears colored with Giemsa. Moreover, successful isolation of clinical isolates of *B. recurrentis* and *B. duttonii*

from patients is now possible (Cutler et al. 1994, 1999). Molecular tools are also available to identify these spirochetes (Marti Ras et al. 1996). However, no extensive studies have been undertaken for formal evaluation and comparison of techniques used for the diagnosis of relapsing fevers.

Detection of *Borrelia* DNA by PCR

In this context, PCR-based methods were expected to provide a solution. More than 20 different targets have been used either on the chromosome or the plasmids. The most commonly reported are *rrs* and *rrl* ribosomal genes, *ospA*, *fla* genes, and the ribosomal spacers *rrf-rrl* and *rrs-rrl*. The sensitivity of DNA detection in skin samples ranges from 50 to 90 percent (Lebech et al. 2000; I. Schwartz et al. 1992). A wide range of PCR success rates is reported in CSF samples. Diagnosis sensitivity of PCR in European patients with neuroborreliosis ranged from 12 to 25 percent (Lebech 2002), whereas in US patients, the sensitivity ranged from 28 percent (Nocton et al. 1996) to 80 percent (Keller et al. 1992). The outcome of PCR in diagnosis is also dependent of the duration of the disease. The rate of PCR positive CSF samples would be higher when the disease duration is <14 days (Lebech 2002). Amplification of *B. burgdorferi* sl DNA from synovial fluid is an important diagnostic tool. By using the *ospA* gene as a target for molecular diagnosis of Lyme arthritis, Nocton et al. (1994) reported a sensitivity of 85 percent. By using the *fla* gene, Jaulhac et al. reported the detection of *B. burgdorferi* DNA in 10 of 11 patients with Lyme arthritis (Jaulhac et al. 1996, 2000; Nocton et al. 1994; Priem et al. 1997). Detection of chromosomal *B. burgdorferi* DNA would be more efficient in synovial tissue than in synovial fluid (Jaulhac et al. 1996). Although urine is an easily available source for PCR, the low sensitivity and overall lack of reproducibility led to this method being abandoned. In using PCR methods for molecular diagnosis of LB, the importance of sample preparation has been emphasized. An improved method could allow a substantial increase in the rate of detection (Bergmann et al. 2002). Otherwise, the hypothesis of target imbalance, suggesting increased sensitivity by using a target with multiple DNA copies such as the plasmidic gene *ospA* (Luft et al. 1992b), has not been confirmed by comparative studies between plasmid-borne and chromosomal genes (Priem et al. 1997).

PCR methodologies have also been proposed in evaluating the effect of antibiotherapy in neurologic manifestations (Nocton et al. 1996) or in cutaneous lesions of EM and ACA (Muellegger et al. 1996). Lastly, since DNA detection does not provide evidence of the viability of spirochetes, Limbach et al. (1999) developed a reverse transcriptase PCR method in a mouse model.

To sum up, results of PCR as diagnostic tools for LB are somewhat disappointing. Despite the method's high sensitivity, the extreme paucity of bacteria in biological samples, particularly in blood samples, is too close by far to the threshold of PCR.

Serology

Whereas diagnosis of EM does not require any serologic confirmation, serological tests are recommended in both the USA and Europe to corroborate the diagnosis of disseminated LB. However, the lack of sensitivity during the early stage of the disease, the lack of specificity, and the inter- and intralaboratory variability of the tests are among the many difficulties that hamper the efficiency of the approach.

To improve specificity, a two-tiered approach has been recommended by the Center for Disease Control composed of an initial test comprising either ELISA or IFA, followed by an immunoblot when the results of the first test is indeterminate or positive (CDC 1995). This two-step strategy also gained wide acceptance in Europe. Specimens negative by a sensitive EIA or IFA need not be tested further.

More detailed serologic criteria have been recommended by the European consortium EUCALB. They are given in Table 2. The minimum standard of at least 90 percent specificity in quantitative assays is recommended.

Considering the geographic pattern of *B. burgdorferi* sl species distribution and strain variability, distinct interpretation criteria of Western blots were proposed for European patients (Hauser et al. 1997; Robertson et al. 2000) and American patients (Dressler et al. 1993; Engstrom et al. 1995). According to the CDC (1995), when Western immunoblot is used during the first 4 weeks of disease onset (early LD), both IgM and IgG procedures should be performed. A positive IgM test result alone is not recommended for use in determining active disease in persons with illness greater than 1 month's duration. If a patient with suspected early LD has a negative serology, serologic evidence of infection is best obtained by testing of paired serum samples. Serum samples from persons with disseminated or late-stage LD almost always have a strong IgG response to *B. burgdorferi* antigens. The immunoblot positivity criteria according to the CDC are given in Table 3. In Europe, several studies have been conducted to formulate the best interpretation rules for the blots (Hauser et al. 1997, 1999; Robertson et al. 2000; Norman et al. 1996; Ryffel et al. 1998). Although no single European rule resulted from these studies, the most discriminatory bands identified were OspC and p41 for IgM, and p83/100, p58, and p39 for IgG (Robertson et al. 2000).

Recently, a C6 peptide ELISA has been developed, based on the conserved region 6 of VlsE lipoprotein (Marques et al. 2002). In humans, the anti-IR6 of VlsE is detected with a very high sensitivity in patients with late LB (Liang and Philipp 1999). The high level of

Table 2 *Serologic diagnosis guidelines according to EUCALB*

Clinical manifestations	Diagnostic guidelines according to EUCALB
EM	Specific IgG and/or IgM found in only 40–60% of untreated cases
	Serology for EM diagnosis not essential
Early neuroborreliosis	Necessary to demonstrate intrathecal antibody production
	A significant change in titer of specific IgG and/or IgM between paired blood samples are supportive for the diagnosis, but serum antibodies can be absent in early cases
Lymphadenosis benigna cutis	A high titer IgM test result or a significant change in titer of specific IgG and/or IgM between paired blood samples are essential for the diagnosis
Chronic neuroborreliosis	Intrathecal antibody production demonstrated by determining the titers of specific antibody in simultaneously drawn blood and CSF samples
	A positive IgM test without the presence of IgG antibodies argues against the diagnosis of chronic neuroborreliosis
Lyme arthritis	Specific IgG antibodies (usually high levels)
	A positive IgM test in the absence of IgG antibodies argues against the diagnosis of Lyme arthritis
Lyme carditis	High titers of IgM or IgG antibodies or a significant change of specific IgG antibody titer in paired serum samples
ACA	Specific IgG antibodies (usually high levels). IgM antibodies may or may not be present.
	A positive IgM test in the absence of IgG antibodies argues against the diagnosis of ACA

Source: vie.dis.strath.ac.uk/vie/LymeEU/diagnosis_serology-diagnostic-guidelines.html

sensitivity and specificity of this new test could circumvent the problems mentioned above and could exempt the two-tiered approach. Results seem promising both in European and American patients.

Due to a lack of sensitivity and specificity, no serological test was available for diagnosing relapsing fevers. However, a serodiagnosis has recently been proposed, based on the glycerophosphodiester phosphodiesterase (Porcella et al. 2000; Schwan et al. 1996), absent from *B. burgdorferi* sl, and which could be useful in the serological survey of louse-borne disease as well as tick-borne relapsing fevers.

Susceptibility to antimicrobial agents

Many studies have been performed to evaluate the susceptibility of *B. burgdorferi* sl to various antimicrobial agents. A macro- or microdilution broth method was commonly used to determine the minimal inhibitory concentration (MIC). However, different methods were used for MIC determinations. Unfortunately, standard methods for determining the minimal bactericidal concentrations (MBC) for slow-growing bacteria such as the *Borrelia* have not been established, thus the methodology must be adapted.

Table 3 *Immunoblot positivity criteria according to the CDC*

IgM	IgG
Two bands among:	Five bands among:
24 kDa or 21 kDa: OspC	18 kDa
39 kDa: BmpA	24 or 21 kDa: OspC
41 kDa: Fla	28 kDa
	30 kDa
	39 kDa: BmpA
	41 kDa: Fla
	45 kDa
	58 kDa (not GroEL)
	66 kDa
	and 93 kDa

In spite of different methodologies and different species or strains used in different studies, there is general agreement on the in vitro activity of most of the commonly used antimicrobials against *B. burgdorferi* sl (Table 4). These spirochetes are susceptible to the macrolides, tetracyclines, semisynthetic penicillins, and the late second- and third-generation cephalosporins. *B. burgdorferi* sl is moderately sensitive to penicillin G and chloramphenicol and relatively resistant to the aminoglycosides, trimethoprim, sulfamethoxazole, quinolones, and rifampin.

Clarithromycin has been shown to be an effective agent for the treatment of early Lyme disease, but warrants further study (Dattwyler et al. 1996).

However, there are a few documented studies concerning the susceptibility in vivo. Despite good activity in vitro, erythomycin is less effective clinically than doxycycline or tetracycline (Rahn and Malawista 1991; Steere et al. 1980; Preac-Mursic et al. 1987). Similarly, penicillin G showed poor activity in vivo (Johnson et al. 1990; Preac-Mursic et al. 1987). Due to its long half-life, its ability to penetrate in inflamed joints and synovial fluid, ceftriaxone is considered effective for the treatment of late Lyme borreliosis.

Only few data are available on the putative sensitivity differences between different species within *B. burgdorferi* sl. Recent studies (Sicklinger et al. 2003) reveal significant differences between MIC and MBC of

Table 4 *In vitro antimicrobial susceptibility of* B. burgdorferi *sl reported in several studies (MIC and MBC in μg/μl)*

Antibiotics	References	Preac Mursic et al. (1987)	Johnson et al. (1990)	Agger et al. (1992)	Dever et al. (1992)	Levin et al. (1993)	Hunfeld et al. (2000)	Sicklinger et al. (2003)
Penicillin G	MIC	0.5–8			0.06			
	MBC		6.4					
Amoxicillin	MIC	0.25–1			0.06	<0.03–0.06	0.06–2	0.015–0.25
	MBC		0.8			<0.03–2.0		0.125–>0.5
Ceftriaxone	MIC	0.06–0.25		0.02	0.03		<0.01–0.125	0.015–0.03
	MBC		0.08	0.02–0.06			1.5–3.0	0.03–0.25
Cefotaxime	MIC	0.06–0.25				<0.03	0.01–0.25	
	MIB					0.03–0.25		
Cefuroxime	MIC			0.09–0.18				
	MBC		1	0.25–0.75				
Ciprofloxacine	MIC	1–4				0.025–2.0		
	MBC					0.5–16		
Ofloxacine	MIC	2–8				0.5–2.0		
	MBC					1.0–8.0		
Doxycycline	MIC				2	0.125–0.5		0.25–4
	MBC		1.6			0.25–4.0		4–16
Erythromycin	MIC	<0.06–0.12			0.03	<0.03–0.06		
	MBC		0.32			0.06–0.5		
Azithromycin	MIC				0.008		<0.015–0.06	0.008–0.015
	MBC						0.5–4	0.0015–0.25

different antibiotics against the three main species. *B. afzelii* is more susceptible to ceftriaxone, *B. garinii* is more susceptible to azithromycin, whereas *B. burgdorferi* ss showed the best susceptibility to amoxicillin (Sicklinger et al. 2003). For other authors, *B. garinii* appeared to be more susceptible to antibiotics (Hunfeld et al. 2000; Preac-Mursic et al. 1996). Moreover, the persistence of *B. garinii* and *B. afzelii* was described in patients despite adequate antibiotherapy (Preac-Mursic et al. 1996).

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Treponema

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INTRODUCTION

Venereal syphilis, the most common of the treponematoses, is caused by *Treponema pallidum* (Smibert 1984) (Table 1). The Spirochetales, to which it belongs, form a distinctive group of bacteria that are commonly given division status. This is based on a number of unique features to be discussed below, as well as sequencing of ribosomal RNA, which shows them to be a unique clade in the Eubacteria (Woese 1987). They have a spiral morphology unique to the group, with a double limiting membrane. The flagella are enclosed between the inner and outer membranes, hence the name ''endoflagella'' for these structures. Because the endoflagella are enclosed between the two membranes, they are not exposed to the external medium and result in a unique form of motility in which the spirochetes are able to move through highly viscous medium (Harwood and Canale-Parola 1984). This allows the members of the family to utilize niches not available to other motile bacteria and may even be of utility for pathogenic spirochetes in penetrating host tissues (Harwood and Canale-Parola 1984). Spirochetes are found in a wide variety of habitats, including environmental samples (especially anaerobic), the termite gut, the rumen of hoofed mammals, and the oral and gastrointestinal cavities of mammals (Smibert 1984). The species that have attracted the most research interest, though, are the human pathogenic members of the genera *Leptospira*, *Borrelia*, and *Treponema*.

The taxonomy of the Spirochetales (or spirochetes) has generally lagged behind that of most other groups of bacteria because of the severe restraints placed on their research because the type species of many of the genera, as well as many of the pathogens, cannot be grown in sustained culture. Even the type species of the free-living genus Spirocheta has never been grown in vitro (*Spirocheta plicatis*). Taxonomic trees have been constructed for the Spirochetales, usually based on ribosomal RNA sequences (Woese 1987). The other genera of spirochetes are generally either microaerophilic or anaerobic and inhabit a wide variety of habitats.

Despite these problems, a considerable amount is now known about the causative agent of syphilis, *Treponema pallidum*. The sequencing of the entire genome (Fraser et al. 1998) has helped resolve some of the problems raised by previous research as well as open up new venues for dissecting the biology of this spirochete. The genome is approximately one mega base in size and contains about 1000 open reading frames; no plasmids or insertion sequences are present. There is no evidence in the genome sequence for any active method of genetic exchange or lateral transmission of DNA sequences from other microorganisms. An excellent review of the biology of *T. pallidum* in light of the completed genome sequence has been published (Norris et al. 2001). The following discussion will look at the more important conclusions about the biology and pathogenesis of *T. pallidum* that can be drawn from the recent data rather

Table 1 *Characteristics of the human treponematoses*[a]

	T. pallidum* subsp. *pallidum	***T. pallidum* subsp. *pertenue***	***T. pallidum* subsp. *endemicum***	***T. carateum***
Disease	Syphilis	Yaws (frambesia, pian)	Endemic syphilis (bejel, dichuchwa)	Pinta (carateum)
Distribution	Worldwide	Tropical areas of both hemispheres	Arid areas, Africa, Middle East, former Yugoslavia	Semiarid areas, Central America
Age of onset	Adolescents, adults	Children	Children to adults	Children, adults
Transmission	Sexual contact	Skin contact	Mucous membrane	Skin contact
Congenital infection?	Yes	No	Rarely	No

a) See US Public Health Service (1968), Perine et al. (1984, 1985), and Engelkens et al. (1991a, b).

than the considerable amount of detailed information that is now available.

Previous work had indicated an aerobic nature for *T. pallidum* based on oxygen consumption and had demonstrated the presence of a complete respiratory chain (Lysko and Cox 1977, 1979). This work is almost certainly artifactual, probably due to contaminating mitochondria from the host in the treponemal preparations that were isolated from rabbit testes. The complete sequencing of the *T. pallidum* genome has shown that no genes are present for cytochromes or other components of the respiratory chain. Energy production appears to be due to substrate-level phosphorylation using the acetate kinase pathway, which is also suggested by the fact that acetate is a major end product of treponemal metabolism (Nichols and Baseman 1975). Since there is ample evidence for the high sensitivity of *T. pallidum* to the products of oxygen reduction such as hydrogen peroxide and the superoxide anion (see below), what the utility of oxygen may be for treponemal metabolism must still be considered an open question. *T. pallidum* is viable and capable of growth only in the presence of host cells that do require oxygen; this fact greatly complicates the argument that *T. pallidum* is directly dependent on oxygen. Many questions are still unanswered on the oxygen problem despite many decades of work on this subject.

In contrast to the work on energy metabolism, previous work showing the absence of enzyme activities for oxygen protective enzymes such as catalase (Steiner et al. 1984a) and superoxide dismutase (Austin et al. 1981) was confirmed by the absence of these genes in the *T. pallidum* genome (Fraser et al. 1998). The absence of catalase or other enzymes capable of removing hydrogen peroxide would seem to account for the high sensitivity to this metabolic byproduct seen in several studies (Steiner et al. 1984a, b) and for the requirement for reducing agents, which has been known for many decades (see Fitzgerald 1981a for an extensive discussion of the reducing agent requirement). The only oxygen protective enzymes present are a possible alkyl hydroperoxidase (Fraser et al. 1998; Norris et al. 2001) and a recently discovered enzyme with superoxide reductase activity (Jovanovi'c et al. 2000; Lombard et al. 2000); the latter enzyme is normally found only in anaerobes. At present the role of these two enzymes, including their regulation and role in the treponemal cell, is unknown.

An interesting hypothesis has been put forward suggesting that *T. pallidum* limits oxygen toxicity by having a paucity of iron-containing protein (Posey et al. 1999). Although interesting, this hypothesis is only weakly supported by the data since a number of potential iron-containing proteins appear to be present in the genome, including a probable bacterioferritin or iron-binding protein (Evans et al. 1995). The absence or presence of free iron in the treponemal cell is of considerable importance in terms of the high sensitivity to oxygen of *T. pallidum*, which has been known for close to a century. Hydrogen peroxide, to which *T. pallidum* is highly sensitive, produces the hydroxyl radical in the presence of free iron (Liochev 1996), which would be expected to produce significant genomic damage when generated inside the cell (Imlay and Linn 1988). Considering the extremely slow rate of DNA repair shown in at least one study (Steiner et al. 1984b), iron in the treponemal cell would be expected to be very deleterious and lead to the rapid death of the treponemes. Bacterial catalase would normally be expected to limit the level of hydrogen peroxide in the cell but since *T. pallidum* contains no catalase (Steiner et al. 1984a; Fraser et al. 1998), sequestration of free iron may be the only way to limit the production of hydroxyl radicals in the cell and prevent genomic damage.

One obvious fact shown by the genome sequence is the heavy dependence on the host for essential small molecules of anabolism (Fraser et al. 1998). *T. pallidum* depends on the host for all purines and pyrimidines as well as all amino acids with the possible exception of proline (Gherardini et al. 1990). *T. pallidum* is missing all of the tricarboxylic acid cycle, which is the source for the carbon skeletons for the majority of these molecules (Moat and Foster 1988), and thus has no capacity for producing them de novo. This is a common factor seen in the completely sequenced genomes from a number of obligate host-associated microorganisms (Andersson

et al. 1998; Cole et al. 2001). *T. pallidum* appears to use only glucose, mannose, and maltose in catabolism with the production of acetic acid through the acetate kinase pathway as its final waste product (Nichols and Baseman 1975; Norris et al. 2001). The presence of this pathway would explain the apparent sensitivity of *T. pallidum* to metronidazole (Wilkinson et al. 1967), although the high level required for inhibition by this antimicrobial drug would suggest it has a limited ability to penetrate the external membranes of the treponeme. All of the small molecules produced by the missing anabolic enzymes have to be provided from the host; genomic analysis of *T. pallidum* indicates it contains a large number of transport systems that would appear to make up for these metabolic deficiencies (Fraser et al. 1998). The importance of individual transport systems (or in some cases even their presence), as well as their relative contribution to treponemal nutrition, still needs to be verified since this work is totally dependent on genomic analysis.

The ability of antimicrobial drugs to penetrate the membranes of *T. pallidum* brings up two important areas of research in syphilis: the nature of the outer membrane and the mechanism of attachment to host cells. It has been known for many years that freshly isolated *T. pallidum* react slowly with high-titer antibody (Nelson and Mayer 1949). This is most notable in the *Treponema pallidum* immobilization (TPI) assay, which requires a 16-h incubation to observe effective immobilization of the freshly isolated treponemes (Nelson and Mayer 1949). The delayed recognition of *T. pallidum* in vitro is not limited to reactions with antibody. In two papers, Alder and co-workers showed that phagocytosis of treponemes proceeds slowly in vitro (Alder et al. 1989, 1990). These workers developed a model system of phagocytosis on a surface in which treponemes were immobilized on a membrane filter and then phagocytized by macrophages from immune animals. Phagocytosis progressed much slower than with control bacteria or particles (Alder et al. 1990), again implying that treponemal antigens were masked by a surface layer that was not recognized by these immune effectors. Both in antibody recognition and in macrophage phagocytosis, the treponemes became more reactive with time in vitro, indicating that the material preventing recognition was labile.

The nature of this surface layer has been an area of active debate, being ascribed to surface mucopolysaccharides (Fitzgerald and Johnson 1979) or adherent host material (Alderete and Baseman 1979) at various times. A number of lipoproteins, which are plentiful in the *T. pallidum* cytoplasmic membrane (Chamberlain et al. 1989; Schouls et al. 1991), were originally described as outer-membrane proteins and thus were presumed to be involved in immune recognition of the treponemes during syphilitic infection. The biological function of these lipoproteins is unknown, but most, or all, are now thought to be anchored by the lipid moiety in the cytoplasmic membrane of *T. pallidum*. Although highly antigenic, none of these lipoproteins has been demonstrated to have an important role in immunity in the course of syphilis. The recently discovered family of 12 *tpr* genes (Fraser et al. 1998), several of which appear to have leader sequences, has been of great interest recently because of their potential for recombination which could be of importance in immune evasion. As of yet though, none has been proven to reside in the outer membrane and their function is unknown.

More recent evidence (Radolf et al. 1989; Walker et al. 1991) indicates that the slow recognition of *T. pallidum* by antibody and/or immune phagocytes relates to the structure of the outer membrane. In both of these papers, freeze fracture studies were used to demonstrate the extremely low number of proteins that appeared to be exposed on the outer membrane of *T. pallidum*. Membranous particles that were presumed to be proteins were up to two logs lower in the outer membrane of *T. pallidum* when compared with *Escherichia coli*. This lack of species specific antigens exposed on the outer membrane could account for the poor clearance of *T. pallidum* seen in several experimental systems, and the inability of passively transferred antibody to eliminate treponemes in experimental systems. It might also account for the fragility of this layer, which can easily be lost during isolation. A highly lipophilic outer membrane that would shield highly antigenic membrane proteins from antibody and complement could prevent or slow immune damage to *T. pallidum* and allow the spirochete to evade the immune system for long periods of time (Radolf 1994). As yet, little is known about the composition and structure of this outer membrane or how it is stabilized. Until the structure of this membrane is better understood, it is unlikely that an effective vaccine candidate will be forthcoming.

Despite the fact that *T. pallidum* is not rapidly phagocytized by macrophages or granulocytes, it does attach readily to host cells in vitro and presumably in vivo since many microscopic studies (both with light and electron microscopy) have shown the treponemes to be predominately in the tissues (Baker-Zander and Sell 1980). *T. pallidum* is only known to attach to one host molecule, the well-studied extracellular-matrix protein fibronectin (Fitzgerald and Repesh 1985; Baugh 1987). Attachment appears to be predominately through the cell-attachment portion of the protein (Thomas et al. 1985). Unlike many other microorganisms that attach to fibronectin, the attachment appears to be readily reversible (Steiner and Sell 1985). Whether the putative adhesin(s) can be used for development of a vaccine that would be capable of blocking tissue invasion is unknown despite the fact that the early work on this interaction was carried out in the 1980s. There is still some controversy on the identification of the adhesin(s) and there are no published reports on the effect of blocking attachment to fibronectin in the host. This is a promising area of

research but at present not enough is known to determine whether interference with attachment has an important role in immunity to *T. pallidum* in human syphilis.

Little else is known about how *T. pallidum* causes an infection in the host and penetrates the host tissues. The publication of the completed genome sequence showed the presence of five putative hemolysins (Fraser et al. 1998), but none of these has convincingly shown to actually be hemolysins or contribute to pathogenesis, and their role in infection (if any) is presently unknown. It was demonstrated a number of years ago that *T. pallidum* can damage tissue culture cells in vitro (Fitzgerald et al. 1982; Wong et al. 1983), but the mechanism of injury to host cells and the treponemal molecules involved have not been determined. The possibility exists that the hemolysins discussed above may be involved in this tissue damage, but this will have to be determined by further research. Since host immune mechanisms are commonly thought to contribute to tissue damage, further work is necessary to determine whether these mechanisms of injury to host cells are important in pathogenesis or simply act to prime the host to cause the damage.

Several of the papers on the toxic effects of treponemes on tissue culture cells also showed that they affected neurons in culture and interfered with neurotransmission (Oakes et al. 1982). Electron microscopy had previously demonstrated the association of treponemes with neurons in experimental animal infections (Ovcinnikov and Delektorskij 1975); thus, this toxicity to, and/or invasion of, neurons could account for the painless nature of treponemal chancres that have not been superinfected with other microorganisms. The reason for this neurotropism/neuroinvasiveness is unknown but if we knew, it would tell us much about the disease process and the reason for the potentially disastrous complications of tertiary syphilis.

EPIDEMIOLOGY

By 1958, with the general use of penicillin, syphilis in the USA had been almost eliminated. However, there have been periodic increases in the number of cases, followed by decreases (Figure 1). Frequently the increases correlated with behavioral changes, such as the use of birth-control pills in the 1960s, gay bath houses in the 1970s and 1980s, and crack cocaine in the 1990s (Brandt 1988; Finelli et al. 1993). In 1990 in the USA, the rate of primary and secondary cases was 20.3 per 100 000, the highest since 1952. Since 1991, the male-to-female ratio has approached one, suggesting that much more of the transmission of syphilis was heterosexual (Figure 1). However, the male-to-female ratio has increased since 1996, reflecting the increasing rates of syphilis in men who have sex with men (MSM). With the increase in acquired syphilis in the heterosexual population there was a parallel increase in the number of infants born with congenital syphilis to a rate of 107.2 per 100 000 live births in 1991. The rates of sexually acquired primary and secondary syphilis for 2000 declined to 2.2 cases per 100 000 population (Centers for Disease Control and Prevention (CDC) 2001). Likewise, the rate of congenital syphilis began to decline in 1992 and has continued to decline to a rate of 13.4 per 100 000 live births in 2000. Outside the USA, reports indicate that syphilis has dramatically increased in the Russian Federation between 1992 and 1996 to 263 cases per 100 000 population (Tichonova et al. 1997). In addition, a resurgence of syphilis is currently being reported in England (Doherty et al. 2002), especially among MSM. In comparison to the USA, the Baltic States, and England, rates of syphilis have progressively declined since the advent of penicillin therapy in other industrialized countries. However, in many nonindustrialized nations, syphilis remains a significant health problem.

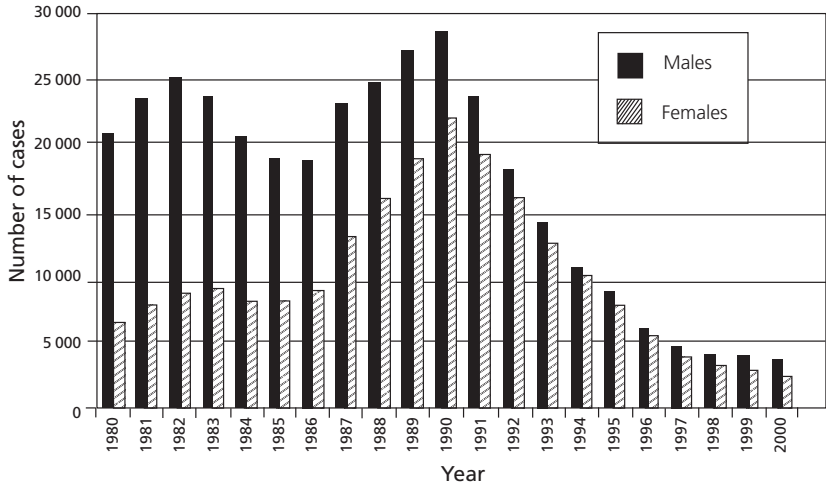

Figure 1 *Number of cases of primary and secondary syphilis in the USA in males and females, 1980–2000*

Table 2 *Stages of infection for sexually acquired syphilis*

Stage	Time post-infection	Symptom	Site
Primary	10–90 days (average 21 days)	Chancre, single or multiple; regional lymphadenopathy	Skin or mucous membrane
Secondary	6 weeks–6 months	Multiple lesions, lymphadenopathy, fever, malaise, condylomata lata	Skin or mucous membrane
		Alopecia	Hair, eyebrows
		Asymptomatic or symptomatic CNS involvement	Meninges
Latent	Early, ⩽1 year; late, >1 year	Asymptomatic	
Late	Months to years		
Benign		Gummatous lesion	Tissue, any organ
Cardiovascular		Aortic aneurysm, tree barking	Aorta
Neurosyphilis		Paresis, tabes dorsalis, dementia	Meningovascular
		Optical atrophy	Eye

CNS, central nervous system.

PATHOLOGY AND MANIFESTATIONS OF SYPHILIS

Untreated syphilis is a chronic disease that progresses through a variety of stages, each with distinct manifestations and pathology, as shown in Table 2.

Early syphilis

Sexual transmission of syphilis requires direct contact with infectious lesions. Approximately 30 percent of people who have sex with an infected partner will develop syphilis (Sparling 1990). Within hours after initial contact with the organism, treponemes disseminate throughout the body and may be detectible by the polymerase chain reaction (PCR) in the blood of people with incubating syphilis (Marfin et al. 2001). The skin surrounding the primary lesion or chancre, which occurs at the point of entry, is edematous and infiltrated with inflammatory cells. The chancre center is mucoid, containing host-produced hyaluronic acid and chondroitin sulfate surrounded by a cellular infiltrate of primarily mononuclear cells; the base of the chancre is fibrotic with neovascularization. The lesions, once present, may spontaneously resolve in 1–5 weeks. During the primary stage, the inguinal lymph nodes may be slightly enlarged but are rarely tender. Serous fluids from the lesion contain numerous treponemes. Humoral antibodies, as detected by the standard nontreponemal and treponemal serological tests for syphilis, usually do not appear until 1–4 weeks after the chancre has formed.

By the secondary stage of syphilis, the organism has invaded every organ of the body and virtually all body fluids. Nonspecific symptoms generally develop 1–5 weeks after the primary lesion has healed and may include fever, headache, sore throat, arthralgias, and anorexia (Table 2). However, a generalized rash, mucous patches, and condylomata lata are the most common characteristic symptoms of secondary syphilis. Epithelial changes for these lesions include infiltration of leukocytes, necrosis of keratinocytes, some thickening of the epithelial cells, and various degrees of hyperkeratosis (Chiu and Radolf 1994). These manifestations spontaneously resolve, usually within 2–6 weeks, but may recur during the first year of infection if the patient is not treated. Circulating immune complexes composed of IgG and C3 (Chiu and Radolf 1994), detected in approximately 80 percent of all secondary cases, may lead to immune complex deposition in the kidneys and subsequent renal damage.

In the early latent stage, the disease progressively becomes a more chronic infection. Duration of infection of 1 year, which is asymptomatic, is arbitrarily defined for epidemiological purposes as early latent stage, although one or more relapses into the secondary stage may occur during this time. Because lesions are not usually present after the first year, the disease is not considered infectious and the term 'late latent' is assigned to this stage. However, in pregnancy, *T. pallidum* can still be transmitted to the fetus up to 4 years after the initial infection, if the initial infection is not treated.

Late syphilis

The pathogenesis of the various forms that the late or tertiary stage of syphilis may take is not completely understood. Often, signs and symptoms of late-stage syphilis occur 10–20 years after the initial infection (Table 2).

An unusual form of late syphilis is benign late. The gumma (lesion) of this stage was previously reported to occur in 16 percent of the cases of untreated syphilis from 2 to more than 40 years after the initial infection (United States Public Health Service 1968). The gummas resemble the granulomata of tuberculosis and

may occur in the skin, bones, mucosae, viscera, muscles, and ocular structure. With osseous gummas, reactive new bone formation and osteolysis are characteristic (Chiu and Radolf 1994). Because organisms are rare and the lesions are characteristic of an inflammatory response, some have suggested that the gummas are a result of hypersensitivity to the few treponemes or *T. pallidum* antigens remaining in foci of long-standing infections. However, treponemes have been detected in gummas using immunofluorescence (Handsfield et al. 1983) and PCR (Horowitz et al. 1994).

In the preantibiotic era, neurosyphilis was reported to occur in 6.5 percent of people with syphilis; however, in a study of a cohort of *T. pallidum*/HIV coinfected patients, the prevalence of neurosyphilis was 3 percent (Brandon et al. 1993). The lesions in the central nervous system in neurosyphilis are also related to the multiplication of treponemes (Fitzgerald 1981b). Although neurological forms of syphilis (e.g. syphilitic meningitis) may develop during the secondary stage, neurosyphilis is usually a complication of late syphilis but may occur as early as 2 years after initial infection. Neurosyphilis may take many forms. All forms have in common chronic meningitis, producing vascular or parenchymatous sequellae in the cerebrum and spinal cord (Swartz 1984). In acute syphilitic meningitis, granular ependymitis and endarteritis may develop, which may lead to thrombosis, vascular occlusion, and cerebral infarction. As a result of obstruction of the cerebrospinal fluid (CSF) flow, several forms of hydrocephalus may result. In generalized paresis, gross findings include cerebral atrophy, demyelinization of the cortical white matter, and varying degrees of thickening of the meninges consistent with chronic meningitis. Cerebral atrophy, particularly of the frontal poles and tips of the temporal lobes, is prominent. Whorls of subependymal astrocytes forming granular ependymitis are a characteristic finding on microscopic examination. Treponemes can be demonstrated in brain tissue of CSF by direct immunofluorescence, PCR (Gordon et al. 1994), or Dieterle silver stain. Nevertheless, symptoms consistent with neurosyphilis may not always be present (Hook and Marra 1992), i.e. asymptomatic neurosyphilis. Asymptomatic neurosyphilis usually is recognized as a disease entity when a lumbar puncture is performed on a person with a reactive serologic test for syphilis and no history of treatment (Centers for Disease Control and Prevention 2002).

Congenital syphilis

In congenital syphilis, the primary stage of the disease is not seen because the organisms directly infect the fetal circulation. Necrotizing funisitis may or may not be present (Schwartz et al. 1995; Guarner et al. 2000). Treponemes, or the effects thereof, are detectable in almost every tissue of the infant. The standard serological tests for syphilis, based on the measurement of IgG, reflect passively transferred antibodies from the mother to the infant, rather than IgM antibodies produced by the infant during gestation. Currently, diagnosis of neonatal congenital syphilis depends on a combination of results from physical, radiographic, serological, and direct microscopic examinations. Clinical signs of congenital syphilis include hepatosplenomegaly, cutaneous lesions, osteochondritis, and snuffles (Kaufman et al. 1977). Although some clinical manifestations may be present at birth, they are more often seen at 3 weeks to 6 months. At birth, up to 50 percent of infants with congenital syphilis are asymptomatic (Kaufman et al. 1977); other stigmata that may develop late include teeth and bone malformation, deafness, blindness, and learning disabilities.

LABORATORY DIAGNOSIS

Various test methods for the diagnosis of syphilis have been developed. These tests fall into four categories:

1. direct microscopic examination, used when lesions are present;
2. nontreponemal tests, used for screening;
3. treponemal tests that are confirmatory or are used for screening; and
4. direct antigen detection tests currently used in research settings and as gold standards for test evaluation.

Direct microscopic methods

When lesions are present, direct detection of the organism is the most specific and easiest means of diagnosing syphilis. A positive result on microscopic examination is definitive evidence of syphilis, if infection with other pathogenic treponemes can be excluded. Also, primary syphilis can be diagnosed by darkfield several days to several weeks before the appearance of reactive serologic tests. However, a negative direct microscopic finding does not exclude the diagnosis of syphilis. Too few organisms may be present to be observed because the lesion may be in the healing stage, or the spirochete may have been altered by systemic or topical treatment. In addition, the sample may be reported as unsatisfactory as a result of the presence of too many blood cells, air bubbles, or tissue fragments. The reported sensitivities of the direct microscopic methods range from 73 to 100 percent (Daniels and Ferneyhough 1977; Romanowski et al. 1987; Ito et al. 1992). When direct microscopic methods are negative, other diseases, such as herpes and chancroid, should be considered because other sexually transmitted diseases are also characterized by lesions.

DARKFIELD MICROSCOPY

Because of their narrow width, treponemes can only be observed with the use of either specialized stains or darkfield microscopy, which is used to visualize live treponemes. Microscopes equipped with a double-reflecting or single-reflecting darkfield condenser are needed to perform the darkfield examination. Illumination for darkfield microscopy is obtained when light rays strike objects in the field at an oblique angle so that no direct rays, but only rays that are reflected from the object, enter the microscope. Therefore, the object itself appears to be illuminated against a dark background. For details of the test see Larsen et al. (1998).

T. pallidum is distinguished from other spiral organisms by the tightness of the spirals and their characteristic corkscrew movement. However, *T. pallidum* subspecies *pallidum* cannot be distinguished from the other pathogenic treponemes. *T. pallidum* is a delicate, corkscrew-shaped organism with rigid, uniform, tightly wound, deep spirals. The characteristic motion of *T. pallidum* is a deliberate forward-and-backward movement with rotation about the longitudinal axis. Organisms easily confused with *T. pallidum* include *T. refringens* and *T. denticola*. Adequate training and experience are necessary to make an accurate diagnosis by darkfield microscopy. The untrained observer may be deceived by artefacts such as cotton fibers and Brownian motion.

Darkfield examination has proved to be diagnostically useful during primary, secondary, infectious relapsing, and early congenital syphilis when moist lesions containing large numbers of treponemes (e.g. chancres, condylomata lata, or mucous patches) are present. Enlarged regional lymph nodes can also serve as a specimen source if the involved node is aspirated and the material obtained is examined. Darkfield examination of lesions of the cervix and vagina are possible if special techniques are used for collection of the specimen.

Because viability of the treponeme is necessary to distinguish *T. pallidum* from morphologically similar saprophytic spirochetes within and near the genitalia due to possible contamination by digestive-tract normal flora, darkfield examination must be performed immediately after the specimen is obtained. Equipment and personnel for darkfield examination must be readily available, or the patient must be sent to a facility where the procedure can be performed.

The proper specimen for darkfield microscopy consists of serous fluid that contains *T. pallidum* but is free of erythrocytes, other organisms, and tissue debris. The lesion should be cleansed only if encrusted or obviously contaminated and only a minimal amount of tap water or physiological saline (without antibacterial additives) should be used. Even the experienced observer may find it difficult or impossible to differentiate *T. pallidum* from saprophytic spirochetes in the mouth; thus, darkfield microscopy should not be used for the examination of samples from oral lesions.

DIRECT FLUORESCENT ANTIBODY TECHNIQUES

The direct fluorescent antibody test for *Treponema pallidum* (DFA-TP) detects and differentiates pathogenic treponemes from nonpathogenic treponemes by an antigen–antibody reaction; thus, the organism is not required to be motile. In addition, because the conjugates used are specific for pathogenic strains of *Treponema*, the DFA-TP is applicable to samples collected from oral, rectal, and intestinal lesions. Because the organism need not be motile, an additional advantage of the DFA-TP over the darkfield is that specimens collected from patients in clinic settings can be submitted to a reference laboratory for examination. Smears may be stained with fluorescein-isothiocyanate (FITC)-labeled anti-*T. pallidum* globulin prepared from the serum of humans or rabbits with syphilis absorbed with Reiter treponemes, or with FITC-conjugated to a mouse monoclonal antibody to *T. pallidum* (Romanowski et al. 1987; Ito et al. 1992). However, even though monoclonal antibodies are used, the test cannot distinguish between pathogenic strains of *Treponema*. The DFA-TP method has been used to detect the presence of *T. pallidum* subspecies in tissues (Hunter et al. 1984; Ito et al. 1992), body fluids (Smith and Israel 1967; Wilkinson 1973), secretions, and lesion exudates (Ito et al. 1992). FITC-labeled polyclonal anti-*T. pallidum* conjugate is available commercially. FITC-labeled monoclonal antibody is available from the CDC.

A combination of the direct fluorescent antibody tissue test for *T. pallidum* (DFAT-TP) and histological stains may be used to examine biopsy and autopsy material for the presence of pathogenic Treponema. Any tissue can be used, but most frequently tissues for paraffin-embedded sections are collected from the brain, gastrointestinal tract, placenta, umbilical cord, or skin. Often DFA-TP is used to diagnose late-stage or congenital syphilis or to distinguish skin lesions of secondary or late syphilis from those of Lyme disease (Ito et al. 1992). Slides prepared from sections are stained with a FITC-labeled monoclonal conjugate as for the DFA-TP, or by an indirect method by using first either an antibody to *T. pallidum* prepared from human serum or from an immunized rabbit or a mouse monoclonal antibody, followed by an FITC-labeled antihuman, antirabbit, or antimouse globulin. A testicular tissue section from a rabbit infected with *T. pallidum* is used as the control. The condition of the initial biopsy specimen or autopsy sample and the thickness of the tissue sections affect the outcome of the test. When the DFA-TP and the Steiner stain, a silver stain, were used to examine umbilical cord

Table 3 *Serological tests for syphilis in the USA*

Nontreponemal	Treponemal
Microscopic tests	Microscopic tests
Venereal Disease Research Laboratory (VDRL) slide	Fluorescent treponemal antibody absorption (FTA-ABS)
Unheated serum reagin (USR)	FTA-ABS double staining (DS)
Macroscopic tests	Macroscopic test
Rapid plasma reagin (RPR) 18-mm circle card	*T. pallidum* particle agglutination (TP-PA)
Toluidine red unheated serum test (TRUST)	Enzyme immunoassay (EIA)

samples, 100 percent agreement between the two tests was found (Schwartz et al. 1995).

IMMUNOHISTOCHEMICAL TECHNIQUES

Immunohistochemical (IHC) staining using enzymes for a color reaction to visualize the treponemes employs the same mouse monoclonal or Reiter absorbed anti-*T. pallidum* globulin that is used in the DFAT-TP. It is a multistep process that uses an enzyme, either peroxidase (Phelps et al. 2000) or alkaline phosphatase (Guarner et al. 2000), conjugated strepavidin, and then a color reaction for visualization of the treponemes in the tissue. Slides are counterstained with hematoxylin, which allows for simultaneous visualization of the treponemes and tissue morphology. Slides are examined using a bright-field microscope. Results of one study undertaken in cases of secondary syphilis indicated that IHC was more sensitive than silver stain (Phelps et al. 2000).

Serological tests

The most common methods for the diagnosis of syphilis are serological tests. These tests detect either antibodies to lipoidal antigens indicative of an infection (nontreponemal methods) or antibodies against specific treponemal antigen (treponemal methods). Those tests that are used for the diagnosis of syphilis in the USA are shown in Table 3. Details of test performance for each of the tests and the methods for specimen collection are found in *A manual of tests for syphilis* (Larsen et al. 1998). Serum is the specimen of choice for all serological tests. However, plasma samples may also be used in the macroscopic nontreponemal card tests. The technician must check the product insert that accompanies the commercial test reagent to be sure that the plasma sample has not exceeded the recommended storage time and that the blood was collected in the specified anticoagulant.

NONTREPONEMAL TESTS

The nontreponemal tests are inexpensive, fast, easy to perform, and excellent for screening purposes. The nontreponemal (reagin) tests measure IgM and IgG antibodies to lipoidal material released from damaged host cells; to lipoprotein-like material; and possibly to cardiolipin released from the treponemes (Matthews et al. 1979). The antilipoidal antibodies are not only produced as a consequence of syphilis and other treponemal diseases, but also may be produced in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs (Catterall 1972). Without some other evidence for the diagnosis of syphilis, a reactive nontreponemal test does not confirm *T. pallidum* infection.

Cardiolipin, when combined with lecithin and cholesterol, forms a serologically active antigen used in the nontreponemal tests. The unheated serum reagin (USR) test antigen is created by the addition of choline chloride and ethylenediaminetetraacetic acid (EDTA) to the Venereal Disease Research Laboratory (VDRL) antigen to stabilize the antigen suspension (Portnoy et al. 1961) and to eliminate the need for heating serum. Plasma was also found to be an acceptable test sample source. In the late 1950s, charcoal particles were incorporated into the USR antigen to aid in reading the reaction (Portnoy et al. 1957). The resulting test was the rapid plasma reagin (RPR) test performed on a plastic-coated card. Additional modifications of the RPR card test resulted in the development of the toluidine red unheated serum test (TRUST) (Pettit et al. 1983). Based on the 2002 College of American Pathologists Syphilis Serology Survey, the RPR card test is the most widely used nontreponemal test in the USA.

Flocculation tests

The flocculation tests (VDRL, USR, RPR, TRUST) can be used as qualitative tests for initial screening or as quantitative tests to determine the efficacy of treatment (Fiumara 1978, 1979, 1980a, b; Pettit et al. 1981; Brown et al. 1985; Romanowski et al. 1991). In the qualitative nontreponemal tests, undiluted serum from a patient is used to measure the presence or absence of antibodies. In the quantitative nontreponemal tests, serial two-fold dilutions are made and the serum is diluted until an end point is reached. Quantitative reactions are reported in terms of the highest (last) dilution in which the specimen is fully reactive. Quantitative tests are more informative than qualitative tests alone. Quantitative

Table 4 *Sensitivities and specificities of the nontreponemal tests*

	% Sensitivity				% Specificity
Test	**Primary**	**Secondary**	**Latent**	**Late %**	**Non-syphilis**
VDRL	78 (74–87)[a]	100	95 (88–100)	71 (37–94)	98 (96–99)
RPR	86 (77–100)	100	98 (95–100)	73	98 (93–99)
USR	80 (72–88)	100	95 (88–100)		99
TRUST	85 (77–86)	100	98 (95–100)		99 (98–99)

RPR, rapid plasma reagin; TRUST, toluidine red unheated serum test; USR, unheated serum reagin; VDRL, Venereal Disease Research Laboratory.
a) Range of sensitivity in Centers for Disease Control and Prevention (CDC) studies.

tests establish a baseline of reactivity from which change can be measured; recent infection can be demonstrated by a four-fold rise in titer and reinfection or relapse can be detected among people with a persistently reactive (serofast) test for syphilis. A four-fold or greater decline in titer following treatment is indicative of successful treatment for syphilis. All serum samples exhibiting any degree of reactivity or roughness should be diluted. Although all four nontreponemal tests have the same level of sensitivity and specificity (Table 4), the levels of reactivity vary. These different levels of reactivity are reflected in the different end-point titers obtained when the same serum specimen is tested by the four tests (Pettit et al. 1981, 1983). Because success or failure of treatment is based on just a two-dilution decrease in titer, the serum samples used as the baseline should be drawn the day treatment is begun. Reactivity levels vary among the tests; therefore, the test used in the initial screening should also be used to monitor treatment.

CSF variations on the VDRL test

The VDRL-CSF test is a variation on the VDRL test for use with spinal fluid in the diagnosis of neurosyphilis. The VDRL antigen is diluted in 10 percent saline to account for the decrease in antibody levels of spinal fluid. The test was designed to be carried out using concavity slides, but standard VDRL slides may be used as well. The test is highly specific for neurosyphilis, but varies from 10 percent sensitivity in asymptomatic individuals to 90 percent in people with symptomatic neurosyphilis. The test can be falsely positive if the spinal tap is contaminated with serum.

Capture-S solid-phase red-blood-cell adherence assay

In 1997 Stone et al. developed a solid-phase red-blood-cell adherence assay for the detection of nontreponemal antibodies in serum or plasma (Stone et al. 1997). The test is based on using a modified VDRL antigen, which has been bound to the wells of a microtiter plate. The patient's serum is added to the well; if nontreponemal antibodies are present, they bind to the VDRL antigen. Bound antibodies are detected using indicator red cells, which have been coated with antihuman IgG and IgM. After addition of the indicator cells, the microtiter plates are centrifuged. The presence of a mat of cells indicates the presence of nontreponemal antibodies in the serum or plasma specimen. The test had 87.1 percent sensitivity in people with untreated syphilis. When a blood donor population was tested, the specificity, based on 10 264 serum samples, was 99.2 percent and the sensitivity was 94 percent. Several hundred tests may be performed in a day using the Capture-S test, and the test has the potential for automation. The test has received Food and Drug Administration (FDA) clearance as a screening test for syphilis in donations received in blood banks.

PROBLEMS ENCOUNTERED WITH THE NONTREPONEMAL TESTS

Three major problems are encountered with the nontreponemal tests: the prozone phenomenon, false-positive reactions, and test interpretation. Serum samples containing large amounts of nontreponemal antibody occasionally demonstrate a prozone reaction in the nontreponemal serological tests. Prozone reactions occur in 1–2 percent of patients with secondary syphilis (Spangler et al. 1964; Jurado et al. 1993). A prozone occurs when an antibody is in excess or blocks the normal antigen–antibody reaction. Initially, these strongly reactive serum samples may show a weakly reactive, atypical, or, on rare occasions, negative rough reaction or grainy appearance in undiluted serum. Upon dilution of a serum exhibiting the prozone reaction, the reactivity will increase and then decrease as the end-point titer is approached. Dilution of the antibody to 1:16 is usually adequate to obtain the proper optimal concentration and a readily detectable reaction. All tests with a 'rough' appearance should be quantitated. A serologist may not detect a prozone reaction because of a lack of reading experience. When fresh serum samples are tested, some exhibit innate roughness. Many serologists compensate for this roughness in their reading and fail to quantitate all serum samples exhibiting a grainy appearance (negative roughs). A second reason that the prozone reaction may not be detected is that the antigen is added to the serum before the sample is spread to fill the circle. The physical spreading of the serum first, before adding the antigen, dilutes the antibodies slightly and frequently prevents the prozone reaction.

The incidence of false-positive reactions depends on the test used and the population studied. False-positive reactions occurring with the nontreponemal tests can be divided into two groups; those that are acute false-positive reactions of <6 months duration and those that are chronic false-positive reactions that persist for >6 months (Jaffe and Musher 1990). Acute false-positive nontreponemal reactions have been associated with hepatitis, infectious mononucleosis, viral pneumonia, chickenpox, measles, other viral infections, malaria, immunizations, pregnancy, and laboratory or technical error (Table 5). Chronic false-positive reactions have been associated with connective tissue diseases such as lupus erythematosus or diseases associated with immunoglobulin abnormalities, which are more common in women; thus, chronic false-positive reactions are more common in women than in men. Other conditions associated with chronic false-positive reactions are narcotic addiction, ageing (Tuffanelli 1966), leprosy, and malignancy (Jaffe and Musher 1990) (Table 5). Human immunodeficiency virus (HIV) infection has not been associated with an increase in false-positive nontreponemal test results in individuals with a low risk of drug addiction (Rusnak et al. 1994). At one time, titer was used to distinguish between false-positive and true-positive results, titers of ⩾8 considered as true positives and those <8 as possible false positives. The titer of false-positive reactions is usually low, but on rare occasions can be extremely high; therefore, the quantitative titer cannot be used to differentiate between a false-positive reaction and syphilis.

The interpretation of nontreponemal test results depends on the population being tested. When the nontreponemal tests are used as screening tests in a low-risk population, all reactive results should be confirmed with a treponemal test. In some low-risk populations, every reactive result may be a false-positive result. Nontreponemal test results also must be interpreted according to the stage of syphilis suspected. A reactive or weakly reactive result can be seen in all stages of syphilis. These results can also indicate a person who is serofast or could represent a false-positive reaction. A nonreactive result generally excludes active infection. However, serological tests may be nonreactive during incubating disease.

A major source of error in the nontreponemal tests is improper temperature of the laboratory, specimens, or reagents. If the temperature of any of these is <23°C, test reactivity is decreased; if temperature is >29°C, test reactivity is increased. Other sources of errors include the testing of hemolyzed specimens (especially in the VDRL-CSF) or contaminated or lipoidal specimens, improper rotation time or speed, and incorrect antigen delivery (Larsen et al. 1998).

Table 5 *Causes of acute and chronic false-positive reactions in the nontreponemal tests*

Acute	Chronic
Hepatitis	Connective-tissue diseases
Viral pneumonia	Immunoglobulin abnormalities
Measles	
Malaria	Narcotic addiction
Pregnancy	Ageing
Infectious mononucleosis	Leprosy
Chickenpox	Malignancy
Other viral infections	
Immunizations	
Laboratory or technical error	

TREPONEMAL TESTS

All treponemal tests use *T. pallidum* or the components thereof as the antigen and are based on the detection of antibodies directed against treponemal components. Treponemal tests are used primarily to verify reactivity in the nontreponemal tests. The treponemal tests may also be used to confirm a clinical impression of syphilis in which the nontreponemal test is nonreactive, but there is evidence of syphilis, such as might occur in late syphilis. Some treponemal tests may also be used as screening tests. Treponemal tests are technically more difficult and costly to perform than nontreponemal tests and preferably should not be used to screen for syphilis. When treponemal tests are used for screening purposes, about 1 percent (Goldman and Lantz 1971) of the general population will have false-positive results. However, a reactive treponemal test result on a sample that is also reactive in a nontreponemal test is highly specific. Since treponemal tests remain reactive despite therapy, they cannot be used to monitor responses to treatment. The tests currently used in the clinical laboratory today are fluorescent antibody tests, agglutination tests, or enzyme immunoassays (Table 3).

Fluorescent treponemal antibody tests

The fluorescent treponemal antibody-absorption (FTA-ABS) test is an indirect fluorescent antibody technique. In this procedure, the antigen used is subspecies *pallidum* (Nichols strain). Plasma cannot be used in the FTA-ABS tests. The patient's serum is first diluted to 1:5 in sorbent (a heat extract from cultures of nonpathogenic Reiter treponemes) to remove group treponemal antibodies that are produced in some people in response to nonpathogenic treponemes. Next, the serum is layered on a microscope slide to which *T. pallidum* has been fixed. If the patient's serum contains antibody, it coats the treponeme. FITC-labeled antihuman immunoglobulin is added and combines with the patient's antibodies adhering to *T. pallidum*, resulting in FITC-stained spirochetes that are visible when examined by fluorescence microscopy. A modification of the standard FTA-ABS test is the FTA-ABS double-stain (DS) test

(Larsen et al. 1998). The conjugate used is rhodamine isothiocyanate-labeled antihuman globulin and the counterstain is FITC-labeled anti-*T. pallidum* conjugate. The counterstain was developed for use with microscopes with incident illumination to eliminate the need to locate the treponemes by darkfield when the patient's nonreactive result is due to the absence of antibodies and not to the absence of treponemes on the slide when it is read with incident illumination. Reactive results of the FTA-ABS tests cover a range of staining intensities from 1+ (reactive minimal) to 4+. Because beaded fluorescence (atypical staining) has been observed in serum from patients with active systemic lupus erythematosus and from patients with other autoimmune diseases, this observation should also be reported.

IgM variations on the FTA test

Several variations on the FTA-ABS test have been used for the diagnosis of congenital syphilis by replacing the IgG conjugate with an IgM conjugate. Only the FTA-ABS IgM is still available for routine use, but it is reported to be both nonspecific and lacking in sensitivity (Alford et al. 1969; Johnston 1972a; Kaufman et al. 1977; Muller 1986). The specificity of the FTA-ABS IgM tests for neonatal congenital syphilis, as well as other indirect immunofluorescent IgM tests for prenatal infections, may be due to IgM antibodies produced in response to passively transferred maternal IgG antibody rather than in response to the infectious agent itself (Reimer et al. 1975). The fractionation of the infant's serum in the FTA-ABS 19S IgM tests appeared to eliminate the majority of the problems with the specificity of the test. However, because of lack of availability of the separation columns used in the test, the test has become too time-consuming and expensive for routine use. Results of the FTA-ABS IgM test for congenital syphilis should not be interpreted without careful repeated clinical assessment combined with serial quantitative nontreponemal tests in the evaluation of the newborn with possible congenital syphilis.

CSF variations on the FTA test

Another variation on the FTA-ABS procedure is the FTA-ABS CSF test for the diagnosis of neurosyphilis. FTA tests used for CSF examination have included techniques that used undiluted CSF (the CSF-FTA) (Harris et al. 1960; Escobar et al. 1970; Wilkinson 1973; Jaffe et al. 1978; Larsen et al. 1985) and tests that used CSF diluted 1:5 with sorbent (the CSF-FTA-ABS test). FTA tests on CSF are more sensitive than the CSF-VDRL slide test. However, the clinical significance of this greater sensitivity remains to be established, because the test may be reactive as a result of antibodies remaining in the CSF of patients who had been adequately treated for early or latent syphilis rather than as a result of neurosyphilis (Jaffe et al. 1978). False-positive results have been reported with the CSF-FTA tests (Escobar et al. 1970; Larsen et al. 1985), which appear to be eliminated by diluting the CSF in sorbent. Several studies appear to support the use of a nonreactive result with the FTA-ABS CSF test to rule out neurosyphilis (Jaffe et al. 1978; Luger et al. 1988; Marra et al. 1995).

The sources for errors are numerous with the FTA-ABS tests and its variations because they are multicomponent tests and each component must be matched with another. Conjugates must be properly titrated and controls for reactive, reactive minimal, nonreactive and nonspecific staining, and sorbent must be included. Slides must be evaluated to ensure that the antigen is adhering. In addition, the microscope must be in proper operating condition with the appropriate filters in place.

Hemagglutination methods

In 1965, Rathlev reported the first reliable application of hemagglutination techniques to the serological diagnosis of syphilis. The antigen used in her procedure was formalinized tanned sheep erythrocytes sensitized with ultrasonicated material from *T. pallidum* (Nichols strain). The presence of treponemal antibodies in the patient's serum was detected by the indirect agglutination of the sensitized erythrocytes and the subsequent formation of a mat of erythrocytes upon their settling. Modifications of Rathlev's procedure followed, including the use of an improved reaction medium (Tomizawa and Kasamatsu 1966) and a sorbent much like the one used in the FTA-ABS test for removal of group treponemal antibodies (Tomizawa et al. 1969). The hemagglutination procedure was first a tube test known as the *T. pallidum* hemagglutination (TPHA) test. Subsequently, reagent for a microvolume hemagglutination test (Cox et al. 1969), the microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP), became commercially available. Other hemagglutination tests for syphilis, based on the use of sensitized turkey, sheep, or chicken cells and performed in microtiter plates, are currently available worldwide. The hemagglutination tests are simpler to perform than the fluorescent antibody tests.

An automated hemagglutination test, the Olympus PK-TP, was developed mainly for use in blood banking. The instrument used is a PK7100 or 7200, an automated pretransfusion blood testing system, which is the basic equipment for blood grouping and typing used by the American Red Cross. In the USA, the use of the test as a screening test is restricted at this time to blood-banking organizations. Early experience with the PK-TP in blood banks (Forbes et al. 1991) indicates that the PK-TP is at least twice as specific as the RPR card test.

Particle agglutination methods

The microhemagglutination test has been modified to use gelatin particles rather than erythrocytes as the

antigen carrier creating the *T. pallidum* particle agglutination (TP-PA) test (Deguchi et al. 1994; Castro et al. 2001). This eliminated the need for preabsorption of the serum to remove heterophile antibodies in the patient's serum sample, making the tests easier to perform. In addition, the current commercially available test (Serodia TP-PA) uses an antigen that is more purified than the one used in the MHA-TP, which helps decrease the nonspecificity. Other than eliminating the preabsorption step, the TP-PA procedure is similar to that of the MHA-TP (Pope and Fears 2000). The TP-PA test has a sensitivity and specificity similar to that of the FTA-ABS test, including cases of primary syphilis.

Latex agglutination methods

In 1985, the first reports of a latex agglutination test for syphilis were published (Asaka 1985). The current commercially available test (Syphilis Fast) uses cloned *T. pallidum* antigens bound to latex particles (Matsumoto et al. 1993; Young et al. 1998). The latex agglutination test is easy to perform and fast, requiring less than 30 min for results. The test is suited for situations where confirmatory test results are needed in a short time frame. The test, which is not available in the USA, has sensitivity and specificity similar to that of the FTA-ABS and TP-PA tests.

Because the tests are based on agglutination, quantitation of treponemal antibodies is possible but has not proved to be useful. Most studies demonstrate no practical relationship between the titer and either the progression of the disease or the clinical stage of syphilis diagnosed; unlike the quantitative nontreponemal tests, the quantitative hemagglutination test does not seem to be useful in post-treatment evaluation (Cox et al. 1971; Johnston 1972b). However, one group found that there was a significant decrease in antibodies following treatment when the MHA-TP or TP-PA was used (Castro et al. 2001). The sources for error with the agglutination tests are usually associated with the use of dusty or improper plates, pipetting errors, and vibrations in the laboratory.

Enzyme immunoassay treponemal antibody tests

The enzyme immunoassay (EIA) was first applied to the area of syphilis serology in 1975 (Veldekamp and Visser 1975). Since that time may other tests using the EIA format have been developed and evaluated as treponemal tests (Pedersen et al. 1982; Pope et al. 1982; Farshy et al. 1985; Young et al. 1992; Nayar and Campos 1993). There are two types of EIA tests available in the USA: one that uses sonicated *T. pallidum* as the antigen (Syphilis-G) and one that uses cloned antigens (Trep-Chek). The cloned antigens most frequently used are the 47-, 17-, and 15-kDa proteins (Young et al. 2000; Sambri et al. 2001). There are some indications that EIA procedures may be less sensitive in early primary syphilis than the RPR or FTA-ABS tests (Gosbell et al. 1999) but are similar to the FTA-ABS in secondary and latent syphilis (Nayar and Campos 1993).

Limitations of the EIA tests are time and cost when small numbers of samples are to be processed. The main advantages of EIA are the capability to automate the test, ability to process large numbers of samples, and an automated objective readout. No longer is the reading of the test subjective, as for the FTA-ABS and agglutination tests, but a spectrophotometric determination is made.

There is increasing use in the USA of the EIA tests as a screening test for syphilis due to the ability to run large numbers of serum samples in a relatively short time in comparison with nontreponemal flocculation tests. The test has also been used in other countries as a screening test (Young et al. 1992). Since the only EIA tests currently available are treponemal tests, the user must remember that treponemal antibodies generally remain for life for the majority of people who have ever had syphilis (United States Public Heath Service 1968). Serum with reactive EIA results should also have a nontreponemal test performed, especially if the clinical history is unknown or suggestive of being a possible contact to someone with syphilis. In populations where the prevalence of syphilis is low, most of the reactive test results will not reflect active syphilis.

IgM variation on the EIA test

A variation on the EIA test is the Captia Syphilis-M test (Ijsselmuiden et al. 1989; Lefèvre et al. 1990; Stoll et al. 1993) for the detection of IgM antibodies in syphilis. Because infected infants can produce IgM in utero after 3 months of gestation and the fetus can be infected with *T. pallidum* at any time during gestation, an IgM EIA test is most useful in congenital syphilis. The test uses antihuman IgM antibody to capture IgM in the patient's serum, followed by the addition of a purified *T. pallidum* antigen to detect those IgM antibodies in the patient's serum directed toward *T. pallidum* (Ijsselmuiden et al. 1989). One study (Bromberg et al. 1993) found the test to be equal in sensitivity to the IgM Western immunoblot in neonatal congenital syphilis but less sensitive than the Western blot in detecting delayed-onset congenital syphilis. Current interpretations of IgM test results are given in Table 6. The interpretation of the test result is linked to the treatment status of the mother and her stage of syphilis.

Immunoblotting

The Western blot technique developed by Hanff et al. (1982) for *T. pallidum* can be used to detect either IgG (Byrne et al. 1992) or IgM (Sanchez et al. 1989) antibodies. To prepare the strips for *T. pallidum* immuno-

Table 6 *Interpretation of IgM test results for the diagnosis of congenital syphilis*

Infant's result	Clinical signs/symptoms in infant	Mother's treatment history	Serodiagnosis
Nonreactive	Present	Untreated or inadequately treated	Congenital syphilis
Nonreactive	Absent	Untreated or inadequately treated early syphilis	Possible incubating congenital syphilis
Nonreactive	Absent	Untreated or inadequately treated latent syphilis	Unknown risk of congenital syphilis
Reactive	Present	Untreated or inadequately treated	Congenital syphilis
Reactive	Absent	Untreated or inadequately treated	Suggestive of congenital

blotting, initially a boiled sodium dodecyl sulfate (SDS) extract of the organism is electrophoresed through a gradient gel. After electrophoresis, a sheet of nitrocellulose is placed on top of the gel and the protein immunodeterminants are electrophoretically transferred to the blot. The blot is cut into strips and incubated with the patient's serum sample. The nitrocellulose strips may be obtained commercially. After incubation of the strips with the patient's serum, antibodies are detected using a second antibody as in an indirect test, usually labeled with an enzyme, and substrate. To date, many investigators (Norris and the *Treponema pallidum* Polypeptide Research Group 1993) and the manufacturer of at least one commercial product agree that detected antibodies to the immunodeterminants with molecular masses of 15.5, 17, 44.5, and 47 kDa appear to be diagnostic for acquired syphilis. The test using IgG conjugate appears to be at least as sensitive and specific as the FTA-ABS tests (Byrne et al. 1992) and efforts have been made to standardize the procedure (George et al. 1991). In addition, various groups have reported the use of cloned proteins (Strugnell et al. 1990; Norris and the *Treponema pallidum* Polypeptide Research Group 1993; Ebel et al. 2000) for Western blotting.

The IgM Western blot may be the most sensitive test currently available for the diagnosis of congenital syphilis (Sanchez et al. 1993; Stoll et al. 1993), especially when used in conjunction with a complete clinical evaluation and other tests (Stoll et al. 1993). Table 6 lists the interpretation of IgM test results, in conjunction with the mother's history.

Immunochromatographic strip tests

Variations on the Western blot include the immunochromatographic strip (ICS) tests, which are commercially available only outside the USA. These tests utilize one or more cloned antigens of *T. pallidum*. The antigen is laid as a stripe on a membrane that has filter pads layered on the top and bottom. A second separate stripe, which serves as the control, is also laid down on the membrane. The sheet is cut into strips for individual tests. The bottom filter contains antihuman conjugate and the indicator, usually colloidal gold, for the reaction. The sample is placed on the bottom filter. The top filter serves to draw the liquid sample and conjugate through the membrane. The tests usually have high specificity, due to the use of cloned antigens. The strips also are considered a rapid test, with results usually obtained within 10–15 min. The strip tests also do not require equipment or electricity. For some tests, a fingerstick sample can be used making the test practical in nontraditional settings.

A recent study compared the utility of a rapid strip test using the 47-kDa protein with the RPR for field screening in rural areas of Gambia (West et al. 2002). Results were compared with an RPR and hemagglutination test done in the laboratory. The RPR and rapid screen test had similar results in field conditions, both having sensitivities of 75–78 percent and specificity of 94–95 percent, based on active syphilis defined as a positive RPR and hemagglutination tests performed in the laboratory. Although neither the RPR nor the strip test used in this study had a high positive predictive value (West et al. 2002), the authors found the strip test easier to use and interpret than the RPR. In another study looking at the same strips (Zarakolu et al. 2002) the agreement between the FTA-ABS and the strip test was 100 percent, while it was slightly more sensitive than the RPR test in the population they tested.

PROBLEMS ENCOUNTERED WITH THE TREPONEMAL TESTS

Although false-positive reactions in the treponemal tests are often transient and their cause is unknown, a definite association has been made between false-positive FTA-ABS test results and the diagnosis of systemic discoid (Kraus et al. 1971; McKenna et al. 1973) and drug-induced varieties of lupus erythematosus (Anderson and Stillman 1978; Monson 1973) (Table 7). Patients with systemic lupus erythematosus can have false-positive FTA-ABS tests that exhibit an 'atypical beading' fluorescence pattern. To resolve these types of false-positive reactions, absorption with calf thymus DNA can be used

Table 7 *Causes of chronic false-positive reactions in the treponemal tests*

Causes of reactions
Systemic lupus erythematosus
Drug-induced lupus erythematosus
Narcotic addiction
Ageing

to remove the anti-DNA antibodies in the serum (Kraus et al. 1971). Unexplained reactive serological results may also occur in elderly patients. Further, some false-positive reactions may be due to the failure of the sorbent used in the tests to remove all cross-reacting group, genus, or family antibodies (e.g. Lyme disease) (Hunter et al. 1986). In these instances, absorption with *Treponema phagendenis* or the use of the agglutination test or immunoblotting may be the only means of differentiating between syphilis and a false-positive reaction. In general, the occurrence of false-positive treponemal tests is rare in 'healthy' people (<1 percent). If at least two treponemal tests, such as the FTA-ABS and agglutination tests, are reactive, the sample is most likely (95 percent) from a person who has or has had syphilis at some point in their lifetime (Rein et al. 1980). However, the diagnosis rests with clinical judgment. False-negative FTA-ABS or DFA-TP tests can occur if the mounting medium has started to deteriorate and the pH has dropped.

Treponemal tests vary in their rates of reactivity in early primary syphilis (Table 8); the varied sensitivities of the treponemal tests in primary syphilis may be related in part to differences between the studies in the time of collection following lesion development. For 85 percent of people successfully treated, test results can remain reactive for years, for some people for a lifetime (Schroeter et al. 1972). In addition, for the diagnosis of late untreated syphilis, reactivity in the treponemal tests may be the only indication of a previous treponemal infection.

Direct antigen detection methods

The shortcomings of the standard tests for syphilis for the diagnosis of early primary, congenital, and neurosyphilis make techniques based solely on the detection of treponemal DNA or antigens very appealing. In order to determine the sensitivity and specificity of these newer techniques, rabbit infectivity testing (RIT) has been used as the gold standard (Grimprel et al. 1991; Sanchez et al. 1993).

ANIMAL INOCULATION

The oldest method for detecting infection with *T. pallidum* is animal infectivity testing. This technique probably offers the most sensitive method for detecting infectious treponemes. Numerous animal species, from hamsters to chimpanzees, have been used either to maintain treponemes or to determine infectivity (Wilcox and Guthe 1966). However, not all animals develop visible signs of infection or reactive serologic tests. The rabbit is the most practical animal because a local lesion can be produced at the site of inoculation, the tissues remain infective for the life of the animal, infection can be transferred from one animal to another using minced lymph nodes or testes, and serological tests for syphilis become reactive following inoculation. Any specimen can be used for RIT as long as the material was collected less than 1 h before inoculation or is flash-frozen immediately after collection and maintained in liquid nitrogen or at temperatures of −78°C or below (Turner and Fleming 1939). Rabbits are usually inoculated intratesticularly or intradermally with the samples suspected of being infective. Detailed methods for the maintenance and passage of *T. pallidum* in rabbits are available from the CDC. The incubation period for the formation of lesions in rabbits is inversely proportional to the size of the inoculum (Magnuson et al. 1948). The sensitivity of RIT approaches 100 percent if the number of organisms in the inoculum exceeds 23 (Magnuson et al. 1956) and the patient has not received antibiotic treatment. Unfortunately this method is expensive, is time-consuming, and may require a month or more before it can be determined to be either positive or negative.

PCR-BASED TESTS FOR DETECTION OF *T. PALLIDUM*

The PCR is highly sensitive for detection of pathogenic microorganisms in clinical material and would appear to

Table 8 *Sensitivities and specificities of treponemal tests*

	% Sensitivity			% Specificity
Test	Primary	Secondary	Latent	Non-syphilis
FTA-ABS	84 (70–100)[a]	100	100	97 (94–100)
TP-PA	88	100	100	98 (95–100)
FTA-ABS DS	86 (69–90)	100	100	98 (97–100)
EIA	90	100	100	99
Western blot	90	100	100	98

EIA, enzyme immunoassay; FTA-ABS, fluorescent treponemal antibody absorption; FTA-ABS, FTA-ABS double staining; TP-PA, *Treponema pallidum* particle agglutination.

a) Range in Centers for Disease Control and Prevention (CDC) studies

be the molecular test of choice for difficult-to-detect microorganisms such as *T. pallidum*. The only tests in clinical use for detection of treponemes in a clinical sample are the darkfield and direct fluorescent antibody tests, which were discussed earlier in this chapter (see sections on Darkfield microscopy and Direct fluorescent antibody techniques, above); both of these are insensitive compared to molecular techniques.

DNA probes have been used in a wide variety of applications including detection of pathogens, of drug resistance, and single-nucleotide polymorphisms (Kricker 1999). This technology is still rapidly developing and many new applications and techniques have been developed over the past several years (Kricker 1999). Unfortunately, no clinically applicable technique for growing *T. pallidum* in vitro has been developed and no in situ technique has been published for detecting *T. pallidum* directly in clinical specimens. To date, no target probe has been evaluated for *T. pallidum* either in clinical materials or for experimental detection. Probe technology has been limited to experimental procedures such as detection of nucleotide polymorphisms between the different subspecies of *T. pallidum* (Antal et al. 2002).

A number of PCR tests have been developed over the past 10 years with a variety of different target genes. The first tests developed were to genes of unknown function, which were presumed to be membrane immunogens. This conclusion was based on the dominant immune response to these antigens during syphilitic infections as measured by seroreactivity on Western blots (Burstain et al. 1991; Wicher et al. 1992; Sanchez et al. 1993). Since membrane proteins of pathogenic microorganisms commonly show variability as a mechanism to avoid immune recognition (Moxon et al. 1994), this approach could have led to difficulties in sensitivity because of potential changes in gene sequence related to immune variability. Although genetic variability is now known for at least one family of *T. pallidum* genes (*tpr* genes) (Fraser et al. 1998), none of the genes used in the assays proposed in these early papers have been shown to vary in sequence. Although the genes used as PCR targets in these early papers were originally reported to be located on the outer membrane and thought to be important in immunity to syphilis (for a review of the early literature on membrane biology of *T. pallidum* see Strugnell et al. 1990), this is now known to be false and probably accounts for the apparent invariant DNA sequences found in these target genes.

The immunodominant 47-kDa antigen gene has been the target for several PCR techniques that were developed in the 1990s (Burstain et al. 1991; Orle et al. 1996). The originally described technique amplified a 658-base-pair fragment of the gene. In several publications (Grimprel et al. 1991; Sanchez et al. 1993) this technique was applied to a number of different clinically derived specimens including serum, amniotic fluid, and CSF. Sensitivity was generally low when compared with the RIT. The amniotic fluid specimens gave reasonably good results but the overall sensitivity was in the range of only 60–67 percent when compared with the RIT; this was caused by low sensitivity of the test with serum and CSF specimens.

There were a variety of difficulties documented in these early papers that could have led to the low sensitivity of PCR when compared with RIT. These problems included the possibility of nonspecific inhibitors in the samples, the large size of the amplicon, and use of inappropriate samples such as serum. Both serum and CSF would be expected to have very low numbers of treponemes and serum is also known to contain inhibitors of PCR. In the paper by Grimprel et al. (1991) the authors used a variety of different DNA isolation techniques to deal with the first of these problems. Of the four methods used, the low-spin separation and alkaline lysis methods gave the results with the greatest concordance between the PCR and RIT. What these results reinforced was the requirement for a good yield of *T. pallidum* DNA from clinical samples; many clinical samples, such as CSF, can have low numbers of treponemes that may result in negative results from samples if DNA yields are not optimal.

The problems related to sample preparation and possible persistence of *T. pallidum* in clinical material was especially evident in the work of Noordhoek et al. (1991) with central nervous system (CNS) specimens. These workers used primers for the gene coding for the 39-kDa basic membrane protein. Initial work showed a lack of specificity when using only one set of primers, but this problem appeared to be overcome by using a second set of primers for amplification of the original PCR amplicons ('nested' primers). This resulted in a second serious problem in that all specimens from confirmed CNS involvement of syphilis resulted in positive PCR results, regardless of the clinical status of the patient; many of the patients had been clinically cured and free of syphilitic symptoms for extended periods of time. Noordhoek et al. (1991) were unable to resolve this paradox, which could have resulted from false positives or persistence of either treponemes or treponemal DNA. Because there is considerable controversy over whether current treatment regimens completely eliminate *T. pallidum* from the CNS (Berry et al. 1987; Johns et al. 1987; Lukehart et al. 1988; Musher 1991), the explanation of these results remains an open question.

The second test that used the 47-kDa protein gene for diagnosis was a multiplex PCR (or M-PCR) by the Roche company, which included *T. pallidum* primers (Orle et al. 1996). This technique used a considerably smaller and different target sequence than that originally described. After amplification from clinical material containing treponemal DNA, a synthetic DNA fragment coated on to a well in a format similar to that used in EIA was used to capture the amplicons. The capture

DNA was then identified by a second probe coupled to an enzyme to allow for colorimetric detection. The enzymatic method of detection significantly increased sensitivity and allowed for automation of the system. The system was termed a multiplex PCR since it was developed to simultaneously detect the presence of *T. pallidum*, *H. ducreyi*, and the herpes simplex virus. In comparison with the standard darkfield examination for the presence of treponemes in presumed syphilitic ulcers, the test proved at least as sensitive and highly specific. Although the procedure appeared promising, it has not been commercially developed and is currently not available on the market.

A recent development in the use of PCR for clinical diagnosis is based on the DNA polymerase I of *T. pallidum* (Liu et al. 2001). The gene coding for this enzyme (*PolA*) has a unique structure; it codes for a number of insertions in the 3′–5′ exonuclease domain not found in other sequenced *polA* genes as well as for an exceptionally high content of the amino acid cysteine (24 residues compared with two in the DNA polymerase I of *E. coli*). Unlike previous PCR methods the target is a well-characterized gene that has a number of unique targets not found in similar genes in other bacteria. It is also a well-known housekeeping gene involved in replication of DNA and DNA repair, and thus would be expected to be present in all strains of *T. pallidum* as well as invariant in sequence. Two different sets of primers were tested against a large range of other microorganisms and clinical material and were shown to react only with genomic DNA from *T. pallidum* (Liu et al. 2001). This test has been used in epidemiological work for the detection of *T. pallidum* in human blood (Marfin et al. 2001) and as a standard for detection of *T. pallidum* prior to molecular typing of *T. pallidum* (Pillay et al. 1998).

Another highly sensitive PCR-based test has been reported recently, which can act as a backup to the more conventional PCR tests. This test uses a reverse transcriptase amplification of rRNA to increase the sensitivity of the test (Centurion-Lara et al. 1997). Because of the large number of copies of rRNA compared with single copy genes used in other clinical PCR-based tests, it should have considerably greater sensitivity. Meeting this prediction, the reverse transcriptase polymerase chain reaction (RT-PCR) proved to be far more sensitive than a PCR using the gene for the 47-kDa protein (the same target used in the Roche and a number of other PCR-based tests) and could amplify targets from all of the clinical isolates tested. The one drawback in this paper was that the test was not performed with actual clinical specimens; all of the clinical isolates had first been inoculated and grown in rabbits and then purified to show sensitivity. The ability to amplify treponemal rRNA in body fluids with different potential inhibitors present was not determined. Because of the need to isolate RNA, it is also more difficult to perform than conventional PCR amplifications and will probably be of greatest utility in samples where the increased complexity is offset by its greater sensitivity.

MOLECULAR TYPING OF *T. PALLIDUM*

A new use for PCR in syphilis has been developed in the past few years with the appearance of a molecular based typing method for use in the epidemiology of syphilis (Pillay et al. 1998). This method involves the amplification of two different genetic loci that demonstrate molecular heterogeneity. The first of these loci is known as the acidic repeat protein gene or *arp* gene. This gene was originally discovered in the Nichols laboratory strain of *T. pallidum*. In this strain the gene contained 14 almost exact repeats of a 60-basepair (bp) sequence. The 20 amino acids coded for by this repeat contain a large amount of the amino acid glutamate, which gives the putative protein a very acid-predicted pI, hence the name acidic repeat protein. Using primers from beyond the repeat region, it was discovered that the number of repeats is variable from one isolate to another and this characteristic has been used as the first step in a two-step strain-typing procedure (Pillay et al. 1998). The second step uses a member of the *tpr* gene family, the *tprJ* gene, which was the original one discovered in this family and is part of a three-member subfamily of these genes. A 2.2-kilobase (kb) fragment of the gene is amplified and then a second amplification produces a 1.8-kb fragment, which is then analyzed by restriction fragment length polymorphism (RFLP). Using this two-step procedure the authors were able to demonstrate 16 different strains from the 46 different isolates that were examined in their original publication.

A second paper related to strain differentiation, in this case differentiating the subspecies of *T. pallidum* (subspecies *pallidum*, *pertenue* and *endemicum*), has also been published (Centurion-Lara et al. 1998). The authors found that while the gene for the highly antigenic 15-kDa lipoprotein from all subspecies was identical, there were two restrictions sites in the DNA surrounding the gene that could be used to differentiate the subspecies of *T. pallidum* as well as the related species *Treponema paraluis-canicoli* (Centurion-Lara et al. 1998). In both *T. pallidum* subsp. *pertenue* and *T. pallidum* subsp. *endemicum* the additional restriction site was the result of a single base-pair substitution. Although only a small number of isolates from each subspecies were tested, this discovery lends hope that a PCR-RFLP method may also be suitable for differentiating subspecies, something that has not previously been possible.

USE AND INTERPRETATION OF SEROLOGICAL TESTS

The results of any testing for syphilis should only be interpreted in the context of complete clinical history.

Table 9 *Criteria for diagnosis of early syphilis*

Primary	
Definitive	
Direct microscopic identification of *T. pallidum* in lesion material, lymph node aspirate, or biopsy section	
Presumptive (requires 1 **and** either 2 or 3)	
1	Typical lesion
2	Reactive nontreponemal or treponemal test and no history of syphilis
3	For people with a history of syphilis a four-fold increase in titer on a quantitative nontreponemal test when results of past tests are compared with recent test results
Suggestive (requires 1 **and** 2)	
1	Lesion resembling a chancre
2	Sexual contact within the preceding 90 days with a person who has primary, secondary, or early latent syphilis
Secondary	
Definitive	
Direct microscopic identification of *T. pallidum* in lesion material, lymph node aspirate, or biopsy section	
Presumptive (requires 1 **and** either 2 or 3)	
1	Skin or mucous membrane lesions typical of secondary syphilis Macular, papular, follicular, papulosquamous, or pustular Condylomata lata (anogenital region or mouth) Mucous patches (oropharynx or cervix)
2	Reactive nontreponemal test titer $\geqslant$1:8, reactive treponemal test, and no previous history of syphilis
3	For people with a history of syphilis, a four-fold increase in the most recent titer when compared with previous results
Suggestive (requires 1 and 2, **and** is made only when serological tests are not available)	
1	Presence of clinical manifestations as described above
2	Sexual exposure within the last 6 months to a person with early syphilis
Early latent	
Definitive	
A definitive diagnosis does not exist because lesions are not present in the latent stage	
Presumptive (requires 1, 2 **and** 3 or 4)	
1	Absence of signs or symptoms
2	Reactive nontreponemal and treponemal test results
3	A history of a nonreactive nontreponemal test in the prior year
4	A four-fold increase in titer compared with the previous test results for people with a history of syphilis or a history of symptoms compatible with syphilis
Suggestive (requires 1 **and** 2)	
1	Reactive nontreponemal test result
2	History of sexual exposure in the preceding year

Sexually acquired syphilis

For the laboratory diagnosis of sexually acquired syphilis, each stage has a particular testing requirement. In the USA, the routine testing scheme has been direct microscopic examination of lesion exudates, followed by a nontreponemal test, which if reactive, is confirmed with a treponemal test (Table 9). Some laboratories with large numbers of samples to test now screen with the EIA followed by a nontreponemal test, simply because the test can be automated.

EARLY SYPHILIS

Criteria for the diagnosis of syphilis are divided into three categories: definitive, presumptive, and suggestive. These criteria are given in Table 9 for the diagnosis of early syphilis. In primary syphilis, the EIA test may be less sensitive than the FTA-ABS and TP-PA, when used to confirm reactivity of the nontreponemal test (Gosbell et al. 1999). As the disease reaches the secondary stage, with few if any exceptions, all serologic tests for syphilis are reactive and treponemes may be found in lesions by direct microscopic examination. Serologic tests are reactive in the early latent stage, but the reactivity in the nontreponemal tests decreases with increasing latency.

Patients with latent syphilis, i.e. with reactive nontreponemal and treponemal tests, with no clinical and historical findings, and with an unknown history of treatment, should be evaluated for potential asymptomatic neurosyphilis.

Table 10 *Criteria for the diagnosis of late syphilis and neonatal congenital syphilis*

Benign and cardiovascular	
Definitive	
Observation by direct microscopic examination of treponemes in tissue sections by direct fluorescent antibody tissue test for *T. pallidum* (DFAT-TP)	
Presumptive (requires 1, 2, **and** 3)	
1	A reactive treponemal test
2	No known history of treatment for syphilis
3	Characteristic symptoms of benign or cardiovascular syphilis
Neurosyphilis	
Definitive (requires 1 **or** 2 **and** 3)	
1	Identification of *T. pallidum* in CSF or CNS tissue by microscopic examination or animal inoculation or by the polymerase chain reaction (PCR)
2	A reactive serum treponemal test
3	A reactive VDRL-CSF test on a spinal fluid sample
Presumptive (requires 1 **and** either 2 or 3)	
1	A reactive serum treponemal test
2	Clinical signs of neurosyphilis
3	Elevated CSF protein or leukocyte count in the absence of other known causes
Neonatal congenital syphilis	
Definitive	
Demonstration of *T. pallidum* by direct microscopic examination of umbilical cord, placenta, nasal discharge, or skin lesion material	
Presumptive (requires 1, 2, **and** 3)	
1	Determination that the infant was born to a mother who had untreated or inadequately treated syphilis at delivery regardless of finding in the infant
2	An infant who has a reactive treponemal test result
3	One of the following additional criteria:
	Clinical signs or symptoms of congenital syphilis on physical examination
	An abnormal CSF finding without other cause
	Reactive VDRL-CSF test result
	A reactive IgM antibody test specific for syphilis

CNS, central nervous system; CSF, cerebrospinal fluid; VDRL, Venereal Disease Research Laboratory.

LATE SYPHILIS

Even when the patient has not received treatment, the nontreponemal tests may be only weakly reactive or even nonreactive in the late stages of syphilis. Because results for approximately 30 percent of patients with late syphilis will be nonreactive by the nontreponemal tests, treponemal test results should be obtained if syphilis in these stages is suspected. However, the sensitivity of the treponemal tests also declines somewhat in the late stages of syphilis (see Table 10).

In the lesions (gummas) of benign late syphilis, few treponemes are found by direct microscopic examination. However, if gummas are present, then a definitive diagnosis is based on the observation of *T. pallidum* in biopsy samples by DFAT-TP (Handsfield et al. 1983) or by a reactive PCR result (Horowitz et al. 1994) (Table 10). Presumptive diagnosis is based on a reactive treponemal test and no known history of treatment for syphilis. Diagnosis of cardiovascular syphilis is made on the basis of symptoms or signs indicative of aortic insufficiency or aneurysm, reactive treponemal test results, and no known history of treatment for syphilis (Bulkey 1984).

Neurosyphilis

CSF examinations, such as the VDRL-CSF slide tests, total protein, and white cell counts, should be performed on the spinal fluid of patients with late latent syphilis and those with clinical symptoms and signs consistent with neurosyphilis (Table 10). Diagnosis of neurosyphilis requires a reactive treponemal test result with a serum sample and a CSF cell count of more than five mononuclear cells per cubic centimeter and CSF total protein in excess of 40 mg/dl.

The VDRL-CSF test should be performed only if the patient's serum treponemal test is reactive. The VDRL is the only test that should be used for testing CSF (Larsen et al. 1985). The results of the VDRL-CSF test are reported as either reactive or as nonreactive. A quantitative test is performed to determine the titer of the antibodies in the CSF by preparing two-fold serial

dilutions in saline. False-positive VDRL-CSF test results have been reported infrequently (Cutler et al. 1954). Because of a lack of sensitivity and specificity the RPR and TRUST card tests cannot be used to test CSF (Larsen et al. 1985). A nonreactive result in the FTA-ABS CSF test (Jaffe et al. 1978; Luger et al. 1988; Marra et al. 1995) may be used to rule out neurosyphilis.

Congenital syphilis

The control of congenital syphilis can be accomplished by serologically screening and treating pregnant women who are infected. Since false-positive nontreponemal test results have been reported in samples from pregnant women (Buchanan and Haserick 1970), it is especially important to confirm a reactive result and, if reactive, the patient should be treated. Also in pregnancy, nontreponemal titers remaining from previous treatment of syphilis tend to increase nonspecifically. This increase in titer may be confused with a diagnosis of reinfection or relapse. An increase in titer may be considered nonspecific if previous treatment can be documented and if positive lesions, a four-fold increase in titer, and a history of recent sexual exposure to a person with infectious syphilis are absent.

When screening for congenital syphilis at delivery, the CDC recommends the testing of the mother's serum rather than cord blood, (United States Public Health Service 1968). Studies have been performed (Chhabra et al. 1993; Stoll et al. 1993) that compared the reactivity of the mother's serum, cord blood, and infant's serum and found that the maternal sample is the best indicator of infection, followed by neonatal serum; cord blood is the least reactive. Infant's serum is the specimen of choice for IgM specific tests. The standard serological tests for syphilis, based on the measurement of IgG, reflect passively transferred antibodies from the mother to the infant, rather than IgM antibodies produced during gestation. Previously it was thought that if the infant's titer was higher than that of the mother's, then the infant had congenital syphilis (Stokes et al. 1944). However, a lower titer in the infant's serum than in that of the mother does not rule out congenital syphilis (Stoll et al. 1993). Examination of serum sample pairs from mothers and infants in cases of congenital syphilis indicated that only in 22 percent of the cases did the infant have a titer higher than that of the mother. The passively transferred antibodies should be catabolized and become undetectable among noninfected infants between the ages of 12 and 18 months (Chang et al. 1995). Criteria for the diagnosis of neonatal congenital syphilis are given in Table 10.

HIV-infected persons with syphilis

In the 1980s, studies showed that 70 percent of serum specimens from homosexual men with acquired immunodeficiency syndrome (AIDS) reacted in the treponemal test for syphilis (Rogers et al. 1983). More recent studies in the heterosexual population indicate that approximately 60 percent of HIV-infected females have reactive syphilis serological test results (Castro et al. 1988); 15 percent of adolescents with reactive serological tests for syphilis were also found to be positive for HIV antibodies (McCabe et al. 1993). Although most HIV-infected people who are also infected with *T. pallidum* appear to display normal serological responses to syphilis and have typical clinical signs (Gourevitch et al. 1993; Rolfs et al. 1997), several exceptions have been published (Dawson et al. 1988; Lanska et al. 1988; Radolf and Kaplan 1988) or reported to the CDC. The problems in the diagnosis of syphilis are:

- confusing clinical signs and symptoms
- lack of serological response in a patient with a clinically confirmed case of active syphilis (Hicks et al. 1987; Gregory et al. 1990)
- failure of nontreponemal test titers to decline after treatment with standard regimens
- unusually high titers in nontreponemal tests (Musher 1991), perhaps as the result of B-cell activation (Lane et al. 1983)
- rapid progression to late stages of syphilis and neurological involvement even after treatment of primary or secondary syphilis (Berry et al. 1987; Johns et al. 1987; Fernandez-Guerrero et al. 1988; Kase et al. 1988; Musher 1991; Gordon et al. 1994)
- disappearance of treponemal test reactivity over time (Haas et al. 1990).

Whether these problems in the diagnosis of syphilis occur more frequently in HIV-seropositive people than in HIV-seronegative people with syphilis is unclear. Aberrant results in the serological tests for syphilis appear to be related to abnormally low absolute CD4 cell counts and are relatively rare (Hicks et al. 1987; Gregory et al. 1990). The diagnosis of syphilis in these cases was supported either by an observation of *T. pallidum* in material from typical lesions or by the appearance of serological reactivity after treatment. The delay in development of a serological response to syphilis, theoretically, should be expected to be low (Rolfs et al. 1997). In addition, several studies (Gourevitch et al. 1993; Jurado et al. 1993; Rolfs et al. 1997) found that end-point titers in the nontreponemal tests are higher among HIV-infected people than among individuals not infected with HIV.

The failure of nontreponemal test titers to decline after treatment with standard therapy has been documented for HIV-seronegative people treated during latent-stage or late-stage syphilis and in people treated following reinfection (Fiumara 1979, 1980a; Romanowski et al. 1991). Therefore, the failure of titers to decline with treatment for syphilis in HIV-infected

people is probably related to the stage of syphilis rather than to HIV status.

Previously, infection with *T. pallidum* has been reported to be immunosuppressive (Jensen and From 1982; Pavia et al. 1976). Recently, Pope et al. (1994) reported that although the percentages of CD4 and CD8 lymphocyte subsets for people with syphilis were within the range of those percentages found in uninfected people, the percentage of CD4 cells were significantly lower ($p < 0.001$) and CD8 cells higher ($p = 0.03$) among patients with syphilis than in the uninfected population. Early findings in the study by Rolfs et al. (1997) indicated that infection with *T. pallidum* in HIV-positive individuals exacerbates the depletion of CD4 receptor cells.

The disappearance of reactivity in treponemal tests after treatment and over time has been reported prior to the identification of HIV-1. Schroeter et al. (1972) reported that 14 percent of the patients with early syphilis lost their reactivity in the FTA-ABS test within 2 years following treatment. However, further studies (Haas et al. 1990) found that specimens from 40 percent of men with AIDS and 7 percent of asymptomatic HIV-seropositive men with histories of treatment for syphilis became nonreactive in the treponemal tests, whereas the treponemal test results of the HIV-seronegative men remained reactive. Loss of reactivity in the treponemal tests was related to decreased total CD4 lymphocytes and CD4:CD8 ratios and a nontreponemal test titer of less than 1:32 at the time of treatment. In the study of Haas et al. (1990), none of the people had active syphilis. On the other hand, when people being treated for active syphilis (with and without HIV infection) were followed up over time (Gourevitch et al. 1993), 16 percent of the patients lost treponemal test reactivity following treatment. The loss of reactivity could not be related to HIV, CD4 count, or stage of syphilis. However, initial low nontreponemal test titer was weakly associated with the loss of treponemal test reactivity. Augenbraun et al. (1998) also found that HIV status did not affect loss of reactivity in the treponemal tests during the first year following treatment.

The literature abounds with descriptions of syphilis in so-called late stage forms in relatively young people who are HIV-seropositive (Johns et al. 1987, Dawson et al. 1988; Fernandez-Guerrero et al. 1988; Kase et al. 1988; Lanska et al. 1988; Radolf and Kaplan 1988), but neurological symptoms may be present among patients with secondary syphilis who are also HIV-seronegative (Merritt et al. 1946). One study (Lukehart et al. 1988) found indications of *T. pallidum* in the CNS of 40 percent of patients with early syphilis. However, no correlation was found between HIV infection and the invasion of the CNS since *T. pallidum* was demonstrated in the CSF of 27 percent of HIV-infected people and 32 percent of those in the study who were HIV seronegative.

TREATMENT AND ANTIMICROBIAL SUSCEPTIBILITIES

Penicillin and its derivatives are the preferred antimicrobial agents for the treatment of syphilis and the other treponematoses (for complete guidelines on treating syphilis see Centers for Disease Control and Prevention (2002) or www.cdc.gov/STD/treatment/). Early syphilis in immunologically competent patients can be treated effectively with a single dose of benzathine penicillin G. Neurosyphilis should be treated with either aqueous crystalline penicillin G or procaine penicillin, because benzathine penicillin does not achieve treponemicidal concentrations in the CNS. In immunologically normal patients, the organisms that remain following treatment are apparently cleared by the host, and the incidence of a relapse is low. Even though most HIV-seropositive people treated with standard regimens for syphilis appear to respond to treatment (Gourevitch et al. 1993; Rolfs et al. 1997), one cannot totally disregard the role of cell-mediated immunity in protecting the person against the progression of syphilis (Metzger 1976) or the interaction of an intact immune system with therapeutic agents (Fernandez-Guerrero et al. 1988). HIV-infected individuals may be susceptible to neurological or ocular complications of syphilis following benzathine penicillin treatment (Berry et al. 1987). Therefore, some authors have recommended that therapy appropriate for neurosyphilis (aqueous penicillin G or procaine penicillin with probenecid) be used for all patients coinfected with *T. pallidum* and HIV, and that HIV-positive patients be examined carefully for clinical signs of neurological involvement (e.g. CSF examination) before and after therapy (Berry et al. 1987; Hook 1989). Late-stage syphilis patients with evidence of neurosyphilis or other forms of active syphilis should have a CSF examination; if neurological involvement is found, some specialists recommend following the IV penicillin treatment with the recommended dose of benzathine penicillin appropriate for tertiary syphilis.

Effective therapy of early syphilis may result in a Jarisch–Herxheimer reaction consisting of fever and local intensification of lesions. This reaction, thought to result from the rapid release of treponemal antigens, occurs within the first 12 h of the initiation of therapy and usually resolves within 24 h. A Jarisch–Herxheimer reaction occurring in pregnant women may cause premature labor, fetal distress, or (rarely) stillbirth. However, the high probability of fetal damage resulting from syphilitic infection in the absence of adequate therapy outweighs this risk.

Doxycycline and tetracycline can be used as alternative therapies for the treatment of penicillin-allergic individuals. Ceftriaxone may be used to treat penicillin-allergic patients with neurosyphilis. However, there is the possibility that cross-reactivity between this anti-

biotic and penicillin may occur in the penicillin-allergic individual. In pregnant women, the only acceptable treatment is the penicillin regimen appropriate for the stage of syphilis. If penicillin allergy is documented by thorough questioning regarding the patient's history and by skin testing, the patient may be desensitized to permit use of penicillin therapy (Centers for Disease Control and Prevention 2002).

Although there are reports of the possibility of erythromycin-resistant strains of *T. pallidum* (Stapleton et al. 1985), antimicrobial susceptibility testing is not straightforward because there is no method for the continuous culture of *T. pallidum*. Various approaches have been used to determine the susceptibilities of representative strains (e.g. the Nichols strain) to antimicrobial agents, including in vitro loss of motility or infectivity, treatment of experimental animal infections, human trials, and examination of the susceptibility of nonpathogenic, cultivable treponemes (Rein 1976). Inhibition of protein synthesis and of in vitro multiplication in a tissue culture system has been shown to be an effective means of determining susceptibility (Stapleton et al. 1985; Norris and Edmondson 1988). In common with other spirochetes, *T. pallidum* was relatively insensitive to seven quinolones tested and to rifampicin. *T. pallidum* strains appear to be uniformly susceptible for penicillin and other β-lactams tested. Ceftriaxone, amoxycillin, and ampicillin are active against *T. pallidum* in vitro and also appear to be effective in the treatment of early syphilis. Amoxycillin and ampicillin offer no theoretical advantages over benzathine penicillin G. Ceftriaxone has a long serum half-life and reaches high levels in the CNS. However, the cost is high, multiple doses are required, and there is a 3–7 percent risk of adverse reactions in penicillin-allergic individuals; in addition, the data on efficacy in patients are limited. Therefore, ceftriaxone is not currently recommended as an alternative therapy for early syphilis. Recent studies have indicated that azithromycin may have utility in treating incubating syphilis (Hook et al. 1999) and early syphilis (Hook et al. 2002). However, there have been recent reports of Azithromycin resistance in *T. pallidum* so caution should be used when treating incubating syphilis using this antimicrobial (Lukehart et al. 2004).

Evaluation after therapy

To follow the efficacy of therapy, patients should be monitored to ensure that signs and symptoms have resolved and that the nontreponemal antibody titer has declined. To monitor the efficacy of treatment, quantitative nontreponemal tests should be performed on the patient's serum samples, which are drawn at intervals of 3 months for at least 1 year. Following adequate therapy for initial episodes of primary and secondary syphilis, there should be at least a four-fold decline in the VDRL titer by the third or fourth month and an eight-fold decline in titer by the sixth to eighth month (Brown et al. 1985). When the RPR card test is used to monitor treatment, the initial four-fold decline in titer may not be observed until the sixth or eighth month following treatment (Romanowski et al. 1991). For most patients treated in early syphilis, the titers decline until little or no reaction is detected after the first 3 years (Fiumara 1978, 1980b; Romanowski et al. 1991). Patients treated in the latent or late stages, or who have had multiple episodes of syphilis, may show a more gradual decline in titer (Fiumara 1979, 1980a). A low titer will persist in approximately 50 percent of these patients after 2 years. As far as can be determined, this persistent seropositivity does not signify treatment failure or reinfection and these patients are likely to remain serofast even if they are retreated.

RELATED TREPONEMES

Yaws, endemic syphilis (bejel), and pinta were common infections before the World Health Organization eradication program that began in 1948. In the early 1950s, it was estimated that more than 200 million people had been exposed to yaws. Areas where the nonvenereal treponematoses are endemic are now more restricted, but decreased surveillance has led to a return of these diseases in many areas. Therefore, the clinical appearance of the lesions formed by *T. pallidum* subspecies *pertenue*, *pallidum*, *endemicum*, and *Treponema carateum*, the anatomical location of the lesion, the mode of transmission, the age of the individual, and the geographical location of the infected individual are the only criteria that can be used to diagnose these infections as separate entities (Perine et al. 1984; Chin 2000) (Table 1). This is especially important because none of the serologic tests for syphilis can distinguish between the diseases caused by the pathogenic treponemes.

Bejel

The lesions of bejel (endemic syphilis), associated with primitive, crowded, and unhygienic conditions, develop after an incubation period of 2 weeks to 3 months. Mucous patches of the mouth usually occur first, followed by moist papules in folds of the skin, and drier lesions of the trunk and extremities. A primary sore is rarely evident. Both depigmentation and hyperpigmentation are common. The late manifestations include destructive lesions of skin, long bones, and nasopharynx similar to gummas of late syphilis (Perine et al. 1984; Chin 2000). However, neurological, cardiovascular, and congenital forms are rarely reported. The disease was once common among children living in poor socioeconomic conditions in the arid regions of the eastern Mediterranean, Asia, and Africa. Bejel still persists in

Chad, Sudan, and Ethiopia and rarely in southern Africa (Meheus and Antal 1992).

Yaws

The primary lesion of yaws, commonly referred to as the mother yaw, occurs following an incubation period of 2 weeks to 3 months. This initial lesion is a papilloma located on the face or extremities. The lesion may slowly proliferate to form a frambesial or raspberry-like lesion or the initial lesion may ulcerate. Secondary lesions of disseminated or satellite papillomata (daughter yaws) form in successive eruptions (crops), with the first crop appearing either before or shortly after the mother yaw has resolved. Other symptoms accompanying the secondary lesions include aching of long bones and inflammation of the fingers and toes. Papillomata and hyperkeratoses that form on the soles and palms are usually very painful and disabling. These lesions heal spontaneously but relapses may occur during the secondary or late phases. The late stage develops in approximately 10 percent of untreated patients. The stage is characterized by destructive lesions of the skin and bones (Perine et al. 1984; Chin 2000). Congenital yaws has not been documented; controversy exists over the occurrence of neurological and cardiovascular forms (Engelkens et al. 1991a). Today, yaws is primarily a disease of children living in the tropical areas of central and west Africa (Meheus and Antal 1992). Some small foci of disease remain in the Americas and on islands of the Caribbean (Meheus and Antal 1992).

Pinta

Perhaps the most distinctive lesions of the pathogenic treponematoses are observed in pinta. First, a primary scaling papule forms 1–8 weeks after infection on the hands, legs, or the back of the foot. Within 3 months to 1 year, a maculopapular, erythematous secondary rash appears that may evolve into tertiary splotches of altered skin pigmentation. Initially the macules are blue, progressing to violet, and then brown, and finally becoming depigmented after months to years. Treponemes are found in the pigmented lesions; the achromic scars are usually free of treponemes. The lesions of the secondary rash most commonly progressing to the late form are frequently found on the face and extremities; lesions in different stages of pigmentation can be observed simultaneously (Perine et al. 1984; Chin 2000). Systemic infection with organ or bone involvement apparently does not occur. Pinta is predominantly a disease found among older children and adults living in the Indian tribes of Mexico and the Amazon basin (Meheus and Antal 1992). Because of the location of the primary lesion, trauma is suspected of providing the portal of entry for the treponemes. Direct and prolonged contact with initial, secondary, and early tertiary lesions appears to be necessary for transmission to take place; however, the mode of transmission is not entirely clear (Engelkens et al. 1991b).

Because yaws, pinta, and nonvenereal syphilis are often childhood diseases, the nontreponemal test titers of adults are expected to be $\leqslant$1:8 for those from a geographical region where endemic treponematoses were virtually eliminated by mass campaigns in the 1950s and 1960s (Antal and Causse 1985; Meheus and Antal 1992). Therefore, any titer >8 is suggestive of venereal syphilis for adults from these regions (Gershman et al. 1992; McDermott et al. 1993). Likewise a titer $\geqslant$8 in a child suggests a resurgence of endemic treponematoses in these particular areas (Perine et al. 1985).

Oral spirochetes

Various spirochetes inhabit supragingival and subgingival plaque in humans; only a small proportion of these have been cultured in vitro and characterized (Choi et al. 1994). Those that have been cultivated and identified to species level are within the genus *Treponema*. Although the species differ from one another slightly in terms of cell diameter and helical configuration, it is generally not possible to identify them on morphological grounds. Biochemical parameters such as growth requirements, carbohydrate fermentation, and enzymatic activities have been used for identifying species (Smibert et al. 1984).

The oral spirochetes are difficult to isolate from healthy gingiva, but they increase both in prevalence and in numbers of organisms present in patients with gingivitis or periodontal disease. Treponemes are detected in subgingival plaque of 88–97 percent of patients with periodontal disease (Moore et al. 1987). *Treponema socranskii* is the most common isolate, followed by *T. denticola* and *Treponema pectinovorum*. The procedures for isolation and characterization of oral spirochetes have been described previously (Smibert and Burmeister 1983; Smibert 1984; Smibert et al. 1984; Moore et al. 1987; Miller et al. 1992). Pathogen-related oral spirochetes (PROS) were found in plaque samples from 11 of 17 patients with necrotizing ulcerative gingivitis and from 10 of 19 patients with periodontitis, but were absent from 24 healthy subjects (Riviere et al. 1991a, 1991b, 1991c; Simonson et al. 1993). PROS have not been cultured in vitro and are identified by their reactivity with monoclonal antibodies specific for *T. pallidum* antigens, including the 35-kDa flagellar sheath protein and a major 47-kDa lipoprotein. Gingivitis and periodontis patients also expressed IgG antibodies reactive with 47-, 27-, 14-, and 12-kDa *T. pallidum* subspecies *pallidum* antigens, whereas a smaller proportion of

healthy subjects had detectable antibodies against only the 47- and 37-kDa antigens (Riviere et al. 1991c). PROS were found in periodontal tissues of ulcerative patients (Riviere et al. 1991b, 1991c) and were capable of migrating through a mouse abdomen wall barrier, an invasive property shared with *T. pallidum* but not with the cultivable oral treponemes (Riviere et al. 1991a). Oral spirochetes reactive with anti-*T. pallidum* monoclonal antibodies were also detected in gorillas and dogs (Simonson et al. 1993). Further studies are in progress to establish the taxonomic relationship between PROS and other spirochetes and to determine whether PROS represent frank pathogens.

Nonpathogenic spirochetes of the genital region

T. phagendenis, *T. refringens*, and *Treponema minutum* are treponemal species that inhabit smegma (sebaceous secretions and desquamated epithelial cells), which is found beneath the prepuce and in other epithelial folds of the genital region. Although *T. phagendenis* and *T. refringens* have been shown by DNA–DNA hybridization to have $\leqslant$5 percent homology with *T. pallidum* (Fieldsteel 1983), comparison of 16S rRNA sequences has indicated that they are related to the pathogenic treponemes. These harmless flora could potentially be misidentified as *T. pallidum* in darkfield preparations from skin sites. Unlike the pathogenic treponemes, these non-pathogenic treponemes are readily cultured (Miller et al. 1992).

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Leptospira

BEN ADLER AND SOLLY FAINE

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THE GENUS *LEPTOSPIRA* AND ITS SPECIES, AND THE ASSOCIATED GENERA *LEPTONEMA* AND *TURNERIA*

The genus *Leptospira* comprises morphologically similar thin helical bacteria (spirochetes). The trivial name used for all members of the genus is leptospira or leptospire (plural leptospires). All are similar morphologically and culturally, but can be grouped serologically by agglutinating antigens into characteristic serovars. More than 200 serovars are recognized. One major serological complex containing most of the leptospires known or suspected to be pathogenic was named the 'interrogans complex'. Later, it became the broad, serologically determined species '*Leptospira interrogans*'. The other major group, containing nonpathogenic leptospires, was previously known as the 'biflexa complex' and was later designated '*L. biflexa*'. The genera *Leptonema* and *Turneria* were first recognized because the type strains were morphologically different from other leptospires. DNA homology studies justified this classification. The leptospires were comprehensively reviewed by Faine et al. (1999).

Definitions

FAMILY AND GENUS

The family Leptospiraceae includes the genera *Leptospira*, *Leptonema*, and *Turneria*, a group of bacteria that separated very early in bacterial evolution (Paster et al. 1984, 1991; Paster and Dewhirst 2000). Characteristically, all members are motile, flexible, helical aerobic bacteria, 6–12 μm long or longer and 0.1 μm in diameter (Johnson and Faine 1984a, b). There is no constant morphological characteristic of each genus, but *Turneria* tends to be shorter. Typically, one or both ends are hooked, but straight variants occur. The helical cell body (cytoplasmic cylinder) is wound around a pair of periplasmic flagella arising from it subterminally, one at each end. The diamino acid in the peptidoglycan is α,ε-diaminopimelic acid. Long chain fatty acids or fatty acid alcohols are used as carbon and energy sources. The guanine + cytosine (G + C) ratio is 35–53 mol%. The formal description of the genus *Leptospira* states that it is gram-negative but difficult to visualize, and oxidase-positive and chemoorganotrophic. It also includes some of the further

information discussed below (Faine and Stallman 1982; Johnson and Faine 1984a).

THE SPECIES OF *LEPTOSPIRA*

The species of leptospires are differentiated by DNA homology under high stringency conditions. Currently, ten named species of leptospires (*alexanderi*, *borgpetersenii*, *fainei*, *inadai*, *interrogans*, *kirschneri*, *meyeri*, *noguchii*, *santarosai*, and *weilii*) are recognized in the 'pathogenic' group, whereas the three species *biflexa*, *hollandia*, and *wolbachia* belong to the saprophytic, 'nonpathogenic' group. In addition, there are at least four species not yet formally named (Brenner et al. 1999). The choice of the names 'interrogans' and 'biflexa' for species of leptospires was unfortunate and their use for both the former serologically defined species and the current genetically determined species is confusing. The few recent comprehensive reviews of the nature and significance of the members of the genus *Leptospira* include those of Adler and Faine (2002), Faine et al. (1999), Levett (2001), Torten and Marshall (1994), and Weyant et al. (1999).

INTRODUCTION AND BRIEF HISTORICAL PERSPECTIVE

Introduction and general perspective

Pathogenic leptospires are important primarily for their ability to cause disease in humans and animals. Nonpathogenic leptospires need to be differentiated from pathogens to avoid confusion in diagnosis and epidemiology. Scientifically, the leptospires are intrinsically important because they are phylogenetically an ancient group of bacteria, unique in their structure, metabolism, and some of their genetic mechanisms.

Leptospirosis is a potentially fatal disease of humans or animals caused by one of the pathogenic leptospires. It is prevalent globally as an acute febrile, sometimes congenital or chronic infection of wild or domesticated animals, mainly mammals and marsupials. Chronically infected animals are survivors of an initial acute infection. Leptospires persist in the renal tubules of kidneys in carrier animals, whence they are excreted in urine into the environment to contaminate water and soil, leading to infection of further animals of the same or different species. In some domestic animals genital carriage and sexual transmission are important. Humans are infected incidentally, affected by an acute febrile, sometimes fatal, clinical illness. Sometimes the disease is subclinical. Humans do not usually become chronic carriers or transmit infection to other humans or animals. Economic loss occurs through animal disease and mortality, loss of productivity, and illness or death in humans. There is a significant social cost of the disease both in humans and animals. Immunity to reinfection with the same serovar or closely related serovars follows infection. Significant topics for microbiological study of leptospires related to the control of leptospirosis include rapid diagnosis, mechanisms of attachment to and persistence in or on tissue cells in chronic carrier infection, and the identity and regulation of antigens important for immunity.

History of the leptospires

The history of leptospires and leptospirosis was reviewed extensively by Faine et al. (1999). Leptospirosis was described as a characteristic febrile jaundice of humans (Weil's disease) during the latter part of the nineteenth century. Its contagious nature and microbial origin were proved independently first in Japan by Inada and colleagues ('*Spirochaeta icterohaemorrhagiae*') in 1915 and soon after in Germany ('*Spirochaeta icterogenes*') by Uhlenhuth and Fromme; both groups isolated, cultivated, and described pathogenic leptospires. Leptospires had been seen, but not cultured, and named *Spirocheta interrogans* by Stimson as early as 1907, in silver stained preparations of liver from a patient believed to have died of yellow fever, whose viral origins were then unrecognized. The patient really had Weil's disease. Later, a saprophytic leptospira found in fresh water was described in 1914 and named *Spirocheta biflexa* but it was not cultivated. Noguchi proposed the name 'leptospira' ('thin spirals') in 1918 following detailed microscopic and cultural observations, believing it to be the cause of yellow fever. Controversy about the role of leptospires in yellow fever persisted until the yellow fever virus was identified and incriminated unequivocally. Microscopically and culturally identical, but antigenically different, leptospires were at first regarded and named as separate species, but later included as serotypes or serovars of a single genus, *L. interrogans*. In the 15 years or so from discovery until the 1930s, many of the important serovars prevalent throughout the world, and their host sources, were discovered (Kmety and Dikken 1993).

Most of the research on leptospires in the next two decades related to the discovery of new serovars, some of them responsible for milder anicteric types of leptospirosis. The low yields from the culture media containing 10 percent rabbit serum were significant impediments to serious research on leptospires as bacteria. There was little progress and much frustration in attempts to understand nutrition or the composition of antigenic fractions, compared with knowledge of other bacteria. Electron microscopy revealed much of the detail of structure during the 1960s and 1970s. The analysis of antigens did not escape from the net of serological classification until Yanagawa and Faine (1966) showed that leptospires were analogous to other bacteria in structure, and that characteristic antigens were

associated with structural elements. A full account history of research in leptospirosis is recounted in Faine et al. (1999)

In the USA, widespread leptospirosis of cattle and pigs due to '*L. pomona*' stimulated significant research into nutrition and cultivation of leptospires, much of it emanating from the laboratory of J.B. Wilson in Wisconsin during the 1960s, and from his former students thereafter. The most important single advance was probably the discovery that the only carbon and energy sources were the long-chain fatty acids essential for growth. They were toxic and had to be presented in a nontoxic form, detoxified by serum or serum albumin or another detoxicant in the medium. Bovine albumin oleic acid culture media improved yields up to 100 times those in rabbit serum medium, especially with aeration, and also allowed the isolation of some strains unable to grow in rabbit serum media. A profusion of isolates of many previously unknown serovars of leptospires necessitated correct identification for diagnosis, prognosis, and epidemiology, and for the development of vaccines. Consequently, leptospirosis researchers developed a preoccupation with serological classification, based only on absorption and cross-agglutination of antisera (Abdussalam et al. 1972). 'Factor analysis' (Kmety 1967) using batteries of absorbed sera to simplify identification was seldom used as it appeared to be more complex than the standard methods. ELISA methods were developed to analyze nonagglutinating as well as agglutinating antigens (Adler et al. 1980) and applied with monoclonal antibodies to identify epitopes involved in immunity, or useful for classification (Adler and Faine 1983; Kolk et al. 1984). For many years leptospires were classified in either the nonpathogenic group as '*L. biflexa*' or the pathogenic group as '*L. interrogans*', the latter term adapted from Stimson's description with the purpose of indicating doubt about the ultimate classification. The current system of genetic classification was adopted in 1994 (Abdussalam et al. 1965, 1972; Ellis 1995).

Much of the work on leptospires and leptospirosis has been supported or stimulated not by physicians or microbiologists but by veterinary public health sources and veterinarians, because the economic consequences of leptospirosis are loss of animal productivity and human food supplies, as well as zoonotic risks to humans. Historically, important developments in the last 20 years dealt with in the text below include: the lipopolysaccharide (LPS) and the LPS derivation of the antigens involved in immunity, and some of the partly characterized epitopes; cloned genes coding for outer membrane and other important proteins; genetic studies of special features of the leptospiral genome. Molecular techniques for identification have allowed genetic speciation on firm grounds and polymerase chain reaction (PCR) methods have been developed for identification and diagnosis.

HABITAT

Leptospires are motile aquatic bacteria whose natural habitat is surface waters, soil, and mud. They feed by attachment to surfaces where long-chain fatty acids are available (Kefford and Marshall 1984). A halophilic leptospire has been isolated from estuarine waters. Leptospires do not survive drying under usual conditions, but lyophilization can preserve them. Pathogenic leptospires enter their hosts through the integument in moist conditions. In the host they circulate and grow in an aqueous milieu and may attach to renal tubular cells in carrier animals, passing in urine into surface waters. Pathogenic leptospires adapt to environmental conditions including the salinity and temperatures of febrile mammals, renal tubular and bladder urine, and soil and surface water. Both pathogens and nonpathogens have been isolated from fast-flowing rivers and still surface ponds. Soil and subsoil type and structure are important for survival in dry periods (reviewed by Faine et al. 1999).

MORPHOLOGY AND VISUALIZATION

Leptospires are helical gram-negative bacteria, so thin that they are hard to see when fixed and stained by conventional bacteriological stains. Their characteristic shape is largely destroyed by heat fixation. Strong stains (carbol fuchsin) and stains that build up on the surface (flagellar stains, Giemsa, silver stains, immunostains) make them appear thicker and improve visualization. Electron microscopy reveals details of morphology. Phase contrast of live cultures is of limited value because the leptospires move rapidly out of phase. The routine method of observation is dark field microscopy of wet preparations. Leptospires pass through bacteriological filters of average pore diameter 0.2 μm.

Shape and structure

The leptospiral cell (Figure 1) is about 10–20 μm long. It comprises a peptidoglycan complex loosely

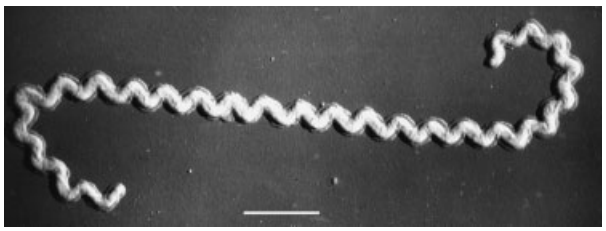

Figure 1 *Shadowed electron micrograph of a typical leptospire (*L. interrogans *serovar* Copenhageni*). The flagella run in grooves in the wall of the helical cylinder from a subterminal insertion near either end. The helical cylinder is surrounded by an outer envelope. Initial magnification ×40 000. Bar = 1.0 μm. (Original electron micrograph by Dr A. Chang)*

referred to as cell wall arranged as a flexible hollow tubular right-handed helix, containing cytoplasmic and genetic material. The amplitude of the coil is about 0.10–0.15 μm and its wavelength is about 0.5 μm. *Leptonema* is generally longer (13–15 μm) than *Leptospira* and has a longer average amplitude of 0.13 μm and wavelength of 0.6 μm; the cells thus appear wider microscopically. By contrast, *Turneria* is shorter (3.5–7.5 μm long) and more tightly coiled (wavelength 0.3–0.36 μm and amplitude 0.13–0.14 μm). Subterminally at either end of the helix a flagellum, essentially similar in structure and function to other bacterial flagella, is inserted in the cell wall and penetrates to the outer aspect of the helix, where it runs through the interior of the coil in a groove to end at about the middle of the leptospire. The two flagella seldom overlap. The flagellar origin appears as a visible bulge below the surface in shadowed electron micrographs. The whole is surrounded by an outer membrane, separated from the cell by a periplasmic space. In stained cross-sections in electron micrographs, the concentric layers are, from the outside, the outer membrane, the periplasmic space, the peptidoglycan, the inner membrane, and the cytoplasmic contents. The flagellum, when included in the plane of the section, is a darkly staining round structure lying in an indentation of the cell wall. Negative stained electron micrographs have revealed more of structure than have shadowed preparations. Cultural conditions and technical procedures affect the appearances of the leptospiral surface on electron microscopy; washing removes much of the outer membrane. There is an unusually high lipid content in leptospires, located mainly in the cell wall or outer membrane. When treated with various detergents or lysozyme the cell relaxes its helical shape and sheds its outer envelope. Gradually, more of the surface layers dissolve as the helix finally flattens in an empty flaccid sac, liberating detectable antigens in the process (Yanagawa and Faine 1966).

SPHEROPLASTS (LEPTOSPIRAL GRANULES)

In some cultures leptospires appear in a 'granular' form. The granules are small cystic structures, about 1.5–2.0 μm in diameter, inside which coiled remnants of the leptospiral cell can be seen. Once thought to be a cystic stage of a life cycle, they are really spheroplasts whose formation can be induced with ethanol, detergents, sodium deoxycholate, high salt conditions, lysozyme or mild heat. They can also be observed in tissues and in phagocytes (Faine et al. 1964). The outer envelope surrounding the spheroplast granules may be removed from them with its antigens largely intact after plasmolysis of the leptospires. Some cultures tend to form spheroplasts spontaneously; these strains probably produce lipase that releases from the medium free, lytic fatty acids not adequately bound to a detoxicant (Figure 2).

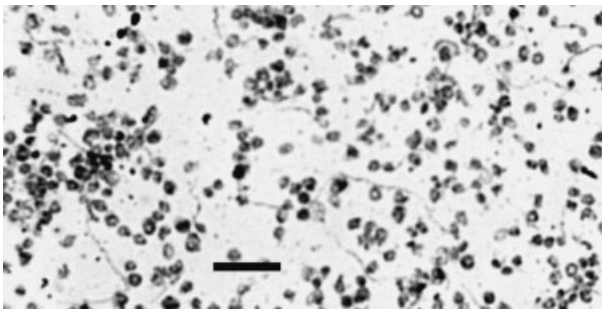

Figure 2 *Spheroplasts ('leptospiral granules') of* L. interrogans *serovar* Copenhageni *formed by heating a culture to 56°C. Thread-like degenerated leptospiral bodies can be seen towards the middle and the lower left corner. Stained with silver stain. Original magnification ×1 000. Bar = 15.0 μm. Adapted from (Faine et al. 1964)*

Flagella

Leptospiral flagella originate in a disc rotor and hooked proximal end structure in the cell wall, similar to other gram-negative bacteria. Flagella may be prepared for study by shearing them off leptospiral cells followed by differential or gradient centrifugation. In such preparations the flagella are seen as tight flat coils. The isolated flagellum is composed of a helically wound central core of proteins, surrounded by an outer sheath. The hook end is usually free of the rotor but may be contaminated with a bleb of lipopolysaccharide at its proximal end. There are associated flagellar antigens. Seven different flagellar proteins have been recognized. There is a 34 kDa protein associated with the core, of 11.3 nm, and a 36 kDa protein associated with the sheath, measuring 21.5 nm. A gene for an endoflagellar subunit protein, *flaB*, was cloned from *L. borgpetersenii* serovar Hardjo, expressed in *Escherichia coli* as a 32 kDa FlaB protein similar in sequence to FlaB proteins described in *Treponema pallidum* (Kelson et al. 1988; Mitchison et al. 1991; Trueba et al. 1992) The FlaB protein structure and its gene sequences are highly conserved throughout the species; a recombinant protein cross-reacted between serovars in immunoblots (Woodward and Redstone 1994). Characteristic flagellar antigen(s) associated with the flagellar sheath (Chang et al. 1974) were subsequently identified as the FlaA protein, which affects flagellar helicity in *Brachyspira* (Li et al. 2000a) and probably in *Leptospira*.

Microscopic appearances

When seen by direct dark field microscopy of wet preparations under optimum conditions, leptospires appear as a series of bright dots arranged in a flexible chain. Typically they are translationally motile and spin fast but spasmodically on their long axes; the loose hooked end looks like a hollow bulb. Occasionally

leptospires bend suddenly along their length in a jerky movement. There are also straight mutant forms, whose flagella are inserted closer to the ends of the cells and which are much less translationally motile, but spin as well as the hooked variety (Figure 3).

It is convenient to observe cultures microscopically in flat drops on a very clean thin slide, without a cover-glass, using a low power, long working distance objective and a low-power dark field condenser of low numerical aperture. For detailed observation of leptospires, a very clean thin slide is required; a drop of the fluid for examination is placed on it and covered with a thin, very clean cover-glass. Any dirt or grease or smear will cause diffraction of light and destroy the contrast required for the darkfield illumination. It is useful but not necessary to seal the edges of the cover-glass to prevent movement in the preparation through convection and evaporation at the edges. The slide is sealed to the surface of an oil immersion darkfield condenser with nonrefracting microscope immersion oil to prevent loss of light by diffraction at air–glass interfaces. An oil immersion objective fitted with a central stop, or a diaphragm to reduce the numerical aperture below that of the condenser is required. Centering of the light path and focusing the condenser are critical for successful observations. After locating the field under low power using dry objectives, a drop of immersion oil is placed on the cover-glass and the oil immersion objective used to focus on the leptospires in the field. Faine et al. (1999) described the technical procedures in detail.

Motility

Leptospires are motile bacteria; generally an immobile leptospire is dead. The characteristic motility of leptospires results from the rotation of the flagellum, generating a helical wave that propels the helical cell in the opposite direction. The precise mechanisms by which flagella generate motion may well vary in different spirochetes (Li et al. 2000b). A mutant of *L. biflexa* lacking flagella retained its helical shape and viability (Picardeau et al. 2001). In fluid media, leptospires can travel about 7–10 μm in a second. In viscous fluid media the speed of movement is increased in proportion to the viscosity. Leptospires are attracted to more viscous environments by 'viscotaxis'.

Recently isolated leptospires growing in media containing blood or serum appear to move faster than laboratory strains, possibly because of the viscosity of the medium. In semisolid agar, in which there are microscopically invisible particles, or in tissues, leptospires can become completely flexible throughout their length and wind through the interstices of the medium in either direction. They resume a rigid conformation and rapid spinning and translational movements as soon as they reach the fluid medium; part of the leptospira can be rigid and rotating while the other end is flexed around particles.

CULTURAL CHARACTERISTICS AND GROWTH REQUIREMENTS

Leptospires are grown routinely in liquid media, occasionally slightly thickened with 0.1–0.2 percent agar. Generally an inoculum of 1–10 percent of the volume of fresh medium is transferred by pipette. Working with liquid media demands the utmost care in their sterilization and quality control and in aseptic techniques, to prevent contamination by extraneous air-borne or filterable water-borne microorganisms, including other leptospires. Colonial growth in solidified media in plates or tubes is primarily employed for research.

Cell division

The stages in division in leptospires can be reconstructed from electron micrographs and direct observation. First, the cell wall constricts about the middle of the length,

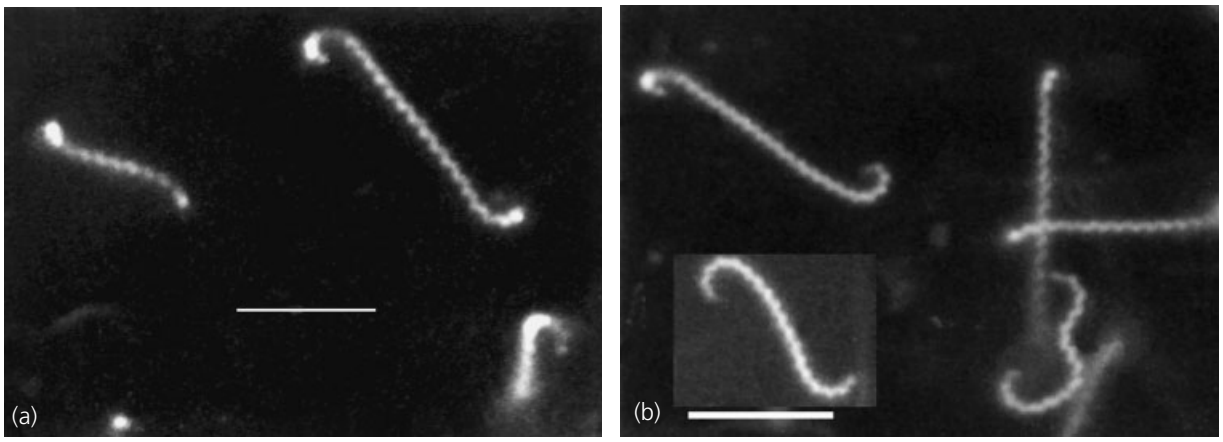

Figure 3 *Typical leptospires (*L. interrogans *serovar* Copenhageni*) seen by high-power dark field microscopy. Initial magnification ×1 000. Original photomicrographs by Drs T. Vinh and S. Faine. Bar = 5.0 μm. Adapted in part from Faine et al. (1999)*

beneath an otherwise intact outer membrane, resulting in fusion of the closed ends to form two new ends, one for each of the new cells. Near the constriction, a new flagellar origin appears from which a flagellum grows towards the middle of the newly formed cell. Last, the outer membrane constricts and pinches off a discrete new cell. About the time of division, the leptospire becomes hyperflexible at the site of future separation; it may bend sharply on itself, while retaining its motility, until the two new cells separate. In media deficient in amount or balance of long-chain fatty acids, leptospires may elongate extremely, or fail to divide (forming long chains), or both. Clearly the nutritional needs for growth (elongation) and division are different. Nothing is known about metabolic or molecular events in cell division specific for leptospires.

Optimum conditions: temperature, pH, osmolarity, and other conditions

TEMPERATURE

Both pathogens and saprophytes grow in the laboratory in an optimum temperature range of 28–30°C, with extremes of 11–42°C. Growth at 11–13°C has been proposed as a phenotypic test for *L. biflexa*. Pathogens grow in mammalian hosts at febrile body temperatures, and in chick embryos and young chicks around 40–42°C, but do not grow well in laboratory media at these temperatures unless they are adapted. In response to a change in temperature, leptospires upregulate the expression of a range of proteins, including homologs of common heat shock proteins in other bacterial species (Ballard et al. 1993, 1998; Nally et al. 2001a, b; Stamm et al. 1991).

pH

Leptospires die in acid conditions at pH below about 6.5, but tolerate more alkaline conditions up to about pH 8.4. Cultures will grow in the range pH 7.2–7.6.

OSMOLARITY

Most of the culture media for leptospires contain a final molarity of salts of about 0.05 M, about one-third of the concentration in other bacteriological media. Pathogens can grow in laboratory media at salt concentrations of 0.85 percent NaCl at 37°C but avirulent pathogens and nonpathogens cannot do so above 30°C (Faine 1959). Halophilic leptospires require sodium ion concentrations of 0.22–0.44 M for growth and can tolerate concentrations up to 0.65–0.75 M (Cinco et al. 1975).

OXIDATION–REDUCTION POTENTIAL

Leptospires are aerobic bacteria that do not tolerate reducing conditions or anaerobiosis, when the oxidation–reduction potential is less than about *E*h - 0.250 mV at pH 7.2.

Growth

Leptospira strains grow slowly, compared with most common pathogenic bacteria; colonies can take from 3–7 days to 3 weeks to appear. Some strains may not grow at all on solid media. The slow growth rate in solid media limits the use of plate counts to measure viability and impedes the rate of progress in molecular studies.

Leptospires grow in still cultures of liquid media at 30°C with a doubling time of 6–8 h under optimum conditions. A 10-fold increase in numbers can be achieved in 24 h. Aeration or agitation increases the yield. Maximum growth can be expected in 3–10 days, depending on the strain and the medium. Maximum growth densities are about 5×10^9–10^{10} leptospires per ml, with best conditions and aeration; cultures are then visually turbid but measurable turbidity is relatively low. *Leptonema* grows rapidly in trypticase–soy broth (TSB) medium to reach maximum density on incubation for 18–72 h at 30°C. The fatty acid concentration is the limiting factor for continuation of growth in cultures of *Leptospira*. Supplementary Tween added to cultures that have exhausted all their available fatty acids will considerably increase the final density. Some cultures die rapidly after the end of the logarithmic phase, because lipases from autolysing leptospires liberate lytic free fatty acids. The doubling time for growth in the intact animal body in experimental infections was 6–8 h.

Growth is measured in liquid media by either direct counts or turbidity. The best way to count leptospires is to use a bacterial counting chamber, or to count them in a measured volume placed under a standard size coverglass, using a calibrated eyepiece graticule micrometer. The culture must be diluted accurately if it is too dense. Motile leptospires are counted as viable; nonmotile and obviously dead and degenerate leptospires are not counted. Motility is seldom so fast that it creates a problem. The usual conventions for counting in counting chambers are observed in relation to leptospires on or overlapping lines (Faine et al. 1999). Leptospiral cultures are much less turbid than those of other bacteria at peak growth. Turbidity measurements are limited in value to estimating the total count, irrespective of viable numbers, in heavily growing cultures at the end of their logarithmic phase, at densities above about 10^8 leptospires per ml, when growth is just easily visible by eye. Nephelometry is more sensitive but limited to cultures denser than 5×10^7 leptospires per ml.

Growth requirements

ATMOSPHERIC CONDITIONS; O_2, CO_2 AND AERATION

Leptospires require an oxygen source, but a measured lower limit of oxygen concentration seems not to have been established. It is assumed that animal tissues in

which leptospires grow and apparently flourish are lower in oxygen than culture media, especially in the presence of inflammation, hemorrhage, cellular damage, and autolysis, and tissue disorganization during the acute stages of infection. Aeration of cultures by sparging or shaking increases growth, although it can cause problems with frothing in protein-rich media. CO_2 is essential, but the limiting amounts are not known.

CARBON SOURCES

Pathogenic leptospires will not grow without long-chain ($C_{12–18}$) unsaturated fatty acids, in whose presence some strains can also utilize saturated fatty acids. *Leptospira biflexa* strains can grow on long- or short-chain, saturated or unsaturated fatty acids. Some pathogens may start to grow sooner from small inocula, especially on solid media, if pyruvate, pyruvate and CO_2, or glycerol are added to the medium (see section on Growth characteristics below). Knowledge of nutritional requirements comes mainly from studies on relatively few isolates made at a time when it seemed that information relevant to any leptospire could be extrapolated to all. Differences in nutrition and metabolism between leptospires may be related more to species than to serovar.

The form of presentation of the fatty acids is important because they are potentially toxic to the leptospires, lysing the cells by destroying the integrity of the outer membrane. Nontoxic 'Tweens', used widely in media as sources of fatty acids, are commercially available sorbitan monoesters of fatty acids; Tween 80 contains oleate, Tween 60 stearate, and Tween 40 palmitate. Their crude industrial form may need to be purified of contaminating free fatty acids and degradation products before use in media. The choice of the chain lengths of the fatty acids and their relative concentrations depends on the strains to be cultivated. In addition, a detoxicant such as bovine serum albumin (BSA), in a final concentration of 1.0 percent, is used to absorb and release fatty acids slowly. BSA may also itself be contaminated with free fatty acids and may need to be purified for use. Likewise, agar used in media may contain contaminating fatty acids which must be removed before use. *Leptonema* will grow without added fatty acids in TSB medium.

NITROGEN SOURCES, PURINES, AND PYRIMIDINES

The only essential nitrogen source is ammonia. Some strains metabolize amino acids, probably producing ammonia by deamination. Urease, inhibited by ammonia, has been described. All *L. biflexa* and other nonpathogens and *Leptonema* can synthesize their own purines and pyrimidines. *Turneria parva* requires purines. In general, all *L. borgpetersenii* and *L. santarosai* and some strains of *L. interrogans* synthesize purines, whereas all the pathogen group require pyrimidines.

OTHER REQUIREMENTS FOR GROWTH

Phosphates, sulfates, ferric iron or hemoglobin (or heme), calcium and magnesium, thiamine and cyanocobalamin are essential. Traces of copper and manganese are also required; these are included in the recipes for oleic–albumin based media, while in serum media they are provided from tap water, serum or other ingredients.

Growth characteristics and appearances of cultures

IN LIQUID MEDIA

Cultures of leptospires in liquid media appear visually clear until densities of about 10^7 per ml are reached. The first appearance of growth is a birefringence on swirling the culture vessel, best seen with oblique lighting against a dark background. Maximum turbidity, well below that achieved by larger bacteria, is reached after about 3–20 days of incubation, depending on the inoculum size, the strain and its adaptation to the medium and conditions, and the growth phase at the time of subculture. There may be a long lag of several days before freshly isolated strains begin to grow. Some cultures autoagglutinate; clumps of actively motile tangled leptospires sink to the bottom of the vessel. Otherwise, leptospires do not settle during cultivation, but precipitates of insoluble salts, fatty acids or amino acids can occur and be a nuisance. Removal of leptospires from cultures for preparation of antigens or for other laboratory manipulations requires a centrifugal force of about 10 000 $\times$ *g* for 15–20 min.

IN SEMISOLID MEDIA

When growing in tubes of media containing agar (0.1 percent or more) after surface or stab inoculation, the distribution of leptospires is restricted mainly to one or more disks 0.1–0.5 mm thick that appear 0.1–5.0 mm below the surface of the medium, although leptospires will often be detected below the disks.

The disks, known as Dinger's disks, represent colonial growth accumulated at a level of optimum oxygenation. If one of the redox indicators 1-naphthol-2-sodium sulphonate indo-2,6-dichlorophenol (*E*h – 0.180 mV at pH 7.2) or 2-6-dichlorophenol-indophenol (*E*h – 0.200 mV at pH 7.2) is incorporated in the medium at a concentration of 1 in 100 000, the blue color of the medium when inoculated discolors around the areas where growth begins, usually at the level where the disks will appear later. Where growth occurs only in disks, the medium gradually decolorizes or changes color from the bottom of the tube upwards as oxygen is absorbed, until the zone below the disks is pale yellow or pink. The medium above the disks remains blue. If single colonies develop in the depths of the medium, the dye fades and becomes pale yellow or pink at the place in the tube

where the gray spherical colonies will later appear. Both disks and colonies may develop in the same tube.

IN AND ON SOLID MEDIA

Leptospires are usually grown on solid media only for research because growth is too slow to be useful for routine diagnosis. Some isolates grow on the surface of media containing 1.5–2.0 percent agar in petri dishes. The agar must not be too stiff for the leptospires to move through it easily and the plates must be sealed or otherwise protected from drying during incubation. Inoculation of a drop with a spreader or by flooding is better than scratching the agar surface when streaking it with a wire loop. Surface colonies are transparent, often very flat, small (less than 2 mm in diameter), and irregular. In less solid media containing 0.8–1 percent agar, growth occurs below the surface, first visible after 3–10 days as a pinpoint hemispherical colony that spreads centrifugally. Colony appearances depend on the density and depth of the agar and the motility of the leptospires. Straight leptospires, not very translationally motile, form small dense gray spherical subsurface colonies (small, opaque, defined border or 'SOD') that do not spread and reach a diameter of 1–3 mm. Leptospires that are conventionally translationally motile may form a larger, fluffy colony (sometimes called hazy growth, or a large, opaque, hazy edge or 'LOH') that may spread rapidly to form a gray ring with a sharply defined edge ('LOD'); this ring may grow wider to occupy a large part of the agar. In a thin layer of medium (2–3 mm) the colony soon looks like a ring of gray with a transparent center and sloping sides, because the medium is not deep enough for continued hemispherical growth. Details are described by Faine et al. (1999). The gray edge indicates a mass of actively motile leptospires; few leptospires, most of them nonviable, will be found internal to the edges of the ring. Incubation to detect growth should be continued for up to 3 weeks. More than one colony type can occur on the same plate. If redox dyes are added to the medium as described above, the color in the medium fades at the points where colonies will appear, forecasting the initiation of colonial growth. A subculture may be taken carefully with a pipette from the spreading edge of the colony. Contaminated cultures or environmental specimens inoculated as a discrete drop on the surface at the centre of the plate can be purified by subculturing leptospires from the edge of the ring of growth.

Culture media

Almost invariably, media contain heat-labile proteins or other ingredients and are sterilized by filtration. Either rabbit or other animal serum, or BSA forms the basis of current culture media. Yields are higher and growth faster in oleate–albumin media. Media free of protein are preferable and available for vaccines and certain other purposes; it is impossible to wash away the last traces of proteins from the culture medium adhering to the leptospiral surface. Liquid media are used for the maintenance of stock cultures and the propagation of cultures used to prepare vaccines and antigens for serological tests.

TECHNICAL NOTES, FORMULATIONS, AND PREPARATION OF MEDIA

Generally, leptospires are grown in tubes of flasks at 28–30°C. The depth of medium in unshaken tubes or flasks should be less than 5 cm; shaking, agitation or sparging with air or oxygen plus CO_2 (with care to minimize frothing) will maximize the yield and growth rate. Cultures should be checked for growth or contamination after 3–4 days and subcultured after 7–21 days; however, leptospires will survive in sealed tubes of medium at room temperature (ideally 20°C, certainly below 40°C) for months or years.

Oleic acid–albumin (OA) media and serum media

Details of composition and preparation of media are found in Alexander (1991) and Faine et al. (1999). All media are hypotonic with respect to mammalian physiological conditions. The most widely used OA medium is EMJH (Ellinghausen, McCullough, Johnson, Harris), based on 1 percent BSA and Tween 80. A BSA stock solution containing other mineral and heat-labile ingredients that should not be autoclaved is sterilized by filtration and added to an autoclaved phosphate-buffered basal medium. Most serum media use a phosphate-buffered base containing a small amount of bacteriological peptone; 10 percent rabbit serum to supply both albumin and fatty acids; and essential supplements such as thiamine, cyanocobalamin, and iron in the form of hemoglobin from lysed erythrocytes. The medium should be just pink and pH 7.2–7.4. It is usually sterilized by final filtration of all the ingredients. A common formulation is Korthof's medium. Nonpathogenic leptospires are generally grown in the same media as pathogens, although *Leptonema* grows well and rapidly in TSB medium.

Special selective and indicator media

Special media were developed empirically to isolate fastidious leptospires, especially *Leptospira interrogans* serovar Bratislava, from pigs and other animals (Adler et al. 1986; Ellis et al. 1985). Composition of media and conditions of cultivation must be adjusted to special needs. Selective media containing one or more of cycloheximide (100 μg/ml), bacitracin (40 μg/ml), 5-fluorouracil (50–1000 μg/ml), nalidixic acid (50 μg/ml), polymyxin-B sulphate (5 units/ml), polymyxin B (2 μg/ml), rifampin (10 μg/ml) or vancomycin (10 μg/ml) have been

recommended to reduce contamination on primary isolation or to purify contaminated cultures. They should not be used routinely as a substitute for careful technique. Subcultures must be made within 4 days.

Protein-free and low-protein media

Media with little or no protein were developed for vaccine production, rather than for nutritional studies. Utmost care is required to minimize toxicity from unwanted fatty acids. Chemical methods or solvents are used to 'clean' Tweens and the specially selected charcoal used as an adsorbent of fatty acids in place of protein. Some of the media used for vaccines are subject to commercial secrecy or patent and have not been published. In order to grow successfully in a protein-free medium, leptospires must first be adapted to it and then subcultured regularly and frequently.

Solid and semisolid medium

Almost any medium may be solidified by the addition of 0.5–1.0 percent agar to allow subsurface colonial growth, or 2 percent agar for surface growth. Growth in and on solid media is described above.

QUALITY CONTROL

It is essential to check the ingredients for culture media for quality before use. BSA is very hygroscopic; it should be kept and used dry, or a correction made for the weight of the water content. Each batch must be tested or previously certified suitable for the growth of leptospires. Some batches can be improved by fat extraction. All chemicals should be analytical grade. Agar should also be tested for freedom from toxic fatty acids. The quality of the water used for protein-free media is critical. The vessels used for preparing media should be dry; water used for solutions must be sterilized to prevent filter-passing leptospires in the water from contaminating the media. After preparation, the media must be checked for sterility by incubation for 3–4 days at 28–30°C as well as at 37°C. The possibility of contamination by leptospires should not be overlooked. The utmost care in labeling during subculture is essential because there are no obvious features to distinguish one leptospire from another. Records of origin of an isolate, its identification and its subculture history are essential. Serological identity should be checked, confirmed, and recorded periodically by agglutination to titer with a reference antiserum. Genetic identity should also be checked periodically where this is appropriate.

METABOLISM

Knowledge of leptospiral metabolism depends largely on studies reported before genetic speciation was known. It may be fallacious to deduce from them that all species of leptospires are metabolically identical. All leptospires are aerobic or microaerophilic chemo-organotrophs and use O_2 as a final electron receptor via cytochromes. Catalase, cytochromes *a*, *c*, and c_1 and oxidases have been identified. Essential long-chain fatty acids, longer than C_{15}, are degraded by β-oxidation for energy. Acid production from sugar fermentation does not occur, but key enzymes in carbohydrate metabolic pathways are present. Sero-specificity depends on the presence of small carbohydrates and their arrangement in the LPS (see the section on Serological groups below). Leptospires cannot synthesize long-chain fatty acids from pyruvate or acetate, but they can shorten long chains; the cellular lipid composition partly reflects the fatty acid composition of the medium. A characteristic component of leptospires, *cis*-11-hexadecenoic acid, is rare in other bacteria. Positionally specific trioleinase lipases are found in all *L. biflexa* and some pathogens, but not in *L. borgpetersenii*, some *L. interrogans*, *L. noguchii*, *L. santarosai*, and *L. weilii*. Hemolytic phospholipases including sphingomyelinase C exist in some pathogens, while *L. biflexa* contains phospholipase A_1 and lysolecithinase. Hemolytic activities dependent on phospholipases are specific for the erythrocytes of the animal concerned, determined by the composition of the dominant phospholipids in its cell membranes. Fatty acid esterases are ubiquitous among leptospires. The genetics and distribution of sphingomyelinase genes have been studied and reviewed (Segers et al. 1992; Segers 1991).

Ammonia is the essential source of nitrogen. In serum media it may be obtained by deamination of amino acids, particularly asparagine. In other media it is provided as ammonium salts. What little there is known about amino acid metabolism is unremarkable except that isoleucine synthesis may follow either the conventional threonine pathway, or an unusual pyruvate pathway, or both; genes for regulating steps in the latter have been identified. A urease, inhibited by ammonia, and various aminopeptidases have been described. Not all leptospires require purines or pyrimidines. Purine metabolism is mentioned below. Methionine biosynthesis in *L. meyeri*, controlled by the *metY* gene, occurs through direct sulphydralation using O-acetylhomoserine as a substrate (Belfaiza et al. 1998).

MOLECULAR BIOLOGY OF *LEPTOSPIRA*

Slow leptospiral growth on solid media and slow, relative metabolic inertness in usual phenotypic tests for other bacteria mean there are few physiological or colonial markers and very few classical genetic studies. Mutants have been selected to grow in media containing either boiled, as opposed to fresh, serum; others grow in antiserum to the parent strain. There are no studies of the genetics of these mutations, or of straight variants, usually forming small colonies, that appear spontaneously in some strains. A straight variant of *Leptonema*

illini was produced using nitrosoguanidine. Virulence is lost on subculture in the laboratory but leptospires able to grow in vivo can be selected from the culture population by inoculating experimental animals. Plasmids and the mechanisms of genetic transfer strongly assumed to occur between leptospires are elusive. Transformation has not been reported among leptospires. It is not yet possible to introduce DNA into pathogenic leptospires artificially and deliberately. A bacteriophage for *L. biflexa* was described, but none has been found that affects any pathogen (Saint Girons et al. 1990, 2000; Saint Girons 1991).

The leptospiral genome and its elements

The chromosome of *Leptospira* is characterized by a G + C content of 35–41 mol%, depending on species, with a genome size originally estimated to be ca. 5 000 kb, measured in pulsed-field gel electrophoresis after digestion with *Not*I, *Nhe*I or *Apa*I (Baril and Saint-Girons 1990). Using mainly the restriction enzymes *Not*I and *Sfi*I, a physical and genetic map *L. interrogans* serovar Pomona was constructed (Zuerner 1991), predicting a genome size of ca. 4 750 kb, similar to that of serovar Icterohaemorrhagiae. The genome consists of two circular replicons, estimated to be approximately 4 750 kb and 350 kb in size (Boursaux-Eude et al. 1998). The complete genome sequences of two very closely related serovars, Lai and Copenhageni, of *L. interrogans*, have been published recently (Nascimento et al. 2004; Ren et al. 2003), revealing almost identical genomes. The large and small replicons comprise 4 330 and 358 kb, respectively. The genomes of two strains of *L. borgpetersenii* serovar Hardjobovis (unpublished) are slightly smaller, with chromosome sizes of 3 614 and 317 kb. The smaller replicon is considered a chromosome because of the presence of essential genes on it. Interspecies comparison reveals regions of colinearity of up to 30 kb, but with large-scale chromosomal rearrangements. A comparison of genetic maps for strains of *L. interrogans* serovars Icterohaemorrhagiae and Pomona indicated considerable heterogeneity and variability within the species *L. interrogans*, that involves rearrangements of large segments of chromosome (Boursaux-Eude et al. 1998; Zuerner et al. 1993).

Both pathogenic and saprophytic leptospires contain two ribosomal 23S rRNA genes (Fukunaga and Mifuchi 1989b). Two genes for 23S rRNA and for 16S rRNA, but only one for 5S rRNA, were found in *L. interrogans* serovar Canicola, each located far from other RNA genes. There were two 5S rRNA genes in *L. biflexa* (Fukunaga and Mifuchi 1989a), one of which was sequenced (Fukunaga et al. 1992). Pathogenic strains of leptospires contained only one highly conserved 5S rRNA gene (Fukunaga et al. 1992). The 23S rRNA was further processed to 14S and 17S rRNA, in a pattern seldom found in prokaryotes, in *L. borgpetersenii* serovars Hardjo (subtype Hardjoprajitno) and Balcanica (Hsu et al. 1990). The two genes for 5S rRNA in *L. biflexa* strains were located several kb apart on the chromosome, with divergent flanking sequences (Fukunaga et al. 1992). Thus, in contrast to the situation in many other bacteria where the 16S (*rrs*), 23S (*rrl*) and 5S (*rrf*) rRNA genes are clustered and cotranscribed, those in *Leptospira* widely scattered on the large chromosome (Baril et al. 1992; Saint Girons et al. 1992).

Although no compound transposons have been found in the genus *Leptospira*, Zuerner (1994) identified and characterized IS*1533* from *L. borgpetersenii*. Genome sequencing has indicated at least 80 intact copies of IS*1533* on the genome. A PCR assay based on IS*1533* sequences was adapted for identification of *L. interrogans* (sensu lato) strains. A similar IS element was reported in serovar Tarassovi (GenBank X77623). An IS*3*-like element, designated IS*1500*, was present only in pathogenic leptospires (Boursaux-Eude et al. 1995). IS*1500* insertions were present only on the large chromosome and appeared to coincide with genomic regions which had undergone rearrangement. This element was also used as the basis for fingerprinting and epidemiological analysis of leptospiral isolates (Zuerner and Bolin 1997).

Interestingly, the serovar Hardjo (type Hardjobovis, but not Hardjoprajitno) LPS biosynthetic locus is bounded by two IS*1533* elements (de la Peña-Moctezuma et al. 1999; Kalambaheti et al. 1999). A second IS element was also found in an intergenic region of the Hardjobovis locus; it appeared to be intact and is related to the IS*5* family, but no functional studies were performed (Kalambaheti et al. 1999). Similarly, an apparently intact IS*1500* element was found to disrupt the *orf35* gene in Ictero No I, but not RGA, strains of serovar Icterohaemorrhagiae; this gene encodes a putative membrane protein within the LPS biosynthetic locus (de la Peña-Moctezuma et al. 2001). It has been speculated that the presence of these IS elements may regulate gene expression leading to differences in LPS structure, but there is no evidence yet for such a role.

The early branching of spirochetes before gram-positive and gram-negative bacteria (Woese 1987) is consistent with their heat shock regulatory mechanisms, which show characteristics of both gram-positive and negative bacteria. The *groE* and *dnaK* operons of *Leptospira* have been identified, cloned, and sequenced (Ballard et al. 1993, 1998; Stamm et al. 1991). The *groE* operon encoding the GroEL and GroES proteins exhibits features found in heat shock operons regulated by either $Sigma^{32}$ or CIRCE (controlling inverted repeat of chaperone expression) (Zeilstra-Ryalls et al. 1991; Zuber and Schumann 1994). These are generally found in gram-negative and gram-positive bacteria, respectively. The CIRCE element was also found upstream of the

dnaK locus, which contained the genes *hrcA*, *grpE*, *dnaK*, and *dnaJ* (Ballard et al. 1998). The genetic organization and regulation of this locus are thus more like those in gram-positive than gram-negative bacteria (Zeilstra-Ryalls et al. 1991; Zuber and Schumann 1994).

Genetic analysis of *Leptospira* has been impeded by the lack of genetic exchange systems. Early reports (Ritchie and Ellinghausen 1966) of bacteriophage-like particles in leptospires were not confirmed, but Saint Girons and her colleagues (1990) isolated three bacteriophages, whose replication was limited to the saprophyte *L. biflexa*. One of these phages, LE1, was shown to replicate as a plasmid in *L. biflexa* and was used as the basis for the first *L. biflexa-E. coli* plasmid shuttle vector, pGKLep4 (Saint Girons et al. 2000). No such system has yet been reported for the pathogenic leptospires. A restriction-modification system has been demonstrated, at least in *L. biflexa* (Brenot et al. 2001).

There are only two reports of gene inactivation by recombination-mediated allelic replacement in *Leptospira*. The kanamycin resistance gene from pGKLep4 was used to inactivate the *flaB* gene, encoding the flagellar subunit protein, in *L. biflexa* (Picardeau et al. 2001). Mutants were nonmotile and lacked flagella and hooked ends, but retained their helical shape. Inactivation of *recA* in *L. biflexa* resulted in reduced growth rate and altered nucleoid morphology (Tchamedeu Kameni et al. 2002). There are no published reports of allelic replacement in pathogenic leptospires.

THE LEPTOSPIRAL SURFACE

The leptospiral outer envelope (OE) generally resembles the outer membrane of gram-negative bacteria; it is also the site of most of the antigens known to be involved in immunity or diagnosis. The main difference is a high lipid content and a relatively low transmembrane outer-membrane protein content in leptospires (Figure 4).

Lipopolysaccharide

Leptospiral LPS comprises the major surface component of leptospires and is the target for agglutinating and opsonizing antibodies (Farrelly et al. 1987). As an agglutinating antigen, LPS is also important for serological classification of leptospires. The structure of LPS remains unknown. However, chemical analysis of leptospiral LPS showed the presence of common hexoses, amino hexoses, and pentoses, and some sugars found more rarely as LPS components, such as xylose and arabinose. The detection of keto-deoxy-octulonate (KDO) in the LPS of *L. interrogans* serovar Copenhageni and *L. borgpetersenii* serovar Hardjo is consistent with a composition similar to that of typical gram-negative LPS.

Genetic analysis has identified the *rfb* loci involved in the biosynthesis of the LPS O-antigen in several leptospiral serovars. The locus in serovar Hardjo contains at least 31 open reading frames (ORF) that encode enzymes involved in the biosynthesis of activated sugars, glycosyl transferases, and sugar processing and transport proteins (de la Peña-Moctezuma et al. 1999; Kalambaheti et al. 1999). Leptospiral LPS appears to be assembled via the classical Wzy (Rfc) dependent pathway. A comparison of the *rfb* loci of 14 other *L. interrogans* serovars revealed conservation at the 3′ end, but genes at the 5′ end vary among serovars, or show more genetic drift (de la Peña-Moctezuma et al. 2001). This region thus appears to encode biosynthetic enzymes and glycosyl transferases responsible for serovar specificity and some genes of unknown function.

Protein and lipoprotein antigens

OmpL1, the first leptospiral surface protein to be identified, is a transmembrane protein with porin activity which exists in a typical trimeric form in the leptospiral outer membrane (Haake et al. 1993; Shang et al. 1995), that contains lower concentrations of proteins than

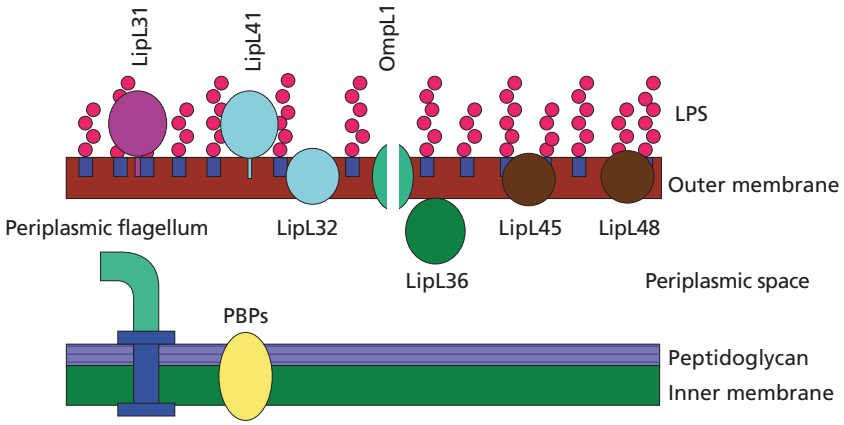

Figure 4 *Diagramatic representation of the leptospiral surface*

typical gram-negative outer membranes. However, the leptospiral outer membrane is abundant in lipoproteins, which are well conserved across the pathogenic species. One of these, LipL36 is not produced during infection, at least in the hamster kidney (Haake et al. 1998), although these results do not preclude a role for LipL36 at other stages of the infectious process. In contrast, the major OMP, LipL32, as well as OmpL1, LipL41, and LPS, are expressed during growth in the hamster kidney (Haake et al. 2000; Zuerner et al. 2000). LipL32 has been investigated as a potential serodiagnostic antigen (Flannery et al. 2001) and was shown to stimulate partial immunity in hamsters (Sonrier et al. 2000). LipL41 and OmpL1 when administered in combination, but not individually, were capable of eliciting an immune response which was partially protective in hamsters (Haake et al. 1999). Several other outer membrane lipoproteins have been identified (Cullen et al. 2002; Matsunaga et al. 2002), some of which show differential expression under a range of environmental conditions. Interestingly, the usual L-X-Y-C consensus sequence for transport and processing of gram-negative lipoproteins varies substantially amongst spirochete lipoproteins and the spirochetal lipobox sequence of L(A/S)-L(V/F/I)-I(V/G)-A(S/G)-C has been proposed (Haake 2000; Zuerner et al. 2000). The properties of leptospiral outer membrane proteins have been reviewed recently (Cullen et al. 2004).

BIOLOGICAL PROPERTIES OF LPS

Despite the structural, biochemical, and immunological similarity of leptospiral LPS to gram-negative bacterial LPS, it is at least 10-fold less toxic for animals or cells. Nevertheless, leptospiral LPS can activate macrophages and act as a B-cell mitogen (Isogai et al. 1990a, b). It has been proposed that the reduced toxic activity is a result of the absence of β-hydroxy-myristic acid. Activation and signaling within macrophages occurs via CD14 and TLR2, rather than via TLR4 as seen for the LPS of gram-negative bacteria (Werts et al. 2001). Notably, TLR2 is commonly used as a receptor for gram-positive bacteria and for other non-LPS components.

Other localized structures and components

A glycolipoprotein (GLP) with a high content of toxic lipids (palmitoleic and oleic acids) is cytotoxic and lethal for laboratory animals. Its cytotoxicity in tissue culture is obscured by the absorption of the toxic lipid into the albumin used in the culture media. Patients and animals produce antibodies to GLP, which can be located in the tissues in infections (Alves et al. 1992; Vinh et al. 1986). GLP acts through its constituent long chain fatty acids (Vinh et al. 1986), and inhibits Na^+, K^+ATPase (Younes-Ibrahim et al. 1997a). Peptidoglycan from leptospires was cytotoxic, inducing the release of TNFα from peripheral blood mononuclear cells (Cinco et al. 1993). Heat shock proteins were located below the surface (Stamm et al. 1991).

CLASSIFICATION

A microorganism with the shape, dimensions, and cultural characteristics of a leptospire is identified provisionally as a member of the Leptospiraceae. Cultural phenotypic characteristics (rapid growth in TSB medium) as well as electron microscopy characterize *Leptonema*. Other isolates look the same microscopically or in culture; they cannot be differentiated by simple biochemical and cultural tests. There are two overlapping systems for further classification, genetic species, and serological typing. Species is determined genetically by DNA homology, 16S rRNA gene sequencing or other molecular methods. Subspecies categories are determined serologically, by serovar, while genetic and serological subtypes are recognized within serovars (Ellis 1995).

Phylogenetic relationships

Spirochetes comprise a phylum of ancient bacteria whose members are very divergent from one another when classified by 16S rRNA relationships. The most deeply branching individual group in the phylum is the family Leptospiraceae; among them the first to branch off is *Leptonema* (Paster et al. 1984, 1991; Paster and Dewhirst 2000). *L. biflexa* and related saprophytes form a group distinct from the pathogenic leptospires, although there is considerable homogeneity between these by DNA homology (Paster et al. 1991). Within the group identified as the 'pathogenic' group (formerly the 'interrogans complex' or '*L. interrogans*' sensu lato), two main branches exist; one contains the species *kirschneri*, *interrogans*, and *noguchii*, the other the species *borgpetersenii*, *santarosai*, and *weilii*. *L. fainei* and *L. inadai* form a distinct group (Perolat et al. 1998). The 'nonpathogenic' group (formerly the 'biflexa complex', '*L. biflexa*' sensu lato) comprised six major branches in addition to *Leptonema* (Ramadass et al. 1990, 1992) (Figure 5).

Genetic classification and species

SPECIES

A species comprises those leptospires whose DNA is 70 percent related and whose related DNA sequences contain 5 percent or fewer unpaired bases (divergence) (Ellis 1995; Watt et al. 1990; Wayne et al. 1987). There are 12 named species, with further groups awaiting classification. Analyses by restriction length fragment polymorphism (RLFP), pulsed-field gel electrophoresis (PFGE) of chromosomal DNA, arbitrarily primed PCR (APPCR), mapped restriction site polymorphisms

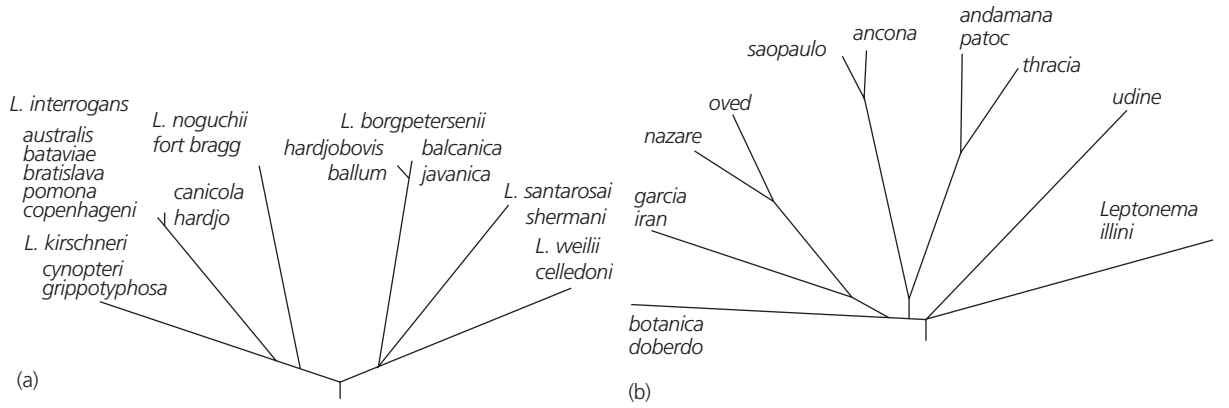

Figure 5 *Phylogenetic relationships of species of leptospires:* **(a)** *pathogenic varieties (adapted from Ramadass et al. (1992));* **(b)** *nonpathogenic varieties (adapted from Ramadass et al. (1990)). Information for the positions of* L. alexanderi *and* L. fainei *and other new species is not available.*

(MRSP), and random amplified polymorphic DNA fingerprinting (RAPD) are easier to perform and give similar results if calibrated (Gerritsen et al. 1995; Herrmann et al. 1992; Ralph et al. 1993). PFGE and RAPD fingerprinting can identify serovars uniquely in some cases. Serotyping identifies the same serovar antigens in more than one species. The recognized species, their G + C percentage ratios and some of the more common or important component serovars are listed with their serogroup identity in Table 1.

Serological groups

Leptospires were classified primarily by serology before the development of genetic methods. An arbitrary but useful definition of serovar was adopted many years ago, that two or more leptospires belong to the same serovar if a residual titer of 10 percent or less of the homologous titer of agglutinating antibodies remains in rabbit antisera after cross-absorption in standardized tests. About 255 serovars have been recorded among the Leptospiraceae. Serovars that are significantly different in their biological and pathogenic properties may be closely related at this 1 log difference level of titer. Some serovars exist in more than one species; that is, the same serovar-specific antigens can be expressed by members of more than one species. Serovars were designated in italic script until recently, when it became customary to use capitalized lower case letters.

The serological identity of an isolate depends on the dominance of one or more epitopes in a surface mosaic of LPS antigens, while the specificity of the epitope depends on its sugar composition and orientation (Ellis 1995; Kmety and Dikken 1993; Midwinter et al. 1994; Vinh et al. 1989). Monoclonal antibodies to LPS epitopes can identify isolates by generating a fingerprint based on agglutination profiles. Serovars that share common (serogroup-reactive) antigens and partially coagglutinate belong to serogroups. Serogroups have no taxonomic status but are useful for facilitating diagnosis and serological identification by antigens common to members of their group. Serological identification is clinically and epidemiologically significant but slow, painstaking, and labor-intensive.

Other phenotypic typing methods

Few phenotypic properties are available for classification. Colonial growth characteristics have not been used. Growth in TSB identifies *Leptonema*. Growth at 13°C (but not at 37°C); growth in one or more of $CuSO_4$, 8-azaguanine or 2,6-diaminopurine; and lipase production are, in general, characteristics of *L. biflexa* and *Leptonema* (Table 1). Flagellar antigens have also been grouped into distinct relationships. Related isolates have similar LPS profiles on polyacrylamide gel electrophoresis (PAGE). For many years it was wrongly assumed that all leptospires were metabolically and probably genetically identical because they appeared to be morphologically and culturally similar, differing from one another only in their serovar-specific antigens. Some early studies should now be repeated using members of different species to understand the extent of intra- and interspecies differences and to test the validity of extrapolating research results to different leptospires.

LABORATORY ISOLATION AND IDENTIFICATION

Laboratories may need to look for leptospires in clinical or pathological specimens from humans or animals, or from environmental sources. They also may need to grow them for research or industrial purposes. Leptospires are so hard to see and so hard and slow to grow that conventional bacteriological diagnostic procedures (microscopy of stained preparations and culture on solid media) are not practicable. Dark field microscopy of diagnostic, including environmental, specimens is

Table 1 *Species, serovars, serogroups, and phenotypes of some leptospires*[a]

Species	Serovar	Serogroup[b]	Serovar	Serogroup
L. alexanderi	Manhao 3	25		
L. biflexa[c,d]	Patoc	20		
L. borgpetersenii[d]	Anhoa	6	Javanica	12
	Arborea	3	Jules	10
	Balcanica	19	Mini	15
	Ballum	3	Sejroe	19
	Guangdong	3	Whitcombi	5
	Hardjo[e]	18	Tarassovi	21
L. fainei	Hurstbridge	23		
L. inadai	Icterohaemorrhagiae	11	Lyme	14
L. interrogans	Australis	1	Kennewicki	16
	Autumnalis	2	Kremastos	16
	Bangkok	1	Lai	11
	Bataviae	4	Lora	1
	Birkini	11	Medanensis	19
	Bratislava	1	Mwogolo	11
	Broomi	5	Naam	11
	Bulgarica	2	Paidjan	4
	Canicola	5	Pomona	16
	Copenhageni	11	Pyrogenes	17
	Djasiman	8	Rachmati	2
	Grippotyphosa	9	Robinsoni	17
	Hardjo[f]	19	Saxkoebing	19
	Hebdomadis	10	Swajizak	15
	Icterohaemorrhagiae	11	Zanoni	17
L. kirschneri	Bim	2	Grippotyphosa	9
	Bulgarica	2	Mwogolo	11
	Butembo	2	Mozdok	16
	Cynopteri	7	Tsaratsova	16
L. meyeri	Ranarum	24		
L. noguchii	Bajan	1	Muenchen	1
	Fortbragg	2		
L. santarosai	Bataviae	4	Pyrogenes	17
	Borincana	10	Tabaquite	15
	Kremastos	10	Trinidad	19
	Maru	10	Weaveri	18
	Navet	21		
L. weilii	Celledoni	5	Mengma	12
	Hainan	5	Sarmin	18
Turneria parva[c]	Parva	22		
Leptonema illini[c]	Illini	13		

a) The species identity of some strains representative of more than 250 recognized serovars in major serogroups is summarized from several sources. The species identity of many more strains awaits formal publication. Isolates were identified initially by serovar. The same serovar occurs in more than one species. Not all serogroups are represented in this table. Complete serovar lists to date were published by Kmety and Dikken (1993) and Faine et al. (1999).
b) Serogroups indicated by the number in the column. 1, Australis; 2, Autumnalis; 3, Ballum; 4, Bataviae; 5, Canicola; 6, Celledoni; 7, Cynopteri; 8, Djasiman; 9, Grippotyphosa; 10, Hebdomadis; 11, Icterohaemorrhagiae; 12, Javanica; 13, Leptonema; 14, Lyme; 15, Mini; 16, Pomona; 17, Pyrogenes; 18, Sarmin; 19, Sejroe; 20, Semaranga; 21, Tarassovi; 22, Turneria; 23, Hurstbridge; 24, Ranarum; 25, Manhao.
c) Nonpathogenic. Includes species *hollandia* and *wolbachia*. Other nonpathogens are listed in Faine et al. (1999).
d) G + C ratio mol% and phenotypes, compiled from various sources. In some cases the only isolate studied was an old laboratory strain. *L. biflexa* G + C: (36.0 ± 0.3)–(37.6 ± 0.7). Phenotype: P −; 11 −; 37 ±; Cu ±; AZ ±; DP +; L +. *L. borgpetersenii* G + C: 39.8 ± 0.3. Phenotype: P +; 11 −; 37 +; Cu −; AZ −; DP −; L ±. *L. fainei* G + C: ? Phenotype P +; 13+, 37?, Cu ?; AZ +; DP ?; L ?. *L. inadai* G + C: 42.6 ± 0.9. Phenotype: P +; 11 −; 37 +; Cu +; AZ +; DP −; L +. *L. interrogans* G + C: 34.9 ± 0.9. Phenotype: P +; 11 −; 37 +; Cu −; AZ −; DP −; L ±. *L. kirschneri* G + C: ?. Phenotype: P +; 13 −, 37 +; Cu ?; AZ −; DP +m; L ?. *L. meyeri* G + C: 35.2 ± 0.2. Phenotype P±11-; 37 +; Cu +; AZ +; DP +; L +. *L. noguchii* G + C: 36.5 ± 1.2. Phenotype: P +; 11 −; 37 +; Cu −; AZ −; DP −; L −. *L. santarosai*: G + C: 40.7 ± 0.5. Phenotype: P +; 11 −; 37 +; Cu −; AZ −; DP +; L −. *L. weilii* G + C: 40.5 ± 0.7. Phenotype: P +; 11 −; 37 −; Cu −; AZ −; DP −; L −. *Turneria parva* G + C: 48.7 ± 0.7. Phenotype: P −; 11 −; 37 +; Cu +; AZ +; DP −; L +. *Leptonema illini* G + C: 54.2 ± 0.2. Phenotype: P −; 11 +; 37 −, Cu −; AZ +; DP +; L +.P, pathogenic; 11, growth at 11°C; 13, growth at 13°C; 37, growth at 37°C; Cu, growth with $CuSO_4$ 100 mg/ml added to the growth medium; AZ, growth with 8-azaguanine 225 mg/ml added to the growth medium; DP, growth with 2,6-diaminopurine 10 mg/ml added to the growth medium; L, lipase trioleinase activity; +, growth recorded or activity present; −, growth or activity not recorded; ±, growth or activity variable among strains; +m, most strains active; ?, unknown or not formally published.
e) Type hardjobovis.
f) Type hardjoprajitno. *L. alexanderi*, serogroup Manhao, serovar Manhao 3, type strain L60^T (ATCC 700520^T) (Brenner et al. 1999) and *L. fainei*, serogroup Hurstbridge, serovar Hurstbridge, reference strain BUT^T (Billinghurst et al. 1994; Perolat et al. 1998) are new species.

technically difficult because particles or other bacteria in the specimens scatter light and impede visualization. Microscopy is presumptive and immediate; culture is definitive if successful, but slow, taking up to 3–4 weeks for growth and days to weeks for identification.

Immunofluorescent staining is rapid and specific if the serovar or serogroup of the leptospires is known or suspected, or pools of antisera are used. Direct PCR can be carried out on specimens. PCR is relatively fast, taking hours or a few days; it is valuable for rapid identification of isolates.

Types of specimens

CLINICAL AND PATHOLOGICAL DIAGNOSTIC SPECIMENS

Similar specimens are, in general, investigated for leptospirosis in humans and in animals. The methods are the same in principle, and almost the same in practice, for both (Faine et al. 1999; Levett 2001; Weyant et al. 1999).

Blood cultures, taken aseptically from febrile patients or animals, are inoculated into EMJH medium containing sodium polyanethane sulfonate (and β-lactamase if treatment with penicillin has begun) at dilutions of at least 1 volume (from 0.1 to 1.0 ml) to 100 volumes of medium. Special media are used for fastidious leptospires. Cerebrospinal fluid (CSF) and urine are cultured similarly; urine specimens must be alkaline when passed and collected aseptically. Cultures are incubated at 30°C and examined macroscopically daily for up to 3 weeks and microscopically to confirm growth when it is suspected, or before discard. Subcultures are made into volumes of 5–10 ml of liquid media with further subcultures every 3–14 days at first; solid media are not used in general. The tissues most usually examined in animals are kidneys, genital tract organs, semen, abortion products, and placentas; examination of human tissues is rare in nonfatal leptospirosis. Urine and autopsy specimens and field specimens from animals are often contaminated; inhibitors such as cycloheximide, bacitracin, neomycin, polymyxin B, and rifampin in the media prevent or retard the growth of other bacteria. Tissues from autopsies, or rarely from biopsies, are ground aseptically with sterile buffered (pH 7.2) saline diluent and the supernatant cultured as above. Carryover of tissue debris can make the medium anaerobic and prevent leptospiral growth (see Faine et al. (1999) and Weyant et al. (1999) for details of diagnostic cultures).

ENVIRONMENTAL SPECIMENS – WATER, MUD, AND SOIL

Leptospires, including pathogens, persist in water, soil, mud, and effluents. Detection by direct dark field microscopy is of limited value. Immunofluorescent staining can detect leptospires without regard to viability. Water can be filtered through a bacteriological filter of 0.22 μm average pore diameter (APD) which will let leptospires through the pores. Soil, mud, and similar samples can be shaken with sterile water and the sediment centrifuged. The supernatant or eluate is filtered as above. The filtrates are cultured in liquid or on solid media. Successful cultures can be achieved by using media with inhibitors (see above). Alternatively, the filtrate or water may be inoculated in a small area in the middle of the surface of a plate of solid medium. After incubation, growth of leptospires will spread in a ring, centrifugally from the inoculation site, allowing subculture from the growing edge. Such cultures need to be checked for purity because more than one strain of leptospires may occur in these specimens. Special high sodium media have been described for culturing halophilic leptospires.

Identification of isolates; typing methods

Cultures, once grown, need to be checked for purity and identity. Purity can be checked by plating on solid media, remembering that a pure culture can have more than one colony type. Colony type is not usually included in criteria for identification or classification. The usual method for testing serological identity is by microscopic agglutination test (MAT).

A reference antiserum is serially diluted in phosphate buffered saline, pH 7.2, usually in two-fold steps starting at one or more steps in a 1 in 10 dilution series, in volumes of 25 μl in microtitration trays. An equal volume of the culture to be tested, standardized for density, is added and the covered tray incubated at a noncritical room temperature or 30°C for 1.5–2.0 h. A loopful from each dilution is examined microscopically under dark field. Alternatively, if tests are performed in flat-bottom trays free of scratches, they may be read directly in the tray using a long working-distance microscope objective. Agglutination is noted and scored as an approximate percentage of the leptospires agglutinated. The end point is 50 percent agglutination. ELISA tests are also used as serological methods for identification, in reactions with standard antisera (Dikken and Kmety 1978; Faine et al. 1999; Levett 2001; Weyant et al. 1999). Serological identification should be done at least to serogroup level, but to serovar level if possible, and in addition, to subtype within the serovar if appropriate. The lengthy standardized procedure begins with MAT using various reference antisera from different serogroups, to find one that agglutinates it to titer or close to titer, repeated similarly with antisera reacting to single serovars. Batteries of pooled sera are used initially. Homologous antisera to the new strain are produced in rabbits. These antisera and the reference antisera to the reacting reference strain are then

absorbed and cross-absorbed with both the new strain and the reacting reference strain. The absorbed antisera are titrated in MAT using homologous and heterologous strain antigens. The new strain is regarded as the same as the old strain if the residual titers after cross-absorption both ways are 10 percent or less. A simplified acceptable procedure is to use batteries of reference monoclonal sera which can identify to at least serogoup level immediately. Since the epitopes are not necessarily characteristic nor unique for a single serovar, pools of monoclonals are used to detect epitopes whose pattern of reaction is specific for a serovar. The use of monoclonal antibodies supplants the methods employing 'factor sera' for 'factor analysis', an analogous form of identification using serovar-specific pools of absorbed antisera.

It is important to identify the species as well as the serovar when describing or researching a leptospire because characteristics specified by species may be different for a given serovar. The reference method for bacterial speciation remains the degree of homology at DNA level (Wayne et al. 1987). However, the procedure is cumbersome and unsuitable as a tool for routine use. RFLP based on PCR amplification of either 16S rRNA or 23S rRNA genes has been shown to group strains in general agreement with their correct species designation (Perolat et al. 1994; Ralph et al. 1993). Probes derived from random primed PCR fingerprints were also able to discriminate at species level (Letocart et al. 1997).

At subspecies level, the first widely used method was restriction endonuclease analysis (REA) of whole genomic DNA (Marshall et al. 1981). Despite the difficulty in reading complex restriction profiles due to the large number of fragments generated, the technique has provided useful epidemiological information. For example, REA first identified the Hardjobovis and Hardjoprajitno subtypes now known to belong to different species, as well as allowing discrimination of three subtypes of Hardjobovis (Thiermann et al. 1986). Similar subtypes have been identified within other serovars. All leptospiral genomes appear to have multiple copies of a range of insertion sequences; some of these have been used as targets for identification and molecular typing schemes (Zuerner et al. 1995; Zuerner and Bolin 1997).

Interestingly, and perhaps unexpectedly, the analysis by PFGE of restriction fragments generated by digestion with rare cutting enzymes such as *Not*I or *Sgr*AI yielded unique patterns for most serovars (Herrmann et al. 1992; Herrmann and Saint-Girons 1993; Herrmann 1994). In reality, PFGE has become accepted as the most appropriate and accurate molecular typing method. A disadvantage is the need for specialized equipment and expertise. For this reason, a range of methods based on random PCR amplification of chromosomal DNA has been reported (Faine et al. 1999; Levett 2001); in general they show good correlation with PFGE and can be performed readily by any laboratory with PCR capacity.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

General and environmental agents

Leptospires and leptonemes are destroyed by heat above about 42°C, acid (below about pH 6.5), alkali (above about pH 8.0), heavy metals, halogens, detergents (including soaps, free fatty acids, bile salts), drying and reducing conditions (*E*h below about −0.300 mV). They can survive chilling to 4°C or freezing to −20°C in tissues (including kidneys in butcher shops). In the laboratory they can be cryopreserved in liquid nitrogen and have been preserved lyophilized. Cultures stored in sealed tubes in the dark at room temperatures below about 40°C remain viable for months; leptospires have been grown after 8 years of storage.

Antibiotics and metabolic antagonists

All classes of antibiotics except chloramphenicol and rifampin kill leptospires. Penicillin and doxycycline are used widely in therapy; the latter has also been used for chemoprophylaxis. Streptomycin and dihydrostreptomycin are used for eliminating leptospires from the kidneys of carrier animals. Resistance to any antibiotic has not appeared as a clinical problem, probably because there is no human-to-human transmission. Individual isolates of leptospires are not usually tested for susceptibility to antibiotics. The selective use of purine and pyrimidine inhibitors for classification has been mentioned above.

PATHOGENICITY AND VIRULENCE FACTORS

This topic is reviewed in detail in Faine et al. (1999) and dealt with in the context of infection, pathogenesis, and diagnosis.

Role in the normal flora of humans

The natural habitat of leptospires is in the environment; they are not known to be part of the normal flora of humans or animals. Leptospires infect humans only by accident of contact with a contaminated environment; spread from humans to other humans is almost unknown apart from congenital infection. Animals that survive acute systemic infection may become renal carriers that excrete leptospires in the urine; the carrier state is not established without prior generalized clinical or subclinical infection.

'Pathogenic' and 'nonpathogenic' leptospires – virulence and virulence factors

Large numbers (approximately 10^{10}) of leptospires of either the nonpathogenic species *L. biflexa* or the pathogenic species are dermonecrotic and cytotoxic. *L. biflexa* and avirulent pathogenic leptospires do not survive for long in the body because naturally occurring immunoglobulins kill them in the presence of lysozyme and complement (Faine and Carter 1968). They are then phagocytosed. Virulent leptospires survive possibly because the antigens reacting with these opsonic immunoglobulins are not expressed or not available on the surface. Virulent leptospires, growing in an animal host, may also be able to survive in macrophages, in which they induce apoptosis (Merien et al. 1998). This activity was lost after a single in vitro passage. An outer membrane lipoprotein LipL36 was synthesized at 30°C but not at 37°C (Nally et al. 2001b), while some other membrane associated proteins or lipoproteins, including LipL41, LipL32, and OmpL1, are produced at either temperature in culture, or only at 37°C and during growth in an animal infection model (Cullen et al. 2002; Haake et al. 1998, 2000; Nally et al. 2001b). LipL41 and OmpL1 were synergistically protective in experimental infections (Haake et al. 1999). The surviving leptospires grow in the blood and selected tissues to a critical concentration correlated with the appearance of lesions. Growth of leptospires in the body stops when opsonizing antibodies appear, 3–10 days after inoculation, followed immediately by clearance by local and central reticuloendothelial phagocytosis. The opsonins are directed to specific LPS epitopes. Nothing is known of the genetics or regulation of expression of the ability to survive natural antibodies (Faine et al. 1999).

The primary lesions in leptospirosis consist of damage to the endothelial cells of small blood vessels, leading to leakage of plasma and hemorrhages. Localized acute inflammation and polymorphonuclear exudates are not seen initially in acute leptospirosis. The consequences of the damage to blood vessels are ischemia to the cells and organs dependent on the disturbed blood supply and the primary and secondary effects of sometimes massive hemorrhage. Almost any organs or body systems can be affected; the process is essentially the same in all animals. Renal tubular necrosis is common, sometimes followed by scarring during repair, and the localization of leptospires on the luminal surface of the tubular cells, where they grow and are excreted in urine (Faine et al. 1999).

The lesions are probably caused by the action of one or more toxins, one of which is a cytotoxic and antigenic GLP (see Chemical composition above) extractable from leptospires, that can be identified in lesions; convalescent patients' antisera react with GLP. GLP activates cytokines (Diament et al. 2002). LPS does not appear to play a significant part in pathogenesis; it is very weakly pyrogenic and does not produce the lesions that GLP does. The effects of GLP may be due to the unusual unsaturated fatty acids of leptospiral origin acting as competitive inhibitors of the incorporation of normally occurring fatty acids in the target cell membranes (Vinh et al. 1986), resulting in a disturbance of Na^+,K^+-ATPase activity in renal tubular cells (Younes-Ibrahim et al. 1997b). Hemolysins (sphingomyelinases) produce holes in erythrocyte and probably other cell membranes (Lee et al. 2002; Thompson 1986).

Microbiological aspects of natural and acquired resistance

Immunity in initial or subsequent infection appears to be wholly humoral and serovar-specific, dependent on opsonization mediated by immunoglobulins directed to LPS epitopes. Phagocytosis depends on antibodies to LPS. Although it is generally agreed that antibodies are of prime importance in protection from initial acute infection, a killed Hardjobovis vaccine stimulated a Th1 response in protected cattle (Naiman et al. 2001).

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Bartonella and *Afipia*

ANNA SANDER

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BARTONELLA

HISTORICAL PERSPECTIVE

The first recognized member of the genus *Bartonella* is *B. bacilliformis*, described by Alberto Barton in 1905. The Peruvian physician observed the presence of intraerythrocytic bacilli in blood smears of patients suffering from Carrión's disease (Barton 1905). Carrión's disease is a biphasic infection that occurs endemically in the valleys of the South American Andes of Peru, Columbia, and Ecuador and is transmitted by the blood sucking sandfly *Lutzomya verrucarum*. The acute stage of the infection is known as 'Oroya fever,' a highly febrile illness followed by a severe acute hemolytic anemia. It was named after a dramatic outbreak during the construction of the Peruvian railroad from Lima to the mining center La Oroya in 1870. More than 8 000 of the 10 000 laborers fell ill and most of them died. Several weeks or months later, many of those workers who survived the acute febrile stage of the illness developed wart-shaped or hemangioma-like skin lesions, the so-called 'Verruga peruana'.

In August 1885, the 26-year-old Peruvian medical student Daniel Alcides Carrión inoculated himself with secretions from a Verruga peruana skin lesion of a 14-year-old patient. Three weeks later he developed high fever followed by severe anemia. Eighteen days later Daniel Carrión died from Oroya fever and demonstrated with this heroical self-experiment the common infectious nature of Oroya fever and Verruga peruana (Schultz 1968; Garcia-Caceres and Garcia 1991).

In 1926, successful cultivation of the causative pathogen on blood containing media was reported and, in honor of Barton, named *Bartonella bacilliformis* (Noguchi and Battistini 1926).

In 1916, during the First World War, a newly recognized disease was first reported from the eastern front of the German army in Wolhynia (today part of the Ukraine) by two German military physicians, Hiss and Werner (Werner and Haenssler 1916). The soldiers suffered from relapsing fever with body temperature up to 40°C (in mean 6–10 attacks), headache, myalgia, bone pain, moderate anemia, and occasionally hepatosplenomegaly. Due to the 5-day periodic recurrence of the fever the illness was known as 5-day-fever, febris quintana or trench fever, but also as Wolhynia fever or Werner–Hiss disease (Munk and da Rocha Lima 1917a, b). To demonstrate the infectious nature of the disease, the two German physicians Benzler and Werner inoculated themselves intramuscularly with blood from a patient suffering from trench fever (Werner et al. 1916). After an incubation period of 20 and 23 days respectively, both physicians fell ill. In contrast to Carrión's disease, however, trench fever is a self-limiting illness and the physicians recovered from their self-experiment.

Henry da Rocha Lima was a Brazilian microbiologist, who worked between 1910 and 1928, together with

Stanislaus von Prowazek, at the Institute for Tropical Medicine in Hamburg, Germany. In 1914, the two microbiologists were told to investigate an outbreak of murine typhus in Cottbus, where both became infected. In contrast to von Prowazek who died on 17 February 1915 of murine typhus, Henry da Rocha Lima survived and described the etiologic agent *Rickettsia prowazekii*. On this occasion, da Rocha Lima was also confronted with trench fever (de Cerqueira Falcao 1966). He recognized very soon that the body louse was the main vector for transmission of trench fever and showed in his investigations that the agent of trench fever (named by him *Rickettsia pediculi*) was found, in contrast to *R. prowazekii*, mainly extracellularly in the gastrointestinal tract of the infected lice. He detected *Rickettsia pediculi* in the body lice of 73 percent of trench-fever patients, whereas only 18 percent of healthy soldiers had been infested with infected lice (Munk and da Rocha-Lima 1917b). In 1961, nearly a half century later, Vinson and Fuller were able to culture the agent of trench fever on blood containing solid media and, in honor of Henry da Rocha Lima, the strain was renamed subsequently in *Rochalimaea quintana* (Vinson and Fuller 1961).

THE GENUS *BARTONELLA*

Members of the family Bartonellaceae belong to the α-2 subdivision of the Proteobacteria, showing a close evolutionary relationship to the human pathogen *Brucella* and to the plant pathogens *Rhizobium meliloti* and *Agrobacterium tumefaciens* (Marston et al. 1999). Depending on the *Bartonella* species, the genome size varies between 1 600 and 2 174 Mb and the G + C (guanine-plus-cytosine) content of the genome ranges from 39 to 41 percent (Roux and Raoult 1995).

In recent years, the classification of the family of Bartonellaceae was revised several times. In 1993, the genus *Rochalimaea*, including the four species *R. quintana*, *R. vinsonii*, *R. henselae*, and *R. elizabethae* was removed from the family Rickettsiaceae and transferred to the family Bartonellaceae, which by then consisted of a single species, namely *B. bacilliformis* (Brenner et al. 1993). Based on the 16S rRNA gene sequencing, DNA hybridization, G + C content, and phenotypic characterization studies, Birtles et al. (1995) proposed the unification of the genera *Grahamella* (consisting of the two species *G. talpae* and *G. peromysci*) and *Bartonella* and defined three new species, namely *B. doshiae*, *B. grahamii*, and *B. taylorii*.

In the following years, the number of identified *Bartonella* species increased considerably. The widespread occurrence of *Bartonella* in the bloodstream of different mammals has resulted in the description of several new *Bartonella* species. Currently, the family Bartonellaceae consists of 20 *Bartonella* species which are listed in Table 1.

Table 1 *The family of Bartonellaceae, their natural reservoir and mode of transmission*

	Bartonella species	Year of description	Reference	Reservoir	Vector
1	*B. bacilliformis*	1905	(Barton 1905)	human	sandfly
2	*B. talpae*	1911	(Brumpt 1911)	mole	unknown
3	*B. quintana*	1917	(Schmincke 1917)	human	body louse
4	*B. peromysci*	1942	(Tyzzer 1942)	deer, mouse	unknown
5a	*B. vinsonii* spp. *vinsonii*	1946	(Baker 1946)	vole	vole ear mite
6	*B. henselae*	1992	(Regnery et al., 1992a; Welch at al., 1992)	cat	cat flea
7	*B. elizabethae*	1993	(Daly et al. 1993)	rat	unknown
8	*B. grahamii*	1995	(Birtles et al. 1995)	mouse, vole	unknown
9	*B. taylorii*	1995	(Birtles et al. 1995)	mouse, vole	unknown
10	*B. doshiae*	1995	(Birtles et al. 1995)	vole	unknown
5b	*B. vinsonii* spp. *berkhoffii*	1996	(Kordick et al. 1996)	dog	ticks
11	*B. clarridgeiae*	1996	(Lawson and Collins 1996)	cat	cat flea
12	*B. tribocorum*	1998	(Heller et al. 1998)	rat	unknown
13	*B. alsatica*	1999	(Heller et al. 1999)	rabbit	unknown
14	*B. koehlerae*	1999	(Droz et al. 1999)	cat	unknown
5c	*B. vinsonii* spp. *arupensis*	1999	(Welch et al. 1999)	mouse	ticks
15	*B. birtlesii*	2000	(Bermond et al. 2000)	mouse	unknown
16	*B. schoenbuchii*	2001	(Dehio et al. 2001)	roe deer	unknown
17	*B. bovis* (=*B. weissii*)	2002	(Bermond et al. 2002)	cattle/cat	unknown
18	*B. capreoli*	2002	(Bermond et al. 2002)	roe deer	unknown
19	*B. washoensis*	2003	(Kosoy et al. 2003)	ground squirrels	unknown
20	*B. chomelii*	2004	(Maillard et al. 2004)	cattle	unknown

Note that 5a, 5b, 5c are subspecies which belong to the same *Bartonella* species.

Microscopically, all *Bartonella* spp. are gram-negative, pleomorphic, slightly curved bacilli of approximately 1–2 – 0.5 μm with a distinct tendency to autoagglutination (Figure 1). Growth of *Bartonella* spp. is obtained on fresh media supplemented with 5 percent sheep or rabbit blood in the presence of 5 percent CO_2. The optimal incubation temperature varies from 25°C (*B. bacilliformis*) to 35°C (other species). The aerobic, highly fastidious organisms are oxidase- and indole-negative, and inert in carbohydrates metabolism. The G + C content ranges from 38.5 to 41 mol%.

Electron microscope examination of *B. bacilliformis*, *B. clarridgeiae*, *B. schoenbuchii*, and *B. capreoli* demonstrated the presence of multiple unipolar flagella. Polar structures resembling fimbriae have been observed in *B. tribocorum* and *B. alsatica*, whereas most strains of *B. henselae* and *B. quintana* have peritrichous pili, which are associated with cytoadherence. *B. bacilliformis* and *B. henselae* (and probably also *B. quintana*, *B. doshiae*, and *B. grahamii*) are known to produce bacteriophage-like particles that package 14 kbp segments of the host chromosome (Anderson et al. 1994; Barbian and Minnick 2000).

RESERVOIR AND MODES OF TRANSMISSION

The natural reservoir of the different *Bartonella* species and the mode of transmission are presented in Table 1. The currently available data indicate a strong association of a given *Bartonella* sp. with a specific host, i.e. *B. bacilliformis* and *B. quintana* with humans, *B. henselae*, *B. clarridgeiae*, and *B. koehlerae* with cats, *B. vinsonii* subsp. *berkhoffii* with dogs and coyotes, *B. alsatica* with wild rabbits, *B. tribocorum* with rats, and *B. schoenbuchii* and *B. capreoli* with roe deer. However, factors which are involved in the determination of host specificity have not been described. Simultaneous infection with more than one *Bartonella* sp. has been demonstrated in cats and rodents (Figure 2).

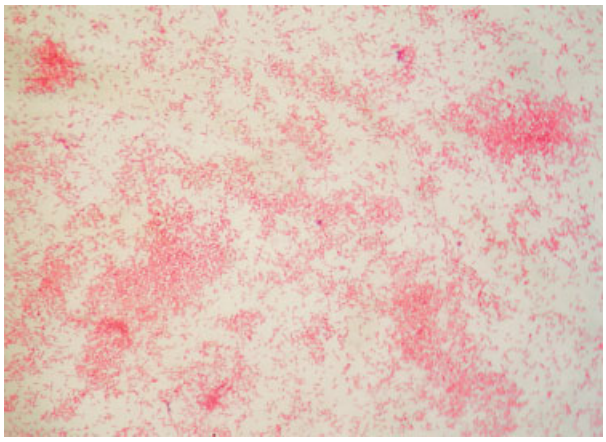

Figure 1 *Microscopic presentation of Gram-stained* B. henselae*: small gram-negative, pleomorphic, slightly curved rods.*

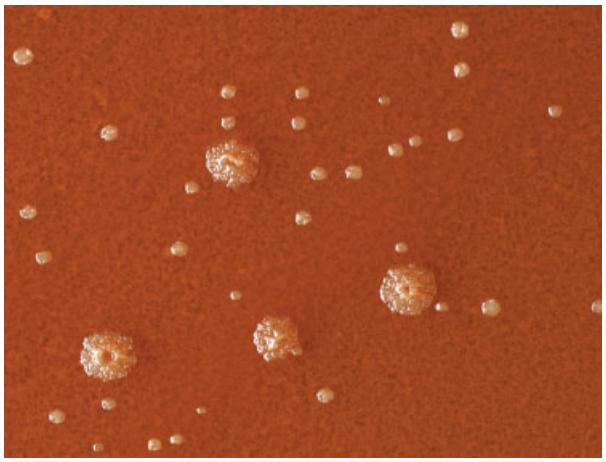

Figure 2 Bartonella henselae *(small colonies) and* B. clarridgeiae *(rough colonies) isolated from a blood culture of a cat.*

Several ectoparasites have been implicated in transmission of *Bartonella* species. *Bartonella bacilliformis* is transmitted to humans by the sandfly *Lutzomyia verrucarum*, *B. quintana* by the human body louse *Pediculus humanus,* and *B. henselae* by scratches or bites from bacteremic cats or by the cat flea *Ctenocephalides felis*.

Bartonella henselae is transmitted from bacteremic cats to specific-pathogen-free cats by the cat flea, and the agent can be cultured from flea feces up to 9 days after feeding on infected blood. Experimental infection of cats by intradermal inoculation with infected flea feces was also shown. In contrast, when uninfected cats were housed with *B. henselae* infected cats in an ectoparasite-free environment, no evidence of *Bartonella* transmission was noted. Although still not proven, there is also strong evidence that transmission of *B. henselae* to humans occurs via infected flea feces, either by contaminated claws or teeth after cat scratches or bites, or directly by inoculation of infected flea feces after flea-bite by scarification of the skin or through conjunctival contact. Although cat fleas frequently infest dogs within a household which also contains infected cats, these organisms have not been isolated from the blood of asymptomatic dogs until now (Breitschwerdt and Kordick 2000).

All *Bartonella* species have been isolated from the bloodstream of their host. Detailed investigations concerning the presence of *B. henselae* in the blood of cats have demonstrated that the bacteria may be found intracellularly in 2.9–6.2 percent of the erythocytes in bacteremic animals (Kordick and Breitschwerdt 1995; Rolain et al. 2001), but also extracellularly in infected animals (Guptil et al. 2000).

In veterinary medicine, the most extensive *Bartonella* studies have involved domestic and feral cats. The rate of bacteremic cats and the seroprevalence of *B. henselae* antigen is much higher (40–70 percent) in cats that live in warm, humid regions of the world, in which severe

flea infestation is expected (Breitschwerdt and Kordick 2000). Feral cats are more likely to be infected than pet cats from the same region. Although some of these animals may be bacteremic over a period of more than 1 year, cats are usually healthy and asymtomatic (Sander et al. 1997).

There is strong evidence that ticks play an important role in the transmission of *Bartonella* species among animals. However, the role of ticks in transmission to humans remains undefined. Elimination of fleas, ticks, and body lice may interrupt transmission of *Bartonella* species to humans and among animals.

Eight of the 20 currently described *Bartonella* spp. are known to be related to human diseases (Table 2).

BARTONELLA BACILLIFORMIS AND CARRIÓN'S DISEASE

Clinical manifestations

B. bacilliformis is a fastidious hemotropic organism which invades and destroys red blood cells. The organism appears to have a restricted geographical distribution to specific altitudes (500–3 200 m) of the mountainous regions in the South American Andes of Peru, Ecuador, and Colombia (Gray et al. 1990; Alexander 1995; Amano et al. 1997; Cáceres-Ríos et al. 1995). However, outbreaks have also been reported in formerly nonendemic areas over recent years and bartonellosis (Carrión's disease) appears to be an emerging disease with an expanding geographical distribution. Outbreaks of bartonellosis were reported in late 1996 in Utcubamba River Valley in the Amazonas Department in Northern Peru (Kosek et al. 2000) and in 1998 in the Urubamba River Valley (Cusco) of Peru (22 case-patients, Ellis et al. 1999), regions not previously considered endemic for this disease, as well as in 1997 in Zamora Chinchipe Province in Ecuador involving 19 cases (Cooper et al. 1997). In a prospective, population-based study of bartonellosis in the Caraz District, an endemic area in Peru, the incidence of bartonellosis was 12.7/1 000 persons/year and the prevalence of asymptomatic *B. bacilliformis* bacteremia was 0.5 percent. The incidence was highest in children, particular young children aged 0–4 years (38/100 person-years) and 5–10 years (16/100 person years). The age-related incidence pattern found suggests an acquired immunity following exposure to this agent (Chamberlin et al. 2002).

Bartonella bacilliformis is transmitted by the bite of the sandfly *Lutzomyia verrucarum*. In the human host, *B. bacilliformis* causes a biphasic disease with two distinct clinical forms.

The first and acute phase of bartonellosis, named Oroya fever, has an incubation period of usually 1–3 weeks (range 7–100 days) and is characterized by fever, headache, hemolytic anemia, pallor, myalgia, and arthralgia. It may also be associated with generalized or regional lymphadenopathy (1.5–3 cm, nonpainful, nonadherent), severe thrombocytopenia, altered mental status, seizures, meningeal signs, hemiparesis, and hypoxic complications of delerium and coma. Funduscopic abnormalities including flame-shaped hemorrhagies, cotton-wool exudates, and papilledema have been described, as well as pericardial and pleural effusions and cardiomegaly (Maguina and Gotuzzo 2000). The severity of this stage ranges from subclinical infection to an acute hemolytic crisis, when *B. bacilliformis* invade up to 90–100 percent of erythrocytes and causes a massive destruction of red blood cells in all organs

Table 2 *Currently recognized human diseases related to* Bartonella *species*

	Bartonella spp.	Associated human diseases	Year of description	Reference
1	*B. bacilliformi*	Carrión's disease: Oroya-fiever, Verruga-Peruana	1870	(Garcia-Caceres and Garcia 1991)
2	*B. quintana*	Trench fever	1916	(Werner et al. 1916)
		Bacillary angiomatosis, peliosis	1990	(Relman et al. 1990)
		Endocarditis	1993	(Spach et al. 1993)
3	*B. henselae*	Cat-scratch disease	1950	(Debré et al. 1950)
		Bacillary angiomatosis, peliosis	1992	(Regnery et al. 1992a; Welch et al. 1992)
		Endocarditis	1993	(Hadfield et al. 1993)
4	*B. elizabethae*	Endocarditis	1993	(Daly et al. 1993)
		Neuroretinitis	1998	(O'Halloran et al. 1998)
5	*B. clarridgeiae*	Cat-scratch disease	1997	(Kordick et al. 1997)
6	*B. grahamii*	Neuroretinitis	1999	(Kerkhoff et al. 1999a)
7a	*B. vinsonii* spp. *arupensis*	Fever, bacteremia	1999	(Welch et al. 1999)
7b	*B. vinsonii* spp. *berkhoffii*	Endocarditis	2000	(Roux et al. 2000)
8	*B. washoensis*	Myocarditis	2003	(Kosoy et al. 2003)

(Reynafarie and Ramos 1961). Untreated, Oroya fever is often life threatening. This acute stage may be accomplished by a variably-induced immunosuppression, and may be associated with the presence of secondary infections. Up to 35 percent of Oroya fever patients may develop infectious complications, including systemic salmonellosis (*Salmonella* Typhi and enteric *Salmonella*), shigellosis, reactivation of toxoplasmosis, disseminated histoplasmosis (*Histoplasma capsulatum*), malaria, and infections caused by *Staphylococcus aureus*, *Enterobacter* spp., *Pneumocystis jiroveci (carinii)* (Colichón and Cuandra 1954; Cuandra 1956; Maguina et al. 2001). After the acute phase, recurring episodes of fever and transitory pain in the bones, joints, and muscles may occur (Ricketts 1949).

Only limited data on the course of bartonellosis during pregnancy are available. One study noted that of five pregnant patients suffering from acute bartonellosis, two died and only one had a successful outcome of pregnancy (Maguina et al. 2001). Bartonellosis seems to bear a great risk for both the pregnant mother and the fetus.

During the Oroya fever outbreak of 1871 a fatality rate of 80 percent was reported and during the Shumpillan outbreak in 1990 (Gray et al. 1990) the patient fatality rate in untreated cases was 88 percent. A recent retrospective hospital-based study reported a mortality rate of 8.8 percent despite appropriate antibiotic treatment (Maguina 1993). In a population-based study during the Utcubamba outbreak in 1996 the case fatality rate differed enormously from those previously reported and was only 0.7 percent. In the same study, the attack rate of Oroya fever was 13.8 percent and that of Verruga peruana 17.6 percent (Kosek et al. 2000).

Following the acute clinical manifestations of Oroya fever, a period of dormancy or latency, generally noted for months, may ensue. Subsequently, patients may develop the second, chronic eruptive stage, named verruga peruana ('Peruvian warts'). Although verruga peruana was originally thought to occur only in patients who had suffered and recovered from Oroya fever, some recent studies found that 5 percent of the patients with Verruga peruana had no history of an acute febrile illness months before the skin eruptions. The acute febrile stage is rarely seen among the natives in endemic regions, on which Verruga peruana is common. Three different types of cutaneous manifestations were observed: (1) the miliary lesions typically consist of multiple 1–4 mm papular, erythematous, round lesions that are frequently associated with pruritus; (2) the nodular (subdermic) lesions appear as a subdermal nodule without changes in the overlying skin, without pruritus and usually few in number; and (3) the mular skin lesions are typically >5 mm in diameter, erythematous, and bleed easily. The verrugas vary in size and number and occur predominantly on the head and distal extremities. Lesions on the conjunctiva and on the nasal and oral mucosa have also been described (Maguina et al. 2001; Welch and Slater 1999).

In a population-based study, the peak prevalence of the appearance of new skin lesions occurred 1 month after the peak prevalence of the febrile illness. The incidence of Verruga peruana was highest in children aged 7–12 years (Kosek et al. 2000). The verrugas may persist for several months to years, but this stage is benign and self-limiting.

In the outbreak study performed by Kosek et al. (2000), 77.5 percent of the native population were found to have evidence of prior infection with *B. bacilliformis*. The seroprevalence ranged from 70.4 percent in children aged <6 years to 85.1 percent in patients aged >55 years. In the same study, the majority (56.4 percent) of seropositive patients reported no illness or new skin lesions during the past year. Serological investigations of healthy volunteers living in an endemic area for *B. bacilliformis* revealed that 45 percent of the people tested were seropositive (Chamberlin et al. 2000).

Public health control measures have included education in endemic areas and the use of insecticides. Visitors going to endemic regions for *B. bacilliformis* should be aware of preventive measures against bartonellosis, e.g. protection against exposure to sandflies, wearing long-sleeve shirts and pants, using insect repellent, and reducing outdoor activities around dusk and dawn when sandflies are most active.

Diagnosis

LABORATORY DIAGNOSIS

For the diagnosis of acute Oroya fever, thin-film blood smears are stained with Giemsa stain and examined by light microscopy for the presence of intra- or extra-erythrocytic coccobacillary bacteria (magnification $\times$1 000). Using bacterial isolation as the standard, sensitivity, specificity, and positive predictive value of thin blood smears were found to be 36, 96, and 44 percent, respectively (Ellis et al. 1999). The percentage of infected red blood cells range from 2 to 100 percent (mean 61 percent) (Maguina et al. 2001) (Table 3).

Only few data concerning the laboratory parameters during bartonellosis have been published. Increased levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin have been reported.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS

B. bacilliformis can be isolated from blood and tissue specimens (spleen, liver, lymph nodes, skin, cerebrospinal fluid (CSF)) on a variety of media containing fresh blood or hemoglobin (e.g. Columbia blood agar

Table 3 *Clinical symptoms in patients with Oroya fever (acute phase of bartonellosis) and verruga peruana (second, chronic stage of bartonellosis) (modified after Ellis et al. 1999 and Maguina et al. 2001)*

Symptom	Oroya fever (%)	Verruga peruana (%)
Fever	99–100	57
Malaise	99	53
Anorexia	93	40
Pallor	85–97	47
Headache	69–80	25
Prostration	69	–
Sweats	56	–
Cardiac murmur	78	6
Tachycardia	56	26
Cough	70	–
Dyspnea	26–45	–
Pulmonary rales	21	–
Emesis	41–65	–
Abdominal pain	46	–
Diarrhea	30	–
Icterus	55–71	1
Hepatomegaly	82	9
Splenomegaly	29	6
Lymphadenopathy	5–69	29
Myalgia	26–50	26
Arthralgia	35–41	47
Edema (pretibial)	20–29	10
Anasarca	9	–
Rash	5	–
Abnormal results of funduscopic examination	15	–
Verruga	0	100
Bleeding of verruga	–	66

–, not reported.

incorporating 10 percent horse blood; Colichon's medium consisting of soy agar plus sheep hemoglobin plus alanine). Cultures should be incubated at 25–28°C for at least 4 weeks in a humid atmosphere. In a study performed by Maguina et al. (2001), the mean time for positive blood cultures was 18 days (range 12–29 days) and isolates of *B. bacilliformis* were also obtained from lymph nodes and from CSF cultures. In the acute phase of bartonellosis, *B. bacilliformis* has been isolated from blood cultures in 71 percent of the cases, whereas in the eruptive-phase bartonellosis thin-film blood smears and blood cultures have been positive in 13 percent of the patients.

Morphologically, *B. bacilliformis* occurs in young cultures mainly in the form of short rods arranged singly, in pairs, in chains, clumps, or chains and clumps. In older cultures coccoid forms predominate. However, there is a considerable degree of pleomorphism. The rods are 0.3–1.5 μm long and 0.2–0.5 μm broad, motile and a tuft of at least ten flagella can be demonstrated at one pole. Ultrathin sections of red corpuscles examined by electron microscopy show that the organisms are situated inside the corpuscles, as well as on the surface, and that they have a trilaminar cell wall.

On solid media, growth occurs either in the form of minute, circular colonies, or as an opaque, finely granular, mucoid film that has a tendency to outgrow the original boundaries of the inoculum. *Bartonella bacilliformis* growth is strictly aerobic at a temperature of 25 to >28°C. It prefers a pH of 7.8, ferments no sugar and survives in semisolid medium at −70°C for years and at 25–28°C for several weeks (Weinman 1968). For biochemical reactions, see also Table 5.

POLYMERASE CHAIN REACTION AND GENOTYPING

Polymerase chain reaction (PCR) methods have been used in few studies to detect *B. bacilliformis* DNA in blood specimens. However, the sensitivity of this method for diagnosis of *B. bacilliformis* infection is currently unknown (Chamberlin et al. 2002). First genotyping studies of isolates obtained from different regions in Peru revealed six genotypes of *B. bacilliformis* (Birtles et al. 2002). Further studies are needed to improve our understanding of the epidemiology and the clinical importance of these different genotypes.

SEROLOGY

Detection of antibodies against *B. bacilliformis* seems to be more reliable in patients suffering from Verruga peruana than in patients with the acute phase of bartonellosis. Mallqui et al. (2000) have described an immunoblot test that uses sonication of whole *B. bacilliformis* and reported 94 percent sensitivity for patients with eruptive-phase bartonellosis and 70 percent sensitivity for patients with acute bartonellosis; in healthy individuals, the test showed 100 percent specificity. A recently developed indirect fluorescence antibody assay (IFA) showed a sensitivity of 82 percent in detecting *B. bacilliformis* antibodies in acute-phase blood samples and a sensitivity of 93 percent when convalescent-phase sera were tested. The specificity of the test was 92 percent and the positive predictive value was 89 percent (Chamberlin et al. 2000). Serological crossreactivity has been noted between *B. bacilliformis* and *Chlamydia psittaci* and has been attributed to similarities in the structure of the lipopolysaccharides (Knobloch et al. 1988).

HISTOPATHOLOGY

Histologically, verruga peruana is characterized by endothelial cell proliferation and neovascularization. Fibrosis and involution occur. In Giemsa-stained verruga biopsy sections, organisms may be seen in the cytoplasm of vascular endothelial cells. The bacteria are best demonstrated by Warthin–Starry staining. Electron microscopic studies have shown that *B. bacilliformis* was found abundantly in the extracellular space of the florid lesions and that no organisms were present in the late resolving subcutaneous nodules (Bass et al. 1997a; Arias-Stella

et al. 1986). The microbial factors responsible for the pathogenesis of these lesions are unclear.

Treatment and susceptibility to antimicrobial agents

B. bacilliformis organisms in vitro are highly susceptible to a large number of antibiotics, including β-lactams, aminoglycosides, chloramphenicol, rifampin, macrolides, tetracyclines, cotrimoxazole, and fluoroquinolones (Sobraqus et al. 1999).

Chloramphenicol, given in a daily dosage of 2 g or more for 10–14 days, was established as the drug of choice for the treatment of Oroya fever in the 1950s (Urteaga and Payne 1955). In a recent study, successful treatment of the acute phase of bartonellosis with chloramphenicol was seen in approximately 95 percent of patients. However, chloramphenicol-resistant cases have been observed and treatment with chloramphenicol did not eliminate the patient's risk of the subsequent development of the eruptive phase of bartonellosis. Successful treatment of acute bartonellosis has also been reported with other antibiotics, e.g. penicillin, streptomycin, erythromycin, tetracycline, and recently with ciprofloxacin (Maguina et al. 2001).

Rifampin at a dosage of 10 mg/kg/day for 10–14 days is the drug of choice for patients suffering from Verruga peruana. Alternatively, streptomycin at a dosage of 15 mg/kg/day for 10 days may be given, but rifampin seems to be more effective (80 percent good response) compared to streptomycin (56 percent good response) (Maguina et al. 2001). More recently, ciprofoxacin (500 mg twice a day for 7–10 days) was successfully used for the treatment of multiple eruptive-phase lesions in adults (Maguina and Gotuzzo 2000).

Pathogenesis

The flagella of *B. bacilliformis* are an important component to allow invasion of human erythrocytes (Scherer et al. 1993). In bacterial culture supernatants, a small water-soluble molecule, called deformin factor or deformin, could be identified and was shown to cause deformation and invagination of red cell membranes, as well as formation of intracellular vacuoles (Derrick and Ihler 2001). Recently, a protein of *B. bacilliformis* with hemolytic activity has been detected (Hendrix 2000).

INFECTIONS DUE TO *BARTONELLA HENSELAE* AND *BARTONELLA QUINTANA*

Clinical manifestations

B. HENSELAE AND CAT-SCRATCH DISEASE

Cat-scratch disease (CSD) was first described in 1950 by Debré, a French physician, who described several patients with suppurative lymphadenitis following cat scratches (Debré et al. 1950). Since then, a large number of viral and bacterial organisms have been suspected as etiologic agents for CSD, including *Chlamydiae*, acid-fast bacteria, *Pasteurella* spp., herpes simplex virus, feline bacteria and viruses, and finally in 1988 *Afipia felis* (English et al. 1988).

In 1992, *B. henselae* was first proposed as the etiologic agent of CSD (Regnery et al. 1992b, c) and thereafter a large number of serologic, culture-, and molecular-based data confirmed this conclusion. Currently, CSD is nearly exclusively associated with *B. henselae*. Reports suggesting additional etiologic agents still await confirmation.

However, a large number of other infective agents are known to cause lymphadenopathy, e.g. staphylococci, streptococci, *Haemophilus* spp., *Mycobacterium* spp., *Francisella tularensis*, *Brucella* spp., *Treponema pallidum*, *Leptospira* spp., *Chlamydia* spp., *Toxoplasma gondii*, *Borrelia* spp., Epstein–Barr virus, cytomegalovirus, *Histoplasma capsulatum*, and others (Ridder et al. 2002; English et al. 1988). All these agents should be considered in the differential diagnosis of infective lymphadenopathy. Lymphoma is the most common malignancy that might be misinterpreted as CSD. So, in cases with atypical clinical presentations, or when the serological results are not indicative, surgical extirpation of the lymph node may be required for histopathological investigations.

Cat-scratch disease occurs worldwide in all ages, in children and adults. Most cases occur in late fall and winter (October–March), when a closer and prolonged contact to indoor cats occurs. Patients with cat-scratch disease are likely to own a cat aged 12 months or younger, and to have been scratched or bitten by a kitten. These cats are often found to be infested with cat fleas. Cat-scratch disease occurring in two or even in three siblings has been reported and was often associated with the acquisition of a new kitten in the household (Gonzalez et al. 2003; Sander et al. 1998c).

Cat-scratch disease is the typical clinical manifestation of a *B. henselae* infection in immunocompetent patients and appears in two different forms.

Typical cat-scratch disease

Typical cat-scratch disease is a benign and self-limited illness. Between 25 and 60 percent of patients report a primary cutaneous granulomatous lesion of 0.3–1 cm in diameter at the site of inoculation (Figure 3a). In a recent study performed by Ridder et al. (2002) only 57 percent of all CSD patients had been exposed to cats, suggesting that other vectors (e.g. cat fleas or even other parasites) may play an important role in transmission of *B. henselae* to humans (Table 4). Of the 61 CSD patients, 54 percent were female and 46 percent were male, and the age range was 4–89 years (median 33.9 years). The primary skin lesions occur 3–10 days after injury (scratches or bites) and precede the onset of

Figure 3 (a) *CSD patient: Primary granulomatous lesion at the site of inoculation a few days after cat scratch.* **(b)** *CSD patient (same patient as presented in panel* **(a)***: Supraclavicular lymphadenitis followed 4 weeks after a cat scratch.*

lymphadenopathy by 1–3 weeks (range 7–60 days). Regional lymphadenpathy is the most common clinical manifestation of CSD and the site of lymphadenopathy depends on the site of inoculation (Figure 3b). The head and neck (28–43 percent) and the axillary (38–49 percent) lymph nodes are most often affected, followed by supraclavicular, inguinal, cubital, epitrochlear, and other localizations. Lymphadenopathy at more than one anatomical site appears in 37–59 percent of all cases. Lymph nodes may increase in size (usually 1–5 cm in diameter or even larger) and number for up to 4–6 weeks. The whole extent of lymphadenopathy can be best documented by ultrasonography (Ridder et al. 2003). The usual duration of lymphadenopathy is approximately 2–4 months, and in some cases even longer. Only one-third of all patients presents with fever up to 40°C in the first days (up to 3 weeks) of the illness. Headache, weakness, weight loss, nausea, vomiting, sore throat, pharyngitis, anorexia, and hepatosplenomegaly may occur. In addition, maculopapular eruptions, erythema nodosum, and thrombocytopenic purpura have been observed (Ridder et al. 2002; Regnery and Tappero 1995; Anderson and Neuman 1997; Bass et al. 1997b; Carithers 1985; Hamilton et al. 1995).

Late in the course of illness, 10–15 percent of the lymph nodes may suppurate. In a recent study acute lymphadenopathy was diagnosed in 64 percent of the CSD patients, 18 percent had suppurated lymph nodes and 18 percent had chronic lymphadenitis of more than 3 months of duration (Ridder et al. 2002).

In summary, CSD must be considered in all cases of regional (acute or chronic) lymphadenopathy. Exposure to cats or cat fleas is the main risk factor. Cat-scratch disease may be more prevalent than previously realized. Unnecessary biopsy of the lymph node may be avoided by use of adequate serological examinations.

Atypical cat-scratch disease

Atypical cat-scratch disease is characterized by unusual manifestations of CSD and occurs in 5–25 percent of all infections. Parinaud's oculoglandular syndrome, neuroretinitis, encephalitis, hepatic granulomas, osteomyelitis, endocarditis, and pulmonary disease may occur, but these complications usually resolve without sequelae.

OCULAR MANIFESTATIONS

Parinaud's oculoglandular syndrome (POGS) occurs in 2–17 percent of all patients and manifests as unilateral, granulomatous conjunctivitis and ipsilateral preauricular lymphadenopathy. POGS occurs after direct inoculation of the organisms into the eye. Recently, further ocular features of *Bartonella* infections have been reported. Mainly unilateral neuroretinitis and endophthalmitis with acute visual loss, optic nerve edema, and macular exudates are typical clinical manifestations (Wear et al. 1985; Grando et al. 1999; Dondey et al. 1997; Le et al. 1994; Kerkhoff et al. 1999b; Reed et al. 1998).

NEUROLOGIC MANIFESTATIONS

Neurologic manifestations associated with CSD occur in 2–3 percent of the patients. Encephalopathy, encephalitis, myelitis, radiculitis, and cerebral arteritis are the most frequently reported symptoms. In patients with CSD encephalopathy symptoms usually appear abruptly. Patiens may develop headaches, convulsions, and may become confused and disorientated. Status epilepticus in children has been reported. A history of cat exposure, regional lymphadenopathy, or a papule on the site of inoculation is often missed and is not essential for the diagnosis. Most patients have an abnormal electroencephalogram, but a normal computed tomographic scan of the brain, and mononuclear pleocytosis in the cerebrospinal fluid is found only in 20–30 percent (Carithers and Margileth 1991; Marra 1995; Noah et al. 1995; Easley et al. 1999).

DISSEMINATED CSD

Disseminated CSD has occasionally been described, presenting with a large variety of clinical symptoms and may be difficult to diagnose. Clinical manifestations

Table 4 *Clinical findings in patients with CSD (modified after Ridder et al. 2002; Tsukahara 2002; Hamilton et al. 1995; Margileth 1993)*

Reference	Ridder et al. (2002)	Tsukahara (2002)	Hamilton et al. (1995)	Margileth (1993)
% of patients	% (of 61)	% (of 619)	% (of 246)	% (of 1 852)
Department	ENT	–	–	Pediatrics
Symptoms of typical CSD:	–	85.6	–	–
Lymphadenopathy (LA) site:	100	92.2	100	100
Head and neck	100	41.6	43	80
Axilla	–	33	38	
Inguinal	–	15.9	20	–
Other sites	–	19.7	13	–
LA at >1 site	59	–	37	34
Acute LA	64	–	–	–
Suppurative LA	18	–	–	15
Chronic LA	18	–	–	–
Primary skin lesion	–	65.1	26	61
Pharyngitis	18	–	13	8
Fever	10	68.9	39	32
Malaise	10	–	34	30
Parotid gland swelling	6.6	–	–	–
Skin rash	3	–	–	5
Laryngitis	1.6	–	–	–
Headache	–	–	6	14
Arthralgia	–	–	–	3
Symptoms of atypical CSD:	–	14.4	–	–
Parinaud's syndrome	10	14.6	–	6–8
Encephalopathy	–	7.9	1	1.5–2.5
Systemic CSD	–	–	–	0.9–7
Neuroretinitis	–	36	–	0.9–7
Erythema nodosum	–	–	–	0.8
Thrombocytopenic purpura	–	2.2	–	0.4
Hepatosplenic involvment	–	13.5	–	0.3
Pneumonia (atypical)	–	–	–	0.2
Osteolytic lesions	–	1.1	–	0.2–1.5
Seizures/coma	–	–	–	3
Exposure to cats	57	75.6	–	95
Gender:				
Female	54	53.7	52	–
Male	46	46.3	48	–
Hospitalized patients	44	–	11	–
Deaths	0	–	0	–
Duration of illness (days)	–	–	–	14–730
Age (in years, mean)	33.9	23	14	–
range	4–89	1-81	1–64	–
⩾18 years	–	39.3	43	–

–, not reported; ENT, Ear, Nose, and Throat Department.

include persistent fever (fever of unknown origin (FUO)), hepatic and splenic abscesses, various skin lesions, arthritis, pneumonia, pleural effusion, thrombocytopenic purpura, and osteolytic lesions. Persistent fever, abdominal pain, and vomiting are important symptoms indicating visceral, especially hepatosplenic, involvement (Waldvogel et al. 1994; Dunn et al. 1997; Sander and Frank 1997; Jacobs and Schutze 1998; Arisoy et al. 1999; Robson et al. 1999; Ventura et al. 1999; Ruess et al. 2000).

B. QUINTANA, TRENCH FEVER, AND BACTEREMIA

Trench fever and its etiologic agent *B. quintana* was first described during the First World War. Very soon, Henry da Rocha Lima identified the body louse as the main

vector of transmission of this pathogen. After the First World War, the incidence of trench fever decreased, and the illness reappeared during the Second World War. Thereafter, living conditions improved and *B. quintana* was nearly forgotten for half a century. A reemergence of *B. quintana* infection was observed in recent years in homeless people and chronic alcohol ill patients. The major predisposing factors are living in poor hygienic conditions and infestation with body lice. In 1999, Brouqui et al. reported that 30 percent of homeless people at the University Hospital in Marseille had high antibody titers against *B. quintana*, 14 percent were bacteremic, and 7 percent had persistent bacteremia. *B. quintana* infections among homeless people and in patients with chronic alcoholism were also described in the United States (Jackson and Spach 1996; Spach et al. 1995). Asymptomatic, prolonged bacteremia (up to 78 weeks) or intermittent bacteremia in homeless people may occur. Treatment with gentamicin and doxycycline was effective in preventing relapses of bacteremia (Foucault et al. 2002). Headaches, severe leg pain, and sweats were the main symptoms associated with bacteremia. In homeless people, *B. quintana* might cause acute, severe illness, but chronic infection with few or no symptoms has often been reported.

It was demonstrated that *B. quintana* invades human erythrocytes. However, in infected people, the proportion of infected erythrocytes is very low, ranging between 0.001 to 0.005 percent without signs of anemia. Bacteria may be detected in erythrocytes by confocal microscopy. However, this method lacks sensitivity and the detection limit is about 5×10^4 colony-forming units (cfu) per ml. Infected erythrocytes each may contain up to five bacteria (Rolain et al. 2002).

Nonhemolytic intracellular colonization of erythrocytes is a unique strategy for bacterial persistence. It facilitates efficient transmission by the body lice and protects from host immune responses, as well as antimicrobial therapy.

BACILLARY ANGIOMATOSIS AND PELIOSIS HEPATIS (*B. HENSELAE* AND *B. QUINTANA*)

In immuncompromised, predominantly in human immunodeficiency virus (HIV)-positive patients, infection with *Bartonella* species manifests with vascular proliferative lesions of the skin, known as bacillary angiomatosis (BA). Skin lesions are solitary or multiple (up to 1 000) and are usually red to purple. They develop from small papules to form nodules or tumors which resemble pyogenic granulomas, hemangiomas, or Kaposi's sarcoma. The lesions are occasionally also found in the lymph nodes, liver or spleen, known as bacillary peliosis hepatis, but rarely found in other organs such as bones, brain, and bronchi (Koehler et al. 1992; Koehler and Tappero 1993). Clinical symptoms like fever, chills, night sweats, vomiting, and weight loss are most often reported along with extracutaneous manifestations. HIV-infected patients with BA are severely immunocompromised and the disease is classified as an AIDS-defining opportunistic infection (Mohle-Boetani et al. 1996). The prevalence of *Bartonella* infection among febrile HIV-infected patients was demonstrated to be 18 percent, and should be considered in the differential diagnosis for patients with fever and late-stage HIV disease (Koehler et al. 2003).

Bartonella henselae and *B. quintana* are equally likely to cause cutaneous bacillary angiomatosis. However, there is a strong predilection for *B. henselae* to cause hepatosplenic and lymph node disease (similar to the organs involved in immunocompetent CSD patients), whereas *B. quintana* is more closely associated with osseous and subcutaneous infections (Koehler et al. 1997).

ENDOCARDITIS (MAINLY *B. HENSELAE* AND *B. QUINTANA*)

Blood culture negative endocarditis occurs between 5 and 30 percent of all cases of endocarditis. Recently, *Bartonella* species have been recognized as one of the various causes of blood culture-negative endocarditis. Recent studies have shown that *Bartonella* endocarditis represents 3 percent of all cases of endocarditis (Raoult et al. 1996; Sander et al. 2001a).

In 1993, Spach et al. reported the first case of endocarditis caused by *B. quintana* in an HIV-infected patient, Hadfield el al. (1993) described the first case of endocarditis caused by *B. henselae* and Daly et al. (1993) the first case of *B. elizabethae* endocarditis. Since then, nearly 100 case reports of *Bartonella* endocarditis have been reported in the literature.

Since *Bartonella* spp. are fastidious and very difficult to culture, serologic investigations for antibodies against *Bartonella* sp. might give the first indication of the presence of *Bartonella* endocarditis and should be included in the diagnostic work up of blood culture negative endocarditis. Endocarditis caused by *B. quintana* is mostly reported in homeless and alcoholic patients with no previous cardiac history, whereas *B. henselae* endocarditis is mostly found in patients with previously known valvular heart disease and contact with cats and/or cat fleas (Fournier et al. 2001). The pathologic changes of the heart valves are suggestive of prolonged infection. Compared with other cases of infective endocarditis, cases of *Bartonella* endocarditis are more fibrotic and calcified, less vascularized, with less extensive vegetation and chronic inflammation (Lepidi et al. 2000).

Diagnosis

The diagnosis of CSD is primarily based on clinical examination and should be suspected in cases of regional lymphadenopathy, primary skin lesions, and exposure to cats or cat fleas. The patients are usually in

good general condition and only about two-thirds of them are afebrile.

LABORATORY DIAGNOSIS

Routine laboratory studies do not provide important clues for the diagnosis of CSD. The illness is usually associated with a normal or slightly elevated white blood cell count. The erythrocyte sedimentation rate and the C-reactive protein may be increased. All other routine laboratory tests are usually within normal ranges. In cases complicated with hepatosplenomegalia, liver function tests may show increased concentrations of alkaline phosphatase, aspartate aminotransferase, and γ-glutamyltransferase.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS

Isolation of *B henselae* from lymph nodes of CSD patients has rarely been reported in the literature and seems not to be a reliable method for the diagnosis of CSD. In most studies, cultures of lymph nodes extracted tissue from CSD patients remained sterile. However, a recent study from Australia reported the isolation of *B. henselae* strains from 20.5 percent of 83 PCR-positive human specimens (Fournier et al. 2002b). In contrast, most isolates of *B. henselae* obtained worldwide have been collected from blood cultures of their natural reservoir, the cat. *Bartonella* isolates of human origin are usually obtained from blood and tissue cultures of immunocompromised patients suffering from bacillary angiomatosis or peliosis hepatis.

Since *Bartonella* species may be intracellular and reside within erythrocytes, blood cells lysis by saponin or by freezing improves their recovery. If EDTA-blood tubes are stored frozen at −65°C for several days, recovery of *Bartonella* isolates is even higher than that from the Isolator™ tubes (Brenner et al. 1997). However, using the lysis-centrifugation method (Isolator™ tubes, Wampole, Cranbury, NJ, USA) is more effective than other conventional automated blood culture systems, e.g. Bact/Alert™ (Organon) (Tierno et al. 1995). If conventional blood culture systems are used, blind subcultures on solid media are required, because the organisms produce little or no CO_2 and bacterial growth will not be indicated. In addition, *Bartonella* species can be detected by routine acridine orange staining of aliquots from aerobe bottles after 8 days of incubation (Larson et al. 1994). Acridine orange stain is known to be more sensitive than the Gram stain. Subcultures with prolonged incubation times (up to 4 weeks) on chocolate agar or Columbia agar base are required for isolation of the strains.

Growth of *Bartonella* species on solid media is hemin dependent. *B. henselae* and *B. quintana* grow well on freshly prepared chocolate agar, Columbia blood agar, brain–heart infusion agar, or trypticase soy agar, supplemented with 5–10 percent sheep blood or rabbit blood. Plates should be incubated in a humid atmosphere containing 5–10 percent CO_2 at 35–37°C for at least 4 (to 8) weeks. On solid media, first visible growth does not occur before 6 to 12 days of incubation. Primary colonies may be very heterogeneous, and appear as very small, smooth colonies and/or as rough, irregular, and cauliflower-like colonies. They are whitish-cream and are usually highly adherent and deeply embedded into the agar. Initially, subcultivation might prove difficult and there is a risk of losing the isolate. However, after three to four subpassages, the organisms become adapted, the colonies become smooth and less adherent, and growth occurs within 3–4 days (Sander 2001).

Isolation of organisms from tissue samples may require co-cultivation in cell cultures such as the human endothelial cell line ECV304 described by Drancourt et al. (1995) or a bovine pulmonary artery endothelial cell line detailed by Koehler et al. (1992), before plating on to solid agar media.

Identification of *Bartonella* species in the routine microbiology laboratory might be difficult. All *Bartonella* species are catalase-, oxidase-, indole-, esculin-, and citrate-negative, and they do not produce acid from carbohydrates such as glucose, lactose, maltose, mannitol, sucrose, fructose, inositol, mannose, raffinose, or rhamnose. Conventional biochemical tests are of little use in the differentiation of the organisms and commercially available identification systems do not contain *Bartonella* species in their database. However, anaerobic identifications systems (e.g. RapID ANA II, Rapid ID 32 A, MicroScan rapid anaerobic identification system, An-IDENT System) are shown to be useful for detection of preformed enzyme profiles for *Bartonella* spp. (Regnery et al. 1992a; Clarridge et al. 1995; Sander 1998). Whole-cell fatty acid analysis (CFA) is useful for identification of *Bartonella* strains at the genus level and there are some characteristic amounts of fatty acid compositions for some species (e.g. *B. elizabethae*, *B. bacilliformis*). However, the different *Bartonella* species are not available in the database and differentiation between *B. henseale*, *B. quintana*, and *B. clarridgeiae* by this method is not possible (Clarridge et al. 1995; Sander 1998). Table 5 details the differential characteristics of *Bartonella* species. There are only small differences between *B. henselae* and *B. quintana* in all diagnostic tests available, and these two species can be distinguished from each other only by molecular based methods. *Bartonella clarridgeiae* differs from *B. henselae* and *B. quintana* by the production of β-NAG in the Rapid ID 32 A test (Figure 4).

POLYMERASE CHAIN REACTION AND GENOTYPING

PCR amplification of a *Bartonella* DNA fragment from human specimens is widely used to diagnose *Bartonella* infections. Several different PCR-based assays have been described using primer pairs with different target

Table 5 *Biochemical reactions of human* Bartonella *species*

	B. bacilliformis	*B. quintana*	*B. henselae*	*B. clarridgeiae*	*B. elizabethae*
Characteristics					
Hemolysis	+	–	–	–	–
Flagella	+	–[a]	–[a]	+	–
Optimal growth temperature (°C)	25–30	35–37	35–37	35–37	35–37
Growth at:					
25°C	+	–	–	–	–
30°C	+	+	+	+	+
36°C	–	+	+	+	+
42°C	–	–	–	–	–
Catalase, oxidase, indole, urea, nitrate reduction, citrate, esculin hydrolysis, gelatine hydrolysis, acid production from carbohydrates	–	–	–	–	–
%Cellular fatty acids:					
16:1 ω7*c*/15 *iso* 2OH	17–21	0–1	0–1	0–1	1
16:0	22–28	15–23	16–23	11–19	24–25
17:0	0	0–5	0–3	0–3	14–19
Summed feature 7 (18:1 ω7*c*/ω9*t*/ω12*t*)	40–52	48–56	48–59	39–68	38–41
18:0	2	16–32	19–36	14–26	11–12
Biotype codes of commercial systems					
RapID ANA II	000 64(7)1	000 671	000 671	004 671	000 671
Rapid ID 32 A	0000 0737 05	0000 0737 05	0000 0737 05	0001 05(7)37 05	0000 0737 05
Rapid -Strep	0040000	0040000	0040000	0040000	0040000

+, positive reaction; –, negative reaction; n.d., not done.
a) Possess pili and may show twitching motility in wet preparations.
Modified after Sander 1998; Sander 2001

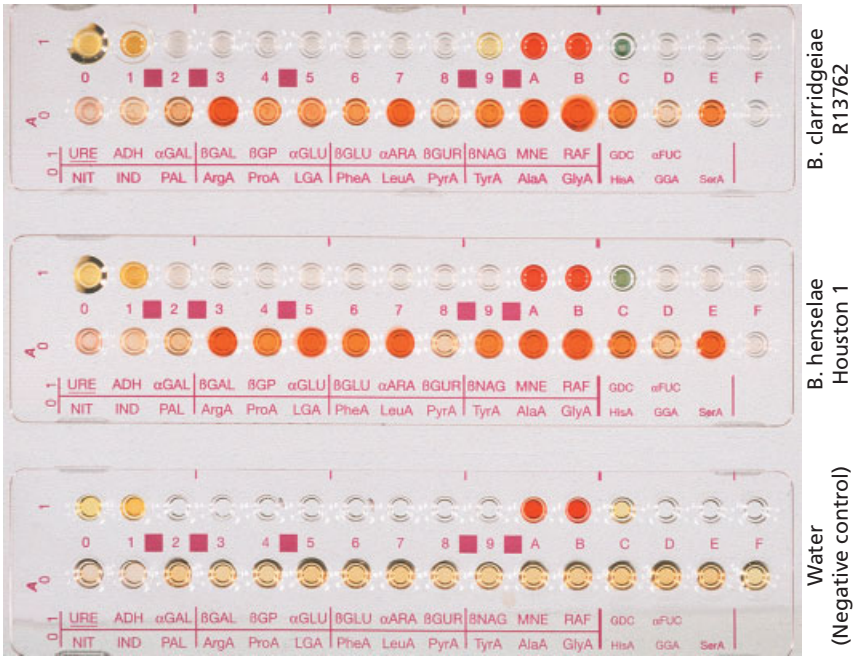

Figure 4 *Biotype codes of* B. clarridgeiae *and* B. henselae *in the Rapid ID 32 A system (bioMrieux). Cultures were grown on chocolate agar plates for 3–4 days; Inoculum size: McFarland 5. Negative control (c).*

genes. However, large differences were found in the sensitivity of these assays, depending on the primers used and on whether fresh (sensitivities ranging from 60 to 100 percent) or formalin-fixed, paraffin-embedded (sensitivities ranging from 43 to 78 percent) tissues were investigated (Sander et al. 1999).

Using a semiquantitative, species-specific PCR enzyme immunoassay, the approximate number of cfu in clinical specimens from patients with CSD or bacillary angiomatosis peliosis (BAP) were calculated to range between 10^3 and 10^6 cfu/ml (Sander and Penno 1999).

A recent study has shown that the results of PCR-based techniques in CSD strongly depend on the duration of disease. *Bartonella henselae* DNA was detected in the extirpated lymph nodes only within the first 6–9 weeks after onset of lymphadenopathy (Ridder et al. 2002). Since CSD lymphadenopathy may last for months, negative PCR results and a positive serology is common in the later stage of the illness.

Based on investigations of different genetic regions, two different genotypes (I and II) of *B. henselae* have been found to be involved in CSD. In different geographic regions, different *B. henselae* genotypes seem to be predominant. In Australia, Germany, and The Netherlands the majority of *B. henselae* isolates from cats belong to genotype II, whereas isolates from humans are mostly identified as genotype I (Fournier et al. 2002b; Sander et al. 1999; Bergmans et al. 1996). In contrast, in Switzerland and France, both cat and human *B. henselae* isolates mainly belong to genotype II (Box et al. 1996; Zeaiter et al. 2002).

However, no differences in the clinical presentation of CSD were found for the different genotypes. In contrast, a recent study suggested that the two *B. henselae* genotypes determined by pulsed field gel electrophoresis (PFGE) may induce different pathological features in HIV-infected patients. Genotype I infected patients were found more likely to have vascular proliferative lesions of the liver and/or spleen than patients with genotype II infections (Chang et al. 2002).

Comparing several different typing techniques for molecular typing of *B. henselae* strains, such as PFGE, repetitive extragenic palindromic (REP)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, or arbitrary primed (AP)-PCR, PFGE was shown to provide the highest discriminatory potential for subtyping of *B. henselae* strains (Sander et al. 1998b).

SEROLOGY

Until recently, CSD was diagnosed by exclusion or by a positive cat scratch disease skin test reaction. However, this test was never standardized, was not commercially available, and is no longer recommended for the diagnosis of CSD. In the last few years, reliable serological tests and molecular based methods have been established worldwide.

Serological testing for *Bartonella* antibodies should be the first step in the diagnosis of typical and atypical CSD and in suspected *Bartonella* endocarditis. A primary negative serological test result does not necessarily exclude a current infection. In some patients, seroconversion may not occur until 3 weeks after onset of disease (Dalton et al. 1995).

Different serological methods, e.g. indirect fluorescence assay (IFA), enzyme immunosorbent assay (EIA), and Western blot have been established for the diagnosis of *Bartonella* infections. The antigen preparation seems to be essential for these tests and antigen extracted from plate-grown *Bartonella* species may lead to false negative results.

IFA is currently the most common test used. It is commercially available and based on antigens prepared from organisms co-cultivated in cell cultures (e.g. Vero cells, human endothelial cell line ECV 304). Comparing different indirect fluorescence assays (e.g. slides from BION Enterprises, Park Ridge Chicago, IL, USA; Focus Technologies (formerly MRL Diagnostic), Cypress, CA, USA; or in-house slides) it was shown that the commercial slides are higly sensitive (85–100 percent), but less specific (73–87 percent) (Maurin et al. 2002; Sander et al. 1998a; Zbinden et al. 1997). Slides for specific detection of *B. henselae* and *B. quintana* are available. However, many studies have demonstrated that serological differentiation between *B. henselae* and *B. quintana* infections is definitely not possible, due to the very high cross-reactivity between both species (95–100 percent). In some cases, titers against *B. quintana* are even higher than those against *B. henselae* in proven *B. henselae* infections (Dalton et al., 1995; La Scola and Raoult 1996; Sander et al. 1998a; Maurin et al. 2002). Similar results have been reported for proven *B. quintana* infections, where 68 percent of patients exhibited higher titers of antibodies to *B. henselae* than to *B. quintana* (Fournier et al. 2002a).

The prevalence of antibodies to *B. henselae* is very high in particular geographic regions. Up to 30 percent of the adult population has detectable IgG antibodies (titers of 1:64 to 1:256) in Europe and no differences between cat owners have been reported (Rath et al. 1997; Zbinden et al. 1997; Sander et al. 1998a). In the United States the seroprevalence in the general population is reported to be 4–6 percent (Zangwill et al. 1993), but healthy family members of patients with CSD may be seropositive in about 30 percent of cases (Demers et al. 1995). Finally, the prevalence of antibodies to *Bartonella* depends on the age of the individuals investigated. A lower seroprevalence of 3.5–13 percent (depending on the test used) was found in children (Sander et al. 2001b).

Due to the high seroprevalence in the general population, the diagnosis of CSD may be difficult in some cases. In general, IFA titers of <1:64 suggest that the patient does not have a current *Bartonella* infection. IgG

antibodies in CSD patients are usually high (⩾1:512), whereas low antibody titers between 1:64 and 1:256 are found not only in CSD patients, but also in up to 30 percent of healthy controls. Therefore, low antibody titers might indicate the beginning or the end of CSD, but may also indicate only prior exposure to *Bartonella* spp. In such cases, a titer increase in a second serum sample (collected 2–3 weeks later) may confirm the diagnosis. Only seroconversion, high IgG titres of ⩾1:512, or a four-fold titer increase in a second serum sample are indicative of CSD. Specific IgM antibodies are less frequently detected in CSD patients and lack of IgM does not exclude a *Bartonella* infection (for a review see Sander et al. 2001b). *Bartonella* endocarditis usually evokes high IgG antibody titers (>1:800). Thus, in cases of culture negative endocarditis high antibody titers may therefore be considered diagnostic (Fournier et al. 2002a).

Western blot analysis and enzyme-linked immunoassays have been described for diagnosis of *Bartonella* infections. Based on whole cell antigen of *B. henselae*, both tests lack sensitivity and specificity, and cross-reactions with different other bacterial infections have been described (*Ehrlichia chaffeensis*, *Mycoplasma pneumoniae*, *Escherichia coli*, *Francisella tularensis*, *Treponema pallidum*, *Chlamydia* spp., *Coxiella burnetii*, and *Rickettsia rickettsii*) (McGill et al. 1998; La Scola and Raoult 1996; Maurin et al. 1997). Western blotting and crossadsorption of the sera with *B. henselae*- and *B. quintana*-antigen suspensions (shaken for 24 h at 4°C) was described to allow identification of the *Bartonella* species in cases of infective endocarditis on a serological basis (Houpikian and Raoult 2003).

Under circumstances where serological testing and the clinical presentation of the patient do not clearly indicate the diagnosis of CSD, fine needle aspiration or surgical removal of the lymph node for histopathological and molecular-based diagnostic procedures is strongly recommended to rule out more severe diseases such as lymphoma or mycobacterial infection. Using a monoclonal antibody against *B. henselae*, which reacts with a *B. henselae* specific 43-kDa epitope, the CSD agent was recently detected by immunofluorescence in lymph node smears of CSD patients (Rolain et al. 2003).

HISTOPATHOLOGY

Histopathological examination of the lymph nodes from patients with CSD shows three evolutionary stages: (1) enlargement of the lymph node caused by the expansion of the cortex and hyperplasia of the germinal centers with epithelioid granulomas and Langhans' giant cells; (2) enlarged granulomas, which become infiltrated with polymorphonuclear leukocytes developing central necrosis with progressive suppuration; and (3) development of pus-filled sinuses and rupture of the capsule may occur. The histopathological presentation of CSD is very similar to those of other granulomatous diseases, e.g. sarcoidosis, infectious mononucleosis, mycobacterial infections, lymphogranuloma venereum, brucellosis or tularemia (Kaschula 1996). Detection of specific bacterial DNA by PCR in any biopsy specimens and serological investigations may be helpful to avoid misdiagnosis in such cases.

Histological findings of BA consist of tumor-like capillary proliferations with plump endothelial cells that protrude into the vascular lumen. These changes are very similar to those seen in verruga peruana and may resemble lesions from Kaposi's sarcoma, pyogenic granuloma, epithelioid hemangioma, and angiosarcoma.

Detection of the *Bartonella* bacilli by Warthin–Starry silver staining of fixed tissue sections in patients with CSD during the early stages of lymphadenopathy was demonstrated, but not in the later granulomatous stage of the illness. In a study performed by Demers et al. (1995), *Bartonella* bacilli were found by this method in only 12.5 percent (four of 32) of patients with CSD and in four of seven patients with BA (Gasquet et al. 1998). In a study performed by Fournier et al. (2002a) argyrophilic organisms have been detected by Warthin–Starry staining in 50 percent of PCR-positive lymph nodes.

In summary, detection of *Bartonella* spp. by culture (extremely unreliable), by Warthin–Starry staining (very insensitive), as well as detection of *Bartonella* DNA by PCR (highly specific, sensitivity depends on the duration of illness) in lymph nodes of patients with CSD seems to be successful only in the first weeks of lymphadenopathy. The early disappearance of the bacteria in the enlarged, persistent lymph nodes, illustrate the importance of highly sensitive and specific serologic methods in the diagnosis of CSD. In more severe and atypical cases, other diseases (e.g. lymphoma) should be excluded by histological examination of the lymph nodes and/or by other additional tests.

Treatment and susceptibility to antimicrobial agents

In vitro, all *Bartonella* isolates were highly susceptible to most antibiotics tested, including macrolides, tetracyclines, aminoglycosides, fluoroquinolones, β-lactams (penicillin G, aminopenicillins, third-generation cephalosporins), trimethoprim-sulfamethoxazole, and rifampin. Vancomycin, which is known to be effective against gram-positive bacteria, shows minimum inhibitory concentration (MIC) close to the breakpoint of 4 mg/l. However, in vivo, treatment of *B. henselae* or *B. quintana* infections may be difficult. Penicillins and first-generation cephalosporins are not effective.

Cat-scratch disease is a self-limiting disease and the effectivity of antimicrobial therapy remains unclear.

In cases of typical CSD, antibiotic therapy is usually not indicated. Antibiotic treatment should be considered in patients with prolonged fever and severe systemic symptoms (usually atypical CSD). Macrolides (erythromycin, azithromycin), tetracyclines, rifampin, and in some cases ciprofloxacin, have been shown to be effective and are considered as the drug of choice for the treatment of CSD. Unfortunately, only one prospective, randomized, double-blind and placebo-controlled study on the treatment of CSD is reported (Bass et al. 1998). Oral azithromycin taken for 5 days (500 mg in the first day, and 250 mg on days 2–5) was shown to be effective (decrease in the size of the lymph node) within the first 4 weeks of treatment. However, new enlarged lymph nodes or lymph nodes increazing in size may appear despite therapy. In both the azithromycin-treated and the placebo-treated group, the lymph nodes disappeared within 4 months.

In contrast to CSD, antimicrobial therapy in immunocompromised patients is absolutely indicated. In patients suffering from BA or peliosis hepatis, erythromycin at a dose of 250–500 mg, four times/day is considered the drug of choice. Newer macrolides and tetracyclines are also effective. The duration of treatment should be at least 2 months, in some cases even longer (Spach and Koehler 1998).

In patients suffering form *Bartonella* endocarditis, surgical valve replacement could not be prevented in most cases despite antbiotic treatment.

Pathogenesis

Bartonella infections are vector-borne diseases. Many species were shown to multiply and persist in red blood cells of their natural hosts. *Bartonella henselae* was found in red blood cells of cats (Kordick and Breitschwerdt 1995; Rolain et al. 2001), *B. tribocorum* in those of rats (Schülein et al. 2001), and *B. quintana* (Rolain et al. 2002) and *B. bacilliformis* (Benson et al. 1986) were shown to invade human erythrocytes. The ability of *Bartonella* to persist in erythrocytes for long periods increases its transmissibility by blood sucking arthropods (cat flea, human body louse, sandfly). On solid media, growth of *Bartonella* species is strongly hemin dependent. For *B. henselae* it was shown that the bacteria are unable to synthesize heme itself. The heme precursor protoporphyrin IX alone, or in combination with $FeCl_2$ or $FeCl_3$, as well as transferrin or lactoferrin, do not support growth (Sander et al. 2000b). A large number of different outer membrane proteins involved in the utilization of heme compounds are known. Recently, Carroll et al. (2000) detected nine different outer membrane hemin-binding proteins from *B. quintana* by hemin blots, including a 30-kDa protein, which showed a close homology to the phage-associated membrane protein Pap31 of *B. henselae* (Bowers et al. 1998). It was demonstrated that the *pap31* gene of *B. henselae* was necessary and sufficient to restore growth of a heme-deficient mutant of *E. coli* (Zimmermann et al. 2003). Pap31 seems to be one of the hemin-binding proteins involved in heme acquisition and may be an important virulence factor in this human pathogen.

A second typical pathogenic feature of *Bartonella* spp. is the ability to induce endothelial cell proliferation and to trigger neoangiogenesis, as observed in bacillary angiomatosis (*B. henselae* and *B. quintana*) and verruga peruana (*B. bacilliformis*). *Bartonella* sp. can provoke angioproliferation by at least two independent mechanisms: directly by triggering proliferation and inhibiting apoptosis of endothelial cells; and indirectly, by stimulating the infected host cells to produce VEGF, the angiogenic vascular endothelial growth factor (Kempf et al. 2001; Schweyer and Fayyazi 2002; Kirby and Nekorchuk 2002; Dehio 2003).

Endothelial interactions involve a unique mode of cellular invasion, the activation of proinflammatory responses and the formation of vasoproliferative tumors (Dehio 2001). *Bartonella*–endothelial cell interaction is not restricted to angiogenesis stimulation. Invasion of endothelial cells was shown for *B. quintana*, *B. henselae*, and *B. bacilliformis* and a proinflammatory activation of endothelial cells was postulated to be a result of receptor–ligand interactions between the activated endothelium and circulating neutrophils (Greub and Raoult 2002). Further research is needed to elucidate the interactions between these pathogens with erythrocytes and endothelial cells.

INFECTIONS CAUSED BY OTHER *BARTONELLA* SPECIES

Bartonella henselae, *B. quintana*, and *B. bacilliformis* are the most common *Bartonella* species associated with human diseases. In recent years, further *Bartonella* species were reported to be pathogenic to humans, most of them isolated from blood cultures of patients suffering from cardiac diseases.

Bartonella elizabethae was first described in 1993 by Daly et al. (1993) and was isolated from a 31-year-old man with fever (39°C) and endocarditis. He reported a 2-month history of fatigue, malaise, anorexia, and shortness of breath accompanied by a weight loss of 9 kg. A two-dimensional echocardiogram revealed large vegetations on the aortic valve and severe aortic regurgitation. Despite therapy with intravenous nafcillin and gentamicin, and lack of fever after 48 h of therapy, he developed increasing congestive heart failure and the aortic valve had to be replaced surgically.

A second case report with serologic evidence (titer 1:128) of a *B. elizabethae* infection was presented in 1998 by O'Halloran et al. from a patient suffering form Leber's neuroretinitis.

Two recent case reports implicated *Bartonella clarridgeiae* as another possible agent of CSD. Based solely on the detection of antibodies, Kordick et al. (1997)

presented the first report of CSD supposed to be caused by *B. clarridgeiae*. A veterinarian was bitten by a kitten and presented symptoms similar to CSD. Initial cultures from the patient and the cat were negative. *Bartonella clarridgeiae* was isolated from the blood of the cat at 1.5 months and again at 4 months after the initial event. The patient developed fever, headache, and lymphadenopathy within 3 weeks of the bite. He did not seroconvert to *B. henselae*, *B. quintana*, or *B. elizabethae* antigens, but demonstrated a reciprocal titer of 1024 against the *B. clarridgeiae* cat isolate.

The second case was presented by Margileth and Baehren (1998). The authors describe a patient with an extensive axillary and chest-wall abscess and the diagnosis based on a seroreactivity only to *B. clarridgeiae*, at a titer of 1:128. However, a culture of the drainage yielded *Streptococcus pneumoniae*. Unfortunately, no molecular based diagnostic tests were applied to confirm the presence of *B. clarridgeiae* DNA in the abscess mass and a possible role of the isolated *S. pneumoniae* in the etiology of this abscess cannot be ignored.

Lack of information on the seroepidemiology of *B. clarridgeiae* complicates an assessment of the organism's true role in infection. Up to now, no culture-proven, or at least PCR-proven, *B. clarridgeiae* infection in humans has been reported and serologic crossreaction between *B. henselae* and *B. clarridgeiae* is seen by IFA. In contrast to *B. henselae*, *B. clarridgeiae* is flagellated. Based on the 41-kDA FlaA protein, an immunoblot was developed and antibodies against this protein were detected in 3.9 percent of patients suffering from lymphadenopathy (Sander et al. 2000a). However, whether antibodies to the FlaA protein of *B. clarridgeiae* are a useful indicator of acute infection remains to be clarified. Future studies are needed to define this organism better as the causative agent of CSD.

Bartonella grahamii was recently detected by PCR analysis from the intraocular fluid of a 55-year-old HIV-negative patient suffering from bilateral neuroretinitis and behavioral changes. The patient was successfully treated with doxycycline (200 mg/day) and rifampin (600 mg/day) for 4 weeks (Kerkhoff et al. 1999a).

B. vinsonii subsp. *berkhoffii* is known to cause bacteremia and endocarditis in dogs. In 2000, Roux et al. described a 35-year-old male patient with a history of feeling unwell for a month, shortness of breath, and chest pain for a week. The patient had both a cat and a dog and was exposed to several other animals. C-reactive protein and erythrocyte sedimentation rate were elevated. Transthoracic echocardiography showed a dilated left ventricle and a bicuspid aortic valve with thickened cusps and significant regurgitation. The patient was afebrile, all blood cultures collected were negative, and no antibiotics were administered. After 3 weeks the patient underwent an aortic valve replacement and *B. vinsonii* subsp. *berkhoffii* was detected by PCR. Anti-*Bartonella* antibodies against *B. henselae* were 1:200, against *B. quintana* 1:400, and against *B. vinsonii* subsp. *vinsonii* 1:100.

Bartonella vinsonii subsp. *arupensis* was isolated in 1994 from the blood culture of a 62-year-old Caucasian cattle rancher from Wyoming, presenting with acute onset of fever (38.3°C), bacteremia, and a relapse of the vasculitis and the associated neurologic manifestations of his Sjögren's syndrome, diagnosed in 1960 (Welch et al. 1999).

Bartonella washoensis was first isolated in 1995 by Regnery from the blood culture of a 70-year-old male resident of Washoe Country, Nevada, who developed fever and myocarditis. The sequences of three genes of this novel species were submitted to GenBank in 1998 and in 2003 the same species was isolated from a California ground squirrel, suggesting that these animals may serve as a reservoir for human infections (Kosoy et al. 2003).

In recent years the recognized diseases caused by *Bartonella* spp. increased continuously. As more laboratories are engaged in the study of these organisms, the spectrum of disease will continue to expand.

AFIPIA

THE GENUS *AFIPIA*

Epidemiology and clinical significance

Detection of *Afipia* species from human specimens is extremely rare and was initially related to CSD. In 1988, *Afipia felis* was first isolated from the lymph nodes of ten patients suffering from CSD (English et al. 1988). Three additional isolates of *A. felis* have been cultured from lymph node aspirates of two children by Brenner et al. (1991). The same authors described two other *Afipia* species (*A. clevelandensis* and *A. broomeae*), as well as three unnamed *Afipia* genospecies (1, 2, and 3). The name *Afipia* is derived from the acronym AFIP, for Armed Forces Institute of Pathology, where the type species was isolated. A further isolate of *Afipia felis* was cultured from a lymph node of a CSD patient in 1998 by Giladi et al. However, in the same study 32 lymph node specimens of CSD patients tested positive for *B. henselae* by PCR, but negative for *A. felis*.

Indirect evidence suggests that *A. felis* may indeed be associated with some human infections. Drancourt et al. (1992) reported a patient with acute meningoencephalitis who seroconverted to positivity for *A. felis* antibodies. Antibodies against *A. felis* have been demonstrated in 17 percent of CSD patients (Fumarola et al. 1994). However, Amerein et al. (1996) found no differences in *A. felis* antibody titers among CSD patients and controls.

Since the first description of *A. felis*, a large number of serological, molecular based, and cultural methods demonstrated that *B. henselae* and not *A. felis* is the main etiologic agent of CSD worldwide. Additionally, there is

Table 6 *Currently known* Afipia *species and the source of isolation*

Species	Source	Reference
A. felis ATCC 53690	Lymph nodes, lymph node aspirates	Brenner et al. (1991); English et al. (1988)
A. clevelandensis ATCC 49720	Tibial biopsy (nosocomial infection)	Brenner et al. (1991); Hall et al. (1991)
A. broomeae ATCC 49717	Sputum	Brenner et al. (1991)
A. broomeae ATCC 49718	Bone marrow	Brenner et al. (1991)
A. broomeae ATCC 49719	Synovium, wrist abscess	Brenner et al. (1991)
Afipia genospecies 1 ATCC 49721	Pleural fluid	Brenner et al. (1991)
Afipia genospecies 2, ATCC 49722	Bronchial washing	Brenner et al. (1991)
Afipia genospecies 3, ATCC 49723	Environmental water	Brenner et al. (1991)
Afipia genospecies 3-related strain (CIP 106343)	Water supply of a hospital	La Scola et al. (2000, 2002)
Afipia birgiae (CIP 106344)	Water supply of a hospital	La Scola et al. (2000, 2002)
Afipia massiliensis (CIP 107022)	Water supply of a hospital	La Scola et al. (2000, 2002)
Afipia felis genospecies A (CIP 106335)	Water supply of a hospital	La Scola et al. (2000, 2002)

ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur, Paris, France.
Modified after Brenner et al. 1991 and La Scola et al. 2002

no evidence indicating that cats represent a reservoir for *A. felis* as they do for *B. henselae*. Therefore, the term 'cat-scratch-disease' should be reserved for infections caused by agents which indeed are transmitted by cats.

The natural reservoir for *A. felis* was unknown for a long time. Recently, successfully isolation of *A. felis* and other *Afipia* species from hospital water in an amoebae co-culture procedure was reported (La Scola and Raoult, 1999; La Scola et al. 2002). Therefore, it cannot be excluded that the reported isolation of *A. felis* from lymph nodes in the three institutions might have been caused by water contamination. However, the clinical relevance of *Afipia* species remains to be further elucidated, and hospital-aquired infections might occur. These organisms may play a role as opportunistic pathogens in respiratory and wound infections in elderly patients and in those with severe underlying medical problems. The currently described *Afipia* species are presented in Table 6.

Cultural and biochemical characteristics

Members of the genus *Afipia* belong to the α-2 subgroup of the Proteobacteria and are gram-negative, oxidase-positive, urease-positive, weakly catalase-positive rods. *Afipia* species have been shown to have similar axenic growth requirements to *Legionella* species and co-culture studies demonstrated that *A. felis* has the ability to grow associated with amebae, indicating that they may exist in aquatic environments (La Scola et al. 2000).

Afipia species appear to be relatively fastidious and difficult to cultivate in the routine laboratory. Original reports on isolation of *A. felis* described successful growth using ground lymph node tissue in brain–heart infusion broth and on brain–heart infusion agar (BHIA) (English et al. 1988; Giladi et al. 1998). In primary cultures, visible growth occurs after 1–2 weeks of incubation at 30–32°C without CO_2. Subcultures should be performed on BHIA or on buffered charcoal yeast extract agar (BCYE) and after several passages, the time to detection of growth is reduced to 3–5 days. *A. clevelandensis* has been recovered on chocolate blood agar at 35°C and on brain–heart infusion agar with blood and potato dextrose agar at 25°C (Hall et al. 1991). Colonies of *Afipia* sp. are gray-white, shiny, convex, opaque with entire edges, measuring 0.5–1.5 mm in width (Figure 5a).

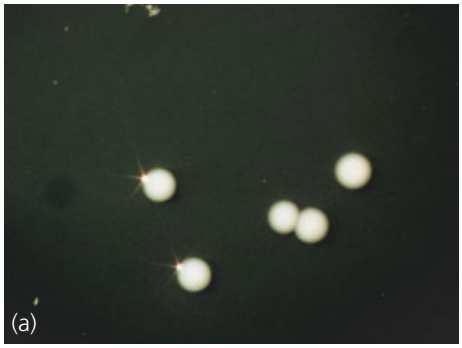

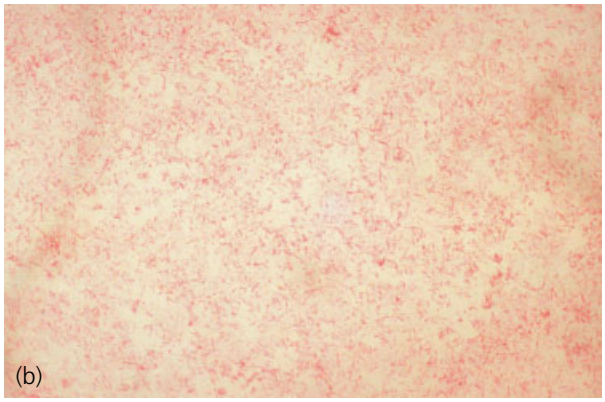

Figure 5 (a) A. felis *colonies grown on BCYE agar for 7 days at 30°C.* **(b)** *Microscopic morphology of Gram-stained* A. felis.

Table 7 *Features of* Afipia *species*

Characteristic[a]	*A. felis*	*A. clevelandensis*	*A. broomeae*	*Afipia* genospecies 1	*Afipia* genospecies 2	*Afipia* genospecies 3	*Afipia* genospecies 3-related strain	*A. birgiae*	*A. massiliensis*
Gram-reaction	–	–	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+
Urea, Christensen[b]	+	+	+ or (+)	+	+	+	+	+	+
Catalase	–/+^{w}	–/+^{w}	+^{w}	+^{w}	+^{w}	+^{w}	+^{w}	+^{w}	+^{w}
Motility	+	+	+	+	+	+	+	–	+
Optimal growth temperature (°C)	25–30	25–30	25–30	25–30	25–30	25–30	25–30	25-30	25–30
Growth on BCYE agar at: 35°C	+	+^{w}	+	+	+	+	–	–	–
37°C	+	–	+	+	+	+	–	–	–
MacConkey agar, growth	–	(+^{w})	–	–	–	–	–	–	–
Growth on: Columbia agar/5% sheep blood	+^{w}	+	–	–	–	+^{w}	+^{w}	–	–
Nutrient broth plus 6% NaCl, growth	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	–	–	–	–	–	+	+	+
H_2S	–	–	–	–	–	–	–	–	–
Esculin hydrolysis	–	–	–	–	–	–	–	–	–
Citrate, Simmons	–	–	–	+	–	–	–	–	–
Assimilation of:									
Glucose	–	–	–	+	+	–	–	–	–
Arabinose	–	–	–	+	+	–	–	–	–
Mannose	–	–	–	+	+	–	–	–	–
Mannitol	–	–	–	+	+	–	–	–	–
N-Acetylglucosamine	–	–	–	+	+	–	–	–	–
Gluconate	–	+	+	+	+	+	–	–	–
Adipate	–	+	+	+	+	+	+	+	+
Malate	+	+	+	+	–	+	+	–	–
Phenylacetate	–	–	+	+	–	+	–	–	–

+, positive reaction within 48 h; –, negative reaction; parentheses, positive reaction in 3–14 days; ^{w}, weak.
a) Incubation at 30°C unless indicated otherwise.
b) Heavy inoculation.
Adapted from Brenner et al. 1991 and La Scola et al. 2002

Additional isolates of *A. felis* have been recovered using a tissue culture system. Sonicated fluid and tissue specimens added to HeLa cell monolayers incubated for up to 18 h at 35°C with subsequent passage on to secondary cell monolayers and on to buffered charcoal yeast extract agar have yielded growth (Brenner et al. 1991; Birkness et al. 1992). It was shown that *Afipia felis* induces uptake by macrophages directly into a non-endocytic compartment and the mechanism of intracellular survival of *A. felis* is due to a phagosome–lysosome inhibiting factor (Brouqui and Raoult 1993; Lührmann et al. 2001).

Microscopically, the organisms vary in staining characteristics from relatively uniform gram-negative rods to pleomorphic slender gram-negative rods with vacuoles. In broth cultures, on prolonged incubation for 3 weeks at 37°C, *A. felis* may demonstrate delicate pleomorphic forms (English et al. 1988) consistent with cell wall-deficient organisms. Giladi et al. (1998) describe the Gram-staining characteristic of their isolate as a small, thin, curved gram-negative bacillus when taken from 5-day-old growth (Figure 5b).

All *Afipia* strains are nonhemolytic and grow at 25–30°C. Restricted growth occurs at 35°C, but strains fail to grow at 42°C or on media containing 6 percent NaCl. With the exception of *A. birgiae*, all strains were motile by means of a single polar or subpolar flagellum.

The biochemical reactions that allow differentiation between the species are given in Table 7. Biochemical tests can be performed by inoculation of API 20NE and API 50CH strips (bioMérieux) according to the manufacturer's instructions, followed by incubation at 30°C for 7–15 days. Strains are negative for hemolysis, arginine dihydrolase activity, esculin and gelatin hydrolysis, β-galactosidase activity, H_2S production and acid production of most carbon substrates, including glucose, fructose, mannose, sucrose, mannitol, and maltose. The G + C content of *Afipia* species varies from 59.3 to 69.0 mol% (Brenner et al., 1991; La Scola et al. 2002).

The profiles of the whole-cell wall fatty acid compositions of members of the genus *Afipia* showed that all strains shared relatively large amounts of hexadecanoic acid ($C_{16:0}$), *cis*-octadec-9-enoic acid ($C_{18:1\omega7c}$), and octadecanoic acid ($C_{18:0}$). With the exception of *A. birgiae*, all strains contained *cis*-hexadec-9-enoic acid ($C_{16:1\omega7c}$) (Brenner et al. 1991; La Scola et al. 2002).

Antimicrobial susceptibility

No standards exist for testing and interpreting in vitro susceptibility data for *Afipia* species and no detailed therapeutic clinical efficacy exists during treatment for putative infections with these organisms.

In vitro testing of *Afipia* strains using the microbroth dilution test method in nutrient broth with an inoculum of 1×10^5–5×10^5 cfu/ml (plates were incubated at 30°C and read 72–96 h later) revealed a high susceptibility to imipenem, aminoglycosides, and rifampin, but intermediate susceptibility or resistance to penicillins, cephalosporines, ciprofloxacin, doxycycline, and cotrimoxazole. Great differences concerning the MICs have been observed among the different species, the most resistant strain seems to be *Afipia* genospecies 1 (La Scola et al. 2002). Brenner and colleagues (1991) used the Sceptor system (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) incubated at 30°C for 48 h to determine specific minimal inhibitory concentrations for all *Afipia* species. Their data demonstrate *Afipia* genospecies 3 to have the lowest MICs among all strains tested. Other species showed higher MICs to most of the antimicrobial agents tested, including first-, second-, and third-generation cephalosporins, ampicillin, and extended spectrum penicillins, β-lactamase inhibitor combinations, aminoglycosides, and tetracycline. The lowest MICs for all species were with imipenem.

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Spirillum minus and *Streptobacillus moniliformis*

SCOTT A. MARTIN

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SPIRILLUM MINUS

Definition

Morphologically this organism is motile and spiral-shaped, but its uncertain taxonomic placement is likely to remain so until appropriate nucleic acid phylogenetic studies can be performed. The likelihood of such studies is uncertain because the organism has not been grown in pure culture. *Spirillum minus* is the etiological agent of rat-bite fever or Sodoku (Hitzig and Liebesman 1944; Roughgarden 1965). This infectious disease is transmitted in the secretions of the mouth or nose or urine of an infected rodent, most commonly via a rat bite.

Introduction and historical perspective

Carter, in 1888, was apparently the first to describe what he termed *Spirillum minor* in wet mounts prepared from the blood of a wild rat. The specific epithet 'minor' is grammatically incorrect and was subsequently changed to *S. minus* (Robertson 1924). This spirillum was initially described as being a rigid cell with two or three spiral turns or planar waves. The cells have blunt or pointed ends, a cell length of 0.8–5 μm, a diameter of approximately 0.2 μm, and a wavelength of 0.8–1.0 μm. The cells are actively motile with bipolar flagella. *S. minus* had been isolated from the blood of infected patients in blood culture medium, but efforts to subculture on solid medium have failed (Roughgarden 1965). The history of this bacterium and its disease is further complicated because rat-bite fever is also known to be caused by *Streptobacillus moniliformis* (Brown and Nunemaker 1942).

Habitat

The principal reservoir of *S. minus* is the rat. Other reservoirs or carriers of the bacterium may be mice, guinea pigs, squirrels, weasels, gerbils, wild and domestic cats, and possibly other carnivores. In infected rats the organism is consistently demonstrated in blood and peritoneal fluid, the conjunctival fluid, and muscles of the tongue. The latter two foci obviously figure prominently in transmission of the spirilla to the bite victim. Carrier rats and mice may occasionally show eye infections (Manouelian 1940). Carrier rates of *S. minus* among rats vary widely, from 0 percent in Atlanta, GA, USA, to 25 percent in London, UK, where serous fluid and blood of trapped rats were examined for the bacterium (Biberstein 1975; McHugh et al. 1985).

Staining reactions and culturability

S. minus is readily stained by aniline stains such as Giemsa's or Wright's stains and Loeffler's methylene blue. The Gram reaction of *S. minus* is not stated in Bergey's *Manual of determinative bacteriology* (Holt et al. 1994). Hitzig and Liebesman (1944) described in detail their investigation of a case of subacute bacterial endocarditis caused by *S. minus*. They described their successful efforts to cultivate *S. minus* from blood of this

patient in two different veal infusion broths in a CO_2 extinction candle-jar and the bacterium was maintained in this environment for 11 months. Microscopic examination of Gram stains of the veal infusion broth cultures showed gram-negative and gram-positive spiral-shaped organisms. These results suggest that *S. minus* is gram-variable. Others have tried to repeat this work. Although successful primary isolations have been reported (Roughgarden 1965), the successful long-term in vitro subcultivations of *S. minus* on solid media and its Gram reaction remain unsolved. A similar lack of data obviously applies to growth requirements, metabolism, pathogenicity, antigenicity, and genetic mechanisms.

Antibiotic susceptibility

In vivo, antimicrobial susceptibility studies stem largely from empirical efficacy studies with penicillin, tetracycline, chloramphenicol, and streptomycin given to experimentally infected mice (Roughgarden 1965). Although these studies were done primarily to determine therapeutic efficacy, they also served to establish a spectrum of antibiotic susceptibility that is still accepted today.

STREPTOBACILLUS MONILIFORMIS

Definition

Streptobacillus moniliformis is a fastidious, highly pleomorphic, aerobic and facultatively anaerobic, gram-negative rod with characteristic yeast-like swellings that readily give rise to L-phase and transitional variants. The organism ferments a range of carbohydrates: it is catalase-negative, oxidase-negative, indole-negative, and urease-negative. The bacillus causes a type of rat-bite fever and is responsible for outbreaks of Haverhill fever. The guanine and cytosine (G+C) content of the DNA is 24 to 26 mol%.

Classification

Currently there is only one species in the genus *Streptobacillus* and the taxonomic position of *S. moniliformis* is uncertain. It appears to have properties more in common with the Mycoplasmatales (Costas and Owen 1987).

Introduction

S. moniliformis, isolated by Levaditi et al. (1925) from the blood of a laboratory worker, is now considered the same organism as *Streptomyces muris ratti* (Schottmuller 1914; Tunnicliff 1916). It is similar to *Haverhillia multiforme* (Parker and Hudson 1926) although the two organisms have not been directly compared. Over the years various synonyms have been associated with this organism (Brown and Nunemaker 1942).

Klieneberger (1935) found that colonies of *S. moniliformis* consisted of two elements: the streptobacillary form and what she took to be a mycoplasma, which she named L-form or L_1. Even though the L_1 appeared not to revert to the streptobacillary form (Klieneberger 1942), it is now regarded as an L-phase variant.

Habitat

The bacterium occurs worldwide as a commensal of the nasopharynx of healthy laboratory rats (Strangeways 1933) and wild rats, sometimes being excreted in the urine. It is also isolated from the nasopharynx of diseased mice and from other rodents.

Morphology

It is a gram-negative, highly pleomorphic bacterium that generally occurs as short coccobacillary forms or rods, appoximately 0.5 μm in width, as well as chains and intertwining wavy filaments (Figure 1a). The filaments may show spherical, oval, or club-shaped gram-variable swellings up to 3 μm in diameter and may give rise to a 'string-of-beads' appearance – hence the description '*moniliformis*' (Latin *monile*, necklace; *forma*, shape or form). In aging cultures, filaments show degenerative changes with the formation of granules and extracellular droplets, irregularity or failure of staining, and fragmentation. In broth medium and in vivo, pleomorphism is less pronounced. The organism is nonmotile. In general, older cultures may not stain well with the Gram stain; Giemsa or Wayson stain may yield better results (Piot 1991).

Culture characteristics and growth requirements

On serum or blood agar it forms circular, translucent colonies with a smooth glistening surface. It is nonhemolytic. Smaller, rougher 'transitional' colonies may be present, and in older cultures mutant L-phase colonies may be recognizable, with their typical 'fried-egg' appearance; these are best seen on a clear medium (Cohen et al. 1968). Blood, serum, ascitic fluid, or synovial fluid, is required for growth. It may appear to be an obligate anaerobe on primary isolation, but it is a facultative anaerobe on subculture. Optimum growth is probably best achieved on chocolate–blood agar or tryptose soy agar, both enriched with 10–20 percent serum and incubated in a microaerophilic atmosphere with additional CO_2 and humidification. Basal medium containing 15 percent sterile defibrinated rabbit blood supports the

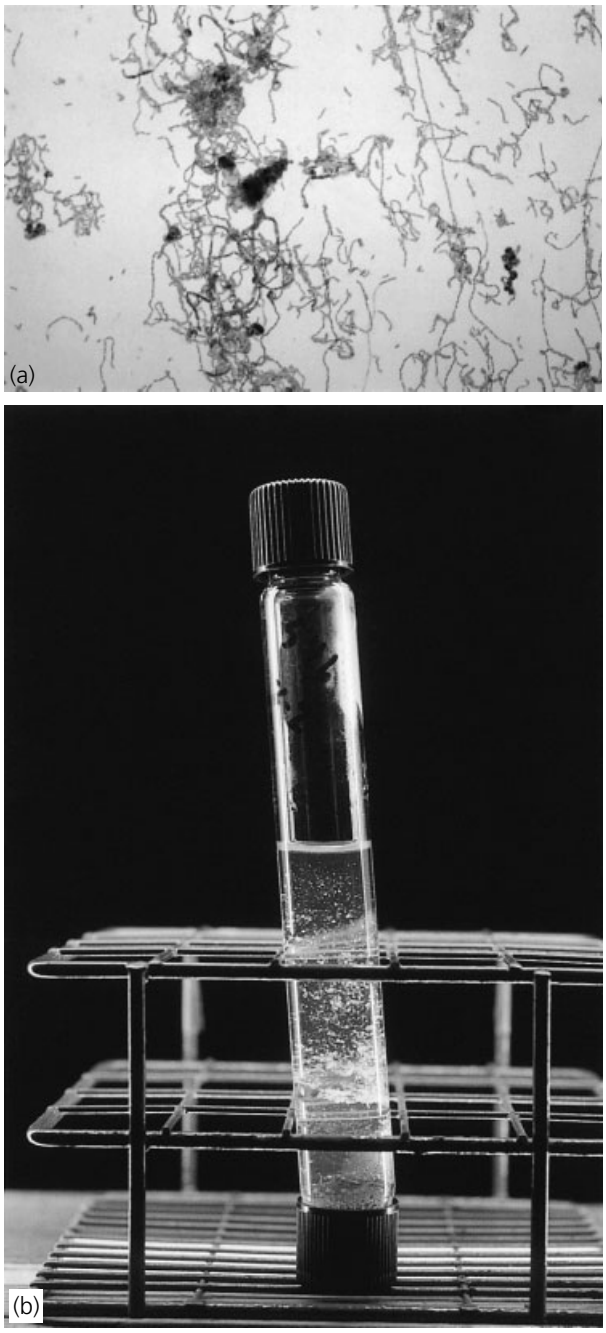

Figure 1 (a) *Typical bacilli (some in chains) and filaments with bulbous swellings (oil immersion; original magnification ×1 000).* **(b)** *Thioglycolate broth with 'puff-ball' colonies. Reproduced with permission from Graves and Janda (2001)*

bacterial-phase organisms well, whereas a clear medium will enhance the isolation of the more nutritionally fastidious L-phase variants (Jenkins 1988). The optimal temperature for growth is 35–37°C, optimal pH 7.4–7.6. The choice of a fluid blood-culture medium is critical: Shanson et al. (1985) recommend brain–heart infusion broth with 0.05 percent cysteine and 2.5 percent Panmede. This could be used in conjunction with a suitable L-phase fluid medium.

Metabolism

The organism is generally nonreactive, with negative reactions for catalase, oxidase, indole production, nitrate reduction, and urease (Table 1). It attacks sugars fermentatively; acid is produced from glucose (without gas), galactose, glycogen, dextrin, raffinose, and starch and variably from fructose, maltose, mannose, salicin, lactose, sucrose, trehalose, and xylose, depending on the basal medium employed. Acid is not formed from sorbitol or mannitol.

The alkaline phosphatase reaction is positive. The end products of glucose metabolism consist mainly of lactic acid with some succinic acid.

Cell-wall and envelope composition; antigenic structure

The major long-chain fatty acids are C(16:0), C(18:2), C(18:1), and C(18:0) (Rowbotham 1983). The electrophoretic profiles of cellular proteins are characteristic and can be used to type strains, 'Haverhill fever' strains being distinguishable from 'rat-bite fever' strains (Costas and Owen 1987).

Strains are antigenically homogeneous, but L-phase variants lack an antigen present in the bacillary form. The species can be identified by fluorescence with a conjugated specific antiserum.

Table 1 *Differential characteristics of* Streptobacillus moniliformis

Characteristic	Reaction
Oxidase	–
Indole	–
Nitrate reduction	–
H_2S production	+ (weak)
Methyl red	–
Hydrolysis of starch	. . .
Hydrolysis of aesculin	V
Hydrolysis of casein	–
Arginine dihydrolase	+
Lysine decarboxylase	. . .
Ornithine decarboxylase	. . .
Glucose O–F test	F
Acid production from	
glucose	+
maltose	V
sucrose	V
lactose	V
mannitol	–
fructose	V
mannose	V
trehalose	V
xylose	V

–, negative reaction; +, positive reaction; F, fermentative; V, variable; . . ., no information or not applicable.

Pathogenicity and virulence factors

Streptobacillary rat-bite fever occurs worldwide and may occur following the bite of a rat or occasionally a mouse, squirrel, weasel, dog, or cat. Most human infections probably occur in children sleeping in rat-infested dwellings. Infections among rodent handlers, laboratory workers, as well as rodent pet owners also occur (Berger et al. 2001). The incubation period is usually 1–4 days and rarely more than 10 days. Onset is typically abrupt with fever, headache, vomiting, and migratory arthralgias and myalgias (Hudsmith et al. 2001). At the time of presentation, the bite wound has generally healed and regional lymphadenopathy is typically not noted. Within 2–4 days after onset, a rash develops over the palms, soles, and extremities. It may present as maculopapular, petechial, or pustular. Symptoms generally resolve over the period of 2 weeks.

Haverhill fever, or erythema arthriticum, is a form of streptobacillary fever that occurs in outbreaks, with vomiting and pharyngitis as prominent symptoms. It appears not to be attributable to direct contact with animals. It was named after Haverhill, Massachusetts, where the first recorded epidemic of the disease occurred (Parker and Hudson 1926); this was thought to be associated with the consumption of raw milk and milk products. The first outbreak in the UK (Shanson et al. 1983) affected pupils and staff of a boarding school in Essex. Of some 700 people exposed, 304 (43 percent) became ill in a period of 10 days (McEvoy et al. 1987).

Adult rats are generally resistant to experimental infection, although newborn rats may develop pneumonia (Strangeways 1933). Mice given the organism intravenously develop either a rapidly fatal septicemia or arthropathy of the lower limbs, with longer survival, the severity of the condition being dose-related (Savage et al. 1981).

Laboratory isolation and identification

Streptobacillary rat-bite fever is usually diagnosed by the isolation of *S. moniliformis* from blood, joint fluid, or pus. If isolation is unexpected, the organism may not be readily identified. Clinically similar conditions such as tularaemia, leptospirosis, rickettsia, and various viral infections should be ruled out. If streptobacillary fever is suspected and routine laboratory cultures are negative, the organism may sometimes be isolated by intraperitoneal inoculation of infected material into mice. Because of the fastidious nature and the slow growth of these gram-negative facultative anaerobic rods and the strict growth requirements, including special media that are not readily available, the diagnosis of streptobacillary rat-bite fever is difficult (Berger et al. 2001).

An enzyme-linked immunosorbent assay (ELISA) for monitoring rodent colonies for *S. moniliformis* has been described by Boot et al. (1993). Twelve strains originating from cases of rat-bite fever and Haverhill fever in humans and from various rodent species showed considerable serological relationship. The ELISA appeared specific since antibodies to *S. moniliformis* were absorbed by autologous and homologous antigen, but not by heterologous bacterial antigens. *Acholeplasma laidlawii* showed a partial serological relationship with *S. moniliformis*.

Recent research has used the polymerase chain reaction (PCR) to detect *S. moniliformis* (Berger et al. 2001; Boot et al. 2002). Berger et al. (2001) were able to detect *S. moniliformis* in blister fluid using their PCR technique, whereas the PCR technique developed by Boot et al. (2002) detected *S. moniliformis* infection in orally or intravenously infected rodents and in five of eight rats exposed to natural infection. Boot et al. (2002) also reported that ELISA and PCR were equally successful in detecting infection in rats.

Graves and Janda (2001) identify or confirm *S. moniliformis* isolates using four tests: (1) a growth requirement of 10–20 percent serum; (2) the presence of typical gram-negative bacilli (sometimes in chains) and filaments with bulbous swellings in a Gram stain (Figure 1a); (3) growth in broth (thioglycolate medium) with 'puff-ball' colony formation (Figure 1b); and (4) a positive direct fluorescent antibody test using an in-house-produced polyclonal antibody. *S. moniliformis* can also be identified by cellular fatty-acid profiling using gas–liquid chromatography (Rygg and Bruun 1992; Pins et al. 1996).

Susceptibility to antimicrobial agents

The bacillary form is sensitive to penicillin and to therapeutically attainable concentrations of cephalosporins, aminoglycosides, tetracycline, erythromycin, and clindamycin; it is less sensitive to chloramphenicol. The L-phase variant is resistant to penicillin but sensitive to tetracycline.

CONCLUSION

S. minus and *S. moniliformis* are the etiological agents of rat-bite fever, a disease that occurs throughout the world in areas where the rat population is heavy and the living conditions of humans are slums. Our knowledge of the fundamental microbiological characteristics of *S. minus* and the epidemiology of rat-bite fever is incomplete. More is known about *S. moniliformis*, but we do not know the true incidence of this disease nor the pathogenic mechanisms of these etiological agents. Clearly this disease and its cause deserve increased attention of microbiologists and infectious disease specialists, not only for the science involved but also to

control a disease that may be more prevalent and harmful than presently known.

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Bacteroides, *Prevotella*, and *Porphyromonas*

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Gram-negative, nonsporing, anaerobic bacilli with rounded or pointed ends, sometimes fusiform, sometimes filamentous, and often pleomorphic, that produce mixed acidic endproducts were previously classified in the genus *Bacteroides*. Recognized as important causes of human and animal infection since the latter part of the nineteenth century, they are also a major component of the normal bacterial flora of humans and animals, colonizing the mucous membranes of the mouth, urogenital, and lower gastrointestinal tract of humans. In animals they are found in the mouth and at various sites in the alimentary tract. Some species metabolize cellulose and other polysaccharides in the rumen of some herbivores; some of the most oxygen-sensitive species have been found in the cecum of chickens and other poultry.

CLASSIFICATION

The isolation and classification of these organisms has presented particular difficulties. These were partly technical, because reliable anaerobic conditions were difficult to achieve and many workers were unable to obtain pure cultures, but they also reflected the diverse properties of members of the group. There was confusion in nomenclature and disagreement between different observers over the description of the same organisms.

The classification of gram-negative anaerobic bacilli has undergone many changes since Veillon and Zuber (1898) named their isolates *Bacillus fragilis*, *Bacillus fusiformis*, etc. This was recognized by Castellani and Chalmers (1919) who proposed the genus *Bacteroides* to contain species of obligately anaerobic nonsporing bacilli. *B. fragilis* was adopted as the type species from the first edition of *Bergey's manual* (1923). Subsequently, Weiss and Rettger (1937) redefined the genus to exclude gram-positive organisms; spindle-shaped gram-negative anaerobic bacilli were placed in the genus *Fusobacterium* by Knorr (1922).

Eggerth and Gagnon (1933) produced a scheme based upon the classification of Castellani and Chalmers (1919) for the identification of strains isolated from the gastrointestinal tract. They defined 18 species on the basis of morphology and carbohydrate fermentation tests, but all of their strains were later found to belong to the *Bacteroides fragilis* group (Holdeman and Moore 1974a).

The system proposed by Prévot (1938) employed generic names that differed from those in general use and included the pigmented species *Bacteroides melaninogenicus* (proposed by Oliver and Wherry in 1921) as *Ristella melaninogenica*. He divided gram-negative anaerobic bacilli into two families, Ristellaceae with five genera (*Ristella*, *Pasteurella*, *Dialister*, *Capsularis*, *Zuberella*) and Sphaerophoraceae with two genera

(*Sphaerophorus* and *Sphaerocillus*). In the sixth edition of *Bergey's manual* (1948) the Bacteroidaceae, comprising the two genera, *Bacteroides* and *Fusobacterium*, was a tribe of the family Parvobacteriaceae. Most of the 23 species of *Bacteroides* were members of the *B. fragilis* group, except for *B. melaninogenicus*, which was described as saccharolytic, and one asaccharolytic species, *Bacteroides caviae*. In the seventh edition of *Bergey's manual* (1957), gram-negative, anaerobic bacilli were reclassified as the family Bacteroidaceae and comprised five genera. The genus *Bacteroides* contained 30 species distinguished by gas production, gelatin liquefaction, cellular morphology, and the production of acid from carbohydrates; two species were motile and two (*Bacteroides coagulans* and *Bacteroides putredinis*) were nonfermentative. There were six species of *Fusobacterium* and two of *Dialister*. The second largest genus was *Sphaerophorus* with 14 species, and aerobic but facultatively anaerobic organisms of the genus *Streptobacillus* were also included in the Bacteroidaceae. In the sixth edition of this book (1974) only three species of *Fusobacterium* and five of *Bacteroides*, and a third genus, *Dialister*, to contain the minute gram-negative bacillus *Dialister pneumosintes*, were included.

The first general agreement on taxonomy and classification was reached at the meeting of the International Commission for Systematic Bacteriology Sub-committee for gram-negative anaerobic rods at Lille in 1967, when new principles for classification were defined (Beerens 1970). These were embodied in the eighth edition of *Bergey's manual* (Holdeman and Moore 1974a). The Bacteroidaceae were divided into three genera: *Bacteroides*, *Fusobacterium*, and *Leptotrichia*. The genus *Bacteroides* contained 22 species in five groups:

1. *B. fragilis* included most of those species described by Eggerth and Gagnon (1933) and previous workers and was divided into five subspecies
2. Phenotypically similar strains that were inhibited by bile included *Bacteroides ruminicola*, *Bacteroides oralis*, *Bacteroides ochraceus*, and *Bacteroides amylophilus*
3. A group of six species that did not produce succinic acid, but were otherwise unrelated
4. Non-saccharolytic non-pigmented strains
5. *B. melaninogenicus* produced black-pigmented colonies on laked-blood agar and was divided into three subspecies.

The genus *Fusobacterium* contained 16 species that formed *n*-butyric acid as a major metabolic product and Vincent's organism (*Fusobacterium plauti-vincenti*) was assigned to the third genus, *Leptotrichia* (Gilmour et al. 1961), under the name *Leptotrichia buccalis*, because it produced lactic, but not *n*-butyric acid as a major product.

In the ninth edition of *Bergey's manual* (Holdeman et al. 1984; Jousimies-Somer et al. 2003), 42 species were assigned to the genus *Bacteroides*. These included eight species previously encompassed by '*B. fragilis*', eight species of saccharolytic nonpigmented oral bacteroides (previously '*B. oralis*'), seven saccharolytic pigmented species (previously '*B. melaninogenicus*'), three species concerned with cellulose degradation in the rumen of herbivores, and nine nonfermentative (asaccharolytic) species of which two, *Bacteroides asaccharolyticus* and *Bacteroides gingivalis*, were pigmented. In addition, three more species were proposed within the *fragilis*-like group of fecal bacteroides (*Bacteroides merdae*, *Bacteroides stercoris*, *Bacteroides caccae*; Johnson et al. 1986), another oral nonpigmented species (*Bacteroides heparinolyticus*; Okuda et al. 1985) and a third asaccharolytic pigmented species (*Bacteroides endodontalis*; van Steenbergen et al. 1984).

The genus *Bacteroides*, which contained species with a mol% content of 26–61, has undergone major taxonomic revisions (Shah 1991a, b). This was inevitable because of the considerable heterogeneity in biochemical and chemical properties that became apparent. The genus now includes species that were formerly described as the '*Bacteroides fragilis*' group. More than 15 new genera have been proposed for organisms formerly classified as *Bacteroides*, most of which are monospecific. However, two genera, *Porphyromonas* and *Prevotella* (Shah and Collins 1989, 1990), contain large numbers of species and appear to be of major clinical importance.

Comparative 16S rRNA sequence analysis has considerably reshaped the taxonomic status of the members of the family Bacteroidaceae. In this chapter, genera that cluster in the *Bacteroides* group of division 2.15 (*Flexibacter–Cytophaga–Bacteroides*) and are designated as *Bacteroides*, *Prevotella*, and *Porphyromonas*, are discussed.

Criteria for classification

Early workers relied almost exclusively upon observations of microscopic and colonial morphology. However, these properties can vary considerably among species depending on their growth conditions. Eggerth and Gagnon (1933) established the first biochemical key for the group, but their tests were performed under varied and often unsuitable conditions. Prévot (1938) used an extensive series of biochemical tests, which included the determination of metabolic endproducts. Conventional bacteriological tests remain an important part of identification schemes for gram-negative anaerobic bacilli (Holdeman and Moore 1974a; Duerden et al. 1976, 1980; Holdeman et al. 1984) and several commercial systems for rapid identification are currently available. Barnes and Goldberg (1968a, b) analyzed the data obtained from phenotypic tests by numerical methods and concluded that the most useful properties were cellular morphology, terminal pH in glucose broth, production

of formic, acetic, propionic, and butyric acids, deamination of threonine, stimulation of growth by 20 percent bile, and inhibition by certain antibiotics.

The short-chain fatty acids formed as endproducts of protein or carbohydrate metabolism were particularly important in the classification of anaerobic bacteria. They were first detected by distillation and paper chromatography (Guillaume et al. 1956), but this was superseded by gas–liquid chromatography (GLC) (Werner 1969; Moore 1970; Carlsson 1973). The family Bacteroidaceae was assigned to two principal genera, *Bacteroides* and *Fusobacterium*, on the grounds that *Fusobacterium* spp. produce *n*-butyric acid as a major product, whereas most *Bacteroides* spp. do not. The third genus, *Leptotrichia*, comprises oral fusiform bacteria that produce lactic acid as the only major product.

Several groups of workers have used comparisons of the mol% G+C content of the DNA to determine similarities and differences between members of the groups of Bacteroidaceae (Sébald 1962; Shah et al. 1976). It was a widely held view that a genus should comprise species that do not differ by more than 10–12 mol% G+C. Thus, the fact that the *Bacteroides* contained species within the range 28–61 mol% G+C indicated that several genera existed within this taxon. DNA–DNA hybridization was used to verify the intraspecies status of several taxa such as the subgroups within the *B. fragilis* group (Johnson 1973; Cato and Johnson 1976) and the subspecies of *B. melaninogenicus* (Coykendall et al. 1980; van Steenbergen et al. 1981; Shah et al. 1982).

In the mid-1970s and 1980s, attention turned increasingly towards chemical analyses of cell constituents for the provision of characters that are useful for classification. Cell wall composition, a major differentiating character among gram-positive taxa, had less impact among gram-negative bacteria. Like most gram-negative microorganisms, *Bacteroides* and *Prevotella* spp. possess a directly crosslinked peptidoglycan based upon *meso*-diaminopimelic acid, whereas some species of *Porphyromonas* appear to have a peptidoglycan structure based upon lysine (Shah et al. 1976; Hammann and Werner 1981). However, the chemical composition and structure of many species remain unknown; thus the full value of this property in the classification of these microorganisms is still to be determined. Multilocus enzyme electrophoresis using oxidoreductases provided a useful means of accentuating the diversity that existed among taxa that were considered to be homogeneous. Thus five electrophoretic patterns were found among *B. melaninogenicus* strains (Shah et al. 1976) that were subsequently assigned to different species, whereas among bacteroides considerable diversity was revealed (Shah and Williams 1982). This technique not only provided evidence of relationships and differences within the genus (Shah and Collins 1983), but also shed light on aspects of intermediary metabolism (Shah and Williams 1987a, b). The black/beige pigments produced by *B. melaninogenicus* and related taxa were regarded as one of the most important distinguishing properties. Chemical analysis revealed that the pigments were porphyrins and not melanin or ferrous sulfide as previously reported and that two major compounds existed (Shah et al. 1979; Shah 1991a). The black pigment was identified as protohemin and the fluorescent light-brown pigment was characterized as protoporphyrin (see Figure 1).

Current studies indicate that several other pigments exist and porphyrin genes are present even among nonpigmented members of the group (Shah and Gharbia, unpublished data). Gram-negative bacteria, particularly anaerobic bacteria, possess a plethora of diverse lipids, such as sphingolipids and plasmalogens (Miyagawa et al. 1978; Shah and Collins 1983). Among the lipids, long-chain cellular fatty acids (Miyagawa et al. 1979; Shah et al. 1982) have provided excellent taxonomic markers and were one of the key chemical characters used to differentiate *Porphyromonas* and *Prevotella*. In addition, analysis of polar lipids (Kunsman 1973; Kunsman and Caldwell 1974; Shah 1991b) and menaquinones (Shah and Collins 1980) have had a significant impact in reshaping the structure of the Bacteroidaceae. Menaquinones are the sole isoprenoid quinones produced by *Bacteroides* spp. and determination of the length of the C-3 polyprenyl side chains have enabled clearer circumscription of several species (Shah and Collins 1980). For example, *B. ochraceus* was shown to differ markedly from *Bacteroides* spp. in possessing menaquinones with six isoprene units. *Bacteroides* spp. and related taxa possess menaquinones with isoprene units in excess of nine. Proteins in whole cell extracts of *Bacteroides* cultures analyzed by discontinuous gradient polyacrylamide gel electrophoresis (PAGE) have been helpful in delineating specific, subspecific, and strain-specific patterns (Strom et al. 1976). Cell surface proteins released by EDTA treatment and mild sonic disintegration, and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), have revealed species-specific patterns among the *Bacteroides* (Poxton and Brown 1979).

The impact of rRNA sequence analysis

Initially, rRNA cataloguing and later, 5, 16, and 23S rRNA sequence analysis, were used to devise a classification system based on natural relationships (Woese 1987). Members of the Bacteroidaceae, like other taxa, were subjected to rigorous analysis and several phylogenetic trees have been constructed (see for example, Lawson et al. 1992; Paster et al. 1994; Shah et al. 1995). In general, ribosomal RNA sequence data have in the main supported previous proposals and there is now general consensus for the genera *Bacteroides*, *Prevotella*,

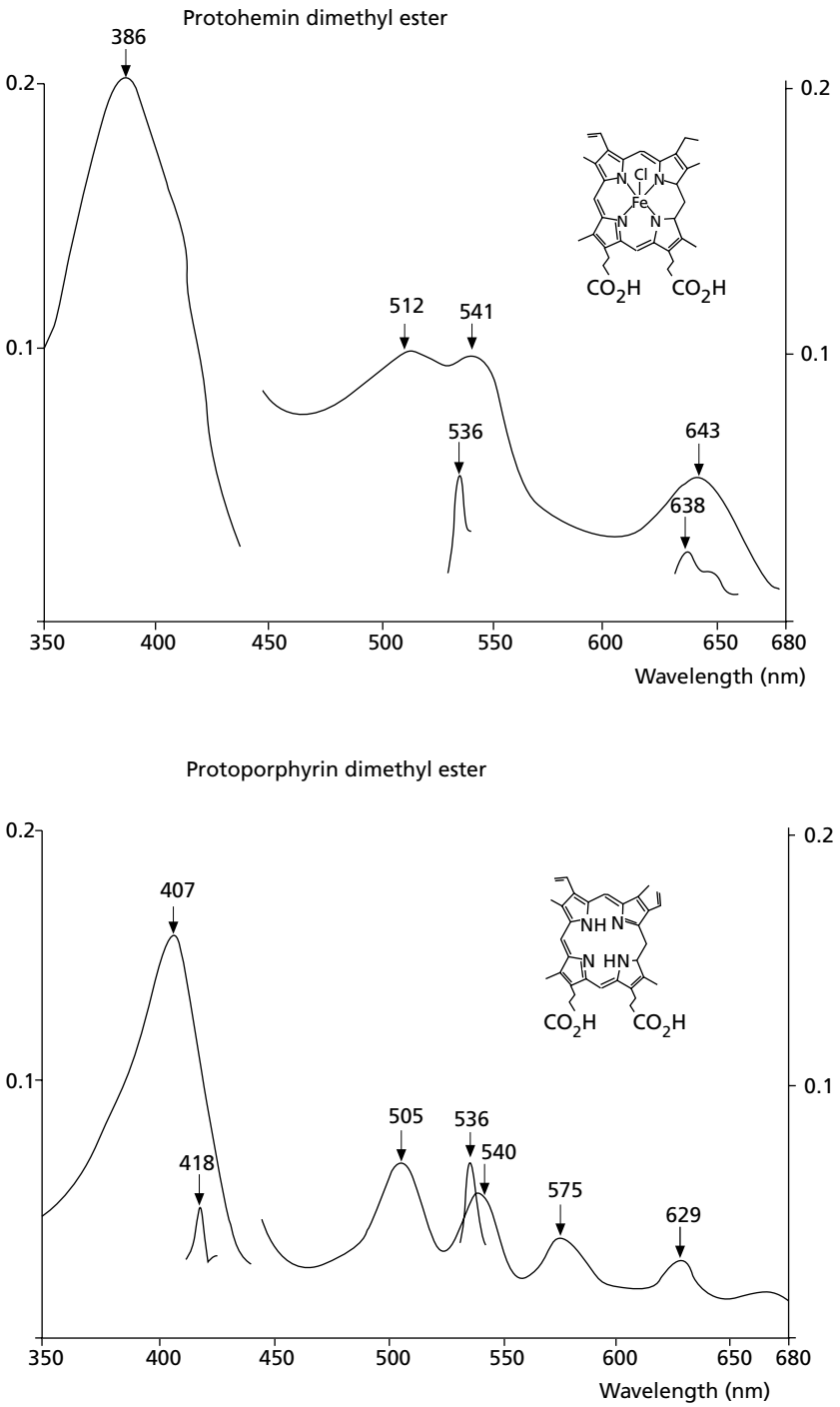

Figure 1 *The electronic spectrum and structure of the two major crystalline pigments (isolated as dimethyl esters) of* Porphyromonas *and* Prevotella *spp. The iron-containing porphyrin gives the cells their characteristic black color on blood agar plates, whereas the light brown, ultraviolet fluorescent colonies which are characteristic of some species such as* P. loescheii *are due to the production of protoporphyrin.*

and *Porphyromonas*. However, some proposals have been made using only partial 16S rDNA sequences, consequently some taxa remain in a state of flux. For example, recent studies using complete 16S rDNA sequences have confirmed that *Bacteroides splanchnicus* does not belong to the genus *Bacteroides*, while *B. merdae* and *Bacteroides distasonis*, which phenotypically resemble other members of the *Bacteroides* belong to separate lineages (see Figure 2).

Prevotella zoogleoformans, which is not inhibited by bile, and *Prevotella heparinolytica* are phenotypically different from other *Bacteroides* species and form distinct groups on the basis of 16S rDNA sequence data. Species of the genus *Prevotella* share a large number of

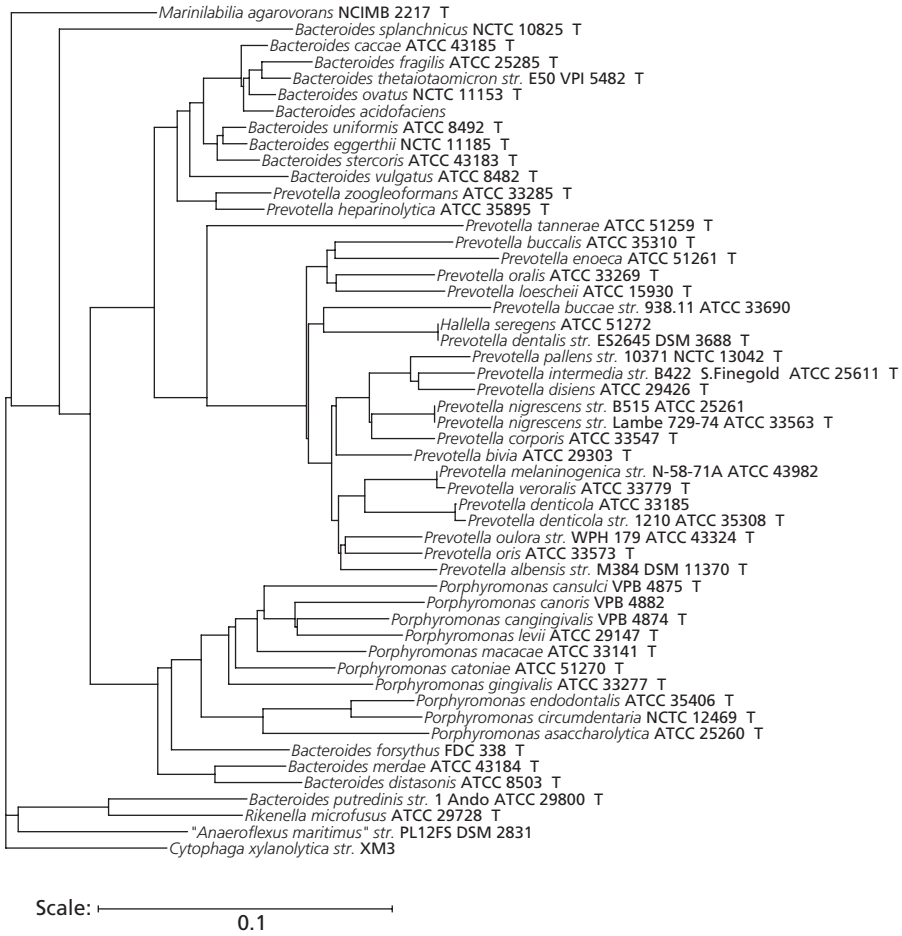

Figure 2 *Unrooted phylogenetic tree showing the position of species of the genera* Bacteroides*,* Prevotella*, and* Porphyromonas*. The tenuous relationship of several species such as* Bacteroides merdae*,* B. distasonis*, and* B. splanchnicus *are shown.* B. forsythus *was recently transferred to a new genus* Tannnerella forsythia *(see Olsen and Shah 2003).*

common traits and demonstrate high 16S rDNA sequence homologies, but there are exceptions. *Prevotella tannerae*, for example, differ from other members in cellular fatty acid composition, shows considerable sequence divergence, and is probably not a *Prevotella*, while *B. forsythus* was recently transferred to a new genus *Tannnerella forsythia* (see Olsen and Shah 2003).

BACTEROIDES

This genus contains gram-negative bacilli of closely related species that share many common properties and a common ecology. They are tolerant of bile and resistant to penicillin and are commensals in the lower gastrointestinal tract in humans. They form a major part of the normal human fecal flora and some species are important pathogens, causing infections particularly after accidental or surgical injury to the gastrointestinal tract or in association with pathological lesions of it. Most of the species described by Veillon and Zuber (1898) and by Eggerth and Gagnon (1933) were members of this group, but its classification was confused. Based on 16S rDNA sequence analysis, the current status of the genus *Bacteroides* is as shown in Table 1.

In the eighth edition of *Bergey's manual* (Holdeman and Moore 1974a) gathered all the members of this group into a single species – *B. fragilis* – with five subspecies: *fragilis*, *vulgatus*, *distasonis*, *ovatus*, and *thetaiotaomicron*. They believed that the species represented a continuum of variants with clusters of strains that were designated subspecies and smaller numbers of intermediate strains. However, Cato and Johnson (1976) found poor DNA homology between the reference strains of the five original subspecies of *B. fragilis*. They proposed that they should be reinstated to specific rank and this was accepted by the International Committee for Systematic Bacteriology (ICSB). Other clusters of strains that fell within the group were given specific status; these included *B. splanchnicus*, *B. merdae*, and *B. stercoris*. The relationship between the species remains the subject of considerable debate. They share many properties, and ecological and epidemiological studies support the concept of a closely related group. The results of a wide

Table 1 *Species of the genus* Bacteroides *with their type strain*

Bacteroides cluster	
Bacteroides caccae	NCTC 13051
Bacteroides eggerthii	NCTC 11155
Bacteroides fragilis	NCTC 9343 – Type species
Bacteroides merdae	NCTC 13052
Bacteroides ovatus	NCTC 11153
Bacteroides splanchnicus	NCTC 10825
Bacteroides stercoris	NCTC 13053
Bacteroides thetaiotaomicron	NCTC 10582
Bacteroides uniformis	NCTC 13054
Bacteroides vulgatus	NCTC 11154
Related cluster	
Bacteroides merdae	NCTC 13052
Bacteroides distasonis	NCTC 11152

range of phenotypic tests on members of the fragilis group form a continuous spectrum with clusters of strains that represent the named species.

Description of the genus

The genus *Bacteroides* comprises small nonmotile gram-negative bacilli and coccobacilli; they are moderately pleomorphic, but long filaments, bizarre shapes, L-forms, and spheroplasts are rare. They grow well in 24–48 h on horse blood agar plates incubated anaerobically at 37°C to form circular, low-convex colonies, 1–3 mm in diameter with an entire edge. The colonies are usually smooth, shiny, translucent, or semi-opaque and gray. Colonies of fresh isolates are often moist and some are mucoid. Most strains do not produce hemolysis, but a few strains are slightly hemolytic and a very small proportion (<1 percent) are α-hemolytic. All species will grow on a medium that contains glucose, hemin, vitamin B_{12}, minerals, ammonium chloride, and a sulfide and is provided with a CO_2/CO_3^- buffer system. They do not use organic nitrogen compounds. They have generally been regarded as catalase-negative, but *B. fragilis* and some other saccharolytic species have been shown to produce small amounts of catalase and superoxide dismutase (Gregory et al. 1977). The G + C content of the DNA is in the range 40–48 mol% and 16S ribosomal RNA analysis revealed over 93 percent similarity among species. All species ferment a range of carbohydrates, including glucose, with the production of acid and gas. The major volatile fatty acid-products of metabolism are acetic and succinic acids; *n*-butyric acid is not produced except by *B. splanchnicus*. Most strains are stimulated by 20 percent bile and are tolerant of the bile salt sodium taurocholate, but inhibited by sodium deoxycholate. They will grow in the presence of Victoria blue 4R (1 in 80 000), but most are inhibited by gentian violet (1 in 100 000) and ethyl violet (1 in 100 000). All strains are resistant to penicillin and to high concentrations (1 000 μg disks) of neomycin and kanamycin. They decarboxylate glutamic acid but threonine is not dehydrogenated, nitrate is not reduced, and urease is not produced. Most strains hydrolyze esculin rapidly and produce acid from xylose. Members of the genus are identified by a small number of variable characteristics that include indole production, esculin hydrolysis, and the fermentation of lactose, sucrose, rhamnose, trehalose, mannitol, salicin, and arabinose (Table 2).

BACTEROIDES FRAGILIS

Isolates of this species cannot be distinguished from other members of the genus by their colonial form, or by their microscopic morphology, except that freshly isolated strains usually form a capsule. *B. fragilis* hydrolyzes esculin and produces acid from glucose, lactose, sucrose, maltose, and usually xylose, but not from rhamnose, trehalose, mannitol, salicin, or arabinose. Indole is not formed and charcoal–gelatin disks are digested slowly or not at all.

B. fragilis forms less than 10 percent of the *Bacteroides* in normal human feces, yet it is by far the most common species of *Bacteroides* to be isolated from infections related to the large intestine (Duerden 1980a). It thus appears to be particularly pathogenic for humans. This may be attributable to its possession of a polysaccharide capsule (Kasper 1976a, b) or to the action of one or more of the extracellular or membrane-associated enzymes that it forms: proteinases, including collagenase, fibrinolysin, hemolysin, neuraminidase, phosphatase, DNAase, hyaluronidase, chondroitin sulfatase, and heparinase (Gesner and Jenkin 1961; Müller and Werner 1970; Rudek and Haque 1976).

The cell wall of *B. fragilis* contains a lipopolysaccharide (LPS) with weak endotoxic activity, the polysaccharide portion of which confers type-specific O-antigenic specificity. A species-specific protein component of the outer membrane has been identified. Its presence may be used for the identification of *B. fragilis*. The pattern of proteins in the outer membrane shown by SDS-PAGE is also species-specific. External to the outer membrane is a thick polysaccharide capsule 1.5–2 times the thickness of the cell wall. It is composed of a high molecular weight polysaccharide ($>7.5 \times 10^3$ kDa). It has antiphagocytic properties and also protects the cell from complement-mediated lysis by antibodies against cell wall antigens. The capsular antigen is species-specific. This capsule is found only in *B. fragilis*. All clinical isolates of *B. fragilis* are capsulate, but the capsule is often lost on repeated subculture. It can be demonstrated in India ink preparations and identified by the Quellung reaction and in an indirect immunofluorescent assay.

Table 2 *Distinguishing characteristics of species of the genus* Bacteroides *and closely related taxa*

Test	*B. fragilis*	*B. vulgatus*	*B. distasonis*	*B. merdae*	*B. caccae*	*B. ovatus*	*B. thetaiotaomicron*	*B. eggerthii*	*B. variabilis*	*B. uniformis*	*B. stercoris*	*B. splanchnicus*
Indole production	–	–	–	–	–	+	+	+	+	+	+	+
Esculin hydrolysis	+	+/–	+	+	+	+	+	+	+	+	+	+
Fermentation of												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	–	+	+	+	–
Rhamnose	–	+	+/–	–	+	+	+	+	+	–	+	–
Trehalose	–	–	+	+	+	+	+	–	–	+	–	–
Mannitol	–	–	–	–	–	+	–	–	–	–	–	–
Xylose	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	–	+	–	–	–	–	+	+	+	+	–	+
Salicin	–	–	+	+	–	+	+	+	–	–	–	–

+, Positive result (growth in tolerance tests); –, negative result.

BACTEROIDES VULGATUS

This species was first isolated from human feces by Eggerth and Gagnon (1933). It is the most common *Bacteroides* species in normal human feces, but is only occasionally implicated in infections. Results of tolerance tests resemble those given by other members of the genus, but approximately 50 percent of strains are tolerant of ethyl violet. Indole is not produced but charcoal–gelatin disks are digested within a few days. Unlike other members of the genus, approximately 50 percent of *B. vulgatus* strains fail to hydrolyze esculin and others do so only weakly.

BACTEROIDES DISTASONIS

This species was described by Eggerth and Gagnon (1933) in their studies of the fecal flora and named after the Romanian bacteriologist A. Distaso. Like *B. vulgatus*, it is a common member of the normal human fecal flora but seldom appears to cause clinical infections. Most strains ferment rhamnose but a significant minority do not. Indole is not produced and charcoal–gelatin disks are digested slowly or not at all.

BACTEROIDES MERDAE

Described by Johnson et al. (1986) in a study of human fecal bacteroides, this species shares the general characters of the genus. Indole is not produced, charcoal–gelatin disks may be digested slowly or not at all, and it is distinguished from other members of the genus by the ability to produce acid from trehalose and salicin, but not from rhamnose or arabinose.

BACTEROIDES CACCAE

B. caccae was described in the same study as *B. merdae* (Johnson et al. 1986), from which it is distinguished by the ability to produce acid from rhamnose, but not from salicin.

BACTEROIDES OVATUS

B. ovatus is one of the less commonly encountered species of the genus. It is not a major component of the normal fecal flora and is isolated only occasionally from clinical specimens. When present in an infection it is usually in large numbers and appears to be a significant pathogen. It produces acid from a wider range of carbohydrates than other members of the genus. The ability to produce acid from salicin and mannitol is used to identify *B. ovatus*. Indole-positive, it digests charcoal–gelatin disks within a few days.

BACTEROIDES THETAIOTAOMICRON

B. thetaiotaomicron was named by Distaso (1912) as a combination of the Greek letters theta, iota, and omicron. It is a common commensal in normal human feces and the second most common species of the genus isolated from clinical infections, where it appears to have a significant pathogenic role. Acid is not formed from mannitol by this species and a variable reaction is obtained with the charcoal–gelatin disk. Some strains digest it readily but others do so only weakly or not at all. In some studies all indole-positive members of the genus that were not *B. ovatus* were assigned to *B. thetaiotaomicron*. However, other indole-positive subgroups or species are known.

BACTEROIDES EGGERTHII

B. eggerthii was named after the American bacteriologist A.H. Eggerth and described by Holdeman and Moore (1974b) following their studies on the human fecal flora. Acid formation from rhamnose but not sucrose distinguishes *B. eggerthii* from other indole-positive species of the genus. Isolates are able to digest charcoal–gelatin disks.

BACTEROIDES UNIFORMIS

This species was described by Eggerth and Gagnon in 1933. The organisms are normally found in human feces, but rarely implicated in clinical infections. In many studies, strains of *B. uniformis* have been included with other indole-positive strains as *B. thetaiotaomicron*. Like *B. variabilis*, some strains that grow well in 20 percent broth are inhibited by sodium taurocholate. Charcoal–gelatin disks are usually digested within a few days.

BACTEROIDES STERCORIS

B. stercoris was described by Johnson et al. (1986) in a study of human fecal bacteroides. Acid is produced from sucrose and rhamnose but not from trehalose, arabinose, or salicin and this distinguishes it from other indole-positive species of the *Bacteroides*.

BACTEROIDES SPLANCHNICUS

B. splanchnicus has been isolated from normal human feces and from several infections related to the lower gastrointestinal tract (Werner et al. 1975); in particular, several strains were isolated in significant numbers from infectious complications of appendicitis. It shares many characters with other members of the *Bacteroides*, but differs from them in one important respect: it is the only fermentative species of *Bacteroides* to form significant amounts of *n*-butyric acid. This it does in addition to forming a variety of other acids including *iso*-valeric, *iso*-butyric, and propionic acids, as well as acetic and succinic acids that are produced by all members of the *Bacteroides*. A minority of strains that grow well in 20 percent bile broth are inhibited by sodium taurocholate. Some strains digest charcoal–gelatin disks, but others do not. This species shares many chemotaxonomic properties with members of the genus *Porphyromonas* and the result of 16S rRNA sequence analysis suggests that it is a separate lineage (Figure 2).

PREVOTELLA

Oliver and Wherry (1921) described gram-negative anaerobic bacilli that produced black pigmented colonies when grown on blood agar. They called their strains *Bacterium melaninogenicum*; subsequently all pigmented strains were assigned to the species *Bacteroides melaninogenicus*. This characteristic appearance was regarded as highly specific and was the sole criterion for differentiation from other *Bacteroides* spp. Confusion surrounded studies on *B. melaninogenicus* because of the diversity of characters among pigmented strains and because of the specific requirements of some strains for growth factors such as vitamin K (Lev 1959), hemin, and sodium succinate. Some *B. melaninogenicus* strains isolated from the normal flora, particularly the gingival crevice, are nutritionally demanding and require a variety of growth factors provided by cocultivation with other bacteria, but as yet are unidentified.

The pigmented strains appeared to share several characters in addition to pigmentation, but there were major differences in metabolic and biochemical activity between groups, and *B. melaninogenicus* was divided into three subspecies: *melaninogenicus*, *intermedius*, and *asaccharolyticus* (Sawyer et al. 1962; Moore and Holdeman 1973). Studies of cell wall composition and DNA base ratios supported this division (Shah et al. 1976). Werner et al. (1971) found that pigmented strains from human feces and from infections related to the lower gastrointestinal tract formed a homogeneous group of asaccharolytic strains and recognized only these as *B. melaninogenicus*. The differences between the subspecies cast doubt upon the validity of assigning all pigmented strains to a single species. The asaccharolytic strains were distinguished from the saccharolytic strains by their production of *n*-butyric acid and also by differences in G + C content of the DNA and in cell wall composition. The ICSB taxonomic subcommittee on gram-negative anaerobic rods (Finegold and Barnes 1977) therefore proposed that the asaccharolytic strains should be classified as a separate species, *B. asaccharolyticus*. Further differences were found subsequently between *B. asaccharolyticus* strains associated with the intestinal flora and with superficial necrotizing infections, and pigmented nonfermentative strains associated with periodontal disease and dental root-canal infections. The latter two groups of isolates were assigned species status as *B. gingivalis* (Coykendall et al. 1980) and *B. endodontalis* (van Steenbergen et al. 1981), respectively, and both were later placed in the genus *Porphyromonas* (Shah and Collins 1988).

The fermentative pigmented species that remained ('*B. melaninogenicus*') was also heterogeneous and was divided into several distinct species. *B. melaninogenicus* subspecies *melaninogenicus* was shown to comprise three species – *B. melaninogenicus* (sensu stricto), *Bacteroides loescheii*, and *Bacteroides denticola* (Holdeman and Johnson 1982) – whereas *B. melaninogenicus* subspecies *intermedius* was divided into two species, *Bacteroides intermedius* and *Bacteroides corporis* (Johnson and Holdeman 1983). Before the elevation to species status, a weakly fermentative subspecies *levii* was proposed. The latter has now been elevated to species rank as *Porphyromonas levii* (Shah et al. 1995) and a second weakly fermentative species, *Porphyromonas macacae* (synonymous with *Porphyromonas salivosa*) has been proposed.

It is now clear that the production of pigmented colonies on media that contain blood has less taxonomic significance than was previously thought and both *Prevotella* and *Porphyromonas* include nonpigmented species. Formerly the *B. melaninogenicus-oralis* group included the fermentative pigmented species and several nonpigmented species that shared many common characters. The species *B. oralis* was originally proposed for nonpigmented bile- and penicillin-sensitive strains of *Bacteroides* isolated from the human mouth (Loesche et al. 1964), but the type strain of *B. oralis* proved to be pigmented and was first reclassified as *B. melaninogenicus* subspecies *melaninogenicus* (Holbrook and Duerden 1974) and subsequently as *B. loescheii* (Holdeman and Johnson 1982). However, other nonpigmented strains were described that corresponded to the original description of *B. oralis* or appeared to be related to it. These were represented by *B. oralis*, *Bacteroides veroralis* (Watabe et al. 1983), *Bacteroides oris*, *Bacteroides buccae* (Holdeman et al. 1982), *Bacteroides zoogleoformans* (Cato et al. 1982), and *Bacteroides buccalis* (Shah and Collins 1981). Two other nonpigmented members of the melaninogenicus-oralis group, *Bacteroides disiens* and *Bacteroides bivius*, were isolated from human clinical infections by Holdeman and Johnson (1977) and have since been found in the normal vaginal flora and infections of the genitourinary tract, and occasionally in the mouth.

Description of the genus

The organisms are gram-negative, obligately anaerobic, nonspore-forming, nonmotile pleomorphic rods. Surface colonies on blood agar plates vary from minute to 2.0 mm in diameter and are generally circular, entire, convex, shiny, and smooth. Colonies on blood agar are translucent, opaque, gray, light brown, or black. Hemolysis is variable. Glucose broth cultures are usually turbid with smooth or stringy sediments, and the terminal pH is between 4.5 and 5.2. The optimum temperature for growth is 37°C, but some strains grow at 25 and 45°C. Growth of most species is inhibited by 6.5 percent NaCl. Hemin and menadione are required for growth of most species. Growth is inhibited by 20 percent (w/v) bile. They are inhibited by sodium taurocholate and

sodium deoxycholate and will not grow in 20 percent bile broth. They are inhibited by most dyes but many strains are tolerant of Victoria blue 4R (1 in 80 000). In antibiotic resistance tests, most strains are sensitive to neomycin (1 000 μg), rifampicin (15 μg), and resistant to kanamycin (1 000 μg) disks. Many strains are sensitive to benzylpenicillin, but some strains of all species of the genus are resistant as a result of plasmid-coded β-lactamase production. Threonine is not dehydrogenated. The major fermentation products are acetic and succinic acid. Occasionally lower levels of *iso*-butyric, *iso*-valeric or lactic acid are produced. Proteolytic activity is variable. Nearly all species are indole-negative. Nitrate is not reduced to nitrite. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The principal respiratory quinones are unsaturated menaquinones, often with 10–13 isoprene units. Both nonhydroxylated and 3-hydroxylated fatty acids are composed of predominantly straight-chain saturated, *anteiso*- and *iso*-methyl, branched-chain types. Sphingolipids are produced. The DNA base compositions are within the range of 40–52 mol% G+C. Other currently described species that conform to the generic description of *Prevotella* are given in Table 3. The genus was emended by Willems and Collins (1995c) to include species that are phylogenetically coherent but possess DNA base compositions within the range 40–60 mol% G+C. The type species is *Prevotella melaninogenica* (Table 4). Characteristics that differentiate species of the genus *Prevotella* are given in Table 3, while those that differentiate the pigmented species (*Prevotella* and *Porphyromonas*) are given in Table 5.

PREVOTELLA MELANINOGENICA

P. melaninogenica is a common commensal in the human gingival crevice and in the vagina and is found in infections related to these sites. Most strains are short gram-negative bacilli or coccobacilli and many are moderately pleomorphic. Colonies are 1–2 mm in diameter, round, convex, and opaque. After incubation for 48 h, they are typically light gray, becoming brown after further incubation. Pigmentation develops more rapidly on lyzed blood agar and varies between strains from light brown to almost black. It begins in the center of the colony and the colonies of many strains have a light brown or pale annulus around a dark-brown center. Charcoal–gelatin disks are digested within a few days. The G + C content of the DNA is 40–42 mol% (Shah et al. 1976). Strains contain a peptidoglycan based upon *meso*-diaminopimelic acid.

PREVOTELLA LOESCHEII

This pigmented species is named after W.J. Loesche. The type strain is ATCC 15930, originally designated *B. oralis*. Pigment production is fairly slow, but is enhanced by growth on lyzed blood agar. Ability to hydrolyze esculin distinguishes it from *P. melaninogenica*. The DNA G + C content is 46–48 mol%.

PREVOTELLA DENTICOLA

Described by Shah and Collins (1981), this pigmented species is found in the gingival crevice and in human clinical specimens from sites in and around the mouth. It is distinguished from *P. melaninogenica* and *P. loescheii* by tests for hydrolysis of esculin and fermentation of cellobiose. The G + C content of its DNA is 49–51 mol%.

PREVOTELLA INTERMEDIA AND *PREVOTELLA NIGRESCENS*

Isolates of these species initially belonged to *P. intermedia*. They are associated with the gingival crevice and are often isolated from cases of gingivitis and other purulent lesions related to the mouth. Most strains are

Table 3 *Characteristics that differentiate nonpigmented* Prevotella *species*

Species	Arabinose	Cellobiose	Lactose	Mannose	Raffinose	Salicin	Xylose	Esculin	Gelatin	Pigment
P. albensis	+	+	+	–	–	+	+	+	+	–
P. bivia	–	–	+	+	–	–	–	–	+	–
P. brevis	+	+	+	+	+	–	–	+	+	–
P. bryantii	+	+	+	+	+	+	+	+	+	–
P. dentalis	+	+	+	+	+	–	–	+	–	–
P. disiens	–	–	–	–	–	–	–	–	+	–
P. enoeca	–	–	+	V	–	–	v	v	+	–
P. heparinolytica	+	+	+	+	–	+	+	+	–	–
P. oralis	–	+	+	+	+	+	+	+	+	–
P. oris	+	+	+	+	+	+	–	+	v	–
P. oulora	–	–	+	+	+	–	–	+	–	–
P. ruminicola	+	+	+	+	+	+	v	+	+	–
P. veroralis	–	V	+	+	+	V	v	+	v	–
P. zoogleoformans	V	V	V	V	v	V	v	+	v	–

+, Positive result; –, negative result; v, variable result.

Table 4 *Species of the genus* Prevotella *with their type strain*

Species	Type strain
Prevotella albensis	NCTC 13060
Prevotella bivia	NCTC 11156
Prevotella brevis	NCTC 13061
Prevotella bryantii	NCTC 13062
Prevotella buccae	NCTC 13063
Prevotella buccalis	NCTC 13064
Prevotella corporis	NCTC 13065
Prevotella dentalis	NCTC 13066
Prevotella denticola	NCTC 13067
Prevotella disiens	NCTC 11157
Prevotella enoeca	NCTC 13068
Prevotella gulae	NCTC 13180
Prevotella heparinolytica	NCTC 13069
Prevotella intermedia	NCTC 13070
Prevotella loescheii	NCTC 11321
Prevotella melaninogenica	NCTC 12963 Type species
Prevotella nigrescens	NCTC 9336
Prevotella oralis	NCTC 11459
Prevotella oris	NCTC 13071
Prevotella oulora	NCTC 11871
Prevotella pallen	NCTC 13042
Prevotella ruminicola	NCTC 13072
Prevotella tannerae	NCTC 13073
Prevotella veroralis	NCTC 13074
Prevotella zoogleoformans	NCTC 13075

short gram-negative bacilli and coccobacilli and many are pleomorphic. The colonies are 1–2 mm in diameter, round, convex, and opaque. After incubation for 48 h, isolated colonies are typically gray, but become uniformly black after further incubation. Some strains are shiny and glistening, but others are dull with a rough, dry surface and these may adhere to the underlying medium. Pigmentation develops more rapidly on lyzed blood agar. Most strains produce clear zones of complete hemolysis in blood agar and are inhibited by Victoria blue 4R. Strains produce indole, readily digest charcoal–gelatin disks and do not ferment lactose. The G + C content of the DNA is 40–42 mol%. Lambe and Jerris (1976) described two distinct serogroups. Johnson and Holdeman (1983) have described two DNA homology groups, which were recently confirmed (Shah and Gharbia 1992) using multilocus enzyme electrophoresis, DNA–DNA homology and subsequently 16S rRNA sequence analysis. This resulted in the proposal of the species *P. nigrescens* for the second homology group.

PREVOTELLA CORPORIS

P. corporis was described by Johnson and Holdeman (1983) to represent strains isolated from clinical human specimens previously considered to be *P. intermedia*, but which do not produce indole or ferment sucrose. The DNA G + C content is 43–46 mol%.

PREVOTELLA ORALIS

Strains that correspond to the original description (Loesche et al. 1964) are an important component of the normal flora of the gingival crevice, and similar strains have been isolated from the normal vagina. Nonpigmented oral strains have also been implicated in infections derived from these sites. All these infections are associated with tissue necrosis and the production of foul-smelling pus. Colonies of *P. oralis* are 1–2 mm in diameter, semi-opaque, and light-buff or gray. After prolonged incubation some become pale brown and some develop a spreading edge with an irregular outline. Most strains are predominantly coccobacillary but a few produce longer, more regular bacilli. Most strains hydrolyze esculin and digest charcoal–gelatin disks within a few days.

PREVOTELLA PALLENS

P. pallens isolates were separated from *P. nigrescens* based on their weak pigmentation of colonies on blood agar plates. A distinct halo of metallic sheen is seen around the colonies and exhibit red fluorescence under UV light. All strains are resistant to vancomycin but susceptible to colistin.

PREVOTELLA VERORALIS

This species differs from *P. oralis* in failing to ferment salicin and has negligible DNA homology with that species (Watabe et al. 1983). It more closely fits the original description of *P. oralis* (Loesche et al. 1964) than the strains now designated *P. oralis*. The DNA G + C content is 42 mol%.

PREVOTELLA BUCCALIS

P. buccalis is indistinguishable from *P. veroralis* in most routine conventional tests and has a similar DNA base composition. However, unlike *P. veroralis*, it produces *iso*-valeric acid as a major metabolic product and it does not hydrolyze starch (Watabe et al. 1983). These differences are supported by lack of DNA homology between *P. buccalis* strains and other *Prevotella* spp.

PREVOTELLA ZOOGLEOFORMANS

This species was described by Weinberg et al. (1937), and Prévot (1938) proposed a new genus *Capsularis* for these strains. Moore et al. (1994) emended the description of the species. No early strains remain extant, but Cato et al. (1982) isolated strains that fulfilled the description from the gingival crevice of patients with periodontal disease. The specific epithet '*zoogleoformans*' represents the character of this organism's growth in broth culture. It forms a viscous, glutinous mass, especially in the presence of fermentable carbohydrate.

Table 5 *Characteristics that differentiate pigmented* Prevotella *and* Porphyromonas *species*[a]

Species and origin	Indole	Lipase	Catalase	Esculin hydrolysis	α-Fucosidase[b]	α-Galactosidase	β-Galactosidase	N-Acetyl-β-glucosaminidase	Chymotrypsin	Trypsin	Glucose	Cellobiose	Lactose	Sucrose	Fatty acids from PYG[c]
Porphyromonas, asaccharolytic or weakly saccharolytic															
Human origin											–	–	–	–	
P. asaccharolytica	+	–	–				+	+	–	+	W	–	W	–	A, P, iv, I, S
P. catoniae[d]	–	–	–	–	+	–	–	–	–	–	–	–	–	–	A, P, ib, B, IV, s
P. endodontalis	+	–	–	–	–	–	–	+	+	–	–	–	–	–	A, P, ib, B, IV, s, pa
P. gingivalis[e]	+	–	–	–	–	–	+	+	–	+	W	–	W	–	A. P, ib, B, IV, s
P. levii-like[e,g]	–	–	–	–	–	–									
Animal origin[g]															
P. canoris	+	–	+	–	–	–	+	+–	–	+	–	–	–	–	A, P, ib, b, IV, s
P. cangingivalis	+	–	+	–	–	–	–	–	–	+	–	–	–	–	A, p, ib, B, IV
P. cansulci	+	–	+[w]	–	–	–	–	–	–	–	–	–	–	–	A, P, ib, B, IV, S, pa
P. circumdentaria	+	–	+	–	–	–	–	–	–	–	–	–	–	–	A, P, ib, b, IV, s, pa
P. crevioricanis	+	NA	–	–	–	NA	NA	–	–	NA	–	–	–	–	A, p, ib, B, IV, s
P. gingivicanis	+	NA	+	–	–	NA	NA	–	–	NA	–	–	–	–	
P. gulae	+	–	+	–	–	–	–[f]	+	+	–	–	–	–	–	A, P, ib, B, IV, s, pa
P. macacae	+	+	+	–	–	+	–[f]	+	+	+	W	–	W	–	A, P, ib, B, IV, s, pa
Prevotella, saccharolytic															
P. corporis	–	–	–	–	–	–	–	–	–	+	+	–	–	–	A, ib, iv, S, (b)
P. denticola	–	–	–	+	+	+	+	+	–	–	+	–	+	+	A, S, (ib, iv, l)
P. intermedia	+	+	–	–	+	–	–	–	–	–	+	–	–	+	A, iv, S, (p, ib)
P. loescheii	–	V	–	+	+	+	+	+	–	–	+	+	+	+	a, S, (l)
P. melaninogenica	–	–	–	–	+	+	+	+	–	–	+	–	+	+	A, S, (ib, iv, l)
P. nigrescens[h]	+	+	–	–	+	–	–	–	–	–	+	–	–	+	A, iv, S, (p, ib)
P. pallens	+	–	–	–	+	–	–	–	–	–	+	–	–	+	A, S, (p, ib)
P. tannerae	–	–	–	–	+	–	+	+	–	–	+	–	+	v	A, iv, S, (ib)

a) See Table 3, footnote a. + [w], most strains positive; some strains weakly positive.
b) Reaction by the API ZYM system or with Rosco Diagnostic Tablets (see Table 4, footnote b). Reactivity in these systems in not always identical (see footnote f).
c) See Table 3, footnote c.
d) Nonpigmented.
e) *P. gingivalis* does not show fluorescence; *P. kevii-like* may show weak or no fluorescence.
f) Negative by the API ZYM system; positive by the Rosco *o*-nitrophenyl-β-d-galactopyranoside test.
g) May be isolated from bite infections; NA, information not available.
h) *P. intermedia* shares the same characteristics. Differentiation based on enzyme electrophoresis, oligonucleotide probe analysis, or arbitrarily primed PCR.

Most strains except the type strain produce indole, ferment cellobiose, and hydrolyze esculin. The major cellular fatty acids are predominantly *iso*-$C_{15:0}$ and *anteiso*-$C_{15:0}$ acids. The G + C content of the type strain ATCC 33285 is 47 mol%. Recently rRNA sequence analysis has indicated a closer affinity with the genus *Bacteroides*.

PREVOTELLA ORIS AND *PREVOTELLA BUCCAE*

These two species accommodate strains of human origin that were previously identified as *B. ruminicola* subspecies *brevis* biovar (biotype) 3. Similar strains have been isolated from chicken ceca. The human isolates are mostly from gingival margins and periodontal pockets and infections at related sites. They differ from *P. oralis* by their ability to ferment pentoses, which is the reason for their earlier confusion with *P. ruminicola*. The DNA G + C content for *P. oris* is 42–46 mol%, whereas for *P. buccae* it is 50–52 mol% (Holdeman et al. 1982).

PREVOTELLA HEPARINOLYTICA

Isolated from patients with periodontitis, this species produces acid from a range of sugars, including pentoses, is indole-positive, hydrolyzes esculin, and produces heparinase (Okuda et al. 1985). rRNA sequence analysis indicates a closer affinity with the genus *Bacteroides*.

PREVOTELLA OULORA

P. oulora is also found in the mouth. It ferments only a limited range of sugars, is indole-negative, and does not hydrolyze esculin. It produces a lipase (Shah et al. 1985).

PREVOTELLA BIVIA

Isolated from human infections by Holdeman and Johnson (1977), this is the most common species of *Prevotella* in the normal human vaginal flora (Duerden 1980b). Most clinical isolates are from infections related to the female genital tract. It cannot be recognized by cell morphology or colonial appearance and does not form pigment, but colonies are often pale brown after prolonged incubation on lyzed blood agar. Few strains hydrolyze esculin, but charcoal–gelatin disks are digested within a few days. The DNA G + C content is 40 mol%.

PREVOTELLA DISIENS

Described by Holdeman and Johnson (1977) at the same time as *P. bivia* and isolated from similar infections and from the normal vaginal flora, strains of *P. disiens* differ from *P. bivia* by their inability to ferment lactose. The DNA G + C content is 40–42 mol%.

PREVOTELLA DENTALIS

Isolates of this species were previously reported as *Hallella seregens* or *Mitsuokella dentalis*. rRNA sequence analysis confirmed that they are similar and are genealogically related to *Prevotella* (Willems and Collins 1995c). Strains are isolated from dental root canals and are distinguished from other *Prevotella* spp. by the typical water drop appearance of its colonies on solid medium. The DNA G + C content is 56–60 mol%.

PREVOTELLA ENOECA

This saccharolytic species was isolated from the gingival crevice of humans. Colonies are small, 1–2 mm in diameter, nonhemolytic, nonpigmented, and strains characteristically ferment glycogen and lactose, but not sucrose, and are unable to ferment gelatin. The endproducts of metabolism are acetic and succinic acids and, in common with most *Prevotella* spp., *P. enoeca* contains *anteiso*-$C_{15:0}$ as its major cellular fatty acid. The G + C content of the type strain ATCC 51261 is 47 mol%.

PREVOTELLA TANNERAE

This species colonizes the human gingival crevice and contains strains that produce small, circular colonies, 1 mm in diameter, that may be black, tan to brown, reddish, or colorless on rabbit blood agar medium. Most strains are unable to hydrolyze esculin, but produce acid from glycogen and lactose. The fermentation acids are mainly succinic and acetic with lower levels of formic, *iso*-valeric and *iso*-butyric acids. Its major cellular fatty acids are *iso*-$C_{15:0}$ and *anteiso*-$C_{15:0}$ acids. The G + C content of the type strain ATCC 51259 is 45 mol%.

PREVOTELLA RUMINICOLA

This species is isolated from the rumen and from the hindgut of many mammalian species. Most strains produce clear zones which are visible after staining with congo red. Most strains ferment arbutin and salicin, and are stimulated by heme in the absence of rumen fluid. The DNA G + C content is 45–52 mol%.

PREVOTELLA ALBENSIS

P. albensis is isolated from the rumen and from the hindgut of many mammalian species. Strains do not ferment melibiose, sucrose, or mannose, but ferment xylose. The DNA G + C content is 39–43 mol%.

PREVOTELLA BREVIS

P. brevis is isolated from the rumen and from the hindgut of many mammalian species. Most strains produce abundant extracellular DNAase activity and do not ferment xylose. Many strains can grow with gum arabic as an energy source. The DNA G + C content is 45–52 mol%.

PREVOTELLA BRYANTII

P. bryantii is isolated from the rumen and from the hindgut of many mammalian species. Most strains

produce abundant extracellular DNAase and ferment xylose. The DNA G + C content is 39–43 mol%.

NEW *PREVOTELLA* SPECIES

Following the completion of this chapter, two new *Prevotella* species: *Prevotella shahii* sp. nov. and *Prevotella salivae* sp. nov. have been proposed (see Sakamoto et al. 2004).

PORPHYROMONAS

Description of the genus

Porphyromonas spp. are gram-negative, obligately anaerobic nonspore-forming, nonmotile rods, or coccobacilli (Table 5). Most cells in broth are small (0.5–0.8 by 1.0–3.5 μm), but occasionally longer cells (4–6 μm) may be formed. Surface colonies on blood agar plates are smooth (rarely rough), shiny, convex, circular, entire, and vary from minute to 3.0 mm in diameter and darken progressively from the edge toward the center after 6–10 days. Eventually the entire colony becomes black due to an overproduction of protoheme. The optimum temperature for growth is 37°C. Growth is not significantly affected by carbohydrates. Nitrogenous substrates such as proteose peptone, trypticase, and yeast extract markedly enhance growth. The major fermentation products from media containing these substrates are *n*-butyric, propionic, and acetic acids with lower levels of *iso*-butyric, *iso*-valeric, succinic, and phenylacetic acids. Some species are strongly proteolytic, whereas others show weak peptidase activities. In general there is a paucity of reliable characters for differentiation of these species (see Table 5), thus new techniques such as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) may be useful in helping to delineate these species (see, for example, Figure 3) (Shah et al. 2000, 2002).

Malate and glutamate dehydrogenases are present in cell extracts. With the exception of a few species isolated from animals, glucose-6-phosphate and 6-phosphogluconate dehydrogenases are absent. Amino acids, particularly aspartic and glutamic acids, are fermented. Indole is produced by most species and nitrate is not reduced to nitrite.

Growth of all the pigmented species is inhibited by vancomycin. The cell wall peptidoglycan contains lysine or *meso*-diaminopimelic acid. The principal respiratory quinones are unsaturated menaquinones. Both nonhydroxylated and 3-hydroxylated fatty acids are present. The nonhydroxylated fatty acids are composed of predominantly *iso*-methyl branched-chain types (*iso*-$C_{15:0}$) and lower levels of straight-chain saturated types. The 3-hydroxylated fatty acids are the straight-chain saturated types. Sphingolipids are produced. The DNA base compositions are within the range 40–55 mol% G + C. Salient phenotypic features of the species of the genus are given in Table 5. The genus was emended recently by Willems and Collins (1995b) to include species that are phylogenetically coherent, but lack some of the key features of the original description of this genus, such as pigment production and their inability to ferment carbohydrates. The type species of the genus *Porphyromonas* is *Porphyromonas asaccharolytica*.

PORPHYROMONAS ASACCHAROLYTICA

These organisms are mainly coccobacilli with only a few slightly longer bacilli. Very small colonies may be visible

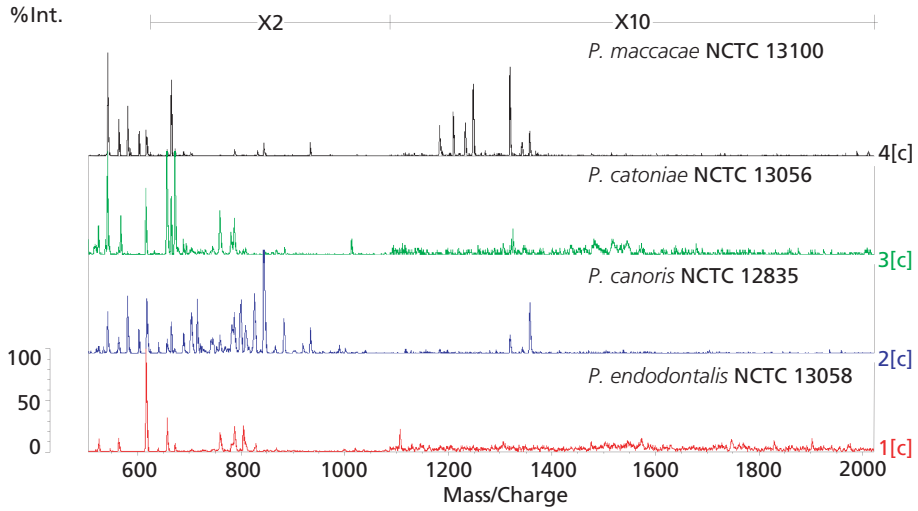

Figure 3 *Differentiation of four species of* Porphyromonas *using MALDI-TOF-MS of intact cell surfaces. The mass ions (m/z value) of the surface components of the cell is given along the horizontal axis, while the mass intensities (0–100 percent) of each spectrum is shown by the peak height. The results show that the mass spectral fingerprint of each species is dintinctive, therefore species may be characterized by reference to a database of existing spectra.*

on blood agar after 24 h, but some strains do not appear for 48 h. They are smooth, shiny, and grey or brown. Pigmentation begins to develop after 3–4 days on whole blood agar, but may be visible in the inoculum after 36–48 h on lyzed blood agar. After 4–5 days, colonies are 0.5–1 mm in diameter, black or very dark brown. The colonies are smooth and shiny; they are often very moist and can be smeared over the agar surface, although the organisms do not produce large amounts of extracellular mucus. Most strains have a characteristically strong, putrid smell. The major endproducts of metabolism in a cooked meat–carbohydrate medium are *n*-butyric and acetic acids together with lower levels of propionic, *iso*-butyric and *iso*-valeric acids. *P. asaccharolytica* is inhibited by bile and bile salts and by dyes, resistant to kanamycin and sensitive to penicillin and rifampicin. Most strains are sensitive to neomycin (1 000 μg per disk), but some are resistant. *P. asaccharolytica* does not agglutinate sheep or horse erythrocytes (Slots and Genco 1979). The G+C content of the DNA is 52–54 mol%. *P. asaccharolytica* is found in normal human feces, but less consistently and in smaller numbers than *Bacteroides* spp. It has also been reported in the normal vagina. It is an important pathogen in mixed infections but is rarely isolated alone. It is found in infections related to the lower gastrointestinal tract and in destructive ulcers and gangrene in diabetics and others with peripheral vascular disease. It should be noted that early work on the experimental pathogenicity of '*B. melaninogenicus*' (Werner et al. 1971) was probably done with *P. asaccharolytica*.

PORPHYROMONAS CANGINGIVALIS

This species is isolated from the subgingival flora of dogs. Cells are coccoids, 0.3–0.6 μm, and occur singly or in clumps. Occasionally filaments up to 16 μm long are observed. On sheep blood agar plates, surface colonies after 2 days are 1 mm in diameter, circular, dome-shaped, nonhemolytic, and smooth. Colonies are poorly pigmented and creamy after 3–5 days and by 10 days are light brown. On egg yolk agar, colonies are yellow or orange. This species produces acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric, succinic, and phenylacetic acids as metabolic endproducts. Strains produce catalase and acid and alkaline phosphatases, but do not produce any proteinases or lipases. The G+C content of the DNA is 49–51 mol%.

PORPHYROMONAS CANORIS

Isolated from the gingival sulcus of dogs, the cells are rod-shaped and are 0.3–0.6 by 0.8–1.5 μm, but filaments up to 16 μm are sometimes present. After growth for 48 h on sheep blood agar plates, surface colonies are 1.5 mm in diameter, circular, rough, and have an orange pigmentation. Between 3 and 6 days colonies exhibit a brick red fluorescence when irradiated with shortwave UV radiation (265 nm), but this changes to a light orange after 13 days. Pigmentation occurs faster with laked blood. The major endproducts of metabolism in cooked meat–carbohydrate medium are acetic, propionic, *iso*-valeric, and succinic acids together with low levels of *n*-butyric and *iso*-butyric acids. Lactate is converted to propionate, but neither pyruvate nor threonine is utilized. Strains are catalase-, acid- and alkaline phosphatase-positive, liquefy gelatin, and possess chymotrypsin activity. Both acid and alkaline phosphatases are produced. A wide spectrum of oxidoreductases are produced, including malate, glutamate, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases. The nonhydroxylated fatty acids are composed of predominantly *iso*-methyl branched types, with *iso*-$C_{15:0}$ acid predominating. The G+C content of the DNA is 49–51 mol%.

PORPHYROMONAS CANSULCI

This species is isolated from the subgingival flora of dogs. Cells are coccoid, 0.3–0.6 μm, and occur singly or in groups. On sheep blood agar plates, surface colonies after 3 days are 1–2 mm in diameter, circular, dome-shaped, and smooth. Colonies are black or brown after 5 days. On egg yolk agar colonies are yellow or orange. This species produces a wide spectrum of fermentation products that includes acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric, succinic, and phenylacetic acids. Neither lactate nor pyruvate is utilized and threonine is not converted to propionate. Strains produce catalase and acid and alkaline phosphatases, but do not produce any proteinases or lipases. The G+C content of the DNA is 49–51 mol%.

PORPHYROMONAS CATONIAE

Isolated from the gingival crevice of humans, strains were of uncertain taxonomic designation initially and were temporarily placed in a group referred to as '*Bacteroides* D26'. These were subsequently assigned to a new genus, *Oribaculum catoniae*, by Moore and Moore (1994), but later transferred to the genus *Porphyromonas* by Willems and Collins (1995b) based on comparative analysis of approximately 96 percent of the total primary sequence of the 16S rRNA. At present, this is the only nonpigmented and saccharolytic member of the genus. Colonies on blood agar plates are 0.5–2 mm in diameter, circular, entire flat to low convex, and transparent. Cells grown in a glucose-containing medium (e.g. peptone–yeast extract–glucose (PYG)) produce a terminal pH of 5.0–5.5 and their metabolic endproducts are mainly acetic and succinic acids with low levels of formic, lactic, and *iso*-valeric acids. The major cellular fatty acids are *iso*-$C_{15:0}$ and *anteiso*-$C_{15:0}$ but substantial levels of *iso*-$C_{13:0}$ are also produced. The DNA base compositions are in the range 49–51 mol% G+C.

PORPHYROMONAS CIRCUMDENTARIA

This species is isolated from soft tissue infections, the gingival margins, and gingival plaques of cats. On sheep blood agar plates, colonies after 72 h are 1–2 mm in diameter, circular, entire, dome-shaped, and are greenish brown, but become creamy brown by 7 days. During this period, colonies exhibit a brick red fluorescence when irradiated with shortwave UV radiation (254 nm). By 14 days, colonies are brown to black. Cells are rod-shaped and are 0.3–0.6 by 0.8–1.5 μm, but filaments up to 10 μm are sometimes present. The major endproducts of metabolism in cooked meat–carbohydrate medium are acetic, *n*-butyric, and *iso*-valeric acids with lower levels of propionic, *iso*-butyric, succinic, and phenylacetic acids. Strains are catalase-positive, produce ammonia, and liquefy gelatin. Both acid and alkaline phosphatases are produced. The G + C content of the DNA is 40–42 mol%.

PORPHYROMONAS CREVIORICANS

P. crevioricans is isolated from the gingival crevicular fluid of beagle dogs. Colonies on rabbit blood agar plates after 72 h are tiny, 0.8–1.5 mm in diameter, circular, entire, dome-shaped, opaque, and brown or black. The major endproducts of metabolism in GAM medium (Nissul, Tokyo, Japan) are acetic, propionic, and *iso*-valeric acids with lower levels of *n*-butyric, *iso*-butyric, succinic, and phenylacetic acids. Malate and glutamate dehydrogenases are present in cell extracts, but glucose-6-phosphate and 6-phosphogluconate dehydrogenases are absent. Cells hemagglutinate sheep erythrocytes. Strains so far tested (Hirasawa and Takada 1994) are susceptible to penicillin (0.5 U per disk), amoxycillin (2 μg per disk), sulbenicillin (5 μg per disk), and erythromycin (0.5 μg per disk). The nonhydroxylated fatty acids are composed of predominantly *iso*-methyl branched types, with *iso*-$C_{15:0}$ acid predominating. The G + C content of the DNA is 44–45 mol%.

PORPHYROMONAS ENDODONTALIS

This pigmented species is more difficult to culture on primary isolation, but becomes easier to maintain with repeated subculture. Initially, colonies are very sticky and adhere strongly to the medium. It is isolated from infected dental root canals with severe periapical destruction (van Steenbergen et al. 1984). Colonies on blood agar plates are shiny, smooth, convex, and circular. Major fermentation products are *n*-butyric and acetic acids. Lower levels of propionic, *iso*-butyric, and *iso*-valeric acids are produced, but phenylacetic acid is not produced. The G + C content of the DNA is 49–51 mol%.

PORPHYROMONAS GINGIVALIS

Cells are nonmotile rods or coccobacilli and are 0.5–2 μm long. Colonies on blood agar are smooth, shiny, convex, and 1–2 mm in diameter and darken from the edge of the colony toward the center between 4 and 8 days. Nonpigmented colonies rarely occur. Growth is markedly affected by the presence of protein hydrolysates such as trypticase, proteose peptone, and yeast extract. Growth is enhanced by 0.5–0.8 percent NaCl. Major fermentation products are *n*-butyric and acetic acids. Lower levels of propionic, *iso*-butyric, *iso*-valeric, and phenylacetic acids are also produced. Cysteine proteinases and collagenases are present. Cells agglutinate sheep erythrocytes. Cell wall peptidoglycan contains lysine as the diamino acid. Both nonhydroxylated and 3-hydroxylated fatty acids are present. The nonhydroxylated fatty acids are composed of predominantly *iso*-methyl-branched types, with *iso*-$C_{15:0}$ acid predominating. The major respiratory quinones are menaquinone with nine isoprene units. The G + C content of the DNA is 46–48 mol%.

PORPHYROMONAS GINGIVICANS

This species is isolated from the gingival crevicular fluid of beagle dogs. Colonies on rabbit blood agar after 72 h are tiny, 0.8–1.5 mm in diameter, circular, entire, dome-shaped, opaque, and brown or black. The major endproducts of metabolism in GAM medium (Nissul, Tokyo, Japan) are acetic, *n*-butyric, and *iso*-valeric acids with lower levels of propionic, *iso*-butyric, and succinic acids. Malate and glutamate dehydrogenases are present in cell extracts, but glucose-6-phosphate and 6-phosphogluconate dehydrogenases are absent. Strains are catalase-positive. Cells neither agglutinate sheep erythrocytes nor exhibit trypsin nor *N*-acetyl-β-glucosaminidase activity. Strains so far tested (Hirasawa and Takada 1994) are susceptible to penicillin (0.5 U per disk), amoxycillin (2 μg per disk), sulbenicillin (5 μg per disk), and erythromycin (0.5 μg per disk). The nonhydroxylated fatty acids are composed of predominantly *iso*-methyl-branched types, with *iso*-$C_{15:0}$ acid predominating. The G + C content of the DNA is 41–42 mol%.

PREVOTELLA GULAE

Described by Fournier et al. (2001), this species was isolated from the subgingival plaque of several animals. Strains are catalase-positive and produce indole. A brown pigment is produced on aging of the cultures, but colonies do not fluoresce under UV light. The DNA G + C content is 51 mol%.

PORPHYROMONAS LEVII

Named after M. Lev, who isolated it from cattle (Lev 1959), this species produces dark brown colonies slowly on blood agar and more promptly on lyzed blood agar. Unlike most pigmented members of the genus, it is weakly fermentative and weakly ferments glucose and lactose, but not other carbohydrates. Charcoal–gelatin disks are digested within a few days. *P. levii* produces

n-butyric acid as its major product of metabolism with moderate levels of acetic, propionic, *iso*-butyric, and *iso*-valeric acids. The nonhydroxylated fatty acids are composed of major levels of *iso*-$C_{15:0}$ and *anteiso*-$C_{15:0}$ acids. The G + C content of the DNA is 45–48 mol%.

PORPHYROMONAS MACACAE

This species is weakly fermentative and has been isolated from periodontal lesions in monkeys (Slots and Genco 1980). Strains are catalase-positive, produce ammonia, and possess both trypsin and chymotrypsin activities. Strains are indole-positive and are further distinguished from *P. levii* by production of propionic acid and not butyric acid as the major metabolic product. This species possesses a wide spectrum of oxidoreductases that include malate, glutamate, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases. The nonhydroxylated fatty acids are composed of predominantly *iso*-methyl branched types, with *iso*-$C_{15:0}$ acid predominating. The DNA G + C content is 43–44 mol% (Coykendall et al. 1980).

THE CURRENT STATUS OF SPECIES THAT PREVIOUSLY BELONGED TO THE GENUS *BACTEROIDES*

The emphasis now is to develop taxonomic schemes that reflect 'natural' or 'evolutionary' relationships among bacteria which has led to re-examination of many taxa that only superficially resemble the generic description of type species. Members of the genus *Bacteroides* have been subjected to rigorous chemotaxonomic analysis and the results from these studies have been largely confirmed by molecular systematic analysis. Thus, the more restricted definition of the genus *Bacteroides* and the proposal of *Prevotella* and *Porphyromonas* have been borne out by data derived from comparative sequence analysis of 16S rDNAs (see Figure 2), whereas others that have been excluded from the genus have been substantiated by these tests (see reviews by Shah and Collins 1983; Shah et al. 1997; Olsen and Shah 2003). The current status of some of these taxa is described below.

Bacteroides ureolyticus and *Bacteroides gracilis*

B. ureolyticus is a nonfermentative, nonpigmented species that characteristically 'pits or corrodes' the agar around the colonies. It was first described by Henriksen (1948) in clinical specimens and later by Eiken (1958) who isolated 21 strains from abscesses, principally from the buccal region, and assigned them to the genus *Bacteroides* as *Bacteroides corrodens*. Khairat (1967) cultivated a similar organism from blood cultures after tooth extraction and also three strains phenotypically similar from the gingival crevice. Reinhold (1966) found it in abscesses and Schröter and Stawru (1970) in diseased tonsils. It was renamed *B. ureolyticus* to clarify the distinction between it and *Eikenella corrodens*, an aerobic and facultatively anaerobic, carbon dioxide-dependent organism that also forms 'corroding' colonies. The two were often confused in early reports (Jackson and Goodman 1978). This species was subsequently shown to share few biochemical and chemical properties with the genus *Bacteroides* sensu stricto (see Shah and Collins 1983) and was excluded from the redefined genus. *B. gracilis* shares a number of common properties with *B. ureolyticus* in that it is nonpigmented, asaccharolytic, microaerophilic, and colonies often show pitting or corroding growth indistinguishable from that of *B. ureolyticus*. However, it is oxidase-negative and does not produce urease, although some strains reduce nitrate to nitrite (Tanner et al. 1981). Chemotaxonomic analyses, DNA–rRNA hybridization, and 16S rRNA sequence analysis have shown that both species belong to the genus *Campylobacter* and *B. ureolyticus* has been formally reclassified in this genus (Vandamme et al. 1995).

Bacteroides praeacutus

Strains have been isolated from human feces and from clinical specimens. *B. praeacutus* differs from other species of *Bacteroides* in being motile by means of peritrichous flagella and differ so markedly in biochemical and chemical properties (e.g. its G + C content is 28 mol%) from other members that it was excluded from the emended description of the genus *Bacteroides* (Shah and Collins 1983) and was reproposed in the genus *Tissierella*, as *Tissierella praeacuta*. The 16S RNA gene sequence has shown that this species is synonymous with *Clostridium hastiforme*.

Bacteroides pneumosintes

B. pneumosintes is a very small bacillus that is filterable through Berkfeld V and N filters and is unreactive in most conventional bacteriological tests. It was described by Olitsky and Gates (1921, 1922) in nasopharyngeal washings from patients in the 1918 influenza pandemic and called *Dialister pneumosintes*. These authors thought that it was a significant pathogen. It has since been isolated from normal persons, but it may be concerned in secondary infections of the respiratory tract, including lung abscesses, and in metastatic brain abscesses. Long considered to be outside the generic description of *Bacteroides* (see Shah and Collins 1983), the genus *Dialister* was revived by Moore and Moore (1994) to accommodate this species. More recently, it was shown by Willems and Collins (1995a) that these microorganisms

belong to the *Sporomusa* sub-branch of the *Clostridium* subphylum.

Bacteroides nodosus

B. nodosus is a significant pathogen, causing foot rot in sheep (Egerton and Parsonson 1966) and possibly also in goats and cattle (Wilkinson et al. 1970). The hoof seems to be its natural habitat. The cells are irregular with terminal enlargements, especially in smears prepared directly from lesions. It is strongly proteolytic and will digest powdered hoof in vitro. The DNA G + C content is 45 mol%. It was reclassified in a new genus, *Dichelobacter*, as *Dichelobacter nodosus*.

Bacteroides putredinis and *Bacteroides microfusus*

Despite being nonfermentative, some strains of *B. putredinis* grow in a glucose broth to produce cells that are either straight or curved with rounded ends. Some cells produce swellings at one end, but spores have not been detected. Strains are proteolytic and some may digest cooked meat. *B. putredinis* produces fibrinolysin and may be isolated from feces, abdominal and rectal abscesses, cases of acute appendicitis, and foot rot in sheep. It has been reported from the human oral cavity. It shares very few characters with members of the genus *Bacteroides* and recent 16S rRNA sequence analysis (Paster et al. 1994) has shown this species to be most closely related to *Rikenella microfusus* (formerly *Bacteroides microfusus*). Both species show close affinity to the genus *Cytophaga*.

Bacteroides ochraceus

This species was shown to be distantly related to the genus *Bacteroides* and to contain several centers of variation. These have now been assigned to the genus *Capnocytophaga* as *Capnocytophaga ochracea* (the type species), *Capnocytophaga canimorsus*, *Capnocytophaga cynodegmi*, *Capnocytophaga gingivalis*, *Capnocytophaga granulosa*, *Capnocytophaga haemolytica*, and *Capnocytophaga sputigena*.

Bacteroides succinogenes and *Bacteroides amylophilus*

Both these species are associated with the rumen and are biochemically and chemically unrelated to the genus *Bacteroides*. They have been reclassified in the genera *Fibrobacter* and *Ruminobacter*, respectively.

Bacteroides furcosus, *Bacteroides termitidis*, *Bacteroides multiacidus*, *Bacteroides hypermegas*

These species share few properties with the genus *Bacteroides* and have been reclassified as *Anaerorhabdus furcosus*, *Sebaldella termitidis*, *Mitsuokella multiacidus*, and *Megamonas hypermegas* (see review, Shah and Gharbia 1997; Shah and Collins 1986). 16S rRNA sequence analysis has shown that some of these genera, such as *Sebaldella* and *Anaerorhabdus*, show a close affinity with gram-positive taxa of the *Clostridium* subphylum. The genera *Mitsuokella* and *Megamonas* cluster with *Selenomonas* and *Pectinatus*.

Prevotella zoogleoformans and *Prevotella heparinolyticus*

These species are classified in the genus *Prevotella* based on their phenotypic properties, but 16S rRNA sequence data suggest that they are distinct lineages (see Olsen and Shah 2003).

ANTIBIOTIC RESISTANCE

Gram-negative anaerobes are sensitive to metronidazole, but are resistant to aminoglycosides, quinolones, and monobactams. Until a few years ago, most members of the Bacteroidaceae were susceptible to antimicrobial agents such as clindamycin, chloramphenicol, erythromycin, and tetracyclines. Over the years antibiotic resistance has been increasingly reported. *Bacteroides* spp. have been shown to acquire resistance rapidly to most beta-lactam antibiotics (minimal inhibitory concentration usually exceeds 16 mg/l). This is associated with the production of broad spectrum beta-lactamases (Olsson et al. 1979). In 1955, the first cases of resistance to penicillin were described (Garrod 1955). A noninducible cephalosporin was isolated from strains of *B. fragilis* and to date 93 percent of resistant strains produce β-lactamases. Similar patterns were reported among *B. ovatus*, *B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis*. Lower incidences of β-lactamases (40–60 percent) have been reported for *Prevotella* and *Porphyromonas*. Pencillinases and cephalosporinases were reported from *P. disiens*, *P. buccae*, *P. oris*, *P. melaninogenica*, and *P. asaccharolytica*. Most β-lactamases produced are constitutive cephalosporinases, although penicillinases and enzymes that hydrolyze cefoxitin and imipenem have been described. Both chromosomally mediated and plasmid-mediated penicillin resistance have been described, with the production of at least six different β-lactamase types.

Resistance to chloramphenicol is mediated by either a nitroreductase or an acetyltransferase. Although both mechanisms have been observed among *Bacteroides*

spp., the acetyltransferase activity is transferable among *Bacteroides* spp. However, the rate of transmission in vivo is apparently low since chloramphenicol resistance is detected in less than 1 percent of isolates. This may be due to the infrequent use of this potentially toxic drug (Jenkin 2001).

Clindamycin is one of the most widely used antimicrobial drugs in the treatment of anaerobic infections. Recent studies suggest that resistance to clindamycin in *B. fragilis* is 5–20 percent, less than 5 percent in other *Bacteroides* spp., and 10 percent for *Prevotella* and *Porphyromonas* species. Resistance is mediated by an RNA methylase that alters the 50S subunit of ribosomal RNA and modifies the site of action of the drug. Resistance is due to the transfer of transposable elements, such as Tn*4351* and Tn*4400* (Shoemaker et al. 1985).

Metronidazole resistance in *Bacteroides* spp. is associated with plasmids that have been shown to transfer to other species via conjugation. Despite the low incidence of resistance to metronidazole, the fact that such resistance is transferable suggests the possibility of increased resistance in the future.

Tetracycline resistance in *Bacteroides* isolates was uncommon, but today all clinical isolates of *Bacteroides* spp. are resistant to tetracycline due to the spread of conjugative elements throughout *Bacteroides* spp. Increased use of tetracyclines for the treatment of periodontal diseases and other oral infections is largely responsible for the spread of resistance among *Prevotella* and *Porphyromonas* spp. Such resistance is mediated by chromosomally expressed ribosomal protection mechanisms. In *Bacteroides* and *Prevotella* spp., conjugative elements harboring the genetic determinants *tetQ* and *tetM* were reported (Salyers and Shoemaker 1995).

Resistance to macrolides is usually plasmid or transposon mediated and is related to lincosamide resistance and results in the dimethylation of the 50S ribosomal subunit. The most frequently detected genes responsible for such activity in *B. fragilis*, *B. ovatus*, and *B.vulgatus* are *ermF* and *ermFS* (Garcia-Rodriguez et al. 1995).

MOLECULAR ANALYSIS

Most of the early work on the genetics of the Bacteroidaceae focused on the genus *Bacteroides*. This is due in part to the fact that members of this genus are among the most oxygen tolerant of all obligate anaerobes, they are not nutritionally fastidious and their growth rates are 1–3 h per generation time. They harbor conjugative plasmids that are transferable to *Escherichia coli* and other members of the Bacteroidaceae. The fact that *Bacteroides* spp. are opportunistic pathogens, *B. fragilis* accounting for almost half of all human anaerobic infections, has promoted interest in its genetics.

The physical and genetic map of *B. fragilis* was compiled using the chromosome of strain YCH46 (Kuwahara et al. 2002). It is a single circular DNA molecule of 5.3 Mb. The physical map was constructed by comparative analysis of the data generated from cross-southern hybridization, partial restriction digest, pulse field gel electrophoresis, and linking clone analysis. The map revealed six rRNA operons (*rrn* loci). The location of ten genes including *recA*, *rprX*, and *tuf* were identified. The loci for the enzymes ATP-synthase, fructanase, neuraminidase, and glutamine synthetase, as well as *ccrA* encoding class beta-lactamases were described. It is envisaged that the localization of genes across the physical map can be linked to the data currently being annotated for the *B. fragilis* and *B. thetaiotamicron* genomovars and will play a significant role in detecting reorganizational events directing the emergence of species (Xu et al. 2003).

Many *Bacteroides* strains have cryptic plasmids (3–6 kb) that are mobilized by conjugative elements. However, clindamycin and erythromycin determinants are harbored on plasmids isolated from *B. ovatus*. Tetracycline resistance determinants were isolated downstream from these genes. However, the gene products of these tetracycline resistance elements are functional only under aerobic conditions.

Conjugative transposons are elements that integrate within the bacterial chromosome but, unlike transposons, do not duplicate the target site. Furthermore, a circular intermediate structure is formed during excision and transfer. *Bacteroides* conjugative transposons usually carry the tetracycline determinant *tetQ* (Salyers and Shoemaker 1995). Some conjugative transposons carry erythromycin-resistance genes that also confer resistance to clindamycin. However, cryptic conjugative elements were detected. Interestingly, strains expressing multiple resistance carry conjugative transposons, which may explain the rapid and widespread occurrence of such resistance among members of the genus *Bacteroides*. In many cases, conjugative transposons can also mediate the excision and mobilization of coresident plasmids.

This transfer is stimulated by the exposure of donors to low concentrations of tetracycline prior to conjugation. This was shown to increase the frequency of transfer by 1 000–10 000-fold. Clindamycin was also shown to stimulate the rate of transfer. *Bacteroides* conjugative elements have been shown to transfer resistance to *Prevotella* and *Porphyromonas* spp. in the presence of tetracycline.

Conjugative transposons are extremely stable and, unlike antibiotic resistance plasmids, are maintained in *Bacteroides* strains in the absence of selection through exposure to antibiotics. Several genes from *B. fragilis*, such as *recA* and sialidases, were expressed in *E. coli*. Therefore, plasmids isolated from *B. fragilis* and *B. thetaiotaomicron* that harbor antibiotic markers were integrated into *E. coli*-based shuttle vectors to construct new systems for the cloning and expression of genes of members of the Bacteroidaceae.

P. gingivalis is an intensively studied species of the genus *Pophyromonas* (Shah 1993) and sufficient genes from a range of strains have been sequenced to enable an insight into the structure of its genome when compared to the sequenced chromosome of strain W83. Once the process is completed, it will have a major impact on resolving the molecular basis of pathogenesis within this species. Early attempts to clone and express surface-associated proteins failed to yield mature proteins and resulted in the rearrangement of the cloned fragments. Random cloning and selection of recombinant was replaced with screening with degenerate primers, constructed N-terminal sequences or conserved motifs of similar proteins. The amplified fragments were sequenced to ascertain their identity and used to screen gene libraries and clinical isolates and better results were obtained. Using this approach, the structure and distribution of the fimbriae-encoding gene was determined (Hamada et al. 1994). Several sequences of the major extracellular proteinase have been reported and deposited in the databank. However, the biochemical and physiological studies support the view that all the extracellular proteolytic activity of *P. gingivalis* is associated with cysteine proteinases (Pavloff et al. 1995). Furthermore, the results of cloning and molecular analysis have revealed that the proteinase forms a complex association with adhesin molecules on the bacterial surface and is responsible for its attachment to erythrocytes.

The metalloenzyme superoxide dismutase (SOD) of *P. gingivalis* has the highest known activity of any *Porphyromonas* or *Prevotella* species. It was reported recently that an increase in temperature from 37 to 39°C resulted in an increase in SOD messenger RNA and a three-fold rise in SOD activity. Molecular analysis revealed that the enzyme is transcribed as single apoprotein monomers, but post-transcriptional modification yields two minor Fe-containing SOD isoenzymes (when cell extracts are maintained anaerobically) and a major Mn-containing SOD (when cells are aerated), both of which are encoded by a single copy on the chromosome, which is a unique feature.

The genes for several unrelated enzymes have been deposited in databases, but the significance of these to the biology of *P. gingivalis* has not yet been clarified. These include a methylase-encoding gene, heat shock protein operon, an insertion element, genes encoding an alkaline phosphatase, and methylmalonyl-CoA mutase (see review by Gharbia et al. 1995).

A unique molecular structure among bacteria was revealed through the study of an adhesin gene from *P. loescheii*. The gene, referred to as *plaA*, possesses two overlapping reading frames which encode an 89 kDa polypeptide (Manch-Citron et al. 1992). The leader sequence and 28 residues of the N-terminus are encoded within frame 1 and a second reading frame is initiated on the complementary strand. Sequencing results and confirmation using polymerase chain reaction (PCR) amplification of several gene fragments strongly suggest a frameshift within *plaA*, which represents an uncommon phenomenon among bacteria. Such a mechanism enables a single gene to encode several proteins, fusion protein transcription, and truncated gene products.

BACTEROIDES, *PREVOTELLA*, AND *PORPHYROMONAS* IN THE NORMAL FLORA

In humans

Gram-negative anaerobic bacilli are a major component of the normal commensal flora of the mucous membranes of the mouth, lower gastrointestinal tract, and vagina. Eggerth and Gagnon (1933) recognized their importance in the fecal flora and Rosebury (1962) stressed their normal occurrence at all three sites. The species of gram-negative anaerobic bacilli found at the three sites form distinct populations that account for the majority of endogenous opportunistic infections.

ORAL MICROFLORA

The mouth does not have a uniform bacterial population. The gingival crevice and subgingival plaque are the principal sites of colonization with gram-negative anaerobic bacilli (Socransky and Manganiello 1971; Hardie and Bowden 1974). Gram-negative anaerobic bacilli are rarely detected in infants before the eruption of teeth. As dentition develops during childhood, the frequency of colonization increases (Sutter 1984). Kelstrup (1966) found that the isolation rate of black-pigmented species rose from 18 percent in children aged 5 years to 70 percent at 16 years. *Prevotella* spp. are present in large numbers in the gingival crevice and subgingival plaque of all healthy adults. Studies have shown that they represent 12 percent (Slots 1977) to approximately 30 percent (Williams et al. 1976; Newman et al. 1978) of the cultivable flora. However, they constitute only approximately 4 percent of the cultivable flora of supragingival plaque in normal subjects (Gibbons et al. 1964), although in institutionalized subjects the proportion may reach 17 percent (Loesche et al. 1972). In the presence of gingivitis and peridontitis their numbers, particularly those of *P. gingivalis* and *P. intermedia*, increase.

Pigmented species were first described in studies of the microflora of mucous membranes, in particular, of the gingival mucosa (Oliver and Wherry 1921). They are useful marker species for the anaerobic flora of the gingival crevice (Holbrook et al. 1978). Pigmented strains constitute 4–6 percent of the cultivable flora of the normal gingival crevice (Gibbons 1974; Williams et al. 1976) and 5–6 percent of the plaque flora from institutionalized subjects (Loesche et al. 1972).

Pigmented *Prevotella* spp. can be isolated from almost all subjects and account for 10–20 percent of the gram-negative anaerobic bacilli, whereas the nonpigmented species account for about 35 percent (Loesche et al. 1972; Gibbons 1974; Duerden 1980c). Species of *B. fragilis* are not part of the normal gingival flora (Hardie 1974).

Gingival strains of Bacteroidaceae are often nutritionally demanding and may be difficult to maintain in pure culture on solid media. Many species are nutritionally interdependent and will grow only in mixed culture, in which satellitism may be demonstrated. Growth is often improved by the addition of 5–10 percent CO_2 in the anaerobic atmosphere (Watt 1973; Stalons et al. 1974) and recognized growth factors include metal ions (Caldwell and Arcand 1974) and hemin (Gibbons and MacDonald 1968; Gilmour and Poole 1970). Most *Prevotella* and *Porphyromonas* spp. also require menadione (vitamin K, Lev (1959)) or its precursors (Robins et al. 1973) and succinate (Lev et al. 1971).

GASTROINTESTINAL MICROFLORA

Gram-negative anaerobic bacilli are generally the most numerous bacteria in the normal flora of the gastrointestinal tract, similar in numbers to the bifidobacteria. The genus *Bacteroides* forms the majority of the cultivable species (Drasar and Hill 1974). The anaerobic intestinal flora develops early in life. In breast-fed infants, bifidobacteria predominate and gram-negative anaerobic bacilli are present in the first week of life only in about 20 percent of such infants. However, more than 60 percent of bottle-fed infants are colonized by *Bacteroides* spp. in the same period. At weaning, the numbers of bifidobacteria decrease, whereas the numbers of *Bacteroides* rise to outnumber bifidobacteria by 3:1 (Long and Swenson 1977). Rotimi and Duerden (1981) reported a heavy growth of *Bacteroides* from 75 percent of 16 infants 5–6 days old who were breast fed, but received supplementary bottle feeds. Obligate anaerobes are seldom found in the normal stomach, duodenum, jejunum, or proximal ileum. *Bacteroides* spp. and *Bifidobacterium* spp. begin to appear only in the distal ileum (Finegold 1977) and they form a major part of the cecal and fecal flora. Viable counts of *Bacteroides* yield 10^{11} organisms per gram wet weight of feces and cecal contents (Drasar 1967; Finegold et al. 1975). The proportion of *Bacteroides* spp. in the fecal flora differs in subjects from different countries and is related to the type of diet. They are found in greater numbers in subjects who consume a mixed western diet that contains comparatively large amounts of fat and stimulates the production of a large volume of bile. Fewer *Bacteroides* spp. are found in subjects from developing countries in Africa and Asia, and from Japan, who consume a mainly vegetarian diet. Gram-positive anaerobes and enterococci are the predominant fecal organisms in these subjects. However, subjects in developed countries who change to a vegetarian diet do not convert their fecal flora to a gram-positive predominance and retain their *Bacteroides* organisms (Drasar 1974).

Some 80 percent of fecal gram-negative anaerobic bacilli are members of the genus *Bacteroides*. The most common species in most subjects are *B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis*. *B. eggerthii* is found regularly, but *B. fragilis* forms only a small proportion of the bacteroides in the normal fecal flora (Werner 1974; Finegold et al. 1975; Moore and Holdeman 1975; Duerden 1980a). Holdeman et al. (1976) identified 1 442 isolates belonging to 101 species from 25 specimens of feces from three men; *B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis* were three of the five most prevalent bacterial species, representing 11.8, 8.9, and 6.0 percent of isolates, respectively, whereas *B. fragilis* represented only 0.3 percent. They also found that the proportion of *B. thetaiotaomicron* isolates rose under stressful conditions. Asaccharolytic species of the Bacteroidaceae form a consistent part of the normal fecal flora, but are present in much smaller numbers than *Bacteroides* (Werner et al. 1971). The most commonly recognized species is *P. asaccharolytica* and the lower gastrointestinal tract is probably the primary habitat of this species. Nonpigmented asaccharolytic species are also present in quite small numbers. They grow slowly to form only small colonies and are difficult to detect.

The gram-negative anaerobic bacilli play an important part in the physiological activities of the large intestine. They are responsible for much of the metabolism of bile salts and bile acids. *Bacteroides* spp. and several clostridia produce enzymes that deconjugate bile salts under conditions of low E_h, and they reduce cholic acid to deoxycholic acid. This is important in the enterohepatic circulation of bile salts. They may also be implicated in the production of carcinogens from bile acids. *Bacteroides* spp. produce dehydrogenases and dehydroxylases that convert bile acids into aromatic compounds and eventually to substituted cyclopentaphenanthrenes, which are known to be carcinogens. Differences in the incidence of colonic carcinoma in various countries are associated with the type of diet consumed and with the relative numbers of gram-negative anaerobic bacilli in the feces (Drasar and Hill 1974).

Several studies have been conducted to provide a comparative analysis of the anaerobic microbiota of the gastrointestinal tract in infants and adults. This is mainly carried out by nonculturable 16S rRNA amplification and random sequencing or hybridization (see Rigottier et al. 2002).

VAGINAL MICROFLORA

The role of the Bacteroidaceae in the normal vaginal flora has been the subject of controversy. The vagina is

not a single environment. The flora of the lower vagina is a mixture of organisms from the vagina with others from the perineum and introitus, whereas the flora of the cervix and fornices more closely represents the true vaginal flora. Moreover, the environment is not constant. The state of the mucosa and secretions changes with age, with each menstrual cycle, and with pregnancy (Hurley et al. 1974).

Gram-negative anaerobic bacilli are common, but not universal, commensals of the cervix and vaginal fornices. The presence of Bacteroidaceae in the vagina was reported first by Burdon (1928). He isolated *P. melaninogenica* from 28 of 35 normal women.

Bacteroides spp. were isolated from cervical cultures in 57 percent (Gorbach et al. 1973) and 65 percent (Sanders et al. 1975) of normal women and from the posterior vaginal fornix in 65 percent and 100 percent of two groups of 20 normal women (Duerden 1980b; Wilks et al. 1984). However, other workers have found them considerably less often: 8.6 percent of 246 preoperative gynecological patients (Neary et al. 1973); 4.6 percent of 500 women attending a family planning clinic; 5 percent of 200 patients attending a gynecological outpatient clinic; 5.4 percent of 280 pregnant women (Hurley et al. 1974); four of 100 pregnant women; and one (4 percent) of 23 normal women in a quantitative study by Taylor et al. (1982). Some of these differences may reflect differences between populations, but most are probably attributable to different methods of investigation and, in particular, to differences in sampling methods and in anaerobic technique. In studies with good anaerobic methods, isolation rates from normal women have been generally higher in qualitative studies (approximately 60 percent, see above) than in quantitative studies – 41 percent (Masfari et al. 1986), 34–40 percent (Bartlett and Polk 1984), 34 percent (Hammann 1982) – in which small numbers of anaerobes may not have been detected. In quantitative studies, mean viable counts of *Bacteroides* spp. in those normal women from whom gram-negative anaerobic bacilli were isolated varied from 10^6 colony-forming units (cfu)/g (Masfari et al. 1986) to $10^{8–9}$ cfu/g or ml (Bartlett and Polk 1984; Wilks et al. 1984) of vaginal secretions. Few consistent variations have been detected in sequential samples taken during the menstrual cycle. Bartlett and Polk (1984) and Wilks et al. (1984) both found that levels of aerobes were higher during the first week of the cycle, but there was little change in the levels of anaerobes. However, *Bacteroides* spp. are reported to disappear during pregnancy and return soon after delivery (Hite et al. 1947; Willis 1977).

The species of Bacteroidaceae isolated in early studies of the vaginal flora were not identified. When detailed identification methods have been used, the predominant species have been members of *Prevotella* spp. *P. bivia* is the most common species isolated in most studies. *P. disiens*, *P. intermedia*, and *P. melaninogenica* are also common. *P. asaccharolytica* can be isolated in smaller numbers. The presence of significant numbers of *Bacteroides* organisms, and in particular of *B. fragilis*, indicates some pathological change in the genital tract.

In animals

Gram-negative anaerobic bacilli are part of the normal flora of intestinal contents and feces in many species of animals from invertebrates to humans. They are an important component of the flora of two specialized gastrointestinal structures, the rumen of ruminant animals and the cecum of poultry.

Species that are now no longer considered as *Bacteroides* spp. are found in large numbers in the rumen: *Fibrobacter succinogenes*, *Ruminobacter amylophilus*, and *P. ruminicola*. *F. succinogenes* is found in most ruminants and attains a viable count of approximately 0.5×10^8 cfu/ml, about 8.5 percent of the total viable count. It is actively cellulolytic and is important in the digestion of cellulose. It also ferments glucose, cellobiose, and several other carbohydrates, producing succinic acid (Hungate 1950). *R. amylophilus* and *P. ruminicola* are not cellulolytic. They are active in the digestion of starch and other carbohydrates. *R. amylophilus* occurs sporadically, but when present may be the predominant starch digester and constitute 10 percent of the total bacteria (Hamlin and Hungate 1956). The fermentative activity of *P. ruminicola* is more varied. It is regularly present and constitutes 6–19 percent of the total viable count.

The rumen bacteria are essential for the nutrition of ruminants. The energy content of the feedstuff is made available to the animal in the form of volatile fatty acids, particularly acetic, butyric, and propionic acids, produced by the bacterial fermentation of cellulose and other carbohydrates. The acids are absorbed through the rumen wall and metabolized by the animal. The removal of these acid products is essential for the continuation of fermentation. In addition, microbial protein constitutes a major part of the ruminants' protein source. Nitrogenous components of the feed are converted to microbial protein during growth and multiplication of the rumen bacteria, which contain approximately 65 percent of protein. In the distal parts of the intestine, dead bacterial cells are digested and provide protein for the animal (Hungate 1966).

In poultry, the cecum is highly developed and has an important role in digestion and absorption. Gram-negative anaerobic bacilli are found in large numbers (approximately $10^{8–9}$/g) in the cecal contents. The most common species is unique to this site – *Megamonas hypermegas* – which ferments a variety of carbohydrates (Barnes and Goldberg 1968a, b).

ANTIMICROBIAL SUSCEPTIBILITY

Susceptibility towards different agents

A number of multicenter studies have been conducted in the United States, Europe (including France, Germany, Great Britain, Sweden, Italy, and Spain), Japan, Australia, Israel, and South Africa in order to survey the in vitro antimicrobial susceptibility of anaerobic bacteria (Falagas et al. 2000). An alarming and gradual increase in resistance rates to several antibiotics have been reported in *Bacteroides* and other anaerobes. These can be classified in four categories according to their effect on the genus *Bacteroides* sensu stricto. The first group is active against almost all (>99 percent) clinical *Bacteroides* isolates and includes metronidazole, chloramphenicol, imipenem, and meropenem. Isolates of *B. fragilis* resistant to this group of antibiotics have been reported sporadically. Furthermore, *B. fragilis* isolates resistant to multiple agents such as metronidazole, imipenem, and amoxicillin/clavulanic acid have been detected (Turner et al. 1995). The second group of agents, which is active against most (95–99 percent) *B. fragilis* isolates comprises the β-lactam/β-lactamase inhibitor combination drugs, i.e. ticarcillin/clavulanic acid, piperacillin/tazobactam, and ampicillin/sulbactam. Many *Bacteroides* strains produce β-lactamases that hydrolyze most β-lactams and addition of β-lactamase inhibitor increases the in vitro activity of β-lactams. *Bacteroides* species other than *B. fragilis* are more apt to be resistant to β-lactam/β-lactamase inhibitor combinations than *B. fragilis* (Snydman et al. 1996). The third group of agents that is frequently active against bacteroides includes clindamycin and cephalosporins such as cefoxitin, cefotetan, ceftizoxime, and cefmetazole. A lower resistance rate to these agents has been observed in *B. fragilis* compared to other bacteroides and there is also considerable interspecies variability in the resistance rate of these drugs between different bacteroides other than *B. fragilis*, as reflected in the susceptibility patterns of *B. fragilis* species isolated from bacteremia (Aldridge et al. 2003). Also among diseased animals (cattle, sheep, goats, horses, pigs, dogs, cats, rodents, zoo animals), resistant strains among *Bacteroides*, *Prevotella*, *Porphyromonas*, and *Fusobacterium* belong mainly to the *Bacteroides* sensu stricto (Even et al. 1998). Among the *Bacteroides*, the highest resistance rates are observed for the following organisms/drug combinations: for clindamycin/*B. distasonis* and *B. ovatus*; for cefoxitin/*B. thetaiotaomicron*, *B. distasonis* and *B. uniformis*; for ampicillin-sulbactam/*B. distasonis*, *B. ovatus*, and *B. uniformis*; and for trovafloxacin/*B. vulgatus* (Aldridge et al. 2001). With few exceptions all agents were highly active against isolates of *Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Peptostreptococcus*. The efficacy of cefoxitin in *Bacteroides* is low with only 43–46 percent of the strains isolated being susceptible (Oteo et al. 2000; Oteo-Iglesias et al. 2002). Fourthly, monobactams, aminoglycosides, and the early quinolones have poor activity against all or most groups of anaerobic bacteria (Goldstein et al. 1993). More recently developed fluoroquinolones, such as trovafloxacin, have improved anti-anaerobic activity. There has been a significant increase in resistance to new fluoroquinolones from 0 percent in 1998 to 12 percent in 2001 (Oteo-Iglesias et al. 2002).

Penicillin, including amoxicillin, is only rarely active against the bacteroides, but is frequently active against *Prevotella* and *Porphyromonas* species (Falagas and Siakavellas 2000). A gradual increase in the rate of resistance to penicillin has been reported over the last 20 years (Niederau et al. 1980; Sedallian and Debrueres 1987; Olsvik and Tenover 1993). Aldridge et al. (2003) found that all blood isolates of bacteroides tested from 1987 to 1999 by the same NCCLS-recommended broth microdilution method were resistant to penicillin G, and 22 percent were resistant to clindamycin. A high prevalence of resistance to clindamycin, reaching 49–56 percent of the isolates in bacteroides has been reported (Oteo et al. 2000; Oteo-Iglesias et al. 2002), while a high incidence of clindamycin (50 to 70 percent) and cefoxitin (31 to 65 percent) resistance was found in *Bacteroides* spp. other than *B. fragilis* (Teng et al. 2002). Blood isolates may be less susceptible to antibiotics than isolates from intraabdominal, obstetric-gynecologic, and other sources of clinically important anaerobes (Aldridge et al. 2001). Beta-lactamase-positive strains may be susceptible to agents such as imipenem and chloramphenicol (Uraz and Turkmen 1999).

There is a synergistic effect between gentamycin and penicillin, clindamycin, or metronidazol against some *Bacteroides* isolates (Brook et al. 1984). Clindamycin, rifampin, nalidixic acid, ticarcillin, erythromycin, and spectinomycin show synergy with metronidazole against less than 50 percent of *B. fragilis* strains (Ralph and Amatnieks 1980). Furthermore, in vitro synergy between trovafloxacin and cefoperazone or ceftazidime has been observed against a small minority of *Bacteroides* spp. (Falagas and Siakavellas 2000).

Mechanisms of resistance

In general, β-lactamase production is the most important cause of β-lactam resistance in anaerobic gram-negative bacteria. All *Bacteroides*, except some strains of *B. distasonis*, produce β-lactamase (Nord 1986; Appelbaum 1992; Rasmussen et al. 1997). Beta-lactamase is also produced by about half of *Prevotella* spp. and about 20 percent of *Porphyromonas* spp. (Falagas and Siakavellas 2000). Bernal et al. (1998) reported that 20 percent of strains identified as *P. intermedia* were β-lactamase-producing, as were 72 percent of *P. nigrescens*.

Carbapenemases have been reported from Japan (Edwards and Greenwood 1996; Bush 1998). Other mechanisms of antibiotic resistance in anaerobes include alteration of penicillin-binding proteins and the blockage of antibiotic penetration to the active site by modification of outer membrane pores (Wexler and Halebian 1990). Tetracycline resistance in anaerobic bacteria is related to blocked antibiotic uptake (Tally et al. 1984). In clindamycin resistance, nonenzymatic alteration of the target site is the usual mechanism (Dalmau et al. 1997), while the rare resistance generally observed for metronidazole is due to decreased uptake of antibiotic and decreased nitroreduction of the compound (Appelbaum 1992). Enhanced pathogenicity has been demonstrated in susceptible strains of *Bacteroides* subjected to low doses of metronidazole (Diniz et al. 2003). Mutational events in the gyrA quinolone resistance-determining region seem to contribute to quinolone resistance in *Bacteroides* spp. (Oh et al. 2001).

PATHOGENICITY

Members of the genera of *Bacteroides*, *Prevotella*, and *Porphyromonas* are part of the normal commensal flora of man and animals. A large number of studies have indicated that the most important pathogens of this group are *B. fragilis* (intestinal) and *P. gingivalis* (oral), consequently most investigations have been focused on these two species.

Environmental factors

Bacterial virulence factors can be modified by a number of environmental parameters such as nutrients, oxygen tension, etc. In the oral cavity, the virulence of *P. intermedia* and *P. nigrescens* was shown to decrease in the presence of glucose, whereas no such effect was observed on *P. gingivalis* (Saito et al. 2001). *P. gingivalis* has an obligate requirement for heme or selected heme- or Fe-containing compounds, however, the regulation of the heme biosynthetic genes remains puzzling (Roper et al. 2000). The influence of heme on the expression of several putative virulence factors in this bacterium has been demonstrated. A 35-kDa protein is one of the hemin-binding proteins in *P. gingivalis* and its binding ability is important for expression of virulence (Shibata et al. 2003). Isolates of *P. gingivalis*, having a low requirement for iron, are likely to have a higher potential for initiating periodontal infections (Grenier et al. 2001).

Microorganisms adapt to environmental cues by turning on and off the expression of virulence genes which allows for optimal growth and survival within different environments (Forng et al. 2000). When *P. gingivalis* in human dental plaque from periodontitis patients were stressed in vitro by a temperature increase of 5°C, a rapid rise occurred in the mRNA associated with the molecular chaperons (*htpG*, *dnak*, *groEL*), SOD (*soda*), and gingipain (*rgp-1*) genes (Shelburne et al. 2002). Furthermore, the levels of stress gene transcription for dnak and htpG were significantly elevated at diseased sites, and grEL gene transcription increase approached statistical significance. There were also correlations between probing depth and increased transcription of *groEL*, *htpG* and *rgp-1* and between disease status and elevated expression of *dnaK* and *htpG*.

Wu et al. (2002) suggested that knockout of *ivi* genes reduced the ability of mutated *P. gingivalis* to survive and cause infection compared to the wild-type strain at the site of infection. Mutants also had lower capacity for invasion.

In a study by Liu and Fletcher (2001a) the *recA* promoter was found to be transcriptionally active during active *P. gingivalis* infection in a murine host. The expression of the *recA* gene is responsive to several environmental signals, but is not regulated by a DNA damage-inducible SOS-like regulatory system (Liu and Fletcher 2001b). The *recA* downstream gene *vimA* (virulence-modulating gene) plays an important role in virulence modulation of *P. gingivalis* W83, possibly representing a novel post-transcriptional or translational regulation of virulence factors in this organism (Abaibou et al. 2001).

Methyl mercaptan is highly toxic and its presence in the periodontal pocket suggests that it could be involved in progression of periodontal disease. *P. gingivalis* is considered as the most potent producer of this substance. Only 7.7 percent of mice infected with strain W83 of *P. gingivalis* survived 4 days after subcutaneous injection, whereas 36 percent of the mice survived injection with a mutant strain deficient in methyl mercaptan production (Nakano et al. 2002). A marked decrease in the formation of methyl mercaptan from L-methionine and decreased virulence was seen in the mutant compared to the wild-type strain (Yoshimura et al. 2000).

Lipopolysaccharide

LPS has been identified as a major factor in bone resorption. Based on the role of IL-1 beta in *P. gingivalis* LPS-induced bone resorption, it was suggested that it may be caused by enhancing osteclastogenesis, not by increasing the osteoclast resorbing activity (Chiang et al. 2002). *P. gingivalis* LPS-induced bone resorption and IL-6 production in the gingival tissue was inhibited with antibody towards CD14 which is a significant receptor for LPS on human gingival fibroblasts and gingival epithelial cells (Wang et al. 2002). LPS from *P. intermedia* participated in periodontal tissue destruction and alveolar bone resorption, but in addition inhibited bone

formation (Pelt et al. 2002). LPS derived from periodontal pathogens also cause imbalance in the regulation of plasminogen activator and plasminogen activator inhibitor in human gingival fibroblasts (Xiao et al. 2001). This may contribute to the destruction of periodontal connective tissue through dysregulated pericellular proteolysis. Urokinase-type plasminogen activator receptor (uPAR) presenting at the cell surface with glycosylphosphatidylinositol anchor is a key component in the plasminogen activator-plasmin system and plays a critical role in extracellular matrix degradation. An increase of the uPAR level by LPS may be important in the progression of periodontal diseases through pericellular proteolysis (Ogura et al. 1999).

P. gingivalis LPS is both agonist and antagonist for p38 mitogen-activated protein kinase activation. LPS from this organism may therefore selectively modify the host response as a means to facilitate colonization (Darveau et al. 2002).

B. fragilis LPS effectively stimulated macrophage inflammatory protein-2 (MIP-2) expression even in the absence of functional TLR4 in the 10ScNCr/23 cell line (Lorenz et al. 2002).

Exotoxins

The enterotoxin of *B. fragilis* is a protease specific for E-cadherin and it promotes the reorganization of the actin cytoskeleton (Richard et al. 1999). Similarly, protease-active protein from *P. gingivalis* induces N-cadherin proteolysis, loss of cell adhesion, and apoptosis in human epithelial cells (Chen et al. 2001). Bacterial toxins that modify the actin cytoskeleton induce various cell dysfunctions including changes in the cell barrier permeability and disruption of intercellular junctions.

Enterotoxigenic *B. fragilis* (ETBF) are present in children and adults. They are distinguished by secretion of a 20 kDa zinc metalloprotease toxin (*B. fragilis* toxin (BFT)) (Sears 2001). Three isotypes of BFT are encoded by distinct *bft* loci which are located in a 6-kb chromosomal region unique to ETBF strains termed *B. fragilis* pathogenicity island (BfPAI). The pathogenicity island and its flanking regions (approximately 700 bp upstream of the *bft* gene) are important to optimal BFT activity (Franco et al. 2002). Interestingly, the BfPAI and its flanking region may be two distinct genetic elements (Franco et al. 1999). Attempts to construct a three-dimensional model of BFT 2 were recently undertaken in our laboratory (Schmid et al. 2004, unpublished) using protein sequence data, crystalline metalloproteinase structures, and Silicon Graphics software (Figure 4).

Adhesion

Bacterial fimbriae play an important role in the interaction between bacteria and host cells or among bacterial cells. *P. gingivalis* fimbriae have been studied most extensively. Its fimbrial structure is composed of 41-kDA fimbrillin proteins. The structure of the fimbrillin gene (*fimA*) shows intraspecies variation, but the common immunological specificities are retained (Hamada et al. 1998). *P. gingivalis* fimbriae have a variety of biological activities, including immunogenicity, binding to various host proteins, stimulation of cytokine production, and promotion of bone resorption. The fimbriae of *P. intermedia* induce hemagglutination reaction, while those of *P. loescheii* cause coaggregation with other bacteria. Also N-acetylneuraminic acid and glucuronic acid are involved in the attachment of *P. gingivalis* to epithelial cells (Agnani et al. 2003).

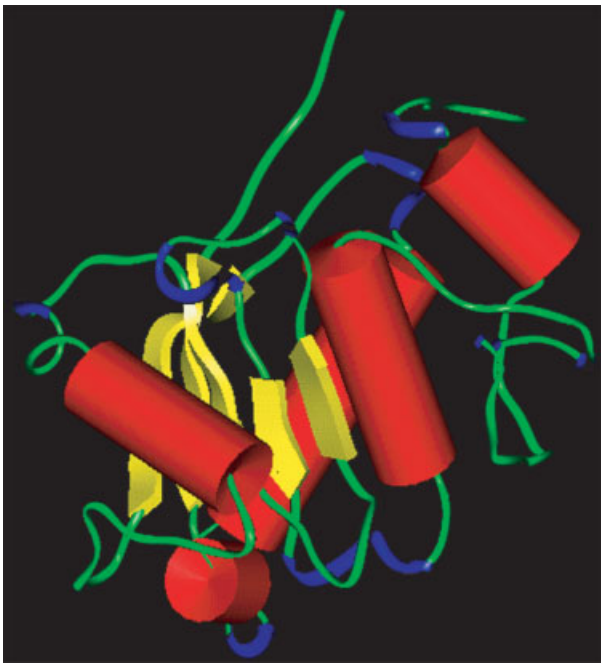

Figure 4 *The deduced three-dimensional crystal structure of the metalloproteinase encoded by* bft. *The α-helices are shown in red, while the β-sheets are in yellow with the arrow tip displaying the direction. The blue strands indicate the turns, while the green strands depict the amino acid residues which, according to computer predictions, do not fold into secondary structures.*

Invasion

Invasion of epithelial cells may be an important virulence factor in bacteria. Dorn et al. (1998) reported that *P. intermedia* can invade an oral epithelial cell line and that the type C fimbriae and a cytoskeletal rearrangement are required for invasion. Entry rates of *P. gingivalis* into gingival epithelial cells are high and rapid and internalized bacteria congregate in the perinuclear region of the cells (Belton et al. 1999). *P. gingivalis* remained inside the epithelial cells for over a 24-h period and induced rearrangement of the actin cytoskeleton along with alteration of the size and shape of the

epithelial cells. In coronary artery endothelial cells, *P. gingivalis* evades the endocytic pathway to lysosomes and instead traffics to the autophagosome (Dorn et al. 2002a, b). Microorganisms associated with root canal infections can invade primary cultures of cardiovascular cells (Dorn et al. 2002c). Further, periodontal pathogens such as *P. gingivalis*, *P. intermedia*, *Actinobacillus actinomycetemcomitans*, and *B. forsythus* are present in atherosclerotic plaque where they may play a role in development and progression of atherosclerosis possibly ending with coronary valvular disease and other clinical sequelae (Mastragelopulos et al. 2002). Long-term systemic challenge with *P. gingivalis* can accelerate atherogenic plaque progression as shown in a heterozygous apolipoprotein E-deficient murine model (Li et al. 2002). *P. gingivalis* also seems to cause lymphocyte apoptosis in peripheral blood mononuclear cells and this may be an important feature of this pathogen in periodontal disease (Geatch et al. 1999).

Abscess formation

B. fragilis is the anaerobe most frequently isolated from intraabdominal abscesses. The abscessogenic nature of *B. fragilis* is related to capsular polysaccharide, a polysaccharide biosynthesis locus being required for virulence (Coyne et al. 2001). Two capsular polysaccharides from *B. fragilis*, PS A1 and PS A1 from strain 9343, have been characterized structurally. Their most striking feature is a zwitterionic charge motif consisting of both positively and negatively charged substituent groups on each repeating unit (Kalka-Moll et al. 2001). The zwitterionic motif is essential in abscess formation.

Neutrophil and monocyte responses

In periodontitis, polymorphonuclear neutrophils (PMNL), primed or stimulated by the presence or persistence of infection, express an elevated and excessive response (Kantarci and Van Dyke 2002). This in turn leads to tissue destruction mediated by neutrophil activity.

The reduced susceptibility to phagocytosis found among some periodontopathogenic anaerobes may account for the differences between invasive and noninvasive stains. Thus a great diversity in phagocytosis susceptibility was determined by estimating the portion of phagocytosing PMNL ranging from 10.6 percent (strain W83) to >99.4 percent (ATCC 33277^T) in *P. gingivalis* and from 15.9 percent (strain MH5) to >95 percent (ATCC 33563^T) in *P. nigrescens* (Conrads et al. 1999). Eick et al. (2000) found that the PMNL of patients with rapidly progressive periodontitis show deficiencies in phagocytic activity and in intracellular killing of periodontopathogenic bacteria.

Proteases/protease inhibitors

P. gingivalis in a nonfermentative species requiring peptides for growth which in vivo are probably derived from the hydrolysis of native proteins by an extracellular proteinase (Shah and Williams 1987a). The enzyme appears to be a major virulence determinant and was fully characterized as a cysteine proteinase and designated the name, gingivain (Shah et al. 1991). This was subsequently confirmed by several workers who then referred to the same enzyme as gingipain.

This enzyme subverts host defense systems with the complement and neutrophils being the main targets (Potempa et al. 2000). They also contribute to local generation of bradykinin and thrombin through uncontrolled activation of the kallikrein/kinin pathway and the coagulation cascade. Bradykinin and thrombin are synergistically working proinflammatory reagents with a strong stimulatory effect on bone resorption. Lourbakos et al. (2001) found that arginine-specific gingipains-R (RgpB and HRgpA) induced an increase in intracellular calcium in human platelets at nanomolar concentrations and caused platelet aggregation with efficiency comparable to thrombin. HRgp may be more important in the virulence of *P. gingivalis* relative to rgpB because of the presence of the hemagglutinin/adhesion domain which can bind phospholipids and modulate enzyme activity (Imamura et al. 2000). Furthermore, gingipains have a strong effect on mechanisms controling host matrix metalloproteinase at the level of gene expression and zymogen activation (Potempa et al. 2000). The action of gingipains and other proteinases produced locally by subgingival bacteria would dysregulate most mechanisms controlling inflammatory reaction.

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Fusobacterium and *Leptotrichia*

TOR HOFSTAD AND INGAR OLSEN

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The genera *Fusobacterium* and *Leptotrichia* include several species of nonsporing and nonmotile gram-negative, pleomorphic rods. They have in common a low guanine plus cytosine (G+C) ratio and there are distinct similarities in whole-cell fatty-acid patterns. *Fusobacterium* spp. are obligately anaerobic, whereas most *Leptotrichia* strains are aerotolerant. In contrast to *Fusobacterium*, all species of which are virtually asaccharolytic, *Leptotrichia* ferments glucose and a range of other carbohydrates. The habitat of both is the mucous membranes of man and animals.

Fusobacterium and *Leptotrichia* are phylogenetically related. Both genera are evolutionarily closer to the gram-positive than the gram-negative bacteria. In the phylogenetic classification of Woese (Woese 1987) *Fusobacterium* and *Leptotrichia* are assigned to a separate division within the Bacteria (Neefs et al. 1993; Olsen et al. 1994).

FUSOBACTERIUM

DEFINITION OF THE GENUS

Fusobacterium spp. are gram-negative rods that are nonsporing, nonmotile, and obligately anaerobic. The cells are often pleomorphic, may be filamentous, and are spindle-shaped with pointed ends ('fusiform') in a few species. Amino acids and peptides are used for energy metabolism. *n*-Butyric acid is a major metabolic product. Threonine is deaminated to propionic acid. The G + C content of DNA is 26–34 mol% and the type species of the genus is *Fusobacterium nucleatum*.

HISTORICAL PERSPECTIVE

The generic name *Fusobacterium* was introduced by Knorr (1923) for obligately gram-negative bacilli that were fusiform. He described three species: *Fusobacterium plauti-vincenti*, *Fusobacterium nucleatum*, and *Fusobacterium polymorphum*. The species name *F. plauti-vincenti* was given to the spindle-shaped organism observed in materials recovered from ulcerative stomatitis by Plaut (1894) and Vincent (1896) and cultivated and named *Bacillus fusiformis* by Veillon and Zuber (1898). This organism is now assigned to the genus *Leptotrichia* under the name *Leptotrichia buccalis*.

Loeffler, in 1884, observed pleomorphic rods in diphtheroid lesions of calves and doves. The organism,

named *Bacillus necrophorus* by Flügge (1886), was cultured by Bang (1890–91) from necrotic lesions of a number of domestic animals and by Schmorl (1891) from an epizootic in rabbits. Prévot (1938) proposed the term *Sphaerophorus* for the pleomorphic anaerobic gram-negative rods that were not fusiform. The seventh edition of the *Bergey's manual* divided the family Bacteroidaceae into three genera: *Bacteroides*, defined as rods with rounded ends; *Fusobacterium*, defined as rods with tapering ends; and *Sphaerophorus*, defined as rods with rounded ends that showed a marked pleomorphism and in which filaments were common. Studies of DNA base ratios (Sebald 1962) and physiological studies performed in the 1960s and the early 1970s indicated that there was no valid basis for separating the genera *Fusobacterium* and *Sphaerophorus*. In the eighth edition of the *Bergey's manual* the valid species included in *Sphaerophorus* were transformed to the genus *Fusobacterium*.

Bergey's manual of systematic bacteriology lists ten *Fusobacterium* species: *F. nucleatum*, *F. gonidiaformans*, *F. varium*, *F. necrophorum*, *F. perfoetens*, *F. naviforme*, *F. russii*, *F. mortiferum*, *F. necrogenes*, and *F. prausnitzii*. Four new species were described subsequently: *F. simiae* (Slots and Potts 1982), *F. periodonticum* (Slots et al. 1983). *F. ulcerans* (Adriaans and Shah 1988), and *F. equinum* (Dorsch et al. 2001). The taxonomic position of *F. perfoetens* is in doubt. The proposed neotype strain (ATCC 29250) (van Assche and Wilssens 1977) is an ovoid coccobacillus distantly related to the other *Fusobacterium* spp. (Conrads et al. 2002). *F. prausnitzii*, which has a DNA G + C composition of 52–57 mol%, is phylogenetically very distant from the genus *Fusobacterium* (Wang et al. 1996; Conrads et al. 2002). *F. alocis* and *F. sulci*, described as new species from the human gingival sulcus (Cato et al. 1985), have been reclassified as *Filifactor alocis* and *Eubacterium sulci*, respectively (Jalava and Eerola 1999).

CLASSIFICATION

The genus *Fusobacterium* is phenotypically relatively homogeneous. Comparison of small-subunit rRNA sequences has revealed levels of sequence similarity that are consistent with a single genus, but intrageneric heterogeneity is evident (Lawson et al. 1991; Tanner et al. 1994). *F. nucleatum*, the species isolated most frequently from man, is divided into five subspecies: *F. nucleatum* subsp. *nucleatum*, subsp. *polymorphum*, subsp. *vincentii*, subsp. *fusiforme*, and subsp. *animalis*, described on the basis of electrophoretic patterns of whole-cell proteins and DNA homology (Dzink et al. 1990) and electrophoretic mobility of two enzymes and DNA homology (Gharbia and Shah 1992). The five subspecies of *F. nucleatum* differed from each other and from the other closely related species by comparison of their 16S–23S internal transcribed spacer regions (Conrads et al. 2002), which supported the validity of previous descriptions. Another potential new subspecies has been isolated from dog- and cat-bite wounds. It is intrinsically quinolone-resistant (Goldstein et al. 2002) and phylogenetically distinct from the five other subspecies. The name '*Fusobacterium canifelium*' has been proposed (Citron 2002). *F. periodonticum* falls phylogenetically within *F. nucleatum* (Jousimies-Somer and Summanen 2002). Animal isolates of *F. necrophorum* fall within two subspecies: *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* (Shinjo et al. 1991), corresponding to biovars A and B. While *F. necrophorum* subsp. *necrophorum* contains the isoleucine and alanine tRNA gene, *F. necrophorum* subsp. *funduliforme* contains the isoleucine tRNA gene (Jin et al. 2002). Southern blot hybridization analysis detected different patterns of leukotoxin A (lktA) hybridizing bands between isolates of the two subspecies of *F. necrophorum* (Narayanan et al. 2001a).

F. mortiferum, *F. varium*, and *F. ulcerans* share several phenotypic properties similar to *F. varium*, but *F. ulcerans* reduces nitrate to nitrite (Citron 2002). It also contains unique fragments of 1000 and 550 bp not present in *F. varium* and *F. mortiferum* (Claros et al. 1999). *F. necrogenes* is distantly related to the other *Fusobacterium* spp. as seen from 16S–23S rDNA spacer region sequences (Citron 2002). *F. gonidiaformans* is genealogically related to *F. necrophorum* (Nicholson et al. 1994).

HABITAT

The fusobacteria are normal inhabitants of the mucous membranes of man and animals. The habitat of *F. nucleatum* and *F. periodonticum* is the human oral cavity; the gingival crevice is the niche of both. *F. nucleatum* is a constant member of the oral microflora of adults and children and is also found in edentulous infants. The relatively frequent isolation of *F. necrophorum* from soft-tissue infections of the oral cavity and the upper respiratory tract, compared with anaerobic infections elsewhere in the body, indicates these regions to be the principal human habitat of this organism. The vagina is likely the primary endogenous site for *F. naviforme* and *F. gonidiaformans* (Hill 1993). The habitat of *F. mortiferum* and *F. varium* is the gastroenteral tract, where they are present in small numbers. *F. necrogenes*, which was originally isolated from a chicken abscess and from cecal contents of ducks, is very rarely isolated from man. The habitat of *F. ulcerans*, isolated from tropical ulcers, is unknown.

F. necrophorum is a normal inhabitant of the alimentary tract of cattle, horses, sheep, and pigs and is frequently isolated from cats and dogs. Reports, mainly from the first part of the twentieth century, indicate its presence in a range of wild animals, including reptiles.

F. russii is normally found in the canine and feline oral flora (Love et al. 1987), but it has also been isolated from human feces. The oral cavity is the likely habitat of *F. equinum* and *F. simiae*, isolated from horse and the stump-tailed macaque (*Macaca arctoides*), respectively. Intragroup DNA homology studies of isolates and of human- and animal-type strains have indicated that similar sites in man and animals may have their own genetic clusters of phenotypically similar fusobacteria (Love et al. 1987).

MORPHOLOGY

Fusobacteria are aflagellar gram-negative rods of varied morphology. The bacterial cells range from short to very long and filamentous; some strains are coccobacilli. Width is variable. The cells may be single, be in pairs end to end, or form long, coiled filaments. Staining may be irregular and spheroplasts are common in some species.

The cells of *F. nucleatum* are slender, spindle-shaped bacilli with tapered or pointed ends, 0.4–0.7 μm thick and 4–10 μm long, appearing singly, in tandem pairs, or in bundles or sheaves of roughly parallel bacilli. Cell length is usually uniform among actively growing cells. The cells may have a beaded appearance due to the presence of intracellular granules. Filaments may be seen in old cultures. *F. periodonticum* and *F. simiae* have a similar cellular morphology. The cells of *F. necrophorum* are pleomorphic, often curved, with rounded and sometimes tapered ends, and they may have spherical enlargements. Free coccoid bodies and, especially, filaments are common. Cell length may vary from coccobacilli to long threads in material from human infections. The organism is a more regular bacillus in young cultures. Except in young cultures, staining is irregular and beaded forms are common. *F. naviforme* strains may have boat-shaped cells. Gonidial forms may be seen in old cultures of *F. gonidiaformans*. *F. varium* is a small bacillus that does not form filaments or spheroplasts. Strains of *F. mortiferum* may be extremely pleomorphic with globular forms, swellings, and threads. Short rods are predominant in cultures of *F. equinum*. The other fusobacteria are pleomorphic filamentous organisms.

CULTURAL CHARACTERISTICS

Fusobacteria are not particularly demanding with regard to a low oxidation–reduction potential. They are, however, killed fairly readily by exposure to oxygen. This is possibly due to their susceptibility to peroxides. Growth is best at 35–37°C and at a pH near 7.

All *Fusobacterium* spp. grow more or less readily on blood agar based on proteose–peptone, tryptone, or trypticase. Good growth is usually obtained in a rich semifluid medium such as brain–heart medium supplemented with yeast extract. Batch cultivation is best performed in a fluid medium containing a reducing agent. If narrow-necked, well-filled containers are used, pre-reduced, anaerobically sterilized (PRAS) media are usually not necessary.

Surface colonies of *F. nucleatum* are 1–2 mm in diameter after incubation for 2 days. They are white to yellow-gray in color, speckled, smooth, or breadcrumb-like. The colonies are usually nonhemolytic. Cultures of *F. nucleatum* produce an unpleasant, but not fetid, smell.

After incubation for 2 days, colonies of animal isolates of *F. necrophorum* are about 2 mm in diameter, flat, circular with scalloped to erase edge, opaque, and white to gray (subsp. *necrophorum*); or 1 mm wide, circular with entire margins, gray, translucent and with smooth surfaces (subsp. *funduliforme*) (Shinjo et al. 1991). *F. necrophorum* subsp. *necrophorum* is strongly β-hemolytic; β-hemolysis is usually weaker in subsp. *funduliforme* and may need incubation for 3 or more days to develop. Cultures of *F. necrophorum* produce a foul, putrid odor. Fluid or semifluid cultures are characterized by abundant production of gas.

The colonial morphology of *F. necrophorum* isolated from human infections is similar to that of *F. necrophorum* subsp. *funduliforme*. The colonies are small and have been described as creamy, opaque, smooth, umbonate or raised, and with entire edges (Hall et al. 1997). The colonial morphology of most other *Fusobacterium* spp. is similar to that of *F. necrophorum* subsp. *funduliforme*.

GENETICS

The genome of *F. nucleatum* (strain ATCC 25586^T) contains 2.17 Mb encoding 2067 open reading frames (ORF) organized on a single circular chromosome with a G + C content of 27 percent (Kapatral et al. 2002). About 3.5 percent of the ORFs are unique to *F. nucleatum*. There are fewer chromosomal clusters between *F. nucleatum* and *Escherichia coli* than between *F. nucleatum* and gram-positive bacteria such as *Clostridium* spp. and *Bacillus subtilis*. This corroborates the allocation, based on 16S rRNA sequences, of *F. nucleatum* in the low-G + C gram-positive bacterial group.

F. nucleatum strain ATCC 25586^T did not contain any plasmids. However, plasmids have been found in other *F. nucleatum* strains (McKay et al. 1995; Haake et al. 2000). Bacteriocin production has been reported in *F. nucleatum* and *F. mortiferum* (Oliveira et al. 1998; Portrait et al. 2000).

Different methods for genotyping, i.e. allozyme electrophoresis, ribotyping, the arbitrarily primed polymerase chain reaction (PCR), and examination of ribosomal RNA gene restriction patterns, have been used for studying heterogeneity (Lawson et al. 1991; George et al. 1997; Morris et al. 1997; Thurnheer et al. 1999), identification and epidemiologic subtyping (Avila-

Campos et al. 1999), and intrafamiliar transmission of oral *F. nucleatum* isolates (Suchett-Kaye et al. 1999). Ribotyping has also been used to demonstrate similarity between *F. necrophorum* isolates from bovine liver abscesses and ruminal contents (Narayanan et al. 1997).

METABOLISM

The core metabolism of *F. nucleatum* is similar to that of *Clostridium*-, *Lactococcus*-, and *Enterococcus* species (Kapatral et al. 2002). More than 137 transporters for the uptake of substrates such as peptides, sugars, metal ions, and cofactors have been detected. Amino acids and small peptides comprise the major sources of energy for all *Fusobacterium* spp., but sugars may be used when amino acids are not available. Acidic and cationic amino acids are the main acids incorporated. Glutamate, histidine, lysine, and serine appear to be key amino acids in *F. nucleatum* (Dzink and Socransky 1990; Rogers et al. 1992). Remaining amino acids are imported as such or as di- or oligopeptides, which are degraded in the bacterial cytoplasm. An active transport of the dipeptide L-cysteinglycine has been detected in *F. nucleatum* (Carlsson et al. 1994). Small peptides may also be cleaved outside the bacterial cell (Bakken et al. 1989b).

Biosynthetic pathways exist for only glutamate, aspartate, and asparigine (Kapatral et al. 2002), and aspartylaspartate and glutamylglutamate can be used as growth substrates for *F. nucleatum* (Takahashi and Sato 2002). Glutamate is a key catabolic substrate in *Fusobacterium* spp. It is catabolized via the 2-oxoglutarate pathway with the production of acetate and butyrate as end products (Gharbia and Shah 1991). Glutamate may also be degraded by the methylaspartate pathway in *F. varium* (Gharbia and Shah 1991). Enzymes representative for the mesaconate pathway for catabolism of glutamate have been detected in *F. varium*, *F. mortiferum*, and *F. ulcerans* (Gharbia and Shah 1991). *F. varium* and *F. mortiferum* also possess enzymes representative of the 4-aminobutyrate pathway. The 3-keto-aminohexamate pathway catabolizes lysine (Barker et al. 1982).

Fusobacterium spp. differ in their ability to use fermentable carbohydrate as energy source for growth (Robrish et al. 1991). *F. nucleatum* and other species utilize glucose for biosynthesis of intracellular glycopolymers that can be degraded to produce energy under conditions of amino-acid deprivation (Robrish and Thompson 1988). The accumulation of glucose is dependent on energy generated by the fermentation of amino acids (Robrish et al. 1987). *F. mortiferum* is an exception in that accumulation of sugars is independent of a fermentable amino acid. Significantly, *F. mortiferum* has the ability to metabolize various sugars as energy sources for growth (Robrish et al. 1991). The range of sugars includes, among others, α- and β-glycosides, which are transported by the phosphoenolpyruvate (PEP)-dependent sugar: phosphotransferase system (PTS). Thus, it utilizes sucrose [glucose-fructose in α(1→2) linkage] and its five isomeric α-D-glucosyl-D-fructoses as energy sources for growth (Pikis et al. 2002). Phospho-β-glucosidase (P-β-glucosidase; EC 3.22.1.86) and 6-phospho-α-D-glucosidase (maltose-6-phosphate hydrolase; EC 3.2.1.122) from *F. mortiferum* have been purified and the encoding genes, *pbgA* and *malH*, respectively, have been expressed in *E. coli* (Bouma et al. 1997; Thompson et al. 1997).

All fusobacteria, except *F. naviforme* and *F. russii*, produce propionate from threonine. Lactate is converted to propionate by *F. necrophorum* and *F. varium*. Fusobacteria form volatile sulfur compounds from cysteine and methionine (Pianotti et al. 1986; Claesson et al. 1990).

CELL-WALL COMPOSITION; ANTIGENIC STRUCTURE

The fusobacteria have a cell-wall structure typical of gram-negative bacteria: an inner (cytoplasmic) and outer membrane, separated by a periplasmic space containing the peptidoglycan layer.

meso-Lanthionine, the monosulfur analog to *meso*-diaminopimelic acid, replaces the latter as the diamino acid present in the peptidoglycan of *F. nucleatum*, *F. gonidiaformans*, *F. necrophorum*, *F. russii*, *F. necrogenes*, *F. simiae*, and *F. periodonticum* (Kato et al. 1981; Vasstrand et al. 1982; Gharbia and Shah 1990). *meso*-Lanthionine is synthesized as such and then incorporated into the peptidoglycan pentapeptide (Fredriksen et al. 1991). *meso*-Diaminopimelic acid is present in the peptidoglycan of *F. varium*, *F. naviforme*, and *F. ulcerans*. The peptidoglycan layer of *F. mortiferum* contains both diamino acids.

The outer membrane is an asymmetric, multilayered membrane consisting of phospholipid, lipoprotein, lipopolysaccharide (LPS), and protein. Fusobacterial LPS shares the general LPS architecture of a heteropolysaccharide, exhibiting O-antigenic specificity, linked to lipid A through 2-keto-3-deoxyoctonate. The lipid A of *F. nucleatum* exhibits close structural relationship to that of enterobacterial LPS and cross-reacts serologically with antibodies to *E. coli* lipid A (Dahlén and Mattsby-Baltzer 1983). *F. necrophorum* subsp. *funduliforme* and human *F. necrophorum* isolates have a rough-type LPS, whereas that of *F. necrophorum* subsp. *necrophorum* is of the smooth type (Brown et al. 1997). The repeat unit of the O-antigenic polysaccharide of *F. necrophorum* LPS is an acid identified as a 2-amino-2-deoxy-2-C-methyl-pentonic acid (2-amino-2-methyl- 3,4,5-trihydroxypentanoic acid), a novel structure not found before in nature (Hermansson et al. 1993).

The dominating protein of the outer membrane of *F. nucleatum*, designated FomA, has a molecular mass of 40 kDa (Bakken et al. 1989a). It is a nonspecific porin

present in the outer membrane as a trimer (Kleivdal et al. 1995). The gene encoding the FomA porin has been sequenced (Bolstad and Jensen 1993a) and a topology model has been made (Bolstad et al. 1994). The deduced topology is similar to that of porin structures from other bacteria. The *fomA* gene has been cloned and expressed in *E. coli* (Jensen et al. 1996; Haake and Wang 1997). Several other outer-membrane proteins have been predicted from the genome sequence, among them some very-high-molecular-weight proteins (Kapatral et al. 2002). FomA and outer-membrane proteins with molecular masses of 55, 60, and 70 kDa seem to be major protein antigens in *F. nucleatum* (Bakken et al. 1989c). Heat-shock proteins (HSP) reacting with anti-*Yersinia enterocolitica* HSP60 and mycobacterial 65 kDa HSP have been detected in *F. nucleatum* (Ando et al. 1995). A homology between *F. nucleatum*, *Mycobacterium leprae*, and *E. coli* HSPs has been indicated (Skar and Bakken 2001).

The cellular fatty acids in the *Fusobacterium* spp. examined are straight-chain saturated and monoenoic acids of chain lengths C_{12}–C_{18} and O-3-hydroxytetradecanoate (Jantzen and Hofstad 1981). O-3-Hydroxyhexadecanoate is distinctive of the oral species *F. nucleatum*, *F. simiae*, and *F. periodonticum* (Jantzen and Hofstad 1981).

Unquestionable fimbriation or capsule formation has not been demonstrated in fusobacteria.

SUSCEPTIBILITY TO ANTIBACTERIAL AGENTS

A varying amount of *F. nucleatum* isolates produce β-lactamase, an activity not reported for other *Fusobacterium* species. A penicillin-hydrolyzing β-lactamase inhibited by clavulanic acid has been isolated from *F. nucleatum* (Tunér et al. 1985). *Fusobacterium* species are fully susceptible to amoxycillin/clavulanate, carbapenems, chloramphenicol, the new quinolone clinafloxalin, linezolide (Goldstein et al. 1999), and nitroimidazoles. Resistance to cefoxitin and clindamycin is very low. Resistance to tetracyclines in *F. nucleatum*, which is common, is due to the presence of the *tetM* gene located on the chromosome. A substantial number of *F. nucleatum* strains harbor the *tetM* determinant on a conjugal transposon (Roberts and Lansciardi 1990). Resistance to glycylglycines has not been observed (Downes et al. 1999). *F. nucleatum* is sensitive to the bactericidal action of protegrins and other antibacterial peptides (Miyasaki et al. 1998).

LABORATORY ISOLATION AND IDENTIFICATION

The isolation of fusobacteria from an anaerobic infection requires proper collection of the specimen, the use of an anaerobic transport vial, optimal anaerobiosis (an anaerobic chamber is not necessary), and a rich medium supplying optimal growth. Fastidious Anaerobe Agar Base (FAA, Lab M) supplemented with 5 percent sheep or horse blood is recommended for culture.

The use of a selective medium is necessary for the enumeration of viable fusobacteria in specimens of normal flora. The addition of josamycin, vancomycin, and norfloxacin plus 5 percent defibrinated horse blood to FAA promotes luxuriant growth of *Fusobacterium* spp. while completely inhibiting most other obligate anaerobes and facultative bacteria (Brazier et al. 1991). Rifampicin blood agar (Sutter et al. 1971) is useful for the selective isolation of *F. varium* and *F. mortiferum*.

All *Fusobacterium* spp. produce butyric acid but no isoacids. They are catalase- and nitrate-negative, sensitive to kanamycin (1000 μg disk) and colistin (10 μg disk), resistant to vancomycin (5 μg disk), and produce a rancid odor. A yellow fluorescence under longwave ultraviolet (UV) light is a common property of fusobacteria grown on blood agar based on FAA.

The characteristic cellular morphology of *F. nucleatum* makes a presumptive identification relatively easy. The fusiform cells of *L. buccalis* are thicker and usually larger (see Table 1) and those of *Capnocytophaga*

Table 1 *Characters differentiating* L. buccalis, F. nucleatum, *and* Capnocytophaga *spp.*

Character	*L. buccalis*	*F. nucleatum*	*Capnocytophaga*
Microscopic morphology (blood agar)	Gram-negative,[a] 5–15 μm long, thick fusiform bacilli	Gram-negative, 3–10 μm long, slender fusiform bacilli	Gram-negative, 3–6 μm long, slender fusiform bacilli
Colonial morphology	Smooth, rhizoid, or convoluted	Smooth, convex, 'flecked' appearance	Flat, rough, spreading margin,[b] adherent; or low-convex, smooth; yellow-pigmented
Growth in air + 5–10% CO_2	+	–	+
Acid from glucose	+	–	+
Production of indole	–	+	–
Main fatty acids from glucose/peptone	Lactic	Butyric	Acetic, succinic

a) Often gram-positive in young cultures.
b) Gliding motility.

spp. generally smaller. Moreover, *L. buccalis* and *Capnocytophaga* spp. are not obligate anaerobes.

The tests shown in Table 2 are useful for the identification of species within the genus *Fusobacterium*. The properties listed for *F. nucleatum* and *F. russii* are shared with *F. periodonticum*. Diagnostic tablets are convenient for the examination of bile resistance, production of alkaline phosphatase, and orthonitrophenol-beta-D-galactopyranoside (ONPG)-test. Commercially available kits based on the detection of preformed enzymes and designed for identification of anaerobes may be of help in supporting a suspected identity of a *Fusobacterium* isolate. The Rapid ID 32A system (bioMérieux), which provides a rapid and reliable method for identification of non-*B. fragilis* group anaerobic gram-negative bacilli to the genus level, discriminates poorly between *F. nucleatum* and *F. necrophorum* (Downes et al. 1999). *Fusobacterium* species differ in their ability to hydrolyze endopeptidase and amino- and diamino peptidase substrates (Beighton et al. 1997).

Determination of the electrophoretic migration of glutamate dehydrogenase and 2-oxoglutarate reductase provides a rapid method for the identification of most *Fusobacterium* spp. (Gharbia and Shah 1990). Identification based on gas–liquid chromatography of cellular fatty acids may be of value in reference laboratories, provided a database is used as a basis for comparison. Of greater potential in the diagnostic laboratory is the use of nucleic-acid probes. A genomic DNA probe has been used successfully to detect *F. nucleatum* directly in samples of subgingival plaque (Lippke et al. 1991) and checkerboard DNA–DNA hybridization has been used to detect *F. nucleatum* and subspecies in periodontal and endodontic infections (Socransky et al. 1998; Sunde et al. 2000). A PCR-amplified nonradioactive probe coding for part of the major 40-kDa outer-membrane protein, the FomA porin, of *F. nucleatum* and seemingly specific for the species when used under condition of high stringency, has been constructed (Bolstad and Jensen 1993b). So far, automated DNA sequencing for identification of particular *Fusobacterium* isolates is best suited for the reference laboratory.

ROLE IN NORMAL FLORA

Coaggregation, adherence of cells of different species or strains among resident bacteria, is an important event in the formation of biofilms such as dental plaque (Kolenbrander and London 1993). *F. nucleatum* is one of the bacterial species most frequently isolated in plaque from healthy sites. The organism coaggregates with several other oral bacterial species and is proposed to act as a bridge between bacteria that adhere early to the tooth surface and the late colonizers (Bolstad et al. 1996). It may be a key organism in the development, maintenance, and stabilization of the microbial flora of the gingival crevice. *F. nucleatum* and *F. periodonticum* coaggregate with *Candida albicans* (Grimaudo and Nesbitt 1997). Coaggregation is a specific process dependent on surface-associated adherence proteins. The nature of the fusobacterial adherence proteins is largely unknown. Galactose and *N*-acetylneuraminic acid-binding adhesins have been identified (Shaniztki et al. 1997, 1998).

The role played by fusobacteria in other ecosystems where fusobacteria are present in low numbers is virtually unknown.

PATHOGENICITY AND VIRULENCE FACTORS

Pathogenic fusobacteria are in particular isolated from inflammatory processes accompanied by necrosis and ulceration. *F. nucleatum* constitutes about two-thirds of the *Fusobacterium* isolates. Next to the most prevalent

Table 2 *Differential characteristics of* Fusobacterium *species encountered in human clinical specimens*

			Production of					Proprionate from	
Species	Bile resistance	Gas in PYG	Indole	Lipase	Hydrolysis of esculin	Phosphatase activity	ONPG	Threonine	Lactate
F. nucleatum	–	–	+	–	–	–	–	+	–
F. gonidiaformans	–	+	+	–	–	–	–	+	–
F. varium	+	+	v	v[1]	–	–	–	+	–
F. necrophorum	–	+	+	+	–	+	–	+	+
F. naviforme	–	–	+	–	–	–	–	–	–
F. russii	–	–	–	–	–	+	–	–	–
F. mortiferum	+	+	–	–	+	–	+	+	–
F. necrogenes[a]	+	+	–	–	+	+		+	–
F. ulcerans[b]	+	+	–	–	–	–	–	+	–

+, Positive reaction for majority of strains (>90%); –, negative reaction for majority of strains (>90%); PYG, peptone-yeast extract-glucose broth; v, variable; v[1], may be positive after prolonged incubation.

a) Rarely isolated from clinical specimens.

b) Isolated from topical ulcers only.

Bacteroides and *Prevotella* species, *F. nucleatum* is the gram-negative anaerobic organism most often encountered in human infections. It is most frequently isolated from infections of the mouth, head, and neck, pleuropulmonary infections, and abscesses of the brain and liver. *F. nucleatum* is one of several bacterial species associated with periodontitis in adults. It causes desulfuration of cysteine and methionine, yielding ammonia, H_2S, methyl mercaptan, and butyrate, which are capable of arresting fibroblast growth and thereby preventing wound healing and aiding penetration of the gingival epithelium (Kapatral et al. 2002). *F. nucleatum* has been exclusively recovered from the amniotic fluid of women with preterm delivery (Holst et al. 1994).

F. necrophorum is the cause of necrobacillosis in man and animals. Lemierre's syndrome is the classical presentation of human necrobacillosis (Lemierre 1936; Kristensen and Prag 2000).This is a specifically recognizable, rare, but serious disease starting with acute sore throat with purulent exudate in a previously healthy young adult who subsequently develops septicemia with disseminated metastatic abscesses, frequently including a septic thrombophlebitis of the jugular vein. *F. necrophorum* is frequently found in specimens from peritonsillar abscesses. The organism is isolated more seldom from purulent infections elsewhere in the body. Human *F. necrophorum* isolates resemble animal strains of *F. necrophorum* subsp. *funduliforme* (Hall et al. 1997). In contrast, necrobacillosis in animals is caused by *F. necrophorum* subsp. *necrophorum*, which is an important animal pathogen that is frequently isolated from necrotic and gangrenous lesions in domestic and wild animals. The most common manifestations associated with the organism are liver abscesses and foot rot in cattle and sheep. *F. necrophorum* subsp. *funduliforme* is a weak pathogen in animals. Human and animal clinical *F. necrophorum* isolates are thus distinct, as are the diseases caused.

The other *Fusobacterium* spp. are occasionally isolated from human infections. *F. russii* may be found in infected dog and cat bites.

Current knowledge of bacterial products or strategies that enable pathogenic fusobacteria to colonize and invade, and to damage the host, is sparse. The role played by the lipopolysaccharide and other cell-wall components in the pathogenesis of anaerobic infections involving fusobacteria has not been resolved. Fc-binding activity in *F. nucleatum* has been associated with polypeptides of 40 and 42 kDa (Guo et al. 2000).

The genome of *F. nucleatum* contains several ORFs that code for putative virulence factors, among them three hemolysins (Kapatral et al. 2002). The organism agglutinates human erythrocytes and attaches to human oral epithelial cells, gingival fibroblasts, and polymorphonuclear leukocytes (Bolstad et al. 1996). *F. nucleatum* also binds to collagen (Xie et al. 1991), fibronectin (Isogai et al. 1992), and a glycosylated protein-rich glycoprotein in the parotid saliva (Gillece-Castro et al. 1991). An immunosuppressive protein, FiP, that arrests human T cells in the mid-G_1 phase of the cell cycle, has been isolated (Shenker and Datar 1995). The encoding gene, *fipA*, has been characterized, cloned, and expressed in *E. coli* (Demuth et al. 1996). It seems that the *fipA* gene is highly conserved among *F. nucleatum* strains and that *fipA* homologs are widely distributed among fusobacteria (Gerardo et al. 2002). Induction of apoptotic cell death in human leukocytes by *F. nucleatum* has been reported (Jewett et al. 2000).

F. necrophorum produces a leukotoxic protein with specificity for ruminant leukocytes (Emery et al. 1984). The toxin has been cloned and sequenced (Narayanan et al. 2001b). It induces activation and apoptosis of bovine leukocytes (Narayanan et al. 2002). Large amounts are produced by *F. necrophorum* subsp. *necrophorum*. The *F. necrophorum* hemolysin is a phospholipase distinct from the leukotoxin (Fifis et al. 1996). A cell-wall preparation from *F. necrophorum* subsp. *necrophorum* has been shown to be collagenolytic (Okamoto et al. 2001).

LEPTOTRICHIA

DEFINITION

Leptotrichia species are gram-negative, large fusiform rods that are nonsporing and nonmotile. They are usually anaerobic on first isolation; most strains grow aerobically in the presence of CO_2. Carbohydrates are used for energy metabolism. Lactic acid is a major metabolic product. The type species of the genus is *L. buccalis* and the G + C content of DNA of the type strain ATCC 14201^T is 25 mol%.

HISTORICAL PERSPECTIVE

The generic name *Leptotrichia* was introduced by Trevisan (1879) for filamentous organisms found in the human mouth. Several years earlier Robin (1853) used the species designation *buccalis* for filamentous forms (*Leptothrix buccalis*) observed in wet mounts of tooth scrapings. *L. buccalis* was first cultured by Wherry and Oliver (1916) and carefully described by Thjøtta et al. (1939). In 1995, another species, *Leptotrichia sanguinegens* ('*Leptotrichia microbii*'), was proposed for a serum-requiring, anaerobic, gram-negative rod isolated from the blood of patients with postpartum and neonatal bacteremia (Hanff et al. 1995). Recently, this organism was transferred to the new genus *Sneathia* as *Sneathia sanguinegens* (Collins et al. 2001). *L. trevisanii* (Tee et al. 2001) and *L. amnionii* (Shukla et al. 2002) have been suggested as additional *Leptotrichia* species. The name *L. trevisanii* was validly published in 2002 (Validation list No. 85 2002), while the name *L. amnionii* has not

been validated so far. Four new *Leptotrichia* species, i.e. *L. goodfellowii*, *L. hofstadii*, *L. shahii*, and *L. wadei* have been established by Eribe et al. (2004).

In *Bergey's Manual of systematic bacteriology*, *Leptotrichia* is classified as the third genus in the family Bacteroidaceae. In the past *L. buccalis* has been confused with *Fusobacterium* spp. and, because of the frequent occurrence in young cultures of gram-positive stained cells, it has also been mistaken for gram-positive bacilli related to lactobacilli. The name *Leptotrichia* has been used for the gram-positive organism termed *L. dentium* by Davis and Baird-Parker (1959) and now classified as *Corynebacterium matruchotii* (Collins and Cummins 1986).

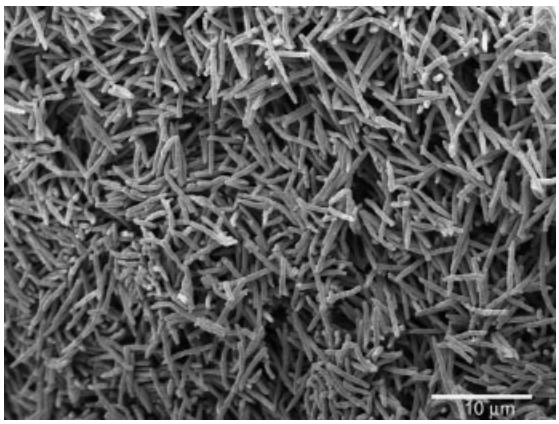

Figure 1 *Scanning electron microscopy of* L. buccalis *cells in a colony*

CLASSIFICATION

Isolates described in the literature as members of the species *L. buccalis* differ with respect to phenotypic characteristics, such as cellular and colonial morphology, aerotolerance, fermentation of sugars, cellular fatty-acid composition, and G + C content (Morgenstein et al. 1980; Duperval et al. 1984; Reig et al. 1985; Hofstad and Naess 1992; Eribe et al. 2002). This indicates heterogeneity of the species, which is being further demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins and random amplified polymorphic DNA (RAPD) (Eribe and Olsen 2002), and results of 16S rRNA-sequencing and DNA–DNA homology confirmed the presence of additional *Leptotrichia* species (Eribe et al. 2004).

HABITAT

The human habitat of *L. buccalis* is the oral cavity, where dental plaque is the principal niche. The organism is present in adults and children and has been found in the mouth of edentulous infants (Könönen et al. 1994), and seems to colonize permucosal implants of edentulous patients (Nakou et al. 1987). *L. buccalis* has also been isolated from the periurethral region of the vagina (Bollgren et al. 1979), from dog bites (Goldstein et al. 1978), and in infections of domestic animals (Hirsh et al. 1979). The role of *L. buccalis* in the normal microflora is unknown.

L. trevisanii was isolated from the blood of a man diagnosed as having acute myeloid leukemia (Tee et al. 2001).

MORPHOLOGY

L. buccalis is an aflagellar, straight or slightly curved rod, 0.8–1.5 µm wide and 5–15 µm long; one or both ends are pointed end or rounded. A very large gram-negative bacillus with one pointed end and one square end is particularly suggestive of *L. buccalis*. In gram-stained smears of 48 h cultures the organism appears as gram-negative cells often with gram-positive granules, singly or in pairs, end to end, and in a 'straw-like' arrangement (Figure 1). Young cultures show a varying proportion of gram-positive cells. Thread forms and chains may be present in smears made from old, filamentous colonies. An unusual ultramicroscopic feature is a ridged surface structure formed by an assay of macromolecules erected on the surface like a fence with closely set vertical pales (Listgarten and Lai 1975). 0.8–0.9 µm wide and 6–13 µm long, fusiform, nonmotile gram-negative cells, many of which are curved, with tapered ends characterizing *L. trevisanii*. (Tee et al. 2001). The cell ends are flattened where they join.

CULTURAL CHARACTERISTICS AND METABOLISM

L. buccalis grows well under anaerobic or microaerobic conditions, but poorly in air even in the presence of CO_2 (Hofstad and Næss 1992). Surface colonies on blood agar medium are 2–3 mm in diameter after incubation anaerobically for 2 days, smooth, colorless, sometimes raised, and with a filamentous edge (Figure 2), nonadherent, and nonhemolytic. After prolonged incubation the surface of the colonies becomes convoluted, resembling that of a human brain.

L. trevisanii grows both anaerobically and under 5 percent CO_2 (in air) on subsequent culture on solid media (Tee et al. 2001). On horse-blood agar plates the colonies are smooth, grayish in color, low convex, and with an erosed edge. They appear with a dark central spot with transillumination and grow to about 2 mm in diameter after 5 days of incubation. Older colonies show a slightly convoluted surface.

L. buccalis is highly saccharolytic. Mono- and disaccharides are fermented with the production of lactic acid alone, or accompanied by trace amounts of acetic and succinic acids. The terminal pH is 5.4 or less. Growth in fluid or semifluid media is enhanced by serum and dependent on the presence in the medium of a fermen-

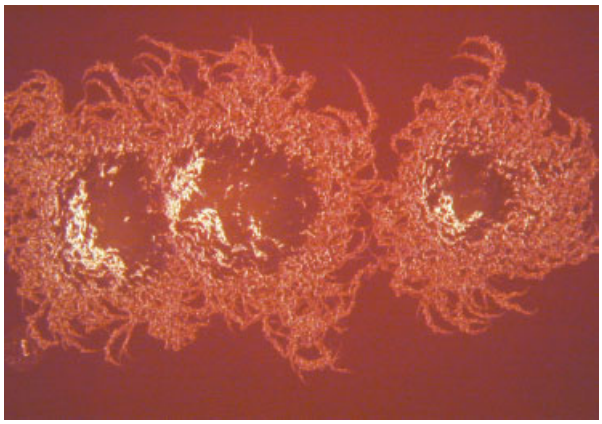

Figure 2 L. buccalis *colonies of 48 h culture (37 °C) on blood agar*

table carbohydrate. Ammonia is not produced. This clearly indicates that carbohydrate is used for energy metabolism. *L. trevisanii* ferments glucose and lactic acid is the major organic end product (Tee et al. 2001).

CELL-WALL COMPOSITION

L. buccalis has a gram-negative cell-wall structure. The diamino acid of the peptidoglycan is *meso*-diaminopimelic acid (Vasstrand et al. 1982). The cellular fatty-acid composition is similar to that of the fusobacteria, with hexadecanoic, octadecanoic, and 3-hydroxytetradecanoic acids as major components (Hofstad and Jantzen 1982). The MIDI (Microbial ID, Inc., Newark, DE, USA) system identified the most abundant cellular fatty acids as $C_{16:0}$ (32–59 percent) and $C_{18:1}$-*cis* 11/t9/t6 fatty acids or an unidentified compound with equivalent chain length of 17.8 (21–42 percent) (Eribe et al. 2002). $C_{12:0}$ (3–16 percent), $C_{15:0}$ DMA or 3-OH-$C_{14:0}$ (4–14 percent), $C_{14:0}$ (3–20 percent), and $C_{16:1}$-*cis* 9 (1–5 percent) were also detected. Seventy percent of the strains contained $C_{18:1}$ –*cis* 9 (1–9 percent) and 62 percent $C_{18:2}$ –*cis* 9,12 (1–6 percent).

LABORATORY ISOLATION AND IDENTIFICATION; SUSCEPTIBILITY TO ANTIBACTERIAL AGENTS

L. buccalis can be isolated from clinical specimens by cultivation anaerobically on blood agar, for instance Brucella blood agar or FAA supplemented with sheep or horse blood. Addition of josamycin, vancomycin, and norfloxacin to the blood-agar medium is recommended for isolation of *L. buccalis* from normal flora. Others use Fusobacterium egg yolk agar for the recovery of *Leptotrichia* (Murray and Citron 1991). A reliable identification can be made from cellular and colonial morphology and a few biochemical tests (Table 1, p. 5).

L. buccalis is susceptible to β-lactam antibiotics, carbapenems, clindamycin, chloramphenicol, tetracyclines, and metronidazole, but resistant to vancomycin and aminoglycosides (George et al. 1981; Weinberger et al. 1991). Most strains are moderately resistant or resistant to erythromycin. The *L. trevisanii* isolate was susceptible to penicillin, metronidazole, and tetracycline, partly resistant to clindamycin, and resistant to erythromycin and gentamycin (Tee et al. 2001).

PATHOGENICITY

Infections with *L. buccalis* are uncommon. Relatively few cases have been described including endocarditis (Hammann et al. 1993; Duperval et al. 1984) and sepsis occurring in immunocompromised, neutropenic patients with mucositis or periodontitis (Reig et al. 1985; Weinberger et al. 1991). The oral cavity may be the source of such infections with the portal of entry being via gingivitis or oral mucosal lesions. The pathogenetic mechanisms of *Leptotrichia* infections are unknown.

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Mycoplasma

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INTRODUCTION

Mycoplasmas are distinguished phenotypically from other bacteria by their minute size and total lack of a cell wall. Taxonomically, the lack of cell walls is used to separate mycoplasmas from other bacteria in a class named Mollicutes (*mollis*, soft; *cutis*, skin, in Latin). The current classification of Mollicutes and the properties distinguishing the established taxa are presented in Table 1. While the trivial terms mycoplasmas or mollicutes have been used interchangably to denote any species included in Mollicutes, the trivial names ureaplasmas, entomoplasmas, mesoplasmas, spiroplasmas, acholeplasmas, asteroleplasmas, and anaeroplasmas are routinely used for members of the corresponding genus. Molecular characterization of the uncultured plant and insect mycoplasma-like organisms (MLO) has provided strong experimental support for their inclusion in the class Mollicutes (see Phylogeny and taxonomy). Consequently, the trivial term phytoplasmas has been proposed to replace the awkward name mycoplasma-like organisms. About 200 established species have already been described within the class Mollicutes, and this number continues to rise, including the still uncultured mollicutes, such as the phytoplasmas and the hemotrophic parasites *Haemobartonella* and *Eperythrozoon* species, recently identified as mollicutes and given the trivial name hemoplasmas (Neimark et al. 2001). The potential number of mollicute species is apparently enormous in light of the most prevalent occurrence of mollicutes in insects as each insect species may have its own unique mollicute flora (Tully and Whitcomb 1992).

The interested reader is referred to a number of books on various aspects of mycoplasmology published during the last decade or so (Maniloff et al. 1992; Razin and Tully 1995; Tully and Razin 1996). The most recent multi-authored treatise covering the molecular biology and pathogenicity of mycoplasmas was published in the summer of 2002 (Razin and Herrmann 2002). Several recent general reviews on the molecular biology, genetics, and pathogenicity of mycoplasmas are also available (Baseman and Tully 1997; Dybvig and Voelker 1996; Razin et al. 1998).

CYTOLOGY

Cellular morphology and cytoskeletal elements

The total lack of a cell wall explains many of the unique properties of the mycoplasmas, such as sensitivity to osmotic shock and detergents, resistance to penicillin, and formation of peculiar fried-egg shaped colonies (Figure 1). Thin sections of mycoplasmas reveal that the cells are built essentially of three organelles: the cell membrane, ribosomes, and a circular densly packed double-stranded DNA molecule, the typical prokaryotic genome (Figure 2). Being devoid of a cell wall, mycoplasmas appear gram-negative when stained by the

Table 1 *Major characteristics and taxonomy of the class Mollicutes*[a]

Classification	No. recognized species	Genome size (kb)	Mol% G + C of genome	Cholesterol requirement	Distinctive properties	Habitat
Mycoplasmataceae						
Genus I: *Mycoplasma*	107 (11)[b]	580–1 350	23–40	Yes	Optimum growth 37°C	Humans, animals
Genus II: *Ureaplasma*	7	760–1 170	25–30	Yes	Urease positive	Humans, animals
Entomoplasmataceae						
Genus I: *Entomoplasma*	6	790–1 140	27–29	Yes	Optimum growth 30°C	Insects, plants
Genus II: *Mesoplasma*	12	870–1 100	27–30	No	Optimum growth 30°C	Insects, plants
Spiroplasmataceae						
Genus I: *Spiroplasma*	34	780–2 220	24–31	Yes	Helical filaments	Insects, plants
Acholeplasmataceae						
Genus I: *Acholeplasma*	14	1 500–1 650	26–36	No	Optimum growth 30–37°C	Animals, plant surfaces
Anaeroplasmataceae						
Genus I: *Anaeroplasma*	4	1 500–1 600	29–34	Yes	Obligate anaerobes, oxygen sensitive	Bovine-ovine rumen
Genus II: *Asteroleplasma*	1	1 500	40	No		
Undefined taxonomic status: Phytoplasma	ND[c] (7)	530–1 350	23–29	ND	Uncultured in vitro	Insects, plants

ND, not determined.
a) Updated and modified from Razin et al. (1998).
b) The number of *Candidatus* species is given in parentheses and includes the haemoplasmas (*Eperythrozoon* and *Haemobartonella*) recently transferred to the genus *Mycoplasma*.
c) The taxonomic status of the uncultured phytoplasmas has not been finally defined; seven *Candidatus* Phytoplasma species have so far been published.

Gram stain. Bounded by a plastic cell membrane only, the dominating shape of a mycoplasma cell is a sphere (0.3–0.8 μm in diameter). However, cells of many mollicutes exhibit a variety of morphological entities, including pear-shaped cells, flask-shaped cells with terminal tip structures, filaments of varying length (some branching), as well as helical filaments (Figures 3–5). Maintaining such shapes in the absence of a rigid cell wall has indicated long ago the presence of a cytoskeleton in mycoplasmas (Razin 1978).

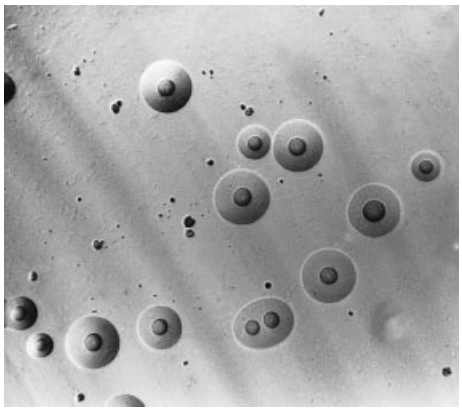

Figure 1 A. laidlawii *colonies on mycoplasma agar (×50) showing the characteristic 'fried-egg' shape, consisting of a central zone embedded in the agar and a peripheral zone on the agar surface.*

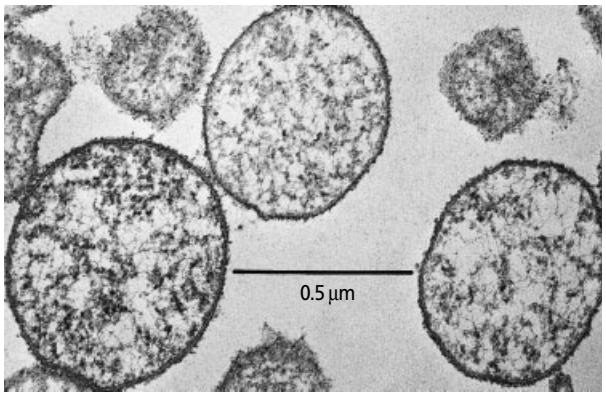

Figure 2 *Electron micrograph of thin-sectioned mycoplasma cells. The cells are bounded by a single membrane exhibiting the characteristic trilaminar shape of sectioned biomembranes fixed in osmium tetroxide. The cytoplasm contains thin threads representing the sectioned chromosome, and dark granules representing ribosomes.*

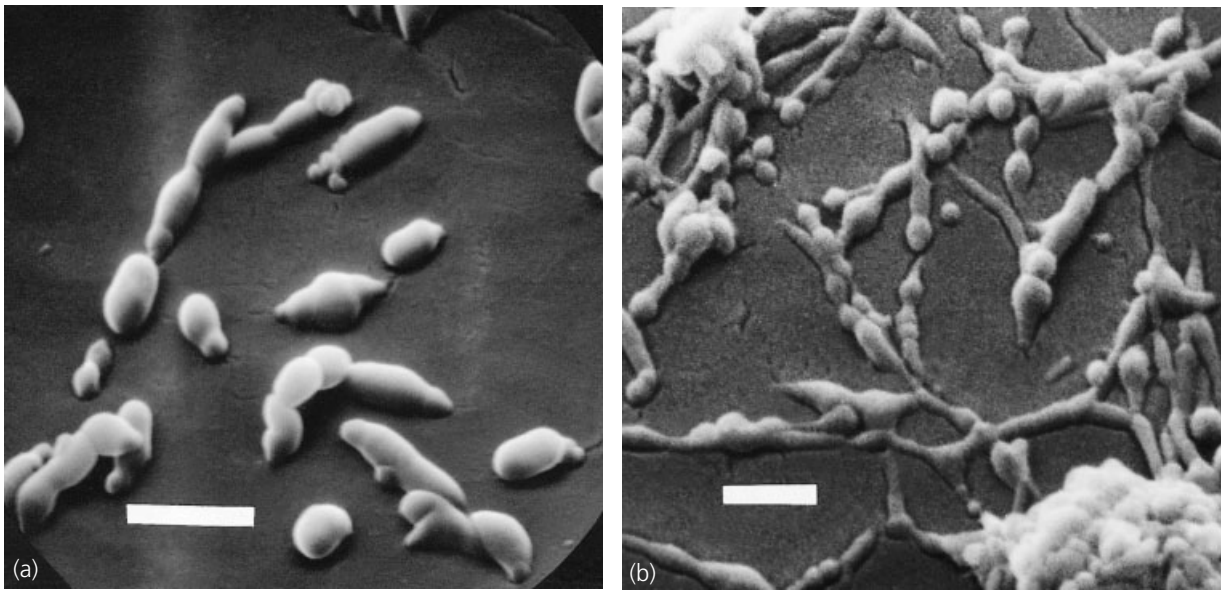

Figure 3 (a) *Scanning electron micrograph of* M. gallisepticum *cells showing the polymorphic shape of the organisms.* **(b)** M. pneumoniae *showing the characteristic elongated cells with tapered tips, as well as spherical cells and microcolonies. Bar = 1 μm.*

An important group of pathogenic mycoplasmas, including *M. pneumoniae* and *M. genitalium*, shares a flask- or club-like cell shape with a protruding tip or bleb structure (Figure 6). These mycoplasmas attach to eukaryotic cells via the tip structure, serving as an attachment organelle (Figure 7; see Adhesion to host cells). Scanning- and transmission-electron microscopy of *M. pneumoniae* cells grown on grids treated with 1 percent Triton X-100 revealed a rod-like tip structure and a network of filamentous strands. Proteins of this so-called 'Triton shell' are apparently cytoskeleton-forming or cytoskeleton-associated proteins (Razin and Jacobs 1992). The cytoskeleton-like structure is thought to function in modulating cell shape, and participate in cell division, gliding motility, and the proper localization of cytadhesins on the mycoplasmal cell surface. Studies by Mayer et al. (1998) suggest that the mollicutes may not be the only bacteria known to possess an internal cytoskeleton.

The genomic analysis of *M. pneumoniae* (Himmelreich et al. 1996) has enabled the identification and molecular characterization of the major protein building blocks of the cytoskeleton of this mycoplasma. Some of these proteins function as surface exposed cytadhesins, while others maintain the proper distribution and/or disposition of the cytadhesins on the mycoplasma membrane, consistent with their presumed scaffolding role (Balish and Krause 2002; Krause and Balish 2001). Recent analysis of the 'Triton shell' by 2D-PAGE and mass spectrometry revealed about 100 protein spots. Regula et al. (2001) identified 41 of them. The identified proteins belonged to several functional categories, mainly energy metabolism, translation, and heat-shock

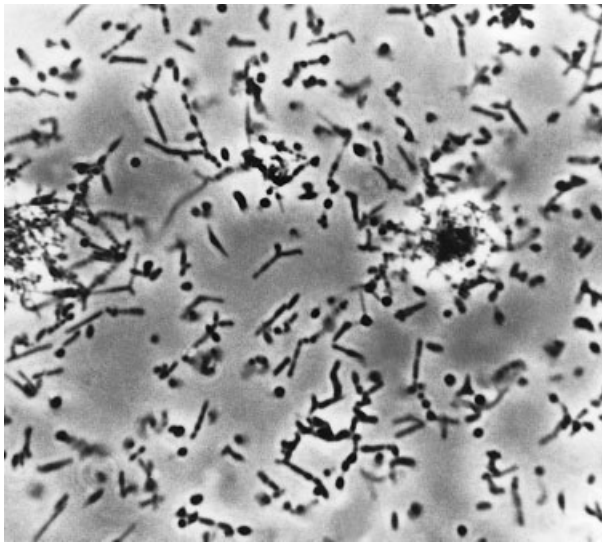

Figure 4 *Phase-contrast light microscopy of* A. laidlawii *cells. Branching filaments and chains of cocci can be seen in these unfixed live broth cultures. Magnification ×1 250.*

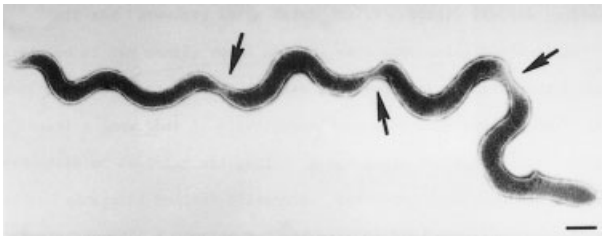

Figure 5 *A negatively-stained helical filament of* Spiroplasma citri *showing constrictions preceding filament division into daughter cells. Bar = 500 nm.*

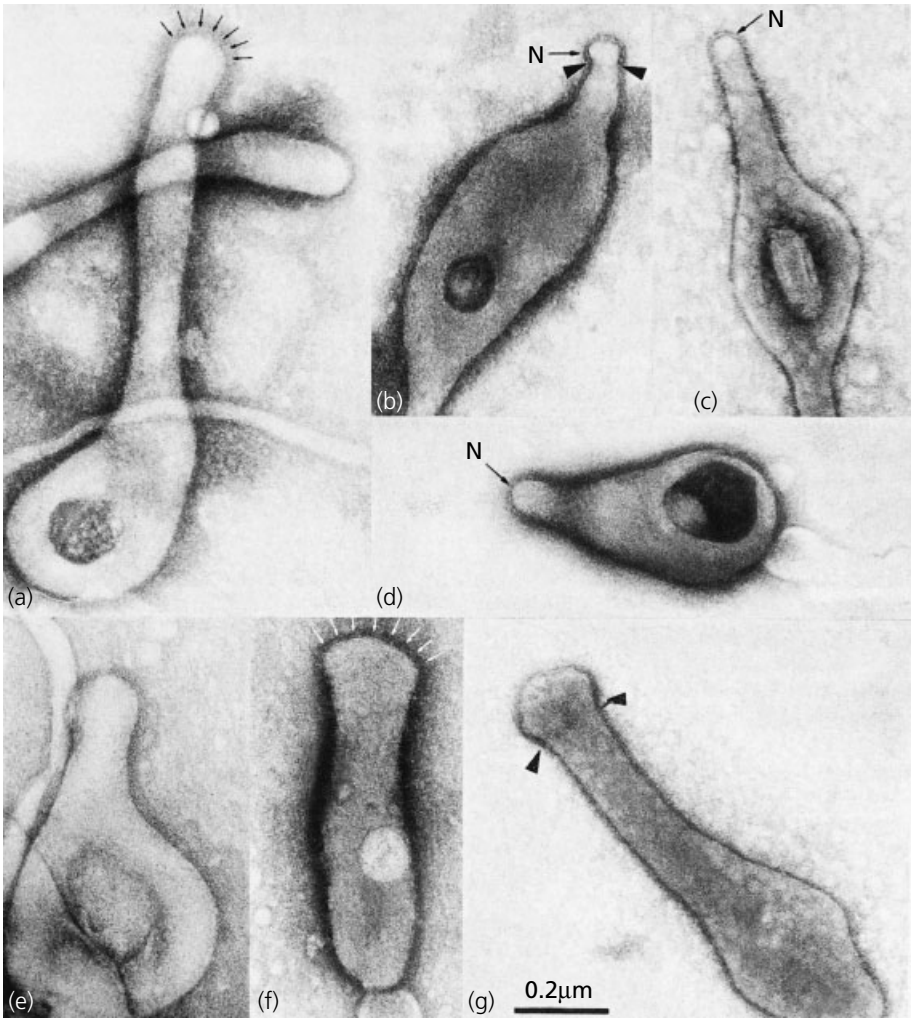

Figure 6 *Electron micrographs of negatively stained mycoplasmas possessing tip structures.* **(a)** M. pulmonis*. Arrows show a thin surface layer on top of a protruding stalk.* **(b)** M. gallisepticum*, showing the distinct bleb structure. The particulate surface nap (N) is not restricted to the bleb structure.* **(c)** M. pneumoniae*. Note the tapered terminal structure covered by the nap (N).* **(d)** M. genitalium*. Arrowhead shows the tip structure covered by a nap (N).* **(e)** M. alvi *showing a well-defined slightly thickened protuberance without surface layer.* **(f)** M. sualvi *showing a distinctly broadened blunt terminal structure with a thin surface layer (arrows)* **(g)** M. mobile*. Note the broadened headlike structure (arrowheads), with no surface layer (Kirchhoff et al. 1984).*

response. In addition, lipoproteins, and most of the proteins involved in cytadherence were found in this fraction (see Adhesion to host cells section and Table 5). Quantitatively, the most prevalent proteins were the heat-shock protein DnaK, elongation factor Tu and subunits α and β of the pyruvate dehydrogenase complex (Regula et al. 2001). Thus, the 'Triton shell' consists of a very heterogenous complex of proteins as could perhaps be expected from a complex cell fraction isolated according to its insolubility at a low concentration of the mild detergent Triton X-100.

Motility

Most mycoplasmas are nonmotile and have no flagella. However, some of the flask-shaped *Mycoplasma* species, including the human and animal pathogens, *M. pneumoniae*, *M. genitalium*, *M. gallisepticum*, *M. pulmonis*, and *M. mobile* exhibit gliding motility on liquid-covered surfaces. *M. mobile*, isolated from fish gills, is the fastest (2.0–4.5 μm/s, which is up to seven times its cell length per second). The mechanism of this peculiar gliding motility is still unknown (Miyata 2002). Although *M. pneumoniae* is motile and exhibits chemotactic behavior, gliding motility genes could not be identified in this mycoplasma, as it is not yet known which genes have to be looked for. Furthermore, none of the components of the chemotactic signal pathway, the Che proteins, which are well conserved among bacteria, as well as any other 'two-component signal transduction system' could be identified in the sequenced *M. pneumoniae* and *M. genitalium* genomes (Himmelreich et al. 1996).

The gliding force of *M. mobile* responsible for the thrust forward reaches up to 27 pN, which is five to seven times higher than that of motor proteins such as

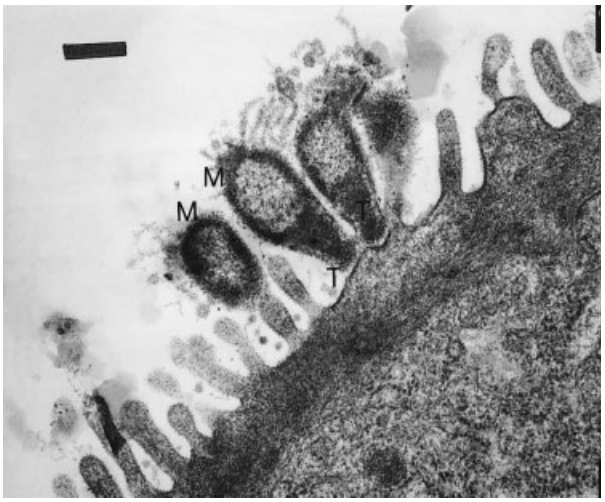

Figure 7 *Thin sections of* Mycoplasma gallisepticum *attaching to tracheal epithelium of chicken 7 days postinfection. The mycoplasmas can be seen to attach through their blebs.* M. mycoplasma*; T, attachment tip. Bar = 0.5 μm. Courtesy of Dykstra and Levisohn.*

myosin and kinensin (Miyata et al. 2002). The force estimate was achieved by trapping gliding *M. mobile* cells, with an attached polystyrene bead, by optical laser tweezers. The ability of the gliding mycoplasmas to cytadhere closely mirrors their ability to bind to glass, a prerequisite for gliding. Because cytadherence is linked to the tip structure it is likely that this attachment organelle plays a major role in the gliding mechanism. In fact, the gliding mycoplasmas always move with the attachment tip organelle at the leading end (Miyata 2002).

Spiroplasma species are unique among mollicutes in having helical morphology (Figure 5), rotary motility, and chemotaxis. Motility is characterized by rapid spinning along the long axis of the cell as well as by flexional or undulating movements of the helical filaments. The spiroplasma cell can essentially be viewed as a membranal tube (about 0.25 μm in diameter) to which a flat cytoskeletal ribbon made of seven parallel paired fibrils (diameter of individual fibril is 45–50 Å) is attached to the inner side of the cell membrane along the shortest helical line. Both tube and ribbon are mutually coiled into a dynamic helix (Trachtenberg and Gilad 2001). Illustrating the minuteness of mollicutes, the size and shape of a whole spiroplasma cell is roughly that of a bacterial flagellar bundle or that of a single eukaryotic flagellum or cilium. In addition to their bare simplicity and structural minimalism, spiroplasma cells are well-defined dynamic helices, amenable to geometrical analysis carried out by Trachtenberg et al. (2003a). Thus, it appears that the scaffolding and the propulsion functions of the cell wall and flagella are both replaced in spiroplasmas by an internal cytoskeleton that functions as a linear motor as well.

Upon cell lysis by deoxycholate, spiroplasmas release the fibrils which consist of a 59 kDa protein. The gene for this protein (*fib*) has been cloned, facilitating the characterization of the structural features of the fibril protein (Williamson et al. 1991). The 59 kDa protein has an equivalent sphere diameter of about 50 Å. Trachtenberg and Gilad (2001) suggest that the fibril protein forms a tetramer, and the tetramer assembles further into flat fibril pairs, seven of which form a flat, monolayered well-ordered ribbon. This structure may function as a linear motor driven by differential and coordinated length changes of the fibrils due to conformational changes of the tetrameric subunits, the shape of which changes from near circular to elliptical. This is the first linear motor described in swimming prokaryotes (Trachtenberg et al. 2003b; Gilad et al. 2003). Berg (2002) takes this point further by proposing a simple way for the ribbon to function in propulsion through bending about two axes in a cyclic manner. The primary effect of this cycle is to roll the spiroplasma cell about its longitudinal axis clockwise. In a viscous medium the helix screws its way like a corkscrew.

Some information on a gene involved in *S. citri* motility has been obtained through transposon mutagenesis (Jacob et al. 1997). An *S. citri* nonmotile mutant was generated by Tn*4001* mutagenesis. The transposon was shown to be inserted into a gene named *scm1*, encoding a putative polypeptide of about 45 kDa with no significant homology to any known protein. The protein was predicted to contain a signal peptide and 10 transmembrane α-helices and a leucine zipper motif. The *scm1* gene was recovered from the wild motile strain, and inserted into an *S. citri* cloning vector. Transfection of the nonmotile mutant with this recombinant plasmid restored motility, indicating that the *scm1* gene product is indeed involved in *S. citri* motility, probably serving as an anchor of the cytoskeletal ribbon to the membrane (Trachtenberg and Gilad 2001). Interestingly, the nonmotile mutant multiplied in the insect vector and was efficiently transmitted to plants, inducing disease. This suggests that spiroplasma motility may not be essential for pathogenicity (Duret et al. 1999).

Cell division

The mode of reproduction of mycoplasmas is essentially not different from that of other prokaryotes dividing by binary fission. The velocity of the DNA replication fork progression in *M. capricolum* was about 6 kb/min, about ten times slower than that of *E. coli*. The time required for one round of chromosome replication was estimated to be about 94 min, and *M. capricolum* is considered among the fastest growing mollicutes (Seto and Miyata 1998). For typical binary fission to occur, cytoplasmic division should be synchronized with genome replication, but in mycoplasmas cytoplasmic division may lag behind genome replication, resulting in the formation of multinucleate filaments. The transformation of mycoplasma filaments into chains of cocci followed by

microcinematography revealed the appearance of constrictions in the cell membrane at about equal distances along the entire length of the filament, a process taking a few minutes (Bredt et al. 1973). While the outline of DNA replication in mycoplasmas is presumably similar to that of walled bacteria, the coupling of chromosome replication and cell division in mycoplasmas is not so simple. The lack of a peptidoglycan layer, which plays important roles in nucleoid partitioning and cell division, argues that the concepts developed for walled bacteria cannot be simply applied to mycoplasmas (Seto and Miyata 1999). Nucleoid movement occurs in combination with cell elongation, but the kind of force that propels and localizes the nucleoids is not known. Mycoplasma cells apparently lack a solid structure like the periseptal annulus in *E. coli*, because they do not have the peptidoglycan layer. It is possible that the fibrillar cytoskeletal structure observed in *M. pneumoniae* and *M. gallisepticum* is involved in nucleoid migration (Seto and Miyata 1999).

The recent sequencing of several mycoplasma genomes (see Genome sequencing section) has provided some information on mycoplasmal genes homologous to cell division genes of wall-covered bacteria. The most important finding, perhaps, is that of the *ftsZ* gene in mycoplasmas (Bork et al. 1995; Fraser et al. 1995; Himmelreich et al. 1996; Wang and Lutkenhaus 1996; Kukekova et al. 1999). In eubacteria the FtsZ protein is a polymer-forming, guanosine triphosphate (GTP)-hydrolyzing protein with tubulin-like elements; it is localized to the site of septation and forms a constricting ring (the Z ring) between the dividing cells. The finding of *ftsZ* in mycoplasmas indicates that it is a highly conserved and ubiquitous gene fulfilling a key role in prokaryote cell division. However, generalizations are risky as *ftsZ* could not be found in *Ureaplasma urealyticum* (Glass et al. 2000) or chlamydia (Galperin 2001). Of the additional genes associated with cell division in eubacteria (*ftsA*, *ftsH*, *ftsI*, *ftsQ*, *ftsW*, *ftsY*) *ftsH* was identified in *M. pneumoniae* (Himmelreich et al. 1996) and *M. capricolum* (Bork et al., 1995), and *ftsY* in *M. genitalium* (Fraser et al., 1995), *M. pneumoniae* (Himmelreich et al. 1996), and *U. urealyticum* (Glass et al. 2000). Applying the proteome approach, Wasinger et al. (1995) proposed the presence of a tubulin-like protein in *M. genitalium*. It has been suggested that the FtsZ protein of prokaryotes is a tubulin homolog, but the tubular structures formed by FtsZ in vitro are substantially different from microtubules, and sequence similarity between FtsZ and tubulin is barely significant (Gupta and Soltys 1996).

An integral part in cell division concerns chromosome replication and segregation into daughter cells. Chromosome replication and partitioning in mollicutes follows the basic principles of binary fission (Miyata and Seto 1999; Miyata 2002). Mycoplasmas carry topoisomerases (see DNA replication and repair section) capable of relaxing superhelical DNA, fulfilling an essential role in cell division (Bailey et al. 1996). In *Mycoplasma* species possessing a tip structure, duplication of this organelle is a prerequisite for cell division. Observation of the cell division process in *M. pneumoniae* suggests that the nascent organelle is formed next to the old one and then migrates to the opposite cell pole before binary fission of the cell takes place (Seto et al., 2001). What is the motive force propelling the migration of the tip organelle? This is certainly an intriguing question for which we have no answer as yet. One possibility is to associate it with the gliding motility of this mycoplasma. Another possibility is to associate it with the filamentous structures taking part in the cell division process (Seto et al. 2001). In conclusion, it appears that on the whole we are still rather far from a clear understanding of the factors coordinating the cell division process in mollicutes, as relevant genetic information has just started to accumulate and is still too fragmentary to be analyzed and evaluated in depth.

CULTIVATION

Several host and cultural factors are important in primary isolation and maintenance of mycoplasmas during early passage levels. These include the chemical and physical suitability of the culture medium, the number of organisms in the tested specimen, and the presence of toxic or inhibitory substances in the inoculum (Taylor-Robinson and Tully, 1998; Tully and Whitcomb 1992). The use of thallium acetate and antibiotics in culture media has made mycoplasma isolation possible in the presence of a variety of other competing organisms in the tested material. Penicillin (500–1 000 units/ml) and polymyxin B (500–1 000 units/ml) have been the drugs of choice for inhibition of either gram-positive or gram-negative prokaryotes and fungi, respectively. Mycoplasmas can often be isolated from diluted but not undiluted infected tissue extracts, suggesting that inhibitors are present in host tissues, like lysolecithin, antimycoplasmal antibodies, and antibiotics (Taylor-Robinson and Tully 1998). It is advisable, therefore, to perform serial dilutions of such extracts in fresh culture media in all primary isolation attempts. Early broth passages of newly isolated mycoplasmas are likely to require prolonged incubation periods, in comparison to the shorter time required for strains well adapted to a particular medium.

The first indication of mycoplasma growth in primary broth cultures is often a slight to moderate pH change of the medium, and in most instances, slight turbidity. Phase-contrast and dark-field microscopy may be effective in monitoring broth cultures, particularly in the case of the motile helical spiroplasmas (Tully and Whitcomb 1992). Checking the agar cultures for the typical fried-egg colonies under a stereomicroscope is the next step. Frequently, the shape of the colonies is not typical

(Taylor-Robinson and Tully 1998). Thus the motile spiroplasmas may form diffuse colonies on the fresh agar surface, lacking the agar-embedded central zone. By increasing the agar concentration, the free water film on the agar surface decreases in thickness. This impedes spiroplasma motility in the film, resulting in the formation of fried-egg colonies (Tully and Whitcomb 1992).

Culture media

A major impediment to mycoplasma research and laboratory diagnosis has been the difficulty of their in vitro cultivation. There is a consensus that only a minority of the mycoplasmas existing in nature have been cultivated so far. For example, despite many efforts for over 30 years, none of the phytoplasmas infecting insects and plants has been cultivated in vitro in an axenic culture (Seemuller et al. 2002). Some of the cultivable mycoplasmas, such as the human respiratory pathogen *M. pneumoniae*, grow very slowly (2–3 weeks incubation at 37°C), particularly on primary isolation. Others, like *U. urealyticum*, grow very fast in vitro, but growth stops abruptly at titers of 10^6–10^7 colony-forming units (cfu) per milliliter compared to 10^9–10^{10} cfu/ml in logarithmic cultures of well-growing mycoplasmas (Razin 1978). The recent mycoplasma genome sequencing projects have provided definitive genetic explanations to the above-mentioned difficulties by demonstrating the remarkable scarcity in mycoplasmas of genes involved in biosynthetic pathways (see Genome sequencing section).

To overcome the assimilative deficiencies of the mycoplasmas, complex media are used for their cultivation. The media are usually based on beef heart infusion, peptone, yeast extract, and serum with various supplements. Efforts to replace the serum component have been continuously made with the aim of reaching a defined growth medium. Serum has been shown to provide, among other nutrients, fatty acids and cholesterol, required for membrane synthesis, in an assimilable nontoxic form. Efforts to substitute the serum component by albumin, fatty acids, and cholesterol solubilized in Tween 80, or by liposomes made of phospholipids and cholesterol, supplemented with serum albumin to neutralize free fatty acid toxicity, have been continued with only limited success (Razin 1978). Nevertheless, several defined culture media were developed for a few fermentative mycoplasmas. The media contain a complex assortment of amino acids, nucleic acid precursors, lipids, vitamins, inorganic ions, together with glucose as an energy source (Hackett et al. 1987). Detailed composition of media for mycoplasmas can be found in the section on cultivation and nutrition in *Methods in mycoplasmology* (Razin and Tully 1983), in Tully and Whitcomb (1992), and in *Molecular and diagnostic procedures in mycoplasmology* (Razin and Tully 1995).

Although the numerous nutritional requirements of mollicutes dictate the need for complex growth media, the notion that the richer the medium the better, may be wrong. It appears, at least in some cases, that the lack of growth of a mycoplasma in a rich medium is not the result of the lack of a specific nutrient, but is rather due to the presence of a component(s) toxic to the mycoplasma. Some *M. hyorhinis* strains, common contaminants of cell cultures, have been known to resist cultivation on conventional mycoplasma media, leading to consider them as particularly fastidious strains. This concept proved to be wrong, as these strains could grow well in a minimal serum-free medium, leading Del Giudice (1998) to suggest that these 'noncultivable' strains are not particularly fastidious, but are more sensitive to inhibitors found in the complex media, mostly as components of peptone and yeast extract. Hence the terms 'noncultivable' and 'fastidious' should be considered as relative terms, that take their meaning only in the context of a specific culture system where growth promoters, and possibly inhibitors, are present.

Generally, mollicutes differ markedly in their atmospheric requirements; while most mollicutes are facultative anaerobes, and usually favor an anaerobic or low-redox-enhanced CO_2 atmosphere on primary isolation, the rumen *Anaeroplasma* and *Asteroleplasma* species are strict anaerobes, very sensitive to oxygen (Table 1). Some mycoplasmas such as *M. hyorhinis* require an aerobic atmosphere. The initial pH of the growth medium should be adjusted to about 8.0 for the fermentative mycoplasmas, and to 6.0–6.5 for the nonfermentative arginine-utilizing mycoplasmas and for the urea-hydrolyzing ureaplasmas. The temperature range for mycoplasma growth varies according to species from about 20–40°C. The optimum temperature for most human and warm-blooded animal mycoplasmas is 36–37°C, while the optimum growth temperature for *M. mobile*, isolated from fish, is 25°C and 30°C for the tortoise mycoplasmas (Brown et al. 2001). Likewise, the optimum temperature for growth of the sterol-requiring entomoplasmas and the sterol-nonrequiring mesoplasmas, isolated from insects and plants, is about 30°C, while the optimum growth temperature of the spiroplasmas and the acholeplasmas lies between 30 and 37°C (Tully et al. 1993).

Cocultivation with cell cultures

A novel approach to improve the chances of in vitro cultivation of fastidious mollicutes is based on coculture with eukaryotic cell lines (cell-assisted growth). In this way, uncultivable spiroplasmas such as the Colorado potato beetle spiroplasma, *S. leptinotarsa*, and the Drosophila sex-ratio spiroplasma, *S. poulsonii*, have been first successfully cocultivated in insect cell lines. Subsequently, primary cultures of these spiroplasmas could be obtained on cell-free media under conditions of low redox potential, enhanced CO_2 atmosphere, and at a pH lower than 7.0, conditions which imitate those

obtained in the insect cell cocultures (Konai et al. 1996). While the use of cocultivation of fastidious spiroplasmas with arthropod cell lines has proved itself in these cases, the choice of an adequate medium for primary isolation and/or subsequent sustained cultivation of these organisms is still much more an art than a science.

Cell-assisted cocultivation, using Vero cell cultures, enabled the cultivation of several strains of the highly fastidious human *M. genitalium* from clinical specimens (Jensen el al. 1996). Also in this case, the mycoplasmas grown in the cell culture (as was indicated by polymerase chain reaction (PCR) monitoring) could be subsequently subcultured in a cell-free medium. The above methodology, as complex as it is, requiring 'heroic' efforts, is certainly inadequate for routine cultivation of fastidious mycoplasmas, leaving the door open for the application of molecular techniques, such as PCR, for detection and identification of fastidious, or the so far uncultivable mycoplasmas (see Diagnosis of infections section).

HABITATS AND ECOLOGY

Mycoplasmas are widespread in nature as parasites of humans (Blanchard and Bebear 2002), mammals (Frey 2002), reptiles (Brown et al. 2001), fish, arthropods, and plants (Seemuller et al. 2002; Tully 1996). The list of hosts known to harbor mycoplasmas is continuously increasing, as does the number of established mollicute species, about 200 so far (Table 1). It is widely agreed that the mollicutes that have already been characterized and taxonomically defined constitute only a part, apparently a minor one, of the mollicutes living in nature (Razin 1992). The higher the number of established mollicute species, the harder it gets to fulfill the minimal requirements for establishing new species (International Committee 1995). A large battery of species-specific antisera and seed are needed for this task. The introduction of molecular tools to taxonomy, including the comparison of 16S rRNA and other conserved gene sequences, genomic restriction patterns, etc. (see Phylogeny and taxonomy and Diagnosis of infections sections) has already been found to be effective in species and strain identification, decreasing the weight of serology in species and strain identification and classification.

Uncultured mollicutes

The successful application of genomic analysis methodology has led to the identification of the uncultured plant and insect MLOs as bona fide mycoplasmas (Phylogeny and taxonomy section), showing the way for confirming the identity of other mycoplasma-like organisms as mollicutes. Thus, the Grey Lung 'virus' disease of mice, caused by an agent resembling mycoplasmas in thin sections of lung material, but resisting cultivation, has been identified as a mycoplasma based on its genome size and 16S rRNA sequence analysis. It has been proposed to classify this organism as '*Candidatus Mycoplasma ravipulmonis*', the '*Candidatus*' taxon being reserved to classification of uncultured prokaryotes (Neimark et al. 1998). Most important is the recent finding, based on 16S rRNA sequence analysis, that *Haemobartonella* and *Eperythrozoon* species, long known as uncultured wall-less pathogenic bacteria, infecting erythrocytes of a wide range of vertebrate hosts, are phylogenetically closely related to Mollicutes rather than to rickettsia, and should, therefore, be reclassified as *Mycoplasma* species (Neimark and Kocan 1997; Rikihisa et al. 1997, see Phylogeny and taxonomy section). The inclusion of these hemotropic agents in Mollicutes represents an entirely new group of pathogens classified as mycoplasmas.

Host and tissue specificity

Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their nutritionally exacting nature and obligate parasitic mode of life. However, there are numerous examples of the presence of mycoplasmas in hosts and tissues different from their normal habitats (Tully 1996). The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, eyes, alimentary canal, mammary glands, and joints. The obligatory anaerobic anaeroplasmas have so far been found in the bovine and ovine rumen only (Table 1). Spiroplasmas and phytoplasmas are widespread in the gut, hemocoel, and salivary glands of arthropods. The spiroplasmas and phytoplasmas may be introduced through sap-sucking insects to the phloem tissues of plants, causing disease (Tully and Whitcomb 1992; Seemuller et al. 2002). Accompanying the increasing numbers of patients suffering from various types of immunodeficiencies, associated with hypogammaglobulinemia, AIDS, treatment with immunosuppressive medication in patients undergoing organ transplantation, more and more reports appear on the isolation of mycoplasmas from organs different from their usual habitats. Thus, mycoplasmas and ureaplasmas belonging to the normal urogenital flora have been isolated from the blood of patients suffering from AIDS or treated with immunosuppressive drugs. Hypogammaglobulinemic and immunocompromised patients become susceptible to infections by the urogenital mycoplasmas *M. hominis* and *U. urealyticum*, spreading into organs such as the respiratory tract and joints (Frangogiannis and Cate 1998; Gass et al. 1996) causing disease in these organs.

Mycoplasmas infecting cell cultures

Cell cultures infected by mycoplasmas constitute an artificial habitat. The serious problems created by the

persistent and hard to detect infections of cell cultures are reflected in the voluminous literature on this subject. Reports from various countries show that 10–87 percent of cell cultures are infected by mycoplasmas (Barile and Rottem 1993; Uphoff and Drexler 2002). The percentage of infected cell cultures depends to a large extent on the population of cell cultures assayed, on the control practices used, and on the efficiency of the assay procedures applied. The *Mycoplasma* species infecting cell cultures have remained essentially the same over the years. *M. hyorhinis*, *M. orale*, *M. arginini*, and *A. laidlawii* being the dominant contaminants. The increasing percentage of cell cultures infected by *M. fermentans*, a mycoplasma incriminated as a cofactor in AIDS and rheumatoid arthritis (see Pathogenicity section), is of particular interest regarding the possible origin of contamination. Elimination of mycoplasmas from infected cell cultures by antimycoplasmal agents is time consuming and is often unsuccessful (Uphoff et al. 2002) probably due to the intracellular location of a small percentage of the contaminating mycoplasmas.

Human and animal mycoplasmas

Table 2 lists the mycoplasmas found in humans. Some of these mycoplasmas may cause disease while others are commensals, constituting part of the normal flora of the oropharynx and the urogenital tract. Some of the human mycoplasmas shown in Table 2 can also be isolated from nonhuman primates. Farm animals, cattle, sheep, goats, horses, swine, chickens, and turkeys harbor a great variety of mollicutes, many of them pathogenic. Mollicutes can also be found in dogs, cats, and small laboratory animals, either as commensals or pathogens. A mollicute flora has been defined in a wide variety of wild animals, including elephants, lions, seals, crocodiles, turtles, and prawns. In essence, mollicutes may be ubiquitous in the animal world. It appears as though the main factor for adding an animal to the long list of animals harboring mycoplasmas is the willingness of a mycoplasmologist to invest the effort required to isolate and characterize mycoplasmas from the animal. Interestingly, the laboratory rabbit represents an exception to this rule, as it appears to be free of mycoplasmas. Lack of a natural mycoplasma flora makes the rabbit a preferred animal for production of specific antisera to mycoplasmas. For detailed descriptions of the mollicute flora of humans and animals see the relevant chapters in *Molecular biology and pathogenicity of mycoplasmas* (Razin and Herrmann 2002).

Mycoplasmas in arthropods and plants

Arthropods are major habitats of mollicutes, including the helical spiroplasmas, sterol-nonrequiring mesoplasmas, sterol-requiring entomoplasmas, and the uncultured phytoplasmas (Table 1). All spiroplasmas are obligately associated with insects, either as commensals, pathogens or mutualists, and may constitute one of the largest microbial genera as a result of their diversification and coevolution with their arthropod hosts (Gasparich 2002).

Spiroplasmas are most frequently found in the insect gut, less frequently in the hemolymph, and occasionally in various organs such as the salivary glands. Spiroplasmas in the insect gut are generally acquired by natural feeding, either from plant tissue, from nectar, or from ingestion of other insects. Some spiroplasmas, like the honeybee spiroplasmas, are pathogenic and may kill their insect hosts, or eliminate the male progeny of infected Drosophila (Williamson et al. 1999), while others are considered commensals, even when present in large numbers in the hemolymph. Spiroplasmas involved in vector-transmitted plant diseases (citrus stubborn, corn stunt) must pass through a complex biological cycle that involves uptake from the sieve cells of the plant phloem and subsequent passage to, or multiplication in, the insect alimentary tract, gut epithelium, basement membrane, hemocoel, and possibly some internal organs. The spiroplasmas apparently enter into insect cells by receptor-mediated endocytosis. In this process the spiroplasmas lose their helicity, and appear as rounded, pleomorphic bodies with a reticulated internal

Table 2 *Site of colonization and pathogenicity of human mollicutes*

Species	Primary site of colonization	Pathogenicity
M. pneumoniae	Lung, oropharynx	Primary atypical pneumonia
M. genitalium	Urogenital tract	Nongonococcal urethritis
M. hominis	Urogenital tract	Usually commensal, may cause infections on reaching internal organs (salpingitis)
M. fermentans	Urogenital tract, respiratory tract	Cofactor in AIDS activation? Arthritis?
M. penetrans	Urogenital tract? Colon?	Cofactor in AIDS activation?
M. salivarium	Buccal cavity	Commensal. Periodontal disease?
M. orale	Buccal cavity	Commensal
Ureaplasma urealyticum	Urogenital tract, placenta	Nongonococcal urethritis, neonatal lung and brain infections

Commensal mycoplasmas less commonly isolated from humans: *M. buccale*, *M. faucium*, and *M. lipophilum* from the oropharynx; *M. primatum* and *M. spermatophilum* from the genitals, and *M. pirum* from blood (see Blanchard and Bebear 2002).

structure, most frequently found in membrane-bound pockets in the insect cell (Kwon et al. 1999). The organisms eventually pass from the hemocoel into the ciliary cells and salivary duct, from which reinoculation of healthy plants takes place. Induction of disease in the plant is the rule. The symptoms in plants include chlorosis, leaf mottling, proliferation of growing points, and general stunting (Tully and Whitcomb 1992).

A rather intriguing issue concerns the possible occurrence and pathogenicity of spiroplasmas in animals. Only one spiroplasma, *S. mirum* strain SMCA has been shown to experimentally infect suckling mice causing cataracts (Gasparich 2002). Claims associating spiroplasmas with transmissible human spongiform encephalopathies have been repeatedly made, the most recent claim being based on positive PCR tests for *S. mirum* 16S rDNA in human brain samples (Bastian and Foster 2001). Spiroplasma-like structures could also be demonstrated in a cataract in a premature baby, a finding supported by positive PCR with the 16S rDNA of *Spiroplasma* group VI (Lorenz et al. 2002). The above findings may be considered at most suggestive, but appear worthy of further study.

The nonhelical mollicutes of the genera *Entomoplasma* and *Mesoplasma* have been found as inhabitants of the insect gut (fireflies, beetles, horseflies, moths, butterflies, and bees). However, some entomoplasmas and mesoplasmas have also been identified on plant surfaces, so that most species in either group can be acquired from various plant surfaces or flowers through insect visitation and feeding excursions (Tully 1996). The largest group of mollicutes inhabiting plants are the uncultured plant-pathogenic phytoplasmas. These mollicutes are transmitted primarily by phloem-feeding leafhoppers and are the causative agents of more than 300 different plant 'yellows' diseases and the list continues to grow. Plants infected by phytoplasmas exhibit an array of symptoms that suggest profound disturbances in the normal balance of plant hormones or growth regulators. Symptoms include virescence (green flowers), phyllody (floral parts develop into leafy structures), sterility of flowers, proliferation of auxilliary shoots (witches broom appearance), abnormal elongation of internodes resulting in slender shoots, generalized stunting, discoloration of leaves and yellowing (Lee et al. 2000; Seemuller et al. 2002).

PHYLOGENY AND TAXONOMY

Phylogeny

The early studies of Woese et al. comparing 16S rRNA sequences of mollicutes have provided the basis for mycoplasma phylogeny. Accordingly, the mycoplasmas arose at a node on the branch of gram-positive bacteria having a low G + C genome. This branch of the *Lactobacillus* group contains *Lactobacillus*, *Bacillus*, and *Streptococcus* species. The closest to mycoplasmas on this branch is a small subgroup of clostridia represented by *Clostridium innocuum* and *C. ramosum* (Figure 8).

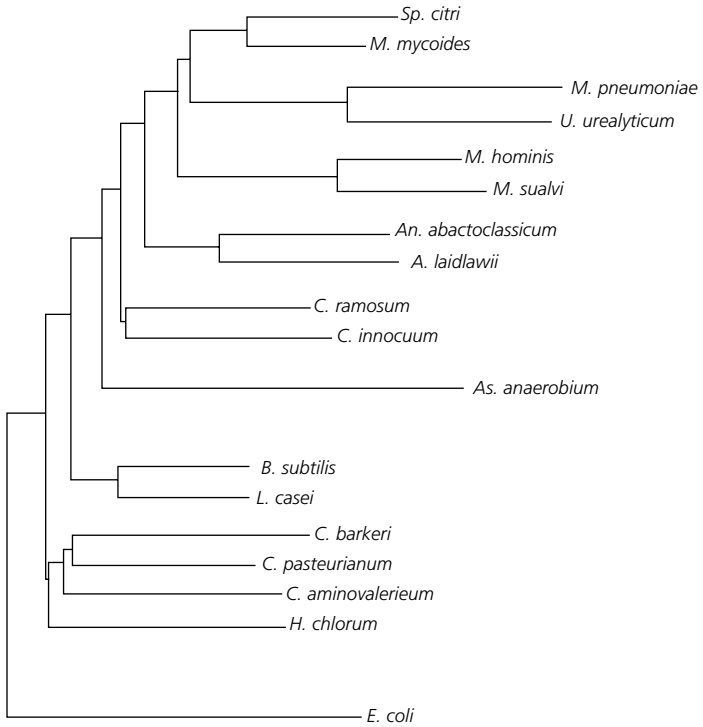

Figure 8 *Schematic dendrogram presentation of the phylogenetic relationship of Mollicutes to some related eubacteria based on 16S rRNA sequences.*

Mycoplasma phylogeny from this node took place by degenerate or reductive evolution, characterized by a rapid tempo, in line with the marked genotypic and phenotypic variability characterizing the mollicutes as a group (Woese 1987). Phylogenetic trees of Mollicutes are still based mostly on 16S rRNA sequences, but several other conserved gene sequences, such as *tuf* genes have been used for this purpose (Kamla et al. 1996). According to 16S rRNA sequences, the Mollicutes are divided into five phylogenetic units (clades): the asteroleplasma, spiroplasma, pneumoniae, hominis, and anaeroplasma clades (Johansson and Pettersson 2002; Maniloff 2002; Weisburg et al. 1989).

A scheme of mycoplasma evolution has been proposed by Maniloff (2002). Accordingly, the ancestral mycoplasma arose from the *Streptococcus* phylogenetic branch about 600 million years ago, having probably a genome of about 2 000 kb. The mycoplasma branch split later into two major branches: one branch led to the *Asteroleplasma*, *Anaeroplasma*, and *Acholeplasma* branches, and the other led to the *Spiroplasma*, *Entomoplasma*, and *Mycoplasma* branches. The phytoplasmas subsequently arose from the *Acholeplasma* branch, and *Ureaplasma* arose from the *Mycoplasma* branch. In both major branches genome reductions had occurred independently during their degenerate evolution. Thus, degenerate evolution of the *Acholeplasma-Anaeroplasma* branch, taking place after the appearance of flowering plants, has led to the phytoplasmas with 600- to 1 200-kb genomes, while evolution of the second major branch produced the *Spiroplasma* branch with 1 000- to 2 000-kb genomes, and the *Entomoplasma*, *Mesoplasma*, *Mycoplasma*, and *Ureaplasma* branches with 600- to 1 200-kb genomes. Clearly, the time at which transition to rapid evolution occurred correlate with major paleontological events in the evolution of the flora and fauna that formed ecosystems for lineages of each branch. Thus, the *Spiroplasma* and *Entomoplasma* transition to rapid evolution occurred about 100 million years ago soon after the appearance of the first flowering plants and their associated insects. The increase in potential hosts presumably provided niches for the selection of new lineages. Although all the above should be taken as hypothetical assessments, they emphasize a cardinal point: the mollicutes are relatively late evolutionary products.

Taxonomy

There is a consensus among bacterial taxonomists that the complete sequences of bacterial genomes will form the basis for phylogeny and, ultimately, taxonomy. Yet, as long as complete genomic sequences are available for a relatively few bacteria only, current bacterial taxonomy, including that of mollicutes (International Committee 1995), relies on the combination of phenotypic characteristics and phylogenetic data based on partial genomic sequences, mostly those of the conserved ribosomal RNA genes. Detailed descriptions and evaluations of current molecular methods in taxonomy, phylogeny, and diagnostics of mollicutes can be found in Razin (1992), in the two volumes of *Molecular and diagnostic procedures in mycoplasmology* (Razin and Tully 1995; Tully and Razin 1996) and in *Mycoplasma protocols* (Miles and Nicholas 1998).

While the availability of complete genomic sequences is expected to provide a sound basis for establishing phylogenetic relatedness among bacterial species and consequently enable the construction of taxonomic entities based on phylogeny, the way to achieve this has not been worked out as yet, as it presents several key problems. Thus, the current somewhat arbitrary and artificial definition of the basic taxonomic entity, that of a bacterial species, includes all strains with approximately 70 percent or higher DNA homology and with 5°C or lower ΔTm (Razin 1992) and over 97 percent sequence similarity of 16S rDNA (Stackebrandt and Schumann 2000). There can be little doubt that this definition will have to be modified once comparison of entire genomic sequences replaces the cumbersome and not too accurate determination of genetic homology based on DNA–DNA hybridization tests. Yet, as long as the number of bacterial genomes sequenced remains small, there is still more than enough time to work out a solution to the above problem. Comparison of the genomic sequences of *M. genitalium* and *M. pneumoniae* (Himmelreich et al. 1997; Herrmann and Reiner 1998) has revealed the significant genomic relatedness of these mycoplasmas, but also underlines the problems taxonomists may face when using comparative genomic data to redefine taxonomic entities, including that of a bacterial species.

Conserved genes as molecular markers

The great weight given to 16S rDNA sequences in mycoplasma phylogeny, taxonomy, and species identification (Razin 1992) has led the International Committee on the Taxonomy of Mollicutes (1997) to recommend the inclusion of the 16S rDNA sequence in any description of a new mollicute species. Johansson has been compiling a database of 16S rDNA sequences of the recognized *Mycoplasma* species deposited at GenBank (Johansson and Pettersson 2002).

A most important contribution of 16S rRNA gene sequences to mollicute phylogeny and taxonomy has been the placing of the uncultured phytoplasmas as a distinct monophyletic clade, within Mollicutes, closely related to the acholeplasmas (Lee et al. 2000; Seemuller et al. 2002). This conclusion has been supported by the small genome size of phytoplasmas, resembling that of the culturable mollicutes (Marcone et al. 1999), by the finding of only two rRNA operons in the phytoplasmas, and a $tRNA^{Ile}$ gene in the spacer region between the 16S

and 23S rRNA genes (Ho et al. 2001; Seemuller et al. 2002), a feature found also in acholeplasmas. The deduced amino acid sequences of some of the highly conserved ribosomal protein genes also indicated that the phytoplasmas are more closely related to *Acholeplasma* than to *Mycoplasma* species, and like acholeplasmas they read UGA as a stop codon rather than a tryptophan codon. Based on comparison of 16S rRNA gene sequences and on sequences of the 16S/23S intergenic spacer regions the phytoplasma clade was divided into 20 subclades (Seemuller et al. 2002). The taxonomic implications of these studies are that the phytoplasmas should be distinguished at the minimal taxonomic level of a genus, and each phytoplasma subclade should represent at least one distinct species (International Committee 1997). However, in the absence of the phenotypic markers used to classify mollicutes, taxonomic affiliations cannot be resolved at the present time in the conventional way. Thus, a provisional classification of the uncultured phytoplasmas has been introduced by applying the *Candidatus* taxonomic category (International Committee 1997). So far seven *Candidatus* species names for phytoplasmas have been published (Table 1; Seemuller et al. 2002).
The phytoplasma case demonstrates well the revolution occurring in bacterial taxonomy, where molecular data suffice for laying the phylogenetic and taxonomic basis for classification of a group of organisms for which very few phenotypic characteristics are available.

The molecular approach to phytoplasma classification has recently also been applied to the uncultured hemoplasmas (Neimark et al. 2001; see Habitats and ecology) comprising of the *Haemobartonella* and the *Eperythrozoon* species. These bacteria have been classified until recently as rickettsiae (order Rickettsiales) due to their small size, staining properties, obligate parasitism, and hemotrophic character. Several of them are transmitted by blood feeding arthropods. However, phylogenetic analyses of 16S rRNA gene sequences have revealed that *Haemobartonella* and *Eperythrozoon* species are not rickettsiae, but are rather mycoplasmas (Neimark and Kocan 1997; Rikihisa et al. 1997) with the highest similarity scores with members of the pneumoniae clade, closest to *M. fastidiosum* and *M. cavipharyngis* (Neimark et al. 2002; Johansson et al. 1999). Since the hemoplasmas have not been cultured as yet, naming of new species should be included in the *Candidatus* category (Neimark et al. 2001, 2002).

The morphology of the hemoplasmas is of small coccoid organisms attached to erythrocytes, while a few may appear free in the erythrocyte cytoplasm. The individual coccoid cells can aggregate to give the appearance of short chains and small clusters of rod-shaped organisms. Electron microscopy shows the coccoid organisms to be about 0.3 μm in diameter with no cell wall. This morphology corresponds well with that of mycoplasmas (Neimark et al. 2002; Messick et al. 2002). Although some *Haemobartonella* and *Eperythrozoon* species can cause overt disease in healthy immunocompetent hosts, the majority of these species produce chronic asymptomatic infections. Clinical disease usually includes hemolytic anemia which varies from mild to severe.

Although the 16S rRNA sequences have proved to be very effective tools in the phylogeny and taxonomy of mollicutes, it has been felt that additional phylogenetic markers are desirable to support the conclusions based on the 16S rRNA data. Generally, 'housekeeping' or constitutive genes, which are expressed in all living organisms because they provide essential functions, such as replication, transcription, and translation, are good candidates for genetic differentiation of species. In fact, such markers have already been applied, including the conserved ribosomal protein genes, the elongation factor EF-Tu (*tuf*) gene, the heat shock protein gene *hsp70*, the *ftsZ* gene, the DNA repair *uvrC* gene, the ribonuclease P (*rnpB*) gene, and the 16S/23SrRNA intergenic sequences (Birkenheuer et al. 2002; Harasawa et al., 2000; Johansson and Pettersson 2002, Razin et al. 1998). Use of these markers has supplemented and complemented the 16S rRNA comparative data. A priori, wobble in the genetic code permits more variations in protein gene sequences, even of highly conserved genes, than is possible in rRNA sequences. Thus, while the ribosomal protein-encoding genes are conserved, they vary in size and primary sequence more than the 16S rRNA genes. Therefore, the ribosomal protein genes have greater potential to reveal variations among closely related strains. Likewise, the intergenic spacer region located between the 16S and 23S rRNA genes, being less subjected to evolutionary constraints than the genes themselves, exhibits greater sequence variation, a property of use in strain differentiation (Harasawa et al. 2000).

Intraspecies genetic heterogeneity

The rather arbitrary and somewhat vague definition of a bacterial species, and even more so the rather frequent and significant chromosomal rearrangements taking place in mycoplasmas (Chromosomal rearrangements section), as well as the rather frequent integration of extrachromosomal elements in the mycoplasmal genome (see Extrachromosomal elements and Insertion-like elements sections) are conducive to the most common finding of genotypic heterogeneity of strains within the same species. Intraspecies genotypic heterogeneity, noticeable through serological testing and electrophoretic analysis of cell proteins, has become much easier to distinguish by applying the much more sensitive molecular tools, such as restriction fragment length polymorphism (RFLP), restriction-modification profiles, conserved gene sequences, and sequence analysis of species-specific protein genes (see Diagnosis of infections section). These tests have provided epidemiolo-

gists with very sensitive tools distinguishing strains of interest in the clinic and in the field. Based on such molecular tools, the clinically important human *Ureaplasma urealyticum*, long known to consist of two clusters of serovars, has recently been divided into two species, *U. urealyticum* and *U. parvum* (Robertson et al. 2002). Another example of clinical importance concerns the difference between the European and African/Australian strains of *M. mycoides* subsp. *mycoides* SC. The African/Australian strains are significantly more virulent than the European strains and cause more serious cases of contagious bovine pleuropneumonia. Recent studies indicate that the chromosome of the European strains lacks an 8.84 kb segment found in the African strains that includes two ORFs of putative ATP-binding cassette (ABC) transporters and several genes encoding membrane proteins and an insertion element. This chromosomal deletion is associated with a deficiency in glycerol uptake and metabolism in the European strains, possibly explaining their lower pathogenicity (Vilei et al. 2000; see Pathogenicity section).

Metabolic and phenotypic markers

The present tendency to depend on direct genomic analysis, made available by the dramatic advancements of molecular genetic methodology, has pushed aside further development of classical taxonomic tools based on determination of nutritional requirements and enzymatic activities (Razin 1992). Even electrophoretic cell protein profiles are now used less extensively as taxonomic tools than in the 1970s and in the early 1980s. The reason being that these profiles reflect the expression of specific genes and are thus liable to changes. It is now possible to clone and sequence the protein genes themselves and thus have a more direct comparative measure. Yet, the recent development of the 'proteome' approach to genomic analysis may revive the use of 2D-electrophoretic patterns of cell proteins as a taxonomic and phylogenetic tool (Weiner et al. 2002).

It should also be pointed out that distinguishing members of the class Mollicutes by their metabolic characteristics has been generally of limited phylogenetic and taxonomic usefulness. There are a few exceptions, including the ability to ferment glucose, hydrolyze arginine and urea, and dependence on cholesterol for growth and anaerobiosis (Razin 1978). Cellular localization of NADH oxidase activity has also been of help in classification of mollicutes at and above the genus level. While *Acholeplasma* species resemble other prokaryotes in having the NADH oxidase localized in the plasma membrane, this enzymatic activity is located in the cytoplasm of *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species. The finding of this activity in the cytoplasm of the *Mesoplasma* and *Entomoplasma* species has helped to distinguish these newly described genera from *Acholeplasma* species (Pollack et al. 1996; Tully et al. 1993). Extensive studies carried out in Pollack's laboratory (reviewed in Pollack 2002b) were aimed at finding the mycoplasmal enzymatic activities useful for the differentiation of the mollicutes genera. Several activities endowed with such a potential were defined, but so far have not been put into general use.

SEROLOGY

At the species level, serologic relatedness has until recently overshadowed all other features used in routine mollicute identification, but the weight given to determination of molecular properties in mollicute classification and identification is steadily increasing (Razin 1992). The high rate of surface antigenic variation characterizing mollicutes (see Antigenic variation) may impose some limitations on the use of monoclonal antibodies to surface antigens as tools in mycoplasma identification (Rosengarten and Yogev 1996), difficulties usually not encountered when polyclonal antibodies are used (International Committee 1997). In order to limit the number of serological tests and, more so, the large number of specific antisera currently required for the definition of new mollicute species, the International Committee on the Taxonomy of Mollicutes (2001) has agreed that based on 16S rRNA sequences it should be possible to place the new isolate in the proper phylogenetic clade and restrict serological cross-testing to members of this clade only.

CHOLESTEROL REQUIREMENT

The weight given to cholesterol requirement in mollicute taxonomy illustrates well the conflict that may occur between taxonomy based on phenotypic characteristics and that based on molecular phylogenetic data. Cholesterol requirement has been considered for a long time as a major criterion in establishing high taxonomic groupings within the Mollicutes. Recent findings weaken the high status of cholesterol requirement in mollicute classification. Thus, the cholesterol-nonrequiring mesoplasmas, considered previously as acholeplasmas, were shown to possess molecular properties very different from those of the classic acholeplasmas, requiring their taxonomic separation (Pollack et al. 1996; Tully et al. 1993). In addition, several *Spiroplasma* species were recently shown to grow in the absence of cholesterol (Tully et al. 1993). These data suggest that nutritional dependency on exogenous sterols is a trait that has arisen independently several times during the evolution of mollicutes, as it occurs in at least three different phylogenetic groups. It thus appears that cholesterol requirement should not be used as a sole definitive criterion in defining higher taxa of the class Mollicutes, but rather serve as one of a matrix of characters in mollicute classification. Consequently, nutritional requirements, though being the most practical traits to

be assessed in the routine laboratory, may not always be reliable indicators of phylogenetic relationships.

Blending taxonomy with phylogeny

While it is generally agreed that basing bacterial taxonomy on phylogeny is both inevitable and advantageous, there is still disagreement among bacterial taxonomists whether the time is ripe for radical changes in bacterial taxonomy (for detailed discussion see Razin 1992). The taxonomic status of the *Mycoplasma mycoides* cluster illustrates the problem well. The Mollicutes phylogenetic tree based on 16S rDNA sequences has suggested that the *M. mycoides* cluster is phylogenetically related to the genus *Spiroplasma* (Weisburg et al. 1989). However, the proposal to reclassify this cluster accordingly has not been adopted by the International Committee (1997) arguing that such a reclassification would create considerable problems in diagnostic veterinary medicine. Likewise, reassignment of all other *Mycoplasma* species to a new genus would have unacceptable consequences in human and veterinary medicine. For the time being a polyphasic classification based only partly on phylogeny is recommended, striving to fit in as much as possible mollicute taxa into phylogenetic classification.

PHYSIOLOGY

Cell membrane proteins

Lacking a cell wall and intracytoplasmic membranes, the mollicutes have only a single type of membrane, the plasma membrane. The ease with which this membrane can be isolated and the ability to introduce controlled alterations in its composition have made mycoplasma membranes effective tools in membrane research (Razin 1978, 1993).

Proteins constitute over two thirds of the mycoplasma membrane mass; the rest being membrane lipids. The structure and function of mycoplasmal membrane proteins have recently been reviewed by Wieslander and Rosen (2002). Membrane lipoproteins have attracted much attention in recent years as their relative abundance in mycoplasma membranes is most remarkable in contrast to the limited number of lipoproteins in membranes of other eubacteria. Based on characteristic lipoprotein-specific features, Himmelreich et al. (1996) annotated 46 putative lipoprotein genes in the *M. pneumoniae* genome, while 21 lipoprotein genes have been annotated in the *M. genitalium* genome (Fraser et al. 1995; Himmelreich et al. 1997). The 25 additional lipoprotein genes in *M. pneumoniae* are only gene amplifications, which are frequently organized in sequential order (Herrmann and Reiner 1998). The *M. pulmonis* genome encodes 56 lipoproteins (Chambaud et al. 2001) while the *U. urealyticum (parvum)* genome appears to be the richest, encoding 74 lipoproteins (Glass et al. 2000). The unusually high number of lipoproteins in mollicutes may be attributed to the absence of a cell wall and a periplasmic space in these organisms. Mollicutes possess typical eubacterial signal peptides that direct the newly synthesized proteins into a secretory pathway for transport across the cell membrane (Yogev et al. 1991b). Still, the mollicute lipoprotein signal peptides are longer than for any other bacteria, a feature possibly associated with their thicker cholesterol-containing cell membrane, requiring longer transmembrane segments (Edman et al. 1999).

For surface-exposed membrane proteins which have to function on the cell outside, acylation of the proteins by long chain fatty acids is an effective way of anchoring the proteins to the cell surface. Cultivation of mollicutes in the presence of labeled palmitate or myristate labels a significant number of membrane proteins. Interestingly, membrane lipoproteins are amongst the most dominant antigens in mollicutes (Razin et al. 1998; Wieslander and Rosen 2002), and a majority of the mycoplasma cell surface antigens known to undergo antigenic variation are lipoproteins (see Antigenic variation section). At least some mycoplasmal membrane lipoproteins, characterized in *M. pneumoniae*, *M. genitalium*, *M. fermentans*, and *M. hyorhinis*, carry only two fatty acid acyl chains linked to the N-terminal cysteine residue via a thioester linkage between diacylglycerol and cysteine, lacking the third fatty acid linked to the amino group of cysteine, normally found in lipoproteins of other eubacteria. In fact, the transacylase responsible for acylation of the amino group of cysteine could not be identified in the *M. pneumoniae* and *M. genitalium* genomic analysis (Calcutt et al. 1999b; Le Henaff et al., 2001; Himmelreich et al., 1996; Himmelreich et al. 1997; Razin et al. 1998). Yet, chemical analysis of spiralin, the major lipoprotein of *S. melliferum*, showed that in addition to the two ester-linked acyl chains, a third acyl chain is amide linked to the cysteine, resembling the classical eubacterial lipoproteins (Le Henaff and Fontenelle 2000; Le Henaff et al. 2000). Similar findings were reported for the acylated lipoproteins of *M. agalactiae* (Le Henaff et al. 2000).

Molecular genetic methodology has recently enabled the preliminary characterization of membrane proteins of the uncultured phytoplasmas. The phytoplasma membrane proteins characterized thus far are apparently not lipoproteins. They appear to possess cleavable N-terminal signal sequences and an hydrophobic C-terminal anchor. The central hydrophilic domain is located on the outside of the cell membrane (Blomquist et al. 2001; Barbara et al. 2002).

Membrane lipids

Virtually all mycoplasma lipids are located in the cell membrane and, as in other biological membranes, consist

of phospholipids, glycolipids, and neutral lipids. A detailed description of the composition, distribution, and biosynthesis of mycoplasmal membrane lipids can be found in Rottem and Kahane (1993). As mentioned in the Genome sequencing section, the mycoplasmas are partially or totally incapable of fatty acid synthesis, and depend on the host, or the culture medium, for their supply. In addition, most mycoplasmas require cholesterol for growth, a unique requirement among prokaryotes (Rottem 2002b). The fatty acid residues of membrane phospholipids and glycolipids, as well as cholesterol, constitute a major portion of the hydrophobic core of the membrane. The dependence of mycoplasmas on the exogenous supply of fatty acids and cholesterol has been one of their greatest advantages as models for membrane studies. The ability to introduce controlled alterations in mycoplasma membrane lipid composition, simply by controlling the composition and content of fatty acids and sterols in the growth medium, has been used most effectively in elucidating the molecular organization and function of the lipids in mycoplasma membranes (Razin 1993; Wieslander and Rosen 2002).

Almost all recent studies concerning mycoplasma lipid biosynthesis and its regulation were carried out on *A. laidlawii*, an organism which has been used for a long time as a model organism, particularly in studies dealing with the physical state of the membrane (Razin 1978, 1993). It was the organism used by Wieslander, Rilfors, Lindblom and their associates at Umea University to show that altering the polar headgroup structure of membrane phospho- and glycolipids, and changing the acyl chain structure, are the two strategies employed by the organisms to adapt their membrane lipid composition to various environmental and physiological conditions (reviewed by Wieslander and Rosen 2002). A common way in prokaryotes to respond to varying growth temperatures is to regulate the degree of unsaturation of the acyl chains of the lipids. *A. laidlawii* cannot synthesize unsaturated fatty acids, and saturated fatty acids are synthesized by it in very limited amounts, when the organism is grown in a thoroughly lipid-depleted medium. Thus, at least one exogenous fatty acid must be supplied in the growth medium in order for the cells to grow.

A. laidlawii exploits a different mechanism to cope with changes in environmental conditions, namely, by altering the proportion of lipids with different polar headgroups. It has been hypothesized and experimentally supported by the Umea group that *A. laidlawii* strain A-EF22 regulates its membrane lipid composition to maintain a proper balance between lipids forming a lamellar crystalline phase, and lipids forming reversed nonlamellar phases. The first acholeplasmal lipid shown to form a nonlamellar phase was monoglucosyl diacylglycerol (MGlcDAG). The other major glycolipid, diglucosyl diacylglycerol (DGlcDAG) resembles the acholeplasmal phospholipids in solely forming the lamellar phase at all temperatures and with all acyl chain compositions. It was then found that the acholeplasma is capable of varying the proportion of the MGlcDAG and DGlcDAG in response to the prevailing growth conditions (Wieslander and Rosen 2002).

In conclusion, the extensive studies of the Umea laboratory show that several basic features of the membrane homeostasis mechanism in *A. laidlawii*, including the maintenance of phase equilibria, spontaneous curvature, and surface charge density of the membrane lipid bilayer, are dependent upon the physical properties of the bilayer and are sensed by the two consecutively acting glucosyltransferases synthesizing the major lipids MGlcDAG and DGlcDAG. While the fine details of the molecular mechanisms regulating the synthesis of the two glucolipids have to be worked out, the experiments with the purified MGlcDAG synthase suggest that enzyme regulation may depend on the enzyme proteins themselves, not requiring a complex multiprotein regulatory mechanism (Berg et al. 2001; Wieslander and Rosen 2002).

Energy-yielding pathways

The small genome size of mollicutes precludes their possession of an extensive range of metabolic activities present in other bacterial groups. Demonstrated metabolic activities appear primarily to be associated with energy generation, rather than with the provision of substrates for synthetic pathways. All the mollicutes examined so far have truncated respiratory systems. They lack a complete tricarboxylic acid cycle and have no quinones and cytochromes, ruling out oxidative phosphorylation as an ATP-generating mechanism (Pollack 2002b). Thus, the energy-yielding pathways of mollicutes produce low ATP yields and relatively large quantities of metabolic end-products, depleting host tissues of the specific substrate metabolized in some cases.

Based on their ability to metabolize carbohydrates, the mollicutes are divided into fermentative and nonfermentative organisms. Members of the fermentative group produce acids from carbohydrates, decreasing the pH of the growth medium. While fermentative mycoplasmas may differ in their ability to utilize sugars other than glucose, this property has only a limited taxonomic value. Interestingly, *M. fermentans* isolates from the urogenital tract of AIDS patients were found to use fructose in preference to glucose (Ozcan and Miles 1999). In man, high fructose concentrations relative to glucose are associated only with seminal vesicle secretions. Thus, fructose-utilizing *M. fermentans* strains may be particularly associated with seminal vesicles and their secretions, and be transmitted in semen.

The sequencing projects of the *M. genitalium* and the *M. pneumoniae* genomes (see Genome sequencing section) showed these mycoplasmas to carry all the

enzymes of the Embden–Meyerhof–Parnas pathway, while the second pathway for metabolizing glucose, the pentose phosphate pathway is incomplete. Pyruvate generated by glycolysis can be further metabolized either to lactate by lactate dehydrogenase, or to acetyl-CoA by the pyruvate dehydrogenase pathway. Most of the nonfermentative mollicutes, and some fermentative species as well, possess the arginine dihydrolase pathway. Arginine hydrolysis by this pathway results in the production of ornithine, ATP, CO_2, and ammonia, raising the pH of the culture medium (Razin 1978). The pathway consists of three enzymes: arginine deiminase, ornithine carbamoyl transferase, and carbamate kinase. The degradation of arginine is coupled to equimolar generation of ATP by substrate level phosphorylation. The possible role of this pathway as a sole energy generating source in nonfermentative mollicutes has been frequently questioned (Himmelreich et al. 1996, 1997; Razin 1978). The demonstration of an arginine-ornithine antiport system in *Spiroplasma melliferum* requiring no ATP for arginine import into the cells, supports an energetic advantage in arginine utilization (Shirazi et al. 1995), but the question of whether arginine degradation can serve as a sole, or even a major, energy-generating mechanism remains unanswered.

The arginine dihydrolase pathway can also be found in some fermentative *Spiroplasma* and *Mycoplasma* species (Appendix in Tully and Razin 1996). In this case, when both glucose and arginine are present in the medium, the acids produced by glycolysis would mask the alkalinization caused by the ammonia liberated on arginine degradation, hampering the test for arginine utilization by the simple pH change assay. Yet, as was shown in a ^{13}C-NMR study, the accumulation of lactate and breakdown of arginine by *M. fermentans* were observed in the simultanous presence of both glucose and arginine, suggesting that glucose utilization has little or no effect on deimination of arginine to citrulline (Olson et al. 1993). Furthermore, proteome analysis of *S. melliferum* showed that the arginine dihydrolase enzyme proteins were constitutively also expressed in the presence of glucose (Cordwell et al. 1997a).

Some mycoplasmas, such as *M. agalactiae*, *M. bovigenitalium*, and *M. bovis* metabolize neither sugars nor arginine, but are capable of oxidation of organic acids (lactate, pyruvate) to acetate and CO_2 (Taylor et al. 1994). The contribution of the oxidation of these substrates to energy provision of the above mycoplasmas should be worked out. Another potential energy-yielding mechanism in mollicutes is based on ATP generation from acetyl phosphate and adenosine 5′-diphosphate by acetate kinase, coupled with acetyl phosphate formation from acetyl coenzyme A (acetyl-CoA) by phosphate acetyl transferase; both enzymes are commonly found in fermentative and nonfermentative mollicutes. Acetyl-CoA can be produced by oxidative phosphorylation of pyruvate by mycoplasmas (Razin 1978). The weight to be given to this pathway in energy metabolism of mollicutes has not been critically evaluated.

Energy metabolism of ureaplasmas presents a special case. Neither glycolysis nor arginine dihydrolase-ATP-generating pathways could be detected in these organisms. Ureaplasmas are unique among the mollicutes in possessing a very potent urease. Although protein and gene analysis of the ureaplasmal urease complex has shown it to resemble other prokaryotic ureases in subunit structure and composition, the specific activity of the ureaplasmal urease is much higher, and was estimated to exceed that of jack bean urease by about 100-fold (Blanchard et al. 1988). More importantly, ureaplasmas appear to be unique among prokaryotes in requiring urea for growth (Razin 1978). Thus, the specific urease inhibitor, flurofamide, inhibits ureaplasma growth (Blanchard et al. 1988). The dependence of ureaplasmas on urea for growth has led to the hypothesis that intracellular urea hydrolysis and the resulting intracellular accumulation of ammonia/ammonium ions is coupled to ATP synthesis through a chemiosmotic type of mechanism (Razin 1978). Experimental support for the generation of a transmembrane potential, with resultant ATP synthesis through the ureaplasmal F_0–F_1-type ATPase, first provided by Romano et al. (1980) was later extended and confirmed by Smith et al. (1993). At an external pH of 6.0, the pH optimum for ureaplasma growth in vitro, urea hydrolysis generated an ammonia chemical potential equivalent to almost 80 mV and, simultaneously, an increase in proton electrochemical potential (Δp) of about 24 mV with resultant de novo synthesis of ATP (Smith et al. 1993). When the external pH of the growth medium reaches about 8.1, due to ammonia released from the cells, the intracellular pH rises to 8.6 and urease activity ceases. ATP generation is blocked at this alkaline pH, leading to the abrupt and steep decline of growth, characterizing the ureaplasmal growth curve. Inhibition of the urease by flurofamide abolished both the chemical potential and the increase of Δp, such that ATP synthesis decreased to approximately 5 percent of the normally obtained levels (Smith et al. 1993; Glass et al. 2000). It is worth mentioning at this point that the pH of the urogenital tract is usually on the acid side of neutrality, corresponding to the pH values optimal for ureaplasma growth, maximum increase in Δp, maximum ammonia chemical potential, maximum urease activity, and maximum ATP generation.

Resembling other eubacteria, mollicutes possess an F-type ATPase (Razin 1978). The number and order of genes of the *atp* operon coding for the F_0F_1 ATPase of mollicutes are identical with those of *E. coli* and *Bacillus subtilis*. However, the b subunit (atpF) of the mycoplasmal ATPase was found to carry the characteristic features of a lipoprotein (Pyrowolakis et al. 1998). This feature, unknown for any other eubacterial ATPase, may explain the long known inability to detach the

mycoplasmal ATPase activity from the cell membrane by varying the osmolarity of the medium or by treating the membrane with EDTA (Razin 1978).

Transport

The expectation to find in *M. genitalium* and *M. pneumoniae* a proportionally high number of genes involved in transport of the many essential nutrients required for growth of these organisms has not been substantiated (Fraser et al. 1995; Himmelreich et al. 1996; Paulsen et al. 1998; see Genome sequencing section). This has led to the notion that at least some of the transport systems, such as those for amino acids and oligopeptides, might not be very specific, an assumption for which little experimental evidence is available thus far (Fraser et al. 2000; Pollack 2002a).

Mycoplasmas, lacking a functional TCA cycle, generate ATP as their primary energy source, and consequently have mostly ATP-dependent permeases, while *B. subtilis* generates a proton motive force (pmf) as its primary source of energy and accordingly utilizes mostly pmf-dependent carriers (Paulsen et al. 1998). The ABC transporter systems are involved in import or export of a large variety of substrates, including sugars, peptides, proteins, and toxins. In fact, the ABC transporters were the most frequent class of proteins found in *B. subtilis* (Kunst et al. 1997) and in *E. coli* (Blattner et al. 1997). Generally, bacterial ABC importers have an ATP-binding domain and membrane spanning domains located on separate polypeptides, whereas the ABC exporters may carry these components on the same or on different polypeptides (Blanchard et al. 1996). Putative ABC transporter genes were reported in *M. hyopneumoniae* (Blanchard et al. 1996), in *U. urealyticum(parvum)* (Glass et al. 2000), *M. pulmonis* (Chambaud et al. 2001), and essentially in all the mollicute genomes sequenced so far; *M. pulmonis* showing the highest number, 56, of genes potentially encoding subunits for ABC transporters. The deduced amino acid sequence of these gene products showed significant homology with the ABC transporter proteins, particularly those of the eukaryotic multidrug resistance (MDR) protein family, a finding previously reported for other bacteria (Paulsen et al. 1998).

M. genitalium and *M. pneumoniae* take up amino acids via two amino acid-polyamine choline (APC) transporters, and they probably accumulate peptides and polymers via two distinct ABC-type systems. These four permeases presumably provide the requisite precursors for protein synthesis (Himmelreich et al. 1996; Paulsen et al. 1998). While trials to identify the substrate-binding domain (OppA) of the ABC oligopeptide transport in *M. genitalium* and *M. pneumoniae* failed (Himmelreich et al. 1996), the study by Henrich et al. (1999) has identified an OppA in the homologous ABC transport system in *M. hominis*, functioning in binding peptides of different length that could endow the transporter with a less restrictive substrate specificity.

The highly efficient phosphoenolpyruvate-dependent sugar phosphotransferase transport systems (PTS) have been reported in mollicutes long ago, and some of their components were partially characterized by classical biochemical methodology (Razin 1978). It appears that *M. genitalium* and *M. pneumoniae* deviate a major fraction of their transport capacity to the task of sugar uptake for the purpose of carbon and energy acquisition. Thus, they possess both glucose and fructose phosphotransferase systems, two sugar permeases of the ABC superfamily, and a nonspecific channel protein of the MIP family, probably capable of transporting small, neutral, straight chain molecules, such as urea, glycerol, and various polyols (Paulsen et al. 1998). Interestingly, a defective PTS system lacking component E1 was found in *U. urealyticum (parvum)* possibly explaining the inability of this mycoplasma to utilize sugars (Glass et al. 2000).

GENETICS

Genome structure

The application of pulse-field gel electrophoresis (PFGE) to mycoplasma genome size determinations has provided a much more accurate and labor-saving procedure than the previously used renaturation kinetics method, resulting in a wealth of genome size data (Neimark and Lange 1990). The data show a continuum of genome sizes among mollicutes, ranging from less than 600 kb to over 2200 kb, with overlapping values between mollicute genera. Thus, genome sizes of *Mycoplasma* species range from 580 kb for *M. genitalium* to 1 380 kb for *M. mycoides* subs. *mycoides* LC, while for the helical *Spiroplasma* species genome size ranges from 780 kb for *S. platyhelix* to 2 220 kb for *S. ixodetis* (Table 1). As a general rule, *Acholeplasma* and *Spiroplasma* species, considered phylogenetically as 'early' mollicutes, have larger genome sizes than *Mycoplasma* and *Ureaplasma* species, considered to be phylogenetically more 'recent' mollicutes (see Phylogeny and taxonomy section). The genome size of representative members of 12 major phylogenetic groups of the uncultured phytoplasmas were recently determined (Marcone et al. 1999). Genome size varied between 530 and 1 350 kb, also showing considerable size variation among members of the same phylogenetic group, resembling in this respect the cultured mollicutes. The Bermuda grass white leaf phytoplasma chromosome of 530 kb is not only the smallest mollicute chromosome found to date, but is also the smallest chromosome known for any living cell. Competing with the mollicutes for the title of organisms with the smallest genome are the *Buchnera* species, with a genome size ranging from

450–670 kb (Gil et al. 2002). However, *Buchnera* species are mutualistic intracellular symbionts of aphids, while *M. genitalium* is a self-replicating bacterium independent on other cells for growth. The size of the *Eperythrozoon suis* genome, reclassified now as a mycoplasma (Phylogeny and taxonomy section) is about 750 kb and that of *Haemobartonella felis* reclassified as *Mycoplasma haemofelis*, is about 1 199 kb (Berent and Messick 2003), that is, well within the range of mollicute genomes (Messick et al. 2000).

The mycoplasma genome is characteristically low in G + C content. With very few exceptions, the G + C content of mycoplasma genomes is within the range of 24 to 33 mol% (Appendix in Tully and Razin 1996). The G + C distribution along the genome is uneven. Thus, while the average G + C content of the *M. genitalium* genome is 32 mol%, the G + C content of its rRNA genes is 44 mol%, and of its tRNA genes 52 mol% (Fraser et al. 1995). The *M. pneumoniae* cytadhesin genes, P1 and ORF6, and their repetitive sequences, exhibit a G + C content as high as 56 mol%, while on the other extreme the origin of replication of this mycoplasma has a G + C content of only 26 mol%, compared to 40 mol% of the entire *M. pneumoniae* genome (Himmelreich et al. 1996, 1997). Consequently, many of the mycoplasmal intergenic regions are richer in A + T than the coding regions, reaching values as high as 80–90 mol% (Dybvig and Voelker 1996; Yogev et al. 1991a). The variable G + C content of coding regions within the mycoplasmal genome has phylogenetic relevance, indicating in the case of the rRNA and tRNA genes their highly conserved nature, and in the case of the cytadhesin genes, their possible exogenous origin (see Adhesion to host cells section).

As in other prokaryotes, some of the adenine and cytosine residues in mycoplasmal genomes may be methylated (Dybvig and Voelker 1996; Razin 1985). In many mycoplasmas the adenine residues at the GATC site are methylated, while in others cytosine residues are methylated. Of special interest is the exclusive methylation of the genomic cytosine residues of *S. monobiae* (MQ-1) when they are located 5′ to guanine (CpG), a methylation trait considered unique to eukaryotes (Renbaum et al. 1990).

Extrachromosomal elements

Among the mollicutes, spiroplasmas and acholeplasmas are most frequently infected by a variety of viruses (phages), whereas very few viruses are known to infect *Mycoplasma* species. The characteristics of mollicute viruses have been described and discussed in detail previously (Maniloff 1992; Renaudin 2002). The mollicute phage DNA genomes range in size from 4 to 40 kb, and may be either circular or linear, and single- or double-stranded (Dybvig and Voelker 1996). Of the very few phages infecting *Mycoplasma* species, the most recent phage discovered is the lysogenic phage MAV1, infecting *M. arthritidis* (Voelker and Dybvig 1999). The finding that this phage is associated with highly arthritogenic strains, and that experimental infection of low-virulence *M. arthritidis* strains with this phage increases significantly their arthritogenicity, suggest that MAV1 carries a virulence enhancing factor. Sequencing of the 15.644-bp MAV1 linear double-stranded genome revealed 15 ORFs. The deduced product of one of the genes, designated *vir*, contained a classic prokaryotic lipoprotein signal sequence. If MAV1 encodes in fact a lipoprotein, it may be speculated that this lipoprotein is incorporated into the *M. arthritidis* cell membrane and in this way induces the conversion of the avirulent phenotype into a virulent one (see also Pathogenicity section).

Plasmids were detected in *S. citri*, and in *M. mycoides* subsp. *mycoides* (Dybvig and Voelker 1996). Extrachromosomal DNA molecules, most prevalent in phytoplasmas of various phylogenetic clades have been postulated to represent plasmids, as no virus particles could be observed by electron microscopy (Rekab et al. 1999). The almost universal occurrence of plasmids in phytoplasmas suggests a function for these molecules. The possibility that phytoplasma plasmids may encode genes associated with pathogenicity (Nishigawa et al. 2002) has been raised, but evidence to support this notion is still missing. The potential of the mollicute phages and plasmids to serve as cloning and shuttle vectors has been a major reason for interest in these elements (see Gene transfer section).

Genome sequencing and gene annotation

Mycoplasmas, carrying the smallest genomes of self-replicating cells, were among the first microorganisms selected for the genome sequencing projects. The complete sequence of the genome of *M. genitalium* was second to that of the *Haemophilus influenzae* genome to be reported (Fleischmann et al. 1995; Fraser et al. 1995). The remarkable power and cost-effectiveness of the new genome sequencing technology have already resulted in the elucidation of the complete genome sequences (as of January 2003) of over 90 eubacteria and archeons (see www.tigr.org/tdb/mdb/). Included among these are the mollicutes *U. urealyticum* (*U. parvum*) (Glass et al. 2000), *M. pulmonis* (Chambaud et al. 2001), and *M. penetrans* (Sasaki et al. 2002). The complete sequencing of the *M. gallisepticum* genome has already been accomplished (Papazisi et al. 2003). The genomes of *M. hyopneumoniae*, *M. mycoides* subsp. *mycoides*, and *S. citri* are at advanced or final stages of sequencing. A large part of the *S. kunkelii* genome has been sequenced and annotated (Bai and Hogenhout 2002).

The voluminous amount of genetic data provided by the genomic projects has opened the way for

'comparative genomics' by which the total genomic complements of organisms could be compared. This provides an opportunity to explore the functional content of genomes and evolutionary relationships between organisms at a new qualitative level. Typically, about 40–60 percent of the genes of a newly sequenced bacterial genome display detectable similarity to protein sequences whose function is at least tentatively known. Gene density is consistent across many bacterial species, with about one gene per kilobase (Fraser et al. 2000). A detailed comparative genomic analysis of *M. genitalium* and its close relative *M. pneumoniae*, compared to that of *H. influenzae* and other eubacteria was presented in Razin et al. (1998). Table 3 summarizes some of the gross properties of the mollicute genomes sequenced so far, in comparison with the *Bacillus subtilis* genome.

Examination of the mycoplasmal genomic data points out the biochemical pathways where economization in genes took place during their reductive evolution, and helps us define the genes which are apparently essential for life of a minimal self-replicating cell. In fact, the definition of the minimal gene set required for life has become a most attractive research subject, applying *M. genitalium* as a prime candidate for this purpose (Hutchison and Montague 2002; Koonin 2000; Razin et al. 1998). Moreover, Hutchison et al. (1999) have promoted the idea of constructing an artificial life form based on the genes found essential for *M. genitalium* survival following global transposon mutagenesis. The essential genes were defined, in part, by the absence of viable mutants containing a transposon insertion within a given coding region. Hutchison et al. identified 243 *M. genitalium* genes in this way whose functions are not essential for cell viability. From this analysis (coupled with statistical and computational analysis) the authors concluded that 265–350 of the 517 genes in *M. genitalium* are essential for its life under laboratory growth conditions, that is, in the presence of a full complement of essential nutrients and in the absence of environmental stress. Recent announcements in public communication media and in *Nature* (Check 2002) and in *Science* (Zimmer 2003) indicate that Venter, Smith and Hutchison have already initiated a most ambitious endeavor directed at the synthesis of an artificial minimal cell. Their idea is to synthesize first an artificial genome, based on the genes found essential for life of *M. genitalium*, and introduce this synthetic genome into *M. genitalium* cells from which the natural genome was removed. Whether the synthetic genome will 'hijack' the cellular machinery of the genome-depleted host cell is, of course, a great mystery. A most interesting article relevant to the issue of the 'minimal cell' concept is that of Kobayashi et al. (2003). By systematic inactivation of *B. subtilis* genes, they could show that only 271 of the 4 112 genes of this bacterium (Table 3) appear essential for its survival and growth under optimal conditions, again demonstrating how little genetic information is theoretically needed for the assembly of a minimal cell.

Comparative genomics

Genomic analysis of *M. genitalium* and *M. pneumoniae* has revealed the scarcity of genes involved in biosynthetic pathways (for detailed discussion see Dandekar et al. 2002 and Razin et al. 1998). The two mycoplasmas have apparently lost all the genes involved in amino acid biosynthesis during their reductive evolution, as well as most of the genes involved in cofactor (vitamin) biosynthesis, so that to cultivate these mycoplasmas in vitro the medium has to be supplemented with the essential amino acids and vitamins. Very significant savings in genetic information have resulted through the loss of the cell wall during mycoplasma evolution. Significant gene saving is also pronounced in lipid metabolism. Most mycoplasmas cannot synthesize any fatty acid and depend, therefore, on the host for their supply (see Membrane lipids section). Being deficient in the ability to regulate membrane fluidity by preferential fatty acid biosynthesis, most mycoplasmas overcome this deficiency by incorporating large quantities of exogenous cholesterol into their membrane. Cholesterol serves as a very effective buffer of membrane fluidity (Razin 1978). Our early studies on mycoplasma nutrition revealed their requirement for the nucleic acid precursors, purines, and pyrimidines. These may be provided by RNA and DNA degraded by the potent mycoplasmal nucleases (Razin 1978). The present genetic data provide an explanation for these observations by

Table 3 *Gross properties of sequenced mollicute and* Bacillus subtilis *genomes*[a]

Property	*U. urealyticum*	*M. pneumoniae*	*M. genitalium*	*M. pulmonis*	*M. penetrans*	*B. subtilis*
No. of base pairs	751 719	816 394	580 074	963 879	1 358 633	4 214 814
G+C content (mol%)	25.5	40.0	32.0	26.6	25.7	43.5
No. of putative protein coding sequences (ORFs)	614	689	484	782	1 038	4 112
No. of ORFs with no functional prediction or database match	295	230	152	204	453	1 722

a) Based on data of Glass et al. (2000), Himmelreich et al. (1996, 1997), Dandekar et al. (2000), Fraser et al. (1995), Chambaud et al. (2001), Sasaki et al. (2002), and Kunst et al. (1997).

revealing the scarcity in mycoplasmas of genes, and consequently of enzymes, responsible for purine and pyrimidine synthesis while genes for salvage pathways utilizing purines and pyrimidines for the synthesis of ribonucleotides and their conversion to deoxyribonucleotides have been detected in mycoplasmas (Fraser et al., 1995; Himmelreich et al., 1996; Tham et al., 1993). It should be emphasized that the picture of extreme reduction in biosynthetic genes, described above for *M. genitalium* and *M. pneumoniae*, is less pronounced on analysis of larger mollicute genomes. Thus, the 1 600 kb genome of *S. kunkelii* carries genes for de novo synthesis of several amino acids and nucleosides, as well as regulatory factors not found in *M. genitalium* and *M. pneumoniae* (Bai and Hogenhout 2002).

The number of genes involved in cellular processes, such as the *fts* genes associated with cell division, heat shock proteins, and genes for chaperone functioning in protein secretion, is definitely smaller in the mycoplasmas as compared to other eubacteria. However, the major chaperone families HSP70 (DnaK) HSP60 (GroEL), and associated components (e.g. DnaJ, GroES, and GrpE) as well as the folding isomerase and the trigger factor are present in *M. pneumoniae* and *M. genitalium*. In essence, all the constituents of a basic protein folding machinery are present (Bang et al. 2000). Yet, the genes for the chaperonins GroES and GroEL were not detected in *U. urealyticum (parvum)* so that different mollicute systems may vary in detail (Glass et al. 2000). The protein secretion system in *M. pneumoniae* is much less complex than in *E. coli*. The channel-forming proteins SecG, SecF, SecE, SecD were not identified in the mycoplasma, leaving only SecA, SecB, SecY, trigger factor, bacterial signal recognition particle, and FtszY as the components of a simplified translocon in *M. pneumoniae* (Himmelreich et al. 1996; Edman et al. 1999; Dandekar et al. 2000).

Many of the regulatory system genes found in other bacteria, such as the two-component signal transduction systems consisting of a sensor and response regulator, were not detected in *M. genitalium* and *M. pneumoniae* as well as the recognizable vestiges of the histidine protein kinases or their target response regulators. Generally, a rough correlation can be observed between genome size and the number of putative protein kinases and protein phosphatases. Thus, while *M. genitalium* carries a single potential protein kinase, *Synechocystis* carries nine potential protein kinases and ten potential protein phosphatases (Shi et al. 1998). *Synechocystis*, with its ability to survive at the barest of environmental resources, light and air, presumably has developed and maintained a more extensive cellular sensory, command, and control apparatus to support its lifestyle than organisms like *M. genitalium* and *M. pneumoniae* that specialized to efficiently exploit more monotonous environmental niches (Shi et al. 1998). The *M. penetrans* genome differs from the other mycoplasma genomes sequenced so far (Table 3) by carrying the genes for a predicted two-component signal system (Sasaki et al. 2002). The absence of identifiable transcription factors from *M. genitalium* is most striking. While *E. coli* has 55 known transcriptional activators and 58 repressors, *M. genitalium*, apart from *nusA* and *nusG*, contains only a single transcription elongation factor (Ouzounis et al. 1996) and the *M. pulmonis* genome was found to carry only two transcription regulation factors (Chambaud et al. 2001). Relevant to this issue is the observation that FruR, the product of the *S. citri* gene *fruR*, acts as an activator of the fructose operon transcription in the spiroplasma. This appears to be the first mollicute operon for which regulation of transcription is documented (Gaurivaud et al., 2000c).

The mollicute genomes sequenced so far are deficient in genes coding for components of intermediary and energy metabolism. Thus, *M. genitalium* and *M. pneumoniae* depend primarily on glycolysis as a means for synthesizing ATP. Genes that encode the components of the pyruvate dehydrogenase complex, phosphotransacetylase, and acetate kinase were also detected, as well as a deficient pentose phosphate pathway (Fraser et al. 1995; Himmelreich et al. 1996). Most striking is the lack of many energy-yielding systems from the mycoplasmas. No tricarboxylic acid cycle, and no quinones nor cytochromes were found in any of the mycoplasmas tested (see Energy-yielding pathways and Transport sections). In terms of reductive evolution strategy, mollicutes may have concentrated two different functions in a single gene during genome size reduction, as evidenced by the malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) example (Cordwell et al. 1997b), where the gene putatively assigned to lactate dehydrogenase in *M. genitalium* and *M. pneumoniae* fulfils both LDH and MDH functions. Along this line, a lipoprotein of *M. hominis* functioning as a cytadhesin, was recently shown to also function as the substrate-binding domain OppA of an oligopeptide permease of *M. hominis* (Henrich et al. 1999). The inability to detect in the mollicute genomes sequenced so far the genes for nucleoside-diphosphate reductase and nucleoside diphosphate kinase was surprising, as these are considered indispensable, ubiquitous, and highly conserved genes, playing a key role in the synthesis of nucleotide triphosphates. A way out of this problem was proposed by Pollack et al. (2002) suggesting that glycolytic kinases are capable of using a variety of purine and pyrimidine di- and trinucleosides as phosphate donors or acceptors rather than ATP and in this way supply the required di- and trinucleotides.

Being dependent on the exogeous supply of many nutrients would predict the need of mycoplasmas for many transport systems. However, the percentage of genes devoted to transport in *M. pneumoniae* and *M. genitalium* is no higher than in *H. influenzae*, while their

absolute number is much lower. The relative small number of transport genes in the two mycoplasmas is even more striking when compared to the 281 transport and binding proteins annotated in *E. coli* K-12 (Blattner et al. 1997) and almost 400 in *B. subtilis* (Kunst et al. 1997). The apparent low substrate specificity of some of the mycoplasmal transport systems, such as those for amino acids (Himmelreich et al. 1996) may contribute to the significant gene saving observed in this category (see Transport section).

The essential role of the basic processes of DNA replication, transcription, and translation, leads us to expect that saving of genes in these categories will be more restricted as compared to metabolic processes. In fact, while the absolute number of genes involved in DNA replication in the mycoplasmas is still much smaller than in *Haemophilus*, the percentage of genes devoted to DNA replication and degradation in *M. genitalium* and *M. pneumoniae* is higher than in *H. influenzae*, pointing to the essential biological role of these genes, so that evolutionary deletion of genes in this category had to be limited. Obviously, it is of interest to define the genes missing from the mycoplasmas, as these are apparently of secondary importance. For example, while the estimated number of genes functioning in DNA repair in *E. coli* is estimated to be about 100 (Blattner et al. 1997), only 13 of the genes in this category were found by Himmelreich et al. (1996) in *M. pneumoniae*. We may assume that those genes detected in the mycoplasmas, including the uracil DNA glycosylase, the ABC exinucleases, and *recA*, must represent the genes really essential for DNA repair (see DNA replication and repair section).

The percentage of genes devoted to transcription is also higher in the mycoplasmas than in other eubacteria though their absolute number is definitely smaller in the mycoplasma. Included in this category are the genes for the RNA polymerase subunits, which are basically similar in mycoplasma and the other eubacteria (see Transcription section). The translation and protein synthesis machinery apparently constitutes the most conserved system in eubacteria, indicating that it represents a very efficient and successful evolutionary development. Thus, in composition, the mycoplasmal translation components very much resemble those of other eubacteria. A marked difference can, nevertheless, be observed in the small copy number of the rRNA and tRNA genes (Table 4 and Ribosomes, ribosomal RNAs and Transfer RNA sections). The mycoplasmas thus appear to possess the minimum machinery needed for protein synthesis. Consequently, protein synthesis and cell replication in mycoplasmas is much slower than in *E. coli*; the velocity of the DNA replication fork was about ten times slower in *M. capricolum* than in *E. coli* (Seto and Miyata 1998). Yet, mycoplasmas appear to grow well in the protective and constant environment of their host and a slow replication rate cannot really be considered as a deficiency of a parasite that can only lose by killing its host. In fact, mycoplasma infections are usually rather mild, and chronic in nature (see Pathogenicity section).

There can be little doubt that the considerable economization in genes depends primarily on the obligate parasitic lifestyle of mycoplasmas. Some genomic price has to be paid for parasitism; that is, mycoplasma cells must possess surface components enabling their attachment to the host cells. In some cases, including *M. genitalium* and *M. pneumoniae*, the mycoplasmas developed special attachment organelles (Cellular morphology and cytoskeletal elements section). Obviously, a significant number of genes is involved in construction of such organelles. Some of these genes have already been identified and characterized, but some have not, and are apparently included in that part of the mycoplasmal coding regions or genes unidentified as yet (see Adhesion to host cells section).

Proteome and transcriptome analyses and bioinformatics

Many ORFs that are identified in the genomic sequence of a particular organism have structurally and function-

Table 4 *Ribosomal RNA operons, transfer RNAs, and UGA codon usage in mollicutes and other eubacteria* [a]

Property	Mollicutes	Other eubacteria	References
No. of rRNA operons	1–2	1–10	Amikam et al. (1984)
5S rRNA length	104–113 nucleotides	>114 nucleotides	Rogers et al. (1985)
No. of tRNA genes	30 (*M. capricolum*) 29 (*M. pulmonis*) 33 (*M. pneumoniae*) 33 (*U. urealyticum*)	84 (*B. subtilis*) 86 (*E. coli*)	Muto and Ushida (2002); Kunst et al. (1997); Chambaud et al. (2001); Glass et al. (2000); Himmelreich et al. (1996); Blattner et al. (1997)
UGA codon usage	Tryptophan codon in *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Mesoplasma* Stop codon in *Acholeplasma* and *Phytoplasma*	Stop codon	Osawa et al. (1992)

a) Adapted and updated from Razin (1985).

ally well-characterized homologs in other organisms and it is possible, therefore, to assign the putative protein structure and function through bioinformatics by similarity searches in protein databases. However, for a large fraction of ORFs neither structure nor function can be assigned by sequence comparisons.

Experimental identification and confirmation of the proposed gene products at the cellular level has become complementary to identification by sequence comparison. Gene expression can be studied either at the level of transcription or at the level of translation. When these analyses are carried out on intact cells the term 'transcriptome' is defined as the sum of all transcripts derived from a genome, while the term 'proteome' refers to the expressed total protein complement of a genome.

Transcriptome analysis uses microarray technology allowing the analysis of gene expression at the transcription levels of thousands of genes simultaneously (Weiner et al. 2002). For this purpose total RNA is isolated from the mycoplasma grown under defined laboratory conditions. The RNA is transcribed into a single-stranded complementary DNA and is labeled with ^{33}P. Specific probes are synthesized for all proposed ORFs by PCR, and the probes are immobilized on nylon membranes, and tested for cross-hybridization with the labeled cDNA. This technique was used to analyze gene expression in *M. pneumoniae* grown under various conditions, such as heat shock, oxidative stress, and growth in the presence of different antibiotics (Weiner et al. 2000, 2002). Of the 688 proposed *M. pneumoniae* genes about 600 were transcribed, but transcription of many genes was temperature dependent and therefore regulated, while numerous genes were constitutively expressed at all temperatures. Interestingly, a substantial proportion of genes with high transcription signals had no known function. Yet, their high expression level suggests that they code for important products (Weiner et al. 2002).

Proteome analysis involves two-dimensional (2D) gel electrophoresis for the separation of proteins according to isoelectric point (pI) and molecular mass, knowledge of the sequences of all cell proteins and mass spectrometry for the characterization of individual proteins. The combination of peptide mass fingerprinting and peptide fragmentation which matches the masses of in-gel proteolytically generated peptides against theoretically digested proteins from the database, has proven to be very effective and reliable (Regula et al. 2000; Weiner et al. 2002; Ueberle et al. 2002).

Mycoplasmas are particularly suitable for proteome analysis, as a high percentage of their proteins can be visualized by 2D-gel electrophoresis, analyzed, and identified. Moreover, the highly conserved nature of a large part of mycoplasmal proteins facilitates their identification. The major limitations of the proteome approach are: (1) Not all cell proteins are expressed at a given time. Thus, it is improbable that an entire proteome can be visualized under a specific set of experimental conditions. (2) The detection threshold of 2D-PAGE is a limiting factor, as a low copy number of protein molecules may not be detected. (3) Proteins with an isoelectric point higher than 11 cannot be separated well in the first dimension of immobilized pH gradients, as well as proteins with five or more transmembrane segments. (4) Presently, proteome analysis is only useful for identifying proteins for which the DNA sequence of their genes is available. (5) Co- and posttranslational modification of proteins may increase the number of protein spots per gene (Ueberle et al. 2002).

The pioneering work on mollicute proteome analysis was carried out by Humphery-Smith and colleagues on *Spiroplasma melliferum* (Cordwell et al. 1997a) and on *M. genitalium* (Wasinger et al. 1995) at a time when complete genome sequences were not available and the sensitivity and accuracy of mass spectrometry methods were inferior to those of today. In a more recent study Wasinger et al. (2000) resolved 427 protein spots in proteome analysis of exponentially-grown *M. genitalium* cells. Of the 201 proteins of sufficient abundance, 158 could be identified by peptide mass fingerprinting. A reduction of 42 percent in protein spots was noticed in post-exponentially grown cells. On the whole, only about one-third of the proteins in the predicted *M. genitalium* proteome could be identified by them. Proteome analysis of *M. pneumoniae* by Regula et al. (2000) characterized about 350 protein spots. It was possible to assign these 350 proteins to 224 genes. Clearly, the *M. pneumoniae* proteome map is still far from being as complete as that of *M. genitalium*. Ueberle et al. (2002) concluded that due to the technical problems discussed above, a complete proteome analysis based on the separation of cell proteins by 2D-gel electrophoresis cannot be achieved at the present, even for simple organisms like *M. genitalium* and *M. pneumoniae*.

DNA replication and repair

The recent mycoplasma genome sequencing projects, as well as the study of Barnes et al. (1994) on *M. pulmonis* DNA polymerases, have considerably clarified the nature of the mycoplasmal DNA polymerases. It appears that the mycoplasmas carry the central enzyme for DNA replication – the DNA polymerase III holoenzyme (Pol III) that has the essential characteristics of the eubacterial gram-positive Pol III. Although the genes responsible for building the major subunits of the holoenzyme, such as the α (*dnaE* or *polC*) and β subunits (*dnaN*) were detected, several other genes responsible for other subunits were missing from the *M. genitalium* and *M. pneumoniae* genomic analysis (Fraser et al. 1995; Himmelreich et al. 1996) indicating a simplified DNA replication complex in the mycoplasmas compared to that of *E. coli* or *H. influenzae*. Yet, most impor-

tantly, the mycoplasmal Pol III α-subunit (about 1 400 amino acids) included the motif for 3′–5′ exonuclease activity typical for Pol C of gram-positive bacteria (Barnes et al. 1994; Himmelreich et al. 1996; Kunst et al. 1997), providing an answer to the question of the way DNA proofreading is done in mycoplasmas.

Barnes et al. (1994) presented experimental evidence for a second enzyme with DNA polymerase activity in *M. pulmonis*. This correlates with sequence data for *M. genitalium* and *M. pneumoniae* indicating that at least these three *Mycoplasma* species carry two DNA polymerase genes, one coding for the larger protein (about 166 kDa) with a 3′–5′ exonuclease activity, with high sequence similarity to the *B. subtilis* polymerase III, and the other gene coding for a Pol III homolog (about 100 kDa) resembling more the *polC* gene from *E. coli*, lacking a 3′–5′ exonuclease domain. This smaller enzyme may correspond to the DNA polymerases lacking exonuclease activity isolated earlier from *M. orale* and *M. hyorhinis* (Razin 1985). The picture appears different for the recently sequenced genome of *Spiroplasma kunkelii* where its larger genome carries a *polA* gene that may encode the full length DNA polymerase I protein, including the proofreading and Klenow domains, resembling in this respect *Streptococcus pneumoniae* (Bai and Hogenhout 2002).

Topoisomerases (topos) catalyze the interconversions of topological isomers of DNA molecules, fulfilling an essential role in DNA replication, transcription, recombination, and repair. The mycoplasmal DNA topos, characterized so far, are the DNA gyrase and topoisomerase IV, both belonging to type II topos. Both mycoplasmal enzymes resemble their eubacterial counterparts in having a tetrameric structure composed of two subunits: A and B in the case of DNA gyrase, and the products of *parC* and *parE* genes in the case of topoisomerase IV (Bebear et al. 1998; Ladefoged and Christiansen 1994). Interest in the molecular characterization of mycoplasmal topoisomerases has been boosted by the fact that these enzymes are the targets of fluoroquinolones, among the most effective antimycoplasmal drugs (Bebear and Bebear, 2002; Kenny et al., 1999; Reinhardt et al., 2002).

The three genes coding for the ABC excinucleases are present in *M. genitalium*, *M. pneumoniae*, and *M. capricolum* so that together with Pol I, helicase II, and ligase, should provide the mechanism for repair of UV damage, such as crosslinking. While dark reactivation of UV damage (excision repair) operates in the above mycoplasmas, photoreactivation may be missing as the photolyase (*phr*) gene could not be identified in *M. genitalium* and *M. pneumoniae*. The RecA protein plays a central role in recombination repair, homologous recombination, and initiation of the SOS response. The *recA* gene has been found essentially in all the mollicutes analyzed so far (Zou and Dybvig 2002). Surprisingly, *S. citri* R8A2 was found to lack a significant portion of the N-terminus of RecA (Marais et al. 1996) affecting its function. However, truncation of RecA is not a characteristic of other *Spiroplasma* species, such as *S. melliferum* (Melcher and Fletcher 1999; Cordwell et al. 1997a). Not surprisingly, searching of DNA repair systems in mollicutes has been associated with the high mutation rates and high tempo of evolution in Mollicutes (Rocha and Blanchard 2002). For an in-depth treatment of the issue of DNA replication and repair in mycoplasmas, the reader is referred to Zou and Dybvig (2002).

Transcription and translation

The DNA-dependent RNA polymerase of mollicutes resembles in subunit structure that of other eubacteria. Thus, the core RNA polymerase of *M. genitalium* (Fraser et al. 1995) and *M. pneumoniae* (Himmelreich et al. 1996) is coded by the conserved genes *rpoA* (α subunit), *rpoB* (β subunit), and *rpoC* (β′ subunit). One of the peculiar properties of mollicutes is their resistance to the antibiotics rifamycin and streptolydigin. Cloning and sequencing of the *rpoB* gene from *S. citri* (Gaurivaud et al. 1996) and *M. gallisepticum* (Skamrov et al. 1996) suggest that minor differences in amino acid sequences in the β-subunit Rif region, the region responsible for rifamycin binding, may confer rifamycin resistance.

Modulation of promoter selectivity of RNA polymerase by replacement of the sigma subunits is an efficient way to alter the global pattern of gene expression in response to changes in environmental conditions. Thus, the *E. coli* genome carries at least six sigma factors, and that of *B. subtilis*, 18 (Kunst et al. 1997). The presence of only one sigma factor in the mycoplasmas (Himmelreich et al. 1996) suggests that the response to external stimuli in these organisms is not controlled by the level of expression of alternative sigma factors. Mycoplasmas have conserved the heat shock response, and heat shock proteins, resembling the eubacterial DnaK (HSP70) and GroEL proteins, have been identified in several mollicutes. The *M. genitalium* and *M. pneumoniae* genomes carry genes for seven heat shock proteins (Fraser et al. 1995; Himmelreich et al. 1996). It has been said that the lack of alternative sigma factors from mycoplasmas, two-component signal transduction systems, or other means of gene regulation common to prototypical walled bacteria is almost as striking as their absence of a cell wall.

TRANSCRIPTION SIGNALS

The mycoplasmal transcription signals generally resemble the classical eubacterial ones. The mycoplasmal-10 (Pribnow box) and to a much lesser extent the -35 regions resemble the eubacterial promoter concensus sequences recognized by the RNA polymerase holoenzyme (Weiner et al. 2000). A concensus

Shine–Dalgarno (SD) sequence could also be identified upstream of the initiation codon (Dybvig and Voelker 1996) though in many cases the SD sequence could not be identified, indicating that signals other than the SD sequence may function as a ribosomal binding site for some mollicute genes. Termination of transcription in mollicutes appears to be independent of the termination factor Rho, as its gene could not be detected in the mycoplasmal genomes analyzed thus far.

RIBOSOMES AND RIBOSOMAL RNAS

The translation machinery of mycoplasmas is rather comprehensive. In *M. pneumoniae* and *M. genitalium* it comprises of 99 proposed genes, constituting about 15–20 percent of the genome's ORFs. The mycoplasmal ribosomes are typically prokaryotic in size, shape, and composition (Razin 1985). The *M. pneumoniae* ribosomal protein genes are organized in operons, retaining some of the gene order found in the *E. coli* and *B. subtilis* genomes. Of the 50 ribosomal proteins found in *E. coli* only the gene for protein S1 could not be detected in *M. genitalium* and *M. pneumoniae*, resembling in this respect a number of gram-positive bacteria, including *Bacillus* (Fraser et al. 1995; Himmelreich et al. 1996). The highly conserved nature of the ribosomal protein genes has also found its use in establishing mollicute phylogeny (see Phylogeny and taxonomy section).

Organization of the mollicute rRNA genes generally follows the characteristic eubacterial order: 16S–23S–5S, functioning as an operon (Razin 1985). Some exceptions to the above classical rRNA gene organization have been reported (Razin et al. 1998; Chambaud et al. 2001). Mollicute genomes carry only one or two rRNA gene sets (Table 4), whereas *Clostridium ramosum* and *Clostridium innocuum*, phylogenetically closest to mollicutes, have four and five sets of rRNA genes, respectively (Bove 1993). Interestingly, phytoplasmas carry two rRNA gene sets (Ho et al. 2001), resembling in this respect their phylogenetic relatives, the acholeplasmas (see Phylogeny and taxonomy section).

TRANSFER RNAS

The number of tRNA genes in mollicutes is kept to a minimum, with very few gene duplicates (Table 4). The set of 29 tRNA genes identified in the *M. pulmonis* genome represents the smallest set of tRNA genes among the sequenced genomes (Chambaud et al. 2001). Accordingly, the number of anticodons in the mycoplasmas is not much higher than in mitochondria and is close to the essential minimum for translation of all the amino acid codons by wobbling (Muto and Ushida 2002).

CODON USAGE

Most mollicutes have genomes with a very low G + C content (Table 1), the outcome of an AT-biased directional mutation pressure (A–T pressure) during their evolution (Muto and Ushida 2002). This has resulted in codon usage favoring synonymous codons with A and T, in particular in the wobble (3′) position. Codon bias in the AT-rich mycoplasmas is not limited to the third nucleotide position, and is evident also in the first and second positions. Mycoplasmas have fewer GGN, CCN, GCN, and CGN codons. Another apparent outcome of codon reassignment under strong A–T pressure is the reassignment in most mollicutes of UGA from a stop codon to a tryptophan codon, a feature found in mitochondria (Osawa et al. 1992). As can be seen in Table 4, not all mollicutes share this property. The phylogenetically early acholeplasmas and phytoplasmas (see Phylogeny and taxonomy section) use the conventional UGG codon for tryptophan, carrying $tRNA^{Trp}$(CCA) and retaining UGA as a stop codon. As a consequence of using UGA as a tryptophan codon, it is difficult to express cloned mollicute genes in *E. coli*. Since *E. coli* regards UGA as a stop codon, translation of a mycoplasmal message in *E. coli* will stop where originally there should be tryptophan, so that mycoplasmal proteins expressed in *E. coli* may be truncated. One way to overcome this difficulty is by using an *E. coli* opal suppressor strain for expression (Renbaum et al. 1990) though efficiency of expression falls considerably with increasing numbers of UGAs in the mycoplasmal message. Another way is to employ *Bacillus subtilis* that carries a tRNA which reads the UGA termination codon as tryptophan (Kannan and Baseman 2000).

The elongation factor genes, *fus*, *efp*, *tsf*, and *tuf*, were identified in the *M. genitalium* and *M. pneumoniae* genomes (Fraser et al. 1995; Himmelreich et al. 1996). Of these, the *tuf* gene, encoding the elongation factor EF-Tu that mediates attachment of amino acyl-tRNAs to the ribosome, has attracted most attention. It has been one of the first mollicute genes to be cloned, sequenced, and expressed in *E. coli* (Yogev et al. 1990). Being a highly conserved housekeeping gene found in all mollicutes examined so far, it has been used as an effective genetic probe in taxonomy and phylogeny of mollicutes (see Phylogeny and taxonomy section).

Gene transfer

The lack of a cell wall in mollicutes would be expected to facilitate the introduction of exogenous DNA into the cells. In fact, conjugative transposition of transposon Tn*916* from *Streptococcus (Enterococcus) faecalis* to *M. hominis* (Roberts and Kenny 1987) and to *M. gallisepticum* (Ruffin et al. 2000) by a spontaneous mating process probably involves transient fusion of the cell membranes at the zone of contact. However, DNA transfer efficiency in these cases was rather low. Increased transformation and transfection efficiencies have been achieved either in the presence of polyethylene glycol (PEG) or by application of the electroporation procedure. It appears that the method of choice

depends on the mollicute species, and on the particular DNA to be transferred (Dybvig and Voelker 1996).

Genetic studies in mollicutes have also been hampered by the paucity of selectable markers (Dybvig and Voelker 1996). Most studies on gene transfer in mollicutes have used as selectable markers the *tetM* tetracycline-resistance determinant found on *Tn916*, or the gentamycin-resistance determinant of *Tn4001*. The *tetM* gene is preferable as a marker, since mollicutes in general are sensitive to tetracycline, while not all mollicutes are sensitive to gentamycin (Dybvig and Voelker 1996). Thus, transposon Tn4001 containing the *tetM* gene constructed by Dybvig et al. (2000) was found effective as a broad host-range vector, as indicated by the successful expression of a lacZ fusion gene in both *M. pulmonis* and *M. arthritidis*.

Gene function can only be ascertained by mutational analysis. The recent deluge of information on putatively identified mycoplasmal genes, based on sequence analysis (see Genome sequencing and gene annotation section), has emphasized the need for complementation of these predictions by mutational analysis. Having efficient transformation procedures at hand, major efforts have been directed at developing cloning and shuttle vectors for mollicutes (reviewed by Renaudin 2002). Transfection experiments with the replicative form (RF) of the *S. citri* virus SpV1, carrying as an insert a segment of the gene coding for the P1 adhesin of *M. pneumoniae* (G-fragment) resulted in the expression of the fragment in the spiroplasma. In this case, the presence of seven UGA codons in the G-fragment insert did not interfere with expression, as *S. citri* can read UGA as tryptophan. Yet, the SpV1-RF/*S. citri* cloning system was deficient as it suffered from the rapid loss of the cloned DNA insert. The loss of the insert was suggested to be the result of illegitimate and homologous recombination (Marais et al. 1993). An improved type of a cloning vector for *S. citri* was subsequently constructed by combining the *oriC* of *S. citri* with *tetM* and a colE1-derived *E. coli* replicon. The artificial recombinant plasmid, named pBOT1, was able to replicate in *S. citri* and then integrate into the spiroplasmal chromosome, being stably maintained there (Renaudin 2002). The pBOT1-derived, recombinant plasmids carrying heterologous DNA inserts were effective as shuttle vectors and, for example, restored fructose utilization in a mutant with a defective fructose operon (Gaurivaud et al. 2000a).

TRANSPOSITION

The first successful transformation of *M. pulmonis* and *A. laidlawii* to antibiotic resistance was achieved by a plasmid, pAM120, harboring the streptococcal transposon Tn916 which contains the tetracycline resistance determinant *tetM* (Dybvig and Cassell 1987). The transposon was excised from the plasmid and integrated into the mycoplasmal chromosome at random sites, rendering the mycoplasmas resistant to tetracycline. Since then, a variety of mollicute species were shown to be transformed to tetracycline resistance by *Tn916* or *Tn1545*, or to gentamycin resistance, using the staphylococcal conjugative transposon *Tn4001*, which contains a gentamycin resistance determinant (Foissac et al., 1997; Voelker and Dybvig 1996).

Transposon mutagenesis has already been applied, rather effectively, to generate mutants of *M. pneumoniae* and *M. genitalium* deficient in cytadherence (Krause et al. 1997) and of *S. citri* mutants deficient in motility and pathogenicity to plants (Foissac et al. 1997; Jacob et al. 1997). Characterization of these mutants has already contributed significantly to the identification of genes and their protein products, associated with the mycoplasmal cytoskeleton and cytadherence (see Adhesion to host cells section), spiroplasmal motility (see Cellular morphology and cytoskeletal elements section) and pathogenicity (see Pathogenicity section). There is no doubt that transposon mutagenesis will continue to be a most useful tool in mollicute genetic studies.

While transposon mutagenesis is either random or directed to specific hot spots in the mycoplasmal genome, Dhandayuthapani et al. (1999) have introduced a technology enabling the targeting of mutagenesis to specific genes or operons. The technique is based on creation and delivery of disruption constructs acting by homologous recombination. The constructed plasmids contain the targeted gene and a gentamycin resistance gene. Electroporation of the disruption plasmid, carrying part of the *mg218 M. genitalium* gene, into wild-type hemagglutination-positive *M. genitalium* permitted the isolation of hemagglutination-negative mutants, resulting apparently from homologous recombination at the mg218 locus by single- or double-crossover events. In this way the role of the mg218 protein product in *M. genitalium* adherence could be demonstrated.

RESTRICTION AND MODIFICATION

Restriction and modification of DNA allow a bacterium to distinguish between its own DNA and any 'foreign' DNA which lacks the characteristic host modification pattern. This difference renders an invading foreign DNA susceptible to attack by restriction enzymes that recognize the absence of methyl groups at the appropriate sites. Hence, these systems may pose a serious barrier to gene transfer. Restriction and modification systems in mollicutes have been reviewed by Sitaraman and Dybvig (2002). Generally, the restriction-modification systems characterized in mollicutes in the past were defined as type II restriction-modification (R-M) systems, in which the endonuclease is separate from the methylase. More recently, *M. pulmonis* was found to possess a more complex type I R-M system, built of the

S, M, and R subunits. These subunits form a holoenzyme which performs both the endonuclease and DNA methylase activities (Dybvig et al. 1998).

CHROMOSOMAL REARRANGEMENTS

The mollicute chromosome is a genetically dynamic structure undergoing frequent rearrangements, insertions, deletions, and inversion of genes, or entire genomic segments. The mollicute genome may carry repetitive elements of various types, such as insertion-like sequences, integrated viral or plasmid genomic segments, or repetitive elements of endogenous origin, being constituted of segments of mycoplasmal genes, as well as recombinases recently identified in mycoplasma genomes (Flitman-Tene et al. 2003; Glew et al. 2002; Sitaraman et al. 2002). These are the elements which facilitate, through homologous recombination, chromosomal rearrangements, as well as loss of genomic material by deletion of intervening sequences during recombination, a process presumably taking place during the reductive evolution of mollicutes (Rocha and Blanchard 2002). The various types and mechanisms of chromosomal gene rearrangements in mycoplasmas have recently been extensively reviewed by Sitaraman and Dybvig (2002). Chromosomal rearrangements that play a crucial role in antigenic variation are discussed in the section on Antigenic variation.

INSERTION-LIKE ELEMENTS

Repetitive elements resembling the large IS*3* family of bacterial insertion sequences appear to be rather prevalent in mollicutes (Razin et al. 1998). However, of these, only IS*1138* was shown to actively transpose in *M. pulmonis* (Bhugra and Dybvig 1993), while all the other elements that failed to show transposition within the mycoplasma chromosome are usually referred to as insertion (IS)-like elements. The *M. pulmonis* IS*1138* is the only mycoplasmal IS element shown to transpose in the *M. pulmonis* chromosome at high frequency. The predicted amino acids encoded by the major IS*1138* ORF share significant similarity with the transposases of the IS*3* family, suggesting that IS*1138* has a potential for development into a cloning vector and/or mutagenesis vehicle in *M. pulmonis*. It should be emphasized that IS*1138* is species-specific to *M. pulmonis*, but it is ubiqutous among its strains (Bhugra and Dybvig 1993).

Another species-specific IS element is IS*1634* that belongs to the IS4 family. It was identified in *M. mycoides* subsp. *mycoides* SC. This pathogenic mycoplasma is distinguished by its exceptionally high number of insertion elements. Its genome was found to carry about 30 copies of IS*1634*, and about 20 copies of another, less specific, IS*1296* element, comprising together about 7 percent of the total genomic DNA (Vilei et al. 1999). One may speculate that the numerous copies of these elements play a role in regulating the expression of the organism's genetic potential, including invasion ability into host tissues, characterizing this highly virulent mycoplasma (Vilei et al. 1999). A new type of IS element, named IS1630, was discovered in *M. fermentans* by Calcutt et al. (1999a). It belongs to the IS30 family of transposons and has the striking ability to target and duplicate inverted repeats of variable length and sequence during transposition. In addition, the IS elements may influence gene expression, either by directly inactivating genes upon insertion or by providing promoters that increase the transcription of downstream genes. Insertion of IS-like elements into mycoplasma genomes can be expected to mediate high-frequency genomic rearrangements. In fact, chromosomal rearrangement activity accompanied insertion of specific IS-like elements into the chromosome of clinical isolates of *M. fermentans*. Insertion occurred at extremely high frequencies, as detected by genomic hybridization analysis of randomly subcloned colonies (Hu et al. 1998).

More recently, *M. fermentans* was also found to carry a large (approximately 23 kb) genetic element that is present in four copies in the *M. fermentans* PG18 chromosome, accounting for approximately 8 percent of the genome (Calcutt et al. 2002). These novel elements resemble conjugative, self-transmissible, integrative elements (named constins) and, surprisingly, are devoid of known integrases, transposases, or recombinases, suggesting that a novel enzyme may be employed in excision and integration of these large elements. The skewed distribution and varied sites of chromosomal integration of the elements among *M. fermentans* isolates, and in two other mycoplasma species, suggest that these elements play a role in promoting genomic and phenotypic variations in mycoplasmas.

PATHOGENICITY

Most mollicutes live as commensals, and in many arthropods they may even be considered as symbionts. In the case of pathogenic mycoplasmas, infections are rarely of the fulminant type, but rather follow a chronic course. Extensive descriptions of the diseases caused by mollicutes to humans, animals, plants, and insects can be found in *The Mycoplasmas* volume IV (Razin and Barile 1985) and volume V (Whitcomb and Tully 1989), in Maniloff et al. (1992) and in Razin and Herrmann (2002).

A variety of human diseases of unknown etiology, are also linked to mycoplasmas. These include the possible role of mycoplasmas as cofactors to AIDS pathogenesis, the Gulf War syndrome, and other diseases of unexplained etiology, such as chronic fatigue syndrome, fibromyalgia, Crohn's disease, and various arthritides (Baseman and Tully 1997; Endresen 2003; Lo et al. 2000). While the association of mycoplasmas with many of these diseases remains doubtful, the possible role of

mycoplasmas in AIDS activation attracted the most attention during the 1990s. The substantial number of studies carried out on the AIDS-associated mycoplasmas, *M. fermentans* and *M. penetrans*, have considerably advanced our knowledge of mycoplasma cell biology and host immune modulation by mycoplasmas, as evidenced in many sections of this chapter. However, the proposed role of mycoplasmas in AIDS activation remains controversial and doubtful (Baseman and Tully 1997; Brenner et al. 1996; Taylor-Robinson and Furr 1998). Mycoplasmas were suspected long ago to be causative agents of rheumatoid arthritis (RA), but solid evidence to support this was lacking. The recent emphasis on the immunomodulatory effects by mycoplasmas (see Immune system modulation section) and more so, detection of mycoplasmal DNA, particularly that of *M. fermentans*, in synovia of RA patients (Schaeverbeke et al. 1999), as well as the prevalence of antibodies to *M. fermentans* in the patients (Horowitz et al. 2000) suggest that in some RA patients *M. fermentans* may play a role in initiating or perpetuating synovitis (Blanchard and Bebear 2002).

Disease manifestations

Table 2 presents the sites of colonization and pathogenicity of mollicutes infecting humans. The best established human pathogen is *M. pneumoniae*. The effects of *M. pneumoniae* on humans include subclinical infection, upper respiratory disease, and bronchopneumonia (reviewed by Jacobs, 2002; Talkington et al. 2001). Most human infections do not progress to a clinically evident pneumonia. When pneumonia occurs, the onset is generally gradual and the clinical picture is one of a mild to moderately severe illness, with early complaints referable to the lower respiratory passages. Radiography frequently reveals evidence of pneumonia before physical signs are apparent. Involvement is usually limited to one of the lower lobes of the lungs, and the pneumonia is interstitial or bronchopneumonic. The course of disease varies; remittent fever, cough, and headache persist for several weeks. One of the most consistent clinical features is long convalescence, which may extend from 4 to 6 weeks. Several infrequent complications following *M. pneumoniae* infections have been noted, including hemolytic anemia, polyradiculitis, encephalitis, aseptic meningitis, and central nervous system illness such as Guillain–Barré syndrome. In addition, pericarditis and pancreatitis have been observed. Although these sequelae have been commonly related to the suspected immunopathology of *M. pneumoniae* disease, *M. pneumoniae* has been increasingly demonstrated in the affected organs by PCR, or in some cases even by culture (Narita et al. 1995; Bencina et al. 2000).

Growing evidence suggests that *Ureaplasma urealyticum* causes nongonococcal urethritis in men free of *Chlamydia trachomatis*, an established agent of nongonococcal urethritis. The wide occurrence of *U. urealyticum* in sexually active, symptom-free adults hampers research in this field. Evidence is based primarily on the production of nongonococcal urethritis symptoms in ureaplasma-free and chlamydia-free volunteers by intraurethral inoculation of *U. urealyticum* (Taylor-Robinson and Furr 1998). Ureaplasmas have also been associated with chorioamnionitis, habitual spontaneous abortion, and low-weight infants (Gerber et al. 2003). *Mycoplasma hominis*, a common inhabitant of the vagina of healthy women, becomes pathogenic once it invades the internal genital organs, where it may cause pelvic inflammatory diseases, such as tubo-ovarian abscess or salpingitis (Taylor-Robinson and Furr 1998).

It has been suggested that *Mycoplasma genitalium*, isolated in 1981 from the urethral discharge of two homosexual men, may account for the tetracycline-responsive, nongonococcal urethritis cases in which chlamydias and ureaplasmas could not be isolated (about 20 percent of all cases). However, *M. genitalium* is so fastidious that very few clinical isolates have so far been made on the best mycoplasma media available. Only the more recent application of specific PCR amplification of the organism's DNA in clinical specimens has provided experimental proof for the relative prevalence of *M. genitalium* in the human urogenital tract and its apparent role in chronic male urethritis (Taylor-Robinson and Furr, 1998; Totten et al. 2001) and possibly also in acute endometritis and cervicitis in women (Cohen 2002; Manhart et al. 2003).

Many *Mycoplasma* species are established pathogens of farm animals, causing contagious pleuropneumonia, mastitis, and conjuctivitis in cattle, goats, and sheep, and chronic respiratory disease and arthritis in swine, chicken, and laboratory animals (Frey 2002).

Molecular basis of mycoplasma pathogenicity

OXIDATIVE DAMAGE AND MEMBRANE FUSION

Potent toxins have not been associated with mycoplasmas. The mildly toxic byproducts of mycoplasma metabolism, such as hydrogen peroxide and superoxide radicals, have been incriminated as causing oxidative damage to host cell membranes. Extensive studies by Almagor et al. (1986) led to the formulation of the following pathological events of *M. pneumoniae* infection (Figure 9). The H_2O_2 and superoxide radicals (O_2^-) continuously generated by the adhering mycoplasmas penetrate into the host cell. As a result of O_2^- accumulation, gradual irreversible inhibition of host cell catalase is induced, thereby causing intracellular H_2O_2 accumulation. This, in turn, may cause product inhibition of the host cell superoxide dismutase. The process is self-perpetuating and results in increased levels of O_2^- and H_2O_2, which induce progressive oxidative damage

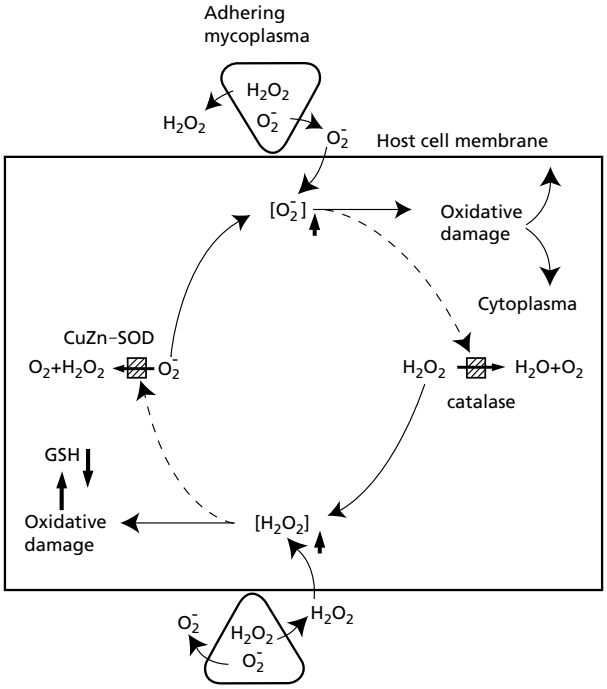

Figure 9 *Schematic presentation of the proposed mechanism for oxidative tissue damage by mycoplasmas. GSH, glutathione; SOD, superoxide dismutase (courtesy of Kahane 1984).*

to vital cell constituents. Thus, malonyldialdehyde, a known oxidative product of membrane lipids, was shown to accumulate in cells infected by *M. pneumoniae*. Supportive of the role of hydrogen peroxide in mycoplasma pathogenicity are the recent findings of Vilei and Frey (2001) showing that the higher virulence of the African strains of *M. mycoides* subsp. *mycoides* SC may be associated with their ability to transport and metabolize glycerol. Peroxide production in glycerol-containing growth media was high for the African strains, but very low for the much less virulent European strains that lack the genes for glycerol transport. A question most frequently asked is how the mycoplasmas, lacking antioxidants like catalase and superoxide dismutase, protect themselves against oxidative stress. The finding by Dhandayuthapani et al. (2001) of methionine sulfoxide reductase in *M. pneumoniae* and *M. genitalium*, an antioxidant enzyme that catalyzes the reduction of methionine sulfoxide residues in proteins to methionine, may play an important role in protecting mycoplasma protein structure from oxidative damage.

The lack of a cell wall in mycoplasmas is expected to facilitate direct contact of the mycoplasma membrane with that of its eukaryotic host, creating a condition which, in principle, could lead to fusion of the two membranes, enabling transfer or exchange of membrane components and 'injection' of mycoplasmal cell constituents into the host cell (Razin et al. 1998; Rottem 2002c). Unique *M. fermentans* glyceroglycolipids containing phosphocholine (Rottem 2002a) exhibit fusogenic properties, in addition to serving as important surface immunogens, inducing cytokine secretion. The possibility that the synthesis of the choline-containing lipids by *M. fermentans* may cause choline depletion in the host has been brought up by Ben-Menachem et al. (2001). Choline is an essential component of eukaryotic cell membranes. *M. fermentans* was found to deplete the choline component of an astrocyte cell culture leading to apoptosis of the cells, an effect that could be prevented by the addition of free choline to the growth medium. Whether choline deficiency can occur in patients infected by *M. fermentans* is obviously a moot point.

INTRACELLULAR LOCATION

Human and animal mycoplasmas were shown to be taken up by polymorphonuclear leukocytes and macrophages (Marshall et al. 1995), and intracellular location of spiroplasmas and phytoplasma within salivary gland cells of insects is an integral part of their life cycle. However, the question of whether animal mycoplasmas can enter epithelial cells has not been easy to resolve, and for a long time this question was answered in the negative. The stimulus to re-examine this issue came from the studies by Lo (1992) showing the intracellular location of *M. fermentans* (strain incognitus) in a variety of nonphagocytic cells in AIDS patients. This finding was strengthened by the discovery of a new human mycoplasma capable of entry into a variety of human cells in vivo and in vitro, named accordingly *M. penetrans* (Lo et al. 1993).

The mechanism of cell entry by mycoplasmas is still unclear. While mycoplasmas, such as *M. penetrans* and *M. genitalium* appear to enter the cells through their specialized tip structure (Jensen et al. 1994; Lo et al. 1993), other mycoplasmas shown to internalize, such as *M. fermentans* and *M. hominis*, have no tip structures (Taylor-Robinson et al. 1991). Following contact of *M. genitalium* with human lung fibroblasts the plasma membrane of the cells appeared to be forced inward to form a cup or a depression. The membrane pockets resembled clatherin-coated pits, suggesting that the mycoplasma might adhere to and enter the cells by a site-directed, receptor-mediated event, resembling cell entry by chlamydia (Mernaugh et al. 1993). Host cell entry appears to depend on the assembly of actin microfilaments and organized microtubules, as cytochalasin D prevented invasion by *M. penetrans* (Rottem 2002c). It appears that *M. penetrans* stimulates host cell phospholipases to cleave membrane phospholipids, thereby initiating a signal transmission cascade. This cascade triggers cytoskeletal rearrangements, allowing the internalization of *M. penetrans*. The presence in the host cell cytoplasm of mycoplasmas, some of which may not even be enclosed within a vacuole, may expose the cytoplasm and the nucleus to mycoplasmal hydrolytic enzymes, such as proteases, nucleases, and phospholipases (Rottem 2002c).

Whether the internalized mycoplasmas replicate intracellularly remains to be resolved. The study by Dallo and Baseman (2000) indicated that intracellular *M. pneumoniae* and *M. penetrans* infectivity in human cells could be detected for over 6 months, while *M. genitalium* survival in the cells was detected by PCR for long periods. Moreover, specific inhibition of eukaryotic nuclear DNA synthesis by aphidicolin enabled the selective detection of mycoplasmal DNA synthesis, supporting their intracellular replication (Dallo and Baseman 2000).

It should be emphasized that intracellular location, if even for a short period, may protect the mycoplasmas against the effects of the host immune system and antibiotics, and to some extent account for the difficulty of eradicating mycoplasmas from infected cell cultures. Thus, intracellular residence, which sequesters mycoplasmas, promotes the establishment of latent or chronic infection states, and circumvents mycoplasmacidal immune mechanisms and selective drug therapies (Bebear and Bebear 2002). Different aspects of the interaction of mycoplasmas with host cells, including fusion of the parasite with the host cell membrane, invasion into host cells, and the implications of these processes on mycoplasma pathogenicity, have been recently reviewed in depth by Rottem (2003).

CLASTOGENIC AND ONCOGENIC EFFECTS

The potent mycoplasmal nucleases, combined with superoxide radicals, may be responsible for clastogenic effects. In fact, reports on chromosomal aberrations, altered morphology, and cell transformation in cell cultures infected by mycoplasmas have appeared rather sporadically since the early 1960s. Interest in this subject has been rekindled recently following the claims that *M. penetrans* may be a cofactor in the induction of HIV-associated Kaposi's sarcoma. Results of experiments with cultured mouse embryo cells reported by Tsai et al. (1995) appeared to support an oncogenic potential of the AIDS-associated mycoplasmas *M. fermentans* and *M. penetrans*. However, instead of acute transformation, a multistage process in promotion and progression of malignant cell transformation with long latency was noted. Only after 18 passages (1 week per passage) of persistent infection with mycoplasmas, an irreversible form of transformation, that included the ability to form tumors in mice, was achieved. It should be pointed out, however, that the tumors had developed very slowly and not in all mice. The irreversible phase of transformation coincided with permanent karyotypic alterations. Once induced, chromosomal alterations continued to accumulate both in cultured cells and in animals without the continued presence of the transforming mycoplasmas. Hence, the mycoplasma-mediated multistage oncogenesis exhibited many characteristics found in the development of human cancer (Lo 2002). Nevertheless, the real biological significance of these findings warrants further investigation.

Another related issue concerns the induction of apoptosis by mycoplasma infection (reviewed by Lo 2002). DNA fragmentation, a common biochemical hallmark of apoptosis, is generally considered to be catalyzed by endogenous endonucleases. In light of the relatively large amounts of nucleases expressed by mycoplasmas (Bendjennat et al. 1999), Paddenberg et al. (1998) tested the effects of *M. hyorhinis* infection of NIH 3T3 cells, checking for parameters characteristic of apoptosis. The infected cells exhibited intranucleosomal DNA degradation into multimers of 200 bp, forming a ladder in agarose gels. Nuclease activities were detected in cell homogenates and culture supernatants. Their mycoplasmal origin was indicated by inhibition of their expression following chloramphenicol treatment of the infected cells. Furthermore, enriched mycoplasmal nucleases, purified from culture supernatants of *M. hyorhinis* and *M. penetrans* could reproduce the apoptotic effects of mycoplasmal infection (Paddenberg et al. 1998; Bendjennat et al. 1999). Apoptotic effects were also observed in lymphocyte and epithelial tumor cell lines infected by mycoplasmas, pointing to the possibility that the numerous cases of 'spontaneous apoptosis' reported in cell cultures are, in fact, the results of an undetected mycoplasma infection (Paddenberg et al. 1998; Stolzenberg et al. 2000). The finding that *U. urealyticum* induces apoptosis in human lung epithelial cells and macrophages may support the proposed role of the ureaplasmas in chronic lung disease of premature infants (Li et al. 2002). Some insight into the mycoplasmal cell components that induce apoptosis has been presented by Into et al. (2002) showing that mycoplasmal membrane lipoproteins induce necrotic and apoptotic cell death in monocytes/macrophages and lymphocytes. Apoptosis was induced by caspases (cysteine proteases) through Toll-like receptor 2 (TLR2)-mediated signaling.

PATHOGENICITY-ASSOCIATED GENES

A promising genetic approach, based on transposon *Tn4001* mutagenesis, has been applied to study pathogenicity factors of *S. citri* (Foissac et al., 1997; Gaurivaud et al. 2000a, b). One of the *Tn4001* mutants did not multiply in the leafhopper vector and, therefore, could not be transmitted to the plant. Another mutant multiplied well in the plant, but did not induce symptoms. It was found that in this nonpathogenic mutant, *Tn4001* was inserted in the spiroplasmal fructose PTS operon, abolishing the ability of the spiroplasma to utilize fructose, probably affecting in this way its pathogenicity. The most likely explanation is that utilization of fructose in the plant sieve tubes by the pathogenic spiroplasmas may interfere with the normal physiology of the plant causing chlorosis, stunting, and wilting (Bai and Hogenhout 2002). It would be of interest to test if

pathogenicity of the mutant can be restored by complementation with the wild-type genes, like the successful restoration of motility in a *Tn4001 S. citri* mutant by complementation with the wild-type genes (Jacob et al. 1997; see Motility section). Continuing this line of research, Jagoueix-Eveillard et al. (2001) embarked on the study of plant genes that are deregulated following infection by mollicutes. These rather preliminary studies revealed that cDNAs of periwinkle plants are differently expressed following infection with *S. citri* or by phytoplasmas. Clearly, much more work has to be carried out to identify the metabolic pathways modified during plant–mollicute interactions, but the genetic approach appears to be very promising.

The lysogenic bacteriophage MAV1 infecting *M. arthritidis* (Voelker and Dybvig 1999; Washburn and Cole 2002) appears to play an important role in the pathogenicity of this murine mycoplasma. All virulent *M. arthritidis* strains carry MAV1 DNA integrated at various sites of the mycoplasmal chromosome, while avirulent strains lack MAV1. Furthermore, the arthritogenic potential of a low-virulence *M. arthritidis* strain could be considerably enhanced by lysogenization with MAV1. The mechanism by which MAV1 enhances virulence is not known. The fact that several MAV1-negative strains are not completely avirulent suggests that MAV1 integration may activate other virulence factors, such as the *M. arthritidis* superantigen MAM (Washburn and Cole 2002), or increase the expression of *M. arthritidis* cytadhesins. The recent identification in MAV1 of a gene, named *vir*, apparently encoding for a lipoprotein, may provide some direction to future research, assuming that the phage-derived lipoprotein is incorporated into the cell membrane of the avirulent *M. arthritidis*, inducing in some unknown way the conversion of the avirulent to the virulent phenotype (Voelker and Dybvig 1999; Tu et al. 2002).

Adhesion to host cells

Most human and animal mycoplasmas adhere tenaciously to the epithelial linings of the respiratory or urogenital tract, rarely invading tissues. Hence, they may be considered as surface parasites. Adhesion of mollicutes to host cells is a prerequisite for colonization by the parasite and for infection. The loss of adhesion capacity by mutation results in loss of infectivity, and reversion to the cytadhering phenotype is accompanied by regaining infectivity and virulence. The best-defined mycoplasmal cytadhesins are those of *M. pneumoniae* and *M. genitalium*. Detailed information on the genes and molecular properties of the encoded cytadhesin proteins of these mycoplasmas (P1, MgPa, and P30) can be found in several reviews (Razin and Jacobs 1992; Razin et al. 1998; Krause and Balish 2001; Balish and Krause 2002).

Although the above-mentioned cytadhesins fulfill the major role in cytadhesion of *M. pneumoniae* and *M. genitalium*, the process appears to be multifactorial, involving in addition to the major cytadhesins, a number of 'accessory' membrane proteins. These 'accessory' proteins act in concert with cytoskeletal elements to facilitate the lateral movement and concentration of the cytadhesin molecules at the attachment tip organelle and provide the scaffolding for the assembly of this organelle (see Cellular morphology and cytoskeletal elements section). Much of the recent effort, forming part of the *M. pneumoniae* genome project, has been directed to the molecular definition of the attachment organelle components, forming part of the mycoplasmal cytoskeleton ('Triton shell'). Some of the genes and the encoded proteins of the *M. pneumoniae* Triton shell (30 kDa; 40 kDa; 90 kDa; P65; P200; HMW1; HMW2, HMW3) have been identified and characterized (Table 5) and have recently been compared to their homologs in *M. genitalium* (Dhandayuthapani et al. 2002). Interestingly, the proteins are proline rich and exhibit repeat sequences and other motifs reminiscent of eukaryotic cytoskeletal proteins. Thus, the C-terminal domain of P30 shows substantial sequence homology with the C-terminus of P1 and exhibits immunological cross-reactivity with fibrinogen, keratin, and myosin, a finding which may be associated with the autoimmune sequelae following *M. pneumoniae* infections (Romero-Arroyo et al. 1999).

The HMW1 protein appears to be responsible for targeting (trafficking) the P1 adhesin to the attachment organelle (Balish et al. 2001) while the 40- and 90-kDa proteins anchor P1 to the membrane of the organelle in a way that allows mutual and intimate interactions of P1

Table 5 M. pneumoniae *membrane and cytoskeletal proteins associated with cytadherence*[a]

Protein	Size (kDa)	Features and presumed role of protein
P1	169	Major adhesin, proline-rich C-terminus
P30	30	Adhesin, proline-rich C-terminus
HMW1	112	Cytoskeletal protein, targeting P1 to attachment tip
HMW2	216	Cytoskeletal coiled-coil protein at attachment tip
HMW3	74	Terminal button and core shaft of attachment tip
B	90	Scaffolding role, anchoring P1 to attachment tip
C	40	Scaffolding role, anchoring P1 to attachment tip
P65	47	Surface protein, proline-rich acidic domain
P200	117	Cytoskeletal protein, role in cytoadherence is uncertain

a) For details see Balish and Krause (2002).

with cytoskeletal forming or associated proteins. Loss of the 40- and 90-kDa proteins by mutation results in the loss of the attachment organelle, cells becoming spherical, and nonadherent (Layh-Schmitt and Harkenthal 1999). The genes for HMW1, HMW3, and P65 are part of the *hmv1* locus, along with the gene for P30. The *hmv2* gene for HMW2 lies about 160 kb from the *hmw1* locus. Yet, transposon insertions into *hmw2* resulted in the simultaneous loss of HMW1, HMW3, and P65, as well as of several other cytadherence-associated proteins, probably due to accelerated endogenous proteolysis (Krause and Balish 2001; Jordan et al. 2001). Thus, it appears that HMW2 functions as a cytoskeletal organizer and is required for the stable maintenance of the above proteins (Fisseha et al. 1999). The complex interrelationships between the components of the attachment organelle underscores the need for the proper ordered assembly of this organelle that must also be coordinated with cell division (Fisseha et al. 1999; Krause and Balish 2001; Seto et al. 2001).

The avian pathogen *M. gallisepticum* also carries an attachment organelle (Figure 6) and its cytadherence, like that of *M. pneumoniae*, is a complex multifactorial process involving the coordinate action of a primary cytadhesin molecule GapA and several additional cytadherence-associated proteins in a manner reminiscent of the complex cytadherence mechanism of *M. pneumoniae*. The gene *crmA* (cytadherence-related molecule A), located immediately upstream of *gapA*, has significant sequence homology to the ORF6 of the *M. pneumoniae* P1 operon (Mudahi-Orenstein et al. 2003; Papazisi et al. 2000; Winner et al. 2003) and must be present together with GapA to mediate cytadherence of *M. gallisepticum* (Papazisi et al. 2002a). An *M. gallisepticum* gene encoding a 105 kDa protein, showed 45 percent homology to the *M. pneumoniae* P1 gene, 46 percent homology to the *M. genitalium* MgPa adhesin gene, and 47 percent homology to the *M. pirum* P1-like protein gene (Goh et al. 1998). Along the same line, the P30 adhesin gene of *M. pneumoniae* shows 40.9 percent identity with the 32 kDa protein gene of *M. gallisepticum*, and 31.4 percent identity with the *M. genitalium* P32 cytadhesin gene (Hnatow et al. 1998). Collectively, these findings suggest the presence of a family of cytadhesin genes conserved among the pathogenic mycoplasmas belonging to the pneumonia phylogenetic clade. One may speculate that these cytadhesin genes originate from the same ancestral operon.

M. hyopneumoniae adheres to the cilia of the respiratory epithelium of swine (Young et al. 2000). The gene coding for the ciliary cytadhesin was cloned and sequenced and its 97 kDa protein product was characterized (Minion et al. 2000). The R1 region near the carboxy terminus of the protein mediates the adhesion of the mycoplasmas to the cilia. Eight R1 repeating units are required for cilia binding. Interestingly, *M. flocculare*, genetically closely related to *M. hyopneumoniae*, lacks the 97 kDa ciliary cytadhesin gene (Blank and Stemke 2001), possibly explaining the lack of pathogenicity of this mycoplasma to swine. Cytadherence of *M. hyopneumoniae* causes a fast increase in the intracellular free Ca^{2+} concentration in the infected porcine ciliated tracheal epithelial cells probably causing damage and loss of the ciliary epithelium (Park et al. 2002).

The receptors on the host cell membranes responsible for mycoplasma attachment, identified so far, are mostly sialoglycoconjugates and sulfated glycolipids (Kitzerow et al. 1999; Razin and Jacobs 1992; Zhang et al. 1994). The glycoprotein fibronectin, known to serve as a host receptor in the binding of streptococci, staphylococci, mycobacteria, borrelia, and treponema appears also to serve as a receptor in the binding of *M. penetrans* (Giron et al. 1996) and of *M. pneumoniae* (Dallo et al. 2002). Somewhat unexpected was the identification of the cytoplasmic elongation factor Tu and the E1β subunit of pyruvate dehydrogenase as the mycoplasmal fibronectin binding proteins. It appears that the *M. pneumoniae* EF-Tu and the pyruvate dehydrogenase subunit, in addition to their major cytoplasmic biosynthetic and metabolic roles, can be translocated to the cell surface and show multifunctionality. Along this line is the recent identification of glyceraldehyde-3-phosphate dehydrogenase of *M. genitalium* acting, in addition to its important metabolic role, as an adhesin to host cell surface mucin (Alvarez et al. 2003). It is worth mentioning that Layh-Schmitt et al. (2000) detected the E1α subunit of pyruvate dehydrogenase as part of a crosslinked complex of the *M. pneumoniae* cytadhesins. What is the weight to be given to the fibronectin cytadhesion system when compared to the well-established specific cytadhesin system (P1, P30, etc.) described above? Does the novel system fulfill an auxilliary role in mycoplasmal attachment to host cells? Obviously, these are issues that require further study.

It may not be surprising that the surface exposed cytadhesin proteins are amongst the most immunodominant mycoplasmal antigens. Furthermore, at least some mycoplasmal cytadhesins, for example, the Vaa proteins of *M. hominis* (Kitzerow et al. 1999), the Vsp proteins of *M. bovis* (Sachse et al., 2000), the MMA2 protein of *M. arthritidis* (Washburn et al. 1998), the pMSA and pMSB hemagglutinins of *M. synoviae* (Bencina et al. 2001), as well as certain hemagglutinnins of *M. gallisepticum* (Boguslavsky et al. 2000) (Table 6) exhibit the phase- and size-variation properties to be discussed in the next section.

Antigenic variation

The mycoplasma plasma membrane, being exposed to the external environment, is the cell organelle that comes into contact with the constituents of the host immune system. Lacking the protection of a rigid cell wall, mycoplasmas are particularly sensitive to growth

Table 6 *Surface variable antigenic systems in mycoplasmas*

Organism	Genes involved	System components	Mechanism of variation
M. hyorhinis	*vlp* gene family	Lipoproteins	Strand slippage (poly A) Yogev et al. (1991a)
M. bovis	*vsp* gene family	Lipoprotein, chimeric lipoprotein	Site-specific inversions homologous recombination (Lysnyansky et al. 2001; Flitman-Tene et al. 2003)
M. agalactiae	*avg* gene family	Lipoproteins	
M. gallisepticum	PMGA (*vlh*) gene family	Lipoproteins	Strand slippage (GAA) (Glew et al. 1998)
	pvpA (single copy)	Integral protein (not lipid-modified adhesin)	Point mutation (GAA) (Boguslavsky et al. 2000)
M. hominis	*lmp* gene family	Lipoproteins (p120; p135)	Unknown; phase-variable masking (Ladefoged and Christiansen 1998)
	vaa single copy gene	Lipoprotein adhesin	Strand slippage (poly A) (Zhang and Wise 1997)
M. fermentans	P78/part of an ABC transporter operon	Lipoprotein	Strand slippage (poly A)(Theiss and Wise 1997)
	malp single copy gene	Lipoprotein	Posttranslational modification (Calcutt et al. 1999b)
M. penetrans	*mpl* genes (P35, P34A,P34B,P38)	Lipoproteins	Promoter inversion (Horino et al. 2003)
M. arthritidis	*maa2* single copy gene	Lipoprotein adhesin	Strand slippage (poly T) (Washburn et al. 1998)
M. mycoides subs. *mycoides* (SC)	*vmm* gene	Lipoprotein	Strand slippage $(TA)_n$ insert in promoter region (Persson et al. 2002)
M. synoviae	*vlh* pseudogene family	Chimeric lipoproteins adhesins	Gene conversion (Noormohammadi et al. 2000)
M. pulmonis	*vsa* gene family	Lipoproteins	Site-specific inversions (Shen et al. 2000)

inhibition and lysis by antibodies and complement. Yet, despite this marked sensitivity, mycoplasma infections are usually chronic in nature, indicating the frequent failure of the host defense mechanisms to eradicate the parasites. It appears that many pathogenic mycoplasmas possess rather sophisticated mechanisms for rapid adaptation to changing microenvironments. Reversible switching of the expression and modification of major membrane protein antigens on the mycoplasma surface provides an effective escape from rapid destruction by the host immune system (reviewed by Yogev et al. (2002)). Considering the chronic nature of mycoplasmal infections, it would appear that the constant selection of subpopulations, rather than adaptation of an entire population to an environmental change, is responsible for the heterogenous populations of these organisms.

Table 6 presents the mycoplasmal antigenic variation systems studied thus far. The data enable some generalizations to be made: (1) Most of the systems consist of lipoproteins exposed on the external cell surface and anchored to the membrane via acyl chains. (2) The lipoproteins, functioning as dominant cell antigens, carry repetitive sequences that can spontaneously vary in size through changes in the number of these motifs (size variation). Moreover, the lipoproteins vary not only in size, but may be subjected to 'on' and 'off' expression states (phase variation). (3) Cloning and sequencing of the lipoprotein genes has revealed that the encoded proteins consist of three regions: the N-terminal leader region, an intermediate domain, and the C-terminal domain comprising of reiterated sequences arranged in tandem. Deletion of the leader sequence on processing of the protein leaves a cysteine residue at the N-terminus free for acylation, preferentially by palmitic and myristic acids. The resulting lipid-modified protein is enchored to the outer surface of the membrane through its lipid moiety. (4) In most cases, each mycoplasma strain carries a family of lipoproteins, encoded by multiple variant genes in several versions. The genes are expressed in various combinations and at varying lengths on the organism's surface. Interestingly, in the case of a large family of genes, only one or two genes are expressed at a given time, the others are translationally silent (Bhugra et al. 1995; Glew et al. 1998; Lysnyansky et al. 1999; Sasaki et al. 2002). (5) In many cases, the surface exposed lipoproteins also function as cytadhesins (Table 6).

The microbial population as a whole may spontaneously and randomly generate distinct cell populations with varied antigenic phenotypes, 'heterotypes', that will

survive the specific host response capable of eliminating the predominant 'homotypes'. The frequency of occurrence of such genetic variants is strikingly high (10^{-4}–10^{-2} per cell per generation, compared to 10^{-6}–10^{-8} of a normal occurring mutation). Lipoproteins may not be the only variable mycoplasma membrane proteins exposed on the cell surface. Thus, the *M. gallisepticum* PvpA is a non-lipid modified, integral phase-variable membrane protein (Boguslavsky et al. 2000; Yogev et al. 2002). Nevertheless, this mycoplasma carries in addition a highly immunogenic lipoprotein, pMGA, undergoing high-frequency phase variation spontaneously and independently (Glew et al. 1998). There is solid experimental evidence for the occurrence of antigenic variation in vivo. Thus, multiple isolates of *M. hominis* obtained from the same joint of a septic arthritis patient during a 6-year period, exhibited antigenic variation, although the strains were indistinguishable by restriction endonuclease analysis (Olson et al. 1991). Field infections of sheep and goats by *M. agalactiae* (Flitman-Tene et al. 2000) and chicken by *M. gallisepticum* (Glew et al. 2000) indicate that high-frequency antigenic phase variation occurs in vivo.

VARIATIONS IN THE *M. PNEUMONIAE* P1 AND P30 ADHESIN GENES

A hot spot for frequent mutation within a structural gene was found in the major cytadhesin gene (P1) of *M. pneumoniae*. Reversible and spontaneous cytadherence-negative and cytadherence-positive mutants were associated with an insertion or deletion of a single nucleotide in a stretch of seven adenines. This mutation resulted in a frameshift and generation of a termination codon causing the premature termination of P1 translation (Su et al. 1989). Large portions of the coding regions of the *M. pneumoniae* P1 operon exist as multiple extragenic copies over the chromosome, named RepMP2/3, Rep MP4, originating in the P1 gene, and Rep MP5 originating in the ORF6 gene of the P1 operon. Relics of these sequences detected in *M. genitalium*, named MgPa repeats, revealed strong sequence similarities to the repetitive DNA sequences from *M. pneumoniae* (Himmelreich et al. 1997). Clinical isolates of *M. pneumoniae* were found to exhibit some nucleotide sequence variation in the P1 gene (Dorigo-Zetsma et al. 2000, 2001). The variable regions in the gene were found to correspond to regions of some of the repetitive elements, suggesting that the P1 gene may vary due to recombination between repetitive regions (Kenri et al. 1999; Su et al. 1993). Accordingly, the *M. pneumoniae* strains have been divided into two groups, I and II (Su et al. 1993).

The 30 kDa (P30) adhesin protein of *M. pneumoniae* also appears to undergo size variation, losing proline-rich repetitive sequences in its C-terminal region. The truncated adhesin showed reduced antigenicity and adhesion capacity. The mechanism underlying the loss of the repeated sequences is not clear, but slipped base pairing during replication or homologous recombination processes between repeated sequences might be the cause of size polymorphism (Layh-Schmitt et al. 1997).

The different genetic mechanisms that form the genetic basis for antigenic variation, including strand slippage, homologous recombination, gene conversions, gene duplications, addition or deletion of tandem repetitive units, and DNA inversions (Table 6) are discussed in detail by Razin et al. (1998) and by Yogev et al. (2002).

Immune system modulation

The complex network of interactions between mycoplasmas and their host immune system involves mycoplasma-induced specific and nonspecific immune reactions. Specific protective defense mechanisms include the production of systemic as well as local anti-mycoplasmal antibodies of different classes and subclasses, stimulation of cell-mediated immunity, and opsonization and phagocytosis of organisms. In some cases, in order for macrophages to kill invading mycoplasmas, some accessory factors provided by the host are required. Thus, alveolar macrophages serving as the primary effector cells in early *M. pulmonis* killing in infected mice depend on the presence in the lungs of the collectin surfactant protein A. Nitric oxide and superoxide radicals produced by the macrophage contribute to the killing either directly or via the production of peroxynitrite ($ONOO^-$) (Hickman-Davis et al. 1999).

The specific reactions elicited by invading mycoplasmas, essential for resistance and protection against mycoplasma infections, have also been shown to play a role in the development of lesions and exacerbation of mycoplasma-induced diseases (Razin et al. 1998). Humoral immunity has a major role in defense against systemic dissemination of mycoplasmal infection, but cellular immune responses may be important in exacerbation of mycoplasmal lung disease. Lack of adaptive response in severe combined immunodeficient (SCID) mice infected with *M. pulmonis* was associated with reduced lung lesion severity and with increased mycoplasmal colonization and disease at extrapulmonary sites. The transfer of naïve spleen cells from immunocompetent mice to *M. pulmonis*-infected SCID mice restored the severity of lung lesions, while the transfer of convalescent anti-*M. pulmonis* serum to the infected SCID mice did not restore lung lesion severity, but prevented arthritis (Cartner et al. 1998).

Although mycoplasmas circumvent phagocytosis (Marshall et al. 1995; Minion 2002), they appear to interact with mononuclear and polymorphonuclear phagocytes, suppressing or stimulating them by a combination of direct and indirect cytokine-mediated effects. For extensive recent reviews on cytokine induction by

mycoplasmas, the reader is referred to Razin et al. (1998) and to Rawadi and Roman-Roman (1998). These immunomodulatory influences depend on both the immune cells and on the mycoplasma species involved. Thus, *M. dispar* manages to bypass phagocytosis by bovine alveolar macrophages because it is covered by a polysaccharide capsule which inhibits activation of macrophages and suppresses the production of TNF-α and IL-1 (Almeida et al. 1992). Other mycoplasmas activate monocytes, macrophages, and brain astrocytes and induce secretion of the proinflammatory cytokines TNF-α, IL-1 and IL-6, chemokines (such as IL-8, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α/β, and granulocyte–monocyte colony-stimulating factors (GM-CSF)), prostaglandins, and active oxygen and nitrogen metabolites (Razin et al. 1998; Ribeiro-Dias et al. 1999; Yirmiya et al., 1997).

In addition to eliciting antimycoplasmal immune responses, mycoplasmas exert a wide range of nonspecific immunomodulatory effects upon cells comprising the immune apparatus (Reyes et al. 1999). Mycoplasmas have been shown to affect the immune system by inducing either suppression, or polyclonal stimulation of B and T lymphocytes, induction of cytokines, increasing cytotoxicity of macrophages, natural killer cells and T cells, enhancing expression of cell receptors, and activation of the complement cascade. The ability of mycoplasmas to immunomodulate host immune responsiveness contributes to their pathogenic properties, enabling them to evade or suppress their host defense mechanisms and establish a chronic, persistent infection (reviewed in Razin et al. 1998; Muhlradt 2002). The cytokine network plays a critical role in the maintenance of immune system homeostasis. The overproduction of some cytokines in response to mycoplasma infections may upset the fragile equilibrium and thereby contribute to triggering or aggrevation of autoimmune diseases (Rawadi and Roman-Roman 1998).

Mycoplasmas are able to stimulate as well as suppress lymphocytes in a non-specific, polyclonal manner, both in vitro and in vivo. It appears that mycoplasmal B cell mitogens differ from bacterial lipopolysaccharides (LPS) in that they are able to activate lymphocytes from C3H/HeJ mice, which respond poorly to LPS, and their inducing potential is unaffected by polymyxin (Brenner et al. 1997). Modulation of monocyte/macrophage activity by mycoplasmas has been demonstrated in vitro by augmented TNF-α-mediated tumoricidal activity towards different tumor cell lines (Razin et al. 1998). The proven capacity of many mycoplasmas to trigger the production of proinflammatory cytokines in vivo may account for much of the pathology observed during mycoplasma infections and might also influence the subsequent interactions of these microorganisms with lymphoid cells (Manimtim et al. 2001). Additionally to affecting various subsets of lymphocytes, mycoplasmas and mycoplasma-derived cell components modulate the activities of monocytes/macrophages and natural killer (NK) cells and trigger the production of a wide variety of upregulating and downregulating cytokines and chemokines (Razin et al. 1998; Rawadi and Roman-Roman 1998). Mycoplasma mediated secretion of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 by macrophages, and of upregulating cytokines by mitogenically stimulated lymphocytes play a major role in mycoplasma-induced immune modulation and inflammatory responses. On the other hand, the ability of some mycoplasmas to induce downregulating cytokines, such as IL-10, IL-13, and possibly also TGF-β may contribute to their ability to evade host immune mechanisms by perturbation of macrophage accessory and phagocytic functions. In this way, decreasing production of proinflammatory cytokines and T-cell proliferation and affecting the balance between different T helper cell subsets. The potential of alternately stimulating or suppressing the immune system would impart a distinct advantage to any pathogen attempting to survive in the hostile and changing environment of the infected host.

As to the mechanism of cytokine induction by mycoplasmas, Rawadi et al. (1998) reported that protein tyrosine kinase is absolutely required by the *Mycoplasma fermentans* lipoproteins (LAMPf) to induce proinflammatory cytokines in monocytic cells. The mycoplasmal membrane lipoproteins bind to a cellular receptor(s) that mediates directly or indirectly protein tyrosine kinase activation. This binding triggers the activation of several signal cascade pathways that lead to the phosphorylation and translocation of transcription factors into the nucleus, resulting in transcription, and/or translation of cytokine mRNAs.

It appears that certain mycoplasmas possess more than a single cell component displaying mitogenic activity, and that mycoplasmal mitogens are not necessarily identical to mycoplasmal cytokine-inducing elements. *M. penetrans* appears to possess several T or B cell mitogens (Brenner et al. 1996; Feng and Lo 1999), including a glycolipid and a major membrane lipoprotein. However, although the mitogenic *M. penetrans* lipoprotein is also highly immunogenic in *M. penetrans*-positive AIDS patients and elicits production of specific antibodies (Lo 1992; Brenner et al. 1996; Feng and Lo 1999), the glycolipid is not recognized in sera of *M. penetrans*-infected individuals.

Of special interest as immunomodulators are the mycoplasmal lipopeptides (MALP) which resemble the classical murein lipoprotein in that they carry a fatty acid-substituted N-terminal S-(2,3-bisacyclooxypropyl) cysteinyl group, but lack the N-acyl long chain fatty acid of the classical bacterial lipoproteins (Muhlradt et al. 1997; Muhlradt 2002). This feature renders these mycoplasmal compounds exceptionally active as in vitro macrophage stimulators. Microgram quantities of the *M. fermentans* MALP-2 were found to stimulate the in vitro, as well as in vivo, release of chemokines from

macrophages, being more potent than LPS on a weight basis (Deiters and Muhlradt 1999; Kaufmann et al. 1999) and the co-administration of MALP-2 with a model antigen resulted in potent enhancement of humoral and cellular antigen-specific immune responses, suggesting the use of the lipopeptide as a promising adjuvant for delivery of vaccine antigens (Rharbaoul et al. 2002). Biogenesis of the MALP-2 lipopeptide from the full-length *M. fermentans* lipoprotein MALP-404 involves the extracellular, site-specific proteolytic processing of the lipoprotein yielding the MALP-2 surface-bound lipopeptide, as well as a stable, released fragment of MALP-404. This defines a new mechanism of 'secretion' in mycoplasmas and a novel means of altering the phenotype of mycoplasmas through posttranslational processing (Davis and Wise 2002). Of phylogenetic interest is the finding that the above mycoplasmal lipoproteins share conserved amino acid motifs with lipoproteins of *B. subtilis*, *Treponema pallidum*, and the relapsing fever *Borrelia* spp. (Calcutt et al. 1999b; Rosati et al. 1999). In summation, the above data indicate that mycoplasmal lipoproteins and lipopeptides are probably the most relevant mycoplasmal components in the early host reaction. The primary target cells are likely to be the alveolar macrophages liberating chemokines that attract further leukocytes. The MALP-2 mediated response being dependent on the signaling molecules Toll-like receptor 2 (TLR2) and the activation of caspases, inducing apoptosis and cell necrosis (Luhrmann et al. 2002; Into et al. 2002; Rawadi 2000).

Another chemically defined immunomodulator, produced by *M. arthritidis*, is MAM, a soluble excreted protein of 26 kDa acting as a superantigen (reviewed in Cole et al. 2000; Washburn and Cole 2002). MAM activates a major fraction of murine T cells and a minor fraction of human T cells, by crosslinking MHC class II to TCR, primarily through zinc-dependent binding to the MHC class II E alpha chain in the mouse and to the DR alpha chain in man (Langlois et al. 2003). Activation of T cells (mainly Vβ6 and Vβ8 TCR subsets in mice and Vβ17 and Vβ3 subsets in humans) induces clonal proliferation and release of lymphokines, including IL-2, IL-4, IL-6, and IFN and nitric oxide (Ribeiro-Dias et al. 2003). It should be emphasized that MAM is the only mycoplasmal superantigen defined thus far. The finding of antibodies to MAM or a portion of the MAM molecule in the sera of RA patients raises the possibility that MAM or MAM-like epitopes crossreactive with MAM may play a role in RA (Cole et al. 2000), but this clearly requires further study before any definite conclusions can be drawn.

Except for the chemically characterized *M. arthritidis* superantigen MAM, the macrophage-stimulating lipopeptides and the phosphocholine-containing phosphoglycolipids of *M. fermentans* (Matsuda et al. 2000; Rottem 2002a) there is a need to identify and isolate additional mycoplasmal cell components that exhibit mitogenic and/or cytokine inducing activities. Likewise, there is very little information on the nature of host cell receptors for the mycoplasmal immune modulators.

EPIDEMIOLOGY AND CONTROL OF INFECTIONS

Transmission

Acquisition of mollicutes can be established by direct contact between hosts or through secondary means, such as aerosols, fomites, food, water, insect vectors, or other carriers, and by nosocomial acquisition, such as organ or tissue transplants (Tully 1996). Many mycoplasmas inhabit the mucous membranes of the respiratory, urogenital, or gastrointestinal tracts of vertebrates, so that the mycoplasmas can be transmitted by direct oral to oral, genital to genital, or oral to genital contact. Thus, mycoplasmas found in the oropharynx may also be found in some cases in the genital tract and vice versa. Respiratory aerosols or fomites apparently play the major role in transmission of respiratory tract mycoplasmas, while neonates can be infected by *U. urealyticum* through in utero infection (Taylor-Robinson and Furr 1998).

Secondary transmission of mycoplasmas can also occur through healthy carriers or through convalescent carriers recovering from acute infections, as apparently occurs in some *M. pneumoniae* respiratory infections in humans (Williamson et al. 1992) and probably in acute and chronic respiratory mycoplasmal diseases in domestic animals. Mollicute transmission after plant grafting of infected tissue to healthy rootstock (Tully and Whitcomb 1992), or through human organ or tissue transplantation have also been described (Tully 1996). Semen contaminated with mycoplasma is an important factor in dissemination of sexually-transmitted diseases in a variety of hosts and should be particularly considered in artificial insemination programs in humans and bovines (Kanakas et al. 1999).

The upper respiratory and genital tracts of human newborns are first colonized with mycoplasmas acquired during passage through the female birth canal. There is now substantial evidence that mycoplasma colonization can occur even earlier in utero by organisms introduced during fertilization or during early pregnancy. Generally, neonatal colonization with both human oral and genital mycoplasmas does not persist and the number and type of mycoplasmas decrease with age. Subsequent reacquisition of the human urogenital mycoplasma flora appears to be directly related to sexual contact (Taylor-Robinson and Furr 1998; Blanchard and Bebear 2002). In vertebrates, acquisition from the maternal genital tract, and in utero transmission (including egg transmission in birds), apparently represents the initial mechanism of infection. Transovarian transmission of spiroplasmas has

been well established in Drosophila species (Williamson et al. 1999).

Host factors also play an important and complex role in determining whether an acquired mycoplasma behaves as a pathogen or is avirulent. Endogenous mycoplasmas may become pathogenic when the human host immune system has been compromised or when surgical intervention or trauma allows mycoplasma commensals to enter the circulation or other host tissues. Common commensals, such as *M. hominis*, *M. salivarium*, and *U. urealyticum* have been shown to induce septicemia and overt mycoplasma invasion of various host tissues and organs, with localization in joints being especially prominent (Blanchard and Bebear 2002).

Since *M. pneumoniae* infections are so common in humans, epidemiological data on these infections are abundant. One of the most puzzling features of *M. pneumoniae* pneumonia is the age distribution of patients. In a survey conducted between 1964 and 1975 of more than 100 000 individuals in the Seattle area, the age-specific attack rate was highest among 5- to 9-year-old children (Foy et al. 1979). Rates of *M. pneumoniae* pneumonia in the youngest age group, 0–4 years old, were about one-half those in school-age children, but considerably higher than in adults. *M. pneumoniae* pneumonia was rarely observed in infants younger than 6 months, suggesting maternally conferred immunity. *M. pneumoniae* accounts for 8–15 percent of all pneumonias in young school-age children. In older children and in young adults, the organism is responsible for approximately 15–50 percent of all pneumonias. Infection with *M. pneumoniae* is worldwide and endemic; it occurs all year round, but shows a predilection for the colder months, apparently because of the greater opportunity for transmission by droplet infection. *M. pneumoniae* appears to require close personal contact to spread; successful spreading usually occurs in families, schools, and institutions. The incubation period is relatively long, ranging from 2–3 weeks. The *M. pneumoniae* disease manifestations and epidemiology have recently been reviewed (Talkington et al. 2001; Jacobs 2002).

U. urealyticum is spread primarily through sexual contact. Colonization has been linked to the frequency of sexual intercourse and the number of sexual partners. Women may be asymptomatic reservoirs of infection (Taylor-Robinson and Furr 1998).

Treatment of infections

The mycoplasmas are sensitive to most broad-spectrum antibiotics, such as the tetracyclines and macrolides, as well as to the newer quinolones, but are resistant to antibiotics that specifically inhibit bacterial cell wall synthesis. Susceptibility profiles of human mycoplasmas to a variety of the 'classical', as well as the newer antibiotics were reviewed by Bebear and Bebear (2002). Recommended guidelines for testing mycoplasma susceptibility to antibiotics are presented by Hannan (2000).

Tetracyclines are widely used in the treatment of genital tract infections, while macrolides are used for respiratory tract infections, mostly by *M. pneumoniae*. Erythromycin, the newer macrolides, the ketolides, and the newer quinolones have equal or sometimes greater activity against mycoplasmas as compared to tetracyclines. Most of the effective antibiotics only inhibit mycoplasma multiplication, and do not kill them. The ketolides and some quinolones have some mycoplasmacidal activity (Taylor-Robinson and Bebear 1997).

Mycoplasmas may develop resistance, either by gene mutation, by acquisition of a resistance gene, or by active efflux of the antimycoplasmal agent (Bebear and Bebear 2002; Pereyre et al. 2002; Raherison et al. 2002; Reinhardt et al. 2002). Thus, integration of the *tetM* gene into the *U. urealyticum* or the *M. hominis* chromosome renders these mycoplasmas resistant to tetracycline. The resistance factor could be transferred from other tetracycline-resistant bacteria by a process resembling conjugation (Roberts and Kenny 1987).

Mycoplasmas may be difficult to eradicate from human or animal hosts or from contaminated cell cultures by antibiotic treatment, either due to resistance of the specific mycoplasma to the antibiotic, lack of mycoplasmacidal activity of the antibiotic, or due to the intracellular location of some of the mycoplasmas. As expected, mycoplasma eradication is particularly difficult in immunosuppressed or immunodeficient patients (Taylor-Robinson and Bebear 1997).

Vaccination

WHOLE CELL VACCINES

The frequent failure of antibiotics and other therapeutic approaches to eradicate mycoplasmas and abort the infectious disease process has led to the conclusion that development of effective vaccines is the most promising approach to control mycoplasma infections. The many efforts to develop effective and safe vaccines to mycoplasmas have been only partially successful. The overall success obtained with formalin-inactivated *M. pneumoniae* vaccines in humans has been extremely disappointing, although inactivated vaccines and live attenuated vaccines have had some success in control of some mycoplasma diseases in animals. No acceptable live vaccine has been developed for human use (reviewed in Ellison et al. 1992). Prior natural infection appears to provide the most effective resistance; however, evidence shows that *M. pneumoniae* infections recur at intervals of several years. These observations suggest that immunity to a single natural infection is of relatively short term, particularly in children, and it may be unrealistic to expect more, or even as much, from artificially induced immunity.

Attenuation of mycoplasma strains tends to reduce virulence and immunogenicity. In most cases, attenuated viable vaccines do not reach the level of protective efficiency required from a commercial vaccine. Killed *M. pneumoniae* vaccines administered intranasally to hamsters are relatively inefficient unless boosted by parenteral inoculation of vaccine (Ellison et al. 1992). Intranasal immunization may be ineffective because the antigenic mass is not retained for a sufficient period in the lungs. On the other hand, parenteral killed vaccines, particularly if combined with adjuvant, do produce adequate protection in terms of reducing pneumonia, although there is only a minimal effect on the number of organisms growing in the lungs. A similar protective effect can be achieved briefly by inoculation with hyperimmune serum. In summary, a single dose of vaccine in a form suitable for clinical use is unlikely to produce lasting immunity to *M. pneumoniae* infection. Stimulation of systemic antibodies may prevent the clinical manifestations of pneumonia, but additional local stimulation with live or killed organisms may be necessary to evoke resistance to colonization.

The advantages of live vaccines are well established, but they frequently suffer from deficiencies. Thus, the live *M. gallisepticum* F-strain provides good protection but may be pathogenic to young chicks. Vaccines based on temperature-sensitive mutants of *M. gallisepticum* are safer than the F-strain, but do not afford the same level of protection. Consequently, Papazisi et al. (2002b) proposed a new approach, based on employing a live, attenuated *M. gallisepticum* strain R_{high} that had lost the *gapA* and *crmA* adhesin genes. Insertion of the adhesin *gapA* gene into the chromosome of the attenuated strain resulted in the expression of a high level of Gap A on the mycoplasma cell surface. Vaccination of chicks with this genetically modified strain produced a high level of antibodies to Gap A, protecting the chicks against infection with the virulent R_{low} *M. gallisepticum*.

PROTEIN VACCINES

An approach worth pursuing is the preparation of vaccines made of antigenic components specifically related to the mycoplasma–host cell interaction, such as mycoplasmal cytadhesins. Guinea pigs preimmunized with purified P1 adhesin protein and subsequently infected with *M. pneumoniae* showed increased levels of *M. pneumoniae*-specific IgG, IgA, and adherence-inhibiting antibodies. However, these animals developed severe lung lesions on challenge, suggesting that it may well be harmful to vaccinate the host with the P1 protein, as it may sensitize the host so that subsequent infection will potentiate host response and lead to a more severe disease (Jacobs et al. 1988; Razin and Jacobs 1992). Another mycoplasmal protein vaccine was developed more recently against *M. hyopneumoniae* (Chen et al. 2001). This vaccine was composed of a recombinant chimera of the nontoxic moiety of *Pseudomonas* exotoxin with a fragment of an *M. hyopneumoniae* cytadhesin overexpressed in *E. coli*. The serological response of pigs to this chimeric protein was promising, but more work is needed to show the effectivity of this vaccine under field conditions.

DNA VACCINES

Genetic immunization (DNA vaccines) is a novel, promising approach to vaccine production that has many of the advantages of live/attenuated vaccines, but with no risk of infection. There is no need for confounding factors, such as adjuvants, hapten carriers, or denaturing agents. It involves introducing DNA encoding a pathogen protein into host cells, using an expression library of the pathogen. The first step in the process is the successful transfection of cells that take up the plasmid. The cDNA, with a strong mammalian promoter in the construct, directs the synthesis of mRNA, leading to the synthesis of the immunogen protein. If the transfected cell is a professional antigen-presenting cell (APC) (e.g. a dendritic cell) that expresses class I and II MHC proteins, as well as costimulatory molecules, the process of antibody production can proceed (Lai and Bennett 1998). The DNA vaccine effectively induces antibodies as well as cytotoxic T lymphocytes. Thus both humoral and cell mediated responses are induced – a distinct advantage. Barry et al. (1995) prepared an *M. pulmonis* library by fusing digested mycoplasmal DNA on to the last exon of the gene encoding the human growth hormone (hGH), using cytomegalovirus as a vector, so that hGH-*M.pulmonis* antigens might be secreted. Theoretically, a library expressing the entire genome of a pathogen could be used as a vaccine. Barry et al. (1995) found that about 1 ng DNA injected into the skin was required to produce an immune response. Mice immunized with *M. pulmonis* libraries and challenged had no culturable mycoplasmas and no lung lesions.

Lai et al. (1994) constructed an *M. pulmonis* genomic library cloned in λgt 11 and transfected into *E. coli*. Clones of *E. coli* expressing a fusion protein reactive with anti-*M. pulmonis* antibodies were transferred orally or intravenously into mice. Expression of fusion proteins in the mice was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) into the drinking water, resulting in local and systemic antibody production and effective protection of the animals against large numbers of virulent *M. pulmonis*. DNA vaccines could also be used to treat animals already infected with *M. pulmonis*. When infected mice were immunized with the DNA vaccine 1 week after infection, the numbers of organisms recovered from tracheolung lavage gradually decreased and were totally eliminated by 4 months (Lai et al. 1997).

It is possible to select the epitope important as an immunogen, by isolating the dominant antigen, prepare

a mAb to it, and use this antibody to select the *E. coli* clone producing the specific fusion protein carrying this epitope. Thus, the DNA vaccine is controllable. Such a specific DNA vaccine was developed for *M. hyopneumoniae*. Following screening of the mycoplasmal clone bank with hyperimmune pig serum, Fagan et al. (1996) identified a clone with a sequence homology to the R2 subunit of ribonucleotide reductase. This clone was expressed in *E. coli* as an 11 kDa protein fused to β-galactosidase. Assessment of the vaccine potential of the fusion protein in pig trials showed the vaccinated pigs to be significantly protected against challenge with a virulent *M. hyopneumoniae*. Use of an attenuated *Salmonella typhimurium aroA* strain as a vector for delivery of the above immunogenic *M. hyopneumoniae* fusion protein did protect mice against experimental infection with *M. hyopneumoniae*, and thus has the potential of providing a cheap and easily administratable oral vaccine for swine (Fagan et al. 1997). Efforts to develop an effective DNA vaccine against *M. hyopneumoniae* are still under way, suggesting the failure of the above-described DNA vaccines to provide satisfactory protection of swine in field trials. A recent trial is that reported by Chen et al. (2003). They constructed a plasmid vector carrying the P42 heat shock protein gene of *M. hyopneumoniae* driven by the potent cytomegalovirus promoter. Injection of the plasmid into mice induced both humoral and cellular responses. The antiserum from the immunized mice inhibited growth of *M. hyopneumoniae*. One can only hope that this vaccine will offer better protection for swine than the previous DNA vaccines.

A novel genetic strategy to control phytoplasma diseases has been based on expression in plants of antibodies (plantibodies) that are able to interfere with multiplication of the pathogen. Le Gall et al. (1998) engineered a gene of a single-chain variable fragment antibody to a major membrane protein of the stolbur phytoplasma. They cloned it in a plasmid of *Agrobacterium tumefaciens* and transformed it to tobacco plants. The transformed plant carried the transgene. When grafted on a stolbur-infected tobacco root stock, the transgenic tobacco shoots grew free of symptoms, while normal tobacco shoots showed severe stolbur symptoms.

There can be little doubt of the great potential of DNA vaccines so that one may expect the rapid development of these new generation vaccines. Whether the high hopes will be substantiated will have to await the results of large-scale field trials.

DIAGNOSIS OF INFECTIONS

Mycoplasma identification and laboratory diagnosis of mycoplasmal infections has been based on the classical bacteriological tests, including morphology, cultural characteristics, physiological, and serological properties. While these tests still occupy a major part in mycoplasma diagnostics, new tests based on the molecular analysis of genomic DNA, ribosomal RNAs, cell proteins, and lipids appear to push aside the classical tests, and one may expect that the molecular tests will shortly become the prevailing tests in mycoplasma identification (Razin 2002). Systematic and detailed description and evaluation of the classical procedures used in mycoplasma identification can be found in the two volumes of *Methods in mycoplasmology* (Razin and Tully 1983; Tully and Razin 1983), while the newer molecular methods are included in the two more recent volumes of *Molecular and diagnostic procedures in mycoplasmology* (Razin and Tully 1995; Tully and Razin 1996). A succinct practical review of current methods applied in routine laboratory diagnosis of mycoplasmal infections, with emphasis on infections in humans, has been published (Waites et al. 2001).

Cultural properties and biochemical tests

The International Committee on the Taxonomy of Mollicutes issued recommended tests for mycoplasma identification, as well as for description of new species of the class Mollicutes (International Committee 1995). The recommended tests include those required to define a new isolate at the higher taxonomic levels (class, order, family), as well as tests for genus and species determination. Thus, a test for sterol requirement, based on growth promotion by cholesterol in the presence of either 0.01 or 0.04 percent Tween 80 (Tully 1995) separates the sterol-nonrequiring *Acholeplasma* and *Mesoplasma* species from the members of Mollicutes requiring cholesterol for growth. Other key tests to identify *Mycoplasma* and *Ureaplasma* species include those for sugar fermentation, arginine, and urea hydrolysis (Razin and Tully 1983).

Serological tests

A wide variety of serological tests have been employed, particularly in mycoplasma species and strain identification. The classical recommended tests include growth and metabolism inhibition by specific antisera, as well as direct and indirect immunofluorescence tests applied to mycoplasma colonies. A combined deformation-metabolism inhibition test system has been used to provide both screening and refined analysis and definition of *Spiroplasma* species (see the relevant chapters in *Methods in mycoplasmology*; Tully and Razin 1983). A variety of other, in some cases more sensitive, tests based on principles of enzyme-linked immunosorbent assay (ELISA), immunobinding, immunoblotting, immunofluorescence, and immunoperoxidase tests have been applied to mycoplasma diagnosis (see section B in Tully and Razin 1996).

A wide variety of serological procedures are based on detection of specific antibodies in sera of humans and animals suffering from mycoplasma infections. The procedures, as well as the evaluation of the results, are discussed in detail in the relevant chapters of *Molecular and diagnostic procedures in mycoplasmology* (Tully and Razin 1996) and in Waites et al. (2001). Serodiagnosis consists of examining serum samples for antibodies that inhibit the growth and metabolism of the organism or fix complement with mycoplasmal antigens. Being the best established and important human pathogen thus far, laboratory diagnosis of *M. pneumoniae* infections has been the subject of numerous studies (reviewed in Daxboeck et al. 2003). Antibody response in mycoplasmal pneumonia is most easily demonstrated by complement fixation (CF), reacting acute- and convalescent-phase sera with intact organisms or their lipid extract as antigen. A four-fold or greater antibody rise is considered indicative of recent infection (Ferwerda et al. 2001), whereas a sustained high antibody titer may not be significant, because a relatively high level of antibody may persist for at least 1 year after infection.

In view of the many limitations of CF tests, they have recently been largely replaced by methods applying alternative technologies, based mostly on enzyme immunoassays (EIA). Efforts were also invested in developing more defined and specific antigens based on immunodominant *M. pneumoniae* membrane proteins, such as P1 (Tuuminen et al. 2001) or the 116 kDa protein (Duffy et al. 1999). A P1-enriched antigen preparation was not only the most sensitive in detecting antibodies at the early stage of infection, but also detected antibodies to *M. pneumoniae* produced during the convalescent phase of infection (Tuuminen et al. 2001). This antigen is suitable for IgG, IgM, and IgA EIA testing (Suni et al. 2001). A combination of *M. pneumoniae*-specific PCR in nasopharyngeal aspirates with an IgM-capture immunoassay in acute phase sera, increases the sensitivity of rapid laboratory diagnosis of *M. pneumoniae*, enabling the attainment of a laboratory result in only 1 or 2 days (Ferwerda et al. 2001).

A variety of rapid tests based on indirect hemagglutination of erythrocytes or latex particles coated with *M. pneumoniae* antigens, or an immunofluorescence assay detecting IgM antibodies to *M. pneumoniae* (Dorigo-Zetsma et al. 1999) have been developed, and some are commercially available. EIA test kits presently achieve the largest market share of commercial mycoplasma serologic tests (Suni et al. 2001; Tuuminen et al. 2001; Waites et al. 2001). In summation, although PCR considerably extends the arsenal of reliable laboratory techniques, serology still retains importance in diagnosis of *M. pneumoniae* infections and of complications associated with *M. pneumoniae* infections. It is well known that IgM antibodies are frequently diagnostic in children because they are most likely experiencing an episode of a primary disease. In adults, on the contrary, IgM antibodies alone may not be diagnostic. Serology using IgA and IgA-antibody detection remains a method of choice for the diagnosis of acute infection in adults (Watkins-Riedel et al. 2001).

Restriction endonuclease analysis

The classic tests for mycoplasma identification and classification have been supplemented by a variety of tests based on genomic DNA analysis. Restriction endonuclease analysis (REA) of the mycoplasma genome provides a convenient and cost-effective means of determining DNA sequence variations among strains of mollicutes species (Razin and Yogev 1995). Variations in the array of fragments generated by a specific restriction endonuclease are called restriction fragment length polymorphisms (RFLP). RFLPs can result from sequence rearrangements, insertion or deletion of DNA segments, or base substitutions within the restriction endonuclease cleavage sites. Restriction endonuclease analysis has become a most useful taxonomic tool, facilitating the identification and classification of mycoplasmal isolates, as well as providing means for evaluating the degree of genotypic heterogeneity of strains within established species (Djordjevic et al. 2001; Razin 1992).

The great advantage of chromosomal restriction endonuclease analysis is that it is universally applicable and sensitive, because the entire genome is evaluated for RFLP, and in addition it is relatively easy to perform. Moreover, unlike SDS-PAGE of mycoplasmal cell proteins, restriction endonuclease analysis is not susceptible to contamination by culture medium contaminants, and requires very little (3–5 μg) of unlabeled DNA per test (Razin and Yogev 1995). A more recently developed technique, named amplified-fragment length polymorphism (AFLP) is a whole genome fingerprinting method based on the selective amplification of restriction fragments. This multistep procedure combines in an elegant manner the power of PCR with the informativeness of REA. AFLP yields more complex banding patterns than other DNA fingerprinting methods, increasing the discrimination between strains. In this way AFLP can be used as a device in studies of epidemiology, pathogenicity, and genetic variability in natural populations of mollicute species (Koktovic et al. 1999).

Southern blot analysis of genomic DNA digested by a restriction enzyme and hybridized with a cloned conserved gene or a specific genomic fragment as a probe, has become a most useful tool in the identification, classification and subtyping of mollicute species (Razin 1992; Yogev and Razin 1995). The fact that the rRNA operons in the various mollicutes differ in restriction sites within the operon and in the flanking sequences results in the production of hybridization patterns specific to different mollicute species or strains.

A restriction enzyme having a 6-bp recognition site will usually cut the one or two mycoplasmal rRNA operons in a few sites, so that the hybridization patterns are usually simple and much easier to compare than the multiband restriction patterns obtained by restriction endonuclease analysis of entire genomic DNA. Southern blot hybridization with cloned rRNA genes as probes has been named 'ribotyping'. However, other conserved genes can be employed as probes, such as the *tuf* gene, encoding the elongation factor EF-Tu. The mollicute genome carries only one copy of this gene, so that the hybridization patterns obtained with restricted mollicute DNAs are also very simple and easy to compare (Razin 1992).

PCR-based diagnostic procedures

The introduction of PCR to diagnostics in the late 1980s pushed aside many of the previously developed DNA probes and commercial kits (Razin 1994). PCR tests are several orders of magnitude more sensitive than those based on direct hybridization with a DNA probe. Moreover, PCR is fast, copying a single DNA sequence over a billion times within 3 hours. Nucleic acid amplification techniques are not limited by the ability of an organism to grow in culture, a feature of paramount importance considering the fastidious nature of some mycoplasmas, such as *M. genitalium*, and the fact that not all mollicutes can be cultured in vitro, notably the phytoplasmas, and the hemoplasmas (*Eperythrozoon* and *Haemobartonella* species, see Phylogeny and taxonomy section). Thus, PCR-amplified 16S rDNA subjected to RFLP analysis has become the method of choice for routine detection, differentiation, and classification of phytoplasmas (Marcone et al. 2000; Seemuller et al. 2002).

PCR methodology is still at a phase of rapid development and new procedures, modifications, etc., are reported almost daily. Detailed descriptions of methodology and evaluation of PCR-based tests in diagnostics can be found in the relevant chapters in *Molecular and diagnostic procedures in mycoplasmology* (Tully and Razin 1996). The first major step in the development of a PCR-based test concerns the selection of the appropriate target sequences for amplification. The sequence to be amplified can be chosen from a published mycoplasmal gene sequence or from a randomly cloned DNA fragment demonstrated to be specific for the mycoplasma to be detected (Kong et al. 2001; Caron et al. 2000). Complete or almost complete sequences of the 16S rRNA genes are now available for almost all the established mollicute species, and can be retrieved from data banks (see Phylogeny and taxonomy section). This facilitates the selection of a variety of target sequences, starting with sequences in the highly conserved regions of the genes, producing primers of wide specificity ('universal primers') which will react with the DNA of any mycoplasma or even with the DNA of other prokaryotes. These may be satisfactory for detection of mycoplasma infection in cell cultures, where the goal is just to screen the cultures for contamination. In case of a need to differentiate the contaminating mycoplasmas, a multiplex PCR system can be employed, consisting of a universal set of primers along with primer sets specific for the mycoplasma species commonly infecting cell cultures (Choppa et al. 1998; Kong et al. 2001). The mycoplasmal 16S rRNA genes carry, in addition to the conserved regions, more specific variable regions, as well as specific 16S–23S intergenic spacer regions. Primers can be selected from these regions with various degrees of specificity, ranging from clusters of species, single species, down to the subspecies level (Kong et al. 2001).

Although conventional PCR tests should, under optimal conditions, detect the DNA of a single mycoplasma, this rarely happens in testing clinical material. Two-step (nested) PCR has been devised to increase sensitivity to a level enabling detection of a single mycoplasma in a clinical sample (Verdin et al. 2000). Another advantage of nested PCR is that the second round PCR serves to confirm the specificity of the first round PCR. Another approach is that termed random amplified polymorphic DNA (RAPD), or arbitrary primer PCR (AP-PCR). It involves PCR amplification with a single arbitrary primer at low stringency, resulting in strain-specific arrays of DNA fragments that can reproducibly distinguish even closely related strains of a species. The simplicity of this procedure, and the availability of commercial kits should facilitate the use of RAPD analysis as a routine procedure for identification of strains of a mollicute species, employing the RAPD patterns to investigate disease outbreaks for epidemiologic tracking (Cousin-Allery et al. 2000).

Comparing PCR results with those of culture and serology

Numerous publications compare the results of the PCR technique with culture and serological testing for *M. pneumoniae* (for references see Razin 2002 and Daxboeck et al. 2003). Most publications indicate the higher sensitivity and the much faster results obtained by PCR (for examples, see Dorigo-Zetsma et al. 1999 and Ferwerda et al. 2001). The sensitivity of PCR assays is usually in the order of magnitude of a few femtograms of mycoplasmal DNA. When translated into numbers of organisms, 1 fg of mycoplasmal DNA is approximately equivalent to the genomic DNA of a single mycoplasma cell, based on a genome size of about 1 000 kb. The ability to detect by PCR a single mycoplasma cell makes PCR the most sensitive detection method available, even more than culture. In theory, a positive culture can

be derived from a single mycoplasma cell. In practice, however, due to many reasons (including mycoplasma cell aggregates, multinuclear filamentous forms, defective or nonviable cells) a positive mycoplasma culture requires an inoculum equivalent to about 100 to 1 000 cells. This is known for other bacteria as well and is the reason for using the expression colony-forming units (cfu) or color-changing units (ccu) rather than number of cells per milliliter (Razin 1994).

Obviously, PCR should be applied in the case of patients with an impaired immune response where serological tests may be much less effective. Positive PCR results for *M. pneumoniae* in culture-negative persons without evidence of respiratory disease suggests either inadequate specificity of the test, persistence of the organisms after infection, or their existence in asymptomatic carriers, making interpretation of such results difficult (Waites et al. 2001). In other words, would such a sensitive technique as PCR lead to 'over-diagnosis' of *M. pneumoniae* infections partly due to its detection in healthy carriers? It is still difficult to answer this question in a definitive manner.

In conclusion, PCR detection of mycoplasmas is still too labor-intensive, expensive, and complex to be carried out routinely in most clinical microbiology laboratories. Development of satisfactory commercial PCR kits may simplify the procedures and bring about better standardization of the technique, and if available at a reasonable cost, PCR could become the major method for diagnosis of mycoplasmal infections.

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Chlamydia

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HISTORIC PERSPECTIVES

The first means to diagnose infections by chlamydia (i.e. by *Chlamydia trachomatis*) was the demonstration of intracytoplasmatic inclusions in smears of eye secretion in cases of 'inclusion conjunctivitis,' a discovery made at the beginning of the twentieth century (Halberstaeter and von Prowasek 1907). During this period it was also demonstrated that parents to babies suffering from this condition also had genital mucosal cells with similar inclusions. The designation of *C. trachomatis* refers to its etiological role in trachoma (Darougar and Jones 1983). Since then, lymphogranuloma venereum (LGV) is also known as a chlamydia infection. A 'rediscovery' of *C. trachomatis* as one of the main causes of nongonococcal urethritis (Dunlop et al. 1972) and cervicitis (Brunham et al. 1984) was made during the 1970s and early 1980s. Complications thereof, such as endometritis and salpingitis (pelvic inflammatory disease (PID)), could be linked to infection by *C. trachomatis* (Mårdh et al. 1977). It was also demonstrated that newborns could develop not only conjunctivitis (Stenberg and Mårdh 1990), but also pneumonia (Beem and Saxon 1977).

During the 1930s, Bedson described the life cycle of chlamydia (i.e. of *Chlamydia psittaci*) (Bedson et al. 1930). The organisms were for some time named Bedsonia after him. It was established that 'parrot fever' or psittacosis/ornithosis was caused by *Chlamydia psittaci*. The agent could be associated with a variety of animals infections and with zoonotic infections, such as abortion and premature birth in women infected by sheep (Johnson et al. 1985), and conjunctivitis due to the feline conjunctivitis agent (Hartley et al. 2001).

In 1976, respiratory tract infections occurred in a Finnish military camp (Kleemola et al. 1988). Isolated agents had similarities to chlamydia strains recovered from the eyes of persons in Taiwan (strain TW-183) and Iran (strain IOL-207) and from a case with acute respiratory tract infection in the USA (strain AR-39). They were regarded variants of agents classified as *C. psittaci*. Initially, these agents were recognized as the TWAR agents (Kuo et al. 1986), now recognized as a separate species, i.e. *C. pneumoniae*. Seroepidemiological studies showed that antibodies to these agents occur more often in persons who later developed heart infarction (Saikku et al. 1988). This observation led to investigations into whether or not there is a correlation between arteriosclerosis and *C. pneumoniae* (Kuo et al. 1993; Campbell and Kuo 2003). If an association, or even correlation, really exists is, however, still an open matter (van der Ven et al. 2002; Broman and Hammerschlag 2002).

In 1957, embryonic hen eggs were used to isolate *C. trachomatis* (T'ang et al. 1957). This latter method was, however, not suitable for routine clinical use, which is why the introduction of tissue cell cultures in 1965 meant a step forward (Gordon and Quan 1965). Later developments involved the use of HeLa 229 cells treated

with DEAE, i.e. a dextran of high molecular weight. Other pretreatments utilized emetin, cytochlasin B, hydrocortisone or iodoxyuridine (IUDR). Irradiation of the cells increased their sensitivity to infection. The need to prepare the cells by irradiation and/or chemicals some days before use meant great restrictions if diagnostic services were to be applied on a large scale. In 1976, a diagnostic breakthrough was made by application of cycloheximide treatment of McCoy cells. This could be made in conjunction with infection of the cells, which made it practical and cheap to use cell cultures (Ripa and Mårdh 1977). By application of this method, screening studies could demonstrate the wide spread of genital chlamydial infections in general populations. Enzyme-linked immunosorbent assay (ELISA), e.g. Chlamydiazyme®, was widely applied in the diagnosis of genital infections by *C. trachomatis* until the end of the 1990s, when nucleic acid-based tests took over in many laboratories. In retrospect, the use of ELISA tests can be regarded as 'the dark age' in chlamydial diagnostic services, as their sensitivity was low, i.e. 50–70 percent. In comparison, adequately performed cell cultures have a sensitivity of at least 85 percent. During the 1990s, kits with amplified nucleic acid tests, such as polymerase chain reaction (PCR) (Livengood and Wrem 2001) and ligase chain reaction (LCR) (Lee et al. 1995) came on the market.

TAXONOMY

The species of the genus *Chlamydia* are shown in Table 1. Three species occur in humans, i.e. *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. The fourth species of genus *Chlamydia,* i.e. *C. pecorum*, is a nonhuman pathogen. The chlamydia organisms belong to the family Chlamydiaceae in order Chlamydiales. On the basis of molecular markers, a new taxonomy for chlamydia organism has been proposed, using the denomination *Chlamydophila* for genus (Everett et al. 1999). These authors proposed the recognition of *C. muridarum* and *C. suis*. However, this taxonomy has not been generally accepted and was opposed by a large group of researchers in the field (Schachter et al. 2001). The old taxonomy is used in this chapter.

C. trachomatis was initially divided into 15 serovars (Table 1). More recently, the proposal of four new subtypes of earlier recognized serovars have been made, i.e. Da, Ga, Ia, and L2a. Serovars of *C. trachomatis* have been identified by microimmunofluorescence (MIF) tests (Wang and Grayston 1982). The subidivision by MIF fitted well with the clinical spectrum of infections caused by the various serovars and with subdivision obtained by genetic sequencing. Differences in the major outer membrane proteins (MOMP) can form a base for subdivision of *C. trachomatis* into different serovars (Caldwell and Schachter 1982). Using antibodies raised against different domains of MOMP, such subdivisions can also be made. PCR-based restriction fragment length polymorphism of outer membrane protein (OMP) 1 means a simpler method for serovar typing than by MIF tests (Frost et al. 1991).

Chlamydia pneumoniae was initially included in *C. psittaci* (Kuo et al. 1986). Structural and antigenic analyses formed the base for separating *C. pneumoniae* into its own species (Campbell et al. 1990).

REPRODUCTIVE CYCLE

Chlamydia organisms, which are intracellular parasites, multiply by binary fission in a cytoplasmatic vacuole which is surrounded by a membrane with similarities to the host cell's cytoplasma membrane (Conati et al. 2002). Chlamydiae are classified as bacteria, but have a unique mode of replication as illustrated in Figure 1.

The infectious particle is the elementary body (EB) (Figure 2). The EB adheres to the host cell with the aid of heparin-sulfate-like glucosaminoglycan molecules which are required for chlamydia to enter a host cell. A number of substances are known to enhance contact between EBs and host cells, i.e. diethylaminoethyl-dextran and iodoxyuridine, which have been utilized in in vitro cultures. The EB is taken up through a vesicle, a phagosome, without phagolysosomal fusion. After an eclipse phase of approximately 12 h, the EB develops into a reticulate body (RB). The EB has a diameter of 200–300 nm, whilst that of the RB is 1 000–1 500 nm. The RB is divided by binary fission inside the vesicle. During the synthesis of substances by the chlamydia cell, the metabolism of the host cell is depressed through influence of the chlamydia parasite. Eighteen to 24 h after the EB has attached, the RBs are undergoing reorganization into EBs. Within another 6 h, the EBs are

Table 1 *Taxonomy of the chlamydia organisms*

Taxonomy	
Order	Chlamydiales
Family	Chlamydiaceae
Genus	*Chlamydia*
Species	*C. trachomatis*, *C. psittaci*, *C. pneumoniae*, *C. pecorum*
Biovars of *C. trachomatis*	trachoma, lymphogranuloma venereum (LGV), mouse pneumonitis agent
Serovars of *C. trachomatis*	A, B, Ba, C, D, E, F, G, H, I, J, K L1, L2, L3

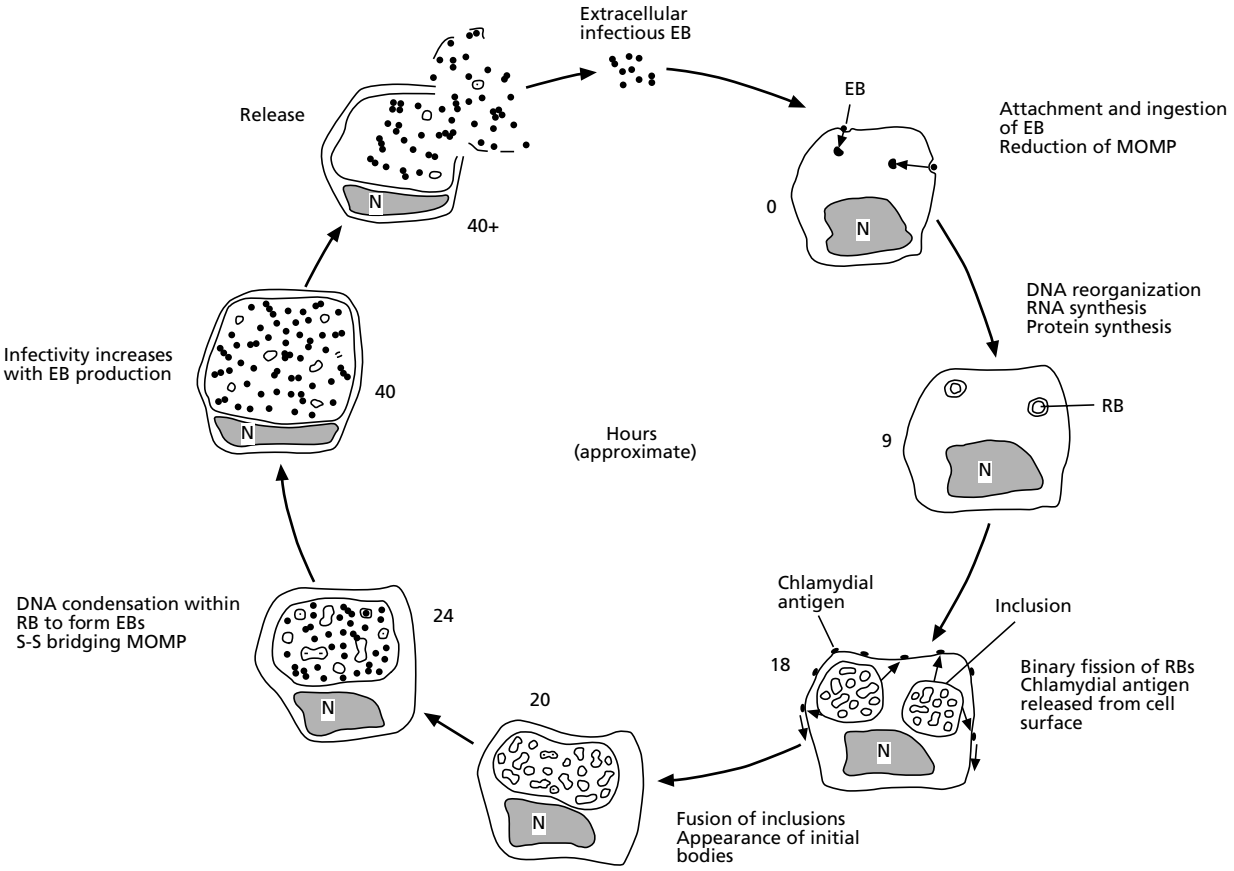

Figure 1 *Schematic drawing of the developmental cycle of* Chlamydia trachomatis *showing uptake of elementary bodies (EBs), formation of reticulate bodies (RBs), multiplication and release of the agent as EBs.*

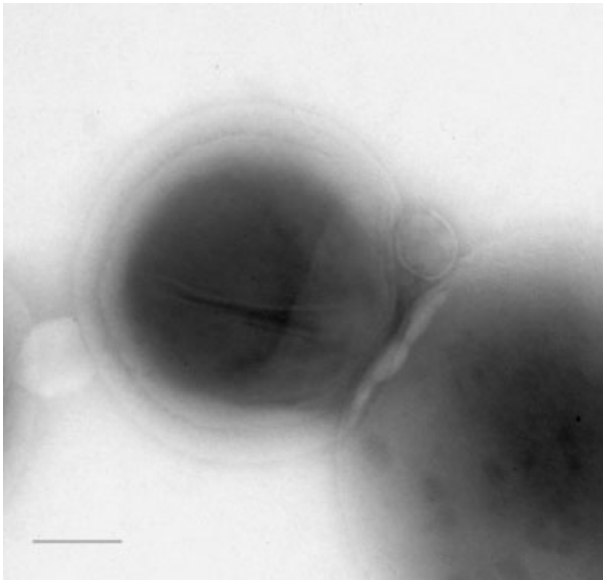

Figure 2 *Electron-microscopic picture of a section of a cell of* Chlamydia trachomatis*, serovar D, attached to carbon-coated grid and stained with ammonium molybdate (with courtesy of S. Birkelund, A.G. Lundmose and G. Christiansen, Aarhus University, Denmark). Bar = 100 nm*

released and the host cell dies. In vivo, the developmental cycle is longer than in in vitro cultures. Its exact time may differ by prevailing circumstances but may be up to a week. At the end of the reproductive phase, the chlamydia inclusion may be so large that it displaces the nucleus of the host cell. This phenomenon may give the impression that the inclusion more or less surrounds the nucleus, like an overcoat. This once gave the organisms their name 'chlamydia,' which in Greek means 'overcoat.'

BIO- AND IMMUNOCHEMISTRY GENETICS

Most bio-, immunochemical and genetic data on chlamydia organisms are derived from studies of *C. trachomatis*. Handling of *C. psittaci* involves risks for laboratory infections and *C. pneumoniae* is difficult to culture.

The chlamydia organisms are energy parasites of eukaryotic cells, which supplies them with ATP, GTP, and UTP. The metabolism of the chlamydia organisms is, otherwise, similar to that of eubacteria (Mårdh et al. 1989).

The EB has a central, electron dense area surrounded by a cytoplasmatic membrane and a double-layered

outer membrane. The outer membrane is made up of up to 50 percent protein and 30 percent lipids as well as fatty acids and carbohydrates (Birkelund et al. 1989; Siridewa et al. 1993; Su et al. 1996; Yxfeldt et al. 1994; Kuo et al. 1996; Stephens et al. 2001; Conati et al. 2002). The EB has a lipopolysaccharide (LPS) surface structure, which is common to all species of the genus *Chlamydia.* The LPS has two antigenic domains. One is shared with gram-negative bacteria while the other is specific for chlamydia organisms. LPS contains a genus specific epitope, i.e. 3-deoxy-D-manno-oct-2-ulopyranosonic acid (KDO). This trisaccharide is highly antigenic. The chlamydial cell wall also contains proteins, e.g. the MOMP, which has a molecular weight of 40×10^6. The MOMP has four variable domains (I, II, III, and IV). Differences between them can be utilized to identify the various species of genus *Chlamydia* (Zhong et al. 1990). Apart from MOMP, there are other proteins on the cell surface, i.e. Omc A and Omc B.

The guanine + cytosine (G + C) content of the DNA of *C. trachomatis* is 44 percent and that of *C. psittaci*/*C. pneumoniae* is 41 percent. The DNA homology is only 10 percent between the two species, while the 16S RNA gene sequence shows over 95 percent homology. The genome of *C. trachomatis* is a double-stranded DNA molecule with a molecular weight of 660×10^6. The entire 1000 kb genome of *C. trachomatis* has been sequenced (Stephens et al. 2002). The structure of MOMP and the sequence of the MOMP genes of *C. psittaci* have been studied (Zhang et al. 1989).

Almost all strains of *C. trachomatis* tested contain additional genetic material in the form of a 7.5 kb cryptic plasmid (Peterson et al. 1990). The nucleic acid sequence is used for diagnostic purposes in PCR and LCR tests (see the section on Diagnosis below). Although only single strains have so far been detected that do not carry this plasmid, individuals harboring such strains will therefore remain undetected in such tests. Obviously, a more general check for the presence of nonplasmid-containing strains of *C. trachomatis* is essential, as long as the main diagnostic tools are based on the nucleic acid sequence in the cryptic plasmids.

PATHOGENESIS AND IMMUNE RESPONSE

Apart from being an intracellular parasite causing cell disruption and death of the host cell (Conati et al. 2002), the pathogenicity mechanisms of chlamydia organisms are not well understood. However, a cytotoxin has been recognized. The LGV serovars are more invasive than the non-LGV serovars, causing ulceration and lymphangitis. The other serovars of *C. trachomatis* (Table 1) cause infection of epithelial linings, such as of the mucosa of the cervical channel, endometrium, uterine tubes, urethra, vas deferens, epididymis, and conjunctiva. Tumor necrosis factor (TNF)-α-induced physiological apoptosis can be inhibited by a chlamydial infection (Fan et al. 1998).

A chlamydial infection may result in proinflammatory cytokine production, along with an immune response to the agent and induce antibodies to heat shock proteins (hsp), e.g. to hsp60 (Witkin et al. 1996). As chlamydia and other bacteria share epitopes with human hsps, it has been speculated that chlamydial infections may cause an autoimmune response. hsp60 production can cause localized inflammatory reactions, e.g. in the uterine tubes. RBs may escape the immune response and thereby explain persistent infections. Formation of new EBs from RBs can induce an immune response and inflammatory reactions resulting in a cyclic process of scarring of the infected tissue. For example, this may eventually lead to partial or total obliteration of the fallopian tubes causing tubal factor infertility or subinfertility. If the vas deferens is affected, the male may transiently be subfertile. Such a pathogenicity mechanism might also explain the lower success rate of in vitro fertilization in women with evidence of a passed genital chlamydial infection. In trachoma, immune reactions contribute to changes resulting in blindness.

Clearance of a chlamydial infection and resistance to reinfection is dependent on T-cell response. CD4- and CD8-positive cells are engaged. Interferon-γ may inhibit multiplication of RBs.

C. pneumoniae can prevent programmed cell death in experimentally infected cell cultures (Carratelli et al. 2002), claimed as a possible pathogenicity principle. A role of chlamydial hps60 in stimulation of an innate immune response to *C. pneumoniae* has been demonstrated (Costa et al. 2002).

CHLAMYDIA TRACHOMATIS

Epidemiology

The World Health Organization (WHO) has estimated that the number of new genital infections by chlamydia has almost reached 100 million annually, which makes these infections one of the most prevalent sexually transmitted diseases (STD). However, the epidemiological situation varies by country (Norman 2002). This can partly be explained by the introduction of national screening programs (Mårdh 2002b). Where such measures have been undertaken, the prevalence of genital chlamydial infections has markedly decreased and so have their complications, e.g. PID (Weström 1998). In the UK, a recent increase in the prevalence of such infections has been seen, whereas in the Scandinavian countries there has been a decrease, although a small increase has been noted in recent years (to some extent due to the introduction of nucleic acid detection methods). It should, however, be observed that population-based data and reliable surveillance systems are

generally lacking (Mårdh 1998). The number of cases detected closely depends on whether screening for chlamydia carriers is performed, as the majority of those who are infected by *C. trachomatis* are more or less asymptomatic and have no reason to consult health providers. The number of genital chlamydial infections detected in a region or country is also dependent on whether or not partner notification is performed (Mårdh 1998). Access to diagnostic services and knowledge of which detection methods are in current use is essential. Some currently used methods have a low sensitivity and/or specificity. This means that either a large proportion of cases remain undetected or are being falsely reported as chlamydia-infected. To sum up, epidemiological data based on population surveys are almost nonexistent and surveillance data must be interpreted with caution.

Young age is identified as a risk factor to contract genital chlamydial infections and also for reinfection (Norman 2002). Thus, in most societies, young teenagers belong to those that should be addressed in preventative work, which has to start at school age to be effective (Mårdh 2002b). With the knowledge that young adults are most commonly infected, screening of middle-aged persons is often neglected. Therefore, the age distribution of documented cases can be misleading for understanding the chlamydia epidemic situation. As for other STDs, a high number of sex partners is, of course, also a risk factor. The transmission rate of genital chlamydial infections is, however, poorly documented.

Trachoma is regarded as the most common cause of preventable blindness (Darougar and Jones 1983). Some decades ago, it was estimated to affect some hundred millions of persons. The so-called 'trachoma belt' covers North Africa and the Sub-Saharan region, Middle East, the dry area of the Indian subcontinent, and South East Asia. Recent society developments and trachoma prevention programs have markedly reduced the prevalence of trachoma (Jones 1975). Ethnic belonging, rather than poor sanitary conditions, seems to play a role for the prevalence of trachoma in displaced persons living in the same camp (Mahmoud et al. 1994a).

Table 2 *Conditions in nonpregnant and pregnant women, in fetuses, in newborns, and in males, in whom infections with* Chlamydia trachomatis *plays an etiological role. Sequelae of these conditions are also listed*

Manifestations	Sequelae
Nonpregnant women	
Cervicitis	Infertility
Endometritis	Chronic abdominal pain
Salpingitis	Ectopic pregnancy
PID	Chronic obstructive lung disease
Periappendicitis	Asthma
Perihepatitis/peritonitis/perisigmoiditis	Trachoma (entropion, pannus, blindness)
Fetus	
Chorioamnionitis ?	
Intrauterine infection of fetuses	
Postpartum PID	
Newborns	
Conjunctivitis in newborns	
Pneumonia in infants	
Males	
Uretritis	
Epididymitis	
Vaseitis	
Both genders	
Proctitis	
Sexually acquired reactive arthritis (SARA)	
Reiter's syndrome	

Clinical syndromes

The manifestations of infections by *C. trachomatis* are shown in Table 2.

GENITAL INFECTIONS IN NONPREGNANT FEMALES, INCLUDING CERVICITIS, ENDOMETRITIS, AND SALPINGITIS (PID)

Chlamydia infections may involve both the lower and upper genital tract of men and women (Mårdh et al. 1977, 1981a; Holmes et al. 1980; Svensson et al. 1981; Ingerslev et al. 1982). In most countries today, *C. trachomatis* is the most common cause of cervicitis. The columnar epithelium of the cervical channel is infected. Mucopurulent discharge from the cervical os is a characteristic finding (Brunham et al. 1984). The majority of chlamydial cervicitis cases is low-symptomatic. Cervical ectopy is not related to an increased risk of contracting chlamydial infection. Tubal infections may spread through the fallopian tubes to intraabdominal sites, such as to the liver surface (Wölner-Hanssen et al. 1981; Mårdh and Wölner-Hanssen 1985).

C. trachomatis is a common etiological agent of endometritis (Mårdh et al. 1981a). Chlamydial endometritis (and salpingitis) is associated with production of chlamydial hsp60 antibodies (Peeling et al. 1997) which elicit a mononuclear cell reaction. An intensive mononuclear cell infiltration can be seen in endometrial aspirates after specific histological staining (Figure 3). Approximately 40 percent of all women with genital chlamydial infections have a PID. In clinical practice it is not possible to determine which patients do have an ascending infection. The infection may spread to the tubes and cause salpingitis (Mårdh et al. 1977; Svensson et al. 1981) (Figure 4). The latter infection may spread further to the abdominal cavity to cause periappendicitis, peritonitis, perihepatitis, perisplenitis, and perisigmoiditis (Wölner-Hanssen et al. 1981; Mårdh and

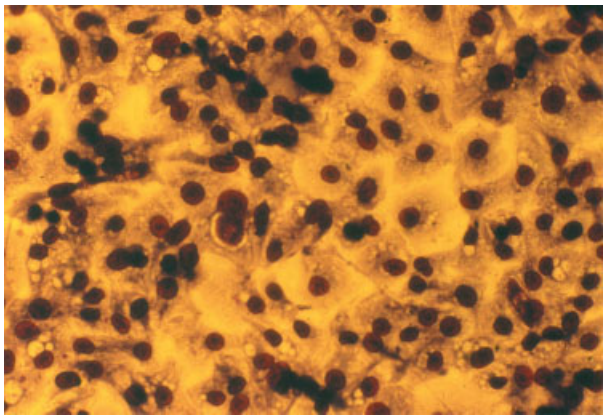

Figure 3 *Chlamydial endometritis with a characteristic plasma cell infiltration*

Wölner-Hanssen 1985) (Figure 5). In chlamydial perihepatitis, the affected woman may present with pain under the right arcus, which can mimic a cholecystitis attack. Tubal infections may result in tubal factor infertility (Henry-Suchet et al. 1981; Svensson et al. 1983; Patton et al. 1994). This risk increases with each episode of salpingitis. In rough terms, the risk for involuntary childlessness is 25, 50, and 75 percent after one, two, and three episodes of PID, respectively. A repeated episode of salpingitis may not necessarily be due to chlamydial infection, but to bacterial infections in a damaged tube. Extrauterine pregnancy (Svensson et al. 1985) is associated with a past chlamydial infection. Chronic abdominal pain may be still another sequelae of PID due to *C. trachomatis*.

Oral contraceptives can influence the natural history of genital chlamydial infections (Svensson et al. 1984) and mask symptoms of chlamydial PID (Mårdh and Hogg 1998). In women with chlamydial salpingitis who are on the pill, spread of the infection into the abdominal cavity is less common than in nonusers.

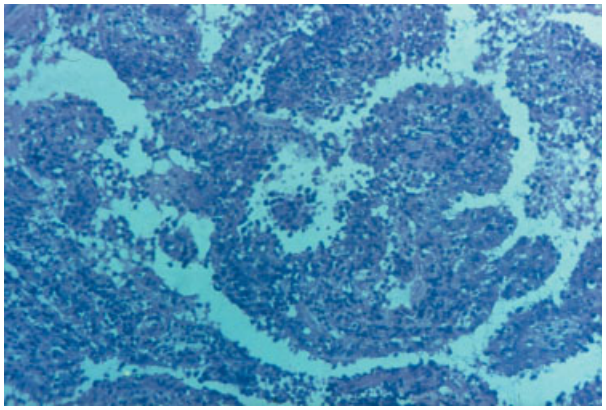

Figure 4 *Histological section of a fallopian tube of a woman with acute salpingitis from whom* Chlamydia trachomatis *was isolated from the tubes and who developed a significant antibody response. The tubes were removed because of severe complications.*

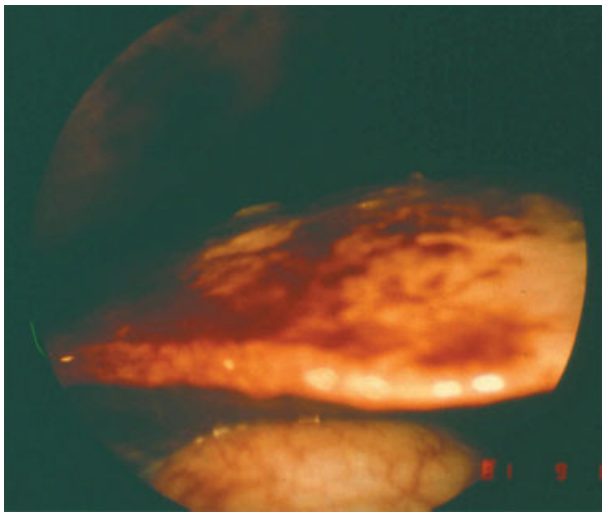

Figure 5 *Chlamydial perihepatitis in a case of chlamydial salpingitis showing the characteristic fibrin deposit on the liver capsule*

NONGONOCOCCAL AND POSTGONOCOCCAL URETHRITIS

The term nongonoccocal urethritis (NGU) was coined to characterize cases of urethral discharge that could not be associated with gonorrhea (Dunlop et al. 1972). When methods to diagnose *C. trachomatis* infection had been established, the true etiology of these cases was discovered. By the end of the 1970s, NGU cases outnumbered those of gonococcal urethritis; a situation which has remained so in most countries. This is also true for urethritis that persists after microbiological, but no clinical cure, of gonorrhea, i.e. for postgonococcal urethritis (PGU). PGU is often due to an infection by *C. trachomatis* not cured by conventional gonorrhoea therapy.

ADVERSE PREGNANCY OUTCOMES

Chlamydial infections are of specific concern in pregnancy (Mårdh 2002a) (Table 2). Such infections have been associated with a number of adverse pregnancy outcomes, such as early and late abortion, stillbirth, prematurity, small-for-date babies, and intrauterine fetal infections. Serological evidence of an intrauterine infection by *C. trachomatis* was found in 10 percent of babies born preterm or in the case of stillbirth, as evidenced from tests of cord blood (Gencay et al. 2000). For any one of these conditions, it is difficult to assess the role, if any, of confounding factors such as genetic polymorphism for interleukine production.

Ectopic pregnancy correlates to a history of PID (Svensson et al. 1985; Brunham et al. 1986) and presense of hsp60 antibodies (Sziller et al. 1998). In countries where nationwide massive screening programs for detection of genital chlamydial infection have been performed, the rate of extrauterine pregnancies has decreased dramatically (Mårdh 2002a).

Infection by *C. trachomatis* may also interfere with in vitro fertilization (IVF) trials (Wölner-Hanssen and Mårdh 1984; Witkin et al. 1994). *C. trachomatis* can attach to, and even penetrate, spermatozoa. hsp60 antibodies to *C. trachomatis* are found in cases subjected to IVF (Spandorfer et al. 1999).

Elective abortion in cervical carriers of *C. trachomatis* involves an increased risk of developing post-abortion PID (Möller and Mårdh, 1980). Therefore, testing women to be subjected to legal abortion has been recommended as a routine measurement.

LGV

LGV is caused by serovars L 1–3 of *C. trachomatis*. LGV is usually found in tropical and subtropical countries (Perine and Stamm 1999). In other regions, it usually occurs as import cases. The incubation period is 1–3 weeks. The first sign of infection is usually a vesicle or an ulcer on the penis or on the fourcette in women (the latter easily gets undetected). Also, extragenital sites such as the fingers may be the primary location. As in syphilis, the ulcer heals. Later, there will be involvement of the inguinal lymph nodes, which occurs after 2–16 weeks. The lymph nodes get swollen, painful, and will suppurate, often through several sinuses. In one third, this phenomenon is seen bilaterally. This is the characteristic 'bubo' of LGV. If the lymphatic nodes under the inguinal ligament are involved, there is the so-called 'groove sign.' In women, the iliac lymphatic glandulae are commonly involved. This second stage of LGV is accompanied by constitutional symptoms, such as chills, headache, myalgias, and arthralgias. When the LGV organisms invade the lymphatic system, it may result in lymphstasis causing elephantiasis of the genital region. There could also be manifestations from several other organs, e.g. the eyes, central nervous system (CNS), heart, and lungs. Rectal strictures and development of rectal and rectal–vaginal fistulae may also be seen.

LGV should be included among differential diagnosis of any genital ulcer if there is any history or epidemiological data supporting the possibility of such an etiology. If case culture is used to diagnose LGV, the entire cell carpet will generally become infected, which can be used as a differential diagnostic marker for LGV. Detection of antibodies to chlamydia LPS has been used to diagnose LGV, as well as PCR and genetic referencing.

CONJUNCTIVITIS

Chlamydial conjunctivitis, also named paratrachoma and inclusion conjunctivitis (usually debuts unilaterally to spread to the contralateral side) is seen in newborns (Figure 6), infants, children, and adults (Figure 7) (Stenberg and Mårdh 1990). It affects the lower lid where follicules are found (Figure 8). It often debuts in the newborn after 1–2 weeks, but may do so within days if the baby has been infected in utero. Chlamydial

Figure 6 *Neonatal conjunctivitis caused by* Chlamydia trachomatis

conjunctivitis is seen in adults with a frequency of one to 200–400 reported cases of genital chlamydial infection. In newborns, the condition is generally self-healing. Credé prophylaxis is ineffective against *C. trachomatis*. The condition is often misdiagnosed and affected persons have often consulted several physicians before the diagnosis is established. Conjunctivitis in a newborn may indicate that the baby or infant also has concomitantly chlamydial pneumonia. It should be noted, however, that half of all neonatal chlamydial pneumonia cases lack signs of conjunctivitis. Chlamydial conjunctivitis resolves over 3 months without treatment. In adults, a concomitant genital chlamydial infection should be searched for (Stenberg and Mårdh 1993). Autoinoculation of the eye with genital secretion is believed to be the most common route of transmission of chlamydial conjunctivitis.

PROCTITIS, EPIDIDYMITIS, AND VASEITIS

C. trachomatis infection can be a cause of proctitis (Stamm et al. 1982). Epididymitis is one complication of chlamydial urethritis, which is most often seen in age groups in which infections with *C. trachomatis* are most

Figure 7 *Chlamydial conjunctivitis in a young man showing intensive congestion*

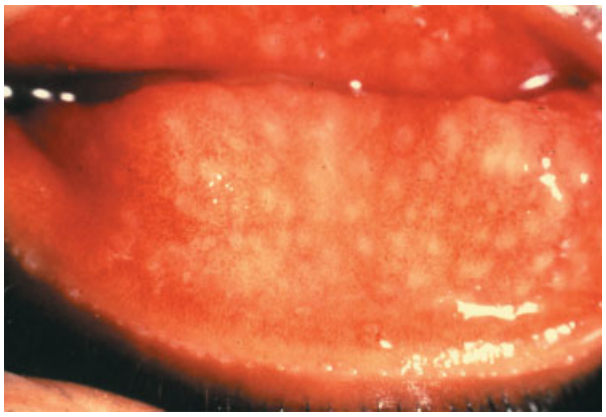

Figure 8 *Chlamydia follicles in the lower lid of a case of inclusion conjunctivitis*

common (Berger et al. 1978). The epididymitis is usually unilateral, which may explain why infertility in males on the basis of a past chlamydial infection is uncommon. It is, however, believed that it may cause transient subfertility. This may also be due to transient occlusion of vas deferens, which can be seen in grivit monkeys experimentally infected with *C. trachomatis* (Möller and Mårdh 1980).

ARTHRITIS AND UVEITIS

Chlamydial arthritis that occurs in conjunction with a genital infection is named sexually acquired reactive arthritis (SARA). Chlamydial urethritis is also one manifestation of nondysenteric Reiter's syndrome, i.e. arthritis, acute anterior uveitis (an intraocular inflammatory disease of the iris or ciliary body of the eye), and urethritis (Keat 1980). SARA is linked to histocompatibility antigen HLA-B27. Chlamydial antigen has been detected in synovial fluid, but whether the chlamydia organisms actually invade joints, and if so, if only transiently, has been discussed for decades. Seroepidemiological studies seem, however, to support an etiological role of *C. trachomatis* in some of these cases.

CERVICAL CANCER

A possible correlation between cervical cancer and genital chlamydia infections has been considered since the beginning of the 1980s (Schachter et al. 1982). Oncogenic types of human papilloma viruses, i.e. types 16/18, closely link the etiology of cervical cancer to infections. These types are involved in at least 90 percent of cervical cancer cases, while several of the other almost 100 HPV types may account for another 5 percent. In a serological study of more than 50 000 Scandinavian women (using sera collected many years previously) who were subjected to cytological screening, antichlamydial antibodies to serotypes G was significantly more common than in controls (OR 6.6 (1.6–27.0)), followed by serotypes I and D (Anttila et al. 2001). The study design did not, however, allow investigation of potential confounding factors such as smoking, sexual risk taking, including number of sex partners, and any difference between healed and nonhealed HPV infections.

NEONATAL INFECTIONS

There are two major manifestations of neonatal chlamydial infection, namely conjunctivitis (Stenberg and Mårdh 1990) and pneumonia (Beem and Saxon 1977), although the lower genital tract may also be colonized. It is believed that the infection is mainly spread from the mother to the infant when the fetus is passing through the birth canal (Schachter et al. 1986). A very early onset may indicate that the child was already infected in utero (Gencay et al. 2000), both in cases with and without premature rupture of the membranes (PROM). There is a comparatively higher chance for an infant to only become transiently colonized in the eyes than to develop clinical chlamydial conjunctivitis (Mårdh et al. 1980b).

Neonatal conjunctivitis often manifests mono-ocularily and the fellow-eye will become affected after 2–7 days in three-quarters of cases, which is earlier than in adults. The eyes of the infant with chlamydial conjunctivitis may be covered by copious discharge. There is usually edema and erythema of the eyelids (Figure 6). There may be bleeding when touching the conjunctival surfaces at examination or during sampling. The nasopharynx is colonized by *C. trachomatis* in one third of cases, which is believed to be the source of lung infections.

A neonatal chlamydial lung infection may easily be overlooked as symptoms are often only vague. The child becomes ill after approximately 6 weeks which is when the parents are likely to be the only observers of the infant's health. The child may be afebrile or only subfebrile. There may be a cough and congestion that the parents often will not recognize as pathologic. Lung auscultation may reveal rales and there may be tachypnea. Chest X-ray shows diffuse infiltrates and hyperinfiltration. Histologically, there are diffuse interstitial and alveolar infiltrates. The child may have eosinophilia (>300 mm^3) and the total serum concentration of IgM may be increased. The presence of antichlamydial IgM antibodies to *C. trachomatis* is a pathognomonic sign. It is notable that *C. trachomatis* pneumonia only occurs in newborns, while *C. pneumoniae* is responsible for chlamydial pneumonia in adults. Neonatal chlamydial pneumonia may involve the risk of developing chronic obstructive lung (COL) disease.

TRACHOMA

Trachoma has been the most common cause of preventable blindness (Darougar and Jones 1983; Jones 1975). The pathological changes in the eyes are more pronounced in the upper than the lower lid. This is in contrast to paratrachoma (inclusion conjunctivitis), where the situation is the opposite (Figure 8). There

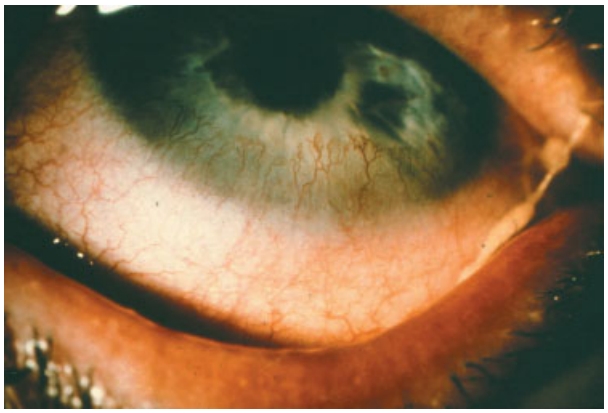

Figure 9 *Pannus formation in a case of trachoma*

are limbal follicules, pannus formation, and scarring, which very rarely occurs in paratrachoma cases (inclusion conjunctivitis). Trachoma may develop over decades and lead to blindness with pannus formation (Figure 9). Immunological, mechanical (entropion causes lesions of the cornea), and infections by other organisms which affect the injured eye makes the condition gradually progress. The prevalence of trachoma is related to poverty, overcrowded living conditions, and poor sanitation. Also, behavior factors may influence the prevalence (Mahmoud et al. 1994a).

CHLAMYDIA PSITTACI

Epidemiology

In the past there was an obvious overdiagnosis of human cases of infections with *C. psittaci* due to crossreactivity with *C. pneumoniae* (TWAR) or even with *C. trachomatis* if based on serology. Birds are the most common source of human infections ('parrot fever') (Olsen et al. 1998), although *C. psittaci* infects a large number of animals. Individuals shown by serological tests to be infected with *C. psittaci* refused to admit that they had had any recent contact with birds or any other animals. Interindividual transfer of infections by *C. psittaci* is a rare event. Spread from animals to humans may be airborne, but often occurs by handling infected material. Infected birds and other non-avian animal species may be healthy carriers of the organism. Persons most vulnerable to contract infections are those plucking and eviscerating birds in butchery plants, i.e. from chickens, turkeys, and ducks (Andrews et al. 1981). Feathers, excrement, nasal secretion, and viscera of infected birds can be the source of infection. Persons owning caged birds may occasionally be infected, as can veterinarians. Human infections have often been associated with the import of pscittacine birds, e.g. of budgerigars and cockatiels. As such birds may spread *C. psittaci*, many countries have strict quarantine regulations for importing caged birds. Other groups in danger of contracting infections are zoological shop personnel and persons connected to pigeon races. A famous epidemic occurred on the Faroe Isles among pickers of seagull eggs. The risk of laboratory infections must also be taken into account as a method of acquiring infections by *C. psittaci*, as must participation in the autopsies of infected animals.

Goats, sheep, pigs, horses, and cattle may be infected by *C. psittaci* (McCauly and Tieken 1968; Dilbeck et al. 1986). Humans are less often infected by these domestic animals.

Clinical conditions in humans

C. psittaci infections may spread from animals to humans, i.e. being the cause of zoonotic infections (Table 3).

PNEUMONIA AND EXTRAPULMONARY MANIFESTATIONS

Lung infections caused by psittacine birds have been referred to as cases of psittacosis, while infections by *C. psittaci* with nonpsittacine strains have been referred to as ornithosis cases.

The incubation period of psittacosis is 1–2 weeks to 1 month (Eidson 2002). The onset can be acute or start gradually. In most cases, there is a nonproductive cough, fever, chills, and headache. Diarrhea and a sore throat may be present. Respiratory symptoms may be absent, but occur in approximately one fifth of cases. Hepatitis, reactive arthritis, and erythema nodosum are less common manifestations. Abdominal symptoms may predominate. There may be splenomegaly. Auscultation may reveal vague rales, which is in contrast to the spread consolidations often seen at chest X-ray. Leukopenia, or at the most a slight increase in the number of white blood cells, are generally present (a hallmark of most human chlamydial infections). Rarely, meningitis, encephalitis, and myocarditis do occur. The infection may be fatal due to cardiovascular or respiratory insufficiency, which occurred in up to one third to one fifth of

Table 3 *Manifestations and consequences of infections by* Chlamydia psittaci *in humans*

Manifestations and consequences of infections
Pneumonia (ornithosis/psittacosis, 'parrot fever')
Placentitis (abortion)
Premature birth
Hepatitis
Conjuctivitis
Myocarditis
Reactive arthritis
Meningitis
Encephalitis

psittacosis cases before the antibiotic era and development of modern intensive care. At present, fatal cases are rare. Autopsy may reveal lung alveoli filled with exudate and extensive signs of pneumonitis.

PLACENTITIS AND ABORTION

Women handling infected sheep may contract infections with the 'ewe abortion agent' resulting in abortion, stillbirth, and premature birth. Humans can contract the infection by lambing sheep or taking care of newborn lambs. Pregnant women may become severely ill (Johnson et al. 1985). Signs of placentitis are found in cases where macroscopic inspection reveals microinfarction areas (Andrews et al. 1981). The histological picture is characterized by signs of infection of the trophoblasts. Caprine *C. psittaci* strains, i.e. '*Chlamydophila abortus*,' serovar 1, can cause placentitis and abortion in women. Infected goats may also abort (Pospischil et al. 2002).

CONJUNCTIVITIS

Cats may infect humans with the chlamydia feline conjunctivitis agent *Chlamydia (Chlamydophila) felis* (Ostler et al. 1969). Intrafamily spread of this eye infection occurs.

Infections in animals

A variety of clinical manifestations can be documented in animal infections by *C. psittaci*, e.g. ocular and central nervous infections, pneumonia, pericarditis, arthritis as well as intestinal (bowel) trachoma, and genital tract infections. Cats may get gastrointestinal infections causing diarrhea (Guillard et al. 1984). Infertility due to *C. psittaci* infection threatens the existence of the koalas in Australia.

CHLAMYDIA PNEUMONIAE

Epidemiology

Antibodies to *C. pneumoniae* is a common finding in population-based surveys. Seroprevalence figures from such studies from USA, Middle America, Spain, Japan, Taiwan, and New Zealand vary betwen 54–59, 62, 63, 48, 75, and 47%, respectively (Saikku et al. 1988). It is estimated that each individual as a mean will contract at least two to three infections by *C. pneumoniae* during their lifetime. It is notable, however, that there are very few isolates of the organism in relation to the claim that *C. pneumoniae* will infect the vast majority of individuals. Men more often have serological evidence of having acquired infections with *C. pneumoniae* than women. This also accounts for smokers. Great variations in the prevalence of *C. pneumoniae* serum antibodies have, however, been reported by time and geographic origin, in both children and adults. The group most commonly affected by *C. pneumoniae* is children 5–15 years of age.

The first evidence of the existence of respiratory tract infections with *C. pneumoniae* was based on an epidemic of such infections in Finnish military barracks in 1976 (Kleemola et al. 1988). In conscripts who had just arrived at the camp, 25 percent had such serum MIF antibodies in contrast to 47 percent of those who had been in the garrison during the epidemic. In another Finnish camp, this latter percentage was 51.

The difficulty of distinguishing a past infection from a current infection by *C. pneumoniae*, and crossreactions with other chlamydial species in serological tests has led some questions to the value of many early seroepidemiological studies. Thus, interpretation of early seroepidemiological studies of pneumonia cases must be made with caution if based on CF tests. This is due to the fact that the test does not differentiate between infections by *C. psittaci* (i.e. ornithosis cases) and infections by *C. pneumoniae*.

Children in developing countries seem to be more commonly affected by pneumonia by *C. pneumoniae* than those in industrialized communities.

Clinical conditions

Proposed and documented manifestations of infections by *C. pneumoniae*, are shown in Table 4.

UPPER RESPIRATORY TRACT INFECTION, INCLUDING CHRONIC BRONCHITIS

Most upper respiratory infections by *C. pneumoniae* seem to be rather mild, particularly in children. Asymptomatic infections do occur (Grayston et al. 1986; Schmidt et al. 2002). The infection is often self-limiting, but persistent infections do occur although they are uncommon in children. Endemic-like situations have been registered from time to time. A correlation between chronic carriership of *C. pneumoniae* and excerbation of chronic bronchitis has been demonstrated, as evidenced from analyses of sputum samples tested by nested touchdown PCR. Prolonged antibiotic courses have been recommended for effective clearance

Table 4 *Manifestations – documented and proposed – and sequelae of infections by* Chlamydia pneumoniae

Documented and proposed manifestations
Rhinitis
Pharyngitis
Pneumonia
Chronic obstructive lung disease (COL, COPD, COAD)
Arteriosclerotic plaque formation
Coronary heart disease
Carotid artery stenosis
Aortic valve stenosis

of *C. pneumoniae* in cases with chronic bronchitis (Blasi et al. 2002).

PNEUMONIA

Infection with *C. pneumoniae* is regarded as one of the three most common etiological agents of community-acquired pneumonia (Monno et al. 2002). Lung infections with *C. pneumoniae* in children share a number of characteristics with that of *Mycoplasma pneumoniae* pneumonia (Nelson 2002). Double infections with the latter organism and *Streptococcus pneumoniae* is also common, which adds to the difficulty of managing such infections based only on case history, symptoms, and signs. Also, chest X-ray and laboratory tests involve differential diagnostic problems. Cough, fever, and fatigue are present in the majority of cases followed by rhinitis, pharyngitis, headache, and myalgia in almost half of all cases developing pneumonia (Table 5), while one or more symptoms of nausea, vomitus, dyspnea are found in approximately one fifth of the patients. Abdominal and chest pain, otitis media, sinusitis, diarrhea, bronchitis, erythema nodosum, exanthema, myocarditis, and pericarditis may be found, but infrequently.

To establish the diagnosis of *C. pneumoniae* pneumonia, serum samples should be collected for serological tests and sputum for antigen detection assays.

CHRONIC OBSTRUCTIVE LUNG DISEASE (COL, COPD, COAD) AND ASTHMA

Chronic infections by *C. pneumoniae* is believed to be involved in the development of COL (COPD, COAD) or chronic acute lung disease (Clementsen et al. 2002; Smieja et al. 2002). Progress of COL is linked to smoking, which is a behavioral factor not yet considered strong enough in studies searching a link between COL and *C. pneumoniae*. Almost half of all smokers will experience COL after 50 years of age. Neonatal pneumonia caused by *C. trachomatis*, like other types of pneumonia affecting babies and infants (Beem and Saxon 1977), may play an etiological role in COL.

Some studies claim an etiological role of *C. pneumoniae* in asthma (Clementsen et al. 2002), while others have not found the seroprevalence of *C. pneumoniae* antibodies to differ between asthmatic patients and controls. Use of corticosteroids in asthmatic patients interferes with the T helper (Th)-1 response and enhances a Th-2 response, which decreases the ability to clear intracellular infections, as that with *C. pneumoniae*. Detection of serum IgA antibodies to *C. pneumoniae* may be used to diagnose chlamydial infection in asthmatic patients. In contrast to coronary disease (see below), detection of chlamydial hsp is not a useful marker of chlamydial infection in patients with less severe asthma.

Table 5 *Symptoms and signs of infections by* Chlamydia pneumoniae *(C = common, i.e. >40 percent, LC = less common 39–11 percent, U = uncommon <10 percent).*

Symptoms and signs of infection	
Cough (C)	Abdominal pain (LC)
Fever (C)	Chest pain (U)
Fatigue (C)	Diarrhea (U)
Myalgia (C)	Exanthema (U)
Nausea (LC)	Arthritis (U)

CORONARY HEART DISEASE AND CONDITIONS AFFECTING NONCORONARY ARTERIES

Associations between *C. pneumoniae* and heart disease have been proposed, i.e. between early impaired coronary flow in heart infarction (coronary artery stenosis) and aortic stenosis. However, other studies deny any such correlation (Janatuinen et al. 2002). Many of the assumed correlations between *C. pneumoniae* and coronary heart disease have also been claimed for *Helicobacter pylori* (Bloemenkamp et al. 2002). hsp60 IgA antibody level is associated with coronary disease, especially in cases with documented *C. pneumoniae* infection and inflammation.

Carotid artery stenosis has been associated with *C. pneumoniae* in some, but not in other, seroepidemiological studies. An association between intimal calcification of femoral arteries in nondiabetic patients and serum antibodies to the organism has been demonstrated.

ARTERIOSCLEROTIC PLAQUE FORMATION

The first observation of a possible association between myocardial infarction and infection by *C. pneumoniae* was based on a serological study of Finnish men publised by Saikku and coworkers in 1988 (Saikku et al. 1988). *C. pneumoniae* infection has been associated with arteriosclerotic plaque formation, e.g. in coronary arteries (Kuo et al. 1993). Others have not found any impaired coronary blood flow in persons with *C. pneumoniae* antibodies (Janatuinen et al. 2002). *C. pneumoniae* antigen has been demonstrated in aortic valves in patients undergoing aortic valve replacement, but others have failed to make such a detection (Rose 2002; Vainio et al. 2002).

A large number of scientific groups have studied the possibility of a correlation between infections by *C. pneumoniae* and arteriosclerosis. However, the results have been contradictory both in seroepidemiological studies and in attempts to demonstrate the agent in arteriosclerotic plaques. The outcome of interlaboratory investigations of the same clinical material has given completely contradictory results, i.e. some laboratories have found evidence of an overall occurrence of *C. pneumoniae* infections in the samples tested, while others completely failed to do so. *C. pneumoniae* seropositivity has been correlated to thrombosis-related microembolization, but not to plaque-related embolization.

TNF-α is involved in the pathogenesis of artherosclerosis, but no link between this factor and *C. pneumoniae* has been demonstrated either to cardiovascular disease or dementia. A role of chlamydial hsp60 as a mediator of inflammatory response during infection by *C. pneumoniae* has been proposed (Costa et al. 2002).

Similar correlations as for *C. pneumoniae* and cardiovascular and other vascular diseases have been made with *Helicobacter pylori* (Fong et al. 2002) and recently also for cytomegalovirus (CMV) (Bloemenkamp et al. 2002) and herpes virus. This has changed the point of argument that rather the total 'infectious load' – perhaps from childhood – is more important for the development of arteriosclerosis than any single agent.

Experimental infection by *C. pneumoniae* in mice (Liuba et al. 2002) and rabbits accelerates arteriosclerotic plaque formation, which has been used as an argument for an etiological link. However, the study designs used did not clearly show if the inoculation dose caused an infection or rather a vaccination. If the latter is true, the possibility of a terrifying side effect of vaccination against *C. pneumoniae* could be assumed.

INFLAMMATORY BOWEL DISEASE

Some studies claim a role of *C. pneumoniae* in inflammatory bowel diseases, but the results are highly conflicting. Studies have confirmed the common presence of *C. pneumoniae* in colonoscopic biopsies in cases of Crohn's disease and ulcerative colitis. However, no significant difference with controls without evidence of any inflammatory bowel disease could be demonstrated (Chen et al. 2002). It is considered that many microbes, including bacteria and viruses, passing the intestine may co-act with chlamydia to produce an inflammatory response promoting development of pathological processes in vessels.

CNS DISEASES AND PERIPHERAL NERVE SYSTEM ENGAGEMENT

As for several other syndromes, a possible correlation between multiple sclerosis (MS) and late onset Alzheimer's disease on one hand, and *C. pneumoniae* on the other, have given conflicting results. A significant difference in the prevalence of antibodies to *C. pneumoniae* in patients with acute ischemic stroke and controls has been detected (Ngeh et al. 2003). However, other recent studies have not come out in the positive for an etiological role of *C. pneumoniae* in any of these CNS conditions.

DIAGNOSIS

Microscopy

In earlier days, Giemsa was used to strain EBs, which are eosinophilic in contrast to the basophilic RBs. Also, dark-field microscopy can be used to detect chlamydia organisms where EBs appear as yellow bodies due to their natural autofluorescence. Microscopy, using monoclonal antibodies to *C. trachomatis* (Tam et al. 1982), is a rapid means of detecting chlamydia EBs in eye secretion in conjunctivitis cases. The sensitivity of immunoflourescence (IF) tests of genital secretion is problematic in more longstanding infections where the number of EBs has often become low. The use of IF tests for urine specimens is also a problem, as there are many artifacts in the samples that can mimic EBs. It is rare to detect chlamydial inclusions in clinical samples. The advantage of IF tests is that the answer may be obtained rapidly. Unfortunately, IF tests have been used by inexperienced staff in many countries, which has resulted in a large number of false-positive diagnoses.

Culture

Commercial interests once colored the debate about which is the best method for diagnosing genital chlamydial infections, in which the value of culture was underscored. The most widely applied culture method in current use is application of cycloheximide-treated McCoy cells (Ripa and Mårdh 1977). Cycloheximide inhibits the protein metabolism of the host cell, thereby favoring growth and multiplication of chlamydia organisms, as being energy-parasites stealing ATP from the host cell. Chlamydial inclusions in cell cultures can be easily detected by iodine staining of the cell carpet, where inclusions stain brown in contrast to the yellow color of the host cells. Inclusions can also be detected by staining with monoclonal antibodies by which they appear intensively apple green (Stamm et al. 1983). Counterstain with rhodamine B gives the cells a red color, which makes the detection of chlamydia-positive cases easier and the picture is attractive to the eye (Figure 10). To establish the diagnosis, at least ten EBs have been recommended to be used as the cut-off

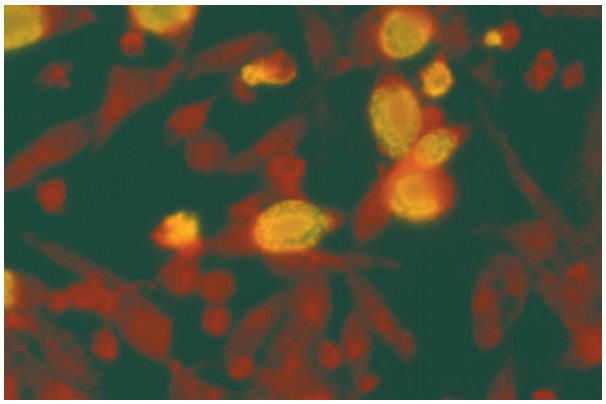

Figure 10 *Intraacytoplasmatic inclusions of* Chlamydia trachomatis *in cycloheximide-treated McCoy cells cultures stained with labeled monoclonal antibodies. Cell carpet counterstained with rhodamin B*

level. A lower cut-off can be accepted, but must be related to the experience of the microscopist. However, a high cut-off will mean that chlamydia-infected individuals may be falsely classified as negative.

The use of both urethral and cervical cultures has been recommended for detection of genital chlamydial infections, increasing the positive rate by approximately 5 percent. However, if taking consecutive samples from the cervix at the same time, the sensitivity of culture studies will also increase. The third consecutive sample collected with cotton-tipped swabs from the cervix yielded the highest positive rate. Use of urine for culture is inadequate, as the positive rate is only 30 percent compared to cervical samples from the same individuals. The current sampling recommendation is to use the first collected cervical swab for gonococcal culture (or discard it) and thereafter collect a sample for chlamydia culture (Mårdh et al. 1981b). The type of sampling swab used is essential, as both cotton and wood sticks can be highly toxic to chlamydia organisms, due to calcium alginate and resins, respectively (Mårdh and Zeeberg 1981).

With the exception of serovars L 1–3, EBs produced in primary cultures of clinical samples do not enter uninfected cells after being released. In the case of LGV, more or less the entire cell carpet may contain inclusions. This characteristic can be used as a diagnostic marker for LGV.

C. psittaci can be grown by the same principles as for *C. trachomatis*, but the high risk for laboratory infections has made serology preferable. *C. pneumoniae* is difficult to get to multiply in tissue cell culture, which may explain the very limited number of strains of this species that have been isolated. Using the same methods as for isolation of *C. trachomatis*, i.e. cycloheximide-treated McCoy cells, the inclusion count of *C. pneumoniae* in primary cultures is extremely low. HeLa cells treated by DEAE-dextran have been proposed to be a better choice, like human lung (HL) cells. The few inclusions that show up are generally small in size (Figure 11). *C. pneumoniae* is also difficult to propagate in tissue cell cultures.

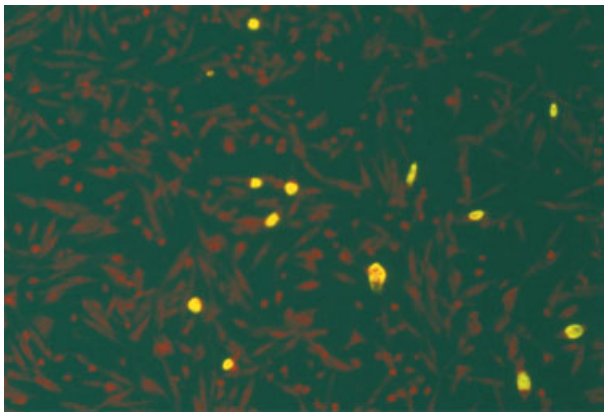

Figure 11 *Intraacytoplasmatic inclusions of* Chlamydia pneumoniae *in McCoy cells stained with immunofluorescent antibodies. Cell carpet counter-stained with rhodamin B.*

Cultures may serve the purpose of allowing rapid test of microbial cure after antibiotic therapy, that is, during the second to third weeks after finishing treatment. DNA tests may then still be positive due to the presence of dead, but not yet shed EBs. The use of cultures as a diagnostic method also offers the possibility of performing antibiotic susceptibility tests of isolated strains and detecting nonplasmid-containing strains.

Geno- and serotyping of clinical isolates

Serotyping of isolates of *C. trachomatis* has so far only been performed in an epidemiological context (Persson 2002). Serovars A, B, Ba, and C of *C. trachomatis* have generally been connected with trachoma, although also D (generally connected with genital infections and inclusion conjunctivitis) may sometimes cause trachoma. Serovar B may also infect genital organs. Serovar E is predominant in nontrachoma areas, e.g. in North America, Europe, and South Pacific. It often outnumbers the prevalence of all other serovars together. Double infections with more than one serovar do occur, but are not common. It seems fair to conclude that there is no strong evidence for a virulent advantage of any of the serovars.

Genotyping by sequencing the chlamydia MOMP gene has revealed subtypes within serovars of *C. trachomatis* (Frost et al. 1991; Pedersen et al. 2000).

ELISA

ELISA tests were widely used to diagnose genital chlamydial infections. Such tests are still in use in economically less developed countries, mainly because of the price being lower than for DNA-based tests. Because of the low specificity, it has been recommended to use confirmatory tests in case of a positive ELISA test, such as in Chlamydiazyme® tests. In ELISA tests, immobilized LPS antibodies bind the LPS antigen. A secondary antibody linked to an enzyme causes a color change that can be registered optically. 'Gray zone' results is a dilemma with these diagnostic tests. The results falling within this zone must be rechecked with confirmatory tests (Mårdh et al. 1990). This delays the return of answers to the clinician. If the cut-off is increased, the sensitivity will, of course, decrease. Other problems are crossreactions with *Staphylococcus aureus* and gram-negative bacterial species, such as *Escherichia coli*. This problem is accentuated when rectal, urine, and pharyngeal samples are to be tested (not recommended).

Nucleic acid and ribosomal tests

At present, nucleic acid amplified tests (NAAT), such as Cobas Amplicor, Roche (PCR) (Livengood and Wrem 2001), LCR (i.e. LcX, Abbott (Lee et al. 1995) and

real-time PCR tests, have been recommended for the diagnosis of genital chlamydial infections. The last method is more sensitive than nested touchdown PCR techniques. PCR and LCR tests for *C. trachomatis* are based on DNA sequences in the cryptic plasmid found in all such strains, but with a few documented exceptions (Peterson et al. 1990). Real-time PCR has been employed to detect *C. pneumoniae* antigen (Bennedsen et al. 2002).

There were initial problems with Taq-polymerase inhibitors and DNA contamination during sample handling, particularly after amplification. Adding inhibitors of potential inhibitors to the kits has solved these problems to a large extent, together with the development of closed sample systems, and chemical sterilization of amplified products. In-house nucleic acid test results from research laboratories that have not taken such precautions must be analyzed with caution. In low prevalence populations, there are problems with the positive predictive value in DNA tests, creating management problems. Clinicians being in charge of patients who mainly belong to a low prevalence group for genital chlamydial infections are recommended retesting of all patients with a positive test result (preferably with another diagnostic test) to avoid false diagnoses.

Chlamydia 16S-rRNA genes have been utilized in recently introduced commercial tests. Transcription-mediated amplification (TMA) tests make use of rRNA as target. These tests have a clinical value similar to that of the DNA-based tests. In the past, Gene-Probe® was the most commonly applied ribosomal RNA test used.

The introduction of DNA and RNA tests meant an increased sensitivity and specificity of chlamydial diagnostic services. Paradoxically, the introduction of automated systems handling NAAT analyses increased the specificity of chlamydial tests in units staffed with inexperienced technicians formerly involved in subjective tests, such as reading of IF tests and cultures.

Some commercial NAAT kits, e.g. for PCR and LCR tests, are offered as a package of tests for both *C. trachomatis* and *Neisseria gonorrhoeae* (Livengood and Wrem 2001).

In LCR tests (Stary et al. 1997), swabs from the vaginal introitus yield a somewhat higher positivity rate than samples collected from the cervix of the same women. Self-swabbing of the introitus by women themselves resulted in an even somewhat higher percentage of positive cases then when the sampling of the same women was performed by medical personnel.

Doctor' s office tests

Doctor' s office tests for the diagnosis of genital chlamydial infections, such as Clearview® and Surecell®, have a low accuracy compared to NAAT tests. One advantage of the former tests is, however, that the result can be obtained when the patient is still at the clinic.

Serology

Tests for antichlamydial antibodies have made use of both chlamydial LPS and MOMP as antigen. Chlamydial LPS is shared by all chlamydial species. As LPS of *C. trachomatis* crossreacts with *C. pneumoniae* and *C. psittaci* and was easy to produce, it was in the past made use of in complement-fixation (CF) tests used for diagnosing LGV and ornithosis/psittacosis (Mårdh et al. 1989). Recombinant MOMP and recombinant OMP-2 have been used to detect antichlamydial antibodies. A species-specific peptide has been identified, which avoids crossreactivity problems in serological assays of chlamydial infections by the various species (Närvänen et al. 1997).

In MIF tests (Wang and Grayston 1982), chlamydia antigen can be applied on a glass slide and a panel of known serovar-specific antibodies can be used to determine the identity of the antigen. Antigens of known serovars of *C. trachomatis* can also be used to test for the presence of serovar-specific antibodies. The use of LPS-free preparations of chlamydia antigen in MIF tests have demonstrated crossreacting antibodies to other chlamydia species being common, particularly during early onset of the immune response (Domeika et al. 1997). Thus, the antibody response becomes more species-specific after the onset of antibody production. A Washington Research Foundation, Labsystems, and MRL Diagnostics kits for MIF tests can be used to detect IgM, IgG, and IgA antibodies to *C. pneumoniae*. The value of real-time quantitative PCR for detection of *C. pneumoniae* has been evaluated (Mygind et al. 2002).

Serology has so far not been a useful means for diagnosing ongoing genital chlamydial infections. The fact that both IgA and IgG antibodies to *C. trachomatis* occur in a high percentage of women with no evidence of a current genital chlamydial infection means that the positive predictive value is unacceptably low. PID patients may lack IgM antibodies to *C. trachomatis*, which might be due to cervicitis which has preceded the tubal infection by some time. Serological tests have been of value in seroepidemiological surveys. Serological tests are, however, of clinical significance in the diagnosis of neonatal pneumonia in which a positive antichlamydial IgM antibody test, is a pathognomonic sign (Persson 2002). The presence of IgA antichlamydial antibodies has been believed to predict a persistent chlamydial infection, as IgA antibodies in general disappear quicker than IgG antibodies. A test for IgG antibodies to *C. trachomatis* has also been argued to be a screening test for tubal occlusion due to a previous chlamydia infection and to support the

diagnosis of ectopic pregnancy. These three latter proposals have, however, been opposed as being clinically useful. Presence of hsp60 antibodies to *C. trachomatis* seems to be a better predictor of tubal factor infertility and ectopic pregnancy than demonstration of MIF antibodies to the agent.

In human *C. psittaci* infections, e.g. in ornithosis/psittacosis, IgM antibodies are often lacking or occur in only low titers, while seroconversion of IgG antibodies can usually be demonstrated. However, a significant IgG antibody change may take up to 3 weeks. Also IgA titer rises can be seen. CF tests using LPS-containing antigen will involve the risk of crossreactivity with other chlamydial species. MIF tests have also been used for antibody detection.

ANTIBIOTIC SUSCEPTIBILITY

Until recently, antibiotic susceptibility tests have played an unimportant role in the management of infections by *C. trachomatis* (Persson 2002) in the belief that all strains are susceptible, at least to erythromycin, azithromycin, and tetracycline drugs, such as doxycycline. However, there are now reports of strains with high minimum inhibitory concentration (MIC) values to erythromycin (i.e. >1 μg/ml) and tetracycline (i.e. >4 μg/ml) (Lefevre and Lepaargneur 1998; Somani et al. 2000). Single strains with a reduced susceptibility to azithromycin and to quinolones, e.g. to ofloxacin, have been linked to therapeutic failure. However, there have been difficulties obtaining interlaboratory consistent susceptibility determinations, which have been an obstacle in the interpretation of such tests and of their clinical significance. Chlamydia organisms lack muramic acid, but the presence of two penicillin-binding proteins may explain their susceptibility to betalactam antibiotics, provided enough high doses are given for at least 2 weeks.

Chlamydia-positive cases at retests have usually been regarded as cases of reinfection or due to compliance problems rather than to antibiotic resistance. However, there seems to be a mandatory task to start to routinely survey the existence of resistant strains of *C. trachomatis* in different geographic locations and to develop surveillance systems for registration of nonsusceptible strains.

Under antibiotic pressure, *C. trachomatis* may, in in vitro tests, develop decreased susceptibility to the drug under test. Such strains usually lose their antibiotic resistance when propagated in absence of the antibiotic. Such heterotypic resistance has, however, not been connected to therapeutic failure (Schmid et al. 2001). Subinhibitory concentrations of betalactam antibiotics may induce RBs which are insensitive to this group of antibiotics. This might explain relapses after use of betalactam antibiotics. RBs are naturally also insensitive to some other antibiotics, which may be another reason for relapses after antibiotic therapy.

ANTICHLAMYDIAL ACTIVITY OF BODY FLUID

Body fluids possess antichlamydial activity, e.g. amniotic fluid (Elbagir et al. 1989a), cervical secretion (Mahmoud et al. 1994b) (with varying effect during the menstrual cycle), saliva (Genc et al. 1994), and seminal fluid (Mårdh et al. 1980a). The seminal fluid activity may explain why chlamydial infections of the male accessory genital glands are rare, compared to PID. The antichlamydial activity of tear fluid (Elbagir et al. 1989b) may explain why transient colonization is more common than conjunctivitis in infants born to mothers with genital chlamydial infection (Mårdh et al. 1980a). The antichlamydial activity of human milk (Elbagir et al. 1990) may also have a protective effect against the development of neonatal chlamydial infections.

THERAPY

Chlamydia trachomatis

Protein inhibitors, e.g. tetracyclines, erythromycin, and azithromycin, are all first-line alternatives in the therapy of chlamydial infections, apart from tetracycline in pregnancy (Brihmer et al. 1995; Carlin and Baron 1996; Magid et al. 1996; Genc 2002; Guaschino and Ricci 2002). Other macrolides, such as roxithromycin and josamycin seem to have a somewhat lower activity than erythromycin, which can be compensated with higher doses and/or prolonged courses (Stenberg and Mårdh 1993).

Azithromycin is a derivative of erythromycin, classified as an azilide antibiotic. It has the advantage that it is rapidly and efficiently taken up by eukaryotic cells, where it can reach chlamydia organisms. Another advantage is that azithromycin can be given as a single dose regimen, i.e. with 1 g. The drug also persists in the body for a period similar to a standard multidose regimen with tetracyline. Azithromycin therapy with this dose can be instituted at the clinic before a chlamydia-positive patient leaves. This means that one can avoid any compliance problem, which often arises with the multidrug intake required when using tetracyclines. Azithromycin can be used in pregnancy (Genc 2002). Doxycycline should be given with 200 mg per day both to men and women; in men for at least 7 days and in women preferably for 10–14 days. Different dose regimens for uncomplicated and complicated genital chlamydial infections in women is artificial, as almost half of all women infected by *C. trachomatis* have a PID (at least endometritis). It is not possible to identify women with PID without performing endometrial aspiration and histological investigation of the aspirate. Such an invasive procedure is unrealistic to perform on a routine basis

and involves risk to facilitate ascendance of chlamydia organisms to the upper genital tract.

Amoxicillin is listed as a second-line therapeutic alternative with a dose regimen of 500 mg three times a day for 7 days. For pregnant women, the best alternative is 1 g of azithromycin or amoxicillin 500 mg for 10 days (Genc 2002). Pregnant women tolerate these drugs better than erythromycin.

Quinolones are chlamydicidal in action (Guaschino and Ricci 2002). However, quinolones are regarded as second-line alternatives in chlamydial infections because of their comparatively low efficacy. Quinolones are contraindicated in pregnancy. Clindamycin, commonly used to treat bacterial vaginosis and to treat upper genital tract infections, has a too low activity against chlamydia organisms to be a drug of choice. This also accounts for chloramphenicol, sulfonamides, and aminoglycosides.

Co-infection with *N. gonorrhoeae* and *C. trachomatis* should be considered in any case in which such infections might be involved (Genc and Mårdh 1997). Some decades ago, roughly one quarter of all cases of either gonorrhea or genital chlamydial infection in Swedish STD and gynecological clinics, had such a double infection.

Treatment guidelines have been given for various situations in genital and allied infections by *C. trachomatis*, e.g. those produced by the Center for Disease Control and Prevention in the USA (CDC Guidelines 2002) and those of the Association of Genitourinary Medicine in UK (National guidelines for 1999).

Local therapy with tetracycline ointment has long been used to treat trachoma (Darougar and Jones 1983), but local therapy with azithromycin is likely to be more effective. General azithromycin therapy is even more effective in this disease (Holm et al. 2001).

Chlamydia psittaci and *Chlamydia pneumoniae*

Human infections by *C. psittaci* should be treated with the same drugs as in infections with *C. trachomatis*, i.e. with one of tetracycline, erythromycin, and azithromycin.

Resolution of pulmonary infection by *C. psittaci* usually starts after 1 week, although it can be longer. Psittacosis resolves in a fortnight to 1 month. Relapses do occur. Reinfection may occur from untreated caged birds. Prophylactic therapy in flocks of birds and non-avian herds infected by *C. psittaci* varies in efficiency.

Treatment of pneumonia with *C. pneumoniae* should be the same as in psittacosis.

Prophylactic antibiotic therapy of persons at risk for coronary heart disease did not result in a decreased rate of heart infarction, and neither did azithromycin prevent coronary heart infarction (Gupta et al. 1997). Treatment, for example with amoxicillin, metronidazole, azithromycin, or omeprazole reduced adverse cardiac events, but the effect was independent of *C. pneumoniae* and *Helicobacter pylori* seropositivity (Stone et al. 2002). Aspirin, and its derivate salicylic acid, had a positive effect on cardiovascular adverse events. The cardioprotective effect of salicylic acid, regarding infections by *C. pneumoniae* and ischemic heart disease, is believed to be mediated by induction of nuclear factor (NF)-kappa B production. This inhibits growth of *C. pneumoniae* and production of interleukines, such as IL-6 and IL-8 (Tiran et al. 2002). Once a month roxithromycin therapy for at least 2 years reduced the progress of peripheral arterial occlusion in a randomized, double-blind, placebo-controlled trial (Wiesli et al. 2002). Likewise, treatment with 150 mg roxithromycin twice daily for 1 month, reduced progress of early carotid artery arteriosclerosis in stroke patients followed-up for 2 years. The effect was best in *C. pneumoniae* seropositive cases (Sander et al. 2002). Reduction or cessation of smoking and regulation of diabetes are among possible confounding factors in heart infarction, the influence of which must be considered in evaluation of prophylactic antibiotic therapy in artery vessel diseases, like the weakness in the diagnostic tools for diagnosing *C. pneumoniae* infections (Broman and Hammerschlag 2002).

CHLAMYDIA SCREENING AND RESCREENING

As the majority of infections by *C. trachomatis* both in men and women are more or less asymptomatic, infected persons have generally no motivation to consult. Therefore, it is essential to perform screening to detect carriers (Genc et al. 1993; Genc and Mårdh 1996; Svensson et al. 1994). This is important as hindering spread of the organism to uninfected individuals will avoid adverse immunological reactions, which may continue even after microbiological cure in people who have contracted the infection.

Screening should be accompanied by partner notification. This is generally highly rewarding, as usually more than half of sexual consorts are chlamydia-positive. There is an obvious need for rescreening persons positive for *C. trachomatis*, as either relapse or reinfection is common in genital chlamydial infections (Mårdh and Persson 2002).

Voided urine or samples from the vaginal introitus collected by self-sampling with mailed or otherwise distributed sampling kits, allow mass screening of populations for *C. trachomatis* (Östergaard et al. 1996).

The pay-off of performing intensive screening programs has been calculated from computer modeling (Genc and Mårdh 1996). In Sweden, just over half a million chlamydia tests were performed for a number of years during the 1980s (in a population of approximately 8.5 million) (Mårdh 2002b). Apart from reduction of the

prevalence of genital chlamydial infections, the number of PID and extrauterine pregnancies decreased sharply after the screening activities (Weström 1998). Screening of selected groups has also proved effective to prevent complications from infections by *C. trachomatis*, for example in women about to undergo abortion (Osser and Persson 1984).

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Rickettsia and *Orienta*

DIDIER RAOULT AND J. STEPHEN DUMLER

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INTRODUCTION

Rickettsiae were historically defined as small gram-negative intracellular bacteria. This definition is very weak and has required that many organisms previously named *Rickettsia* needed to be reclassified. This includes *Coxiella burnetii* (first named *Rickettsia diasporica*), *Neorickettsia sennetsu* (first named *Rickettsia sennetsu* and then *Ehrlichia sennetsu*), *Ehrlichia canis* (first named *Rickettsia canis*), and *Bartonella quintana* (first named *Rickettsia quintana*, and lately *Rochalimaea quintana*), among others. The past 20 years have seen significant changes in the field. Three major changes include the reclassification of the Rickettsiales, the description of many new emerging rickettsial diseases, and the beginning of the genomic era for intracellular bacteria.

The universal gene encoding for 16S rRNA has been widely used for the classification of bacteria. It is particularly useful for intracellular bacteria such as rickettsiae because so few phenotypic criteria are available. Many genera were reclassified following 16S rRNA sequencing. The *Orienta* genus was created from an independent branch of the *Rickettsia* genus phylum (Tamura et al. 1995). The 'ehrlichia' group was recently reclassified (Dumler et al. 2001) into four genera, including *Ehrlichia* and *Anaplasma*, which are associated with ticks, *Neorickettsia*, which is associated with helminths, and *Wolbachia*, which are associated with both arthropods and helminths (Figures 1 and 2). The genus *Cowdria* has been abandoned and many species have been reclassified into other genera. The *Bartonella* genus is a close neighbor in the alpha group of proteobacteria and now comprises the former *Rochalimeae* species, *Grahamella*, and *Wolbachia melophagi*. *Coxiella* and *Rickettsiella* are now in the gamma group of proteobacteria closely related to *Legionella* species and *Francisella tularensis*. *Wolbachia persica* is a species in the genus *Francisella*. The more distantly related *Eperythrozoon* and *Haemobartonella* genera, including erythocytic animal pathogens that were classified with *Anaplasma*, are in fact closely related to mycoplasmas and issue from the gram-positive lineage.

Until the use of cell cultures and molecular amplification with typing for *Rickettsia*, identification was based on culture in animals and embryonated eggs. The description of new *Rickettsia* species was very slow. Since 1980, many new *Rickettsia* and rickettsial diseases have been found and more rickettsioses and ehrlichioses have been reported (Table 1). The new genetic tools, as well as the use of cell culture assays, have allowed for the discovery of many new rickettsioses and ehrlichioses in the past 20 years (see Table 1) (Raoult and Roux 1997), including three ehrlichioses and ten rickettsioses that have since been described. Three major conditions determined the discovery of new rickettsioses. Some were discovered following clinical descriptions in

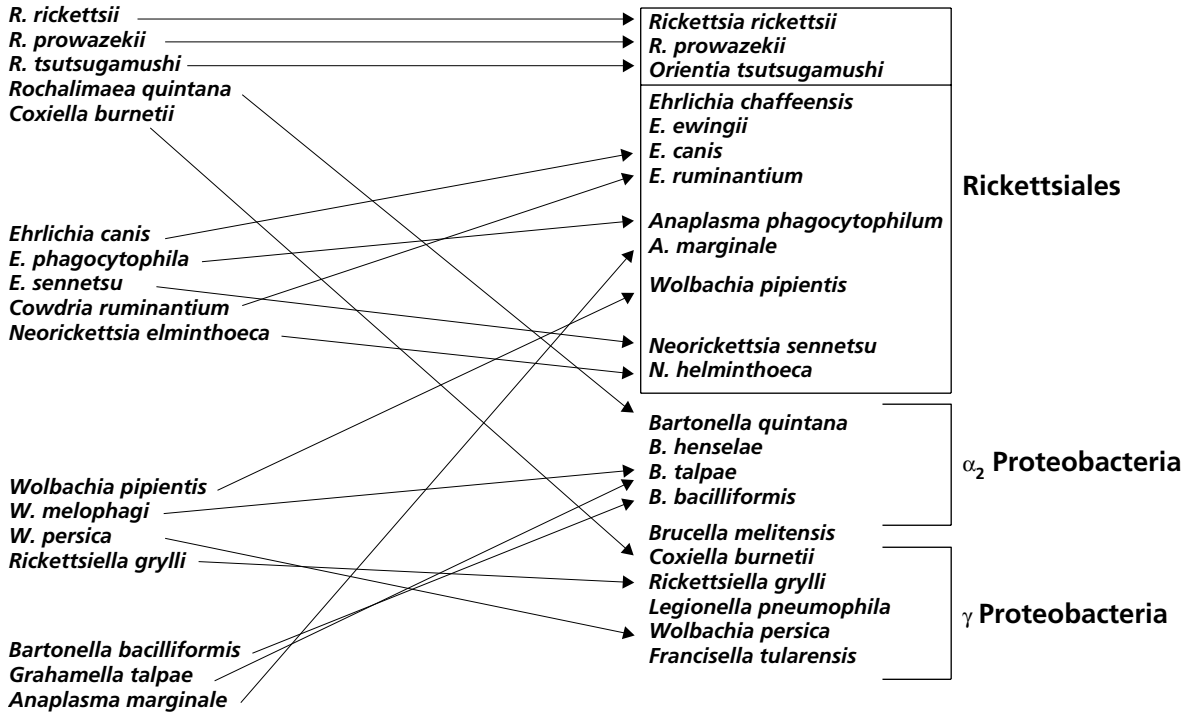

Figure 1 *Current classification of the Rickettsiales (right column) compared with the former classification (from 1984) (left column).*

countries where spotted fever was unknown (Japan and *R. japonica* (Mahara 1997), Flinders' Island and *R. honei* (Stenos et al. 1998), and Russia and Astrakhan fever (Tarasevich et al. 1991)). Some were recognized after bacterial identification based on culture and polymerase chain reaction (PCR) in places where the new pathogen was confused with another known rickettsial pathogen (*R. africae* (Raoult et al. 2001b), *R. mongolotimonae* (Fournier et al. 2000a), and *R. aeschlimanii* (Raoult et al. 2002b) with *R. conorii*, *R. felis* (Schriefer et al. 1994) with *R. typhi*). Finally, some were identified by the collaborations of physicians and microbiologists who faced atypical or unknown diseases (*R. slovaca* (Raoult et al. 2002a) and *R. helvetica* (Fournier et al. 2000b) (Raoult and Roux 1997)).

Many *Rickettsia* in their arthropod hosts were initially identified long before their implication as a human pathogen (Raoult and Roux 1997). The denomination 'nonpathogenic' rickettsia is therefore highly speculative and has later been proven untrue in many instances. For example, *Coxiella burnetii* and *Legionella pneumophila* were considered 'nonpathogenic.' Among rickettsiae, *R. felis* and *R. africae* were identified in their vectors long before being implicated as human pathogens.

Two species, *R. prowazekii* (Andersson et al. 1998) and *R. conorii* (Ogata et al. 2000, 2001), were among the first 50 sequenced bacterial genomes. They are considered paradigms of the intracellular bacteria with small genomes. Genomic studies have shown that *R. prowazekii* should be considered as a subset of *R. conorii* that has incompletely evolved in its current niche, as attested by the large proportion of noncoding DNA in the former species. This noncoding DNA consists mainly of remnants of genes found in *R. conorii*. Genome analysis showed that both rickettsiae have multiple copies of ATP-ADP translocase genes (a parasitic enzyme that allows the importation of adenosine triphosphate (ATP)), spot T (an alarmone that is secreted in other microorganisms with starvation), and proline/betain transporters. The analysis of *R. conorii* also exhibited two new strategies of bacterial evolution. Palindromic repeats that can integrate into coding genes were found (Ogata et al. 2000, 2002). The different steps of evolution from complete gene to a gene remnant through a 'splitting' phenomenon were also described (Ogata et al. 2001). Genome analysis of metabolic pathways that are lacking in Rickettsia may provide a rational basis for design of medium culture for the axenic growth of *Rickettsia* species, an ultimate goal for rickettsiologists.

HISTORY

Typhus is suspected to be the cause of many huge, historical outbreaks including the plague of Athens, as described by Thucidides in the fifth century BC. However, it is still controversial whether this event can truly be attributed to typhus. One theory proposes that typhus was imported from America after Columbus' return. This theory matches with the fact that the only nonhuman reservoir identified currently is in America in flying squirrels (Dasch et al. 1978). The first description of a disease compatible with typhus was made by

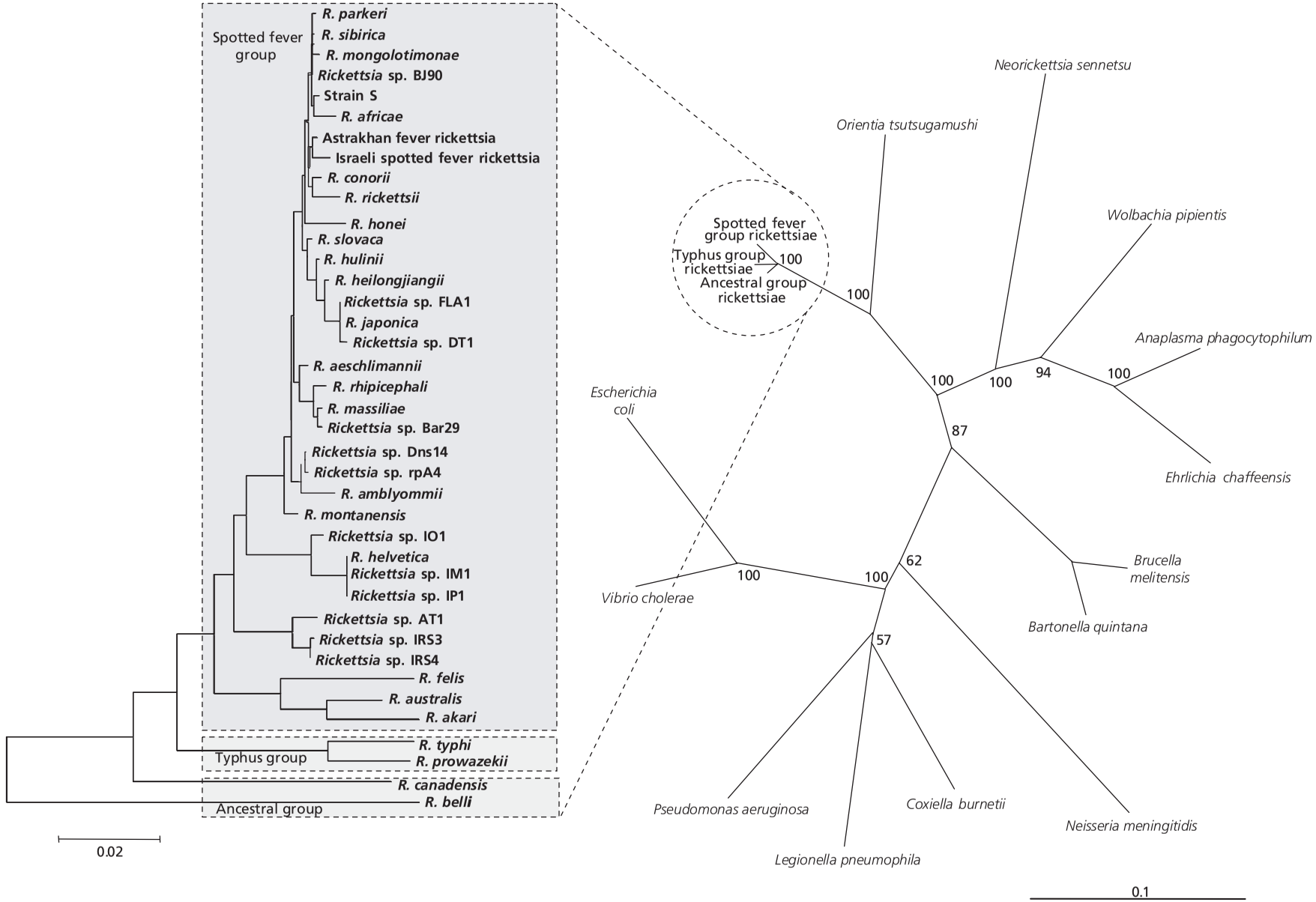

Figure 2 *Phylogenic tree including Rickettsiales and bacteria previously included in this group. The large tree (right) is obtained by the neighbor-joining method based on 16S r RNA; the left tree is based on citrate synthase and rOmpA.*

Fracastor. Typhus was included with typhoid in the group of diseases in which patients suffered fever and typhos (which in Greek means 'change in mental status'). Several authors, including Boissier de Sauvage in 1760 (Weiss, E. 1988), proposed a scheme to differentiate these two diseases (see Table 1). Typhus was first properly described by Von Hielderbrand in 1810 (Weiss, E. 1988) after the outbreak in Napoleon's army (Weiss, E. 1988; Weiss, K. 1988). Gerhard (Weiss, E. 1988) described the pathological differences in these two diseases in 1832. It is generally accepted that typhus and louse-transmitted diseases killed more than half of the 700 000 men of Napoleon's army during the Russian campaign in 1812 (Zinsser 1935). It also killed millions during the First World War and five million Russians during the Bolshevik revolution. During the Second World War, outbreaks were noted in concentration camps in North Africa and in Italy. The association of

Table 1 *Historical data on diseases caused by* Rickettsial *species (first and senior authors)*

Year	Discovery	Author	Reference
1760	Description of exanthematic typhus	Boissier de Sauvage	Weiss, E. 1988
1879	First report of scrub typhus	Nagayo	Rehacek and Tarasevich 1988
1899	Description of RMSF	Maxcy	Weiss, E. 1988
1908	Tick role in RMSF	McCalla	Weiss, E. 1988
1909	Role of body lice in typhus	Ch. Nicolle (Nobel Prize)	Raoult et al. 1997
1909	Description of MSF	Conor et al.	Raoult et al. 1997
1910	Serology test based on Proteus	Wilson	Weiss, E. 1988
1911	Isolation of *Rickettsia rickettsii*	Ricketts	Weiss, E. 1988
1911	Isolation of *R. prowazekii*	Ch. Nicolle	Raoult et al. 1997
1916	Weil–Felix test	Weil and Felix	Weiss, E. 1988
1921	Identification of *R. typhi*	Mooser	Weiss, E. 1988
1925	Description of the 'tâche noire' in MSF	Pieri	Raoult et al. 1997
1930	First isolation of *O. tsutsugamushi* (*R. orientalis*)	Nagayo	Rehacek and Tarasevich 1988
1930	Role of chiggers in scrub typhus	Kawarimura	Rehacek and Tarasevich 1988
1930	Role of fleas in murine typhus	Dyer	Weiss, E. 1988
1932	Isolation of *R. conorii*	Brumpt	Raoult et al. 1997
1935	Description of Siberian tick typhus	Shmatikov et al.	Rehacek and Tarasevich 1988
1938	Isolation of *R. sibirica*	Krontovuka et al.	Rehacek and Tarasevich 1988
1946	Description of Rickettsialpox	Huebner	Weiss, E. 1988
1946	Isolation of *R. akari*	Huebner	Weiss, E. 1988
1946	Isolation of *R. australis*	Plotz and Smadel	Weiss, E. 1988
1946	Queensland tick typhus	Plotz and Smadel	Weiss, E. 1988
1968	Isolation of *R. slovaca*	Brezina et al.	Rehacek and Tarasevich 1988
1974	Culture of *R. conorii* Israel	Goldwasser	Raoult et al. 1997
1979	Isolation of *R. helvetica*	Burgdofer and Peter	Raoult et al. 1997
1984	Japanese spotted fever	Mahara	Raoult et al. 1997
1985	Culture of *R. heilongjanhensis*	Lov	Raoult et al. 1997
1989	Culture of *R. japonica*	Udida and Walker	Raoult et al. 1997
1990	Isolation of *R. africae*	Kelly	Raoult et al. 1997
1999	Astrachan fever	Tarasevitch and Raoult	Raoult et al. 1997
1991	Flinders' Island spotted fever	Stewart	Raoult et al. 1997
1992	First case of infection by *R. africae*	Kelly and Raoult	Raoult et al. 1997
1992	Culture and identification of *R. conorii* caspian	Tarasevitch and Raoult	Raoult et al. 1997
1992	Culture of *R. honei*	Baird et al.	Raoult et al. 1997
1993	Culture and identification of *R. sibirica mongolotimonae*	Yu and Raoult	Raoult et al. 1997
1994	First case of flea-borne spotted fever	Schriefer and Azad	Schriefer et al. 1994
1996	Infection by *R. sibirica mongolotimonae*	Raoult et al.	Raoult et al. 1996
1997	First infection by *R. slovaca*	Raoult et al.	Raoult et al. 2002b
1997	Culture of *R. aeschlimannii*	Beati and Raoult	Raoult et al. 2002b
2000	First case of acute infection by *R. helvetica*	Fournier and Raoult	Fournier et al. 2002
2000	Culture of *R. felis*	Raoult et al.	Raoult et al. 2001a
2002	First case of infection by *R. aeschlimannii*	Raoult et al.	Raoult et al. 2002b

MSF, Mediterranean spotted fever; RMSF, Rocky Mountain spotted fever.

fever, rash, and neurologic involvement became the hallmark of rickettsial diseases. Rocky Mountain spotted fever in 1899 and Mediterraneous spotted fever were described based on these basic clinical findings.

The etiologic agents of these diseases were not found easily. They do not grow in basic axenic media. *Proteus* species were isolated from the urine of patients with typhus and were erroneously considered to be the etiological agents. By chance, they cross-react with rickettsia and were used in the first serological assay (the Weil–Felix test). Culture of the rickettsial agents was difficult but was finally obtained in laboratory animals at the beginning of the twentieth century (see Table 1). Rickettsial diseases have been among the most deadly in history. It was also very dangerous to work on these diseases and two famous rickettsiologists, Ricketts and Von Prowazek, died of typhus. Da Rocha Lima named the causative agent of typhus in their honor: *Rickettsia prowazekii*.

Charles Nicolle identified the role of body lice in the transmission of typhus and its agent, and the role of ticks in transmission of the causative agent of Rocky Mountain spotted fever was definitively established by H. Ricketts (see Table 1). He was also the first to isolate *Rickettsia rickettsii*.

Rickettsiae were grown first in animals. Guinea pigs (for *Rickettsia* spp.) and mice (for *Orientia*) were used. The scrotal reaction of interperitoneally infected guinea pigs allowed differentiation between *R. typhi* (first named *R. mooseri*) and *R. prowazekii*. As animals shed living organisms, manipulation of rickettsia was very dangerous. Until the discovery of the role of chloramphenicol in the treatment of typhus during the Second World War by Woodward, it remained a dangerous field (Weiss, K. 1988).

MICROBIOLOGY

The rickettsiae are intracellular parasites. They are small gram-negative bacteria that live freely in the cytoplasm of their infected eukaryotic host cells. Rickettsiae induce phagocytosis and rapidly escape from the phagocytic vacuole to live in the cytoplasm. Escape from the phagosome may be due to activation of a phospholipase. Phospholipase A2 was believed to be involved (Walker et al. 2003); however, a phopholipase A2 gene is not present in the genomes of *R. prowazekii* or *R. conorii*. It may be that phospholipase D, which is secreted by *R. conorii*, is in fact the key enzyme for intracellular parasitis (Renesto et al. 2003). Spotted-fever-group rickettsiae can grow in both the cytoplasm and the nucleus of infected cells, and they can also escape from living cells. Typhus-group rickettsiae grow in the cytoplasm only and are released when the heavily infected host-cell membranes are destroyed. This difference is determined by the intracellular motility of SFG rickettsiae, which is in turn determined by polymerization of host actin (Teysseire and Raoult 1992; Teysseire et al. 1992). *R. typhi* have limited motility and actin polymerization does not occur in *R. prowazekii*-infected cells. By comparing the genomes of *R. prowazekii* and *R. conorii*, it was speculated that a gene encoding for a protein resembling ActA that is involved in *Listeria monocytogenes* may be responsible for the ability to polymerize actin (Ogata et al. 2001); this theory has been confirmed (Harlander et al. 2003; Gouin et al. 2004).

Rickettsiae are 0.3–0.5 μm in diameter and 1–2 μm in length. As they do not stain well with Gram stain, other stains are preferable. The Giménez technique is used for *Rickettsia* species and Giemsa is used for *Orientia*. Rickettsiae can be grown in animals, including guinea pigs for *Rickettsiae* and mice for *Orientia*, as well as in embryonated eggs and cell cultures.

CLASSIFICATION

The genus *Rickettsia* was first divided into three different groups: spotted fever, typhus, and scrub typhus. *Rickettsia tsutsugamushi*, the agent of scrub typhus, has been found to be distinct enough by 16S rRNA gene sequence comparisons to warrant transfer into a new genus, *Orientia*, which was created to include the single species, *Orienta tsutsugamush* (Tamura et al. 1995). Presently, the genus *Rickettsia* is still made up of the typhus group (TG) and the spotted fever group (SFG). However, *R. canadensis*, which is transmitted by ticks and for which pathogenicity in man is unknown, along with *R. bellii*, does not belong to either of these groups. Several of the bacteria in these groups are pathogenic and are transmitted to man by arthropod bites or arthropod feces (Table 2). The TG includes *R. prowazekii*, the agent of the epidemic typhus, transmitted by the body louse, and *R. typhi*, the agent of murine typhus that is transmitted by fleas. The SFG includes an increasing number of serotypes following the improvement of approaches for rickettsial isolation and a more widespread interest in arthropod-transmitted disease (Raoult and Roux 1997). New SFG rickettsiae have been identified by culture or PCR from humans, including Israeli tick typhus rickettsia in Israel, Portugal, and Italy, *R. africae* in Africa, *R. japonica* in Japan, '*R. felis*' in Europe and America, the Astrakhan fever rickettsia in Russia, *R. honei* on Flinders' Island, Australia, and '*R. mongolotimonae*', *R. slovaca*, and *R. aeschlimannii* in France. Several rickettsia were isolated from ticks only: *R. massilliae* in France and in Greece, Bar 29 in Spain, *R. helvetica* in Europe and Japan, Strain S in Armenia, *R. peacockii*, '*R. cooleyi*,' and '*R. amblyommii*' in the USA, and *R. sibirica* HL-93 in China. Other rickettsiae were characterized in insects, but their culture has been impossible until now: AB bacterium, pea aphid rickettsia, and PTB bacterium were characterized in *Adalia bipunctata*, *Acythsiphon pisum*, and *Empoasca*

Table 2 *Classification of rickettsial strains*

Taxon	Vector	Geographic distribution
Spotted fever group		
R. conorii (malish)	*Rhipicephalus, Haemophysalis*	Mediterranean Sea, Black Sea, Africa
R. conorii strain M1	*Rhipicephalus*	Georgia
R. conorii strain Indian tick typhus rickettsia	*Rhipicephalus*	India
Astrakhan fever tick	*Rhipicephalus*	Astrakhan region (Russia)
Israeli tick typhus rickettsia	*Rhipicephalus*	Israel, Portugal, Italy
R. sibirica	*Haemophysalis, Dermacentor*	China, Russia, Pakistan
R. mongolotimonae	*Haemophysalis*	France, Inner Mongolia
R. africae	*Amblyomma*	Africa
R. parkeri	*Amblyomma*	USA
Strain S	*Rhipicephalus*	Armenia
R. slovaca	*Dermacentor*	Eurasia
R. honei	*Rhipicephalus*?, *Ixodes*?	Tasmania, Thailand
R. rickettsii	*Dermacentor, Amblyomma*	America
R. japonica	*Dermacentor, Ixodes*	Japan
R. heilongjanghensis	*Dermacentor*	Russia, China
R. paecockii	*Dermacentor*	USA
R. massiliae	*Rhipicephalus*	France, Portugal, Greece
Bar 29	*Rhipicephalus*	Spain
R. rhipicephali	*Rhipicephalus*	USA, Portugal, France
R. aeschlimanii	*Hyalomma*	Africa
R. montanensis	*Dermacentor*	USA
MO Aa	*Amblyomma*	USA
WB-8-2	*Amblyomma*	USA
'*R. amblyommii*'	*Amblyomma*	USA
R. helvetica	*Ixodes*	Switerzland, France, Sweden
R. akari	*Allodermanyssus sanguineus*	Worldwide
R. australis	*Ixodes*	Australia
'*R. felis*'	*Ctenophalides felis*	Worldwide
Unnamed group		
R. canadensis	*Haemophysalis*	Canada
R. bellii	*Argas, Ornithodoros, Dermacentor, Haemophysalis*	USA
AB bacterium	*Adalia bipunctata*	England
Pea aphid rickettsia	*Acrythosiphon pisum*	USA
PRB bacterium	*Empoasca papaye*	Puerto Rico, Costa Rica
'*R. cooleyi*'	*Ixodes*	USA
Typhus group		
R. prowazekii	*Pediculus humanus corporis*	Worldwide
R. typhi	*Xenopsylla cheopis*	Worldwide
Scrub typhus		
Orienta tsutsugamushi	*Leptothrombidium*	Asia, Oceania/entry>

papaye, respectively (Raoult and Roux 1997; Parola and Raoult 2001; Roux and Raoult 1999).

PHENOTYPIC IDENTIFICATION AND CLASSIFICATION INSIDE THE GENUS *RICKETTSIA*

As rickettsiae are difficult to grow and purify, studies have been very limited. Until recently, rickettsial phylogeny was nonexistent and classification had been based on morphological, pathogenic, ecological, and antigenic criteria, as they could not be characterized by the phenotypic criteria traditionally used in bacterial taxonomy.

The differentiation of the groups within the genus *Rickettsia* has historically been based on several factors (Weiss and Moulder 1984): (1) the intracellular position in the nucleus and the cytoplasm for the SFG rickettsiae and *R. canadensis*, but only in the cytoplasm for *R. prowazekii* and *R. typhi*; (2) an optimal growth temperature of 32°C for the SFG (or 28°C for *R. felis*) and 37°C for the TG; (3) a G + C percent of DNA of 32–33

percent for the SFG and of 29–30 percent for the TG; (4) the cross-reaction of sera from patients with rickettsial infection with the somatic antigens of strains of *Proteus*, including OX19 for TG and *R. rickettsii* and OX2 for SFG. Serologic cross-reactions were also noted between the *R. canadensis* group and *R. prowazekii* and *R. typhi* on the one hand and between the representatives of the SFG on the other hand. Complement fixation was the first test used among SFG to differentiate between Mediterranean spotted fever and Rocky Mountain spotted fever (Plotz et al. 1944). *R. sibirica*, *R. akari*, and *R. australis* were then also differentiated (Pickens et al. 1965). Toxin neutralization tests were successfully applied to the differentiation of the same rickettsiae (Bell and Stoenner 1960). The advent of the density gradient centrifugation purification method enabled the separation of rickettsiae from host-cell component (Weiss et al. 1975) and has allowed the study of rickettsial proteins. Rickettsiae have been differentiated on the basis of their protein profiles in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), including analysis of all rickettsia for which culture was possible. Western blot performed with antisera from convalescent humans or animals demonstrates reactivity with two proteins (now called rOmpA and rOmpB) for the SFG rickettsiae and *R. canadensis* and only with rOmpB for the TG rickettsiae. These outer-membrane proteins were shown to have molecular weights characteristic for each species (Gilmore and Hackstadt 1991). The indirect microimmunofluorescence (MIF) serologic typing test with mouse sera was developed in 1978 and was the reference method for the identification of new SFG rickettsiae for more than 10 years (Philip et al. 1978). The antigenic determinants for this serotyping scheme are rOmpA and rOmpB; two groups were confirmed, the TG, including *R. prowazekii*, *R. typhi*, and *R. canadensis*, and the SFG, in which ten serotypes were described. Two strains of *R. rickettsii*, Hlp-like and R-like, and isolates obtained from *Dermacentor occidentalis* in California clustered in the same serotype, but their antigenic profiles were different. Some antigenic relatedness among serotypes was identified. In one subgroup were included *R. sibirica*, *R. parkeri*, and *R. conorii*, which were found more distant. Another subgroup included *R. montanensis*, *R. australis*, and isolates obtained from *Ixodes pacificus* in Oregon, but these serotypes were only remotely related to one another. *R. rhipicephali* and *R. akari* did not clearly belong to any subgroup.

Anacker et al. (1987) demonstrated species, subspecies, and subgroup antigenic determinants on rOmpA and rOmpB by reactivity with monoclonal antibodies against *R. rickettsii*. Most of the species accepted by taxonomists could be differentiated, except for *R. akari* and *R. australis*, which reacted only with monoclonal antibodies to the group-specific lipopolysaccharide (LPS)-like antigen. Xu and Raoult (1998) determined taxonomic relationships among the SFG rickettsiae by comparative analysis of immunogenic epitopes reactive against a panel of 98 monoclonal antibodies. Monoclonal antibodies were directed against *R. africae*, *R. conorii*, *R. massiliae*, *R. akari*, *R. sibirica*, and *R. slovaca*. Neither monoclonal antibodies reactive with the LPS-like antigen nor those reactive with the major outer-membrane proteins reacted with *R. bellii*, confirming that this species is not an SFG rickettsiae. The taxonomic relationships determined among the SFG were consistent with those previously determined by genomic criteria, which are discussed below. Monoclonal antibodies also demonstrated that *R. felis* is antigenically related to SFG rickettsia (Fang and Raoult 2003).

Antibiotic susceptibility also allows differentiation of several groups. The rickettsiae of the *R. massiliae* group, of which *R. aeschlimanii* is a human pathogen (Raoult et al. 2002b), are resistant to erythromycin and rifampin. The other representatives of the genus *Rickettsia* are resistant to erythromycin and susceptible to rifampin (Rolain et al. 1998).

Considering modern biosystematics including phylogeny and population genetics, it is evident that the present classification and nomenclature of the genus *Rickettsia* are inadequate. The division of the genus in TG and SFG is not accurate based on gene phylogeny. *R. canadensis* and *R. bellii* should be excluded from the TG and the SFG, respectively.

Bacterial taxonomy should reflect classification and nomenclature of the different representatives and should allow identification of unknown organisms (Table 3). Polyphasic taxonomy, which integrates phenotypic and phylogenetic data, seems to be the best approach for rickettsial taxonomy, as demonstrated for other bacetrial genera (Vandamme et al. 1996). Serotyping by microimmunofluorescence and monoclonal antibodies are available. However, as these antibodies are often directed against rOmpA and rOmpB outer-membrane proteins, the information they give is redundant with those of genotypic approaches based on study *ompA* and *ompB* sequences, which are less subjective. Whole-cell protein patterns for the rickettsiae can be compared when culture is possible. Several methods should be considered. A standard definition determines that different strains belong to the same species if they share 70 percent or greater DNA–DNA relatedness (Wayne et al. 1987). However, the 70 percent cutoff value is not absolute. For rickettsia as an example, when applying this criterion, *R. rickettsii*, *R. conorii*, *R. sibirica*, and *R. monatensis* would be a single species (Walker 1989). An alternative phylogenetic species concept could delineate a species by comparison of percent gene similarities. The comparison of 16S rRNA sequences is not a good taxonomic approach for analysis of the genus *Rickettsia* because more than 97 percent similarity exists between any two taxa. However several other gene sequences have been determined for most species and

Table 3 *Genome size, G + C percent content, DNA–DNA homology, and antibiotic susceptibility for different representatives of the genus* Rickettsia

Representative	Genome size (kb)	G + C%	Erythromycin susceptibility	Rifampin susceptibility
R. conorii	1 237	32–33	R	S
R. rickettsii	1 272	32–33	R	S
R. sibirica	1 243	32–33	R	S
R. massiliae	1 370	NT	R	R
R. rhipicephali	1 256	32–33	R	R
R. monatensis	1 250	NT	R	R
R. akari group				
R. akari	1 229	29–30	R	S
R. australis	1 269	NT	R	S
R. prowazekii	1 119	29–30	S	S
R. typhi	1 099	29–30	S	S
R. canadensis	NT	29–30	R	S

NT, not tested; R, resistant; S, sensitive.

could be used to establish a method for rickettsial classification and nomenclature, including *ompA*, *ompB*, *gltA*, 17-kDa lipoprotein gene, and gene D (Sekeyova et al. 2001) (see Figure 1). Antibiotic susceptibility patterns also provide an interesting approach for confirming phylogenetic groups, as determined for rifampin resistance in the *R. massiliae* group. A remaining problem is that rickettsiae are intracellular bacteria, and for some, culture is not yet possible. Thus, for these bacteria, gene sequence comparisons are without doubt the only basis for taxonomy.

In conclusion, based on current knowledge, it is possible to differentiate four groups in the clade *Rickettsia–Orientia* (see Figure 1).

1. *Orienta*, the agent of Scrub typhus.
2. The typhus group with *R. prowazekii* and *R. typhi*.
3. The spotted fever group, which is very homogeneous with most of the agents of tick-borne rickettsioses (see Figure 2), including *R. rickettsii*, *R. sibirica*, *R. africae*, *R. conorii*, *R. slovaca*, *R. japonica*, *R. heilongjanghensis*, *R. honei*, and several independent species: *R. aeschlimanii*; *R. akari*, *R. australis*, and *R. helvetica*.
4. A fourth group, previously named the ancestral group (Fuerst and Poetter 1996), comprised of *R. canadensis* and *R. bellii*.

GENETICS

The annotated genome sequence of *R. prowazekii* was published in 1998 (Andersson et al. 1998) and that of *R. conorii* was published in 2001 (Ogata et al. 2001). Both have a single small unique chromosome, but each presents different features (see Table 4). The *R. prowazekii* genome not only is smaller, but also has a smaller coding content and a theoretical coding capacity that is only 61 percent of that of *R. conorii*. Rickettsial genomes also exhibit a previously unknown phenomenon called repeat parasitism, whereby palindromic repeats are inserted into various coding and noncoding regions. The predicted proteins encoded by the parasitized genes appear functional (Ogata et al. 2000). A comparative analysis of the two genomes shows that no gene was transferred since the separation of the two species, an estimated 80 million years ago. Moreover, the genomes express a perfect colinearity that allows an analysis of gene evolution. It was shown that some genes were progressively destroyed. First, a stop codon was insterted in the gene, but the two resulting open reading frames (ORF) were transcribed, them smaller ORFs (those with proteins predicted to be under 70 amino acids long) were no longer transcribed. These ORFs would then be degraded into noncoding remnants. Among the *R. conorii* genes in *R. prowazekii* genomes, 200 such remnants were identified.

Rickettsiae have paralog gene families that may transduce important functions. An ATP/ADP translocase gene is present in four copies and encodes a parasitic enzyme exclusively found among *Chlamydia*, *Rickettsia*, and plants. Four spotT copies are also found, which encode proteins that play a major role in processes related to bacterial starvation. Both rickettsiae also have several copies of genes encoding ProP, a transporter that plays a role in response to osmotic changes. Multiple genes of outer-membrane proteins of the Sca family

Table 4 *Comparison between* Rickettsia conorii *and* Rickettsia prowazekii

Species	*R. conorii*	*R. prowazekii*
Genome size (bp)	1 268 755	1 111 523
G + C content (%)	32.4	29.0
ORFs	1374	834
Coding content (%)	81	76

were found (five in *R. conorii* and four in *R. prowazekii*). Three of these proteins were previously identified as major antigens (rOmpA, rOmpB, and gene D).

METABOLISM AND COMPOSITION

Compared with bacteria that grow in axenic medium, little information is available about the metabolism of rickettsiae (Winkler 1990). *Rickettsia prowazekii* has been studied most extensively. ATP/ADP translocase, a unique parasitic enzyme, was identified that allows the importation of ATP from the host cytosol. Genome analysis predicts that several functions are missing (Ogata et al. 2001). For energy metabolism, *R. prowazekii* and *R. conorii* lack glycolysis, sugar nucleotide, and pentose phosphate pathways. For biosynthesis functions, both lack purine and pyrimidine ribonucleotide biosynthesis enzymes. Many amino acid biosynthetic pathways, and other gene families, including those for glutamate, serine, histidine, aromatic amino acid, and branched-chain amino acid biosynthesis, are lacking. Folic acid and cofactor enzymes, such as biotin and riboflavin (for *R. prowazekii* only) are also lacking.

The composition of the cell walls of rickettsiae is typical for that of proteobacteria (Winkler 1990), including peptidoglycan, lipopolysaccharide, and an outer membrane with only a few identified proteins. A family of 5 *Omp* (Sca family) has been identified by genome sequencing (Ogata et al. 2001). *sca5* encodes rOmpA and is present only in SFG rickettsiae; *sca4* encodes rOmpB and is present in SFG and TG but is absent in *R. bellii* and *R. canadensis*; *sca3* or gene D is also absent in the *R. bellii* group; and *sca1* and *sca2* have been detected in *R. conorii* and *R. prowazeki* (Ogata et al. 2001). These genes are also found in all other rickettsiae in a truncated form (unpublished). *rOmp*A and *rOmp*B are immunodominant proteins against which protective antibodies may be generated. As such, they are potential vaccines. Thus, OMPs determine the species-specific antigenicity and LPS determines the group-specific antigenicity.

Orientia tsutsugamushi does not exhibit a typical cell wall (Kawamura et al. 1995) and neither peptidoglycan nor LPS have been detected. One cross-reaction identified in *O. tsutsugamushi* is that used in the Weil–Felix test with *Proteus mirabilis* OXK.

PATHOGENICITY AND VIRULENCE FACTORS

The natural history of rickettsial diseases involves several steps (Walker et al. 2003). Transmission is provided by either saliva inoculation (tick-, mite-, or flea-transmitted), by autoinoculation after scratching lesions contaminated by insect feces (epidemic and murine typhus), or by aerosol (epidemic typhus by inhalation of louse feces, of other rickettsioses in the laboratory, or in the context of bioterrorism). After entry through the skin, mucous membranes, or lung, the rickettsiae will spread, usually through the lymphatics from the portal of entry. Target cells are the endothelial cells (with the exception of *R. akari* and *O. tsutsugamushi*, for which the monocyte is also a target). Rickettsiae enter host cells by phagocytosis. Adhesion may involve a rickettsial outer-membrane protein such as *rOmp*A and *rOmp* (Li and Walker 1998). After internalization, the phagocytic vacuole is rapidly lysed (Teysseire et al. 1995) and rickettsiae escape the phagocytic digestion to multiply freely in the cytoplasm (and the nucleus for spotted fever rickettsia). SFG rickettsiae can spread from cell to cell by actin mobilization (Teysseire et al. 1992; Heinzen et al. 1993). The infected cell is injured via lipid peroxidation of host-cell membranes (Silverman and Santucci 1988). In order to exploit the cell for its own growth, *R. rickettsii* inhibits cell apoptosis by upregulating NF-κB activation (Clifton et al. 1998). Escape from the vacuole is suspected to be mediated by an enzyme, and indirect evidence implicates a phospholipase A (Silverman and Santucci 1988; Walker et al. 2002). A gene encoding for a phospholipase D has been found in the genome of *R. prowazekii* and *R. conorii*, and preliminary data show that it may be a key factor in virulence (Renesto et al. 2003). The immune response to rickettsiae is mainly based on cytokine-mediated intracellular killing and clearance by cytotoxic CD8 lymphocyte (Feng and Walker 1996). In mice, interferon gamma (IFN-γ) and tumor neciosis fact alpha (TNF-α) synergistically activate nitric oxide synthase-2-dependent killing. Antibodies to outer-membrane proteins play a major role in protection against rickettsiae. The immune control of rickettsiae is not sterilizing. *R. prowazekii* survive in apparently cured patients who may relapse, years later, with the influence of stress. Persistence, late relapse, and chronic diseases have not been established for other rickettsioses.

TOOLS FOR THE DIAGNOSIS OF RICKETTSIOSES

The biological tools for diagnosis have changed significantly in recent years. The main difference comes from the application of molecular biology techniques, the generalized use of PCR amplification, and microcell culture using shell vial methodologies (Raoult and Roux 1997)

Isolation of Rickettsiae

Rickettsiae are typically identified with Giménez staining, although some other bacteria also retain the basic carbol fuchsin stain and must be distinguished on the basis of culture requirements. Rickettsiae have beenisolated by several different methods. Animal inocula-

tion has been widely used, originally with guinea pigs and subsequently using rats and voles. Embryonated eggs have also been widely used. However, cell culture is currently the most widely used system for primary isolation. Rickettsial cell culture isolation differs from that used for viral isolation, since antibiotics, with the exception of cotrimoxazole, cannot be used (Kelly et al. 1992). Tick or mammalian cell lines have been used. We have used a microculture system to isolate rickettsiae from human blood and other sources (Bacellar et al. 1995; Balayeva et al. 1994; Eremeeva et al. 1994; Marrero and Raoult 1989). The shell vial assay was adapted from a commercially available method for cytomegalovirus culture with early antigen detection. Isolation of rickettsiae by cell culture is now performed routinely in our laboratory using heparinized (leukocyte cell buffy coat) blood, skin biopsy samples before antibiotic therapy, or arthropods (Marrero and Raoult 1989; Espejo-Arenas and Raoult 1989). Many rickettsiae, including *R. conorii*, *R. rickettsii*, *R. massiliae*, *R. aeschlimannii*, *R. slovaca*, *R. helvetica*, '*R. mongolotimonae*,' and *R. africae*, have been isolated by this method. It has also been used in Zimbabwe (Kelly et al. 1992) and in Portugal (Bacellar et al. 1995). Although this assay is useful, for unknown reasons about one-third of the isolates are lost on passage (La Scola and Raoult 1996). However, the importance of culture cannot be underestimated, since obtaining an isolate from a tick or a patient is the ultimate goal in rickettsial disease description. Recently, we faced an unsolved problem in trying to grow *R. felis*. This rickettsia was reported to grow in Vero cells at 37°C (Radulovic et al. 1995) but was lost under these conditions. We were unable to grow it from infected fleas in our routine shell vial assay over a period of 1 year. After multiple attempts, we tried isolation of *R. felis* at 28°C, as its arthropod hosts are fleas (Raoult et al. 2001a). Flea-associated microorganisms, such as *Yersinia pestis*, frequently grow better at this temperature. As HEL cells do not grow well at 28°C, we used XTC2 cells, an amphibian cell line that grows well at 28°C and is used for arbovirus culture. This technique allowed easy isolation of *R. felis* (Raoult et al. 2001a). Subsequently, we tried without success to grow it at 37°C (La Scola et al. 2002). We believe that this organism does not grow at 37°C and that the first report of its cultivation reflected contamination by *R. typhi* (La Scola et al. 2002).

Currently, when an isolate has been obtained, the best method of identification is gene-based. Citrate synthase gene (*gltA*), the 16S rRNA gene, 17-kDa lipoprotein gene, and outer-membrane protein genes (*rOmpA*, rOmpB, and gene D) are most frequently used for this purpose (Raoult and Roux 1997). However, a clear genetic definition of a new species is currently lacking. Traditionally, the *Rickettsia* species definition was based on serotyping by microimmunofluorescence (MIF) (Sekeyova et al. 1998). This technique takes a long time, requires a pool of antisera, and lacks reproducibility.

Serology

Serological assays are the simplest diagnostic tests to perform. The Weil–Felix test was the first to be used and involves antigens from three *Proteus* strains: *P. vulgaris* OX2, *P. vulgaris* OX19, and *P. mirabilis* OXK. Its use in the diagnosis of rickettsioses is based on serological crossreactions (Raoult and Dasch 1995). The test lacks sensitivity and specificity but has historically been used for laboratory diagnosis and provides evidence of newly encountered rickettsioses.

Today, the most commonly used serological test is MIF. It is reliable but does not allow differentiation of infection among the SFG rickettsia (Hechemy et al. 1986; Hechemy and Michaelson 1985). The Western blot immunoassay (Raoult and Dasch 1995) allows differentiation among the SFG, provided that acute-phase sera are used. The test detects two types of antigens, high-molecular-weight outer-membrane protein (rOmpA and rOmpB) and LPS-like antigen. The high-molecular-weight outer-membrane proteins are species-specific (Beati et al. 1994; Raoult and Dasch 1995) and provide the basis for rickettsial serotyping (Philip et al. 1978). If sera are collected very early in infection, strong homologous reactions are often observed, making a specific diagnosis possible (Raoult et al. 2001c). However, as this rarely occurs, more specific methods are needed. Cross-absorption studies are useful, especially if complemented by Western blotting (Brouqui et al. 1997) (see Figure 3). This is the case for typhus, because in 50 percent of patients, serum antibody titers were the same when tested using *R. prowazekii* and *R. typhi* antigen (La Scola et al. 2000). Although this technique is accurate, it is also very expensive and time-consuming, since a large number of rickettsiae are required for each absorption (La Scola et al. 2000).

It must be emphasized that serological testing can be considered only the first step in diagnosis or in recognition of a rickettsial disease. In general, direct evidence of the identity of a rickettsial pathogen is required before purported new syndromes, new manifestations, or new areas of endemic infection can be defined. This evidence should be based on a combination of culture, microscopic, or genetic detection techniques and not solely on serology. In fact, the literature is replete with serologically based reports of new diseases or new clinical forms of disease that can only be viewed with some degree of skepticism (Raoult and Roux 1997). Recently, crossreactions were identified between *Legionella* and *Rickettsia* species (Raoult and Dasch 1995). In conclusion, MIF remains the basic technique. It is useful to test several antigens simultaneously to help identify the causative species, which usually exhibits a

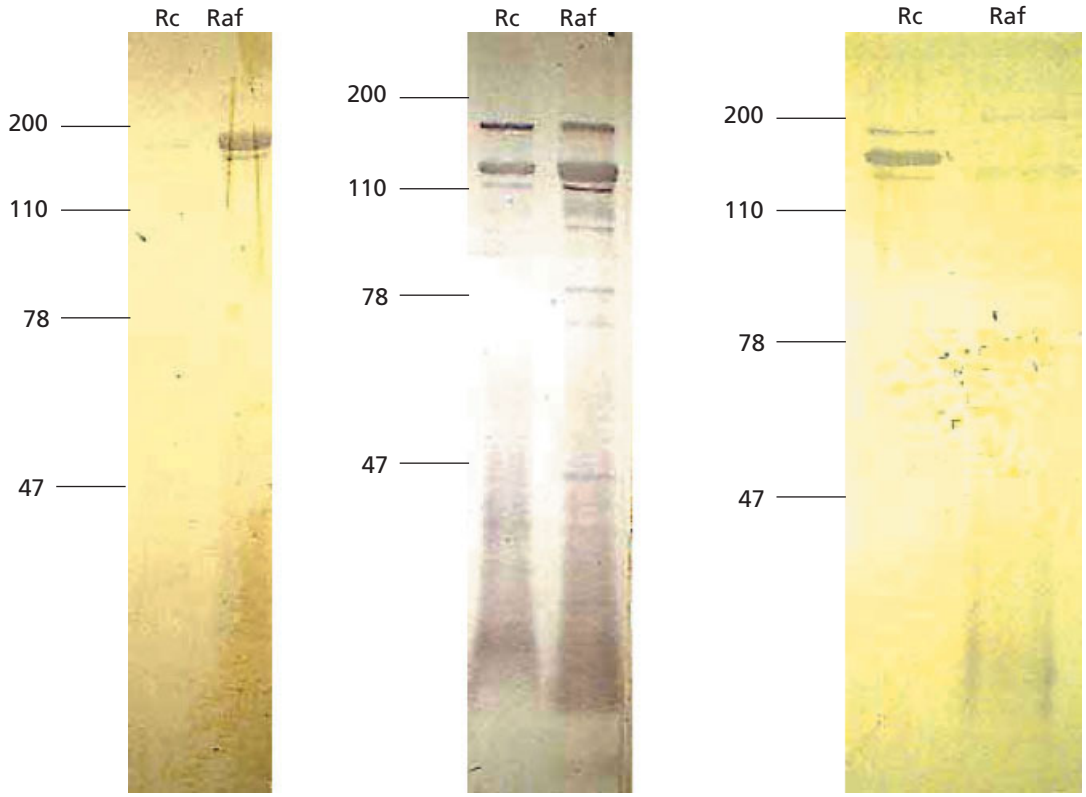

Figure 3 *Western blot comparing* Rickettsia conorii *and* Rickettsia africae.

higher titer. Determining the presence of IgM is useful to confirm recent infection. Usually, diagnostic cutoffs are 64 or 128 for IgG antibodies and 32 or 64 for IgM antibodies.

PCR-based detection of Rickettsiae

Rickettsiae may be detected by PCR amplification from blood, skin biopsy samples, and arthropod tissues. To preclude PCR inhibition and to enhance sensitivity, specific procedures must be used prior to amplification from blood. Cells are sedimented and the leukocytic cell buffy coat is used for rickettsial testing. Although heparinized blood is used for cell culture, it is preferable to use blood collected in ethylenediaminetetraacetic acid (EDTA) or sodium citrate, because heparin inhibits PCR and is difficult to neutralize. PCR amplification must be performed before initiation of antibiotic treatment and before antibody becomes detectable. Fresh tissues are preferred for this procedure, but paraffin-embedded tissues and even specimens fixed and mounted on to slides may be used (Stein and Raoult 1992). Although rarely present in Rocky Mountain spotted fever and not always present in other rickettsioses, the tâche noire (eschar) is the most useful biopsy sample to assay (Williams et al. 1994). Tâche noire samples are the best for detection of SFG rickettsiae because the tissue contains more bacteria than are present in blood; several isolates have been characterized by this approach (Brouqui et al. 1997; Raoult et al. 1996). PCR amplification of tâche noire or blood samples can be very useful because infection can be detected before cell culture is positive or seroconversion has occurred.

PCR-based methods for the detection of rickettsiae are attractive as they not only circumvent the need for culture but also possibly offer more sensitive and specific alternatives. Rickettsial DNA can also be detected in ticks (Gage et al. 1994; Gage et al. 1992), fleas, and lice by PCR-based amplification method (Higgins and Azad 1995). Detection strategies based on recognition of sequences within the 16S rRNA gene (Roux and Raoult 1995; Pretzman et al. 1995), and genes encoding the 17-kDa lipoprotein (Anderson and Tzianabos 1989; Baird et al. 1996; Balayeva et al. 1995), citrate synthase (Schriefer et al. 1994; Raoult et al. 1997; Wood et al. 1987), and rOmpB (Raoult et al. 1997; Gilmore et al. 1991) and rOmpA (for SFG rickettsiae (Raoult et al. 1996; Niebylski et al. 1997; Roux et al. 1996) outer-membrane proteins have been described. Since none of the PCR assays to date is specific for individual rickettsial species, reaction products must be further analyzed to identify the species being detected. Approaches involving either restriction endonuclease analysis or nucleotide sequence determination have been described.

The major problem when using PCR is the risk of contamination. To circumvent this problem, we sequence all amplicons and consider a diagnosis only when either the sequence is found for the first time in the laboratory or when at least two different target genes have been amplified and sequenced. Regardless, negative controls that mimic typical specimens should be used every third or fifth sample. Amplification should not be detected in any negative control before interpretation of results.

In order to increase the specificity of PCR, a new technique termed 'suicide PCR' was described. This technique was first introduced to detect *Y. pestis* in ancient remains (Raoult et al. 2000). In this technique, a target sequence is selected that has never before been used in the laboratory and is used for a single assay only. As no prior contamination is possible, highly sensitive techniques, such as nested PCR, are therefore applicable with limited risk. This technique has been used for the diagnosis of *R. africae* (Raoult et al. 2001b) and *R. slovaca* (Raoult et al. 2002a), which illustrates the greater robustness and potential than regular PCR. The availability of the complete genome sequences of *R. prowazekii* (Andersson et al. 1998) and *R. conorii* (Ogata et al. 2001) provides a large potential complement of target sequences for such studies.

Immunological detection of Rickettsiae

Skin biopsy specimens have been used in the diagnosis of rickettsioses since the early work of Woodward (Walker et al. 1978b; Woodward 1973). Samples can be tested as fresh frozen sections or after fixation and paraffin embedding. Clinical samples obtained at autopsy can be tested in the same manner as skin biopsy specimens (Walker et al. 1978a, 1981).

The use of methods incorporating specific polyclonal or monoclonal antibodies allows detection of rickettsiae in blood or tissue (George et al. 1992). This diagnostic approach allows the confirmation of infection in patients before seroconversion and permits rapid specific treatment. The method can also be used to diagnose rickettsial infection in fixed tissues retrospectively (Paddock et al. 2003).

Ticks collected for attempted isolation of Rickettsiae should be kept alive before being tested. If transport or long-term maintenance is needed, a humidified chamber is useful. While the ticks are still alive, the hemolymph test should be performed following surface sterilization (Burgdorfer 1970). In this procedure, one tick leg is amputated, allowing the collection of a drop of hemolymph, which can be applied on to a glass slide and subjected to either Giménez staining (Gimenez 1964) or immunodetection methods. Ticks should then be dissected (Kelly et al. 1991; Peter et al. 1990). Tick tissues, including the reproductive organs, can be carefully removed for further testing. Immunological detection methods can incorporate polyclonal or monoclonal antibodies, the latter of which can be used to determine the infecting species. Immunofluorescent labels have been widely used in conjunction with these antibodies, but immunoperoxidase labels and detection systems appear to allow a better microscopic definition of cells around the detected Rickettsiae (Dumler et al. 1990). *R. typhi* can also be detected in infected fleas by enzyme-linked immunosorbent assay (ELISA) (Dobson et al. 1989).

HABITAT AND EPIDEMIOLOGY

Rickettsiae live in association with arthropods (Rehacek and Tarasevich 1989). In some instances, this association is stable and the Rickettsiae are transmitted transovarially from the female to progeny. This is true for many tick-associated Rickettsiae, such as *O. tsutsugamushi* and *R. akari* in mites, and for *R. typhi* and *R. felis* in fleas. *R. prowazekii* kills its arthropod host and is not transmitted to its progeny (Houhamdi et al. 2002). The spectrum of possible infected artropods is not known. For example, human lice can be infected by *R. typhi* (Houhamdi et al. 2003), *R. akari* (Rehacek and Kovacova 1976), and *R. rickettsii* (unpublished data). Ticks can be artificially infected with *R. akari* (Rehacek and Kovacova 1976) and have been found naturally infected with *R. prowazeki* (Raoult and Roux 1997). Typically, there is a limit to the tick species that can harbor specific serotypes of Rickettsiae. However, specific coevolution of ticks and Rickettsiae has not been demonstrated. Some Rickettsiae (such as *R. rickettsii*) have a large range of hosts, whereas others are restricted to a single host.

The geographical and temporal distributions of rickettsioses and ehrlichioses are mainly determined by the distribution of their vectors (Table 2, Figure 4). Louse-transmitted diseases are found worldwide. The human louse is common to all humans. Lice parasitize poor people, preferentially in cold places, and specifically during times of war. Common fleas such as cat and dog fleas (*Ctenocephalides felis* and *C. canis*), as well as rat fleas (*Xenopsylla cheopis* and *Pulex irritans*), are distributed worldwide, as are the diseases (murine typhus and flea-borne spotted fever) caused by the Rickettsiae that they transmit. Tick species are highly dependent on the environment and very few are distributed worldwide, with the exception of *Rhipicephalus sanguineus*, the brown dog tick and vector of *Rickettsiae conorii* (in the old world). Therefore, tick-transmitted diseases are usually restricted to a part of the world where transmission by the local fauna is possible. For example, no SFG rickettsioses are currently found in either Europe or the USA.

Tick behavior may determine the target human population and seasonality of disease, as well as influencing the clinical presentation. For example, some *Derma-*

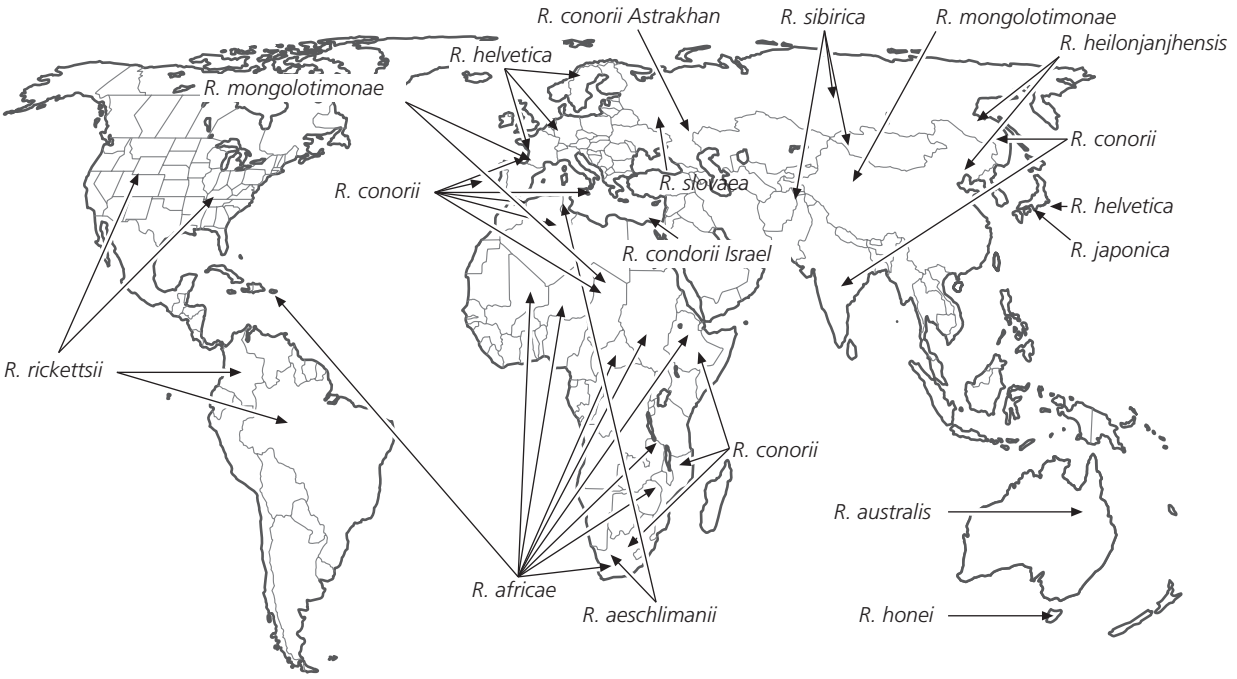

Figure 4 *Geographical distribution of tick-borne rickettsioses.*

centor species may wait for hosts with an ambush strategy, falling from as much as 1 m on to a hair-bearing host (Parola and Raoult 2001). Therefore, *Dermacentor* species frequently bite above the hairline and children are a primary target. As a consequence, *Dermacentor*-transmitted rickettsioses, such as Rocky Mountain spotted fever and infection by *R. slovaca*, more frequently involve children than do other rickettsial diseases (Raoult et al. 2002a). *Amblyomma* ticks are aggressive hunters that frequently attack in groups. This behavior may lead to grouped cases and detection of more than one inoculation eschar per patient. Wide variations in the annual incidence of tick-transmitted diseases such as Rocky Mountain spotted fever and Mediterranean spotted fever have been observed. A worldwide increase in the 1970s was noted, followed by a decrease in the 1980s that is not understood. Several hypotheses suggested such factors as increased exposure, better use of diagnostic tests, or a shift from effective to ineffective, empirically prescribed antibiotics (Walker and Raoult 1995) (see Table 5).

CLINICAL MANIFESTATIONS

Rocky Mountain spotted fever

Rocky Mountain spotted fever (Table 5) is the most severe of all rickettsioses. It is caused by *R. rickettsii* and is currently the only tick-transmitted rickettsiosis recognized in America, where it is prevalent in 44 US states and in Central and South America (Argentina, Brazil, Colombia, Costa Rica, Mexico, and Panama). The vectors are mainly *Dermacentor andersonii* (wood tick) in the western USA, *D. variabilis* (the American dog tick) in the eastern, southern, and Midwest USA, and *Amblyomma cajennense* in Central and South America (De Lemos et al. 2001; Sexton and Kaye 2002). There are 500–1 000 cases annually in the USA, with a majority diagnosed during late spring and summer (Paddock et al. 2002). The disease is relatively more prevalent in children under 10 years of age.

Rocky Mountain spotted fever starts with fever and headache after an incubation of 2–14 days. The fever is high and associated with nonspecific symptoms and signs including malaise, nausea, myalgias, anorexia, vomiting, and diarrhea. The classical triad of fever, headache, and rash is present in only 44 percent of confirmed cases. The rash is initially macular and appears first on the ankles and wrists and then becomes generalized. The rash can evolve from pink to petechial and purpuric. A rash can be absent, and 'spotless' fever represents 34 percent of cases.

The disease results to various degrees from increased vascular permeability in multiple organs that can lead to multiple organ dysfunction syndrome. In severe forms, patients suffer edema, hypovolemia, hypoalbuminemia, and hypotension, leading to shock. In these cases, necrosis and gangrene of the extremities can occur. In some instances, noncardiogenic pulmonary edema develops and causes death. Renal failure could result from hypovolemia and shock, or from acute tubular necrosis, and may require hemodialysis. Frequent neurological manifestations include confusion, lethargy, and

Table 5 *Main characteristic rickettsioses*

Bacteria	Disease	Rash	Specificity	Inoculation eschar	Multiple eschars	Draining lymph-adenopathy	Mortality (without treatment) (%)
R. rickettsii	Rocky Mountain spotted fever	Yes	Purpuric (50% cases)	Very rare	No	No	20–40
R. conorii	Mediterranean spotted fever	Yes	Purpuric (rarely)	Yes	No	Rare	1
R. conorii Israel	Israeli spotted fever	Yes	Rarely purpuric	No	No	No	<1
R. conorii Astrakhan	Astrakhan fever	Yes	No	Rare	No	No	No
R. conorii ITT	Indian tick typhus	Yes	May be purpuric	Rare	No	No	Low
R. africae	African tick-bite fever	Rare (<50%)	Vesicular	Yes	Yes	Yes	Very low
R. slovaca	Spotted fever	Rare	–	Yes	No	Yes	No
R. aeschlimannii	Spotted fever	Yes	–	Yes	No	Yes	?
R. sibirica	Siberian tick	Yes	No	Yes	No	Yes	Low
R. australis	Queensland tick fever	Yes	Vesicular	50% cases	No	Yes	Low
R. honei	Flinders Island tick typhus	Yes	–	Yes	No	Yes	Low
R. japonica	Japanese or oriental spotted	Yes	No	Yes	No	No	Low
R. helvetica	Fever	No	–	No			No
R. heilongjanhensis	Spotted fever	Yes	Maculopapular	Yes	No	Yes	No
R. mongolotimonae	Spotted fever	Yes	Few spots lymphangitis	Yes	Yes	Yes	No
R. akari	Rickettsial pox	Yes	Vesicular	Yes	Yes	Yes	Low
R. prowazekii	Epidemic typhus	Yes	Petechinal	No	No	No	20
R. typhi	Murine typhus	Yes	Macular	No	No	No	Low
O. tsutsugamushi	Scrub typhus	Yes	Macular	Yes	Yes	Yes	1–15
R. felis	Flea-borne spotted fever	Yes	–	Yes	Possible	Maybe	?

stupor. Cerebrospinal fluid (CSF) exhibits pleocytosis, usually a few monocytic cells (10–100), indicative of meningitis in one-third of cases. Heart involvement can cause dysrhythmia. Liver involvement manifests as an increase in serum transaminases in one-third of patients and as jaundice in eight percent. The intestinal tract involvement manifests as abdominal pain, diarrhea, vomiting, and, in severe cases, bleeding (which can lead to death). Ocular involvement includes conjunctivitis and retinal abnormalities.

Laboratory examination reveals a normal number of white blood cells, but bands and immature myeloid cells are often observed. Thrombocytopenia is seen in 30–50 percent of cases and may be marked in severe disease. Anemia may develop. Coagulopathy with decreases in clotting factors (infrequently including fibrinogen), and prolonged coagulation times may contribute to bleeding; hypoalbuminemia may be present; and proteins of acute phase response increased (C-reactive protein, ferritin, fibrinogen). Hyponatremia and hypocalcemia may also be noted. Increased concentrations of serum enzymes such as aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), lactate dehydrogenase, and creatine phosphokinase are common.

The evolution of Rocky Mountain spotted fever depends strongly on timing of diagnosis and antibiotic treatment. The spontaneous evolution is frequently fatal; the current fatality rate is 2.4 percent based on a 4-year national survey in USA (27 deaths were attributable to the disease during this period), but local figures may be considerably higher. Chloramphenicol is associated with a poorer outcome than doxycycline (Holman et al. 2001).

Other tick-borne rickettsioses

Rickettsia conorii (Table 5) causes Mediterranean spotted fever. The species comprises a group of different but closely related serotypes; the type strain is Malish. Other regional serotypes include Israel (in Israel and southern Europe), Astrakhan (in Russia), and Indian

tick typhus *Rickettsia* (in India) (Raoult and Roux 1997). Mediterranean spotted fever resembles Rocky Mountain spotted fever but is milder. A malignant form of the disease with a purpuric rash and shock has been described in alcoholic, diabetic, and elderly patients (Raoult et al. 1986). The typical clinical presentation is that of a patient with fever, rash, and tâche noire, a black eschar at the site of the tick bite. The tâche noire is found in 50–80 percent of cases. The rash is frequently papular, which led to one of the names of the disease, Boutonneuse fever. Israel spotted fever (Yagupsky and Wolach 1993) and Astrakhan fever (Tarasevich et al. 1991) appear milder than typical MSF, and tâche noire is frequently lacking.

R. africae, which causes African tick bite fever, may be responsible for the most frequent tick-borne rickettsiosis worldwide. The Rickettsiae are transmitted by the African ticks, *Amblyomma hebraeum* and *A. africanum*, which attack in groups and lead to a high prevalence of autochthonous infection in rural Africa and in travelers (Raoult et al. 2001b). The tick attacks typically generate grouped cases. In half of cases, more then one tâche noire is detected (see Figure 5). The disease is milder than Mediterranean spotted fever. Fever is frequently absent; a rash is observed in only one-half of the patients and may be vesicular. Tâches noire are frequently observed on lower limbs and are often associated with lymphadenopathy in the groin.

Japanese spotted fever (Mahara 1997), caused by *R. japonica*, and Siberian tick typhus, caused by *R. sibirica* (Rehacek and Tarasevich 1988), both resemble mediterranear spotted fever. *R. australis* (Queensland tick typhus) and *R. honei* (Flinders Island spotted fever) cause diseases resembling Mediterranean spotted fever, but the rash can be vesicular (Graves and Stenos 2003).

Figure 5 *Patient with* Rickettsia africae *infection. Note the presence of the two tâches noire.*

Infections caused by '*R. mongolotimonae*' resemble Mediterranean spotted fever, but in some cases exhibit specific clinical features characterized by tâche noire, groin lymphadenopathy, and draining lymphangitis (Fournier et al. 2000a). *R. slovaca* causes a disease apparently common in Europe (Hungary, France, Spain) (Raoult et al. 2002a), but is more prevalent in children and women, contrary to other tick-borne rickettsioses. It rarely produces exanthema, and the typical clinical picture includes an erythematous skin lesion at the site of the tick bite (usually in the scalp) and tender cervical lymphadenopathy. Residual alopecia can be observed at the site of the tick bite (see Figure 6). *R. aeschlimanii* infection resembles that caused by *R. conorii*. *R. heilongjiangensis* causes fever, rash, tâche noire, and draining, tender lymph nodes. It is prevalent in Siberia and China (unpublished data).

The diagnosis of Rocky Mountain spotted fever and other tick-borne rickettsioses is similar, using mainly serology indirect fluorescent antibody (IFA) and PCR or immunohistochemistry of skin lesions (see Tools for the diagnosis of rickettsioses), with the exception of *R. slovaca* infection, in which the serological response is weak (perhaps because of it lacks generalized infection) but may be diagnosed by PCR of skin lesions or lymph node aspirates (Raoult et al. 2002a).

Murine typhus

Humans often come into contact with rat fleas, which are distributed worldwide in domestic environments. Murine typhus is prevalent in hot and humid areas. In the USA, 50–100 cases are reported yearly, mainly in southern California and southern Texas. A transmission cycle also involving opossums and cat fleas (*C. felis*) has been demonstrated (Dumler et al. 1991).

The incubation period ranges from 8 to 16 days. The disease begins with abrupt fever, nausea, vomiting,

Figure 6 *Residual alopecia of a patient with* Rickettsia slovaca *infection.*

myalgias, arthralgias, and headache. A rash is observed in 40–50 percent of patients, on average 6 days after the onset. The rash consists of pink macules, which can evolve into a maculopapular rash (Tselentis et al. 1986) that does not involve the face, palms, or soles. In severe cases, it can become purpuric. Pneumonia can be observed in severe cases, and, when severe, respiratory distress can lead to intubation and artificial ventilation. Neurological symptoms range from confusion and stupor, to coma and seizures in severe forms. Cerebral hemorrhage may occur. Digestive tract involvement can manifest as vomiting, abdominal pains, jaundice, and, in severe cases, gastric hemorrhage (Table 5).

A moderate increase in serum liver enzymes is common. In patients with severe disease, hyponatremia and hypoalbuminemia are observed. The prognosis is usually favorable, but ten percent of patients require intensive care and one percent die.

Flea-borne spotted fever caused by *R. felis* is a new, incompletely defined disease. The bacterium is found in fleas in the USA, Peru, Europe, and Africa. Seven cases have been reported from Texas, Mexico, Brazil, France, and German (Raoult et al. 2001a; Richter et al. 2002; Zavala-Velasquez et al. 2000). All reported cases exhibited fever, a rash was detected in six out of seven, and an inoculation eschar was observed in some cases.

Epidemic louse-borne typhus

The human body louse lives in clothes and multiplies rapidly with cold weather and lack of hygiene. Their detection and prevalence reflects the low socioeconomic status of certain members of a society. Louse-borne typhus is prevalent during war, in poor countries, and even among the homeless populations of affluent societies, including those in the USA and Europe. An outbreak of typhus involving 100 000 people was reported in 1997 during the civil war in Burundi (Raoult et al. 1998), and cases were reported in Russia (Tarasevich et al. 1998), Peru (Raoult et al. 1999), USA, Algeria (Niang et al. 1999), and France (unpublished) in the 1990s (Raoult and Roux 1999). The epidemiology of *R. prowazekii* is mainly related to humans as reservoirs and lice as vectors. However, flying squirrels in the USA constitute a sylvatic reservoir and generate a few cases of typhus (Bozeman et al. 1975). The patient is usually contaminated by infected louse feces (in which *R. prowazekii* can survive for weeks) (Houhamdi et al. 2002), through aerosols, or by skin autoinoculation. Patients who recover from typhus may harbor the bacterium in a dormant form, leading to relapse years later. The relapsing form is named Brill–Zinsser disease (Stein et al. 1999). During the relapse, a bacteremia occurs, which may allow the start of a new outbreak if lice bite the patient.

Typhus (Table 5) begins abruptly with fever, headaches, and myalgias. Pneumonia is common. Neurological involvement manifests as stupor, confusion, or coma. A rash is observed in 20–80 percent of the patients and is more rarely observed on dark skin. It usually starts in the axilla and then spreads. It is macular and evolves to purpuric in severe cases. In some cases, diarrhea and jaundice are reported. Splenomegaly is infrequently found. In severe cases, shock is observed; the spontaneous fatality rate in the absence of specific antibiotic therapy is 20–30 percent.

Brill–Zinsser disease is milder and frequently undiagnosed, as the rash can be absent. The patient interview may reveal prior exposure to lice and a diagnosis of typhus years earlier.

Sylvatic typhus in the USA is caused by a variant *R. prowazekii* and is also milder. The most prominent clinical features are neurological. Only a few cases have been described and nearly all occurred in areas east of the Mississippi, where the eastern flying squirrel is found.

Rickettsialpox

Rickettsialpox (Table 5) is caused by *R. akari*, which is transmitted by mites of the house mouse (*Mus musculus*). After 7–10 days' incubation, fever, rash, and an inoculation lesion are observed. The local lesion may be vesicular or an eschar and is associated with tender, draining lymph nodes. The rash can also become vesicular (smallpox rickettsia was another name of *R. akari*). The disease is mild.

Scrub typhus

Scrub typhus is a common disease caused by *O. tsutsugamushi*. It is prevalent in eastern Asia and Oceania when chiggers (larval mites) are common (Kawamura et al. 1995). Local residents and tourists may be exposed to chigger bites. The real prevalence of scrub typhus is not known, but in Thailand, for example, it is with leptosporis one of the two most important causes of infection in hospitalized patients. During the Vietnam War, scrub typhus was a major cause of fever in American soldiers (Deller and Russell 1967; Berman et al. 1973). It is also common in India, but apparently grossly underreported (Mathai et al. in press).

The major symptoms of scrub typhus (Table 5) are fever, mental status changes, headache, inoculation eschar, and lymphodenopathy (Tattersall 1945; Berman and Kundin 1973). Ten or more days after exposure to a focus of scrub typhus, the disease onset is abrupt, with fever, headache, and myalgias. One or more inoculation eschars are found at the site of the mite bite in half of all cases (Kawamura et al. 1995). The rash is macular, pale, transient, and easily missed. Lymphadenopathy can

be tender and is sometimes limited to the site of the mite bite. Hepatomegaly, general lymphadenopathy, and splenomegaly can be observed. Mental changes are frequent and range from slight intellectual blunting to coma or mania (Tattersall 1945).

In severe cases, evolution to multiple organ dysfunction syndrome with hemorrhage can be observed. After apparent recovery and following short treatment courses, relapses frequently occur. Relapse disease is usually less severe than the first attack. Scrub typhus is associated with a reduction of viral load with human immunodeficiency virus (HIV-1)-infected patients and has been observed to restore the immune potential of infected patients (Watt et al. 2000b). There is a wide variation in prevalence of cutaneous symptoms as well in severity and fatality rate (Kawamura et al. 1995). Prevalence varies from 3 percent in Taiwan to 60 percent on the northern coast of Japan. Scrub typhus is still a life-threatening disease despite the availability of efficient treatment. During pregnancy, scrub typhus frequently causes abortion (Mathai et al. in press).

ANTIBIOTIC SUSCEPTIBILITY TESTING

Because of their obligate intracellular lifestyle, susceptibility of Rickettsiae to antibiotics cannot be assessed by conventional microbiologial tests (Parola et al. 2002; Raoult and Maurin 2002a, b; Maurin and Raoult 1998, 2002a, b; Walker and Sexton 2002).

Arthropod model

Infected body lice have been used in an in vivo model to test the antibiotic sucsceptibility of *R. prowazekii* to antibiotics. Lice usually die from infection with *R. prowazekii*. Infected lice received either doxycycline or ripamcin. For both antibiotics, inhibitions of rickettsial growth and a delay in death of infected body lice were obtained.

Embryonated egg model

In the embryonated egg model, Rickettsiae are injected into the yolk sacs of eggs. This results in death of the embryo after an interval of several days. Antibiotics are administered by the same route within the first hour following inoculation. Antibiotic activity is deduced based upon the differences in mean survival time of infected embryos that received antibiotics as compared with infected controls. By this method, and confirming with Giménez staining of yolk sacs, penicillin G and the aminoglycosides streptomycin and gentamicin are not effective. Oxytetracycline and, to a lesser extent, chlortetracycline and chloramphenicol, were considered rickettsiostatic. Erythromycin was found to be effective against *R. prowazekii*. However, Weiss and Dressler (1960) demonstrated that in vitro, *R. prowazekii* could become resistant to this antibiotic after only three passages.

Cell culture models

Plaque formation in infected cell cultures was first used for enumeration of viable Rickettsiae and was later adapted for the determination of in vitro antibiotic susceptibility. The plaque assay system is recommended. Cell monolayers (usually Vero cells) are grown in Petri dishes and are infected by incubation with a rickettsial inoculum while rocking to enhance Rickettsia–cell interactions. Infected cells are then overlaid with minimal Eagle medium (MEM) containing 2 percent fetal calf serum and 0.5 percent agar. Antibiotics are added at different concentrations at the same time, whereas no antibiotics are added in drug-free controls. The Petri dishes are incubated for 4 days at 37°C in a 5 percent CO_2 atmosphere, and the cell monolayers are then stained with neutral red dye allowing visualization of plaques. Minimum inhibitory concentrations (MIC) are defined as the lowest antibiotic concentration allowing complete inhibition of plaque formation, as compared with the drug-free control. Ives et al. recently described a new cell culture system in which inhibition of rickettsia proliferation by antibiotics was determined by comparing rickettsial growth in infected Vero cell cultures incubated in the presence of antibiotics to that in drug-free control (Ives et al. 1997). Infected cells were enumerated by indirect fluorescent antibody tests. Kelly et al. (1995) reported the use of flow cytometry to quantify the antibiotic susceptibility of *O. tsutsugamushi*. We have reported the more rapid and efficient measurement of rickettsial DNA concentrations by real-time quantitative PCR using a Light Cycler to evaluate rickettsial growth inhibition by antibiotics (Rolain et al. 2002).

Chloramphenicol, doxycycline, tetracycline, minocycline, and rifampin are effective against *R. rickettsii*, with MICs ranging from 0.005 to 1 μg/ml (see Table 6). Penicillins and cephems were considered poorly effective, although in earlier experiments, Wisseman et al. demonstrated partial inhibition of rickettsial growth and spheroplast-like rickettsiae when using high doses of penicillin (Wisseman et al. 1974). The newer macrolide compounds, roxithromycin, azithromycin, and clarithromycin, have been tested in the immunofluorescent antibody assay (Ives et al. 1997). Clarythromycin was the most effective compound, with MICs ten times lower than that of *erythromycin* (0.25 and 2 μg/ml, respectively). The new ketolide compound, telithromycin, displayed an MIC of 0.5 μg/ml. Nalidixic acid produces only partial inhibition of plaque formation by *R. prowazekii*, whereas the fluoroquinolone compounds were more effective. In vitro, quinolones are poorly effective on *R. tsutsugamush* (Kawamura et al. 1995). A preli-

Table 6 *Antibiotic susceptibility of rickettsiae to antibiotics minimum inhibitory concentrations (MIC) in μg/ml) (based on Rolain et al. 1998, 2000, 2002; Kelly et al. 1995; Watt et al. 1999)*

	Spotted fever group rickettsiae	*R. aeschlimanii*	Typhus group rickettsiae	*O. tsutsugamushi*
Doxycycline	0.06–0.25	0.06–0.25	0.06–0.125	0.02–0.9
Thiamphenicol	0.5–4	0.5–2	1-2	0.2–0.4
Rifampin	0.03–1	2–4	0.06–0.25	0.06
Erythromycin	2–8	1.8	0.125–1	>8
Clarithromycin	0.5–4	0.5–2	0.5–1	
Telithromycin	0.5–1	–	0.5	
Ciprofloxacin	0.25–1	0.25–1	0.5–1	6–24
Ofloxacin	0.5–2	0.5–1	1	3–24

minary report claimed that fluoroquinolones were effective in a mouse model, but this has not been confirmed. Moreover, ciprofloxacin is not effective in treating patients with scrub typhus (Mathai et al. in press). Azithromycin was effective for scrub typhus in a mouse model (Watt et al. 1999). Many new macrolide compounds exhibit very good in vitro efficacy and should be tested to find alternatives for doxycycline.

TREATMENT

Doxycycline is the drug of choice for the treatment of the rickettsioses. It was demonstrated that a single dose cured patients suffering from epidemic typhus and Mediterranean spotted fever. For other rickettsial diseases, a 7–14-day regimen is recommended. For children, doxycycline should also be used. It has been demonstrated that staining of teeth is unlikely to appear when fewer than five therapeutic courses of doxycycline are prescribed in childhood (Fort and Benincovi 1969). Chloramphenicol is an alternative treatment to doxycycline in pregnancy (Watt et al. 1996) and in people with tetracycline hypersensitivity. It is less efficient than doxycycline in treating Rocky Mountain spotted fever (Walker and Sexton 2002).

Macrolide antibiotics have been tested for treatment of rickettsial diseases. Erythromycin should be avoided as it is ineffective for treatment of typhus and spotted fever (Maurin and Raoult 2002a). Clarithromycin (Cascio et al. 2001), azithromycin (Meloni and Meloni 1996; Cascio et al. 2002), and josamycin (Bella et al. 1990) were reported effective in the treatment of Mediterranean spotted fever. Azithromycin was also used with success for scrub typhus (Choi and Pai 1998). Macrolides can be used in pregnant women.

Rifampicin theoretically offers an alternative to doxycycline as MICs are low, except for *R. aeschlimanii*. However, a randomized trial performed in children suffering Mediterranean spotted fever showed that it was less efficient than doxycycline (Bella et al. 1991). Rifampin was shown to be effective in treating scrub typhus and should be recommended in pregnant women or in patients potentially infected with doxycycline-resistant strains (Watt et al. 2000a).

Quinolones exhibit some activity on Rickettsiae. Their use was proposed for the treatment of Mediterranean spotted fever (Gudiol et al. 1989) but should be avoided for treatment of typhus as a patient receiving ciprofloxacin died (Zanetti et al. 1998). In vitro results for *O. tsutsugamushi* are discrepant, but treatment failures for scrub typhus in pregnant women have been reported (Mathai et al. in press).

In conclusion, doxycycline currently remains the drug of choice for the rickettsioses. New macrolide compounds, such as telithromycin, that show great in vitro activity should be evalutated as alternative drugs.

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Ehrlichia and *Anaplasma*

J. STEPHEN DUMLER AND DIDIER RAOULT

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INTRODUCTION

In the past, the taxonomy of the rickettsiae and what were broadly known as ehrlichiae was based principally on comparisons of phenotypic characteristics. More so than in other bacteria, historical, geographical, and environmental considerations also played important roles in classification. More recently, classification of rickettsiae and ehrlichiae has been based increasingly on DNA sequences, particularly on *rrs* (16S rRNA) and *groEL* gene sequence comparisons. As a result of genetic analyses, a realignment of the families *Rickettsiaeceae* and *Anaplasmataceae* was recently proposed (Dumler et al. 2001). The new classification schema eliminates the tribe structure of the *Rickettsiaceae* family by moving the remaining members of the prior tribe *Ehrlichieae* and placing them into the family *Anaplasmataceae*. The genetic analyses also revealed misalignment among the existing genera in the previous tribe *Ehrlichieae*, such that some members of the genera *Ehrlichia* and *Cowdria* were reassigned to other genera in *Anaplasmataceae*, with the creation of several new genus and species combinations. The final result of the realignment was the inclusion of four genera in the family *Anaplasmataceae*: *Anaplasma*, *Ehrlichia*, *Wolbachia*, and *Neorickettsia*; the genus *Cowdria* was eliminated and its sole species, *C. ruminantium*, was reassigned into the genus *Ehrlichia* as *E. ruminantium* nov. comb. Prior members of the genus *Wolbachia*, *W. persica* and *W. melophagi*, were reassigned on the basis of DNA sequence comparisons into the genera *Francisella* and *Bartonella*, respectively. The genera *Haemobartonella* and *Eperythrozoon* formerly assigned within the *Anaplasmataceae* were reassigned to the *Mollicutes* (mycoplasmas). *Aegyptianella* spp. that infect avian, reptile, and amphibian erythrocytes are likely to be species in the genus *Anaplasma*, but more study is needed. The common underlying nongenetic characteristic of the *Anaplasmataceae* is that the bacteria reside within a vacuole in the cytoplasm of the infected host cell, and that host cells generally involve both arthropod cells and mammalian cells of hematopoitic origin, except for some *Wolbachia* that reside exclusively in insect cells, and some *Wolbachia* and *Neorickettsia* that may also involve cells in helminths, including nematodes and trematodes, respectively. Pathogens of mammalian cells are found in the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia*, although a role for *Wolbachia* spp. in human

pathogenicity is becoming increasingly appreciated. The general properties of *Anaplasmataceae* that infect mammalian cells are presented in Table 1.

HISTORICAL PERSPECTIVES

Some of the *Anaplasmataceae* are very important pathogens of man and animals. At the time when Howard Taylor Ricketts was conducting his ground-breaking working on the 'virus' of Rocky Mountain spotted fever in Montana of the USA, Sir Arnold Theiler was describing small darkly stained structures, marginal points, near the edges of erythrocytes from cattle with redwater, a febrile hemolytic disease first identified in tick-exposed South African cattle. He reasoned that these marginal points were nondescript parasites that infected erythrocytes as did *Plasmodium* spp., as was the prevailing view in his day, and coined the name

Table 1 *Diseases and habitats of species of the ehrlichias*

Genetic group species	Animals affected	Disease	Invertebrate host	Geographical distribution
Anaplasma				
Anaplasma phagocytophilum	Humans, horses, dogs, ruminants, llamas	Tick-borne fever (ruminants), anaplasmosis (humans); formerly granulocytic ehrlichiosis (HGE, equine granulocytic ehrlichiosis)	Ticks (*Ixodes persulcatus* group, including *I. scapularis*, *I. pacificus*, *I. ricinus*, *I. persulcatus*, *I. spinipalpus*, and *I. trianguliceps*)	North and South America, Europe, Asia, Africa
Anaplasma platys	Dogs	Canine cyclic thrombocytopenia	Ticks?	North and South America, Asia, Europe, Australia
Anaplasma bovis	Cattle	Bovine ehrlichiosis	Ticks (*Hyalomma*)	Africa, Middle East, Europe, North America?
Anaplasma marginale	Cattle	Anaplasmosis	Ticks (*Dermacentor*, *Boophilus*), tabanid flies	Worldwide
Ehrlichia				
Ehrlichia canis	Dogs, humans	Canine monocytic ehrlichiosis, tropical canine pantocytopenia	Ticks (*Rhipicephalus sanguineus*)	Worldwide
Ehrlichia chaffeensis	Humans, dogs	Human monocytic ehrlichiosis	Ticks (*Amblyomma americanum*)	USA, Asia?
Ehrlichia ewingii	Dogs, humans	Canine granulocytic ehrlichiosis, ehrlichiosis 'ewingii'	Ticks (*Amblyomma americanum*)	USA
Ehrlichia muris	Mice	Unnamed	?	Japan
Ehrlichia ruminantium	Ruminants	Heartwater	Ticks (*Amblyomma hebraeum*)	Sub-Saharan Africa, Carribean Islands
Neorickettsia				
Neorickettsia sennetsu	Humans	Sennetsu ehrlichiosis	Fluke?	Japan, Malaysia
Neorickettsia risticii	Horses	Potomac horse fever, equine monocytic ehrlichiosis	Trematode (*Juga* spp.) cercaria, aquatic insects	USA, Europe
Neorickettsia helminthoeca	Dogs	Salmon poisoning, Elokomin fluke fever	Fluke (*Nanophyetus salmincola*)	Pacific northwest of North America
SF agent	Humans, dogs	Hyuganetsu disease	Fluke (*Stallantchasmus falcatus*)	Japan
Wolbachia				
Wolbachia pipientis-like symbionts	Arthropods, isopods	Cytoplasmic incompatibility, sex-ratio distortion	Seven orders of insects, *Armadillium*	Europe, USA
Wolbachia pipientis-like symbionts	Humans?	Contributes to inflammation with lymphatic filariasis?	Filarial nematodes	Tropical areas

HGE, human granulocytic ehrlichiosis.

Anaplasma marginale. Similar inclusions in febrile animals that were located within central regions of erythrocytes were denoted as *Anaplasma centrale*, although he was unable to discern other differences. By 1925, Cowdry had described the bont tick (*Amblyomma hebraeum*)-transmitted rickettsial agent of heartwater, a severe bovine illness in sub-Saharan Africa characterized by encephalitis and cardiac tamponade, and called it *Rickettsia ruminantium*. Tick-borne fever, a febrile condition of ruminants in Europe that was associated with multiple secondary infections, was described in 1932 and Gordon et al. implied a rickettsial agent as its cause before the name *Rickettsia phagocytophila* was eventually settled on in 1940. While this was occurring, Donatien and Lestoquard described several unique rickettsial infections of dogs and cattle in Algeria in 1936, and named them *Rickettsia canis* and *Rickettsia bovis*, respectively. The same year, Hertig named small bacteria in insect tissues as *Wolbachia*, in honor of S. Burt Wolbach, the pathologist who first demonstrated rickettsiae in tissues from infected patients. In 1938, Tyzzer described a parasite of blood neutrophils in North American *Microtus* and *Peromyscus* spp. that probably represented the same agent identified by Gordon in 1932; named this *Cytoecetes microti*, a genus name still preferred by some in the field. In the cumbersome task of taxonomic classification, Moshkovski recalled the early work of Kurlov, a Russian scientist working under the direction of Paul Ehrlich at the beginning of modern microbiology. Kurlov was assigned to study the causes of anemia in guinea pigs by examination of stained blood films. Although well recognized as noninfectious now, the Kurlov body was sufficiently similar in morphology to the intracytoplasmic inclusions seen with many of these infections that Moshkovski created a new genus in honor of Ehrlich, and assigned into it many of the species once classified as *Rickettsia*, to create the new combinations *Ehrlichia canis*, *Ehrlichia phagocytophila*, and *Ehrlichia bovis*; he also created the new genus *Cowdria* to accommodate the former *Rickettsia ruminantium*. Philip in 1953, and Misao and Kobayshi in 1954, described unique rickettsial species of dogs and humans that did not have strong tick or arthropod associations but in fact had closer epidemiological ties with the consumption of raw fish and, in the case of *Neorickettsia helminthoeca*, with trematodes that infest the fish and are consumed by the dog. Subsequent discoveries were classified as *Ehrlichia* spp. if the bacteria clustered in a vacuole in a peripheral blood leukocyte (*Ehrlichia equi* (Stannard et al. 1969), *Ehrlichia platys* (French and Harvey 1983), *Ehrlichia risticii* (Holland et al. 1985), *Ehrlichia chaffeensis* (Anderson et al. 1991), *Ehrlichia ewingii* (Anderson et al. 1992b), and *Ehrlichia muris* (Wen et al. 1995a), among others). A reclassification proposal in 2001 (Dumler et al. 2001) established the existing taxonomic structure and new combinations.

HABITAT

A common feature of the habitat of the pathogenic *Ehrlichia* and *Anaplasma* spp. is the association with ticks and vertebrate animals. Humans and some animals are accidental hosts that then develop clinical signs; such findings suggest that coevolution has permitted persistent infections in some reservoir hosts that do not result in clinical manifestations or signs of disease, such as with some cervids or small mammals. There are, however, considerable differences in the details of such associations, and each species (or groups of closely related species) needs to be discussed separately (Table 1).

Various microorganisms living in intracellular symbioses with arthropods cause sex-ratio distortion and alteration of sex determination of their hosts, such as parthenogenesis, female-biased sex ratios, and sterility of offspring of crosses between infected males and uninfected females. Most such microorganisms are relatives of the species *W. pipientis* (Werren et al. 1995). Hertig first described this species in 1936 as occurring in the gonads and gut epithelium of the mosquito *Culex pipiens*. Some *Wolbachia* spp. identified in filarial nematodes may influence successful development between larval instar stages and maturation into adults. The presence of *Wolbachia* bacterial components in the filariae is also suspected to contribute to a pro-inflammatory response that could exacerbate lymphatic or venous obstruction, or contribute to the post-inflammatory fibrosis that complicates filariasis (Taylor 2002).

A common feature of the tick-associated *Ehrlichia* and *Anaplasma* species is the inefficient transmission from one generation of ticks to the next. This ineffective transovarial transmission implies a reliance on horizontal transmission (tick–vertebrate host–tick) that further suggests the need for persistent infection, probably persistent bacteremia, of reservoir host vertebrates.

E. canis, a worldwide pathogen of dogs, is transmitted by the brown dog tick, *R. sanguineus*. Similarly, *A. variegatum* and *A. hebraeum* ticks transmit *Ehrlichia ruminantium* to ruminants. *E. chaffeensis*, which occurs most frequently in southeastern and south-central USA, and *Ehrlichia ewingii*, a human and canine pathogen also in the midwest USA, have been shown to occur in the tick vector, *Amblyomma americanum* (Lockhart et al. 1996). These two species may be difficult to differentiate by serological analysis, which has shown evidence of human infection in Europe, Asia, and Africa (Dumler and Bakken 1995). The former species *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the unnamed agent of human granulocytic ehrlichiosis (HGE) have been combined into a single species, *Anaplasma phagocytophilum* that is responsible for human anaplasmosis (formerly HGE) in the northern USA and Europe; *Ixodes persulcatus* group ticks including *I. scapularis* in the eastern USA,

I. pacificus in the western US, *I. ricinus* in Europe and Asia, and *I. persulcatus* in Asia transmit the bacterium. Other tick hosts that do not bite man, such as *I. spinipalpis*, may also transmit *A. phagocytophilum* among small mammals and other animals (Richter et al. 1996). The habitats of other *Anaplasmataceae* are not well understood or studied. For *Neorickettsia*, ticks are not part of the transmission cycle, and fluke transmission has been proven for *N. helminthoeca*, *N. risticii*, and the *Neorickettsia* sp. in *Stellantchasmus falcatus* (SF agent) (Wen et al. 1996) and is suspected in the case of *N. sennetsu*. An important role exists for trematode metacercariae, which carry *N. risticii* to aquatic insects that are accidentally ingested; the importance of this cycle for other *Neorickettsia* spp. has not been evaluated.

MICROENVIRONMENT AND MORPHOLOGY

Species of the genera *Ehrlichia*, *Anaplasma*, and *Neorickettsia* prevent phagosome fusion with lysosomes and thus remain in a favorable environment for growth. A few species, such as *A. phagocytophilum*, *E. ewingii*, and *E. ruminantium*, invade and propagate in granulocytes, a biological niche unique among all bacteria, while *Anaplasma marginale* and *Anaplasma platys* grow within erythrocyte and platelet vacuoles, respectively. *Ehrlichia ruminantium* parasitizes predominantly endothelial cells in vivo, whereas in vitro it grows well in macrophage cultures. Similarly, *E. chaffeensis* may be propagated in a variety of cell lines, including macrophages, endothelial cells, and even fibroblasts.

The mechanisms by which vacuoles that contain *Anaplasmataceae* avoid fusion with lysosomes have been studied only for *N. risticii* (Rikihisa 1991a), *N. sennetsu* (Barnewall et al. 1999), *A. phagocytophilum* (Barnewall et al. 1999; Webster et al. 1998), and *E. chaffeensis* (Barnewall et al. 1997). All three of these species enter early endosomes; the vacuoles that contain *A. phagocytophilum* do not mature or accumulate additional markers of endosomes, whereas both *E. chaffeensis* and *N. sennetsu* accumulate other markers of early endosomes but never mature into later stages such as sorting or late endosomes or phagolysosomes (Barnewall et al. 1997; Webster et al. 1998). *E. chaffeensis* specifically abrogates vacuolar maturation by directing the accumulation of all cellular transferrin receptors to the parasitophorous vacuole. The net result is the shuttling of the infected vacuole into a receptor-salvage/recycling pathway that does not fuse with lysosomes.

Infection of canine monocytes with *Ehrlichia*, *Anaplasma*, and *Neorickettsia* spp. is effected by small round bacteria 0.5 μm in diameter. Upon entry into the vacuole, they increase in size and divide by binary fission to form clusters in a membrane-bound vacuole, usually referred to as morulae. Individual bacteria have typical gram-negative trilaminar outer and inner membranes. The various genera and species of *Anaplasmataceae* vary in the size of the morulae, some ultrastructural features, and time of release of the individual bacteria (Chaichanasiriwithaya et al. 1994; Popov et al. 1995). The morphology of ehrlichial morulae and individual bacteria is illustrated in Figure 1. Two distinct ultrastructural morphologies have been identified, including small cells with densely packed chromatin (dense core) and larger forms with loose, reticulated chromatin (reticulate). Despite the similarity to the life-stage forms of chlamydiae, both dense-core and reticulate forms divide by binary fission, although some evidence suggests that dense-core cells selectively express adhesins for binding to cells prior to internalization (Popov et al. 2000).

Anaplasmataceae bacteria vary from 0.2 to 1.5 μm in diameter and usually are coccoid or ellipsoid in shape, although a variety of unusual shapes and forms may be observed. Flagella and pili are not present; in vitro genetic recombination has not been demonstrated, although natural recombination of certain antigen encoding genes is probably a critical factor for prolonged in vivo survival in vertebrate reservoirs. Ultrastructural studies confirm that members of the genera *Ehrlichia*, *Anaplasma*, *Wolbachia*, and *Neorickettsia* contain chromosomal structures, ribosomes, and other subcellular organelles (Popov et al. 1995). As in the *Rickettsiaeceae*, bacteria in the family *Anaplasmataceae* are surrounded by an outer envelope (cell wall) similar to that of gram-negative bacteria. In general, both the inner and the outer leaflets have similar dimensions, unlike the situation for *Rickettsiaceae*. Moreover, lipopolysaccharide (LPS) is not present in *Anaplasmataceae*, although spheroplasts form in the presence of some antibiotics that target peptidoglycan, suggesting its presence in the cell wall (Rikihisa 1991a; Lin and Rikihisa, 2003). In some preparations, extensions of the bacterial membrane may allow the bacteria to become wrapped in several layers or to develop invaginations into the bacterial protoplasm (Popov et al. 1995). *Ehrlichia* spp. but not *Anaplasma* spp. possess intravacuolar fibrillar and tubular structures that may contain bacterial antigens. Some strains of *A. marginale* possess appendages extending from the surface of the endocytic vacuole into the host cell cytoplasm that also integrate bacterial antigens.

STAINING PROCEDURES

Romanowsky stains, such as Giemsa or Wright, or rapid methods are used frequently for light-microscopic visualization of *Anaplasmataceae*. Using this method, the microorganisms are easily recognized in tissue culture cells or blood smears, although the abundance of other intracellular granules and structures renders this less useful for histological sections. Acridine orange staining combines visualization with an assessment of physiological activity because active, but not dead, micro-

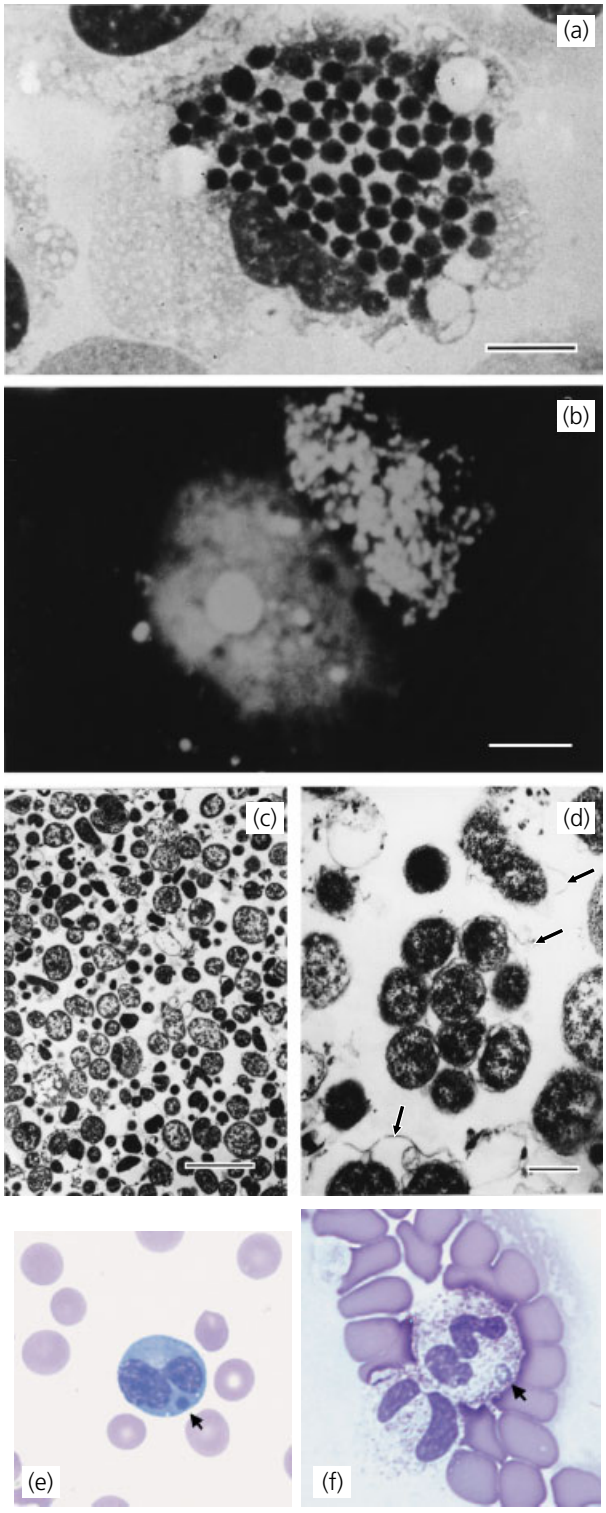

Figure 1 *The morphology of ehrlichiae.* **(a)** E. canis *in primary canine monocyte cultures, stained by Wright–Giemsa. Bar = 10 μm;* **(b–d)** E. risticii *in mouse macrophage.* **(b)** *Stained by acridine orange. Note clump of organisms within the cytoplasm. Bar = 10 μm.* **(c)** *Note variety of morphological forms. Bar = 3 μm.* **(d)** *Note retention of morula structure and some host membranes (arrows), even in purified preparation. Bar = 0.5 μm.* **(e)** E. chaffeensis *morula (arrow) in a peripheral blood monocyte from a patient with human monocytic erlichiosis.* **(f)** A. phagocytophilum *morula (arrow) in a peripheral blood neurophil from a patient with human granulocytic anaplasmosis.*

organisms, fluoresce an intense bright orange to red color against a yellow-green background. An ultraviolet microscope is required for this procedure, which will also stain most other bacteria and eukaryotes. Fluorescent antibody tests permit simultaneous detection and identification of the rickettsiae, depending in part upon the specificity of the antibody reagents used. *Ehrlichia* and *Anaplasma* cells have also been visualized in tissue sections by immunohistochemical methods that employ specific antibodies and by methods that use the in situ hybridization of specific nucleic acid probes (Dumler et al. 1991; Walker and Dumler 1997; Dawson et al. 2001).

LABORATORY ISOLATION, CULTURAL CHARACTERISTICS, AND GROWTH REQUIREMENTS

Of the classical methods for recovery of rickettsiae from clinical and environmental samples, the most successful for recovery of *Ehrlichia*, *Anaplasma*, *Wolbachia*, and *Neorickettsia* spp. is cell culture. Many species have not yet been propagated in vitro and depend upon propagation by continuous inoculation of infectious materials, usually blood, into susceptible animals. Moreover, *Wolbachia* spp. are particularly recalcitrant to in vitro growth as a lytic phase in rarely observed. A further difficulty in dealing with *Anaplasmataceae* is the inherent difficulty in deriving clonal populations, as monolayer plaques are not produced or are inapparent. *Ehrlichia*, *Anaplasma*, and *Neorickettsia* spp. have been grown in a variety of cells, including L929, BGM, mouse embryo, Vero, P388D1, DH82, bovine endothelial, tick embryo IDE8, HL-60 human promyelocytic leukemia, THP-1 human myelomonocytic leukemia, KG-1 human granulocytic leukemia, and mouse M1 myeloid leukemia cells, and in co-cultures of erythrocytes and endothelial cells (Rikihisa 1991a; Chen et al. 1995; Goodman et al. 1996; Wen et al. 1996). *Wolbachia pipientis*-infected mosquito cell cultures are available for study, and methods for infection of other cells have been described (O'Neill et al. 1997; Fenollar et al. 2003).

REPLICATION

Replication of *Ehrlichia*, *Anaplasma*, *Wolbachia*, and *Neorickettsia* spp. occurs by binary fission within the endosome. Although both dense-core and reticulate forms may divide, dense-core forms seem to predominate late in infection, suggesting a role in infectivity, perhaps owing to an enrichment in adhesins that facilitate entry into new host cells after discharge from the infected cell. Host cells infected with *Anaplasmataceae* show little cytopathology until cytoplasmic vacuoles are filled with microorganisms and the cells burst or the infected vacuole fuses to the cell membrane in an exocytic-like event.

METABOLISM

Limited information is available on the metabolism of *E. sennetsu* and *E. risticii* (Weiss et al. 1989). Isolated ehrlichiae resemble rickettsiae in being nonglycolytic and in carrying out oxidative phosphorylation and adenosine triphosphate (ATP) synthesis in the presence of citric-acid-cycle intermediates, particularly glutamine, when incubated at neutral pH. The genomic sequences of strains of *Ehrlichia chaffeensis*, *Ehrlichia ruminantium*, *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Neorickettsia sennetsu*, and *Wolbachia* spp. are nearing completion and should allow a comprehensive overview of metabolic pathways among these bacteria.

CLASSIFICATION

Classification of rickettsiae has undergone significant change since publication of the last edition of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984). In the newest edition (Garrity et al. 2005), the family *Rickettsiaceae* has been limited by reassignment of the genera *Ehrlichia*, *Neorickettsia*, *Cowdria*, and *Wolbachia* into the family *Anaplasmataceae*, also eliminating the tribe schema. In the reclassification, several new genus and species combinations were created, and additional members of *Anaplasmataceae* (the genera *Haemobartonella* and *Eperythrozoon*) were reclassified into the *Mollicutes*. The taxonomic standing of some genera such as *Aegyptianella* is still unresolved, although recent data suggest correct placement in the *Anaplasmataceae* family and a close relationship to the genus *Anaplasma* (Rikihisa et al. 2003). A current phylodendrogram based upon neighbor-joining analysis of informative positions of 16S ribosomal RNA genes of recognized species of *Anaplasmataceae* and their relationship to some *Rickettsia* species and *Escherichia coli* as an outgroup is shown in Figure 2. Similar trees result from the analysis of sequences of both *groESL* and *gltA* (citrate synthase) (Dumler et al. 2001; Sumner et al. 1997; Inokuma et al. 2002). Some authors suggest alternative classification; thus, the final schema has not yet been decided (Uilenberg et al. 2004).

GENOME

Increasing information about the genomes and genomic structure of the *Anaplasmataceae* is becoming available. The sizes of the genomes in this family vary from small for *Neorickettsia* (860–900 kbp), to moderate for *Ehrlichia chaffeensis* (1236 kbp), *Anaplasma marginale* (1250 kbp), and *Anaplasma phagocytophilum* (1580 kbp), and highly variable for *Wolbachia* spp. (950–1660 kbp). Similarly, the %G + C varies from very low (32–39 percent) for *Ehrlichia* spp. to high (56 percent) for *Anaplasma marginale* (Alleman et al. 1993). The molecular differentiation of *Anaplasmataceae* depends primarily on sequences of *rrs* (16S rRNA) and *groEL* genes (Dumler et al. 2001). Whereas new isolates and molecular fingerprints allow differentiation based upon sequences of these highly conserved genes, antigenic and biological properties are generally consistent (Dumler et al. 1995b, 2001; Wen et al. 1995a, 1995b, 1996). *E. risticii* isolates differing in pathogenicity have been distinguished by restriction fragment length polymorphism (RFLP) analysis of genomic DNA probed with specific antigen gene probes or a partial *rrs* (16S rRNA gene) polymerase chain reaction (PCR) products (Chaichanasiriwithaya et al. 1994; Vemulapalli et al. 1995). Sequences of the *rrs* (16S rRNA gene) and *rrl* (23S rRNA gene) and the bacterial cell division gene *ftsZ* have provided evidence of multiple clades or lineages of *Wolbachia*-like symbionts and bacteria in a wide diversity of invertebrate species as well as helminths (Werren et al. 1995; Bandi et al. 1998; Lo et al. 2002). This monophyletic grouping contains up to six distinct internal clades, several of which are specifically associated with insects such as fruit flies, beetles, and mosquitoes, or with nematode helminths such as *Brugia*, *Onchocerca*, and *Dirofilaria*. Lineages of the *Wolbachia* spp. are exceptionally closely associated with their hosts suggesting long-term coevolution. The rRNA operons of *W. pipientis* are unusual in that *rrs* (16S rRNA) is unlinked to *rrl/rrf* (23S-5S rRNA) operon, and neither is it linked to tRNA-encoding sequences, each is present in a single genomic copy, and the antitermination boxes usually present in eubacteria are absent (Bensaadi-Merchermek et al. 1995). *Wolbachia* isolates from *Culex* and *Ephestia* differed in sequences flanking the rRNA operons although their rRNA operon sequences and organization were conserved. It is anticipated that a wealth of data regarding the overall genomic structure and biological capabilities will become available when genome sequences for several *Ehrlichia* spp (*E. chaffeensis*, *E. ruminantium*), *Anaplasma* spp. (*A. marginale*, *A. phagocytophilum*), *Wolbachia* spp., and *Neorickettsia sennetsu* are released.

An increasing number of genes of *Anaplasmataceae* have been cloned and evaluated as part of analyses of immunological reactions and as antigens for serodiagnostic tests. For *Neorickettsia*, these include the 44-, 51-, 55-, 70-, and 85-kDa antigen genes of *N. risticii* (Dutta et al. 1991; Vemulapalli et al. 1995) and the *groEL* of *N. risticii* and *N. sennetsu*, as well as the *gltA* (citrate synthase) or *N. risticii*. Many more genes have been cloned and analyzed for the *Ehrlichia* genus, including *rrs* (16S rRNA), the *groE* operons, *gltA*, 120- and 140-kDa antigen genes of *E. chaffeensis* and *E. canis*, and multigene families that are regular components of the genomes of *Ehrlichia*, *Anaplasma*, and *Wolbachia* spp. (Sumner et al. 1993; Yu and Walker 1995; Ijdo et al. 1998; Jiggins et al. 2002). Initially identified by the immunodominance of these protein antigens

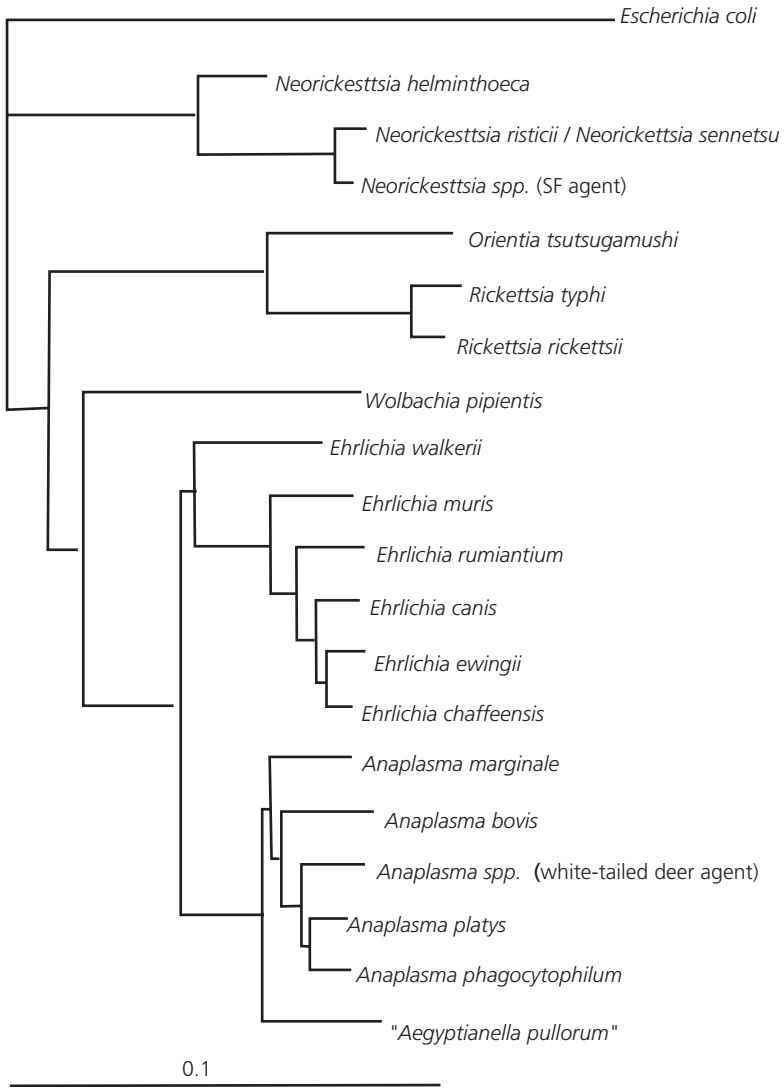

Figure 2 *Phylogenetic relationships among current members of the family* Anaplasmataceae. *The tree was generated by clustal alignment of* rrs *(16S rRNA gene) sequences from the organisms shown using the neighbor-joining method. The bar indicates the number of substitutions per 1000 bp.*

using polyclonal sera and later using monoclonal antibodies, the antigens encoded by these genes have a conserved domain referred to in the National Center for Biotechnology Information's (NCBI) Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) as 'pfam01617.8, Surface_Ag_2;' other proteins that contain this domain include the phase-variable integral membrane opacity (Opa) porin proteins of pathogenic *Neisseria* spp. that also mediate pathogen-host–cell interactions. The domain architecture has been detected in protein families denoted as major surface protein-2 (MSP-2) in *Anaplasma marginale*, *Anaplasma ovis*, and *Anaplasma centrale*, as major surface protein-4 (MSP-4) in *Anaplasma marginale*, as major surface protein-2 (Msp2), P44, HGE-44, and '44-kDa antigen' in *Anaplasma phagocytophilum*, as P28 or Omp-1 in *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia muris*, and *Ehrlichia ewingii*, as MAP1 and MAP2 in *Ehrlichia ruminantium*, and as outer-membrane protein precursors or *Wolbachia* surface proteins (Wsp) in *Wolbachia* spp. pfam01617.8, Surface_Ag_2 sequences have not yet been detected in *Neorickettsia*. The prevailing pattern of the pfam01617.8, Surface_Ag_2-related gene families is the presence of multiple paralogs within the genome, and the gene structure is characterized by conserved regions that flank hypervariable sequences. In *E. chaffeensis* and *E. canis*, the *p28* paralogs are tandemly arranged in a single genomic cluster and each possesses unique promoters that could allow the genes to become transcribed independently in vitro and possibly in vivo. In *Anaplasma marginale*, and probably in *A. phagocytophilum*, the paralogs are distributed through the genome in small clusters of three to six paralogs that lack individual promoters. It is now believed that a single or small number of promoter sites are present in the *Anaplasma* genome, and that expression of individual paralogs may

require gene conversion. An in-depth analysis of the structure of the pfam01617.8, Surface_Ag_2 paralogs of *Ehrlichia* and *Anaplasma* indicates the presence of additional conserved sites that may allow both gene conversion and intragenic recombination, and that successive combinatorial changes could yield enormous genetic and antigenic diversity. The underlying potential genetic heterogeneity is not limited to intact paralogs, even if combinatorial processes allow frequent recombination among these 'pseudogenes.' An analysis of the nearly complete *A. phagocytophilum* genome for Msp2-like sequences in contiguous open reading frames of at least 100 amino acids identified at least 52 paralogs – an enormous genetic reservoir that may in part explain the success of these bacteria despite highly restrictive niches (Scorpio et al. 2004).

The major function of the pfam01617.8, Surface_Ag_2 family is not known, but roles in antigenic diversity and as adhesins are suggested by extrapolation from *Neisseria*. In fact, antigenic diversity is a well-established principle for survival of *A. marginale* infection of cattle, and increasing evidence suggests that similar genetic changes that promote antigenic diversity may play a role in immune evasion for *Ehrlichia chaffeensis*, *Ehrlichia canis*, and *A. phagocytophilum*. The preferential expression of some paralog-encoded antigens in mammalian cells in vitro, or with in vivo mammalian or tick infections, suggests other potential roles. MSP2 of *A. marginale* forms membrane complexes with at least four other surface-exposed proteins, and limited evidence suggests that adhesion to erythrocytes occurs via this complex. Similarly, Msp2 of *A. phagocytophilum* in part mediates adhesion of the bacterium to granulocyte surfaces, in particular platelet selectin glycoprotein ligand-1 (PSGL-1), proving a role for this protein as an adhesin. Given the degree of relationship among the pfam01617.8, Surface_Ag_2-like sequences, it is not surprising that variable degrees of antigenic cross-reactivity are detected, more among phylogenetically close *Anaplasmataceae*; in fact, the tremendous potential for heterogeneity of surface antigens suggests that at least some of the serological cross-reactivity may be due to the expression of other antigenically similar proteins.

Other genes of potential importance have been identified and cloned. *Ehrlichia chaffeensis* and *E. canis* possess genes that encode immunodominant 120- and 140-kDa glycoprotein antigens, respectively, that may also be adhesins (McBride et al. 2000; Popov et al. 2000), and similar sequences are identified in the genes that encode 100-kDa (*gra*) and 130-kDa (*rea*) antigens of *A. phagocytophilum* (Storey et al. 1998). Another shared genomic characteristic is the presence of eight genes (four each of *virB* and *virD*) that encode the machinery for type IV secretion and that are clustered in two separate loci in the genomes of both *E. chaffeensis* and *A. phagocytophilum* (Ohashi et al. 2002). The genes encoding these putative virulence factors are colocalized and polycistronically transcribed with *sodB* and an *msp2* paralog. As the superoxide dismutase product of *sodB* possesses a metal ion cofactor, metal-rich environments probably regulate the gene, such as in proximity to or within a eukaryotic vacuole. The resulting transcription of *msp2* may permit enhanced expression of this adhesin. A similar genomic arrangement has been described for *Wolbachia* spp., although transcriptional analysis has yet to be conducted (Masui et al. 2000). *A. phagocytophilum ankA* encodes a 160-kDa protein antigen called AnkA that has at least 11 tandemly repeated ankyrin motifs and that translocates into the nucleus of the infected neutrophil and complexes with host-cell chromatin (Storey et al. 1998; Caturegli et al. 2000). The function of this protein is not known, but it is reasonably conserved regardless of geographic origin.

CELL-WALL COMPOSITION AND ANTIGENIC PROPERTIES

Ultrastructural studies indicate that the cell wall of *Anaplasmataceae* is similar to that of other gram-negative bacteria, but no obvious peptidoglycan layer is visible. Although biological responses characteristic of exposure to LPS have been demonstrated for *Wolbachia* spp. (Taylor et al. 2000), genes encoding biomachinery are absent from the genomes of *E. chaffeensis*, *A. phagocytophilum*, and *W. pipientis* (Lin and Rikihisa 2003; Wu et al. 2004). Techniques that examine the components of the cell wall have shown the presence of a number of integral membrane proteins, the most abundant of which are the pfam01617.8, Surface_Ag_2 proteins, although numerous other proteins can be visualized. A total of 18 *N. risticii* proteins were identified by surface iodination and radioimmunoprecipitation, nine of which were prominent when examined using homologous polyclonal sera. Antisera from different species, as well as from individual animals, exhibited differences in reactivity to *E. risticii* antigens. *N. risticii* and *N. sennetsu* share many antigens, including those of 110, 70, 58, and 44 kDa (Dasch et al. 1990; Shankarappa et al. 1992), but in accordance with phylogenetic placements, most react with *N. helminthoeca* and only a few react with *E. canis* (Rikihisa 1991b; Shankarappa et al., 1992). In fact, *N. sennetsu* immunization protects ponies against challenge with *N. risticii* and elicits strong serologic reactivity to the shared 44-kDa protein. However, mice that were protected against *N. risticii* challenge by a killed homologous vaccine reacted primarily to 33-, 57-, and 100-kDa antigens (Rikihisa 1991c).

In the genus *Ehrlichia*, a number of antigens can be detected in whole-cell immunoblot preparations. *E. chaffeensis* has 20 protein antigens detectable with homologous antisera. Cross-reactions occur most strongly with antisera to other *Ehrlichia* spp., including *E. canis*, *E. ewingii*, *E. ruminantium*, and *E. muris*.

Based upon immunofluorescence reactions using whole cells rather than defined proteins, serologic reactions among *Ehrlichia* spp. can not be easily distinguished, even with differential titers. Western blot analyses reveal a broad range of serologically cross-reactive proteins, including some shared broadly among bacteria outside of the *Ehrlichia* genus (e.g. GroESL heat-shock proteins), some apparently unique to the genus, and even some that weakly react with antigens expected to be unique for the species (Unver et al. 1999; Bowie et al. 1999). Study of the antigenic specificity of the pfam01617.8, Surface_Ag_2 proteins for individual *Ehrlichia* spp. has revealed contradictory results, although serologic cross-reactions are not often detected. However, the phylogenetic relationships among members of the genus and the high degree of pair-wise nucleotide and predicted amino-acid similarity in the pfam01617.8, Surface_Ag_2 gene and protein conserved regions suggests a low but real likelihood of shared or common epitopes. For *Ehrlichia*, the only known potential adhesin is the gp120 of *E. chaffeensis*, which is also the only protein expressed to a higher degree on dense core cells (Yu et al. 2000; Popov et al. 2000).

Anaplasma marginale is the best antigenically characterized species of the genus. It has six distinct major surface proteins (MSP1a, MSP1b, MSP-2, MSP-3, MSP-4, and MSP-5) associated with the three potential adhesins, MSP1a, MSP1b, and MSP2 (Vidotto et al. 1994; McGarey and Allred 1994a; McGarey et al. 1994b). These proteins interact in the membrane and can be co-immunoprecipitated after treatment with cross-linking reagents. Under nonreducing conditions, MSP2 and MSP5 form disulfide-linked multimers (Vidotto et al. 1994). MSP2 migrates as multiple spots on two-dimensional gels (Zakimi et al. 1994). MSP1 complexes consist of a heterodimer of both disulfide and noncovalently associated MSP1a and 1b polypeptides. *A. marginale* bind to erythrocytes and tick cells in vitro via MSP1, and MSP1 antibody inhibits tick-cell infectivity consistent with earlier observations suggesting a direct role as an adhesin (de la Fuente et al. 2001; McGarey and Allred 1994a; Blouin et al. 2003). *A. phagocytophilum* also possesses a large number of antigens visualized in immunoblots. The most quantitatively and immunologically dominant protein is Msp2, which varies in size from 40 to 50 kDa and in structure on strains from different geographical regions. Msp2 expression also changes over generations owing to gene conversion and recombination of Msp2 gene sequences or modifications in *msp2* mRNA (Caspersen et al. 2002; Barbet et al. 2003). Park et al. (2003) described the role of *A. phagocytophilum* Msp2 as an adhesin for binding to neutrophils, suggesting the importance of a complex of major surface proteins for binding by *Anaplasma* spp. AnkA is a 155–160-kDa protein (Storey et al. 1998; Massung et al. 2000) that is secreted from *A. phagocytophilum* and eventually gains access to the host cell nucleus where it binds to the chromatin (Caturegli et al. 2000). AnkA antigens are not detected in *Ehrlichia* spp. As for *Ehrlichia* and *Neorickettsia*, *Anaplasma* also possess antigenic GroESL that cross-reacts widely with other *Anaplasmataceae* and many other unrelated bacteria.

Frequent serologic reactions between *A. phagocytophilum* and *Ehrlichia* spp. are detected, largely owing to reactions between heat-shock proteins that share similar epitopes, but potentially to a lesser degree by virtue of epitopes shared on pfam01617.8, Surface_Ag_2 proteins (Wong et al. 1997; Unver et al. 2001a). In fact, the prior taxonomic classification scheme was based in part upon degrees of serologic cross-reactivity, and despite unique taxonomic positions and the lack of shared pfam01617.8, Surface_Ag_2 proteins, serologic cross-reactivity between *Ehrlichia*, *Anaplasma*, and *Neorickettsia* spp. has been demonstrated (Chen et al. 1994b; Rikihisa et al. 1994; Wen et al. 1995a; Dumler et al. 1995a). Regardless, most notable specific reactions appear directed toward the pfam01617.8, Surface_Ag_2 proteins, including: (i) P28 in *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, and *E. ruminantium* (MAP1); (ii) Wsp (28 kDa) in *Wolbachia* spp.; and (iii) Msp2 (40–50 kDa) in *Anaplasma marginale* and *A. phagocytophilum*. Although no pfam01617.8, Surface_Ag_2 protein homolog has yet been identified in *Neorickettsia*, this genus demonstrates the presence of dominant 80-, 70-, and 64-kDa protein antigens, of which the latter probably represents GroESL (Rikihisa 1991b). Other surface antigenic proteins of importance include a low-molecular-weight protein (approximately 20 kDa) called MSP5 in *A. marginale* and *A. centrale*, and MAP2 in *E. canis*, *E. chaffeensis*, and *E. ruminantium*. These proteins possess a conserved domain shared by SCO1/SenC/PrrC proteins that are involved in involved in biogenesis of respiratory and photosynthetic systems of α-proteobacteria, *Rickettsia conorii*, and *Rickettsia prowazekii* and also in mitochondria. The function in *Anaplasmataceae* is not known.

Considerable study has been made regarding the protective nature of immune reactions toward antigens of the *Anaplasmataceae*. Most study has been directed at veterinary control of heartwater (*E. ruminantium*) and anaplasmosis (*A. marginale*). The consensus of opinion now favors an important role for the generation of activated macrophages through the action of interferon-γ (IFNγ) that either acts directly on infected cells or facilitates destruction of antibody opsonized bacteria during the extracellular transition (Palmer et al. 1999; Akkoyunlu and Fikrig 2000; Banerjee et al. 2000; Van Kleef et al. 2002). Other observations focus upon specific antigens that induce antibody and immune responses that protect against virulent challenge. These studies implicate series of major surface antigens, usually of low molecular size (13–18 kDa and the 31-kDa MAP1) for *E. ruminantium* (Van Kleef et al. 2002)

and usually directed against the MSP1 complex (MSP1α and MSP1β) for *A. marginale* (Brown et al. 2001). Of interest is the observation that passive antibody administration abrogates *E. chaffeensis* and *A. phagocytophilum* infection in mice (Winslow et al. 2000; Sun et al. 1997). Moreover, the use of monoclonal antibodies as passive immunization to *E. chaffeensis* pfam01617.8, Surface_Ag_2 protein P28 provides protection whereas those to *A. phagocytophilum* pfam01617.8, Surface_Ag_2 protein Msp2s are only variably protective (Li et al. 2001; Kim and Rikihisa 1998). Mouse models also illustrate the important role of IFNγ in clearance of bacteria in both *E. chaffeensis* and *A. phagocytophilum*, although emerging data support a direct role for the immune response in the development of histopathologic lesions, clinical signs, and disease (Akkoyunlu and Fikrig 2000; Martin et al. 2000, 2001).

IDENTIFICATION AND TYPING METHODS

Morphologic methods are not specific enough alone to differentiate among *Ehrlichia*, *Anaplasma*, or *Neorickettsia* spp. The infected host and infected cell provide abundant information but rarely provide sufficiently specific information for a definitive identification. For example, *A. marginale* and *A. centrale* are located as clusters of bacteria in bovine erythrocytes, *Ehrlichia chaffeensis* and *N. sennetsu* are identified as bacterial clusters in human peripheral blood monocytes, *Anaplasma phagocytophilum* and *Ehrlichia ewingii* are identified in human or other mammalian neutrophils, *Ehrlichia ruminantium* may be observed as clusters in bovine endothelial cells, while *A. platys* and rarely other *Ehrlichia* and *Anaplasma* spp. are found in platelets. *Wolbachia* spp. have only been visualized in arthropod and nematode tissues. However, the abundance of newly identified species in *Anaplasmataceae*, and the overlapping hosts and host cell range, have reduced this simple morphological scheme for differentiation to a screening tool only.

Originally, identification of the various members of *Anaplasmataceae* in clinical or tick samples was based primarily on indirect fluorescent antibody reactivity with specific sera. Recently, molecular methods and DNA sequencing have supplanted these less specific identification tools (Dumler et al. 2001; Inokuma et al. 2002). PCR primers specific for nearly every member of the *Ehrlichia*, *Anaplasma*, and *Neorickettsia* spp. have been developed and described, including the conserved targets *rrs* (16S rRNA gene), *groESL* operon, *gltA* (citrate synthase), and a variety of antigen-encoding genes, especially those that encode pfam01617.8, Surface_Ag_2 proteins (Anderson et al. 1991; Massung and Slater 2003; Olano and Walker 2002; Inokuma et al. 2002; Cheng et al. 2003). Each target has advantages and disadvantages: genes involved in housekeeping function and RNA transcription are generally present as single copies or in low copy numbers, whereas several multigene families possess species-specific sequences that are present in multiple copies in the genome, facilitating sensitive identification. As the level of genetic similarity among many species is high, it is advantageous to confirm the identity of a specific molecular identification by sequence analysis.

Serologic tests generally identify *Anaplasmataceae* within the genus; however, cross-reactions out of the genus, especially between *Anaplasma* and *Ehrlichia* spp., are frequent. Thus, protein immunoblotting has been particularly useful in demonstrating antigen variation and differences between genera, between species, and even among strains within species. For example, *N. risticii* strains that vary in pathogenicity may be partly differentiated by a panel of monoclonal antibodies that identifies unique epitopes on the 51- and 85-kDa proteins (Chaichanasiriwithaya et al. 1994; Vemulapalli et al. 1995). Reactivity of sera with pfam01617.8, Surface_Ag_2 protein antigens of *E. chaffeensis*, *E. canis*, and *E. ruminantium* (P28 or MAP1) or with *A. phagocytophilum* (Msp2 or P44) has been suggested as a specific means to distinguish between infections by these organisms (Dumler and Bakken 1995). However, the use of cloned recombinant proteins for detection of serological responses has been confusing and disappointing probably owing to the high degree of genetic and antigenic diversity of immunodominant targets such as pfam01617.8, Surface_Ag_2 protein antigens. Moreover, no specific means exist for differentiation of strains with varying degrees of virulence, although recent evidence suggests that some genetic markers may allow differentiation of *A. phagocytophilum* strains pathogenic for humans (Massung et al. 2000; Petrovec et al. 2002). (Table 2).

TRANSMISSION, PATHOGENESIS, AND CLINICAL MANIFESTATIONS

Ehrlichia genus human infections

Human infection by members of the genus *Ehrlichia* has been limited to *E. chaffeensis*, *E. ewingii*, and, in a single patient, *E. canis* (Olano and Walker 2002; Buller et al. 1999; Perez et al. 1996).

HUMAN MONOCYTIC (MONOCYTOTROPIC) EHRLICHIOSIS (*EHRLICHIA CHAFFEENSIS*)

The vast majority of cases have resulted from infection by *E. chaffeensis*, a bacterium that infects predominantly monocytes in the blood and mononuclear phagocytes in tissues and organs. Thus, this infection has been named human monocytic (or monocytotropic) ehrlichiosis (HME). Unfortunately, most diagnoses of *E. chaffeensis* infection are established based upon the detection of

Table 2 *Clinical manifestations of human infections by* Ehrlichia *and* Anaplasma

Disease	Human monocytic ehrlichiosis	Ehrlichiosis 'ewingii'	Human anaplasmosis (formerly human granulocytic ehrlichiosis)
Causative agent	*Ehrlichia chaffeensis*	*Ehrlichia ewingii*	*Anaplasma phagocytophilum*
Severity	Moderate to severe	Mild to moderate	Mild to severe
Fever, headache, myalgias	Yes	Yes	Yes
Rash	36–40%	0%	$\leqslant$10%
Type of rash	Maculopapular or petechial	None described	Maculopapular
Respiratory tract involvement	25%	0%	10–20%
CNS involvement	20%	0%	rare
Thrombocytopenia	72%	75%	78%
Leukopenia	60–74%	75%	53%
Case-fatality ratio (%)	2%	0%	<1%

CNS, central nervous system.

E. chaffeensis antibodies in convalescence, and it is now increasingly recognized that this serological approach is not highly specific for differentiation of HME from infections caused by *E. ewingii* or *A. phagocytophilum*. It is estimated that approximately 10 percent of some serologically diagnosed *E. chaffeensis* infections may actually result from *E. ewingii* (Buller et al. 1999), and it is equally well recognized that between 10 and 40 percent of sera that react with *E. chaffeensis* cross-react with *A. phagocytophilum* (Wong et al. 1997). Despite these caveats, increasing knowledge about the infection in humans is accumulating. HME is characterized as an 'influenza-like' infection, and most patients present with fever, headache, and myalgias (Olano and Walker 2002). Approximately half or less develop manifestations attributable to the gastrointestinal system (nausea, vomiting, diarrhea, abdominal pain), and cough or radiographic evidence of respiratory involvement is observed in about 25 percent. Of interest is the frequent (20 percent) development of central nervous system (CNS) manifestations, often confirmed by cerebrospinal fluid (CSF) pleocytosis. Skin rash is much less common than with vasculotropic rickettsioses, and is present as a macular, maculopapular, or petechial rash on the trunk or extremities in 30–40 percent of patients, but only in 6 percent at presentation. Children more frequently develop rash with HME, up to 100 percent in some series (Schutze and Jacobs 1997). Characteristic laboratory findings including the presence of leukopenia in 60–74 percent, thrombocytopenia in 72 percent, and mild to significant elevations in serum transaminase activities in up to 90 percent of infections. Both leukopenia and thrombocytopenia are most often mild to moderate, but profound reductions ($<1 \times 10^9$ white blood cells (WBC)/L or $<10 \times 10^9$ platelets/L) are sometimes observed. An interesting set of hematological abnormalities may occasionally occur, including very early neutropenia and lymphopenia, followed by a left shift despite the leukopenia. After about 1 week, a reactive atypical lymphocytosis may be present; the atypical lymphocytes are phenotypically γδT cells (Caldwell et al. 1995). Although nonspecific, anemia is also often detected. Despite the frequent (90 percent of patients) mild to moderate elevations in serum aminotransferase levels, hepatic function is usually not impaired (Smith-Sehdev and Dumler 2002). Rarely, marked elevations occur, suggesting the possibility of viral hepatitis. In addition, increases in serum alkaline phosphatase occur in 25–60 percent and may suggest cholestasis (Nutt and Raufman 1999).

HME is a relatively severe infection that results in approximately 60 percent of patients requiring hospitalization, and an average of 3 weeks before return to normal activities. The case fatality rate is between 2 and 3 percent, and severe complications include meningoencephalitis, acute respiratory distress, hepatitis and hepatic failure, toxic or septic shock-like condition, renal failure, cardiomyopathy, coagulopathy and hemorrhage, fulminant infection in immunocompromised patients (HIV, organ transplantation, corticosteroid therapy), and the development of opportunistic infections by fungi and viruses.

Pathological samples from patients with HME demonstrate no vasculitis but have evidence of diffuse tissue mononuclear phagocyte activation with occasional granuloma formation and mononuclear phagocyte infiltrates in tissues (Walker and Dumler 1997; Dumler et al. 1993; Marty et al. 1995; Smith-Sehdev and Dumler 2002). Scattered perivascular lymphohistiocytic infiltrates in liver, lung, brain, and other tissues are evidence of disseminated low-level inflammation. Normocellular and hypercellular bone marrows are typical, favoring the concept of leukopenia and thrombocytopenia caused by peripheral sequestration or consumption rather than bone-marrow suppression (Dumler et al. 1993); similarly, the level of bacteremia is insufficient to explain leukocyte destruction, especially given the pancytopenia of multiple hematopoietic lineages that are not suscep-

tible to *E. chaffeensis* infection. Focal hepatocyte necroses with or without adjacent inflammatory foci may explain elevations in serum hepatic transaminases. *E. chaffeensis* are only infrequently observed in tissue samples, except when obtained from immunocompromised individuals who often develop fulminant infection (Paddock et al. 1993; Marty et al. 1995; Smith-Sehdev and Dumler 2002). In some patients, significant liver injury with focal hepatocellular necrosis and cholangitis with cholestasis are observed (Smith-Sehdev and Dumler 2002). The discrepancy between the quantity of bacteria in the tissues and the degree of pathologic injury suggests that the pathogen triggers an immunopathologic response rather than directly causing tissue injury. Thus, the current working hypothesis about pathogenesis with *E. chaffeensis* is that infection perturbs mononuclear phagocyte function, important regulators of proinflammatory response, thus allowing inflammation to proceed out of proportion to the level of infection. Both humoral and cellular immunity are likely to participate in recovery and eradication of infection, but macrophage activation by IFNγ is probably pivotal (Winslow et al. 2000; Barnewall and Rikihisa 1994). In fact, *E. chaffeensis* infection in the absence of opsonizing antibody suppresses cytokine production by macrophages and promotes a moderate anti-inflammatory response by production of interleukin-10 (IL-10) (Lee and Rikihisa 1996). Dichotomously, macrophage infection in the absence of immune response also promotes penetration of infected cells through endothelial cells, fixing subsequent proinflammatory responses into target tissues and organs (Park et al. 2003). Internalization of *E. chaffeensis* opsonized by antibody leads to a marked proinflammatory response heralded by production of TNFα, IL-1β, and IL-8 (Lee and Rikihisa 1997).

'EHRLICHIOSIS *EWINGII*' (*EHRLICHIA EWINGII*)

During routine application of PCR diagnostics for HME in an endemic region, it was discovered that approximately 10 percent of patients with *Anaplasmataceae* DNA in blood were not infected with *E. chaffeensis*, and sequence analysis revealed human infection by *Ehrlichia ewingii*, only previously known as a pathogen of canine neutrophils. To avoid increasing confusion of disease names, some have elected to call this condition 'ehrlichiosis ewingii.' Three of the first four patients were immunocompromised and infection of peripheral blood neutrophils was detected in two of these patients. Since that time, *E. ewingii* has been implicated in a limited number of human infections, in both immune-competent and immune-suppressed individuals (chiefly HIV-infected). As *E. ewingii* and *E. chaffeensis* are close phylogenetic relatives, there is considerable serologic cross-reactivity; thus, serologic testing based upon whole cell methods is unable to distinguish *E. ewingii* from *E. chaffeensis* infections. However, in seropositive convalescent sera tested in protein immunoblots, the absence of reactivity with the P28 of *E. chaffeensis* and *E. canis* suggests *E. ewingii* infection. Overall, clinical manifestations are similar to those observed with *E. chaffeensis*, and are dominated by fever, with fewer patients having headache, myalgias, and malaise. Leukopenia, thrombocytopenia, and elevated serum hepatic transaminase levels are also observed. The infection appears to be less severe than for *E. chaffeensis*, but too few cases have been identified and described to make any certain conclusions. Pathological investigations of humans with *E. ewingii* infection have not been reported.

Persistent, asymptomatic *Ehrlichia canis* infection in a human

An *E. canis* strain was isolated from a seropositive, asymptomatic human in Venezuela (Perez et al. 1996). The patient was seropositive for at least 1 year, and no antibiotic therapy was given before blood was obtained for culture. It is reported that morulae were observed in leukocytes in the peripheral blood, and the patient's laboratory examination was unremarkable with the exception of a relative (50 percent) lymphocytosis. The identity of the isolate was confirmed by comparison with an *E. canis* isolate obtained from seropositive Venezuelan dogs (Unver et al. 2001b).

Anaplasma genus human infections

HUMAN ANAPLASMOSIS (FORMERLY GRANULOCYTIC EHRLICHIOSIS) (*ANAPLASMA PHAGOCYTOPHILUM*)

Human infection by members of the genus *Anaplasma* has so far been limited to only *Anaplasma phagocytophilum*. Unlike *E. chaffeensis*, but similar to *E. ewingii*, *A. phagocytophilum* infects predominantly cells derived from bone marrow myeloid cells, chiefly neutrophils in the blood and perhaps immature myelomonocytic precursors in the bone marrow (Bakken et al. 1994; Bakken and Dumler 2000). Initially, infection of humans was recognized as caused by an 'ehrlichia,' and because of the neutrophil host cells, the disease was called human granulocytic ehrlichiosis (HGE). More than 10 years have passed since the distinction of *A. phagocytophilum* infections from those caused by *E. chaffeensis* in humans; however, many diagnoses are still miscategorized as a result of incomplete diagnostic evaluation based upon flawed serological principles and studies that do not differentiate the serologically cross-reactive species. The situation is even more complex for *A. phagocytophilum* infections because of nomenclature difficulties. For several years, the specific identity of the bacterial cause of HGE was unclear. In 2001, the taxonomy of the HGE agent and closely related species

assigned to the genus *Ehrlichia* was revised to reflect a more accurate genetic phylogeny in the genus *Anaplasma*, where the HGE agent, *Ehrlichia equi*, and *Ehrlichia phagocytophila* were unified under the single species name, *A. phagocytophilum*. Although the name is more accurate, the accomplishments of Stannard, Gribble, and Madigan, who investigated *E. equi*, should not be forgotten (Stannard et al. 1969; Madigan and Gribble 1987). Moreover, the new taxonomic position creates a discrepancy in the disease name that formerly reflected the genus *Ehrlichia*. A more rational and increasingly accepted designation for HGE is human anaplasmosis, as it will be called here.

Human anaplasmosis, as for related infections, is characterized as 'influenza-like,' with a clinical presentation dominated by fever, and, less often, rigors; approximately 65–85 percent have headache, and myalgias are frequent. Gastrointestinal system involvement with nausea, vomiting, diarrhea, or abdominal pain is present in about one-third to one-half of patients, and cough or radiographic evidence of respiratory involvement is observed in about 30 percent (Bakken and Dumler 2000; Aguero-Rosenfeld et al. 1996). About 17 percent also present with some degree of confusion, but objective evidence of CNS infection with CSF pleocytosis is very rare (Bakken and Dumler 2000; Lee et al. 2000). Skin rash is seen also very infrequently, not more often than 10 percent of patients, and in many of these the skin is involved as erythema migrans because of cotransmitted *Borrelia burgdorferi* (Aguero-Rosenfeld et al. 1996). Laboratory findings include leukopenia in 53 percent, thrombocytopenia in 78 percent, and mild to significant elevations in serum transaminase activities in up to 90 percent of infections (Bakken et al. 2001). A majority of patients have either a normal leukocyte or platelet count at presentation, and their nadirs occur after about 5 and 7 days, respectively. During the first week, many patients will also have a left shift, giving the impression of a relative neutrophilia if a careful evaluation is not conducted (Bakken et al. 2001). During the second week, a reactive lymphocytosis may be present as also seen in HME. Despite the frequent (90 percent of patients) mild to moderate elevations in serum aminotransferase levels, hepatic function is usually not impaired. The C-reactive protein is often elevated with anaplasmosis and may assist in differentiation from viral infections (Bakken et al. 1996; Lotric-Furlan et al. 2000).

Human anaplasmosis varies in severity from mild, possibly subclinical, to severe and fatal (Olano and Walker 2002; Bakken and Dumler 2000; Blanco and Oteo 2002). For reasons that have yet to be defined, infections in the upper midwest USA are more severe than in the eastern USA, and these are more severe than observed in Europe. In the USA, more than half of patients require hospitalization, and up to 17 percent are admitted for care into an intensive care unit. The average interval of hospitalization is 6 days. The case fatality rate is <1 percent, but severe complications include opportunistic fungal and viral infections, severe nosocomial infections, acute respiratory distress, toxic or septic shock-like condition, renal failure, rhabdomyolysis, myocarditis, neuropathies, coagulopathy, and hemorrhage. Because *A. phagocytophilum* is transmitted by the bites of *Ixodes* spp. ticks that also harbor a variety of human pathogens, coinfections with such agents as *B. burgdorferi* or *Babesia microti* have been reported (Krause et al. 2002; Moss and Dumler 2003; Nadelman et al. 1997). The concept of enhanced clinical severity of these infections during human anaplasmosis is unproven, although animal models have provided compelling data suggesting this possibility. In theory, European patients in tick-borne encephalitis (TBE) endemic regions could be at risk for concurrent TBE and anaplasmosis (Lotric-Furlan et al. 2000).

Little histopathologic examination has been conducted in tissues from patients with anaplasmosis. Most examinations are performed in post-mortem examinations and are confounded by pathology resulting from opportunistic or nosocomial infections. However, sufficient examination has been conducted to determine that the overall histopathologic changes are similar to those observed with natural and experimental equine and canine infections and are also similar to changes observed with infection by *E. chaffeensis* (Walker and Dumler 1997; Lepidi et al. 2000). The major findings are in mononuclear phagocyte organs, where histopathologic evidence of macrophage activation is present. Such findings include mixed infiltrates with mononuclear phagocytes and lymphocytes, especially in the liver, where small nodular lymphohistiocytic lesions in hepatic lobules may be observed. On occasion, these nodules may also contain a single or several apoptotic cells, presumably hepatocytes, and a few neutrophils. The vast majority of these lesions lack any infected cells (Lepidi et al. 2000). The spleen often has an increase in foamy-appearing macrophages containing phagocytosed material, including erythrocytes, leukocytes, and platelets. The spleen is the organ within which most infected cells can be detected, but these are infrequent and not associated with the hemophagocytic cells (Walker and Dumler 1997). On occasion, lymph nodes may demonstrate paracortical hyperplasia – evidence of a T-lymphocyte mediated immune response. Bone marrow has been examined in only a small number of cases and is usually normocellular (Lepidi et al. 2000). The most dramatic findings in post-mortem examinations is the identification of opportunistic infections, which include fungal (disseminated infection and necrotizing esophagitis caused by *C. albicans*, *C. neoformans* pneumonia, invasive pulmonary aspergillosis) and viral infections (herpes simplex virus necrotizing esophagitis) (Bakken et al. 1996). Two patients with infectious esophagitis exsanguinated from their esophageal ulcers. One patient developed myocarditis of undetermined etiology but

suggestive of a viral infection (Jahangir et al. 1999). Although a higher level of bacteremia is detected with human anaplasmosis than with HME, the quantity of bacteria is still insufficient to explain the leukopenia, thrombocytopenia, and anemia (Walker and Dumler 1997; Lepidi et al. 2000). These features are best explained by the presence of activated and hemophagocytic macrophages, in spite of the paucity of infected cells that are usually detected in tissues. As in HME, this discrepancy suggests an immunopathologic mechanism, and experimental infections in mice confirm this possibility (Martin et al. 2001). Similar to the *E. chaffeensis* pathogenesis working hypothesis, infection with *A. phagocytophilum* leads to dichotomous neutrophil responses. Infected neutrophils are in part activated so that chemokines, but not proinflammatory cytokines, are produced (Klein et al. 2000), and cells show evidence of degranulation and secretion of metalloproteases and other biologically active products (Choi et al. 2003). In contrast, infected neutrophils are unable to (i) bind to endothelial cells owing to a loss of surface selectins (Choi et al. 2003), (ii) transmigrate endothelial barriers to the same degree as infected cells (Park et al. 2003), and (iii) engage in phagocytosis and to generate antimicrobial killing to the same degree as uninfected cells (Woldehiwet 1987; Banerjee et al. 2000). Humans with *A. phagocytophilum* infection develop high blood levels of IFNγ, and peripheral blood mononuclear cells from infected people release proinflammatory cytokines on exposure to *A. phagocytophilum* antigens (Dumler et al. 2000; Kim and Rikihisa 2000). In experimental mouse infections, IFNγ is critical for reduction of *A. phagocytophilum* bacterial load (Akkoyunlu and Fikrig 2000) but is also the major contributor to tissue histopathology and presumably disease manifestations (Martin et al. 2001). Thus, infected cells recruit mononuclear phagocytes and lymphocytes to promote inflammatory responses, but are unable to participate in either control of infectious agents or in regulation of the inflammatory response. As neutrophils are key modulators of inflammatory mediators, this situation allows a poorly regulated proinflammatory response that could account for many of the clinical manifestations and sequelae of human anaplasmosis.

Neorickettsia genus human infections

The only recognized species of *Neorickettsia* to produce clinical disease in humans is *N. sennetsu*. The infection results in a mononucleosis-like illness called sennetsu fever in Japan (Misao and Kobayashi 1955). There is also evidence for *N. sennetsu* infection of humans in Malaysia, but only a very small number of cases have been adequately proven and described altogether. The worldwide distribution of *N. risticii*, a very closely related species, suggests that human infection may occur more broadly as well. Sennetsu fever is relatively mild and no fatalities have been recorded. The pathogenesis in humans is essentially unstudied, but murine models have provided some insight. Clinical manifestations in humans include fever, malaise, and anorexia with lymphadenopathy. The presence of these findings and increased concentrations of peripheral blood atypical lymphocytes lead to clinical confusion with infectious mononucleosis. *N. sennetsu* has been isolated from blood, bone marrow, and lymph nodes of patients (Misao and Kobayashi 1955). Hyuganetsu, which is reported to be caused by the SF (*Stellantschasmus falcatus*) agent *Neorickettsia* species, appears to be clinically indistinguishable from sennetsu fever (Fukuda 1958; Fukuda and Yamamoto 1981); the genetic similarity of the SF agent to *N. sennetsu* argues that this is likely to be correct. In animals infected with either *N. risticii* or *N. helminthoeca*, the clinical manifestations often include severe diarrhea, but this has not been reported with human infections.

Wolbachia-associated human disease

The notion that *Wolbachia* spp. may contribute to human disease derives from the frequent detection of these endosymbionts in the tissues of nematodes that are the cause of filariasis, including river blindness and elephantiasis. These processes are associated with severe inflammation that leads to obstructive and fibrotic manifestations. In fact, such inflammatory reactions are also associated with nematodal death, either spontaneously or with therapy. The recent recognition that much of the inflammatory response is driven by 'endotoxic' activities, including activities related to CD14 and toll-like receptor-4, which derive from the endosymbiont *Wolbachia* spp., suggests that antimicrobial therapy directed at reduction in nematode bacterial burdens would be beneficial (Taylor 2002; Hoerauf et al. 1999). Considerable effort will be required in the future to conduct definitive studies, but the reward, if the hypotheses are validated, is the prevention of millions of deaths each year.

EPIDEMIOLOGY AND NATURAL HISTORY

Ehrlichia chaffeensis and *Ehrlichia ewingii*

As of the end of 2002, the Centers for Disease Control (CDC) had reported 1417 cases of HME in the USA in *Morbidity and Mortality Weekly Reports* since reporting began in 1987. In spite of the relatively low incidence that is calculated based upon these passively collected figures, a retrospective analysis by the CDC has revealed average annual rates of 5.53 cases per million in Arkansas, with the highest incidence of disease in the

adjacent south midwestern states of Missouri and Oklahoma, but also including high rates in Illinois, Kentucky, Indiana, and Tennessee. As for Rocky Mountain spotted fever, the incidence of HME is also high in the southeastern and mid-Atlantic USA, including North Carolina, Virginia, Maryland, and New Jersey. It is likely that the low reported incidence of infection in adjacent states is the result of under-recognition and nonmandatory reporting by the USA. The average age of infected patients is approximately 45 years, and the gender ratio is 2M:1F. However, severe and even fatal infections have been described in children. Between 60 and 80 percent of patients recognize a tick bite prior to onset of symptoms.

The specific distribution of HME results from the association of several factors, including the widening distribution of the tick vector, *Amblyomma americanum*, and its major host, *Odocoileus virginianus*, the white-tailed deer (Childs and Paddock 2003). *A. americanum* is an aggressive host-seeking tick that will feed on many different hosts, including man. All three life stages of *A. americanum*, larvae, nymphs, and adults, can feed on white-tailed deer. The rapid expansion of white-tailed deer populations and geographic range, and the high seroprevalence of infection and evidence of persistent *E. chaffeensis* bacteremia in the absence of any clinical signs make these the most significant reservoir hosts (Ewing et al. 1995). However, the promiscuous feeding behavior of *A. americanum* ensures that many other species may become infected, including canids (domestic dogs, coyotes, wolves, foxes), raccoons, opossums, goats, and potentially *Peromyscus leucopus* (white-footed mice), widening the potential spectrum of reservoir hosts (Childs and Paddock 2003; Paddock and Childs 2003). A potential role for avian species that may serve as hosts for immature *A. americanum* stages needs more investigation. Several factors may account for the recent emergence of HME including the increasing abundance of ticks and white-tailed deer, and the increasing overlap of human and deer habitats in the USA. An important role for dogs must be considered as these animals may become persistently and subclinically infected and are known vehicles for the transport of infected ticks into the domestic environment. Currently, only limited data suggest that *E. chaffeensis* occurs outside of North America, where the only known competent vector (*A. americanum*) exists. However, increasing interest and investigation have yielded compelling data about the presence of *E. chaffeensis* or similar species in ticks or as human infections in Asia, Africa, South America, and Europe (Paddock and Childs 2003). The epidemiological and ecological conditions for *E. ewingii* infection are nearly identical to those of *E. chaffeensis* owing to the shared tick vector, *A. americanum* (Childs and Paddock 2003; Yabsley et al. 2002). A few studies have documented the presence of *E. ewingii* DNA in from 5 to 20 percent of white-tailed deer in the midwest and southeastern USA (Yabsley et al. 2002; Steiert and Gilfoy 2002).

Anaplasma phagocytophilum

As of the end of 2002, the CDC had reported 1 423 cases of human anaplasmosis in the USA since the infection was first recognized in 1990. As for the low incidence of HME based upon similar passive reporting, average annual rates as high as 15.9 and 8.8 cases per million population have been reported for Connecticut and Wisconsin, respectively (McQuiston et al. 1999). An evaluation of incidence based upon active case surveillance has revealed much higher incidence rates; for example, the average annual incidence of infection in Spooner County, Wisconsin, from 1991 through 1996 and around Lyme, Connecticut, in 1998 was 58 cases and 51 cases per 100 000 population, respectively (Bakken et al. 1996; Ijdo et al. 2000). The highest rates of incidence for human anaplasmosis are found in the northern USA, including the northeastern states of Connecticut, New York, Rhode Island, Massachusetts, New Jersey, and Pennsylvania, and in the upper midwest states of Wisconsin and Minnesota, although a small focus of infection has also been described in northern California (Foley et al. 1999; Fritz et al. 1997). Although tick-borne fever of ruminants and related *A. phagocytophilum* infections of horses and dogs have been known in Europe for many years, human infection appears to be mild and often subclinical (Blanco and Oteo 2002). Although ample evidence of human infection based upon serological analyses is present in Europe, and despite the frequent presence of *A. phagocytophilum* in *Ixodes ricinus* ticks that are known to bite and transmit disease agents to humans, only 15 cases of human anaplasmosis have been reported from Europe, and most were identified in Slovenia or Sweden (Blanco and Oteo 2002; Lotric-Furlan et al. 1998; Bjoersdorff et al. 2002). The reasons for the discrepant occurrence of human disease between North America and Europe are unclear, but genetic analyses indicate that differences in the bacteria between these locations may be a fruitful area for investigation (Petrovec et al. 2002; Caturegli et al. 2000; Massung et al. 2000). As for HME, the average age of human anaplasmosis patients is more than 40 years, with twice as many infections in men. Pediatric infections occur and may also be severe (Lepidi et al. 2000; Moss and Dumler 2003; Arnez et al. 2001). Tick bites are documented in about three-fourths of all diagnosed patients.

The distribution of *A. phagocytophilum* infections in humans parallels that of other major *Ixodes*-associated zoonoses such as Lyme disease, babesiosis, and TBE. The *Ixodes persulcatus* group of ticks is distributed widely around the world, and important vectors of human infection include *I. scapularis* in the eastern

USA, *I. pacificus* in the western USA, *I. ricinus* in Europe and western Asia, and *I. persulcatus* in Asia. These ticks characteristically feed on several hosts, with each life stage having a range of preferred hosts. Thus, a variety of potential reservoirs for *A. phagocytophilum* exist, including small mammals such as *P. leucopus* (white-footed mouse) in the eastern USA, *Neotoma fuscipes* (the dusky footed woodrat) in the western USA, *Apodemus* spp. voles in Europe, and a variety of cervids (white-tailed deer, Columbian black-tailed deer, roe deer, red deer) or ruminants (goats, sheep, cattle), and as diverse as bear, canids, and even birds. Currently the best evidence for an animal reservoir exists for small mammals such as mice and voles (Telford et al. 1996; Des Vignes and Fish 1997) and cervids (Belongia et al. 1997; Alberdi et al. 2000; Foley et al. 1998), both of which are frequently infected, sustain prolonged bacteremia, and develop no clinical signs (Stuen et al. 2001; Magnarelli et al. 1999). Owing to a worldwide rapid expansion in deer populations as well as expansions in tick populations that infest deer and small mammals, the emergence of anaplasmosis can be in part understood.

Neorickettsia sennetsu

Tick transmission of *N. sennetsu* was suspected for many years, owing to an erroneous assumption regarding a close relationship to tick-borne rickettsioses and ehrlichioses. However, soon after the identification of *N. sennetsu* in humans, some epidemiological data suggested an association with consumption of red mullet fish. Although unproven for *N. sennetsu*, the related *N. helminthoeca* and the very similar *N. risticii* are both transmitted to mammalian hosts through the consumption of trematode-contaminated materials, such as raw fish, fresh-water snails, or insects with an aquatic stage that harbor *Neorickettsia*-infected trematode cercariae.

SPECIMEN COLLECTION AND DIAGNOSIS

Because of the nonspecific clinical manifestations of human *Ehrlichia* and *Anaplasma* infections, laboratory testing is needed to confirm an etiologic diagnosis. Many techniques have been developed and evaluated, but serological testing remains the most simple and effective approach to diagnosis. Cultivation techniques have improved but are largely limited to major reference and public health laboratories. Unfortunately, serologic methods generally require a comparison of acute and convalescent sera, and culture often requires a week to more than a month before results are obtained. Molecular techniques are becoming increasingly used and are important to establish an early diagnosis at a time when therapeutic decisions are being made. However, neither serology nor isolation allows a diagnosis to be made early enough to affect patient management.

Rapid methods that may suggest a diagnosis should be attempted, such as simple microscopic examination of a Romanowsky-stained blood film. Morulae can be detected in peripheral blood leukocytes of patients with HME or anaplasmosis, but this technique is extremely laborious and is only about 2 percent sensitive for HME and between 25 and 75 percent sensitive for human anaplasmosis (Dumler and Bakken 1995). Many laboratories now offer direct PCR amplification of *E. chaffeensis* or *A. phagocytophilum* nucleic acids from the acute-phase blood of suspected patients. This approach does not identify the closely related *E. ewingii*. Thus, some laboratories have devised 'universal' amplification methods that target regions specific for *Anaplasmataceae* on highly conserved genes, and follow a positive test with a second specific PCR or with sequence analysis of the amplicon from the universal method. Each has advantages and disadvantages: the universal approaches are generally less sensitive than specific amplifications, but can detect a broader range of microbes; a direct amplification approach allows a more rapid identification rather than the universal approach, which requires a second stage of analysis. A significant potential advantage of the universal approach is the ability to recognize new infectious agents that may be related to the species of interest. Sensitivity and specificity of the diagnostic molecular reactions may differ considerably depending upon the target gene selected, the method of DNA preparation, and the quality and integrity of the sample to be tested.

Serological assays

The indirect fluorescent antibody (IFA) procedure remains the most widely used technique for the serodiagnosis of HME and human anaplasmosis, but other techniques are now being assessed, with variable success (Walker 2000; Walls et al. 1999; Aguero-Rosenfeld et al. 2000; Brouqui et al. 2001; Childs et al. 1999; Dawson et al. 1990). Because of the high degree of cross-reactivity between *E. chaffeensis* and *E. ewingii*, and *E. chaffeensis* and *A. phagocytophilum*, methods to improve specificity have been attempted, including protein immunoblots and recombinant protein serology. Enzyme-linked immunosorbent assays (ELISA) are extremely sensitive but have not been well developed for these organisms. Important criteria for selecting appropriate serological tests include the sensitivity, specificity, reproducibility, and predictive value. No careful assessment of the IFA method for testing *E. chaffeensis* antibodies has been conducted, but some analyses suggest a minimum sensitivity of 88 percent for IgG IFA and 86 percent for IgM IFA; specificity is not known, and cross-reactions with *A. phagocytophilum*, *Rickettsia* spp., *C. burnetii*,

Brucella spp., *Borrelia burgdorferi*, Epstein–Barr virus (EBV) infection, and rheumatoid factors are known to occur (Dawson et al. 1990). Much more study has been conducted on these features of IFA serology for *A. phagocytophilum* antibodies (Bakken et al. 2002). Overall, the sensitivity of IFA for human anaplasmosis is reported to be between 82 and 100 percent for IgG IFA and 17 and 37 percent for IgM IFA. The overall specificity also varies from 82 to 100 percent for both IgG and IgM IFA; however, significant cross-reactivity with *E. chaffeensis* confounds specificity and requires that IFA for both *A. phagocytophilum* and *E. chaffeensis* be performed; a four-fold higher titer is suggestive of the serodiagnosis. Unfortunately, a small proportion of patients have antibodies of similar titers for both agents that do not allow a definitive serodiagnosis. Another consideration for serological testing of human anaplasmosis is the frequent coinfection with *Borrelia burgdorferi*. It has still not been resolved whether *A. phagocytophilum* can also elicit a positive serum test for Lyme disease in the absence of *B. burgdorferi* infection. As for all serological tests, there are a number of conditions that have also resulted in false-positive serological results, including Rocky Mountain spotted fever, murine typhus, Q fever, EBV infection, rheumatoid factors, antinuclear antibodies, and antineutrophil cytoplasmic antibodies (ANCA).

Most samples should be tested for both IgG and IgM antibodies, the latter after removal of IgG from an aliquot of serum. While it is advocated that specific diagnostic titers be established by individual laboratories or according to the manufacturer's instructions for kits, the most useful criterion for a definitive serodiagnosis is the demonstration of a four-fold increase in titer between acute and convalescent sera, with a minimum titer of at least 64 or 128. When a single serum is all that is available, a single high titer, usually 128 or greater, provides some level of serologic confidence. While *E. chaffeensis* and *A. phagocytophilum* have only recently been described as human infections and prior serologic assays used surrogate antigens, the use of specific diagnostic antigens is encouraged to minimize false-negative results. Unfortunately, *E. ewingii* has not yet been cultivated in vitro; thus, all serologic tests for this agent are based upon cross-reactions with the related *E. chaffeensis* or *E. canis*. Up to 40 percent of patients with HME and human anaplasmosis have detectable antibodies at presentation, and nearly 100 percent are seropositive within 1 month of onset (Dawson et al. 1990; Bakken et al. 2002).

A number of investigators have assessed recombinant proteins as potential specific serological tools for HME and human anaplasmosis (Lodes et al. 2001; Unver et al. 2001a; Magnarelli et al. 2001; Alleman et al. 2000; Tajima et al. 2000; Ijdo et al. 1999; Yu et al. 1999; Zhi et al. 1998). While these investigations hold some promise, none has proven significantly superior to IFA, and the antigenic variability of the major immunodominant antigens (pfam01617.8, Surface_Ag_2 proteins) confounds selection of a single protein that would be effective broadly.

Factors for consideration in serologic testing include the predictive value of positive and negative tests that are based upon the sensitivity, specificity, and prevalence of the infection in the population to be tested. Unfortunately, the precise prevalence for these infections is not known and is probably significantly underestimated by current national reporting practices. Based upon prevalence data even in highly endemic areas, the positive predictive value conducted in the absence of any additional selection of the population would be unacceptably low, under 1 percent. Thus, all sera obtained for this testing must be carefully selected only from patients with a reasonably high probability of infection. This selection criterion has the effect of increasing the prevalence of infection in the tested population and will significantly improve the positive predictive value. Likewise, a negative test under these circumstances also provides very valuable information.

Molecular diagnostic assays

The real revolution in diagnosis of infections by *E. chaffeensis*, *E. ewingii*, and *A. phagocytophilum* has resulted from the application of PCR amplification of organism-specific nucleotide sequences (Anderson et al. 1992a; Chen et al. 1994a; Buller et al. 1999). The same genes that have been extensively used for phylogenetic and taxonomic purposes are also often used as diagnostic targets. The process of nucleic acid amplification is also highly sensitive, theoretically increasing the starting number of target sequences more that one million times to a level that can be easily detected by routine electrophoresis in agarose gels. The molecular size of the amplified DNA can be compared to a known control for specificity, or a highly specific nucleotide probe can be used in a Southern blot method to further enhance specificity. Methods to improve diagnostic sensitivity also include the selection of targets that are present in multiple copies in the *Ehrlichia* or *Anaplasma* genome.

A variety of gene targets have been applied for diagnosis of HME, including the *E. chaffeensis rrs* (16S rRNA gene) (Anderson et al. 1992b), *groESL* operon (Sumner et al. 1997), *gltA* (citrate synthase) (Inokuma et al. 2002), *p28* multigene family conserved regions (Ohashi et al. 1998), *gp120*, and *nadA* (Carpenter et al. 1999), among others. The advantage of the *p28* family (pfam01617.8, Surface_Ag_2 protein gene) is that 21 distinct copies in the genome possess highly conserved sequences that can be targeted for enhanced analytical sensitivity. However, an important feature of these diagnostic tests is the ability to identify infected individuals

regardless of the analytical sensitivity of the assay applied. For *E. chaffeensis rrs* PCR, the overall sensitivity has been estimated at between 60 and 90 percent (Anderson et al. 1992b; Everett et al. 1994). For *E. ewingii*, little information is available about the sensitivity and specificity of molecular diagnostics (Buller et al. 1999). The limited numbers of human infections have been identified based upon amplification of *rrs* with universal primers followed by sequence analysis. However, specific regions in the *rrs*, as well as sequences of the *groESL* and *p28* genes, have been published and should provide good alternatives for development of highly specific and sensitive assays (Paddock et al. 2001; Sumner et al. 2000; Gusa et al. 2001).

Similarly, the diagnosis of human anaplasmosis is often procured using molecular tests, including targets on *rrs*, *groESL*, *gltA*, *ankA*, and *msp2* (*p44*, HGE-44), among others (Chen et al. 1994a; Edelman and Dumler 1996; Sumner et al. 1997; Inokuma et al. 2002; Walls et al. 2000; Ijdo et al. 1998). As for *E. chaffeensis*, the Msp2 (pfam01617.8, Surface_Ag_2 protein) gene family includes at least 22 and perhaps more than 52 paralogs in the *A. phagocytophilum* genome (Scorpio et al. 2004), a desirable diagnostic target. The *rrs* PCR has been estimated to be at least 60 percent sensitive (Edelman and Dumler 1996; Horowitz et al. 1998); however, PCR based upon the multicopy *ankA* doubled the number of detections among a seropositive cohort, confirming the significant improvement in diagnostic sensitivity with a multicopy gene target (Walls et al. 2000).

Culture-based assays

Both *E. chaffeensis* and *A. phagocytophilum* have been recovered by in vitro cultivation methods from whole blood or isolated leukocytes from infected humans (Dawson et al. 1991; Goodman et al. 1996). *E. ewingii* has still not been cultivated in vitro. As in vitro methods for primary recovery of these agents from patients' blood requires at least several days to several weeks of active incubation in cell culture, these methods are not usually applicable to routine diagnostic laboratories (Horowitz et al. 1998). Regardless, culture methods are a mainstay of bacterial diagnosis, and the recovery of isolates provides a useful reference for investigation of variants and their disease-causing properties. *E. chaffeensis* is ordinarily isolated in cultures of peripheral blood mononuclear cell fractions incubated with the canine histiocyte cell line, DH82. Other cell lines have been used, including THP1, HL-60, U937, and others, but most isolates have been recovered in DH82 cells. Initially, recovery times were expected to require 30 days or longer, but improving experience has reduced that interval to approximately 10–14 days. Cell cultures are examined two or three times weekly by Romanowsky stains, by immunofluorescence using specific antibodies, or by PCR. When visualized by Romanowsky stains or immunofluorescence, the culture can be expanded and stocks prepared for frozen storage or definitive confirmation of species by PCR and sequence analysis of several genes.

A. phagocytophilum can be cultivated from human peripheral blood as well (Goodman et al. 1996; Asanovich et al. 1997). The most frequent cell line used for primary inoculation is HL-60, a human promyelocytic leukemia cell line. Other cells infrequently used include THP1 and KG-1. Recovery times vary but tend to be shorter than for *E. chaffeensis*, with the average culture recovery requiring approximately 7–10 days. One advantage of the HL-60 cell line is that it is maintained as suspensions and is not highly susceptible to the toxic effect of lysed erythrocytes as is the DH82 cell line. Thus, a simple protocol for recovery of *A. phagocytophilum* involves direct inoculation of a small volume of ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood into a culture of HL-60 cells.

SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Physical stability and chemical inactivation

Ehrlichiae are highly sensitive to environmental conditions, as they are unable to survive outside of the host cell for long periods of time. Long-term maintenance is usually achieved by maintaining highly infected cultured cells in $\geqslant$30 percent serum with 10 percent DMSO at -70°C, or preferably in liquid nitrogen. Purification of individual bacteria from host cells poses even greater challenges because of the tenacity with which the bacteria bind to host vacuolar membranes and their associated proteins and nucleic acids. Physical inactivation with ordinary laboratory disinfectants (10 percent bleach, 70 percent ethanol, etc.) or heat inactivation (autoclaving) are highly effective.

Sensitivity to antibiotics

The in vitro antibiotic susceptibility of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *Neorickettsia* sennetsu have been determined (Rikihisa 1991a; Brouqui and Raoult 1992; Klein et al. 1997; Maurin et al. 2003). Doxycycline and rifampin exert bactericidal effects whereas chloramphenicol, erythromycin, cotrimoxazole, penicillin, and gentamicin are not inhibitory. Ciprofloxacin was bacteriostatic for *E. chaffeensis* only at high concentrations but very effective against *E. sennetsu*. Other fluoroquinolones, such as trovofloxacin, may be effective in vitro against *A. phagocytophilum* (Klein et al. 1997), but a mutation in the quinolone-resistance-determining region of *gyrA* (DNA gyrase

gene) in *Ehrlichia* spp. makes these resistant to most or all drugs in this class (Maurin et al. 2001). Similarly, both *Anaplasma* and *Ehrlichia* spp. seem to be highly resistant to even new-generation macrolides. Practical in vivo experience with antibiotics gained during the treatment of a wider range of human and veterinary diseases caused by the ehrlichiae is in agreement with the in vitro data (Dumler and Bakken 1995). Doxycycline is generally effective when given in an early stage of infection. Tetracyclines have also been used to eliminate *W. pipientis* from arthropods to demonstrate their role in various manifestations of cytoplasmic incompatibility (Holden et al. 1993), and trials for reduction of inflammatory sequelae that results from nematodes containing *Wolbachia* in lymphatic filariasis also show promise (Hoerauf et al. 1999). In vivo resistance of *Ehrlichia* and *Anaplasma* spp. to chloramphenicol, coupled with in vitro inefficacy and anecdotal evidence of chloramphenicol failure in patients, suggests it is prudent to use doxycycline rather than chloramphenicol, especially given the potential for fatal outcome.

Treatment

All members of *Anaplasmataceae*, including the three important human pathogens *E. chaffeensis*, *A. phagocytophilum*, and *E. ewingii*, are susceptible to antibiotics. Prompt administration of appropriate therapy is critical to minimize the degree to which host inflammatory and immune mechanisms are driven toward pathologic consequences and severe disease. Thus, doxycycline is the drug of choice for treating HME, *ehrlichiosis ewingii*, and human anaplasmosis, although the empirical success of rifampin in the treatment of human anaplasmosis during pregnancy (Buitrago et al. 1998) also agrees with in vitro susceptibility data. Doxycycline is preferred because of the twice per day dosage and the very high therapeutic window. Although many pediatricians have been reluctant to use doxycycline in children under 8 years of age, the American Academy of Pediatrics now advocates the use of doxycycline to treat ehrlichiosis (and anaplasmosis) (American Academy of Pediatrics 2000). No clinical studies have examined the efficacy of either doxycycline or rifampin, but the potential adverse effects of doxycycline in pregnancy have led to the successful use of rifampin in that situation. Adults should receive 200 mg of doxycycline twice per day, orally, every 6 h. Children should receive 3–4 mg/kg of doxycycline every 24 h in two doses, not to exceed 300 mg per dose. Patients who are unable to take doxycycline orally should receive intravenous treatment. The duration of therapy should extend for at least 3–5 days after defervescence, for a total of not less than 5–7 days, but many prefer to treat for a total of 21 days to provide adequate therapy for the potential of cotransmitted *B. burgdorferi* with *A. phagocytophilum*. Some standard therapies for Lyme disease (amoxicillin or ceftriaxone) are not effective for anaplasmosis or HME. Clinical improvement is rapid in properly treated patients. Most patients with HME or human anaplasmosis report resolution of fever and other clinical manifestations within 48–72 h. Relapse or reinfection has only rarely been reported.

Prevention

Because tick-borne *Anaplasmataceae* infections occur when humans intrude on areas infested with reservoir animals and ticks, the best prevention is avoidance of such areas. Control of ticks, small mammal and rodent populations, and deer populations is probably important for prevention, but studies to extend these findings from Lyme disease have not been conducted. Personal protective measures include clothing treated with permethrin and diethyltoluamide may reduce the frequency of tick bites. Prompt detection and removal of ticks is a useful and cost-effective preventive measure. People who must enter tick-infested areas should wear light-colored clothing to facilitate detection of ticks. Embedded ticks should be removed as soon as possible as a short interval is required prior to transmission from the tick into the host. Ticks may be removed with tweezers by grasping at the skin surface and pulling with slow, steady traction. Careful disinfection of the attachment site with soap and water is also a useful precaution.

TICK TESTING AS A PREDICTOR OF DISEASE

An emerging concept in tick-transmitted infections is the determination of whether an attached tick is infected, and the use of this information to guide in prophylactic therapy. A rational approach requires information about whether prophylactic therapy is useful and the likelihood of transmission occurring from an infected tick. The latter concept also requires knowledge regarding the interval of tick attachment needed prior to transmission of *Ehrlichia* or *Anaplasma* spp. Experimental transmission of *E. chaffeensis* by infected *A. americanum* ticks has been conducted, but data regarding the 'grace period' have not been generated. Although *A. americanum* is also the suspected vector of *E. ewingii*, experimental and timed transmission studies have not been conducted. Transmission of *A. phagocytophilum* from infected ticks to mice begins within 24 h, when as little as 30 percent to as many as 67 percent of mice acquire infection (Hodzic et al. 1998; des Vignes et al. 2001). Thus, unlike the situation for *B. burgdorferi*, daily tick checks and removal may not be adequate to prevent transmission. As the majority of these studies were conducted by cofeeding multiple ticks on a single susceptible host, the proportion of ticks capable of trans-

mitting *A. phagocytophilum* is not known. In fact, the US military maintains a facility for testing ticks removed after attachment to soldiers. The prevalence of *E. chaffeensis* in attached *A. americanum* is 15 percent, and of *A. phagocytophilum* in *I. scapularis* is 2 percent (Stromdahl et al. 2001); regardless, both rates far exceed the reported numbers of cases of either HME or human anaplasmosis in the US military services, suggesting that this approach poorly correlates with subsequent disease. Moreover, few data are available regarding the prophylactic efficacy of treatment during the incubation period of HME and human anaplasmosis, although a single patient who received prophylactic doxycycline after an *A. americanum* bite seroconverted to *E. chaffeensis* and never became ill. Currently, too little information is available to make specific recommendations regarding the feasibility of tick testing to guide decisions about antibiotic prophylaxis.

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Coxiella burnetii

NIGEL F. LIGHTFOOT AND GRAHAM LLOYD

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INTRODUCTION

Coxiellia burnetii is an intracellular, gram-negative acidophile bacterium that replicates in high numbers within the phagolysosome of its eukaryotic host and is the etiological agent of Q fever. An Australian physician, Edward Derrick, described a febrile illness amongst abattoir workers in 1935 as 'query (Q) fever' (Derrick 1937). Derrick's initial attempts to isolate the causative agent proved unsuccessful. However, clinical material obtained from ill workers in a meat-packing plant in Brisbane, Australia reproduced the disease in laboratory guinea pigs. Infected guinea pig liver sent to MacFarlane Burnet, confirmed the disease in laboratory animals. Impression smears of infected mouse spleen revealed large intracellular vacuoles containing small rod-shaped organisms that Burnet and Freeman characterized as rickettsia. At the same time, in Hamilton, Montana, an unexpected febrile illness was also produced in guinea pigs during a Rickettsia spotted fever study. The causative agent was referred to as 'Nine Mile agent' and later named *Rickettsia diaporica* because, unlike other rickettsial agents, it was filterable (Davis and Cox 1938). All attempts to grow the agent on axenic media failed, but during these studies a febrile illness displayed, through a laboratory-derived infection, a confirmed link between Q fever and Nine Mile agent. Blood from one of the patients produced a similar febrile illness in guinea pigs. Davies and Cox demonstrated that convalescent guinea pigs previously infected with Burnet's spleen sample were immune to challenge by the Nine Mile agent, thus demonstrating antigenic crossreactivity between the two agents. In 1938, the causative agent was assigned a new genus and species name, *Coxiella burnetii*, in recognition of the work by Cox and Burnet (Weiss 1992).

CLASSIFICATION

In the past, the taxonomy of coxiella was closely linked to the rickettsiae and was principally based on comparisons of phenotypic characteristics. More so than in other bacteria, historical and environmental considerations also played important roles in classification. In the eighth edition of *Topley & Wilson*, the principal genera discussed were *Rickettsia*, *Rochalimaea*, and *Coxiella*. More recently, classification of rickettsiae and other bacteria have been increasingly based on DNA sequences, particularly on 16S and 23S rDNA sequence comparisons. The genera *Rickettsia* and *Ehrlichia* belong in distinct branches of the α subdivision of Proteobacteria (Roux and Raoult 1995; Stothard and Fuerst 1995), whereas *Rochalimaea* belong in the α_2 subdivision and are most closely related to the agrobacteria and rhizobacteria. Recent phylogenetic analysis of the 16S rRNA sequences has resulted in the reclassification of

C. burnetii to the γ subgroup of Proteobacteria, thus establishing it being closely related to *Legionella pneumophila* and *Wolbachia persica* (Weisburg et al. 1989). While *Rickettsia* species replicate in the host cytoplasm, *C. burnetii* displays both phenotypic and genetic similarities to *L. pneumophila*, including intracellular growth in a membrane-bound vacuole and sequence similarities for a variety of genes (Mo et al. 1995).

HABITAT

Coxiella burnetii is a category B bioterrorism agent that is highly infective to both humans and livestock (Martin and Raoult 1999). The agent is widespread in the natural environment due, in part, to its high degree of stability under conditions unfavorable for growth and to its transmission from animal to animal and to humans, primarily by aerosol (Raoult and Marrie 1995). It has been reported from virtually every part of the world. A tick host (*Dermacentor andersonii*) has been implicated to play an important role in natural infection, but many vertebrate animals are also heavily infected and play a role in transmission. *C. burnetii* is often an occupational disease in individuals working in slaughterhouses. The bacterium is particularly abundant in the placenta of a parturient animal. Parturient cats in a household can also cause infection.

MICROENVIRONMENT AND MORPHOLOGY

C. burnetii is a unique bacterium that combines adaptation to the acid environment of the phagolysosome with a developmental cycle that includes transverse binary fission and sporogenesis. The developmental cycle is described in detail by McCaul and Williams (1981). In addition to the schematic representation of the cycle, the great variety of forms in a purified preparation and a cell undergoing cell division and containing a spore is shown. The usual smears of *C. burnetii* infected cells display a great variety of forms; the cells which have not yet divided are usually small rods (approximately 0.3 by 1.0 μm) which double in size or develop a spore at one end or both. The smears also contain spores in various stages of development. In contrast to other rickettsiae, coxiellae have some gram-positive surface components but also, on repeated cultivation in the laboratory, may display lipopolysaccharide (LPS) phase variation. Phase I cells are infectious for animals, while phase II cells usually appear upon repeated cultivation in the yolk sac of chicken embryos. Phase II cells do not have the full complement of properties required for survival in macrophages. The adaptation to an acid environment is demonstrated through detecting many metabolic activities only when the pH is 5 or lower (Redd and Thompson 1995).

LABORATORY ISOLATION, CULTURAL CHARACTERISTICS, AND GROWTH REQUIREMENTS

The yolk sacs of embryonated chicken embryos have proved a good medium for the isolation of Q fever. Eggs are inoculated when the embryos are 5–7 days old. The top of the egg above the air sac is surface-sterilized and a hole is punched aseptically. A 0.2–0.4 ml inoculum is delivered with a 22-gauge needle long enough (2–3 cm) to penetrate into the yolk sac. The *Coxiella* inoculum is adjusted to kill 30 percent of the embryos in 5–8 days. Suspending fluids for such harvests are brain–heart infusion (BHI) broth or sucrose phosphate glutamate (SPG) solution, consisting of 0.22 M sucrose, 0.1 M potassium phosphate, 5 mM potassium glutamate, pH 7.0. The pools of yolk sacs and their suspending medium are homogenized in bottles containing 5 mM glass beads, dispensed in vials and used as inocula or purified for use in physiological or antigenic studies. For further details of these procedures, see Dasch and Weiss (1992).

Cell cultures offer many opportunities for rapid isolation and identification of coxiella from infected animals or patients. The shell vial culture technique has the advantage that it concentrates a moderate volume of inoculum over a small monolayer of highly susceptible cells on a coverslip. It enhances the infection by centrifugation (usually at 700 *g* for 1 h) prior to incubation. After centrifugation, the supernatant is removed and replaced by an appropriate medium, such as Eagle's minimal essential medium (MEM) containing 10 percent fetal bovine serum. If several vials are treated with the same inoculum, a duplicate coverslip can be examined a few days after inoculation and the rickettsiae identified by the indirect fluorescent antibody method. After a satisfactory level of infection is reached, the remaining vials can be used for passage of the coxiella into tissue culture flasks for the production of larger volumes. This method has been used to identify *C. burnetii* from human blood in cases of acute disease and suspected chronic endocarditis (Musso and Raoult 1995).

Isolation of *Coxiella* by plaque formation is usually undertaken on monolayers of chicken embryo fibroblasts but can also be done on L929 or Vero cells (Figure 1). The tissues are obtained from decapitated 10-day-old chicken embryos, thoroughly minced in Earle's balanced salt solution (EBSS), and the cells are further separated from each other by digestion with 0.25 percent trypsin for 20 min at 37°C. Trypsinization is terminated by the addition of fetal bovine serum. Clumps are removed by filtration over sterile cheesecloth and the isolated cells are sedimented by centrifugation and resuspended in MEM containing 10 percent fetal bovine serum. The suspension is dispensed into 25-cm^2 tissue culture flasks, 5 ml per flask, and confluent monolayers are allowed to form by incubation overnight at 37°C. The samples to be

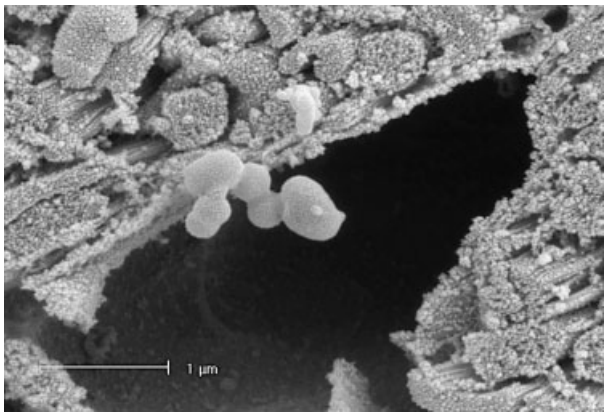

Figure 1 *Scanning electron micrograph of* C. burnetii *cells with dewaxed sections of guinea pig heart 9 days after aerosol infection. Courtesy of B. Dowestt, S. Lever and G. Lloyd, HPA Porton, UK*

plaqued are diluted serially in BHI and, after the supernatant fluid has been removed, 0.1 ml volumes are added to the monolayers. Plaquing efficiency is increased by gentle rocking of the flasks for 60 min or by moderate centrifugation of the inoculum over the cells. Finally, the cultures are overlaid with an appropriate medium (such as RPMI 1640 or Medium 199 containing glutamine and 0.8 percent agarose). Spotted Fever Group rickettsial plaques are recognized 4–7 days post-inoculation and are 2–3 mm in diameter. Plaque isolation is particularly valuable for the isolation of phase I and phase II variant forms of *C. burnetii*.

For the mass cultivation of coxiellae, irradiated mouse L929 or Vero cells are often used in large flasks. When growth is sufficiently abundant, the cells are harvested with glass beads. The suspension is then centrifuged and the pellet resuspended in SPG for freezing or in BHI for further propagation. For additional information, see Dasch and Weiss (1992) and Williams et al. (1992a)

MORPHOLOGY – GENOME AND PLASMIDS

For many years, *C. burnetii* was thought to be relatively homogeneous, except for antigenic phase variation, and the same organism was believed to cause both acute and chronic disease. Recent genetic studies have shown that *Coxiella* is not homogeneous, but it appears that the same genotype can cause both types of disease. *Coxiella* isolates exhibited 14 distinct restriction fragment length polymorphism (RLPF) patterns of genomic DNA by pulsed field gel electrophoresis (PFGE), giving a genomic size of 1.4–1.9 Mb, in good agreement with the 1.7 Mb estimated by reassociation kinetics (Thiele et al. 1993; Thiele and Willems 1994). The DNA base composition of *Coxiella* is 42 percent G + C. Plasmids have been discovered in *C. burnetii*. Initially, six different genomic groups were distinguished among American isolates by the type of plasmid and RFLP pattern obtained using *Eco*RI or *Bam*HI digested total DNA separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver stained. A 36 kb plasmid, QpH1, was present in three genomic groups with isolates from ticks or animals or causing acute illness in humans, the 39 kb plasmid, QpRS, was present in an isolate causing chronic disease, and the 51 kb plasmid, QpDG, was present in feral rodent isolates of unknown pathogenicity. Another chronic disease isolate group contained both QpRS and QpH1 related plasmid sequences integrated into the chromosome. No unique sequences common to the plasmidless isolates and QpRS have been found that might cause 'chronic' disease. About 30 kb of core plasmid sequence is conserved in all genomic groups, whereas the remainder is plasmid specific. Isolates varying only in phase state or passage history exhibit minimal differences. These plasmid sequences are not known to encode virulence factors but their conservation in all genetic groups suggests that they are essential to the survival of *C. burnetii*. European isolates were also found to contain QpH1 sequences whereas a Namibian isolate contained QpRS sequences. More recently, the association of specific plasmid type with pathogenicity has been questioned as only nine of 30 European isolates, including both acute and chronic isolates, had sequences specific to QpH1 (Stein and Raoult 1993; Thiele and Willems 1994). Similarly, a new 33.5 kb plasmid, QpDV, found in isolates from both acute and chronic disease, has been described (Valkova and Kazar 1995). Both the 90–95 percent DNA–DNA homologies exhibited between different genomic groups and the greater than 99 percent 16S rDNA sequence similarity in different isolates of *C. burnetii* argue for their retention as a single species (Hendrix et al. 1991; Stein et al. 1993).

Thirty-seven open reading frames (ORF) have been identified in QpH1 (Thiele and Willems 1994). ORF cbhE′ encoded a 39.4 kDa hydrophilic protein unique to QpH1. ORFs related to *Pseudomonas* proteins, ferripyochelin-binding protein (FBP), 32.4 kDa protein, and replication proteins, to *Escherichia* DNA helicase I and SopA protein, to *Agrobacterium* replication protein RepB and to bacteriophage λ tail assembly protein K were recognized. FBP plays a role in iron acquisition and may be a virulence determinant in *Pseudomonas*. QpH1 proteins with homology to the F plasmid partition proteins SopA and SopB were found to be also conserved in the plasmids QpRS, QpDV, and QpDG, but absent in plasmidless isolates (Lin et al. 1995). The QpRS-specific gene *cbbE′* was not found in plasmidless isolates and encoded a 55 kDa protein which was a surface-exposed outer-membrane protein of unknown function.

The rRNA operon of *C. burnetii* is organized in the order 16S–23S–5S rDNA (Afseth et al. 1995). The 16S–23S rDNA spacer is *Coxiella* specific and encodes tRNA

genes for isoleucine and alanine. The 23S rDNA contains an unusual 444 bp intervening sequence which has a stem-loop structure due to flanking 28 bp inverted repeats. The absence of intact mature 23S rRNA suggests the loop is excised. Approximately 19 copies of a 1450 bp repetitive element were found in *C. burnetii*, including one associated with the *groESL* heat shock protein operon (Hoover et al. 1992). The element had terminal seven and internal 12 bp inverted repeats at each end and encoded an ORF for a 367 amino acid basic polypeptide with a DNA binding helix-turn-helix and a leucine zipper motif, suggesting it may be a transposase. Ten French isolates with eight different PFGE patterns each contained this repetitive element (Thiele and Willems 1994). Three independent copies of this element each had additional nonhomologous internal 85–93 bp inserts with strong dyad symmetry demarked by identical 7 bp inverted repeats (Hoover et al. 1992). The *gltA-sdh* intergenic region was found to contain three similar, nearly perfect, inverted repeats (Heinzen et al. 1995). A probe to the terminal 7 bp inverted repeat sequence hybridized to multiple sequences in four different genomic groups. Whether the inverted repeat sequences have important regulatory functions is unknown.

Although *C. burnetii* is rather unique in its life-style, it retains many genetic functions that are organized similarly to those of other γ Proteobacteria such as *Escherichia coli*; this permits their cloning and identification by functional complementation of mutants and by sequence homology. Characterized genomic regions include a TCA cycle gene cluster similar in arrangement to that of *E. coli* (Heinzen et al. 1995) and the aspartate carbamylase catalytic subunit. The *sdhCDAB* gene cluster encodes succinate dehydrogenase enzyme complex and is adjoined by the 2-oxoglutarate dehydrogenase and citrate synthase genes. Other characterized genes express iron containing superoxide dismutase, homologs of *E. coli* heat shock GroES and GroEL proteins, the heat shock protein DnaJ, and the *mucZ* gene product which has some homology to *dnaJ* and activates capsule synthesis in *E. coli*. An autonomous replication sequence was characterized that contains ORFs with homology to genes often found near bacterial chromosomal origins, including *rpmH*, *rnpA*, 9 kDa, and 60 kDa membrane proteins. Some information has been obtained for genes encoding a protein similar to the *Legionella* macrophage infectivity potentiator (Mip) protein (Mo et al. 1995), a protein with homology to bacterial sensor proteins found in two-component regulatory systems (Mo and Mallavia 1994), and a 27 kDa surface-exposed loosely associated outer-membrane protein Com1 (Hendrix et al. 1993). The *rnc* locus of *C. burnetii* contained genes with protein homology to RNAase III, RecO, and Era of *E. coli*; *rnc* suppressed capsule synthesis in *E. coli* even though *C. burnetii* does not contain a capsule. Era is a 35 kDa GTP binding protein that is essential for cell viability. RNAase III is a double-stranded RNA-dependent endoribonuclease. RecO function is involved in DNA recombination and repair.

STRUCTURE–CELL WALL COMPOSITION AND ANTIGENIC PROPERTIES

Consistent with the phylogenetic distance of *Coxiella* from the other rickettsiae, it is antigenically distinct from them. Although the gram-negative cell wall structure and composition of *C. burnetii* have been studied intensively (Williams and Waag 1991), an accurate inventory of the cell wall components and their function is difficult because of the distinct morphological forms present in its complex developmental cycle, the unique virulence-associated LPS phase variation, the difficult physical solubility properties of individual components in the cell envelope, and the range of analytical methods used in cell fractionation. The structure of the LPS is not completely known, but phase I and II differences in chemical composition and size are compatible with a smooth to rough transition where the O side polysaccharide undergoes significant truncation while the core region which contains KDO and lipid A is retained. The neutral sugars virenose and dihydrohydroxystreptose are the major antigenic sugars in the O side chain but they are not unique to *Coxiella*. The branched chain fatty acid composition of the LPS is unique to *Coxiella* although other pathogenic bacteria share some components. Phase I LPS is relatively nontoxic compared with *Salmonella* LPS.

Formalin-treated phase I whole cell vaccines have been widely used in animals and are effective in man, while phase II whole cells are considerably less effective (Ackland et al. 1994). Unfortunately, the phase I whole cell vaccines occasionally induce erythema, induration, granulomas, and sterile abscesses in some individuals, and such severe local and systemic reactions in immune individuals that they must be prescreened by skin test. Consequently, a chloroform–methanol residue (CMR) vaccine was developed which retained good efficacy for animals and man and fewer adverse reactions (Williams et al. 1992b; Fries et al. 1993). This vaccine retains the LPS and the majority of cellular proteins, but is greatly reduced in phospholipids. Trichloroacetic acid extracts containing phase I LPS and purified phase I LPS were protective in mice but not phase II LPS or polysaccharide or lipid A fractions of phase I or II LPS (Gajdosova et al. 1994). Phase I cells contain both immunosuppressive and immunostimulatory molecules that are in part disassociated by chloroform–methanol extraction (Williams and Waag 1991; Tokarevich et al. 1993). Both tumor regression and antiviral activity of phase I and CMR fraction have been observed (Zvilich et al. 1995).

Various protein antigens have been identified by radioimmunoprecipitation of surface radioiodinated proteins and immunoblotting (Williams and Waag 1991). The best characterized is a 29 kDa protein P1 present in phase I and II cells, but not in the small dense cell type or endogenous spore (McCaul et al. 1991). It is both immunogenic and protective for animals (Gajdosova et al. 1994; Lukacova et al. 1994). The 29 kDa protein P1 may be identical to the cloned 27 kDa Com1 protein, or several antigenic proteins of similar size are present since another 29 kDa lipoprotein, P2, has also been described. Zhang et al. (1994) also found a 67 kDa outer-membrane protein which was protective in guinea pigs and mice. The conserved 62 kDa protein of *Coxiella* is homologous to the widely conserved chaperonin 60 heat shock protein GroEL. Its expression is regulated by heat in *E. coli* and by heat or 10 percent ethanol in acid-activated *Coxiella*. It is present on the surface of phase II but not phase I cells. The 62 kDa protein is immunogenic since it is recognized by sera from acute and chronic Q fever patients, but it is probably not protective. Whether combinations of purified LPS and antigenic protein or peptide components will offer advantages over CMR or whole cell vaccines is unresolved.

TRANSMISSION, PATHOGENESIS, AND CLINICAL SYMPTOMS

Transmission

Q fever is usually transmitted by the inhalation of small particle aerosols that originate from infected animals. Q fever is enzootic among domestic animals, especially sheep, cattle, and goats. Infected cats have also been reported as sources of infection (Marrie 1988). *C. burnetii* is found in high concentrations within the placenta of infected females and is shed at parturition. *C. burnetii* is extremely stable under environmental conditions, is highly infectious, can be broadly disseminated by dust, dried manure, or other materials, and presents an infectious hazard in areas where animal husbandry is practised. Diseases occur sporadically, but outbreaks are common, particularly among abattoir workers. Outbreaks of Q fever have also occurred in research facilities where sheep are used as experimental animals (Meikeljohn et al. 1981). Consumption of raw milk from infected animals has also been responsible for a number of human infections (Fishbein and Raoult 1992).

The incubation period varies from 9 to 28 days, averaging 18 to 21 days and is dependent on the number of organisms that initially infect the patient. Onset is abrupt with fever, severe headache, chills, severe malaise, myalgia, confusion, sore throat, chills, and, often, chest pains. Fever may rise to 40°C (104°F) and may persist for 1 to >3 weeks. A nonproductive cough and X-ray evidence of pneumonitis often develop during the second week of illness in 30 to 50 percent of symptomatic infection. In severe cases, lobar consolidation usually occurs, and the gross appearance of the lung may resemble that of bacterial pneumonia. Histological changes in Q fever pneumonia resemble those of psittacosis and some viral pneumonia as demonstrated by an intense interstitial infiltrate around the bronchioles and blood vessels which extends into the adjacent alveolar. The bronchial luminar may also show extensive invasive polymorphonuclear leukocytes. Approximately a third of patients with protracted Q fever develop hepatitis, characterized by fever, malaise, hepatomegaly with right upper abdominal pain, and possibly jaundice. Liver biopsy specimens show diffuse granulomatous changes, and *C. burnetii* may be identified by immunofluorescence. Headache or respiratory signs are frequently absent. Lobar pneumonia may be particularly severe in aged or debilitated patients. In general, most patients will recover to good health within a few months without treatment. Approximately 1–2 percent patients who contract Q fever may die.

There are also several forms of chronic Q fever with chronic hepatitis and endocarditis. Chronic Q fever is characterized by an infection that lasts longer than 6 months. Patients who have had acute Q fever may develop the chronic form as soon as 1 year, or as long as 20 years, after initial infection. A serious complication of Q fever is endocarditis, generally involving the aortic heart valve and less commonly the mitral valve (Figure 2). Most people who develop chronic Q fever have a pre-existing valvular heart disease or have a history of vascular graft. Transplant patients, cancer suffers, and those with kidney disease are also at risk of developing chronic Q fever. As many as 65 percent of persons with chronic Q fever may die of the disease.

Those patients who fully recover from infection may possess lifelong immunity against reinfection.

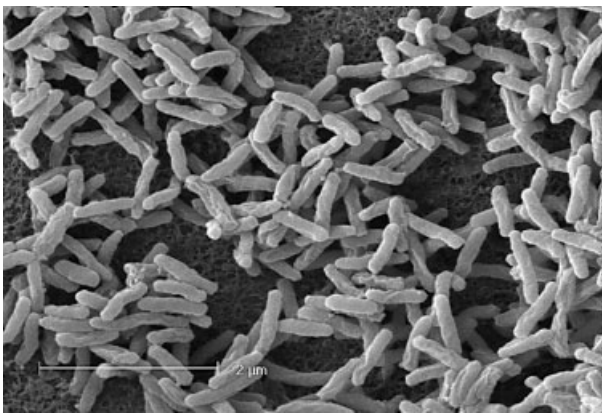

Figure 2 *Scanning electron micrograph of* C. burnetii *cells grown in Vero Cells. Courtesy of B. Dowestt, S. Lever and G. Lloyd, HPA Porton, UK*

Pathogenesis

C. burnetii parasitizes the acidified phagolysosomal compartment of its host cells (Baca et al. 1994). Both catabolic and anabolic activity, including the synthesis of DNA and protein, can be measured in defined medium at acidic pH (Thompson 1991; Redd and Thompson 1995). Following acid activation 34, 24, and 12 kDa proteins are exported by *Coxiella* but their function is unknown. The *Coxiella* 24 kDa Mip protein possesses peptidyl-prolyl-*cis-trans*-isomerase activity and is an exported protein, suggesting that it has a role in protein folding and trafficking (Mo et al. 1995). Whether Mip is one of the exported acid activation proteins is unknown. In *Legionella* and *Chlamydia*, Mip proteins have been found to have roles in establishing infection of host cells. Phase II cells more readily attach to L cells than phase I organisms and while purified phase I LPS did not inhibit attachment of *Coxiella* to host cells, protease treatment of host cells abolished attachment (Baca et al. 1993). Heat- or formaldehyde-inactivated *Coxiella* could attach and enter cells whereas inhibitors of host cell phagocytosis (such as NaF and cytochalasins B and D) abolished rickettsial uptake. This suggests *Coxiella* is passively phagocytosed. *C. burnetii* also binds C-reactive protein, complement components, and normal immunoglobulin which may facilitate cell infection in vivo (Williams et al. 1989). *Coxiella* adhesins or proteins essential for survival in the phagolysosome have not been identified conclusively, although roles for superoxide dismutase, catalase, and acid phosphatase have been suggested (Baca et al. 1994). The acid phosphatase activity blocks superoxide anion production by stimulated human neutrophils. In *Salmonella*, acid phosphatase encoding genes are regulated by *phoQ* and *phoP* genes which enhance survival in macrophages. A similar sensor-like protein has been described in *Coxiella* (Mo and Mallavia 1994). Phase II cells are sensitive to complement-mediated killing while phase I cells are not, suggesting that phase I LPS mediates resistance. Phase I LPS may mask underlying antigens from exposure to the immune system since ^{125}I-labelled antiphase II antibodies bound 10- and 44-fold more to trichloroacetic acid-extracted phase I cells and to phase II cells, respectively, than to native phase I cells (Hackstadt 1988), thus explaining, in part, the slow phase I serological response in *Coxiella* infection. LPS is an important virulence factor since intrastrain LPS phase I, intermediate, and phase II variants differ significantly in virulence in guinea pig and mouse models (Kazar et al. 1993).

LABORATORY DIAGNOSIS

There are significant risks associated with direct isolation of *C. burnetii* and any attempts should be undertaken under biosafety level three laboratory containment. Direct isolation of *C. burnetii* is successfully achieved through the inoculation of cell monolayers of Vero cells or J774.16 mouse macrophages cells in shell vials (Raoult et al. 1991; Spyridaki et al. 2002). The direct identification of *C. burnetii* is most easily accomplished by direct immunofluorescent antibody staining of organisms in biopsies or after isolation (Musso and Raoult 1995). A monoclonal antibody suitable for this purpose has been described. Alternatively, polymerase chain reaction (PCR), using primers specific for superoxide dismutase, a conserved plasmid sequence, or a large conserved repetitive element (Thiele and Willems 1994; Spyridaki et al. 2002) may be done. Chromosomal DNA and plasmid-based typing has been used extensively to differentiate isolates. Protein profiles and Western blotting of proteins have not been very useful for typing strains, although distinct LPS profiles may be obtained, particularly among phase variants (Kovacova et al. 1994). Monoclonal and polyclonal sera can be used for distinguishing LPS variants (Yu and Raoult 1994).

Most diagnoses of Q fever infection are based on a variety of retrospective serological procedures based on indirect immunofluorescence employing phase I and phase II antigens (Uhaa et al. 1994; Waag et al. 1995; Hamzie et al. 2003). The responses to the different phase I and II antigens is important in establishing a diagnosis and reflects the different clinical presentations of the disease. In acute cases of Q fever, the presence of IgM and IgG phase II antibodies is generally detected during the second week of illness. Evidence of chronic Q fever is demonstrated by the presence of high levels of IgG anti-coxiella phase I antibodies. These antibodies require longer to appear and indicate continued exposure to the bacteria. Increased levels of phase 1 IgG and IgA antibodies indicate the possibility of Q endocarditis (Camacho et al. 1998). More recently, the value of molecular techniques has been evaluated and data indicate that the use of PCR techniques could be of value during the first 2 weeks of the disease and not used 4 weeks post onset, when the serology is highly sensitive. (Fournier and Raoult 2003). This has been extended to the early detection of clinically and histologically silent Q fever endocarditis accidentally diagnosed by PCR targeting eubacterial 16S rDNA on the mitral valve removed after surgery (Issartel et al. 2002).

TREATMENT – SENSITIVITY TO ANTIBIOTICS

Since *C. burnetii* does not multiply in axenic media, animals, embryonated chicken eggs, and cell culture methods have been used for testing antibiotic susceptibility. All of these methods suggest that *C. burnetii* is more resistant to antibiotics like chloramphenicol, erythromycin, and streptomycin. Egg survival and suppression of illness in animals are less useful than growth in tissue culture for gauging whether antibiotics

are bactericidal rather than bacteriostatic. In eggs, clindamycin, erythromycin, viomycin, cycloserine, and cephalothin were ineffective, while rifampicin, trimethoprim, doxycycline, and oxytetracycline were bacteriostatic. Chlorotetracycline-resistant isolates of *C. burnetii* have been isolated by serial passage in eggs in the presence of low concentrations of antibiotic; one Cyprus isolate with enhanced natural resistance to tetracyclines was found.

Suppression of primary shell vial growth in HEL cells of 13 *C. burnetii* isolates was used to evaluate bacteriostatic properties of antibiotics (Raoult 1993). Amikacin and amoxycillin were never effective. Co-trimoxazole, rifampicin, doxycycline, tetracycline, minocycline, sparfloxacin, and quinolones PD127,391 and PD131,628 were always effective. Variable resistance was noted with ofloxacin, pefloxacin, chloramphenicol, ceftriaxone, fusidic acid, and erythromycin. The fluoroquinolones ciprofloxacin, pefloxacin, and fleroxacin were bacteriostatic in eggs and Vero cells (Keren et al. 1994). Assays employing persistently infected dividing L929 cells initially suggested that rifampicin and the quinolones pefloxacin, ofloxacin, and ciprofloxacin were bactericidal but that doxycycline was not. However, this could not be confirmed for any of these antibiotics when cell growth was inhibited by cycloheximide. Using this assay or direct enumeration of viable *Coxiella* exposed to antibiotic for 24 h after infection in P388D1 macrophage cells, the lysosomotropic agents amantadine, chloroquine, or ammonium chloride were used to alkalinize the pH 4.8 *C. burnetii*-containing phagolysosome to pH 5.3, 5.7, or 6.8 (Maurin et al. 1992). Pefloxacin and doxycycline were bactericidal at the more alkaline pH whereas rifampicin was not. Clarithromycin, a macrolide erythromycin derivative that can be tolerated during childhood and pregnancy, exhibited good bacteriostatic activity against four genomic types but it was not tested in the presence of lysosomotropic agents for bactericidal activity (Maurin and Raoult 1993).

Since acute Q fever is often self-limiting, bacteriostatic antibiotics are often effective. A dose of 100 mg of doxycycline taken twice daily for 15–21 days is a frequently prescribed therapy. Chronic Q fever can be managed with bacteriostatic antibiotics but the patient may not be cured unless a bactericidal regimen is employed. Although differential resistance of isolates from chronic disease patients has been noted, this may be due to changes in host cell (tetracyclines) or isolate-specific permeabilities (fluoroquinolones) (Yeaman and Baca 1991). Two different treatment protocols have been successfully evaluated: (1) doxycyline in combination with quinolines for at least 4 years; and (2) doxycycline combination with hydroxychloroquine for 1.5–3 years. The second therapy leads to fewer relapses, but requires routine eye examinations to detect accumulation of chloroquine. Surgery to remove damaged valves may be required for some cases of *C. burnetii* endocarditis. Cotrimoxazole treatment was found to be effective during pregnancy preventing abortion although it did not significantly reduce colonization of the placenta (Raoult et al. 2002).

EPIDEMIOLOGY AND CONTROL

For Q fever, the only known important natural infection mechanism in man is by the inhalation route and human to human transmission has only occurred rarely. Experiments carried out in the 1950s examined the infectious doses by the inhalation route in guinea pigs and man (Tigertt and Benenson 1956; Tigertt 1959) and determined that one organism could initiate infection in the guinea pig and that the infectious dose in man was ten organisms. The incubation period at this level was 17 days and decreased to 10 days with an infecting dose of 10^5 organisms. The agent used was *C. burnetii* AD strain which had been isolated from milk in California. It was found possible to suppress completely any clinical disease in man exposed by the inhalation route with oxytetracyline administered during the incubation period.

C. burnetii is maintained as a zoonotic infection through an extensive reservoir in mammal, bird, and arthropod species. Wild rodents appear to be a significant reservoir and household pets, such as cats, can transmit infections during parturition. Cattle, sheep, and goats are often infected without clinical disease and *C. burnetii* are shed in their feces, milk, and birth products. Workers involved in animal rearing, dairy farming, or animal slaughter therefore have a significant risk (Woldehiwet 2004). Outbreaks have normally resulted from occupational exposure involving veterinarians, abattoir workers, sheep and dairy workers, livestock farmers, and researchers (Marmion and Stoker 1958; Thomas et al. 1995).

Some outbreaks have been extensive and less easily explained and thought to be due to the survival of the organism in the environment, airborne spread, and very low infective dose. Five cases of clinical Q fever were identified in schoolchildren exposed to goats and raw goats milk, and further investigations in 461 pupils revealed 80 who had past evidence of infection (Jorm et al. 1990). The largest outbreak of Q fever in the UK occurred in Birmingham in 1989, 147 cases were identified but none had direct animal contact. Investigations revealed the probable source of the outbreak to be airborne spread of organisms from farmland during gale force winds (Hawker et al. 1998). Other outbreaks have been associated with deployed troops in Bosnia and windborne spread probably from a sheep farm by helicopter down draught air disturbance (Splino et al. 2003). Intriguingly, office renovation and the repeated drilling of contaminated straw-containing boards has been proposed as the source of 95 cases of acute Q fever in an office setting in South Wales (Von Woerden et al. 2004).

A number of measures has been initiated post outbreak or are routinely put in place in endemic areas which range from educating the public to vaccinating research or abattoir workers.

Control

C. burnetii is highly resistant to elevated temperatures, desiccation, osmotic shock, ultraviolet light, and chemical disinfectants (Scott and Williams 1990). Coupled with its infectivity for humans of 1–10 organisms, contamination of food, buildings, and pastures, it has frequently led to human and animal infections. Highly effective disinfectants of suspended organisms were 70 percent ethanol, 5 percent chloroform, and 5 percent EnviroChem, whereas Alcide, sodium hypochlorite, benzalkonium chloride, Lysol, and 5 percent formalin were not. Paraformaldehyde gas and ethylene oxide sterilization of surface-applied organisms was inefficient under low humidity conditions. It is probable that metabolic dormancy at pH 6 and the developmental cycle which generates spores and small dense forms both contribute to the physical and chemical resistance of *C. burnetii* to inactivation.

Control by reducing the risks has been approached by issuing advice to animal workers that, if they have pregnant partners, care should be taken with hygiene after occupational exposure by hand washing and removing contaminated clothing for separate washing.

A vaccine has been developed in Australia and is used for prevention of infection in populations at risk (Marmion et al. 1984).

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Introduction

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The publication of this immunology volume as a separate entity in *Topley & Wilson's Microbiology and Microbial Infections* is a welcome innovation and a reflection of extensive progress in our understanding of the immune system in recent decades. Nevertheless there are still enormous gaps in our knowledge that need to be investigated before we will be able to manipulate immune responses predictably to our advantage. Recent advances are widely distributed in numerous different journals and publications, and this makes it difficult to keep up with so much new information relating to the complex and now vast field of immunology. Present knowledge relied on a whole range of interdisciplinary research activities such as molecular and cellular biology, protein chemistry, proliferation and differentiation signals, regulation of gene expression, membrane biology, etc. Hence this well-referenced book will be most useful to many investigators.

The editors have collected 46 chapters written by different experts and have assembled much basic knowledge to update us on both innate and adaptive (or acquired) immune responsiveness. The innate cellular responses provide the first line of defense against invading microbes. By production of mediators when activated they set the scene for the activation of the adaptive responses with specificity for the challenging antigen(s). Type I interferons are produced very rapidly in many infections; natural killer (NK) cells and their subsets, γ/δ cells, complement components, phagocytes and granulocytes (i.e. macrophages and neutrophils; are of central importance in innate responsiveness by formation of various chemokines and cytokines (interleukins). Pattern recognition receptors (such as Toll-like receptors (TLR)) are expressed by certain cell types. They recognize particular groups of microbes or their components and play a major role in early cell signaling events. Although the innate immune responses do exert some control over the pathogen load early in infection, they are rarely sufficient to totally clear intracellular microbial infections, be they caused by viruses or parasites.

Adaptive immunity depends on a highly complex and intricate network of interactions between different cell types and their subpopulations, regulated by their production of mediators, i.e. cytokines, chemokines, interferons type I and II . The adaptive immune responses are driven to a large extent by antigenic challenge resulting in the clonal expansion, differentiation, and maturation of diverse clones of antigen-specific B cells to form circulating antibodies (humoral immunity) and of antigen-specific cell-mediated responses by thymus-derived T cells. These vary in phenotype, cytokine formation, and effector function. In this brief introduction it is impossible to do justice to all the main players in this amazingly multifaceted system. However, this volume contains detailed articles on the many cell types involved, the lymphocytes and their subpopulations differing in phenotype and function, their lifestyle, and their antigen recognition receptors and patterns.

In addition the main antigen-presenting cells (APC), i.e. dendritic cells and macrophages, are essential to activate B and T cells from their resting and naive state.

The T cells are divided into two major subpopulations according to their phenotype, the expression of the CD4 and CD8 surface markers, as well as their functional differences. Chapters 19 and 21 convey the antigen-processing pathways and the assembly of the major histocompatibility complexes (MHC) with antigen-derived fragments for recognition by the T-cell receptors. $CD4^+$ T cells recognize antigen-derived fragments in conjunction with MHC class II molecules on the cell surface while $CD8^+$ cytotoxic

T cells (CTL) see antigen-derived peptides in association with MHC class I. The MHC gene complex is of course highly polymorphic and the binding of diverse antigen fragments to the polymorphic MHC molecules varies in different haplotypes in the population. Both T and B cells are the source of many cytokines and chemokines when they recognize antigen. Nonantigen-specific B and T cells are also recruited by chemokines into infected or damaged areas, and become activated by bystander cytokines in the environment of lymphoid organs to further enhance inflammation.

Excessive levels of certain immune responses and resulting cytokines can cause serious immunopathology as seen, for example, in certain infections, autoimmune disorders, allergic reactions, or hypersensitivity reactions. Thus it is not sufficient to activate and induce immune responses, but, to prevent lymphoproliferative disorders and overactivation of cells in the lymphoid tissues or infected organs, it is also essential that the immune cells can be down-regulated. Regulatory T cells and their formation of inhibitory cytokines (such as the interleukin (IL)-10 and transforming growth factor (TGF)-β) or apoptotic signals fulfill this need.

Chapters 40–46 are devoted to recent approaches used in efforts to develop new vaccines. There are no effective vaccines as yet against serious health threats caused by some extensively studied and fully characterized pathogens, as well as by newly emerging infectious agents. However, this is an area that still requires intensive research and study. Each infectious microbe is a law unto itself and differs in tissue tropism; it is endowed with different mechanisms to evade immune control, varies in its host–pathogen relationship, lifestyle, and the effects of host genetic influences. Therefore each microbial infection has to be examined as such and there are no obvious shortcuts. Not all immune responses are beneficial and deleterious reactivities have to be avoided. Clearly, there is a need to define appropriate protective responses against intracellular infections. We would like to be left also with immune memory (i.e. antigen-specific lymphoid cells that respond more rapidly and are present at a higher frequency than found in naive hosts) for defense against subsequent challenges with the same microbes. The induction of antibodies alone is not necessarily the answer to all our prayers. Chapter 18 addresses the therapeutic application of monoclonal antibodies.

This *Immunology* volume provides basic information on broad aspects of immunology that must be taken into consideration when dealing with infectious diseases and so many clinical problems and disorders involving lymphoid cells and pathological inflammatory responses. This volume will be invaluable to students and post-doctoral investigators, as well as experts, microbiologists, immunologists, basic or clinical scientists, and clinicians in many fields.

History

ANNE MARIE MOULIN

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IMMUNOLOGY WITH AN EMPHASIS ON INFECTIOUS DISEASES: A RETROSPECTIVE

The modern science of immunology stemmed from the germ theory of diseases at the end of the nineteenth century and then developed in close connection to it. At that time, immunology was decisively empowered by the early successes of rabies vaccine and anti-diphtheria serotherapy and contributed to establish bacteriology as one of the pillars of modern medicine.

In the twentieth century, immunology acquired a growing autonomy and distanced itself from the domain of infectious diseases. Immunology dealt with a growing number of affections with no infectious agent identified, or even hypothesized. The enlargement of the definition of antigenicity, addressing a whole array of molecules (Landsteiner 1936), helped to emancipate immunology from its historical origins.

Immunology as the 'science of the immune system' acquired connections with other systems in the body such as the endocrine or the nervous apparatus. The immune system played a major role in the selection of species and individuals and the present ontogeny reflected these developments. Immunology, the 'latest language of medicine' as tentatively named (Moulin 1991), could claim to be becoming the science of the 'system of systems', because the immune system was increasingly shown interacting with other vital functions and regulating them.

The dream of the eradication of microbes was nurtured by the success over smallpox and infectious diseases, and the observation of the world's spectacular demographic expansion. Yet, in the immunology of the last quarter of the twentieth century, the share of research linked to infectious diseases declined. Some scientists, focusing on idiotypic–anti-idiotypic relations, went so far as to discard the primacy of infections in the understanding of immune responses, and set forth the distinction between a so-called peripheral system dealing with external pathogens, corresponding more or less to the infection-oriented immunological tradition, and a central part, or immunological core, described as a self-centered network (Coutinho et al. 1984).

These last years have been marked by a revived interest in infectious diseases. In sharp contrast with the previous period, the last decade of the twentieth century has witnessed a growing anxiety over the so-called return of infectious diseases, and disenchantment over the perspectives of a rapid and complete eradication. The fight against ancient and new plagues has been resumed with accrued vigor and traditional tools have been refurbished with the help of all-powerful molecular biology.

Inflammation, an antiquated concept taking us back to Celsius and Roman times, is again finding a central

place in immunology. Inflammation associated to infection is a crossroad phenomenon that includes secretion of multiple factors. A whole network of molecules, entertaining complex relationships with each other, thus contributes to the permanent homeostatic control of the body and adjustment essential to life (Canguilhem 1966).

The immunology of infectious diseases has been rehabilitated in a new guise, most notably expressed in a molecular language. Immunology, once described as a science attached to the ideal of specificity, or the 'science of boundaries' trespassed only in rare cases (Moulin 1997), is by now deeply concerned with the exchange of biological material across the species barrier. The trends of innate and acquired immunity, dubbed natural and adaptive immunity, go intertwined through recent immunological literature, opening a flow of speculations over evolution, selection, and survival.

THE 'RETURN' OF INFECTIOUS DISEASES

With the development of microbiology at the end of the nineteenth century, the initiative seemed to be in the medical camp for ever, and a definitive advantage given to the doctors' side. The isolation and cultivation of germs made plausible the hope of destroying them.

The eradication goal was immediately perceived as at hand not only by journalists but also by scientists (Gradman 2000). Louis Pasteur set the tone by predicting the demise of tuberculosis, rabies, plague, and cholera. Although Pasteur admitted that new plagues were to come, he was confident that, as they would show up, fresh science would take over. This fighting adopted almost mystical tones. Scientists crusaded to relieve humankind from the original sin lurking in the body in the guise of germs. Not that the physicians of the past had ignored any ontology of disease. On the contrary, they had all the time been familiar with the general idea of the 'seeds of the disease' (Nutton 1983). But so long as this theory was of little practical value for curing patients, this had been a mere speculative luxury.

Operating in the wake of World War II, the World Health Organization (WHO) decided to give a decisive impulse to the eradication of infectious diseases. Its officers launched the campaign against smallpox which led to the announcement of its eradication in 1979 and provided the model, to be followed by tuberculosis, malaria, poliomyelitis, and other diseases.

Although immunology provided a whole line of vaccines, antibiotics, by alleviating the burden of infections, reduced the pressure on immunologists to explore the inadequacies of the immune response. Immunologists interacted more closely with biological research in embryology, neurology, cognitive sciences, cancer studies, and pharmacology, and provided tools and concepts to other disciplines.

Between the 1980s and the present time, the period under scrutiny in this chapter, immunology faced events that boosted and remodeled its activity in the domain of infections.

Today, in Africa and Asia, countries still bear an enormous infectious burden and AIDS has shortened by many years the gain in life expectancy, which immunization and modern medicine had helped to procure. In contrast, in industrialized countries, less than 10 percent of mortality is attributed to infectious diseases, and even AIDS has only globally induced a rise of about 1 percent. Yet, infectious diseases have stepped again to the forefront for many reasons:

- As a result of the neglect of immunization and political unrest, some ancient plagues have burst out again in Africa. Yellow fever erupted in Brazil, in urban settings, in 1998. A threat weighs over Asia, historically free from yellow fever without demonstrable immunity to it, in spite of the presence of other flaviviruses. Hemorrhagic dengue is now expanding in South America and the Caribbean.
- The microbial world has displayed a previously unheard of diversity, including new classes of pathogens, among which bacteria such as legionellae, viruses such as the bunyaviruses, the parvoviruses, the arenaviruses to which belong the viruses of hemorrhagic fevers (Machupo, Junin, Lassa, Sabia), the filoviruses (Ebola, Marburg), and above all the retroviruses.

Systematic investigation in birds and rodents around the world has detected thousands of viruses that have no ascertained pathogenic role. Field studies represent at best a glimpse into the diversity of viruses circulating among rodents, vectors, and mammals – a potential reservoir of pests for the rest of the living world.

Defying the dogmas of molecular biology, unconventional particles of poorly defined status, such as prion proteins, have been involved in the genesis of neurological disorders (Gajdusek 1977). With the demonstration of the transmissibility of the bovine encephalitis to humans, natural order was challenged, because the species barrier is no longer an absolute protection. Inasmuch as the tomato spotted wild virus, a bunyavirus akin to the Rift Valley fever virus, is adapted to plants, plant viruses could grow on vertebrate cells.

Laboratories and hospitals are no longer sanctuaries. Hospitals, considered once as hotbeds of infection, a threat to neighborhoods, reappear as dangerous places. Nosocomial infections are scaled up to 10 percent of the registered infections, even in Africa, although with germs differing from those in the West. It is in a hospital, in 1976, that the Ebola epidemic burst out, as well as a few months later, in Yambuku in Zaire, and in 1995 again in Zaire.

In the laboratories of the industrialized world, accidents may happen. The Marburg virus, close to the Ebola virus, draws its name from the city where it made its way from monkey cells used for vaccine production

in 1967. Until recently, there have been reported cases of infected people among the staff handling the Rift Valley fever virus, identified in 1983 and extremely contagious. The creation of high security laboratories (P4) illustrates the difficulty of manipulating ultra-virulent and unidentified infectious material. But there are no P4 laboratories close to the infection foci in Africa. Moreover, growing resistance of microbes to antibiotics instilled the idea that no defense line would soon remain available. In a pre-vision of this therapeutic collapse, the mechanisms of immunity should be revisited, and immunologically inspired treatments investigated more carefully.

The return of infectious diseases, at the beginning of the new millennium, has roused an anxiety aggravated by the recent threat of bacteriological warfare. Mass media currently discuss germ pulverization in the atmosphere; they evoke the mad scientist working for terrorists, flooding germs and toxins in water pipes, inventing genetically modified microorganisms, resuscitating the smallpox virus in populations deprived of immunity.

The 'Conquest of Diseases' (Winslow 1943) has been replaced by the idea of a 'victory à la Pyrrhus' (Ameisen 1999, p. 158), with nobody vanquishing or being vanquished. Epiloguing on this 'return', which typically reveals postmodern distress in a 'global' world, we have to admit that the notion of 'new virus' actually reflects our previous ignorance and witnesses the extension of our inventory of the living world. It is as much the vanishing of an illusion, thanks to the improvement of our detection tools, as the awareness of a grim reality. It is not only the immune system that has been taken off guard; many systems of surveillance in the world have been dismantled, in the former Soviet Union, for example, or in Africa.

This so-called return, be it the emergence of radically new plagues, or the re-emergence of previously tamed epidemics, confronts long-term germ evolution with the quickening step of human history (Moulin 1996b). The relationship between humans and the environment has been transformed, some say perverted, by human activity, and a debate bears on the necessity of rethinking a new alliance or even a 'new contract' (Serres 1990) between 'Man' and 'Nature', which he possesses far less than he is possessed by it.

The AIDS epidemics has provided an unmatched opportunity for dissecting the immune mechanisms. The virus, when dormant in the cells of the immune system, is relatively unobnoxious for most human tissues, with perhaps an exception for the nervous system, but it is deleterious to the immune system and transforms the body into a 'city without walls' (Epicurus) ready for 'opportunistic' attacks. Our present pessimism is rooted in our failure to produce a vaccine rapidly, in spite of the unprecedented research effort. We still miss a satisfactory animal model and an anchorage in the natural history of the disease, documenting spontaneous cure. Virus lines developed in the laboratory swiftly behave like 'circus strains', to use a phrase coined by French biologist Charles Nicolle, too obedient to the dompter's whistles and falling rapidly apart from wild viruses. After the introduction of active tritherapy, much is expected from the manipulation of the immune system, giving it a chance to take over when specific therapy is discontinued.

THE NEW AGE OF INFECTION AND IMMUNITY

The development of monoclonal antibody technique has followed a paper published by Cesar Milstein and Georges Köhler in 1975 in *Nature* and crowned by the Nobel prize. This technique, initially conceived by Milstein to explore the immunoglobulin genes and the diversity of antibody formation, turned out to be available for all kinds of studies. Hybridoma technology (the name appeared 2 years later) has resulted in immortal cell lines producing, at will, any human or murine antibody of chosen specificity, a wonderful tool for genetics and immunogenetics. The T4 count (CD4 cells still rank among the major indicators of the risk for opportunistic infections) was defined with the help of monoclonal antibodies (Cambrosio and Keating 1995). Flux cytometry uses monoclonal antibodies to mark cells and the cell sorter has turned into a quantitating machine (Cambrosio and Keating 1994). Novel ligands for cell-surface molecules can be identified using high-avidity recombinant reagents displayed on fluorescent beads. These reagents allow description of interactions between cells and molecular analogs of receptors or targets (Wedderburn and Dianda 2000).

Genetics had at first been little concerned with infectious diseases, being absorbed in the model of 'innate errors of metabolism' (Garrod 1909) and research on diseases recognized for a long time as being hereditary, based on family pedigrees and aiming at genetic counseling. Innovations included karyotyping techniques and the selection of new animal models that mimic human pathologies. Standard mice were produced between the two world wars by the Jackson Laboratory, established by the geneticist Little (Löwy and Gaudillière 1998). The center has become the world leader in the study and production of inbred, genetically homologous strains of mice.

'Knock-out' (KO), with artificially defective genomes, have provided exquisite tools for the analysis of the immune system (Mak et al. 2001). Studies have shown that the immune system finds a new balance when it loses some component. The deletion of a gene coding for an important molecule of immunity may have consequences in a remote sector of physiological life, e.g. mice missing the *PrP* gene, which are insensitive to prion infection, display anomalies in systems other than the immune system. But genetic manipulations do not

necessarily result in gross alterations in development and survival. The block that occurs to some pathways through genetic defects does not necessarily compromise the formation of innate or even acquired immunity, suggesting flexibility and redundancy as major features of the immune system, which is critical for survival.

In the two last decades, the actors of the immune system, studied with new tools, have multiplied. The immunological band once cartooned by Gershon in the 1970s has by now expanded to the dimensions of a symphonic orchestra (Gershon 1980).

In the mouse, two functionally different CD4 populations were described in 1989: T-helper 1 (Th1) and T-helper 2 (Th2) (Mosmann and Coffmann 1989). The T-/B-cell dichotomy, corresponding more or less to the historical split between the cellular and humoral response, has mirrored itself in a new subdivision in the T-cell population between Th1 and Th2 subsets, key tuned to different classes of pathogens, extra- and intracellular. Characteristically, this division was based on different patterns of lymphocyte secretions. The observation was soon extended to the human case (Romagnani 1996).

Another new subset of cells has been dendritic cells. When they were described by Paul Langerhans in the dermis (Langerhans 1868), they had nothing to do with immunity. Langerhans called them dendritic because of their aspect, which was reminiscent of nerve cells, and hypothesized that they belonged to the nervous system. The French dermatologist Darier later noticed their frequency in inflammatory lesions (Darier 1900). Dendritic cells are scattered throughout the body, close to the main portals of microbe entry. Although the phenomenon called crosspriming or crosspresentation was described in 1976 (Bevan Cross 1976), their role was diversely interpreted before being viewed as essential to the onset and modulation of the immune response. In lymph nodes, they are sufficiently 'professionalized' (Amigorena 1999, p. 932) to instruct naïve lymphocytes and present antigens from infected cells, in association with class I molecules, to T-cytotoxic lymphocytes. In AIDS, they are among the first cells to capture the virus in contaminated fluids. This strategic position makes them appear as good targets for potential vaccines at an early stage of primoinfection.

Dendritic cells identify pathogen agents via toll-like receptors, so called because of an analogy with proteic receptors in *Drosophila*, which are mediators of embryogenesis. Toll receptors recognize molecular patterns shared by numerous pathogens of the gram-negative families (Rock et al. 1998). The activation of these receptors in mammals leads to the production of antimicrobial peptides such as cecropin and defensin.

The genetic diversity of B lymphocytes results primarily from the recombination of the many genes harvested in our body libraries. To build the receptors that display specific targets on their shields, lymphocytes make use of enzymes under the dependence of two genes called recombinant-activating genes (RAG). These genes, shared by all species endowed with T lymphocytes such as mammals, birds, and some fish, probably derive from a very primitive microorganism, a transposon, that was integrated into the human genome millions of years ago.

In 'the golden age of genetics and the dark age of infectious diseases' (Tibayrenc 2001), how did immunology evolve? The biologist Paul Ewald sharply subtitled his popular book *Plague Time* (Ewald 2002) *The new germ theory of disease*. Post-modern immunology features:

- a new definition of pathogens and pathogenicity
- a focus on infection currently redefined
- a genetic agenda for the assessment of the germ/host relationship.

The nature of the pathogen has diversified. RNA viruses are particularly proteiform, because they mutate rapidly when they multiply in the host cells and because their polymerase, an enzyme essential in the synthesis of nucleic acids, does not entail any mechanism for repairing errors. RNA viruses, numerous in plants and animals, are good candidates for the etiology of many chronic affections such as multiple sclerosis or Parkinson's disease.

Genetic recombinations frequently happen between two subpopulations of one microbial species, in a vector or a host, with unpredictable consequences for pathogenicity.

The nature of pathogens has become elusive. It is not excluded that viroides, small monocatenary pieces of RNA involved in transmissible diseases in plants, play a role in humans and animals. In the cerebral tissue of patients suffering from Creutzfeldt–Jakob dementia, the PrP protein results from the alteration of a normal protein. Although the modalities of infection by prions remain far from understood in their entirety, the infectious role of proteins is now well established.

Some pathogens had been known about for a long time in the animal kingdom, but damaged immunity in humans (whatever the cause, and they are many in the modern world, such as chemotherapy, transplantation, severe congenital defects) procures them new opportunities. These 'opportunist infections' are best illustrated in the case of microsporidia.

Microsporidia are unicellular parasites that are widespread in the animal kingdom. The German zoologist Naegeli described corpuscles, which he called *Noema bombyci*, under the skin of the silkworms in rearings. Babiani suggested, in 1882, the name microsporidia because of their tiny dimensions. Their position in taxonomy is still controversial. The absence of mitochondria favors a 'regressive evolution', with loss of important organelles. With the help of the high-resolution electronic microscope, it became possible to

investigate the pathogenic power of this supposedly benign parasite. The first human case described was in a patient stricken with AIDS (Desportes et al. 1985). The story of this patient, whom the author came to know personally, was most unfortunate because for a long time there was a failure to attribute the disease correctly to a transfusion in Haiti. Many cases have been reported since in AIDS and immunosuppressed patients. In athymic or severe combined immunodeficient (SCID) mice, parasites invade the whole organism and infections of microsporidia are lethal.

The very notion of infection has lost its clear outlook (Evans 1993). Medical interest has moved from canonical descriptions of infections, almost in Hippocratic format, to the search for all possible symptoms. Computer technology has played a crucial role in this shift from clinical to bibliographic knowledge. Primoinfection linked to a given pathogen is shown to generate a broad spectrum of situations, from the healthy carrier to acute and severe forms, and to chronic diseases pursuing their course in the absence of a detectable pathogen. Murine models, in the case of leishmania infections, for example, illustrate the clinical diversity of human forms, from the benign oriental sore to deadly kala-azar.

The opposition between infection and hereditary disease is no longer clear cut. The transmissible spongiform encephalitis such as that of Creutzfeldt–Jakob disease exists under two forms: the infectious one, including cases that result from treatment using growth hormone extracted from patients who died with dementia, and the hereditary one.

Since the late 1980s, the role of chronic infections as facilitators of more severe diseases has been discussed: they would weaken the immune system by overstraining or overstimulating its capacity to respond. In 1989, in order to explain HIV diffusion, Luc Montagnier suggested such a role for mycoplasma infections. Later, sexually transmitted diseases associated with *Chlamydia* have been incriminated. The general idea of an infection ecosystem in unstable equilibrium in populations, or 'pathocenosis', as put forward by the late historian Mirko Grmek (1969), has led to the global incrimination of chain reactions in the immune system without assigning the first signal.

New immunology addresses the question why an epidemic intervenes in the course of the silent co-evolution of germs and their potential hosts. Closer articulation of the innate response, allegedly more primitive, and the adaptive response, claimed to be the privilege of the most recent species, has reinforced the link between our sophisticated organisms and the rest of the living world, and restored a more balanced view of the respective advantages and disadvantages among species. Already sobered by moral considerations on the cyclic return of the 'brute beast' in humans (Nicolle 1933), our positivist optimism has been dampened by the discovery of the highly innovative capacity of germs and their ability to exploit molecular niches in the intimacy of the body.

To address these challenges, immunology operates within the framework of the selective theory (Silverstein 1989), which still prevails, even though it has lost its original simplicity since Burnet offered the idea that the antigen selects the corresponding clone, which subsequently expands T cells to identify the molecules of the self carried by the histocompatibility complex on antigen-presenting cells. As far as immunity is concerned, the differences in repertoires between individuals account for both the havoc caused by epidemics and the survival of a few. Differences between high and low responders were suggested by Ludwig Hirschfeld in his seminal book, *Konstitutionserologie* (Hirschfeld 1928). Biological individuality is considered alternately as an obstacle to general strategies of immunization, leading some authors to recommend immunization 'à la carte', and as a providential reservoir of biodiversity, illustrating the leibnizian principle of 'multiple splendor' in 'Creation' (Moulin 2001) and the maximization of good.

The history of primoinfection is one of mounting a powerful immune response that involves all known components of immunity, from the old crones, antibodies and cells, to the creatures of novel immunology, cytotoxic cells, Th1 and Th2, from Metchnikoff's phagocytes to dendritic cellules and natural killers, from Jules Bordet's complement to cytokines and lymphokines.

Today, knowledge about HIV infections illustrates the general development of such knowledge about the immune system, the vulnerability of which was thus exposed. The way HIV infection proceeds fatally toward a chronic form by escape from the immune system is extreme and cannot constitute the general model of all kinds of primoinfection. Yet it has reshaped our understanding of the encounter between germs and organisms that may shelter them.

Transmission of germs and genes, now more integrated than in the past, constitutes a general and unifying perspective. A century and half after the beginning of bacteriology, there is space for multicausal explication. With the emergence of notions such as genetic pattern or pathogenicity islets in the genome, which mean more than constitution, terrain, soil, natural immunity, predisposition, etc., immunogenic knowledge and epidemiological data point to the inequality of bodies, without denying the role played by lifestyle.

THE MOLECULARIZATION OF IMMUNOLOGY

Immunology has entered the modern era by exploring the genetic aspects of the immune response and by adopting molecularization. Molecular immunology is convenient shorthand for the study of the molecules of

the immunological orchestra and their interactions (Moulin and Silverstein 1990), with the tools of molecular biology. This abbreviation refers to both an ideal and an enterprise in the making.

The molecularization of immunology raises the question of the changing position of immunology among other biological disciplines. Did it significantly help it to solve physiological issues and open new diagnostic and therapeutic vistas in diseases (Panem 1984)? In the 1980s, cancer was the main target of the 'immunological revolution' (Fridman 2000): molecularized immunology was expected to open a 'fourth line' of therapies, after radiotherapy, chemotherapy, and surgery (Löwy 1997). In the 1990s, infectious diseases were reconsidered as possible beneficiaries of advances in the field, from a curative and also a preventive viewpoint (vaccines).

The breaking of the genetic code and the identification of the nucleic acids as the support of heredity have been presented as *The Eighth Day of Creation* (Judson 1979). But it was only the beginning of an era that saw biologists manipulating gene segments and expressing them in vectors. The colossal enterprise of the Human Genome Project, in contrast to the medical programs for eradicating diseases, has been regularly ahead of its deadlines. Mapping the human genome has become the totem of the new molecular biology, in a planetary vision (Jordan 1995).

If the development of restriction enzymes from the 1975s onwards marked an important milestone of molecular genetics, the 1975 hybridoma paper by Köhler and Milstein (1975), ending with a few lines on the applications for the production of antibodies of medical interest, is considered as a similarly crucial step in the molecularization of immunology, in terms of phenomenological analysis, and production of tools and therapies. Either ancillary to molecular biology or bringing its own recipes, immunology has come into the scientific mainstream. Immunologists can pursue the historical mission that its pioneers had assigned themselves. The goal of specificity, a major tenet of early immunology (Mazumdar 1995), is alleged to be reached thanks to elegant techniques allowing the dissection of the immune response into all its components and the identification of all relevant pathways. Since the 1980s, molecular biology has allowed the localization of genes for capsules, toxins, adherence factors, invasion and survival cell factors on plasmids, bacteriophages, and even on chromosomes, leading to a description of 'pathogenicity islets' and the identification of a whole array of molecules involved in infection and immunity.

Interferons were first described by Isaacs and Lindenmann in 1957, referring to the phenomenon of viral interference. They were used as antitumoral agents only some 30 years later. They are today considered as both constitutive and induced immunity factors.

David (1966) and Bloom and Bennett (1966), independently hypothesized that a substance, formed by lymphoid cell–antigen interaction, inhibits macrophage migration. Bloom called this factor migration inhibiting factor (MIF). In 1969, Dumonde et al. suggested the term 'lymphokines' for substances different from classic antibodies, generated by antigen-activated lymphocytes.

In 1975, while investigating a viral etiology of leukemia in Gallo's laboratory, Morgan and Ruscetti noted that media from phytohemagglutinin (PHA)-stimulated lymphocytes were capable of prolonging the growth of cells from leukemic cells in continuous cultures. The new molecule (called T-cell growth factor (TCGF)), and not the antigen, allowed cultured normal lymphocytes to maintain their functions. In 1978, a murine cytotoxic cell line was used as a bioassay for testing this lymphokine. An assembly of immunologists recognized the identity of thymocyte mitogenic factor, killed helper factor, and lymphocyte mitogenic factor, which have previously been described, and co-chromatograph with TCGF. The subsequent cloning and purification of interleukin (IL) 2 by Taniguchi et al. in 1983 confirmed that all these activities were ascribed to a single molecule. IL-2 is presently one of the best-known molecules. B-cell-stimulating lymphokines, such as IL-4, IL-5 (also active on eosinophils), and IL-6, were successively defined. Human lymphokines were thought to be similar to their mouse homologs, although most experiments needed to prove it were impossible for obvious ethical reasons.

The availability of a variety of hemopoietic growth factors has allowed the development of in vitro approaches to the differentiation of hemopoietic lineages. These factors regulate genes that are required for the maintenance of viability during proliferation and differentiation. With the advances in modern biotechnology, many of them have become available in sufficient amounts and purity to study their effects in vitro and in vivo. It was expected that knowledge gained from these studies would lead to new approaches to the treatment of human disease.

As more factors were identified and cloned, the complexity of their effects and interactions was perceived. 'Cytokines', immunologically active proteins that bind to specific receptors on target cells, can be produced by a wide variety of cells, e.g. IL-1, stimulated by lipopolysaccharide (LPS) and a component of Gram-negative bacteria, is secreted by monocytes; many other cells can, however, make IL-1. Among many bacterial products, the ubiquitous LPS (Besredka 1906) has been characterized as a potent stimulus of both immunity and inflammation (Cavaillon and Le Garrec 1998).

As growth factors, cytokines have been particularly useful for facilitating the culture of cells reputed to be difficult to grow in vitro. With further recognition of the numerous cell subsets involved in immunity, additional ingredients were poured into the 'cauldron'. How these molecules are classified has currently depended on whether an endocrinologist, immunologist, or hematolo-

gist described them first. Later, their protean properties have somewhat bewildered researchers, who are always eager to advertise them as potential immunostimulants or more generally immunomodulators, but embarrassed for classifying them and putting them into a hierarchy. The availability of purified recombinant factors, the development of radioligand assays, and the production of monoclonal antibodies to surface determinants have permitted quantitative study of the chemistry of ligand–receptor interactions. The information contained within the structure of the factor is received by the receptor and rapidly translated into a limited repertoire of chemical internal signals that affect cellular functions.

Cytokines characteristically act at a site distant from the cells that produce them, witnessing the potential of the immune response to reach target organs far from the microbial entry. However, a regulation is required to provide balances and checks of cytokine production. Cytokines are now viewed as the key to the understanding of the inflammatory reaction and the regulation of the immune response. Anti-inflammatory cytokines, such as the 'quintet' of IL-4, IL-10, IL-13, interferon-α and the transforming growth factor (TGF) would have the capacity to repress proinflammatory cytokines elaborated by activated macrophages.

Chemokines, described in 1992, are a family of small proteins that recruit circulating cells and play an important role during inflammatory reactions. Their function is chemotaxis or attraction exerted on cells. Chemokines play also a role in angiogenesis, collagen production, and proliferation of hemopoietic cells. This family includes numerous members, among which is IL-8, and has raised a considerable interest, because of its potential therapeutic action in AIDS.

In contrast to the ideal of narrow specificity, the system is redundant. Chemokines are promiscuous in receptor usage – several cells produce several chemokines with an overlapping sector of action. The robustness of biochemical pathways renders their output relatively insensitive to change. The natural polymorphism occurring in an outbred cell population does not usually affect the immune response for this reason. No chemokine is uniquely active on one leukocyte, and usually a given leukocyte population has receptors for and responds to different molecules. Most known receptors interact with multiple ligands, and most ligands interact with more than one receptor.

Generally speaking, it is impossible to discriminate between 'good' and 'evil' cells and molecules. Infection is a drama with successive steps where immediate destruction of viruses by specific cytotoxic cells may be associated with negative indirect effects on non-infected cells. Antigen–antibody complexes can protect viruses hidden in lymph nodes. Although chemokines can stick to ligands, preventing infection of the cells by viruses, cytokines can diminish the regeneration of cell lines, and trigger destruction in neighboring cells.

The molecular biologist Jean-Pierre Changeux has characterized the immune system as an allosteric orchestra, to emphasize the role of allostery or alternate changes in chemical structure, which induces functional diversity, as illustrated by hemoglobin in the Wyman–Changeux–Monod model. Shared signals, structural analogies in receptors forming families that overlap the species barrier, manifest the unity of the molecular language in which infection and immunity are described today.

AN EMPIRICAL VIEW OF THE IMMUNE SYSTEM

In 1990, the author wrote, with Silverstein in a book dedicated to 'The role of cells and cytokines in immunity and inflammation, that ''the past decade has been marked more by technologic than by conceptual developments''' (Moulin and Silverstein 1990, p. 3).

A tentative model of disease has been based on dysfunctional production of and responsiveness to cytokines. The administration of LPS reproduces some of the disease events by inducing an outburst of cytokines. Coordinated reactions, facing danger, involve a reorganization of self-perception and behavior. Anorexia, sleepiness and drowsiness bordering on comatose states, onirism, and retreating from the world had been described for a long time by patients themselves. Infectious disease has been one of the most studied human behaviors. A masterpiece of Elizabethan literature in the Renaissance is John Donne's *Reflections upon Emergent Occasions*. Donne wrote, during a typhus epidemic, his detailed personal account of the events accompanying disease, fever, and delirium, and the effects of consulting the physician and the priest.

The cold/hot opposition was among the basic tenets of the humoral theory that for centuries shaped medical thinking. Fever has remained the clinical hallmark of inflammation, and is narrowly associated with disease, trauma, or infection. The physiology of fever is better understood in the context of recent immunology: cytokines are responsible for fever induction and the behavioral changes during the acute phase of infection.

In response to a stimulus such as LPS, the body's thermostatic control center within the hypothalamus increases its set-point temperature. The body then increases its core temperature to match the new level. Physiological (vasoconstriction, reduced sweating) and also behavioral modifications (drinking warm liquids, putting on warmer clothes) drive up the body's temperature. When the fever breaks, the body temperature again is too high relative to the new set-point, and physiological and behavioral modifications result in a lowering of core temperature, increased blood flow in the skin, sweating, and the desire to drink cold liquids. Fever is a central episode of immunity and infection. Clinical considerations on the onset and rhythm of fever

paroxysms remain a decisive component of the biomedical enterprise in the era of the cytokine orchestra.

At the onset of the disease, inflammation is a nonspecific restorative response of tissues to soluble chemical mediators produced after injury. If the harmful agent is not removed or the disequilibrium persists, a pathological state of chronic inflammation ensues. If the inflammatory cycle is broken, the host is restored to its preinflammatory state of physiological equilibrium, but, as the French philosopher of medicine Georges Canguilhem stated in many of his writings, there is no return to cellular innocence.

IN SEARCH OF A MECHANISM UNIFYING IMMUNOLOGY

The tantalizing identification of multiple factors in constant interaction has resulted in a 'balkanization' of research, i.e. division of research into small separate sections. It has blurred the primitive simplicity of the immune response with its two arms – the informative and the effective – once gracefully linked in a kind of reflexive arc.

The distinction between self and nonself is still very much there, even if many doubts are expressed about its pertinence. Frank Mcfarlane Burnet had long ago envisioned the possibility of accounting for the nature of the self in precise molecular terms, before retreating to use of the phrase in a metaphorical sense. Zinkernagel and Doherty's (1979) demonstration that the T-lymphocyte receptor reacts with a polypeptide attached to a major histocompatibility complex (MHC) molecule reinforced the notion of the recognition of nonself in the context of self. At the onset of molecular immunology, there were attempts to describe more precisely the self as the 'peptidic self' (Kourilsky and Claverie 1988).

Generally speaking, however, the self/nonself distinction functions more as a general background than as an effective theoretical construct (Moulin 1994), an indication that, although many questions remain to be clarified, most immunological reactivity is oriented for the sake of survival of the organism. It does not radically differ from expressions such as 'genes' or 'immunity', the meaning of which is constantly fluctuating. Although it has been said that 'The self is constantly defined anew, which is another way of saying it doesn't really exist at all' (Richardson 1996), the reference to self and nonself ensures communication between generations of scientists and between scientists and their public.

The last few decades have seen an outburst of data where it is difficult to single out unambiguous signals. Epistemologists insist on the dependence of scientists, more than theory, on the experimental systems that they handle on a daily basis (Rheinberger 1997). It had been suggested for a long time that tolerance can be induced by small as well as overwhelming doses of antigen, but never has it been made clearer that the self/nonself distinction is made empirically, case by case, and the meaning of immunological phenomena is practically determined. For Zinkernagel (2000), there is nothing absolute: immunity is, in his own words, a matter of dose, space, and time. Philippe Kourilsky (1998), inaugurating the first chair of 'molecular immunology' in France in 1998, stated the same idea:

> Specificity in the immune system, more than on the complementarity of structures, is founded on the dynamic of cellular activations: some defence mechanisms are deprived of specificity and acquire it only by operating at the right time, at the right place.

This perspective is not as empirical as it appears at first sight. It simply moves the emphasis to the importance of microenvironment and local determinism. Cells and factors are not identical because, following a 'leibnizian' doctrine of individuality, they are always located in space and time. Obviously, we miss an all-encompassing and simultaneous view of all compatible phenomena in the realm of immunity – the vision of GOD (for Generator Of Diversity), to recall Melvin Cohn's jocular and unmistakably serious address. Any activation can result in overreaction and destruction of antigen-stimulated cells. The recognition of autoreactivity as a physiological phenomenon resulting in autoimmunity has illustrated this empirical turn.

The identification of autoreactive cells within the lymphocyte repertoire recalls the interpretation of these cells playing a role in body homeostasis, such as immunocircuits controlling the antibody level, e.g. through idiotypic interactions. Autoimmunity became pathogenic only when effector cells penetrated organs, favored by microchanges in the environment.

It is currently being suggested (and has been suggested for decades) that infections may cause autoimmune diseases. The molecular basis still remains to be established in many cases, but it is a fascinating hypothesis, also available for many idiopathic diseases. The scenario would be the following: molecular mimicry between a pathogen-derived antigen and a host antigen would set off subliminal autoimmunity.

T-cell receptor recognition of antigen looks highly degenerate. In experimental models, myelin basic protein, involved in multiple sclerosis, may display similarities with peptides presented by MHC structures. Mimicry of human sphingolipids by lipopolysaccharides from *Haemophilus influenzae* may provoke the Guillain–Barré syndrome or Lewis-like polysaccharide antigens from *Helicobacter pylori* lead to atrophic gastritis in humans. The analogy can go beyond primary amino acid sequence homology and includes shapes in space (e.g. between arrangements of a three-domain coil of HIV-1 glycoproteins and contact areas of IL-2 with the α, β, and γ chains of the IL-2 receptor), considerably extending the possibilities of chain reactions, even if one

does not know to what extent in vitro studies can be extrapolated to autoimmunity in humans: 'Molecular mimicry is round every corner' (Regner and Lambert 2001, p. 185). The production of proinflammatory cytokines would play a decisive role. A genetic component might determine susceptibility to autoimmune diseases, and environmental factors play a role. Everything is possible if the worst does not necessarily happen. In the empirical frame of mind presented above, it has been suggested that autoimmunity can benefit self-maintenance and even be 'a cause of health' (Schwartz and Cohen 2000).

As autoimmune diseases involve stimulation against certain antigens in the individual, no wonder some concerns have arisen about the flirtation with infection called immunization or vaccination, which might also contribute to an autoimmune effect (Shoenfeld and Aron-Maor 2000). Vaccination does not differ substantially from casual immunization through infection, in that it stimulates various pathways depending on both the antigen and the host, and irreversible disorder may be created. The examples of hepatitis B and multiple sclerosis have recently been scrutinized, and the impact of immunization programs on the repertoire of the immune responses in populations taken seriously. The multiplication of vaccines has raised the iconoclastic question of whether they still offer the best strategy for preventing infections. Unfortunately, most of the time, autoimmune diseases have over the last few years been associated with immunization based almost exclusively on case reports – the weakest of scientific evidence.

Although immunization remains a major tool in the management of public health, the consideration of individual reactions, in conformity with the principle of precaution, is forcibly discussed in industrialized countries, where there is a move in favor of immunization 'à la carte'. These considerations reflect the individual and liberal mood of the last few years, with uncertainty about the consequences of this change, at economic, symbolic, or demographic levels.

In the sound and fury of molecular signaling, immunology remains in search of an innovative theoretical framework that would shelter its empirical work and substantiate its claims for solving medical problems and offering clues to general physiology, beyond the sole study of infectious diseases. Not satisfied with the elusiveness of the self/nonself topic, some immunologists have searched for phenomena that convey a general physiological meaning. These attempts have generated much interest and debate and also some confusion; if they stimulated imagination, they fell short of revolutionizing immunology.

The 'danger theory' brought forth by Polly Matzinger has been a flamboyant and significant episode of this quest for a new unifying trend. Matzinger considers that her 'danger theory' has undermined Burnet's classic view of self/nonself discrimination and the paradigm of clonal selection that ruled immunology for more than 30 years. She suggests that, in place of being sensitive to the discrimination between self and nonself, the immune system is in a state of permanent alarm, detecting cellular lesions, which she labels 'danger', after various events, ranging from exposure to tetanus toxin, to a pollutant interfering with the environment, and consequently reacting. In the nineteenth century, physiologists included, in the definition of inflammation, besides Celsius' triad of 'rubor–dolor–edema' (redness, pain, swollenness), '*functio laesa*' or impaired function. Matzinger aimed to discard teleological explanations and introduce simple mechanisms to account for accumulated data in cellular and molecular immunology. The use of the danger notion remains, however, as contextual and teleological as the self/nonself distinction and, even if it clashes with the almost excessive mystique of the self (Silverstein 1997), it does not induce any revolutionary changes in immunologists' theoretical thinking.

The emphasis on apoptosis has also played a role in bringing together different axes of research. The formation of small fragmented bodies, which contain remnants of nuclei, has been documented for a long time in scattered single cells. They were described for the first time in the liver in yellow fever as 'Councilman's bodies'. The Greek word *apoptosis*, used to describe the dropping of petals from flowers or leaves from the trees in autumn, was applied in 1972 by Kerr and collaborators to controlled cell deletion (Kerr et al. 1972) – a genetically programmed cell death, recognized as a vital biological phenomenon. The structural changes would comprise the formation of apoptotic bodies, and their subsequent degradation and phagocytosis.

Apoptosis appears to play an opposite, but also complementary, role to mitosis in the regulation of cell populations. It is determined by intrinsic clocks that are specific to the cell type involved, and plays an important role in the regulation of cell numbers in a variety of tissues under both physiological and pathological conditions. In 1991, Clem and Fechheimer described, in baculovirus (an insect virus), two proteins that inhibit molecules triggering cellular death. One of them at least would belong to a large molecular family operating throughout species. Most viruses such as the human papilloma virus, responsible in the long term for the occurrence of genital cancers, interfere with gene reparation and apoptosis. Anti-infectious defense, aging, and carcinogenesis seem so closely interrelated that any therapeutic or even preventive strategy is at risk of provoking undesired effects.

Apoptosis bears some analogies with Pasteur's hypothesis of 1882 that bacteria invading the organisms rapidly run short of food, a scorched earth tactic on the part of the organism. This theory was rapidly discarded and replaced by a more positive theory focusing on Metchnikoff's phagocytosis. Cell suicide triggered during primoinfection comes very close to the

idea of a mechanism that allows the pathogen to starve to death.

Apoptosis has been put in sharp focus by contemporary studies of the immune system. Immortalized cells may transform into cancer cells and give birth to lymphomas, if the immune system is weakened or strained. Apoptosis offers a clue to the mechanisms that determine whether the immune response will be positive or negative, activation or tolerance. In 1995, Douglas and Thomas Ferguson offered two mirroring images of apoptosis: in sanctuaries such as the eye and brain or the pregnant woman's womb, the cells of the immune system are forced to commit suicide, whereas, in the immune system, it is the infected cells that are forced to commit suicide (Green 1998).

Apoptosis captured the imagination in the scientific race for new ideas. *La Sculpture du Vivant* by Jean-Claude Ameisen, dedicated to apoptosis in immunology, refers to an episode of Odyssey on Ulysses in the Messina Straits (Ulysses attached to the mast in the ship listens to the mermaids' songs and wants to die with them; the sailors who do not hear them go on rowing). The myth illustrates the modern science of biological signals, the contradictory or alternate messages of which determine cell behavior.

With apoptosis as the key concept to the understanding of immunity as interference with cell suicide, we have to pay attention to the merging of two functions that are historically claimed by immunologists: infection control and cancer surveillance. The molecular description of a cycle of gene repressors leading to cellular death enables reconsideration of Burnet's past effort to associate them. The new orientation in anti-infectious strategy is no longer the mere destruction, either chemical or cellular, of the pathogen, but the exploitation of programmed suicide, by sending the cells a signal that is ordinarily repressed. The link between potent life and death metaphors, and the schemes of thought favored by scientists will remain a matter of debate for those who wish to elucidate the mechanisms of discovery and serendipity in research.

THE MOLECULARIZATION OF VACCINOLOGY

If new immunology is still uncertain about its theoretical foundations (is the self more than philosophical platitudes?), can it at least rely on its medical practical successes in the molecular era?

'The 1990s will be remembered as the decade of vaccines', a researcher from Connaught laboratories exclaimed in 1995, in Canada, during a congress on vaccines (Klein 1996, p. 295). And he went on to explain how research would update the classic vaccines and deliver new ones. The vaccine model had unified and stimulated the field of immunity studies and powerfully motivated scientists and governments. But the old models of war against germs appeared inappropriate in a sophisticated era where cognitive modes of thought tended to replace bellicose metaphors (Fox-Keller 1995).

Although the notion of species fixity has always been controversial (Amsterdamska 1987), vaccines were first conceived on the basis of a supposedly stable bacterial order. This order has been shattered. The constant mutations of flu viruses oblige us to invent new vaccines each winter. Bacteria or viruses, we have learnt, contain revertant individuals displaying the virulence of wild strains, or a new virulence, after mutations induced by the environment or through hybridization with other strains in a propitious environment. Reversion to virulence and polio cases that happen after oral immunization are probably the result of recombinations in the gut of the three vaccinal strains, or possibly between vaccinal viruses and other enteroviruses. A genetic drift makes strains of pertussis, the agent of whooping cough, diverge from the vaccinal prototypes, increasing the burden of the vaccinal enterprise.

The whole historical enterprise of immunization, notwithstanding its numerous 'hazards', was rather successful in the long run. Yet, molecularization was expected to fare better still and provide safer vaccines in diseases that were already controlled. Overall, it was advertised as coping with diseases refractory to immunization, such as leprosy or dengue fever, or new plagues such as AIDS.

Molecular vaccinology stands in sharp contrast to the former empirical methods of pathogen attenuation and is celebrated as the achievement of Landsteiner's ideal, with the replacement of crude preparations by molecules of exquisite specificity. Technical advances in gene splitting, gene sequencing, and protein engineering would allow the identification and optimization of new protective antigens against bacterial, viral, and parasitic diseases, on the basis of the most recent knowledge on the immune response pathways.

As vaccines played an important role in the popularity of immunology, although the development of immunology followed rather than caused the advent of early vaccines, molecularization – the shift from mere empiricism to rational design – was considered as the historical opportunity for immunology to produce at last ideal vaccines.

Several pathways offered for molecularization deserve comment:

- The first is the cloning of genes coding for virulence factors, islets grouping all sites involved in cellular invasion, toxin production, for the rational development of knock-out bacterial strains, or their introduction in vectors (Kourilsky 1987). Although BCG, administered soon after birth, avoids meningitis and disseminated infections in infants, it protects imperfectly against pulmonary tuberculosis in adult life. The new BCG projects plan to replace one of the most controversial vaccines by a modern one (Palfy et al. 1999).

- The second pathway is the recombinant subunit vaccines, the prototype for which is the hepatitis B vaccine.
- Genetic immunization is presented as an elegant way of making muscular tissue produce a vaccine.
- Finally, synthetic vaccines would mark the peak of innovation. The computerized invention of vaccinal structures, e.g. polypeptidic antigens, flatters the esthetic taste and comforts the quest for vaccines tailored to each individual's genetic make-up and lifestyle.

Adenoviruses were the first vectors in favor. They had been used as oral vaccines for respiratory infections since 1969. Then came the poxviruses (Panicali and Paoletti 1982). Cowpox, in use for two centuries, had allowed smallpox eradication. The first recombinant successfully produced as a vaccine was the canarypox rabies vaccine for dogs.

One of the great successes of molecular immunology was the production of recombinant vaccine against hepatitis B (Blumberg 1975). The research was fueled by anxiety linked to the use of hepatitis B carrier plasmas and the threat of transmission of an unknown virus undetected by the tests and undestroyed by inactivation techniques (Muraskin 1995). The recombinant vaccine obtained in vitro by expression of hepatitis B antigen in yeasts was licensed in 1986 (McAleer et al. 1984). Polysaccharidic vaccines protecting against capsular bacteria such as *Neisseria meningitidis* or *Haemophilus infuenzae* were also produced.

The programs have led more to a gathering of an unprecedented amount of information on immunity than to solving the practical issues that they had in mind. In spite of the adoption of current methods of molecular biology, reviews of the processes employed concluded that the choice of various preparations was still empirical. More clinical trials, especially in infants, were necessary to help understand 'conjugate immunology' (Moreau 1996, p. 148).

Expression of the foreign antigen by the carrier is not sufficient to elicit an immune response. The presentation of the antigen to the immune system depends on its physical location on the bacterial surface, and its accessibility is crucial for the magnitude of the immune response. Rather than choosing attenuated pathogens, it has been suggested that commensal microorganisms should be used to deliver vaccine antigens, but they then have to compete with the local flora. Molecular biology forces us to enter a realm always further away, toward an artificially produced environment. This strategy requires surveillance of its short- and long-term consequences, in terms of infection, morbidity, and mortality rates. Are we prepared to sustain this challenge, especially in countries where health services are inadequate or disorganized through internal or external conflicts?

In spite of its proponents' enthusiasm, it appears that the revolution has not yet borne all the expected fruit. Parasitic diseases do not give way, even the leishmaniases that had fostered well-founded hopes, since the early attempts at empirical immunization went back to the eighteenth century in the Middle East. The WHO has recently encouraged clinical trials for a modernized version of this legacy of the past (Mudabber 1989).

Genetic vaccines, although made attractive by their simple, cheap, and easy manufacture, seem to be inefficient and raise numerous queries about the advent of genes into the microenvironment of the body. The old adjuvant, alum, which is still in use, was recently charged as provoking untoward effects. There is an agreement about its replacement by synthetic molecules: this means that research starts again from zero, with clinical trials required for assessing the new adjuvants.

The rational approach paradoxically does not guarantee that the deleted bacterium will produce the desired immunity. Although usually passage of strains through animals leads to attenuation, biological engineering modifies pathogenicity without offering parallel constant immunogenicity. In any case, the new vaccine must be tested in phase I studies, without the benefit of more than one century of experimentation and results in the field of immunity. Molecularization leads to purity that is not synonymous with antigenicity, an event that could be expected, after revisiting early studies on haptens by Karl Landsteiner at the turn of the twentieth century. Landsteiner expressed the necessity of coupling small well-known chemicals, which he labeled as 'haptens', with large molecules playing an elusive role (carriers) in the triggering of the immune response.

The cytoxic lymphocyte (CTL) epitopes, supposed to provide clues for the treatment of cancer and of chronic infectious diseases, are not necessarily potent antigens, and subunit and peptide vaccines often behave as poor antigens.

The unification of vaccines under the banner of molecular immunology has not been completed to date, never mind the economic problems linked to the cost of new technologies and the difficulty of replacing old vaccines by new ones, bearing in mind modern regulations about innovation and their approval.

If molecularization has not resulted in a general program for safe and effective vaccines, is this for structural or historical reasons? The optimists argue that we just have to wait for appropriate tools and molecules, exquisite specificity retaining its long-term promises. The pessimists argue that immunization has reached its historical limits and will fail when confronted with pathogens that have evolved, or rather coevolved, with potent mechanisms for using the organism's cells and the immune system itself to survive and direct the components of the organisms to their profit: incorporation in the cell genome, fixation on receptors, use of chemokines as growth factors or decoys. Failures would occur

inexorably and the number of invading viruses (the 'emergence' phenomenon) would rise, to the point where it may be discussed whether we have reached a limit along this pathway in our capacity to deal with infections (Moulin 1996a). Have we not dealt in the first place with the easiest-going pathogens, those secreting a toxin (diphtheria, tetanus)? Does it remain possible to reach those that are more or less integrated into the nuclei of our cells? Killing infected cells may go too far and trigger an array of inflammatory phenomena.

This could change the very meaning of immunization and point to relative rather than absolute prevention, as is actually the case in AIDS trials where vaccine may be administered in the course of infection, to help the organism to cure the disease or reduce its severity. But this change in the biological meaning of vaccines would entail a dramatic revision in both popular and medical perceptions of immunization as a tool of public health, and would probably undermine its current applications.

THE IMMUNE SYSTEM AS A SUBSYSTEM OR A PART OF THE WHOLE

Jerne and others had already described formal analogies between the nervous and the immune systems, and suggested mathematical modeling for the sake of comparison (Lefkovits 1980). Molecularization of immunology, with the emergence of multiple families, has led to the relativization of the frontiers between organs and awareness of the narrow relationships among organs, cell lines, and systems. Compared with the nervous system, its paragon and model, the immune system, is first characterized by the mobility of its elements. Fridman (1992) called it the mobile brain. Cells of the immune system move continuously and this movement is essential for immunosurveillance and clonal selection, 'in the sea of microbes, toxins, parasites and mutagens we call Earth' (Anderson 1990, p. 14).

The immune system shares more than cursory similarities with other systems such as the endocrine or nervous systems. Bidirectional communication between them seems to occur as a result of common hormones and receptors. Revisited in the era of molecular biology, the list is impressive and ever increasing. Not only do neuroendocrine and immune tissues produce, communicate, and regulate a battery of informational molecules, but also there is growing evidence that these systems may use similar, if not identical, molecules to achieve their ends. Receptors for neuroendocrine molecules on immune cells and for lymphokines on neuroendocrine cells provide ample means for the two systems to interact. Cytokines are secreted by immunocompetent and glial cells. Neuroendocrine factors such as corticotropin-releasing hormone or growth hormone could have been qualified as cytokines if they had initially been described by immunologists, and molecules that were seemingly typically immune could have been qualified as neuropeptides if there had been timely identification by neurobiologists.

Receptors for cytokines present in the brain emit information that modulates the reaction to infection by pathogens. There are ligands for norepinephrine (noradrenaline) and other neuropeptides on the surface of macrophages, lymphocytes, and other immunocompetent cells. Immunoblotting has allowed the identification of receptors on immune and neural cells, ascertaining their molecular identity. β-Endorphin, which participates in analgesia during stress, can link with opioid receptors on lymphocytes (Dantzer et al. 1999). The humoral immune response is accompanied by an activation of the hypothalamic–hypophyseal–cortical–adrenocorticotropic axis and the rise of cortiscosterone in blood. A lesion of the hypothalamic–hypophyseal axis by radiofrequency suppresses the cytotoxic activity of natural killer (NK) cells in mice (Blalock 1997). Immune cells behave like a sensorial organ that is able to convey information to the brain.

These data suggest the importance of taking seriously not only the unity of the organism, but also the difficulty of interfering with its inner organization.

IMMUNOLOGY OF INFECTION: A POST-MODERN PERSPECTIVE

The immune system is a biomedical construct, the elaboration of which has proceeded over several periods, during which the understanding of its functions and its composition have been radically modified: after defense against germs, these functions were the self/nonself discrimination, tumor surveillance, participation in homeostasis, etc.

During the second half of the twentieth century, the microbial element lost its importance as the most immediate correlate of immune function, and the immune system appeared more and more able to respond to various stimulations and to interact with other systems that share the bodily space.

As we began to consider the complexity of microorganisms, and their considerable flexibility, resistance, and capacity to escape, disenchantment has set in to match the ingenious nature of parasites and viruses, strategies have become more sophisticated, clever, and costly. This observation opens on human history and evolution of the living world that coexist and collide under our eyes.

How will the new immunology meet the challenge of the dark infectious age agents? Not only had the microbial world accrued diversity, but also the pathways of pathogens into the organism were known to be multiple: how does the immune system deal with pathogens ranging from telluric bacteria, such as the tetanus bacillus secreting a toxin that can be absorbed *per os* without causing the least trouble, to viruses able to

invade the genome of the immune system and remain there silent for long periods of time before bursting out?

The return of infectious diseases has prompted the need for fresh knowledge and the urge to deepen both immunological and genetic components of the immune response. Not only did it revive the idea that heredity and immunity, or vertical and horizontal transmission, are two faces of the same coin, it also led to the idea that immunity is strongly integrated into many physiological and pathological responses.

With the new challenges posed by the return of infectious diseases, now that we are aware of the two-edged nature of most of our therapeutic tools and the ambivalence of most drugs, we can start to appreciate how the scientific revolution that is ritually celebrated has so far been incomplete, and to measure the extent of the work that lies ahead of us. There is a huge need for specific studies, but integrated approaches are also badly needed. It is a concern for the author of this chapter that the historical and philosophical approach attempted allows us to cast a critical glance over our accumulated knowledge and the seemingly impressive breakthroughs that deepen our knowledge, but also to move the future targets forward.

There is no such thing in history as a return to a former perspective. Even if the rallying cry of the fight against infectious diseases has sounded over these last few years for immunologists, it is by no means a return to the past. Contemporary immunology, with an emphasis on infectious diseases, can indeed be redefined as an immunity of infection rather than immunology of infections. Although infectious diseases are many, this chapter on the recent past of immunology spells 'infection' in the singular. Infection does not point to a separate chapter on pathology, but is a central episode corresponding not only to traditional infectious diseases but to many other disorders, from poorly understood autoimmune syndromes to diseases once labeled neurological, endocrine, or otherwise, and cancers. The advances made in the understanding of the immune system illuminate some crucial events in the genesis of disease and the fabric of normal life through all kinds of encounters with pathogens both internal and external. The immune system can be assimilated, in a sixth sense, for sensing pathogens, the key organ of interaction between the organism and its environment. The central position of immunology today in medicine echoes the centrality of infection as a crossroads in the economy of the organism.

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Phagocytes part 1: Macrophages

GORDON D. BROWN AND SIAMON GORDON

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Macrophages (Mϕ) play an essential role in anti-infectious immunity. They are key players in the innate immune system, detecting and responding to microbial invasion, restricting the spread of microbes, and recruiting other immune cells to the site of infection. Mϕs can act as accessory cells in secondary lymphocyte activation and as effector cells in cell-mediated immunity, and participate in humoral immunity by eliminating foreign antigens. The functions of Mϕs are enhanced when these cells are activated; thus activation represents a crucial step in anti-infective immunity and defects in this process result in an increased susceptibility to pathogens. Mϕs produce a number of anti-inflammatory molecules, which contribute to the resolution of inflammatory responses, and are also involved in many essential homeostatic functions, such as the clearance of apoptotic cells.

In this chapter we present an overview of Mϕ functions, as they relate to anti-infectious immunity. We describe the origins and types of Mϕs, the mechanisms they use for microbial recognition, uptake, and killing, as well as describing their interactions with the adaptive immune system through the release of cytokines and other soluble factors, and through the presentation of antigens. We explore Mϕ activation, their role in homeostatic processes, as it relates to infection, and briefly discuss Mϕ-induced pathology. With such an important role in anti-infective immunity, the Mϕ also provides an ideal niche for pathogens able to modify Mϕ functions, and we demonstrate some of the mechanisms that pathogens use to achieve these goals.

ORIGINS OF MACROPHAGES

Macrophages originate from blood monocytes which are generated in the bone marrow from monoblasts and promonocytes. Monocytes enter the bloodstream constitutively throughout life, accounting for 1–6 percent of the total normal adult white blood cell count (Ross and Auger 2002). These cells leave the bloodstream by adhering to endothelial cells through interactions of a variety of surface receptors, including β_1- and β_2-integrins, such as VLA-4 and LFA-1, which interact with immunoglobulin-domain-containing endothelial receptors, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Springer 1994; Yusuf-Makagiansar et al. 2002). Pathogen-derived molecules, such as lipopolysaccharide (LPS), and a number of inflammatory cytokines cause upregulation of these receptors, resulting in increased leukocyte delivery to sites of infection and inflammation (Roebuck and Finnegan 1999). After adherence, monocytes pass between the endothelial cells by diapedesis, involving interactions with other molecules such as CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)), and subsequently migrate into tissues where they differentiate into mature macrophages (Muller and Randolph 1999). A diagrammatic repre-

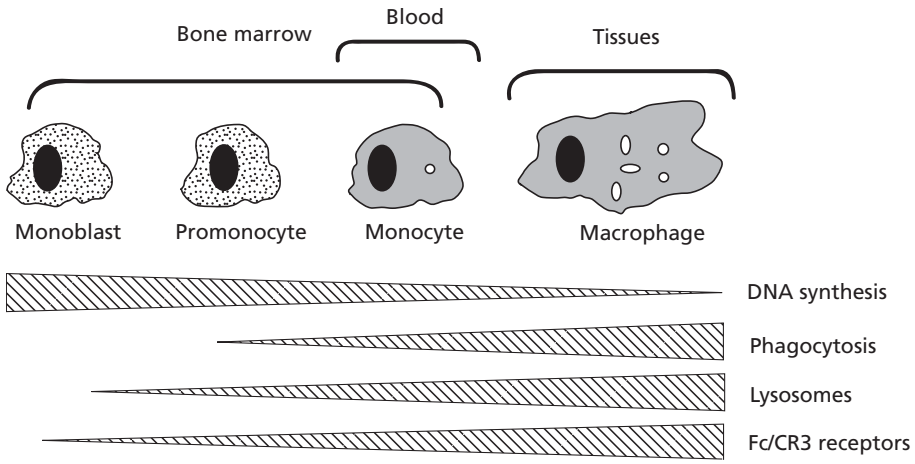

Figure 1 *The origins and characteristics of cells giving rise to tissue macrophages. (Adapted with permission from Auger and Ross 1992.)*

sentation of the development of tissue macrophages and some of their characteristics is shown in Figure 1.

Tissue macrophages display great functional and morphological heterogeneity, depending on their functional state as well as their interactions with other cells and molecules in the local microenvironment (Gordon 1999, 2001, 2003b). The characterization of these cells has been made possible by the development of tools such as monoclonal antibodies that recognize distinct macrophage proteins (Gordon et al. 1992; Gordon 1999). Resident populations of Mϕs are found at or close to portals of entry (e.g. lung, gut, skin), as sinusoidal cells in liver (Kupffer cells), as stromal cells in bone marrow, and spleen and lymph nodes, and as part of the neural parenchyma (microglia) (Crocker et al. 1991; Perry et al. 1993). They display considerable phenotypic microheterogeneity, e.g. in splenic red pulp, white pulp, and marginal zone, where blood-borne pathogens interact with specialized Mϕ subpopulations and other immune cells (Kraal 1992; Martinez-Pomares and Gordon 1999). An analogous subpopulation of Mϕs is present in the subcapsular region of lymph nodes, encountering potential antigens and dendritic cells in afferent lymph. An example of macrophage heterogeneity in the spleen is shown in Figure 2.

Monocytes can also differentiate into dendritic cells (DCs), specialized cells that have the unique ability to migrate out of tissues to secondary lymphoid organs and prime naïve T cells (Mellman and Steinman 2001). These cells are found in most tissues, such as the Langerhans' cells of the skin, and display functional and morphological heterogeneity. They have two distinct functional stages, based on their ability to take up and process antigens (the immature stage) and their ability to migrate to the lymphoid organs and activate antigen-specific T and B cells, directing the generation of the adaptive immune response (the mature stage). Although possessing unique abilities, many of the functions of DCs are similar to those found in macrophages, including their ability to recognize and internalize pathogens and their ability to recruit other immune cells, such as macrophages, neutrophils, natural killer (NK) cells and immature DCs, to the sites of infection. Aspects of this are covered below and in other chapters.

RECOGNITION OF PATHOGENS

During initial infection, the recognition of pathogens by macrophages and other phagocytes is reliant on germline-encoded molecules, termed pattern recognition receptors (PRR) (Janeway 1992). These molecules do not undergo the somatic mutation characteristically required by the adaptive immune response. As the name implies, these receptors recognize conserved microbial structures (the pathogen-associated microbial patterns (PAMP)), such as lipoteichoic acid (LTA) of gram-positive bacteria, LPS of gram-negative bacteria, and β-glucan of fungi. By recognizing these conserved structures, the host can recognize a variety of microbes with a limited set of receptors. Expression of these receptors is varied in different macrophage populations and can also be regulated by cytokines and other immune-modulating agents, including microbial components such as LPS (McKnight and Gordon 1998). In addition to pathogen recognition, the PRRs also determine the mechanism and route of cellular uptake, the response mediated by the Mϕ, and ultimately the immune response generated toward the pathogen.

The PRRs are either found free in the serum, as membrane receptors, or located intracellularly. A list of selected molecules in each of these classes, as well as their microbial ligands, is shown in Table 1. The serum-derived PRRs include the collectins, pentraxins, and complement, which coat the microbe (opsonization), allowing recognition and binding by opsonic receptors on host phagocytes. Specific antimicrobial antibodies,

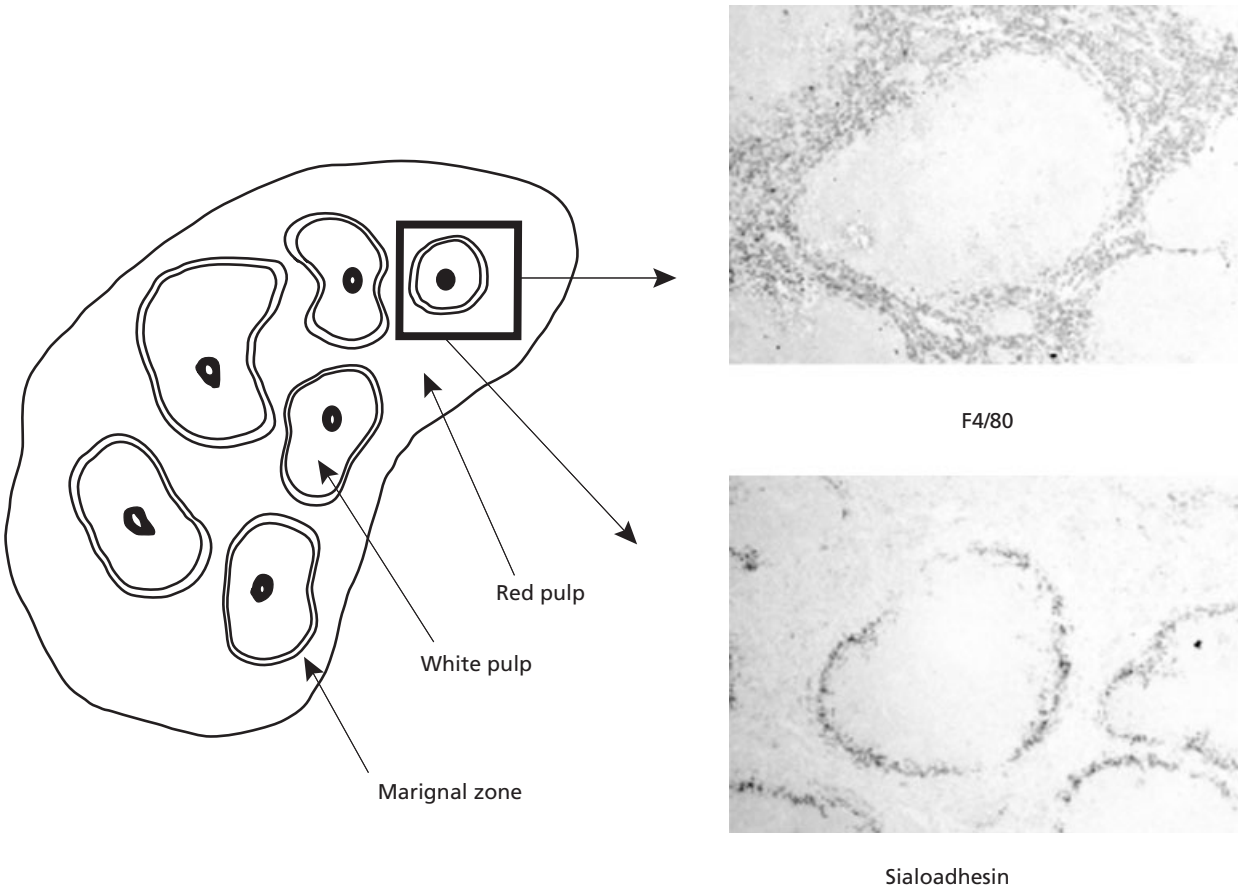

Figure 2 *Mouse spleen showing macrophage heterogeneity. Red pulp macrophage (Mϕ) can be detected with F4/80, whereas metallophilic Mϕs (in the marginal zone) can be detected with the anti-sialoadhesin antibody (3D6). (Images courtesy of Dr Luisa Martinez-Pomares, University of Oxford.)*

produced after the induction of acquired immunity, enhance the opsonic recognition of microbes. Although some of the opsonic receptors on host cells have been described, including the complement receptors and those that recognize the Fc portion of antibodies (the so-called Fc receptors) (Ravetch and Clynes 1998), many are still unknown. The nonopsonic or direct recognition and binding of microbes is mediated by a variety of membrane-bound PRRs on phagocytes, including classic and nonclassic C-type lectins, leucine-rich proteins, scavenger receptors, and integrins. Intracellular PRRs include the recently described 'nucleotide-binding oligomerization domain (NOD)' proteins as well as a variety of proteins involved in the recognition of viruses. It should be remembered that the in vivo recognition of microbes probably involves coordinated recognition by many of these receptors, e.g. binding of *Mycobacterium tuberculosis* to macrophages has been shown to involve the complement and mannose receptors, as well as CD14 and the surfactant protein A (collectin) receptor (Ernst 1998).

Although less well characterized, molecules other than the PRRs can also be used by pathogens to bind to and/or enter Mϕs. This is especially true for viruses that make use of cellular molecules, such as the proteoglycans, or other receptors, such as the chemokine receptor CCR5 used by HIV (Miller and Gordon in press). The use of these 'nonimmune' molecules to gain entry into cells enables pathogens to bypass the antimicrobial effects normally generated by the PRRs.

While the binding and internalization of microbes is mediated by various PRRs, generation of the appropriate responses has been ascribed to a recently identified family of receptors, the Toll-like receptors (TLRs). These receptors were originally identified by homology to a receptor in *Drosophila*, and are a family of receptors with homology to the interleukin (IL) 1 (IL-1) receptor (Hoffmann and Reichhart 2002). The various TLR family members can distinguish between various pathogens, often by acting in combinations, and initiate signaling cascades similar to those induced by the IL-1 receptor, via MyD88 and IRAK to activate the factor NFκB, resulting in the production and/or release of proinflammatory mediators and cytokines (Medzhitov 2001; Underhill et al. 1999; Ozinsky et al. 2000). Although a number of TLRs and their ligands have been identified, they all appear to

Table 1 *Microbial pattern recognition molecules*

Location	Family	Member(s)	Selected microbial ligand(s)	Reference(s)
Serum	Collectins	SP-A, SP-D	Influenza A virus, herpes simplex virus, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Escherichia coli*, *Pneumocystis carinii*, *C. neoformans*, *Aspergillus fumigatus*	Crouch (1998); Haagsman (1998)
		Mannose-binding lectin	HIV, influenza A virus, *S. aureus*, *Neiserria meningitidis*, *Chlamydia pneumoniae*, *Candida albicans*, *Cryptococcus neoformans*, *A. fumigatus*	Kilpatrick (2002)
	Pentraxins	C-reactive protein, serum amyloid P	*S. aureus*, *E. coli*, *Streptococcus pyogenes*, *N. meningitidis*, *A. fumigatus*, *C. albicans*, *Plasmodium falciparum*, *influenza A virus*, LPS	Noursadeghi et al. (2000); Szalai (2002)
	Complement	C1q	Antibody-coated microbes, *Listeria monocytogenes*, *Legionella pneumophila*, *E. coli*, HIV	Nicholson-Weller and Klickstein (1999); Stoiber et al. (2001)
		C3	Microbial surfaces	Taylor et al. (1998)
	Lipid transferases	LBP	LPS	Jack et al. (1997)
Membrane bound	Classic C-type lectins	Mannose receptor	*Candida albicans*, *P. carinii*, *Mycobacterium tuberculosis*, *K. pneumoniae*, *Leishmania donovani*, HIV-1, zymosan	East and Isacke (2002)
		DC-SIGN	HIV, Ebola virus, *Leishmania* spp.	Geijtenbeek et al. (2002)
	Non-classic C-type lectins	Dectin-1	β-Glucans, zymosan, *Saccharomyces cerevisiae*, *C. albicans*	Brown and Gordon (2001)
	Leucine-rich proteins	CD14	*E. coli*, LPS, LTA, peptidoglycan	Landmann et al. (2000)
		Toll-like receptors (1–10)	LPS, LTA, zymosan, bacterial lipoproteins, peptidoglycan, viral proteins, flagellin, bacterial DNA	Medzhitov (2001)
	Scavenger receptors	SR-A (I and II), LOX-1, MARCO	*E. coli*, *S. aureus*, *L. monocytogenes*, *M. tuberculosis*, *Enterococcus faecalis*, *N. meningitidis*, LPS, LTA, bacterial DNA	Peiser et al. (2002); Shimaoka et al. (2001); Sankala et al. (2002)
	Integrins	CR3, CR4	Complement-coated microbes, LPS, LPG, *C. albicans*, *M. tuberculosis*, *C. neoformans*	Ehlers (2000); Ingalls and Golenbock (1995); Taborda and Casadevall (2002)
Intracellular	NODs	NOD-1, NOD-2	LPS, *S. flexneri*	Inohara et al. (2002)
	Interferon-induced proteins	PKR, OAS, ADAR1	Viral dsRNA	Samuel (2001)
		Mx GTPase	Viral–protein complexes	Ponten et al. (1997)

LPG, lipophosphoglycan; LPS, lipopolysaccharide; LTA, lipotechoic acid; NODs, nucleotide-binding oligomerization domains.

signal via the same pathway and it is therefore not yet understood how specific responses to each pathogen are initiated. There is some evidence that response specificity is mediated by specific intracellular adaptors, such as TIRAP, and also by the PRRs involved in recognition and binding (Engering et al. 2002; Horng et al. 2002).

It should be remembered that Mϕs also possess many other surface receptors that play a role in the response to infection, but are not involved in microbial recognition. These receptors include the cytokine and chemokine receptors, as well as receptors involved in migration, adhesion, and antigen presentation. Some of these aspects are covered in more detail below and in later chapters.

INGESTION OF MICROBES

Macrophages have a prodigious capacity to ingest microbes and are capable of engulfing very large particles. This ability may be the result of their, apparently unique, ability to utilize membrane from the endoplasmic reticulum (ER) for this uptake (Gagnon et al. 2002). Ingestion is initiated by binding of microbes to the Mϕ surface receptors, which triggers the transmembrane activation signals leading to their internalization. For microbes larger than 0.5 μm, internalization occurs through a heterogeneous actin-dependent process called phagocytosis (Aderem and Underhill 1999). Many of the details of these processes are incompletely understood and made more

complex by the fact that microbes are recognized by more than one receptor, each of which may be dictating a different mechanism of uptake. The receptors themselves may also interact with other surface receptors, such as CD172a (signal regulatory protein alpha (SIRPα)) (Oldenborg et al. 2001), either stimulating or inhibiting uptake.

The understanding of phagocytosis, first observed by Elie Metchnikoff in the 1890s, is currently based on the study of two receptors, the Fcγ and complement receptors. The Fcγ receptor (FcγR) mediates what is known as the zipper model of phagocytosis, whereby actin-rich pseudopods extend over the particle surface through the sequential binding of cell surface receptors to the microbe, ultimately engulfing the entire particle (Griffin et al. 1975, 1976). This process is initiated by receptor clustering, occurring from interactions with the microbe, and leads to the phosphorylation of tyrosine residues in specific motifs (the immunoglobulin gene family tyrosine activation motifs) of the cytoplasmic tail of the receptor (Daeron 1997). These phosphorylation events are dependent on cytoplasmic enzymes, the src kinases, and result in the sequential recruitment and signaling via a variety of other molecules, including Syk, Cdc42, and Rac, which results in actin assembly, membrane protrusion, pseudopod extension, and finally closure of the phagocytic cup (Caron and Hall 1998; Aderem and Underhill 1999).

Complement receptor (CR)-mediated phagocytosis is less well understood but is known to require an additional stimulus to occur, such as tumor necrosis factor α (TNF-α) or attachment to extracellular matrix proteins. In contrast to the FcγR, the particle is not engulfed by pseudopods but rather appears to sink into the cell, forming a phagosome where the membrane is less adherent, held by point-like contacts to the microbe (Kaplan 1977; Aderem and Underhill 1999). CR-mediated phagocytosis also requires intact microtubules, uses different signaling molecules, such as Rho, and is not blocked by inhibitors of tyrosine kinases (Allen and Aderem 1996; Caron and Hall 1998).

Microbes can be ingested through a variety of other mechanisms, including macropinocytosis, coiling phagocytosis, and endocytosis. Macropinocytosis describes a process whereby membrane ruffles form a large spacious vacuole around the adjacent microbe, leading to subsequent ingestion (Rittig et al. 1999). In coiling phagocytosis, there is an initial extension of a single pseudopod, which wraps around the microbe. Subsequent pseudopods form, giving the characteristic whorl-like appearance, which ultimately fuse to form the phagosome (Rittig et al. 1999). Endocytosis is a heterogeneous group of cellular processes used for the internalization of particles smaller than 0.5 μm, such as viruses, and includes caveolae- and clathrin-mediated endocytosis (Gruenberg 2001; Shin and Abraham 2001; Sieczkarski and Whittaker 2002).

Receptor-mediated endocytosis and phagocytosis are used for homeostatic functions by Mϕ and to clear up the debris released by infection, including necrotic and apoptotic cells. In addition, these mechanisms are used or subverted by a variety of pathogens, the study of which has given many insights into the molecular mechanism underlying these processes.

After internalization, the phagosome matures through a number of sequential steps involving interactions with the endocytic pathway (Desjardins et al. 1994). This consists of extensive vesicle budding and fusion, controlled by a variety of cytoplasmic proteins including the rab guanosine triphosphatases (GTPases) (Aderem and Underhill 1999). During maturation the phagosome moves along the microtubule network from the periphery to a perinuclear location. As it matures, the phagosome acquires hydrolytic enzymes, such as cathepsin-D, and the proton pump adenosine triphosphatase (ATPase), which contributes to a lowering of the phagosomal pH. The late stages of maturation involve fusion with lysosomes generating a low pH phagolysosomal compartment containing a variety of degradative lysosomal hydrolases (Alvarez-Dominguez et al. 1999). The process of maturation therefore generates an increasingly antimicrobial environment in which the ingested microbe is ultimately killed and digested. A simplified schematic diagram of these processes is shown in Figure 3.

ANTIMICROBIAL MECHANISMS

Macrophages possess a variety of antimicrobial mechanisms including the use of toxic metabolites, enzymes, and peptides, as well as through physical restraint of the pathogens, by ingestion into phagosomes, or through the formation of a granuloma. Most organisms are killed within the phagosome after phagocytosis, through a number of mechanisms that can work synergistically. The maturing phagosome generates an increasingly antimicrobial environment, achieved through lowering of the phagosomal pH, limitation of nutrients required for microbial growth, and generation of reactive metabolites. After fusion of the phagosome with lysosomes, there is also the acquisition of hydrolytic enzymes, antimicrobial proteins, and peptides, such as the membrane-permeabilizing peptide defensins, which have a broad spectrum of microbial targets, and lysozyme, which attacks the peptidoglycan layer of bacterial cell walls.

The confinement of microbes within the phagosome acts as a physical barrier to the microbe, limiting the availability of essential nutrients required for microbial growth, such as iron. The supply of nutrients to the phagosome is further restricted by active mechanisms within the cells. The transferrin receptor, for example, which transports iron via the endosomal pathway, is downregulated upon infection whereas another molecule, natural resistance-associated macrophage protein-1

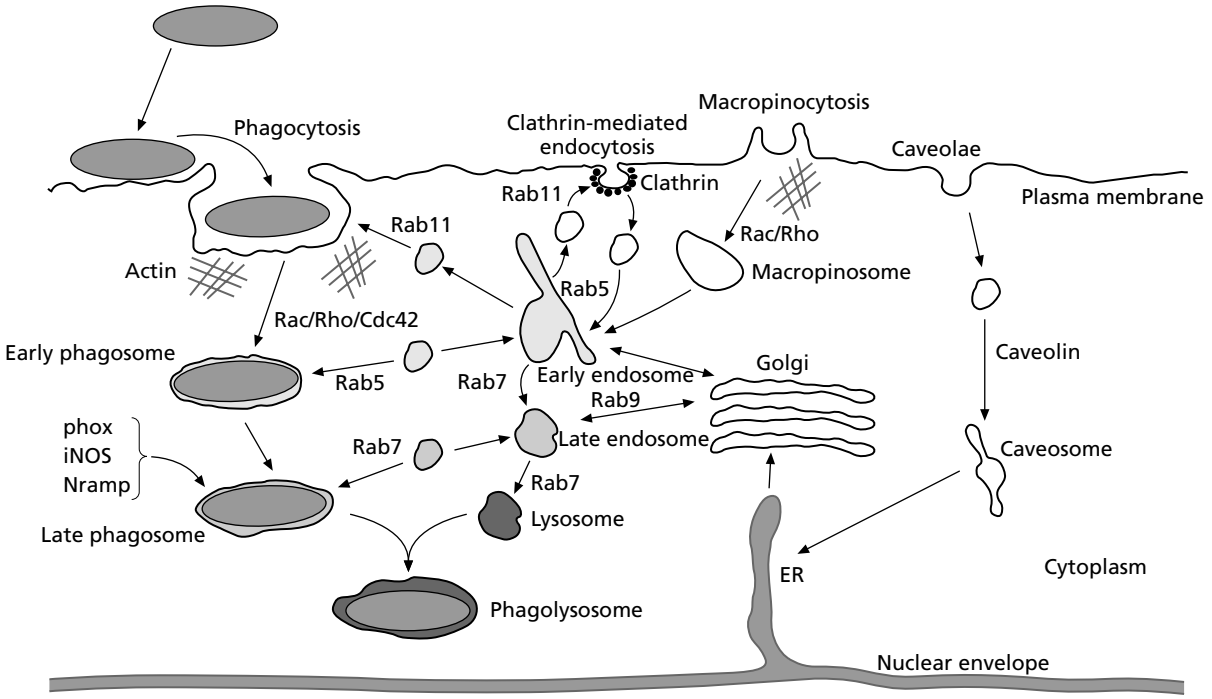

Figure 3 *A schematic representation depicting the various routes used by macrophage (Mϕ) to internalize microbes. Some of the molecules associated with this process are also indicated. (Adapted in part from Sieczkarski and Whittaker 2002.)*

(Nramp-1), is recruited to the phagosome, ensuring removal of iron and other essential divalent cations (Gruenheid et al. 1997; Sunder-Plassmann et al. 1999; Forbes and Gros 2001).

The production of reactive oxygen intermediates (ROIs), described as the respiratory burst of Mϕs, is a very efficient antimicrobial defense. Superoxide ion ($O_2^{-\bullet}$) is generated by the activated, membrane-associated, protein complex, the phagocyte reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (phox), which transfers electrons from NADPH to O_2. Toxic oxidants, including hydroxyl radicals and hydrogen peroxide, are then generated in the phagosomal lumen, and kill the internalized organism (Hampton et al. 1998). The phox proteins may also assemble in the plasma membrane, resulting in the generation of extracellular toxic oxidants, which may also contribute to pathology. Superoxide ions can also act as a signal for the activation of granule proteases in neutrophils (Reeves et al. 2002). Activation and localization of the oxidase complex are determined by the cellular receptors involved in recognition of the pathogen, and involve numerous signal transduction cascades that result in phosphorylation of one of the cytosolic phox proteins ($p47^{phox}$) (Karlsson and Dahlgren 2002). Chronic granulomatous disease (CGD) is a disease characterized by recurrent infections with a number of pathogens, and results from mutations in the phox proteins, highlighting the importance of this antimicrobial mechanism for the host (Goldblatt and Thrasher 2000).

Myeloperoxidase (MPO) is a major neutrophil granule protein that is also found in monocytes. MPO generates hypochlorous acid from hydrogen peroxide, one of the most potent antimicrobial agents produced (Winterbourn et al. 2000). Although MPO is not produced in Mϕs, it can be taken up via the macrophage mannose receptor and is known to augment macrophage antimicrobial activity (Shepherd and Hoidal 1990; Marodi et al. 1998).

The inducible nitric oxide synthase (iNOS) of phagocytes is another important enzyme contributing to the antimicrobial actions of Mϕs. Induction of iNOS leads to the production of nitric oxide (NO), through the oxidative deamination of l-arginine, which subsequently reacts with superoxide or thiol groups to produce antimicrobial compounds, such as peroxynitrite and nitrosothiols (Burgner et al. 1999). The production of NO by iNOS requires homodimerization and interaction with many other molecules, including calmodulin, and NADPH. Microbial products, such as LPS, and cytokines, such as TNF-α or interferon γ (IFN-γ), can act either individually or synergistically to induce the production of iNOS (MacMicking et al. 1997). The use of iNOS inhibitors and knockout mice has clearly demonstrated the importance of this antimicrobial mechanism in the control of many infections (Wei et al. 1995).

Macrophages possess two other notable features that play a role in antimicrobial activities, that of cellular activation and apoptosis. Apoptosis, or programmed cell death, is an important defense mechanism limiting the spread and growth of intracellular organisms. Mϕs, which themselves undergo apoptosis during certain infections, are key players in the clearance of apoptotic bodies and are able to induce this process in other cells (Geske et al. 2002). The role of apoptosis in infection is not always so clear, however, because in some cases apoptosis appears to favor the pathogen (Weinrauch and Zychlinsky 1999; Navarre and Zychlinsky 2000). Finally, macrophage activation is extremely important for the control of infection, because activation, via cytokines or microbial products, enhances the antimicrobial activities of these cells.

MACROPHAGE ACTIVATION

The ability of macrophages to become activated is critical for the resolution of infection. This term has been loosely applied to describe the selective, stereotypical responses of Mϕs to different stimuli, mainly of a microbial or immunological nature. Recent gene array studies have started to illustrate the diversity of genes expressed in various infectious and other experimental models. Earlier phenotypic characterization made it possible to discern the following types of cell activation: innate activation, classic immune activation, and alternate activation. Finally, the control of this process, through the deactivation of Mϕs, is critically important to reduce pathology.

Innate activation, induced by microbial products and intact microorganisms, is mediated by specific receptors and signaling pathways described already, and results in altered pro- and anti-inflammatory metabolite secretion, enhanced expression of co-stimulatory molecules, and induction of plasma membrane receptors such as MARCO, a type A scavenger receptor (Gordon 2001). Thus, antimicrobial and immune functions are induced, as well as cell migration and dendritic cell maturation.

Classic immune activation was originally described by Mackaness as enhanced, antigen non-specific, antimicrobial activation of Mϕs during *Listeria monocytogenes* and BCG (mycobacterial) infection (Gordon 1999, 2003b). This can now be ascribed to IFN-γ, acting on specific Mϕ receptors and their signaling pathway (Gordon 1999, 2003b). IFN-γ is produced by natural killer (NK) cells and activated T lymphocytes in response to cytokines produced by antigen-presenting cells (APCs), IL-12, IL-18, and IL-15. Genetic studies in mouse and humans have confirmed the importance of this pathway in cellular immunity to infection by mycobacteria and other selected pathogens. Secondary deficiency contributes to opportunistic infections in AIDS. In addition, IFN-γ induces major histocompatibility complex class II (MHCII) and iNOS expression and primes Mϕs to secrete high levels of mediators in response to microbial stimuli.

Alternate immune activation can be induced by the cytokines IL-4 and IL-13, acting on a common receptor subunit and resulting in a distinct program of gene expression in Mϕs to promote humoral immunity, especially against extracellular parasites (Gordon 2003a). Phenotypic markers include elevated MHCII, enhanced mannose receptor, and, in some situations, giant cell formation. Certain chemokines (MDC and TARC) and a range of other markers are also selectively upregulated. Inducible NOS is downregulated, whereas l-arginine metabolism is switched to favor collagen production by fibroblasts, and repair.

Deactivation is mediated by cytokines such as IL-10 and transforming growth factor β (TGF-β) which profoundly downregulate Mϕ proinflammatory, immunological, and cytotoxic activities (Gordon 2003a). The balance between activation and deactivation is critical in preventing host injury, as illustrated in inflammatory bowel disease and genetic deficiency of the above cytokines. New findings have highlighted the importance of inhibitory receptors on the Mϕ surface, such as SIRPα, which limit activation of Mϕ. Distinct receptor pairs that regulate Mϕ activation include CD200/CD200R – microglia become spontaneously activated in CD200-deficient mice; experimental autoimmune disease is regulated, in part, by this pathway. Important intracellular regulatory systems include the suppressors of cytokine synthesis (SOCS) family and type I interferons. The uptake of apoptotic cells downregulates inflammatory responses of Mϕs.

SECRETION OF SOLUBLE MEDIATORS AND OTHER FACTORS

In addition to the secretion of ROIs, described above, Mϕs produce and secrete a variety of other products either constitutively or after stimulation. These include factors that are involved in homeostasis, such as pro- and antiangiogenic factors controlling vascular supply, or those, such as lysozyme, that are involved in combating infection. Other secretion products include enzymes, such as lysosomal hydrolases, proteases, and lipases, some of which help to maintain extracellular matrix balance, and promote and interact with plasma protein cascades (coagulation, fibrinolysis, complement, kinin generation) in response to injury (Gordon 2003b). Mϕs produce complement components, which opsonize microbes and are major producers of arachidonic acid intermediates (Ross and Auger 2002).

Macropages produce cytokines and chemokines, which recruit other immune cells to the site of infection and which, along with antigen presentation to lymphocytes, help direct the generation and type of the adaptive immune response. Cytokines are central molecules of the immune response, forming a complex network that

Table 2 *Selected cytokines produced by or having an effect on macrophages (Mϕs)*

Cytokine	Major producer	Effect
IL-4/IL-13	T cells	Alternate Mϕ activation
		↑ MHCII, B7-1, B7-2
		↑ giant cell formation
		↓ IL-1, TNF-α, IL-6, IL-12
IL-10	Mϕ	Mϕ deactivation
	DC	↓ IL-1, TNF-α, IL-6, IL-12
	Activated T and B cells	↓ MHCII, B7-1, B7-2
IL-12	Mϕ	T-cell proliferation
	DC	$CD4^+$ Th1 differentiation
	Others	↑ IFN-γ
		↑ CTL activity
IFNγ	T cells	Mϕ and PMN activation
	NK cells	$CD4^+$ Th1 differentiation
		B-cell isotype switching (IgG2a, IgG3)
		↑ NK cell cytolytic activity
		↑ vascular adhesion molecules
		↑ MHCI and MHCII
		↑ TNF-α, IL-1, IL-12, IFN-β
		↓ IL-10
IL-18	Mϕ	T-cell proliferation
		$CD4^+$ Th1 differentiation
		↑ IFNγ
		↓ IL-10
IFN-α/β	Mϕ	Mϕ and NK activation
	Others	↑ CTL activity
		↑ MHCI
TNF-α	Mϕ	Mϕ and PMN activation
	Others	Inflammatory cell recruitment
		↑ vascular adhesion molecules
		↑ MHCI
		↑ acute phase proteins
		↑ T-cell apoptosis
TGF-β	Platelets	Monocyte activation
	Mϕ	Mϕ deactivation
		↑ extracellular matrix proteins
		↑ selected growth factors

CTL, cytotoxic T lymphocyte; DC, dendritic cell; Th1, T-helper cell 1; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; PMN, polymorphonuclear neutrophil; MHC, major histocompatibility complex; Mϕ, macrophage; NK, natural killer; TGF, transforming growth factor; TNF, tumor necrosis factor.

mediates and regulates many local and systemic immune functions. Cytokines bind to their cognate cell surface receptors and signal through a variety of pathways, including the Janus-family kinase (Jaks) and signal transducer and activator of transcription (Stat) proteins (Imada and Leonard 2000). The properties and characteristics of a few selected cytokines are listed in Table 2. Many of those listed are involved in macrophage activation (IL-4, IL-13, IL-12, IL-18, and IFN-γ) or deactivation (IL-10 and TGF-β). Cytokines, such as TGF-β, also play an important role in the suppression and resolution of the immune response (Ashcroft 1999).

Chemokines, or chemotactic cytokines, are small, structurally related peptides that are recognized by seven transmembrane G-protein-coupled receptors on their target cells (Thomson 1998). They are involved in a variety of homeostatic functions, including organogenesis and hemopoiesis, but also function to recruit neutrophils, monocytes, immature dendritic cells, and activated T cells to the sites of infection (Olson and Ley 2002). Chemokines are highly basic proteins and can bind to glycosaminoglycans on extracellular matrix surfaces, generating gradients that attract leukocytes and promote their attachment and diapedesis through vascular endothelium, as described above. Chemokines are divided into four families, based on the number and position of conserved cysteines: C, CC, CXC, and the CX3C chemokines. In general, CXC chemokines, such as IL-8, attract neutrophils, whereas CC and C chemokines, such as monocytic chemotactic peptide 1 (MCP-1)

and lymphotactin, attract monocytes and lymphocytes. Chemokines can also induce the release of proinflammatory mediators and stimulate the respiratory burst.

ANTIGEN PRESENTATION

Antigen presentation, along with cytokine production, is a critical component of innate immunity required for the generation of the adaptive immune response and resolution of most infections (Fearon and Locksley 1996). Mϕs present antigens only to primed T cells, whereas DCs have the ability to prime naïve T cells (Mellman and Steinman 2001). Antigen presentation results in the generation of pathogen-specific immunity and specific subsets of T cells. These subsets include the $CD4^+$ T cells, which are involved in the resolution of intracellular (T-helper (Th) 1 cells) and extracellular (Th2 cells) pathogens, and $CD8^+$ T cells, which are involved in killing cells infected with cytosolic pathogens. Both $CD4^+$ and $CD8^+$ T cells can activate the antimicrobial activities of Mϕs, although $CD4^+$ Th2 cells normally mediate Mϕ-independent responses to extracellular parasites, such as helminths.

Antigen presentation requires the noncovalent association of the antigen with MHC molecules. This association occurs via two main routes, through loading of antigens onto MHCII, which have been ingested and degraded in the lysosomes, and loading of antigens onto MHCI, which are derived from the cytosol. This is an oversimplified scheme, however, because lysosomally derived antigens can be loaded on MHCI molecules, although the mechanisms involved are less clear. Antigens loaded onto MHC molecules are normally peptides, but lipids and glycolipids can be presented through MHC-like molecules to T cells. We describe the various cellular mechanisms in antigen loading and presentation to T cells, including a brief description of the co-stimulatory molecules and cytokines involved.

MHCI is expressed by nearly all cells and presents cytosolic peptides to cytotoxic $CD8^+$ T cells (Guermonprez et al. 2002). These peptides are generated by the proteasome and are translocated into the ER by transporter associated with antigen processing (TAP) protein where they associate with the MHCI molecules before transport to the cell surface. As the presented peptides originate from the cytosol, MHCI presentation is important in the control of cytosolic pathogens, such as viruses.

MHCII is expressed by Mϕs, DCs, and B cells and presents exogenously derived peptides to $CD4^+$ T-helper cells (Guermonprez et al. 2002). MHCII is upregulated after activation of Mϕs and on maturation of DCs. Exogenous antigens released from the degradative late endosomal and/or phagosomal compartments are thought to be loaded onto MHCII in the MIIC compartment. MHCII molecules are associated with an invariant chain (Ii), which prevents association with endogenous peptides during synthesis in the ER and Golgi body. Ii is removed by proteolysis and the actions of an auxiliary molecule, HLA-DM, in the MIIC compartment, allowing the binding of foreign peptide and transport to the cell surface. In certain circumstances peptides can be also be loaded onto MHCII in the ER, early endosomes, and at the plasma membrane (Robinson and Delvig 2002). As peptides derived from the endosomal/phagosomal pathway are presented through MHCII, this presentation pathway is, however, critical for the control of intracellular pathogens.

Antigen can also be presented to T cells through two other classes of molecules: MHCIb and CD1 (Porcelli and Modlin 1999; Schaible et al. 1999). MHCIb present short *N*-formylmethionine-containing peptides of bacterial and/or mitochondrial origin to $CD8^+$ T cells. Although MHCIb is structurally similar to MHCIc, the processing and peptide-loading mechanisms are unclear and may be TAP independent. Also structurally related to MHCI is the CD1 family of proteins, consisting of five members, four of which (CD1a, -b, -c, -e) are found only in humans. Most of these proteins, expressed mainly by activated Mϕs, DCs, and B cells, are capable of presenting microbial lipid and glycolipid antigens, such as mycolic acids from mycobacteria, to cytotoxic $CD4^-$ $CD8^-$ T cells and possibly also $CD8^+$ T cells. Antigen loading is TAP independent.

In addition to T-cell receptor (TCR) recognition of antigen presented on MHC molecules, the activation and proliferation of T cells requires a 'second' signal (Guermonprez et al. 2002). This additional signal is mediated by co-stimulatory molecules on the APCs, including CD40, B7-1, and B7-2 which interact with CD40L, CD28, and CTLA4 on the lymphocytes. These interactions activate the APCs and mediate the type of $CD4^+$ T-helper cell (Th1 or Th2) produced.

The differentiation of $CD4^+$ T cells into Th1- or Th2-type cells is also mediated by cytokines. Activation of DCs in the periphery and crosslinking of CD40 during the presentation of antigens induce IL-12 production, which biases the development of naïve T cells to the Th1 type, suppressing the production of Th2-type cells. Conversely, IL-4 stimulates the production of Th2-type cells, while suppressing Th1-type development. The driving factors behind Th2 development are still unknown, leading some investigators to propose that Th2 is the default pathway. The role and generation of Th1 and Th2 T cells in infection are discussed in greater detail in later chapters.

Macrophages and other APCs have the ability to ingest apoptotic or necrotic cells and present the antigens they contain in a process known as cross-presentation, or crosspriming (Heath and Carbone 2001). These antigens can be presented on both MHCI and MHCII molecules by a process that is influenced by

several factors in vivo, including antigen dose, age of the host, and tissue type. It should be noted that cross-presentation of cellular antigens can also lead to T-cell tolerance (cross-tolerance).

SUBVERSION OF MACROPHAGE FUNCTION BY PATHOGENS

Although Mϕs are very efficient at recognizing and destroying microbes, pathogens have evolved numerous strategies to avoid or subvert the antimicrobial functions of these leukocytes. Pathogens can evade or promote recognition through specific PRRs, and they can block or modify the phagocytic pathway, the antimicrobial mechanisms, the production of soluble mediators, and the ability of Mϕs to present antigens. Studies of these microbial subversion strategies have, however, greatly aided our understanding of the basic mechanisms underlying the cellular processes in Mϕs and other leukocytes.

Pathogens have developed mechanisms to avoid phagocytosis or to direct phagocytosis through specific receptors. Phagocytosis mediated by FcγR, but not CR3, for example, results in the production of proinflammatory mediators and induces the respiratory burst (Underhill and Ozinsky 2002). Consequently, CRs are commonly used by intracellular pathogens, such as *Mycobacterium tuberculosis*, to gain access to host cells (Ernst 1998). Pathogens may induce entry into macrophages, such as salmonellae, which stimulate macropinocytosis by transferring proteins into the host cytosol using a type III secretion system (Goosney et al. 1999). Other pathogens actively block phagocytosis, such as *Yersinia* which use a type III secretion system to transfer proteins to block the signaling mechanisms that control cytoskeletal rearrangements (Fallman et al. 1995; Cornelis 2002).

Macrophages also provide numerous niches for intracellular pathogens, which have evolved a variety of mechanisms for survival within these cells. Some pathogens interfere with phagosomal maturation, such as *Mycobacterium tuberculosis*, which arrests maturation at an early stage (Armstrong and Hart 1971). Pathogens such as *Legionella*, are able to convert the phagosome to a unique vacuole, whereas others, such as *Shigella* spp., escape the phagosome completely and reside in the host cytosol (Portnoy et al. 1988; High et al. 1992). Some pathogens, such as *Leishmania* and *Coxiella* spp., have even adapted to life within the harsh environment of the phagolysome (Chang and Dwyer 1976; Heinzen et al. 1996; Rabinovitch and Veras 1996).

The antimicrobial mechanisms of Mϕs present other obstacles that have been overcome by pathogens, leading to the development of a number of resistance mechanisms. Pathogens overcome the acidification of the phagosome by excluding the vesicular proton pump ATPase, such as occurs with mycobacteria, or escape into the cytoplasm, as mentioned above (Sturgill-Koszycki et al. 1994). Essential nutrients can be acquired by, for example, secreting siderophores, which bind iron with a higher affinity than host molecules, such as the mycobactins and exochelins of mycobacteria (De Voss et al. 1999). The respiratory burst can be avoided by entry through specific receptors, as discussed, but it can also be suppressed by inhibiting the activation cascades, as occurs with *Yersinia* spp. (Cornelis 2002). Microbes, such as salmonellae and mycobacteria, protect themselves from toxic oxidants and reactive nitrogen intermediates by producing enzymes, such as catalase, glutathione reductase, alkyl hydroperoxide reductase, and superoxide dismutase, which break down these compounds (Storz et al. 1990; Manca et al. 1999).

Pathogens are able to modulate the ability of Mϕs to interact with the rest of the immune system, by affecting their ability to produce cytokines and to present antigens. Pathogens such as the pox and herpes viruses produce virokines and viroceptors, which mimic cytokines and cytokine receptors, whereas pathogens such as mycobacteria can deactivate Mϕs, making them refractory to activating cytokines, and induce the production of the anti-inflammatory cytokine, TGF-β (McFadden et al. 1998; Mosser and Karp 1999). Viruses and bacteria have also been shown to prevent surface expression of both MHCI and MHCII, or they can produce super-antigens that can cause extensive nonspecific T-cell activation (Herman et al. 1991; Schaible et al. 1999; Yewdell and Bennink 1999; Papageorgiou and Acharya 2000). Thus, pathogens have not only evolved mechanisms to subvert Mϕ function, they have also evolved mechanisms to modulate the entire immune response.

ROLE OF MACROPHAGES IN HOMEOSTASIS

Macrophages, as a system, display features consistent with a major role in tissue homeostasis. They are constitutively dispersed throughout the body, in the absence of inflammation, and can be recruited in increased numbers to sites of local injury and infection. They are relatively long-lived cells, remaining biosynthetically active, display a range of plasma membrane receptors for recognition of endogenous and exogenous ligands, and are able to secrete a wide variety of products in response to stimulation. Through these activities they regulate the activity of many cell types in their local microenvironment, as well as systemically. Here we touch briefly on selected aspects to illustrate diverse homeostatic aspects of Mϕ function particularly relevant to infection.

As discussed above, resident tissue Mϕs show great variation, the functions of which are not well understood, but can be broadly interpreted as homeostatic. In bone marrow, for example, stromal Mϕs play a tropic role in hemopoiesis by interacting with developing myeloid and erythroid cells (as well as removing

erythrocyte nuclei) (Crocker et al. 1991). In lung, alveolar Mϕs contribute to surfactant metabolism as well as host defense. In the central nervous system, microglia persist throughout adult life, closely associated with neurons and astrocytes, possibly contributing to neurotransmitter catabolism (Perry et al. 1993).

Macrophages play a major role in the uptake and destruction of apoptotic, senescent, and necrotic cells, through a range of scavenger and other receptors (Fadok et al. 2001; Peiser et al. 2002). Apart from their phagocytic and surface immune interaction molecules such as MHC and co-stimulatory antigens, Mϕs are well placed to remove a wide range of ligands from the circulation, or extravascular tissue fluids by phagocytosis and endocytosis. Examples include classic reticuloendothelial cell clearance functions of Kupffer cells, often shared with liver sinusoidal, true endothelial cells. Thus many lysosomal hydrolases express a terminal saccharide recognized by the Mϕ mannosyl/fucosyl receptor (East and Isacke 2002). Other important host-protective endocytic clearance systems include CD91 (α_2-macroglobulin–protease complexes) and CD163 (haptoglobin–hemoglobin complexes) (Binder et al. 2001; Kristiansen et al. 2001). The products of activated neutrophils can also be removed by several of the above receptors.

Macrophages express a variety of constitutive and inducible metabolic activities that help to catabolize substrates such as hemoglobin, and inactivate chemical compounds including drugs. Mϕs contribute to bile pigment formation through hemoxygenase and other enzymes, and can express high levels of cytochrome P450. They contribute to iron homeostasis through a variety of pathways.

MACROPHAGE-INDUCED PATHOLOGY

Many of the properties of Mϕs, described above, can be readily extrapolated to pathological responses to infection and metabolic diseases in which Mϕs play a central role, with or without contributions from other cells (Gordon 1999, 2001, 2003b). We illustrate the range of diseases by selected examples, in which Mϕs are overactive or deficient in function. These include chronic infections and/or inflammatory diseases, such as tuberculosis and rheumatoid arthritis in which Th1 responses and TNF-α production predominate, and a range of storage diseases, such as atherosclerosis.

Granuloma development is a characteristic of mycobacterial infection. Persistence of a microbial stimulus, in Mϕs themselves, can result in the focal accumulation of newly recruited monocytes and their differentiation into Mϕs (epithelioid and Langhans' giant cells), together with T lymphocytes, other myeloid cells, and fibroblasts. The granuloma Mϕs express a range of cell activation markers including lysozyme and proinflammatory cytokines (TNF-α, IL-1β, IL-6), as well as IL-10. TNF-α is essential for granuloma formation, as shown in anti-

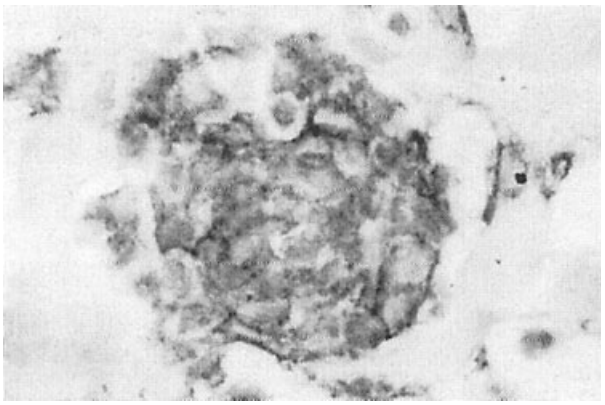

Figure 4 C. parvum-*induced granuloma in the liver stained with F4/80. The majority of the cells in the granuloma stain positive for F4/80, as do some adjacent Kupffer cells.*

body and genetic depletion studies. The type 3 complement receptor is also important in mobilization of monocytes to form granulomas. A granuloma stained with F4/80 is shown in Figure 4.

The immunologically primed host is exquisitely sensitive to LPS or microbial stimuli, which readily precipitate a septic shock syndrome, mediated through CD14 and TLR pathways, with diffuse intravascular coagulation and vascular collapse, mainly through the actions of cytokines such as TNF-α and IL-1β on the endothelium. Infection by a range of other intracellular pathogens mentioned above can give rise to widespread local and systemic inflammatory sequelae, including wasting, fibrosis, and secondary amyloidosis, as well as immunosuppression.

Other types of modified chronic inflammation in which Mϕs are prominent include atherosclerosis and, perhaps, Alzheimer's disease. Accumulation of intra- or extracellular lipoprotein or fibrillar deposits in large arteries or brain, respectively, results in persistent Mϕ secretion of tropic and destructive products. Classic Mϕ storage diseases include lysosomal storage of macromolecules caused by genetic enzyme deficiency and iron overload. Such Mϕs can become potent secretory sources of enzymes that serve as markers (e.g. chitotriosidase in Gaucher's disease), or mediators of tissue injury (elastase, collagenase).

Deficiencies of Mϕ function include failure to clear apoptotic cells, which may contribute to autoantigen formation in diseases such as systemic lupus erythematosus, and the inability to destroy opportunistic pathogens in AIDS. Rare inherited deficiencies of respiratory burst components result in inefficient killing of a range of microorganisms, in chronic granulomatous disease.

CONCLUSION

We have briefly highlighted the various roles that Mϕs play in anti-infectious immunity and have shown that,

through their ability to recognize, ingest, and destroy microbes, as well as recruit other immune cells to the sites of infection, Mϕs are key cells in innate immunity. We have demonstrated that Mϕs play an essential role in specific immunity by modulating this response through the release of cytokines, and other soluble mediators, and acting as effector cells and accessory cells in secondary lymphocyte activation. Although macrophages are essential for the control of infection, we have also noted some of their homeostatic functions and their ability to induce pathology.

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Phagocytes part 2: Neutrophils

ANTONIO FERRANTE

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INTRODUCTION

The importance of neutrophils in host defense is best demonstrated in hereditary and acquired neutropenias, conditions that place an individual at risk of life-threatening infections. Neutrophils devote their activity mainly to producing oxygen-reactive species, which combine with granule constituents to form very potent microbicidal systems. This was first appreciated in the late 1950s when it was discovered that respiration was not dependent on mitochondria. Through the combined efforts of a number of independent investigators, not only was the identification of the release of hydrogen peroxide and superoxide discovered as a major feature of the cell (Paul and Sbarra 1968; Babior et al. 1973), but also the special location of the unassembled and assembled components of the oxidase were described (reviewed by Heyworth et al. 1999). A much more comprehensive understanding of the biochemistry of the oxidase came through the efforts of Segal and colleagues (1978), who identified a *b*-type cytochrome which formed a key component of the oxidase system of the cell and which was found to be the defect in some patients who had chronic granulomatous disease (CGD) whose cells failed to generate oxygen radicals and kill some bacteria. Subsequently, the complexity of the oxidase became evident with the identification of several different components that exist in a dormant form until phosphorylated and assembled at the phagosome membrane. The understanding of the generation of antimicrobial substances by neutrophils was further extended by the work of Klebanoff (1968), who demonstrated the reaction of the azurophilic granule enzyme, myeloperoxidase (MPO), with H_2O_2 and chloride to yield hypochlorous acid.

During this time, excitement about the characteristics of the respiratory burst of neutrophils overshadowed many other important discoveries such as the identification of antimicrobial cationic proteins in granules by Zeya and Spitznagel (1966), and it was not until the 1990s, when Lehrer and Ganz (1990) purified these as defensins, that major interest developed.

The discovery in the late 1970s and early 1980s of the role of specific colony-stimulating factors (CSFs) in the regulation of neutrophil hemopoiesis through the efforts of Metcalf and colleagues (Metcalf 1989) opened new vistas in granulocyte biology with potential therapeutic applications in neutrophil disorders.

In the 1980s, the author's group and others discovered more intriguing properties of this cell, that of priming. In this process, the neutrophil interacts with cytokines such as tumor necrosis factor (TNF) and gains significantly greater antimicrobial powers (Ferrante and Mocatta 1984; Klebanoff et al. 1986). Thus it became evident for the first time that the neutrophil can be educated by factors released by tissues, endothelial cells,

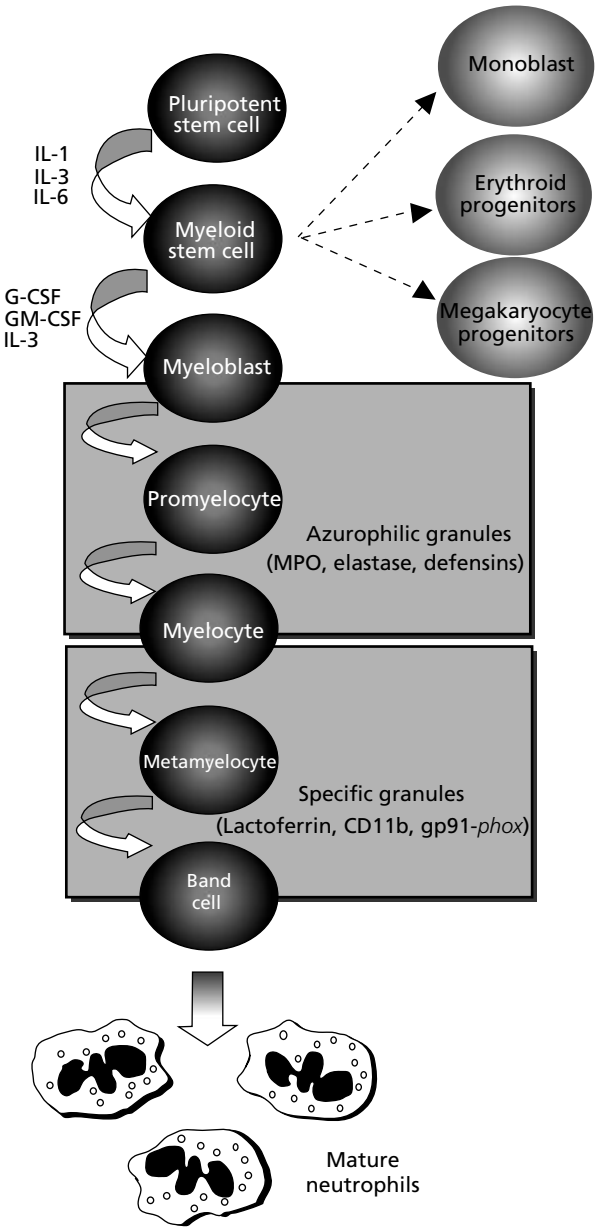

Figure 1 *Scheme showing the different stages of neutrophil development in the bone marrow from stem cells. Cytokines that promote granulocyte development are outlined. Note the initial appearance of azurophilic granules and later expression of specific granules. Some of the markers specific for the different granules are also shown.*

lymphocytes and monocytes in the way that macrophages were shown to be activated by interferon-γ in the 1970s.

When Tiku et al. (1986) reported that zymosan-stimulated neutrophils released interleukin (IL) 1 (IL-1), it was appreciated that neutrophils were able to promote the immunological response through the release of cytokines that regulate macrophage and lymphocyte function.

The ability of neutrophils to migrate rapidly into tissue at infection sites, release oxygen reactive species, and degranulate in order to kill bacteria needs to be regulated because of their tissue-damaging properties. The discovery that neutrophils die by programmed cell death (apoptosis), which protects against tissue damage mediated by this cell, has contributed significantly to our understanding of the mechanism of regulation of the inflammatory reaction.

The concept of neutrophil emigration from blood to tissues under a gradient of molecules had already been put in place in the mid- to late 1800s by the founding fathers, such as Metchnikoff (1893). Since then, substantial complexity has been added to this concept. This includes the array of the chemotactic factors generated during inflammation, the mechanisms of cell movement, and the interactions required between neutrophils and the endothelium for emigration into tissues.

Although the neutrophil has always been considered the 'poor cousin' of the mononuclear phagocyte because of its short life and inability to be educated to perform many of the intricate functions of the macrophage necessary to develop a strong immunological defense system, it is evident that singular properties of the neutrophil are becoming apparent. This chapter covers the properties of neutrophils in general and attempts not only to describe properties that are considered classic from the times of Elie Metchnikoff, but also to illustrate those properties that have only recently been appreciated.

NEUTROPHIL DEVELOPMENT

The bone marrow is involved in the production of neutrophils. This is the site for proliferation of neutrophil precursors and maturation to the mature stage (Figure 1) (Bainton 1999). Once cells evolve from pluripotent stem cells to progenitor cells, they can no longer differentiate into any type of blood cell and will differentiate into the mature cells. Hemopoiesis is regulated by a number of cytokines. Four of these, the CSFs, IL-3 (multi-CSF), macrophage (M)-CSF, granulocyte–macrophage (GM)-CSF, and G-CSF regulate development of leukocytes (Metcalf 1989). The granulocyte lineages come under the control of G-CSF, GM-CSF, and IL-3. These factors regulate the production of neutrophils.

During neutrophil development in the bone marrow, at different stages the cell acquires functional properties necessary for optimal antimicrobial activity (Figure 1) (Borregaard and Cowland 1997). As constituents of granules such as MPO and eventually lactoferrin are synthesized, they are packaged in their respective granules. Components of the oxidase, such as the gp91^{phox} and functional surface receptors (CD18, CD11b) which ligate complement-opsonized bacteria, also become expressed at the plasma membrane of these cells as the cells differentiate toward the mature neutrophil. The granules to appear first are the azurophilic (primary) granules containing MPO and elastase, morphologically

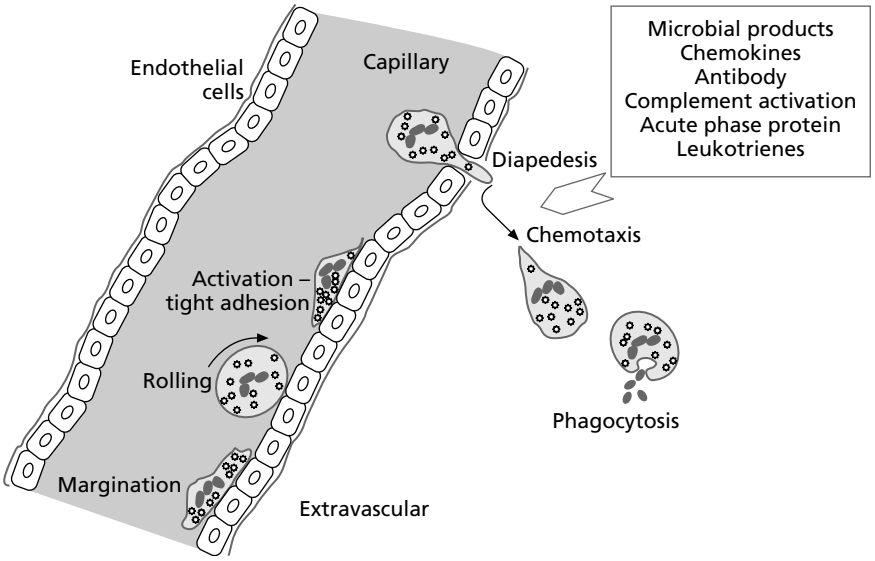

Figure 2 *Diagrammatic representation of early events in the recruitment of neutrophils to inflammatory sites. Stimulation of both neutrophils and the endothelium will promote the process of rolling, attachment, and transendothelial cell migration. The nature of the inflammatory response and mediators produced dictates a preference for selective recruitment of neutrophils at the level of neutrophil receptors for promoting adhesion and chemotaxis and, also, endothelial cell activation.*

seen at the promyelocyte stage. The secondary granules (specific) characterize the myelocyte stage. Once the cell lineages reach the metamyelocyte stage, the cells cease to divide.

MOBILIZATION TO SITES OF INFECTION AND DAMAGED TISSUES

Accumulation of neutrophils at infection foci and sites of tissue damage begins with margination, whereby the neutrophils attach to the capillary wall and have the potential to migrate out of the blood vessel (Gallin and Snyderman 1999) (Figure 2). This is followed by diapedesis, a step in which the cells squeeze through gaps in the endothelial cell layer. Their chemotactic properties enable the cells to migrate preferentially towards the source of infection and local tissue damage, and may experience migration inhibition to enable retention of cells at the infection site.

To achieve cell accumulation in tissues, a number of complex interactions occur between receptors on neutrophil surfaces and various soluble or cell surface ligands, which stimulate the cells to respond (Gallin and Snyderman 1999). Bacterial infiltration of tissues leads to the release of microbial products (e.g. lipopolysaccharide (LPS)), which can promote neutrophil accumulation in tissues themselves, via their ability to activate the endothelium and/or act as chemotactic factors. These microbial products and structures can also promote the generation of chemotactic active peptides of complement such as C5a and stimulate tissues to produce chemokines (cytokines such as IL-8) and the leukotriene, LTB_4, a chemotactic factor produced during the metabolism of arachidonic acid via the lipoxygenase pathway. Some cytokines such as TNF can also cause migration inhibition, especially at concentrations likely to be generated at inflammatory sites (Ferrante 1992).

Although many of the properties of the mediator effects on cells are reasonably well understood, how the network of interactions coordinately brings about a physiological response and when this response becomes uncontrolled to give rise to pathophysiology are far from clear and continue to be an area of research interest.

The cell cytoskeleton

The intracellular components are embedded in a supporting frame, the cytoskeleton, which consists of microfilaments, microtubules, intermediate filaments, and the microtrabecular lattice. Second messengers link the surface ligand-binding receptor to the cytoskeleton. Changes in this cytoskeleton then form the basis for cell movement, phagocytosis, granule movement, receptor recycling, and activation of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Edwards 1994; Stossel 1999).

Microfilaments found predominantly beneath the plasma membrane are considered to be the most dynamic of the components of the cytoskeleton. Microfilaments are composed of actin molecules that exist as monomers (G-actin), which can reversibly assemble into actin filament (F-actin). Actin polymerization is initiated by the activation of actin monomers by divalent cations. A range of actin-binding proteins is involved in maintaining actin in an unpolymerized state in cells and promoting rapid assembly during cell activation. Microtubules consist of α- and β-tubulin subunits arranged in a

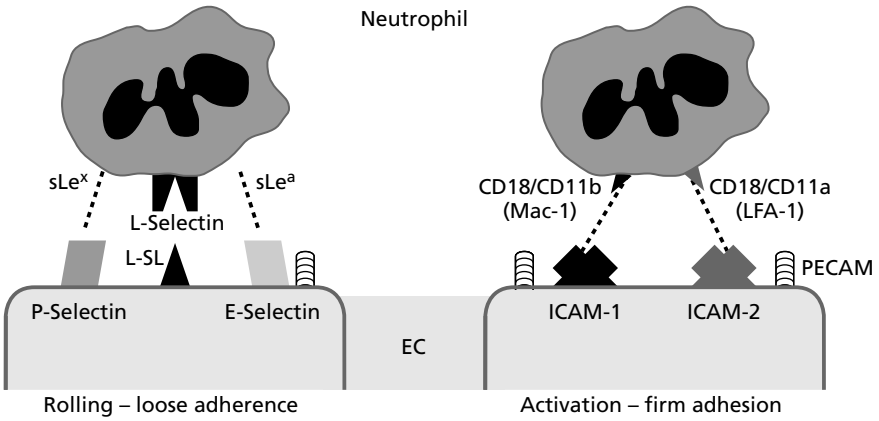

Figure 3 *The receptor ligand involved in the interaction of neutrophils with endothelial cells. Interaction of the tetrasaccharides sialyl Lewis^a (sLe^a), sLe^x, L-selectin with the counter-receptor/ligand, E-selectin, P-selectin, and L-selectin ligand (L-SL) promotes loose adherence and enables the neutrophils to roll. Endothelial cell (EC) activation induces/upregulates expression of cell adhesion molecules, ICAM-1/-2 enabling interactions with β_2-integrins and promoting firm adhesion of the cell to the endothelial surface. PECAM then facilitates transendothelial cell migration.*

helical structure. Assembly of these is increased during cell activation. Assembly is upregulated by cyclic guanosine monophosphate (cGMP) and decreased by cyclic adenosine monophosphate (cAMP) and Ca^{2+}. Neutrophils also have intermediate filaments, which consist of vimentin molecules that can be rapidly polymerized. Their function is not clearly known but they may be involved in connecting the peripheral region of the cell to the central regions. The microtrabecular lattice, an intricate network of fine strands, is also part of the cytoskeleton. Actin is the major polypeptide of this lattice. Cytoplasmic granules can be found associated within this lattice.

Neutrophil–endothelial cell interactions

Recruitment of neutrophils to sites of inflammation requires a series of events involving rolling, activation, adhesion, and transendothelial cell migration, which are depicted in Figure 2 and 3. The type of inflammation may dictate the cell types that leave the circulation and migrate into tissues which is in part a reflection of the type of adhesion molecules upregulated, i.e. members of the common cell adhesion molecule (CAM) family, apart from their selection by specific chemokines.

The events seen at inflammatory sites are the emigration of neutrophils from the circulation in the postcapillary venules and their loose and reversible adhesion to the endothelial cells (see Figure 2). This interaction, which mainly involves the selectin family and the sialylated Lewis-bearing ligands (see Figure 3), enables the cells to roll under the influence of forces of blood flow (Robinson et al. 1999). This interaction leads to neutrophil activation as such and indirectly by mediators released by the endothelial cells. Integrins are upregulated to high-affinity states, rolling ceases, and the cells show tight binding to the endothelium through the counter-receptor, intercellular adhesion molecule (ICAM)-1, and ICAM-2 (members of the immunoglobulin gene superfamily) (see Figure 3) (Kishimoto et al. 1999).

The neutrophils advance to crawl/squeeze between the endothelial cell junctions in an amoeboidal manner (Muller 1999). This transendothelial cell migration is highly dependent on platelet endothelial cell adhesion molecule-1 (PECAM-1). Having traversed the intercellular junctions, neutrophils need to migrate across the basal lamina. This again involves recognition molecules such as PECAM-1 and either physical penetration or degradation of the matrix by release of proteases.

Chemotaxis

Neutrophils are quick to respond to a gradient of chemical agents generated at a site of infection and tissue damage. These chemotactic agents bind to receptors on the cell surface to bring about a network of interactions that promotes directional movement of the cell toward the source of the chemical agent (Uhing and Snyderman 1999). This involves the recognition of a concentration gradient spanning the front and rear ends of the cell, and a combination of calcium mobilization, actin, actin-binding proteins, and proteins that regulate the cytoskeleton of the cell.

After the binding of chemotactic agents to neutrophils, the cell undergoes a shape change, characteristically bipolar. The leading edge of the cell orients toward the source of chemotactic factor and forms a pseudopod or lamellapodium that adheres to the substratum. Toward the rear of the cell can be found the nucleus and the contractile uropod exhibiting retraction fibers, which enable the cell to adhere. A repeated sequence of lamellapodia extension towards the chemo-

tactic source and uropod retraction towards the cell body leads to cell migration.

RECOGNITION AND PHAGOCYTOSIS OF MICROBIAL PATHOGENS

Innate immunity in vertebrates expresses primitive forms of recognition system that involve recognition of structures generally based on their charge and special arrangement of chemical groups (Gallin and Snyderman 1999). These possibly evolved from scavenger properties of primitive cells such as amoebae. Thus carbohydrate residues on the surface of a pathogen may be recognized by receptors that perform scavenger functions. These types of recognition system may play an important role at sites in which complement and antibody levels are very low or absent.

Neutrophils display a wide range of receptors on their surface, which enable a highly regulated attack on microorganisms, although such receptors will also direct the neutrophil attack toward damaged tissue. Several well-characterized receptors are outlined in Table 1. These receptors are set into operation when bacteria are opsonized with antibody and/or complement components (Greenberg 1999), enabling neutrophils to attach to the bacteria via the Fc portion of the immunoglobulin molecule involving FcγR or FcαR. Deposition of complement components on the surface of bacteria leads to binding of neutrophils to bacteria via the complement receptors, CR1, CR3, and CR4. The ligation of these receptors promotes the phagocytosis of the microorganisms and their killing by stimulating the neutrophil respiratory burst and degranulation.

The binding of antibody to the surface antigens of microbial pathogens leads to the exposure of a region in the Fc domain of the immunoglobulin molecule, which can be recognized by the Fc receptors (FcRs) on the neutrophil. There are three types of FcγR on neutrophils recognized to date: FcγRI (CD64), FcγRIIA (CD32), and FcγRIIIB (CD16). The function of these is to recognize the Fc domain of IgG, but their ability to do this is dependent on various physiological and pathophysiological conditions. The FcγRI is a glycosylated 72-kDa transmembrane protein which is absent in blood neutrophils but is upregulated and detected following pre-exposure to interferon-γ (IFN-γ) which involves transcription. This contrasts with the regulation of FcγRIIIB, which can be upregulated by release from granules and downregulated by shedding. The FcγRI, in comparison to FcγRIIA and FcγRIIIB binds monomeric IgG with high affinity.

Table 1 *Functional receptors mediating phagocytosis of opsonized particles and activation in neutrophils*

Receptor type	Ligand
FcγRI (CD64)[a]	Fc domain of IgG
FcγRIIA (CD32)	Fc domain of IgG
FcγRIII B (CD16)	Fc domain of IgG
FcαR (CD89)	Fc domain of IgA
CR1 (CD35)	C3b
CR3 (CD11b/CD18)	C3bi
CR4 (CD11c/CD18)	C3bi

a) CD classification of Fcγ and CR on neutrophils.

The significance of FcγRI on neutrophils is not clear because it is expressed by neutrophils only after long-term exposure to IFN-γ. However, this may argue for an important role for neutrophil activation and priming in a similar manner to mononuclear phagocytes (Ferrante 1992). Similarly, the role of FcγRIIIB in neutrophils is not clear because it lacks a transmembrane or cytoplasmic domain, suggesting that it is not involved in intracellular signaling for a respiratory burst and/or degranulation. But, because it is expressed in abundance on neutrophils, its main function may be the binding and removal of immune complexes. Presumably FcγRIIIB promotes the binding of bacteria but it needs to cooperate with FcγRIIA to stimulate the relevant intracellular signals.

FcαR which recognizes the Fc domain of IgA is also expressed by neutrophils. IgA in high concentration opsonizes and facilitates microbial phagocytosis and activation of the neutrophil respiratory burst through this receptor. This is likely to be an important defense mechanism in mucosal surfaces where the complement concentration is likely to be too low to promote phagocytosis.

During inflammation, whether induced by microbial pathogens or altered tissues, complement is activated in a cascade manner to generate components that are recognized by neutrophils, some of which act as opsonins to promote phagocytosis. The component C3 is of vital importance because it generates fragments, C3b and C3bi, which when deposited on bacteria facilitate recognition by neutrophils usually through the receptors CR1, CR3, and CR4 (Greenberg 1999) (see Complement). Whether particles are coated with C3b or C3bi is dependent on whether complement is activated via the classic or the alternate pathway. CR1 (LAF-1) binds C3b primarily and has very weak affinity for C3bi. In contrast CR3 (Mac-1) binds to C3bi deposited on bacteria with very high affinity. CR4 also probably recognizes C3bi. Neutrophils can recognize microbial surfaces in the absence of antibody and complement by an opsonin-independent system that may involve CR3 receptors recognizing other residues.

Appropriate recognition and adherence of neutrophils to bacteria and fungi lead to the engulfment of these microbes, thereby confining and limiting their harmful effects. This involves the formation of a pseudopod, which extends around the surface of the bacteria in a 'zipper hypothesis' manner to confine the organisms within a vacuole. The cell is then able to deal with the engulfed bacteria by a combination of pH changes in the

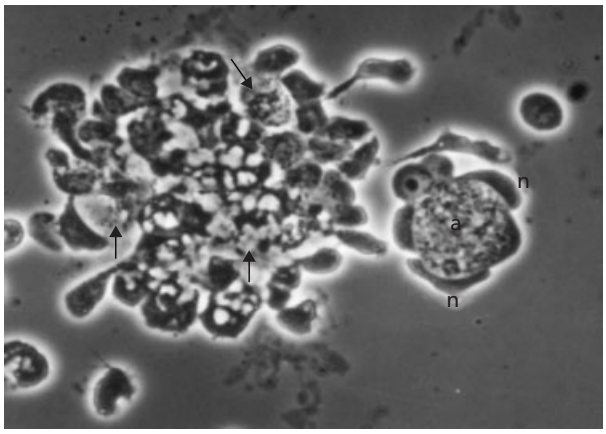

Figure 4 *The interaction of neutrophils with pathogenic free-living amoebae in the peritoneal cavity of mice immune to* Naegleria fowleri. *Mice were injected intraperitoneally with living amoebae and, after 8 h, the peritoneal content harvested and examined. A motile amoeba* **(a)** *is seen with attached neutrophils* **(n)**. *Several amoebae are seen highly vacuolated or lysed (→) because these are attacked by neutrophils.*

phagocytic vacuole and the release of toxic oxygen intermediates and granule constituents, some of which have enzymatic properties. A unique interaction between neutrophils and parasites has been described in which the neutrophil extracts *Plasmodium falciparum* from within an erythrocyte without lysis of the erythrocyte (Kumaratilake et al. 1994).

Frustrated phagocytosis has been found to occur when a particle is too large to be engulfed by the neutrophil. This may occur with some large unicellular protozoan parasites such as amoebae (Figure 4) and in the case of multicellular parasites and hyphae of *Candida albicans*. Interaction with altered tissues may also lead to an event of frustrated phagocytosis. In these situations, a 'partial vacuole' is formed in which the environment seen in a complete phagocytic vacuole is created. The reactive oxygen species and granule constituents are released and able to damage and lyse the cells. Usually a number of neutrophils will attack one multicellular parasite and presumably it is the net effect of surface parasite damage induced by several neutrophils that leads to parasite death. In autoimmune diseases such as glomerulonephritis and rheumatoid arthritis, the antigen–antibody complexes deposited on the membranes and their ability to activate complement promote this neutrophil-mediated cellular cytotoxicity.

ANTIMICROBIAL ARMORY OF THE NEUTROPHIL

Once neutrophils localize at an infection site and come into contact with bacteria, the cells are stimulated to produce an array of microbicidal substances within seconds and minutes. The neutrophil microbicidal systems can be divided into the nonoxidative and oxidative components. Depending on the type of bacteria or parasite, either one of these components may be effective or, in other cases, both systems are seen to operate synergistically.

Nonoxidative antimicrobial systems

Neutrophils contain two major granules, specific and azurophilic granules which can be distinguished by the presence of different constituents (Table 2). The constituents present in the azurophilic granules have primarily an antimicrobial function. The ability of these constituents to kill microbial pathogens contrasts with a mechanism by which organisms can be killed in their absence, simply by being confined in a phagocytic vacuole and destroyed by the microbe's own products. Evidence for a role of nonoxidative mechanisms in neutrophil-mediated microbial killing is seen from experiments where neutrophils from patients with CGD, lacking NADPH oxidase activity, while unable to kill some bacteria can still kill others. In addition, others have found that crude fractions of neutrophils were able to kill gram-negative bacteria (Borregaard and Cowland 1997). Granule constituents play various roles in nonoxidative killing of microorganisms. These mechanisms are continuing to be an area of major research interest. Some of the granule enzyme components show direct antimicrobial activities and in purified forms can kill bacteria. Others, although not directly microbicidal, are involved in the digestion of the bacteria. Nonenzymatic granule proteins have also been shown to be highly antimicrobial. The following azurophilic granule constituents are of interest in relation to nonoxidative killing of microbial pathogens:

- Defensins, cationic peptides of 3.5–4.5 kDa, have a broad-spectrum antimicrobial activity (Lehrer and Ganz 1990). Both α- and β-defensins have been found

Table 2 *An abridged summary of the two major neutrophil granule constituents*[a]

Azurophilic/primary granules	Specific/secondary granules
Myeloperoxidase	Lysozyme
β-Glucuronidase	Lactoferrin
Elastase	Vitamin B_{12}-binding protein
Cathepsin G	Receptors – CR3, fMLP receptor
Defensins	Components of NADPH oxidase
BPI	
Lysozyme	

fMLP, formyl–methionine–leucine–phenylalanine or f-Met-Leu-Phe; BPI, bactericidal permeability-increasing protein.
a) Note that these two granules need to be considered along with a tertiary granule (gelatinase granules) and secretory vesicles.

in these neutrophil granules. The peptides show cytotoxic activity towards fungi, parasites, and gram-negative bacteria. They are released into the phagocytic vacuole during phagocytosis of bacteria.

- Serprocidins such as elastase, azurocidin, and cathepsin G, which have microbicidal activity, are also contained in azurophilic granules (Edwards 1994). This microbicidal activity is independent of proteolytic action.
- Cathepsin G is active against a range of gram-positive and gram-negative bacteria, particularly against *Neisseria gonorrheae* and fungi.
- A highly cationic protein (bactericidal/permeability – increasing protein) with marked bactericidal activity, particularly towards gram-negative bacteria, is also found in azurophilic granules (Elsbach and Weiss 1983). The molecule inserts into bacterial outer membranes causing increased permeability.

Oxidative systems

THE OXYGEN-DEPENDENT RESPIRATORY BURST

When phagocytes adhere to bacteria, one of the most dramatic responses seen is the oxygen-dependent respiratory burst and the generation of a spectrum of reactive oxygen intermediates (ROIs) which are either directly or indirectly microbicidal. The enzyme responsible for this is NADPH oxidase, which consists of several different components that assemble on the phagosome membrane (Heyworth et al. 1999). When neutrophils are stimulated most of the glucose is oxidized via the hexose monophosphate shunt which is closely linked to the reduction of oxygen by the NADPH oxidase. The initial product of the respiratory burst is the production of superoxide (O_2^-).

It was only a few decades ago that the unique characteristics of the neutrophil respiratory burst were first described. It was shown that bacterial killing still occurred in the presence of cyanide, suggesting that mitochondria and the citrate cycle were not involved, consistent with the finding of very few active mitochondria in neutrophils. The ATP generation comes mainly from glycolysis, which does not require oxygen, and this is considered as an advantage because neutrophils need to kill microorganisms in oxygen-depleted tissue sites. Later, it became evident that the majority of the oxygen consumed by the neutrophil was converted to O_2^- (Barbior et al. 1973; Fridovich 1978). Thus, during a respiratory burst, glucose is oxidized via the hexose monophosphate shunt, generating NADPH which together with oxygen provides the source of electrons to produce superoxide anion. From O_2^- several ROIs are formed in the phagocytic vacuole. These are shown below.

$$\underset{\text{(Superoxide)}}{O_2^-} + \underset{}{O_2^-} + 2H + \xrightarrow[\text{Dismutase}]{\text{Superoxide}} \underset{\text{(Hydrogen Peroxide)}}{O_2 + H_2O_2}$$

Haber–Weiss reaction:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \underset{\text{(Hydroxyl radical)}}{OH^{\cdot}} + \underset{\text{(Hydroxyl ion)}}{OH^-}$$

The activation of the respiratory burst oxidase can be demonstrated in the reduction of nitroblue tetrazolium (NBT) by the cells which causes the dye to change from a yellow, soluble compound to a blue–black, formazan-insoluble substance in the cell (Figure 5).

COMPONENTS AND ASSEMBLY OF NADPH OXIDASE

NADPH is essentially dormant and becomes rapidly activated after the stimulation of appropriate intracellular signals by agonists ligating the cell surface receptors on neutrophils (Heyworth et al. 1999). Activation occurs once the various components become rapidly assembled at the plasma membrane (vacuole membrane), involving both membrane and cytosolic components of this system (Figure 6). Within the membrane is a unique cytochrome b_{245} (Segal and Jones 1978), a heterodimer of two subunits, $gp91^{phox}$ and $p22^{phox}$, suggested to contain two different types of redox centers. There is one flavin adenine dinucleotide (FAD) domain and at least two heme prosthetic groups (Babior and Kipnes 1977; Segal et al. 1992). The three cytosolic proteins are believed to be necessary to associate with the cytochrome at the membrane, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, forming the active enzyme system (Wientjes et al. 1993; Heyworth et al. 1999). Other constituents such as the small GTP-binding proteins are also involved (Heyworth et al. 1999). The oxidase is strategically located to generate ROIs in the vacuole containing the ingested bacteria. Consequently, the binding site for the substrate, NADPH, is on the cytoplasmic side and superoxide is formed at the phagosome membrane side (Figure 6).

MYELOPEROXIDASE–H_2O_2–HALIDE SYSTEM

Myeloperoxidase is present in very high amounts in neutrophil azurophilic granules. During phagocytosis, activation of the respiratory burst is accompanied by degranulation, resulting in release of H_2O_2 and MPO into the phagosome. Interestingly, some of the bacteria phagocytosed can also be a source of H_2O_2. Chloride is then oxidized to hypochlorite (HOCl) by the MPO and H_2O_2. The system produces other toxic agents, including chloramines, hydroxyl radicals, and singlet oxygen.

$$H_2O_2 + 2Cl^- \xrightarrow{MPO} 2HOCl$$

This system causes destruction of microorganisms in phagocytic vacuoles in two main ways: by halogenation and by oxidation (Klebanoff 1968, 1999); it has been

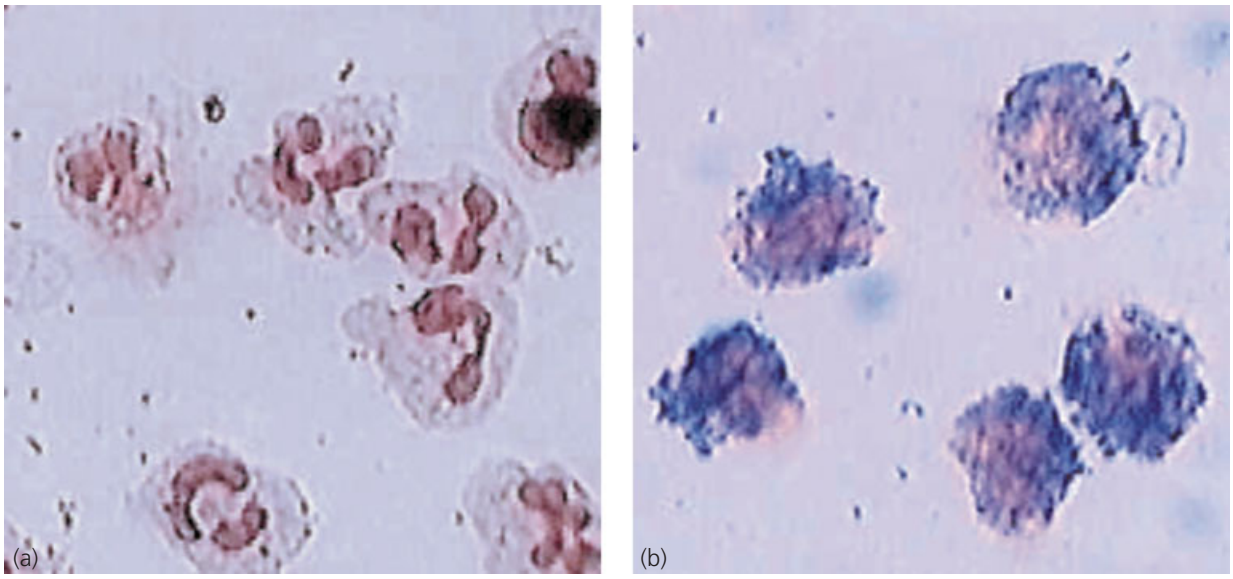

Figure 5 *Oxidative burst activity in stimulated neutrophils shown by the nitroblue tetrazolium (NBT) reduction assay.* **(a)** *Non-stimulated neutrophils lacking positive NBT staining compared with phorbol myristate acetate (PMA)-activated neutrophils* **(b)**, *which show the blue–black formazan deposits.*

shown to damage/kill a wide range of microorganisms, including bacteria, protozoan parasites and metazoan parasites (Klebanoff 1999), as well as tumor tissues during inflammation (Klebanoff 1999). Bacterial toxins are also inactivated by the MPO–H_2O_2–halide system.

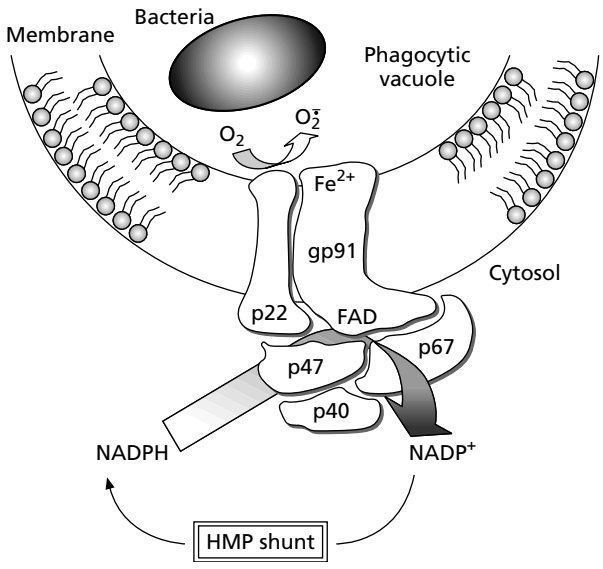

Figure 6 *The positioning of the activated oxidase in the membrane of the phagocytic vacuole and the link between the oxidation of NADPH and the generation and release of superoxide into the bacteria-containing phagocytic vacuole. The components of the NADPH oxidase are also depicted. The cytochrome* b *$gp91^{phox}$ and $p22^{phox}$ are localized in the membrane and require the recruitment of at least three cytosolic components, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ for the complex to act as the oxidase. The substrate for oxidase is generated when glucose is oxidized via the hexose monophosphate (HMP) shunt. In this reaction two electrons are transferred from the NADPH to FAD, followed by the reduction of Fe^{3+} to Fe^{2+} and direct binding of the reduced hemes to oxygen molecules to form superoxide.*

INTRACELLULAR SIGNALING AND NEUTROPHIL FUNCTIONS

The chemoattractant coupling of the surface receptor is coupled to the intracellular second messengers by the heterotrimeric G-proteins (guanine nucleotide-binding proteins). The second messengers, inositol triphosphate (IP_3), Ca^{2+} mobilization, phosphatidic acid (PA), arachidonic acid (AA), and cyclic nucleotides, have all been shown to participate in cell movement. Antibody- and/or complement-opsonized microorganisms ligate the FcγR and CR3 receptors and induce signal transduction mechanisms, involving G-proteins, protein kinases, and phospholipids, which control phagocytosis, activation of NADPH oxidase, and degranulation.

Nonstimulated neutrophils express a transmembrane FcγRIIA and FcγRIIIB, which is the GDP-dissociation inhibitory factor GPI linked to the membrane. Ligation and crosslinking of these receptors stimulates tyrosine phosphorylation as well as phospholipase Cγ. This then leads to the activation of protein kinase C (PKC), Ca^{2+} mobilization, and activation of phosphotidylinositol-3-kinase (PI3-K) signaling pathways. These molecules are then responsible for activating downstream signals such as the mitogen-activated protein (MAP) kinases, leading to a network interaction to stimulate functional responses including phagocytosis, activation of the respiratory burst, and degranulation. The complement receptors such as CR1 and CR2 are likely to associate with the Fcγ receptors to promote these responses.

Ligation of the CR3 (CD18/CD11b) on neutrophils leads to the activation of upstream signals including PI3-K. A number of intracellular molecules have been shown to regulate the various neutrophil functions. The PI3-K, a family of kinases, phosphorylates inositol-containing phospholipids to form phosphatidyl inositol. Two classes of PI3-K have been identified in neutrophils: the classic class Ia (p85/p110) heterodimer and a G-protein βγ subunit-regulated PI3-K. PI3-K is required for stimulating the respiratory burst and degranulation.

Protein kinase C is a family of serine/threonine kinases that resides in the cytoplasm and translocates to the particulate fraction (plasma membrane and cytoskeleton associated) after neutrophil stimulation. Translocation of PKC is closely associated with its activation. Approximately 12 different PKC isozymes have been described and these will vary from cell type to cell type. They are also activated differently by individual agonists and various isozymes may be responsible for different cellular functions. In neutrophils the expression of PKCα, β1, β11, δ, and ξ has been reported. Evidence has been presented that PKC is required for the neutrophil functions of chemotaxis, respiratory burst, and phagocytosis.

There are three distinct families of MAP kinases recognized: the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38. The role of JNK in neutrophil function has not been identified because various agonists fail to activate JNK, unless special conditions are met such as pre-adherence of cells and addition of protease inhibitors (Avdi et al. 2001).

ERK1 and ERK2, which can be activated via various receptors including FcγR, have been shown to play a role in the activation of the oxidase by phosphorylating $p47^{phox}$, a cytosolic component of the oxidase. More recent studies have, however, demonstrated that the ERK cascade plays a major role in chemokinesis and a lesser role in bacterial and fungal killing by neutrophils (Hii et al. 1999).

Of the four recognized isoforms of p38, neutrophils express the α and δ isoforms. Activation of p38 can be achieved by both G-protein-coupled receptors as well as non-G-protein-coupled receptors. In terms of neutrophil functions, p38 plays a role in chemotaxis, respiratory burst, degranulation, and apoptosis.

NEUTROPHIL PRIMING

There is now overwhelming evidence that neutrophils can be educated for increased performance in their antimicrobial activity (Coffey 1992). A variety of exogenous and endogenous mediators has been shown to prime neutrophils for increased antimicrobial function but perhaps the most interesting have been the cytokines (Ferrante 1992). Priming of neutrophils requires short-term exposure to cytokines and in most cases is optimal with a 30-min pre-treatment time (Ferrante et al. 1993b). Cytokine-primed neutrophils are then able to respond more effectively to a challenging agonist, be it soluble or particulate, including microbial pathogens. This has been shown as an increased expression of functional surface receptors (CR3, FcγR), increased particle adhesion and phagocytosis, increased release of ROIs and degranulation, and increase in microbial killing or tissue damage (Klebanoff et al. 1986; Ferrante et al. 1987; Ferrante 1989).

Many cytokines have been shown to alter neutrophil responses, in terms of priming. These include TNF-α, TNF-β, GM-CSF, G-CSF, IL-1, IFN-γ, IFN-β, IL-2, and IL-8 (Coffey 1992). Receptors for many of these cytokines have been described on neutrophils. Treatment of neutrophils with some of these cytokines alone produces little response, fulfilling the role as primers and enabling the cell to undergo rapid stimulation upon engagement of microbial pathogens (Ferrante 1992). Increases in formyl-methionine-leucine-phenylalanine (fMLP) receptor, CR3, and FcγR expression have been described during the priming phase (Klebanoff et al. 1986; Kumaratilake et al. 1995). Priming of neutrophils can also be achieved by exposing the cells to cytokine-rich supernatants from stimulated T cells or macrophages, or from purified cytokine from these cells (Kumaratilake et al. 1991; Ferrante 1992) and even after direct contact with activated T lymphocytes (Zhang et al. 1992). Peripheral blood mononuclear cells stimulated with heat-killed *Staphylococcus aureus* produce high amounts of TNF-α and IL-1. These supernatants were found to prime neutrophils for increased killing of opsonized *S. aureus* and this property was primarily caused by TNF-α (Ferrante 1992). Table 3 outlines the properties of TNF-α more extensively.

An examination of the various models of microbial killing by cytokine-primed neutrophils shows that, in models such as the killing of opsonized, pathogenic, free-living amoebae, e.g. *Naegeria fowleri*, unprimed neutrophils have little effect and only attain amoebicidal properties once primed with cytokines (Ferrante and Mocatta 1984). In other cases, the neutrophils kill the bacteria, such as *S. aureus*, but this killing can be markedly improved by priming the neutrophils (Ferrante et al. 1993b). Interestingly, Kowanko et al. (1996) showed that such priming for killing of *S. aureus* was oxygen-dependent, but oxygen-independent for killing of intraerythrocytic asexual blood stages of *Plasmodium falciparum*. In the case of blood stages of *P. falciparum*, the merozoite and asexual intraerythrocytic stages, TNF-primed neutrophils will also show enhanced killing in the absence of opsonins, although it is much more marked when the parasites have been opsonized with antibody and complement (Kumaratilake et al. 1991, 1992; Ferrante et al. 1993a). This suggests that priming may also enhance killing when recognition is via a pattern-recognition system.

Table 3 *Properties of TNF-primed neutrophils*

Increased expression of CR3, FcγR, fMLPR
Inhibited migration
Increased agonist-induced respiratory burst in response to:
fMLP
opsonized bacteria
opsonized yeast/fungi
opsonized parasites
Increased agonist-induced degranulation in response to:
fMLP
opsonized bacteria
opsonized yeast/fungi
opsonized parasites
Increased microbial killing, e.g. for
Staphylococcus aureus
Streptococcus pneumoniae
Candida albicans
Plasmodium falciparum merozoites and intraerythrocytic asexual blood stage
pathogenic free-living amoebae
Increased tissue damage:
articular cartilage

fMLP, formyl–methionine–lysine–phenylalanine or f-Met-Lys-Phe; fMLPR, fMLP receptor; TNF, tumor necrosis factor.

Neutrophil priming by other exogenous and endogenous inflammatory mediators, apart from cytokines, also occurs, e.g. fMLP, a microbial peptide, is a poor neutrophil stimulator of respiratory burst and degranulation but a strong chemotactic agent and a strong neutrophil priming agent for the former two responses. This thus enables cells to arrive at inflammatory sites relatively controlled and primed to respond to bacteria. Products of the cyclooxygenase pathway, in particular LTB_4 and 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), will also act as neutrophil priming agents to increase neutrophil adhesion, superoxide production, and degranulation (Powell et al. 1997; Ferrante et al. 1999).

ROLE OF NEUTROPHILS IN MICROBIAL DEFENSE

The importance of neutrophils in immunity to bacterial and fungal infections has been appreciated for over a century and their role in immunity to protozoan and metazoan parasites has been more recently recognized. The first line of evidence for a role of neutrophils in resistance against infection is demonstrated by the susceptibility of microbial pathogens to various products of neutrophils. Second, provided that the relevant opsonic factors are present, neutrophils have been shown to engulf bacteria such as *S. aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* and also fungi such as *Candida albicans*. Asexual intraerythrocytic blood stages of *P. falciparum* are readily engulfed and destroyed by neutrophils (Ferrante et al. 1993a). Larger unicellular (amoebae) and multicellular (such as nematodes, microfilaria, etc.) parasites are, however, killed by an extracellular mechanism.

There is an overwhelming demonstration of the accumulation of neutrophils at sites of microbial pathogens in the tissues. These include numerous types of bacteria, fungi, and parasites. It is not uncommon to find neutrophils with engulfed bacteria at these sites. Furthermore, a range of microbial products promotes neutrophil accumulation at inflammatory sites.

Experiments of nature demonstrate an important role of neutrophils in resistance against infection. Neutropenia, whether hereditary or drug induced, leads to increased risk of infection and death from overwhelming infection. Neutropenic patients are in particular susceptible to both bacterial and fungal infections. Several neutrophil functional defects also predispose children to severe/recurrent life-threatening infection.

In experimental animal models of infection, the role of the neutrophil has also been established. Experimentally induced neutropenia makes animals highly susceptible to bacterial, fungal, and parasitic infections. In some cases, such as infections with nematodes and pathogenic free-living amoebae, depletion of neutrophils from immune mice renders them highly susceptible to these infections (Penttila et al. 1985; Ferrante 1992).

Primed neutrophils show increase antimicrobial activity. In vitro there is evidence that neutrophil-mediated killing of microbial pathogens can be increased dramatically by priming with cytokines such as TNF, GM-CSF, IL-1, LTs, and IFN-γ (Coffey 1992; Tan et al. 1995). In some cases neutrophils fail to show any significant killing of the microorganism unless the cells have been primed with these agents (Ferrante 1992). It is interesting that human peripheral blood mononuclear cells stimulated with heat-killed *S. aureus* generate supernatants that are very effective neutrophil-priming agents, and this activity can be ablated by treatment with anti-TNF antibodies (Ferrante 1992). The role of TNF-primed neutrophils in microbial killing has been extended to several bacteria, including *H. influenzae* and *Legionella pneumophila* (Blanchard 1992; Tan et al. 1995).

Interestingly, neutrophils recovered from immune mice can kill microbial pathogens at significantly greater rates (Ferrante 1992; Penttila et al. 1984). In other studies with experimental infection models, it has been shown that infusion of cytokines increases the resistance against bacterial, fungal, and parasitic infection. Furthermore, immunized mice resistant to microbial pathogens can be severely compromised by treatment with anti-TNF or anti-GM-CSF antibodies (Coffey 1992; Ferrante 1992).

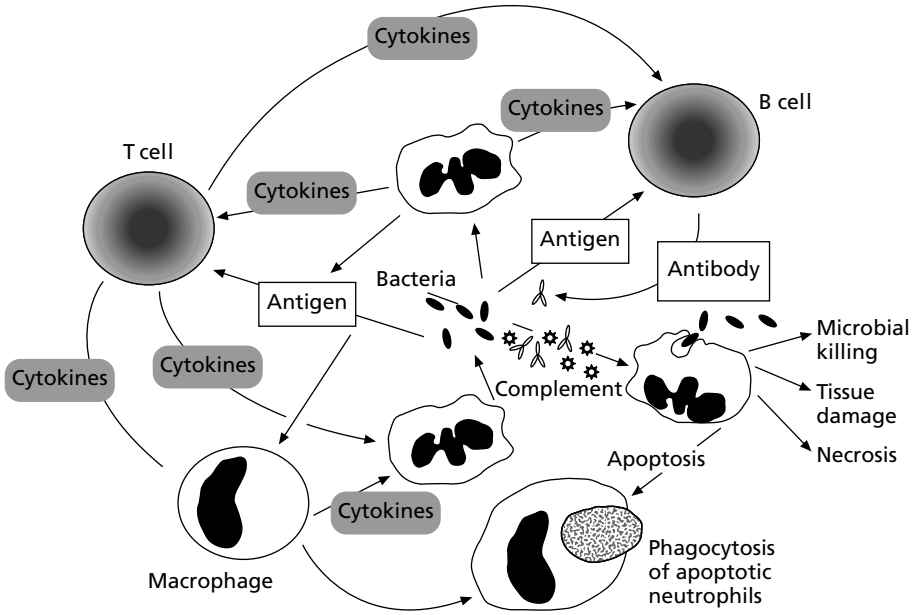

Figure 7 *An abridged version of the intricate interrelationship of neutrophils, lymphocytes, and mononuclear phagocytes. The main function of the neutrophil is to phagocytose microbial pathogens. Phagocytosis is promoted by coating of the bacteria with antibody and complement. Neutrophil recognition of particles, phagocytosis, and killing is greatly accelerated by priming neutrophils with cytokines released by T lymphocytes and macrophages. Downregulation of tissue damage by activated neutrophils is achieved through the stimulation of apoptosis in neutrophils and engulfment by macrophages. The likely contribution of neutrophils to T- and B-lymphocyte responses is also demonstrated in this scheme.*

APOPTOSIS

Activation of a cell-intrinsic suicide program results in programmed cell death or apoptosis, which can be distinguished from degenerative cell death or necrosis. The dying cell is characterized by several properties such as shrinkage, crenation, condensed chromatin, and DNA fragmentation. Apoptosis is controlled by many types of endogenous and exogenous mediators. Neutrophil apoptosis is considered a host tissue-protective property because these cells lose their functional receptors and ability to produce toxic products, and are eventually engulfed and removed by macrophages (Fanning et al. 1999). This aging fate of neutrophils was first indicated by Metchnikoff (1893).

Neutrophils reaching inflammatory sites engulf and kill microbial pathogens and die at these sites. Death via necrosis leads to the release of tissue-damaging products. Thus, the physiological response, besides initiating events that lead to microbial killing, also ensures the subsequent initiation of apoptotic signals to downregulate the powerful oxidative and nonoxidative responses of the neutrophils and finally leads to the clearance of neutrophils from these sites (Newman et al. 1982). Inflammatory leukocytes, such as activated neutrophils, express Fas, a death factor receptor. Ligation of this receptor transduces the intracellular signals for apoptotic death (French and Tschopp 1997). Apoptotic signals in neutrophils lead to a loss in their ability to respond to inflammatory mediators because their cell surface receptors are downregulated. There is also a major loss in chemotactic, phagocytic, degranulation, and respiratory burst function. Further isolation from their toxic products results from the engulfment of the apoptotic neutrophils by macrophages. Essentially, this is a means of limiting tissue injury as demonstrated in Figure 7. It is believed that apoptotic neutrophils are recognized by either the exposure of phosphatidylserine on their surface and/or expression of a thrombospondin-binding moiety that recognizes thrombospondin and allows binding to the vitronectin (CD16) receptor on macrophages (Fadok et al. 1992).

Inflammatory fluids contain many different factors some of which protect against apoptosis. The cytokines G-CSF, GM-CSF and IL-2, as well as LPS and glucocorticoids, inhibit neutrophil apoptosis. However, conflicting data have been presented for TNF and fMLP.

PRODUCTION OF INFLAMMATORY MEDIATORS

Neutrophils play a central role in the inflammatory response in contributing to the inflammatory mediator network by releasing a range of mediators, including oxygen radicals, enzymes such as MPO, LTs, and prostaglandins (PGs) as well as a range of cytokines (Table 4). In this manner, neutrophils could have a significant influence on the acute and chronic inflammatory responses, as well as the immune response, by regulating T lymphocyte, macrophage, and antigen-presenting cell functions (Figure 8).

Table 4 *Products of stimulated neutrophils which may regulate inflammation and immune responses*

Class of mediator	Mediator
Lipids	PAF, AA, LTB4, PGE2, lipoxins, TXA4, 5-oxo-ETE
Cytokines	IL-1, TNF-α, IFN-α, IL-8, IL-6, TGF-β, IL-12, IL-10, IL-3, G-CSF, M-CSF, MCP-1, MIP-2, IL-1α

AA, arachidonic acid; CSF, colony-stimulating factor; G-CSF, granulocyte CSF; M-CSF, macrophage CSF; IFN, interferon; IL, interleukin; LT, leukotriene; MCP, monocytic chemotactic peptide; MIP, macrophage inflammatory protein; PAF, platelet-activating factor; PG, prostaglandin; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; TGF, transforming growth factor; TNF, tumor necrosis factor; Tx, thromboxane.

Lipid inflammatory mediators

Through the action of a number of cellular enzymes, such as phospholipase (PLA_2), cyclooxygenases, and lipoxygenases, neutrophils release a range of mediators (see Table 4). Several mediators produced are known for their marked effects on inflammation and immune responses (Haeggstrom and Serhan 1999). These include arachidonic acid (AA), LTB_4, PGE_2, and platelet-activating factor (PAF), all of which regulate neutrophil, eosinophil, basophil, T-lymphocyte, macrophage, and endothelial cell function.

Platelet-activating factor plays a role in both acute and chronic inflammation. It stimulates a range of neutrophil activities including chemotaxis, aggregation, adherence, respiratory burst, and degranulation. LTs have diverse effects and may promote the pathophysiological process during infection by stimulating endothelial cell function, upregulating macrophage, and lymphocyte functions. This contrasts with their role in promoting pathophysiology in rheumatoid arthritis, airway diseases such as asthma and adult respiratory distress syndrome, allergic inflammation, and cystic fibrosis (Penrose et al. 1999).

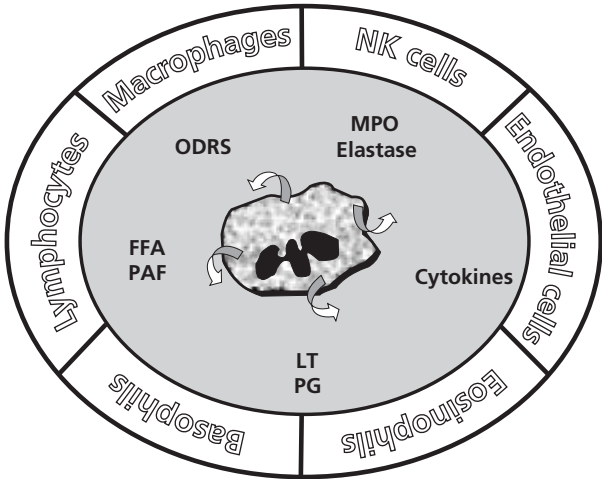

Figure 8 *Diagrammatic summary of mediators released by stimulated neutrophils and the cells that these may regulate. FFA, free fatty acid; LT, leukotriene; MPO, myeloperoxidase; NK, natural killer; PAF, platelet-activating factor; PG, prostaglandin.*

Prostaglandins, produced during the oxidation of AA via the cyclooxygenase pathway, have a wide range of effects and play a role in both physiological and pathophysiological responses (Griffith 1999). These regulate pain, edema, fever, and granulocyte, macrophage and lymphocyte function. PGE_2 suppresses neutrophil functions, chemotaxis, and respiratory burst. It also inhibits the activation of mononuclear phagocytes, depressing TNF synthesis. Inhibition of T-cell function, lymphoproliferation, and cytokine production are also inhibited by PGE_2, in particular the Th1 cytokines.

Cytokines

Although mature neutrophils have been considered as end cells not capable of RNA and protein synthesis, more recent evidence suggests that they can be induced to synthesize new proteins (Cassatella 1999). Most interesting has been the discovery that neutrophils can produce a wide range of cytokines (see Table 4). In this manner, the cells can regulate immune reactions. Although these cytokines are produced in very low amounts by neutrophils compared with macrophages, with a predominance of neutrophils in some inflammatory sites, it is possible that neutrophils release effective amounts of these cytokines to control other cells of the inflammatory response. Through the ability to release the spectrum of cytokines outlined in Table 4, neutrophils have the potential to control migration of mononuclear cells into tissues as well as to promote their activation. Thus, T lymphocytes as well as B lymphocytes may be under neutrophil control through the release of cytokines such as IL-1, IL-6, IL-10, IL-3, etc. Similarly, these and others will influence the function of mononuclear phagocytes and APCs. In this manner neutrophils that arrive early in inflammatory sites and antigen injection sites are likely to have some influence on the inflammatory response. The examination of the spectrum of cytokines produced by neutrophils shows that these may be of either Th1 or Th2 type, suggesting that the neutrophil could influence the type of immune response elicited by microbial antigens. Similarly, there are cytokines that regulate macrophage functions.

Interestingly, apart from a wide range of compounds able to stimulate cytokine production by neutrophils, there is also evidence that different stimulators may

cause the production of different types of cytokines and that both extrinsic and intrinsic stimulators can induce cytokine production in these cells (Cassatella 1999). Extrinsic agents include bacteria, fungi, and viruses and fMLP. Intrinsic stimulators include LTB_4, PAF, C5a and of course the cytokines themselves. The ability to produce IL-1 receptor antagonist (IL-1ra) enables the neutrophil to regulate the activity of the pyrogenic cytokine, IL-1.

Neutrophils, through the release of IL-8 (a CXC chemokine), can regulate the migration of other neutrophils and stimulate these cells for antimicrobial functions. As IL-8 is also chemotactic for T lymphocytes and basophils, neutrophils could be involved in exerting influence on these cell types during inflammation. Other CXC chemokines produced by neutrophils that have effects similar to IL-8 on neutrophils are growth-related gene product α/β (GROα/β) and IFN-γ-inducible protein (IP-10) which are mainly chemotactic for natural killer (NK) cells, and may therefore be important in the recruitment of these cells, as well as T cells and monocytes. Neutrophils can also secrete CC chemokines, such as macrophage inflammatory protein 1-α/β (MIP-α/β), which have potent chemotactic properties for monocytes and T lymphocytes.

The three cytokines that are classically referred to as the pyrogenic cytokines, TNF-α, IL-1, and IL-6, can also be produced by neutrophils. Thus, there is the potential for neutrophils to mediate pathogenesis by releasing these cytokines. Both TNF-α and IL-1 are also known to prime neutrophils for increased antimicrobial activity and it is possible that neutrophils may autoregulate their antimicrobial functions in this manner. The ability of these cytokines to regulate lymphocyte activation and differentiation enables neutrophils also to influence this property of the immunological system. The expression of the CD30 ligand (CD30L), a member of the nerve growth factor (NGF)/TNF receptor super family) on neutrophils suggests that neutrophils could interact via CD30 on T lymphocytes to promote activation of these cells. Neutrophils can also produce IL-1ra, which controls the effects of IL-1. This represents another level of regulation of inflammation and pathogenesis by neutrophils. A variety of agents is also capable of inducing the production of IL-1ra in neutrophils. The influence that stimulated the effects that neutrophils may have on the development of the Th1 versus the Th2 response can be extrapolated from their reported ability to produce and release IL-12, which induces IFN-γ production from NK cells and T lymphocytes, and a cytokine that facilitates Th1 lymphocyte responses and thereby suppresses IgE formation.

Although the amount of cytokine produced by neutrophils compared with macrophages is very small, in a number of inflammatory conditions neutrophils are present in substantial numbers and may contribute to immunoregulation through the release of selective cytokine patterns. Important consideration needs, however, to be given in specific situations, e.g. the ability of neutrophils to produce IFN-α in similar levels to that produced by monocytes when infected with Sendai virus suggests an important role for neutrophils in defense against viruses.

NEUTROPHIL IMMUNODEFICIENCY DISEASES

Congenital defects

The spectrum of primary neutrophil disorders is listed in Table 5. These range from disorders in which the numbers of neutrophils are too low to cope with infections to others that show functional defects of chemotaxis, adhesion, respiratory burst, and degranulation (Quile et al. 1996).

NEUTROPENIA

Leukocyte counts of below 1500 cells/mm^2 will increase the risk of infection in a progressive manner as the numbers continue to decrease below this level. A dramatic increase in incidence of infection is seen once the levels are below 500 cells/mm^2. Hereditary neutropenia occurs, ranging from severe, as seen in infantile genetic agranulocytosis, to moderate, as in familial neutropenia. The former disorder is a result of maturation arrest that is inherited in an autosomal recessive manner. Patients with hereditary neutropenia experience severe infection and death in infancy. Another neutropenia characterized by the periodic disappearance of neutrophils from the blood is cyclic neutropenia, an autosomal dominant disorder. During the period of low neutrophil counts, the patient can present with cutaneous infections and associated fever and malaise. Overcoming the neutropenic oscillations can be achieved with G-CSF or prednisolone treatment.

LEUKOCYTE ADHESION DEFICIENCY

Two types of leukocyte adhesion deficiency (LAD) have been described. In type 1 there is a defect in the integrin molecule. This is the result of an abnormal β-chain synthesis presenting as a spectrum of mutations in the β gene. Although the α chain is produced in normal amounts, the assembly of the α and β chains is poor and is poorly transported to the cell surface. The neutrophils have impaired adherence, chemotaxis, and respiratory burst in response to complement- but not antibody-coated particles. Although cells from these individuals display normal rolling adhesion, they cannot bind firmly to venule walls and hence are unable to migrate out of the blood vessels. This is an autosomal recessive disease after a history of consanguinity. Patients are likely to present with prolonged, as well as recurrent, infections with *Pseudomonas* and *Staphylococcus* spp. during

Table 5 *Summary of primary immunodeficiencies of neutrophils*

Disease	Function affected
Neutropenia	
Infantile genetic agranulocytosis	
Familial neutropenia	
Cyclic neutropenia	
Leukocyte adhesion deficiency	
Type 1 (CR3 deficiency)	Adhesion, chemotaxis, aggregation, spreading
Type 2 (sialyl Lewis deficiency)	Selectin ligation, rolling, adhesion
Chronic granulomatosis disease	
Membrane defects of	Oxidase (lack of production of oxygen reactive species)
$gp91^{phox}$	
$p22^{phox}$	
Cytosol defects of	
$p47^{phox}$	
$p67^{phox}$	
Chédiak–Higashi syndrome	Granules (giant lysosomal granules): decreased chemotaxis, degranulation and bactericidal activity, increased H_2O_2 production, deficient elastase and cathepsin G
Specific granule deficiency	Specific granules are absent; decreased chemotaxis, O_2^- production, bactericidal activity
Myeloperoxidase (MPO) deficiency	Absent or low MPO
Hyper-IgE (Job's) syndrome	Chemotactic defect

infancy. Poor wound healing can also occur which can manifest in delayed umbilical cord separation. Infection sites include the intestinal tract, soft tissues, and mucosal surfaces. Those individuals who survive infancy are likely to develop acute gingivitis and eventually gingival hypertrophy. Patients with the moderate phenotype may survive to adulthood. It has been estimated that two-fifths of patients with type 1 LAD die before 2 years of age.

In LAD type 2, the defect is an absence of expression of the primary selectin ligand sLe^x on the neutrophils. This is an autosomal recessive disorder in which poor random mobility and chemotactic responses by neutrophils are exhibited. However, the respiratory burst response to opsonized particles does lie within a normal range. The neutrophils fail to adhere to stimulated venules and lack rolling adhesion. As the integrin expression is normal the neutrophils still adhere to blood vessel walls. Patients present from early childhood with recurrent bacterial infections.

CHRONIC GRANULOMATOUS DISEASE

Chronic granulomatous disease is a group of disorders of phagocyte oxidative respiratory burst which can be inherited either in an X-linked or an autosomal recessive manner. The basis of this deficiency is the result of a defect in one of several components that make up the oxidase. Abnormalities in the membrane-bound component $gp91^{phox}$ of cytochrome b_{558} give rise to the most common form of CGD (55–60 percent). Defects of the light chain of the membrane cytochrome, $gp22^{phox}$ have also been described with an approximate frequency of 5 percent. Defects in the cytosolic components $p47^{phox}$ and $p67^{phox}$ account for 30–35 and 5 percent of the CGD, respectively.

Chronic granulomatous disease is manifested clinically by the development of serious infections in early childhood. Pneumonia, skin and soft tissue infections, lung abscesses, osteomyelitis, and hepatic abscesses may be common. The majority of the infections can be accounted for by *S. aureus* and *Serratia burkholderia* and, less commonly, by infection with *Aspergillus* and *Nocardia* spp. Management of patients with CGD primarily involves aggressive antibiotic cover, surgical drainage, and even white cell transfusion. Prophylactic IFN-γ has recently been employed which has reduced the occurrence of these life-threatening infections. It is recommended that IFN-γ be used in conjunction with trimethoprim–sulfamethoxazole. Efforts are being made at present to use gene therapy to correct the defect in CGD, with some early success with retroviral-mediated gene therapy for $p47^{phox}$ deficiency.

CHÉDIAK–HIGASHI SYNDROME

Neutrophils from patients with Chédiak–Higashi syndrome (CHS) show abnormal (large) granules as a result of fusion of two granules. Although the cells can phagocytose particles, chemotaxis and microbial killing

are abnormal. The respiratory burst and its ability to produce oxygen-reactive species is normal but the release of MPO is delayed. The neutrophils show deficiency in cathepsin G and elastase. There is abnormal fusion of granules, possibly as a result of defects in membrane fluidity and the microtubules. The bactericidal activity of the cell is greatly diminished as a consequence of a delay in the delivery of granule constituents to the phagocytic vacuole. Apart from neutrophils, defective functions of other cells of the immune system occur, impaired NK cell activity, and antibody-dependent, cell-mediated cytotoxicity. Cytotoxic T-cell activity is also affected. Decreased neutrophil and monocyte migration and reduced bactericidal activity are likely to explain the delayed inflammatory response and the increased susceptibility to infections expressed by patients with CHS, who usually die from infections at an early age.

SPECIFIC GRANULE DEFICIENCY

Patients with an absence of specific granules have been described. Their neutrophils lack specific granule constituents such as lactoferrin and vitamin B_{12}-binding protein. However, it has also been found that the cells are devoid of defensins, which are major components of the azurophilic granule. Thus, there is likely to be a more general defect. As a result of the lack of specific granules, upregulation of functional surface receptors is compromised. It is not surprising to find that neutrophils have chemotactic defects. Patients present with recurrent infections.

MYELOPEROXIDASE DEFICIENCY

This is the most common of the neutrophil disorders. This deficiency may go unnoticed because, in many individuals, the susceptibility to infection is not easily recognized and only a few cases have been reported with serious infections, mainly candidiasis. Killing of microorganisms is delayed because of the absence of MPO. Other functions such as chemotaxis, phagocytosis, respiratory burst, and degranulation are normal. Superoxide levels may be increased because of the lack of MPO and this may compensate for the lack of killing of bacteria via the MPO–H_2O_2–halide system.

HYPER-IgE SYNDROME (JOB'S SYNDROME)

Patients with extremely high levels of IgE have depressed neutrophil chemotaxis and depressed acute inflammation. One of the suggested causes of this is the release of histamine. These patients present with recurrent cutaneous and sinopulmonary infections. Characteristically, the infections are caused by *S. aureus*, but in some cases *H. influenzae* causes pulmonary infections. T lymphocytes from these patients have also been reported to be deficient in IFN-γ production and it has been suggested that an imbalance between IL-4 and IFN-γ production leads to downstream switching of immunoglobulin genes, giving rise to elevated IgE. Although some sporadic cases have been described, hyper-IgE syndrome is an autosomal dominant disease.

Acquired immunodeficiencies of neutrophils

Secondary immunodeficiency of neutrophils, as with primary immunodeficiency, spans a wide range, but will usually occur in combinations because of the nonspecific nature of the causative agent. This includes malnutrition, cancer and chemotherapy, infection, immaturity of the immune system in neonates, and deterioration as a result of age. The hallmark of these diseases is the increased severity and recurrence of infection, as for primary immunodeficiencies, although it is considered not to be as severe.

Drug therapy is responsible for acquired neutropenias, seen in patients undergoing chemotherapy for immunological and neoplastic disorders. Idiosyncratic reaction may occur when drugs such as penicillin, cephalosporins, vancomycin, sulfonamides, and phenothiazines are used. With antibody-mediated neutrophil damage or hypersplenism, neutropenia is experienced because of sequestration of the neutrophils in splenic tissue (Palmblad et al. 2001).

Diseases in which circulating immune complexes occur, including rheumatoid arthritis, systemic lupus erythematosus, etc., can show a high rate of oxidative respiratory burst and granule release, and decrease in chemotactic responses. Other forms of serum chemotactic inhibitors have been found. Bactericidal products may cause neutrophil chemotactic abnormalities as seen, for example, in some patients with gingival infections with *Bacterioides ochraceus* (Clark et al. 1977).

Treatment of these infections restores chemotactic responsiveness. IgG antineutrophil antibodies have also been implicated with neutrophil chemotactic responsiveness seen in patients with recurrent skin infections. Other agents that can cause decreased chemotactic responsiveness include the drugs amphotericin B and tetracyclines, and alcohol. Other causes of acquired chemotactic immunodeficiencies include malnutrition, physiological immaturity of the neonate, viral infections, sepsis, and thermal injury. In leukemia, thermal injury, various autoimmune conditions, sepsis, and viral infections, the neutrophil microbicidal activity can also be significantly reduced. Viral infections can lead to decreased neutrophil functions and associated risk of bacterial infections (Faden and Ogra 1986). Thus, during epidemics of influenza, deaths have been associated with bacterial pneumonia. Viruses have been shown to depress neutrophil chemotaxis, phagocytosis, and respiratory burst/intracellular killing.

A range of bacteria as well as fungi and parasites cause depressed neutrophil chemotaxis, phagocytosis, and oxidative burst. The pneumococcal toxin, pneumolysin was found to cause marked inhibition of neutrophil-mediated killing of *S. pneumonia* and the respiratory burst of the cell (Paton and Ferrante 1983).

Therapies for neutrophil disorders

Marked neutropenia and severe neutrophil function defects lead to life-threatening infections (Walsh et al. 1994). Antibiotic prophylaxis is used in most cases, although the development of antibiotic-resistant organisms presents another problem. Other forms of therapies include neutrophil transfusion, bone marrow transplantation, and cytokine infusions.

Neutrophil transfusion is of possible benefit to neutropenic patients with infections, although the results from some of the clinical trials have questioned whether the patients actually benefit. Nevertheless, benefits can be achieved if a careful approach is taken (Strauss 1999). There are various explanations for the inconsistencies in results with this type of therapy, including the severity of the infection and the various antibiotics used. Thus transfusions should be used in combination with the various therapies, antibiotics, immunoglobulin, and recombinant growth factors. The decision to proceed with neutrophil infusion should be guided by ensuring that relevant preparation methods are used and effective doses are given to patients. Neutrophils prepared from donors who have received G-CSF seem to give a more favorable response.

Bone marrow transplantation has been used successfully in LAD and CGD and more recently hemopoietic progenitor cell transfusions have become an attractive option. The treatment of patients with recombinant hemopoietic growth factors such as G-CSF is very attractive; it has proved useful to enhance bone marrow recovery as a consequence of chemotherapy and was beneficial in protecting against infection in these patients. According to reported information, low-dose G-CSF in patients with cyclic and idiopathic neutropenia is effective in elevating circulating neutrophil numbers and decreasing the episodes of fever and infections. In congenital neutropenia high doses of G-CSF are needed.

Trials have also been conducted on the use of G-CSF in non-neutropenic patients with pneumonia. These patients experienced an increase in neutrophil levels as well as clinical improvement. In addition, cytokine therapy has been used in CGD. The incidence of life-threatening infection in CGD patients can be markedly reduced with prophylactic IFN-γ. Both the number and the severity of infections are dramatically reduced (Quie et al. 1996). The action is most likely through a multiple effect, including restoration of some functional oxidase activity, macrophage granule stimulation, and increased expression of functional receptors such as FcγR. This cytokine is recommended in conjunction with antimicrobial agents as a prophylactic for CGD.

Laboratory diagnosis of neutrophil deficiencies

The various neutrophil immunodeficiency syndromes can be easily diagnosed by a series of laboratory tests (Metcalf, Gallin et al. 1986; Kuijpers et al. 1999; Virella 1999). These tests can range from very simple procedures to those requiring much more sophistication. Although some tests will measure a specific pathway, others measure the activity of several pathways culminating in a final event. When the defect in neutrophil function is severe, patients will usually present with recurrent and life-threatening infections. An outline of the tests used to assess the competence of neutrophils in a patient is summarized in Table 6. Usually evaluations would proceed to this group of tests when recurrent bacterial and fungal infections in patients cannot be explained by neutropenia, defects of antibody or complement, and T-cell abnormalities.

Leukocyte adhesion deficiency can be diagnosed by functional and flow cytometric analysis and the two forms, LAD1 and LAD2, should be differentiated. In LAD1 there is deficient expression of the α and β chains

Table 6 *Neutrophil functional tests for diagnosis of primary immunodeficiency*

Function	Tests
LAD1	Adhesion of neutrophils
	Flow cytometry
LAD2	Flow cytometry
Chemotaxis	Migration under agarose
	Boyden chamber
	Modified/commercial Boyden chamber
Phagocytosis	Uptake of yeast particles/by microscopy
	Uptake of particles by flow cytometry
	Radiometric assay
	Erythrocyte lysis assay
NADPH oxidase	NBT assay
	Chemiluminescence (lucigenin)
	$O_2^{\cdot-}$ or H_2O_2 assays
	HMP shunt
MPO	Quantitative leukocyte iodination assay
	MPO enzyme assay
	Histochemical assay
Microbicidal activity	Bactericidal assay
	Fungicidal assay

HMP, hexose monophosphate; LAD, leukocyte adhesion deficiency; MPO, myeloperoxidase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium.

of the integrins, and hence antibodies to these surface molecules can be used in conjunction with flow cytometry to diagnose this deficiency. Similarly the detection of the surface expression of sLe^x is a diagnostic tool for LAD2. The ability for neutrophils from these patients to adhere to plasma-coated surfaces in microtiter plates can be measured when these cells are either treated or not treated with agonists such as TNF or fMLP. The ability to respond to complement-coated particles that are ligated to neutrophils via the CR3 receptor can also be diagnostic for LAD1. Because these cells are unable to adhere to substrates they will also show a deficiency of chemotaxis.

Chemotactic assays have now been relatively standardized and defects of chemotaxis can be identified by these assay methods. The two methods that are usually used are the technique of migration under agarose and in chemotactic chambers, developed by Boyden and referred to as the Boyden chamber (Boyden 1962), and modifications thereof (Rot 1999). In the former assay, wells are cut into agarose into which cells and a chemotactic agent such as fMLP can be applied. As the fMLP diffuses through the agarose, a chemotactic gradient is set up which attracts the cells and the distance migrated can be measured microscopically.

The Boyden chamber and related chambers consist of two compartments separated by a filter that contains pores to enable neutrophils to crawl through from one chamber toward the chemotactic agent in the other chamber. The distance moved or number of cells moved into the filter is measured to ascertain the chemotactic response. Once again fMLP is used most commonly as the chemotactic agent but others include LTB_4 and complement, e.g. C5a, IL-8.

Measurement of the phagocytic activity of the cell can be quite difficult because it is difficult to distinguish between adhered and engulfed particles. Some techniques can be rather subjective when microscopic counting of internalized particles is conducted. The particles used include zymosan (yeast), *C. albicans*, and erythrocytes. These are opsonized for promoting recognition by neutrophils. One means of distinguishing between adhered and ingested particles is to use radiolabeled *S. aureus* and then eliminate those that have not been ingested by treating with lysostaphin. A radiometric technique has also been described that takes into consideration that there is little RNA synthesis in neutrophils. In this assay, after an incubation period to allow phagocytosis to take place, [^{3}H]uridine, which is readily taken up by non-engulfed bacteria or fungi, is added to the cultures. The amount of inhibition of uridine uptake measures an index of phagocytic power. Other systems make use of erythrocytes and hypotonic lysis of nonphagocytosed erythrocytes. A flow cytometry method has also been developed which makes use of fungi or bacteria with fluorescent labels and quenching of any nonengulfed bacteria with trypan blue.

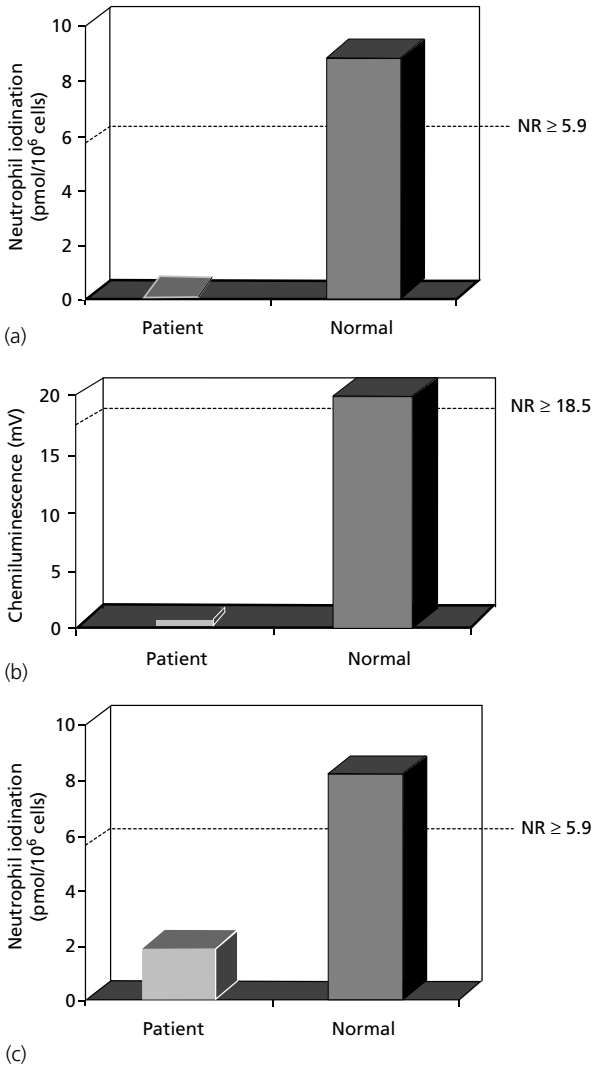

Figure 9 *The neutrophil iodination reaction in chronic granulomatous disease (CGD) and myeloperoxidase (MPO)-deficient neutrophils.* **(a)** *Lack of chemiluminescence response in neutrophils from patients with chronic granulomatous disease stimulated with formyl-methionine–leucine–phenylalanine (fMLP).* **(b)** *Poor leukocyte iodination reaction in the same cells stimulated with opsonized zymosan.* **(c)** *Depressed leukocyte iodination reaction in neutrophils from an MPO-deficient patient. NR, normal range.*

Defects in the oxidase can be assessed by a range of assays. The cells fail to undergo respiratory burst activity and hence do not produce oxygen-derived reaction species. Various assays are available for measuring O_2^- and H_2O_2. A standard assay for O_2^- is the superoxide dismutase-inhibitable cytochrome *c* reduction assay, which measures a color change that is read spectrophotometrically (Figure 9). In another system, a lucigenin-dependent chemiluminescence assay is used for measuring the superoxide produced by cells. This assay is facilitated by the used of a luminometer which dark-adapts the cells and is able to inject various components into the assay mixture.

The classic test for GGD and a detective oxidase system is the nitroblue tetrazolium (NBT) dye reduction. This is readily performed on cells adherent to microscope slides. A stimulus is used to activate the oxidase. In this reaction, the pale-yellow NBT is reduced to form a blue formazan. The cells positive for this reaction are scored microscopically. Normal cells will have more than 98 percent of the cell staining positively for NBT reduction while neutrophils from CGD patients lack staining. As a defect of the oxidase is being sought, phorbol myristate acetate (PMA) is the preferred stimulus which bypasses the surface receptor and stimulates the assembly of the oxidase by activating PKC. This excludes other possible defects such as β_2-integrin deficiencies, which can give rise to lack of activation of the oxidase because of particle adhesion defects. Then, by using opsonized zymosan in combination with PMA, defects of adhesion and ingestion can be diagnosed by the same testing system.

Flow cytometers are now readily available and a fluorescence assay that is based on the oxidation of an intracellular 2′,7′-dichlorofluorescein diacetate to the highly fluorescent 2′,7′-dichlorofluorescein can also be used to detect oxidase activity in stimulated neutrophils. Other fluorescence indicators include dihydrorhodamine 123. Both PMA and opsonized zymosan can be used to stimulate the respiratory burst.

Myeloperoxidase deficiency is the most common neutrophil disorder and various methods can be used to detect the presence or absence of MPO as well as to quantify MPO levels. This includes histochemical staining of the enzyme in cells or examining a leukocyte extract for the enzyme. A convenient method is the quantitative leukocyte iodination reaction (see Figure 9). This takes into consideration both the release of MPO and the production of H_2O_2 (i.e. oxidase activity). Thus, in conjunction with a measurement of the oxidase assay such as NBT or chemiluminescence, the assay can be a first-line screen for MPO deficiency (see Figure 9).

Neutrophil microbial killing assays are important because these represent the net effect of all the cellular components and, if the appropriate opsonin and microorganisms are chosen, this will detect most of the neutrophil defects such as LAD, MPO deficiency, NADPH oxidase defects, degranulation deficiency, and phagocytosis defects. The particles used will usually be complement-opsonized bacteria or fungi. Samples taken over the period of 1 hour for bacteria and 2 hours for fungi can be used to determine both the amount and the rate of killing, e.g. with MPO deficiency, neutrophils will show a delay in killing of *C. albicans*. For killing of bacteria, *S. aureus* is usually chosen. As this bacterium can produce H_2O_2, the lack of NADPH oxidase activity can be overcome. Thus, it is essential that the *S. aureus* used is catalase positive.

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Basophils and eosinophils

EDWARD E.S. MITRE AND THOMAS B. NUTMAN

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Basophils and eosinophils are leukocytes with large cytoplasmic granules that originate from $CD34^+$ progenitor cells. Although both are present at tissue sites of inflammation in allergic and helminthic disease, basophils reside predominantly in the circulation, whereas the majority of eosinophils are found in the tissues. Both cell types have lifespans of a few days, which can be prolonged through the actions of the interleukins IL-3, IL-5, and other growth factors; there is a large overlap in the mediators that cause them to traffic from the circulation to sites of inflammation. Both cell types release multiple inflammatory mediators, with histamine being the primary effector molecule of basophils and granule proteins being the primary effector molecules of eosinophils. Basophils typically degranulate through an IgE-dependent pathway, whereas eosinophils are most often activated by cytokine–cytokine receptor interaction. Although both cells are classically considered part of the innate immune system, they also modify the adaptive immune system through the release of cytokines and chemokines. In addition, histamine released from basophils has multiple immunomodulatory effects, and eosinophils have the ability to process and present antigen. Finally, basophils and eosinophils are clearly involved in the immune response to helminth infections and play a role in HIV and respiratory virus infections.

BASIC BIOLOGY

Basophils

MORPHOLOGY

Basophils are the least common white blood cell, making up 0.5–1 percent of the circulating peripheral blood cell population and accounting for 0.33 percent of the nucleated cells in the marrow (Juhlin 1963). They have lobulated nuclei of various forms and contain multiple prominent amorphous granules that stain purple to red with blue aniline dyes (Figures 1a and 2a). This staining pattern is the result of the presence within the granules of highly sulfated proteoglycans which are often complexed to proteases, histamine, and cytokines.

GROWTH AND DIFFERENTIATION

Basophils, as well as eosinophils, mast cells, monocytes, and neutrophils, originate from pluripotential $CD34^+$ progenitor cells found in cord blood, peripheral blood, and bone marrow (Kirshenbaum et al. 1991; Metcalf 1989). Basophils and eosinophils are believed to derive from a subset of $CD34^+$ cells that are also interleukin (IL) IL-3Rα^+ and IL-5R$^+$ (Boyce et al. 1995). The existence of a common progenitor cell for eosinophils and basophils is supported by the finding of granulocytes with hybrid eosinophil/basophil phenotype in vivo in patients with chronic myelogenous leukemia (Weil and Hrisinko 1987) and in vitro in cultures of peripheral blood and bone marrow from normal human donors (Leary and Ogawa 1984; Denburg et al. 1985). There is also evidence that basophils share a common progenitor with mast cells. Traditionally, basophils have been thought to arise from a separate cell lineage than mast cells, the progenitor cells of which are $CD34^+/CD38^+$ (Kempuraj et al. 1999) or $CD34^+$/c-kit$^+/CD13^+$ (Kirshenbaum et al. 1999). Recently, however, a new monoclonal antibody (97A6 – which targets ectonucleotide pyropho-

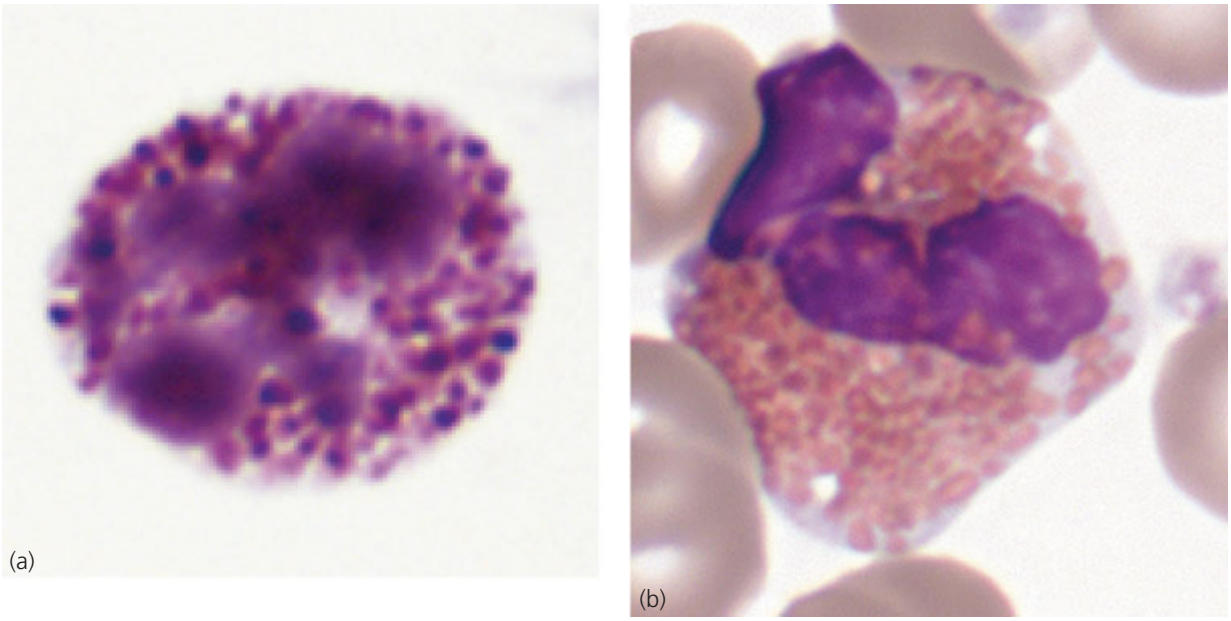

Figure 1 (a) *May–Grunïwald stain of a human peripheral blood basophil at 1000 × magnification with typical multilobulated nucleus and multiple large purple-staining granules of variable size.* **(b)** *Wright–Giemsa stain of a human peripheral blood eosinophil viewed at 1000 × demonstrating the characteristic bilobed nucleus and red-staining granules.*

sphatase/phosphodiesterase 3) has been described that is specific for mast cells, basophils, and their precursors (Buhring et al. 1999, 2001), suggesting that mast cells and basophils arise from a similar lineage.

The principal cytokine required for basophil growth and differentiation is IL-3 (Valent 1995); 9.5–50 percent of cord blood and bone marrow cells differentiated in the presence of IL-3 becomes basophils, with the remaining cells consisting of eosinophils, neutrophils, and macrophages (Saito et al. 1988). Growth of chronic myelogenous leukemia basophils, and of the human basophil-like cell line KU 812, is supported by IL-3 through high-affinity binding sites (Valent et al. 1989, 1990); moreover, humans treated with recombinant human IL-3 (rhIL-3) develop basophilia (Ganser et al. 1990). Studies with IL-3 knock-out (KO) mice have

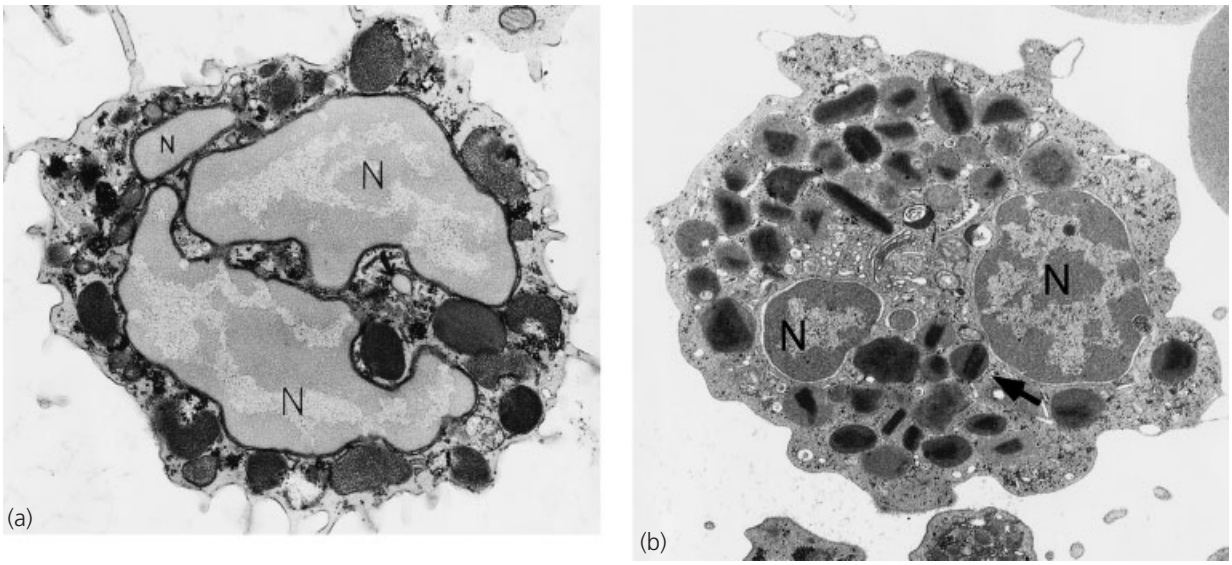

Figure 2 (a) *Electron micrograph of a human blood basophil with characteristic multilobulated nucleus (N) and numerous large secretory granules filled with electron-dense particles. Another granule type is small, rests near the nucleus, and contains homogenous, poorly electron-dense material (arrow). Focal electron-dense particles and aggregates of glycogen are present in the cytoplasm. ×77000 (With permission, Dvorak et al. 1996).* **(b)** *Electron micrograph of a human blood eosinophil with bilobed nucleus (N) and several characteristic specific granules with dense crystalloid cores (arrow). ×77000 (Image courtesy of William Riemenschneider NHLBI, NIH.)*

demonstrated that IL-3 is not required for development of baseline basophil numbers in the bone marrow and circulation, but is necessary for the enhanced basophil production seen in mouse models of helminth infection (Lantz et al. 1998).

Cytokines other than IL-3 also contribute to basophil development. Differentiation of the human basophil-like cell line KU 812 can be promoted by IL-6 and tumor necrosis factor α (TNF-α) (Nilsson et al. 1994); granulocyte–macrophage colony-stimulating factor (GM-CSF) and possibly IL-5 can cause basophil differentiation and increased production from the peripheral blood (Hutt-Taylor et al. 1988; Saito et al. 1988; Denburg et al. 1991). Although they do not have much of an effect when acting alone, nerve growth factor (NGF) and transforming growth factor β (TGF-β) can act in synergy with GM-CSF and IL-3, respectively, to enhance basophil growth (Matsuda et al. 1988; Tsuda et al. 1991; Sillaber et al. 1992).

NATURAL HISTORY

Basophils, which primarily remain in the circulation after maturation in the bone marrow, can infiltrate tissues at sites of active inflammation in parasitic diseases (Cross et al. 1987), asthma (Kepley et al. 2001), and allergy (Mitchell et al. 1982; Guo et al. 1994). The typical basophil has a relatively short lifespan, ranging from a few days to a few weeks. IL-3, IL-5, and NGF all improve the basophil lifespan in culture, with IL-3 improving viability the most (Miura et al. 2001). Whereas basophils cultured in media alone die within 3 days, basophils cultured with IL-3 have > 95 percent viability at 3 days (Miura et al. 2001). The basophil lifespan is also prolonged slightly by GM-CSF but not by IL-4, G-CSF, M-CSF (Yamaguchi et al. 1992), or stem cell factor (Dvorak et al. 1994). Basophils differentiated in vitro appear at about 1 week, peak at 3 weeks, and then decrease in number thereafter (Kirshenbaum et al. 1989). Basophils usually die through apoptosis, which can be induced by glucocorticoids (Yoshimura et al. 2001) and inhibited by IL-3 (although not by IL-5 or GM-CSF) (Zheng et al. 2002).

Eosinophils

MORPHOLOGY

Eosinophils have bilobed nuclei and large cytoplasmic granules that stain with eosin and other acidic dyes (see Figures 1b and 2b). Specific granules have dense crystalloid cores and store the major secretory effector proteins as well as various enzymes and cytokines. Another population of granules, so-called primary granules, contain Charcot–Leyden crystal protein. Smaller granules contain acid phosphatase and arylsulfatase, and non-membrane-bound organelles called lipid bodies contain arachidonic acid, 5-lipoxygenase, and cyclooxygenase (and are probably the main sites of prostaglandin and leukotriene production in the eosinophil) (Weller and Dvorak 1994). Eosinophils also have vesiculotubular structures that contain cytochrome b_{558}, a component of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that enables eosinophils to produce superoxide upon activation.

GROWTH AND DIFFERENTIATION

Like basophils, eosinophils develop in the bone marrow from pluripotent $CD34^+$ cells in approximately 1 week. Differentiation into eosinophils is primarily guided by IL-3, IL-5, and GM-CSF. Of these, IL-5 is the most powerful inducer of eosinophilopoiesis, as the usual bone marrow eosinophilia that develops in sensitized mice challenged with allergen is almost completely abrogated by treatment with anti-IL-5, yet hardly diminished by either anti-IL-3 or anti-GM-CSF (Tomaki et al. 2002). Eosinophilopoiesis is not dependent exclusively on these three cytokines, because mice that lack the IL-5 receptor (Foster et al. 1996) or the common receptor β chain for these three cytokines (Nishinakamura et al. 1995) are able to produce eosinophils, although the basal level is lower than normal and the mice are unable to increase their eosinophil counts in response to helminths or allergic challenge. Unlike IL-3 and GM-CSF, which promote the development of multiple lineages, IL-5 acts most specifically on eosinophils. IL-5 is made predominantly by type II $CD4^+$ T cells, but can also be made by natural killer (NK) cells, $CD8^+$ T cells, and eosinophils.

Eosinophilopoiesis can be inhibited by IL-12. Mice lacking IL-12 have higher bone marrow eosinophil counts than control mice (Zhao et al. 2000), and treatment of allergic mice with intrapulmonary IL-12 decreases bone marrow eosinophil counts by up to 45 percent (Rais et al. 2002). IL-12 may inhibit eosinophil production by upregulation of interferon γ (IFN-γ), because IL-12 inhibition of eosinophilopoiesis does not occur in IFN-γ KO mice (Rais et al. 2002).

NATURAL HISTORY

After differentiation, a pool of mature eosinophils remains in the bone marrow and can be rapidly released after IL-5 or eotaxin administration (Palframan et al. 1998a, 1998b). Eosinophils typically circulate for 3–26 hours in the circulation before migration into tissues, where the vast majority of eosinophils exist and where they survive for several days to weeks. In vivo studies of mice infected with *Angiostrongylus cantonensis* show that eosinophils taken from the site of infection have prolonged survival compared with that of peripheral blood eosinophils (Sugaya et al. 2001). The eosinophil lifespan is prolonged by IL-5, IL-3, and GM-CSF, each of which prevents apoptosis of eosinophils in in vitro culture systems (Simon and Blaser 1995). In the absence

of stimulating cytokines, eosinophils soon undergo apoptosis (Walsh 1997). Eosinophils survive up to 10 days in the presence of IL-5 but only up to 4 days in its absence (Yamaguchi et al. 1991); 53 percent of eosinophils cultured in vitro with IL-3 are viable at 14 days, whereas only 10 percent are viable in its absence (Rothenberg et al. 1988). After 7 days of culture with GM-CSF, 43 percent of human eosinophils remain alive, whereas most eosinophils cultured in media alone are dead (Owen et al. 1987). IL-5, IL-3, and GM-CSF most probably prolong eosinophil survival by inhibiting apoptosis by signaling through the β subunit of their receptors, which they all share. This β subunit interacts with Lyn and syk tyrosine kinases involved in anti-apoptotic signaling in eosinophils (Yousefi et al. 1996).

Eosinophil survival can also be prolonged by IL-15 (probably through induction of autocrine GM-CSF production by eosinophils) (Hoontrakoon et al. 2002), TNF-α (Valerius et al. 1990), nitric oxide (Beauvais et al. 1995), and engagement of CD40 (Bureau et al. 2002). Although IFN-γ and IFN-α are generally considered capable of prolonging eosinophil survival (Valerius et al. 1990), one study showed that interferons diminished eosinophil survival (Morita et al. 1996).

Eosinophils usually die through apoptosis. This occurs in the absence of activating cytokines and is most probably induced through events mediated by the Fas receptor (CD95) (Matsumoto et al. 1995), which eosinophils express on their surface (Druilhe et al. 1996). Apoptosis of eosinophils can also be induced by glucocorticoids (Meagher et al. 1996), TNF-α (Fujihara et al. 2002), TGF-β_1 (Atsuta et al. 1995), and through ligation of CD69, a type II membrane antigen expressed on cytokine-stimulated eosinophils (Walsh et al. 1996). The recent finding that CD69 expression on eosinophils of patients with asthma increases after stimulation with a specific inhalation challenge suggests that CD69 may play a role in a negative feedback loop in eosinophilia (Pignatti et al. 2002).

Eosinophils that have undergone apoptosis are ingested by macrophages using macrophage surface proteins CD36 and $\alpha_v\beta_3$ (Stern et al. 1996), thus preventing unwanted deleterious release of toxic intracellular products upon eosinophil death.

CELL TRAFFICKING

Trafficking of basophils and eosinophils from the peripheral circulation to sites of inflammation occurs through a multistep process (Springer 1994), with leukocytes rolling on endothelial cells through binding of selectins, firmly adhering to endothelium via integrins, transmigrating through the endothelial cell layer by diapedesis, and finally proceeding to sites of inflammation through the effects of chemotactic agents. The exact mechanisms by which this occurs have been studied in greater detail in eosinophils than in basophils. As a result of the large number of adhesion and chemotaxis factors common to both cell types (Tables 1 and 2), it is likely that trafficking of basophils and eosinophils is quite similar. The extent of the overlap between the pathways of basophil and eosinophil recruitment and the extent to which these cell types are recruited independently of one another are areas of active investigation (Bochner and Schleimer 2001). Factors that alter expression of adhesion molecules and the relative importance of different chemotactic factors with regard to each cell type are discussed below.

Basophils

BASOPHIL ADHESION

Expression of basophil adhesion molecules is modulated by several factors. Pretreatment with IL-1 and TNF-α (Bochner et al. 1988), as well as FcεR1 aggregation (Bochner et al. 1989), enhances the ability of basophils to bind to endothelial cells, probably by upregulating CD18 on basophils. IL-3 increases basophil adhesiveness to endothelial cells, possibly by increasing CD11b – a molecule that can bind to intercellular adhesion molecule 1 (ICAM-1), fibrinogen, and complement component C3bi (Bochner et al. 1990) – on basophils. In addition to CD11b, basophil activation also increases surface expression of CD11c and αd, two other β integrins (Bochner 2000).

The type 2 cytokines IL-4 and IL-13 increase the ability of basophils and eosinophils to bind to endothelial cells by increasing endothelial cell expression of vascular cell adhesion molecule 1 (VCAM-1), to which basophils and eosinophils, but not neutrophils, can bind (Columbo et al. 1992; Bochner et al. 1995).

CHEMOTAXIS

Multiple factors – including cytokines, chemokines, complement components, and lipid products – serve as basophil chemoattractants. Of the CC chemokines, RANTES and monocytic chemotactic peptide (MCP-3) 3 have the greatest ability to attract basophils, followed by MCP-1 and macrophage infectivity potentiator 1α (MIP-1α) (Dahinden et al. 1994). In terms of the effects of the CC chemokines on basophils, RANTES acts mostly as a chemoattractant, MCP-1 as an inducer of basophil degranulation, and MCP-3 as a combination, serving as both a strong chemoattractant and an inducer of basophil degranulation (Rot et al. 1992; Dahinden et al. 1994). MCP-2, another CC chemokine, is also chemoattractive for basophils (Weber et al. 1995). Stromal cell-derived factor 1α (SDF-1α), a CXC chemokine that is the only known ligand for CXCR4, has been shown to recruit basophils with even greater potency than RANTES and MCP-1 (Jinquan et al. 2000). Of the

Table 1 *Basophil and eosinophil adhesion molecules and chemotaxis factors*

Adhesion molecules	Ligands	Adhesion molecule expression by Basophils	Eosinophils
Integrins			
β_1 *(VLA family)*			
$\alpha_4\beta_1$ (CD49d/CD29)	VCAM-1, fibronectin	+	+
$\alpha_5\beta_1$ (CD49e/CD29)	Fibronectin	+	+
$\alpha_6\beta_1$ (CD49f/CD29)	Laminin	–	+
β_2 *family*			
LFA-1 (CD11a/CD18)	ICAM-1, -2, -3	+	+
Mac-1 (CD11b/CD18)	C3bi, ICAM-1, fibronectin	+	+
P150,95 (CD11c/CD18)	C3bi, others	+	+
αd (αd/CD18)	ICAM-3, VCAM-1	+	+
β_7 *family*			
$\alpha_4\beta_7$	MAdCAM-1, VCAM-1, fibronectin	+	+
Ig gene superfamily			
ICAM-1 (CD54)	LFA-1, Mac-1	+	+
ICAM-3 (CD50)	LFA-1	+	+
PECAM-1 (CD31)	CD31	+	+
ICAM-2 (CD102)	LFA-1	+	–
Selectins			
L-Selectin (CD62L)	GlyCAM-1, CD34, MAdCAM-1	+	+
PSGL-1 (CD162)	P-Selectin	+	+
Sialyl Lewis-X (CD15s)	E-Selectin, P-selectin	+	+
Sialyl-dimeric Lewis-X	E-Selectin	+	+
Lewis-X (CD15)	P-Selectin	–	+
Others			
Pgp-1 (CD44)	Hyaluronic acid	+	+
Siglec-8	Sialic acid	+	+

Table 2 *Basophil and eosinophil adhesion molecules and chemotaxis factors*

Chemotaxis factors	Predominant recepter	Recepter expression by Basophils	Eosinophils
Chemokines			
MIP-1α, RANTES, MCP-2[a]	CCR1	+	+
Eotaxin, MCP-3, MCP-4, RANTES	CCR3	+	+
SDF-1α	CXCR4	+	+
MCP-1	CCR2	+	–
MIP-3α	CCR6	+	–
Cytokines			
IL-3	IL-3R	+	+
IL-5	IL-5R	+	+
IL-8	CXCR1, CXCR2	?	?
GM-CSF	GM-CSFR	+	+
TNF-α	TNF-αR	–	+
Others			
C3a	C3aR	+	+
C5a	C5aR (CD88)	+	+
fMLP	fMLPR	+	+
PAF	PAFR	+	+
Prostaglandin D_2	CRTH1	+	+
Leukotriene B_4	LTB_4R	?	+

See text for abbreviations.
a) MCP-2 can also use CCR2B and CCR3 as functional receptor.

cytokines, IL-3 and GM-CSF are strong basophil chemoattractants, and IL-5 and IL-8 are weak ones (Tanimoto et al. 1992; Yamaguchi et al. 1992).

Eotaxin, eotaxin-2, and eotaxin-3, which are usually considered primarily as eosinophil chemoattractive proteins, are also potent basophil chemoattractants (Yamada et al. 1997; Dulkys et al. 2001; Menzies-Gow et al. 2002). Other basophil chemoattractants include the complement components C3a and C5a, leukotriene B_4 (LTB_4), and platelet-activating factor (PAF) (Lett-Brown et al. 1976; Tanimoto et al. 1992). Recently, prostaglandin D_2 has been shown to induce migration of basophils and eosinophils through the CRTH2 receptor (Hirai et al. 2001).

The ability of basophils to migrate toward chemoattractants C5a, IL-3, and IL-8 is inhibited by dexamethasone (Yamaguchi et al. 1994).

Eosinophils

ADHESION

Recruitment of eosinophils out of the circulation begins with binding of VLA-4 ($\alpha_4\beta_1$) and selectins on eosinophils to VCAM-1 and E-selectin present on activated endothelial cells (Sriramarao et al. 1994, 2000; Ulfman et al. 1999). The ability to bind to VCAM-1, in particular, allows eosinophils and basophils to be selectively recruited from the circulation, because neutrophils lack VLA-4 (Walsh et al. 1991).

Once tethered, eosinophils can be acted upon by inflammatory mediators to upregulate integrins and other cell adhesion molecules to enable firm adhesion. IL-8, MCP-1, C3a, C5a, and PAF can all induce activation-dependent adhesion of rolling eosinophils, probably through upregulation of β_2-integrins that can bind to ICAM-1 on endothelial cells (Discipio et al. 1999; Gerszten et al. 1999; Ulfman et al. 2001; Broide 2002). Although CD11b–CD18 binding to ICAM-1 is considered important for firm adhesion, it is not necessary for tissue eosinophilia, because patients with CD18 deficiency still have eosinophils present in inflamed tissues (Anderson et al. 1985). After firm adhesion, cells then diapedese across the endothelial wall. In addition to inducing firm adhesion, C5a has been shown to enable eosinophils to migrate across the vascular endothelium (Discipio et al. 1999). Once across, leukocytes migrate through tissues under the guidance of chemoattractants.

Eosinophil-binding molecules are affected by numerous factors. As with basophils, IL-4 increases selective recruitment of eosinophils from the circulation by specifically upregulating expression of VCAM-1 on endothelial cells and enhancing VLA-4/VCAM-1 binding (Moser et al. 1992; Schleimer et al. 1992). IL-4, as well as TNF-α, has also been shown to upregulate VCAM-1 expression by lung fibroblasts (Sabatini et al. 2002). TNF-α also causes lung fibroblasts to increase ICAM-1 expression and to release the eosinophil chemoattractants MCP-1 and eotaxin. IL-4 and IL-13 have been shown in a mouse model of *Onchocerca volvulus* keratitis to recruit eosinophils to the cornea through upregulation of ICAM-1 expression on limbal vascular endothelial cells (Berger et al. 2002).

Other cytokines that can affect eosinophil adhesion include IL-3 and GM-CSF, which were shown to upregulate expression of VLA-4 and VLA-5 on eosinophils in a study of patients infected with *Onchocerca volvulus* (Brattig et al. 1995). This study also found that infective onchocerca larvae had fibronectin deposited on their surface, raising the possibility that eosinophils bind directly to helminths through fibronectin, a known ligand for VLA-4 and VLA-5.

Together, TNF-α and C5a increase eosinophil attachment to human bronchial epithelial cells threefold through upregulation of $\alpha_5\beta_1$ integrin, which appears to bind fibronectin (Burke-Gaffney et al. 2002).

In a sheep model of trichostrongylus infection, eosinophils at sites of helminth infection exhibit increased CD18 expression compared with circulating eosinophils (Stevenson et al. 2001). One agent that has been shown to increase CD18 and CD11b expression on eosinophils is leukotriene D_3 (LTD_3), which probably does so through the cysLT-1 receptor and β_2 integrin (Nagata et al. 2002).

The eotaxin proteins can also affect eosinophil adhesion. Eotaxin-1 has been shown to enhance binding of eosinophils to VCAM-1 (Kitayama et al. 1997). Eotaxin-2 appears to cause eosinophils to shift from VCAM-1 to ICAM-1 adhesion, possibly acting to enable firm adhesion after eosinophils have been tethered (Tachimoto et al. 2002). Eotaxin-2 also plays a role in eosinophil deadhesion (Tachimoto et al. 2000), whereas eotaxin-3 induces transmigration of eosinophils across the endothelium (Cuvelier and Patel 2001).

CHEMOTAXIS

Eotaxin

Initially isolated from the bronchiolar fluid of sensitized guinea pigs, eotaxin-1 is an 8- to 9-kDa protein with an amino acid sequence very similar to that of the CC chemokines MCP-1, MCP-2, and MCP-3 (Jose et al. 1994). Eotaxin is a powerful and specific eosinophil chemoattractant (Jose et al. 1994; Garcia-Zepeda et al. 1996). It acts by binding to CCR3, which is also the receptor for RANTES, MCP-2, MCP-3, and MCP-4 (Marleau et al. 1996; Alkhatib et al. 1997; Heath et al. 1997), and its production probably occurs via activation of the transcription factor NF-κB (Jedrzkiewicz et al. 2000). The importance of eotaxin and other CC chemokines in eosinophil recruitment is underscored by the finding that CCR3 KO mice fail to recruit eosinophils to the small intestine and skeletal muscle even after

infection with *Trichinella spiralis* (Gurish et al. 2002). Eotaxin can be made by pulmonary epithelial cells, because eotaxin mRNA is detectable in human epithelial cell lines after stimulation with TNF-α and IL-1β (Lilly et al. 1997). Production of eotaxin-1 by human airway fibroblasts is increased by TGF-β and IL-13 (Wenzel et al. 2002). IL-4, another type 2 cytokine, has differential effects on the production of eotaxin, upregulating its production by human lung epithelial cells and downregulating its production by peripheral blood mononuclear cells (PBMCs) (Nakamura et al. 2001). This differential effect probably serves to increase eosinophil recruitment out of the peripheral circulation and into the tissues.

Mice lacking the eotoxin gene have lower baseline levels of peripheral blood eosinophils and less eosinophilia in response to antigen challenge than normal mice (Rothenberg et al. 1997; Matthews et al. 1998). The findings that plasma eotaxin levels are significantly elevated in patients with acute asthma compared with age-, sex-, and ethnicity-matched patients with stable asthma (Lilly et al. 1999) and that bronchoalveolar lavage (BAL) levels of eotaxin increase after segmental allergen challenge (Lilly et al. 2001) suggest that eotaxin contributes to the eosinophilia in asthma and allergic diseases of humans. Intradermal injection of eotaxin and eotaxin-2 into atopic and nonatopic human volunteers results in infiltration of eosinophils, basophils, neutrophils, and macrophages, as well as in histological changes consistent with mast cell degranulation (Menzies-Gow et al. 2002).

As discussed in the previous section, eotaxin also mediates eosinophil adhesion. Notably, the ability of eotaxin to upregulate CD11b is three times greater in IL-5 transgenic mice than in normal mice, a finding that suggests synergy between eosinophil-specific cytokines such as IL-5 and chemokines in terms of inducing eosinophil aggregation (Kudlacz et al. 2002). Glucocorticoids, which suppress eosinophilia, have been shown to suppress eotaxin mRNA expression (Lilly et al. 1997).

Eotaxin-2 and eotaxin-3 (so named because of functional, not structural, similarities to eotaxin) are other CC chemokines that mediate their effects through CCR3. They serve as chemoattractants for both eosinophils and basophils and have been shown to have similar efficacy for inducing calcium flux, activating Gi proteins, and inducing superoxide anion generation as eotaxin (Forssmann et al. 1997; Dulkys et al. 2001; Badewa et al. 2002).

Interleukin 5

In addition to supporting differentiation and growth of eosinophils, IL-5 is a strong eosinophil chemoattractant (Lampinen et al. 2001). Although IL-5 does not appear to regulate baseline eosinophil blood counts, as IL-5 KO mice have normal basal peripheral eosinophil counts (Kopf et al. 1996), IL-5 is one of the major factors responsible for recruiting eosinophils into tissues. IL-5 KO mice sensitized to ovalbumin do not develop the eosinophilia, hyperreactivity, and lung damage that normal sensitized mice do after allergen challenge (Foster et al. 1996). Overexpression of IL-5 in transgenic mice results in increased numbers of eosinophils in the gastrointestinal mucosa compared with those in control mice (Mishra et al. 2002), and treatment of patients with asthma with monoclonal anti-IgE antibody results in significant decreases in sputum eosinophils (Fahy et al. 1997).

Although important, IL-5 is not an absolute requirement for the recruitment of eosinophils into tissues, because pulmonary eosinophilia with morphologically normal eosinophils occurs in the lungs of IL-5KO mice infected with pneumonia virus of mice (PVM) to the same degree as in those of control mice (Domachowske et al. 2002).

Other chemotaxis factors

Many other factors have been shown to serve as eosinophil chemoattractants, including the complement components C3a and C5a, the CC chemokines MCP-2, MCP-3, and RANTES, and the cytokines GM-CSF, IL-2, IL-3, and IL-8 (Resnick and Weller 1993; Noso et al. 1994; Schweizer et al. 1994; Weber et al. 1995; Discipio et al. 1999). It has been shown that LTB_4 and PAF can induce eosinophil chemotaxis (Klein et al. 2001; Oliveira et al. 2002), and TNF-α has been show to recruit eosinophils in ulcerative colitis (Lampinen et al. 2001). Ligation of SDF-1α to CXCR4 also induces eosinophil migration (Nagase et al. 2000). Although not directly acting on eosinophils, intranasal delivery of IL-13 into mice causes lung and airway eosinophilia by increasing IL-5 and eotaxin production (Pope et al. 2001). Finally, it has been shown that eosinophils can accumulate at sites of helminth infection even in mice lacking B and T cells, suggesting that eosinophils may be directly recruited by some helminth molecule or some factor released by normal tissue in response to a helminth infection (Shinkai et al. 2002).

CELL FUNCTION

Basophils

As a result of their ability to bind IgE, individual basophils can specifically recognize and be activated by many different antigens. Activation of basophils causes release of preformed and newly synthesized inflammatory mediators that play important roles in vascular reaction, exudation, leukocyte accumulation, and wound healing. In addition, basophils have the ability to produce and release cytokines and to phagocytose particles.

ACTIVATION

IgE-mediated activation

The most important molecules for basophil function are their high-affinity IgE receptors (FcεR1s). These receptors are highly specific for IgE and bind them in a 1:1 ratio, with the Cε3 domain of the Fc portion of the IgE antibody serving as the principal binding site for FcεR1 (Weetall et al. 1990). When a multivalent antigen binds to several IgE molecules, the receptors aggregate and initiate cell activation, stimulating mediator generation and release (Turner and Kinet 1999). Basophil degranulation occurs very quickly, with crosslinking of IgE causing half-maximum histamine release from basophils in 7 minutes (Kuna et al. 1992).

Although maximal histamine response occurs when about 10 percent of the FcεR1 is aggregated, aggregation of as few as 100 receptors is enough to activate a basophil (Menon et al. 1984). As basophils can express well over 200 000 FcεR1s per cell (MacGlashan et al. 1997), a single basophil can theoretically hold enough different IgE molecules to enable that basophil specifically to recognize and be activated by well over 1000 antigens.

FcεR1 upregulation on basophils is mediated by the interaction of IgE with FcεR1 (MacGlashan et al. 1999). As a result of this, the number of FcεR1s expressed on basophils is proportional to the amount of IgE in the serum (MacGlashan et al. 1997). IL-3 and IL-5, but not NGF, also probably induce FcεR1 upregulation, because they have been shown to induce FcεR1β mRNA expression in basophils in culture in vitro (Miura et al. 2001).

Human FcεR1 can be expressed as a tetrameric ($\alpha\beta\gamma_2$) or trimeric ($\alpha\beta_2$) structure (Kinet 1999). There is a large range in the ratio of the FcεR1 α:β subunits in basophils from different human donors (Saini et al. 2001). As the β subunit of FcεR1 amplifies IgE-triggered signaling (Lin et al. 1996; Dombrowicz et al. 1998) and increases surface FcεR1 expression (Donnadieu et al. 2000), the finding of different FcεR1β:FcεR1α ratios suggests that basophil responsiveness is modulated in part by changes in this ratio.

In addition to FcεR1, human basophils also express FcγRIIb, a low-affinity IgG receptor. In vitro studies using human basophils have shown that co-aggregation of FcγRIIb and FcεR1 molecules decreases IgE-dependent activation (Daeron et al. 1995).

Non-IgE activation

Basophils can be activated by a number of substances through IgE-independent mechanisms. These factors, known as histamine-releasing factors, include complement proteins, human histamine-releasing factor (HRF), chemokines, cytokines, substance P, PAF, contrast media, ionophores, opiates, the bacterial peptide formyl-methionine-leucine-phenylalanine (f-Met-Leu-Phe or fMLP), and HIV glycoprotein 120.

The complement proteins C3a, C4a, and C5 cause basophil as well as mast cell degranulation (Schulman et al. 1988; Bischoff et al. 1990b). They may have a role in anaphylactoid reactions and are therefore known as anaphylatoxins. It has been suggested that anaphylatoxins may be activated in immune complex-mediated diseases, reactions to iodinated contrast media, and reactions to dialysis tubing. C3a requires the presence of IL-3 or GM-CSF to enable basophils to release histamine (Bischoff et al. 1990b). In addition to histamine release, C5a in the presence of IL-3 can cause long-lasting release of leukotriene C_4 (LTC_4), IL-4, and IL-13 by continuous signaling through the C5a receptor (Eglite et al. 2000).

Histamine-releasing factor (p23) is made by lymphocytes of atopic children and causes histamine and IL-4 release from a subset of basophils in patients with allergy (MacDonald et al. 1995; Schroeder et al. 1996). Initially believed to act through IgE, it has recently been shown to activate basophils in an IgE-independent manner, although its basophil receptor has yet to be identified (Wantke et al. 1999).

Of the CC chemokines, MCP-1 and MCP-3 are the most potent HRFs described (Dahinden et al. 1994). MCP-1 has been shown to be equivalent to IgE and C5a in its ability to cause basophils to degranulate, and it induces histamine release from basophils within 30 seconds (Alam et al. 1992; Kuna et al. 1993). Other CC chemokines that induce basophils to release histamine include MCP-2, RANTES, and MIP-1α (Alam et al. 1994; Dahinden et al. 1994). Of interest, although it is a weak inducer of basophil degranulation, MCP-2 also causes basophils not pretreated with IL-3 to lose their ability to be degranulated by MCP-1 and MCP-3, suggesting that MCP-2 may act as a functional inhibitor of MCP-1 and MCP-3 (Weber et al. 1995). SDF-1α, a CXC chemokine, induces histamine release from basophils as well as MCP-1 does (Jinquan et al. 2000).

Other less potent basophil activators include fibroblast-induced cytokine (Alam et al. 1994), MIP-1α, connective tissue-activating peptide III (CTAPIII), secretory IgA (Iikura et al. 1998), neutrophil-activating peptide-2 (NAP-2) (Kuna et al. 1993), and stem cell factor (Columbo et al. 1992). IL-18, a proinflammatory cytokine that activates NK cells and Th1 responses, induces basophils to release histamine, IL-4, and IL-13 when co-cultured with IL-3 (Yoshimoto et al. 1999). When IL-18 is given with IL-12, however, it inhibits IgE-triggered histamine activation (Yoshimoto et al. 1999).

Degranulation

Basophils release granule contents in two different ways. Anaphylactic degranulation (AND), the typical way in which basophils degranulate after IgE-mediated activation, occurs through granule-to-plasma membrane fusion and allows for very rapid release of large amounts of

granule materials (Dvorak et al. 1980). Piecemeal degranulation (PMD), on the other hand, occurs through trafficking of small vesicles from granules to the plasma membrane and results in a slow, piecemeal loss of granule contents (Dvorak et al. 1976). The most effective trigger for AND is anti-IgE (then MCP-1, then recombinant HRF or rHRF), whereas rank orders for PMD and granule-vesicle attachments are MCP-1 > anti-IgE > rHRF (Dvorak et al. 1996). Although morphologically they appear very different, AND and PMD actually represent anatomical extremes of a continuum of basophil degranulation, because, at very high rates of PMD, vesicles form into tubules by vesicle-to-vesicle fusions, allowing for direct granule-to-plasma membrane fusion, leading to the morphological release pattern termed AND (Dvorak et al. 1991).

Activation markers

CD63 has been the most commonly used marker of basophil activation. IgE-dependent activation of basophils is associated with upregulation of CD63, and the kinetics of this upregulation parallel those of histamine release from basophils (Knol et al. 1991). Increased expression of CD63 on basophils as detected by flow cytometry after in vitro allergen challenge correlates strongly with in vitro assays of histamine and LTC_4, release and has been shown to correlate with a history of allergy to both pollen (Paris-Kohler et al. 2000) and hymenoptera venom (Sainte-Laudy et al. 2000). In addition to CD63, activated basophils also upregulate several β_2-integrins (Bochner 2000).

As CD63 and integrins are not specific only for basophils, studies for other markers of activation are being actively pursued. One possible candidate is E-NPP3 (CD203c), a type II transmembrane protein exclusively expressed on basophils, mast cells, and their $CD34^+$ precursor cells (Buhring et al. 1999, 2001). E-NPP3 has recently been shown to be upregulated on peripheral blood basophils from sensitized individuals after exposure to antigen (Hauswirth et al. 2002).

Degranulation potentiators

Major potentiators of basophil degranulation include IL-3, IL-5, GM-CSF, mast cell growth factor (c-kit ligand), and NGF (Bischoff and Dahinden 1992; Kuna et al. 1993). Although none of these by itself induces basophil degranulation, they all potentiate the effects of factors that cause basophil degranulation. IL-5 enables NAP-1 and C3a to cause basophils to degranulate (Bischoff et al. 1990a) and primes basophils for IgE-dependent histamine release (Kuna et al. 1993). The ability of IL-5 to potentiate the basophil response to C5a is unclear, with studies showing both increased degranulation in response to C5a (Bischoff et al. 1990a) and no change (Miura et al. 2001). IL-3 enables IL-8 and RANTES to induce basophil degranulation (Fureder et al. 1995) and enables C5a-induced LTC_4 generation (Miura et al. 2001). Secretory IgA causes degranulation only after basophils have been exposed to IL-3, IL-5, or GM-CSF, suggesting that the intracellular signaling that primes the basophil for degranulation occurs through the shared β chain of these cytokine receptors (Iikura et al. 1998).

Other factors that potentiate IgE-mediated histamine release from basophils include recombinant human stem cell factor, IL-1α, IL-1β, mast cell growth factor (c-kit ligand), and insulin-like growth factor II (Massey et al. 1989; Columbo et al. 1992; Kuna et al. 1993).

IL-8 inhibits CTAPIII- and IL-3-dependent histamine release from basophils, although it does not inhibit anti-IgE- or fMLP-induced basophil degranulation (Kuna et al. 1991).

EFFECTOR MOLECULES

Basophils release both preformed and newly synthesized inflammatory mediators (Table 3). Preformed inflammatory mediators include histamine, chondroitin sulfates, neutral protease, elastase, β-glucuronidase, major basic protein, cathepsin G-like enzyme, Charcot–Leyden crystal protein, tryptase, chymase, and carboxypeptidase. Basophils also store basogranulin, the function of which is unknown, and IL-4. Factors that basophils synthesize on activation include LTC_4, PAI-1, IL-4, IL-13, and MIP-1α.

Histamine

Basophils are the predominant source of histamine in human peripheral blood (Porter and Mitchell 1972).

Table 3 *Basophil effector molecules*

Basophil effector molecules
Preformed inflammatory mediators
Histamine
Chondroitin sulfates
Neutral protease with bradykinin-generating activity
Elastase
β-Glucuronidase
Major basic protein
Cathepsin G-like enzyme
Charcot–Leyden crystal protein
Tryptase
Chymase
Carboxypeptidase A
Factors produced upon basophil activation
Leukotriene C_4
Plasminogen-activator inhibitor 1
Cytokines
IL-4 (preformed and *de novo* synthesis on activation)
IL-13 (*de novo* synthesis on activation)
Chemokines
MIP-1α (*de novo* synthesis on activation)

Histamine is made from histidine through the action of histidine carboxylase (Bauza and Lagunoff 1981). Circulating basophils have an average concentration of 1 pg histamine per basophil (Schulman et al. 1983; Fox et al. 1985). Histamine binds to the carboxyl groups of proteins and proteoglycans in secretory granules through ionic forces. During degranulation, histamine dissociates from the proteoglycan–protein complex by cation exchange with extracellular sodium at neutral pH. Histamine probably acts close to its site of release, because it is degraded into either methylhistamine or imidazole acetic acid within minutes of release. Histamine affects cells through its interactions with cell-specific H_1-, H_2-, and H_3-receptors (Black et al. 1972; Arrang et al. 1987). H_1-receptors induce vascular permeability, dilate arterioles, and stimulate intestinal and bronchial smooth muscle contraction. H_2-receptors stimulate gastric acid production by parietal cells, increase mucus secretion, cause endothelial cells to release prostacyclin (PGI_2 – a potent inhibitor of platelet aggregation), and modulate the immune response by inhibiting secretion from cytotoxic lymphocytes as well as neutrophils and basophils. H_3-receptors modulate neuroconduction by affecting neurotransmitter release in the central and peripheral nervous systems. Histamine also has specific immunomodulatory effects that are discussed below.

Other effector molecules

The effects of LTC_4 overlap significantly with those of histamine, because it causes smooth muscle contraction, increased vascular permeability, and mucus secretion. As it is not preformed, LTC_4 is released several hours after basophil activation and, thus, is a contributor to the late-phase response seen in allergic reactions. MIP-1α is a potent inflammatory mediator that stimulates further histamine release, causing a positive feedback loop of basophil degranulation (Li et al. 1996b).

C5a stimulation of the basophil cell line KU 812 results in production of plasminogen activator inhibitor-1, suggesting that basophils may play a role in the modulation of fibrinolysis (Wojta et al. 2002). Basogranulin is a recently identified basic protein that appears to be unique to basophils, is located in secretory granules, and is secreted (McEuen et al. 1999, 2001). Its physiological role is as yet undetermined.

Eosinophils

Upon activation, eosinophils migrate towards chemoattractants, release stored granules, and produce reactive oxygen metabolites. Eosinophils can also phagocytose foreign antigens and other materials, but do not do so as efficiently as neutrophils.

ACTIVATION

Non-Ig-mediated activation

Eosinophils are predominantly activated by non-Ig-dependent mechanisms. IL-3, IL-5, and GM-CSF, in addition to prolonging eosinophil survival, can all activate eosinophils (Owen et al. 1987; Rothenberg et al. 1988; Yamaguchi et al. 1988). In addition to its role as an eosinophil chemoattractant, eotaxin also activates eosinophils, causing calcium mobilization, production of reactive oxygen species, and upregulation of CD11b through pertussis-sensitive signaling pathways (Tenscher et al. 1996). SDF-1α, through ligation of CXCR4, also causes intracellular calcium fluxes in eosinophils (Nagase et al. 2000).

Eosinophils have receptors to many other cytokines, including IL-1α, IL-2, IL-4, IL-16, stem cell factor (c-kit), TNF-α, IFN-α, and IFN-γ, although the exact effects of these cytokines on eosinophils are not yet known.

In addition to responding to signals made by other cells, eosinophils may be able to respond directly to pathogens. While eosinophils have been shown to express mRNA for Toll-like receptors 1, 2, 4, 7, 9, and 10 (Plotz et al. 2001; Nagase et al. 2003), the functional significance of these receptors on eosinophils remains unclear. While an initial study found that LPS was able to activate eosinophils (Plotz et al. 2001), two subsequent studies have found no effect of LPS on eosinophils (Sabroe et al. 2002; Nagase et al. 2003). Interestingly, R-848, a ligand of TLR7 and TLR8, has been demonstrated to prolong survival and induced superoxide generation in eosinophils (Nagase et al. 2003). These data suggest that eosinophils have the ability to directly respond to pathogens.

Ig-mediated activation

Eosinophils have both high-affinity FcεRI and low-affinity FcεRII IgE receptors on their surface. One study showed that binding of IgE to FcεRI mediated the release of eosinophil peroxidase (EPO) from activated eosinophils (Khalife et al. 1986). A more recent study, however, showed that eosinophils have almost no surface expression of FcεRI (Seminario et al. 1999). Rather, eosinophils contain large quantities of FcεRIα (but no FcεRIβ) intracellularly and release this into the supernatant (Seminario et al. 1999). As FcεRIα binds to IgE, extracellular release of FcεRIα may serve as a way to inhibit IgE-mediated events by binding up free IgE.

Eosinophils express both IgA and IgG receptors on their surface. Of the IgG receptors, eosinophils primarily express FcγRII (CDw32), the low-affinity IgG receptor, although they also express small amounts of FcγRI (CD64), a high-affinity IgG receptor, and can express FcγRIII after exposure to IFN-γ. Crosslinking of either the IgG or IgA receptors stimulates rapid eosinophil

degranulation (Abu-Ghazaleh et al. 1989; De Andres et al. 1997; Motegi and Kita 1998), but these processes also cause the eosinophils to die and may not be physiological (Weiler et al. 1996). Thus, the role IgG and IgA receptors play in human eosinophil function remains unknown.

Degranulation

Unlike basophils, the exact mechanisms by which eosinophils undergo degranulation have not yet been elucidated. Electron microscopic analysis suggests that eosinophilic granules release their contents into small vesicles that transport them to and release them at the cell surface, akin to piecemeal degranulation in basophils (Dvorak and Weller 2000; Karawajczyk et al. 2000). In a mouse model of Nippostrongylus infection, it has been shown that, although not necessary for eosinophil accumulation at sites of helminth infection, $CD4^+$ T cells are required to enable eosinophil degranulation (Shinkai et al. 2002). The signal transmitted by $CD4^+$ T cells to cause eosinophil degranulation in this model is not known, but even $CD4^+$ T cells deficient in IL-4, IL-13, and the signal-transducing element Stat6, were able to mediate degranulation (Shinkai et al. 2002).

Once activated, eosinophils take on characteristic morphological changes with generation of cytoplasmic vacuolizations, fewer specific granules, and increased numbers of lipid bodies. Activated eosinophils are less dense and more metabolically active than unstimulated eosinophils. These hypodense eosinophils have been described in both allergic and parasitic diseases and exhibit increased surface expression of several proteins, including CD25, CD69, HLA-DR, ICAM-1, FcγRIII, and β_2-integrins (Mawhorter et al. 1996; Bochner 2000). To date, however, no unique marker of eosinophil activation has been described.

EFFECTOR MOLECULES

Effector molecules that eosinophils release include granule proteins, Charcot–Leyden crystal protein, lipid mediators, and enzymes (see Table 4), all of which are released into the extracellular space upon activation via degranulation.

Major granule proteins

Major granule proteins include major basic protein (MBP), EPO, eosinophil-derived neurotoxin (EDN), and ECP. All are located in specific granules, have markedly basic isoelectric points, and have a broad range of direct in vitro toxicity against many organisms (including helminths) and normal cells. MBP, so named because of its predominance in specific granules, is located in the central core of the specific granule, whereas the others are found in the matrix of the granule around the core. Although MBP can be found in low amounts in basophils (and EDN and ECP can be found in neutrophils) (Sur et al. 1998), eosinophils are the primary source of these proteins. In addition to its direct cytotoxic effects, MBP induces histamine release from basophils and mast cells, and superoxide production from neutrophils (Moy et al. 1990).

Eosinophil peroxidase catalyzes conversion of hydrogen peroxide and halides such as bromide and

Table 4 *Eosinophil effector molecules*

Eosinophil effector molecules
Major granule proteins
Eosinophil-derived neurotoxin
Eosinophil peroxidase
Major basic protein
Eosinophil cationic protein
Primary granule protein
Charcot–Leyden crystal protein
Lipid mediators
Leukotriene C_4
Lipoxins
5-HETE
5,15- and 8,15-diHETE
15-Oxo-ETE
Platelet-activating factor (PAF)
Prostaglandins E_1 and E_2
Thromboxane B_2
Enzymes
Acid phosphatase
Arylsulfatase
Catalase
Hexoseaminidase
Histaminase
Lysophospholipase
Phospholipase D
Oxidative products
Hydrogen peroxide
Hydroxyl radical
Superoxide anion
Singlet oxygen
Cytokines
GM-CSF
TGF-α
TGF-β_1
TNF-α
IFN-γ
Nerve growth factor (NGF)
Neurotrophin 3
Interleukins IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-16
Chemokines
IL-8
MIP-1α
RANTES
MCP-3S
Eotaxin

See text for abbreviations.

chloride to hypohalous acids. EDN and ECP are ribonuclease A superfamily members; however, it is not clear what role their enzymatic ability serves, because the cytotoxic activity of ECP does not require its ribonuclease activity (Rosenberg 1995). EDN, named because of its neuropathic effect on rabbits, has never been shown to be neurotoxic for humans.

Charcot–Leyden crystal protein

Located in primary granules (Dvorak et al. 1988), Charcot–Leyden crystal (CLC) protein makes up 7–10 percent of the eosinophil total protein. CLC is a lysophospholipase, which suggests that eosinophils have a role in lipid and membrane metabolism (Weller et al. 1984). X-ray crystallography of CLC shows a structural similarity to galectins (β-galactoside-binding proteins) (Leonidas et al. 1995). CLC is able to bind specifically to β-galactoside sugars, but a natural ligand for CLC has yet to be found (Dyer and Rosenberg 1996).

Other effector molecules

Eosinophils store esterified arachidonic acid in their lipid bodies. Arachidonic acid metabolism through the 5-lipoxygenase pathway results in production and release of LTC_4, which causes vasoconstriction, bronchoconstriction, and smooth muscle contraction (Lewis and Austen 1984). Arachidonic acid metabolism through 15-lipoxygenase results in lipoxins, and metabolism by cyclooxygenase pathways produces prostaglandin E_2 (PGE_2) and thromboxane B_2. Eosinophils also release several oxidative products (including hydrogen peroxide, superoxide anion, and hydroxyl radical and singlet oxygen) and release PAF (Lee et al. 1984).

IMMUNOMODULATORY CAPABILITIES

Although basophils and eosinophils are typically thought of as cells of the innate immune system, they also serve to modify the adaptive immune system by releasing cytokines. In addition, histamine released by basophils has immunomodulatory effects, and eosinophils have the ability to process and present antigen.

Basophils

CYTOKINE RELEASE

Basophils release both IL-4 and IL-13. IL-4 induces $CD4^+$ T-helper (Th) 0 cells to become Th2 cells, and both cytokines can, in combination with ligation of CD40, cause B cells to switch to IgE isotype. Of all PBMCs, basophils are the only ones with the ability to provide IL-4 early in response to a stimulus (Gibbs et al. 2000), releasing preformed IL-4 within 5–10 minutes of IgE stimulation (Gibbs et al. 1996). After activation, basophils also synthesize IL-4 and IL-13 *de novo*, with time-course experiments showing a second peak of IL-4 release after 4 hours and a first peak of IL-13 release at 24 hours (Gibbs et al. 1996).

IL-4 release is increased in basophils exposed to IL-3 but not to basophils exposed to IL-5, GM-CSF, or NGF (Brunner et al. 1993; Dahinden et al. 1997). IL-13 production is also enhanced by IL-3 (Li et al. 1996a). There are probably some differences in the regulation of IL-4 and IL-13 production by basophils, because only IL-13 is secreted from basophils after stimulation with IL-3 alone and IL-4 production is inhibited by the immunosuppressant FK506, whereas IL-13 production is not (Redrup et al. 1998). Also, 20 hours after activation, the amount of IL-4 released from basophils after stimulation with IL-3 and anti-IgE correlates strongly with the percentage of histamine secreted, whereas the amount of IL-13 released does not (Redrup et al. 1998).

Eotaxin also modulates basophil cytokine production, augmenting antigen-dependent IL-4 production and release from basophils by two- to fourfold and lowering the threshold for basophil activation and IL-4 production by 40-fold (Devouassoux et al. 1999). Other factors that enhance basophil IL-4 production include the chemokines MCP-2, MCP-3, MCP-4, RANTES, and eotaxin-2, all of which act on basophils through CCR-3 (Devouassoux et al. 1999), and the recombinant HRF (rp21) (Schroeder et al. 1997).

As they can express CD40 ligand as well as release IL-4 and IL-13, basophils have the ability to induce B cells to switch to IgE isotype (Gauchat et al. 1993), potentially creating a positive feedback loop whereby IgE-dependent activation of basophils results in the production of more IgE by B cells.

ANTIGEN PRESENTATION

Unlike eosinophils, there is no evidence that basophils can act as antigen-presenting cells (APCs).

IMMUNOMODULATORY PROPERTIES OF HISTAMINE

Basophils can modulate the immune response through secreted histamine. Histamine modulates the expression and action of several cytokines, having been shown to increase production of IL-2, IL-5, IL-6, IL-8, IL-10, IL-11, IL-16, GM-CSF, and RANTES and to downregulate production of IL-2, IL-4, IL-12, IFN-γ, and TNF-α (Igaz et al. 2001).

Signaling through the different histamine receptors mediates different effects on the Th response. Triggering of the H1R on $CD4^+$ T cells enhances Th1-type responses, causing increased IFN-γ production and subsequent downregulation of Th2-type responses, whereas triggering through the H2R downregulates both Th1 and Th2 responses (Elenkov et al. 1998; Jutel et al. 2001). As the Th2-type response is integral to the production of IgE through the actions of IL-4 and IL-13, the downregulation of the Th2 arm of the cellular

immune system through H_1- and H_2-receptors may serve as a negative feedback on the response that initially accounted for the production of IgE which led to the basophil degranulation.

It is clear that histamine affects a variety of cell types and modulates the Th1/Th2 balance at several different points. Histamine affects monocytes by decreasing their production of IL-12 (van der Pouw Kraan et al. 1998) and inhibiting ICAM-1 expression induced by IL-18 (Takahashi et al. 2002). Through interaction with the H_2-receptor, histamine causes increased IL-10 and decreased IL-12 production by dendritic cells (Mazzoni et al. 2001). Histamine also increases the release of IL-10 from unstimulated and LPS-stimulated human alveolar macrophages by PGE_2 and nitric oxide (NO) production (Sirois et al. 2000). One study suggests that many of the effects of histamine may be the result of its upregulation of IL-18 production, because addition of anti-IL-18 abolished all cytokine changes caused by histamine in in vitro studies of PBMCs (Kohka et al. 2000).

Eosinophils

CYTOKINE RELEASE

Eosinophils release a multitude of cytokines (see Table 2), many of which amplify a type 2 response. Eosinophils produce and release IL-1α, which can work in concert with APCs as a stimulator for Th2 lymphocytes (Del Pozo et al. 1990; Weller et al. 1993). In murine schistome granulomas, eosinophils are the predominant source of IL-4. Eosinophils also release IL-3, IL-5, and GM-CSF (Kita et al. 1991; Moqbel et al. 1991; Broide et al. 1992; Desreumaux et al. 1993), the cytokines most responsible for eosinophilopoeisis in the bone marrow, suggesting that eosinophil degranulation results in increased eosinophil production. In a murine model of Nippostrongylus infection, eosinophils accumulate at sites of helminth infection and express IL-4 even in mice lacking B and T cells, raising the possibility that eosinophils may even have the capacity to initiate a Th2 response (Shinkai et al. 2002).

In addition to contributing to the Th2 response, several cytokines released by eosinophils play a direct role in the inflammatory response. IL-1α and TNF-α, both secreted by eosinophils (Del Pozo et al. 1990; Weller et al. 1993; Finotto et al. 1994), are potent inflammatory agents that can enhance local blood flow, activate macrophages, and induce fibroblast growth. TGF-α and TGF-β, also released by eosinophils, are potent promoters of fibroblast growth and collagen synthesis and may play a role in the tissue fibrosis that occurs in diseases of chronic eosinophilic inflammation (Todd et al. 1991; Finotto et al. 1994).

It has recently been shown that eosinophils synthesize and release NGF and neurotrophin-3 (Kobayashi et al. 2002), factors that are essential for the survival and development of peripheral neurons. Although the physiological relevance of this finding has yet to be determined, it suggests that eosinophils may be involved in processes such as guiding tissue homeostasis and development. Alternatively, as NGF can activate eosinophils (Solomon et al. 1998), release of NGF by eosinophils may serve as a means by which eosinophil activation is amplified in an autocrine fashion.

ANTIGEN PRESENTATION

Eosinophils express MHC class II (HLA-DR) when cultured with GM-CSF in vitro (Lucey et al. 1989). In vivo, eosinophils taken from sites of inflammation in patients with asthma, allergy, and chronic eosinophilic pneumonia also express MHC class II (Hansel et al. 1991; Beninati et al. 1993). Evidence that eosinophils can function as APCs comes from in vitro and mouse models showing that eosinophils can stimulate antigen-specific T-cell proliferation (Del Pozo et al. 1992; Hansel et al. 1992; Weller et al. 1993). The extent to which eosinophils actually present antigen in vivo is unclear, especially as they are not particularly efficient at phagocytosis, often a first step required for antigen presentation.

BASOPHILIA AND EOSINOPHILIA AS DISEASE MARKERS

Eosinophilia, which is usually defined as an eosinophil count > 500 cells/mm^3, is a common finding in helminth infections, allergy, asthma, and several other diseases. Basophilia is a rare finding in all disease processes except for chronic myelogenous leukemia. Tables 5 and 6 list the infectious and non-infectious diseases in which basophilia and eosinophilia have been reported to occur.

ROLE IN INFECTIOUS DISEASES

The majority of research on eosinophils and basophils has focused on their roles in diseases such as asthma and allergy. In addition to these states, however, it is clear that basophils and eosinophils play large roles in the immunological response to infectious diseases – especially to helminths and possibly to viruses. Despite decades of study, however, the exact function that these cells serve in the response to infection remains unclear.

Helminth infections

BASOPHILS

Several factors suggest that basophils play an important role in the immune response to helminth infections. While a recent retrospective study suggests that basophilia does not occur in helminth infections of humans (Mitre and Nutman 2003), it clearly occurs in animal

Table 5 *Diseases associated with basophilia*

Diseases associated with basophilia
Infectious diseases
Helminths (in animal models, not reported in humans)
Tuberculosis
Viruses
Influenza[a]
Smallpox[a]
Varicella-zoster[a]
Non-infectious causes
Allergic reactions to medication or food
Diabetes mellitus
Erythroderma
Estrogen administration
Hodgkin's disease
Hypothyroidism
Iron deficiency
Juvenile rheumatoid arthritis
Myeloproliferative/neoplastic diseases
Chronic myelogenous leukemia
Myelofibrosis
Polycythemia vera
Primary thrombocythemia
Serum sickness
Ulcerative colitis
Urticaria

a) Mentioned in other reviews; primary sources not found.

models of parasitic infection. One of the earliest reports of this phenomenon came from Chan in the 1960s, who showed that bone marrow and circulating basophil counts increase within 48 hours of subcutaneous injection of Ascaris body (Chan 1965) and ova fluid (Chan 1968) into guinea pigs. Since then, several other investigators have reported similar findings in several different animal models of parasitic infection. *Trichostrongylus colubriformis* infection has been shown to increase basophil numbers in the bone marrow, small intestine, and peripheral circulation of guinea pigs (Rothwell and Love 1975). Infection of rats (Ogilvie et al. 1978; Roth and Levy 1980; Kasugai et al. 1993) and gerbils (Okada et al. 1997) with *Nippostrongylus brasiliensis* resulted in up to a 50-fold increase in peripheral basophil counts at 2 weeks. *Trichinella spiralis* infection in rats (Ogilvie et al. 1980) and guinea pigs (Lindor et al. 1983) also results in a marked basophilia that precedes the onset of eosinophilia by approximately 1 week. Fasciola infection of guinea pigs is associated with a chronic peripheral basophilia that is detectable up to 4 months after infection (Conboy and Stromberg 1991), and strongyloides infection of *Erythrocebus patas* monkeys causes peripheral basophilia that is occasionally detectable for more than a month after infection (Harper et al. 1984).

Basophils clearly respond to helminth infections, because basophils from patients infected with *Toxocara*, *Ascaris*, *Onchocerca*, *Wuchereria*, *Strongyloides*, and *Schistosoma* spp. have been shown to release histamine in response to parasite antigen (Ottesen et al. 1979; Genta et al. 1983; Hofstetter et al. 1983; Nielsen et al. 1994; Gonzalez-Munoz et al. 1999). Helminth infections are associated with high levels of IgE (Rossi et al. 1993; Estambale et al. 1995; Ramirez et al. 1996), and the amount of histamine released by basophils in response to toxocara antigen is proportional to the serum concentration of toxocara antigen-specific IgE (Nielsen et al. 1994).

In addition to high levels of IgE, human helminth infections are also associated with increased production of IL-4, the prototypical Th2 cytokine. Basophils from filaria-infected patients have been shown to release IL-4 in response to infective stage (L3) larvae and filarial antigen (King 2001), and basophils from non-infected individuals have been shown to release IL-4 after stimulation with schistosoma egg antigen (Falcone et al. 1996). Although basophils make up only a small percentage of peripheral white blood cells, they have the ability specifically to recognize many different antigens by virtue of binding different IgE antibodies on their surface. Consequently, it is possible that the number of basophils that specifically recognize and are activated by helminth antigen may equal or even exceed the number of antigen-specific T cells responding to helminths. Indeed, results from a recent study showed that the number of basophils producing IL-4 in response to filarial antigen was usually equal to or greater than the number of IL-4-producing T-cells in filaria-infected patients (Mitre et al. 2004). Whereas filarial antigen was only shown to cause basophil activation in filaria-infected patients with filarial-Ag specific IgE, it has been demonstrated that a glycoprotein from *Schistosoma mansoni* eggs can cause basophils to degranulate and release IL-4 by binding and crosslinking non-antigen-specific IgE (Falcone et al. 1996; Haisch et al. 2001), raising the possibility that some helminth antigens may act as a type of 'super-allergen' by being able to bind directly to the nonvariable portion of IgE. This suggests that, in some cases, basophils may serve as the initial source of IL-4, providing the stimulus for T cells to differentiate toward a Th2 phenotype.

Similarly, homologs of mammalian, translationally controlled tumor protein, a calcium-binding protein that directly stimulates histamine release from basophils, have recently been cloned from *Schistosoma mansoni* (Rao et al. 2002) and the filarial parasites *Wuchereria bancrofti* and *Brugia malayi* (Gnanasekar et al. 2002).

EOSINOPHILS

Eosinophilia is a frequent finding in invasive helminth infections; such eosinophilia is probably mediated by IL-5, because treatment of mice with anti-IL-5 results in ablation of helminth-induced eosinophilia (Coffman et al.

Table 6 *Diseases associated with eosinophilia*

Infectious diseases	Non-infectious causes
Bacteria	**Allergic diseases**
Resolving scarlet fever	Allergic rhinitis[a]
Tuberculosis[a]	Asthma[a]
Fungi	Atopic dermatitis[a]
Allergic bronchopulmonary aspergillosis[a]	Medication allergy[a]
Coccidioidomycosis	Urticaria/angioedema[a]
Helminths	**Dermatological diseases**
Angiostrongyliasis[a]	Atopic dermatitis[a]
Anisakiasis[a]	Bullous pemphigoid
Ascariasis[a]	Urticaria/angioedema[a]
Capillariasis[a]	**Immunodeficiencies**
Clonorchiasis[a]	Wiskott–Aldrich syndrome
Coenurosis[a]	Hyper-IgE (Job's) syndrome
Cysticercosis	Autoimmune lymphoproliferative syndrome (ALPS)
Dicrocoeliasis[a]	**Rheumatological diseases**
Echinococcosis[a]	Eosinophilic fasciitis[a]
Echinostomiasis[a]	Hypersensitivity vasculitis
Enterobiasis	Rheumatoid arthritis
Fascioliasis[a]	**Myeloproliferative/neoplastic diseases**
Filariasis[a]	Eosinophilic leukemia[a]
Gnathostomiasis[a]	Hodgkin's disease
Heterophyiasis[a]	Idiopathic hypereosinophilic syndrome
Hookworm[a]	Kimura disease
Hymenolepsiasis[a]	Lymphomas
Metagoniamiasis[a]	Myeloid leukemia
Opisthorciasis[a]	Solid tumors
Paragonomiasis[a]	**Gastrointestinal diseases**
Schistosomiasis[a]	Celiac sprue
Sparganosis[a]	Eosinophilic gastroenteritis[a]
Strongyloidiasis[a]	Inflammatory bowel disease
Trichinosis[a]	**Respiratory diseases**
Trichuriasis[a]	Asthma[a]
Visceral larva migrans[a]	Allergic rhinitis[a]
Protozoa	Churg–Strauss syndrome
Isosporiasis	Eosinophilic pneumonia
Viruses	**Miscellaneous**
HIV	Eosinophilic myalgia syndrome
Respiratory syncytial virus	Graft-versus-host disease
	GM-CSF therapy
	Histiocytosis
	Hypereosinophilic syndrome
	IL-2 therapy
	Toxic oil syndrome

a) Disease in which eosinophilia commonly occurs.

1989; Sher et al. 1990). After treatment of human patients infected with onchocerciasis or lymphatic filariasis, serum IL-5 levels increase and circulating eosinophil levels decrease, suggesting that eosinophils are recruited out of the circulation and into the tissues by IL-5 in response to dying helminths (Limaye et al. 1991, 1993; Cooper et al. 1999). In addition to increased production and recruitment of eosinophils by IL-5, eosinophilia in response to helminth infections may be the result of a prolonged eosinophil lifespan, because eosinophils from splenic granulomas of *Schistosoma mansoni*-infected mice have been shown to be resistant to apoptosis in vivo and protected from Fas–FasL-mediated apoptosis by the absence of Fas ligand expression (Rumbley et al. 2001).

Studies in mice and in vitro clearly show that eosinophils are toxic to helminths. Culture of freshly isolated human eosinophils with antibody-coated *Schistosoma*

mansoni larvae results in death of 14 percent of the parasites. After addition of IL-3, the eosinophils kill over 50 percent of the *S. mansoni* larvae (Rothenberg et al. 1988). IL-5 can also enhance the toxicity of eosinophils against *S. mansoni* (Mazza et al. 1991).

Binding of CD11b and VLA-4 on the eosinophil surface to C3a and plasma fibronectin deposited on the cuticular surface of *Nippostrongylus brasiliesis* larvae after experimental infection of transgenic IL-5 mice allows eosinophils to adhere directly to the larvae (Shin et al. 2001). Electron microscopy reveals that the cuticular surface of *N. brasiliensis* larvae in this model becomes damaged from electron-dense materials released from the eosinophils. Indeed, purifed eosinophil granule proteins have been shown to have direct toxicity on various helminths, including *Trichinella spiralis* (Hamann et al. 1987), *S. mansoni* (Ackerman et al. 1985), *Brugia malayi* (Hamann et al. 1990), and *Trypanosoma cruzi* (Molina et al. 1988). Eosinophil toxicity of helminths has been shown to occur via antibody-dependent (Butterworth et al. 1974) and antibody-independent (Shin et al. 2001) mechanisms.

Whether eosinophils actively protect against helminth infections remains controversial. Several studies using rabbit anti-mouse eosinophil serum, which depletes mice of circulating eosinophils, showed that eosinophils probably have a protective effect against Schistoma, Trichinella, and Trichostrongylus infections (Mahmoud et al. 1975; Grove et al. 1977; Gleich et al. 1979).

In contrast to these findings, more recent studies using anti-IL-5 antibodies to deplete circulating eosinophils in experimental helminth infections have shown mixed results in terms of the importance of eosinophils in mediating helminth protection. Mouse models of Schistosoma, Trichinella, and Trichuris infections found no differences in parasite burden in eosinophil-depleted mice compared with control mice (Sher et al. 1990; Herndon and Kayes 1992; Betts and Else 1999). On the other hand, elimination of eosinophils using IL-5-neutralizing antibodies, although not affecting the number of tissue-migrating larvae recovered from the lungs in primary infection with *Strongyloides venezuelensis*, does result in greater numbers of adult Strongyloides worms in the intestine, suggesting that eosinophils are important in the control of infection, although perhaps not during the initial establishment of infection (Korenaga et al. 1994). Also, anti-IL-5-treated mice become susceptible to re-infection with *Onchocerca lienalis*, a helminth to which mice are usually resistant (Folkard et al. 1996).

Other approaches to ascertain the role of eosinophils in response to helminth infection also come to different conclusions. Mice deficient in IL-5 cannot develop circulating or tissue eosinophilia. These IL-5 KO mice have been shown to have similar susceptibility to *Mesocestoides corti*, *Hymenolepis diminuta*, and *Fasciola hepatica* as control mice (Kopf et al. 1996; Ovington and Behm 1997). *Toxocara canis*, *Strongyloides ratti*, and *Heligmosomoides polygyrias* infections, however, appear to be more severe and cause greater tissue damage in IL-5-deficient mice than in control mice (Ovington and Behm 1997). In one study, IL-5 transgenic mice, which have high baseline peripheral blood eosinophil levels, were shown to have the same susceptibility to infection with *Schistosoma mansoni* as control mice (Freeman et al. 1995). Another study, however, demonstrated that IL-5 transgenic mice have greater resistance against helminths, with a sevenfold decrease in the worm recovery rate 60 days after infection with *Litomosoides sigmodontis* compared with control mice (Martin et al. 2000). In an ex vivo gut loop experimental infection of *Fasciola hepatica*, protection correlated strongly with the frequency of eosinophils in the gut (Van Milligen et al. 1999). CCR3 KO mice, which are unable to recruit eosinophils to the small intestine and skeletal muscle after infection with *Trichinella spiralis*, exhibit normal rejection rates of adult worms from the small intestine but have greater total numbers of cysts in the skeletal muscle and these cysts exhibit less necrosis than those of normal mice (Gurish et al. 2002).

There are several possible explanations for the discrepant results of studies of eosinophils and helminth infections. It may be that eosinophils play different roles in different animal species. Moreover, there are significant differences between human and mouse eosinophils: murine eosinophils lack Fcε receptors (de Andres et al. 1997), and there are large differences between the amino acid sequences of mouse EDN and ECP compared with human EDN and ECP (Larson et al. 1996; Nittoh et al. 1997). Also, eosinophils may be efficacious against some helminth infections (or some stages of the infections) but not all. To date, one of the major barriers to studying the role of eosinophils in helminth infections has been the lack of a specific method for selective depletion of eosinophils. A monoclonal antibody to CCR3 has recently been developed and been shown selectively to deplete eosinophils in mice (Grimaldi et al. 1999). Studies using this antibody are currently under way and will, it is hoped, better elucidate the role of eosinophils in the immune response to helminths.

Viral infections

BASOPHILS

Although the role, if any, that basophils play in host defense against HIV is unknown, it is clear that basophils may be an important reservoir for HIV infection. Basophils express CD4 as well as the HIV co-receptors CCR3 and CXCR4, can be infected with M-tropic HIV in vitro, and have been shown to be infected with HIV in many patients with AIDS (Li et al. 2001). HIV-1 Tat protein upregulates CCR3 expression on basophils, increasing the likelihood that the virus successfully

infects the cells (Marone et al. 2001). IL-16, which can bind to CD4, makes basophils less susceptible to infection with M-tropic HIV (Qi et al. 2002). Notably, HIV-1 gp120 can act as a 'super-allergen' by interacting with the heavy chain variable 3 (V[H]3) region of IgE to induce cytokine and histamine release from FcεR1$^+$ cells (Marone et al. 2001). The physiological significance of this phenomenon is unknown.

Basophils also interact with respiratory viruses. In vitro, infection of human PBMCs with respiratory viruses results in enhanced IgE-mediated histamine release from basophils (Chonmaitree et al. 1988), and experimental infection of allergic rhinitis patients with rhinovirus 16 results in increased plasma histamine levels (Calhoun et al. 1991). Rhinovirus infection of the basophil cell line KU 812 augments anti-IgE-induced histamine release and increases maximal IL-4 and IL-6 production by basophils (Hosoda et al. 2002). These results suggest that some respiratory viruses may trigger asthma attacks by causing release of histamine and type 2 cytokines from basophils. The effect that the basophils have in terms of host defense in these infections is not known.

EOSINOPHILS

Asymptomatic eosinophilia occurs in 18 percent of patients with HIV (Caterino-de-Araujo 1994), and occurrence of eosinophilia in HIV correlates with low CD4$^+$ cell counts (Smith et al. 1994; Tietz et al. 1997). Eosinophilia in HIV patients, however, is neither a useful clinical marker for eosinophil-related diseases nor a predictive factor for the future course of HIV infection. In a study of blood counts in HIV patients over a 4-year period, no etiological agent could be found in patients with eosinophilia (Cohen and Steigbigel 1996). Other than a moderate increase in the prevalence of rash, a case–control study of HIV patients with and without eosinophilia found no significant clinical correlation between eosinophilia and other diseases such as parasitic infection, allergic reaction, or malignancy (Skiest and Keiser 1997).

Like basophils, eosinophils express CD4 and CCR3 and can be infected by macrophage-tropic strains of HIV (Weller et al. 1995; Alkhatib et al. 1997). Eotaxin, which binds to CCR3, diminishes HIV entry into eosinophils (Alkhatib et al. 1997).

Some in vitro data suggest that eosinophils may aid in eliminating HIV. EPO has been shown to have marked antiviral activity against HIV-1 (Klebanoff and Coombs 1996), and eosinophils have been shown to inhibit cellular transduction by a mouse retrovirus through a mechanism dependent on eosinophil ribonucleases (Domachowske and Rosenberg 1997); however, subcutaneous injection of IL-3 into humans with HIV, which resulted in a 17-fold increase in absolute eosinophil counts, caused no significant quantitative changes in viral load, suggesting that IL-3 and corresponding eosinophilia do not enhance HIV elimination in humans (Scadden et al. 1995).

Eosinophils may also play a role in the pathogenesis of respiratory syncytial virus (RSV) infection. Eosinophils are often found in nasal washes of children with RSV (Zhao et al. 2002), and are occasionally found in increased numbers in the peripheral circulation as well (Ehlenfield et al. 2000). Whether eosinophils serve a protective role against RSV has not yet been completely determined, although such a role has been postulated (Rosenberg and Domachowske 2001). The tissue eosinophilia induced by RSV infection may, however, exacerbate asthma. The degree of eosinophilia in nasopharyngeal secretions correlates with the degree of asthma exacerbation in children with asthma who are infected with RSV, but not influenza (Zhao et al. 2002). In addition, higher peripheral blood eosinophil counts at the time of RSV bronchiolitis are associated with an increased risk of developing childhood asthma (Ehlenfield et al. 2000).

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Natural killer cells

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DEFINITION AND GENERAL CHARACTERISTICS OF NATURAL KILLER CELLS

Historical perspective

Natural killer cells were discovered in the early 1970s, through their capacity to kill certain tumor cells in cytotoxicity assays in vitro. In the search for evidence of a specific immune response in patients or experimental animals with tumors, several groups observed a cytotoxic activity by lymphocytes isolated from the blood or the spleen. It was soon realized that this activity occurred also in healthy individuals, and had nothing to do with a specific tumor response. Although some considered this activity as 'nonspecific background', others went on to analyze the phenomenon in more detail. This research soon provided evidence that the cytotoxicity was mediated by a previously undescribed, lymphocyte-like cell with unique characteristics, and the term natural killer (NK) cell was then coined (Herberman et al. 1975a, b; Kiessling et al. 1975a, b). Equally important, it became clear that the activity observed in vitro correlated with natural resistance to transplantable tumors in vivo in animals (Kiessling et al. 1975c). The NK cells were soon also identified as the effector cell responsible for a peculiar form of bone marrow transplant rejection that had puzzled immunologists for a decade (Kiessling et al. 1977). The notion of one novel cell type with in vivo relevance triggered further research, mainly in tumor immunology, but eventually also in infection immunology. Evidence for a role of NK cells in the early defense against viral infections soon emerged. Other researchers also observed effects mediated by NK cells in bacterial and parasite infections. The microbiology-oriented NK cell research has been particularly important for defining additional effector functions of NK cells. Their capacity to secrete cytokines such as interferon (IFN)-γ is in many situations more important than the killer function that forms the basis of their name.

It took a relatively long time before NK cell recognition could be defined in molecular terms. Research on inhibitory, major histocompatibility complex (MHC) class I-recognizing receptors, as postulated in the 'missing self' model, provided a platform for identification of the first NK cell receptors (Kärre 1985, 2002; Ljunggren and Kärre 1990). Intensive research during the 1990s has defined a large number of activating and inhibiting receptors (Moretta et al. 1993, 2001; Yokoyama and Plougastel 2003). A challenge for the current and coming years of research is to transform the reductionist study of individual receptors into a comprehensive view of how these molecules interact in decision processes created by different challenges, especially different microbial agents.

Identification and isolation of NK cells – cell surface markers

There is no single cell surface molecule or other feature that can be used to identify all NK cells, and

nothing but NK cells. Any efforts to define or isolate these cells must therefore rely on a combination of characteristics. NK cells are of lymphoid lineage and are often associated with a special morphology, that of the large granular lymphocyte (LGL) (Figure 1) (Timonen and Saksela 1980). In the normal blood of healthy individuals, there is a good correlation between NK cells and this morphological phenotype. However, not all NK cells are LGLs, and not all LGLs are NK cells.

Natural killer cells are usually defined through a combination of cell surface markers – in the human as $CD56^{+}CD16^{+}CD3^{-}$ immunoglobulin (Ig) cells with the morphology of lymphocytes (Moretta et al. 1994; Carson and Caligiuri 1996). CD56 is the neuronal cell adhesion molecule (N-CAM), but it is unclear whether and how this marker relates to the function of NK cells. In the mouse, the allotypic marker NK1.1 is used instead of CD56, although the two markers are completely unrelated (Yokoyama 1999). NK cells express a number of additional adhesion molecules, cytokine receptors, and cognate recognition molecules (Tables 1 and 2), many of which are discussed further later in the chapter in relation to the functional aspects.

The NK cells represent about 5–15 percent of the lymphocytes in the blood, and about 1 percent of the lymphocytes in the spleen. These are the two usual sources for isolation of NK cells in the human and mouse, respectively. An enriched or purified population requires cell sorting by flow cytometry or immunomagnetic beads, based on the combinations of surface marker characteristics discussed above. NK cells can also be isolated from the thymus, liver, and lymph nodes, although they are rare in these tissues.

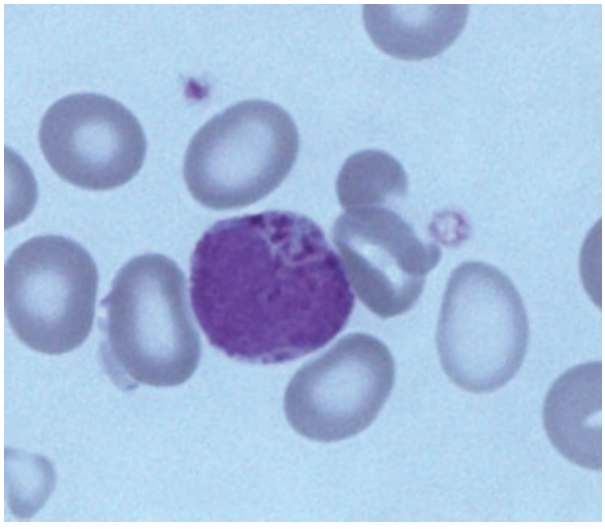

Figure 1 *A large granular lymphocyte. Blood smear – large granular lymphocyte. (Photograph kindly provided by Dr V. Krenn, Charité, Berlin.)*

Table 1 *Cell surface molecules expressed by natural killer (NK) cells*

Antigen	Ligand
CD2	CD58, CD48
CD11a/LFA-1	ICAM-1, ICAM-2
CD11b/Mac1	ICAM-1, ICAM-2, complement
CD16	IgG
CD27	CD70
CD28	B7–1, B7–2
CD40L	CD40
CD44	Hyaluronic acid
CD45/B220	
CD49b/DX5	
CD49e	Collagen, laminin
CD56 (human)	
CD62L	
CD69	–
CD94	
IFN-αβR	IFN-α, IFN-β
IL-2Rβ	IL-2, IL-15
IL-12R	IL-12
IL-18R	IL-18
Asialo-GM1 (rodent)	

ICAM, intercellular adhesion molecule; IFN, interferon; Ig, immunoglobulin; IL, interleukin.

NK cells do not display the typical clonal receptor distribution seen in T and B cells, and they do not rely on rearrangement of gene segments in a germline repertoire for generation of receptors. Hence, their development is not disturbed in severe combined immunodeficient (SCID) or RAG gene-deficient mice (Yokoyama 1999). However, NK cells are heterogeneous in their receptor repertoire, in the sense that different cells express different combinations of activating and inhibitory receptors. In addition, a functional heterogeneity is emerging, at least in the human. The majority of blood NK cells express moderate levels of CD56 in combination with various molecules of the killer cell immunoglobulin receptor (KIR) family (Cooper et al. 2001). This population also expresses high levels of perforin, a pore-forming protein secreted on triggering of the killer machinery. In addition, there is a small subpopulation of blood NK cells that expresses high levels of CD56 in combination with the inhibitory receptor NKG2A, and no receptors of the KIR family. This subset has low perforin levels and seems to be specialized for high cytokine secretion rather than direct killing. The latter subset may be the only NK cells present in the lymph nodes, an organ that was initially thought to be completely devoid of NK cells (Ferlazzo et al. 2004). NK cells are also observed in the capillary beds of the lungs and liver, and they can evidently deliver effector functions in the skin as well as in the peritoneal cavity.

Table 2 *Mouse strains useful for studies of natural killer (NK) cells*

Mutation	Development	Comment
Beige	Normal	Defect in natural killing
Tgϵ26	Compromised	Lack NK and T cells (transgenic for human CD3e
Ly49A#7-tg	Compromised	NK-cell deficiency (accidentally)
IL-15	Compromised	Development and homeostasis
$\gamma c^{-/-}$	Compromised	Reduced cytotoxicity and cytokine production
$Ikaros^{-/-}$	Compromised	Reduced cytotoxicity
$Ets1^{-/-}$	Compromised	Reduce cytotoxicity and cytokine production
$PU.1^{-/-}$	Compromised	Normal cytotoxicity
$IRF1^{-/-}$	Compromised	Reduced cytotoxicity
$ID2^{-/-}$	Compromised	compromised cytotoxicity
$MITF^{-/-}$	Normal	Reduced cytotoxicity and cytokine production
$CEBP\gamma^{-/-}$	Normal	Reduced cytotoxicity and cytokine production
$CD3e^{-/-}$ $Fc\epsilon RIg^{-/-}$	Normal	Compromised specific killing
$Syk^{-/-}$ $ZAP70^{-/-}$	Normal	Compromised specific killing
$DAP12^{-/-}$	Normal	Cytotoxicity and cytokine production compromised
$Fyn^{-/-}$	Normal	Non-MHC receptor-mediated cytotoxicity compromised
$STAT\text{-}1^{-/-}$	Normal	Impaired rejection of tumors
dnSHP1-tg	Normal	Non-MHC receptor-mediated cytotoxicity compromised
$Perforin^{-/-}$	Normal	Perforin-independent cytotoxicity (Fas-L, TRAIL)

γc, common cytokine receptor γ chain; CEBP, CCAAT/enhancer binding protein; Ets1, E26 avian leukemia oncogene 1; Fas-L, Fas ligand; ID2, inhibitor of DNA binding 2; IRF1, interferon regulatory factor 1; MITF, microphthalmia-associated transcription factor; ZAP70, ζ-chain associated protein 70 kDa; DAP12/KARAP, killer cell activating receptor associated protein; STAT, signal transducer and activator of transcription; dnSHP1, dominant-negative SH_2-domain-containing protein tyrosine phosphatase; TRAIL, tumor necrosis factor (TNF)-related, apoptosis-inducing ligand. For further information see (Colucci et al. 2003).

Effector functions

As indicated by their name, the ability to kill various target cells is a central functional feature of NK cells. They possess several different mechanisms to do this, most of which they share with cytotoxic T lymphocytes. The most important one is perforin-dependent killing (Young and Cohn 1986). Perforin is a pore-forming protein, which is stored in the secretory granules of NK cells. When killing is triggered, the secretory apparatus is oriented toward the interface with the target cell, and the granule contents are released in the 'immune synapse' between the two cells (Davis et al. 1999; Henkart 1999; Lieberman 2003). The granules contain additional proteins, such as the serine proteases granzyme A, B, and C. These are introduced into the target cell via the pores formed by perforin, and subsequently activate apoptotic pathways. These, as well as osmotic effects created by the perforin-induced pores, eventually lead to target cell death. The process involves considerable temporospatial specificity – one NK cell can bind and initiate a 'synapse' with several cells simultaneously, and still kills only those that present the appropriate triggering signals; the others are left intact (Eriksson et al. 1999).

This rapid and efficient killer mechanism, allowing elimination of numerous target cells within minutes to hours, is defective in perforin-deficient mice. The NK cells from such mice can still kill many target cells, although this usually requires longer observation times (12–24 h) (Wallin et al. 2003). This killing is dependent on membrane-bound death receptors such as Fas ligand (Fas-L or CD178) and tumor necrosis factor (TNF)-related, apoptosis-inducing ligand-dependent receptors (Arase et al. 1995; Kayagaki et al. 1999). These interact in a cognate fashion with Fas and tumor necrosis factor-related, apoptosis-inducing ligand (TRAIL) and can thereby activate apoptosis in target cells expressing these molecules. Such mechanisms may dominate in certain situations, even when the NK cells express perforin.

Natural killer cells can also secrete cytokines, particularly IFN-γ and TNF-α (Trinchieri et al. 1984; Degliantoni et al. 1985). These cytokines may be directly cytotoxic in certain situations, but they are mainly viewed as regulators of other cellular functions. In this way NK cells may contribute to host defense by influencing infected cells and other white blood cells as well as noninfected cells outside the immune system, but which nevertheless are important for the inflammatory response (e.g. endothelial cells) (Trinchieri 1995; Biron et al. 1999). For example, IFN-γ induces increased expression of a number of cell surface molecules, including MHC class I and II products and intracellular proteins involved in antigen processing. IFN-γ can also induce intracellular pathways that limit the replication or even lead to the direct elimination of microorganisms. It can influence the profile of the ensuing specific

adaptive response, deviating the T cells toward a T-helper (Th) type 1 response. Other cytokines that can be secreted by NK cells include the interleukin (IL)-10, IL-13, and granulocyte–macrophage colony-stimulating factor (GM-CSF).

The soluble effectors expressed by NK cells also include molecules with antimicrobial activity. The peptide granulysin is one of the granule components, allowing NK cells to exert direct bactericidal effects (Krensky 2000). Finally, a relatively recently discovered effector function involves cognate receptors that can stimulate T cells directly. The cell surface molecule 2B4 and its ligand CD48 appear to play a central role here (Lee et al. 2003).

Many stimuli can induce several parallel effector functions, e.g. the recognition of target cells via activating and inhibitory receptors will in many cases lead to cytotoxicity as well as IFN-γ secretion. Other stimuli, such as cytokine receptors, may elicit more restricted action.

Methods to study NK cell function in vitro and in vivo

Natural killer cell activity is often measured with the type of cytotoxicity assays that allowed their discovery (Kiessling et al. 1975a, b; Herberman et al. 1975a, b). These are usually based on assessing the proportion of target cells that releases an intracellular marker as a sign of cell damage or death after incubation with a cellular preparation containing NK cells. The target cells are usually 'labeled' with the marker before the assay, e.g. with the isotope ^{51}Cr that binds to intracellular proteins and is therefore minimally released during 4–8 h used for the assay, unless the target cells are killed.

Some target cells, e.g. normal resting cells, label poorly or are difficult to prepare to give minimal 'spontaneous release' of the isotope in the absence of killer cells. An alternative in such cases in experimental animal research is a type of assay based on 'in vivo elimination'. Target cells labeled with isotope or fluorescent dyes are inoculated into mice, usually intravenously, and the numbers of surviving cells are measured by sacrificing the animals and counting radioactivity or fluorescent cells in different organs (Riccardi et al. 1979). These assays have the advantage of demonstrating killer activity directly in vivo, which is usually more efficient than the in vitro activity. When used with tumor cells, the most efficient NK cell-mediated elimination is usually observed in the lungs, where many tumor cells are initially trapped in the capillary bed (Smyth et al. 2002). This type of assay has so far been used only to a limited extent in microbiological NK research.

A more complex, but of course highly relevant, way to study NK cells is to assess their influence on the incidence or outcome of disease or a treatment. This has been done extensively in relation to infectious agents as well as tumors. In such studies, it is of course vital to have parallel control animals in which NK cells are defective in some way or totally depleted. Measuring the survival or replication of the microorganism, and/or signs of disease and ultimately death of the infected animal, can then follow infection. Some examples on the role of NK cells in infections are discussed further below. In cancer research, the outcome can be measured by complete rejection or outgrowth of a subcutaneously inoculated tumor (Kiessling et al. 1975c). This requires the use of titrated, low doses of tumor inocula, but gives the advantage of demonstrating that NK cells can 'finish the job' and eradicate the last tumor cells (Kärre et al. 1986). Alternately, higher tumor doses can be used, and the outcome assessed by measuring the tumor size or number of metastases at given time points. NK cells are particularly efficient at preventing hematogenous metastases to the lungs (Smyth et al. 2002), which may reflect their efficient activity in the blood and the capillary beds. NK cells have been studied extensively in experimental bone marrow transplantation in lethally irradiated mice (Kiessling et al. 1977; Bennett et al. 1995; Barao and Murphy 2003). The outcome is then evaluated about a week after the transplantation, and based on assessing the spleen colonization by transplanted cells. This is done either by counting colonies or by measuring how an injected isotope is taken up specifically by proliferating hematopoietic cells.

Controls used to assure that effects are caused by NK cells are usually based on removal of the NK cells from the cell population or the animal. In this case, most investigators use treatment with a monoclonal antibody or antiserum directed against a cell surface marker of NK cells, such as DX5, NK1.1, or asialo GM1 (Ehl et al. 1996). As these reagents are not entirely specific for NK cells, additional controls may be required, e.g. NK 1.1 is expressed on a subpopulation of T cells, the so-called NK-T cells, and it may therefore be appropriate to perform the experiment also in mice lacking such cells. There is, to date, no natural mutant or intentionally constructed gene knock-out mouse with a complete and selective lack of NK cells. However, an experiment aimed at producing a transgenic strain unexpectedly led to a complete NK cell defect in one founder, presumably as a result of the insertion site of the transgene (Kim et al. 2000). The strain derived from this founder is a useful tool to study development of NK cells, but can also be used as a negative control for NK cell effects in vivo, particularly in long-term experiments where it is difficult to maintain absence of NK activity by repeated injections of antibodies (Table 3). Several genetically manipulated mouse strains with an approximately normal size of the NK cell population, but with defects in single receptors or signal transduction pathways affecting NK cell functions, have been established (Colucci et al. 2003). These strains will be useful to dissect the importance of different NK functions in

Table 3 *Activating and inhibiting receptors of natural killer (NK) cells and expression on T cells*

Receptor	mu	hu	Ligand	References	T cells
Inhibiting					
Ly49	+	–	MHCI	Anderson et al. (2001); Kane et al. (2001)	+ ($CD8^-$ effector/memory subset)
Ly49A			D^d, D^k, (D^b)	Karlhofer et al. (1992)	
Ly49C			K^b, (K^d, D^d, D^k, D^b)		
Ly49G			D^d, L^d		
Ly49I			K^d, MCMV m157	Arase et al. (2002); Smith et al. (2002)	
Ly49O			D^b, D^d, D^{dk}, L^d		
Ly49V			H-2^b, H-2^d, H-2^k		
Ly49B, -E, -F, -J, -Q, -S, -T			Specificity n.d.		
KIR	–	+	HLA cl.I	Lanier (1998); Natarajan et al. (2002)	+ ($CD8^-$ effector/memory subset)
2DL2, 2DL3			HLA-C group 1[a]		
2DL1			HLA-C group 2[a]		
3DL1			HLA-Bw4		
3DL2			HLA-A3, -A11		
2DL5, 3DL7					
CD94/NKG2A/B	+	+	Qa-1, HLA-E, HLA-E/UL40	Braud et al. (1998); Tomasec et al. (2000)	+ ($CD8^-$ subset, all activated)
KLRG1/MAFA	+	+			+ ($CD8^-$ effector/memory subset)
LIR1/ILT2	+		HCMV UL18	Reyburn et al. (1997)	+ ($CD8^-$ subset)
NKR-P1D	+	+	Clr-b	Iizuka et al. (2003)	
Activating					
Ly-49[b]	+	–		Anderson et al. (2001); Kane et al. (2001)	
Ly-49D			H-$2D^d$, ligand on CHO cells	Idris et al. (1999)	–
Ly-49H			MCMV m157	Arase et al. (2002); Smith et al. (2002)	
Ly-49P			D^d		
Ly-49R			D^d, D^k, L^d		
Ly-49W			H-2^d, H-2^k		
Ly-49L, -M, -U			Specificity n.d.		

(*Continued over*)

Table 3 *Activating and inhibiting receptors of natural killer (NK) cells and expression on T cells (Continued)*

Receptor	mu	hu	Ligand	References	T cells
KIR	–	+	HLA cl.I	Lanier (1998); Natarajan et al. (2002)	+ ($CD8^-$ memory subset)
2DS2			HLA-C group 1		
2DS1			HLA-C group 2		
2DL4			HLA-G		
2DS4			HLA-C		
2DS3, 2DS5, 3DS1					
CD94/NKG2C	+	+	Qa-1, HLA-E	Braud et al. (1998); Vance et al. (1999)	+ ($CD8^-$ subset)
CD94/NKG2E/H	+	+	Qa-1	Vance et al. (1999)	+ ($CD8^-$ subset)
NKG2D	+	+	Rae-1, H60; MIC-A/B, ULBP	Raulet (2003)	+ ($CD8^-$ subset; hu-all, mu-effectors)
NKp30	+	+	Ligand on iDCs	Moretta (2002)	–
NKp44	–	+	Influenza HA		–
NKp46	+	+	Influenza HA	Mandelboim et al. (2001)	–
NKR-P1A	+	+			
NKR-P1C/NK1.1	+	–			+ ($CD8^+$ effector/memory subset)
Co-receptors					
NKp80		+		Moretta et al. (2001)	+ subset
2B4	+	+	CD48	Moretta et al. (2001)	+
DNAM-1		+	Nectins		+
NTB-A		+			

Further inhibiting receptors: P75/AIRM, IRp60, LAIR-1, gp49B1.
CHO, Chinese hamster ovary; CMV, human cytomegalovirus; HA, hemagglutinin; iDCs, immature dendritic cells; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell immunoglobulin-like receptors; MCMV, murine cytomegalovirus; n.d., not defined yet.
Low-affinity ligands are indicated in parenthesis.
a) HLA-C: group 1 – Ser-77, Asn-80 in Cw1, Cw3, Cw7, Cw8; group 2 – Asn-77, Lys-80 in Cw2, Cw4, Cw5, Cw6.
b) As 'activating' have been designated Ly49 receptors (1) for which stimulatory activity has been revealed or (2) that have no ITIMs and a positively charged residue in the transmembrane segment (Anderson et al. 2001; Kane et al. 2001).

complex in vivo scenarios, e.g. in infectious diseases. Similarly, mice defective in genes critical for various effector functions can be used to study exactly how NK cells contribute to a complex response or development of disease.

There are mice with a profound immune deficiency that involves NK cells as well as other cells, e.g. mice deficient for the common γ chain of cytokine receptors (γc) (Table 3). Despite such a broad defect, these mice can be used for the study of NK cells by adoptive transfer of isolated NK cells. Cytokine-stimulated NK cells can also be used in adoptive transfer to improve the resistance to tumor cells in normal mice.

Ontogeny and regulation

Like other white blood cells, NK cells are derived from hematopoietic stem cells in the bone marrow. In the fetus, NK cells and other lineages also develop in the yolk sac, liver, and aorta–gonad–mesonephros region. Studies in mice indicate that generation of NK cells may be critically dependent on the microenvironment of the bone marrow during adult life. Mice treated with the marrow-ablating agents ^{89}Sr and estradiol, in which other pathways of myelo- and lymphopoiesis are taken over by the spleen, show severely impaired NK cell development (Colucci et al. 2003). Stroma cells of the marrow appear to have a critical role, providing cytokines as well as cognate interactions required for NK cell development.

Through the use of well-defined culture conditions, it is possible to generate NK cells from hematopoietic cells from different sources in the mouse as well as in the human (Mrozek et al. 1996; Williams et al. 1997; Colucci et al. 2003). Multiple intermediate stages have been proposed. Although these differ between species, source of stem cells, and culture conditions, a general pathway emerges that is remarkably similar in the human and the mouse. This simplified scheme has three phases.

The first phase leads to NK cell precursors, via initial commitment to the lymphoid lineage and subsequently to the NK cell lineage. The NK precursors in the human as well as in the mouse express the IL-2 receptor IL-2Rβ, but lack many of the typical NK cell markers. The second phase leads to immature NK cells, during which the NK precursors gradually acquire surface markers and several functions of NK cells. NKRP-1 and CD2 molecules appear early, followed by DX5 (mouse) or CD56 (human), in parallel with the development of the cytolytic potential. The MHC receptors are acquired late; those of the NKG2 family precede those of the Ly49 (mouse) and KIR (human) families. In the third and final phase NK cells are exported to the periphery. Phase 1 and at least part of phase 2 can be achieved in culture by the correct cytokine combinations, without the need for stromal cells. The cytokines stem cell factor (SCF) (also known as c-KIT ligand), fetal liver kinase 2 (FLK2) ligand (also known as FMS-like tyrosine kinase 3 ligand or FLT3L), and IL-7 can drive the cells through phase 1, although none of these is essential by itself for NK development in vivo (Williams et al. 1997). IL-15 is critical in phase 2 (Mrozek et al. 1996). The latter requirement may explain the block in NK cell development in mice with common cytokine receptor γ-chain deficiency – this chain is an essential part of the IL-15 and IL-2 receptor. Although high doses of IL-2 can substitute for IL-15 in vitro, there is overwhelming evidence that in vivo development is critically dependent on IL-15, and not at all on IL-2. Under the influence of IL-15 in vitro, NK cells acquire cytotoxic capacity, but they still lack several characteristics of mature NK cells, such as receptors of the Ly49 or KIR families. Complete maturation requires interaction of NK cell precursors with MHC and possibly other molecules on bone marrow stromal cells, in order to develop and fine-tune the receptor repertoire (Roth et al. 2000; Raulet et al. 2001). The latter aspect is discussed separately.

Developmental processes are regulated by transcription factors, some of which can act as crucial master switches in hematopoietic lineage commitment (Colucci et al. 2003). Mice deficient in the Ikaros gene show a block in lymphoid development, including T, B, and NK cells, whereas myelopoiesis is unaffected (see Table 3). NOTCH-1 and PAX5 act as master switches for the development of T cells and B cells, respectively. Mutations in these genes do not affect NK cell development. There is evidence that ID molecules (inhibitors of DNA-binding proteins), which negatively regulate the basic-loop-helix (bHLH) family of DNA-binding proteins, are important to favor the NK over the T- and B-cell lineage (Yokota et al. 1999). Deficiency in several other transcription factors can partially impair NK cell development, or skew the receptor repertoire (Colucci et al. 2003).

Natural killer cell precursors can be isolated from the thymus. It is, however, not clear whether this signifies a closer relationship to the T-cell lineage. In any case, the thymus plays no critical role in NK cell development; athymic patients (di George syndrome) and mice (nu/nu genotype) have normal if not increased NK cell numbers and activity (Yokoyama 1999).

Once mature, NK cells have a lifespan of at least several weeks. They circulate in the blood and can enter mucosae as well as parenchymatous organs. A special subset is detected in the placenta (Moffett-King 2002). During local infection or inflammation, NK cells may infiltrate the site by attraction through chemokines and the use of integrins such as very late antigen 4 (VLA-4) (or CD49d/CD29) (see Table 1). The homeostatic survival may, like NK development, depend critically on IL-15. Adoptive transfer experiments suggest that mature NK cells are blocked from dividing under normal conditions, but homeostatic proliferation can

take place if the number of NK cells is low (Prlic et al. 2003). There is a vast proliferation on overproduction of the cytokine IL-15 in vivo and NK cells may become dominant in the spleen (Fehniger and Caligiuri 2001). In vitro, NK cells can be induced to proliferate by IL-2. As a result of the constitutive expression of IL-2Rβ, they are the major responding cell population when normal blood or spleen cells are exposed to high doses of IL-2, in what has been termed lymphokine-activated killer (LAK) cultures (Rosenberg 1988; Yokoyama 1999). Hence, they are the major effector cells in the anti-tumoral responses observed after adoptive transfer of LAK cells in experimental and clinical situations (Smyth et al. 2002). The physiological role of IL-2 in NK cell regulation and proliferation is unclear, but it is not a critical factor for development or proliferation of NK cells in vivo (Colucci et al. 2003). There is evidence for nonspecific, cytokine-driven, as well as specific, cognate, recognition-driven proliferation of NK cells during viral infection (Biron et al. 1999).

Many cytokines, chemokines and other mediators can increase the cytotoxic activity of NK cells. Type I IFNs secreted in response to virus infection or artificial stimuli, such as poly(I:C) and tilorone, are frequently used to optimize NK activity in murine experimental models (Biron et al. 1999; Yokoyama 1999). IL-12, IL-18, IL-21, as well as several chemokines can all act to increase NK cytotoxic function (Trinchieri 2003b). Combinations of one or more of these factors often show potentiating effects. Some pathways of regulation have a more profound influence on certain NK cell functions. There is, for example, dichotomy in murine cytomegalovirus (CMV) infection where IL-12 appears more important for NK cell-derived IFN-γ, whereas type I IFNs induce the cytotoxic function. NK cytotoxicity can be down-regulated by IL-10 and transforming growth factor β (TGF-β) (Biron et al. 2002).

Various conditions, diseases, diets, and activities – from high yogurt consumption to laughter and space trips – have been investigated with respect to their influence on NK activity. It is difficult to discern any specific patterns or regulatory pathways from the literature. In general, NK activity may vary considerably and appears relatively easy to modulate on a short-term basis. The functional impact of such spontaneous or induced alterations is unclear.

SPECIFICITY OF NK CELLS – A BALANCE BETWEEN ACTIVATING AND INHIBITORY RECEPTORS

The 'missing self' model

It is now clear that the specificity of NK cells depend on the integration of different recognition events mediated by activating as well as inhibitory receptors. This basic paradigm was first formulated within the 'missing self' model for NK cell recognition (Figure 2) (Karre 1985, 1997, 2002; Ljunggren and Kärre 1990). Briefly, this model proposed that one (but not the only) function of NK cells is to recognize and act on information that is missing in the target and present in the host rather than the opposite, which is how T cells work. More specifically, the information in question would be host MHC class I molecules. An immune mechanism eliminating cells without, or with too few, MHC class I molecules could have evolved as an alternative, as well as a necessary complement to T cells: an alternative because aberrant cells do not always express novel epitopes – loss of gene products might represent an early or even unique change in infected or transformed cells; complementary, because without MHC class I molecules, no antigens would be presented to CD8 T cells, which open for microbial escape mechanisms targeting this pathway. The postulated recognition strategy would also eliminate cells from other individuals, as a result of lack of host MHC molecules. Exposure to such cells occurs frequently in certain invertebrates, for which it has long been recognized that they possess graft rejection mechanisms based on self-recognition (Kärre 2002). Transfer of cells between individuals can also occur in

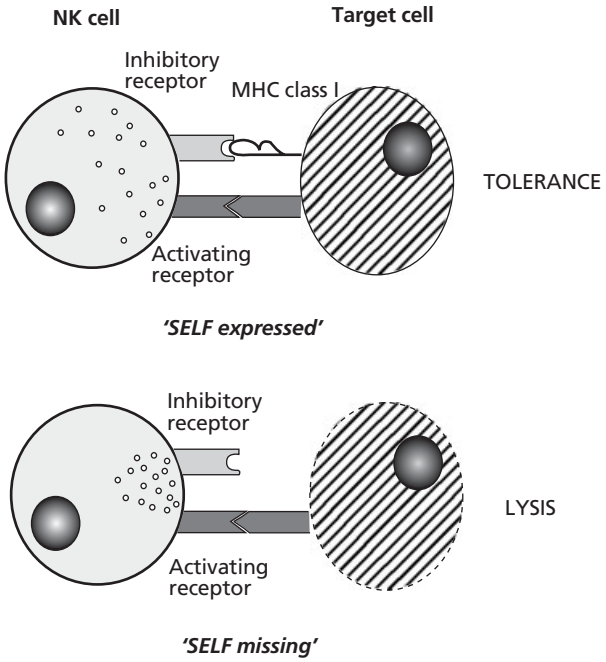

Figure 2 *The 'missing self' model. The expression of major histocompatibility complex class I (MHC class I) (*SELF expressed*) on a potential target cell can be sensed by inhibitory natural killer (NK) cell receptors. Tolerance is caused by dominance of inhibitory signals over NK cell triggering signals exerted by activating receptors engaging ligands on the potential target cell. Reduced or no MHCI expression (*SELF missing*) leads to a reduced or lack of inhibitory signals. Consequently activating signals dominate and killing of the target cell is triggered (Kärre 1985; Ljunggren and Kärre 1990).*

mammals, e.g. during intimate contacts or pregnancy, and this could represent a way for spreading infections. It can thus be argued that mechanisms in 'primitive' defense systems may have evolved further and adapted to the MHC system of mammals in the form of missing self-recognition.

This model provided a number of testable predictions (Kärre 2002), many of which are alluded to in the sections on cancer and transplantation in this chapter. A key prediction related to the main mechanistic model for missing self-recognition, depicted in Figure 2, is based on the idea that NK cells depend on two (sets of) receptors, one activating and one inhibiting. The activating receptor(s) were postulated to recognize ubiquitously expressed ligands, present on most normal cells. This would allow NK cells to bind most cells in the body and receive a preliminary triggering signal. The ultimate decision would then be taken by the inhibitory receptors, recognizing one or more self MHC class I ligands of the host. This would inhibit the triggering signal. If critical class I molecules were missing or too few in number, the inhibitory signal would be insufficient, and lysis of the target cell would proceed by default. This predicted the existence of MHC class I allele-specific receptors on NK cells, and that antibodies prepared against such receptors should induce lysis (by blocking of the inhibitory recognition) rather than block it. This experimental prediction was confirmed with the identification of two different inhibitory receptor families in the human and mouse, respectively (Karlhofer et al. 1992; Moretta et al. 1993, 1994; Yokoyama 1999). The use of antibodies against such receptors allowed a further dissection of specificity and eventually the identification of activating receptors. Although the basic platform provided by the missing self model has turned out to be correct, it is now clear that the picture is immensely more complex, with a variety of activating and inhibitory receptors balancing each other. The decision does not always lie with the inhibitory receptors – some of the activating receptors recognize ligands expressed only by infected or stressed cells, and they can induce NK effector functions even if the target cell expresses self MHC class I molecules (Moretta et al. 2001; Raulet 2003; Yokoyama and Plougastel 2003). Most of the known NK receptors are summarized in Table 4; the most important ones are discussed in more detail below.

Inhibitory receptors

Natural killer cells express inhibitory receptors that recognize MHC class I molecules with varying degrees of allele specificity. Interestingly, these receptors are encoded by two entirely different gene families in the human and in the mouse – the KIR and the Ly49 receptors (Ly49R) in the lectin-like superfamily, respectively (Moretta et al. 2001; Yokoyama and Plougastel 2003). Even though the proteins are in two different structural groups, the receptor systems in the two species show remarkable similarities at the functional level. Both types of receptor recognize a subgroup of MHC class I alleles. Both deliver inhibitory signals by immunotyrosine inhibitory motifs (ITIM) (with the sequence V/I–x–Y–x–x–L/V) in the cytoplasmic tail, which on phosphorylation can recruit phosphatases acting on substrates in the signaling pathways initiated by activating receptors (Lanier 2003). Both are encoded in complex gene families showing considerable polymorphism between individuals, and also include activating receptors of similar specificity and structure, except for the ITIM in the cytoplasmic tail (Trowsdale et al. 2001; Yokoyama and Plougastel 2003).

Table 4 *Key features of signal transduction in natural killer (NK) cells*

Receptor	Module/adaptor	Downstream elements
$Ly49_{inhibiting}$	ITIM	SHP1
$Ly49_{activating}$	+TM/DAP12	SYK, ZAP70
$KIR_{inhibiting}$	ITIM	SHP1
$KIR_{activating}$	+TM/DAP12	SYK, ZAP70 – PLCγ1, ERK1/2
CD94/NKG2A	ITIM	SHP1
CD94/NKG2C, -E	+TM/DAP12	SYK, ZAP70
NKG2D	$+TM/DAP10_{(YINM)}$[a], $+TM/DAP12_{(ITAM)}$[b]	SLP-76, PI3K, Grb2 – Vav, PLCγ2, SYK, ZAP70
2B4	SAP or SHP1; LAT	PI3K
NKp30	FcεRIγ, CD3ζ	SYK, ZAP70
NKp44	+TM/DAP12	SYK, ZAP70
NKp46	FcεRIγ, CD3ζ	SYK, ZAP70
Nkrp-1c	FcεRIγ, CD3ζ	SYK, ZAP70
CD16	FcεRIγ, CD3ζ	SYK, ZAP70 – PI3K, PLCγ2, Vav

ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell immunoglobulin receptor; LAT, linker for activation of T cells; PI3K, phosphatidylinositol 3-kinase; PLCγ1, phospholipase C γ1, SAP, SLAM-associated protein; SHP1, SH_2-domain-containing protein tyrosine phosphatase; +TM, positively charged residues in the transmembrane portion; ZAP70 – ζ chain-associated protein 70 kDa. More detailed information is provided in Colucci et al. (2002), Lanier (2003), and Trinchieri (2003a).

a) Resting and activated NK cells.

b) Activated NK cells.

Both types of receptor are expressed in complex patterns within one individual, yielding NK cells or clones or subpopulations with different but partly overlapping combinations of receptors (Raulet et al. 2001; Parham and McQueen 2003). Most importantly, both types of receptor allow the NK cells to distinguish between cells with normal or insufficient self MHC class I expression, by inhibiting effector function in response to the former but not to the latter. In the following, the mouse and human receptors are discussed separately, with emphasis on the features that distinguish them. It is nevertheless clear that the unifying functional aspects dominate. It is a conundrum why evolution has selected two different complex receptor systems with such similar functions in different species.

HLA CLASS I RECOGNIZING KIR RECEPTORS

As evident from their name, the KIR belong to the immunoglobulin superfamily of genes. The KIR genes are located within a larger gene complex termed the leukocyte receptor cluster (LRC) on chromosome 19q13.4 (Trowsdale et al. 2001). Fourteen different expressed KIRs have been described so far (Lanier 1998; Parham and McQueen 2003). The inhibitory receptors have two or three Ig-like folds, and the commonly used terminology is partly based on this feature (e.g. KIR2DL1 means KIR of 2 Ig-like domains, with long cytoplasmic tail including ITIM). As summarized in Table 2, the 2D KIRs distinguish two major subgroups of HLA-C alleles on the basis of a dimorphism involving residues 77 and 80. The KIRs bind on top of the MHC class I molecule, interacting with both α helices and the peptide (Boyington and Sun 2002; Natarajan et al. 2002) (Figure 3). This explains why the peptide bound by the MHC molecule can influence the KIR recognition, although there is no generalized difference between self and nonself (e.g. microbe-derived) peptides. The KIR3DL1 receptor recognizes a subgroup of HLA-B molecules with the Bw4 serological motif determined by residues 77–83, in a manner that can also be influenced by the bound peptide.

A given NK clone can express between 0 and 3 inhibitory KIRs. This expression pattern appears stable within each clone (Lanier 1998). The composition and expression pattern of the KIR-gene complex differ among individuals (Vilches and Parham 2002). The expression pattern is genetically determined; it is stable within one individual and similar in monozygotic twins but, surprisingly, it is not strongly influenced by the HLA type of the individual. The issue of receptor repertoires in rela-

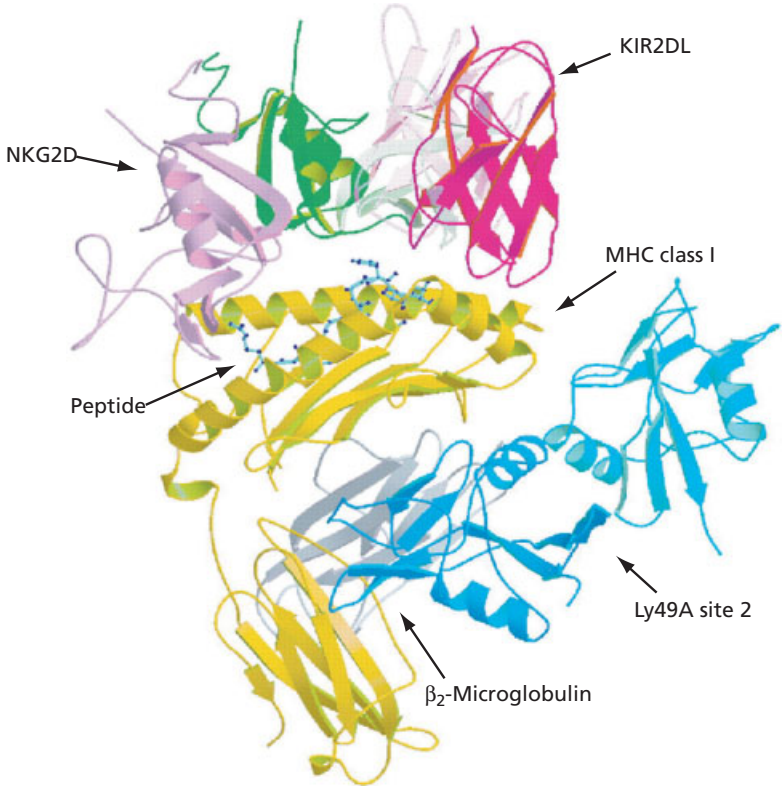

Figure 3 *Structural aspects of major histocompatibility complex class I (MHC class I) recognition by three natural killer (NK) receptors. Binding sites for Ly49A, KIR2DL, and NKG2D on MHC class I (shown is H-2DS^d). Ly49A interacting at site 2 is depicted in cyan/light blue, KIR2DL in magenta, and NKG2D in green/pink. (Figure reproduced with permission from Natarajan et al. (2002) © 2004 Annual Reviews. For detailed discussions see Boyington and Sun (2002); Natarajan et al. (2002), and Dam et al. (2003).)*

tion to tolerance and disease susceptibility is discussed in more detail below.

In addition, the LRC contains the *LIR/ILT* gene family, which includes inhibiting as well as activating receptors (Borges and Cosman 2000; Colonna et al. 2000; Trowsdale et al. 2001). They differ from KIRs in the expression pattern, and are found mainly on other cells in the immune system (Natarajan et al. 2002), e.g. LIR-1/ILT-2 is expressed by B cells, macrophages, and dendritic cells, and only on a smaller fraction of NK cells. This receptor can recognize several different MHC class I molecules but, interestingly, its affinity for the human cytomegalovirus (HCMV) protein UL 18 is 1000-fold higher (Chapman et al. 1999). LIR-2/ILT-4 also bind to MHC class I molecules, whereas the specificity of other members of this family is elusive.

H-2 CLASS I RECOGNIZING LY49 RECEPTORS

The *Ly49* genes are encoded within the NK gene complex (NKC) of distal chromosome 6 in the mouse, together with several other genes that also belong to the lectin superfamily (Yokoyama and Plougastel 2003). There are, however, structural features of Ly49 molecules that argue against a true lectin function, i.e. carbohydrate recognition. A total of 16 full-length Ly49 genes have been identified; the number of genes varies between different mouse strains (Kane et al. 2001). They are expressed as homodimers with type II membrane orientation on the cell surface (Lanier 1998). Like the KIR family, the Ly49 family also includes genes that encode activating receptors.

The Ly49 receptors bind mouse MHC class I molecules with varying degree of allele specificity (see Table 4) (Kane et al. 2001). The Ly49A receptor, co-crystallized with its ligand, surprisingly revealed two sites of interaction: 'site 1' at the top of the groove near the N terminus of the peptide, and 'site 2' below the antigen-binding groove, with several contact residues in the $\alpha 3$ domain and β_2-microglobulin (Kane et al. 2001; Natarajan et al. 2002). Although 'site 1' was initially believed to represent the physiological binding, multiple lines of evidence now argue in favor of 'site 2' being the important recognition zone (Dam et al. 2003). This puts the issue of allelic specificity in focus, because many contact residues at 'site 2' are nonpolymorphic. The most likely explanation is that alterations in polymorphic residues distant from the contact zone can nevertheless affect it by structural changes transmitted through the molecule, ultimately affecting the orientation of nonpolymorphic contact residues. Even polymorphic residues in the floor of the antigen-binding groove can influence allele specificity, which may explain why certain Ly49 receptors are influenced by the nature of the peptide bound to the groove. Perhaps the opposing ways by which KIR and Ly49 molecules bind and distinguish different MHC class I molecules may explain why the two species have evolved different types of receptors.

Each NK cell expresses between 0 and 6 Ly49 receptors, the average estimated as being 2.6 different ones per cell (Kubota et al. 1999; Raulet et al. 2001). The development of the inhibitory receptor repertoire is discussed below.

HLA-E/QA-1 RECOGNIZING NKG2A RECEPTORS

NKG2A receptors are also encoded in the NKC, but, in contrast to the Ly49 family, they and the family of genes they belong to are encoded in the syntenic human chromosome 12p13 (Yokoyama and Plougastel 2003). NKG2A, with two intracytoplasmic ITIMs, is expressed as a heterodimer together with CD94, also encoded within the NKC (Carretero et al. 1997; Lanier 1998). NKG2A recognizes the nonclassic conserved MHC class I molecule HLA-E or its homolog Qa-1 in the mouse. These ligands are not transported from the endoplasmic reticulum (ER) to the cell surface unless they bind peptides with specific anchor motifs, present mainly in leader sequences of polymorphic MHC class I molecules (Braud et al. 1998). Paradoxically, the loading of such leader sequences in HLA-E/Qa-1 molecules requires the ER membrane-bound peptide transporter associated with antigen presentation (TAP). The NKG2A receptor will thus allow the scanning for expression of MHC class I molecules and/or TAP on potential target cells. Reduced production of such molecules will indirectly lead to insufficient HLA-E/Qa-1 expression, which is required to inhibit via the NKG2A receptor (Leibson 1998).

The division of labor between NKG2A and KIR/Ly49 receptors is not clear. Perhaps they are used as complementary tools to sense intact antigen-processing/MHC class I machinery. NKG2A can be expressed together with one or several KIRs, but they can also be expressed in the absence of KIR.

INHIBITING RECEPTORS WITH OTHER SPECIFICITIES

The inhibiting receptors in the mouse also include NKR-P1B and -D. The latter recognizes a lectin expressed by dendritic cells. Interestingly, the gene for this inhibitory ligand is closely linked to the receptor, suggesting that tolerance in this system evolved by linked inheritance of receptor and ligand (Iizuka et al. 2003). In the human, an Ig-like molecule termed p75/AIRM has been identified as an ITIM containing inhibitory NK receptors. It is a member of the sialoadhesin family in the LRC, and it is also expressed by myeloid cells. It mediates sialic acid-dependent recognition but the functional specificity is unknown (Vitale et al. 2001).

The existence of inhibitory receptors with specificities other than MHC class I suggests that the 'missing self' concept may be extended to include situations where target cells need to be protected from NK cells, although MHC class I expression is suboptimal. One

obvious example is the red blood cell. It is not known how erythrocytes escape NK lysis. So far, unknown inhibitory receptor–ligand pairs represent one possibility, but it is also possible that red blood cells do not express adhesion molecules and activating ligands for initial binding and triggering of NK cells.

Activating receptors

ACTIVATING RECEPTORS IN THE KIR AND LIR FAMILIES

Several of the inhibitory KIRs have closely related homologs, which, however, differ in that they only have a short cytoplasmic tail, without ITIMs. These receptors are designated with an S (for short cytoplasmic tail) instead of a D, e.g. KIR2DS1. They possess a charged amino acid in the intramembrane-spanning domain (Lanier 1998), allowing the association with the adaptor molecule KARAP/DAP-12 that couples to activating signal transduction pathways through immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic tail (Tomasello et al. 2000; Lanier 2003). These are based on the semiconserved peptide sequence YxxL(I)$x_{6–8}$YxxL(I) (Lanier and Bakker 2000). Cross-linking of the activating KIRs thus leads to triggering of NK cells, although the role for this type of recognition for overall NK cell function is not clear. It could represent one of several activating recognition systems for ligands expressed by most cells, and necessary for NK cells to mediate surveillance for the missing self. A more popular idea is that the activating KIRs directly recognize ligands preferentially expressed by infected or stressed cells – either 'altered self' in the form of MHC class I/peptide combinations, or pathogen-encoded, MHC class I-like molecules. There is evidence that activating KIRs may have lower affinity for MHC class I ligands than inhibitory KIRs, and that they are mostly expressed together with the inhibitory homolog (Khakoo and Brooks 2003). These aspects could be important to avoid autoreactivity. As discussed below, the activating KIRs appear to undergo rapid evolution, in line with the idea of an evolutionary pressure created by pathogens.

Some receptors in the LIR/ILT family, such as LIR-6a/b and LIR7/ILT1, share the features of activating KIRs, i.e. a short cytoplasmic tail with a charged amino acid in the membrane-spanning region (Borges and Cosman 2000; Colonna et al. 2000). The function of activating LIRs is not known.

ACTIVATING RECEPTORS IN THE LY49 FAMILY

The Ly49 family also includes activating receptors. Ly49H and Ly49D are the most well characterized (Nakamura et al. 1999; Yokoyama and Plougastel 2003). Like the activating KIRs, they lack ITIMs (despite a long cytoplasmic tail) and instead harbor a charged amino acid in the membrane-spanning region. Similar to activating KIRs, this allows them to signal through the adaptor molecule KARAP/DAP-12 (Lanier and Bakker 2000). Ly49H recognizes a murine cytomegalovirus (MCMV)-encoded ligand, and this receptor is indeed the product of a CMV resistance gene studied for a long time in the mouse strain C57BL, as discussed below (Scalzo et al. 2003). Ly49D, also associating with DAP-12, can bind to the murine MHC class I molecule H-$2D^d$. Ly49D$^+$ NK cells from mice lacking H-$2D^d$ can use the receptor to kill cells that express this MHC allele (Nakamura et al. 1999). An appealing hypothesis that integrates these seemingly disparate specificities of Ly49H and Ly49D, addressing the function and evolution of activating of MHC class I-recognizing receptors in general, is discussed below.

NKG2D: AN ACTIVATING RECEPTOR RECOGNIZING LIGANDS INDUCED IN STRESSED, INFECTED, AND TRANSFORMED CELLS

NKG2D is closely linked genetically to NKG2A and -C, but only distantly related to them with respect to sequence homology (28 percent). Furthermore, it is expressed as a homodimer rather than complexing with CD94. It is expressed by T cells (all in the human and by subsets of γδT and activated αβT cells in the mouse) and by activated macrophages (Raulet 2003). Through a charged amino acid in the membrane-spanning region, it can associate and induce activating signals through the adaptor molecule DAP10 in T cells, which enables it to work as a co-receptor for T-cell receptor (TCR) triggering. In NK cells it can act as an activating receptor on its own. This is the result of two splice variants, allowing association with KARAP/DAP12 as well as DAP10 (Lanier 2003). NKG2D can recognize a large variety of ligands distantly related to MHC class I, expressed mainly on certain stressed, infected, or transformed cells. The ligands for human NKG2D include MICA and MICB, expressed on stressed epithelial cells, and the CMV-encoded proteins ULBP1–3. Murine NKG2D binds to Rae-1 and HL-60 expressed by stressed cells and certain tumor cells, and a recently identified ligand termed mouse ULBP-like transcript-a (MULT-1) (Raulet 2003). The last appears to be constitutively expressed in many tissues. Structural studies of NKG2D co-crystallizing with its ligands have provided an explanation of how this receptor can bind to different ligands (see Figure 3) (Natarajan et al. 2002). NKG2D was initially thought to be completely independent of inhibitory signals; it now appears that these signals can inhibit activation of NKG2D, but less efficiently than other activating receptors (Pende et al. 2001). Stressed normal cells can therefore be killed through recognition by NKG2D even if they express considerable levels of MHC class I molecules. NKG2D and its ligands provide many exciting lines of research in infection and tumor immunology, discussed further in the specialized sections below.

ACTIVATING RECEPTORS WITH UNKNOWN SPECIFICITY, INCLUDING RECEPTORS RECOGNIZING NON-MHC MOLECULES ON NORMAL CELLS

The 'missing self' model postulates that the activating NK cell receptors recognize ligands on most normal cells. There is strong evidence that NK cells can indeed kill normal cells, which lack MHC class I, and which also lack the stress-induced ligands for NKG2D discussed previously. Thus, there must be other activating receptors. Some of the receptors discussed here play a role in the surveillance of normal cells, but it must be emphasized that the exact (combination of) receptors used in different situations remain to be clarified. One of the reasons for this may be that NK cells use several receptors in a parallel and redundant fashion, which makes it difficult to prove the role of one by targeting it genetically or in antibody blockade experiments. It is therefore interesting that several activating receptors were identified in an experimental approach with xenogeneic effector target cell combinations, in order to minimize the number of receptor–ligand pairs involved. Using this approach, antibodies binding to the three so-called NCRs, NKp30, NK p44, and NKp46, were identified (Moretta et al. 2001). All NK cells, in humans as well as in mice, express NKp30 and NKp46. The former is the main activating receptor in NK cell interactions with dendritic cells (Moretta 2002). NKp46 is involved in recognition of normal as well as many tumor cells, and there is also evidence that it can recognize influenza virus-infected cells via sialic residues of the hemagglutinin (Mandelboim et al. 2001). The level of NKp46 varies between NK cells, and this correlates with their cytotoxic potential. Only human activated NK cells express NKp44 (Moretta et al. 2001). Its ligands are unknown.

There is intensive ongoing research on the specificity and expression patterns of these activating receptors, as well as their role in recognition of various target cells. A frequently used strategy to define the specificity of receptors is to produce multimeric, soluble proteins to identify ligands in different types of cells. This is not always as straightforward as it sounds – the geometry and affinity of these constructs may not always allow ligand-specific binding above background levels. The final answer as to which activating receptors NK cells use in different situations must be based on methods allowing the direct measurement of ligands on target cells, as well as antibody cocktails combining different antibodies to completely block the critical interactions (Moretta et al. 2001).

Transduction and integration of signals after NK cell recognition

The ligation of activating and inhibitory receptors must be transduced to intracellular signals, and these must be integrated and read out to activate different NK cell programs. This is an intensive area of research, and only a fragmentary picture is currently available (Colucci et al. 2002; Lanier 2003). Upon ligation of inhibitory receptors, the phosphorylated ITIMs in their cytoplasmic tails recruit -SH2-domain-containing tyrosine phosphatase (SHP-1) and other phosphatases. These are thought to act on substrates that have been phosphorylated in the proximal part of activating pathways. There are multiple, parallel, proximal signaling pathways for activating receptors, which generally signal through noncovalently associated adaptor molecules. This association commonly involves an aspartic acid residue in the transmembrane region of the adaptor molecule, interacting with a positively charged amino acid in corresponding domain of the ligand-binding receptor. Some of the adaptor molecules, such as CD3ζ, FcεRIγ, and KARAP/DAP-12, contain ITAMs in their cytoplasmic tails. Ligation of an NK receptor linked to an ITAM-containing adaptor initiate proximal signaling events in a protein tyrosine kinase (PTK) cascade similar to ITAM-dependent mechanisms in T- and B-cell activation. It involves activation of PTK in the Src family, leading to activation of ZAP70 and Syk in the Syk PTK family (Colucci et al. 2002). DAP10, one of the adaptor molecules associating with NKG2D, lacks ITAMs (Lanier 2003). Its cytoplasmic tail instead contains the motif YINM, which can bind and activate phosphatidylinositol 3-kinase (PI3K) (Trinchieri 2003a).

Further downstream, additional intracellular adaptor molecules are activated, e.g. linker for activation of T cells (LAT), SLP 76, phospholipase Cγ (PLCγ1), PI3K, and Vav family members of guanine nucleotide exchange factors. Examples of receptors interacting with ITAM-containing molecules are CD16, NKp46, and NKp30, as well as the activating KIRs and Ly49Rs (Yokoyama 1999; Moretta et al. 2001; Colucci et al. 2003).

Table 4 summarizes the pathways known so far, including examples of interacting receptors, adaptor molecules, and some information on downstream signaling. The multiple pathways may just represent redundancy as a result of evolutionary tinkering, but it is also possible that they are needed to preserve hierarchies of interactions and specific consequences of certain interactions. As a result of these multiple, partly parallel pathways, specific defects caused by gene deletion or pharmacological intervention may in many cases have few if any consequences for overall NK function, whereas T- and B-cell function and even development may be severely affected. The highly NK-sensitive target cell YAC-1 is, for example, killed efficiently by mice with defects in Syk, ZAP70, or both (Colucci et al. 2002). One may speculate that this and other 'prototype' NK targets were once chosen initially as highly reliable for NK assays, just because they express multiple

activating ligands, although that was of course not known at the time. Some NK functions appear to rely critically on one pathway, e.g. Ly49H–KARAP/DAP-12, relevant for mouse CMV infection (Yokoyama and Plougastel 2003). Cytokines and adhesion molecules can also trigger or modulate NK function; this is regulated by pathways common to many white blood cells.

As more is learned about activating and inhibiting receptors, tools must be developed to dissect the complexity created by multiple inputs. Can all pathways interact and, if so, are the signals simply additive or subtractive? Are some activating receptors specialized in the sense that they are less (or not at all) influenced by inhibitory receptors, or activate only some effector function? This analysis will require imaging techniques to assess intracellular clustering of receptors and signaling molecules, as well as mathematical models for prediction and interpretation of the final outcome in effector function in response to different inputs. A few studies on inhibitory and activating immune synapses between NK cells and target cells have been published (Davis 2002). Although this field is just starting, it is clear that inhibitory receptors and their MHC class I ligands cluster in the center of the synapse in many situations.

Expression of NK receptors on T cells

Many of the NK receptors discussed above can also be expressed by subpopulations of T cells under certain conditions (Mingari et al. 2000; McMahon and Raulet 2001; Young and Uhrberg 2002; Trinchieri 2003a). One may indeed question the term NK receptors, which is usually based on the notion that the given receptor was first identified on NK cells. A detailed discussion is beyond the scope of this chapter, but an overview is integrated into Table 2. Suffice it to say that the function of these receptors is not clear; it has been proposed that these receptors can be used to fine-tune the action of T cells with respect to effector function, proliferation, survival, or homeostasis. A common theme is that they appear to act exclusively as co-receptors for activation or inhibition; they can thus act only by superimposing signals on those triggered via TCR recognition.

Polymorphism and complexity: implications for receptor repertoires, tolerance, and disease associations

As evident from the discussion of each NK receptor type, there are several levels of polymorphisms and complexity in this system. There are multiple genes encoding activating and inhibiting receptors within one individual. Some of these genes are present in all individuals and are conserved; some are present in all individuals, but with different alleles; some are expressed only in some individuals (Vilches and Parham 2002; Parham and McQueen 2003). Furthermore, within one individual, different NK cells express different combinations of receptors (Raulet et al. 2001; Hsu et al. 2002; Vilches and Parham 2002). Most of the mechanisms behind these patterns, as well as the functional implications, are unknown. In the following, the first section reviews a number of solid observations about NK repertoires. The next two sections deal with interpretations and hypotheses about this complexity, first in relation to tolerance development and then in relation to resistance against disease associations and evolutionary pressures.

DEVELOPMENT OF RECEPTOR REPERTOIRES

Natural killer cells acquire their MHC class I-specific receptors relatively late during ontogeny. The development of the receptor repertoire has been studied mainly in mice (Raulet et al. 2001). Some key observations from such studies are described below, with reference to human studies whenever there are similar or different data available.

The patterns with different combinations of receptors expressed by each NK cells arise from the basic notion that each receptor gene is either on or off in a given cell. This ‘variegated’ pattern of Ly49Rs and KIRs is determined at the gene expression level in a partly stochastic process. Some transcription factors influencing this process have been identified, but the detailed regulation remains unclear (Held et al. 1999). The receptor genes are usually, but not always, expressed in a monoallelic fashion, indicating that the stochastic mechanisms determining whether a particular gene is on or off apply to different loci as well as to different alleles at one locus. As a result, the proportion of cells in an individual that express a given receptor can vary between 5 and 80 percent. Expression of multiple different receptors on one cell follows the ‘product rule’. This means that the probability for simultaneous expression of two different Ly49Rs (as well as the KIRs) can be calculated by multiplying the proportion of cells that express each single receptor (Raulet et al. 2001).

The NK cells in a newborn mouse or human cord blood initially express NKG2A/CD94, but not Ly49Rs or KIRs. The Ly49Rs then appear sequentially in certain patterns, Ly49A usually preceding Ly49C and Ly49G2, but this order is not absolute. Interaction with MHC class I molecules on stromal cells influence this process (Roth et al. 2000), in a manner that is not well understood, but which reduces the number of cells expressing several receptors of the same specificity. After 3–4 weeks, the Ly49R expression has developed to the adult pattern, which means that each NK cell expresses at least one and often several receptors (Raulet et al. 2001).

Concurrently, the proportion of NKG2A-positive cells falls to approximately 50 percent in the mouse, whereas the majority of human NK cells express this receptor throughout life. Once established, the receptor expression of each cell appears stable. Long-term follow-up of human NK clones in vitro shows a similar pattern.

NK CELL REPERTOIRE IN RELATION TO AUTOLOGOUS MHC MOLECULES

Most NK cells have the potential to kill according to the 'missing self' model, i.e. their interactions with most normal cells are ultimately controlled by the inhibitory MHC class I receptors. As the latter, at least the ones in the KIR and Ly49R families, are allospecific, there is a risk for autoreactivity. How can 'tolerance' be ascertained in the system, given the complexity described above? Not only do receptors differ between individuals, and between NK cells within the individual, but in addition, the MHC class I ligands are extremely polymorphic and not genetically linked to the receptor genes. The ability to be triggered by activating ligands expressed by most cells must thus somehow be matched somatically with the expression of inhibitory receptors independently recognizing segregating ligands on autologous cells. How this is achieved has so far remained an enigma.

There are several theoretical possibilities. A simple positive selection model is based on stochastic expression of different receptor combinations in each NK cell, followed by a selection step in which only those receptors recognizing that at least one self MHC class I molecule during development receives a survival signal. Models based on this principle predict that the presence of a given MHC class I gene product should select for increased numbers of NK cells with this specificity. However, there is little or no influence at all of HLA on the KIR repertoire; in the mouse, H-2 has a minor influence on the frequency of NK cells with given Ly49 receptors, but in the opposite direction: the number of cells expressing a given receptor are marginally, but reproducibly, fewer if their ligands are present in the host. MHC class I-deficient mice therefore have somewhat more cells expressing any given Ly49R than normal mice (Raulet et al. 2001).

A second possibility, 'the sequential receptor expression' model, postulates that developing NK cells express and test receptors against the environment in sequential and cumulative order, in a process that is terminated as soon as one receptor detects self-ligands in the environment. This predicts a reduced probability for expression of multiple different receptors, which can recognize the same ligand in each NK cell. There is supporting evidence for this in normal mice as well as in transgenic mice where one receptor is forced on the whole NK cell population. The mechanisms limiting expression of several receptors for the same self-ligand appear to act late in differentiation in an interaction between NK cells and stromal cells. It may represent a way to render NK cells as 'useful' as possible, because limited inhibitory receptor expression specific for any MHC product would lead to more sensitive detection of cells in which this particular product is partly down-regulated (Raulet et al. 2001; Salmon-Divon et al. 2003).

Despite these observations, the complete available data do not fit the simplest form of the sequential receptor expression model, which would predict that all NK cells in MHC class I-deficient mice or humans should express all inhibitory receptors (and this should still not be sufficient for tolerance). However, these individuals have normal numbers of NK cells, which display the usual variegated expression pattern of Ly49Rs and KIRs, and which are tolerant to autologous cells (Raulet et al. 2001). They retain other cytotoxic NK functions against certain tumor cells and antibody-coated target cells in antibody-dependent cell-mediated cytotoxicity (ADCC) assays.

It must thus be questioned whether tolerance is based solely on inhibitory, MHC class I-recognizing receptors. In the human, complete analysis of in vitro cultured NK clones from a few individuals indicate that each NK cell expresses at least one self-receptor (Valiante et al. 1997). To be conclusive as to whether inhibitory receptors can account for tolerance, corresponding studies must be done on freshly derived cells. These have so far been performed only in mice, and always reveal a fraction of cells where no known self-receptor is expressed (Raulet et al. 2001). However, it can be argued that this observation is also inconclusive until there is a complete panel of receptors identified (and the corresponding reagents).

The currently most accepted, modified model is based on the idea that the sequential receptor expression of inhibitory receptors is only one of several contributing to tolerance, and that it acts only during a limited time window in differentiation. It would therefore not allow expression of all receptors in one NK cell. Additional mechanisms to consider in conjunction with this model, or independently, are those that do not rely on inhibitory MHC class I-specific receptors. Some NK cells could thus acquire tolerance by expression of another type of inhibitory receptor(s), or by down-regulating the receptors or critical links in signal transduction pathways that are responsible for the initial activation in missing self-recognition (Held et al. 2003).

It should be possible to identify these mechanisms by comparative transcriptomic or proteomic profiles on NK cells from mice that differ only in one MHC class I allele (and, hence, NK cell tolerance to cells that lack this allele). The most dramatic influence of MHC type on NK receptors observed so far is a down-regulation of Ly49R (by 30–90 percent) when their high-affinity ligands are present in the host (Olsson et al. 1995; Raulet et al. 2001). This appears to result partly from a direct, post-translational response to interaction with the

ligand, most probably leading to internalization or shedding of the receptor. It has not been observed with the KIR in humans. Although this down-regulation leads to enhanced sensitivity to detect impaired expression of the corresponding allele, potentially making the NK cell more 'useful', the functional significance of this phenomenon is unclear. Finally, there is evidence that the receptor levels of NK cells are influenced by the ligand expression of the NK cell itself (Kase et al. 1998; Doucey et al. 2004). This has led to speculations that Ly49Rs can interact with their ligands in '*cis*', and that this may be involved in regulation or tolerance (Held et al. 2003).

Whether tolerance is based on alteration of activating or inhibitory pathways, one must finally consider whether it is irreversible, or whether mature NK cells can alter their specificity once they are mature. In mice with mosaic MHC class I expression where a part of the cells lacked one self-allele out of three expressed by the remaining cells, NK cells were nonreactive to both MHC class I phenotypes but retained the ability to kill cells lacking all MHC class I alleles (Johansson et al. 1997). When NK cells of the two MHC phenotypes were separated and cultured in IL-2 in vitro, the ones with complete expression of three alleles acquired the ability to kill the cells expressing only two alleles. Although this demonstrates that some tolerant cells are preserved and not deleted, it remains to be investigated whether tolerance is actually reversible in vivo.

In summary, it seems plausible that NK cells acquire tolerance by a combination of mechanisms influencing activating as well as inhibiting mechanisms. This can be manifested at the level of gene expression, but post-translational mechanisms, where the cellular distribution and even membrane topology for receptor expression patterns influence the specificity, are not excluded. Further research is required to understand the general principles behind the development of receptor repertoires and tolerance in the NK system.

NK RECEPTOR GENES: EVOLUTIONARY ASPECTS AND IMPLICATIONS FOR DISEASE ASSOCIATIONS

Figure 4 provides a schematic overview of the two major families. The activating and the inhibiting receptors in the NKG2 family have been largely conserved. The mouse has four genes in the Nkrp1 family, the human only one (Yokoyama and Plougastel 2003). There are several other more or less related genes encoded in the NKC, such as CD69, an early activation marker for lymphocytes, including NK cells. The NK cells in these two species, as well as in the rat, are thus similar in many respects. However, there is a dramatic difference in the expansion of the Ly49 family in the mouse and rat. The Ly49L gene in humans does not encode a functional product, and this gene family therefore plays no role in humans.

The human genome instead contains the gene families in the LRC on chromosome 19 (Figure 4) (Trowsdale et al. 2001). For both KIR and Ly49R families, there is considerable polymorphism and evidence that they undergo rapid evolution – the KIRs may even be evolving at a higher pace than human MHC genes (Kane et al. 2001; Hsu et al. 2002; Vilches and Parham 2002). It is believed that infectious agents drive this evolution, although the mechanisms are not well understood. One key to the problem may lie in the function of the MHC class I-specific activating receptors in each family, which, at least in humans, are the genes that show the highest diversity. It has been proposed that their specificity goes beyond MHC class I molecules, and that they may recognize pathogen-encoded ligands, either in association with MHC molecules or by themselves. The latter is certainly the case for the activating Ly49H receptor in the mouse, as discussed in the section on CMV.

The considerable polymorphism and dynamic evolution of the KIR genes in the human population opens the possibility for studies of disease associations. Individuals differ in the number of loci as well as the alleles carried by these loci. Certain typical 'haplotypes' (combinations of loci and their respective alleles) are now being defined, and observed to be present in different frequencies in different ethnic groups (Hsu et al. 2002; Parham and McQueen 2003). Do different genes or haplotypes affect the incidence or course of infectious, malignant, or autoimmune diseases and is there an impact on the outcome of transplantation? Some associations, open for different mechanistic interpretations, have been reported (Table 5) and more are probably waiting in the wings in this intensive research field.

NK CELLS IN INFECTIONS

Virus infections

CLINICAL OBSERVATIONS

The picture emerging from the study of natural infections and their correlation with impaired NK activity in patients suggests that NK cells may be particularly important for the defense against viruses in the herpes family (Biron et al. 1989; Cerwenka and Lanier 2001; Orange et al. 2002). These have in common that they remain latent with no or little viral replication after the primary infection; they may be reactivated and cause opportunistic infections under conditions where the immune system is compromised, e.g. in malignant or infectious diseases and transplantation. This group includes, among others, varicella-zoster virus (VZV), herpes simplex type 1 virus (HSV-1), Epstein–Barr virus (EBV), and HCMV. The evidence is often anecdotal and sometimes difficult to interpret, because impaired

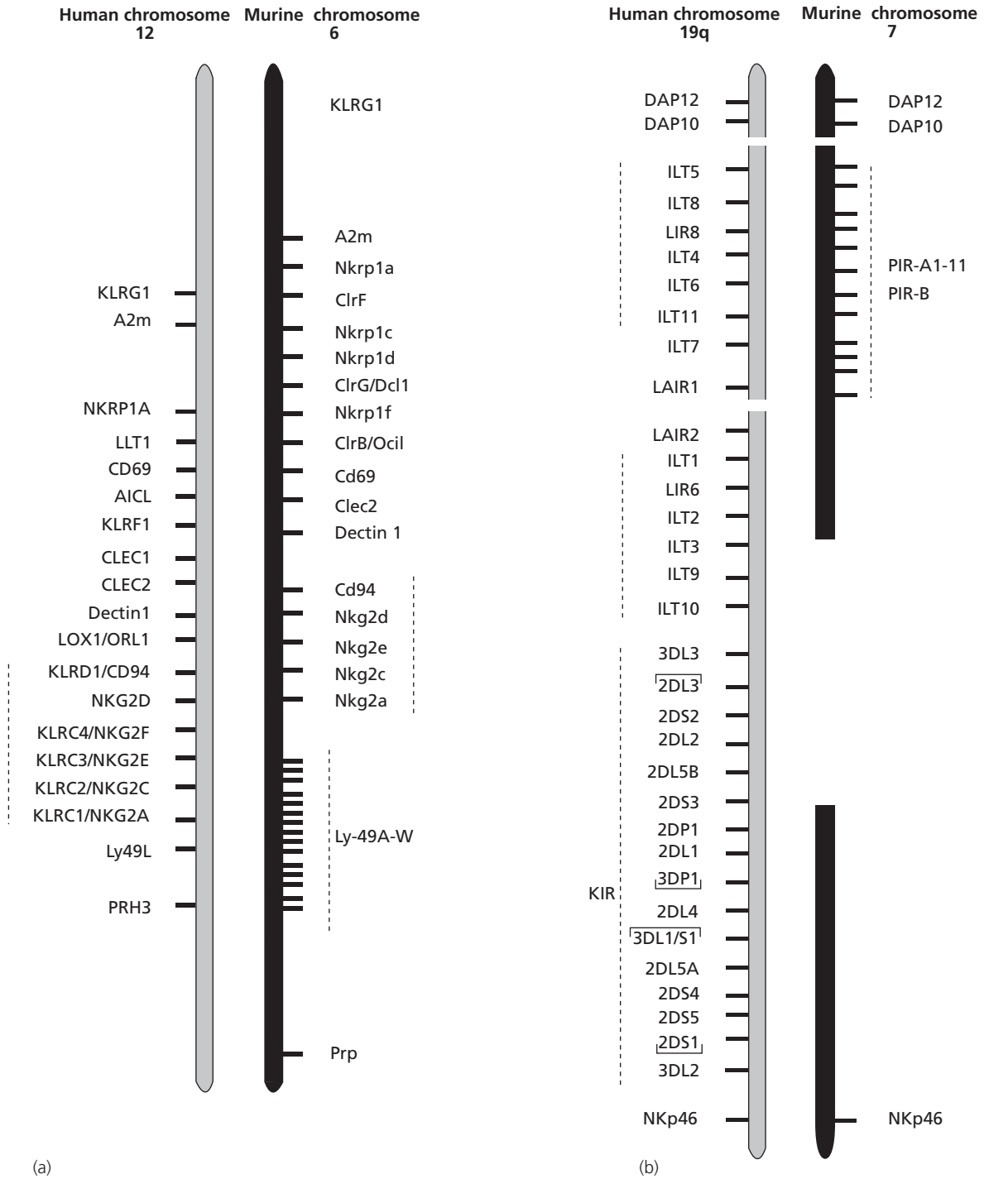

Figure (a) *The natural killer (NK) gene complex (NKC) and* **(b)** *the leukocyte receptor cluster (LRC). The NKC is present on human chromosome 12p13.1 and mouse distal chromosome 6 whereas the LRC is on human chromosome 19q13 and mouse chromosome 7 (Trowsdale et al. 2001; Hsu et al. 2002; Yokoyama and Plougastel 2003). Dashed vertical lines denote the NKG2/CD94-, the Ly49-, the LIR/ILT-, the PIR-, and the KIR-gene families. The brackets comprise the variable KIR gene regions, opposed to the three conserved framework genes* 3DL3, 3DL2, *and* 2DL4 *(Vilches and Parham 2002). Positions of genes are not drawn to scale.*

NK cell function may be the nonspecific consequence of infectious disease rather than its specific cause. Furthermore, NK deficiency is usually relative rather than absolute, and often associated with other immune defects. A typical example is the X-linked lymphoproliferative syndrome (Moretta et al. 2001), resulting from defective control of EBV, which is associated with perturbed function of NK cells as well as of T cells as a result of a defect in the signaling lymphocyte activation molecule-associated protein (SAP), involved in regulation of the signaling lymphocyte activation molecule (SLAM) pathway.

Table 5 *Killer cell immunoglobulin receptor (KIR) polymorphisms and disease associations*

KIR	Disease association
KIR3DS1	HLA-Bw-80Ile^{+} HIV-1-infected individuals – delayed progression to AIDS
	In case of HLA-Bw-80Ile absence KIR3DS1 is associated with more rapid progression (Martin et al. 2002a)
KIR2DS2	Development of rheumatoid vasculitis associated with 2DS2^{+} CD4^{+} CD28^{null} T cells (Namekawa et al. 2000)
KIR2DS1, KIR2DS2	Associated with psoriatic arthritis, but only when the HLA ligands for their homologous inhibitory receptors were absent (Martin et al. 2002b)
KIR2DS2	Susceptibility to develop type 1 diabetes when present with group 1 HLA-C ligands (van der Silk et al. 2003)

There is an often cited example of an NK-deficient patient that is particularly informative (Biron et al. 1989). A 13-year-old girl presented with an unusually severe chickenpox (varicella) infection. She was found to have a complete and selective lack of NK cells. Other cells of the innate immune system were normal, and so were T- and B-cell responses, which eventually resolved the infection. Within 5 years, she developed first a severe HCMV infection and then a disseminated HSV-1 infection. Siblings and relatives showed normal NK cell development and function, and the mechanism behind this immune defect remains a mystery. Such total and selective NK cell deficiency is apparently extremely rare, and one may ask why. It may be that it is usually lethal in the intrauterine or early postnatal period. Another possibility is simply that it requires a combination of genetic defects for which the probability is extremely low. For future research it may be important to identify patients with more discrete effects, e.g. patients who retain a normal or close to normal NK cell number that, however, lacks one or more receptor or effector functions.

Patients with human immunodeficiency (HIV) infection and/or acquired immune deficiency syndrome (AIDS) represent one of the most well-studied patient categories in clinical immunology laboratories. A consistent pattern in these patients is that NK cell activity is reduced (Scott-Algara and Paul 2002). This can occur relatively early in infection, and often without diminished NK cell numbers in the blood. The functional defect appears more prominent for the direct NK cytotoxicity than the ADCC activity mediated through a FcγRIII receptor. There may be an association with reduced expression levels of the activating receptors NKp30 and NKp46 (De Maria et al. 2003; Mavilio et al. 2003). NK cells may contribute to various defense mechanisms, including production of chemokines blocking co-receptors for viral infection; NK cells may also be important for control of human herpesvirus 8 (HHV-8) and Kaposi's sarcoma (Cerwenka and Lanier 2001). The Nef gene product of the virus down-regulates HLA-A and -B expression, but does not affect HLA-C and -E molecules, ligands for the inhibitory NK receptors KIR2D and NKG2A (Cohen et al. 1999; Cerwenka and Lanier 2001; Mavilio et al. 2003).

EXPERIMENTAL STUDIES

The role of NK cells has been studied in a variety of animal models, mainly in mice, and usually by depleting the NK cells before injection of a relatively high dose of pathogenic virus. Normal mice respond with increased NK cell activity, locally and systemically, to most if not all viruses 2–4 days after infection (Tay et al. 1998; Biron et al. 1999). Early cytokines, particularly type I interferons and IL-12, are important factors for this rapid augmentation of NK activity. In some virus infections, such as MCMV, Pichinde virus, HSV-1, influenza virus, Coxsackie virus, vaccinia virus, ectromelia virus, encephalomyocarditis virus, and Theiler's virus, the early NK defense is crucial. NK cell-depleted mice show dramatically increased viral replication locally or systemically (Biron et al. 1999). Depending on the infection dose and the virus, the NK cell-depleted mice may die within one week, whereas normal mice limit the replication and survive until the adaptive T- and B-cell response reaches effective activity and clears the infection. As discussed in more detail in relation to MCMV, NK cells contribute to early defense by direct killing of infected cells, as well as by secretion of chemokines and cytokines, particularly IFN-γ (Biron et al. 1999).

With other viruses, NK cell depletion has no effect on the course of the infection, even though there may be strong initial augmentation of NK cell activity. A typical example in this category is lymphocytic choriomeningitis virus (LCMV), which otherwise is extremely sensitive to immune mechanisms and cytotoxic T cells (Tay et al. 1998; Biron et al. 1999). The reasons why certain viruses are 'NK insensitive' are not known. It may relate to whether NK cells can specifically target the virus-infected cells. Antiviral NK cell-mediated defense through cytotoxicity implies that NK cells should be able to recognize the infected cells and specifically eliminate them. In many cases, virus-infected human or murine cells are more NK susceptible than non-infected controls, but this is not a general rule. For certain viruses, infected cells may even become more resistant. Viruses may affect NK sensitivity by a variety of molecular mechanisms, including reduced MHC class I expression, leading to killing by recognition of 'missing self'. There is good evidence for this in the

case of HSV-1- and vaccinia virus-infected cells (Orange et al. 2002). The pattern with HCMV and MCMV, known to possess multiple mechanisms for interference with the MHC class I pathway, is more complex, as discussed in detail below.

NK CELLS IN CMV INFECTION: MOLECULAR ASPECTS OF RECOGNITION, EFFECTOR FUNCTION, AND ESCAPE

MCMV and HCMV are the most well-studied infections with respect to NK cells, and several interesting molecular mechanisms have been revealed. These closely related viruses are therefore discussed in more detail, as an example of the complex pattern that may emerge in co-evolution of host and virus. HCMV and MCMV establish latent infections, and their large DNA-based genomes encode many proteins that are not required for replication in host cells in vitro, but act instead to modulate and interfere with host immune responses, including NK cells.

Mice with NK cell deficiency caused by low age, experimental depletion, or gene defects are highly sensitive to experimental infection with MCMV (Tay et al. 1998; Lee et al. 2002). Adoptive transfer of NK cells can restore resistance. NK cells appear to mediate their effect by at least two mechanisms: perforin-dependent cytotoxicity, which is the more important mechanism in the spleen, and IFN-γ secretion, which is crucial to limit viral replication in the liver (Biron et al. 1999; Lee et al. 2002). The cytotoxic response depends on type I interferons acting through the STAT-1 signaling pathway in NK cells, whereas the cytokine response depends on IL-12 produced by macrophages and acting through STAT-4 (Biron et al. 2002). IFN-γ can exert its effect directly on hepatocytes to limit viral replication, but may also act by stimulating macrophages for inducible nitric oxide synthase. There is thus an important crosstalk between NK cells and macrophages in the liver, emphasized also by a crucial role of the macrophage-secreted chemokine macrophage inflammatory protein 1-α (MIP-1α) for NK cell migration into the liver during infection (Lee et al. 2002).

How can NK cells target CMV-infected cells? Given that both HCMV and MCMV possess several different genes for interference with the MHC class I pathway, NK cells are important innate effector cells against infection in both species, and the particularly strong CMV resistance in the mouse strain C57Black has for a long time been shown to map to a locus within or close to the Ly49 family (CMV1), it has been speculated whether 'missing self' recognition plays an important role (Lee et al. 2002). However, C57Black mice deficient for MHC class I molecules, the NK cells of which cannot perform missing self-recognition, show a strong NK-mediated resistance to the infections. Research in recent years has instead identified the activating Ly49H receptor as the product of the CMVR1 locus (Yokoyama and Scalzo 2002). Resistance is impaired in mice treated with Ly49H antibodies, and in mice deficient in the adaptor molecule crucial for Ly49H signaling, KARAP/DAP12 (Sjolin et al. 2002; Yokoyama and Plougastel 2003). The Ly49 receptor acts by recognizing the virus-encoded, GPI membrane-attached molecule m157 on infected cells (Arase et al. 2002; Smith et al. 2002). This viral gene, which shows distant homology to MHC class I genes, is not required for virus replication in vitro, and one may wonder why and how the virus has evolved it, because it acts to limit spread of the virus. An attractive model proposes that m157 evolved as a ligand to target the inhibitory MHC class I-recognizing receptor Ly49I (Yokoyama and Scalzo 2002), thereby allowing the virus to escape missing self-recognition in spite of its interference with host cell MHC class I molecules. The Ly49H receptor would represent the evolutionary countermove of the host, resulting in the selection of a receptor gene where recombination and other mechanisms would have maintained recognition of m157, but not of host MHC class I molecules, and switched the intramembrane/cytoplasmic domains to convey activating rather than inhibiting signals.

This scenario may be difficult if not impossible to test, but it has profound implications for our understanding of infections as an evolutionary pressure for the NK cell system. MCMV carries at least half a dozen additional genes encoding distant MHC class I homologs (Yokoyama and Scalzo 2002), the function of which remain to be determined. Similarly, mice as well as humans have a number of activating MHC class I receptors in the Ly49 and KIR families with poorly understood function (see Table 2). It may be that the inhibitory MHC class I-specific receptors provide a platform for development of viral escape strategies that can easily be turned into counter-weapons in the next host, or in the next evolutionary step. The intensive research on activating KIRs, and their possible association with diseases (Martin et al. 2002a, b; Namekawa et al. 2000; van der Slik et al. 2003) (Table 5), may give important clues in this research field.

One may still ask why CMV interference with MHC class I recognition is not sufficient to induce NK cell susceptibility. This is probably a result of a variety of parallel viral mechanisms that interfere with NK cell recognition and triggering (Figure 5). MCMV and HCMV both possess genes, *UL16* and *m152* respectively, the products of which bind to and prevent cell surface expression of ligands for the activating NKG2D receptor, such as MIC, ULBP1 and -2 in humans, and Rae-1 and H-60 in mice (Cerwenka and Lanier 2001; Krmpotic et al. 2002; Orange et al. 2002; Dunn et al. 2003; Lodoen et al. 2003; Rolle et al. 2003). Even if the NKG2D receptor is not believed to be the primary activating receptor for 'missing self' surveillance of normal cells, there is evidence that the activating ligands of this

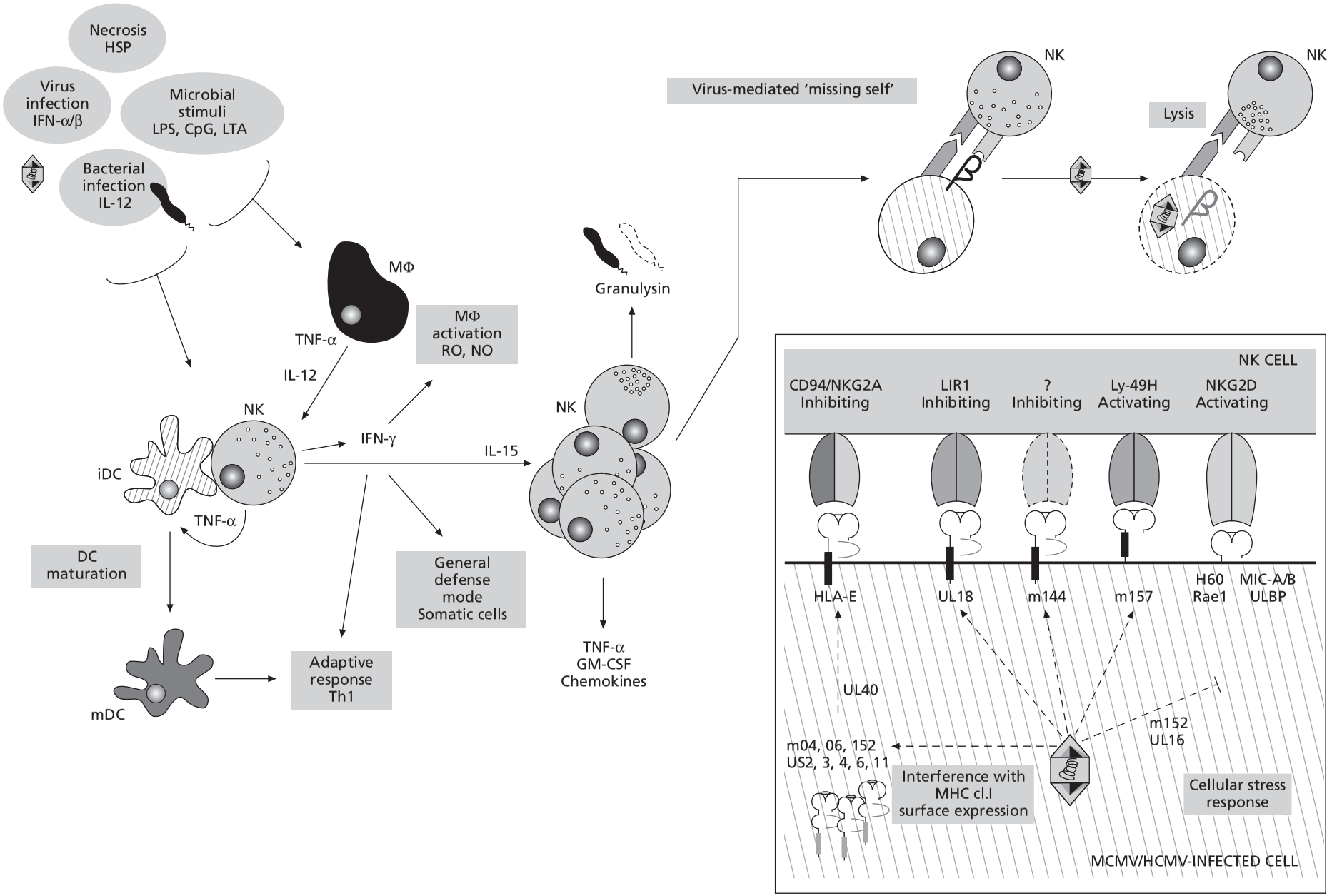
Necrosis
HSP
Virus
infection
IFN-α/β
Microbial
stimuli
LPS, CpG, LTA
Bacterial
infection
IL-12
MΦ
TNF-α
IL-12
MΦ
activation
RO, NO
NK
IFN-γ
iDC
TNF-α
IL-15
DC
maturation
General
defense
mode
Somatic cells
mDC
Adaptive
response
Th1
Granulysin
NK
TNF-α
GM-CSF
Chemokines
Virus-mediated 'missing self'
NK
Lysis
NK
NK CELL
CD94/NKG2A
Inhibiting
LIR1
Inhibiting
?
Inhibiting
Ly-49H
Activating
NKG2D
Activating
HLA-E
UL18
m144
m157
H60
Rae1
MIC-A/B
ULBP
UL40
m152
UL16
m04, 06, 152
US2, 3, 4, 6, 11
Interference with
MHC cl.I
surface expression
Cellular stress
response
MCMV/HCMV-INFECTED CELL

receptor, which are induced during HCMV infection, play a more prominent role for NK cell recognition of infected cells. This implies that the virus in parallel down-regulates the activating ligands that are important in normal cells, but, as these are not completely known, this hypothesis remains to be tested.

Furthermore, the HCMV gene *UL40* provides a signal sequence that can bind to and promote expression of HLA-E, which may counteract the reduced expression of this ligand for the inhibitory NKG2A receptor caused by HCMV products that interfere with TAP function (Braud et al. 2002; Orange et al. 2002). HCMV also contains the gene *UL18*, which binds to the LIR-1 receptor on NK, B and dendritic cells with 1000-fold higher affinity than MHC class I molecules (Chapman et al. 1999). The UL18 protein has been proposed to act as a decoy ligand that inhibits NK cells (Reyburn et al. 1997), but this is controversial and the pathophysiological role of this viral product is not clear (Orange et al. 2002).

The overall picture emerging in the case of CMV is that the NK cell defense is important, because the virus has evolved different mechanisms to avoid it. The recognition strategy of NK cells with multiple activating and inhibitory receptors has probably emerged in dynamic co-evolution with CMV and other infectious agents, and it continues to provide a complex platform for ongoing evolution where activating and inhibitory receptors and their ligands interact.

Bacterial infections

Several effector functions of NK cells contribute to antibacterial responses. Granulysin, a membrane-perturbing peptide component of human (but not mouse) NK cell granules, is directly active against gram-positive and gram-negative bacteria, including *Mycobacterium tuberculosis* (Krensky 2000). NK cells can also lyse target cells infected with bacteria, e.g. *Shigella*, *Listeria*, *Salmonella*, *Legionella*, and *Mycobacterium* spp. (Bancroft 1993; Yokoyama 1999). Finally, by secretion of IFN-γ, they can activate macrophages and induce more efficient intracellular and extracellular elimination of bacteria, e.g. through reactive oxygen metabolites and reactive nitrogen intermediates. As IFN-γ can also deviate the ensuing adaptive response toward a Th1 type, NK cells cannot contribute only to early innate resistance; they also provide a link to adaptive responses against bacteria. In this context, one may also note that NK cells interact efficiently with dendritic cells (DC) (Moretta 2002; Zitvogel 2002). Upon infection with *M. tuberculosis*, DCs develop the capacity to activate NK cells, but they also become resistant to NK cell attack. The activated NK cells can, however, kill uninfected, immature DCs. This process, which involves altered expression levels of DC ligands for the NKp30-activating receptor and NKG2A-inhibiting receptor, may be important for optimal antigen presentation to T cells.

Although depletion of NK cells in experimental infection in mice led to enhanced growth of *Mycobacterium avium* (Florido et al. 2003), there is so far no model where NK cells have an absolutely critical role in immunocompetent animals. This is presumably because numerous partly overlapping mechanisms cooperate in antibacterial resistance. In several infections, the contribution of NK cells is most clearly recognized in the absence of one or more other immune components. A well-studied example is resistance to *Listeria monocytogenes* in SCID mice (Bancroft 1993). NK cells are critical for the early limitation of bacterial spread. Under the influence of IL-12 and other cytokines produced by infected macrophages, NK cells are induced to secrete IFN-γ, which in turn activates the macrophages. Their induced antibacterial pathways can limit the spread of bacteria, but T cells are required ultimately to resolve the infection. Similar schemes can be drawn for several other intracellular bacteria, e.g. *M. tuberculosis*.

It is also uncertain whether there is a particularly prominent role for NK cells in any disease in the clinical panorama. Bacterial infections, particularly in the respiratory tract, have been reported in certain patients with defects of dysregulation of NK cells, but it cannot be excluded that these reflect secondary infections

Figure 5 *A consensus model for the role of natural killer (NK) cells in infections. During infection NK cells can be activated either directly by recognition of microbial components or indirectly by cytokines (interferon IFN-αβ, interleukin IL-12) produced from infected cells or activated macrophages (Mϕ) (Biron et al. 1999; Trinchieri 2003b). The interaction with immature dendritic cells (iDC) encountering antigens or pathogens leads to reciprocal activation, driving, on the one hand, maturation of DCs, which is crucial for induction of the adaptive immune response, and, on the other, NK cell activation (Moretta 2002). This is accompanied by strong NK cell-derived IFN-γ production, most probably IL-15-driven NK cell proliferation and ordered NK cell recruitment to the site of infection, which is orchestrated by chemokines. Although IFN-γ is crucial for induction of a variety of defense programs exerted by macrophages and also somatic cells, NK cells contribute to pathogen destruction by cytotoxic mechanisms. Missing or altered self as a consequence of pathogen invasion can be recognized by NK cells and induces killing of the infected cell. The elaborate interplay between NK cells and* murine *or* human cytomegalovirus *(MCMV and HCMV) is illustrated (Cerwenka and Lanier 2001; Braud et al. 2002; Orange et al. 2002). The virus interferes with MHCI surface expression (m04, -06, -152; US2, -3, -4, -6, -11), expresses MHCI-like surrogates (m144, m157, UL18), and interferes with surface expression (m152, UL16) of cellular 'stress'-indicative molecules (H60, Rae-1, MIC-A/B, ULBP). The viral MHCI-like m144 protein binds to an unknown inhibitory receptor, whereas the viral m157 protein is recognized by the activating Ly49H receptor, which significantly contributes to resistance against MCMV in certain mouse strains (Arase et al. 2002; Smith et al. 2002). Cells expressing 'stress'-indicative molecules (*altered SELF*) are recognized via the activating receptor NKG2D (Raulet 2003).*

complicating an initial viral disease (Orange et al. 2002). The lack of evidence for an essential role of NK cells or any other innate component in an infection does not mean that these are unimportant. There are large individual variations in the severity of initial disease developing before the sterilizing adaptive responses step in. Future research in bacterial diseases will address this if such patterns can be correlated to selective defects of, for example, NK receptor pathways.

Upon bacterial infection, NK cells may not only become activated indirectly via cytokines secreted by macrophages and DCs. Cognate interactions involving the sensing of reduced MHC class I expression of infected macrophages may occur with *Salmonella*, *Yersinia*, and *Chlamydia* spp. (Kirveskari et al. 1999). Through the Toll-like receptor 9, NK cells can be directly activated by unmethylated CpG sequences in bacterial DNA (Ashkar and Rosenthal 2002). There is also evidence that human NK cells can use the activating NKp46 receptor to directly recognize and kill *M. tuberculosis*-infected monocytes, without concurrent down-regulation of MHC class I molecules (Vankayalapati et al. 2002). The future research on NK cells may well identify several direct molecular recognition pathways for bacteria or bacteria-infected cells.

PARASITE INFECTIONS

The parasites represent a very heterogeneous group of organisms. Nevertheless, common patterns emerge with respect to the role of NK cells in infections, some resembling those seen with NK responses to intracellular bacteria (Figure 5). Infected human or murine hosts often show an early augmentation of NK cell activity, e.g. in toxoplasma, leishmania, and trypanosoma infection (Scott and Trinchieri 1995). This activation is partly indirect via cytokines such as IL-12 and TNF-α secreted by infected macrophages. In addition, there is evidence for direct recognition of parasites, their products or infected cells. Human NK cells can be stimulated by the leishmania lipophosphoglycan via Toll-like receptor 2 (Becker et al. 2003), and they can also recognize *Plasmodium falciparum*-infected erythrocytes by a so far unknown receptor (Artavanis-Tsakonas and Riley 2002). Apart from activation, there may also be local recruitment of NK cells; these represent the second wave of infiltrating cells (after granulocytes) at the site of leishmania infection in mice.

As a consequence of activation, NK cells can secrete cytokines to stimulate macrophage-mediated elimination of intracellular and extracellular parasites. Leishmania antigens can also induce a proliferative response of NK cells in vitro. Furthermore, activated NK cells can destroy infected host cells as well as extracellular parasites, e.g. tachyzoites of *Toxoplasma gondii* and trypomastigotes in Chagas' disease. As with many intracellular bacteria, IFN-γ appears to be a key cytokine (Cardillo et al. 1996), even if clear evidence for the in vivo role of NK cells may require models where other components are defective. Infection of mice with *T. gondii* may serve as an example. In β_2-microglobulin-deficient mice, with defective T-cell responses, there is a strong proliferative response of NK cells (Denkers et al. 1993). These eventually become the dominating cell population in the spleen and secrete high levels of IFN-γ, acting to limit the infection. IL-15 may play a role for this expansion of the NK cell population. In immunocompetent mice, NK cells contribute to the IFN-γ response, as do $CD8^+$ T cells, which are required to clear the infection.

As with other microorganisms, one focus of future research will be the identification of parasite structures that can be directly recognized by activating (or inhibiting) receptors of NK cells. Parasites represent a particular challenge in such studies, given their complex life cycles, with extracellular and intracellular stages, and in several cases also mechanisms for antigenic variation.

NK CELLS IN CANCER

Natural killer cells were discovered by virtue of their capacity to kill cancer cells. Furthermore, it was the correlation between strong NK activity and resistance to tumor transplants in mice that stimulated further interest in the field, at the early stage when this cytotoxic activity was frequently questioned as an in vitro artifact. There is now overwhelming evidence from animal models that NK cells can reject transplantable tumors of various histological types (Smyth et al. 2002). As expected from an innate nonadaptive mechanism, NK cell effects operate early, within hours to days of tumor inoculation, and they can be overruled by increasing the dose of transplanted tumor cells (Riccardi et al. 1979; Kärre et al. 1986; Smyth et al. 2002).

In some situations, the NK resistance is particularly efficient. These include host tumor combinations allowing 'missing self' recognition of the tumor, e.g. a F1 hybrid or transgenic host expressing MHC class I molecules that are not present in the tumor (Kiessling et al. 1975c) (discussed further below), or grafts of tumor cells with impaired MHC class I expression (Kärre et al. 1986; Smyth et al. 2002). However, efficient rejection can take place with, as well as without, alterations of the MHC phenotype of the tumor, e.g. the murine lymphoma RMA is not efficiently rejected by NK cells in the autologous C57Black strain. Strong rejection is, however, seen with sublines of the lymphoma such as RMA-S, selected for reduced MHC class I expression, as well as with sublines with normal MHC class I expression, but transfected with Rae-1, the ligand for the activating NKG2D receptor (Kärre et al. 1986; Raulet 2003). Other tumor lines are rejected because they

express Rae-1 constitutively. Furthermore, NK-mediated resistance is most efficient in models assessing hematogenous metastasis, usually to the lungs (Hanna 1982; Smyth et al. 2002). This correlates with the rapid NK cell-mediated elimination that can be observed in the lungs in short-term in vivo assays with isotope- or fluorescence-labeled tumor cells. Rapid, perforin-dependent cytotoxicity is thus a major, but not the only, mechanism in tumor cell rejection by NK cells. In its absence, Fas ligand-dependent killing can be observed, even though it is slower and appears less efficient in terms of the number of tumor cells that can be cleared (Screpanti et al. 2001; Wallin et al. 2003). It has also been proposed that NK cells can interact in concert with adaptive response mechanisms in tumor immunity. One obvious example is ADCC. NK cells can exert antitumor effects via this mechanism, at least in situations where monoclonal antibodies are used for treatment (Ravetch and Bolland 2001; Smyth et al. 2002). Another possibility is that early NK cell killing of tumor cells can promote antigen uptake and presentation by DCs, which will lead to more efficient T-cell responses.

There is limited information on the capacity of NK cells to eliminate primary tumor cells in the autochthonous host, as a result of the lack of good experimental models. Transgenic mice developing certain types of tumors at high frequency may become a useful tool here. It is clear that normal NK cell-defective mice to not develop spontaneous tumors in dramatic excess, arguing against classic immunosurveillance as the major function of NK cells. This does not exclude that they may be exploited for cancer therapy. Clinical studies may provide more answers in this context. The NK activity of cancer patients is often weakened. This observation is, however, difficult to interpret – it may simply reflect a consequence of advanced disease.

Adoptive transfer of NK cells was an inherent part of clinical trials with LAK cells performed during the 1980s. These were based on activation and expansion of lymphocytes from cancer patients in vitro, in medium containing relatively high doses of IL-2 (Rosenberg 1988; Smyth et al. 2002). Reinfusion of the cells led to documented shrinkage of tumor deposits in a minor part of the patients, and anecdotal dramatic responses. The available evidence suggests that NK cells were the dominant effector cells in the heterogeneous LAK cell populations, in the human as well as in the murine models providing the platform for this therapy (Whiteside et al. 1998; Wu and Lanier 2003). The therapy was abandoned as a result of limited efficacy, considerable safety problems with severe side effects often requiring intensive care, and development of novel cytokine modalities. However, these efforts demonstrated that activated, expanded, and reinfused NK cells could exert some antitumoral effects in a clinical setting, an important milestone. This has led to several protocols to activate and/or transfer NK cells for cancer therapy. In today's perspective, it is clear that many protocols are based on quite nonspecific stimulation of the NK system, with little attention being paid to specificity and tumor phenotype. In the future, it is reasonable to expect new modalities based on advances in understanding the molecular aspects of NK recognition, e.g. it has recently been reported that colon cancer cells may not only express the NKG2D ligand MICA, but may also shed this molecule in the circulation, where it can block recognition as well as down-modulate the activating NKG2D receptors (Salih et al. 2002). In future studies it may be more critical to assess that the tumor and NK cells in trials express the correct receptor–ligand pair, and take action to diagnose and remove soluble blocking factors. Another example where (mis)matching for missing self-recognition can be used to obtain antitumor effects is discussed below.

NK CELLS IN TRANSPLANTATION

The first thorough studies of an in vivo phenomenon associated with NK cells, at least with respect to genetics, was initiated more than 10 years before the discovery of the NK cells themselves. In the 1960s, it was recognized that lethally irradiated mice, whose adaptive T- and B-cell responses had been abrogated, could still reject MHC-mismatched bone marrow grafts. The mismatch combinations included not only completely allogeneic grafts (A/A rejecting B/B) but also parental grafts into F1 hybrid hosts (A/B rejecting B/B). The latter combination should not lead to T-cell-mediated rejection according to the classic transplantation laws, and this type of rejection was coined as 'hybrid resistance' (Cudkowicz and Bennett 1971). It was also demonstrated against tumor grafts and, in the rat, against grafts of normal lymphocytes. On the discovery of NK cells, it was soon demonstrated that they are the effector cells behind this peculiar type of rejection (Kiessling et al. 1977; Barao and Murphy 2003).

Natural killer cell-mediated rejection of allogeneic and parental grafts is controlled by the MHC region, but it was initially believed that MHC class I molecules were not involved. One major model proposed a new type of recessive, MHC-linked hemopoietic histocompatibility (Hh) locus (Yu et al. 1992). Today, the phenomena are interpreted within the paradigm of MHC class I receptors on NK cells, and missing self-recognition in particular: a graft of MHC type B/B lacks A alleles expressed by an allogeneic A/A as well as an F1 A/B host. NK cells with an inhibitory receptor for A may therefore not be inhibited by the B/B graft, leading to rejection (Kärre 1985; Kärre et al. 1986; Barao and Murphy 2003). Strong evidence for this has been provided by studies of mice made transgenic for a new MHC class I allele (the NK cells of which reject bone marrow from wild-type cells) and mice with deficient MHC class I expression (the bone marrow of which is rejected by NK cells from

wild-type mice) (Ohlen et al. 1989; Bix et al. 1991). However, missing self-recognition does not dominate in all combinations; NK cells can also use activating MHC class I-recognizing receptors to recognize an allogeneic graft, and this is the dominant mechanism in certain combinations (Nakamura et al. 1999; Smyth et al. 2002). The role of NK cells in rejection of organ grafts, where rejection is observed only in allogeneic, and not F1 anti-parental, combinations is less clear. They can infiltrate transplanted kidneys and hearts, and it has been proposed that they may attack allogeneic cells; alternatively they may regulate the T-cell responses that are initiated.

It is not clear whether recipient NK cells can affect the outcome of hematopoietic transplantation in the human. However, the role of NK cells in the grafted marrow or stem cells is now the focus of intensive studies. This started with a retrospective analysis of the outcome of so-called haploidentical transplantations between siblings or between parents and children, where one MHC haplotype is shared and the other not (A/B to B/C). One set of HLA-B- and -C-encoded ligands for inhibiting KIRs are thus matched, but the remaining ones may result in a combination where the graft carries an inhibitory KIR ligand that is absent from the host. The murine studies predict that this should lead to NK recognition in the graft-versus-host direction. A subgroup of transplantations representing this combination did indeed show fewer relapses of myeloid leukemia (compared with groups with complete match or KIR ligands present in the recipients but absent from the donor) (Farag et al. 2002; Ruggeri et al. 2002). Parallel studies of human NK cells in vitro or transferred to mice with human leukemia supported the role of an NK-mediated graft-versus-leukemia effect. Surprisingly, there was a reduced incidence of graft-versus-host disease in the same combinations. Experimental studies indicate that this may have been caused by efficient NK cell removal of host DCs, which initiate graft-versus-host reactivity. Follow-up studies, where the same type of mismatches occur in transplantations between unrelated individuals, have given divergent results, possibly as a result of the different recipient conditioning and graft T-cell depletion protocols involved (Davies et al. 2002; Giebel et al. 2003). The role of allogeneic, and particularly KIR-ligand-mismatched, NK cells will undoubtedly be addressed in many different clinical settings in the future, including the use of allogeneic donor lymphocyte infusions and minitransplantations for the treatment of solid tumors (Farag et al. 2002).

NK CELLS IN PREGNANCY

In immunological terms, the fetus can be regarded as an allograft. One of its HLA haplotypes is from the father, and thus foreign to the mother's immune system. The fetal–maternal interface in the decidua must provide tolerance and allow trophoblast invasion, but not beyond certain limits; defense against infections must also be provided. Cells of the NK lineage are the dominating lymphocytes in the decidua, and they may play an important role in one or several of these processes (Moffett-King 2002). The uterine NK (uNK) cells are of a particular phenotype, resembling the minor $CD56^{bright}$ subset in the blood. Their number in the endometrium starts to increase already during the post-ovulatory, secretory phase of the menstrual cycle. The uNK cells normally die as menstruation starts but, if an implantation occurs, they continue to expand and dominate the deciduas until midgestation, when placentation is complete. They then gradually disappear. The expansion of the population may be driven by IL-15 and prolactin, produced by stromal cells under the influence of the pregnancy hormone progesterone (Moffett-King et al. 2002). There are very few T cells in the decidua and no B cells.

The functional role of uNK cells is not clear. It is, however, intriguing that the MHC class I molecules expressed by the extravillous trophoblast in the decidua are HLA-E, HLA-G, and HLA-C, all of which can be recognized by different NK receptors (Moffett-King 2002). The uNK cells all express the inhibitory receptor NKG2A (recognizing HLA-E), like the $CD56^{bright}$ cells in the blood. However, the uNK cells differ from the latter in that a high proportion also expresses KIR, especially the ones recognizing HLA-C alleles. It has been proposed that inhibitory receptors prevent uNK cell lysis of trophoblast, but antibody-blocking studies in vitro do not support this. An alternate possibility is that the receptors regulate cytokine production. The uNK cells can produce the same cytokines as blood NK cells, and the invading trophoblast cells have receptors for many of these (e.g. IFN-γ, CSF1, and TNF). In addition, uNK cells produce several factors involved in angiogenesis and vascular stability. One hypothesis is that NK cells regulate trophoblast invasion and a process intimately connected to this, the transformation of maternal blood vessels in the decidua (Moffett-King 2002; Moffett-King et al. 2002). This is required for adequate fetal blood supply. Dysregulation of this process may lead to the condition preeclampsia, with risk for growth retardation of the fetus or still birth.

There are major differences in the anatomy of placentation between species, and it is therefore difficult to extrapolate observations from murine models to humans. There are some common factors and some intriguing observations from studies in mice (Croy et al. 2003). NK cells also represent the dominating lymphocyte in the mouse decidua, and they appear to be recruited from secondary lymphoid tissues during pregnancy. Mice lacking NK (and uNK) cells breed, and these cells are therefore not essential for implantation, decidualization, and completion of pregnancy. However, in the absence of uNK cells, pregnant mice show

several decidual abnormalities at midgestation, such as thick-walled decidual spiral arteries with narrow lumen diameters and a dramatic acellularity of the decidua (Moffett-King 2002; Croy et al. 2003). Studies of gene defective mice have shown that uNK cell-produced IFN-γ may be a central cytokine for vascular modifications (dilatation, elongation, and branching) of spiral arteries.

In conclusion, studies from humans and mice indicate that uNK cells play a role in regulating the trophoblast invasion and vascular transformation in the decidua. Although they are not absolutely required under normal conditions, they may be critical in pregnancies complicated by other factors.

NK CELLS IN AUTOIMMUNE DISEASES

Natural killer cell numbers and functions are often altered in patients with autoimmune disease. As in cancer and infection, such findings are not easy to interpret, because they may represent secondary alterations resulting from, for example, metabolic or other disturbances in the disease. Infiltration of autoimmune target organs by NK cells has been observed in, for example, pancreatic islets in diabetes and the synovial fluid in rheumatoid arthritis (French and Yokoyama 2004). In the latter case, all the infiltrating NK cells are of the $CD56^{bright}CD16^{low}$ cytokine-producing phenotype, which is rare in the blood. Their role in the pathogenesis of rheumatoid arthritis is unclear.

There are two rare clinical conditions where a role for 'missing self' recognition by NK cells has been proposed as responsible for autoreactivity. In the 'bare lymphocyte syndrome', there is complete lack or severely impaired MHC class I expression on cells. This can be caused by different gene defects, one of which affects the so-called TAP. When TAP expression is impaired, the HLA molecules become unstable as a result of deficient loading of peptides in the ER. A subgroup of patients with TAP deficiency suffers from necrotizing granulomatous lesions in skin and lungs, characterized by infiltration of NK cells and T cells, and leading to clinical manifestations in these organs (Zimmer et al. 1998; Moins-Teisserenc et al. 1999). There is indirect evidence that these lesions are caused by autodestructive NK cells that can recognize the 'missing self' phenotype of cells in the target tissue. There must be additional factors required for triggering of this condition, because it does not develop in all patients with the bare lymphocyte syndrome; even patient's siblings who have inherited the TAP deficiency may not develop this pattern. Most MHC class I-deficient patients thus acquire NK cell tolerance toward their own cells, just as mice with the corresponding defects. One possibility is that certain infectious agents can break this tolerance, which could explain why only certain TAP-deficient individuals are affected.

It has also been proposed that the red cell aplasia associated with benign or malignant LGL expansions may be caused by 'missing self' recognition of erythroblasts (with poor MHC class I expression) by the KIRs expressing LGLs (Lamy and Loughran 2003). Such LGL expansions may consist of T cells (αβor γδ) or NK cells, but both populations can express KIRs.

Several experimental studies have addressed the role of NK cells in animal models of autoimmunity by depleting them just before the induction or during progression of disease. Although corresponding studies in cancer, infection, and transplantation in most cases show impaired reactivity against the target or in some cases no effect, but never evidence for increased reactivity, any of the three outcomes can be observed in autoimmune models (Baxter and Smyth 2002; Flodstrom et al. 2002; French and Yokoyama 2004). There is as yet no clear-cut pattern with respect to type or localization of disease – opposite effects of NK cell depletion have even been reported in different studies of the same disease model (experimental allergic encephalomyelitis). These divergent observations are not surprising, given the complexity in the responses leading to autoimmune manifestations. The effect may thus depend on whether NK cells have a role early in induction or deviation of adaptive responses, or late as effector cells in target tissues, e.g. in a model of myasthenia gravis, NK cells promoted disease by cytokines, which skewed the response toward Th1 and production of autoantibodies to the acetylcholine receptor (Shi et al. 2000).The most consistent results have been observed in diabetes models, where NK cells in three of three cases seemed to promote disease (Flodstrom et al. 2002). The mechanisms behind this are not known, although there is evidence that NK cells can kill pancreatic β cells in vitro, suggesting a direct role as effector cells. NK cells can kill several additional normal cell types in vitro, e.g. oligodendrocytes and neurons.

In conclusion, NK cells may contribute to or prevent autoimmune disease at several levels, including cytokine-mediated stimulation, suppression or deviation of adaptive responses, and cytokine- or perforin-dependent destruction of cells in target organs. One important focus of future research is to reveal the receptors and ligands involved.

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Pattern recognition

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BRIEF HISTORIC INTRODUCTION

'Putrid poison', 'sepsin', and the birth of microbiology

After a long time of devastating infectious diseases, limiting the average life-time expectancy of the population worldwide until the late nineteenth century, several key findings by research pioneers in Europe not more than 120 years ago led the way to the understanding of basic principles of infection, and to the development of successful prevention and treatment strategies over the last decade. Even before the discovery of living microorganisms as the cause of infectious diseases inaugurating the discipline of microbiology, it was postulated by Panum that a 'putrid poison' was responsible for the lethal outcome of an infection (Panum 1874). Ernst von Bergmann of the Charité, Berlin in the late nineteenth century believed that a chemically defined substance was responsible for fatal infections, which he termed 'sepsin' (Bergmann and Schmiedeberg 1868).

Of course it was Louis Pasteur and Robert Koch in their ground-breaking discoveries who revealed that microorganisms are the cause of infectious diseases and they founded the new discipline of microbiology (reviewed in: Dubos 1995; Brock 1999). Although especially Koch in Berlin isolated several important bacteria presenting proof that they were the cause of disease, it still for a long time remained unclear how a given pathogen would cause a specific disease and it took a long time to define both the 'toxic' elements of microorganisms and the host's reaction patterns involved in the disease (reviewed in Beutler and Rietschel 2003).

Host–pathogen interaction as cause of many diseases of importance

Introduction of hygiene measures preventing spread of infectious diseases and the development of powerful antibiotics during the twentieth century reduced the lethality of infectious diseases in the industrial world dramatically. However, even today, certain diseases caused by microorganisms remain a clinical problem despite the highest hygiene standards and broad-spectrum antibiotics: sepsis and septic shock caused by microorganisms are still a major threat in intensive care units and statistically sepsis is a major killer in the western world (Hotchkiss and Karl 2003). Apparently, here the host reacts early to pathogens and induces a response, unfortunately not leading to limitation of the disease but to a critical state.

On the other hand, chronic inflammatory reactions account for a large number of diseases that are also particularly difficult to treat, and that lead to major

financial healthcare burdens. It is very likely that microorganisms persisting within the host account for many of these diseases, although they are not directly treatable by antibiotics (Relman 2002). Other important clinical syndromes characterized by a distinct host reaction include different forms of toxic shock syndrome, chronic parasitic diseases, or virally caused cancers, to name just a few.

Molecular mechanisms of pathogen recognition

The two reaction patterns as examples mentioned above of septic shock, on the one hand, and of chronic inflammation, on the other, depict the wide spectrum of host–pathogen interactions that can take place during an infection, and for a long time the following questions were asked:

- How is this interaction of the pathogen and the host determined?
- What molecules are involved on both sides?
- Can this reaction pattern be influenced?
- Could this be the basis for a therapy for infectious or inflammatory diseases?

Only recently, key molecular elements of the host detection system of innate immune cells were discovered with the family of Toll-like receptors (TLR), which are discussed in detail in this chapter. Furthermore, soluble molecules with the ability to detect pathogens and intracellular molecular elements of the innate immune system have been discovered, which are also outlined.

Microorganisms and their hosts – the 'battle of the genomes'

The last 120 years of microbiology since Koch have also led to the discovery of a large multitude of microorganisms displaying a wide variety of genomic organization and phenotypic appearance. Even though just a small portion of the existing microorganisms is pathogenic for humans, there is still an extremely wide variety of potentially pathogenic microorganisms. The human host, on the other hand, possesses a much larger genome compared with the microbes; however, as was revealed recently, it is smaller than expected with limited variation even between humans and other mammals. As a consequence any host organism must be overwhelmed by the task of recognizing the huge variety of potentially pathogenic microorganisms.

It has thus been postulated, and confirmed by research, lately, that the host's recognition system searches for molecular *patterns* present on distinct families of pathogens. The host apparently has sensors for these 'pathogen-associated molecular patterns (PAMP)' described in the next section and this interaction is a key step in pathogenesis of related diseases. The following section describes how the host, with the help of soluble and cellular recognition molecules, senses foreign material or dangerous situations in order to mount an appropriate response.

MICROBIAL TOXINS

Lipopolysaccharide as paradigm for pathogen-associated molecular patterns

It took several more years until the first theories on 'toxins', postulated in the pre-microbiological era by Panum and von Bergmann, were substantiated by direct evidence for the existence of a microbial substance causing the host's immune response. It was Pfeiffer who, for the first time, showed that even in the absence of live bacteria a toxic potential was present originating from bacteria and not being a protein (reviewed in Rietschel and Cavaillon 2002). This work later led to the discovery of endotoxin or lipopolysaccharide (LPS), now viewed as a classic PAMP inducing a strong activation of the innate immune system via defined molecular structures as outlined in detail below (Rietschel et al. 1996; Raetz and Whitfield 2002).

Lipopolysaccharide has a long history of excellent, thorough chemical analysis, and synthetic LPS was shown to elicit similar responses to isolated bacterial LPS in host cells. With the discovery of lipopolysaccharide-binding protein (LBP) and CD14, two of the first 'pattern-recognition receptors (PRR)' were discovered, 20 years ago, and functionally characterized in 1990 (Schumann et al. 1990; Wright et al. 1990). Further biomedical research on LPS also recently led to the discovery and functional analysis of the important PRR family of TLRs (reviewed in Beutler 2003).

A large variety of PAMPs on pathogenic microorganisms

Meanwhile, numerous pathogenic bacteria have been studied extensively, and have been analyzed chemically and genetically. Both secreted toxins, and elements of their cell walls have been well characterized and shown to bear the potential to induce innate immune responses. In addition, it has been shown that bacterial and viral nucleic acids, i.e. GC-rich oligonucleotides, can be sensed by the host's innate immune system leading to cellular activation (reviewed in Wagner 2002). These bacterial elements have furthermore been shown to be important co-stimulatory factors for inducing the adaptive immune responses, and thus are key elements as adjuvants for immunization.

MOLECULAR PATTERNS OF PATHOGENS

Are the patterns really known?

Until now it is still quite puzzling that, although certain patterns, such as LPS, present in all gram-negative bacteria, are characterized and well defined, other potentially immunostimulatory ligands of microbial source with the ability to activate immune cells are quite diverse, and seem to include such structurally distinct molecules as proteins, nucleic acids, and simple carbohydrates. Although this is still the focus of research, some molecular patterns, such as lipid structures, have been identified and are discussed at the end of this chapter.

The process of pattern recognition may also be very likely to change over time when microorganisms and the host live in symbiosis. Furthermore, selective pressure caused by antimicrobial agents may lead to rapid changes in the gene expression pattern of the pathogens and, as a consequence, to a shift in PAMP expression. Evasion and escape mechanisms of the microorganisms may lead to additional variations in patterns expressed on the surface, yet the host, in order to survive, still has to retain the ability to detect them. Novel gene array techniques may aid in analyzing this delicate balance of genetic adaptation between host and pathogen (Whitney et al. 2003). Furthermore, it has become clear that redundant pattern-recognition systems exist in order to ensure survival of the host in a changing environment.

Is pattern recognition part of the problem or part of the solution?

Despite the large success of recent research regarding both the host's and the pathogen's molecular 'arsenal', it is still not clear whether PAMPs are elements that are instrumental for the microbe to attack the host and improve survival of the pathogen, or whether the host's detection of these molecules is a key defense mechanism helping to fight off the microbes. Inflammation may be part of a successful defense reaction, and at the same time also contribute to the pathophysiology of disease. Recent results with LBP knock-out mice point in the direction that pattern recognition by the host is needed in order to combat live pathogens successfully (Jack et al. 1997; Wurfel et al. 1997; Heinrich et al. 2001; Fierer et al. 2002). Mice lacking certain PRRs were found to be unresponsive to isolated PAMPs and exhibited a worsened outcome when inoculated with whole bacteria (summarized in Werling and Jungi 2003).

Lately, studies investigating frequent single nucleotide polymorphisms (SNP) in genes encoding for TLRs in humans have been helpful for analyzing the role of these PRRs in health and disease (Lazarus et al. 2002). Analyses on the correlation of these SNPs with susceptibility to infectious diseases, discussed in detail in the final section of this chapter, are, however, controversial: although some reports show increased disease susceptibility when a PRR is genetically defective, one recent study revealed a protective effect of a TLR-4 SNP; these results were, however, obtained not for an acute infectious disease, but for an inflammatory disease, atherosclerosis (Kiechl et al. 2002).

Commensals, pathogens, and chronic inflammation

Recent research has focused on questions about the potentially beneficial role of commensal bacteria and the role of their host interaction – or lack thereof – for health and disease. Every human carries in the epigastrium more bacterial than human cells and this physiological flora clearly has important physiological functions. Why these commensals, despite carrying many of the known PAMPs, do not induce inflammatory responses is currently not known, but may be caused by a different arsenal or expression pattern of PRRs in the epigastrium (Melmed et al. 2003).

An overreaction to harmless commensals, on the other hand, has been suggested to be pathogenic in certain chronic inflammatory diseases of the gastrointestinal tract. The delicate balance between a strong defense reaction followed by the elimination of microorganisms and a 'silent symbiosis', based on the lack of early detection and interaction, most probably plays a key role in chronic inflammatory diseases in general (Kanai et al. 2002). These questions are not discussed in detail in this chapter because of a lack of space. They are closely related to topics of 'molecular mimicry' of microorganisms and the subject of autoimmune diseases, which are broadly covered in other chapters of this volume.

INNATE IMMUNITY

Adaptive and innate immunity

The discovery of the complex cellular system of the adaptive immune system during the twentieth century led to the broad application of one of the most important and successful concepts in modern medicine – immunization. The adaptive defense system, which is extensively covered in other parts of this volume, as we now know, needs repeated encounters of the host with the pathogen, and it cannot account for the rapid and potentially dramatic early reactions seen during acute infectious diseases.

In 1884, almost simultaneous with the groundbreaking discoveries of Koch and Pasteur described above, Metschnikoff discovered and described the phenomenon that certain cells within the host, which he

called 'phagocytes', are able to detect and destroy foreign structures (Metschnikoff 1844). He also recognized that phagocytes of animals immunized against *Bacillus anthracis* were able to clear bacteria faster compared with their non-immune counterparts.

With these observations, Metschnikoff founded the basic principle of innate immunity, a defense system within the host that rapidly detects foreign microorganisms, kills them, and, if necessary, evokes further adaptive mechanisms against them. Meanwhile we know that, in addition to macrophages, granulocytes and dendritic cells (DC) also contribute to this part of the immune system, as discussed in detail below.

Self, nonself, and danger

To recognize invading organisms, innate immune cells need receptors that are able to distinguish self from nonself. These receptors should be germ-line encoded in order to allow for a rapid response, i.e. within hours, in contrast to the more time-consuming processes of adaptive immunity. Furthermore, these receptors should recognize common patterns not present within the host, which are vital for the invading organisms, and therefore cannot easily be switched off. The innate immune system then activates and controls the adaptive immunity bridging these two systems (Medzhitov and Janeway 1997).

As mentioned above, these patterns are now commonly referred to as PAMPs, but it should be noted that they are expressed not only by pathogens, but also by commensals. The so-called PRRs are able to recognize a wide array of different PAMPs, including proteins, polysaccharides, glycolipids, lipids, and nucleic acid, as discussed below. Notably, some PRRs also detect endogenous ligands. These findings may be explained by the recently proposed 'danger model' (Matzinger 2002), which, in contrast to the 'self–nonself' model explained before, states that PRRs not only recognize structures of invading organisms, but also recognize molecules associated with dangerous circumstances in common, including, for example, breakdown products of necrotic cells (Figure 1). Recent studies confirm this hypothesis by showing that only structures released by necrotic (or 'attacked') cells, but not apoptotic (or 'selfinduced death') cells, share this feature (Sauter et al. 2000).

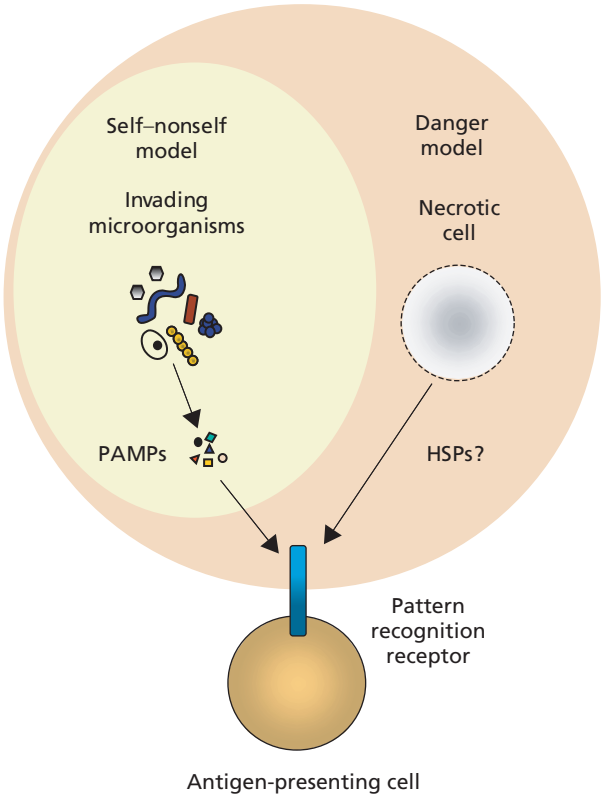

Figure 1 *General principles of pattern recognition by antigen-presenting cells (APC). APCs such as macrophages or dendritic cells (DC) play a key role in early innate immunity. They recognize molecular patterns associated with infection or danger via pattern recognition receptors (PRR). According to the self–nonself model, PRRs exclusively recognize pathogen-associated molecular patterns (PAMP), followed by the initiation of immune responses. The proposed danger model suggests that, in addition to PAMPs, endogenous molecular patterns associated with infection or trauma, e.g. partial structures of necrotic cells or released heat shock proteins (HSP), bind to PRRs, initiating an immune response.*

CELLS OF THE INNATE IMMUNE SYSTEM

Antigen-presenting cells

Cells participating in innate immune recognition share the ability of detecting invading microorganisms via PRRs, followed by phagocytosis and digestion. Next, partial structures are shuttled to the outside of the cell in order to present them to other immune cells via major histocompatibility complex (MHC) class I and II molecules (Thery and Amigorena 2001). The complex process of antigen presentation is described in detail in other chapters of this volume. Immune cells sharing this feature are commonly referred to as antigen-presenting cells (APC).

Antigen-presenting cells include granulocytes, monocytes, and macrophages, as well as DCs (Underhill et al. 1999a). As a result of their capacity to present antigen and thus to orchestrate the induction of the adaptive immune response, DCs are currently studied extensively in almost all fields of immunology, including tumor immunology (Banchereau and Steinman 1998). To give an overview of these cells centrally involved in pattern recognition and innate immunity, Figure 2 shows how hemopoietic stem cells differentiate early on into common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP).

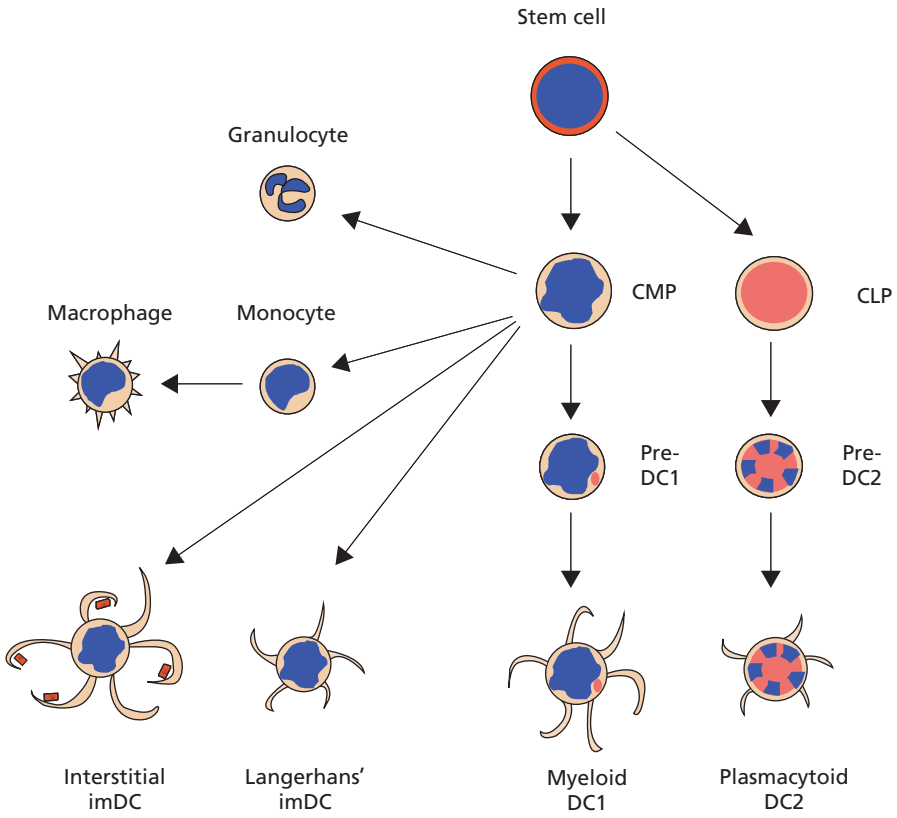

Figure 2 *Overview on the hemopoiesis of antigen-presenting cells. Hemopoietic stem cells differentiate into common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP). CMPs give rise to granulocytes and monocytes, which are able to differentiate into tissue macrophages. CMPs can also differentiate into immature dendritic cells (imDC). The imDCs found in the skin are referred to as Langerhans' imDCs, whereas those migrating to the dermis and other tissues are called interstitial imDCs. These are, in contrast to the Langerhans' cells, capable of large-scale phagocytosis and produce interleukin 10 (IL-10), leading to activation of T and B lymphocytes. CMPs are furthermore progenitors of the so-called premature myeloid or DC type 1 cells (pre-DC1), which, similar to their immature interstitial counterparts, are capable of phagocytosis. Premature plasmacytoid or DC type 2 (pre-DC2) cells, in contrast, are unable to phagocytose microorganisms; instead they are producers of the type 1 interferons, i.e. interferon-α and -β.*

Hemopoiesis of dendritic cells

Different classes of DCs have been described and are found in specific tissues of the host: CMPs differentiate into granulocytes, monocytes, and $CD34^+$ late progenitor cells. The last include two subsets: CLA^+ and CLA^- cells. CLA^+ cells differentiate into $CD11c^+CD1a^+$ Langerhans' cell precursors, which migrate into the skin and develop into Langerhans' immature DCs (imDC). CLA^- cells differentiate into $CD11c^+CD1a^-$ interstitial DC precursors, which travel to the dermis, becoming interstitial dendtritic cells. This subset of imDCs is capable of ingesting large quantities of microbial material and initiating IgM production by B cells. CMPs also differentiate into pre-DCs type 1 or monocytic pre-DCs, whereas CLPs differentiate into pre-DCs type 2 or plasmacytoid DCs. Pre-DCs type 1 express antigens related to monocytes, including CD14, CD11, CD13, and CD33 (Liu et al. 2001), whereas pre-DCs type 2 bear markers related to lymphocytes, such as pre-T-antigen and Spi-B (Spits et al. 2000; Bendriss-Vermare et al. 2001). In general, pre-DCs type 1 are capable of phagocytosis of microorganisms, whereas pre-DCs type 2 synthesize large amounts of class 1 interferons, with implications for immune responses to viral infections, as discussed in detail below (Kadowaki and Liu 2002).

PATTERN RECOGNITION RECEPTORS

Basic characteristics

A growing number of PRRs have been identified and are summarized in Table 1. Generally two classes can be distinguished: one binds microbial structures leading to detoxification and inhibition of cellular responses. The other group is involved in the induction of signal transduction and the stimulation of responses of the innate immune system. Cellular PRRs are generally found within the membrane of any host cell; however, they are predominantly found in APCs. Furthermore, intracellular forms of PRRs have been described with the function of binding microbial structures after phagocytosis of microorganisms. Finally, soluble PRRs, with

Table 1 *Overview on pattern recognition receptors*

Receptor	Origin	Ligands	Function
Cell surface			
Type 3 complement receptor	Monocytes/macrophages	Complement opsonized particles	Phagocytosis
Scavenger receptors	Macrophages	Anionic polymers	Clearance/phagocytosis
C-type lectins	Macrophages	Carbohydrates with mannose residues	Phagocytosis
CD14	Monocytes/macrophages	LPS, LTA, lipoproteins, peptidoglycan	Cytokine induction
Toll-like receptors	Various, including monocytes/ macrophages, dendritic cells, endothelia	Various, including compounds derived from bacteria, virus, fungi, and protozoa	Cytokine induction
Intracellular			
BPI	Neutrophilic granulocytes	LPS	Killing of engulfed gram-negative bacteria
nod1/nod2	Various, including monocytes/ macrophages and epithelia	Peptidoglycan	Innate immune responses
Soluble			
LPS-binding protein	Acute phase protein synthesized in the liver	LPS, LTA	Cytokine induction
CRP	Acute phase protein synthesized in the liver	Phosphorylcholine	Activation of the classic complement pathway
Soluble CD14	Monocytes/macrophages	LPS, LTA, lipoproteins Peptidoglycan	Cytokine induction

According to Medzhitov et al. (1997), PRRs can be classified regarding their distribution in cell-surface, intracellular and soluble PRRs. This table gives an overview on some PRRs, their origin and their function. Note that, in some cases, origin and function are currently still the subject of controversial discussion.
BPI, bactericidal/permeability increasing protein; CRP, C-reactive protein; LBP, LPS-binding protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid.

the function of binding and presenting the microbial target to cellular receptor or enabling phagocytosis (opsonic function), have also been described. A common feature of all PRRs is that they recognize common structures of invading microorganisms, followed by the initiation of immune responses. PRRs can be distinguished into cell-surface, intracellular, and secreted PRRs (Medzhitov 2001) (Table 1).

Cell-surface PRRs

Besides the TLR family, which is discussed in detail later, other cellular receptors for microbial ligands have been described, and three examples are given here. A membrane-bound PRR is the type 3 complement receptor, a β_2-integrin also known as CD18/CD11b. It is present on macrophages, enabling phagocytosis of complement-opsonized particles or whole pathogens (Ross 2000). CD18/CD11b has been proposed as being involved in LPS-mediated signal transduction in cooperation with CD14; however, these results are still not conclusive (Flo et al. 2000). Furthermore, the large family of scavenger receptors is expressed not only by macrophages but also by certain endothelial cells. They bind to a wide array of polyanionic ligands, including exogenous molecules such as LPS of gram-negative bacteria or endogenous compounds, i.e. low-density lipoprotein (LDL) (Krieger and Stern 2001). Interaction of bacterial ligands with members of the scavenger receptor family does not induce signal transduction, but leads to detoxification of the ligands.

Another group of cell surface PRRs is the C-type lectins, including mannose receptors and denditric cell-specific ICAM-grabbing nonintegrin (DC-SIGN). Mannose receptors are expressed by macrophages, DCs, and some endothelial cells, whereas DC-SIGN is restricted to DCs (Geijtenbeek et al. 2002). They bind mannosyl/fucosyl as well as glucosamine residues (East and Isacke 2002), which are commonly found on the bacterial surface. The interaction of mannose receptors with pathogens, affecting intracellular signaling, is not fully elucidated by now, however, there is evidence that virulent strains of *Mycobacterium tuberculosis* are phagocytosed via a pathway involving mannose receptors, whereas their avirulent counterparts are not (Kang and Schlesinger 1998).

Intracellular PRRs

BACTERICIDAL/PERMEABILITY INCREASING PROTEIN

Bactericidal/permeability-increasing protein (BPI) is a predominantly intracellular protein with the ability to bind LPS with high affinity (Weiss et al. 1978). It can

therefore be referred to as an intracellular PRR. BPI is found within the primary granules of polymorphonuclear neutrophils (PMN), has bactericidal properties toward gram-negative bacteria, and apparently binds whole bacteria or LPS within the phagocytic compartment of the cell in order to kill engulfed bacteria. BPI is one of the first PRRs that entered clinical trials (Neuprex) in order to modulate the innate immune response brought about by LPS during sepsis, which is discussed in the final chapters (Elsbach and Weiss 1998).

Recently, intracellular PRRs termed 'NOD1' and 'NOD2' were described with the ability to participate in the induction of programmed cell death. It has been suggested that NODs, which share structural similarity with TLRs, are involved in intracellular recognition of LPS and other bacterial partial structures; however, these results still have to be confirmed (Inohara and Nunez 2001). The next section covers some of the results from basic science on NOD2 and the final section of this chapter summarizes the findings of the relationship between NOD2 polymorphisms and Crohn's disease. TLR-9 is another intracellular PRR that recognizes bacterial DNA, which is discussed separately later under Recognition of bacterial DNA by TLR-9.

PRR ROLE FOR NOD2/CARD 15?

A member of a recently discovered, growing family of proteins containing a so-called caspase-recruitment domain (CARD) involved in apoptosis regulation, originally termed 'nucleotide-binding oligomerization domain (NOD)', has recently been postulated to recognize LPS and bacterial muramyldipeptide (MDP) intracellularly (Girardin et al. 2003; Inohara et al. 2003). NOD proteins are related to the family of membrane-bound R-proteins of plants involved in plant host defense (Dangl and Jones 2001). Several NOD proteins in humans have been identified and NOD1 (also called CARD 4) has been implicated with LPS recognition, whereas NOD2 (also called CARD 15), although previously suggested also to recognize LPS, has recently been shown to be involved in MDP recognition (Inohara and Nunez 2001; Inohara et al. 2003).

The NOD proteins all contain leucin-rich repeat (LRR) domains and share homology with the apoptosis regulator apoptotic protease-activating factor-1 (Apaf-1) (Bertin et al. 1999). NOD2/CARD 15 is expressed only by monocytes and recently has gained attention because of a correlation of SNPs with the incidence of Crohn's disease and a rare disease called Blau syndrome (arthritis, uveitis, and skin rash) in humans (see details later under The NOD2/*CARD15* SNPs). A frameshift mutation here leads to a truncated protein that is apparently unable to respond to LPS or MDP (Ogura et al. 2001b; Girardin et al. 2003; Inohara et al. 2003).

The CARD-containing proteins and certain TLRs signal via a CARD-containing serine/threonine kinase termed 'RICK/Rip2/CARDIAK', leading to NF-κB translocation (Kobayashi et al. 2002). The family of CARD/NOD proteins, in summary, are related to TLRs and CD14 with regard to their structure of LRRs, and may serve as intracellular sensors for PAMPs.

Soluble PRRs

SOLUBLE CD14

CD14 is a PRR present on monocytes, macrophages, granulocytes, and DCs, attached to the cell via a phosphatidylinositol (PI) anchor that is unable to transduce signals into the cell (Goyert et al. 1988; Pugin et al. 1994). Cellular CD14 has been shown to be part of a lipid raft allowing for clustering of several PRRs upon ligand stimulation (Pfeiffer et al. 2001). This may serve the function of concentrating bacterial ligands locally in order to be presented to a signal-transducing receptor such as members of the TLR family (Schmitz and Orso 2002).

A second, slightly smaller, form of CD14 termed 'soluble CD14' (sCD14) is released into serum, and both forms play a crucial role in pattern recognition (Schumann and Latz 2000). CD14 has been shown to bind to LPS, a glycolipid present in the outer membranes of gram-negative bacteria (Wright et al. 1990; Rietschel et al. 1996; Alexander and Rietschel 2001). CD14 is also involved in recognition of other bacteria and in initiation of phagocytosis. It leads to an accumulation of PAMPs, enabling recognition by the signal-transducing molecules of the TLR family (Schumann et al. 1994; Landmann et al. 2000). CD14 contains LRRs, a structural characteristic typical of many PRRs including the TLRs addressed in detail below under The family of Toll-like receptors (Ziegler-Heitbrock and Ulevitch 1993).

LIPOPOLYSACCHARIDE-BINDING PROTEIN

During cell growth and death, gram-negative microorganisms release LPS (Brandtzaeg et al. 1989), and in serum it is primarily recognized and bound by LPS-binding protein (LBP), (Figure 3), another soluble PRR. LBP is mainly synthesized in the liver, catalyzing the transfer of LPS to CD14 (Schumann et al. 1990) and enabling the cells to respond with, for example, cytokine release (Figure 3 and Table 1) (Alexander and Rietschel 2001). LBP is a typical acute phase protein that is induced upon stimulation during the acute phase response via interleukin 1 (IL-1), IL-6, and dexamethasone (Schumann et al. 1996). It belongs to a family of proteins that share the ability to bind lipid-like structures including another LBP called BPI, cholesterol ester-transfer protein (CETP), and phospholipid-transfer protein (PLTP) (Kirschning et al. 1997).

Epithelial cells both of intestinal origin and from the respiratory tract have been found to be sources of LBP as well (Vreugdenhil et al. 1999; Dentener et al. 2000). Both CD14 and LBP have been found to be involved in

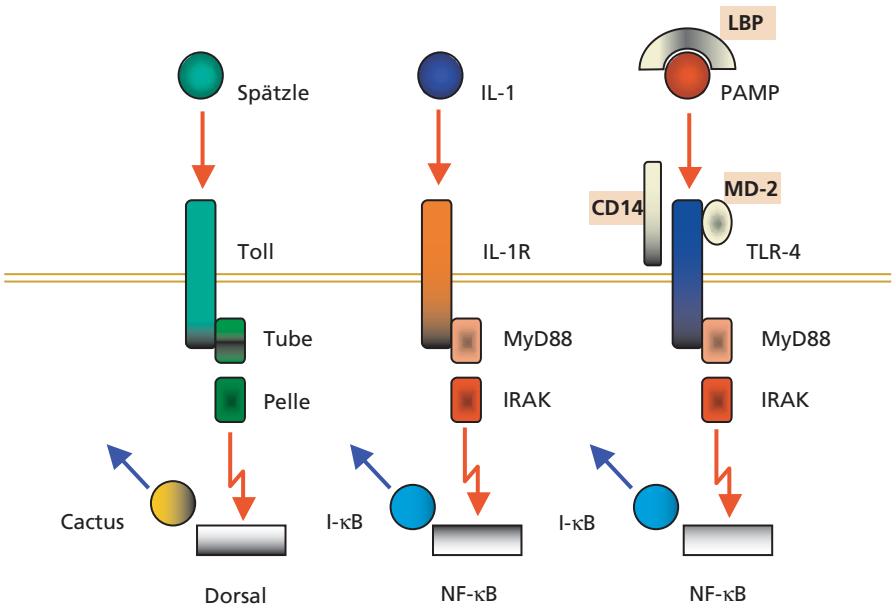

Figure 3 *Comparison of signal transduction via drosophila Toll-like receptors (TLR), interleukin 1 receptor (IL-1R), and TLR-4. In* Drosophila*, signal transduction via Toll is initiated by the cleavage of the endogenous ligand* spätzle*, which binds to Toll, initiating a signal transduction cascade involving the adapter molecule* tube *and the kinase* pelle*. After the inhibitory factor* cactus *has dissociated, the transcription factor dorsal translocates into the nucleus, initiating certain transcriptional events. This pathway – because of the sequence homologies – shares striking similarities with the known IL-1R, as well as with the recently identified TLR-4 signaling pathway (almost similar among all other members of the TLR family). After the receptor has recognized its ligand (IL-1 or lipopolysaccharide (LPS)), signaling in both cases involves the adapter molecule MyD88 and the kinase IRAK. The inhibitory factor IF-κB dissociates and the transcription factor NF-κB translocates to the nucleus inducing immune responses. An equivalent of the protease cascade, leading to release of the endogenous ligand* spätzle *in the IL-1/TLR pathway has not yet been found. Instead, for recognition of LPS, several soluble pattern recognition receptors (PRR) are involved with LPS-binding protein (LBP), transferring LPS to the receptor complex, which in addition to TLR-4 also involves CD14 and MD-2.*

recognition of other bacterial structures, such as spirochetal glycolipids, pneumococcal peptidoglycan, lipoteichoic acid (LTA), and others (Perera et al. 1997; Kurt-Jones et al. 2000; Schroder et al. 2000, 2003).

Recently LBP was found to serve a dual role during infection, depending on the local concentration in relation to the concentration of the bacterial ligand. Although small concentrations of LBP enable host cell responses to LPS, high concentrations of LBP, as present in human serum, inhibited cellular LPS-induced signaling (Lamping et al. 1998; Hamann et al. 2000; Zweigner et al. 2001). The soluble PRR LBP may serve both of the functions of innate immunity mentioned above. Early detection of pathogens and induction of the immune response, and fighting off the bacteria and inhibiting its host effects, depend on its concentration, which is tightly regulated during the acute phase.

THE FAMILY OF TOLL-LIKE RECEPTORS

General characteristics of TLRs

BASIC LESSONS LEARNED FROM FLIES

Drosophila Toll was discovered in the early 1980s during a mutagenesis screen by Anderson and Nüsslein-Vollhard (1984). It was further characterized as a factor crucial for dorsoventral axis formation within the drosophila embryo and includes a total of 12 genes (Hashimoto et al. 1988). It is said that the German researcher and Nobel laureate Christine Nüsslein-Volhard, after discovery of this factor, spontaneously shouted 'Toll!', which in German means 'great!'. Toll activation in *Drosophila* also involves a ligand termed *spätzle*, the adaptor protein *tube*, the protein kinase *pelle*, as well as the transcription factor *dorsal* and its inhibitor *cactus* (Medzhitov and Janeway 1998) (see Figure 3).

In the late 1990s it was found that there are striking sequence similarities between all members of this pathway and the mammalian interleukin 1 receptor (IL-1R) signal transduction pathway (Medzhitov et al. 1997). It was postulated that *toll* may also play a role in the innate immunity of *Drosophila*. This was supported by the finding that fruit flies deficient of members of this pathway are highly susceptible to fungal infections, however, immunity against bacteria is not impaired (Lemaitre et al. 1996). Another member of the *toll* family, 18-wheeler, is apparently involved in bacterial recognition (Williams et al. 1997), although the signaling pathways involved are different from *toll* (Medzhitov 2001). A further demonstration of an immune function of *toll* relates to its involvement in the induction of antimicrobial peptides, i.e. Drosomycin and Metchnikowin (reviewed in Imler and Hoffmann 2002, 2003).

FIRST EVIDENCE FOR THE ROLE OF TLRS IN MAMMALS

The previous results, and the fact that IL-1 stimulation of human cells leads to similar effects as compared with stimulation with bacterial ligands, led to studies investigating the potential role of mammalian homologs of *toll*, also called 'Toll-like receptors' in innate immunity (Rock et al. 1998). First reports concluded that human TLR-2 was involved in LPS-mediated signaling (Kirschning et al. 1998; Yang et al. 1998). Later it was found that these studies may have been performed in the presence of lipoproteins, structures now known to be classic TLR-2 agonists. Extensive positional cloning studies that analyzed LPS-hyporesponsive C3H/HeJ mice and the formerly termed LPS gene, leading to hyporesponsiveness to LPS, led to the finding that these mice carried a mutation in the gene encoding for TLR-4. These studies and the development of the TLR-4 knockout mouse revealed that TLR-4 was the long-sought-after LPS gene responsible for detection of LPS (Poltorak et al. 1998; Beutler et al. 2001). It is now agreed that TLR-2 recognizes a wide variety of other bacterial ligands, mainly of gram-positive pathogens (see below under Ligands of the heterodimeric TLR-2–TLR-1–TLR-6 complex, and Table 2) and some rare and atypical forms of LPS.

STRUCTURE OF TLRs

All TLRs described by now are type I transmembrane receptors characterized by the presence of LRRs within the extracellular domain (O'Neill and Dinarello 2000). The role of LRRs in TLR function is currently not completely elucidated. However, proteins exhibiting LRRs, e.g. ribonuclease inhibitors (Kobe and Deisenhofer 1995), have been shown to exhibit a 'horseshoe-like' structure (Figure 4), with the LRRs being relevant for interaction with a proteinaceous ligand. It should be noted that other PRRs besides the TLRs, such as CD14, the NODs, and RP105, share the LRR architecture (Kirschning and Schumann 2002). It is thus tempting to speculate that this structural feature is involved in pattern recognition brought about by these proteins.

Toll-like receptors all possess an intracellular signaling region exhibiting homologies to IL-1R, also referred to as Toll/IL-1R receptor (TIR) domain (see Figure 3). There are a total of 20 proteins with TIR domains so far identified in the human genome, and many TIR-domain-containing proteins have been described in other species including plants (O'Neill 2002). The structure of the TIR domain of TLR-1 and -2 has recently been solved; it contains a crucial region for signaling, as the mutation of the LPS-unresponsive HeJ mouse is located in this region (Xu et al. 2000). Signal transduction events initiated by TLR–ligand interaction are discussed in detail later under Signal transduction cascade induced by TLR-ligand interaction. It may be noteworthy that RP105, a receptor associated with MD-1 and also containing LRRs, lacks a TIR domain. Apparently, most of the TLRs described form heterodimers in order to induce signaling, TLR-4 most probably forming homodimers.

Table 2 *Toll-like receptors and their agonists: an overview*

Receptor	Ligand
TLR-1	Diacylated lipoproteins/lipopeptides (TLR-1/2 heterodimers)
TLR-2	Lipoproteins/lipopeptides (various)
	Peptidoglycan (gram-positive bacteria)
	Lipoteichoic acid (gram-positive bacteria)
	Lipoarabinomannan (mycobacteria)
	Spirochetal glycolipids (*Treponema* spp.)
	Zymosan (crude candida particles)
	Atypical lipopolysaccharide (LPS) (*Leptospira interrogans*)
	Atypical LPS (*Porphyromonas gingivalis*)[a]
	Glycoinositolphospholipids (*Trypanosoma cruzi*)
	Neisseria porins
	Heat shock protein (HSP) 70 (host)
TLR-3	Double-stranded RNA
TLR-4	LPS (gram-negative bacteria)[b]
	Respiratory syncytial virus fusion protein
	HSP 60 (*Chlamydia* spp.)
	β-Defensins (host)
	HSP 60 (host)
	HSP 70 (host)[c]
TLR-5	Bacterial flagellin
TLR-6	Triacylated lipoproteins/lipopeptides (TLR-1/6/ heterodimers)
TLR-7	Imidazoquinolines (antiviral compounds)
	Loxoribine (antiviral compounds)
	Single-stranded V-rich RNA (in mice)
TLR-8	See TLR-7
TLR-9	CpG DNA
TLR-11	Uropathogenic *E. coli* (in mice)

Ligands are ordered according to the different members of the TLR family. Note that some reports have been questioned.
a) Synthetic LPS of *P. gingivalis* has been reported to act as a TLR-4 ligand.
b) According to some investigators, LPS is the sole ligand for TLR-4.
c) Human HSP 70 has been reported to be devoid of stimulating capacities in the absence of contaminating LPS.

TLR TISSUE DISTRIBUTION

Toll-like receptors are not equally distributed among APCs, with monocytes as well as monocytoid or type 1 DCs expressing a rather wide variety of TLRs, whereas plasmacytoid or type 2 DCs preferentially express TLR-7 and TLR-9 (Kadowaki et al. 2001) (Table 2). Other cells types not exclusively contributing to innate immunity have also been described as expressing different TLRs, e.g. endothelial cells predominantly exhibit TLR-4, whereas TLR-2 is usually weakly expressed (Faure et al. 2000), a pattern that is also found in intestinal epithelial cells (Melmed et al. 2003). TLR-3 expression appears to be restricted to DCs, whereas TLR-9 is

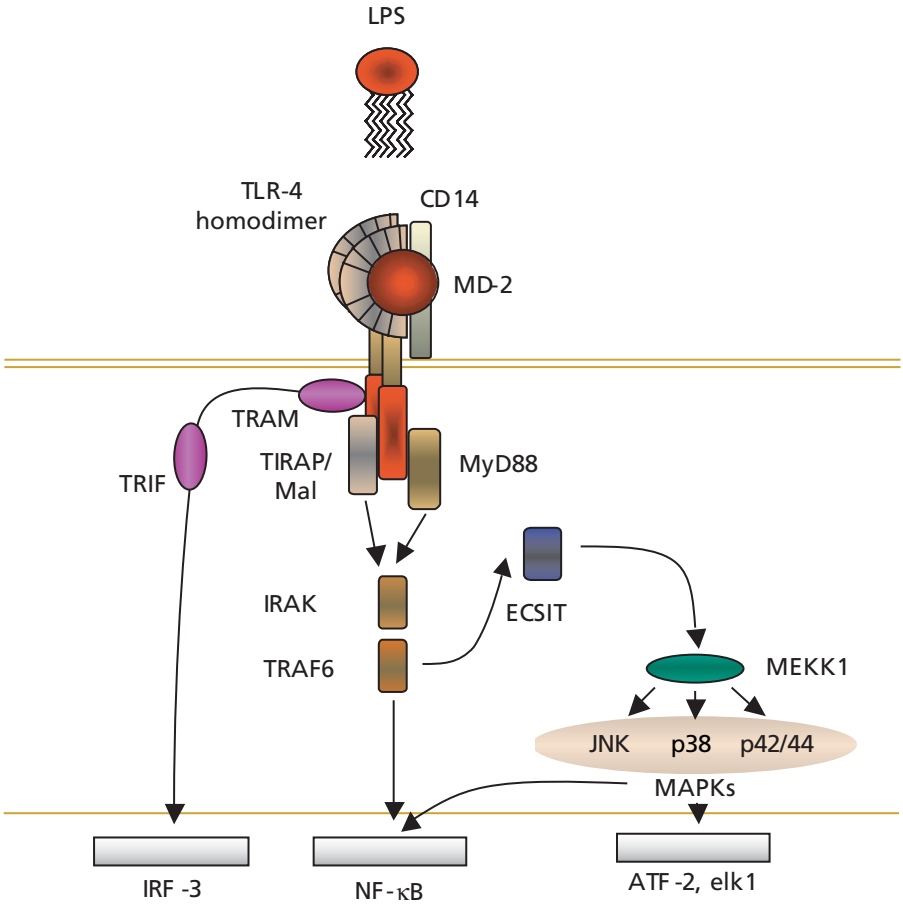

Figure 4 *Signaling pathways of Toll-like receptor 4 (TLR-4). Lipopolysaccharide (LPS) interacts with its receptor complex consisting of CD14, MD-2, and a TLR-4 homodimer or multimer. There are several pathways leading to signal transduction into the nucleus, which are discussed in detail later under Signal transduction cascade induced by TLR-ligand interaction. The MyD88-dependent pathway, in addition to MyD88, involves IRAK, TRAF-6, and the transcription factor NF-κB. TRAF6 also interacts with ECSIT, in turn leading to activation of MAP/erk kinase kinase (MEKK1) and subsequently several MAPKs, involving p38, p42/44, and JNK. Diacylated amphiphilic ligands, such as MALP-2 or lipotechoic acid (LTA) interacting with TLR-2/TLR-6 heterodimers, have been shown to activate several other transcription factors, including ATF-2 and elk1. There are at least two MyD88-independent signal transduction pathways: One involves TIRAP/Mal, which activates IRAK and the other downstream elements known from the MyD88-dependent pathway. Furthermore, activation of the TLR-4 receptor complex via recently identified signaling elements TRAM and TRIF leads to activation of Interferon regulatory factor 3 (IRF-3).*

detectable predominantly on B cells and, as mentioned above, type 2 DCs (Muzio et al. 2000). TLR-2 has been shown to be expressed, or upregulated, within the brain (Koedel et al. 2003). The following sections discuss the characteristics of particular TLR subfamilies in detail; a general overview on the agonists found for the given TLRs is given in Table 3.

TLR-1, TLR-2, TLR-6, and TLR-10

LIGANDS OF THE HETERODIMERIC TLR-2–TLR-1–TLR-6 COMPLEX

Toll-like receptor 2 was the first member of the TLR family shown to be involved in pattern recognition (Kirschning et al. 1998). Over the last few years TLR-2 has been found to be involved in recognition of microorganisms of almost all types including, for example, peptidoglycan and LTA of gram-positive bacteria (Schwandner et al. 1999; Opitz et al. 2001), bacterial lipopeptides of *Borrelia* and *Mycoplasma* spp. (Lien et al. 1999), lipoarabinomannan of mycobacteria (Means et al. 1999), and zymosan of *Candida* spp. (Underhill et al. 1999b) (reviewed in Kirschning and Schumann 2002). TLR-2 apparently does not interact with MD-2 but, as CD14 has been shown to play a crucial role in cytokine induction by LTA, peptidoglycan, and lipopeptides (Weidemann et al. 1997; Sellati et al. 1998; Hermann et al. 2002), it is very likely that CD14 is also part of the TLR-2 receptor complex. TLR-2, next to its cell surface expression, also appears intracellularly, but only after phagocytic processes (Underhill et al. 1999b).

The high degree of homology among TLR-2, TLR-1, and TLR-6 corresponds to the fact that these receptors are able to form heterodimers (Ozinsky et al. 2000). Apparently, LTA and mycoplasma lipopeptide

Table 3 *Ligands of Toll-like receptors classified according to their origins*

Organism	Substance class	Ligand	Receptor
Bacteria	Glycolipid	LPS (gram-negative)	TLR-4
		LPS *L. interrogans*	TLR-2
		LTA (gram-positive) lipoarabinomannan (mycobacteria)	TLR-2
		Spirochetal glycolipids (*Treponema* spp.)	TLR-2
	Lipoprotein/lipopeptide	Lipoprotein (*Borrelia burgdorferi*)	TLR-2/-1
		Mycoplasma spp. (lipopeptide)	TLR-2/-6
	Nucleic acid	CpG	TLR-9
	Protein	Flagellin	TLR-5
		Neisseria porins	TLR-4
		Chlamydia HSP	TLR-2
	?	Uropathogenic *E. coli*	TLR-11
Fungi	Various	Zymosan	TLR-2
Protozoa	Glycolipids	Glycoinositolphospholipids	TLR-2
Virus	Nucleic acid	Double-stranded RNA	TLR-3
		Single-stranded RNA	TLR-7/-8
	Protein	RSV fusion protein	TLR-4
Endogenous	Saccharides	Hyaluronic acid oligomers	TLR-4
		Heparan sulfate	TLR-4
	Proteins	HSP 60 and 70	TLR-4
		HSP 70	TLR-2
		β-Defensins	TLR-4
Others		Paclitaxel	TLR-4
		Antiviral compounds	TLR-7/-8

TLR ligands are classified according to their origin. Ligands derived from bacteria, viruses, fungi, and parasites are listed. Note that there are also reports on endogenous ligands of TLRs derived from the host, not listed here.
HSP, heat shock protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; RSV, respiratory syncytial virus.

(MALP-2) are recognized by dimers consisting of TLR-2 and TLR-6 (Morr et al. 2002; Takeuchi et al. 2001), whereas borrelia lipoproteins and their synthetic lipopeptide counterparts, respectively, interact with TLR-2/TLR-1 dimers (Takeuchi et al. 2002). Taken together, these data indicate that diacylated amphiphilic molecules (i.e. MALP and LTA) in common are recognized by TLR-2/TLR-6, whereas triacylated molecules (i.e. borrelia lipoproteins) are recognized by TLR-2/TLR-1 (Figure 5). Until now, however, no ligand has been described for TLR-10. As this putative receptor exhibits high homology to TLR-1 and TLR-6, it is tempting to speculate that it also forms heterodimers with TLR-2.

IS THERE STILL EVIDENCE FOR TLR-2 BEING AN LPS RECEPTOR?

There have been several reports on 'atypical' LPS molecules interacting with TLR-2 and not with TLR-4. These reports mainly focused on the LPS of *Leptospira interrogans* and *Porphyromonas gingivalis* (Hirschfeld et al. 2001; Werts et al. 2001). In this context, the term 'atypical' describes a lipid A portion of LPS being less phosphorylated and lacking secondary fatty acids compared with the well-characterized Enterobacteriaceae lipid A. However, *Porphyromonas gingivalis* LPS has been described to act as a sole TLR-4 agonist after extensive purification, and synthetic preparations of its lipid A also do not display any interaction with TLR-2 (Ogawa et al. 2002).

Toll-like receptor 3

Toll-like receptor 3 has been shown to recognize double-stranded RNA (dsRNA), which is found during viral infections (Alexopoulou et al. 2001). This evidence was obtained by transfection of a TLR-3-negative cell line, rendering it responsive to dsRNA, as well as studies on TLR-3-deficient mice, which were more susceptible to administered dsRNA in comparison to their wild-type counterparts.

Signal transduction involves MyD88 and NF-κB, or the MyD88-independent TICAM-1 pathway (see later under Signal transduction cascade induced by TLR-ligand interaction). Double-stranded RNA, or its synthetic counterpart, polyinosinic–polycytidylic acid [poly(I):poly(C)] (Alexopoulou et al. 2001) is known to be a potent inducer of type I interferons, such as interferon-α (IFN-α) and IFN-β, which are mainly synthesized by plasmacytoid DCs. However, these cells do not express TLR-3 (Kadowaki et al. 2001). Thus, dsRNA may in addition interact with other receptors. Furthermore, TLR-3, predominantly expressed in monocytoid DCs, may also bind other as yet unidentified ligands. TLR-3 stimulation has recently been

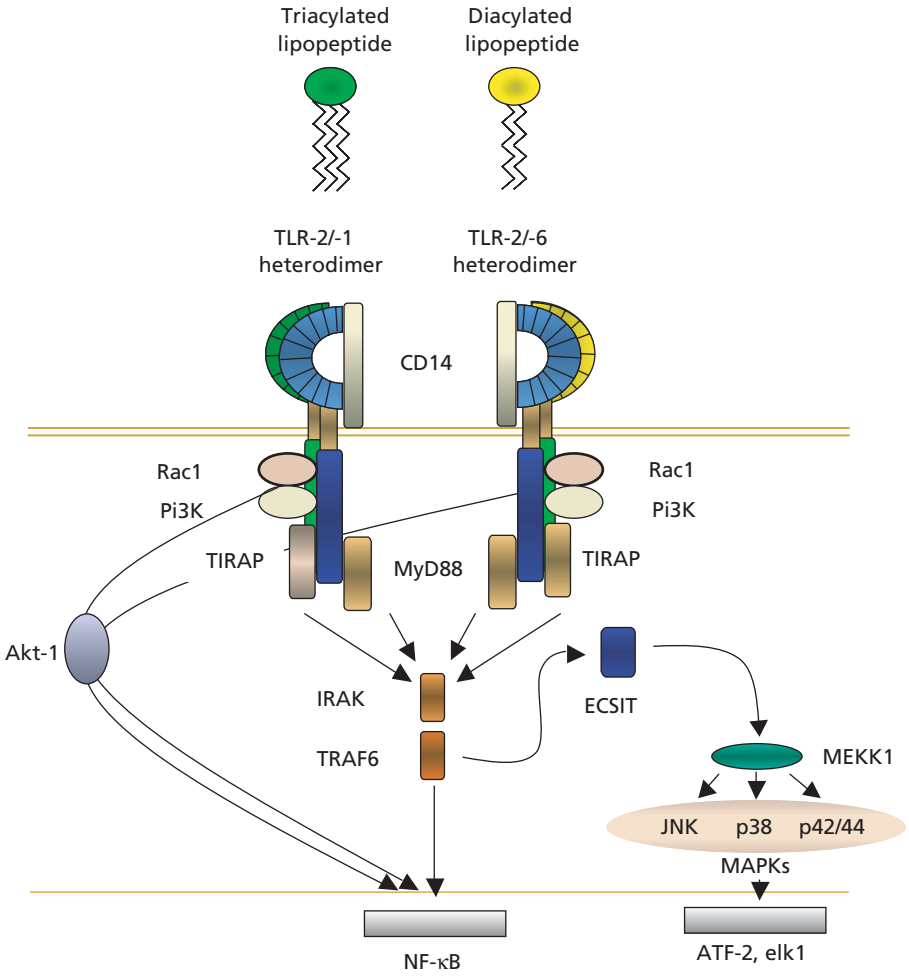

Figure 5 *Toll-like receptor 2 (TLR-2)-associated signaling pathways. The TLR-2 receptor complex consists of a heterodimer and apparently involves CD14, as well as TLR-1 or TLR-6. Triacylated amphiphilic molecules, i.e. lipopeptides based on the lipoprotein basic structure of* Borrelia burgdorferi *lipoproteins, interact with TLR-2/TLR-1 heterodimers, whereas diacylated amphiphilic ligands, such as MALP-2 or lipoteichoic acid (LTA), interact with TLR-2/TLR-6 heterodimers. Signal transduction involves the MyD88-dependent pathway or the TIRAP/Mal pathway, largely MyD88 independent (see Figure 4). Furthermore, NF-κB translocation is brought about by a signaling cascade involving phosphatidylinositol 3 (PI-3) kinase and Rac1, forming a complex with TLR-2, and AKT. Note that this pathway has also been demonstrated for TLR-4.*

found to induce a complex set of antiviral responses, which are more efficient in fighting viruses compared with stimulation of TLR-4 (Doyle et al. 2003).

Toll-like receptor 4

THE LPS RECEPTOR

With the exception of the LPS–TLR-2 interaction mentioned above, TLR-4 is currently viewed as the sole receptor for bacterial LPS (Beutler et al. 2001). The term 'receptor' appears premature because LPS binding to TLR-4 has not been shown yet; however, binding of LPS to MD-2, a TLR-4 adaptor protein, could be demonstrated (Viriyakosol et al. 2001). It is most likely that TLR-4 forms homodimers or multimers and this receptor complex also involves CD14 and the adaptor molecule MD-2 (da Silva Correia et al. 2001).

Despite the previous finding that TLR-2 is involved in LPS recognition, numerous studies have provided evidence that TLR-4 is the main cellular 'receptor' for LPS (Chow et al. 1999; Heine et al. 1999). TLR-4, therefore, has been found to contain the ability to discriminate LPS types differing in their lipid content within their lipid A moiety, leading to divergent levels of activity (Lien et al. 2000). Transfection of TLR-4 from different species was able to transfer the ability of certain types of LPS to act as active agonist, inactive non-competitive molecule, or antagonist (Poltorak et al. 2000). It has also been shown, by laser scanning microscopy, that the CD14–TLR-4–MD-2 complex specifically travels within the cell to and from the Golgi apparatus transporting LPS, a process that is, however, separate from the induction of signal transduction (Latz et al. 2002).

The crucial role of MD-2 for LPS recognition by TLR-4 could be demonstrated by transfection experiments

(Shimazu et al. 1999), as well as by studies employing MD-2-mutant cell lines (Schromm et al. 2001). Recently, the generation of MD-2-deficient mice clearly showed that MD-2 is absolutely essential for host LPS recognition (Nagai et al. 2002). MD-2 has been shown to form dimers; however, the monomeric form seems to be important for LPS recognition (Re and Strominger 2002). The principle of TLR-4 and MD-2 forming a receptor complex exhibits striking similarities with RP105, the LRR-containing receptor present on B lymphocytes that is associated with an MD-2-related adaptor protein called MD-1 (Miyake and Shimazu 1998). RP105, however, is the only LRR-containing molecule lacking the TIR domain described later, which has been found to be essential for signaling (Miyake 2003).

NON-LPS LIGANDS OF TLR-4

Following the finding that LPS is an agonist for TLR-4, other potential ligands have been proposed to interact with the TLR-4 receptor complex, which are, apart from paclitaxel (Kawasaki et al. 2000), proteins. These include bacterial chlamydia heat shock proteins (HSP) (Costa et al. 2002), viral respiratory syncytial virus (RSV) fusion protein (Kurt-Jones et al. 2000), as well as endogenous compounds, especially host HSPs (Ohashi et al. 2000) (summarized in Table 3). It should be noted that studies of interactions of TLR-4 with proteins expressed in gram-negative bacteria, such as *E. coli*, are often hampered by contaminating LPS. Therefore, results obtained with recombinant proteins have to be interpreted carefully, and recent studies came to the conclusion that some of the effects described were mediated by contaminating LPS (Bausinger et al. 2002).

However, RSV-F protein, after protease digestion, exhibited a diminished activity, indicating an interaction of this proteinaceous ligand with TLR-4. For the large and quite diverse family of HSPs, which may be viewed as sensors for necrosis, it has been shown that they also activate innate immune cells (reviewed in Vabulas et al. 2002). For HSPs of both bacterial and mammalian origin, TLR involvement in signaling has been proposed, with human HSP60 and Gp96 being the most prominent ones (Vabulas et al. 2001). The regulation of the host's innate immune response by proteins released by cells may be explained by the above-mentioned 'danger model', with HSPs indicating tissue injury or imbalance of hemostasis. HSPs activate macrophages and DCs and evidence has been obtained that both CD14 and TLR-2, as well as TLR-4, may be involved (Asea et al. 2002).

Toll-like receptor 5

BACTERIAL FLAGELLIN IS THE AGONIST OF TLR-5

Many enteric bacteria possess flagella responsible for motility and directed movement. The flagellum consists of a filament and a hook with the filament being made up of 11 flagellin monomers (reviewed in Smith and Ozinsky 2002). This 55-kDa flagellin protein has been shown to be a potent inducer of inflammation. Flagellin has been recently demonstrated to act as an agonist for another member of the TLR family, TLR-5 (Hayashi et al. 2001). Besides many enteric gram-negative bacteria, gram-positive *Listeria* spp. and spirochetes also contain flagellin, potentially leading to an interaction with TLR-5.

The signal transduction cascade, as has been shown for the other members of the TLR-family, involves MyD88 and NF-κB (Hayashi et al. 2001). Next to mammalians, flagellin is also detected by the immune system of insects and plants. In *Arabidopsis*, a receptor-like kinase termed FLS2 has been identified which interacts with flagellin (Gomez-Gomez and Boller 2000). Interestingly, FLS2 exhibits similarities with TLRs over the presence of LRRs within the extracellular domain. Therefore, recognition of bacterial flagellin appears to be a ubiquitous mechanism of innate immune responses.

POTENTIAL CLINICAL IMPLICATIONS OF TLR-5 FOR GASTROINTESTINAL DISEASES

Intestinal epithelia are only weakly responsive to LPS; however, they react with strong cytokine release on exposure to flagellin (Gewirtz et al. 2001b). TLR-5 appears to be expressed exclusively on the basolateral surface of intestinal cells (Gewirtz et al. 2001a). Therefore, flagellin has to be translocated from the luminal to the basolateral surface in order to elicit immune responses. Pathogenic *Salmonella* spp. translocate into the epithelium, whereas commensal *Escherichia* spp. do not (Gewirtz et al. 2001a). Thus, basolateral expression of TLR-5 might represent a mechanism to distinguish pathogenic from colonizing bacteria. Furthermore bacteria have developed mechanisms to regulate flagellin expression in order to evade TLR-5 recognition, i.e. the human pathogen *Listeria monocytogenes* suppresses flagellin expression at 37°C, whereas the non-pathogenic species *L. innocua* does not (Kathariou et al. 1995).

Toll-like receptors 7, TLR-8, and TLR-9

IMIDAZOQUINOLINES AS LIGANDS FOR TLR-7 AND TLR-8

Toll-like receptors TLR-7, TLR-8, and TLR-9 are closely related. TLR-7 and TLR-8 have been shown to recognize antiviral compounds, the imidazoquinolines, such as imiquimod and R-848 (Hemmi et al. 2002; Jurk et al. 2002), which are commonly used for treatment of anogenital warts caused by human papilloma virus. Recently it has been shown that TLR-7 and -8 recognize single-stranded, V-rich viral RNA (Heil et al. 2004; Diebold et al. 2004; Lund et al. 2004). TLR-9 recognizes bacterial DNA as outlined below. Signal transduction

through these receptors, as has been shown for TLR-4 and TLR-2, also involves MyD88 (Hemmi et al. 2000, 2002).

RECOGNITION OF BACTERIAL DNA BY TLR-9

Over the few last years the discovery was made that bacterial DNA differs from eukaryotic DNA in that it exhibits unmethylated CG-rich regions (commonly referred to as 'CpG'). Oligonucleotides containing certain typical nucleotides have been shown to exhibit extensive immunostimulatory capacities (Sparwasser et al. 1997; Krieg 2002). TLR-9 recognizes bacterial DNA, as revealed by studies employing TLR-9-deficient mice that could not be stimulated by CpG oligonucleotides for proinflammatory activation (Hemmi et al. 2000).

TLR-9 is predominantly expressed in plasmacytoid DCs (pre-DC2), and stimulation with CpG-DNA leads to production of high levels of IL-12, initiating a Th1 response. This makes CpG motifs that interact with TLR-9 interesting candidates for immunization strategies (Wagner 2002). In addition, CpG motifs may in the future play a role in gene therapy and cancer immunization strategies (Krieg 1999). It is obvious that, during infections treated by antibiotics, bacterial DNA is released; however, the precise role of CpG-DNA in the pathogenesis of inflammatory diseases or septic shock is still not completely elucidated. In contrast to other TLRs, TLR-9 is not found on the cell surface, but within endosomes (Ahmad-Nejad et al. 2002).

SIGNAL TRANSDUCTION CASCADE INDUCED BY TLR–LIGAND INTERACTION

As mentioned above, there are numerous homologies in the signaling pathways of human TLR activation, drosophila Toll protein, and even plant R-protein signaling (Lemaitre et al. 1996; Kimbrell and Beutler 2001). All systems initially appear to require LRR-containing molecules and, in order to initiate signaling, the presence of TIR domains is essential (Fluhr and Kaplan-Levy 2002). In addition, the human IL-1 signaling pathway displays many similarities to the TLR signaling, which may reflect the ability of the host to amplify certain responses to PAMPs via host molecules such as IL-1, released by immune cells (O'Neill and Dinarello 2000). The homologies among vertebrates, insects, and plants are numerous, and are present basically in every step of signaling. In the following section only the main pathways of TLR signaling in humans are shown.

Myd88-dependent pathways

The signal transduction cascade activated upon interaction of LPS with the TLR-4–CD14–MD-2 receptor complex, as an example for typical and largely similar TLR signaling, is shown in Figure 4. MyD88 is an adaptor molecule containing a TIR domain involved in signaling induced by many receptors of the TLR family (Takeuchi and Akira 2002). A difference exists in the additional utilization of the Mal/TIRAP protein mentioned in detail below and termed 'MyD88 independent' (reviewed in O'Neill 2002). Next to the TIR domain, MyD88 also contains a 'death domain' that is found in the downstream signaling molecule IL-1R-associated kinase (IRAK) (Cao et al. 1996). MyD88 activation after interaction of PAMP ligands with its specific TLR and subsequent multimerization is followed by an activation of IRAK and TNF receptor-associated factor 6 (TRAF-6), a member of a family of proteins involved in TNF signaling (Lomaga et al. 1999). Upon this activation, NF-κB dimers are formed involving the NF-κB-inducing kinase (NIK) (Malinin et al. 1997). After inactivation of inhibitory factor I-κB by I-κB kinase (IKK) (DiDonato et al. 1997) NF-κB translocates to the nucleus initiating transcription of genes encoding for, for example, proinflammatory cytokines.

Recruitment of TRAF-6 also leads to activation of 'evolutionarily conserved signaling intermediate in Toll pathways (ECSIT)', which in turn activates mitogen-activated protein (MAP)/extracellular stress-related kinas (eerk) kinase kinase (MEKK1), thus forming a link between Toll-mediated signaling and the MAP kinase pathways (Kopp et al. 1999). These pathways are commonly referred to as 'MyD88-dependent pathways' and seem to be involved in signal transduction by several other TLRs. Furthermore, there is evidence for another signal transduction pathway involving Rac1/PI3K/AKT, leading to the translocation of NF-κB (Arbibe et al. 2000). This pathway has originally described for TLR-2 ligands; however, recent data suggest that it may also play a role in TLR-4-mediated signaling (Ojaniemi et al. 2003).

MyD88-independent

The observation that MyD88-deficient mice at least partially respond to LPS led to the hypothesis of MyD88-independent signal transduction by TLR-4 (Kawai et al. 1999). Meanwhile, a novel TIR-domain-containing adaptor protein (TIRAP) has been described, which has been termed by others 'MyD88 adaptor-like' (Mal) (Horng et al. 2001). As TIRAP/Mal plays no role in IL-1R-mediated signaling, it represents a bifurcation between these pathways. The TIRAP/Mal knock-out mouse confirmed this bifurcation and led to the discovery that TIRAP/Mal is apparently involved in TLR-4, TLR-2, TLR-1, and TLR-6 signaling, but not in signaling brought about by activation of TLR-5, TLR-7, and TLR-9 (Horng et al. 2002; Yamamoto et al. 2002).

Recently, another MyD88-independent pathway has been discovered which leads to the activation of interferon regulatory factor-3 (IRF-3, Kawai et al. 2001). At least

three other TIR-domain-containing adaptor proteins exist (reviewed in O'Neill et al. 2003). Two of them, termed TRAM and TRIF have recently been shown to mediate the Myd-88 independent activation of IRF-3.

LINKS TO ADAPTIVE IMMUNITY

Antigen presentation

One major task of the cells of the innate immune system is to screen for pathogen-associated patterns and subsequently detect and kill invading microorganisms as first-line defense. APCs, as mentioned above (
Processing and presentation of the antigen by the class II histocompatibility system; and MHC class I antigen processing system), also act as a link between innate and adaptive immunity by presenting antigens to lymphocytes, which is extensively covered in other chapters of this volume. This link is in particular represented by immature imDCs (see above under Cells of the innate immune system), with interstitial imDCs being most important. These cells reside within peripheral tissues and scan their environment via endocytosis. After having contact with a wide variety of ligands, mainly via TLRs, they undergo maturation (reviewed in Banchereau and Steinman 1998).

PAMPs recognized by imDCs involve LPS, CpG-DNA, lipoproteins, and peptidoglycan (reviewed in Takeda et al. 2003). Maturation involves the expression of MHC molecules as well as CD80/CD86; furthermore proinflammatory cytokines, such as IL-12, are secreted. Mature DCs travel to lymph NODs and present processed antigen to T lymphocytes, initiating adaptive immune responses.

Type 1 interferons

Another link between innate and adaptive immunity is represented by plasmacytoid or type 1 pre-DCs. These cells, as shown above, express TLR-7 and TLR-9 and secrete type 1 interferons upon stimulation with adequate ligands (Kadowaki and Liu 2002). These cytokines, besides directly inhibiting viral replication and stimulating macrophages and NK cells, also activate T and B lymphocytes. They enhance proliferation of CD8 T lymphocytes, enhance the survival of T cells in general, and elicit antibody production by B lymphocytes. Obviously, type 1 interferons are crucial for immune responses against viral infections; however, they have also been described to act against bacteria and protozoa.

A TWO-STEP MODEL OF PATTERN RECOGNITION

Although the discoveries of the last few years improved understanding of pattern recognition, the general mechanism is far from being completely understood. As outlined in previous chapters, the diversity of microbial ligands is large and there are only first real molecular similarities, which allow for a definition of 'patterns'. Here, a hypothetical two-step model is proposed where, on one the hand, charged molecules are sensed, followed by a fine recognition system focusing on atypical lipids.

As described above, the PRR CD14 is able to bind a variety of ligands, including LPS, LTA, and triacylated lipopeptides. The structure of these ligands is quite different: LPS is a glycolipid with a lipid part and a polymeric part made up of carbohydrates (Raetz and Whitfield 2002). The lipid part of LPS, lipid A, is substituted with up to six fatty acids and exhibits a high degree of polarity as a result of its phosphate content (Figure 6). In contrast, LTA of *Staphylococcus aureus* is a glycolipid with a lipid anchor, being composed of glycerol and two fatty acids (diacylglycerol) and fewer phosphate charges. The polymeric region mainly consists of polyglycerophosphate, partially substituted with carbohydrates (Schroder et al. 2003). Lipopeptides are not glycolipids at all, but are amphiphiles bearing diacylglycerol linked to a peptide chain via cysteine. All these molecules are recognized by CD14 and LBP. Therefore, LBP and CD14 may represent a 'first-line' pattern recognition system shuttling amphiphiles to their receptor complexes.

Cellular PRRs such as the TLRs are able to discriminate further these molecular patterns with TLR-4 interacting with LPS, whereas TLR-2/TLR-6 dimers interact with LTA (and diacylated lipopeptides) and TLR-2/-TLR-1 dimers interact with triacylated lipopeptides (Takeuchi et al. 2001). Following this principle, TLRs would be regarded as 'second-line' PRRs initiating a more specific response to different patterns. This is reflected by the fact that the TLR-4-mediated response differs from that initiated by TLR-2 by involving IRF-3. A similar model can be proposed for the TLRs discriminating different types of nucleic acid which are TLR-3, TLR-7, and TLR-9.

CLINICAL IMPLICATIONS AND CONCLUSIONS

Exploitation of pattern recognition for therapy?

As a strong inflammatory reaction is widely viewed as playing a role in the pathology of sepsis and septic shock, interfering with innate immune responses has for many years been the focus of experimental sepsis therapy. Some of these approaches, which all failed to improve sepsis outcome, now show surprising results in the treatment of chronic inflammatory diseases. Interfering with TLR signaling is thus very likely to be the focus of a next generation of novel therapeutic strategies for both types of diseases (Beutler 2003).

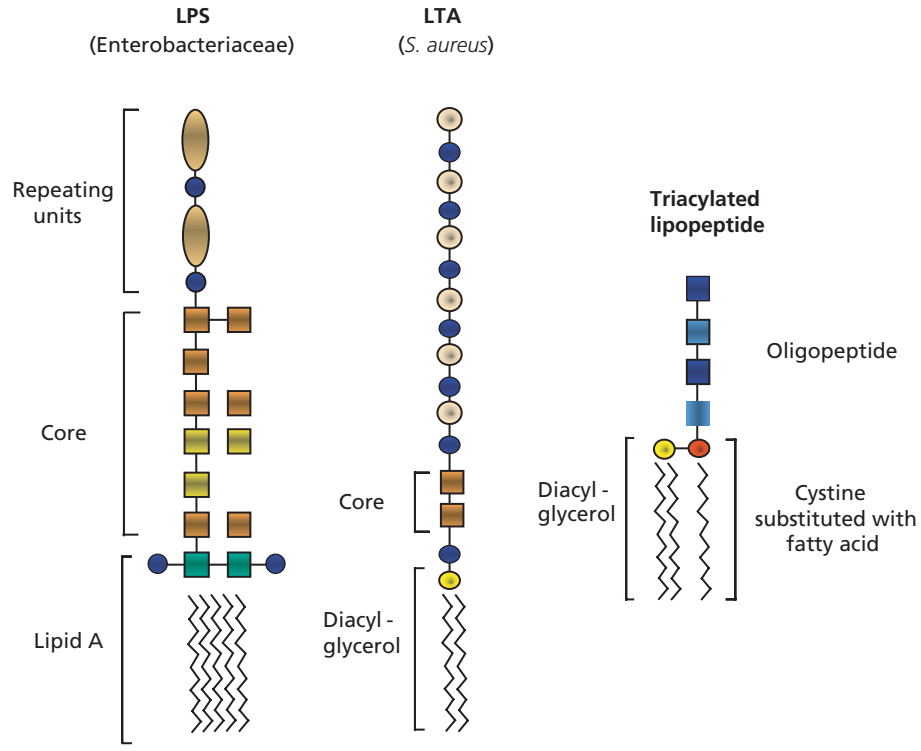

Figure 6 *Structural comparison of CD14 ligands. To attempt to define the structural requirements of a pathogen-associated molecular pattern (PAMP), here the simplified structures of lipopolysaccharide (LPS, as found in Enterobacteriaceae), lipoteichoic acid (LTA, from* Staphylococcus aureus*) and triacylated lipopeptide are compared: LPS is a glycolipid composed of a lipid part termed 'lipid A', a carbohydrate core region, and carbohydrate repeating units. Lipid A part contains up to six fatty acids. Major changes in this composition largely reduce the immunostimulatory capacity of LPS. LTA is glycolipid with a simple diacylglycerol lipid anchor, a small core region, and a polymeric backbone of polyglycerophosphate. In contrast, triacylated lipopeptides are diacylglycerols linked to cystine, which is substituted by a third fatty acid and linked to an oligopeptide.*

The LPS-inhibiting PMN protein BPI binds bacterial LPS, and in addition is bactericidal. BPI does not transfer LPS to any other receptor, leading to subsequent signal transduction and cytokine release, and therefore a potential therapeutic effect during infections with gram-negative bacteria was assumed. The recombinant amino-terminal part of BPI (rBPI21) has been applied to children with meningococcemia, which is usually associated with high levels of LPS in the bloodstream. Within the first phase I/II trials positive effects of administration of rBPI21 were observed (Giroir et al. 1997), and a phase III study came to the result that complications were less frequent among children receiving rBPI (Levin et al. 2000).

Although final results are currently not available, the concept of an early blocking of a key PAMP appears to be promising. Application of high concentrations of LBP may be an additional approach for inhibiting LPS-induced inflammatory responses (Lamping et al. 1998; Zweigner et al. 2001). LBP as an acute phase serum protein has a longer half-life compared with BPI and also inhibits effects of LPS and other PAMPs in concentrations found in serum. The host releases large quantities of LBP during the acute phase, apparently to achieve inhibition of inflammation brought about by PAMP–PRR interaction.

Genetic polymorphisms of PRRs and their impact on disease

Genetic polymorphisms, also referred to as 'nucleotide polymorphisms' (SNP), are frequent variations within a gene, which are found at least among 1 percent of individuals within a population. SNPs may have an impact on protein function; however, most SNPs reported so far are 'silent'. The relationship of genetic variations among humans and their susceptibility to disease is currently often investigated in order to identify high-risk populations and subsequently to be able to prevent diseases in these populations by specific prophylaxis.

CD14 AND LBP SNPs

During the past few years, several SNPs of PRRs were reported, including CD14 and LBP. For CD14, two polymorphisms within the promoter were reported, with one (Cys159Thr) being correlated with incidence of septic shock (Gibot et al. 2002), whereas another (Cys260Thr) apparently had no impact on the incidence of sepsis in trauma patients (Heesen et al. 2002). With regard to LBP, two SNPs were identified which were both correlated with a worsened outcome in patients with sepsis (Hubacek et al. 2002).

TOLL-LIKE RECEPTOR SNPS

Shortly after the identification of TLR-4 as the receptor for LPS, SNPs within the TLR-4 gene were described. These polymorphisms, Asp299Thr and Thr399Ile, were initially reported to be associated with decreased asthmatic reactions to inhaled LPS (Arbour et al. 2000). Meanwhile, evidence has been presented that the Asp299Thr SNPs may also be associated with the incidence of sepsis (Lorenz et al. 2002). These SNPs were mainly investigated with regard to a potential increase of susceptibility to infection. However, a decreased incidence of TLR-4 SNPs was found among patients with atherosclerosis (Kiechl et al. 2002), indicating that the limited recognition of pathogens may influence the course of a disease in a beneficial manner. SNPs were also reported for TLR-2 (Lorenz et al. 2000) and TLR-9 (Lazarus et al. 2003); however, their potential impact on human infectious diseases has not been conclusively investigated yet.

THE NOD2/*CARD15* SNPS

A striking correlation between an SNP of a PRR and disease susceptibility has been found for several mutations in the NOD2/*CARD15* gene and the incidence of Crohn's disease, which is a chronic inflammatory disease of the gastrointestinal tract occurring as frequently as 1 per 1000 in western countries (Hugot et al. 2001; Ogura et al. 2001a). Furthermore, the same mutations were found to be associated with 'Blau syndrome', a rare autosomal dominant disease consisting of uveitis, arthritis, and skin rash (Miceli-Richard et al. 2001). As mentioned above, several lines of evidence point in the direction that NOD2/*CARD15* acts as an intracellular PRR recognizing LPS or bacterial MDP (Bonen et al. 2003; Girardin et al. 2003; Inohara et al. 2003).

On the other hand, the NOD proteins similar to many of the related family members contain the caspase recruitment domain centrally involved in apoptosis regulation. It has thus been suggested that disturbance of NOD2 does not change pattern recognition but changes apoptosis regulation (Beutler 2001). This would correspond to the fact that anti-TNF treatment is highly efficient in Crohn's disease patients because TNF is a major apoptosis regulator. Recently, for Crohn's disease another, NOD2-independent genetic linkage has been found; the nature of the gene is, however, currently not known (Hampe et al. 2002).

Studies with different ethnic groups confirmed a role of NOD2/*CARD15* for Crohn's disease, ruling out an involvement in ulcerative colitis (Hampe et al. 2001). Ulcerative colitis, in contrast, was recently found in a small study to be associated with a polymorphism in the CD14 promoter, further supporting the concept of PRR SNPs being responsible for chronic inflammatory diseases of the gastrointestinal tract (Obana et al. 2002). The variation among different ethnic groups is reflected by one study showing the absence, in Japanese Crohn's disease patients, of the NOD2/*CARD15* mutations found in European populations (Kimouchi 2003). A recent study revealed an additional SNP in NOD2/*CARD15* among Ashkenazi Jews which also predisposes to the development of Crohn's disease (Sugimura et al. 2003).

New members of the CARD-containing protein family have recently been identified and several missense mutations have been found to be associated with rare inflammatory diseases, such as Muckle–Wells syndrome, familial cold urticaria, and chronic infantile neurological cutaneous and articular syndrome (McDermott 2002; Albrecht et al. 2003).

Most studies performed so far that investigate the potential effect of SNPs on the course of a particular disease focused on genetic variations of only a few proteins, including cytokines. However, the development and course of a bacterial infection are most probably a multifactorial event. Therefore, future studies investigating several SNPs in parallel and employing high-throughput technologies such as gene arrays may clarify whether SNPs of PRRs really influence the incidence and course of infectious and inflammatory diseases.

CONCLUSION

Pattern recognition is a key feature of the host's repertoire to react early to encounters with pathogens. Innate immunity most probably has an impact on both acute infectious disease complications, such as septic shock, and chronic illnesses caused by microorganisms. Innate immune responses are elicited by microbial compounds released from organisms, and thus cannot be 'treated' successfully by the application of antibiotics. APCs possess the ability to check the environment for these and other danger signals and orchestrate a response bridging innate and adaptive immunity. Major discoveries of the last 5 years include central host receptors of the Toll-like receptor family and their function. These results may lead to novel intervention strategies in acute and chronic infectious diseases that are currently not treatable by antibiotics. Genomic analyses confirm a central role of PRRs for incidence and course of infectious diseases. Intense basic and clinical research, with the help of modern molecular technologies, is, however, needed in order to exploit this growing field of immunology successfully.

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Apoptosis

ANTOINE GALMICHE AND ARTURO ZYCHLINSKY

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INTRODUCTION

In multicellular organisms, cells often die by apoptosis. This form of death occurs as a physiological event in the organism's tissues, where it ensures the elimination of unwanted cells and maintains tissue homeostasis. Apoptosis is an active process, i.e. the cell itself produces the effector proteins that kill it. For this reason, apoptosis is often compared to a cell 'suicide' (Hengartner 2000).

Characteristic morphological alterations accompany apoptosis (Mills et al. 1999; Hengartner 2000). In the nucleus, the chromatin condenses and accumulates at the level of the nuclear membrane. Cytoplasmic shrinkage and plasma membrane blebbing are also evident. Membrane blebs can ultimately pinch off the cell, forming vesicles called apoptotic bodies which contain cell organelles and nuclear material. These morphological alterations are distinct from the ones observed during necrosis, a form of cell death that follows accidental injury. In contrast to necrosis, there is no cytoplasmic swelling, and the integrity of the cell organelles is conserved until the late stages of apoptosis.

During apoptosis, the nuclear DNA is cleaved at the internucleosomal region, generating fragments ranging from 180 to 200 base-pairs. These fragments produce a typical 'ladder' when resolved on agarose gels. The surface of the cell is also modified. Phosphatidylserine, an amino-phospholipid normally restricted to the inner leaflet of the plasma membrane, flips to the outer leaflet. These changes allow phagocytes to recognize and remove apoptotic cells efficiently. Until their latest stages, apoptotic cells, however, display no changes of their plasma membrane integrity. This characteristic, together with the efficient clearance process mediated by phagocytes, ensures that the apoptotic cell does not release its contents.

THE INDUCTION OF APOPTOSIS: EXTRINSIC VERSUS INTRINSIC PATHWAYS

Apoptosis can occur as a consequence of: (1) extracellular stimulation via the engagement of death receptors present at the surface of the cell (e.g. Fas/CD95 or tumor necrosis factor (TNF) receptor), (2) changes in the transcriptional control (glucocorticoids), (3) disruption of organelles or DNA lesions induced, for example, by ionizing radiation or chemotherapy reagents, and (4) absence of trophic factors (reviewed in Hengartner 2000).

These stimuli activate two major cellular pathways controlling the occurrence of apoptosis (Figure 8.1): The extrinsic pathway is activated by extracellular stimuli such as the binding of death receptor ligands to their cognate receptors on the cell surface. In contrast, the intrinsic pathway is initiated as a consequence of many cell stresses (Figure 1). Although these two pathways are initiated and controlled in distinct fashions, two systems play a key role in their transduction: (1) mito-

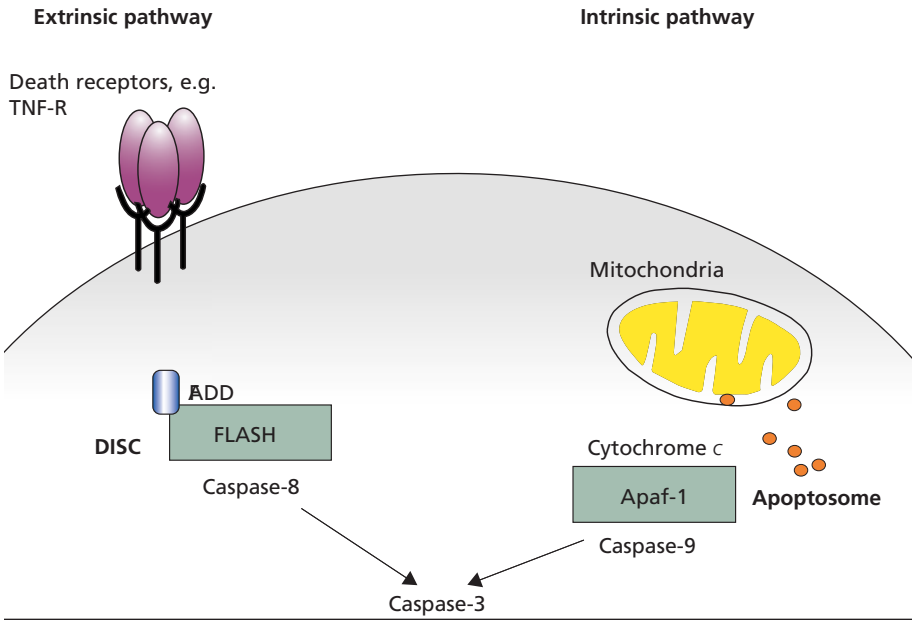

Figure 1 *Key components of the apoptotic pathway in mammalian cells. Two major pathways control the occurrence of apoptosis: one, called the intrinsic pathway, is activated by extracellular stimuli, e.g. the binding of death receptor ligands to their cognate receptors on the cell surface. These receptors transduce their death signal via adaptor proteins. These adaptor proteins mediate interactions between participants of the apoptotic cascade, e.g. the adaptor protein FADD is recruited by the activated tumor necrosis factor α (TNF-α) receptor. It in turn recruits and activates the initiator caspase caspase-8. In contrast, the intrinsic pathway is initiated as a consequence of cell stresses. A key role is played by the mitochondrial release of factors such as cytochrome c, activating the formation of a protein complex called apoptosome. The apoptosome contains the initiator caspase caspase-9 complexed with apoptosis protease-activating factor-1 (Apaf-1). Both the intrinsic and the extrinsic pathways activate the common downstream executioner caspase caspase-3. This executioner caspase is ultimately responsible for the cleavage of many cellular substrates.*

chondria and (2) caspases. In the following paragraphs, we summarize what is known about these two systems.

MITOCHONDRIA

Mitochondria are membrane organelles localized in the cytoplasm of most eukaryotic cells. They are formed by two membranes delineating an intermembrane space and the mitochondrial matrix. Mitochondria contain multiple enzyme and protein complexes involved in respiration and energy metabolism, at the level of the inner membrane and in the matrix. They also contain several factors, the release of which into the cytosol contributes to apoptosis: cytochrome *c*, the flavoprotein AIF, Smac/Diablo, HtrA2/Omi, endonuclease G, and some pro-caspases (Kroemer and Reed 2000). Cytochrome *c* is the mitochondrial factor with the best documented contribution to apoptosis induction. Upon its release from the mitochondrial intermembrane space, it serves as a cofactor for the assembly of the apoptosome and the activation of caspase-9 (see later). The mitochondrial outer membrane (MOM) is normally only permeable to solutes of small size, i.e. molecular weight or $M_r < 1.5$ kDa, and not to proteins such as cytochrome *c* (Kroemer and Reed 2000). An increased permeability of the MOM occurs early in the course of apoptosis. How apoptogenic stimuli are converted into changes in the permeability of the MOM is controversial, but different models have been proposed (Kroemer and Reed 2000), implicating either the formation of pores in the MOM or its complete rupture.

The proteins of the Bcl-2 family play a key role in regulating mitochondrial permeability (Cory and Adams 2002). In mammals, there are at least 20 relatives of the protein Bcl-2 (Figure 2). These proteins share at least one conserved Bcl-2 homology (BH) domain, BH1–4. Members of the Bcl-2 family can be classified into different subtypes: the most evident classification is based on their opposing functions: (1) the pro-survival family and (2) the pro-apoptotic family.

The pro-survival members of the Bcl-2 family

Bcl-2 and Bcl-XL are the best-characterized members of this family. These proteins share a carboxy-terminal membrane-targeting sequence. Bcl-XL is predominantly located in the MOM, whereas Bcl-2 has a broader membrane distribution, including the endoplasmic reticulum and nuclear envelope (Kaufmann et al. 2003). The pro-survival members of the Bcl-2 family are probably required in every cell of the organism in order to

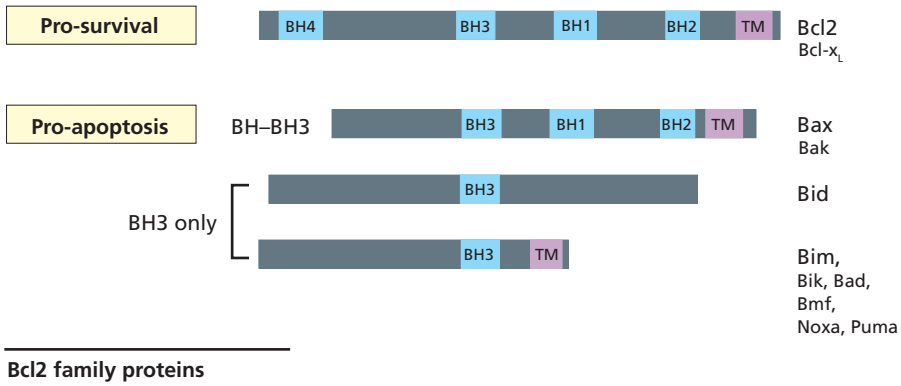

Figure 2 *Bcl-2 proteins. Proteins of the Bcl-2 family are often classified into anti- and pro-apoptotic subfamilies. They share some conserved domains (BH1–BH4), and hydrophobic carboxy-terminal membrane-targeting domain. The BH1–BH3 domains allow the members of the Bcl-2 protein family to associate to each other. The BH3 domain is present in all the members of the Bcl-2 family – it is essential for the pro-apoptotic function of the proteins Bax and Bak. The members of the BH3-only subfamily, such as Bad and Bid, function upstream of Bax/Bak. The BH3-only proteins constitute cellular sentinels, being able to activate the Bax/Bak system in response to a variety of death-inducing stimuli. Bcl-2 contains a BH4 domain which allows its interaction with other proteins. A caspase-cleavage site in this region eliminates this domain, thereby converting Bcl-2 into an apoptosis-promoting protein.*

prevent the spontaneous occurrence of apoptosis. Therefore, they play a critical function in tissue homeostasis.

The pro-apoptotic members of the Bcl-2 family

This family can be further separated into a BH3-only subfamily (Bad, Bid, Bim, Bmf, Noxa, Puma), and a BH1–3 subfamily (Bax and Bak) (reviewed in Cory and Adams 2002). The proteins Bax and Bak constitute the BH1–3 subfamily. Genetic evidence supports their role as core components of the apoptotic machinery, because Bax/Bak double knock-out cells are resistant to most apoptotic stimuli (Wei et al. 2001). The members of the BH3-only subfamily function upstream of Bax/Bak (Cheng et al. 2001; Zong et al. 2001). The BH3-only proteins constitute cellular sentinels, able to activate the Bax/Bak system in response to a variety of death-inducing stimuli. In a living cell, they are normally kept in check by different mechanisms, including sequestration at the subcellular level (interaction with the cell cytoskeleton in the case of Bim and Bmf), post-translational modifications (phosphorylation in the case of Bad, proteolytic processing in the case of Bid), or regulation at the transcriptional level (Noxa and Puma) (review in Cory and Adams 2002).

Kuwana et al. (2002) recently reported the use of a cell-free assay using purified components to reconstitute the permeation of the MOM by the protein Bax in vitro. In this system, Bax added to liposomes mimicking the composition of the MOM could permeate them to large-molecular-weight dextran molecules. This process occurred in the absence of any intrinsic mitochondrial proteins, but required the lipid cardiolipin (Kuwana et al. 2002). During apoptosis, protein efflux from mitochondria might be a consequence of local reorganization of the lipids in the MOM, driven by the proapoptotic protein Bax. This lipid reorganization might also cause the mitochondrial fragmentation that occurs during apoptosis (Karbowski et al. 2002).

The members of the pro-survival family of Bcl-2 proteins are able to bind directly and counteract the effect of the pro-apoptotic family members. In addition, they could protect cells from apoptosis independently of their mitochondrial effect (Kaufmann et al. 2003). Although the mechanisms involved are not yet clear, they may directly inhibit caspases (Marsden et al. 2002).

Clearly, many different events take place in the apoptotic mitochondria, and it is not yet possible to establish a causality link between them. Lately, a phenomenon known as the mitochondria-permeability transition (MPT) occurs. It is caused by the opening of a pore formed at the contact of the mitochondrial outer and inner membranes. This pore results in a drop in mitochondrial inner membrane polarity and swelling of the matrix, causing the rupture of the MOM and its non-selective permeabilization (review in Kroemer and Reed 2000).

CASPASES

Caspases (*c*ysteine *as*partyl-specific *prot*e*ase*) are a family of proteases that play a critical role in apoptosis (review in Earnshaw et al. 1999). As their name indicates, they are cysteine proteases that cleave their substrates after an aspartate residue. Caspases are initially produced as inactive zymogens. They share a common structural organization in three domains: an amino-terminal prodomain, and two catalytic domains p20 and p10 (Figure 3).

Depending on the level at which they participate in the apoptotic program, caspases are classified as initiator or executioner. Initiator caspases play a role in the early

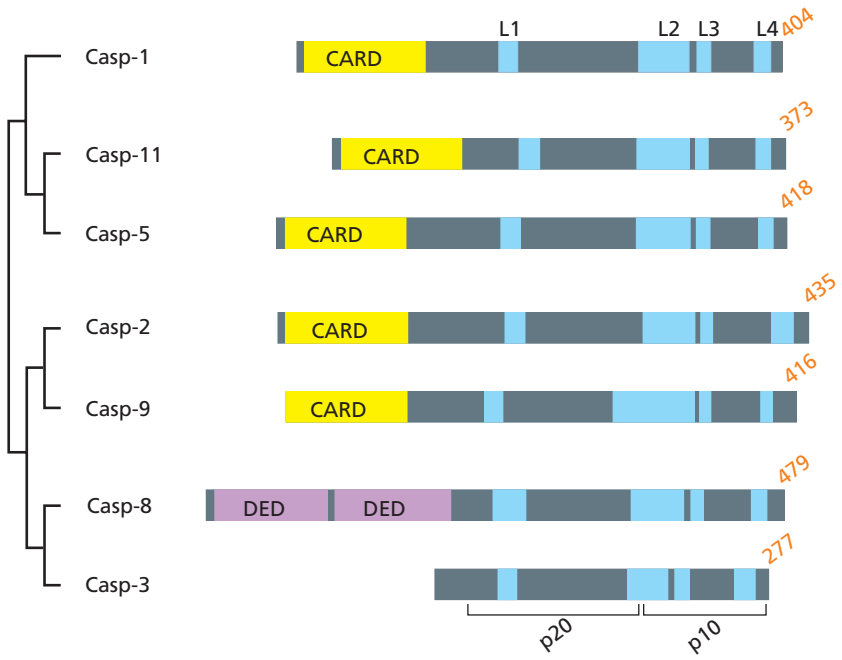

Figure 3 *Structure of caspases. Caspases are cysteine proteases that cleave their substrates after an aspartate residue. They are initially produced as inactive zymogens, and share a common structural organization in three domains: an amino-terminal prodomain, and the catalytic domains p20 and p10. Prodomain sequences vary among the members of the caspase family. In contrast to effector caspases such as caspase-3, initiator caspases such as caspase-9 or -1 contain a long amino-terminal prodomain. These prodomains contain one of the protein–protein interaction modules of the death adaptor family – death-effector domain (DED) in the case of caspase-8, caspase activation and recruitment domain (CARD) for other caspases: 1, 2, 9. These domains mediate homotypic interactions between the caspases themselves and with adaptor proteins. They therefore play a critical role in the activation of initiator caspases. (Adapted from Shi 2002.)*

phase of apoptosis, at the time the cell becomes committed to death. In contrast, executioner caspases are activated downstream of initiator caspases, and they are responsible for the morphological alterations that characterize apoptosis. These two types of caspases rely on two important mechanisms for their activation (review in Shi 2002): proteolytic cleavage of their constitutive domains and protein–protein interactions.

Proteolytic cleavage between their constitutive domains

Cleavage can be either autocatalytic or performed by other caspases. Proteolysis is the usual activation mode for the effector caspases, the prototype of which is caspase-3. Activated effector caspases cleave many proteins of the cytoskeleton, nucleus as well as other cellular organelles, thereby resulting in the characteristic hallmarks of apoptosis.

Protein–protein interactions

This activation mode is encountered with initiator caspases, which contain one of the protein–protein interaction modules of the death adaptor family in their prodomain. Members of this family are the death domain (DD), death effect or domain (DED), caspase activation and recruitment domain (CARD), and the newly described pyrin domain (PYD). Although they share no sequence homology, these domains have a common structural organization. Work so far suggests that they mediate homotypic interactions, typically between caspases and adaptor proteins (review in Shi 2002). These homotypic interactions between members of the death effector family are of paramount importance for the assembly of the large protein complexes that activate initiator caspases.

Here, we summarize only the data about the assembly of the death-inducing signaling complex (DISC), a complex that activates caspase-8, and the apoptosome, a complex that activates caspase-9. Other protein complexes contribute to the activation of caspase-1 (Martinon et al. 2002) and caspase-2 (Read et al. 2002), and are not described here.

The DISC is a large protein complex that activates caspase-8. It assembles as a consequence of the interaction between death ligands, such as TNF-α or Fas ligand, and their cognate receptors on the cell surface. TNF receptor family members contain a DD in their cytoplasmic tail, which allows them to recruit the adaptor protein FADD. FADD in turn recruits FLASH, a protein that assembles in a platform that recruits and activates caspase-8 by means of homotypic DED interactions (Imai et al. 1999).

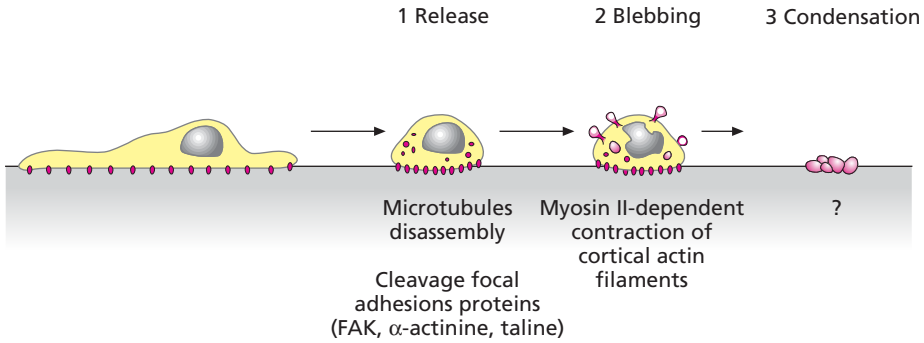

Figure 4 *The cytoplasmic execution phase of apoptosis. The cytoplasmic execution phase of apoptosis can be schematically divided into distinct steps: (1) a first step of release of the cell from its environment. The cell loses its attachment to extracellular matrix and to other cells, and thereby adopts a round appearance. The cleavage of some focal adhesion proteins, and a disassembly of the microtubule network, account for these modifications; (2) a step of blebbing, driven by the myosin II-dependent contraction of the cortical actin cytoskeleton; (3) ultimately, blebbing ceases and the cell leaves apoptotic bodies as only remnants. At that time, actin and microtubule cytoskeletons are disassembled or degraded. (Adapted from Mills et al. 1999.)*

The apoptosome is a cytoplasmic complex that assembles as a consequence of the release of cytochrome *c* from mitochondria. The cytoplasmic protein apoptosis protease-activating factor-1 (Apaf-1) is its main component. It recruits caspase-9 after binding cytochrome *c* in the presence of ATP/dATP. Pro-caspase-9 contains a CARD that mediates a specific interaction with the CARD of Apaf-1 (Shiozaki et al. 2002). A first determination of the structure of the apoptosome revealed that the Apaf-1/cytochrome *c* complex is a wheel-shaped oligomer, composed of seven Apaf-1 subunits (Acehan et al. 2002). The multimerized Apaf-1 forms a platform on which caspase-9 can dock and become activated in a CARD-dependent fashion.

Recently, a new potential aspect of caspase regulation has been highlighted: their subcellular compartmentalization. This could be an important determinant in their activation and substrate specificity, e.g. intracellular filaments recruit activated caspase-8 (Siegel et al. 1998). Nuclear import could also play a role in the compartmentalization of caspases. Candidate nuclear localization sequences (NLS) have been identified in the prodomains of caspase-1 (Mao et al. 1998) and caspase-2 (Baliga et al. 2002).

BASIC CELLULAR MECHANISMS AND REGULATION

Although caspase activation and mitochondrial permeabilization are involved in the changes observed in apoptotic cells, it is unclear to what extent these two systems are involved in the initial decision to die, and how much they contribute to reaching the 'point of no return' in the execution of death. Evolutionary considerations point to the importance of mitochondria in phylogenetically ancient organisms (Leist and Jaattela 2001). In mammals, however, this remains a controversial issue, and caspases might play a role upstream of mitochondria as initiators of the stress-induced apoptosis (Lassus et al. 2002). Nevertheless, there is a large consensus to attribute a role in the morphology of the dying cell to caspases.

Living cells normally prevent the spontaneous activation of the apoptotic systems. Among the proteins mediating this inhibitory effect, inhibitors of apoptosis (IAPs) are direct inhibitors of caspases (Shi 2002). All members of this family contain one or more BIR (baculovirus IAP repeat) domains, a domain of about 70 amino acids forming a zinc-coordinating fold. The proteins XIAP, c-IAP1, and c-IAP2 are direct caspase inhibitors: they bind to and inhibit caspases-3, -7, and -9. In addition, XIAP, c-IAP1, and c-IAP2 possess a RING-finger domain at their carboxy-termini. The RING-finger domain endows these proteins with a substrate-specific ubiquityl ligase activity toward caspase-3 and -7 (Jesenberger and Jentsch 2002). Ubiquitinylation is a post-translational modification of proteins consisting of the covalent attachment of ubiquitin, a small protein of 76 amino acids. Ubiquitin often targets proteins for degradation by the proteasome, a large cytoplasmic protease. Therefore, IAPs exert both direct and indirect inhibitory effects on caspases, by regulating their activity and controlling their destruction by the proteasome.

Decoy proteins also prevent the spontaneous activation of the apoptotic machinery in living cells. They exploit the use of protein domains involved in the apoptosis transduction pathway. Decoy proteins containing these domains prevent the spontaneous assembly of the caspase-activating complexes. This strategy is typically illustrated by the FLICE-inhibitory proteins (FLIP), proteins that possess DEDs and compete with caspase-8 for incorporation into the DISC.

Other systems appear to regulate cellular susceptibility to apoptosis although they are not directly part of its core machinery. The transcription factor NF-κB controls the synthesis of many regulators of the apoptotic pathway, such as members of the Bcl-2 or IAP family, and it therefore plays a major regulatory role during apoptosis (Karin and Lin 2002).

Once the initial brakes are relieved, activated caspases mediate the programmed demise of the cell. As more and more caspase substrates are described, we are gaining a better understanding of the key events responsible for the disposal of the cellular contents.

Important events take place in the nucleus. The DNase caspase-activated DNase (CAD) is activated as a consequence of the degradation of ICAD, its associated inhibitory protein, by caspase-3 (Enari et al. 1998; Sakahira et al. 1998). Activation of CAD is responsible for the cleavage of chromosomal DNA at the internuclosomal region, generating the typical DNA fragments visualized as a ladder on an agarose gel. Nuclear lamins, which are components of the nuclear membrane, are also cleaved. Lamin cleavage is probably partly responsible for chromatin condensation during apoptosis (Ruchaud et al. 2002).

The cytoskeleton participates actively in the morphological modifications that characterize apoptosis (Figure 4, review in Mills et al. 1999). In fibroblasts committed to apoptosis by death receptor activation, plectin constitutes an early substrate for caspase-8 (Stegh et al. 2000). Plectin, a member of the family of plakins, serves as a link among the three major constituents of the cell cytoskeleton: actin microfilaments, microtubules, and intermediate filaments. The cleavage of plectin is responsible for the early reorganization of the cytoskeleton observed in dying cells (Stegh et al. 2000). In epithelial cells, intermediate filaments (mostly of the cytokeratin family) are an early substrate of effector caspases (review in Oshima 2002). The cleavage of cytokeratins by activated caspases provides an interesting paradigm: in addition to contributing to the programmed dismantling of the cell, it probably provides a positive feedback for further caspase activation (Oshima 2002).

Membrane organelles such as the Golgi apparatus undergo fragmentation during apoptosis. The cleavage of protein p115, a key vesicle tethering protein required for the maintenance of this organelle, contributes to this fragmentation process (Chiu et al. 2002). Here, again, a cleavage product of p115 plays a key role by further amplification of the apoptotic response.

Taken together, the preliminary analysis of the apoptotic dismantling of the cell is consistent with the view that this process is highly organized, its multiple aspects being highly coordinated.

WHAT HAPPENS AFTER DEATH?

Apoptotic cells are efficiently recognized and removed by phagocytes (review in Savill and Fadok 2000). It is widely accepted that dying cells exhibit molecules that signal for their identification and clearance. A large number of cell adhesion molecules could participate in this process, although it is not yet clear which candidates really play a role in vivo.

A change in the distribution of phosphatidylserine (PtdSer), a phospholipid normally restricted to the inner leaflet of the plasma membrane, probably constitutes an important event for the recognition of apoptotic cells. In addition to the appearance of PtdSer on the outer face of the plasma membrane, apoptosis leads to the inappropriate surface localization of molecules that are normally restricted to internal cellular compartments. Many candidate receptors for PtdSer have been identified. The only candidates for which an in vivo contribution has been documented are the product of the proto-oncogene Mer (Scott et al. 2001), and the milk fat globule epidermal growth factor (EGF) 8 (MFG-E8), a secreted glycoprotein that might bridge PtdSer with integrins on macrophages (Hanayama et al. 2002). It is likely that they contribute in a cell- and tissue-specific fashion to the recognition of apoptotic cells.

Interestingly, the report of Brown et al. (2002) recently modulated the classic view that only 'positive' signals are responsible for the clearance of apoptotic cells. These authors showed that viable cells can deactivate macrophage phagocytic ability: a 'repulsive' signal follows the initial contact with the macrophage, and it is mediated by the homophilic interaction of the CD31 molecule present on both the macrophage and its prey. Apoptosis-induced disablement of this signal would promote the engulfment of non-viable cells (Brown et al. 2002).

The interface between the dying cell and the macrophage that attempts to eliminate it is an active one (review in Conradt 2002). The recognition of a dying cell triggers its phagocytosis by means of activation of effectors such as the small GTPase Rac. In return, the recognition of dying cells by macrophages could participate in the completion of the apoptotic program (Conradt 2002). Apoptotic cells signal not only toward macrophages, but also toward their neighbors: early during the apoptotic program, dying epithelial cells provoke their extrusion by an active, Rho GTPase-dependent contraction mechanism performed by their neighbors (Rosenblatt et al. 2001).

Traditionally, the recognition of apoptotic material is considered to have mostly anti-inflammatory and immunosuppressive effects (Savill and Fadok 2000). This view has been challenged recently and more work is clearly needed to determine how apoptosis influences the immune reaction. As the receptors involved in the clearance of apoptotic cells are better characterized, the role of apoptosis in inflammation and immunity will be better understood (Savill et al. 2002).

BACTERIAL-INDUCED APOPTOSIS

Bacterial pathogens induce apoptosis by a variety of mechanisms (Weinrauch and Zychlinsky 1999). One mechanism is the production of bacterial toxins, either pore-forming or with an intracytoplasmic mode of action.

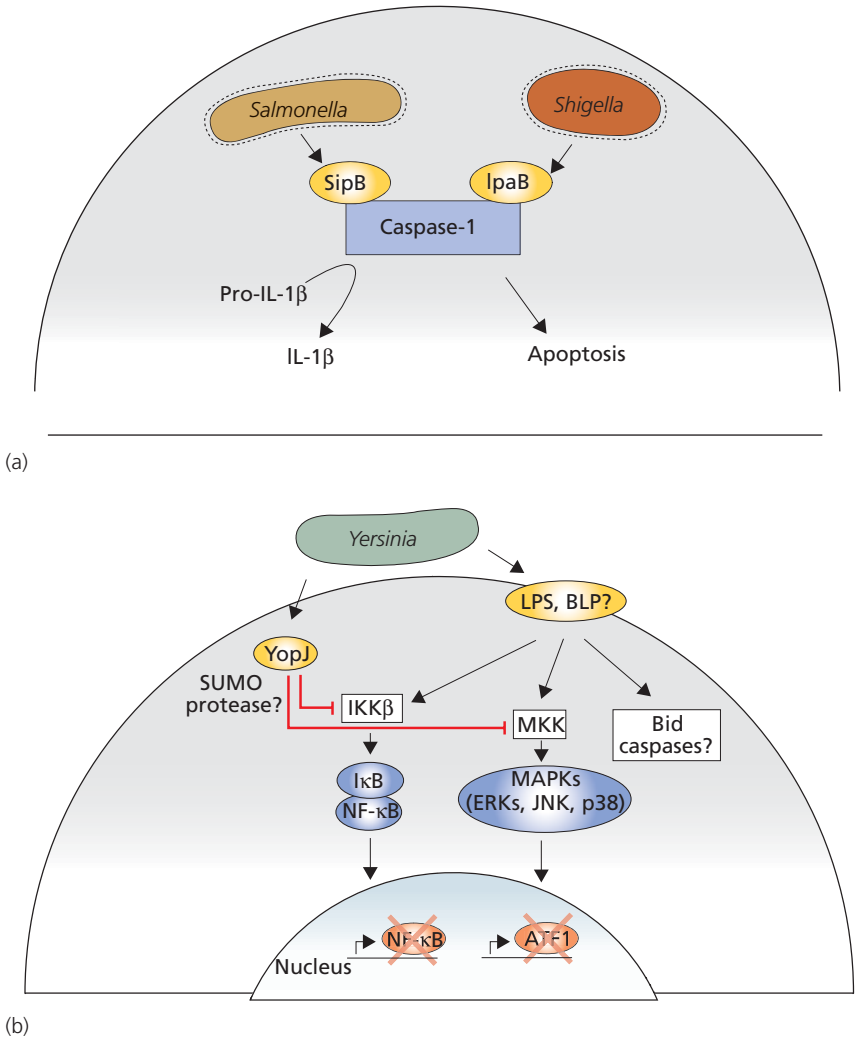

Figure 5 *Bacterial-induced apoptosis.* **(a)** Salmonella *and* Shigella *spp.-induced apoptosis.* Salmonella *and* Shigella *spp. provoke the death of macrophages by introducing, respectively, the proteins IpaB and SipB into their cytoplasm. The apoptosis that ensues is dependent on caspase-1. It is characterized by the simultaneous production and release of the proinflammatory cytokines interleukin (IL)-1β and IL-18. The molecular detail of the interactions between IpaB or SipB and caspase-1 are still unknown.* **(b)** Yersinia *spp.-induced apoptosis. The factor YopJ accounts for the ability of* Yersinia *spp. to kill its host's macrophages. YopJ has recently been shown to act as a cysteine protease, having the ubiquitin-like molecule SUMO as substrate. YopJ disrupts this post-translational modification. As a consequence, it inhibits the mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways. These two pathways are used by the activated macrophages to produce proinflammatory cytokines, i.e. tumor necrosis factor (TNF)-α. As a result, macrophage death is not accompanied by the production of inflammatory mediators. Other bacterial factors recognized by surface receptors of the Toll-like receptor (TLR) family, such as bacterial lipopolysaccharide or lipoproteins, probably play a cofactor role in macrophage death induced by* Yersinia *spp.*

Other bacterial products can be secreted and translocated into host cells by means of specialized secretion systems, such as type III secretion. We have chosen to discuss here three model microorganisms, *Salmonella*, *Shigella*, and *Yersinia* spp., which induce apoptosis of macrophages as part of their pathogenic strategy.

Shigella spp are the etiological agent of bacterial dysentery, a diarrheal syndrome. This intestinal infection is an acute, inflammatory disease caused by an acute destruction of the colonic epithelium. In contrast, *Salmonella typhi* disseminates in the immune tissues of its host, causing systemic infections such as typhoid fever in humans. Early during the course of its infection, *Shigella* spp. induce apoptosis of macrophages in the lymphoid follicles of the intestinal mucosa. The bacterial protein IpaB is responsible for macrophage death. IpaB is introduced into the cytoplasm by a type III secretion apparatus. Macrophage apoptosis induced by the bacteria of the genus *Salmonella* is mediated by the bacterial protein SipB, which shares more than 40 percent sequence identity with IpaB (Hersh et al. 1999). Both IpaB and SipB induce macrophage apoptosis by activating caspase-1: macrophages from caspase-1 knock-out mice survive infection by *Shigella* (Hilbi et al. 1998) or *Salmonella* spp. (Hersh et al. 1999). In addition to its role in macrophage death, caspase-1 is also a

cytokine processor (it was initially named interleukin 1β (IL-1β)-converting enzyme, or ICE): its activation provokes, together with macrophage death, the release of the proinflammatory cytokines IL-1β and IL-18.

Caspase-1 knock-out mice infected with *Shigella flexneri* do not develop the acute inflammation that is characteristic of shigellosis (Sansonetti et al. 2000). These mice are also much less sensitive than their wild-type counterparts to oral infection, but not to intraperitoneal infection, by virulent *Salmonella* spp. (Monack et al. 2000). Caspase-1 activation and macrophage apoptosis are therefore important events in the course of shigella and salmonella infections. Both these bacteria use macrophage apoptosis as a virulence strategy to initiate an inflammatory response that probably favors their invasion of the intestinal mucosa.

The *Yersinia* genus offers another example of bacterial-induced macrophage programmed cell death. It includes three species that are pathogenic in humans: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. Whereas *Y. pestis* is the causative agent of the plague, *Y. enterocolitica* and *Y. pseudotuberculosis* cause gastrointestinal syndromes, lymphadenitis, and septicemia. These symptoms appear as a consequence of the ability of *Yersinia* spp. to survive inside the lymphatic tissue of the host. In contrast to *Shigella* or *Salmonella* spp., *Yersinia* spp. are extracellular pathogens. They are able to induce macrophage apoptosis, and this apoptotic response is dependent on the bacterial protein YopJ/P (YopJ in *Y. pestis* and *Y. pseudotuberculosis*, YopP in *Y. enterocolitica*). In the macrophage cytosol, YopJ/P blocks the activation of the transcription factor NF-κB and the activity of a family of kinases: the MAPKs (Figure 5). It probably does so by cleaving the protein SUMO, a ubiquitin-like molecule that is covalently added to numerous regulatory proteins (Orth et al. 2000). NF-κB controls the transcription of genes that promote cell survival and the production of proinflammatory cytokines. In contrast to *Shigella* or *Salmonella* spp., by preventing the activation of NF-κB, *Yersinia* spp. induce macrophage apoptosis without causing inflammation. This response probably represents an adaptation to the extracellular lifestyle of *Yersinia* spp., which renders these bacteria extremely sensitive to inflammatory mediators such as IL-8, interferon-γ (IFN-γ)- or TNF-α. The simultaneous elimination of phagocytic cells and the suppression of proinflammatory cytokine production promote systemic dissemination of *Yersinia* spp. (Monack et al. 1998).

Apoptosis induction is not only induced by bacteria for purposes of pathogenicity, but is also an immune response used by the host against bacteria, in order to limit the extension of the infection. In mammals, this mechanism might particularly operate at the level of epithelia. In a mouse model of *Pseudomonas aeruginosa* pneumonia, Grassme et al. (2000) observed that bronchial cell apoptosis mediated by the cell death receptor CD95 allowed an increased survival of the animals. Apoptosis might contribute to the elimination of cells that are in contact with germs and to the prevention of bacterial dissemination. Apoptosis might therefore serve as an important mechanism preventing the establishment of 'sanctuaries' for pathogens.

Apoptosis is probably not only a mechanism that limits the pathogen's niche. It might also contribute to the initiation of a specific immune response. Following the apoptosis of *Salmonella typhimurium*-infected macrophages, bacterial antigens can be efficiently taken up and presented by bystander dendritic cells (Yrlid and Wick 2000). It is not yet clear how much the processing of antigens obtained from apoptotic macrophages contributes to the activation of antibacterial immune mechanisms. However, during the course of bacterial infection, macrophages could, by committing 'altruistic' suicide, (1) favor the recruitment of potent bactericidal effectors such as polymorphonuclear cells to the site of infection and (2) help deliver antigenic material in an efficient way to other immunocompetent cells. Apoptosis might therefore play a role in the immune response against some bacterial pathogens.

CONCLUSION

Major progress has been achieved in the field of apoptosis during the last decade. While the existence of programmed cell death has gained widespread acceptance, it has been possible to identify some key components of the death pathway. Apoptosis is now acknowledged as an important step in the pathogenicity of many diseases, including bacterial infections.

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Complement

B. PAUL MORGAN

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INTRODUCTION

Complement (C) is a central component of innate immunity, playing important roles in defense against pathogens and the handling of dead and dying cells and cell debris. It also influences adaptive immunity, providing a bridge between these defense systems. The C system has been reviewed and revisited scores of times over the past three decades and it is not my intention in this chapter to rehash the reviews that have gone before. Instead, I will summarize what I believe is essential for an understanding of the system and then focus on more recent developments that have made the study of C exciting again, particularly those of relevance to human disease. Along the way I will, of course, show my own biases and give undue attention to aspects that I find personally fascinating. I hope that these excursions will be excused and perhaps even entertain.

Complement comprises a group of 17 soluble plasma proteins that interact with one another in three distinct enzymatic activation cascades (the classical alternative and lectin pathways) and in the nonenzymatic assembly of a cytolytic complex (the membrane attack pathway) (Figure 1 and Table 1). It is rigidly controlled by a battery of at least 10 regulatory proteins (CReg) present in plasma and on cell membranes which together prevent damage to self and rapid consumption of C in vivo. In the following sections, the anatomy of the C system, its activation and control, the physiological and pathological roles of C and its activation, the diseases associated with defective C function, and therapies targeting C are described.

HISTORICAL ASPECTS

Complement was discovered during the last decade of the nineteenth century from studies of the capacity of immune sera to kill bacteria and lyse foreign erythrocytes. Two serum factors were required for these bactericidal and hemolytic activities: a heat-stable factor present only in immune serum and a heat-labile factor that was present in both immune and nonimmune sera (Ehrlich and Morgenroth 1899, 1900). The heat-stable factor, termed 'immune body' by Ehrlich, was of course antibody present only in the immune serum. The factor destroyed by warming the serum (a critical cut-off of 56°C was demonstrated later), Bordet and, later, Ehrlich considered 'a complement' to the killing capacity of the immune bodies, coining the name we now all know and love (Bordet 1900). Another important finding from these early studies was the demonstration that C was used up or consumed during lysis of erythrocytes, indicating that it was more than the accessory factor postulated by Ehrlich, and rather an active participant in the lytic reaction, defined by Muir as 'that labile substance of normal serum which is taken up by the combination of an antigen and its antisubstance' (Muir and Browning 1904). The multi-component nature of C was first demonstrated by Ferrata (1907) in the first decade of the twentieth century. He showed that C comprised both water-soluble (euglobulin) and insoluble (pseudoglobulin) components, later termed C′1 and C′2 respectively. Application of other fractionation techniques identified further 'components', termed C′3 and C′4, so

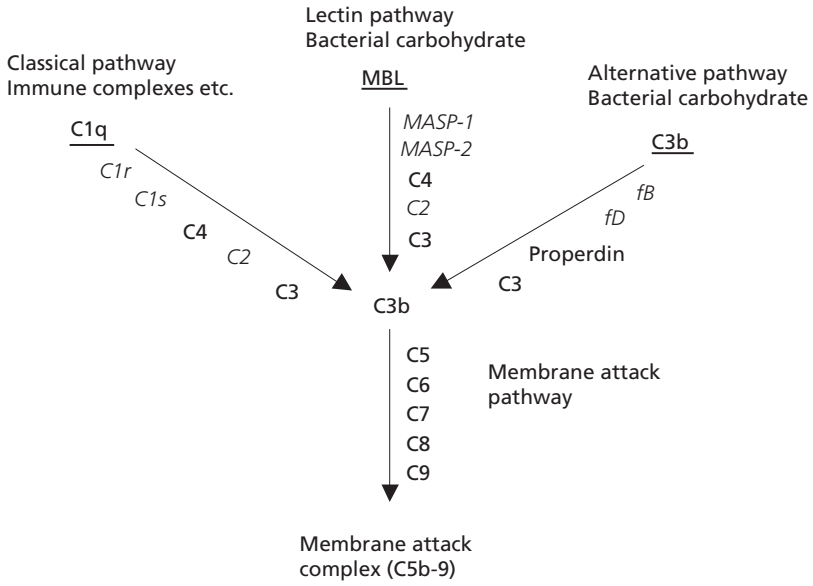

Figure 1 *The complement system. The constituent pathways of the C system and the component proteins are shown in sequential order. The initiating component of each pathway is underlined. Components that are enzymes or proenzymes are shown in italics. Abbreviations are as in the text.*

that by the mid-1920s it was clear that C comprised at least four different and separable activities from serum (Whitehead et al. 1925). However, the nature of these activities was unknown. Definitive evidence that at least some of the 'components' were protein in nature was first provided by Pillemer in his seminal work during the 1940s and 1950s, applying the new electrophoretic and ultracentrifugation techniques (Pillemer et al. 1954). These studies also revealed the presence of a second activation pathway, distinct from the antibody-triggered 'classical' pathway of Ehrlich, which was termed the 'alternative' pathway (Pillemer et al. 1954; Blum et al. 1959). Over the next two decades, pioneering use of separation methods by the group of Muller-Eberhard provided further dissection of the C system with the identification of at least nine protein components by the mid-1960s (Muller-Eberhard 1969). Complementary functional analyses, particularly from the groups of Mayer and Lepow, established the enzymatic nature of C activation and the reaction sequences from which the pathways emerged (Mayer 1965).

THE ANATOMY OF THE C SYSTEM

Complement can be activated in several different ways, reflecting the various targets for the system. The hemolytic and bacteriolytic phenomena observed by Bordet and Ehrlich were dependent on antibody that triggers activation of the classical pathway. However, pathogens and other 'foreign' targets activate C in the absence of antibody via two more recently described pathways, the alternative pathway and the lectin pathway.

Activation of the classical pathway

The classical pathway is 'classically' triggered by antibody attached to particulate antigen, although many other substances, including components of damaged cells, bacterial lipopolysaccharide (LPS), C-reactive protein (CRP), and nucleic acids, can also trigger the classical pathway in an antibody-independent manner. Attachment of immunoglobulin (IgG) antibody in sufficient density on the particle surface creates an environment in which the first component of C, C1, can bind. Not all subclasses of IgG antibodies are capable of binding C1; sequence differences in the antibody Fc (Cγ2 domain) regions influence the C1-binding site such that, in humans, IgG1 and IgG3 are strong binders, IgG2 binds weakly, and IgG4 does not bind. Classical pathway-activating capacity correlates precisely with C1 binding (Lucisano Valim and Lachmann 1991). IgM antibody is a strong binder of C1 and, because of its multimeric (pentameric) nature, does not require a high surface density to activate C. Conformational changes occur in the IgM molecule on attachment to antigen, making the Fc (Cμ3 domain) regions available for binding C1 (Perkins et al. 1991). C1 is a large multicomponent complex (molecular weight or M_r about 800 kDa), comprising a single molecule of the recognition unit, C1q, and two molecules each of the enzymatic units, C1r and C1s (Arlaud et al. 1989). The C1q molecule is a member of a growing family of proteins, the collectins, which have both collagen-like and lectin-like domains (Holmskov et al. 1994). Six subunits, each composed of three homologous chains (A, B, and C), associate along an extended collagenous tail and then

Table 1 *The component proteins of the C system*

Component	Structure	Sub-component	Plasma concn (mg/l)	(μmol/l)
Classical pathway				
C1	Complicated molecule, composed of three proteins, C1q (460 kDa), C1r (80 kDa), C1s (80 kDa) in a complex (C1qr2s2)	C1q	180	0.40
		C1r	34	0.43
		C1s	31	0.39
C4	Three chains (α, 97 kDa; β, 75 kDa, γ, 33 kDa); from a single precursor		600	2.93
C2	Single chain, 102 kDa		20	0.20
Alternative pathway				
fB	Single chain, 93 kDa		210	2.26
fD	Single chain, 24 kDa		2	0.08
Properdin	Oligomers of identical 53 kDa chains		5	0.09
Lectin pathway				
MBL/MASP	Complicated molecule, composed of three proteins, MBL (about 600 kDa), MASP-1 (93 kDa), MASP-2 (76 kDa) in a complex. Excess of MASP-1 over MBL in plasma	MBL	1[a]	0.002[a]
		MASP-1	6	0.07
		MASP-2	nk	–
Common				
C3	Two chains: α, 110 kDa, β, 75 kDa		1300	7.03
Terminal pathway				
C5	Two chains: 115 kDa, 75 kDa		70	0.39
C6	Single chain, 120 kDa		65	0.54
C7	Single chain, 110 kDa		55	0.50
C8	Three chains: α 65 kDa; β 65 kDa; γ 22 kDa		55	0.36
C9	Single chain, 69 kDa		60	0.89

a) The plasma concentration of MBL varies widely among normal individuals. The proteins that constitute the classical, alternative, and membrane attack pathways are listed. MBL, mannin-binding lectin; MASP, MBL-associated serine proteases; nk, not known. Modified from: Morgan, B.P. and Harris, C.L. 1999: *Complement regulatory proteins*. London: Academic Press.

separate to form the six globular heads, each of which represents a binding site for antibody Fc. Between the heads sits the enzymatic core, the $(C1r:C1s)_2$ complex (Figure 2). C1r and C1s are homologous single-chain molecules of M_r 80 kDa, which associate with one another and with C1q in a Ca^{2+}-dependent complex (Reid 1986; Reid and Day 1989). Upon binding to antibody through several head groups, conformational changes occur within the C1q molecule which in turn cause conformational alterations in the C1r proenzyme, triggering autocatalytic activation by cleaving a single site (R^{446}–I^{474}) in the molecule. Active C1r cleaves C1s at a single site (R^{422}–I^{423}) to activate the C1s serine protease (Arlaud et al. 1989). C1s in the activated C1 complex will cleave and activate the next component of the classical pathway, C4.

C4 is a large, plasma protein (M_r 200 kDa) containing three disulfide-bonded chains (α, β, and γ) (Schreiber and Muller-Eberhard 1974; Janatova and Tack 1981). C1s cleaves C4 at a single site (R^{737}–A^{738}), near the amino-terminus of the α chain, releasing a small fragment, C4a (M_r about 9 kDa), and exposing a labile, reactive, thioester group in the α chain of the large fragment, C4b. The thioester, formed between residues C^{991} and Q^{994} in the sequence GCGEQT, is key to the way that C acts selectively on targets (Dodds et al. 1996; Law and Dodds 1997). The exposed thioester in C4b forms covalent amide or ester bonds with exposed amino or hydroxyl groups, respectively, on the activating surface, locking the molecule to the surface. The two isotypes of C4, C4A and C4B, although identical apart from four clustered amino acids in the α chain, differ in their

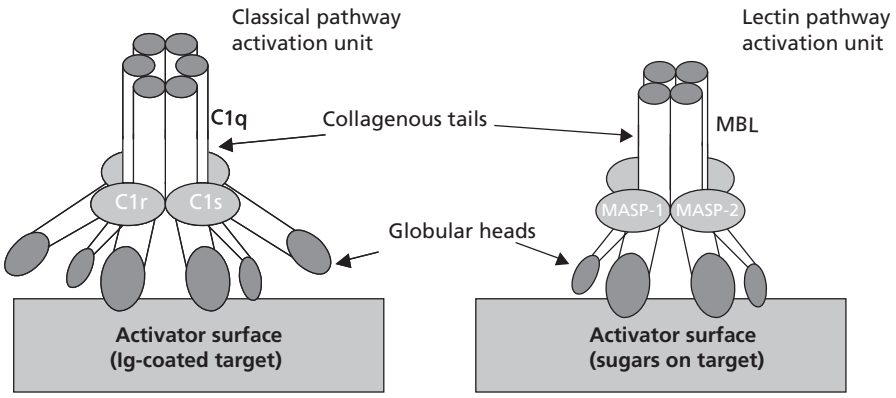

Figure 2 *The initiating molecules of the classical and lectin pathways. The activating unit of the classical pathway comprises one molecule of the multimeric C1q (itself made up of six subunits, each composed of three polypeptide chains), together with two molecules each of C1r and C1s arranged as a tetramolecular complex close to the heads of C1q. The activating unit of the lectin pathway has a very similar structure, comprising one molecule of mannan-binding lectin (MBL) (itself made up of between two and six subunits), together with two molecules each of MBL-associated serine proteases (MASP) MASP-1 and MASP-2 arranged as a tetramolecular complex close to the heads of MBL. Each unit binds an activating surface through its globular head groups, thereby triggering activation of the enzymatic components of the complex.*

surface-binding proclivities such that C4A preferentially binds amino groups and forms amide bonds whereas C4B favors hydroxyl groups and forms ester bonds (Tosi et al. 1985). The terminology used for C4 represents one of the many opportunities for confusion in the complement system, the lower case suffixes a and b denoting the fragments generated on cleavage of C4 and the upper case suffixes A and B the isotypes, both of which are cleaved by C1. Attachment of C4b is an inefficient process because the thioester is rapidly inactivated in the fluid phase by hydrolysis, effectively limiting the spread of 'active' C4b to the area surrounding the activating C1. C4b bound close to C1 provides a receptor for the next component of the classical pathway, C2, and presents it for cleavage by C1.

C2 is a single chain plasma protein of molecular weight 102 kDa (Kerr and Porter 1978). In the presence of Mg^{2+} ions, the proenzyme C2 binds membrane-bound C4b and is cleaved by C1s in an adjacent C1 complex at a single site (R^{223}–K^{224}). Cleavage is absolutely dependent on the association of C2 with C4b and fluid-phase C2 is not cleaved by C1. The larger carboxy-terminal fragment, C2a, containing the serine protease domain, remains attached to C4b to form the C4b2a complex, the next enzyme in the classical pathway, whereas C2b is released. Again, terminology is confusing. For most cleavages in C activation, the larger fragment that is required for propagation of activation is termed the 'b' fragment; for historical reasons, C2 does not follow this pattern.

C3, a 185-kDa two-chain molecule, is the most abundant of the C components (1–2 mg/ml in serum) and is essential for activity of all activation pathways (Lambris 1988). C3 attaches loosely to the C4b2a complex and is cleaved at a single site in the α-chain (R^{726}–S^{727}) by the C2a enzyme. Cleavage releases a small, biologically active peptide, C3a (77 amino acids; M_r 9 kDa), from the amino-terminus of the α chain and exposes in the large fragment, C3b, a labile thioester group formed between residues C^{988} and Q^{991} in the sequence GCGEQN. The thioester shares the properties of that in C4b and is similarly susceptible to inactivation by hydrolysis. C3b binds covalently via the thioester either to the activating C4b2a complex or to the adjacent membrane (Kozono et al. 1990; Ebanks et al. 1992). C3b bound to the activating C4b2a complex creates a new enzyme, C4b2a3b, the C5-cleaving enzyme (convertase) of the classical pathway (Kinoshita et al. 1988).

C5 is a two-chain protein of molecular weight 190 kDa, present in plasma at about 75 μg/ml (Tack et al. 1979). C5 is structurally related to C3 and C4 but lacks the thioester so it cannot bind covalently to surfaces. C5 attaches to C3b in the C4b2a3b convertase and is cleaved at a single site in the α chain (R^{733}–L^{734}) by C2a in the complex. A small, glycosylated fragment, C5a (74 amino acids; M_r about 10 kDa), is released from the amino terminus of the α chain, the large fragment, C5b, remaining attached to the convertase.

Activation of the alternative pathway

The alternative pathway provides a rapid, antibody-independent route for activation and amplification of C on foreign surfaces (Figure 3). The efficiency of alternative pathway activation results in large part from the fact that it is in a constant state of low-level or 'tickover' activation which targets all exposed surfaces (Lachmann and Hughes-Jones 1984; Law and Dodds 1990). As in the classical pathway, C3 is the central player, but three

unique proteins, factor B (fB), factor D (fD), and properdin, are also involved. During tickover, C3 in plasma is hydrolyzed to form a metastable $C3(H_2O)$ molecule which, in the presence of Mg^{2+} ions, binds fB, a single-chain (M_r 90 kDa) protein closely related to C2 and performing, in the alternative pathway, a role analogous to that of C2 in the classical pathway. Once bound to $C3(H_2O)$, fB is cleaved by fD, a highly specific and constitutively active serine protease (M_r 25 kDa) present in plasma at very low concentrations (2 μg/ml). FD cleaves fB in the $C3(H_2O)$ complex at a single site (R^{234}–K^{235}), releasing the smaller fragment Ba (M_r 30 kDa) and exposing in the larger fragment, Bb, a cryptic serine protease domain (Gotze 1986). The fluid-phase C3 convertase thus formed can now cleave plasma C3 at a single site identical to that described for the classical pathway, releasing C3a and exposing the thioester in C3b that allows covalent binding to surfaces. C3b binds indiscriminately to surfaces but further propagation continues only on so-called 'activator' surfaces such as invading bacteria. Healthy self-cells are 'nonactivator' and do not favor propagation. On activator surfaces, covalently bound C3b binds fB, enabling fD cleavage to form a surface C3bBb enzyme that cleaves more C3b, driving amplification and coating of the activating particle with C3b. Self-cells are nonactivators in part because of the expression of membrane C regulators, described below. However, it is now clear that the fluid-phase C regulator factor H (fH) is the main discriminating factor, binding and inhibiting C3bBb enzymes on nonactivators but ignoring those on activator surfaces (Jokiranta et al. 1996). Properdin, a single-chain plasma protein, also contributes to the survival of C3bBb on activator surfaces by binding and stabilizing the clustered complex (Lambris et al. 1984).

A proportion of the nascent C3b formed will attach directly to the C3bBb enzyme, creating in the trimolecular complex, $(C3b)_2Bb$, a binding site for the next component, C5, and simultaneously altering the substrate specificity of the enzyme to a C5 convertase. This enzyme binds and cleaves C5 in precisely the same way as described above for the classical pathway C5 convertase.

The lectin pathway

This newly characterized antibody-independent pathway of C is perhaps better described as a C1 bypass route because, apart from the initiating component, it is identical to the classical pathway (Gadjeva et al. 2001). The role of C1 is played by another complex molecule, structurally related to C1 and comprising a collectin recognition unit and associated serine proteases. The recognition unit, mannan-binding lectin (MBL) is a high-molecular-weight serum lectin, made up of between two and six structural units, each a trimer of a single 32-kDa chain. The subunits associate in a collagenous tail and separate into heads that form the recognition units (see Figure 2). MBL binds the simple carbohydrates

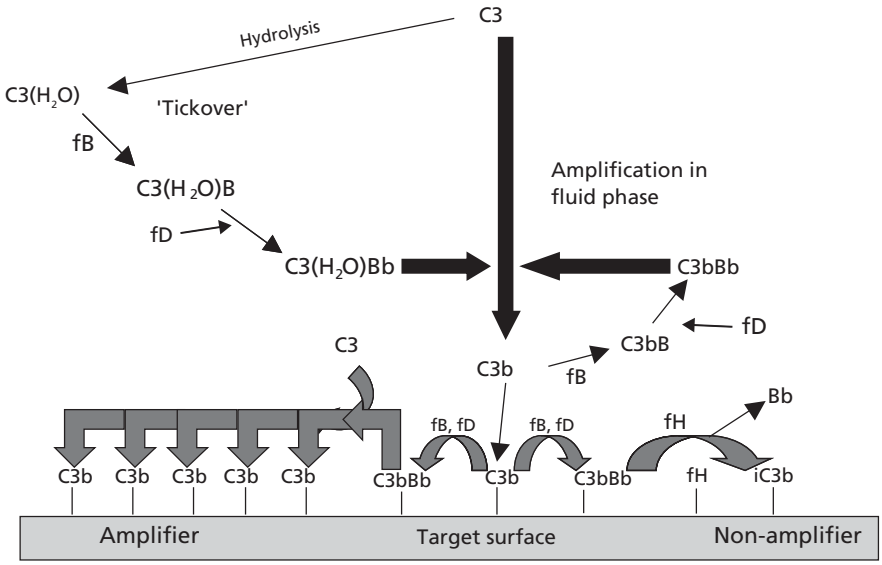

Figure 3 *The alternative pathway activation loop and the role of fH. Activation begins in the fluid phase with the slow, spontaneous hydrolysis of C3 to form $C3(H_2O)$. Binding of fB to $C3(H_2O)$ and subsequent cleavage by fD generates a fluid-phase convertase, $C3(H_2O)Bb$, that cleaves C3 to C3b. Some of the C3b formed will bind fB and perpetuate activation in the fluid phase but a proportion will bind surrounding surfaces and form C3 convertases. On nonactivating surfaces (self-cells), fH (present in the fluid phase and on the surface) inactivates the bound convertase and stops further activation. On activator surfaces, amplification proceeds unhindered and the surface rapidly becomes coated with C3b.*

mannose and *N*-acetylglucosamine present on the cell walls or envelopes of diverse pathogens, including bacteria, yeast, fungi, and viruses (Holmskov et al. 1994; Reid and Turner 1994). The enzymatic activity resides in MBL-associated serine proteases (MASP) MASP-1 and MASP-2, structural and functional homologues of C1r and C1s. Binding of the MBL–MASP complex to targets causes autolytic cleavage at a single site in MASP-1 and MASP-2 to unmask the serine protease domains. Although the precise roles of the two proteases are unclear, the weight of evidence suggests that MASP-2 in the complex is the C2-cleaving moiety. The activated MBL–MASP complex cleaves C4 and subsequently C2 in a manner identical to that described for C1 in the classical pathway. There is in vitro evidence that MASP-1 directly cleaves C3, bypassing the requirement for C4 and C2. The physiological relevance of this observation is not known. Recent evidence indicates that the ficolins, a family of lectin-like proteins present in plasma, can substitute for MBL and form C-activating complexes with the MASPs (Matsushita and Fujita 2001).

The membrane attack pathway

The membrane attack or terminal pathway involves the noncovalent association of C5b with the four terminal C components, C6, C7, C8, and C9, to form an amphipathic membrane-inserted complex, the membrane attack complex (MAC) (Muller-Eberhard 1986). The four terminal components are homologous molecules that have arisen by gene reduplication during evolution, a fact that is apparent from studies of the primitive C system of sharks (Jensen et al. 1981). Sharks generate a functional MAC from a single terminal component that most closely resembles human C9. The added complexity in mammals probably provides a more efficient targeting system to limit damage to self. The first step in MAC assembly involves the capture by C5b, still attached to the C5 convertase, of C6, a large single chain protein (M_r 120 kDa) present in plasma at about 50 μg/ml (DiScipio and Hugli 1989). A binding site newly exposed in C6 enables the C5b6 complex to capture the next component in the sequence, C7, a single chain protein (M_r 90 kDa; about 90 μg/ml in plasma) (DiScipio et al. 1988; DiScipio 1992a). Capture of C7 triggers release of the trimolecular complex from the convertase, and creates a binding site for the next component (C8), and a labile hydrophobic binding site through which C5b67 can bind tightly to the membrane. As a result of the lability of the membrane-binding site and the presence of plasma inhibitors (described below, under The membrane attack pathway), most of the C5b67 formed does not attach to membrane but 'decays' to an inactive complex that can be detected in plasma as an index of C activation. C5b67 that does bind membrane becomes tightly associated but does not disrupt the bilayer. C8, a large, heterotrimeric plasma protein (150 kDa; 80 μg/ml in plasma), binds C7 in membrane-associated C5b67, causing the tetrameric C5b-8 complex to insert deeper in the membrane and creating binding sites for the final terminal component, C9 (M_r 70 kDa; 60 μg/ml in plasma) (Sodetz 1989). C9 undergoes a major conformational change upon binding C5b-8, from a compact, globular shape to an extended conformation that traverses the membrane. As well as disrupting the integrity of the bilayer, this event exposes C9 binding sites for additional C9 molecules which in turn unfold, insert in the membrane, and recruit more C9 until a rigid ring structure, the classical MAC, has assembled (Podack and Tschopp 1984). The MAC ring, comprising between 12 and 18 C9 molecules, surrounds a pore through which ions and water can pass, causing osmotic lysis of the target. It should be noted that complete rings, the hallmark of the MAC, are not essential for this lytic process; even complexes containing as few as two or three C9 molecules cause considerable bilayer disruption, generating functional pores.

The active products of C

The physiological roles of C are mediated by the products of the pathways described above. In the activation pathways, the small fragments of C3 and C5 shed upon enzymatic cleavage, C3a and C5a, are powerful cell activators whereas the large fragments of C3 and C4 bound to the activating surface, C3b and C4b, direct phagocyte attack. The MAC can cause direct lysis of targets but may also trigger activation events in cells.

The opsonic C fragments

During C activation, pathogens become coated with C3b, C4b, and their breakdown products, a process termed 'opsonization'. These large fragments bind specific C receptors on phagocytes, triggering internalization and destruction of the pathogen. The important phagocyte receptors are C receptor 1 (CR1, CD35), which binds C3b and C4b, and CR3 (CD11b/CD18) and CR4 (CD11c/CD18) which both bind inactivated C3b (iC3b) (Krych et al. 1992). CR1 is a member of the regulators of C activation (RCA) family and, indeed, also functions as a C regulator (see below) (Rey-Campos et al. 1990). CR3 and CR4 are members of the integrin family of cell surface receptors (Myones et al. 1988). C3 fragments iC3b and C3d also bind another receptor, CR2 (CD21), an RCA family member structurally related to CR1 (Krych et al. 1992). CR2, absent on phagocytes but expressed on B cells, some T cells, follicular dendritic cells, and a few other cell types, plays

important roles in the response to antigen, described below, under Role of C in the adaptive immune response. CR1 is also expressed on erythrocytes where it plays an important role in the transport of immune complexes (Madi et al. 1991; Pascual and Schifferli 1992). Immune complexes, coated with C3b through classical pathway activation, bind to CR1 on the erythrocyte, effectively removing the immune complex from the circulation – the immune adherence phenomenon. Immune complexes are stripped from erythrocytes passing through the spleen and liver and transferred to fixed macrophages, expressing C receptors and antibody Fc receptors, which endocytose and destroy the complex. The mechanism by which the immune complex is released from erythrocyte CR1 and the fate of CR1 during this process of immune complex stripping are still the subject of debate. Early studies suggested that CR1 was cleaved from the erythrocyte and lost during delivery; indeed, the reduced expression of CR1 on erythrocytes in systemic lupus erythematosus (SLE) was ascribed to this process (Davies et al. 1990, 1992). However, others showed that there was little or no loss of CR1 during passage of immune complex-loaded erythrocytes through the spleen and proposed a dynamic binding model for erythrocyte transport of immune complexes (Schifferli and Peters 1983; Schifferli and Taylor 1989). C3b attached to CR1, a cofactor for fI, is rapidly cleaved to iC3b, thereby releasing the attachment, only for another C3b to take up the attachment. In the spleen, macrophages expressing multiple C receptors and Fc receptors compete to capture the immune complex without loss of erythrocyte CR1. To complicate things still further, more recent elegant studies of immune complex handling in nonhuman primates clearly show that proteolysis of CR1 occurs together with delivery of the immune complex, brought about by specific proteases on the macrophage surface after the immune complex-laden erythrocyte has been captured through macrophage Fc receptors (Nardin et al. 1999).

The anaphylactic and chemotactic C peptides

Several active fragments are released into the fluid phase during C activation. C3a and C5a are structurally similar peptides (77 and 74 amino acids, respectively) released by the convertases of the activation pathways that mediate biological effects through distinct cell receptors, the C3a receptor (C3aR) and C5a receptor (C5aR, CD88) (Hugli 1984; Gerard and Gerard 1994; Ames et al. 1996). These are both members of the large family of seven-transmembrane, G-protein-coupled receptors and signal upon binding their respective ligands. C5a is arguably the most biologically active of the activation products. Binding of C5a to its receptor on neutrophils and other phagocytes triggers cell activation, chemotaxis towards the source, generation of reactive oxygen species, release of toxic cytoplasmic granule contents (enzymes, histamine, leukotrienes, etc.), and upregulation of adhesion properties. The net result is the recruitment of 'angry phagocytes' to the site of complement activation. C5aR is expressed on numerous other cell types, including astrocytes, smooth muscle cells, and endothelia, but the effects of C5a on these cells are still unclear. C3aR is also expressed on phagocytes – in high number on eosinophils and basophils, but in very low number on neutrophils (Zwirner et al. 1999). Binding of C3a activates cells abundantly expressing C3aR, but has little or no role in neutrophil activation. As with C5a, the effects of C3a on the many other cell types that express the receptor are unclear. The effects of C3a and C5a are focally and temporally limited because they are rapidly inactivated by a plasma enzyme termed carboxypeptidase N (anaphylatoxin inactivator). This enzyme cleaves the carboxy-terminal arginine (Arg) to generate the 'desArg' forms (C3a-desArg, C5a-desArg), thereby completely inactivating C3a and reducing the activity of C5a by one or two logs.

An intriguing story has emerged over the last decade linking C3a-desArg with the regulation of fat metabolism (Cianflone et al. 1994). The story began with the identification of a plasma protein, termed acylation stimulating protein (ASP), which stimulated triglyceride synthesis in fibroblasts and adipocytes in vitro. Characterization of purified ASP revealed that it was identical to C3a-desArg! This, together with the demonstration that adipose tissue was a major site of synthesis of fD (originally termed 'adipsin' in this context) and also produced C3 and fB, led to the proposal that a novel pathway involving local alternative pathway activation in adipose tissue to produce C3a-desArg (ASP) was a major homeostatic factor for fat metabolism (Sniderman and Cianflone 1997). In support of this proposal, a putative receptor for C3a-desArg was demonstrated on adipocytes. Doubt has been cast on the central role of this 'adipsin-ASP' pathway in fat metabolism, perhaps most persuasively from those who point out that C3 deficiency in humans and mice is not associated with deficits in lipid metabolism (Kildsgaard et al. 1999).

Of the other fragments and complexes generated during activation, the C4a fragment, although structurally related to C3a and C5a, does not appear to have any biological role in humans. The fragment Ba released during alternative pathway activation has been implicated as an immunosuppressive agent, but the evidence is weak and the mechanisms undefined (Oppermann et al. 1991). The fluid-phase product of terminal pathway activation, SC5b-9 (also called terminal C complex (TCC)), has been shown to activate both neutrophils and endothelia, although no receptor for this complex has yet been identified (Tedesco et al. 1997; Dobrina et al. 2002).

Role of C in the adaptive immune response

The activities described above, opsonization of immune complexes and pathogens, chemotactic attraction and activation of phagocytes, and other cell activation events, can be considered as the 'classical' consequences of C activation, acting to dispose of pathogens and other toxic entities. It is now apparent that there are other, less well-defined but equally important roles of C activation that contribute to homeostasis and the immune response.

A role for C in the adaptive immune response to antigen was first suggested by Pepys in a landmark paper in 1974. Pepys (1974) showed that the antibody response to T-cell-dependent antigens was severely blunted in C-depleted mice, implying a role for C in the immune response to antigen. The mechanism underlying this seminal and controversial finding was not identified until the early 1990s when Tedder, Fearon, and co-workers identified a critical role for CR2 (Tedder et al. 1986; Matsumoto et al. 1991; Fearon 1991). They showed that CR2 expressed on B cells is present in a complex with CD19 and other signaling molecules and plays a key role in the B-cell response to antigen (Figure 4). Particulate antigens coated with C3d, the cell-bound end-product of C3b inactivation, will bind both through the B-cell receptor and through CR2, delivering additional stimuli to the B cell and lowering the threshold of cell response to antigen by several orders of magnitude (Tedder et al. 1997; Fearon and Carroll 2000). CR2 on follicular dendritic cells plays a similar, though less well-defined, role and appears to be required for efficient antigen trapping. This key role of C3d in the generation of an efficient immune response is now being exploited in immunization studies where C3d coupled to antigen acts as a molecular adjuvant (Dempsey et al. 1996). C thus provides a strong link between the innate and adaptive immune systems that had previously been considered primarily as discrete, non-interacting systems.

Role of C in apoptosis

Cell death follows one of two pathways: either necrosis involving disruption of the plasma membrane by an external agent and release of intracellular contents, or apoptosis involving the triggering of an endogenous cell death program that leaves the plasma membrane intact and does not release cell contents. Given the lytic capacity of the MAC, a role for C in necrosis is easy to envi-

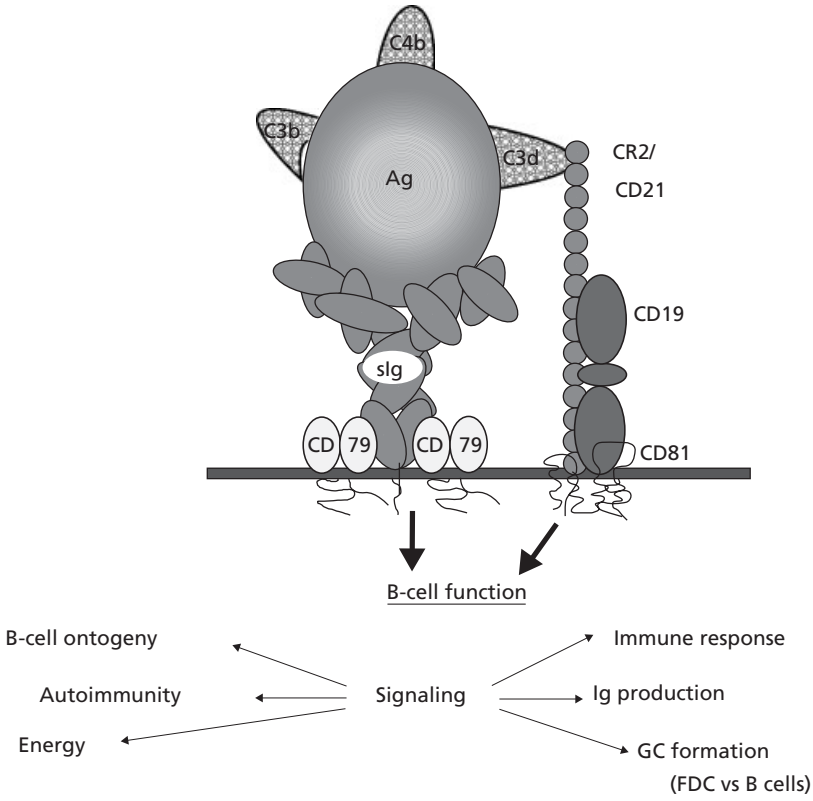

Figure 4 *The role of C in the B-cell response to antigen. The antigen (Ag) or immune complex becomes coated with C3b and other C fragments. Enzymatic degradation of C3b leaves C3d fragments, the ligand for CR2, attached to the activating complex. When antigen binds the specific B-cell membrane receptor (slg), CR2 together with its associated molecules (CD19, CD81) is recruited through binding of C3d. The net result is that signals are transmitted to the B cell both through the B-cell receptor complex and through the CR2/CD19/CD81 complex, thereby lowering the threshold for B-cell response to antigen.*

sage. Indeed, in numerous models of ischemic injury where necrotic cell death is predominant, an important role for C, and specifically the MAC, has been demonstrated (see section on Complement in specific pathologies). Necrotic cell debris triggers further inflammation and C activation, in part through binding of the C activator, CRP, to the cell debris (Griselli et al. 1999). This in turn exacerbates injury in the models. Apoptosis, in contrast, is a 'clean' process that does not involve the release of cell contents and is not proinflammatory. Apoptotic cells will eventually undergo necrotic changes, making efficient removal by phagocytes an essential part of the process. Although C opsonization is an excellent means of targeting phagocytosis, it is somewhat counterintuitive to suggest that the proinflammatory C system might be involved in apoptosis. Indeed, numerous non-C recognition and uptake systems have been identified for the safe removal of apoptotic cells (Savill et al. 1993; Savill and Fadok 2000). Nevertheless, there is abundant evidence from in vitro studies that cells undergoing apoptosis activate C and are opsonized for uptake by phagocytes using the opsonic C receptors described above (Navratil and Ahearn 2001; Mold and Morris 2001). The relevance of opsonization to removal of apoptotic cells in vivo is a continuing source of controversy and the activating triggers a source of debate (Medzhitov and Janeway 2002). Early studies indicated that apoptotic cells activated the alternative pathway (Matsui et al. 1994), whereas later work demonstrated binding of C1q and MBL to sites on the apoptotic blebs, implicating the classical and lectin pathways (Navratil et al. 1999, 2001; Mevorach 2000; Ogden et al. 2001). Whatever the activating pathway, the consequences of C activation will be the same. Opsonization may aid clearance but, if activation were to proceed through to MAC formation, then lysis would ensue with unwanted inflammatory consequences. It has been suggested that activation of the terminal pathway on apoptotic cells is restricted, although no mechanism has yet emerged (Gershov et al. 2000).

Further evidence implicating the classical pathway in apoptotic cell clearance came from analysis of mice deficient in C1q. The mice developed an autoimmune glomerulonephritis with an accumulation of apoptotic cells in the kidney, apparently caused by defective clearance (Botto et al. 1998). These symptoms closely resembled those seen in C-deficient individuals with SLE or lupus-like disease, and provided a new concept of how C deficiency causes SLE, defects in clearance of apoptotic cells rather than immune complexes being key (Taylor et al. 2000). Perhaps the best analogy for the role of C, and specifically the classical pathway, in the prevention of SLE is that suggested by Walport (2001a), the 'waste disposal' hypothesis. This suggests that activation of C on immune complexes, apoptotic cells, and perhaps other debris labels these items as garbage for rapid and efficient clearance by phagocytes. Failure of this system, because of either C deficiencies or a garbage load that exceeds the labeling capacity of the system, will lead to the accumulation of garbage with resultant disease.

C activation products have also been implicated as modulators of apoptosis. C5a has been shown to protect neutrophils from apoptosis (Lee et al. 1993; Perianayagam et al. 2002), but accelerate apoptosis in thymocytes (Guo et al. 2000; Riedemann et al. 2002). In neurons, different studies have reported C5a-induced protection from (Mukherjee and Pasinetti 2001) or induction of (Farkas et al. 1998) apoptosis. The MAC can also influence apoptosis. Using C6-deficient rats in models of renal disease, the MAC was shown to drive apoptosis of mesangial and endothelial cells (Sato et al. 1999; Hughes et al. 2000). In contrast, exposure to nonlethal MAC attack of Schwann cells and oligodendrocytes, the myelin-producing cells of the peripheral and central nervous systems, respectively, protected against apoptosis (Dashiell et al. 2000; Soane et al. 2001). The overall picture is one of confusion, with different cell types responding in dramatically differing ways, the only common thread being that C activation products exert an influence, positive or negative, on the apoptotic process.

REGULATION OF C

It is evident from the above description of the active products that the C system carries with it the potential to harm self, hence the much-repeated adage that C is a 'double-edged sword'. To minimize damage to self, C is tightly controlled at multiple stages in the pathway by regulatory proteins present in plasma and on cell membranes (Table 2 and Figure 5) (Morgan and Harris 1999).

Regulation in the activation pathways

The first step of the classical pathway is regulated by C1-inhibitor (C1inh), a serine protease inhibitor (serpin), present in plasma at about 150 μg/ml. C1inh binds activated C1 and removes C1r and C1s from the complex (Davis 1988, 1989). C1inh forms a covalent complex comprising two molecules of C1inh and one each of the target proteases C1r and C1s ($C1inh_2C1rC1s$), and is itself cleaved in the process, therefore acting as a suicide pseudosubstrate inhibitor. C1inh is the only plasma inhibitor of activated C1 but also regulates kallikrein in the contact activation system of kinin generation and factors XIa and XIIa in the coagulation system. C1inh may also act to stabilize the proenzymic C1 complex by binding C1 in plasma, thereby reducing the likelihood of activation (Ziccardi 1982).

Although still a matter of debate, the weight of evidence indicates that C1inh also regulates the initial step in the lectin pathway (Matsushita et al. 2000). Regulation by C1inh mirrors that described for C1, acting to remove the proteases MASP-1 and MASP-2

Table 2 *The regulatory proteins of the C system*

Regulator	Structure/function	Plasma concn/tissue distribution
Activation pathways		
C1inh	Single chain, two-domain heavily glycosylated, approx. M_r 104 kDa. Suicide inhibitor of activated C1	150 μg/ml (approx. 2 μmol/l)
fH	Single chain, 20 SCRs, approx. M_r 155 kDa; cofactor and decay accelerator for AP convertases	550 μg/ml (approx. 3.5 μmol/l)
fHL-1	Single chain, 7 SCRs, approx. M_r 42 kDa, cofactor and decay accelerator for AP convertases, adhesion	30 μg/ml (approx. 0.7 μmol/l)
C4BP	Oligomer comprising 7 α chains (8 SCRs) and 1 β chain (3 SCRs), approx. M_r 550 kDa. Cofactor and decay accelerator for CP convertases	250 μg/ml (approx. 0.45 μM)
fI	Two-chain from single chain precursor, serine protease, approx. M_r 90 kDa. Cleavage of C3b/C4b in convertases	35 μg/ml (approx. 0.40 μmol/l)
DAF	Single chain, 4 SCRs, GPI tail, approx. M_r kDa. Decay of CP and AP convertases	Broadly distributed, all blood cells, endothelia. Soluble DAF in plasma
MCP	Single chain, 4 SCRs, tm tail, approx. M_r 60 kDa (two major isoforms), cofactor for CP and AP convertases	Broadly distributed, not on human E
Terminal pathway		
S-protein	Single chain, 75 kDa, binds C5b-7 complex and prevents membrane insertion	350 μg/ml (approx. 4.5 μmol/l)
Clusterin	Two chains (α and β each approx. 40 kDa) from single chain precursor. Binds C5b-7 complex and prevents membrane insertion	200 μg/ml (approx. 3 μmol/l)
CD59	Single chain, compact, globular, GPI tail, approx. M_r 20 kDa, heavily glycosylated. Inhibits MAC assemby	Broadly distributed, all blood cells, endothelia, etc.

For references, see the text.
AP, alternative pathway; CP, classical pathway; DAF, decay accelerating factor; GPI, glycosyl phosphatidylinositol; SCRs, short consensus repeats

from the activated MBL–MASP complex. C1inh forms covalent complexes with the MASPs, although the precise stoichiometry of these complexes is uncertain (Petersen et al. 2000). Regulation of MBL–MASP

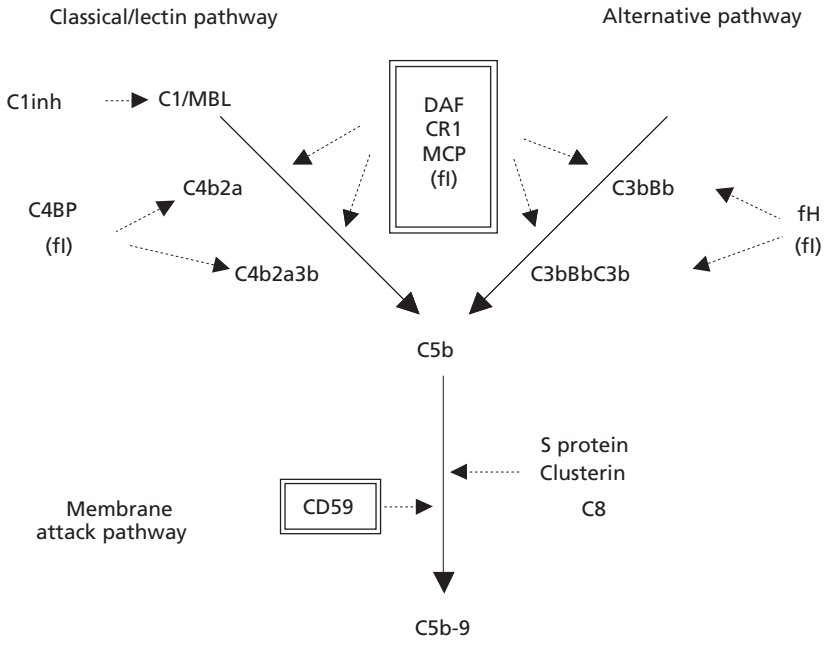

Figure 5 *C regulators. The fluid-phase and membrane regulators of C are illustrated, with broken arrows indicating the points in the pathway at which they exert their effects. Membrane regulators are boxed. Abbreviations as in the text.*

activity by α_2-macroglobulin, a plasma protease inhibitor structurally related to C3/C4, has also been reported in vitro, and complexes of MBL–MASP with α_2-macroglobulin have been detected in plasma (Terai et al. 1995). The physiological relevance of this interaction is unknown.

Control of the C3 and C5 convertases is provided by fI, a highly specific serine protease, which, in the presence of essential cofactors, cleaves the α chains of C3b and C4b to inactivate the convertases (DiScipio 1992b). In plasma, two proteins act as cofactors for fI, factor H (fH) in the alternative pathway and C4-binding protein (C4BP) in the classical pathway (Dahlback 1983). Both fH and C4BP also inhibit by accelerating the decay of the convertases. fH is the index member of a large family of structurally related proteins (Zipfel et al. 1999). One of these, fH-like-1 (fHL-1) arises by alternative splicing in the *fH* gene and regulates C. The others, fH-related proteins 1–5 (fHR-1 to fHR-5) are encoded by linked genes in the RCA cluster; their function is still under study.

On the membrane, decay accelerating factor (DAF) (CD55) acts to accelerate the decay of convertases, whereas membrane cofactor protein (MCP, CD46) acts as cofactor for the cleavage of C4b and C3b by fI, irreversibly inactivating the enzyme (Lublin and Atkinson 1990). Both are widely distributed, present on most cell types, although MCP is conspicuously absent from erythrocytes in humans. CR1, a large transmembrane protein mentioned above as a C fragment receptor, is also a regulator of C with both decay accelerating and cofactor activities (Holers et al. 1986). It is expressed on erythrocytes, B cells, polymorphonuclear leukocytes, follicular dendritic cells, and several other cell types. The active sites for C regulation in CR1 are placed a considerable distance from the membrane in the large CR1 molecule, provoking the suggestion that CR1 acts extrinsically to regulate C activation on surrounding cells and surfaces.

With the exception of fI, all of the regulators of C3/C5 convertases and several of the receptors for fragments of C3, C4, and C5 are members of a gene family, called the RCA family, encoded in the RCA locus on chromosome 1q32 (Hourcade et al. 1989). All contain multiple copies of a conserved structural unit, the short consensus repeat (SCR), comprising a compact motif of 60 amino acids with conserved disulfide bridges. Some RCA proteins, such as fH, are made up entirely of SCR units, whereas others contain additional domains performing other roles. The SCR unit is also found in non-RCA molecules that interact with C3, C4, or C5, including the C proteins C1r, C1s, C2, fB, C6, and C7.

Control in the membrane attack pathway

The membrane attack pathway is also regulated by inhibitors present in the fluid phase and on membranes. The fluid-phase C5b-7 complex is the target of S-protein (vitronectin) and clusterin, abundant plasma proteins (each about 0.2–0.5 mg/ml) that, among their many other roles, help regulate C activation by binding the hydrophobic site in C5b-7 and preventing its association with membranes (Podack and Muller-Eberhard 1979; Tschopp and French 1994). Binding of C8 to the fluid-phase C5b-7 complex also blocks attachment to the membrane. On the membrane, CD59, a small, compact, glycolipid-anchored membrane protein unrelated to the RCA-encoded regulators, binds tightly to C8 in the C5b-8 complex and perhaps also to C9 in the C5b-9_1 complex, thereby blocking further incorporation of C9 and assembly of the MAC (Lachmann 1991; Morgan 1999).

COMPLEMENT DEFICIENCIES

Deficiencies of almost every C protein and regulator have been described, giving a wide range of signs and symptoms, depending on the pathways affected by the deficiency (Figure 6). These 'experiments of nature' give important information on the physiological roles of C and have pointed the way to some surprising discoveries.

Classical pathway deficiencies

Deficiencies of components of the classical pathway (C1, C4, or C2) are strongly associated with a syndrome clinically indistinguishable from SLE, a consequence of the failure of immune complex solubilization (Walport and Lachmann 1990). The frequency and severity of disease are greatest with deficiencies of one of the subunits of C1 (C1q, C1r, C1s), closely followed by total C4 deficiency (Kolble and Reid 1993). The incidence of SLE is almost 100 percent in C1q deficient subjects, close to 70 percent in those deficient in C1r and/or C1s, and 75 percent in total C4 deficiency. C4 is unusual in that it is encoded by duplicated genes differing in only a few amino acids but yielding proteins (C4A and C4B) that are functionally distinct (Schifferli and Paccaud 1989). As a result of the presence of four loci, complete deficiency of C4 is exceedingly rare, but null alleles at one or more of the four C4 loci are common, giving rise to partial or complete deficiencies of either C4A or C4B. Deficiencies of each isotype have been associated with an increased incidence of lupus-like disease, bacterial infection, and numerous other disorders. Deficiency of C2 is the most common homozygous C deficiency in white people but infrequently causes disease – most C2 deficiencies have been identified by chance in healthy individuals. About 10 percent of C2-deficient individuals present with SLE. The strong association of immune complex disease with classical pathway deficiencies is a consequence of the failure of the 'waste

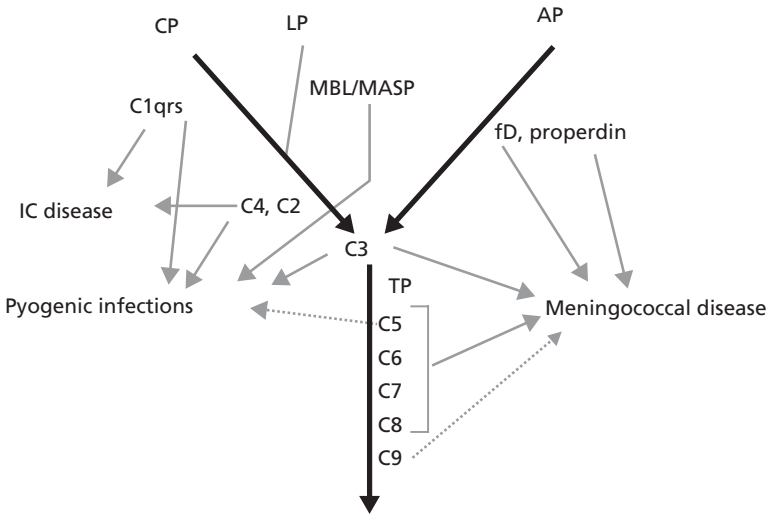

Figure 6 *C deficiencies. The clinical sequelae of deficiencies in different parts of the C system are summarized. AP, alternative pathway; CP, clasical pathway; IC, immune complex; LP, lectin pathway; other abbreviations as in the text.*

disposal' role of the classical pathway described in earlier. Bacterial infections are not usually a prominent feature of deficiencies in the classical pathway, illustrating the dominant role of the alternative pathway in dealing with bacteria.

Alternative pathway deficiencies

Deficiencies of alternative pathway components are rare. A few individuals deficient in fD have been described, all of whom have presented with severe and/or recurrent neisseria infections, usually meningococcal meningitis (Biesma et al. 2001). Homozygous deficiency of fB has not been reported, leading to the assumption that the defect was lethal. However, the mild phenotype observed in fB-deficient mice indicates that survival is possible in the absence of fB (Matsumoto et al. 1997).

Deficiency of the positive regulator, properdin, is the most common disorder of the alternative pathway and is also associated with meningococcal infection (Sjoholm et al. 1982). Properdin is encoded on the short arm of the X-chromosome and deficiencies are therefore found only in males. Three distinct forms of the deficiency have been described: type 1, the most common, is associated with complete absence of the protein, type 2 with low levels (1–10 percent) of apparently active protein, and type 3 with normal levels of a functionally impaired protein (Sjoholm et al. 1988a, b; Fijen et al. 1999). Affected individuals usually present with fulminant meningococcal disease that is frequently fatal. Disease onset occurs later than is usual for meningococcal disease (average age of onset 14 years) and is frequently caused by unusual serotypes of the meningococcus. Survivors rarely get recurrence because the antibody response developed during first infection is protective. Screening of male siblings is essential when properdin deficiency is identified because immunization can forestall the risk of a potentially fatal outcome.

Lectin pathway deficiencies

Defects in the lectin pathway are surprisingly common. Indeed, discovery of the pathway followed analyses in a cohort of infants presenting with recurrent bacterial infections. These infants exhibited a reduced capacity of their serum to opsonize yeast particles with C3b, initially ascribed to a defect in the alternative pathway (Soothill and Harvey 1976, 1977). Population screens identified this opsonic defect in up to 5 percent of the population, the large majority of whom had no history of recurrent infections. On closer analysis, no specific alternative pathway deficiency was identified in these individuals. More than a decade later, an association of the opsonic defect with a deficiency of MBL, the C1q-like lectin that is the recognition unit of the lectin pathway, was demonstrated (Super et al. 1989; Turner 1991). About 5 percent of the population in northern Europe have little or no MBL in plasma; in almost all cases, one of three point mutations in the *mbl* gene is present, each causing a single amino acid substitution in the collagen-like domain (Lipscombe et al. 1996; Summerfield et al. 1997). These substitutions interfere with the oligomerization of MBL monomers into the functional protein. Plasma levels in homozygotes are essentially zero, whereas, in heterozygotes, plasma levels vary from about 10 to 50 percent of normal, depending on the polymorphism, although functional activity may be much lower because oligomers containing mutant chains are functionally impaired. Clinical sequelae of MBL deficiency were initially considered to be restricted to infants; however, recent studies have demonstrated that MBL deficiency is also a risk factor for infection in

immunodeficient adults and may be associated with increased disease severity in autoimmunity (Sumiya and Summerfield 1996; Petersen et al. 2001).

Inherited and acquired deficiencies of C3

C3 is the lynchpin of the C system, an essential component of all activation pathways and vital for efficient opsonization of bacteria. Deficiency is associated with a marked susceptibility to bacterial infections and, because of the critical role of C3 fragments in responses to antigen, a generalized reduction in immune responsiveness (Botto and Walport 1993). Some C3-deficient individuals also develop glomerulonephritis or lupus-like illness as a result of defects in immune complex handling. Skin rashes are a frequent feature, particularly during infective episodes. The large majority of C3-deficient individuals present with severe, recurrent infections early in life that can be fatal. However, prophylactic antibiotic therapy can now rescue such individuals, enabling survival into adulthood.

Secondary deficiencies of C3 resulting from consumption can also occur. C3 consumption associated with deficiencies of C regulators is discussed below; here, other factors that cause pathological consumption of C3 are introduced. Perhaps the most clinically relevant are autoantibodies, termed C3 nephritic factors (C3NeF), which bind and stabilize the C3 convertase of the alternative pathway, C3bBb (Walport et al. 1994). C3NeFs usually occur in the absence of other features of autoimmunity and the underlying trigger for their production is unknown. Although C3 is consumed systemically and plasma C3 levels can be very low, the dominant clinical consequence is membranoproliferative glomerulonephritis. Less commonly, C3NeFs cause an unusual and distressing syndrome, partial lipodystrophy, in which near-total fat loss occurs, predominantly in the upper body (Mathieson et al. 1993). Adipose tissue is targeted because it is the major site of synthesis of fD in the body. C3bBb complexes can thus form readily in the tissue and, in the presence of a C3NeFs, these complexes are stabilized and cause local activation of C sufficient to kill adipocytes. The gradient of fat loss, with the face and upper extremities most affected, reflects a differential capacity of adipose tissue to produce fD (Mathieson et al. 1993). Rarely, autoantibodies that bind and stabilize the classical pathway convertase C4b2a, C4 nephritic factors (C4NeF), occur; like C3NeFs, these consume C3 and are associated with glomerulonephritis and also lupus-like pathology (Daha et al. 1976).

Terminal pathway deficiencies

Deficiencies of terminal pathway components (C5, C6, C7, C8, or C9) cause susceptibility to infection with organisms of the genus *Neisseria* – meningococcal meningitis and systemic infection with the meningococcus (Wurzner et al. 1992). This reflects the role that the MAC plays in bactericidal activity against this group of organisms. It should, however, be stressed that most individuals with terminal pathway deficiencies remain healthy and are ascertained by chance. Deficiency of C5 causes loss not only of MAC function but also of the potent chemotactic and cell-activating fragment C5a. Despite this, the spectrum of disease in C5-deficient individuals does not differ from that in deficiencies of other terminal components. C6 deficiency is the second most common C deficiency in caucasians; other terminal pathway deficiencies are rare in this group. Deficiency of C9 deserves special mention for three reasons: first, because the link with meningococcal disease, although still present, is weaker, probably because C9-deficient serum kills bacteria, albeit inefficiently (Pramoonjago et al. 1992). Second, because it is extremely common in Japan where 0.1 percent of the population are deficient in C9. Virtually all Japanese C9-deficient individuals carry the same defect, a stop mutation early in the coding region (D^{95}→Stop) (Horiuchi et al. 1998). Almost 7 percent of the Japanese population are carriers for this mutation. Third, the C9 concentration in neonates is extremely low, reaching adult levels a few months after birth (Lassiter et al. 1992; Hogasen et al. 2000). Neonates are thus likely to have reduced capacity to form MAC and may as a consequence be more susceptible to infection with *Neisseria* spp.

Deficiencies of C regulators

The activation pathways are regulated from the fluid phase by the serpin C1inh and by the enzyme fI, together with its cofactors, fH in the alternative pathway and C4BP in the classical pathway. Deficiency of C1inh deserves particular attention because it causes a syndrome termed hereditary angioedema (HAE), a relatively common, distressing and potentially lethal disorder characterized by episodic painless swelling of areas of the skin or mucosa (Carugati et al. 2001). Involvement of the laryngeal mucosa can cause asphyxia and gut involvement may mimic an acute abdominal emergency. HAE is unusual in that symptoms manifest in individuals heterozygous for defects in the C1inh gene. C1inh is the sole plasma regulator of activated C1 and also regulates activation in the contact and coagulation systems. Because C1inh is a 'suicide inhibitor', consumed when it interacts with its enzyme target, plasma levels reflect a balance between ongoing consumption and biosynthesis. A decreased rate of biosynthesis in the face of sustained consumption will therefore rapidly result in very low plasma levels of C1inh. In heterozygotes, synthesis will be reduced by around 50 percent but plasma levels of C1inh may be 10

percent or less. Attacks often follow minor trauma that initiates activation of the C and contact systems, normally held in check by C1inh. The products of uncontrolled activation, particularly bradykinin and fragments of C2, cause local increased vascular permeability that underlies the tissue edema. HAE affects about 1 in 50 000 people (Davis 1988); more than 20 percent of cases are the result of new mutations and therefore lack a family history of disease (Agostoni and Cicardi 1992). The most common form of HAE, type 1, involves mutations in the C1inh gene that yield little or no protein. Measurement of C1inh protein and function correlate and are both low. In some 15 percent of cases the mutated gene yields an abnormal, functionally compromised protein usually resulting from point mutations that cause amino acid substitutions at or near the active site of the enzyme (Davis et al. 1993). The abnormal protein is detected in antigenic assays, giving normal or elevated serum concentrations and masking the deficiency (type 2 HAE). Functional assays are essential to make the diagnosis. Angioedema indistinguishable from that in HAE may also occur in older individuals, usually in association with lymphoproliferative disease and, occasionally, other malignancies (Cicardi et al. 1996). Here, low level activation of the classical pathway by the large tumor mass and resultant consumption of C1inh causes a secondary deficiency (acquired angioedema (AAE)). Rarely, AAE may occur, in the absence of malignancy, caused by the presence of blocking autoantibodies against C1inh (Mandle et al. 1994).

The factors fI and fH collaborate to control the alternative pathway convertases in the fluid phase and on surfaces. In the absence of either, control fails causing unregulated activation of C and consumption of C3 and other components. Individuals deficient in fI or fH thus have profound secondary deficiency of C3 and the clinical consequences are essentially identical to those described for C3 deficiency. Individuals deficient in fH often have, in addition, renal disease, most commonly membranoproliferative glomerulonephritis or the hemolytic uremic syndrome (HUS). The association of idiopathic (noninfectious) HUS with fH deficiency is of particular interest. Although the link was first noted in patients with low plasma levels of fH, it has now become clear that apparently trivial mutations in the carboxy-terminal region of fH, which have little effect on the plasma level of the protein, can cause HUS, perhaps by interfering with the capacity of fH to bind surfaces (Taylor 2001; Zipfel 2001).

Deficiency of C4BP is rare and the few reports in the literature are lacking in information. Various autoimmune-like symptoms and angioedema have been seen in individuals with complete or partial deficiencies of C4BP but no clear evidence of causative association has emerged.

On membranes, DAF and MCP, the latter as a cofactor for fI, control the activation pathway convertases. No reports of MCP deficiency exist in the literature. DAF deficiency can occur as a global absence of DAF as a result of mutations in the *Daf* gene, or absence from circulating cells in the syndrome paroxysmal nocturnal hemoglobinuria (PNH), secondary to defects in glycosyl phosphatidylinositol (GPI) anchor synthesis. In PNH, erythrocytes and other circulating cells arising from the mutant hemopoietic stem cell clone lack all GPI-anchored proteins on the membranes (Rosse and Ware 1995). The absence of the C regulators DAF and CD59 renders the cells susceptible to C damage in the circulation, causing hemolysis, thrombosis, and leukocyte activation. Erythrocytes are particularly affected because they naturally lack MCP and are thus essentially devoid of protection from C. Several DAF-deficient individuals have been described, recognized because of the absence of the Cromer blood group antigens (the *Inab* phenotype), known to reside within DAF (Merry et al. 1989; Telen and Green 1989). Individuals deficient in DAF were healthy apart from a vague association with intestinal problems in some families. In particular, DAF-deficient individuals did not have intravascular hemolysis or other evidence of C damage to circulating cells.

No reports of complete deficiencies of S-protein (vitronectin) or clusterin, the fluid-phase inhibitors of the terminal pathway, have been published. These proteins have numerous biological roles outside the C system that probably make them indispensable. On the membrane, CD59 regulates the terminal pathway (Davies and Lachmann 1993). As noted above, PNH cells are deficient in CD59 and DAF as a result of defective GPI anchor synthesis and are, as a consequence, susceptible to C damage. A single individual with global absence of CD59 caused by *Cd59* gene mutation has been reported, presenting at a young age with symptoms compatible with PNH (Yamashina et al. 1990). Taken together with the lack of symptoms associated with DAF deficiency, these observations make it clear that CD59 is crucial for survival of cells in the circulation.

COMPLEMENT IN SPECIFIC PATHOLOGIES

Inappropriate activation of C occurs in a large number of inflammatory, iatrogenic, ischemic, and other diseases and, in many, is a major contributor to the initiation or perpetuation of tissue damage (Table 3). Pathological effects are mediated by precisely the same mediators that are responsible for the protective roles of C described above. The anaphylactic and chemotactic peptide C5a drives inflammation by recruiting and activating neutrophils, C3a may cause pathological activation of other phagocytes, the MAC may kill or injure cells, and so on. Evidence for a role of C may be sought in humans by looking for the products of C activation either in the tissues or in plasma. In many cases, animal

Table 3 *Examples of diseases in which C is implicated*

Disease	Evidence of C involvement
Immune-mediated diseases	
Rheumatoid arthritis (RA)	Consumption of plasma C in acute disease; C activation products in synovial fluid and synovial membrane; C inhibition (various agents) suppresses disease in animal models of rheumatoid arthritis (RA); C deficiency suppresses disease in mouse models of RA
Membranoproliferative glomerulonephritis (MPGN)	Abundant deposits of C activation products, including membrane attack complex (MAC), in glomeruli; C inhibition (various agents) suppresses disease in models of MPGN; intrarenal administration of C regulators suppresses disease in models
Multiple sclerosis (MS)	C activation products in CSF and around areas of demyelination in the CNS; C inhibition (various agents) suppresses disease in animal models of MS; C deficiency suppresses disease in rodent models of MS
Myasthenia gravis (MG)	Abundant C activation products at motor end plate during attacks; C deficiency or depletion abrogates disease in rodent models; C inhibition (CVF; sCR1) suppresses disease in models of MG
Degenerative diseases	
Alzheimer's disease	C activation products in and around plaques in CNS; anti-inflammatory therapy appears to slow progression
Ischaemia–reperfusion injuries	
Myocardial infarct (MI)	Reperfusion associated with C activation in ischemic area; abundant deposits of C activation products in and around infarct; C inhibition at time of reperfusion in models reduces size of infarct
Stroke	Evidence as described above for MI

For references, see the text.

models have proved invaluable in dissecting the roles of C in diseases. Here, no attempt is made to discuss all of these diseases but instead a few examples of disease groups where C plays a part are given.

Complement in SLE and related syndromes

Systemic lupus erythematosus (SLE) is characterized pathologically by the presence of immune complex deposits in affected tissues such as the kidney and skin, which are invariably associated with deposition of C components. Plasma C activity and plasma levels of specific components, particularly C3 and C4, are low in severe disease, reflecting the massive C consumption that occurs in the tissues. These observations have generated the consensus view that C activation drives tissue damage in SLE and related diseases. However, this consensus is difficult to reconcile with the knowledge that disease closely resembling SLE occurs in individuals deficient in components of the classical pathway, where the capacity to generate the toxic products of C activation will be impaired (Navratil et al. 1999). A better explanation for the occurrence of SLE in C-deficient and C-sufficient individuals is provided by Walport's 'waste disposal' hypothesis (Walport 2001b). This suggests that a crucial role of C is the safe disposal of immune complexes and apoptotic cells via binding of C1 and C4 and subsequent engulfment by macrophages. In C deficiency the process fails because of a global lack of these components. In idiopathic SLE it fails because consumption of C causes a localized, secondary deficiency of C1 and C4. In either situation, accumulation of 'garbage' in the tissues is toxic and initiates further tissue damage.

An additional precipitating factor in some individuals with idiopathic SLE is the presence of autoantibodies against the collagen-like region (CLR) of C1q (anti-C1q CLR), which are thought to affect C1 function (Strife et al. 1989; Wener et al. 1989). The presence of anti-C1q CLR antibodies correlates strongly with the severity of renal disease in SLE. These autoantibodies are also found in association with several other pathologies, including membranous glomerulonephritis, rheumatoid vasculitis, and hypocomplementemic urticarial vasculitis (HUVS). Virtually all patients with HUVS have anti-C1q CLR antibodies and the presence of these antibodies has been used as a diagnostic test for this syndrome (Wisnieski and Naff 1989; Wisnieski et al. 1995).

Complement in ischemia–reperfusion injuries

Many common conditions are caused by a transient or permanent loss of blood supply to tissue. Examples include myocardial infarction (MI), ischemic strokes, and malfunction in newly transplanted organs. In transient ischemia, much of the damage is done during reperfusion rather than in the ischemic period. Restoration of blood flow, either spontaneous or achieved

through thrombolytic therapy, is accompanied by tissue damage and destruction that often extend far beyond the margins of the ischemic area. One of the major tissue-damaging factors is C activation. Dead and damaged cells are efficient activators of C and local activation during reperfusion of ischemic tissue will cause further tissue damage, propagating the injury into surrounding viable tissue. In MI, the infarct and surrounding 'penumbra' of damaged but viable tissue contains abundant deposits of C activation products, also detectable in plasma for days after the episode (Kilgore et al. 1994). CRP is also deposited in and around the infarct where it binds to damaged cells and triggers further activation of C (Griselli et al. 1999).

Similar evidence is apparent in stroke where the effects of C activation may be magnified because of the remarkable sensitivity of brain cells to C damage (Singhrao et al. 2000). In transplanted organs, the degree of C activation and tissue damage correlate closely with the ischemic interval between organ harvest and implantation. The involvement of C during the brief window of reperfusion in all these injuries presents an ideal opportunity for the use of anti-C agents. Early studies, using the C-depleting agent cobra venom factor (CVF) showed a marked protective effect when given before reperfusion in a baboon model of MI (Crawford et al. 1988). CVF pre-treatment also markedly increased graft survival in rodent models of heart and kidney transplantation (Thomas et al. 1977; Forbes et al. 1978). CVF is a toxic and highly antigenic agent that inactivates C by depleting the system to exhaustion and it is therefore not suitable for use as a therapeutic. Modern C therapies have also been used in ischemia–reperfusion models. A recombinant soluble form of the C receptor CR1 (sCR1), an efficient inhibitor of C in vivo, markedly reduces infarct volume when given at the time of reperfusion in a rat model of MI (Weisman et al. 1990).

Complement in neurological diseases

Complement has been implicated in diverse disorders of the nervous system, including such conditions as stroke, traumatic injury, demyelinating disorders, and neurodegenerative diseases. A summary of the information from the last two groups of diseases follows.

Demyelination in the central nervous system causes multiple sclerosis (MS), a distressing, progressive disease that causes severe disability and even death in sufferers. The underlying cause of MS remains the subject of debate, although it is now broadly accepted that there is an underlying autoimmune component. T cells infiltrating the areas of pathology in the brain are prime suspects. Indeed, in animal models of MS, disease can be transferred by T cells from affected animals to naïve recipients. A role for C in MS was first suggested by demonstrating C consumption and C activation products in plasma and cerebrospinal fluid (CSF) (Morgan et al. 1984; Mollnes et al. 1987). Others showed C deposition in and around plaques in MS brain (Compston et al. 1989) and demonstrated that myelin in vitro directly activated C via the classical pathway (Liu et al. 1983). Recently, detailed clinical and histopathological studies have divided MS into several subgroups, C activation being a major feature in some but not all the groups (Lucchinetti et al. 2000). Decomplementation or therapy with anti-C agents reduced myelin injury in rodent models of MS, further implicating C in myelin loss (Linington et al. 1989; Piddlesden et al. 1994). Demyelination in the peripheral nervous system causes several related pathologies, the most common of which is the Guillain–Barré syndrome (GBS). A role for C was first suggested from demonstrations of the capacity of peripheral nerve myelin to activate C (Koski et al. 1985). C activation products were found in CSF (Sanders et al. 1986) and peripheral nerve (Hafer-Macko et al. 1996) from GBS patients, and decomplementation inhibited disease in the rodent model of GBS (Vriesendorp et al. 1995).

Taken together, these data make a persuasive case for the use of anti-C therapy in at least some forms of demyelinating diseases. The problem is that none of the currently available agents is suitable for long-term therapy, so treatment in MS will probably have to await the development of better drugs. GBS, unlike MS, is a relatively acute disease, most patients making a complete or partial recovery from the demyelinating episode. Anti-C therapy given early in the course of disease might have particularly beneficial effects and could be undertaken with contemporary agents.

To suggest that the innate immune system contributes to degenerative disorders of the nervous system smacks of heresy, yet there is a substantial and growing body of evidence to support the suggestion. Neurodegeneration underlying Alzheimer's disease and related syndromes is, despite an enormous research effort over the past decade, poorly understood. Many mechanisms have been proposed but none has yet proved to be the key to understanding and preventing loss of neurons. The first suggestion of a role of C came from histological studies that showed deposits of C activation products in and around the plaques and tangles that typify the brain in Alzheimer's disease (McGeer et al. 1991, 1994; Eikelenboom et al. 1992). The β-amyloid peptide that is present in plaques activates C in an antibody-independent manner. These findings have led several workers in the field to suggest that Alzheimer's disease is a low-grade inflammatory disease and that anti-inflammatory or anti-C therapy would slow or reverse cognitive decline in Alzheimer's disease (McGeer and McGeer 1999). Trials of anti-inflammatory agents have begun and time will tell whether this approach is likely to be of benefit. Studies in the many animal models of Alzheimer's disease may also help establish whether C plays a significant role in neurodegeneration.

Complement in iatrogenic syndromes

With increasingly sophisticated interventions, iatrogenic or treatment-caused injuries are becoming more frequent. Here, focus is given to one group of injuries where C is known to have a role and anti-C therapies might prove beneficial.

Numerous interventions involve exposure of blood to a foreign surface in an extracorporeal circuit. In renal dialysis, blood is exposed to the dialyzer membrane, usually made of cellulosic materials such as cuprophane, or polymeric materials such as polysulfone. All these materials are, to a greater or lesser extent, bioincompatible and trigger activation of many plasma effector systems, including C. Activation of C and generation of C activation products, particularly C5a, activates neutrophils and other cells in the plasma and can cause fever, neutropenia, and other unwanted effects. The problem can be circumvented to a degree by choosing the least activating surface or by precoating the dialyzer membrane with either albumin or plasma proteins. Membranes that are used several times are always most activating on first use because of this surface-coating phenomenon. C activation can be reduced by pretreating the membrane with heparin, an inhibitor of C activation. C activation may be even more marked in cardiopulmonary bypass circuits, where not only the extensive surfaces of membranes and tubing, but also the plasma-expanding fluids and the gas–liquid interface in the oxygenator, may all activate C (Mollnes et al. 1991; Mollnes 1997). Secondary activation of neutrophils and other blood cells, and generation of inflammatory cytokines, will contribute to the injury and it is not uncommon for patients coming off bypass to be severely compromised for several days, necessitating prolonged intensive care – the post-bypass syndrome. Again, choice of the least activating system and precoating of circuits with protein and/or heparin may reduce activation in the circuit and minimize iatrogenic injury (Svennevig et al. 1993; Fosse et al. 1994). Studies in vitro and in animal models indicate that administration of anti-C agents during the period of bypass may be of great benefit in reducing the risk of unwanted sequelae (Gillinov et al. 1993; Moat et al. 1993; Finn et al. 1996; Lazar et al. 1999).

COMPLEMENT THERAPIES

It should be clear from the previous sections that an ability to manipulate the C system therapeutically might be of considerable benefit in many diseases. Until very recently, no agents suitable for the inhibition of C in humans were available. Crude methods using agents that consumed C (CVF) or, at high doses, expressed a modest anti-C activity (e.g. low-molecular-weight heparins) had been used in animals with some promising results. However, problems of toxicity, antigenicity, or other unwanted effects rendered such agents unusable in humans. The breakthrough came with the demonstration, in the early 1990s, that an engineered recombinant soluble form of a natural C regulator, CR1, was a powerful inhibitor of C activation both in vitro and in vivo (Weisman et al. 1990). Over the last decade, the field of C therapies has exploded and we now have numerous agents on the verge of application in the clinic (Table 4).

There are various considerations to be taken into account when developing anti-C therapies. These include the side effects of long-term systemic inhibition of the C system, choice of the most efficient point at which to inhibit C, rapid clearance of reagents in vivo, and the high cost of biological therapies. Inhibition of C will render the recipient less capable of fending off bacterial infections, may predispose to immune complex diseases, and could cause defects in immune tolerance. Although short-term inhibition, as might be used in acute situations, is unlikely to cause many problems, long-term therapy for chronic conditions might well do so. Judicious choice of the stage of inhibition in the C pathway may reduce the risk. Given that so many of the 'physiological' effects of C are mediated by C3 and its fragments, agents that inhibit after C3 cleavage are unlikely to cause significant detrimental effects. Considerations of half-life and cost also relate primarily to long-term uses. A variety of strategies has been proposed to create longer-acting, cheaper anti-C agents for use in chronic conditions.

Recombinant soluble forms of the naturally occurring C regulators

As noted above under Complement therapies, sCR1 (TP10), generated by removing the membrane-anchoring domain from CR1, led the new wave of anti-C agents and has proved efficacious in a large and diverse range of disease models. sCR1 inhibits formation of the C3 and C5 convertase enzymes, preventing C3b opsonization, C5a generation, and MAC formation. It has been awarded 'orphan drug' status from the Food and Drugs Administration (FDA) for therapy of adult respiratory distress syndrome (ARDS) and as a treatment for infants undergoing cardiac surgery (Zimmerman et al. 2000; Rioux 2001). Numerous modifications have been made to sCR1 to alter or improve efficacy in vivo. Removal of the amino-terminal seven SCRs generates a reagent termed sCR1[desLHR-A], an alternative pathway-specific inhibitor which permits continued classical pathway activation for immune complex handling (Murohara et al. 1995; Scesney et al. 1996). Both sCR1 and sCR1[desLHR-A] have been derivatized with the sialyl Lewis^x (sLe^x) carbohydrate antigen, a ligand for E- and P-selectins (Rittershaus et al. 1999). These agents

Table 4 *Current C therapies*

Agent	History and status	Pros	Cons
sCR1 (TP10)	The first of the new generation of anti-C therapies; used in many models; first in clinical trials. Now superseded?	Proof of concept; works across species	Expensive Systemic
SLe^x-sCR1 (TP20)	First of the modified sCR1 agents; binds endothelium at sites of inflammation, tested in many models, no clinical trial	May be more 'site specific' than sCR1	Expensive Systemic
APT-070	Truncated, membrane-targeted sCR1 derivative, tested in several models, early stages of clinical trials	'Site specific'?, retained at injection site? Made in bacteria so cheaper?	Unproven Systemic
h5G1.1 and derivatives	Recombinant scFv of anti-C5 monoclonal antibody (mAb); permits opsonization; effective in several models; well advanced in clinical trials	Long half-life compared with sCR1; relatively cheap; nearest to the clinic. The mAb-based therapies well accepted	Systemic
C5aR antagonists	Several agents vying for this niche; attractive drug target; many positive results in models	Small molecule agents (peptides and others); may be inexpensive, may work orally or topically	Unproven
Small molecule antagonists of components	Numerous agents, best explored is the C3 inhibitor Compstatin; good results in models, no trials	May be inexpensive, may be active orally or topically	Unproven

For references to these agents, see the text.

showed enhanced efficacy in murine models of stroke and ischemia–reperfusion injury and rat models of ARDS, probably as a result of the combined activities of localizing C inhibition to the endothelial cell membrane and blockade of leukocyte adhesion to the endothelium (Huang et al. 1999; Mulligan et al. 1999; Schmid et al. 2001).

A truncated form of sCR1, comprising the amino-terminal three SCRs, has been successfully expressed and refolded from *Escherichia coli* and shown to retain activity (Dodd et al. 1995; Mossakowska et al. 1999). Chemical modification of the carboxy-terminus with an 'addressin', comprising a membrane-targeting peptide and a lipophilic acyl chain, myristate, results in a powerful regulator of C activation (Smith and Smith 2001). The 'addressed' reagent, termed APT070, is 100-fold more active than the parent molecule and has proved to be beneficial in various models of disease including arthritis and ischemia–reperfusion (Linton et al. 2000).

Recombinant, soluble forms of MCP and DAF have also been generated and proved effective at inhibiting C in vitro and in various models of C-mediated disease (Moran et al. 1992; Christiansen et al. 1996b). Unlike sCR1, which has both cofactor and decay-accelerating activity, sDAF and sMCP have only one anti-C activity. The sCR1 form is a more effective fluid phase inhibitor than either sDAF or sMCP and mixtures of the two agents are more effective than either alone, illustrating cooperative activities (Christiansen et al. 1996a). A hybrid comprising sMCP fused to the amino terminus of sDAF, termed C activation blocker-2 (CAB-2), is a potent inhibitor both in vitro and in models (Higgins et al. 1997; Kroshus et al. 2000).

The membrane MAC regulator, CD59, has also been generated as a soluble form (sCD59) (Sugita et al. 1994; Quigg et al. 2000). However, sCD59 is a poor inhibitor of C in whole serum, a consequence of its short plasma half-life and tendency to bind plasma lipoproteins.

Antibody-based therapies

Antibodies targeting either C components or C receptors have attracted considerable attention as potential therapies, buoyed by the many other antibody-based agents that have already reached the clinic. Perhaps the most successful of the current anti-C agents is an antibody, h5G1.1, that binds C5 and prevents its enzymatic cleavage (Thomas et al. 1996). This agent does not prevent cleavage of C3, so opsonization of pathogens and immune complexes with C3b can occur, whereas generation of C5a and MAC are prevented. A recombinant, humanized, single-chain Fv of this antibody has been used in clinical trials for treatment of ARDS and ischemia–reperfusion injury, and has been particularly successful in cardiopulmonary bypass at reducing the post-bypass syndrome (Thomas et al. 1996; Fitch et al. 1999). Other antibodies have been generated that target other components of the terminal pathway (Rollins et al. 1995), C fragments such as C3a and C5a (Czermak et al. 1999), or the receptors for these fragments (C3aR, C5aR) (Morgan et al. 1993).

Small molecule inhibitors

A different approach to the generation of anti-C agents has been to seek small molecule inhibitors. Such agents

potentially have major advantages over biological agents in terms of cost and ease of administration but are not without problems. Several groups have probed libraries of phage-display random peptides to identify agents. This approach has identified a cyclic 13-residue peptide, termed compstatin, which binds C3 and prevents its cleavage by convertase enzymes (Sahu et al. 1996; Nilsson et al. 1998; Fiane et al. 1999), and peptides that inhibit C1 activation (Roos et al. 2001). Other approaches have identified small molecule serine protease inhibitors specifically targeting C1s and factor D (fD) in the classical pathway and alternative pathway, respectively (Buerke et al. 1995; Kimura et al. 1998; Ueda et al. 2000). Numerous small molecule agents, peptide and non-peptide, targeting the C5aR (C5aR antagonists) have been developed and shown to be effective in models of ischemia–reperfusion injury and shock, and in the reverse passive Arthus reaction (Heller et al. 1999; Short et al. 1999; Riley et al. 2000; Strachan et al. 2001). Therapeutic effects have been demonstrated with some of these C5aR antagonists after oral or topical administration; such agents may be important therapeutic agents for the future.

CONCLUDING REMARKS

Complement, now over 110 years old, has finally come of age. The influence of C in many different areas of immunology and cell biology is now being realized; in fact, one would be hard pressed to identify a more protean part of the immune system. It should be clear from what has gone before that C is core to innate immunity, casts a large influence over adaptive immunity, plays key roles in cell death and clearance of debris, and is a major pathological factor in diverse diseases. The field is now ready to shed its slightly shady past and is prepared to be accepted as a 'respectable' branch of immunology. Welcome it with open arms!

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Antimicrobial peptides: defensins and cathelicidins

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Leukocytes, inflammatory exudates, and many secretions exert antimicrobial activity that results from the individual and combined effects of their constituents. The responsible components may include enzymes such as lysozyme, metal-binding proteins such as lactoferrin, highly cationic proteins such as histones or fragments thereof, various lipopolysaccharide (LPS)-binding molecules, and antimicrobial peptides. Surprisingly, there is large interspecies variation in the number and nature of these effector molecules, and considerable cell-to-cell and tissue-to-tissue variation within any given species. This chapter covers two large groups of antimicrobial peptides found in mammals: defensins and cathelicidins.

We define antimicrobial peptides as molecules with fewer than 100 amino acids, which exist in host defense settings, and exhibit antimicrobial activity under locally prevailing conditions. Antimicrobial peptides are ancient weapons of host defense, and many have been found in organisms that antedate mammals. A partial roster of these antimicrobial peptides includes the microhalocins of Archaebacteria (O'Connor and Shand 2002), the bacteriocins (Diep and Nes 2002), and lantibiotics (Pag and Sahl 2002) of Eubacteria, the amoebapores of protozoa (Leippe 1999), the cysteine-rich 'defensins' of plants (Broekaert et al. 1995; Garcia-Olmedo et al. 1998; Thomma et al. 2002), and numerous peptides of insects and other invertebrates (Dimarcq et al. 1998; Bulet et al. 1999). Readers seeking a broader view of antimicrobial peptides should consult the cited references, and the many fascinating studies in *Drosophila* spp. (Hoffmann and Reichhart 2002; Tzou et al. 2002).

Much information about mammalian antimicrobial peptides has come from studying the leukocytes found in blood and their precursors in the bone marrow. In humans, the most abundant blood leukocytes are granulocytes – cells with cytoplasm that contains multiple granules. Those most important for antimicrobial defense are variously called neutrophils, 'polys' – shorthand abbreviation of their more formal (and formidable) name: polymorphonucleated neutrophilic granulocytes (PMN). The several thousand small granules within the cytoplasm of each human PMN are tiny storage organelles that contain a variety of peptides and proteins, many with antimicrobial properties. The PMNs of rabbits and chickens have such an abundance of highly cationic peptides in their granules that they appear eosinophilic after conventional blood staining (Wetzel et al. 1967; Kogut et al. 1994; Stabler et al. 1994). These cells, often called 'heterophils' or pseudo-eosinophils, are the functional equivalent of the human PMN.

The cytoplasmic granules in human PMNs are of several types, and they behave differently after the cell ingests microbes or detects molecular signals (e.g. *N*-formylated peptides, complement-derived chemotaxins, etc.) denoting their presence. The PMN's primary ('azurophil') granules are formed early during its differentiation in the bone marrow, and these granules largely transfer their contents to intracellular phagocytic vacuoles. The secondary ('specific') granules of PMNs form after the azurophil granules and contain a substantially different set of proteins and peptides. Most of the contents of specific granules are secreted outside the PMN; whereas defensins are stored in azurophil

granules, cathelicidins are stored in the specific granules. As a result of these storage and delivery patterns, defensins and cathelicidins operate in environments that differ in ionic composition, pH, local peptide concentration, presence or absence of serum proteins, etc. Not surprisingly, they have different structures and properties.

DEFENSINS

Defensins constitute a large and growing family of antimicrobial peptides, the members of which have been identified in mammals, birds, and some reptiles. All defensins have a largely β sheet structure that is stabilized by a tri-disulfide framework formed by six paired cysteines. These peptides were initially called 'lysosomal cationic proteins' and were first noticed in studies of rabbit and guinea-pig granulocytes (Zeya and Spitznagel 1966a, 1966b). Technical advances in peptide purification and characterization allowed more detailed chemical descriptions of the rabbit molecules by the mid-1980s (Lehrer et al. 1983; Selsted et al. 1983, 1984), and homologous peptides were identified soon thereafter in human leukocytes (Ganz et al. 1985; Selsted et al. 1985a). Defensins are also produced by epithelial cells (Ouellette et al. 1989) and may contribute prominently to innate mucosal host defense in the respiratory, reproductive, and gastrointestinal tracts (Huttner and Bevins 1999).

Structural considerations

Cysteine-rich antimicrobial peptides that are also called defensins occur in the hemolymph of insects (Boman and Hultmark 1987; Hoffmann et al. 1996) and in various plant tissues (Broekaert et al. 1995; Garcia-Olmedo et al. 1998). In antifungal plant defensins, the peptide backbone is stabilized by four cystine disulfide bonds, rather than the three intramolecular S-S bonds present in mammalian defensins. At least 13 putative defensin genes have been identified in *Arabidopsis thaliana*, and these genes may encode 11 different defensins. The evolutionary relationship of insect and plant defensins to those of vertebrates is uncertain.

A short α-helical domain is present in several human β defensins (Hoover et al. 2000; Sawai et al. 2001; Schibli et al. 2002), insect defensins (Cornet et al. 1995; Bulet et al. 1999), and some plant defensins (Fant et al. 1998). However, the disulfide connectivity of insect defensins (cysteines 1–4, 2–5, 3–6) differs from that of any mammalian defensin. Structures of several α and β defensins have been solved by two-dimensional nuclear magnetic resonance (NMR) and X-ray crystallography (Selsted et al. 1985a; Bach et al. 1987; Pardi et al. 1988, 1992; Zhang et al. 1992; Zimmermann et al. 1995; Hoover et al. 2000; Sawai et al. 2001; Schibli et al. 2002). Although the cysteine spacing of mammalian α and β defensins differs considerably, and their connectivity differs slightly (Figure 1), all contain a triple-stranded β sheet with a distinctive 'defensin fold'. The six cysteines of α defensins are linked in a 1–6, 2–4, 3–5 pattern (Selsted and Harwig 1989), whereas a 1–5, 2–4, 3–6 pattern is found in β defensins (Tang and Selsted 1993). As cysteines 5 and 6 are positioned right next to each other in both α and β defensins, this connectivity difference has little impact on their backbone structure (Zimmermann et al. 1995). Several human β defensins and insect defensins are illustrated in Figure 2. Recent forays into the human genome have revealed a large number of β-defensin genes and/or pseudogenes, the integrity and functionality of which remain to be determined (Schutte et al. 2002).

Defensin evolution

The presence of β-defensin-like peptides in snake venom (e.g. growth-arresting peptide and crotamines (Zhao et al. 2001b)) suggests that some of these host defense peptides can be used against targets considerably larger than bacteria, fungi, and viruses. Three members of a structurally unique defensin subfamily were recently identified in leukocytes and bone marrow of the rhesus macaque (Tang et al. 1999; Leonova et al. 2001). Called rhesus theta (θ) defensins (RTD), these molecules are cyclic octadecapeptides with three intramolecular disulfide bonds and a β-sheet structure (Tang et al. 1999). Their production involves post-translational splicing of two nonapeptide segments, each derived from a truncated, α-defensin-like precursor (Figure 3). The adjoining chromosomal locations of their genes, and

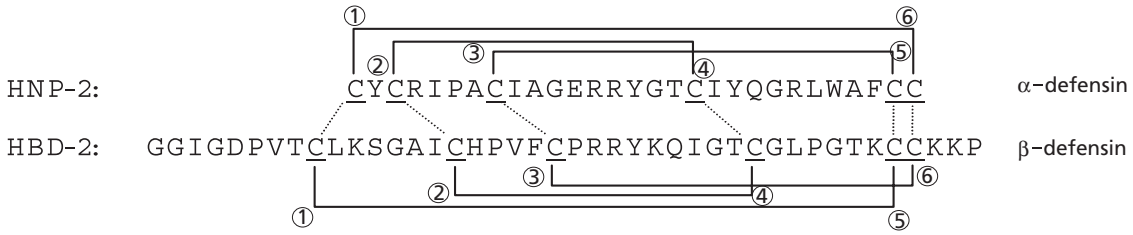

Figure 1 *α and β defensins. Human neutrophil peptide-2 (HNP-2), an α-defensin, and human β-defensin-2 (HBD-2) are shown. Both contain six cysteines, that have been numbered from the amino terminus. The cysteines of α and β-defensins have a different pairing pattern, as described in the text, and β defensins are somewhat larger.*

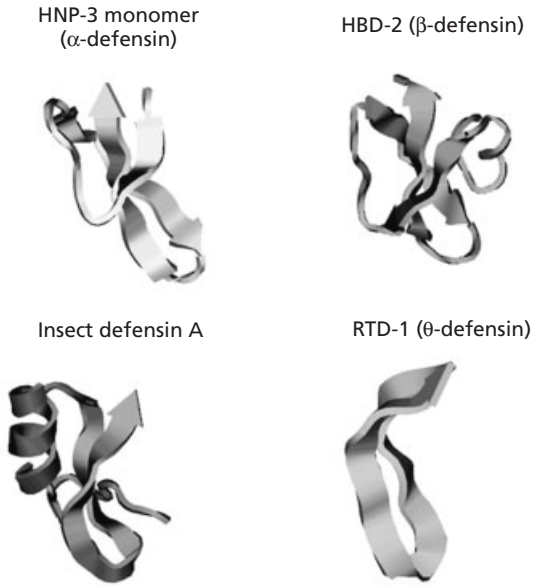

Figure 2 *Backbone structures of four different types of defensins. The α-, β-, and θ-defensin peptides expressed by animals arose from a common ancestral gene, the relatedness of which to insect defensin genes is unknown.*

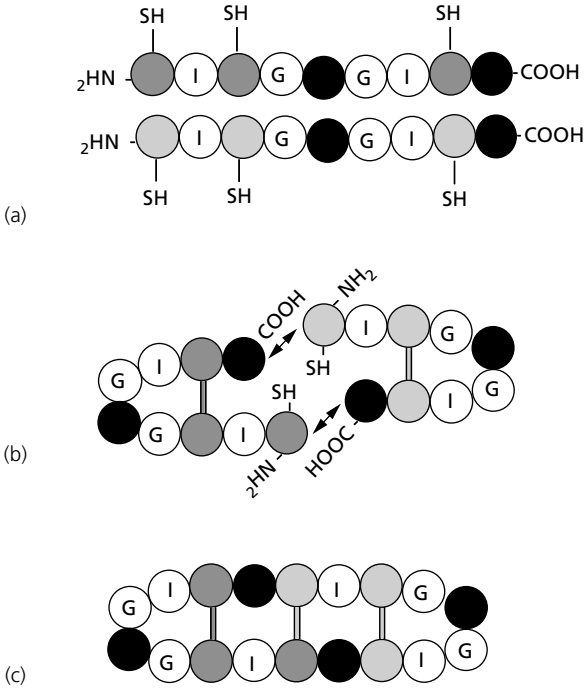

Figure 3 *Making a θ defensin. In these cartoons, each circle represents an amino acid residue identified either by single letter code (G, glycine; I, isoleucine) or color (black, arginine; gray, cysteine). The two identical nonapeptides in* **(a)** *correspond to a portion of the retrocyclin-1 precursor described in the text. In* **(b)**, *the double-headed arrows show where these nonapeptides will be spliced to form the cyclic octadecapeptide (θ defensin) shown in* **(c)**. *The events shown in this figure are illustrative – the actual intracellular biochemistry of θ-defensin production remains to be established.*

the structural similarity of their peptides or peptide precursors, make it virtually certain that α, β, and θ defensins arose from a common ancestral gene (Liu et al. 1997).

Just as the existence of β defensins in birds and reptiles identifies them as the oldest vertebrate defensin subfamily, the initial appearance of θ defensins in Old World Monkeys marks them as the youngest subfamily. We have identified intact *DEFT* (θ defensin) genes in several Old World Monkeys, siamangs (a lesser ape), and orangutans. In contrast, we found that the corresponding *DEFT* genes of humans, gorillas, and chimpanzees contained a mutation (a stop codon in the signal sequence) that prevented translation. From phylogenetic considerations, we infer that a hominid ancestor who lived between 7.5 and 10 million years ago sustained the mutation that removed θ defensins from the innate host-defense arsenal of his or her descendants. θ defensins are interesting for many reasons, not the least of which is their ability to inhibit the uptake of HIV-1 by otherwise susceptible $CD4^+$ target cells (Cole et al. 2002).

Variability of expression

Although α- and/or β-defensin peptides probably exist in all mammals and birds, relatively few different species have been studied to date. Defensin expression by neutrophils demonstrates remarkable interspecies variability. Whereas the PMNs of humans, rats (Eisenhauer et al. 1989, 1990), and rabbits contain large amounts of α defensins, the PMNs of cattle (Selsted et al. 1993) and chickens (Harwig et al. 1994) have only β defensins, and murine PMNs contain neither α nor β defensins. Several other mammals, including pigs, sheep, goats, and horses, have PMNs that lack defensins. In some of these animals (e.g. pigs and sheep), the PMNs may compensate by expanding their cathelicidin content. In other species, PMNs may rely more on oxidative microbicidal mechanisms mediated via reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Wientjes and Segal 1995) and/or inducible nitric oxide synthase (MacMicking et al. 1997; Sethi and Dikshit 2000).

The highest local concentrations of defensins (>10 mg/ml) occur in the storage organelles (granules) of leukocytes (Ganz 1987). When leukocytes ingest microbes and sequester them within phagocytic vacuoles, fusion of the granule and vacuole membranes deposits the defensins and other contents directly on the captured microbe. As little 'empty' space exists in phagocytic vacuoles, ingested microbes are exposed to very high peptide concentrations which, by favoring defensin oligomer/multimer formation, could enhance their efficacy and override any inhibitory effects of the intravacuolar ionic milieu or pH.

Paneth cells are specialized host-defense cells that occupy the base of the crypts in the small intestines of humans and other omnivores. Their prominent secretory granules contain α defensins, and these peptides are secreted into the narrow crypt lumen after bacteria or bacterial LPS enters the intestine. Paneth cell secretion is also stimulated by cholecystokinin or parasympathomimetic agents (Satoh 1988; Satoh et al. 1989). Intestinal crypts are the exclusive sites where the epithelial cells that line intestinal villi arise. Consequently, anticipatory secretion of defensins by Paneth cells may protect this vital cell nursery from adventurous microbes that enter the crypts. Given the crypt lumen's narrow dimensions, its post-secretory defensin concentration may resemble that found in PMN phagocytic vacuoles (Ayabe et al. 2000).

Skin keratinocytes and various epithelia produce β-defensins constitutively (Valore et al. 1998) or in response to infection or inflammation (Harder et al. 2001; Schonwetter et al. 1995). On average, the concentration of defensins in these epithelia ranges from 10 to 100 μg/ml (Harder et al. 1997; Shi et al. 1999), but may be much higher locally if the peptides are unevenly distributed. In the human skin, human β-defensin-2 production is massively induced by interleukin-1 released from myeloid cells, and is secreted by lamellar bodies into the intercellular space between differentiating keratinocytes (Liu et al. 2002, 2003; Oren et al. 2003). Keratinocytes that overexpress human β-defensin-1 (HBD-1) show greater expression of keratin 10, a differentiation marker, suggesting that HBD-1 may enhance cell keratinocyte differentiation (Frye et al. 2001).

Although murine PMNs lack leukocyte defensins and rat PMNs have them in abundance, the Paneth cells of both species express multiple α defensins, and their epithelial tissues express many β defensins. In some cases, defensin expression may be induced by a combination of a specific cell type and local environment. Inflammatory macrophages are leukocytes that arise from circulating blood monocytes which differentiate under the influence of local tissue signals. Rabbit alveolar (lung) macrophages contained two α defensins (NP-1 and NP-2) in amounts approaching their abundance in rabbit neutrophils, but no defensins were present in peritoneal macrophages from the same animals (Ganz et al. 1989). Although defensin expression in monocytes, macrophages, and lymphocytes of some mammals can be detected by highly sensitive techniques (Ryan et al. 1998; Agerberth et al. 2000; Duits et al. 2001), high levels of defensins within macrophages have been documented only in the rabbit alveolar macrophage (Patterson-Delafield et al. 1980, 1981). Some peculiarities in the variable pattern of defensin expression may be driven by evolutionary pressure from species-specific or organ-specific pathogens.

Antimicrobial and antiviral properties

Most defensins display microbicidal activity against bacteria and fungi, especially if tested under low ionic strength conditions or with low concentrations of divalent cations, plasma proteins, or other interfering substances (Selsted et al. 1985b; Lehrer et al. 1988). Under optimal conditions, defensin-mediated antimicrobial activity is evident at peptide concentrations in the low micromolar range. Increasing the medium's salinity inhibits the antimicrobial efficacy of defensins against many organisms, especially gram-negative bacteria. Certain plasma proteins bind defensins, e.g. α_2-macroglobulin (Panyutich and Ganz 1991), and are also inhibitory. In general, metabolically active bacteria are much more sensitive to defensins than bacteria made inactive by nutrient deprivation or metabolic inhibitors. Some bacteria, e.g. *Brucella* and *Burkholderia* spp., are intrinsically resistant to defensins and many other antimicrobial peptides (Martinez et al. 1995; Jones et al. 1996).

Infection by enveloped viruses, such as herpes simplex and vesicular stomatitis virus (Lehrer et al. 1985; Daher et al. 1986), and by adenovirus, a nonenveloped virus (Gropp et al. 1999; Bastian and Schafer 2001), is inhibited by defensins. Human α defensins HNP-1, -2, and-3 suppressed HIV replication (Nakashima et al. 1993). Their production by αβ-$CD8^+$ T cells may promote resistance to developing AIDS (Zhang et al. 2002).

As some defensins are cytotoxic to mammalian cells in vitro (Lichtenstein et al. 1986, 1988; Lichtenstein 1991; Zhang et al. 2001), especially when they are tested at high concentrations or under serum-free conditions, it is possible that defensins may contribute to inflammatory tissue damage in vivo.

ANTIMICROBIAL MECHANISMS

An indicator bacterium (*Escherichia coli* ML-35) (Lehrer et al. 1989) and the K562 mammalian cell line (Lichtenstein 1991) became permeable to small molecules, such as sugars and trypan blue, after being treated with α defensins. In the bacteria, the onset of permeabilization coincided with inhibition of RNA, DNA, and protein synthesis, and decreased bacterial viability as assessed by colony-forming assays. In the K562 cell line, permeabilized cells could be rescued for up to 1 h by removing the defensin. There is evidence that intracellular sites of action also contribute to cell death (Gera and Lichtenstein 1991; Lichtenstein 1991).

In experiments with artificial membranes composed of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine in a 2:2:1 ratio, defensins NP-1 (rabbit) and HNP-1 (human) formed voltage-dependent channels, requiring negative potential on the membrane side opposite to where defensins were applied (Kagan et al. 1990). Evidently, insertion of defensin molecules

into the membrane is aided when transmembrane electrical forces attract the positively charged defensin molecule. Unlike melittin, a bee-venom peptide that indiscriminately rendered vesicles composed of neutral or anionic phospholipids permeable, defensins were much more active against vesicles containing negatively charged phospholipids (Fujii et al. 1993).

In general, the membranolytic activity of defensins on phospholipid vesicles was diminished by increased salt concentrations, supporting the importance of electrostatic interactions between the cationic (positively charged) defensins and the anionic (negatively charged) head groups of membrane phospholipids. In other experiments, large unilamellar vesicles composed of palmitoyloleoylphosphatidylglycerol (POPG), a negatively charged phospholipid) were permeabilized by human defensin HNP-2 and adding neutral phospholipids to the lipid mix inhibited both defensin binding and permeabilization (Wimley et al. 1994). The importance of anionic phospholipids in defensin–membrane interactions was also shown by calorimetric measurements of phase transitions in membranes (Lohner et al. 1997). In the aggregate, these studies show that both externally applied and local electric fields influence the ability of defensin molecules to insert into a target membrane.

It is less certain what happens once the defensin molecules are in the membrane. The observed leakage of dye markers from liposomes implies the formation of stable or transient transmembrane pores. (We use the term 'pore' to refer to any type of ion or water-permeable structure or lesion within the membrane.) For some defensins, the release of internal markers from each vesicle appeared to occur in an all-or-none fashion (Wimley et al. 1994), suggesting the formation of stable pores with a diameter estimated at 2.5 nm by using markers with different sizes. The authors proposed a model of a defensin pore – a hexamer of dimers that could generate an opening of the observed size. However, stable pore formation is not the only mechanism of defensin interaction with membranes. The more cationic rabbit defensins induced a partial release of markers from individual vesicles, indicating that the pores formed were not stable. Possibly, electrostatic repulsion between the highly cationic rabbit defensin molecules destabilizes the pores.

MICROBIAL RESISTANCE

Specific mechanisms that confer increased bacterial resistance to defensins have been identified by insertional mutagenesis. Disruption of the two-component transcriptional regulator phoP-phoQ increased the sensitivity of *Salmonella* spp. to defensins and other cationic peptides (Groisman et al. 1989, 1992; Miller et al. 1990; Miller 1991). The phoP-phoQ directly regulates multiple genes involved in resistance to cationic peptides and also exerts some of its activity by modulating a second two-component regulator, PmrA-PmrB. The function of the downstream genes includes covalent modification of LPSs that decreases their affinity for cationic peptides (Guo et al. 1998) and expression of membrane proteases that degrade cationic peptides (Guina et al. 2000). In *Neisseria gonorrhoeae*, a bacterium that is naturally resistant to defensins (Qu et al. 1996), the energy-dependent efflux system *mtr* increases the resistance to protegrins, potently antimicrobial β-hairpin peptides originally isolated from pig neutrophils (Shafer et al. 1998). In staphylococci, the disruption of either of two genes, *dlt* or *MprF*, increased sensitivity to defensins (Peschel et al. 1999, 2001). The *dlt* gene is required for covalent modification of cell wall teichoic acid by alanine, and *MprF* is needed for covalent modification of membrane phosphatidylglycerol with l-lysine. By decreasing the negative charge of the cell wall and bacterial membrane, respectively, these mutations diminish their attraction for the cationic defensins. Homologs of these resistance genes exist in many bacterial species, indicating that similar mechanisms may be widespread.

OTHER ACTIVITIES

Various defensins have chemotactic activity for monocytes, T lymphocytes, and dendritic cells (Territo et al. 1989; Chertov et al. 1996; Yang et al. 1999, 2000). Human β defensins-1 and -2 (HBD-2) attract memory T cells and immature dendritic cells, apparently by binding to the chemokine receptor CCR6 (Yang et al. 1999). Although the physiological significance of this interaction is uncertain, the high concentrations of HBD-2 in inflamed skin might allow this defensin to compete effectively with the natural chemokine ligand (variously named CCL20, LARC, or MIP-3α) despite the latter's higher affinity for the CCR6 receptor. Recent structural analysis of CCL20 pointed out remarkable similarities to HBD-2 in the putative receptor-binding region of CCL20. The role of this region in the chemotactic activity of HBD-2 needs to be confirmed by mutating the amino acid residues suspected in its interaction with CCR6. Human neutrophil defensins HNP-1–3 were reported to be chemotactic for monocytes (Territo et al. 1989), naive T cells, and immature dendritic cells (Yang et al. 2000) but a specific receptor remains to be identified. Murine β-defensin-2 acted like an in vivo adjuvant for anti-tumor immunization, and induced dendritic cell maturation by activating Toll-like receptor-4 (TLR4), leading to a type 1 polarized adaptive response in vivo.

Certain defensins, especially rabbit NP-3A, can oppose the action of adrenocorticotropic hormone (ACTH) by binding to ACTH receptor without activating it (Tominaga et al. 1990). NP-3A was called Corticostatin-1 (CS-1) by some investigators (Zhu et al. 1989; Solomon et al. 1991) who failed either to recognize or to acknowledge that their sequences were identical.

By inhibiting production of cortisol (an immunosuppressive hormone) in vivo, this property might enhance the net response to infections. Tominaga et al. (1992) used an antiserum against synthetic rabbit NP-3a (CS-1 in their terminology) to examine the peptide's distribution in several rabbit tissues, including the hypothalamic–pituitary–adrenal axis. Lung and spleen had the highest levels of immunoreactivity, and relatively high levels were also detected in the pituitary, adrenal medulla, and small intestine, although not in the hypothalamus. Plasma levels of NP-3A averaged 7.8 ng/ml, and increased to 185.4 ng/ml with infection. Levels of NP-3A in the adrenal gland, small intestine, and hypothalamus also increased in rabbits with active inflammation. Although these data suggest that NP-3A (CS-1) may affect the hypothalamic–pituitary–adrenal axis in an endocrine or paracrine manner, the physiological role – if any – of corticostatic activity remains to be demonstrated.

Nanomolar concentrations of some defensins activate nifedipine-sensitive calcium channels in mammalian cells (MacLeod et al. 1991; Bateman et al. 1996). Certain mouse Paneth cell defensins (called 'cryptdins' to commemorate their presence in murine small intestinal crypts) activate chloride secretion, most probably by forming channels in the apical membrane of epithelial cells (Lencer et al. 1997; Merlin et al. 2001). This activity is limited to a subset of cryptdins, and its structural basis is not yet known. It is difficult, at present, to reconcile the multiple and varied activities of defensins with any simple receptor–ligand model.

We have recently discovered that certain α and θ defensins interact with various sugars and that this lectin-like property underlies their high-affinity binding to functionally significant glycoproteins and glycolipids. As immunoglobulins, cellular receptors, and viral envelope proteins used to promote cell entry are frequently glycosylated, this property (sugar binding) may affect many activities of defensins, especially their antiviral properties.

Defensin biosynthesis

In humans, multiple genes encoding various α and β defensins are clustered on chromosome 8p23 (Sparkes et al. 1989; Harder et al. 1997; Liu et al. 1997, 1998; Frohlich et al. 2001). Additional defensin-related genes were identified in four other clusters, so that, overall, as many as 30 transcribed defensin genes may exist in 'mice and men' (Schutte et al. 2002). It has been difficult to map the primary defensin locus in 8p23, presumably as a result of its polymorphic nature and because individuals and their chromosomes differ in the number of copies of certain defensin genes (Mars et al. 1995). α defensins are generally encoded as a tripartite pre-propeptide sequence, wherein a 90–100 amino acid precursor contains an amino terminus consisting of a 19 amino signal sequence, an anionic propiece of about 45 amino acids, and a carboxy-terminal mature cationic defensin of about 30 amino acids (Daher et al. 1988) (Figure 4). In many cases, the charges of the propiece and the mature defensin approximately balance (Michaelson et al. 1992), and this arrangement may be important for folding and/or to prevent intracellular interactions with membranes (Liu and Ganz 1995; Valore et al. 1996).

α-defensin synthesis by PMNs takes place in the bone marrow, in precursor cells called promyelocytes (Yount et al. 1995; Arnljots et al. 1998; Cowland and Borregaard 1999). Mature neutrophils in blood or inflamed tissues contain large amounts of defensin peptides, but no longer synthesize significant amounts of the peptides or their RNA. In myeloid cell lines, the defensin signal sequence is rapidly removed during synthesis. Subsequent proteolytic processing takes many hours, and is completed in maturing cytoplasmic granules (Valore and Ganz 1992). Defensin processing is a very efficient process, because only small amounts of incompletely processed intermediates are detectable in mature neutrophils (Harwig et al. 1992).

Processing the α-defensins (cryptdins) of murine intestinal Paneth cells requires a metalloprotease called matrilysin (MMP-7), because mice with homozygous disruption of the matrilysin gene fail to process Paneth cell pro-defensins past the removal of the signal sequence. In humans, processing of Paneth cell defensins takes place after secretion and involves Paneth cell trypsin (Ghosh et al. 2002). β-defensin precursors are structurally less complex than α-defensin precursors, having a signal sequence, a short or absent propiece, and a C-terminal mature defensin domain. It is not yet clear why β-defensin precursors can lack an anionic propiece and α-defensin precursors apparently must have one.

Defensin sequences and composition

Except for the conservation of their cystine framework and a few additional residues, the amino acid sequences of mature defensins are highly variable. Clusters of positively charged amino acids are characteristic of most α and β defensins but their specific distribution within the defensin molecule is inconstant (Figure 5 and see Figure 4).

STORAGE AND DELIVERY

In leukocytes and Paneth cells of the small intestine, defensins are stored in granules. These subcellular storage organelles are rich in negatively charged glycosaminoglycans (Spicer et al. 1967; Olsson 1970; Fromm et al. 1995; Hileman et al. 1998). With the exception of chicken gallinacins, these α and β defensins contain arginine as the predominant cationic amino acid. In contrast, β defensins that are secreted from epithelial cells contain similar amounts of arginine and lysine. The preferential use of arginine in defensins stored in

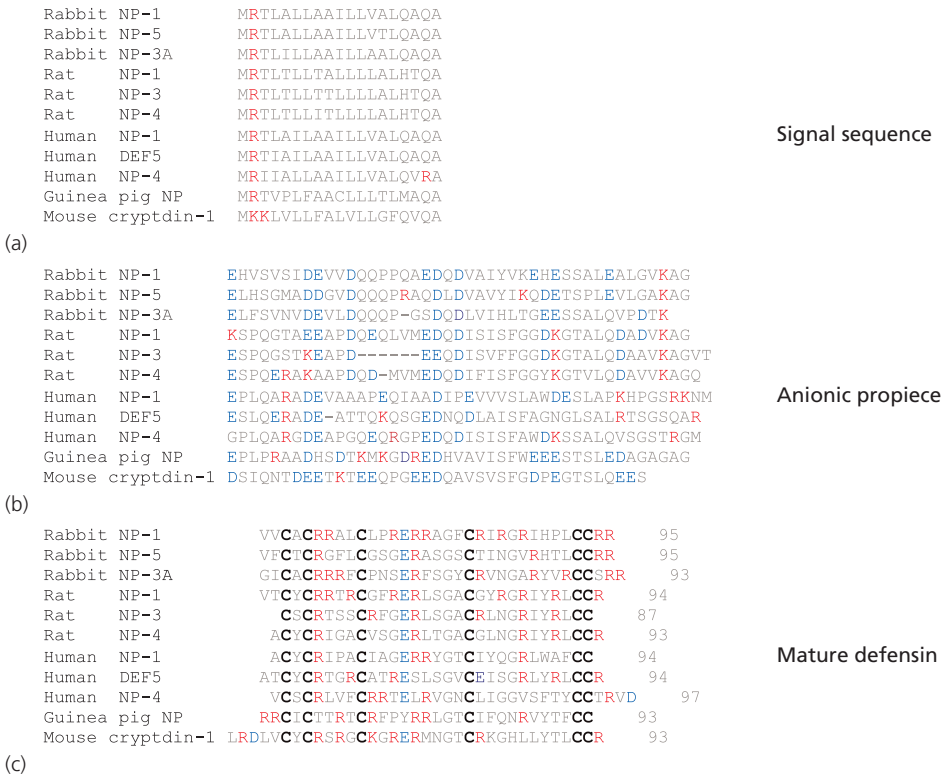

Figure 4 *Sequences of α-defensin precursors. Eleven α defensins from four species are shown. Positively charged amino acids, arginine (R) and lysine (K), are red; negatively charged residues, aspartic (D) or glutamic (E) acid, are blue; cysteine (C) is black. Note that:* **(a)** *the signal sequences are highly conserved;* **(b)** *the long propiece has a net negative charge; and* **(c)** *the mature defensin domain contains 29 (human NP-1) to 35 (mouse cryptdin-1) residues, including six conserved cysteines, and has a net positive charge.*

granules may reflect the constraints imposed by packing defensin molecules into the glycosaminoglycan matrix of granules. Alternately, synergistic interactions between defensin and myeloperoxidase, or its downstream products (e.g. HOCl), might have favored the selection of arginines over lysines. Myeloperoxidase is present in mammalian neutrophils but is absent from those of chickens and other fowl (Penniall and Spitznagel 1975; Rausch and Moore 1975).

STRUCTURE–FUNCTION CONSIDERATIONS

At present, no single mechanism explains how defensins kill bacteria, or even if the critical lethal event that defensins inflict is membrane permeabilization. In part, the present uncertainty reflects the diversity in net charge, amino acid sequence, and quaternary structure (monomers versus dimers) found among defensins. It seems likely that these differences evolved to allow defensins to recognize and target bacteria with different cell wall or membrane structures, or different patterns of metabolism or growth. Furthermore, although interactions between defensins with model membranes have been examined (Wimley et al. 1994), they may provide only a first approximation of the events that transpire in more complex biological systems. Further work in this area is needed.

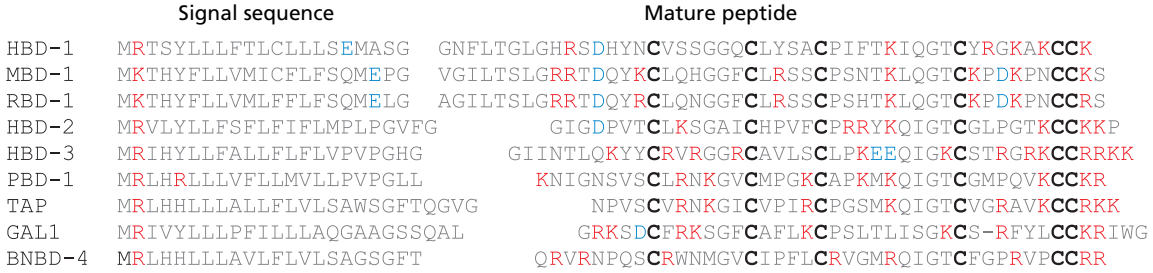

Figure 5 *Sequences of β-defensin precursors. Nine peptides from six species are shown. β-Defensin precursors are considerably shorter than α-defensin precursors, primarily as a result of the presence of a much smaller propiece. Mature β-defensin peptides are positively charged (especially so in HBD-3 or TAP – transporter associated with antigen processing), are longer than α defensins, and the spacing of their six conserved cysteines differs from that seen in α defensins.*

CATHELICIDINS

Cathelicidins are bipartite molecules with a conserved amino-terminal 'cathelin' domain containing about 100 amino acid residues (Zanetti et al. 1995, 2000). The word cathelin is an acronym for cathepsin L inhibitor, and was chosen to commemorate the domain's homology to cystatins, a family of proteins that inhibit cysteine proteases. This inference was supported by later X-ray crystallography and NMR studies (Sanchez et al. 2002a, 2002b). These studies revealed that the cathelin domain and other cystatin family members had similar backbone folds; namely an amino-terminal helix surrounded by a four-stranded β sheet. In addition, the core hydrophobic residues and disulfides of cathelin and cystatins were conserved. Although the function of the cathelin domain remains uncertain, it may protect cathelicidin precursors from premature proteolytic activation during their targeting, packaging, and storage. In many cathelicidins, the carboxy-terminal domain has both antimicrobial and LPS-binding properties. Although the antimicrobial properties of cathelicidins remain latent until the C-terminal peptide is separated from the cathelin domain by limited proteolysis (Zanetti et al. 1993), the LPS-binding properties may not require this activation step (Zarember et al. 2002).

Cathelicidins with short antimicrobial domains

Cathelin-associated antimicrobial peptides vary considerably in size and structure. The smallest are the 12-residue dodecapeptides (also called cyclic 'bactenecins') found in cattle and sheep. The peptides have similar sequences – RLCRIVVIRVCR (bovine) and RICRIIFLRVCR (sheep) – and contain a disulfide bond between their cysteine residues. Bovine indolicidin has 13 residues (ILPWKWPWWPWRR), including 5 tryptophans. Its small size and unusual composition has made it a favorite for analog design and structure–activity studies. NMR studies of indolicidin performed in a membrane-like environment composed of dodecylphosphocholine micelles revealed a well-defined, extended backbone between residues 3 and 11, with half-turns at residues Lys5 and Trp8. The peptide's central hydrophobic core, which is proline and tryptophan rich, was bracketed by positively charged side chains closer to its ends. All but one tryptophan side chain folded flat against the backbone, imparting a wedge shape to the molecule. Indolicidin's preferred location in micelles and lipid bilayers was at the membrane interface. It bound purified LPS with high affinity, and permeabilized the outer and inner membranes of *E. coli* (Falla et al. 1996). When a sufficiently electronegative potential (threshold −70 to −80 mV) was applied to the distal face of a model planar lipid bilayer, the peptide induced transmembrane currents and formed discrete channels with a conductance ranging from 0.05 to 0.15 nanosiemens (nS).

Tritrpticin (VRRFPWWWPFLRR) is a 13-residue antimicrobial peptide with a precursor that is expressed by porcine leukocytes as part of the pro-piece of a 79-residue proline and phenylalanine-rich peptide called prophenin (Harwig et al. 1995). The initial studies of tritrpticin showed activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphyloccus epidermidis*, *Proteus mirabilis*, group D streptococci, and *Aspergillus fumigatus*. The peptide was bactericidal rather than bacteriostatic and its activity was completely inhibited by 2 mmol/l $MgCl_2$ (Lawyer et al. 1996).

In sodium dodecylsulfate micelles, tritrpticin's adjacent turns cluster its hydrophobic residues and separate them from the positively charged arginines, making the peptide amphipathic (Schibli et al. 1999). Spin-label studies showed tritrpticin near the micelle surface, with its aromatic side chains equally partitioned into the hydrophilic–hydrophobic interface and its tryptophan residues inserted. The tryptophan side chains inserted more deeply in vesicles that contained phospholipids with anionic phosphatidylglycerol head groups (Schibli et al. 2002).

Cathelicidins with β-sheet antimicrobial domains

To date, protegrins are the only cathelin-associated antimicrobial peptides with a β-sheet structure. Three protegrin peptides (PG-1,-2, and-3 were purified from porcine leukocytes, and two additional protegrins (PG-4 and-5) were identified by cloning studies. Porcine protegrins contain 16–18 amino acid residues, including four cysteines that form two intramolecular disulfides. These disulfides are essential to preserve optimal broad-spectrum antimicrobial activity in physiological saline. Although protegrins are much smaller than defensins, they show substantial sequence similarity to rabbit defensin, NP-3a. Relatively low concentrations of protegrins (<5 μg/ml) rapidly kill a very wide range of bacteria and fungi, including organisms that are resistant to conventional antibiotics. These properties, in combination with their small size and relatively simple production by direct chemical synthesis, has led to their use in mechanistic studies and as templates for pharmaceutical development (Bellm et al. 2000). Protegrins PG-1 and PG-3 have immunomodulatory properties, because both initiated the processing of pro-interleukin-1β from LPS-stimulated human monocytes, resulting in the efficient release of mature interleukin 1β (IL-1β) (Perregaux et al. 2002).

Cathelicidins with proline-rich antimicrobial domains

The cathelicidins of pigs and ruminants include several with remarkably proline-rich (40–50 percent proline!)

antimicrobial domains (Table 1), with either arginine or phenylalanine the next most abundant residue. In bovine bactenecin-7 (Bac7), most prolines are evenly spaced and separated by a single nonproline residue. Bovine Bac5 and porcine PR-39 contain several repeats of a 'PPXX' motif (i.e. two prolines followed by two nonproline residues). The initial 60 residues of prophenins 1 and 2 are repeated proline-rich decamers. Both of these prophenin isoforms were found in porcine lung surfactant preparations prepared by chloroform/methanol extraction (Wang et al. 1999) and contained, in addition to the expected components (98 percent phospholipids and 1–2 percent surfactant-associated proteins B and C), three prophenin-related molecules. These were: full-length prophenin-1; FPPPPPFRPPP FGPPRF – the carboxy-terminal octadecapeptide of prophenin-1; and a variant of prophenin-2 with an amino-terminal pyroglutamic acid extension. These components may have contributed to the antimicrobial properties of the preparation, the commercial equivalent of which (Curosurf) has been given to tens of thousands of pre-term infants with respiratory distress syndrome since its European introduction in 1992.

PR-39, a proline- and arginine-rich cathelicidin peptide found in porcine PMNs, has no known human counterpart. The peptide was initially detected as a result of its antimicrobial properties, but was subsequently reported to modulate proteoglycan production in wounds (Gallo et al. 1994), to promote leukocyte chemotaxis (Huang et al. 1997), to interact with intracellular -SH_3 proteins (Chan and Gallo 1998), and to inhibit superoxide production by PMNs (Shi et al. 1996). Expression of PR-39 and protegrin by porcine bone marrow increased substantially after experimental infection with *Salmonellas typhimurium* or in vivo administration of LPS (Wu et al. 2000). In vitro, expression of PR-39 and protegrin by cultured porcine bone marrow cells increased in response to IL-6 or all-*trans*-retinoic acid. The PR-39 and protegrin genes have upstream binding sites for IL-6 and retinoic acid, as well as potential C/EBP-binding motifs (Wu et al. 2000).

Cathelicidins with α-helical domains

Most cathelin-associated antimicrobial peptides are α helical. Typically, such peptides have 23–37 residues and a net positive charge of +6 or more (see Table 1). In addition, many possess an amidated C terminus – a feature that further increases overall cationicity. SMAP-29, an α-helical cathelicidin-derived peptide of sheep, has unusually potent, broad-spectrum antimicrobial activity in vitro (Travis et al. 2000). Brogden and associates induced a *Mannheimia haemolytica* pneumonia in lambs and, 24 h later, administered 0.5 mg SMAP-29 directly to the affected part of the lung (Brogden et al. 2001; Kalfa et al. 2001). Twenty-four hours later, the experimental and control animals were euthanized and their lungs examined. The peptide-treated animals showed reduced inflammation and consolidation, and had lower numbers of bacteria in their bronchoalveolar lavage fluid.

Sites of cathelicidin synthesis and storage

The neutrophils of cattle store cathelicidin peptides in large cytoplasmic granules. The stored peptides remain inactive until their cathelin and antimicrobial domains

Table 1 *Sequences of selected cathelicidin peptides*

Structure	Species	Sequence
α Helical		
LL-37	Human	**LLG**D**FFRKSKEKIG**KEF**K**RIVQRIKDFLRNLVPRTES
RL-37	Rhesus	RLGNFFRKVKEKIGGGLKKVGQKIKDFLGNLVPRTAS
CRAMP	Mouse	**LL**RKGGE**K**IGE**K**----**LKKIGQKIK**NFFQK**LVP**QPEQ
Proline rich		
Bac-5	Cow	RFR**PP**IRR**PP**IR**PP**FY**PP**FR**PP**IR**PP**IF**PP**IR**PP**FR**PP**LG**P**F**P***
PR-39	Pig	RRR**PR**P**P**YL**PR**P**R**P**PP**FFP**P**RL**PP**RI**PP**GF**PP**RF**PP**RF**P***
Prophenin-1	Pig	AF**PPP**NV**P**G**P**R[F**PPP**NF**P**G**P**R]$_3$F**PPP**NF**P**G**PP**F**PPP**IF**P**G**P**WF**PPPPP**FR**PPP**FG**PP**RF**P***
Cyclic dodecapeptide		
Bactenecin	Cow	**R**LC**RI**VVI**RVCR**
Bactenecin	Sheep	**R**ICR**I**IFL**RVCR**
β Hairpin		
Protegrin (PG)-1	Pig	**RGGRLCYCR**RRFCV**CVGR***
Protegrin (PG)-4	Pig	**RGGRLCYCR**GWIC**FCVGR***
Tryptophan rich		
Indolicidin	Cow	ILPWK**WP**W**W**PW**RR***
Iritrpticin	Pig	VRRFP**WWW**PFL**RR**

In the proline-rich peptide sequences, the proline residues are emboldened to accentuate their motifs. In the other peptide groupings, identical residues are emboldened, and similar residues are underlined. The asterisk (*) signifies carboxy-terminal amidation

are separated proteolytically by neutrophil elastase (Scocchi et al. 1992), which also activates cathelicidins in porcine neutrophils (Panyutich et al. 1997; Cole et al. 2001). In contrast, human PMNs activate their cathelicidin, hCAP-18, with another serine protease – protease 3 (Sorensen et al. 2001).

The cytoplasmic storage organelles of human and bovine PMNs differ in morphology and content. In human neutrophils, the primary ('azurophil') granules contain α defensins along with cathepsins, bactericidal/permeability-increasing protein (BPI), lysozyme, and several catalytically active neutral serine proteases (elastase, cathepsin G, and protease 3). The secondary ('specific') granules store hCAP-18, the cathelin-containing precursor of LL-37, along with lactoferrin, additional lysozyme, and B_{12}-binding protein. Bovine PMNs (and those of other ruminants) have an additional class of storage organelles called 'intermediate' granules. (Gennaro et al. 1983; Baggiolini et al. 1985). Intermediate granules serve as the main repositories for cathelicidins, are larger and more numerous than primary or secondary granules, and release their contents in response to phagocytosis or soluble stimuli (Gennaro et al. 1983; Baggiolini et al. 1985).

In an in vitro experimental system wherein hCAP-18 was misdirected into azurophil granules, it underwent premature proteolytic activation and released LL-37 (Bulow et al. 2002). Peptide purification and cloning have revealed at least seven different cathelin-associated antimicrobial peptides in bovine neutrophils: cyclic dodecapeptide, indolicidin, three α-helical peptides (BMAP-27, BMAP-28, and BMAP-37), and two proline-rich molecules (Bac5 and Bac7) (Scocchi et al. 1997). Similar studies in pigs have identified five protegrins, three proline-rich molecules (PR-39, prophenin-1, and prophenin-2), and three α-helical peptides (PMAP-23, PMAP-36, and PMAP-37) (Gennaro and Zanetti 2000; Zanetti et al. 2000).

CATHELICIDINS OF MICE AND MEN

In contrast to numerous cathelicidins in pigs, cattle, and ruminants, the human genome encodes only a single cathelicidin, hCAP-18, the antimicrobial domain of which is the 37-residue, α-helical peptide called LL-37. The gene is expressed constitutively in human neutrophils and the resulting propeptide is stored in its secondary (specific) granules. The murine homologue of hCAP-18 is CRAMP, an acronym for 'cathelin-related antimicrobial peptide (CRAMP)'. Adult mice express CRAMP transcripts in bone marrow, testis, spleen, and stomach. Their CRAMP gene maps to a region of mouse chromosome 9 that is syntenic to the loci of porcine and human cathelicidin genes. NMR spectroscopy of CRAMP in a membrane-mimetic solvent system revealed two amphipathic α helices (Leu4 to Lys10 and Gly16 to Leu33), connected by a flexible region between Gly11 and Gly16. Truncated CRAMP variants that contained its amphipathic carboxy-terminal region (Gly16 to Leu33) retained considerable antibiotic activity.

In human neutrophils, hCAP-18 and lactoferrin are about equally abundant on a molar basis (Sorensen et al. 1997). Other sites of hCAP-18 expression include epididymis, spermatids, various epithelial cells, and some lymphocytes. Expression of hCAP-18 in skin is inducible. The carboxy-terminal domain of murine CRAMP shows considerable homology to LL-37 but is somewhat shorter. Synthetic CRAMP inhibited the growth of a variety of bacterial strains (minimum inhibitory concentrations 0.5–8.0 μmol), and 1.0 μmol CRAMP permeabilized the inner membrane of *E. coli*. Knockout mice that do not express mCRAMP, the murine homolog of LL-37, show undue susceptibility to infection by group A streptococci (Nizet et al. 2001).

In addition to its antimicrobial properties, LL-37 binds bacterial LPS and can induce chemotaxis via the formyl peptide-like receptor-1 (De et al. 2000). A few mammalian α-helical cathelicidin peptides are shorter than LL-37 (e.g. porcine PMAP-23 and bovine BMAP-27), but most are about as long. Whereas the α-helical cathelicidin peptides of cattle and sheep have amidated carboxyl termini, those of rodents and primates do not. Although all the peptides shown in Table 1 are cationic, their net positive charges vary from +3 in porcine PMAP-37 to +14 in PMAP-36. Human LL-37 has 11 positively charged lysine and arginines, but its five negatively charged residues reduce its net charge to +6 and contribute to its poor activity against *Staphylococcus aureus* in media with normal or elevated concentrations of NaCl (Zhao et al. 2001a).

CATHELICIDIN GENES

Like other porcine and human cathelicidin genes (Agerberth et al. 1996; Gudmundsson et al. 1996), porcine protegrin and prophenin genes contain four exons and three introns (Zhao et al. 1995a, 1995b). Exon I encodes the signal sequence and the first 37 amino acid residues of the cathelin domain. Exons II and III code for 36 and 24 additional cathelin residues, respectively. Exon IV contains the final few cathelin residues followed by the protegrin sequence. This quadripartite gene structure suggests that expression of structurally diverse antimicrobial peptides on a common, cathelin-containing precursor occurred after mutational translocation of a suitable peptide domain into exon IV. Circumstantial evidence for such events is embedded in the sequences of the PR-39 and prophenin genes (Zhao et al. 1995a).

The upstream region of the PR-39 cathelicidin gene contains several potential recognition sites for NF-IL6 and acute phase response factor (APRF), as well as binding motifs for NF-κB, granulocyte–macrophage colony-stimulating factor (GM-CSF), and NF-1 – factors

that regulate gene responsiveness to cytokines and acute phase response factors. Site-directed mutagenesis studies also revealed a negative regulatory element in the 5′-flanking region of the PR-39 gene, suggesting a mechanism for its tissue-specific and age-dependent repression (Wu et al. 2000, 2002).

Bovine or porcine leukocytes each contain at least 10 different cathelicidins (Zanetti et al. 1995) whose structurally diverse C termini contain α-helical, β-sheet, proline-rich, tryptophan-rich, or other molecules (e.g. bactenecin dodecapeptide). In contrast to this polypharmacy, human and murine PMNs each contain a single cathelicidin: hCAP-18 in humans and CRAMP in mice. Neither human nor mice skin cells (keratinocytes) express hCAP-18 or CRAMP constitutively. However, production of both peptides is induced soon after injury (e.g. sterile incision) or infection. As it takes a few hours for epithelial cells to begin substantial cathelicidin production, the rapid infiltration of granulocytes affords protection in the interim (Hoffmann et al. 1996). LL-37 and HBD-2 are abundant in psoriatic skin, but their concentration in atopic skin lesions is considerably lower, perhaps contributing to diminished resistance to *S. aureus* infections in this setting (Ong et al. 2002).

The attributes of LL-37 and other cathelicidins may extend well beyond their core missions of exerting direct antimicrobial activity, and neutralizing the toxicity of LPS. Normal seminal plasma contains levels of hCAP-18 (40–140 μg/ml) that are 70-fold higher than those in blood plasma. Much of this hCAP-18 is on sperm cells, which carry about 6.6 million hCAP-18 molecules each (Andersson et al. 2002). Spermatozoa undoubtedly acquire their hCAP-18 coating in the epididymis, because high level hCAP-18 expression was noted in the body and tail of the epididymis (Malm et al. 2000). As the epididymis of humans (and mice) also expresses β-defensin genes, sperm may start their perilous journey equipped with something of a 'chemical condom' to dissuade any microbial hitchhikers from accompanying them on their journey (von Horsten et al. 2002; Yamaguchi et al. 2002). Alternately, the peptide could participate in later events directly associated with fertilization.

Studies by Oppenheim and associates have delineated a role of LL-37 and defensins as signals that link innate and adaptive immune responses. LL-37 acted via the Gi protein-coupled FPRL-1 (formyl peptide-like receptor-1) (Yang et al. 2001a, b) to induce a chemotactic response in human PMNs, monocytes and lymphocytes. The relatively high concentration of LL-37 needed to induce optimal chemotactic responses in peripheral blood monocytes was 10 μmol/l (about 50 μg/ml) which is likely to occur, if at all, only at sites of inflammation.

LL-37 was detected in freshly isolated lymphocytes and in supernatants of human T and natural killer (NK) cells grown with IL-2 for 5 days in vitro (Agerberth et al. 2000). Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated LL-37 expression in B cells, γ, δ T cells, cloned NK cells, and monocytes, but not in αβ T-cell lines. Transcription and secretion of LL-37 by primary cultures of human lymphocytes responded to IL-6 and interferon-γ. Consequently, LL-37 (and defensins) may also contribute to the antibacterial activity of T cells and NK cells.

Some pathogenic bacteria may undermine host defense by inhibiting local production of antimicrobial peptides, including LL-37, e.g. early in human shigella infections expression of LL-37 and human β-defensin-1 was reduced or eliminated, with shigella plasmid DNA possibly being one of the mediators (Islam et al. 2001). If this were to promote bacterial adherence and invasion, it might enhance virulence.

CONCLUDING REMARKS

Interest in antimicrobial peptides has increased during the past decade, and they are now recognized as fundamental elements of the innate immune system. In addition to having direct effects on microbes, some of these peptides have prominent antiviral properties. In the case of α and θ defensins, our recent (and still largely unpublished) studies have found strong correlations between their antiviral activity and their ability to act as lectins that bind carbohydrate moieties present on viral glycoproteins and their cellular receptors. One wonders if the ability to recognize carbohydrates is also involved in their signaling and co-stimulatory properties. It seems likely that many surprises about the properties of these very old molecules will emerge during the decades to come.

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Lipid mediators: leukotrienes, prostanoids and other lipids

CHARLES W. PARKER

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The lipid mediators are a complex group of biologically active metabolites with diverse effects on cellular metabolism, which particularly target the immune system, airways, and microvasculature. Most of them are produced from arachidonate in response to an immunological or nonimmunological stimulus. They tend to have a short half-life and to act in a paracrine or autocrine fashion. Arachidonate metabolites are typically present as mixtures in areas of immunological inflammation. They vary considerably in their cell(s) of origin, time of appearance, susceptibility to inhibition by pharmacological agents, potency, stability, and biological action, frequently exhibiting antagonistic effects (Parker 1984). None of them is stored in significant quantities and they are primarily products of activated rather than resting cells. They represent an important group of regulatory or modulatory agents whose actions in the control of cellular function are still being unraveled. Although this chapter emphasizes their role in immunological inflammation, gene disruption studies indicate that arachidonate metabolites have functions in virtually every organ, tissue, and cell (Funk 2001).

ARACHIDONATE METABOLITES

Major pathways of arachidonate metabolism

Arachidonate is a C_{20} fatty acid with double bonds at 5–6, 8–9, 11–12, and 14–15 positions (numbering from the carboxyl end). The multiplicity of double bonds helps explain its versatility as a biosynthetic precursor. As discussed below, the two major routes of arachidonate metabolism are the lipoxygenase and cyclooxygenase pathways. Lipoxygenases in mammalian cells act primarily at the 5–6, 12–13, or 15–16 double bonds of arachidonate (termed 5-, 12-, and 15-lipoxygenases, respectively). The five lipoxygenases produce the 5-hydroperoxide eicosatetraenoate (5-HPETE) that is metabolized to the slow-reacting substances (LTC_4, LTD_4, and LTE_4) and LTB_4 (collectively termed 'leukotrienes') (Figure 1). Cyclooxygenases oxidize and cyclize arachidonate, producing the endoperoxides PGG_2 and PGH_2 which are metabolized to the D, E, and F prostaglandins (PGD_2, PGE_2, and PGF_2), prostacyclin (PGI_2), and thromboxane A_2 (TxA_2) (Figure 2).
The term prostanoid is often used to describe the various cyclooxygenase products. As a result of the C_{20} fatty acid structure from which they are derived, the metabolites of arachidonate in the two pathways are collectively termed 'eicosanoids'.

Production of free arachidonate

Lipoxygenases and cyclooxygenases act largely or entirely on nonesterified arachidonate. As almost all of the arachidonate in cells is stored in ester linkage in phospholipids or triglycerides and much of the free arachidonate in the blood is bound to plasma proteins, esterified arachidonate must be released enzymatically at the time of cell activation. Recent evidence indicates

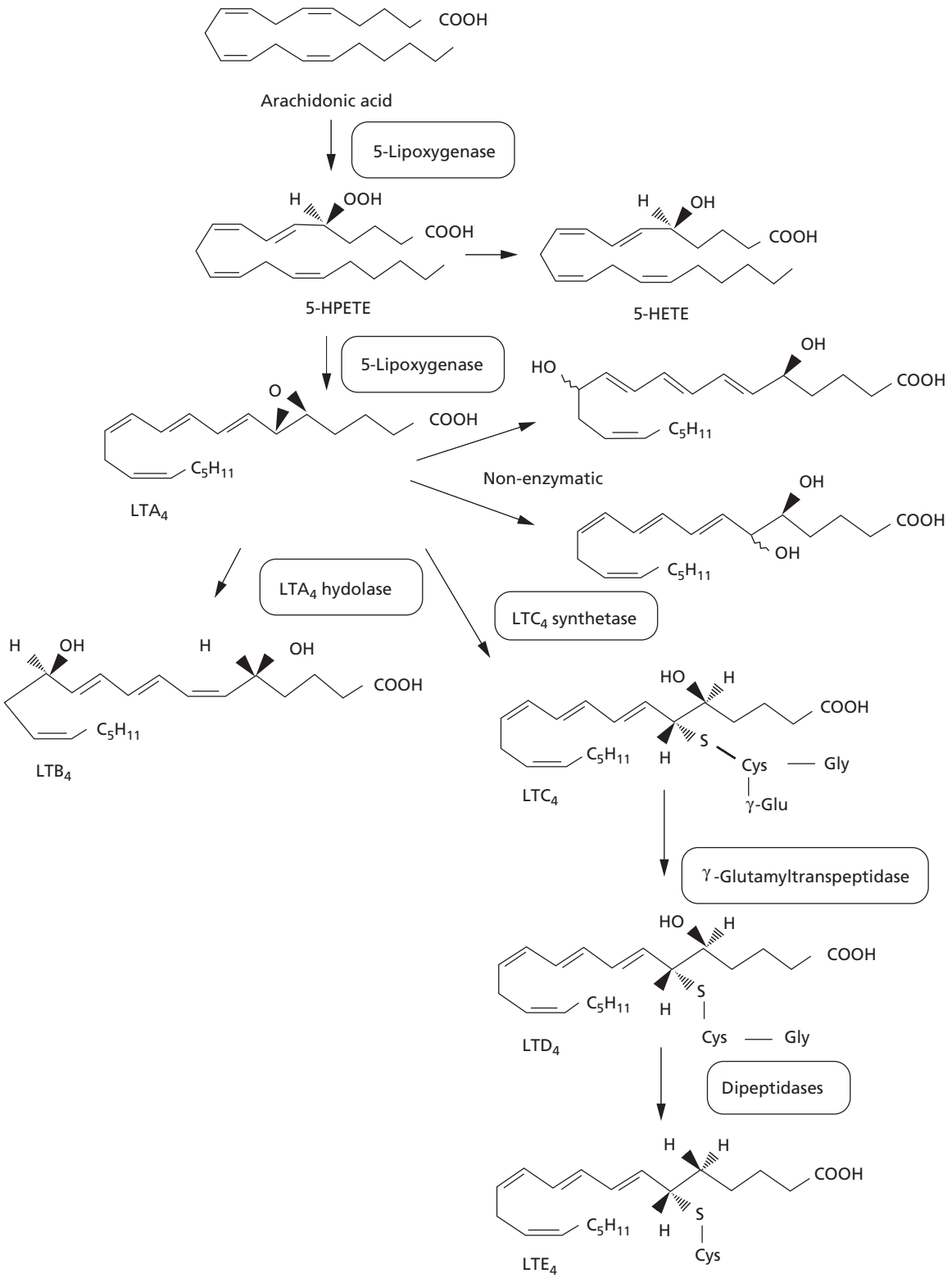

Figure 1 *Biosynthesis of leukotrienes. The enzymes involved are shown in boxes. HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxide eicosatetraenoate; LT, leukotriene. (Redrawn from Figure 2, p. 5, of Nicosia et al. (2001) with the permission of the publisher Academic Press, Orlando, Florida.)*

that cytosolic phospholipase A_2 is an important enzyme in arachidonate release. Cells lacking this enzyme generally fail to produce eicosanoid metabolites (Funk 2001). Depending on the cell type, cell activation results in

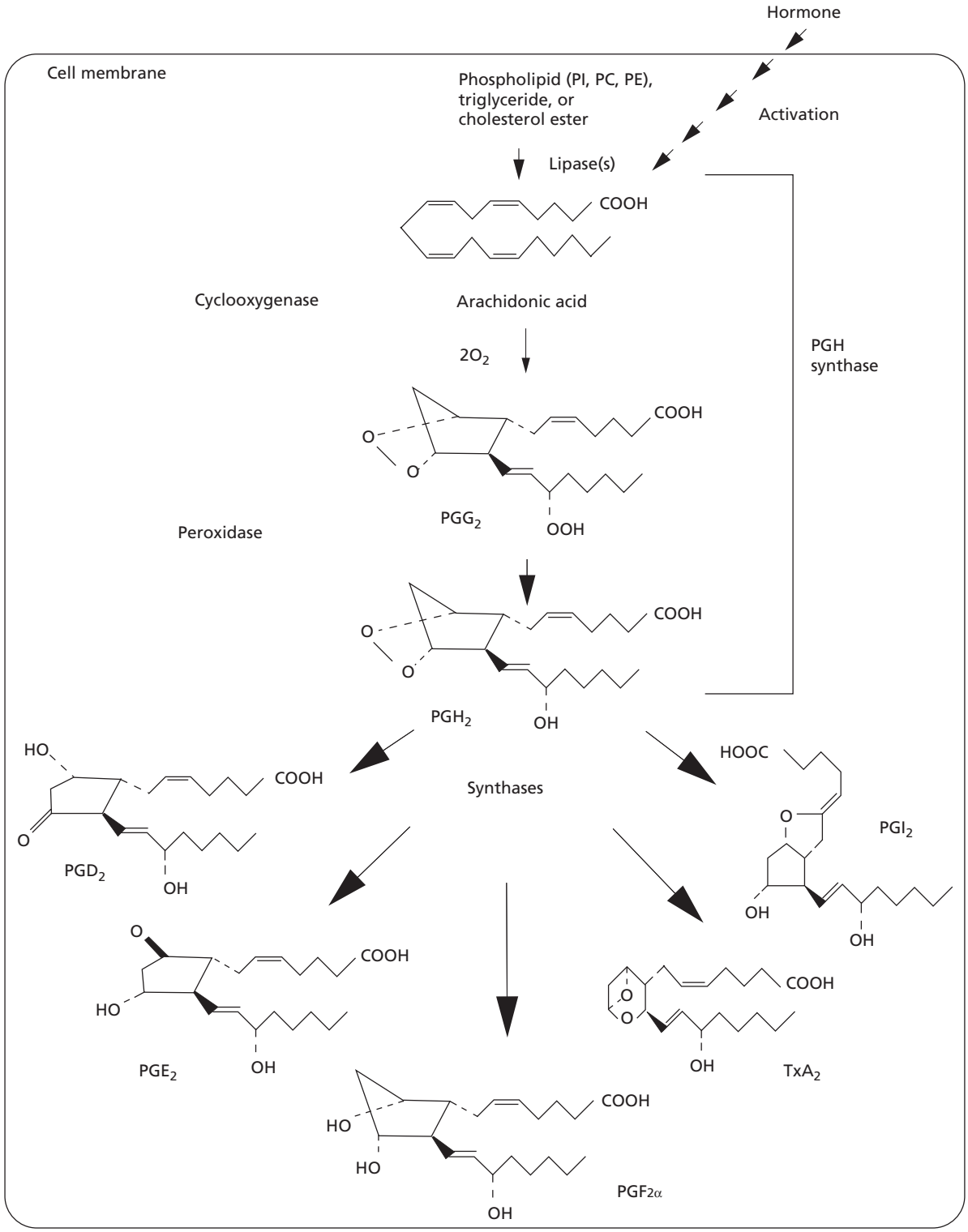

Figure 2 *Biosynthesis of prostanoids. The stimulus may be a hormone, antigen (antibody), or cytokine. Pg, prostaglandin; PGI , prostacyclin; TxA_2, thromboxane A_2. (Redrawn from Smith and Dewitt (1996), Figure 1, p. 168, with the permission of the publisher Academic Press, Orlando, Florida.)*

localization of this enzyme to the nuclear envelope, Golgi apparatus, or endoplasmic reticulum where it is thought to act locally to release arachidonate to nearby enzymes.

An important mechanism of glucocorticoid action is to inhibit phospholipase A_2 activity, decreasing the availability of free arachidonate, although eicosanoids vary

with respect to their susceptibility to steroid inhibition of their biosynthesis (Bisgaard 2000).

Lipoxygenases

Lipoxygenases add molecular oxygen to *cis*,*cis*-1,4-pentadiene moieties on long chain fatty acids, converting them to hydroperoxides and producing a rearrangement of the double bonds (Galliard 1975). Under special circumstances, fatty acid esters and alcohols are also substrates for these enzymes. The initial enzyme involved in leukotriene biosynthesis is a 5-lipoxygenase (Borgeat and Samuelsson 1979; Jakschik et al. 1977; Parker 1984; Samuelsson et al. 1987). This enzyme is primarily seen in mast cells, macrophages, eosinophils, and polymorphonuclear leukocytes, although basophils, endothelial cells, and epithelial cells also have activity (Parker 1987). It converts arachidonate sequentially to 5-HPETE and the 5,6-epoxide, LTA_4; LTA_4 is in turn converted enzymatically or nonenzymatically to LTC_4 (which has a glutathionyl side chain in thioether linkage at C_6), LTB_4 (a particular 5,12-diHETE diastereoisomer) or other diHETEs (see Figure 1). 5-HETE is also formed, probably primarily from 5-HPETE. The relative proportion of products depends on the cell type. Neutrophils contain leukotriene A_4 hydrolase which hydrolyzes LTA_4 to LTB_4, an important product in leukotriene function (Funk 2001). Mast cells contain LTC_4 synthase, which catalyzes the conjugation of LTA_4 to glutathione; neutrophils lack this enzyme and therefore produce little or no LTC_4.

The lipoxygenases that have been carefully studied all contain at least one atom of nonheme iron per enzyme molecule and this is also true of 5-lipoxygenases (Funk 2001). Lipoxygenases are subject to autocatalysis and autoinhibition, probably both caused by peroxides. Lipoxygenase product formation is rapid for the first 5–10 min after cell activation and then stops almost completely, even in the presence of excess exogenous arachidonate. In macrophages pretreated with lipopolysaccharide (LPS), 5-lipoxygenase activity can be downregulated for many hours apparently as a result of induction of nitric oxide synthesis (Coffey et al. 2000). The 5-lipoxygenases that have been characterized are calcium-dependent enzymes with a monomeric molecular weight of about 80 000 (Samuelsson et al. 1987). They have a segment in the N-terminal region capable of binding two calcium ions (Hammarberg et al. 2000). In nonactivated cells, most of the enzyme is in the cytosol in an inactive form, although depending on the cell some of it may be in the nucleoplasm. When the cell is activated and Ca^{2+} enters the cell, cytosolic 5-lipoxygenase presumably binds some of the Ca^{2+} and localizes in the perinuclear membrane where it associates with an activating protein (FLAP) and assumes full activity (Peters-Golden and Brock 2001).

Slow-reacting substances (leukotrienes C, D, and E)

Once LTC_4 is formed it is metabolized by peptidases to LTD_4 (cysteinyl-glycyl leukotriene) and LTE_4 (cysteinyl leukotriene). Collectively LTC_4, LTD_4, and LTE_4 constitute the activity originally described as slow-reacting substance (SRS). SRS was first described in 1938 as an activity obtained from guinea-pig lungs perfused with cobra venom enzymes which produced a characteristic, slowly evolving contraction of isolated guinea-pig ileal smooth muscle (Feldberg and Kellaway 1938). It was later shown to be present in sensitized guinea-pig lungs perfused by antigen. Working with a line of rat basophilic leukemia cells (RBL-1) that contain IgE receptors, our laboratory identified SRS as a mixture of arachidonate metabolites produced through the 5-lipoxygenase pathway (Jakschik et al. 1977; Parker 1982). We later showed that each of the SRS species had a sulfur-containing side chain and identified two of the three side chains as glutathione and cysteine (reviewed in Parker 1982). We also reported that the immunologically and nonimmunologically induced forms of SRS were identical in structure (Watanabe and Parker 1980). Samuelsson and his colleagues placed the sulfur at the 6 position and determined the location of the double bonds (Murphy et al. 1979). Orning et al. (1980) and Morris et al. (1980) definitively identified the cysteinyl-glycyl form of the molecule, for which we had earlier obtained suggestive evidence. Proof of structure and assignment of double bond configuration of LTC_4 were obtained independently by Hammarstrom et al. (1979) and Rokasch et al. (1980) by organic synthesis.

Once LTC_4 is formed, it is rapidly released into the cell medium, where it is normally metabolized to LTD_4 and eventually to LTE_4 by enzymes present in plasma, leukocytes themselves, and tissues (reviewed in Parker 1984). Release into the medium apparently involves the multidrug resistance transporter protein (Qian et al. 2001). The conversion to LTD_4 can involve conventional γ-glutamyl transferases, although recent evidence also implicates a distinct protease with a similar action (Drazen 2000). Once formed, LTD_4 is less rapidly converted to LTE_4 by various dipeptidases. These conversions are associated with substantial changes in biological activity. In chopped lung fragments or isolated leukocytes suspended in medium without serum or plasma proteins, the conversion to LTD_4 is ordinarily well under way within the first 3–5 min and, by 15 min, LTD_4 is the predominant SRS species that is present (Parker 1984). LTE_4 is generally not the most prominent SRS species until after 30 min. The sulfoleukotrienes can also undergo double bond isomerization or oxidation of the sulfur, leading to changes in biological activity; SRS metabolites and SRS itself (particularly LTE_4) are excreted to a significant extent in the urine

and bile in humans, and the levels of urinary LTE_4 correlate relatively well with disease activity in patients with asthma, especially if they are aspirin-sensitive (Bisgaard 2000; Nicosia et al. 2001).

The biological activity of SRS that has received most attention is its spasmogenic action on smooth muscle (reviewed in Parker 1983, 1984, 1987). Concentrations of LTD_4 as low as 0.1 pmol/ml produce readily detectable contractile responses on guinea-pig ileal and tracheal smooth muscle strips. On a molar basis, LTD_4 is about 60–100 times more active than histamine on the guinea-pig ileum. LTC_4 and LTE_4 are somewhat less active (by factors of 2 and 10, respectively, in the ileal system) but are still considerably more potent than histamine. In addition to its high potency, SRS produces an exceptionally sustained contractile response normally without tachyphylaxis, raising the possibility of a particularly prolonged action in vivo. Spasmogenic effects are produced on both the central and peripheral airways in humans and animals. LTC_4 and LTD_4 have been reported to constrict and dilate, respectively, cutaneous blood vessels, and decrease systemic blood pressure (Drazen et al. 1980). They also enhance cellular adherence, microvascular edema, mucus hypersecretion, and chemotaxis (especially of eosinophils) (James and Sampson 2001). The cysteinyl leukotrienes also promote granule enzyme release, although they are much less potent than LTB_4 in this regard (Goetzl 1980). Effects of leukotrienes (Öhd et al. 2000) and a 12-HPETE metabolite (Gu et al. 2001) on cell survival have also been described which is of interest with regard to the nuclear localization of active 5-lipoxygenase. The cysteinyl leukotrienes also exert a sustained effect on Purkinje nerve cell activity, which together with other evidence raises the possibility of important effects in the nervous system (Samuelsson et al. 1987).

Two types of receptors for cysteinyl leukotrienes have been identified and characterized: Cys LT1 and Cys LT2 (Drazen 2000; Maekawa et al. 2001). The Cys LT1 receptor is expressed in airway smooth muscle, macrophages, monocytes, eosinophils, and vascular endothelial cells. It has a higher affinity for LTD_4 than the other leukotrienes. The Cys LT2 receptor has an approximately equal affinity for LTD_4 and LTC_4. It is represented in peripheral blood leukocytes including eosinophils as well as in the lung, heart, adrenal medulla, and brain. Both types of receptors are members of the G-protein superfamily. The level of Cys LT1 receptor expression in human monocytes is upregulated by IL-4 and IL-13, suggesting a mechanism by which these cytokines might increase LTD_4 responsiveness and magnify the local response in IgE-mediated allergy (Thivierge et al. 2001).

There has been considerable activity in the pharmaceutical industry to develop drugs that prevent LTC_4 and LTD_4 formation or action. Both 5-lipoxygenase inhibitors and leukotriene LT1 receptor antagonists are now available for treatment of patients with respiratory allergy in Europe and the USA, and have been shown often to help relieve symptoms in chronic and acutely induced asthma (Nicosia et al. 2001). However, not all chronically asthmatic patients respond well and, as standard regimens not using these agents are often effective, more studies are needed for better definition of their precise role in treatment.

Dihydroxyeicosatetraenoic acids

LTB_4 is produced enzymatically from LTA_4 by a specific hydroxylating enzyme, although nonenzymatic hydrolysis may play a minor role (Samuelsson et al. 1987). When LTB_4 is purified from biological samples, several diastereoisomers of LTB_4 can be seen by high-pressure liquid chromatography or thin layer chromatography. These products appear to arise primarily by racemization of LTB_4 during purification. In general, the diastereoisomers are less active in biological systems than LTB_4 (Ford-Hutchinson et al. 1980).

As with cysteinyl leukotrienes, the major cell sources for LTB_4 in vitro are neutrophils, eosinophils, mast cells, monocytes, and basophils (Parker 1987; Nicosia et al. 2001), and the maximal response occurs within the first 5–10 min after cell activation. In neutrophils, much of the LTB_4 produced initially remains associated with the cells, but ultimately the bulk of the LTB_4 appears in the medium either as LTB_4 itself or as one of its metabolites (Stenson and Parker 1979). At 37°C human neutrophils rapidly metabolize LTB_4 to its 20-OH and 20-COOH derivatives, both of which are substantially less active in chemotaxis and granule enzyme release than LTB_4 itself.

LTB_4 stimulates chemokinesis in human neutrophils at concentrations of 1 nmol/ml or even lower (Ford-Hutchinson et al. 1980; Parker 1984), although substantially higher concentrations are required if plasma albumin, which binds LTB_4 is also present. The two major diastereoisomers of LTB_4 are considerably less active in this system. Although neutrophils are especially sensitive to LTB_4, cell movement is also stimulated in eosinophils, lymphocytes, and monocytes, indicating a general effect on leukocyte migration. LTB_4 also stimulates chemotaxis, although higher LTB_4 concentrations are needed (Goetzl 1980). At low nanomoles per milliliter concentrations LTB_4 also enhances lysosomal enzyme release from neutrophils, although as a rule this effect has required the concurrent presence of cytochalasins, casting some doubt on its physiological relevance. LTB_4 has little effect on vascular permeability but can enhance leukocyte adherence and emigration from the vasculature, resulting in part from upregulation of the expression of b_2-integrins (Wallace and Ma 2001).

Although early evidence indicated the presence of specific receptors for LTB_4 on human neutrophils (Kreisle and Parker 1983), it is only recently that the

genes involved have been identified. There is a high-affinity receptor termed BLT1 on leukocytes which acts though G-coupled proteins to stimulate chemokinesis, chemotaxis, and granule secretion (Serhan and Prescott 2000). Another LTB receptor, BLT2, has a considerably lower affinity for LTB_4 but is much more widely distributed. Evidence for the importance of LTB_4 and the BLT1 receptor in in vitro models of inflammation has been obtained in genetically manipulated mice that overexpress or fail to express this receptor (Serhan and Prescott 2000). One of the effects of overexpression of the BLT1 receptor was enhanced leukotriene biosynthesis, consistent with other evidence that LTB_4 is primarily a proinflammatory mediator. Specific antagonists for these receptors are under development.

Monohydroxyeicosatetraenoic acids

5-Hydroxyeicosatetraenoic acid (5-HETE) is quantitatively the major 5-lipoxygenase product obtained from leukocytes and mast cells (Parker 1984; Stenson and Parker 1979). It is formed from 5-HPETE and LTA_4 by spontaneous or enzymatic reduction. Peroxidases appear to be the primary enzymes involved in the enzymatic reduction. The mono-HETEs are less potent than SRS and LTB_4 in their known biological activities. Nevertheless, 5-HETE, 5-HPETE, and to a lesser extent the other mono-HETEs have significant chemokinetic and granule enzyme-releasing activities (Goetzl 1980; Stenson and Parker 1980). Although low micromolar concentrations are required to produce these effects, it is possible that the mono-HETEs are primarily active in the cells in which they originate. In neutrophils 5-HETE alone induces the release of specific granule enzymes. In mast cells and basophils, 5-HETE enhances antigen-induced histamine release but is not active by itself. Although the mechanism of 5-HETE action is not known, it and other monohydroxyl C_{20} acids have the very interesting property of being incorporated covalently into phospholipids and triglycerides (Stenson and Parker 1979). Even though the extent of this incorporation appears to be quite limited, localization of 5-HETE containing phospholipids in critical areas of lipid membranes could have important effects on local membrane fluidity and function (Parker 1984).

Other lipoxygenases and their products

The lipoxins are eicosanoids produced primarily in leukocytes by the combined or sequential action of 15- and 5-lipoxygenases (Samuelsson et al. 1987; Levy et al. 2001); 15-lipoxygenase activation is induced as a delayed response in leukocytes during inflammatory responses, caused at least in part by exposure to PGE_2 generated locally during cell activation (Levy et al. 2001). Once 15-HPETE has been produced, it is further metabolized to lipoxins, a group of 5,15-dihydroxy fatty acids with at least one additional hydroxyl group attached to the fatty acid chain; lipoxin formation sometimes involves collaboration between nearby leukocytes containing different lipoxygenases. Similar to PGE_2, lipoxins have both pro- and anti-inflammatory actions. Levy et al. (2001) have emphasized the anti-inflammatory effects of lipoxins proposing that they act primarily in the later stages of inflammation to reduce leukocytic infiltration and function. This may occur in part through an ability of lipoxins to stimulate phagocytosis of apoptotic leukocytes, removing them from the local environment.

Cyclooxygenases

In the cyclooxygenase pathway, oxygen is added enzymatically to arachidonate to form an unstable endoperoxide, PGG_2, with a cyclopentane ring derived from carbons 8–12 and a hydroperoxy group at the 15 position (Moncada et al. 1980; Calder 2001). The hydroperoxy group is then reduced by the same enzyme, producing PGH_2. Cyclooxygenase is frequently abbreviated as COX or PGHS for PGH synthetase. COX activity requires the presence of molecular oxygen and is promoted by a prosthetic heme group bound tightly to the enzyme (Smith and Dewitt 1996). Although COX exists in cells in a proactive state, there appears to be a requirement for at least some peroxide for enzyme activity. As the lipoxygenase system generates hydroperoxy fatty acids, which may in turn be reduced by glutathione peroxidase and other peroxidases, both of these enzymes may indirectly affect COX activity. There are two major classes of COX enzymes: COX-1 and COX-2; COX-1 is primarily a constitutive enzyme broadly represented in organs and tissues (Gianoukakis et al. 2001). It is responsible for basal prostaglandin (PG) synthesis. COX-2 is usually induced by synthesis from scratch of the COX-2 protein by hormones, growth factors, and cytokines. Induction occurs in immunocytes during immunological activation and is the major source of the increase in PGE_2 that accompanies immunological inflammation (Calder 2001). The COX-1 and COX-2 proteins are encoded by two very similar but distinct genes. The crystal structures of the two enzymes are almost identical but a critical amino acid difference leads to a larger substrate-binding pocket in COX-2 (Funk 2001). The two enzymes appear to be primarily localized in the endoplasmic reticulum and nuclear envelope, respectively (Smith and Dewitt 1996; Funk 2001), which may have important functional implications (see below).

PGG_2 and PGH_2 have half-lives in aqueous solution of about 5 min. Depending on the cell or tissue, PGH_2 may be converted to a PG, TxA_2, or PGI_2 and sometimes a mixture of two or more of these products is formed (Kleeberger and Freed 2000). PGG_2 can be

metabolized directly to PGD_2. Which of these products is made depends on the presence and level of activity of the appropriate enzyme systems (thromboxane synthetase, prostacyclin synthetase, 11-keto-isomerase, 9-keto-isomerase, and 9-keto-reductase) (reviewed in Parker 1984). In general, nonsteroidal anti-inflammatory agents such as aspirin and indometacin act primarily by blocking the COX, thereby reducing PGG_2 and PGH_2 formation. Therefore, they inhibit the synthesis of all of the eventual COX products. Nevertheless, the PG, TxA_2, and PGI_2 pathways are not necessarily inhibited equally. The degrees of inactivation depend in part on the relative K_ms of the individual enzymes involved in these subsequent metabolic conversions. Aspirin has a unique mechanism of action through acetylation of a critical serine residue on the enzyme. Most COX inhibitors act more or less equally on the COX-1 and COX-2 enzymes. Selective inhibitors of COX-2, rofecoxib (Vioxx) and celecoxib (Celebrex), are now in clinical use and still more selective inhibitors are under development (Funk 2001). The use of the selective COX-2 inhibitors reduces gastrointestinal side effects such as bleeding but the long-term advantages (and disadvantages) of such drugs are still not fully elucidated, particularly in individuals with other chronic diseases. None of the nonsteroidal anti-inflammatory agents can be regarded as completely specific for COX.

Prostaglandin metabolism and actions

The PGs are C_{20} fatty acids with a cyclopentane ring (see Figure 2). The E, F, and D PGs vary in the arrangement and type (hydroxy or keto and hydroxy) of substituents on the cyclopentane ring (Moncada et al. 1980). PG formation is associated with the loss of two double bonds, so prostaglandins derived from arachidonate have two remaining double bonds (e.g. PGE_2). PGH_2 is converted to PGD_2 and PGE_2 by a 11-keto-isomerase and 9-keto-isomerase, respectively. Both of these enzymes require glutathione. In addition to PGH_2, PGG_2 may be a substrate for 9-keto-isomerase. To a limited extent these conversions can be nonenzymatic because PGs can be formed by spontaneous breakdown of the endoperoxides. The existence of an isomerase for $PGF_{2\alpha}$ is doubtful. $PGF_{2\alpha}$ may be formed from PGE_2 by 9-keto-reductase or by direct reduction of PGH_2.

The PGs have long been known to affect blood vessel tone and promote smooth muscle contraction or relaxation, and they were originally postulated to be circulating hormones. However, it is now known that most of the PG in the circulation is rapidly metabolized, and it is generally agreed that PGs do not act as hormones affecting distant tissues (Moncada et al. 1980), except perhaps under exceptional circumstances. The major catabolic pathway for PG is by oxidation of the 15-OH group to the corresponding ketone by prostaglandin 15-OH-dehydrogenase (Kleeberger and Freed 2000). The 15-keto metabolite is then reduced to a 13,14-dihydro derivative. Subsequent metabolism is by β and terminal oxidation (Parker 1984). Enzymes that catalyze PG degradation are widely distributed in the body. One of the most active regions is the lung and, for example, more than 90 percent of infused PGE_2 is inactivated during a single passage through the lungs.

Leukocytes show marked differences in their abilities to produce PGs (Parker 1984; Kleeberger and Freed 2000; Funk 2001). Macrophages and monocytes make large amounts of PGE_2, substantial amounts of $PGF_{2\alpha}$, and very little or no PGD_2; neutrophils make moderate amounts of PGE_2 and little or no $PGF_{2\alpha}$ or PGD_2; mast cells make large amounts of PGD_2 and little or no $PGF_{2\alpha}$ or PGE_2. Resting lymphocytes or lymphocytic cell lines have been reported to make PGE_2 and less frequently to make $PGF_{2\alpha}$, but the amounts are quite small relative to monocytes and macrophages, and contamination of unstimulated lymphocyte preparations by monocytes may account for most or all of the PGs that are formed by these cells (Goldyne and Stobo 1979). However, when lymphocytes are stimulated by mitogens or appropriate cytokines, rapid induction of the COX-2 enzyme, with corresponding increases in PGE_2 production, may be seen, although the amounts of PGE_2 formed are not usually large. Originally TNF-α, IL-1, and IFN-γ were found to be stimulatory and IL-4, IL-10, and IL-13 inhibitory to enzyme induction (Berg et al. 2001). There is now evidence that IL-10 can be a potent inducer of COX-2 and PGE_2 production in LPS-stimulated mouse splenocytes. The induction is apparently produced indirectly through increases in cytokines that promote PGE_2 production. Surface stimuli including cytokines and antigens may also promote the production and release of PGs from monocytes and mast cells. As discussed below, the predominant effect of local increases in PGE_2 production may be to down-regulate ongoing immune responses.

One of the problems in interpretation of the data on PG formation has been the variation in results in different laboratories in the quantities or even the spectrum of arachidonate metabolites formed by a given cell type or mixture (Parker 1984). A study in human peripheral blood monocytes done many years ago (Bockman 1981) indicates that some of the discrepancies may involve differences in cellular sources and subsequent handling. The pattern of arachidonate metabolites formed by human peripheral blood monocytes cultured in vitro varied markedly with the duration of the culture following isolation of monocytes by cellular adherence. TxA_2 and PGEs were the major products on the first day of the culture. On the second day, PGEs were the major arachidonate metabolites and TxA_2 production was markedly diminished. Further changes in the spectrum of eicosanoids may be seen because monocytes continue to be maintained in tissue culture (Funk 2001).

Cellular differentiation, selective cell loss, restraints placed on the cells by the nutrient medium provided, and the accumulation of stimulatory and inhibitory cytokines may all affect arachidonate metabolite formation in this situation.

The biological effects of the PGs on immune function are quite diverse. PGE_2 is often the most active of the prostaglandins. In relatively high concentrations PGE_2 induces immature thymocytes and immature B lymphocytes to differentiate and acquire the functional, morphological, and immunological characteristics of mature lymphocytes (reviewed in Parker et al. 1974; Parker 1984), e.g. the addition of exogenous PGE_2 to thymocytes in vitro results in an increase in thymocyte cyclic adenosine monophosphate (cAMP) levels and thymocyte proliferation (Scheid et al. 1975). Other agents raising cAMP in these cells produce a similar response. PGE_2 affects erythropoiesis through direct effects on red cell precursors as well as by releasing erythropoietin from the kidney (Parker 1984). Some leukocyte precursors are not stimulated by PGE_2, e.g. macrophage colony formation is markedly inhibited. PGE_2 may also affect the differentiation of more mature cells, e.g. by the induction of collagenase secretion in apparently mature monocytes.

Once cells have matured, PGE_2 is primarily inhibitory exerting a variety of effects on leukocyte function, limiting inflammation (reviewed in Parker 1984; Calder 2001). PGE_2 has been reported to inhibit: (1) T- and B-cell proliferation; (2) leukocyte chemotaxis, chemokinesis, aggregation, spreading, phagocytosis, and oxidative metabolism; (3) cell-mediated cytotoxicity either by natural killer or cytotoxic T cells; (4) the release of inflammatory mediators from mast cells, basophils, eosinophils, and neutrophils; (5) production of cytokines, particularly IL-1, IL-2, IL-6, TNFα, and IFN-λ (Calder 2001) (although it has been suggested that PGs promote the T-helper (Th)2 cell pathway, in our laboratory PGE_2 and orally effective PGE_1 analogs are rapid and potent inhibitors of murine splenocyte IL-4 production, in vitro, apparently through a direct suppressive effect on IL-4 mRNA levels (Parker et al. 1995); and (6) inhibition of MHC antigen expression. Most of these effects are probably exerted directly on the responding cell preparations but indirect effects involving nearby cytokine producing cells undoubtedly also occur. While some of the inhibitory effects of PGE_2 require relatively high PGE_2 concentrations, others are produced at concentrations likely to be present at inflammatory sites in vivo. In a delicately regulated immune response, even relatively modest inhibitions may be significant, particularly if they are exerted selectively. In pharmacological concentrations in experimental animals, PGE and PGE analogs markedly suppress experimental inflammatory responses such as allograft rejection, immune complex glomerulonephritis, and the Arthus reaction (early work is reviewed in Stenson and Parker 1982). Orally effective PGE_1 analogs are being evaluated for their possible usefulness in a variety of therapeutic situations in humans.

Prostaglandins have proinflammatory as well as anti-inflammatory effects. Exogenously administered PGE_2 induces fever, erythema, increased vascular permeability, and vasodilatation (Moncada et al. 1980). PGE_2 is a very potent vasodilator. The dilatation is exerted throughout much of the microvascular bed. Although exogenously added PGs do not induce pain by themselves, they can markedly potentiate the pain and edema caused by bradykinin and histamine (Parker 1984). PGE_2 is found in increased quantities in inflammatory exudates at concentrations that can produce the classic signs of inflammation. Moreover, PGE_2 appears in experimentally induced inflammatory exudates over a time course consistent with its role as a mediator and PGE_2-binding proteins administered to arthritic joints have been shown to reduce local inflammation. A role for PGE_2 in arthritis seems particularly likely and this is supported by the widespread use of COX inhibitors in therapy in humans. PGs may also play a role in asthma and inflammatory bowel disease (Berg et al. 2001). $PGF_{2\alpha}$, 15-keto- $PGF_{2\alpha}$, and PGD_2 are potent constrictors of bronchial smooth muscle in humans and other animals (O'Byrne 1997). Subthreshold amounts of $PGF_{2\alpha}$ and PGD_2 increase overall airway reactivity. The bronchoconstriction involves both a direct effect on smooth muscle and an indirect effect through cholinergic pathways. PGE_2 and PGI_2 are bronchodilatory: PGE_2 may act in part by inhibiting the release of acetylcholine from airway cholinergic nerves.

By 2001 there were nine known PG receptor subtypes including four binding PGE_2 (EP_1–EP_4), two binding PGD_2 (DP_1 and DP_2), and one each binding $PGF_{2\alpha}$ (FP), PG_2 (IP), and TxA_2 (TP) (Funk 2001). All except DP_2 are members of the G-protein-coupled superfamily with seven transmembrane-spanning domains. Marked interspecies differences have been observed in the cellular distribution and function of some of these receptors (Kleeberger and Freed 2000). Most are located in the plasma membrane but several, including EP_1, are (or can be) in the nuclear envelope (Bhattacharya et al. 1998; Funk 2001). Selective effects involving individual PGE receptors have already been described, and it seems almost certain that the pattern will become more complex as studies continue. PGE_2 exerts most of its physiological effects through changes in intracellular cAMP. In many of the tissues or isolated cell systems in which it has been studied, PGE_2 stimulates adenylyl cyclase and increases intracellular cAMP concentrations. Many of its effects can be mimicked by cAMP agonists and analogs. However, effects through the EP_1 receptor apparently occur via increases in intracellular Ca^{2+} (Funk 2001) and stimulation through the EP_3 receptor is associated with decreases in cAMP. The F-type prostaglandins are generally less effective in raising cAMP

than the PGEs and, in a few tissues, stimulate increases in cGMP instead of cAMP; PGI_2, TxA_2, PGH_2, and fatty acid hydroperoxides may also affect cAMP or cGMP levels (Parker 1984).

Changes in the level of expression or location of the prostanoid receptors with time during an immune response in conjunction with evolving patterns of eicosanoid biosynthesis could importantly alter cellular responsiveness. Although PGs are normally thought of as having primarily a paracrine function, the evidence that the COX-2 enzyme is mainly localized in the perinuclear membrane where PG receptors may also be present is of interest for a possible additional autocrine action of PGs in the nucleus, perhaps affecting cellular replication or differentiation (Smith and Dewitt 1996). In this connection, cAMP localization has been demonstrated in the nucleus of human lymphocytes both in adenylyl cyclase studies of isolated nuclei and by cAMP immunofluorescence (Wedner and Parker 1977). Although nuclear cAMP accumulation was not stimulated by PG, only PGE_1 was studied and PG receptors might have been lost during the procedure used for the isolation of nuclei.

Thromboxanes

Thromboxane A_2 is a chemically labile C_{20} fatty acid with a six-membered oxygen-containing ring originally characterized structurally in platelet supernatants by Hamberg and Samuellsson (1974). It is produced from PGH_2 by the enzyme thromboxane synthetase. Thromboxane synthetase also catalyzes the conversion of PGH_2 to 12-hydroxy-5, 8, 10-heptadecatraenoic acid (HHT), a C_{17} hydroxy fatty acid formed by splitting off C9, C10, and C11 of the cyclopentane ring. The two products, TxA_2 and HHT, are formed in comparable amounts; HHT is unique in that it is the only simple hydroxylated eicosanoid that is not a lipoxygenase product. TxA_2 has a half-life in aqueous solution of about 30 s, spontaneously degrading to thromboxane B_2 (TxB_2) by the addition of water. As a result of this lability, it is almost always TxB_2 that is measured when thromboxane metabolism is studied.

Apart from platelets, macrophages and monocytes are the major known biosynthetic sources of TxA_2 (O'Byrne 1997). Mast cells and lymphocytes are poor sources of TxA_2, neutrophils make moderate amounts and platelets can account for about 95 percent of thromboxane detected in serum (Wallace and Ma 2001).

Stable endoperoxides mimicking TxA_2 action, inhibitors of TxA_2 synthetases, and TxA_2 receptor antagonists are now available and have helped to elucidate thromboxane's role (O'Byrne 1997). TxA_2 is a very potent vasoconstrictor, particularly in the rabbit aorta, but is also very effective in constricting tracheal and bronchial smooth muscle and has vasoconstrictor activity throughout the vascular system. Roles in asthma and in early and late bronchial responses to antigen have been suggested, but there appear to be major species differences and the effects of TxA_2 inhibitors in these situations have not been dramatic; TxA_2 has been implicated in late cutaneous responses to antigen in human skin. The best-defined functional action of TxA_2 on circulating cells is its ability to promote platelet aggregation (Hamberg and Samuellsson 1974). It may also have a role in neutrophil adherence. Inhibitors of thromboxane synthesis have been reported to reduce lectin-induced mitogenesis in human T cells in an apparent relationship with their potency as thromboxane inhibitors, raising the possibility that the thromboxanes play a positive role in mitogenesis (Parker 1984), but there has been little recent interest in this possibility. In contrast to PGI_2 and PGE_2, TxA_2 lowers intracellular cAMP concentrations, at least in platelets.

Prostacyclin

Prostacyclin synthetase catalyzes the conversion of PGH_2 to PGI_2, which in turn spontaneously degrades to 6-keto-$PGF_{1\alpha}$ by the addition of water (Moncada et al. 1980). Its half-life in aqueous solution at 37°C and neutral pH is several minutes or less. There is an interesting interaction between the lipoxygenase and cyclooxygenase pathways in that prostacyclin synthetase is inhibited by the hydroperoxy intermediates of the lipoxygenase pathway (Stenson and Parker 1982).

Prostacyclin is formed in vascular endothelium, macrophages, and mast cells (Humes et al. 1977; Kleeberger and Freed 2000). PGI_2 and TxA_2 frequently have adversarial roles and the relative amounts of these two agents may serve as a normal physiological control mechanism. PGI_2 is a potent vasodilator and inhibitor of platelet aggregation and, given intravenously in small quantities, produces systemic hypotension. Levels of the prostacyclin metabolite, 6-keto- $PGF_{1\alpha}$, are elevated in the inflammatory exudates of chronic granulomas (Parker 1984). In addition to its potency as a vasodilator, PGI_2 increases vascular permeability directly and potentiates the increased vascular permeability induced by other inflammatory mediators. PGI_2 is also a potent inducer of pain. Most or all of PGI_2's effects on tissue and cells appear to be mediated through increases in cAMP.

OTHER LIPIDS WITH A KNOWN OR POSSIBLE SIGNALING ROLE

Isoprostanes

Isoprostanes are isomers of conventionally derived PGs, which are produced primarily by a free radical attack on esterified polysaturated fatty acids in cell membranes,

especially arachidonate (Pratico et al. 2001). They are generated in situ under conditions of oxidant stress and then released, presumably by phospholipase. The F_2 isoprostanes (isomers of $PGF_{2\alpha}$, designated as F_2-iPs) have been the most frequently studied, but analogous isomers exist for other PGs and LTs. In addition to being markers for lipid peroxidation a number of the isoprostanes have reasonably potent biological activities, e.g. $iPF_{2\alpha}$ III produces bronchoconstriction, vasoconstriction, and platelet aggregation, apparently by stimulation of the TxA_2 receptor, at relatively low concentrations. The role of isoprostanes in inflammatory processes in vivo is still uncertain.

Lipid metabolites resembling eicosanoids

In general, LTs and PGs are produced predominantly from arachidonate. However, long-chain polyunsaturated fatty acids other than arachidonate also have to be considered as sources of biologically active lipids. They are present in cell membranes and may be substrates for enzymes in the various lipoxygenase and COX pathways, depending on their availability for rapid release as free fatty acids, structure (location of the double bonds, number of carbon atoms), and the capabilities of the individual enzymes. The resulting products are as a rule formed in relatively limited quantities. Moreover, they are altered in their double bond structure or number of carbons and tend to be less potent biologically than the usual LTs and PGs (Calder 2001). They may assume increased importance when the fatty acid content of cells has been manipulated in vivo by diet. Animals on diets rich in eicosapentaenoic, docosahexaenoic, oleic, or linoleic acids in general show less active responses in experimental models of inflammation than animals on the usual laboratory diets. Known or suspected mechanisms include decreases in the arachidonate content of cells that reduce its availability as a substrate, competitive inhibition of arachidonate-metabolizing enzymes by nonarachidonate fatty acids and their metabolites, and possible effects through alterations in cellular function, which may include changes in enzyme or receptor expression directly affecting eicosanoid metabolism or action. A considerable literature already exists on the possible usefulness of such diets in treating immunological diseases in humans.

Platelet-activating factor

Platelet-activating factor (PAF) is a family of structurally closely related, chemically stable neutral lipids containing a glyceryl core, a phosphorylcholine head group at the 3 position, one of several long-chain alkyl ethers (especially hexadecyl or octadecyl) at the 1 position, and an esterified acetyl group at the 2 position (Pinckard et al. 1982). 1-Alkyl,2-acyl-phosphatidylcholines, in which the acyl group is oxidatively fragmented, may also have PAF-like activity (Prescott et al. 2000). Studies of the aggregation response of rabbit platelets with analogs of PAF indicate that all three of the groups attached to the glyceryl backbone are important for its aggregating activity (Demopoulos et al. 1979). Replacement of the alkyl group at the 1 position by a carboxylic acid ester of the same chain length results in a 250-fold decrease in activity. The naturally occurring analog of PAF with a 16:0 alkyl group has about five times the activity of 18:0 alkyl-PAF. Replacement of the 2-acetyl group with butyryl reduces activity about 10-fold, whereas lyso-PAF has no activity even at very high concentrations. Removal of the choline or phosphorylcholine group results in a decrease in activity of more than 1000-fold.

Platelet-activating factor is not prestored. There are two known pathways of PAF biosynthesis. One is a pathway from scratch in which the final step is the addition phosphorylcholine to 1-alkyl,2-acetyl-glycerol (Watson and Snapper 1997). Such synthesis can account for most of the low-grade constitutive synthesis of PAF that is seen in some tissues. In the second, generally more important remodeling pathway, preexisting 1-alkyl,2-acyl-phosphatidylcholine is deacylated at the 2 position to form the corresponding lysophosphatidylcholine (or lyso-PAF) which in turn is acetylated by an acetyl-CoA-lyso-PAF-acetyl transferase to form PAF. The remodeling pathway predominates when PAF is generated in response to an acute or chronic cellular stimulus. As with the eicosanoids, cytosolic phospholipase A_2, which preferably acts on *sn*-2-arachidonyl residues in phospholipids, plays a key role (Prescott et al. 2000). Thus eicosanoid and PAF biosynthesis tend to occur together.

Production of PAF in response to cellular stimuli occurs rapidly, with significant activity usually being generated in less than a minute. Originally PAF action was thought to require its release from cells. Although activity does appear in the medium of cells stimulated in vitro and the free PAF is active, the extent of this release varies with the cell type. Vascular endothelial cells retain most of their PAF activity on their surfaces (Prescott et al. 2000). In this position the PAF interacts with PAF receptors on nearby intraluminal leukocytes, resulting in their immobilization and metabolic activation. P-selectin participates importantly in the immobilization process. PAF production tends to fall off rapidly, probably resulting in part from limitations in alkylphospholipid precursor availability, which varies considerably in different tissues. Once formed the PAF is rapidly degraded, much of it within a few minutes. The major mechanism for inactivation both inside and outside the cell is enzymatic deacetylation. Extracellular PAF acetyl hydrolases are present primarily in the lipoprotein fractions of human and animal plasma (Pinckard et al.

1982). At least three isoforms of the enzyme exist intracellularly (Prescott et al. 2000) and all forms of the enzyme are serine proteases inactivated by diisopropylfluorophosphate. As PAF production falls off rapidly with time, PAF is quickly degraded and receptor desensitization tends to occur rapidly, PAF actions typically are brief. However, in ongoing inflammatory responses in vivo newly arrived cells may help continue the response.

Platelet-activating factor is produced in many different cell types and tissues, usually in response to an immunological or nonimmunological stimulus. Immunological stimuli producing PAF include antigen and antibody, cytokines, and other mediators. Immunocytes including monocytes, macrophages, neutrophils, basophils, and eosinophils (Watson and Snapper 1997) are significant sources with small amounts being made by lymphocytes, mast cells, and platelets. Vascular endothelial cells are an important site of PAF production. The best-characterized effects of PAF (and stable PAF-like agonists) are on vascular reactivity and inflammation (Watson and Snapper 1997; Prescott et al. 2000; Wallace and Ma 2001). PAF stimulates: (1) platelet aggregation; (2) leukocyte aggregation, chemotaxis, and secretion; (3) airway and intestinal smooth muscle contraction; and (4) microvascular leakage and spasm. PAF may also induce the production of more effective action by other mediators and cytokines. There is substantial species variation in PAF actions. Aggregation responses of rabbit platelets and neutrophils occur at subnanomolar concentrations of PAF but human platelets are considerably less susceptible. The rapid intravenous infusion of as little as 2.5 μg of PAF in adult rabbits results in the almost immediate induction of marked neutropenia, thrombocytopenia, hypotension, and respiratory obstruction, culminating in respiratory arrest and death within minutes (McManus et al. 1980). At lower, nonfatal doses of PAF, the decreases in circulating platelets and neutrophils are reversible but animals show aggregates of platelets and neutrophils in pulmonary vessels and pulmonary arterial hypertension. Similar changes occur in sensitized rabbits undergoing fatal or nonfatal systemic anaphylaxis. Most of the effects of systemically injected PAF in experimental animals probably do not involve mechanical obstruction as such. In addition to direct effects on vessel walls by PAF, PAF may in part act by stimulating the secretion of TxA_2 and LTs (Pinckard et al. 1982) by platelets and leukocytes. The bronchoconstrictor effects of aerosolized PAF also appear to have complex mechanisms. PAF may have physiologically significant actions on cutaneous vascular reactivity. On a molar basis PAF is 10–1000 times more potent than histamine in producing acute weal-and-flare reactions in human and animal skin. Important actions outside the immune system in the brain and elsewhere are also suspected (Prescott et al. 2000).

The PAF receptor is a G-linked protein that is expressed on the surfaces on a variety of cell types (Prescott et al. 2000) and the receptor has a number of conformational states with various affinities. Most of the available PAF antagonists act by reversibly competing with PAF for the receptor. They include products present in Chinese herbal medicines that have long been used in the treatment of asthma (Watson and Snapper 1997). Despite evidence that PAF may participate in the pathogenesis of asthma, used by themselves, PAF antagonists have not been shown to be consistently helpful in the treatment of asthma.

Despite its potency and diverse actions PAF is produced in association with other mediators and is rapidly metabolized, complicating studies of its role in vivo; however, studies on genetically manipulated animals with increased or absent PAF receptor expression, or impaired PAF degradation, are now available (Prescott et al. 2000). Taken together with pharmacological studies with PAF receptor antagonists and inhibitors of PAF synthesis, they indicate that the PAF system is probably not normally essential for physiological well-being. However, PAF clearly can have substantial modulating roles affecting airway reactivity, anaphylaxis, endotoxemia, and local inflammation. Not enough is known about the role of PAF in local resistance to serious acute and chronic infections. Excess PAF activity is a potential problem and humans with defective PAF catabolism appear to be at increased risk for severe asthma and neonatal necrotizing enterocolitis.

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The acute phase response

JOHN G. RAYNES

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INTRODUCTION

In this chapter the aim is to explain the changes that occur as a local inflammatory response becomes sufficiently strong to start causing systemic changes. The term 'acute phase response' is frequently used to encompass all the changes that occur in different organs when inflammation becomes systemic, indeed some of the most common manifestations of severe illness are components of the acute phase response. Fever, somnolence, and confusion represent some of the behavioral or physiological changes that are easily observed, whereas analysis of the biochemical, immunological, or metabolic alterations has demonstrated an array of changes that take place.

The acute phase response (APR) is a conserved response and it is rare to find individual patients who have an inability to mount components of the response. This is not surprising as the response is vital for homeostatic effects and protection against infection. There is an argument that one of the most powerful selective pressures evolutionarily is infection so that, whether or not the acute phase function response was directly protective or homeostatic, most of the responses seen are likely to counter infection. Over the last few years, the amount of investigation into the APR has been dwarfed by the efforts afforded to the cytokine initiators of the APR. These are the proinflammatory cytokines that generate the APR. These cytokines and other inflammatory mediators, which act at a local level, are also responsible for effects at the systemic level. In this chapter the role of these inducers is reviewed, concentrating on systemic responses rather than purely localized responses. The APR could be considered to include circulating cytokine responses to inflammation because they are also part of the overall systemic inflammatory response. One further aspect of the acute phase response is that many of the changes that occur and are present in an acute episode of inflammation will also be present in a chronic inflammatory disease. Thus, the name acute phase response is not really ideal because it is also a phenomenon of chronic inflammation, and an alternative term is the 'systemic inflammatory response syndrome (SIRS)'. This term is often used to describe severe clinical situations, however, and sepsis is used if the cause is infectious.

One of the most interesting aspects of the APR is the increase in acute phase proteins (APP) synthesized mainly in hepatocytes (Kushner 1982). Although normally considered to be a function of the liver, the definition of APP is not entirely limited to hepatic synthesis, because many APPs are synthesized outside the hepatocyte. Aspects of the APR are found in many different cells and sites in the body.

In this review, the APR to inflammation of any cause is considered although the concentration is on infectious examples where appropriate. It has to be remembered, however, that some of these proteins described are likely to contribute to homeostasis in many noninfectious situations and many may have more than one function. The aim is to cover mechanisms by which responses are stimulated, what is known about the func-

Table 1 *Major aspects of the acute phase response*

Metabolic aspects
Loss of muscle and negative nitrogen balance (protein catabolism)
Decreased gluconeogenesis (lactate, etc. no longer converted to glucose)
Osteoporosis (in chronic inflammation)
Increased hepatic lipogenesis
Increased lipolysis in adipose tissue
Increased triglycerides in serum and decreased high-density lipoprotein
Cachexia
Increased requirement for glutamine in fast-dividing immune cells, possibly leading to deficiency of glutamine
Micronutrient changes
Reduced serum zinc, iron, and copper
Reduced plasma retinol
Increased glutathione concentrations
Hemopoietic changes
Reduced red blood cell numbers
Increased neutrophils
Increased platelets
Neuroendocrine changes
Fever
Somnolence
Increased corticotropin-releasing hormone, corticotropin, and cortisol
Increased secretion of arginine vasopressin
Decreased insulin-like growth factor 1
Increased secretion of catecholamines
Hepatic changes
Positive and negative acute phase proteins
Increased metallothionein, heme oxygenase, etc.

tion of these responses, and where responses may malfunction. The major aspects are summarized in Table 1.

INDUCTION OF THE ACUTE PHASE RESPONSE

Induction of systemic inflammation

The APR is a nonspecific response in that it can be initiated by many different stimuli although, regardless of stimulus, the magnitude of the response is dependent on the extent of injury. Such injury may be caused by burns, irradiation, surgical or physical trauma of tissue injury or infection. Infection is often a strong inducer of the response, and particularly bacterial infection, because of the range of powerful immune stimulators that bacteria possess. More recently, when more sensitive assays were used to measure markers of inflammation such as C-reactive protein (CRP), associations were observed in a number of diseases that were previously not thought of as inflammatory in nature, such as atherosclerosis (Haverkate et al. 1997; Libby et al. 2002). This may be extended to other diseases in time. At the lower end of the spectrum of stimulus, strenuous exercise such as marathon running (Vider et al. 2001) or some psychiatric illnesses or stress (Maes et al. 1997) may cause a mild APR, at least detectable through the more sensitive indicators of the acute phase.

The initial stimulus for the APR may be physical trauma, through other means of tissue damage, or through adaptive immunological mechanisms including allergen and IgE-mediated mechanisms, immune complex activation of phagocytic cells, or T-cell-mediated recognition. Alternately, infection can be recognized by an increasing range of innate mechanisms such as endotoxin or peptidoglycan activation through Toll-like receptors (TLR) or other pattern recognition molecules. It is now known that such pattern recognition molecules can recognize many different foreign structures including nucleotide, protein, carbohydrate, and lipid structures. Following such stimuli, tissue injury may at first cause changes in vascular permeability through short-lived lipid mediators such as prostaglandins and leukotrienes, which allow fluid to leak from the post-capillary venules into the tissues. This is accompanied by a chemotactic stimulus from a variety of factors that bring inflammatory cells such as neutrophils, and subsequently macrophages, into the inflammatory site. The chemotactic stimulus works in conjunction with expression of adhesion molecules on the endothelium and leukocyte to control this cellular influx. These include the chemokines such as interleukin-8 (IL-8) which attracts neutrophils. The inflammatory cells themselves contain or synthesize a number of proinflammatory activators, many small and short-lived, but in addition some proinflammatory cytokines are generated that have the ability to transmit the inflammatory signal over much larger distances.

Different aspects of the APR take a different length of time to be observed following a defined stimulus. The induction of some of the APPs, for instance, may take between 24 and 72 hours to reach their peak (Figure 1b) whereas effects such as fever and malaise may be more rapidly observed. The main reason for such delays is that several rounds of inducible protein synthesis may be required. The proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1α or IL-1β) can be generated within an hour or two of the start of the stimulus. TNFα is usually induced (largely through post-transcriptional regulation) earlier than IL-1β but both then induce a number of other cytokines (Figure 1a). If the stimulus for inflammation continues to be present at a sufficient level then the proinflammatory cytokines TNFα and IL-1β (more than IL-1α) are released into the circulation and induce responses at distant sites. In addition to release of such cytokines, early responses involve the release of a range

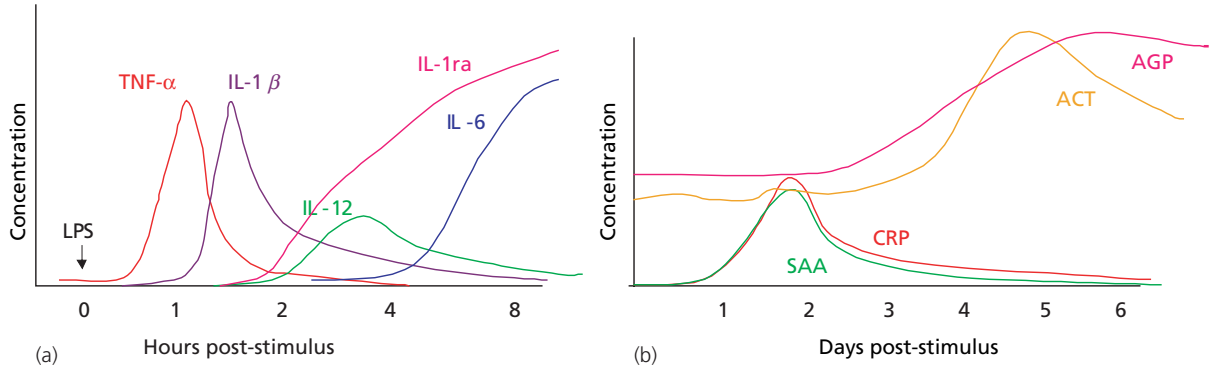

Figure 1 *Time course of induction of* **(a)** *cytokines and* **(b)** *acute phase proteins after an acute inflammatory stimulus such as lipopolysaccharide (LPS). ACT, antichymotrypsin; AGP, acid glycoprotein; CRP, C-reactive protein; IL, interleukin; SAA, serum amyloid A; TNF, tumor necrosis factor.*

of different chemokines, each with their own spectrum of cell attraction properties. These chemokines influence the range of cells entering the site of inflammation and thus the course of the inflammatory response. These early cytokines will in turn give rise to other cytokines that they induce at local and distal sites, such as IL-6 which frequently mediate cellular responses (Figure 1a). Other cytokines take rather longer to be synthesized and in some cases are no longer proinflammatory and some, such as IL-10 and IL-1 receptor antagonist (IL-1ra), are definitely anti-inflammatory. The overall effect is sometimes referred to as a cytokine 'storm' – a useful phrase because it reflects the battery of new stimuli to which cells are subjected.

Proinflammatory cytokines and the acute phase proteins

As an example of how the APR is generated hepatocyte production of APPs is the main focus because this is comparatively well known. Other aspects are considered later. The induction of the APR in the liver was studied extensively as a model of gene expression. The major cytokine involved in induction of the APR is IL-6. IL-6 was originally also called hepatocyte-stimulating factor (among other names), based on its inducing properties (Gauldie et al. 1987). This cytokine is the most prominent member of a family of cytokines (oncostatin M, leukemia inhibitory factor, ciliary neutrotrophic factor, IL-11, and cardiotropin 1) that can all induce the APR. This family of cytokines binds a family of receptor complexes containing a cytokine-specific receptor component chain, as well as a common component chain called gp130. It is the gp130 that has the major signaling role in the complex, which responds by transmitting a signal when homodimerized (Paonessa et al. 1995). Interestingly, the soluble IL-6R (sIL-6R) acts as an agonist and continues to help induce responses to IL-6 if gp130 is expressed on the cell surface (Mackiewicz et al. 1992). In addition, the sIL-6R–IL-6 complex has a longer half-life, thus stabilizing the activity of IL-6 (Peters et al. 1996). The soluble gp130, on the other hand, inhibits the response (Jostock et al. 2001). The other major inducer of the APR is IL-1. This cytokine family has many members but three are relevant to the induction of APPs. IL-1β is probably the most important inducing form because it is the major serum form of active IL-1. IL-1α is thought to be more important at a local level and the other form of IL-1 is an antagonist form called interleukin-1 receptor antagonist (IL-1ra) which can bind to receptor but does not activate or recruit the accessory chain (Hannum et al. 1990; Greenfeder et al. 1995). IL-1β binds to two receptors: IL-1R1, which is capable of inducing response, and IL-1RII, which is a decoy receptor with no ability to signal (Colotta et al. 1993). Very few receptors need be occupied for a cell to respond to this cytokine but receptor numbers are regulated during the APR. Responses to IL-1 are thus kept under control by a number of systems (Mantovani et al. 2001).

The APPs have been classified as class I or class II based on the cytokines to which they respond (Figure 2). The class I APPs are induced by IL-1-type cytokines such as IL-1β or IL-1α (as well as TNFα and TNFβ) and IL-6. The TNF cytokines are included in this definition because they induce some of the same signaling and transcription factors as IL-1. The class II APPs, on the other hand, are induced almost exclusively by IL-6 and other members of that family of cytokines (mainly through IL-6RE which bind the STAT transcription factors). In some cases IL-1 may even reduce responses, for instance although IL-6 induces fibrinogen this is inhibited by IL-1β or TNFα (Rokita et al. 1994). The use of mouse IL-6 knock-out animals has confirmed the important role of IL-6 but this revealed another feature of the APR. Although turpentine-induced inflammation could not induce an APR in these animals, induction with lipopolysaccharide (LPS) led to an almost normal APR. This may be caused by induction of a different range of cytokines that can make up for the lack of IL-6 (Fattori

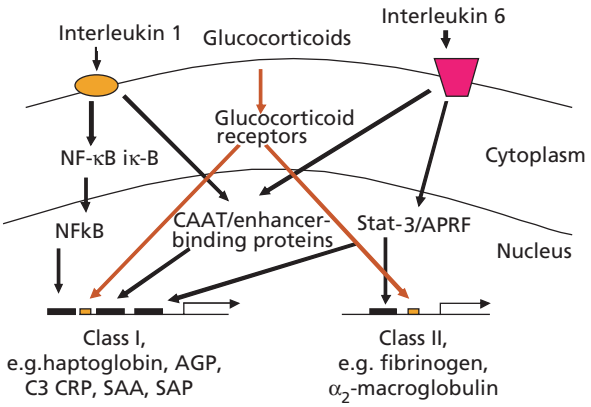

Figure 2 *Simplified pathways of induction of acute phase protein genes. AGP, acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A; SAP, serum amyloid P.*

et al. 1994; Kopf et al. 1994). The likely candidates are other members of the IL-6 family of cytokines but leukemia inhibitory factor (LIF) and IL-11 are not likely to be involved (Gabay et al. 1996). Interestingly, the same sort of pattern is seen in IL-1β knock-out mice with responses to turpentine being lost but LPS maintained (Zheng et al. 1995). IL-6 production in response to IL-1 is diminished after turpentine injection. Other knock-out mice lacking IL-1-converting enzyme (caspase-1) show no reduction in responses to LPS or local turpentine (Fantuzzi et al. 1997). The loss of the signaling molecule gp130 in mice leads to severe abnormalities in development and immune cells making interpretation difficult. It has been seen, however, that loss of the signaling component STAT3 (downstream of gp130 signaling) leads to complete loss of induction of some class I acute phase genes which have regulatory elements for C/EBP transcription factors. Type II APPs were reduced at 24 hours but earlier responses are maintained (Alonzi et al. 2001). Some APPs (complement C3 and SAA3) have no STAT3 regulatory regions and are not affected by its loss. Adaptor proteins that bind STAT3 such as STAP-2 (BKS) also have significant roles in regulating signaling for APP synthesis (Minoguchi et al. 2003). Synthesis of each APP gene is regulated both transcriptionally and post-transcriptionally. The transcriptional regulation is controlled by the range of nuclear factors that bind to the promoter regions of the protein gene. Some of these are induced by IL-6 such as NF–IL–6 and others such as NF-κB are induced by TNFα or IL-1β (Edbrooke et al. 1989; Akira et al. 1990). Often such factors may act synergistically, e.g. the combinations of IL-6 and IL-1 for synthesis of serum amyloid A (SAA). In addition, some proteins are also strongly regulated post-transcriptionally by stabilization of the mRNA and this occurs for instance for α acid glycoprotein (AGP) (Shiels et al. 1987). The regulation of each protein is, however, complex and unique, e.g. a range of transcription factors has been identified in the induction of SAA genes including NF-κB, C/EBP, AP-2, Sp1 and YY1, and SAF (Uhlar and Whitehead 1999).

Lipopolysaccharide has been reported to activate hepatocytes directly, although it is hard to rule out the possibility that this is an indirect effect through LPS-induced cytokines. Thus, some questions remain that are unresolved with regard to induction of the hepatic response. Can the hepatocyte, if it expresses Toll-like receptor 4 (TLR4), respond to LPS directly? And if not, what role do the nonparenchymal cells of the liver have in directing the hepatocyte response? In particular, what contribution to the IL-6 stimulus to the hepatocyte comes from the Kupffer cell or possibly even the endothelial cell or fibroblast, and what contribution comes from circulating cytokine? Histological studies of mRNA or protein synthesis in liver sections has revealed a pattern of synthesis that involves initial synthesis in periportal regions followed by spread to perilobular, midlobular, and centriolobular regions until, at peak serum CRP, almost all hepatocytes are synthesizing CRP (Kushner and Feldmann 1978) (Figure 3). It should also be remembered that control can be exerted at the secretion stage as, for instance, for CRP (Yue et al. 1996). As referred to later, under Protein metabolism, the hepatocyte requires an increased supply of

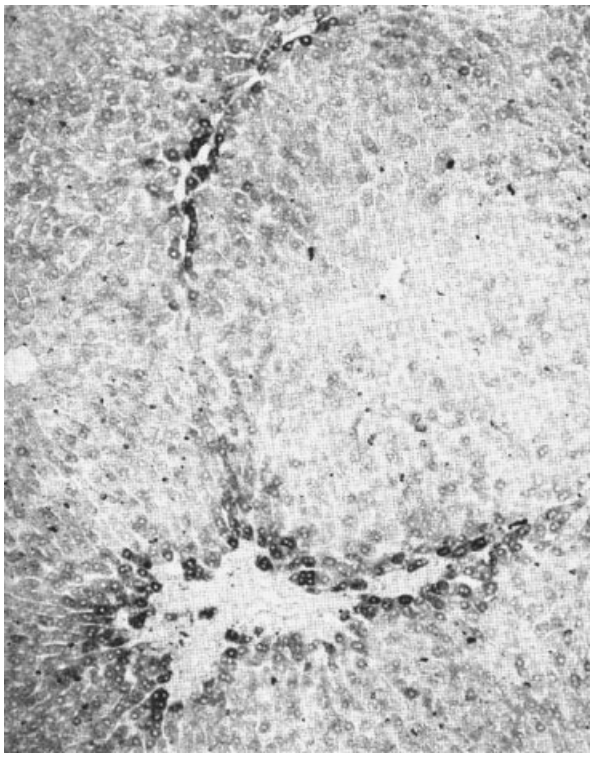

Figure 3 *Immunoenzymatic identification of hepatocytes producing C-reactive protein (CRP) 8 h after an inflammatory stimulus. Perilobular and periportal areas show intense cytoplasmic staining. (Reproduced from Kushner and Feldmann 1978, with permission.)*

amino acids for this increased burden of protein synthesis, and thus amino acid uptake by hepatocytes is increased.

Influence of inhibitory or modulating factors on APPs

Although glucocorticoids are generally an anti-inflammatory influence, it is also clear that they enhance synthesis of many APP genes (Baumann et al. 1989). The presence or absence of glucocorticoid response elements controls the extent of response to glucocorticoids which varies between proteins with, for instance, AGP being very strongly dependent on these hormones.

As IL-1 is a major inducing factor, it is not surprising that IL-1Ra would have an inhibitory action because it increases at a later time point in the APR subsequent to IL-1β. IL-1ra can reduce synthesis of APPs (Bevan and Raynes 1991) and, in a mouse model, completely prevented SAA induction, showing that IL-1 is required for SAA synthesis (Grehan et al. 1997). The hepatocyte makes a significant contribution to plasma IL-1ra (Gabay et al. 2001) and would thus be an autocrine regulatory response. The liver makes this IL-1ra in response to IL-6 whereas IL-1ra from other tissue appears to be independent of IL-6 (Gabay et al. 2001).

TNFα has an ability to induce responses in vitro and would clearly do so indirectly in vivo. Transforming growth factor β (TGFβ) and interferon-α and -γ have been demonstrated to have some ability to induce APPs (Ramadori et al. 1988; Mackiewicz et al. 1990). Other factors with an influence are hepatocyte growth factor and retinoic acid (Koj et al. 1995). Insulin has a downregulatory effect on APP synthesis (Campos and Baumann 1992).

It has recently been found that a member of the IL-10 family of cytokines, IL-22, has the ability to induce an APR in hepatocytes. This cytokine, produced mainly by T cells, can interact with IL-22 receptor together with IL-10R2 to induce STAT1 and STAT3 and hence induce a range of proteins such as SAA, haptoglobin, and antichymotrypsin (Dumoutier et al. 2000a, 2000b). As yet the extent of IL-22 contribution to the APR is not known. The effects of IL-22 are also not restricted to the hepatocyte because it has been shown that epithelial cells can also respond and IL-22 may thus have pleiotropic effects. These and the APR induction are antagonized by a soluble receptor which acts as a binding protein or by receptor antagonism (Dumoutier et al. 2001; Wei et al. 2003).

Effects of nutrient deficiency or modulation

Protein synthesis by hepatocytes represents a considerable drain on amino acid precursors and total hepatic protein synthesis during inflammation. It might be expected that it would therefore be absent or greatly reduced in nutritional deficiency. However, this appears not to be the case, because in severe protein malnutrition the APR is often seen in equal or higher levels compared with individuals with normal weight for height, although such studies do not take into account a reduced resistance to infection in such individuals. In fact, when faced with an equivalent stimulus in human studies there is a reduction in response in malnutrition following, for example, immunization protocols (Doherty et al. 1993) or in animal studies (Jennings et al. 1992; Lyoumi et al. 1998). Nevertheless the response is still a strong one and is essentially maintained. The reduction is probably at the level of cytokine production rather than hepatocyte response because serum IL-6 was reduced as well as APPs (Doherty et al. 1994). This provides further evidence for the essential role of the APR and APPs in homeostasis or resistance to infection. It is often assumed that the concentration of the APPs is dependent on the rate of synthesis; however, there is evidence that the rate of clearance may be different in protein malnutrition (Morlese et al. 1998). It is also clear that certain proteins such as fibrinogen may be more severely affected by protein malnutrition than others (Morlese et al. 1998) and that the malnutrition itself, in the absence of other stimuli, may in some cases cause an increase in certain proteins (Lyoumi et al. 1998). The effects of other forms of nutritional deficiency on the APR are less well characterized, although in the reverse direction the APR can certainly alter micronutrient availability (see later, under Micronutrient changes). Low levels of vitamin E may lead to a more pronounced inflammatory response, perhaps because of a reduced ability to control oxidants.

Another factor that can modulate APRs through their effect on cytokine production is the amount of *n*-3 and *n*-6 polyunsaturated fatty acids in the diet. Fish oils that are rich in *n*-3 fatty acids reduce the proinflammatory cytokine production of IL-1 and TNFα in comparison with *n*-6 fatty acids, which increase cytokine responses (Calder and Grimble 2002). The effect of the *n*-3-rich oils is sufficient to improve the status of patients with diseases with inflammatory pathology such as rheumatoid arthritis and psoriasis or asthma and in animal studies with LPS (Calder and Grimble 2002)

Induction of other aspects of the APR

Fever has been shown to be induced by administration of proinflammatory cytokines (called endogenous pyrogens) but appears to need IL-6, which acts downstream of the potent pyrogens IL-1α or IL-1β. When the cytokines reach the central nervous system they generate prostaglandins, particularly PGE_2 in the organum vasculosum of the lamina terminalis, which reset the

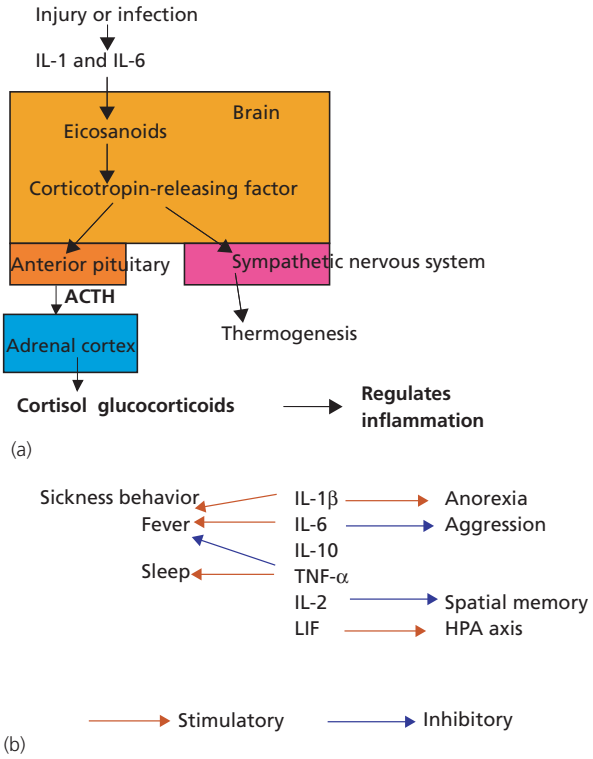

Figure 4 *Induction of fever and glucocorticoid downregulation of inflammation through brain responses to proinflammatory cytokines. HPA, hypothalamo–pituitary–adrenal; IL, interleukin; LIF, leukemia inhibitory factor; TNF, tumor necrosis factor.*

temperature set point (Figure 4a). Mice lacking the prostaglandin receptor EP_3 are unable to generate a febrile response (Ushikubi et al. 1998). Although other pathways may ultimately also act, they all generate a response in the thermoregulatory center in the anterior hypothalamus. Although the original model suggests that the cytokine originates from the periphery, there are several possible pathways and local cytokine synthesis is likely. It is even possible that endotoxins may act directly in a similar way to that proposed for hepatocyte responses. Responses have been linked to a number of different cytokines using inhibition or addition experiments, but direct effects are difficult to demonstrate. The use of knock-out mice has suggested that IL-6 and IL-1β are involved in the induction of fever (Zheng et al. 1995; Kluger et al. 1998; Netea et al. 2000; Inui 2001) whereas others such as TNFα or IL-10 appear to reduce fever (Leon et al. 1997; Leon 2002). Recently it was shown that vagotomy reduces the extent of fever in response to intra-abdominal IL-1 (Watkins et al. 1995). The sensory input of afferent vagal nerves can thus alter fever responses in the brain and this is dependent on the site and type of stimulation. The interaction between neural and immune inflammation was recently reviewed (Tracy 2002).

Studies using knock-out mice also suggest that anorexia and somnolence are regulated by IL-1β and IL-6 (Figure 4b, reviewed in Inui 2001). IL-1 appears to be important for anorexia and TNFα is thought to be needed for sleep responses. Lethargy may be caused by IL-1 because mice lacking type I IL-1 receptor do not show lethargy responses and these responses do not require IL-6 (Leon et al. 1997).

Little information is available on the proinflammatory cytokine regulation of hemopoiesis and they are likely to work through growth factors (see later under Hemopoietic responses). Thrombocytosis is regulated by IL-6 mainly through thrombopoietin (Kaser et al. 2001).

ACUTE PHASE PROTEINS

Acute phase proteins have many different functions and in this review have been organized into functionally related groups, although there is some doubt about the role of many proteins and to which category they should belong. It should be remembered that there is no requirement that a particular protein should only have one function. The choice of whether to include proteins that are made mainly outside the liver or in nonhepatic cells, or when to start considering proteins as part of the acute phase, is a difficult one. In some respects a review of the acute phase should consider TNFα and other cytokines released as early components of this system. However, this review focuses a few hours after the initial stimulus and includes proteins that are increased by at least 25 percent or more during inflammation and that are at least, in part, made in hepatocytes. Also included are proteins that show substantial increases in concentration in human sera and/or increases in several other species, which may be made in other cells that might be used to follow inflammation. It has been shown recently, using oligonucleotide array hybridization, that 7 percent of hepatic genes were responsive to LPS (Yoo and Desiderio 2003), demonstrating the importance of this response in liver function and confirming the impression that many such genes are related to host defense and immunity, metabolism, or coagulation.

Acute phase proteins are produced at a time when the host might be expected to require a downregulatory force on the systemic inflammation that generated the APR. Thus many of the proteins are thought to have an anti-inflammatory function (Tilg et al. 1997). It is interesting that, although similar proteins are found in most species, there is considerable variation between the concentrations of the proteins, and variations between which are strongly or weakly induced, e.g. CRP, haptoglobin, AGP, and α_2-macroglobulin, have very different patterns of expression in humans, mice, rabbits, and rats.

In general APPs are well conserved and many have ancestors in teleosts and elasmobranchs (Bayne and Gerwick 2001). There are some human APP deficiencies

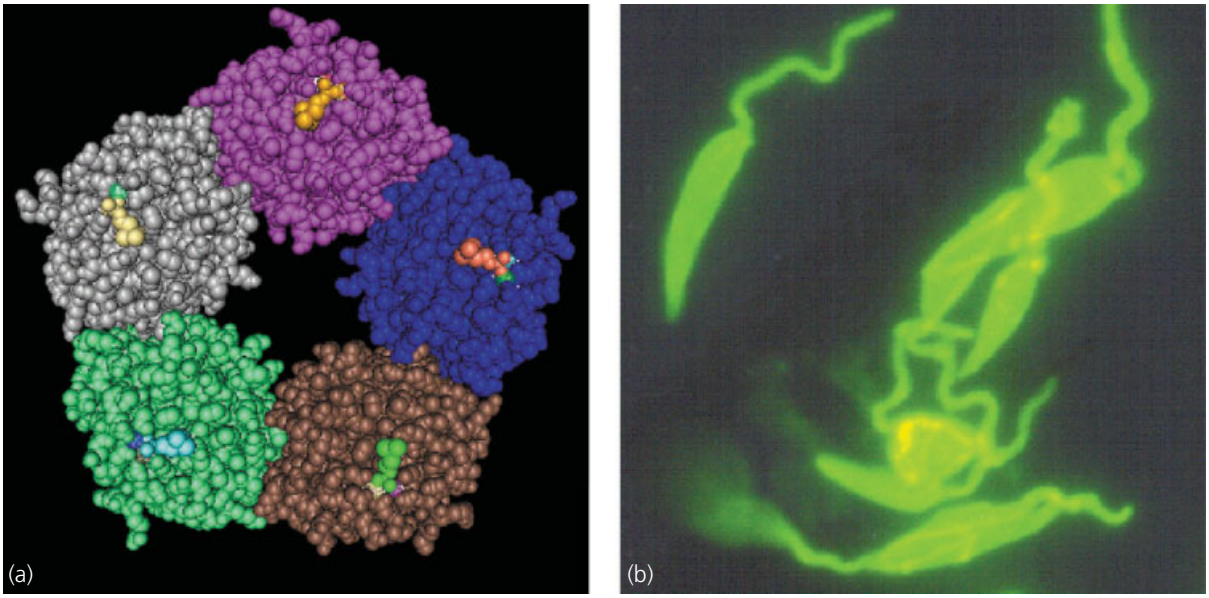

Figure 5 (a) *Structure of C-reactive protein (CRP);* **(b)** *binding of CRP to* Leishmania mexicana *demonstrated by immunofluorescence.*

which have provided information about function and knock-out mice have also provided information. APPs will now be considered according to groups with related function.

Innate immune proteins

C-REACTIVE PROTEIN AND OTHER PENTRAXINS

C-reactive protein was originally discovered over 70 years ago and given its name because it interacted with the phosphorylcholine-containing polysaccharide and lipoteichoic acid found on *Streptococcus pneumoniae* (Tillet and Francis 1930). CRP is the best known of the APPs because it is regularly used as a marker of systemic inflammation in clinical settings and can increase from approximately 1 μg/ml to 500 μg/ml in severe inflammation (see later under Clinical use of APPs and inflammation markers). The protein is, however, a member of a family of proteins (pentraxins) that includes homologs of similar size to CRP such as serum amyloid P (SAP) component or homologous domains found as part of larger proteins such as long pentraxins (PTX3). Other species usually show either SAP or CRP to be an APP. In mice, for example, CRP is present at low levels and SAP is a major APP whereas human SAP concentrations change little in inflammation (Pepys and Baltz 1983). CRPs are found in arthropods that lack an acquired immune system and often several copies are found; in the horseshoe crab these have different binding specificities (Iwaki et al. 1999). Human PTX3 demonstrates small increases in concentration during the acute phase (Muller et al. 2001b).

The common feature of these proteins is their structure which is pentameric (see Figure 5a). Crystal structures are now available for both CRP and its homolog SAP (Emsley et al. 1994; Shrive et al. 1996). They both possess the ability to bind to phosphorylated ligands such as phosphorylcholine or phosphorylethanolamine in a calcium-dependent manner. However, this is not the only ligand because phosphorylated carbohydrates can also bind (Culley et al. 2000). Such ligands are found on microorganisms including *Leishmania* spp. (see Figure 5b) One feature of these interactions is that the avidity of CRP for monomeric ligand is usually low and, only when such ligands are expressed as an array, e.g. on the surface of a microorganism, are high avidities generated. The binding specificity of SAP includes heparin, a 4,6-cyclic acetal of pyruvate, 6-phosphorylated mannose, and 3-sulfated saccharides (Hind et al. 1984; Loveless et al. 1992). CRP, SAP, and PTX3 have all been shown to interact with apoptotic cells (Gershov et al. 2000; Rovere et al. 2000; reviewed in Nauta et al. 2003). The mechanism of binding for CRP may be related to binding to damaged membranes which has been observed in a number of other systems where membrane integrity or composition is altered (Li et al. 1994).

It is clear that CRP can activate complement through the classical pathway but it has been shown that CRP activates the pathway only as far as C5 (Mold et al. 1984). The C1q binding site is found on the opposite face to the phosphorylcholine- or calcium-dependent ligand-binding face. CRP can directly interact with cellular receptors such as FcγRI (Marnell et al. 1995; Bodman-Smith et al. 2002) and perhaps also FcγRII. The result of CRP interaction with a range of micro-

organisms is increased phagocytosis by cells such as macrophages and neutrophils, either directly or indirectly through complement. These ligands on infectious organisms may allow CRP to be protective against disease as in the case of *Streptococcus pneumoniae* (Yother et al. 1982; Du Clos and Mold 2001), but may not always be so in cases of *Leishmania donovani* when CRP binding to lipophosphoglycan on the parasite increases access to the macrophage as an important part of its life cycle (Culley et al. 1996). There is little or no evidence for opsonization of microorganisms through SAP binding, although recently it has been suggested that SAP can cause phagocytosis of its ligand zymosan through murine Fc receptors (Mold et al. 2001).

The role of slightly higher baseline CRP concentrations as a predictor of heart disease is a fascinating new observation (Haverkate et al. 1997; Libby et al. 2002). It is currently important to discover whether this is a causal observation or merely a marker of some other inflammation-driven cause of atherosclerosis. A potential mechanism may be through low-density lipoprotein (LDL) uptake into monocytes or through activation of endothelial (CRP induces tissue factor) or other cell types at the lesion site.

PTX3 lacks the ability to bind to phosphorylcholine ligand but retains an affinity for carbohydrate and also binds to macrophages and can protect against fungal infection (Garlanda et al. 2002).

COMPLEMENT COMPONENTS

A number of the complement cascade proteins are APPs including the central component C3. The complement cascade acts as an innate immune and acquired immune pathway, being triggered by both antibody and the innate proteins mannose-binding lectin (MBL) and ficolin. The alternative pathway is activated by charged surfaces often presented by microbes. MBL is itself an APP (Ezekovitz et al. 1988). The complement components (see Complement) that are induced are C1q, C3, C4, C9, factor B, C1 inhibitor, and C4-binding protein. Some of these are required to replenish protein depleted by activation and usage of complement as is observed after, for example, a severe burn injury. APPs such as C4-binding protein are required as controlling proteins.

Mannose-binding lectin is a member of a family of collectins that all bind to carbohydrate on pathogens and direct these pathogens for opsonization through complement after association with a protease called mannose-binding protein-associated serine protease (MASP), which activates C1q (Matsushita and Fujita 1992). This protein is a decamer of units that have globular lectin domains and collagenous tails (Figure 6) (Peterson et al. 2001). The binding to a variety of carbohydrate structures such as mannose or *N*-acetylglucosamine on microbes can lead to phagocytosis. Besides activating complement a direct receptor for MBL has been implicated but not defined as yet. A fairly common mutation in one of the alleles leads to large reduction in secreted active protein and such individuals are more at risk of certain infections, particularly at the ages of 2–4 years before an appropriate humoral immunity has been attained (Sumiya et al. 1991). MBL is one of a number of proteins that can lead to improved presentation to T cells (Figure 7).

LIPOPOLYSACCHARIDE-BINDING PROTEIN

Lipopolysaccharide-binding protein (LBP) is found constitutively at about 5–10 μg/ml but can increase 10-fold during severe inflammation. LBP has been shown to bind to LPS and transfer it to CD14 and TLR4, and consistent with this LBP can increase the activity of LPS to activate $CD14^+$ cells by more than 100-fold (Schumann et al. 1990). However, LBP also transfers LPS into high-density lipoprotein (HDL) within which environment LPS is almost completely without activating effect (Wurfel et al. 1994). As the LBP interacts mainly with the lipid A portion of the LPS, it is unlikely to bind directly to bacteria and opsonize them. Further information of its role comes from LBP-deficient mice which can clear LPS normally but need LBP for an inflammatory response to LPS. Furthermore, these mice are more susceptible to intraperitoneal salmonella infection (Jack et al. 1997). In the *salmonella* infections, TNFα synthesis was reduced and evidence was presented that LBP contributed towards TNFα activation of polymorphs (Fierer et al. 2002).

SECRETORY PHOSPHOLIPASE A_2

Phospholipase A_2 (PLA_2) is an enzyme that generates fatty acid and a lysophospholipid from phospholipid. The enzyme exists in a cytosolic form and as a secreted version ($sPLA_2$), and there are several different isoforms of each. The products are important for signaling pathways and generation of lipid inflammatory mediators and for this reason have been considered as potential targets for anti-inflammatory drugs. The secreted PLA_2 is an APP (Andreani et al. 2000), of which the most studied form, $sPLA_2$ type IIA, is a potent anti-streptococcal and anti-staphylococcal enzyme (Weinrauch et al. 1998). It is also thought to be coordinated with other prostaglandin synthesis enzymes in order to play a role in inflammation. The effects and potential functions of such enzymes are clearly wide ranging and a range of different receptors is being characterized (reviewed in Lambeau and Lazdunski 1999; Murakami et al. 2000).

Protease inhibitors

There are a number of protease inhibitors present in plasma that have a spectrum of activity and many of

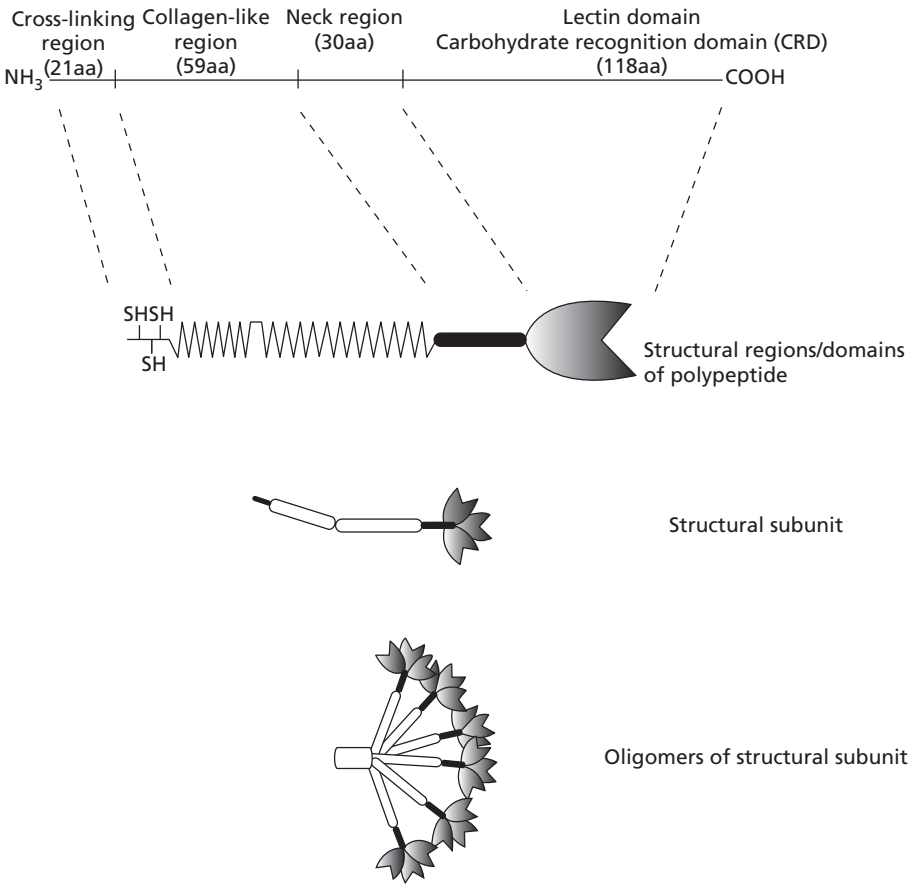

Figure 6 *Structure of mannan-binding lectin (MBL) showing structural domains, subunits, and oligomeric organization. (Redrawn from Peterson et al. 2001.)*

which increase in response to inflammation. They generally increase at later time points (2–5 days). Names of the protease inhibitors have often been given on the basis of the protease that allows demonstration of inhibitory activity rather than the actual protease that they inhibit in vivo. Although a major role is to inhibit endogenous proteases and thus control inflammation and tissue clearance before remodeling, it has also been proposed that some functions may relate to control of microbial proteases (Hiemstra 2002). Many of these protease inhibitors are serine protease inhibitors of a family called serpins. Some of these inhibitors have particular functions such as plasminogen activator inhibitor, antithrombin III, and C1q inhibitor which are therefore considered in other sections.

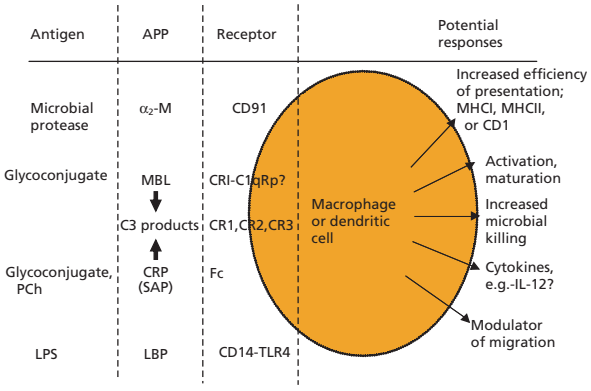

Figure 7 *Diagram depicting the different acute phase proteins that deliver particular microbial products to macrophages or dendritic cells. This can have a number of different potential outcomes, all of which have been described for at least one of the uptake pathways.*

α_1-ANTITRYPSIN OR α_1-PROTEASE INHIBITOR

α_1-Protease inhibitor (α_1-PI) is defined as a serpin because it is structurally related to members of a family of serine protease inhibitors. Already present at approximately 2–3 mg/ml it can increase fourfold. The name α_1-PI is to be preferred to antitrypsin because its actual antiprotease effect is more likely to be on neutrophil elastase and cathepsin G or protease 3. This glycoprotein is synthesized mainly by hepatocytes but also by macrophages and neutrophils. Occasionally we can learn about function from deficiency diseases and this is the case for α_1-PI (Carrell and Lomas 2002). The disease leads to a greatly increased risk of emphysema or liver disease. The

emphysema is the result of the lack of control of proteases, and the liver disease also caused by a problem with misfolding and protein aggregates accumulating in the hepatocyte endoplasmic reticulum (Lomas et al. 1992). Thus, a major function of this protein is to prevent degradation of elastase in the lung. Treatment with replacement α_1-PI was able to reduce frequency of lung infection in α_1-PI-deficient patients (Lieberman 2000). After complex formation with protease, a large change in the protease structure is generated (Huntington et al. 2000) and clearance follows by endocytosis through the serpin enzyme complex (SEC) receptor (Perlmutter et al. 1990; Joslin et al. 1992).

α_1-ANTICHYMOTRYPSIN

This protein is increased to a greater extent (up to sixfold) than other slow responding human APPs such as α_1-PI (see Figure 1b). Again, the specificity for serine proteases and localization is such that it is more likely to inhibit cathepsin G and mast cell chymases. Interestingly a point mutation in α_1-antichymotrypsin (α_1-ACT) can lead to reduced concentrations in plasma and a deficiency disease similar to that seen with α_1-PI. This is only seen with particular mutations (Faber et al. 1993). As with other serpinopathies (of C1 inhibitor or antithrombin III) many α_1-ACT mutations do not lead to such pathology. This is likely to be because they are produced at 10 percent of the rate and degradative pathways can handle the inappropriately folded protein. Interestingly, the presence of an APR may precipitate pathology when the rate of synthesis is increased, although this is based on a limited number of observations.

α_2-MACROGLOBULIN

This is a very large protein with an interesting mechanism of inhibition. It is so large that it can be visualized by electron microscopy and it acts to trap the protease when the protease cleaves a bond on the internal aspect of one of the faces of the protein. The change in conformation of the α_2-macroglobulin reveals a site for interaction with a receptor called LDL receptor-related protein or CD91 (Moestrup and Gliemann 1989) (see Figure 8). CD91 has been identified as a receptor site for heat shock protein (HSP)-mediated binding and endocytosis. The result of this is that α_2-macroglobulin may also be an immune component because it improves adjuvanticity through cross-presentation (Binder et al. 2001) (see Figure 7).

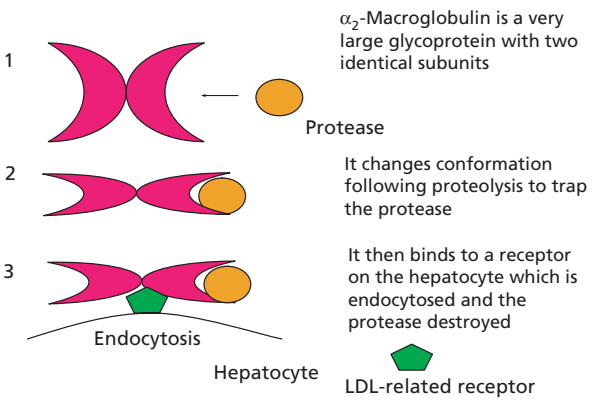

Figure 8 *Diagram depicting the mechanism by which α_2-macroglobulin captures proteases and delivers them from endocytosis into macrophages and other cells expressing CD91 or low-density lipoprotein (LDL)-related receptor.*

Transport proteins

FERRITIN

Ferritin is composed of two sorts of protein subunit – H and L – which combine together to form a complex of 24 subunits that form an apoferritin shell which can hold up to 4500 iron atoms. It is present inside the cell and acts mainly as a storage protein rather than a transporter, but smaller amounts are also found in the serum. The serum form is thought to be derived from the L subunit and is normally poor in iron. Hepatocyte cytoplasmic ferritin is also predominantly the L form. Its primary function is to sequester iron and, by reducing availability for microbial growth, this helps control infection. However, ferritin should be considered as a member of a group of proteins that together with transferrin and transferrin receptor work to make available the iron necessary for many of the host of mammalian iron-containing proteins. Ferritin synthesis is induced in a post-transcriptional way by intracellular iron availability. It is also induced by proinflammatory cytokines in a complex way which also involves increased secretion (Torti and Torti 2002), although IL-4 and IL-13 may also regulate ferritin produced in macrophages (Weiss et al. 1997). The function and regulation of iron availability appear to be linked with another APP called hepcidin which is produced by hepatocytes and macrophages (Nemeth et al. 2003)

HAPTOGLOBIN

This glycoprotein is mainly synthesized in hepatocytes but some is also made in other cell types. Its function appears to be to bind to free hemoglobin and prevent damage, particularly renal damage, which would be caused by free hemoglobin, and then to recycle the iron and reduce availability for microbes. There are two alleles (designated 1 and 2) which give rise to polymeric molecules with properties that diverge considerably (Langlois and Delanghe 1996). Allelic associations exist with infections, atherosclerosis, and autoimmune disease. The haptoglobin and hemoglobin complex interacts with a receptor recently identified as CD163 (Kristiansen et al. 2001) which clears the iron mainly to macrophages. However, the haptoglobin-deficient mouse shows no

major symptoms until placed under strong hemolytic stress, when it displays renal injury as a result of oxidative damage (Lim et al. 1998). Extrahepatic synthesis in lung macrophages may hint at other functions.

HEMOPEXIN

Hemopexin has the ability to bind heme released from hemoglobin or other heme-containing proteins, thus acting as a second-line defense against hemoglobin damage. As for haptoglobin, this reduces the opportunity for free iron to cause oxidative damage and also reduces the ease with which microorganisms can have access to iron for growth. It has been proposed that, once heme has been bound with high affinity (dissociation constant $K_d < 1$ pmol/l), a site is revealed that allows binding to a hepatocyte receptor; however, it is unclear if this is responsible for the total uptake by hepatocytes (Noyer et al. 1998). Heme would then be catabolized to bilirubin, biliverdin, and iron, with hemopexin and the receptor recirculating. Deletion of the gene for hemopexin generated mice that had normal handling of iron under unchallenged conditions, but the mice were less able to cope with hemolysis (Tolosano et al. 1999). Although haptoglobin- and hemopexin-deficient mice have increased amounts of the other protein (perhaps to compensate) the double knock-out has a similar phenotype to the single knock-outs. The delivery of heme to cells for destruction is part of a system that includes heme oxygenase which acts to reduce oxidative stress from iron inside the cell (see later, under Liver protective responses).

GC GLOBULIN

Group-specific component (Gc globulin), also known as vitamin D-binding protein, is only a very minor acute phase reactant. Its function appears to be twofold: it acts in clearing actin, a protein that has been implicated in the formation of microthrombi (Lee and Galbraith 1992). Increased tissue damage releases large amounts of actin that need to be cleared, and this first function is achieved in conjunction with the muscle cell-derived gelsolin. Initially after trauma, levels are reduced, presumably reflecting functional depletion and subsequently rise following IL-6 effects on hepatocytes. A second function, as its other name suggests, is as a transporter of vitamin D_3 and its major metabolites including the active 1α,25-dihydroxyvitamin D_3 (Daiger et al. 1975). When circulating, only a small percentage of the circulating protein has vitamin D bound; nevertheless it appears to control availability and uptake by cells. Other reports have suggested an immunomodulating function.

Procoagulant/fibrinolytic proteins

In the acute phase, the balance between the different factors that promote or clear clot formation is one that shifts towards the procoagulant. The process of coagulation is linked to inflammation because coagulation increases inflammation and inhibition of this coagulation can reduce the severity of inflammation which involves coagulation. In addition to the proteins mentioned below, factor VIII and von Willebrand factor are APPs induced by IL-6.

FIBRINOGEN

A range of different mutations in humans has led to rare afibrinogenemias where complete lack of fibrinogen is associated with bleeding disorders. Alternately less severe dysfibrinogenemias have different presentations and patterns of pathology. Fibrinogen is composed of two sets of three different sorts of chains – lack of any one of these leads to deficiency (Doolittle 1984). As well as being an important protein for blood clot formation with the platelet plug, it has a role in inflammation, wound healing, cell migration, and cell proliferation. These properties are thought to be related to interactions with integrins on endothelial cells or (CD11b/CD18) on leukocytes.

This protein is required for wound healing and appropriate cell distribution and migration during the wound healing process (Drew et al. 2001), which was studied in knock-out mice. Although mainly synthesized in the liver, extrahepatic synthesis occurs, e.g. in lung epithelial cells.

PLASMINOGEN AND PLASMINOGEN ACTIVATOR

This protein is a zymogen that is cleaved by the plasminogen activators – urokinase (uPA) or tissue type (tPA) – to form plasmin, an enzyme that cleaves fibrin and other extracellular proteins to remove clots. Upregulation of both plasminogen and plasminogen activator and the variety of other coagulation/fibrinolysis proteins does not necessarily favor enhanced thrombosis (Esmon 2000), but may rather represent a replacement of consumed protein.

PROTEIN S

Activated protein C and protein S work together as an anticoagulant and an anti-inflammatory agent. When small microthombi of fibrin and thrombin are deposited in inflammation or infection then thrombin/thrombomodulin activates the protein C (an antithrombotic serine protease). This led to a trial in patients with severe sepsis of an activated protein C that showed a small improvement in mortality from sepsis compared with placebo, a rare finding because many attempts to improve outcome in sepsis have failed (Bernard et al. 2001; see also Shock/sepsis). Further studies are needed to confirm efficacy.

VITRONECTIN AND PLASMINOGEN ACTIVATOR INHIBITOR TYPE I

Although mostly synthesized in the liver, mRNA for vitronectin is found at lower levels in the brain, heart, and adipose tissues. It is stimulated by IL-6 and protein can increase by two- to threefold (Seiffert et al. 1994). Vitronectin is a multifunctional adhesive glycoprotein that forms a complex with plasminogen activator inhibitor type I (PAI-1), which stabilizes the function of the latter. PAI-1 binds to plasminogen activators such as tPA to generate a stable inactive complex. Vitronectin also helps PAI-1 binding to fibrin. In addition PAI-1 acts as an antiadhesive factor for cell adhesion involving vitronectin and integrins or the urokinase receptor. PAI-1 deficiency in humans is associated with bleeding disorders, indicating a role in stable clot formation.

ANTITHROMBIN III

Antithrombin II is a negative APP, which by its reduction leads to a more procoagulant state. Antithrombin III-deficiency diseases are characterized by thromboembolism (Mackie et al. 1978). It is a serpin that inhibits thrombin and other proteases that cause coagulation.

Regulatory molecules released into the circulation during the APR

IL-1 RECEPTOR ANTAGONIST

The IL-1Ra, as its name implies, prevents IL-1 action by competing for available receptor. Originally found as a cytokine generated by the same cells that make the active form, but temporarily delayed in response, it has more recently been described as an APP under different regulation in hepatocytes (Gabay et al. 2001).

MACROPHAGE MIGRATION INHIBITORY FACTOR

With the similarity of migration inhibitory factor (MIF) to procalcitonin, it appears that MIF acts as an APP in septic or immune inflammation rather than trauma-induced inflammation (Beishuizen et al. 2001). MIF is an interesting protein which appears to have many immune and inflammation-related functions. Its function may relate to reducing glucocorticoid effects on the inflammatory response (Baugh and Bucala 2002). It appears to mediate shock responses in reaction to Gram-positive and -negative bacteria, and mice that lack MIF are resistant to LPS-induced shock (Bozza et al. 1999). The mechanism of action of MIF remains somewhat unclear despite existing in large extracellular amounts, and no cellular membrane receptor has been identified. It is also found intracellularly and an intracellular ligand, Jab1, has been linked to some responses (Kleemann et al. 2000).

SOLUBLE ADHESION MOLECULES

Soluble intercellular adhesion molecule (sICAM) or soluble vascular cell adhesion molecule (svCAM), which are immunoglobulin adhesion molecule family members, and soluble E selectin are all found in increased amounts in plasma after any infectious and inflammatory stimuli that give rise to systemic effects (Kulander et al. 2001; Reinhart et al. 2002). Their function may relate to an ability to inhibit cellular extravasation.

ANGIOTENSINOGEN

Angiotensinogen is largely produced in the liver, although other tissues can make it and it is cleaved by renin to angiotensin II, which is a powerful vasoconstrictor that controls salt and water homeostasis (Morgan et al. 1996). Other studies have suggested that it may also have proinflammatory effects on vascular endothelia (Brasier et al. 2002).

Other APPs of uncertain function

SERUM AMYLOID A

Serum amyloid A (SAA) protein is a major APP in humans but it is unclear what the true function for this protein might be. SAA was first discovered as the serum component precursor of amyloid AA (Rosenthal et al. 1976). Since then several members of the family have been found. Human SAA is a gene family including two acute phase forms, SAA1 and SAA2, as well as a constitutive form SAA4 (Uhlar and Whitehead 1999). During normal conditions the most abundant isoform is SAA4, but during severe inflammation this protein increases only slightly with the major forms becoming SAA2 and particularly SAA1 which can increase 1000-fold up to 500 μg/ml. All forms of the protein are found associated with HDL to which it is reversibly bound (more than 95 percent in complexed form). This association with HDL has led to the idea that SAA function might be linked to that of HDL. When SAA associates with HDL other lipoproteins such as apolipoprotein A1 are displaced (Coetzee et al. 1986). Nevertheless, despite the demonstration that SAA has effects on lipid transport between HDL and various cells and effects on enzyme activities, no clear role has been demonstrated. Changes in lipid metabolism are relatively small and perhaps to be expected from any protein that displaces other proteins and becomes a major protein (up to 50 percent or more) in the total HDL protein in severe inflammation. In vivo cholesterol transport was not altered by SAA1 overexpressed through an adenoviral construct, although the constitutive form did alter lipoprotein profiles (Kindy et al. 2000).

An alternate hypothesis for SAA function is that it is involved in immune function as a chemotactic agent. Evidence for this has been gathered from experiments

that show movement of a variety of cells in response to lipid-free SAA (Xu et al. 1995). As further support for this, interaction with a G-protein-coupled receptor FPRL1 has been suggested (Su et al. 1999). Surprisingly HDL inhibits this function and, if this is a major function, it is unclear why hepatocytes would make so much SAA. An interaction with the RAGE receptor has also been suggested for the amyloidogenic form of SAA (Yan et al. 2000).

SAA3 is synthesized extrahepatically in a number of cells such as macrophages and adipose cells (Meek and Benditt 1986). It has recently been demonstrated that adipose cells synthesize SAA3 in response to hyperglycemia (Lin et al. 2001). In addition epithelial tissues were found to express SAA1, SAA2, and SAA4 under normal physiological states (Urieli-Shoval et al. 1998).

α_1-ACID GLYCOPROTEIN

Acid glycoprotein (AGP) (also called orosomucoid) is highly acidic with a p*I* of between 2.8 and 3.8 as a result of approximately 60 percent of its mass being made up of N-linked glycans (Fournier et al. 2000). It is a member of a class of protein called the lipocalins, which also includes α_1-microglobulin and retinol-binding protein. In plasma it is an abundant protein (see Table 1), which can increase up to fivefold from 0.3 to 0.6 mg/ml.

A large proportion of the research on this protein is related to its ability to bind and alter the bioavailability of a wide range of drugs. This is because a number of sites are available to interact with drugs of both basic and neutral lipophilic character and even acidic drugs. Some of the more important drugs bound are propranolol, verapamil, and quinine (Israili and Dayton 2001). The fact that AGP increases severalfold during an APR may thus alter the availability of drug and pharmacokinetics in the acute phase as compared with the normal dosage of drug (e.g. quinine). This ability to carry small molecules may be part of AGP's function and certainly AGP can be seen to be associated with steroid hormones such as progesterone. However, several other possible functions exist. The N-linked sugars themselves change during the APR becoming shorter, whereas in pregnancy they are found to be longer, perhaps reflecting the rate of synthesis (Raynes 1982). The glycosyl transferases responsible for full glycosylation of proteins are also induced by cytokines, but their induction takes place over a slow time course (Mackiewicz et al. 1987). This phenomenon is not restricted to AGP. A further change to the carbohydrate that depends on activation status of secreting cells is the presence of a sialyl Lewis X structure on a percentage of AGP glycans, although this too is not restricted to AGP (De Graaf et al. 1993). It is possible that this acts to inhibit selectin-mediated effects at the endothelium. An immunomodulating capacity has been reported; however, this is still contentious and, although AGP is reported to prevent TNFα or LPS septic shock under some circumstances, this is not always observed in different models. Other reported observations include an inhibition of lymphocyte proliferation and a selective alteration in permeability of endothelia.

One alternative possibility that is attractive and for which there is limited evidence is that it may act to bind and inactivate pathogens that would use such N-linked glycans to bind to endothelia or gain entry to cells, e.g. a nonspecific resistance to infections such as that with *Klebsiella pneumoniae* (Hochepied et al. 2000). This may be related to the property of certain pathogen adhesins to bind to N-linked sugars.

CERULOPLASMIN

This protein is named after its blue color as a result of the copper it contains. It requires the copper for function but is not involved in transport of this metal despite the fact that it contains the bulk of plasma copper. Rather it has ferroxidase activity and is involved in the release of iron from stores. This property came to light following the discovery of a neurodegenerative deficiency disease (aceruloplasminemia) (Yoshida et al. 1995; Hellman and Gitlin 2002).

Negative APPs

There is no obvious reason why a protein such as albumin should be reduced in the APR; nevertheless this process is a controlled one in which the synthesis rate is reduced. This active downregulation is through transcriptional and post-transcriptional control (Perlmutter et al. 1986). The most plausible reason for this is that, although there is an overall increase in protein synthesis rate by the hepatocyte during the APR, the required switch towards the upregulated proteins requires extra capacity which can be achieved only by downregulating synthesis of this major serum protein.

Transthyretin was previously called prealbumin and functions to transport retinol-binding protein and thyroxine. Transferrin transports iron but it is reduced in the acute phase, presumably to reduce availability of iron for microorganisms that usually have a greater affinity for the iron it carries. The transferrin receptor is responsible for uptake into those tissues that require the iron.

Liver protective responses

Following inflammatory stimulation the hepatocyte also starts to produce a number of other enzymes and proteins that have host-protective function. Inducible nitric oxide synthase (iNOS) was first identified in hepatocytes and has an ability to protect the hepatocyte against injury in severe sepsis. Raised plasma nitric

oxide is observed in a number of inflammatory conditions. Another enzyme produced in hepatocytes, as well as other cells such as endothelial cells, is heme oxygenase which has been demonstrated to have a protective role against stress from oxidative conditions and to reduce inflammation (Willis et al. 1996). This enzyme is responsible for reducing the effects of iron released from heme-containing enzymes (Ferris et al. 1999). Its main role may lie in protecting not only hepatocytes but also endothelial cells from injury.

Metallothionein is an intracellular low-molecular-weight protein that appears to have several functions, including protection against heavy metal and oxidant damage, or metabolism and control of zinc (Coyle et al. 2002). Two isoforms are induced in hepatocytes in response to IL-6, as well as other stimuli such as metals or drugs. An increase in zinc content in hepatocytes is mirrored by a hypozincemia.

Clinical use of APPs and inflammation markers

The most commonly used marker of APR has traditionally been the erythrocyte sedimentation rate (ESR), which is simple to perform but suffers from problems that affect results, such as anemia, and an increase with age. It has been considered that the major factor behind the ESR is the concentration of fibrinogen, which is slower to react than some of the faster APPs. Mainly for these reasons and the increase in sensitivity of assays, CRP is currently the best marker of severity of inflammation. There are, however, exceptions to the rule that CRP reflects inflammation in, for example, systemic lupus erythematosus (SLE) when CRP levels are not increased (Pepys et al. 1982). As a measure of inflammation, SAA may be slightly more sensitive for particular diseases (Malle and de Beer 1996) but is more difficult to measure for a number of technical reasons, including a difficulty in generating good antibodies that are specific for the acute phase form, and do not recognize the constitutive form. Certain cytokines have been reported to have levels that correlate with severity of inflammation but in general they suffer from problems of rapid fluctuation, with the result that even the better ones, such as IL-6, are unlikely to be used in preference to CRP. In the future other markers may be used that provide more information than the current nonspecific ones, e.g. procalcitonin may be useful because it reacts faster than CRP and is induced only by infection and not by other sterile inflammatory stimuli (Chirouze et al. 2002; Reinhart et al. 2002). Alternately, proteins specific for damage to particular organs or cells have been investigated, although to date little improvement on CRP has been found either for any specific inflammatory disease or to be specific to particular cell types or locations. Neonatal tissues also appear capable of an APR and APPs can be used as markers of infection (Dollner et al. 2001).

High-sensitivity CRP assays that measure higher levels within the normal range revealed that higher CRP was a risk factor for coronary heart disease. In addition, statin therapy, which reduces coronary events, also reduces CRP (Ridker et al. 2001). Following this a number of potential markers of vascular inflammation have been examined, e.g. sICAM-1, IL-6, TNFα, and soluble P-selectin, all of which indicate an increased vascular risk in a number of clinical settings (reviewed in Blake and Ridker 2001). The APR is largely unaffected by age although baseline levels of CRP increase slightly with age. CRP has been identified at lesion sites; however, other APPs such a fibrinogen are also associated with atherosclerosis. As CRP is raised in a number of subclinical conditions, the value of such low level CRP determinations may be more than currently appreciated.

The APR may have effects on other markers used to assess, for example, nutritional status. Vitamin A measurement in serum is also somewhat confused by the fact that the carrier transthyretin and retinol-binding protein are negative APPs and thus serum carriage of vitamin A may be reduced as a result. Transferrin receptor and ferritin may be used to measure iron deficiency but transferrin receptor may have the advantage that it is not subject to the confusion caused by potential APP responses of the ferritin.

HEMOPOIETIC RESPONSES

Lymphocytes

The APR includes increases in glucocorticoids and catecholamines, which have considerable effect on the thymus causing a large increase in apoptosis (Haeryfar and Berczi 2001). This may reflect the greater importance of diverting resources to the immediate and innate immune mechanisms rather than promoting long-term adaptive immune responses.

Polymorphonuclear cells

The leukocytosis that occurs in even comparatively mild inflammatory conditions results in an increased release of cells from the bone marrow and a consequent rise in the concentration in the circulation. As the movement from the bone marrow to the circulation under the control of cytokines, such as IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), and G-CSF and others, continues and severity of inflammation increases, less mature forms ($CD34^+$) can migrate into the circulation (Liu et al. 1997). The increase in numbers thus outweighs the disappearance of these cells into the site of inflammation or increased apoptosis rates. This

response provides rapid supplementation of cells, the function of which is to kill microorganisms. The cytokine control of release of cell type is best seen in asthma or helminth infections. IL-6-deficient mice have normal circulating neutrophil numbers but do not show leukocytosis to *Listeria monocytogenes* and are thus more susceptible to this infection (Dalrymple et al. 1995).

Other cells

Although there are no initial changes in red cell numbers after a prolonged inflammatory response resulting from autoimmune stimuli, chronic infection, or cancer, a reduced number of erythrocytes are seen in circulation which is often called the anemia of chronic disease. This results from a combination of factors such as reduced iron, removal of cells by phagocytosis, reduction in synthesis rate in bone marrow, and reduced erythropoietin, several of which are influenced by proinflammatory cytokines (Bron et al. 2001).

Circulating monocytic cells are increased during the first 24 h and this increase comes from an increased rate of release from the bone marrow, which cannot be caused by the stores of cells because there are limited supplies, but rather result from an increased cell cycle turnover. This is controlled by M-CSF as has been seen in phase I trials of M-CSF (Weiner et al. 1994).

Platelets are also increased during an inflammatory state and thrombocytosis correlates with IL-6. Thombocytosis is controlled by thrombopoietin (TPO), which is removed by platelets themselves. IL-6 does not appear directly to regulate platelet counts but rather induces TPO because IL-6 induction of platelet increases can be prevented by neutralization of TPO (Kaser et al. 2001). TPO may thus be considered an APP (Cerutti et al. 1999).

METABOLIC CHANGES

Lipid metabolism

The lipoprotein changes that occur in different species can vary, so the following references are all to humans except where indicated. One of the major changes is the hypertriglyceridemia, largely because of the increase in very-low-density lipoprotein (VLDL) through reduced clearance or increased production (Feingold et al. 1992). Driven by cytokines such as TNFα, IL-1, and IL-6, the hepatocyte increases synthesis of fatty acid which is esterified and incorporated into VLDL. At high concentrations, LPS from bacterial infection has the ability to inhibit lipoprotein lipase, an enzyme responsible for clearance of triglyceride-rich VLDL (Feingold et al. 1992). There are other changes in the VLDL during inflammation, such as an increased sphingolipid content, which also reduce clearance (Krauss et al. 1990). At the same time there is a decreased hepatic lipase and tissue expression of apolipoprotein E.

HDL and LDL are decreased during an APR whereas VLDL levels are increased. The HDL apolipoproteins apo-AI and apo-AII are both negative APPs and HDL content is decreased and replaced with other proteins such as SAA and apo-J. The role of apo-J is not known. The HDL-cholesterol is thus also reduced. The HDL that circulates is depleted in cholesterol ester but has higher free cholesterol, triglyceride, and sphingolipid (Auerbach and Parks 1989).

During an APR the anti-inflammatory and antioxidative properties of HDL are reduced. The reason for this is not entirely clear but a possible explanation is that it may be related to a decrease in content of certain enzymes such as paraoxanase or PAF acetylhydrolase (Feingold et al. 1998). Equally it may be that HDL takes up a number of inflammatory lipids or oxidized lipids released into the circulation. The cholesterol ester and total cholesterol content of acute phase HDL is lower than normal, although the reason is uncertain and the free cholesterol, triglyceride, and sphingolipid are increased.

Protein metabolism

The most obvious change that occurs dependent on proinflammatory cytokine action is muscle protein degradation, although a decreased rate of both myofibrillar and sarcoplasmic protein synthesis also contributes. There is a negative nitrogen balance that occurs in systemic inflammation which results in body weight loss. The cytokines responsible for this weight loss are thought to be TNFα, IL-1, IL-6, etc. The degradation of muscle protein provides amino acids and the hepatocyte increases amino acid uptake in order to cope with the increased protein synthesis and secretion (reviewed in Fischer et al. 1995). The synthesis of such an array of APPs is thought to give rise to a deficiency in sulfur-containing amino acids which are in particular demand (Grimble and Grimble 1998). Nevertheless, as previously indicated by the fact that the APPs are still synthesized in protein malnutrition states, APP synthesis is largely preserved. Rather the lack of these amino acids tends to affect glutathione synthesis to a greater extent (Grimble and Grimble 1998). Another amino acid of particular importance during inflammation is glutamine, which is essential for immune function in critically ill individuals (Andrews and Griffiths 2002).

Micronutrient changes

There are several levels of interaction between micronutrients and inflammation because the inflammation itself can reduce availability of micronutrients, and in

the reverse direction micronutrient deficiency may alter the severity of inflammation and the ability to generate inflammatory mediators. It is perhaps also necessary to say that assessment of micronutrient status is frequently based on blood analysis and this, as has already been shown, is heavily dependent on carrier proteins in the serum. An example of this is vitamin A which is reduced in serum during inflammation because of reduced levels of carrier protein; this must be taken into account when assessing vitamin status. The deficiency of vitamin A does, however, also lead to a more inflammatory response (Wiedermann et al. 1996) – an effect that may be the result of an alteration of cytokines and expansion of myeloid cells (Kuwata et al. 2000).

Other micronutrients affected by inflammation include the levels of zinc, iron, and copper which are all decreased by inflammation. Again many of these reductions may not reflect whole body levels but are likely to reflect available levels for peripheral or immune cells. The decrease is related to the increased production of metallothionein in the liver (Cousins and Leinart 1988). The effect of zinc depletion may have adverse effects on the immune system and may relate to a switch to a more Th2-like phenotypic response to infection (Prasad 2000). Selenium has recently been shown to be important for effective responses, particularly to viral illness (Beck and Levander 2000), and is also reduced during acute inflammation (Maehira et al. 2002); this may also be the reason for reduced glutathione through alteration in selenium availability for selenium-containing antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase. Selenium deficiency appears to reduce the synthesis of a set of hepatocyte-produced proteins in rats (Fischer et al. 2001).

HORMONAL CHANGES

In inflammatory conditions, the major endocrine changes are increased plasma concentration of glucagon, glucocorticoid, and catecholamine. One of the major changes that occurs is stimulation of the hypothalamic, pituitary, and adrenal glands in an ordered manner. This occurs after cytokine stimulation and involves alteration in certain hormones and leads to the induction of the controlling glucocorticoids such as prednisolone or cortisol (see Figure 4, p. 6). Loss of mediators such as corticotropin-releasing hormone reduces but does not prevent responses (Jacobson et al. 2000). Cytokines also induce the production of corticotropin-releasing factor (CRF). This has recently been found to be important not just for its ability to generate ACTH in the pituitary and then in turn corticosteroid in the adrenal cortex, but also because it can act on immune cells and has an anti-inflammatory action on a number of immune cells that express one of the receptors for CRF (Casadevall et al. 1999). The changes in such hormones can stimulate the metabolic changes seen in infection or inflammation (Bessey et al. 1984). Although the glucocorticoids increase in the acute phase there is a reduced production of mineralocorticoids and androgens. Triiodothyronine (T3) and thyroxine (T4) are reduced during the acute phase (Woloski and Jamieson 1987). Sex steroids are also changed, with some increased and some decreased.

Immunostimulatory hormones such as prolactin, growth hormone (GH), and insulin-like growth factor 1 (IGF-1) are suppressed. The reduction in the concentrations of GH and IGF-1 or the reduction in the ability of cells to respond may be part of the reason for the protein imbalance that leads to catabolism of muscle and synthesis of APPs.

Arginine vasopressin (AVP) is stimulated by IL-6 and this causes hyponatremia by increasing water reabsorption. AVP is also synthesized within the hypothalamus in response to cytokines and acts in a synergistic way with CRF (Chikanza et al. 2000). Prolactin is synthesized in the pituitary, and both prolactin and AVP have generally proinflammatory effects (Chikanza et al. 2000). CRF also induces α-melanocyte-stimulating hormone (αMSH) in the pituitary, which is an anti-inflammatory hormone acting on several tissues.

Procalcitonin is a 13-kDa precursor of the hormone calcitonin. The gene *calc-1* gives rise to an intracellular protein that can be secreted. Following infection-induced inflammation it becomes elevated about 4 h after endotoxin administration and is back to normal after about 24 h; as previously indicated under Clinical use of APPs and inflammation markers, it may have value in that its induction appears to be specific to infectious inflammation. Under normal conditions, expression is limited to the thyroid but in sepsis all tissues and particularly parenchymal cells produce it (Muller et al. 2001a), although the reason for infection specificity is not clear. Its function is also somewhat unclear, with some reports suggesting that it may have cytokine/chemokine-like properties and others hormonal properties. It appears to reduce survival in models of systemic infection (Nylen et al. 1998).

Leptin is a hormonal protein that is synthesized in the adipocyte which at least in some circumstances appears to behave as an APP. The 16-kDa protein is similar in structure to the IL-6 family (Fantuzzi and Faggioni 2000). The leptin receptor present on target cells also has similarity to IL-6 receptors because it is a gp130-containing complex; despite this leptin has not been shown to have any ability to induce hepatic APP synthesis. The role that led to the gene's discovery in obese mice is one of lipid metabolism control. This relates to the fact that larger fat stores produce more leptin, which then regulate appetite and energy expenditure through specific areas of the brain. Conversely, when leptin is low hunger is stimulated, and this may relate to the fact that hunger is depressed during inflammatory episodes when leptin is stimulated (Faggioni et al. 2001).

However, this is not the only function because the obese leptin-deficient mice have several other defects. It has been demonstrated that immune responses are also inhibited in the absence of leptin such as reduced phagocytosis by macrophages, which leads to a impaired host defense to gram-negative pneumonia (Mancuso et al. 2002).

FEVER AND OTHER BEHAVIORAL CHANGES

The process of fever is thought to be beneficial because overall it is more harmful to the microorganism than the host and may put additional stress on the microorganism inducing, for example, heat shock proteins (Hasday et al. 2000). Some animal models support an increased resistance at higher temperature. An alternate concept is that it favors mammalian membrane function.

It is reasonable to assume that somnolence will reduce energy expenditure to allow redirection of resources to fight infection and for tissue repair. It is possible that similar arguments might also account for anorexia, because a fit host would have sufficient protein resource to survive a sufficient time to control infection.

CONTROL OF SYSTEMIC INFLAMMATION

Current new therapies designed to inhibit inflammation are often intended to have an effect at local level to control damage done to specific organs or structures as, for example, in rheumatoid arthritis. In part this research is driven by the fact that the current most powerful anti-inflammatory drugs are steroids, which have considerable metabolic side effects. It is also clear, however, that these treatments also reduce the inflammation systemically. The range of different approaches to inhibit inflammation has also increased. Recently the use of soluble TNF receptor or humanized monoclonal antibody to TNFα has been shown to be an effective treatment for rheumatoid arthritis, Crohn's disease, and psoriasis (Elliott et al. 1994; Moreland et al. 2001). Currently inhibitors of intracellular signaling pathways such as the MAP kinase pathway are in trial. All of these approaches can reduce the APR including the APPs, leukocyte activation and migration, fever, etc. Not all these approaches will have exactly the same inhibitory effect but, with the reduction in inflammation, there may also come a risk that removal of some of these responses may have deleterious effects. At least at some level all these effects are likely to be beneficial to host function, particularly in terms of response to infection; to remove them may therefore increase risks of infection, although at least for TNFα blockade this appears to be a rare phenomenon.

The statins introduced to reduce cholesterol in atherosclerosis have been found to have an ability not only to reduce LDL cholesterol but also to reduce CRP and other APPs such as fibrinogen and SAA (Staels et al. 1998; Ridker et al. 2001). This control may be exerted through the peroxisome proliferator receptor α (PPARα), which not only alters lipid metabolism but has an anti-inflammatory role. It is possible that such effects are through transcription factors that induce CRP (Kleeman et al. 2003). In any event such treatment may be useful in other diseases that have an inflammatory component such as Alzheimer's disease, diabetes mellitus, or osteoporosis.

DISORDERS OF THE ACUTE PHASE RESPONSE

AA Amyloidosis

Serum amyloid A can, when present in high amounts for sufficient length of time (as, for example, in chronic diseases such as Crohn's disease or rheumatoid arthritis), give rise to deposits of a structurally altered AA amyloid protein that is resistant to degradation. This is thus one of the many forms of the amyloidosis diseases in which stable protein fibrils are deposited in vital organs. Although many of these amyloidoses can resolve they are capable, for example in AA amyloid, of destroying renal function and causing death if the balance remains in favor of deposition. These proteins are linked by the ability to switch conformation towards a β-pleated sheet-rich form that has resistance to degradation. In the case of SAA this is often accompanied by a cleavage of approximately 30 amino acids from the C-terminal end of the protein. Techniques to allow visualization of deposition of amyloid has demonstrated that AA amyloid deposits can regress (Hawkins et al. 1993) and more recently that depletion of the SAP component has the potential to be therapeutically useful (Pepys et al. 2002)

APP deficiences

As previously mentioned, deficiency in some anti-proteases leads to disease (see α_1-Antitrypsin or α_1-protease inhibitor). So far no patient with a deficiency of certain proteins such as CRP has been reported. However, the number of reports of deficiencies of particular APPs is increasing and, together with information from knock-out mice, the role of some of the proteins is in many cases not only confirming previous ideas but also providing some unexpected new observations.

Osteoporosis

Associated with increased proinflammatory cytokine levels, the balance of bone resorption and synthesis moves towards resorption. There is thus a tendency

towards osteoporosis. A number of proinflammatory cytokines and some newly discovered members of the TNF family regulate this process (Rodan and Martin 2000).

Systemic inflammation and shock

The ultimate effect of an uncontrolled APR is shock (see Shock/sepsis). Associated with such severe systemic inflammation, a further complication that may arise is acute respiratory distress syndrome. Sufficiently severe inflammation can also have the effect of apparently reducing immune responses (Lederer et al. 1999; Faunce et al. 2003). Lung function is compromised and so far one of the more promising ways of treating this is the use of neutrophil elastase inhibitors – a number of other potential treatments such as PAF antagonists having failed to have significant effect in clinical trials (Eaton and Martin 2002). There is considerable interest in some proteins that behave as APPs which seem to amplify the systemic inflammation. One such is high mobility group 1 protein (HMGB1) which can activate cells when released from necrotic cells. It is normally a nuclear protein which when released can behave as a cytokine (Andersson et al. 2002; Scaffidi et al. 2002). It is possible that this and other late mediators of inflammation might be more effective targets.

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Chemokines

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Chemokine is a short form for chemotactic cytokine. The term was coined at a meeting in Baden near Vienna after the International Immunology Congress of Budapest (Lindley et al. 1993). Only few chemokines were known at that time and receptors for interleukin (IL)-8 had just been cloned and shown to belong to the family of G-protein-coupled receptors (GPCR) (Holmes et al. 1991; Murphy and Tiffany 1991). The importance of the new class of cytokines and their function as chemoattractants was already obvious, but no one in the field had anticipated the enormous and rapid development of research that led to a new understanding of leukocyte traffic (Baggiolini 1998; Loetscher et al. 2000; Sallusto et al. 2000; Gerard and Rollins 2001; Mackay 2001; Moser and Loetscher 2001; Murphy 2001; Thelen 2001). Chemokines are produced by leukocytes and tissue cells either constitutively or after induction, and act locally in a paracrine and possibly autocrine fashion on cells that express the appropriate GPCR. Chemotaxis is their main common effect.

A systematic nomenclature for chemokines was established recently (Zlotnik and Yoshie 2000). It is based on the principle adopted for chemokine receptors, which are defined as CXC, CC, XC, and CX_3C followed by R and a number. Chemokines are defined by the same structure-related acronyms followed by L (for ligand) and their gene number. Chemokine genes were originally designated as *SCY* (for small, secreted cytokines) and numbered chronologically. The systematic nomenclature is particularly useful for chemokines that were discovered in recent years, which have often been given more than one name. For IL-8, the monocyte chemoattractant proteins (MCP), RANTES, the macrophage infectivity potentiators (MIP), and other chemokines of the early days, however, the traditional names are still in current use (Tables 1 and 2 and Figure 1).

CHEMOKINES AND THEIR STRUCTURES

Chemokines are small soluble proteins with four conserved cysteines and molecular weights in the range 7–15 kDa (Baggiolini et al. 1994, 1997). They are synthesized with a leader sequence of 20–25 amino acids, which is cleaved off before release. Two main subfamilies, CXC and CC chemokines, are distinguished according to the position of the first two cysteines, which are adjacent (CC) or separated by one amino acid (CXC). The cysteines form two disulfide bonds linking Cys1 to Cys3 and Cys2 to Cys4, which keep the chemokines in a stable three-dimensional conformation that is required for receptor binding and activation. This structure has been resolved by nuclear magnetic resonance (NMR) in solution and X-ray crystallography. Chemokines have a prominent core made of anti-parallel β strands and the connecting loops, a short, conformationally disordered amino-terminal region (3–10 amino acids) and a carboxy-terminal α helix which usually consists of 20–30 amino acids, but can exceed 50. The NMR structure of stromal cell-derived factor 1 (SDF-1) (CXCL12) is presented as an example in Figure 2.

In concentrated solution and on crystallization several chemokines form dimers (Clore et al. 1990; Baldwin

Table 1 *Human CXC chemokines and their receptors*

Systematic and original names		Gene locus	Receptor
CXCL1	GRO	4q21.1	CXCR2
CXCL2	GROβ	4q21.1	CXCR2
CXCL3	GROγ	4q21.1	CXCR2
CXCL4	PF4	4q21.1	Unknown
CXCL5	ENA-78	4q21.1	CXCR2
CXCL6	GCP-2	4q21.1	CXCR1, CXCR2
CXCL7	NAP-2	4q21.1	CXCR2
CXCL8	IL-8	4q21.1	CXCR1, CXCR2
CXCL9	Mig	4q21.1	CXCR3
CXCL10	IP10	4q21.1	CXCR3
CXCL11	I-TAC	4q21.1	CXCR3
CXCL12	SDF-1α/β	10q11.21	CXCR4
CXCL13	BCA-1	5q31.1	CXCR5
CXCL14	BRAK	Unknown	Unknown
CXCL16		Unknown	CXCR6

For abbreviations see the text.

et al. 1991). The mode of aggregation is strikingly different for CXC and CC chemokines, as illustrated in Figure 3 for IL-8 (CXCL8) and MIP-1α (CCL3). Studies of IL-8 (CXCL8), GROα (CXCL1), and NAP-2 (CXCL7) show that CXC chemokines associate along their first β strands via hydrogen bonds and amino acid side chain interactions (Baggiolini et al. 1997), whereas CC chemokines, e.g. MIP-1β (CCL4) (Lodi et al. 1994) and RANTES (CCL5) (Chung et al. 1995; Skelton et al. 1995) dimerize by interacting with their amino-terminal regions. It is believed that the distribution of hydrophobic areas on the surface, which is different for CXC

Table 2 *Human CC chemokines and their receptors*

Systematic and original names		Gene locus	Receptor
CCL1	I-309	17q11.2	CCR8
CCL2	MCP-1	17q11.2	CCR2
CCL3	MIP-1α, LD78	17q12	CCR1, CCR5
CCL3L1	LD78β	17q12	CCR1, CCR5
CCL4	MIP-1β	17q12	CCR5
CCL5	RANTES	17q12	CCR1, CCR3, CCR5
CCL7	MCP-3	17q11.2	CCR1, CCR2, CCR3
CCL8	MCP-2	17q11.2	CCR1, CCR2, CCR3, CCR5
CCL11	Eotaxin-1	17q11.2	CCR3
CCL13	MCP-4	17q11.2	CCR1, CCR2, CCR3
CCL14	HCC-1	17q12	CCR1
CCL15	HCC-2, Lkn-1, MIP-1δ	17q12	CCR1, CCR3
CCL16	HCC-4, LEC	17q12	CCR1
CCL17	TARC	16q13	CCR4
CCL18	PARC, DC-CK1, AMAC1	17q12	Unknown
CCL19	ELC, MIP-3β, exodus-3	9p13.3	CCR7
CCL20	LARC, MIP-3α, exodus-1	2q36.3	CCR6
CCL21	SLC, 6Ckine, exodus-2	9p13.3	CCR7
CCL22	MDC, STCP-1	16q13	CCR4
CCL23	MPIF-1, CKβ8	17q12	CCR1
CCL24	Eotaxin-2, MPIF-2	7q11.23	CCR3
CCL25	TECK	9p13.3	CCR9
CCL26	Eotaxin-3	7q11.23	CCR3
CCL27	CTACK, ILC	9p13.3	CCR10
CCL28	MEC	5p12	CCR3, CCR10

For abbreviations see the text.

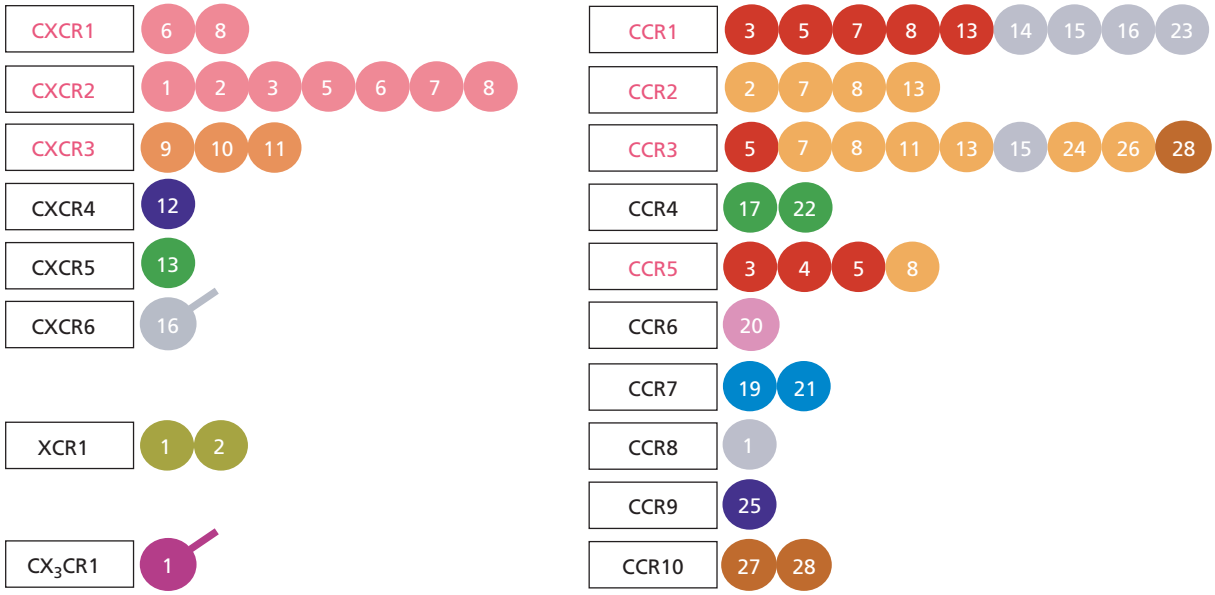

Figure 1 *Human chemokines and chemokine receptors: chemokines including the lymphotactins are presented as circles. Circles with a bar represent membrane-anchored chemokines. The numbers within the circles correspond to the chemokine numbers in the systematic nomenclature (see Tables 1 and 2). The colors of the circles indicate chemokines with structural similarities. Receptors for inflammatory chemokines are presented in red.*

and CC chemokines, determines the mode of dimer formation (Covell et al. 1994; Clore and Gronenborn 1995). The distribution of the inner hydrophobicity clusters, by contrast, is similar for chemokines of both subfamilies, explaining the common mode of folding of monomers.

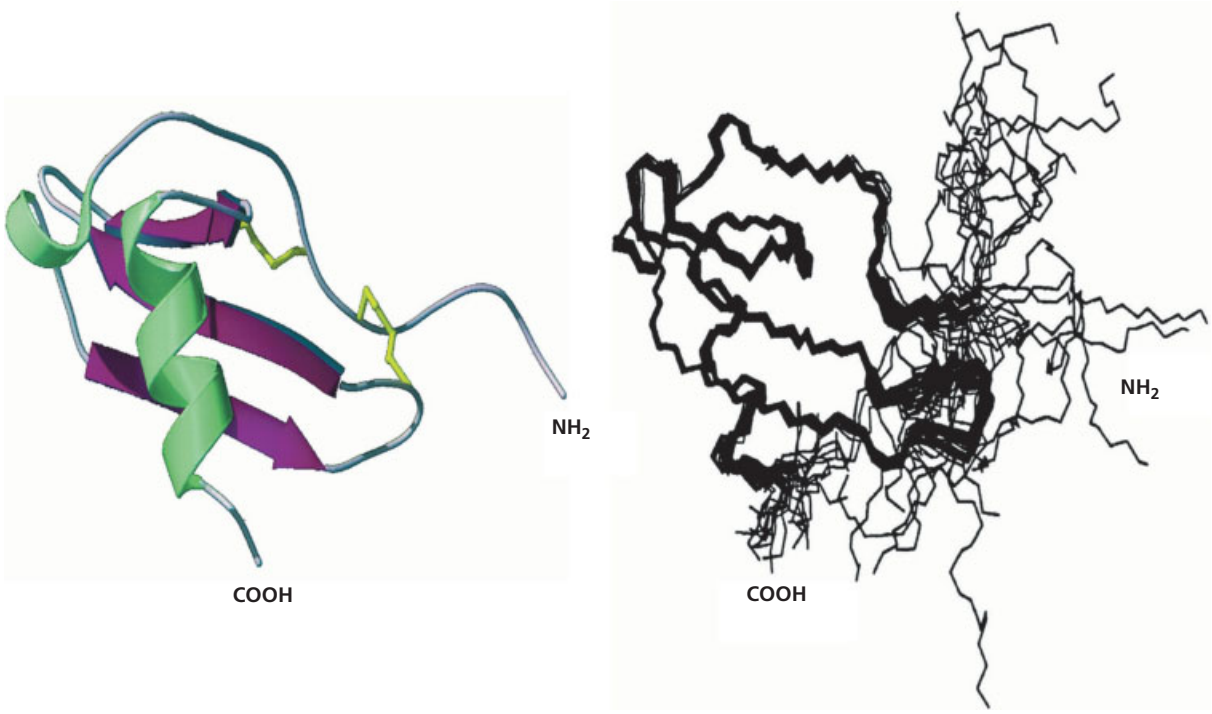

Figure 2 *Three-dimensional structure of the CXC chemokine SDF-1 (CXCL12) as obtained by NMR spectroscopy in solution. The model on the left shows the core consisting of anti-parallel β strands and the connecting loops, which is kept together by the disulfide bonds (in yellow), and presents an idealized view of the amino-terminal sequence and the carboxyl-terminal α helix. On the right, 30 single NMR views are projected on to each other. This presentation highlights the rigidity of the core and the structural disorder of the terminal regions. (Reproduced with permission from Crump et al. (1997).)*

Figure 3 *Dimer formation by CXC and CC chemokines as illustrated by IL-8 (CXCL8) and MIP-1α (CCL3). CXC chemokines associate along their first β strands (Gerber et al. 2000), whereas CC chemokines associate by interacting with their amino-terminal regions (Czaplewski et al. 1999). The mode of dimer formation is believed to reflect the different distribution of surface hydrophobicity areas in CXC and CC chemokines. The monomers of both subfamilies, by contrast, fold in the same manner because the distribution of the internal hydrophobicity clusters is similar.*

Aggregation was originally considered an important property and it was suggested that chemokines, like some cytokines, act as dimers (Baggiolini et al. 1997). It was eventually realized, however, that the biological activities of chemokines are observed at nanomolar concentrations, which are too low for stable aggregation to dimers (Burrows et al. 1994; Clark-Lewis et al. 1995). To demonstrate that IL-8 (CXCL8) can function as a monomer, an analogue of the 72 amino acid form was synthesized with *N*-methyl-leucine instead of leucine at position 25 to disturb hydrogen bonding and prevent dimerization. Although the analog remained monomeric, it was as active as wild-type IL-8 (CXCL8) (Rajarathnam et al. 1994). Similar results were obtained with monomeric forms of MIP-1α (CCL3) (Graham et al. 1994) and it was shown that some chemokines, MCP-3 (CCL7) for instance, do not dimerize (Kim et al. 1996). As monomeric chemokines have full biological activity and aggregation occurs at concentrations that are much higher than those required for a chemotactic response, there is little doubt today that the biologically relevant conformation of chemokines is the monomer (Clark-Lewis et al. 1995).

Two variants of the chemokine structure paradigm have been described: lymphotactin (XCL1, XCL2) with two instead of four conserved cysteines (Kennedy et al. 1995) and fractalkine or neurotactin (CX_3CL1), a membrane-bound mucin bearing an amino-terminal chemokine-like domain with three amino acids between the first two cysteines (CX_3C motif) (Bazan et al. 1997; Pan et al. 1997).

RECEPTOR BINDING AND ACTIVATION

The receptor for chemotactic *N*-formyl-methionyl peptides was cloned more than a decade ago (Boulay et al. 1990) and found to have a heptahelical structure and to couple to heterotrimeric GTP-binding proteins. The cloning of the first chemokine receptors, two heptahelical proteins with high affinity for IL-8 (CXCL8), followed soon thereafter (Holmes et al. 1991; Murphy and Tiffany 1991). Meanwhile six human receptors for CXC chemokines and ten for CC chemokines, in addition to the receptors for lymphotactin and fractalkine, have been characterized (Murphy et al. 2000; Murphy 2002). The study of receptor selectivity and expression in different types of leukocytes has provided considerable insight into the mechanism for the specific recruitment of different types of leukocytes and the regulation of leukocyte traffic in health and disease. Blood phagocytes, for instance, express different types and combinations of chemokine receptors. CXCR1 and CXCR2 are found exclusively in neutrophils, the front-line cells for antibacterial defense. Eosinophil and basophil granulocytes as well as monocytes share CCR1, but can be recruited selectively by chemokines acting on CCR3, for eosinophils and basophils, or CCR5 for monocytes (Figure 4).

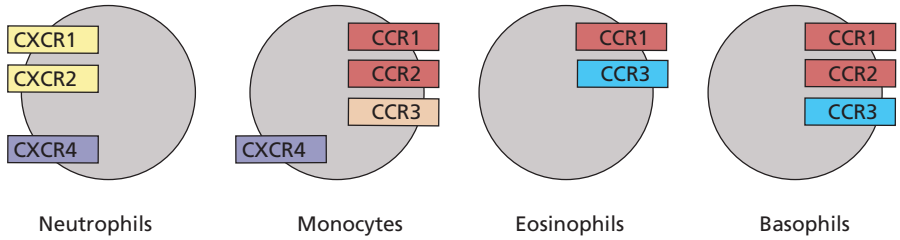

Figure 4 *Chemokine receptor expression in human phagocytic leukocytes.*

The search for sequence domains that are involved in receptor recognition and activation began after the characterization of the receptors for IL-8 (CXCL8) and related chemokines. These studies revealed the importance of the amino-terminal region and showed that a sequence of three residues, Glu-Leu-Arg, the so-called ELR motif, immediately preceding the first cysteine, is essential for IL-8 (CXCL8) activity (Clark-Lewis et al. 1991, 1993; Hébert et al. 1991). The ELR motif is conserved in all IL-8 (CXCL8)-related chemokines, the growth-related oncogene (GRO) proteins (CXCL1–3), ENA-78 (CXCL5), granulocyte chemoattractant protein 2 (GCP-2) (CXCL6), and neutrophil-activating protein 2 (NAP-2) (CXCL7), and is necessary for activation of CXCR1 and CXCR2 (Baggiolini et al. 1994, 1997). Neither receptor, however, responds to linear or cyclic oligopeptides enclosing the Glu-Leu-Arg-Cys-X-Cys sequence or to unrelated chemokines such as MCP-1 (CCL2) and interferon-inducible protein 10 (IP10) (CXCL10) that were modified by insertion of an ELR-containing amino-terminal sequence (Clark-Lewis et al. 1993). These observations indicated that other recognition sites are required. Using mutagenesis or chemical synthesis of analogues of the 72-residue form of IL-8 (CXCL8), such sites were identified in the loop region following the second cysteine (residues 10–17) and in the sequence immediately preceding the third cysteine (Clark-Lewis et al. 1994, 1995). Discrete differences between IL-8 (CXCL8) and its analogs, e.g. NAP-2 (CXCL7) and GROα (CXCL1) (Kim et al. 1994), determine the binding selectivity for CXCR1 and CXCR2.

Similar studies performed with several other CC and CXC chemokines confirmed the importance of the amino-terminal region for receptor activation (Loetscher and Clark-Lewis 2001; Fernandez and Lolis 2002). In most cases, however, receptor-triggering motifs could not be unequivocally identified. MCP-1, for instance, requires the entire sequence of 10 amino acids preceding the first cysteine for full activity on CCR2. Potency drops considerably after truncation or elongation of the amino-terminal sequence, but the amino-terminal residue can be replaced by several amino acids without affecting activity (Clark-Lewis et al. 1995). MCP-1, MCP-2, MCP-3, and MCP-4 (CCL2, CCL8, CCL7, and CCL13), which are all potent agonists for CCR2, share the amino-terminal Gln-Pro motif and have other similarities within the first 10 residues. Despite these analogies, the structure–activity relationships are far from clear. In contrast to MCP-1 (CCL2), all three analogs act on additional receptors, CCR3 in particular. On the other hand, MCP-1 (CCL2) itself becomes a high-affinity ligand for CCR3 after deletion of glutamine at the amino terminus, which causes a 50-fold drop in affinity for CCR2 (Weber et al. 1996).

Of particular interest are the structure–activity relations studies on SDF-1 (CXCL12) (Crump et al. 1997; P. Loetscher et al. 1998a), the exclusive ligand for CXCR4, a receptor that is widely expressed in leukocytes and tissue cells and functions as a co-receptor for HIV (Murphy et al. 2000). The first amino-terminal residues of SDF-1 (CXCL12), Lys1-Pro2, are essential for receptor triggering: deletion of Lys1 dramatically decreases activity and the deletion of both amino acids fully inactivates the chemokine. As for IL-8 (CXCL8), some residues in the loop region of SDF-1 (CXCL12) immediately after the second cysteine (residues 12–17, RFFESH motif) are required for receptor binding (Crump et al. 1997). The importance of the amino-terminal domain of SDF-1 is underscored by an astonishing observation: GROα (CXCL1), a selective ligand of CXCR2, and IP10 (CXCL10), a selective ligand of CXCR3, can be converted into agonists for CXCR4 by substituting their amino-terminal domain with the first 17 residues of SDF-1 (CXCL12). Interestingly, CXCR4 triggering was also obtained with an oligopeptide consisting only of the first 17 residues of SDF-1 (P. Loetscher et al. 1998a), which folds in a similar way to the wild-type chemokine (Elisseeva et al. 2000).

All studies of structure–activity relationships indicate the importance of the amino-terminal domain of CXC and CC chemokines for receptor recognition and activation (Clark-Lewis et al. 1995; Loetscher and Clark-Lewis 2001). Two separate or partly overlapping sites of interaction with the receptors can be distinguished, one in the amino-terminal sequence preceding the first cysteine and the other within the exposed, conformationally rigid loop after the second cysteine (N loop). Both sites are small (consisting of few amino acids) and are kept in close proximity by the disulfide bonds (see Figure 2).
Clark-Lewis and colleagues (Crump et al. 1997) have proposed that the receptor recognizes first the binding site within the loop region, which functions as a *docking*

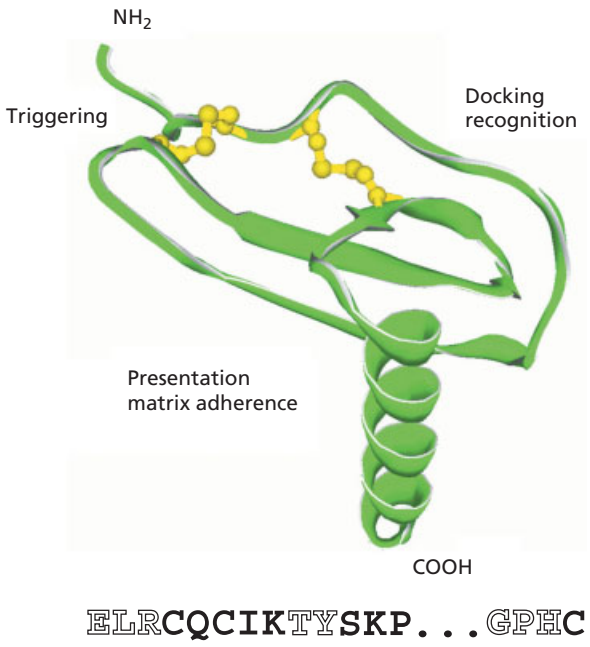

Figure 5 *Chemokine-binding domains: chemokines interact with their receptors by means of two discrete, contiguous, or slightly overlapping domains. The receptor is recognized via a binding site within the loop region, which appears to function as a* docking domain*. This interaction restricts the mobility of the chemokine and presumably facilitates the proper orientation of the amino-terminal* triggering domain *which activates the receptor. Chemokines tend to bind to glycosaminoglycans on the surface of cells or in the extracellular matrix via basic residues of the core and the carboxy-terminal region. Bound chemokines retain their full chemotactic activity.*

domain. This interaction restricts the mobility of the chemokine and presumably facilitates the proper orientation of the amino-terminal *triggering domain* that activates the receptor (Figure 5).

INTRACELLULAR SIGNALING

Chemokine signaling was first studied in human neutrophils stimulated with IL-8 (CXCL8). It was found that all functional responses were blocked when the cells were pre-treated with *Bordetella pertussis* toxin, indicating that the receptor couples to GTP-binding proteins of the Gi type (Thelen et al. 1988), which eventually turned out to be the rule for all chemokine receptors (Murphy et al. 2000). When a chemokine binds to its receptor, heterotrimeric Gi proteins are activated: GDP, which is bound to the inactive trimeric complex, is exchanged for GTP, and the complex dissociates into its α subunit bound to GTP and the $\beta\gamma$ subunit. The release of the $\beta\gamma$ subunit is essential for the stimulation of chemotaxis (Neptune and Bourne 1997), which involves the activation of the rho GTPases Rac and CDC42, the Wiscott–Aldrich syndrome protein (WASP), and the Arp2/3 complex, and leads to actin polymerization (Cory and Ridley 2002). In addition the $\beta\gamma$ subunit activates two major signal transduction enzymes: a phospholipase C ($PLC\beta_2$ and-β_3) which is specific for phosphatidylinositol, and a phosphatidylinositol-3-hydroxyl kinase ($PI3K\gamma$). The phospholipase cleaves phosphatidylinositol 4,5-bisphosphate, yielding two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglygerol (DAG). IP_3 induces the release of Ca^{2+} from intracellular stores, leading to a characteristic, transient rise of the free calcium concentration, and DAG activates several isoforms of protein kinase C (PKC). The IP_3 kinase generates phosphatidylinositol 3,4,5-trisphosphate and initiates the activation of another kinase, PKB. There is evidence that the $\beta\gamma$ subunit of the Gi protein may activate other signaling kinases, such as the MAP kinase, but the mechanism and the role of this step are still unclear (Thelen 2001). The signaling that is initiated with the exchange of GTP for GDP and the consequent dissociation of the α and $\beta\gamma$ subunits is terminated by the hydrolysis of GTP to GDP and the re-association of the $\beta\gamma$ subunit with the GDP-bound α subunit. Newer findings suggest that the α subunit is more than a binding moiety that regulates the activity of the $\beta\gamma$ subunit, and signals on its own by activating tyrosine kinases (Thelen 2001).

The signal transduction events induced by IL-8 (CXCL8) appear to correspond to those observed after stimulation with the classic chemotactic agonists such as fMet-Leu-Phe (formyl-methionine-leucine-phenylalanine) and C5a (Baggiolini and Kernen 1992). Using Jurkat cells stably transfected with CXCR1 or CXCR2 copy DNA (cDNA), it was shown that both receptors respond equally well to IL-8 (CXCL8), as judged by the activation of p42/p44 MAP kinase (Jones et al. 1995), Ca^{2+} mobilization and chemotaxis (Loetscher et al. 1994a), and that the two IL-8 receptors function independently of each other. By blocking one or the other receptor in neutrophils using monoclonal antibodies it was found that phospholipase D activation and the respiratory burst are mediated by CXCR1 only (Jones et al. 1996). In agreement with these observations, it was reported that phospholipase D is activated by stimulation with IL-8 (CXCL8), but not with GROα (CXCL1) or NAP-2 (CXCL7) (L'Heureux et al. 1995).

In contrast to other chemokines, which induce short-lived responses, SDF-1 (CXCL12) was found to mediate the prolonged activation of protein kinase B and the MAP kinases ERK1/2 via CXCR4 (Tilton et al. 1997). The sustained effect on intracellular signaling reflects the continuous interaction of CXCR4 with its ligand. Such activity may be required for the retention pre-B cells at their sites of maturation within the bone marrow (Ma et al. 1999). Prolonged activation of protein kinase B was shown to protect cells from apoptosis (Marte and Downward 1997). Prolongation of cell survival is an as yet unexplored effect mediated by CXCR4, which is widely expressed in leukocytes and nonhemopoietic cells.

It was recently reported that after stimulation chemokine receptors form homo- or heterodimers (Mellado et al. 2001). Up to now, this mechanism has not received general acceptance and its role in the initiation of chemokine responses has been questioned (Thelen and Baggiolini 2001). The existence of GPCR dimers is documented by studies showing that aggregation may pre-exist or occurs slowly after stimulation (Bouvier 2001; Pierce et al. 2002), suggesting that dimerization is too slow as an initiating event.

ASSESSING CHEMOKINE ACTIVITIES

The most common test for chemokine activity is chemotaxis in a two-well chamber where leukocytes, which are placed in the upper well, migrate through a porous polycarbonate membrane in response to chemokines supplied in the lower well. The cells sense chemokine molecules that diffuse across the membrane and move toward higher concentrations of the attractant. The pores are always smaller than the migrating cells, and their diameter is chosen depending on the properties of the cells, in particular their size and mobility. Migration is assessed by counting the cells that have crossed the membrane and stick to its lower surface or fall into the fluid of the lower well. Coating of the lower membrane surface with collagen is used to increase sticking for the purpose of recovering migrated cells on the lower surface of the filter. The crossing of a polycarbonate membrane is a simple model of diapedesis, the migration of leukocytes through the endothelial layer and the basement membrane of microvessels, which is the first step of the intervention of host-defense cells in infection and inflammation.

The dependence of the chemotaxis response in vitro on chemokine concentration is usually bimodal: the number of migrating cells increases up to an optimum concentration and then falls, because over-stimulation increasingly leads to adherence (Baggiolini et al. 1994). Migration may also be assessed in vivo by monitoring the infiltration of leukocytes into a tissue area or a body cavity where a chemokine is injected. The infiltration can be quantified by image analysis of histology or cytochemistry specimens (Uguccioni et al. 1999), by counting the leukocytes collected in an exudate or by measuring the radioactivity of tissue sites in animals supplied with radioactively labeled leukocytes (Colditz et al. 1989). Furthermore, vital microscopy may be used in microcirculation experiments to observe chemokine-induced rolling and adhesion of leukocytes on the endothelial surface (Stein et al. 2000; Warnock et al. 2000).

The migration across the wall of microvessels and within the tissues along a chemokine concentration gradient is a complex function resulting from the combination of discrete responses. Some of these responses can be measured in real time as in vitro correlates of chemotaxis. Early studies on neutrophils stimulated with IL-8 (CXCL8) have shown that chemokines induce rapid, active changes in cellular shape as a result of the polymerization and depolymerization of actin (Thelen et al. 1988). Other characteristic effects are the upregulation and activation of integrins through which the leukocytes adhere to endothelial cells before emigration (Springer 1994), the release of granule contents, e.g. proteases from neutrophils, monocytes, $CD8^+$ T lymphocytes, and natural killer (NK) cells, histamine from basophils and cytotoxic proteins from eosinophils, the production of bioactive lipids, and the formation of oxygen radicals during the respiratory burst (Baggiolini et al. 1994, 1997). The release responses are observed at higher chemokine concentrations and are enhanced when the leukocytes are exposed to bacterial toxins or inflammatory cytokines. It must be assumed that release occurs once the leukocytes have reached their target, e.g. a site of infection and inflammation, where they stop migrating and perform antimicrobial and other functions in host defense (Baggiolini et al. 1994, 1997).

As already mentioned the second messenger inositol trisphosphate releases Ca^{2+} from intracellular stores, leading to a transient rise of its free concentration in the cytosol, which is one of the earliest cellular responses to chemoattractants (von Tscharner et al. 1986a, 1986b). With appropriate instruments one can measure the rate of the increase of Ca^{2+}-related fluorescence, which reflects the intensity of chemokine effects in real time and is the most reliable method to assess concentration–response relationships. We have used this assay extensively for the analysis of chemokine receptor selectivity and desensitization (Uguccioni et al. 1995, 1996; P. Loetscher et al. 1994b, 1996; Forssmann et al. 1997; Bardi et al. 2001).

CHEMOKINE ACTIONS ON PHAGOCYTES

As shown in Figure 4, phagocytes rely on a small repertoire of chemokine receptors, CXCR1 and CXCR2 for neutrophils, CCR1, CCR2, CCR3, and CCR5 for eosinophils, basophils, and monocytes. They are expressed constitutively and are always detectable and functional on the phagocytes that circulate in the blood. The receptors are ready for rapid interventions in response to chemokines induced in endothelial cells and in the tissues under pathological conditions by inflammatory stimuli such as bacterial toxins, inflammatory cytokines, such as IL-1, tumor necrosis factor (TNF), and interferons, hemopoietic growth factors, bioactive lipids, oxygen radicals, etc. (Baggiolini et al. 1994). Even though receptor numbers may change when the cells are activated (Bonecchi et al. 1999, 2000), their repertoire remains characteristic for each type of phagocyte. The system that drives phagocyte recruitment is redundant: most receptors recognize more than one chemokine and several chemokines act on multiple receptors (see

Figure 1). CXCR4, the selective receptor for SDF-1 (CXCL12), which is found in neutrophils and monocytes, makes an exception, because its role for these cells is unknown. In addition, phagocytes express several other receptors for nonchemokine ligands, like the complement product C5a, *N*-formyl-methionyl peptides, and several bioactive lipids, which may all contribute to phagocyte recruitment in inflammation (Boulay et al. 1997).

The ligands of CXCR1, CXCR2, CCR1, CCR2, CCR3, and CCR5 are collectively referred to as *inflammatory chemokines* because they are released in pathological conditions and disturbances of tissue homeostasis leading to inflammation. As a rule these chemokines are not detectable in healthy tissues. Numerous publications document the expression of CXC and CC chemokines at sites of inflammation, infection, and immune reactions. The main sources are different types of tissue cells, tissue macrophages, mast cells, but also the phagocytes that enter the inflamed tissues, which amplify in this way inflammatory and host-defense reactions by contributing to further cell recruitment (Baggiolini et al. 1994, 1997).

Inflammatory chemokines act as well on lymphocytes and other effector cells of acquired immunity. $CD4^+$ and $CD8^+$ T cells (Loetscher et al. 2000; Sallusto et al. 2000), NK cells (Maghazachi and Al-Aoukaty 1998), and immature dendritic cells (Sozzani et al. 1999) can express CCR1, CCR2, CCR3, and CCR5, and are thus readily attracted into inflamed tissues. In addition T and NK cells express CXCR3, which is upregulated in lymphocytes together with the other receptors for inflammatory CC chemokines indicated, and respond to a monokine induced by interferon γ (Mig), IP10, and interferon-inducible T-cell α-chemoattractant (I-TAC) (CXCL9, 10, and 11, respectively), which are induced in inflamed tissues by interferon γ (IFN-γ). CXCR3 is the only receptor for inflammatory chemokines that is not found in phagocytes.

CHEMOKINE ACTIONS ON LYMPHOCYTES

Memory and effector functions

In contrast to phagocytes, which leave the bloodstream to enter tissues where they perform host-defense functions and eventually die, but never return to the blood, lymphocytes circulate continuously through lymphoid and nonlymphoid tissues in search of antigens to provide specific protection against pathogens (Mackay 1993; Butcher and Picker 1996). When naïve T cells, in secondary lymphoid tissues, encounter an antigen they are activated, differentiate into effector or memory cells, and proliferate. Unlike their naïve precursors, effector and memory lymphocytes usually migrate into nonlymphoid tissues, which are the prime sites of entry for microorganisms and other foreign antigens. They have, however, the ability to home again into secondary lymphoid tissues for renewed episodes of activation. Recent research has shown that the various re-circulation itineraries are regulated by the expression of chemokine receptors on the lymphocytes and of the appropriate chemokines in the homing areas as well as adhesion molecules (Sallusto et al. 2000; Von Andrian and Mackay 2000; Moser and Loetscher 2001).

Early studies had suggested that T lymphocytes isolated from the blood respond poorly to chemokines. When T cells, however, are kept in culture in the presence of IL-2 the expression of several receptors for inflammatory chemokines, namely CCR1, CCR2, CCR5, and CXCR3, is markedly enhanced and migration in responses to the respective ligands, such as RANTES (CCL5), MCP-1 (CCL2), MIP-1β (CCL4), and IP10 (CXCL10), increases progressively (M. Loetscher et al. 1998; P. Loetscher et al. 1996). IL-4, IL-10, and IL-12 have similar effects but are less potent, whereas other cytokines known to act on T lymphocytes (IL-13, IFN-γ, IL-1β, and TNFα) are inactive. The effect is reversible: Receptor expression and responsiveness are rapidly lost when IL-2 is withdrawn, and are fully restored when it is added again (P. Loetscher et al. 1996). Rapid down-regulation of receptors for inflammatory chemokines is also observed when IL-2 is replaced by anti-CD3 alone or in combination with anti-CD28, mimicking activation via the T-cell receptor. These observations created much interest and several laboratories began systematically to study chemokine receptor expression in relation to T-lymphocyte differentiation.

T-lymphocyte-dependent immune responses rely on helper cells of T-helper (Th)1 or Th2 phenotype with different functional properties (Mosmann and Coffman 1989; Abbas et al. 1996), which develop from naïve precursors after antigen recognition and priming. Th1 cells secrete IFN-γ and lymphotoxin and enhance cell-mediated immunity to intracellular pathogens, whereas Th2 cells secrete IL-4 and IL-5, and regulate allergic responses and humoral immunity against parasites. A lymphocyte infiltrate may be of Th1 or Th2 type depending on the pathological conditions, and it was natural to assume that the selective recruitment of cells of either phenotype is driven by the chemokine receptors that they bear and the chemokines expressed in the diseased tissues.

CCR3, a receptor that was originally identified in eosinophils and basophils and that binds several chemokines including the eotaxins (see Figure 1), is expressed on a subset of blood T lymphocytes which produce IL-4 and IL-5 and thus qualify as Th2 cells (Sallusto et al. 1997). CCR3 is also expressed at high levels in Th2-type cells, which are obtained by culturing umbilical cord blood lymphocytes activated with phytohemagglutinin in the presence of IL-4 and neutralizing anti-IL-12 antibodies (Sallusto et al. 1997, 1998), and in cloned, allergen-

specific T cells with a Th2 phenotype (Gerber et al. 1997). CCR3-positive Th2 cells are recruited together with eosinophils to sites of allergic inflammation, and are likely to support eosinophil survival by secreting IL-4 and IL-5 (Gerber et al. 1997). Without questioning the fact that CCR3 is found in Th2 and not in Th1 cells, it is important to note that the level of CCR3 expression in Th2 lymphocytes was shown in several studies to be moderate and variable, suggesting that CCR3-bearing cells constitute a Th2 subpopulation (Loetscher et al. 2000).

CCR4 and CCR8 are also considered as characteristic for Th2 lymphocytes (D'Ambrosio et al. 1998; Zingoni et al. 1998; Imai et al. 1999). Both receptors are transiently upregulated in Th2 cells after stimulation in culture with anti-CD3 and anti-CD28. In contrast to CCR8, which is present exclusively in activated Th2 cells, CCR4 appears to be expressed constitutively and is also found in Th1 cells on activation with anti-CD3 and anti-CD28 (D'Ambrosio et al. 1998).

CCR5 is expressed preferentially in Th1 cells (Annunziato et al. 1998; Bonecchi et al. 1998; P. Loetscher et al. 1998b; Siveke and Hamann 1998). It is detected in Th1 and Th2 cells obtained from umbilical cord blood by treatments inducing polarization, but is not expressed in cloned Th2 cells (P. Loetscher et al. 1998b; Sallusto et al. 1998). Th1 lymphocytes also express high levels of CXCR3, the receptor for Mig, IP10, and I-TAC (CXCL9, 10, and 11, respectively) (Cole et al. 1998; P. Loetscher et al. 1998b; Qin et al. 1998). We and others have found that CXCR3 is present in both types of helper cells after proliferation induced with IL-2 (Annunziato et al. 1998; P. Loetscher et al. 1998b), whereas preferential expression in Th1 cells has been observed under other conditions (Bonecchi et al. 1998; Sallusto et al. 1998). Cytochemical analysis shows that the abundant Th1 lymphocyte infiltrate in the synovial pannus of rheumatoid arthritis joints is strongly positive for CCR5 and CXCR3, whereas CCR3 is virtually absent (P. Loetscher et al. 1998b; Qin et al. 1998).

A similar chemokine receptor pattern is observed in cytotoxic T cells (Tc): CCR5 is present in Tc1 whereas enhanced levels of transcripts for CCR3, CCR4, and CCR8 are characteristic for Tc2 cells (D'Ambrosio et al. 1998). It was recently reported that CXCR6 is preferentially expressed in blood Th1 and Tc1 cells and that CXCR6-positive $CD4^+$ and $CD8^+$ T cells are enriched in the synovial fluid of rheumatoid arthritis joints (Kim et al. 2001b). Such observations have been confirmed and extended in several studies emphasizing the role of chemokines and their receptors in directing lymphocyte traffic under physiological and pathological conditions (Sallusto et al. 2000; Von Andrian and Mackay 2000; Moser and Loetscher 2001).

A particularly important role in lymphocyte traffic and for the identification of cells with distinct functional properties is ascribed to CCR7. Sallusto et al. (1999) have defined two subsets of human memory T lymphocytes, one comprising cells without immediate effector properties, termed central memory T cells (T_{CM}), which are CCR7 positive, and the other comprising IL-4 and IFN-γ producing, perforin-positive cells, termed effector memory T cells (T_{EM}), which lack CCR7, but bear inflammatory chemokine receptors including CXCR3 and CCR5. The receptor profile underscores the different role played by these cells in a secondary immune response. T_{EM} cells can be recruited rapidly into inflamed tissues for immediate defense, whereas T_{CM} cells, which represent a clonally expanded memory cell pool, enter lymph nodes after a secondary antigen challenge, where they may stimulate dendritic cells to produce IL-12, provide help to antigen-specific B cells, and generate a new wave of effector T cells (Sallusto et al. 1999; Masopust et al. 2001; Reinhardt et al. 2001).

The concept of central and effector memory lymphocytes has recently been questioned in a number of reports. According to the model, CCR7 expression should be lost in memory $CD4^+$ T lymphocytes homing into the skin and the gut, and in fully differentiated, cytokine-producing Th1 and Th2 cells. However, in human blood, most memory $CD4^+$ lymphocytes with skin- or gut-homing markers (CLA^+ and $\alpha4\beta7^+$, respectively) were reported to bear CCR7 (Campbell et al. 2001), and the same was observed for a substantial fraction of IFN-γ- or IL-4-producing effector $CD4^+$ T lymphocytes (Kim et al. 2001c; Debes et al. 2002). It was also reported that CCR7-positive $CD4^+$ and $CD8^+$ memory T lymphocytes, arising in mice after viral infection, produce IFN-γ and exhibit lytic activity, respectively (Unsoeld et al. 2002). As they bear CCR7, such cells retain the capacity to re-circulate through secondary lymphoid tissues. The apparent discrepancy between the original and the subsequent reports should be clarified by further studies.

Much attention has recently been given to CXCR5, the selective receptor for BCA-1 (BLC in the mouse, CXCL13), to identify lymphocytes with distinct homing and effector properties (Mackay 2000; Kim et al. 2001a; Moser et al. 2002). CXCR5 is expressed in mature, circulating B lymphocytes and a subset of memory T cells (Förster et al. 1994; Breitfeld et al. 2000; Schaerli et al. 2000; Kim et al. 2001d). It is present in about 20 percent of the blood $CD4^+CD45RO^+$ T lymphocytes, which also express CCR7 but do not produce effector cytokines, suggesting that they qualify as T_{CM} cells. $CXCR5^+$ and $CCR7^+$ blood lymphocytes are considered as the precursors of the follicular B-helper T (T_{FH}) cells found in the B-cell areas of secondary lymphoid tissues. In the tonsils, most $CD4^+$ memory T cells express CXCR5. They have a B-helper function, which is particularly evident in $CD57^+$ T cells from germinal centers, and markedly enhance antibody production when co-cultured with B cells (Breitfeld et al. 2000; Schaerli et al. 2000; Kim et al. 2001d). Naïve blood T lymphocytes

rapidly acquire CXCR5 on activation in vitro with antigen-presenting dendritic cells and irreversibly lose expression of this receptor after terminal differentiation to effector cells (Schaerli et al. 2001). Together, these observations show that CXCR5-bearing T lymphocytes constitute a unique subset that is distinct from Th1 and Th2 cells.

Homing into lymphoid tissues

The role of chemokines in lymphocyte homing has already been addressed in the context of immune defense, but it is important to highlight their critical involvement in the formation and the renewal of secondary lymphoid tissues, e.g. lymph nodes and Peyer's patches, through the recruitment of naïve cells, a process that depends on two chemokine receptors, CCR7 and CXCR5, and their ligands (Sallusto et al. 2000; Von Andrian and Mackay 2000; Moser and Loetscher 2001).

The recognition that chemokines direct the homing of lymphocytes into secondary lymphoid tissues goes back to a remarkable experiment by Lipp and colleagues (Förster et al. 1996) who found that deletion of the gene of the then putative chemokine receptor termed 'BLR1' or 'MDR15', which was cloned from Burkitt's lymphoma (Förster et al. 1994) and human monocytes (Barella et al. 1995), resulted in impaired formation of Peyer's patches and inguinal lymph nodes. B cells lacking BLR1 were unable to enter their area of destination. The ligand for BLR1 was soon found to be a chemokine, termed 'BCA-1' or 'BLC' (CXCL13), and the receptor was renamed CXCR5 (Gunn et al. 1998; Legler et al. 1998). BCA-1/BLC (CXCL13) is selectively expressed in the B-cell follicles and deletion of the *BLC* (CXCL13) gene in mice yields the same phenotype as the deletion of the CXCR5 gene (Ansel et al. 2000).

Subsequent studies elucidated the role of CCR7, which binds two chemokines – secondary lymphoid tissue chemokine (SLC) (CCL21) and Epstein–Barr virus-induced receptor ligand chemokine (ELC) (CCL19) – and is expressed in all naïve and part of memory T lymphocytes as well as on B lymphocytes (Loetscher et al. 2000; Sallusto et al. 2000). In response to SLC (CCL21), which is expressed in high-endothelial venules, T lymphocytes enter secondary lymphoid tissues and are then attracted within the parafollicular area by SLC (CCL21) and ELC (CCL19), which are co-expressed there. The receptor and the ligands are essential for the homing and distribution of T lymphocytes as illustrated by deletion of the corresponding genes. Mice lacking CCR7 present morphological abnormalities, reflecting a disturbed homing of T cells into lymph nodes, and are unable to mount adequate antibody and delayed-type hypersensitivity responses (Förster et al. 1999). Similar disturbances are observed in mice with the *plt* (paucity of lymph node T cells) syndrome resulting from defective SLC (CCL21) and ELC (CCL19) expression (Gunn et al. 1999; Stein et al. 2000; Warnock et al. 2000).

Despite the disturbance of T lymphocytes homing, *plt* mice have a nearly normal B-lymphocyte recruitment into secondary lymphoid organs (Nakano et al. 1997). CCR7-deficient B lymphocytes transferred into normal mice, however, accumulate less efficiently in lymph nodes and Peyer's patches (Förster et al. 1999). Using transferred chemokine receptor-deficient and chemokine-desensitized B lymphocytes, it has recently been shown, by intravital microscopy, that B-cell homing into lymph nodes depends on two receptors – CCR7 and CXCR4 – and that, in addition, CXCR5 is important for B-cell homing into Peyer's patches (Okada et al. 2002). As shown by experiments in mice, the positioning of B cells in lymphoid tissues depends on responsiveness to BLC (CXCL13), on the one hand, and ELC and SLC (CCL19, CCL21), on the other, which are expressed in the B- and the T-cell areas, respectively (Reif et al. 2002). Naïve B cells, which bear CXCR5, migrate into the B-cell area in response to BLC (CXCL13). After antigen encounter and activation, however, B cells upregulate CCR7 and are attracted by SLC (CCL21) and ELC (CCL19) to the boundary between the B-and T-cell area (Reif et al. 2002).

In contrast to B2 cells (the conventional B cells), B1 cells home preferentially to the peritoneal and pleural cavities where macrophages and omentum cells constitutively produce BLC. B1 cells are a significant source of serum antibodies and make a major contribution to natural antibody production (low-affinity immunoglobulin M or IgM). In mice lacking BLC (CXCL13), the immigration of B1 cells into the peritoneal and pleural cavities is impaired and the production of natural and antigen-specific antibodies is markedly decreased (Ansel et al. 2002). Additional evidence for the important role of BLC (CXCL13) in homing of B1 cells is provided by the observation that B1 cells accumulate in the kidney and the thymus of aging BWF1 mice as a result of the abnormal expression of BLC (CXCL13) by myeloid dendritic cells (Ishikawa et al. 2001).

Another aspect of homing is the relocation of cells within a lymphoid tissue during maturation and differentiation as observed for thymocytes, which change receptor expression as they move from cortical to intermediate and then medullar sites (Campbell et al. 1999b; Annunziato et al. 2001; Ansel and Cyster 2001). Chemokines that are selectively produced in the different thymic areas are believed to retain the thymocytes as long as they express the proper chemokine receptor. A similar mechanism may operate in lympho- and myelopoiesis. Before being recognized as a chemokine, SDF-1 (CXCL12) was described as a factor with co-stimulatory effects on B-cell precursors (Tashiro et al. 1993; Nagasawa et al. 1994). We found that this chemokine attracts pre- and pro-B cells in contrast to more mature forms,

and suggested that it could stimulate lymphopoiesis by attracting maturing cells in the vicinity of growth factor-producing stromal cells (D'Apuzzo et al. 1997; Baggiolini 1998). In agreement with this hypothesis, deletion of CXCR4 in mice resulted in enhanced escape of B-cell precursors into the circulation (Ma et al. 1999).

Homing into nonlymphoid tissues

Chemokines and their receptors are also involved in the regulation of T-cell traffic through nonlymphoid tissues. It has been suggested that memory T cells show a selective tropism for specific peripheral tissues and re-circulate preferentially through the tissues where they have acquired their immunological memory (Mackay 1993). Good examples are the skin-homing and gut-homing memory T cells, which are characterized by the expression of adhesion molecules, the cutaneous lymphocyte-associated antigen (CLA), and the integrin $\alpha_4\beta_7$, which co-determine their homing selectivity. CLA binds to E-selectin expressed on endothelial cells in the skin, whereas $\alpha_4\beta_7$ binds to mucosal addressin-cell adhesion molecule-1 (MadCAM-1) expressed in the endothelia of mucosal tissues. The homing preference of the cells is also reflected by the chemokine receptors that they express, CCR4, CCR9, and CCR10 in particular, and by the production of the corresponding chemokines in the tissues.

CCR4 is present on all CLA-positive and other memory T cells, but absent from $\alpha_4\beta_7$ memory T cells (Campbell et al. 1999a; Andrew et al. 2001). TARC (CCL17), one of CCR4's two selective ligands, is expressed by normal and inflamed endothelium of the skin, but not of the intestine (Campbell et al. 1999a). About a third of CLA^+ T cells also bear CCR10 and thus migrate in response to its two ligands, CTACK (CCL27) and MEC (CCL28) (Morales et al. 1999; Homey et al. 2000, 2002; Pan et al. 2000; Wang et al. 2000). CTACK (CCL27) is a skin-associated chemokine (Morales et al. 1999) that is produced in the basal layers of the epidermis and is upregulated in inflammatory skin diseases (Homey et al. 2002). In two different mouse models of allergen-challenged skin, anti-cutaneous T-cell attracting chemokine (CTACK) antibodies markedly inhibited the recruitment of lymphocytes and tissue swelling (Homey et al. 2002). A role for CCR4 and CCR10 in T-cell recruitment into the inflamed skin is suggested by a recent observation in delayed-type hypersensitivity. The immigration of T lymphocytes was not appreciably affected by antibodies neutralizing the CCR10 ligand CTACK (CCL27), but complete inhibition was observed when transferred CCR4-deficient T cells were used, suggesting that both receptors are involved concomitantly (Reiss et al. 2001).

CCR9 and its ligand thymus-expressed chemokine (TECK) (CCL25) are implicated in the homing of $\alpha_4\beta_7^+$ T cells to the gut (Zabel et al. 1999; Kunkel et al. 2000). CCR9 is expressed in $\alpha_4\beta_7^+$ T cells, and TECK (CCL25) is produced by epithelial cells of the small intestine, in particular in the crypt region, which is closely associated with the vessels involved in lymphocyte recruitment that express MadCAM-1 (Kunkel et al. 2000; Wurbel et al. 2000). TECK (CCL25) is also detected in small intestine endothelial cells (Papadakis et al. 2001). Several investigations have shown that nearly all T cells in the small intestine express CCR9, underscoring the possible importance of CCR9 and TECK (CCL25) for the lymphocyte traffic in the gut (Zabel et al. 1999; Kunkel et al. 2000; Papadakis et al. 2001).

CHEMOKINES IN DISEASE

The analysis of tissues, exudates, and body fluids by immunochemistry, cytofluorimetry, and in situ hybridization highlights the role of chemokines in infectious and inflammatory pathologies. Receptors for inflammatory chemokines are commonly detected in the lesions of chronic inflammatory diseases, such as rheumatoid arthritis, lupus erythematosus, ulcerative colitis, Crohn's disease, multiple sclerosis, chronic bronchitis, sarcoidosis, and arteriosclerosis. The patterns of expression reflect the peculiarities of the underlying pathology: CCR1, CCR2, CCR5, and CXCR3 are highly expressed in chronic lesions with a predominant infiltration of mononuclear phagocytes and Th1 lymphocytes, CCR3 is enhanced in allergic pathologies with participation of eosinophils and Th2 lymphocytes, whereas CXCR1 and CXCR2 dominate in bacterial infections and acute inflammation. Especially in chronic processes the analysis of receptors and their ligands reveals an apparent redundancy of chemokine involvement in pathology. In animal models, deletions of chemokine or receptor genes and selective receptor antagonists have been used to explore the pathophysiological role of single chemokines and receptors. The issue of chemokine hierarchy in host defense and inflammation, which is highly relevant for the design of therapeutic approaches targeting chemokines, is addressed in a recent review (Gerard and Rollins 2001).

Chemokines and viral infections

Although infections by bacteria and fungi elicit inflammatory responses by inducing chemokine production either directly or on mediation by inflammatory cytokines such as IL-1 and TNF, herpes viruses, pox viruses, and retroviruses have evolved means to subvert the chemokine defense system to their own advantage. The genome of these viruses can encode chemokines, chemokine receptors, structurally unrelated chemokine receptor ligands, and chemokine-binding proteins. These gene products may be expressed on the viral surface or by the cells that the viruses infect. The most impressive case of viral interaction with the chemokine system is the mechanism that enables HIV to enter CD4-bearing

cells. Gp120, a viral envelope protein with no structural similarity to chemokines, binds first CD4 and then a chemokine receptor, usually CCR5 or CXCR4. This interaction exposes the fusogenic extremity of another surface protein, gp41, initiating the fusion of the viral envelope with the membrane of the target cell, which becomes infected. The interaction process leading to fusion and viral entry has not been clarified beyond the definition of the molecules that are necessary on the surface of the virus and the target cell. Several points of contact are assumed between the interacting moieties and formation of aggregates has been suggested, as recently reviewed (Murphy 2001). Co-receptor recognition, the choice between CCR5 and CXCR4, depends on the structure of the variable region of the V3 loop of gp120. In addition to clarifying the mechanism of HIV entry, the discovery of the second binding site, which remained elusive for long after the recognition that CD4 was important, helped to define R5 and X4 HIV strains, which bind CCR5 and CXCR4, respectively, and strains binding to both co-receptors, termed 'R5X4' (Berger et al. 1999). The relevance of chemokine receptors for the infection with HIV is underscored by the observations that individuals with an inactive form of CCR5 (CCR5Δ32) are resistant.

It was reported that gp120, in addition to docking on to CCR5 or CXCR4, also induces signaling (Davis et al. 1997) and thus may contribute to recruitment of additional target cells and/or influence cellular immune defense. A similar interference is ascribed to another HIV protein, the transcription factor Tat, which is released into tissue fluids and has been reported to attract leukocytes bearing CCR2 or CCR3 and to block CXCR4 (Murphy 2001).

Multiple, more direct interferences with the chemokine system were described for human herpesviruses 6 and 8 (HHV6 and 8), and human *cytomegalovirus* (HCMV) which all encode chemokines and chemokine receptors. The receptors have evolved for broad activities and are either constitutively active, similar to ORF74 encoded by HHV8 and expressed in Kaposi's sarcoma (Arvanitakis et al. 1997), or responsive to multiple human chemokines, such as ORF U51 encoded by HHV6 (Milne et al. 2000) and US28 encoded by HCMV (Gao and Murphy 1994). HCMV encodes two CXC chemokines, vCXC1, which is selective for CXCR2, and vCXC2, which has not been characterized (Penfold et al. 1999). The best known viral chemokines vMIP-I, -II, and-III are encoded by HHV8 and interact, as agonist or antagonists, with several CCRs in addition to CXCR4 (Kledal et al. 1997; Stine et al. 2000).

Studies on inflammatory diseases in animal models

In recognition of the inflammatory nature of arteriosclerosis and the importance of monocytes, which infiltrate the arterial wall and progressively transform into macrophages and foam cells (Ross 1999), research has focused on chemokines attracting monocytes. Although, as in other chronic inflammatory conditions, several chemokines are expressed in arteriosclerotic plaques and the underlying arterial wall tissue, deletion of the genes encoding CCR2 in murine models of the disease results in a marked reduction of fatty streak and plaque formation (Boring et al. 1998; Dawson et al. 1999). Remarkably, the pathological process is also attenuated in animals with a deletion of the MCP-1 gene, indicating that other MCPs cannot compensate for the lack of MCP-1 (CCL2) (Gu et al. 1998; Gosling et al. 1999).

The pathology of experimental allergic encephalomyelitis in mice also appears to depend on chemokines acting on mononuclear cells. Deletion of CCR2, the receptor for MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), and MCP-4 (CCL13), protects the animals against the relapsing central nervous system (CNS) inflammation induced by immunization with myelin components (Fife et al. 2000; Izikson et al. 2000), suggesting that monocytes and T lymphocytes, which respond to CCR2 ligands, are particularly important for disease progression. By contrast the development of the disease was not, or only minimally, affected by deleting the genes for MIP-1α (CCL3), RANTES (CCL5), and other chemokines (Gerard and Rollins 2001). These observations suggest that some chemokines, even if expressed in inflammatory lesions, play only a minor role in pathology or act in subordination to other chemokines. This conclusion is supported by studies showing that the encephalomyelitis was not appreciably affected by deletion of the *CCR1* or the *CCR5* genes, suggesting that neither receptor is important for the recruitment of mononuclear cells (Gerard and Rollins 2001). Unfortunately, the role of CXCR3, a receptor that is exclusively expressed in activated T lymphocytes, and its exclusive ligands, Mig, IP10, and I-TAC (CXCL9, -10, and -11, respectively), has not yet been investigated.

Experimental transplantation is a very useful model because of its close relationship to the clinical situation. The introduction of the graft elicits an almost immediate inflammatory response, which is characterized by the expression of IL-8 (CXCL8), MCP-1 (CCL2), and their analogs. This reaction is not related to the specific process of graft rejection and may be induced by inflammatory mediators produced as a consequence of transplant reperfusion (Hancock et al. 2000a). A reaction against the graft follows after the local induction – in the graft and the host tissue – of chemokines, which attract mononuclear cells via CCR1, CCR2, CCR5, and CXCR3. Deletion of any of these receptors retards graft rejection, but the most pronounced effect is observed after deletion of CXCR3 (Gao et al. 2000; Hancock et al. 2000a, b, 2001), confirming that activated $CD4^+$ and $CD8^+$ T cells, which characteristically express high levels

of CXCR3 (M. Loetscher et al. 1996), are essential for the immune reaction leading to rejection. The apparent involvement of CCR1 and CCR2, which are not selective for the effector T-cell population, suggests a role for other players, or simply reflects the concomitant occurrence of an unspecific inflammatory reaction.

An alternate way to assess the role of chemokines in pathology is the use of receptor-blocking agents. In contrast to gene deletion, the intervention with antagonists can be timed at any stage of the pathological process allowing exploration of preventive as well as curative effects. Several models of chronic inflammatory joint diseases, such as the adjuvant-induced arthritis in rats, collagen-induced arthritis in mice, and chronic polyarthritis of the MRL-lpr mouse, are considered as correlates of human rheumatoid arthritis. These models are widely used in the pharmaceutical industry for the assessment of anti-inflammatory drugs, and are of obvious interest for the study of chemokines. Two chemokine receptor antagonists, an amino-terminally truncated MCP-1 (CCL2) (Gong et al. 1997) and Met-RANTES (Met-CCL5) (Plater-Zyberk et al. 1997), were successfully applied in these models to prevent the development of inflammatory lesions and to attenuate the already established disease. These observations confirm the major role of the MCP receptor, CCR2, in the development and maintenance of chronic inflammation, and suggest the involvement of CCR1 and possibly CCR5.

Studies in patients

Despite the frequent, almost obligatory expression of chemokines in diseased tissues, the information about their role in human pathology is rather limited. It follows that the task of chemokine expression studies in human disease is to identify chemokines that are chiefly involved in steering the process of host defense and inflammation for two main reasons: a better understanding of pathogenic mechanisms and the identification of ligands and receptors as targets for therapeutic intervention.

Immunocytochemistry and in situ hybridization of tissue biopsies show that several inflammatory chemokines are usually expressed concomitantly. As chemokines act in the microenvironment of the cells that release them, the study of their potential role in pathology must be based on observations in situ. Discrete evaluation of lesional and nonlesional areas of the same tissue usually discloses important differences and provides a good internal control for the changes occurring in the affected tissues. A random assessment does not take into account the focal nature of inflammation, and measurements of chemokine concentrations in exudates and other body fluids yield only a pale reflection of the disease process. The approach in such investigations may be exemplified by a study of biopsies of inflamed and normal areas of the gut mucosa at different stages of ulcerative colitis (Uguccioni et al. 1999). The local levels of chemokines were quantified by determining the numbers of chemokine-producing cells using image analysis. It was not attempted to quantify the *intensity* of chemokine staining within the cells or in the extracellular space because we found it virtually impossible to obtain reproducible results in standard specimens. Within the lesions, the numbers of cells expressing IL-8 (CXCL8), MCP-1 (CCL2), and MCP-3 (CCL7) were strictly correlated with disease severity, as assessed by clinical criteria, but the frequency of cells producing IL-8 (CXCL8) was about tenfold higher than those producing MCPs. IP10 (CXCL10)-positive cells also increased with pathology, but a significant expression of this chemokine was also observed in nonlesional biopsies, a finding that was not studied further. MIP-1α (CCL3), a chemokine acting via CCR1 and CCR5, was also studied, but no positive cells were detected. These observations indicate that leukocytes bearing CCR2, CXCR1, CXCR2, and CXCR3, i.e. monocytes, activated T cells, and neutrophils, are most likely to be recruited into the lesions, which is confirmed by histology. CCR1 and CCR3, which bind MCP-3 (CCL7), may also be involved in the immigration of mononuclear cells. The redundancy in the expression of inflammatory chemokines is well documented in human disease and animal models. In consideration of the chemokine expression pattern observed in ulcerative colitis, CCR2, the receptor for MCPs, which is expressed in monocytes and all IL-2-activated T cells (Figure 6 and see Figure 4), appears to be of particular relevance.

The only known chemokine receptor defect in humans results from the expression of an inactive variant of CCR5, CCR5Δ32. The defect is relatively frequent in individuals of Caucasian descent who do not present an increased susceptibility to infectious diseases (see 'Chemokines and viral infections' above). Several studies have shown that carriers of the Δ32 allele are partially resistant to chronic inflammatory diseases. It was found that they have a delayed onset of multiple sclerosis (Barcellos et al. 2000). Even homozygotes, however, can develop the disease, indicating that a functional CCR5 is not essential for the occurrence of this particular pathology (Bennetts et al. 1997). In a comparison of about 700 patients with rheumatoid arthritis, 100 patients with lupus erythematosus, and 800 controls, no difference was found in the frequencies of the normal and defective CCR5 allele, but no patient was homozygous for the defective receptor (Gòmez-Reino et al. 1999). Another study of 163 patients with rheumatoid arthritis revealed no difference in the hetero- and homozygous incidence of the CCR5 defect as compared with the healthy population. Carriers of the Δ32 allele, however, showed a tendency to milder disease (Garred et al. 1998).

Svanborg and her colleagues (Frendeus et al. 2000) have studied the role of IL-8 receptor expression for

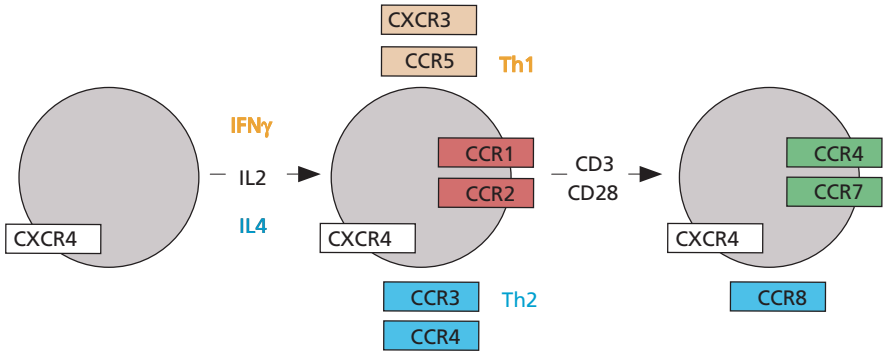

Figure 6 *Chemokine receptor expression in human T lymphocytes. Blood T lymphocytes usually express low levels of chemokine receptors and respond poorly to chemokines. Receptor expression depends on maturation, activation, and differentiation (see text).*

antibacterial defense by comparing the coping of normal and IL-8 receptor-deficient mice with urinary tract infections. The susceptibility of receptor-deficient mice (Godaly et al. 2000; Hang et al. 2000) motivated a study of CXCR1 expression in neutrophils of normal and pyelonephritis-prone children, whose neutrophils were found to have significantly lower CXCR1 levels than those of healthy controls. This observation offers an explanation for the recurrence of pyelonephritis in certain individuals and may lead to the characterization of a genetic polymorphism of CXCR1.

A highly interesting case of chemokine involvement in infection-induced human pathology is documented by a study of chemokine expression in *Helicobacter pylori* gastritis (Mazzucchelli et al. 1999). The comparison of normal and infected mucosa shows that the B-cell-attracting chemokine (BCA-1) (CXCL13) is abundantly and exclusively expressed at the sites of formation of the ectopic follicles, which are characteristic for the disease. The staining for the chemokine coincides with the staining for its receptor, CXCR5, in the immigrated lymphocytes. SLC, by contrast, which is expressed constitutively in the extrafollicular areas of lymphoid tissues together with ELC and is prominent in high-endothelial venules, was not detectable in and around the mucosal follicles, and was restricted to the endothelia of some microvessels. It may have a role in the extravasation of B cells which are then attracted into the ectopic follicles by BCA-1 (CXCL13). Mucosa-associated lymphoid tissue (MALT) lymphomas are a frequent complication of *H. pylori* infection. Interestingly the transformed cells produce high amounts of BCA-1 (CXCL13) and express CXCR5, suggesting that the B-cell-attracting chemokine, in the tumor, may act in an autocrine fashion and influence the proliferation of lymphoma cells. The pathological formation of follicle-like structures was later found to be associated with BCA-1 (CXCL13) expression in Sjögren's syndrome (Amft et al. 2001) and rheumatoid arthritis (Shi et al. 2001) as well.

Luther et al. (2000) have reported the formation of ectopic lymph node-like structures in pancreas islets after expression of BLC, the murine homolog of BCA-1 (CXCL13), in transgenic mice. This study shows that immigrating CXCR5-positive B cells cluster to form follicles. The process of ectopic lymphoid neogenesis, however, goes beyond the accumulation of B cells observed in *H. pylori*-induced gastritis and other human inflammatory diseases. The transgenic animals also express SLC in the parafollicular area leading to the formation of a T-cell zone with high-endothelial venules and lymphotoxin, possibly as a consequence of the exaggerated production of BLC.

CHEMOKINE RECEPTOR ANTAGONISTS

The idea that chemokine antagonists could have therapeutic effects in inflammatory diseases by preventing leukocyte infiltration was proposed soon after the discovery of IL-8 (CXCL8) (Baggiolini et al. 1989). It was then shown that CXCR1 and CXCR2 could be blocked with IL-8 (CXCL8) analogs obtained by truncation or minimal modification of the amino-terminal triggering sequence, and that the same principle could be applied to MCP-1 (CCL2) and RANTES (CCL5) to block the respective CC chemokine receptors (Baggiolini et al. 1994, 1997). The interest in the antagonists grew considerably with the recognition that all chemokines act via heptahelical, Gi-protein-coupled receptors (Murphy et al. 2000) and the discovery that human immunodeficiency viruses bind to chemokine receptors to infect leukocytes and viral entry is prevented by competing chemokines (Berger et al. 1999) and chemokine antagonists (Arenzana-Seisdedos et al. 1996; Simmons et al. 1997). In the same context, chemical substances with known HIV-suppressing activity were tested for chemokine antagonism and it was shown that chemokine receptors could be blocked with small, chemokine-unrelated molecules (De Clercq 2000; Gerlach et al. 2001). These observations boosted the search for nonpeptide chemokine antagonists using high-throughput screening of chemical libraries, with the aim of discovering new drugs for chronic inflammatory diseases.

The hypothesis that antagonists could be effective in the therapy of chronic inflammatory diseases was validated in animal models using amino-terminally modified MCP-1 (CCL2) or RANTES (CCL5). Daily injections of the CCR2 antagonists MCP-1 (9–76), obtained by omission of the first eight amino-terminal residue of synthetic MCP-1 (CCL2), prevented or strongly inhibited the development of the chronic arthritis that arises spontaneously in MRL-lpr mice, and showed considerable anti-inflammatory effects even in animals that had already developed the disease (Gong et al. 1997). Similar preventive and therapeutic effects were observed on repeated injections of Met-RANTES (Met-CCL5) in mice with collagen-induced arthritis (Plater-Zyberk et al. 1997). Met-RANTES also prevented the infiltration of inflammatory cells and significantly reduced vascular injury and tubular damage in renal allografts performed in Lewis RT1 rats (Grone et al. 1999). Met-RANTES is not a true antagonist, because it retains different levels of agonistic activity on the RANTES receptors, CCR1, CCR3, and CCR5 (Proudfoot et al. 1999).

Synthetic, chemokine-unrelated antagonists

The development of chemokine antagonists became a major goal of the pharmaceutical industry. The focus is on receptors that mediate leukocyte infiltration in inflammatory diseases as reflected by the patent applications, which mainly claim activities against CXCR1, CXCR2, CXCR3, CCR1, CCR2, and CCR3. Other, more restricted targets are CXCR4 and CCR5, the two main HIV co-receptors. Several, mostly polycyclic, compounds have been identified in broad screening programs as blockers of single or multiple receptors (Horuk and Ng 2000).

Among the compounds that are ready for studies in patients, the CCR1 antagonist BX471 (Berlex) may soon yield the proof of concept for the use of chemokine antagonists in the therapy of inflammation. It is a potent inhibitor of chemokine activities with K_i values around 1 nmol/l, is apparently selective for CCR1 (Liang et al. 2000), and inactive on adrenergic, muscarinic, dopamine, serotonin, bradykinin, leukotriene B_4 (LTB_4), and neuropeptide receptors. In animal models of disease BX471 was moderately effective in preventing graft rejection in rodents (Horuk et al. 2001b) and was found to act in synergy with the immunosuppressant cyclosporin (Horuk et al. 2001a, 2001b). It also reduced leukocyte infiltration and the development of renal fibrosis after unilateral ureter ligation in mice (Anders et al. 2002). Somewhat related to BX471 is a xanthene derivative, from the Banyu Company, that blocks CCR1 and CCR3. The combination of CCR1 and CCR3 antagonism may be therapeutically desirable for allergic inflammation because both receptors are prominent on eosinophil and basophil leukocytes. Highly selective antagonism for CCR3 was obtained, however, in the same laboratories with benzothiazole derivatives with IC_{50} (concentration of 50 percent inhibition) values for inhibition of binding and function in the low-nanomolar range (Naya et al. 2001; Sabroe et al. 2000). Naphthalene compounds synthesized by Smith Kline Beecham also showed potent blocking effects on CCR3 and only weak interaction with CXCR1, CXCR2, CCR1, CCR7, and the receptors for C5a, C3a, and LTD_4 (White et al. 2000; Dhanak et al. 2001).

Interesting studies were performed with spiropiperidine compounds from Roche (e.g. RS-102895, RS-504393), binding CCR2 but not CCR1, CCR3, or CXCR1 and inhibiting the activity of MCP-1 (CCL2) and its analogs, which are major mediators in chronic inflammatory diseases (Mirzadegan et al. 2000). These compounds were shown to interact with the acidic residue Glu291 at the extracellular end of the seventh transmembrane helix of CCR2, which is important for the high-affinity binding of MCP-1 (CCL2). The compounds, however, were equally or even more potent as blockers for α_1-adrenergic receptors (Mirzadegan et al. 2000), possibly jeopardizing their use as MCP blockers.

In view of their HIV co-receptor function, CCR5 and CXCR4 became prime targets for the development of synthetic antagonists. TAK-799, a compound by Takeda, with high affinity for CCR5 and CCR2 with K_i values of about 1 and 20 nmol/l, respectively, and no effects on CCR1, CCR3, CCR4, and CXCR4 (Baba et al. 1999; Shiraishi et al. 2000) was reported to prevent the binding of the HIV surface protein gp120 by interacting with a cluster of residues at the outer end of the transmembrane helices 1, 2, 3, and 7 of CCR5 (Dragic et al. 2000). CCR5 antagonists without relevant inhibition of CCR2 were synthesized by Ono (Maeda et al. 2001), Schering-Plough (Strizki et al. 2001), and Merck (Finke et al. 2001). Cyclams and bicyclams, on the other hand, are potent antagonists for CXCR4 (De Clercq 2000). Binding-site analysis shows that bicyclams interact with two acidic residues, Asp171 and Asp262, located at the extracellular end of transmembrane domains 4 and 6 of CXCR4, which are both necessary for high-affinity blockade of the ligand-binding pocket (Gerlach et al. 2001).

Missed targets

So far the development of synthetic low-molecular-mass antagonists for CXCR1, CXCR3, and CCR2 has not been as successful. IL-8 (CXCL8), the major ligand for CXCR1, is expressed ubiquitously and most prominently in inflamed tissues, and must be regarded as a primary mediator of neutrophil recruitment in conditions such as infarction and reperfusion, ulcerative colitis, alcoholic hepatitis, early graft rejection, etc., for which CXCR1 antagonists are therapeutically desirable. Antagonists for CXCR3, the receptor for Mig, IP10, and I-TAC

(CXCL9, -10, and -11, respectively) appears equally important, because they would be ideal drugs for preventing the recruitment of activated T lymphocytes in autoimmune diseases and at sites of transplant rejection. Finally the need for selective CCR2 antagonists is underscored by many observations in experimental pathology, including studies on receptor and ligand gene deletions, indicating a primary role for MCP-1 in chronic inflammatory diseases (Gerard and Rollins 2001).

Research and development in this important area of drug discovery have concentrated so far on the search of monoselective compounds. In inflamed tissues, however, one usually observes the concerted expression of several chemokines, suggesting that inhibitors of multiple receptors may be better drugs. Autoimmune inflammation may be treated more efficiently by drugs that block CCR1, CCR2, and CXCR3, rather than with antagonists for a single receptor. Similarly, the combined blockade of CCR1, CCR2, and CCR3 may be the best therapeutic strategy in allergic inflammation. The comparison of the therapeutic efficacy of antagonists for single and multiple receptors in animal models will be useful for directing drug discovery programs.

Natural chemokines as antagonists

Recent observations have shown that some natural chemokines have agonistic as well as antagonistic activities. In a study of chemokines acting on receptors that are differentially expressed in Th1 and Th2 lymphocytes, we found that the agonists for CXCR3, Mig, IP10, and I-TAC (CXCL9,-10, and-11, respectively) are antagonists for CCR3 (Loetscher et al. 2001). They compete for the binding of chemokines to CCR3-bearing cells, and inhibit calcium mobilization and migration induced by CCR3 agonists such as eotaxin-1 (CCL11), eotaxin-2 (CCL24), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), and RANTES (CCL5). As a result of their dual properties the attractants of Th1 lymphocytes acting via CXCR3 can inhibit the recruitment of Th2 lymphocytes by blocking CCR3, and in this way enhance the polarization of T-cell recruitment. Other natural chemokines with agonistic and antagonistic activities are eotaxin (CCL11), which attracts eosinophils, basophils, and Th2 lymphocytes via CCR3 while blocking CCR2 (Ogilvie et al. 2001), and MCP-3 (CCL7), a potent agonist for CCR1, CCR2, and CCR3 that blocks CCR5 (Blanpain et al. 1999). The inhibition of CCR2-, CCR3-, and CCR5-mediated responses represents a true antagonism as indicated by the fact that binding of the inhibitor does not induce receptor internalization. More work must be done to assess the role of the combination of triggering and inhibiting effects of single chemokines on different receptors, which impresses as a possible mechanism for increasing the selectivity of leukocyte recruitment in disease.

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Type I interferons and receptors

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In the 1930s several investigators described the phenomenon of viral interference, whereby the infection of an animal by a virus seemed somehow to protect it against subsequent infection by another virus (Findlay and Mac Callum 1937; Schlesinger 1959; Dianzani 1975). In 1957 Isaacs and Lindenmann (1957; Davies et al. 1982) found an agent of viral interference: a protein, released by cells exposed to a virus, which enabled other cells to resist viral infection. They called it interferon. The great promise of interferon as an antiviral agent was evident from the moment of its discovery, which was supported by an independent report of similar findings by Nagano and Kojima (1958). The promise was enticing because interferon is not directed against any one virus but rather protects cells against a wide range of viruses.

The interferons represent proteins with antiviral activity that are secreted from cells in response to a variety of stimuli (Pestka 1981, 1986; Pestka et al. 1987). There are at least six classes of interferons (IFN): alpha (α), beta (β), gamma (γ), tau (τ), omega (ω), and kappa (κ). The interferons are divided into two groups, designated type I and type II interferons (Pestka et al. 1987). IFN-γ is the only type II interferon whereas the type I interferons consist of five major classes: IFN-α, IFN-β, IFN-ω, IFN-τ, and IFN-κ. There is only one human Hu-IFN-β (Hu-IFN-β), one Hu-IFN-ω, and one Hu-IFN-κ, but a family of multiple IFN-β species exists. It is unlikely that any human IFN-τ exists. IFN-τ was described first as ovine trophoblast protein-1 and is found in ungulates, where it is required for implantation of the ovum (Roberts et al. 1991). Hu-IFN-κ, although it exhibits low specific antiviral activity, is expressed in human keratinocytes (LaFleur et al. 2001).

In general, exposure of human cells to viruses and double-stranded RNAs induces the production of IFN-α and IFN-β species, although IFN-ω and IFN-κ can be produced by appropriate cells. The ratio of IFN-α to IFN-β produced by cells varies with the tissue of origin and the species of the organism. T lymphocytes produce IFN-γ when stimulated with antigens to which they are immune, or when stimulated with mitogens such as staphylococcal enterotoxin A or the combination of phytohemagglutinin and phorbol esters. Most of the interferons in therapeutic use are produced through genetic engineering (Pestka 1981, 1983b, 1986; Pestka et al. 1987). As the first biotherapeutic approved, IFN-α paved the way for the many new biotherapeutics to follow. Nevertheless, we have only touched the surface of understanding the multitude of human interferons.

Many procedures have been described for the partial purification of human and animal interferons (Berg 1982; Pestka 1981, 1983b, 1986; Pestka et al. 1987). Although partial purification of the interferons as bands on a sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was reported by a number of groups, it was not until 1978 and thereafter that any

interferon was purified to homogeneity in sufficient amounts for its chemical and physical characterization (Rubinstein et al. 1978, 1979a, 1981; Stein et al. 1980; Friesen et al. 1981). The introduction of reverse phase and normal phase high-performance liquid chromatography (HPLC) to the purification of proteins (Rubinstein et al. 1978, 1979a, 1981; Stein et al. 1980; Friesen et al. 1981) led to the first successful purification of these proteins so that sufficient amounts were available without detergent for their chemical, biological, and immunological studies. Various affinity purification techniques, particularly antibody affinity chromatography were utilized to purify human leukocyte (α) and fibroblast (β) interferons (Knight 1976; Berthold et al. 1978; Cabrer et al. 1979; Zoon et al. 1979; Allen and Fantes 1980; Okamura et al. 1980; Berg and Heron 1981; Kawade et al. 1981; Zoon 1981b; Knight and Fahey 1982; Pestka et al. 1987).

PURIFICATION OF HUMAN LEUKOCYTE INTERFERON α

Since their discovery, many attempts have been made to purify the interferons, with little success until 1978. In fact, interferon used in experiments as well as in initial human clinical trials was essentially a crude protein fraction, less than 1 percent of which by weight consisted of interferon. As a result of the use of such crude interferon-containing material, it was not clear what activities of these preparations were caused inherently by the interferon present and what activities were caused by the numerous other contaminating proteins. By definition, the antiviral activity was the result of the interferon. However, these crude preparations exhibited antiprotozoal and antibacterial activities, inhibited cellular growth (antiproliferative activity), and blocked antibody synthesis, and were ascribed many other activities. However, without purified interferon, it was not possible to demonstrate definitively whether or not a particular activity was the result of the interferon protein molecule itself. Accordingly, it was essential to obtain purified interferon to determine what activities were an inherent part of the interferon molecule. As very little was known about the size and structure of the interferons, the isolation of purified interferons established their chemical composition and structure as well as their biological activities.

Production

We began purification of interferon from human leukocytes in 1977. This interferon was produced by incubating human white blood cells with Newcastle disease virus or Sendai virus for 6–24 h (Familletti and Pestka 1981; Familletti et al. 1981a; Hershberg et al. 1981; Waldman et al. 1981). The procedure was a combination of techniques that have previously been reported (Wheelock 1966; Cantell and Tovell 1971). The antiviral activity was found in the cell culture medium after overnight incubation of the leukocytes as illustrated in Figure 1. We substituted milk casein for human or bovine serum in the culture medium as had been described (Cantell and Tovell 1971). The use of casein, a single protein, instead of serum, which contains many different and uncharacterized proteins, simplified the initial concentration and purification steps. We used leukocytes from normal donors as well as from patients with chronic myelogenous leukemia. These leukemic cells make large amounts of human leukocyte interferon when induced with Newcastle disease virus or Sendai virus (Hadhazy et al. 1967; Lee et al. 1969; Rubinstein et al. 1979a; Familletti et al. 1981a).

The cytopathic effect inhibition assay for interferon as originally described took 3 days. Other assays for interferon were even longer. A more rapid assay was necessary to proceed with the purification expeditiously. A cytopathic effect inhibition assay that could be done in 12–16 h was developed (Familletti et al., 1981b; S. Rubinstein et al., 1981) and accelerated the purification immensely.

High-performance liquid chromatography for protein purification

As classic techniques for protein purification were not successful in the purification of the human interferons,

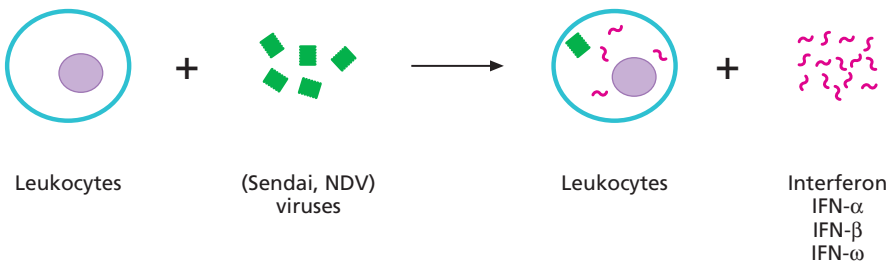

Figure 1 *Production of leukocyte interferon with Newcastle disease virus (NDV) and leukocytes. Leukocytes, the white blood cells of peripheral blood, were obtained from normal donors or patients with chronic myelogenous leukemia. The cells were washed, placed in culture medium with Newcastle disease virus (or with Sendai virus), and incubated overnight at 37°C. Interferon is found in the culture medium after incubation. The cells and virus can be removed by centrifugation, leaving the culture medium containing interferon.*

we applied HPLC to the purification process. Udenfriend and co-workers (Udenfriend et al. 1972; Stein et al. 1973; Bohlen et al. 1975) had developed sensitive fluorescent techniques for detection of amino acids and peptides, and had achieved the separation of peptides by reverse-phase HPLC. However, separation of proteins had not yet been accomplished. In the early experiments, there was uniform failure to achieve any purification of proteins with reverse-phase HPLC because the interferon activity was constantly lost. At the time, increasing the ethanol concentration was used to elute proteins and this was tried for interferon and other proteins without success. It was necessary to use a less polar solvent to elute interferon. Although there was initial hesitancy to use *n*-propanol above 20 percent (v/v) and other organic solvents because of the limited solubility of proteins in such solvents, it was found that *n*-propanol gradients effectively eluted interferon and other proteins without noticeable precipitation at the concentrations employed (Rubinstein et al. 1978, 1979b; S. Rubinstein et al. 1981; Rubinstein and Pestka 1981). Furthermore, by changing the pH of the elution buffer, a completely different separation could be achieved during elution of the same reverse-phase column with *n*-propanol. As subsequently demonstrated with fibroblast interferon, (Friesen et al. 1981) a large number of different columns and solvent systems could be used to effect resolution of proteins. By applying normal-phase chromatography with a diol silica column between the two reverse-phase columns, it was possible to use just three sequential HPLC steps to purify human leukocyte interferon to homogeneity. Sufficient amounts were purified in high yield for initial chemical characterization of the protein and for determination of amino acid composition. The amino acid composition of the human leukocyte interferon species γ2 was the first reported for any purified interferon (Rubinstein et al. 1979b).

The initial steps for purification of IFN included selective precipitations and gel filtration (Figure 2) followed by HPLC. The HPLC steps were reverse-phase chromatography (Figure 3) at pH 7.5 on LiChrosorb RP-8, normal partition chromatography on LiChrosorb Diol (Figure 4), and reverse-phase chromatography at pH 4.0 on LiChrosorb RP-8 (Figure 5). Gradients of *n*-propanol were used for elution of interferon from these columns (Figures 3-5). The overall purification was about 80 000-fold and the specific activity of purified interferon was 2–4 $\times$ 10^8 units/mg (Rubinstein et al. 1979b). Interferon prepared by this procedure yielded a single band of molecular mass (M_r) 17 500 on PAGE. The antiviral activity was associated with the single protein band (Rubinstein et al. 1978). The specific activity of this peak was 4 $\times$ 10^8 units/mg.

Several reports had previously described HPLC of proteins, mainly on ion exchange and size exclusion columns (Chang et al. 1976; Regnier and Noel 1976). However, these systems were either not commercially available or had a low capacity. With proper choice of eluent and pore size, octyl and octadecyl silica could be used for high-resolution reverse-phase HPLC of both peptides and proteins. Accordingly, with *n*-propanol as eluent, the use of LiChrosorb RP-8 (octyl silica) columns for protein fractionation was a major factor in the success of the purification (Figure 5). In addition, LiChrosorb Diol, which is chemically similar to glycophase resins that have been used for exclusion chromatography of proteins, was introduced as a support for normal partition chromatography of proteins (Figure 5b). High recoveries of interferon activity were obtained in each chromatographic step, a requirement when small amounts of initial starting material are present. Although the initial experiments were

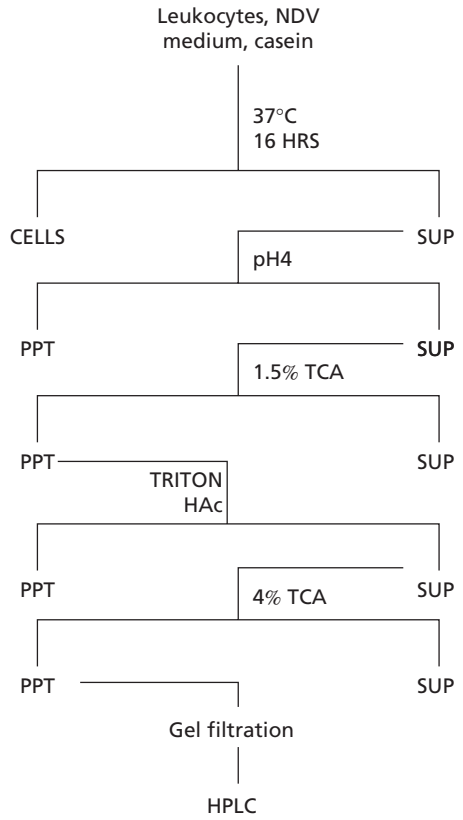

Figure 2 *Flow chart of initial steps in purification of leukocyte interferon. Cells and debris were removed by low-speed centrifugation from the medium containing interferon. Casein was used as a serum substitute. By acidification of the medium to pH 4 with hydrochloric acid, the bulk of the casein, which precipitated, was removed from the interferon, which remained in solution. The interferon was concentrated by two steps involving precipitation with trichloroacetic acid. The concentrated solution containing relatively crude interferon was separated into components of different sizes by gel filtration on Sephadex G-100 in the presence of 4 mol/l urea. Details of these procedures have been described (M. Rubinstein et al. 1979b, 1981a; Familletti et al. 1981a; Hershberg et al. 1981; Waldman et al. 1981). HLPC, high-performance liquid chromatography; NDV, Newcastle disease virus; Ppt, precipitate; Sup, supernatant; TCA, trichloroacetic acid.*

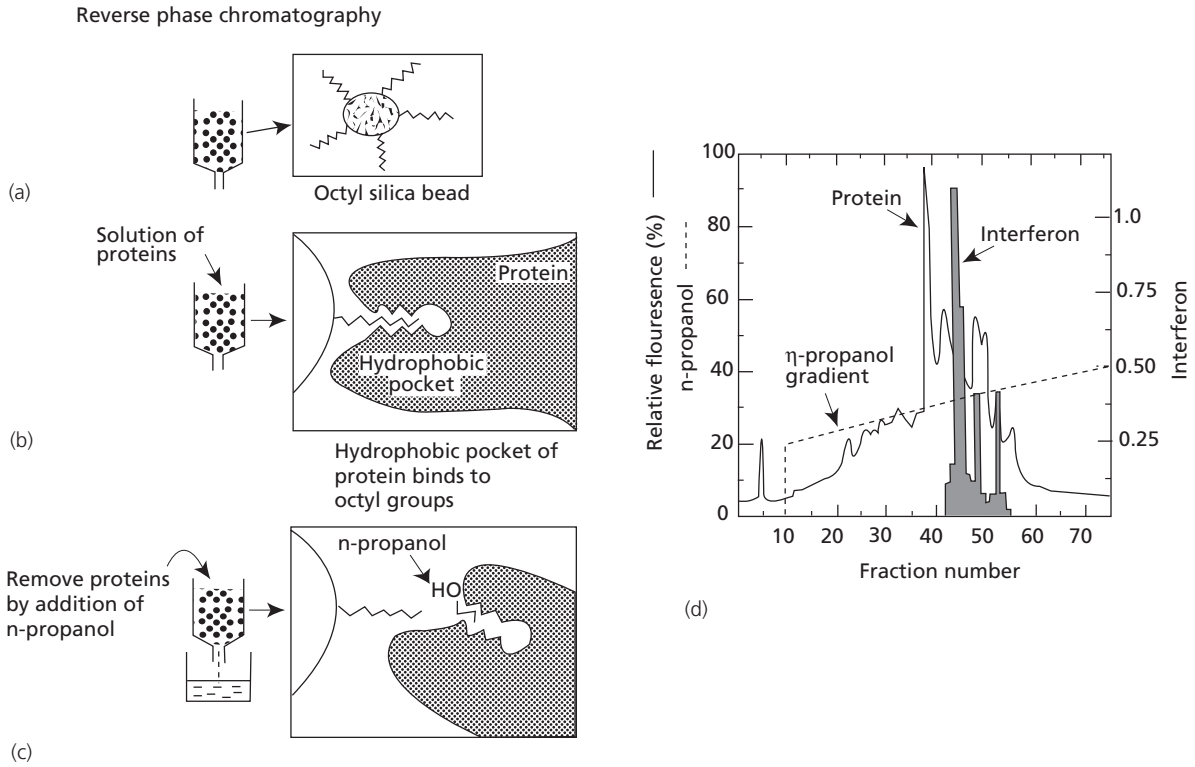

Figure 3 *High-performance liquid chromatography of leukocyte interferon on reverse-phase columns. Columns of porous silica to which octyl groups were bound were used to separate leukocyte interferons from other proteins and to resolve the individual leukocyte interferons. The proteins enter the interstices within the silica particles where the hydrophobic areas of the protein bind tightly to the octyl groups. The nonpolar portion of* n*-propanol can interact with interferon, releasing interferon and other proteins from the column. By eluting the proteins with an increasing gradient of* n*-propanol, the proteins are released in order of their increasing hydrophobicity. Those proteins binding most tightly are released only after high concentrations of* n*-propanol or other appropriate organic solvents are used (Pestka 1983b).*

performed with leukocytes from normal donors (Rubinstein et al. 1978, 1979b), it was found that leukocytes from patients with chronic myelogenous leukemia (CML), who were undergoing leukapheresis to lower their peripheral white blood cell counts, were a rich source of interferon that appeared to be essentially identical to the human leukocyte interferon purified from leukocytes from normal donors (Rubinstein et al. 1979a). As with HPLC of interferon from normal leukocytes on the Diol column (Figure 5b), three major peaks of activity, labeled α, β, γ^2, were observed with interferon prepared from CML cells. (The natural interferons which were isolated from the mixture present in leukocyte interferon by HPLC (Rubinstein et al. 1978, 1979b, 1981; Pestka 1983b) were then designated α1, α2, β1, β2, β3, γ1, γ2, γ3, γ4, γ5, and δ. Unfortunately, the same Greek letters were later used to designate leukocyte, fibroblast, and immune interferons, respectively, as α, β, γ.) Although the protein profiles were almost identical, the activity profiles showed that the amount of activity under peak γ was lower in preparations from leukemic cells compared with normal leukocytes (Rubinstein et al. 1979a, 1979b). However, even from normal leukocytes, the ratio of peaks α, β, and γ varied from one preparation to another. Human lymphoblastoid interferon produced by suspension cultures of Namalva cells was purified by a combination of immunoaffinity chromatography and other methods by Zoon et al. (1979).

The amino acid composition of human leukocyte interferon purified by HPLC as described above (Rubinstein et al. 1979b) shows similarity with human lymphoblastoid interferon (Zoon et al. 1979) and one of the types of mouse interferon (Cabrer et al. 1979). It should be noted that the terms 'leukocyte' and 'lymphoblastoid' interferons simply designate the source of the interferons. The major component of both these preparations consists of Hu-IFN-α species; a minor component of Hu-IFN-β and Hu-IFN-ω is also present in these preparations. As a result of the fact that multiple interferon classes were present in interferons produced from these cell sources, a new nomenclature was proposed to designate the species of interferon rather than the source of the interferon (Interferon Nomenclature 1980). The designation IFN-α was suggested for the major class of interferons present in leukocyte and lymphoblastoid interferon preparations. Hu-IFN-β was the designation for the major interferon produced by human fibroblasts

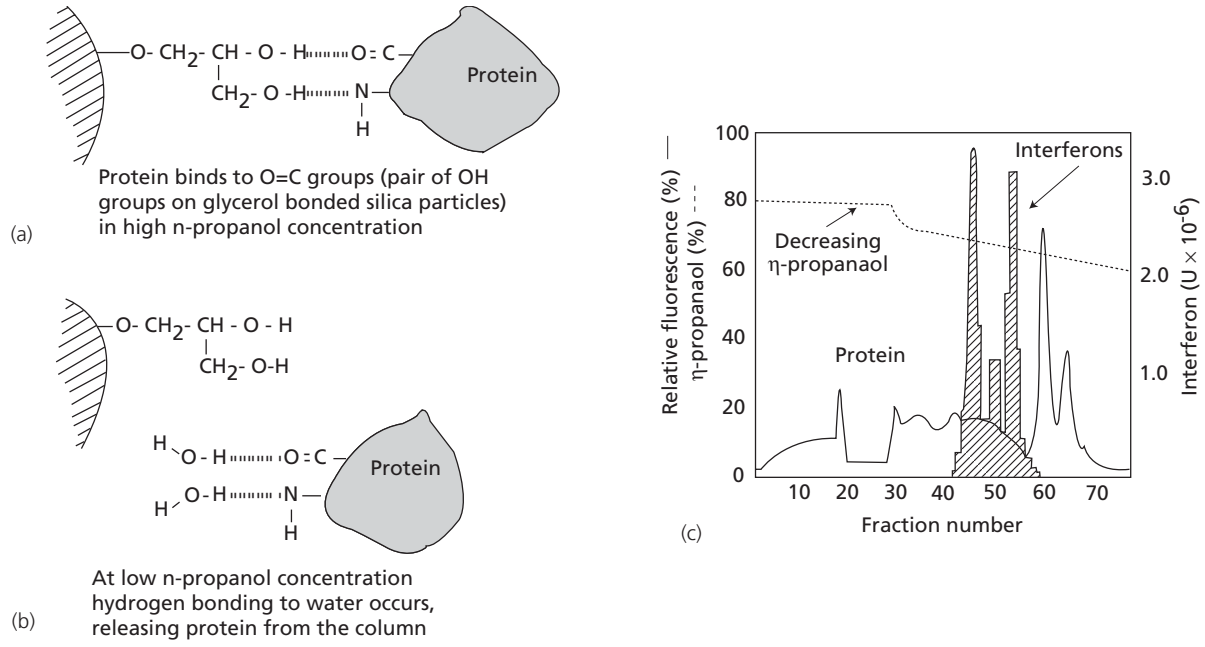

Figure 4 *Hydrogen-bonding high-performance liquid chromatography on diol silica. Hydrogen bonding of proteins to columns containing glycerol covalently attached to silica was used to separate interferon species. As hydrogen bonds are relatively weak bonds and are readily made to the water in which the protein is dissolved, in order to force proteins to bind to the diol silica, it was necessary to use very high concentrations of* n*-propanol. Upon reduction of the* n*-propanol concentration, proteins are released from the column in order of their increasing ability to form hydrogen bonds with the diol groups. Those proteins released first form the weakest hydrogen bonds.* **(a)** *Protein binds to diol groups (pair of OH groups on glycerol-bonded silica particles) in high* n*-propanol concentration.* **(b)** *At low* n*-propanol concentration hydrogen bonding to water occurs, releasing protein from the column. Multiple molecular species of human leukocyte interferon (α, β, and γ) are shown in the figure. These designations should not be confused with the subsequent nomenclature of IFN-α, IFN-β, and IFN-γ assigned to leukocyte, fibroblast, and immune interferons, respectively.*

in response to induction with poly(I)·poly(C). Hu-IFN-γ was the designation given for human immune interferon.

Multiple species of leukocyte interferon

During the purification of leukocyte interferon, it became evident that there were multiple species (see Figures 4 and 5). Ten separate species listed as α1, α2, β1, β2, β3, γ1, γ2, γ3, γ4 and γ5 were purified (Table 1). These are all α interferons under the current nomenclature. The designations initially used refer to the peaks obtained on chromatography. It was astonishing at the time, particularly after peptide mapping and sequencing of some of these species, to find a family of proteins that exhibited different activities uncovered (see below).

Human leukocyte interferon is heterogeneous and several bands containing antiviral activity ranging in M_r from 15 000 to 21 000 were observed on SDS–PAGE (Stewart 1974). Heterogeneity of human leukocyte interferon was also observed by isoelectric focusing (Stewart et al. 1977) and several types of chromatographic procedures (Chen et al. 1976; Jankowski et al. 1976; Torma and Paucker 1976; Grob and Chadha 1979; M. Rubinstein et al. 1981). As noted above, our initial work with HPLC revealed three major groups of interferon species that were labeled α, β, and γ according to their order of elution from a LiChrosorb Diol (polar-bonded phase) column (M. Rubinstein et al. 1979b, 1981). These groups were further resolved into several homogeneous components. Although others had reported heterogeneity in crude human leukocyte interferon preparations (Chen et al. 1976; Jankowski et al. 1976; Torma and Paucker 1976; Grob and Chadha 1979), this was not thought to be caused by amino acid sequence heterogeneity. In fact, it had been reported by a number of groups that leukocyte interferon contained carbohydrate and that heterogeneity was the result of differences in the carbohydrate content of the protein (Bose et al. 1976; Bose and Hickman 1977; Bridgen et al. 1977; Stewart et al. 1977). Thus, the well-established heterogeneity of human leukocyte interferon was attributed to differences in the degree of glycosylation. However, five purified species of leukocyte interferon examined contained no detectable carbohydrate: the amino sugar content of each species analyzed (α1, β1, β2, β3, and γ3) was determined to be much less than one residue of either glucosamine or galactosamine per molecule of interferon (M. Rubinstein et al. 1981). Allen and Fantes (1980) also found no carbohydrate on the species of leukocyte interferon that they purified. We therefore concluded that, contrary to the prevailing dogma, human leukocyte interferon is

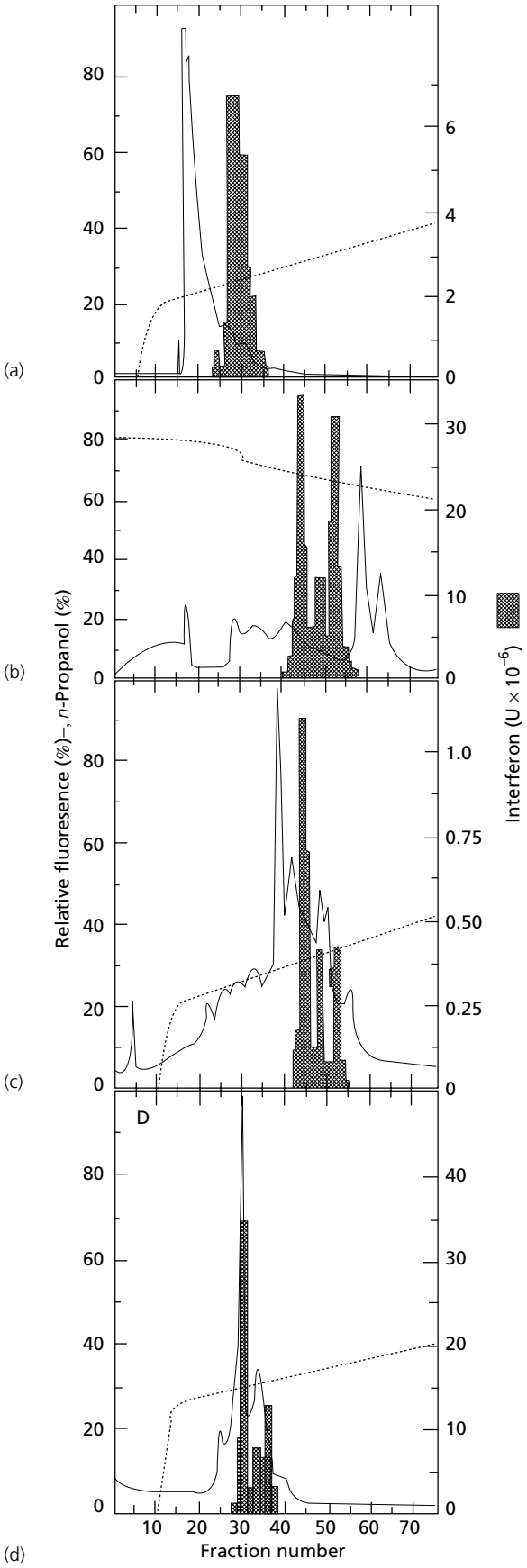

largely devoid of carbohydrate. Furthermore, because peptide mapping and sequencing revealed significant structural differences among the species, we concluded that leukocyte interferon represents a family of homologous proteins. By analogous procedures, additional leukocyte interferon species were isolated from cultured myeloblasts (Hobbs et al. 1981; Hobbs and Pestka 1982). Since our initial purification, other reports (Allen and Fantes 1980; Berg and Heron 1981; Zoon 1981a, b) have also described multiple species of leukocyte interferon.

Amino acid sequences of leukocyte interferons

As only relatively small amounts of each species were isolated in these early experiments, it was difficult to obtain information about their amino acid sequences. Determinations of the amino acid sequences of the amino-terminal end of human IFN-α were reported (Levy et al. 1980; Zoon et al. 1980), and that of IFN-β as well (Knight et al. 1980; Okamura et al. 1980; Stein et al. 1980; Friesen et al. 1981). It was clear that all the amino acid sequences obtained for human fibroblast interferon were identical and that we all had purified and sequenced the same protein. At the time, it was comforting to know that the new microsequencing procedures were dependable. However, it was striking that the amino-terminal sequences of individual species of human leukocyte interferon were different (Levy et al. 1980; Zoon et al. 1980). The differences confirmed that the leukocyte interferons consisted of a family of closely related proteins. We subsequently isolated a human interferon DNA recombinant (Maeda et al. 1980, 1981; Pestka 1983b), the coding sequence of which was virtually identical to the sequence of one of our purified proteins.

Levy et al. (1981) and Shively et al. (1982) reported amino acid sequences of three species of human leukocyte interferon. Additional sequences were reported by Zoon (1981a). Allen and Fantes (1980) reported the sequences of tryptic fragments obtained from a mixture of several leukocyte interferon species. All these sequences were sufficiently different to establish very clearly the concept of a family of closely related proteins. As described above (Maeda et al. 1980; Pestka 1983b), the first clone of human leukocyte interferon isolated in our laboratory was almost identical in

Figure 5 *High-performance liquid chromatography of interferon. Chromatography on* **(a)** *LiChrosorb RP-8 at pH 7.5;* **(b)** *LiChrosorb diol at pH 7.5; and* **(c)** *LiChrosorb RP-8 at pH 4.0 of the γ peak of part* **(b)**. **(d)** *Rechromatography on LiChrosorb RP-8 of the major activity peak of* **(c)**. *The conditions were similar to those of step* **(c)**. *Several preparations carried through step* **(c)** *were pooled (1.3 × 10⁷ units) and applied to the last column. The gradations on the abscissa correspond to the end of the fractions. The solid lines on the graph represent protein as measured by the fluorescamine method (Rubinstein et al. 1979b; Pestka 1983b).*

Table 1 *Human leukocyte interferons first purified*

Species	Molecular mass	Specific activity on bovine MDBK cells (units/mg × 10^{-8})	Specific activity on human AG-1732 cells (units/mg × 10^{-8})
α1	16 500	2.6	2.6
α2	16 200	4	3
β1	16 200	3.4	4.4
β2	16 500	4	2
β3	21 000	4	3
γ1	17 700	2.6	2
γ2	17 700	4	1.5
γ3	17 200	3.5	0.15
γ4	21 000	3.5	4
γ5	16 500	0.9	0.02

Data taken from (M. Rubinstein et al. 1981). The species designations refer to high-performance liquid chromatography peaks not what is now termed IFN-α, IFN-β, and IFN-γ.

sequence to human leukocyte interferon-α2 and interferon-β species, which were, however, 10 amino acids shorter than expected from the DNA sequence. It should be noted that during this time several groups (Iwakura et al. 1978; Kawakita et al. 1978; Maeyer-Guignard et al. 1978; Cabrer et al. 1979; Kawade et al. 1981; Maeyer-Guignard 1981) reported the purification of mouse interferons, and some amino acid sequences were also reported (Taira et al. 1980).

Carbohydrate content

The carbohydrate content of the species of human IFN-α, which were derived from patients with CML and from normal donors, was determined (Labdon et al. 1984). Amino sugar content was measured by HPLC and fluorescamine detection of acid hydrolysates of each sample (Labdon et al. 1984). O-linked glycosylation was also detected by a combination of HPLC, enzymatic analysis, and SDS–PAGE (Adolf et al. 1991). Two species showed significant amounts of glucosamine (Labdon et al. 1984). Most of the purified species of leukocyte interferon from a myeloblast cell line were also tested and two species were found to contain sugar residues. These forms also differed from the CML interferons in that they revealed the presence of greater amounts of galactosamine. The apparent lack of carbohydrate in some of the higher-molecular-mass species of interferon implicated factors other than glycosylation in the molecular-mass differences. The results indicated that some species of IFN-α are glycosylated to various degrees. It was later shown that a natural form of Hu-IFN-α2 was O-glycosylated (Adolf et al. 1991) and that Hu-IFN-ω is glycosylated (Adolf et al. 1990). Considering that the recombinant human IFN-α species produced in *Escherichia coli* do not contain carbohydrate, it was useful to discover that most of the human IFN-α species were devoid of carbohydrate.

PURIFICATION OF HUMAN FIBROBLAST INTERFERON-β

Throughout this chapter, the details focus on Hu-IFN-α because of space limitations. However, sufficient references to the other type I interferons are provided to make the chapter comprehensive.

Native human IFN-β is the major interferon produced by human fibroblasts in response to induction with poly(I)·poly(C) (Havell and Vilcek 1972; Knight 1976; Berthold et al. 1978; Stein et al. 1980; Friesen et al. 1981; Leong and Horoszewicz 1981; Van Damme and Billiau 1981). Figure 6 illustrates the production of IFN-β from human fibroblasts. The media from fibroblasts are collected and the native Hu-IFN-β was partially purified by a number of procedures (Knight 1976; Berthold et al. 1978; Cabrer et al. 1979; Stein et al. 1980; Friesen et al. 1981). It was purified to homogeneity with the use of HPLC (Stein et al. 1980; Friesen et al. 1981). Native IFN-β was found to be glycosylated. The partially purified material was used in initial clinical trials, but only after it was produced by recombinant DNA technology did major clinical trials proceed.

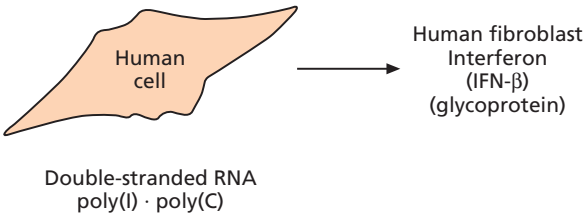

Figure 6 *Production of fibroblast interferon (IFN-β). Human fibroblasts were grown in culture and then exposed to the double-stranded RNA, poly(I)·poly(C), and cycloheximide for about 4 h at which time actinomycin D was added. At about 6 h the medium was replaced to remove the poly(I)·poly(C), cycloheximide, and actinomycin D. The culture fluid containing fibroblast interferon was harvested at 24 h.*

The recombinant human IFN-α species

As recombinant DNA technology offered an opportunity to produce large amounts of Hu-IFNs economically, many scientific teams set out to clone them in bacteria. Several groups achieved the isolation of recombinants for several Hu-IFN-α species (Maeda et al. 1980; Nagata et al. 1980) and for IFN-β (Derynck et al. 1980; Goeddel et al. 1980a; Houghton et al. 1980; Maeda et al. 1980; Taniguchi et al. 1980), arriving at their goals by somewhat different but analogous approaches. The cloning and expression of Hu-IFN-αA as an illustration of these procedures are described.

Isolation of Hu-IFN DNA sequences was a formidable task because it meant preparing DNA recombinants from cellular mRNA that was present at a low level. This task had never been accomplished previously from a protein with an unknown structure. In addition, in order to reconstruct DNA recombinants that would express natural IFN, it is useful to know the partial amino acid sequence of the proteins, particularly at the amino- and carboxy-terminal ends. Without this information synthesis of natural Hu-IFN in bacterial cells would not have been possible. Thus, purification of the Hu-IFNs and determination of their structure (M. Rubinstein et al. 1978, 1979b, 1981b; Zoon et al. 1979; Allen and Fantes 1980; Knight et al. 1980; Levy et al. 1981; Hobbs and Pestka 1982; Shively et al. 1982) assisted us in these efforts.

To isolate recombinants containing the human DNA corresponding to IFN-α, we used a number of procedures. First, it was necessary to isolate and measure the IFN mRNA. This was accomplished several years earlier when IFN mRNA was translated in cell-free extracts (Pestka et al. 1975; Thang et al. 1975) and in frog oocytes (Reynolds et al. 1975; Cavalieri and Pestka 1977; Cavalieri et al. 1977a, 1977b). The next step was to prepare sufficient mRNA from cells synthesizing IFN, and this was accomplished with both fibroblasts and leukocytes (Familletti et al. 1981a; McCandliss et al. 1981a). A library of complementary DNA (cDNA) was prepared from a template of partially purified mRNA isolated from human leukocytes synthesizing IFN. The next and hardest part of the procedure was to find in this vast library of recombinant plasmids those that contained DNA encoding IFN. We devised an indirect two-stage procedure to identify clones containing interferon sequences. In the first stage, we screened all the bacterial colonies to find those with cDNA made from the RNA of induced cells; among these there might have been some carrying IFN cDNA. We therefore screened all the recombinants for their ability to bind to mRNA from cells synthesizing IFN (induced cells), but not to mRNA from uninduced cells (those not producing IFN). To do this, individual transformant colonies were screened by colony hybridization for the presence of induced, specific sequences with ^{32P}-labeled IFN mRNA (mRNA from induced cells) as the probe. In the presence of excess mRNA from uninduced cells, recombinants that were representative of mRNA sequences existing only in induced cells should be evident on hybridization. This screening procedure allowed us to discard about 90 percent of the colonies: as their plasmids carried no induced cDNA, these could not encode IFN (Maeda et al. 1980, 1981).

In the second stage, we identified those recombinants containing the IFN DNA sequences among the remaining 10 percent. To do this, we pooled the recombinant plasmids in groups of 10 and examined these for the presence of IFN-specific sequences by an assay that depends on hybridization of IFN mRNA to plasmid DNA (Maeda et al. 1980; McCandliss et al. 1981b). Plasmid DNA from 10 recombinants was isolated and covalently bound to diazobenzyloxymethyl (DBM) paper. The mRNA from induced cells was hybridized to each filter. Unhybridized mRNA was removed by washing. After the specifically hybridized mRNA was eluted, both fractions were translated in *Xenopus laevis* oocytes. Once a positive group had been found (one in which the specifically hybridized mRNA yielded IFN after microinjection into frog oocytes), it was necessary to identify the specific clone or clones containing IFN cDNA. The 10 individual colonies were grown, the plasmid DNAs were prepared, and each individual DNA was examined by mRNA hybridization as above. By these procedures a recombinant, plasmid 104 (p104), containing most of the coding sequence for a Hu-IFN-α was identified (Maeda et al. 1980). The DNA sequence was determined and found to correspond to what was then known of the amino acid sequence of purified Hu-IFN-α (Levy et al. 1980, 1981). The cDNA insert in p104 contained the sequence corresponding to more than 80 percent of the amino acids in IFN-αA, but not for those at its amino-terminal end. It was, therefore, used as a probe for finding a full-length copy of the IFN cDNA sequence which could be used for expression of Hu-IFN-αA in *E. coli*. In addition, p104 DNA was used to isolate DNA sequences corresponding to other IFN-α species directly from a human gene bank.

Examination of the coding regions of the IFN-α genes that were isolated in our laboratory and others have shown that these correspond to a family of homologous proteins, the IFN-α species (Rubinstein et al. 1979b; Pestka 1983b), which are closely related to each other and yet each is unique in its amino acid sequence. Thus, the previously discovered heterogeneity in Hu-IFN-α was shown to be at least in part the result of distinct genes representing each expressed Hu-IFN-α sequence. The cloned Hu-IFN-αA, which was the first one that we isolated, corresponds to the natural Hu-IFN-α species which we purified from the mixture present in IFN-α by HPLC and termed α2 (see above). By similar proce-

dures to those described for 104, p101 was shown to contain the sequence for Hu-IFN-β (Maeda et al. 1980). Thus, the nucleotide sequences coding for Hu-IFN-α and Hu-IFN-β were identified.

The recombinant human IFN-α genes and proteins

A summary of the IFN-α genes and proteins reported is given in Table 2. There are in essence 14 human genes that comprise the IFN-α family. Minor variants consisting of one or two amino acid differences account for the multiple alleles (Pestka 1983a, 1983b, 1986; Diaz et al. 1994). Excluding the pseudogene *IFNAP22*, there are 13 genes. One of them, *IFNA10* is also a pseudogene in one allelic form. There are 13 proteins expressed from these genes. The protein produced from gene *IFNA13* is identical to that produced from *IFNA1*. Thus, there are 12 separate IFN-α proteins (and allelic forms) produced from these 14 genes (Table 2).

ACTIVITIES OF THE PURIFIED IFN-α SPECIES

After purification of the IFN-α species, we determined their activities (see Table 1). It was evident that each interferon species exhibited a distinct profile of antiviral activity (AV) on human AG-1732 and bovine MDBK cells (Evinger et al. 1980a, b, 1981; Pestka et al. 1980a, b; Evinger and Pestka 1981; S. Rubinstein et al. 1981). The overall ratios varied over a range of 200-fold. This presaged the idea that each IFN-α species had a different activity profile. When the profile of activities was expanded to evaluate antiproliferative activity (AP) (Table 3) and natural killer (NK) cell stimulatory activity (Table 3), it was observed that there was no clear correlation among the activities. Thus, the IFN-α family represents related and homologous proteins each exhibiting a unique activity profile. This is illustrated further below.

Table 2 *Human interferon α genes (14) and proteins (12/13)*

Genes	Proteins
IFNA1	IFN-αD, IFN-α1
IFNA2	IFN-αA (IFN-α2a), IFN-α2 (IFN-α2b), IFN-α2c
IFNA4	IFN-α4a (IFN-α76), IFN-α4b
IFNA5	IFN-αG, IFN-α5, IFNα61
IFNA6	IFN-αK, IFN-α6, IFN-α54
IFNA7	IFN-αJ, IFN-αJ1, IFN-α7
IFNA8	IFN-αB2, IFN-αB, IFN-α8
IFNA10	IFN-αC, IFN-α10, IFN-αL, IFN-α6L
IFNA13	IFN-α13 (sequence identical to IFN-α1)
IFNA14	IFN-αH, IFN-αH1, IFN-α14
IFNA16	IFN-αWA, IFN-α16,IFN-αO
IFNA17	IFN-αI, IFN-α17, IFN-α88
IFNA21	IFN-αF, IFN-α21
IFNAP22	IFN-αE

Table 3 *Relative activities of human leukocyte interferons*

Species	AV	AP	NK
α_1	59	22	Low
α_2	68	12	Low
β_1	100	ND	Low
β_2	45	100	High
β_3	68	6	High
γ_1	45	12	High
γ_2	34	47	Low
γ_3	3.4	16	Low
γ_4	91	5	Low
γ_5	0.45	75	Low

AV, relative antiviral activity; AP, relative antiproliferative activity; NK, relative natural killer cell activity; ND, not done. The activities are relative, with the value 100 arbitrarily set for the interferon with the highest activity.

After the production and purification of the recombinant IFN-α species, a number of activities were determined. The sequences of various IFN-α species were described (Goeddel et al. 1980b; Goeddel and Pestka 1982, 1989; Henco et al. 1985). As can be seen in Table 4, the hybrid IFN-αA and IFN-αD molecules vary markedly in their AV, AP, and NK cell activity (Herberman et al. 1982a; Ortaldo et al. 1982, 1983a, 1983b, 1984; Rehberg et al. 1982). It is possible to change radically the properties of an interferon by making hybrids and generate properties that neither one of the parental molecules exhibits, e.g. the hybrid Hu-IFN-αA/D (*Bgl*) constructed from IFN-αA and IFN-αD exhibits high activity on mouse cells, a property neither of the parental molecules exhibits (Rehberg et al. 1982). As with the natural IFN-α species, the ranges in the activity ratios vary about 100-fold among the hybrid species (Table 4). A hybrid between Hu-IFN-αB2 (α8) and Hu-IFN-αD (α1) was constructed (called Hu-IFN-αB/D) and tested in humans with results comparable to the approved recombinant interferons (Gangemi et al. 1989; von Wussow et al. 1991; Hochkeppel et al. 1992; Ritch et al. 1992; Schellekens et al. 1996). Consensus IFN (IFN-con1) is a synthetic, non-naturally

Table 4 *Ratio of specific molecular activities*

IFN-α	AV/AP	AV/NK
A	0.38	41
D	0.80	1.3
A/D (*Bgl*)	0.59	95
A/D (*Pvu*)	0.14	3.2
D/A (*Bgl*)	1.7	6.0
D/A (*Pvu*)	0.14	13
A/D/A	0.60	1.8

AV, relative antiviral activity; AP, relative antiproliferative activity; NK, relative natural killer cell activity.

Table 5 *Effect of interferons on human natural killer (NK) cell activity*

	IFN-α (units/ml)
αA, α2	2
αB, α8	1
αC, α10	2
αD, α1	7
αF, α21	89
αI, α17	11
αJ, α7	> 10 000
αK, α6	29

The values in the table represent the concentration for 50 percent stimulation of natural killer cell activity. Data from Ortaldo et al. (1984).

occurring IFN-α hybrid designed by assigning each of the most frequently observed amino acids appearing at each position in the Hu-IFN-α subtypes (Klein et al. 1988). For the most part it is almost a hybrid between IFN-αA and IFN-αF with only five differences, several of which were introduced for purposes of cloning. As a result of the large number of amino acid differences between the hybrid IFN-α species and the naturally occurring IFN subtypes, there is the potential for greater immunogenicity than with the natural IFNs in long-term treatment. However, clinical studies with both the IFN-αB/D and IFN-αCon1 molecules suggests that immunogenicity is not a significant problem. Among the normal IFN-α species (Table 5), the individual species differ by over 10 000-fold in NK activity alone (Ortaldo et al. 1983a, 1984). IFN-αJ (IFN-α7) exhibits virtually no NK activity (Table 5) and can act as an antagonist of NK activity, blocking other IFN-α species from stimulating NK cells (Ortaldo et al. 1984).

FUNCTIONAL UNIT OF IFN-α

As we did not know whether IFN-α functioned as a monomer or a dimer, we used electron bombardment to determine the functional unit (Pestka et al. 1983; Kempner and Pestka 1986). The results are summarized in Figure 7. The functional unit of IFN-α was shown to be a monomer, whether the species were produced in *E. coli* or by human leukocytes (Pestka et al. 1983; Kempner and Pestka 1986). The functional unit of IFN-β appears to be a dimer (Pestka et al. 1983; Kempner and Pestka 1986) whereas that of IFN-γ was shown to be a tetramer (Pestka et al. 1983; Kempner and Pestka 1986; Langer et al. 1994). One crystal structure of human IFN-α2b suggested a dimer structure (Radhakrishnan et al. 1996). However, the solution structure is consistent with a monomer (Klaus et al. 1997). These results need to be understood in terms of the receptor–ligand interaction. The structural results suggest that IFN-α and IFN-β interact with the receptor differently,

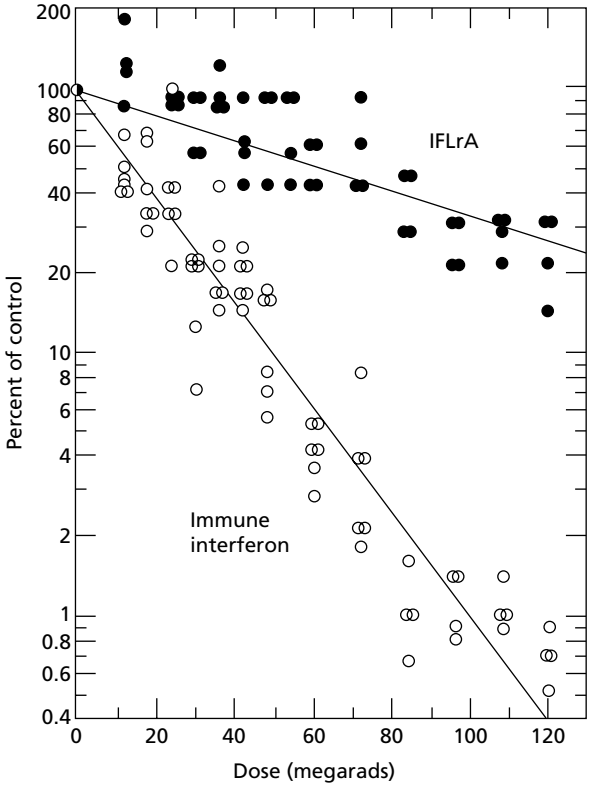

Figure 7 *Electron bombardment of recombinant human leukocyte (Hu-IFN-αA) and immune (IFN-γ) interferons. Data from Batcheler et al. (1986) and Fish et al. (1986). The molecular weight estimates for the functional units of the recombinant proteins are as follows: IFN-αA, 20 000; IFN-γ, 73 000. The monomer molecular masses are: IFN-αA, 19 219; IFN-γ, 17 126.*

which will very probably explain some of their functional differences.

General remarks

The IFN-α family consists of 13 expressed alleles producing 12 different proteins that exhibit remarkably different activity profiles. For the most part, the IFN-α species are not glycosylated although some contain carbohydrate. The recombinant proteins produced in *E. coli* have properties essentially equivalent to the proteins produced by human cells. Predominantly, only one recombinant IFN-α protein is used therapeutically (IFN-α2a, IFN-α2b, and IFN-α2c, allelic variants) so that the remaining IFN-α species remain an untapped reservoir of opportunity. Why the body produces so many of these interferons is an unanswered question. As our understanding of the mechanism of their receptor interactions develops, some of these answers should be forthcoming.

Although purification of the interferons to homogeneity remained elusive for about two decades after their discovery, they are now available in purified form. The largest amounts available are the species

produced in *E. coli*. For purification, immunoaffinity chromatography has proved to be generally useful both on a laboratory and on a commercial scale. Nevertheless, techniques other than immunoaffinity chromatography have also proved to be successful. The availability of these proteins for laboratory and clinical studies has already catalyzed extensive new developments with them. The interferons are approved therapeutic agents worldwide. As the understanding of these agents becomes more extensive, we will gain new insights into their actions and develop new applications for their use.

RECOMBINANT HUMAN IFN-β

There is only one human IFN-β gene. The IFN-β was cloned by a number of laboratories (Derynck et al. 1980; Goeddel et al. 1980a; Houghton et al. 1980; Taniguchi et al. 1980) and expressed in *E. coli* and in eukaryotic vectors for expression in mammalian cell such as mouse or Chinese hamster ovary (CHO) cells (Canaani and Berg 1982; Zinn et al. 1982; Higashi et al. 1983; Maroteaux et al. 1983). IFN-β is active when expressed with or without carbohydrate. Similar to IFN-α, IFN-β exhibits antiviral and antiproliferative activity. The major use of IFN-β has been in the treatment of multiple sclerosis (Keegan and Noseworthy 2002), but the mechanisms by which it functions to ameliorate this disease are far from understood.

INTERFERON RECEPTORS

As noted above, the interferons are divided into two groups designated type I (IFN-α, IFN-β, IFN-κ, IFN-ω, and IFN-τ) and type II (IFN-γ) (Pestka 1981, 1986, 1997; Pestka et al. 1987; LaFleur et al. 2001). The past and current designations of the interferons are given in Table 6. Although leukocyte and fibroblast interferons were the original designations based on the source of the interferons, they usually consisted of multiple components dependent on the method of production. In general, leukocyte interferon obtained from white blood cells predominantly contained IFN-α species, but small quantities of IFN-β and IFN-ω were also present. Human fibroblast interferon consisted of IFN-β when induced by poly(I)·poly(C), but contained both IFN-α and IFN-β when induced by viruses, although IFN-β was predominant.

Similar to most cytokines and growth factors the actions of interferons are mediated by interaction with specific cell-surface receptors and the Jak–Stat signal transduction pathway (Aguet 1980; Lengyel 1982; Pestka et al. 1987; Langer and Pestka 1988; Sen and Lengyel 1992; Darnell et al. 1994; Uzé et al. 1995), although other pathways are increasingly being delineated (Raz et al. 1994; Durbin et al. 2000; Dalod et al. 2002). Competition binding studies demonstrated that type I IFNs share the same receptor complex, whereas type II IFN (IFN-γ) binds to a distinct receptor (Branca and Baglioni 1981; Pestka et al. 1987; Langer and Pestka 1988; Flores et al. 1991; Li and Roberts 1994; Alexenko et al. 1995). Components of both these classes of receptors were cloned. With the cloning of the receptor components, the mechanism of interferon action and the resultant signal transduction events were delineated in remarkable detail. Two transmembrane chains of the type I receptor complex (Uzé et al. 1990; Cleary et al. 1992, 1994; Domanski et al. 1995; Novick et al. 1994; Soh et al. 1994c; Lutfalla et al. 1995; Cook et al. 1996) and two chains of the type II receptor complex (Rashidbaigi et al. 1986; Jung et al. 1987; Aguet et al. 1988; Gray et al. 1989; Hemmi et al. 1989, 1994b; Kumar et al. 1989; Munro and Maniatis 1989; Cofano et al. 1990; Soh et al. 1993, 1994a) were characterized and cloned. As the IFN-γ–receptor complex (type II) has been characterized much better than the type I interferon–receptor complex, the IFN-γ receptor is discussed as a preface to the type I receptor. Understanding of the molecular events from ligand binding to signal transduction has elucidated several new paradigms that provide a basis for understanding the family of multichain cytokine receptors and the specificity of signal transduction. The IFN-γ–receptor complex has thus provided the basic model for other members of the family of class II cytokine receptors including the type I interferon and interleukin (IL) 10 receptor complexes.

Table 6 *Classes of interferons*

Original designation	Greek name	Current designation	Type
Leukocyte	Alpha	IFN-α	Type I
Fibroblast	Beta	IFN-β	Type I
Leukocyte (II)	Omega	IFN-ω	Type I
Keratinocyte	Kappa	IFN-κ	Type I
Trophoblast	Tau	IFN-τ	Type I
Immune	Gamma	IFN-γ	Type II

Receptor nomenclature

The designations of the interferon receptor components are given in Tables 7 and 8. These receptor complexes consist of two or more components. It also appears that the individual components may contribute to one extent or another to ligand binding so that designations such as an α subunit for the ligand-binding component and the β subunit for the signal transduction subunit are not warranted. The subunits are named in the order in which they were cloned and discovered as distinct entities. Alternate designations for the subunits that have been used are also given (Tables 7 and 8).

Table 7 *Designations of human interferon receptor components: type I interferon (α/β/ω/τ) receptor*

Chain or mRNA	Gene	References
Hu-IFN-αR1, Hu-IFN-αR1a	*IFNAR1*	Uzé et al. (1990)
Hu-IFN-αR1s, Hu-IFN-αR1b (splice variant missing exons IV, V)	*IFNAR1*	Cleary et al. (1994); Cook et al. (1996)
Hu-IFN-αR2a (soluble form)	*IFNAR2*	Novick et al. (1994)
Hu-IFN-αR2b (short form)	*IFNAR2*	Novick et al. (1994)
Hu-IFN-αR2c (long form)	*IFNAR2*	Domanski et al. (1995); Lutfalla et al. (1995)
αYAC (F136C5)	*IFNAR1* and *IFNAR2*	Soh et al. (1994c)

Table 8 *Designations of human interferon receptor components: type II interferon (IFN-γ) receptor*

Chain	Gene	References
Hu-IFN-γR1 (Hu-IFN-γRα; ligand binding chain of receptor)	*IFNGR1*	Aguet et al. (1988); Kumar et al. (1989); Hemmi et al. (1989); Gray et al. (1989); Munro and Maniatis (1989); Cofano et al. (1990)
Hu-IFN-γR2 (accessory factor-1; AF-1; Hu-IFN-γRβ; second chain of receptor)	*IFNGR2*	Soh et al. (1994a); Hemmi et al. (1994b)

THE TYPE II INTERFERON (IFN-γ) RECEPTOR

Chromosomal localization of the IFN-γ receptor ligand-binding chain and discovery of two chains required for activity

It was shown by study of ligand-binding competition that the IFN-γ receptor was distinct from that of the receptor that bound IFN-α and IFN-β (Branca and Baglioni 1981). Through somatic cell genetic techniques, Rashidbaigi et al. (1986) demonstrated that the gene for the ligand-binding chain of the human IFN-γ receptor was localized to human chromosome 6 (specifically 6q). Nevertheless, although somatic cell hybrids that contain this region of, or the entire, chromosome 6 exhibited excellent binding of IFN-γ, the ligand was unable to initiate any biological activities. This study led Jung et al. (1987, 1988) to the discovery that an additional component located on human chromosome 21 (specifically 21q) was required for function through the IFN-γR1 chain encoded on human chromosome 6. Thus, two species-specific components were involved as part of the functional IFN-γ receptor: the ligand-binding chain of the receptor, IFN-γR1; and the second chain of the receptor, IFN-γR2, we initially designated accessory factor-1 (AF-1) that is required for signal transduction through the receptor. These somatic cell genetic experiments led to the discovery of the location of the ligand-binding chain (IFN-γR1) to chromosome 6 and the location of the second chain, IFN-γR2, to human chromosome 21 and set the stage to isolate and clone these components. The comparable mouse chains were localized to mouse chromosomes 10 and 16, respectively (Mariano et al. 1987, 1996). Tables 7 and 8 summarize the suggested nomenclature for these chains.

Subsequent to the localization of the ligand-binding chain, both the human (Aguet et al. 1988) and mouse (Gray et al. 1989; Hemmi et al. 1989; Kumar et al. 1989; Munro and Maniatis 1989; Cofano et al. 1990) IFN-γR1 chains were cloned and shown to bind ligand. In addition, these studies confirmed our observations that the ligand-binding component was insufficient for generating a biological response. To generate a biological response, it was necessary to have the ligand-binding chain plus the second chain IFN-γR2 (AF-1). It should be noted that the biological response measured in these assays was induction of class I major histocompatibility complex (MHC) antigen expression. By reconstituting functional activity with the cloned Hu-IFN-γR1 and human chromosome 21, it was definitively demonstrated that the ligand-binding chain was the necessary and sufficient component contributed by human chromosome 6 (Jung et al. 1990). It was thus concluded that the functional receptor consisted of two chains, which are now designated IFN-γR1 and IFN-γR2 (see Table 7).

Study of chimeric human and mouse IFN-γR1 chains demonstrated that the ability of mouse and human IFN-γ species to stimulate MHC class I antigen induction in various cells transfected with the chimeric receptors required the homologous extracellular domain of the receptor, e.g. the chimeric receptor HMM (human extracellular, murine transmembrane, and murine intracellular domains, respectively) was able to respond to Hu-IFN-γ only in mouse cells or hamster cells containing human chromosome 21 or 21q (Gibbs et al. 1991; Hemmi et al. 1992; Hibino et al. 1992; Kalina et al. 1993). Modification of the mouse IFN-γR1 chain by

site-specific mutation was reported by Lai (1994) to yield an interesting ectodomain mutant chain (His196-Ala) that was able to bind ligand, but was not able to support signal transduction as measured by MHC class I antigen induction, growth inhibition, or activation of Stat1α. This appears to be the first ectodomain mutant that alters signal transduction, but not ligand binding. It should provide some interesting insight into how this His196 of the IFN-γR1 chain is involved in supporting signal transduction. Perhaps, this region of the receptor is required for interaction with the IFN-γR2 chain.

The IFN-γR1 chain

The IFN-γR1 chain serves to bind ligand. The functional architecture of the IFN-γR1 intracellular domain is concentrated in two specific regions. Distal from the membrane, a five-residue sequence (Y^{457}DKPH^{461}) is required for all signal transduction (Cook et al. 1992; Farrar et al. 1991, 1992). This sequence is completely conserved in human and mouse receptors. Y^{457} is phosphorylated on ligand binding, and serves as a recruitment site for Stat1α (Greenlund et al. 1994). The function of the other residues in the YDKPH motif are not known, but D^{458} and H^{461} are required (Cook et al. 1992; Farrar et al. 1992). Specific mutations have shown that the carboxy-terminal 29 amino acids have no apparent function (Cook et al. 1992). A membrane-proximal L^{266}PKS^{269} motif is required for receptor activity and Jak-1 binding (Kaplan et al. 1996).

The second receptor chain (IFN-γR2)

The second chain of the IFN-γ receptor was isolated by Soh et al. (1993, 1994a); Cook et al. (1994), and Hemmi et al. (1994b). The cDNA clones encode the necessary species-specific factor and are able to substitute for human chromosome 21 to reconstitute the Hu-IFN-γ receptor-mediated induction of class I HLA antigens. However, the factor encoded by the cDNA does not confer full antiviral protection against encephalomyocarditis virus (EMCV), suggesting that an additional factor encoded on human chromosome 21 may be required for reconstitution of antiviral activity against EMCV (Soh et al. 1993, 1994a; Cook et al. 1994; Lembo et al. 1996). Similar observations were made with the mouse IFN-γR1 and IFN-γR2 chains. Substitution of each of the tyrosine residues of the intracellular domain by phenylalanine did not alter the ability of the mouse IFN-γR2 chain to support signal transduction (Hemmi et al. 1994a). Kotenko et al. (1995) showed that the intracellular domain of the Hu-IFN-γR2 chain lacking the terminal 49 residues was totally inactive in MHC class I antigen induction, Jak1 and Jak2 phosphorylation, Stat1α activation, and tyrosine phosphorylation of the IFN-γR1 chain. Furthermore, cells expressing the Hu-IFN-γR2 chain alone did not crosslink or bind ligand; however, when both Hu-IFN-γR1 and Hu-IFN-γR2 chains were co-expressed in hamster cells, the association constant for binding of ligand increased about threefold and a specific band of a crosslinked Hu-IFN-γ–Hu-IFN-γR2 complex was observed (Kotenko et al. 1995). This indicated that the two chains of the receptor must be in close proximity as suggested by experiments with chimeric receptors noted above. Jak2 was found to associate with the IFN-γR2 intracellular domain (Kotenko et al. 1995). Residues 263–267 and 270–274 of the intracellular domain of IFN-γR2 act as a Jak2-binding site (Bach et al. 1996). The cytoplasmic domains of the IFN-γR2 subunits, similar to the cytoplasmic domains of the IFN-γR1 chains, can be interchanged between species with no loss of biological activity, confirming that the species-specific interaction of the IFN-γR1 and IFN-γR2 chains involves only the extracellular domains of the two proteins (Muthukumaran et al. 1996).

The functional IFN-γ receptor complex

A model for the functional IFN-γ receptor is shown in Figure 8. The IFN-γR1 chains bind the ligand whereas the IFN-γR2 chains serve to complete the complex for signal transduction.

Specificity of ligand binding

There are two distinct interactions that underlie species specificity: the binding of ligand to the IFN-γR1 chain, and the interaction of the IFN-γR1 and IFN-γR2, independent of ligand. The interaction of IFN-γ with the IFN-γR1 chain is highly species specific. Human IFN-γ stimulates no activity on rodent cells whatsoever, and rodent IFN-γ exhibits no activity on human cells. This effect is attributed to the interaction of the ligand with the IFN-γR1 chain. The presence of the second chain IFN-γR2 may increase the overall affinity of the ligand to the receptor complex slightly (Lai 1994; Kotenko et al. 1995; Marsters et al. 1995).

Specificity of the interactions of the two chains

Chimeric receptors were constructed between the mouse and human IFN-γR1 chains (Gibbs et al. 1991; Hemmi et al. 1992; Hibino et al. 1992; Kalina et al. 1993). The extracellular, transmembrane and intracellular domains were swapped between the human and mouse IFN-γR1 chains. The chains were introduced into mouse–human and hamster–human somatic cell hybrids that contained human chromosome 21, which expressed the Hu-IFN-γR2 chain. These results established that the extracellular domains of the two chains must be matched from the same species to enable the ligand to initiate signal transduction.

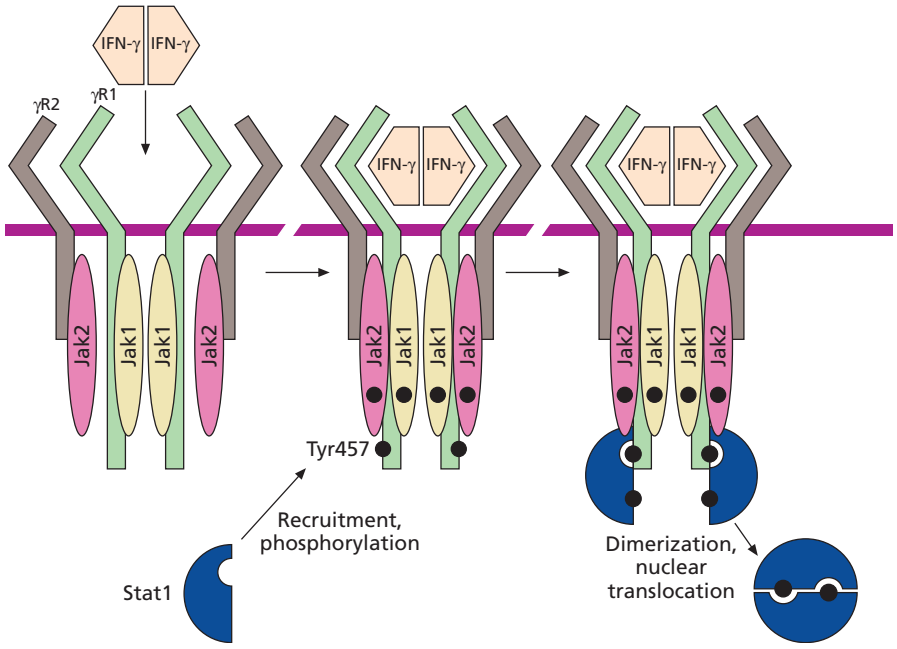

Figure 8 *Model of the IFN-γ receptor complex and signal transduction. In the three-dimensional structure it is likely that the extracellular domains of both IFN-γR1 and IFN-γR2 chains contact the ligand, IFN-γ.*

In a complementary series of experiments with hamster–mouse somatic cell hybrid cells, it was shown that mouse IFN-γ could activate the hybrid cells only when the mouse chromosome 16 was present in the hybrids and the IFN-γR1 chain contained the extracellular mouse domain of this chain (Hibino et al. 1992; Mariano et al. 1996).

Specificity of signal transduction

Each cytokine that utilizes the Jak–Stat signal transduction pathway activates a distinct combination of members of the Jak and Stat families. Thus, the Jaks, the Stats, or both could contribute to the specificity of ligand action. With the use of chimeric receptors involving the interferon-γ receptor (IFN-γR) complex as a model system, Kotenko et al. (1996) demonstrated that Jak2 activation is not an absolute requirement for IFN-γ signaling. Other members of the Jak family can functionally substitute for Jak2. IFN-γ can signal through the activation of Jak family members other than Jak2 as measured by Statlα homodimerization and MHC class I antigen expression (Figure 9). This indicates that Jaks are interchangeable in the Jak–Stat signal transduction pathway. The necessity for the activation of one particular kinase during signaling can be overcome by recruiting another kinase to the receptor complex (Figure 9). The results suggest that the Jaks do not contribute significantly to the specificity of signal transduction.

The Stats represent proteins containing -SH_2, -SH_3, and DNA-binding domains (for reviews, see Darnell et al. 1994; Fu 1995). The highly selective and specific interaction between Stat -SH_2 domains and the phosphotyrosine-containing Stat recruitment sites on the intracellular domains of the cytokine receptors determines which Stats are to be recruited to a particular receptor complex (Heim et al. 1995; Stahl et al. 1995). Thus, the major specificity of the pathway probably results in large part from the specificity of the Stat recruitment sites on the receptor chains. Other molecules that interact with Jaks and Stats may contribute to the specificity and range of the interaction (Pollack et al. 1995; Collum et al. 2000).

Rationale for the multichain receptor

In the current model, binding of IFN-γ causes oligomerization of the two IFN-γ receptor (IFN-γR) subunits, receptor chain 1 (IFN-γR1, the ligand-binding chain) and the second chain of the receptor (IFN-γR2), and causes activation of two Jak kinases (Jak1 and Jak2) as illustrated in Figure 8. In contrast, the erythropoietin receptor (EpoR) requires only one receptor chain and one Jak kinase (Jak2). Chimeras between the EpoR and the IFN-γR1 and IFN-γR2 chains demonstrated that the architecture of the EpoR and the IFN-γR complexes differs significantly (Muthukumaran et al. 1997). Although IFN-γR1 alone cannot initiate signal transduction, both the chimeric EpoR/γR1 (extracellular/intracellular) homodimer and the EpoR/γR1–EpoR/γR2 heterodimer generate responses characteristic of IFN-γ in response to Epo. Thus, the configuration of the extracellular domains influences the architecture of the intracellular domains.

In contrast to the growth hormone (GH) receptor (de Vos et al. 1992) and the EpoR (Watowich et al. 1992)

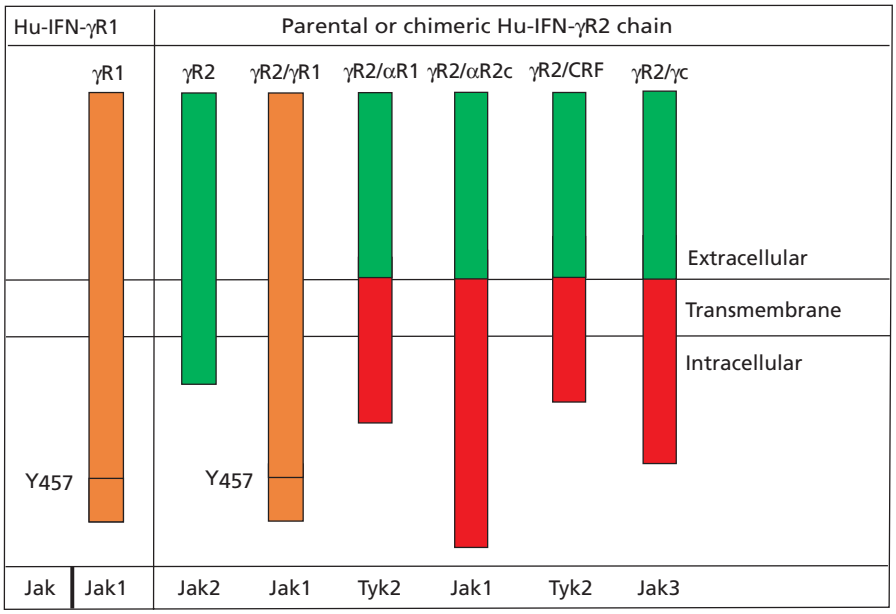

Figure 9 *Structure of chimeric receptors. Hu-IFN-γR1 (γR1) and Hu-IFN-γR2 (γR2) are the intact chains of the human IFN-γ receptor complex. All chimeric receptors have the extracellular domain of the human IFN-γR2 and the transmembrane and intracellular domains of different human receptors: γR2/γR1, Hu-IFN-γR1; γR2/αR1, Hu-IFN-αR1 (Uzé et al. 1990); γR2/αR2, Hu-IFN-αR2c (Domanski et al. 1995; Lutfalla et al. 1995); γR2/CRF, CRFB4 (Lutfalla et al. 1993); γR2/γC, IL-2 receptor γ_C chain (Takeshita et al. 1992). The short form Hu-IFN-αR2b binds Jak1 (Novick et al. 1994), but does not support signal transduction.*

complexes, when one IFN-γ homodimer binds two IFN-γR1 molecules, the two receptor subunits do not interact with one another and are separated by 2.7 nm (Walter et al. 1995) at their closest point. Therefore, although the IFN-γR1 chain possesses both a Jak1-association site and a Stat1α-recruitment site, alone it is unable to transduce a signal on homodimerization because the two Jak1 kinases are not in physical proximity to permit transphosphorylation (Figure 10a). Crystallographic analysis of the IFN-γ–IFN-γR1 complex suggests that each monomer of the IFN-γ homodimer binds one IFN-γR1 and one IFN-γR2 subunit (Walter et al. 1995). Thus the signal-transducing complex of IFN-γ consists of the IFN-γ homodimer bound to two IFN-γR1 and two IFN-γR2 chains (see Figures 8 and 10b) which recruit Jak1 and Jak2, respectively (Kotenko et al. 1995, 1996; Muthukumaran et al. 1996, 1997) and Jak2 phosphorylates Jak1, after which either kinase phosphorylates Tyr-457 of the IFN-γR1 chain. The phosphorylated segment of each IFN-γR1 chain recruits Stat1α, which is then phosphorylated by Jak1 or Jak2, and released to dimerize and form the active Stat1α. In contrast, with the EpoR/γR1 dimer, two Jak1 kinases are brought sufficiently close together to activate one another (Figure 10c). In the case of the EpoR/γR1–EpoR/γR2 dimer, one Jak1 and one Jak2 are in close apposition for Jak2 to phosphorylate Jak1 and initiate efficient downstream signaling events (Figure 10d). A major function of receptor dimerization is to bring two receptor-associated kinases together for transactivation and phosphorylation of the receptor chains. The cytoplasmic domain of the IFN-γR2 subunit serves to bring Jak2 kinase into the signal transduction complex (see Figures 8 and 10b) because the IFN-γR1 chain alone cannot bring Jak1 into sufficient proximity to initiate signal transduction.

For hormones, growth factors, and cytokines, the conversion of the extracellular ligand-binding event to the intracellular signal involves a change in the structure of the receptor. Depending on the ligand, this can take the form of receptor homodimers (Epo, GH), heterodimers (ciliary neurotropic factor (CNTF), leukemia inhibitory factor (LIF)), homotrimers (tumor necrosis factor (TNF)), and more complex assemblies (Stahl and Yancopoulos 1993). The IFN-γR2 subunit is a helper receptor subunit with a Jak2-association site, but no Stat-recruitment site; its intracellular domain can be substituted with the cytoplasmic domain of any receptor subunit that can bring a Jak kinase to the IFN-γ receptor complex to support signal transduction (Kotenko et al. 1996; Muthukumaran et al. 1996).

We propose that the multichain cytokine class II receptors have two major chains exemplified by the IFN-γ receptor complex (see Figure 10b). The ligand-binding chain (IFN-γR1) and the accessory chain (AC) (IFN-γR2; helper receptor) serve as a foundation for the functional IFN-γR complex (Kotenko et al. 1995, 1996; Muthukumaran et al. 1996, 1997). The geometry of the IFN-γR1 chain is such that its homodimerization yields a nonfunctional intracellular receptor complex. The accessory chain completes this function (see Figure 10b). The question arises of why

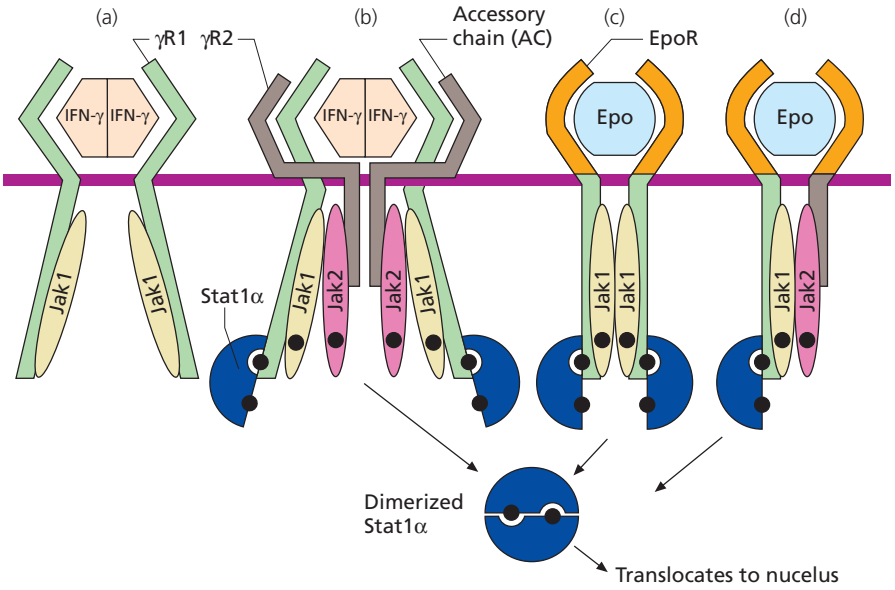

Figure 10 *Schematic representation of receptor complexes.* **(a)** *The IFN-γR1 homodimer bound to IFN-γ. The cytoplasmic domains of the two chains are too far apart to permit transactivation of the two Jak1 kinases.* **(b)** *The active heteromeric IFN-γ receptor complex with two IFN-γR1 and two IFN-γR2 subunits per complex. The IFN-γ homodimer binds to two IFN-γR1 chains, followed by its interaction with two IFN-γR2 chains. The associated Jak2 and Jak1 kinases activate one another by transphosphorylation, with subsequent phosphorylation and dimerization of Stat1α.* **(c)** *The EpoR/IFN-γR1 homodimer which, unlike the IFN-γR1 homodimer, permits transactivation of the two Jak1 molecules.* **(d)** *The structure of the heterodimer of EpoR/IFN-γR1 and EpoR/IFN-γR2 which is the putative active receptor complex.*

two separate chains should have evolved when one in the correct configuration would suffice. We postulate that the presence of two distinct chains provides more effective control and fine-tuning of responses to ligand, e.g. the differences in response of T-helper (Th)1 and Th2 cells to IFN-γ results from the lack of expression of the IFN-γR2 chain in the Th1 subset (Adolf et al. 1990, 1991) and allows exquisite fine-tuning of sensitivity to IFN-γ. It is also possible that receptors with multiple chains could recruit additional factors into the complex to generate a wider variety of intracellular signals. This could explain how receptors with multiple subunits could activate a greater number of specific pathways and signals than those with fewer elements in the receptor complex.

THE TYPE I INTERFERON (IFN-α/β/ω) RECEPTOR

Chromosomal localization, antibodies, and cDNA clones

Somatic cell genetic studies with human–rodent hybrid cells containing various combinations of human chromosomes have provided evidence that the presence of human chromosome 21 confers sensitivity of the rodent cells to human type I interferons (Tan et al. 1973; Slate et al. 1978; Epstein et al. 1982; Raziuddin and Gupta 1985). It was also demonstrated that antibodies to human chromosome 21-encoded cell surface components were able to block the action or binding of Hu-IFN-α to cells (Revel et al. 1976; Shulman et al. 1984). Later, Langer et al. (1990) demonstrated that 3x1S irradiation-reduced hamster–human somatic hybrid cells containing about 3 Mb (megabases) of chromosome 21q around 21q22.1 (Jung 1991; Soh et al. 1994c) were able to bind ^{32}P-labeled Hu-IFN-αA and generate a complex of about 150 kDa when crosslinked to the cell surface. During this time, the gene for the Hu-IFN-αR1 receptor chain was mapped to the 3x1S region (21q22.1) (Lutfalla et al. 1990). The paradoxical observation that Chinese hamster ovary 3x1S cells could bind Hu-IFN-αA, whereas the expression of the cloned receptor cDNA (Hu-IFN-αR1) in mouse cells did not confer binding to Hu-IFN-αA, suggested that the 3x1S region of human chromosome 21 contains other subunits of the receptor complex. This assumption was supported by the identification of two separate components after immunoprecipitation of ^{125}I-labeled Hu-IFN-αA–receptor complexes from 3x1S cell extracts with anti-IFN-α receptor antibody (Colamonici et al. 1990, 1992; Colamonici and Domanski 1993). These two subunits differ from the cloned Hu-IFN-αR1. The monoclonal antibodies (MAbs) against one subunit (110 kDa) and the recombinant Hu-IFN-αR1 receptor locked the biological activity of type I interferons whereas MAbs against the second subunit did not (Colamonici et al. 1990, 1992; Benoit et al. 1993; Colamonici and Domanski 1993), suggesting that the type I IFN receptor consists of at least two

different subunits. Antibodies to Hu-IFN-αR1 blocked the activity of various type I interferons (Uzé et al. 1991). Thus, it was likely that the Hu-IFN-αR1 molecule was at least one component of the type I IFN receptor.

Mouse cells with functional Hu-IFN type I IFN receptors were first isolated by Jung and Pestka (1986) and Revel et al. (1991). The mouse cells transfected with total human DNA exhibited the properties of a type I interferon receptor (also designated Hu-IFN-αR, Hu-IFN-βR, or Hu-IFN-α/βR) in that they responded to two type I interferons tested: Hu-IFN-α and Hu-IFN-β. Although primary transformants were obtained by these groups, no molecular clone was obtained that provided type I interferon receptor activity. Employing the procedures of Jung and Pestka (1986); Uzé et al. (1990), by switching the selection procedure from Hu-IFN-αA and Hu-IFN-β to Hu-IFN-αB2 (a more species-specific Hu-IFN-α), were able to obtain a molecular clone which they designated the type I IFN receptor. However, as shown by them and other groups, this cDNA clone, when expressed in mouse or hamster cells, did not bind all type I interferons other than Hu-IFN-αB2 and yielded very little response even to Hu-IFN-αB2. However, Hu-IFN-αR1 expressed in xenopus oocytes to minimize the possibility of endogenous receptor components can bind and be covalently crosslinked to Hu-IFN-αA and Hu-IFN-αB, although to a small degree (Lim et al. 1994). None of the other Hu-IFN-α species tested seemed to activate the mouse cells expressing this cDNA clone. During this period, however, a number of groups obtained antibodies, suggesting that the type I receptor consisted of multiple components, a hypothesis consistent with the inability of Jung and Pestka (1986) to obtain stable secondary transformants. A comparison of hamster cells with the 3x1S region of human chromosome 21 and cells transfected with the Hu-IFN-αR1 cDNA showed that other components were involved in the type I interferon receptor. Hamster cells with the 3x1S region of human DNA responded to Hu-IFN-αA and Hu-IFN-β, whereas mouse or hamster cells with the Hu-IFN-αR1 cDNA did not respond to low concentrations of these interferons (Langer et al. 1990; Soh et al. 1994c).

As noted above, cloning of the first human type I IFN receptor chain (IFN-αR1) was reported on the basis of rendering mouse cells sensitive to Hu-IFN-αB2 (Uzé et al. 1990). However, when mouse cells were transfected with the Hu-IFN-αR1 cDNA, they did not exhibit binding and antiviral protection with type I IFN subtypes other than Hu-IFN-αB2. Similarly, human cells transfected with the homologous cloned mouse Mu-IFN-αR1 receptor cDNA showed antiviral protection activity with only Mu-IFN-α11. However, the expression of this Mu-IFN-αR1 cDNA in murine L1210 R101 cells resistant to type I IFNs and lacking mRNA for this Mu-IFN-αR1 receptor component showed antiviral protection in response to all type I Mu-IFNs tested (Uzé et al. 1992). In contrast, it was reported that, when CHO-K1 cells were transfected with the cloned Hu-IFN-αR1 cDNA, no induction of 2′,5′-oligoadenylic acid synthetase activity was observed after treating cells with Hu-IFN-αA and Hu-IFN-αB2 (Revel et al. 1991).

In affinity, crosslinking experiments, ^{125}I-labeled Hu-IFN-α–receptor complexes with an M_r of 80 000 (Hannigan et al. 1986), 210 000 (Colamonici et al. 1992), 260 000 (Vanden Broecke and Pfeffer 1988), or 300 000 (Raziuddin and Gupta 1985) were observed, in addition to the major complex that migrates as a broad band with an M_r of 140 000–150 000. These observations strongly suggested that other subunits, components or accessory proteins, are involved in ligand binding and in signal transduction in response to type I IFNs (Colamonici and Pfeffer 1991; Mariano et al. 1992; Abramovich et al. 1994b) as described for the IFN-γ receptor (Jung et al. 1987; Cook et al. 1992, 1994; Soh et al. 1993, 1994a).

Functional YAC selection and screening

Jung et al. (1987, 1988, 1990; Jung 1991) attempted to obtain the gene and/or cDNA clone for Hu-IFN-γR2 in studies that served as a foundation for isolation of a genomic clone encoding the functional type I IFN receptor complex. However, the use of cDNA expression libraries, cosmid genomic clones, and total human DNA proved inadequate to obtain a molecular clone for Hu-IFN-γR2. We thus turned to the use of yeast artificial chromosomes (YAC) because they contain large inserts of human DNA. YAC cloning techniques allow the cloning of DNA fragments from 100 to 2000 kb. (Burke et al. 1987; Chumakov et al. 1992a). The large insert size not only facilitates physical mapping (Chumakov et al. 1992b), but also makes it possible to express genes that are larger than permitted by conventional cloning procedures and regions of chromosomes containing multiple genes. To use YACs for fusion to mammalian cells and selection, it was necessary to devise and construct plasmids that would insert mammalian markers into the YACs so that the appropriate cells could be selected (Soh et al. 1994b; Emanuel et al. 1995). In addition, we designed vectors that would fragment the YAC clones into more manageable smaller ones (Cook et al. 1993, 1994; Emanuel et al. 1995). By examining a number of YACs containing human DNA inserts from this region, we were able to find one that contained the gene for the Hu-IFN-γR2 chain. Having successfully used this approach to obtain a functional Hu-IFN-γ receptor, we then applied this YAC technology to examine the type I interferon system.

A functional human interferon type I receptor

With the knowledge that the 3x1S region of human chromosome 21 contained the genes for the type I inter-

feron receptor, we screened three YAC clones from this region. Two YACs were initially screened by a primer pair derived from the 524-5P probe (*21S58* locus) near the cloned Hu-IFN-αR1 receptor gene (Lutfalla et al. 1992). Tassone et al. (1990) reported that the two loci (*21S58* and *IFNAR*) were located within 170 kb of each other. It was also reported that the *CRFB4* gene identified by the 524-5P probe was located at less than 35 kb from the Hu-IFN-αR1 gene (Lutfalla et al. 1993). The αYAC containing the gene for the cloned Hu-IFN-αR1 receptor cDNA was selected for expression into hamster cells based on the assumption that all the genes involved in forming a functional type I IFN receptor might be encompassed on this YAC. The objective was to obtain a YAC clone that would support type I human interferon activity when transferred into hamster cells. We found one YAC clone that fit our criteria. It contained the genes for the cloned Hu-IFN-αR1 receptor subunit and other genes in the 3x1S region of chromosome 21. In addition, the introduction of this YAC clone, designated αYAC, into hamster cells rendered the cells much more sensitive to Hu-IFN-αA and Hu-IFN-αB2, and somewhat more sensitive to Hu-IFN-ω and Hu-IFN-β (Soh et al. 1994c). We thus hypothesized that this YAC clone must contain multiple genes required to reconstitute a fully functional type I IFN receptor.

Antiviral protection of hamster cells containing the Hu-IFN-αR1 cDNA or the αYAC

The hamster cells fused to spheroplasts with the αYAC containing the type I interferon receptor complex showed increased sensitivity to Hu-IFN-αA and Hu-IFN-αB2 in antiviral protection against EMCV and vesicular stomatitis virus (VSV) when compared with the parental hamster 16-9 cells or hamster cells transfected with the Hu-IFN-αR1 receptor cDNA (Soh et al. 1994c). The hamster cells containing the αYAC exhibited large increases in sensitivity to Hu-IFN-αA and Hu-IFN-αB2. Furthermore, there was substantial increase in sensitivity to Hu-IFN-β and Hu-IFN-ω. When the sensitivity of the cells to the interferons was evaluated, in all cases hamster cells with the αYAC showed substantial increase in sensitivity to all type I interferons ranging from about threefold to more than 100-fold. There was little or no increased sensitivity of the comparable cells containing the cloned Hu-IFN-αR1 cDNA.

Induction of class I MHC surface antigens

Subclones isolated from transformed hamster 16-9 cells, which express the human HLA-B7 antigen, fused to the αYAC or transfected with the Hu-IFN-αR1 cDNA exhibited HLA-B7 induction as a function of IFN concentration for all of the type I IFNs tested. Hu-IFN-αA, Hu-IFN-αB2, Hu-IFN-β, and Hu-IFN-ω had little or no effect on the parental hamster 16-9 cells or hamster 16-9 cells transfected with the Hu-IFN-αR1 cDNA. However, hamster cells fused to the αYAC showed enhanced levels of HLA-B7 antigens in response to Hu-IFN-αA, Hu-IFN-αB2, Hu-IFN-β, and Hu-IFN-ω. Treatment of hamster cells fused with the αYAC with as little as 1 unit/ml of Hu-IFN-αA, Hu-IFN-αB2, Hu-IFN-ω, or Hu-IFN-β gave a significant HLA-B7 induction whereas parental hamster 16-9 cells or 16-9 cells transfected with the Hu-IFN-αR1 cDNA did not show any significant induction at this level (Soh et al. 1994c).

Cells containing the αYAC can bind ^{32}P-labeled Hu-IFN-αA and ^{32}P-labeled Hu-IFN-αB2

The hamster cells with the αYAC bound ^{32}P-labeled Hu-IFN-αA-P1 and ^{32}P-labeled Hu-IFN-αB2-P quite well. There was a small level of binding of ^{32}P-labeled Hu-IFN-αB2-P to cells containing the Hu-IFN-αR1 cDNA, but no binding of ^{32}P-labeled Hu-IFN-αA-P1 was seen. Furthermore, competition binding studies with ^{32}P-labeled Hu-IFN-αA-P1 as the labeled ligand showed that Hu-IFN-αA, Hu-IFN-αB2, Hu-IFN-β, and Hu-IFN-ω all compete effectively for ^{32}P-labeled Hu-IFN-αA-P1 binding to hamster cells with the αYAC as well as to human Daudi lymphoblastoid cells (Soh et al. 1994c). The specific binding of Hu-IFN-αA and Hu-IFN-αB2 to cells containing the αYAC and the competition by all type I interferon classes together with the activity data indicate that the human DNA insert in this YAC contains the genes encoding the subunits that comprise a functional type I receptor. As the Hu-IFN-αR1 gene is included in this DNA insert, one or more additional genes complementing the cloned Hu-IFN-αR1 receptor encoded on this YAC enable the cells to bind these interferons effectively (Soh et al. 1994c).

Proof that the IFN-αR1 component is part of the type I receptor complex

A large number of experiments were consistent with the concept that the IFN-αR1 chain was one component of the type I receptor complex. IFN-α-resistant K562 cells can bind type I IFNs, but are restored to IFN-α sensitivity by the introduction of Hu-IFN-αR1, without any apparent change in receptor number or affinity, suggesting a role for Hu-IFN-αR1 in cellular signaling (Colamonici et al. 1994a). Evidence for the role of IFN-αR1 in signal transduction includes the enhanced sensitivity to IFNs seen in some transfection studies, sometimes in the absence of enhanced IFN binding (Colamonici et al. 1994a; Constantinescu et al. 1994) and the interaction of IFN-αR1 with the intracellular tyrosine kinase Tyk2,

required for IFN-α responses (Velazquez et al. 1992; Barbieri et al. 1994; Colamonici et al. 1994b).

Direct proof of the requirement for the IFN-αR1 chain for type I interferon receptor function was demonstrated by experiments disrupting the IFN-αR1 gene. Homozygous deletions of *IFNAR1* in mice (IFN-αR$^{0/0}$) were reported to cause enhanced susceptibility to several viruses and eliminate antiproliferative activity of IFN-α and IFN-β (Müller et al. 1994; Hwang et al. 1995). Primary embryo fibroblasts from such mice lack type I IFN-stimulated resistance to viruses and inducibility of 2′,5′-oligoadenylic acid synthetase. Using homologous recombination targeted to the αYAC, Cleary et al. (1994) produced a deletion within the human IFN-α receptor (Hu-IFN-αR1) gene which eliminated exon II of the gene. The resultant αYAC was transferred to CHO cells. This deletion effectively eliminated the ability of type I interferons to induce MHC Class I antigens and exhibit antiviral activity, which are properties of the fully functional parental YAC clone (Soh et al. 1994c). By subsequent transfection of the cDNA for Hu-IFN-αR1 into cells containing the αYAC, antiviral activity and ability of Hu-IFN-α to stimulate MHC class I antigens was successfully reconstituted. The Hu-IFN-αR1 subunit thus plays a critical role in the functional human type I IFN receptor complex, the components of which are encoded on this YAC. In addition, as binding of ligands is retained in the cells containing the YAC with the deletion, it was clear that one or more additional subunits encoded on the YAC are responsible for ligand binding and activity.

Proof that the IFN-αR2c chain is required for function of the type I interferon receptor

After our studies describing the αYAC, a second chain, IFN-αR2b, which was suggested as another component of the type I interferon receptor complex, was reported, but it appeared to exhibit no intrinsic activity alone or together with the Hu-IFN-αR1 chain (Novick et al. 1994). The human gene *IFNAR2* encoding this chain is located on the αYAC and also localizes to the 3x1S region of chromosome 21. This Hu-IFN-αR2b chain was reported to bind type I interferons and antibodies to this chain were able to co-immunoprecipitate Jak1 (Novick et al. 1994). However, expression of IFN-αR1 or IFN-αR2b chains, or the combination of both, in hamster cells was not able to reconstitute functional human receptor activity (Mariano et al. 1994; Soh et al. 1994b; Kotenko et al. 1995). In addition, the CHO cells containing the ΔαYAC with the disrupted *IFNAR1* gene retained the ability to bind Hu-IFN-αA and Hu-IFN-αB2 (Cleary et al. 1994). This paradox was partially resolved when U5 cells (Darnell et al. 1994) that do not respond to type I interferons were able to be reconstituted to respond to the interferons by introduction of the αYAC (J. Cook, S. Pestka, I. Kerr, and G. Stark, unpublished data). Unfortunately, no stable transformants were obtained with the αYAC in U5 cells. The data indicated, however, that another component encoded on the αYAC was necessary for response to type I interferons. This component was subsequently cloned (Domanski et al. 1995; Lutfalla et al. 1995) and shown to be a long form of the Hu-IFN-αR2b chain called Hu-IFN-αR2c (see Table 7). All three chains (Hu-IFN-αR2a, -αR2b, and -αR2c) are encoded by the same *IFNAR2* gene. The Hu-IFN-αR2c gene contains a large intracellular domain compared with the short domain of the Hu-IFN-αR2c chain. The Hu-IFN-αR2c chain reconstitutes U5 cells for responses to type I interferons. The Hu-IFN-αR2a protein is a soluble form of the receptor consisting of the extracellular domain of the Hu-IFN-αR2b and Hu-IFN-αR2c chains.

Hamster cells containing the αYAC exhibit the properties expected for a functional type I human IFN receptor complex. The YAC provides genes which are necessary and sufficient to encode this functional type I interferon receptor complex as measured by three distinct biological assays and by binding of both ^{32}P-labeled Hu-IFN-αA and ^{32}P-labeled Hu-IFN-αB2 to cells fused to this αYAC.

Diversity of the interaction of type I interferons with the receptor

As noted above CHO cells containing the YAC F136C5 (αYAC) respond to all type I human interferons including IFN-αA, IFN-β, and IFN-ω. The αYAC contains at least two genes encoding IFN-αR chains that are required for response to type I human interferons: Hu-IFN-αR1 and Hu-IFN-αR2c. We previously isolated a splice variant of the Hu-IFN-αR1 chain designated Hu-IFN-αR1s or Hu-IFN-αR1b (Cleary et al. 1992) (see Table 7). CHO cells containing the disrupted αYAC, which contains a deletion in the Hu-IFN-αR1 gene, ΔαYAC, were transfected with expression vectors for the Hu-IFN-αR1 and Hu-IFN-αR1s chains. With these cells, two type I interferons were identified that can interact with the splice variant (Hu-IFN-αR1s) and with the Hu-IFN-αR1 chains: Hu-IFN-αA and Hu-IFN-ω. Two other type I interferons – Hu-IFN-αB2 and Hu-IFN-αF – are capable of signaling through the Hu-IFN-αR1 chain only, and cannot utilize the splice variant Hu-IFN-αR1s. Hu-IFN-αR1 and Hu-IFN-αR1s differ in that the latter is missing a single subdomain of the four subdomains of the receptor extracellular domain encoded by exons 4 and 5 of the Hu-IFN-αR1 gene. Therefore, different type I interferons require different subdomains of the Hu-IFN-αR1 receptor chain.

These results with the various forms of the Hu-IFN-α receptor components generally demonstrate differences

in interaction of all the type I interferons with the receptor that reflect the differences in the activity profiles of the interferons. Specifically, some type I interferons (e.g. Hu-IFN-B2, Hu-IFN-αF) require the two subdomains absent in the splice variant (Figure 11) and some can function without these domains (e.g. Hu-IFN-αA, Hu-IFN-ω) as described by Cook et al. (1996). The results indicate that the various Hu-IFN-α species and other type I interferons interact with the receptor differently, thereby accounting for their differential activities. The conservation of the multitude of type I interferons throughout evolution of the mammals is consistent with unique functional roles for each of the type I interferons.

THE CYTOKINE TYPE 2 RECEPTOR FAMILY

The IFN-αR1, IFN-αR2, CRFB4, IFN-γR1, and IFN-γR2 chains are members of the cytokine type 2 receptor family as described by Bazan (1990a, b) and by Thoreau et al. (1991), who proposed that the interferon receptors as well as other receptors for cytokines and some growth factors are composed of two folding domains that comprise the ligand-binding site, which resides in the crevice between the folds. The primary cytokine–receptor interaction was suggested to involve one face of the ligand whereas another face of the bound cytokine can interact with accessory binding components. A summary of these receptors for the interferon-related receptor components is illustrated in a recent review (Kotenko and Pestka 2000). These homologies relate the interferon receptor components to the fibronectin type III structure, which in turn relates all these structures to the immunoglobulin superfamily.

SIGNAL TRANSDUCTION

All type I IFNs activate Jak1 and Tyk2 tyrosine kinases during signal transduction, leading to formation and activation of ISGF3 (IFN-α-stimulated gene factor 3), DNA-binding complexes consisting of Stat1 and Stat2 transcriptional factors and p48 DNA-binding protein from the IFN regulatory factor (IRF) family of proteins (Fu et al. 1990, 1992; Schindler et al. 1992; Veals et al. 1992; Müller et al. 1993). Two subunits of the type I IFN receptor complex were identified: Hu-IFN-αR1 and Hu-IFN-αR2c and its variants as described above (Novick et al. 1994; Domanski et al. 1995; Lutfalla et al. 1995).

Some Stats are activated by a number of cytokines; others are highly specific. Stat2 has been shown to be activated only in response to type I IFN (Fu et al. 1992). A number of studies were made to define how Stat1 and Stat2 are recruited to the type I IFN receptor complex (Uddin et al. 1995; Domanski and Colamonici 1996; Yan et al. 1996; Li et al. 1997). Although it is clear that both receptors are necessary for signaling of type I IFNs, because disruption of any one of them completely abolishes the ability of cells to respond to type I IFNs (Hwang et al. 1995; Lutfalla et al. 1995), the reports about their role in Stat recruitment were inconclusive. The IFN-αR1 chain as well as both forms of the IFN-αR2 chain, the IFN-αR2c and the IFN-αR2b chain, were reported to associate with Stat proteins (Uddin et al. 1995; Domanski and Colamonici 1996; Krishnan et al. 1996; Yan et al. 1996; Yang et al. 1996).

The difficulty in evaluating the contribution of each chain of the IFN-α receptor complex to signal transduction is the result of the lack of cell lines without the endogenous receptor chains and in the cross-species activity of the type I IFNs. To overcome these limita-

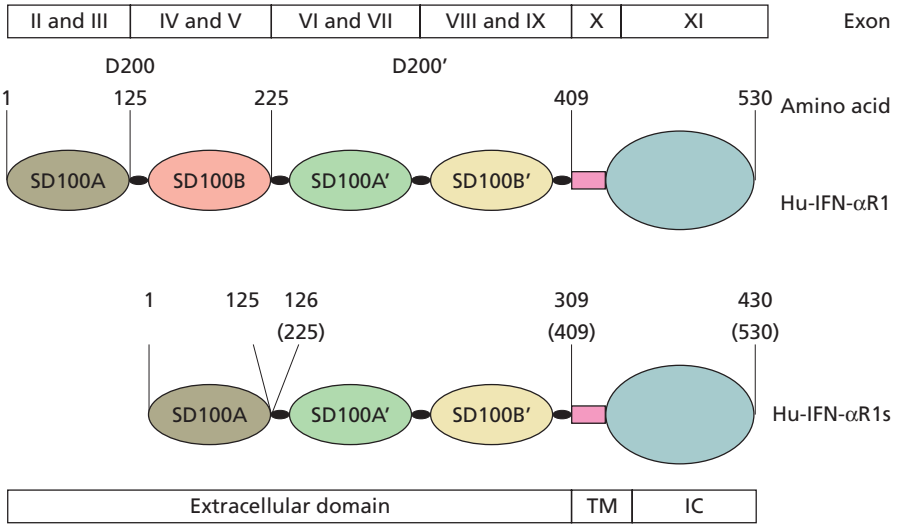

Figure 11 *Schematic Illustration of the Hu-IFN-αR1 chain and the splice variant Hu-IFN-αR1s. The domains of the Hu-IFN-αR1 chain are shown. The splice variant chain, Hu-IFN-αR1s, lacks exons 4 and 5.*

tions we used chimeric receptors with the extracellular human IFN-γ receptor chains expressed in hamster cells because Hu-IFN-γ is highly species specific and does not activate the endogenous hamster IFN-γ receptor. This strategy with the use of chimeric receptors (Kotenko et al. 1999) permitted us to show that the IFN-αR2c chain alone is necessary and sufficient for recruitment of Stat1 and Stat2.

Results of other workers have implicated all the receptor subunits of the IFN-α receptor complex (the IFN-αR1, IFN-αR2b, and IFN-αR2c chains) in Stat activation (Uddin et al. 1995; Domanski and Colamonici 1996; Yan et al. 1996; Li et al. 1997). The IFN-αR1 chain was reported to bind Stat2 and Stat3 in a ligand-dependent manner through the phosphorylated Tyr466 and the phosphorylated Tyr527, respectively (Yan et al. 1996; Yang et al. 1996; Li et al. 1997). It was originally demonstrated that the peptide containing phosphorylated Tyr466 (P-Tyr466) can inhibit type I IFN signaling in permeabilized cells and specifically interact with the -SH_2 domain of Stat2, but not of Stat1 (Yan et al. 1996). Later the P-Tyr466 peptide, which was one amino acid longer, was shown to interact with both Stat1 and Stat2 proteins (Li et al. 1997). However, mutation of all four tyrosine residues within the IFN-αR1 intracellular domain to phenylalanine resulted in a functional receptor (Gibbs et al. 1996), demonstrating that phosphorylation of the IFN-αR1 chain is unnecessary for the generation of a biological response. The IFN-αR2c chain was shown to bind both Stat1 and Stat2 in a ligand-independent manner (Domanski and Colamonici 1996; Li et al. 1997). As Stat1 activation by type I IFNs is Stat2 dependent (Improta et al. 1994; Leung et al. 1995), it was proposed that Stat1 is recruited to the complex through Stat2 (Leung et al. 1995; Li et al. 1997). The short form, the IFN-α2b chain, was reported to associate with Stat2 in a ligand-dependent manner (Uddin et al. 1995). However, most experiments were performed in cells where the endogenous type I receptor subunits were present. It is thus likely that endogenous components interacting with the heterologous type I interferon components in the host cells contributed to the results previously reported.

In our experiments with chimeric receptors, we could isolate the contributions of endogenous and exogenous components which was not possible previously (Kotenko et al. 1999). The chimeric receptors with the IFN-γR2 extracellular domain and intracellular αR1 and αR2c intracellular domains were expressed in hamster cells expressing the Hu-IFN-γR1 chain. The γR2/αR1 and γR2/αR2c chimeras rendered 16-9 cells sensitive to Hu-IFN-γ as measured by IFN-γ-induced MHC class I antigen expression and Stat1α activation as did the intact γR2/γR2. The γR2/αR2b was unable to support IFN-γ signaling because the IFN-αR2b intracellular domain does not associate with any kinase as we demonstrated previously (Fu 1995). The IFN-γ-induced activation of Stat1α DNA-binding complexes in hamster cells expressing chimeric receptors correlated with MHC class I antigen induction; however, formation of the IFN-γ-induced ISGF3 DNA-binding complexes was detected only in γR2/αR2c cells (Kotenko et al. 1999). Thus, we demonstrated that the Stat2 protein can be recruited and activated in cells expressing the chimeric receptor complex where the γR2/γR2 chain is substituted by the γR2/αR2c chimeric chain, but not by the γR2/αR1 or the γR2/αR2b chimeric chains, demonstrating that recruitment and activation of Stat2 occur through the intracellular domain of the IFN-αR2c chain. In addition, the γR2/αR2c chimeric chain expressed in these cells was able to support IFN-γ-induced antiviral protection against EMCV.

In the γR2/αR2c cells the presence of the Hu-IFN-γR1 chain, which recruits Stat1α to the receptor complex, did not allow us to answer the question of whether or not the presence of the IFN-αR2c intracellular domain is sufficient only for recruitment, and activation of Stat1α and Stat2 for formation of the ISGF3 DNA-binding complexes and the resultant biological activities. To define the requirements for Stat2 recruitment further, we switched to a hamster cell line expressing the Hu-IFN-γR2 chain which does not recruit any Stats, unlike the IFN-γR1 chain. Thus, the chimeric receptors with the IFN-γR1 extracellular domain expressed in these cells were the only chains that could contribute to Stat recruitment. Therefore, MHC class I antigen induction in these cells could serve as a marker of Stat activation. The cells expressing the γR1/γR1 and γR1/αR2c chimeras demonstrated IFN-γ-induced MHC class I antigen expression, whereas the γR1/γR1 and γR1/αR2b cells did not (Kotenko et al. 1999). Similarly, Stat1α DNA-binding complexes and small amounts of Stat3 DNA-binding complexes were activated in the γR1/γR1 and γR1/αR2c cells, but not in the γR1/γR1 and γR1/αR2b cells (Kotenko et al. 1999). However, ISGF3 DNA-binding complexes were induced only in γR1/αR2c cells (Kotenko et al. 1999). Thus, the presence of the IFN-αR2c intracellular domain as the only Stat-recruiting domain in the chimeric receptor complex is sufficient for Stat1, Stat2, and Stat3 recruitment, ISGF3 DNA-binding complex activation, and induction of MHC class I antigens. Only when the IFN-αR2c intracellular domain was present in the chimeric receptor was IFN-γ able to induce antiviral protection in cells.

We therefore conclude that all Stats activated by type I IFNs, Stat1, Stat2, and Stat3 are activated through the IFN-αR2c intracellular domain (Figure 12). The IFN-αR1 intracellular domain does not recruit Stats, but supports type I IFN signal transduction by bringing Tyk2 tyrosine kinase to the receptor complex and modulates type I IFN signaling. Stat recruitment by the type I IFN receptor complex is solely a function of the IFN-αR2c intracellular domain and the IFN-αR2c chain is sufficient and necessary for recruitment of Stat1, Stat2, and Stat3.

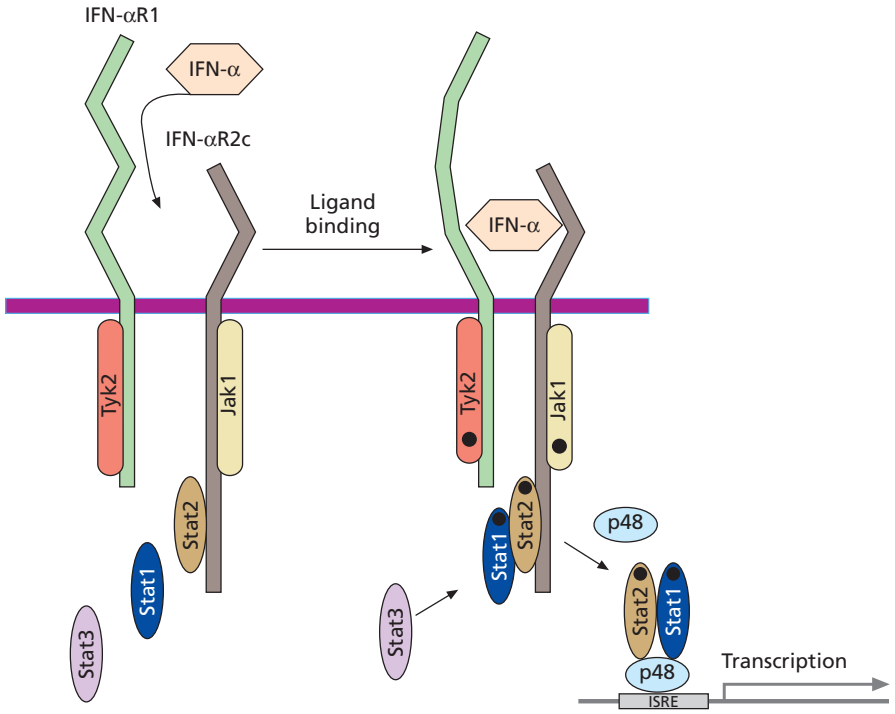

Figure 12 *Model of type I interferon (IFN)–receptor complex and signaling. Ligand binding to the subunits of the type I IFN receptor complex, the IFN-αR2c and the IFN-αR1 chains, initiates the cascade of signal transduction events. All Stats involved in IFN-α signaling are activated through the intracellular domain of the IFN-αR2c chain.*

Receptor and signal transduction endnote

Although two major components of the type I interferon receptor have been identified, a number of results suggest that there are other components yet to be delineated (Abramovich et al. 1994a; Cook et al. 1996; Hertzog et al. 1994). The crystal structures of Mu-IFN-β (Mitsui et al. 1993; Senda et al. 1995) and Hu-IFN-α2b (Radhakrishnan et al. 1996) and solution structure (Klaus et al. 1997) provide a foundation to develop an understanding of the interferon–receptor interactions further. The beginning of the elucidation of the type I interferon receptor is just starting with undoubtedly many surprises to come.

THERAPEUTIC USE OF TYPE I INTERFERONS

Interferons have been used in clinical trials for about three decades, but were not approved by regulatory agencies until the 1980s. The recombinant human interferons (IFN-α, IFN-β, and IFN-ω) and bovine IFN-τ have been used in clinical trials since 1981. Hu-IFN-αA (Hu-IFN-α2a) was the first recombinant interferon approved for clinical trial; and both Hu-IFN-αA (Hu-IFN-α2a) and Hu-IFN-α2 (Hu-IFN-α2b) were approved by regulatory agencies in many countries about the same time in the mid-1980s. The first approvals were for hairy cell leukemia and Kaposi's sarcoma. Since that time, they have been found to be useful in a variety of cancers and viral diseases. The Hu-IFN-αs have been approved for the treatment of a broad range of cancers such as CML (Talpaz et al. 1987; Faderl et al. 2000) and metastatic malignant melanoma (Sun and Schuchter 2001; Gray et al. 2002; Kilbridge et al. 2002; Kirkwood 2002; Kirkwood et al. 2002). They have been used in many clinical protocols for the treatment of other cancers with significant effects. In some cancers a small subset of patients seems to respond, but the genotype or phenotype of the responders has not been identified. It would be a major advance if the responders could be identified by genomic or other markers, a goal that many investigators are pursuing. Clinical trials continue in many cancers such as renal cell cancer (Hofmockel et al. 1997; Dutcher et al. 2001; van Herpen and De Mulder 2002) and many others (Cooper 1991; Einhorn and Strander 1993; Portillo Martin et al. 1995; Pfeffer et al. 1998; Ravandi et al. 1999; Borden et al. 2000; Oberg 2000; Eliason 2001; Jonasch and Haluska 2001; Brassard et al. 2002; Nathan and Eisen 2002). A summary of the approved therapeutic indications for interferons is given in Table 9. Local regional therapy has been attempted (van Herpen and De Mulder 2000), but effective delivery or sustained-release systems have not yet been developed.

Although interferons were first discovered because of their antiviral activity, they have not yet been used

Table 9 *Therapeutic activities of interferons*

Approved therapeutic interventions	
Hairy cell leukemia[a]	Kaposi's sarcoma[a]
Renal cell leukemia[a]	Non-Hodgkin's lymphoma[a]
T-cell lymphoma[a]	Multiple myeloma[a]
Laryngeal papillomatosis[a]	Malignant melanoma[a]
Bladder cell carcinoma[a]	Colon carcinoma[a]
Condyloma scuminatum[a]	Rhinovirus (prevention)[d]
Chronic hepatitis B[a]	Chronic hepatitis C (NANB)[a]
Hemangiomas[a]	Chronic myelogenous leukemia[a]
Malignant carcinoid tumor[a]	Crohn's disease[d]
Chronic granulomatous disease[b]	Diabetic retinopathy[d]
Cervical intraepithelial neoplasia[c]	Multiple sclerosis[c]
Carcinoid tumors[d]	Mycosis fungoides[d]
Ovarian cancer[d]	Polycythemia vera[d]
Skin cancer[d]	Thrombocytosis[d]

a) IFN-α, approval in the USA and/or other countries.
b) IFN-γ, approval in the USA and other countries.
c) IFN-β, approval in the USA and/or other countries.
d) IFN-α, not approved.

generally to treat many viral diseases. However, remarkably, virtually every class of viruses has one or more members that are sensitive to interferon in culture or in animals (Table 10). This leaves open the possibility that most viral diseases will be able to be prevented and/or treated with an appropriate interferon.

The interferons have been useful in the treatment of hepatitis. Specifically, the IFN-α species have been used effectively and are approved for the treatment of chronic hepatitis B (Haria and Benfield 1995; Woo and Burnakis 1997; Grob 1998; Marques et al. 1998; Mazzella et al. 1999; Alberti et al. 2002; Perrillo 2002) and hepatitis C (Vithanomsat et al. 1984; Yamamoto et al. 1992; Davis 1994; Iino et al. 1994; Trepo et al. 1994; Weiland 1994; Haria and Benfield 1995; Roffi et al. 1995; Romeo et al. 1995; Saracco and Rizzetto 1995; Zein and Rakela 1995; Jonas 1996; Alberti et al. 1997; Cooksley et al. 1997; Woo and Burnakis 1997; Hanley and Haydon 1998; Barnes et al. 1999; Shiffman 1999; Markland et al. 2000; Pianko and McHutchison 2000; Yasuda et al. 2000; Diamond and Harris 2001; Hu et al. 2001; Lyra and Di Bisceglie 2001; Sagmeister et al. 2001; Wilkinson 2001; Hino et al. 2002; Katze et al. 2002; Anderson and Rahal 2002). Certain strains of hepatitis C virus are more resistant to treatment than others. These can be identified by genotyping the strains (Martell et al. 1992; Okamoto et al. 1992; Hino et al. 1994; Tsubota et al. 1994; Enomoto and Sato 1995a, 1995b; Enomoto et al. 1995, 1996; Gale et al. 1997; Arens 2001). Studies have correlated a region of the hepatitis C viral nonstructural protein 5A (NS5A) with sensitivity and resistance to interferon. Protein NS5A was found to repress interferon-induced protein kinase, PKR, which is one of the mediators of antiviral activity of interferon. Furthermore, it has been possible to begin to correlate single nucleotide polymorphisms in patients with hepatitis C with their ability to respond to interferon (Hijikata et al. 2001). This will probably be improved immensely in the next few years.

Although interferon has been approved for the treatment of few other infectious diseases (e.g. condyloma acuminatum (Rockley and Tyring 1995), genital warts caused by human papilloma virus type 6 and 11, and labial and genital herpes (Ophir et al. 1995; Glezerman et al. 1988)), it has been shown to be effective for the treatment of laryngeal papillomatosis (Deunas et al. 1997; Haglund et al. 1981; Lundquist et al. 1984) and the prevention of common colds produced by rhinoviruses (Scott et al. 1982; Hayden and Gwaltney 1983; Phillpotts et al. 1983, 1984; Hayden et al. 1986; Higgins et al. 1986), although it has not been approved for these and other viral diseases. Very probably the type I interferons would be efficacious in the treatment and prevention of many viral diseases once appropriate delivery systems have been developed. Moreover, it has been suggested that interferon will be used for many infectious diseases in the future because it has potent immunomodulatory activities (Masihi 2000, 2001). That fact that interferon is active in blocking propagation of all classes of viruses (see Table 10) is tantalizing and may suggest that it will take some novel initiatives to make better use of the interferons to treat viral diseases, e.g. IFN-α is effective in substantially inhibiting retroviruses in tissue culture (Sen et al. 1984; Sperber et al. 1992), but has little effect in the treatment of diseases caused by retroviruses such as HIV – a paradox that has not been adequately addressed.

IFN-β has been found to be effective in relapsing–remitting multiple sclerosis (Silberberg 1994; Goodin 2001; Calabresi 2002; Keegan and Noseworthy 2002; Wingerchuk and Noseworthy 2002) and is currently approved for treatment. Various preparations of IFN-β are in use and all are effective, although no treatment can eliminate the disease. The mechanism by which interferon works is not clear, but it was suggested that IFN-β inhibits migration of activated T cells into the central nervous system (Leppert et al. 1996).

The detailed biological effector mechanisms that result in the various activities of the interferons are surprisingly not defined in most cases. What has made this exceptionally difficult is that the mechanisms vary from animal species to species and from one cell type to another even in the same species. It is clear that the Jak–Stat pathway is involved in many of the mechanisms, as demonstrated by analysis of mice deficient in various components of this pathway, e.g. Stat1 is required for protection of mice from microbial and viral pathogens (Durbin et al. 1996; Meraz et al. 1996). However, the downstream events required for protection against specific viruses and other pathogens is still not

Table 10 *Effect of interferons on viral propagation and in patients*

Virus family	Inhibited by interferons	Major viruses studied with interferon	References
Picornaviridae	Yes	Rhinovirus, cardiovirus (encephalomyocarditis virus)	Scott et al. (1982); Hayden and Gwaltney (1983); Phillpotts et al. (1983, 1984); Hayden et al. (1986); Higgins et al. (1986); Sperber et al. (1993b); Soh et al. (1994c); Yasuda et al. (2000)
Caliciviridae	Yes	Feline calicivirus (FCV)	Fulton and Burge (1985); Mochizuki et al. (1994)
Astroviridae	Not tested		Not tested
Togaviridae	Yes	Sindbis, Semliki forest virus	Imanishi et al. (1981); Weigent et al. (1981); Lloyd et al. (1983); Yasuda et al. (2000)
Flaviviridae	Yes	Hepatitis C virus, Dengue, West Nile, yellow fever	Vithanomsat et al. (1984); Yamamoto et al. (1992); Iino et al. (1994); Trepo et al. (1994); Weiland (1994); Haria and Benfield (1995); Roffi et al. (1995); Romeo et al. (1995); Saracco and Rizzetto (1995); Zein and Rakela (1995); Jonas (1996); Alberti et al. (1997); Cooksley et al. (1997); Woo and Burnakis (1997); Hanley and Haydon (1998); Barnes et al. (1999); Shiffman (1999); Markland et al. (2000); Pianko and McHutchison (2000); Yasuda et al. (2000); Diamond and Harris (2001); Hu et al. (2001); Lyra and Di Bisceglie (2001); Sagmeister et al. (2001); Wilkinson (2001); Anderson and Rahal (2002); Katze et al. (2002); Hino et al. (2002)
Coronaviridae	Yes	Infectious bronchitis virus (IBV; poultry), mouse hepatitis virus	Aurisicchio et al. (2000); Pei et al. (2001)
Arteriviridae	Yes	Porcine reproductive and respiratory syndrome virus (PRRSV)	Albina et al. (1998); Buddaert et al. (1998)
Rhabdoviridae	Yes	Vesicular stomatitis virus	Familletti et al. (1981b); Imanishi et al. (1981); Langford et al. (1981); S. Rubinstein et al. (1981); Arnheiter and Haller (1983); Soh et al. (1994c); Yasuda et al. (2000)
Filoviridae	Yes	Marburg, Ebola	Jahrling et al. (1999); Bray (2001); Kolokol'tsov et al. (2001)
Paramyxoviridae	Yes	Respiratory syncytial virus (human, bovine), Newcastle disease virus	Imanishi et al. (1981); Strube et al. (1985); Marcus and Sekellick (2001)
Orthomyxoviridae	Yes	Influenza viruses	Machida et al. (1980); Imanishi et al. (1981); Sedmak and Grossberg (1981); Isomura et al. (1982); Arnheiter and Haller (1983); Saito et al. (1983); Hayden et al. (1984); Phillpotts et al. (1984); Ransohoff et al. (1985); Sasaki et al. (1985); Strube et al. (1985); Sun et al. (1986); Arnheiter and Haller (1988)
Bunyavididae	Yes	Bunyamwera bunyavirus	Weber and Elliott (2002); Weber et al. (2002)
Arenaviridae	Yes	Lymphocytic choriomeningitis	Andrei and De Clercq (1993); Djavani et al. (2001)
Bornaviridae	Yes	Borna disease virus	von Rheinbaben et al. (1985); Hallensleben and Staeheli (1999); Staeheli et al. (2001)
Reoviridae	Yes	Reovirus, rotavirus	Petersen et al. (1997); Marcus and Sekellick (2001)
Retroviridae	Yes	Human immunodeficiency virus, feline immunodeficiency virus, many others	Sen et al. (1984); Wells et al. (1991); Sperber et al. (1992, 1993a)
Papovaviridae	Yes	SV-40, polyoma virus, papillomavirinae	Gotlieb-Stematsky et al. (1966); Oxman et al. (1967); Mallucci and Taylor-Papadimitriou (1973); Birg and Meyer (1975); Yakobson et al. (1977); Tevethia et al. (1979); Haglund et al. (1981); Brennan and Stark (1983); Lundquist et al. (1984); Strander (1986); Lambropoulos and Koliais (1989); Einhorn et al. (1991); Browder et al. (1992); Melkova and Esteban (1994); Rockley and Tyring (1995); Deunas et al. (1997); Aaltonen et al. (2002)

(*Continued over*)

Table 10 *Effect of interferons on viral propagation and in patients (Continued)*

Virus family	Inhibited by interferons	Major viruses studied with interferon	References
Adenoviridae	Yes	Adenovirus	Pusztai and Szabo (1978); Langford et al. (1983); Mistchenko et al. (1987)
Parvoviridae	Yes	Canine parvovirus	Minagawa et al. (1999)
Herpesviridae	Yes	Herpes simplex, Epstein–Barr virus, cytomegalovirus	Falcoff et al. (1968); Langford et al. (1983); Andersson et al. (1985); Batcheler et al. (1986); Domke-Opitz et al. 1986; Babiuk et al. (1987); Fish et al. (1986); Scheck et al. (1986); Leventon-Kriss et al. (1987); Glezerman et al. (1988); Ophir et al. (1995); Ryman et al. 2000; Yasuda et al. (2000); Carr and Noisakran (2002); Cull et al. 2002; Katze et al. (2002)
Poxviridae	Yes	Vaccinia, mouse pox	Rosel and Jungwirth (1983); Rodriguez et al. (1991); Karupiah et al. (1993); Melkova and Esteban (1994); Baron et al. (2002); Katze et al. (2002)
Hepadnaviridae	Yes	Hepatitis B virus	Haria and Benfield (1995); Woo and Burnakis (1997); Grob (1998); Mazzella et al. (1999); Alberti et al. (2002); Perrillo (2002)

defined, and the mechanisms differ from virus to virus. Furthermore, it is becoming increasingly clear that other pathways independent of the Jak–Stat pathway can initiate signal transduction by the type I interferons, e.g. cells from mice lacking Stat1 can still respond to type I interferons: IFN-α and IFN-β can modulate proliferative responses in phagocytes from Stat1-deficient mice (Gil et al. 2001). Understanding of these events is further complicated by the multiplicity of the type I interferons (see Table 6) that exhibit different activities (see Tables 3-5), although they interact with the same receptor. This is an area that needs to be explored in great detail to understand this family of proteins and their mechanisms of action.

The side effects of most biotherapeutics are significant and limit the dose of most cytokines. Interferon is not an exception, with side effects that often require reducing dosage or ceasing treatment (Nathan et al. 2002; Raanani and Ben Bassat 2002). So far the mechanisms by which interferons produce side effects have not been determined. It would be a major advance to have interferon molecules with little or no toxicity.

Patients treated with protein molecules often develop serum antibodies. If the antibodies neutralize the activity of the proteins, they can cause a relapse. This has been shown in many diseases treated with interferons (Nolte et al. 1994; Antonelli et al. 1997; Dianzani and Pestka 1997; Viscomi et al. 1999).

In carrying out clinical trials with interferons, it is useful to follow the activity in patients and in culture by biological assays. A number of these have been developed (Meager 2002), but no single assay or set of assays currently provides a standard assessment of activity in patients. Many molecules have been reported to stimulate production of interferons and other cytokines. One of these, imiquimod, has been approved as a topical agent for the treatment of external and anal genital warts (Dockrell and Kinghorn 2001).

As noted above, interferon is already used extensively for a wide range of diseases. Nevertheless, because the type I interferons have broad activities, it is likely that they will be used to a greater extent in the future, e.g. these interferons activate cytotoxicity of many cells of the immune system such as NK cells, T cells, macrophages, and dendritic cells (Herberman et al. 1981, 1982a, 1982b; Ortaldo et al. 1982, 1983a, 1983b; Li et al. 1990). Interferon has enormous anti-angiogenic activity, perhaps, the highest activity of any known biotherapeutic (White et al. 1989; Appelbaum 1990; White 1990; Kaban et al. 1999; Ozawa et al. 2001). Furthermore, interferons enhance the expression of tumor-associated antigens on the cell surface of cells enormously in culture and in patients (Greiner et al. 1984, 1985, 1987, 1992; Ozzello et al. 1995). This increase in the surface expression of the tumor-associated antigens often turns the heterogeneous population of tumor cells with low levels of the antigens and few cells carrying these surface antigens into a population of cells that are all expressing these surface tumor antigens. The cells are, therefore, recognized more efficiently by the immune system and by cytotoxic cells activated to destroy cells expressing these antigens. Together with the direct antiproliferative activity of interferons on the tumor cells, all these actions make interferon a most promising agent to treat cancers in general. The challenge is to be able to use this enormous potential of the interferons without the debilitating side effects. Appropriate technology to localize the interferons in tumors could overcome the problem of systemic side effects. Overall, it is highly likely that interferon will play a major role in the next generation of novel antitumor and antiviral therapies.

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Cytokines

SERGIO ROMAGNANI

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Cytokines are important signaling molecules produced by cells involved in inflammation, immunity, differentiation, cell division, fibrosis repair, and many other functions. Although cytokines share properties in common with hormones that are produced continuously and interact with them, there are also notable distinctions. Hormones are typically produced by specialized cells and released into the bloodstream and so can act at a distance from the source, in an 'endocrine fashion'. In contrast, cytokines usually act at short range, a few cell diameters apart, as in a 'paracrine' or 'autocrine' manner. The major differences between hormones and cytokines are summarized in Figure 1.

Today, we are confronted by considerably more than 100 cytokines as structurally identified molecules (Oppenheim and Feldman 2000). A great proportion of these molecules have recently been characterized because of their chemotactic activity and, therefore, they have been named as chemotactic cytokines or chemokines (Zlotnik and Yoshie 2000). These are considered separately (see Chemokines). Some other cytokines are not discussed here, because either they mainly act as membrane rather than soluble signals or their activity is only minimally involved in the function of the immune system. Usually cytokines are grouped in different families according to structural similarity of ligands and/or receptors. Despite the fact that the same cytokine may exhibit different functions and that the same function may be exerted by different cytokines, we have tried to group cytokines into five different families based on their most impressive or better-known function (Table 1). This type of classification is certainly arbitrary and questionable, but it may be of some usefulness from a didactic point of view.

GROWTH FACTORS FOR HEMOPOIETIC PRECURSORS, MYELOID CELLS, AND THROMBOCYTES

Definitive hemopoiesis is a complex cellular process that is regulated, in part, by hemopoietic growth factors (HGF) and the bone marrow microenvironment, which act to promote the survival, proliferation, and differentiation of hemopoietic stem cells (SC) and their progeny. HGFs are produced by accessory or stromal cells (macrophages, fibroblasts, endothelial cells, and adipocytes) in the bone marrow and elsewhere, and interact with specific receptors expressed on hemopoietic cells.

Stem cell factor

The main HGF is stem cell factor (SCF). SCF is also known as steel factor (SF), because it was mapped to the Sl locus, Kit ligand (KL), and mast cell growth factor (MCGF) (Lyman and Jacobsen 1998). SCF is produced in both soluble and membrane-bound forms. These isoforms arise from two differentially spliced complementary DNAs (cDNA). One cDNA encodes a precursor of 273 amino acids that contains a cleavable N-terminal signal peptide of 25 amino acids and gives rise to the transmembrane protein with 248 amino acids. The gene for SCF might be located on the distal end of

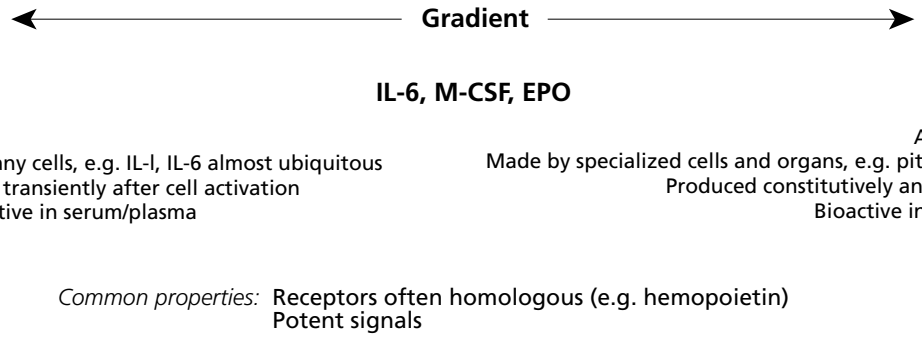

Figure 1 *Differences between cytokines and hormones. EPO, erythropoietin; IL, interleukin; M-CSF, macrophage colony-stimulating factor. (Redrawn from Oppenheim and Feldman 2000, with permission.)*

the long arm of human chromosome 12 (Keller and Linnekin 2000).

Analysis of the expression of the gene for SCF in the developing embryo suggests that this gene has multiple functional roles in development, e.g. the gene for SCF is expressed along the migratory pathways for stem cells that give rise to hemopoietic, melanocyte, and germ-cell lineages. Specifically, SCF has been detected in the genital ridge of day 10 embryos, whereas its receptor (SCFR) is expressed in the migrating germ cells. SCF expression in the genital ridge decreases early after the beginning of gestation, but remains high in the developing gonads during sexual differentiation (both in the testes and in the ovary). This pattern of expression suggests that SCF is involved in regulating the migration, proliferation, and differentiation of germ cells (Matsui et al. 1990). Moreover, SCF is expressed in mesenchymal cells located in the limb bud where melanocyte precursors colonize, whereas SCFR is expressed on the migrating cells. The expression of SCF persists during and after stem cell colonization of the limb buds. SCF is also expressed in fetal liver, which is the migratory site for hemopoietic SCs in the developing embryo. As would be predicted, there is a marked reduction in the number of SCFR-positive SCs that migrate to the fetal livers of Sl/Sl embryos (mutants that have deletion of the transmembrane and cytoplasmic domains of SCG). SCF mRNA transcripts have also been detected in other tissues in the developing embryo, including the spinal cord, forebrain, cerebellum, and olfactory bulbs, suggesting that SCF might play a role in the developing CNS. Although there are no gross neurological defects in Sl mutant mice, Sl/Sl mutant mice show a defect in hippocampus-dependent learning (spatial learning). In the adult, SCF is expressed by stromal or accessory cells (endothelial cells, monocytes, and fibroblasts) in the adult, where these cells constitute the hemopoietic microenvironment (Broudy 1997). SCF is also produced by intestinal epithelial cells, Sertoli cells, and follicular cells that surround oocytes in the gonads, thymic stroma, and brain cells, including those that constitute the olfactory bulb, thalamus, cerebellum, and brain. SCF expression has also been detected in human $CD34^+$ cells and keratinocytes in the skin (Ratajczak et al. 1995).

Stem cell factor plays an essential role in the survival, growth, and differentiation of cells responsible for hemo-

Table 1 *Classification of cytokines based on their major functional activities*

Growth factors for hemopoietic precursors, myeloid cells and thrombocytes
Stem cell factor (SCF)
Colony-stimulating factors (CSFs): interleukin (IL)-3, granulocyte (G)-CSF, macrophage (M)-CSF, GM-CSF
IL-5
IL-11
Growth and differentiation factors for lymphoid cells
IL-2
IL-4
IL-7
IL-9
IL-15
IL-21
IL-27
Proinflammatory cytokines
IL-1α and IL-1β
IL-6
Tumor necrosis factor (TNF)-α
Lymphotoxins (LT)α and LTβ
Interferon (IFN)-γ
IL-12
IL-16
IL-17 family
IL-18
IL-23
IL-25
Anti-inflammatory and regulatory cytokines
IL-1 receptor antagonist (IL-1Ra)
IL-10
IL-13
Transforming growth factor (TGF)-β
Chemotactic cytokines (chemokines)
(See Chemokines)

poietic, germ-cell, and melanocyte cell development during both embryonic development and adult life In particular, SCF is essential for the migration and homing of SCs to their appropriate developmental sites; moreover, SCF directly promotes the survival of these stem-cell populations (Keller and Linnekin 2000).

The receptor for SCF, SCFR, is expressed on most hemopoietic progenitors but is not expressed on their differentiated progeny, including mature myeloid and monocytic cells or lymphoid and erythroid cell populations; however, its expression is maintained at high levels on mast cells. In this regard, SCF promotes the survival, proliferation, and differentiation of mast cells in vitro. In addition, SCF, in combination with the interleukin (IL)-3 and IL-4, promotes the proliferation of mast cells and their progenitors. SCF can promote the adhesion of mast cells, hemopoietic progenitor cells, and cell lines to fibronectin or vascular cell adhesion molecule 1 (VCAM-1) expressed on endothelial cells by activating VLA-4 and VLA-5 integrin expression on progenitor cells. A single injection of SCF to normal mice promotes a dose-dependent increase in total number of peripheral blood leukocytes, including neutrophils and immature myeloid cells, but not circulating platelets or red cells (Galli et al. 1994). The in vivo hemopoietic effects of SCF are summarized in Table 2.

Although no abnormalities involving the SCF genetic loci have been reported, locally high concentrations of soluble SCF have been found in lesions of human cutaneous mastocytosis. This disease is characterized by accumulations of mast cells, as well as increases in the production of epidermal melanin similar to that observed in transgenic animals which expressed SCF transgenes in keratinocytes. This has led to the hypothesis that locally produced SCF can promote mast cell hyperplasia (Keller and Linnekin 2000).

Table 2 *In vivo hemopoietic effects of stem cell factor*

Cell type		Response
LTRC	Expansion	+
	Mobilization	+
Primitive/committed progenitors	Expansion	++
	Mobilization	++
Red blood cells	Reticulocytes	+/NE
	Hematocrit	+/NE
Platelets	Megakaryocytes	+/NE
	Platelets	+/NE
White blood cells	Total number	+
	Granulocytes	+
	Monocytes	+/NE
	Lymphocytes	+/NE
Mast cells	Number	+
	Activation	+
Dendritic cells	Number	NE

+, increase; NE, no effect; +/NE, effect found in some, but not all species investigated.Modified from Lyman and Jacobsen (1998).

Colony-stimulating factors

Hemopoietic activity is also the main activity of the so-called colony-stimulating factors (CSF). CSFs comprise four cytokines, named multi-CSF or IL-3, granulocyte (G)-CSF, CSF-1 or macrophage (M)-CSF, and granulocyte–macrophage (GM)-CSF. The major activity of CSFs is the development of colonies from precursors of the granulocyte and monocyte–macrophage lineage.

INTERLEUKIN 3

IL-3, also known as multi-CSF, is a typical member of the family of four helix-bundle cytokines. The mature human IL-3 is a polypeptide of 133 amino acids and the mature murine IL-3 has 140 amino acids. The human gene for IL-3 is on chromosome 5q21 (Schrader 2000).

IL-3 is synthesized and released by antigen-activated T lymphocytes, as well as by mast cells and eosinophils following antigen crosslinking of cytophilic antibodies bound to Fc receptors present on their surface (Schrader 2000).

IL-3 stimulates the growth, differentiation, and survival of pluripotential hemopoietic SCs and many of their progeny, including multipotential progenitors and progenitors committed to individual cell lineages and mature cells (Bukowski et al. 1996). These include SCs able to originate all lymphohemopoietic cell lineages and multipotential progenitors capable of generating neutrophils, macrophages, megakaryocytes, and erythroid cells. More committed progenitors targeted by IL-3 include those giving rise to in vitro colonies of eosinophils, neutrophils, macrophages, megakaryocytes, mast cells, and erythroid cells. IL-3 also supports the survival of mature mast cells, eosinophils, basophils, and megakaryocytes, and also augments the effector functions of basophils, mast cells, and eosinophils.

The effects of IL-3 depend on where it is released. The local release of IL-3 in mucosal surfaces or in lymph nodes results in the generation of mucosa-type mast cells from undifferentiated, committed, mast-cell progenitors present in these tissues. Systemic release of IL-3 results in increases in the number of hemopoietic progenitor cells and mast cell precursors in the spleen, as well as increases in megakaryocytes, neutrophils, and mast cells.

IL-3 appears to have a role in the response to certain parasites, probably because of its ability to increase the numbers of infection-stimulated mast cells and basophils. Administration of recombinant IL-3 by subcutaneous injection increases the levels of neutrophils and eosinophils, as well platelet counts, lymphocyte numbers and numbers of $CD34^+$ cells in the peripheral blood

(Schrader 2000). Side effects induced by IL-3 include headaches, flu-like symptoms, fever, and rashes. In general, clinical application of IL-3 has been poor.

G-CSF

G-CSF is a 25-kDa glycoprotein that regulates production of neutrophilic granulocytes, and activates mature neutrophils. Its gene is located on human chromosome 17q21-q22 (Nagata 2000).

Monocytes, macrophages, endothelial cells, and fibroblasts are induced to express G-CSF by various stimuli (Metcalf and Nicola 1985). Some carcinoma cells and tumor cell lines produce G-CSF constitutively. G-CSF has a unique receptor which mediates colony formation of neutrophilic granulocytes in semi-solid cultures of bone marrow cells.

Unlike other CSFs, such as GM-CSF and IL-3, G-CSF is rather specific to progenitor cells of neutrophilic granulocytes (Nicolas et al. 1983). G-CSF not only stimulates proliferation and differentiation of progenitors, but also prolongs the survival of mature neutrophils and enhances their functional capacity. Mice lacking the G-CSF gene show chronic neutropenia (Figure 2), are deficient in granulocyte and macrophage progenitor cells, and show impaired neutrophil mobilization (Lieschke et al. 1994; Basu et al. 2002), whereas administration of G-CSF causes increase in granulocyte numbers (Welte et al. 1987) and long-term exposure to

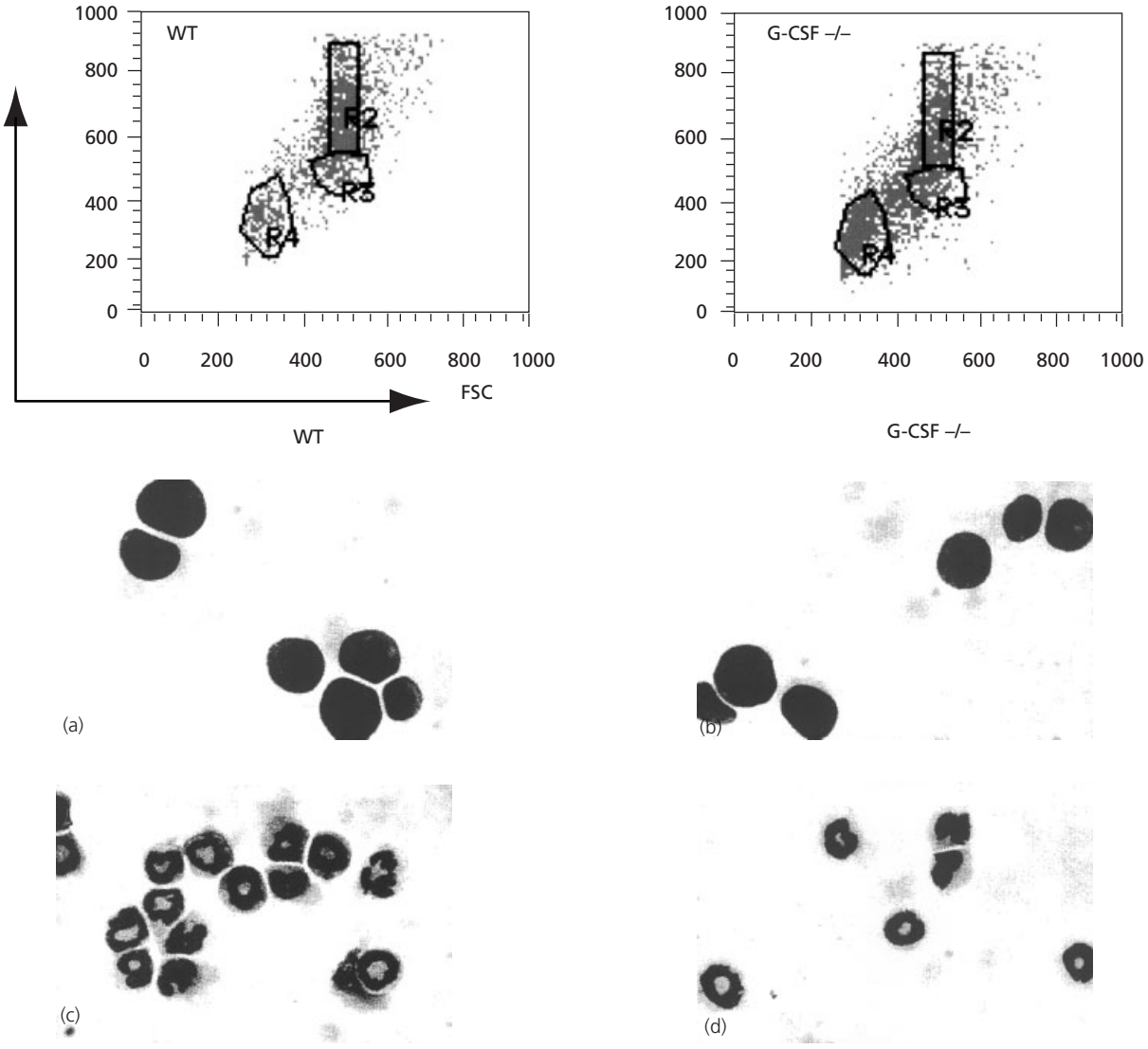

Figure 2 *Essential role of G-CSF in neutrophil development. Dot–blot presentation of forward scatter (FSC) versus side scatter (SSC) of bone marrow cells from wild-type (WT) and granulocyte colony-stimulating factor (G-CSF)-deficient (G-CSF$^{(-/-)}$) mice. R2, granulocytes; R3, blasts; R4, lymphocytes. Cells in regions R2 and R3 were sorted, and cytospin preparations of these cells were stained with May–Grünwald–Giemsa. Panels* **(a)** *and* **(b)** *represent cells in region R3, and panels* **(c)** *and* **(d)** *represent cells in region 2. (Redrawn from Basu et al. 2002, with permission.)*

G-CSF of transgenic mice causes sustained granulocytosis (Chang et al. 1989).

G-CSF is effectively used to stimulate granulopoiesis in neutropenic patients. G-CSF is also being administered to patients with cancer receiving chemotherapy or radiotherapy with or without bone marrow transplantation, to patients receiving immunosuppressive agents after organ transplantation, and to patients with cyclic neutropenia. In these last patients, without altering the cyclic nature of this disease, G-CSF elevates neutrophil levels during the nadir phase and therefore prevents many of the symptoms of the disease (Nagata 2000).

M-CSF

M-CSF, initially known as CSF-1, is a 80- to 100-kDa glycoprotein or 130- to 160-kDa chondroitin sulfate-containing proteoglycan, or it is expressed as a membrane-spanning 68- to 86-kDa glycoprotein on the surface of cells. The human M-CSF gene is approximately 21 kilobases (kb) in length, comprising 10 exons, and is localized to human chromosome 1p13-p21 (Stanley 1994).

M-CSF is expressed in most tissues, including submaxillary gland, lung, spleen, kidney, lymph nodes, brain, liver, testis, and ovary. Several types of normal cells synthesize M-CSF, including fibroblasts, endothelial cells, bone marrow stromal cells, osteoblasts, thymic epithelial cells, keratinocytes, astrocytes, myoblasts, mesothelial cells, liver parenchymal cells, thyrocytes, and adipocytes. M-CSF is also synthesized by ovarian granulosa cells, oviduct epithelium, and in large amounts by uterine epithelial cells during pregnancy (Arceci et al. 1992).

M-CSF is the primary regulator of the survival, proliferation, and differentiation of mononuclear phagocytes, including tissue macrophages and osteoclasts. It has also been shown to stimulate $CD5^+$ B lymphocytes to develop into biphenotypic B/macrophage cells. Cells requiring M-CSF for their development may regulate, via tropic and/or scavenger functions, bone resorption, male fertility, the thickness of the dermis, and neural processing. In the female reproductive system, M-CSF regulates the development of macrophages and the function of nonmononuclear phagocytic cells.

GM-CSF

GM-CSF is a protein of 144 amino acids, containing a leader sequence of 17 amino acids, two intramolecular disulfide bonds, and two potential sites of *N*-glycosylation, as well as sites of *O*-glycosylation. The apparent molecular weight of the mature glycosylated proteins is 13 kDa. The GM-CSF gene maps to chromosome 5q31.1 (Nicolas 2000) and it is very tightly linked to the IL-3 gene. There is also a cluster of cytokine (M-CSF, IL-4, IL-5) and receptor (*c-fms*) genes in the same area.

GM-CSF is a product of activated T lymphocytes, fibroblasts, endothelial cells, macrophages, and stromal cells, but also B lymphocytes, mast cells, eosinophils, blast cells, and osteoblasts. The major stimulant for T-cell production of GM-CSF is engagement of the T-cell receptor (TCR) (TCR) by antigen. In addition, the cytokines IL-1 and IL-2 and infection with the human T-cell lymphotropic virus (HTLV) also increase GM-CSF production by T cells. For B lymphocytes, antigen receptor activation (e.g. by *Staphylococcus aureus*) and bacterial lipopolysaccharide (LPS) or phorbol esters induce GM-CSF synthesis. For mast cells, degranulating agents, such as anti-IgE and calcium ionophores and, for osteoblasts, LPS and parathyroid hormone, are effective. For macrophages, fibroblasts, and endothelial cells, the major inducers of GM-CSF synthesis are LPS, IL-1, and tumor necrosis factor (TNF), as well as phorbol esters (Metcalf and Nicola 1995).

The effects of injected GM-CSF consist of a rapid and profound decrease in white blood cell counts, resulting from sequestration within the lungs followed by a progressive rise in circulating neutrophils, eosinophils, monocytes, and progenitor cells for all lineages (colony-forming cells) (Metcalf and Nicola 1995). These rises are, however, significantly less than those seen with injected G-CSF. GM-CSF has also been used to mobilize hemopoietic SCs into the peripheral blood for subsequent autologous bone marrow transplantation (ABMT). Such SCs induce a more rapid recovery of both neutrophils and platelets than is achieved by ABMT, but G-CSF exhibits a greater mobilizing efficiency than GM-CSF even for this purpose. Some studies have begun to appear in human beings evaluating the use of GM-CSF as an antiviral vaccine adjuvant for immunization against hepatitis B and influenza, with encouraging but preliminary results. Trials have also been initiated in which GM-CSF is administered after surgical resection of melanoma tumors or with passive (antimelanoma or antineuroblastoma antibodies) or active (autologous melanoma cells with BCG) immunization against tumor cells (Nicolas 2000).

Interleukin 5

IL-5 is an important growth and differentiation factor for eosinophil granulocytes. It is a glycoprotein with an M_r of 45 kDa and is unusual among the T-cell-produced cytokines in being a disulfide-linked homodimer (Gretchen et al. 2000). It is the most highly conserved member of a group of evolutionally related cytokines, including IL-3, IL-4, IL-13, and GM-CSF, which are closely linked on human chromosome 5.

T cells are an important source of the cytokine (Sanderson 1992). However, IL-5 mRNA production has also been demonstrated in mast-cell lines and human Epstein–Barr virus (EBV)-transformed B cells. Furthermore, eosinophils themselves have been demonstrated to produce IL-5 (Broide et al. 1992).

IL-5 plays a unique and specific role in the control of eosinophil production and differentiation (eosinophil differentiating factor (EDF)). It is also able to activate basophils, but is probably not the major factor controlling basophil production. The effect on eosinophil production is relatively direct. When IL-5 is expressed, eosinophils are produced, and when IL-5 expression is inhibited by drugs or gene knock-out eosinophil production and survival virtually cease. The role of IL-5 in eosinophilia (Figure 3), coupled with a better understanding of the part played by eosinophils in the development of tissue damage in chronic allergy, has made IL-5 a major target for a new generation of antiallergic drugs (Schwenger et al. 2000).

Interleukin 11

IL-11 is an important growth factor for thrombocytes. The mature IL-11 protein is a highly conserved, cationic protein of 178 amino acids. The IL-11 gene consists of five exons and four introns which contain 7 kilobase-pairs (kbp) of genomic DNA. The gene for IL-11 has been localized to the long arm of chromosome 19 at band 19q13.3–q13.4. The IL-11 gene is in close proximity to the gene that codes for the subunit of protein kinase

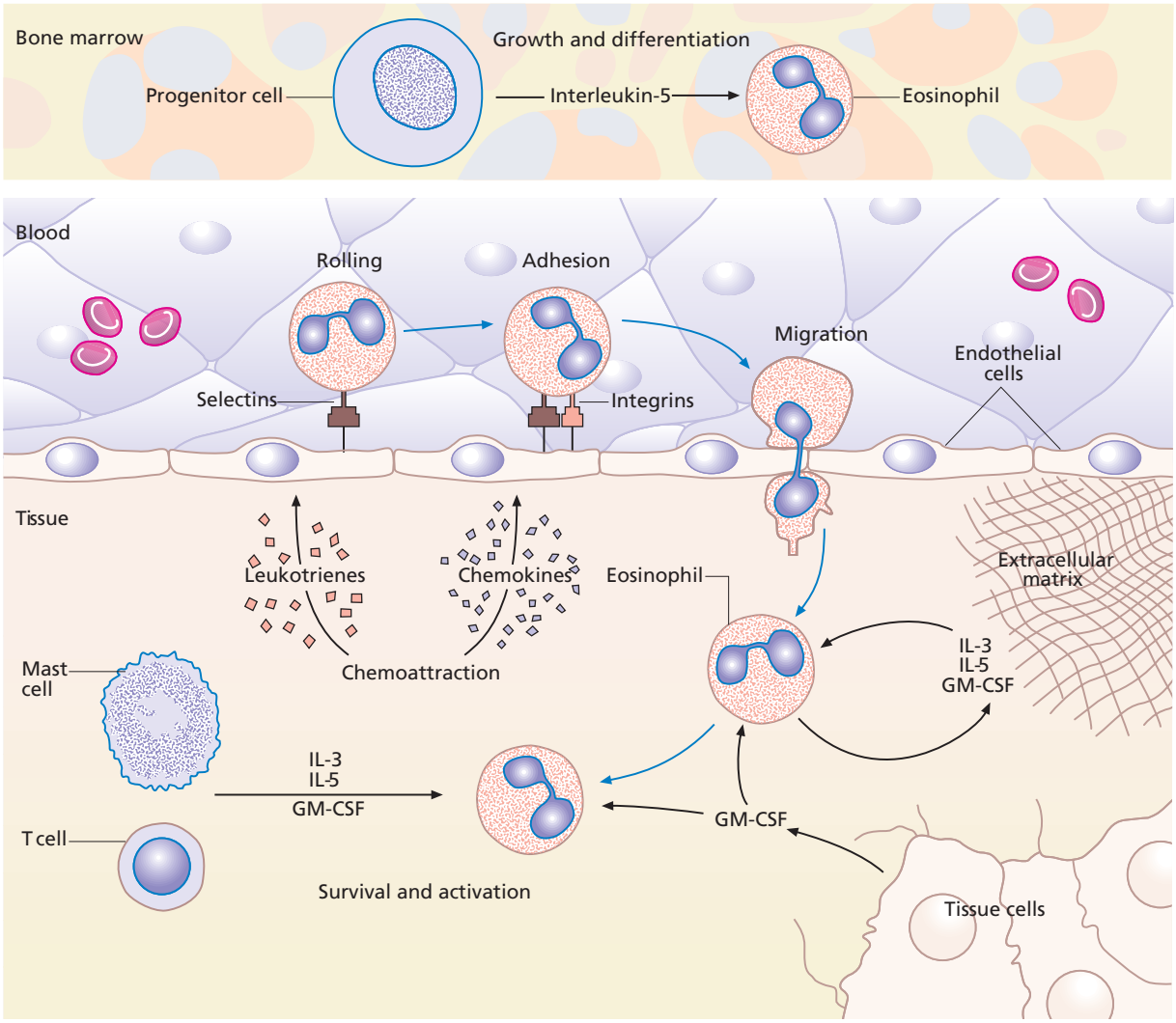

Figure 3 *Different activities of interleukin IL-5 on eosinophil granulocytes and in processes involved in eosinophilia. Eosinophilia develops in the bone marrow in response to the stimulation of progenitor cells by IL-5. Mature eosinophils in the blood adhere to endothelial cells through the interaction of selectins and integrins with endothelial receptors for these molecules. On exposure to chemoattractant mediators, eosinophils undergo diapedesis between endothelial cells and migrate into the tissues. The accumulation of eosinophils is regulated by the generation and survival and activation factors (IL-3, IL-5, and granulocyte–macrophage colony-stimulating factor [GM-CSF]) by T cells and mast cells. In response to extracellular matrix components, eosinophils themselves can also generate the cytokines and prolong their survival. (Redrawn from Rothenberg 1998, with permission.)*

C, as well as the genes for several zinc finger proteins (Keith 2000).

Basal and inducible IL-11 mRNA expression can be detected in fibroblasts, epithelial cells, chondrocytes, synoviocytes, keratinocytes, endothelial cells, osteoblasts, and certain tumor cells and cell lines (Table 3).

IL-11 has several activities. Human IL-11, in synergy with IL-3, IL-4, IL-6, or SCF, supports murine primitive hemopoietic cell. IL-11 can induce production of the same acute phase proteins (APP) as IL-6. IL-11 also acts on adipocytes and osteoclasts, and reduces proinflammatory mediator production by LPS-activated macrophages. IL-11 plasma levels are significantly elevated in patients with severe thrombocytopenia secondary to myeloablative therapy or in those with immune thrombocytopenia. Platelet counts and endogenous IL-11 levels after myeloablative therapy were inversely related. Therefore, it is suggested that induction of IL-11 expression is a physiological response to thrombocytopenia in humans and that IL-11 plays a role in the regulation of megakaryocytopoiesis and thrombopoiesis during acute thrombocytopenia (Goldman 1995). Although administration of IL-11 as a single agent to normal animals primarily stimulated megakaryocytopoiesis and thrombopoiesis, in myelosuppressed animals IL-11 stimulated multilineage recovery of hemopoietic progenitor cells. Mice receiving subcutaneous human IL-11 before whole-body radiation exposure had increased numbers of surviving crypts. IL-11 produces intestinal mucosal tropic effects in rats with experimental short bowel syndrome. Administration of IL-11 was also shown to enhance survival in a murine model of radiation-induced pulmonary injury (radiation pneumonitis). IL-11 has been recently approved for use in the prevention of the severe thrombocytopenia that occurs after cancer chemotherapy. Studies of cells and tissues from other organ systems indicate that IL-11 is active in protection and restoration of the gastrointestinal mucosa, and has major effects as an immunomodulating agent as well as activity in bone metabolism (Keith 2000).

Table 3 *Tissue/cell types expressing interleukin IL-11*

Tissue	Cell type/cell lines
CNS	Hippocampal neurons
	Spinal motor and sympathetic neurons
	Astrocytic glioblastoma
Thymus	Myeloid? (T2)
Lung	Fibroblasts, epithelial cells
Muscle cells	
Bone	Fibroblasts, osteosarcoma cell lines, osteoblasts
Connective tissues	Chondrocytes, synoviocytes
Vein endothelial cells	
Uterus	Trophoblast, endometriotic, and endometrial tissues
Skin	Keratinocytes, melanoma cell lines
Sarcoidosis	Multinucleated giant cells
Testis	Round spermatids

From Du and Williams (1997).

GROWTH AND DIFFERENTIATION FACTORS FOR LYMPHOID CELLS

Interleukin 2

IL-2 was the first interleukin to be identified and characterized at the molecular level. IL-2 is a small (15.5 kDa) globular glycoprotein of 133 amino acids. The IL-2 gene is located in chromosome 4, band q28 (Smith 2000).

For a long time, the only cells known to express the IL-2 gene following antigen or polyclonal stimulation were T cells (Smith 1980). More recently, however, dendritic cells have also been shown to express the IL-2 gene (Granucci et al. 2003). Of note, IL-2 shares a receptor chain (γc) with other cytokines, i.e. IL-4, IL-7, IL-9, IL-15, and IL-21 (Figure 4), whereas its α chain (known as TAC) is specific.

One of the major activities of IL-2 is to promote the clonal expansion of both $CD4^+$ T-helper (Th) cells

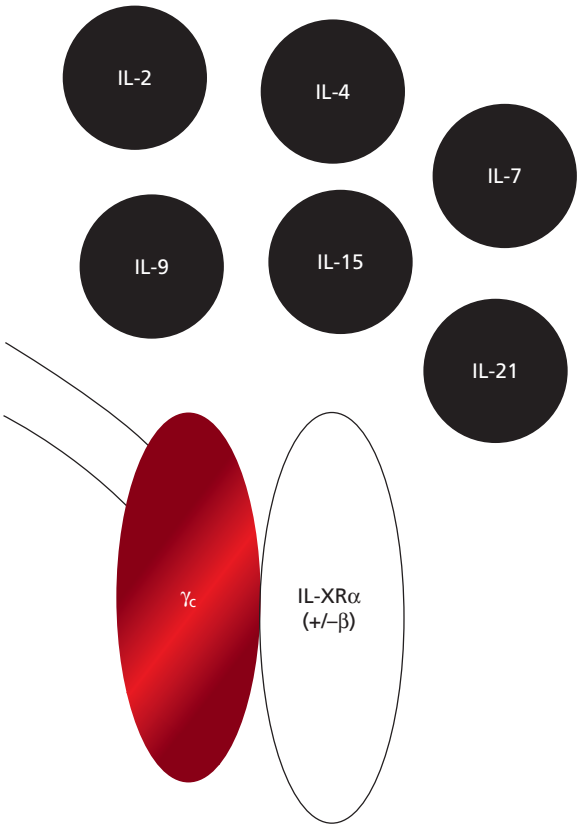

Figure 4 *Interleukins IL-2, IL-4, IL-7, IL-9, and IL-15 share the γ chain of their receptor. (Modified from Fry and Mackall 2002.)*

and $CD8^+$ cytotoxic T lymphocytes (CTLs). IL-2 is obligatory for the generation of both type 1 T-helper (Th1) and type 2 (Th2) cells and potentiates the production of the characteristic cytokines released by these differentiated Th cell subsets. IL-2 also synergizes with both interferon-γ (IFN-γ) and IL-12 in augmenting natural killer (NK) cell cytolytic activity. In addition, IL-2 also triggers the feedback downregulation of the expanded, differentiated effector cells. First, IL-2 is produced only transiently after antigen activation and, therefore, its positive effects rapidly disappear. Second, IL-2 withdrawal results in apoptosis, so that many of the antigen-specific effector cells are deleted as soon as there is no longer any antigen-induced IL-2 production. There are also other ways in which IL-2 gives negative signals. One is via the IL-2-promoted expression of CTLA-4, a negative regulator of T-cell receptor signals, which competes with the positive, costimulatory molecule, CD28. Thus, instead of leading to continuous cytokine production, the IL-2-promoted expression of CTLA-4 results in suppression of IL-2 gene expression. Also, IL-2 promotes the expression of Fas ligand (FasL), which activates programmed cell death via Fas, expressed to a greater extent on antigen-activated $CD4^+$ T cells (Smith 2000).

Deletion of the IL-2 gene has no effect on lymphocyte development, so that at birth IL-2 knock-out mice have normal numbers of T cells, B cells, and NK cells in both primary and secondary lymphoid compartments. However, during postnatal development a progressive lymphoid hyperplasia occurs, and cells accumulate in the secondary lymphoid tissues with an activated surface phenotype. Concomitantly, an autoimmune hemolytic anemia ensues, and mice begin to die from anemia within the first few weeks of life. Mice surviving beyond this initial phase subsequently develop colonic enteritis (Schorle et al. 1991)

IL-2 has been the first interleukin to be used therapeutically in patients with cancer. IL-2 was initially administered in these patients at very high doses intravenously. This kind of treatment regimen resulted in severe systemic toxicity ('cytokine syndrome'). IL-2 therapy results in an antitumor response in about 15 percent of individuals suffering from renal cell carcinoma, with about 10 percent of individuals achieving a long-term, complete response (Rosenberg et al. 1987), but the mechanisms responsible for this antitumor response still remain obscure, even after 15 years of IL-2 therapy. More recently, IL-2 has been used in the treatment of individuals infected with HIV (Kovacs et al. 1996). The use of the anti-TAC monoclonal antibody to prevent IL-2 binding to its high-affinity receptor, has recently been shown to be an effective immunosuppressant therapy for the prevention of allograft rejection (Vincenti et al. 1998). Accordingly, all of these approaches underscore the central role of IL-2 in the generation of an effective immune response, and the effectiveness of immunosuppressive therapies that block either IL-2 production or activity.

Interleukin 4

IL-4 is a 20-kDa secreted glycoprotein. The IL-4 gene resides on the long arm of chromosome 5 at position 5q21 within a complex that contains genes for other cytokines. The IL-4 gene is closely linked to the IL-13 gene, which lies immediately downstream of the IL-4 locus. The gene for another Th2 cytokine, IL-5, is also closely linked. The IL-3 and GM-CSF genes are located in this chromosomal region as well. The close proximity of the cytokines IL-4, IL-13, and IL-5 suggests that there may be some type of locus control to allow for coordinate expressions of these cytokines in Th2 cells (Keegan 2000).

IL-4 expression is induced in T cells and highly induced in differentiated Th2 cells and $NK1.1^+$ T cells in response to stimulation via the TCR. IL-4 message is also expressed in mast cells, basophils, and eosinophils in response to stimulation through the cell surface receptor for IgE.

IL-4 can elicit many diverse biological responses. These responses include costimulation of B-cell and T-cell proliferation, the protection of cells from spontaneous and induced apoptosis, and the regulation of chloride ion transport by intestinal epithelial cells. However, the dominant function of IL-4 is its ability to promote the differentiation of naïve $CD4^+$ T cells into the so-called Th2 profile of cytokine production (Figure 5). As known, Th2 cells secrete a set of cytokines, including IL-4, IL-5, IL-6, IL-10, and IL-13, that tend to favor a phagocyte-independent immune response while suppressing the phagocyte-dependent immune response controlled by Th1 cells. The second dominant function of IL-4 is its ability to drive immunoglobulin class switching to the IgG1 and IgE isotypes in mice and to the IgG4 and IgE isotypes in humans (Paul 1991). The ability of IL-4 to regulate the Th cell phenotype has direct relevance to the outcome of several diseases. Excessive IL-4 production by Th2 cells has been associated with elevated IgE production and allergy. In addition, a strong tendency towards Th2 cell differentiation can result in a failure to cure certain infections efficiently by intracellular pathogens such as *Leishmania major*, and lead to immunopathology. IL-4 also has a number of effects on monocytes. It increases expression of MHC class II molecules and IL-1Ra, while downregulating production of the proinflammatory cytokines, IL-1, TNF-α, IL-6, and IL-8. IL-4 is a growth and survival factor for mast cells. It enhances VCAM-1 expression on endothelial cells and downregulates IL-8 production. IL-4 induces eotaxin production by lung, and calcium ion secretion by gut, epithelial cells (Keegan 2000).

IL-4 has been used to treat a number of disease models in mice. It has been the most successful at

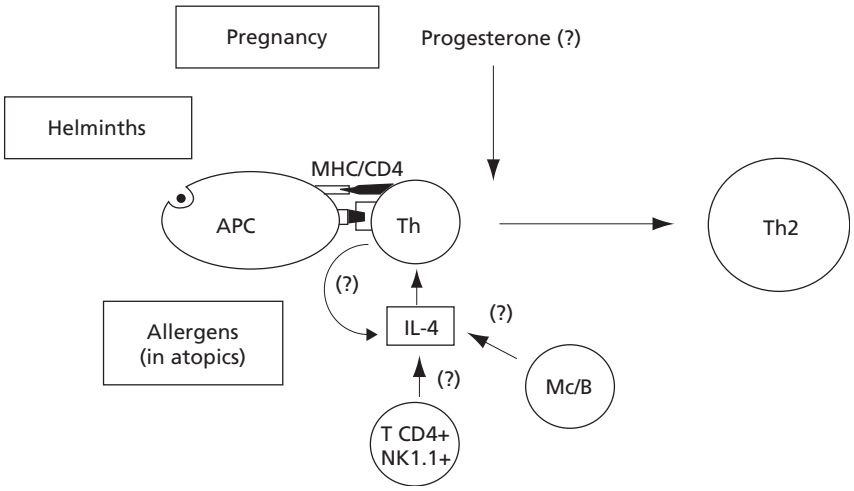

Figure 5 *Role of interleukin IL-4 in the differentiation of naive T-helper (Th) cells into Th2 effectors. IL-4, produced by natural killer NK.1.1$^+$ T cells, mast cells/basophils, but mainly by naïve Th cells themselves, at the beginning of the specific immune response, is required for the differentiation of these last cells into Th2 effectors. APC, antigen-presenting cell. (Redrawn from Romagnani 1998, with permission.)*

ameliorating autoimmune diseases that are caused by activated Th1 cells. IL-4 treatment prevents the development of diabetes in NOD mice, as well the development of experimental autoimmune encephalitis (EAE) or neuritis. Although initially characterized as a growth and survival factor, IL-4 has been shown to suppress the growth of some cancer cells in vivo. This inhibition is seen in the absence of T cells and depends on the infiltration of eosinophil granulocytes and the inhibition of tumor angiogenesis. IL-4 has been used systemically as an aid to cell-based therapy for cancer. Bolus injection of IL-4 results in flu-like symptoms, gastrointestinal upset, asymptomatic liver damage, severe allergy-type symptoms in the nasal mucosa, and vascular leak syndrome. However, IL-4 does not have apparent toxicity if administered locally (Saleh et al. 1999).

Interleukin 7

IL-7 is a single-chain glycoprotein of 25 kDa. The human IL-7 gene is located on chromosome 8, bands q13 (Spits 2000).

The main cell types that express the gene are bone marrow stroma, thymic stroma, intestinal epithelial cells and keratinocytes, mature but not immature dendritic cells, dendritic cells derived from CD34$^+$ cord blood cells cultured with GM-CSF and TNF-α, and platelets (Wiles et al. 1992; Watanabe et al. 1995).

Gene-targeting studies in the mouse have demonstrated that IL-7 is a major factor involved in development of T and B lymphocytes. In humans, IL-7 plays an essential role in the development of T cells (Figure 6).
IL-7 also has effects on human B-cell precursors, but it does not seem to be essential for human B-cell development. Whether IL-7 is actually a growth factor for immature thymocytes is somewhat controversial, but it is well established that IL-7 acts as a growth/maturation factor for human thymocyte precursors. IL-7 has also been reported directly to augment cytolytic activity in human NK cells, even if less efficiently than IL-2. Several reports have documented effects of IL-7 on both development and function of cells of the myeloid lineages. IL-7 also enhances myeloid colony formation from CD34$^+$ cells in vitro and may play a role in the function of mature monocyte–macrophages by inducing secretion of cytokines and increasing tumoricidal activity (Spits 2000).

The activities of IL-7 on T cells have led to the proposal that IL-7 can be used to overcome immunodeficiencies in certain situations by accelerating T-cell development, expanding the pool of newly developed T cells, and promoting functional maturation of T cells. However, more studies are necessary to evaluate the effects of IL-7 on immune reconstitution.

Interleukin 9

IL-9 protein sequence contains 144 residues with a signal peptide of 18 amino acids. The human IL-9 gene is a single copy gene and was mapped on chromosome 5, in the 5q31q35 region, which contains various growth factor and growth factor receptor genes such as IL-3, IL-4, IL-5, GM-CSF, and CSF-1 receptor (CSF-1R) (Renauld 2000).

So far, IL-9 expression seems to be mainly restricted to activated CD4$^+$CD45RO$^+$ T cells (Houssiau et al. 1995), HTLVI- infected T-cell leukemias (Kelleher et al. 1991), and Hodgkin's cell lines (Merz et al. 1991). Polyclonal T-cell stimulants induce a substantial IL-9 expression by T-cell-enriched lymphocyte populations, and more specifically, by CD4$^+$CD45RO$^+$ T cells. IL-9 expression appears in the late stages of T-cell activation, with a peak at 28 h. IL-2 was identified as a major

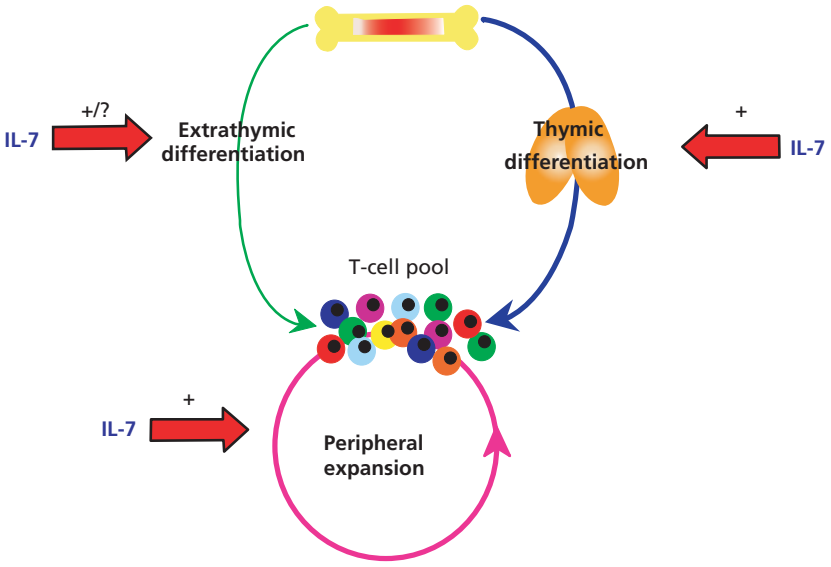

Figure 6 *Modulation of T-cell regenerative pathways by the interleukin IL-7. After T-cell depletion, regeneration of the peripheral T-cell pool can occur through multiple mechanisms. Thymic differentiation is the predominant pathway through which new T cells are generated if thymic capacity is sufficient. However, with diminished thymic function related to therapy-related toxicity, or age-related decline, the peripheral expansion of remaining mature T cells can substantially regenerate the T-cell pool. Extrathymic differentiation from bone marrow progenitors is a relatively minor pathway through which new T cells develop. IL-7 can profoundly increase thymic differentiation, peripheral expansion, and potentially extrathymic differentiation pathways to T-cell regeneration. (Redrawn from Fry and Mackall 2002, with permission.)*

mediator of IL-9 expression, because anti-TAC antibodies completely block this process.

Several studies indicate that IL-9 not only is involved in the late growth of T cells, but also induces the proliferation and differentiation of mast cells. Other potential biological targets for IL-9 include B lymphocytes, eosinophils, and hemopoietic progenitors. IL-9 might play an important role in the immune response against intestinal parasites such as helminths, probably by contributing to the development of mucosal mast cell hyperplasia induced by worm infections. Resistance to *Trichuris muris* was found to correlate with the production of IL-5 and IL-9 in mesenteric lymph nodes. The existence of an IL-9-mediated autocrine loop has been suggested for Hodgkin's disease. In addition, both genetic and experimental evidence points to the implication of IL-9 in the pathogenesis of asthma (Figure 7).
The potential activity of IL-9 in autoimmunity models is illustrated by preliminary data obtained in nonobese diabetic (NOD) mice (Renauld 2000).

Interleukin 15

IL-15 is a 15-kDa glycoprotein whose mature form consists of 114 amino acids. The IL-15 gene was mapped to chromosome 4q31. The classic long (48 amino acids) signal peptide associated with all secreted IL-15 is encoded by a 1.6 kbp cDNA (Waldmann and Tagaya 2000).

There is widespread constitutive expression of IL-15 mRNA in a variety of tissues (Waldmann and Tagaya 2000). IL-15 stimulates the proliferation of activated $CD4^+$ and $CD8^+$ T cells and dendritic epidermal T cells. IL-15 does not have any effect on resting B cells, but it induces proliferation and immunoglobulin synthesis by B cells costimulated with phorbol myristate acetate or an immobilized anti-IgM antibody. However, the most critical function of IL-15 is a pivotal role in the development, survival, and activation of NK cells (Figure 8).
IL-15 also has unique functions on nonlymphoid cells, including mast cells and muscle, brain, and microglial cells. The action of IL-15 on T cells, B cells, and NK cells can be blocked by selective antibodies to the IL-2/15Rβ chain. IL-15 acting through the IL-2/15Rβ and γc receptors leads to the induction of the expression of IL-2Rα by T lymphocytes.

Failure to produce IL-15 has not been demonstrated in disease states, although presumably it would be associated with the absence of NK cell development. Abnormalities involving increased IL-15 expression have been reported in inflammatory autoimmune diseases. IL-15 may precede tumor necrosis factor-α (TNF-α) in the cytokine cascade and suggest a role for IL-15 in development of rheumatoid arthritis, as well as in other inflammatory diseases, including active ulcerative colitis, Crohn's disease, type C chronic liver disease, sarcoidosis, T-cell-mediated alveolitis, and multiple sclerosis. Furthermore, the observation that IL-15 stimulates mast cell proliferation suggests a potential role for this cytokine in mastocytosis. IL-15 was shown to help correct impaired proliferative responses of $CD4^+$ lymphocytes studied ex vivo from HIV-infected individuals. Thus, as IL-15 is associated with less severe capillary leak syndrome than

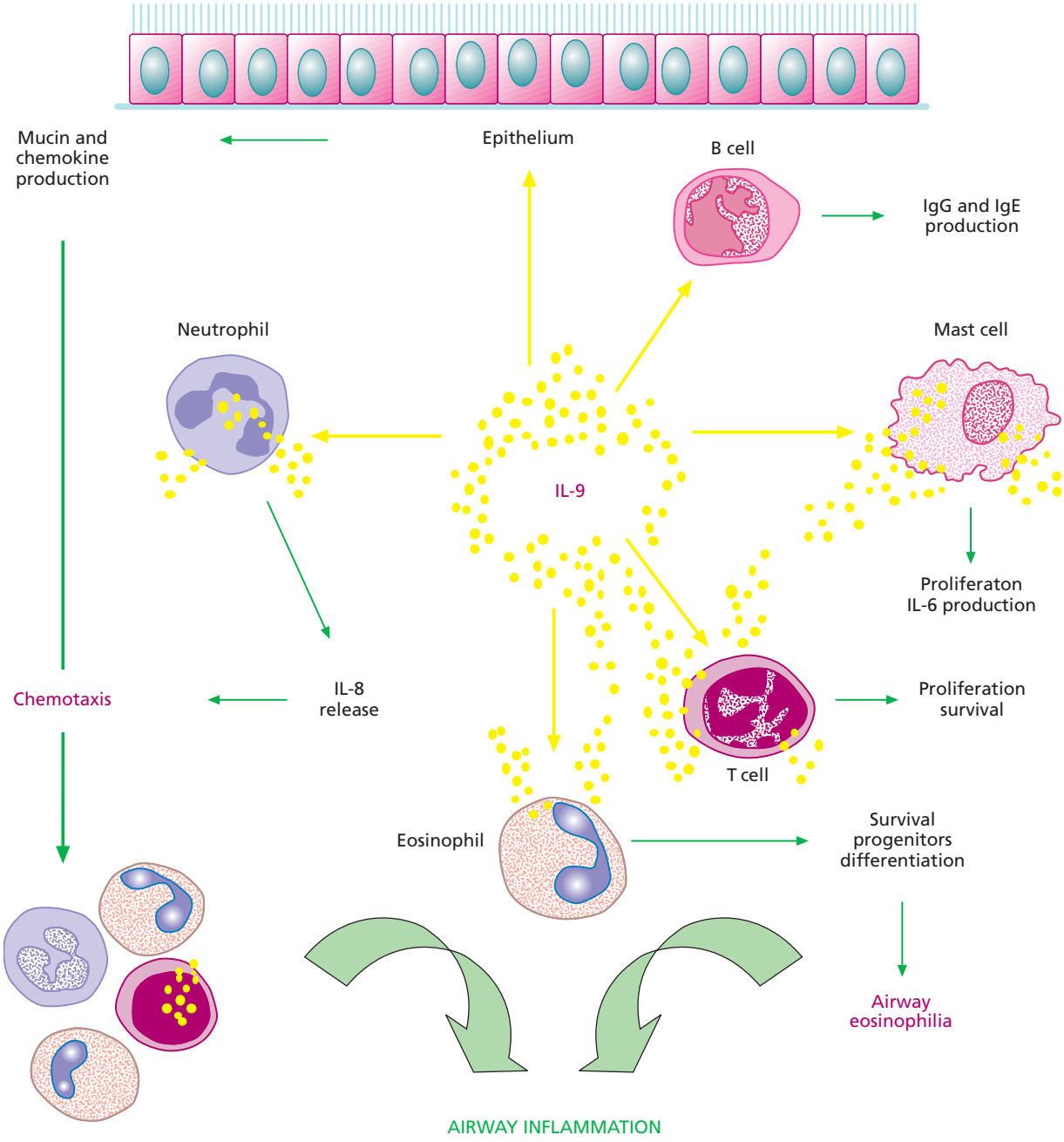

Figure 7 *Role of the interleukin IL-9 in pathophysiological manifestations of allergic inflammation and bronchial asthma. IL-9, a T-helper 2 (Th2) cytokine, has a pleiotropic activity on inflammatory and structural cells associated with asthma, such as mast cells, T cells, eosinophils, and epithelial cells. Within the lung, IL-9 is mainly produced by T cells and, to a lesser extent, by granulocytes. The effect of IL-9 leads to various immunological processes, including eosinophil survival, chemokines, and mucus production. These pathways may provide a potential mechanism to explain airway inflammation. (Redrawn from Soussi-Gonoussi et al. 2001, with permission.)*

the IL-2 molecule, it could provide an alternate therapeutic option to IL-2 in the treatment of patients with select tumors or AIDS (Waldmann and Tagaya 2000).

Interleukin 21

IL-21 is a four-helix-bundle cytokine with 131 amino acids that has significant sequence with homology to IL-2, IL-4, and IL-15. The IL-21R has highest homology to the IL-2Rβ chain and the IL-4Rα chain. On ligand binding, IL-21R has been reported to associate with the common γ chain, a property that it shares with receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. The main source for IL-21 seems to be activated T lymphocytes. IL-21 apparently influences the functions of B cells, T cells, and NK cells (Parish-Novak et al. 2000). Moreover,

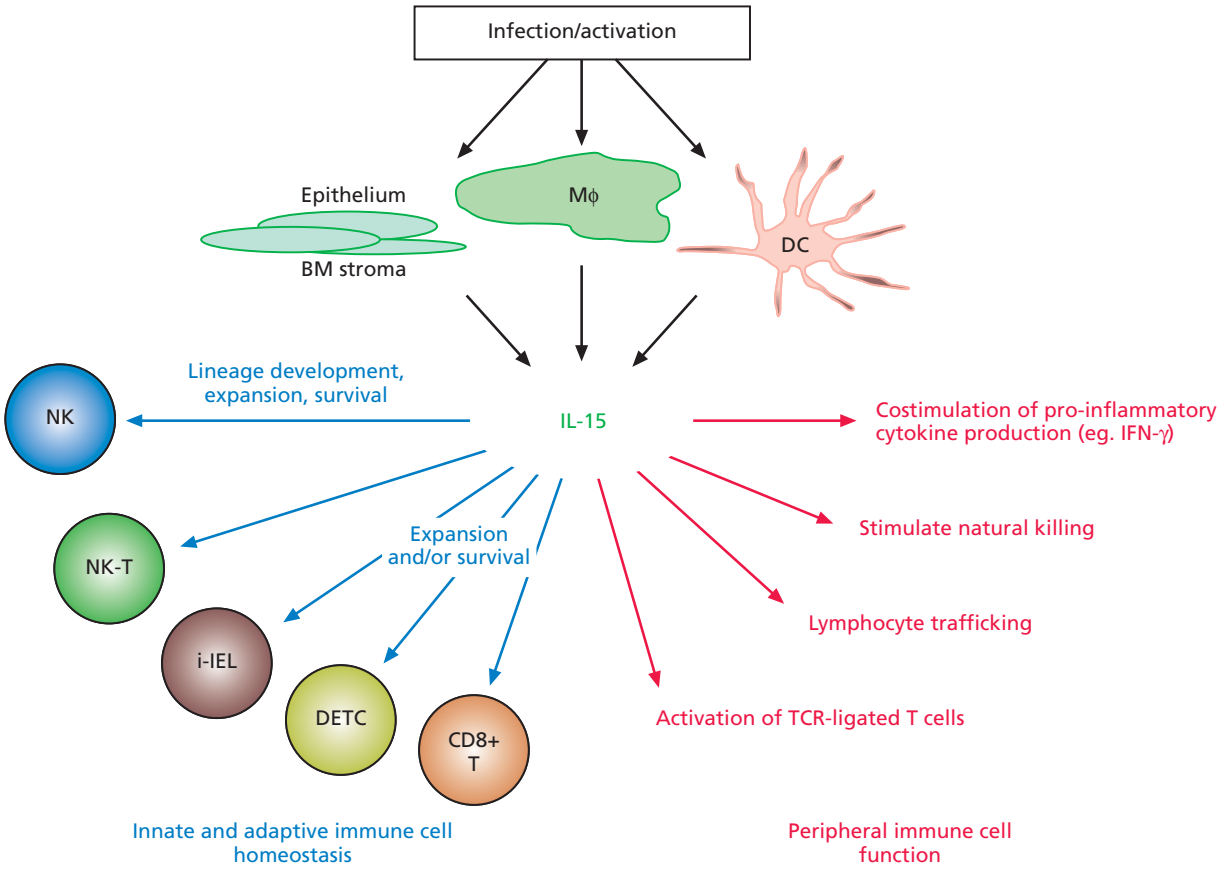

Figure 8 *Role of interleukin IL-15 in natural killer (NK) cell development, lymphocytes homeostasis, and peripheral immune functions. Multiple cell types elaborate IL-15, including activated monocytes/macrophages, dendritic cells, and epithelium, as well as constitutive production by bone marrow stromal cells. IL-15 has a critical role in the development of NK lineage, as well as in its survival, expansion, and function. Other innate lymphocyte (NK-T, CD8αα intestinal intraepithelial lymphocytes, TCRγδ DETCs) and memory phenotype CD8$^+$ T cells depend on IL-15 for survival or expansion. DETC, dendritic epidermal T-cells; IFN, interferon; Mϕ, macrophage; TCR, T-cell receptor. (Redrawn from Fehniger and Caligiuri 2001, with permission.)*

IL-21 has recently been shown to exert a proliferative effect on human myeloma cells and to inhibit the apoptosis of the IL-6-dependent human myeloma cell lines with a potency close to that of IL-6 (Brenne et al. 2002).

Interleukin 27

IL-27 is a new heterodimeric cytokine composed of EB13 (an IL-12 p40-related protein) and p28 (a new IL-12 p35-related polypeptide). It represents an early product of activated antigen-presenting cells (APC) and induces the proliferation of naïve, but not memory, CD4$^+$ T cells. It also synergizes with IL-12 for the development of Th1 cells via the receptor TCCR/WSX-1 (Pflanz et al. 2002).

PROINFLAMMATORY CYTOKINES

Interleukin 1

There are two distinct species of IL-1, which have been named as IL-1α and IL-1β, respectively. IL-1α is initially synthesized as a (31-kDa) precursor molecule without a signal peptide, mature IL-1α being a 17.5-kDa molecule. Calpain, a calcium-activated cysteine protease, is responsible for the cleavage of the IL-1α precursor (Dinarello 2000b). IL-1β is initially synthesized as a precursor molecule (31 kDa) without a signal peptide. Mature IL-1β is a 17-kDa molecule. The IL-1β-converting enzyme (ICE) is primarily responsible for cleavage of the precursor intracellularly, but other proteases can also process the IL-1β precursor into an active cytokine (Dinarello 2000a, 2000b).

Blood monocytes and tissue macrophages are the main sources of IL-1α and IL-1β. However, unlike IL-1β, IL-1α is found constitutively expressed also in keratinocytes and other epithelial cells. Virtually all microbes and microbial products induce the production of both IL-1α and IL-1β. The main differences between the two cytokines are summarized in Table 4.

IL-1 induces neutrophilia and the production of acute phase proteins (APP), and the injection of modest doses of IL-1 into mice results in fever, anorexia, and the production of circulating IL-6. The most dramatic

Table 4 *A comparison of interleukins IL-1α and IL-1β*

IL-1α	IL-1β
Pro-IL-1α is active	Pro-IL-1β is inactive
Active membrane form is IL-1α	Membrane IL-1β not observed
Mature IL-1α does not circulate	Mature IL-1β circulates
Nuclear localization	No nuclear localization
Intracellular role for pro-IL-1α	No intracellular role for pro-IL-1β
Neutralizing autoantibodies	Non-neutralizing autoantibodies
Calpain cleavage of pro-IL-1α	ICE and PR-3 cleavage of pro-IL-1β
No disease link with calpain expression	Disease link with ICE expression
No correlation	Correlation with bone resorption
IL-1α knock-out mice normal	IL-1β knock-out mice resistant to disease
Anti-IL-1α ineffective in CIA	Anti-IL-1β effective in CIA
IL-1α expression in AML absent	IL-1β expression in AML present
No data	ICE inhibition→reduced brain ischemia
No data	ICE inhibition→reduced AML proliferation
No data	ICE antisense→reduced AML proliferation

AML, acute myeloid leukemia; CIA, collagen-induced arthritis; ICE, IL-1β-converting enzyme; PR-3, a compound used for cleavage.
From Dinarello (2000b).

responses to the pharmacological effects of IL-1 are observed in humans. The role of IL-1 in human disease states is best revealed by the response of humans to parenterally administered IL-1. Chills and fever are observed in nearly all patients. The febrile response increases in magnitude with increasing dose, and chills and fever can be abated with indometacin (indomethacin) treatment. IL-1 is also a potent immunoadjuvant for several tumors. IL-1 has been administered to patients during receipt of ABMT. The treatment with IL-1 of ABM or SC transfer resulted in an earlier recovery of thrombocytopenia compared with controls (Dinarello 2000b).

Interleukin 6

Human IL-6 is a protein of 186 amino acids glycosylated at positions 73 and 172. It is synthesized as a precursor protein of 212 amino acids. At least five different molecular forms of IL-6 with molecular masses from 21 to 28 kDa are expressed in monocytes. The IL-6 gene has a length of approximately 5 kb and maps to human chromosome 7p21 between the markers D7S135 and D7S370 (Matsuda and Hirano 2000).

IL-6 is produced by a variety of cell types. The main sources are macrophages, fibroblasts, and endothelial cells, but also T and B cells. The IL-6 receptor consists of two subunits: the α chain (IL-6R), an 80-kDa transmembrane glycoprotein that binds IL-6 with low affinity, and the β chain (gp130), a 130-kDa transmembrane glycoprotein that binds to the IL-6–IL-6R heterodimer to form the high-affinity signal transducing complex (Matsuda and Hirano 2000).

IL-6 induces immunoglobulin production by normal activated or EBV-transformed or leukemic B cells. IL-6 is involved in T-cell activation, growth, and differentiation. IL-6 synergizes with IL-3 to induce the proliferation of murine pluripotent hemopoietic progenitors in vitro. Human megakaryocytes were demonstrated to express IL-6 receptor and produce IL-6, suggesting that IL-6 regulates the terminal maturation of megakaryocytes in an autocrine manner. However, the most important function of IL-6 is to induce the biosynthesis by hepatocytes of APPs, such as fibrinogen, α1-antichymotrypsin, haptoglobulin, serum amyloid A, and C-reactive protein (CRP) (Geiger et al. 1988). As a result of the ability of IL-6 to act as a growth factor for plasmacytoma, myeloma, and hybridoma cells, different inhibitors of IL-6, such as anti-IL-6 monoclonal antibody and mutated IL-6, might be used to treat patients with multiple myeloma, Castleman's disease, and rheumatoid arthritis (Hirano 1998).

TNF-α

TNF-α under denaturing conditions is about 17 kDa. Under native conditions, however, TNF-α is a trimer with an approximate molecular mass of 50 kDa. The TNF-α gene is localized on human chromosome 6 in a region between p23 and q12 (p21.1 and p21.3) (Aggarwal et al. 2000).

Although first isolated from a macrophage-like cell line, it is now clear that TNF is produced by a wide variety of different cell types, including macrophages and monocytes, T, B, and NK cells, astrocytes, fibroblasts, basophils, mast cells, Kupffer cells, smooth muscle cells, and epidermal cells, as well as neoplastic cells from breast tumor, ovarian tumor, prostate tumor, pancreatic cancer, glioblastoma, melanoma, and leukemia. However, monocytes/macrophages are prob-

ably the major sources of TNF in most conditions (Aggarwal and Vilcek 1992).

Tumor necrosis factor binds to two different receptors referred to as p60 (also called p55, or type I or CD120a) and p80 (also called p75, or type II or CD120b) based on their molecular weight. These two receptors are homologous in their extracellular domains, but distinct in their intracellular domains. The p60 receptor is expressed on all cell types, whereas the p80 form is expressed chiefly on cells of the hemopoietic and immune system, as well as on endothelial cells. The binding of TNF to its receptor can be displaced by lymphotoxin, suggesting that they have a common receptor.

The true normal physiological role of TNF in vivo is unclear. TNF is an inflammatory cytokine, because of its chemotactic activity on monocytes and neutrophils. Moreover, stimulation with TNF induces phagocytosis, and adherence of these cells to endothelial cells, as well as generation of free radicals of oxygen, superoxide anion, and hydrogen peroxide. Therefore, it is believed that TNF is important for protection against bacterial, fungal, parasitic, and perhaps even viral infections and other stressful stimuli. Besides hemorrhagic necrosis, whether TNF is able to block tumorigenesis and metastasis in vivo is still not fully understood (Lejeune et al. 1998). Administration of TNF to various animals leads to hemorrhage, necrosis, local inflammation, shock, and death. Prolonged exposure to TNF causes anorexia, loss of body weight, dehydration, and loss of body proteins and lipids. This condition, called cachexia, may occur during chronic parasitic, bacterial, and viral infections. The symptoms also appear in cancer patients. The mediator involved in this process was named cachectin, later recognized to be the same as TNF. In severe diseases such as AIDS, the TNF level is significantly increased when the signs and symptoms of cachexia develop. Stimulation of cultured human endothelial cells with TNF induces procoagulant activity. TNF-induced endothelial cell activation leads to the structural reorganization of the endothelium, resulting in vascular leakiness that is partly caused by its capacity to upregulate vascular endothelial growth factor (VEGF). The main activities of TNF-α are summarized in Figure 9. TNF is considered as a major mediator of septic shock, because it is overproduced during sepsis and administration of high doses of TNF-inducing LPS causes shock and tissue injury that are identical to sepsis (Aggarwal et al. 2000).

Lymphotoxins

Lymphotoxin (LT) was initially discovered as a secreted cytotoxic/cytostatic factor produced by activated lymphocytes. It was then recognized as a member of a large superfamily (Table 5). A distinct cell surface form of LT was defined as a heterotrimer made up of a subunit of LT with a dimer of a 33-kDa protein, designated LTβ, the third member of the family. The soluble LT is known as LTα, which was previously called TNF-β. The cell surface form of LT is formed by two subunits, LTα and LTβ. Two distinct heterotrimers are formed, LTαβ2 (major form) and LTα2β1. LTα is TNF splicing factor (SF)1 and LTβ is TNFSF3. LTα is a secreted glycoprotein of 171 amino acids with a secondary structure of antiparallel sandwich structure which assembles into a relatively compact trimer, a feature common to all members of the TNF superfamily. LTβ is a type II transmembrane glycoprotein (N-terminal cytosolic tail), which lacks a signal cleavage sequence and is retained in the membrane. LTβ is biologically active as a heterotrimer with LTα. The LTαβ ligand remains anchored to the cell membrane and, as such, acts in a localized fashion that requires cell-to-cell contact. By contrast, LTα lacks a retained transmembrane domain and is exclusively secreted as a homotrimer. TNF, LTα, and LTβ form a contiguous cytokine locus that resides on chromosome 6p21.3 (chromosome 17 in mouse) within the major histocompatibility gene complex (MHC). The LTα, LTβ, and TNF genes show similar intron and exon organization (Wallach et al. 1999), which is largely conserved for all members of the TNF superfamily.

The main sources of LTα and LTβ are activated T and B lymphocytes, but LTs are also produced by NK and lymphokine-activated killer (LAK) cells in response to IL-2. LTα binds to the receptors TNFRI and TNFRII with high affinity, and to HVEM with low affinity; LTα2β1 binds to the LTβR, whereas the LTα2β1 complex binds LTβ R weakly (25 nmol/l), but TNFRI and TNFRII with high affinity (Ware et al. 1998).

The in vitro biological responses to LTα and LTβ are very similar. However, based on dose–response curves, LTα is typically less potent compared with TNF in inducing most functions. LTα behaves as a partial agonist of TNF in some assays, such as induction of MHC molecules, intercellular adhesion molecule 1 (ICAM-1), and other types of markers of inflammatory reactions. LTα1β2 displays even weaker potency for induction of proinflammatory markers on various cell types, including endothelial cells. LTs play a role in the initiation of inflammation, which presumably contributes to the protection against microbes. LTα can also induce expression of adhesion molecules and chemokines, whereas LTαβ does not appear to be able to induce adhesion molecules, but can induce chemokines. LTα and LTβ may play normal roles in tumor immunity. LTα induces apoptosis (programmed cell death) in several tumor lines in vitro and LTα1β2 induces killing of a more limited array of tumor cells. This effect is the result of the action of a distinct types of receptor; LTα uses TNFRI, a death domain proapoptotic signaling receptor that directly activates the caspase pathway,

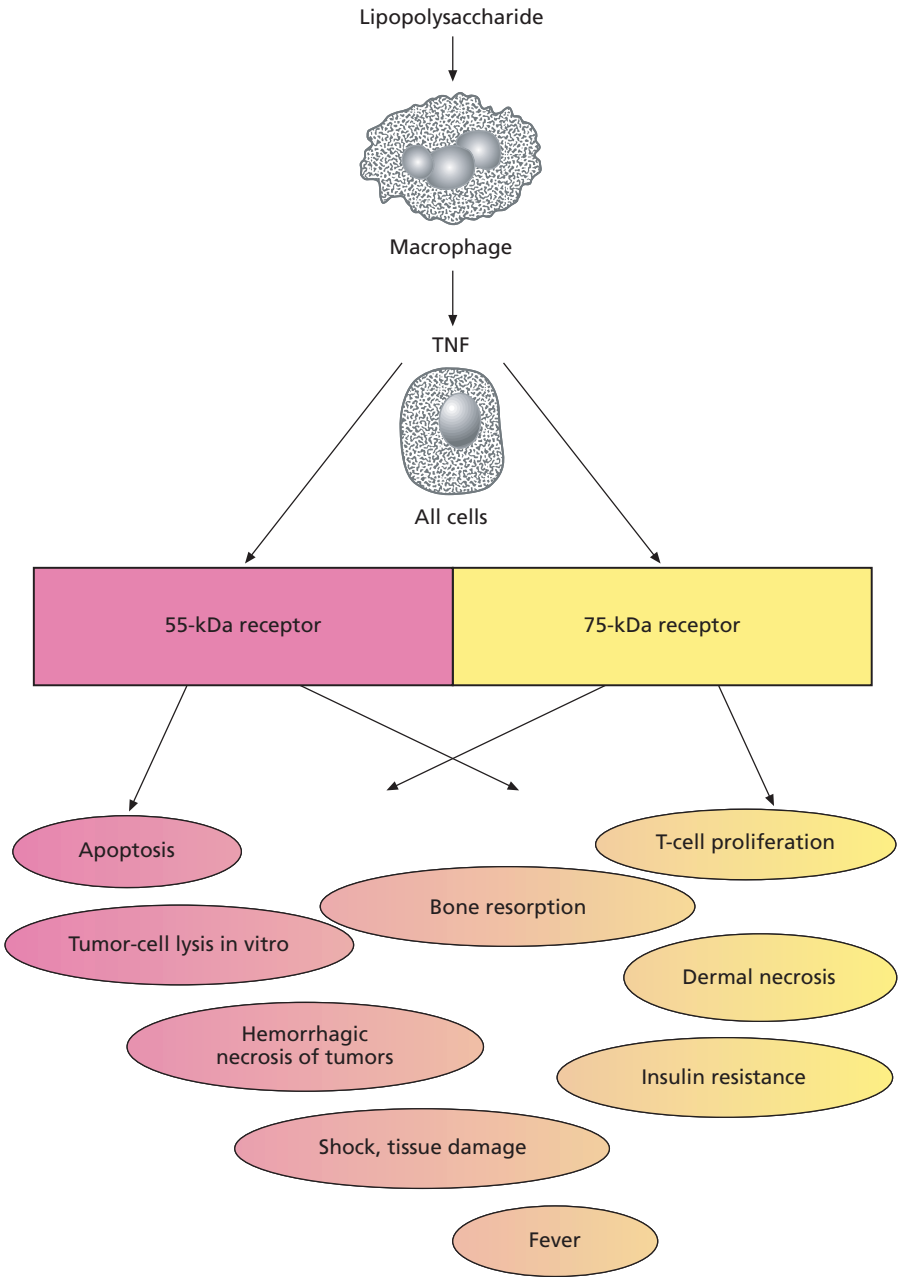

Figure 9 *Range of actions of tumor necrosis factor α (TNF-α). In response to inflammatory stimuli, such as lipopolysaccharide (LPS), macrophages produce TNF-α. TNF-α binds to receptors present on virtually all cells throughout the body, causing a variety of reactions. (Redrawn from Bazzoni and Beutler 1996, with permission.)*

whereas LTα1β2 uses the LTβR, a TRAF-binding receptor and a potent activator of apoptotic inhibitory pathways, such as NF-κB transcription factor (Ware et al. 1998). LT gene-deficient mice lack most lymph nodes, do not have Peyer's patches, and have disorganized spleens (Alimzhanov et al. 1997). LTs are elevated in several human diseases, including multiple sclerosis, juvenile rheumatoid arthritis, juvenile spondylarthropathy, and Crohn's disease, suggesting an important role of LTs in these disorders.

Interferon-γ

In its biologically active form, IFN-γ is a 34-kDa homodimer stabilized by noncovalent forces. The IFN-γ gene maps close to the D12S335 and D12S313 microsatellites and shows physical linkage with the *MDM2* oncogene on chromosome band 12q15 (Billiau and Vandenbroeck 2000).

Interferon-γ is a typical lymphokine, being produced almost exclusively by NK cells and certain subpopula-

Table 5 *Members of the tumor necrosis factor (TNF) ligand and TNF receptor family*

Ligand	Source of ligand	Receptor	Distribution of receptor
TNF-α	Macrophages	55-kDa	Many cell types
LTα	Macrophages	55-kDa	Many cell types
LTβ	T cells, others	75-kDa	T cells, B cells, others
Fas-L	T cells, others	LTβ receptor	Many cell types
NGF	NA	NGF receptor	Neurons, others
C40L	T cells	NGF receptor	B cells, T cells
CD27L	T cells	CD27	T cells
CD30L	T cells	CD30	T cells, B cells, others
OX-40L	T cells	OX40	T cells
4-1BBL	T cells	4-1BB	T cells

LT, lymphotoxin; NA, not applicable; NGF, nerve growth factor. Reproduced from Bazzoni and Beutler (1996), with permission.

tions of T cells. Both $CD4^+$ and $CD8^+$ lymphocytes can produce IFN-γ, but only Th1 cells produce IFN-γ following appropriate stimulation, whereas Th2 cells do not. IFN-γ acts on cells by inducing increased expression of several genes, the spectrum of which varies depending on the cell type concerned and the presence of other cytokines, some of which (e.g. IFN-γ) synergize with IFN-γ, whereas others (e.g. IL-4) antagonize its activities. Receptors for IFN-γ occur on virtually all cells of the body, so that many organs and systems undergo the action of IFN-γ (Billiau and Vandenbroeck 2000).

Interferon-γ regulates cellular activities responsible for inflammation, in particular the activation state of macrophages and endothelial cells. It also regulates the antigen-specific immune response by affecting both APCs and antigen-recognizing lymphocytes. IFN-γ has an important role in the generation of NO. Another important function of IFN-γ is the induction of MHC class II antigens on many, although not all, cell types, thus enhancing or inducing the ability of these cells to present foreign antigens. IFN-γ can also increase the expression of MHCI antigen. It is recognized as the most important cytokine converting macrophages from a 'resting' to an 'activated' state (macrophage activating factor (MAF)). IFN-γ and IL-4 antagonize each other in a variety of systems, and it has been established that antibody responses in fact depend on the balance between two categories of cytokines, IFN-γ and IL-2 produced by Th1 cells and IL-4, IL-5, IL-6, and IL-10 produced by Th2 cells. In this context, IFN-γ suppresses IgG1 and IgE and stimulates IgG2a antibody formation. Thus IFN-γ is not only important in the initial phase (aspecific inflammatory and antigen presenting) and in the middle phase (expansion and differentiation of antigen-reactive lymphocyte clones), but also in the end phase (sustained or final inflammation) of immune reactions. Defects in the IFN-γ mechanism are associated with severe impairment of resistance to infections caused by viruses and certain bacteria, in particular those normally killed by activated macrophages. Massive production of IFN-γ, occurring as part of so-called acute cytokine release syndromes, is often associated with severe systemic manifestations, such as generalized bleeding and lethal shock. IFN-γ has the potential to protect the host against virus infection, not only because of its regulatory activity on immunocytes, but also by virtue of its direct antiviral effect on most cell types. IFN-γ contributes to protection against tumors, as shown by studies in which the rejection of some immunogenic autologous or isologous tumors was abrogated by administration of neutralizing anti-IFN-γ antibodies. In children suffering from exceptional susceptibility to mycobacterial infections, inherited deficiencies in the IFN-γ receptor system have recently been described (Billiau and Vandenbroeck 2000).

Interleukin-12

IL-12 is a 70-kDa heterodimer composed of two disulfide-linked chains of 35 kDa (p35) and 40 kDa (p40). The genes encoding the two heterologous chains of IL-12 are located on different chromosomes. In humans, p40 is located in the 5q31–q33 region, whereas p35 has been mapped to 3p12–p13.2. Secretion of the biologically active IL-12 heterodimer (p70) is regulated at the level of p40 chain transcription. The p40 gene is highly inducible and expressed only in IL-12-secreting cells, whereas the p35 gene is expressed ubiquitously (Esche et al. 2000).

IL-12 was initially recognized as an inducer of IFN-γ synthesis by resting human peripheral blood mononuclear cells (PBMC) in vitro and as being capable of synergizing with IL-2 to increase cytotoxic lymphocyte responses. IL-12 also represents a growth factor for activated $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, and most importantly it promotes Th1-specific immune responses (Manetti et al. 1994) (Figure 10). Thus, IL-12 enhances host defenses against organisms that are controlled by cells mediating the delayed hypersensitivity response of T cells.

Dendritic cells are the most powerful producer of IL-12 when stimulated with CD154 (CD40L) or IL-12. Epidermal Langerhans' cells express IL-12 p40 mRNA,

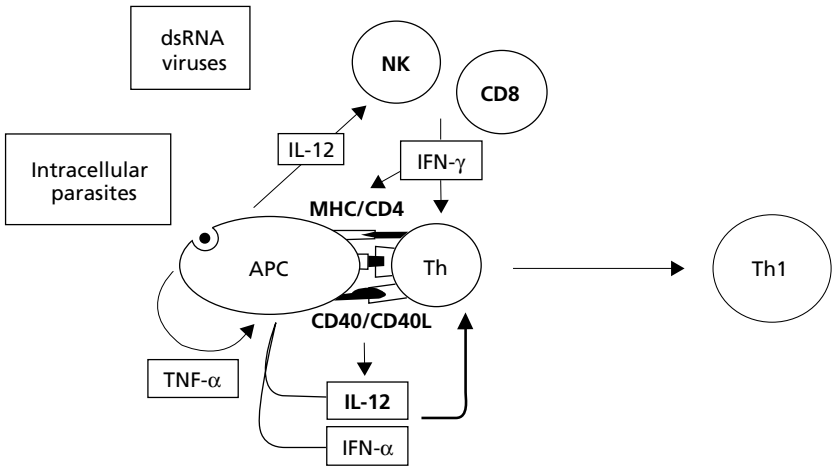

Figure 10 *Role of IL-12 in the differentiation of naive T-helper (Th) cells into Th1 effectors. Intracellular bacteria and some viruses strongly activate dendritic cells and macrophages to produce interferon α (IFN-α) and IL-12, as well as natural killer (NK cells) to produce IFN-γ. Under these microenvironmental conditions and in the absence of IL-4, naïve Th cells responsible for the subsequent specific immune response differentiate into Th1 effectors. (Reproduced from Romagnani 1998, with permission.)*

as well as p40 and functional p70 protein. Stimulated macrophages produce both p40 and p70. Although monomeric p40 is typically secreted in large excess over the p70 heterodimer, only the latter is biologically active. In humans, the homodimer binds with a much lower affinity than the heterodimer and therefore acts as an antagonist only at much higher concentration. IL-12-induced production of large amounts of IFN-γ from resting and activated T and NK cells requires the presence of low levels of both TNF-α and IL-1. IL-12 is also capable of inducing its own inhibitor IL-10, a negative-feedback mechanism that limits ongoing T-cell activation. IL-12 promotes the generation and potentiates the activity of cytotoxic T lymphocytes (CTL) and LAK cells (Trinchieri 1998).

This array of activities suggests clinical potential for IL-12 as an anti-infective and antitumor agent. The lack of IL-12R expression indeed results in a human immunodeficiency characterized by strong reduction in resistance to infections caused by intracellular bacteria. The attempt to administrate IL-12 in patients with cancer has been hampered by adverse events that are mostly IFN-γ dependent and include fever, chills, fatigue, headache, nausea, vomiting, cough, myalgia, dizziness, insomnia, anemia, neutropenia, lymphocytopenia, thrombocytopenia, hyperglycemia, liver function test abnormalities, rhinitis, stomatitis, and colitis. Three deaths have also been reported in renal carcinoma patients receiving IL-12, but prior exposure to IL-12 has been shown to protect against IL-12 toxicity (Leonard et al. 1997).

Interleukin 16

IL-16 is a 55-kDa protein which appears as a single 17-kDa band on sodium dodecylsulfate (SDS) gel electrophoresis, prompting the hypothesis of a monomeric polypeptide autoaggregated into tetramers. The IL-16 gen is located in chromosome 15q26.1 (Center et al. 2000).

IL-16 is synthesized by a variety of cell types although it was first identified as a $CD8^+$ lymphocyte cell product. $CD8^+$ T cells release IL-16 in response to stimulation by mitogens, antigens, or vasoactive amines such as histamine and serotonin (Laberge et al. 1996). For histamine, H_2-type receptors are required based on inhibition of secretion by H_2-, but not H_1-, receptor antagonists. Histamine has no effect on the secretion of IL-16 from $CD4^+$ T cells. Serotonin has similar effects on $CD8^+$ T cells via S2 receptors, but no effect on $CD4^+$ T cells.

IL-16 was initially described as a chemoattractant with specificity for $CD4^+$ T cells. It was later observed that IL-16 is also a potent chemoattractant for all peripheral immune cells expressing CD4, including $CD4^+$ monocytes, eosinophils, and dendritic cells. The role of IL-16 in vivo remains to be elucidated; however, one possibility is that it contributes at least in part to antigen-independent, nonclonal recruitment and priming of $CD4^+$ cells in inflammatory processes, whereas the same cells become unresponsive to specific antigen stimulation. This condition may result in the increase of the number of viable cells recruited to an inflammatory site by reducing the susceptibility of those cells to antigen-induced cell death (AICD). As a result of these activities, IL-16 has to be considered as a proinflammatory cytokine. It may play an important role in the pathogenesis of diseases characterized by $CD4^+$ cellular infiltration, such as bronchial asthma and granulomatous diseases (Center et al. 2000).

Interleukin 17 family

IL-17 is secreted, after cleavage of a signal peptide of 23 amino acids, as a glycoprotein homodimer of

155 amino acids with an M_r ranging from 22 to 15 kDa. IL-17 gene has been mapped to chromosome 2 (2q31).

IL-17 appears to be mostly expressed by activated $CD4^+$ T cells, in particular by CD4/$^+$CD45RO 'memory' T cells. All T-cell activation signals upregulate IL-17 gene transcription. Analysis of T-cell clones suggests that both Th1 and Th2 $CD4^+$ subsets can express IL-17 at comparable levels. The IL-17R is ubiquitously expressed.

IL-17 was found to exert biological effects on cells of hemopoietic origin and stromal cells, to induce the secretion of IL-6 by skin and lung fibroblasts and by normal and rheumatoid synoviocytes. It also stimulates the production of IL-8, PGE_2, G-CSF, and leukemia inhibitory factor (LIF) by rheumatoid synoviocytes, upregulates the expression of ICAM-1 by foreskin fibroblasts, stimulates the production of NO by normal and osteoarthritic human articular chondrocytes, and increases their production of IL-1, IL-6, stromelysin, the inducible NO synthase (iNOS), and cyclooxygenase 2 (COX-2). IL-17 stimulates the production by endothelial and epithelial cells of several chemokines. IL-17 increases the secretion of G-CSF by human synoviocytes and by murine fibroblasts, leading fibroblasts to support the growth and differentiation of $CD34^+$ hemopoietic progenitors into mature neutrophils. IL-17 cooperates with TNF-α, IFN-γ, and IL-1 to induce the secretion of IL-6 by rheumatoid synoviocytes and with IFN-γ to enhance the production of IL-6 and IL-8 and to upregulate the expression of ICAM-1 and HLA-DR by human keratinocytes (Yao et al. 1995a, b). Thus, IL-17 mainly exhibits proinflammatory and hemopoietic activities, which appear to play an upstream role in T-cell-triggered inflammation and hemopoiesis, by stimulating stromal cells to secrete other cytokines and growth factors. The hemopoietic effect of IL-17, and in particular its ability to trigger indirectly an acute neutrophilia, might have therapeutic applications in the context of immunosuppression, i.e. after bone marrow transplantation. On the other side, IL-17 might represent a target for therapeutic inhibition in T-cell-dependent autoimmune diseases, chronic inflammatory conditions of the lung, skin, and intestinal tract, organ graft rejection, and some cancers (Lebecque et al. 2000).

More recently, three IL-17-associated molecules have been described, which have been called IL-17B, IL-17C, and IL-17F. Although also these new members can promote inflammation and hemopoiesis, some of the activities of IL-17B and -C are distinct from those described for IL-17, e.g. IL-17B and -C do not bind to IL-17R and only promote the expression of TNF-α and IL-1β, but not of IL-6, IL-8, and G-CSF. IL-17F-induced substantial increases in the mRNA for inflammatory cytokines and chemokines, including IL-6, IFN-γ, CXCL10, and CXCL9 (Li et al. 2000; Shi et al. 2000; Starnes et al. 2001).

Interleukin 18

IL-18 is a protein consisting of 193 amino acids showing homology with IL-1. IL-18 is processed by a protease-like ICE. Moreover, the receptor for IL-18 was shown to be a member of the IL-1 receptor family. The IL-18 gene is located on chromosome 11q22.2.3, closely linked to the dopamine receptor D2 (DRD2) locus, whereas the IL-18R gene maps to chromosome 2q11, on which human IL-1 family members and IL-1R type I are located (Okamura et al. 1995).

A wide range of cell types, including mononuclear cells, keratinocytes, osteoblastic cells, intestine epithelial cells, dendritic cells, chondrocytes, and neuroblastomas, express IL-18. However, IL-18 is mainly produced by activated macrophages or dendritic cells.

IL-18 was originally discovered as an IFN-γ-inducing factor for $CD4^+$ T cells. IL-18 by itself, as IL-12, induces only small amounts of this cytokine. However, combined stimulation of IL-18 and IL-12 induces remarkably higher production of IFN-γ. The underlying mechanism for this strong synergism is that IL-12 induces the IL-18R on $CD4^+$ T cells. $CD8^+$ T cells are also an important source of IFN-γ, and the same synergistic mechanism with IL-12 seems to be operating on $CD8^+$ T cells, although IL-18 also directly activates the cytotoxicity of $CD8^+$ T cells (Biet et al. 2002). When IL-18 was discovered, it was considered to be another factor that could differentiate naïve Th cells into Th1 effectors, such as IL-12. More recent studies have revealed that IL-18 itself has no ability to drive the differentiation of Th1 cells, but strongly induces them to express their functions and induce synthesis of IFN-γ or Fas ligand. However, surprisingly, it was recently found that IL-18, as IFN-γ, also enhances production of the Th2 cytokine IL-13 in T and NK cells. These findings suggest that IL-18 may be involved in the amplification, as well as the regulation, of Th1 responses through the induction of Th2 cytokine production. As with its actions on T cells, IL-18 alone, as well as IL-12 alone, induces NK cells to produce only a little IFN-γ, but again IL-18 and IL-12 synergize in the induction of IFN-γ by NK cells. The main targets of IL-18 and its mechanisms of action are summarized in Figure 11.

Since IL-18 induces IFN-γ production, it is obvious that this cytokine is involved in the defense mechanism against intracellular parasites, such as mycobacteria and *Listeria* spp. Recently, IL-18 was shown to contribute also to host defenses against *Salmonella typhimurium*, *Yersinia enterocolitica*, *Cryptococcus neoformans*, and some viruses, such as Epstein–Barr virus, influenza A virus, Sendai virus, herpes simplex virus, vaccinia, and encephalomyocarditis. Although IL-18 exhibits antitumor activity against several types of cells, the mechanism and mediating factors seem to be different for each type of tumor cell. Tumors transfected with

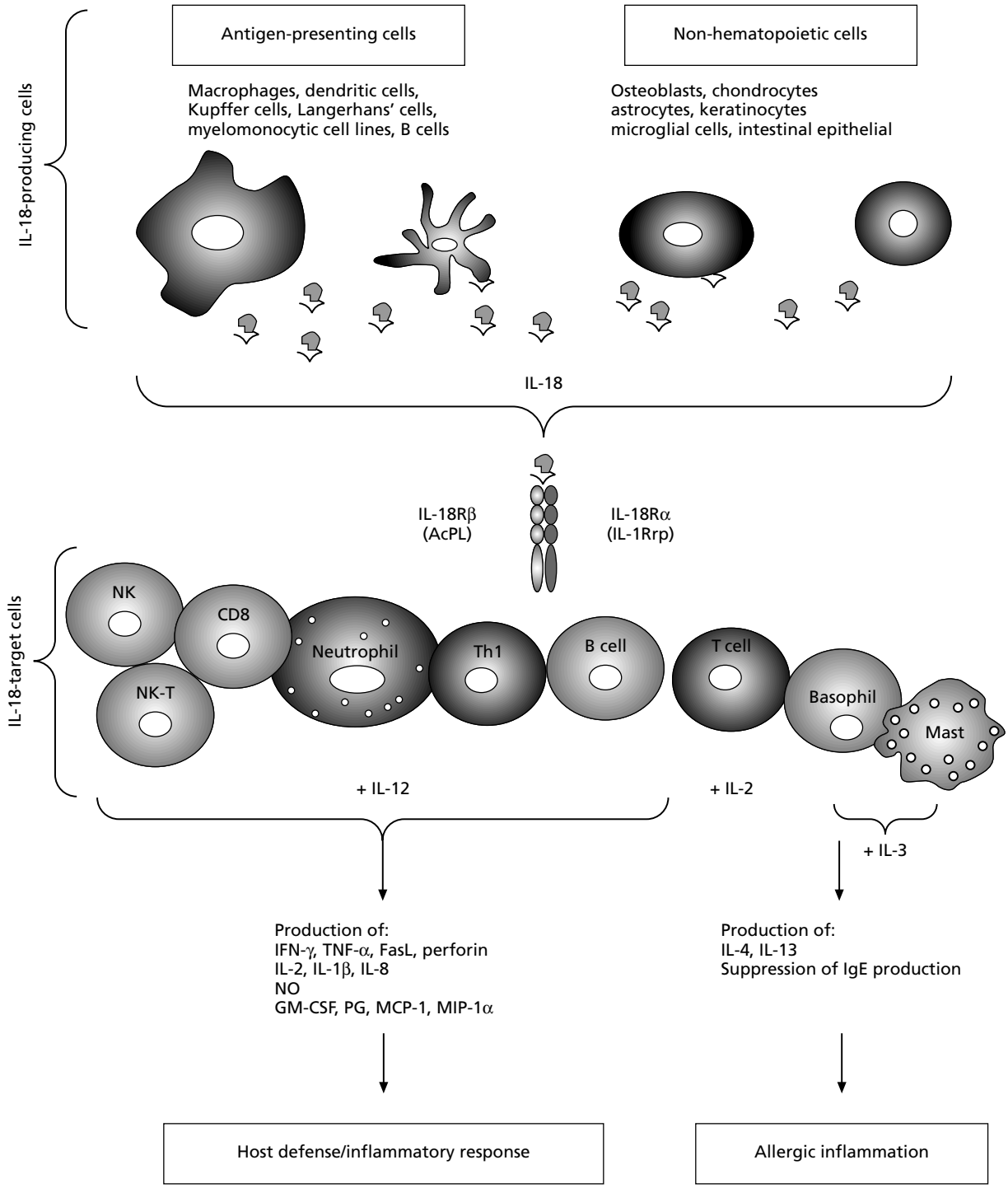

Figure 11 *Cellular sources and the targets of interleukin IL-18. Both antigen-presenting cells (APC) and nonhemopoietic cells produce IL-18. Synergy between IL-18 and IL-12 induce T-helper type 1 (Th1) differentiation increases the cytotoxic response of $CD8^+$ T cells, natural killer (NK) cells, and NK T cells and their production of interferon γ (IFN-γ), which results in the activation of macrophages. IL-18 has also the potential to induce Th2 cells in a IL-4-independent manner. In response to IL-18 and IL-3, mast cells and basophils produce large amounts of IL-4 and IL-13. Administration of a mixture of IL-12 and IL-18 inhibits IgE production. GM-CSF, granulocyte–macrophage colony-stimulating factor; MCP, monocytic chemotactic protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor. (Redrawn from Biet et al. 2002, with permission.)*

IL-18 gene exhibited reduced tumorigenicity and exhibited immunoprotective effects against parental tumor cells in an IFN-γ-dependent manner. IL-18 is also involved in the pathogenesis of chronic inflammatory and autoimmune diseases, such as type 1 diabetes mellitus, experimental autoimmune encephalo-

myelitis, and chronic active hepatitis (Okamura et al. 2000).

Interleukin 23

The newly discovered cytokine IL-23 shares some in vivo functions with IL-12. This factor, which was termed p19, showed no biological activity itself, but combined with the p40 subunit of IL-12 to form a novel, biologically active compound. IL-23 binds to IL-12Rβ1, but fails to engage IL-12Rβ2 (Oppmann et al. 2000).

IL-23 is secreted by activated dendritic cells, induces the activation of the transcription factor Stat4 in stimulated T cells, a strong proliferation of memory T cells, and IFN-γ production by the same cells (Belladonna et al. 2002).

Interleukin 25

IL-25 is a recently discovered cytokine showing close homology with the other members of IL-17 family. IL-25 gene expression was detected following *Aspergillus* and *Nippostrongylus* infection in the lung and gut.

IL-25 induces responses similar to those mediated by Th2 cells, including IL-4, IL-5, IL-13, and eotaxin production, which is followed by eosinophil infiltrate and airway hyperreactivity (Hurst et al. 2002).

ANTI-INFLAMMATORY AND REGULATORY CYTOKINES

Interleukin receptor antagonist

IL-1R antagonist (IL-1Ra) is a member of the IL-1 family. Three forms of IL-1Ra have been described, two of them being expressed as intracellular proteins (icIL-1RaI and icIL-1RaII) and one being secreted (sIL-1Ra). The three forms of IL-1Ra result from the same gene. The function(s) of the intracellular forms of IL-1Ra are still elusive, whereas sIL-1Ra binds competitively to IL-1RI without inducing signal transduction and thus inhibits IL-1α and IL-1β actions. Mature sIL-1Ra (sIL-1Ra) is a protein of 152 amino acids which is generated by the cleavage of a hydrophobic signal sequence of 25 amino acids from a cytoplasmic precursor of 177 amino acids. The IL-1Ra gene is referred to as IL-1RN and has been mapped in the long arm of chromosome 2 at band q11 (Burger and Dayer 2000).

It is very likely that all cell types able to produce IL-1α and/or IL-1β also express sIL-1Ra or icIL-1Ra or both forms. However, most of the studies have reported the expression of sIL-1Ra in monocyte/macrophages, neutrophils, and fibroblasts and of icIL-1Ra in epithelial cells. In the brain, IL-1Ra is constitutively expressed by cell types other than those expressing IL-1, such as neurons of the paraventricular nucleus and supraoptic nucleus. The sIL-1Ra competitively binds the IL-1RI without inducing signal transduction. It is therefore likely that sIL-1Ra regulates (inhibits) cellular functions affected by IL-1α and/or IL-1β (Figure 12). The function(s) of intracellular forms of IL-1Ra are less obvious. One possibility may be that icIL-1Ra counteracts intracellular IL-1α activities and destabilizes and/or degrades mRNAs induced by IL-1α. More recently, it has been shown that resting and cytokine-stimulated human pulmonary epithelial cells release icIL-1RaI in the extracellular space, where it can antagonize cell surface IL-1R. Therefore, icIL-1Ra might be an epithelial store of IL-1Ra liable to immediate release. The role of IL-1Ra in normal physiology of healthy humans remains unclear. The serum levels of sIL-1Ra are elevated in many pathologies (Arend et al. 1998). Therefore, although IL-1Ra was inefficient in sepsis treatment, clinical trials are currently undertaken in inflammatory diseases.

Interleukin 10

IL-10 belongs to the family of long-chain cytokines. The mature IL-10 protein consists of 160 amino acids, Ser1 being the N-terminal residue. The predicted molecular weight of hIL-10 is 18 647 Da and it runs as a single species with an apparent molecular weight of 17 kDa in SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Biologically active IL-10 is a homodimer. IL-10 is located on chromosome 1q. The human IL-10 gene spans 4.7 kb and consists of five exons separated by four introns.

IL-10 is expressed by naïve and memory T cells derived from either peripheral blood or cord blood, from T-cell clones belonging to Th1, Th2, and from T-regulatory 1 (Tr1) subsets, NK cells, and B cells derived from peripheral blood, tonsils, or spleen. A viral homolog of IL-10 has been found in the EBV genome. The EBV-encoded IL-10 (viral IL-10) is located on the BCRF sequence (the gene sequence of the Epstein–Barr virus) and the protein has maintained some, but not all, the biological activities of its cellular counterpart. IL-10 has 90 percent homology at the amino acid level with viral IL-10 (de Waal Malefyt 2000).

IL-10 regulates many aspects of inflammatory and immune responses and acts on both hemopoietic and nonhemopoietic cells. It has dominant suppressive effects on the production of proinflammatory cytokines by monocytes and neutrophils, and downregulates the expression of activating and costimulatory molecules on monocytes and dendritic cells (Fiorentino et al. 1991). This is the reason why it has also been defined as ‘macrophage deactivating factor’. IL-10 strongly inhibits the production of cytokines and chemokines by activated monocytes/macrophages. In addition, IL-10 enhances the production of IL-1Ra expression, as well

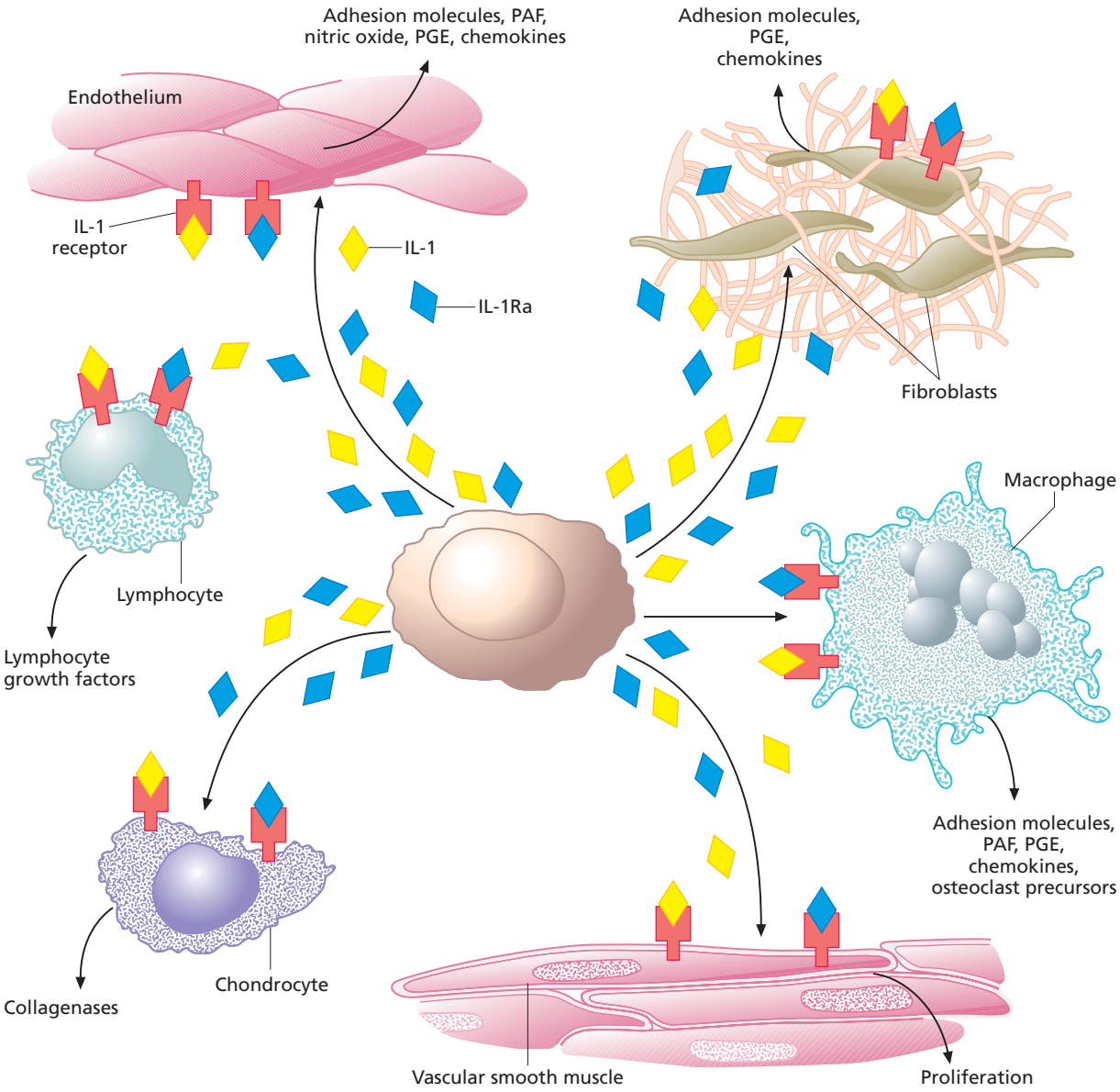

Figure 12 *The actions of interleukin 1 (IL-1) and its receptor antagonist (IL-1Ra). IL-1 activates its receptor on cells resulting in various functions. When IL-1R is occupied by the IL-1Ra, IL-1 cannot bind and thus cannot elicit a biological response. This is a natural balance between the proinflammatory activities of IL-1 and the ability of IL-1Ra to keep those activities in check by occupying IL-1Rs. PAF, platelet-activating factor; PGE, prostaglandin E. (Redrawn from Dinarello 2000c, with permission)*

as of soluble p55 and p75 TNFRs, indicating that IL-10 induces a shift from production of proinflammatory to anti-inflammatory mediators. IL-10 also inhibits expression of MHCII antigens, CD54 (ICAM-1), CD80 (B7), and CD86 (B7.2) on monocytes, even following induction of these molecules by IL-4 or IFN. IL-10 inhibits production of PGE_2, another proinflammatory mediator, as well as the ability of monocytes/macrophages to modulate turnover of extracellular matrix, an effect that is mediated by the inhibition of the production of gelatinase and collagenase, and the increase in the production of tissue inhibitor of metalloproteases. IL-10 also inhibits LPS-induced CRP biosynthesis.

Furthermore, IL-10 inhibits production of IL-12 and expression of costimulatory molecules on various types of dendritic cells. IL-10 also inhibits the production of chemokines, proinflammatory cytokines, and mediators of granulocyte survival, thus limiting the duration of inflammatory responses. IL-10 exhibits growth factor activities on B cells and mast cells, and inhibits or enhances the activities of $CD4^+$ and $CD8^+$ T cells depending on their activation conditions. IL-10 enhances the survival of normal human B cells (depending on their activation state), which correlated with increased expression of the antiapoptotic protein Bcl-2 and strongly increases the proliferation of human B-cell

precursors and mature activated B cells. Such effects of IL-10 may play a role in pathogenesis of systemic lupus erythematosus (SLE), where positive correlations between serum IL-10 levels and severity of disease, and between the production of IL-10 and autoantibodies by the SLE patient's B cells, have been demonstrated. IL-10 strongly inhibits cytokine production and proliferation of T cells and T-cell clones activated in the presence of APCs. This effect of IL-10 results mostly from its downregulatory effects on APC functions. The effects of IL-10 on individual cell types suggest that it could have potent anti-inflammatory/immunosuppressive activities in vivo in chronic inflammatory disorders, autoimmune disorders, and infectious diseases (de Waal Malefyt 2000).

Interleukin 10-related cytokines

Recently, five novel IL-10-related molecules and their receptors have been identified in blood mononuclear cells. These cytokines have been named IL-19, IL-20, IL-22, IL-24, and IL-26 (Gallagher et al. 2000; Dumoutier et al. 2000, 2001; Blumberg et al. 2001; Caudell et al. 2002; Wolk et al. 2002). Similar to IL-10, they are secreted α-helical proteins, the amino acid sequences of which are up to approximately 30 percent identical to those of IL-10 and comprise a definite position for cysteine. Of note, the encoding genes are located in the humane genome in two clusters, one comprising the genes for IL-10, IL-19, IL-20, and IL-24 on chromosome 1q32, and another comprising the IL-22- and IL-26-encoding genes located on chromosome 12q1 (Dumoutier et al. 2001)

IL-19 and IL-20 were found to be preferentially expressed in monocytes, whereas IL-22 and IL-26 (AK155) expression was exclusively detected in T cells, especially on Th1 polarization, and in NK cells (Wolk et al. 2002). IL-24 (melanoma differentiation-associated gene 7) expression was restricted to monocytes and T cells. Detection of these molecules in lymphocytes was predominantly linked to cellular activation. With regard to T cells, IL-26 was primarily produced by memory cells, and its expression was independent of costimulation.

In contrast to the extensively studied IL-10, the knowledge of the biology of the novel IL-10 homologs is still fragmentary. Preliminary functional data exist for IL-20, IL-22, and IL-24. Overexpression of IL-20 induced neonatal lethality, psoriasis-like skin abnormalities, lack of adipose tissue, and elevated apoptosis of thymic lymphocytes. IL-22 was suggested as playing a role in inflammatory processes through the observation that it induces acute phase reactant production in a hepatoma cell line and in vivo. Overexpression of IL-24 via adenovirus gene transfer induced growth inhibition in various tumor types. No function is known for IL-19 and IL-26 as yet.

However, although immune cells exhibit a differential expression of the IL-10 homologs, in contrast to IL-10 that is produced by and acts on all monocytes, NK cells, B cells, and T cells, these cells do not seem to be the major target population of these molecules.

Interleukin 13

IL-13 is secreted as a monomeric peptide of 10 kDa (McKenzie and Matthews 2000). Of note, the amino acid sequences of IL-13 and IL-4 are approximately 30 percent homologous, a level of similarity that is not evident among any other interleukins. The human IL-13 gene is located on the long arm of human chromosome 5 (5q31), where it is closely linked to the genes encoding IL-4, IL-5, IL-3, and GM-CSF. Indeed, the IL-13 gene maps approximately 12 kb upstream of the IL-4 gene (Figure 13) and there is a coordinate regulator of IL-4, IL-13, and IL-5 (Loots et al. 2000).

IL-13 is mainly produced by $CD4^+$ Th2 cells and clones. However, Th1 cell clones, Th0 cell clones, and $CD8^+$ T-cell clones, have also been shown to express IL-13 mRNA. Primary mouse mast cells and mouse and human mast cell lines have also been shown to express IL-13 in response to activation, and primary mouse NK cells and the human NK cell line NK3.3 secrete IL-13 in response to activation. IL-13 gene transcription has also been detected in a number of B-cell malignancies (McKenzie and Heath 1996).

IL-13 has been shown to have anti-inflammatory functions on monocytes and macrophages and to induce B cells to proliferate and isotype switch to the production of IgE. Blockage of IL-13 activity has been shown to inhibit the pathophysiology of asthma. Studies using IL-1 gene-deficient mice have identified impaired Th2 cell development in the absence of IL-13 and indicated an important role for IL-13 in the expulsion of parasitic gastrointestinal helminths. The anti-inflammatory function of IL-13 is manifested in its ability to suppress the expression of cytokines such as IL-1α and IL-1β, TNF-α, IL-8, and IL-12. Significantly, by inhibiting IL-12, it may also act to skew the T-helper populations away from a Th1 phenotype and towards a Th2 phenotype. IL-13 expression correlates strongly with the occurrence of allergic asthma and atopy, and the associated expression of IgE. Recent studies using mouse models of experimental nairway hypersensitivity have demonstrated that IL-13 plays a central role in these responses. On the other side, the anti-inflammatory roles of IL-13 may be important in controlling inflammation, such as that encountered in osteoarthritis. Interestingly, IL-13 has recently been demonstrated to be secreted by, and stimulatory for the growth of, Reed–Sternberg cells. Administration of recombinant IL-13 to IL-13-deficient mice or severe combined immunodeficient (SCID) mice resulted in improved expulsion of *Nippostrongylus brasiliensis* from helminth-infected animals, although the

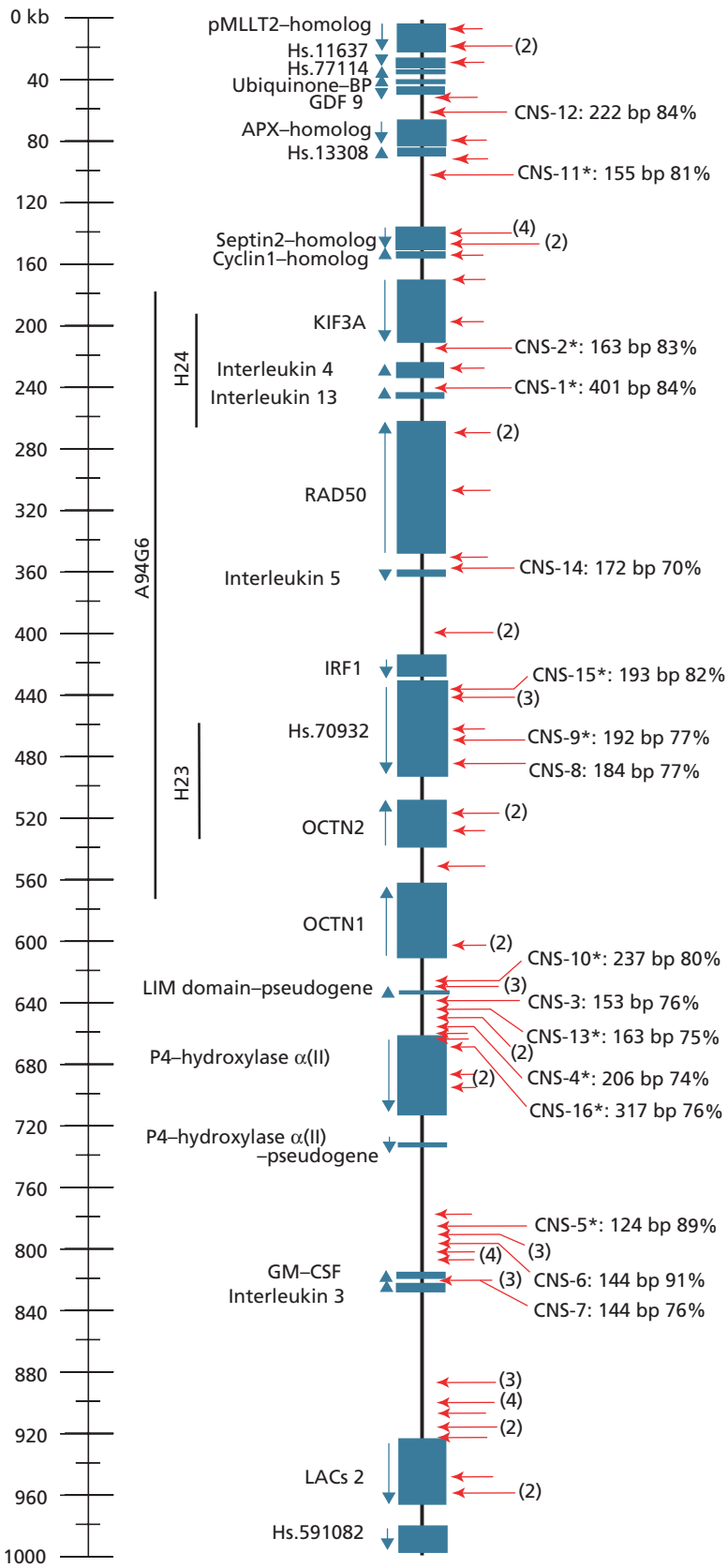

Figure 13 *The chromosomal association of interleukin IL-13 with other T-helper type 2 (Th2) cytokines, as shown by the physical map of the 1-Mbp human 5q31 region. (From Loots et al. 2000.)*

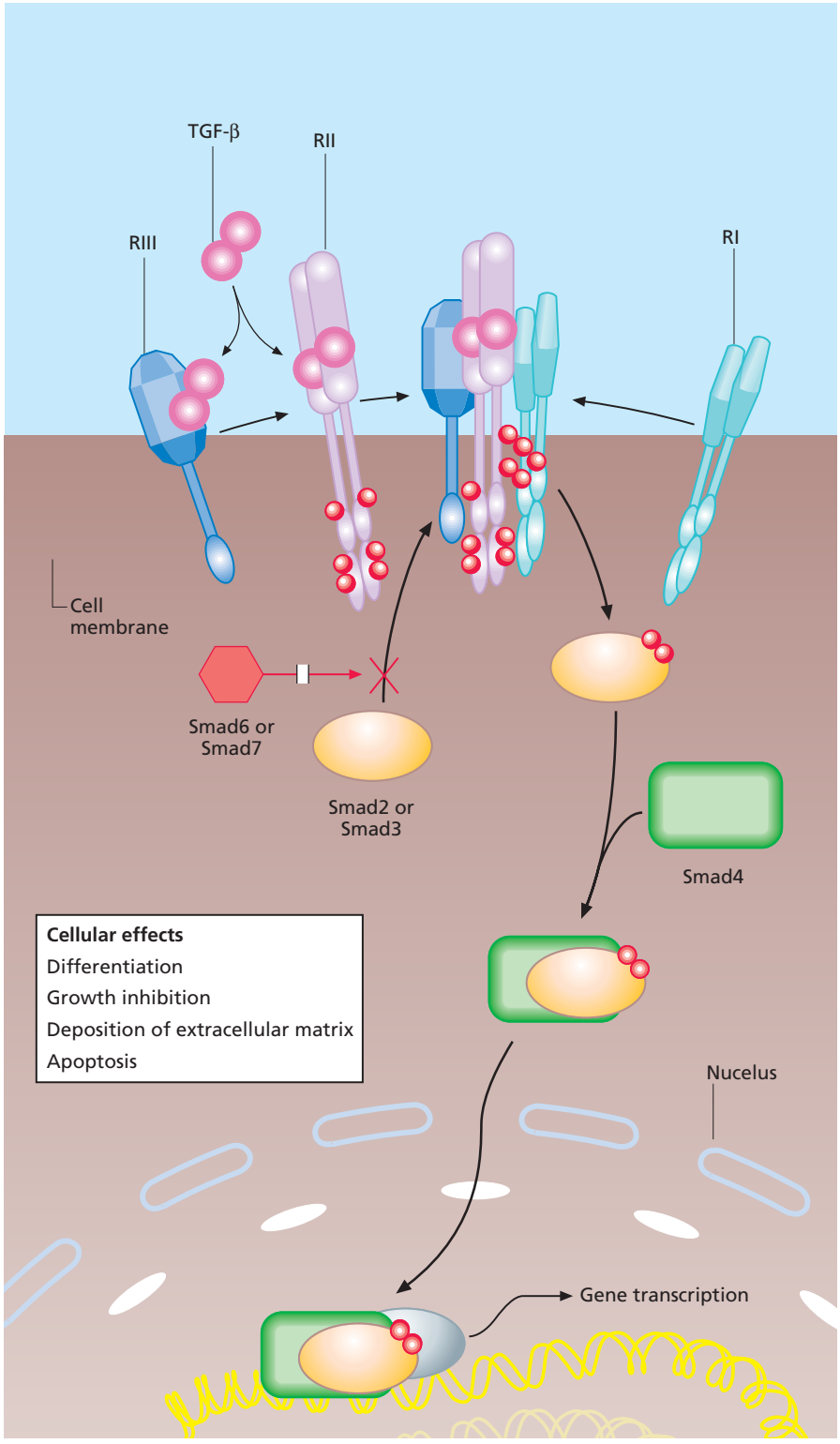

Figure 14 *Mechanisms of signal transduction and cellular effects of transforming growth factor β (TGF-β). In the extracellular space TGF-β binds either to the type III TGF-β receptor (RIIi), which presents it to the type II receptor (RII), or directly to RII on the cell membrane. The binding of TGF-β to RII then leads to binding of the type I receptor (RI) to the complex, and the phosphorylation of RI initiates the cascade of transcription factors that regulate the transcription of TGF-β-responsive genes and mediate the effects of TGF-β at the cellular level. (Redrawn from Blobe et al. 2000, with permission.)*

mechanism for this clearance is still unclear. As a result of its anti-inflammatory roles, IL-13 treatment has been used in models of collagen-induced arthritis and uveitis, and LPS-induced endotoxemia (McKenzie and Matthews 2000).

TGF-β

Three different isoforms of TGF-β (TGF-β_1, TGF-β_2, and TGF-β_3) exist as stable 25-kDa homo- or heterodimers crosslinked by a single disulfide bond that is formed by cysteine 77 of each monomeric unit (Flanders and Roberts 2000). TGF-β is secreted in noncovalent association with its own prodomain (latency-associated protein (LAP)) in a 'latent' form that is unable to bind receptors until activated. Whereas in other TGF-β family members the divergent N-terminal region of the unprocessed precursor serves only to facilitate proper folding and secretion of the mature peptides, for the TGF-βs, the LAP protein serves a unique role of conferring latency and effectively masking the receptor-binding domains of the molecule (Gleizes et al. 1997). The three TGF-β species are encoded by unique genes located on different chromosomes. All three genes share a similar intron/exon structure with a total of seven exons spanning greater than 100 kb of genomic DNA.

TGF-β is widely expressed in embryo tissues, whereas the major sources in adults are platelets, bone, and spleen (Thompson et al. 1989).

As almost every cell type can express receptors for TGF-β, its physiological and pathophysiological roles are extensive. The activity of the TGF-βs that distinguishes them from most other cytokines is their ability to limit cell growth. This growth inhibitory action is quite broad, targeting epithelial cells, endothelial cells, and hemopoietic cells. TGF-β also regulates the differentiated function of immune cells, acting as a strong suppressor of activation of T cells and of antibody secretion by B cells. Other major cellular targets for TGF-β action include its effects on chemotaxis (fibroblasts, lymphocytes, macrophages, and neutrophils) and its ability to control cellular differentiation, apoptosis, and extracellular matrix production. The last is the most prominent effect of TGF-β on mesenchymal cells, and is manifested by enhanced expression of extracellular matrix proteins, suppression of expression of matrix-degrading proteases, induction of expression of protease inhibitors, and regulation of expression of integrin receptors. Together, these effects result in increased accumulation of extracellular matrix and in cellular interaction with matrix (Figure 14). These many and varied cellular functions of TGF-β underlie its pathophysiological roles in fibrotic diseases, carcinogenesis, wound healing, autoimmune diseases, and parasitic diseases. TGF-β can be either synergistic and antagonistic to the action of other cytokines/growth factors, depending on the cellular context. Thus, TGF-βs play critical roles in normal physiology, most prominently in branching morphogenesis, in the transformation from epithelial to mesenchymal, control of cell growth, chemotaxis, and elaboration of extracellular matrix (Letterio and Roberts 1998).

The widespread expression of TGF-β coupled with its multiple bioactivities leads to its involvement in the pathology of many human diseases, such as fibrotic disorders, acute experimental allergic encephalomyelitis, and collagen-induced arthritis. TGF-β antagonists may be useful not only in these conditions, but mainly in reducing tumor growth, possibly by inhibiting the immunosuppressive effects of TGF-β. Clinical applications for TGF-β are limited by the difficulty to find optimal methods of delivery and ensure its bioavailability (Flanders and Roberts 2000)

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Antibodies and B lymphocytes

WILLIAM CUSHLEY AND GILLIAN BORLAND

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Antibody molecules are the functional products of the humoral arm of the mammalian immune system. They are glycoprotein molecules found mainly in the γ fraction of serum, and are members of the immunoglobulin (Ig) family of serum proteins. Although Ig molecules exist as five classes, each with distinctive structural and functional properties, all Ig molecules conform to a common unit structural theme, the four-chain model (Figure 1), which provides a molecular explanation for their immunological activities. Antibody molecules are synthesized by B lymphocytes.

The humoral response is one arm of the specific, adaptive, immune response, and is characterized by the diversity of individual specific antibodies that it can produce, immunological memory, and production of antibodies of the most appropriate type to combat particular pathogens. The humoral response interacts with the cellular immune response and also interfaces with components of the nonspecific immune system. Thus, the nonspecific immune system, which shows no specificity or immunological memory, possesses potent cytotoxic power in the form of phagocytic cells and the components of the complement cascade. The humoral response has essentially no toxic capacity in its own right, but uses antibody specifically to recruit the cytotoxic power of the nonspecific system and focus it at the pathogen target.

The humoral immune response displays characteristic features. After initial challenge with antigen, levels of antibody of the IgM class rise over some 4–7 days; this is the primary humoral response. If antigenic challenge persists, antibodies of other classes, notably IgG, are produced; this is the secondary response. Second or subsequent challenge with the same antigen elicits a response that is more rapid than the primary response, and generates greater quantities of specific antibody of greater quality (i.e. different class and higher affinity) from those found in the primary response. The capacity to respond to re-challenge by a given antigen is long-lived and resides in memory B cells. The secondary response encompasses antibody responses, the major components of which are of the IgA, IgD, IgE, or IgG classes.

IMMUNOGLOBULIN STRUCTURE

The four-chain model

The four-chain model describes the overall structure of IgG molecules (Porter 1962), and this structural theme is common to all five Ig classes. The general features of the four-chain model are illustrated in Figure 1, using human IgG1 as an example. Antibody molecules are composed of two identical heavy chains, disulfide bonded to two identical light chains in such a way that the amino terminus of each heavy and light chain is juxtaposed in three-dimensional terms to generate the

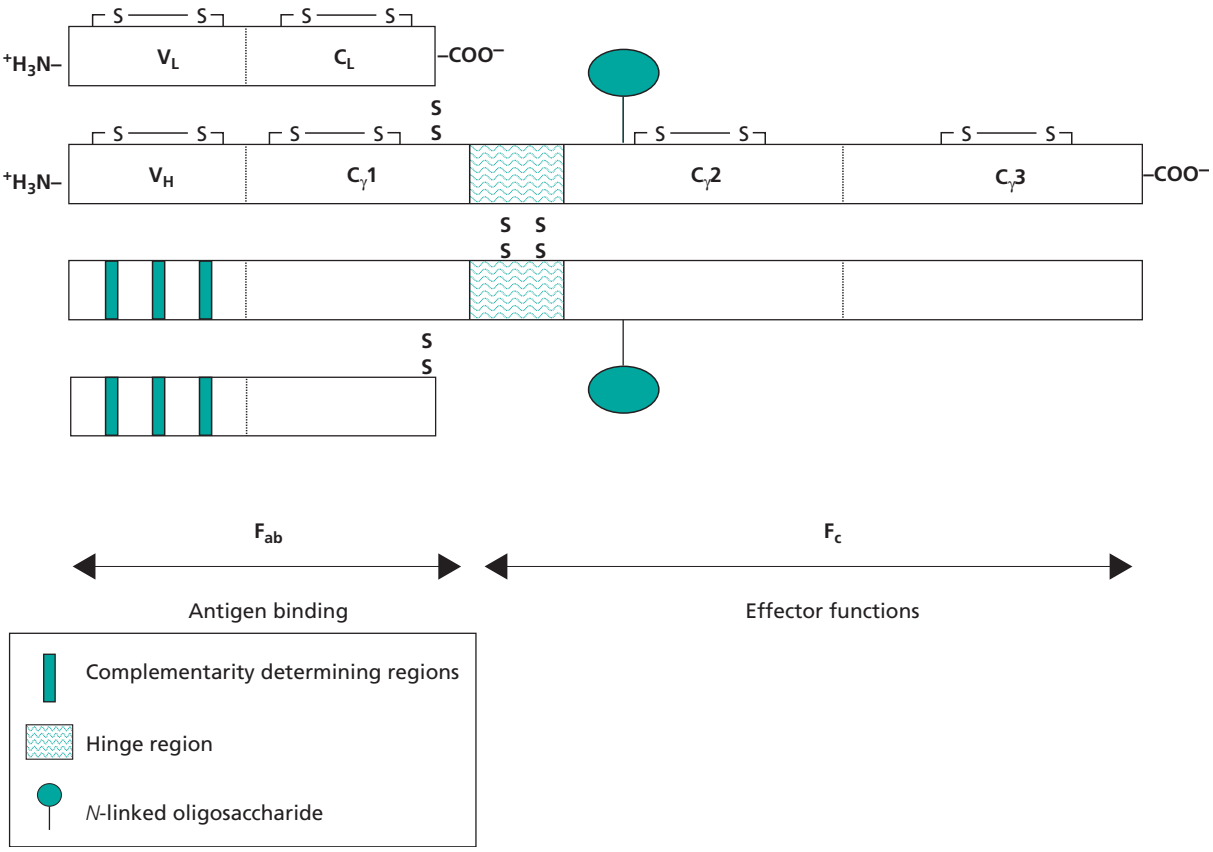

Figure 1 *The four-chain model: the arrangement of an IgG1 molecule is shown. The hypervariable regions/complementarity determining regions are shown as solid rectangles in the V domains. The hinge region is illustrated as a patterned box and N-linked oligosaccharide by a filled circle. The locations of the Fab and Fc regions are noted.*

antibody-combining site. Interchain disulfide bonds link not only the heavy and light chains but also the two heavy chains together. The IgG molecule is bivalent with respect to antigen binding (Edelman and Poulik 1961; Porter 1962).

Controlled proteolysis of IgG molecules has provided insights into structure–function relationships by investigation of the distinct fragments of Ig molecules generated (see Figure 1). Papain cleaves the intact IgG molecule to release three fragments of approximately equal molecular weight. Two of these fragments, derived from the amino terminus of the molecule, are identical and, because they retain antigen-binding capacity, are referred to as Fab (fragment antigen binding) fragments. Fab fragments are monovalent with respect to antigen binding and, as such, are unable to form immunoprecipitates in solution or in semi-solid media. The remaining product of papain digestion, the Fc (fragment crystalline) piece, is derived from the C terminus of the intact IgG molecule, and has no antigen-binding capacity. It is, however, of considerable importance in expression of antibody effector functions.

Pepsin digestion of intact IgG yields quite different proteolysis products. A large fragment of about 100 kDa is the main product, the remaining material usually being small peptides. The l00-kDa fragment is derived from the amino terminus of the intact IgG, has antigen-binding capacity, and is bivalent (i.e. possesses two equivalent antigen-combining sites). This is the $F(ab)'_2$ fragment.

Primary structure

Primary protein structure is the linear arrangement of amino acids in the polypeptide chain. Initial sequencing studies of Ig molecules were performed using Bence–Jones proteins, which are monomeric or dimeric light chains derived from the urine of patients with multiple myeloma (Edelman and Gally 1962). Primary sequence analysis of Bence–Jones light chains revealed that they were divisible into two distinct regions of approximately equal size. Thus, from amino acid 1 (the amino-terminal residue) to amino acid 107, the sequence of one Bence–Jones protein was different from that of any other Bence–Jones protein with which it was compared, whereas the sequences from residues 108 to 214 were essentially identical for all Bence–Jones proteins. These observations led to the division of light chains into variable (V) and constant (C) regions on the basis of the variability of amino acid sequence within the protein (Milstein 1966).

A similar structural division can be made for heavy chains. As described for the light chains, the amino-terminal regions of the heavy chains possess considerable variability from one chain to another, but the carboxy-terminal regions are relatively constant. In the case of the heavy chains, however, the variable region extends from residue 1 to residue 113, and the constant region from residue 114 to the carboxyl terminus, which may be some 400 amino acid residues distant in the primary sequence. Furthermore, when the sequences of heavy chain C regions are analyzed, it is clear that there are areas of significant homology of primary sequence along the length of the C region. The sequence can be subdivided into units of homology of approximately 100–110 amino acids. The functional importance of these domains is discussed below in the context of tertiary structure.

The V regions of both heavy (V_H) and light (V_L) chains have numerous amino acid substitutions when individual polypeptide chains are compared. However, plots of variability of amino acid versus position in the primary sequence reveal that the distribution of amino acid substitutions is not random, and that the variability is clustered in small regions of the primary structure. These areas of the V region are the hypervariable regions and they provide a molecular explanation for antibody specificity. Thus, the V_L and V_H regions of an individual antibody molecule are unique and the recognition of antigen by that antibody is similarly distinctive. As the hypervariable regions govern the specificity of the antibody molecule, they are sometimes referred to as complementarity determining regions (CDR).

Both heavy and light chains possess three CDRs. The heavy chain CDRs are located at positions 26–36, 55–65, and 95–105 in the primary sequence, and are referred to as CDR H1–3, respectively. The light chain CDRs (L1–3) are positioned at residues 25–35, 54–67, and 94–108. The above numbering is based on the EU myeloma protein (Edelman et al. 1969). Outwith the hypervariable regions, or CDRs, the primary sequence of the V region is largely conserved between molecules. These conserved regions are crucial in the maintenance of the higher structure of the V_H and V_L regions and are therefore termed 'framework regions'.

Secondary and tertiary structure

Secondary structure is the relationship between amino acids located some distance apart in the primary structure, the interactions of which give rise to periodic structural motifs in the folded protein molecule (e.g. α helix or β-pleated sheet structures). Ig molecules contain no α-helical structures but are rich in β-pleated sheets. Tertiary structure also describes the spatial relationship of amino acids which are distant in the primary sequence and, for the purposes of this discussion, refers to those cysteine residues in the primary sequence that form intrachain disulfide bridges, giving rise to individual domains within the Ig molecule.

The 'Ig-like fold' or domain is a characteristic higher structural motif of Ig structure. The primary structural data indicate that the sequences of Ig chains are divisible into homology units of approximately 110 amino acids in length. Within each of these units lies a single disulfide bridge, enclosing 65–75 residues in the V regions and 52–59 residues in the C-region domains. Clearly, the location of the cysteine residues is critical in domain formation, and this is reflected not only in the conservation of this amino acid at defined positions in the polypeptide chain, but also by the conservation of primary sequences surrounding the cysteine groups participating in intrachain disulfide bond formation.

The domain structure produced by disulfide bond formation is rich in β-sheet structure, with each domain in both the V and the C regions of the Ig molecule containing two β sheets. In each β sheet, the polypeptide structure may be considered as layers of overlapping sequence lying parallel to the long axis of the domain, one layer containing three strands of polypeptide and the other four strands. The polarity of adjacent, individual strands is anti-parallel, and the two layers of β strands are joined by the single disulfide bond. Multiple, hydrophobic, amino acid, side-chain groups protrude into the space between the layers.

Quaternary structure

Quaternary structure reflects the interaction between distinct polypeptide subunits of a multidomain protein. In Ig molecules the interaction between particular domains is well defined and correlates well with biological activity. Thus, V_H and V_L domains interact to yield the correct conformation for generation of the antigen-combining site. C_L and C_H domains also interact. In the C region, like domains interact. Thus, Cγ2 interacts with Cγ2 and Cγ3 with Cγ3. However, the interaction does not always involve direct contact between the faces of the two participating domains. In IgG molecules the Cγ2 domains are separated from each other by the large oligosaccharide unit located in this domain, whereas Cγ3 domains interact via contacts between faces of the two domains.

The forces that stabilize domain–domain interactions are noncovalent in character and involve weak intermolecular physical forces (i.e. hydrophobic interactions, salt bridges, hydrogen bonding). Although the physical forces that mediate domain interaction are identical for V and C domains, the faces of the protein that interact are distinct. Thus, V domains interact via those layers of the domain containing three strands of peptide sequence, whereas the C domains interact via the layers containing four strands of polypeptide.

The detailed higher structure of Ig molecules is discussed in under 'Structural biology of Ig and Ig receptors' below.

Other structural features

The hinge region of IgG molecules is of critical importance to their function; located between the Cγl and Cγ2 domains, it is rich in proline and cysteine residues. The size of the hinge region varies from one IgG subclass to another, being 10–20 residues in length in all subclasses except IgG3 where it is 60 residues long (Figure 2). This area of the Ig molecule has considerable segmental flexibility, which is important in the expression of the effector functions of IgG molecules, e.g. the greater the segmental flexibility of a given immunoglobulin isotype, the more likely it is that complement can be effectively fixed (Oi et al. 1984). The positioning of the hinge region is such that it allows the Fab arms of the IgG to adopt a wide range of orientations with respect to each other; this feature also explains the characteristic 'Y' shape of IgG molecules visualized in antigen-binding experiments in the electron microscope (Valentine and Green 1967). The hinge region is the location of the inter-heavy chain disulfide bonds in the IgG molecule, the exact number of which varies between IgG subclasses. Thus, IgG1 and IgG4 have two interchain disulfide bridges, IgG2 has four, and IgG3 15. The region is also exposed to the solvent, with the consequence that proteolytic enzymes may attack it and generate fragments as described earlier in this chapter.

Ig molecules contain varying amounts of carbohydrate. In most instances the glycans are attached to asparagine residues (so-called N-linked oligosaccharides), but carbohydrate is also found linked to the hydroxyl groups of serine or threonine residues (O-linked sugars) in human IgD molecules and in the hinge region of human IgA molecules. In IgG molecules the principal site of glycosylation is asn297, and the N-linked oligosaccharide is usually a complex structure with two branches of linear sugars originating at a single point, although the precise oligosaccharide structure can vary enormously between individual IgG molecules. The presence of certain types of carbohydrate structure has been correlated with particular autoimmune disease states (Parekh et al. 1985), but the role of the N-linked glycans themselves in disease pathogenesis remains unclear. The other Ig isotypes are multiply glycosylated (Winkelhake 1978).

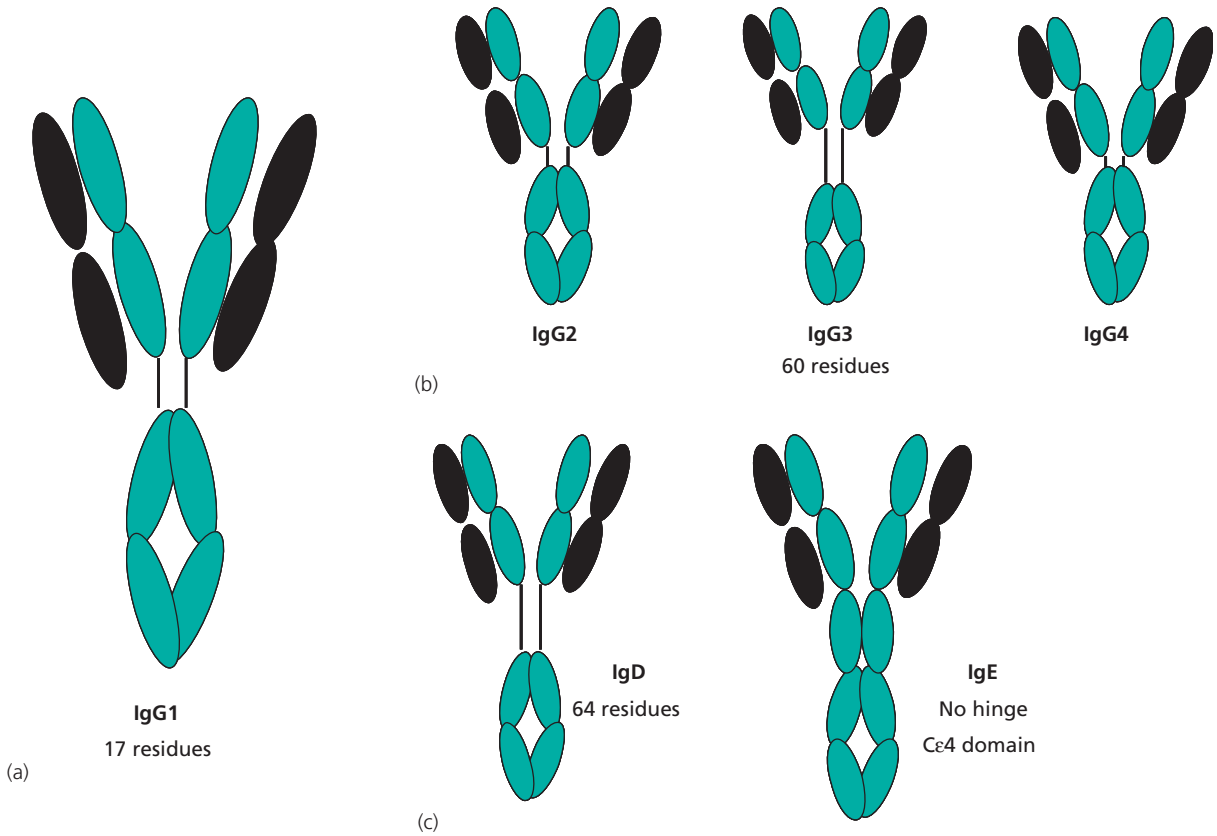

Figure 2 *Monomeric immunoglobulins: the layout of the domains in the heavy and light chains of IgG, IgD, and IgE molecules is illustrated. The light chain domains are illustrated as filled, black ovals in all cases. IgG1 is illustrated as the largest element in* **(a)**, *and each of the other IgG subclasses is illustrated with the hinge regions illustrated as solid lines of different sizes* **(b)**. *IgD and IgE molecules are shown in their 'extended' forms* **(c)**.

IMMUNOGLOBULIN CLASSES

Ig molecules exist as five classes, or isotypes, in humans, each of which has characteristic structural features and particular immunological activities. The serological marker that defines Ig class or subclass is the isotype, and the terms 'isotype' and 'class' (and 'subclass') are used interchangeably. All isotypes have a four-chain unit as their fundamental structural feature, and each class is named for the heavy chain of the molecule. Thus, IgG is named for the γ (gamma) heavy chain, IgM for the μ (mu) chain, and IgA for the α (alpha) chain. The principal molecular properties of the five human Ig classes are detailed in Table 1, and are illustrated diagrammatically in Figures 2 and 3.

IgM

IgM is a pentameric structure comprising five identical four-chain units, i.e. it has 10 identical binding sites. The μ heavy chain has five domains, V_H plus four C regions (Cμl, Cμ2, Cμ3, and Cμ4), and lacks a hinge region. The pentameric structure is stabilized by disulfide bonding between adjacent Cμ3 domains, and by the presence of the J (joining) chain. A single J chain is disulfide bonded close to the C terminus of the IgM pentamer (Chapuis and Koshland 1974). The critical cysteine residue (Cys575) is part of an 18 amino acid carboxy-terminal peptide extension located immediately following the Cμ4 domain, and this residue forms disulfide bonds either with J chain or with cysteines located in an identical position in other μ chains of the pentameric complex (see Figure).

IgM is the principal component of the primary humoral response. As a result of its large size (970 kDa, 19 S), it is located mainly in the bloodstream. It is decavalent, leading to highly avid binding of antigens (thereby overcoming the potentially low affinity of the antibody–antigen interaction), and is efficient in both opsonization and complement fixation.

IgG

IgG is the main class of Ig in serum. As will be obvious from the detailed discussion of the four-chain model, it exists as a molecule of about 146–160 kDa (7 S) in serum, and is an abundant component of the secondary humoral immune response. This class of Ig is found not only in the bloodstream itself, but also in extravascular spaces. It is transported across the placental membrane and is therefore responsible for passive immunity in the fetus and neonate (see Figure 4).

There are four major subclasses of the IgG isotype in humans, each distinguished by minor variations in amino acid sequence in the C region and by the number and location of disulfide bridges (Figure 5 and see Figures 1 and 2). The structural variations have consequences for biological activity. Thus, the IgG1 and IgG3 isotypes are efficient in fixation of complement, whereas IgG2 and IgG4 subclasses are less effective in activating complement. There is a similar hierarchy among IgG subclasses in binding to different types of the Fc receptor (see 'Receptors for immunoglobulin' later).

IgA

IgA is found in two forms in the body: in serum where it occurs as a monomer (160 kDa, 7 S), and on secretory surfaces where it exists as a dimeric molecule (385 kDa, 11 S) (see Figure 3). The dimeric form is known as secretory IgA (sIgA) and is found in association with J chain and a secretory component, the latter being involved in transport of the IgA to the secretory surfaces. Secretory component is noncovalently associated with the IgA molecules in the sIgA complex. The α chain has three C domains and, as with μ chains, possesses an 18 amino acid carboxy-terminal sequence allowing disulfide bonding to the J chain. A small hinge region is also present. There are two subclasses of IgA, IgA1, and IgA2, distinguished by their distribution and

Table 1 *Molecular properties of immunoglobulins*

	IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	sIgA	IgD	IgE
Heavy chain	μ	γ_1	γ_2	γ_3	γ_4	α_1	α_2	α_1/α_2	δ	ϵ
Mol. wt (kDa)	65	51	51	60	51	56	52	52 or 56	70	72.5
Assembled form	$(\mu_2L_2)_5$ J[a]	γ_2L_2[b]	γ_2L_2	γ_2L_2	γ_2L_2	α_2L_2	α_2L_2	$(\alpha_2L_2)2$ J SC[c]	δ_2L_2	ϵ_2L_2
Mol. wt. (kDa)	970	146	146	160	146	160	160	385	188	184
Sedimentation co-efficient	19S	7S	7S	7S	7S	7S	7S	11S	7S	8S
Valency for Ag	10	2	2	2	2	2	2	4	2	2
Serum concentration (mg/ml)	1.5	9	3	1	0.5	3	0.5	0.05	0.03	0.00005

a) Mol. wt. of J chain is 15kDa
b) Written as γ_2L_2 for convenience; formal structural formula is $(\gamma_1)_2L_2$
c) Mol. wt of secretory component (SC) is 70 kDa.

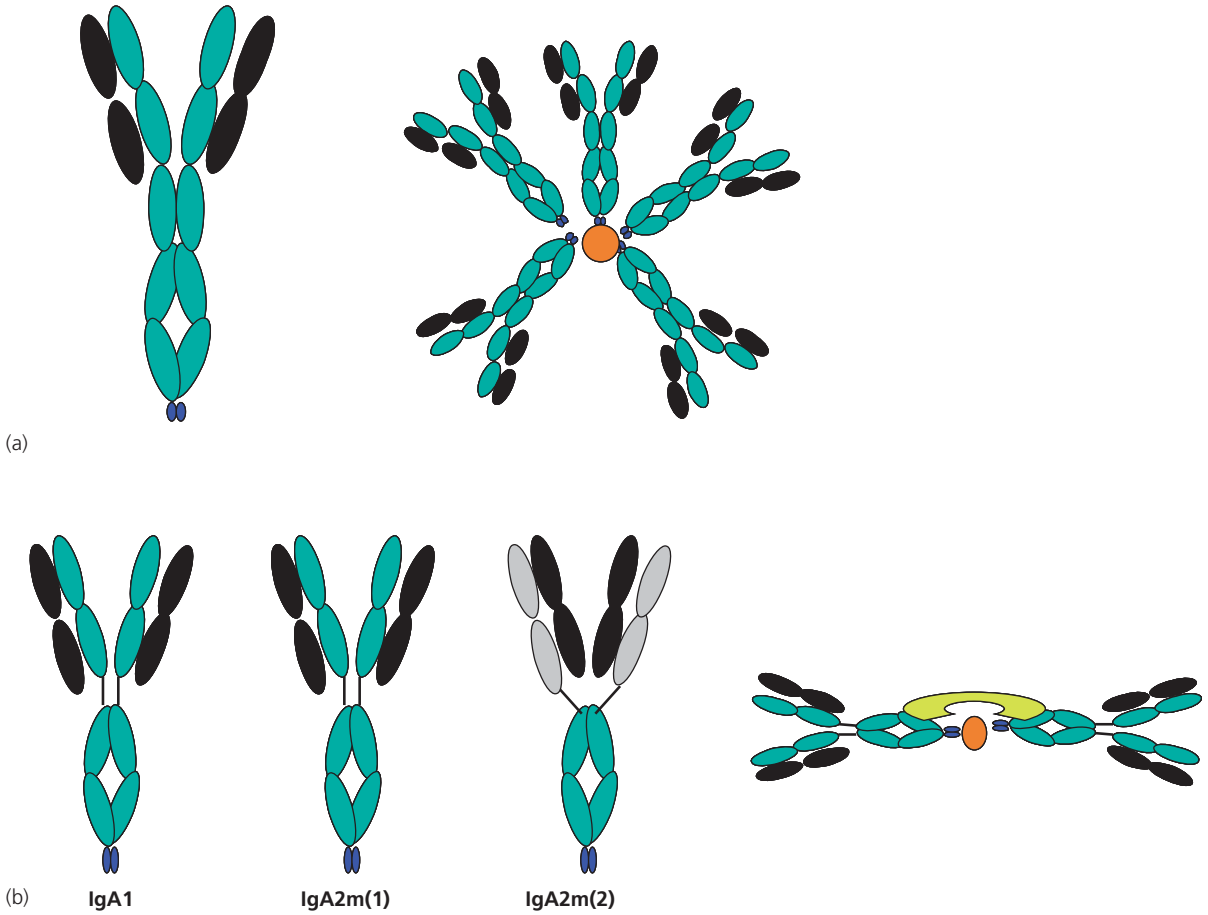

Figure 3 *Polymeric immunoglobulins: the monomeric forms of IgM* **(a)** *and IgA* **(b)**, *based on the four-chain model, are shown. Note the different location of the light chains in the IgA2m(2) molecule (an allele of IgA2). Polymeric forms of both Igs are illustrated, with a pentameric structure for IgM and a dimer for IgA. On polymeric IgM and IgA, J chain is shown as a red circle and on IgA, secretory piece is illustrated as a yellow 'C' shape bridging the two IgA monomers. The μ and α heavy chains each possess a small region of 18–20 residues that facilitates binding of J chain; this is illustrated as a small blue oval on the figures.*

arrangement of disulfide bonds. IgA1 is the predominant form of IgA found in serum (about 85 percent of total), whereas IgA1 and IgA2 isotypes are present in roughly equal proportions in sIgA; both the α1 and α2 heavy chains can interact with J chain and secretory component.

IgA is a component of the secondary humoral response. The principal antigens that elicit an IgA response are microorganisms in the gut (e.g. antigens introduced by foodstuffs) or the airways. IgA cannot cross the placenta, and so has no role in passive immunity in the fetus. However, sIgA can be passed to the neonate during lactation and is therefore of significant protective value because the IgA transferred by the mother will reflect immune responses to pathogens present in the environment throughout the period of breast-feeding.

IgE

IgE (about 184 kDa, 8 S), historically referred to as reaginic antibody, is present in very small amounts in normal individuals, but levels are increased in patients with allergic conditions (e.g. hay fever) or those with parasitic infections. The ϵ (epsilon) chain has four C domains (see Figures 2 and 5), and its Fc structure is specialized for interaction with high-affinity receptors for the Fc piece of IgE present on mast cells and basophils. Crosslinking of IgE bound to such Fc receptors leads to degranulation of the cell, with subsequent release of histamine and other pharmacological mediators (Ishizaka et al. 1971; Sutton and Gould 1993; Metzger 1994, 1999), triggering a series of physiological reactions that can result in anaphylaxis. Thus, IgE is a mediator of type I hypersensitivity reactions. IgE may have originally evolved to combat parasitic infestations (e.g. by helminths), and the development of anaphylactic responses was an unfortunate byproduct. IgE does not fix complement via the classic pathway but may activate the alternate pathway. It is found in roughly equal proportions in the bloodstream and extravascular space.

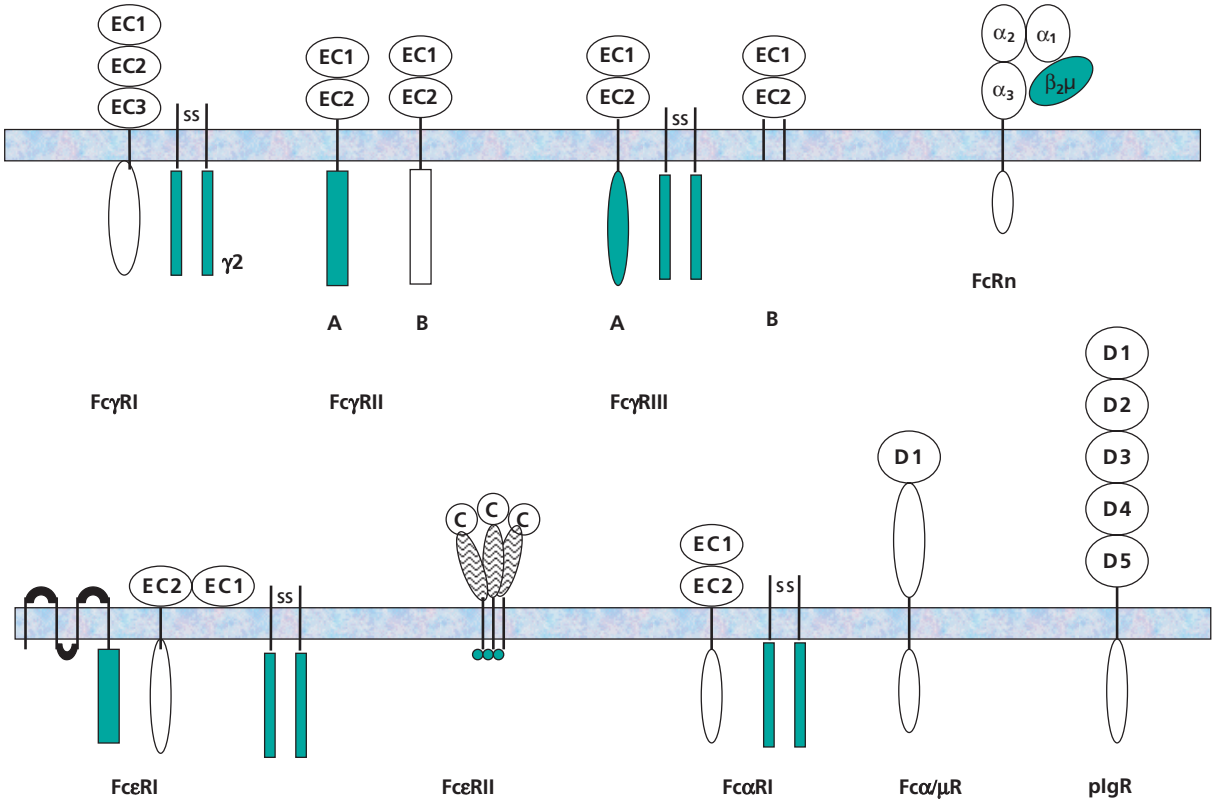

Figure 16.4 *Immunoglobulin receptors: the main classes of Fc receptor for IgG, IgA, IgM and IgE are illustrated. The upper panel shows receptors for IgG, and the lower panel receptors for other Ig isotypes. Each Ig-like extracellular domain (EC, or D in the main text) is shown as a circle, and the domains are numbered from the amino terminus of the receptor molecule. Note that FcγRIIIB is linked to the membrane via a glycophosphatidylinositol (GPI) anchor. ITAM (immunoreceptor tyrosine-based activation motif) and ITIM (immunoreceptor tyrosine-based inhibitory motif) intracellular domains are represented as filled and open rectangles, respectively. Intracellular domains with no or undefined signaling functions are shown as open ovals. FcεRIγ is depicted in a 'lying-down' orientation. For FcεRII, the C-type lectin domain is depicted as 'C' and the oval domain represents the stalk region.*

IgD

IgD is present in very low concentrations in the serum, and its exact functional role is unknown. The human δ heavy chain has three C domains (see Figure 16.2). IgD is most frequently found on the cell membrane of B lymphocytes, where its main function appears to be that of a cell membrane receptor for antigen. Human IgD does have a long hinge region of some 64 residues, and is rich in O-linked oligosaccharide groups; the extent of glycosylation explains its high apparent molecular mass (about 175 kDa). Unusually, the hinge region is encoded by two separate exons in the Cδ gene (White et al. 1985). The mouse, by contrast, has only two constant region domains, Cδ1 and Cδ3 (Preud'-homme et al. 2000). The functions of IgD are not greatly understood, although there are good numbers of IgD^+ plasma cells in nasal and tonsillar tissue. In disease terms, high levels of IgD are found in an autosomal recessive condition, hyper-IgD syndrome, which is linked to a mutation in mevalonate kinase (Drenth et al. 1999), but the links between Ig and isoprenoid synthesis are not clear.

Other immunoglobulin components

Light chains are found in two forms, kappa (κ) and lambda (λ), and any single antibody molecule contains only one type of light chain. The proportion of κ:λ in the human is 2:1, but this ratio varies considerably between species. The κ and λ proteins are products of independent genes located on different chromosomes.

J chain is a glycoprotein of about 15 kDa associated with polymeric Igs (i.e. IgM or IgA, see Figure 16.3), which serves to stabilize the structure of the polymerized immunoglobulins (Chapuis and Koshland 1974). Secretory component is a glycoprotein of about 70 kDa found almost exclusively in sIgA (see Figure 16.3), although association of secretory component with IgM, to yield secretory IgM, has been described in patients with IgA deficiency (Thompson 1970). The association of secretory component with IgM molecules is a reflection of the initial identity of secretory component as a polymeric immunoglobulin receptor (pIgR) on the basolateral surface of epithelial cells. Thus, secretory component, an important component of the sIgA molecule, is not a biosynthetic product of B cells. The immunobiology of

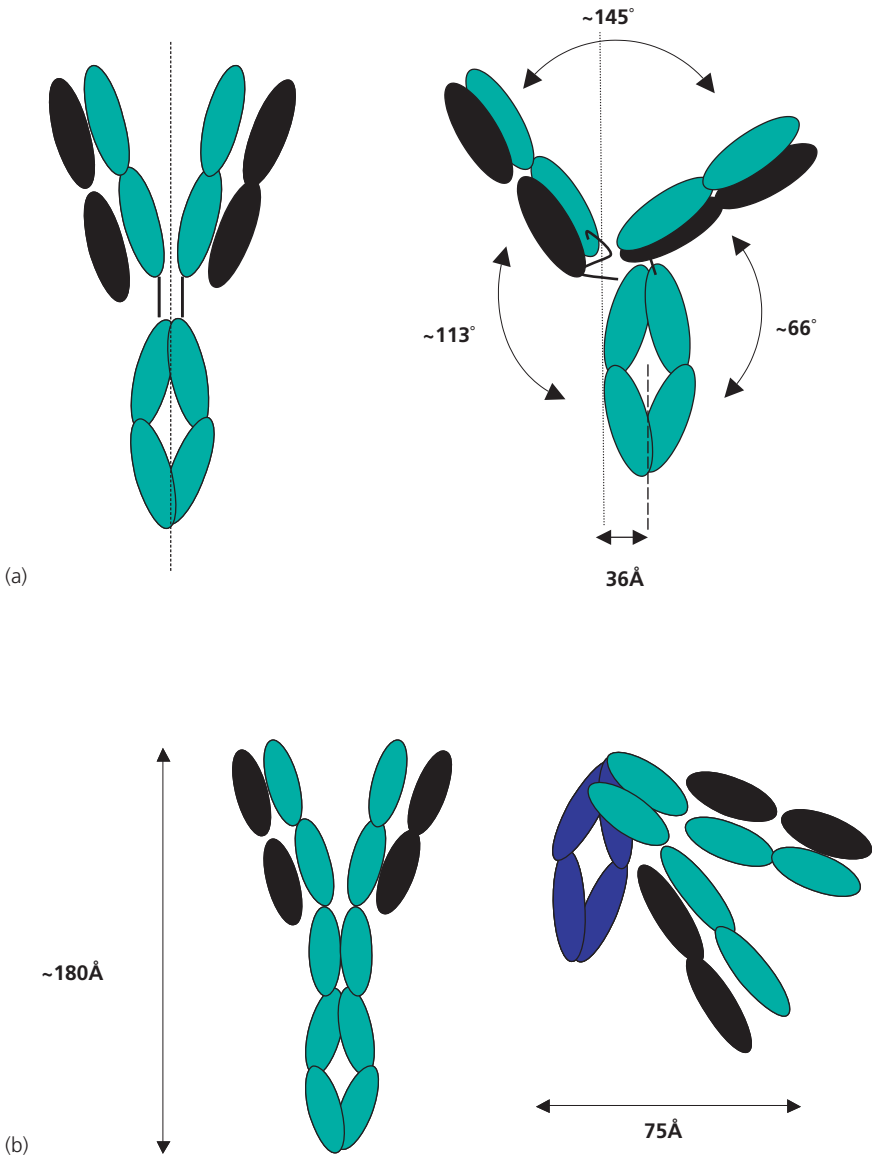

Figure 5 *Structures of IgG and IgE re-visited: the disposition of domains on IgG* **(a)** *based on crystallographic data from the b12 monoclonal antibody (MAb) are sketched and approximate sizes and relative angles of domains illustrated. The broken lines in the IgG cartoons represent the main axis of symmetry through a 'symmetrical' IgG molecule, to illustrate the 3.2 nm offset of the Fc domain in b12. Equivalent cartoons for IgE* **(b)** *are based on crystallographic analysis of the Cε2/Cε4 Fc region of human IgE. For IgE Cε2–Cε4, the Cε3 and Cε4 domains are shown in blue.*

pIgR is discussed under 'The polymeric immunoglobulin receptor' later).

Immunoglobulin metabolism

Immunoglobulins are being constantly synthesized, secreted into the circulation, and catabolized (Table 2). Once secreted into the extracellular environment, each Ig isotype has a characteristic metabolic half-life, with IgG molecules tending to be the longest-lived species, and IgE molecules being most rapidly cleared from the circulation (Table 2). The rate of synthesis of IgG molecules in humans is 33 mg/kg per day, i.e. more than 2 g/day for a 70-kg man. IgG molecules are generally relatively long-lived in the circulation, with a half-life of approximately 21 days; IgG3 has a half-life of 7 days. The fractional catabolic rate of IgG molecules, or the percentage turned over per day, is of the order of 17 percent (7 percent for IgG3). During an active immune response, the amount of specific antibody synthesized in terms of micrograms of protein is quite small, and represents only a minor fraction of the total IgG present in the serum. Therefore, initiation of a vigorous humoral immune response does not make a large impact on the total serum concentration of Ig. The catabolism of Ig is regulated by the Fc region and involves both protein and oligosaccharide components (Winkelhake 1978).

Table 2 *Metabolism of immunoglobulins*

	IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgD	IgE
Serum concentration (μg/ml)	1500	9000	3000	1000	500	3000	500	30	0.05
Distribution (%)[a]	80	45	45	45	45	42	42	75	50
Synthetic rate (mg/kg per day)	3.3	33	33	33	33	24	24	0.4	0.002
Fractional catabolic rate (%)[b]	8.8	7	7	17	7	25	25	37	71
Half-life (days)	10	21	20	7	20	6	6	3	2

a) Distribution is expressed as the percentage of intravascular immunoglobulin.
b) Fractional catabolic rate is expressed as the percentage of the intravascular pool catabolized per day.

Membrane immunoglobulin

B lymphocytes express a receptor form of antibody, membrane immunoglobulin (mIg), on their cell surfaces. As required by the clonal selection hypothesis, the receptor antibody is identical in essentially all respects to the antibody that will be secreted by the B cell on contact with antigen. The sole difference is the presence of a hydrophobic sequence at the carboxy-terminus of the heavy chain which allows stable insertion into the plasma membrane. The mIg forms part of the B-cell antigen receptor complex (BcR), and is found associated with other proteins at the cell membrane. It should be noted that the mIg molecule is always found as a single four-chain unit; thus, primary B cells synthesizing IgM express a single four-chain mIgM unit at their cell surface although, once activated by antigen, they secrete a polymeric IgM containing five four-chain IgM units. The mIg-associated proteins are transmembrane proteins of 34 kDa (Ig-α) and 37 kDa (Ig-β) which have functions in efficient transport of the mIg molecule to the cell surface and in linking the BcR to the intracellular signaling machinery of the B lymphocyte. The Ig-α and Ig-β proteins form a disulfide-bonded complex with each other, and associate noncovalently with the mIg molecule (Reth 1995). The mechanisms of intracellular signaling triggered by the BcR are extremely complex, and involve recruitment of soluble protein tyrosine kinases, activation of inositol lipid hydrolysis, and calcium mobilization. The biological response of the B cell to binding of antigen to the BcR depends on the state of differentiation of the B cell (see The B cell antigen receptor).

BIOLOGICAL EFFECTOR FUNCTIONS OF IMMUNOGLOBULINS

Range of effector functions

Antibody molecules by themselves are not directly cytotoxic toward invading pathogens. In most cases, the humoral immune system depends on the recruitment of other cytotoxic systems that can eliminate invading organisms, once specifically directed to the target by antibody. Consequently, antibody molecules participate in a wide variety of effector functions (Table 16.3). These activities are triggered after binding of specific antigen, and can involve a wide range of serum proteins, lymphocytes, and nonspecific inflammatory cells. Thus, the complement cascade is readily activated by immune complexes containing antibodies of particular subclasses. The Fc portion of immunoglobulins can be bound by specific receptors on the plasma membranes of a wide range of cells, and this interaction has consequences for phagocytosis of target organisms, or for direction of antibody-dependent cellular cytotoxicity (ADCC) for the destruction of cellular targets. Studies with fragments of Ig molecules and with monoclonal antibodies (MAb) to specific domains of the IgG molecule have demonstrated convincingly that the expression of

Table 3 *Effector functions of immunoglobulins*

	IgM	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	sIgA	IgD	IgE
Complement fixation	+++	++	+	+++	–	+[a]	–	–	–	–
Placental transfer	–	–	+	–	+	–	–	–	–	–
Binding to Mononuclear cells	–	+	–	+	–	–	–	–	–	–
Polymorphonuclear cells	–	+	–	+	–	–	–	–	–	–
Basophils and mast cells	–	–	–	–	–	–	–	–	–	+++
T and B lymphocytes	+	++	–	++	–	+	+	–	–	+

a) Activation of the alternate pathway.

effector functions is mediated by the Fc portion of the immunoglobulin molecule.

Neutralization

Neutralization is a direct consequence of the binding of antibody to an antigen and is an important means by which antibodies protect the host against disease. Many pathogens mediate their effects by elaborating toxic agents that bind to specific receptors on target tissues to induce a biological response or, in the case of viruses and intracellular bacteria, utilize cell-surface structures to gain entry to cells where replication can occur. In such circumstances, a protective antibody is one that specifically interferes with the interaction of the toxin or pathogen with a cell-surface receptor. In an immune response to a single agent, a large array of antibodies is produced which can bind at distinct sites (called epitopes or determinants) on the foreign protein or pathogen. Taking a toxin as an example, if an antibody binds to the toxin at an epitope that is required by the toxin for interaction with a cell-surface receptor, the antibody sterically hinders this binding of the toxin. The antibody is said to neutralize the biological activity of the toxin because the toxin cannot bind to the receptor and so no pathology or disease develops. The same applies to neutralizing antibodies specific for viruses; if antibody blocks the interaction of a virus with a receptor, the virus cannot enter a cell and consequently no replication occurs and no overt disease develops. A goal of vaccine strategies is therefore to induce the production of neutralizing antibodies in the immunized host. This has been studied at the crystallographic level using the b12 antibody directed against the HIV gp120 protein (Saphire et al. 2001b). The data show clearly that the binding site of the antibody, particularly the CDR3 of the heavy chain, mimics the CD4 natural ligand for gp130 by having a tryptophan residue protruding into space to fill a pocket in gp130 normally occupied by phenylalanine of CD4 (see 'Structure of human IgG1: the b12 anti-HIV-gp120 MAb' later).

Complement fixation

The classic pathway of complement activation is readily triggered by immune complex formation, although not all isotypes are capable of participating in this reaction (see Table 3). Thus, IgM is a potent activator of complement but IgA is unable to activate the classic pathway. It should be noted, however, that aggregated IgA, and possibly IgE, molecules may activate the alternate pathway of complement fixation. The capacity to activate the complement cascade depends on the ability to interact with the Clq component and, in the case of IgG molecules, on the segmental flexibility of the molecule.

The point of interaction between IgG and Clq has been mapped to three residues: Glu318, Lys32, and Lys322 in the three-strand face of the Cγ2 domain (Duncan and Winter 1988). The carbohydrate group of IgG, located on Asn297, influences the effectiveness of complement fixation because absence of the oligosaccharide leads to a decrease in activation of the classic pathway, an effect that may be explained by a threefold increase in the dissociation constant for the interaction between IgG and Clq when nonglycosylated IgG molecules are used (Leatherbarrow et al. 1985). The biology of complement is discussed in detail in Complement.

RECEPTORS FOR IMMUNOGLOBULIN

Antibody molecules express a range of effector functions that are concerned with the elimination of the pathogen or antigen from the host. These functions can range from simple neutralization of function of an invading particle, to bringing about destruction and clearance of a pathogen by either activation of complement or binding to receptors for the Fc regions of individual immunoglobulin isotypes. Fc receptors (FcR) have a range of roles in the immune response, are generally isotype-specific in their binding to Ig, and show a high affinity for binding to the Fc regions of their target Ig (Table 4 and see Figure 4). Some FcRs show a preference for Ig in immune complexes whereas others can bind monomeric Ig with high affinity, and many FcRs exist, not only as membrane-associated molecules but also as soluble proteins that retain Ig-binding activity and can compete with the cell-surface receptor for Ig binding. Most obviously, perhaps, FcRs can behave as vehicles for mediating uptake of antibody-coated pathogens leading to intracellular destruction of the organisms, although FcRs can also be linked to exocytotic responses that allow cells rapidly to release pharmacological mediators and/or toxic proteins toward a pathogen in a tightly focused manner. Finally, FcRs perform regulatory roles in ongoing immune responses, serving to maintain overall homeostatic control of humoral responses to pathogens.

Receptors for IgG

There are three main subsets of receptors specific for the Fc region of IgG molecules (FcγR), each with distinctive subclass-binding preferences and tissue distributions. Each FcγR type exists in several isoforms, being encoded by separate individual genes located at chromosome 1q and/or by alternate splice variants of those structural genes. All FcγR species belong to the Ig-like domain superfamily and possess two or three extracellular Ig-like domains.

Table 4 *Properties of human Fc receptors*

Ig bound	Receptor	CD	Gene	Chromosome	Transcripts	Proteins (kDa)	K_d (l/mol)	Isotype preference	Signaling	Complex
IgM	Fcα/μR			1q32.3		70	3×10^9 (IgM), 3×10^8 (IgA)	IgM > IgA		
IgD	FcδR					70				
IgG	FcγRI	CD64	FcγRIA	1q21	FcγRIa	60–70	10^8–10^9	3 > 1 > 4 >> 2	γ	$\alpha\ \gamma_2$
			FcγRIB		FcγRIb1	60–70	10^8–10^9			
					FcγRIb2	60–70	10^8–10^9			
			FcγRIC		FcγRIc	60–70	10^8–10^9			
	FcγRII	CD32	FcγRIIA	1q23-4	FcγRIIa1	35–40	10^7	3 > 1 >> 2, 4	ITAM	
					FcγRIIa2	35–40	10^7			
			FcγRIIB		FcγRIIb1	35–40	10^7		ITIM	
					FcγRIIb2	35–40	10^7			
					FcγRIIb3	35–40	10^7			
			FcγRIIC		FcγRIIc	35–40	10^7		ITAM	
	FcγRIII	CD16	FcγRIIIA	1q23-4	FcγRIIIa	25–30	$1–3 \times 10^7$	1 = 3 >> 2, 4	γ (CD3ζ)	$\alpha\ \gamma_2$, $\alpha\ \zeta_2$, $\alpha\ \zeta\gamma$
			FcγRIIIB		FcγRIIIb	20–25		1 = 3 >> 2, 4		
	FcRn					45 + 12	$2–5 \times 10^7$			
IgA	FcαR	CD89		19		55–75	10^6		γ	$\alpha\ \gamma_2$
IgE	FcϵRI						10^{10}		γ	$\alpha\ \beta\ \gamma_2$
	FcϵRII	CD23	FcϵRII	19	FcϵRIIa	45	10^7			
					FcϵRIIb	45	10^7			
IgM, IgA	pIgR					100–105	2×10^9 (IgM), 10^8 (IgA)	IgM > IgA		

ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; pIgR, polymeric Ig receptor.

FcγRI (CD64)

FcγRI (CD64) is the high-affinity IgG receptor, capable of binding monomeric IgG molecules. There are three structural genes encoding FcγRI (CD64), FcγRIA, -B, and -C (Ernst et al. 1992). These genes give rise to primary protein products of 30–40 kDa which are subsequently heavily glycosylated to yield mature glycoproteins of about 60–70 kDa (Ernst et al. 1998). The FcγRIA and -C genes each gives rise to a single transcript and protein product, named FcγRIa and FcγRIc, respectively, whereas the FcγRIB gene directs the synthesis of two RNA and protein products, FcγRIb1 and FcγRIb2 (Ernst et al. 1998). The FcγRIa membrane protein has three extracellular V-like Ig domains (EC1–3), whereas the FcγRIb and FcγRIc proteins possess only two such domains and lack the EC3 domain. FcγRI is the sole member of this family that is capable of binding monomeric IgG molecules, and is regarded as the high-affinity IgG receptor (association constant K_a roughly 10^8–10^9/mol per l) (Allen and Seed 1989; Shopes et al. 1990). FcγRI binds monomeric IgG1, -3, and -4 subclasses, with a preference for IgG3 and IgG1 molecules, but binds IgG2 only very weakly.

FcγRI molecules are type I integral membrane glycoproteins anchored to the cell surface via a single transmembrane helix, and they possess a large intracellular domain (Allen and Seed 1989). However, there are no signaling motifs found in this domain and the FcγRI molecule must associate noncovalently with a disulfide-bonded dimer of the γ chain of the high-affinity IgE receptor, in order to trigger intracellular signaling pathways in FcγRI$^+$ cells (Ernst et al. 1993; Scholl and Geha 1993). The γ chains possess immunoreceptor tyrosine-based activation motifs (ITAM) that recruit the necessary protein tyrosine kinases for initiation of intracellular signaling following receptor cross-linking (see The B cell antigen receptor). The stoichiometry of the FcγRI receptor complex is $\alpha\gamma_2$. The presence of an ITAM within the FcγRI complex allows this FcγR to function as an 'activating' receptor on monocytes/macrophages, granulocytes, natural killer (NK) cells, platelets, and certain lymphocyte subsets. Interaction of these receptors with IgG activates phagocytosis and uptake of the immune complex (or opsonized target), secretion of inflammatory mediators (cytokines and lipid mediators), and release of other toxic agents required to bring about ADCC. Alterations to patterns of gene expression in activated cells also occur. The principal functions of FcγRI are in uptake of immune complexes, delivery of ADCC to opsonized target, initiation of inflammatory responses, and, in an immunoregulatory context, delivery of antigen in immune complexes into processing pathways for subsequent presentation to T cells.

The binding site on IgG is located in the Cγ2 domain and the lower hinge region of IgG also contributes to FcγRI–IgG interaction. The capacity of IgG to interact with the high-affinity monocyte FcR is a particular property of the Cγ2 domain of the molecule (Woof et al. 1986) and Leu235 in the Cγ2 domain is critical in the interaction (Duncan et al. 1988). As with the IgG–Clq interaction, the carbohydrate groups seem to be necessary for optimal receptor–ligand interaction, because absence of oligosaccharides severely reduces binding of IgG to FcR; the precise molecular role of the oligosaccharides is not well understood. Binding of monomeric IgG to the receptor drives internalization, but the FcγRI-IgG complexes are recycled to the plasma membrane. It is only when FcγRI molecules are cross-linked that the internalized complexes are targeted to intracellular vesicles involved in protein hydrolysis or oxidative killing of organisms (Harrison et al. 1994). The FcγRI–IgG interaction follows a 1:1 stoichiometry.

FcγRII (CD32)

As with the FcγRI family, three independent structural genes at chromosome 1q23-24 encode FcγRII transcripts and proteins (Ernst et al. 1992). The FcγRIIA gene encodes two transcripts (FcγRIIa1 and FcγRIIa2), the FcγRIIB gene three transcripts, whereas the FcγRIIC gene yields only a single transcript (Dijstelbloem et al. 2001). The FcγRII family bind monomeric IgG with relatively low affinity (K_a roughly 10^7/mol per l) and so are unlikely to bind IgG in a monomeric form (Hulett and Hogarth 1994). The mature cell surface receptor glycoproteins have M_r values in the 35 000–41 000 range, with a single transmembrane helix. All isoforms possess two Ig-like extracellular domains and, in striking contrast to FcγRI (and FcγRIII) proteins, the cytoplasmic domains of FcγRII proteins contain ITAM or tyrosine-based inhibitory (ITIM) motifs that allow these receptors to link to intracellular signaling cascades without the need for association with other membrane proteins (Daeron 1995; Van den Herik-Oudijk et al. 1995). The FcγRII proteins display subtle differences in IgG subclass-binding specificity, but there is a general preference for IgG3 and, to a lesser extent, IgG1, with binding of IgG2 and IgG4 being of much lower affinity (Dijstelbloem et al. 2001). As with FcγRI, the binding site on IgG involves the Cγ2 and lower hinge regions of IgG, with residues 234–237 being important for the interaction.

Each FcγRII protein type displays a characteristic pattern of expression. FcγRIIa is expressed at good levels on monocytes and macrophages, and on neutrophils, but is essentially absent from NK cells and lymphoid cells. FcγRIIb proteins are found on monocytes and macrophages, but not on neutrophils, NK cells, or T cells. FcγRIIb is, however, expressed on both B lymphocytes and follicular dendritic cells, where it has key roles in positive and negative regulation of B-cell responses.

FcγRIII (CD16)

The two highly homologous FcγRIII genes are also found at chromosome 1q23-24, and encode mature proteins of quite different character; there are no reports of alternate transcripts from either of the FcγRIIIA or FcγRIIIB genes (Ravetch and Perussia 1989). Both protein products have external regions of about 180 amino acids arranged as two Ig-like domains, have a low to medium affinity for monomeric IgG (K_a roughly 1–3 $\times$ 10^7/mol per l) and so are likely to bind IgG only in immune complexes or aggregates (Ghirlando et al. 1995). As with other FcγRs, there is a preference for binding of IgG3 and IgG1 subclasses over the IgG2 and IgG4 isotypes. Receptor–ligand complexes show a 1:1 stoichiometry (Ghirlando et al. 1995), and the target region of IgG for binding of FcγRIII is as described for FcγRI and FcγRII.

The FcγRIIIa protein is a heavily glycosylated transmembrane glycoprotein, containing two extracellular Ig-like domains and a small cytoplasmic domain that lacks ITAM and/or ITIM motifs (Ravetch and Perussia 1989); FcγRIIIa must, therefore, associate with other chains possessing such motifs to gain signaling capability. This is provided either by the γ chain of the high-affinity IgE receptor (Hibbs et al. 1989), as is observed with FcγRI, or by the ζ chain of the CD3–TcR complex (in NK cells – Lanier et al. 1989). FcγRIIIa is expressed on neutrophils and eosinophils and also by NK cells; activated macrophages in the tissues are also FcγRIIIa$^+$, although the receptor is absent from monocytes in the peripheral circulation. FcγRIIIB, by contrast, encodes a smaller protein product, FcγRIIIb, which is associated with the plasma membrane via a glycophosphatidylinositol (GPI) linkage (Ravetch and Perussia 1989), a feature that mandates both a lack of a cytoplasmic domain and ease of release from the membrane via the action of phospholipases. FcγRIIIb is expressed principally by neutrophils, but its expression is also induced in eosinophils by IFNγ.

Neonatal FcRn

A final group of receptors for IgG found on endothelial and epithelial cells in some tissues are the 'neonatal FcR' class (FcRn), so called in recognition of their discovery in intestinal epithelial cells of neonatal rats (Jones and Waldmann 1972). These receptors also possess Ig-like domains, but are strikingly structurally related to MHC class I molecules. The functions of FcRn include transport of IgG from the gut lumen to the bloodstream, transfer of maternal IgG across the placental barrier to the neonatal circulation, and possibly maintenance of overall levels of IgG in the circulation. Uniquely among Fc receptors, the FcRn molecules bind ligand as a homodimeric receptor and show a notable pH dependence for ligand binding.

FcRn

FcRn is a noncovalent heterodimeric complex comprising the FcRn α chain (Simister and Mostov 1989a, b), a type I transmembrane glycoprotein, and the peripheral membrane protein β_2-microglobulin (Simister and Rees 1985). The organization and structural features of FcRn are therefore very similar to those of a class I MHC antigen. The FcRn α chain is 45–50 kDa and has three external domains – α1, α2, and α3 – with the last, most proximal membrane being a classic C-region type Ig domain (Simister and Mostov 1989a, b). The α1 and α2 domains have four β strands and a long α-helical region, highly reminiscent of the peptide-binding grooves of MHC antigens, but the α helices in FcRn are very close together and this precludes formation of a 'groove-like' structure (Raghavan et al. 1993; Burmeister et al. 1994a).

FcRn has an intermediate affinity for monomeric IgG (K_a roughly 2–5 $\times$ 10^7/mol per l) (Wallace and Rees 1980; Raghavan et al. 1995) and, unlike other FcγR species, appears to function as a homodimer with a receptor–IgG binding stoichiometry of 2:1. Surface plasmon resonance studies indicate that FcRn monomers have a 100-fold reduced affinity for IgG compared with FcRn dimers (Raghavan et al. 1995). The FcRn dimers are formed via contacts between the α3 and β_2-microglobulin domains and it is believed that dimer formation is dependent on binding of IgG (in a manner similar to that observed in, for example, the growth hormone–growth hormone receptor interaction). Crystallization studies suggest that the FcRn dimer adopts an unusual configuration in its asymmetrical binding of IgG molecules (Burmeister et al. 1994a, 1994b). Thus, receptor contacts with IgG were at the interface of the Cγ2 and Cγ3 domains and were made by only one of the two FcRn molecules in the complex via residues at the edge of the α1/α2 domains and the amino-terminal region of β_2-microglobulin (Burmeister et al. 1994b). The region bound by FcRn is distinct from that bound by other FcγR species and is more akin to that bound by, for example, protein A from the Cowan I strain of *Staphylococcus aureus* (Deisenhofer 1981). The contacts between FcRn and the Cγ2 and Cγ3 interface region involve conserved histidine residues in both partners (His250 and -251 in FcRn and His310 and -433 in IgG) (Burmeister et al. 1994b). The model further suggested that, in order to bind IgG, the dimer of the FcRn molecule would be positioned such that its long axis lay parallel to the plane of the plasma membrane. In this model, the FcRn dimer is 'lying down' in order to capture ligand, and this contrasts with the 'standing up' orientation of class I MHC molecules (and indeed many other FcγR species) where the ligand-binding site projects away from the membrane distal end of the molecule (Burmeister et al. 1994b). Robust evidence that Fc receptors can adopt 'lying down' configurations is provided by the

X-ray crystallographic analysis of the FcεRI–IgE Fc interaction (see 'The interaction of IgE with soluble FcεRIα', below).

FcRn molecules are found in intestinal epithelial cells (Jones and Waldmann 1972), the yolk sac in mice (Ahouse et al. 1993), certain subsets of hepatocytes (at least in rats), and human placenta (Story et al. 1994). FcRn has an intermediate affinity for IgG, but shows a pronounced pH dependence in ligand binding. Binding is optimal and stable at pH 5–6, but is reduced up to 100-fold as pH increases beyond neutrality (Story et al. 1994). This ensures that IgG is bound at the apical (or luminal) surface of intraepithelial lymphocytes (IEL) in the gut where the pH is low, moved across the cell by transcytosis and released by the receptor at the basolateral surface of the cell (blood side) where the pH is neutral. However, there is no pH gradient across the placental barrier and, in this instance, it is proposed that IgG is bound by FcRn in intracellular acidic vesicles which are subsequently targeted to the basolateral face of the cell for release into the fetal circulation (Story et al. 1994). FcγRII is thought to be responsible for the initial binding and uptake of IgG on placental cells and delivery of the IgG to the appropriate intracellular vesicles for interaction with FcRn. The pH sensitivity of FcRn function is explained by the presence of histidine residues in the region of contact between the receptor and IgG. Once IgG is released, the FcRn dimer dissociates and is re-cycled for further rounds of transcytotic delivery of IgG, a situation that contrasts with IgA transport via the polymeric Ig receptor (see 'The polymeric immunoglobulin receptor' below).

IgE receptors

IgE is found at low levels in the blood in normal individuals and, functionally, is responsible for initiating powerful local (and sometimes systemic) acute inflammatory responses (Metzger 1994, 1999; Sutton and Gould 1993). There are two receptors for IgE distinguished by profoundly different structures, cellular functions, tissue distribution, and, importantly, affinities for IgE. The effects of IgE-mediated allergic reactions are almost entirely explained by the biology of the high-affinity IgE receptor, and it is fair to note that the efforts to understand the structure and function of this receptor have driven forward our understanding of receptors for other Ig isotypes.

FcεRI

FcεRI is the high-affinity receptor for IgE, and is a tetrameric complex of three transmembrane glycoprotein chains (Blank et al. 1989): the α chain, responsible for IgE binding, the β chain, and a dimer of the γ chain which is responsible for activation of intracellular signaling via its ITAM motifs. The affinity of FcεRI for IgE is sufficiently high (K_a roughly 10^{10}/mol per l) to dictate that monomeric IgE is bound by the receptor (Beavil et al. 1993); this partly explains the observation that serum IgE levels are low because, in fact, most of the IgE is essentially irreversibly bound to FcεRI$^+$ cells. FcεRI is expressed on mast cells, basophils, and eosinophils, where crosslinking of receptors leads to an exocytotic response, and also on dendritic and monocytic cells where a role in antigen processing and presentation is more likely.

The FcεRI α chain possesses two extracellular Ig-like domains: a transmembrane region and modest cytoplasmic domain of about 25 residues (Kinet et al. 1987). The cytoplasmic domain lacks signaling motifs, but linkage to signaling pathways is provided via the ITAM motifs found in the β and γ chains of the receptor complex (Nadler et al. 2000). The β chain belongs to the tetraspanin family of membrane proteins, passing through the membrane four times, and both the amino and carboxyl termini of the β chain are exposed in the cytoplasm (Kinet et al. 1988). The carboxy-terminal cytoplasmic domain contains an ITAM motif. The γ chain possesses a small extracellular domain, a transmembrane region, and a large cytoplasmic domain that contains ITAM motifs. The dominant FcεRI stoichiometry found on mast cells and basophils is $\alpha\beta\gamma_2$, but other cell types where the IgE receptor may be involved in antigen processing rather than exocytosis (e.g. monocytic cells) show a different FcεRI stoichiometry of $\alpha_{2\gamma}$.

FcεRII (CD23)

FcεRII is the low-affinity receptor for IgE, and is expressed on a range of cells of hemopoietic origin (Delespesse et al. 1992; Bonnefoy et al. 1997). It is a 45-kDa type II transmembrane glycoprotein and is expressed as a noncovalent homotrimeric assembly at the plasma membrane. The affinity for IgE is significantly lower than that of FcεRI (K_a roughly 10^7/mol per l) (Sutton and Gould 1993). FcεRII is encoded by a single gene at chromosome 19p21 (Soilleux et al. 2000) that gives rise to two isoforms – FcεRIIa and FcεRIIb (Yokota et al. 1988) – that differ by six or seven residues at their cytoplasmic amino terminus, and are proposed to be linked to distinct signaling and uptake pathways (Yokota et al. 1992). In human B cells, the two isoforms are subject to distinct patterns of transcriptional regulation (Ewart et al. 2002). FcεRII is unusual among the Fc receptors in that it is not a member of the Ig superfamily, but rather has features that suggest that it is a member of the calcium-dependent family of C-type animal lectins (Delespesse et al. 1992; Bonnefoy et al. 1997). The membrane form of FcεRII/CD23 can be released as several soluble proteins (soluble CD23 or sCD23) and these can act as cytokine-like molecules (Gordon et al. 1989), influencing IgE synthesis and apoptosis in mature B cells (Liu et al. 1991a; Flores-

Romo et al. 1993) and pre-B cells (White et al. 1997), and proinflammatory cytokine synthesis in monocytic cells (Lecoanet-Henchoz et al. 1995; Hermann et al. 1999). The findings of two FcεRII isoforms and cytokine-like activities of sCD23 are apparently unique features of the human FcεRII proteins.

The binding site for FcεRII on IgE is, like that for FcεRI, located in the Cε3 domain of IgE. The precise location of the contact sites for FcεRII is not precisely defined. Soluble CD23 molecules can bind IgE and compete with FcεRI for IgE binding, via simple steric hindrance, thereby providing a degree of antagonism for loading of mast cell FcεRI with IgE. In this context, it is unsurprising that atopic individuals not only have high serum IgE levels, but also have elevated concentrations of sCD23 in their serum (Yanagihara et al. 1990; Wilhelm et al. 1994). FcεRII molecules can bind IgE and on crosslinking can rapidly take up the FcεRII–IgE immune complexes, and FcεRII is required for IgE-dependent antigen processing and presentation. Ligation of FcεRII with IgE–allergen complexes delivers a negative regulatory signal to B cells, downregulating production of IgE (Luo et al. 1991). Mice lacking FcεRII fail to show a negative regulation of IgE antibody responses (Yu et al. 1994). The structural basis for FcεRII function remains to be elucidated.

IgA receptors

IgA is the most abundant Ig class in the body, having a relatively short half-life coupled with a high synthetic rate. IgA functions in several tissue compartments, namely the blood, gut, and airways, and requires receptors both for performance of effector functions and for transport from sites of synthesis to secretory surfaces. IgA therefore interacts with the polymeric Ig receptor (discussed in detail below), an IgM receptor that has specificity for both IgM and IgA (see under IgM receptors), and also with an IgA-specific receptor that assists in the protective functions of IgA. There are also data to suggest that the transferrin receptor can act as a receptor for IgA1 (Moura et al. 2001).

FcαRI (CD89)

FcαRI is an IgA-specific receptor expressed on monocytic cells, neutrophils, and eosinophils (van Egmond et al. 2001). The receptor is a complex comprising the CD89 molecule (the receptor α chain), a transmembrane glycoprotein comprising two Ig-like extracellular domains, a transmembrane helix, and a small cytoplasmic domain (Maliszewski et al. 1990), in noncovalent association with a dimer of the γ chain found in FcεRI, FcγRI, and FcγRIIIa complexes (van Egmond et al. 1999). CD89 is encoded on chromosome 19 (Martin et al. 2002). As in the other receptors, it is the γ chain that is responsible for initiation of signaling in cells following activation of the IgA receptor. The affinity (K_a roughly 10^6/mol per l) is relatively low (Wines et al. 1999), suggesting that IgA will bind to the receptor only in complexes or aggregates rather than in a monomeric form. Both IgA subclasses are bound equally well by FcαRI, a result consistent with the mapping of the binding site, on IgA itself, to the interface between the Cα2 and Cα3 domains (Pleass et al. 1999).

IgA is classically considered to be a 'non'-inflammatory immunoglobulin isotype. However, given the pattern of expression of FcαRI, it is difficult to envisage how FcαRI and FcγRI, both using the γ chain as signal initiator, would generate anti- and proinflammatory responses, respectively, after activation. It is therefore unsurprising that $Fc\alpha RI^+$ neutrophils can both phagocytose IgA-coated particles (Weisbart et al. 1988) and generate a subsequent powerful oxidative burst (Keler et al. 2000). $Fc\alpha RI^+$ cells can also mediate ADCC-type lysis of tumor cells (Keler et al. 2000), and FcαRI is important in IgA-dependent antigen processing and presentation (Geissmann et al. 2001). The FcαRI complex should therefore be regarded as an 'activatory' FcR in the same way as FcγRI, although FcαRI may act as an overtly proinflammatory receptor in a more limited set of circumstances.

IgM receptors

IgM is the first Ig isotype to be produced in an immune response, and levels of IgM are known to be important in feedback homeostatic regulation of antibody responses. This observation suggests that receptors for IgM are expressed on a range of cell types. There are two main types of IgM receptor, a GPI-linked IgM-binding protein found in activated human B cells and a receptor, Fcα/μR, that binds both IgM and IgA, but is distinct from CD89/FcαRI and, with the exception of a small conserved motif, from pIgR.

Fcα/μR

Fcα/μR is a transmembrane glycoprotein of 535 amino acids, encoded by a gene located at chromosome 1q32.3, with no significant homology to proteins already recorded in public databases, apart from single Ig-like domain in the extracellular region (Shibuya et al. 2000). The protein is heavily glycosylated and has a molecular mass of about 70 kDa. The Ig-like domain of Fcα/μR has a stretch of some 25 residues that shows strong conservation with the polymeric Ig-binding site found in the most membrane distal Ig-like domain of the poly-Ig receptor (pIgR). This site is regarded as a potential binding site on Fcα/μR for IgM and IgA. Overall, however, Fcα/μR has less than 10 percent homology to pIgR.

The Fcα/μR molecule binds IgM with high affinity (K_a roughly 3×10^9/mol per l), and the murine receptor can bind mouse, human and rat IgM molecules with broadly

similar affinity. Fcα/μR also binds IgA, but with a lower, intermediate affinity (K_a roughly 3×10^8/mol per l). The receptor is capable of mediating endocytic uptake of IgM-coated particles, raising the possibility that this receptor could deliver antigen in IgM immune complexes to the antigen-processing machinery of the cell. It remains unclear whether ligation of Fcα/μR on monocytic cells leads to proinflammatory cytokine synthesis, and whether IgA-coated targets can be internalized by Fcα/μR$^+$ cells (Shibuya et al. 2000). The cytoplasmic domain contains no ITAM/ITIM motifs and it is reasonable to expect that Fcα/μR will associate with other membrane proteins in order to activate intracellular signaling pathways in the cells expressing it. However, there are no data to suggest that Fcα/μR associates with the γ chain found in other FcR complexes. In hemopoietic tissue, the receptor is expressed in B cells, including B-cell precursors, and on macrophages, but is absent from granulocytes, T lymphocytes, and NK cells. In nonlymphoid tissue, Fcα/μR transcripts are detected in liver, kidney, intestinal tissue, testis, and placenta, and also on thymic stroma (Shibuya et al. 2000). The functions of Fcα/μR in these tissues remain to be fully characterized.

IgD receptors

Although the existence of IgD receptors (IgD-R) is well documented, the formal molecular characterization of these binding proteins has proved a difficult problem (Preud'homme et al. 2000). The receptors expressed on human T cells appear to bind both membrane-associated IgD and secreted IgD and, in the case of the latter type, monomeric and polymeric (i.e. complexed or aggregated) forms (Coico et al. 1988b). Thus, monomeric IgD tends to downregulate IgD-R expression, whereas higher oligomers tend to enhance its expression (Coico et al. 1987, 1988a). The molecular mass of the receptor is about 70 kDa in human cells (the murine receptor appears to be different, containing two components), but there are no details of its primary or higher structural features (Tamma and Coico 1992). The receptor species appear to bind to O-linked oligosaccharides of the IgD molecule, a feature that accounts for the ability of IgD-R also to bind IgA1 molecules (Swenson et al. 1998). In the murine system the interaction of IgD-R with Ig requires Ca^{2+}, suggesting a C-type lectin interaction (Swenson et al. 1993). The paucity of molecular details makes difficult the task of defining functions for IgD-R. However, it is likely that the main roles of this receptor are in the antigen presentation process. The observation that monomeric IgD can drive IgD-R downregulation suggests that the receptor protein or complex has at least intermediate affinity for IgD, otherwise monomeric IgD would not bind for a sufficient time to be internalized. If the IgD-R is endocytosed, as seems likely in light of the trafficking of other receptors, crosslinked receptors may be internalized and routed to processing compartments for assembly of peptides into MHC molecules. Other data suggest that IgD-R can interact with glycans on membrane IgD on B cells and so enhance cell–cell contact between T cells and B cells. The molecular basis for all the potential functions of IgD-R requires cloning of receptor complementary DNAs (cDNA).

The polymeric immunoglobulin receptor

The secretory surfaces of the gut and airways are protected by secretory IgA (sIgA) (Johansen et al. 2000) or, in the case of individuals with selective IgA deficiencies, secretory IgM (sIgM) (Thompson 1970). In each case, the newly synthesized Ig must be transported from the site of synthesis across a cellular barrier in order to reach the tissue space where its protective function can be expressed. This transport is achieved via transcytosis and is mediated by the pIgR. In this elegant process, the pIgR collects the polymeric Ig at the basolateral surface of the cell, transports it via the cell cytoplasm to the apical surface, and is then itself cleaved and released from the cell (Mostov et al. 1984; Johansen et al. 2000). The cleaved pIgR molecule remains bound to sIgA (-M) as secretory component (SC) and now offers a degree of protection against proteolysis in the airway or gut microenvironment.

pIgR

Like most other Ig-binding proteins, pIgR is a member of the Ig-domain superfamily. The mature pIgR has a molecular mass of 100–105 kDa, and contains five Ig-like domains in the extracellular region of the receptor (named D1–D5), a transmembrane helix, and a cytoplasmic domain (Mostov et al. 1984). The five extracellular domains are very similar to V-like Ig domains (Mostov et al. 1984), and the cytoplasmic domain contains a targeting motif that ensures targeting of pIgR to the basolateral surface of the cell after biosynthesis (Casanova et al. 1991). There are separate motifs that facilitate rapid endocytosis after binding of pIgR and inhibition of unwanted delivery of the endocytosed complex to lysosomal compartments during transcytosis. The D1 domain is the main site of binding of polymeric Ig, although the remaining extracellular domains also contribute to binding. Pentameric IgM and dimeric IgA bind to pIgR in different ways, and association constants for the interaction of pIgR with each isotype are distinct (K_a roughly 2×10^9/mol per l for IgM (Goto and Aki 1984) and roughly 10^8/mol per l for IgA (Kuhn and Kraehenbuhl 1979)). Possession of J chain within the polymeric Ig appears to be a requirement for interaction with pIgR. Polymeric, J-chain-containing IgA molecules

bind pIgR via D1 (Coyne et al. 1994), but also appear to require contacts between the α chain and the D2 and D3 domains of pIgR for stable receptor interaction (Norderhaug et al. 1999). A disulfide bond is formed between Cys467 in pIgR and Cys311 in the Cα2 domain of the IgA molecule (Fallgreen-Gebauer et al. 1993), which gives further stability to the complex. In contrast, binding of IgM to the pIgR appears to require only the D1 domain (Roe et al. 1999); there appears to be no need for contacts between the μ chain and D2/D3 domains, nor is a μ chain–pIgR disulfide bond formed (Fallgreen-Gebauer et al. 1993). The site of interaction of IgA α chains with pIgR appears to map to the Cα3 domain, with Cα2 also likely to be involved (Geneste et al. 1986).

Although it is indisputable that J chain is required for interaction of polymeric Igs with pIgR, what is less definitively clear is whether J chain itself makes direct physical contact with pIgR (Johansen et al. 2000). Data from knock-out mice lacking either J chain (Hendrickson et al. 1995) or pIgR (Johansen et al. 1999), in which both strains fail to transport Ig, argue in favor of a direct interaction. The capacity of anti-J-chain antibodies to block the interaction of polymeric IgA and pIgR are also consistent with a direct interaction between pIgR and J chain (Brandtzaeg 1975). However, phage display experiments demonstrating a strong interaction between Cα3 peptides and pIgR argue against a mandatory requirement for J chain for the interaction of polymeric Igs with pIgR (Hexham et al. 1999). The precise role of J chain remains to be defined, but it is possible that it interacts with polymeric Ig to create a conformation that can be bound effectively by pIgR.

STRUCTURAL BIOLOGY OF IMMUNOGLOBULINS AND IMMUNOGLOBULIN RECEPTORS

Elucidation of protein structure via X-ray crystallographic analysis invariably provides great insights into the molecular basis of protein function. The structures provide significant insight into the functions of Ig molecules and their receptors.

Structure of human IgG1: the b12 anti-HIV-gp120 MAb

The b12 human IgG1κ antibody was isolated from a phage-display combinatorial library prepared from bone marrow cells derived from an HIV-positive donor. The antibody recognizes an epitope on the gp120 protein that partially overlaps the site recognized by CD4, and the MAb can neutralize HIV infectivity by many strains both in vitro and in in vivo animal models (Burton et al. 1994).

The b12 IgG1κ molecule is highly asymmetrical in shape and structure (Saphire et al. 2001a,b). The molecule as a whole lies somewhere between a 'T' or 'Y' shape, but the Fc region does not lie centrally beneath the Fab arms. Rather, it is shifted significantly to one side of the central axis of symmetry of a Y-shaped IgG molecule and lies largely under only one of the Fab arms (Figure 5). Thus, one Fab arm sits on top of the Fc region and can make physical contact with it (via Cγ2), whereas the second Fab arm makes no contact at all with the Fc domains. In detailed terms (Saphire et al. 2001a, 2001b), the Fab arms lie at an angle of 143–148° relative to each other with a distance between the two apices of the combining sites on the Fab arms of 17.1 nm. The Fc region is offset by 3.2 nm from the central axis of the molecule and is twisted by almost 90° relative to the major axes of the Fab arms. The Fab arms are also rotated relative to each other, such that the light chains appear on opposite sides of the heavy chain in the intact molecule. The V_H–V_L and CH_1–C_L domains in the Fab arms have a unique 'elbow' angle between their individual axes of symmetry.

In the Fc region, the two Cγ3 domains pack closely with essentially complete symmetry, whereas the Cγ2 domains make few protein–protein contacts as a result of the presence of the N-linked oligosaccharides in the space between the domains. The Fc region can show rotational and translation shifts relative to the Fab arms, meaning that different angles exist between this region and the two Fab arms. This has consequences for the disposition of the hinge region where the hinge angle in a single molecule can be quite different. In the case of the b12 IgG1κ molecule, one hinge angle is acute (66°) and the other obtuse (113°) (see Figure 5).

The b12 molecule allowed good visualization of the hinge region in an intact human IgG molecule (Saphire et al. 2001a, b). The 17-residue hinge of IgG1 molecules is functionally split into three regions: an upper hinge close to the Cγ1 domain, a core hinge, and a lower hinge immediately proximal to the Cγ2 domain. The upper hinge allows rotation and other movement of the Fab arm, and the lower hinge serves the same function relative to the Fc region; the core hinge is cysteine and proline rich and is therefore involved in interchain disulfide bridging between the γ heavy chains. Surprisingly, however, the crystal structure of IgG1κ reveals that only one of the two possible disulfide bonds in the hinge region actually forms; the Cys226 residues form a disulfide, but the Cys229 residues do not. In general terms, the b12 hinge region appears to have an extended conformation, with the upper and lower hinges being somewhat disordered in the crystal structure and the core hinge having some polyproline helical character.

The combining site structure of the b12 molecule departs from the conventional view of a large flat area available for surface-to-surface contacts between the antibody and the protein antigen. The b12-combining site could be considered as a large 'spike' flanked by two platforms. The spike region is contributed by the long

CD3 of the heavy chain (CDR H3), and one platform of 1.1 nm width is composed of the remaining two heavy chain CDRs and the second platform of 0.7 nm width by the three light chain CDRs. The CDR H3 is longer than normal, a feature common to anti-CD4 neutralizing antibodies and, in the combining site, protrudes 1.5 nm above the plane of the low, light chain 'platform' and is tipped by Trp100 (Saphire et al. 2001b). In complex with the gp120 monomer, 103 nm of gp130 and 104 nm of the b12-combining site are buried, and the intercalating residues on the two proteins are completely complementary. In particular, the Trp100 residue of CDR H3 of the b12 antibody protrudes precisely into the same pocket that would be occupied by a critical residue of CD4 (Phe43) – a feature that helps to explain the capacity of b12 to act as a potent neutralizing antibody.

The availability of the b12 structure allows comparisons to be made with other complete structures (e.g. of murine IgG2a and IgG1 molecules) or partial structures (Saphire et al. 2002). The human structure differs profoundly, but a common feature is the extent of asymmetry in the structures. Thus, although the primary structures of the two 'halves' of the IgG molecules are identical, they have completely different structures. In terms of general layout, murine IgG1 is more Y-shaped, with the angle between the Fab arms being 115°, whereas the murine IgG2a has a more T-like organization with an angle of 172° between the Fab arms. The Fab 'elbow' angles in murine IgG1 appear to be identical, whereas the IgG2a equivalents are slightly different (143° and 159°). Significant differences also exist in the position of the Fc region relative to the Fab arms. Thus, in b12, the Fc region is twisted and offset by 3.2 nm relative to the plane of the Fab arms, whereas murine IgG2a is shifted 2.6 nm and away from both Fab arms such that essentially no contact between the Fc and Fab arms takes place. The Fc 'elbow' angles of the murine IgG1 and IgG2a molecules are in close agreement with the b12 Fc elbow angle, being 177.2° and 176.6°, respectively. The hinges of murine IgG1 (19 residues) and IgG2a (12 residues) are broadly similar to the b12 structures, showing extended conformations with different torsion angles in the two heavy chains. However, in contrast to b12, all three possible interchain disulfide bridges in both murine Ig hinges are formed (Saphire et al. 2002).

Structure of the IgE Fc region

Biophysical studies clearly indicate that the IgE molecule in solution is 'bent' in conformation such that the distance between the tips of the Fab arms and the tail of the Fc region is approximately 7.5 nm (see Figure 5), in both free and receptor-bound forms (Zheng et al. 1991, 1992). Recent success in crystallizing the Fc region (Cε2–Cε4) of human IgE provides insight into the structural basis for IgE 'bending' and begins to explain the asymmetry of the interaction of IgE with its high-affinity receptor (Wurzburg et al. 2000; Wan et al. 2002; Wurzburg and Jardetzky 2002).

The IgE Fc region contains three C-type Ig domains and has no hinge region, with the Cε2 domain occupying this position in the IgE molecule. The Cε3 and Cε4 domains can be regarded as broadly similar to the Cγ2 and Cγ3 domains of IgG, with Cε4 domains being essentially symmetrical and making extensive protein–protein contacts, and the Cε3 domains being held apart in space by oligosaccharide groups. In crystals of the Cε3/Cε4 protein (Wurzburg et al. 2000), the Fc region adopts a relatively 'closed' configuration relative to IgG Fc, but this structure 'opens up' somewhat upon interaction with the FcεRI, with the two Cε3 domains moving 1 nm apart (Wurzburg et al. 2000; Garman et al. 2000). The IgE Fc also shows considerably greater flexibility than that described for IgG Fc regions (Wurzburg et al. 2000).

However, it is in the Cε2/Cε4 Fc structure, where a pronounced bend occurs at the region that links the Cε2 domains to Cε3, that the most striking feature of the IgE Fc is found (Wan et al. 2002). The Cε2 domains associate with a high level of symmetry, adopt classic Ig-like folding patterns, but unusually use polar contacts (rather than nonpolar) to stabilize the domain interaction. The linker region between Cε2 and Cε3 is dramatically bent, folding acutely through 62° and back onto the Cε3 and Cε4 domains (Wan et al. 2002) (see Figure 5). A consequence of this acute folding back is that the Cε2 domain of one ε chain makes extensive contacts with both the Cε3 and Cε4 domains of the second ε heavy chain, whereas the second Cε2 domain makes rather few contacts with the more carboxy-terminal domains. The contacts made between Cε2 and the Cε3/4 region are therefore highly asymmetrical. The interaction buries a total 39.0 nm^2 of solvent-exposed surface on the Cε2/Cε4 molecule (30.6 nm^2 on Cε3 and 24.1 nm^2 on Cε4, respectively) and a total of six hydrogen bonds and three salt bridges are formed. Experiments with purified Cε2 and Cε3/Cε4 proteins in vitro suggest that the affinity of interaction between these domains is in the micromolar range, a value consistent with the area of surface interaction. A final notable feature of the asymmetry of the IgE Fc region relates to the two disulfide bridges in the Cε2 domains. These disulfides are formed by Cys241 and Cys328 in the ε heavy chain, but, rather than forming 'parallel' bonds, the disulfides 'crossover' in the structure, i.e. in each case, Cys241 interacts with Cys328 to form the disulfide bond (Wan et al. 2002).

The interaction of IgE Fc with soluble FcεRIα

The recent crystallization of a soluble FcεRI α chain–IgE Fc complex reveals the basis for IgE binding to the

receptor (Garman et al. 2000). The complex stoichiometry is 1:1, there is no role for carbohydrate groups on either IgE or the receptor in the interaction, and there is little change in the receptor after ligand binding. Contacts are made exclusively between the Cε3 domain and the α chain; although the Cε4 domain provides a dimerization platform for IgE Fc, neither this domain itself nor the Cε3/Cε4 interface region makes physical contact with the receptor. The Cε3 domain binds at two distinct sites on the receptor, the first site being along one edge of the D2 domain, and the second being located at the apex of the interface between the D1 and D2 regions (Garman et al. 2000). These data explain earlier studies which suggested that mutant α chains lacking the D1 domain still bound IgE but with vastly reduced affinity; both sites are needed for high-affinity binding. The binding site along the edge of the D2 domain is centered around tyr131, while site two involves four tryptophan residues located some distance apart in the primary sequence (Trp87, Trp110, Trp113, and Trp156).

IgE adopts an unusual 'bent' conformation in solution, and retains this when bound to FcεRI (Zheng et al. 1991, 1992). The bending of IgE results in the molecule presenting a convex and a concave face, with only the convex face presenting a functional binding site for the FcεRI complex. This explains the observed FcεRI–IgE stoichiometry of 1:1. The data suggest that the FcεRI complex adopts a 'lying-down' conformation on the cell surface such that the Cε2–Cε3 interface region is readily available for interaction with the bent IgE ligand (Garman et al. 2000).

The interaction of IgG Fc with soluble FcγRIII

The molecular basis of the interaction of the Fc region of IgG with an Fc receptor was visualized using a soluble, two-domain form of the FcγRIII molecule (Sondermann et al. 2000). The partners interact with a K_a of roughly 5×10^5/mol per l, which is lower than found with intact proteins. The data obtained are entirely consistent with results from mutagenesis studies identifying particular residues as being critical for the interaction. As with the structural biological analysis of IgG, the analysis of the Fc–FcR interaction again shows a large element of asymmetry in the behavior of the proteins.

In the crystal structure, the FcγRIII molecule uses principally domain 2 to make contact with the Fc molecule, and only two residues from the linker between the first and second receptor domains also participate in making contacts with the Fc piece (Sondermann et al. 2000). The key regions of the Fc piece involved in the receptor interaction are the Cγ2 domain and the lower hinge region; as expected Leu-234 and Leu-235 residues of the Fc piece make contact with receptor elements. The contact between the receptor and the Fc region Cγ2 domains is asymmetrical, with one Cγ2 domain having more surface area buried in the interaction (23.34 nm^2) than the other (18.70 nm^2).

The contact region involves five discrete stretches of three to four amino acids of the receptor domain binding to three regions of the Cγ2 domain: the Leu234–Ser239 region of both lower hinges and an *N*-acetylglucosamine group of the Fc region carbohydrate (Sondermann et al. 2000). The physical interactions are mainly van der Waals' and hydrogen bonds, many of which are contributed by residues 234–239 in the lower hinge. These latter lower hinge regions are one of two main regions of contact in the crystal, the other being Pro329 of one Cγ2 domain which is found in a 'proline sandwich' secured tightly by two tryptophan residues (Trp89 and -110) of the receptor.

The interaction of the Fc piece with the receptor causes large changes in the structures of each participant (Sondermann et al. 2000). In the unliganded receptor, the elbow angle between the two domains is about 70°, but this opens up on interaction with the Fc region to some 80°. The Fc piece also 'opens up' on binding to the receptor, with the tips of the two Cγ2 domains being pushed 0.7 nm further apart than is observed in the free Fc structure. The interaction of the *N*-linked oligosaccharides in the Fc piece is also altered with the terminal mannose residues moving from 0.28 nm to 0.53 nm apart. One consequence of the conformational change and asymmetrical nature of the interaction is that binding of a second FcR molecule to the IgG Fc region is precluded, thereby enforcing a 1:1 stoichiometry of interaction of receptor and IgG.

CELLULAR BASIS OF ANTIBODY PRODUCTION

Theories of antibody production

The primary structural analysis of Ig molecules indicates that the humoral immune system is capable of recognizing a vast array of antigens in a specific manner. Two schools of thought emerged to account for this diversity: instruction of the immune system by each antigen or selection of pre-existing antibody by antigen. Evidence to support the selective theory was provided by studies of denatured and reduced Fab fragments of purified anti-RNAse antibody (Haber 1964). Reactivity to RNAse was always recovered after renaturation in the absence of RNAse, indicating that antigen (RNAse) did not 'instruct' the antibody to recognize it. Thus, the information for antibody specificity must be contained in the primary structure of the immunoglobulin.

The clonal selection theory (Burnet 1959) was developed to provide a framework to explain the synthesis of antibody molecules by B lymphocytes. All B lympho-

cytes, whether responding to antigen for the first time or daughter memory cells undergoing activation in response to re-challenge, obey the rules of this theory. The principal tenets of this theory are:

- The capacity to respond to a given antigen exists before antigen exposure.
- Each B lymphocyte possesses a single receptor specificity for antigen.
- The binding specificity of the receptor is identical to that of the antibody that the cell will secrete in response to antigen binding.
- Specific antigen is the sole signal for clonal expansion.
- The antibody produced by a cell is subject to allelic exclusion (i.e. only one set of antibody-determining genes is activated).

B lymphocytes are the cellular site of synthesis of antibody molecules. B lymphocytes, like all blood cells in mature mammals, initiate their development in the bone marrow and are derived originally from pluripotent hemopoietic stem cells. Some early B-cell progenitors are found in fetal liver and the yolk sac and, in avian species, B cells develop in the cloacal organ, the bursa of Fabricius. Moreover, in murine models, there are good data demonstrating the development of a self-repopulating B-cell subset (variously called Ly-1$^+$, CD5$^+$, or B-1 B cells) in the peritoneal cavity. In all cases, however, the B-cell development program is a multistage process, and regulation of differentiation is achieved by stepwise expression of key genes, accurate rearrangement of Ig genes, cell–cell adhesion and soluble signals from bone marrow stromal elements or other cell types in the periphery, and contact with antigen (Figure 6).

Antigen-independent B-cell development

The development of mature B cells from pluripotent hemopoietic stem cells is a complex process during which regulated rearrangement of the germline Ig heavy (*Igh*) and Ig light (*Igl*) genes takes place. B-cell differentiation can be divided into two anatomically and functionally distinct stages. The first phase of development is antigen independent, occurs in the bone marrow, and is concerned with generation of a specific receptor for antigen (i.e. successful somatic recombination of Ig heavy and light chain genes). The second phase of B-

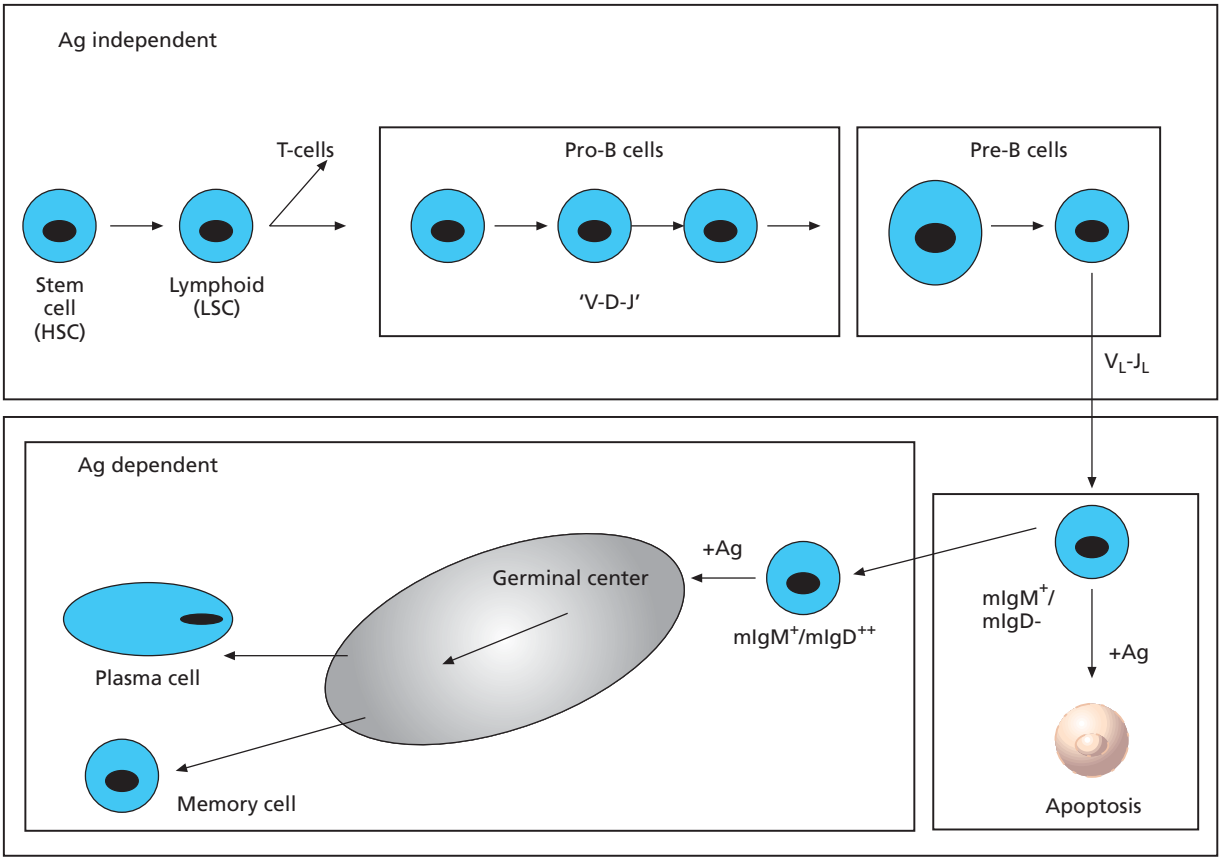

Figure 6 *A route map of B-cell differentiation: the main stages of B-cell development are shown. The upper panel illustrates the antigen-independent phase of development, occurring largely in the bone marrow, whereas the lower panel describes antigen-dependent events in the periphery.*

lymphocyte development is antigen dependent and occurs in the peripheral lymphoid system. In this second phase of development, the fate of B cells expressing clonally distributed receptors for antigen (i.e. membrane Ig or 'BcR') is inextricably linked to contact with specific antigen. This contact can result in clonal deletion or anergy, clonal expansion or prevention of apoptosis, and recruitment into the memory B-cell pool, with the outcome being dependent on the stage of differentiation of the B cell upon contact with antigen. A number of checkpoints and selection steps ensure that only cells expressing functional Ig heavy and light chains, which can associate into cell-surface IgM that is not autoreactive, are allowed to progress to the mature B-cell stage.

The first step in progression along the pathway of antigen-independent B-cell development in the bone marrow is differentiation of pluripotent stem cells into $CD34^+$ bipotential lymphoid stem cells (LSC). These LSCs can, depending on the transcription factors activated in this cell, become either a T-cell precursor ($CD2^+$ or $CD7^+$) or a B-cell precursor ($CD19^+$). The earliest stage at which cells committed to the B lineage can be detected is known as the pro-B-cell stage. These cells express some B-cell-specific markers (e.g. CD19), and undergo the process of *Igh* rearrangement (Hardy et al. 1991). Once the *Igh* locus has been successfully rearranged, the resulting μ chain is expressed on the surface as part of the pre-B-cell receptor (pre-BcR, described below), and the cells undergo the burst of rapid proliferation that is characteristic of the early (large) pre-B-cell stage (Kurasuyama et al. 1994). This stage of proliferation means that each productively rearranged IgH chain is duplicated in many daughter cells and has the opportunity to associate with many different IgL chains; the *Igl* locus is rearranged during the next stage of development, that of the small (late) pre-B cell. The cytokine interleukin IL-7, produced by bone marrow stromal cells, is important during these early stages of B-cell development in the mouse, particularly in its ability to stimulate proliferation of the cells (Corcoran et al. 1996). Mice deficient in IL-7, the IL-7 receptor α chain, or the cytokine receptor common γ chain (γc) have severely impaired B-cell development (Peschon et al. 1994; Cao et al. 1995; von Freeden-Jeffry et al. 1995). However, IL-7 appears to be much less important during human B-cell development, and has only a weak proliferative effect on normal human B-cell precursors in vitro (Wolf et al. 1991). One form of the disease severe combined immunodeficiency (SCID) is caused by a lack of the γc chain required for IL-7 signaling, but although they have few T cells or NK cells, patients with this disease have normal peripheral B-cell numbers (Noguchi et al. 1993).

Productive rearrangement of *Igl* leads to the cell surface expression of heavy (μ) and light (κ or λ) chain on the cell surface as intact IgM, at which point the cells enter the immature B-cell stage. This is the stage at which negative selection of autoreactive B cells occurs, resulting in B-cell tolerance. Thus, only non-self-reactive B cells are able to exit the bone marrow and enter the periphery, where they complete their development into mature B cells, which express cell-surface IgM and IgD and can be activated on binding of antigen.

Selection during antigen-independent B-cell development

Selection of B cells occurs throughout their development. One of the most important and best-characterized selection events is for pre-B cells, which are capable of expressing the pre-BcR. Once IgH rearrangement is complete, μ chain is expressed on the cell surface as one component of the pre-BcR; the other components are the surrogate light chains λ_5 and $V_{pre\text{-}B}$, products of B-cell-specific, non-rearranging genes, and the signaling components Igα and Igβ. Only μ chains resulting from in-frame *Igh* recombination and capable of binding κ or λ light chain after *Igl* rearrangement will be able to associate with the rest of the pre-BcR and allow signaling through this receptor. Productive rearrangement and the resulting pre-BcR signaling lead to proliferation of the cells (Kurasuyama et al. 1994); those cells with non-productive rearrangements cannot form the pre-BcR and/or signal through that complex, and so will not progress further through the B-cell developmental pathway. Proliferation induced by pre-BcR signaling appears to be self-limiting, as production of the λ_5 surrogate light chain component ceases once the pre-BcR is expressed on the cell surface. Thus, the expression level of the pre-BcR and the proliferative signals transduced by it reduce as proliferation progresses, until the cells no longer receive the proliferative signal and so exit the cell cycle. No ligand has yet been identified for the pre-BcR, and it has been speculated that no such ligand exists. Thus, it is possible that it is simply the expression of the pre-BcR on the cell surface that causes the complex to signal, rather than binding of an extracellular ligand to the complex.

The importance of signaling through the pre-BcR is demonstrated by the effect of loss of activity of the Bruton's tyrosine kinase (Btk), a defect that results in X-linked agammaglobulinemia (XLA) (Vetrie et al. 1993). Btk is an important component of the pre-BcR (and, indeed, of the mature BcR) signaling pathway and its loss results in a defect in the ability of pro-B cells to progress to the pre-B-cell stage and proliferate (Nomura et al. 2000). Thus, XLA patients have reduced numbers of pre-B cells (and therefore also of mature B cells), with the severity of the reduction in numbers varying depending on the precise Btk mutation inherited by the patient (Hashimoto et al. 1999). Interestingly, loss of Btk function in mice, by either natural mutation or targeted gene disruption, results in a much less severe phenotype than seen in human XLA (Scher 1982;

Kerner et al. 1995). These data suggest a lesser reliance on Btk in early murine B-cell development than in human B-cell development.

Expression of the intact μ chain and the pre-BcR also leads to allelic exclusion at the *Igh* locus; recombinase activating gene products (RAG) 1 and 2 are degraded, and presumably alterations in transcription factor expression at the pro-B- to pre-B-cell transition reduce the accessibility of the *Igh* locus, preventing further recombination (Grawunder et al. 1995). Thus, only a single productively rearranged Igh locus is present in each pre-B cell. The ability of a productively rearranged *Igh* locus to induce allelic exclusion has been convincingly demonstrated using mice transgenic for a particular V_HDJ_H gene combination: the only μ chain expressed is that of the transgene, indicating that its presence and ability to associate in the pre-BcR prevent rearrangement of either of the endogenous IgH alleles (Nussenzweig et al. 1987).

The next stage at which B cell selection takes place is after rearrangement of the *Igl* locus. In a manner similar to that of selection of pre-B cells, which can successfully associate intact μ chain in the pre-BcR, only those cells in which the product of the newly recombined κ or λ light chain gene can associate with existing μ chain to allow expression of IgM on the cell surface in association with Igα and Igβ and form the mature BcR can progress further. The signal resulting in this positive selection is not known, but successfully selected cells go on to become surface-expressing immature B cells, and undergo the next level of selection.

So far, cells have only been selected for the ability to express intact and functional μ and κ/λ chains; there has been no negative selection to remove those cells that express autoreactive receptors. This occurs at the immature cell stage. Interestingly, it seems that not all autoreactive cells are immediately deleted; instead, cells expressing self-reactive surface IgM can enter a process known as 'receptor editing', during which secondary rearrangements at the *Igh* and/or *Igl* loci change the antigen specificity of the BcR expressed (Tiegs et al. 1993). If receptor editing does not succeed, self-reactive immature B cells undergo anergy or apoptosis on encounter with antigen. This contrasts with the ability of mature B cells to become activated on encounter with antigen: mature cells become activated, whereas immature cells die by apoptosis. The differences in signaling mechanisms underlying these radically different responses to antigen at the immature and mature B-cell stages are considered in detail in The B cell antigen receptor.

Transcriptional control of B-cell development

So far, the mechanisms of B-cell development, but not of the control of the developmental pathway, have been discussed. This takes place through the expression of various transcription factors, which regulate the activation of lineage-appropriate genes, the repression of genes associated with development of other hemopoietic lineages, and through control of the accessibility of the *Igh* and *Igl* loci to the recombination machinery. Several of the most important and best-studied transcription factors are considered here.

PU.1

PU.1 is a member of the Ets family of transcription factors, and is expressed in monocytic, granulocytic, and lymphoid lineages (Klemsz et al. 1990). Ets sites, which can be bound by PU.1 in association with various other proteins, are found in a number of genes important in B-cell development, including *Igh* and *Igl* genes, RAG1, components of the pre-BcR, Btk, and terminal deoxynucleotidyl transferase (TdT) (Kozmik et al. 1992; Omori and Wall 1993; Ha et al. 1994; Fitzsimmons and Hagman 1996; Muller et al. 1996). PU.1-deficient mice lack both B- and T-cell precursors, and die before birth (Scott et al. 1997), but experiments in which PU.$1^{-/-}$ embryonic stem cells or fetal liver cells were transferred to irradiated normal animals suggests that this transcription factor is especially important in the development of a multipotential lymphomyeloid precursor cell (Scott et al. 1997). Although PU.1 is expressed in cells from various hemopoietic lineages in normal animals, it appears to play a role in lineage commitment. Retroviral transfer of PU.1 to PU.$1^{-/-}$ lymphomyeloid progenitor cells from fetal liver rescued development of both B cells and macrophages, but the macrophages expressed significantly higher levels of PU.1 than the B cells (DeKoter and Singh 2000); these experiments also demonstrated that overexpression of PU.1 inhibits B-cell development. Thus, PU.1 operates very early in hemopoietic development, at the level of multipotent precursors and lineage commitment.

IKAROS AND AIOLÖS

Ikaros is mainly expressed in hemopoietic cells, particularly in B-cell precursors, suggesting a role for Ikaros in B-cell development. Ikaros-deficient mice lack fetal B and T cells, but only peripheral B cells are missing from the adult mice (Wang et al. 1996). The block in B-cell development caused by loss of Ikaros is before the pro-B-cell stage (Wang et al. 1996), and Ikaros-binding sites have been identified in the promoters of RAG1, Igα, V_{preB}, λ_5, and TdT genes (Georgopoulos et al. 1997). In the TdT promoter, the Ikaros-binding site overlaps an Ets-binding site, suggesting that Ikaros and Ets transcription factors may compete for binding here (Ernst et al. 1996).

Aiolös is a member of the Ikaros family of proteins but is expressed later in development than Ikaros itself (Morgan et al. 1997). Aiolös is first detected in

committed B- and T-cell precursors, and its expression levels increase as the cells mature (Morgan et al. 1997). Aiolös-deficient mice have increased numbers of pro- and pre-B cells, suggesting a defect in differentiation of the cells to the later stages of antigen-independent development (Wang et al. 1998). Such mice also show defects in mature B-cell functions, including isotype switching in the absence of immunization, proliferation in response to very low levels of BcR signaling, and the formation of B-cell lymphomas (Wang et al. 1998). In contrast to Aiolös$^{-/-}$, Ikaros$^{+/+}$ mice, animals that are Aiolös$^{-/-}$ Ikaros$^{+/-}$ have decreased numbers of both pre-B and mature B cells (Cortes et al. 1999), suggesting that these two transcriptional regulatory proteins act together. In fact, the two proteins are expressed in a complex in both developing and mature B-cell populations. Mature B cells in Aiolös$^{-/-}$ Ikaros$^{+/-}$ mice are also more readily activated than those derived from Aiolös$^{-/-}$ Ikaros$^{+/+}$ mice (Cortes et al. 1999), indicating that the two proteins may also act in concert to regulate signaling thresholds for B-cell activation through the BcR.

E2A

Alternate splicing leads to the production of two proteins from the *E2A* gene, known as E12 and E47 (Murre 1994). These basic helix–loop–helix transcription factors are members of the E protein family (also known as class I HLH proteins), the members of which homo- or heterodimerize (to other E proteins or to members of the class II HLH family) to allow binding to the sequence CANNTG, known as an E-box site. This sequence is found in the promoter of a number of B-lineage genes, including Igα and λ_5 (Sigvardsson et al. 1997). E12 and E47 are ubiquitously expressed, but are especially important in B-cell development, where B-cell-specific phosphorylation allows the E47 protein to form a DNA-binding homodimer (Sloane et al. 1996). In other tissues, E proteins such as E12 and E47 usually heterodimerize with class II HLH proteins; thus such homodimerization is tissue specific. E12 and E47 act early in B-cell development, and mice deficient in E2A have B cells that are blocked at the very early pro-B cell stage, before DJ_H rearrangement, and have virtually no mature B cells (Bain et al. 1994). These cells also do not express proteins associated with B-cell commitment and development, such as Igα and RAG1, underlining the important role played in early B-cell development by the E2A proteins (Bain et al. 1994, 1997; Zhuang et al. 1994).

The early and profound block in B-cell development seen in E2A$^{-/-}$ mice means that these mice cannot be used to identify genetic targets of the E12 and E47 transcription factors, or their potential roles in later B-cell development. Thus, alternate strategies, such as transfection of non-B-lineage cell lines with E12 or E47, must be employed. Such studies have shown that, in various cell lines, expression of E47 results in production of germline Ig transcripts, the RAGs and TdT, suggesting an involvement in regulation of Ig rearrangement as well as lineage commitment (Schlissel et al. 1991; Choi et al. 1996; Romanow et al. 2000). Indeed, when E47 and the RAG genes are expressed in a human embryonic kidney carcinoma cell line, both *Igh* DJ and Igκ VJ rearrangements are seen (Romanow et al. 2000). Rather than the transcription factors acting directly in V(D)J recombination, however, it appears that binding of E2A proteins to Ig enhancers increases the accessibility of these loci to the recombination machinery. Transfection of E12 into a macrophage cell line that has rearranged *IgH* and Igκ genes, but which does not express cell surface IgM, results in the induction of a number of B-cell-specific genes, including RAG-1, EBF, λ_5, and *Pax5* (Kee and Murre 1998). It is also notable that there is a gene dosage effect for E2A proteins in B-cell development. Thus E2A$^{+/-}$ mice have 50 percent of the numbers of B cells found in E2A$^{+/+}$ animals (Zhuang et al. 1994). This effect is explained by the Id protein family discussed below.

Another means of investigating the roles of E2A proteins in B-cell development is to transfect members of an E2A-inhibiting family known as Id proteins into E2A-expressing cells. These Id proteins are related to the E proteins, containing the domain that allows them to heterodimerize with E proteins but not the DNA-binding domain (Sun et al. 1991). Thus, expression of Id proteins causes E proteins to be 'trapped' in non-DNA-binding E–Id heterodimers, effectively inhibiting the function of the E proteins. Mice expressing an *Id1* transgene exhibit the same block in B-cell development as E2A-deficient mice, and the severity of the block is dependent on the level of expression of the transgene, i.e. on the amount of E2A proteins that can be sequestered by *Id1* (Sun et al. 1991).

EBF

As mentioned above, transfection of cell lines with E2A proteins has shown that these transcription factors can induce production of early B cell factor (EBF) (Kee and Murre 1998). This is supported by the observation that, although EBF-deficient mice, similar to E2A$^{-/-}$ mice, exhibit an early block in B-cell development (Lin and Grosschedl 1995), the few B-cell precursors that are found express E12 and E47, indicating that E2A expression is not dependent on EBF expression (Lin and Grosschedl 1995). A number of B-cell-specific genes have binding sites for EBF in their promoters, including Igα, Igβ, and λ_5 (Kee and Murre 1998), indicating that expression of these proteins may be directly regulated by EBF. EBF and the E2A transcription factors appear to act together to regulate certain B-cell-specific genes, such as the components of the surrogate light chain, and binding sites for both EBF and E47 are present in the λ_5 promoter (Sigvardsson et al. 1997).

PAX5/BSAP

Expression of the *Pax5* gene, encoding the B-cell stimulatory/activatory protein (BSAP) protein, is regulated by the E2A and EBF transcription factors; EBF binds to a site in the *Pax5* promoter, and activates transcription. The BSAP protein is expressed in all committed B cells from the pro-B stage until activation of mature B cells; plasma cells do not express this transcription factor (Busslinger and Urbanek 1995). Interestingly, BSAP appears to function as both a transcriptional activator and a repressor: it activates genes for B-cell-specific proteins such as CD19 and the surrogate light chains, but represses genes associated with other hemopoietic lineages (Kozmik et al. 1992; Nutt et al. 1998, 1999; Wallin et al. 1998). This dual function is dependent on *Pax5* protein levels in the cell; transcriptional activatory sites have a higher affinity for *Pax5* than repressive sites (Wallin et al. 1998), and so the activation functions of this transcription factor can be carried out when the protein is present at lower levels than is necessary for repression. B-cell development in mice deficient in *Pax5* is blocked at the pro-B-cell stage (Nutt et al. 1997), i.e. slightly later in the differentiation pathway than is seen in the E2A$^{-/-}$ and EBF$^{-/-}$ mice; this is consistent with the regulation of *Pax5* by EBF. The role of *Pax5*/BSAP in B-cell commitment has recently been demonstrated in vitro, using pro-B cells from *Pax5*$^{-/-}$ mice. Under suitable culture conditions, these cells, which appear to be committed to the B-cell lineage, can differentiate into a variety of other hemopoietic lineages, including macrophages, granulocytes, and T cells (Nutt et al. 1999). Thus, expression of *Pax5* appears to be vital to allow very early B cells to commit irreversibly to the B lineage.

Thus, B-cell commitment and development are controlled by the sequential expression of a number of transcription factors that control the developmental pathway by activating those genes required for B-cell differentiation and repressing lineage-inappropriate genes.

Antigen-dependent B-cell development

Recirculating mature B cells in the periphery die after several weeks or months unless they encounter an antigen containing epitope(s) recognized by the cell's BcR. Numbers of mature B cells are maintained by the continual replacement of 'old' B cells by newly developed cells that have emigrated from the bone marrow.

B cells can be activated in either the presence or absence of help from T cells, depending on the type of antigen encountered. T-independent (TI) antigens can themselves provide the co-stimulatory signals required for B-cell activation, either through binding non-BcR cell-surface receptors that cause B-cell activation, or through extensive crosslinking of the BcR by highly repetitive antigens. TI antigens usually lead to low-affinity IgM responses. Mature B cells that are activated by T-dependent antigen in the presence of co-stimulatory signals undergo further differentiation, which allows the cells to:

- introduce mutations into their IgH and IgL chain genes to increase affinity for the antigen (somatic hypermutation)
- change the isotype of the antibody produced (class or isotype switching)
- become either antibody-secreting plasma cells or memory B cells.

This affinity maturation of B-cell responses occurs in specialized structures within lymph nodes known as germinal centers, and allows the humoral response to become faster and more efficient on future contact with the same antigen.

B-CELL ACTIVATION

Most immune responses require both contact between, and activation of, B and T cells recognizing the same antigen (although not necessarily the same epitope on that antigen). Binding of specific antigen by B cells leads to signaling through the BcR, which activates the B cell. In addition, the antigen–BcR complex is internalized by the B cell; the antigen is processed and then presented on the cell surface in association with MHC class II molecules. In the T-cell area of the lymph node, antigen-presenting B cells interact with antigen-specific CD4^{+} helper T cells, which have been primed by encounter with the antigen presented on MHC class II by interdigitating dendritic cells in the lymph node. Only B cells that interact with T cells that have been activated by the same antigen will go on to differentiate further.

The interaction of B cells with antigen-specific T cells involves a number of receptor–ligand pairs on the two cell types in addition to the MHC class II–T cell receptor interaction. Various adhesion molecule pairs, such as leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1), each of which is expressed on both B and T cells, contribute to stabilizing the interaction. These adhesion molecules can also transduce signals into the cells that contribute to B-cell activation, although the signaling pathway(s) used is not clear.

Perhaps the most important co-stimulatory interaction that takes place during B-cell activation is that of CD40 (on B cells) and its ligand (CD40L or CD154) on activated T cells and follicular dendritic cells (FDC) (Gordon and Pound 2000). Engagement of CD40 by its ligand promotes a variety of functions involved in enhancement of B-cell responses, including proliferation, antibody synthesis, cytokine production, and isotype switching. Once activated, B cells are predisposed to apoptose in the absence of survival signals at various checkpoints during antigen-dependent differentiation;

signaling though the CD40–CD40L interaction is an important survival signal for the cells and inhibits apoptosis of germinal center B cells in vitro. The importance of CD40-mediated signaling is underlined by the effects of a mutation in the gene for CD40L, which results in the immunodeficiency disease X-linked hyper-IgM syndrome (Kroczek et al. 1994). Patients with this disease have impaired antibody responses, mainly caused by an inability of B cells to undergo class switching and affinity maturation, resulting in overproduction of low-affinity IgM antibodies (see 'Mechanisms of somatic recombination' below). Additional signals are received by B cells through interactions between B-cell surface CD80 (B7–1) or CD86 (B7–2) and T-cell CD28 (Jeannin et al. 1997). These interactions are also important in the co-stimulation of T cells by signaling through CD28. CD80 and CD86 are expressed on centrocytes, but not on centroblasts.

Once B cells are activated by encounter with antigen-specific, activated, helper T cells, they can differentiate immediately into antibody-secreting plasma cells. These plasma cells will usually produce only low-affinity antibody, of the IgM isotype, but allow specific antibody to be secreted while other B cells undergo additional differentiation to produce higher-affinity antibodies of different isotypes. Those activated B cells that do not develop into plasma cells at this stage enter the primary follicle in the lymphoid tissue, adjacent to the T-cell area where they have been activated, and initiate the formation of a germinal center (Maclennan 1994). The germinal center consists of a dark zone containing centroblasts (B cells rapidly proliferating and undergoing somatic hypermutation) and a light zone containing centrocytes (B cells undergoing selection for high-affinity BcRs after somatic hypermutation has taken place).

SOMATIC HYPERMUTATION, CLASS SWITCHING, AND SELECTION

The process of somatic hypermutation introduces mutations into the IgH and IgL V(D)J regions; these are usually point mutations, although deletions and duplications are also occasionally found (Goossens et al. 1998). The mechanism by which mutation occurs is not well understood, and although upregulation of the RAG genes is found in germinal center B cells (Han et al. 1996), the type of gene rearrangement seen in earlier stages of B-cell development does not seem to occur. There is some evidence to suggest that mutations are introduced by a form of error-prone, DNA-repair system. Whatever the method used, the process of hypermutation introduces mutations into the V(D)J region at a rate of approximately 1 change per 10^3 base-pairs/cell per generation (McKean et al. 1984).

Introduction of mutations does not necessarily lead to increased affinity for the antigen; many mutated BcRs will have reduced affinity or will even become potentially autoreactive. Thus, some form of selection must operate to allow those B cells with increased affinity to survive, and those with reduced affinity or altered antigen specificity to be lost. As mentioned above, germinal center B cells are predisposed to apoptose in the absence of survival signals, and these survival signals are dependent on the ability of the BcR to interact with antigen and of the cell to present antigen to specific T-helper (Th) cells and receive survival signals from them (Liu et al. 1991b). Therefore, after somatic hypermutation has taken place, the centroblasts move from the dark zone of the germinal center to the light zone, where they become centrocytes and can interact with mature FDCs present in this area. These FDCs express a number of cell-surface receptors, including complement receptors and Fc receptors, which allow them to bind large quantities of immune complexes and retain them for long periods of time on the cell surface. Centrocytes compete for access to these immune complexes, resulting in rescue signals (e.g. through the CD40–CD40L interaction (Gordon and Pound 2000)) from the FDCs for only those centrocytes expressing the highest affinity BcRs. Those cells that are selected (i.e. those with high-affinity BcRs) are able to acquire and process the antigen from the FDCs and present it on class II MHC to T cells, receiving a further survival signal from the antigen-specific Th cells at this stage. These two stages of selection ensure that only cells with high-affinity BcRs, which recognize antigen that is also recognized by T cells, are allowed to survive passage through the germinal center; other cells die by apoptosis. This prevents the survival of centrocytes that recognize self-antigen after somatic hypermutation, because, even if the self-antigen is present on FDCs, no T cells specific for that antigen will be present.

As discussed earlier, antibody molecules can carry out different effector functions by utilizing different C regions in the *IgH* gene. Switching is dependent on engagement of B-cell CD40 by its ligand (Kawabe et al. 1994), and the production of different cytokines by Th cells which induce switching to specific Ig isotypes, e.g. activation of B cells in the presence of IL-4 leads to production of IgE (Th2-type response), whereas IFN-γ causes switching to IgG3 and $IgG2_2$ (Th1-type response). The molecular mechanisms underlying immunoglobulin isotype switching are discussed under 'Class switch recombination' below.

B cells that have undergone somatic hypermutation and class switching can either form high-affinity plasma cells in the lymph nodes or bone marrow, which secrete large amounts of specific, high-affinity antibody of the appropriate isotype for 2–3 weeks before dying, or become long-lived memory B cells. These memory B cells retain the antigen specificity and antibody isotype determined by their differentiation in the germinal center, and remain in the lymph node until they again encounter antigen. At this time, they become activated

more quickly than naïve B cells, allowing a faster response to antigen; they also have greater capacity for presenting antigen to T cells, through higher expression of MHC class II and co-stimulatory molecules such as CD80 and CD86.

IMMUNOGLOBULIN GENES AND ANTIBODY DIVERSITY

Antibody diversity

The adaptive immune system must be able to generate a very large number of different antibodies (or BcRs and hence B lymphocytes) in order to recognize all possible antigens that may be encountered by the host. The same is true of T-cell receptors (TcR). However, the size of the genome is finite, and so genetic mechanisms must exist to enable a large number of gene products (Ig chains) to be assembled from a limited number of genes. How is this achieved?

The pioneering experiments of Tonegawa and his colleagues demonstrated that the genetic elements encoding V and C regions of the polypeptide are located some distance from each other in the DNA of non-B cells, but are fused together in antibody-synthesizing B cells (Hozumi and Tonegawa 1976). Indeed, in the case of the genetic material encoding V-region sequences, multiple separate elements were shown to be involved. Thus, for κ chains, there are multiple V genes (250–300) and four functional J 'mini-genes'. V and J genes are separated by a great distance in germline DNA but, in a B cell, one V element is found joined to a single J element, giving a continuous unit of VJ information, i.e. V and J elements undergo somatic recombination. The joining of any V gene to any J gene (or DJ for heavy chains) results in deletion of all intervening DNA. A simple arithmetic calculation reveals that between 1000 and 1200 different VJ units can be formed by somatic recombination at the κ locus. A similar rearrangement of V and J information is observed at the λ locus (Figure 7).

At the heavy chain locus, there is an additional mini-gene family located between the V and J genes, called the D (diversity) genes. Two somatic recombination events are therefore required at the heavy chain locus to give a complete and active V gene. These occur in the order of one D and one J element being recombined to give a DJ unit, followed by fusion of this DJ gene to a single V gene to give a continuous *VDJ* gene (Coleclough et al. 1981). Clearly, therefore, the capacity to rearrange members of mini-gene families randomly greatly expands the final number of total genes and proteins available as antibodies or BcRs. Finally, somatic recombination is independent of antigen, and successful recombination on one of the two chromosomes bearing a given Ig gene prevents rearrangement on the other chromosome (allelic exclusion), thus conforming to the rules of the clonal selection theory.

V(D)J recombination: signals and rules

The recombination process is controlled by highly conserved nucleotide sequences flanking the genes to be rearranged. For V genes examination of sequences lying 3′ to the end of the coding elements revealed the presence of a conserved heptameric sequence linked by a spacer to a similarly highly conserved nonameric sequence. These are called recombination signal sequences (RSS). The size of the spacer between the heptanucleotide and nonanucleotide RSSs is always either 12 ± 1 or 23 ± 1 nucleotides, even though the sequence itself is not highly conserved. Indeed, recombination can occur only between mini-genes flanked by RSSs with different-sized spacers, i.e. one partner must have a 12-nucleotide spacer and the other a 23-nucleotide spacer. This is the so-called 12/23 rule. The recombination process is mediated by the RAG products, and is not exact, and shifts in the frame of recombination can increase diversity. In the case of fusion to the D genes, the enzyme terminal deoxyribonucleotidyl transferase can add nucleotides (usually G) to 3′-hydroxyl groups a nontemplate-directed manner; these are referred to as Nucleotide (N) regions. There are also small regions of palindromic repeats found at V(D)J joints and these are referred to as P sequences.

Mechanisms of somatic recombination

The V(D)J recombination process can be divided into four main steps: alteration of chromatin structure to allow access of the recombination machinery; recognition and binding of RSS; cleavage of DNA; and DNA repair and closure of joints. The joints formed are a 'coding joint', which brings together V and J sequences, and a signal joint which forms a closed circle containing the intervening DNA lost as a function of the recombination reaction. Signal joints are always precise, whereas coding joints are variable, a feature that is important in generation of antibody (and TcR) diversity. The proteins participating in V(D)J recombination are drawn from both lymphocyte-specific enzymes required for RSS recognition and cleavage and from ubiquitously expressed proteins involved in DNA repair and chromatin dynamics.

Highly condensed chromatin is likely to impede access of RAG complexes to Ig genes and associated RSS motifs (Roth and Roth 2000). Indeed, in in vitro assays mononucleosomes are able to inhibit the recombination reaction (Kwon et al. 1998; McBlane and Boyes 2000), largely by inhibiting the binding of the RAG complex to the RSS (McBlane and Boyes 2000; Kwon et al. 2000). Studies of histone acetylation have suggested that the

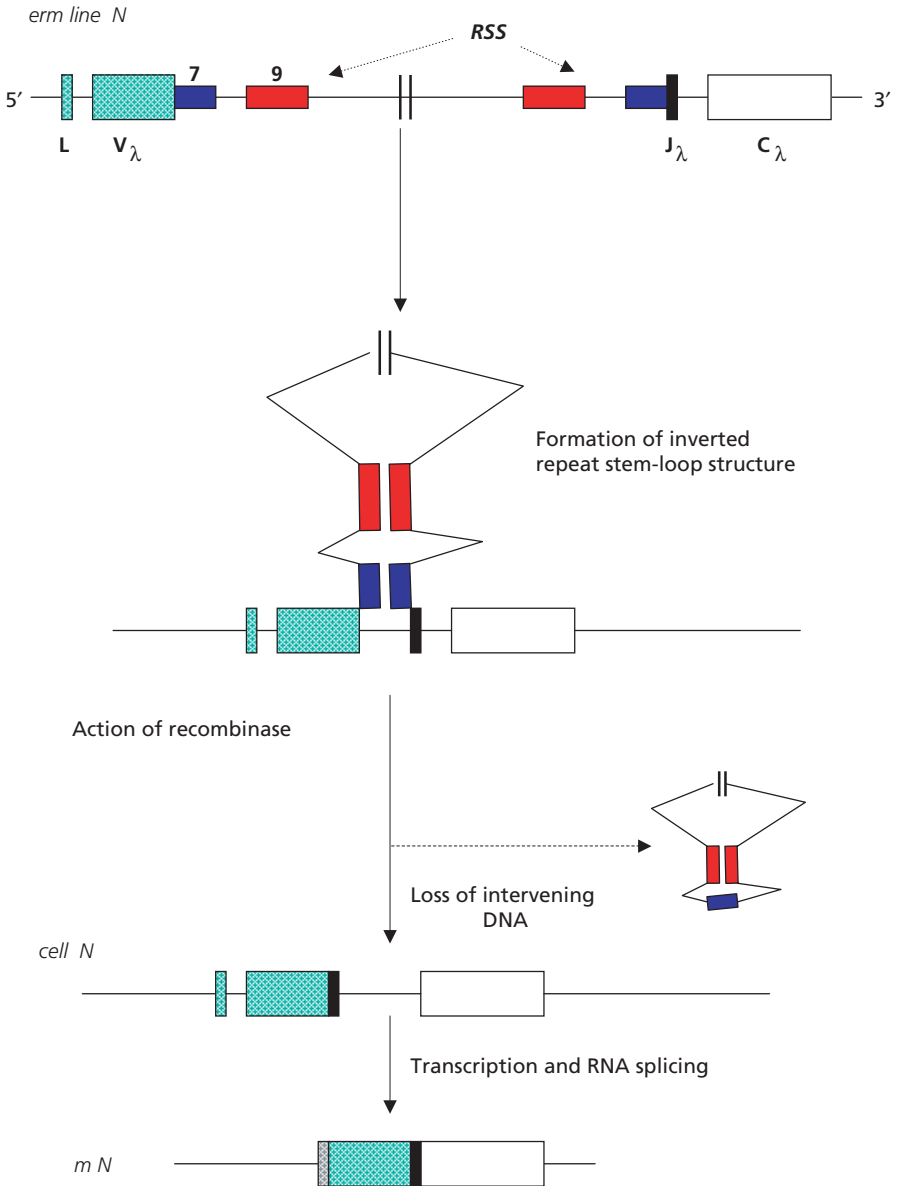

Figure 7 *Immunoglobulin λ chain gene rearrangement: the organization of the Igλ locus in germline and B-cell DNA is illustrated. Vλ and its associated leader peptide exon (L) are shown as stippled boxes, Jλ as a solid black box and Cλ as a solid white box. The heptameric and nonameric RSS are shown in blue and red, respectively. Intronic sequences are depicted by the thin line, and exons by boxes.*

level of acetylated histone H3 correlates with the degree of V(D)J recombination observed in vivo, indicating that local chromatin remodeling caused by acetylation favors recombination (McMurry and Krangel 2000). In many models, histone acetylation is greatly increased in those parts of the chromatin where transcription is viable.

Using the λ light gene as the model (see Figure 7), a large single-stranded stem-loop structure is formed by pairing of complementary heptanucleotide and nonanucleotide RSSs separated by 12 and 23 spacer sequences. This structure is bound by a tetrameric RAG1–RAG2 protein complex consisting of two molecules of each RAG (Bailin et al. 1999; Sadofsky 2001). RAG1 has demonstrable affinity for the nonanucleotide RSS (Difilippantonio et al. 1996; Mo et al. 1999), but it remains to be demonstrated unequivocally that RAG2 binds DNA (although this seems likely (Mo et al. 1999)). The enzyme complex introduces a single nick in each DNA strand, precisely cleaving the phosphodiester bond between the final base of the coding sequence and the first base of the heptameric RSS (McBlane et al. 1995). The free hydroxyl group at the 3′ of the coding strand now attacks the phosphodiester bond at the same position on the opposite strand, forming a closed hairpin loop at the coding joint. Plainly, hairpins are formed at both the V and the J coding joints, and the remaining

products are blunt and 5′-phosphate-bearing ends of the signal joint. The signal joints are precisely closed in a process requiring the RAG proteins, but, more importantly, the normal enzymes of DNA repair (Ku proteins, XRCC4, and DNA ligase IV) (Jeggo 1998).

In order for V-J joining to proceed, the hairpin loops must now be opened. As with signal joints, the RAG and Ku proteins appear to be mandatory for hairpin opening, but the process also requires DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the Artemis double-stranded DNA repair protein (Schlissel 2002). Artemis itself possesses a Mg^{2+}-dependent exonuclease activity but normally has no endonuclease activity. However, Artemis forms a complex, both in vivo and in vitro, with DNA-PKcs and is a substrate for the kinase activity of DNA-PKcs (Ma et al. 2002). In this complex, phosphorylated Artemis now expresses an endonuclease activity and is capable of opening hairpin loops using water as nucleophile for cleavage of target phosphodiester bonds. Support for the hypothesis that the Artemis–DNA-PKcs complex opens hairpins at V and J coding joints is provided by the rare human condition of radiation-sensitive SCID (RS-SCID). Fibroblasts from RS-SCID patients have a specific defect in V(D)J recombination in that no coding joints are formed, implying an inability to open hairpins; the sole genetic defect in RS-SCID is the Artemis enzyme activity (Moshous et al. 2001).

The action of the Artemis–DNA-PKcs complex opens the hairpin loops, leaving overhanging ends, generally of two to seven nucleotides (Livak and Schatz 1997; Schlissel 1998), bearing 3′-OH groups. These 3′-OH groups are the target for action of other enzymes that can add nucleotides via either template-dependent or nontemplate-directed mechanisms. The enzyme TdT adds bases to free 3′-OH groups in a nontemplate-directed manner, with a preference for guanine; these additional sequences are referred to as N regions. A second group of 'extra' nucleotides (i.e. bases not attributable to the germline V or J sequences) are the P nucleotides. These short stretches of up to four bases are palindromes of sequences at the end of coding joints and are thought to be generated by repair of overhanging ends during hairpin opening. Both N and P nucleotides contribute greatly to junctional diversity and, together with the inherent imprecision of V(D)J joining itself, are an important contribution to maximizing the possible range of antibodies that can be generated from a fixed number of germline mini-genes.

The final stage of VDJ recombination is the closing of the coding joint, and this proceeds by using the ubiquitously expressed enzymes involved in DNA repair and nonhomologous end joining (NHEJ). The key proteins involved are the XRCC4 protein in a complex with DNA ligase IV, as are the members of the Ku autoantigen family (Jeggo 1998). The overhanging ends of the opened hairpins will be repaired, either by exonuclease action to trim the ends or by polymerase action to fill the overhang with complementary nucleotides, before final joining of blunt ends. Any overhanging flaps that remain after trimming or in-filling are removed by flap endonucleases such as the fen endonuclease, or possibly by the RAG enzymes themselves. Ku binds exclusively to ends of DNA molecules in a sequence-independent manner; there are also no structural restrictions on Ku binding and so it can associate with blunt or overhanging ends (Smith and Jackson 1999). Binding of Ku allows other proteins to bind, including DNA-PKcs, and the XRCC4–DNA ligase IV complex and, indeed, Ku promotes the ligase activity of the latter complex (Jones et al. 2001). Recent structural analysis of Ku indicates that it can form a bridge over broken DNA in such a way that not only can it bind the free end of the DNA molecule (Walker et al. 2001; Jones et al. 2001), but it can also allow access of other proteins to the nucleotide strands to be joined and repaired. In V(D)J recombination, Ku is likely to serve to bind the blunt ends of the coding joints and recruit the XRCC4–DNA ligase IV complex to them (Ramsden and Gellert 1998; Nick McElhinny et al. 2000). It is the action of DNA ligase IV that ultimately forms the phosphodiester bond joining the V and J segments to yield a somatically recombined VJ unit.

A number of disease states provide compelling evidence for the involvement in the recombination process of the various proteins discussed above. Thus, there is ample evidence from knock-out mice that absence of *RAG-1* or *RAG-2* genes causes severe immunodeficiency usually manifest as a lack of both B and T cells, and the same is true of human RAG null mutations. However, RAG mutations that result in defective but not inactive proteins give rise to Omenn's syndrome (Villa et al. 1998), an immunodeficiency that is characterized by a lack of B cells and the presence of some T cells (Noordzij et al. 2000). As noted above, Artemis deficiency results in an unusual SCID disease (RS-SCID) and, intriguingly, the original *scid* mice accumulate unopened hairpin coding joints and rarely make coding joints (Roth et al. 1992); the defective gene in *scid* mice encodes DNA-PKcs (Schlissel 2002). Deficiencies in any of the Ku family proteins or in XRCC4 or DNA ligase IV all give rise to striking immunodeficiencies of differing levels of severity, usually coupled with defective DNA-repair and developmental abnormalities in a range of tissues. The data from disease states therefore underscore the importance of both lymphoid-specific and ubiquitous protein factors in V(D)J recombination.

Class switch recombination

Immunological memory in the humoral response is reflected in the production of higher affinity antibodies of non-IgM isotypes (e.g. IgG). The *Igh* locus comprises

the V, D, and J gene clusters, plus groups of exons encoding the individual domains of particular heavy chains (Figure 8). Increased affinity is a function of somatic hypermutation of V region sequences and selection of antigen-mediated selection of high affinity clones in germinal centers (see 'Antigen-dependent B-cell development' above). Isotype switching at the level of the DNA results from movement of the *VDJ* gene from its original position just 5′ to the Cμ gene to a new position immediately 5′ to, for example, the Cγ gene. All intervening DNA is deleted during this rearrangement process, called switch recombination, but the molecular signals are different from those controlling VDJ and VJ recombination events (Harriman et al. 1993; Kinoshita and Honjo 2001). The exception to this is the simultaneous production of mIgM and mIgD by mature B cells. The synthesis of the δ heavy chain does not involve switch recombination, but is a result of RNA splicing of a long transcript containing *VDJ*, all the Cμ information, and the Cδ gene (Blattner and Tucker 1984).

The 5′-flanking region of each C_H gene cluster contains a 'switch' recombination region (or S region). Unlike V(D)J recombination, there are no tightly defined DNA sequences for the S regions, although these areas do contain many tandemly repeated and palindromic sequences. As class switching always involves changing from synthesis of IgM (and IgD) to a second isotype located 3′ to the Cμ exons, it follows that Sμ is mandatory for pairing with downstream S regions of selected C_H exons. Indeed, deletion of the Sμ region greatly impairs class switching (Luby et al. 2001), and deletion of individual S regions blocks switching to the associated isotype (Gu et al. 1993; Jung et al. 1993; Kinoshita et al. 1998). As indicated in 'Antigen-dependent B-cell development', cytokines direct class switching and, at the molecular level, this is reflected in local alterations in chromatin structure. Thus, IL-4 promotes IgE synthesis and, in B cells undergoing such switching, IL-4 promotes synthesis of a sterile transcript comprising the Iε region plus the four Cε exons, indicative of a local opening of chromatin structure (Stavnezer-Nordgren and Sirlin 1986; Yancopoulos et al. 1986). Transcription is required for class switching to occur. Similarly, transforming growth factor β stimulation results in transcription of the Iα and Cα domains (Nakamura et al. 1996).

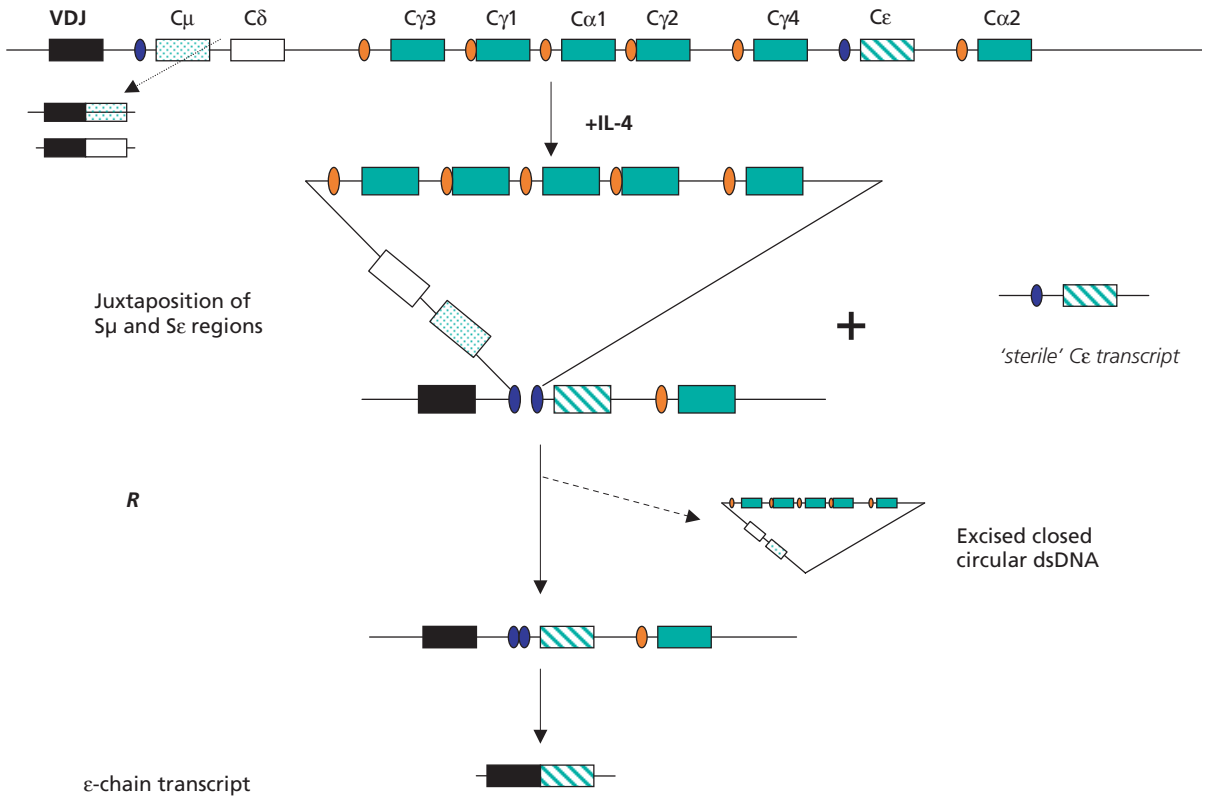

Figure 8 *Immunoglobulin isotype switching: the layout of the human* Igh *locus is shown and the S sequences at each* C_H *gene are illustrated as small red circles except those of Sμ and Sε which are blue. The figure depicts switching from IgM(D) synthesis (white and stippled boxes) to IgE production (hatched box) under the influence of interleukin 4 (IL-4) showing movement of the somatically recombined* VDJ *unit from 5′ of Cμ/Cδ to just 5′ of Cε, with concomitant loss of intervening DNA. Sterile and productive transcripts from the Ig locus during the isotype switch process are shown.*

Once the target C_H gene has been chosen (e.g. Cε), the Sμ and Sε regions are brought into close proximity so that the components of the recombination system can act. The putative switch recombinase cleaves the DNA, joins the S regions together (so bringing the *VDJ* unit close to the Cε exons), and removes the intervening DNA as a looped-out, closed, circular DNA (see Figure 8). The absence of defined 'switch recombination sequences' suggests that the recombinase machinery might recognize higher structural features of the DNA rather than sequence elements (Kinoshita et al. 1998; Tashiro et al. 2001). Current models suggest that the feature recognized is a single-stranded stem-loop structure not dissimilar to that recognized in V(D)J rearrangement. Such structures are favored by the presence of short inverted repeat sequences, such as those found in S regions.

Analysis of the junction regions in switch recombination products indicated the presence of both deletion of DNA sequences and duplication of others at the junctions (Lee et al. 1998; Kinoshita and Honjo 2001). These data indicate that the recombinase machinery introduces staggered cleavages or nicks into the two strands of DNA. Such staggered cleavage products could be acted on by either single-stranded nucleases or error-prone polymerases (e.g. TdT) to repair the ends before closure by the same NHEJ DNA-repair machinery that seals V(D)J joints.

Not only is transcription of the targeted C_H gene required for isotype switching, but *de novo* protein synthesis is also mandatory (Muramatsu et al. 1999), indicating the requirement for transcription and translation of new gene products during the switching process. One such gene is the AID product, whose expression is limited to B cells and, more importantly, to those B cells in germinal centers (Muramatsu et al. 1999). Over-expression of AID enhances class switching in in vitro models, and mice lacking the gene ($AID^{-/-}$) fail to produce IgG, IgA, or IgE, indicating that class switching has been abolished. Moreover, $AID^{-/-}$ animals show essentially no somatic hypermutation in response to immunization, suggesting that AID has a role in both class switching and somatic hypermutation. In humans, the hyper-IgM syndrome (HIM) manifests itself by over-production of IgM and a lack of other Ig isotypes. There are two forms of the disease HIM1: an X-linked condition linked to a failure to produce CD40L (Gordon and Pound 2000), and HIM2, an autosomal recessive condition mapping to 12p13, the location of the AID gene (Revy et al. 1998). $AID^{-/-}$ (Muramatsu et al. 2000) and HIM B cells (Revy et al. 2000) can perform VDJ recombination normally and, on cytokine stimulation, synthesize 'sterile' transcripts, suggesting that AID is required for the latter stages of switch recombination. This is entirely consistent with the cytidine deaminase activity of both murine and human AID (Muramatsu et al. 1999), a function that suggests a potential role for AID in RNA editing.

BcR heavy chains and Ig synthesis

Clonal selection requires the receptor Ig to have the same structure as the antibody that the B cell will later secrete. B cells simultaneously synthesize discrete mRNAs that program the synthesis of the structurally distinct receptor and secretory forms of their heavy chain polypeptide (Singer et al. 1980). This is again accomplished by RNA splicing, in this case by selective use of two small exons located at the 3′ end of the C gene for each heavy chain isotype, which encode hydrophobic sequences allowing stable insertion into the plasma membrane; these are the M (membrane) exons (Alt et al. 1980). Heavy and light chain mRNA molecules are synthesized on distinct membrane-bound polyribosomes and, in common with all secretory and membrane proteins, the nascent chains are guided across the endoplasmic reticulum membrane by a hydrophobic sequence, the signal peptide, which is proteolytically removed during transit across the membrane. Glycosylation of the nascent chain occurs co-translationally and assembly of four-chain units occurs in the lumen of the endoplasmic reticulum; in the case of IgM and IgA molecules, polymerization, and addition of J chains are late events, probably occurring immediately before the final secretory event. Ig synthesis proceeds in different B cells at distinct rates that reflect the state of differentiation of the B cell. Thus, a small resting B cell synthesizes 10^6 Ig molecules per day, mostly for use as receptor Ig, whereas a plasma cell can produce up to 2000 Ig molecules per second, essentially all of which is of the secretory form.

The active Ig gene is transcribed into a primary nuclear RNA (nRNA) transcript that contains not only the coding elements (exons) for V and C regions but also all the noncoding sequences (introns). Introns are found between the recombined VJ or VDJ unit and its appropriate C gene and, in the case of heavy chains only, between the exons that encode the individual domains of the heavy chain polypeptide. Introns are removed by RNA splicing to yield an mRNA molecule where all the V and C information is continuous. This is translated to yield the functional Ig polypeptide.

IMMUNOTECHNOLOGY: GENERATION AND USES OF MONOCLONAL ANTIBODIES

The specificity of antibodies makes them excellent tools for use in diagnostics and therapy. The critical advance that revolutionized the diagnostic and therapeutic application of antibodies was the development of MAbs (Kohler and Milstein 1975, 1976; Kohler et al. 1976). In short, this technology allows the establishment of an immortal cell line that secretes a single antibody of defined specificity. In practical terms, the procedure

exploits the enormous specificity present in a sample of normal lymphocytes and the capacity for infinite growth found in B-cell tumors.

In practice, normal lymphocytes from an immunized host are fused to continuously growing tumor cells in vitro to make a hybrid cell. The tumor cells are derived from plasmacytomas, a tumor of myeloma cells, and so have a highly developed secretory apparatus; sublines are generally chosen on the basis of an inability to synthesize their own heavy and light chains. As normal murine B lymphocytes are highly prone to apoptosis in vitro, any that do not fuse to the tumor cells die after a few days in culture. The tumor cells used in the fusion are chosen on the basis of a defect in nucleotide metabolism (a lack of the enzyme hypoxanthine–guanine phosphoribosyl transferase (HGPRT)), which means that addition of nucleotide analogues to the cultures will selectively cause death of the tumor cells. The normal lymphocytes possess HGPRT activity and when fused to a tumor cell contribute both their antibody specificity and their normal nucleotide metabolism to gain the immortal properties of the myeloma. It is this combination that allows only the hybrid cells to survive in the presence of the selecting drug. The hybrids, called 'hybridomas', can be cloned at limiting dilution and screened for the desired MAb specificity. Once a suitable clone is identified, it can be grown in large cultures, and gram quantities of homogeneous antibody produced.

Monoclonal antibodies have found many applications in medicine. Perhaps the most obvious example of an immunodiagnostic product is the pregnancy testing kit available at most pharmacists which employs MAbs to human chorionic gonadotropin (hCG) and yields a definitive result in under 5 min. In this system, MAbs to two distinct epitopes on the hCG molecule are generated, one for 'capture' and the second for detection. The capture MAb is immobilized on a plastic surface and binds any hCG present in a morning urine sample. In contemporary kits, the flow of fluid solubilizes the second MAb, directed against a distinct hCG epitope. This second MAb has an enzyme coupled to it that will convert a colorless substrate also present in the solubilized material into an insoluble product that precipitates around the zone where the first MAb is immobilized. In practice, this is visualized as a colored line. An insoluble precipitate can form only if the second MAb is retained in the 'sensor' area, an event that can occur only if hCG is itself captured by the first MAb. MAbs specific for a range of cell-surface markers are used in the diagnosis of leukemia (e.g. the presence of CD10, or c-ALLA, on cells derived from acute lymphoblastic leukemia patients, or the $CD5^+/CD23^+$ phenotype of B-chronic lymphocytic leukemia cells), and for accurate quantitation of analytes in tissue samples.

The application of MAbs in vivo as a therapeutic agent faces the hurdle of hypersensitivity reactions in the patient. Thus injection of murine Ig into an individual will provoke an anti-murine IgG response that will cause severe immune complex disease on subsequent exposure to the murine MAb (see Airway hypersensitivity and Autoimmunity). To overcome this, murine MAbs can be humanized by 'grafting' the CDRs from a useful murine MAb on to the backbone of a human IgG molecule. A similar strategy can be used to take useful sequences from antibody-combining sites selected by phage display methods, to generate a therapeutically useful product (e.g. the b12 IgG1κ molecular discussed earlier). These molecular manipulations go some way to alleviating the potential problems of host hypersensitivity, but this remains a significant factor in management of patients treated with MAbs.

Immunotherapeutics using antibodies envisages infusion of MAbs, either alone or coupled to radionuclides or potent biological toxins, as a means of eliminating tumor cells or alleviating other conditions (see Monoclonal antibody therapy). Several MAbs have met with notable success in the treatment of neoplastic and other diseases, although it is fair to note that the precise in vivo mechanisms by which they provide clinical benefit remain unclear. In allograft patients, a particular problem is rejection of the grafted tissue by host T cells. Two humanized MAbs, basiliximab and daclizumab, directed against the CD25 molecule (a component of high-affinity IL-2 receptors (Cushley et al. 2002)) have been used to suppress acute rejection in renal allograft patients (Waldmann 2000). The MAbs appear to function by blocking the capacity of IL-2 receptors to send mitogenic signals to host T cells, thereby blocking the early proliferative phase of the T-cell response to the allograft. Neither MAb elicits severe side effects in the patients, and daclizumab is notably effective when used in conjunction with immunosuppressive drugs such as cyclosporin. In the context of neoplasia, rituximab, an anti-CD20-specific reagent is widely used in treatment of non-Hodgkin's lymphoma and other B-cell neoplasias (McLaughlin 2002), with encouraging results. As the MAb appears to act by reducing (neoplastic) B-cell numbers, there are also suggestions that rituximab might be applied to eliminate or reduce the high levels of $CD20^+$ autoreactive B cells in patients with inflammatory autoimmune disease. Finally, in the specific case of rheumatoid arthritis, infliximab, an anti-tumor necrosis factor reagent, is used successfully to ameliorate disease, and finds application in other inflammatory disorders, including Crohn's disease (Graninger and Smolen 2002).

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The B-cell antigen receptor

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The major function of B lymphocytes is to make antibodies. The control of antibody production is a complex process, but a central element of that control, as initially enunciated by the clonal selection theory, is that contact of antigen by the B-cell antigen receptor (BCR) promotes activation of the B cell, which, if coming in the presence of sufficient additional signals such as those produced by helper T cells, leads to the production of antibodies.

Studies in recent years have revealed that the BCR plays a pervasive role in B-cell biology, driving nearly every step in the development of B-lineage cells into mature B cells, in addition to being a critical element in the triggering of B-cell activation. Also, the BCR plays a key role in promoting B-cell survival at several stages of B-cell life. These functions of the BCR are summarized in Figure 1.

STRUCTURE AND SYNTHESIS OF THE BCR

The BCR is a complex between the membrane form of immunoglobulin (mIg) and two signaling chains, called Igα and Igβ (or CD79a and CD79b) (Figure 2). Recognition of antigen by the mIg unit of the BCR leads to signaling via the cytoplasmic domains of Igα and Igβ.

The membrane and secreted forms of Ig made by a particular B cell differ only at the carboxy-terminal end of the heavy chain. This is achieved by differential transcriptional termination and RNA-processing events, giving rise to a distinct mRNA for each type of heavy chain. As a result, individual B cells have the same specificity in their antigen receptors and in the antibody that will be secreted after B-cell activation and terminal differentiation to the plasma cell state.

The mRNA encoding the secreted form of the heavy chain is made by usage of the first of two polyadenylation sites. Usage of this site occurs when transcription terminates before the two exons that encode the transmembrane and cytoplasmic exons. This early termination gives rise to an mRNA that encodes the secretory form, whereas usage of the second polyadenylation site permits RNA splicing which cuts out the sequence that is found only in the secretory form and also removes stop codons (Calame 2001). RNA splicing of this longer transcript produces mRNA encoding the membrane form of the heavy chain. The membrane forms of heavy chains contain at their carboxyl terminus a transmembrane domain and a very short cytoplasmic domain, ranging from 3 to 28 amino acids, depending on the isotype of heavy chain (Reth 1992). Whereas naïve B cells make primarily the membrane form of Ig, terminal differentiation of B cells into high rate antibody-secreting cells (plasma cells) leads to synthesis of much more of the secretory form and less of the membrane form, thereby leading to production of secreted antibodies.

Although the short (three amino acids) cytoplasmic domain of mIgM and mIgD is not known to have any function other than positioning the transmembrane domain relative to the membrane, the longer cytoplasmic domains of the switched isotypes could engage signaling components. In transgenic mouse experiments,

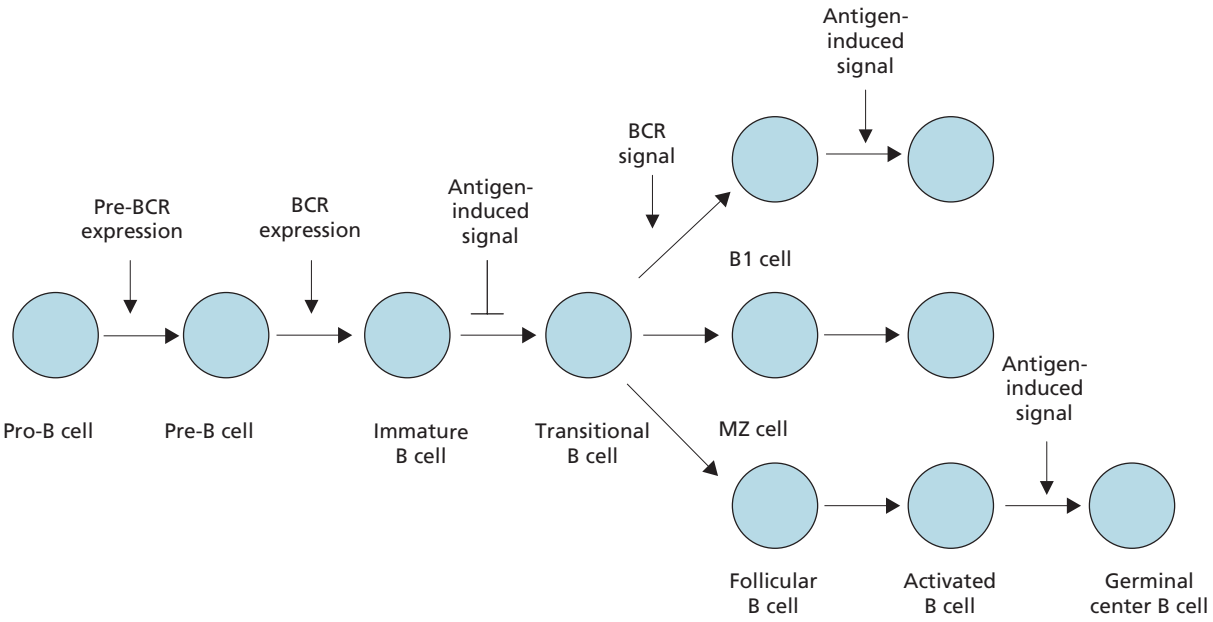

Figure 1 *Regulation of B-cell development, survival, tolerance, and activation by the B-cell antigen receptor (BCR). Shown are stages of B-cell development from the pro-B-cell stage (μ chain negative) to the three mature B cell types, B1 cells, marginal zone B cells and follicular B cells. Signals from the pre-BCR or BCR, either generated at a low level as a result of expression ('tonic' signaling) or induced by contact with a self or foreign antigen, regulate the steps shown. Arrows indicate stimulation of the step shown, whereas a perpendicular line indicates inhibition. Low-level BCR signals from weak responsiveness to self-antigens are probably required for maturation of all three B-cell types from transitional B cells, as well as their survival (not shown). Stronger BCR signals ('antigen-induced signal') promote activation of mature B cells, as well as entry of follicular B cells into germinal center reactions and survival of centrocytes.*

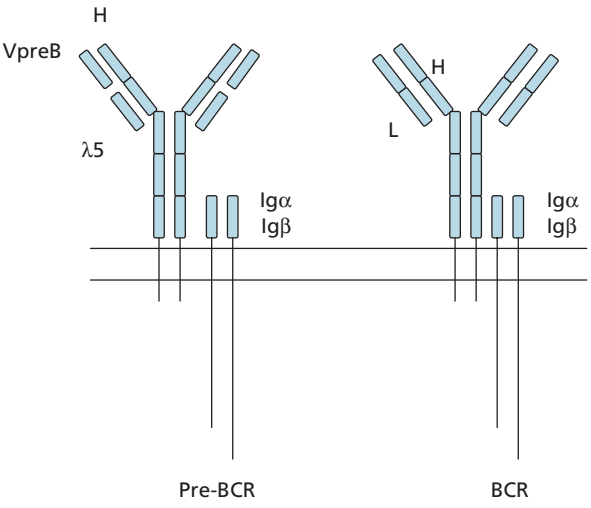

Figure 2 *Structure of the pre-BCR and the B-cell antigen receptor (BCR). The pre-BCR consists of a pair of identical μ heavy chains, each of which is bound to the two surrogate light chains, Vpre-B1 and λ5, and to a disulfide-linked heterodimer of Igα and Igβ. The cytoplasmic domains of these latter two molecules have ITAM sequences, which are primarily responsible for mediating signaling. The BCR is a similar structure, with rearranged κ or λ light chains instead of surrogate light chains. In mature B cells, BCRs with μ or δ heavy chains are found and isotype-switched B cells have BCRs with γ, α, or ε heavy chains complexed with light chains and Igα/Igβ.*

anti-lysozyme IgG1 transgenic mice were found to give rise to much larger T-cell-dependent antibody responses than equivalent IgM transgenic mice as a result of a greater proliferative burst and enhanced plasma cell differentiation. These properties could be conferred by the transmembrane domain plus the cytoplasmic tail of the γ1 heavy chain fused to the extracellular domain of the μ heavy chain (Martin and Goodnow 2002). It seems likely that this functional effect depends primarily on the cytoplasmic domains, because gene-targeted mice, in which there has been a deletion of the IgG1 cytoplasmic tail (Kaisho et al. 1997) or the IgE cytoplasmic tail (Achatz et al. 1997), show considerably decreased antibody production of those isotypes. Thus, the longer cytoplasmic tail of the isotype-switched Ig heavy chains promotes B-cell proliferation, survival, and antibody production and may account for the hallmark properties of memory B cells.

The pre-BCR and B-cell development

Pre-B cells have a signaling receptor related to the BCR called the pre-BCR. This receptor is a complex of Igα/Igβ, Ig heavy chain, and two polypeptides that take the place of light chain and hence are called surrogate light chains (see Figure 2). These two polypeptides, called λ5 and Vpre-B, each has an Ig domain related to light chain C and V regions, respectively. The pre-BCR plays

a critical role during B-cell development by generating a signal to indicate the presence of a properly rearranged heavy chain. When an IgH gene rearrangement occurs that creates an in-frame μ heavy chain gene, this leads to synthesis of Ig heavy chain and formation of the pre-BCR, which then sends a signal inducing further developmental progression toward the B-cell state (see Figure 1).

The function of the pre-BCR has been extensively studied by targeted gene mutations in mice (Kurosaki 2002a; Meffre et al. 2000). Ablation of the IgH locus or of the *rag1* or *rag2* genes required for V(D)J recombination causes a complete block in B-cell development in which $B220^+$ ($CD45R^+$) B-cell precursors fail to progress beyond the pro-B-cell stage, characterized as being positive for the S7 epitope of the CD43 molecule. This epitope is created by a specific carbohydrate modification of the CD43 polypeptide backbone (Shiota et al. 1994). Pro-B cells also have the functional property that they will proliferate in contact with suitable stromal cells derived from bone marrow, but will not proliferate in response to interleukin 7 (IL-7) in the absence of contact with stromal cells. Expression of a properly rearranged μ heavy chain and subsequent pre-BCR signaling induces enhanced responsiveness to IL-7 (Reichman-Fried et al. 1993; Fleming and Paige 2001). This developmental transition is also characterized by expression of CD25 (the α chain of the IL-2 receptor), and loss of expression of c-kit, a transmembrane tyrosine kinase receptor for a hemopoietic growth factor. Pre-BCR signaling also retargets the V(D)J recombinase away from the IgH locus and toward the IgL loci (Schlissel and Stanhope-Baker 1997). This is important for insuring that a given B cell expresses only a single Ig heavy chain, a phenomenon referred to as allelic exclusion.

Other mutations that affect pre-BCR assembly or signaling lead to impairment in the developmental transition from pro-B cell to pre-B cell (Figure 3). These include mutations of the IgH locus or of λ5, combined deletion of the cytoplasmic domains of Igα and Igβ (Kraus et al. 2001), and mutations in a variety of signaling components (Kurosaki 2002a; Meffre et al. 2000). The last mutations are discussed in greater detail below. Individual deletions of the cytoplasmic domain of Igα or Igβ have partial, but relatively mild, phenotypes, indicating a redundancy in the signaling capabilities of these two BCR-associated proteins.

BCR sequences that mediate intracellular signaling

The BCR complex forms in the endoplasmic reticulum, where mIg and Igα/Igβ subcomplexes are retained by chaperones until assembly occurs. Assembly allows the signaling-competent BCR to traffic to the cell surface. Igα and Igβ each contain an amino-terminal single Ig domain extracellular domain, a transmembrane domain,

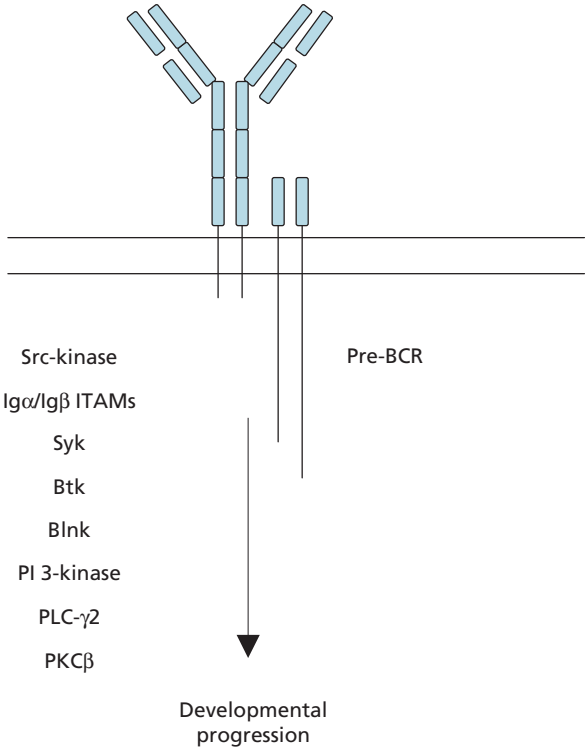

Figure 3 *Signaling events required for pre-BCR signaling. Genetic experiments have probed the signaling events required for the pre-BCR to induce developmental progression from the pro-B cell stage to the pre-B cell stage. Signaling components or sequences required for this transition are shown. In addition, μ heavy chain is required as is the λ5 surrogate light chain.*

and a moderate length cytoplasmic domain (about 50 amino acids long) containing immunoreceptor tyrosine-based activation motif (ITAM) sequences. ITAMs are found also in subunits of the T-cell receptor (TCR), the activating Fc receptors, and activating natural killer (NK) and myeloid cell receptors, and are responsible for the signaling functions of these receptors. Signaling is initiated by phosphorylation of the tyrosines in the ITAM motifs, as described in greater detail below. Targeted mutations of mice that mutate ITAM tyrosines of Igα or Igβ lead to defects in B-cell development, presumably as a result of defects in signaling by the pre-BCR and/or the BCR (Kraus et al. 2001; Kurosaki 2002a).

Higher order structure of the BCR

Recent studies have indicated that the BCR exists in the B-cell membrane as a higher oligomer of the basic $H_2L_2Ig\alpha/\beta$ unit (Schamel and Reth 2000; Matsuuchi and Gold 2001; Reth 2001). The number of units within these higher-order structures has not been defined, but unliganded BCR complexes are submicroscopic and thus are probably relatively small oligomers. These oligomers are composed of single isotypes of mIg (e.g. individual oligomers contain all mIgM or all mIgD on a naïve B cell expressing both IgM and IgD).

The functional consequence of the oligomeric structure of the BCR has likewise not been clearly defined, but there are two leading hypotheses. The first is that these oligomers hold the BCR in a conformation that sends a tonic signal which promotes B-cell developmental events and survival of mature B-cell types (see Figure 1) (Reth 2001). According to this hypothesis, antigen binding distorts these oligomers and thereby results in a qualitative change in the nature of BCR signaling events. This qualitatively different BCR signal would promote activation of mature B cells or deletion of transitional B cells. The clearest precedent for this model is distortion of IgM pentamers on antigen binding which initiates the complement cascade. However, at this point there is little evidence for two qualitatively distinct modes of BCR signaling.

The alternate hypothesis also holds that, in the absence of antigen, there is a low level of tonic BCR signaling which mediates various developmental transitions and survival of mature B cells. However, this hypothesis postulates that antigen induces clustering of BCRs, which triggers a higher level of the same signaling events. The latter hypothesis has been the more traditional view of how BCR signaling is triggered and in general can explain many of the phenomena regarding the relationship between the oligomeric state of antigens and their ability to induce stronger or weaker immune responses, e.g. antigens able to crosslink the BCR strongly (e.g. capsular polysaccharides of bacteria and capsid proteins of highly ordered virus particles (Mond et al. 1995; Bachmann and Zinkernagel 1996)), can induce antibody responses in the absence of helper T cells, whereas most protein antigens, which have a lesser potential for inducing BCR clustering, require T-cell help to generate an antibody response.

The clustering hypothesis is compatible with the idea that the BCR pre-exists in higher-order oligomers, although clearly it does raise questions about why the oligomers signal at only a low rate, whereas further clustering triggers a much higher amount of signaling. This issue may relate to lipid raft localization, which is triggered by clustering of the BCR with a ligand (see below). Moreover, given the complexity of BCR signaling events (DeFranco 1997; Kurosaki 2002b) and the observations that some signaling events participate in some biological responses to the BCR and not others (Richards et al. 2001), it may be that tonic signaling generates a subset of the signaling reactions induced by antigen-induced clustering, e.g. the signals may have some qualitative differences in addition to quantitative differences.

According to the clustering hypothesis for BCR signaling, what is the purpose of the higher-order oligomeric structure of the BCR before antigen encounter? It is likely that clustering of oligomers instead of monomeric BCR units ($H_2L_2Ig\alpha/\beta$) would amplify the signal at low doses of antigen as clustering would bring together oligomers, each with multiple Igα/β signaling modules (Matsuuchi and Gold 2001). Indeed direct evidence for this possibility has been obtained in experiments using coexpression of two distinguishable forms of Igα, one of which had an external FLAG-epitope tag (Schamel and Reth 2000). Stimulating only the FLAG-tagged BCRs by using an anti-FLAG antibody revealed tyrosine phosphorylation not only of the FLAG-tagged Igα molecules, but also of the untagged Igα molecules. Presumably this is because oligomers contain both types of Igα molecules. Thus, BCRs that are not directly bound by the stimulating antibody are activated to signal.

BCR SIGNALING

Antigen-induced clustering of the BCR leads to rapid tyrosine phosphorylation of the ITAM tyrosines by the Src family tyrosine kinases expressed in B cells, Lyn, Fyn, and Blk. Why this occurs is not clearly established, but it may relate to the microdomains of the plasma membrane referred to as lipid rafts. Lipid rafts are enriched in cholesterol, sphingolipids, and a variety of lipid-modified proteins, all of which associate with one another in a dynamic fashion to form subdomains that vary in size from about 70 nm in diameter to almost 1 μm (Pierce 2002). In unstimulated B cells, these structures are relatively small and dispersed, although they may account for as much as 30 percent of the plasma membrane surface area. Lyn, Fyn and Blk are highly enriched in lipid rafts, whereas before stimulation the BCR is found in the nonraft region of the bilayer. Upon stimulation, the BCR rapidly enters or associates with lipid rafts, and this association apparently does not require phosphorylation of the ITAMs of the BCR (Cheng et al. 2001).

Why antigen-induced multimerization of the BCR induces it to associate with lipid rafts is not known. One hypothesis is that the BCR has a weak affinity for lipid rafts as an oligomer, and multimerization increases that interaction, making it more stable. An argument against this possibility is that the association of antigen-ligated BCRs with lipid rafts occurs in mature B cells but not in immature B cells (Sproul et al. 2000; Chung et al. 2001), suggesting that there is some way of regulating this association. A second hypothesis is that a small subset of BCR molecules are preassociated with lipid rafts and multimerization brings non-lipid draft BCRs together with raft BCRs, with the resulting aggregate now associated with rafts. In this case, a regulated post-translational modification or protein–protein association could be responsible for directing some BCRs to lipid rafts before stimulation.

Activation of protein tyrosine kinases by the BCR

A major consequence of BCR association with lipid rafts is likely to be a closer proximity of the BCR and Src

family tyrosine kinases located in the lipid rafts and hence increased tyrosine phosphorylation of the ITAMs. Phosphorylation of both tyrosines of a single ITAM triggers the binding of a second type of intracellular protein tyrosine kinase, Syk (Figure 4). This binding is mediated by association of the two SH2 (Src homology 2) domains of Syk with the doubly phosphorylated ITAM sequence. The ITAM sequence is essentially a tandem SH2-binding site, with the amino acids between the two SH2-binding motifs (Y-PxxL/I where Y-P means a phosphorylated tyrosine) extending a sufficient distance to permit the ITAM to bind simultaneously to both SH2 domains of one Syk molecule, as demonstrated by structural studies (Futterer et al. 1998).

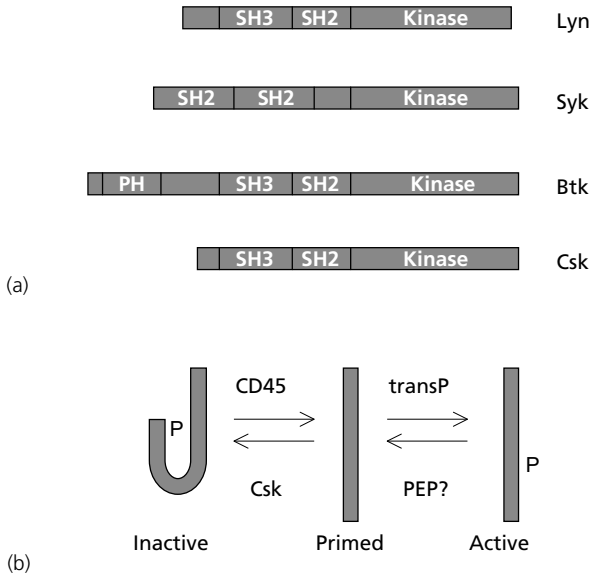

Figure 4 *Tyrosine kinases involved in B-cell antigen receptor (BCR) signaling.* **(a)** *Structures of the four major types of intracellular protein tyrosine kinases that participate in or regulate BCR signaling. Lyn, Fyn, and Blk are members of the Src family of tyrosine kinases, which have amino-terminal unique sequences with lipid attachment sites (myristate in all cases and palmitate in the cases of Lyn and Fyn). The unique region is followed by Src homology 3 (SH3) and Src homology 2 (SH2) domains, a kinase domain, and a negative regulatory tyrosine near the carboxyl terminus (not shown). Syk, like ZAP-70 has two SH2 domains, an interdomain region with sites of regulatory tyrosine phosphorylation, and a kinase domain. Btk, a Tec family kinase, has an amino-terminal pleckstrin homology (PH) domain that mediates regulated binding to membranes via PIP_3, followed by SH3, SH2, and kinase domains. Csk is responsible for phosphorylating the negative-regulatory site on Src family kinases and has a structure similar to Src, but lacking the amino-terminal fatty acylation sites and the negative regulatory tyrosine.* **(b)** *Regulation of Src family tyrosine kinases by tyrosine phosphorylation. Csk phosphorylation of the carboxy-terminal negative regulatory site of Lyn converts it to an inactive state in which the SH2 domain is bound to the C-terminal tyrosine phosphate and the kinase domain is inactive. CD45 is responsible for removing the carboxy-terminal tyrosine phosphate. Lyn lacking the carboxy-terminal phosphate is in a primed but low activity state. Transphosphorylation of a tyrosine residue in the activation loop of the kinase domain leads to the fully active state. The tyrosine kinase PEP is a leading candidate for removing this activating phosphorylation and it forms a complex with Csk, suggesting coordinate regulation converting active Src family kinases to the inactive state. This inactivation may occur via a feedback inhibition mechanism, because Src family kinases apparently are responsible for phosphorylating Cbp, which recruits Csk to the membrane.*

Once Syk has docked on to the oligomerized BCRs in the lipid rafts, it becomes activated. The binding of an ITAM to the Syk SH2 domains appears to be partly responsible for this activation by allosteric means (Rowley et al. 1995; Shiue et al. 1995). In addition, Syk is activated by tyrosine phosphorylation of a tyrosine in the kinase domain activation loop (Kurosaki et al. 1995). Syk activity appears to increase by at least 10-fold and, of course, Syk activity is also localized by its association with the BCR. Indeed, fluorescence microscopy demonstrates a dramatic movement of Syk from the cytosol to the plasma membrane after BCR crosslinking (Ma et al. 2001), and this association is primarily associated with lipid raft subdomains of the plasma membrane (Gupta and DeFranco 2003). Similarly, a dramatic increase in tyrosine phosphorylation of proteins can also be seen to occur coincident with lipid raft microdomains. Thus, BCR signaling appears to be occurring primarily in or adjacent to lipid rafts.

In addition to the activation of Syk, there is also a rapid increase in the activity of Src family tyrosine kinases on stimulation of the BCR (Saouaf et al. 1994). This increase may result partly from association of Src family tyrosine kinases with ITAM half-sites via their single SH2 domains (Law et al. 1993; Clark et al. 1994). Alternatively, it may relate to decreased inhibition of Src kinase activity by Csk, which phosphorylates the C-terminal negative regulatory tyrosine of Src family kinases (Figure 4). Csk localization is regulated and, in T cells, Csk is present initially in lipid rafts bound to a protein called Csk-binding protein (Cbp) (also called PAG). Upon TCR stimulation, Csk is released from Cbp by dephosphorylation of Cbp (Torgersen et al. 2001). Whether or not this occurs in B cells is not yet known. Finally, greater removal of Csk phosphorylations by the transmembrane protein tyrosine phosphatase CD45 could be an important means of regulating activity of Src family tyrosine kinases (Figure 4).

Formation of signaling complexes

Once Syk is activated, it phosphorylates the key signaling adapter molecule BLNK (also called SLP-65) (Kelly and Chan 2000; Kurosaki 2002b). BLNK is a cytosolic molecule containing a carboxy-terminal SH2-domain and multiple tyrosines that become phosphorylated and then serve as binding sites for SH2-containing signaling molecules (Fu et al. 1998). How BLNK is localized to signaling receptors is not entirely clear at this point. In TCR signaling, the adapter SLP-76 is

closely analogous to BLNK and it is recruited to participate in TCR signaling by binding to a phosphorylated tyrosine in a transmembrane lipid raft-localized protein called linker for activation of T cells (LAT). Genetic evidence demonstrates that both SLP-76 and LAT are required for TCR signaling (Leo and Schraven 2001). Moreover, SLP-76 and LAT, but not either molecule alone, can replace BLNK to permit BCR signaling in DT-40 chicken B cells mutated in BLNK (Wong et al. 2000). These observations suggest that there may be a B-cell-specific equivalent of LAT and a candidate molecule called LAB (linker for activation of B cells) or NTAL (non T-cell activation linker) has been found (Brdicka et al. 2002; Janssen et al. 2003). An alternative possibility is that the BCR itself recruits BLNK to participate in BCR signaling and indeed there is evidence that BLNK's SH2 domain can bind well to a non-ITAM tyrosine (Y204) of Igα when this tyrosine becomes phosphorylated (Engels et al. 2001; Kabak et al. 2002).

The importance of BLNK for BCR signaling has been established by genetic experiments in chickens (Fu et al. 1998) and mice (Pappu et al. 1999; Hayashi et al. 2000; Xu et al. 2000), and by the discovery of rare immunodeficiency patients with agammaglobulinemia who have loss of function mutations of BLNK (Minegishi et al. 1999). Mice lacking BLNK have a strong block in B-cell development at the pro-B cell stage, probably as a result of a defect in pre-BCR signaling. An equivalent defect is seen in mice with genetic ablation of Syk (Cheng et al. 1995; Turner et al. 1995) or of the three main Src family tyrosine kinases expressed in B cells (Lyn, Fyn, and Blk) (Saijo et al. 2003) (see Figure 3).

Phosphorylation of BLNK leads to formation of a signaling complex that includes a third type of intracellular protein tyrosine kinase, Btk (see Figure 4), and phospholipase Cγ2 (PLCγ2) (Kurosaki 2002b). In this complex, Btk becomes activated, apparently by phosphorylation by Syk, whereupon it phosphorylates PLCγ2, activating its catalytic function. Thus, BLNK both recruits PLCγ2 to the membrane where its substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) is located and promotes its activation (Figure 5). These combined actions induce PLCγ2 to hydrolyze PIP_2, yielding diacylglycerol, which activates protein kinase C

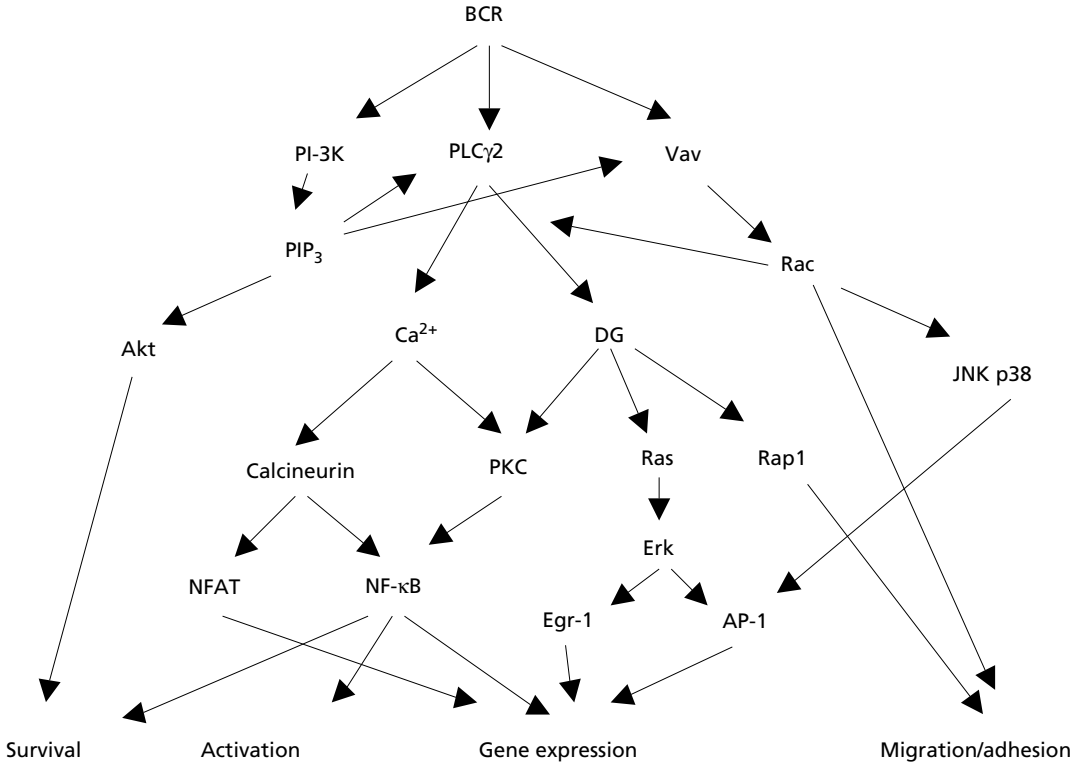

Figure 5 *Networks of signaling reactions activated by the B-cell antigen receptor (BCR). Clustering of the BCR causes it to associate with lipid rafts, which contain Lyn, Fyn and Blk. These Src family tyrosine kinases phosphorylate the BCR ITAMs, inducing recruitment of Syk (not shown), with each of its SH2 domains binding to one-half of the phosphorylated ITAM. Activated Syk and/or Src family tyrosine kinases phosphorylate adapter molecules (see text) such as Blnk and BCAP, leading to activation of PI 3-kinase, PLC-γ2 and the guanine nucleotide exchange factor Vav. PIP_3 generated by PI 3-kinase plays an important role in activation of PLC-γ2 and Vav, as well as the protein kinase Akt, which promotes survival. PLC-γ2 hydrolyzes the phospholipid PIP_2, generating calcium and diacylglycerol (DG). These reactions lead to activation of the transcription factors NFAT, NF-κB, and AP-1 and the induced expression of the transcription factor Egr-1. Also activated are the small-molecular-mass GTP-binding proteins Ras, Rac, and Rap1, which regulate gene expression via MAP kinases and also regulate actin networks and integrin adhesiveness, controlling adhesion, and migration.*

(PKC) isoforms, and inositol 1,4,5-trisphosphate (IP_3), which opens channels releasing stored calcium into the cytoplasm. Depletion of intracellular calcium stores results in the opening of plasma membrane Trp1 channels to keep intracellular free calcium levels elevated (Mori et al. 2002).

The recruitment of Btk to the plasma membrane requires not only BLNK but also phosphoinositide 3-kinase (PI 3-kinase) (Kurosaki 2002b). PI 3-kinase phosphorylates PIP_2 to yield phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 in turn provides a binding ligand for Btk. Thus, Btk is recruited to participate in BCR signaling by the combined action of BLNK and PI 3-kinase.

Btk and a number of other signaling proteins are attracted to the membrane by PIP_3 via homologous domains, called pleckstrin homology (PH) domains. Among these PH domain- containing signaling proteins are the protein kinase Akt and its upstream activating kinase PDK-1. Akt is thought to be important for promoting B-cell survival.

Mice or humans with mutations in Btk, the PI 3-kinase p85α subunit, or PLCγ2 exhibit impairment in B-cell development that is similar to that seen in the Blnk and Syk deficiencies (Fruman et al. 2000), presumably reflecting the participation of these components in pre-BCR signaling (see Figure 3).

Activation of PI 3-kinase

Activation of PI 3-kinase by the BCR is also mediated by adapter proteins. On BCR oligomerization, the transmembrane protein CD19 becomes phosphorylated on tyrosines in its cytoplasmic domain which serve as recruitment sites for PI 3-kinase and another signaling molecule Vav (see below) (Kelly and Chan 2000). CD19 is part of the complement receptor 2 (CR2) complex and acts as a co-receptor for BCR signaling, as discussed below.

A genetic deficiency in CD19 in mice compromises BCR signaling (Engel et al. 1995; Rickert et al. 1995), although less so than mutation of PI 3-kinase or Btk, suggesting that there are other mechanisms for activating PI 3-kinase. Two other adapter proteins have been found to bind PI 3-kinase after BCR stimulation: B-cell adapter for PI 3-kinase (BCAP) (Okada et al. 2000) and Grb-2-associated binding protein 1 (Gab1) (Ingham et al. 2001). PI 3-kinase activation is compromised in BCAP mutants of the chicken DT-40 B-cell line (Okada et al. 2000), but not in splenic B cells from mice lacking BCAP. These mice do exhibit defects in B1 B-cell development, greatly decreased antibody responses to T-independent type II antigens (see below), and somewhat decreased numbers of mature B cells (Yamazaki et al. 2002). Moreover, the BCAP-deficient B cells have poor calcium elevation and poor proliferative responses, although decreased activation of PI 3-kinase was not evident (Yamazaki et al. 2002). These defects are less than those seen in PI 3-kinase p85α subunit or Btk mutant mice. It could be that CD19 and BCAP represent independent routes for BCR-induced activation of PI 3-kinase, or alternately that they act in concert or that BCAP functions for activation of PLC-γ2 independently of PI 3-kinase.

BCR signaling induces phosphorylation of Gab1 along with binding of PI 3-kinase. So far, the effects of removing Gab1 on B-cell function have not been established, but overexpression of Gab1 in the WEHI-231 B-cell line potentiated BCR-induced activation of PI 3-kinase (Ingham et al. 2001). BCR stimulation induces Gab1 translocation from the cytosol to the plasma membrane and this translocation requires the PH domain of Gab1, which binds PIP_3. Therefore it has been proposed that Gab1 is an amplifier of PI 3-kinase activation in BCR signaling. Initial activation of PI 3-kinase via CD19 or BCAP is postulated to result in production of some PIP_3, which would attract Gab1 to the membrane, where it would become tyrosine phosphorylated and recruit additional PI 3-kinase (Ingham et al. 2001).

Signaling events activated by the BCR

As described above, BCR stimulation leads to activation of PI 3-kinase and PIP_2 hydrolysis. BCR stimulation also leads to activation of Vav1, Vav2, and Vav3, which are exchange factors for Rac/Rho family GTPases, activation of Ras, and downstream activation of a variety of transcription factors, including NFAT, NF-κB, AP-1, and Egr-1 (DeFranco 1997; Kurosaki 2002b).

VAV

Vav exists as three isoforms: Vav1, Vav2, and Vav3. Genetic experiments have demonstrated the importance of Vav1 and Vav2 in murine B cells (DeFranco 2001) and Vav3 in DT-40 chicken B cells (Inabe et al. 2002). Vav1 is thought to be activated as a consequence of its recruitment either to the cytoplasmic tail of CD19 or to BLNK, in either case via an SH2-mediated interaction. How Vav2 and Vav3 are activated is less clear. Deficiency of Vav1 in mice leads to loss of B1 B cells, as does loss of CD19, whereas Vav2-deficient mice have decreased T-cell-independent type 2 and T-cell-dependent antibody responses. These findings indicate that Vav1 and Vav2 have distinctive functions (DeFranco 2001). The Vav1/Vav2 double knockout mice have severe defects in B-cell development, maturation in the periphery, and BCR signaling, indicating that many of the functions of Vav1 and Vav2 are overlapping.

The three Vavs all function as activators of Rac family GTPases. Among the downstream consequences of Vav activation are modest activation of the JNK and p38 mitogen-activated protein (MAP) kinases, stimulated

actin polymerization, and activation of PIP 5-kinase, which replenishes PIP_2 to permit rapid PIP_2 hydrolysis by PLCγ2. Indeed *vav1*$^{-/-}$*vav2*$^{-/-}$ B cells exhibit very poor calcium responses to BCR stimulation, indicating weak PIP_2 hydrolysis (DeFranco 2001). Vav3 has been reported to activate PI 3-kinase in chicken B cells downstream of activation of Rac1 (Inabe et al. 2002). As Vav has a PH domain that binds to PIP_3 resulting in increased activity for activation of Rac, it may be that Vav activation of PI 3-kinase is part of a positive feedback amplification loop (Kurosaki 2002b).

RAS

Another key signaling reaction activated by the BCR involves the small GTPase Ras. BCR signaling promotes the loading of GTP on to Ras, which converts it to the active configuration (DeFranco 1997; Gold 2002). Activated Ras has a number of different downstream effects, but the best characterized is the activation of the Erk MAP kinases. MAP kinases are involved in activation of a number of transcriptional pathways.

The mechanism by which the BCR activates Ras is not well established. A major mechanism for the activation of Ras by many receptors involves tyrosine phosphorylation of binding sites for the adapter molecule Grb2, either on a receptor or on the adapter molecule Shc. In most cells, Grb2 is pre-bound to Sos, a guanine nucleotide exchange factor for Ras. Recruitment of Sos to the plasma membrane brings it in close proximity to Ras, where it can induce Ras to release GDP and bind GTP. In lymphocytes, however, Grb2 and Sos are not present in a complex before stimulation (Ravichandran et al. 1995; Harmer and DeFranco 1997). BCR stimulation induces a large increase in Shc phosphorylation and there is formation of a Shc/Grb2/Sos complex, which is localized to the plasma membrane (Saxton et al. 1994; Harmer and DeFranco 1997).

Given the important role of Grb2 and Sos in activating Ras in other systems and the strong tyrosine phosphorylation of Shc in response to BCR stimulation, it has been attractive to consider this to be the mechanism by which BCR stimulation leads to Ras activation. However, genetic studies in DT-40 chicken B cells have found that Grb2 and Shc are not necessary for the activation of Ras via the BCR (Nagai et al. 1995). Thus, the importance of Shc for BCR signaling remains unclear. Interestingly, most of the phosphorylated Shc in BCR-stimulated B cells is in a complex with SHIP (Harmer and DeFranco 1999), an SH2-domain containing inositol phosphatase which is active in removing the 5-phosphate from PIP_3, and thereby countering at least some of the actions of PI 3-kinase (see below), so Shc's primary role may reflect feedback inhibitory events rather than Ras pathway activation.

Recently, a second mechanism for activating Ras in some cell types has been established. Diacylglycerol generated from PIP_2 breakdown can recruit a distinct type of guanine nucleotide exchange factor for Ras, called RasGRP (Ebinu et al. 2000). In this activation mode, Ras activation is downstream of PIP_2 hydrolysis. This possible mechanism for BCR activation of Ras is supported by studies in DT-40 cells, which have shown the importance of phospholipase (PLC)γ2 for activation of the Ras pathway (Hashimoto et al. 1998), a result that would not be expected if Shc/Grb2/Sos were responsible for BCR-induced Ras activation.

In addition to Ras, Rac family GTPases are also activated by the BCR, as indicated by Vav activation (Hashimoto et al. 1998). Rac family GTPases regulate actin polymerization and activation of JNK and p38 MAP kinases, as described above.

A third type of small GTPase that is activated by BCR signaling is Rap1 (McLeod et al. 1998). Activated Rap1 increases integrin binding to their ligands and thereby promotes cell adhesion and possibly migration (Gold 2002). Activation of Rap is thought to be a consequence of diacylglycerol elevation resulting from PLC-γ2 activation.

Activation of transcription factors downstream of BCR signaling

A key signaling pathway downstream of the BCR is the activation of the NF-κB transcription factors, which are dimers of two types of subunits: the p50 (p50 or p52) and the p65 type (p65/RelA, c-Rel, or RelB). Mice deficient in the c-Rel and p50 subunits of NF-κB have normal lymphocyte development but are severely defective in B- and T-cell activation (Pohl et al. 2002). Thus, antigen receptor activation of NF-κB is necessary for lymphocyte activation. How antigen receptors activate NF-κB is not well established, but some of the players are known. In T cells, PKCθ is required for TCR activation of NF-κB (Arendt et al. 2002). The analogous function in B cells is performed by PKCβ (Saijo et al. 2002; Su et al. 2002).

Another signaling component that has been found to participate in antigen receptor activation of NF-κB is the adapter molecule Bcl-10. Mice lacking Bcl-10 have a lymphocyte phenotype that is very similar to that of NF-κB knockouts: normal lymphocyte development, but a failure of lymphocyte activation (Ruland et al. 2001). Bcl-10 is an adapter molecule with an amino-terminal domain with considerable homology to caspase recruitment domains (CARD) of other proteins such as Apaf-1. Bcl-10 was initially discovered as being encoded by a gene translocated to the Ig locus in a subset of B-cell lymphomas (Willis et al. 1999), so overexpression of Bcl-10 probably contributes to lymphoma formation. The opposite hypothesis has also been advanced, e.g. that translocation to the Ig locus leads to inactivation of Bcl-10 by somatic mutation (Willis et al. 1999), but

translocations are not always accompanied by somatic mutations in Bcl-10, so the model that Bcl-10 overexpression contributes to lymphoma formation, probably via activation of NF-κB, seems most likely.

Bcl-10 is thought to function in a complex with CARD11 (also called CARMA1) or related proteins containing CARD, PDZ, SH3, and guanylate kinase domains (Pomerantz et al. 2002). In addition to CARD11, which is highly expressed in lymphoid tissues, this subgroup of the membrane-associated guanylate kinase (MAGUK) family of molecular scaffolds includes CARD10 (Bimp1), and CARD14 (Bimp2) (Bertin et al. 2000, 2001; Gaide et al. 2001, 2002; McAllister-Lucas et al. 2001; Wang et al. 2001). Other members of this signaling complex are less well defined, but may include a protein with caspase-like, Ig-like, and death domains called mucosa-associated lymphoid tissue 1 (MALT-1) (Akagi et al. 1999; Morgan et al. 1999). The gene for MALT-1, similar to that for Bcl-10, is translocated to the Ig loci in a subset of MALT lymphomas.

As in T cells, elevation of intracellular calcium resulting from BCR engagement leads to activation of NFAT transcription factors (Dolmetsch et al. 1997), although their downstream targets in B cells are not well characterized. One target appears to be the gene encoding CD5 (Berland and Wortis 1998), which is a negative regulator of BCR signaling (see below). Curiously, mice genetically modified to ablate both NFATc1 and NFATc2 have hyperactivated B cells and elevated levels of IgG1 and IgE (Peng et al. 2001). NFATc2 ablation alone resulted in increased B-cell proliferation (Caetano et al. 2002).

Ets transcription factors such as Ets-1 are also likely to be regulated by calcium elevation in B cells (Fisher et al. 1991); in the case of Ets-1, calcium leads to its inactivation. Mice in which the Ets-1 gene has been ablated exhibit elevated numbers of IgM-secreting plasma cells (Bories et al. 1995), indicating that Ets-1 probably plays a role in maintaining B cells in a resting state in the absence of antigenic stimulation. Two other Ets family transcription factors important in B cells are PU.1 and Spi-B (Su et al. 1997), apparently by regulating expression of c-Rel, a NF-κB family member (Hu et al. 2001).

Another transcription factor that plays an important role in mediating the response to BCR signaling is Egr-1, which is an early response gene that is activated via the Ras pathway and serum response element (SRE) sequences in B cells (McMahon and Monroe 1995). Downstream of Egr-1 appear genes encoding two cell adhesion molecules, ICAM-1 and CD44 (Maltzman and Monroe 1996b, 1996a). MEK (or MAP kinase/ERK kinase) inhibitors, which block the Ras/Erk pathway just upstream of Erk, block activation of Egr-1, upregulation of these adhesion molecules, and B-cell proliferation induced by BCR signaling (Richards et al. 2001).

B-cell co-receptors

The concept of co-receptors was initially developed to describe the role of CD4 and CD8 in T-cell activation. Both the TCR and the co-receptors bind to the same ligands, MHC–peptide complexes. Clustering of TCR and co-receptor by binding to the same ligand allows their cytoplasmic domains to cooperate to generate stronger TCR signaling reactions than would occur without the co-receptor. Thus, one can view the role of the co-receptor as providing additional information about the ligand that is being recognized by the TCR. B cells also have co-receptors which provide important additional information about the nature of molecules that bind to the BCR or the type of cell on which the antigen is found.

The CR2 (also called CD21) complex is a positively acting co-receptor for B cells. CR2 forms a complex with CD19 and CD81. If complement fragments are deposited on an antigen, then the antigen is much more effective at inducing an immune response, as a result of the ability of the CR2/CD19/CD81 complex to boost suboptimal BCR signaling, e.g. immunization of mice with a lysozyme fusion protein containing ligands for CR2 allowed it to elicit a strong antibody response at 1000–10 000 × lower concentration of immunizing antigen compared with normal lysozyme (Dempsey et al. 1996). This enhanced B cell activation is thought to result from phosphorylation of the cytoplasmic tail of CD19 on sites that permit recruitment of Vav and PI 3-kinase, two signaling components described above (Fearon and Carroll 2000). CD19 also binds to Lyn and promotes its activation (Fujimoto et al. 2000); it helps maintain the BCR in lipid rafts (Cherukuri et al. 2001b) and enhances loading of antigenic peptides on to class II MHC molecules for antigen presentation to T cells (Cherukuri et al. 2001a).

B cells also express several co-receptors that decrease B-cell activation, analogous to the regulation of NK cell killing by NK cell inhibitory receptors. The best understood example is FcγRIIB. Co-engagement of the BCR and FcγRIIB blocks B-cell activation when a naïve B cell encounters antigen in the form of immune complexes with IgG. This is a circumstance when an immune response has already been made, so FcγRIIB mediates a negative feedback loop, and indeed mice lacking FcγRIIB exhibit enhanced antibody responses, particularly at later times (Takai et al. 1996). The inhibitory function of FcγRIIB is circumvented when a germinal center B cell contacts antigen on the surface of a follicular dendritic cell, probably because these cells have a very high density of FcγRIIB and therefore bind the Fc portions of the IgG in the immune complexes on their surface (Tarlinton and Smith 2000).

The inhibitory effect of co-engagement of FcγRIIB with the BCR is the result of a sequence motif in the

cytoplasmic domain of FcγRIIB, called the immunoreceptor tyrosine-based inhibitory motif (ITIM), consisting of I/V/L/SxYxxL/V. When FcγRIIB and the BCR are brought together by an IgG-containing immune complex, kinases associated with the BCR, of which Lyn is the most important (DeFranco et al. 1998), phosphorylate the tyrosine in the FcγRIIB ITIM. This phosphorylation serves as a site to recruit SHIP, a phosphatase that removes the 5-phosphate from PIP_3, and thereby counters the positive action of PI 3-kinase for B-cell activation (Ono et al. 1997; Kurosaki 2002b). SHIP also forms a complex with $p62^{dok}$ and RasGAP, so it appears also directly to downregulate Ras activation. Indeed, mice lacking $p62^{dok}$ fail to suppress Erk activation (Tamir et al. 2000; Yamanashi et al. 2000).

A second established negative co-receptor of B cells is CD22, which is an Ig superfamily member specific for some sialic acid-containing glycoproteins, and thus a member of the family of proteins called Siglecs (Tsubata 1999; Crocker and Varki 2001). Although the role of CD22 in regulating B-cell activation is not well understood, it has been proposed that it inhibits BCR signaling when the B cell recognizes an antigen on the surface of a host cell, rather than on the surface of a microbe that does not express sialic acid (Lanoue et al. 2002).

Other inhibitory receptors on B cells include PIR-B, CD72, and CD5 (expressed by B1 cells and anergic B cells), although their functions remain to be elucidated.

BCR ANTIGEN UPTAKE FUNCTION

The BCR, in addition to its signaling function, also plays a key role in B-cell activation by its function as an uptake receptor, greatly increasing the amount of antigen presented by B cells that can bind the antigen via their BCR versus B cells that cannot. This antigen is routed to the correct intracellular compartment, e.g. the late endosomes, where it is processed to peptides and loaded on to MHC class II molecules. Therefore, the efficient uptake and presentation of antigen allow antigen-specific B cells to present that antigen to helper T cells and thus allow for antigen-specific T cell-dependent antibody responses.

This function of the BCR is heavily dependent on the Igα/Igβ heterodimer and optimally requires the cytoplasmic domains of both these chains. Thus, the cytoplasmic domains of Igα and Igβ seem to provide complementary functions, in addition to the redundant functions of the ITAMs of these two polypeptides (Siemasko and Clark 2001). The mechanisms of these complementary functions for antigen uptake and trafficking to the correct compartment inside the B cell are not known.

FUNCTIONS OF THE BCR IN B-CELL MATURATION AND ACTIVATION

As described above, pre-BCR signaling events are required for B-cell development. In contrast, too much BCR signaling in immature B cells arrests their maturation and blocks exit from the bone marrow. These cells undergo further VJ recombination events at the light chain loci, a process called receptor editing (Nemazee 2000). If antigen specificity is changed by these rearrangements such that the B cell is no longer self-reactive, it can presumably exit the bone marrow and resume maturation. Once in the periphery, transitional B cells must choose between additional tolerance fates (clonal deletion or clonal anergy) and three possible mature cell types: B-1 cells, marginal zone B cells, and follicular recirculating B cells (also called B-2 cells). The BCR is thought to play important roles in all of these cell fate decisions (Meffre et al. 2000; Cariappa and Pillai 2002; Gold 2002; Kurosaki 2002a). How this occurs is poorly understood because it seems unlikely that simple quantitatively different thresholds for each possible fate can explain these different outcomes. In any case, once mature B cells are present, they require a low level of BCR signaling for continued survival (Lam et al. 1997). A higher level of BCR signaling leads to activation of the B cell, as is summarized briefly here.

In mature B cells that encounter antigen, BCR signaling is important for initial activation of the B cell. If the antigen is highly polymeric in nature, such as a repetitive carbohydrate epitope on a bacterial cell surface or a highly repetitive protein determinant on a virion, it will induce very strong signaling reactions and this induces a T-cell-independent mode of B-cell activation. This rapid IgM immune response is produced by marginal zone B cells and B-1 cells, and appears to serve an early defense role in response to infection (Bachmann and Kopf 1999; Martin and Kearney 2001).

Most antigens induce a lower level of BCR signaling, and B-cell activation in this case is dependent on productive interactions with antigen-specific helper T cells. BCR signaling induces follicular B cells to migrate to the outer T-cell zone of the lymph node, Peyer's patch, or splenic white cord. Some of the helper T cells activated by antigen-presenting dendritic cells also migrate to this same region, where they scan B cells for the requisite MHC class II–peptide complexes. If the helper T cell finds a B cell that presents the correct peptide–MHC complex, this leads to a stable interaction lasting many hours. Marginal zone B cells can also participate in T-cell-dependent antibody responses.

Among the signals that the activated helper T cell provides for the antigen-stimulated B cell are CD40L and cytokines such as IL-4 and IL-5. Interestingly, CD40L stimulation, in addition to being a key activation signal, also induces Fas on the B cell and makes it

susceptible to Fas-induced apoptosis. BCR signaling protects against Fas-induced death in this circumstance (Rathmell et al. 1995; Rothstein et al. 1995), and this may be one way that anergic B cells are prevented from making T-cell-dependent antibody responses, because anergic B cells signal poorly through their BCR (Cooke et al. 1994).

Another function of CD40L is to induce the B cell to initiate a germinal center reaction, where there is both class switching and somatic hypermutation followed by affinity selection. The affinity selection step is believed to require both BCR signaling function and BCR antigen uptake function. Antigen in the germinal center is held on the surface of follicular dendritic cells and B cells with high-affinity BCRs can interact with this antigen and receive a survival signal. In addition, these B cells extract the antigen from the follicular dendritic cells, take it up and present it to helper T cells, which have also migrated into the germinal center (Batista et al. 2001). These helper T cells provide an essential role in promoting activation of these germinal center B cells. Therefore, the BCR again plays a critical role in the germinal center reaction, the products of which are high-affinity isotype switched antibody produced by long-lived plasma cells and high-affinity memory B cells.

CONCLUSION

The BCR is composed of the membrane-bound form of Ig bound to a heterodimer of Igα and Igβ (CD79a and -b). Membrane Ig has the antigen-binding function of the receptor whereas the cytoplasmic tails of Igα and Igβ mediate the signaling function. Pre-B cells express a related receptor called the pre-BCR, which differs from the BCR in that it has the two surrogate light chains, Vpre-B1 and λ5, in place of the Ig light chains.

The pre-BCR and the BCR function in two ways: by generating tonic signals that induce developmental transitions and/or provide survival signals, and by generating stronger signals on antigen-induced clustering. The latter type of signaling causes developmental arrest of developing B cells in the bone marrow, tolerance-related effects such as deletion and anergy of immature B cells in the periphery, and activation of mature B cells. Little is known about the events important for tonic signaling, with the exception of pre-BCR signaling, which has been genetically studied in detail, as illustrated in Figure 3.

Antigen (or anti-Ig)-induced signaling is understood in outline and many of the signaling reactions are reasonably well defined. BCR clustering leads to movement of clustered receptors into lipid raft microdomains of the plasma membrane, where signaling molecules such as Src family tyrosine kinases are localized. This leads to phosphorylation of ITAM sequences in the Igα and Igβ cytoplasmic domains, which in turn recruits the Syk tyrosine kinase. Once activated, Syk phosphorylates downstream signaling components that mostly function by assembly into signaling complexes. Signaling events activated include PIP_3 generation, PIP_2 hydrolysis, calcium elevation, activation of small GTPases including Ras, Rac, and Rap1, and eventually activation of transcription factors that lead to alterations in gene expression. Much remains to be learned about how downstream events are mediated, particularly those related to the biological functions of the BCR.

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Monoclonal antibody therapy

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INTRODUCTION

The possibility of using antibodies in the treatment of diseases was first recognized in the 1890s when it was shown that antibodies to bacterial exotoxins were responsible for immunity to tetanus and diphtheria, and that antisera to cholera vibrios could transfer immunity to naive animals (Silverstein 1988). At the start of the twentieth century, until the advent of microbial chemotherapy, antibodies played a major role in the treatment of a wide range of infectious diseases. More recently polyclonal antibodies are routinely used for tetanus, rabies, hepatitis B, and other infectious diseases (Oral and Akdis 2000), as well as for the prevention of immunization of the mother in cases of rhesus incompatibility (Moise 2002).

Therapy in most cases has been with polyclonal antibodies, produced either by immunization of animals with the appropriate antigen or by purification of human immunoglobulin (Ig). A new opportunity was offered by the invention in 1975 of the hybridoma approach to isolating monoclonal antibodies of a desired specificity (see

Antibodies and B lymphocytes) (Kohler and Milstein 1975). It is worth noting (see below) that monoclonal antibodies are not inherently better than polyclonal antibodies; indeed for many applications polyclonal antibodies are more potent. The big advantage of monoclonal antibodies is that it is possible to produce essentially unlimited amounts of antibody with a predetermined specificity.

Polyclonal antibodies versus monoclonal antibodies

It is often assumed that monoclonal antibodies are to be preferred to polyclonal antibodies for most clinical applications. However, although there are very good practical reasons to opt for monoclonal antibodies, there can be advantages to using polyclonal antibodies. Hybridoma technology is one of the possible ways of generating monoclonal antibodies; there are others, such as phage display, which are discussed below. Indeed tumors of antibody-producing cells produce monoclonal antibodies, although normally of unknown specificity. Monoclonal antibodies, as implied by their name, are derived from a single clone of B cells. As such they react with a single epitope on the target. Polyclonal antibodies, on the other hand, react with a range of epitopes. This has the advantage that it is harder for the target (e.g. an infectious pathogen or neoplastic cell) to mutate in order to escape from attack by a polyclonal antibody, because it would need to alter many epitopes. It also makes polyclonal antibodies more robust at reacting with different strains of infectious agent. The ability of polyclonal antibodies to bind to multiple epitopes makes it easier for them to precipitate out molecules from solution, because they can form a lattice with the target molecules. In general the target can also get coated with more antibody molecules (including those of different isotypes) when a polyclonal antibody is used, making recruitment of effector mechanisms more efficient.

The main advantage of monoclonal antibodies produced by hybridoma technology is that it is possible to make large quantities of the antibody. Polyclonal antibodies are derived by bleeding immunized animals and purifying the antibodies obtained from the sera. The amount of antibody that can be obtained from one animal is limited, and once it has been used up the process has to be repeated – resulting in considerable batch-to-batch variability. This also militates against a

thorough characterization of the antibody, because much of it would be used in the characterization process. In the case of a monoclonal antibody it is worth investing considerable time and effort into characterizing the antibody specificity because it is possible to generate as much antibody as is required. In addition, monoclonal antibodies are valuable when an antibody against a single antigen that is difficult to isolate in a pure enough form to immunize an animal is required. Finally, the use of monoclonal antibodies that can be produced in defined cell lines in vitro obviates many of the safety considerations inherent in using antibodies isolated from animals (including humans) that may carry infectious organisms.

When monoclonal antibodies were first described there was considerable optimism that they would form the next generation of therapeutic agent, acting as 'magic bullets' that could be used to seek out and destroy target cells. This was fuelled by early data from lymphoma, in which the first patient treated with an anti-idiotypic antibody specific for his tumor cells went into long-term complete remission (Miller et al. 1982). However, after a period in which there was considerable investment in antibody-based therapies with little return, disillusionment spread and there was a belief that those antibodies would play little role in the clinic. In recent years the picture has changed (Glennie and Johnson 2000; Carter 2001). There has been a considerable increase in the number of antibody molecules licensed by the US Food and Drug Administration (FDA) (Table 1) and the antibody field is now one of the fastest growing areas of the pharmaceutical industry.

One of the problems with our thinking about the use of antibodies is probably with the term 'magic bullet', which has been overused in many papers, grant applications, and business proposals over the years. It is a somewhat unfortunate term, even if originally coined by the father of antibody therapy, Paul Ehrlich, to describe the targeting of toxic substances with antibodies. The term 'magic bullets' suggests a guided missile, and that if such an antibody was injected into a patient it would somehow sense where its target is and home to, and kill, it. In fact, administration of antibodies to a patient results in their distribution throughout the body, and if they happen to bump into their target they will bind to it (for a while). It could be argued that this inappropriate term has befuddled our way of thinking about antibody therapy.

In this chapter, some of the reasons why it has taken 25 years for antibodies to make an impact are discussed, and some of the more futuristic applications of these molecules considered.

HOW ANTIBODIES KILL TARGETS

One of the important questions in antibody therapy is how antibodies work in therapeutic settings. In general this can be divided into three categories: those that rely on the natural functions recruited by antibodies, those that use the ability of antibodies to bind to soluble or cell surface receptors, and those that harness artificial effectors.

Recruitment of natural effectors

COMPLEMENT

The Fc region of antibody molecules is capable of recruiting the complement cascade, as described in much more detail in Complement and reviewed in Walport (2001a, b). This has several consequences, including the release of soluble mediators such as C5a, which act as proinflammatory anaphylatoxins, and the coating of the target with molecules such as C3b, which serve as opsonins, aiding recognition and phagocytosis by macrophages and other phagocytes. In addition, the deposition of the membrane attack complex (the final step of which involves polymerization of C9) can lead to osmotic lysis of the target cell.

In addition to promoting inflammation and destruction of the target cells, complement has a number of other important functions. Complement deposition can reduce the size of immune complexes that form between antibodies and antigens (Miller and Nussenzweig 1975). In addition complement is involved in the processing and clearance of immune complexes, e.g. in patients with C2 deficiency no splenic uptake of immune complexes is observed (Davies et al. 1993). More recently complement has been implicated in the clearance of apoptotic bodies (Taylor et al. 2000). Finally, complement deposition is also important in the generation of antibody responses after immunization (Pepys 1974), in part because of the presence of complement receptors on the surface of follicular dendritic cells and also because the binding of C3d by CD21 (complexed to CD19) on B cells is important in B-cell activation (Fearon and Carroll 2000).

Thus, complement activation is important not only because of its potential direct role in the destruction of the target pathogen, but also because it is involved in the clearance of the resulting debris from the body and the immunization of the individual against the pathogen.

Fc RECEPTOR-MEDIATED KILLING

A wide range of cells expresses receptors for the Fc region of the antibody molecule (FcR), and interaction of the Fc with the FcR has a range of effects (Ravetch and Bolland 2001). These can include the promotion of phagocytosis (e.g. by macrophages and other phagocytes; see Phagocytes part 1: Macrophages), the induction of antibody-directed cellular cytotoxicity (ADDC) (by natural killer (NK) cells; see Natural killer cells), the release of inflammatory mediators (e.g. following interaction through the FcεR of mast

Table 1 *Antibodies approved for clinical use* [a]

***Trade name* (Proper name)**	**Indications for use**	**Specificity**	**Nature**	**Manufacturer**	**Date approved**
Avastin (Bevacizumab)	Metastatic colorectal cancer	VEGF (vascular endothelial growth factor)	Humanized	Genentech	2004
Bexxar Tositumab and I^{131} Tositumab	$CD20^{+}$ NHL	CD20	^{131}I labeled / unlabeled murine	Corixa	2003
Campath (Alemtuzumab)	B-CLL	CD52	Humanized	ILEX	2001
CEA-scan (Arcitumomab)	Imaging colorectal carcinoma	CEA	^{99m}Tc labeled Fab′ murine	Immunomedics	1996
Erbitux (Cetuximab)	Colorectal cancer	Epidermal growth factor receptor	Chimeric	ImClone	2004
Herceptin (Trastuzumab)	Breast cancer	HER–2/*erb* B2	Humanized	Genentech	1998
Humira (Adalimumab)	Rheumatoid arthritis	TNF	Human (phage derived)	Abbott Laboratories	2002
Leukoscan (Sulesomab)	Imaging inflammation/ infection in osteomyelitis	NCA 90 (granulocyte marker)	^{99m}Tc labeled Fab murine	Immunomedics	1997 EU
Myoscint (Imciromab pentetate)	Cardiac imaging	Cardiac myosin	^{111}In labeled Fab murine	Centocor	1996
Oncoscint (Satumomab pendetide)	Imaging colorectal/ovarian carcinomas	TAG-72	^{111}In labeled murine	Cytogen	1992
Orthoclone (muromonab-CD3)	Renal allograft rejection	CD3	Murine	Ortho	1986
Panorex (Edrecolomab)	Rectal/colon cancer	17-1A	Murine	Johnson & Johnson	1995 (Germany)
Prostascint (Capromab pendetide)	Imaging prostatic cancer	Prostate-specific membrane antigen	^{111}In labeled murine IgG	Cytogen Corp	1996

(*Continued over*)

Table 1 *Antibodies approved for clinical use* [a] *(Continued)*

Trade name **(Proper name)**	**Indications for use**	**Specificity**	**Nature**	**Manufacturer**	**Date approved**
Raptiva (Efalizumab)	Psoriasis	CD11a	Humanized	Genentech	2003
Remicade (Infliximab)	Rheumatoid arthritis and Crohn's disease	TNF	Chimeric	Centocor	1999
ReoPro (Abciximab)	Cardiac intervention, unstable angina	Glycoprotein IIb/IIIa (GPIIb/IIIa) receptor of human platelets	Fab fragment of chimeric antibody	Centocor	1997
Rituxan/Mabthera (Rituximab)	Non-Hodgkins lymphoma	CD20	Chimeric	Genentech/Hoffman La-Roche	1997
Simulect (Baxsiliximab)	Renal transplantation	CD25	Chimeric	Novartis	1998
Synagis (Palivizumab)	Respiratory syncytial viral infection (RSV)	F protein of RSV	Humanized	MedImmune (USA)Abbot (EU)	1998
Verluma (Nofetumomab)	Imaging small cell lung cancer	40 kDa carcinoma associated antigen	^{99m}Tc labeled Fab fragment of murine antibody	Dr Karl Thomae GmbH	1996
Xolair (Omalizumab)	Asthma	IgE	Humanized	Genentech	2003
Zenapax (Daclizumab)	Renal allograft rejection	CD25	Humanized	Hoffman La Roche	1997
Zevalin (Ibritumomab Tiuxetan)	Non Hodgkins lymphoma	CD20	^{111}In and ^{90}Y labeled murine antibody	IDEC	2002

a) The table lists the majority of current approved drugs (withdrawn drugs not given) as of 2004. The date of first approval is shown, there may have been subsequent approvals for other indications. Approval date is shown for the USA unless otherwise indicated. Data from web and personal communications.

cells and basophils; see Basophils and eosinophils), and clearance from the circulation by cells of the reticuloendothelial system (see Phagocytes part 1: Macrophages). In some cases these functions will be enhanced by complement activation. In addition the interaction of the Fc region with FcRs is important in controlling humoral immunity (Ravetch and Bolland 2001).

GENERAL CONSIDERATIONS FOR THE RECRUITMENT OF NATURAL EFFECTORS

One of the features of the use of monoclonal antibodies is that a single Ig isotype is employed. This is either of rodent origin or, after antibody engineering, may be a human isotype. It is clearly important to select an appropriate isotype for the recruitment of the appropriate effector functions. This assumes that the important effector function is known and often this can be difficult to determine, e.g. in the case of neoplastic disease there is still little consensus as to whether recruitment of complement or ADCC is important in treatment. The ability of different rodent or human Fc regions to recruit human effectors has been well described; however, the situation is not simple. There are polymorphisms in FcR that influence their ability to bind to particular subclasses of antibody; e.g. the FcγRII (CD32) receptor is polymorphic in the human population, with one form (H131) binding to IgG2 and the other (R131) not (Warmerdam et al. 1990). The allotype of the antibody can also be important in its ability to bind to FcR (Greenwood and Greenwood 1993). Other factors can also determine the ability of an antibody to recruit an effector function, such as the distance from the epitope to the cell surface, as well as the orientation and density of the epitope (Bindon et al. 1988). It is therefore important to determine for each individual antibody what effector functions it can recruit.

Blocking or activation by antibodies

Until recently the main focus has been on the use of antibodies as killing agents, using natural or artificial effector mechanisms. More recently we have come to an appreciation that they can act to modulate cellular responses, either by blocking the interaction of soluble molecules, such as cytokines, with their receptors, or by binding to molecules on the cell surface that then signal to the cell.

BLOCKING BY ANTIBODIES

Antibodies are widely used in experimental settings to block the action of cytokines or other soluble molecules. This can be done using either antibodies against the soluble molecule or a blocking antibody against the receptor.

In the clinic, the most advanced version of this application for cytokines is the administration of antibodies to tumor necrosis factor-α (TNF-α) in the treatment of rheumatoid arthritis and other inflammatory disorders (Taylor 2000). The first such trial involved a chimeric anti-TNF-α antibody (cA2, infliximab) (Elliott et al. 1993), which was followed by a number of trials (some in combination with drug therapy (Elliott et al. 1994a, b; Maini et al. 1999; reviewed in Feldmann and Maini 2001). The overall picture is that most patients (50–60 percent) respond rapidly to treatment with infliximab, with an improvement in both symptoms and clinical signs of disease. However, when treatment is stopped patients eventually relapse, necessitating prolonged or repeated therapy. A range of anti-TNF-α agents has been produced, including humanized antibodies (CDP571), phage-derived antibodies (D2E7), and fusion proteins between the Fc region of the antibody with the p75 (etanercept) or p55 (lenercept) TNF receptors (reviewed in Feldmann and Maini 2001). TNF-α blockade has also been shown to have benefit in other inflammatory diseases, including Crohn's disease (Targan et al. 1997) and Jarisch–Herxheimer reactions (Fekade et al. 1996). Similar strategies have also been used in septic shock, although with less success (McCloskey et al. 1994), whereas treatment of multiple sclerosis with TNF-α-blocking agents appears to be deleterious (Lenercept Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group 1999).

Antibodies have also been used against other cytokines, such as interleukin-2 (IL-2). The alternative approach is to target the receptor for the cytokine, such as the CD25 receptor for IL-2, which has been used to downregulate immune responses in graft rejection. An antibody against endotoxin (HA-1A, Centoxin) was developed for use in septic shock, but was withdrawn when phase III trials demonstrated an increased mortality in patients treated with the antibody (McCloskey et al. 1994). Antibodies are also routinely used to reverse drug toxicities (such as ouabain).

As well as the modulation of cytokines, antibodies can block the access of toxins (such as diphtheria and tetanus) to receptors on cells. Similar blocking can prevent molecules on the surface of viruses from engaging receptors on the surface of cells. However, in many cases the viruses 'hide' the interaction site on their protein either in a buried location or by masking it with another protein. One of the advantages of some of the antibody engineering approaches outlined below is that it may be possible to make artificial antibodies that can bind to these buried interaction sites.

Antibodies can also block interactions between cell surface receptors. Anti-CD3 was the first antibody licensed for clinical use, and is used to block T-cell responses during graft rejection. In experimental settings, nondepleting antibodies against CD4 will block

T-cell responses by interfering in the signaling events leading to T-cell activation, whereas antibodies to adhesion molecules on either endothelial cells or leukocytes will block the recruitment of inflammatory cells.

SIGNALING BY ANTIBODIES

Antibodies are also able to signal cells by cross-linking surface receptors. The importance of this in therapy was realized in patients treated with anti-idiotypic antibody for lymphoma. It was shown in a retrospective study that cells from patients who responded to treatment with antibody showed evidence of signaling (tyrosine phosphorylation) after binding of anti-idiotypic antibody to tumor cells, whereas those that showed little or no clinical benefit did not show such signaling (Vuist et al. 1994). It is also likely that the actions of rituximab (anti-CD20) in lymphoma and trastuzumab (anti-Her2/neu) in solid tumors are in large part the result of their ability to signal to the neoplastic cells (Johnson and Glennie 2001; Yarden and Sliwkowski 2001). This can lead to synergy with other forms of therapy, such as chemotherapy, because the signaling can sensitize the tumor cells to the drug (Demidem et al. 1997).

In addition to direct signaling on the target cell, it is also possible to see an effect resulting from signaling to a nontarget cell, e.g. in an animal model of lymphoma it was shown that anti-CD40 antibodies were highly effective in tumor treatment, but only in situations when the animals had a high tumor burden (French et al. 1999; Tutt et al. 2002). Dissection of the mechanisms involved demonstrated that this was because the anti-CD40 antibody was interacting with CD40 on dendritic cells, rather than the tumor cells. The dendritic cells were activated by this interaction, and then presented tumor antigens to T cells – efficiently immunizing the mice against the lymphoma. Similar strategies could be considered in other settings where immunization is needed.

It should be noted that the signaling function can be reduced by the generation of $F(ab')_2$ molecules which lack the Fc region. The reason for this is that the Fc region can help in the signaling effect by interaction with FcR^+ cells that cross-link the antibody, so increasing its potency (Lane et al. 1991).

Artificial effector functions

The concept of antibodies as delivery agents has proved highly attractive. Thus antibodies have been conjugated to a wide range of toxic agents or other molecules in order to target the molecule to where it is needed. Some of the most common of these are discussed below. The main drawbacks with this approach are that antibody targeting, although capable of high specificity, is relatively poor at delivering large numbers of antibody molecules to the target site. In clinical trials in neoplastic disease, typically 0.01 percent of the injected dose localizes per gram of tissue (Esteban et al. 1987). This is one of those times when the concept of the antibody as a magic bullet is perhaps misleading because it suggests that a large proportion of the antibody molecules administered will reach the target site. Thus, the molecule being targeted needs to be capable of being effective in low amounts. The other side of this equation is that most of the antibody never reaches the target site. Thus, if the antibody is conjugated to a toxic molecule there is the possibility of nonspecific damage to healthy tissues.

TOXINS AND DRUGS

Toxins are one of the most obvious types of molecule to target with antibodies (Figure 1). There is a vast range of such toxins, derived from plants (e.g. ricin, saporin, gelonin), from bacteria (e.g. diphtheria and pseudomonas exotoxins), and from fungi (e.g. α-sarcin) (Thrush et al. 1996; Kreitman 1999). These molecules typically enzymatically inactivate protein synthesis, and so are highly potent, with one molecule of the toxin being capable of killing a cell. These toxins have been conjugated to antibodies, to form immunotoxins, using either chemical or genetic means.

The major problem faced with the use of immunotoxins is that it is not sufficient simply to deliver the toxin to the surface of the cell; it must enter the cytosol to be effective. Natural toxins have a range of mechanisms by which they do this, e.g. ricin, which is probably the most commonly used plant-derived toxin, is made of two chains joined by a disulfide bond (Olsnes and Kozlov 2001). The A chain is the enzymatically active chain. The B chain binds to the cell surface and facilitates entry of the A chain into the cytosol. If immunotoxins are made that consist of an intact A and B chain, they are effective, but also show considerable nontargeted toxicity because the B chain binds to all cells. However, if the B chain is removed, the toxin no longer binds to irrelevant cells, but the immunotoxin is relatively poor at entering the target cells. Several strategies have been used to block or modify the cell-binding chain, while leaving the internalization functions of the B chain intact (Thrush et al. 1996). Similar approaches have also been used with the bacterially derived toxins, although the binding, internalization, and enzymatic regions are normally found on the same polypeptide chain.

The importance of cytosolic entry also means that some antigens are better for immunotoxin delivery than others. These antigens generally internalize after binding of antibodies into endosomes. After acidification of the endosomes, toxins can escape into the cytosol. However, this remains an inefficient process, e.g. in in vitro experiments carried out using bispecific antibodies to target a toxin, saporin to lymphoma cells, it was shown that

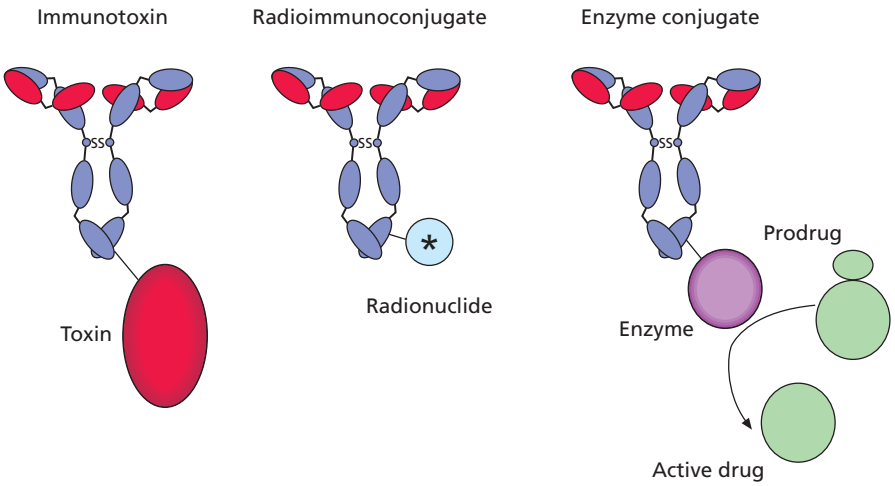

Figure 1 *Immunoconjugates: in the text three basic types of immunoconjugate are described. The first is the immunotoxin, in which the antibody is conjugated to a toxin molecule. This can lead to selective delivery of the toxin to the target cell. The second are radioimmunoconjugates, in which a radioisotope (suitable for therapy or imaging) is used. Although the targeting of the conjugate to the target is specific, the effect of the radiation will spread from that site, depending on the pathlength. Finally antibody–enzyme conjugates can be used to convert harmless prodrugs into active drugs at the target site. This leads to amplification of the targeting effect, with many copies of the drug created by each enzyme.*

constructs directed against CD22 were highly effective at killing, whereas those against CD19, CD37, and surface Ig less so. For 50 percent killing of the tumor cells it was necessary to target around 1 000 toxin molecules per cell using CD22. With the other targets, far more toxin was needed – more than 90 000 molecules in the case of CD37 (Bonardi et al. 1993). Therefore, claims about the potency of the toxins used in immunotoxins need to be treated with caution; they are incredibly toxic but only once they are in the cytosol of the cell.

The second problem about many of these toxins is that they are very immunogenic (see below for why immunogenicity is a problem). Indeed in laboratories making ricin, hypersensitivity reactions to the toxin are more problematic than the toxicity. This limits the number of times an immunotoxin can be administered. For this reason there is increasing interest in mammalian DNase and RNase molecules which have low immunogenicity (Newton and Rybak 2001). These work by degrading all the DNA or RNA in the cell, and again are highly potent if they can enter the cell.

A final problem with immunotoxins is that many of them have side effects, e.g. a limiting feature of treatment with ricin A chain is vascular leak syndrome, caused by interactions of the toxin with endothelial cells (Schindler et al. 2001). Elegant mutational analysis has allowed identification of the regions involved in this, and it may prove possible to mutate out this feature of the molecules.

In a similar manner to toxins, a number of research groups have targeted drugs using antibodies. However, a drawback of this approach is that normally a relatively large number of drug molecules is needed to see an effect. In some cases it may be possible to target enough of the drug to improve the therapeutic window of efficacy.

RADIOIMMUNOCONJUGATES

Conjugation of radionuclides to antibodies can be used for two purposes: therapy and imaging (see Figure 1). In the case of therapy a radionuclide with a short pathlength (e.g. β emitters) is used so that the damage is limited to the targeted area. For imaging, radionuclides with long pathlengths are used (γ emitters), which can be detected by an appropriate camera.

One feature of radioimmunoconjugates is that, even if the antibody is targeted specifically to a cell bearing the appropriate antigen, the killing mechanism spreads from that targeted cell. This can lead to unwanted toxicity to normal tissues. However, it can be an advantage because the radiation may kill cells that need to be killed, but are not targeted by the antibody. This might include the blood vessels feeding a tumor or tumor cells that the antibody cannot reach.

Imaging with antibodies may prove useful in a number of settings. It may localize where disease activity is, monitor its response to therapy, or even assess the nature of the disease, using specific antibodies. In most cases the antibodies used are directed against tumor cells. One exciting alternative is to image E-selectin expression on endothelial cells. E-selectin is an adhesion molecule that is expressed only on activated endothelium, seen at sites of inflammation. Not only is it a highly specific marker of inflammation, but, being expressed on the luminal surface of endothelium, it is highly accessible to antibodies in the vasculature. Antibodies to E-selectin have been used to image rheumatoid

arthritis and inflammatory bowel disease, as well as to locate occult infection in patients with acquired immunodeficiency syndrome (AIDS) (Chapman et al. 1996).

Most imaging is done with γ-emitting antibodies, which can be detected with a gamma camera, using standard nuclear medicine facilities such as single photon emission computed tomography (SPECT). However, there are alternatives, including the delivery of positron emitters for positron emission tomography (PET) and magnetic contrast agents for magnetic resonance imaging (MRI) (Weissleder and Mahmood 2001). Antibodies have even been conjugated onto bubbles, with a view of developing targetable contrast agents for ultrasonography (Villanueva et al. 1998). Each imaging modality has particular advantages, giving complementary information for clinical diagnosis. The ability to use antibodies to localize the expression of molecules on cells has enormous potential not only for clinical diagnosis but also for the careful monitoring of novel therapies.

ENZYME CONJUGATES

One of the most promising, although also complex, forms of immunotherapy relies on targeting an enzyme within a tumor, often termed 'ADEPT'. Once the enzyme–antibody conjugate has been allowed to localize to the target site, an inactive prodrug is given that is converted by the enzyme into its active form (see Figure 1). There are several such enzyme–prodrug combinations that have been developed (Niculescu-Duvaz et al. 1999; Syrigos and Epenetos 1999). In many cases the prodrug is a modified version of a standard drug already in use.

There are several advantages to this approach. The first is that both agents administered to the patient (antibody–enzyme conjugate and prodrug) are essentially nontoxic, and only become toxic when combined. If a drug is chosen that has a short half-life, the toxicity is limited to close to the site of activation. The second is that targeting a single enzyme molecule can facilitate the production of thousands of drug molecules; there is therefore an inbuilt amplification step. In addition in most cases (in neoplastic disease) the drug is more cytopathic to tumor cells than normal cells, so reducing the toxicity to normal tissue around the activation site. At the very least, this approach should improve the therapeutic window of drugs.

The drawback to this approach is the complexity. Not only are there two agents that need to be optimized, but also the timing of the administration of the antibody–enzyme conjugate and the prodrug needs to be optimized. The prodrug cannot be administered while there is free antibody–enzyme in circulation, otherwise drug activation will not be localized to the tumor. To avoid waiting a long time for unbound conjugate to clear, most groups are devising methods to clear the antibody–enzyme from the circulation. Administering a second antibody (e.g. anti-enzyme, anti-idiotypic, or anti-mouse) that binds to the conjugate and causes clearance can do this. Alternatively, the glycosylation status of the antibody can be used to ensure rapid hepatic clearance.

Thus, although attractive, ADEPT has still to make a major impact in the clinic. It is perhaps surprising that all the attention has focused on neoplastic disease for this approach to therapy. It might be thought that this would be useful for focusing the action of anti-parasitic or anti-inflammatory drugs to sites where they are needed.

BISPECIFIC ANTIBODIES

Natural antibodies are monospecific, i.e. they have a single form of the antigen-binding site. Bispecific antibodies are an artificial form of the antibody that has two distinct antigen-binding sites (Figure 2).

There are three ways to prepare bispecific antibodies: chemical, hybrid hybridoma, and recombinant. Chemical methods (reviewed in Segal et al. 1992) range from relatively crude crosslinking with molecules, such as *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), which yield a heterogeneous product, to highly precise production of clinical grade material, e.g. by conjugation through the hinge-region cysteines of Fab′ molecules (Glennie et al. 1987). Hybrid hydridoma technology relies on the fusion of two monoclonal antibody-producing hybridomas (Milstein and Cuello 1983). The advantage over the chemical approach is that the result is a cell line that secretes the bispecific antibody on a permanent basis. However, because of random assortment of the two heavy and two light chain domains, a large number of antibody species is produced – only one of which is bispecific. It is necessary to find ways of purifying the desired molecule from the others. The final way to generate bispecific antibodies is by recombinant DNA technology (George and Huston 1997); a number of constructs have been made, mostly based on the basic scFv unit (as discussed below). The final choice as to which method is used will depend on the amount of material needed, the grade it has to be made to, and also whether it is important that the molecule is monovalent or polyvalent to the antigens (i.e. the number of antigen-binding sites present).

Bispecific antibodies can be specific for soluble molecules (Figure 2). In this way they are essentially acting as immunoconjugates, and toxins, drugs, and radionuclides have also been targeted in this way (French et al. 1995). It is not necessary to make a conjugate between the antibody and the other effector molecule, which can damage the effector. It would theoretically be possible to administer the bispecific antibody and the effector molecule separately, which, if the effector has a short half-life, should reduce toxicity.

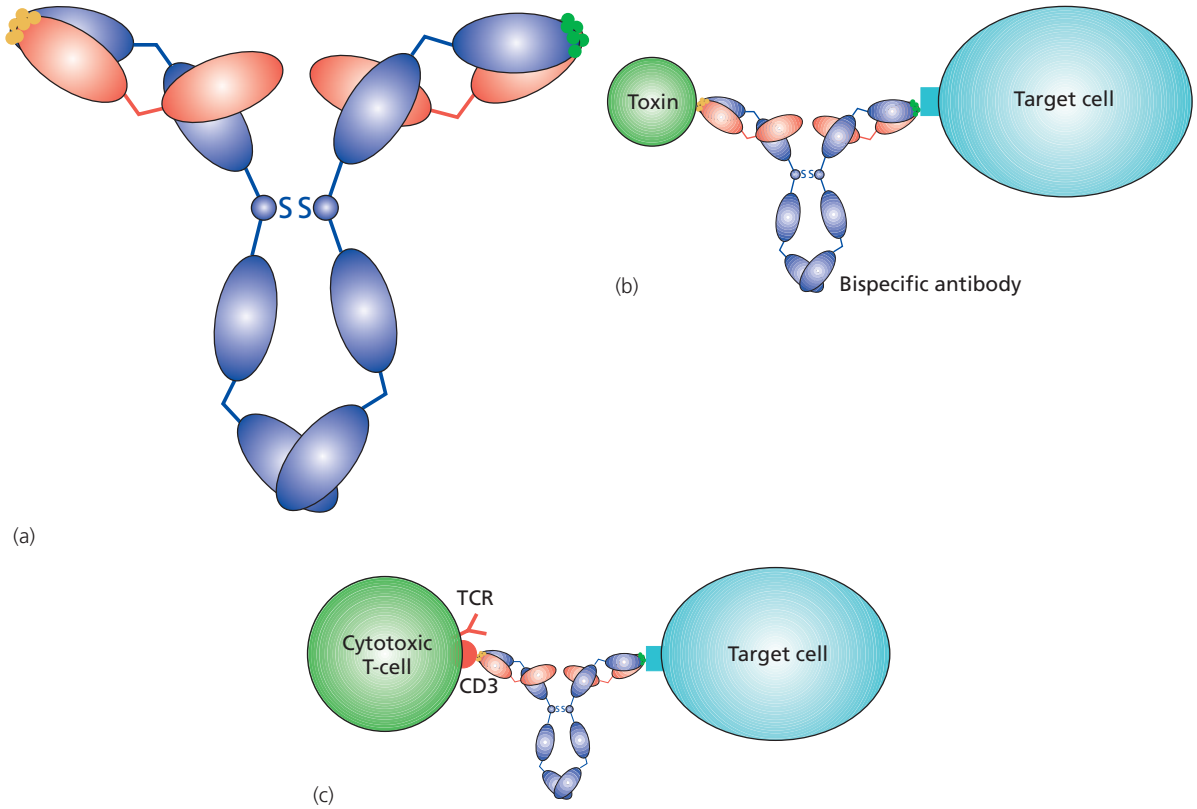

Figure 2 *Bispecific antibodies have two distinct antigen-binding sites, as illustrated here* **(a)** *using different colours for the CDR regions. They can be used to target soluble molecules or effector cells to the target site as shown for* **(b)** *targeting toxins and* **(c)** *cellular cytotoxicity.*

However, the pharmacokinetics of antibody delivery would mean that this is an inefficient process. This process is also highly dependent on the affinity and kinetics of the bispecific antibody to both the target cell and the therapeutic molecule (George et al. 1995a).

In addition to targeting effector molecules, bispecific antibodies can also be used to target immunogens to antigen-presenting cells, to boost immune responses (Snider and Segal 1989), e.g. bispecific antibodies specific for hen egg lysozyme and major histocompatibility (MHC) class II molecules on antigen-presenting cells (as well as some other molecules) have been shown to be effective in vivo at immunizing animals (Snider et al. 1990).

The above applications for bispecific antibodies are essentially similar to those for immunoconjugates. However, one way in which bispecific antibodies can be used is to target cells (Karpovsky et al. 1984; Perez et al. 1985) (Figure 2). In this case the bispecific antibody has to be specific for a molecule on the effector and the target cells. The effector cell could include T cells, neutrophils, NK cells, or any other cell capable of mediating an appropriate response. The bispecific antibody serves not only to bring the two cells together, but also to trigger the effector cell. For that reason the antibody must interact with a 'trigger molecule' on the effector cell, such as CD3 on T cells or FcγRIII (CD16) on NK cells.

The main application of this approach is to kill neoplastic or virally infected cells. The advantage of this strategy is that the effector cells are appropriate for the targets (e.g. the main function of CD8 cells is to kill virally infected cells). They are therefore efficient at the task.

Bispecific antibodies can also be used to activate cells, e.g. combinations of bispecific antibodies can be used that engage not only the trigger molecule on the effector cells, but a co-stimulatory molecule to activate the cell (such as CD28) (Grosse-Hovest et al. 1999). It is even possible to generate trispecific antibodies that engage two molecules on the effector cell, a triggering molecule and an activating molecule (Tutt et al. 1991).

There have been a number of clinical trials using bispecific antibodies (Segal et al. 1999; van Spriel et al. 2000). Some of these have shown promising results; however, progress is slow. The reasons for this are likely to lie in the complex nature of the therapy being attempted; it is necessary to ensure that the antibody and the effector cells both reach the target tissue and that the effector cells are adequately activated. However, the ability to target effector cells that are

optimal for the killing of the target cell remains highly attractive.

OTHER CONJUGATES

Although the above strategies represent the main ones being developed or used for therapy, the ability to conjugate antibodies using chemical or molecular techniques can be applied to an enormous range of partners, e.g. antibodies linked to magnetic beads can be used to purge bone marrow of malignant cells (Gee et al. 1992). Antibodies have also been used to target cytokines such as IL-2 to stimulate immune cells at specific sites (Boleti et al. 1995). In addition antibodies can also be used to target gene therapy vectors, both viral (Harari et al. 1999) and nonviral (Tan et al. 2003), to specific cells.

PROBLEMS WITH ANTIBODY THERAPY

Given the plethora of different strategies described above, it has to be asked why antibody therapy is not more successful. Some of the major problems with antibody therapy are given here and, in the next section, the genetic strategies that can be used to overcome these are considered.

Immunogenicity

Nearly all monoclonal antibodies produced by hybridoma technology are of mouse or rat origin. This means that when they are introduced into patients they are immunogenic. They therefore elicit an immune response, which can be seen as a human anti-mouse (or rat) antibody (HAMA) response. The production of HAMA has two consequences. First, the antibodies bind to the therapeutic antibody, making it ineffective by blocking it and removing it from circulation. Second, the formation of immune complexes with the antibody can lead to serum sickness (type III hypersensitivity). Similar responses against the effector molecule in immunoconjugates (such as toxin molecules) can have a similar effect. The result of this is that it is frequently impossible to give repeated administrations of antibodies to patients.

Pharmacokinetics

Antibodies are large molecules compared with most drugs (around 150 kDa). This means that they are relatively poor at penetrating solid tissue, especially in cases where the tissue has a high interstitial pressure (such as tumors). This limits their ability to get to where they are needed. In addition the large size of antibodies (as well as their interaction with the Brambell receptor FcRn – Junghans 1997) means that they have a long half-life ($t_{1/2}$). The $t_{1/2}$ of a human IgG1 antibody in a patient is around 21 days. Although most murine antibodies have a shorter half-life in patients, this means that large amounts of unbound antibody remain in the circulation for significant periods after administration. If the antibody is conjugated to a cytotoxic agent, this increases the nonspecific toxicity. In addition, for other applications, such as imaging, the unbound antibody provides a background that needs to clear in order for the specific bound antibody to be detected.

Loss of antigen

If the target loses the antigen recognized by the antibody, it can escape attack. This can occur (for both pathogens and neoplastic cells) by mutation of the antigen. In some parasites there are well-described strategies for switching surface antigens to enable them to escape antibody attack. In addition, the loss of antigen can be temporary, caused by modulation of surface antigen (patching, capping, and internalization or shedding of antigen complexed with antibody).

Nature of antigen

Some of the antigens chosen for antibody therapy are not effective because they are not expressed on all the cells (perhaps because of differential expression through the cell cycle). In addition, as discussed above for the immunotoxins (see section on Toxins and drugs), some antigens are better at delivering effector molecules to the cell than others. Expression of the same (or cross-reactive) epitopes on other cells can also cause problems, e.g. ricin A chain containing-immunotoxin was infused into five patients with breast cancer, most of whom developed marked fluid overload and debilitating sensorimotor neuropathies. This was probably the result of an unexpected recognition by the antibody of a molecule on Schwann cells, which resulted in demyelination of the patients' neurons (Gould et al. 1989).

GENETIC ENGINEERING

Many of the problems outlined above have been tackled using the tools of antibody genetic engineering. There are three aspects of antibody production that genetic engineering has influenced: the selection of novel antibodies, the design and construction of 'improved' antibody molecules, and the inexpensive production of antibodies using mammalian, eukarytotic, and prokaryotic cells (outside the scope of this chapter, but reviewed in Verma et al. 1998).

Production of new antibodies

Hybridoma technology has proved a powerful tool for generating monoclonal antibodies. However, it has some severe limitations. The first of these is that it is limited to the production of mouse, rat, or hamster antibodies. Although some groups have been able to use hybridoma

technology to make antibodies from other species (including humans), for a variety of technical reasons (difficulty of immunizing, problems with fusion partners) this has never been routine.

The second problem relates to the way in which hybridoma technology is used to screen for antibodies. This relies on screening individual wells containing hybridomas to see whether they contain an antibody of the desired specificity. This process is inherently inefficient, and so it is difficult to obtain antibodies that are rare. To give a practical illustration of this, if we want to isolate a hybridoma that is present at a frequency of 1/10 000 one would need to screen in the order of 100 96-well plates in order to find it, and that is assuming that every well contains one hybridoma.

This has led to a number of approaches for generating novel antibodies, such as phage display (McCafferty et al. 1990; Clackson et al. 1991) and ribosome display (Hanes and Pluckthun 1997; He and Taussig 1997). Of these, phage display is the most commonly used. In addition, transgenic mouse technology has allowed the production of human antibodies using conventional hybridoma technology (Bruggemann et al. 1991).

PHAGE DISPLAY

Filamentous bacteriophages (such as M13) are single-stranded DNA viruses that infect bacteria such as *E. coli*. They are long phages, typically 1 μm, and around 6–7 nm in diameter. The single-stranded DNA is covered with coat proteins, in particular the major coat protein pVIII, which is present at around 2 700 copies per phage, and a number of minor coat proteins of which the most important for our purposes is pIII, which is present at around three to five copies at one end of the phage.

To carry out phage display it is necessary to make recombinant antibody fragments (see section on Antibody fragments) fused to one of the coat proteins of the phage (for a review of this technology, see Winter et al. 1994; George and Epenetos 1996). Several fragments have been fused to a number of coat proteins, but, for simplicity, scFv fragments fused to pIII are considered here. This is probably the most commonly used format, although many groups use Fab fragments.

In the basic protocol (Figure 3), B cells are used as a source of mRNA. This is reverse transcribed to cDNA and polymerase chain reaction (PCR) used to isolate the gene fragments encoding the heavy and light chain variable domains. These are cloned into the phage as scFv (or Fab) fragments fused to the gene encoding pIII. When these phages are expressed in bacteria they are generated as phage particles that express antibody fragments on their surface (McCafferty et al. 1990). The result of this is a library of phages expressing a large number of antibody molecules. It is now possible to take this library and incubate it with immobilized antigen (e.g. coated onto plastic). Phages expressing the antibody of interest will bind to the antigen; the rest will be washed away. Repeating this panning procedure allows the isolation of phages bearing the appropriate antibody (and also containing the gene encoding for that antibody) (Clackson et al. 1991).

Looking at the steps in the process in some more detail, the source of variable region gene can be B cells from any species from which it is possible to amplify the genes – including humans. In addition, the animal can be either immunized or 'naïve' to the antigen in question. In many cases the source of the genes is synthetic, using artificial sequences. Indeed some groups have moved beyond the antibody, and are making antigen-binding molecules based on different scaffolds, such as fibronectin-derived domains (Koide et al. 1998). This opens up the possibility of making antigen binders with a different number of complementarity-determining regions (CDR) regions, or with different sized CDR regions (long protuberant CDR may be useful to bind to buried epitopes on viruses as discussed earlier).

The size of the library is a very important factor in the process. Typical libraries will have 10^7–10^9 different antibody molecules. It is also possible to use genetic tricks to make libraries that have close to 10^{11} different antibody molecules (Griffiths et al. 1994). To put that in context, the number of different B cells in a mouse is probably around 10^8. Given that a mouse is capable of making an antibody against just about any foreign antigen, this means that it is highly likely that a library will contain an antibody of the desired specificity. The other advantage of large libraries is that the antibodies isolated are of higher affinity than those isolated from smaller libraries (Griffiths et al. 1994).

The affinity selection can take place in a number of ways. In general, purified antigen is coated on to plastic plates. Alternatively, selection can be done in solution, using biotinylated antigen followed by the addition of streptavidin-coated beads to pull the antigen–phage complex out of solution. However, selection can be done on the surface of cells (Palmer et al. 1997), on crude extracts, tissue sections (Owens et al. 2000), and even in vivo, after injection of the library into an animal (Johns et al. 2000). This latter approach allows the isolation of antibodies reactive with tissue-specific markers on endothelium. Selection can also involve negative selection steps, to remove unwanted specificities, or sequential selection on different antigens (e.g. different strains of human immunodeficiency virus (HIV) to deliberately select for antibodies capable of cross-reacting with both antigens or the use of blocking antibodies to direct the antibodies away from a particular epitope (Ditzel et al. 1995).

Once the antibody has been selected it can be modified to give it appropriate effector functions (see 'Effector functions'). However, in many cases, especially

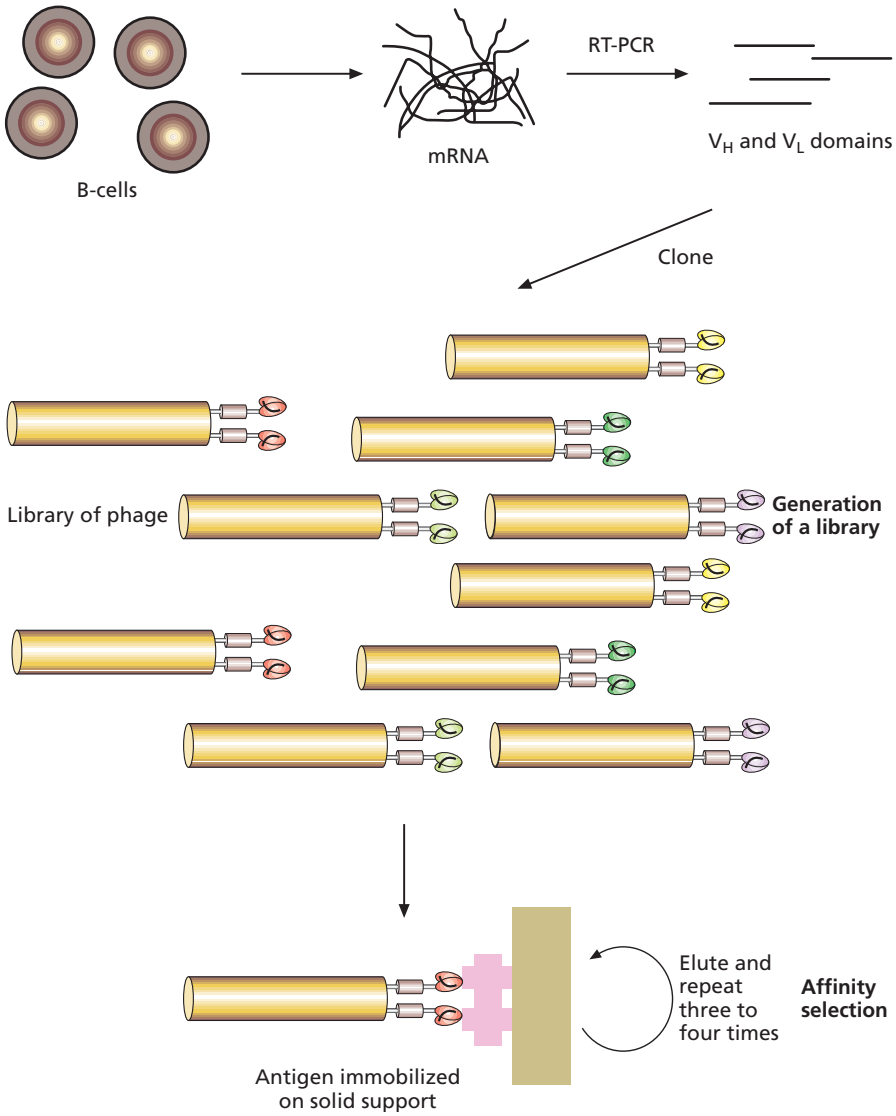

Figure 3 *In phage display technology V region genes are isolated from B cells (or artificially made) and are constructed to form a library of antibody fragments (illustrated here as scFv), displayed on the surface of filamentous phage as a fusion with a coat protein (in this case pIII). The library consists of a large number of phages, each with different antibodies on their surface, and carrying in the genome of the phage particle, the gene encoding that antibody. As a result, if affinity selection techniques are used, it is possible to isolate phage-expressing antibodies that bind to the antigen in question. This allows isolation of the gene for that antibody. The selection process has to be repeated a number of times, resulting in the isolation of an antibody specific for the antigen. RT-PCR, reverse transcriptase polymerase chain reaction.*

from small libraries, the affinity of the antibody is too low for practical use. It is possible to improve the affinity by random mutation of the antibody (using error-prone PCR or passing the phage through a mutator strain of bacteria), followed by further selection on antigen (Marks et al. 1992b). In addition, shuffling the heavy and light chains with novel heavy and light chains, and repeating the selection, can also improve affinity (Marks et al. 1992a). This process can be highly effective; in one case the affinity of anti-HIV antibodies was improved using a variety of mutational approaches from the already high affinity of 6.3 nmol/l to 15 pmol/l (Yang et al. 1995).

The process described above is highly reminiscent of the natural life history of B cells and antibody production. In the bone marrow a large library of naïve B cells is produced with different antibodies on their surface. Antigens are used to affinity select those B cells with the appropriate specificity. These are then randomly mutated and subject to further selection to affinity mature the response. The shuffling of heavy and light chains is even similar to the process of receptor editing, which may play a role in the improvement of antibody affinity (George and Gray 1999).

What are the advantages of phage display? The ability to use human or even artificial antibodies is important.

The process is also better at the isolation of rare antibodies. As antibodies are selected using an affinity technique, so long as the selection system is robust, it is possible to isolate very rare antibodies quickly. In addition, the library has never undergone negative selection or tolerance in vivo. It is therefore possible to isolate antibodies against antigens to which the animals are tolerant (Griffiths et al. 1993). In some cases this has made it possible to produce antibodies that recognize the same antigen in a variety of species (Palmer et al. 1999).

TRANSGENIC ANIMALS

One method for producing human antibodies using hybridoma technology has been to make animals in which their native Ig loci have been replaced using knockout and transgenic approaches with the human counterparts (Lonberg et al. 1994; Bruggemann and Neuberger 1996; Mendez et al. 1997; Nicholson et al. 1999; Tomizuka et al. 2000). These animals, when immunized, produce human antibodies. The antibodies undergo somatic mutation and, if the genes for the appropriate heavy chain isotype are present, can class switch. It is therefore simple to use such immunized animals to generate human monoclonal antibodies using conventional hybridoma technology.

If such transgenic approaches could be applied to larger animals, such as pigs, sheep, or cattle, it might be possible to use them as a source of therapeutic polyclonal human antibodies. It would be conceivable to immunize such animals with virus or toxoids, and to harvest the antibody from them. This should have the advantage of reducing the risks of using material derived from humans, as well as allowing the production of large quantities of defined material.

Immunogenicity

Among the first constructs to be made were those aimed at tackling the immunogenicity of rodent (mostly murine) antibody molecules. The most commonly used methods for doing this are chimerization and humanization, although other procedures such as veneering have been described. There are additional strategies for reducing the immunogenicity, including the production of nonbinding antibody molecules and removal of T-cell epitopes (termed 'deimmunization').

CHIMERIZATION

The structure of the antibody molecule is made up of domains, which essentially fold as independent units. It is therefore relatively easy to swap domains from one antibody to another. Chimeric antibodies are created by replacing all the rodent constant region domains with those of humans, resulting in a molecule where 8 of 12 of the domains are human (Figure 4) (Boulianne et al. 1984; Morrison et al. 1984). These antibodies have considerably reduced immunogenicity in patients when compared with their murine counterpart. In addition, the antibodies have human Fc regions (which can be chosen at will), and so will in general be better at interacting with human effector mechanisms than murine Fc regions.

HUMANIZATION

Although the majority of a chimeric antibody is human in sequence, the variable domains remain rodent in origin. However, most of the variable domains serve no antigen-binding function, but act as a platform for the six CDRs (or hypervariable loops) to form the antigen-binding site. This has led to the process of humanization, in which just the hypervariable loops from the rodent antibody are transferred onto human variable domains (Jones et al. 1986; Reichmann et al. 1988). The result is an antibody that is essentially totally human.

The process of humanization relies first on the identification of human variable domains that are similar in structure to the original rodent variable domains. The CDRs can then be transferred using standard recombinant approaches. When this is done, it is often found that the antibodies have a lower affinity than the parental antibody. Judicious mutation of some of the framework residues that interact with the CDRs will normally restore the affinity. Although this process was originally somewhat hit and miss, there is now a considerable body of experience that ensures that humanization is a robust and relatively routine procedure (Emery and Harris 1995).

Although humanization might seem optimal, it is important to note that the resulting antibodies are still immunogenic (Clark 2000). This is because the antigen-binding site is immunogenic and capable of eliciting an anti-idiotypic response. This is not unique, of course, to humanized antibodies; natural human antibodies are equally capable of inducing anti-idiotypic antibodies. There is no evidence that the CDRs of humanized antibodies are any more (or less) immunogenic than the CDRs of human antibodies. It is also important to realize that, although there might be theoretical advantages of humanization over chimerization, there is little evidence that the humanized antibodies are better than chimeric antibodies (Clark 2000). The immunogenicity of the six CDR loops is probably considerably greater than that of the relatively few framework residues that differ between rodent and human.

VENEERING AND DEIMMUNIZATION

Veneering antibodies relies on the realization that, if the surface of a murine antibody variable domain is compared with its human equivalent (and ignoring the

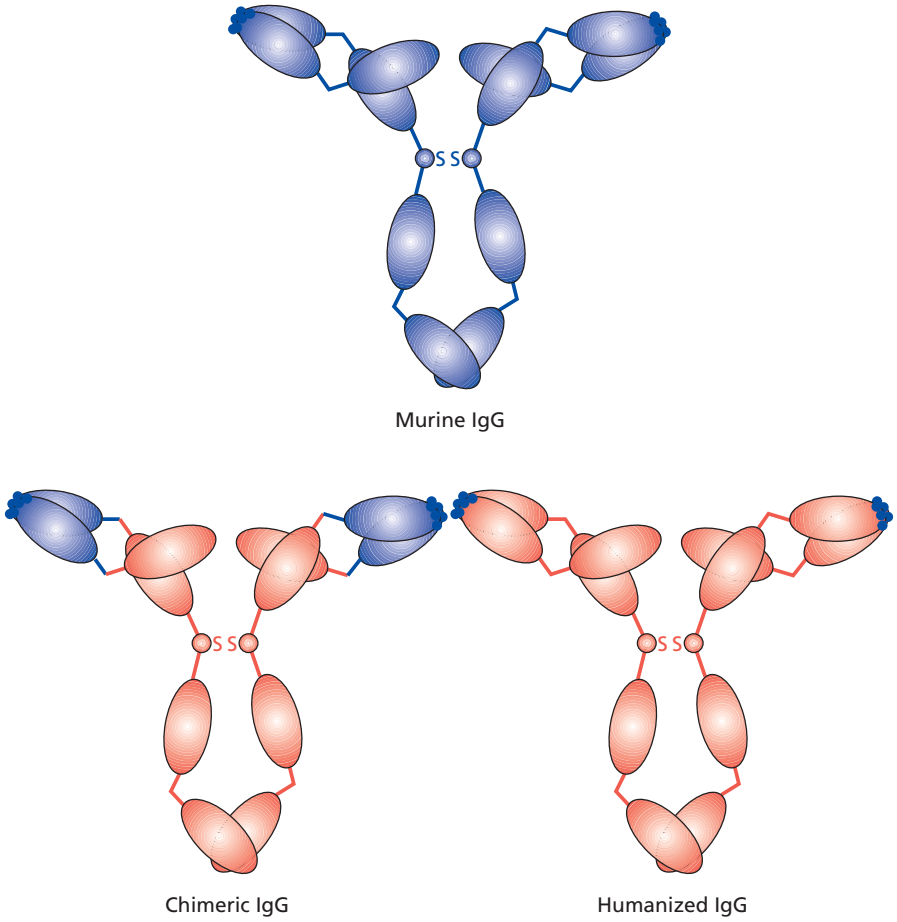

Figure 4 *Reducing immunogenicity: most monoclonal antibodies are totally rodent in origin (shown as blue). However, it is possible to replace parts of the molecule with human sequences (red). In the simplest form chimeric antibodies contain the rodent variable domains and human constant domains. In humanized antibodies all the domains are human in sequence, but the complementarity-determining regions (CDR), shown as circles, are replaced by those from the parental antibody.*

CDR regions), relatively few residues are different (Padlan 1991). Given that HAMA will recognize the surface of these domains, mutation of these few residues from the mouse to the human sequence should abolish the ability of HAMA to bind to the antibodies (Roguska et al. 1994). This approach has been termed 'veneering', because it is similar to the practice in furniture making of covering cheap wood with a veneer of expensive wood. The mouse variable domain is covered with a veneer of human amino acids.

The complementary approach to this has been termed 'deimmunization', in which it has been reasoned that to generate a HAMA response it is necessary for the relevant B cells to obtain T-cell help. As the T-helper cells will see peptide bound in the groove of MHC II molecules, and as this binding is dependent on motifs of amino acids, it should be possible to mutate out these MHC-binding motifs, making an antibody that it invisible to T cells.

Both of these approaches are elegant. However, given the success of humanization and chimerization, it is likely that they will not have a major impact except, possibly, as a way to overcome restrictive patents on the other strategies. It is possible that deimmunization may be effective in preventing anti-idiotypic responses (which require T-cell help), in which case there would be a clear advantage for this approach. However, this has yet to be shown.

TOLERANCE INDUCTION

All of the approaches outlined above are designed to make the antibody invisible to the immune system. An alternative would be to tolerize the patient to the antibody being given. One approach to this is based on the observation that the administration of soluble antibodies to patients (or animals) is normally tolerogenic. However, administration of antibodies that bind to cell surfaces is immunogenic. It is possible to produce antibodies that have mutations in their CDR loops and so no longer bind to their antigen. Intravenous injection of these antibodies (at least in animal models) tolerizes the animal to the antibody, allowing subsequent administration of the complete antigen-binding antibody with no induction of HAMA (Gilliland et al. 1999).

Antibody fragments

As noted above the large size of antibody molecules ensures that they have a long half-life and are relatively poor at penetrating solid tissue. For many circumstances this is an advantage, e.g. if the object is to maintain high levels of a blocking antibody for prolonged periods. However, in many cases it would be preferable to have antibodies that are cleared rapidly. The main approach to doing this has been to make recombinant antibody fragments, which are smaller than the native molecule.

It is fairly easy to make the range of antibody fragments (Fab, Fab′, F(ab′)$_2$ – Figure 5) that are available using conventional enzymatic digestion of antibodies. It is also possible to make novel molecules, normally based on the Fv fragment of an antibody that consists of the two variable domains of an antibody. This is the smallest antibody fragment that retains an intact antigen-binding site (25 kDa, as compared with 150 kDa for IgG). It is possible to make Fv fragments by enzymatic digestion of some antibodies (Inbar et al. 1972); however, the molecules are normally unstable because the two domains are free to dissociate (in an Fab fragment the heavy and light chains are held together by a disulfide bonds as well as noncovalent interactions that are predominantly between the two constant domains). There has been interest, therefore, in strategies to make artificial Fv-based molecules.

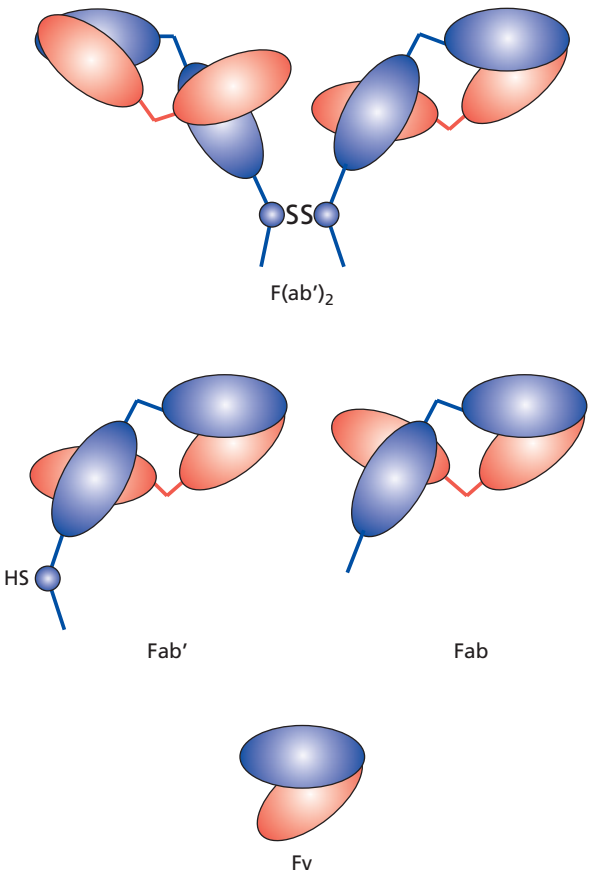

Figure 5 *Antibody fragments: the whole immunoglobulin IgG molecule can be enzymatically digested to produce F(ab′)$_2$, Fab′, Fab, and Fv fragments of the molecule, which contain intact antigen-binding sites but are smaller than the parental molecule. These are also accessible using recombinant approaches. However, the Fv in particular is unstable, because there are no disulfide bonds and only weak noncovalent interactions to prevent the two chains from dissociating.*

Fv-BASED CONSTRUCTS

The aim of all Fv-based constructs is to stabilize the interactions between the two variable domains without destroying the antibody-binding site. The first type of molecule in which this was achieved was the single chain Fv (scFv or sFv, commonly referred to as a single chain antibody). In this molecule a peptide linker is used to join the C terminus of one domain with the N terminus of the other (Bird et al. 1988; Huston et al. 1988) (Figure 6). The (linear) distance that needs to be spanned is around 3–4 nm (Huston et al. 1996a), and to do this the linker is typically around 15 amino acids; it has been designed or selected to be highly flexible (the archetypal sequence being $(Gly_4Ser)_3$, with the glycines providing maximal flexibility – Huston et al. 1988). However, many other linker sequences have been designed or selected by phage display (Turner et al. 1997). The format of the scFv can be V_H–linker–V_L, or V_L–linker–V_H, whereas some scFvs perform better in one orientation than the other; in most cases the choice is unimportant.

The scFv has the advantage of being a robust building block for the generation of targeting molecules, in large part because of its simplicity; it is a single polypeptide chain encoded by a single gene construct. The pharmacokinetics of the scFv are as might be expected from its small

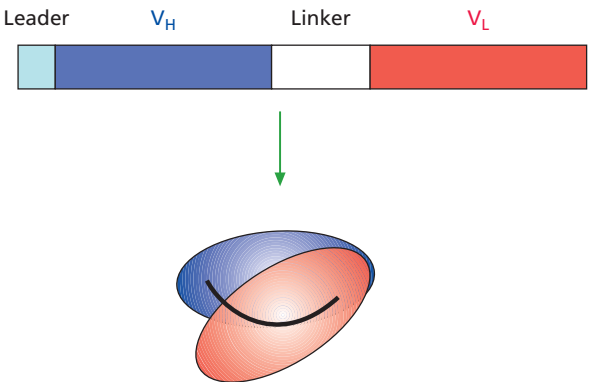

Figure 6 *The single chain Fv (scFv) consists of the V_H and V_L domains of the antibody, stabilized by a peptide spacer between the N terminus of one domain and the C terminus of the other. The molecule is encoded by a single gene, which translates into a single polypeptide chain.*

Table 2 *Half-lives of antibody fragments*

	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)
IgG	39	113
F(ab′)$_2$	26	12
Fab′	9.2	88 min
scFv	3.7	90 min

The data taken from Milenic et al. (1991) shows the half-life of different versions of the CC49 antibody. These are given as two phases, the α phase (roughly corresponding to equilibration into the extravascular volume) and the β phase (clearance from the body). The scFv and Fab′ fragments show much more rapid equilibration and clearance.

size, with a very short clearance rate (Table 2) (Milenic et al. 1991) and good penetration of solid tissue. A phage-derived scFv with specificity for carcinoembryonic antigen has been used to image patients with neoplastic disease, with a half-life of around 5 h (Begent et al. 1996). In this case the antibody was better at imaging tumors than conventional antibodies. Similarly, a scFv specific for the C5 component of complement has a half-life of 7–14 h in patients (Fitch et al. 1999).

Other Fv-based molecules have been produced. The main alternative is the disulfide-stabilized Fv (dsFv) (Figure 7). In this molecule single mutations have been made that have changed an amino acid residue on the interface between the two variable domains into cysteine (Glockshuber et al. 1990). The molecules are then free to pair and make a disulfide bond that stabilizes the interaction between the two domains. Clinical trials have been described in which dsFvs fused to toxins have been administered to patients with hematological malignancies (Kreitman et al. 2001).

SMALLER FRAGMENTS

Although the Fv is the smallest fragment of a human antibody that retains an intact antigen-binding site, attempts have been made to decrease the size of recombinant antibodies still further. Although an intact antigen-binding site has six CDRs that can contribute to the interaction with antigen, in many cases not all are needed. Thus, some antibodies show preferential usage of the heavy chain CDRs. This has led to the production of single domain antibodies (dAb), in which just one variable domain (normally the V_H) is produced (Ward et al. 1989). In some cases, dAbs show binding to antigen. However, there are two drawbacks to this approach. The first is that there is likely to be a loss of affinity as half the CDRs are removed. This might be overcome by techniques such as phage display. The major problem is that the dAb contains a surface that is normally hidden by interaction with the other variable domain, and this surface contains hydrophobic amino acids that normally contribute to stabilization of the interaction between the domains. However, in a dAb they can cause the fragment to become highly 'sticky' and also prone to aggregate.

One solution to this has come from the realization that members of the cammelid family (camels and llamas) have antibodies that consist of just the heavy chain (Hamers-Casterman et al. 1993). This feature is

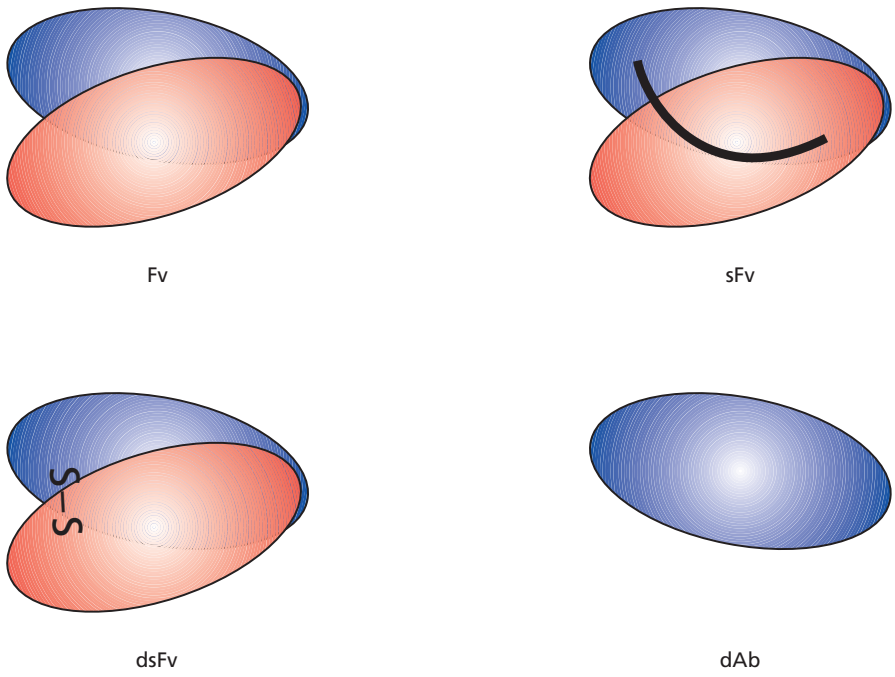

Figure 7 *Other Fv-based molecules: in addition to the scFv other molecules have been made with the aim of exploiting the antigen-binding site of the Fv. These include the disulfide-stabilized Fv (dsFv) in which a disulfide bond in the interface of the two domains prevents the V_H and V_L from dissociating. In addition the single domain antibody (dAb) contains just one domain (normally the V_H).*

also seen in other species such as sharks (Pilstrom 2002). The equivalent surface of the heavy chain variable domain is adapted to working in the absence of a partner V_L, and so is not 'sticky'. It is therefore possible to make dAbs from such species, as well as to consider mutating the human V_H domain to make it more 'camel like' (Davies and Riechmann 1994),

The ultimate in small recognition units is a single CDR. Several groups have devised peptide sequences based on the CDR of an antibody that retains a degree of antigen binding (Zhang et al. 1996; Laune et al. 1997; Park et al. 2000). In most cases these peptides are cyclic, thus somewhat constraining their conformation. In most cases the affinity of the peptides for their antigen is relatively low. However, they are inexpensive to make and can be administered in large quantities to compensate for this. Although it is unlikely that they will replace antibody-like molecules for most targeting purposes, the use of antibody technologies to identify and isolate CDRs that can be used for specialized applications (such as blocking viral entry) is an interesting route to drug development.

Effector functions

The small antibody fragments described above have the ability to target their antigen, but carry no effector function (other than blocking). In addition, most of the fragments described above are monovalent and so are not capable of cross-linking their antigen (and also suffer from a loss of avidity). It is therefore necessary to modify them, normally by fusing them to alternative molecules. In the following section, several examples normally based on the scFv molecule (as that is the most commonly used) are given. However, most of the approaches can also be applied to other fragments (Fab, dsFv, dAb, etc.).

FUSION PROTEINS

The simplest way to endow an antibody fragment with function is to fuse it to the molecule with the desired function (Figure 8) (Neuberger et al. 1984). The scFv is very tolerant of fusions to both the N terminus and C terminus (or indeed both), retaining its affinity for antigen, although this is not always true of the fusion partner.

The partner protein can be just about any protein, including toxins (Chaudhary et al. 1989), enzymes, and cytokines (Boleti et al. 1995), as described earlier for conventional conjugates. They can also be viral coat proteins (e.g. pIII in filamentous phage, see section on Phage display). In some cases it is also possible to use peptides with a function, e.g. a short peptide has been designed that chelates the radioisotope ^{99m}Tc (George et al. 1995b). The scFv incorporating this peptide can, in a single, rapid, one-tube process, bind to and chelate the radioisotope. The interaction is highly stable and allows the site-specific labeling of recombinant scFv (or other proteins), making them highly suitable for imaging applications (George et al. 1995b; Huston et al. 1996b).

RECOMBINANT BISPECIFIC ANTIBODIES

Conventional methods of making bispecific antibodies have been described above, but recombinant antibody fragments also offer very convenient building blocks for making these molecules (George and Huston 1997). A variety of fragments has been described, some of which are shown in Figure 9.

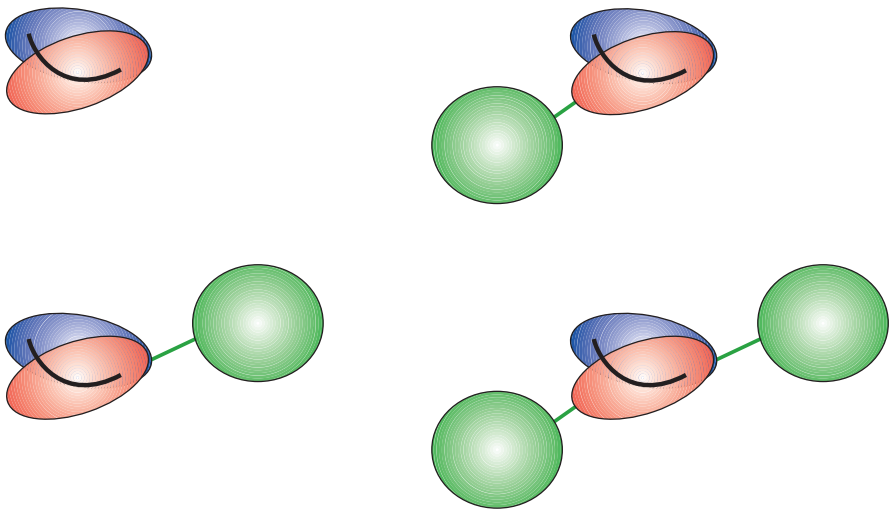

Figure 8 *Fusions with scFv: functional properties can be given to the scFv antibody by fusing the molecule with another protein or peptide (shown in green). This fusion can be N- or C-terminal to the scFv (or even at both ends). In general the scFv is tolerant to such additions, although the function of the effector protein may depend on the orientation.*

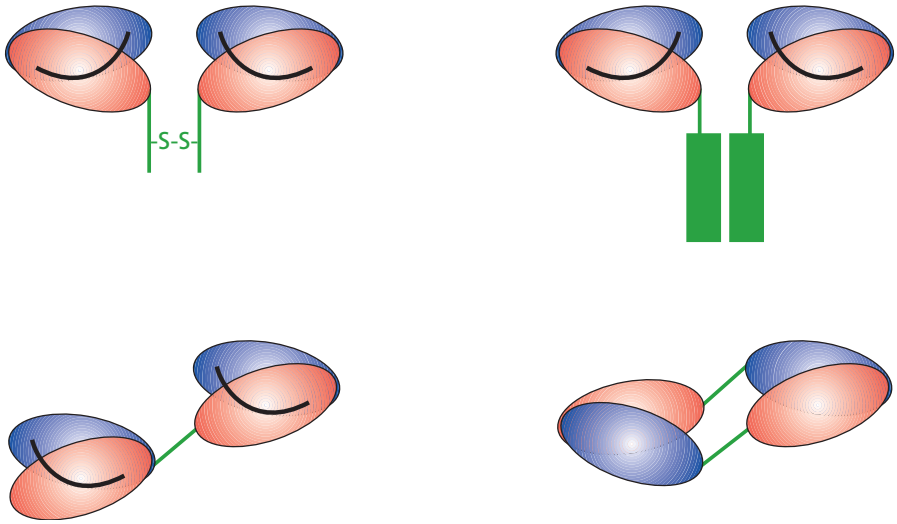

Figure 9 *Recombinant bispecific constructs: there are several ways in which the scFv (or equivalent) antibody fragments can be used to generate bispecific antibodies. These include using disulfide bonds to join two fragments together, fusion to domains (such as leucine zippers) that cause heterodimerization, use of a peptide linker to join two scFvs, or the formation of diabodies in which two polypeptide chains associate in a head-to-tail conformation.*

The simplest approach is to add a cysteine residue to the scFv to allow dimerization (or chemical conjugation) (McCartney et al. 1995). It is possible to fuse the scFv with domains in order to encourage heterodimerization, as opposed to homodimerization. These are often based on leucine zipper motifs, such as jun and fos, which naturally heterodimerize. Fusion of scFv or other antibody fragments with such zippers results in the formation of bispecific antibodies (Kostelny et al. 1992; de-Kruif and Logtenberg 1996).

The second approach is simply to fuse two scFv together, to form a single chain bispecific antibody, which is expressed on a single polypeptide chain. Although this is an elegant approach, in many cases the yields of such molecules are low (Andrew et al. 1991; Huston et al. 1991; Kurucz et al. 1995).

The final approach that is described is the formation of diabodies (Holliger et al. 1993; Whitlow et al. 1994). The scFv molecule is designed with a long enough linker so that the V_H and V_L domains interact with each other. However, if the linker is shortened this is not possible, and in such cases the domains interact with their partner domains on other molecules, forming two chain diabodies. With the appropriate choice of molecule, it is possible to generate bispecific diabodies. Indeed, this approach can be taken further to create trivalent triabodies (Hudson and Kortt 1999).

All of the strategies described above for generating bispecific antibodies can also be used to make bivalent scFv. This might have advantages in terms of the affinity of the interaction with cell surfaces (Adams et al. 1998), and also allow cross-linking of the target molecules and therefore signaling.

MODIFICATION OF TARGET CELLS

The above approaches all rely on administering the recombinant antibody as a protein. However, it is also possible genetically to modify cells so that they express the antibody of interest in a manner that modulates their phenotype or function. One example of this is to express scFv fragments as fusion proteins with molecules involved in signaling (such as the ζ chain of the T-cell receptor (TCR)–CD3 complex) (Eshhar et al. 1993; Eshhar 1997). If such a scFv-ζ fusion is expressed in a T cell, the T cell can become activated upon encounter with the antibody (so-called T bodies). This approach has been applied to other cell types, and may form a useful therapy for neoplastic disease as well as against virally infected cells.

The scFv can also be expressed in an intracellular localization, where they can bind to their antigens. They are directed to the appropriate site by fusion with signal peptides (e.g. KDEL which retains or retrieves the molecule to the endoplasmic reticulum). This can result in 'knocking out' expression of a particular protein or blocking the function of that molecule (Figure 10) (Richardson et al. 1995). However, in the context of infectious disease it can also be used to block viral infection (often termed 'intracellular immunization'). This has been considerably developed in the context of plant infections, but has also been applied to human settings, e.g. in the case of HIV, intracellular antibodies have been generated to a wide range of the viral proteins. Expression of these proteins can prevent functional virions being assembled and so block infection (Mhashilkar et al. 1995; Marasco et al. 1999; Poznansky et al. 1999). This opens the possibility of gene therapy in

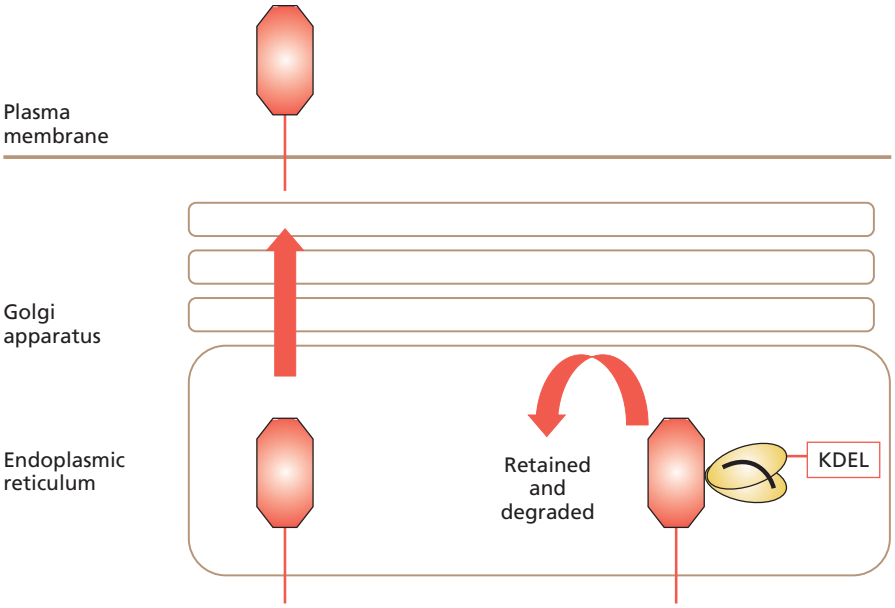

Figure 10 *Intracellular antibodies: normally (shown on left) molecules destined for secretion from a cell or expression on the cell membrane are synthesized in the endoplasmic reticulum. They then pass through the Golgi apparatus to the cell surface. On the right is shown the intervention possible using an intracellular antibody. An antibody (scFv in this example) is expressed in the cell fused to the KDEL sequence. This causes the scFv to be retained in or retrieved to the endoplasmic reticulum. There they bind their target antigen, preventing them from moving to the Golgi and to the cell surface – eventually targeting them for degradation.*

which bone marrow cells are made resistant to infection by expression of intracellular antibodies.

CONCLUSION

A quarter of a century after the description of a generally applicable method for making monoclonal antibodies, they are only now beginning to take their place in the clinic. Why did this process take so long? Many of the reasons were scientific, and the problems and solutions have been discussed above. In addition, there are many commercial obstacles that scientists and clinicians are often rather naïve about, which slow down the development of antibodies (for a fascinating account of the development of one antibody, CAMPATH-1, see Hale and Waldmann 2000). It should perhaps be remembered that it does take time to develop new classes of therapeutic agent. Even penicillin, which is often seen as a remarkable wonder drug that achieved widespread and rapid acceptance, took some time to be developed. Alexander Fleming first noted the death of bacteria on the Petri dish in 1928. It was first tested in patients in 1941 and was in routine clinical use by 1945. This represented a period of scientific advance (including the isolation of the active molecule) as well as industrial development (to produce the product in large enough amounts), which was accelerated by the wartime demands. One of the problems with the cycles of hype and cynicism that surround new forms of therapy is that it can disrupt the steady and sustained effort that is needed to achieve success.

What does the future hold? The current antibodies that have achieved licensing by the FDA are mostly relatively conventional (albeit humanized or chimerized). In the pipeline are the products of phage display and more radical antibody engineering. These are likely to start to make an impact over the next decade, although it is difficult to tell how great this will be. Although the tools of molecular biology do offer really exciting possibilities, it should be remembered that technology developed in the nineteenth century (polyclonal antibodies) is still the most commonly used for the production of clinical reagents. We must adopt the new technologies where appropriate, while not being dazzled by their novelty into applying them in an inappropriate manner.

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Processing and presentation of antigen by the class II histocompatibility system

EMIL R. UNANUE

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INTRODUCTION

Antigen presentation involves the interaction between the T cell and an antigen-presenting cell (APC) responsible for the uptake and intracellular handling of the protein. All T cells recognize antigen molecules that are presented in the context of histocompatibility molecules. The molecules encoded in the major histocompatibility gene complex (MHC) bind peptides derived from the fragmentation of the protein antigen and present these to T cells. It is the peptide–MHC complex, which constitutes the antigenic determinant, that the T cell recognizes. It follows that all proteins need to be processed by an APC in order for peptides to be generated. Without antigen presentation there is no recognition of the protein by the lymphocyte system (for a historical summary, see Unanue 2002). The two major stable sets of T cells, the CD4 and the CD8 T cells, each recognize peptides that are bound to different sets of MHC molecules. The set of MHC class I (MHCI) molecules present peptides to the CD8 T cells, whereas the MHCII molecules present peptides to the CD4 T cells. The MHCI molecules, examined in (see MHC class I antigen processing system), mainly sample the cytosol of the APCs, whereas in contrast the MHCII molecules preferentially sample the vesicular system. In this way, the MHC molecules contain peptides from proteins that have entered the two major cellular compartments of the APCs. Thus, viruses, intracellular bacteria, or parasites that enter the cytosol are recognized primarily by the CD8 T cells which recognize the peptide antigen–MHCI complex (Rock and Goldberg 1999; Yewdell and Bennink 1999). In contrast, soluble proteins or proteins derived from phagocytosed organisms are presented to CD4 T cells that recognize the peptide–MHCII complex. There are other MHC molecules expressed in lower amounts which can present certain unique molecules, e.g. the MHCI-like molecule H-2M3, binds to small hydrophobic peptides that contain formlymethionine at its amino terminus (Lindahl et al. 1997). The CD1 molecules bind to glycolipids, including those derived from *Mycobacterium tuberculosis* (Porcelli et al. 1992). T cells directed to these MHC complexes may have a role in antimicrobial immunity. This chapter, based on a previous review (Unanue 2001), considers antigen processing and presentation by the MHCII molecules.

It is important, in the context of microbial immunity, to understand how the immune system has evolved to deal with either protein or polysaccharide antigens. The former involve the T cells, as indicated above, by recognition of products of antigen processing, as well as B cells, which can recognize the proteins before catabolism. The end result of a productive encounter with protein antigens is both the production of antibody and the development of a T-cell response – cellular immunity. The T-cell response is long-lived and heightens on repeated immunization, a fundamental feature of prophylactic immunization – the recall response associated with immunization. With the T-cell response

Table 1 *Responses to carbohydrates and protein antigens*

	Pure polysaccharide	Protein
B-cell recognition	Yes	Yes
T-cell recognition	No	Yes
Binding to MHC molecules	No	Yes
Activation of CD4 T cells	No	Yes
T-cell memory	No	Yes
Delayed sensitivity response	No	Yes
Antibody affinity changes	No	Yes

there follows the capacity to activate macrophages and to form the immune granulomas. An important clinical demonstration of the T-cell response is the 'delayed sensitivity' skin reaction that takes place on injection of a minute amount of the antigen into the skin of the immunized individual. This small indurated lesion indicates that the patient has encountered the microbe, and has activated T cells. With regard to the B-cell response, it is characterized by high-affinity antibodies of the various subclasses, which is an indication of affinity-driven maturation by T-cell 'help'.

In contrast, pure carbohydates do not stimulate T cells and only induce a B-cell response that is limited in the range of its response. Immunological memory is poor. There is no delayed sensitivity response. The antibody response shows poor affinity maturation and is mostly represented by IgM antibodies.

This difference in T-cell responses between proteins and pure polysaccharides is explained because carbohydrates do not interact with MHC molecules (Harding et al. 1991; Ishioka et al. 1992). Without an MHC molecule to serve as a carrier of the foreign entity, T cells will not recognize it. Knowing this difference, vaccines against bacterial polysaccharides are now produced by conjugating the carbohydrate to an immunogenic 'carrier' protein, in order also to elicit a T-cell response (Schneerson et al. 1980; Insel and Anderson 1986). Table 1 compares both responses.

THE INNATE SYSTEM OF DEFENSE, APCS, AND SYMBIOSIS WITH T CELLS

The APC cellular system is essential for antigen presentation. Three main cells are involved in presentation; two are derived from the monocyte lineage: the dendritic cells (DC) and the macrophages. They belong to the 'innate' cellular system that operates independently of the lymphocyte lineage (Janeway and Medzhitov 2002). These cells sample and handle moieties from the extracellular milieu, and are therefore essential APCs. The second APCs are the B cells which are involved in the interaction with CD4 T cells during antibody formation.

There is an intimate, truly symbiotic relationship between the innate cellular system and the lymphocyte (Unanue 1981, 1997). The innate system is represented by cells of monocyte lineage: the granulocytes and the natural killer (NK) cells. Only those cells derived from the monocytes are APCs. The cells of the innate system sample the environment, constituting the earliest system of defense that reacts to the presence of diverse pathogens. The innate system is phylogenetically older and represented by the amebocytes and phagocytes of invertebrates (Hoffmann et al. 1999). This is the cellular system first described by Metchnikoff in his classic studies of the phagocytes as an essential defense cellular system (Metchnikoff 1968). In vertebrates, the innate system can effectively handle a number of pathogens but it is incapable of resolving the infections entirely, and importantly will show the property of neither specificity in recognition nor immunological memory.

The phagocytes recognize many bacteria and microorganisms through 'pattern recognition' in which the polysaccharides, lipoproteins, and other moieties are seen as a whole (see Pattern recognition). Phagocytes contain a number of cell surface molecules that allow them to handle a wide range of pathogens (see below). Phagocytes, and in general the cells of the innate system, obey chemical signals from a variety of secreted polypeptides – the family of cytokines and chemokines produced by the lymphocytes (Buchmeier and Schreiber 1985; reviewed in Luster 2002). As a result, the innate system heightens its capacity to deal with, and eliminates, a number of pathogens once the lymphocyte enters the scene. The lymphocyte system, on the other hand, can effectively eliminate pathogens, have exquisite specificity of recognition, and importantly shows memory – the basis of prophylactic immunization. The operation is, however, truly symbiotic, inasmuch as the lymphocyte is blind to antigens unless the antigen molecules are presented by the phagocytes (referring to DCs and macrophages), by way of their MHC molecules, during antigen presentation. During handling of the antigen molecules, the phagocytes also release a number of cytokines, which help in the early recruitment and activation of the lymphocytes (Hsieh et al. 1993) (Figure 1). As the phagocyte system needs to be 'activated' through a number of cytokines elaborated by the lymphocytes, the loop of cell-to-cell interaction is evident, each cell critically depending on the other. The communication between the two interacting cellular systems is by way of antigen presentation via MHCII molecules as well as by cytokines. It is antigen presentation that imparts specificity to the system because the recognition of the presenting antigens recruits the specific clones of T cells. A comparison of the two recognition systems is shown in Table 2.

A highly informative experimental model of infection that brings out the symbiotic relationship between the innate and adaptive system is that of *Listeria mono-*

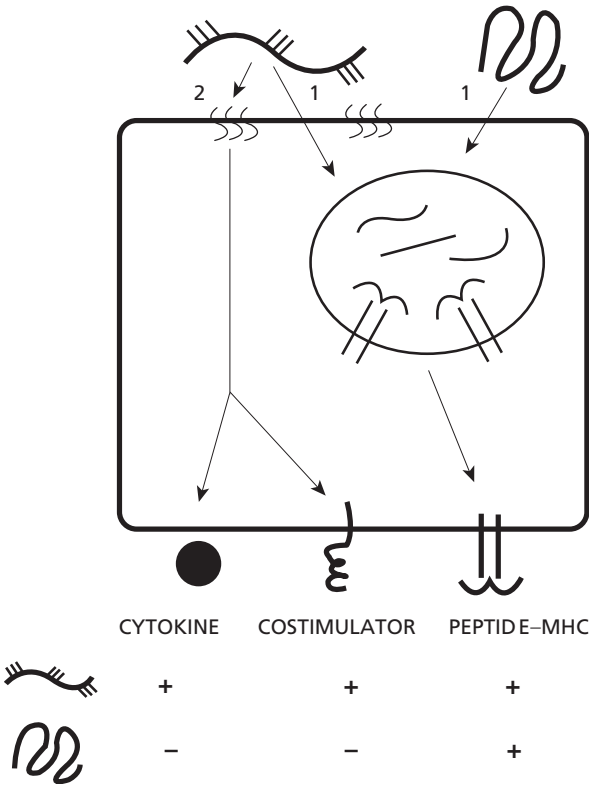

Figure 1 *Graphic representation of the results of two kinds of interactions of an antigen-presenting cell (APC). A bacterium (left side, top) will generate peptides after it is internalized (pathway indicated as 1), but will also activate (pathway 2) Toll-like receptors (TLRs). Pathway 1 will yield peptide–MHC complex to T-cell recognition. Pathway 2 will result in secretion of soluble cytokines and the presence of membrane costimulatory molecules. All three components are required for optimal T-cell activation. A protein (right, top) will give rise to peptide–MHC complex after it is processed, but will not induce cytokine or costimulation and will not productively activate T cells.*

cytogenes infection in the inbred mouse (Mackanness 1962; reviewed in Unanue 1997). *Listeria* species is an intracellular pathogen that is taken up by macrophages where it escapes from the phagocytic vacuole to multiply in the cytoplasm. *Listeria* infection proceeds through various stages: first there is an early neutrophil component that partially contains the growth particularly in the liver; this is followed by a phase in which the macrophages curb *Listeria* replication while also releasing inflammatory cytokines such as the interleukins IL-1, IL-12, and IL-18 and tumor necrosis factor (TNF). These cytokines then lead to an activation of various cells including NK cells as well as CD4 and CD8 T cells. The last are activated by presentation of *Listeria* antigens and then participate in the complete elimination of the bacteria. Mice that only have an innate system, as happens in mice with the severe combined immunodeficiency (SCID) mutation, which lack lymphocytes, will not result in sterilizing immunity although the growth of *Listeria* monocytogen is partially contained. SCID mice will become carriers of the infection but will eliminate *Listeria* sp. completely if reconstituted with T cells. Both CD4 and CD8 kill *Listeria* monocytogen found in phagocytes or epithelial or mesenchymal cells.

With regard to APCs, four conditions are required for them to be effective in antigen presentation. First, the cells must have the capacity to take up proteins, which they do by various molecular systems. Second, the APCs must have the internal cellular machinery to enable them to process proteins. All cells have a lysosomal system that allows them to handle endocytosed proteins. Thus, as far as their capacity to present is concerned, it will depend to a major extent whether the MHC molecules are expressed. Indeed, many cell lines that do not express MHCII molecules will do so if transfected with the genes encoding MHC genes. Lastly, for effective antigen presentation APCs must also express other molecules that allow for stimulation of the T cell.

Dendritic cells derive from the blood monocyte and inhabit many tissues, where they are highly effective in antigen presentation (Inaba et al. 1983; Banchereau and Steinman 1998; Lanzavecchia and Sallusto 2001; Guermonprez et al. 2002). DCs circulate in small numbers in blood and are rapidly mobilized to sites of infection. Monocytes also differentiate to DCs as they cross into the tissues. In the lymphoid organs, DCs constitute a network of interdigitating cells located mainly in the deep cortex of the lymph nodes, or the periarteriolar sheath of the spleen. DCs express receptors for some chemokines such as CCR7 which regulates their migration into lymphoid organs (Tang and Cyster 1999). DCs are found in the skin, as Langerhans' cells, situated between the keratinocytes: on interaction with antigens that contact the skin, they migrate into the draining node and there recruit specific T cells. DCs are also found in the thymus where they play a vital role in the process of presentation of autologous proteins. DCs are divided into two subsets called myeloid and lymphoid,

Table 2 *Comparison of innate and adaptive immunity*

	Innate	Adaptive: B cell	Adaptive: T cell
Antigen recognition	Nonclonal	Clonal	Clonal
Antigen receptors	Many	Single	Single
MHC involvement for recognition	No	No	Yes
Memory	No	Yes	Yes

A clonal system refers to B and T cells, each of which express only one set of antigen receptors having unique specificity. The adaptive system is therefore a selection by antigen of those B and T cells with receptors of high affinity. The cells of the innate system can identify more than one foreign antigen.

distinguished by the presence of the C8 α protein, in the latter.

Dendritic cells are highly effective in interacting with T cells (Lanzavecchia and Sallusto 2001). Their expression of both class I and II MHC molecules is high and constitutive. DCs also express adhesion and costimulatory molecules. DCs show long cytoplasmic extensions which favor the intimate physical contact with T cells. DCs can be generated from bone marrow cells in the presence of the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) (Inaba et al. 1992; Sallusto and Lanzavecchia 1994). These in vitro cells can present antigens and have been used in transplantation studies to immunize, particularly in cancer immunotherapy (Nestle et al. 2001).

Dendritic cells evolve through various stages of maturation (Thery and Amigorena 2001; Guermonprez et al. 2002). The immature DCs express low levels of adhesion and costimulatory molecules, although their processing of antigens is not impaired. On interaction with a variety of stimuli, DCs increase their expression of the adhesion and costimulatory molecules and become more effective in antigen presentation. Among the stimuli are cytokines such as TNF-α or IL-1, both gram-positive and -negative bacteria, and their products, such as endotoxins, double-stranded RNA, and interaction with the CD40 ligand (Inaba et al. 1993; Cella et al. 1996, 1999; Randolph et al. 1998).

Macrophages were the first cells to be identified as APCs (Unanue 2002). Derived from monocytes by way of interaction with the cytokine CSF-1 (or M-CSF), macrophages inhabit many tissues, particularly around blood vessels where they sample the extracellular fluid (see Phagocytes part 1: macrophages). Macrophages have an important effector function in their uptake and handling of microbes. Their microbicidal activity is readily enhanced by contact with cytokines particularly with interferon-γ and TNF. Macrophages secrete a large number of important cytokines that are involved in the early stages of an immune response. Macrophages differentiate in tissues influenced by the local environment of the tissue. They express low levels of MHC molecules and their antigen-presenting function can be limited unless activated by interferon, which markedly increases their expression.

B cells express constitutively both class I and II molecules and have an important antigen-presenting role in the interaction with CD4 T cells (Chesnut et al. 1982; MacPherson et al. 1999). This interaction leads to antibody formation in a two-step process: first, B cells bind the antigen by way of their specific membrane-bound antibody (Lanzavecchia 1990), after which the protein is processed, leading to the expression and presentation of the peptide–MHC complex; second the cloned T cell then recognizes the complex leading to their reciprocal activation. The final result is a reciprocal activation of both cells: the T lymphocyte will continue its activation while the B cell proliferates and differentiates, evolving into an antibody-forming cell. The end product is the release of antibody molecules that are identical in their specificity to the original antibody on the B-cell surface. These antibodies can be directed to conformational determinants of the protein. Thus, the result is the release of antibodies to conformational determinants of the protein and T cells that recognize linear sequences of the same. Immature B cells have limited antigen-presenting capacity, which increases as the B cell is activated.

ANTIGEN PRESENTATION IN THYMUS AND PERIPHERAL LYMPHOID ORGANS

Antigen presentation takes place during the response to foreign molecules that enter the tissues, including the lymphoid tissues. The encounter of the phagocytes with antigen results in the selection of T cell, with complementary receptors to the peptide–MHC complex displayed on the APC surface. The exact events involve several steps where cytokines and chemokines are released, resulting in increases in vascular permeability and cell migration to the foci of antigen–APC interaction. The end result is the recruitment of the antigen-specific T-cell clones, leading to their proliferation and the release of cytokines. Clonal enlargement results in an increase in specific lymphocytes and a more effective process for elimination of the foreign antigen. The cytokines released by T cells markedly influence the cellular environment, fostering growth and differentiation as well as activation of phagocytes. From this process, the phagocytes change their behavior to a cell more active in cytocidal activity and capable of eliminating a range of microorganisms.

T cells also encounter peptide–MHC during their normal process of maturation in the thymus gland. The nature of this encounter is very different from that described above, in that the peptides that form the complex do not derive from foreign molecules (which normally do not enter the thymus gland), but from the processing of self-proteins. It is this encounter that influences the fate of the early T cells during its normal process of differentiation. The thymus contains in its cortex a network of epithelial cells that express MHCII molecules (in contrast to most epithelial cells which do not). It also contains DCs particularly in the area between cortex and medulla, and in the medullary area itself. As the thymocyte matures, it expresses by random rearrangements of its receptor genes, antigen receptors (T-cell receptor (TCR)) that may or may not have specific complementarity to the autologous peptide–MHC complexes. This normally engages amino acid residues from the peptide, and also segments of the MHC molecules itself, around the area that binds the peptide. Thus, it is a receptor with a dual specificity (Garcia et al. 1996; Garboczi et al. 1996; Reinherz et al. 1999).

Three developments take place during the differentiation of the T cell, depending on the nature of the TCR and the MHC molecules with which it can interact (reviewed in von Boehmer 1990; Robey and Fowlkes 1994; Jameson et al. 1995; Borowski et al. 2002). A T cell with a TCR that does not match or complement the autologous peptide–MHC dies by 'neglect'. A T cell that encounters a perfect match will also die, a process called 'negative selection' (Kappler et al. 1987; Kisielow et al. 1988a, 1988b). Through this negative selection event, a number of autoreactive T cells are eliminated and therefore autoimmunity is prevented. In both these processes the T cell dies by apoptosis. Finally, a T cell with a receptor that matches the MHC, but matches the peptide poorly, matures, leaves the thymus, enters the recirculating pool of blood T cells, and seeds the lymphoid organs (Kisielow et al. 1988b). This process of 'positive selection' results in the survival and selection of T cells with TCRs that interact with self-MHC molecules, and particularly with peptides other than the autologous ones presented in the thymus, i.e. peptides from foreign proteins.

This process of T-cell maturation, in which T cells are selected to recognize a peptide and its own MHC molecule, explains the important phenomenon known as 'MHC restriction', which is characteristic of antigen presentation (Rosenthal and Shevach 1973; Zinkernagel and Doherty 1974). The mature T cells only recognize peptides derived from proteins presented by an APC that displays its own MHC molecules. In an outbred population, where the MHC shows marked allelic polymorphism, a T cell with specificity for a protein processed by its own APCs will not recognize the protein presented by an APC of a different individual. The fundamental reason is that the T cell is constrained as a result of the nature of the thymic selection process in recognizing its own MHC proteins. The phenomenon of MHC restriction was discovered for both CD4 and CD8 T cells when antigen-specific cells from one inbred strain of experimental animals were combined in culture with APCs of strains with different MHC alleles. (The experiments that discovered restriction in the MHCII molecule are shown in Table 3.) The same results were obtained when studying T- and B-cell interactions (Katz and Unanue 1973). Apart from the fact that this phenomenon was instrumental in pointing to the basic rules of APC–T-cell interaction, it also has practical applications. The clinical relevance is to the use of antigen-specific T cells for immunotherapy of patients with infectious diseases. For example, specific T-cell clones that can be generated ex vivo will not be useful unless they are transplanted in individuals who share the same MHC alleles.

MHCII MOLECULES

Every species contains an MHC gene complex that encodes for a number of class I and class II molecules. In humans, the complex is the HLA gene complex encoded in chromosome 6. The HLA gene complex includes genes that encode for the α and β chains of MHCII molecules, for the heavy chain of several MHCI molecules and genes for several complement proteins. The MHCII molecules contain two transmembrane chains, termed α and β, that assemble to create the peptide-binding site. The MHC of the mouse is H-2, discovered first by transplantation biologists who identified it as a system of cell-surface antigens with multiple alleles, responsible for tissue rejection.

The major function of class I and II molecules is to bind peptides, creating the complex that forms the substrate for T-cell recognition (Babbitt et al. 1985). One important feature of the MHC gene complex is its large degree of allelic polymorphism. The allelic differences are accounted for by several amino acids located in the region of the molecules that bind to peptides. The evolutionary pressure for diversification of the MHC and its selection is probably accounted for by microbial and parasitic infections (reviewed in Klein et al. 1993; Dawkins et al. 1999; Parham 1999). The argument goes

Table 3 *Rosenthal–Shevach experiment showing major histocompatibility complex (MHC) restriction for the presentation of purified protein derivative (PPD)*

Macrophages		T lymphocytes (cpm × 10^{-3})		
Strain	**PPD**	**Strain 2**	**Strain 13**	**Strain (2 × 13) F1**
2	–	0.9	5.7	1.6
2	+	26.4	8.6	7.0
13	–	4.6	1.7	1.8
13	+	3.1	19.9	7.8
(2 × 13) F1	–	1.9	4.3	1.7
(2 × 13) F1	+	12.4	11.8	12.6

The two inbred strains of guinea pigs, named 2 and 13, differ at their class II MHC gene locus. The guinea pigs were immunized with dried tubercle bacillus in water in oil emulsion. Their T cells were isolated and cultured with macrophages from normal guinea pigs, in the absence or presence of PPD of *Mycobacterium tuberculosis*. Strain 2 lymphocytes responded only to PPD presented by macrophages of strain 2, and the same applied to lymphocytes from strain 13. Note the mild mixed lymphocytic reaction when lymphocytes from 2 or 13 are incubated with macrophages of strains 13 or 2, respectively. Modified from Rosenthal and Shevach (1973).

that the more polymorphism there is in the species, the more the diversity of peptides is selected from microbes, and the better protected is the species to face infectious agents. Examples are known of HLA alleles favoring protection against malaria and leprosy (Piazza et al. 1973; Hill et al. 1991). Conversely, situations in nature that have reduced MHC polymorphism translate into enhanced susceptibility to infection (O'Brien and Yuhki 1999). This occurs in some examples of cheetahs, tigers, and Florida panthers (O'Brien and Yuhki 1999).

MHC molecules are peptide-binding molecules that select peptides from intracellular digestion of the protein (Babbitt et al. 1985; Bjorkman et al. 1987b; Unanue 2002). Both MHC molecules contain a single binding site that accommodates peptides of varying lengths. The MHCI molecule is made up of a single transmembrane glycopolypeptide – a heavy chain of about 44 kDa that folds in its amino terminus to create the binding site. It also contains a small polypeptide called β_2-microglobulin that gives structure to the heavy chain. The MHCII molecules contain two extracellular transmembrane chains, α and β, of about 20–30 kDa, which together create the binding site. The binding site of both class I and II molecules is similar with a platform of β-pleated sheets and two helical walls, between which is located the peptide (Bjorkman et al. 1987a, 1987b; Stern et al. 1994; Madden 1995).

Each MHC molecule binds one peptide, usually in the range of a nanomolar or micromolar affinity constant. The interaction of MHC molecules with peptides has been studied in binding interactions using purified molecules and peptides (Babbitt et al. 1985, 1986; Buus et al. 1987a, 1987b). By creating single or multiple changes in the composition of the peptides, the specificity of the interactions and the contributions of a given residue to binding strength can be determined. Peptides usually bind with a slow on-rate but, once bound, the dissociation rate from the complex is slow. It translates into peptides in the live APCs being maintained for a relatively long period of time.

The structural studies of MHC molecules containing peptides are important for understanding the nature of the binding (Brown et al. 1993; Stern et al. 1994; Fremont et al. 1998a, 1998b; Scott et al. 1998; Latek and Unanue 1999). In the MHC molecules, the various residues responsible for the allelic differences are situated in sites combining class I and II MHC molecules. These various residues constitute allele-specific binding sites or 'pockets' which accommodate different amino acids from the peptide. For each MHC allelic form, there are usually four to five sites: P1, P4, P6, P7, and P9. These accommodate the various side chains of the peptide, which is stretched through the combining site. For many alleles one or two of these pocket sites are mainly responsible for both binding the peptide and its specificity. The binding results from two main interactions, that of the peptide amino acid side chains with the pockets and that of the peptide backbone with conserved residues of the MHC binding sites, through which a large network of hydrogen bonds is created (Chicz et al. 1992; Hammer et al. 1992; Hunt et al. 1992; Sinigaglia and Hammer 1995; Suri et al. 2002).

What are the features of peptides presented by the class II molecules that CD4 T cells recognize? Peptides presented by class II MHC molecules are usually longer than 10 amino acids whereas those presented by class I are eight to nine residues in length. The peptides contain a 'core' of nine amino acids and flanking residues of varying lengths (reviewed in Latek and Unanue 1999). In the peptide core sequence, some amino acid side chains interact with the allele-specific pockets, mentioned above. The flank residues are important in contributing to binding strength and to the stability of the MHC–peptide complex. Class II peptides vary in the length of their flanks; in fact many peptides are selected as families made of the core with flanks of varying length. Two to three amino acid side chains of the peptide are solvent exposed and potentially capable of binding to the TCR.

It is important to note that the MHC molecules bind to self and foreign peptides equally well (Babbitt et al. 1986). This is evident from the description of thymic maturation, which involves interaction with self-peptides. It follows that, because self-proteins are always normally being processed, there is always the potential to develop autoimmunity. Indeed, a series of autoimmune diseases are known to develop in which autoreactive T cells recognize self-peptides bound to MHC molecules.

The process of protein processing and presentation between class I and II molecules differs in important respects. Suffice it to state here that class I molecules have evolved to recognize peptides resulting from cytosolic catabolism, which are then taken into the endoplasmic reticulum where the complex is assembled (Townsend et al. 1986). Class II molecules, as indicated before, mainly recognize peptides derived from the intravesicular processing and are loaded in acid lysosomal-like compartments. The nature of the peptides recognized by both systems differs.

Peptides bind with different degrees of affinity, depending on their constellation of amino acid side chains and the nature of the particular class II molecule. Some do not bind at all and to these the immune system will not mount an immune response (Levine et al. 1963; McDevitt and Sela 1965; reviewed in Benacerraf 1981; McDevitt 2002). It is the fact that some MHC alleles bind peptides poorly whereas others bind strongly that led to the discovery of the role of the MHC gene complex in the immune response. The MHC had been previously examined only in the context of nonphysiological reactions, those of tissue transplantation. When studying responses to small polypeptides of limited heterogeneity, it soon became apparent that some inbred strains of mice responded strongly whereas others made poor responses

(McDevitt 2002). Such a trait was found to be autosomal dominant and mapped to H-2, and in particular to a new region, later found to encode for the murine class II molecules (McDevitt and Chinitz 1969). These studies were seminal in calling attention to the importance of H-2 in regulating immune responses to proteins.

The nature of the peptides bound to MHC molecules during processing can now be studied by electrospray tandem mass spectrometry (Chicz et al. 1992; Hunt et al. 1992) (Figure 2). MHC molecules are isolated from APCs and denatured to release the peptides, which are then analyzed and sequenced. One can identify peptide families with as many as 40 members, and some represented in very large amounts (Latek and Unanue 1999). This approach has now been used to identify the precise peptides selected during the processing of viruses and bacteria. Examination of peptides selected by different MHC alleles indicates the preference for each allele to select peptides based on particular sequence motifs. These motifs are based on the presence of amino acids in which side chains enter into interaction with the specific binding loci. For any given protein, some peptide segments are selected during processing and bound to the MHC molecules whereas others are not recognized or recognized very weakly, and rapidly dissociate.

The peptides bound to MHCII molecules derive from extracellular proteins, as expected. Most peptides derive from the processing of membrane proteins, e.g. transferrin receptor or invariant chain, or even from the α and β chain of the class II molecule itself. Peptides also derive from proteins of the cytosol. These probably enter the vesicles by autophagy.

As the peptides bind to class II molecules, a number of the side chains of their component amino acids become solvent exposed and can potentially contact the TCR (Allen et al. 1987). In class II-bound peptides, these solvent-exposed residues are consistently placed at the P1, P2, P3, P5, and P8 positions (the MHC anchors placed at P1, P4, P6, P7, and P9). The TCR interacts with some of the residues of the solvent-exposed residues. Their interactions tend to be of low affinity. Structural studies indicate that both the peptide and the MHC are contacted by the two chains of the TCR (Garboczi et al. 1996; Garcia et al. 1996; Reinherz et al. 1999; Hennecke and Wiley 2001; Rudolph and Wilson 2002). The TCR adopts a canonical conformation in which its long contact axis is diagonal or orthogonal to the peptide–MHC. Part of the TCR contacts the helices of the MHC, particularly the complementary determinant regions (CDR) 1 and 2, which are germline encoded. (The CDRs are the regions of the TCR responsible for their binding specificity.) On the other hand, the CDR3 segment, where extensive variations exist, contacts the middle regions of the peptide. The structural studies have therefore validated the concepts derived from the findings of MHC restriction that the TCR has double specificity, one for the MHC, another for the peptide. One interpretation is that the contacts of CDR1 and CDR2 are responsible for positive selection, whereas it is CDR3 interaction that offers the peptide specificity.

STAGES IN ANTIGEN PRESENTATION

There are several stages in presentation. The antigen molecules need to be taken into vacuoles where they are processed, resulting in the generation and display of the

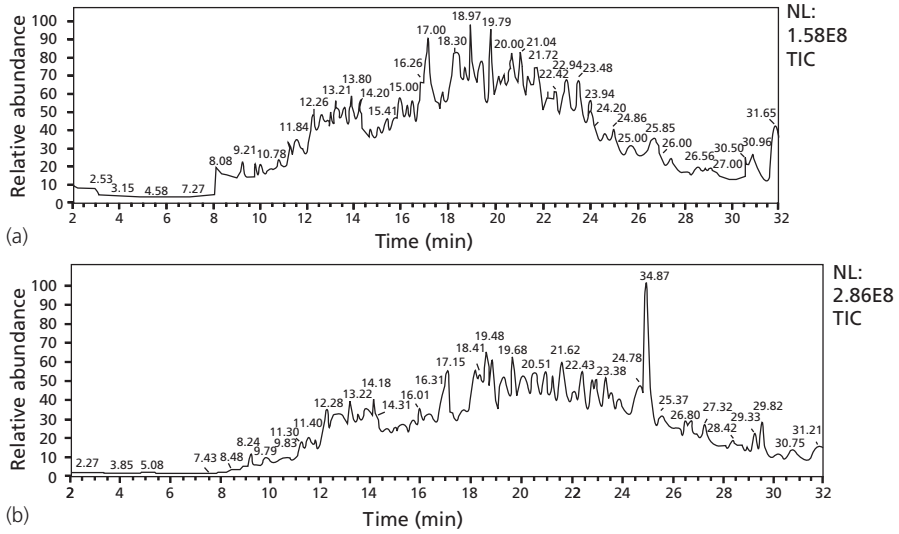

Figure 2 *Biochemical analysis of peptides extracted from the class II MHC molecule I-A . The two panels represent the separation of peptides by reverse phase chromatography. The top panel indicates the many peptides are bound to class II molecules. In the lower panel, the antigen-presenting cells (APC) were given lysozyme. Note the large peak at 25 min: it is the dominant peptide processed from lysozyme. (Redrawn from Gugasyan et al. 2000, with permission.)*

peptide–MHC complex. Following the display, there is the encounter with the T cells that have receptors specific for the complex. Recognition by the T cell is followed by activation depending on a number of important variables.

Antigen-presenting cells can present peptides from free proteins, or from proteins that form part of the structure of microbes or unicellular organisms. APCs can also interact directly with peptides found in extracellular fluids, and can also present proteins from cells or cell material that has been phagocytosed. (The term 'crosspresentation' or 'crosspriming' has been used to describe T-cell activation by a cell that lacks MHC molecules, but can be taken up when dead by an APC, which then presents its antigens.)

Protein antigen can enter the APC by fluid phase endocytosis or by ligand receptor internalization (reviewed in Unanue 2001). Under both conditions, the internalized protein eventually encounters the MHCII molecules, although the amount of uptake is favored in the case of receptor-mediated uptake. Uptake of viruses, bacteria, or other pathogens can be mediated by a variety of receptors such as the Fc and the complement receptors; or other surface receptors such as the scavenging receptor or those that recognize various polysaccharides. Together with bacteria, an important component of the recognition is the Toll-like receptor (TLR) (Medzhitov et al. 1997; Takeuchi et al. 1999; reviewed in Krutzik et al. 2001; Janeway and Medzhitov 2002). TLRs are involved in signaling during the interaction of the bacterium or its products with the surface of the phagocytes. Each TLR has a preference for the microbial ligands.

For B cells, the receptor for the protein antigen can be the specific membrane-bound immunoglobulin (Lanzavecchia 1990). Uptake by specific B cells can increase the efficiency of presentation by about a 1000-fold over uptake by fluid phase endocytosis. It is the uptake of antigen by specific immunoglobulin of B cells, and the subsequent presentation of its peptides to T cells, that form the basis of B cell–T cell interaction that leads to antibody formation.

The processing of proteins and the loading of MHC molecules with peptides are fast events (Table 4). Usually by an hour or less after uptake, complexes of peptide and MHCII molecules can be found on the cell surface (Ziegler and Unanue 1981; Cresswell 1994; Watts 1997, 2001). The proteins are usually processed in deep vesicular compartments of the APC, which are rich in lysosomal proteases as well as having a reducing activity. A number of lysosomal proteases are involved in the proteolytic processing (reviewed in Villadangos et al. 1999; Watts 2001). Alkalinization of the acid proteases of the APC results in inhibition of antigen processing (Table 4). The protein antigens have to be denatured and/or partially fragmented before interaction with MHC molecules. A scenario is that the protein, or a large polypeptide derived from it, is unfolded, after which the MHC binds to a preferred segment. The peptide segment is then protected from catabolism (Donermeyer and Allen 1989). Enzymes with amino- and carboxy-peptidase activity proceed to trim the segment of the polypeptide that extends beyond the combining site (Nelson et al. 1997). The reduction and partial fragmentation of the protein give it sufficient conformational flexibility to allow for binding to class II molecules. Although peptides and partially denatured proteins can be bound by class II molecules, most globular proteins in their native state do not bind. As indicated in Table 4, peptides can bind to class II molecules and these do not require any further handling. In fact, peptides can bind to surface MHCII molecules directly.

Proteins that are denatured meet the nascent MHCII molecules, recently arrived from the endoplasmic reticulum (ER) via Golgi vesicles (reviewed in Pieters 2000; Unanue 2002). The lysosomal vesicle that receives the antigen also receives the nascent MHCII molecules. MHCII molecules are transported from ER–Golgi to the loading vesicle together with another polypeptide called

Table 4 *Experiments indicating the processing of protein antigens for class II major histocompatibility complex (MHC) presentation*

Antigen-presenting cell display					
Protein	**Peptide**	**Time (min)**	**Treatment**	**Fixation**	**Peptide–MHC**
1 +	–	0	–	–	No presentation
2 +	–	60	–	–	++++
3 +	–	60	Chloroquine	–	+/–
4 +	–	60	–	Fix at time 0	No presentation
5 +	–	60	–	Fix at 60 min	+++
6 –	+	0	–	–	+++
7 –	+	0	Chloroquine	–	+++
8 –	+	0	–	Fix at time 0	++

A protein antigen requires a period of time (indicated as 60 min) before presentation takes place to T cells. Inhibition of intracellular proteolysis by alkalinization of the vesicular pH by chloroquine inhibits presentation (no. 3). Cells that processed the protein and displayed it in their surface MHC molecules can be fixed and still present to T cells (no. 4). Peptides do not need processing (nos 6–8).

the invariant chain (so called because it does not show the allelic polymorphism of the class II molecules). The invariant chain is about 33 kDa, but shows some heterogeneity, with post-translational changes and various different splicing forms (Cresswell 1996). Three invariant chain forms a complex with three α–β dimers of MHCII. The function of the invariant chain is to direct the MHC molecule to the loading vesicle (Lotteau et al. 1990). It also folds over the peptide-binding site, and in this way blocks the binding to it of peptides found in the ER (Roche and Cresswell 1990). Once the proteins arrive at the loading vesicle, the invariant chain is degraded by acid proteases, particularly involving cathepsin S (Riese et al. 1996), liberating the peptide-binding site of the class II molecules, which can then receive peptides from its surrounding (Villadangos et al. 1997).

Peptide loading is markedly favored by a second MHC-like molecule named HLA-DM (Cho et al. 1991; Kelly et al. 1991). DM interacts with class II but does not bind peptides (it is also made of two chains, but has a combining site that is closed) (Fremont et al. 1996; Mosyak et al. 1998). The function of DM is a catalytic one, favoring the rapid dissociation of weak peptides and fostering the binding and stability of strong binding ones. In this way, DM serves to edit peptide selection, favoring the binding of peptides with strong affinities (Morris et al. 1994; Sloan et al. 1995).

Finally, it should be noted that microbes affect intracellular processing, attempting to divert the presentation to T cells. One mechanism is to inhibit fusion of the phagocytic vacuole with lysosomes avoiding processing. This inhibition occurs most notably with *Mycobacterium tuberculosis* (Armstrong and Hart 1975; Ferrari et al. 1999). A number of viruses affect primarily the processing by way of MHCI presentation.

APC INTERACTIONS WITH T CELLS

T cells contact the APC bearing the peptide–MHC complex and establish firm contact that lasts for several hours. During the time of contact, molecular events take place in the T cell that lead to their activation. This contact is in the immunological synapse. At the synapse, there is an early and rapid rearrangement of the TCR, and its accompanying components, with their coalescence into a tight compact area (Shaw and Dustin 1997; Grakoui et al. 1999; Shaw and Allen 2001; Krummel and Davis 2002). Likewise, the peptide–MHC complex of the APC also reorganizes into a single large aggregate. In the synapse, the TCR moves towards the center of it whereas molecules that favor adhesion, such as the intercellular adhesion molecule (ICAM), move towards the periphery of the complex. This segregation of the membrane molecules allows for effective contact of each of the complementing pairs of molecules of the T cell and the APC. The relatively small TCR and the peptide–MHC spans about 12 nm, whereas very large molecules such as CD43, CD44, and CD45 move toward the periphery of the synapse and, by doing this, do not hinder the interaction. The synapse forms part of the events that favor the transcriptional activation of the TCR (Lee et al. 2002). The thinking behind this is that the TCR may contact the complexes several times in a cycle of binding/dissociation. Eventually, the TCRs are internalized and lost (Lanzavecchia et al. 1999).

The amounts of peptide–MHC complex that stimulate a T cell have been determined in some systems (Latek and Unanue 1999). They vary depending on the state of activation of the T cell. In unstimulated T cells the amounts are about 200–300 complexes per APC. This number is reduced greatly, to about 10 or even less, in already activated or 'memory' T cells. Note should be made of the high sensitivity of the immature thymocyte to MHC–peptide complexes. The estimates are that only two or three are needed for negative selection and killing of an immature thymocyte. Thus, central thymic recognition is highly susceptible to low numbers of complexes, as expected for a system that needs to eliminate self-reactive T cells.

The presentation of a peptide–MHC complex by an APC, and its engagement by a specific TCR, result in the activation of the T cell only under suitable conditions of cellular activation brought about by an inflammatory response. These conditions are those that lead to a change in the APC, in turn leading it to express a number of auxiliary molecules that foster the subsequent activation of the T cell. In situations where the number of peptide–MHC complexes is limiting and the APC is in a 'resting' state, the T cell will not respond or even undergoes a process of negative response called anergy (see Figure 1, p. 377).

The current thinking follows the 'two-signal hypothesis' (Bretscher and Cohn 1970): TCR engagement by itself is not sufficient to drive the T cell into proliferation and differentiation, and the additional participation of auxiliary molecules plays a role in what is a highly complex series of cellular events. Note that, in cell cultures, engagement of the TCR, say with an antibody, will not suffice to drive the T cell to proliferate unless a second antibody is added to one of the costimulator molecules (in this example to CD28). Engagement of the TCR without costimulation may result in anergy (Jenkins and Schwartz 1987).

Three sets of molecules foster the activation of a T cell on interaction with an APC: the membrane 'costimulator' molecules, which drive the T cell to enter the DNA cycle, activate some cytokine genes, and maintain their viability; adhesion molecules which serve to maintain the physical interaction of the two cells; and the cytokines which are released by the activated APCs and modulate the activation and differentiation of the T cell.

A number of cell surface molecules have been identified and are known to play an important role as costimulators (Linsley et al. 1990; Gross et al. 1990; Turka et al.

1990; Gimmi et al. 1991; Linsley and Ledbetter 1993; Sperling and Bluestone 1996). The CD28 molecule, a homodimer with a single immunoglobulin-like domain, is expressed on the T cell in small amounts, but increases during the activation process (Sharpe and Freeman 2002). The complementary ligand of CD28 is made up of two molecules on the APC, termed B7-1 (CD80) and B7-2 (CD86). Both are expressed in small amounts, but markedly increase their expression if the APCs are activated. Engagement of CD28 fosters T cell–APC adhesion, and increases the transcription of a number of cytokine genes, by activating a number of intracellular transcription systems including the Lck and itk molecules and the Jun and NF-κB pathways. Engagement of CD28 also increases the survival of the T cell through the expression of antiapoptotic molecules (such as BCl-xL). The end-result is a stronger response of the T cell in terms of both its growth survival and its production of cytokines such as IL-2. Mice with created mutations of the CD28 or B7 molecules respond poorly to antigen molecules.

Expression of the B7 family is fostered by activation of the APCs, by cytokines, particularly by TNF, and importantly by interaction with microbes and their products through the TLR molecules that activate the NF-κB pathways of transcription. B7-1 and B7-2 are also induced as a result of interactions involving another critical pair of surface molecules, the CD40 molecule expressed on B cells, DCs, and monocytes (Grewal and Flavell 1998). CD40 has structural homology to the TNF receptor family. Its complementary molecule is a member of the TNF family, the CD40-ligand molecule expressed on T cells in small amounts. Engagement of CD40 on DCs leads to a heightened expression of B7-1 and B7-2. Engagement of CD40 on the B cells is a critical step, because it results in their proliferation and differentiation to antibody-secreting cells. (The importance of the CD40–CD40-ligand molecules can best be found in children with mutations in the gene for the CD40-ligand, called 'hyper-IgM syndrome': its T cells are poor in providing helper activity, which clinically translates into problems in antibody formation against infections with a number of microbes.)

Other surface molecules are involved in fostering the APC interaction with T cells. In humans, the CD2 molecules on T cells also play a critical role akin to CD28. Also participating in the interaction are molecules that foster the adherence of the various cells. Among the adhesion molecules, the intercellular adhesion molecule 1 (ICAM) molecule in APCs is particularly important, fostering the contact with the α_2-β_1-integrins of T cells.

Soluble molecules also influence the outcome of the T cell–APC interaction. Cytokines produced by the APC influence the program of gene expression on T cells in important ways (Hsieh et al. 1993). Among the cytokines that are released on interaction of APCs with microbes, particularly bacterial products, are TNF, IL-1, IL-12, and IL-18. The last two influence the expression of interferon-γ by the CD4 T cell, a program of differentiation called 'Th1' (which contrasts with situations in which the cytokine Il-4 drives the expression of IL-4) (reviewed in Farrar et al. 2002; Murphy and Reiner 2002).

Finally, it is critical to stop the APC–T-cell interaction and regulate the extent of activation of the T cell. The interaction ends, of course, as the stimulus, the antigen molecules, becomes progressively more limiting and finally disappears. Also controlling the interaction are negative regulatory molecules. The molecule CTLA-4 is expressed briefly in the activated T cell. Its engagement by the B-7 molecules on APCs dampens T-cell activation. (Mouse strains with an absence of functional CTLA-4 show marked hyperproliferation of lymphocytes (Thompson and Allison 1997).) Cytokines also dampen the activity of APCs, and this most prominently includes IL-10 and transforming growth factor β.

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CD4$^+$ T lymphocytes

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INTRODUCTION

CD4$^+$ T cells are the conductors of the immunological orchestra, being not only responsible for many important effector functions, but also necessary for the activation of many other aspects of the immune response, including B lymphocytes, CD8$^+$ T lymphocytes, macrophages, and other inflammatory cells (Figure 1) (Boes and Ploegh 2004). Thus, there are few immune responses that do not involve CD4$^+$ T cells at some stage, a role that is underlined by the life-threatening susceptibility to infection seen in patients with a deficiency of this population in, for example, human immunodeficiency virus (HIV) infection.

NATURE AND ROLE OF CD4$^+$ T LYMPHOCYTES

CD4$^+$ T cells account for 60–70 percent of the T lymphocytes found in the bloodstream and in most organized lymphoid organs, including the spleen and lymph nodes, as well as the lymphoid tissues associated with the mucosal surfaces such as the Peyer's patches, appendix, and tonsils. Mature CD4$^+$ T cells are characterized by the expression of CD3, an αβ T-cell receptor (TCR) and the CD4 molecule, a four-domain monomeric member of the immunoglobulin (Ig) superfamily that binds major histocompatibility complex class II (MHC II). CD4 is also the primary receptor for HIV entry, binding the gp120 surface protein. Most resting CD4$^+$ T cells also express other pan-T-cell markers such as CD2, CD5, and CD28, many of which are important during the process of activation (see below). Unlike immature T cells in the thymus, mature CD4$^+$ T cells cannot express the CD8 molecule.

By virtue of the presence of CD4, the vast majority of this population recognizes peptide antigen when presented by MHC II molecules on the surface of an accessory cell, although there is an unusual subset of 'natural' CD4$^+$ T cells that accounts for less than 5 percent of the population and which recognizes nonclassic major histocompatibility complex (MHC) molecules such as CD1 (see below). Only a few cell types can be involved in the activation of CD4$^+$ T cells by foreign antigen – dendritic cells (DC), B lymphocytes, and activated macrophages. During inflammation,

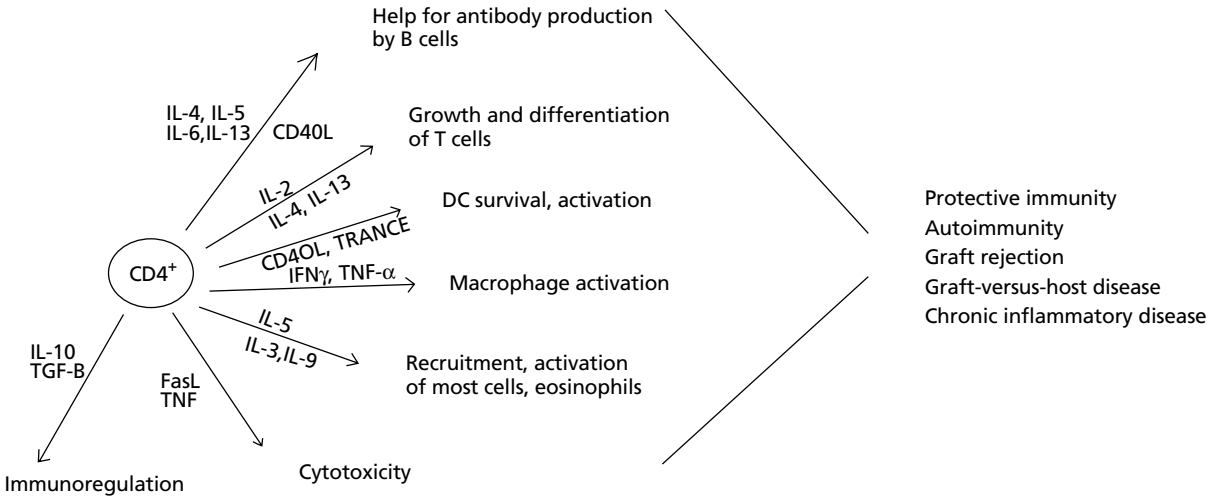

Figure 1 *Functions and roles of CD4+ T lymphocytes in protective immunity and immunopathology. DC, dendritic cell; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.*

other cell types can be induced to express MHC II, but the role of these cells in presenting antigen to CD4+ T cells in vivo is unclear.

As discussed elsewhere in this volume, antigens that are presented on MHC II molecules are derived from proteolytic processing mechanisms that generate 12–24 amino acid peptides within the endocytic pathway of the antigen-presenting cell (APC). As a result, CD4+ T cells are geared to recognize antigens that are taken up into APCs by phagocytosis, endocytosis, or pinocytosis. Thus, CD4+ T cells are important in protective immunity against extracellular pathogens such as pyogenic bacteria, large parasites, and other free-living organisms. In addition, intracellular pathogens such as *Mycobacterium*, *Salmonella*, and *Leishmania* spp., which survive in and colonize endosomes or phagosomes, can be recognized by CD4+ T cells (Boes and Ploegh 2004). Indeed, it is this group of infections that causes the biggest problem in CD4 deficiency states. Nevertheless, it is important to note that CD4+ T cells also play a prominent and often essential role in responses to other types of intracellular pathogens, which do not invade endosomes and characteristically stimulate MHC I-restricted T-cell responses (e.g. viruses). This may be secondary to the uptake of infected and/or damaged cells by phagocytic cells such as DCs or macrophages, or to the recycling of surface-expressed viral proteins via the endocytic pathway.

CD4+ T cells also play critical roles in most immune responses of pathological importance, including organ-specific autoimmune disease, chronic inflammatory disease, and other hypersensitivity disorders. In addition, alone or together with other lymphocytes, CD4+ T cells are important in the rejection of allogeneic transplants and in graft-versus-host disease after bone marrow transplantation (Figure 1).

LIFE HISTORY OF CD4+ T LYMPHOCYTES

Similar to all peripheral αβ T cells, CD4+ T cells differentiate from common lymphoid progenitor cells in the thymus, mostly during fetal and early postnatal life (Figure 2). The thymic processes of negative and positive selection ensure that the only CD4+ T cells that survive and are exported to the periphery have a TCR that can recognize foreign antigens when presented by self MHC II antigens, but cannot recognize self-tissue antigens (reviewed in Goldrath and Bevan 1999; Starr et al. 2003; Mathis and Benoist 2004). Positive selection occurs because maturing T cells are programmed to die by apoptosis unless they receive a signal from their TCR after it has interacted with self-peptides presented by self-MHC molecules, probably on the network of epithelial cells present in the thymic cortex. In turn, if the TCR has a very high affinity for self-MHC and peptide presented by thymic DCs, the cell will undergo negative selection ('deletion'), again by the induction of apoptosis. The exact timing of these events and their precise anatomical location are still somewhat controversial, but both selection processes occur soon after the T cell has acquired a CD3–TCR complex and when it still expresses both CD4 and CD8 markers ('double positives'). Thereafter, one of these markers is lost permanently as a result of silencing of the appropriate promoter; T cells with a TCR that recognizes peptides on MHC II molecules lose CD8 and retain the CD4 molecule; conversely, T cells with a TCR that recognizes peptides on MHC I molecules lose CD4 and retain CD8 (Figure 2). After commitment to the CD4 lineage, the single positive T cells enter the thymic medulla, where they spend a further period of 5–6 days, before emigrating to the thymus-dependent

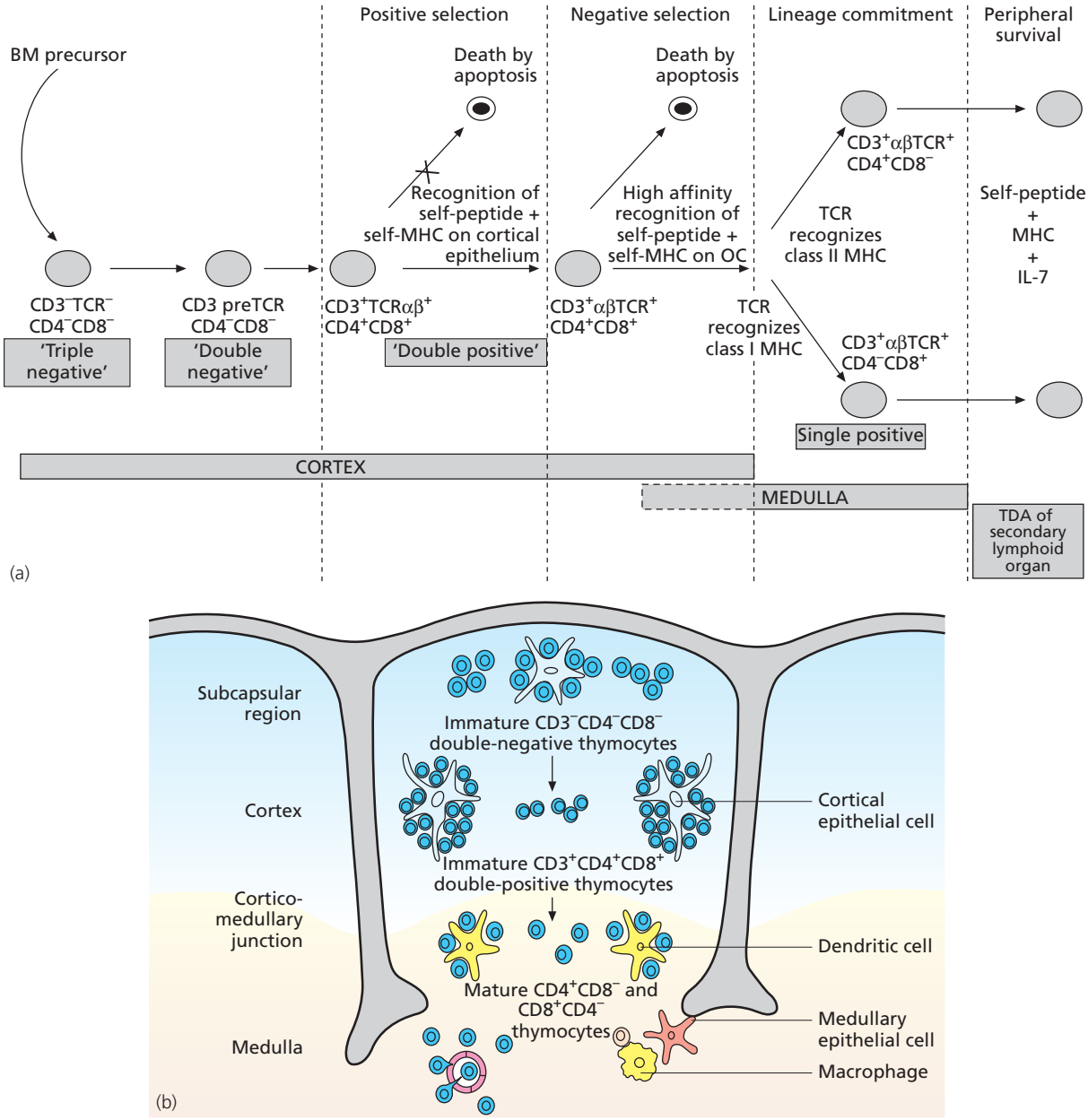

Figure 2 *Selection and differentiation pathways of T lymphocytes. Lymphoid precursor cells derived from the bone marrow enter the thymic cortex during fetal life and are induced to begin expansion and differentiation under the influence of soluble mediators and cell-surface molecules expressed by cortical epithelial cells and mesenchymal cells. At this time, the cells do not express a T-cell receptor (TCR), CD3, or CD4 and CD8 ('triple negative'), but they then rapidly acquire a pre-TCR composed of a TCR β chain and the pre-Tα chain, together with low levels of CD3 ('double negative'). Successful expression of this pre-TCR induces expression of both CD4 and CD8, and the mature αβ TCR replaces the pre-TCR. These 'double-positive' cells are now subjected to positive and negative selection, in which only those T cells expressing a TCR capable of recognizing self-peptides + self-MHC on cortical epithelium (positive selection), but which cannot recognize self-peptides + self-MHC strongly on dendritic cells (negative selection), are allowed to survive. This small fraction of cells (2–3 percent of total output) is then restricted to recognizing peptides from foreign antigens that are in complex with self-MHC molecules, but cannot recognize self-antigens with sufficient strength to cause autoimmunity. These selection processes probably occur mainly in the thymic cortex* **(b)**, *although the medulla may also play a role. After selection, T cells with a TCR that recognizes MHC I down-regulate the CD4 molecule and retain CD8 expression, whereas T cells with a TCR that recognizes MHC II down-regulate the CD8 molecule and retain CD4 expression. The resulting single positive T cells now remain in the medulla for a short period of further maturation, before exiting the thymus to recirculate from blood to the thymus-dependent areas (TDA) of secondary lymphoid organs. There they are maintained alive by continual low-level stimulation of their TCR ('tickling') by self-peptide + self-MHC on antigen-presenting cells in the tissues and by the cytokine interleukin 7 (IL-7). The insets show the histological appearance of the normal thymus, illustrating the cortex (C) and medulla (M), and a schematic representation of the local differentiation events. (From Janeway et al. 2001.)*

areas (TDA) of secondary lymphoid organs via the bloodstream.

At this time, the CD4+ T cells are referred to as 'naive', because they have not yet encountered antigen; naive T cells are in a resting state and can survive in this way for many years without stimulation by foreign antigen, or without constant output from the thymus. It was previously believed that outside stimuli were not required for this long-term persistence and that it was an inherent property of naive T cells. However, it is now clear that such T cells require continual stimulation via their TCRs to survive in the periphery (Goldrath and Bevan 1999). This does not involve foreign antigen, but seems to require recognition of the same self-peptide–MHC II complex that was responsible for positively selecting the T cell in the thymus. It is believed that DCs in secondary lymphoid organs are responsible for presenting these complexes and other factors, including cytokines such as interleukin (IL) 7 may also be involved (Figure 2) (Marrack and Kappler 2004; Stockinger et al. 2004). As a result of this 'tickling' by self-antigen under physiological conditions, the T cell does not usually undergo clonal expansion or become fully activated; instead it receives survival signals that allow it to persist in the peripheral tissues in a resting state. Interestingly, however, if the same CD4+ T cells are in an immunocompromised host, they can proliferate extensively under the influence of the same signals in an attempt to fill up the space in the 'empty' immune system. This has led to the concept that the normal immune system is tightly regulated to maintain a pre-set number of T cells and that this is determined by competition for a finite amount of MHC–peptide ligands and other survival signals. The phenomenon of 'homeostatic proliferation' is also important in understanding how the immune system may compensate for insults such as irradiation, chemotherapy, or HIV infection especially in adults, where the thymus is almost inactive.

ACTIVATION OF CD4+ T LYMPHOCYTES

Inappropriate CD4+ T cell activation can have serious consequences for the body because of the effects that these cells can have on surrounding tissues via the production of cytokines and activation of nonspecific effector cells. For these reasons, the activation of naive CD4+ T cells is subject to a number of stringent controls, including restrictions in the type and location of cells that can present antigen with MHC II, as well as a requirement for a sequence of highly defined signals to occur before full stimulation of the T cell is achieved (Boes and Ploegh 2004). For a complex antigen to be recognized by CD4+ T cells, it must be internalized by an appropriate APC, degraded into peptides in an endosomal compartment, and loaded on to newly synthesized class II MHC molecules, which are then expressed on the surface of the APC. In addition, the APC must express a number of nonpolymorphic accessory molecules that trigger specific receptors on the T cell and provide 'co-stimulation' of the signal obtained via the TCR (Sharpe and Freeman 2002) (Figure 3).

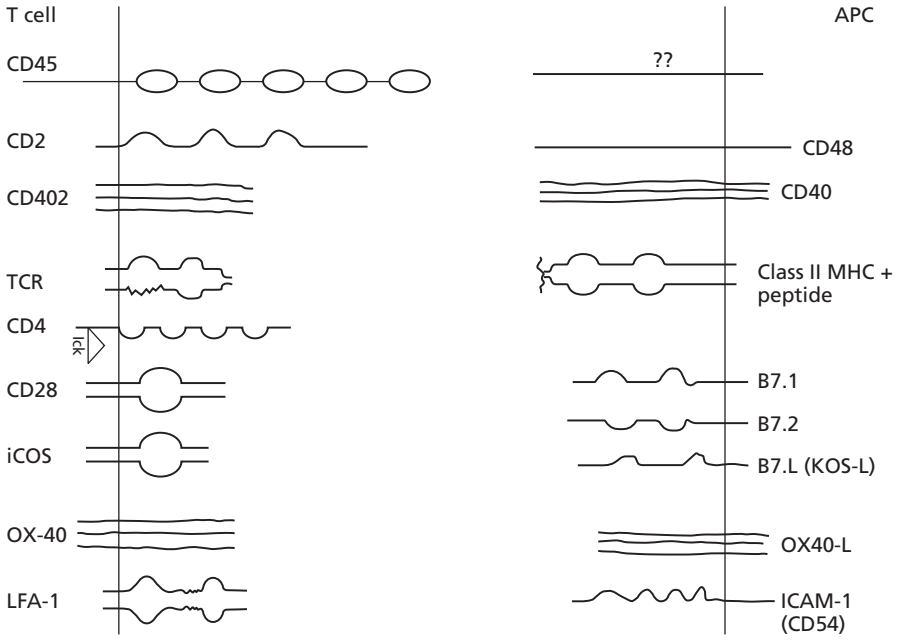

Figure 3 *Co-stimulatory molecules involved in T cell–antigen-presenting cell (APC) interactions and in the formation of the immunological synapse. ICAM, intercellular adhesion molecule; iCOS, inducible co-stimulatory molecule; MHC, major histocompatibility complex; TCR, T-cell receptor. See text for details.*

DENDRITIC CELLS AND PRIMING OF CD4+ T LYMPHOCYTES

In most immune responses, priming of CD4+ T cells occurs in the thymus-dependent areas (TDA) of secondary lymphoid tissues and the APCs are DCs. DCs are a specialized group of MHC II expressing leukocytes, which can be found in most tissues of the body, but they are most prominent in secondary lymphoid organs. There may be a number of different DC subsets and their exact functions may vary with their anatomical location, but their overall properties make them ideal candidates for initiating and directing CD4+ T-cell responses. They have several distinct, but related, roles (Banchereau et al. 2000; Guermonprez et al. 2002; Steinman et al. 2003). First, they are extremely effective at taking up and processing complex antigens for MHC II-restricted presentation. In addition, they are unique among APCs in their ability to patrol the tissues and solid organs of the body, before carrying antigen to the TDA of lymphoid organs where they meet naive T cells (Itano et al. 2003; Boes and Ploegh 2004). DCs are also capable of expressing a wide range of co-stimulatory molecules and can secrete soluble mediators that help in the activation and differentiation of T cells. Finally, and perhaps most importantly, all these DC functions are exquisitely sensitive to modulation by environmental signals derived from pathogens and damaged tissues, allowing DCs to act as the critical cell in linking innate and adaptive immunity. Essentially, they recognize threats to the body ('danger') and translate them into inflammatory signals that, together with specific antigen, the immune system can respond to in an appropriate manner (Banchereau et al. 2000).

The life history and functions of DCs have been elucidated in recent years, using a combination of in vivo and in vitro approaches (Figure 4). The tissue-resident DC is typified by the Langerhans' cell (LC) found in the epidermis of the skin, but analogous cells can be found in other epithelial surfaces and in solid organs. These cells are relatively immature, quiescent cells that are derived from a common myeloid precursor circulating in blood, the role of which is to continually sample the local environment. This reflects an extremely high and constitutive ability to take up exogenous materials by a variety of different mechanisms, including endocytosis, phagocytosis, and macrophagocytosis. However, at this stage, LCs and equivalent DCs are not effective APCs. Although they express MHC II, most of this is retained intracellularly within vesicular compartments and the small amount that reaches the cell surface is recycled rapidly. DCs remain in this quiescent state until exposed to products of pathogens or other inflammatory stimuli (Table 1). These include bacterial cell wall components such as flagellin, lipopolysaccharide (LPS), and other glycolipids, as well as bacterial DNA, viral nucleic acids, heat shock proteins, and a variety of cytokines released by infected or damaged cells, including IL-1, IL-6, and tumor necrosis factor (TNF) α. By binding to appropriate Toll-like receptors (TLR) (Barton and Medzhitov 2002) or cytokine receptors, these so-called 'danger signals' alert the DCs to the presence of potential harm and stimulate a number of coordinated, activation-related events in the DCs (Banchereau et al. 2000). Endosomal pH levels fall and acidic endosomal protease activity is increased, leading to increased availability of antigenic peptides as a result of enhanced processing of ingested antigen. Stable peptide–MHC II complexes are then exported to the cell surface and remain there for considerable periods of time (48 h or more). In parallel, the endocytic activities of the DCs fall dramatically, meaning that the cell focuses its MHC II processing and presentation activities on the antigen(s) that have provoked the threat of danger. At the same time as these changes in intracellular function, the DCs become motile and alter their expression of chemokine receptors, so that they leave the tissue via afferent lymphatic vessels and migrate to the draining lymph node. The most important chemokine receptor involved in these processes is CCR7, which binds the chemokines CCL (ELC) and CCL (SLC), which are produced in the TDA of lymphoid organs, and are responsible for recruiting

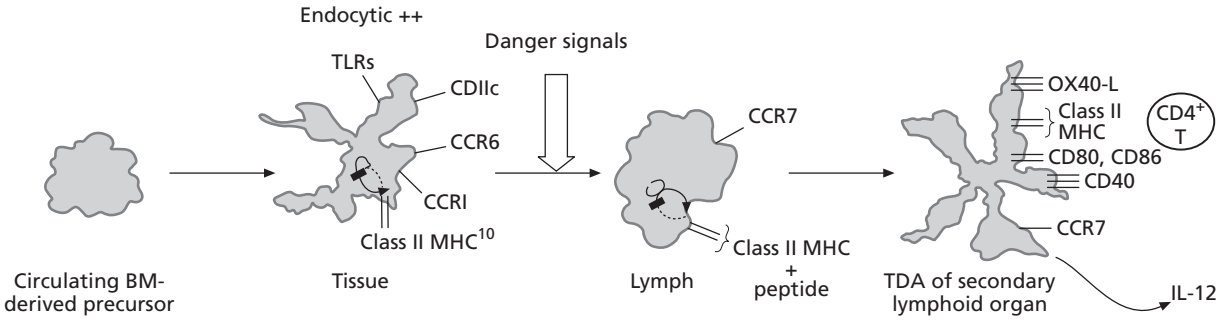

Figure 4 *Life history of dendritic cells (DCs). DCs derived from circulating bone marrow-derived precursors (myeloid or lymphoid) enter tissues in a relatively immature state; stimulation by pathogen-derived factors or inflammatory mediators then induces them to mature and emigrate from the tissue to the thymus-dependent area (TDA) of draining lymphoid organs via the afferent lymphatics. BM, bone marrow; IL, interleukin; MHC, major histocompatibility complex; TLR, Toll-like receptor. See text for details.*

Table 1 *Danger signals for dendritic cells*[a]

	Receptor
Pathogen associated molecule	
Bacterial lipopeptide	TLR-1
Bacterial lipopolysaccharide	TLR-2, TLR-4, CD14
Double-stranded RNA	TLR-3
Flagellin	TLR-5
Peptidoglycan	TLR-6
Bacterial DNA	TLR-9
Endogenous molecule	
Heat shock proteins	CD91
TNF-α	Type 1 TNF receptor (p55)
IL-1	IL-1 receptor
Histamine	H_1 histamine receptor

IL, interleukin; TNF, tumor necrosis factor.
a) These may be pathogen-associated molecular patterns (PAMPS) that normally interact with members of the Toll-like receptor (TLR) family, or endogenous mediators released during tissue.

both DCs and naive CD4+ T cells to this site (Randolph 2001). Quiescent DCs in tissues do not express CCR7, but have receptors for chemokines produced by tissue cells such as keratinocytes and intestinal epithelial cells (CCR6), as well as for chemokines produced during inflammation (CCR1, CCR2, CCR5). After activation, the expression of these receptors is inhibited, partly because activated DCs produce inflammatory chemokines that induce downregulation of their own receptors; in parallel, CCR7 expression is induced. As a result, the activated DC loses its ability to localize in tissues or inflamed sites and acquires the ability to migrate specifically to the TDA of lymph nodes (Figure 4).

The other important effect of DC activation is to induce the expression of the co-stimulatory molecules required for full activation of T lymphocytes, including CD80 (B7.1), CD86 (B7.2), and CD40. In addition, they become capable of producing immunomodulatory cytokines such as IL-12 (Figure 4). Together with all the other changes discussed above, the result is that DCs arriving in the TDA of lymphoid organs are now fully competent to activate and polarize effector T-cell responses. In return, the DCs receive signals from the T cell that promote DC survival and activate them further, much of which is mediated by CD40 ligand, which is in turn upregulated on the activated T cell (Banchereau et al. 2000).

DCs are not a homogeneous population and a number of different subsets have been described on the basis of origin, tissue distribution, differentiation status, and function (Banchereau et al. 2000; Guermonprez et al. 2002). In many species, it appears that DCs may develop from either myeloid or lymphoid precursors and in humans these are referred to as 'monocyte-derived' and 'plasmacytoid' DCs. The exact relationship between the subsets is not yet clear, but their potential importance is that different populations of DCs may have distinct effects on CD4+ T cells, selectively stimulating T-helper (Th) 1 or Th2 responses, or even T-cell tolerance. In addition, they may localize preferentially in different tissues.

OTHER TYPES OF ANTIGEN-PRESENTING CELL

It is now considered that DCs are the principal, if not the only, APCs capable of priming CD4+ T cells in vivo. However, other MHC II-expressing cells can act as APCs under some circumstances, especially in the later stages of primary immune responses or in secondary responses. Although macrophages normally degrade materials to the amino acid level, macrophages activated by microbial stimuli and/or interferon (IFN)-γ express high levels of MHC II and co-stimulatory molecules. Such macrophages may be able to sustain ongoing CD4+ T-cell responses, or even initiate them. B lymphocytes possess many of the features of APCs, including the constitutive expression of MHC II, the ability to process proteins in the endocytic pathway, and the expression of co-stimulatory molecules. In practice, the resting B lymphocytes found in normal lymphoid tissues cannot prime naive CD4+ T cells, but, as the response matures, direct presentation of antigen by activated B cells in germinal centers is essential for previously primed CD4+ T cells to help the differentiation of antibody-producing B lymphocytes. This presentation reflects the very high efficiency of antigen-specific B cells in internalizing and processing their cognate antigen via surface Ig. As the frequency of antigen-specific B cells is increased after primary immunization, these cells may also contribute to the initiation of secondary immune responses by presentation to CD4+ T cells.

Many tissue cells can express MHC II during inflammation, probably under the influence of IFNγ. This is exemplified by epithelial cells in the intestine, stomach, kidney, and thyroid during chronic inflammation, as well as by the β cells of the pancreatic islets in type 1 diabetes and by vascular endothelial cells in many different organs. The role of these cells in T-cell-mediated immune responses is controversial, because they usually do not express co-stimulatory molecules. Nevertheless, previously activated CD4+ T cells do not require as much co-stimulation as naive T cells and therefore MHC II-expressing tissue cells may sustain ongoing inflammation or protective immunity. Alternately, some workers believe that presentation of antigen by such APCs represents an attempt to downregulate the immune response by inactivating CD4+ T cells (see below) (Schwartz 2003).

SUPERANTIGENS

A specialized group of proteins can activate CD4+ T cells in an MHC II-restricted fashion, without the need for processing or co-stimulation. These are the 'superantigens', which act by forming a direct connection

between nonpolymorphic regions on the MHC II molecule and certain families of TCR (Sundberg et al. 2002). Both $CD4^+$ and $CD8^+$ T cells can be activated in this way, but most superantigens act predominantly on $CD4^+$ T cells. Most are also microbial products and they are capable of activating up to 25 percent of all T cells because of their ability to bind to framework regions on the TCR distant from the MHC–peptide-binding site. Although their recognition by T cells does not require proteolytic processing, superantigens must be presented with appropriate MHC molecules on the surface of APCs, in the same way as conventional peptide antigens. Superantigens have proved extremely useful experimental tools for dissecting T-cell functions and tolerance, as the responding families of T cells can be readily identified using appropriate specific antibodies. In addition, they may contribute to the pathogenesis of the infections caused by the various organisms by virtue of their ability to activate large numbers of T cells, stimulating the production of large amounts of proinflammatory cytokines. The best example is TSST-A, which causes the toxic shock syndrome associated with vaginal infection by *Staphylococcus aureus* after chronic tampon retention, and the others may have analogous effects.

ACCESSORY MOLECULES AND $CD4^+$ T-CELL ACTIVATION

As we have noted, several nonpolymorphic molecules on $CD4^+$ T cells are essential for effective activation by appropriate peptide–MHC complexes (see Figure 3). This partly reflects the fact that the TCR itself does not undergo major conformational change after binding to antigen and also has very short cytoplasmic regions, meaning that it is incapable of initiating intracellular signaling on its own. In addition, each TCR–MHC–peptide interaction is of relatively low affinity and may be extremely transient. Thus, additional molecules are required both to stabilize the binding of the T cell to an APC carrying specific antigen and to assist the biochemical signaling pathways.

The CD3 complex that is physically linked to the TCR on all T cells is critical to T-cell signaling, but does not contribute to the interaction with the APC and its functions are considered in T cell receptors. CD4 itself is the most important of the other molecules involved in these functions. It is a single chain molecule of 55 kDa, comprising four Ig superfamily domains, the outermost of which binds to nonpolymorphic residues on the α_2 domain of the MHC II molecule, distant from the peptide-binding groove. It is likely that each CD4 molecule may bind two separate MHC II molecules, each of which may bind a specific TCR, thus leading to the formation of 'pseudo-dimers'.

The binding of CD4 has two major effects on the T cell. First, it stabilizes the binding between the T cell and the APC, effectively increasing the affinity of the TCR for MHC–peptide and reducing the off-rate of this interaction. The second critical function of CD4 is to initiate tyrosine kinase-mediated signaling via the TCR. CD4 associates with the lck tyrosine kinase in the cell membrane and, after binding to the same MHC II as that bound by the TCR, CD4 brings lck into contact with the CD3–TCR complex, allowing lck to phosphorylate and activate the CD3ζ chain. This is the first and critical step in TCR-mediated signaling (see T cell receptors) and an analogous function is served by CD8 binding to MHC I on $CD8^+$ T cells.

Many of the remaining nonpolymorphic co-stimulatory molecules belong to the CD28 or TNF/TNF receptor families (Sharpe and Freeman 2002; Croft 2003). CD28 is present on all mature $CD4^+$ T cells and is a homodimeric molecule; each of the chains of this molecule is a 44-kDa Ig superfamily molecule comprising a proximal Ig C-like domain and a distal Ig V-like domain. It binds to two related ligands present on professional APCs: B7.1 (CD80) and B7.2 (CD86). They are homologous to each other and to CD28. The B7 molecules appear to dimerize on the APCs, each unit binding a separate CD28 molecule, creating a zipper-like binding lattice that provides a highly stable and effective cell–cell interaction.

Co-ligation of CD28 after TCR–peptide–MHC binding is essential for most aspects of $CD4^+$ T-cell activation, including clonal expansion, effector T-cell differentiation, germinal center formation, and memory cell generation. The exact mechanisms of action of CD28 are still unclear, but it is a potent signaling molecule that can activate a number of intracellular pathways in the T cell, some of which are unique to CD28, including the activation of phosphatidylinositol-3-kinase. Together, these effects amplify and sustain signaling via the TCR pathway, leading to increased IL-2 production, prolongation in the lifespan of IL-2 mRNA, and improved $CD4^+$ T-cell survival, probably involving upregulation of anti-apoptotic molecules such as bcl_{XL} and of the telomerase enzyme that assists telomere repair in dividing cells. In the absence of these CD28-mediated effects, productive T-cell activation does not occur and $CD4^+$ T cells are effectively blinded to the antigen.

A molecule related to CD28, which plays somewhat similar roles at a different stage of the immune response, is the inducible co-stimulatory molecule (iCOS). It is highly homologous to CD28, but is expressed only after CD28-mediated activation of $CD4^+$ T cells and binds to a B7-related ligand (B7RP1) on B lymphocytes. The primary function of iCOS appears to be in promoting the differentiation of effector T cells after initial clonal expansion has occurred and it co-stimulates the production of cytokines such as IFNγ, IL-4, IL-5 and IL-10, but not IL-2. It is also important in determining the outcome of T-cell–B-cell cooperation and in germinal center formation.

CD40 ligand (CD40L) is the most critical of the TNF-related molecules involved in $CD4^+$ T-cell activation. A homotrimeric molecule expressed on activated $CD4^+$ T cells, it binds CD40 on DCs and B cells, and is required for T-cell help of B-cell differentiation (see below), as well as for the production of IL-12 and differentiation of IFNγ-producing Th1 cells in most immune responses. Its expression on $CD4^+$ T cells requires activation via TCR and CD28, and ligation of CD40 on DCs or macrophages stimulates the expression of B7 molecules and the production of IL-12. Thus, CD40L plays an essential role in positive feedback stimulation of $CD4^+$ T cells and APCs, as well as in delivery of effector T-cell function. Mice lacking CD40L have a severe deficiency in IL-12 production and Th1-mediated defenses against intracellular pathogens.

Other related molecules involved in the co-stimulation of $CD4^+$ T cells include OX40, 4-1BBL, and TRANCE, which bind the DC molecules OX40L, 4-BB, and RANK respectively. The specific functions of these molecules are still under investigation, but they may overlap or synergize with those of the CD40L–CD40 interaction. In addition, ligation of RANK by TRANCE appears to play a unique role as a survival signal for DCs after interaction with antigen-specific T cells.

A number of adhesion molecules act as accessory factors during $CD4^+$ T cell–APC interactions, particularly in the initial, antigen-independent stage before specific TCR–peptide–MHC binding. These include intercellular adhesion molecule (ICAM)-3, and its interaction with its ligand DC-SIGN may be the first event in the binding of T cells to DCs. Leukocyte function antigen 1 (LFA-1) and its ligand ICAM-1 (CD54) are expressed on both T cells and APCs, and they play an essential role in forming the highly organized binding complex (the 'immunological synapse') that is responsible for focusing the TCR on relevant MHC II–peptide complexes (see Processing and presentation of antigen by the Class II histocompatibility system). Other accessory molecules include CD2, which is expressed on all T cells and binds to ICAM-3 and CD48 on APCs. As well as acting as adhesion molecules, ligation of all these receptors can also enhance signaling within the T cell.

TOLERANCE AND IMMUNITY AS ALTERNATIVE OUTCOMES OF $CD4^+$ T-CELL ENCOUNTER WITH ANTIGEN

Productive activation of $CD4^+$ T cells is not the only possible consequence of presentation of MHC II–peptide complexes and, under some circumstances, the T cell may be inactivated, leading to the phenomenon of specific immunological tolerance. In this situation, the T cell not only fails to make an effective primary response to antigen, but is also unresponsive to subsequent re-stimulation with the same antigen, even if it is presented under conditions that would normally induce a strong immune response. Tolerance is critical for preventing immune responses to tissue antigens by self-reactive $CD4^+$ T cells that have escaped deletion in the thymus. It is also useful for preventing inappropriate responses to harmless foreign antigens, particularly when such responses could lead to tissue damage. Antigens of this kind include the fetus, food proteins, aerosol antigens, and commensal bacteria.

There are many theories and mechanisms to explain the induction of tolerance rather than productive immunity in $CD4^+$ T cells (Table 2). As all the antigens (self and foreign) that induce tolerance share the property of not stimulating inflammation and so not inducing the activation of APCs, it seems that tolerance develops when the antigen is presented by APCs that lack appropriate co-stimulatory molecules. Under these conditions, the $CD4^+$ T cell is partly activated after recognizing its specific peptide–MHC II complex on the APC, but does not receive the additional accessory stimuli necessary for full IL-2 production (Schwartz 2003). As a result, the T cell undergoes cell cycle arrest, probably mediated in part by accumulation of inhibitory factors within the cytoplasm, and it fails to make sufficient cell divisions to ensure an adequate primary response. In addition, the aberrant signaling in the T cell renders it unable to respond to subsequent stimulation via the TCR, permanently preventing effector cell differentiation. Despite these defects, these so-called 'anergic' T cells may also retain some functional capabilities, including the production of cytokines such as IL-10 and transforming growth factor (TGF) β, allowing them to inhibit the functions of other T cells and APCs. Such 'regulatory T cells' (T_{reg}) appear to play an important role in maintaining many forms of tolerance in vivo, because they can prevent the activation of naive, fully responsive $CD4^+$ T cells when antigen is next encountered (Maloy and Powrie 2001). A number of such T_{reg} cells have been described, of which the most studied recently is the $CD4^+CD25^+$ subset, which appears to be a naturally occurring regulatory cell derived from the thymus and which controls immune responses to self-antigens in peripheral tissues by as yet ill-defined mechanisms (Sakaguchi 2004). Others include the Tr1 cell, which differentiates in response to foreign antigen under the influence of environmental IL-10 and acts by producing IL-10 itself (Roncarolo et al. 2001).

Current evidence suggests that the APCs responsible for inducing tolerance in $CD4^+$ T cells may not be completely lacking in accessory molecules and that the process requires an alternate set of co-stimulatory events (Greenwald et al. 2002). Of these, the best understood is that involving CTLA-4, a homolog of CD28 that is expressed mainly on activated $CD4^+$ T cells, but which also can be involved in the earliest phase of T-cell interaction with APCs. Similar to CD28,

Table 2 *Potential mechanisms of tolerance*

Mechanisms	
Clonal deletion	Self-antigens in thymus
	Very high doses of peripheral antigens?
	Apoptosis after ligation of TCR
Clonal anergy	Self-antigens in thymus
	Self-antigens in periphery
	Harmless foreign antigens in periphery (foods, commensals, etc.)
	TCR ligation in absence of co-stimulation (or with dominant negative co-stimulation by, for example, CTLA-4) → partial activation, failure to transcribe IL-2 gene, defective clonal expansion, and no proliferation/IL-2 production on challenge
Regulatory T cells	T cells actively inhibit priming/functions of other, naive T cells
	Self-antigens in thymus – $CD4^+CD25^+$ T cells
	Self + foreign antigens in periphery
	$CD4^+CD25^+$ T cells generated by aberrant selection in thymus
	Regulatory T cells induced in periphery by resting APCs or APCs conditioned by, for example, IL-10
	$CD4^+CD25^+$ T cells act by cytokines, cell-cell contact
	Peripheral T_{reg} act by producing IL-10/TGF-β (Tr1, Th3)
Clonal ignorance	Antigen sequestered from T cell and not recognized by TCR
	Self-antigens such as lens protein, testis
	Specific T cells still present and potentially reactive

See text for details.
APC, antigen-presenting cell; IL, interleukin; TCR, T-cell receptor; TGF, transforming growth factor.

CTLA-4 binds CD80 and CD86 and was originally thought to have identical co-stimulatory functions. However, the observation that CTLA-4 knock-out mice developed fatal a lymphoproliferative disease led to the recognition that the principal role of CTLA-4 is to downregulate $CD4^+$ T-cell responses. The exact mechanisms responsible for this effect are not yet known, but it is clear that ligation of CTLA-4 transmits an inhibitory signal to the T cell and that this dominates over other, potentially stimulatory, signals. This can occur late in T-cell responses, when it is important to prevent over-activation of the immune response; this is probably the explanation for the high expression of CTLA-4 on activated T cells, when CD28 is also decreasing. In addition, CTLA-4 has higher affinity for CD80 and CD86 than CD28, meaning that CTLA-4 binding will predominate when the co-stimulatory molecules are expressed at low levels on APCs. As discussed, this is most likely to occur when APCs have not been activated adequately and it has been shown that the induction of tolerance in $CD4^+$ T cells requires early ligation of CTLA-4. Thus, CTLA-4 and CD28 play overlapping, but opposing, roles in the response of $CD4^+$ T cells to antigen, allowing the T cell to discriminate between dangerous antigen presented in the context of inflammation and self or harmless foreign antigens to which tolerance is necessary.

An additional co-stimulatory molecule on T cells that appears to play a preferential role in the induction of tolerance in $CD4^+$ T cells is PD-1, which is a CD28/CTLA-4 homolog that binds PDL-1 and PDL-2 on APCs. Its functions are not yet fully understood, but seem to involve activation of intracellular phosphatases that inactivate the tyrosine kinase signaling pathways of TCR-mediated activation.

DIFFERENTIATION AND FUNCTIONS OF TH1 AND TH2 CELLS

As noted above, $CD4^+$ Th cells are central to the normal immune response and are essential for protective immunity against a wide range of potentially pathogenic organisms. However, $CD4^+$ T cells can be further subdivided depending on the pattern of cytokines they produce and the type of immune response they support. The cardinal marker of Th1 T cells is the secretion of IFNγ. This cytokine is essential for the cell-mediated immune responses generally required for protection against intracellular pathogens such as *Leishmania* spp. and mycobacteria. In contrast, Th2 cells were defined by their production of IL-4 and a role in helping humoral immunity and responses against extracellular pathogens such as helminths (Abbas et al. 1996). Th1 and Th2 responses tend to be reciprocally regulated and their importance has been highlighted in a number of disease models where susceptibility or resistance to a particular pathogen is dependent on the ability to mount the appropriate Th1 versus Th2 response. Dysregulation of these responses may also be involved in a number of inflammatory disorders.

A number of models have been proposed to explain the process by which a naive precursor CD4+ T cell can become either a Th1 or a Th2 cell (Murphy and Reiner 2002; O'Garra and Robinson 2004). Distinct precursors may exist and must become either Th1 or Th2. Alternately, cells could switch between the Th1 and Th2 phenotype, but can express only one particular phenotype at a time. However, it appears from studies in a number of TCR transgenic systems, where cells of the same specificity can mount a Th1 or a Th2 response, depending on the priming conditions, that the most likely explanation is that a common precursor exists which can differentiate into either Th1 or Th2 (Figure 5).

A large body of work has shown that cytokines are centrally important in causing naive CD4+ T cells to differentiate into IL-4- or IFNγ-producing cells. Cytokines may exert their polarizing influence by acting directly on T cells or via APCs, and may have one or all of the following effects: promote differentiation of precursor Th cell towards a particular phenotype, cause preferential proliferation of a particular phenotype (i.e. expansion not differentiation), or inhibit either of the above for a particular subset, thereby leading to dominance of the other.

The heterodimeric cytokine IL-12 is a potent inducer of IFNγ production by both natural killer (NK) cells and T cells, and is the principal Th1-inducing cytokine. Many adjuvants and microbial products stimulate IL-12 production from macrophages and DCs and it interacts directly with naive Th cells. Indeed, functional receptors for IL-12 are present on recently activated, uncommitted cells and Th1 cells but are lost from differentiated Th2 cells. IL-18 is an IL-1-related cytokine that assists the IL-12-induced differentiation of Th1 cells, although it may also enhance Th2 responses under some conditions. Other cytokines that may be involved in Th1 cell development are IL-23 and IL-27, both of which are partly related to IL-12. IFNγ also plays a role in the development of Th1 and Th2, partly because it inhibits the clonal expansion of IL-4-producing Th2 cells and

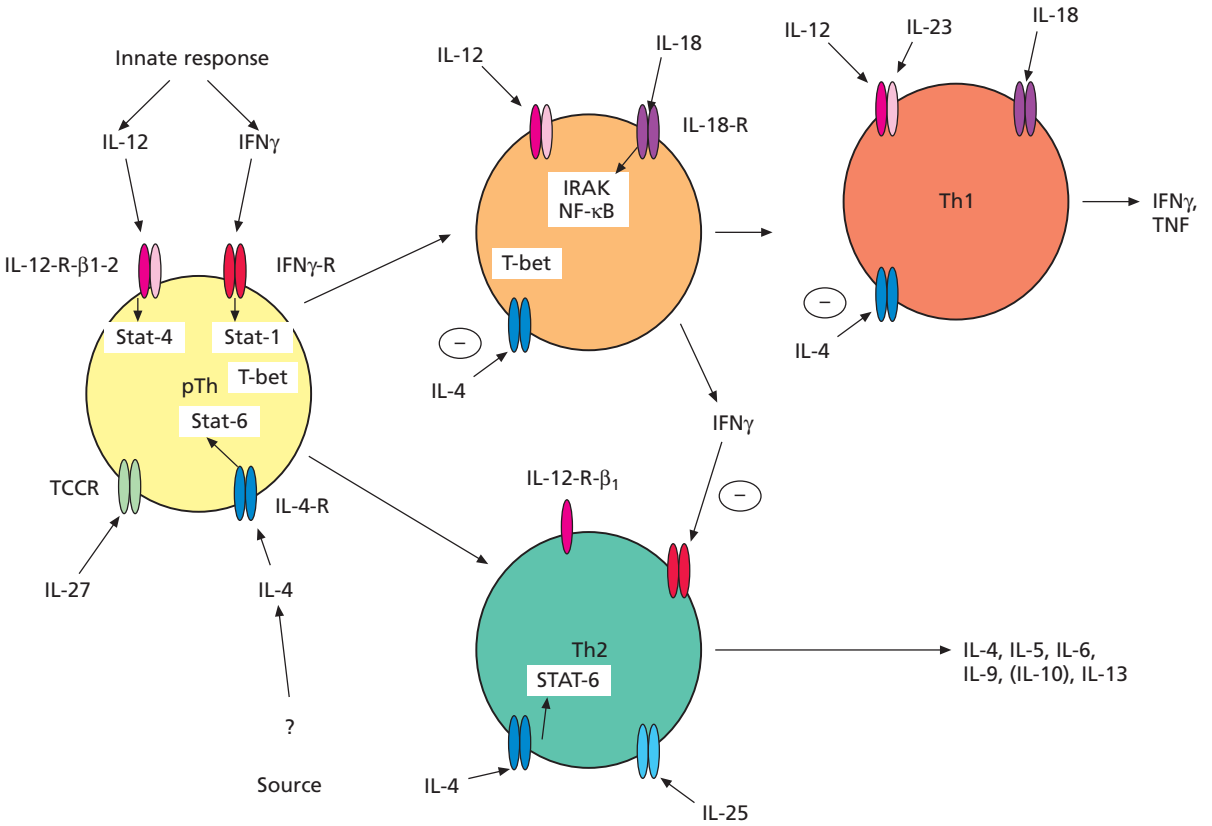

Figure 5 *Naive CD4+ T cells can differentiate into either T-helper 1 (Th1) or 2 (Th2) cells after recognition of antigen, depending on signals derived from the local microenvironment. Interleukin IL-12 released by macrophages and dendritic cells (DCs) in response to, for example, intracellular pathogens binds to the IL-12-R-β1/-2 receptor, triggering signaling through Stat-4 and the consequent production of interferon-γ (IFNγ) and activation of the T-bet transcription factor. This leads to increased expression of the IL-18-R, allowing IL-18 released from a wide variety of cell types (including potential APCs) to enhance the production of IFNγ and further differentiation to the Th1 lineage via activation of NF-κB. Th1 cells maintain expression of the IL-12-R-β1/-2 chains, allowing continued positive feedback via IL-12 and the synergistic effects of IL-23 derived from cells such as macrophages and DCs. Th2 differentiation is dependent on IL-4, but its source early in the response is unknown. IL-4 acts by activating transcription factors such as Stat-6 and GATA-3. Developing Th2 cells lose expression of the IL-12-R-β-2 chain making them unresponsive to IL-12/IL-23, but maintain expression of the IFNγ-R, which accounts for the inhibitory effect of IFNγ on Th2 cell differentiation.*

also because it enhances IL-12 production by APCs and maintains the expression of functional IL-12 receptors on $CD4^+$ T cells.

IL-4-producing Th2 cells emerge if IL-4 is present during priming, particularly if this is accompanied by the suppression of the appearance of IFNγ-producing cells. IL-4 appears to act directly on T cells and, once established, the IL-4-producing phenotype appears to be very stable. Another Th2 product, IL-10, diminishes IL-2 and IFNγ production by primed T cells, but, in contrast to IL-4, it appears that IL-10 acts via the APCs by suppressing production of the Th1-polarizing cytokine IL-12. Overall, it appears that IL-10 may contribute to the differentiation towards Th2 but it is not essential; this is supported by the fact that IL-10 knockout mice are capable of mounting Th2 responses.

WHAT ARE THE SOURCES OF THE CYTOKINES THAT POLARIZE TH1 AND TH2 RESPONSES IN VIVO?

Dendritic cells and to some extent macrophages are the sources of the IL-12 essential for the development of Th1 cells. However the in vivo source of IL-4 to promote differentiation into Th2 is more contentious. Naive T cells do not make appreciable amounts of IL-4 and differentiated Th2 cells would make a contribution only in an already primed animal. However, $NK1.1^+$ T cells, which recognize antigens presented in association with the nonpolymorphic β_2-microglobulin-associated molecule, CD1, can produce considerable amounts of IL-4 very rapidly on activation (Chen et al. 1997). However, the role of these cells remains controversial because Th2 responses can still be induced in the absence of these cells. Alternately, cells of the mast cell/basophil lineage may be the source of IL-4, possibly after triggering of the FcεRI or FcγRII/III receptors. Although this would require pre-existing IgE or IgG1 antibodies, mast cells and basophils can be activated nonspecifically by agents such as helminth products.

OTHER FACTORS IN CONTROL OF $CD4^+$ T-CELL DIFFERENTIATION

A number of factors have been implicated in the polarization of naive $CD4^+$ T cells into Th1 or Th2 cells, including the nature of the APC, the dose of antigen, and the co-stimulatory molecules involved (O'Garra and Robinson 2004).

Initial ideas suggested that DCs were responsible for polarizing Th1-cell responses, whereas presentation of antigen by B lymphocytes induced Th2-cell responses preferentially. However, it now appears that all APCs can initiate either form of T-cell response, although different populations of APCs may influence the subsequent maintenance of the distinct forms of response. This may reflect differential production of and/or response to polarizing cytokines, or via the expression of different co-stimulatory molecules (see below). Recently it has been proposed that the distinct lineages of DCs (lymphoid vs myeloid: DC1 vs DC2) may promote the selective differentiation of Th1 and Th2 respectively.

However, again it seems that this is an oversimplification and that all DC subsets may promote either Th1 or Th2 responses under appropriate circumstances. Recent studies indicate that DCs exposed to the cytokine thymic stromal lymphopoietin, produced by tissue cells from atopic individuals, induce Th2 differentiation, suggesting that this may be a corollary for IL-12 and Th1 differentiation in allergic disease (Soumelis et al. 2002). Indeed by altering the affinity of the peptide interaction with the class II molecule or the TCR it has been found that an overall lower affinity favors Th2 priming, whereas Th1 responses dominate if there is high affinity. These findings can be summarized as follows:

Co-stimulation

It has already been suggested that APCs may play a role in the differentiation of Th subsets and that they may do this via their expression of co-stimulatory molecules. Most work in this area has concentrated on the interaction of the well-known co-stimulatory molecules CD28 and CTLA-4 with their ligands B7-1 (CD80) and B7-2 (CD86). However, a variety of blocking or 'knock-out' studies in a wide range of experimental models in vitro and in vivo have yielded conflicting results (O'Garra and Robinson 2004). The confusion is probably related to the fact that, as noted above, the initial interaction of CD28 (or CTLA-4) with its B7 ligands is required for T-cell priming but it may then have differential effects in the expansion and persistence of Th1 and Th2 responses. The role of CTLA-4 in Th1/-2 polarization is also complicated by the negative regulatory effects of this molecule.

It has become apparent recently that a third signal may also be required to elicit co-stimulation for T cells and this may be mediated by iCOS, which is a homolog of CD28, and does not bind either B7-1 or B7-2 but rather interacts with B7-related protein 1 (B7RP-1). This ICOS is not present on naive T cells but is induced on all activated T cells. The signaling of iCOS appears to be involved the generation and maintenance of both Th1 and Th2 responses, but may be of heightened importance in the latter because iCOS expression is sustained on Th2 cells after chronic stimulation.

Thus, the overall affinity of the interaction resulting from the net effects of ligand density, affinity with APCs and TCR, and co-stimulation may be important in determining the type of response initiated but must be viewed in the context of the cytokine environment induced in vivo. It now seems likely that the convergence point for all of these influences is the signal transduction across the cell membrane and this is discussed in greater detail in T cell receptors.

Genetic background

A final, and relatively poorly defined, influence on Th polarization is that of non-MHC genes. Different inbred strains of mice have long been known to express distinct resistant or susceptible phenotypes to a variety of infections (e.g. *Leishmania major*, *Trichuris muris*) even when they are of the same MHC genotype, implying that genetic influences other than the MHC may be important. Studies using TCR transgenic animals backcrossed onto congenic strains that have the same MHC II antigens but different background genes have demonstrated that these genes influence naive T-cell polarization. The effect in this system appeared to be at the level of the T cell because mixing APCs and T cells from the different strains always led to the production of the cytokine profile associated with the T cell rather than the APC. Thus, it appears that T cells on a BALB/c background default to a Th2 phenotype as a result of greater initial IL-4 production and a decrease in IL-12 responsiveness resulting from the loss of IL-12-Rβ_2 expression. In contrast, animals on the congenic B10.D2 background do not display early IL-4 production and maintain IL-12-Rβ_2 expression and IL-12 responsiveness to develop a default Th1 phenotype.

PHENOTYPIC MARKERS OF TH1 AND TH2 CELLS

One of the major hindrances to progress in the study of Th1 and Th2 polarization has been the lack of reliable phenotypic markers of the two subsets. However, recent studies have identified several candidate molecules (Sallusto et al. 1998). The ligands for P- and E-selectin are preferentially expressed on Th1 cells and facilitate differential migration of these cells into inflammatory sites such as inflamed joints or sensitized skin (Austrup et al. 1997). The migration of Th2 cells, eosinophils, and basophils into tissues undergoing allergic reactions appears to be mediated by the eotaxin (a chemokine) receptor, CCR3, which is expressed on all of these cells but not Th1 cells (Sallusto et al. 1998). Another chemokine receptor, CCR5, may be restricted to Th1 cells whereas the IL-1-R homolog, ST2L, may occur only on differentiated Th2 cells.

B-CELL HELPER FUNCTION OF CD4+ T CELLS – THE GERMINAL CENTER REACTION

The antibody response is an essential component of the immunological armory. In many cases the development of antibody-producing plasma cells from B cells is highly dependent on help from T cells, as evidenced by the lack of B-cell responses in congenitally athymic animals that lack T cells. For an antibody response to occur, rare antigen-specific T and B cells must encounter the antigen that they both recognize and each other. This is achieved in germinal centers in the secondary lymphoid organs (MacLennan 1994).

As discussed above, following exposure to antigen it is transported to the secondary lymphoid tissues, either in blood/lymph or by specialized antigen-transporting cells (e.g. Langerhans' cells in skin). The activated, laden APC has now upregulated receptors (e.g. CCR7) for chemokines (e.g. CCL19, CCL21), which are produced by the stromal cells and other APCs in the thymus-dependent T cell area of the lymph node (paracortex), and is thus attracted to this area. Recirculating naive T cells also express CCR7 and therefore also localize in the paracortex, optimizing the chance of them encountering their cognate antigen (Sallusto et al. 2000). The naive T cell can now 'see' antigenic peptide presented to it in the context of MHC II by the antigen-laden APC. This results in upregulation of co-stimulatory molecules such as CD40L, the production of cytokines, and further migration. Naive B cells also recirculate but they express a chemokine receptor (CXCR5) that is specific for a chemokine (CXCL13). CXCL13 is preferentially produced by the network of follicular DCs that forms the basic structure underlying the B-cell areas of the lymph nodes or primary follicles. Within the specialized B-cell follicle, the naive B cell has also acquired antigen via its surface immunoglobulin (sIg) by this stage, which leads to its activation. It is important to note that although there are very few B cells of any particular antigen specificity they have an enhanced capacity to acquire specific antigen because of their surface receptor. The activated B cells now upregulate their expression of CCR7 and are drawn towards the T-cell area of the lymph node. Antigen-specific T and B cells now meet at the border between the paracortex (Garside et al. 1998) and the follicle, as a result of what has been termed 'a balanced responsiveness' to chemokines produced in each area. Thus, sampling and transport of foreign molecules from all over the body to secondary lymphoid 'depots' that are under constant surveillance by recirculating, naive lymphocytes has maximized the opportunity for these cells to encounter their specific antigen. Furthermore, coordinated expression of chemokines and their receptors has brought activated antigen-specific T and B cells into close proximity within the lymph node (Cyster 2003).

A range of membrane-associated and soluble molecules is now employed to help T and B lymphocytes 'talk' to each other in a variety of ways. Most of these 'conversations' take place within and around the developing germinal center (Cyster 2003). Germinal centers are the specialized anatomical microenvironment where activated B cells proliferate and attempt to increase the affinity of their antibody for the eliciting antigen in the process known as affinity maturation (MacLennan 1994).

Germinal centers are derived from the primary follicles, which, as noted above, are the sites to which naive recirculating B cells migrate as they pass through the lymphoid tissue. Primary follicles contain resting B cells clustered around a network of processes extending from follicular dendritic cells (FDC) (Cyster 2003). FDCs are important in attracting B cells to follicles, but also play an important role in affinity maturation by holding intact antigen and/or antigen–antibody complexes on their surfaces for long periods of time.

To make an antibody response a B cell needs to hypermutate, survive, proliferate (clonally expand), differentiate, and secrete antibody. T cells help B cells to do all of these things. B cells that have acquired antigen via their specific receptor (sIg) are now further activated by presenting peptides from that antigen on their surface and undergoing a cognate interaction with Th cells specific for the same antigen. The necessity for this cognate interaction is demonstrated by the facts that the correct antigen and restriction element (MHC II) are required to elicit T-cell help for B cells. This is important to prevent nonspecific bystander activation of B cells, which could lead to autoantibody production. These activated B cells within the primary lymphoid follicle now start dividing to form germinal centers, and these tightly packed, proliferating B lymphocytes, which no longer express sIg, are referred to as centroblasts, and form the dark zone of the germinal center (MacLennan 1994).

For affinity maturation to occur, variability is randomly generated in the B-cell receptors and those with the highest affinity are selected. Random point mutations are targeted to the V-region genes of the rapidly dividing centroblasts in the process of somatic hypermutation. Newly configured mutant receptors are now expressed as sIg on the progeny of the centroblasts, which are known as centrocytes. Centrocytes subsequently migrate towards the FDC network in the light zone of the germinal center where they can encounter Th cells that have also migrated there. Centrocytes die by apoptosis within a short time of expressing their new sIg unless it is bound by antigen; then they are contacted by an activated Th cell expressing the survival signal CD40 ligand (CD40L). The random process of somatic hypermutation also means that the sIg expressed on the centrocytes derived from a particular progenitor may bind and take up antigen more or less efficiently than that expressed on its precursor. Centrocytes with mutations that result in a loss of the ability to bind antigen die by apoptosis. However, if the mutant sIg binds antigen well, the centrocyte is induced to express the *bcl-xl* gene, the product of which inhibits apoptotic cell death, and the cell is rescued. It is in this competitive environment that centrocytes with the highest affinity receptors bind and take up antigen and move to the outer edge of the light zone, where activated Th cells expressing CD40L are concentrated, whereas those with low-affinity receptors lose out and die by apoptosis (Rathmell et al. 1996). If the centrocyte and Th cell are specific for epitopes from the same antigen, they undergo a cognate interaction during which they exchange signals that promote their further proliferation and differentiation. The B cells become either memory cells or antibody-secreting plasma cells and may have also undergone isotype switching in response to cytokines secreted by Th cells. The involvement of T cells in the selective process in the germinal center prevents survival of centrocytes that have acquired specificity for self-antigens.

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MHC class I antigen processing system

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EVOLUTIONARY CONTEXT: OVERVIEW OF THE ADAPTIVE CELLULAR IMMUNE SYSTEM

Many infectious agents are obligate intracellular parasites. All viruses meet this description, as do many pathogenic prokaryotes and unicellular eukaryotes. Cellular organisms can also be opportunistic intracellular interlopers, particularly if it enables them to escape the clutches of the humoral immune system. Cells are not wholly defenseless against intracellular microorganisms, although the capacity of such innate immune mechanisms is relatively limited.

An important part of the innate response of infected cells is the manufacture and release of cytokines (most commonly α- and β interferons). In many circumstances, this provides the initial warning of the invasion to the organism. At this juncture two strategies are conceivable: the immune system could indiscriminately target the general vicinity of the infected cell and implement an anti-pathogen program akin to carpet bombing. As immune effector mechanisms can have deleterious effects on cells (the ultimate being death, of course) this would come with a high cost in collateral damage. Alternately, the immune system can selectively target infected cells for individual attention. This is a sufficiently attractive possibility for vertebrate organisms to make an enormous evolutionary investment to achieve it.

The essential problem faced by the immune system is the efficient identification of infected cells in a spatially complex organ. Any reasonable solution entails endowing immune cells with the ability to detect the presence of intracellular pathogens without disrupting the integrity of cells harboring the agent. This was achieved by the evolution of a molecule that carries small bits of information encoded by the pathogen to the surface of the infected cell for perusal by immune cells bristling with receptors which co-evolved the capacity to decode the information. The information-carrying molecule is known as a major histocompatibility complex (MHC) class I molecule. The information takes the form of pathogen-encoded oligopeptides (usually 8–11 residues in length). These are generated by the antigen-processing machinery, which, like class I molecules, is constitutively expressed by virtually all cells in a vertebrate organism. Decoding is performed by a receptor (the αβ T-cell receptor (TCR) expressed by cells (the $CD8^+$ T cell) charged with the task of finding and modifying cells expressing nonself peptides.

This is an impressive achievement, but the system has a potential flaw: pathogens can evolve to mimic self-peptides and avoid detection. To discourage this, an additional level of complexity was added. The value added by this modification is best illustrated by its high cost. Alleles of MHC class I genes evolved that present distinct sets of peptides. Such alleles are present in the population of most vertebrate species at sufficient frequencies to make it likely that any two individuals present substantially different sets of peptides on their class I molecules. This is the inexpensive part.

As every American parent with college-age children painfully knows, life's real expense comes in education. Since the immune system in any individual cannot anticipate which class I molecules it must recognize, its TCRs cannot be hard-wired into the genome. Rather, evolution invented thymic selection to select, among T-cell clones

expressing different TCRs, for those that are capable of recognizing self class I molecules with the proper affinity.

The thymus is more akin to a mortuary than a kindergarten, because the vast majority of T cells entering cannot meet the exacting standard of expressing a TCR that binds to class I molecules with the intermediate affinity required to enable self–nonself discrimination. Given that humans can generate up to 10^9 T cells per day (at birth, the peak of thymic activity), which represents 2–5 percent of immature thymocytes entering the thymus, the daily carnage amounts to perhaps 2×10^{10} cells (50 g or so) (Haynes et al. 2000)! Waste is a theme that we return to when discussing the generation of antigenic peptides.

$CD8^+$ T cells are but half of a dynamic duo, their partners being $CD4^+$ T cells. The siblings are charged with largely distinct tasks. $CD8^+$ T cells serve as a principal immune effector mechanism for fighting viruses and other intracellular parasites. $CD4^+$ T cells are preoccupied with regulating the activities of other immune cells, including their $CD8^+$ T-cell siblings. $CD4^+$ T cells also recognize oligopeptides (occasionally the same peptides as $CD8^+$ T cells), but presented by MHC class II molecules (dead ringers for class I molecules structurally, but very different in primary sequence). $CD8^+$ and $CD4^+$ T cells acquire their TCRs from a single gene pool. The discrimination of class I versus class II molecules results from the expression of the CD8 versus CD4 co-receptors. The interaction of CD8 and CD4 with class I and class II molecules, respectively, is required for positive thymic selection, and also enhances the interaction of mature $CD4^+$ and $CD8^+$ T cells with antigen-presenting cells (APC).

Humans possess about 10^{11} mature T cells, representing approximately 10^8 clones with distinct TCRs (making an average clone size of 1000) (Arstila et al. 1999; Img et al. 2000). Each T cell expresses approximately 10^5 TCRs. T cells can be triggered by APCs expressing about 10–100 class I molecules containing an appropriate peptide. This is truly an amazing feat because, first, the T cell must sort through the roughly 10^5 class I molecules on the APC surface that contain inappropriate peptides, and, second, the affinity of the TCR for its cognate peptide–class I complex is such that contact is maintained only for the order of a few seconds to minutes.

It is important to distinguish two types of $CD8^+$ T-cell activation. 'Afferent' refers to the activation of resting naïve or memory $CD8^+$ T cells. 'Efferent' refers to the activation of $CD8^+$ T-cell effector function by target cells. In both cases activation requires ligation of the TCR by an appropriate MHC–peptide complex. Afferent activation requires additional 'co-stimulatory' signals that are transmitted by molecules normally expressed only by professional APCs (pAPC). The most important co-stimulatory interaction is that of CD28 on the $CD8^+$ T cells with CD80 or CD86 on the pAPC. The pAPCs are bone marrow-derived cells that normally activate $CD8^+$ T cells in lymphoid organs, typically a local draining node or the spleen. These include macrophages, B cells, and dendritic cells (DC). Unlike other cell types, pAPCs constitutively express MHC class II molecules, which enables them to present antigens to $CD4^+$ T cells, and to serve as a bridge between $CD4^+$ and $CD8^+$ T cells.

DCs are believed to play a particularly important role in generating $CD8^+$ T-cell responses. Large numbers are present in tissues, where they can form an interdigitating network. Such networks have been shown to exist in the skin (where the DCs are known as Langerhans' cells) and the respiratory tract. At the early stages of inflammation DCs 'mature', altering their phenotype and physiology as they migrate via lymphatics to local draining lymph nodes, where they activate T cells. Importantly, pAPCs are able to present both 'endogenous' viral antigens, i.e. peptides synthesized by the cell as a result of viral infection, as well as 'exogenous antigens', peptides, or polypeptides acquired from other virus-infected cells. This latter pathway is known as 'crosspriming', and it appears to be the principal pathway used to induce $CD8^+$ T cells when viruses are unable to initiate infection in pAPCs, or when viruses express gene products that interfere with antigen presentation by infected pAPCs.

A critical feature of $CD8^+$ T-cell responses to viruses (and probably other pathogens) is that only a tiny fraction (ranging from a few to perhaps 50 or so) of the thousands to hundreds of thousands of different peptides (depending on the complexity of the virus) generated by infected cells induce measurable $CD8^+$ T-cell responses. This phenomenon, known as immunodominance, is caused by a number of factors (Yewdell and Bennink 1999). The most important is that that only about 0.5 percent of peptides of the correct length bind to any given class I molecule allele with sufficient affinity ($K_D > 5 (10^{-7})$) to produce enough complexes to trigger $CD8^+$ T-cell activation. Second, the TCR repertoire can distinguish as foreign only about 50 percent of viral peptides that bind to class I molecules at or above this threshold affinity. Third, the antigen-processing machinery is limiting for production of 80 percent of the determinants that bind to class I molecules, mostly as a result of difficulties in proteolytic generation of determinants. Fourth, strong $CD8^+$ T-cell responses to a given determinant suppress responses to other determinants (this is known as immunodomination). Finally, to maximize replication, many viruses shut down the synthesis of host proteins, including class I molecules. If the supply of class I molecules is limiting, determinants from viral proteins expressed late in the infectious cycle will have no means of presentation to $CD8^+$ T cells (this applies only to cases in which $CD8^+$ T-cell activation requires presentation of endogenous antigens by pAPCs).

ANTIGEN PROCESSING: STEP BY STEP

The tripartite complex

CLASS I STRUCTURE

Class I molecules recognized by TCRs have three noncovalently bound chains. In order of size they consist of an oligopeptide of 8–12 residues, an invariant small soluble subunit (termed 'β_2-microglobulin'), and a membrane-bound integral membrane glycoprotein (termed 'α chain') encoded by highly variable genes. α chains consist of a short carboxy-terminal short domain, a typical transmembrane hydrophobic region, a relatively large extracellular domain, and an amino-terminal signal peptide that is cleaved upon import of class I molecules into the endoplasmic reticulum (ER). The three-dimensional structures of the extracellular domains of numerous class I allomorphs have been determined at high resolution by X-ray crystallography (the intracellular and transmembrane domains are excluded to enable crystal formation) (Madden 1995) (Figure 1). The stalk of the molecule is formed by the domain most proximal to the membrane, which is composed of residues encoded by the third of the seven exons that encode the α chain (and therefore termed the 'α3 domain'). The α3 domain makes important contacts with β_2-microglobulin and is the region that interacts with CD8. Residues from the first two exons (α1 and α2) combine to form the region most distal from the membrane, which is at its tip and contains the peptide-binding groove. The groove consists of two anti-parallel α helices which border a floor consisting of 7-β sheets. This region of the molecule contains most of the polymorphic residues, which are concentrated in areas of the groove that contact peptides.

α Chains are encoded by the MHC, termed human leukocyte antigen (HLA) in humans (Figure 2) and H-2 in mice, located, respectively on chromosomes 6 and 17. Three loci in the HLA encode class I genes, termed HLA-A, -B, and -C, whereas H-2 has two or three such loci (depending on the strain), termed H-2K, -D, or -L. At each α chain locus there are thousands of alleles present at appreciable frequencies in the entire

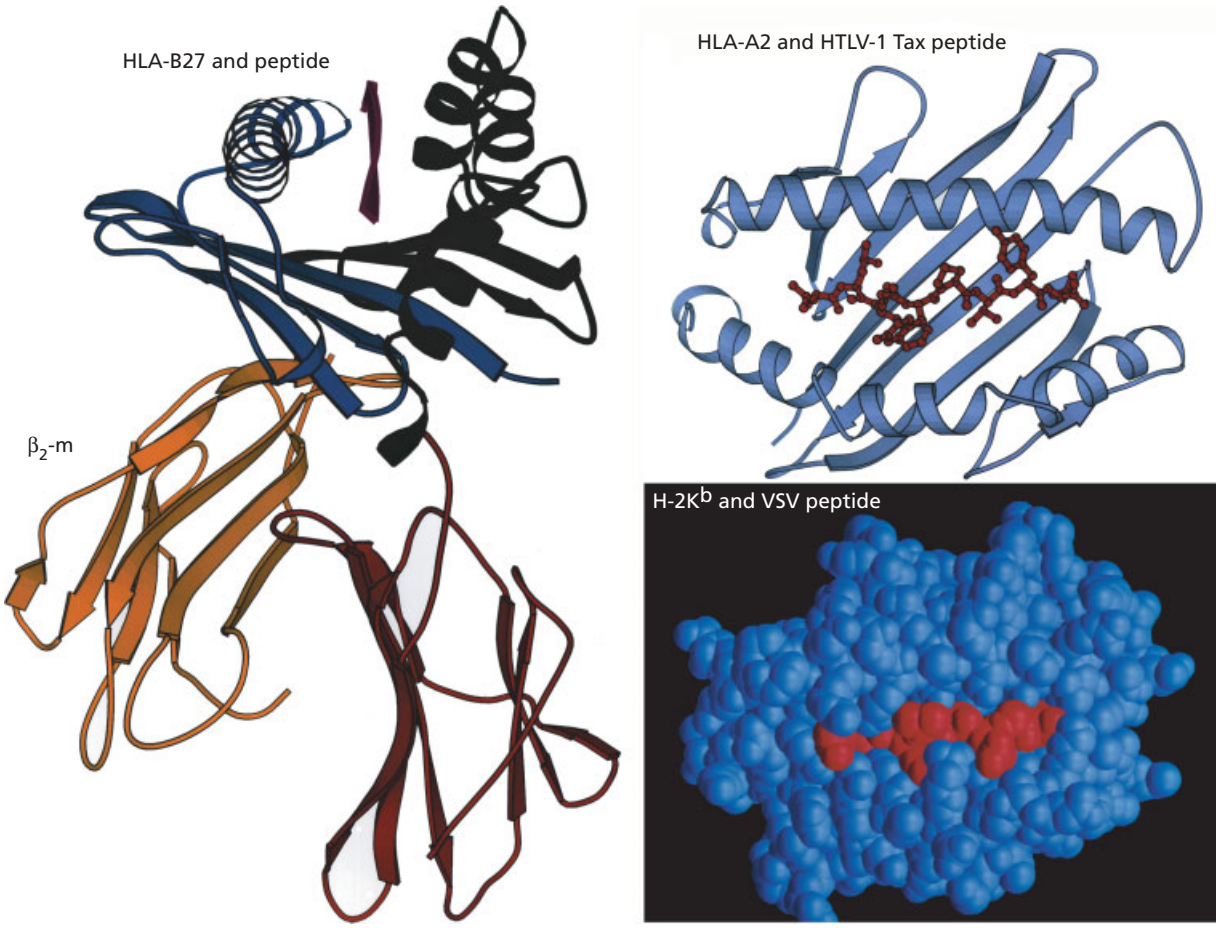

Figure 1 *Three-dimensional structure of MHC class I and class II molecules: structure of α-carbon backbone of HLA-B27 molecule (left) bearing an antigenic peptide (short red ribbon) in the groove, as determined by X-ray crystallography of water-soluble molecules released from the cell surface by protease digestion. Molecule is oriented with top toward the T-cell receptor (TCR). On the right are ribbon (top) and space-filling (bottom) models of peptides snug in the grooves of the class I molecules as they would be seen by the TCR.*

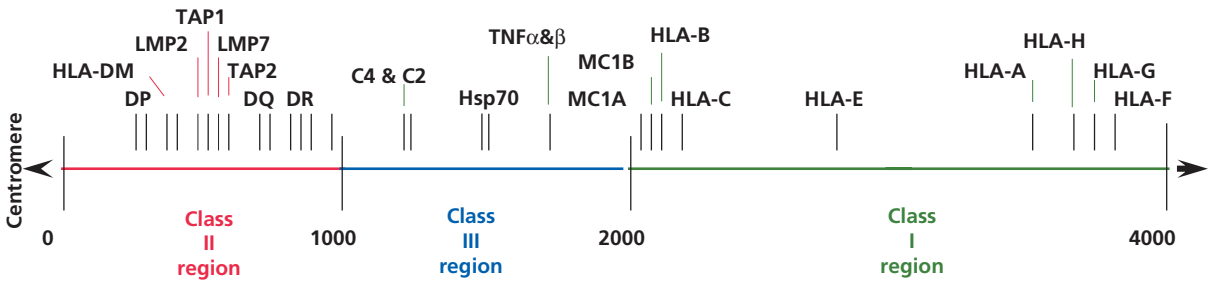

Figure 2 *Genetic map of HLA complex.*

human population. Individual ethnic groups often possess certain alleles that are present at higher frequencies than in the overall population. As the functions of HLA-A, -B, and -C molecules are similar, it is useful to lump together all the alleles produced by the three loci under the term 'allomorph'. HLA allomorphs are designated by a logical series of numbers (mercifully) that distinguish allelic alterations in nonsynonymous and synonymous alteration in coding regions and alterations in noncoding regions (see Table 1), whereas H-2 allomorphs are identified by a bewildering array of capital letters and superscripts, a nomenclature worthy of Torquemada.

The α chains under discussion are components of 'classical' class I molecules, also known as class Ia molecules (referred to throughout this chapter simply as class I molecules). In addition, there are a number of other homologous genes encoding α chains that generate 'nonclassic' or class Ib molecules. In general, class Ib genes are much less polymorphic than class Ia genes (although there are exceptions). The HLA encodes five such molecules: HLA-E, -F, and -G, which bind β_2-microglobulin, and MHC class I chain-related (MIC) gene products MICA and MICB, which do not (MIC is also of interest because its genes are highly polymorphic). All these genes have immunological functions that for are the most part independent of recognition by T cells with αβ TCRs. In addition, genes outside the MHC encode class Ib molecules: these include genes with immunological functions (such as *CD1*) and those apparently without direct immune function (such as *HFE*, which functions in iron metabolism). Mice have more than 40 genes encoding class Ib α chains; current understanding of the functions of these molecules ranges from fragmentary to nil.

CLASS I PEPTIDE LIGANDS

Binding rules

Peptides recovered from class I molecules are a highly heterogeneous mixture. Given a cell with 10^5 class I molecules of a given allomorph, there will be perhaps 10 000 distinct peptides presented. The dominant anchor residues of peptides bound by numerous HLA class I allomorphs have been determined by Edman sequencing of peptide pools recovered from purified molecules (Engelhard 1994; Rammensee et al. 1995) (the exact sequence of peptides of sufficient abundance can be determined directly by mass spectroscopy). In recent years, mass spectroscopy has been increasingly used to identify peptide ligands, as a result of the rapid technical advances in mass spectroscopy and greater access to advanced mass spectrometers. To date, however, only a few antigenic peptides from viruses have been identified in this manner.

The binding of peptides to class I molecules displays two important features. First, main chain atoms at the carboxyl and amino termini of the peptide make energetically important bonds with invariant residues at the ends of the groove (Bouvier and Wiley 1994). Peptides of fewer than eight residues cannot simultaneously contact both ends of the groove. Elongation of peptides also prevents these interactions because only a limited amount of kinking is allowed. Consequently, class I peptides exhibit little size heterogeneity, with about 90 percent of the peptides recovered from class I molecules falling within the range 8–11 residues in length, depending on the class I allomorph and the ability of peptides to bend in the groove. Second, the residues that demonstrate the most polymorphism among allomorphs form two to three deep pockets in the bottom of the groove which accommodate a highly restricted set of amino acids from bound peptides. As a result, each class I allomorph demonstrates a distinct preference for peptides that possess the complementary 'dominant anchor' residues, one of which is usually located at the carboxyl terminus (Table 1).

If antiviral $CD8^+$ T cells are known to be specific for a given viral protein presented by an allomorph with defined anchor residues, predictive algorithms (based largely on dominant anchor residues) can be used to identify a reasonably small number of candidate peptides from the protein (depending on the size of the viral protein, there are usually two to five such peptides) (Rammensee et al. 1997). These peptides can then be synthesized and used to determine the precise peptide recognized by virus-specific $CD8^+$ T cells. This method is used routinely, and is able to identify about 50 percent

Table 1 *Human MHC class I-binding peptide motifs*

	Position									
HLA molecule	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
HLA-A1			D, E						Y	
HLA-A*0201		L							L	
HLA-A*0205									L	
HLA-A3		L, V, M							K, Y, F	
HLA-A*1101									K	K
HLA-A24		Y							I, L, F	
HLA-A*3101,2									R	
HLA-A68.1		V, T							R, K	
HLA-B7		P							L, F	
HLA-B8			K		K, R				L	
HLA-B*2702		R							F, Y, I, L, W	
HLA-B*2705		R							L, F	
HLA-B*3501		P							Y, F, M, L, I	Y
HLA-B*3701		D						F, M, L	I, L	
HLA-B*3801									F, L	
HLA-B*39011		R, H							L	
HLA-B*3902		K, Q							L	
HLA-B40		E							L	
HLA-B*4402,3		E							F, Y	
HLA-B*5101,2,3		A, P, G							F, I, V	
HLA-B*5201								I, V	I, V	
HLA-B53		P								
HLA-B*5801		A, S, T							F, W	
HLA-B60 (B*40012)		E							L	
HLA-B61 (B*4006)		E							V	
HLA-B62 (B*1501)		Q, L							F, Y	
HLA-B*7801		P, A, G								
HLA-Cw*0301									L	
HLA-Cw*0401		Y, P, F							L, F	
HLA-Cw*0602									L	
HLA-Cw*0702									Y	

Human MHC molecules are termed 'human leukocyte antigens' (HLA). There are three loci encoding class I molecules, HLA-A, B, and C. Originally HLA molecules were typed serologically; such antigenically defined gene products are identified by one or two digits. More recently, HLA gene products are identified indirectly by genotyping. This provides much greater precision and revealed that previously identified alleles comprised a number of closely related genes. HLA genes (as opposed to gene products) are designated by an asterisk, and are numbered with up to seven digits. Closely related alleles share the first two digits and are discriminated by the last two digits (the remaining three digits are used to designate synonymous alterations in coding regions, or changes in noncoding regions), e.g. there are 30 known genes closely related to the canonical HLA-A2; these are numbered A0201 to A0230. HLA-C genes are given a gratuitous 'w' to avoid confusion with complement components. The anchor residues favor binding to various human class I allomorphs as determined by pooled sequencing of peptides eluted from purified class I molecules. The presence of anchor residues at consecutive positions at residues at consecutive positions at the carboxyl terminus reflects heterogeneity in peptide length, and not consecutive anchor positions.

of the determinants recognized by antiviral $CD8^+$ T cells. If this method fails, overlapping peptides covering the entire protein can be screened for activity. Empirically it has been found that peptides of 13 residues overlapping by 9–10 residues work well.

Exogenous peptide presentation: practical issues

All cells expressing class I molecules possess cell-surface class I molecules capable of binding peptides present in the extracellular fluids. Free peptides are probably rarely present in sufficient concentrations in vivo during a virus infection to sensitize bystander cells. Although the physiological significance of such peptide-receptive (PR) cell surface class I molecules is uncertain, PR class I molecules enable the use of synthetic peptides to sensitize cells to activate $CD8^+$ T cells for in vitro assays (lysis, intracellular cytokine staining, ELISPOT), and to stimulate secondary in vitro responses (an oft-used method to generate $CD8^+$ T cells specific for a known peptide, and an alternate method for determining which peptides are recognized by $CD8^+$ T cells). The expression of cell surface PR molecules can be enhanced by culturing cells at 25–28°C in media supplemented with 2–5 μg/ml human β_2-microglobulin.

Immunogenic peptides typically bind to class I molecules with a K_D of $<10^{-7}$ mol/l. Using optimized systems, most antiviral effector $CD8^+$ T cells require only 10–1000 peptide–class I complexes for activation. Translated into practical terms, incubating cells with sub-nanomolar (often picomolar) concentrations of peptide is sufficient for activation of effector $CD8^+$ T cells. This enables screening of peptides with 13 residues, based on the generation of fragments by serum proteases in culture media (Kozlowski et al. 1992), or low-affinity binding of extended suboptimal peptides. It also means that for screening purposes highly purified peptides need not be used (though, if impure peptides are used to generate $CD8^+$ T cells, they may induce $CD8^+$ T cells that are specific for contaminants (Chen et al. 1996)). Note that great care has to be taken when working with synthetic peptides corresponding to naturally processed peptides, which when present in a 1 mmol/l stock solution often represent a billionfold excess. Care must also be taken when weighing out the peptides, which are notoriously susceptible to floating away on errant air currents, where the tiniest speck can contaminate fluids. If possible, the balance should not be located in rooms used to perform assays or culture target cells. Incubation of cells with a vast excess of peptide can lead to spurious recognition by $CD8^+$ T cells specific for other determinants. Consequently, it is important to establish that $CD8^+$ T cells recognize cells sensitized with a concentration of peptide that results in the generation of a physiological number of peptide–class I complexes; usually this means incubating cells with sub-nanomolar peptide concentrations. Ultimately, definitive evidence that $CD8^+$ T cells recognize a given determinant in a protein requires the demonstration that mutating the corresponding residues in a protein modifies antigenicity. Lamentably, this rigorous approach is rarely applied.

Determinants that fail to be identified using synthetic peptides may possess post-translational modifications required for binding to class I molecules and/or recognition by the cognate TCR. These include phosphorylation, glycosylation, or deglycosylation of N-linked oligosaccharides, which converts asparagine residues that serve as the branch point for N-linked oligosaccharides into aspartic acid (Skipper et al. 1996; Andersen et al. 1999; Zarling et al. 2000). Cysteine-containing peptides (about 15 percent of all antigenic peptides) present special challenges as a result of the highly reactive nature of the free sulfhydryl group. $CD8^+$ T cells may recognize cysteine-crosslinked peptide dimers, cysteinylated peptides, or other undefined post-translational modifications (Meadows et al. 1997; Chen et al. 1999). Another potential reason for failing to locate a determinant in a protein is that the peptide is encoded by an alternate reading frame (Mayrand and Green 1998).

Although the basis for the antigenicity of synthetic peptides is nearly always their binding to cell-surface PR molecules, unless the cells are incubated with peptides at low temperature, peptides will traffic to endosomal compartments as a result of constitutive endocytosis and also to the ER (Day et al. 1997), where they can associate with resident PR molecules.

CLASS I ASSEMBLY AND SURFACE EXPRESSION

As with most integral membrane proteins, α chains are co-translationally inserted into the ER via the translocon. HLA α chains acquire a single N-linked oligosaccharide during translocation, whereas H-2 α chains are either di- or triglycyosylated. Newly synthesized α chains initially bind to calnexin, an abundant general purpose ER molecular chaperone which, like other chaperones, is thought to facilitate proper folding by preventing inappropriate interactions between hydrophobic domains (Williams and Watts 1995) (Figure 3). Within 5–10 min, α chains associate with β_2-microglobulin, and are transferred from calnexin to a complex of calreticulin, ERp56 (two more general purpose chaperones), and tapasin, a chaperone dedicated to class I molecules (Pamer and Cresswell 1998). ERp56 catalyzes the proper disulfide bond formation in α chains (Hughes and Cresswell 1998), whereas tapasin mediates the binding of the assembly complex to transporter associated with antigen processing (TAP). $\alpha\beta_2$-microglobulin heterodimers remain in the ER tethered to TAP until their release is triggered by peptide binding (Ortmann et al. 1994; Suh et al. 1994). Class I departure from the ER appears to be subject to further quality control inspection at ER exit sites (Spiliotis et al. 2001). Depending on the class I allomorph and the supply of peptides (and probably other undefined factors), class I export from the ER can take between 10 min and several hours. Transport to the cell surface via the Golgi complex occurs over a period of 10–20 min. The degree of association of class I molecules with tapasin (and therefore TAP) varies considerably among allomorphs (Peh et al. 1998). Why this should be remains an interesting mystery. Although tapasin is not absolutely required for peptide binding, it facilitates the loading of high-affinity peptides through an undefined mechanism (Garbi et al. 2000; Grandea et al. 2000).

'High-affinity' peptide binding refers to a K_D of $<10^{-7}$ mol/l. Peptide ligands that bind with this affinity will not dissociate from class I heterodimers for hours, and those with higher affinities will on average probably remain bound to class I until the molecule is shed from the cell surface or destroyed after internalization into lysosomes, which occurs with a half-time of the order of tens of hours. It is likely, however, that a substantial portion of peptides binds class I molecules less stably. Once peptides dissociate from cell surface class I molecules, class I molecules are unstable and unfold with a time of about 10 min. Just before unfolding β_2-microglobulin dissociates from heavy chains, which denature rapidly in its absence. This process is reversible, which is

why β_2-microglobulin promotes the formation of PR molecules. β_2-Microglobulin is naturally present in human and mouse serum and plasma (and fetal bovine serum) at concentrations that create such PR class I molecules. Increasing the β_2-microglobulin concentration enhances the formation of PR molecules, as discussed above. Human β_2-microglobulin is preferred for this purpose because of its commercial availability and because it binds more tightly to both human and mouse α chains than mouse β_2-microglobulin, and it is thus more efficient at creating PR class I molecules.

Generating peptide ligands from biosynthesized proteins

SOURCE OF ANTIGENIC PEPTIDES

The process by which peptides are derived from proteins and delivered to class I molecules (or, for that matter, MHC class II molecules) is termed 'antigen processing' (Yewdell and Bennink 1992; Germain and Margulies 1993; Heemels and Ploegh 1995; Pamer and Cresswell 1998; Yewdell et al. 1999a). The classic class I antigen-processing pathway begins in the cytosol (see Figure 3). Proteins can reach the cytosol by virtue of being either synthesized by ribosomes (endogenous antigens) or physically introduced into the cytosol from extracellular fluids (exogenous antigens).

Antigenic peptides can be derived from any type of viral or cellular protein, including those located in the cytosol, nucleus, mitochondria, and various membranes. Even secreted proteins can be highly antigenic. How are peptides generated from these disparate protein sources? A possible clue comes from the kinetics of presentation of newly synthesized viral proteins to $CD8^+$ T cells, which can occur within an hour of adding a virus to cells. Given the time needed for viral penetration and transcription (using DNA or negative strand RNA viruses) and transport of class I–peptide complexes from the ER to the cell surface, this leaves only about 30 min for the virus to begin to synthesize its proteins. If viral proteins were simply entering the steady-state pool of proteins present in a cell, and had to compete with cellular proteins for access to class I molecules, it is difficult to understand how presentation could occur so rapidly.

One potential solution would be that peptides are derived from viral proteins that are much less stable than the cellular proteins with which they compete for access to MHC class I molecules. But, in fact, antigenic peptides are usually derived from viral proteins that are extremely stable when measured by standard means. Another solution, originally proposed by Van Pel and Boon (1989), is that antigenic peptides are derived from short-lived, misbegotten out-of-frame polypeptides that result from errors in transcription or translation. This probably occurs (Mayrand and Green 1998), but it does not account for the vast majority of peptide ligands that are translated in the correct reading frame (Rammensee et al. 1997).

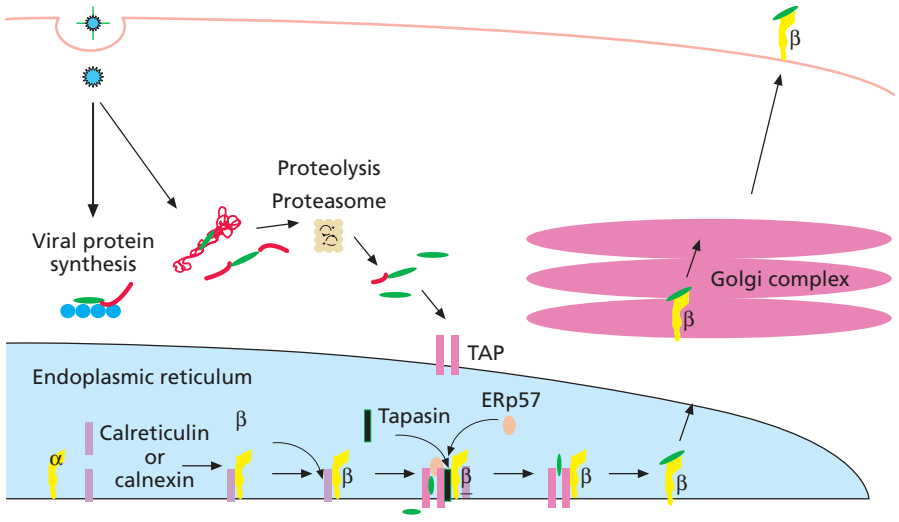

Figure 3 *Class I antigen-processing pathway: at the top left of the figure, a virion is shown entering the cell via endocytosis, and beneath it a viral capsid (blue burr with white border) is shown entering the cytosol (with enveloped viruses this is a consequence of fusion of viral and endosomal membranes; with nonenveloped viruses, the virus directly breaches the cellular membrane). In most situations, the major source of antigenic peptides (green oval) is protein synthesis (major arrow emanating from capsid), but peptides can be derived from capsid proteins if sufficient quantities are delivered to the cytosol. Many, and perhaps most, peptides are probably derived from defective forms of newly synthesized proteins (Schubert et al. 2000). Proteasomes are the major producer of peptide in the cytosol, but other proteases may also contribute. In particular, aminopeptidases can trim proteasome products. Cytosolic peptides of 8–17 amino acids are transported into the endoplasmic reticulum (ER) by TAP (transporter associated with antigen processing), where trimming by aminopeptidases can also occur. Peptides bind to newly synthesized class I $\alpha\beta_2$-microglobulin heterodimers, which are released from TAP and transported via the Golgi complex to the cell surface for immune perusal.*

An alternate solution is that protein biosynthesis, like all biological processes, is imperfect. A certain percentage of ribosomal products must be defective in some way and not reach their stable conformation. It has been known for years that introducing alterations in proteins, which increases their degradation in cells, increases their presentation to $CD8^+$ T cells (Tevethia et al. 1983; Townsend et al. 1986, 1988), with the implication that imperfect forms of proteins generated from wild-type genes would be a preferred source of antigenic peptides. Many different types of errors can be envisaged in mRNA generation (misincorporation of nucleotides, missplicing), or in the process of protein synthesis itself that results in the misincorporation of amino acids, premature termination, or deletion of residues. In addition, many proteins are members of multi-subunit complexes, and there must be imbalances in synthesis that result in an excess of certain subunits which are unstable in the absence of their normal partners. Additional mistakes must be made in protein targeting, such that proteins are delivered to the wrong organelle, where they misfold and are targeted for destruction. The folding of nascent proteins in the crowded environment of the cytosol is more difficult than folding in a homogeneous solution in vitro, and there are ample opportunities for the process to go awry, particularly for large multi-domain proteins.

The products of these errors can be grouped together as a source of antigenic peptides, because they will all presumably be degraded by proteasomes and other cellular proteases contributing to quality control. Such substrates have been termed defective ribosomal products (DRiP) (Yewdell et al. 1996). The beauty of DRiPs for the immune system is that they enable MHC class I molecules to monitor protein synthesis rates in cells and not protein concentrations. In this way, viral infections could be detected as soon as possible, particularly as many viruses rapidly monopolize the protein synthesis machinery to produce their own gene products. This would explain the rapid detection of virus-infected cells by $CD8^+$ T cells and also the generation of antigenic peptides from a huge variety of viral proteins, regardless of their apparent metabolic stability or intracellular targeting.

Note that DRiPs are not exclusively limited to viral proteins, but rather they are an inevitable consequence of synthesizing *any* protein. Indeed, the average viral protein may be less prone to DRiP formation than the average cellular protein. An additional strength of the DRiP hypothesis is that it provides an explanation for why class I peptide ligands are not biased toward originating from metabolically unstable proteins. There is experimental evidence to support the idea that DRiPs provide a significant source of peptide ligands to MHC class I molecules. First, 30 percent or more of the total pool of newly synthesized cellular proteins appear to be degraded within a few minutes of their synthesis, as inferred by the increased recovery of radiolabeled proteins from cells treated with proteasome inhibitors. An increased fraction of newly synthesized proteins recovered from proteasome inhibitor-treated cells were polyubiquitylated, as predicted (the ubiquitin–proteasome system is discussed below) (Schubert et al. 2000). Second, blocking protein synthesis reduces the supply of peptide ligands for TAP or class I molecules (Reits et al. 2000; Schubert et al. 2000). Third, using cells expressing a gene under the control of a repressible promoter, the rate of peptide generation from the gene is proportional to the amount of mRNA, not the level of the source protein (Khan et al. 2001). Fourth, presentation of viral peptides is tightly linked to the rate of translation of the source gene product and not the steady state level of stable protein (Princiotta et al. 2003).

This is not to say that DRiPs are the only source of antigenic peptides; clearly they are not. For all we know, on average, peptides might be more efficiently produced from long-lived proteins. Indeed, it is to be expected that the DRiP rate will vary tremendously between different proteins. For proteins with low DRiP rates, the bulk of peptides will probably be derived from the classic turnover of proteins, and vice versa for proteins with high DRiP rates.

What about secreted proteins?

Many viral proteins are targeted to the ER for secretion or insertion into membranes used for viral maturation. Depending on the cell type, a high percentage of cellular proteins can be similarly targeted. Despite their targeting, antigenic peptides can be derived from these proteins with similar efficiency as nuclear or cytosolic proteins. How are such proteins processed?

As discussed below, the secretory pathway has a limited capacity for generating peptides from full-length proteins. Consequently, in a high percentage of cases, processing of ER-targeted proteins occurs in the cytosol (this represents a subset of DRiPs). There are two general mechanisms for delivering such proteins to the cytosol: either they are diverted to the cytosol on their way to the ER or they are returned to the cytosol after visiting the ER. Although only a few studies have examined this question, it appears that the latter route is the more traveled (Bacik et al. 1997; Mosse et al. 1998).

This is consistent with the recent appreciation that degradation of ER proteins occurs in the cytosol after the re-export of the protein through the translocon (Brodsky and McCracken 1999). Ironically, even class I molecules are degraded through this mechanism (Hughes et al. 1997), a process greatly accelerated by the action of some viral proteins (Wiertz et al. 1996).

THE UBIQUITIN–PROTEASOME PATHWAY

The axe

Proteasomes are the principal mechanism used by eukaryotic cells to degrade proteins. In so doing, they

provide a major source of class I peptide ligands or their precursors (Rock and Goldberg 1999). Proteasomes are ancient structures, present in all eukaryotes and in many prokaryotic species as well (Voges et al. 1999). They are abundant in eukaryotic cells, e.g. HeLa cells contain approximately 500 000. They are present in both the nucleus and the cytosol, where many bind to the ER: where they may be involved in process of retro-translocation of misfolded ER proteins to the cytosol.

Proteasomes are remarkable machines, capable of degrading a wide variety of protein substrates; indeed, few proteins (if any) are known to be resistant to the action of proteasomes. The cleavage reactions occur in a barrel-like structure known as the 20-S proteasome, and regulatory subunits that attach to each end of the 20-S proteasome. The 20-S proteasomes contain 14 distinct subunits arrayed in a four-ring structure of the type, $\alpha_7\beta_7\beta_7\alpha_7$. Three of the β subunits in each inner ring are known to be catalytically active, with their active sites facing a central chamber in which proteins are degraded.

Given their ability to degrade virtually anything, 20-S proteasomes are potentially extremely dangerous to cells, and safeguards prevent such proteolytic rampages. The catalytic subunits are synthesized with pre-sequences that must be cleaved during the assembly process to activate proteolytic activity. The barrels are closed at both ends, and they must bind to a 19-S regulatory particle for protein substrates to gain access to the central chamber. The 20-S subunits bind to one 19-S subunit on each end to form the 26-S proteasome – the active form of proteasomes in cells. Why proteasomes exhibit dyad symmetry is unclear. The generation of a proteolytic chamber from symmetrical components is a parsimonious solution which is used by organisms to generate other barrel-like assemblies used for protein degradation or folding. The binding of two 19-S subunits may simply follow from the symmetry of 20-S proteasomes and not add much to proteasome function. Alternately, if the 19-S subunit processing of the protein is the rate-limiting step in protein degradation, having two 19-S submits may optimize the use of the 20-S subunit.

Unlike most proteases, the 26-S proteasome degrades substrates in an energy-dependent manner that utilizes adenosine triphosphate (ATP). ATP is exclusively consumed by the 19-S subunit, which recognizes potential substrates bearing polyubiquitin chains, unfolds the substrates, and feeds them, spaghetti like, into the barrel of the 20-S proteasome via the narrow portal that is opened as part of the process (Glickman et al. 1999). At some point before this event, the polyubiquitin chains are removed from the substrate and the ubiquitin is returned to the pool of ubiquitin monomers for reuse.

Proteasomes come in multiple forms. Indeed, the initial evidence implicating proteasomes in antigen processing was the discovery of the MHC-encoded proteasome subunits and that their expression (and that of a third subunit) is enhanced by exposure of cells to interferon γ (IFN)-γ or tumor necrosis factor (TNF)-α, the predominant cytokines released by activated $CD8^+$ T cells (Monaco and Nandi 1995) (these cytokines upregulate transcription of genes encoding each of the components of the class I processing pathway, which share common promoter elements). When induced, these subunits replace constitutively expressed subunits to create 20-S 'immunoproteasomes', which appear to be better at producing peptides favored by MHC class I molecules (Tanaka and Kasahara 1998). Such immunoproteasomes can be created only from scratch, because subunit exchange in mature proteasomes is not known to occur. As proteasomes are extremely long lived (half-life of about 10 days), cells exposed to cytokines will possess a mixture of standard proteasomes and immunoproteasomes for a very long time. Although some defined determinants are produced more efficiently by immunoproteasomes, other determinants are produced less efficiently.

Cytokine-exposed cells also produce 11-S regulators, which can take the place of 19-S regulators on 20-S proteasomes. Expression of 11-S regulators has been shown to favor antigen presentation through undefined mechanisms (Rechsteiner et al. 2000). It has been proposed that 11-S and 19-S regulators bind to a single 20-S subunit to form hybrid proteasomes, with the 11-S regulator serving to attach proteasomes to TAP and thereby increase the efficiency of peptide delivery.

The handle

Ubiquitin is a 76-residue protein that is remarkably well conserved among eukaryotes – yeast ubiquitin differs from human ubiquitin by only three conservative substitutions. It is extremely abundant in cells (about 10^8 copies per HeLa cell (Haas and Bright 1985)), and its many known cellular functions that revolve around covalent conjugation to the ϵ-NH_2 groups of lysine residues (or the amino terminus of proteins) via its carboxyl terminus (Hershko and Ciechanover 1998). Ubiquitin itself is ubiquitylated and, when polyubiquitin 'trees' containing four or more ubiquitins are present on proteins, they bind to 19-S regulators that deliver the protein to proteasomes for destruction, whereas the ubiquitin is recycled (Thrower et al. 2000). Given its dire consequences, polyubiquitylation of proteins would be expected to be a highly regulated and complicated affair and, indeed, numerous gene products are devoted to ubiquitin conjugation. For obscure reasons, cells are not content to add a single ubiquitin tree to proteins destined for destruction. Rather, polyubiquitylation is highly heterogeneous, and trees of different sizes are added to multiple substrate sites in what appears to be an irregular manner, such that polyubiquitylated proteins usually migrate as a ladder (or smear) in sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, the process is not

irreversible, and enzymes capable of removing ubiquitin from proteins (ubiquitin hydrolases) are highly active in cells (Wilkinson 2000). Polyubiquitylation is not an absolute prerequisite for protein degradation by proteasomes. There are only a few specific examples of such ubiquitin-independent targeting at present, but this could well prove to be a major source of proteasome substrates (Verma and Deshaies 2000; Benaroudj et al. 2001).

Role of proteasomes in generating antigenic peptides

The contributions of proteasomes to antigen processing, and indeed mammalian cell physiology, have been defined largely through the use of membrane-permeant, low-molecular-weight inhibitors of the proteolytic activities of 26-S proteasomes (Bogyo et al. 1997; Goldberg and Rock 2002). These include natural products of microorganisms and synthetic oligopeptide-based compounds. The former usually exhibit far more specificity for the proteasome than the latter. There are two major problems in using proteasome inhibitors to study cellular processes. First, proteasome inhibitors, like other inhibitors, cannot be absolutely specific for a single target, and they must interact with other cellular targets. It is possible to control for nonproteasome-related effects in that proteasome inhibitors with distinct chemical structures give similar results. Second, proteasomes play a key role in many cellular processes, and blocking them rapidly induces a number of serious secondary effects. These effects include depletion of the pool of free ubiquitin available for protein conjugation, induction of molecular chaperones, inhibition of protein synthesis, and interference with the cell cycle and signaling. That some of these effects are likely to be caused by an accumulation of ubiquitylated substrates awaiting destruction does not simplify matters. These effects can be minimized (although not necessarily made insignificant) by minimizing the time for which cells are exposed to proteasome inhibitors.

Proteasome inhibitors have been used to demonstrate that proteasomes are the principal cytosolic protease used by cells for generating MHC class I peptide ligands (Rock et al. 1994; Rock and Goldberg 1999). Peptide generation is maintained, however, in the presence of proteasome inhibitors, but at a lower rate. This varies in a class I allomorph-specific manner. There are many examples of antigenic peptides whose generation is unimpeded or even enhanced by proteasome inhibitors. In the latter case it has been documented for several examples that proteasomes can function to destroy determinants. These findings have two interpretations that are not mutually exclusive. First, alternate cellular endoproteases may participate in antigen processing. Second, it may not be possible completely to block proteasome function with the inhibitors currently in use. This may be the result of either resistant activities in individual proteasomes or the existence of a subset of proteasomes immune to the effects of the inhibitors, resulting perhaps from inaccessibility.

In vitro studies with isolated 20-S proteasomes have shown that proteasomes can generate the precise peptides presented by MHC class I molecules. This process is not very efficient, however, and extended versions, particularly peptides with amino-terminal extensions, are more likely to be produced. The 20-S proteasomes from yeast generate a similar set of peptides to mammalian proteasomes, deflating the hopes of immunologists that proteasomes (at least standard proteasomes) evolved for the purpose of generating antigenic peptides. Given its numerous subunits, it might be expected that proteasomal cleavage be governed by complex rules. Proteasomes do not disappoint in this regard. Proteasome activity for individual peptide bonds is influenced by amino acids on both sides of the cleavage site, as wells as flanking residues, with the greatest influence mediated by those within five residues of the cleavage sites. Work is progressing on developing predictive algorithms for the peptides that are liberated by proteasomes (Altuvia and Margalit 2000; Kuttler et al. 2000).

ROLE OF NONPROTEASOMAL CYTOSOLIC PROTEASES IN ANTIGEN PROCESSING

Trimming enzymes

Proteasomes need not generate the precise peptides presented by class I molecules. A considerable body of evidence indicates that peptides can be trimmed by aminopeptidases in the cytosol (York et al. 1999) and ER (Yewdell et al. 1999a), whereas carboxypeptidase trimming seems to be highly unusual. A candidate cytosolic aminopeptidase is leucyl aminopeptidase, because its expression is enhanced by IFN-γ and TNF (Beninga et al. 1998). Other aminopeptidases (bleomycin hydrolase and puromycin-sensitive aminopeptidases) are known to be capable of trimming amino-terminal extensions from defined antigenic peptides (Stoltze et al. 2000). The ER contains two aminopeptidases (ERAP1 and ERAP2) that are largely, if not exclusively, dedicated to trimming antigenic peptides (Serwold et al. 2002; York et al. 2002).

One of the most striking features of antigen processing is that the recovery of antigenic peptides from cells is dependent on expression of a class I molecule that binds the peptide with more than the threshold affinity. Two mechanisms potentially contribute to this phenomenon (Falk et al. 1990):

1. class I molecules are required for the generation of antigenic peptides by serving as a template for the action of trimming exopeptidases
2. peptides are rapidly destroyed unless they are protected by class I molecules.

It appears that the second possibility plays a major role in the phenomenon. Class I molecules are still required for the recovery of antigenic peptides when the antigenic peptides are synthesized as cytosolic minigene products corresponding to the exact peptide. Moreover, peptides are rapidly degraded by cytosolic extracts and rapidly exported from the ER unless they bind to class I molecules. Peptides can be destroyed by the very aminopeptidases involved in trimming, and also by endopeptidases active on oligopeptides. One such endopeptidase known to be capable of degrading antigenic peptides and their precursors is thimet oligopeptidase (Silva et al. 1999). Peptide destruction is probably a major contributor to the low efficiency of antigen processing discussed above.

Proteasome understudy?

As mentioned above, the generation of many class I peptide ligands is insensitive to proteasome inhibitors. Two groups reported that it is even possible to adapt cells to resist the cytotoxic effects of proteasome inhibitors (Glas et al. 1998; Geier et al. 1999). In both studies the adapted cells demonstrated enhanced expression of tripeptidyl peptidase (TPPII), which was proposed as a substitute for the functions of the proteasome. This was unexpected because proteasomes are essential for the viability of yeast. Subsequently it was shown that cells adapted to proteasome inhibitors retain proteasome activity and that such activity contributes to antigen processing and protein degradation and is required for viability (Princiotta et al. 2001). School is still out on the possible contributions of TPPII to antigen processing and overall protein degradation, though a recent report suggests that a majority of proteasome products must be further degraded by TPPII to be eligible for binding to class I molecules (Reits et al. 2004).

SHEPHERDING PEPTIDES TO THE PROMISED LAND: TAP

Endoplasmic reticulum trimming by aminopeptidases is possible because extended peptides have access to the ER. Peptides are transported from the cytosol to the ER by TAP, which is a member of a large family of prokaryotic and eukaryotic proteins (the ABC, or ATP-binding cassette, family) that function in the transmembrane transport of a wide variety of substrates (Elliott 1997). TAP is formed by the association of two homologous subunits termed TAP1 and TAP2 which rapidly assemble in the ER. Each subunit possesses multiple (9–10), short, membrane-spanning domains attached to a globular carboxy-terminal, cytosolic, nucleotide-binding domain.

As with other family members, TAP-mediated peptide transport is driven by hydrolysis of ATP or other trinucleotides. TAP preferentially transports the types of peptides favored by class I molecules, particularly in its requirements that peptides be at least eight residues in length. The upper limit of peptide length is messier, but the efficiency drops off considerably when peptides are longer than 16 residues or so, although even those with 40 residues may be transported at low efficiency (Koopmann et al. 1996).

TAP does not transport all peptides with equal efficiencies. Alterations at all positions can influence TAP-mediated transport. The greatest preferences are demonstrated for carboxy-terminal residues; indeed TAP from different species demonstrates a marked preference for the types of carboxyl termini preferred by their respective class I molecules (in humans, hydrophobic or positively charged residues) (Momburg et al. 1994; Schumacher et al. 1994). Thus, longer peptides transported into the ER by TAP are more likely to possess amino-terminal extensions than carboxy-terminal ones. Next in importance are the first three residues from the amino terminus. Similar preferences exist for peptides of different lengths. This suggests a model in which efficient TAP recognition of peptides entails interaction with the peptide's three amino-terminal residues and carboxy-terminal residue, and the presence of no less than 4, and no more than 12, internal spacer residues. Interestingly, despite the fact that proline at position 2 is a dominant anchor residue for numerous human class I allomorphs, this greatly disfavors TAP-mediated transport of many peptides. This suggests that, for these peptides, trimming of amino-terminal extensions in the ER is particularly important.

TAP polymorphism in rats greatly influences the peptides delivered to class I molecules (Howard 1995). Although human TAP genes display limited polymorphism, the known alleles do not appear to differ greatly in their specificities (Obst et al. 1995). Thus, it does not appear that TAP polymorphism plays a major role in influencing the response of individuals to viral proteins. A splice variant of human TAP2 has been described that possesses an altered carboxyl terminus (Yan et al. 1999). This looks to modify the specificity of TAP, but its effects in vivo await further characterization.

TAP-INDEPENDENT PROCESSING OF ENDOGENOUS ANTIGENS

As mentioned above, class I molecules are bound to TAP (via tapasin) until released by peptide binding. It is thought that this facilitates their association with peptides transported by the same complex, thereby greatly increasing the effective peptide concentration. TAP is not, however, essential for peptide loading on to class I molecules. Peptides targeted to the ER are able to associate efficiently with class I molecules in cells lacking TAP (Anderson et al. 1991). Indeed, in studies using recombinant vaccinia virus expressing various forms of a peptide antigen, peptides targeted to the ER have been found to be more immunogenic in mice than

cytosolic peptides biosynthesized in similar amounts, or the full-length gene viral proteins products that are the natural source of the peptides (Restifo et al. 1995). It has also been found that cytosolic antigens can be presented to a limited extent in a TAP-independent manner by an unknown mechanism. Although these exceptions are interesting and possibly of practical importance (using ER-targeted peptides for immunization), it should be emphasized that TAP plays a critical role in the presentation of most viral determinants. TAP is also crucial for T-cell development, because TAP-deficient individuals demonstrate greatly decreased levels of T cells (de la Salle et al. 1994; Gadola et al. 2000). Curiously, such individuals are relatively healthy, having mainly recurrent localized bacterial infections.

CONTRIBUTION OF SECRETORY PROTEASES TO ANTIGEN PROCESSING

Many proteins targeted to the ER immediately lose their amino-terminal signal sequences, presumably as a result of the action of signal peptidase. Further proteolysis of these peptides enables their binding to class I molecules, particularly if class I allomorphs favor binding of hydrophobic peptides. HLA-A2 is the prototype for these allomorphs, and many of the peptides that it presents are derived from signal sequences. Signal sequence presentation is central to the function of some class Ib molecules, which present leader peptides from class I molecules to natural killer (NK) cells.

The presentation of such leader peptides can be either TAP independent or TAP dependent. In the latter case, presentation can be either proteasome dependent or independent. Thus, signal peptides can be produced strictly by secretory proteases or a combination of secretory and cytosolic proteases.

From a functional standpoint, using ERAP the ER is able to trim amino-terminal extensions of TAP-transported peptides efficiently, while still having a very limited capacity for removing carboxy-terminal residues. Peptides transported into the ER independently of TAP appear to be handled in a similar manner.

The secretory pathway displays a limited capacity for generating determinants from very large substrates, presumably as a result of endopeptidases acting in concert with aminopeptidases. Such endopeptidases are not necessarily limited to the ER: furin, an endopeptidase activated only in the trans-Golgi complex, is involved in the generation of several defined peptides, which are presumably generated and loaded in the trans-Golgi complex (Gil-Torregrosa et al. 2000).

Generating peptide ligands from exogenous proteins

As mentioned at the start, the activation of naïve $CD8^+$ T cells requires pAPCs that express the proper co-stimulatory molecules (at least according to dogma). Obviously, such cells will present peptides from endogenous antigens synthesized during a viral infection. But, what if the virus cannot (or will not) infect the appropriate APC? And what about nonviral pathogens that do not hijack host ribosomes for their own nefarious ends? Can naïve $CD8^+$ T cells respond under these conditions?

The answer to this question is yes. This pathway was initially discovered by Bevan, who reported that $CD8^+$ T cells can be induced against antigens introduced into mice by adoptive transfer of MHC-mismatched cells, terming the phenomenon 'crosspriming' (Bevan 1976). Underlying this are a number of potential mechanisms that also serve as the basis for the immunogenicity of protein-based vaccines that elicit $CD8^+$ T-cell responses. The true nature of the crosspriming pathway in vivo remains to be determined, although it is likely that it reflects the operation of many (or all) of the mechanisms defined below.

TWO PATHS TO RIGHTEOUSNESS

The processing of exogenous antigens can be divided into two distinct pathways. First, antigens can access the cytosol where they are treated in a manner similar to endogenous antigens. Three basic routes into the cytosol are possible.

The first is traversing cell membranes as a result of properties associated with the substance, e.g. viruses or liposomes can fuse with cell membranes, delivering their contents to the cytosol. Other viruses directly penetrate cellular membranes. In the case of abundant viral core proteins, a sufficient quantity may enter the cytosol to produce enough MHC–class I complexes to trigger $CD8^+$ T cells (Yewdell et al. 1988; Riddell et al. 1991). Bacteria that reside in endosomal compartments secrete proteins that translocate endosomal members to gain access to the cytosol where they can alter cellular function (Pamer et al. 1991). Some proteins have sequences that enable them to cross the plasma membrane (Chikh et al. 2001). These sequences can enable even large proteins to cross membranes.

The second route entails entry from endosomes that leak by accident or by design. Highly active phagocytic cells create macropinosomes which enable substrate access to the cytosol (Norbury et al. 1995). DCs possess an apparently unique endocytic compartment that delivers substances of about 40 kDa to the cytosol (Rodriguez et al. 1999).

The third route is retrograde transport from endosomes to the Golgi complex and then to the ER, where they follow the route traversed by endoplasmic reticulum-associated degradation (ERAD) substrates back to the cytosol. Many toxins are thought to enter cells via this route (Lord and Roberts 1998).

Peptides can also be generated from exogenous antigens outside the APC or in endosomal compartments of

APCs and loaded on to cell surface or endosomal PR class I molecules. This pathway resembles the classic class II antigen-processing pathway. The pAPCs are particularly adept at such nonclassic class I antigen processing.

GENERATION AND TRAFFICKING OF PR CLASS I MOLECULES

Within 60 min of their departure from the ER, a substantial fraction of class I molecules lacks peptide ligands. These molecules may never have been loaded, may have been loaded with a lower affinity ligand, or may have suboptimally bound a peptide that normally binds with higher affinity. This is the source of PR class I molecules, the properties of which are described above. Interference with peptide supply by blocking proteasomes or inactivating TAP actually *decreases* the number of PR class I molecules (Day et al. 1995), presumably because less poorly loaded class I molecules leave the ER. The percentage of such poorly loaded molecules can be very high, representing even 95 percent of class I molecules exported from the ER (Su and Miller 2001).

Such PR molecules are present throughout the secretory pathway. As discussed above, exogenous β_2-microglobulin and/or low temperatures stabilize PR molecules. PR molecules stabilized in this manner also participate in the presentation of exogenous antigens processed in endosomal compartments (Song and Harding 1996; Schirmbeck et al. 1997). This latter statement alludes to the trafficking of class I molecules to endosomes. Class I molecules derived from the cell surface have been found in endosomal compartments that contain class II molecules in a number of cell types, including DCs (Geuze et al. 1985; Chiu et al. 1999; Gromme et al. 1999; Castellino et al. 2000; Kleijmeer et al. 2001; MacAry et al. 2001). A number of class I allomorphs have been reported to bind peptides at the acidic pH characteristic of endosomes (Stryhn et al. 1996), and the generation of peptide class I complexes in endosomes has been visualized (Castellino et al. 2000).

It is uncertain how class I molecules are targeted to endosomal compartments. Several class I allomorphs have been reported to associate with the invariant chain (Cerundolo et al. 1992), and the invariant chain has been reported to direct HLA-B27 to endosomal compartments (Sugita and Brenner 1995). The trafficking of class I molecules from endosomes to the plasma membrane of DCs is upregulated upon DC maturation (MacAry et al. 2001). The mechanism underlying this and nearly every aspect of the intracellular trafficking of class I molecules to and from endosomes remains to be characterized.

Class I molecules are also present in exosomes: small vesicles generated in the lumen of large endosomes by a pinching-off process with the topological consequence of orienting luminal domains of integral membrane proteins (similar to class I molecules) on the outer surface of the exosome. After the fusion of multivesicular endosomes with the plasma membrane, exosomes are released by cells. Originally described in reticulocytes, exosomes are also known to be produced by B cells and DCs. Exosomes are rich in MHC class II and co-stimulatory molecules, and are able to able to stimulate $CD4^+$ (Raposo et al. 1996) and $CD8^+$ T cells (Zitvogel et al. 1998). The physiological significance of exosomes in immune responses remains to be established.

ANTIGEN PROCESSING IN ENDOSOMAL COMPARTMENTS

The possibility of endosomal processing of MHC class I antigens was first shown using antigen coupled to beads (Rock et al. 1990), and has now been extended to a large number of antigens, including bacteria, viruses, virus-like particles, and aggregated proteins (reviewed in Yewdell et al. 1999b). Although 'peptide regurgitation' (i.e. cell-surface PR class I binding of peptides generated in endosomal compartments) (Pfeifer et al. 1993) has been shown to occur under some conditions, it is likely that peptides generated in endosomes bind to endosomal class I molecules. Although every cell type is probably capable of endosomal processing, it occurs more efficiently in pAPCs. The extent to which this is caused by the increased endocytosis versus enhanced trafficking of class I molecules to and from endosomes versus enhanced peptide generation versus facilitated loading remains to be established. Indeed, precious little is known about endosomal processing of class I peptides, and the nature of the proteases and mechanism of peptide loading are completely undefined.

WHAT DO MOLECULAR CHAPERONES DO?

In searching for factors that are able to prime the immune system to reject transplanted tumors, Srivastava and colleagues discovered that the molecular chaperone gp96 purified from tumor cells is able to induce protective immunity against tumor challenge (Li et al. 2002). These findings have been extended to show that several different molecular chaperones are able to induce $CD8^+$ T-cell responses to defined antigens when the chaperones are purified from cells expressing the antigen or loaded with antigenic peptides (Li et al. 2002). Notably, the responses induced by molecular chaperones are quite weak in comparison to virus infections, and even to other exogenous antigen preparations. The jury is still out on the physiological role of molecular chaperones in the crosspriming phenomenon (Wallin et al. 2002), though recent evidence disfavors their participation in crosspriming (Norbury et al. 2004; Wolkers et al. 2004).

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The role of CD8 T cells in the control of infectious disease and malignancies

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INTRODUCTION

CD8 T cells are a subset of T lymphocytes that are responsible for cellular immune responses. CD8 T cells recognize specific peptide epitopes presented by class I major histocompatibility complexes (MHC) on the surface of pathogen-infected or malignant cells using T-cell receptors (TCR) and CD8 molecules. TCRs consist of α and β chains, which associate with CD3 molecules. TCRs associating with CD3 bind to peptide and the polymorphic region of MHC, whereas CD8 binds to the nonpolymorphic region. After recognition of peptide–MHCI complexes and binding to several adhesion molecules on pathogen-infected and malignant cells, CD8 T cells are able to protect against pathogens and malignancies by secreting soluble mediators and inducing cytotoxicity. In this chapter, the role of CD8 T cells in the control of infectious diseases and malignancies, the induction of CD8 T-cell responses, and the protective mechanisms mediated by CD8 T cells are discussed.

CD8 T LYMPHOCYTES IN THE CONTROL OF INFECTIOUS DISEASES

CD8 T-cell immunity to viruses

Unlike extracellular pathogens, viruses are obligate intracellular pathogens, the multiplication of which occurs inside infected cells; once released from infected cells, mature virions will infect other normal cells. Although virus-specific antibodies can clear extracellular virus particles by neutralization or other mechanisms, antibodies may not be able to effectively eliminate intracellular virus, particularly as some viruses can be transmitted by fusion between infected and normal cells (Spickett et al. 1989; Rausch et al. 1992; Kahn et al. 1999). When virus infection occurs, viral antigens are processed into short peptides, which are presented by MHCI molecules (Townsend and Bodmer 1989), CD8 T cells are able to recognize these peptide epitopes presented by MHCI molecules. The level of CD8 T-cell responses also increases earlier than that of other adaptive immune responses. The level of CD8 cytotoxic T-lymphocyte (CTL) activity can be measured by in vitro cytotoxicity assays or cytokine analysis. After recognition of peptide–MHC complexes presented by virus-infected cells, CD8 CTLs are capable of inhibiting intracellular virus proliferation by cytotoxicity or the activity of secreted cytokines (Guidotti and Chisari 2001). Virus-specific CD8 CTL responses induced by different vaccine constructs have been demonstrated to protect against several virus infections in animal models (Schulz et al. 1991; Ciernik et al. 1996; Hsu et al. 1998, 1999; Leifert et al. 2001; Li et al. 2001a; Nass et al. 2001; Gierynska et al. 2002). Whether or not the level of CTL activity or the frequency of CD8 CTLs is important in

conferring protection needs further investigation in situations where protective immune responses have been induced after immunization.

Different viruses also have a variety of ways to escape CD8 T-cell responses (Phillips et al. 1991; Goulder et al. 1997; McMichael and Phillips 1997; McMichael and Rowland-Jones 2001), which could result in persistence of virus infection or reinfection within the population. Viruses with a potentially high mutation rate, such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), hepatitis C virus (HCV), and influenza virus with antigenic variations within the epitopes, are able to avoid CTL recognition. These observations demonstrate the protective role of CD8 CTLs (Price et al. 2000; Erickson et al. 2001; McMichael and Rowland-Jones 2001). Herpesviruses can interfere with the MHCI processing and presentation pathway (Yamashita et al. 1993; Hill et al. 1995) and respiratory syncytial virus infection can suppress CD8 T-cell memory responses (Chang and Braciale 2002). HIV, adenovirus, and cytomegalovirus (CMV) can down-regulate MHCI molecule expression on the cell surface (Yamashita et al. 1993; Pereira et al. 1995; Mahr and Gooding 1999; Reusch et al. 1999; McMichael and Rowland-Jones 2001). However, little is known about the dynamics of immune responses after infection with mutant viruses. Specificity, cytokine expression and frequency of CD8 T-cell responses to wild-type viruses may be different from those to mutant viruses during infection. The results of studies of the differences in CD8 T-cell responses after infection with wild-type virus and mutant virus could help in the design of promising and effective vaccine constructs and new immunization strategies.

CD8 T-cell immunity to bacteria

Most pathogenic bacteria can survive and proliferate outside cells. However, when intracellular bacteria, such as mycobacteria and *Listeria* spp., cause infection, CD8 T cells could play an important protective role (Gellin and Broome 1989; Harty et al. 1992; Clemens and Horwitz 1996; Sturgill-Koszycki et al. 1996). Mycobacteria are acid-fast intracellular pathogens and, after infection, they can survive in certain endosomal compartments (Sturgill-Koszycki et al. 1996). Mycobacterial antigens are processed in these compartments into peptide fragments that are presented by MHCI molecules to CD8 T cells. Both αβ- and γδ T cells have been shown to play a protective role against mycobacterial infections (Ladel et al. 1995; D'Souza et al. 1997). CD8 CTLs can mediate protective immunity against mycobacteria by granule-mediated cytolysis or by the release of cytokines, such as interferon-γ (IFN-γ) (Harty et al. 1992; Ehrt et al. 2001). IFN-γ and interleukin-12 (IL-12) are also involved in granulomatosis and the generation of protective cellular immune responses (Cooper et al. 1997). Protective CD8 CTL responses against mycobacteria can be induced by a variety of vaccine preparations, including recombinant viral vaccines and DNA vaccines (Lowrie et al. 1997; Zhu et al. 1997).

Listeria monocytogenes is a gram-positive intracellular bacterium that causes listeriosis (Gellin and Broome 1989). Infection with live *L. monocytogenes* induces antigen-specific CD8 T-cell responses, with peak responses 7–9 days and 5 days after reinfection (Busch et al. 1998). Antigen-specific CD8 T-cell responses induced by live *L. monocytogenes* provides protective immunity against subsequent infection. On the other hand, immunization with heat-inactivated *L. monocytogenes* could not provide effective protection against subsequent infections, although the size of the antigen-specific CD8 T-cell population was increased to the same extent as that in immune animals (Von Koenig et al. 1982; Lauvau et al. 2001). However, in situations where CD8 T-cell responses did confer protective immunity against intracellular bacteria after infection or immunization, the soluble mediators secreted by CD8 T cells participating in protection still remain unknown.

CD8 T-cell immunity to parasites

Most pathogenic parasites have complicated life cycles with different developmental stages (Markell and Voge 1981). After infection with intracellular parasites, parasitic antigens are processed and presented as MHCI–peptide complexes in infected cells, which are recognized by CD8 T cells at certain stages in some cases. Thus, CD8 T cells are able to protect against malaria infection at the liver stages (Hoffman et al. 1989; Romero et al. 1989; Charest et al. 2000). Antigen-specific CD8 T-cell responses protect against *Toxoplasma gondii* parasites after immunization (Khan et al. 1994; Nielsen et al. 1991, 1999). In CD8-deficient or CD8-depleted animals, the growth of leishmania parasites cannot be controlled, which indicates the protective role of CD8 T cells in this infection (Belkaid et al. 2002). The protective ability of CD8 T cells against a variety of parasitic infections has also been demonstrated by the adoptive transfer of parasite-specific CD8 T cells from immune animals (Nielsen et al. 1991). As a result of their complicated life cycles, many parasitic antigens from parasites are expressed in different stages and, as an added complication, malaria parasites can also infect red blood cells, which are cells that do not express MHCI–peptide complexes (Urban et al. 1999). The question of whether there are malaria antigens expressed in red blood cells capable of regulating CD8 T-cell responses has yet to be answered.

Parasites have also developed different ways of escaping immune responses (Borst 1991; Hall and Joiner 1991; Barral-Netto et al. 1992), including antigenic variation, modulation of cytokine expression, avoidance of

killing by oxygen metabolites or hydrolase, and alteration of the function of antigen-presenting cells (APC). Whether certain parasites have also developed the same strategies as used by viruses to escape CD8 T-cell responses remains to be investigated.

CD8 T LYMPHOCYTES IN THE CONTROL OF MALIGNANCIES

CD8 T-cell immunity to tumors

Historically, several lines of evidence have shown the existence of tumor-specific immunity. Clinical evidence has also shown that tumors sometimes regressed in cancer patients after bacterial infection and the growth of tumors is suppressed after transplantation into an MHC-incompatible host in experimental models. The immune surveillance hypothesis was proposed to explain the role of immune responses in the control of tumors (Boon et al. 1994). Although immune responses are able to control tumors, they always eventually escape immune attack in the absence of the use of further and better treatment (Marincola et al. 2000). In contrast to MHCII molecules, which are mainly expressed on specialized APCs, most cells express MHCI molecules, including tumor cells. With their ability to recognize specific peptide–MHC complexes, CD8 CTLs are considered to be important effectors of cellular immunity against tumor cells. To induce specific CD8 CTL responses against tumor cells, the identification of tumor antigens is important and this also has importance for the development of immunotherapeutic approaches. Several tumor antigens have been identified that have the ability to induce protective tumor-specific CD8 CTL responses, e.g. the melanoma antigens, MAGE, BAGE, and GAGE, are expressed in melanoma cells but not in normal cells (Boon et al. 1994). Many of the melanoma-associated antigens are derived from normal nonmutated melanocyte differentiation antigens, including MART-1, gp-100, and tyrosine-related protein-2 (Zeh et al. 1999).

The lack of appropriate tumor antigens has impeded the development of effective immunotherapeutic approaches to other tumors. To induce protective CD8 T-cell immunity to other tumors, tumor-associated antigens that are overexpressed selfantigens in other tumors, such as mdm and WT-1, have been used in attempts to generate protective tumor immunity (Sadovinikova and Stauss 1996; Gao et al. 2000; Ohminami et al. 2000; Oka et al. 2000). As these overexpressed tumor antigens are expressed in normal cells at lower levels, there is a possibility that deleterious autoimmune responses could be induced when effective tumor-specific T-cell responses are generated against them. Furthermore, the induction of tolerance could also impair the maintenance of T-cell responses against tumors using these overexpressed self-proteins as tumor antigens.

Tumor immunotherapy by CD8 T cells

Tumor immunotherapy can provide an alternative method to traditional therapeutic approaches. Several immunotherapeutic approaches have been developed to generate humoral or cellular immunity to tumors, including the induction of protective cellular immune responses by immunization with tumor antigens and the generation of tumor-specific CTL lines or clones with APCs expressing tumor antigens (Matis et al. 1986). Different tumor antigen preparations have been developed for immunization purposes (Oka et al. 2000). Tumor antigens formulated in different adjuvants, expressed in viral vectors or nucleic acids, and expressed in specialized APCs have been used to induce tumor-specific CD8 CTL responses (Ciernik et al. 1996). CD8 CTL peptide epitopes identified from tumor antigens have been used to generate CD8 CTL lines or clones for tumor immunotherapy (Sadovinikova and Stauss 1996; Zeh et al. 1999). CD8 CTL epitopes have also been used for the construction of vaccines to generate protective CD8 CTL responses. Several vaccine preparations and immunization approaches, including the induction of CD4 T-cell responses and the inclusion of cytokines, chemokines, and costimulatory molecules in the vaccine design, have been used to improve the effectiveness of tumor-specific CD8 CTL responses. In animal models, the induction of tumor-specific CD8 CTL responses by immunization and the transfer of tumor-specific CTL lines or clones have had some success in the control of tumor growth, although the control of growth was not complete. Variations in the effectiveness of immunotherapy to different tumors using CD8 T cells have also been described. Similar to pathogens in some aspects, tumors are also able to avoid immune responses by modulating the expression of MHC molecules, cell surface molecules, and cytokines (Marincola et al. 2000; Buggins et al. 2001; Ibrahim et al. 2001). The investigation of the mechanisms used by specific cells to evade immune responses could lead to the design of better therapeutic strategies. However, the mechanisms used by CD8 CTL to control tumor cells are also not clear other than their cytolytic activity and cytokine expression.

THE PROTECTIVE MECHANISMS INVOLVING CD8 T LYMPHOCYTES

Soluble mediators secreted by CD8 T cells

CD8 T cells protect against infection by intracellular pathogens via cytolytic and noncytolytic mechanisms (Barth et al. 1991; Tuttle et al. 1993; Yang and Walker 1997; Guidotti and Chisari 2001; Binder and Griffin 2001; Russell and Ley 2002). The noncytolytic mechanisms are mediated by cytokines, chemokines, and other

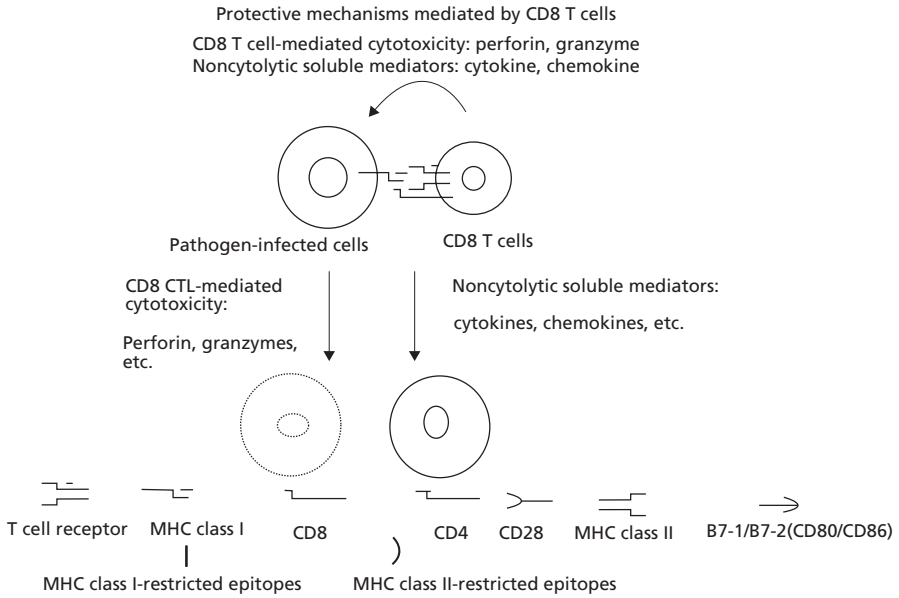

Figure 1 *CD8 T cells can protect against pathogens by secreted noncytolytic soluble mediators or granule-mediated cytolysis. Noncytolytic soluble mediators include cytokines or chemokines that inhibit proliferation or infection of intracellular pathogens. Granules containing perforins, granzymes, etc. induce cytolysis of pathogen-infected cells. CTL, cytotoxic T lymphocyte.*

soluble mediators secreted from CD8 T cells (Figure 1). The involvement of these noncytolytic mechanisms in protection against pathogens was observed in experiments by using particular cytokine gene-deficient animals or depletion of particular cytokines by a specific antibody in animal models (Klavinskis et al. 1989; Tishon et al. 1995; Hsu et al. 1998). Using these approaches, it has been shown that certain cytokine-deficient CD8 T cells are less efficient in the control of infection with some pathogens than with others (Harty et al. 1992; Tishon et al. 1995). CD8 T cells secrete several antiviral cytokines after infection or peptide epitope stimulation (Klavinskis et al. 1989; Hsu et al. 1998; Patterson et al. 2002). IFN-γ and tumor necrosis factor α (TNF-α) secreted by CD8 T cells are able to reduce virus infection by intracellular or extracellular mechanisms, to recruit and activate macrophages, T cells, or natural killer (NK) cells to perform their effector function. IFN can directly inhibit virus replication by activating the oligoadenylate synthetases, Mx proteins, double-stranded-RNA-activated protein kinase, and the double-stranded-RNA-specific adenosine deaminase systems (Silverman 1994; Patterson et al. 1995; Haller et al. 1998; Terenzi et al. 1999). Oligoadenylate synthetases suppress viral replication by induction of several cellular RNases that degrade viral transcripts. Double-stranded-RNA-activated protein kinase and adenosine deaminase inhibit viral protein synthesis. IFN-γ and TNF-α are also able to regulate antigen processing and presentation, enhance the expression of MHC molecules on the cell surface, and recruit macrophages, NK cells, and T cells into virus infection sites to perform their antipathogen effector function.

During pathogen infection, CD8 T cells are capable of making and secreting many cytokines, chemokines, and other soluble mediators with as yet unknown functions (Toso et al. 1995; Wagner et al. 1998). Anti-HIV-specific CD8 T cells can produce unidentified noncytolytic factors that suppress HIV viral replication in vitro after antigen recognition (Toso et al. 1995). Intracellular bacteria and parasites are also inhibited by several cytokines secreted from CD8 T cells. The detailed intracellular events that inhibit intracellular bacteria or parasites are less clear than those that inhibit intracellular viruses. Although the intracellular mechanisms that inhibit viral replication have been well studied, there are still intracellular events that are not very clear other than the suppression of viral RNA or protein synthesis. The induction of the expression by intracellular molecules, which play an important role in the inhibition of intracellular bacteria and parasites, has been suggested by several authors (Seguin et al. 1994; Scheller et al. 1997). Further investigation of the intracellular events that protect against infection of intracellular bacteria, parasites, or viruses by cytokines could lead to the design of effective therapeutic approaches. Whether there are unidentified soluble molecules produced by CD8 T cells that protect against different virus infections represents a new area to be explored.

CD8 T-cell-mediated cytotoxicity

CD8 CTLs can mediate cytolysis of pathogen-infected or malignant cells by granule exocytosis of perforin, granzymes, and other molecules (Russell and Ley 2002). CD8 CTLs are also able to lyse the pathogen-infected or

malignant cells by the Fas/Fas ligand pathway (Figure 1). After T-cell activation, granules including perforin, granzyme, and other components are synthesized and reside in the cytoplasm. In the recognition of pathogen-infected cells, granules are fused with cell membranes at the contact sites of the tight junction between CD8 T cells and target cells. Once granules are released from CD8 T cells, perforin polymerizes to insert into the cell membrane and induces membrane damage in the presence of calcium (Tschopp et al. 1989; Muller and Tschopp 1994). Although the function of perforin is similar to that of complement in inducing membrane damage, perforin alone is not sufficient to induce apoptosis of the target cells. Perforin alone was thought to be capable of causing apoptosis of the target cells; however, several authors have suggested that the pore created by perforin is too small to allow large molecules, granzymes, and other components to pass into the target cells to induce apoptosis (Podack et al. 1985; Browne et al. 1999). This debate does not change the fact that the function of perforin is to induce membrane damage. However, the role of perforin in cytotoxicity mechanisms induced by granule exocytosis remains to be defined.

Granzymes are serine proteases in the cytotoxic granules: granzyme A is the tryptase that prefers to cleave protein substrates in the position of arginine or lysine residues (Odake et al. 1991). Granzyme B is similar to the capases that prefer to cleave protein substrates in the position after aspartic acid residues (Odake et al. 1991). The function of granzyme A and other granzymes in cytotoxic granules has not been defined. Granzyme B cleaves procapase-3 and other procaspases in vitro (Sarin et al. 1998; Yang et al. 1998; Sutton et al. 2000), which could also be required for activation of caspase-3 to cleave other substrates in the target cells. After granule exocytosis by CD8 CTL, the cascade effect of substrate digestion by granzymes could result in apoptosis of target cells. Granzyme B induces nuclear DNA fragmentation in cells that are functionally caspase deficient, which suggests that this enzyme can directly activate apoptotic nucleases (Sutton et al. 2000). One of the apoptotic nucleases was identified as caspase-activated DNase (Enari et al. 1998). Granzyme B can enter the nucleus without the involvement of perforin or the alteration of the nuclear pore (Jans et al. 1996). In one of the pathways of granzyme-induced apoptosis, the involvement of mitochondria has been suggested because Bcl-2 can block death induced by perforin and granzymes (Russell and Ley 2002).

Granzyme A is less efficient at inducing apoptosis than granzyme B and the cellular pathways used by granzyme A to induce apoptosis are different from those by granzyme B. Granzyme A neither activates caspases nor induces cleavage of granzyme B substrates, caspase-3, DNA-dependent protein kinase (DNA-PK), or Poly-ADP-ribose-polymerase (PARP). Granzyme A does not result in oligonucleosomal DNA degradation, but only induces single DNA breaks, indicating that granzyme A activates different nucleases from those activated by granzyme B (Shi et al. 1992). The mechanisms used by CD8 CTLs to avoid cell death after granule exocytosis have been studied for many years and CD8 CTLs contain an inhibitor of granzyme, B, known as protease inhibitor 9 (PI-9) (Sun et al. 2001). When murine homologues of this inhibitor, SPI-6 and PI-9 are overexpressed in target cells, they protect these cells from death (Medema et al. 2001). The detailed mechanisms of the intracellular cascade events resulting in the induction of apoptosis of target cells are still unclear. Several viruses can encode inhibitors of granzyme B to inhibit both Fas- and granzyme-mediated lysis, including the poxvirus-encoded cytokine response modifier A (CrmA) and the granzyme B inhibitor which interacts with granzyme B and several capases to inhibit CTL-mediated lysis (Tewari et al. 1995). A study of the mechanisms used by viruses or tumor cells to evade CTL-mediated lysis by granzymes may well provide information that could help in the design of better immunization and therapeutic approaches.

THE DEVELOPMENT OF VACCINES FOR THE INDUCTION OF CD8 T-CELL RESPONSES

Inactivated vaccines

For many years, inactivated vaccines have been prepared from whole virions treated with chemicals, such as formalin, that reduce their infectivity and maintain their ability to stimulate protective immunity (Eller 1969; Fulginiti et al. 1969; Kapikian et al. 1969; Kim et al. 1969). Inactivated pathogens are the only vaccines available to stimulate protective antibody responses for many diseases. Although inactivated vaccines were thought to maintain the immunogenicity to stimulate immune responses with minor damage to the structure of viral proteins, formalin-inactivated vaccines are ineffective at generating protective, conformation-dependent, antibody responses and cellular immune responses mediated by CD8 CTLs (Chargelegue et al. 1998; Hsu et al. 1999). There are also other disadvantages of inactivated vaccines: virulent pathogens could still be infectious if the quality of vaccine preparation is not well controlled; and the contamination of vaccine preparations with materials derived from tissue culture could result in hypersensitivity reactions (Eller 1969; Chanock et al. 1992; Wertz and Sullender 1992). Indeed, the use of formalin-inactivated respiratory syncytial virus vaccines induced exacerbated disease during a subsequent epidemic (Fulginiti et al. 1969) and the pathological effects induced by formalin-inactivated respiratory syncytial virus vaccine have been extensively studied

(Openshaw et al. 2001). A detailed analysis of the factors that result in pathological consequences following the use of formalin-inactivated vaccines is important for the development of new vaccines and immunization strategies to generate protective immunity without exacerbated diseases.

Attenuated vaccines

Attenuated vaccines are mutant live pathogens that have similar antigenicity to wild-type pathogens and induce no pathogenic effects (Wegmann et al. 1986). Mutant live pathogens are selected by attenuating strains of wild-type pathogens in serial cell culture or by genetic manipulation (Beck et al. 1986; Wertz and Sullender 1992; Khattar et al. 2001; Bukreyev et al. 1999, 2001; Stittelaar et al. 2002). Many of the currently available vaccines to prevent infectious diseases are live attenuated vaccines (Table 1) that are considered to induce protective humoral and cellular immune responses similar to those induced by the wild-type virus but without pathogenic consequences. In contrast to inactivated vaccines, live attenuated vaccines are able to induce pathogen-specific CD8 CTL responses. There is very little information about the differences in cellular immune responses mediated by CD8 CTL responses generated by attenuated vaccines and wild-type pathogens, and a comparison of these differences will facilitate the development of better vaccines and immunization approaches to avoid any pathogenic consequences of immunization.

Genetic manipulation approaches can be used to insert foreign genes with immunoregulatory functions, such as cytokines, into the pathogen genome, to delete the genes related to pathogenic effects, to introduce mutations into the genome of pathogens to enhance protective immunity, and to avoid exacerbated pathogenesis (Bukreyev et al. 1999, 2001; Khattar et al. 2001). Although the attenuated vaccines currently in use have given encouraging results in conferring protective immune responses against many infectious diseases, there is an urgent need to develop alternative vaccines to prevent infection with mutant pathogens. There is a possibility that attenuated vaccines may revert to the virulent strain or could be contaminated with virulent pathogens after serial cultivation in tissue culture or in the infected population. Investigations into the long-term pathogenic effects of immunization with attenuated vaccines will be important for the design of improved vaccines.

Subunit vaccines

Subunit vaccines mainly comprise protein or carbohydrates expressed by pathogens that are recognized by antibodies, T lymphocytes, or other immune cells (Zuckerman 1986; Jones et al. 1996; Lingappa et al. 2001). Many viral proteins expressed on the surface of virions have been selected as subunit vaccines for the induction of protective antibody responses (Zuckerman 1986; Schirmbeck et al. 1994; Li et al. 1998; Bembridge et al. 2000). Surface and internal virus proteins are capable of generating protective T-cell responses (Schirmbeck et al. 1994; Li et al. 1998; Bembridge et al. 2000). Although capsular carbohydrate antigens are used as subunit vaccines to generate immune responses (Jones et al. 1996; Lingappa et al. 2001), they are not effective at generating antibody responses without the inclusion of a protein carrier or appropriate adjuvant formulations. Designing appropriate immunization strategies and vaccine constructs to generate anticarbohydrate immune responses is important for the development of vaccines against pathogenic bacteria with capsular carbohydrate antigens. T-cell recognition of carbohydrates has also been suggested by several authors (Carbone and Gleeson 1997; Galli-Stampino et al. 1997; Deck et al. 1999; Haurum et al. 1999), and that MHCI molecules present glycopeptides to CD8 T cells. Immunization with MHC-restricted binding glycopeptides generates

Table 1 *Vaccines in use to prevent infectious diseases*

Prevention of infectious diseases	Vaccines in use	Route of immunization	CD8 CTL induction
Poliomyelitis	Live attenuated	Oral	Yes (++++)
MMR			
Measles	Live attenuated	Parenteral	Yes (++++)
Mumps	Live attenuated	Parenteral	Yes (++++)
Rubella	Live attenuated	Parenteral	Yes (++++)
Hepatitis B	Subunit vaccines	Intramuscular	(–)?
Purified HBsAg from healthy carrier			
HBsAg from recombinant DNA in yeast			
Haemophilus influenzae b	Subunit vaccines		(–)?
Capsular carbohydrate conjugate vaccine			
Meningococcal C	Subunit vaccines		(–)?
Capsular carbohydrate conjugate vaccine			

(++++), vaccines are efficient at induction of CD8 CTL responses. (–)?, considered to be inefficient at inducing CD8 CTL responses. The ability of the vaccines (hepatitis B subunit vaccines) to induce CD8 CTL responses is not conclusive in the literature. HBsAg, hepatitis B surface antigen.

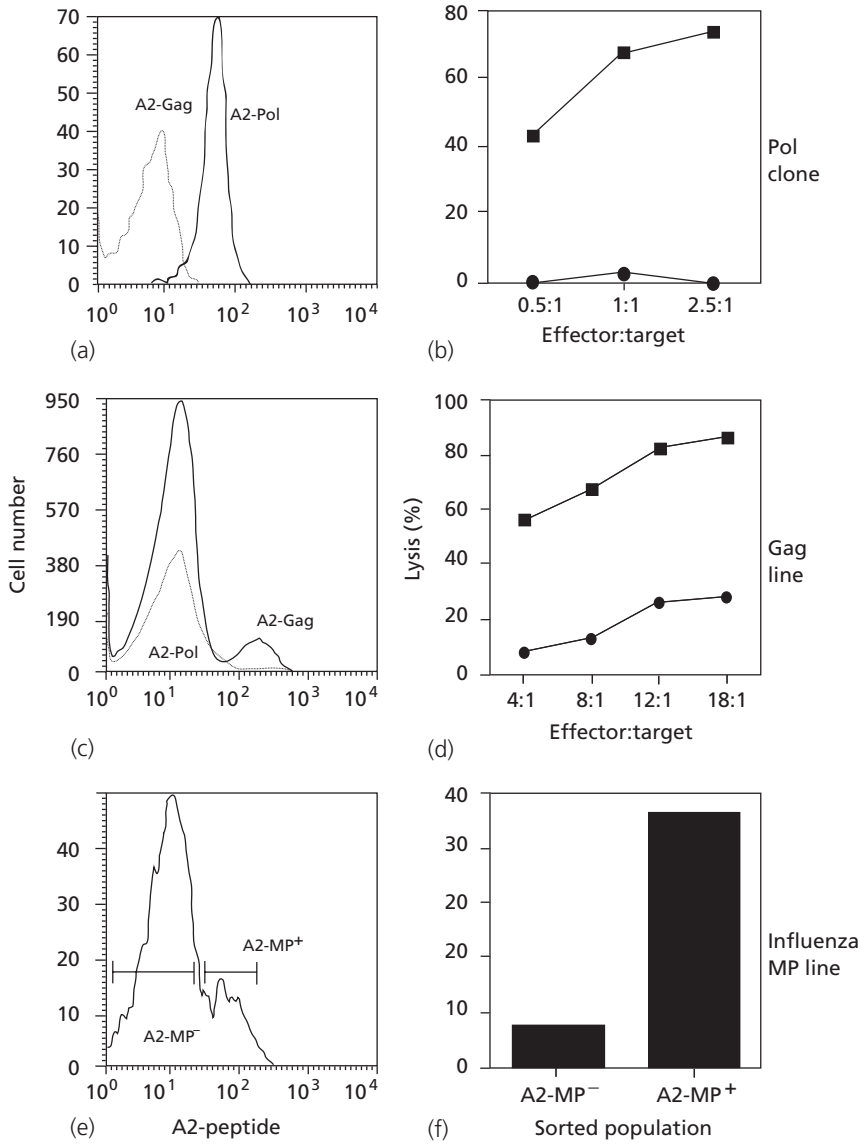

Figure 2 *Staining by MHC–peptide tetramers correlates with peptide-dependent cytotoxicity. Flow cytometric analysis (18) of CD8+ T cells (17, 20, 30) from* **(a)** *clone 20 stained with A2-Pol (solid line) and A2-Gag (dotted line) tetramers,* **(c)** *HIV-Gag-specific cytoxic T lymphocyte (CTL) line 868 stained with A2-Gag (solid line) and A2-Pol (dotted line) tetramers, and* **(e)** *an HLA-A2-restricted influenza matrix peptide CTL line (PG-001), stained with A2-MP tetramers and sorted into A2-MP+ and A2-MP− populations, as indicated. Cytotoxicity assays with* **(b)** *clone 20 showed specific killing of autologous* Epstein–Barr virus*-transformed B cells pulsed with Pol peptide (closed squares), but not target cells without added peptide (closed circles).* **(d)** *The 868 Gag-specific CTL line killed cells pulsed with the Gag peptide (closed squares) but not target cells without added peptide (closed circles).* **(f)** *The sorted populations from* **(e)** *were assayed for killing of MP-pulsed target cells at an effector:target ratio of 1:1. The same ratio, in cells not treated with peptide, with no killing of target cells was seen. (From Altman et al. 1996 with permission.)*

glycopeptide-specific, MHC-restricted, CD8 CTLs (Speir et al. 1999); however, the function of glycopeptide-specific CD8 T cells in the control of infection with pathogens expressing carbohydrates is not completely understood.

Advances in molecular biology have facilitated the development of subunit vaccines in which the proteins of pathogens are purified as protein subunit vaccines, expressed with other proteins as fusion protein subunit vaccines, or inserted into a variety of vectors as recombinant subunit vaccines (Gherardi et al. 1999; Berzofsky et al. 2001). However, immunization with purified soluble proteins from pathogens is not effective at inducing CD8 T-cell responses in the absence of appropriate adjuvant formulation (Schirmbeck et al. 1994; Speidel et al. 1997; Sheikh et al. 1999). The use of some subunit proteins has resulted in the induction of exacerbated disease after infection.

Priming with G protein derived from respiratory syncytial virus resulted in eosinophilia during subsequent infection (Openshaw et al. 1992). Although different genetic, physical, or chemical modifications of purified

proteins have been used to improve humoral and cellular immune responses after immunization, these approaches have tended to enhance one arm of immune responses while inhibiting the other (Speidel et al. 1997). Whether antigen processing and presentation of fusion protein vaccines are similar to those of protein antigens expressed on pathogens is not clear. The conformation of epitopes of fusion proteins may also be different from that of protein antigens expressed on pathogens. Pathogen proteins are inserted and expressed in different vectors, including viral, bacterial, and plasmid DNA vectors, as recombinant subunit vaccines. Recombinant subunit vaccines using live viral or bacterial vectors have the disadvantage that the boosting is ineffective because immune responses could be directed to the vectors rather than to the expressed pathogen proteins (Fuller et al. 2002; Harrington et al. 2002). Mutant pathogens with antigenic variations could also escape from the immune responses generated by recombinant vaccines expressing proteins from wild-type pathogens.

Epitope-based vaccines

Epitopes are small fragments of antigens that are recognized by specific antibodies produced from B lymphocytes (B-cell epitopes) or presented by MHC to TCRs of T lymphocytes (T-cell epitopes). Most epitopes are peptides derived from proteins of pathogens or specific antigens. CD8 T-cell peptide epitopes consist of 8–12 amino acids that bind to MHCI molecules (Townsend et al. 1989). Epitope-based vaccines consist of T- or B-cell epitopes that are made as synthetic peptides or synthetic oligonucleotides expressed by plasmid DNA or a variety of recombinant vectors (Chargelegue et al. 1998; Hsu et al. 1998, 1999; Berzofsky et al. 2001; Wee et al. 2002). Synthetic peptides, epitope-based DNA, or recombinant vectors expressing T-cell epitopes have all been shown to be capable of inducing protective CD8 CTL responses.

Synthetic peptides alone are often not able to induce effective immune responses without modification by the inclusion of additional sequences or the use of specific adjuvant formulations. Several sequence modifications and different adjuvants have been applied to the enhancement of induction of CD8 CTL responses (Gao et al. 1991; Hsu et al. 1995; Franke et al. 1997). Thus, the addition of T-helper epitopes, fusion peptide, lipophilic molecules, and signal peptides to CD8 CTL epitopes has resulted in enhanced specific CD8 CTL responses (Shirai et al. 1994; Hsu et al. 1995; Hiranuma et al. 1999). One of the advantages of the use of epitope-based vaccines is to allow T-cell epitopes to bind to MHC molecules bypassing some of the steps in protein antigen processing and presentation pathways. The flexibility of the design of epitope-based vaccines also allows peptide epitopes to be delivered to particular intracellular organelles to activate specific protective immune responses. Epitope-based immunization strategies could also be used to explore several unclear areas in immunology at the intercellular level, such as the extent of the TCR repertoire, the importance of the affinity–avidity of T-cell recognition, and the role of CD4–CD8 and CD8–APC interactions. The information from these areas can aid the design of a new generation of epitope-based vaccines and immunization strategies.

THE INDUCTION OF CD8 T-CELL RESPONSES

The role of CD4 T cells

In contrast to CD8 T cells, CD4 T cells recognize peptide epitopes bound to MHCII molecules presented by specialized APCs, such as dendritic cells or macrophages (Allen et al. 1985). CD4 T cells are able to improve CD8 T-cell immunity against pathogens and malignant cells after infection or immunization (Berzofsky et al. 2001); however, the mechanisms used by CD4 T cells to increase CD8 T-cell activity are not clear. CD4 T cells could simply expand the frequency of CD8 T cells or alter the function of CD8 T cells and vice versa. Indeed, CD8 T-cell responses can be expanded or suppressed via the action of cytokines or other soluble mediators from different types of CD4 T cells (Aung and Graham 2000; Aung et al. 1999; Sato et al. 2001). Specialized APCs express MHCI and MHCII molecules that are capable of presenting peptide epitopes to CD4 and CD8 T cells (Figure 3). CD8 T-cell responses could also be augmented by the microenviroments of cell–cell contacts between CD8 T cells, CD4 T cells, and specialized APCs. Induction of protective CD8 T-cell immunity by specialized APCs in the absence of CD4 T-cell responses has also been described (Sato et al. 2001; Wang et al. 2001; Diehl et al. 2002). Different vaccine preparations have been used to induce specific CD8 T-cell responses and, in many cases, vaccines including components for the induction of CD4 T-cell responses have resulted in enhanced specific CD8 CTL responses. It should be noted that CD8 CTL responses could also be induced in the absence of CD4 T-cell responses (Figure 3). The induction of CD8 T-cell responses with peptides bound with higher affinity to MHCI molecules depends less on the presence of exogenous CD4 T-cell peptide epitopes than CD8 epitopes bound with lower affinity to MHCI molecules. Whether or not other factors contribute to the induction of effective CD8 T-cell immunity by specialized APCs without CD4 T cells needs further investigation.

The role of antigen-presenting cells

CD8 T cells can recognize APCs expressing MHCI–peptide molecules on the cell surface. Several APCs,

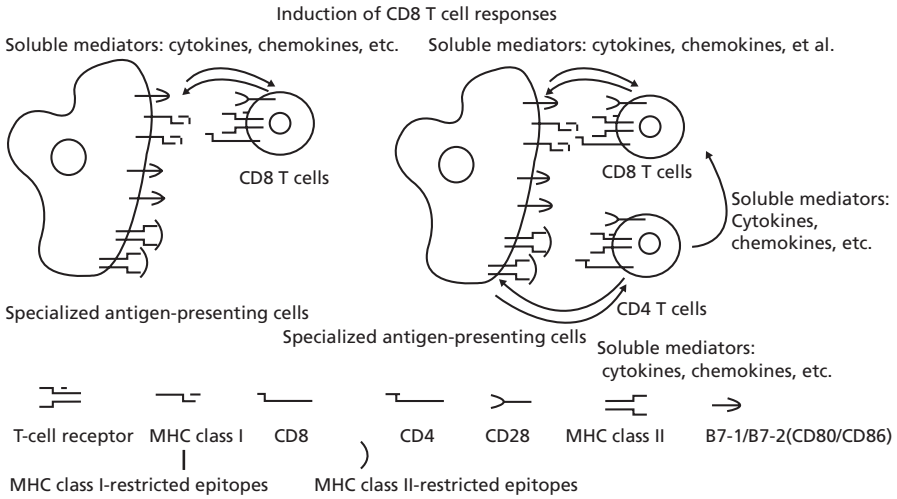

Figure 3 *Specialized antigen-presenting cells (APC) present MHCI- and MHCII-restricted epitopes to CD8 and CD4 T cells. CD8 T-cell responses are induced by cell-to-cell contact of CD4, CD8, and specialized APCs upon costimulatory signals or be soluble mediators, CD8 T-cell responses are also capable of being induced in the absence of CD4 T cells.*

such as fibroblasts, epithelial cells, endothelial cells, and different specific cells, are recognized and lysed by CD8 CTLs in vitro using CTL assays (Bennink et al. 1982; Gooding and O'Connell 1983; Townsend et al. 1986; Li et al. 2001b). MHCI-restricted antigen processing and presentation by specialized APCs, such as dendritic cells or macrophages, is required to induce and activate CD8 T-cell responses, as indicated in many publications. In these studies, several different ways to prepare and purify dendritic cells were used to induce protective CD8 T-cell responses (Steinman 1991; Galibert et al. 2001; Manz et al. 2001). There is limited information available to determine whether variations in methods for generation and purification of dendritic cells could alter their immunological function.

Specialized APCs, which some pathogens can directly infect, are capable of presenting MHC–peptide complexes to CD8 T cells through the endogenous MHCI processing and presentation pathway (Bennink et al. 1982; Townsend et al. 1986, 1989; Moore et al. 1988; Pamer and Cresswell 1998; Rock and Goldberg 1999). For other pathogens and particulate antigens, specialized APCs could present MHCI–peptide complexes to CD8 T cells via exogenous pathways that normally present MHCII–peptide complexes to CD4 T cells (Yewdell et al. 1988; Schirmbeck et al. 1995; Rock and Goldberg 1999; Pamer and Creswell 1998; Rock and Goldberg 1999; Stober et al. 2002). In classic MHCI endogenous antigen processing pathways, cytoplasmic antigens are processed into peptides by ubiquitin- and proteosome-mediated proteolysis (Rock et al. 1994; Rock and Goldberg 1999). Processed peptides are transported into the endoplasmic reticulum by TAP-dependent or TAP-independent pathways. After peptide epitopes are selected and bound to MHCI molecules, MHCI–peptide molecules are transported to the Golgi body and then to the cell surface. In the alternate exogenous MHCI pathway, exogenous antigens are endocytosed by specialized APCs into endosomal compartments. Protein antigens are processed by endosomal proteases into peptide fragments and processed peptides are transported into the cytoplasm to enter the endoplasmic reticulum or into other organelle compartments to associate with MHCI molecules. The detailed intracellular pathways of MHCI molecules are not totally clear, and whether the peptide repertoires produced by classic endogenous pathways are different from those produced by alternate exogenous pathways is not known. Specialized APCs stimulate effective specific CD8 CTL responses against pathogens or tumors, when pulsed with peptides and particulate protein antigens or by transfection with recombinant vectors in vivo or in vitro (Porgador and Gilboa 1995; Schirmbeck et al. 1995; Luft et al. 2002; Stober et al. 2002). Specialized APCs are able to express MHCI, MHCII, costimulatory, and other surface molecules. A knowledge of the differences in expression of surface molecules and cytokines between specialized APCs and other APCs is required in order to regulate the immunostimulatory function of specialized APCs for immunotherapy and the generation of protective immunity.

The role of cytokines

Several cytokines are involved in the induction of CD8 T-cell responses after pathogen infection or immunization. Many cytokines have already been included in the design of vaccines and adjuvant formulations to enhance the induction of CD8 CTL responses (Ahlers et al. 2001; Sato et al. 2001; Staats et al. 2001; Cho et al. 2002; Luft et al. 2002; Padovan et al. 2002). Some cytokines, such as interleukin 2 (IL-2), directly stimulate CD8 T-cell proliferation and activation. Granulocyte–monocyte

colony-stimulating factor (GM-CSF) and IL-12 are able to upregulate the expression of receptors or surface molecules on CD8 T cells, to induce T-helper cell responses or to induce the cascade effects of cytokine expression from other cells (Gerrard et al. 1987; Ahlers et al. 2001; Berzofsky et al. 2001). IL-12 is able to enhance the induction of CD8 CTL responses and the production of IFN-γ Gately et al. 1994) and GM-CSF increases the number of infiltrating APCs and enhances the expression of IFN-γ and IL-12 receptors (Ahlers et al. 2001; Bukreyev et al. 2001). In addition, cytokines can also act synergistically on the induction of CD8 T cell responses (Mehrotra et al. 1995; Ahlers et al. 2001; Berzofsky et al. 2001). Type 1 IFNs are required for bone marrow- or resident monocyte-derived dendritic cells to stimulate CD8 CTL responses (Palmer et al. 2000; Luft et al. 2002; Montoya et al. 2002; Padovan et al. 2002). Both type 1 and type 2 IFNs regulate MHCI-restricted antigen processing and presentation (Atta et al. 1995; Keskinen et al. 1997). IL-4 has been found to suppress or enhance CD8 CTL activity in different experimental conditions (Bembridge et al. 1998; Aung et al. 1999; Aung and Graham 2000). Finally, cytokines are also involved in pathogenesis of diseases after pathogen infection (Peebles et al. 2000; Openshaw 2002).

Whether cytokines enhance the induction of CD8 CTL responses through the regulation of antigen processing and presentation is an important area to be explored. In several publications, a significant effect on immune responses was observed in the situation of IFN-γ deficiency, which is consistent with the observation that IFN-γ has an effect on the antigen processing and presentation pathway (Hsu et al. 1998; Guidotti and Chisari 2001; Nakajima et al. 2001), and induces the expression of subunits of proteasomes that are responsible for proteolysis in the MHCI-processing pathway (York and Rock 1996; Pamer and Cresswell 1998; Rock and Goldberg 1999). Type 1 IFNs increase the expression of MHCI and MHCII molecules and activate the effector function of T cells, NK cells, and macrophages (Guidotti and Chisari 2001). Whether the mechanisms by which type 1 IFN regulate MHCI expression are similar to those mediated by IFN-γ is not clear.

The role of co-stimulatory and surface molecules

T-cell activation requires the signal derived from the recognition of specific MHC–peptide complexes by TCRs and also additional costimulatory signals provided by accessory molecules on T cells and specialized APCs. CD28 is a well-known costimulatory molecule expressed on naïve or primed T cells, which bind to B7-1 (CD80) or B7-2 (CD86) on APCs (Schwartz 1992; Azuma et al. 1993; Freeman et al. 1993; Lenschow et al. 1996; Alegre et al. 2001). After recognition of specific MHC–peptides and costimulatory molecules, T cells proliferate and produce IL-2. In the absence of costimulatory signals, the engagement of TCRs and MHC–peptide complexes could result in anergic T cells. Costimulatory signals also promote T-cell survival by inducing the expression of antiapoptotic Bcl-2 (Boise et al. 1993). The mechanisms by which costimulatory signals provided by CD28–B7 ligation facilitate T-cell proliferation and differentiation are still not clear. The signals from CD28–B7 ligation may increase the TCR signaling pathway or stimulate different pathways from TCR signaling (Vola and Lanzavecchia 1996). Costimulatory signals were found to reduce the number of TCRs required for stimulation of cytokine production and proliferation at the cellular level. There are other positive signals provided by activating receptors including NKG2D to facilitate CD8 T-cell responses and tumor immunity (Diefenbach et al. 2001). Besides positive costimulatory signals, inhibitory receptors including CTLA-4 were also found to inhibit T-cell activation by suppressing IL-2 and IL-2 receptor expression (Alegre et al. 2001).

The movement of T or B lymphocytes into lymphoid organs is mediated by adhesion molecules on lymphocytes interacting with receptors on the high endothelial venules (HEV). Many experiments prove that T or B cells prefer localization to specific tissues. Lymphocytes isolated from gut-associated lymphoid tissue tend to localize to Peyer's patches after being reinfused, whereas splenocytes prefer migrating to the spleen. Immune responses in nonlymphoid tissues induce the movement of lymphocytes into nonlymphoid tissues. Cytokines induce the expression of adhesion molecules on endothelium which mediate the migration of lymphocytes into the inflammation tissues (Issekutz 1992). Different cytokines induce expression of different adhesion molecules that control different stages of lymphocyte migration (Issekutz 1990). ICAM-1 (CD54) and ICAM-2 (CD102) expressed on endothelial cells are receptors for LFA-1 on leukocytes (Bevilacqua 1993). The expression of adhesion molecules can improve the interaction of T cells with endothelium and target cells. Several publications have indicated that the lack of expression of some adhesion molecules resulted in diminished immune responses.

THE MAJOR HISTOCOMPATIBILITY COMPLEXES AND TCRS

The binding affinity of peptides to MHC

MHCI molecules consist of a heavy chain and β^2-microglobulin. β^2-Microglobulin associates noncovalently with the extracellular region of the heavy chain to form the peptide-binding region. After peptide binding, stable peptide–MHCI complexes are presented on the cell

surface. In TAP-deficient RMA-S cells, MHCI complexes are not stable at 37°C without optimal peptide binding (Townsend et al. 1989; Cerundolo et al. 1990). MHCI molecules are detectable at 26°C with specific antibody in RMA-S cells, which are used to screen H-2 K^b-binding peptides. To measure the binding affinity of peptides to MHC, MHCI heavy chains are purified to associate with β^2-microglobulin and labeled peptides (Quesnel et al. 1996). The alternative approaches are to label heavy chain or β^2-microglobulin for the measurement of peptide-binding affinity. The binding affinity of peptides to MHC and the stability of MHC–peptide complexes have been shown to affect the number of MHC–peptide complexes expressed on the cell surface. The binding affinity to MHC also affects the immunogenicity of peptide epitopes for the induction of CD8 CTL responses (Sette et al. 1994; van der Burg et al. 1996). The actual amino acid residues that are important for the binding of peptide epitopes to MHC with appropriate affinity need further investigation. The minimal number of specific MHC–peptide complexes required for activation of CD8 CTL-mediated cytolysis has been suggested to be from one to a few hundred (Christinck et al. 1991; Kageyama et al. 1995; Sykulev et al. 1996). Several factors affect the antigenicity and immunogenicity of peptide epitopes, including the density and number of specific MHC–peptide complexes; however, the number and density of these complexes required for the induction of CD8 CTL responses and CD8 CTL-mediated lysis of target cells are not clear.

The binding affinity and avidity of MHC–peptide complexes to TCRs

Soluble MHC–peptide complexes can be produced with purified MHC heavy chain, β^2-microglobulin, and peptides, and the binding affinity between TCRs and MHC–peptide complexes has been measured with purified MHC–peptide complexes and TCRs. The binding affinity between TCRs and MHC–peptide complexes is lower than that between antibody and antigen (Matsui et al. 1991). The detection of the number of specific CD8 T cells is difficult using specific monomeric MHC–peptide complexes resulting from low binding affinity. Although the number and density of MHC–peptide complexes required for the activation of CD8 CTL-mediated lysis of target cells are not clear, T-cell recognition of specific MHC–peptide complexes could involve multiple binding of TCRs (Altman et al. 1996; Reich et al. 1997; Rubio-Godoy et al. 2001; Demotte et al. 2002; Dutoit et al. 2002; Knabel et al. 2002; Valmori et al. 2002; Wu et al. 2002). Multimeric MHC–peptide complexes have been developed to measure the frequency of specific CD8 T cells (Altman et al. 1996; Rubio-Godoy et al. 2001; Dutoit et al. 2002; Knabel et al. 2002; Valmori et al. 2002) (see Figure 2, p. 425). However, appropriate methodology to measure the avidity of T-cell recognition of specific MHC–peptide complexes on the surface of target cells by TCRs is still not available. In several studies, high-avidity CD8 CTL lines or clones that recognize the target cells pulsed with lower concentration of specific peptides have been suggested to provide better protection in vivo (Alexander-Miller et al. 1996; Herbert et al. 1999; Derby et al. 2001). Many hypotheses have also been proposed to explain the avidity of the recognition of MHC–peptide complexes by CD8 CTL, including the binding affinity of TCR to MHC–peptide complexes, maturation of the functional avidity of T-cell recognition and the number of TCRs required for recognition (Vola and Lanzavecchia 1996; Reich et al. 1997; Holler et al. 2001; Slifka and Whitton 2001). The mechanisms by which avidity plays a role in the recognition of MHC–peptide complexes by CD8 T cells and the mediation of cytolysis and cytokine production remain to be defined.

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Unconventional T cells

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DEFINING UNCONVENTIONAL T CELLS

It is now evident that both B lymphocytes and T lymphocytes need to be subdivided into distinct classes. For B cells, these classes comprise B1 cells commonly enriched in the peritoneum, and circulating B2 cells. As the distinct features of B1 cells have only become apparent in the past 25 years, as their functions remain enigmatic, and as B2 cells fully satisfy conventional assays for B-lymphocyte activity, B2 cells are most commonly regarded as 'conventional B cells', with B1 cells being viewed as 'unconventional'. This view of B1 cells as peripheral to core B-cell function is encouraged by their apparently varied representation in different species, and their presence in humans is still uncertain. Likewise, it is now common to view T cells as comprising 'conventional', circulating αβ T cells, defined by expression of the heterodimeric αβ T-cell receptor (TCR), and 'unconventional T cells' which, like B1 cells, are of enigmatic function and highly varied in their representation in different species. Nevertheless, any view of immunology that discounts unconventional T cells from playing critical roles in host defense is ill-judged, ignoring their evolutionary conservation over at least 400 million years, and the emerging evidence that they have potent immunoprotective and immunoregulatory roles that are germane to infectious disease, tumor surveillance, and autoimmunity.

The contrast of unconventional T cells with conventional T cells inevitably requires clear operational criteria for conventional T-cell activity. These are: (1) recognition of peptide antigens presented by highly polymorphic major histocompatibility complex (MHC) proteins; (2) negative selection during development by high-affinity engagement of self-antigens; (3) antigen priming in the secondary lymphoid tissues (lymph nodes and spleen) by 'professional' antigen-presenting cells (APC); and (4) commitment of a subset of the primed T-cell repertoire to the long-term memory pool that composes cellular immunity. Collectively, unconventional T cells appear to fail most (perhaps all) of these four criteria. Thus, (1) they are not restricted by conventional, highly polymorphic MHC; (2) it is not certain that they are negatively selected by engagement of autoantigens during development, but rather may be positively selected; (3) their patterns of systemic circulation are commonly (perhaps invariably) distinct, with greater constitutive association with tissues rather than with lymph nodes, spleen, and bone marrow; and (4) the evidence that they contribute to long-term memory is equivocal, with the greater mass of data implying that their primary contribution is to the early phases of immune responses, preceding the activation of conventional T cells.

By these operational criteria, the activities of unconventional T cells would seem complementary to those of conventional T cells. Such complementarity could provide a powerful argument for the cells' evolutionary conservation. At the same time it is unwise to view unconventional and conventional T cells as operating entirely independently in their contributions to immune responses. Growing evidence indicates that unconventional T cells have a potent capacity to regulate conventional T-cell responses, and are thus important factors to consider in the understanding and treatment of immunopathologies such as inflammation, allergy, and antigen-specific autoimmunity.

SUBTYPES OF UNCONVENTIONAL T CELLS: γδ T CELLS

Perhaps the most galvanizing development in the recognition of T-cell heterogeneity was the discovery in 1984 of a TCR gene cDNA (complementary DNA), TCR γ, that encoded neither the TCR α chain nor the TCR β chain (Saito et al. 1984). Within 3 years, the existence of TCRγδ$^+$ cells was firmly established. Antigen recognition by TCRγδ is critically different from that of conventional αβ T cells. In particular, mice that are deficient in polymorphic MHC class I and/or II molecules show substantial defects in circulating αβ T cells, whereas the γδ cell compartment is largely unaffected (Correa et al. 1992; Bigby et al. 1993). The fact that before 1984 no one had predicted the existence of γδ cells strongly suggested that conventional assays for T-cell activity – primarily the development of antigen-specific immunity – were fully satisfied by αβ T cells. In turn, this suggested from the outset that γδ cells use their unconventional TCR specificities to contribute to immune responses in unconventional ways: much work over the past 15 years has supported this idea. To consider γδ cells more clearly, one must recognize that the γδ cell compartment itself is made up of several distinct subcompartments.

TCRγδ$^+$ dendritic epidermal T cells

Murine dendritic epidermal T cells (DETC) are arguably the prototypical nonconventional T cell, and their study introduces many features of nonconventional T cells more broadly. DETCs are an example of intraepithelial lymphocytes (IEL): rather than circulating, they are constitutively associated with a single tissue, which, in the case of DETCs, is the epidermis. Unlike conventional T cells, which are round cells dominated by their nucleus, DETCs appear in situ as dendritic cells that collectively form an anastomosing network in contact with the majority of basal keratinocytes. Several experiments over the past decade have demonstrated that DETCs can directly engage heat-shocked or malignantly transformed keratinocytes, provoking cytokine release and/or target cell killing (Havran et al. 1991). Thus, DETCs as well as other IELs have been considered to contribute to a 'first line of defense' (Janeway et al. 1988). There is, by contrast, little evidence that DETCs interact with professional APCs, such as Langerhans' cells which prime the conventional T-cell response.

DETC progenitors are the first to colonize the murine fetal thymus (Havran and Allison 1988) and, consistent with their failure to engage conventional class I or class II MHC, they complete development without the acquisition of CD4 and CD8 expression which is a signatory feature of conventional αβ T-cell development. Seemingly, they leave the thymus after positive selection, but until their TCR ligand is characterized the details of this event will remain opaque. The basis for homing to and residence within the skin has also not been elucidated, but it is likely that different molecules contribute to the two processes. Once the perinatal wave of skin colonization by DETCs has occurred, the thymus loses its capacity to regenerate DETCs (Ikuta et al. 1990). Possibly, stem cells from which DETCs can be replenished exist in some compartment of the skin (e.g. the dermis), but if this is the case, those progenitors, like DETC themselves, are vulnerable to surface ultraviolet irradiation, because such treatment causes long-term DETC ablation (Aberer et al. 1986).

Similar to other thymocytes, developing DETC progenitors utilize recombinase-activating gene (RAG)-mediated somatic DNA recombination to generate a TCR. By the quasi-random and imprecise joining of any one of a large number of variable (V) gene segments with any one of a large number of diversity (D) gene segments, which is in turn joined to any one of a large number of junctional (J) gene segments, RAG-mediated recombination can generate immensely diverse receptor repertoires characteristic of the T- and B-cell compartments (Tonegawa 1983). In curious contrast, DETCs express a monomorphic Vγ5/Vδ1 TCR (Asarnow et al. 1988).

Although its ligand is currently not elucidated, the simplicity of the DETC TCR is consistent with the hypothesis that DETCs recognize a generic self-antigen, induced on infected or transformed epithelial cells by a wide range of cellular insult (Janeway et al. 1988). The basis for this hypothesis is that IELs resident within epithelia will not have the opportunity to sample millions of different antigens presented by APCs in the lymph nodes of infected mice, but must therefore rely on activation by a self-antigen that is expressed selectively on infected or transformed epithelial cells.

The TCR is not the only lymphocyte surface molecule that may be engaged in this fashion. Activated DETCs, along with other cytolytic T cells and natural killer (NK) cells, express an activating receptor, NKG2D, that engages any one of a number of nonconventional MHC class IB gene products, e.g. Rae-1 (mouse and human), H60 (mouse), ULBP (human), and MICA/MICB (human) (Bauer et al. 1999; Cerwenka et al. 2000; Diefenbach et al. 2000; Cosman et al. 2001). These molecules resemble conventional MHC class I proteins in general structural features, but most probably do not present antigenic peptides (Li et al. 1999, 2002). As peptide binding stabilizes conventional class I and II MHC proteins, the stable display of peptide–MHC complexes by an APC signifies its 'immunological visibility' to conventional T cells. By contrast, the immunological visibility of cells expressing peptide-independent MHC-related molecules to NKG2D$^+$ lymphocytes is regulated at least in part by strictly inducible gene expression.

Thus, Rae-1 is expressed primarily in the embryo (Zou et al. 1996), and at negligible levels in normal adult tissue (Cerwenka et al. 2000; Diefenbach et al. 2000), but its expression increases in vivo in skin epithelial cells exposed to transforming chemicals (Girardi et al. 2001). Likewise, the expression of MICA/B is upregulated on heat-shocked or transformed epithelial cells of the human gut (Groh et al. 1996, 1999) where there is an additional population of resident TCRγδ$^+$ cells (see below). Currently, the cell signaling pathways that tightly regulate the expression of 'stress-inducible' class IB MHC are unknown.

DETCs can also express receptors, notably Ly49E (Van Beneden et al. 2002), that are associated with NK cells, and not commonly expressed by conventional T cells. The engagement of such receptors limits T-cell activation, implying that the immunological visibility of skin epithelial cells also relies on overcoming the engagement of inhibitory receptors, either by reducing or altering the expression of their ligands, or by quantitatively overwhelming the cell via activating receptor engagement.

There has to date been little effective testing of the roles of DETCs in the protection of mouse skin against infection. Nevertheless, the physiological relevance of DETC engagement of transformed keratinocytes was strongly implied by the finding that TCRγδ-deficient mice are substantially more susceptible to multiple, diverse protocols for the induction of squamous cell carcinoma (Girardi et al. 2001). Indeed, the mice are more reproducibly susceptible than are TCR αβ–deficient mice. These studies potentially provide a model for the protective activities of TCRγδ$^+$ IELs against the development of solid tumors in humans (see below).

Once activated, DETCs, as with many other nonconventional T cells, display a clear potential for cytolytic and T-helper (Th)1-type effector responses that can account for their local protective activities. In addition, DETCs and other IELs express well-characterized chemokines, such as RANTES and lymphotactin (Boismenu et al. 1996), as well as enigmatic molecules such as thymosin β4 (Shires et al. 2001), which have potent anti-inflammatory activities in certain assays. Perhaps consistent with this, DETCs and other IELs have immunoregulatory activity and, on certain genetic backgrounds, DETC deficiency leads to striking pathological inflammatory infiltrates of the skin which are provoked by αβ T-cell responses to environmental antigens (Girardi et al. 2002). Indeed, on particular genetic backgrounds, the pathognomonic outward appearance of TCRγδ$^{-/-}$ mice is visible to the naked eye. Although the TCR is apparently involved in immunoregulation by DETCs, the relevant ligand is again unelucidated. Indeed, immunoregulation might occur following direct interaction of DETCs with activated infiltrating lymphoid cells, or as an indirect by-product of TCR-mediated DETC engagement of activated epithelial cells.

Importantly, the phenotypic analysis of TCRγδ$^{-/-}$ mice informs us that DETCs provide a multifaceted protection of epithelial integrity, ranging from the eradication of dysregulated epithelial cells to the inhibition of tissue disruption by systemic lymphoid cells. A further contribution of DETC activity to the maintenance of epithelial integrity is evident in the reported expression by DETCs of fibroblast growth factors that promote keratinocyte growth (Boismenu and Havran 1994; Jameson et al. 2002). Although this may appear paradoxical given the capacity of DETCs to kill epithelial cells, the data are collectively interpreted to suggest that, after target cell eradication, DETCs subsequently contribute to wound healing.

Clearly, the initial view of DETCs as cells that use a monomorphic TCR to target dysregulated epithelial cells as part of a pseudo-innate immune response is insufficient to accommodate the complexity of DETC functions. Conceivably, despite their expression of a single TCR, DETCs may comprise functionally distinct subsets, capable of cytolysis, immunoregulation, and epithelial cell growth promotion, respectively. This hypothesis is being tested at present.

Although DETCs capably illustrate the impressive functional potential of 'local', tissue-associated T cells, the general relevance of the lessons learned has been questioned, given that DETCs are not obviously conserved in humans. Indeed, some have wondered whether 'DETC functions' are an idiosyncrasy of relatively short-lived mammals, such as rodents, which have large litter sizes, placing less emphasis on the long-term survival of the individual (sometimes cited as the key provision of antigen-specific memory in the conventional adaptive immune system). This is clearly not the case, because DETCs are conserved in cattle, large mammals with long lifespans that are more similar to humans than to mice. Therefore, the probability exists that 'local immune functions' exist in human skin, but are provided by other cell types. Thus, it has been reported that a unique TCR γδ$^+$ subset resides in the dermis of healthy human skin (Holtmeier et al. 2001), and there are increases in cutaneous γδ cells in scleroderma, an inflammatory pathology characterized in part by fibrotic wound healing, and possibly promoted by human γδ cells. Given the potential of DETCs to be activated via the NKG2D receptor, it is also possible that local immune function in human skin is provided by NK cells or myeloid cells that can likewise use the NKG2D pathway to respond to epithelial cell dysregulation and/or activated inflammatory infiltrates. In sum, the study of DETCs emphasizes the need for better understanding of local immune function in cutaneous immune responses in humans.

A second important contribution of DETCs to the study of human immunology is the potential to improve our understanding of other epithelia that also harbor TCRγδ$^+$ IELs, both in mice and in humans.

TCRγδ$^+$ IELs in other tissues in mice and humans

In mice, the functional and molecular relatedness of IELs that characterize different epithelia – the skin, reproductive tissue, tongue, intestine, and (reportedly) lung – has yet to be firmly established. Unlike the case of DETCs, direct equivalents of such cells are evident in certain human tissues, e.g. the human small intestine. Given the very large surface area of the human gut (>200 m^2), it is likely that IELs will account numerically for a major fraction of human T cells.

Similar to DETCs, the TCRs of IELs in different tissues show limited diversity. Murine vaginal and tongue-associated IELs express a single predominant Vγ chain (Vγ6) (Lafaille et al. 1989) distinct from that in skin (Vγ5) (Asarnow et al. 1988) and the gut (Vγ7) (Bonneville et al. 1988; Goodman and Lefrancois 1988; Kyes et al. 1989; Takagaki et al. 1989). In the human gut, IELs express Vγ1 (Deusch et al. 1991), by contrast to Vγ2 expressed by most systemic γδ cells. The intestinal IELs of mice and humans show some junctional diversity and some variation in Vδ usage, but the biological significance of this vis-à-vis antigen recognition is unresolved.

In the mouse, the IELs that populate the different tissues develop in sequential 'waves' in the fetal and neonatal thymus, with those in the skin preceding those in the reproductive tissue, which precede those in the gut (Havran and Allison 1988). Thus, it has been proposed that the association of particular TCRs (especially Vγ genes) with particular tissues reflects the sequential activation of gene segment usage. Whether or not this is true, the same association has quite naturally provoked the concept that each TCR recognizes a tissue-specific 'stress antigen' that would serve as a generic beacon of epithelial distress. The likelihood that each class of γδ TCR recognizes a clearly distinct entity is high because, by contrast to the usual situation in conventional T cells and B cells, individual Vγ genes share little homology and are very distinct from each other in the complementarity determining regions (CDR) CDR1 and CDR2 which would be predicted to form part of the antigen-binding surfaces (Arden et al. 1995b).

As was the case with DETC, there is scant evidence for a critical immunoprotective role of TCRγδ$^+$ IELs against pathogens that either enter or reside within epithelia. Indeed, whereas αβ T-cell-deficient mice showed high susceptibility toward a natural parasitic infection of the intestinal epithelium, the immunoprotective role of γδ cells was only exposed in mice that also lacked αβ T cells (Smith and Hayday 2000). Such data have encouraged some scientists to regard TCRγδ$^+$ cells as inferior to αβ T cells in their value to the host. However, when infected with a natural parasite of the intestinal epithelium, γδ-cell-deficient mice are not without phenotype: rather, they primarily show a defect in immunoregulation, manifest in aggressive αβ T-cell-dependent immunopathology (Roberts et al. 1996).

Also paralleling the findings with DETCs, human Vγ1/Vδ1$^+$ IELs isolated from surgical specimens of bowel cancer would kill the cancer cells after engagement of the NKG2D ligand, MICA (Groh et al. 1998). Vγ2/Vδ2$^+$ cells would not do this, provoking the concept that MICA engages TCRγδ as well as NKG2D (Groh et al. 1998; Wu et al. 2002). A precedent for such ligand promiscuity might be found in the binding of conventional MHC class I simultaneously to TCRαβ and to CD8, or in the binding of human HLA-C or murine Qa-1 to TCRαβ and to NK receptors (see below). Indeed, cytolysis by Vδ1$^+$ IELs could be inhibited with anti-MICA and anti-TCRγδ monoclonal antibodies. To clarify the capacity of the Vγ1/Vδ1 TCRs to bind MICA, a T-cell line that does not express NKG2D was transfected with TCR chains from MICA-responsive γδ clones or MICA-unresponsive clones (Wu et al. 2002). Cells expressing TCRs derived from responsive clones bound MICA tetramers, whereas those transfected with TCRs derived from unresponsive clones did not. Clearly, if these data are correctly interpreted, it should be possible to demonstrate direct binding of MICA to a soluble Vγ1/Vδ1 TCR by surface plasmon resonance.

The same experimental approach might usefully support the evidence that human T-cell clones expressing collectively Vγ1 paired with Vδ1.4 or Vδ2 bind to CD1c (Spada et al. 2000). CD1 proteins constitute a further subset of nonpolymorphic class IB MHC molecules. There are two groups of CD1 proteins: group 1 includes CD1a, Cd1b, and Cd1c, which collectively are found in humans but not in mice; group 2 includes CD1d which is conserved (Gumperz and Brenner 2001). All CD1 proteins have deep hydrophobic grooves that permit them to present lipid molecules to reactive TCRs (see below).

In sum, TCRγδ$^+$ IELs in the gut display both protective and immunoregulatory functions. The molecular potential for such actions was readily revealed by the comprehensive cataloguing of genes expressed by murine gut TCRγδ$^+$ IELs. These analyses undertaken by microchip array and by serial analysis of gene expression (SAGE) have shown that more than 2 percent of IEL mRNA is devoted to the expression of the cytolytic effector molecules, granzymes A and B, and the immunoregulatory chemokine, RANTES (Shires et al. 2001).

Systemic γδ cells

γδ cells can be found in the peripheral blood, spleen, and lymph nodes, but again their representation varies greatly from very rare (humans and mice) to more than 50 percent of systemic CD3$^+$ cells (calves). Nevertheless,

the low numbers of systemic γδ cells in humans can increase substantially, particularly during bacterial infections, to more than 40 percent of $CD3^+$ (PBL). Systemic γδ cells, similar to $TCR\gamma\delta^+$ IELs, use limited Vγ and Vδ pairings, but, as these pairings are dissimilar from those used by $TCR\gamma\delta^+$ IELs, systemic γδ cells and $TCR\gamma\delta^+$ IELs may recognize distinct classes of antigen. Moreover, in contrast to $TCR\gamma\delta^+$ IELs, systemic γδ cells often display substantial junctional diversity in their TCRs. Indeed, the junctional diversity of TCRγδ may be more diverse than either TCRαβ or Ig.

Additional evidence that systemic γδ cells may not be limited to the recognition of MHC-like structures is provided by an indepth analysis of TCR structure. The CDR1 and CDR2 regions of γ and δ chains are longer than those of TCRα and -β (Arden et al. 1995a; Clark et al. 1995), and although the CDR3 regions of TCRα and -β chains are limited to 6–12 nucleotides (interpreted as the constraint imposed by binding to the relatively 'flat' peptide/MHC surface), the CDR3 of TCRδ, similar to that of IgH, is extremely varied, (6–22 nucleotides), suggesting no constraining topology of γδ ligands (Rock et al. 1994). Nevertheless, how such diversity translates into antigen specificity is not yet clear.

The crystal structure of an isolated human Vδ3 chain showed that CDR1 and CDR3 assumed a conformation similar to TCRαβ, whereas CDR2 assumed a conformation more like IgH (Li et al. 1998). More recently, a structure of a complete human Vγ2Vδ2 TCR confirmed that CDR2 of Vδ2 more closely resembled IgH than TCRαβ, although the significance of this with respect to antigen binding is also unclear (Allison et al. 2001).

Consistent with their failure to recognize antigen restricted by polymorphic MHC class I or II, systemic γδ cells in mice and humans usually express neither CD4 nor CD8, and derive directly from $CD4^-CD8^-$ ('double-negative') thymocytes. In humans, the bulk of systemic γδ cells arise from fetal thymic progenitors.

In the mouse, about 0.5 percent of systemic γδ cells bind to the nonconventional, peptide-independent β_2-microglobulin-associated MHC class 1B molecule, T10/T22 (Crowley et al. 2000). To date, this is the only γδ TCR reactivity for which a direct biochemical interaction has been shown. Using a murine γδ T-cell hybridoma, a reactivity to MHC class II has also been reported, although recognition does not focus on the peptide-containing α1α2 helical groove that is seen by TCRαβ (Chien et al. 1996). Systemic murine γδ cells have also been proposed to recognize the nonclassic class I molecule, Qa-1 (Vidovic et al. 1989), although this was in the context of presenting a nominal, nonphysiological antigen comprising a mixture of polymers of glutamic acid, aspartic acid, and tyrosine.

In humans but not mice, a highly unconventional TCR specificity has been reported in the capacity of peripheral blood, $V\gamma2V\delta2^+$ cells to be stimulated by low-molecular-mass, nonpeptidic chemicals such as isopentenyl pyrophosphate (Tanaka et al. 1995) and, at much higher concentrations, alkylamines (Bukowski et al. 1999) (Figure 1). These chemicals can be isolated from extracts of bacteria, parasites, and algae, but at least some are absent from vertebrates. Although direct chemical binding to the TCR has not been shown, the possibility exists that these chemicals are relatives of 'true' high-affinity antigens which, when clearly identified, will permit direct binding to be shown. In this vein, human $V\gamma2V\delta2^+$ cells are strikingly responsive to a mutant strain of *Escherichia coli* defective in the *lytB* gene, an essential enzyme in the 2-C-methylerythritol-4-phosphate (MEP) pathway of isoprenoid biosynthesis (Altincicek et al. 2001). The hyperresponsiveness presumably reflects reactivity toward an accumulated, biologically active intermediate that has been identified as a pyrophosphated molecule of 262 Da (Eberl et al. 2002).

Such specificities may enable systemic $V\gamma2V\delta2^+$ cells to recognize microbial products directly, but, if this is the case, it remains uncertain how the low-molecular-mass, hapten-like antigens can activate the T cell, conventionally achieved via TCR crosslinking of at least a TCR dimer. Likewise, how would an activated T cell focus in on a target, now thought to require synapse formation with an engaged cell? Possibly the low-molecular-mass antigens are presented by a novel antigen presentation system. Consistent with this hypothesis, the recognition by human γδ cells of another class of low-molecular-mass chemicals, the aminobisphosphonates, requires a myeloid-presenting cell (Miyagawa et al. 2001).

As well as being produced by microbes, some low-molecular-mass chemicals that stimulate systemic γδ cells may be products of increased metabolism, e.g. intermediates in protein farnesylation in actively growing cells. Moreover, alkylamines can be generated by catabolism of the amino acid theamine, which exists in tea. The biological relevance of these aspects is not elucidated, although it has been proposed that peripheral blood γδ cells might be primed by dietary components to respond rapidly to microbes and/or transformed cells. Reactivity of peripheral blood γδ cells to tumors was reported over a decade ago in the recognition of Daudi B-cell lymphomas by $V\gamma2V\delta2^+$ cells (Fisch et al. 1990). However, in this case the recognition was proposed to be of surface-bound heat shock proteins (HSP).

Murine γδ cells derived from the thymus have also been reported to recognize HSPs (O'Brien et al. 1989), although there is again no biochemical evidence for this as yet. Moreover, it has become increasingly clear that surface-bound HSPs can be engaged by a variety of non-TCR receptors on lymphoid and/or myeloid cells (Srivastava 2002). Thus, the mechanism and significance of HSP recognition by γδ cells remains uncertain. As systemic γδ cells are much more abundant in bovine

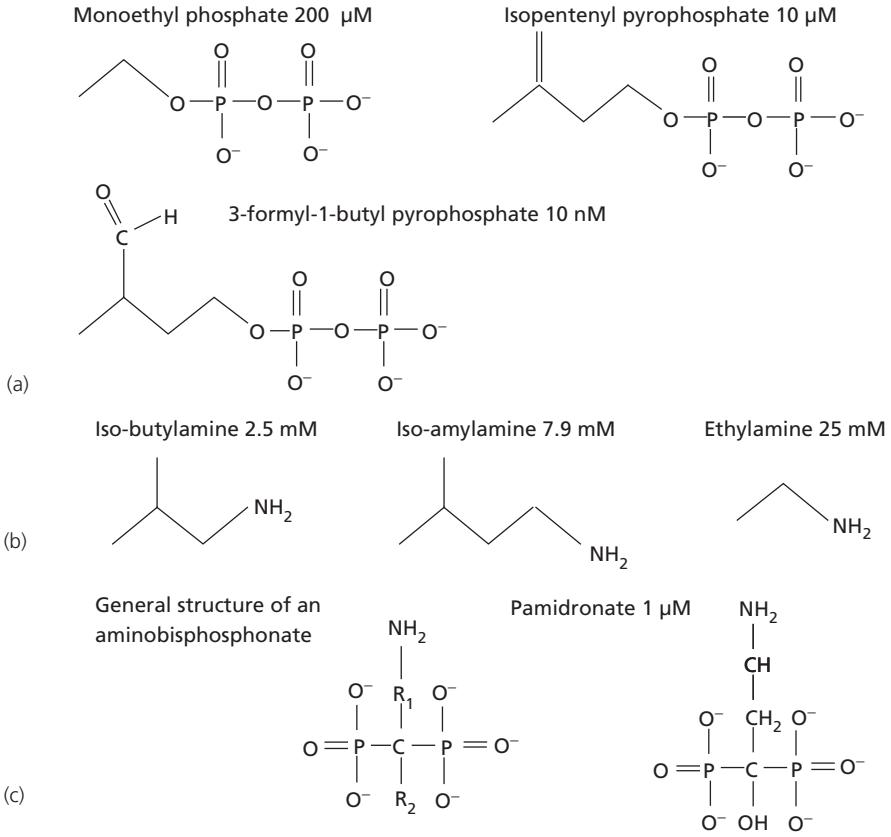

Figure 1 *Low-molecular-mass antigens for γδ cells. Structures of* **(a)** *phosphate antigens,* **(b)** *alkylamines, and* **(c)** *aminobisphosphonates. Active concentrations are indicated.*

species, particularly during the first year of life, it would be interesting to determine those cells' reactivities. Although such studies are ongoing, emerging data suggest that those cells too are unconventional, not restricted to conventional MHC.

The biological functions of systemic γδ cells have again been assigned as cytolytic and/or immunoregulatory. In certain strains of mice infected with Coxsackie virus B3, a Th1-mediated myocardial inflammation develops that is dependent on γδ cells. Rather than being directly causative of the inflammation, the role of γδ cells is to eradicate, by fas-mediated cytolysis, virus-responsive Th2 cells (Huber et al. 1999). A broader involvement of unconventional cytolytic T cells in immune regulation is apparent from the identification of regulatory $CD8^+$ αβ T cells, described in a later section.

The activation of human $TCR\gamma\delta^+$ PBLs by low-molecular-mass microbial antigens has been linked to the dramatic expansions that the cells may show in cases of bacterial infection. Human γδ cells reactive to low-molecular-mass mycobacterial antigens express cytolytic chemicals such as granulysin, and may target mycobacteria-infected macrophages. Although a critical contribution of systemic γδ cells to protection against mycobacteria has not been clearly validated, and although no mouse γδ cells have been identified that respond to low-molecular-mass chemicals, γδ-deficient mice inoculated with mycobacteria via aerosols showed altered, disseminated granulomas, consistent with the cells' role in regulating activities of other cells of the immune system (D'Souza et al. 1997). Thus, there are good grounds for viewing γδ cells as an important component of the host response to mycobacteria in mice and humans.

In the rare instances that critical contributions of γδ cells to host protection have been documented in the mouse, they primarily expose a role for γδ cells early in infection, before the priming and expansion of conventional MHC-restricted T cells in the lymph nodes. Thus, it can be considered that such nonconventional T cells collaborate with NK cells in limiting the spread of infection, before the development of antigen-specific immunity. In support of this, there has as yet been no identification of a cloned murine γδ cell line that will transfer antigen-specific immunity upon adoptive transfer to a naïve recipient. Recently, the presence of immunoprotective, mycobacteria-reactive $TCR\gamma\delta^+$ memory cells was reported in primates (Shen et al. 2002), but until we have an improved molecular definition of memory cells (that will probably emerge from contemporary genomic and proteomic studies), the

contribution of γδ cells to a memory pool remains uncertain.

Moreover, one possibility relating to γδ cells and other nonconventional T cells is that they are positively selected on autoantigens in the thymus, rather than the negative selection imposed on conventional T cells that are autoreactive. Were this to be the case, the cells could emerge from the thymus in a primed state, capable of very rapid responses to antigen in the periphery. Such a situation has recently been described for unconventional *Listeria*-reactive αβ T cells (see below). Clearly, rapid responses cannot depend on extensive clonal expression of an effector cell (because there would not be time to achieve this). Consistent with this, γδ cell responses to putative antigens, such as low-molecular-mass chemicals or HSPs, seem to occur at the population rather than at the clonal level. Such behavior is consistent with the hypothesis (discussed above) that the fundamental contribution to antigen recognition may be conferred by the CDR1 and CDR2 regions of the Vγ and Vδ gene segments used by the responding populations.

SUBTYPES OF UNCONVENTIONAL T CELLS: αβ T CELLS

TCRαβ$^+$ IELs

The murine intestinal IEL compartment is composed numerically of three main subsets: TCRγδ$^+$ IELs, and two subsets of αβT cells expressing the CD8αα homodimer and the CD8αβ heterodimer, respectively. The CD8αβ heterodimer is the form of CD8 expressed by conventional, systemic, MHC class-I-restricted αβ T cells. Consistent with this, TCRαβ$^+$ CD8αβ$^+$ IELs share many properties with conventional T cells. By contrast, CD8αα expression on systemic T cells is rare and, by several criteria, TCRαβ$^+$CD8αα$^+$ IELs are non-conventional T cells, with much in common with TCRγδ$^+$ IELs. Again, their representation in different species varies, with these IELs being rarer than TCRγδ$^+$ IELs in the adult human gut.

TCRαβ$^+$CD8αα IELs are not restricted by conventional class I or II MHC. Although there are as yet no biochemical data on TCRαβ$^+$CD8αα specificity, the cells, like TCRγδ$^+$ IELs, may recognize nonclassic MHC proteins, transcriptionally upregulated as part of a response to 'stress'. Indeed, their development is strongly regulated by Qa-2 (Das et al. 2000), an MHC class 1B molecule, which similar to MICA and Rae-1 does not require peptide loading by transport associated with antigen processing (TAP) but, unlike MICA and Rae-1, is paired with β_2-microglobulin.

To date, no examples of pathogen-specific, TCRαβ$^+$CD8αα$^+$ IELs have been described. Like TCRγδ$^+$ IELs, some TCRαβ$^+$CD8αα$^+$ IELs may mature in situ, in extrathymic cryptopatches that were recently identified in the murine small intestine (Kanamori et al. 1996). However, under normal circumstances, most probably develop in the thymus. The cells' gene expression profile, sampled as a population, is almost identical to that of TCRγδ$^+$ IELs, with strong potential for cytolysis, and the production of Th1 cytokines and chemokines. In addition, both sets of nonconventional T cells express poorly characterized molecules (e.g. interleukin (IL) 16 and Toll-like receptor (TLR) 8) that may contribute substantially to the delivery and regulation of their effector function (Shires et al. 2001). Not surprisingly, analyses of animal physiology have shown that TCRαβ$^+$CD8αα$^+$ IELs (similar to TCRγδ$^+$ IELs of the gut and of the skin) downregulate tissue inflammation provoked by conventional αβ T cells (Poussier et al. 2002). Both nonconventional TCR αβ$^+$ IELs, and TCR γδ$^+$ IELs may be regulated in part by inhibitory NK cell receptors, such as Ly49E which are expressed on a subset of each cell type.

Unconventional systemic αβ T cells: CD1d-reactive cells

The prototypic αβ T cell exhibiting limited TCR diversity is the NKT cell that expresses the NK1.1 marker (CD161) and that in mice and humans recognizes the conserved group 2 CD1 antigen, CD1d. Most NKT cells express an invariant TCRα chain (Vα14/Jα281 in mouse; Vα21/JαQ in humans), paired with a limited repertoire of TCRβ chains (MacDonald 2002). The development of multivalent CD1d reagents has identified CD1d reactivity in a broader set of cells than those expressing only the signatory TCR and NK1.1. Moreover, not all NK1.1$^+$ cells react to CD1d. Thus, the value of the term 'NKT' is operationally useful, but should not be overinterpreted.

Following the identification of murine NKT cells, autoreactive against CD1d (Bendelac et al. 1995), it was observed that the cells' responses were enhanced by the glycosylated sphingolipid α-galactosylceramide (α-GalCer), a model antigen derived from a marine sponge (Kawano et al. 1997) (Figure 2). Other α-linked glycolipids activate NKT cells, whereas β-linked glycolipids, which more closely resemble mammalian lipids, do not. α-linked glycolipids are not normally found in mammalian cells, but may be found in particular fetal tissue, in the kidney or intestine, or in transformed cells (Gumperz and Brenner 2001). Physiological antigens presented by CD1d are only now being identified (Zhou et al. 2004), although at least some are thought likely to be self-encoded because NKT cells are present at essentially normal levels in germ-free mice. The prospect that more than one self-antigen may be recognized is suggested by the fact that a panel of NKT clones showed quite different responses to cells transfected with CD1d,

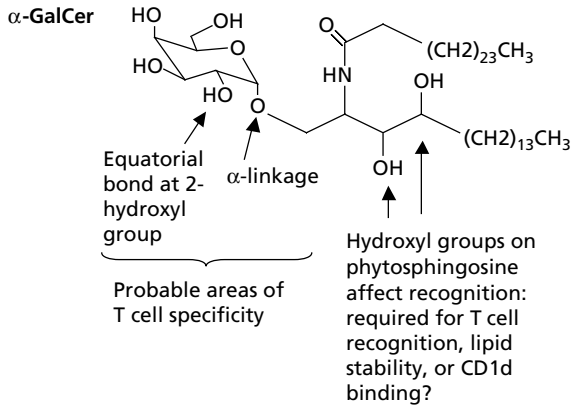

Figure 2 *Murine NKT cells are reactive to α-galactosylceramide (α-GalCer) presented by CD1d. NKT cells show fine specificity for the α anomer of galactose, and for an equatorial hydroxyl group at carbon 2. Removal of the hydroxyl groups at positions 2 and 3 of the phytosphingosine also prevented recognition, but this could be the result of decreased lipid stability or CD1d presentation.*

probably reflecting a spectrum of reactivities to different CD1d–lipid complexes displayed by the different transfectants.

CD1d-reactive cells have been assigned a plethora of functions, with a capacity to secrete large amounts of cytokines, including IL-4, IL-10, and interferon γ (IFN-γ) implicating the cell in immunoregulation (Gumperz and Brenner 2001). The NKT cytokine spectrum also implies that some nonconventional T cells are not constrained by the Th1/Th2 paradigm. Altered development of autoimmune disease has been reported in NKT-deficient animals such as $CD1d^{-/-}$ mice, on the appropriate genetic backgrounds. After infection with *Borrelia burgdorferi* (the etiological agent of Lyme disease), $CD1d^{-/-}$ mice displayed an altered (rather than a diminished) immune response (Kumar et al. 2000). Collectively, these data have strongly suggested that NKT cells respond rapidly to perturbation (e.g. infected or transformed cells), by coordinating the actions of other cells of the immune system. Thus, as with γδ cells, these unconventional T cells may make use of limited TCR diversity to recognize generic beacons of host dysregulation and activate immune responses, in a manner not dissimilar from the actions of dendritic cells or macrophages. Interestingly, large numbers of NKT cells are found in the liver, which is the organ responsible for releasing acute phase response mediators.

This notwithstanding, the biology of NKT cells may differ from that commonly attributed to γδ cells in that NKT cells emerge relatively late in thymic ontogeny, compared with the fetal and perinatal emergence of $TCR\gamma\delta^+$ IELs. Given this, the raison d'être of NKT cells may be to regulate conventional αβ T cells, rather than to contribute intrinsic immunoprotective capacities, as has been proposed for $TCR\gamma\delta^+$ cells. Perhaps consistent with this, the positive selection of NKT cells in the thymus is critically influenced by $CD4^+CD8^+$ 'double-positive' thymocytes, which are signatory progenitors of conventional αβ T cells (Bendelac 1995).

CD1a, -b, -c reactive αβ T cells

Numerous T cells have been identified that are specific for various glycolipid antigens presented by CD1a, CD1b, or CD1c. The cells express diverse TCRs, and have high specificity for the surface-exposed carbohydrate heads of glycolipid antigens that are anchored by their acyl chains deep within the hydrophobic core of the antigen-presenting pocket (Figure 3). CD1b will present mycobacterial cell wall-derived glycolipids, but CD1 molecules may also present endogenous ligands. In sum, the biological distinction between self and nonself that is a core parameter of conventional αβ T-cell activity remains enigmatic for the bulk of CD1-reactive T cells, as it does for γδ cells.

Like γδ cells, unconventional CD1-reactive T cells are primarily Th1/cytolytic $CD4^-CD8^-$ (double-negative (DN)) cells, but are not exclusively so. Hence, their true representation, based exclusively on the analysis of the DN T-cell population, may have been underestimated, particularly in infections. As CD1a, -b, and -c genes are absent from the mouse genome, a role for CD1-reactive cells in infection has been inferred from human studies showing that cells reactive to CD1-presented mycobacterial isoprenoid lipid antigens are readily detectable in people with recent *Mycobacteria tuberculosis* infections. Of note, efficacious immune responses to mycobacteria have been associated with group 1 CD1 antigen expression by dendritic cells (DC).

Unlike expression of MHC class II recognized by conventional T cells, CD1 is highly expressed on immature DCs, before their activation and migration from the periphery to the lymph nodes. The potential therefore exists to activate CD1-reactive T cells directly in peripheral tissues, further supporting the view that unconventional T cells complement conventional T-cell activities by contributing locally to an early phase of the immune response. Interestingly, the different forms of CD1 appear to associate with lipid antigens in different intracellular compartments (Jayawardena-Wolf and Bendelac 2001; Winau et al. 2004), suggesting that a spectrum of antigen-processing events may stimulate different subsets of CD1-reactive cells to respond to either endogenously synthesized antigen (in transformed or infected cells) or exogenously acquired antigen (after phagocytosis).

In sum, CD1-reactive cells compose a potent and versatile response repertoire, and it therefore seems all the more surprising that this system is not conserved in mice. Arguably, the relatively higher abundance of $\gamma\delta^+$ cells in mice may partially substitute for the function of

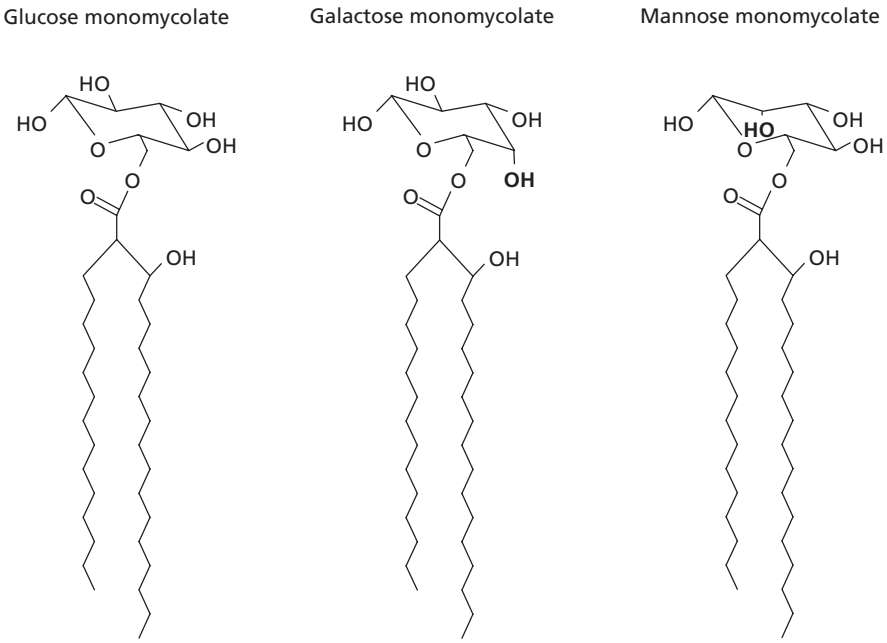

Figure 3 *αβ T cells exhibit fine specificity for glycolipid antigens presented by CD1b. Some αβ T cells recognize derivatives of mycolic acid, but not mycolic acid derivatized with galactose or mannose, which are stereoisomers of glucose and differ from glucose in the orientation of a single hydroxyl group.*

CD1-reactive cells, and CD1 reactivity of $\gamma\delta^+$ cells has been reported, ironically in humans (see above). Nevertheless, the reason that the specific constitutions of unconventional T-cell repertoires seem so variable across species remains poorly understood.

MR1-reactive αβ T cells

Another population of unconventional αβ T cells has been described in the lamina propria of the intestine in mice, humans, and cattle. These T cells express an invariant Vα7Jα33 TCR in humans (the homologous Vα19Jα33 in mice and cattle) (Tilloy et al. 1999). It was noted that this population was present in MHC class I and CD1 double-deficient mice, MHC class II-deficient mice, and TAP-deficient mice, but was missing in $\beta_2m^{-/-}$ mice. Therefore, candidate nonclassic MHC class I molecules were tested as potential ligands. As a result, this invariant T-cell population was found to be dependent on the presence of the nonclassic MHC class I molecule MR1 (Treiner et al. 2003). MR1 mRNA was previously reported to be expressed ubiquitously (Yamaguchi et al. 1997), although no antibody was available to confirm the distribution of the MR1 protein. It has since been found that, despite widespread mRNA expression, the MR1 protein is expressed only on the surface of activated B cells in the lamina propria. These B cells expressing MR1 activate the invariant Vα7Vα19 T cells to secrete transforming growth factor-β (TGF-β), which causes the B cells to class switch to IgA, the appropriate isotype for mucosal surfaces (Lantz, unpublished) (Figure 4). These recent findings concerning MR1-reactive αβ T cells parallel the findings from γδ cell biology in emphasizing the association of unconventional T cells with tissues and tissue-specific processes.

Qa-1 reactive/HLA-E reactive CD8αβ$^+$ αβ T cells

Other αβ T-cell populations are restricted by MHC class IB molecules of the H-2Q region, the counterpart of which in the human is the HLA-E molecule. After infection of mice with *Salmonella typhimurium*, large numbers of H-2Qa-1 reactive cells were described, whereas HLA-E-reactive T cells have been described in humans infected with *Mycobacterium tuberculosis*. Commonly, such unconventional MHC class Ib-reactive CD8αβ$^+$ αβ T cells appear to be constitutively activated, before infection, and may thus be primed to contribute to primary protection at early times after infection (see next section). However, an additional role has been proposed for MHC class Ib-reactive CD8αβ$^+$ αβ T cells, following the identification in the mouse of cells that respond to a Vβ8-derived peptide presented by Qa-1 on seemingly conventional Vβ8$^+$ CD4$^+$ TCRαβ$^+$ T-helper cells (Jiang and Chess 2000) (Figure 5). This CD8αβ$^+$ αβ T-cell response, which can likewise be elicited by irrelevant cells co-transfected with the Qa-1 and TCR Vβ8 chain genes, results in the cytolysis of the CD4$^+$ T cell, thus downregulating the associated immune effector response.

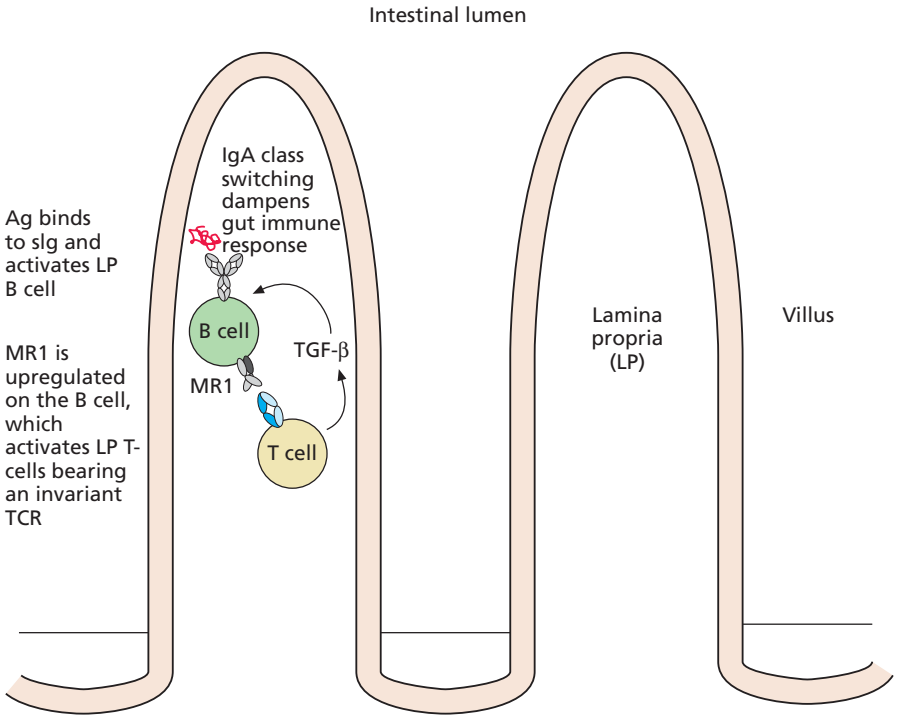

Figure 4 *MR1 is a ligand for a conserved population of lamina propria T cells. Expression of MR1 is upregulated on B cells on antigen binding to surface immunoglobulin. MR1 is recognized by T cells expressing an invariant Vα7Jα33 in humans (the homologous Vα19Jα33 in mice and cattle). On MR1 recognition, these T cells produce transforming growth factor β (TGFβ), which induces class switching to IgA, the appropriate isotype for the intestine; as well as preventing hyperactivation of the gut immune system. Ag, antigen; slg, surface immunoglobulin.*

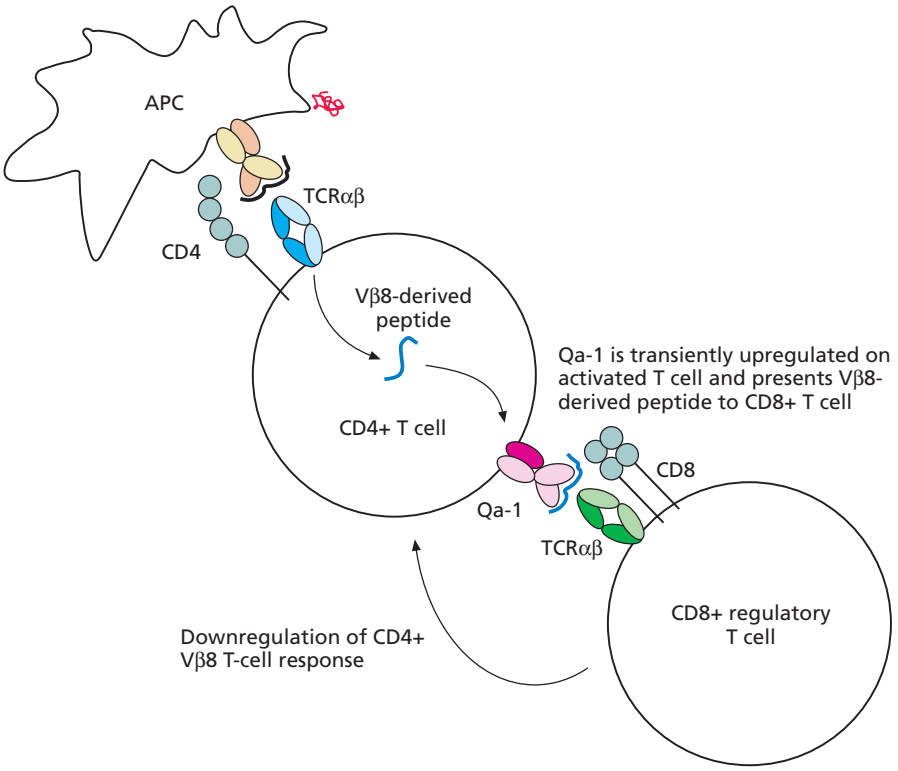

Figure 5 *Recognition by regulatory $CD8^+$ T cells of T-cell receptor (TCR)-derived peptides presented on Qa-1. Activated $CD4^+$ T cells transiently upregulate the nonclassic MHC class I molecule Qa-1, which presents peptides derived from the TCR Vβ8 chain. Qa-1 peptide complexes are recognized by $CD8^+$ regulatory T cells which downregulate the CD4 T-cell response. APC, antigen-presenting cell.*

Evidence that $CD8^+$ cytolytic T cells downregulate immune responses to self and to foreign antigens has been presented in many experimental systems for many years, e.g. mice induced to develop experimental allergic encephalomyelitis (in many regards, an animal model for multiple sclerosis (MS)) by immunization with myelin basic protein peptides conventionally show marked periods of disease remission, reminiscent of those that characterize MS and other autoimmune diseases in humans. These are eradicated by either antibody-mediated, or genetic depletion of, $CD8^+$ T cells, leading to unrelenting disease progression (Jiang et al. 1992; Koh et al. 1992). The prospect that such $CD8^+$ T-cell-mediated regulation might target $CD4^+$ T cells in an antigen receptor-specific fashion was emphasized by the finding that the immune response to hen egg lysozyme is reduced in strains of mice that, paradoxically, have more TCR Vβ genes. This immune deficiency is rescued by depletion of $CD8^+$ T cells, indicating that the regulatory cells are targeting $CD4^+$ T cells that express particular TCR Vβ genes (Jiang and Chess 2000).

One would assume, from first principles, that the cytolysis of $CD4^+$ $TCR\alpha\beta^+$ T-helper cells must be carefully regulated. In this light, Qa-1 has some interesting properties. First, it is only expressed on $CD4^+$ T cells after activation and, second, its expression is transient, thereby limiting the potential for regulation to a short window. Third, Qa-1 presenting leader peptides of conventional MHC class I molecules (Qdm) in addition engages an NKG2A/CD94 NK inhibitory receptor that is commonly expressed on activated $CD8^+$ T cells (Aldrich et al. 1994). The same is true for HLA-E, in humans. Thus, one might envision that, when a $CD4^+$ T cell expresses an excess of Qa-1.Qdm over Qa-1.TCR, the regulatory $CD8^+$ T cell is inhibited, whereas, when Qa-1.TCR is in excess, it is activated to target the $CD4^+$ T cell. Interestingly, immune regulation by $CD8^+$ T cells seems primarily to target $CD4^+$ Th1 cells rather than $CD4^+$ Th2 cells. The opposite has been claimed for the *fas*-mediated downregulation of autoreactive Th2 cells by systemic γδ cells (see above).

Unconventional peptide-reactive αβ T cells

Contemporary genomics analyses have revealed an increasing number of unconventional, MHC class 1B genes that are candidate ligands for further unconventional T-cell reactivities. One such reactivity is the αβ T-cell response to bacterial *N*-formylated peptides (Nfp), presented by H2-M3. As the initiator methionine groups in eukaryotic proteins are very rarely *N*-formylated, the recognition of Nfp might be regarded as a form of pathogen-associated molecular pattern recognition.

H2-M3 Nfp-reactive cells have been described in mice infected with *Mycobacterium tuberculosis* and *Listeria monocytogenes* and they may form a part of the T-cell response to a broad spectrum of bacteria. In *Listeria*-infected mice, multivalent cell-staining reagents comprising H2-M3 Nfp tetramers detected reactive αβ T cells early in the response, before the clonal expansion of conventional, MHC-peptide reactive αβ T cells. Consistent with this, the cells constitutively display markers of activation (e.g. $CD44^{hi}$). Two additional aspects of the tetramer-reactive cells are reminiscent of other unconventional T cells: (1) they have cytolytic and Th1 effector potential; and (2) they contribute substantially to primary responses, but, after secondary infection, their contribution is eclipsed by the effector responses of conventional $CD8\alpha\beta^+$ αβ T cells (Kerksiek et al. 1999). Also complying with the hypothesis that unconventional T cells differ from conventional T cells in their selection during development, it was recently shown that the progenitors of H2-M3 Nfp-reactive cells will positively select on autologous MHC class Ib antigens expressed in the thymus by hemopoietic cells, and that, contingent upon this, the cells immediately acquire an activated phenotype before thymic emigration (Urdahl et al. 2002).

Unconventional 'β-only' T cells

When a mutation was experimentally introduced into the mouse TCRα chain, by gene-by-gene knockout technology, a small population of peripheral, $CD4^+$ $TCR\beta^+$ cells was found in the resultant mice (Mombaerts et al. 1992). The potential for TCR β chain expression in the absence of TCRα exists via the formation of either TCRβ/β homodimers, or a complex of TCRβ with an alternately spliced product of the pre-Tα chain which can ordinarily pair with TCRβ during thymocyte development (Barber et al. 1998). As for other nonconventional T cells, these 'β-only' cells have potent Th1 activity and are in part responsible for pathogen-responsive bowel inflammations that develop in $TCR\alpha^{-/-}$ mice but not in $TCR\beta^{-/-}$ mice, which would lack $TCR\beta^{+\alpha-}$ cells.

Naturally, β-only cells have generally been regarded as an idiosyncrasy of $TCR\alpha^{-/-}$ mice, but their existence in normal animals should not be discounted. In thymocyte development, TCRβ-gene rearrangement precedes TCRα-gene rearrangement and the formal possibility therefore exists for cells that do not productively rearrange either TCRα-chain allele to mature as β-only cells. Although such cells would be thought unlikely to receive essential signals for positive selection on MHC, the fact that such cells are present in the periphery of $TCR\alpha^{-/-}$ mice clearly indicates the potential for some 'β-only cells' to mature. Given their functional potency, particularly their capacity to respond to microbial antigens, their existence in humans needs to be more thoroughly tested.

Regulatory CD4$^+$ CD25$^+$ αβ T cells: putative unconventional T cells

The diversity of unconventional T cells that has been considered in this chapter provokes the concept that unconventional T cells are not particularly rare, and that there may be other sets of T cells that are currently poorly understood, which may ultimately be assigned as unconventional. In this regard, much attention has recently been paid to a set of immunosuppressive T cells that can be found in mice and humans within the CD4$^+$CD25^{hi} subset. Although these markers alone are insufficient to identify a pure population of regulatory T cells (e.g. they also identify activated effector cells), they identify a subset of cells in the thymus that seemingly acquires an activated phenotype immediately on maturation. Perinatal thymectomy in certain strains of mice (e.g. Balb/c) selectively ablates such cells, provoking spontaneous, multiorgan, autoimmune disease. There remains much to learn about these regulatory cells, and there is a high likelihood that they are heterogeneous in phenotype. Nevertheless, evidence is gathering that at least some of them may comply with the operating definitions of unconventional T cells proposed at the beginning of this chapter. Specifically: their antigen specificity may be primarily selfreactive, not necessarily restricted to conventional MHC; their development, as stated, seems to be distinct from that of conventional αβ T cells; their recirculation patterns are ill-defined but evidence suggests that they may function primarily within tissues; and their potential to contribute to antigen-specific memory is also as yet unclear. The potential that such cells, with proven capacity to regulate immune responses in vivo would be assigned to the unconventional T-cell compartment further highlights the prudence of paying greater attention to the representation of unconventional T-cell subsets in health and disease.

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T-cell receptors

MARC BONNEVILLE

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Even long after the characterization of immunoglobulins and B-cell receptors, the molecular basis of antigen recognition by T cells has remained a major enigma for immunologists. Both the exquisite antigen specificity of T cells and their ability to mount memory responses suggested that, as with B cells, T cells recognized antigens through structurally diverse and clonally distributed receptors, then referred to as T-cell receptors (TCR). However, the unsuccessful attempts to identify idiotypic determinants shared by antibodies and TCRs and the fundamental difference between B-cell and T-cell antigen recognition processes, unveiled by the work of Zinkernagel and Doherty, fostered intensive debates in the 1970s about the TCR nature and structure. Addressing this issue turned out to be particularly challenging but was finally settled in the 1980s with the molecular characterization of the TCR genes. During the last decade, biophysical studies of TCR–pMHC (peptide major histocompatibility complex) interactions and structural analyses of trimolecular TCR–peptide–MHC complexes have brought insights into the fine mechanism of TCR recognition and T-cell activation, which are now viewed as dynamic processes relying on TCR conformational fits and complex interplays between immune receptors and signaling molecules within specialized supramolecular arrangements.

ISOLATION OF T-CELL RECEPTORS: AN HISTORICAL OVERVIEW

In several early 1970s studies, Zinkernagel and Doherty demonstrated that T-cell recognition of virally infected cells requires both viral antigens and expression of self-major histocompatibility complex (MHC) molecules by infected cells (Zinkernagel and Doherty 1974). By analyzing the responses of hybridomas obtained by fusion of T cells with distinct antigen and MHC specificities, Kappler and Marrack's group then showed, in agreement with the so-called 'altered self-model', that this 'self-MHC restriction' involves a single receptor that is able to recognize self-MHC molecules modified by or complexed to the antigen (Kappler et al. 1981). The first hints at the TCR structure were obtained by Allison and colleagues, who generated antisera specific to surface receptors expressed by restricted sets of T-cell clones and demonstrated the heterodimeric nature of these 'clonotypic' receptors (McIntyre and Allison 1983). Several groups showed later on that these heterodimeric receptors, called TCR$\alpha\beta$, were tightly associated on the cell surface with a multimeric transduction complex called CD3 (for a review, see Clevers et al. 1988). Identification of the first TCR gene was achieved in 1984 by the groups of Davis and Mak (Hedrick et al. 1984; Yanagi et al. 1984). The elegant strategy designed by Davis and colleagues to isolate candidate TCR genes relied on

two main assumptions: first that the putative TCR genes should be specifically transcribed in T cells and, second, that as with immunoglobulin (Ig) genes, TCR genes should undergo somatic rearrangement. Thus they prepared complementary DNA (cDNA) from a T-cell clone and enriched for T-cell-specific genes by removing the cDNA species that hybridized to B-cell messenger RNA (mRNA). The remaining cDNAs were then used as probes to detect, by Southern blot analysis, genes with patterns that are specifically modified in mature T cells. Following this approach, this group isolated a cDNA showing significant sequence homology with Ig genes, which turned out to encode the TCRβ chain. While looking for the missing α-chain gene (which was isolated a few months later), Tonegawa and colleagues fortuitously identified, in the same year, another gene specifically rearranged in T cells called γ (Saito et al. 1984). Discovery of this 'orphan' gene suggested the existence of new class of TCR, which was totally unexpected from previous immunological studies, and the existence of which remained elusive during the following 2 years. Postulating that, as with the αβ TCR, the γ gene product should form a TCR associated with the CD3 complex, Brenner and colleagues used CD3-specific monoclonal antibodies to immunoprecipitate a new heterodimeric receptor expressed on a distinct set of T cells (Brenner et al. 1986). The TCR gene coding for the missing δ chain was finally identified in 1987 by Davis's group (Chien et al. 1987).

GENOMIC ORGANIZATION AND SOURCE OF TCR DIVERSITY

As for Ig genes, functional TCR genes are generated through somatic recombination of gene segments that are originally separated on the chromosome. This rearrangement process, which is tightly controlled in a lineage- and stage-specific manner, allows the production of myriads of distinct receptors. Similar to antibodies, TCR diversity originates both from the combinatorial usage of VDJ gene segments picked out from a repertoire of distinct elements and from extensive sequence modifications of the rearranged VDJ genes which are mediated by the recombination machinery.

Organization of TCR loci

There are four TCR gene loci in the germline DNA, each comprising arrays of distinct V, J, and in some cases D elements. V elements can be grouped into families, which correspond by convention to sets of V genes with nucleotide sequences that exhibit more than 75 percent homology. V-gene segments have two exons, D and J segments a single exon, and C-gene segments three to five exons. A schematic representation of the organization of TCR loci is represented in Figure 1. Detailed description of the VDJ sequences and nomenclatures can be obtained from the ImmunoGenetics database (www.imgt.cnusc.fr:8104).

THE TCRβ LOCUS

The TCRβ locus is located on chromosome 6 in the mouse and 7 in humans. It encompasses a region of grossly 600 kb in humans. All Vβ genes are clustered in the same chromosomal region except *BV30*, which is located downstream of the Cβ2 element and in opposite transcriptional orientation. In humans, there are 67 Vβ elements (of which 41–47 are functional) clustered into 32 Vβ families (Rowen et al. 1996). The 3′ part of the TCRβ locus corresponds to a duplication of an ancestral region comprising one Dβ segment, six functional Jβ segments, and a Cβ gene. The two Cβ genes differ by four amino

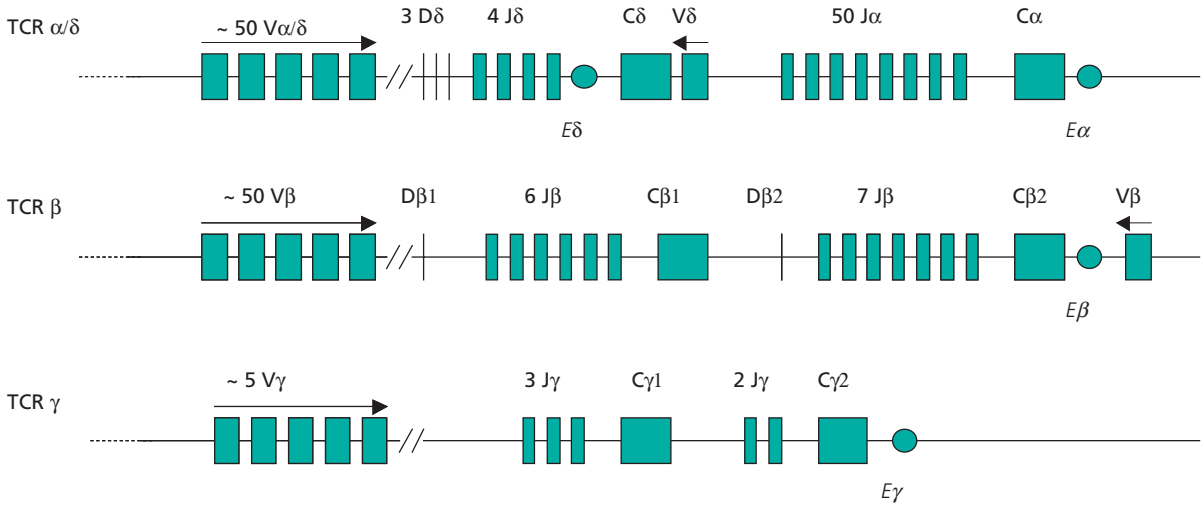

Figure 1 *Genomic organization of human T-cell receptor (TCR) gene loci. See the text for details. The numbers mentioned in the figure take into account functional V- and J-gene segments only. V-gene transcriptional orientation is indicated by arrows. Circles correspond to enhancer elements.*

acids only, and appear functionally equivalent. In this regard, unlike Ig genes, rearranged TCR genes do not undergo isotype switching (which consists of replacing the constant region of a rearranged Ig gene by another constant region, generally endowed with distinct functional properties). A β enhancer, which plays a key role in the control of TCRβ locus transcription and rearrangement (see 'Control of TCR gene assembly' below), is located between the Cβ2 element and the downstream BV30 element (Gottschalk and Leiden 1990).

THE TCRα/δ LOCUS

The TCRαδ locus is located on chromosome 14 in rodents and humans, and encompasses a chromosomal region of roughly 1 Mb. It comprises 45–47 functional Vα segments grouped into 41 families in humans. There are 50 functional Jα elements and a single Cα gene. The TCRδ locus is located within the α locus, between the Vα and Jα segments. This peculiar location has important implications because any VJα rearrangement leads to deletion of the δ locus. Together with transcriptional regulation processes detailed below, this mechanism prevents co-expression of α and δ chains and ensures mutual expression of αβ and γδ TCR in a given T cell. The human δ locus comprises three Dδ segments (two in rodents), four Jδ segments (two in rodents), and one Cδ segment. The Vδ and Vα elements are interspersed, which theoretically permits their rearrangement to either DJδ or Jα elements. Although this happens to be the case for some V segments (Miossec et al. 1990), the sets of Vα/δ elements used to form αβ and γδ TCR are generally distinct and only eight Vα/δ elements are used to a significant extent by γδ cells. Among these, one Vδ segment (DV3) is located between the Cδ and Jα elements, in an opposite transcriptional orientation. The rules governing usage of V-gene segments by either the α or δ chain are still unclear but presumably complex. It is likely that both cell-selection processes and locus accessibility constraints contribute to the biased expression of particular Vδ regions by most γδ T cells (see below). Several regulatory sequences controlling TCRα/δ locus transcription and rearrangement have been identified: a TCRα enhancer located downstream to the Cα gene (Ho et al. 1989), the T-early α (TEA) element (which controls rearrangements involving the most upstream Jα segments) between the Cδ and Jα elements (de Chasseval and De Villartay 1993; Wilson et al. 1996), a TCRα silencer (called BEAD-1 for blocking element α–δ 1) immediately upstream from TEA (Zhong and Krangel 1997) and a TCRδ enhancer upstream from the Cδ gene (Redondo et al. 1990).

THE TCRγ LOCUS

Located on chromosome 13 in the mouse and 7 in humans, the γ locus is spread over 160 kb in humans and 200 kb in the mouse. Its genomic organization significantly differs between these two species. In the mouse, there are four clusters presumably resulting from duplication events, which each comprise a single J and C segment together with one to four Vγ segments. A γ enhancer is found downstream to Cγ1 and Cγ3 and upstream from Cγ2 (Vernooij et al. 1993). The human γ locus comprises 11–14 Vγ segments (of which four to six are functional) grouped into four families, and two downstream clusters of J- and C-gene segments (Lefranc and Rabbitts 1989). A γ enhancer and two silencers are found downstream to Cγ2 (Lefranc and Alexandre 1995).

TCR rearrangements

Each TCR chain is encoded by multiple VDJ gene segments which are originally separated on the chromosome. During T-cell development these elements are joined to form a single exon coding for the variable domain of each recognition subunit. The V domain of TCRα and γ chains, like Ig light chains, is encoded by V and J elements, whereas the V domain of TCRβ and δ chains, like Ig heavy chains, is encoded by V, D, and J elements. V(D)J recombination is initiated by recognition and cleavage of double-stranded DNA at conserved sites, named recombination signal sequences (RSS), that immediately flank the V, D, and J elements (Tonegawa 1983). The RSSs are highly conserved in TCR and Ig genes, and each consist of a palindromic 7-base-pair (bp) sequence (consensus 5′-CACAGTG) and an A/T-rich 9-bp sequence (consensus 5′-ACAAAAACC), separated by a poorly conserved spacer, the length of which is either 12 ∓ 1 or 23 ∓ 1 bp (Figure 2a). As for Ig genes, recombination between two TCR-gene segments follows the 12/23 rule, i.e. recombination occurs only between an RSS with a 12-bp spacer and one with a 23-bp spacer (Early et al. 1980) (Figure 2b). The enzymatic machinery mediating TCR and Ig gene rearrangements involves both lymphoid cell-specific recombinases called RAG1 and RAG2 (Schatz et al. 1989; Oettinger et al. 1990), and ubiquitous enzymes of the DNA-repair machinery (for recent reviews, see Grawunder et al. 1998; Fugmann et al. 2000). The recombination process is outlined below and depicted in Figure 3.

V(D)J assembly is initiated by the recognition of the 12-bp and 23-bp RSSs by the RAG proteins, which bring the RSS into close juxtaposition (synapsis) (step no. 1). This step involves two ubiquitous enzymes promoting DNA bending (called high mobility group (HMG)-1 and -2 proteins), which stabilize interactions between RAG proteins and RSSs and ensure the 12/23 rule (Van Gent et al. 1997). RAG proteins then introduce a precise nick on one DNA strand between the RSSs and its flanking element, rapidly followed by cleavage of the other strand (step no. 2). At the end of this first phase, the

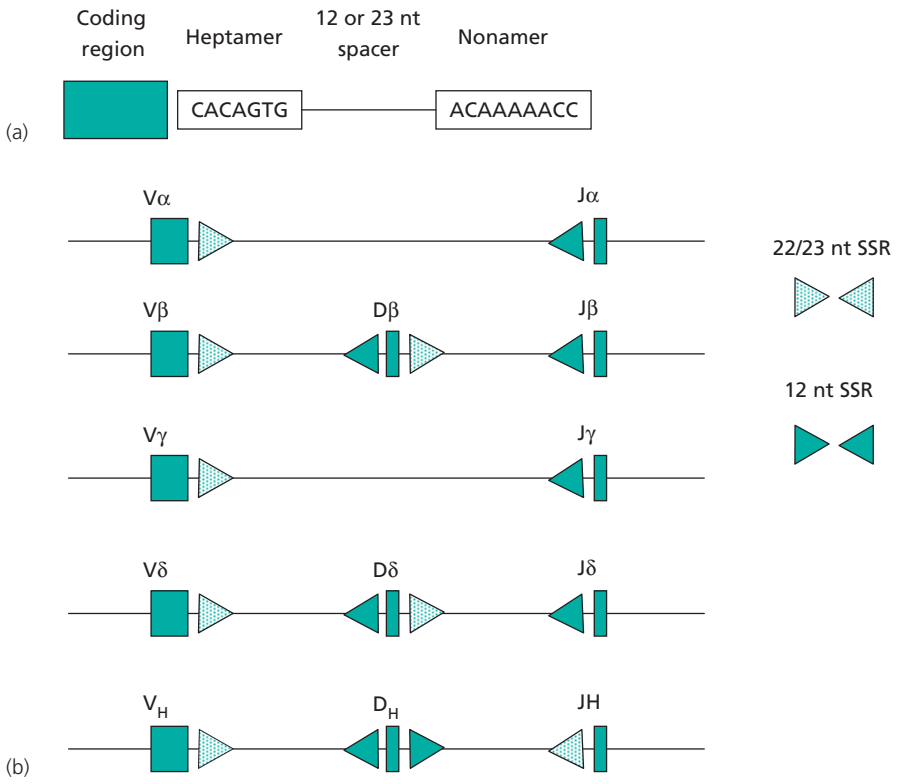

Figure 2 *Structure of recombination signal sequences and distribution within the T-cell receptor (TCR) and immunoglobulin (Ig) gene loci. See the text for details.*

recombinases catalyze by a transesterification mechanism both the generation of two open signal strands and the formation of a hairpin between the two open coding strands (McBlane et al. 1995).

The second phase of the recombination reaction is more complex and involves both the recombinases and a larger set of enzymes involved in the repair of DNA double-stranded breaks. The signal ends are joined

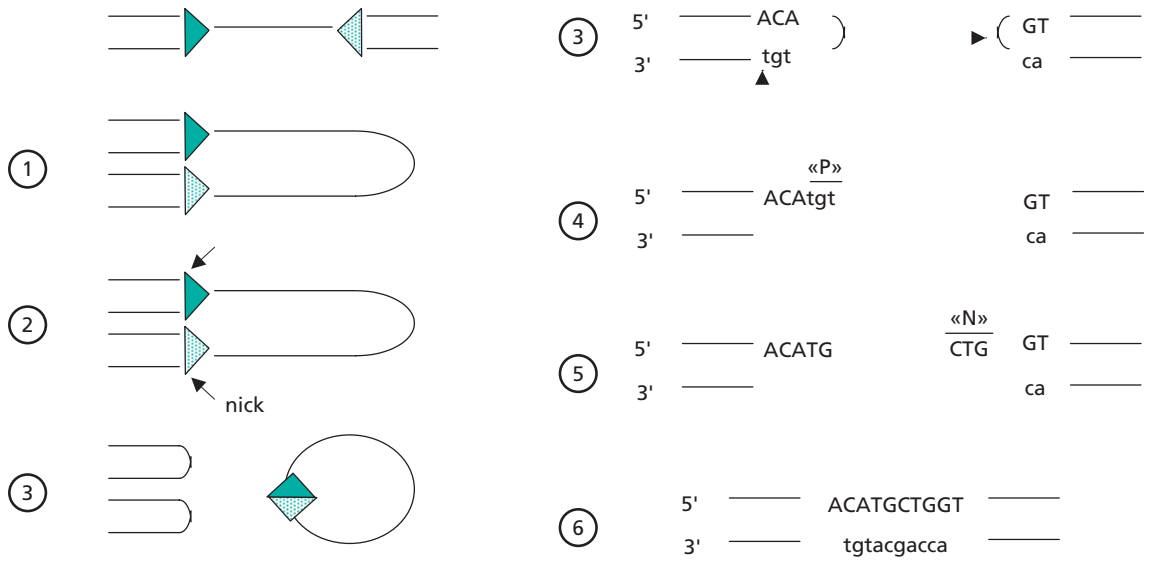

Figure 3 *The V(D)J gene recombination reaction. See the text for details. Dark and light triangles correspond to 12 bp and 23 bp recombination signal sequence (RSS). Step 1: synapsis. Step 2: nicking of the RSS. Step 3 (bottom left): generation of hairpins at the coding ends, formation of signal joints in excised circular DNA. Step 3 (up right): hairpin nicking resulting in nibbling of coding ends or addition of 'P' nucleotides (step 4). Step 5: addition of 'N' nucleotides. Step 6: filling and ligation of the coding joints.*

head to head, leading to formation of a 'signal joint' (step no. 3). This process is catalyzed by the RAG proteins and the ubiquitous HMG and Ku DNA-repair proteins (Agrawal and Schatz 1997). On the other side of the recombination complex, the hairpin formed between the two coding ends is opened through introduction of a nick, possibly catalyzed by the recombinases themselves (Shockett and Schatz 1999) (step no. 3). This nicking process is quite imprecise because it occurs at the tip of the hairpin, the coding strand, or the noncoding strand. This generates, respectively, full-length coding ends, deletions on the coding end, or addition of so-called palindromic (or 'P') nucleotides derived from the noncoding strand (Lafaille et al. 1989; Lieber 1991) (step no. 4). In mice deficient for the catalytic subunit of DNA–protein kinase (DNA-PKcs) or for Ku proteins, there is an almost complete abrogation of T- and B-cell production, accompanied by accumulation of uncleaved hairpin coding ends in developing lymphocytes (Lieber et al. 1988; Zhu et al. 1996). This indicates that DNA-PKcs and Ku proteins are both required for RAG-mediated hairpin opening, and probably act by stabilizing interactions between the RAG protein and the coding end-complex. After hairpin opening, the coding ends may undergo an exonucleolytic processing controlled in part by the DNA-PK, which results in significant nibbling of the rearranged VDJ elements. Variable numbers of so-called 'N' nucleotides may be added in a template-independent fashion at the coding ends (step no. 5). This reaction is catalyzed by a lymphoid cell-specific polymerase called terminal deoxynucleotidyl transferase (TdT) (Komori et al. 1993), which acts in conjunction with DNA-PKcs and Ku proteins (Bogue et al. 1997). As for B cells, TdT expression is repressed during embryonic life, resulting in lack of 'N' additions within the rearranged TCR genes produced at that stage. The physiological significance of the regulated expression of TdT during development is still unclear. As suggested by analysis of fetal γδ T-cell development in in vitro thymic organ cultures, TdT repression may favor generation of particular fetal T-cell subsets with conserved TCR and possibly mandatory functions (Ikuta et al. 1990). Alternately lack of TdT activity may prevent development of particular lymphoid subsets which might be deleterious during fetal life.

In the last step of the recombination reaction, the sense and antisense strands of the processed coding ends tend to align along short homologous regions of two to three nucleotides (homology-guided recombination) (Feeney 1992). As discussed earlier, the homology-guided recombination process favors the production of TCR with particular junctional sequences, which may represent a significant fraction of the fetal TCR repertoire (Itohara et al. 1993). The double-stranded DNA junctions are reconstituted through the combined action of yet undefined polymerases and nucleases (step no. 6). Final ligation of the coding strands is performed by the ligase IV/XRCC4 complex, presumably recruited by the Ku proteins (Nick McElhinny et al. 2000).

Modalities of TCR recombination

As for Ig genes, the intervening DNA sequence separating the rearranged TCR gene segments has two possible outcomes (Figure 4). When the rearrangement involves

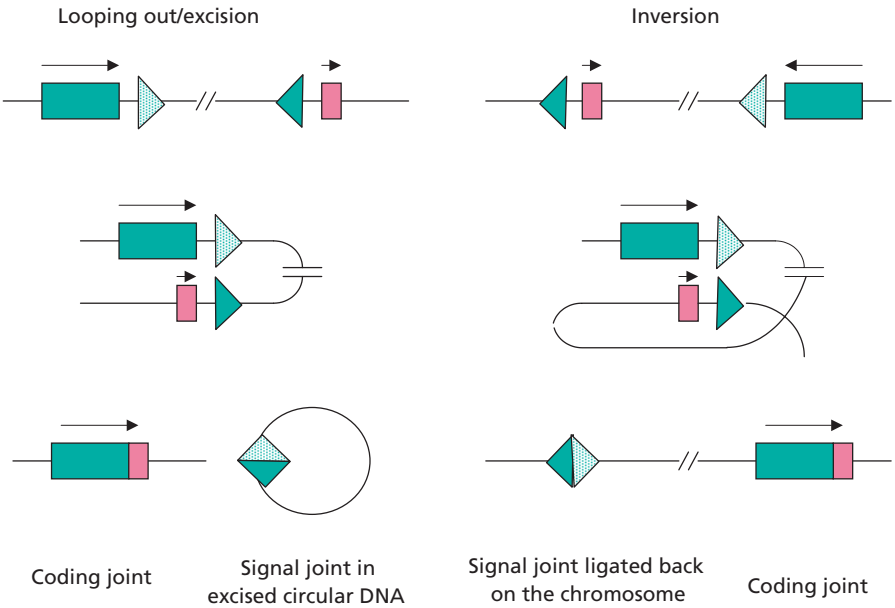

Figure 4 *Recombination modalities. See the text for details. Rectangles: coding regions. Triangles: recombination signal sequences (RSSs). Arrows: transcriptional orientation.*

elements in the same transcriptional orientation, which is the classic situation, the intervening DNA stretch between the rearranged gene segments is removed in the form of circular excision products, generated on head-to-head ligation of the RSS (looping out/excision model) (Okazaki et al. 1997). These circular DNAs are specifically found within developing lymphocytes and, thus, can be used as markers to follow T-cell regeneration, e.g. in HIV patients and bone marrow recipients. When the rearrangement involves gene segments in opposite transcriptional orientation (as is the case for the most downstream V elements in the TCRβ and TCRδ loci), the intervening DNA is ligated back and translocated on the chromosome through a chromosomal inversion mechanism (Malissen et al. 1986).

Although recombination events classically involve gene segments belonging to the same locus, they sometimes occur between distinct TCR loci, TCR and Ig loci, or TCR or Ig loci and other genomic elements. These interlocus recombinations or 'trans-rearrangements' are mediated through recognition of classic RSS and/or related or cryptic RSS motifs that are present throughout the genome. The peculiar configuration of the TCRα/δ locus naturally favors rearrangements of a given Vα/δ element to either DJδ or Jα segments (Miossec et al. 1990). Rearrangements between human TCRβ and TCRγ loci, which are both located on chromosome 7 but in distinct regions and in opposite orientations, may occur at high frequencies in T cells from healthy donors and patients with ataxia telangiectasia (Lipkowitz et al. 1990). These trans-rearrangements lead to expression of functional Vγ(Dβ)JβCβ chains that pair with classic TCRα chains, and thus significantly contribute to TCR repertoire diversification (Davodeau et al. 1994; Retière et al. 1999). Finally some lymphoid tumors may originate from illegitimate recombinations between an RSS within a TCR or Ig locus and a cryptic signal sequence close to an oncogene, e.g. Burkitt's lymphomas result from rearrangements between the protooncogene *c-myc* and RSS flanking Jα segments, leading to constitutive expression of *c-myc* expression under the control of the α enhancer (Bernard et al. 1988).

Sources of TCR diversity

Although the number of germline V(D)J elements is much lower in TCRs than in Ig loci, several mechanisms operate during TCR gene assembly to yield a high degree of diversity within the junctional regions of the rearranged VJ or VDJ genes.

COMBINATORIAL DIVERSITY

TCR combinatorial diversity is determined by the number of random V(D)J combinations yielding an αβ or γδ TCR. Although lower than Ig, the combinatorial diversity of αβ TCR appears to be much higher than that of γδ TCR. The random assembly of TCR VJα and VDJβ genes can generate, grossly, 2500 (i.e. 50×50) and 1200 (i.e. $50 \times 2 \times 12$) possible combinations respectively. Considering a random association of α and β chains, this yields up to 3×10^6 distinct TCRs. By contrast, the total number of VJγ/VDJδ combinations is around 3000 only (i.e. $(6 \times 5) \times (10 \times 3 \times 4)$) in humans. An additional source of combinatorial diversity is provided by the peculiar arrangement of the 12- and 23-bp RSSs in TCR genes which permits, unlike Ig genes, joining of several D-gene segments in tandem (see Figure 2b). Such joining events, yielding VDDJ or even VDDDJ junctions, are frequently seen within rearranged TCRδ genes and drastically broaden the junctional length distribution of the δ chains (Chien et al. 1996).

JUNCTIONAL DIVERSITY

VDJ assembly is associated with important modifications of the coding ends of the rearranged segments (see Figure 3). First, the imprecise cleavage of hairpin coding ends may lead to either 'P' nucleotide additions or nibbling of V(D)J sequences. Second, further VDJ nibbling may occur at a later stage of the recombination reaction by exonucleolytic processing. Third, addition of variable numbers of 'N' nucleotides by TdT may further increase junctional sequence diversity. All these mechanisms operate in both TCR and Ig gene rearrangements. However, although 'N' additions occur in rearranged Ig heavy chain genes only, they are observed in all rearranged TCR genes. Reading frame changes resulting from nucleotide addition and trimming may generate nonproductive rearrangements but, in the case of VDJ rearrangements, they may also generate new junctional sequences encoded by alternate reading frames. In this regard, unlike D_H segments, both the Dβ and Dδ segments can be read in all three reading frames. Altogether nucleotide addition and nibbling at the V(D)J joints generate an enormous diversity in these regions. Considering that up to six nucleotides can be added at each junction, the number of distinct junctional sequences that can be produced in the order of 10^7 to 10^{11}.

SOMATIC HYPERMUTATION

In B cells, additional diversity is generated in the rearranged V(D)J genes by somatic hypermutation (see Antibodies and B Lymphocytes). By contrast, TCR genes do not undergo somatic hypermutation. This probably ensures that the T-cell repertoire is not modified after its thymic selection and avoids generation of cells with unwanted self-reactivity.

FACTORS RESTRICTING TCR DIVERSITY

The combined effects of combinatorial and junctional diversification processes theoretically permit the production of up to 10^{16} distinct TCRs in a given individual. However, this number is probably largely overestimated for several reasons. Assembly of V(D)J segments is not

random because, in general, not all gene segments are equally accessible to the recombination machinery at a given time point. In particular the most downstream V and upstream J elements tend to be rearranged first during ontogeny (Thompson et al. 1990), probably reflecting progressive 'opening' of the TCR locus under the control of proximal *cis*-acting elements. This is also illustrated by the programmed rearrangement of different sets of VDJ gene segments at particular T-cell developmental stages (Goldman et al. 1993; Itohara et al. 1993), which favors generation of distinct waves of γδ TCR with restricted combinations of VJγ and VDJδ elements. Finally, both the repression of TdT activity during fetal life and homology-guided recombination further restrict the diversity of the fetal repertoire, which is sometimes dominated by few 'canonical' TCR sequences (Itohara et al. 1993).

CONTROL OF TCR GENE ASSEMBLY

Assembly of TCR and Ig V(D)J segments is regulated in a tissue-, lineage-, and stage-specific fashion. The restricted expression of RAG proteins in lymphoid cells at a particular stage of their development confers both the tissue- and stage-specificity of the recombination process. In addition, the restricted activation of Ig and TCR gene rearrangements in respectively T and B cells is controlled by *cis*- and *trans*-acting elements that regulate the 'accessibility' of TCR and Ig gene loci to the recombinases (Sleckman et al. 1996).

Regulation of TCR gene assembly during T-cell development

T-cell development is a highly complex and ordered epigenetic process that allows efficient generation and highly restricted TCR expression in the appropriate cell lineage, as well as selection of TCR with 'useful' (i.e. self-MHC-restricted) and 'harmless' (i.e. nonself-reactive) specificities. Importantly a tight control of the accessibility of the various TCR loci to the recombination machinery, which is transiently activated during the pre-T-cell stage, leads to a highly ordered activation of TCR rearrangements in the developing T cell (Figure 5) and early commitment of precursor cells to either the αβ or the γδ lineage (Figure 6).

INITIATION OF TCR REARRANGEMENTS AND PRE-TCR FORMATION

Intrathymic T-cell maturation can be subdivided into discrete developmental stages characterized by a particular TCR rearrangement status and phenotypic profile (Shortman and Wu 1996). In the mouse the earliest precursors, which are CD4/CD8 double negative (DN), are further split into four subsets based on CD44 and CD25 expression. RAG protein expression is initiated in $CD44^+CD25^-$ (DN I) precursors (Wilson et al. 1994). VJγ, partial VDδ or DJδ and few complete VDJδ rearrangements are first detected at the $CD44^+CD25^+$ (DN II) stage (Livak et al. 1999). Most VJγ, VDJδ, and partial DJβ rearrangements take place during transition to the $CD44^-CD25^+$ (DN III) stage (Tourigny et al. 1997). Complete VDJβ rearrangements are initiated in $CD44^+CD25^+$ cells and are completed in $CD44^-CD25^-$ (DN IV) cells, where productive VDJβ rearrangements are primarily found. VJα rearrangements are first detected in $CD44^-CD25^-$ cells but mainly take place at the following $CD4^+CD8^+$ stage.

A similarly ordered rearrangement of TCRδ, γ, β, and α genes has been more recently documented in human thymocytes (Blom et al. 1999). The TCRβ chains, which are produced at a stage when the α locus is still in germline configuration, pair with a surrogate α chain called

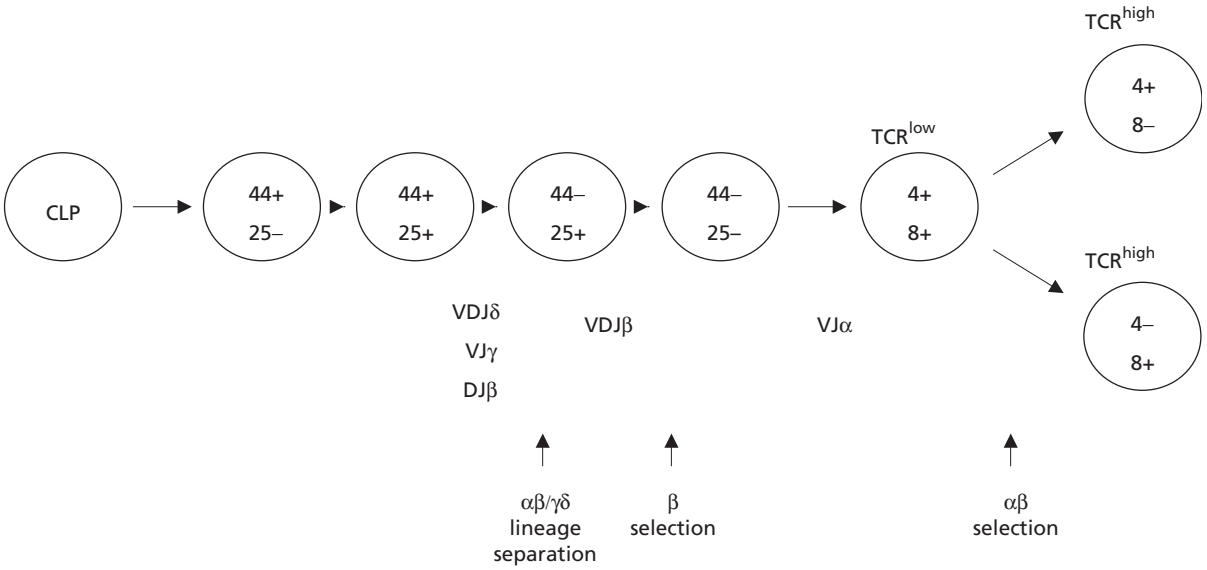

Figure 5 *Intrathymic development of murine T cells. See the text for details. CLP, common lymphoid progenitor.*

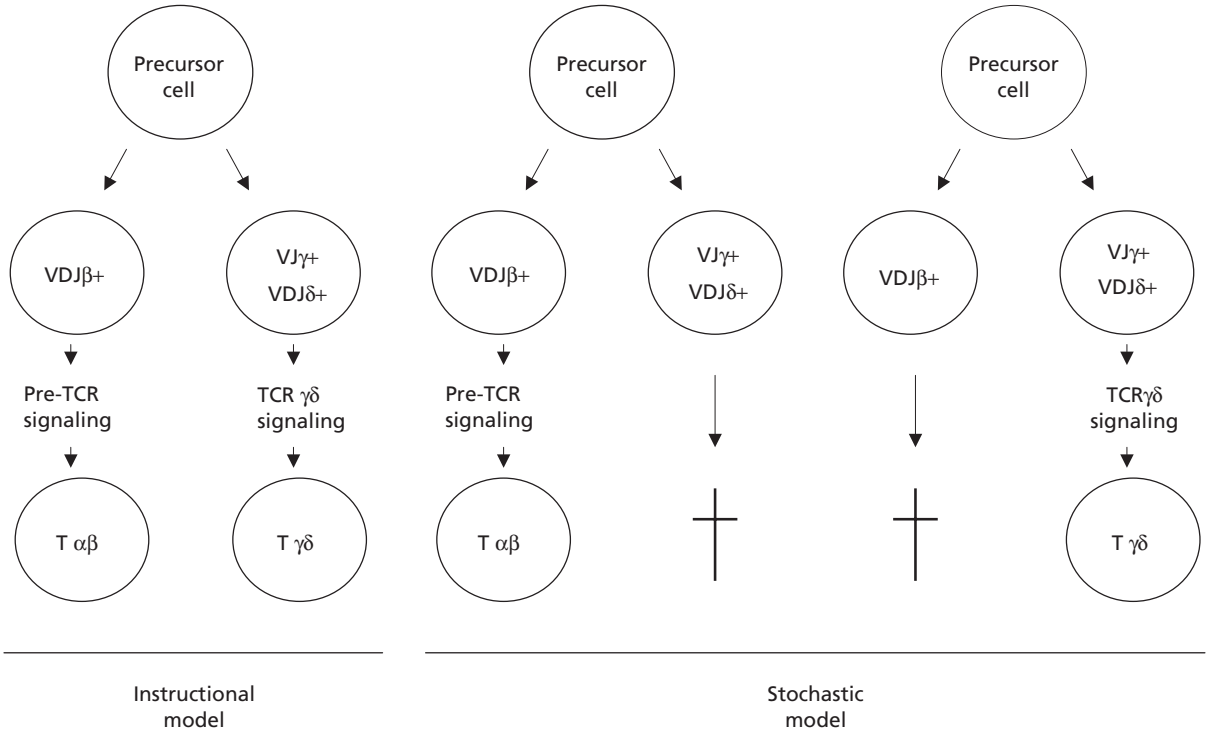

Figure 6 *Models of αβ/γδ lineage separation. See the text for details.*

pTα to form a 'pre-TCR' (Saint Ruf et al. 1994). The pTα is encoded by a non-rearranging gene, the expression of which is transiently switched on during T-cell development. It comprises a single Ig-like ectodomain, a transmembrane region with a positively charged amino acid, and a short intracytoplasmic region. Similar to the TCR, the pre-TCR is associated with a complex of CD3 subunits that mediates signal transduction (see 'TCR structure' below). Signaling through the CD3/pre-TCR complex induces transient but massive thymocyte proliferation, paralleled by rapid loss of CD25 and acquisition of CD4, CD8, CD2, CD5, and CD69 markers (Levelt et al. 1995). The mere expression of pre-TCR/CD3 complexes (i.e. without engagement by a putative thymic ligand) is probably sufficient to induce the transition from the DN to the $CD4^+CD8^+$ stage (Irving et al. 1998). Signaling through the pre-TCR (also called 'β selection') represents a key developmental checkpoint, which permits selective expansion and subsequent maturation of T-cell precursors that have generated a functional TCRβ chain. Consistent with a such a critical role played by the pre-TCR, T-cell development is arrested at the $CD44^-CD25^+$ stage in mice deficient for the RAG, β, pTα, CD3γ, or CD3ϵ proteins (for a recent review, see Sebzda et al. 1999).

EXPRESSION AND INTRATHYMIC SELECTION OF αβ TCR

During the proliferation phase induced by pre-TCR signaling, recombinase activity is drastically reduced as a result of phosphorylation and accelerated degradation of the RAG2 protein (Lin and Desiderio 1994). Re-expression of RAG proteins at this stage seems to be controlled by specific *cis*-acting elements located upstream of the RAG2 promoter (Yu et al. 1999). RAG expression and 'opening' of the α loci allow VJα rearrangements and expression of αβ TCR, which then undergo intrathymic selection. According to the widely accepted affinity/avidity model, the outcome of the $CD4^+CD8^+$ thymocyte will depend on the strength of the avidity signal delivered by its TCR, which integrates both the affinity of the TCR for self-pMHC complexes and the number or density of TCR/pMHC interactions. Below a given avidity threshold, the thymocyte dies by neglect. Above it, the thymocyte is positively selected, i.e. it receives a maturation signal that triggers RAG protein shut-off, TCR upregulation, and modulation of either CD4 or CD8 co-receptors. Finally thymocytes showing very high avidity for self-pMHC are negatively selected, i.e. they receive an apoptotic or inactivating signal leading to their deletion or anergization. Altogether these thymic selection processes, while avoiding production of self-reactive T cells, allow generation of a functional T-cell repertoire with significant affinity for self-MHC (Sebzda et al. 1999).

TCR ALLELIC EXCLUSION

As a result of their diploid nature, T-cell precursors can produce up to four distinct αβ TCRs. T-cell clones

generally express a single β chain, which indicates that, similar to Ig genes, the TCRβ locus exhibits allelic exclusion (for a review, see Malissen et al. 1992). Endogenous VDJβ, but not DJβ, gene rearrangements are inhibited in mice expressing a functional TCRβ transgene, suggesting a tight exclusion mechanism whereby generation of a productive VDJβ rearrangement blocks subsequent V to DJβ rearrangement on the other allele (Uematsu et al. 1988). The increased frequency of precursors carrying two complete VDJβ rearrangements in pTα-deficient mice suggests a key implication of the pre-TCR in the negative feedback loop leading to allelic exclusion (Aifantis et al. 1997). Therefore TCRβ exclusion can be explained by the fact that, in the presence of pTα, any functional β chain, irrespective of its specificity, forms a pre-TCR that systematically generates an allelic exclusion signal. Its precise mechanism is still unclear but probably relies on both a transient reduction of the recombinase activity during the proliferation phase that follows pre-TCR signaling and concomitant inhibition of β locus accessibility to recombinases (Sleckman et al. 1996). Unlike β chains, expression of two distinct α chains is quite frequent within mature T-cell clones, indicating a less stringent rearrangement control in the α loci (Padovan et al. 1993). Accordingly, T cells from mice expressing a functional TCRα transgene frequently express endogenous α genes (Borgulya et al. 1992). TCRα allelic inclusion is explained by the fact that the TCRα rearrangement arrest signal, which leads to recombinase shut off in the developing thymocyte, is only generated after TCR engagement by a positively selecting ligand (Turka et al. 1991). Therefore in a T cell that expresses two distinct αβ TCRs, only one is likely to be functional, i.e. restricted by self-MHC molecules.

αβ/γδ LINEAGE SEPARATION

Targeted inactivation of pTα or β genes in the mouse abrogates development of αβ T cells and drastically inhibits production of double-positive thymocytes. By contrast it has no effect on the generation of γδ T cells, which indicates that γδ T-cell development does not require a pre-TCR (Fehling et al. 1995). Accordingly, unlike β and α genes, activation of γ and δ gene rearrangements occurs concomitantly during development, suggesting that γδ T cells do not undergo a 'preselection' process. αβ and γδ T cells derive from a common progenitor and probably diverge during the transition from the DN III to the DN IV stage (Mertsching et al. 1997). Analyses of the TCR rearrangement status of developing and mature T cells suggest several models of αβ/γδ lineage separation (see Figure 6). According to the 'stochastic' model, precursor cells are precommitted to one or the other lineage before TCR rearrangement and expression. This assumption is supported by studies documenting transcriptional silencing of pTα or α genes in γδ T-cell precursors (Capone et al. 1993; Mertsching et al. 1997) or silencing of some γ genes in αβ T cells (Ishida et al. 1990).

'Instructive' models postulate that the fate of a given precursor is determined by the expressed TCR, i.e. generation of productive γ and δ rearrangements and subsequent expression of γδ TCR engage the precursor toward the γδ lineage, whereas generation of a productive β rearrangement and pre-TCR expression engage the precursor toward the αβ lineage. Among these last models, the 'separate lineage' model proposes that αβ T cells primarily develop from precursor cells that have failed to produce a functional γδ TCR, assuming that VJγ and VDJδ rearrangements precede VDJβ rearrangements during ontogeny (Dudley et al. 1995). According to the 'competitive' model, which seems better supported by recent studies (Livak et al. 1995; Aifantis et al. 1998), VJγ, VDJδ, and VDJβ gene rearrangements take place concomitantly in the same precursor cell, the fate of which is determined by the TCR or pre-TCR that is first produced. Actually, none of these models provides fully satisfactory explanations for all the experimental data obtained so far. It seems likely that the modalities of αβ/γδ lineage separation differ from one subset to another and are possibly determined by a mix of stochastic and instructive events, as is the case for the differentiation of $CD4^+CD8^+$ thymocytes into either $CD4^+CD8^-$ or $CD4^-CD8^+$ cells (see $CD4^+$ T lymphocytes and The role of CD8 T cells in the control of infectious disease and malignancies).

Factors controlling TCR accessibility to recombinases

Expression of transfected RAG proteins in nonlymphoid cells leads to rearrangement of plasmid-like recombination substrates, but not to endogenous TCR or Ig genes (Schatz et al. 1992). Thus, unlike developing lymphocytes, the antigen-receptor genes of nonlymphoid cells are not accessible to the recombination machinery. The fact the Ig and TCR gene rearrangements are restricted respectively to B and T cells suggests differential control of the accessibility of these antigen receptor loci in the corresponding lymphoid precursors (Sleckman et al. 1996). The mechanisms controlling TCR locus accessibility are not completely understood. VDJ recombination closely correlates with changes in chromatin structure, changes in gene methylation status, and transcriptional activation of TCR (or Ig) gene loci (Sleckman et al. 1996). Active transcription of germline V segments coincides with their rearrangement, as clearly illustrated by analysis of the TCRγ locus, where production of germline Vγ transcripts tightly correlates with the ordered rearrangement of these segments during development (Goldman et al. 1993). Along this line analyses of transgenic recombination substrates suggest a key role played by *cis*-acting transcriptional

enhancer elements in regulating, in a tissue-, lineage- and stage-specific fashion, recombination of a given TCR or Ig gene locus, e.g. rearrangements of endogenous α genes occur at a later developmental stage than for endogenous β genes. Accordingly β transgenes carrying an α enhancer rearrange later than the same transgenes with a β enhancer, suggesting a direct role of enhancer elements in dictating the stage specificity of VDJ recombination (Capone et al. 1993). However, recombination and rearrangement are not always strictly correlated. In particular, deletion of the β enhancer inhibits both TCRβ transcription and rearrangements. However, RAG-mediated cleavage of VDJβ elements still occurs but ligation of the coding end is prevented. This suggests that the β enhancer regulates the late stages of the recombination reaction but to a lesser extent the locus accessibility to recombinases (Hempel et al. 1998).

Transcriptional regulation is mediated in part by interactions between *trans*-acting DNA-binding proteins with specific motifs in *cis*-acting promoter and enhancer elements. This process turns out to be particularly complex and involves the combinatorial action of several ubiquitous transcriptional factors (Ernst and Smale 1995). As a result of this complexity, it has not been possible yet to identify specific combinations of transcriptional factors able to regulate, in a lineage- or stage-specific fashion, TCR or Ig gene rearrangements, e.g. mutations in the binding sites for *c-myb* and CBF/PEBP2 transcription factors within the δ enhancer abrogate recombination of a TCRδ minilocus. However, replacement of the δ enhancer by a DNA fragment carrying both *c-myb* and CBF/PEBP2 sites does not restore recombination, indicating the need for additional factors for proper regulation of δ rearrangements (Lauzurica et al. 1997).

Besides transcriptional activation, occurrence of VDJ rearrangements is tightly associated with remodeling of chromatin structure, possibly under the direct control of TCR enhancers. In particular histone hyperacetylation and associated chromatin decondensation strongly correlate with activation and/or completion of VDJ rearrangements (McMurry and Krangel 2000). Gene methylation, which is associated with transcriptional silencing, also prevents TCR rearrangements (Hsieh and Lieber 1992). TCR locus demethylation, which is concomitant to VDJ recombination, seems again directly regulated by the enhancers (Kirillov et al. 1996).

Finally *cis*-acting elements blocking TCR accessibility have been identified in the TCRα and γ loci (Ishida et al. 1990). These 'silencers' may selectively repress, in either a stochastic or instructive manner recombination of a given locus in precursor cells committed to the reciprocal cell lineage (see 'Regulation of TCR gene assembly during T-cell development' above).

TCR STRUCTURE

Overall structure of the T-cell recognition complex

TCR CHAINS

αβ and γδ TCRs are expressed in a mutually exclusive fashion on T cells. In adult thymus, blood, spleen, and lymph nodes, the vast majority of T cells express αβ TCRs in rodents and primates. By contrast γδ T cells are greatly enriched in epithelial sites where they sometimes make up all $CD3^+$ T cells (see Unconventional T cells). The apparent molecular mass (M_r) of α and β chains, which are both glycosylated, is around 42–48 kDs (Clevers et al. 1988). The M_r of γ and δ chains ranges from 30 to 60 kDa and from 40 to 60 kDa, respectively. The isoelectric point of γ and β chains is basic and that of α and δ chains acidic. Each TCR chain possesses a short cytoplasmic tail, a hydrophobic transmembrane region and a large extracellular portion comprising two Ig-like domains, a variable and a constant domain which are respectively distal and proximal to the membrane (Figure 7).

As with Ig, the variable domain is encoded by two to three gene segments that form a single exon after somatic rearrangement (Figure 8). The amino-terminal part of the variable domain is encoded by the V-gene segment whereas its carboxy-terminal part is encoded by either a single J segment (for α and γ) or D and J segments (for β and δ chains). The length of the V region is around 75–95 amino acids, the J region 15 amino acids, and the D region 5 amino acids. The constant domain is encoded by a C gene comprising three to four exons. The first exon codes for the globular domain stabilized by an intrachain disulfide bond, which is slightly longer (125–135 amino acids for β) or shorter (85 amino acids for α) than the Ig domain (around 110 amino acids). The second exon codes for a 'connecting' region of six amino acids, which frequently comprises a cysteine involved in the covalent binding of the two TCR chains. This cysteine is absent in the duplicated or triplicated exon II of the human Cγ2 gene, but the corresponding γ chain can still form functional TCR by pairing noncovalently with the TCRδ chain (Lefranc and Rabbitts 1989). The transmembrane region of each TCR subunit (21–22 amino acids long) is encoded by a third exon. Although strongly hydrophobic, this region comprises one or two positively charged residues that establish electrostatic interactions with acidic residues located in the transmembrane region of several subunits of the CD3 signal transduction complex. Mutation of these residues abrogates CD3/TCR assembly (Hall et al. 1991). The short TCR chain intracytoplasmic region (4–12 amino acids) is

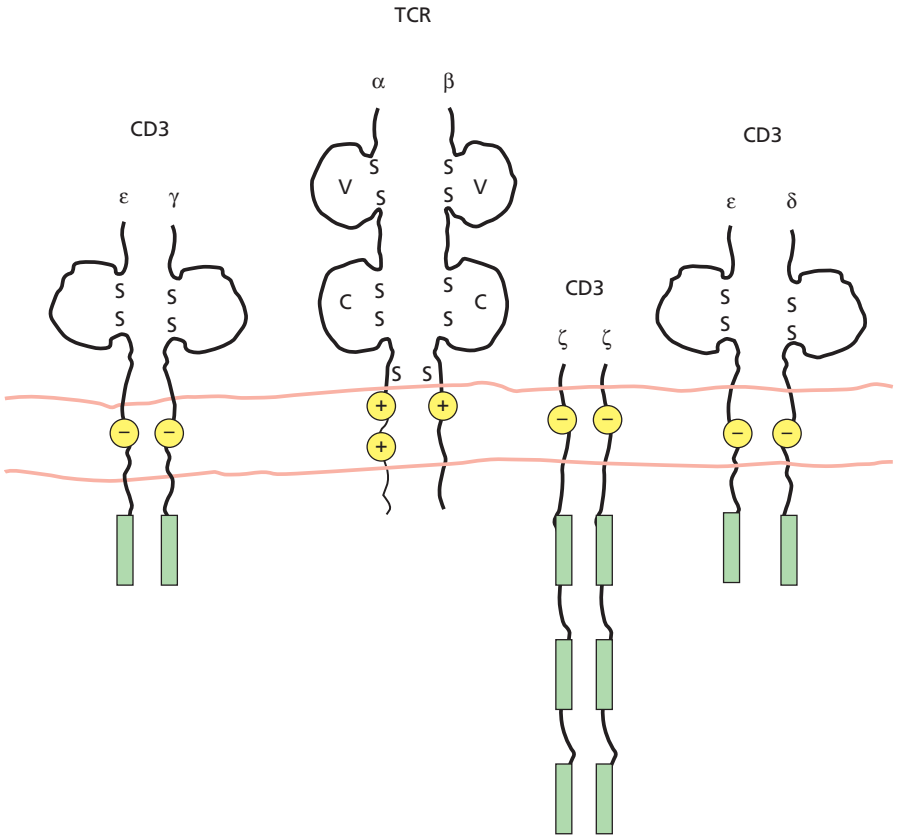

Figure 7 *Overall structure of the T-cell receptor (TCR)/CD3 complex. See the text for details. − and + symbols correspond to acidic and basic residues located within the transmembrane region of TCR and CD3 chains. ITAMs located on the intracellular portion of CD3 subunits are symbolized by rectangles. S-S, disulfide bridges.*

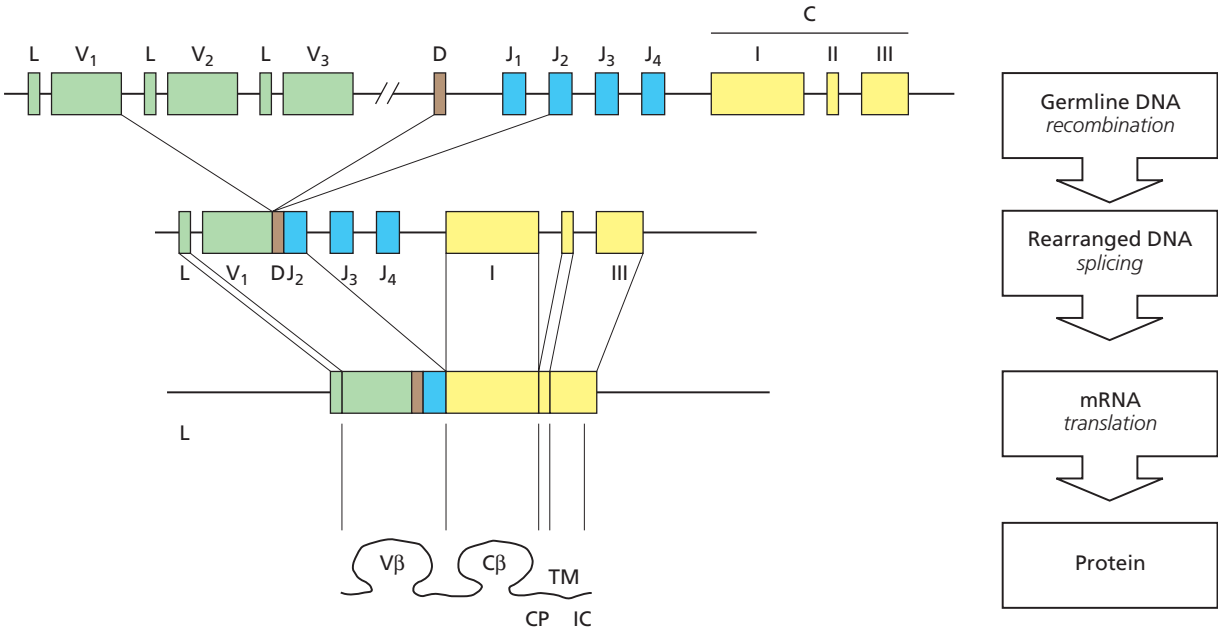

Figure 8 *Biosynthesis of a T-cell receptor (TCR)β chain. See the text for details. I, II, III, exons of the Cb gene; CP, connecting peptide; IC, intracytoplasmic region; TM, transmembrane region.*

encoded by either exon III (for Cβ genes) or a fourth exon (for Cα genes) (Figure 8).

THE CD3 TRANSDUCTION COMPLEX

None of the TCR subunits possesses a cytoplasmic tail long enough to permit signal transduction after antigen recognition. This function is mediated by a multimeric complex called CD3, which is closely associated with the αβ and γδ TCR and required for TCR surface expression (Sussman et al. 1988; Hall et al. 1991) (see Figure 7). In this way, the TCR complex appears very similar to the BCR complex, which consists of an antigen recognition unit (the membrane-bound immunoglobulin) associated with a transduction complex composed of Igα and Igβ subunits (see Antibodies and B lymphocytes). Five distinct CD3 subunits, called γ, δ, ϵ, ζ, and ν, have been biochemically identified within the CD3 complex (Borst et al. 1983). In most instances, the CD3 complex is composed of four distinct subunits (γ, δ, ϵ, and ζ) that are organized in three dimeric transduction modules: γϵ, δϵ, and ζζ (Alarcon et al. 1991). CD3γ, -δ, and -ϵ are encoded by highly homologous genes located within a 50-kb region on chromosome 11 in human and 9 in the mouse (Letourneur et al. 1989). The CD3ζ gene is located on chromosome 1 and can undergo an alternate splicing in the mouse, which leads to generation of either a CD3ζ or a CD3ν protein (Jin et al. 1990). Although the CD3 γ, δ, and ϵ chains possess a single Ig-like ectodomain of 80 amino acids, the CD3-ζ and ν chains have a very short (nine amino acids) extracellular region. All CD3 subunits contain a negatively charged acidic residue (Asp) in their transmembrane region, which ensures proper assembly of the CD3/TCR complex (see above). The intracytoplasmic tail of all CD3 subunits contain one (for CD3γ, -δ, or -ϵ) to three (for CD3ζ) consensus motifs called immuno-tyrosine-based activation motif (ITAM) (Reth 1989). These motifs, also found in a variety of other immune receptors (such as Igα/β and some Fc receptors), play a key role in signal transduction by interacting, once phosphorylated, with specific tyrosine kinases required for the initiation of the signaling cascade (like ZAP-70 in T cells) (see also The B cell antigen receptor). The composition and stoichiometry of CD3 complexes may differ from one T cell to another. In the mouse, about 5–10 percent of $CD3^+$ lymphocytes express ζν heterodimers instead of ζζ homodimers (Baniyash et al. 1988). Although ν is not found on human T cells, some $CD3^+$ T cells in both species may utilize the FcϵRIγ subunit instead of the ζ chain (Koyasu et al. 1992). The qualitative/quantitative differences of the signals initiated by CD3 complexes containing ζζ, ζν, or FcϵRIγ dimers are still unclear. Finally the composition of CD3 complexes associated with αβ and γδ TCR show differences: unlike αβ TCR complexes, γδ TCR complexes lack CD3δ and rapidly recruit FcϵRIγ on T-cell activation (Hayes and Love 2002).

THE CD4 AND CD8 CO-RECEPTORS

Interactions between the αβ TCR and pMHC complexes are classically strengthened by the CD4 or CD8 co-receptors, which bind to the conserved part of MHC class II and class I molecules respectively. As detailed in CD4+ T lymphocytes and 22, The role of CD8 T cells in the control of infectious disease and malignancies, these co-receptors also play a key role in the initiation of signal transduction by recruiting $p56^{lck}$, which then mediates early phosphorylation of ITAM on the CD3 subunits. As the CD4 and CD8 co-receptors modulate the avidity of TCR/pMHC interactions, they play a critical function during the intrathymic selection of the TCR repertoire and the terminal maturation of CD4/CD8 double-positive thymocytes into CD4 or CD8 single-positive T cells (Ellmeier et al. 1999).

TCR crystallographic analyses

αβ TCRS

Although Ig can interact with high affinity with a wide variety of native antigen, αβ TCRs must recognize subtle differences between MHC molecules loaded with distinct peptidic antigen. This task is facilitated by the concentration of the TCR structural diversity at the center of the TCR/pMHC interface. As with Ig, diversity of rearranged TCR genes is not evenly distributed within the variable region but is instead clustered in so-called hypervariable (HV) regions. There are two HV regions within the V gene segment (HV1, HV2) and a third one corresponding to the V(D)J junction. These three regions code, respectively, for the complementarity determining regions (CDR)-1, -2 and -3 that form the antigen-binding site (see below). A fourth hypervariable region (HV) 4 has been identified in the V region, which forms an additional loop involved in superantigen binding (see 'Structural basis of T-cell antigen recognition' below and see Superantigens). Consistent with earlier predictions, the overall TCR structure is very similar to that of an Ig Fab fragment (Figure 9) (for a recent review, see Garcia et al. 1999).

The C and V domains of both TCR chains adopt a classic 'Ig fold' composed of a sandwich of two β sheets with five inner and four outer β strands for the V domains, and four inner and three outer strands for the carboxyl domains (see also Antibodies and B lymphocytes, and The B cell antigen receptor). As for Ig, the CDR loops protrude on top of the V domains to form the antigen-binding site. TCRs also show several distinctive structural features, listed below. The Cβ domain carries a large protruding loop

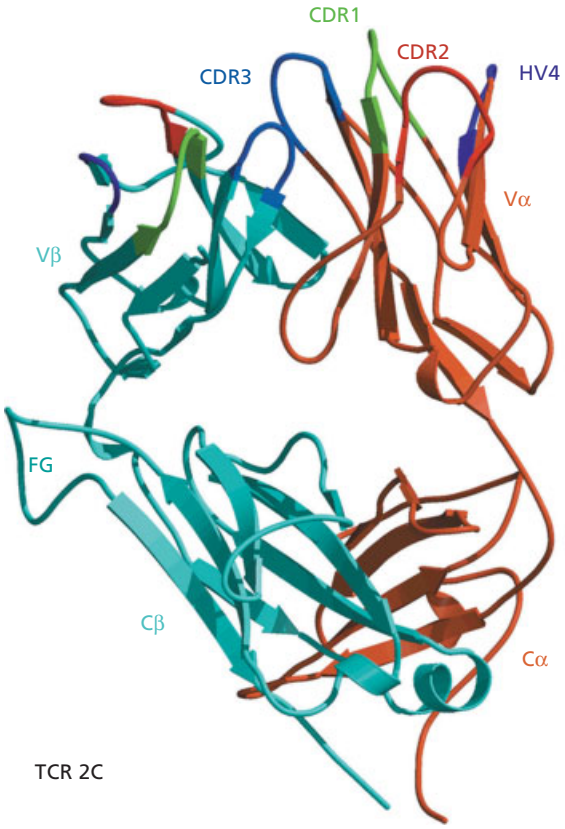

Figure 9 *Three-dimensional structure of an αβ T-cell receptor (TCR). See the text for details. Structure of a murine TCR. (Kindly provided by J.B. Reiser and D. Housset, Grenoble, France.)*

between the F and G strands, the significance of which remains unclear. It may facilitate interactions with either some CD3 subunits (Ghendler et al. 1996) or co-receptors. The elbow angle between Vβ and Cβ is fairly constant (between 140° and 150°) contrasting with the much higher flexibility of Ig, with elbow angles ranging from 120° to 225°. Furthermore, the Cα domain shows some structural differences with canonical Ig folds and in particular lacks a C′ and C″ strand. This unusual feature is anticipated from the primary Cα sequence because only 50 amino acids separate the two cysteines, forming the intradomain disulfide bridge in the Cα domain, compared with around 65 amino acids for the Cβ or Ig constant domains. The antigen-binding site of αβ TCR is made of six hypervariable (CDR) loops, three from each Vα and Vβ domain. CDR1 and CDR2 are located in the periphery whereas the two CDR3 are located centrally. While the CDR1 and CDR2 from the Vα and Vβ domains seem to adopt a limited set of canonical conformations, the CDR3α and CDR3β show substantially different conformations and undergo extensive conformational changes upon pMHC bin ing (see below). In this regard, the lack of canonical CDR3 structures is fully consistent with the extensive sequence diversity of the VJα and VDJβ joints (see 'Genomic organization and source of TCR diversity' above).

γδ TCRS

The sole γδ TCR structure resolved so far (Allison et al. 2001) has a highly distinctive overall shape, when compared with αβ TCR or Fab fragments. This unusual shape is produced by a small elbow angle and a small VγCγ interdomain angle, which are both the smallest ever seen within Fabs and TCR structures. Both the Cδ and Cγ domains adopt a canonical Ig fold but the Cγ domain, which is more homologous to Cβ than Cα, does not have the long FG loop found in the Cβ domain. The antigen-binding surface of the γδ TCR appears much rougher than that of αβ TCR. This peculiar feature seems to result mainly from the unusual positioning of the CDR2δ (also seen in crystals of another Vδ domain (Li et al. 1998)), and to large protrusions formed by the CDR3γ and CDR3δ loops. Together with parts of the CDR1 and CDR2 loops, the CDR3 loops form a deep cleft that may constitute the antigen-binding site. Consistent with an Ig-like mode of antigen recognition by γδ TCRs (Chien et al. 1996; see Unconventional T cells), the jagged antigen-binding surface of the γδ TCR resembles that of an antibody specific to small proteins. However, general predictions about the topology of γδ TCR/antigen interactions, based on analysis of a single unbound γδ TCR, should be taken with caution, particularly in light of the dramatic conformational modifications occurring on the CDR3 loops of several αβ TCR on binding to their pMHC complexes (Reiser et al. 2002).

STRUCTURAL BASIS OF T-CELL ANTIGEN RECOGNITION

Crystallographic analysis of antibody TCR bound to pMHC complexes

As the vast majority of amino acid variations lie in the V(D)J junctional regions, which are equivalent to the centrally located CDR3 within the Ig antigen-binding site, it has been proposed that the CDR3β loops from the Vα and Vβ domains line up above the antigenic peptide, whereas the less diverse CDR1 and CDR2 interact with the MHC molecule (Davis and Bjorkman 1988). As suggested by TCR and MHC mutagenesis studies (Jorgensen et al. 1992; Sant'Angelo et al. 1996) and more recently confirmed by crystallographic analyses of several TCR/pMHC structures (Garcia et al. 1999), some but not all of these assumptions proved to be correct.

Determination of the crystal structure of TCR/pMHC complexes has represented a particularly challenging task, not only because of the difficulties in producing and purifying large quantities of TCR and pMHC and generating stable trimolecular complexes, but also because of the crystallographic problems associated with analysis of such large macromolecular complexes. This explains why only a handful of high-resolution TCR/

pMHC complex structures have been reported so far. Despite this, several general principles for recognition of pMHC by TCR can already be drawn from the few structures determined to date (Figure 10).

OVERALL TOPOLOGY

The TCR adopts a diagonal orientation over the pMHC complex in all the TCR/pMHC class I and class II structures resolved so far (Garcia et al. 1996, 1999) (Figure 10a, b). This particular topology probably maximizes the cooptation between the MHC and TCR surfaces, because it allows the relatively flat TCR binding surface to lie between the two opposite high points of the MHC α helices (Garboczi et al. 1996). In all instances, the Vα domain is located over the amino-terminal half of the peptide and the Vβ domain over the carboxy-terminal half. Here again this orientation is likely to be conserved, in order to ensure proper positioning of the CD4 and CD8 co-receptors within the TCR/pMHC complex.

CDR FOOTPRINTS ON THE PMHC

Both CDR3 loops lie centrally and establish contacts almost exclusively with the antigenic peptide (Figure 10a, b). The CDR1α and β interact with both MHC and peptide residues whereas the CDR2 contacts almost exclusively the MHC α helices. Early crystallographic studies suggested a dominant contribution of the Vα (over the Vβ) CDRs to TCR/pMHC interaction, and frequent involvement of conserved residues at the tip of CDR1α and CDR2α loops in TCR/MHC contacts at conserved MHC areas, thus supporting a key role for

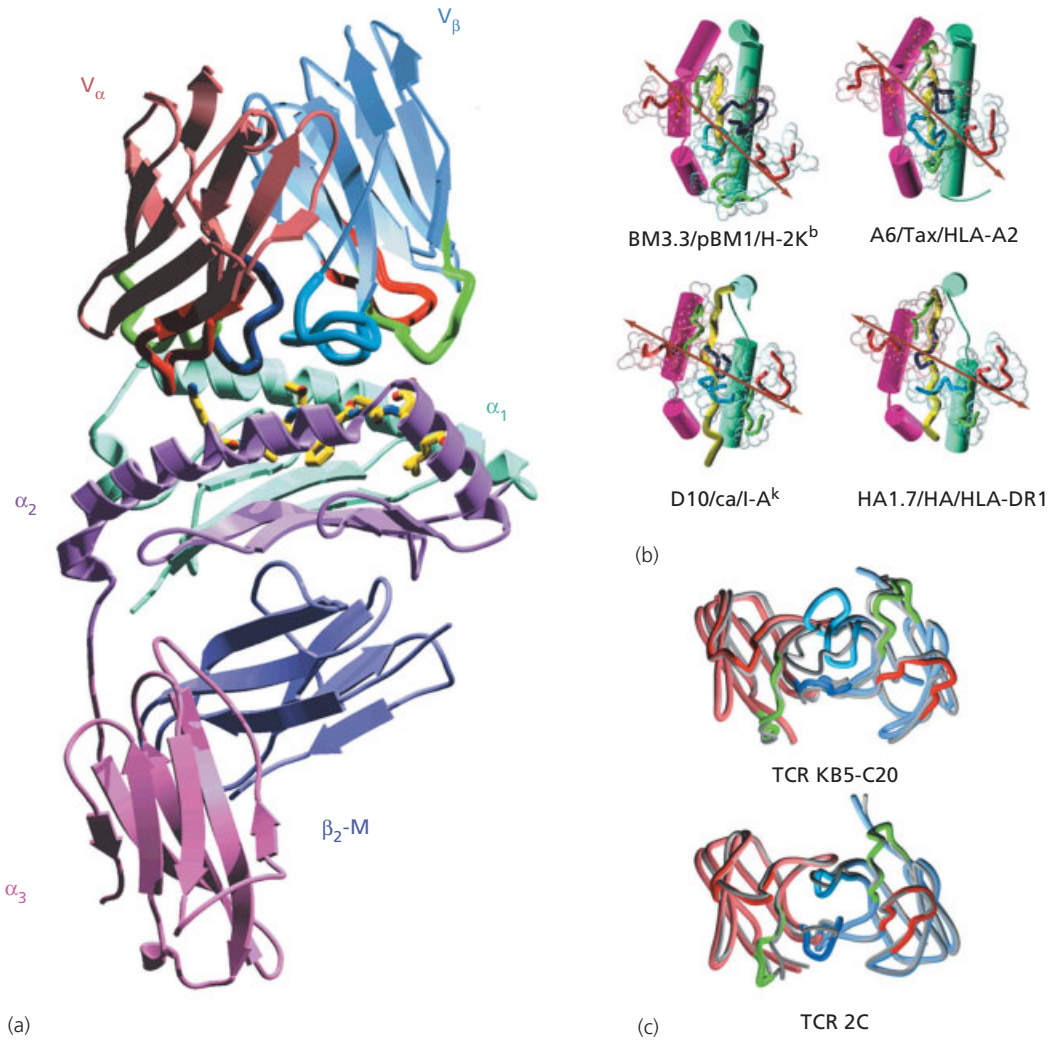

Figure 10 *Structure and overall topology of the T-cell receptor (TCR)/pMHC complex. See the text for details.* **(a)** *Overall structure of a TCR/pMHC complex (CDR1 in green, CDR2 in red, CDR3 in blue, peptide in yellow).* **(b)** *Diagonal orientation of TCR over pMHC. Upper left: murine TCR/pMHC class I, upper right: human TCR/pMHC class I, lower left: murine TCR/pMHC class II, lower right: human TCR/pMHC class II (for CDRs and peptide, same color code as in* **(a)**). **(c)** *Comparison of the CDR conformation of TCR either unbound (gray) or bound to pMHC (same color code as in* **(a)** *and* **(b)**). *Note the significant conformational modifications of the CDR3. (Kindly provided by J.B. Reiser and D. Housset, Grenoble, France.)*

the Vα domain in driving the TCR orientation over the pMHC complex (Garcia et al. 1998). This hypothesis has been more recently challenged by the balanced contribution of Vα and Vβ domains to pMHC interaction observed in additional structures, and the significant tilting of the TCR over the 'MHC saddle' from one complex to another (Reiser et al. 2000, 2002). Importantly these studies have failed to identify a truly conserved TCR footprint on the MHC shared by all TCR/pMHC class I complexes. Thus, there is so far no straightforward structural explanation for the inherent MHC reactivity of the TCR documented by biological studies (Zerrahn et al. 1997).

MHC VS PEPTIDE INTERFACE

Clearly the dominance of TCR contacts with the MHC helices should provide enough binding energy between the TCR and MHC heavy chain to allow proper TCR docking on the complex and subsequent scanning of its peptide content (see below). Furthermore comparison of bound versus unbound TCR indicates large conformational changes occurring mainly on the CDR3 loops, which may compensate for the relatively poor shape complementarity between the peptide and the TCR loops (Garcia et al. 1998; Reiser et al. 2002) (Figure 10c). Hence, this suggests an important plasticity of the T-cell recognition process, and provides a likely molecular explanation for the ability of a given T cell to recognize, in a degenerate fashion, large sets of peptides during its intrathymic positive selection or its peripheral activation.

ALLOREACTIVITY

A large fraction of T cells (up to several percent) cross-reacts to allogeneic MHC molecules. The molecular basis of this phenomenon, called alloreactivity, has been deciphered during the last decade and turns out to cover distinct types of interactions. In most cases, recognition of allogeneic cells appears mildly affected by the peptidic content of the recognized MHC molecules, suggesting highly degenerate low-affinity interactions between TCR and a large fraction of allogeneic pMHC complexes. Some alloreactive TCRs may also recognize, with much higher affinity and in a more peptide-dependent fashion, allogeneic MHC alleles related to self-MHC (Obst et al. 2000). Comparison of TCR bound to a positively selecting pMHC complex or an allogeneic pMHC suggests that some interactions with MHC in the former case can be 'mimicked' by interactions occurring with the peptide in the latter case (Garcia et al. 1998; Reiser et al. 2000). As a result of the heterogeneity of alloreactive responses documented by biological studies (see above), other molecular explanations (such as weak cooptation between the TCR and pMHC surfaces associated with increased peptide degeneracy) seem highly likely although they need confirmation through resolution of additional structures.

Structure of αβ TCR/superantigen complexes

Antigens are not always recognized in a form of MHC-bound peptide because some of them may directly bind to the TCR and MHC class II molecules in a native form. This particular class of TCR ligands, which is produced by a variety of microbial and viral pathogens, are called superantigens (SAg) because they can activate a substantial fraction of naive T cells. As briefly mentioned below and detailed in Superantigens, the TCR-binding modes of antigen and SAg are drastically different because MHC class II-bound SAgs can engage all TCRs sharing a particular Vβ (or Vγ) region, irrespective of their Vα (or Vδ) regions and their VDJβ (or VJγ) junctional sequences. This explains why a given SAg can activate up to several percent of the total T-cell repertoire in naïve individuals. Crystallographic analysis of TCR/SAg and SAg/MHC class II complexes has clearly confirmed the peculiar topology of these interactions (Li et al. 1999). Although different SAgs bind to class II MHC in very different ways, their mode of interaction with the TCR appears quite conserved: in most instances, SAgs establish contacts with the HV4 as well as the CDR1 and CDR2 loops of the Vβ chain. This is in stark contrast with TCR/pMHC complexes, for which pMHC contacts involve residues located on all six CDRs but not HV4 (see above). Importantly the orientation of the TCR over the MHC molecule within the TCR/SAg/MHC complex is very different from that in TCR/pMHC complexes, and does not permit proper interaction with the bound peptide, which is consistent with previous observations demonstrating TCR/SAg/MHC interactions irrespective of the bound peptide (Li et al. 1999).

A DYNAMIC VIEW OF THE T-CELL ANTIGEN RECOGNITION PROCESS

Analysis of TCR/pMHC interactions

AGONISM VS ANTAGONISM

TCR engagement by pMHC complexes does not always lead to full T-cell activation. In particular alteration of the antigenic peptide sequence may affect T-cell functions in very different ways. Although some peptide mutants referred to as full agonists retain the ability to activate all T-cell effector functions, others referred to as weak or partial agonists may induce decreased T-cell responses or some but not all effector functions (Evavold and Allen 1991). In extreme cases, T-cell response to agonist peptides may be inhibited after

exposure to some altered peptide ligands referred to as antagonists (for a review, see Sloan-Lancaster and Allen 1996). The physiological relevance of these nonproductive or suboptimal TCR/pMHC interactions is still unclear. They may affect T-cell responses against infections and/or may contribute to the selection of the T-cell repertoire as well as peripheral T-cell homeostasis. From a mechanistic point of view, antagonist peptides do not seem to compete with agonist peptides for binding to MHC molecules. Instead antagonists may engage either sterile or abortive TCR interactions (Sloan-Lancaster et al. 1994; Madrenas et al. 1995), thus decreasing the number of productive TCR/agonist interactions and/or triggering an inhibitory signal that dominates the activating signal induced by the agonist. Some biophysical studies, detailed below, have brought important insights into this phenomenon although we do not have yet a clear picture of what precisely is going on.

αβ TCR/PMHC-BINDING STUDIES

Analyses of TCR/pMHC interactions have lagged far behind those of antigen–antibody interactions because of two major technical obstacles. First, the membrane-bound nature of the TCR and MHC molecules, and the relative instability of TCR heterodimers when expressed under soluble forms, have greatly hampered their large-scale production and purification. A second problem lies in the affinity of pMHC/TCR interactions, which have turned out to be in the lower micromolar range, and measurable only using recently available surface plasmon resonance technologies. Indeed the affinity of TCR/pMHC interactions (K_d values in the order of 10^{-4}–10^{-7}) is on average lower than that of antibody–antigen interactions (K_d values of 10^{-6}–10^{-10}). Although the on-rates of TCR/pMHC interactions are quite heterogeneous (from 10^3 to 2.10^5 mol/l per s), their off-rates are very fast and remain in a relatively narrow range (from 0.2 to 0.005/s at 25°C). These fast off-rates have led to the proposal that T-cell activation by a small number of pMHC complexes would be made possible by the transient engagement of many different TCRs within a short time lag (Valitutti et al. 1995). However, the correlation between the strength of the T-cell response and the off-rates of TCR/pMHC interactions is still being debated. In their 'serial TCR engagement' model, Lanzavecchia and colleagues propose that lower TCR/pMHC off-rates should lead to reduced T-cell activation as a result of decreased numbers of serially engaged TCR within a given time frame (Viola and Lanzavecchia 1996). However, this hypothesis is difficult to reconcile with the inverse correlation observed by other groups between TCR/pMHC dissociation rates and T-cell responsiveness (for a recent review, see Davis et al. 1998). Indeed the TCR-binding affinity for strong pMHC agonists appears to be generally much higher than for antagonists. Furthermore, binding of TCRs to weak agonists and antagonists may show very similar affinities but different dissociation rates, which can be up to 10-fold higher in the latter than in the former case (Matsui et al. 1994; Lyons et al. 1996). Molecular explanations for this phenomenon are still pending but recent studies suggest that antagonist peptides prevent efficient TCR clustering and subsequent maturation of the immunological synapse (see above).

Although crystallographic studies allow identification of all the residues involved in a given interaction, many of these interactions can be neutral (i.e. mutation of the corresponding residues may have no impact on the binding affinity). Systematic mutagenesis analyses performed on either the TCR or the pMHC complex have allowed mapping of energetic 'hotspots' involving only a small fraction of interacting residues identified by X-ray analyses (Baker et al. 2001). Interestingly, the nature of TCR/MHC and TCR/peptide interactions may fundamentally differ, as suggested by another recent mutagenesis study (Wu et al. 2002). Mutations introduced within the antigenic peptide have a dramatic effect on the stability of the TCR/pMHC complex. This decreased affinity, when measurable, seems mainly accounted for by a marked increase of the dissociation constant, with only a minor effect on the association constant. By contrast, mutations on the MHC molecule have significantly less impact on the stability of pMHC/TCR interaction and, in most instances, this effect results from a decrease of the association constant. These results suggest a two-step mechanism for T-cell antigenic recognition. In a first step, interactions between TCR residues on the CDR1 and CDR2 loops and MHC residues would permit docking of the TCR on the MHC molecule, and scanning of its peptide content. In a second step pMHC/TCR binding would be stabilized on forming a complex via an induced conformational fit of the CDR3 loops, already documented in several crystallographic studies (Garcia et al. 1998; Reiser et al. 2002). This two-step process is also consistent with the entropic changes of TCR/pMHC interactions reported in several thermodynamic analyses (Willcox et al. 1999; Boniface et al. 1999).

γδ TCR/ANTIGEN-BINDING STUDIES

Unlike αβ T cells, γδ T cells do not generally recognize peptide antigens bound to classic MHC class I and class II molecules. As detailed in Lymphocyte mediated cytotoxicity, γδ TCRs seem to interact with native antigen in an Ig-like fashion. This assumption is consistent with the extensive structural heterogeneity of the TCRγδ ligands identified so far, which include native MHC or MHC-like proteins, viral glycoproteins, Ig, heat shock protein (HSP), and small phosphorylated esters commonly produced by microorganisms (Chien et al. 1996). The sole biophysical binding study performed to

date with a γδ TCR directed against an 'empty' MHC class I-like molecule has revealed several unusual features. The affinity of this interaction appears much higher than that of αβ TCR/pMHC interactions, resulting mainly from a very low dissociation rate (Crowley et al. 2000). As, in this particular case, the MHC class I-like molecules recognized by the γδ TCR are expressed at high densities on target cells, γδ T-cell activation is probably induced after stable engagement of a large number of TCRs by their ligands. These high avidity/high affinity interactions might be common within nonconventional T cells, as suggested by another recent binding study performed with an αβ TCR specific for CD1d/glycolipid complexes (Sidobre et al. 2002).

The immunological synapse

Although the half-lives of TCR interactions are of the order of seconds, the duration of signaling required for T-cell activation is of the order of hours, thus suggesting specific mechanisms permitting sustained TCR interactions with their specific pMHC complex. This is achieved through the formation of supramolecular activation clusters (SMAC), which consist in a highly organized clustering of TCR, co-receptors, co-stimulatory, and adhesion receptors as well as signaling molecules within lipid rafts enriched for glycolipids and glycosphingolipids (Monks et al. 1998; Grakoui et al. 1999) (Figure 11).
These SMACs of immunological receptors, which form within minutes after contact between the T cell and the antigen-presenting cell (APC), are classically referred to as the immunological synapse. Although a detailed description of the formation and function of the immunological synapse is clearly beyond the scope of the present chapter, several general features of the immunological synapse are outlined here.

T-cell activation and immunological synapse formation can be grossly split into four distinct temporal stages corresponding to: (1) the initial T-cell polarization events taking place shortly after APC encounter; (2) the segregation of adhesion molecules within early supramolecular clusters leading to enhanced T-cell adhesion to APC; (3) early TCR-signaling leading to formation of the immature immunological synapse; and finally (4) immunological synapse maturation associated with sustained TCR signaling (Bromley et al. 2001).

T-CELL POLARIZATION

Initial adhesion of the T cell to the APC is accompanied by rapid membrane reorganization and T-cell polarization, presumably in response to chemokines (Sanchez-Madrid and del Pozo 1999). Subsequent signaling through heterotrimeric G protein then triggers actin polymerization and integrin activation. The engaged integrins can in turn promote the formation of membrane protrusions tipped by TCR-enriched membranes, which are probably involved in the initial scanning of pMHC complexes and set the initial stages of immunological synapse formation (Dustin and Cooper 2000).

T-CELL ADHESION TO APCS

Effective adhesion between the T cell and the APC requires rapid segregation of adhesion and recognition receptors within these early supramolecular arrangements. Although surface molecules with large extracellular domains (such as CD43 and CD45) are excluded, smaller adhesion receptors such as CD2 and LFA1 are clustered in the center of the structure and form tight interactions with their counterreceptors (CD58 and intercellular adhesion molecule 1 (ICAM-1), respectively) (Sperling et al. 1998; Grakoui et al. 1999; Leupin et al. 2000).

IMMUNOLOGICAL SYNAPSE FORMATION AND MATURATION

In its initial stage, the immunological synapse is made up of an adhesion molecule cluster surrounded by a ring of engaged TCRs. Along immunological synapse maturation, the structure is inverted: the engaged TCR/pMHC complexes progressively move toward the center of the SMAC (cSMAC) whereas the adhesion molecules move to its periphery (pSMAC) (Grakoui et al. 1999). Although it seems clear that the earliest TCR signaling events induce the cytoskeletal changes required for immunological synapse maturation, the parameters (quantitative or qualitative) controlling sustained signaling within the mature immunological synapse are still poorly defined. Stable formation of the immunological synapse seems to be associated with the recruitment of specific kinases required for full T-cell activation (such as protein kinase C) (Monks et al. 1998). Furthermore CD28, which segregates within the cSMAC in the mature immunological synapse, probably plays an important role in the potentiation of TCR signaling because its co-engagement with the TCR has been shown to sustain TCR-induced tyrosine phosphorylations (Viola et al. 1999). As already mentioned, nonproductive TCR/pMHC interactions (e.g. with high off-rates as observed with some antagonist peptides) may dramatically reduce the density of TCR/pMHC complexes within the mature immunological synapse and thus affect sustained signaling.

CONCLUDING REMARKS

Recent studies are shedding light on several common principles that underlie T-cell antigen recognition and its translation into particular biological outcomes. Recognition of pMHC by TCRs turns out to be a complex multistep process, which involves induction of conformational changes of TCRs on binding and rapid recruitment of adhesion and co-stimulatory receptors within highly

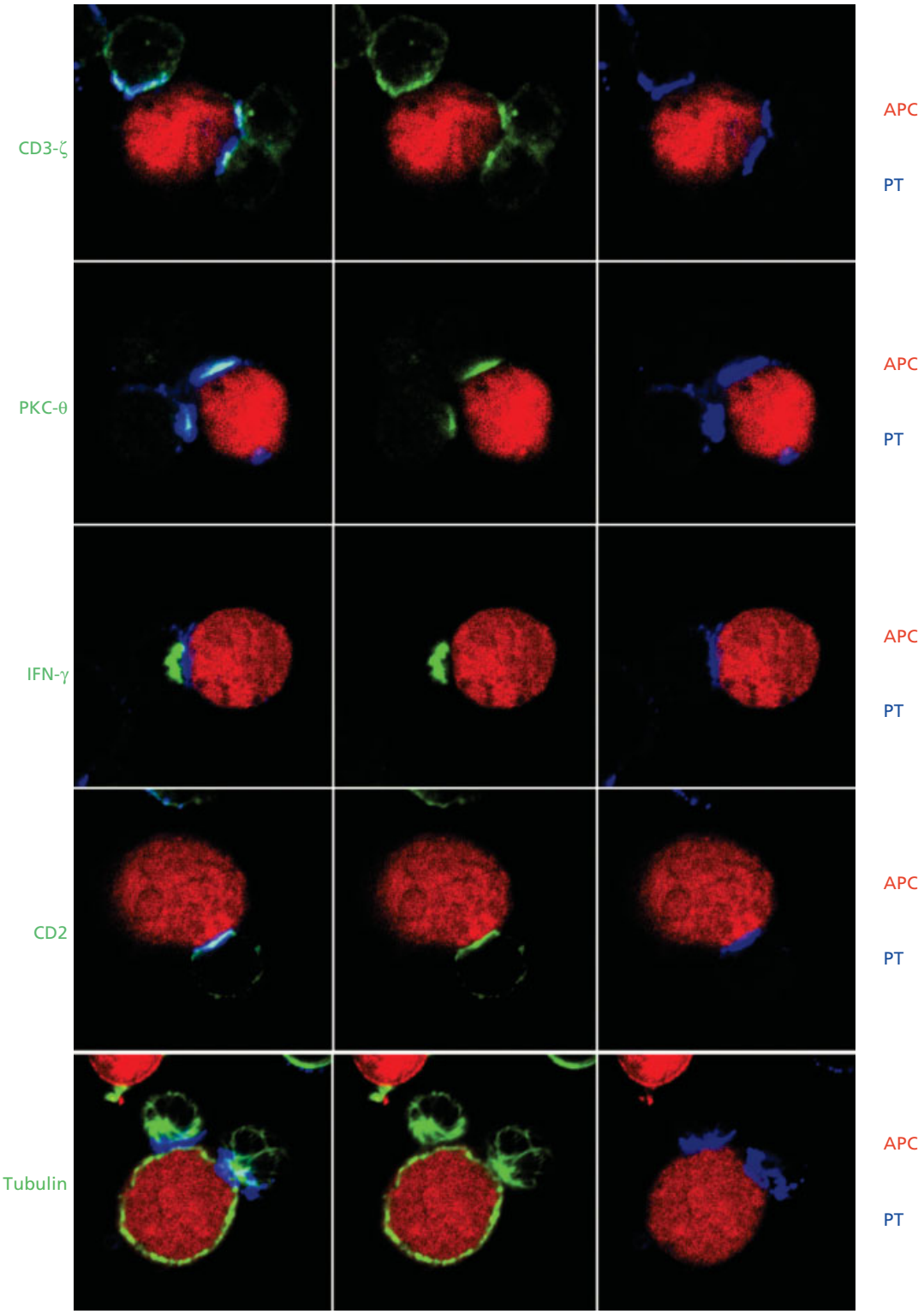

Figure 11 *Clustering of surface receptors and signaling molecules within the immunological synapse. See the text for details. APC, antigen-presenting cell (in red). PT, phosphotyrosine (in blue). Note the co-clustering (light blue) of several surface receptors (CD3-ζ, CD2) and phosphorylated tyrosine kinases (protein kinase CΘ) at the contact point between the T cell and the APC, and the polarized secretion of interferon IFN-γ. (Kindly provided by S. Mueller and S. Valitutti, Toulouse, France.)*

organized membrane receptor clusters. Naturally, there are still several challenging issues that should be addressed by future work. Structural explanations for the inherent MHC reactivity of TCR are still lacking and will certainly need resolution of additional TCR/pMHC structures. Biophysical and thermodynamic

analyses of TCR/pMHC interactions should help clarify the mechanisms underlying the biological effects of altered peptide ligands. Finally a precise understanding of how the T cell integrates the activating and inhibitory signals that are concomitantly generated during encounter of APCs will be another major challenge, which will probably require the combined use of sophisticated molecular, biochemical, and morphological approaches.

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Lymphocyte-mediated cytotoxicity

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Cytotoxic lymphocytes and their killing machinery, the cytolytic granule, are a critical component of the immune response. They are required for the removal of intracellular pathogens and for immune surveillance against tumors. This function necessitates the use of a fail-safe system, embodied by perforin and granzymes that act cooperatively in the clearance of even non-cooperative cells, such as virus-infected and transformed tumor cells. Although cytotoxicity and perforin have a well-understood functional role in the effector pathway of cyototoxic lymphocyte (CTL) and natural killer (NK) cells, it is only now being appreciated that perforin also has important functions in the induction of the adaptive CTL response. Finally, perforin and the granule exocytosis pathway of cytotoxicity are also critical for maintaining homeostasis of the CD8 T-cell compartment and downregulation of CTL memory responses. Cytotoxicity and perforin thus play a complex role in induction of CTL, in their effector function, and in the homeostasis of the CTL response. We are only beginning to understand the implications of these observations. Controlling and manipulating cytotoxicity are the holy grail of tumor immunology and antiviral therapy; there is every hope that further understanding of cytotoxicity will bring this goal within our grasp.

CYTOTOXIC LYMPHOCYTES

Two types of lymphocytes exist with regard to cytotoxicity: constitutively cytotoxic cells and inducibly cytotoxic lymphocytes. NK, NK-T, and γ,δ-T cells have constitutive cytotoxic activity and are able to kill targets without the need for induction. In contrast, naïve CD4 and CD8 T cells require activation and clonal expansion, accompanied by differentiation into CTLs. This process is driven by antigen stimulation with the help of several co-stimulatory molecules, and is accompanied by the acquisition of cytoplasmic granules containing the molecules responsible for target cell lysis, principally perforin and granzymes. Memory CTLs are similar to naïve T cells with regard to cytotoxic activity, except that their reacquisition of cytotoxicity is much more rapid than that of naïve cells.

The ability to kill a given target cell is determined by the specificity of cell surface receptors, whereas the killing process itself is unspecific. Once a killer cell has recognized a cell as a target, the killing machinery (the cytolytic granules) is engaged and kills the target indiscriminately and independently of target cooperation. The decision to kill is made by recognition rather than by downstream events. This decision tree is quite unlike the decision of a target to commit apoptosis, which

depends on apoptotic receptor engagement *and* downstream signaling events. Apoptotic receptor engagement can indeed result in cell proliferation via NFκB activation. Targets recognized by CTLs or NK cells are not asked for compliance in dying; they are killed by multiple lytic, apoptotic, and nonapoptotic pathways without recourse to protection.

Recognition of targets by CTLs is achieved by the T-cell receptor binding to major histocompatibility complex (MHC) peptide complexes, triggering tight binding to the target, and killing by granule exocytosis. NK cells, NK-T cells, and γ,δ-T cells recognize a variety of surface molecules, not all of which have been defined. Killing by NK cells is, however, primarily determined by the engagement, or lack thereof, of dominant NK cell inhibitory receptors binding to the MHC class I molecules. If the target expresses normal levels of MHC the dominant killer cell inhibitory receptors will prevent activation of the cytolytic program by dephosporylation of intracellular substrates, even when positively signaling receptors are engaged. CTLs thus kill by MHC recognition whereas NK cells kill only in the absence of MHC molecules. This complementarity of recognition by CTLs and NK cells is an important mechanism to prevent the evasion of virally infected cells from the immune response.

Viruses must express viral genes and proteins during replication and some of the viral peptides will be presented by MHC and detected by CTLs. Virus-mediated downregulation of MHC molecules effectively prevents detection by T cells; however, absence of MHC allows NK cell killing. Viruses are therefore challenged to evolve mechanisms by which both NK cell and CTL evasion is achieved. Two mechanisms are known: cytomegalovirus (CMV) downregulates MHC expression of infected cells, thereby avoiding T-cell detection; in order to avoid killing by NK cells, CMV encodes a viral class I MHC molecule that is expressed on the infected cell and recognized by the inhibitory receptors of the NK cell, thereby inhibiting the killing activity. HIV nef downregulates HLA-A and -B molecules but not HLA-C molecules. As T-cell responses are primarily restricted by HLA-A and -B, CTLs will not be able to recognize HIV-infected cells; as HLA-C engages a large pool of NK cells through the inhibitory receptors, HIV-infected cells are also protected from NK cell attack.

It is evident from these examples that the primary function of cytotoxic lymphocytes is to clear our body of virus-infected cells and other intracellular parasites; this biological task is under continued evolutionary pressure by evolving pathogens. As the genesis of tumors is accompanied by multiple mutations and generation of neoantigens, oncogenic transformation may also be considered as parasitization of cells. As we shall see this process is under surveillance by cytotoxic cells protecting us from carcinogens and spontaneous tumor formation. Autoimmunity is the result of dysregulated immune responses and is frequently accompanied by infiltration of tissues with cytotoxic cells. Cytotoxic cells are generated after allogeneic bone marrow transplantation and contribute to graft-versus-host disease. In allogeneic organ transplantation invasion of cytotoxic cells is one of the hallmarks of organ rejection.

CTLS AND NK CELL MORPHOLOGY AND FUNCTION

Cytotoxicity is a specialized secretory process and CTLs and NK cells are secretory cells (Dennert and Podack 1983; Podack and Dennert 1983; Henkart et al. 1984; Podack and Konigsberg 1984; Henkart 1985; Podack 1985, 1989; Podack and Kupfer 1991; Podack et al. 1991; Kagi et al. 1994, 1996; Berke 1995; Liu et al. 1995; Podack, 1995a, b; Griffiths 1997; Zhang et al. 2001; Blott and Griffiths 2002; Djeu et al. 2002. Secretion of stored granules, also known as granule exocytosis, is the hallmark of the cytolytic process. Secretory cells come in three flavors (Figure 1):

1. Constitutively secreting cells: constitutive secretion describes a secretory process occurring continuously without external trigger. Antibody secretion by B cells is the classic example. Membrane-bound vesicles containing the cargo bud of the trans-Golgi network fuse with the plasma membrane, releasing their cargo or inserting membrane proteins into the plasma membrane.
2. Regulated secretion of storage granules is dependent on triggers and Ca^{2+} signals for release of intracellularly stored products. Secretory vesicles budding of the trans-Golgi network fuse with the secretory granule, where the cargo materials are concentrated with the aid of proteoglycans. This type of secretion is typical for endocrine and exocrine glands. In these cells lysosomes and granules are clearly distinct and distinguishable organelles receiving input from the Golgi apparatus by separate vesicular pathways.
3. Regulated secretion of secretory lysosomes (granulosomes) is the process used by cytolytic lymphocytes. Although all cells have lysosomes, only specialized cells have secretory lysosomes. Secretion is triggered by external stimuli and Ca^{2+} signals. Secretory lysosomes are specialized organelles receiving input from the Golgi body through the mannose-6-phosphate receptor pathway used by lysosomes and from the plasma membrane through endocytic vesicles and endosomes. Only lymphocytes, granulocytes, mast cells, platelets, melanocytes, and some renal epithelial cells are equipped with secretory lysosomes (Dennert and Podack 1983; Griffiths 1997; Pham and Ley 1997; Simon et al. 1997).

CTLs and NK cells are indistinguishable by electron microscopic morphology (Figure 2) (Henkart et al.

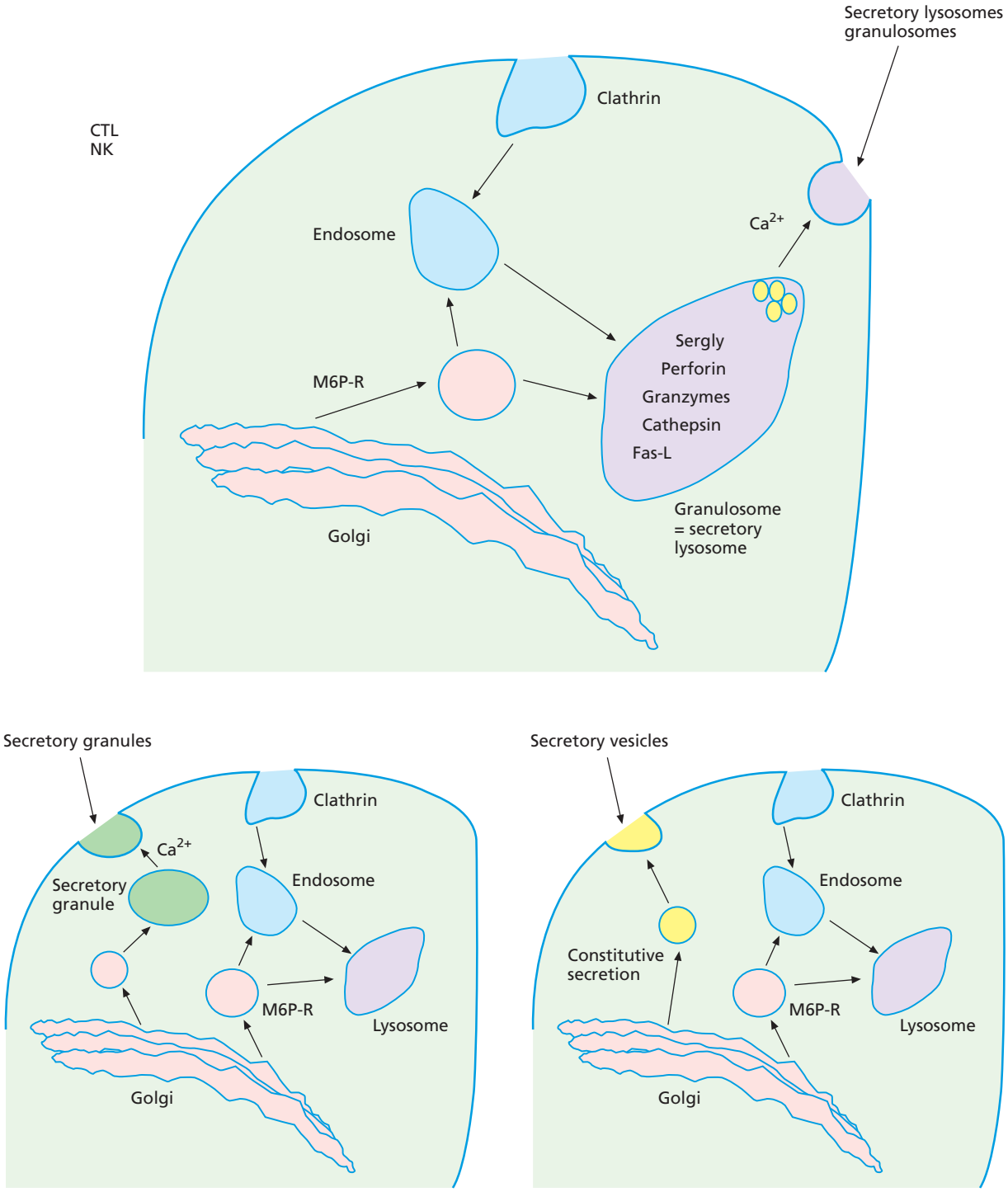

Figure 1 *Three types of secretory cells. Top: secretory lysosomes (granulosomes), this type of regulated secretion is used by cytotoxic lymphocytes (CTLs) and natural killer (NK) cells. Lower left: regulated secretion of storage granules, used by exocrine and endocrine glands. Lower right: constitutive secretion used, for example, by antibody-secreting B cells.*

1984; Podack 1995b; Uellner et al. 1997). The cells are characterized by a reniform or lobed nucleus, a well-developed active endoplasmic reticulum, a pronounced Golgi apparatus, and large granules (secretory lysosomes, granulosomes). The granulosomes are characterized by a dense core and areas containing vesicular, apparently membrane material. The significance of the vesicular material is not known; however, it may indicate storage of membrane-bound mediators such as the Fas ligand (Fas-L).

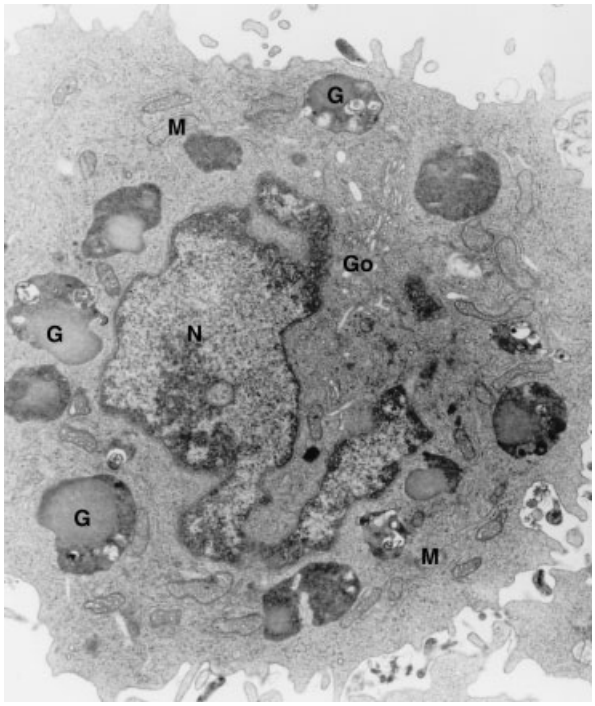

Figure 2 *Morphology of cytotoxic lymphocyte. G, granulosome; Go, Golgi body; M, mitochondria; N, nucleus.*

KILLING

On target recognition and T-cell receptor (TCR) triggering the cytolytic synapse is formed. The synapse is bordered by a tight adhesion ring composed of integrins (lymphocyte-mediated cytotoxicity 1 (LFA-1)) on the CTL side and of corresponding ligands (intercellular adhesion molecule 1 (ICAM)) on the target. The integrins are anchored via talin to the cytoskeleton of the CTLs. From inside the adhesion ring signals emanate via TCR-associated kinases and activate Erk-kinase, which in combination with Ca^{2+} fluxes results in a complex reorientation of the killer cells. The Golgi body and the microtubule-organizing center (MTOC) are brought to the area of contact between killer and target, and vectorial migration of granules toward the synapse is initiated (Figure 3). Only individual or few granules are secreted before the synapse is broken, allowing the CTLs to attack many targets in succession without having to replenish the granule supply. The process of synapse formation, reorientation, and secretion is completed in 2–3 min and is followed immediately by violent blebbing of the target cell, indicating that the death process has begun. Isolation of membrane fragments after the killing event shows numerous membrane pores, suggesting that the killer cell has perforated the target membrane and that this event is a critical element in the cytolytic process (Figure 4).

How is the target cell killed and how is the killer cell protected from its own killing molecules? Before we can answer this question, we must study what is being secreted in the form of granule exocytosis to cause this violent and rapid demise of the target cell.

COMPOSITION OF CYTOLYTIC GRANULES OF CTLS AND NK CELLS

Given that cytolytic granules are granulosomes they contain typical lysosomal components such as

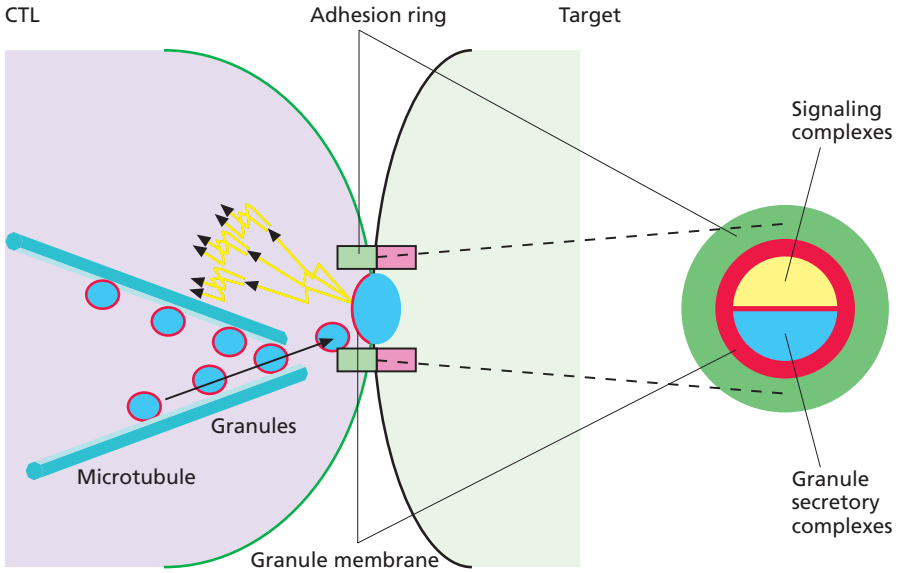

Figure 3 *Cytolytic synapse: the adhesion ring is depicted in side view (left) and en face (right panel). Inside the adhesion ring the localization of granule components and signaling components is shown. The granule membrane, lining the synapse on the cytotoxic lymphocyte (CTL) side, is shown in red.*

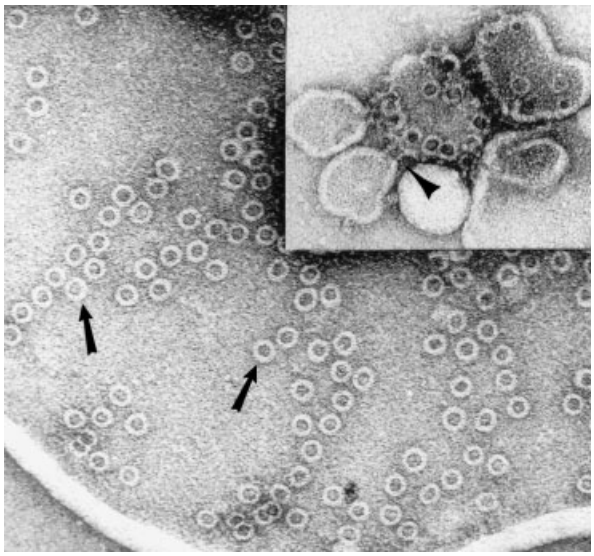

Figure 4 *Poly-perforin complexes on target membranes visualized by negative staining electron microscopy. Arrow: complex seen in top view; arrow head on inset: complex seen in side view (profile view).*

cathepsins, glucosaminidase, hexosaminidase, and other acid hydrolases. In addition granulosomes contain specific components not found in lysosomes that are designed for the killing process. The CTL and NK cell-specific components are perforin, the granzymes, Fas-L, granulysin, and serglycine as packing material.

Serglycine

Serglycine is a proteoglycan with a 14-kDa peptide core, which is heavily glycosylated with highly negatively charged chondroitin sulfate and heparan sulfate side chains, in a 250-kDa complex. Serglycine, similar to other granule proteoglycans, packed into dense highly concentrated packages allows condensation of granule contents, mainly granzymes and perforin. This effect is achieved through the formation of salt bridges between the negative charges of the proteoglycan, with positive charges on granzymes and perforin eliminating water molecules and reducing the colloid osmotic pressure. Granule contents are, then, large heteromultimeric complexes composed of the proteoglycan core with complexed perforin and granzymes, which are secreted en bloc.

Perforin

Perforin is a pore-forming protein sharing homology with complement components C6, C7, C8α, C8β, and C9 (Podack and Tschopp 1982; Henkart et al. 1984; Young et al. 1986; Podack and Kupfer 1991; Henkart 1997; Ponting 1999; Rukavina and Podack 2000; Smyth and Trapani 2001). The region of homology has been named the membrane attack complex/perforin (MACPF) domain; modeling studies suggest that this domain forms two amphipathic helices which are involved in transmembrane insertion and channel formation. Genome sequencing of several organisms has revealed additional MACPF-containing proteins and that the MACPF domain has been conserved from prokaryotes through plants and *Drosophila* spp. to the vertebrate system (Figure 5). Interestingly, the only prokaryotes containing proteins with MACPF domains to date are *Chlamydia pneumoniae* and *C. trachomatis*, both highly pathogenic organisms with intracellular localization.

Perforin is a 70-kDa protein synthesized as a hydrophilic, glycosylated monomer and it is directed from the trans-Golgi to the granulosome using as yet undefined targeting signals. Perforin on its C-terminus contains a C2 domain that is 100 amino acids long, which is characteristic for phospholipid-binding proteins (Jiang et al. 2001). On secretion and exposure to Ca^{2+} perforin binds to phospholipid head groups, triggering its ability to form multimers and insert itself into the hydrocarbon core of the membrane (Figure 6). Polymerization and membrane insertion have to go hand in hand, because, once polymerized, poly-perforin is unable spontaneously to insert into phospholipid bilayers. Perforin shares with the complement proteins above the remarkable biochemical ability to undergo a dramatic hydrophilic-to-amphiphilic transition which allows it to form transmembrane pores. The fully assembled pore is made up of about 20 perforin protomers, forming a tubular (cylindrical) complex that harbors a 16 nm-wide aqueous core. The poly-perforin cylinder is about 16 nm tall and inserted 4–6 nm deep in the membrane.

Perforin pores may be formed with as few as two or as many as 20 perforin molecules. Membrane pores allow the equilibration of ionic gradients, including Ca^{2+}, across the plasma membrane and result in the loss of membrane potential. Larger pores allow the efflux even of macromolecular cellular constituents. Membrane pores must be repaired, or else the cell would die via disruption of the normal cellular homeostasis. Membrane repair is achieved by endocytosis of the membrane patch containing the pore. The signal for the repair process comes from the Ca^{2+} influx across the pore. Endocytosis has two consequences: first, it is accompanied by pinocytosis of the liquid adjacent to the pore which will now be filling the endosome. As this liquid originates from the exocytosed granulosome and contains granzymes and other products, these components are now internalized in the target cell. Second, the pore formed by perforin persists in the endosome, interfering with acidification and normal endosome traffic, and may be responsible for endosomolysis which releases the endosome contents into the cytoplasm of the target cell where they initiate apoptotic and nonapoptotic cell death (Masson et al. 1986).

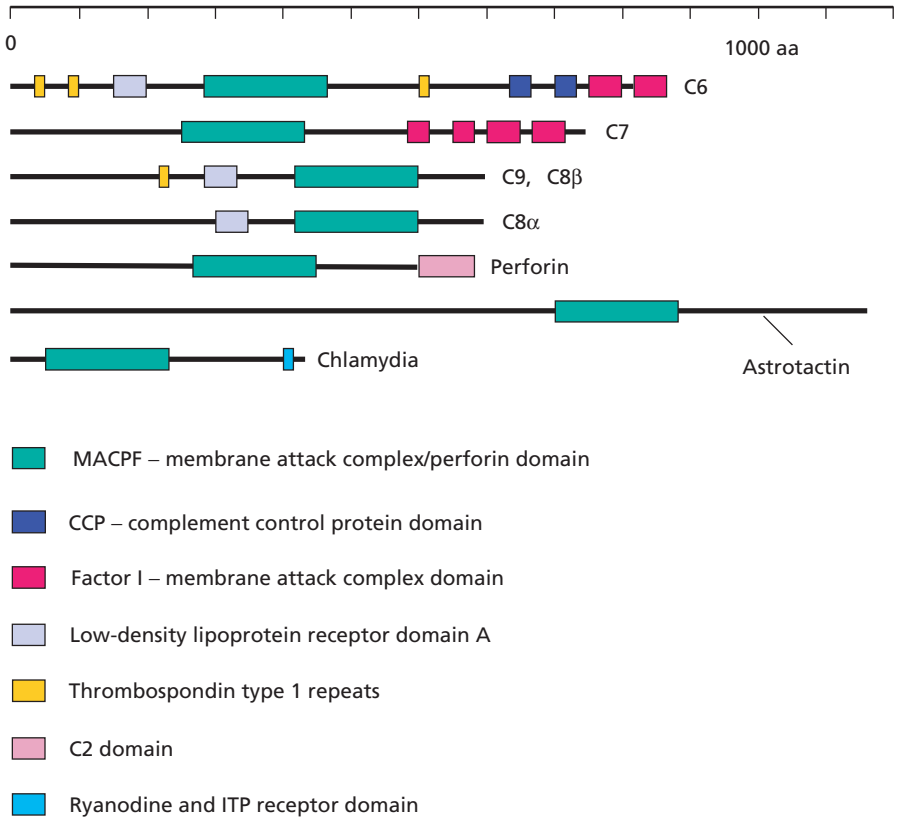

Figure 5 *Proteins sharing the membrane attack complex/perforin (MACPF) domain.*

Granzymes

Granzymes make up quantitatively the largest mass of the granule contents (Masson et al. 1986; Masson and Tschopp 1987; Shiver et al. 1992; Berke 1995; Beresford et al. 1997; Henkart 1997; Shiver et al. 1992; Darmon et al. 1996; Froelich et al. 1996; Atkinson et al. 1998; Kataoka et al. 1998; Metkar et al. 2002; Wallich et al. 2001; Zhang et al. 2001). Granzymes are serine esterases with narrow substrate specificities. They are transported to granulosomes by the mannose-6-phosphate receptor pathway typical for other lysosomal components including cathepsins, glycosylases, and other lysosomal enzymes. After exocytosis, granzymes gain entry into the target cell cytoplasm via perforin-dependent endocytosis and endosomolysis. In the target cytoplasm are the specific substrates for the granzymes. Granzymes always act in concert, depending on the collection of granzymes contained in the cytolytic granule. Humans have five granzymes, described below.

Granzyme B is a serine esterase with specificity of cleavage next to acidic (aspartate) residues (Masson et al. 1986; Masson and Tschopp 1987). Within the target cell it cleaves caspases and Bid (BH3 domain binding B-cell lymphoma [bcl]-2-like proapoptotic protein) and CAD (caspase-dominated DNAase) and sets off the apoptotic cascade including the mitochondrial pathway and the direct pathway of ICAD/CAD activation resulting in internucleasomal DNA cleavage and the generation of typical DNA ladders. The downstream activation of apoptotic mediators by granzyme B avoids some of the upstream regulators of apoptosis but is subject to blockade by bcl-2 and crmA. Granzyme B can bind to mannose-6-phosphate receptors expressed by certain cells and enter the cell by receptor-mediated endocytosis. However, in order for granzyme B to exit the endosome into the cytoplasm, perforin-mediated endosomolysis is essential.

Granzymes A and K (Masson et al. 1986; Masson and Tschopp 1987; Nakajima et al. 1995; Mullbacher et al. 1996; Beresford et al. 1997; Wilharm et al. 1999; Davis et al. 2001; Fan et al. 2002) are esterases with specificity for basic residues (arginine, lysine). They enter the target cell in a perforin-dependent process together with granzyme B and other granule components. Granzyme A mediates target apoptosis, including membrane blebbing and disruption of the nucleus. However, caspases are not activated and DNA laddering is not observed. Instead single-stranded DNA breaks are responsible for DNA breakdown. Granzyme A interacts with the nucleosome assembly complex that is involved in altering chromatin structure, to make it accessible for transcription and for single-stranded DNA excision-repair enzymes. It appears that the last activity is altered

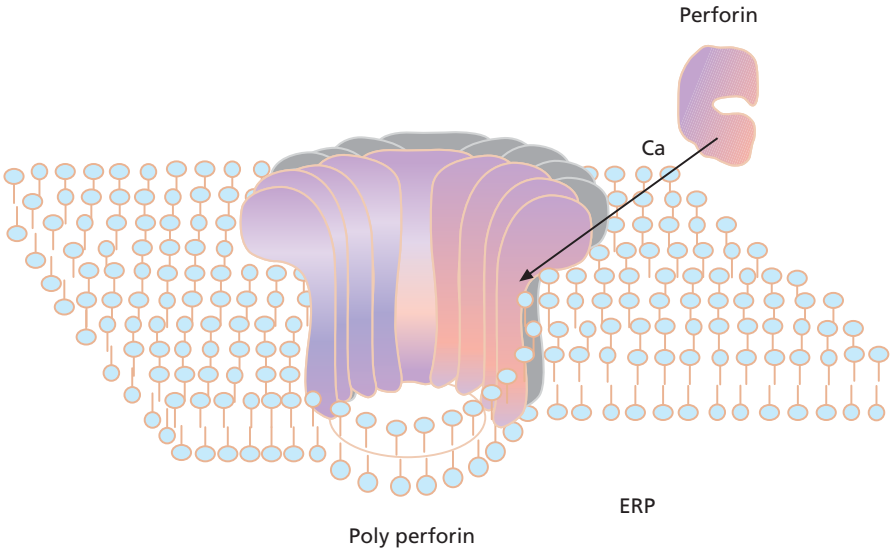

Figure 6 *Schematic view of perforin binding to phospholipid via Ca^{2+} and inserting itself as polymeric complex into the lipid bilayer.*

by granzyme A and results in single-stranded DNA breaks. Although granzyme K has similar enzymatic specificity to granzyme A, it is not known what its intracellular substrates are.

Granzymes H and M have chymotryptic specificity, with granzyme M also cleaving next to methionines. Granzyme H is expressed primarily by CTLs whereas granzyme M is exclusively expressed by NK cells. Granzyme H has been reported not to mediate cell death or apoptosis on internalization by target cells. Its function, similar to that of granzyme M, remains to be defined.

Mice, in addition to granzymes A, B, K, and M, express granzymes C, D, E, F, and G, but not granzyme H. This greater polymorphism suggests that mice have to combat a larger array of intracellular pathogens than humans.

Granulysin

Granulysin is found only in human granules. It is distantly related to saponins and has antibacterial activity for mycobacteria. It acts via membrane association and disordering of the lipid bilayer through ionic interaction. On delivery into cells it has specificity for mitochondria and triggers apoptosis and disrupts electron transport.

Fas ligand

Fas-L mediates apoptosis on Fas engagement by activation of caspase 8, and is the classic apoptotic mediator (Takahashi et al. 1994; Nagata and Suda 1995; Nagata 1996; Kataoka et al. 1998; Nagata 1998; Bossi and Griffiths 1999; Barry and Bleackley 2002). Apoptosis is a carefully regulated process subject to multiple regulatory checkpoints. Fas-L has two different localizations for expression. In granulosome-containing CTLs Fas-L is routed from the Golgi to the granule. The targeting information is contained in the proline-rich intracellular domain of Fas-L. In other cells Fas-L is found on the cell membrane. This distinct distribution explains why Fas-L killing of targets by CTLs is MHC restricted because it is exocytosed similar to or together with granule exocytosis only on appropriate signaling by the TCR. In other cells the surface expression of Fas-L allows triggering of apoptosis in Fas-carrying cells without additional triggering. The presence of Fas-L in cytolytic granules links this apoptotic mechanism directly to granule exocytosis.

KILLER CELL SELF-PROTECTION

How is the CTL or NK cell protected from perforin and the highly toxic molecules secreted during the cytotoxic event? First, the microanatomy of the cytolytic synapse confines the action of perforin and directs it toward the target membrane. The adhesion ring around the synapse is so dense that lateral diffusion will be restricted. Therefore, only the granule membrane lines the synapse on the CTL side. It is likely that the granule membrane is impervious to perforin action, and this hypothesis has recently been attributed to cathepsin B which is found coating the granule membrane and not allowing perforin to insert (Balaji et al. 2002). Second, the half-life of perforin after exposure to Ca^{2+} is very short and measured in seconds, because Ca^{2+} induces polymerization and polymerized perforin is not membrane active. Polymerization and membrane insertion have to proceed in a concerted action. Finally, CTLs and other hemopoietic cells express many serpins. Serpins are serine protease inhibitors first described and analyzed in serum

as trypsin and thrombin inhibitors. They form 1:1 covalent complexes with the protease that are resistant to dissociation by denaturing agents. Serpins are highly specific substrates for the respective serine protease. On cleavage of a loop in the serpin, a dramatic and rapid conformational change takes place in the serpin molecule, which distorts the active site of the protease to such an extent that the cleavage dissociation reaction cannot take place and the protease remains trapped in the complex. PI-9 is a human intracellular serpin, expressed by killer cells, dendritic cells, and other hemopoietic cells, that inhibits granzyme B with high specificity (Hirst et al. 2001). Surprisingly, although caspases and granzyme B have similar substrate specificities, PI-9 is a much better inhibitor for granzyme B than for caspases. In the mouse at least six additional serpins have been identified, and it is likely that there may be a specific serpin for each of the granzymes described above.

TARGET CELL DEATH

CTL- and NK cell-mediated killing unleashes several cell death pathways simultaneously via the granule exocytosis mechanism (Figure 7). The mechanism by which perforin mediates entry of other granule components into the target cytoplasm may be compared with a very sophisticated injection mechanism entailing plasma membrane pore formation and endosomolysis. Receptor-mediated endocytosis of granule contents is also possible but perforin is still required to liberate the granule contents and allow their exit into the cytoplasm where they mediate destruction. The following events, each potentially leading to death, occur contemporaneously upon granule exocytosis:

- Perforin pore formation alone, if extensive enough, can kill cells. More commonly, however, perforin is the catalyst for uptake of granule components and their release into the target cytoplasm.
- Granzyme A mediates cell death by single-stranded DNA breaks, and interference with DNA repair and nucleosome assembly.
- Granzyme B results in caspase and Bid cleavage, setting off two apoptotic cascades that result in CAD activation and DNA laddering.
- Granzymes H, K, and M (NK) are likely to contribute to cell death although their mode of action is as yet unknown.
- Granulysin sets off mitochondrial death and disruption of energy generation by associating with the outer mitochondrial membrane.
- Not studied yet are the effects of typical lysosomal constituents, including acid hydrolases which presumably gain entry into the target cell and may well have lethal effects.
- Finally, in concert with perforin and the pathways depending on it, Fas-L is exocytosed and gains surface expression to engage the Fas receptor in order to trigger conventional apoptosis; the Fas ligand pathway, in contrast to the molecular pathways described above, is not dependent on the availability of perforin.

This listing, presumably still incomplete, shows the barrage of biological weapons brought to bear on the annihilation of target cells. Target cells are recognized because they are parasitized by viruses or oncogenes that strive to evade death and to make the target noncompliant to normal apoptotic signals. By employing multiple death pathways it will be very difficult for the virus, or other parasite, to block all pathways simultaneously, and noncompliance can be overcome by the killer cell.

ROLE OF PERFORIN IN THE INDUCTION OF CTL ACTIVATION

CTLs and NK cells are effector cells mediating effector responses in the efferent arm of immunity by killing target cells via perforin and producing cytokines, principally IFN-γ (Beresford et al. 1997; Bossi and Griffiths 1999; Smyth et al. 2000; Haddad et al. 2001; Hirst et al. 2001). Both cytotoxicity and IFN-γ production are essential for tumor rejection and viral clearance. However, perhaps surprisingly, cytotoxic activity mediated by NK cells is also required in the afferent arm of immunity for induction of CTL responses. Two mechanisms have been defined: (1) CD70-expressing tumor cells are killed by NK cells via CD27 engagement. Killing by NK and IFN-γ production is absolutely required for the induction of a CTL response and establishment of tumor-specific memory (Smyth et al. 2001; Kelly et al. 2002). (2) Heat shock proteins are released on trauma or necrosis and chaperone peptides including potential antigens. Dendritic cells and macrophages have receptors for heat shock proteins, thereby endocytosing the molecules along with bound peptides. The peptides are channeled into the class I MHC antigen-presenting pathway and are available for CTL recognition. However, T-cell activation requires the presence of NK cells and the action of perforin and IFN-γ. In the absence of perforin-containing NK cells, a CTL response does not ensue; NK and dendritic cells undergo reciprocal activation which is needed to permit T-cell activation and clonal expansion.

PERFORIN FUNCTION IN HOMEOSTASIS

During and after an immune response T cells need to expand and contract rapidly, respectively. Clonal contraction is part of the homeostatic regulation required to maintain a balanced T-cell repertoire. Fas and Fas-L, other tumor necrosis factor (TNF) receptors, and cytokines participate in homeostatic regulation of T

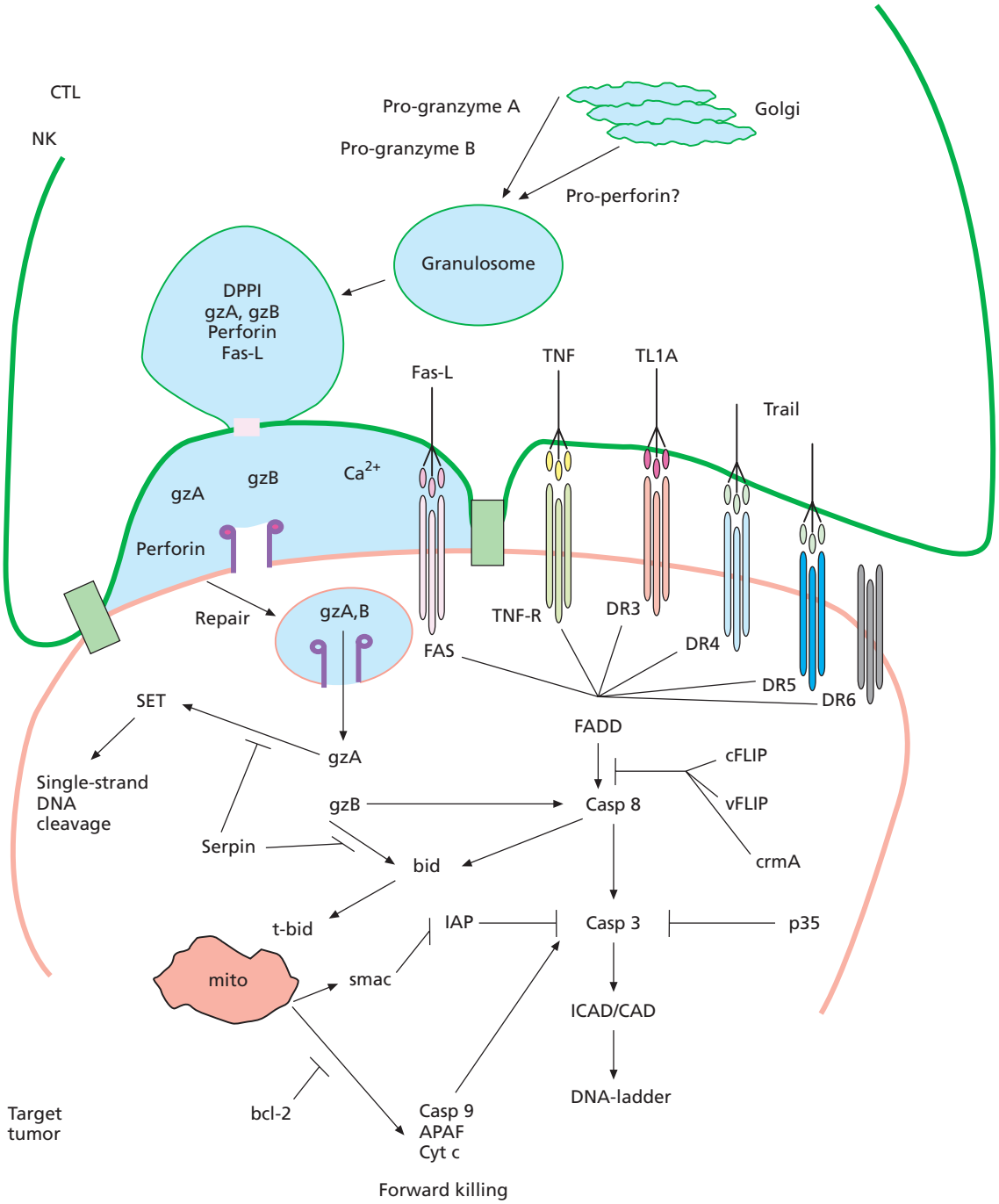

Figure 7 *Granule-mediated target killing: events in the target cell. See text for abbreviations.*

and B cells. Cytotoxicity mediated by perforin and associated with granules is required for homeostasis of CD8 CTLs and monocytes/macrophages. This function of perforin is seen only after viral challenge or in combination with other genetic defects. In murine models, the combined deficiency of perforin with Fas-L or Fas is much more severe than that of Fas-L or Fas deficiency alone (Spielman et al. 1998). Perforin/Fas-L deficiency results in early death and organ infiltration with CD8 cells and macrophages, indicating a critical role for perforin in their homeostasis (Figure 8). Similarly, perforin deficiency in humans causes childhood death by lymphoproliferation of CD8 cells and macrophage-type cells called histiocytes (see below) (Stepp et al. 1999; Fadeel et al. 2001). Homeostatic dysregulation in perforin deficiency is noted only after repeated antigenic (viral) challenge, suggesting dysregulation of the memory CTL pool.

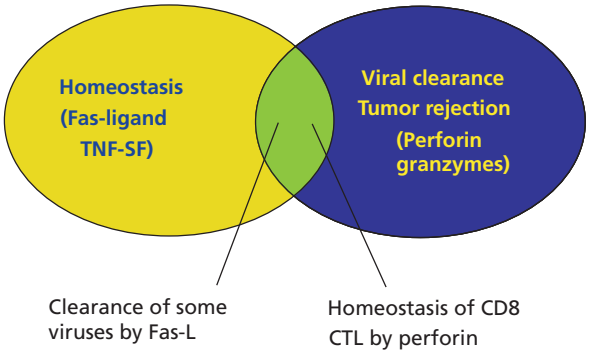

Figure 8 *Diagram of separate functions of perforin and Fas ligand (Fas-L) in immune defense and homeostatic regulation. Overlapping function in clearance of some viruses by Fas-L, and homeostatic regulation of CD8 cytotoxic lymphocytes (CTLs) by perforin.*

HOMEOSTATIC FUNCTION OF FAS AND FAS-L

Fas-L can induce apoptosis in Fas-expressing cells. Fas or Fas-L deficiency in mice results in lymphoproliferation and grossly enlarged lymph nodes by 6–12 months of age (Nagata and Suda 1995). Lymphoproliferation can be associated with autoimmunity in certain genetic backgrounds. The largest group of expanded T cells is found in the CD4/CD8$^-$, B220$^+$ population. Cells of the monocyte/macrophage lineage are only moderately expanded. Fas and Fas-L are important for antigen-induced cell death during secondary stimulation of T cells, and are believed to facilitate and maintain peripheral tolerance of T cells for self-antigens. CD4 cells are more susceptible to Fas-L-mediated deletion than CD8 cells, and Fas/Fas-L may be more important for CD4 T-cell homeostasis than for CD8 T cells. In humans, mutations in Fas or Fas-L give rise to autoimmune syndromes. The dysregulated removal of activated T cells results in the maintenance of selfreactive cells causing autoimmunity. It is not clear at the current time which cell types need to express Fas-L to maintain homeostasis; candidates are T cells or antigen-presenting cells.

GENETIC DEFECTS OF CYTOTOXICITY

Perforin deficiency in mice

Perforin is constitutively expressed in NK cells, NK-T cells, and γδ-T cells, all of which comprise a segment of the innate immune system. Although NK-T and γδ-T cells have TCRs, the TCR chains are relatively invariant and do not recognize MHC peptide complexes, although they recognize relatively invariant MHC class Ib molecules in association with lipids or carbohydrates. In αβ-T cells perforin is induced on T-cell activation and clonal expansion. The site with the highest level of perforin expression under physiological conditions is the decidua in the pregnant uterus. The decidua is enriched with decidual NK cells which express very high levels of perforin and may contribute to protection against infection. Perforin deficiency in mice that are kept free of pathogens has no obvious phenotype and does not disrupt the process of a normal pregnancy. However, on viral challenge perforin-deficient mice exhibit increased morbidity and mortality, emphasizing the importance of perforin in protection from viral infection. Perforin-deficient cells exhibit normal clonal expansion and tissue infiltration; however, viral clearance is impaired in the absence of perforin. Perforin is thus critical for the cytolytic process in vivo that eliminates the viruses. Perforin-deficient CTLs and NK cells in vitro have greatly diminished cytolytic activity and are only able to induce apoptosis in targets via Fas-L, TNF, or TNF-related apoptosis-inducing ligand (TRAIL) (Kagi et al. 1996).

Perforin-deficient mice develop spontaneous tumors with high incidence, lymphomas, and adenocarcinomas of the lung, indicating that perforin is important for immune surveillance against spontaneous tumors (Smyth et al. 2000; Smyth and Trapani 2001). Perforin-deficient mice are unable to reject challenge with immunogenic tumors when compared with wild-type mice. Controlled treatment with carcinogens demonstrates a higher frequency and earlier incidence of tumors in perforin-deficient mice in comparison to wild-type mice (van den Broek and Hengartner 2000; van den Broek et al. 1996). It is evident from these observations that perforin is an important mediator of tumor immunity.

Perforin is frequently found expressed at sites of autoimmune reactivity. Accordingly perforin deficiency can ameliorate certain autoimmune states, e.g. perforin-deficient non-obese diabetic (NOD) mice have a much lower and later incidence of diabetes than perforin-sufficient NOD.

Allogeneic bone marrow transplantation is complicated by the development of graft-versus-host disease arising from grafted T cells activated by the recipient's alloantigens. By transplanting perforin-deficient T cells the onset of graft-versus-host disease is dramatically delayed but then sets in with full force and lethality, suggesting that perforin contributes to the initiation of the immune response (Baker et al. 1996, 1997; Jiang et al. 2001). In allogeneic organ transplantation, perforin-expressing cells are found during transplant rejection. However, perforin deficiency does not noticeably affect organ rejection, indicating multiple redundant pathways (Atkinson et al. 1998; Kataoka et al. 1998; Ponting 1999).

Perforin-deficient mice have an impaired ability to downregulate CTLs on chronic infection and repeated in vivo stimulation, indicating a participation of perforin in CD8 CTL homeostasis (Edwards et al. 1999; Harty et al. 2000). This is more apparent when perforin deficiency is

combined with Fas or Fas-L deficiency. Perforin/Fas-L double-deficient mice die at about 10 weeks of age of an autoimmune syndrome characterized by organ infiltration with macrophages and expansion of CD8 and CD4 cells, with an inversion of the normal CD4:CD8 ratio (Spielman et al. 1998). The mechanism by which perforin contributes to CTL downregulation is not clear, but two hypotheses have been discussed:

FRATRICIDE

On CTL killing of target cells, the CTLs can pick up target antigens, process the antigens, present them via MHC-I, and thereby become a target itself for other CTLs. In the presence of perforin CTLs would kill each other and thereby downregulate their activity.

KILLING OF ANTIGEN-PRESENTING CELLS

CTL activation and clonal expansion is mediated by antigen-presenting cells. On acquisition of cytotoxicity CTLs are perfectly able to kill antigen-presenting cells, thereby removing the initial signal for their own generation. In the absence of perforin the elimination of antigen-presenting cells is impaired, thereby providing chronic stimulation of CTLs and disruption of CTL homeostasis.

PERFORIN DEFICIENCY AND DEFECTS IN CYTOTOXICITY IN HUMANS

The syndrome of familial hemophagocytic lymphohistiocytosis (FHL) is a lethal inheritable disease associated with dysfunctional cytotoxicity of NK cells and CTLs (Stepp et al. 1999). The disease is characterized by early childhood onset, frequently after viral infections, and accompanied by lymphocytic proliferation and infiltration of tissues with histiocytes. The last cell type represents a late-stage monocyte/macrophage-derived cell characteristic for this syndrome. Recently, several etiological causes of the syndrome have been identified and can be subgrouped into several categories.

About 30 percent of the cases of FHL are caused by perforin deficiency. The remaining cases come from several disorders associated with dysfunctional secretory lysosomes, including granulosomes. Secretory lysosomes are found in lymphocytes and NK cells, granulocytes, mast cells, platelets, and melanocytes. Dysfunction of the secretory or trafficking pathway of secretory lysosomes may therefore affect other cellular systems, in addition to the cytolytic machinery.

The Chédiak–Higashi syndrome is an autosomal recessive disorder causing hypopigmentation of the skin, eyes, and hair, prolonged bleeding times, recurrent infections, abnormal NK cell function, and peripheral neuropathy. Morbidity results from patients succumbing to frequent bacterial infections or to lymphoproliferation. The *CHS1/LYST* gene encoding a cytosolic protein of 430 kDa is dysfunctional. The exact function of this gene is not known except that it participates in the biogenesis/secretion of secretory lysosomes. A similar defect is found in Beige mice. NK cells and polymorphonuclear granulocytes (Figure 9) have grotesquely enlarged granules which, however, cannot be secreted and hence NK cell activity is diminished. The inability of granule secretion is associated with the inability to use perforin for killing and in this regard appears as functional perforin deficiency with the same consequence of hemophagocytic lymphohistiocytosis resulting in death.

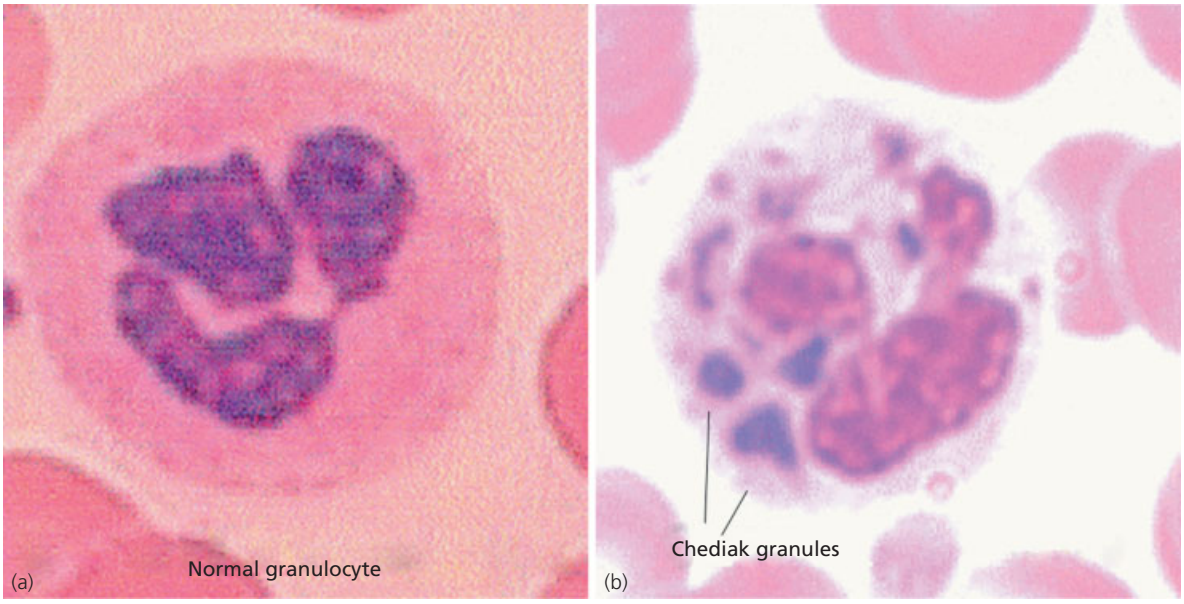

Figure 9 *Gigantic granules in Chediák–H igashi syndrome.* **(a)** *Normal granulocytes.* **(b)** *a polymorphonuclear granulocyte with enlarged granules is shown.*

Griscelli's disease is an autosomal recessive disorder associated with hypopigmentation and occurrence of acute phases of uncontrolled lymphocyte and macrophage activation, ultimately causing death. Several genetic variations of the disease exist. One form of the disease is caused by mutations in Rab27a, a protein involved in vesicle traffic and required for granule exocytosis in lymphocytes and for melanocyte function. Evidence in Ashen mice suggests that Rab27b can substitute for Rab27a, but that Rab27b is not expressed in lymphocytes and melanocytes. CTL granules appear normal in Griscelli's syndrome; they migrate to the cytotoxic synapse; exocytosis does not take place, however, as a result of the absence of Rab27a (Haddad et al. 2001; Stinchcombe et al. 2001).

Patients with Hermansky–Pudlak disease have primarily bleeding abnormalities and ocular albinism. The syndrome includes mutations of at least three different gene products affecting vesicular traffic. One of the mutations in the Rab geranylgeranyl transferase α subunit reduces Rab prenylation and is responsible for defects in platelet granule synthesis. Cytolytic CTL granules fail to polarize properly, although their exocytosis appears normal. The murine model for this disease is found in gunmetal mice.

GRANZYME DEFICIENCIES

Granzyme deficiencies have been created in mice by gene targeting but so far have not been described in human populations. Granzyme A and granzyme B deletions have been described in the literature (Pham and Ley 1997; Simon et al. 1997). However, as granzyme B is on the same chromosome with, and upstream of, granzymes C, D, E, and F, the insertion of a neocassette also inactivated downstream granzyme expression. Re-examination of the granzyme B targeting using the Cre/lox system showed that most of the effects attributed to granzyme B knockout may have been caused by the concurrent extinction of granzyme C.

Granzyme A-deficient mice have no overt phenotype and no defect in the cytotoxic activity of T and NK cells. Similarly, granzyme B-deficient mice appear normal and their cytotoxic activity in vitro is not diminished. However, when DNA release is measured by using granzyme A- or B-deficient CTLs a reduction and delay in DNA release is observed, indicating impaired DNA degradation.

As mentioned above, it is likely that granzymes have evolved to defeat viral escape mechanisms. Therefore it might be expected that granzyme deficiencies may be associated with increased susceptibility to certain viral infections and mortality. Indeed, by titrating the viral dose and measuring disease and mortality, an important function for granzymes in antiviral defense can be demonstrated. Ectromelia is the mouse pox virus which is highly virulent in mice. Wild-type mice are susceptible to infection, but lethality requires an infective dose of more than a million viral units. Perforin-deficient mice are highly susceptible and succumb to disease from as little as one viral unit. Granzyme A- and B-deficient mice exhibit susceptibility between these two extremes (Mullbacher et al. 1996, 1999). Interestingly, the ability to clear virus infection and to reject tumors does not correlate (Table 1). Although granzymes clearly are important for viral protection they seem to be dispensable for tumor rejection. RMAS is an MHC-negative tumor that requires NK cells for rejection whereas the K1735 melanoma is rejected by CTLs. In both cases and in immune surveillance against methylcholanthrene-induced carcinomas, perforin is required but granzymes A and B are not.

DPP1 DEFICIENCY (CATHEPSIN C)

Dipeptidyl peptidase is required for the cleavage of the pro-dipeptide of all granzymes. In the absence of this cleavage granzymes remain inactive as zymogens. CTLs with deficiency of the dipeptidyl peptidase have the hallmarks of combined granzyme A/B deficiency. However, dipeptidyl peptidase is also important for activation of proteases in mast cells, granulocytes, and other cells. In humans dipeptidyl peptidase deficiency, the Papillon–Lefevre syndrome, or keratosis palmoplantaris with periodontopathia is an autosomal recessive disorder. Patients suffer early loss of teeth, apparently from chronic infection of the periodontium.

Table 1 *Role of perforin and granzymes in viral clearance and tumor rejection*

Deficiency	Lethal viral dose (mouse pox virus)	Rejection of RMAS tumor	Rejection of K1735 melanoma	Immune surveillance, carcinogenesis
Wild type	$>10^6$	Yes	Yes	Normal
Perforin	1	No	No	Impaired
Granzyme A	10^5	Yes	Yes	Normal
Granzyme B[a]	10^4	Yes	Yes	Normal
Granzyme A + B[a]	1	Yes	Yes	Normal

a) Granzyme B deficiency includes cluster deficiency.

Cytotoxicity and antiviral defense

Viruses are intracellular parasites infecting the victim's own cells. Antibodies and pattern recognition receptors used for recognition and defense against extracellular parasites, primarily bacteria and bacilli, are not much use against intracellular parasites, which can be removed only by the destruction of the infected cells. This is achieved in two ways: by suicide (apoptosis) of the infected cell and, in the case of failure of suicide, by the cytotoxic lymphocyte (Mullbacher et al. 1996; Edwards et al. 1999; Harty et al. 2000; Wallich et al. 2001).

A primary defense system against viral spread is the interferon system. Double-stranded RNA or DNA of the viruses is recognized by DNA- or RNA-dependent protein kinase which turns on INF-α and -β with the resultant interferon secretion signaling via interferon receptors to the infected and neighboring cells. The signals result in shutdown of protein synthesis and transcription of antiviral genes that block viral replication and viral clearance, or in apoptosis of the infected cell. Many virus infections are contained at this stage. Other viruses have evolved to overcome the initial antiviral response and block apoptosis of the infected cell. The interferon secretion, however, also activates NK cells which are attracted and activated and begin killing infected cells that fail to undergo apoptosis. It is apparent that successful killing must overcome the anti-apoptotic activities of the virus that have allowed it to begin to replicate. How does the NK cell recognize the infected cell? First, some viruses downregulate MHC molecules to avoid T-cell detection; this is the signal for NK cells to start killing such cells. Second, infected cells frequently express new gene products (stress genes, e.g. Rae-1β) that are recognized by positively signaling receptors on the NK cell (NKG2D) and initiate cytotoxicity. NK cell killing and antigen release, together with IFN-γ production, bring dendritic cells to the site of action. Dendritic cells further activate NK cells and recruit the CTL response. The fact that the virus has survived in the infected cell up to this point is testimony to its ability to block apoptotic pathways. The virus now needs to focus on evading detection by NK and T cells and/or to block the cytotoxic assault. Herpes viruses have very large genomes devoted largely to the purpose of evading immune recognition and to thwarting the cytotoxic response. Indeed many herpes viruses are never totally eliminated and persist as latent infections that can flare up at any time that the CTL response is suppressed by medical immune suppression or other natural causes. Pox viruses focus on blocking cytotoxicity; they express powerful inhibitors for caspases (crmA) and granzymes (serpin homologues). However, the evolution of several granzymes in the arsenal of the cytotoxic response seems to be the response to this family of viruses allowing survival of the host, even though with great difficulty.

It is self-evident that only those species able to resist and overcome viral attack were able to survive and reproduce, and likewise that viruses had to continue to evolve new strategies to prevent obliteration. This struggle continues today, best exemplified in the HIV epidemic and the trend of new viruses emerging.

CYTOTOXICITY AND ANTI-TUMOR DEFENSE

Perforin is a critical part of immune surveillance against spontaneous tumors and protection against carcinogens. In addition perforin is required for the rejection of tumors by NK cells and CTLs whereas granzymes appear dispensable. One might conclude that it is easier to kill tumor cells than virus-infected cells. So, then, why are tumors not eliminated by the immune system? The answer is that the tumor has developed elaborate pathways to evade or suppress the immune system. Tumors do not secrete interferons that would alert the NK response; indeed the lack of an interferon response makes many tumors susceptible to viruses that are easily suppressed by normal cells. The lack of arousal of an NK response seriously hampers the harnessing of dendritic cells and a CTL response. The lack of signals emanating from the tumor also prevents CTLs from homing in on the tumor. The tumor, unlike viral infection, is not perceived by the immune system as dangerous. Again, unlike viral infection and replication, tumor progression and stepwise mutation are slow. Tumors express many negative regulators of the immune response. CTL responses to viruses entail a rapid burst of clonal expansion followed by an equally rapid clonal contraction, leaving behind a few memory cells that can respond again and faster in a second viral attack. The clonal contraction, mediated for CD8 CTL cells by perforin and for CD4 cells by Fas-L, is absolutely necessary to avoid autoimmunity and lymphoproliferation. Tumors express genes that can interfere with clonal expansion and/or cause premature clonal contraction and downregulate cytotoxic activity, thereby allowing their continued survival. The challenge for tumor immunologists is the ability not only to develop vaccines that generate activated NK cells and specific CTLs, but also to find ways to direct these cells to the tumor site.

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Immunological memory

SHANE CROTTY AND RAFI AHMED

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The primary function of the immune system is to defend against microbial pathogens. It accomplishes this task at two levels: first, by generating a specific immune response against the invading pathogen and controlling the infection; and second, by remembering this first encounter and mounting an accelerated immune response upon re-exposure to the same pathogen. This rapid recall response can either completely prevent disease or greatly lessen the severity of clinical symptoms. The first documentation of 'immune memory' dates back to the time of the Greek historian Thucydides, who recorded that the 'same man was never attacked twice' while describing the plague of Athens in 430BC (Finley 1951). It is remarkable that we knew nothing about the immune system or about microbes when Thucydides made his astute observations on immune memory; it would be more than 2000 years before we gained an appreciation of the immune system and learnt that microbes cause infectious diseases. However, it is now well established that memory and specificity constitute the two defining features of adaptive immune responses.

The focus of this chapter is on the principles of immune memory. This chapter is divided into four parts: (1) a historical perspective of vaccination; (2) an overview of protective immunity to microbes; (3) a discussion of the current models of memory T- and B-cell differentiation; and (4) an overview of the mechanisms involved in maintaining immunological memory.

HISTORY OF VACCINATION

Immunological memory is the basis for vaccination; the discipline of vaccinology originated from the observation that previous exposure to a disease conferred resistance to a subsequent episode. Thus, the principle of vaccinology was that, if somehow the first episode of a disease could be attenuated, one would be protected against the more severe form of the disease. The earliest recorded attempts to put this into practice were made in China and India around AD 1500 when pustular material from smallpox patients was inoculated into healthy people (Fenner et al. 1988). This process, called 'variolation', caused smallpox but, for reasons that are not fully understood, the disease was usually milder. More importantly, 'variolated' individuals were protected against naturally acquired smallpox. However, this approach did not gain popularity and was eventually discontinued because the degree of morbidity and mortality associated with variolation was simply too variable and too high to be acceptable.

The major breakthrough came with the classic experiments of Jenner in 1796. Jenner had noticed that milkmaids who were commonly infected with cowpox were spared the ravages of smallpox. Jenner reasoned that inoculation of cowpox pustular material might prevent smallpox. In many ways, vaccinology was born in 1796 when Jenner inoculated young James Phipps with cowpox virus and subsequently prevented smallpox, thereby showing that exposure to a related but less pathogenic virus could confer protection against a more virulent virus. However, despite this remarkable achievement, the field of vaccinology stood still for the next 50–75 years. This lack of progress was primarily the result of the fact that it was still not proven that microbes caused smallpox, or any disease. Thus, there was no clear scientific basis for developing additional vaccines.

Things changed dramatically in the second half of the nineteenth century as a result of the pioneering work of Pasteur, Koch, Ross, von Behring, Ehrlich, and others. These scientific giants not only proved that microbes cause infectious diseases, thus laying the foundations of immunology and microbiology, but their work also resulted in the development of successful vaccines against rabies, typhoid, cholera, plague, and diphtheria (Plotkin and Orenstein 1999). It is only fitting that the first Nobel Prize in Medicine and Physiology, given in 1901, was awarded to von Behring for his work on diphtheria. Several additional vaccines were developed during the first half of the twentieth century, including the highly effective yellow fever virus vaccine by Theiler, for which he was awarded the Nobel Prize in 1951.

Until 1950 all viral vaccines were prepared in either mammalian or avian whole tissues. In that year Enders and colleagues showed that viruses can be grown in vitro in cell culture, which opened enormous scientific possibilities and facilitated the development of many of our currently used vaccines (Table 1), including vaccines against polio virus, measles, mumps, and rubella. This technology continues to be the most common method of manufacturing viral vaccines to this day.

Major advances have occurred during the past 25 years in our ability to 'cut and paste' genes, yet only two currently licensed vaccines are made using recombinant DNA technology – the hepatitis B vaccine and the Lyme disease vaccine. However, it is expected that almost all future vaccines will rely on recombinant DNA technology to some extent. A multitude of expression vectors (bacterial, viral, 'naked' DNA) have been developed and considerable effort has gone into optimizing their in vivo expression and methods/routes of immunization (Table 2; see Part VII: Vaccines). It is likely that these will become the main technologies for our future vaccines. Indeed, several recombinant vaccines are currently in human clinical trials. It remains to be seen which of these vectors will prove to be the most effective. The crucial properties will be the ability to target antigen-presenting cells (APC), the level and duration of gene expression in vivo, the ease of constructing the vector, and issues such as cost and safety of the vectors.

Table 1 *General approaches for vaccines*

Type of vaccine	Comments	Examples	
		Viral	Bacterial
Live attenuated organism	The organism is attenuated such that it does not produce clinical disease but still retains sufficient immunogenicity and replicative capacity to induce protective immunity	Polio (Sabin, OPV), measles, mumps, rubella, yellow fever, varicella (chickenpox)	Bacilius Calmette–Guérin (BCG)
Killed organism	The vaccine consists of the whole organism, which has been chemically inactivated to prevent replication, but the immunogenic structure of the organism is preserved	Polio (Salk, IPV), influenza, Japanese encephalitis, rabies, hepatitis A	Pertussis (whole), cholera
Antigenically related organism	This is the strategy used by the original vaccine	Cowpox or vaccinia, for smallpox	None
Subunit vaccines	The vaccine consists of subcomponents of the organism. These subcomponents are usually either surface proteins of a virus, or proteins and/or polysaccharides of bacteria	Hepatitis B	*Haemophilus influenza*b, pneumococci, meningococci, typhoid, pertussis (acellular), Lyme disease
Antitoxins	The vaccine consists of inactivated toxin (toxoid) that is no longer toxic but still retains its immunogenic structure	None	Tetanus, diphtheria, anthrax
Vectored vaccines	These vaccines are currently in development. These strategies use known immunogenic and safe viruses or bacteria as engineered vectors to express immunogenic proteins from pathogens of interest (see Table 2)	None licensed, but several in phase I and II clinical trials	
Nuclei acid (DNA) vaccines	These vaccines are currently in development. The vaccine consists of plasmid DNA capable of expressing the antigen of interest when introduced into cells	None licensed, but several in phase I clinical trials	

Table 2 *Recombinant delivery systems for future vaccines*[a]

Delivery systems
Plasmid 'naked' DNA
Bacterial vectors
Listeria spp.
Mycobacterium spp.
Salmonella spp.
Shigella spp.
Viral vectors
Adenoviruses
Alphaviruses
Lentiviruses
Picornaviruses
Poxviruses
Retroviruses

a) This is a partial list of the many bacterial and viral vectors currently being tested.

PROTECTIVE IMMUNITY

There has been great interest in determining the relative importance of humoral versus cellular responses in protective immunity. This has resulted in much debate and considerable experimentation to assess the role of T- and B-cell responses in protection from reinfection. When examining this issue, one must remember that antibodies and T cells have evolved to perform entirely different functions. The business of antibodies is to deal with the microbe itself (i.e. free virus particles, bacteria, and parasites) and that of T cells is to deal with infected cells. As T cells can only recognize microbial antigens in association with host MHC molecules, the free virus particles or bacteria are invisible to them. Thus, antibody provides our only specific defense against free microbial organisms, and therefore the importance of pre-existing antibody in protective immunity against infectious diseases cannot be overemphasized. In fact, antibody is likely to be the sole mechanism of protective immunity against bacteria and parasites that have an exclusively extracellular lifestyle. However, the equation begins to change for viruses and for those bacteria and parasites that can survive or replicate intracellularly. Although antibody again provides the first line of defense against such infections, frequently the inoculum is not entirely neutralized by the pre-existing antibody. This is where the T cells come into play, because their main function is surveillance of infected cells. T cells are divided into two subsets, $CD4^+$ and $CD8^+$ T cells, and both of these subsets play a role in protective immunity. They do this by either killing the infected cell and/or by releasing cytokines that stop or slow growth of the microbe. In addition to these effector functions carried out by both $CD4^+$ and $CD8^+$ T cells, $CD4^+$ T cells also play an important role in providing help for antibody production and help for generating $CD8^+$ T-cell responses.

Thus, based on these basic principles, protective immunity to extracellular pathogens depends primarily upon B cells and $CD4^+$ T cells (as helpers), whereas a concerted effort by B cells, $CD4^+$ T cells (as both helpers and effectors), and $CD8^+$ T cells is required to control intracellular pathogens. Although this generalization is reasonably accurate, there are several examples where microbial infections have been controlled exclusively by antibody (i.e. passive antibody transfer) or by $CD8^+$ T cells (i.e. after immunization by a vaccine containing only the $CD8^+$ T-cell epitopes) (Ahmed and Biron 1999). These experiments clearly show that there are situations where either humoral or cellular immunity can be sufficient for protection. The correct conclusion from such experiments is that, in the given situation, just the $CD8^+$ T-cell response or the antibody response was adequate for protection. However, it is crucial to realize that it is misleading to conclude from this type of experiment that the missing response does not play a role in protective immunity. The danger of reaching such a conclusion is that potentially useful immune responses may get excluded from the design of a vaccine.

Similarly, when designing a vaccine it is prudent to look beyond the type of immunity generated during the natural infection, e.g. antibody plays a minimal role in controlling an acute primary lymphocytic choriomeningitis virus (LCMV) infection in mice, or in conferring protection from re-infection ($CD8^+$ T cells are the major players in both instances) (Buchmeier and Zajac 1999). Yet, passive transfer of neutralizing antibody can confer a reasonable degree of protection (Buchmeier and Zajac 1999). A similar situation exists for *Listeria monocytogenes*. *Listeria* species has long been considered to be an infection controlled exclusively by T cells, but a recent study has shown that antibody is also effective in eliminating it (Edelson et al. 1999). These findings have obvious implications for vaccine design, e.g. although very little neutralizing antibody is generated during the course of HIV infection, immunization with the right form of HIV envelope protein may induce neutralizing antibody that would be useful in conferring protective immunity (Mascola et al. 2000; Burton 2002). Thus, the right strategy for a HIV vaccine would be to stimulate both the humoral and cellular arms of the immune system.

What is the basis of protective immunity? Is it protection from infection or from disease? In the strictest sense, protection from infection would mean that there was sufficient pre-existing antibody to neutralize/opsonize all of the pathogen in the inoculum and there was no further amplification of the microbe. This is frequently referred to as sterilizing immunity. Does this ever happen? The precise answer is not known, but it is likely that it does happen regularly when the inoculum size of a pathogen is very low. However, when challenged with a substantial inoculum of pathogen, it is more likely that a substantial fraction of the virus/

bacteria gets neutralized and the remaining fraction that is able to initiate infection is quickly controlled by the rapid anamnestic response of memory T cells and/or memory B cells (Ahmed and Gray 1996; McChesney et al. 1997). Overall, this is a pretty good scenario. First, the infection is rapidly controlled and there is no disease; second, the virus is fully eliminated by the memory response; and third, this low-grade, transient, subclinical infection serves as a natural 'booster' to the immune system.

MEMORY T- AND B-CELL DIFFERENTIATION

Before considering models of memory B- and T-cell differentiation it is important to appreciate a fundamental difference between cellular and humoral immunity. Microbial infections usually induce both T- and B-cell long-term memory. However, the nature of T- and B-cell memory is different (Figure 1 and Table 3).

B-cell memory is usually manifested by continuous antibody production, even after resolution of the disease. Prolonged antibody production, lasting for several years after infection or immunization, has been observed in humans as well as in experimental systems (Scheibel et al. 1966; Simonsen et al. 1984; Kjeldsen et al. 1985; Cohen et al. 1994; Slifka et al. 1995; Bottiger et al. 1998; Crotty et al. 2003; Hammarlund et al. 2003). In contrast, the effector phase of the T-cell response is short-lived (a few weeks), and 'memory' in the T-cell compartment results from the presence of quiescent memory T cells, which are found at higher frequencies than naïve cells and can respond faster and develop into effector cells (i.e. cytoxic T lymphocyte (CTL) or cytokine producers) more efficiently than can naïve T cells (Kedl and Mescher 1998; Bachmann et al. 1999a; Cho et al. 1999; Zimmermann et al. 1999; Barber et al. 2003). This dichotomy in effector function in the humoral and cellular memory pools is a feature of most immune responses to immunizations or acute infections, and it makes teleological sense. Sustained secretion or overproduction of cytokines can have deleterious effects, and the presence of fully active killer memory T cells could result in severe immunopathological damage if some of these CTLs were crossreactive with selfantigens. Thus, maintenance of T-cell immunity by sustaining the effector phase would carry a high price tag. As memory T cells can rapidly develop into effectors and quickly gain access to sites of infection, it is not essential in most instances to maintain effector T cells to provide protection.

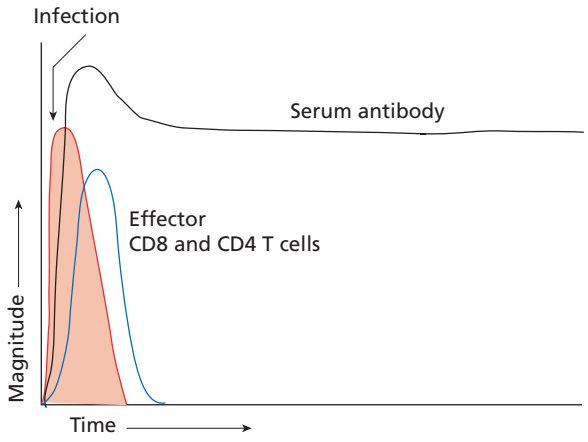

Figure 1 *The nature of effector T- and B-cell responses is different. Most microbial infections induce prolonged serum antibody responses that can persist for months or years after resolution of the infection. In contrast, effector T-cell responses (i.e. active killer cells and cytokine producers) are short-lived and are seen only during the acute phase of infection.*

Table 3 *Distinction between effector B and T cells*

Effector B cells
Plasma cells are end-stage-differentiated cells that constitutively produce antibody in the absence of antigen.[a] A proportion of plasma cells can live for extended periods in the bone marrow. These long-lived plasma cells are an important source of antibody in the serum and can also contribute to antibody in the mucosa. This pre-existing antibody forms the first line of defense against infection and is a key aspect of protective immunity against microbial infections
Effector T cells
Effector T cells secrete cytokines and can kill infected cells. In contrast to plasma cells, T cells maintain effector functions only in the presence of antigen. In the absence of antigen, effector T cells either die or differentiate into memory T cells. This also contrasts with plasma cells that are fully differentiated end-stage cells. Plasma cells do not give rise to memory B cells

a) Antigen is needed for differentiation of naïve or memory B cells into antibody-secreting plasma cells but is not required for maintaining antibody production by fully differentiated plasma cells.

B-cell memory

The two critical cell types involved in B-cell memory are memory B cells and plasma cells. The major differences in naïve B cells, memory B cells, and plasma cells are summarized in Tables 4 and 5 and the lineage interrelationships of these three cell types are shown in Figure 2. In this simple model, after the initial activation of naïve B cells (by antigen and T-cell help), activated B cells undergo clonal expansion. This initial proliferation is observable as histologically defined regions of B-cell clusters, known as extrafollicular foci, within the T-cell-rich areas of secondary lymphoid organs. During this initial phase of activation, B cells can class switch from IgM to IgG or other isotypes if stimulated strongly. In addition, it is generally thought that there is no or minimal somatic mutation/affinity maturation of the B-cell receptor at this stage of the B-cell response (Jacob and Kelsoe 1992; MacLennan

Table 4 *Differences between naive and memory B cells, in humans*

Naïve B cells	Memory B cells
No somatic mutation or affinity maturation of B-cell receptor	Somatic mutation and affinity maturation of B-cell receptor
Not isotype switched; mostly IgM^+ IgD^+	~40% isotype switched; ~60% IgD^-: 40% IgM^+ IgD^+ 40% IgG^+ or IgA^+, IgD^- 20% IgM^+ IgD-
$CD27^-$	$CD27^+$ More efficient antigen-induced proliferation? (Martin and Goodnow 2002)

1994); however, this has been challenged by a recent study in an autoimmune mouse model (William et al. 2002). Importantly, most of the antibody-secreting plasma cells generated from B cells in extrafollicular foci are thought to be short-lived, with a half-life of 3–7 days (McHeyzer-Williams 1997; Ho et al. 1986).

An important aspect of the model described in Figure 2 is affinity maturation of memory B cells and the generation of long-lived plasma cells. After the initial phase of B-cell proliferation and differentiation in extrafollicular foci, some B cells migrate back to the follicle (the B-cell regions of secondary lymphoid organs), and there is a second phase of clonal expansion of activated B cells within the specialized microenvironment of a germinal center (GC) (Jacob and Kelsoe 1992; MacLennan 1994; Tsiagbe and Thorbecke 1998). It is normally within the GCs that affinity maturation occurs, and B cells selected within the GC mature into memory B cells or plasma cells (Berek et al. 1985; Jacob et al. 1991). Additional class switching occurs in the GC. GCs contain antigen-specific CD4 T cells, and follicular dendritic cells (FDC) that retain depots of antigen for stimulation and selection of high-affinity B cells. Germinal center B cells proliferate rapidly (of the order of 6–7 h per division – Liu et al. 1991) and undergo positive selection by B-cell receptor binding to antigen attached to FDCs, as well as by B-cell interactions with antigen-specific CD4 T cells (Kosco et al. 1988; MacLennan 1994). A great deal of B-cell apoptosis occurs in GCs, as many of the B cells that undergo somatic hypermutation end up with nonantigen-specific or nonfunctional B-cell receptors (MacLennan 1994; McHeyzer-Williams et al. 2001; Ho et al. 1986).

There is evidence to suggest that plasma cells derived from high-affinity, isotype switched B cells tend to be longer-lived and reside in the bone marrow (Manz et al. 1997, 1998; Slifka et al. 1998). Teleologically, the basic model of a B-cell response generating short-lived plasma cells first and long-lived plasma cells and memory B cells later is appealing because, in an infection, it is valuable to get antipathogen antibodies produced as rapidly as possible (which is done by those antigen-specific B cells that differentiate into plasma cells early in extrafollicular foci). But it is also important to evolve high-

Table 5 *Differences between memory B cells and plasma cells*

	Memory B cell	Plasma cell
Location	Reside primarily in the secondary lymphoid organs	Reside primarily in the bone marrow
Function	Important for replenishing the pool of plasma cells and memory B cells. Do not actively secrete antibody, but respond to antigen by rapidly dividing and giving rise to more memory B cells and also differentiating into plasma cells	Terminally differentiated cells that constitutively produce antibody. Not stimulated by antigen (low to no surface B cell receptor). Do not divide
Markers[a]		
Surface Ig	++++	+/–
B220 (mouse only)	++++	+/–
CD19	++++	+/–
CD20 (human only)	++++	+/–
MHC class II	++++	+/–
CD138 (syndecan-1)	–	++++
Blimp-1	–	++++

a) List of markers commonly used to distinguish between memory B cells and plasma cells in mice and humans (not a comprehensive listing). All of the markers listed here except for Blimp-1 are expressed at the cell surface and can be used to separate plasma cells from memory B cells.

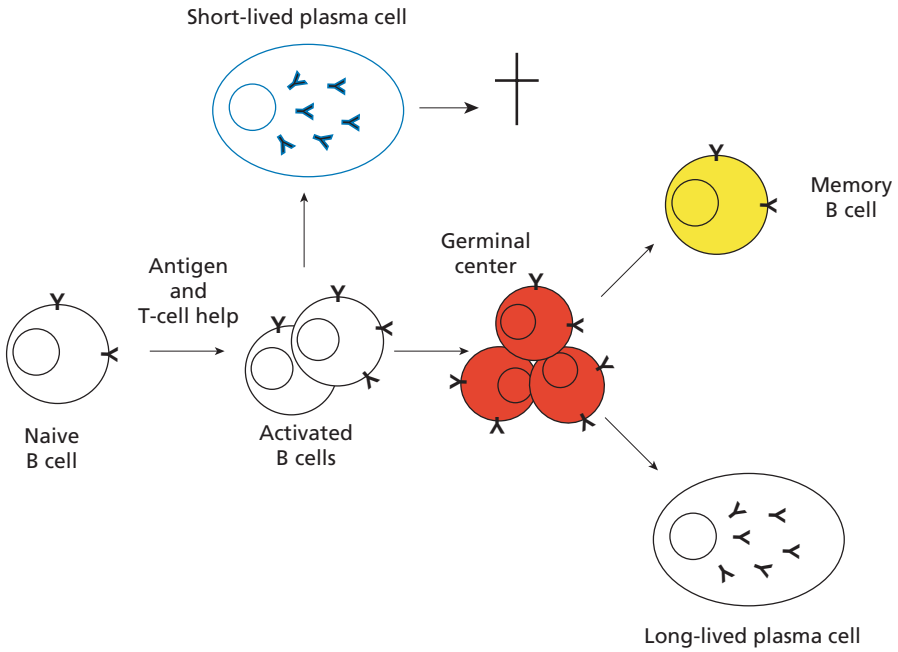

Figure 2 *Model of memory B-cell differentiation. After antigenic stimulation, naive B cells undergo clonal expansion and form clusters of activated B cells known as extrafollicular foci. These activated B cells can either differentiate into short-lived plasma cells or migrate back into the follicle and initiate a germinal center reaction. After proliferation and affinity maturation, germinal center B cells produce both long-lived plasma cells, which in turn produce high-affinity antibodies, and memory B cells which have high-affinity B-cell receptors.*

affinity antibodies that are much more efficient at controlling and eliminating the pathogen (which is done by the antigen-specific B cells that initiate GCs).

The kinetics and anatomical location of antibody production after an acute viral infection in mice are shown in Figure 3. The IgM response is transient but the serum IgG response is long-lived and can persist for several years. Antibody is initially produced by short-lived plasma cells present in the secondary lymphoid organs. The plasma cell response in these tissues peaks during the first 2 weeks and then declines within 2–4 weeks after infection (Slifka et al. 1995). As splenic plasma cell populations decline, antigen-specific plasma cells begin to migrate to and/or accumulate in the bone marrow compartment. After the GC reaction subsides, the bone marrow becomes the predominant site of antibody production, with 80–90 percent of the host's plasma cells located in this compartment (Slifka et al. 1995, 1998).

Upon reinfection, antibody responses occur rapidly in the spleen and lymph nodes, because most of the memory B cells reside in these organs. After secondary infection, the memory B cells in spleen and lymph nodes proliferate and/or differentiate into plasma cells, resulting in a transient increase in the number of antibody-secreting cells (ASC) in the spleen. However, once the secondary infection has cleared, this response subsides and, once again, the bone marrow becomes the predominant site of antibody production. It is worth noting the anatomical segregation between plasma cells and memory B cells. One way of looking at this is to consider the spleen and lymph nodes as 'factories' where

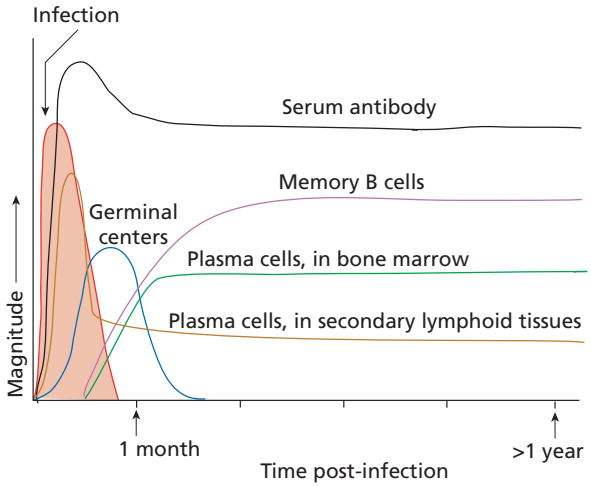

Figure 3 *Kinetics and anatomical site of B-cell responses after infection. Initial antibody production is by short-lived plasma cells in the spleen and lymph nodes. Germinal centers form and are maintained for approximately 2–6 weeks after antigen clearance. Long-lived plasma cells and memory B cells are produced from the germinal centers. Most of the long-lived plasma cells migrate to and reside in the bone marrow, though some stay in secondary lymphoid organs. Long-lived plasma cells in the bone marrow are generally responsible for most of the maintenance of serum antibody levels. Most of the memory B cells reside in secondary lymphoid organs long term, although some migrate to and reside in the bone marrow.*

B-cell differentiation into plasma cells occurs, and the bone marrow as the storehouse where the products (i.e. plasma cells) are kept. It makes sense that memory B cells should reside in the vicinity of the factories so that they can be quickly mobilized into action as the need arises, and that the plasma cells are shipped out to the bone marrow to make room in the factory. Plasma cells residing in the bone marrow go on with their business of making antibody, which ends up in the blood and can protect against reinfection. Note that some plasma cells are expected to reside in mucosal tissues and produce IgG and IgA which is secreted to protect the mucosal surfaces, and it should also be pointed out that serum antibody (IgG) can enter the mucosa by transudation and it is therefore possible that plasma cells residing in the bone marrow also contribute to the antibody present at mucosal sites.

The factors that are critical for generation of B-cell memory and plasma cells can be divided into three groups: (1) genes essential for FDC development, (2) B-cell intrinsic factors, and (3) CD4 T-cell intrinsic factors. TNFR1, LTα, and LTβ are essential for FDC development. CD40, CD19, and AID are examples of critical B-cell intrinsic factors (see Tsiagbe and Thorbecke 1998, for a more extensive list). CD28, B7-1/2, ICOS, and CD40L are examples of CD4 T-cell intrinsic factors required for the generation of GCs and development of B-cell memory (McAdam et al. 2001; Tafuri et al. 2001; Borriello et al. 1997; Xu et al. 1994). Interestingly, the intracellular signaling protein SAP (SLAM-associated protein; also known as SH2D1A), which is defective in individuals with the genetic disease X-linked lymphoproliferative (XLP), is essential in CD4 T cells for the generation of GCs and B-cell memory, but not for most other CD4 T-cell functions (Crotty et al. 2003b).

A separate set of genes has been identified that is specifically required for plasma cell differentiation, and XBP-1 and Blimp-1 are two critical B-cell transcription factors controlling the plasma cell differentiation program (Shaffer et al. 2001, 2002; Zhan et al. 2002). Within GCs, multiple factors play a role in determining the ratio of memory B cell versus plasma cell production, including Bcl-6, interleukin 10 (IL-10), OX40, and others (Tsiagbe and Thorbecke 1998).

In summary, immunological memory in the B-cell compartment consists of memory B cells and plasma cells – two distinct cell types with different anatomical locations and very different functions (Table 5). Memory B cells are located primarily in the secondary lymphoid organs and are present at much higher frequencies than naïve B cells of the same specificity. In addition to this quantitative advantage over naïve B cells, memory B cells are also qualitatively different and produce antibody of much higher affinity. However, memory B cells do not actively secrete antibody (i.e. they are not effector cells). Their major function is to make rapid recall responses to infection by quickly dividing and differentiating into plasma cells, as well as initiating new GCs. The rapid rise in antibody concentration upon reinfection is the result of memory B-cell differentiation into new antibody-secreting plasma cells. In contrast to memory B cells, plasma cells cannot be stimulated by antigen either to divide or to increase their rate of antibody production. These plasma cells are terminally differentiated cells that constitutively produce and secrete antibody. Plasma cells are extraordinarily efficient in their ability to make antibody; it has been estimated that plasma cells can secrete >1000 antibody molecules per second (Helmreich et al. 1961; Hibi and Dosch 1986). As pre-existing antibody provides the first line of defense against infection by microbial pathogens, the importance of plasma cells in protective immunity cannot be overstated. In fact, it could be argued that plasma cells may be the single most important cell type in protective immunity to infections.

T-cell memory

T-cell memory does not have a plasma cell equivalent, i.e. an effector cell that can continue its effector function in the absence of antigenic stimulation. In contrast to plasma cells, effector T cells elaborate their effector functions (i.e. cytokine secretion and killing of infected cells by release of secretory granules containing perforin/granzymes) only in the presence of antigen (see Figure 1 and Table 3). These cells exhibit an on–off lifestyle with antigen regulating this switch (Badovinac et al. 2000; Slifka and Whitton 2000). If effector T-cell responses are only transient, then what is the cellular basis of long-term T-cell immunity? It is well established that T-cell memory, as assessed by accelerated recall responses in vivo, is long-lived and is seen after most microbial infections (Ahmed and Gray 1996; Zinkernagel et al. 1996; Dutton et al. 1998; Freitas and Rocha 2000). These rapid recall responses to reinfection are the result of both quantitative and qualitative changes in the pathogen-specific T cells. On primary infection there is substantial expansion of the pool of both CD8 and CD4 T cells specific to the pathogen (Figure 4). Several studies have shown that the size of the memory T-cell pool is determined by the burst size during the expansion phase (Figure 5) (Hou et al. 1994; Busch et al. 1998; Murali-Krishna et al. 1998; Butz et al. 1998). In the case of some acute viral infections, the increase in the number of antigen-specific T cells can be as great as 1000- to 5000-fold (Murali-Krishna et al. 1998; Ahmed and Biron 1999). This numerical advantage alone can make an enormous difference to the recall response, but recent studies have shown that memory T cells are also qualitatively distinct from naïve T cells and can respond with much quicker cytokine secretion and killing on re-exposure to antigen (Zimmerman et al. 1996; Kedl and Mescher 1998; Bach-

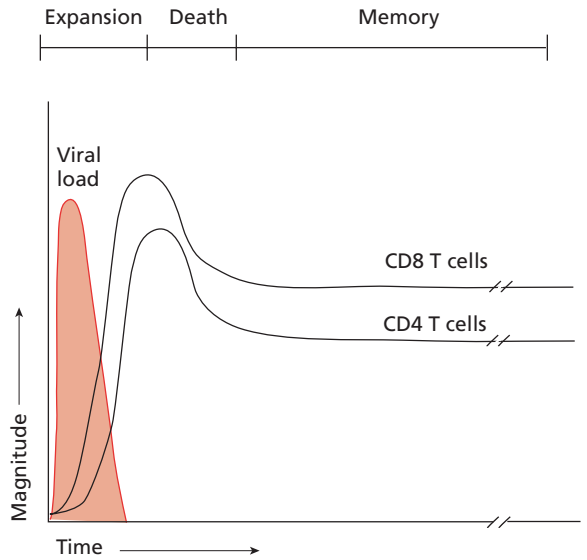

Figure 4 *Antiviral CD8 and CD4 T-cell responses. The three phases of the immune response are indicated at the top. The increase in cell number during the 'expansion' phase is the result of clones of T cells undergoing cell division. Soon after the virus is cleared, there is a 'death' phase characterized by decreasing numbers of virus-specific T cells caused by apoptosis. Following the death phase, the number of virus-specific T cells stabilizes and can be maintained for extended periods. This is the 'memory' phase. The CD4 T-cell response is similar to the CD8 T-cell response except that the magnitude of the CD4 response is usually lower and slightly delayed, and the death phase can be more protracted than the CD8 response.*

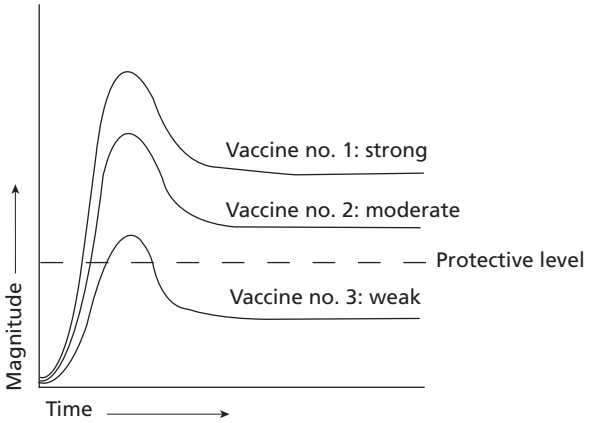

Figure 5 *The size of the memory T-cell pool is determined by the clonal burst size during the expansion phase. In this figure, the lines represent the T-cell responses induced by three different vaccines. Vaccine number 1 induces the largest expansion of T cells and hence generates the largest pool of memory T cells, vaccine no. 2 is next, and vaccine no. 3 is the weakest. The asterisk denotes the minimum number of antigen-specific T cells required for protective immunity. In this scenario, vaccines nos 1 and 2 will confer long-term immunity whereas protective immunity induced by vaccine no. 3 will be of short duration. The main reason for the failure of vaccine no. 3 is a smaller burst size during the expansion phase. Note that the maintenance of the memory T-cell pool is similar for all three vaccines.*

mann et al. 1999a, 1999b; Cho et al. 1999; Barber et al. 2003).

In fact, this faster responsiveness is clearly the defining functional characteristic of memory T cells (Table 6). Another important property of memory T cells is their ability to extravasate into nonlymphoid tissues; several recent studies have shown that a subset of memory T cells (termed 'effector' memory) are present in many peripheral tissues and also at mucosal sites (Sallusto et al. 1999; Masopust et al. 2001; Reinhardt et al. 2001). Thus, it is the combination of increased numbers of antigen-specific T cells, their faster responsiveness, and their better location (i.e. near sites of microbial entry) that underpins T-cell memory, and explains how memory T cells confer long-term protective immunity (Lau et al. 1994; Ahmed and Gray 1996; Zinkernagel et al. 1996; Dutton et al. 1998; Freitas and Rocha 2000).

It is well established that memory B cells and plasma cells differentiate along separate arms of a bifurcated pathway, and some of the conditions and signals that direct naïve B cells into the memory versus effector pathways are being defined (McHeyzer-Williams and Ahmed 1999; Calame 2001; Fearon et al. 2001). Our understanding of the memory T-cell differentiation pathway is less clear, but over the past few years there has been substantial progress in defining the lineage interrelationships of naive, effector, and memory T cells (Champagne et al. 2001; Lanzavecchia and Sallusto 2001; Kaech et al. 2003). Also, several markers have been identified that distinguish these three cell types (Table 7) (Ahmed and Gray 1996; Zinkernagel et al. 1996; Dutton et al. 1998; Freitas and Rocha 2000;

Table 6 *Defining characteristics of memory B and T cells*

Memory B cells
The hallmark of memory B cells is affinity maturation of the B-cell receptor. This means that the antibody produced by these cells (after their differentiation into antibody secreting cells) is of higher affinity. Thus, the fundamental difference between naïve and memory B cells is that the memory B cells can make a better 'product'. It is also likely that memory B cells respond to antigen stimulation more efficiently than naïve B cells
Memory T cells
The hallmark of memory T cells is faster responsiveness on re-encountering the antigen. In some instances the response is so rapid (i.e. cytokine production in less than 4 h) that memory T cells give the illusion of being effector cells. The effector molecules (cytokines, perforin, granzymes, etc.) of naïve and memory T cells are identical. Thus, the fundamental difference between memory and naïve T cells is that the memory T cells are a much more efficient 'factory' than naïve cells, although the product is the same. Also, unlike naïve T cells, which are located mostly in lymphoid tissues, a subset of memory T cells ($CD62^{lo}$ $CCR7^{-}$) is present in nonlymphoid tissues and at mucosal sites and can immediately confront the invading pathogen

Harrington et al. 2000). Possible models of memory T-cell differentiation are shown in Figure 6. It is unlikely that the 'divergent pathway' (model 1 – Figure 6a) represents a major pathway of memory T-cell differentiation because recent experimental evidence favors the linear differentiation model (model 2 – Figure 6b). Studies using transgenic T cells, or genetic techniques to mark antigen-specific T cells, have shown that memory T cells are derived from effector cells (Jacob and Baltimore 1999; Opferman et al. 1999). However, this simple linear differentiation model does not directly account for the fact that most effector cells undergo apoptosis and only a fraction (about 10 percent) of these cells survive to become memory cells. A second approach, using experiments involving adoptive transfer of effector T cells into naïve mice, showed that memory T cells probably arise directly from this population (Opferman et al. 1999; Hu et al. 2001; Kaech et al. 2003).

The 'decreasing potential hypothesis' (model 3 – Figure 6c) incorporates a mechanism for discriminating between effectors that preferentially die and those that preferentially survive and differentiate into memory cells (Kaech et al. 2002). According to this model the balance between effector cells and memory cells is governed by the duration and level of antigenic stimulation. Cells become more and more terminally differentiated with prolonged antigenic stimulation and this is accompanied by an increasing susceptibility to apoptosis and a decreasing potential for memory cell development. This model also explains the phenomenon of clonal deletion/exhaustion that occurs during chronic viral infections with a high antigen load (Moskophidis et al. 1993; Zajac et al. 1998; Ahmed and Biron 1999). Finally, the last memory differentiation model we would like to discuss incorporates the development of 'central' and 'effector' memory T cells (Figure 7) (Sallusto et al. 1999). In this model, a shorter duration of antigenic stimulation favors the development of 'central' memory cells whereas longer duration favors 'effector' memory cells. 'Effector' memory cells ($CCR7^{-}$, $CD62L^{lo}$) are characterized by rapid acquisition of effector function and are found predominantly in nonlymphoid tissues, whereas 'central' memory cells ($CCR7^{+}$, $CD62L^{hi}$) are located within lymphoid tissues and presumably respond slower than 'effector' memory cells. However, it should be noted that even the 'central' memory cells respond much faster than naïve T cells. There are some interesting parallels between the 'decreasing potential' model and the model incorporating the development of 'central' and 'effector' memory T-cell subsets. In both models, the duration of antigenic stimulus regulates differentiation and a more prolonged antigenic stimulus favors cells with a more effector phenotype (i.e. terminally differentiated cell) (Kaech et al. 2002).

MAINTENANCE OF IMMUNOLOGICAL MEMORY

How is immunological memory maintained? This question has fascinated immunologists, microbiologists, and infectious disease specialists for many years, and there has been great interest in understanding the mechanisms that maintain long-term protective immunity. This infor-

Table 7 *Markers that distinguish naıve,¨effector, and memory T cells*[a]

	Naive	Effector	Memory
Group A			
CD44	Low	High	High
CD11a (LFA-1)	Low	High	High
Ly-6C	Low	High	High
CD122 (IL-2Rβ)	Low	High	High
Group B			
CD25 (IL-2Rα)	Low	High	Low
CD43 (high M_r form)	Low	High	Low
CD80 (B7-1)	Low	High	Low
CD38	Low	High	Low
MHC class II	Low	High	Low
Group C			
CD62L (L-selectin)	High	Low	High/Low
CCR7	High	Low	High/Low
CD45RA	High	Low	High/Low
CD45RO	Low	High	Low/High

a) Not a comprehensive list but illustrates the three patterns of changes seen as naïve T cells go through the naïve→effector→memory transition. Some surface markers (group A) are expressed at low levels in naïve cells, then upregulated upon antigen activation (effector cells) and maintained at high levels in memory T cells. Other markers as shown in group B are only transiently upregulated in effector cells and can be useful in discriminating between memory and effector T cells. Finally, a third group of markers (group C) show a much more gradual change during the effector-to-memory transition and have turned out to be very useful in identifying different subsets of memory T cells, i.e. lymphoid versus nonlymphoid. This group of markers may also be useful in identifying the various stages of memory T-cell differentiation.

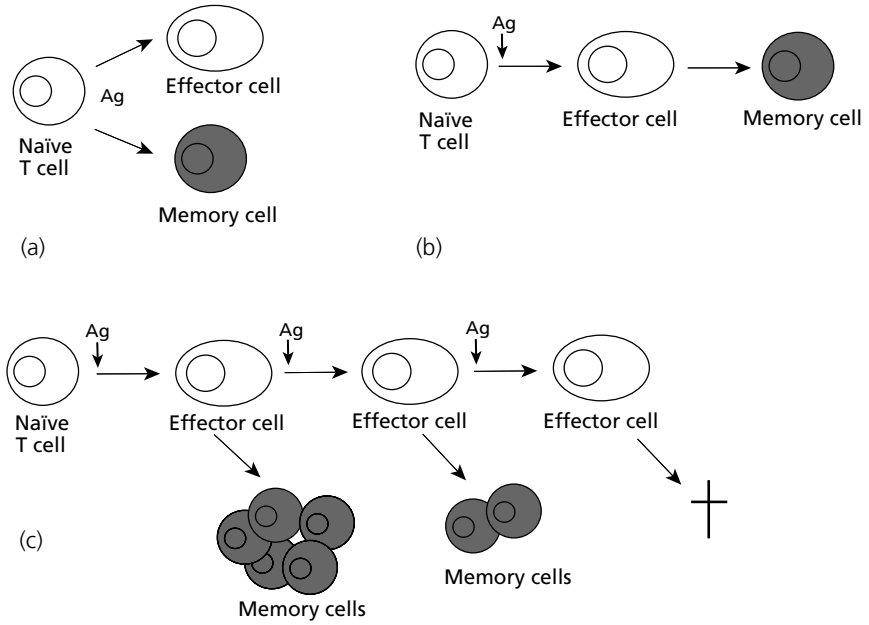

Figure 6 *Models of memory T-cell differentiation.* **(a)** *Model 1 represents the B-cell/germinal center paradigm of dichotomy in memory and effector pathways.* **(b)** *Model 2 is the more traditional view of memory T-cell differentiation representing a linear differentiation pathway.* **(c)** *Model 3 is a variation of model 2 and takes into account the finding that only 5–10 percent of the effector cells survive to become memory T cells. In this model, progress toward terminal differentiation (driven by antigen – Ag) is accompanied by increased susceptibility to apoptosis and a decreased potential for memory cell development. In all of these models the effector cell represents a transient population whereas the memory cells survive for long periods. On re-exposure to antigen, the memory T cells can develop into effector cells and also generate more memory cells.*

mation is important for a fundamental understanding of both immunological memory and vaccine development.

The mechanisms involved in sustaining immunological memory are listed in Table 8. These can be divided into two categories: antigen dependent and antigen independent. One of the most effective means of maintaining immunity is periodic re-exposure to the pathogen. Such reinfections are usually asymptomatic or produce only mild clinical symptoms and serve as a natural 'booster' to the immune system. The importance of this mechanism is documented by epidemiological studies which show that protective immunity is maintained for longer periods in individuals living in areas endemic for a given disease. Having said this, it is equally, if not more, important to point out that there are several striking examples of long-term protective immunity in the absence of re-exposure to the pathogen. Three such examples are given in Table 9. These observations were crucial in shaping our ideas about immunological memory because they showed that the immune system could remember an encounter that occurred many years before and that there are mechanisms for sustaining this long-term memory (Panum 1847; Paul et al. 1951; Sawyer 1931).

Among the 'not reinfection' mechanisms of maintaining immunity, two are antigen dependent: microbial

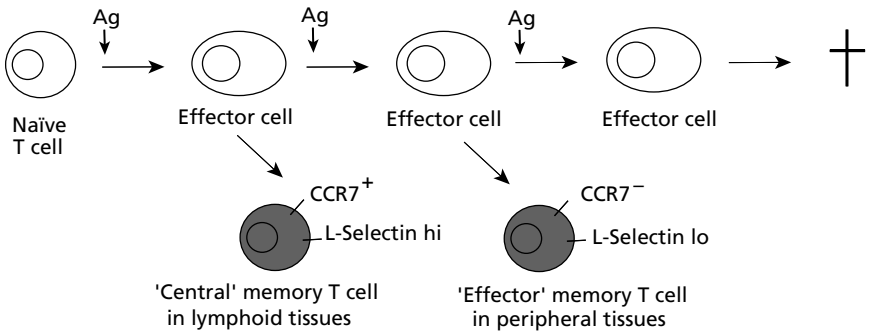

Figure 7 *Model of memory T-cell differentiation incorporating the development of 'central' and 'effector' memory T cells. In this model a short duration of antigenic stimulation favors the development of 'central' memory cells whereas longer duration favors differentiation to 'effector' memory T cells. Ag, antigen.*

Table 8 *Maintenance of immunological memory*

Antigen-dependent
1. Re-exposure to pathogen can serve as a periodic natural booster to the immune system
2. Low grade chronic/latent infection. Many pathogens can persist at low levels and provide a continuous/intermittent stimulus to memory T and B cells
3. Antigen–antibody complexes on follicular dendritic cells can persist for extended periods and provide a reservoir of antigen that possibly stimulates memory B cells and CD4 T cells
Antigen-independent
1. Long-lived plasma cells can constitutively produce antibody, sustaining serum antibody levels in the absence of antigen
2. Memory B and T cells can persist and maintain their numbers in an antigen-free environment. Memory T cells can undergo homeostatic proliferation to replenish their pool; this homeostatic proliferation does not require stimulation with antigen
3. Memory CD8 and CD4 T cells can retain their rapid responsiveness in the absence of antigen and are able to confer protective immunity

There are antigen-dependent and antigen-independent mechanisms of maintaining long-term immunity to infection.

persistence and antigen depots. A number of microbes can persist at low levels in healthy individuals and this chronicity can provide either a continuous or an intermittent antigenic stimulus to the immune system. This can be an effective mechanism of maintaining a higher frequency of antigen-specific T and B cells but requires a careful balance between pathogen levels and immune responses. This critical balance is necessary to avoid excessive immunopathology and also to avoid overstimulation of the antigen-specific T cells to such an extent that these cells get deleted or functionally exhausted (Moskophidis et al. 1993; Zajac et al. 1998). Pathogens that cause latent infection with periodic reactivation, such as the herpesviruses (Epstein–Barr virus (EBV), herpes simplex virus (HSV), varicella-zoster virus (VZV)) exhibit a lifestyle that is perhaps most conducive to providing an antigenic stimulus for maintaining long-term immunity.

The second antigen-dependent mechanism, antigen depots, relies on the ability of FDCs to trap antigen–antibody complexes on their cell surface and retain them for extended periods of time (Mandel et al. 1980; MacLennan 1994; Bachmann et al. 1996; Zinkernagel et al. 1996; Dutton et al. 1998; Ahmed and Biron 1999; Freitas and Rocha 2000). FDCs express Fc receptors on their cell surface and antigen–antibody complexes can bind to these Fc receptors. It appears that FDCs do not internalize these antigen–antibody complexes but instead display them on their cell surface for long periods, thus promoting maintenance of GCs and continued affinity maturation of B cells. It is generally agreed that antigen is required to maintain GC reactions, to sustain the rapid B-cell proliferation, provide a substrate for positive selection, and prevent apoptosis (MacLennan 1994). Some studies have suggested that antigen can persist on FDCs for more than a year (Mandel et al. 1980). Data from the only study directly tracking antigen in GCs after a viral infection suggest that GCs are sustained by antigen on FDCs for about 30 days after clearance of the virus, after which there is a greater than 90 percent drop in the number of GCs and a greater than 90 percent drop in the amount of viral antigen bound by the FDCs (Bachmann et al. 1996).

It is unlikely that viral particles or protein antigens in vivo can survive the wear and tear of antibody binding for long before being broken into pieces or destroyed. It has been postulated that this trapped antigen directly stimulates the specific B cells and can also be acquired by the B cells, processed and then presented via MHC class II molecules to CD4 T cells (Mandel et al. 1980; MacLennan 1994). Thus, it appears that FDC-trapped antigen can be used for stimulating both memory B cells and CD4 T cells. Many interesting questions about FDCs remain unanswered. What is their half-life? Do these cells divide? What prevents the antigen–antibody complexes from being degraded and/or internalized by the FDCs? What is the half-life of antigen presented by an FDC? It will be important to study FDCs in more detail for better understanding of their role in maintaining B- and T-cell memory.

During the past decade major advances have been made in identifying antigen-independent mechanisms of maintaining immunological memory (Ahmed and Gray 1996; Dutton et al. 1998; Freitas and Rocha 2000; Marrack et al. 2000; Lanzavecchia and Sallusto 2001; Sprent and Surh 2001). These studies were greatly facilitated by the development of new and sensitive techni-

Table 9 *Long-term immunity in the absence of re-exposure to the pathogen*

Infection	Duration of immunity (years)	Reference
Measles on the Faroe Islands	65	Panum (1847)
Yellow fever virus in Norfolk, VA, USA	75	Sawyer (1931)
Polio in remote Eskimo villages	40	Paul et al. (1951)
Inactivated poliovirus immunization in Sweden	30+	Bottiger et al. (1998)
Immune memory after smallpox vaccination	30–70+	Crotty et al. (2003a), Hammerlund (2003)

ques for assessing T- and B-cell functions at the single cell level and, even more importantly, by the ability physically to identify antigen-specific T cells using MHC class I and class II tetramers (Altman et al. 1996; Crawford et al. 1998; Murali-Krishna et al. 1998; Butz and Bevan 1998). Also, the use of transgenic T and B cells in mouse model systems has allowed us to monitor the immune response in vivo as naïve cells get activated and differentiate into effector and memory cells (Kearney et al. 1994; Zimmermann et al. 1999). Using a combination of these approaches to analyze a variety of immune responses in mice (using both infectious and noninfectious model systems), it is now clear that both memory CD4 and CD8 T cells, as well as memory B cells, can persist in the absence of antigen (Hou et al. 1994; Lau et al. 1994; Mullbacher 1994; Ahmed and Gray 1996; Tanchot et al. 1997; Dutton et al. 1998; Markiewicz et al. 1998; Murali-Krishna et al. 1999; Swain et al. 1999; Freitas and Rocha 2000; Marrack et al. 2000; Maruyama et al. 2000; Lanzavecchia and Sallusto 2001; Sprent and Surh 2001). It has also been shown that memory T cells can undergo homeostatic proliferation to replenish their numbers and that this proliferative renewal does not require stimulation with antigen (Tanchot et al. 1997; Murali-Krishna et al. 1999; Swain et al. 1999; Sprent and Surh 2001; Kassiotis et al. 2002). Taken together, these experimental studies done in mice have unequivocally shown that memory B and T cells can persist for extended periods (2 years or more) in the absence of antigen.

What about the situation in humans? Here the results are less definitive, but there are data showing long-term memory under conditions where antigen persistence is unlikely, e.g. it has been shown that vaccinia virus-specific memory CD8 T cells can be detected in individuals immunized more than 30 years earlier (Demkowicz et al. 1996; Crotty et al. 2003a; Hammarlund et al. 2003). It is unlikely that this long-term CTL memory is the result of antigenic stimulation, because vaccinia virus does not cause a chronic or latent infection in humans and there is no possibility of re-exposure to vaccinia virus, because vaccination against smallpox virus was discontinued in 1977. There are also studies documenting persistence of memory CD4 T cells to tetanus toxoid many years after immunization (Helms et al. 2000). There is also data showing that memory B cells persist for greater than 50 years after smallpox vaccination (Crotty et al. 2003a). Nevertheless, there is clearly a need for more detailed analysis of memory responses in humans. It would also be worthwhile to pursue such studies in nonhuman primates.

What factors control antigen-independent maintenance of memory B and T cells? The answer to this question is unclear for memory B cells, but this issue of homeostatic maintenance of memory has received a great deal of interest in the study of memory T cells, and it is now clear that cytokines (such as IL-15 and IL-7) play a major role in controlling homeostatic proliferation (Sprent and Surh 2001; Becker et al. 2002; Goldrath et al. 2002; Kaech et al. 2002; Kieper et al. 2002; Tan et al. 2002).

It is clear that there are antigen-independent mechanisms for maintaining memory B and T cells, but what about antibody production? The traditional view has been that plasma cells are short-lived cells (half-life estimates ranging from 3 days to at most 2–3 weeks), and that continuous antigenic stimulation of memory B cells is essential to replenish the pool of rapidly dying plasma cells and thereby maintain antibody production. This view has been challenged by recent studies showing that plasma cells can live for extended periods and that some plasma cells can survive for the life of the mouse (Manz et al. 1997, 1998; Slifka et al. 1998; Helms et al. 2000). This finding may provide an explanation for the remarkable longevity of antibody responses seen in humans after certain acute infections and immunizations (Ahmed and Gray 1996; Slifka and Ahmed 1998). Many acute viral infections induce serum antibody responses that persist for decades (Plotkin and Orenstein 1999), but what is even more striking is that some nonreplicating antigens can also induce long-term humoral immunity. Perhaps the most impressive demonstration of this phenomenon is the large study of poliovirus immunity done in Sweden (Bottiger et al. 1998). The Swedish population is almost ideal for such a study, for several reasons: (1) poliomyelitis has been eliminated in Sweden since 1962; (2) only inactivated poliovirus vaccine (IPV) (the Salk vaccine) has ever been used in the Swedish population; (3) the vaccine is provided by a single, standardized supplier; (4) the final booster immunization is given at the young age of 5 years; (5) the enterovirus infection burden in Sweden is extremely low, and there are relatively few opportunities for introduction of poliovirus from foreign sources; and (6) Sweden has excellent healthcare records and public health surveillance. Given these factors, it is striking to see that, when the Swedish population was surveyed for poliovirus immunity in 1991, substantial antipoliovirus antibody titers were detected in all age groups (Bottiger et al. 1998). Interestingly, there was virtually no difference in serum antibody titers among the different age groups (all at >10 years after immunization), indicating that antipoliovirus antibody titers are stably maintained in the absence of additional immunizations or exposure to live virus (Bottiger et al. 1998). In addition, it is well described that people immunized with diphtheria or tetanus toxoid can have circulating antibody for more than 25, or even 50, years (Scheibel et al. 1966; Simonsen et al. 1984; Kjeldsen et al. 1985; Cohen et al. 1994; Bottiger et al. 1998).

CONCLUSION

Not all infections and vaccines induce long-term antibody responses and there are several instances where antibody responses decay rapidly (Plotkin and Orenstein

1999). The underlying reasons for this are not well understood. Are there only certain conditions and/or antigen formulations that can result in the generation of long-lived plasma cells? One possibility is that T-cell help is necessary and that only high-affinity B cells can differentiate into long-lived plasma cells, whereas low-affinity B cells give rise to short-lived plasma cells. What are the factors that regulate plasma cell survival? Is residence in the bone marrow essential for plasma cell survival? These issues about plasma cells need to be addressed to gain a better understanding of long-term humoral immunity.

In conclusion, in this chapter we have attempted to give an overview of the principles of immunological memory to infection. This remains one of the most exciting areas of immunology and infectious diseases and there are many challenges ahead. Although considerable progress has been made in the past few years, and some of our questions have been answered, many more remain, e.g. what is the molecular definition of a memory T cell? Are there memory specific genes? How are these cells 'wired', making them so different from naïve cells (Bachmann et al. 1999b)? What controls the homeostasis of memory T and B cells? How many memory cells can we accommodate as we age and encounter multiple infections (Selin et al. 1999)? What signals regulate the differentiation program of memory cells (Kaech and Ahmed 2001; Lanzavecchia and Sallusto 2001; van Stipdonk et al. 2001; Wong and Pamer 2001)? Answers to these and many other questions are needed to define the cellular and molecular basis of immunological memory.

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Lymphocyte homing

MICHEL AURRAND-LIONS AND BEAT A. IMHOF

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THE CONCEPT OF RECIRCULATION: LYMPHOCYTES VERSUS LEUKOCYTES

The immune system protects the body from invading microorganisms and parasites and surveys the tissues for expression of aberrant selfantigens. The principal players in this defense system are the leukocytes, and they are well equipped to cope with virtually any pathogen. However, most pathogens have the advantage of an extremely high proliferation rate, and prevention of systemic infections and damage requires appropriate rapid responses from innate and adaptive immune systems. Although all the cells of the innate immune system are able to mount fast responses against various antigens, the response of the adaptive, specific branch is greatly hampered by the low number of cells specific for a given antigen. To counterbalance this disadvantage, cells of the adaptive immune system, i.e. lymphocytes, follow distinct migration and recirculation routes in order to maximize a possible encounter with their cognate antigens and to be strategically positioned at sites of antigen entry.

Cells of the innate immune system are the first effector cells that arrive at the site of infection or tissue damage. As they constitute a uniform antigen nonspecific pool, they can be rapidly recruited in large numbers and, having exerted their function, they are deleted. The recirculation of lymphoid cells contrasts markedly to the unidirectional migration of cells of the innate immune system from blood into tissue. Lymphocytes have a complex migration pattern that is strongly dependent on their state of activation and memory. They have the ability to recirculate, i.e. to re-enter the systemic blood circulation from tissues and migrate to a distant site in the body (Figure 1). The reason for this recirculation behavior is presumably the relatively low number of antigen-specific lymphocytes that can be generated for each of the millions of antigens. Positive and negative selections of antigen receptors produce a vast number of specificities, but necessarily only a very small number of cells for any given specificity. Therefore, the migration of lymphocytes is highly organized to ensure an efficient exposure of lymphocytes to their cognate antigens in secondary lymphoid organs. Moreover, clones with relevant specificities, i.e. receptors that recognize antigenic epitopes of foreign organisms, are not lost after their engagement, but are preserved as memory cells for more rapid and stronger responses on reinfection with the same organism. This generation of memory within the specific immune system is the rationale for immunization and it can persist and protect an organism for the rest of its life. Thus, the constitutive recirculation of lymphocytes through secondary lymphoid tissues is distinct from the leukocyte recruitment occurring at sites of inflammation where vascularized tissues respond to damages.

MIGRATION OF LYMPHOCYTES

The initial experiments to study the origin of cells in the lymph (hence the name lymphocytes) were conducted

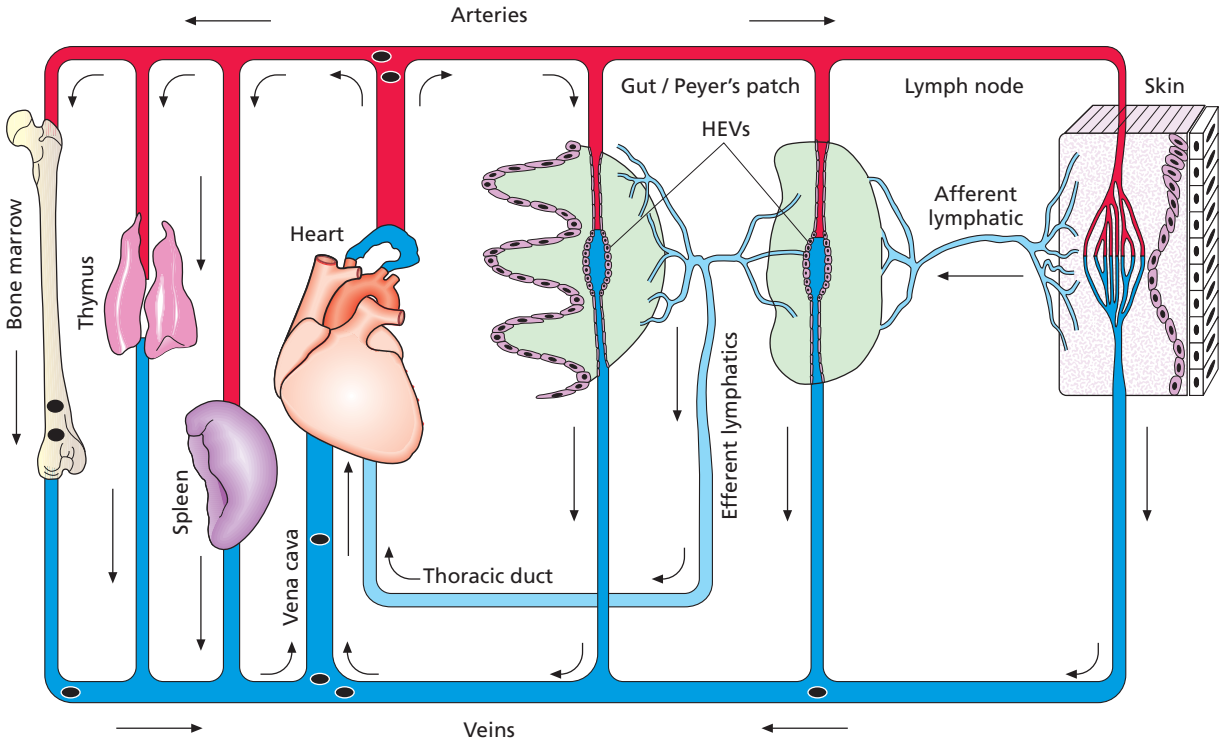

Figure 1 *Routes of lymphocyte migration and recirculation. The diagram depicts blood and lymphatic connections between primary and secondary lymphoid organs. The naïve lymphoid cells are located in the thymus (T cells) or bone marrow (B cells) and reach the secondary lymphoid organs as indicated. Lymphoid cells circulating through the blood system enter in organs through postcapillaries venules (skin) or high endothelial venules (HEVs) in Peyer's patches and lymph nodes. The second system used by lymphocytes is the lymphatic system (in black), which drains immune cells from peripheral tissues through lymph nodes and mucosa-associated tissues (MALT) back to the blood via the thoracic duct.*

by James Gowans and colleagues (Gowans 1957; Gowans and Knight 1964). They were the first to show that cells in the lymphatics are blood-borne cells that have the ability to circulate between blood and lymph in order to seek out their cognate antigen, constituting the so-called immune surveillance (Pabst and Binns 1989; Salmi and Jalkanen 1997). Westermann and Pabst have estimated that, in humans, about 5×10^{11} lymphocytes leave the blood each day to enter various organs and then return to the blood (Westermann and Pabst 1990, 1996). In lymphoid organs, lymphocyte extravasation occurs in the postcapillary venules (Gowans and Knight 1964) at specialized postcapillary vascular sites called high endothelial venules (HEV) (Anderson and Anderson 1976; Anderson et al. 1976). These represent the major constitutive extravasation route in vivo (Lasky 1992), and most recirculating lymphocytes selectively bind to the endothelium of HEVs, while ignoring normal vascular endothelium (Mackay et al. 1990). In humans, HEVs are found in all secondary lymphoid organs except the spleen. Endothelial cells of HEVs have a cuboidal appearance that differs considerably from the flat morphology of other endothelial cells (Girard and Springer 1995). In addition to this characteristic morphology, endothelial cells of HEVs differ from others by the specific expression of several genes increasing their adhesive properties for lymphocytes (Girard et al. 1999). Consequently, leukocytes are more likely to collide with, adhere to, and migrate across endothelial cells of HEVs than any other nonstimulated resting endothelial cells (Bjerknes et al. 1986; Johnson-Leger et al. 2000). This constitutive specialization of HEVs to sustain leukocyte recirculation contrasts sharply with the situation at inflamed sites, where the damaged tissue triggers new adhesive properties in the adjacent endothelium in order to induce directional migration of lymphocytes toward the site where the immune effector function takes place (Muller 2002).

STRUCTURE AND ORGANIZATION OF LYMPHOID TISSUES

Lymphoid structures are divided into primary and secondary lymphoid organs. The primary lymphoid organs are the thymus and the bone marrow in mammals, which constitute the respective sites of differentiation for T and B cells. Their function is to sustain the lymphoid differentiation toward the stage of acquisition of immunocompetence, e.g. expression of functional antigen-specific receptors on the surface of lymphoid cells. Such cells are named naïve. Precursor lymphoid cells, also termed 'stem cells', are located in the bone

marrow and differentiate in B cells or T-precursor cells, before leaving this organ and seeding secondary lymphoid organs in the case of B cells or thymus in the case of T-precursor cells. Although the migration of T-precursor cells to the thymus is essential for T-cell differentiation, studies of the molecular mechanisms of homing have been hampered by the minute number of precursor T cells leaving the bone marrow and seeding the thymus. Once naïve cells have completed their differentiation in primary lymphoid organs, they specifically migrate to secondary lymphoid organs.

The secondary lymphoid organs encompass the spleen, peripheral and mesenteric lymph nodes, mucosa-associated lymphoid tissue (MALT) including tonsils, and gut-associated lymphoid tissue (GALT) such as Peyer's patches and the appendix. Functionally, the lymphoid organs can be classified into three distinct groups: MALT traps antigens that enter the body through various mucous membrane surfaces, peripheral lymph nodes (PLN) collect antigen from the intercellular tissue fluids, whereas the spleen filters blood-borne antigens (see Figure 1). Although important morphological differences exist among the different secondary lymphoid organs, they all have the common function of concentrating B, T and antigen-presenting cells at unique sites in order to mount efficient immune responses. This is reflected by the presence of discrete structures named lymphoid follicles that can be distinguished histologically in all secondary lymphoid organs. They are especially abundant in mucosal surfaces and consist of aggregates of various lymphoid cells surrounded by a network of draining lymphatic capillaries. These structures are surrounded by a fibrous capsule and contain distinct regions of B- and T-cell activity. Lymph nodes are the most complex secondary lymphoid organs, organized in distinct microenvironments for antigen presentation and lymphocyte recirculation. Afferent lymphatic vessels collect the lymph, antigen-presenting cells (dendritic cells), and lymphocytes from tissue and drain them to peripheral lymph nodes or to intestinal/mesenteric lymph nodes. At the same time, blood-borne lymphocytes enter these lymph nodes via specialized postcapillary venules, the HEVs, in order to encounter the resident cells as well as the cells entering via the afferent lymph. The lymph nodes thus constitute a point where the pathways for antigens and specific effector cells converge. Efferent lymphatic vessels drain the nodes and carry lymph and lymphocytes via the thoracic duct back to the systemic blood circulation into the subclavian vein.

The spleen is adapted to respond to systemic infections by filtering the blood and trapping blood-borne antigens. In contrast to all the other secondary lymphoid organs, the spleen is not connected to the lymphatics and therefore does not directly communicate with the tissues. Furthermore, the spleen lacks HEVs: circulating cells and antigens have unhampered access to this organ through arterioles that terminate open-ended in the sinuses of the marginal zone and through the lack of HEVs. As a result of the high blood flow, the spleen has by far the highest lymphocyte throughput of all lymphoid organs and thus is the organ that lymphocytes pass most frequently.

MOLECULAR MECHANISMS OF LYMPHOCYTE MIGRATION

The multistep paradigm

In the early 1990s, Eugene Butcher and Tim Springer (Butcher 1991; Springer 1994) proposed a multistep paradigm for leukocyte recruitment governed by a different class of adhesion molecules. Although specific molecular interactions have been demonstrated for subsets of leukocytes, and resting and inflammatory situations, leukocyte recruitment always occurs in four different steps, namely: tethering and rolling, triggering, firm adhesion, and transmigration (Butcher 1992). The four different steps involve different classes of adhesion molecules with specific properties. Although additional adhesion molecules have been involved in the initial step, leukocyte rolling is mainly mediated by weak interaction of selectins with their carbohydrate ligands on opposing cells. The rolling is a reversible step but is a prerequisite to the following triggering step by chemokines, leading to leukocyte integrin activation and firm adhesion. This is followed by the final transmigration step, also called diapedesis, which consists of ameboid movement of leukocytes between tightly apposed endothelial cells. This final step involves an active remodeling of interendothelial junctions and has recently received new insights with the discovery of proteins specifically involved in transmigration (Vestweber 2002). Although the models for constitutive and inflammatory lymphocyte recruitment are similar, different adhesion molecules are specifically expressed at sites of constitutive lymphocyte recirculation (HEV) or at sites of inflammatory-induced lymphocyte recruitment (Dailey 1998; von Andrian and Mackay 2000). The specific migration route taken by lymphocytes is controlled by the combined vascular expression of L-selectin ligands, E- and P-selectins, chemokines, integrin ligands, and interendothelial junctional molecules. Mirroring this endothelial diversification, lymphocyte subpopulations express themselves as specific sets of selectin, selectin ligands, chemokine receptors, integrins, and integrin ligands.

Tethering/rolling

The initial rolling step of lymphocytes along the vessel wall is known to depend mainly on weak interactions between selectins and their ligands. Three selectins,

named respectively L-, E-, and P- selectins for leukocytic, endothelial, and platelet selectins, have been identified (Vestweber and Blanks 1999). They all interact with carbohydrate structures and are involved in the regulation of both naive and effector/memory lymphocyte migration. As a result of their anchorage to the actin cytoskeleton, they initiate the adhesion cascade by slowing down the leukocyte along the vascular bed (Dwir et al. 2001) in order to allow the triggering and firm adhesion to occur. In addition, the engagement of selectins leads to signal transduction inside the rolling cells, which may participate in integrin activation (Sikorski et al. 1996; Simon et al. 2000). In contrast to the specific involvement of selectins in lymphocyte rolling, the integrin $\alpha_4\beta_7$ can contribute either to lymphocyte rolling or to firm adhesion (Holzmann et al. 1989; Berlin et al. 1993).

Tethering/rolling of naive lymphocytes

L-selectin is constitutively expressed on virtually all naïve lymphocytes, a portion of memory T cells, neutrophils, eosinophils, and monocytes (Lewinsohn et al. 1987), and is responsible for trafficking of naïve lymphocytes through lymph nodes (Berg et al. 1989). More precisely, L-selectin is implicated in the rolling of naïve lymphocytes along HEVs as demonstrated by real-time intravital video microscopy studies in animals treated with antibody against L-selectin (von Andrian 1996). This C-type lectin interacts with sialyl-Lewis^x structures (sLe^x) found on peripheral lymph node addressins (PNAd) expressed on HEVs such as GlyCAM-1, CD34, podocalyxin, Sgp200, and MAdCAM-1. The optimal binding of L-selectin to its ligands requires sialylation, fucosylation, and carbohydrate sulfation of these ligands by enzymes expressed in HEVs, as demonstrated by the reduced lymphocyte homing to lymph nodes observed in animals deficient for α(1,3)-fucosyltransferase (FucTVII) or *N*-acetyl glycosamine-6-sulfotransferase (HEC-GlcNAc6ST) (Maly et al. 1996; Hemmerich et al. 2001). It is therefore important to consider that optimal L-selectin activity requires expression of PNAd core proteins and post-translational modifying enzymes. Although interaction of L-selectin with its ligands accounts for most lymphocyte entry in lymph nodes, the homing to the mucosal associated tissue in the gut (gut-associated lymphoid tissue or GALT), especially to the Peyer's patches, is only moderately affected in animals deficient for L-selectin, fucosyl, or sulfotransferases (Arbones et al. 1994; Maly et al. 1996; Hemmerich et al. 2001). This is the result of the binding of $\alpha_4\beta_7$ integrin to MAdCAM-1 and to the expression of MAdCAM-1 by HEVs of Peyer's patches (Berlin et al. 1993). Indeed, mesenteric lymph nodes present the features of Peyer's patches and peripheral lymph nodes because they express only MAdCAM-1 on some HEVs, whereas others express MadCAM and L-selectin (Streeter et al. 1988a, 1988b; Berg et al. 1993). However, one could keep in mind that integrin $\alpha_4\beta_7$ represents one of the rare exceptions in which an integrin is used by lymphocytes for rolling.

The significance of integrin $\alpha_4\beta_7$ in lymphocyte homing is best shown in mice that are deficient in the integrin β_7 subunit. The GALT in these mice is severely impaired as a result of the failure of lymphocytes to migrate into the lamina propria, Peyer's patches, and the intraepithelial lymphocyte (IEL) compartment (Wagner et al. 1996). This lymphocyte's homing deficiency is not caused by a defect on HEVs, because naïve β_7-deficient lymphocytes transferred into normal mice normally home to peripheral and mesenteric lymph nodes via L-selectin, but fail to migrate to mucosal effector sites of the gut, i.e. Peyer's patches, lamina propria, and the IEL compartment (Lefrancois et al. 1999).

Tethering/rolling of antigen-primed lymphocytes

Once a naïve lymphocyte encounters its cognate antigen in lymphoid organs, it activates and undergoes dramatic changes in the expression of surface adhesion molecules as it differentiates into an effector or memory lymphocyte (Jung et al. 1988; Picker et al. 1990b; Williams and Butcher 1997). Although subtle differences in expression of adhesion molecules can be found between different effector/memory cell subpopulations (Sallusto et al. 1999; Campbell et al. 2001), the loss of L-selectin expression and the upregulation of ligands for E- and P-selectin by antigen-experienced cells prevent their entry into lymphoid organs (Rigby and Dailey 2000; Hafezi-Moghadam et al. 2001). Notably, this dogma has been recently revised with the identification of memory cell subpopulations that express high levels of L-selectin, migrate toward lymphoid organs, and are called central memory cells (Sallusto et al. 1999; Campbell et al. 2001). Upon leukocyte activation, the downregulation of L-selectin involves transcriptional regulation and protein shedding by surface metalloproteases (Kishimoto et al. 1989; Preece et al. 1996). According to these changes, tethering and rolling of lymphocytes in inflammatory conditions mainly involve P- and E-selectins induced on vascular endothelium by inflammatory stimuli. P-selectin was originally identified in α granules of platelets and later found to be expressed in Weibel–Palade bodies of endothelial cells (Hsu-Lin et al. 1984; McEver et al. 1989). On appropriate stimulation with inflammatory factors such as histamine or thrombin, degranulation of these compartments leads to the rapid expression of P-selectin on the vascular cell surface (Hattori et al. 1989) making it available for interacting with the ligand P-selectin glycoprotein ligand-1 (PSGL-1) or CD24 (the old name was heat stable antigen or HSA) expressed on

the surface of effector lymphocyte subpopulations (Aigner et al. 1995; Borges et al. 1997). Although P-selectin is expressed within minutes of stimulation on the cell surface, E-selectin expression on endothelial cells is transcriptionally regulated by inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), or lipopolysaccharide (LPS), leading to expression within 4–24 h (Bevilacqua et al. 1987). Once expressed on the cell surface, E-selectin interacts with ligands expressed on leukocytes, namely E-selectin ligand-1 (ESL-1), PSGL-1, or L-selectin (Vestweber and Blanks 1999). Therefore one important aspect of lymphocyte migration toward the site of inflammation is the expression of E- and P-selectin ligands, among which PSGL-1 is the best characterized. Similar to the binding of L-selectin to the sugar moiety of PNAds, the binding of E-selectins to PSGL-1 requires carbohydrate modifications whereas P-selectin binding requires additional tyrosine sulfations (Pouyani and Seed 1995).

The physiological importance of post-translational modifications of PSGL-1 on lymphocytes is best demonstrated by the functional characterization of an inducible carbohydrate epitope expressed on PSGL-1 and named cutaneous lymphocyte-associated antigen (CLA) (Picker et al. 1990a; Berg et al. 1991). Expression of CLA on T-memory lymphocytes has been found to correlate with the ability of these cells to home to inflamed skin. CLA is a carbohydrate epitope attached to PSGL-1 by fucosyltransferase VII (Fuhlbrigge et al. 1997). The CLA-epitope mediates cell binding to E-selectin expressed on endothelial cells in inflamed skin (Picker et al. 1991) and thereby the recruitment of memory T cells. Differential regulation of CLA seems to occur during the activation of naïve T lymphocytes in peripheral lymph nodes (Borowitz et al. 1993; Picker et al. 1993). Following these initial findings, several studies have shown that the expression of L-selectin and E- and P-selectin ligands, in combination with chemokine receptor expression, can be used to subdivide the different effector and memory lymphocytes subsets upon antigenic challenge (Tietz et al. 1998; Sallusto et al. 1999; Campbell et al. 2001).

This contrasts with integrin β_7 expression which appears sufficient to sustain the migration of lymphocytes to the GALT upon antigenic activation (Rott et al. 1997, 2000; Williams and Butcher 1997; Rose et al. 1998). The unique migration pattern of $\alpha_4\beta_7$-expressing cells is confirmed by the fact that orally administrated antigens result in a higher incidence of antigen-specific cells expressing $\alpha_4\beta_7$ compared with systemic antigen exposure which generates antigen-specific cells within the L-selectin expressing compartment (Lakew et al. 1995; Kantele et al. 1997).

Although this is beyond the scope of this chapter, the reader should be aware that additional adhesion molecules have been implicated in the initial rolling step of lymphocytes (Table 1). This has been demonstrated by using blocking reagents for L-selectin function and the discovery of alternate molecular pathways using CD44, VAP-1, or α_4 integrins (Stoolman 1989; Picker et al. 1989; Alon et al. 1995; Salmi et al. 1997). Although $\alpha_4\beta_1$, CD44, and VAP-1 are all involved in adhesion of lymphocytes on inflammatory endothelium (Elices et al. 1990; Salmi et al. 1993; Maiti et al. 1998), their relative contributions to constitutive versus antigen-primed lymphocyte rolling remain to be formally established in vivo.

FIRM ADHESION: INTEGRINS GUIDE TO THE 'NO RETURN POINT'

In contrast to the rolling step supported by several classes of adhesion molecules, the subsequent lymphocyte arrest is almost exclusively mediated by activated integrin receptors (see Table 1). They constitute the most versatile adhesion molecules because their adhesive properties are regulated with the state of cell activation and because of the nature of activation signals to which leukocytes are exposed (Alon and Feigelson 2002). Integrins are formed of an α and a β subunit, and activation signals change their shape leading to increased affinity of integrins for their ligands (Shimaoka et al. 2002). This is known as a mechanism of inside-out signaling. At the same time, ligand engagement by integrins leads to signal transduction inside the cells in a process called outside-in signaling. Integrin activation occurs within fractions of seconds on chemokine triggering or ligand engagement, implying that most of the circulating lymphocytes express integrins in a nonactivated, resting state. Therefore, leukocyte integrins are exclusively triggered if the rolling step has occurred and if chemokines and endothelial ligands are locally expressed at the site of lymphocyte extravasation.

Among the different heterodimers, $\alpha_L\beta_2$ (LFA-1, CD11a/CD18), $\alpha_4\beta_1$ (VLA-4), and $\alpha_4\beta_7$ (LPAM-1) integrins are the key molecules that mediate firm adhesion of rolling lymphocytes by engagement with their counterreceptors, which include members of the immunoglobulin superfamily (IgSf) (Staunton et al. 1988). The latter can be tentatively classified into two groups: IgSf molecules that are constitutively expressed by endothelial cells at specific sites, such as MAdCAM-1 or intercellular adhesion molecule 2 (ICAM-2) (Staunton et al. 1989; Briskin et al. 1993), and those that are induced by inflammatory stimuli on endothelial cells such as vascular cell adhesion molecule 1 (VCAM-1) or intercellular adhesion molecule 1 (ICAM-1) (Marlin and Springer 1987; Elices et al. 1990). However, it has become clear that this classification is not absolute because constitutive expression of ICAM-1 and VCAM-1 on HEVs has been reported (Tamatani et al. 1991; Sasaki et al. 1996; Faveeuw et al. 2000). ICAM-1, ICAM-2, and intercellular adhesion molecule 3 (ICAM-3) are counterreceptors for $\alpha_L\beta_2$ integrin whereas VCAM-1 binds weakly to $\alpha_4\beta_7$ and is the main ligand

Table 1 *Cell adhesion molecules involved in lymphocyte homing*

Cell adhesion molecule	Expression	Ligand	Adhesion step	Implicated in
Integrins				
LFA-1 ($\alpha_L\beta_2$, CD11a/CD18)	Most leukocytes	ICAM-1, -2, -3	Tight adhesion	General role in extravasation
$\alpha_4\beta_1$ (VLA-4)	Lymphocytes	VCAM-1, fibronectin	Tethering and tight adhesion	Leukocyte homing to inflamed tissue
$\alpha_4\beta_7$	Lymphocytes	MadCAM-1, fibronectin, VCAM-1	Tethering and tight adhesion	Lymphocyte homing to MALT
Selectins				
L-Selectin (MEL-14, CD62L)	Most nonactivated leukocytes	sLe^x on GlyCAM, CD34 and MadCAM-1	Tethering	Lymphocyte homing to LNs, leukocyte homing to inflamed tissue
P-Selectin (CD62)	Activated platelets, inflammatory endothelium	PSGL-1	Tethering	Leukocyte recruitment by inflamed tissue
E-Selectin (CD62E)	Inflammatory endothelium	CLA, PSGL-1, ESL-1	Tethering	Leukocyte recruitment by inflamed tissue
Ig superfamily				
ICAM-1	Inflammatory endothelium, activated lymphocytes	LFA-1, $\alpha_M\beta_2$	Tight adhesion	Leukocyte recruitment by inflamed tissue
ICAM-2	Endothelium and resting lymphocytes	LFA-1	Tight adhesion	General role in leukocyte extravasation
VCAM-1	Inflammatory endothelium	$\alpha_4\beta_1$, $\alpha_4\beta_1$	Tight adhesion	Leukocyte recruitment by inflamed tissue
MAdCAM-1	Mucosal endothelium	L-selectin, $\alpha_4\beta_7$	Tethering and tight adhesion	Lymphocyte recruitment to mucosal tissue
PECAM	Endothelium, leukocytes, platelets	PECAM, $\alpha_V\beta_3$	Diapedesis	Monocyte recruitment to inflamed tissues
JAM-1	Endothelium, epithelium, leukocytes, platelets	JAM-1, LFA-1	Tight adhesion and diapedesis	General role in leukocyte extravasation
JAM-2 (also called JAM3)	Endothelium, activated lymphocytes, platelets	JAM-2, VE-JAM, Mac-1	Adhesion and diapedesis	General role in leukocyte extravasation
Carbohydrate ligands of selectins				
GlyCAM (glycosylation dependent CAM)	High endothelial venules	L-Selectin	Tethering	Recruitment of naïve lymphocytes to lymph nodes
CD34	Endothelium	L-Selectin	Tethering	Recruitment of naïve lymphocytes to lymph nodes and inflamed tissue
CLA (cutaneous lymphocyte antigen, carbohydrate structure on PSGL-1)	Activated lymphocytes	E-Selectin	Tethering	Homing of lymphocytes to inflamed skin
MAdCAM-1 (mucosal addressin CAM)	Mucosal endothelium	L-Selectin	Tethering	Recruitment of lymphocytes to MALT
PSGL-1 (P-selectin glycoligand-1)	Myeloid, lymphoid and dendritic lineages	P-Selectin, E-selectin	Tethering	Homing to inflamed tissue
Others				
CD43	All leukocytes except mature B cells	Sialoadhesin-1	Negative regulation of tethering	Leukocyte migration and activation
CD44	Broad distribution	Hyaluronan	Tethering	Function in cell migration to inflamed areas
VAP-1	High endothelial venules, inflammatory endothelium	Amine oxidase activity on primary amino groups	Tethering	Recruitment of naïve lymphocytes to lymph nodes and inflamed tissue

Subdivisions are presented according to the molecular nature of the different protein, and their classification in different protein families. Although different classes of adhesion molecules mediate tethering, firm adhesion and diapedesis of lymphocytes involve exclusively integrins and their counterreceptors. See text for abbreviations.

(with fibronectin) for $\alpha_4\beta_1$ integrin. Therefore, $\alpha_4\beta_1$ is predominantly involved in the migration of lymphocytes to sites of effector functions, whereas $\alpha_L\beta_2$ plays a dual role in constitutive and inflammatory induced migration.

Firm adhesion of naïve lymphocytes

The importance of $\alpha_L\beta_2$ (LFA-1) integrin in homeostatic homing of lymphocytes has been demonstrated in α_L-deficient mice that show reduced numbers of lymphocytes in PLNs (Berlin-Rufenach et al. 1999). LFA-1-mediated adhesion is clearly a downstream event of rolling mediated by L-selectin and does not occur with nonrolling cells (Warnock et al. 1998). Interestingly, residual homing to lymph nodes in LFA-1-deficient mice appears to depend on lymphocyte α_4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) and VCAM-1 expressed by HEVs. However, the expression of VCAM-1 on HEVs under noninflammatory conditions is still controversial (Hahne et al. 1993; May et al. 1993; Berlin et al. 1995; Henninger et al. 1997). Real-time video microscopy studies have shown that the sticking fraction of lymphocytes on HEVs of peripheral lymph nodes (which do not express MAdCAM-1 and cannot undergo $\alpha_4\beta_7$-mediated adhesion) is not affected by blocking antibodies against α_4 integrin (Warnock et al. 1998).

In contrast to $\alpha_4\beta_1$, the dual role of $\alpha_4\beta_7$ integrin in rolling and firm adhesion of naïve lymphocytes on MAdCAM-1 is clearly established (Berlin et al. 1995). This was demonstrated using preactivated naïve lymphocytes which require only $\alpha_4\beta_7$ for arrest on the HEVs of Peyer's patches (Bargatze et al. 1995). Therefore, the specific engagement $\alpha_L\beta_2$ or $\alpha_4\beta_7$ with its respective ligand depends mainly on activation signals received by transmigrating cells (Campbell et al. 1998), as illustrated by numerous studies showing that the signals required for integrin clustering and stable adhesion differ between integrin α_4 and integrin β_2 (Constantin et al. 2000; Ding et al. 2001; Shamri et al. 2002).

Firm adhesion of antigen-primed lymphocytes

Once lymphocytes are activated in secondary lymphoid organs, they change their expression pattern of chemokine receptors and adhesion molecules, as demonstrated by phenotypic analysis of surface antigenic markers. Although the original names 'Very late Antigens, VLA' are no longer relevant for most integrins, $\alpha_4\beta_1$ (VLA-4) expression increases after long-term T-cell activation (Hemler 1990). In contrast, $\alpha_L\beta_2$ (lymphocyte function antigen-1 (LFA-1)) was initially identified as a T-cell costimulatory molecule involved in T-cell effector function (Kaufmann et al. 1982), before being shown to participate in lymphocyte adhesion to ICAM-1 and ICAM-2 on endothelial cells (Dustin and Springer 1988; van Kooyk and Figdor 2000). Therefore, in addition to selectin, the migration of lymphocytes at sites of inflammation is regulated by integrin engagement with their ligands ICAM-1 and VCAM-1. The latter are expressed within hours on endothelial cells stimulated by inflammatory chemokines, but their expression peaks within days (Haraldsen et al. 1996). The importance of ICAM-1 and VCAM-1 in lymphocyte migration at inflammatory sites has been formally demonstrated using antibodies and small molecules that inhibit the ligand-binding activities of the integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$, thus dampening inflammatory reactions (Isobe et al. 1992; Kakimoto et al. 1992; Yednock et al. 1992; Issekutz 1993). The central role of LFA-1 engagement with its counter-receptor ICAM-1 in inflammatory reactions is further demonstrated by the decrease in allogeneic response and delayed-type hypersensitivity in mice with a genetically engineered ICAM-1 expression deficiency (Sligh et al. 1993; Xu et al. 1994). The same transgenic approach has also been used to inactivate the different integrin chains (β_2, α_L, α_4, and β_1) and VCAM-1, confirming to some extent the role of these molecules in lymphocyte migration and inflammatory reactions (Arroyo et al. 1996; 1999; Coxon et al. 1996; Huleatt and Lefrancois 1996; Berlin-Rufenach et al. 1999; Potocnik et al. 2000). However, in the cases of α_4 and VCAM-1 deficiencies, unexpected defects in placental and heart development have also been reported (Kwee et al. 1995; Yang et al. 1995), suggesting a pleiotropic role of integrins in cell migration, differentiation, and organogenesis.

CHEMOKINES SHOW THE WAY

Although selectins and integrins are key mediators of cell arrest along the vasculature, they are not sufficient in determining the specificity of lymphocyte migration. The ability of a circulating leukocyte to employ integrins for tight adhesion is determined by the expression of chemokine receptors that, on ligand binding, signal to and thereby activate the available integrins (Table 2).

Interestingly, chemokines and their receptors show a much higher degree of tissue specificity than the adhesion molecules. As a result of the slow, selectin-mediated rolling (see above), the lymphocyte's chemokine receptors interact with chemokines presented by the glycocalyx of the endothelium. This mechanism collects the tissue-specific information by triggering $G_{\alpha i}$-protein-linked signaling events which activate the leukocyte integrins. Activated integrins then mediate tight adhesion of the lymphocyte to vascular endothelium of tissue specific lymphoid organs, and this is followed by migration into the tissue (Pachynski et al. 1998). The secretion of chemokines by inflammatory tissue may bypass tissue homeostatic lymphocyte homing. This can occur anywhere in the body and it efficiently redirects the migration of lymphocytes. Furthermore, the ability of lymphocytes to respond to

Table 2 *Chemokine receptors and their ligands*

Receptor	Ligands (old name)	Expression profile lymphocytes	Functional role for lymphocytes	Condition of function
CXCR3	CXCL9 (MIG)	Activated Th1 cells < Th2, B cells	Recruitment of lymphocytes to Th1-type inflammation	Inflammation
	CXCL10 (IP-10)	NK cells		
	CXCL11 (I-TAC)			
CXCR4	CXCL12 (SDF-1)	Progenitor cells, resting T and B cells	Emigration of B cells from bone marrow, stem cell, and thymocyte homing	Constitutive
CXCR5	CXCL13 (BCA-1, BLC)	B cells, memory T cells	Lymphocyte migration to B-cell follicles	Constitutive
CXCR6	CXCL16	Memory T cells	Migration of Th1 cells to inflammatory tissue	Inflammation
CCR1	CCL3 (MIP-1α)	Memory T cells	Migration of T cells to inflammatory tissue	Inflammation
	CCL5 (RANTES)			
	CCL7 (MCP-3, MARC)			
	CCL14 (HCC-1, CKβ1)			
	CCL15 (MIP-5, HCC-2, Lkn-1)			
	CCL16 (HCC-4, LEC, Mtn-1, LCC-1)			
	CCL23 (MPIF-1, ckβ8-1, ckβ-8)			
CCR3	CCL5 (RANTES)	Activated Th2 > Th1 cells	Migration of T cells to Th2-type inflammation	Inflammation
	CCL7 (MCP-3, MARC)			
	CCL8 (MCP-2)			
	CCL13 (MCP-4)			
	CCL15 (HCC-2, Lkn-1, MIP-5)			
	CCL24 (MPIF-2, eotaxin-2)			
	CCL26 (MIP-4α, eotaxin-3)			
CCR4	CCL17 (TARC)	Activate Th2 > Th1 cells	Migration of T cells to Th2-type inflammation	Inflammation
	CCL22 (MDC, STCP1, abcd-1)			
CCR5	CCL3 (MIP-1α)	Progenitors, activated Th1 cells	Migration to Th2-type inflammation	Inflammation
	CCL4 (MIP-1β)			
	CCL5 (RANTES)			
CCR6	CCL20 (LARC, MIP-3αEXODUS)	Memory T cells	Migration to inflammatory skin and other tissue	Inflammation
CCR7	CCL19 (ELC, MIP-3β, β11)	Resting and activated T cells, B cells	Migration to secondary lymphoid organs	Constitutive
	CCL21 (6Ckine, SLC, ckβ9)			
CCR8	CCL1 (I-309)	Th2 cells	Migration to Th2-type inflammation	Inflammation
	CCL4 (MIP-1β)			
CCR9	CCL25 (TECK, ckβ15)	$\alpha_4\beta_7^+$ T cells, thymocytes, IgA-secreting B cells	Homing to the gut, thymocyte selection	Constitutive
CCR10	CCL27 (CTACK, ILC, ESkine)	CLA^+ T cells	Homing to skin	Constitutive
XCR1	XCL1 (lymphotactin, ATAC)	Recent activated T cells, B cells		Inflammation
	XCL2			
CX3CR1	CX3CL1 (fractalkine, neurotactin)	Activated T cells	Migration to inflammatory tissue	Inflammation

Chemokine receptors are proteins that contain a seven-transmembrane ('serpentine') spanning domain, which can associate with trimeric G-proteins of the Gαi family. Upon chemokine binding, these receptors trigger a $G_{\alpha i}$-mediated signaling cascade that can lead to the activation of leukocyte integrins (Zlotnik and Yoshie 2000). See text for abbreviations.

chemokines depends on the expression of the appropriate chemokine receptor (Table 2).

Homeostatic homing of lymphocytes

MIGRATION OF NAÏVE LYMPHOCYTES TO SECONDARY LYMPHOID TISSUE

On the search for antigens, thymus-emigrating naïve T cells have to enter secondary lymphoid organs. They enter through postcapillary venules made of high cuboidal cells, the HEVs. The HEVs themselves and nonlymphoid cells in T-cell-rich areas of lymph nodes, spleen, and Peyer's patches produce the chemokine CCL21/SLC (Gunn et al. 1998b). It triggers rapid activation of integrins LFA-1 and $\alpha_4\beta_7$ and arrests rolling lymphocytes by signaling through the CCL21/SLC receptor CCR7. T lymphocytes deficient in CCR7 show very poor homing to lymph nodes and Peyer's patches (Nakano et al. 1997; Campbell et al. 1998; Gunn et al. 1998b, 1999) and plt mice, which lack CCL21/SLC, show impaired lymphocyte homing (Gunn et al. 1999). After entering the lymphoid organ T cells they have to be correctly positioned. The chemokine receptor CCR7 is also involved in this positioning mechanism but here it uses both ligands, CCL21/SLC and CCL19/ELC (Cyster 1999). Endothelial and stromal cells in T-cell zones produce CCL21/SLC, and dendritic cells and macrophages in T-cell areas make CCL19/ELC. Some of the CCL19/ELC, however, is transcytosed across to HEVs and has also a small effect on T-cell entry into the organ (Baekkevold et al. 2001).

The chemokine responsible for entrance of naïve B cells into lymphoid organs is not known. It is certainly different from CCL21/SLC because absence of CCR7 and/or CCL21/SLC does not dramatically change B-cell homing (Forster et al. 1996; Gunn et al. 1999). Inside the organ, however, B cells use CXCL13/BLC and its receptor CXCR5 to migrate into lymphoid follicles (Forster et al. 1994, 1996; Ansel et al. 2000). Stromal cells and follicular dendritic cells produce CXCL13/BLC in B-cell-rich lymphoid follicles (Gunn et al. 1998a). On B-cell activation the expression of the CXCL13/BLC receptor, CXCR5, is downregulated, probably allowing emigration – release of activated or memory B cells from follicular zones and lymphoid organs.

Polarization and tissue-specific imprinting of lymphocytes in secondary lymphoid organs

NAÏVE LYMPHOCYTES

Naïve lymphocytes enter the different secondary lymphoid organs such as spleen, lymph nodes, Peyer's patches, or tonsils more or less randomly via the chemokine CCL21/SLC and its receptor CCR7. Encounter of antigens leads to activated/memory lymphocytes and to reprogramming of homing properties corresponding to the lymphoid organ microenvironment (Campbell and Butcher 2002; Kunkel and Butcher 2002). It is therefore conceivable that leukocyte subset-specific expression of chemokine receptors may help selectively to recruit only certain subsets of cells to peripheral or lymphoid tissues, e.g. T-cell activation in mucosal lymph nodes induces loss of CCR7 and the expression of CCR9, a receptor for the chemokine CCL25/TECK. This chemokine is abundantly produced by the small intestine and partly by the thymus, and leads to homing of activated or memory T cells to the gut. Alternately, T-cell activation in skin-draining lymph nodes leads to expression of CLA (a skin-specific selectin-binding molecule – see above) and the chemokine receptors CCR4 and CCR10 (Campbell et al. 1999; Kunkel and Butcher 2002). These receptors bind the chemokines CCL17/TARC and CCL27/CTACK respectively. Both chemokines are constitutively produced by cutaneous epithelial cells and induce skin homing of lymphocytes expressing CCR4 and CCR10.

ANTIGEN-ACTIVATED LYMPHOCYTES

Activated/memory T cells were first thought to be mostly CCR7, i.e. unable to re-enter lymphoid organs. This was revised by the finding that $CCR7^+$ and $CCR7^-$ cells existed in these populations and led to the concept that central and effector memory T cells exist (Sallusto and Lanzavecchia 2000). In addition to the tissue-specific chemokine receptors, central memory cells retain their expression of CCR7 and L-selectin and can home back to lymphoid organs to participate in activation of naïve B and T cells. However, this concept has recently been challenged by the finding that cytokine-producing effector cells express CCR7 and migrate back to lymphoid organs (Kim et al. 2001; Debes et al. 2002; Unsoeld et al. 2002). Nevertheless, a large proportion of effector memory or long-term-activated cells lack CCR7 and L-selectin, and migrate to peripheral organs. Both populations may migrate to sites of inflammatory reactions. The $CD4^+$ T cells are of either central or effector subtype whereas $CD8^+$ T cells are mostly of effector type.

T-HELPER 1 VERSUS T-HELPER 2 LYMPHOCYTES

In addition to tissue-specific imprinting activated/memory, CD4 T cells in lymphoid organs polarize their function into T-helper 1 (Th1) and T-helper 2 (Th2) cells (Kim et al. 2001). The Th1 cells secrete cytokines such as lymphotoxin and IFN-γ to promote mainly antiviral cellular immune responses. Th2 cells release IL-4, IL-5, IL-6 and IL-13 to stimulate humoral immunity and allergic reactions, and to augment innate immune reactions. The expression pattern of chemokine receptors not only distinguishes the major lymphocyte subsets

from one another, but might also be in fact the key for differential migration characteristics of Th1 versus Th2 cells (Sallusto et al. 1998). Analysis of chemokine receptors on in vitro cultured lymphocytes has attributed expression of CXCR3 and CCR5 to Th1 and of CCR3 and CCR4 to Th2 (Sallusto et al. 1999). However, analysis of primary human T-helper cells revealed a chemokine expression pattern that was slightly more complex (Kim et al. 2001). It showed that the only chemokine receptors that defined helper cell populations were CXCR3 on Th1 cells, and nonexclusive but prevalent expression of CCR4 on Th2 cells. Different subgroups of Th1 cells showed various expression levels of CCR2, CCR5, and CXCR6. With the exception of CCR4 none of the chemokine receptors was exclusive for Th2 cells, including CCR3 for which data are controversial (Imai et al. 1999; Romagnani et al. 1999). The receptors CXCR5, CCR7, and CCR6 were negatively regulated in respect to T-cell polarization, i.e. these receptors are expressed by T cells with reduced polarity such as Th0 cells. Recent data also showed that there are more $CCR7^+$ than $CCR7^-$ T-helper cells in the blood, and the authors concluded that CCR7 expression may be sufficient to enter lymphoid tissue but its presence did not prevent homing of polarized cells into peripheral, nonlymphoid tissue (Kim et al. 2001).

B LYMPHOCYTES

B lymphocytes show less subtle polarization than T cells. However, when B-cell precursors mature into antibody-secreting plasma cells they home to the splenic red pulp, lymph node medullary cords, and bone marrow, where they become resident. Upon differentiation plasma cells express the CXCL12/SDF receptor CXCR4 and they downregulate the CXCL13/BLC and CCL19/ELC receptors, CXCR5 and CCR7, respectively (Wehrli et al. 2001; Hauser et al. 2002; Kunkel and Butcher 2002). Thus, responsiveness to CXCL12/SDF-1 allows B plasma cells to home to secondary lymphoid organs and the bone marrow. Natural antibody-producing B1 cells, however, maintain CXCL13/BLC receptors and use them for migration to the peritoneal cavity and the omentum (Ansel et al. 2002).

Migration of antigen-primed lymphocytes to inflammatory tissue

Inflammatory reactions locally induce the production of inflammatory chemokines by endothelial cells, epithelial cells, dendritic cells, resident macrophages, or other cells. Thereby, the type of inflammatory response is important and selects for the type of chemokines to be produced, e.g. acute Th1 reactions start by bacterial or viral infections that induce the secretion of IFN-γ, TNF-α, IL-1, or IL-12. These inflammatory cytokines stimulate the production of the CXCR3-binding chemokines CXCL10/IP-10, CXCL9/MIG, and CXCL11/I-TAC, and they attract mainly CXCR3-expressing Th1 cells. In contrast, allergic Th2 inflammatory reactions are induced by the cytokine IL-4 which leads to the production of the CCR4-binding chemokines CCL22/MDC or CCL17/TARC, attracting CCR4-expressing Th2 cells. However, subgroups of Th1 and Th2 cells express additional chemokine receptors that are less exclusive (see above). This has the consequence that lymphocytes recruited by inflammatory reactions show mixed populations with prevalent tissue-specific Th1 or Th2, skin- or gut-seeking cells.

DIAPEDESIS

Following rolling, triggering and firm adhesion is a final irreversible step of transmigration, also called diapedesis. The pathway used by transmigrating cells to cross the vascular barrier is still a matter of debate because there is evidence for both transcellular and paracellular migration of leukocytes (Muller 2001; Indrasingh et al. 2002). Therefore, the molecular mechanisms involved in this final step are still poorly understood even if recent findings suggest that molecules concentrated at interendothelial junctions regulate leukocyte transmigration, thus favoring the hypothesis that lymphocyte transmigration occurs at interendothelial contacts (Dejana et al. 2001; Aurrand-Lions et al. 2002; Luscinskas et al. 2002; Vestweber 2002). So far, interendothelial adhesion molecules playing a role in the control of leukocyte transmigration can be classified as follows:

- Proteins concentrated in intercellular contacts and interacting with counterreceptors on transmigrating cells such as PECAM-1/CD31, CD99 and JAMs (Muller et al. 1993; Martin-Padura et al. 1998; Cunningham et al. 2002; Schenkel et al. 2002).
- Structural molecules specifically incorporated in endothelial adherens or tight junctions which are involved in endothelial barrier function, such as VE-cadherin, occludin, or claudins (Allport et al. 2000; Burns et al. 2000; Kucharzik et al. 2001).

In contrast to claudin and JAM family members, which are not equally expressed by all endothelial cells (Morita et al. 1999; Palmeri et al. 2000; Aurrand-Lions et al. 2001; Liang et al. 2002), CD99 and VE-cadherin may play a role in both constitutive and inflammatory lymphocyte recirculation because they are expressed by the entire vascular tree. Therefore, one current hypothesis is that there is heterogeneity in the molecular composition of interendothelial junctions which are more or less permissive to lymphocyte transendothelial migration. Furthermore, the combination of intercellular adhesion molecules found in interendothelial contacts varies with endothelial activation such as inflammation. Indeed, it has been described that inflammatory activation induces interendothelial junctional remodeling and

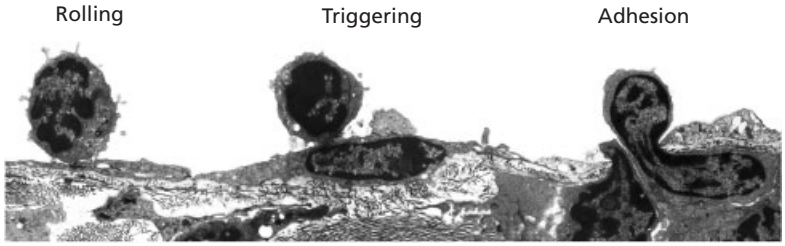

LN	L-Selectin/PNAd	CCR7/CCL21	LFA-1/ICAM-2
GALT	L-Selectin/MAdCAM-1 Integrin $\alpha_4\beta_7$/MAdCAM-1	CCR7/CCL21 CCR9/CCL25	LFA-1/ICAM-2 Integrin $\alpha_4\beta_7$/MAdCAM-1
Skin	CLA/E-selectin	CCR4/CCL17 CCR10/CCL27	LFA-1/ICAM-2
Inflammation	PSGL-1/P-, E-selectin	CXCR3/CXCL9,10,11 CCR4/CCL17, 22	LFA-1/ICAM-1, -2 $\alpha_4\beta_1$/ VCAM-1

Figure 2 *The multistep paradigm. In the first step, cell adhesion molecules on leukocytes interact loosely with their ligands on the endothelial surface, leading to rolling and slowing down of the leukocyte in the bloodstream. This allows chemokines presented by the endothelial glycocalyx to bind to leukocyte chemokine receptors, thereby triggering the activation of leukocyte integrins. Such activated integrins bind to their respective ligands on the endothelial cells, arrest the leukocyte, and eventually mediate diapedesis. In this sequential process, each step depends on the preceding step, starting with the tethering that induces leukocyte rolling. Therefore, only the expression of a distinct combination of chemokine receptors and cell adhesion molecules allows a leukocyte to adhere to and transmigrate the endothelium at a given site. Inversely, the endothelium can selectively recruit a certain leukocyte subset by expressing the appropriate combination of cell adhesion molecules and chemokines. The boxes below the flow scheme indicate the cell adhesion molecules and chemokine receptors and their ligands (leukocyte/endothelium) implicated in each step of this adhesion cascade in different tissues. LN, lymph nodes; GALT, gut-associated lymphoid tissue; infl., skin and general inflammatory situations.*

redistribution of VE-cadherin, PECAM-1, and JAM away from interendothelial contacts (Romer et al. 1995; Rival et al. 1996; Ozaki et al. 1999; Wong et al. 1999; Shaw et al. 2001). This contributes to the specific regulation of lymphocyte emigration. Such a concept has been reinforced by the findings that JAM is a ligand for the lymphocyte integrin $\alpha_L\beta_2$ (LFA-1), and that its subcellular redistribution to the apical surface of endothelial cells influences its function in lymphocyte transendothelial migration (Ostermann et al. 2002). In contrast to the adhesive interaction of JAM with LFA-1, PECAM-1 homophilic interaction regulates integrin activation in endothelial cells or transmigrating leukocytes (Tanaka et al. 1992; Piali et al. 1993; Zhao and Newman 2001), suggesting that interactions between endothelial and transmigrating cells may have a dual role in adhesion and cellular signaling. Dissecting the specific and sequential contribution of adhesion molecules to this complex signaling network will certainly improve our understanding of the specificity of lymphocyte recirculation.

CONCLUSION

The scope of this chapter was to outline the concept and mechanisms of lymphocyte homing and recirculation and to discuss the underlying molecular functions of cell adhesion molecules, chemokines, and chemokine receptors. As suggested by the pioneering studies on lymphocyte recirculation, the integrated function of the immune system requires highly efficient and specific adhesive mechanisms. These properties are illustrated by the multistep paradigm where a lymphocyte can proceed to the next step only if the previous one occurred successfully. The repertoire of molecular specificity is given by the panel of adhesion molecules that support rolling and firm adhesion, but is also greatly enhanced by the complex network of chemokines/chemokine receptors (Figure 2), which constitute the fine-tuning of the specific migration. Finally, the last transmigration step starts to be analyzed with molecular tools, but its contribution to specificity, irreversibility, and control of lymphocyte recirculation is still poorly understood.

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The mucosal immune response

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INTRODUCTION

That a highly integrated and finely regulated mucosal immune system exists alongside and separate from the peripheral immune system might at first seem redundant and puzzling. Why should such a separate and sophisticated system be necessary when the peripheral immune system already seems to ensure immunity for the host? The mucosal areas exceed over 400 m^2, which is 200 times larger than the skin surface, and this large area is consistently exposed to environmental antigens, allergens, and pathogenic microorganisms (McGhee et al. 1999). Thus, the mucosal immune system provides the first line of defense for the host. It is not, therefore, difficult to imagine that effective mucosal immune responses represents a central pathway to combat infectious diseases, including bioagents of mass destruction, e.g. category A microorganisms/exotoxins. There can be no doubt about the sophistication and elegance of the mucosal immune system. It presents a well-tuned, two-part defense, one more structured and localized and another more diffuse. In the first, foreign antigens are encountered and selectively taken up into highly structured sites for the initiation of both mucosal and systemic immune responses. In the second, diffuse collections of effector cells such as B and T lymphocytes, differentiated plasma cells, macrophages (MΦ), and dendritic cells (DC), as well as eosinophils, basophils, and especially mast cells, come into play. Together, the two produce either mucosal and serum antibody responses and T-cell-mediated immunity (CMI), on one hand, or systemic anergy, commonly termed 'oral' or 'nasal tolerance', on the other. Such a separate and sophisticated immune system may well have evolved as a major defense mechanism against mucosally encountered infectious agents as well as to attenuate mucosal inflammation through tolerance induction. This chapter focuses on the cellular and molecular aspects of the mucosal immune response, including the innate and acquired responses as well as cross-talk between them.

THE INNATE MUCOSAL DEFENSE SYSTEM

Innate defenses comprise a network of cells and molecules that encounter foreign antigens before the acquired immune response. Innate mucosal immunity includes the physical barrier provided by epithelial cells and cilia movement, mucus production, secreted molecules with antibacterial activity, and the cytolytic activity of natural killer (NK) cells. In concert, these innate mechanisms provide a first line of defense against exogenous antigens, allergens, and invading pathogens. These mechanisms also set the stage for the development of antigen-specific immune responses to antigens/pathogens that can be eliminated at the level of the epithelium itself.

Antimicrobial peptide defensins

A growing number of antimicrobial molecules, generally termed 'defensins', are produced by cells of the mucosal epithelium which contribute to the natural protection of mucosal surfaces. Defensins exhibit antimicrobial effects similar to antibiotics. These molecules are β-sheet peptides that generally contain between 30 and 40 amino acid residues and can be divided into α- and β-defensins (Figure 1). The α-defensins are smaller, because of two contiguous cysteine residues, whereas β-defensins have cysteines separated by six amino acids; as a result of disulfide bonding the defensins are complexly folded microcyclic molecules. Paneth cells in the crypt regions of the small intestine possess secretory granules that are rich in α-defensins, often termed 'cryptidins', and additional antimicrobial peptides are secreted into the lumen of the crypts. It should be noted that the crypt regions are normally sterile; however, infection, e.g. by gram-negative pathogens, may result in cryptidin production to combat the pathogen. Two α-defensins, the human intestinal defensins (HD) HD-5 and HD-6, were identified in Paneth cells and the human reproductive tract (Ouellette 1997; Porter et al. 1997) (Figure 1). α-defensins are also secreted by tracheal epithelial cells and they are homologous to peptides that function as mediators of nonoxidative microbial cell killing in human neutrophils (human neutrophil peptides (HNP)) (Ouellette 1997; Porter et al. 1997). The β-defensins, and in particular human β-defensin (HBD)-1, are expressed in the oral mucosa, trachea, and bronchi, as well as mammary and salivary gland epithelial cells in humans (Zhao et al. 1996; Singh et al. 1998; Mathews et al. 1999) (Figure 1).

Defensins contribute to the protection of mucosal surfaces through their direct antimicrobial activity. It has been shown that two to four defensin molecules interact to form multimers that insert into the cell membrane of the target bacteria, where they induce membrane disruption. The resultant channel formation allows leakage of essential bacterial components and leads to death. The fact that adenovirus infection is impaired in cells expressing HBD-1 or HD-5 suggests that the microbicidal activity of mucosal defensins also applies to viruses (Gropp et al. 1999). There is abundant evidence that defensin expression is upregulated during infection, e.g. although bronchoalveolar lavage (BAL) fluids from normal patients express only HBD-1, those of patients

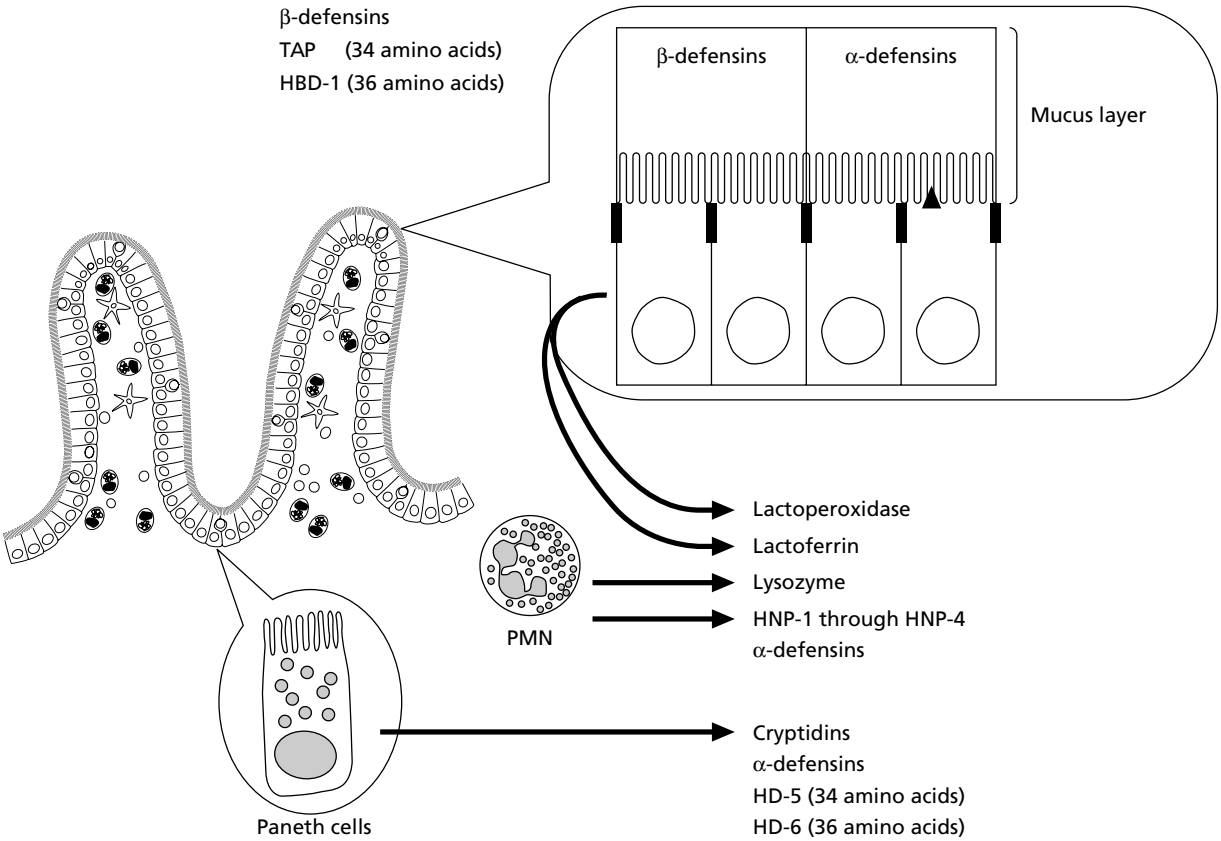

Figure 1 *Innate mucosal host defense factors: a thick coat of mucus prevents penetration of macromolecules and potential pathogens. The crypt regions contain Paneth cells, which produce cryptins (α-defensins). The β-defensins are products of epithelial cells and form a defensin network. Other innate factors such as lysozyme, lactoperoxidase, lactoferrin, and secretory leukocyte protease inhibitor (SLIP) also serve in antimicrobial defense. HBD, human β defensin; HD, human defensin; HNP, human neutrophil peptide.*

with cystic fibrosis (CF) or inflammatory lung diseases express both HBD-1 and HBD-2 (Singh et al. 1998). This expression of both HBD-1 versus HBD-2 in CF patients suggests a protective role for HBD-2 in inflammation. On the other hand, normal expression of HBD-1 may protect in the absence of inflammation. Mechanisms that control activation of defensins are poorly understood. Inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor α (TNF-α), and lipopolysaccharide (LPS) have been shown to play a role in the induction of some defensins (Singh et al. 1998; Mathews et al. 1999). Metalloprotease matrilysin was recently reported to co-localize with α-defensins in murine Paneth cells (Wilson et al. 1999). Matrilysin was found to cleave the pro-segment from cryptidin precursors (Wilson et al. 1999), suggesting that this molecule regulates the antimicrobial activity of intestinal defensins. Recent studies showed that trypsin plays an important role as a prodefensin convertase in human Paneth cells in the regulation of innate immunity in the small intestine (Ghosh et al. 2002).

Lactoferrin, lysozyme, lactoperoxidase, and antimicrobial innate defense molecules

Other potent antimicrobial innate defense molecules are produced by epithelial cells and serve in natural host defense. These molecules include lactoferrin, lysozyme, the peroxidases, secretory phospholipase A_2, and cathelin-associated peptides. Some of these molecules are secreted by intestinal Paneth cells; all are produced by polymorphonuclear leukocytes (PMN), suggesting that both cell types play important roles in innate defense of mucosal surfaces. Lactoferrin is a member of the transferrin family and shares structural homologies with transferrin and ovotransferrin. This molecule is found in exocrine secretions including milk and saliva, and in lymphocytes of many species where it mediates a number of biological effects including antimicrobial properties. Epithelial cells do not produce lactoferrin; however, they express the lactoferrin receptor. Gastric pepsin hydrolysis of either human or bovine lactoferrin generates a 25-residue peptide called lactoferricin with potent antimicrobial activity (Bellamy et al. 1992). However, not all the 25 residues of lactoferricin are required for antimicrobial activity because an 11-residue peptide containing 5 of the 8 basic residues in bovine lactoferricin displays antimicrobial properties similar to those of the 25-residue peptide (Kang et al. 1996).

Lysozyme is a highly cationic, low-molecular-weight protein that enzymatically cleaves the bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid which stabilizes bacterial peptidoglycans. High concentrations of lysozyme (1 209–1 325 μg/ml) are found in tears. Other secretions that contain lysozyme include saliva, human colostrum, serum, and urine (Pruitt et al. 1990). Human milk is particularly enriched in lysozyme with concentrations ranging from 20 to 245 μg/ml depending on the lactation period (Goldman et al. 1982). These concentrations are about 3000-fold higher than those found in cows' milk. Gram-positive bacteria are most susceptible to the antimicrobial activity of lysozyme, whereas gram-negative bacteria are generally resistant to this effect largely as a result of the protective effects of the outer membrane of LPS that masks the underlying peptidoglycan layer.

Peroxidase activity measured in many mucosal secretions is derived from an enzyme, human lactoperoxidase (hLPO), synthesized in the exocrine glands and secreted into mucosal surfaces. The PMNs also contribute to the peroxidase activity through secretion of myeloperoxidase (MPO) and eosinophil peroxidase (EPO). In this regard, human milk contains at least two peroxidases, the MPO from milk leukocytes and the hLPO produced by mammary gland ductal cells; both display similar properties to the human salivary peroxidases (hSPO) (Pruitt et al. 1991). Peroxidases protect by catalyzing the peroxidation of halides (Cl^-, Br^-, and I^-) and thiocyanate (SCN^-) ions to generate reactive products with potent antimicrobial properties. In the absence of halides and SCN^-, peroxidases can behave as catalases. These two properties of peroxidases prevent the accumulation of toxic products of oxygen reduction.

Secretory leukocyte protease inhibitor (SLPI), a 12-kDa nonglycosylated serine antiprotease, is a potent elastase, trypsin, and chymotrypsin inhibitor found in fluids lining mucosal surfaces, including the respiratory tract, salivary glands, and nasal and cervical mucosa (Mooren et al. 1983; Thompson and Ohlsson 1986; Franken et al. 1989; Hubbard and Crystal 1991; Westin et al. 1994). Thus, it has been shown that SLPI is produced by mucosal epithelial cells as well as goblet cells (Fryksmark et al. 1982; Maruyama et al. 1994; Asano et al. 1995). SLPI has been extensively studied for the protection it gives to the large airway epithelium of the human lung against neutrophil elastase. Thus, it has been suggested that SLPI would be a potential therapeutic tool in inflammatory lung diseases such as CF and pulmonary emphysema (Stolk et al. 1995). Further, SLPI-directed therapeutic genes showed potential roles for directing toxicity to carcinoma tissues (Garver et al. 1994).

Recently, human saliva itself showed an inhibitory effect for adherent primary monocytes infected with HIV-1Ba-L, and it has been shown that this anti-HIV activity was caused by SLPI (McNeely et al. 1995). In this regard, SLPI proved to be a potent inhibitor of translation in vitro. The mechanism of translation inhibition was deduced from in vitro experiments showing that SLPI bound to mRNA and interfered with the interaction of RNA-metabolizing enzymes, such as RNase (Miller et al. 1989). Furthermore, it has been suggested

that several inflammatory mediators such as TNF-α and phorbol myristate acetate (PMA) enhanced SLPI mRNA levels (Maruyama et al. 1994); however, the precise regulatory mechanism for the production of SLPI, such as the contribution of immune regulatory T cells and their derived cytokines, still remains unclear.

THE MUCOSAL EPITHELIUM BRIDGES INNATE WITH ACQUIRED MUCOSAL IMMUNITY

A major hallmark of the mucosal immune system is the presence of a mucosal intranet consisting of epithelial cells, αβT-cell receptor positive (TCR^+) T-helper 1 (Th1), T-helper 2 (Th2) cells, γδTCR^+ T cells and IgA-committed B cells for the induction and regulation of antigen (Ag)-specific secretory IgA (S-IgA) antibodies (Ab) in external secretions. The epithelium that covers mucosal surfaces largely consists of columnar epithelial cells because it is the major point of contact between localized environmental Ags in the gastrointestinal (GI) tract lumen and the underlying mucosal immune system. Epithelial cells not only provide a physical barrier but also produce and express a variety of glycoproteins, peptides, cytokines, and receptors that are important components of mucosal immunity. In addition, this epithelial layer contains a large number of $CD3^+$ T cells commonly termed intraepithelial lymphocytes (IEL). Both intestinal epithelial cells (IEC) and IELs are considered key players in the mucosal intranet and their contact leads to inter- and intracellular molecular communication. Thus, lymphoepithelial interactions contribute both to the maintenance of mucosal immunity and to homeostasis against infection.

Epithelial cells as a physical barrier

Mucosal surfaces are covered by a layer of epithelial cells that prevent the entry of exogenous antigens into the host. The physical protection of the largest mucosal surface, the GI tract, involves a monolayer of tightly joined absorptive epithelial cells termed 'enterocytes' which constitute a highly specialized selective barrier that allows the absorption of nutrients while preventing the entry of pathogens (Fujihashi and Ernst 1999). The intestinal epithelium also contains goblet cells, Paneth cells, and tuft cells, as well as undifferentiated crypt epithelial cells and IELs which contribute to the homeostasis of the epithelium and also serve physical barrier functions. The barrier effect of IECs is facilitated by the mucus blanket covering these cells and prevents the penetration of microorganisms and diffusion of molecules toward the intestinal surface. Mucus consists of glycoproteins of various molecular sizes (Roussel et al. 1988) secreted by goblet cells. These molecules resemble glycoprotein and glycolipid receptors that occur on enterocyte membranes and thus they tend to interfere with the attachment of microorganisms. In the human GI tract, the mucus blanket is approximately 100 μm in thickness. In the stomach and colon, however, this mucus blanket may range from 50 to 450 or 110 to 160 μm, respectively (Kerss et al. 1982; Sandzen et al. 1988). The continuous renewal of the epithelial cell layer also contributes to the barrier effect because damaged or infected enterocytes are replaced by crypt epithelial cells which differentiate into enterocytes as they migrate toward the desquamation zone at the villus tip. This process results in the complete renewal of the absorptive enterocyte layer every 2–3 days.

The epithelia of the other mucosal surfaces, including the oral cavity, pharynx, tonsils, urethra, and vagina, are covered by multilayered squamous epithelial cells. These epithelia lack tight junctions but provide a permeability barrier by secretion of a coating of glycolipoprotein into the intercellular space of the lower stratified layers (Farbman et al. 1988). A mucus blanket of approximately 2 μm covers the respiratory epithelium. Additional barrier effects are provided by the renewal of exposed epithelial cell layers by cells from subjacent layers. The final barrier provided by epithelial cells involves their transport of polymeric IgA (pIgA) via the pIg receptor (pIgR) with intracellular neutralization of viral particles (see below).

Cytokine and chemokine responses to mucosal pathogens

It has now been established that freshly isolated human colonic epithelial cells expressed mRNA for IL-6 and IL-8 (Jung et al. 1995). Further, epithelial cell lines have been useful for investigating the mechanisms that lead to proinflammatory cytokine and chemokine responses following a mucosal infection with pathogenic bacteria or viruses (Figure 2). In response to stimuli such as an *Escherichia coli* infection, epithelial cell lines of both intestinal (HT-29 and Caco-2) and urinary tract origin (A-498 and J82) produce IL-6 (Hedges et al. 1992; Agace et al. 1993). Further, fimbriae from *E. coli* also induce IL-6 production in these cell lines, suggesting that adhesive properties of the bacteria play an important role in the induction of this inflammatory cytokine (Figure 2). In addition, it has been shown that mucosal epithelial cell lines produce IL-8 after stimulation with *E. coli*, *Salmonella* spp., or *Listeria monocytogenes* (Agace et al. 1993; Eckmann et al. 1993a) (Figure 2). Others have shown that the adhesion of *S. typhimurium* to T84 human colonic epithelial cell lines induces the production of IL-8 (McCormick et al. 1993) and is associated with an increased transepithelial migration of neutrophils, although this process appears not to be regulated by IL-8 (McCormick et al. 1993). In addition, it has been reported that the mouse small intestinal

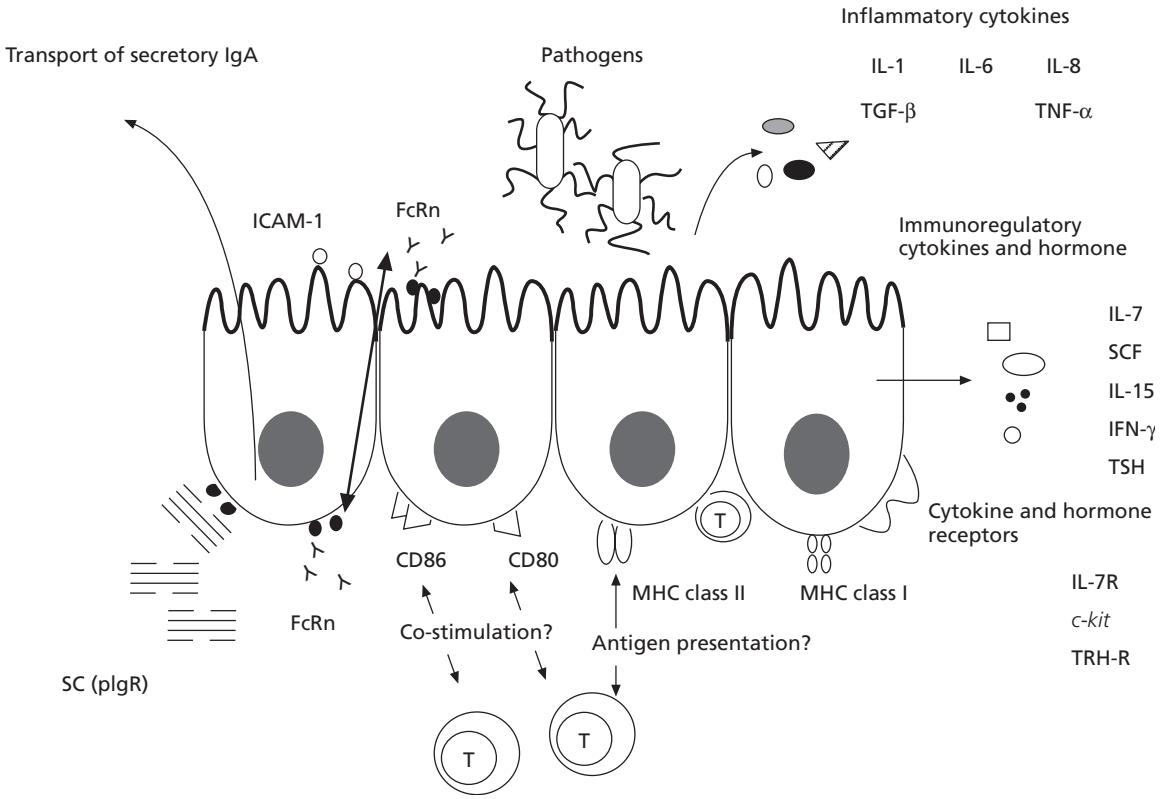

Figure 2 *Molecular interactions at the epithelial surface: the mucosal epithelial cells are essential for bridging innate and acquired immunity. The epithelial cells play important roles in both innate and acquired immunity. Cytokines and chemokines are induced by pathogens. Toll-like receptors (TLRs), which play key roles for pathogen-associated virulence factors are expressed by mucosal epithelial cells. The epithelial cells express accessory molecules (major histocompatibility complex [MHC] class I, II, CD80, CD86, CD1, and TL) and serve for antigen-presenting cell (APC) functions. Finally, antigen-specific secretory antibody (S-IgA) as well as IgG transport functions of the epithelial cells indicate essential roles in acquired immunity. ICAM, intercellular adhesion molecule; IFN, interferon; SC, secretory component; SCF, stem cell factor; TGF, transforming growth factor; TNF, tumor necrosis factor; TRH, thyroid-releasing hormone; TSH, thyroid-stimulating hormone.*

cell line, MODE-K, produces stem cell factor (SCF) when infected with *S. typhimurium* (Klimpel et al. 1996) (Figure 2). Furthermore, invasive strains of bacteria, such as *S. dublin*, *Shigella dysenteriae*, *Yersinia enterocolitica*, *L. monocytogenes*, and enteroinvasive *E. coli*, induce colonic epithelial cell lines (T84, HT29, and Caco-2) to produce a characteristic array of proinflammatory cytokines, including IL-8, monocyte chemotactic protein-1 (MCP-1), granulocyte–macrophage colony-stimulating factor (GM-CSF), and TNF-α shortly after infection/stimulation (Jung et al. 1995) (Figure 2). A similar cytokine array was produced after infection with *Chlamydia*, but chemokine production was delayed and was dependent on infection of cultured cells (Rasmussen et al. 1997). *Helicobacter pylori* infection of gastric epithelium cell lines (Kato III, AGS, or MKN28), but not nongastric epithelial cell lines, induced IL-8 mRNA and protein synthesis (Crowe et al. 1995; Sharma et al. 1995). Immunohistochemical staining of human colon has demonstrated that IECs can express IL-6 and isolated IECs stimulated with LPS have been shown to contain mRNA for both IL-1 and IL-6 (Mayer et al. 1990). It was also shown that cholera toxin (CT)-induced inflammatory cytokines, such as IL-1 and IL-6, in the rat IEC line, IEC-17 (Bromander et al. 1993). Further, increased levels of SCF were also produced by the MODE-K cell line when stimulated with CT (Klimpel et al. 1995). Finally, respiratory syncytial virus (RSV) induced selective production of the chemokine RANTES in upper airway epithelial cells (Saito et al. 1997). Taken together, these studies suggest that epithelial cells are major players in both the inflammatory process and immunity, where they produce anti-inflammatory cytokine pathways.

The secretion of these factors is likely to be mediated initially in large part by interactions between components of microbes and pattern recognition receptors on the cell surface of the epithelium, which stimulate intracellular signals to initiate transcription of genes associated with the inflammatory cascade (Fearon and Locksley 1996). Recently, Toll-like receptors (TLR) have been shown to be key molecules for pathogen-associated virulence factors (e.g. LPS, peptidoglycan, and lipoprotein) and are considered to be major players in

innate immunity (Medzhitov et al. 1997; Gerard 1998). In this regard, it has been shown that TLRs are expressed by mucosal epithelial cells in the oral cavity, and the GI and respiratory tracts (Cario et al. 2000; Akira 2001; Asai et al. 2001; Wang et al. 2002) (Figure 2), e.g. individual bacterial components bind to specific TLRs, such as peptidoglycan (TLR-2 and TLR-4), lipoteichoic acid (TLR-2), or LPS (TLR-4) (Hoshino et al. 1999; Qureshi et al. 1999; Shimazu et al. 1999; Yoshimura et al. 1999). In addition, other TLRs bind to lipoteichoic acids from gram-positive bacteria (TLR-3), bacterial flagellin (TLR-5), and microbial CpG motifs of DNA (TLR-9) (Akira 2001). In the case of TLR-4, LPS recognition is coupled to CD14 binding wherein LPS bound to CD14 interacts with a TLR-4/MD-2 protein heterodimer (Asai et al. 2001). As breast milk contains soluble CD14 for the innate recognition of bacteria, it is possible that $CD14^-$ epithelial cells recognize LPS through soluble CD14 from milk (Labéta et al. 2000). The TLRs initiate an intracellular signaling cascade and induce activation of NF-κB, which regulates the epithelial cell cycle and subsequent production of soluble mediators (Aradhya and Nelson 2001).

Epithelial cell accessory molecules and antigen-presenting cell functions

In addition to the production of cytokines, IEC lines express adhesion molecules known to be required for antigen-presenting cell (APC) interactions with lymphocytes (see Figure 2). Both intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen (LFA)-3 were constitutively expressed at low levels by human intestinal epithelial cell lines and expression of ICAM-1 was enhanced by exposure to the inflammatory cytokines interferon γ (IFN-γ), TNF-α, IL-1β, and IL-6 (Kvale et al. 1992). It was also shown that ICAM-1 expression on the apical side of epithelial cells was upregulated after infection with invasive pathogenic bacteria (Huang et al. 1996). Furthermore, gastric epithelial cell lines, as well as freshly isolated gastric epithelial cells, expressed both B7-1 (CD80) and B7-2 (CD86) (Ye et al. 1997) (see Figure 2). Thus, B7-2 expression was increased after crosslinking major histocompatibility complex (MHC) class II on IFN-γ-treated epithelial cells and in cells pretreated with IFN-γ and infected with *H. pylori*. These observations provide support for the finding that rat IECs present processed antigen to antigen-specific $CD4^+$ T cells (Brandeis et al. 1994). Taken together, the findings that epithelial cells express the adhesion molecules ICAM-1 and LFA-3 as well as B7-1 and B7-2, and present antigen to sensitized $CD4^+$ T cells, suggests that IECs probably play an important role in the maintenance of mucosal immune responses in mucosal effector sites and especially in the IEL compartment. It is also likely that IECs exhibit APC functions responsible for some forms of T-cell anergy in mucosal tolerance.

It has been shown that both intestinal and respiratory epithelial cells present antigen in both classic and nonclassic fashions to mucosal T cells (see Figure 2). Immunohistochemical analysis revealed that normal human IECs from the small intestine expressed HLA-DR, but less HLA-DP, and this MHC II expression was increased in inflammatory bowel disease (Mayer et al. 1991). Further, direct evidence for APC function was provided with studies of freshly isolated rat IECs and transfected epithelial cell lines, which presented a soluble allopeptide to primed T cells in an MHC II-restricted manner (Brandeis et al. 1994). Although uptake of antigen occurred at both apical (normal) and basal (inflammation) sides of IECs, peptide presentation to T cells through MHC II was seen only at the basolateral surface (Hershberg et al. 1998). Finally, nonclassic MHC I-restricted IEC presentation has been described. Thus, IECs express CD1d constitutively, which in turn is regulated by IFN-γ that also enhances classic MHC I and II molecule expression (Blumberg et al. 1991; Sydora et al. 1996a). The murine CD1 (mCD1) homolog of human CD1d may also bind to peptide antigen (Sydora et al. 1996b). Further, it was shown that mCD1 can bind to $CD8^+$ IELs, i.e. γδ and αβ TCR^+ T cells which express CD8αα homodimers (Sydora et al. 1996b; Gapin et al. 1999). Low numbers of CD1d-restricted NK T cells have been defined in the epithelium using CD1d tetramers (Matsuda et al. 2000). Other nonclassic MHC I molecules expressed by IECs have been directly linked to novel forms of co-stimulation (Leishman et al. 2001; Roberts et al. 2001). The expression of the thymus leukemia (TL) antigen by mouse IEC has been linked to ligation of CD8αα expressed on a subset of IELs, which then delivers a growth-promoting signal to the epithelium (Leishman et al. 2001).

Polymeric IgA and IgM, and monomeric IgG epithelial cell transport

A major function of epithelial cells in mucosal immune responses is the active transcellular transport of pIgA produced in mucosal and glandular tissues to the mucosal surface (see Figure 2). The molecule responsible for transport of pIgA into mucosal secretions is the pIgR (Mostov et al. 1980, 1984; Krajci et al. 1992). A major hallmark of the mucosal immune response is the presence of Ag-specific S-IgA Abs in external secretions. As discussed above, S-IgA Abs in humans occur as S-IgA1 and S-IgA2 subclasses, with a preponderance of the former in the upper respiratory tract (URT) and upper GI tract (GIT), and the latter in the lower ileum and large intestine (Mestecky and McGhee 1987; Brandtzaeg 1994). Plasma cells in lamina propria and

acinar regions of exocrine glands produce pIgA associated with the 15.6-kDa peptide J chain (Brandtzaeg 1974; Mestecky and McGhee 1987). Initially, it was thought that J chain was involved in polymerization of intracellular monomeric IgA (mIgA) or IgM (mIgM) (Brandtzaeg and Prydz 1984); however, recent studies indicate that aberrant polymers of IgM occur in the absence of J chain (Niles et al. 1995). Further, J chain knock-out mice exhibit IgA in external secretions; however, only mIgA is transported in this mouse (Hendrickson et al. 1995, 1996). Taken together, this suggests that J chain is required in the formation of pIgA and the correct pentameric form of IgM that enables the molecule to bind to pIgR on the basolateral side of the epithelium (Brandtzaeg 1974; Brandtzaeg and Prydz 1984; Hendrickson et al. 1995, 1996; Niles et al. 1995).

As indicated above, epithelial cells in glands and in basolateral crypts of the GIT and URT produce the full-length 100-kDa pIgR which associates with the nonserosal surface of epithelial cells (Mostov 1994). The binding of pIgA/pIgM is followed by endocytosis and transcytosis across the epithelial cell (Eckmann et al. 1993a). During transcytosis, disulfide bonds form between pIgR and pIgA and secretion of S-IgA follows cleavage of a 20-kDa component of pIgR and exocytosis – the entire process requiring less than an hour (Mestecky and McGhee 1987; Mostov 1994) (see Figure 1). The disulfide-bonded portion of the pIgR remains attached to the Ab molecule, termed secretory component (SC), stabilizes S-IgA and S-IgM, and renders the molecule more resistant to proteolytic digestion (Mestecky and McGhee 1987; Mostov 1994). This transport process is highly efficient and a normal adult produces 2.8–3.0 g S-IgA/day in the GIT alone.

A number of studies have provided evidence that IEC lines produce cytokines and express cytokine receptors and adhesion molecules that may affect the induction of mucosal immune responses. When human IECs were examined for their ability to produce cytokines, both cell lines and freshly isolated human IECs were shown to produce IL-8 (Eckmann et al. 1993a, b; Schüerer-Maly et al. 1994), and the former also expressed mRNA for IL-1α, IL-1β, IL-10, and TNF-α, but not for IL-2, IL-4, IL-5, IL-6, or IFN-γ (Eckmann et al. 1993a, b). It has now been shown that IFN-γ and TNF-α induced HT-29 cells to increase membrane-associated pIgR (Sollid et al. 1987; Kvale et al. 1988). Further, IL-4 enhanced the expression of surface pIgR, and this cytokine acted in synergy with IFN-γ to induce high levels of pIgR on HT-29 cells (Phillips et al. 1990). Taken together, these results suggest that IECs have the ability to produce cytokines that could play a role in the induction and maintenance of mucosal immune responses and inflammation.

The epithelium also expresses the neonatal Fc receptor for IgG (FcRn). It has been shown that FcRn is responsible for the passive acquisition of IgG neonatally in rodents (Israel et al. 1997; Zhu et al. 2001). Thus, it was predicted that FcRn expression was strictly limited to neonatal life. However, recent evidence indicates that this molecule is also expressed by adult human epithelium and by Mϕs in the intestinal lamina propria. When expressed postnatally in adult humans, FcRn may serve in a capacity to provide for the continuous presence of IgG Abs to prevent microbial infections. The FcRn binds monomeric IgG in a pH-dependent process (pH 6 on, pH 7.4 off) because of histidine residues in the Fc region of IgG. In contrast to the transport associated with pIgR, the pathway associated with FcRn goes from basal to apical as well as from apical to basal (Dickinson et al. 1999) (see Figure 2). In addition, the FcRn is not associated with proteolytic cleavage and is thus available for several rounds of transport. It is predicted, therefore, that the FcRn is a major player in the steady-state distribution of IgG on both sides of the epithelial barrier. Most feel that paracellular transport of this macromolecule does not occur because of the molecular exclusion of the tight junctions.

AN IEL–IEC INTRANET

Origin and function of intestinal IELs

The mucosal epithelium, an important interface between the host and its environment, contains a large number of $CD3^+$ T cells which are commonly termed IELs (Ernst et al. 1985; LeFrançois 1991; McGhee et al. 1994) (Figure 3). As the name IEL indicates, these lymphocytes reside on the basolateral surface of epithelial cells covering the GI, nasal, and reproductive tracts. It has been estimated that one IEL can be found for every four to six epithelial cells (Ferguson and Parrott 1972; Crowe and Marsh 1994). Thus, tremendous numbers of $CD3^+$ T cells are situated in the mucosal epithelium where they are continuously exposed to mucosally encountered antigens. Although human IELs share several unique features with mouse IELs, the phenotype of cell surface antigens is different, e.g. the most striking characteristic of mouse IELs is the presence of high numbers of γδ T cells (Ernst et al. 1985; McGhee et al. 1994); however, most human IELs express oligoclonal αβTCRs (Ebert 1994; Halstensen and Brandtzaeg 1994) (Figure 3). Despite the numerous studies that have focused on a better understanding of the ontogeny of murine IELs, only limited information is currently available about the origin and development of human IELs. Morphological studies have suggested that human IELs originate from cells in the lamina propria. Thus, it has been shown that they possess trailing cytoplasmic pseudopods in the direction of lamina propria (Orlic and Lev 1977). Further, the epithelial basement membrane around the site of IEL migration has specific pores

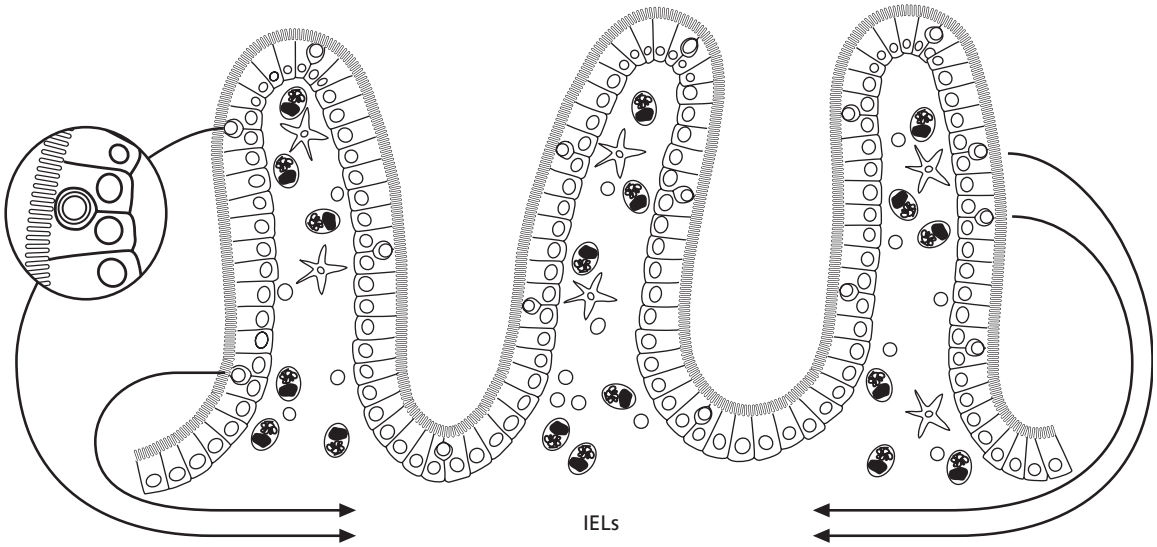

Figure 3 *Intraepithelial lymphocytes (IELs). Unique effector T-cell subsets are present in the epithelium. In this regard, the origin and immunological functions of IELs are distinct from other mature T cells. IECs, intestinal epithelial cells; TCR, T-cell receptor.*

(Toner and Ferguson 1971). These findings suggest that human IELs not only migrate from the lamina propria, but also have the potential to migrate back to this region. These observations further suggest that potential extrathymic development of human IELs may occur. Indeed, human IELs express CD7 (Fcμ receptor) which is also expressed on $CD3^-$ pre-T cells in the thymus (Malizia et al. 1985). These $CD7^+$ IELs contain small but significant numbers of $CD3^-$ IELs. In the case of γδ T cells in human IELs, Vδ2 expression also supports the possibility of extrathymic development. It has been shown that this T-cell population is rare in the gut although most fetal γδ IELs express the Vδ2 segment (Spencer and MacDonald 1994). In contrast, other reports showed that human IELs contain an equal frequency of Vδ1 and Vδ2. Despite the lack of a clear answer for the origin of human IELs, recent reports strongly suggest that patches that occur in the crypt region, termed 'cryptopatches', in the intestinal lamina propria of mice do not express lineage markers, including CD3, B220, Mac-1, Gr-1, and TER119, or *c-kit*$^+$ cells that allow differentiation into either αβ or γδ IELs (Kanamori et al. 1996; Saito et al. 1998).

IELs may be assumed to be a primary source of effector T cells in the natural environment of the mucosal epithelium. Such effector cells form a first line of defense against translocation of the mucosal microflora and invading microbial pathogens. The $CD8^+$/IEL T-cell subset is associated with cytotoxic activity (Ernst et al. 1985; LeFrançois 1991; McGhee et al. 1994), e.g. human $CD8^+$ IELs possess cytotoxic activity and kill a tumor epithelial cell line by mechanisms that are totally different from NK-type activity (Ebert 1994; Spencer and MacDonald 1994). It is important to note that cytotoxic activity in human $CD8^+$ IELs is restricted to epithelial cells. These findings clearly suggest that human $CD8^+$ IELs play an immune surveillance role in order to identify and remove defective or infected IECs. Cytotoxic activity in IELs has also been studied in detail in the mouse system. Thus, CTL activity for both αβ and γδ IELs was shown by use of a re-directed cytotoxic cell assay in hybridoma cell lines producing monoclonal antibodies (mAb) specific for $CD3^+$, $\alpha\beta TCR^+$, or $\gamma\delta TCR^+$ cells as targets (LeFrançois and Goodman 1989; Guy-Grand et al. 1991). Furthermore, it has been shown that MHC I-restricted virus-specific cytoxic T lymphocytes (CTL) can be induced in the intestinal epithelium of mice (London et al. 1987; Offit and Dudzik 1989).

Certain subsets of IEL T cells have been shown to contribute to regional host defense against intestinal infections with bacterial pathogens (Yamamoto et al. 1993; Emoto et al. 1996; Roberts et al. 1996). During

Listeria monocytogenes infection, IFN-γ production and target cell lysis by $CD8^+$ αβ T cells were essential for complete (sterile) clearance of this pathogen (Kaufmann 1993). It was also shown that both $CD4^+$ αβ and γδ T cells contribute to optimal protection after systemic challenge with *L. monocytogenes* (Kaufmann 1993). Studies in TCR-deficient mice suggest an important role for γδ T cells in immune responses to both intracellular bacteria and parasites (Mombaerts et al. 1993; Tsuji et al. 1994; Ladel et al. 1995). The γδ T cells were required for control of mycobacterial infection (Mombaerts et al. 1993) and also contributed to immunity after *Plasmodium yoelii* immunization, because γδ T-cell-defective ($TCR\delta^{-/-}$) mice failed to respond to these intracellular pathogens and succumbed to infection (Tsuji et al. 1994). The γδ T cells also play an accessory role in the late stages of protective immune responses to *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) (Ladel et al. 1995). Taken together, these findings show that intraepithelial T cells can serve as effector cells to protect from mucosal parasitic, bacterial, and viral infections.

The vast majority of γδ T cells occur in the epithelium of the small intestine of normal mice and as such could provide a key role in the maintenance of mucosal immunohomeostasis, in addition to regulation of immune responses to intracellular bacteria and parasites. Our previous studies suggested that γδ IEL T cells are important for the maintenance of mucosal IgA Ab responses in the presence of systemic unresponsiveness (or oral tolerance) induced by oral immunization (Fujihashi et al. 1992). Indeed, it was shown that γδ T cells serve an important regulatory role for mucosal IgA responses, because $TCR\delta^{-/-}$ mice possess normal numbers of functional αβ T cells, but display markedly decreased IgA Ab responses after oral immunization (Fujihashi et al. 1996b). Furthermore, recent studies have suggested that γδ T cells play distinct roles in the induction of both low- and high-dose oral tolerance (Ke et al. 1997; Fujihashi et al. 1999). Thus, γδ T cells play a significant immunoregulatory role in IL-10-mediated, low-dose oral tolerance induction, but are not essential participants in the induction of systemic tolerance to orally introduced Ags given in larger doses (Fujihashi et al. 1999).

IEL and IEC crosstalk at the intestinal mucosal barrier

It is logical to consider that epithelial cells and IEL T cells in mucosal tissues cross-regulate each other in order to provide a cellular and molecular network to induce and maintain appropriate immunological homeostasis. Thus, as discussed above, IECs and $CD8^+$ IEL T cells elicit increased levels of MHC I and CTL activity, respectively, after infection by pathogenic viruses. It has been shown that IEL T cells are also capable of producing an array of cytokines including transforming growth factor β (TGF-β) and IFN-γ which in turn induce high levels of MHC expression on IECs (Taguchi et al. 1990b; Barrett et al. 1992; Fujihashi et al. 1993a, b). Further, IEL T-cell-derived cytokines, including IFN-γ, IL-4, and TNF-α, have been shown to influence IEC functions such as the production of the pIgR (Kvale et al. 1988; Phillips et al. 1990). Intraepithelial γδ but not αβ T cells have been shown to moderate the growth and differentiation of IECs by the production of keratinocyte growth factor (Boismenu and Havran 1994) (Figure 4). Studies in $TCR\delta^{-/-}$ mice have emphasized the important role played by IEL γδ T cells in IEC growth. In $TCR\delta^{-/-}$ mice, the turnover of IECs in intestinal villi and the levels of MHC II expression were both lower than in mice of the same genetic background (Komano et al. 1995). These findings suggest that intraepithelial γδ T cells provide cytokines essential for the growth and proper functioning of IECs.

Regulation is bi-directional, because epithelial cells can also influence γδ T-cell development and function. It was recently demonstrated that cytokine signaling between IL-7 and its receptor (IL-7R) contributes to development and activation of IEL γδ T cells. It was shown that both human and murine IECs contain specific mRNA for IL-7 (Watanabe et al. 1995; Fujihashi et al. 1996a) (Figure 4). Flow cytometry analysis of IL-7R expression revealed that a subset of γδ T cells exhibit high mRNA levels (Fujihashi et al. 1996a) (Figure 4). Thus, co-cultivation of intestinal γδ T cells and recombinant IL-7 results in the activation of T cells for proliferative responses (Fujihashi et al. 1996a). Stimulatory signals provided by IL-7, and by the IL-7R pathway (between epithelial cells and γδ T cells), also induce IL-2R expression on γδ T cells. In addition, IL-2 provides a synergistic activation signal for further activation of γδ T cells. Mice that lack the common cytokine receptor γ chain (γc), which is a part of both IL-2R and IL-7R (Noguchi et al. 1993), manifest complete loss of γδ T cells (Cao et al. 1995; He and Malek 1996; Maki et al. 1996). Furthermore, although studies have also provided evidence for a lack of γδ T cells in IL-7 and IL-7R gene-deleted ($IL\text{-}7^{-/-}$ and $IL\text{-}7R^{-/-}$) mice (Cao et al. 1995; He and Malek 1996; Maki et al. 1996; Moore et al. 1996), direct comparison of $IL\text{-}7^{-/-}$ and $IL\text{-}7R^{-/-}$ mice provides a unique opportunity to demonstrate the importance of this pathway for intestinal γδ T-cell development. Intestinal γδ T cells were reduced when the IL-7 gene was deleted and completely disappeared in the absence of IL-7R (Fujihashi et al. 1997). These findings support the idea that epithelial cell-derived IL-7 is an essential cytokine for the development and activation of neighboring $IL\text{-}7R^+$ γδ T cells.

In addition to the IL-7-corresponding receptor cascade, a study with WBB6/F1-W/W^v (*c-kit*) and WCB6/F1-Sl/Sl^d (SCF) mutant mice has suggested that

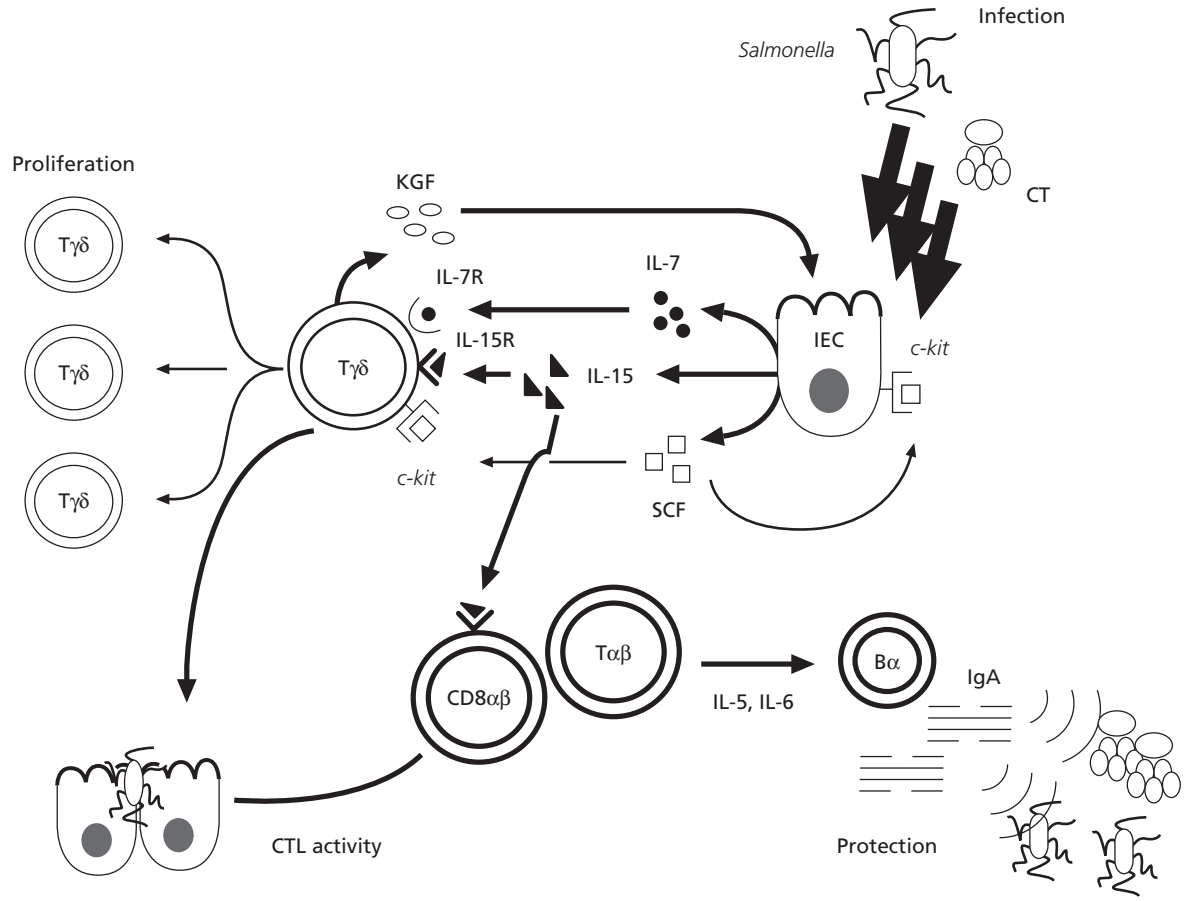

Figure 4 *The molecular intranet: a cytokine and growth factor intranet between epithelial cells and intraepithelial lymphocytes (IELs). Bi-directional regulation occurs between epithelial cells and γδ T cells for development and function. Intraepithelial γδ T cells have been shown to moderate the growth and differentiation of epithelial cells by the production of keratinocyte growth factor (KGF). Further, epithelial cells provide essential cytokines, interleukin IL-7, stem cell factor (SCF), IL-15 for γδ T cells, and thyroid-stimulating hormone (TSH) and IL-15 for CD8 αβ T cells. CTLs, cytotoxic T lymphocytes; IECs, intraepithelial cells.*

SCF and *c-kit* interactions also play important roles in the development and maintenance of IELs γδ T cells (Puddington et al. 1994) (Figure 4). Further, this study demonstrates that neighboring epithelial cells were able to produce SCF (Puddington et al. 1994) (Figure 4). Thus, although normal levels of γδ T cells were detected in IELs of both SCF and *c-kit* mutant mice at 4–8 weeks of age, decreased numbers of this T-cell subset were noted at 16 weeks of age (Puddington et al. 1994). These observations indicate that cross-talk between γδ IELs and IECs is essential for homeostasis of the mucosal immune system in the GIT. As SCF and *c-kit* are the products of IECs and IELs, respectively, this cytokine-signaling pathway from epithelial cells to intestinal γδ T cells is essential for the growth of mucosal γδ T cells.

The SCF and *c-kit* network has also been shown to be important for intestinal fluid secretion induced by *Vibrio cholerae* (Klimpel et al. 1995). Using a ligated intestinal loop model, it was shown that both *Sl/Sld* and *W/Wv* mice produced significantly less intestinal fluid in response to CT challenge than did control mice. The interaction of SCF with its receptor was further shown to be important in salmonella infections (Klimpel et al. 1996). Both human and murine IEC lines show enhanced SCF synthesis and expression of mRNA after exposure to *Salmonella* spp. (Klimpel et al. 1996). Thus, pretreatment of these cell lines with SCF increased their resistance to salmonella invasion (Klimpel et al. 1996). Further, when *c-kit* mutant and control mice were challenged with oral *S. typhimurium*, *W/Wv* mice were more susceptible to infection than were controls (Klimpel et al. 1996). These results show that interactions between SCF and *c-kit* play an essential role as a first line of defense at the mucosal epithelium. In the case of salmonella infection, it has been shown that IL-15 preferentially induces T-cell growth in peritoneal γδ T cells (Nishimura et al. 1996). These studies suggest that IL-15 is a potential growth factor for intestinal IEL γδ T cells. Indeed, other studies showed that IL-15 promoted the growth of IEL γδ T cells (Inagaki-Ohara et al. 1997). Further, it has been shown that gene deletion of the

IL-15 and IL-15R pathway resulted in reduced numbers of IEL γδ T cells in the intestinal epithelium (Lodolce et al. 1998; Kennedy et al. 2000).

Although IEC and IEL γδ T-cell interactions have thus far been the focus of this discussion, the role of IEL αβ T cells should not be ignored. This T-cell subset has the capacity to respond to IL-7 and SCF produced by IECs. In addition to participating in these cytokine signal pathways, IEL αβ T cells and IECs form a unique local hormone network (Wang et al. 1997). Thus, neuroendocrine hormones play an important role in the development of IEL CD8αβ$^+$, αβ T cells (Wang and Klein 1994, 1995). Mouse IECs express thyrotropin-releasing hormone (TRH) receptor and produce thyroid-stimulating hormone (TSH) upon TRH signal transduction, whereas IELs express TSH receptor (TSH-R)-specific mRNA (Wang et al. 1997). The phenotypic analysis of IELs in *hyt/hyt* mice, which have congenital point mutation in the TSH-R gene, revealed significant reductions in CD8αβ and αβ T-cell subsets but not the CD8αα fraction (Wang et al. 1997). In addition, recent studies showed significant development of CD8αβ$^+$, NK1.1$^+$ T cells in the IELs and subsequent induction of small intestinal inflammation by intestinal epithelium-specific IL-15 transgenic mice (Ohta et al. 2002). These results suggest the existence of additional important lymphoepithelial interactions, especially those involving intraepithelial CD8αβ$^+$, αβ T cells, and their important role in mucosal homeostasis and immunity. Taken together, epithelial cells and intraepithelial T cells are major players in the formation of a mucosal intranet and can reciprocally regulate each other via select cytokines and corresponding receptor interactions in order to provide and maintain an effective host defense system in the mucosal environment.

INDUCTIVE AND EFFECTOR LYMPHOEPITHELIAL SITES OF THE MUCOSAL IMMUNE SYSTEM

Organization of the mucosal immune system

The mammalian host has evolved organized secondary lymphoid tissues in the URT and GIT regions which facilitate antigen uptake, processing and presentation for induction of mucosal immune responses. Collectively, these tissues are termed 'mucosal inductive sites' (Figure 5). Although the gut-associated

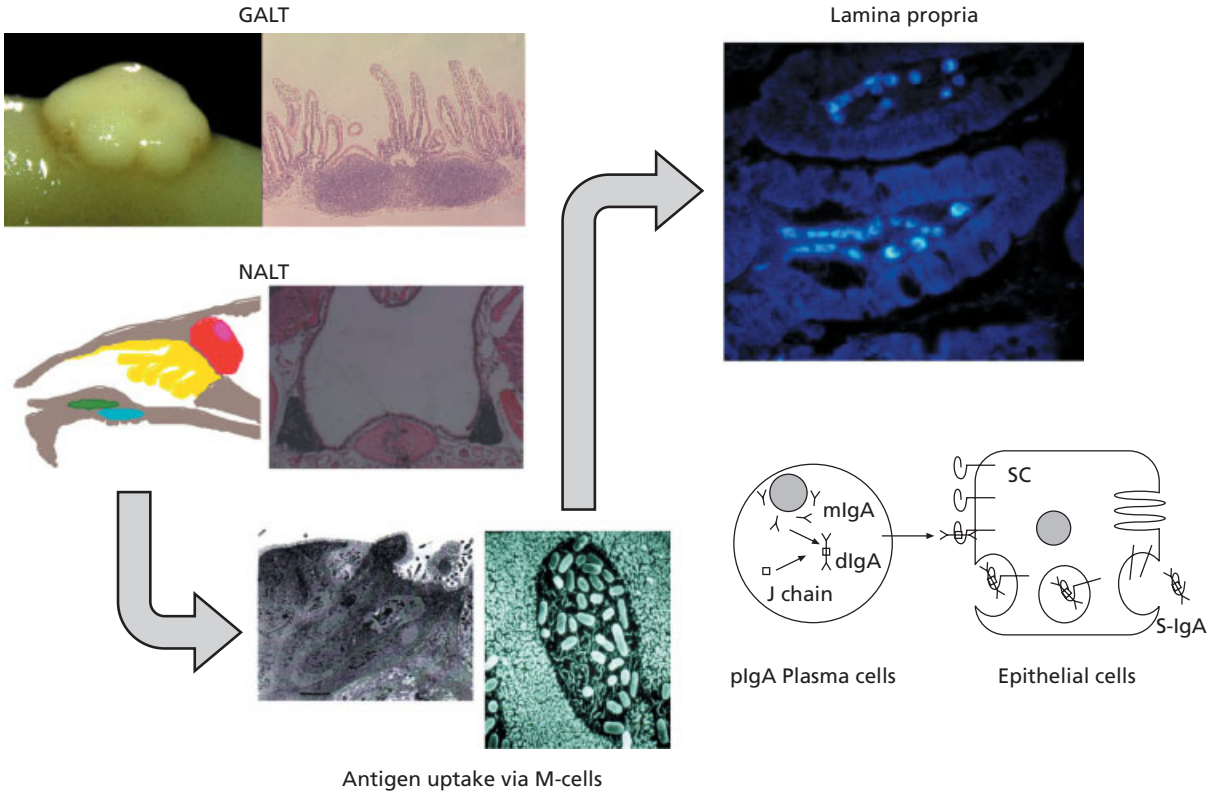

Figure 5 *The common mucosal immune system (CMIS): the mammalian host has evolved organized secondary lymphoid tissues, termed inductive sites in the upper respiratory and gastrointestinal tract regions which facilitate antigen uptake, processing, and presentation for induction of mucosal immune responses. Subsequently, antigen-activated and memory B- and T-cell populations emigrate from the inductive environment via lymphatic drainage, circulate through the bloodstream, and home to mucosal effector sites. SC, secretory component; S-IgA, antigen-specific secretory IgA antibody.*

lymphoepithelial tissues (GALT), e.g. Peyer's patches, the appendix, and smaller lymphoid aggregates called solitary lymph nodes (SLN), appear to be major inductive sites in all of the most common experimental mammalian systems, the degree of bronchus-associated lymphoepithelial tissue (BALT) developed at airway branches for defense against intranasal/inhaled antigens differs considerably among species (Pabst 1992; Kolopp-Sarda et al. 1994). Instead, the major inductive tissues for nasal/inhaled antigens in humans, primates, mice, and rats appear to be the palatine tonsils and adenoids (nasopharyngeal tonsils), which together form a physical barrier of lymphoid tissues termed 'Waldeyer's ring', now more frequently referred to as a nasopharyngeal-associated lymphoepithelial tissue (NALT) (Bernstein 1992; Kuper et al. 1992). To summarize, NALT and GALT in humans, mice, and primates and NALT, BALT, and GALT in other experimental mammalian systems comprise a mucosa-associated lymphoepithelial tissue (MALT) network (Bienenstock et al. 1978; Mestecky and McGhee 1987), the integration of which is only partly understood.

There are two major features that distinguish MALT from the other systemic lymphoid tissues. First, the epithelium that separates the tissue from the lumen contains a specialized cell type now called a microfold or M cell which is closely associated with lymphoid cells. Together this epithelial cell network is termed the follicle-associated epithelium (FAE). Second, MALT contains organized regions that include a subepithelial area (dome), B-cell zones with germinal centers containing IgA-committed B cells (surface IgA^+ ($sIgA^+$) B cells) and adjacent T-cell regions with APCs and high endothelial venules (HEV). Naive, recirculating B and T lymphocytes enter MALT through HEVs. Antigen-activated and memory B- and T-cell populations then emigrate from the inductive environment via lymphatic drainage, circulate through the bloodstream, and home to mucosal effector sites (via the common mucosal immune system (CMIS)) (Figure 5). These effector sites include more diffuse tissues where antigen-specific T and B lymphocytes ultimately reside and perform their respective functions (i.e. CMI, CTL, and regulatory functions or antibody synthesis) to protect mucosal surfaces.

Mucosal inductive sites

Mucosal inductive sites of the GIT include Peyer's patches, the appendix and SLNs, which collectively comprise the GALT (Bienenstock et al. 1978; Mestecky and McGhee 1987), while the tonsils and adenoids, or NALT, probably serve as the mucosal inductive sites for the URT, the nasal/oral cavity, and the genitourinary tract (Bernstein 1992; Kuper et al. 1992). The most extensively studied mucosal inductive tissues are the Peyer's patches of the murine GI tract, although, in recent years, several groups have also characterized NALT, albeit to a lesser extent than GALT, and salient characteristics of both are presented.

GUT-ASSOCIATED LYMPHOEPITHELIAL TISSUE

The initial steps involved in murine Peyer's patch development have been studied in some detail in mice. A cluster of $VCAM\text{-}1^+/ICAM\text{-}1^+$ cells occurs in the upper small intestine beginning at embryonic day –15 to –16 followed by the presence of cells expressing the IL-7 receptor ($IL\text{-}7R\alpha^+$) at day −17.5 (Adachi et al. 1997, 1998; Yoshida et al. 1999) which appear to be the anlage of the patch. Mice defective in IL-7Rα gene expression fail to form mature GALT (Yoshida et al. 1999). It now appears that IL-7–IL-7R triggering results in upregulation of $LT\alpha_1\beta_2$ membrane expression by lymphoid cells, including those in developing Peyer's patches (Rennert et al. 1996; Alimzhanov et al. 1997; Koni et al. 1997; Futterer et al. 1998). Further, mice that lack LT-α_1, LT-β_2 or that have been treated in utero with a fusion protein of LT-β receptor Ig (LT-β-R-Ig) fail to develop Peyer's patches or systemic lymph nodes (De Togni et al. 1994; Rennert et al. 1996; Alimzhanov et al. 1997; Koni et al. 1997; Futterer et al. 1998). In addition, lymphoplasia (*aly/aly*) mice, with a mutation in the NF-κB-inducing kinase (Shinkura et al. 1999), which appears to act downstream of LT-$\alpha_1\beta_2$-LT-βR signaling, also fail to develop Peyer's patches (Shinkura et al. 1999). There is recent evidence that a lymphoid progenitor cell from fetal liver expresses α4β7 and migrates to the Peyer's patch anlage where they ultimately develop into T cells, NK cells, DCs, and lymphotoxin (LT)-lineage cells (Yoshida et al. 2001). In humans, the Peyer's patches develop during prenatal life, a situation also seen in sheep, pigs, dogs, and horses (Griebel and Hein 1996).

Murine Peyer's patches contain a dome, underlying follicles (B-cell zones with germinal centers) (Figure 6a), and parafollicular regions enriched with T cells (Figure 6b). Originally, the specialized epithelial cell covering MALT was termed a 'FAE cell' because it characterized the organized lymphoid tissues in the GI tract and exhibited pinocytotic activity (Bockman and Cooper 1973). However, the M cell was later named for its unique topical morphology (microfold/membranous) (Owen and Jones 1974) and others now commonly describe the epithelium covering MALT as an FAE type because of characteristic M cells, lack of goblet cells, and close association with lymphoid cells (Wolf and Bye 1984; Farstad et al. 1994; Gebert et al. 1996; Neutra et al. 1996). The surface of the dome region is covered by the specialized FAE, 10–20 percent of which is made up of M cells that exhibit thin extensions around lymphoid cells (Bockman and Cooper 1973; Owen and Jones 1974; Wolf and Bye 1984; Farstad et al. 1994; Gebert et al. 1996; Neutra et al. 1996). These

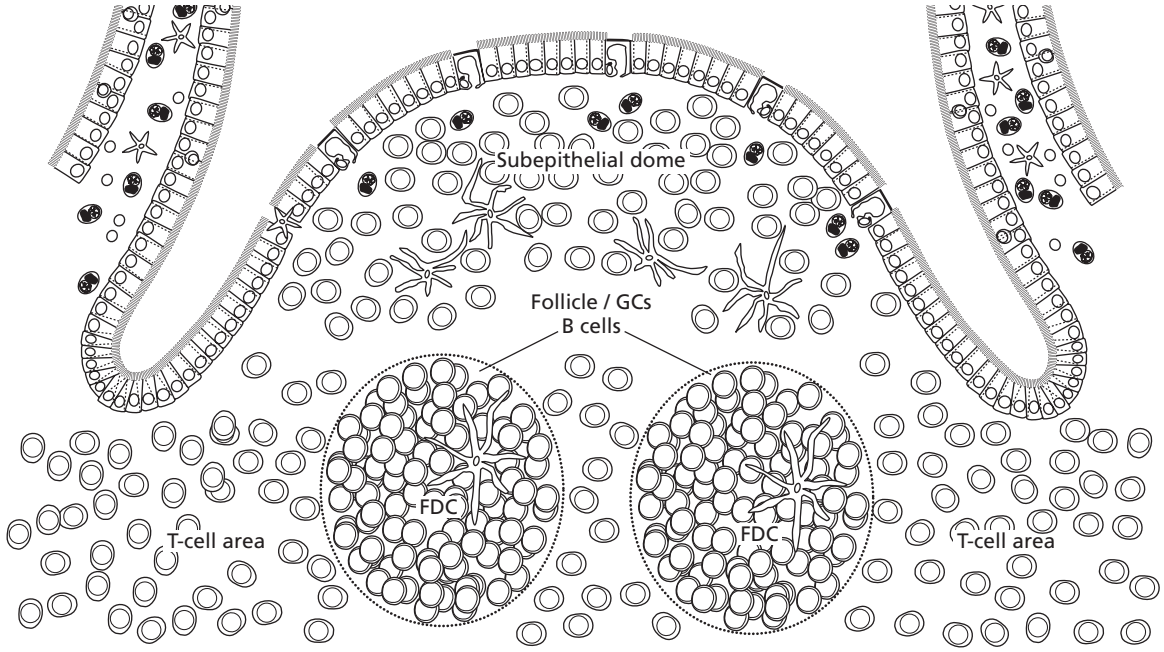

Primary follicle (at birth) sIgM$^+$, sIgD$^+$
Secondary follicle (by 1wk), GCs,
FDCs (LTα1β2 – LT-βR)
GCs are CD40–CD40L dependent

TGF- $\beta \rightarrow \mu \rightarrow \alpha$ switching in GCs
Memory sIgD$^-$, sIgM$^+$ or sIgA$^+$ induction
α4β7 / LFA-1 expression (MAdCAM-1/ICAM-1)

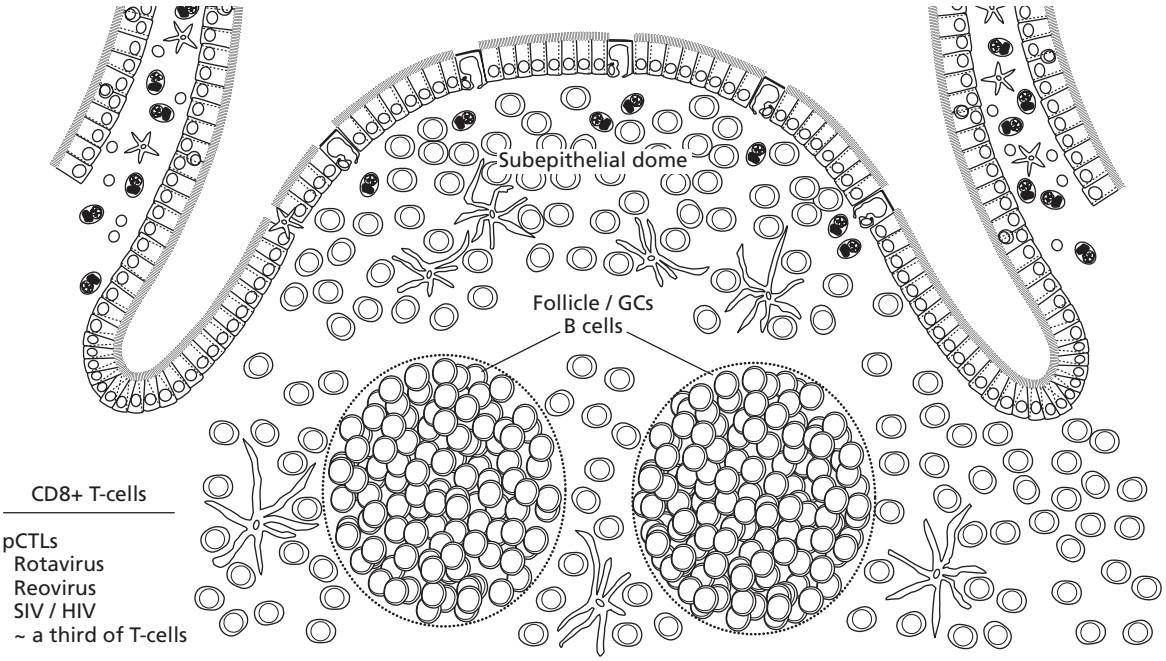

CD8+ T-cells

pCTLs
Rotavirus
Reovirus
SIV / HIV
~ a third of T-cells

Characteristics of DCs

CD11b$^-$, CD11c$^+$, CD8α^+ (lymphoid)
in vitro express:
DEC205$^+$, class II$^+$,
B7-1, B7-2, produce IL-10

CD4$^+$ Th cells

~ 70% of T cells
~ 70% CD4$^+$ Th2 type
~ 30% CD4$^+$ Th1 type

Figure 6 *Mucosal inductive sites: the immunological features of typical gut-associated lymphoreticular tissue (GALT), i.e. Peyer's patches.* **(a)** *B-cell zones are located beneath the dome area of the Peyer's patches and contain germinal centers (GCs) where significant B-cell division is seen.* **(b)** *All major mature T-cell subsets are found in the parafollicular area. Over 97 percent of T cells express the $\alpha\beta$ T-cell receptor. FDC, follicular dendritic cell; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; MIP, macrophage inflammatory protein; SIV, simian immunodeficiency virus; Th, T-helper cell; TGF, transforming growth factor.*

extensions, which almost surround B and T lymphocytes and occasional macrophages (MΦ), form an apparent pocket (Farstad et al. 1994; Gebert et al. 1996; Neutra et al. 1996). The M cells, which have short microvilli, small cytoplasmic vesicles, and few lysosomes, are adept at uptake and transport of luminal antigens, including proteins and particulates such as viruses, bacteria, small parasites, and microspheres (Wolf and Bye 1984; Ermak et al. 1995; Gebert et al. 1996; Neutra et al. 1996). Investigators in this field disagree on whether M cells are able to process and present antigen. Some believe that antigen uptake by M cells and transcellular passage result in delivery of intact antigen into the underlying lymphoid tissue (Wolf and Bye 1984; Gebert et al. 1996). Others, however, contend that findings such as M-cell expression of MHC II molecules and acidic endosomal–lysosomal compartments suggest that M cells may also be involved in antigen processing and presentation (Allan et al. 1993). It is possible that the nature of endocytosed antigen influences M-cell activation and their potential to express MHC II molecules. In an important and elegant in vitro study, human Caco-2 cells, which are more immature enterocytes, differentiated into M-like cells when treated with mouse Peyer's patch T and B cells or with a human B-cell line (Raji) (Kerneis et al. 1997). Mice that lack B cells (termed μMT) were also shown to exhibit fewer M cells and less well-developed FAE and Peyer's patches than normal mice (Golovkina et al. 1999). These findings suggest that lymphocytes and especially B cells possess signaling molecules that induce M-cell differentiation.

In addition to serving as a means of transport for luminal antigens, the M cells also provide an entry way for pathogens. Invasive strains of *S. typhimurium* initiate murine infection by invading the M cells of the Peyer's patches (Jones et al. 1994). Although M cells are able to transport luminal antigen, noninvasive strains of *S. typhimurium* cannot penetrate M cells and thus are avirulent. Reoviruses also initiate infection of the mouse through the M cell (Wolf et al. 1981), an ability that has been associated with the reovirus sigma protein (Nibert et al. 1991). As discussed in more detail below, NALT also has a lymphoepithelium with M cells, and the tubercle bacillus *M. tuberculosis* uses this cell type for entry into the host with subsequent uptake in draining lymph nodes (Teitelbaum et al. 1999). Identification of bacterial and viral virulence factors associated with invasion or infection of M cells may provide tools to construct more efficient attenuated bacterial or viral vectors (see below) or to target mucosal vaccines to the inductive environment of MALT. Further, it is possible that M cells are also involved in the induction of mucosally-induced tolerance (e.g. oral tolerance). If antigen deposition via MALT is essential for induction of systemic unresponsiveness, it is likely that optimization of antigen delivery vehicles (including live vectors) that target M cells may improve immunization schema for development of mucosally induced tolerance.

The underlying dome region of the Peyer's patch consists of sparse plasma cells, as well as B and T lymphocytes (Gebert et al. 1996; Adachi et al. 1997), and this suggests that immediate antigen presentation may occur in the dome area after antigen uptake. It is also possible that T- and B-cell interactions in the dome area provide necessary protection for the Peyer's patch. The presence of MΦs (Sminia and van der Ende 1991) including the tingible body type suggests that significant apoptosis occurs, but is not yet proven. An immunohistological study has called into question whether dome MΦs are indeed a major cell type (Kelsall and Strober 1996). This important study has described a major APC population in the dome with characteristics of DCs (Kelsall and Strober 1996) (Figure 7). Interestingly, the dome DCs were $N418^+$ (anti-CD11c) and could be differentiated from more classic DCs present in the interfollicular area (T-cell zone), demonstrating that at least two DC subsets occur in key antigen-sampling areas of the Peyer's patch (Kelsall and Strober 1996). This study also suggested that fewer numbers of $B220^+$ B cells occur in the dome area of mouse GALT. Studies of the lymphocyte populations associated with human M-cell pockets, the area where luminal antigen may first be recognized by T and B lymphocytes, have also provided evidence for a characteristic T-cell distribution, e.g. M-cell pockets in human GALT contain approximately equal numbers of $CD3^+$ T and $CD19^+$/$CD20^+$ B lymphocytes with fewer $CD68^+$ macrophages (Farstad et al. 1994). Of the T cells in this location, approximately 75 percent exhibited a T-helper cell phenotype.

Distinct follicles (B-cell zones) are located beneath the dome area of the Peyer's patches and contain germinal centers where significant B-cell division is seen (Figure 6a). These germinal centers, which contain most of the $sIgA^+$ B cells (Butcher et al. 1982; Lebman et al. 1987; George and Cebra 1991; Weinstein and Cebra 1991; Weinstein et al. 1991), are considered to be sites where frequent B-cell switches to IgA and affinity maturation occur. However, unlike immune lymph nodes and the spleen in the systemic compartment, plasma cell development does not occur. All major T-cell subsets are found in the T-cell-dependent areas adjacent to follicles. The parafollicular T cells are mature and more than 97 percent of these T cells use the αβ heterodimer form of the TCR (Figure 6b). Approximately two-thirds of Peyer's patch αβ TCR^+ T cells are $CD4^+$, $CD8^-$ and exhibit properties of Th cells, including support for IgA responses (Kiyono et al. 1982a). Approximately one-third of the αβ T cells in GALT are $CD4^-$, $CD8^+$; this T-cell subset contains precursors of CTLs (London et al. 1987, 1990), whereas other $CD8^+$ T-cell subsets appear to contribute to mucosally-induced tolerance (see below).

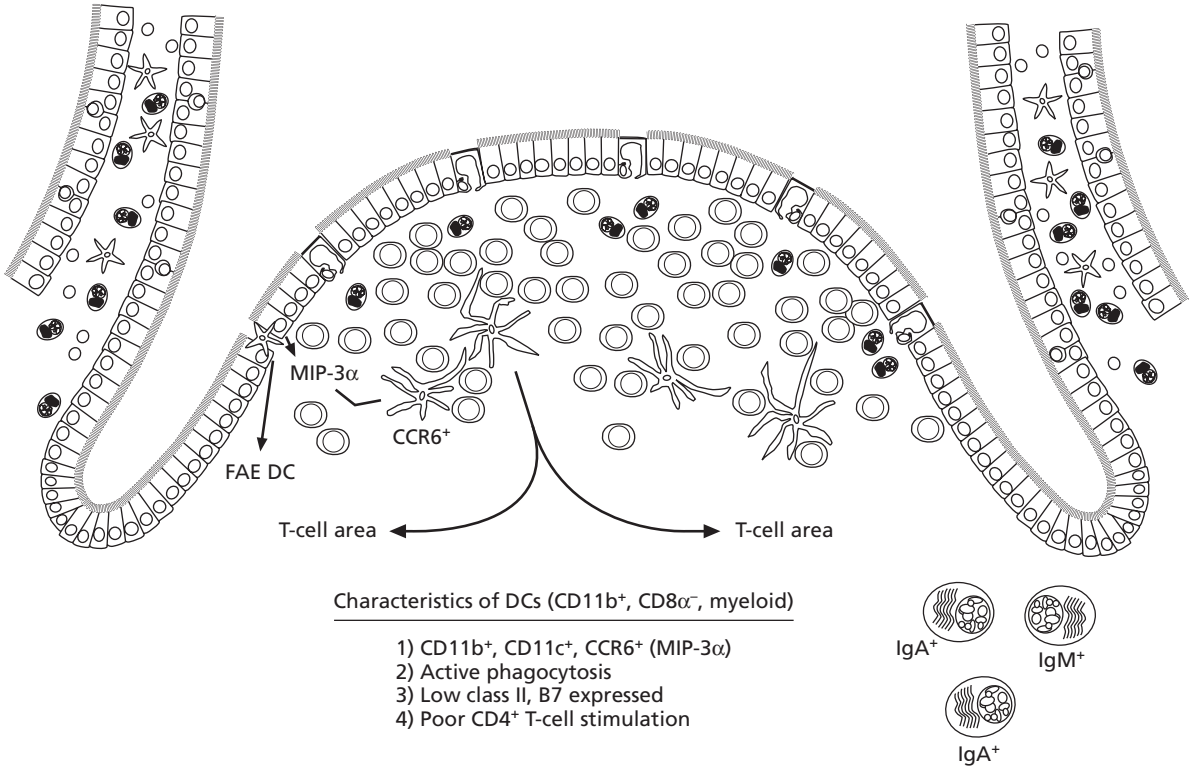

Figure 7 *Gut-associated lymphoreticular tissue (GALT) compartments: the subepithelial dome region of the Peyer's patch consists of sparse plasma cells, as well as B and T lymphocytes. A major antigen-presenting cell (APC) population in the subepithelial dome with characteristics of dendritic cells (DCs) plays a role in immediate antigen presentation in the dome area after antigen uptake. FAE, follicle-associated epithelium.*

NASAL-ASSOCIATED LYMPHOEPITHELIAL TISSUE

Although the mouse has been the major model used to study Peyer's patches, the human tonsils are the most accessible secondary lymphoid tissue for study of NALT and, as such, a great deal is known about the component cells (see Figure 5). Although the palatine and nasopharyngeal tonsils (adenoids) are largely covered by a squamous epithelium and are often not appreciated as mucosal tissues, the palatine tonsils usually contain 10–20 crypts that increase their surface area. The deeper regions of these crypts contain M cells, which may take up encountered antigens (Owen and Nemanic 1978; Allan et al. 1993). The tonsils contain all major classes of APCs including DCs and Langerhans' cells, MΦs, class II^+ B cells and antigen-retaining, follicular DCs in B-cell germinal centers (see Figure 2). Tonsillar APCs are capable of inducing T-cell proliferative and cytokine responses following in vitro restimulation with appropriate vaccines such as tetanus and diphtheria toxoid and purified protein derivative (PPD) of *M. tuberculosis*.

Approximately half of tonsillar cells are B lymphocytes and mainly occur in follicle-containing germinal centers. Most human tonsillar B cells are actually surface IgG-positive ($sIgG^+$); however, significant numbers of $sIgM^+$ and $sIgA^+$ B cells are also present. Further, in situ staining of B-cell blasts/plasma cells indicate a predominance of IgG blasts in germinal centers and of plasma cells in the parafollicular area. The overall ratio of Ig^+ cells was 65:30 for IgG versus IgA (Brandtzaeg et al. 1978; Boyaka et al. 2000). The human palatine tonsil also contains a distinct subepithelial B-cell population which differs from both germinal center and follicular mantle B cells (Dono et al. 1996a, b). This subset may represent the homolog of the extrafollicular B cells of the splenic marginal zone (Dono et al. 1996a, b). It is not accurate to suggest that the tonsils are only a mucosal IgA-inductive site, because of the presence of B cells committed to other isotypes, especially for IgG subclasses. Approximately 40 percent of tonsillar cells are T cells and more than 98 percent express the αβ TCR. Further, somewhat higher CD4:CD8 ratios are found in tonsils (3:1) when compared with peripheral blood or murine Peyer's patches. In summary, the tonsils clearly exhibit not only features of mucosal inductive sites, but also characteristics of effector sites with high numbers of plasma cells. The role of the tonsils in host mucosal immunity after intranasal immunization is not yet established.

To understand the precise contribution of NALT to the induction of IgA responses to inhaled antigens, studies in both mice and rats have established a NALT-like structure (Kuper et al. 1992; Asanuma et al. 1995;

Wu et al. 1996). The NALT consists of bilateral strips of nonencapsulated lymphoid tissue underlying the epithelium on the ventral aspect of the posterior nasal tract and exhibits a bell-like shape in cross-sections (Kilian and Russell 1994; Wu et al. 1997; Hiroi et al. 1998). Although dense aggregates of lymphocytes have been observed in the NALT of normal mice, germinal centers are absent, but could be induced by nasal application of antigen (Hiroi et al. 1998). Thus, uncommitted B cells ($sIgM^+$) have been found in high proportions (80–85 percent), whereas low numbers of $sIgA^+$ and $sIgG^+$ B cells (3–4 percent and 0–1 percent, respectively) have been noted in mononuclear cells isolated from NALT (Wu et al. 1996; Hiroi et al. 1998). In contrast to GALT where a high frequency (10–15 percent) of $sIgA^+$ B cells occur, NALT was found to contain fewer IgA-committed B cells. Despite this, nasal immunization induces much higher numbers of IgA^+ than IgG^+ NALT B cells in the memory compartment, showing the propensity of NALT for mucosal S-IgA Ab responses (McGhee et al. 1989). Characterization of isolated NALT mononuclear cells revealed that approximately 30–40 percent of these cells are $CD3^+$ T cells with a CD4/CD8 ratio of about 3.0 (Wu et al. 1996, 1997; Hiroi et al. 1998). The majority of NALT $CD3^+$ T cells co-express CD45RB, suggestive of naïve, resting T cells (Wu et al. 1996, 1997; Hiroi et al. 1998). As transcriptional single cell analysis revealed the expression of mRNA for both Th1 and Th2 cytokines, the majority of $CD4^+$ T cells are considered to be Thp or Th0 types (Hiroi et al. 1998). Further, stimulation via the TCR–CD3 complex resulted in differentiation of both Th1- and Th2-type cells. These results support the notion that NALT exhibits characteristics of mucosal inductive sites.

Mucosal effector tissues

After initial exposure to antigen in MALT, mucosal lymphocytes leave the inductive site and home to mucosal effector tissues. This pathway, which results in immunity at several mucous membrane sites, is referred to as the common mucosal immune system (CMIS) (see Figure 5). Effector sites for mucosal immune responses include the lymphoid cells in the lamina propria (LP) regions of the GI, URT, and reproductive tracts as well as secretory glandular tissues such as mammary, salivary, and lacrimal glands (Adachi et al. 1997). In addition, most evidence suggests that the lymphocytes that reside in the epithelium (i.e. IELs) also serve as effector cells; however, it has been difficult to define IEL functions precisely (see Figure 3). Antigen-specific mucosal effector cells include IgA-producing plasma cells as well as B and T lymphocytes. IgA is the primary Ig involved in protecting mucosal surfaces and is locally produced in effector tissues (Adachi et al. 1997). Again, the presence of antigen-specific S-IgA Abs at mucosal surfaces other than the inductive site where antigen uptake initially occurred is definitive evidence of the CMIS. Thus, it would suggest that immunization of either NALT or GALT could induce mucosal immune responses in all mucosal effector tissues.

Effector mechanisms employed to protect mucosal surfaces include CTLs, as well as effector $CD4^+$ Th cells for CMI (Th1) and for S-IgA Ab (Th2) responses (McGhee et al. 1989; Kilian and Russell 1994). Indeed, both CTL and S-IgA Ab responses have been associated with protection against infection at mucosal surfaces and both may be important for resistance to, or more importantly prevention of, mucosal infection with viruses including HIV (Staats et al. 1994). Although little information about protective $CD4^+$ Th1-CMI responses is available, mucosal CMI responses appear to be important in tolerance and in control of infections by intracellular pathogens.

It must be remembered, however, that effector sites, which must serve as a barrier against numerous environmental foreign antigens and mucosal pathogens with which the inductive sites need not contend, will offer mechanisms of protection significantly different from those seen in the inductive sites. The high concentration of IgA plasma cells (estimated at $>10^{10}$ IgA plasma cells per meter of human small intestine (Brandtzaeg and Farstad 1999)) has traditionally been viewed as the most distinctive trait of the immunity offered at these effector sites (Figure 8). As discussed below, the murine GI lamina propria has an almost equal distribution of peritoneal B1 B-cell-derived IgA plasma cells. Further, the lamina propria of the gut also contains more than 50 percent IgA plasma cells that are B2 GALT B-cell derived (see below). However, as important, if not more so, are the large numbers of B and T lymphocytes (e.g. lamina propria lymphocytes (LPL)), more than 60 percent of which are T cells (James et al. 1986; Abreu-Martin and Targan 1996). The importance of these cell types is discussed in detail below.

When presented with an environmental antigen, epithelial cells endocytose it and in some cases themselves express MHC II molecules, processing antigens with subsequent association of immunogenic peptides with MHC II (Mayer and Shlien 1987). It has also been shown that Langerhans-like cells occur on the luminal side of the intestine at epithelial junctions between epithelial cells which could also provide accessory functions (Huang et al. 2000). When confronted with microorganisms and even with soluble proteins that can transverse the tight junctions between epithelial cells, the APCs at the effector sites may process them and so induce B- and T-cell responses. Some have suggested that MHC II^+ $sIgA^+$ B cells may bind antigen through endocytic pathways, process, and present peptides to $CD4^+$ Th cells. Macrophages in LP regions could also function in this manner for more complex antigens.

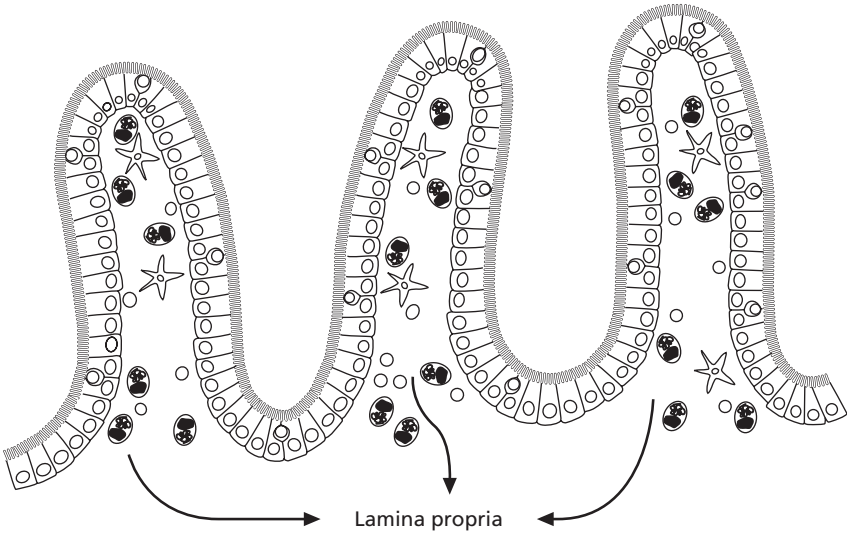

B lineage	
$CD5^+$	~ 50%
$CD5^-$	~ 50%
Memory	~ 90%
Naive	<5%
Plasma cells	20%
IgA^+	79–90%
IgM^+	11–18%
IgG^+	3–5%

T lineage	
$\alpha\beta TCR^+$	95–75%
$\gamma\delta TCR^+$	3–5%
$CD4^+$ Th:	60–70%
Th1	~ 40%
Th2	~ 60%
$CD8^+$ CTL	30–40%
Memory	~ 95%
Naive	~ 5%
Activated, $CD25^+$ blasts	20%

Figure 8 *Structural features and cellular characteristics of mucosal effector sites. The lamina propria is equally populated by B1 and B2 cells, both of which differentiate into IgA^+ plasma cells. Note that memory B and T lymphocytes occur in this compartment.*

Freshly isolated mouse intestinal LP $CD4^+$ T cells contain high numbers of IL-5-secreting Th2-type cells in addition to IFN-γ-secreting Th1-type cells, suggesting that the effector regions of the mucosal immune system are somewhat biased toward a Th2 phenotype (Taguchi et al. 1990a, b). Further, recent findings obtained by a single cell reverse transcriptase polymerase chain reaction (RT-PCR) analysis of $CD4^+$ T cells from murine nasal passages revealed a high frequency of $CD4^+$ T cells expressing Th2 cytokine-specific mRNA (Hiroi et al. 1998). In summary, mucosal effector tissues contain all the necessary cellular components including epithelial cells, Th1/Th2 type $CD4^+$ T cells, CTLs, and IgA-producing cells for a multilayer barrier against the numerous environmental foreign antigens and mucosal pathogens.

MUCOSAL T CELLS AND CYTOKINES

T cells and cytokines that influence IgA switching

It is known that cytokines exert profound influences on B-cell switching from sIgM, sIgD expression to downstream isotypes including IgG subclasses, IgE, and IgA. Clear evidence was presented that clones of T cells from murine GALT, when mixed with noncommitted $sIgM^+$ B cells, induced isotype switching to B cells expressing sIgA (Kawanishi et al. 1983a, b) (see Figure 6a). More recent studies have revealed that T-cell–B-cell interactions support B-cell switches and have postulated a major role for the CD40 receptor on germinal center B cells with CD40L on activated T cells (Fuleihan et al. 1993; Banchereau et al. 1994; MacLennan 1994). In terms of cytokines involved in IgA switching, the most definitive studies to date suggest that transforming growth factor-β1 (TGF-β1) is the major cytokine (Coffman et al. 1989; Sonoda et al. 1989) (see Figure 6a). The first studies showed that addition of TGF-β1 to LPS-triggered mouse splenic B-cell cultures resulted in switching to IgA, and IgA synthesis was markedly enhanced by IL-2 (Lebman et al. 1990a) and IL-5 (Sonoda et al. 1989). The effect of TGF-β1 was on $sIgM^+$, $sIgA^-$ B cells and was not the result of selective induction of terminal B-cell differentiation. It was shown that TGF-β1 induced sterile Cα germline transcripts (Coffman et al. 1989; Lebman et al. 1990b), an event that clearly precedes actual switching to IgA. Importantly, TGF-$\beta^{-/-}$ mice exhibit low levels of IgA^+ plasma

cells in effector sites and of S-IgA in external secretions, providing evidence that TGF-β1 is also important for μ → α switching in vivo (van Ginkel et al. 1999).

Although many presume that isotype switching to IgA (i.e. μ → α) occurs in mucosal inductive sites such as GALT, and that terminal differentiation into plasma cells producing IgA is a major event in effector sites, only indirect evidence is at hand to support these assumptions. In this regard, most studies of μ → α switching have been performed with nonmucosal lymphoid cells, e.g. splenic B cells, whereas in vitro studies of B-cell differentiation to IgA synthesis normally employ Peyer's patch B cells (a mucosal inductive site) to support the idea that this also occurs in effector mucosal sites, such as LP and exocrine glands. Moreover, cytokine knock-out mice have been employed to determine the relevance of particular cytokines for mucosal immunity, an approach discussed below. That this dogma of μ → α switching occurs only in mucosal inductive sites is challenged by several recent findings. The activation-induced cytokine deaminaseAID) gene, initially discovered in germinal center B cells (Muramatsu etal. 1999) has been cloned from B-lymphoma cells stimulated with CD40L, IL-4, and TGF-β1 which were undergoing μ → α switches (Muramatsu etal. 2000). Over-expression of AID in μ^+ B-lymphoma cells resulted in spontaneous class switching from IgM to IgA in the complete absence of TGF-β1 or other cytokines (Muramatsu etal. 2000). Mice defective in the AID gene ($AID^{-/-}$) and humans with AID deficiency exhibit a hyper-IgM syndrome with no evidence of downstream switching (Muramatsu et al. 2000; Revy et al. 2000). However, recent studies revealed that $AID^{-/-}$ mice have a subset of $B220^+$ surface IgA^+ B cells in lamina propria (an effector site), and the presence of circles of 'looped out' DNA suggest that μ → α switching had just occurred in this site (Fagarasan et al. 2001). Most recent reports have shown the presence of isolated lymphocyte follicles (ILF) in the LP that contain $sIgA^+$ B cells (Hamada et al. 2002). Thus, it is still possible that these ILFs are the sites of IgA switching. Along these lines, it was also recently revealed that B-cell-deficient, μMT mice also exhibit LP IgA^+ plasma cells, suggesting that switches to IgA can occur even during pre-B-cell development (Macpherson et al. 2001). These intriguing studies have used mouse models where class switching in germinal centers is absent. Thus, μ → α B-cell switches may occur throughout the mucosal immune system and in the complete absence of germinal centers.

T cells and their derived cyokines for IgA synthesis

$CD4^+$ T cells and cytokines are essential for the generation of IgA-producing cells, e.g. depletion of $CD4^+$ T-cell subsets in vivo with mAbs or by knockout of the CD4 co-receptor gene markedly affects mucosal immune responses (Mega et al. 1991; Hörnquist et al. 1995). Loss of $CD4^+$ T cells is associated with diminished levels of IgA^+ plasma cells (Mega et al. 1991) and with deficient Th-cell-regulated IgA responses (Hörnquist et al. 1995). For B-cell terminal differentiation, IL-5 and especially IL-6, possibly in combination with other cytokines, appear essential for the continued presence of plasma cells undergoing high-rate secretion of IgA antibodies (Beagley et al. 1988, 1989; Harriman et al. 1988) (see Figure 6a).

Earlier studies revealed that addition of culture supernatants from DC–T-cell clusters, T-cell clones, or T-cell hybridomas to cultures of Peyer's patch or splenic B cells resulted in enhanced secretion of IgA (Spalding et al. 1984; Kiyono et al. 1985). One cytokine responsible for this activity was subsequently shown to be IL-5 (Coffman et al. 1987; Murray et al. 1987; Beagley et al. 1988; Harriman et al. 1988; Lebman and Coffman 1988; Lebman et al. 1990a). Removal of $sIgA^+$ B cells from Peyer's patch (PP) B-cell cultures abrogated IgA synthesis, demonstrating that this cytokine affected post-switched, IgA-committed B cells (Murray et al. 1987). No in vitro stimulus was required for Peyer's patch B cells and IL-4 did not further enhance the effect of IL-5 (Lebman and Coffman 1988). Taken together, these results suggest that IL-5 induces $sIgA^+$ B cells that are in cell cycle (blasts) to differentiate into IgA-producing plasma cells (Figure 9). Interestingly, another B-cell population, the peritoneal cavity B-1 cells, has been shown to contain precursors of lamina propria IgA^+ plasma cells (Kroese et al. 1989). Human IL-5 is thought to act mainly as an eosinophil differentiation factor and thus may have little effect on B-cell isotype switching and differentiation. It has been reported, however, that human B cells, when stimulated with the bacterium *Moraxella (Branhamella) catarrhalis*, could be induced by IL-5 to secrete IgA, and also possibly to undergo isotype switching to IgA (Benson et al. 1990). This effect could not be demonstrated using other more conventional B-cell mitogens, a finding that demonstrates the importance of the primary in vitro activation signal for B-cell switching.

The cytokine IL-6, when added to Peyer's patch B cells in the absence of any in vitro stimulus, causes a marked increase in IgA secretion with little effect on either IgM or IgG synthesis (Beagley et al. 1989) (Figure 9). In these studies, IL-6 induced two- to threefold more IgA than IL-5 (Beagley et al. 1989). The removal of $sIgA^+$ B cells abolished the effect of IL-6, demonstrating that, similar to IL-5, this cytokine also acted on post-switched B cells. Of relevance was the finding that human appendix $sIgA^+$ B cells express IL-6 receptors, whereas other B-cell subsets present do not. Further, appendix B cells were induced by IL-6 to secrete both IgA1 and IgA2 in the absence of any in

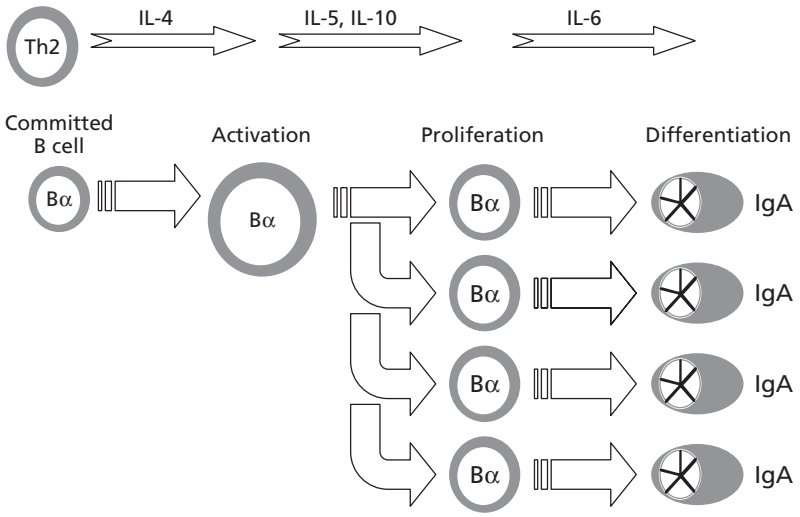

Figure 9 *Cytokine cascades in mucosal IgA immunity. T-helper 2 (Th2)-type cyokines clearly support the induction of IgA antibody responses. Interleukin-4 (IL-4) activates post-switched IgA-committed B cells and IL-5 induces sIgA$^+$ B cells which are in cell cycle (blasts) to differentiate into IgA-producing cells. IL-6 induces a marked increase in IgA secretion (two- to three-fold more IgA secretion than IL-5) when added to Peyer's patch B cells in the absence of any in vitro stimulus.*

vitro activation (Fujihashi et al. 1991) (Figure 9). This effect was also shown in IgA-committed B cells, again demonstrating the importance of IL-6 for inducing the terminal differentiation of sIgA$^+$ B cells into IgA-producing plasma cells. An additional Th2 cell cytokine, IL-10, has also been shown to play an important role in the induction of IgA synthesis in humans (DeFrance et al. 1992; Nonoyama et al. 1993; Briere et al. 1994). Taken together, these findings demonstrate that Th2 cell cytokines, such as IL-5, IL-6, and IL-10, all play major roles in the induction of IgA plasma cell responses.

INDUCTION OF MUCOSAL IMMUNITY AND VACCINES

Immune responses expressed in mucosal tissues are typified by S-IgA Abs (McGhee et al. 1999). This S-IgA constitutes the predominant Ig class in human external secretions, and is the best-known entity providing specific immune protection for mucosal tissues (Figure 10). The resistance of S-IgA Abs to endogenous proteases makes Abs of this isotype uniquely well suited to protect mucosal surfaces. Specific S-IgA

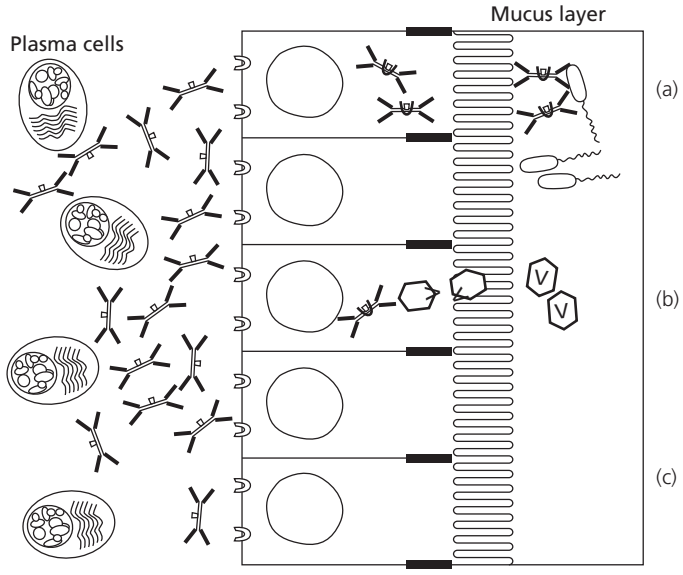

Figure 10 *The multiple functions of IgA: immunobiological function of antigen-specific secretory IgA antibody (S-IgA) for mucosal immunity. S-IgA constitutes the predominant immunoglobulin class in human external secretions and is the best-known entity providing specific immune protection for mucosal tissues.* **(a)** *Inhibition of adherence:* S. mutans, Vibrio cholerae, Shigella *spp.*, Escherichia coli, Giardia *spp.*, Haemophilus influenzae, *others;* **(b)** *Intracellular neutralization of:* rotavirus, Sendai virus, reovirus; **(c)** *removal of pIgA immune complexes.*

Abs provide classic 'immune exclusion' of noxious antigens. In addition, S-IgA prevents adherence of bacterial pathogens to epithelial cells and may also neutralize viral particles. Mucosal S-IgA Abs can serve as antitoxins to neutralize bacterial exotoxins, including enterotoxins and other potentially harmful molecules. In addition, S-IgA Abs may interfere with the utilization of growth factors for bacterial pathogens in the mucosal environment. Furthermore, it now appears that pIgA Abs, in the process of transport through the epithelial cell via the polymeric Ig receptor (pIgR), can also function to neutralize viruses and may also transport Ag–IgA complexes into external secretions (Ostov and Kaetzel 1999; Zhang et al. 2000). Taken together, it is essential to induce S-IgA Ab responses in order to provide effective mucosal immunity to environmental antigens, allergens, and infectious pathogens.

Mucosal APCs

Antigen presentation in mucosal tissues reflects the complexity of immune responses that occur in these sites. Indeed, the site of mucosal antigen uptake (i.e. M cells or epithelial cells) and the nature of antigen presentation that takes place will determine the outcome of the immune response. It is now well accepted that large macromolecules are taken up by M cells in the GI tract. It is controversial as to whether M cells express MHC II molecules and present antigen to immune effector cells (Hirata et al. 1986; Bjerke et al. 1993). However, as mentioned earlier, the presence of all three major APC types (i.e. memory B cells, MΦs, and DCs) in the dome epithelium of Peyer's patches make it likely that antigen uptake occurs immediately after release from M cells. Immunohistology of murine Peyer's patches have shown that $N418^+$, $2A1^+$, NLDC-145^-, $M342^-$ DCs form a dense layer of cells in the subepithelial dome (SED), just beneath the follicle epithelium where $CD3^+$ and $CD4^+$, but not $CD8^+$, T cells occur (Kelsall and Strober 1996) (see Figure 7). Another subset of DCs, $N418^+$, $2A1^+$, NLDC-145^+, and $M342^+$ DCs, was identified in the interfollicular T-cell regions where $CD3^+$, $CD4^+$, and $CD8^+$ T cells reside (Kelsall and Strober 1996). In contrast with DCs in the dome region, which express the phenotype of immature DCs (i.e. high endocytic activity and low levels of MHC and B7 molecule expression), those in the T-cell areas are mature, interdigitating DCs with low endocytic activity and high levels of MHC I and II as well as B7 molecules (Kelsall and Strober 1996). The presence of these separate populations of DCs suggest that antigen is taken up by M cells and is first endocytosed in the dome region by DCs which may migrate to T-cell areas to become mature DCs.

It has recently been shown that Peyer's patch DCs preferentially promote Th2-type cells (Iwasaki and Kelsall 1999), suggesting that these cells also influence the nature of immune responses initiated in the gut. Few MΦs occur in the SED; however, both B cells and MΦs were found in the interfollicular T regions of Peyer's patches and lamina propria of intestinal villi. In addition, memory B and T cells occur in the pockets formed by M cells, and may be initial sites of immune responses (Yamanaka et al. 2001). DCs are also found in the NALT where they seem to play the same role as in the GALT. In fact, DCs are found in human tonsils (Bernstein 1999). It has been shown that DCs are recruited into the respiratory epithelium during acute immune responses induced by bacterial, viral, and protein antigens (McWilliam et al. 1996). In this regard, although freshly isolated rat respiratory DCs preferentially supported $CD4^+$ Th2-type responses, induction of Th1-type cytokine-mediated immune responses was also seen after stimulation with antigens (Stumbles et al. 1998). Studies of this nature suggest a further compartmentalization/specialization for antigen presentation by classic mucosal APCs; however, much is still unknown about the transport of antigen from M cells to DCs, MΦs, and memory B cells.

Mucosal immunization

It is generally agreed that mucosal adjuvants or delivery systems are essential in order to induce mucosal immune responses. In this regard, two bacterial enterotoxins (i.e. CT and LT from *E. coli*) are now established as very effective mucosal adjuvants for the induction of both mucosal and systemic immunity to co-administered protein antigens. To circumvent toxicity linked to these enterotoxins, mutant CT (mCT) and mutant LT (mLT) molecules devoid of their toxic activity were generated by site-directed mutagenesis. This approach involves the introduction of single amino acid substitutions in the active site (i.e. the site responsible for the ADP-ribosylation activity) of the A subunit of CT or LT or in the protease-sensitive loop of LT. Further, attenuated pathogenic bacteria such as recombinant (r) *Salmonella* were also used as a mucosal delivery system for the induction of S-IgA Ab responses to delivered antigen.

It is now realized that the nature of the delivery system as well as the route of immunization influences the nature of Th cell subsets induced and markedly affect the outcome of systemic and mucosal immunity, e.g. use of native CT or nontoxic mutants of CT with vaccines given orally tends to induce $CD4^+$ Th2-type cells with characteristic serum IgG1, IgG2b, IgE, and IgA as well as mucosal S-IgA Ab responses (Xu-Amano et al. 1993; Marinaro et al. 1995) (Figure 11). On the other hand, oral immunization with recombinant bacteria, e.g. r*Salmonella*-expressing proteins, tends to induce not only $CD4^+$ Th1-type cells and CMI responses but characteristic $CD4^+$ Th cells as well. These $CD4^+$ Th cells produce cytokines such as IFN-γ and IL-10 which appear to support mucosal S-IgA Ab responses

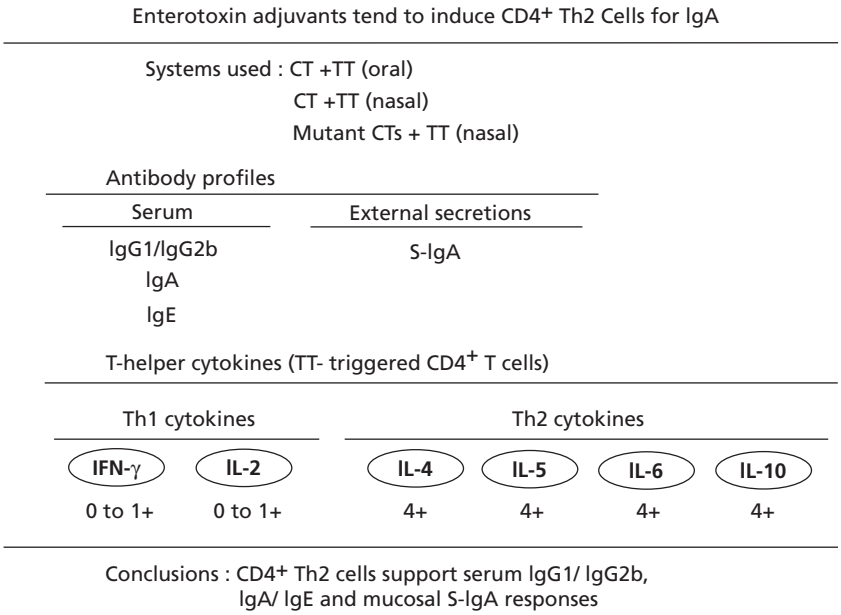

Figure 11 *T-helper 2 (Th2) cells for IgA responses. Th2-type cytokines mediate S-IgA Ab response induction. Use of native cholera toxin (CT) or nontoxic mutants of CT with vaccines given orally tend to induce CD4+ Th2-type cells with characteristic serum IgG1, IgG2b, IgE, and IgA as well as mucosal S-IgA responses.*

(Okahashi et al. 1996; VanCott et al. 1996). In this section, we have selected studies that illustrate the development of oral and nasal immunization strategies using either mucosal adjuvants or live vectors for mucosal vaccine development.

ORAL IMMUNIZATION

We have purposely selected a small subset of studies to illustrate the nature of murine immune responses which result from oral immunization with microbial vaccines. It is important to emphasize that immune responses vary depending on the nature of antigen used as well as mouse strains selected for study. However, proteins such as tetanus toxoid (TT) or hen egg white lysozyme (HEL) with CT as mucosal adjuvant induce predominantly serum IgG1 and IgE antibodies (Snider et al. 1994; Marinaro et al. 1995) (Figure 11). Further, in one study it was shown that systemic challenge of mice orally immunized with HEL and CT led to a fatal anaphylactic response (Snider et al. 1994). Thus, all studies that use CT or its mutant derivatives should be concerned with possible untoward reactions which may occur as a result of induction of IgE Ab responses. Oral immunization of mice with keyhole limpet hemocyanin (KLH) or TT and CT as adjuvant has been shown to induce Ag-specific $CD4^+$ Th cells with a characteristic Th2 phenotype (Wilson et al. 1991; Xu-Amano et al. 1993; Marinaro et al. 1995) (Figure 11), e.g. both PP and lamina propria lymphocytes, when restimulated with KLH, produced significant quantities of IL-4 and IL-5, with minimal levels of IFN-γ and IL-2 (Wilson et al. 1991). Thus, several studies now support the notion that oral immunization with soluble proteins and CT as adjuvant induces Th2-type responses, which provide help for characteristic serum IgG1 and IgE as well as mucosal S-IgA Abs. There is evidence that orally co-administered CT can also induce antigen-specific $CD4^+$ Th1-type responses, e.g. mice given a soluble preparation of *Toxoplasma gondii* on three occasions at 10-day intervals harbored splenic *T. gondii*-specific T cells (Bourguin et al. 1993). Further, in vitro restimulation resulted in increased levels of both IFN-γ and IL-2, whereas IL-4 and IL-5 levels were similar to those seen in mice orally immunized with *T. gondii* only (Bourguin et al. 1993).

A more revealing study used the mouse parasite *Trichuris muris* in a soluble form with CT and assessed IFN-γ and IL-5 levels as markers for Th1- or Th2-type responses respectively (Robinson et al. 1995). Interestingly, oral *T. muris* plus CT induced T cells that produced IL-5 before parasite challenge, but which switched to IFN-γ production after *T. muris* infection. This shift suggests that the infection itself results in $CD4^+$ Th1-type responses, even in the presence of a predominant Th2-type response. One study has shown that oral immunization with parasite antigen and CT elicits both serum IgG and mucosal IgA Ab responses (Zhang et al. 1995). This study, which also assessed anti-parasite Abs in bile (where IgA was the predominant isotype), revealed that oral immunization normally induces serum IgA, and in experimental species such as mice, rats, and rabbits, where the serum IgA is predominantly polymeric, the pIgA is transported into the GI tract. Thus, all studies that assess intestinal IgA responses should ensure that the response actually occurs locally and

therefore perform assays to detect IgA-producing plasma cells in lamina propria regions of the GI tract.

Attenuated avirulent salmonella strains have received considerable attention as mucosal vaccine delivery vectors for recombinant proteins associated with virulence (Chatfield et al. 1993; Curtiss et al. 1993; Roberts et al. 1994; Doggett and Brown 1996). After oral administration, *Salmonella* spp. replicate directly in the mucosa-associated tissues (e.g. PP) and thereafter disseminate via the MALT to systemic sites (e.g. spleen). This characteristic dissemination pattern of growth in both mucosal and systemic sites allows *Salmonella* spp. to induce broad-based immune responses, including cell-mediated as well as serum IgG and mucosal S-IgA Ab responses. Although a large number of genes from bacteria, viruses, parasites, and mammals have been expressed in attenuated r*Salmonella* spp. (Curtiss et al. 1993; Roberts et al. 1994), few studies have fully characterized both T- and B-cell responses to the expressed protein Ag. In particular, the balance between Ag-specific $CD4^+$ Th1 and Th2 cells, and their subsequent influence on subclass-specific IgG and mucosal IgA responses, have received little attention in these systems. Such clarity is paramount to the development of delivery protocols that will provide the appropriate immune response to a given pathogen.

A pioneering study in this area showed that mice given an oral attenuated *S. typhimurium* (expressing leishmania surface protein gp63) elicited $CD4^+$ T cells that produced IFN-γ and IL-2, but not IL-4 (Yang et al. 1990). The results at first might appear puzzling. As it appears that mucosal S-IgA responses are dependent on Th2 cells and cytokines such as IL-5, IL-6, and IL-10, by what mechanisms do these attenuated r*Salmonella*-expressing foreign protein antigens induce S-IgA responses? Recent studies have addressed this issue by use of r*Salmonella* spp. expressing the *Tox C* gene of TT. Oral administration of r*Salmonella Tox C* resulted in predominant serum IgG2a and IgG2b anti-TT as well as mucosal S-IgA anti-TT Ab responses (VanCott et al. 1996) (Figure 12). Further, splenic and PP $CD4^+$ T cells selectively produced IFN-γ and IL-2 as well as the Th2-cytokine IL-10 (VanCott et al. 1996). Also, Mϕs from these mice produced heightened levels of IL-6 (VanCott et al. 1996) (Figure 12). Clear verification that IL-4 is not involved was shown by oral immunization of IL-4 knockout ($IL\text{-}4^{-/-}$) mice which produced serum IgG2a and mucosal S-IgA Abs. Interestingly, $CD4^+$ T cells in these mice exhibited two cytokine patterns, a Th1-phenotype as well as T cells that produced IL-6 and IL-10, but not IL-5 (Okahashi et al. 1996) (see Figure 11). The latter subset of Th2 cells, which produce only IgA-enhancing cytokines, has been termed 'level 2 Th2-type cells' in contrast to so-called 'level 1 Th2-type cells' producing an array of IL-4, IL-5, IL-6, and IL-10.

NASAL IMMUNIZATION

Nasal antigen plus mucosal adjuvant delivery has emerged as perhaps the most effective route for induction of both peripheral and mucosal immunity. Again, most studies can be divided into those that use soluble vaccine components with mucosal adjuvants such as CT

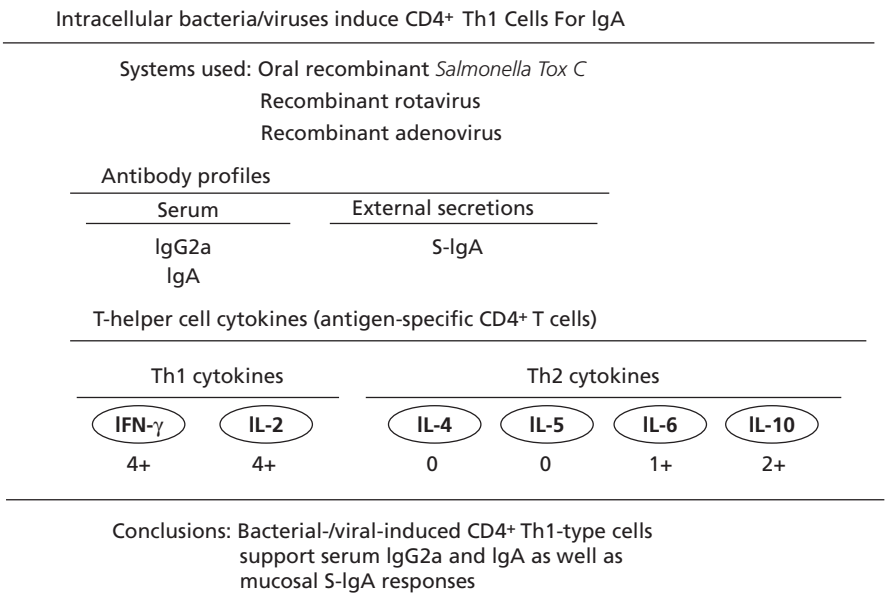

Figure 12 *T-helper 1 (Th1) cells for IgA responses: induction of S-IgA Abs via Th1-type responses. Oral administration of recombinant* Salmonella Tox C *resulted in predominant serum IgG2a and IgG2b anti-tetanus toxin (TT) as well as mucosal S-IgA anti-TT Ab responses. Further, splenic and Peyer's patch $CD4^+$ T cells selectively produced interferon-γ and interleukin-2 as well as the Th2 cytokine interleukin-10.*

(as well as protein–CT-B conjugates) versus studies with attenuated vectors. A series of extensive studies using the influenza virus model demonstrated that nasal immunization with influenza vaccine provides more effective protective immunity than oral immunization (Hirabayashi et al. 1990; Tamura and Kurata 1996). Nasal immunization with trivalent influenza vaccines in the presence of CT-B containing a trace amount of A subunit provides cross-protection against a broad range of viruses (Tamura et al. 1992). It was also shown that nasal immunization with influenza vaccine together with LT-B (containing a trace amount of the LT) induces antigen-specific immune responses in humans (Hashigucci et al. 1996). These findings show that an appropriate nasal vaccine can provide effective immunity against viral infection.

With regard to vectors, most studies have been limited to respiratory tract viral pathogens, which have been subjected to attenuating mutations or genomic deletions. It is interesting that recombinant adenoviruses (r-Ad), currently used for gene therapy, are also candidates for mucosal delivery of vaccine proteins. In most current protocols, vaccine is instilled into each nostril (usually 10 μg/nostril) and inhaled, resulting in effective delivery of vaccine presumably into NALT. Larger aliquots are subsequently swallowed, resulting in oral immunization. For this reason, any nasal protocol cannot avoid some oral delivery, although most studies of oral immunization employ gastric intubation to avoid potential nasal immunization. It should also be noted that two routes of pulmonary immunization have been used: intratracheal delivery versus nasal instillation. In general, it appears that intratracheal immunization tends to induce responses in the lower lungs and associated cervical and other draining lymph nodes, whereas nasal immunization sensitizes NALT and associated cervical lymph nodes.

Several studies can be used to illustrate principles associated with nasal immunization. In one, nasal administration of inactivated RSV with CT resulted in nasal IgA and serum IgG anti-RSV responses (Reuman et al. 1991). Analysis of IgG subclasses suggests that both IgG1 and IgG2a are induced (Reuman et al. 1991). Interestingly, infection with RSV and subsequent Th1-type responses are characterized by a favorable outcome, whereas Th2-type responses are associated with significant pathology (Graham et al. 1991a, b). Nasal immunization with the C fragment of TT (fragment C) and CT as adjuvant resulted in serum antibody responses characterized by comparable IgG1, IgG2a, and IgG2b anti-fragment C titers, suggesting that the use of CT as an adjuvant with fragment C induces a response characterized by both Th1- and Th2-type responses (Roberts et al. 1995). However, when fragment C is administered nasally with pertussis toxin (PT) or a mutated form of PT known as PT-9K/129G as mucosal adjuvants, anti-fragment C IgG1 predominates, suggesting that an immune response biased toward the Th2-type has been induced (Roberts et al. 1995). Direct comparisons between CT and PT or PT-9K/129 in this study are difficult because the use of CT is associated with much more potent anti-fragment C IgG responses. Further investigation will be needed to determine the role that adjuvants, the vaccine antigen, and the route of immunization play in induction of $CD4^+$ Th1- and Th2-type responses.

Mutants of CT constructed by substitution of serine with phenylalanine at position 61 (mCT-S61F) and glutamate by lysine at position 112 (mCT-E112K) in the ADP-ribosyltransferase activity center of the CT gene from *Vibrio cholerae* 01 strain GP14 display no ADP-ribosyltransferase activity or enterotoxicity (Yamamoto et al. 1997b). These mutants have been studied as potential nasal adjuvants. The levels of antigen-specific serum IgG and S-IgA Abs induced by the mutants are comparable to those induced by wild-type CT and significantly higher than those induced by recombinant CT-B (Yamamoto et al. 1997a, b). Further, the mCT-E112K, like mCT, induces Th2-type responses through a preferential inhibition of Th1-type $CD4^+$ T cells. Mutations in other sites of the CT molecule were reported to induce nontoxic derivatives but the adjuvant activity was also affected.

Mutant LT molecules, whether possessing a residual ADP-ribosyltransferase activity (e.g. LT-72R) or totally devoid of it (e.g. LT-7K and LT-6K3), can function as mucosal adjuvants when intranasally administered to mice together with unrelated antigens (Rappuoli et al. 1999). However, discrepant results were reported when two nontoxic mLTs were tested for their adjuvanticity after oral immunization of mice. Thus, although mutant LT-E112K (bearing a substitution in the active site of the A subunit) was unable to amplify the response to keyhole limpet hemocyanin when both were given orally to C57BL/6 mice, the mutant with a substitution in the protease-sensitive region, LT-R192G, retained the ability to act as a mucosal adjuvant (Dickinson and Clements 1995). Whether the type of the substitution played a role in the reported discrepancies remains to be elucidated. As LT induces a mixed $CD4^+$ Th1-type (i.e. IFN-γ) and Th2-type (i.e. IL-4, IL-5, IL-6, and IL-10) response (Takahashi et al. 1996), one might envision the use of mutants of LT when both Th1- and Th2-type responses are desired.

MUCOSALLY-INDUCED TOLERANCE

Mucosal tolerance

In addition to the beneficial induction of Ag-specific S-IgA and serum IgG Ab responses after mucosal immunization, the mucosal route of Ag delivery can also induce another type of immune response, namely the induction of systemic unresponsiveness (e.g. oral tolerance). Thus, mucosal Ag delivery can either up- or down-regulate

systemic immune responses. As discussed above, for the purpose of mucosal vaccine development against infectious diseases, the goal is induction of both mucosal and systemic immunity in order to provide two layers of protection. In contrast, inhibition of Ag-specific immune responses in systemic compartments by mucosal Ag delivery is important for the prevention of over-stimulation of responses and frequently encountered hypersensitivity responses to food proteins and allergens. Further, this system could potentially be applied to the prevention and possibly the treatment of autoimmune diseases by feeding relevant Ags.

Oral administration of a single high-dose or repeated oral delivery of low doses of proteins have been shown to induce systemic unresponsiveness, presumably in the presence of mucosal IgA Ab responses (Challacombe and Tomasi 1980; Tomasi 1980; Mowat 1987; Weiner et al. 1994; Czerkinsky et al. 1999; Garside et al. 1999; Mayer 2000; Fujihashi et al. 2001a) (Figure 13). These immunologically distinct responses in mucosa-associated versus systemic-associated lymphoid tissues were originally termed oral tolerance (Tomasi 1980). More recent studies have shown that the nasal administration of proteins also induces systemic unresponsiveness (Hoyne et al. 1993; McMenamin and Holt 1993; Metzler and Wraith 1993; Tian et al. 1996; Higuchi et al. 2000) and has led to the more general term 'mucosal tolerance' to include nasal or oral Ag induction of unresponsiveness.

Mucosal administration of protein Ag is a long-recognized method of inducing peripheral unresponsiveness or tolerance (Wells 1911). This is a unique immune reaction and is characterized by the fact that experimental animals fed large quantities of protein Ag become refractory or have a diminished capability to develop an immune response when re-exposed to that same Ag introduced by the systemic route (e.g. by parenteral injection) (Tomasi 1980; Mowat 1994). This unique response is an important natural physiological mechanism whereby the host presumably avoids development of hypersensitivity reactions to many ingested food proteins and other antigens (Garside et al. 1999). Thus, tolerance (or systemic unresponsiveness) represents the most common response of the host to our environment. The continuous ingestion of several thousand different food proteins is but one important example, whereas tolerance to our indigenous microflora that colonize the large intestine represents another major example. It is also useful to consider that induction of mucosal and systemic immunity by oral immunization is rather difficult and requires use of potent mucosal adjuvants, vectors, or other special delivery systems (see previous section). Further, the development of mucosal tolerance against pollen and dust antigens could also be essential for the inhibition of allergic reactions, including IgE-mediated hypersensitivity.

Most now agree that mucosal tolerance is mediated by T cells which are involved in the generation of active suppression or clonal anergy and/or deletion (Friedman and Weiner 1994), e.g. high doses of antigen given by the oral route induced clonal deletion or anergy (Whitacre et al. 1991; Melamed and Friedman 1993; Chen et al. 1995b) characterized by the absence of T-cell proliferation and diminished IL-2 production, as well as IL-2R expression. Frequently administered, low doses of antigen, however, induced active suppression by $CD4^+$ or $CD8^+$ T cells, which secreted cytokines such as

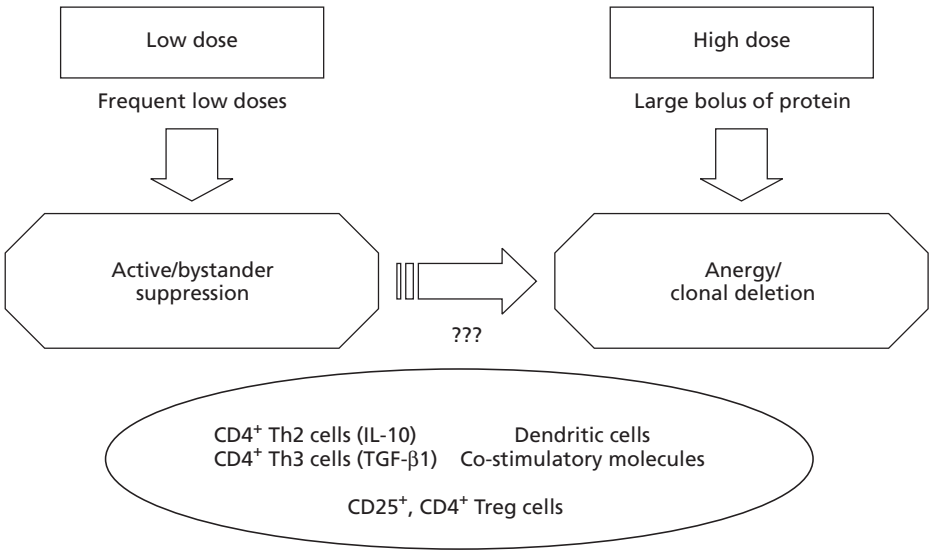

Figure 13 *Proposed mechanisms for mucosal tolerance: oral administration of a single high-dose or repeated oral delivery of low doses of proteins have been shown to induce systemic unresponsiveness. High doses of antigen given by the oral route induced clonal deletion or anergy characterized by the absence of T-cell proliferation and diminished interleukin 2 (IL-2) production as well as IL-2 receptor expression. Frequently administered low doses of antigen induced active suppression by $CD4^+$ or $CD8^+$ T cells which secreted cytokines such as transforming growth factor-β, and IL-10.*

TGF-β, IL-4, and IL-10 (Khoury et al. 1992; Chen et al. 1995a) (Figure 13). It is interesting to note that the latter scenario involves cytokines that are also known to upregulate IgA production (Czerkinsky and Holmgren 1995) and is thus compatible with the observation that mucosal immune responses and systemic tolerance may develop concomitantly (Asano et al. 1995; McGhee et al. 1999). As oral tolerance is specific for the antigen initially ingested or inhaled, and thus does not influence the development of systemic immune responses against other antigens, its manipulation has become an increasingly attractive strategy for preventing and possibly treating illnesses associated with or resulting from the development of untoward immunological reactions against specific antigens encountered or expressed (autoantigens) in nonmucosal tissues.

Mechanisms to explain oral tolerance

In the late 1970s and early 1980s, mucosal immunologists had already made attempts to investigate the possible mechanisms of oral tolerance at a time when the immune system was not characterized at the cellular and molecular levels (Thomas and Parrott 1974). Although several possible mechanisms (e.g. B-cell tolerance, anti-idiotypic antibody, intestinal antigen-processing events for tolerogen, and APCs) have been shown to be involved in the induction of oral tolerance (Mowat 1994), the most compelling evidence to date suggests that T cells are the major cell type involved in the induction of mucosally induced tolerance. In earlier work, it was shown that systemic unresponsiveness was induced by the adoptive transfer of T cells from rats orally fed bovine serum albumin (Thomas and Parrott 1974). Subsequently, a large number of studies demonstrated that oral immunization of protein antigen induces $CD4^+$ Th cells in mucosa-associated tissues that support IgA responses, whereas suppressor T cells were induced in systemic compartments such as spleen that downregulate Ag-specific IgM, IgG, and IgE responses (Mattingly and Waksman 1978; Ngan and Kind 1978; Kagnoff 1980; Kiyono et al. 1980, 1982b; Richman et al. 1981; Mowat et al. 1988), e.g. oral feeding of ovalbumin to mice led to the generation of Th cells supporting IgA responses and suppressor T cells for IgG and IgE responses in GALT (Mattingly and Waksman 1978; Ngan and Kind 1978; Mowat et al. 1988). Further, the former T cells for IgA responses remained in Peyer's patch, whereas the suppressor T cells migrated into the systemic compartment (e.g. spleen). These observations were considered to be logical explanations for cellular mechanisms of oral tolerance where Peyer's patch-derived $CD4^+$ Th cells supported IgA responses, whereas splenic T-suppressor cells induced systemic unresponsiveness. This must now be re-evaluated, because it has been shown that administration of a large dose of ovalbumin to mice followed by attempts at oral immunization with ovalbumin plus CT resulted in unresponsiveness in both mucosal and peripheral immune compartments (Kato et al. 2001).

A role for αβ T cells in oral tolerance

The αβ TCR^+ T cells appear to be the major players in downregulation of systemic immune responses to orally administered antigens. Most also agree that the status of oral tolerance can be explained by (1) clonal anergy, (2) clonal deletion of T cells, or (3) active suppression by T-regulatory (Treg) cells through the secretion of inhibitory cytokines (Whitacre et al. 1991; Miller et al. 1992; Gregerson et al. 1993; Melamed and Friedman 1993, 1994; Friedman and Weiner 1994; Garside et al. 1995; Hirahara et al. 1995). Low doses of oral antigen tend to favor the latter form of inhibition, whereas higher doses of feeding induce clonal anergy of immunocompetent T cells (Gregerson et al. 1993; Friedman and Weiner 1994; Garside et al. 1995; Hirahara et al. 1995). These two forms of oral tolerance are not mutually exclusive and may occur simultaneously after oral administration of antigens.

T-CELL ANERGY

Anergy is defined as a state of T-cell unresponsiveness characterized by the lack of proliferation and IL-2 synthesis, and by diminished IL-2R expression (Schwartz 1990), a condition reversed by pre-culturing T cells with IL-2 (DeSilva et al. 1991). Oral tolerance to a large dose of ovalbumin Ag-induced anergy in ovalbumin-specific T cells (Melamed and Friedman 1993) (see Figure 13). Further, oral myelin basic protein (MBP) diminished IL-2 and IFN-γ synthesis (Whitacre et al. 1991). These findings suggest that Th1-type T cells may be susceptible to the induction of anergy after oral feeding. To support this, it has been shown that Th1-type cells appear to be more sensitive to the induction of tolerance in vitro than Th2-type cells (Williams et al. 1990). In vivo evidence has demonstrated that Th1 cells are likely to be anergized in oral tolerance (Burstein and Abbas 1993). This may be an oversimplification because it has been shown that oral tolerance can be induced in mice defective in Th1 ($STAT4^{-/-}$) or Th2 ($STAT6^{-/-}$) cells (Shi et al. 1999). Further, in order to identify which lymphocyte compartment (e.g. $CD4^+$ versus $CD8^+$ T cells) preferentially mediates the induction of oral tolerance, cell transfer experiments were recently performed using severe combined immunodeficiency (SCID) and *nu/nu* mice (Hirahara et al. 1995). Adoptive transfer of splenic lymphocytes from mice orally tolerized with bovine α-casein resulted in the induction of tolerance in these immunocompromised mice. It was shown that oral tolerance was induced by anergized $CD4^+$ but not $CD8^+$ T cells. Taken together, a form of oral tolerance can be achieved by the induction of anergic $CD4^+$ T cells in the systemic compartment.

AN IMBALANCED TH1/TH2 CYTOKINE NETWORK

The induction of oral tolerance can also be explained by dysregulation of homeostasis between Th1- and Th2-type cells, e.g. preferential activation of Th2 cells may lead to downregulation of Th1 CMI responses by Th2 cytokines such as IL-4 and IL-10 (Burstein and Abbas 1993). In addition, Th1-type cells are much more sensitive to anergy induction after oral administration of protein antigens. These findings suggest that oral tolerance is associated with selective downregulation of Th1 cells by Th2 cells via their respective cytokines in the systemic immune compartment. This possibility is consistent with the fact that oral tolerance has more profound effects on Th1-regulated CMI responses than on Th2-mediated antibody responses. However, recent studies have shown that feeding high doses of ovalbumin inhibited production of both Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-5, and IL-10) cytokines and resulted in the reduction of IFN-γ- and IL-4-dependent antigen-specific IgG2a and IgG1 Ab responses, respectively (Garside et al. 1995). These findings indicate that both subsets of Th cells are involved in the induction of oral tolerance and both are downregulated. Oral ovalbumin induced brisk IFN-γ production with inhibition of the IgG-enhancing cytokine IL-4 by Th2 cells, leading to reduced B-cell responses, although no oral tolerance was seen in IFN-γ knockout mice (Kweon et al. 1998). Further, repeated oral administration of high doses of ovalbumin to ovalbumin-specific TCR Tg mice resulted in an IFN-γ-dominated immune responses in the Peyer's patches (Marth et al. 1996). Taken together, the immunological consequences of systemic B-cell tolerance induced by a high dose of oral Ag could be the result of IFN-γ-mediated immune regulation, with significant suppression of Th2-type cells.

T-regulatory cells in oral tolerance

The finding that $CD4^+$ T-cell clones were generated after induction of oral tolerance to MBP clearly led to the description of a new phenotype of regulatory T cells. Clones of $CD4^+$ T cells were MBP specific and, of 48 clones assessed, 42 produced the active form of TGF-β1. Six clones produced high levels of TGF-β1 with essentially no IL-4 or IL-10 (Chen et al. 1994). On the other hand, five clones produced high IL-4 and IL-10, which is of course typical of Th2-type cells. The authors suggested the existence of a TGF-β1-producing, regulatory T cell involved in control of mucosal immune responses and named it Th3 (Chen et al. 1994).

The Treg cells appear to control mucosal immunity, tolerance, and inflammation to a higher degree than comparable Treg cell types in peripheral lymphoid tissues. Generally speaking, Treg cells do not proliferate well in vitro, a characteristic reminiscent of anergic T cells. In fact, cloned anergic T cells can suppress immune response in vivo and this appears to be partly caused by effects on APCs (Taams et al. 1998; Chai et al. 1999; Vendetti et al. 2000). Thus, anergic T cells downregulate DC expression of CD80 and CD86 in a contact-specific fashion (Vendetti et al. 2000). Further, anergic T cells have been shown to produce IL-10, which is a major characteristic of some $CD4^+$ $CD25^+$ Treg cells (Sundstedt et al. 1997; Buer et al. 1998; Sakaguchi 2000; Shevach 2000). Thus, it appears that anergic T cells, through production of IL-10 (and perhaps through other mechanisms), become Treg cells that suppress immune responses to other antigens (Buer et al. 1998), a process sometimes termed 'infectious tolerance' or 'bystander suppression'. Despite their poor proliferative responses to Ag, it has been possible to induce populations of T-cell clones after incubation with IL-10 and alloantigen in humans (Groux et al. 1996) or to ovalbumin peptide in DO11.10 mice (Groux et al. 1997). The T-cell clones obtained had similar properties, including secretion of high levels of IL-10, some production TGF-β1, with no IL-4 synthesis and poor proliferative responses (Maloy and Powrie 2001). Cells with these characteristics are now termed T-regulatory one cells (Tr1). Thus far, Tr1 and Th3 cells have in common the production of TGF-β1 (Th3) or TGF-β1 + IL-10 (Tr1) with suppressive-type properties.

In a hapten-induced model of colitis, it was shown that 2,4,6-trinitrobenzene sulfonic acid (TNBS) coupled to mouse colonic (self) proteins, when given orally, led to oral tolerance to hapten and a failure to induce TNBS colitis (Neurath et al. 1996). Interestingly, both Peyer's patch and lamina propria $CD4^+$ T cells, when stimulated via the TCR, produced high levels of TGF-β1, IL-4, and IL-10, suggesting that Th3 and possibly Tr1 cells regulate this type of tolerance (Neurath et al. 1996). Direct evidence for intestinal tolerance to luminal bacteria in humans was provided by the finding that lamina propria $CD4^+$ T cells responded poorly to *E. coli* proteins, a condition reversed by anti-IL-10 or anti-TGF-β1 mAb treatment (Khoo et al. 1997). The best direct evidence for induction of $CD4^+$ $CD25^+$ Treg cells by oral Ag was provided by an adoptive transfer model of DO11.10 TCR ovalbumin Tg T cells. Transfer followed by either intravenous or oral ovalbumin led to an increase in ovalbumin-specific, $CD4^+$ $CD25^+$ T cells (Thorstenson and Khoruts 2001). It should be emphasized that this last study used 25 mg ovalbumin in drinking water over an 18-h period, suggesting a relatively high oral dose of Ag-induced Treg cells (Thorstenson and Khoruts 2001).

A distinct role for γδ T cells in low-dose oral tolerance

It is well established that mucosal immune compartments such as the intestinal epithelium contain large

numbers of γδ T cells in addition to αβ T cells, as does the lamina propria region of the small intestine (Aicher et al. 1992). As γδ T cells are localized in mucosa-associated tissues, we hypothesized that mucosal γδ T cells could be involved in immune responses in the lamina propria of normal mice, e.g. IEL T cells from mice immunized orally with sheep erythrocytes (SRBC) were separated into γδ and αβ T cells. When purified γδ and αβ T cells were adoptively transferred to mice orally tolerized with SRBCs, a conversion of systemic unresponsiveness to IgM, IgG, and IgA anti-SRBC Ab responses was achieved in mice that received γδ but not αβ T cells (Fujihashi et al. 1992). A more recent study has also demonstrated that γδ T cells isolated from mucosa-associated tissues of mice immunized orally with protein vaccine (B subunit of LT from *E. coli* – LT-B) exhibited a similar activity, i.e. IEL γδ T cells from LT-B-fed mice (which were tolerized) abrogated systemic unresponsiveness after adoptive transfer to syngenic mice orally tolerized with LT-B (Takahashi et al. 1995). Thus, γδ T cells appear to be important in the maintenance of mucosal IgA responses and possibly systemic unresponsiveness. In this regard, studies demonstrated that γδ T cells are essential for the induction of oral tolerance using mAb anti-γδ TCR-treated and TCR$\delta^{-/-}$ mice (Ke et al. 1997). Further, a distinct role was suggested for γδ T cells in the induction of high- versus low-dose oral tolerance. Although γδ T cells play a major immunoregulatory role for the induction of low-dose oral tolerance via upregulation of IL-10 synthesis, this T-cell subset is not involved in high-dose oral tolerance (Fujihashi et al. 1999) (see Figure 13).

A role for Peyer's patches in oral tolerance

The precise site of Ag uptake in the GIT during oral tolerance induction has not been firmly established. At least three possibilities could be proposed and none is mutually exclusive (Rubas and Grass 1991). First, Ag may be pinocytosed into the epithelial cells themselves and interactions with IELs may influence oral tolerance. Second, Ag may selectively enter the GALT via M cells and lead to APC–T-cell interactions which downregulate T-/B-cell responses. Finally, oral Ag may not perturb the GIT immune system at all, but simply enter and cross the epithelium in a paracellular fashion and reach the bloodstream, where tolerance would be induced. Some investigators have suggested that organized lymphoid tissue in the GIT was not required for oral tolerance to ovalbumin, because B-cell-defective mice, which contain poorly developed Peyer's patches, were fully tolerized at the level of T cells (Alpan et al. 2001). Although the study claimed that GALT was absent, others have shown that some remnants of Peyer's patches are seen in μMT mice (Golovkina et al. 1999). Others have also shown that direct injection of Peyer's patches induced oral tolerance (Chen et al. 2000). The availability of mice without Peyer's patches has allowed reinvestigation of the notion that GALT may be involved in oral tolerance (Figure 14). In one study, it was shown that mice lacking GALT but retaining mesenteric lymph nodes (MLN) could be orally tolerized to ovalbumin (Spahn et al. 2001). In other studies, mice lacking Peyer's patches but retaining MLNs were found to be resistant to oral tolerance to protein (Fujihashi et al. 2001b); however, these mice showed normal mucosal S-IgA Ab responses to oral protein given with CT as adjuvant (Yamamoto et al. 2000). One cannot yet conclude whether GALT is a strict requirement for oral tolerance to proteins.

Oral tolerance in humans

Increasing attention is being paid to oral tolerance and the role that it could play in the prevention or treatment of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and uveitis, as well as type 1 diabetes and contact hypersensitivity (Weiner 1997). Indeed, humans fed the neoantigen KLH developed systemic unresponsiveness evaluated by delayed-type hypersensitivity and T-cell proliferative responses (Husby et al. 1994). However, B-cell responses were primed in both systemic and mucosal sites (Husby et al. 1994). In more recent studies, humans naturally ingesting the dietary Ags bovine γ-globulin, ovalbumin, and soybean protein developed a T-cell tolerance characterized by anergy (Zivny et al. 2001). Antigen-specific Th3 cells secreting TGF-β have been observed in the blood of multiple sclerosis patients orally treated with a bovine myelin preparation (Fukaura et al. 1996), demonstrating that oral administration of autoantigen can induce autoantigen-specific TGF-β-secreting cells in a human autoimmune disease.

Pilot clinical trials of oral tolerance have been conducted in patients with autoimmune diseases and promising clinical benefits have been reported (Weiner 1997; Czerkinsky et al. 1999). Despite encouraging results that have been reported regarding oral delivery of autoantigens for the prevention and treatment of autoimmune diseases, a follow-up study provides an unfortunate alternative possibility. Oral feeding of autoantigen in mice resulted in the generation of antigen-specific $CD8^+$ CTL responses, which could lead to the aggravation of autoimmune disease (Blanas et al. 1996). Thus, one must also keep in mind that oral administration of autoantigen may induce undesirable $CD8^+$ CTLs that may worsen the disease instead of preventing the development of autoimmune diseases.

Oral desensitization to nickel allergy was reported to reduce nickel-specific T cells and the cutaneous form of eczema (Bagot 1995). Oral Ags had earlier been

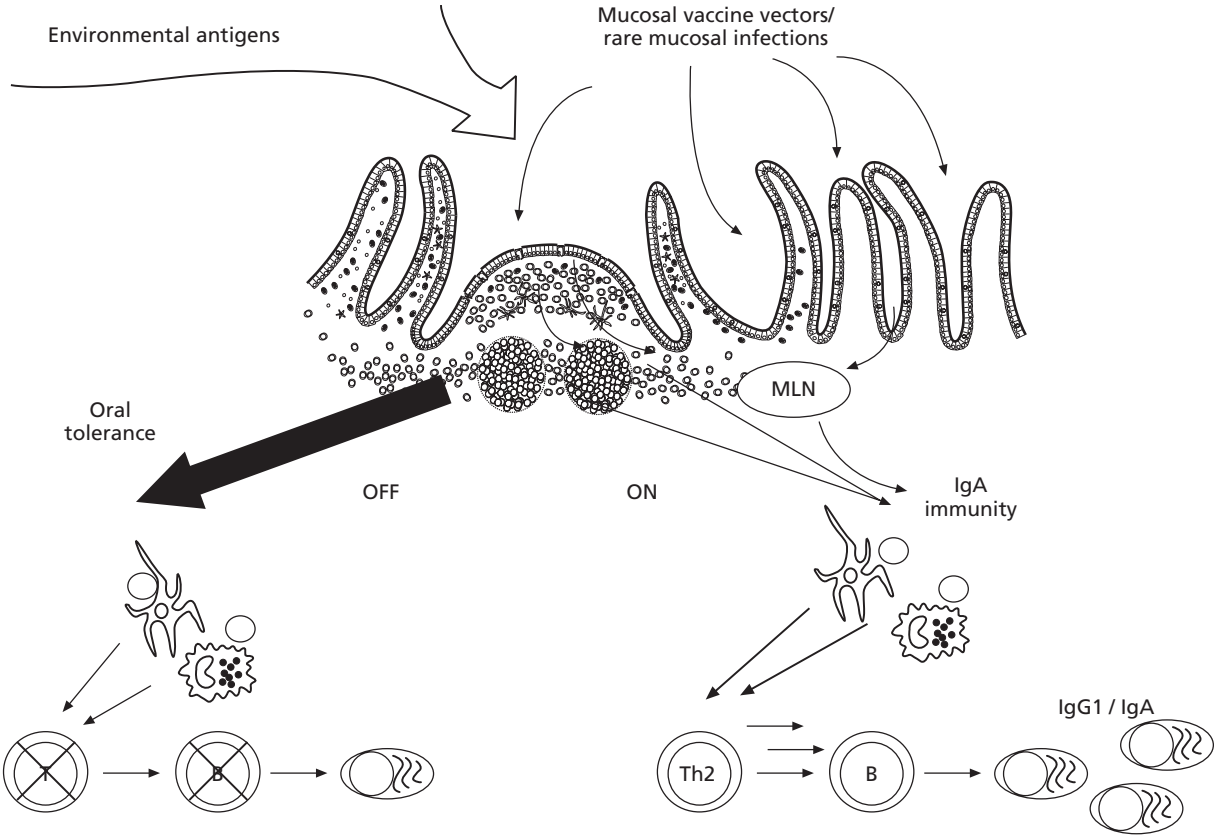

Figure 14 *Regulation of mucosal immunity and tolerance: mucosal homeostasis provided by mechanisms of tolerance including oral tolerance may be common and important mucosal immune responses. On the other hand, induction of vaccine-specific IgA responses at mucosal surfaces requires mucosal adjuvants or delivery systems.*

proposed to prevent or even treat allergic reactions to common allergens such as house-dust mite and grass pollens (Wortmann 1977; Rebien et al. 1982). However, this has not yet reached fruition. Thus, the experience of most investigators is that, once an immune response has been induced, it cannot be reversed through mucosal tolerance. Although the above examples indicate that oral tolerance offers promise for inducing antigen-specific immunological tolerance, its therapeutic potential remains limited by practical problems. Indeed, large quantities of orally administered antigens (e.g. milligrams in mice, grams in humans) are required to induce systemic unresponsiveness in experimental animals and humans. Taken together, these initial human trials have shown that oral tolerance, which paralleled results in animal models, could be induced in humans. However, a consistent clinical efficacy remains to be shown in human diseases.

CONCLUSIONS

The mucosal immune system is maintained through well-organized, interactive, innate, and acquired immune compartments. Thus, mucosal surfaces, which cover huge areas, are protected in concert by the two arms of this defense system, respectively. In addition, most studies clearly suggest that the main players in innate immunity serve as a bridge with acquired immunity, e.g. IECs produce antimicrobial peptides such as defensins. Further, an array of cytokines and chemokines is also produced by epithelial cells and these molecular responses subsequently induce expression of MHC I and II as well as co-stimulatory molecules, which are key factors for the APC-directed initiation of acquired mucosal immune responses. In addition, cross-talk by the epithelium to IEL T cells again bridges with acquired immunity. In this regard, IECs and IEL T cells cross-regulate each other in maintaining mucosal homeostasis at the barrier interface.

It is obvious that S-IgA Abs play central roles in mucosal immunity. Thus, large efforts have been made to define how S-IgA Ab responses are regulated. The organized mucosal inductive and effector tissues are essential elements and play key roles in the induction and regulation of S-IgA Ab responses. Inside these tissues, $CD4^+$ T cells and their derived cytokines are essential for B-cell switching to sIgA expression, stimulation, growth, and terminal differentiation into IgA-producing plasma cells. Induction of Ag-specific IgA Ab responses at mucosal surfaces is a rather difficult task

(see Figure 14). In this regard, potent mucosal adjuvants or former pathogens, including recombinant delivery systems, are essential for mucosal vaccine development, and have been extensively studied. To date, only mucosal immunization (oral, nasal, and rectal routes) successfully induces S-IgA Ab responses. Effective mucosal vaccines provide two layers of defense, the first at mucosal surfaces (S-IgA) and the second in systemic lymphoid sites (plasma IgG). The occurrence of Ag-specific S-IgA Ab responses is a relatively rare type of immune response. Thus, mucosal homeostasis provided by mechanisms of tolerance including oral tolerance may be a more common and important type of mucosal immune response (see Figure 14). In summary, the major goals in the past, the present and the future will be to elucidate the cellular and molecular mechanisms involved in the induction and regulation of opposite types of mucosal immune responses, i.e. S-IgA immunity and mucosal tolerance (see Figure 14).

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Superantigens

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'Superantigens' is the designation for a heterogeneous group of proteins that use a common, extremely efficient mechanism of T-lymphocyte stimulation. They bind to MHC class II molecules on antigen-presenting cells and to variable parts of the T-cell receptor on $CD4^+$ and $CD8^+$ T cells, thus mimicking the recognition of specific antigen. The prototype superantigen is the staphylococcal enterotoxin B, member of a family of genetically related pyrogenic exotoxins produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. This principle of T-lymphocyte stimulation has been evolved several times independently by infectious pathogens.

The unusual mechanism of T-lymphocyte stimulation has been explored at a molecular level and it has been shown that the pathogenic effects of these molecules are the result of their ability to stimulate a large fraction of T cells. Consequences of confrontation of the body with superantigenic toxins are:

1. shock, mediated by a massive liberation of cytokines from cells of the immune system;
2. immunosuppression, mainly caused by an uncoordinated activation of the immune system and a massive deletion of T cells;
3. autoimmunity resulting from bypass activation of autoreactive T and B cells.

INTRODUCTION

Antigen recognition by T lymphocytes is one of the most sensitive assays in biology. With its antigen receptor the T cell can respond to only a few antigenic peptides presented by an antigen-presenting cell (APC). This unique sensitivity is provided by a set of molecular mechanisms that intracellularly amplify the initial signal given by one or only a few T-cell receptors (TCR). Superantigens have been adapted in evolution to use the T-cell machinery to become most effective T-cell stimulators. Although there are different types of superantigens that have been independently generated several times in evolution and that are completely unrelated to each other, they all use the same molecular mechanism: they bind to major histocompatibility complex (MHC) class II molecules on APCs and to the TCR, and thus crosslink the TCR to the MHC molecule of the APC. Therefore, by closely mimicking antigen recognition they use most of the amplification mechanisms that the T cell uses normally. Thus, a hallmark of all superantigens is their enormous potency: they are active in concentrations of 10^{-9} mol/l. This means that a few picograms per milliliter can still effectively stimulate T cells in culture. Another characteristic feature of superantigens is their use of variable determinants on molecules of the immune system: all superantigens known so far bind to the variable part of the TCR β chain (Vβ); thus each superantigen stimulates a particular fraction (up to 30 percent) of the T-cell repertoire. On the MHCII molecule there are different binding sites used by different superantigens, some superantigens even involve the antigenic peptide in their binding.

This principle of T-lymphocyte stimulation has evolved several times independently. Infectious pathogens as different as gram-positive cocci, gram-negative rods, a mycoplasma, and retroviruses produce superantigens that are apparently completely unrelated to each other. Obviously, this very special mechanism of

Table 1 *Some candidate superantigens produced by microorganisms*

Producing pathogen	Protein	Other activities	Superantigen?
Staphylococcus aureus	SEA-E, SEG-P		Yes
	TSST-1		Yes
	ETA, ETB	Proteases	?
Streptococcus pyogenes	SPEA, C, G–J		Yes
	SSA		Yes
	SMEZ		Yes
	SPEB	Protease	No
	M protein	Receptor	No
	MF ('SPEF')	Nuclease	No
Clostridium perfringens	Enterotoxin[a]	Pore former	No
Pseudomonas aeruginosa	Exotoxin A[b]	ADP-ribosyltransferase	No
Yersinia pseudotuberculosis	YPM		Yes
Yersinia enterocolitica	Undefined[c]		?
Mycobacterium tuberculosis	Undefined[d]		?
Mycoplasma arthritidis	MAS		Yes
Toxoplasma gondii	Undefined[e]		?
MMTV	orf		Yes
HERV	orf		Yes

a) Bowness et al. (1992), McClane (1994), Krakauer et al. (1997).
b) Legaard et al. (1991).
c) Stuart and Woodward (1992).
d) Ohmen et al. (1994).
e) Denkers et al. (1994).

T-cell stimulation has been developed by very different pathogens independently in evolution so it is likely that additional pathogens will also use it. Therefore, in the last few years, there have been a surprisingly large number of reports describing novel superantigens in various viruses, bacteria, or protozoa. With one exception, the lectin *Urtica dioica* agglutinin of the stinging nettle (Saul et al. 2000), all molecules that have been proposed to be superantigens are produced by infectious pathogens. (The lectin has two saccharide-binding sites and its superantigenic properties arise from the simultaneous fixation of glycans on the TCR and MHC molecules.) The long list of candidate superantigens includes a wide range of bacteria, protozoa, and viruses, some of which are listed in Table 1. In most cases the evidence is still indirect or the initial report has not been confirmed by an independent study. This review limits the discussion to those superantigens that are beyond any doubt superantigens.

PYROGENIC EXOTOXINS OF *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PYOGENES*

Genes and molecules

The pyrogenic exotoxins (PET) produced by these two genera of gram-positive cocci comprise a highly polymorphic family of genetically related toxins consisting of the staphylococcal enterotoxins (SE) and the toxic shock syndrome toxin-1 (TSST-1) produced by *Staphylococcus aureus* and streptococcal pyrogenic exotoxins (SPE) produced by *S. pyogenes*. *S. aureus* is widespread among humans and animals and many of these toxins are also present in *S. aureus* strains from other species. They are heat-stable and protease-resistant molecules of molecular mass 22–28 kDa.

Historically, the staphylococcal enterotoxins were defined by their ability to cause emesis and diarrhea in humans and monkeys. They are a major cause of food poisoning worldwide. Initially, five major serotypes SEA, SEB, SEC, SED, and SEE have been described that were serologically defined by the lack of cross-reaction of specific antisera. TSST-1 was initially designated SEF, but was later renamed TSST-1 because of its association with toxic shock syndrome – and because the initial finding of emetic activity could not be confirmed (Reiser et al. 1983). Currently the staphylococcal enterotoxins include in addition SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, and SEP (Jarraud et al. 2001; McCormick et al. 2001).

The SPEs are usually discussed separately from the SEs because they are derived from another genus and an enterotoxic activity has not been described. However, as such activity can be detected only in humans or monkeys it is not clear whether this putative difference has been sufficiently investigated. From a genetic point of view the separation of the SPEs from the SEs is not appropriate because some SPEs are more related to SEs than to other

Table 2 *Grouping of members of the staphylococcal enterotoxin superfamily according to genetic relatedness (Proft et al. 1999; McCormick et al. 2001)*

Group	Members		Common properties
I	TSST-1	TSST-1 and variants	
II	SEB-like	SEB, $SEC_{(n)}$, SSA, SPEA, SEG	Central cysteine loop
III	SEA-like	SEA, SEE, SEJ, SED, SEH	Central cysteine loop, zinc-binding site
IV	SPEC-like	SPEC, SPEJ, SMEZ, SPEG	Zinc-binding site
V	SPEH-like	SEK, SPEH, SEP, SEI, SEL	Zinc-binding site

Not yet classified: SEM, SEN, SEO (Jarraud et al. 2001).

SPEs. The members of the SE family can be divided into five subgroups (Table 2) according to their sequence relatedness (Betley et al. 1992; Munson et al. 1998; Proft et al. 1999, 2001; McCormick et al. 2001).

Homologies vary considerably within the groups. A common feature of all PETs is the overall molecular architecture: globular two-domain proteins of about 22–29 kDa with a long solvent accessible α-helix in the center of the molecule. Although some PETs, such as TSST-1 or SPEC, share little similarity with any other PET, there is sufficient sequence homology among all PETs to suggest that streptococcal and staphylococcal PETs have evolved from a single ancestor gene. This is in line with the finding that staphylococcal and streptococcal toxins are mixed within the different subgroups. Moreover, as other 'accessory proteins' are not required for bacterial growth many of the PETs are encoded on mobile genetic elements – those for SEA, SEE, SPEA, and SPEC are carried within bacteriophage DNA and for SED and SEJ on plasmid DNA (Betley et al. 1992; Johnson and Schlievert 1984; Weeks and Ferretti 1984; Goshorn and Schlievert 1989). Others are encoded within pathogenicity islands on the chromosome, e.g. TSST-1, SEB, or SEC (Novick et al. 2001). The streptococcal superantigen SSA is more related to SEB and SEC than to other streptococcal PETs, suggesting an intergeneric transfer in that it appears to have been acquired by several different clonal lineages of *S. pyogenes* during the twentieth century (Reda et al. 1994).

A characteristic feature of SEs and SPEs is their extensive polymorphism; there are many alleles of some toxins known which differ in only few amino acids, e.g. three subtypes of SEC have been numbered (SEC1–3) and a number of variants of SEC of *S. aureus* strains derived from humans, dogs, cattle, and food have been cloned (Marr et al. 1993). The variants differ only in a few amino acids but may differ in their biological activity (Bohach 1997). Several isolates of SEC3 that are serologically indistinguishable differ in several mutations. SEA and SEE have very high homology and show strong serological crossreaction; thus SEE could even be classified as a variant of SEA. TSST-1 produced by *S. aureus* strains associated with humans has a p*I* of 7.0 whereas *S. aureus* isolates from sheep produce the variant TSST-0 with a p*I* of 8.6 (Ho et al. 1989) which differs in only seven amino acids from TSST-1, but differs dramatically in its biological activity (Murray et al. 1994). Four different variants of SPEA have been described (Nelson et al. 1991) and three variants of SSA (Reda et al. 1994).

A particularly interesting toxin is the streptococcal mitogenic exotoxin Z (SMEZ) of *S. pyogenes* (Kamezawa et al. 1997). It forms a polymorphic family with wide allelic variation leading to significant antigenic variation (Proft et al. 1999, 2000). Currently more than 25 alleles have been identified (Proft et al. 1999, 2000; Gerlach et al. 2000). All SMEZ variants are powerful stimulators of human Vβ4 and Vβ8 T cells and significant production of SMEZ occurs in about 70 percent of all *S. pyogenes* isolates. The gene encoding SMEZ is chromosomal and almost invariably present in all group A streptococci, making it the most common of all PET genes. The extensive degree of variation can be taken as evidence that SMEZ provides an advantage to the producing *S. pyogenes* and that new variants arise as a result of the need to escape neutralizing antibodies (Proft et al. 2000). It is interesting and still unexplained, in spite of this variation, that all SMEZ alleles target Vβ4 and Vβ8 T cells. It has been speculated that these T cells may have a special function in host defense against *S. pyogenes* (Proft et al. 2000). This conserved Vβ specificity is in contrast to, for example, SEA and SEE which in spite of having 85 percent amino acid identity vary in their Vβ specificities (Hudson et al. 1993).

The frequency of enterotoxin producers among clinical *S. aureus* isolates has been determined in several studies (Lehn et al. 1995). Depending on the origin of the isolates approximately 40–65 percent of *S. aureus* strains produce a PET. Most commonly SEA is produced (20–25 percent of strains), followed by TSST-1 (13–23 percent), SEB (7–13 percent), SEC (5–9 percent), SED (3–6 percent), and SEE (>1 percent). Five to 8 percent produce two enterotoxins, usually SEA plus TSST-1 or SEA plus SED, which is a much higher frequency than would have been expected from respective individual frequencies. Isolates producing toxins, e.g. SEG, SEH, or SEI are extremely rare. Data about the production of SPEA and SPEC by *S. pyogenes* strains isolated

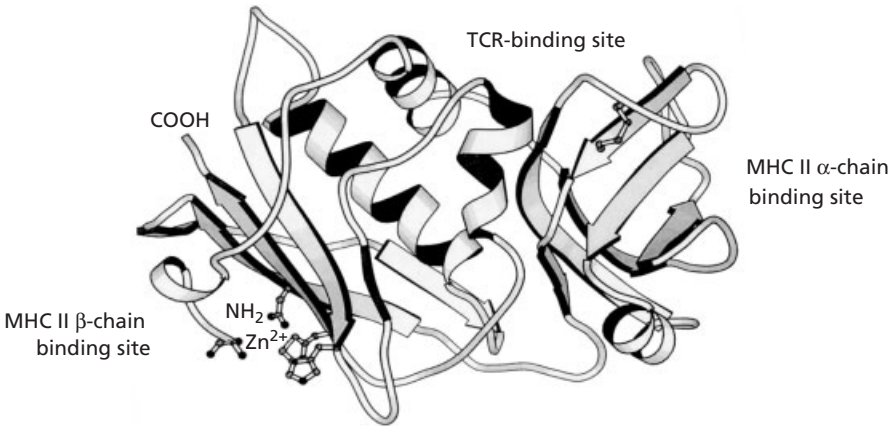

Figure 1 *Polypeptide fold of the staphylococcal enterotoxin A molecule. The binding sites for the T-cell receptor TCR Vβ, for the MHCII α chain and the Zn^{2+}-dependent binding site for the MHCII β chain are indicated. The drawing was made according to the published three-dimensional structure of staphylococcal enterotoxin A (Schad et al. 1995).*

from various sources vary considerably, reflecting the variability of clinical sources and the sensitivity of detection, because *S. pyogenes* produces lower amounts of SPE than *S. aureus* of SE. It is remarkable that the *smeZ* and *speG* genes are present in almost all group A streptococci in contrast to the other toxins (Proft et al. 2000). Information about the purification of the natural proteins has been reviewed (Alouf et al. 1991).

For many members of the SE family (the staphylococcal SEA, SEB, SEC2, SED, SEH, and TSST-1, and the streptococcal SPEA, SPEC, SPEH, SMEZ-2, and SSA) crystal structures are now available (Swaminathan et al. 1992; Prasad et al. 1993; Acharya et al. 1994; Papageorgiou et al. 1995; Schad et al. 1995). More recently, the crystal structures of several toxins, e.g. SEB, TSST-1, SEA, complexed with the human MHCII molecule HLA-DR1 have been determined and have – together with a large number of studies using mutated toxins or class II molecules – illustrated the molecular mode of binding of the toxins to the class II molecule (Jardetzky et al. 1994; Kim et al. 1994).

In spite of their low level of overall sequence homology the PETs show a similar overall structure: they are kidney-shaped, tightly packed molecules with a complex tertiary intertwined folding and are composed of two domains both containing β-sheets and α-helical structures (Figure 1). An interdomain groove is the TCR-binding site; distinct MHCII-binding sites have been identified. Each toxin has individual surface features explaining the individual mechanism of action and the low or absent serological crossreactivity between toxins.

Fraser et al. first described, for SEA, that some superantigens bind a Zn^{2+} ion by two histidine residues and an aspartate residue; in the case of SEA residues this was His-187, His-225, and Asp-227. This binding of Zn^{2+} is required for binding to MHCII molecules (Fraser et al. 1992; Abrahmsen et al. 1995; Hudson et al. 1995). A similar Zn^{2+}-binding motif is present in SEC but not in SEB and is apparently not related to functional activities (Bohach 1997).

Molecular mechanism of action

BINDING TO MHC CLASS II MOLECULES

A hallmark of superantigens is their requirement for MHCII molecules on presenting cells for optimal stimulation of T cells (Fleischer and Schrezenmeier 1988), reflected by their binding to MHCII molecules (Fischer et al. 1989; Fleischer et al. 1989; Fraser 1989; Scholl et al. 1989). Binding differs from normal presentation of processed peptide antigen because superantigens bind as unprocessed proteins outside the antigen-binding groove of the class II molecule (Jardetzky et al. 1994; Kim et al. 1994). Fixed MHCII-positive cells present superantigens effectively to T cells (Fleischer and Schrezenmeier 1988). However, although this basic mechanism is shared by all superantigens individual members of the PETs have developed unique modes of interaction with the class II molecule. Most intriguing are the many different strategies that various PETs use to accomplish MHC binding. They exhibit different binding sites and binding mechanisms and, by using two different binding sites or by forming homodimers, they can crosslink MHC molecules.

The staphylococcal enterotoxins and the SPEs SSA and SPEA have a generic binding site for the MHC α chain in their N-terminus. The toxins of groups IV and V as well as SEH appear not to use or possess this binding site, but use a high-affinity binding site for the β chain via zinc.

The crystal structures of the complex between HLA-DR1 and SEB or TSST-1 shows that SEB and TSST-1 bind to overlapping regions on the monomorphic α chain. Both toxins use residues of the N-terminal domain for this interaction. SEB binds at the side of the molecule leaving the peptide binding site and the peptide exposed (Jardetzky et al. 1994). This binding is of low affinity and it has been suggested that, in the trimolecular complex of SEB, class II molecule, and TCR, the TCR will interact with the class II molecule to

stabilize the complex (Seth et al. 1994). TSST-1, in contrast, binds on the top of the α chain and covers the α chain α-helix peptide-binding site (Kim et al. 1994). Binding to the class II molecule does not change the conformation of TSST-1 (Mitchell et al. 1997). Although the binding sites of SEB and TSST-1 overlap on the class II molecule the two toxins do not compete for binding. This is because the peptide within the antigen-binding groove influences the binding of TSST-1 (von Bonin et al. 1995; Wen et al. 1996). TSST-1 binds only to a subset of class II molecules containing the appropriate peptides to which SEB does not bind. Thus, TSST-1 exploits the extreme variability of the MHC-bound peptides for its superantigenic toxins. The crystal structure of SEH (which does not bind to the α chain) complexed with HLA-DR1 also shows an interaction of the superantigen with the peptide (Petersson et al. 2001). The same may be true for other PETs of this group.

Members of groups III, IV, and V use another binding site at the polymorphic β chain of the class II molecule involving the Zn^{2+}-binding site. This binding is of high affinity and uses a bridge between the Zn^{2+} bound to, for example, SEA (via His-187, His-225, and Asp2-27) and His-81 of the MHCII β chain (Abrahmsen et al. 1995; Hudson et al. 1995). Details of this interaction are described in reviews (Svensson et al. 1997; Papageogiou and Acharya 2000).

Members of the SEA group (except SEH) use this mechanism in addition to the α-chain-binding site. Thus, SEA and similar toxins are able to bind with two distinct binding sites to two different sites on the class II molecule and are, as a consequence, able to crosslink class II molecules on the surface of the presenting cell on its own, in the absence of a T cell. It has been shown that this crosslinking leads to transduction of signals in the cell, resulting in cytokine production in monocytes or costimulation of B cells (Mourad et al. 1990; Fuleihan et al. 1991). SEA competes with SEB and TSST-1 for binding.

Some PETs have the ability to form homodimers and in this way form functionally divalent molecules that are also able to crosslink class II molecules (Papageorgiu and Acharya 2000). The streptococcal SPEC forms a noncovalent homodimer which binds only to the β chain of the class II molecule by a Zn^{2+}-mediated binding. The low-affinity α-chain-binding sites of SPEC are covered by the dimerization (Roussel et al. 1997; Li et al. 1997, 1998). SED has been reported to form a zinc-dependent homodimer (Al-Daccak et al. 1998).

NON-MHC RECEPTORS

Under certain conditions SEs have also been shown to activate T cells in the absence of class II molecules on accessory or target cells. Cytotoxic T cells can, for example, lyse target cells that do not express class II molecules; however, much higher concentrations of SEs are required (Dohlsten et al. 1991; Herrmann et al. 1991). This is not surprising given the fact that PETs can interact directly with the TCR (see below), so a superantigen bound to any surface can in principle activate T cells. Whether there is a second specific receptor or whether the SEs nonspecifically stick to the surface of certain cells is unclear. Additional receptors have also been postulated, e.g. on endothelial cells or on thrombocytes, but have not been characterized (reviewed in Fleischer and Hartwig 1992). It is unclear whether such non-MHC receptors play any physiological role.

INTERACTION WITH THE TCR

The major interaction site on the αβ TCR is the variable part of the β chain (Vβ). Stimulation of murine or human T cells with a given toxin in vitro (Table 3), leads to selective expansion of $CD4^+$ and $CD8^+$T cells carrying certain Vβ (Janeway et al. 1989; Kappler et al. 1989; White et al. 1989). A similar preference (for Vγ) was reported for the response of γδ T cells. Transfer of a given Vβ or of an epitope of the hypervariable region 4 of the Vβ, transfers the specific response of the T cell to the recipient T cell (Choi et al. 1990b; Fleischer et al. 1996). The hypervariable region 4 is not involved in peptide-antigen recognition and is located on an exposed lateral region. It is important to note that different Vβs bind to different toxins with different affinities, so T cells with the highest affinity for a given toxin are preferentially expanded in bulk culture. This notion explains that T cells with certain Vβ can respond to a toxin that are not preferentially expanded in such bulk cultures (Fleischer et al. 1991, 1996).

From structural and mutational data a model of the ternary complex of SEB, TCR, and MHCII molecule was hypothesized. The complementarity-determining regions of the TCR are oriented over the MHC peptide-binding site, with the Vβ domain bound to SEB and the Vα domain above the class II β1 domain (Jardetzky et al. 1994). This model is consistent with the findings that the TCR α chain and the polymorphic MHC allotype influence the response of the T cell to some superantigens. In the ternary complex involving SEB the TCR interacts directly with the class II molecule, this is not the case with, for example, SEH (Petersson et al. 2001). Thus individual superantigens vary in the way they crosslink TCR and MHC II.

More recently, a soluble TCR Vβ chain has been used to analyze the binding of PET. The soluble β chain had the same specificity for different PETs as the T cell from which the β chain was derived. Affinities ranged from 0.9 μmol/l to 140 μmol/l, similar to what had been found for the affinities of the TCR to specific peptide/MHC complexes (Li et al. 1998). The three-dimensional structure of the complex between a soluble TCR β chain and

Table 3 *Preferential stimulation of human and murine T cells by bacterial toxins*

	Reported preference for T cells expressing TCR Vβ of	
Toxin	**Human T cells**	**Mouse T cells**
SEA	1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9.1, 18	1, 3, 10, 11, 12, 17
SEB	3, 12, 13.1, 13.2, 14, 15, 17, 20	3, 7, 8.1, 8.2, 8.3, 11, 17
SEC1	3, 12, 13.1, 13.2, 14, 15, 17, 20	3, 6.4, 12, 15
SEC2	12, 13.1, 13.2, 14, 15, 17, 20	3, 8.2, 10, 17
SEC3	12, 13.2, 14, 15, 17, 20	3, 5, 12, 13.2
SED	5, 12	3, 8.2, 8.3, 11, 17
SEE	5, 6, 8, 18	11, 15, 17
SEG	3, 12, 13.1, 13.2, 14, 15	
SEH		No stimulation
SEI	1, 5, 6, 9, 23	
SEJ	2, 3, 12, 14, 17	
SEK	5.1, 5.2, 6.7	
SEL		
SEM	6, 7, 8, 9, 18, 21	
SEM		
SEN		
SEO	5, 7, 9, 22	
TSST-1	2	3, 4, 15, 17
SPEA	12, 14, 15	8.2
SPEC	1, 2, 5.1, 10	No stimulation
SPEG	**2.1**, 4.1, 11.2, 19.1, 12, 3	
SPEH	**2.1**, 7.3, 9.1	
SPEI	5.3, 6.9, 9.1, **18.1**, 22	
SPEJ	2, 3, 12, 14, 17 (**2.1**, 8.1)	
SPEK		
SPEL		
SMEZ	4, 8	NA
SSA	1, 3, (5.2), 15	NA
YPMa	3, 9, 13.1, 13.2	7, 8.1, 8.2, 8.3
YPMb	3, 9, 13.1, 13.2	NA
MAS	3.1, 5, 7, 8, 10, 11.1, 12.1, 14, 17.1, 20	5.1, 6, 8.1, 8.2, 8.3

Data are compiled from the literature. Note that determinations have been made in many cases with native and not with recombinant proteins; therefore Vβ specificities of SE and SPE may be affected by contaminations with other toxins, e.g. stimulation of human $V\beta2^+$ and $V\beta8^+$ T cells reported for native SPEA (Abe et al. 1991), and of $V\beta8^+$ T cells reported for native SPEC (Tomai et al. 1992), is not found with recombinant toxins (Braun et al. 1993). Vβ profiles were assayed by polymerase chain reaction or by Vβ-specific antibodies.

SEC3 was determined. The complementarity-determining region (CDR) 2 of the β chain and, to lesser extents, CDR1 and the hypervariable region 4 (HV4) bind in a cleft between the small and large domains of the SE (Fields et al. 1996).

In spite of the requirement for class II molecules in T-cell stimulation, the toxins can also stimulate T cells in the absence of class II molecules. SE-mediated cytotoxicity has been found against several class II-negative target cells (Dohlsten et al. 1991; Herrmann et al. 1991). SEA covalently linked to antibodies against any cell surface molecule can direct lysis of cytotoxic cells to class II-negative target cells (Dohlsten et al. 1991) and toxin bound to plastic can under certain conditions activate T cells (Fleischer et al. 1991). It is noteworthy that some PETs, e.g. SPEC and SPEG and SEH do not stimulate mouse T cells at all, although they do bind to murine MHCII molecules (Proft et al. 1999; Petersson et al. 2001).

The molecular mechanism of T-cell stimulation is thus a multivalent crosslinking of TCR molecules with MHCII molecules on the presenting cell. This crosslinking of the TCR, however, requires – at least at low toxin concentrations – additional adhesion molecules, such as CD2, CD11a, or CD28. The CD4 or CD8 molecules appear not to be required, although in some cases CD4 interaction augments the response (Sekaly et al. 1991). By using this molecular mechanism the superantigen uses the most efficient way to stimulate T lymphocytes. The use of class II molecules as specific receptors restricts the T-cell interaction to the professional APCs, most probably the dendritic cells because macrophages are dispensable to T-cell activation in vivo (Bette et al. 1993).

Biological significance of pyrogenic exotoxins

ROLE OF PYROGENIC EXOTOXINS AS VIRULENCE FACTORS

Although superantigens act on T cells of different species, it appears that they work best in the species that is a natural host for the producing pathogen. The toxins derived from *S. aureus* and *S. pyogenes* (bacteria not found in wild mice) stimulate human T cells much more efficiently than murine T cells, whereas the opposite is true for the mitogen derived from *Mycoplasma arthritidis* (a natural pathogen for rodents) (Fleischer et al. 1991). TSST-1 or SEC produced by ovine strains of *S. aureus* is much more efficient on ovine T cells than on human T cells (Lee et al. 1992; Marr et al. 1993). This suggests that these molecules have been adapted in evolution to the MHC and TCR molecules of the natural host. Acquisition by gene transfer of toxin genes and their subsequent mutation is probably the mechanism of such adaptation, such as transfer of *tst* genes from human to ovine and bovine *S. aureus* strains.

For several reasons it may be expected that the production of a superantigen offers an evolutionary advantage for the respective pathogen. First, the molecular mechanism of action, the crosslinking of TCR Vβ with class II molecules, has evolved several times separately in evolution. Second, the extensive genetic polymorphism of the PETs argues that the presence of multiple forms of a superantigen is advantageous for the producing species. Finally, it appears that superantigens have been adapted by the producing pathogen to the host's immune system, because they have the highest mitogenic potential in the immune system of their respective natural host.

Thus, the evolutionary advantage can be expected to be directly or indirectly linked to the T-cell stimulatory function of superantigens. This has indeed recently been shown for the mouse mammary tumor viruses: T-lymphocyte stimulation is required for effective multiplication and transmission of the virus as discussed below (Held et al. 1994). For the gram-positive cocci there is so far no clear evidence where the advantage of T-cell stimulation lies. The decisive mechanisms against these extracellular bacteria are granulocytes, antibodies, and complement. It is, however, noteworthy that production of a superantigen leads to delayed clearance of *S. aureus* in infected mice (Rott and Fleischer 1994). Moreover, it has been repeatedly shown that humoral and cellular immune responses are diminished if an SE is injected along with a conventional antigen.

ROLE IN PATHOGENICITY

Given the extremely low concentration of picograms per milliliter required for T-cell stimulation and the large amounts of toxin secreted by some strains of staphylococci and streptococci into the culture medium, it is clear that a small focus of bacterial infection can be sufficient to induce a general activation of the immune system and a massive expansion of responding T cells. In patients with toxic shock caused by TSST-1-producing *S. aureus*, $V\beta^+$ cells expand to up to 70 percent of all peripheral blood T cells (Choi et al. 1990a). Consequences of such polyclonal T-cell stimulation can be shock, immunosuppression, and autoimmunity.

The shock-like symptoms induced by these toxins are caused by a massive release of lymphokines (e.g. tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-2 (IL-2)) and monokines (IL-12). Critical mediators in this shock-like symptom are apparently TNF-α and IFN-γ, both of which are produced by T lymphocytes (Miethke et al. 1992; Bette et al. 1993). The same mediators are involved in the septic shock induced by endotoxin of gram-negative bacteria. It appears that, although distinct cell populations are stimulated in gram-positive and gram-negative shock, a similar cytokine cascade is initiated that ultimately leads to the same terminal events. It is remarkable and still not explained in detail that lipopolysaccharide (LPS) and superantigen potentiate their effects if acting simultaneously in vivo.

Toxic shock syndrome (TSS) is observed during human infections with *S. aureus* and *S. pyogenes*. It is interesting to note that not all SEs have been implicated in such diseases. So far TSST-1 and SEB are almost exclusively involved in staphylococcal toxic shock. Although the gene for TSST-1 is widely distributed among staphylococci, a single *S. aureus* clone accounts for the majority of toxic shock cases in five countries (Musser et al. 1990). This indicates that not the superantigen alone but rather a combination of virulence factors determines pathogenicity. A greater heterogeneity was found with *S. pyogenes* isolates from patients with streptococcal TSS-like syndrome. Here, the ability to cause this syndrome is associated with all of the known SPEs, but SPEA-producing bacteria cause a high proportion of cases. Interestingly, two of four *speA* alleles were present in single bacterial clones that caused the great majority of these cases (Nelson et al. 1991). As a result of the higher invasiveness of *S. pyogenes* streptococcal TSS is associated with higher mortality and certain M types are more strongly associated with TSS than others (reviewed by McCormick et al. 2001).

Immunosuppression can result because stimulation of many T cells will impede the coordinated immune response. Moreover, a toxin can induce anergy and death by apoptosis in those T cells responding to it. After injection of an SE into mice, the initially responding T cells are partially deleted and those remaining in the spleen no longer respond to staphylococcal

enterotoxin B or to anti-TCR antibodies. It is also conceivable that activated $CD8^+$ T cells could destroy APCs or B lymphocytes that have toxin molecules bound to their class II antigens.

ASSOCIATION WITH AUTOIMMUNITY

The polyclonal activation of T cells by PETs could bypass the control of autoreactive T cells or helper T cells could be focused on autoreactive B cells and induce autoantibody production. This may be of relevance, e.g. in streptococcal diseases that are associated with autoimmune phenomena. Stimulation of autoreactive B cells by crossreactive epitopes of, for example, M proteins and the generation of help from T cells stimulated by erythrogenic toxins may provide a pathogenic mechanism. The requirement for such an interaction of two different pathogenicity factors of group A streptococci could explain why such poststreptococcal autoimmune diseases are relatively rarely found. In experimental models it was shown that control of autoreactive T cells could be bypassed by injection of a superantigen (Schiffenbauer et al. 1998). An ongoing autoimmune reaction against brain or joint antigens was aggravated by the injection of a superantigen (Cole and Griffiths 1993; Schiffenbauer et al. 1998). Moreover, a resolved experimental allergic encephalomyelitis could be re-exacerbated by injection of a superantigen stimulating the appropriate autoreactive T cells. Moreover in naïve animals a polyclonal T cell activation by a superantigen could prime Vβ-specific T-cell reponses to a selfantigen by a non-Vβ-specific mechanism of 'innocent' bystander activation (Rott et al. 1995).

Interest has been focused on the findings of an increase in $V\beta2^+$ and $V\beta8^+$ T cells in Kawasaki's syndrome, an acute multisystem vasculitis in children. Staphylococci and streptococci producing PETs have been isolated from these patients, their pathogenic relevance is, however, still unclear (Freeman and Shulman 2001).

In patients with atopic dermatitis, a chronic inflammatory skin disease associated with pruritus and elevated serum IgE, persistent colonization with *S. aureus* is a well-known feature. There is evidence that SEs produced by the staphylococci have the potential to trigger the chronic T-cell-mediated skin inflammation (Herz et al. 1998). It is also a common observation that exacerbations of psoriasis are often associated with streptococcal infections and a causal link has been suggested (Ortonne 1996).

THE ENTEROTOXIC ACTIVITY

The staphylococcal enterotoxins are named for their ability to induce a gastrointestinal illness on oral uptake of a few micrograms within 2–4 h in primates. Studies with mutant molecules (Hufnagle et al. 1991; Harris et al. 1993) and with chemically modified SEs (Alber et al. 1990) indicate that the enteropathogenicity of SEs is not caused by an action on T cells. It has been suggested that the symptoms of the gastrointestinal intoxication are caused by leukotrienes and histamine released from mast cells in response to substance P in the mucosa (Alber et al. 1989). The receptors involved are still obscure.

MYCOPLASMA ARTHRITIDIS SUPERANTIGEN

Mycoplasma arthritidis is a pathogen for rodents and induces an acute inflammatory infection in rats and mice. In mice this inflammation is followed by a chronic joint disease. Cell-free supernatants of *M. arthritidis* contain a potent T-cell mitogen, acting on T cells of several species. Although this mitogenic principle has been known of for more than 15 years (Cole and Atkin 1991), only recently has the protein been cloned (Cole et al. 1996). This is a result of the small amounts of M. arthritidis superantigen (MAS) in the mycoplasma culture and of the lability and adhesive property of MAS. MAS is a hydrophobic and basic peptide that is heat labile at 56°C and susceptible to serine proteases. The sequence reveals a protein unrelated to any other superantigen with 153 amino acids and a calculated molecular mass of 25 kDa and a very basic p*I* of 10 (Cole et al. 1996).

Mycoplasma arthritidis superantigen has all the functional properties of superantigens (Matthes et al. 1988; Cole et al. 1990). Its T-cell stimulatory properties are strictly dependent on the presence of H2-IE molecules or HLA-DR molecules on the stimulating cells. MAS stimulates both $CD4^+$ and $CD8^+$ T lymphocytes and responsive γδ T cells have been described. There is a preferential stimulation of mouse T cells expressing Vβ6, 8.1, 8.2, and 8.3, and of human T lymphocytes expressing Vβ17.1 (also designated 19.1). In addition, there are other TCRs that respond to MAS, but with lower affinity (Fleischer et al. 1991).

Injection of MAS into experimental animals induces a mild toxic shock syndrome and pronounced T-cell suppression (Cole and Wells 1990). This is only found in MAS-responsive mouse strains that possess H2-IE molecules. The chronic joint disease is induced only by infection with *Mycoplasma* itself or by intra-articular injection of MAS. The role of the superantigen in the induction of the arthritic disease is still not clear. It is noteworthy that MAS injection induces a flare-up in mice that have recovered from a collagen-induced arthritis. It has been noted that some parameters of the *M. arthritidis*-induced disease can also be found in rheumatoid arthritis and that antibodies to MAS are present in some patients with this disease. An involvement of MAS-like superantigens in the pathophysiology of rheumatoid arthritis has been suspected (Knudtson et al. 1997).

YERSINIA PSEUDOTUBERCULOSIS MITOGEN

The three pathogenic species of *Yersinia*, *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*, produce many virulence factors required for survival in the body. *Y. enterocolitica* and *Y. pseudotuberculosis* have been reported to produce superantigens. Although the putative superantigen of *Y. enterocolitica* still awaits isolation and confirmation, a superantigen designated Y. pseudotuberculosis mitogen (YPM) has been purified and cloned (Ito et al. 1995; Miyoshi-Akiyama et al. 1995). YPM is synthesized as a precursor of 151 amino acids which is cleaved to a mature protein of 14 kDa and 131 amino acids. It has no homology with any other superantigen. The protein has all the characteristics of a superantigen in vitro and in vivo. It is produced by certain serotypes of *Y. pseudotuberculosis* with distinct geographical distribution. The gene encoding YPM was detected in 95 percent of clinical isolates of *Y. pseudotuberculosis* from Japan or the far eastern Russian states but only in 17 percent of European clinical isolates (Yoshino et al. 1995). Recently, a variant YPM was isolated and cloned (Ramamurthy et al. 1997). The two YPMs (designated YPMa and YPMb) have 83 percent homology. A third variant was recently described. This indicates that YPM forms a polymorphic family as the SE.

The infection with *Y. pseudotuberculosis* is characterized by acute mesenteric inflammation and lymphadenopathy and systemic symptoms such as fever and leukocytosis. In patients with *Y. pseudotuberculosis* infections an elevation of Vβ3-bearing T cells has been found (Abe et al. 1997). Several immunopathological diseases can follow an infection with *Y. pseudotuberculosis* such as reactive arthritis or uveitis and erythema nodosum. Moreover, Kawasaki's syndrome has been linked to *Y. pseudotuberculosis* infections. A role of the superantigen YPM in the pathology of this infection is conceivable but still not clear. It has been shown in mice that YPM-deficient *Y. pseudotuberculosis* bacteria have a reduced virulence (Carnoy et al. 2000).

MOUSE MAMMARY TUMOR VIRUSES

The first viral superantigens (vSag) described were those encoded by mouse mammary tumor viruses (MMTV), retroviruses that cause mammary carcinomas in mice. In fact these powerful stimulators were known since 1973 as minor lymphocyte-stimulating antigens (Mls) but remained enigmatic (Janeway 1991) until their association with MMTV was found (reviewed in Acha-Orbea and MacDonald 1995).

The superantigen is encoded in an open reading frame encoding approximately 320 amino acids located adjacent to the 3′ long terminal repeat. As with the bacterial superantigens there is extensive polymorphism. All MMTV superantigens show a high degree of sequence conservation with the exception of the carboxy-terminus which shows extensive polymorphism. This part encodes the putative Vβ-binding part of the molecule. The viral superantigen is synthesized as a glycosylated type II membrane protein with an extracellular carboxy terminus and is then proteolytically processed. In CHO cells the superantigen is cleaved by the endoprotease furin and a carboxy-terminal 18-kDa fragment has been shown to be expressed on the surface. Endoproteases are also possibly able to cleave the superantigen (Denis et al. 2000).

It has been noted that the vSags have functional and structural similarities with the MHCII-associated invariant chain Ii. Both are class II membrane proteins of similar size and both associate with class II molecules inside the cell. Both possess a strong and invariant class II-binding motif, the invariant chain Ii has the CLIP sequence, and the vSag has a class II-binding motif at a location corresponding to CLIP in the Ii (Hsu et al. 2001). This motif is conserved in all MMTV superantigens. A model for the association of vSag with class II molecules implies that vSag competes with Ii for binding to the peptide groove of class II αβ dimers in the endoplasmic reticulum. In the trans-Golgi network the vSag is cleaved by furin, yielding the 18-kDa carboxy-terminal fragment of the superantigen that is bound outside the peptide-binding groove (Hsu et al. 2001). After transport to the cell surface the vSag–class II complex is accessible for T cells. The fate of the remaining portion of the vSag is still unclear.

When taken up by the newborn mouse with the maternal milk, the virus infects naïve B cells and immature dendritic cells (DC) (Vacheron et al. 2002) which express the viral superantigen. Infected B cells stimulate T cells carrying the appropriate Vβs which in turn stimulate infected B cells to proliferate and increase exponentially in number (Held et al. 1993). This allows the virus to persist until the mammary gland has matured. Infected mammary epithelial cells produce virus, allowing the transfer to the newborn mouse. Proviral insertion in epithelial cells can lead to activation of endogenous cellular oncogenes and cell transformation.

Mice harboring integrated endogenous MMTVs or a transgenic superantigen delete T cells carrying the Vβ that has reacted with the vSag of the endogenous MMTV. These mice are protected against infectious MMTV with specificity for the same Vβ (Golovkina et al. 1992). MMTVs in which the vSag has been deleted are still fully infectious but are not able to reach the mammary gland to effect transmission.

HUMAN ENDOGENOUS RETROVIRUSES

After the description of the MMTV superantigen many groups searched for viral superantigens in humans. There were a number of reports that described imbal-

ances in Vβ expression in certain human diseases. However, although they were published in first-class journals they remained either unclear or unconfirmed. Rabies virus (Lafon et al. 1992), HIV (Imberti et al. 1991), cytomegalovirus (CMV) (Dobrescu et al. 1995), or Epstein–Barr virus (EBV) (Sutkowski et al. 1996), for example, were proposed to encode superantigens. However, it was not possible to identify clearly the respective gene in these viruses, e.g. by recombinant expression.

Several groups have also provided indirect evidence for the involvement of superantigens in human diseases. In all these cases an imbalance in the Vβ-composition of T cells from patients has been found, e.g. two groups have demonstrated a selective increase in T cells carrying Vβ14 in the synovial fluid of patients with rheumatoid arthritis (Kotzin et al. 1993). Furthermore, an imbalance in the Vβ repertoire of T cells has repeatedly been reported in patients with AIDS, although not confirmed by all investigators. It is still unclear, however, if this is the result of a superantigen produced by HIV or of secondary infection or a nonsuperantigen-dependent mechanism. Although the idea that superantigens are at work in such diseases is very exciting, one has to bear in mind that preferential stimulation of certain Vβ-bearing T cells can also occur during a normal immune response (Boitel et al. 1992).

More recently, however, compelling evidence has been presented by Conrad et al. (1994) that a human endogenous retrovirus encodes a superantigen. The N-terminal moiety of the envelope gene of a polymorphic and defective provirus originally named IDDMK1,2 encodes an MHCII-dependent Sag. This virus, now designated HERV-K18.1, is one of three alleles of the HERV-K18 family of proviruses, each of which encodes a superantigen with specificity for Vβ7 and Vβ13 T cells.

Conrad et al. (1994) had previously observed that $V\beta7^+$ T cells were selectively expanded among islet-infiltrating T cells in two individuals in the early phase of type 1 diabetes mellitus. Moreover there was evidence of positive selection, after exposure to diabetic islet cell membrane preparations, of $V\beta7^+$ T cells from peripheral blood lymphocytes of individuals who did not have diabetes. They proposed that expression of the superantigen from the HERV-K18.1, induced in professional APCs, leads to β-cell destruction via the systemic activation of autoreactive T cells (Conrad et al. 1997).

Surprisingly, however, several other groups have not been able to find evidence for a superantigen in HERV. Lapatschek et al. (2000) amplified by polymerase chain reaction (PCR) and cloned into open reading frames of eukaryotic expression vectors, which were identical or very similar to IDDMK1,2. Although they were able to demonstrate mRNA expression and protein production in A20 B-lymphoma cells they could not find any evidence that the open reading frame (orf)-stimulated human or murine T cells in a Vβ-specific fashion.

An intriguing observation was that expression of HERV-K18 Sags was inducible by IFN-α and this is sufficient to stimulate Vβ7 T cells to levels comparable to transfectants constitutively expressing HERV-K18 Sags (Stauffer et al. 2001). This link between viral infections and IFN-α-regulated Sag expression could be a mechanism through which environmental factors may cause disease in genetically susceptible individuals. This observation was extended to show that EBV transcriptionally activated the viral superantigen, suggesting that the previously described EBV-associated superantigen activity was caused by the EBV-induced activation of the endogenous retrovirus (Sutkowski et al. 2001).

It has therefore been proposed that the superantigen activity found with several human viruses may not be caused by a viral gene product but by the activation, e.g. via IFN, of endogenous retroviral superantigen expression (Woodland 2002). This could explain the failure to identify molecularly such superantigens in rabies virus, CMV, and EBV. It could also explain the Vβ imbalances found in a number of human autoimmune diseases or HIV infection.

MORE SUPERANTIGENS?

In addition to the members of the PET family, there are other staphylococcal and streptococcal proteins genetically unrelated to the enterotoxins that have been proposed as superantigens, such as the streptococcal M protein, the streptococcal erythrogenic toxin B, and the staphylococcal exfoliative (epidermolytic) toxins. However, the evidence discussed below suggests that these molecules are not superantigens but that their mitogenicity is the result of artifacts (Fleischer et al. 1995).

The staphylococcal exfoliative toxins

The exfoliative toxins A and B of *S. aureus* (ETA and ETB) share a high-sequence homology but are serologically unrelated. They are able to dissociate intercellular adhesion molecules in the skin leading to subgranular epidermolysis. Epidermolytic toxin A is synthesized as a precursor molecule of 31 kDa which is subsequently cleaved to the mature polypeptide of 27 kDa. Both ETA and ETB have an intrinsic esterase activity and are homologous to the staphylococcus V8 protease (Bailey and Redpath 1992). They share the catalytic triad at the protease active center. Mutation of the serine of the active center leads to loss of epidermolytic activity in mice. ETA was reported to be a superantigen stimulating $V\beta2^+$ T cells (Marrack and Kappler 1990).

However, it was subsequently shown that ETA does not bind MHCII molecules (Herrmann et al. 1989) and that recombinant ETA produced in S. aureus had no

mitogenic activity toward human peripheral blood mononuclear cells (PBMC) or Vβ2$^+$ T cells (Fleischer and Bailey 1991). More recently, it was described that both ETA and ETB in recombinant form stimulated human Vβ3, -12, -14, -15, and -17 T cells, a profile that is very similar to that of SEB and SEC. MHCII binding was not investigated. In in vivo tests for pyrogenicity only ETB, but not ETB, showed pyrogenic activity (Monday et al. 1999), one of the hallmarks of superantigens. The question if the exfoliative toxins are superantigens (or T-cell-specific mitogens at all) is thus not unequivocally settled.

M proteins and SPEB of *S. pyogenes*

M proteins of *S. pyogenes* are major virulence factors of group A streptococci. They are receptor proteins that show multiple binding to different plasma proteins and protect streptococci against phagocytosis. They form a family of closely related proteins protruding from the cell surface. The variable M-terminal portion of the molecule carries the M-type-specific antigen epitopes. M-protein fragments produced by limited proteolytic digestion of whole streptococci with a molecular mass of 33 kDa (pepM5) have been proposed to be superantigens and to stimulate Vβ2$^+$, 4$^+$, and -8$^+$ human T cells (Tomai et al. 1991, 1992).

If, however, M5 protein or pepM5 is thoroughly purified, it is no longer mitogenic (Fleischer et al. 1992; Degnan et al. 1997). The stimulatory activity for Vβ2$^+$ and Vβ8$^+$ T cells could be separated from each other, suggesting that the superantigenic activity of M protein type 5 could be caused by contamination with at least two different, possibly novel, streptococcal PETs (Schmidt et al. 1995). Recombinant M5 protein or its fragments were not mitogenic (Robinson et al. 1991; Schmidt et al. 1995; Degnan et al. 1997).

Taken together, the available evidence strongly suggests that mitogenicity of M-protein preparations is not the result of an intrinsic activity but of contaminating SPEs, some of them not known at the time of the reports. The Vβ pattern matches well the pattern of SPEC, SPEG, and SMEZ.

The same situation was found with the streptococcal erythrogenic toxin B (SPEB) which is identical to the precursor of the streptococcal protease. It is a molecule of 371 residues with 40 kDa of molecular mass and is successively processed to the mature protease of 253 amino acids and 27 kDa. All group A streptococci have SPEB, and there is only one nonpolymorphic gene for this molecule present in the genome. SPEB has no homologies to any member of the staphylococcal enterotoxin family (Betley et al. 1992). SPEB has been reported to stimulate selectively Vβ8$^+$ human T cells. On proper purification of SPEB, however, it became evident that the superantigenic properties of these molecules are caused by contamination with a novel Vβ8-stimulating PET designated SPEX (Braun et al. 1993) and recombinant SPEB was not mitogenic (Gerlach et al. 1994). The Vβ8-stimulator SPEX is identical to a variant of SMEZ (Kamezawa et al. 1997) and has subsequently been cloned (Gerlach et al. 2000).

The mitogenic factor of *S. pyogenes*

A protein of 26 kDa, the so-called mitogenic factor (MF), or SPEF (Norrby-Teglund et al. 1994), was purified from *S. pyogenes* strain NY5 (the prototype strain from which SPEA, SPEB, SPEC, and SMEZ were originally purified). MF is not polymorphic and is elaborated by all group A streptococci, but not by other streptococci. Interestingly, it has enzymatic activity: it is a nuclease (Iwasaki et al. 1997) and is identical with DNase B. The Vβ profile of the stimulated human T cells was recently described to be Vβ2, -4, -8, -15, and -19, and is surprisingly similar to that of pepM5 (Norrby-Teglund et al. 1994). We have not been able to detect mitogenicity with recombinant MF and have recently shown that SPEC and SMEZ are the contaminants causing mitogenicity of MF (Gerlach et al. 2001).

Taken together, these results raise an important caveat for the work with T-cell mitogens from *S. aureus* and *S. pyogenes*. As SE and SPE are active in concentrations of a few picograms per milliliter, the sensitivity of T cells to these stimulators is several orders of magnitude higher than the sensitivity of any biochemical or serological test and contamination with an SE or SPE is extremely difficult to exclude. Such a contamination can even occur if a recombinant protein is purified on columns previously used for purification of natural enterotoxin. Moreover, given the high polymorphism and unequal distribution of the PET it is surprising that nonpolymorphic proteins, such as SPEB or MF, present in all *S. pyogenes* isolates are superantigens. A number of different proteins of *S. pyogenes* have been purified from culture supernatants and have been found to behave like superantigens (Fleischer et al. 1995). The possibility that *S. pyogenes* has evolved several unrelated superantigens is exciting but we should await the confirmation of their activity using recombinant proteins.

The surprising finding that the unique molecular mechanism of T-cell stimulation has been developed completely independently by infectious agents of four very different classes, gram-positive cocci, gram-negative bacteria, *Mycoplasma*, and retroviruses, has led to the expectation that additional superantigens produced by other infectious pathogens would be found. This expectation together with the appealing designation 'superantigens' has induced a boom in reports of putative novel superantigens, usually in high-impact journals. However, the number of reported candidates exceeds by far the number of superantigens that are likely to exist.

Even a lectin has been reported to act as a superantigen (Saul et al. 2000) but this has not been confirmed by an independent group. For several candidates it is already now reported that attempts to confirm the initial claim have failed or have prompted contradiction. We have, for example, not been able to find any superantigenic activity with purified enterotoxin of *Clostridium perfringens*, both purified from *Clostridium perfringens* cultures and as a recombinant molecule from *Escherichia coli* (Krakauer et al. 1997), and also not with exotoxin A of *Pseudomonas aeruginosa* (unpublished observations). Likewise the initial report on a superantigen in *Toxoplasma gondii* was never confirmed (Denkers et al. 1994), and also one in *M. tuberculosis* (Ohmen et al. 1994).

CONCLUSION

The superantigens described in this chapter constitute the most efficient T-lymphocyte stimulators known. Although their exact benefit as virulence factors for the production of bacteria is not established, it is obvious that their induction of immunosuppression, destruction of APCs, and T-cell anergy should be of advantage to the infecting microorganisms. The manifold mechanisms of interaction with variable target structures of the immune system, the MHCII molecule and the TCR, allow a maximum of biological efficacy combined with a minimum of immunological crossreactivity. This is the basis for the extensive polymorphism of the superantigens that provides the producing species with a multitude of non-crossreacting toxins. The polymorphism is probably maintained and extended by permanent modifications of the superantigen genes through mutations and selection for variants that are optimally stimulatory for the host's immune system.

The determination of the complete genome sequence of *S. aureus* has revealed a cluster of genes called the *sets* (Staphylococcal enterotoxin-like toxins) that are resident in a pathogenicity island SaPIn2 and share structural homology to the SEs and SPEs and in particular to TSST-1 (Williams et al. 2000). They form a highly polymorphic family with more than a dozen members and several members are found within a given *S. aureus* strain. The encoded proteins that are secreted have the three-dimensional structure characteristic of the SEs but do not have the classical functions of superantigens, such as mitogenicity, pyrogenicity or the enhancement of endotoxic shock (Arcus et al. 2002).

Recently, Fraser and coworkers have been able to discover a biological function for an SET. SET1 was found to bind to IgA and to inhibit polyclonal IgA binding to Fc-receptors for IgA on myeloid cells. Moreover, SET1 also binds complement C5 with nanomolar affinity and inhibits complement-mediated lysis (Langley et al. in press). This is an exciting discovery showing that superantigen-related molecules that apparently have evolved from a common ancestor target components of the innate immune system and contribute to virulence and pathogenicity.

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Immunogenetics

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Knowledge of the human genes involved in determining infectious disease susceptibility will enhance our understanding of the mechanisms of protective immunity and facilitate development of new treatment strategies and prophylactic measures. In some cases, targeting individuals at high risk of chronic manifestations of disease may, where resources are limited, provide a way forward. It is becoming increasingly simp9le to use the multiple polymorphisms available in immune response genes; hence risk prediction will become a realistic possibility in the near future. However, a plethora of small studies appearing in the literature, some of which have little power to reveal minor genetic effects, at present gives an incomplete picture. This chapter concentrates on human rather than murine studies, and common rather than rare genetic variants. It is necessarily a personal view, and does not attempt to list every known gene of interest or every disease investigated. At the start it seems appropriate to highlight the rapid developments not only in human genetics, but also in pathogen sequencing. In the future it will be fruitless to study human or pathogen variation in splendid isolation. Variation in the pathogen may affect virulence and the host immune response. Clearly human and pathogen genetic variations are inextricably linked in determining disease outcome, and their relative importance will vary with disease.

SIMPLE VERSUS COMPLEX INHERITANCE

There are rare single gene disorders that have contributed to understanding of the role of immune response genes and immunological function in infection. Investigations of single gene disorders show that individual mutations may not be the sole determinants of phenotype. Other influences can modify the phenotype, and disease may have a threshold for manifestation. Hence, some disorders that have been regarded as simple Mendelian disorders are now better described as 'complex' traits. A range of mutations, such as seen in the tumor necrosis factor (TNF) receptor gene *TNFRSF1A*, means that some mutations result in immune dysfunction, and others may be low penetrance (i.e. not always expressed) rather than benign mutations (Aksentijevich et al. 2001). Similarly, development of an infectious disease, or position on the disease spectrum, is likely to be caused by an accumulation of predisposing factors, including susceptibility alleles. Most variation in disease susceptibility will be 'multifactorial' i.e. with both genetic and environmental influences. Genetic variation is likely to be polygenic, with many contributing genes and disease resulting from multiple common variants in polymorphic genes, rather than from rare mutations. Examples of genes that were

initially shown to have rare deleterious mutations, and are now studied for common nonpathogenic variants, are the interferon receptor genes *IFNGR1* and *IFNGR2* (Gao et al. 1999). It is likely that most variation underlying disease is caused by mildly deleterious alleles. The frequency of susceptibility alleles may be high, and there is likely to be allelic heterogeneity between populations (Pritchard 2001). For immunogenetic studies, assumptions are often made of low allelic heterogeneity within loci, which, when combined with a lack of knowledge of linkage disequilibrium, makes the interpretation of results challenging.

DEFINING CLINICAL PHENOTYPE

One of the key issues for genetic studies is that of defining the clinical phenotype. Most investigations are conducted using qualitative traits, e.g. disease versus absence of disease. However, quantitative traits are also employed, particularly for immune responsiveness in relation to disease. Nongenetic effects, such as routes of infection, age, and sex, must also be accounted for. Although genetic studies have the advantage that DNA may be sampled at any time, and do not require active disease, it is clear that samples pertaining to chronic disease states are more often collected, whether for family or case–control studies. A major problem for an infectious disease is ensuring that disease-free individuals are really resistant, rather than simply not exposed to the infection. For many infections exposure is hard to quantify, although for mycobacterial infections a positive skin test response to crude mycobacterial antigen can be used as an indicator of exposure. Some of the most effective studies do not concern susceptibility to disease itself, but to type of disease, which is often related to the ability to mount an appropriate immune response. Consequently, diseases that have a spectrum of severity, such as mycobacterial and protozoal diseases, have been relatively well studied.

TESTING FOR GENETIC CONTROL

Familial clustering of disease, twin studies, and racial differences may all point to genetic control. Adoptee studies are rare and not appropriate for infectious diseases. A statistic commonly associated with multifactorial conditions is λ_s, the risk to a sibling of an affected case compared with the population prevalence – the higher the value, the larger the role of genetics. However, shared sibling environment makes such estimates unreliable and they are not in common usage for infectious diseases. Nutritional status and co-infections may also be confounding factors. Another statistic is heritability: the proportion of phenotypic variation resulting from genetic causes. It is often used for quantitative traits such as pathogen burden or immune responses. The heritability of immune responses to many pathogen antigens tends to be high, with some of this being the result of non-major histocompatiblity complex (MHC) control.

Segregation analyses are formal tests of the fit of family data to models of inheritance. The occurrence of disease at random in the population is tested using a sporadic model. Rejection of a sporadic model shows a nonrandom distribution of disease. Further models can then be tested, comparing specific modes of inheritance (e.g. single gene control) with polygenic and nongenetic models. Although many segregation analyses for infectious diseases have favored single major gene control, this may reflect the choice of models that have been tested and the limitations of analytical methods.

APPROACHES FOR GENE IDENTIFICATION

Having established that there is genetic control, there are two approaches to gene identification: candidate gene testing and genome scanning. Candidate genes may be genes relevant to the immune response, associated with other similar diseases, implicated in murine model systems or within regions highlighted by genome scans. Genome scanning, as the name implies, covers the entire genome equally in the search for regions controlling susceptibility. Ideally we would like to ascertain not only the number of genes contributing to infectious disease susceptibility but also how they interact – additively or multiplicatively? The relative risk of disease for a particular gene will contribute to the ability to detect its effect. In general methods may loosely be categorized as based on linkage or association, the latter relying on linkage disequilibrium (Ott and Hoh 2000). Whereas linkage is employed for genome scanning, association is often the method of choice for a candidate gene approach. Investigators of other multifactorial conditions, particularly those studying autoimmunity and type 1 diabetes mellitus, pioneered methods of analysis.

LINKAGE

Linkage studies are used to identify broad regions of the genome involved in disease susceptibility. Historically, linkage formed part of a positional cloning strategy. However, with the sequencing of the human genome the necessity for positional cloning has been reduced. Laboratory mice, which can be maintained in environmentally controlled conditions, have been important in linkage mapping and locus identification, because there is much conserved synteny between murine and human genomes (groups of genes in which the order is conserved). Use has been made of recombinant inbred, congenic, and more recently recombinant congenic strains (Fortin et al. 2001b). Recombinant congenic strains are derived from backcrossing of a background strain and a donor strain, followed by brother–sister

mating where each resulting strain carries about 10 percent of one parental strain on the genetic background of the other. These strains are used when a limited number of genes have a major effect on phenotype (Malo and Skamene 1994). Transgenic and knock-out mice have been used to confirm findings. Some of the genes identified in mouse models have been catalogued (McLeod et al. 1995), but only selected examples are mentioned in this chapter.

Microsatellite markers, particularly dinucleotide repeats, are usually the polymorphisms of choice for linkage studies. This is because they are highly polymorphic, as measured by polymorphism information content (PIC) or heterozygosity; hence they maximize the information from a given dataset. Haplotypes, using more than a single polymorphic site, can also be used to improve information content. Repeatability of linkage results within a population is essential, and genome scans often adopt a staged approach, with initial results tested in other independent samples. Most genetic markers are expected to be in Hardy–Weinberg equilibrium, and deviations could be indicative of selection or genotyping errors.

There are two methods of linkage analysis: model dependent, where a mode of inheritance must be specified, and model free. The LOD score method is used for parametric, model-dependent analysis and is powerful. A LOD score is the logarithm of odds ratio, where the ratio is the likelihood of the observed results given linkage to a given locus at a recombination fraction of <0.5, over the likelihood under a null hypothesis of no linkage. A maximum likelihood ratio can be expressed as a probability or p value (Nyholt 2000). A LOD of 3 has been used as significant evidence of linkage for mendelian traits, and a LOD of -2 as indicative of no linkage. Multipoint analysis, which combines information from multiple loci to localize a disease susceptibility locus on a genetic map, can result in increased power, but such methods are affected by pedigree size and incomplete pedigrees. Use of single large pedigrees, with or without inbreeding, is uncommon for infectious disease (Farrall 1993). Parametric analysis is thought to be sensitive to model misspecification; however, Greenberg et al. (1998) suggested that the critical factor in LOD score analysis is the mode of inheritance for the linked locus, not that of the disease or trait itself. It is easy to examine parent-of-origin effects using parametric methods. This has been employed in studies of asthma and atopy. True combined segregation and linkage analysis, where the mode of inheritance of a disease is assessed at the same time as linkage status of a disease susceptibility locus, is also a parametric method. It is more common for studies to employ a previously ascertained mode of inheritance when assessing linkage. This is simpler but more limited.

Nonparametric methods are not model dependent and often use affected sibling pairs rather than large pedigrees. This use of only affected individuals neatly gets round problems of exposure for infectious disease. Such methods examine alleles or haplotypes from the parents shared by affected sibs (identical by descent (IBD)). Correction may be made for multiple sib-pair comparisons within a family. A contrasting method examines alleles shared by affected relative pairs (identical by state (IBS)). IBS methods have been regarded as less powerful than IBD methods, because they may lose information, although the choice depends on the family structure available. For high relative risk and PIC values distant relatives are better for affected pair methods, whereas for smaller relative risks and PIC values sibs are best (Risch 1990). Attempting to circumvent some of the problems are programs such as GENEHUNTER, which uses all pedigree members in a multipoint approach (Kruglyak et al. 1996). Kruglyak et al. (1996) argue that their nonparametric linkage (NPL) method shows little loss of power compared with parametric methods and is less sensitive to misspecification of allele frequencies, for pedigrees of moderate size.

There are a number of linkage methods appropriate to the study of quantitative traits based on robust regression and powerful variance component methodologies (Sham et al. 2002). In some instances extreme discordant or concordant sibs are selected. Variance component methods are often used, looking at allele-sharing probabilities and the contribution to population variance. Variance may be partitioned into that caused by environmental effects, a major gene, plus other genes, and methods are implemented by programs such as GENEHUNTER (Pratt et al. 2000).

When genome scanning, approximately 300 microsatellite markers are tested for linkage. The amount of correction of the statistical significance of genome scanning results needed to allow for multiple comparisons is controversial; methods are reviewed, and accepted thresholds for genome-wide significance and corrections for multiple testing discussed (Nyholt 2000). Overly cautious approaches will cause loose linkages to be missed; however, enthusiastic reporting will cause confusion. Point-wise and genome-wide significance levels differ (Lander and Kruglyak 1995). Some believe that the degree of type 1 error for claims of linkage and association in complex inheritance is unacceptably high (Morton 1998).

An interesting question arises over the power needed to detect necessary versus susceptibility loci (Greenberg 1993). Whereas linkage is ideal for looking for loci that are necessary if not sufficient, it may be less than ideal for loci that are neither necessary nor sufficient, i.e. susceptibility loci. If the allele confers less than 10 times an increase in risk, as is the case for almost all infectious disease loci, linkage is hard to use. The numbers used for affected sib-pair genome scanning are often in the region of 200 sib-pairs plus parents (800 individuals). Population size and levels of inbreeding affect numbers

required for all types of linkage and association studies. In large populations heterogeneity is often addressed by studying large numbers of families; however, in founder populations there is presumed to be decreased heterogeneity and fewer susceptibility alleles (Ober and Cox 1998).

ASSOCIATION

Association methods are often used after linkage studies for fine mapping, or for candidate gene approaches. They rely on linkage disequilibrium – the nonrandom assortment of alleles at adjacent loci within the population. The closer together two loci are, the less likely it is that a particular haplotypic combination will be separated by recombination. Such combinations may also result from admixture, selection, or drift. One cannot assume that the polymorphism under investigation is itself functionally relevant, but rather that it is used as a marker for a small region. As association methods rely on the presence of linkage disequilibrium, they are applicable only over short genetic distances. Many association studies use a case–control design. Typically a chi-squared test is employed, and Fisher's exact test may be required, depending on allele frequencies. It is possible to look at association with alleles (if the Hardy–Weinberg equilibrium holds), genotypes, or carriage (presence versus absence) of a particular allele. Whereas genotypic association is most appropriate for many loci (Sasieni 1997), carriage is appropriate for studies of human leukocyte antigen (HLA). Stratification of data, e.g. by HLA type, to look for more minor genetic influences is often used. Approaches for association mapping, analogous to multipoint linkage mapping, still need to be developed. The degree of linkage disequilibrium will determine the density of markers required to examine a region. Linkage disequilibrium is not uniform over the genome, with estimates ranging from 500 to <50 kb (Abecasis et al. 2001). In addition, linkage disequilibrium appears to be highly structured into discrete blocks, separated by hot spots of recombination (Goldstein 2001). Population size and population history will also affect the degree of linkage disequilibrium. In isolated populations there are limited ancestral haplotypes, and if a mutation is new there will be high linkage disequilibrium. However, results to date suggest that genetic isolates (unlike for linkage) will not prove more useful than larger populations for linkage disequilibrium mapping (Eaves et al. 2000), i.e. levels of linkage disequilibrium are equivalent across populations of varying sizes.

Case–control studies are potentially affected by population stratification; this occurs where the frequencies of both the disease and unlinked alleles vary between populations, and can lead to false-positive or false-negative results. The extent of problems caused by population structure has been questioned, but, to avoid such problems, the transmission disequilibrium test (TDT) is increasingly used to test for association. It is a family-based method, which observes transmission of alleles from heterozygous parents preferably to a single affected child, and is typically implemented in a variety of programs such as ETDT (Sham and Curtis 1995). Transmissions may be from the paternal or maternal side. Although originally described by Spielman et al. (1993) as a test of linkage in the presence of association, the tightness of linkage inferred by this method is debatable (Whittaker et al. 2000); however, as a test of association it avoids problems of population subdivision and admixture (Ewens and Spielman 1995). If parents of affected offspring are not available, deduction of missing parental genotypes can introduce bias, and use of unaffected siblings as controls has been suggested (Curtis 1997; Spielman and Ewens 1998). The haplotype relative risk (HRR) method also gets round population stratification, by using the set of alleles that were not transmitted as controls (Knapp et al. 1993). The TDT, together with the HRR, is the most widely used family-based test for linkage disequilibrium and association (Spielman and Ewens 1996). However, this approach may be less powerful than that of a case–control. TDT can also be performed for quantitative traits (Allison 1997; Rabinowitz 1997; Abecasis et al. 2000).

Association methods are more powerful than linkage, as confirmed by stimulation studies comparing TDT and affected sib-pair methods (Risch and Merikangas 1996). Knapp (1999) gives further consideration to power approximations for the TDT. Sample sizes for TDT have been calculated (Wang and Sun 2000). As for linkage analysis, the need for correction of multiple comparisons when interpreting case–control and family-based association studies has been much discussed. The Bonferroni correction method is most widely used (Bland and Altman 1995) and also the Holm method (Aickin and Gensler 1996). Exactly when to correct has been debated and correction is certainly not uniformly applied (Nyholt 2001). There are now many disease associations in the literature, in many populations. Heterogeneity of findings, even to the extent of positive versus no association in the same population group, is commonplace, and probably reflects both publication bias and the small size of many studies. Recent guidelines for the interpretation of association studies suggest that results should be confirmed with a second study, with $p < 0.001$, similar to the two-stage genome scans for linkage. Meta-analyses are likely to be needed to confirm associations: recent meta-analyses of genetic association studies suggest that only 20–30 percent of association studies are significant and 16 percent of reported associations are supported by further studies (Ioannidis et al. 2001, 2003; Lohmueller et al. 2003). Although not considering specifically infectious disease associations, such studies suggest that many of the reported associations will prove to be false.

The future will bring genome scanning by association using single nucleotide polymorphisms (SNP). It is estimated that there is more than 1 SNP per 1000 base-pairs, or more than 3 million in the genome. They may occur in coding sequences and are more stable than microsatellites. Large-scale identification and screening for SNPs is now possible (Wang et al. 1998, see www.ncbi.nlm.nih.gov/SNP/). How polymorphic and densely spaced should SNPs be for a genome scan? One suggestion is that 7–900 moderately polymorphic SNPs, and a map of 1 500–3 000 SNPs, would be superior to the current microsatellite linkage strategy (Kruglyak 1997). Alternatively, a level of linkage disequilibrium of 3 kb would suggest that half a million SNPs are required (Kruglyak 1999)! Use of haplotypes may reduce the number of SNPs required for screening of complex disease (Johnson et al. 2001), as demonstrated by looking at the haplotypic structure of 5q31 (Daly et al. 2001). However, the nonuniformity of linkage disequilibrium in different regions of the genome (Collins et al. 1999) suggests that SNPs for whole genome scanning will have to be suitably chosen region by region.

SINGLE GENE CONTROL OF THE IMMUNE RESPONSE AND BEYOND

It is beyond the scope of this chapter to catalogue primary immunodeficiencies that have been well reviewed elsewhere (Fischer et al. 1997). In general these are rare disorders, in some cases recognized as single gene disorders through pedigree analysis. Many are lethal and some disorders are associated with multiple rare mutations. In other single gene disorders, the gene may be localized but not identified. However, it is worth noting that, according to the defect, infection is likely to be with characteristic pathogens. Some of these opportunistic infections are not usually associated with pathogenicity. Defects of the adaptive immune response include defects in MHC, particularly defects in expression such as bare lymphocyte syndrome (BLS), defects in T-cell development and function, such as the severe combined immunodeficiencies, e.g. X-SCID, and defects in B-cell function with numerous irregularities in immunoglobulin production. Defects also occur in the innate immune response such as those of phagocytic cells, e.g. chronic granulomatous diseases. Somewhat more common are deficiencies in the classic and alternative complement pathways. Whereas defects in classic components tend to result in the autoimmune disorder systemic lupus erythematosus, defects in the membrane attack complex predispose to pyogenic bacteria (Walport 1993). Single gene disorders affecting nonimmune response genes are also associated with particular pathogens. Individuals homozygous for mutations in *CFTR*, resulting in cystic fibrosis, are susceptible to *Pseudomonas aeruginosa*.

Interest in MHC class II expression was the result of expression being inducible by stimuli such as interferon γ (IFN-γ). Cells in BLS have no MHC II molecules. The story of BLS is pleasing for geneticists because heterogeneity in BLS was characterized by classic genetic complementation analysis in culture. There is a defect at the level of transcription, associated with the highly conserved X and Y boxes in the promoters of all class II genes. Complementation group A is caused by the absence of the *trans*-acting factor CIITA (MHC class II transactivator), which is a non-DNA-binding co-activator, necessary for activity of the class II promoter. Complementation groups B and C, on the other hand, are the result of deficiencies in the X-box-binding protein RFX, which is composed of a 75-kDa and a 36-kDa subunit. Failure of RFX to bind to the X box leaves the class II promoter unoccupied, and is associated with change in chromatin structure and the loss of DNase I hypersensitive sites (Reith et al. 1995; DeSandro et al. 1999).

X-linked SCID is the most common form of severe combined immunodeficiency. T and natural killer (NK) cells are affected whereas B-cell function is largely intact. The interleukin-2 (IL-2) receptor is made up of three chains: α, β, and γ (now named 'common γ' or γc). The γc chain is responsible for increasing the binding affinity of the receptor and is involved in intracellular signal transduction; it is also an essential component of other cytokine receptors: IL-4R, IL-7R, IL-9R, and IL-15R. Mutations in the gene for γc result in X-SCID (Sugamura et al. 1996; Cacalano and Johnston 1999). It appears that the primary mechanism of X-SCID is through the absence of the IL-7R necessary for T-cell development (Puel et al. 1998). Mutations in the α chains of both the IL-2R (Sharfe et al. 1997) and the IL-7R (Roifman et al. 2000) also occur. Cytokine receptors are often heterodimers or heterotrimers, and the phenomenon of chain sharing is not uncommon (e.g. the IL-4R chain occurs in both the IL-4 and the IL-13 receptors). This means that their binding specificities may have some overlap, but also that mutation in one chain may have far-reaching consequences. Mutations in signaling molecules, e.g. the Janus kinases, associated with these receptors are responsible for similar phenotypes. A few single gene defects, such as that identified in *IFNGR1*, are described together with the effect of more common variants of the same gene.

Defects in immunoglobulin (Ig) production are characterized by too much or too little of a particular Ig class or subclass. Some of these are caused by failure in class switching. Hyper-IgE syndrome presents as an autosomal dominant disorder with variable expressivity and is rare (Grimbacher et al. 1999a, b). In contrast IgA deficiency is not a single gene disorder and shows some MHC involvement (Vorechovsky et al. 1999). With a prevalence of 1 in 600, such disorders are not likely to be major contributors to disease patterns, but unusual

clinical manifestations of an infectious disease may be associated with them. Of more relevance to this chapter are the studies of common variants that control Ig levels. Total IgE levels are the best studied, as a result of involvement in asthma and atopy, and are considered together with the genes on 5q31–q33. A genome scan has been carried out implicating a region of chromosome 13 in control of total IgA levels (Wiltshire et al. 1998).

Aside from investigation of Ig levels, there have been few attempts to examine common genetic control of the immune system, independently of infection. Genetics has a major influence on the variation in peripheral T-cell subset numbers: Hall et al. (2000) reported heritabilities of 65 percent for CD4:CD8 T-cell ratio, about 50 percent for absolute lymphocyte, $CD3^+$ and $CD4^+$, counts, and 56 percent for $CD8^+$ numbers. The human NK-cell repertoire is influenced by variation in *KIR* (coding for killer cell Ig-like receptor) and HLA class I (Shilling et al. 2002). Murine studies have shown that the IgE-suppressive activity of IFN-γ is polymorphic and controlled by a gene outside *H-2* (Matsushita and Katz 1992). Use of an infection-free phenotype, IL-12 responsiveness, and therefore subsequent ability to mount a T-helper 1 (Th1) or T-helper-2 (Th2) response implicated the Th2 gene cluster (Gorham et al. 1996). Examination of the effects of genetic variation on protein production, especially cytokine production, has proved fraught with problems (Nieters et al. 2001). This contrasts with some success in tying in polymorphisms with other immune response phenotypes, and is in part the result of problems associated with laboratory techniques, e.g. use of bulk cultures (Zipp et al. 2001). Proliferative responses to crude and defined antigens such as vaccine candidates, and skin test responsiveness, have been the subjects of limited study in the recent past. As anticipated there is some influence of HLA, but non-MHC genes are also implicated (Jepson et al. 1997a; Blackwell et al. 1999).

COMMONLY INVESTIGATED GENES

Commonly investigated genes for susceptibility/resistance to particular pathogens contribute to both innate and acquired immunity. The genes described below are those most widely investigated and/or genes localized to clusters of immune response genes, which have often arisen through gene duplication.

HLA class I and class II

Classic HLA molecules are particularly relevant to genetic studies through their role in antigen presentation, and consequent influence on responsiveness to infection and immunization. The human MHC, on chromosome 6p21.31, is arguably the most interesting region in immunogenetics because this is the most polymorphic

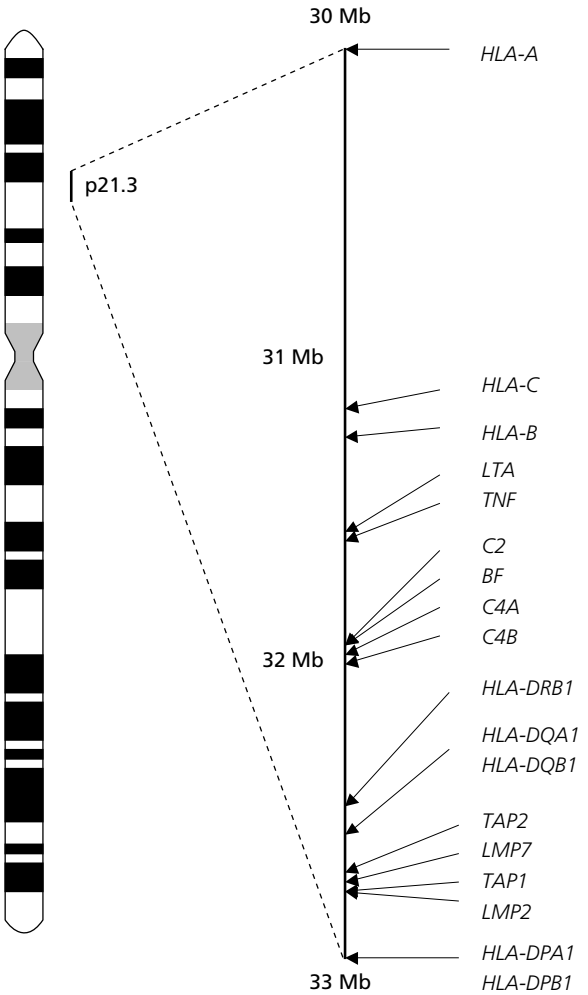

Figure 1 *Chromosome 6: the MHC*

region of the genome, some genes having over 200 allelic variants (Figure 1). This multiple allelic diversity contrasts with the mechanisms for generation of diversity in Ig and T-cell receptor molecules, where single proteins are produced from several recombined gene segments. Almost all early immunogenetic studies concerned HLA, and analytically HLA provided the first group of highly polymorphic markers. HLA molecular structure, complexity, and allelic variation are well described (Marsh et al. 2000).

HLA class I was originally investigated genetically through its impact on transplantation, although class II molecules also have significant impact on graft rejection. Typically HLA class I was typed by serological methods, and HLA class II was typed by the mixed lymphocyte reaction. Nowadays, the vast majority of HLA typing is done by polymerase chain reaction (PCR) using sequence-specific primer or sequence-specific oligonucleotide methodology. The improved resolution of DNA-based typing has contributed to the subdivision of previously described HLA alleles, e.g. *HLA-DR2* comprises *HLA-DRB1*15* and *HLA-DRB1*16*, which

complicates comparisons across studies. Peptide-binding specificities of alleles have been described. In 1999, ahead of publication of the human genome sequence, a complete sequence and gene map of a human MHC were reported (MHC Sequencing Consortium 1999). At this time there were 224 identified loci, of which it was predicted that 128 were expressed, about half of which had immune function. Many of these genes were identified as a result of the sequencing and many remain of unknown function. As the entire region contains over 200 genes in 3–4 Mb of DNA, the study and use of haplotypes and linkage disequilibrium are very important. HLA associations have been reported for the disease per se, position on the clinical spectra of disease, and ability to respond to crude and defined antigens; both qualitative and quantitative traits. For many infectious diseases, it remains the only region investigated to date.

HLA study design typically uses a case–control approach. Results are expressed as a relative risk of disease when carrying a particular allele. However, there were also early investigations of haplotype sharing among affected siblings, akin to current sib-pair analysis, which tackled the variable penetrance associated with susceptibility genes for complex disease. For the first and best-known HLA association, although more than 90 percent of patients with ankylosing spondylitis have *HLA-B27*, only a small percentage of individuals with *HLA-B27* will get clinical disease. In this way many early disease associations were reported, the most striking being for autoimmune disease. Many such conditions may have infectious triggers, or are more directly linked to known pathogens, such as rheumatoid and reactive arthritis, inflammatory diseases such as Crohn's disease and inflammatory bowel disease, and periodontal disease. HLA associations sometimes differ from other genetic associations because, as a result of the role of the HLA molecules in antigen presentation, a single copy of a particular allele suffices. HLA control of responses to particular antigens is best characterized for the immediate hypersensitivity response to allergens. The influence of HLA on specific IgE responsiveness to a wide variety of allergens has been known for many years, e.g. there are a number of MHC II-restricted epitopes in *Dermatophagoides pteronyssinus* allergen 1 (Higgins et al. 1994) and allergen 2 (Verhoef et al. 1993). However, although there is some evidence for HLA influence on *Der p*-specific IgE responsiveness, other loci are also involved, and genome scanning has implicated several loci (Hizawa et al. 1998a, b).

Although common haplotypes are known to occur, the HLA region is large enough for recombination to be seen and linkage disequilibrium to be incomplete. Linkage disequilibrium mapping, combined with examination of peptide-binding specificities, have been required to ascertain a complete picture. Perhaps the best example is for type 1 diabetes mellitus where a strong *HLA-DR3/DR4* association was known to occur. *HLA-DR3/DR4* alleles were thought to be too common to be disease-susceptibility alleles. In fact these alleles are in linkage disequilibrium with alleles at *HLA-DQB1*, which is the disease locus. Pinpointing disease susceptibility-causing mutations remains a problem in such a complex region.

The MHC has short sequence motifs, which essentially remain unaltered over millions of years. The class I and II regions, unlike the class III region, have many pseudogenes, possibly giving rise to new alleles via gene conversion. Class I and II arose by multiple duplication events (Shiina et al. 1999; Beck and Trowsdale 2000) and further duplication has resulted in differing copy number among haplotypes, which in turn may diverge to new functions. Those for *HLA-DRB* are the best known. How is allelic diversity maintained? The extreme polymorphism observed is likely to be a result of susceptibility/resistance to infectious agents. An excess of heterozygotes has been observed, suggesting balancing selection (Apanius et al. 1997; Jeffrey and Bangham 2000). Two theories are heterozygote advantage, where possession of two different alleles is optimal, and frequency-dependent selection, where low-frequency alleles are an advantage against pathogen-evasion strategies. One possible mechanism for the maintenance of diversity is through odor-mediated mate choice for mates of a different MHC type, hence maintaining heterozygosity. Selection may also promote different combinations of alleles or haplotypes. However, linkage disequilibrium and conservation of haplotypes will be influenced by clustered hot spots of recombination within the MHC (Jeffreys et al. 2001).

Class III genes – *TNF/LTA*

The class III region is the most diverse and gene-dense region, and may be the oldest because the origins of the MHC precede adaptive immunity.

For autoimmune, and later infectious diseases susceptibility, the most investigated cytokine genes are those coding for the macrophage proinflammatory cytokines tumor necrosis factor α (TNF-α) and IL-1. The gene for TNF-α (*TNF*) lies in the class III region between the gene *LTA* coding for lymphotoxin-α (also known as TNF-β) and *LTB* (Figure 1). This region is sometimes referred to as the class IV region. There are several polymorphisms in the *TNF* promoter, the best known of which is TNF_{-308}, alleles at which are often simply referred to as *TNF*1* and *TNF*2* (Knight and Kwiatkowski 1999; Ruuls and Sedgwick 1999). There is some confusion in the early literature because the first observed polymorphism, an *NcoI* restriction fragment length polymorphism (RFLP), was assumed to lie within *TNF*, and production of TNF-α appeared to relate to allelic variants. This polymorphism turned out to be in

the first intron of *LTA* (Messer et al. 1991): the then described *TNFB*1* resulted in an asparagine at amino acid position 26, whereas *TNFB*2* resulted in a threonine. This variant was reported by Messer et al. (1991) to determine TNF-β rather than TNF-α production. Although TNF_{-308} has been the focus of attention, the *LTA NcoI* RFLP has also been employed as a result of more informative allele frequencies. There is linkage disequilibrium reported between these two polymorphisms in a number of populations: *TNF*1/LTA*1* and *TNF*2/LTA*2* being more common than expected by random assortment.

Many early class I and II associations reported for disease susceptibility could be caused by linkage disequilibrium between these loci and those of the class III region, such as *TNF* and *LTA* (Jacob et al. 1990), e.g. the uncommon allele TNF_{-308}*2, which is often correlated with disease, shows very strong association with *HLA-A1*, *HLA-B8*, and *HLA-DR3* alleles (Wilson et al. 1993). Dissecting genetic effects in the region remains a problem. There have been numerous attempts to correlate particular alleles with function (e.g. Skoog et al. 1999). Many of the problems have related to study design, and the ability to demonstrate stable interindividual differences in cytokine production is rare (Molvig et al. 1988; Louis and Franchimont 1998). Despite issues over methodology (Kroeger et al. 1999), and diverse results (Brinkman et al. 1996; Wilson et al. 1997), undoubtedly confounded by attempting to examine the effects of a single base change on varied haplotypic backgrounds, TNF_{-308}*2 often shows higher production of TNF-α than TNF_{-308}*1 (Hajeer and Hutchinson 2001). TNF-α binds to two receptors TNF-R1 and TNF-R2, coded for elsewhere in the genome, both of which can be cleaved to release soluble receptors. The receptor genes have received less attention than the cytokine genes themselves, which is perhaps surprising in view of their role in the apoptotic pathway. Other class III genes include several coding for complement components.

The *IL1* gene cluster

The *IL1* gene cluster on chromosome 2q13 comprises *IL1A*, *IL1B*, and *IL1RN*, coding for IL-1α, IL-1β, and the IL-1 receptor antagonist, respectively (Figure 2). About 200 kb separates *IL1B* and *IL1RN* (Nicklin et al. 1994). A number of base change, microsatellite, and variable number tandem repeat (VNTR) polymorphisms have been identified in this region (Nothwang et al. 1997). It has been speculated, and occasionally demonstrated, that polymorphisms in each of these three genes may be functional, i.e. influence cytokine production (Pociot et al. 1992; Bailly et al. 1993; Danis et al. 1995). Such studies are difficult with a family of genes that are regulated at transcriptional, translational, and post-translational levels (Arend et al. 1998). The *IL1* gene cluster does show linkage disequilibrium (Cox et al. 1998), e.g. the $IL1B_{-511}$*2/$IL1RNV_{NTR}$*2 haplotype has been observed to be more common than anticipated by random assortment. These two alleles are thought to produce high levels of IL-1β and IL-1RA respectively. Although findings with this gene cluster are not so striking as those for the HLA class III region, *IL1* genes seem to be very important for control of certain infections, e.g. *IL1RN* in susceptibility to *Listeria monocytogenes* (Hirsch et al. 1996).

Some of the genes coding for the family of IL-1 receptors are also to be found on the long arm of chromosome 2, proximal to *IL1A*, *IL1B*, and *IL1RN* (Dale and

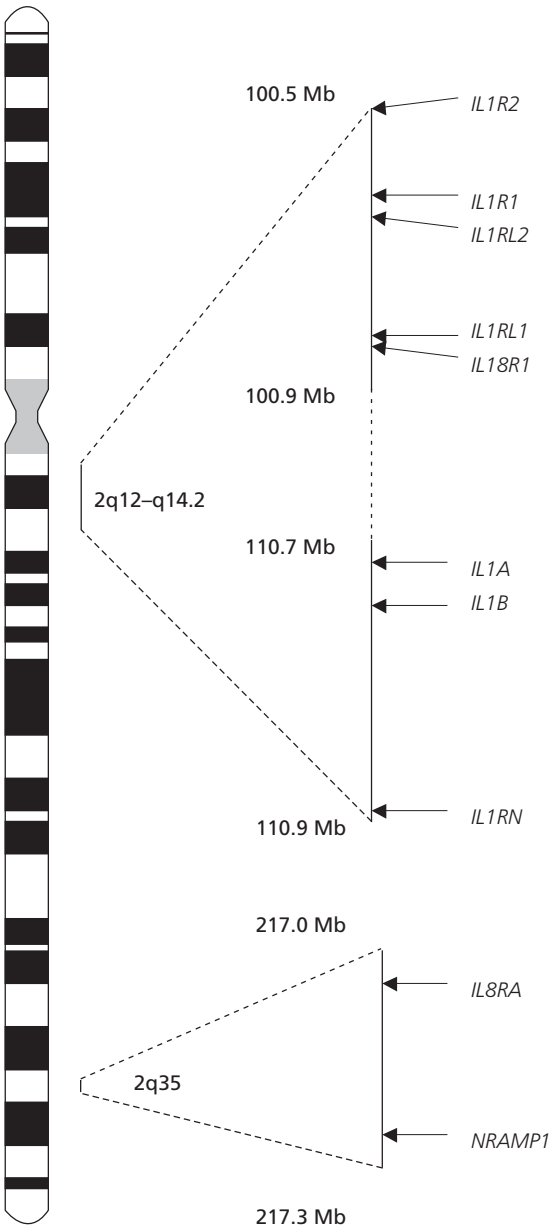

Figure 2 *Chromosome 2: the* IL1 *gene cluster and* NRAMP1.

Nicklin 1999). The most investigated are *IL1R1* and *IL1R2*. Another gene, *IL1RL1*, codes for the IL-1 receptor-related protein 1, which is a component of the IL-18 receptor. These genes are part of the larger IL-1 receptor/Toll-like receptor superfamily and additional members are still being described (O'Neill and Dinarello 2000). The proteins have three extracellular Ig-like domains. The soluble part of the type 1 IL-1 receptor has been crystallized together with the IL-1RA (Schreuder and Tardif 1997). As for *TNF*, the receptor genes have received less attention than the cytokine genes themselves, despite early indications that genetic variation of Toll-like receptor 4 may influence risk of gram-negative infections (Agnese et al. 2002). There are polymorphisms available for study, e.g. in the promoter of *IL1R1* (Bergholdt et al. 2000), although association studies with autoimmune conditions have been largely unrewarding. The IL-1 cytokines and receptors necessitate dissection of sometimes small genetic effects in a network of complex regulation and overlapping specificities.

SLC11A1 (*NRAMP1*, *Lsh/Ity/Bcg*)

SLC11A1, otherwise known as *NRAMP1*, is the best example of a locus that was originally investigated in the context of susceptibility to pathogens, rather than for its role in coding for an immune response protein (Figure 2). A number of inbred mouse strains have different susceptibilities to the intracellular macrophage pathogens *Salmonella typhimurium*, *Leishmania donovani*, and *Mycobacterium* spp. This genetic effect is remarkable because there is a clear dichotomy between resistant and susceptible strains, apparently under single gene control, with susceptibility proving recessive. Although the genes were named as *Ity*, *Lsh*, and *Bcg* respectively, following the cloning of *Nramp1* (natural resistance-associated macrophage protein) in 1993 by Vidal et al., it was proven, using a gene knockout and transfection, that the resistance to these intracellular macrophage pathogens was controlled by a single locus (Barton et al. 1995; Vidal et al. 1995). The human gene was named *NRAMP1*, now renamed *SLC11A1*, and lies on chromosome 2q35 near to *IL8R*. *Nramp1* has pleiotropic effects on macrophage function. The murine-resistant allele upregulates production of TNF-α and IL-1 and expression of MHC class II, and hence antigen-presenting capability. *Nramp1* codes for an integral membrane protein, with homology to transporters, and susceptibility is determined by a single nonconservative glycine/asparagine amino acid substitution (Vidal et al. 1993). Although the structure suggests a transport function, and despite identification of *Nramp1* as part of a family of transporter molecules (Cellier et al. 1995), the precise function has proved elusive. Subsequently localized to the late endosomal and lysosomal compartments, the protein is now thought to be an iron transporter (Blackwell and Searle 1999). The murine polymorphism has not been observed in human populations, highlighting potential problems of generalizing across species. However, cloning of the human homolog has been accompanied by identification of a number of other coding and noncoding polymorphisms (Cellier et al. 1994; Blackwell et al. 1995; Liu et al. 1995). One of these, a dinucleotide repeat in the human promoter, has been associated with functional differences in allelic expression (Searle and Blackwell 1998). There have been numerous attempts to demonstrate a clear role for *NRAMP1* in susceptibility to pathogens (Skamene et al. 1998, described below) and fewer studies examining its role in autoimmunity (Shaw et al. 1996; Blackwell and Searle 1999). One allele of the promoter polymorphism shows some association with autoimmune disease susceptibility and another allele with infectious disease susceptibility. However, findings are weak, relative to the murine system. This gene is being examined in other relevant species such as reservoir hosts (Altet et al. 2002).

Mannose-binding lectin and complement genes

Genes for classical, alternative, and membrane attack complex components are distributed throughout the genome. However, they occur in gene clusters, and there is substantial evidence of gene duplication and duplication of particular exons, e.g. those responsible for thioester bond formation, which enable key complement components to attach to the cell surface (Campbell et al. 1988; Schneider and Wurzner 1999). The genes *C2*, *C4A*, and *C4B*, and the gene coding for factor B, lie within the class III region of the MHC (see Figure 1). A variety of complement deficiency disorders is known, under single gene control. Unlike many 'single gene disorders', heterozygotes are relatively common, particularly for C2 deficiency: 1–1.5 percent of Caucasians have a *C2* null gene (Colten 1992). As mentioned above, complement deficiencies are associated with the autoimmune disorder systemic lupus erythematosus and infection with pyogenic bacteria (Walport 1993). Of the complement genes that have been investigated, predominantly those in the classical pathway, some, but not all, show polymorphic coding variation (Schneider and Wurzner 1999). As complement receptors, particularly those for C3, can act as receptors for invading microorganisms, and have a significant impact on infections such as HIV-1, it is to be expected that variation in the genes could impact on disease susceptibility (Fearon and Locksley 1996; Speth et al. 1997). However, despite a long history of research into variation in the complement system, these genes remain relatively poorly investigated with respect to infectious disease. There is also strong linkage disequilibrium between complement genes, especially

those in the class III region, hence, as for *TNF/LTA* and HLA class II genes, it is difficult to dissect genetic effects. Studies are rarely large enough to establish more than preliminary associations, and reported associations may merely reflect linkage disequilibrium with another nearby gene.

Mannan-binding protein/mannose-binding lectin (MBP/MBL) binds to terminal mannose groups on the surfaces of bacteria and enhances uptake by phagocytes through opsonization. Its structure is like that of C1q and, together with associated proteases, it can initiate the classical pathway in the absence of antibody. As it recognizes a broad range of infectious agents and is part of the early innate immune response, variation in this molecule may be critical in disease susceptibility (Fearon and Locksley 1996; Ezekowitz 1998). Polymorphisms in exon 1 of *MBL* (*MBL2*, chromosome 10q11.2-q21), at codons 52, 54, and 57, lead to low or absent serum MBL. The codon 54 mutation results in an unstable protein that is unable to activate complement. The effects of these mutations are detectable in the heterozygous state. As the frequency of these mutations is high, inevitably this has raised the question of selection. However, no selective advantage has been demonstrated to date (Ezekowitz 1998). There is also variation in the promoter region (Madsen et al. 1998). A number of population studies have attempted to correlate *MBL* haplotypes with varying concentrations of MBL in Old World and New World populations (Madsen et al. 1998; Turner et al. 2000; Boldt and Petzl-Erler 2002). The role of *MBL* variation in susceptibility to infectious disease has received considerable attention (Hill 1998).

The chromosome 5 gene cluster and other genes influencing IgE

The main stimulus for research into genetics of the 5q region has been its connection with the most common group of multifactorial disorders in western populations: asthma and atopy. Looking at linkages for asthma and atopy from the Asthma Gene database, chromosome 5 is most commonly identified (see www.cooke.gsf.de/asthmagen/main.cfm). For asthma/atopy, and latterly for infectious disease susceptibility, studies have concentrated on linkage rather than association. There are many reviews of the genetics of asthma and atopy available (Anderson and Cookson 1999). Genes present in the extended 5q31–q33 region include cytokine genes typically associated with a Th2 response: *IL3*, *IL4* (Arai et al. 1989), *IL5*, *IL9*, *IL13*, and also the gene coding for the p40 subunit of IL-12 (*IL12B*, Sieburth et al. 1992) (Figure 3). Other genes of interest include *IRF1* (interferon regulatory factor), *CSF2* (granulocyte–macrophage colony-stimulating factor (GM-CSF)), *CD14* (Goyert et al. 1988), *ADRB2* (β_2-adrenergic receptor), and *CSF1R*. A detailed physical map is available (Frazer et al. 1997). It appears there have been a number of gene duplication events, although some require rapid sequence divergence (van Leeuwen et al. 1989). New genes are being identified for asthma that may have direct links with infectious disease susceptibility. Identification of the *Tim* gene family in the region determining the *Tapr* locus (T-cell and airway phenotype regulator) used mice congenic for the chromosomal region homologous with human 5q23-q35 (McIntire et al. 2001).

The wealth of candidate immune response genes perhaps accounts for why studies either fail to develop beyond linkage approaches or are isolated gene association studies. There are a number of SNP and microsatellite polymorphisms in and between genes in this region (Noguchi et al. 2001). A few have been used extensively in study of disease, some of which are proposed to have functional consequences, such as the IL-13-coding polymorphism (Heinzmann et al. 2000). Where polymorphic sites are sufficiently dense haplotypes have been derived (Drysdale et al. 2000; Graves et al. 2000). Although linkage disequilibrium does occur in this region, unlike for HLA, there have been few attempts to characterize patterns (Takabayashi et al. 1999). There is a potential role of this gene cluster in regulation of Th1/Th2 responses, despite indications that the Th1 versus Th2 split is not controlled by *IL4* directly (Guler et al. 1999). Recent research has centered on control of the genes in this region, in particular the region between *IL4* and *IL13*. By comparing human and mouse sequences, a 401-bp-conserved noncoding sequence CNS-1 immediately downstream of *IL13* has been identified (Loots et al. 2000). Experiments in mice suggested this region as a coordinate regulator of *IL4*, *IL13*, and *IL5* spanning 120 kb. The capacity to develop Th2 cells was compromised in vitro and in vivo in the absence of CNS-1, despite retaining some capacity to produce IL-4 (Mohrs et al. 2001). Chromatin accessibility is likely to be important in cytokine expression (Agarwal et al. 1999; Hural et al. 2000; Guo et al. 2002).

Total IgE has been known to be under genetic control for many years with heritability estimates in excess of 50 percent (Bazaral et al. 1974; Blumenthal et al. 1981). Total IgE is perhaps the best characterized of the multiplicity of asthma/atopy traits. The best linkage known for control of a quantitative immune trait is the linkage of total IgE to the chromosome 5q31 gene cluster, first described by Marsh et al. (1994) in Amish sib-pairs. This linkage has been confirmed in a number of populations (Xu J. et al. 2000) and not detected in a few (Laitinen et al. 1997). In the context of total IgE, *IL4*, *IL13* (Graves et al. 2000; Liu et al. 2000), *CD14* (Baldini et al. 1999), and *ADRB2* (Dewar et al. 1997) have all been studied. There are sufficient data in the literature for meta-analysis, which will hopefully contribute to elucidation of which genes and how many genes are important for disease states in this region (Collins et al. 2000).

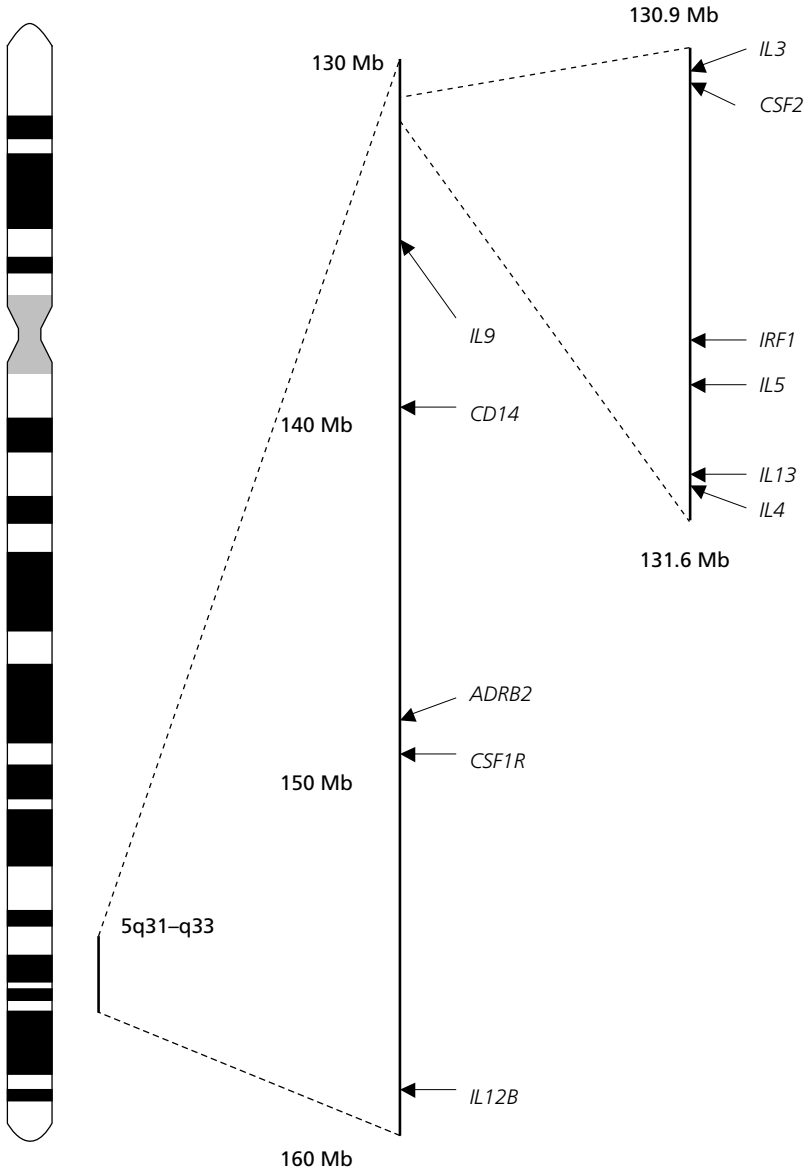

Figure 3 *The chromosome 5 gene cluster.*

The 5q cluster is also implicated in a number of inflammatory diseases some of which may have infectious disease etiology. A gene *IBD5* (inflammatory bowel disease 5) confers susceptibility to Crohn's disease and lies in 5q31. Use of a TDT approach gave strong evidence that a common 5q31 haplotype conferred susceptibility to Crohn's disease, although causal mutations remain to be identified (Rioux et al. 2001).

Familial eosinophilia is a rare autosomal dominant disorder. A genome scan provided evidence for linkage of this trait to chromosome 5q31–q35 (Rioux et al. 1998). There were no functional mutations in *IL3*, *IL5*, or *CSF2*, suggesting a defect elsewhere in this region. This may prove comparable to the *IFNGR1* story where there are both rare deleterious mutations and common less or nonpathogenic variants. The region proves to be of interest for helminth infections associated with IgE and eosinophilia.

Variation in receptors for the 5q-encoded cytokines is also implicated in asthma and atopy. The IL-4 and IL-13 receptors are inextricably linked through the sharing of a common IL-4Rα chain and overlapping specificities (IL-4 binding to both receptors) (Callard et al. 1996). The IL-4Rα chain has been extensively investigated in the context of asthma and atopy. There are a number of common coding polymorphisms known, of which the most studied are an extracellular variant Ile50Val (Mitsuyasu et al. 1998, 1999) and a cytoplasmic variant Gln576Arg (Hershey et al. 1997; Grimbacher et al. 1998; Wang et al. 1999). There is some evidence for a functional effect of each polymorphism: Arg576 was associated with increased expression of CD23 after IL-4

stimulation of mononuclear cells, and Ile50-transfected cells showed augmented responses to IL-4, which appeared not to change chain expression but to augment STAT6 activation. However, initial findings have not been entirely supported, and recent studies have suggested that variation at more than one site is necessary for functional effects, e.g. at both positions 50 and 576, or positions 503 and 576 (Kruse et al. 1999). Functional significance of variants within this gene remains topical. In addition, many associations have been reported between alleles at various positions, but with variation between studies. Analysis of *IL4RA* haplotypes has been of some value. The IL-4R story is a good example of confusion through examination of associations, and potential functionality of particular variants, in isolation. However, with more polymorphisms being identified, in both the promoter (Hackstein et al. 2001) and coding (Deichmann et al. 1997) regions, further consideration of haplotypes should prove informative (Risma et al. 2002). Using variants in both *IL13* and *IL4RA* there are early indicators of gene–gene interactions (Howard et al. 2001).

Variants in *IL12B* and receptors for Th1-type cytokines, have been studied primarily in the context of mycobacterial infection and are considered under the appropriate section.

A refined segregation analysis was compatible with more than one gene regulating total IgE (Xu et al. 1995). As total IgE is likely to be a multifactorial trait, a number of other genes have secondarily been implicated, which include *FCER1B*, *IL4RA*, *TNF*, loci on chromosomes 7 (Xu J. et al. 2000) and 12 (Barnes et al. 1996; Nickel et al. 1997; Xu J. et al. 2000), *INFGR1* and *IFNGR2* (Gao et al. 1999), and an *IL18R* splice variant (Watanabe et al. 2002). The gene for the β chain of the high-affinity IgE receptor, *FCER1B*, is on chromosome 11q13, and there is linkage of several asthma/atopy traits to this region in some populations. The original report was notable for its analysis of maternal transmission (Cookson et al. 1992). Three identified coding variants of *FCER1B* have shown some associations, but the precise effect of these polymorphisms is unclear (Furumoto et al. 2000; Hizawa et al. 2000). That results are somewhat mixed for Ile181Leu and Val183Leu, but less so for Glu237Gly, may be indicative of population heterogeneity. There have been very few attempts at haplotype construction across polymorphisms in this region. Other genes in this region, such as *CC16*, may be implicated. Together with the chromosome 5 cluster, 11q13 is the most investigated region in the context of asthma/atopy.

The chromosome 17 genes

Genes on the long arm of chromosome 17 have received less attention than the chromosome 5 cluster (Figures 4 and 30.5). However, with increasing interest in chemokines and chemokine receptors, largely stimulated by HIV-1 studies, and the occurrence of multiple candidate genes, it is likely that this region will prove critical in determining susceptibility to infectious disease.

Receiving the most attention to date is *NOS2A*, the gene coding for inducible nitric oxide (NO) synthase. There are three genes coding for nitric oxide synthase, but only the inducible form maps to this region (Marsden et al. 1994; Xu et al. 1994). As NO is a killing mechanism for intracellular macrophage bacteria and protozoa, a rate-limiting enzyme responsible for NO production is clearly important. Several variants in *NOS2A* have been widely investigated in relation to infectious disease susceptibility (Hobbs et al. 2002; Xu W. et al. 2000).

A number of small inducible cytokine genes named '*SCYA*', now renamed as chemokine CC motif ligand 'CCL', also occur in this region. One such molecule is the proinflammatory chemoattractant RANTES (*SCYA5*). Relatively late appearing mRNA transcripts are upregulated in cells stimulated with TNF-α and IL-1β, and TNF-α induces transcriptional activation of *SCYA5*, although regulatory mechanisms may be tissue-specific (Nelson et al. 1993). Also coded in this region are two related C-C chemokines macrophage inflammatory protein 1α(MIP-1α, *SCYA3*) and 1β (MIP-1β, *SCYA4*). The genes are 14 kb apart, arranged in a head-to-head fashion, in keeping with their coordinate regulation. In addition there are reduplicated loci in this region, the presence of which varies between individuals (Irving et al. 1990). Syntenic, but proximal to *SCYA3* and *SCYA4*, between the genes for RANTES and monocyte chemotactic protein-1 (MCP-1) (*SCYA2*), are the genes coding for MCP-2 (*SCYA8*) and MCP-3 (*SCYA7*) (Opdenakker et al. 1994). Breakpoints in the region are associated with several malignancies. It is reasonable to assume, as for C-X-C chemokine genes on chromosome 4q13–q21, C-X-C chemokine receptor genes on chromosome 2q34–q35, and C-C chemokine receptor genes on chromosome 3p21.3, that the origins of these molecules are through gene duplication. This is in keeping with repeat and palindromic sequences in the region (Opdenakker et al. 1994). Another locus in this cluster is eotaxin (*SCYA11*), also stimulated by TNF-α and IL-1α, with eosinophil-specific chemotactic ability and close homology to MCP-4 (*SCYA13*) (Bartels et al. 1996). As for the other genes in this cluster, eotaxin shows polymorphic variation (Miyamasu et al. 2001), in this case associated with plasma eotaxin levels and eosinophil counts, and may account for some of the genetic component proposed to determine eosinophil levels (Holberg et al. 1999).

Other polymorphisms

A number of other immune response genes have been proposed as candidates for resistance/susceptibility to

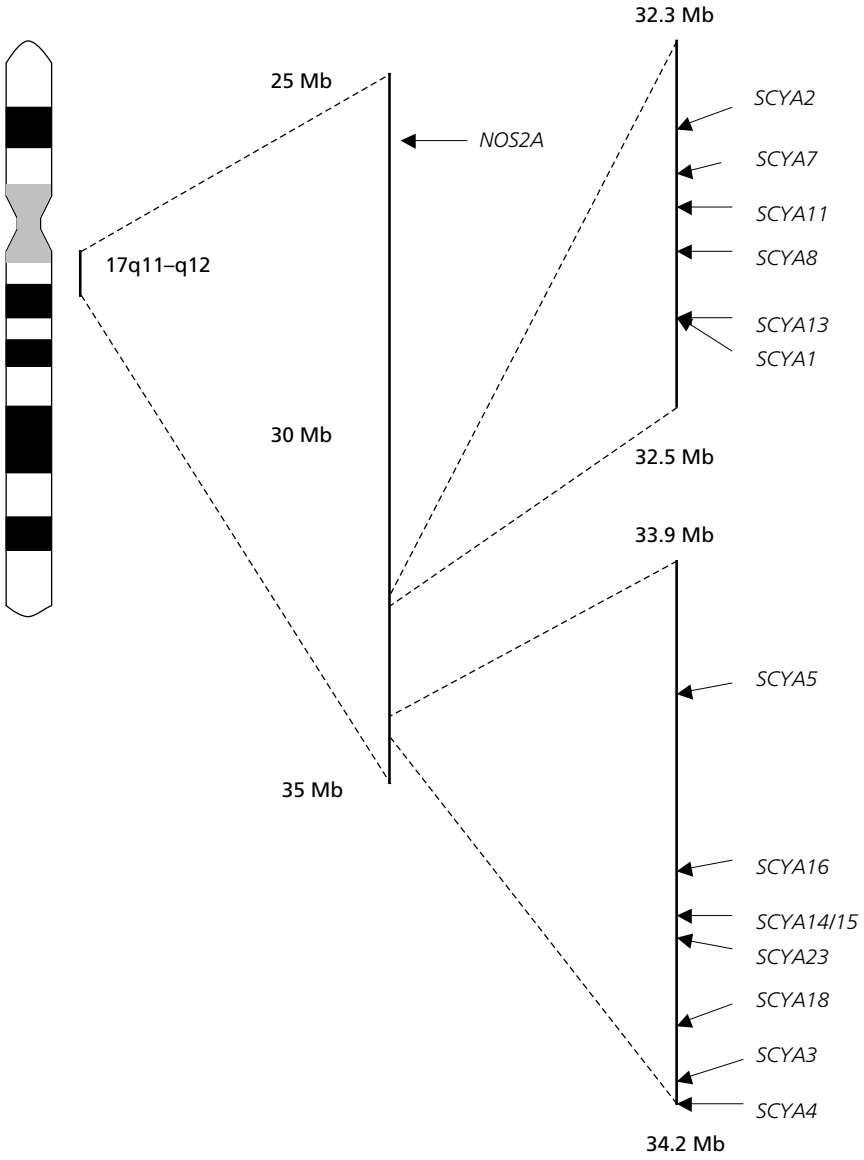

Figure 4 *Chromosome 17: genes on the long arm*

infectious disease, although the number of studies to date is limited. These include gene clusters coding for members of the Ig superfamily: Ig gene allotypes, T-cell receptor genes, and Fc receptor genes (including *CD32*) (Figure 6). However, the literature on T-cell receptor gene usage outweighs the information on polymorphic diversity. Such gene clusters do not display the high allelic diversity of the MHC. Among the cytokine genes most commonly investigated in an infectious disease context are those coding for IFN-γ (*IFNG*, 12q14), transforming growth factor β (TGF-β1) (*TGFB1*, 19q13), and IL-10 (*IL10*, 1q31–q32). Multiple polymorphic sites allow the construction of haplotypes. There has also been much interest in a variety of receptors, ligands, and adhesion molecules: CTLA-4 (*CTLA4*, 2q33), intercellular adhesion molecule 1 (ICAM-1) (*ICAM1*, 19p13), CD36 (*CD36*, 7q11), DARC (*FY*, 1q21–q22), and the vitamin D receptor (*VDR*, 12q12–q14). All of these, with the exception of *VDR*, have been studied primarily in the context of malaria. The active form of vitamin D is 1,25-dihydroxycholecalciferol, an immunomodulatory molecule, and the *VDR* gene has been considered for several infectious diseases.

Aside from the general web-based databases that catalogue microsatellite and single nucleotide polymorphisms, there are a few more specialized databases. Many of the polymorphisms that have been investigated for multifactorial diseases are in cytokine genes and have been collated (Bidwell et al. 1999a, b; Haukim et al. 2002). This specialist database has the advantage of providing detailed information on functionality and past disease associations. More generally, the On-line

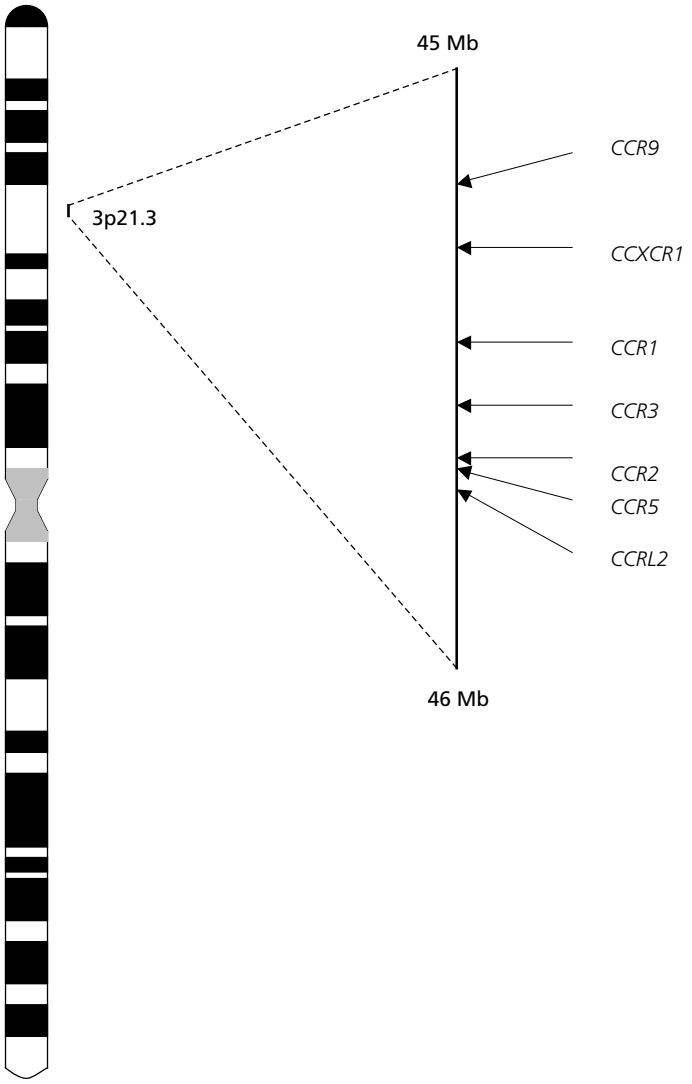

Figure 5 *Chromosome 17: genes on the short arm*

Mendelian Inheritance in Man (OMIM) site allows keyword searching and a history of genes and their variants. Whether polymorphisms are of functional relevance is a difficult issue. Clearly polymorphisms in regulatory regions may influence gene expression, and coding variation resulting in amino acid substitutions may affect function. Suffice it to say that there is very little good evidence of functional importance for most polymorphic sites.

Interestingly some cytokine genes, *IL2* and *IL4*, have been shown to be expressed monoallelically in single cells (Matesanz et al. 2000). This phenomenon of allelic exclusion is usually associated with the Ig molecules, where allelic exclusion is clearly necessary if a single cell is to produce a single Ig molecule. The reason for allelic exclusion in cytokine production is less clear. The mechanism appears to be through asynchronous DNA transcription (Mostolavsky et al. 2001).

VIRAL INFECTIONS

The number of human genetic studies on susceptibility to most viral infections is not high. This is surprising because receptor-mediated viral entry provides a number of good candidate genes. Studies are much farther forward in mice, although there are some limitations on simple extrapolation from mouse to humans (Brownstein 1998).

HIV

Human studies have concerned susceptibility to HIV-1 rather than HIV-2. There are a number of reviews available (Rowland-Jones 1998; Kaslow and McNicholl 1999; Michael 1999). Interest is focused in several areas: those individuals who remain seronegative despite

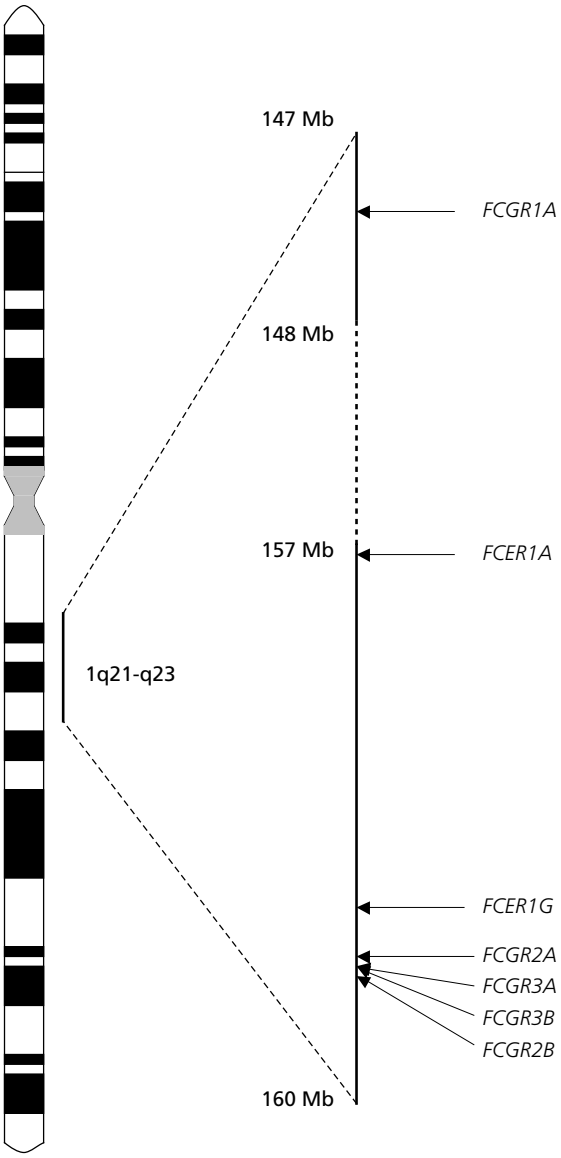

Figure 6 *Chromosome 1*

repeated exposure, differences in progression of disease after seroconversion, pediatric infection, and vertical transmission (Matte and Roger 2001), and latterly an interest in susceptibility to the various opportunistic infections. To a limited extent we now have the ability to collect genetic information from patients to give potential indicators as to disease progression (Carrington et al. 2001). HIV-1 uses co-receptors to enter host cells. The first co-receptor identified was CXCR4, then known as fusin, necessary for invasion of syncytium-inducing, T-cell-tropic isolates into $CD4^+$ T cells in the later stages of infection. Recombinant fusin enabled CD4-expressing nonhuman cells to support HIV-1 fusion. Antibodies to fusin blocked cell fusion and infection with normal $CD4^+$ human cells (Feng et al. 1996). CXCR4 is the receptor for stromal-derived factor 1 (SDF-1).

There is a greater literature concerning CCR5, rapidly implicated after CXCR4 (Deng et al. 1996; Dragic et al. 1996), as a necessary co-receptor for entry of nonsyncytium-inducing isolates into macrophages in the early stages of infection. Discovery was followed by description of the mechanism of entry: high-affinity binding of gp120 to CD4 causes conformational changes in gp120, creating a recognition site for CCR5 (Trkola et al. 1996; Wu et al. 1996). CCR5 is the receptor for the CC chemokines MIP-1α, MIP-1β, and RANTES, the genes of which have been studied in relation to HIV-1. Other members of this receptor family, CCR3 (the receptor for eotaxin) and CCR2b, have emerged as potential alternative co-receptors to CCR5.

Samson et al. (1996) showed that a variant 32-bp deletion in *CCR5* makes up about 10 percent of alleles in western Caucasian populations, but was absent from west and central African and Japanese populations. This leads to a truncated protein that is not functionally expressed (Liu et al. 1996). In HIV-infected Caucasians no homozygotes for *CCR5Δ32* were found, and the frequency of heterozygotes was 35 percent lower than in the general population. As primary infections were thought to be with nonsyncytium-inducing strains, initial observations suggested that such homozygotes would be totally refractory. Indeed cells from a homozygous individual were resistant to infection by macrophage tropic viruses. However, a handful of exceptions are now taken to be infection with syncytium-inducing or dual tropic strains utilizing CXCR4. Heterozygotes are not resistant but have slower disease progression, again protection being against nonsyncytium-inducing strains (Michael et al. 1997). Other mutations in *CCR5* occur with lower frequency and, although less is known of their effects, some produce functional alterations. Mutations within the *CCR5* promoter are likely to have less easily detectable effects than those resulting in truncated proteins. The ease with which mutations have been identified in *CCR5* argues for selective pressure favoring these alleles (Blanpain et al. 2000). The *CCR5Δ32*-containing ancestral haplotype has been estimated to be 700 years old, and this recent emergence is consistent with a strong selective event such as a plague epidemic (Stephens et al. 1998). CCR3 and CCR5 are both expressed in microglial cells of the central nervous system (CNS), and are thought to be responsible for transmission of macrophage tropic strains within the CNS (He et al. 1997). *CCR3* also shows several genetic variants (Zimmermann et al. 1998).

A mutation in the gene coding for CCR2b results in a conservative valine-to-isoleucine substitution at codon 64. Although described as affecting HIV progression, this gene is in linkage disequilibrium with *CCR5*, both being on chromosome 3 and only 20 kb apart.

Hence, for these mutations, causal effects can be hard to disentangle. Smith et al. (1997) showed that the *CCR5Δ32* allele is almost in complete linkage disequilibrium with *CCR2-64I*: the two mutations are not found together on the same chromosome. In European populations about 20 percent of the population will carry one of the two mutant alleles (Romano-Spica et al. 2000). However, unlike *CCR5Δ32*, *CCR2-64I* does occur in African populations (Su et al. 1999), where there are slow progressors, despite the much shorter median time from seroconversion to acquired immunodeficiency syndrome (AIDS).

A point mutation in the 3′UTR of the gene coding for SDF-1α, the ligand for CXCR4, *SDF1-3′A* has a recessive protective effect, unlike the effect of *CCR2–64I* which is dominant. Homozygotes make up about 1 percent of Caucasian populations and these people show a much slower disease progression. The frequency of *SDF1-3′A* varies worldwide with high frequencies in Oceanian populations (Su et al. 1999). An *SDF1* heterozygous genotype is one factor implicated in infectivity and hence in mother-to-offspring transmission (John et al. 2000).

Genetic variation has also been studied with respect to other chemokine ligands including MIP-1α, RANTES, and eotaxin (Liu et al. 1999; Modi et al. 2000; Gonzalez et al. 2001). Two polymorphisms in the *SCYA5* promoter at positions −403 and −28 result in GC, AC, and AG haplotypes. The −28G allele, which occurs at a frequency of 17 percent in noninfected Japanese, was associated with reduced $CD4^+$ lymphocyte depletion rates in HIV-1-infected individuals and increased transcription of the RANTES gene (Liu et al. 1999). Homozygosity for the *SCYA5* AC haplotype was associated with increased risk of acquiring HIV-1 as well as accelerated progression in Europeans, but not African–Americans, despite a high prevalence of the haplotype in people of African origin (Gonzalez et al. 2001).

In contrast to the work on chemokines, there has been little consideration of the effects of cytokine polymorphism on HIV-1 infection (Nakayama et al. 2002; Shin et al. 2000). In general findings are weak and concern disease progression. Similarly, there are few studies on the role of genes involved in innate immunity and immunomodulation such as *NRAMP1* (Marquet et al. 1999), *MBL* (Pastinen et al. 1998), and *VDR* (Hill 1998). In a Colombian population four *NRAMP1* genotypes were associated with altered risk of infection. Three genotypes were associated with reduced relative risks (0.24–0.35), but were in strong linkage disequilibrium, and one genotype was associated with an increased relative risk (2.29) (Marquet et al. 1999). Heterozygotes for a *VDR* polymorphism are at reduced risk of infection (Hill 1998).

In contrast there are many observations concerning HIV-1 susceptibility and progression according to HLA type. Findings are variable, which may be in part accounted for by diverse ethnic background (Al Jabri 2002). *HLA-B*35-Cw*04* is associated with rapid progression in white Europeans and, although cytotoxic T lymphocytes (CTL) have been observed to recognize epitopes in the context of *HLA-B*3501*, analysis of subtypes suggests that a single amino acid change may be key (Gao et al. 2001). Lack of alleles *HLA-B*35* and *HLA-Cw*04*, together with full heterozygosity at HLA class I loci, has been associated with extended survival (Carrington et al. 1999). It is interesting in this context that lack of diversity at chimpanzee class I loci has been highlighted in relation to their natural resistance to AIDS: it has been proposed that a selective sweep has occurred as a result of exposure to simian immunodeficiency virus (SIV) or a closely related retrovirus (de Groot et al. 2002). Another well-characterized haplotype, *HLA-A1-B8-DR3*, is associated with rapid progression, and some autoimmune conditions (Candore et al. 1998; Price et al. 1999). Such associations with extended haplotypes raise the possibility of the involvement of class III loci such as *TNF* (Khoo et al. 1997).

Finally, there are some studies concerning the susceptibility to opportunistic infections associated with HIV-1, e.g. inflammatory cytokine and Fc receptor genes have been examined for influence on susceptibility to Kaposi's sarcoma, itself associated with human herpesvirus 8, with some significant associations (Foster et al. 2000; Lehrnbecher et al. 2000).

HTLV-1 and HTLV-2

Human T-cell lymphotropic virus (HTLV)-1 and HTLV-2 are retroviruses where, from rodent models, it may be expected that host genetic background will influence clinical manifestations. HTLV-I causes adult T-cell leukemia/lymphoma (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1 uveitis (HU) in a small percentage of infected carriers. Most human genetic studies have been on the MHC. HLA haplotypes have been examined for ATL patients, and a TNF_{-857} allele, associated with enhanced production of TNF-α, correlated with enhanced susceptibility to ATL in Japanese (Tsukasaki et al. 2001). Class II alleles differ among patients developing ATL or HAM/TSP (Manns et al. 1998). An HLA effect for HAM/TSP would be consistent with the suggestion that pathology for this condition is the result of an exaggerated CTL response (Bangham et al. 1999). Concurrence of HAM/TSP and HU is relatively common, but concurrence of either with ATL is rare. *TNF* promoter alleles at positions −1031 and −863 have been proposed as risk factors for HU (Seki et al. 1999). Although many of these studies have considered linkage disequilibrium, elucidation of genetic effects is still in the early stages.

Respiratory syncytial virus

Respiratory syncytial virus infection results in bronchiolitis in a proportion of infected children. Pathology is correlated with neutrophils and IL-8 production. An *IL8* promoter allele was associated with increased IL-8 production in LPS-stimulated blood, and was more frequently transmitted by parents to offspring with bronchiolitis (Hull et al. 2000). This observation has been confirmed and a detailed haplotypic analysis of the region conducted using six SNPs. The putative disease allele resided on two haplotypes, only one of which was implicated in disease, suggesting that the original polymorphism was not functional (Hull et al. 2001). Consistent with observations from other loci, Europeans have few common haplotypes differing at multiple sites whereas Africans show greater haplotypic diversity. There is also some evidence for a role of *IL4* (Choi et al. 2002), and possibly *IL4RA*, in severe bronchiolitis (Hoebee et al. 2003).

In addition to the retroviruses described above, a group of viruses showing latent or asymptomatic infections, with later pathological consequences in a subset of individuals, have been considered for human immunogenetic determinants. These include hepatitis B and hepatitis C, Epstein–Barr virus (EBV), papilloma viruses, cytomegalovirus (CMV), and herpesviruses.

Hepatitis B and hepatitis C

In mice, two non-*H-2*-linked genes have been proposed to determine susceptibility to hepatitis viruses (*Hv1* and *Hv2*; McLeod et al. 1995). Host genetics may be involved in several aspects of infection. Infection may be transmitted from an infected mother to a child perinatally, and there are racial differences in the frequency of perinatal transmission, implicating genetic control. Twin studies in China employed several different markers of hepatitis B (HBV) infection (Lin et al. 1989). No significant difference in the concordance of HBV infection was observed in the monozygotic and dizygotic twins; however, significant differences were seen between twins and controls. Highly significant differences were seen in the concordance of carrier status between monozygotic and dizygotic twins, indicating that genetics may be involved in the persistence of hepatitis B. MHC genotype is a risk factor for developing persistent infection. In addition, the MHC may be involved in nonresponsiveness to immunization. Both carriage of certain MHC alleles and MHC heterozygosity may be important in protection from persistent infection. In a west African population, where 90 percent of adults have been infected and 15 percent have persistent infection, there was some heterozygote advantage for the class II but not for the class I region, as well as a protective association with *HLA-DRB1*1302* (Thursz et al. 1997; Hill 1998). Two further studies have shown this allele or its supertype *HLA-DR6* to be protective. However, in a small Qatari study *HLA-DR2* was associated with clearance and *HLA-DR7* with persistence (Almarri and Batchelor 1994). Heterozygote advantage was confirmed in a study of largely African–American intravenous drug users, which found persistence associated with *HLA-DQA1*0501, DQB1*0301*, and class II homozygosity (Thio et al. 1999). The different allelic associations seen in this study were ascribed to the different age of initial infection. In addition to class II, *TNF* promoter and *VDR* alleles have been associated with HBV clearance (Hill 1998; Bellamy et al. 1999), and in Africans, but not other populations, *MBL* does not appear to influence the risk of HBV persistence (Bellamy et al. 1998b).

A particularly interesting set of questions relates to the chronic persistence of HBV as a risk factor for hepatocellular carcinoma (Wild and Hall 1999). It is possible that even apparently immune individuals are responding to sequestered virus, and risk factors for hepatocellular carcinoma include not only persistent infections such as HBV, but also other lifestyle, environmental, and genetic aspects. It seems likely that HLA determines development of hepatocellular carcinoma in hepatitis B antigen-positive patients (Donaldson et al. 2001). Susceptibility is also reported to be associated with genetic variation in the enzymatic detoxification of aflatoxin B1 (McGlynn et al. 1995), and variation in the gene for cytochrome P450 1A1 (*CYP1A1*), involved in transforming tobacco polycyclic aromatic hydrocarbons into carcinogenic metabolites, affects the risk of hepatocellular carcinoma in smokers (Yu et al. 1999). The complex mechanisms and interactions of these many factors remain to be elucidated.

The more recently described hepatitis C (HCV), like hepatitis B, is successfully cleared only in a minority of individuals. In the persistently infected, some remain asymptomatic whereas others progress to hepatocellular carcinoma. Again the role of HLA has been examined at a number of stages: susceptibility itself, viral clearance, progression to hepatocellular carcinoma, and response to treatment (Donaldson 1999). The first of these questions is dogged by the bugbear of all infectious disease immunogenetics, exposure. The second is the best studied, e.g. in the UK Minton et al. (1998) reported *HLA-DRB1*1101* (a *DR5* allele) and *HLA-DQB1*0301* to be associated with clearance of hepatitis C. This supports a previously observed protective effect of *HLA-DR5* (Zavaglia et al. 1996), although haplotypic analysis suggests *HLA-DQB1*0301* as being key (Alric et al. 1997; Donaldson 1999). However, a number of studies do not fit with such associations (e.g. Lechmann et al. 1999), although some of these studies have relatively small sample sizes. A suggestion that some of the disparities were the result of ethnic diversity (Thio et al. 2001) has stimulated some discussion (Sarmiento et al. 2002). Kuzushita et al. (1998) examined the effects of

HLA on progression to liver damage in HCV-infected subjects and showed that haplotypes that included *HLA-B*54* were associated with liver damage, whereas haplotypes that included *HLA-DRB1*1302-DQB1*0604* were associated with low hepatitis activity. The less frequent alleles of the TNF_{-308} and TNF_{-238} polymorphisms were correlated with development of cirrhosis in hepatitis C carriers (Yee et al. 2000); however, *TNF* has not been studied in conjunction with class I and II alleles. Sustained response to antiviral therapy has been associated with *IL10* but not *TNF* haplotypes (Yee et al. 2001). Outcomes of hepatitis C infection have also been correlated with variation in the gene for the low-density lipoprotein receptor (*LDLR*), apparently through effects on immune function rather than receptor status (Hennig et al. 2002).

Patients with liver–kidney microsomal type 1 antibodies often have detectable hepatitis C. In the presence of hepatitis C, a correlation was seen between the presence of antibodies and *HLA-DR7* in a Spanish study (Jurado et al. 1997), whereas *HLA-DR3* correlated with serum cryoglobulinemia and *HLA-DR4* with serum autoantibodies in a Chinese population (Hwang et al. 2002). The etiologically distinct autoimmune hepatitis, which is not caused or triggered by viral infection, also has well-characterized HLA associations. In addition non-HLA genes may be involved: in studies of *IL1*, *IL10*, *TNF*, and *CTLA4*, susceptibility to type 1 autoimmune hepatitis was associated with *CTLA4* alleles and TNF_{-308}*2 which is interdependent with *HLA-DRB1*0301* (Cookson et al. 1999; Djilali-Saiah et al. 2001).

Other viral infections

A number of other viruses are also associated with specific malignancies. As for hepatitis, there is often evidence for both genetic susceptibility to the virus itself, and genetic susceptibility to malignancy after infection. However, these questions are sometimes difficult to unravel.

Epstein–Barr virus is associated with a variety of malignancies. Interestingly, from a geneticist's point of view, racial groups show clear differences in the type of malignancy, with Burkitt's lymphoma in African populations and nasopharyngeal carcinoma in east Asian populations. It has been proposed that pathogenesis is a result of a failure of EBV-specific immunity (Niedobitek et al. 2001). EBV is ubiquitous and there has been some consideration as to the mechanism by which HLA could control primary infection, either as a co-receptor for viral entry (Haan and Longnecker 2000) or by influencing T-cell precursor frequencies (Toussirot et al. 1999). *IL10* promoter polymorphisms appear to influence susceptibility to primary infection (Helminen et al. 1999, 2001). HLA class I and II, *TNF*, and *IL10* may all play a role in susceptibility to EBV-associated gastric carcinoma (Koriyama et al. 2001; Wu et al. 2002b). *HLA-A*0207*, which is common in Chinese but not Caucasian populations, is associated with nasopharyngeal carcinoma (Hildesheim et al. 2002). *HLA-A*0207* is in linkage disequilibrium with *HLA-B*4601*, a previously identified risk factor, and *HLA-A*1101* is protective. A recent genome scan of Chinese families with nasopharyngeal carcinoma, and using parametric linkage analysis, identified a single region on chromosome 4, with a LOD score of 3.67 to marker D4S405, which was increased by incorporation of information on EBV antibody titer (Feng et al. 2002).

The human papilloma viruses (HPV) infect the squamous epithelial cells of skin, the oral cavity, and the cervix, leading to warts and in some cases cancer. The viral E6 oncoprotein binds to the tumor suppressor p53 and induces degradation. The degradation is affected by a Pro/Arg variation at position 72 in p53, with homozygosity for the Arg variant resulting in increased susceptibility to HPV-16/-18 infection and oral carcinogenesis (Nagpal et al. 2002). Similarly, there is genetic predisposition to cervical cancer (Magnusson and Gyllensten 2000), with MHC involvement, although findings are mixed. *HLA-B53* was found to be at increased frequency in patients with cervical neoplasia and HPV of types other than 16 or 18, whereas *HLA-B55* was increased in HPV-negative neoplasia patients (Krul et al. 1999). MHC II associations have also been reported. In the context of immunization, some HPV-16 E7 oncoprotein epitopes bind to the products of several *HLA-A2* alleles (Ressing et al. 1999).

HLA associations for infection with other human viruses have been identified. HLA restriction of the immune response to dengue virus may affect vaccine development (Loke et al. 2001), and known variation in response to immunization, correlated with HLA restriction, such as in the case of measles, may influence worldwide population vaccine efficacy (Poland 1999). Other genes may influence viral outcome, such as *VDR* and *FCRG2A* in susceptibility to severe dengue hemorrhagic fever (Loke et al. 2002).

In a number of instances, murine studies are well ahead, as with the murine leukemia virus-mapped genes (Malo and Skamene 1994; McLeod et al. 1995). There are at least two regions controlling CMV in mice: the MHC and *Cmv1*. *Cmv1*, an autosomal dominant gene, controls virus replication in the spleen and mediates its effects through NK cells. MHC associations in human CMV infection are also seen and could be caused partly by an effect of the proinflammatory *TNF* gene (Hurme and Helminen 1998). Influenza is controlled by *Mx1* in mice and has been cloned (Malo and Skamene 1994). In susceptible laboratory strains there is a deletion of exons 9–11, or alternatively a point mutation in exon 10, resulting in a stop codon. *Mx1* is controlled by interferons α and β, and has been highly conserved in

evolution, being present in all eukaryotes. A gene *Mx2* has also been identified that controls vesicular stomatitis virus in mice. The human homologs will be of interest. Of topical interest is host genetic susceptibility to West Nile Virus, a flavivirus, where a mutation in the gene coding for IFN-γ-inducible 2′,5′-oligoadenylate synthetase is associated with murine susceptibility (Samuel 2002).

Prions

The *PRNP* gene encodes the prion protein (PrP). Classification of prion diseases has been as genetic, infectious, or sporadic; however, all involve modification of PrP (Pruisner and Scott 1997). Approximately 10 percent of prion disease is inherited. There are several mutations in *PRNP* available for study (Kovacs et al. 2002), e.g. the murine equivalent of the PrP P102L mutation, associated with Gerstmann–Straussler syndrome, alters incubation time of transmissible spongiform encephalopathy (Manson et al. 1999). Most notably, all patients to date with variant Creutzfeldt–Jakob disease, and many with sporadic Creutzfeldt–Jakob disease, are homozygous *PRNP* for a methionine at position 129, whereas heterozygotes are protected (Palmer et al. 1991; Windl et al. 1996; Zeidler et al. 1997). The kuru epidemic in New Guinea also preferentially affected individuals with the *PRNP129* M/M genotype, resulting in complete loss of this genotype from affected communities (Lee et al. 2001). Undoubtedly *PRNP* does not act as a sole determinant of disease. Studies in other species, including mice, have searched for additional genetic components. A PrP-like protein, doppel protein, is coded for by *PRND*, close to *PRNP*. Whether variants of *PRND* will influence human prion disease directly, or merely reflect variation in *PRNP*, remains to be established (Peoc'h et al. 2000; Schroder et al. 2001). A recent study implicated HLA in variant Creutzfeldt–Jakob disease, with patients showing a reduced frequency of *HLA-DQB1*0301/4/9* (Jackson et al. 2001). This was an exciting finding, because it was thought to explain why many putatively infected, *PRNP*-susceptible, individuals did not develop disease. However, this finding has not been supported (Laplanche et al. 2003; Partanen 2003; Pepys et al. 2003).

BACTERIAL INFECTIONS

Intracellular bacteria

A number of intracellular bacteria have been investigated for control of infection by host genetic factors. In particular, there have been many studies of bacterial pathogens of macrophages, and these studies may be considered together with those concerning protozoal macrophage parasites such as *Leishmania* spp. Mycobacterial species have been a focus of attention and *Mycobacterium tuberculosis* and *Mycobacterium leprae* are considered separately.

Whereas common genetic variation has been proposed to influence the major infectious diseases, the mycobacteria also provide good examples of rare host deleterious mutations resulting in disease caused by poorly pathogenic bacteria (Ottenhoff et al. 2002). The main infections observed are disseminated *Mycobacterium bovis* BCG and *Mycobacterium avium*, although infections with *Salmonella* spp. also occur (Jouanguy et al. 1999a). The underlying phenotype is of defective bacterial killing resulting from a failure to produce or respond to IFN-γ, and fatal infection is common. Deficiencies may be partial or complete, and inherited in either a recessive or a dominant fashion. These deficiencies are often caused by mutations in cytokine receptor genes, and a variety of mutation types occur (Ottenhoff et al. 2002). The first mutations were identified in Maltese and Tunisian patients (Jouanguy et al. 1996; Newport et al. 1996), where genome scanning for regions of homozygosity in Maltese individuals pinpointed a premature stop codon in *IFNGR1* (Newport et al. 1996). Functional studies confirmed the genetic observations (Newport et al. 1996; Altare et al. 1998b). Later studies identified a deletion hotspot in *IFNGR1* downstream from the mutations first identified (Jouanguy et al. 1999b). These studies were rapidly followed by the identification in other patients of IL-12R deficiency, as a result of premature stop codons in the gene for the β1 subunit of the IL-12R (*IL12RB1*) (Altare et al. 1998a; de Jong et al. 1998). A recent report of complete IL-12Rβ1 deficiency in 41 patients from 17 countries reported a fair prognosis and discussed the redundancy of IL-12 in immunity (Fieschi et al. 2003). To date, mutations have been identified in three further genes, encoding IL-12 p40 (*IL12B*), IFN-γR2 (*IFNGR2*), and STAT1. The p40 subunit of IL-12 principally interacts with the β1 subunit of the IL-12R. Other cytokines and receptors may also have a phenotypic role, such as IL-23, which shares the p40 subunit with IL-12, and IL-23R, which shares IL-12Rβ1 with IL-12R (Ottenhoff et al. 2002). Overlapping cytokine and receptor specificities may account for the appearance of mutations in only a proportion of subunits.

Phenotypic severity in such patients correlates with the degree of impairment of IFN-γ pathways. Complete deficiency of IFN-γR1 and IFN-γR2 are the most severe (Ottenhoff et al. 1998). However, disease phenotype heterogeneity is consistent with involvement of other genetic or environmental factors, in addition to primary mutations (Picard et al. 2002). Indeed such rare deficiencies may be regarded as being at one end of a continuous spectrum of disease severity, with common variation contributing to responses to more pathogenic organisms at the other end of the spectrum. Unlike

variation in proinflammatory cytokine genes, and type 2 cytokine and receptor genes, there is little evidence that common variation in type 1 cytokine pathways plays a significant role in noninfectious disease susceptibility.

Studies of common variation in other candidate genes for the less common mycobacterial pathogens, such as *NRAMP1* in *Mycobacterium avium-intracellulare* infections, sometimes associated with HIV, and *Mycobacterium ulcerans* infections, not known to be HIV associated, have not shown allelic associations (Huang et al. 1998; Stienstra et al. 2001).

Leprosy

The clearly defined spectrum of clinical disease in leprosy, from lepromatous to tuberculoid forms, and the slow progression of disease, has long attracted the interest of geneticists. There is also evidence that the incidence of leprosy infection is much higher than the incidence of clinical leprosy, with 95 percent of those infected proposed to have persistent or self-curing subclinical infection (Fine 1983). A genetic predisposition to leprosy has been speculated upon for more than 50 years (Fine 1981; Schurr et al. 1991), with observations of racial differences and familial clustering, although it has been difficult to differentiate between genetic effects and shared environment. Further support for the role of genetics was obtained using twin studies, where concordance in monozygotic twins is consistently higher than in dizygotic twins (Schurr et al. 1991). Studies implicate genetic predisposition to position on the leprosy spectrum as well as to the disease itself. More formal proof has been obtained from family studies, using a variety of methods including complex segregation analyses, carried out on a number of American and Asian populations (Haile et al. 1985; Shields et al. 1987; Abel and Demenais 1988; Wagener et al. 1988; Abel et al. 1992b; Feitosa et al. 1995; Shaw et al. 2001). All studies have demonstrated the importance of host genetics, but, whereas some results suggest major gene control of susceptibility to leprosy itself, there has been no consensus as to the precise mode of inheritance, or to genetic control of severity. Such mixed results may be accounted for in part by limitations of the methods employed, partly by population heterogeneity and partly by strong environmental confounding factors (Feitosa et al. 1995), e.g. segregation analyses rely on liability classes, which should be derived from accurate epidemiological information. Early papers tested only single gene and multifactorial models, whereas a later study used the program COMDS which allowed testing of an oligogenic model (major gene plus additional genetic effects, Shaw et al. 2001). In this study, data from 76 Brazilian multicase leprosy pedigrees supported a two-locus model as the best fit: a recessive major gene plus a recessive modifier gene.

There has been a single recent human genome scan for susceptibility to leprosy (Siddiqui et al. 2001). This study was carried out using 245 affected sib-pairs from an Indian population where leprosy was predominantly tuberculoid. Significant linkage was obtained for a single chromosomal region, 10p13. Surprisingly, there was no linkage to HLA. Further evaluation with 140 additional families, for a region that had shown weak evidence of linkage in the genome scan, identified linkage to a second region at 20p12 in families from Tamil Nadu, but not Andhra Pradesh (Tosh et al. 2002). Transmission disequilibrium testing was employed for fine mapping of the chromosome 20 region, and showed that a marker, D20S835, was associated with protection against leprosy, and presumably lies close to the susceptibility locus. Neither 10p13 nor 20p12 contains obvious candidate genes. These results remain to be investigated in other populations.

The earliest linkage studies of HLA and leprosy involved the observation of nonrandom segregation of parental HLA haplotypes (de Vries et al. 1976; Fine et al. 1979), whereas later studies have also employed parametric linkage approaches (Haile et al. 1985; Dessoukey and El-Shiemy 1996; Shaw et al. 2001), in some instances using information on mode of inheritance derived from the same population. Linkage to the HLA region has been observed in Surinamese, Indian, Egyptian, and Brazilian families. Maximum LOD scores are found at a recombination fraction of 0, directly implicating the MHC (Shaw et al. 2001). Evidence for HLA control has often been found using tuberculoid siblings, but, as this is often the most common type of leprosy, further conclusions on the role of HLA are difficult. A 1976 study reported an increase in haplotype sharing between siblings with similar leprosy type, whereas siblings with different types of leprosy shared haplotypes less often than expected (de Vries et al. 1976). This led to the suggestion that two HLA genes were involved, determining susceptibility to leprosy itself and severity of leprosy, respectively, an idea that remains to be confirmed or refuted.

Some of the many HLA association studies have been reviewed (Todd et al. 1990; Meyer et al. 1998). Results have been variable, and such inconsistencies may reflect the lack of power of studies, variable selection of controls, and statistical false positives, in addition to population heterogeneity. HLA class I associations have not been highly reproducible, and perhaps the only study of note is an *HLA-A11* association which has been reported with erythema nodosum leprosum, seen in some multibacillary patients (Agrewala et al. 1989). The clearest associations, found in several ethnic groups, are for MHC class II: *HLA-DR2* is often associated with both lepromatous and tuberculoid leprosy, and *HLA-DR3* is associated with tuberculoid leprosy. *HLA-DQw1* may also be a susceptibility allele (Jawinska and Serjeantson 1988; Todd et al. 1990). Further resolution

of susceptibility and resistance class II haplotypes has been achieved in later studies, and some HLA influence, at least on disease severity, seems certain.

A number of studies have also been performed on HLA control of in vitro immune responsiveness, usually using crude mycobacterial antigens. However, HLA-restricted mycobacterial epitopes might be expected to elicit protective or suppressive immune responses. Although little is known about the nature of such epitopes (Meyer et al. 1998), potential leprogenic motifs are suggested from the observation that arginine residues in the peptide-binding groove of *HLA-DRB1* at codons 13 or 70–71 in pocket 4 conferred a relative risk for tuberculoid leprosy of 8.8 in Indian leprosy patients compared with healthy controls (Zerva et al. 1996). Eleven *HLA-DRB1* motifs positively associated with leprosy in a Nigerian population tended to have basic residues at codons 13, 70, 71, 74, and 86, resulting in a net positive charge, whereas five negatively associated motifs were characterized by acidic residues and a net negative charge (Uko et al. 1999).

It is still possible that observed associations are merely a reflection of linkage disequilibrium occurring within the HLA region, such as with the *TAP2* locus within the class II region showing allelic association with tuberculoid leprosy (Rajalingam et al. 1997). However, studies on *TNF* in the class III region suggest that there may be multiple susceptibility genes in the HLA region. In a study of Indian leprosy patients TNF_{-308}*2 was found at increased frequency in lepromatous but not tuberculoid patients (Roy et al. 1997), an allele commonly thought to be associated with high TNF-α production. In contrast, use of the transmission disequilibrium test for Brazilian leprosy families revealed an association of TNF_{-308}*1 for all types of leprosy (Shaw et al. 2001). Two-locus transmission disequilibrium testing for the class III region identified *TNF*1/LTA*2* as a susceptibility haplotype, *TNF*2/LTA*2* as a resistance haplotype, and *TNF*1/LTA*1* as not associated with leprosy. This confirms that the TNF_{-308} polymorphism is not the whole story. The complex influence of such haplotypes is in accordance with studies on other infectious diseases and this region. *TNF* is also an excellent candidate gene for nerve damage and leprosy reactions, commonly associated with high levels of circulating TNF-α, although no convincing associations have been shown to date (Sarno et al. 2000). There have been no association studies for the other proinflammatory cytokine IL-1. A recent study of Toll-like receptor 2 suggests a promising avenue for exploration (Kang and Chae 2001).

NRAMP1 might be expected to play a role in human susceptibility to leprosy, because susceptibility to *M. lepraemurium* is determined by a single amino acid change in *Nramp1* in inbred mouse strains. Analysis of *NRAMP1* haplotype sharing in predominantly Vietnamese leprosy families, containing both lepromatous and tuberculoid patients, showed significantly nonrandom sharing (Abel et al. 1998), conflicting with two earlier small studies that failed to find evidence of linkage (Shaw et al. 1993; Levee et al. 1994). However, parametric LOD score analysis, on an extended haplotype, showed only very weak evidence for linkage, and no specific allelic associations were identified. A Malian association study found that, for one *NRAMP1* polymorphism, in the 3′UTR, heterozygotes were more common in lepromatous than tuberculoid patients (Meisner et al. 2001). This finding was not reproduced in Indian patients (Roy et al. 1999). No associations were found for leprosy as such. From limited information, it does not appear that *NRAMP1* is a major player in human susceptibility to *M. leprae*. An investigation of skin test responsiveness to lepromin, using the predominantly Vietnamese families, showed linkage of positive Mitsuda reaction to *NRAMP1* (Alcais et al. 2000), building on the observation of familial aggregation of positive Mitsuda reaction in Brazilians (Feitosa et al. 1996). Although linkage was independent of leprosy status, the suggestion that a Mitsuda reaction can be used as a predictor of susceptibility to lepromatous disease makes precise interpretation of these findings difficult.

The *VDR* codon 352 polymorphism has also been examined in Indian leprosy patients (Roy et al. 1999). The homozygous genotype previously associated with low bone mineral density, enhanced clearance of hepatitis B and resistance to pulmonary tuberculosis (TB) was more common in tuberculoid patients than in controls. In contrast, homozygosity for the opposing allele was more common in lepromatous patients than in controls.

Tuberculosis

Studies of genetic susceptibility to *M. tuberculosis* are somewhat fewer than those for *M. leprae*. This may be the result of a less marked contribution of HLA, problems of ensuring reliable diagnosis, and a less polarized spectrum of disease. Almost all genetic studies concern pulmonary TB, and other disease manifestations have been sadly neglected. Twin studies do suggest genetic control of pulmonary TB, with concordance in monozygotic twins being substantially higher than in dizygotic twins (Comstock 1978; Fine 1981; Bellamy and Hill 1998). However, estimates of heritability vary widely. There is a single formal study of genetic control of susceptibility to TB, where complex segregation analysis pointed to oligogenic control of TB in a Brazilian population (Shaw et al. 1997). The lack of more formal proof is surprising in view of efforts expended in gene identification.

The majority of gene studies have employed a candidate gene approach, with HLA, including *TNF*, the *IL1*

gene cluster, *NRAMP1*, *MBL*, and *VDR*, all receiving attention. In particular, investigation of *NRAMP1* was triggered by the observations of single gene control of mycobacterial species made in mice more than 20 years ago. Recently, a genome scan in mice has suggested further regions (Lavebratt et al. 1999). However, although murine genome scan results on mycobacterial and leishmanial species are correlated, there is no obvious correlation with patterns seen in humans. A human genome scan using Gambian and South African sib-pairs, and a derivation of homozygosity mapping, dependent on common ancestry, showed suggestive evidence for linkage of TB to chromosomes 15q and Xq (Bellamy et al. 2000). Fine mapping of the 15q11–13 region implicated a gene *UBE3A* coding for ubiquitin protein ligase E3A, or a close flanking gene (Cervino et al. 2002). It remains to be seen whether regions identified from genome scans prove to be more important than previously studied candidate genes, or whether such regions turn out to be study-specific.

As for many infectious diseases HLA has been a candidate region for control of susceptibility. In the case of TB this has been supported by murine studies. However, fewer human studies have been conducted than for leprosy. Most studies have been carried out in association with pulmonary TB and, although results are not consistent between small population groups and there are potential problems of population stratification, a number of studies have reported an association with *HLA-DR2* (later *HLA-DRB1*1501*, reviewed in Meyer et al. 1998). Studies in Indian populations have genotyped additional loci within the class II region (*HLA-DQA1*, *-DQB1*, and *-DPB1*), in an effort to determine susceptibility haplotypes (Mehra et al. 1995; Ravikumar et al. 1999). Consideration of *TAP* genes, also within the class II region, points to a complex picture of haplotypes contributing to disease susceptibility (Rajalingam et al. 1997). TB is a common opportunistic infection in immunosuppressed patients and it is likely that HLA associations in nonimmunosuppressed and immunosuppressed cases will differ. Whereas *HLA-DQA1*0101*, *HLA-DQB1*0501*, and *HLA-DRB1*1501* were risk factors for TB in nonimmunosuppressed Mexican patients, *HLA-DRB1*1101* appeared to influence disease in HIV-infected patients (Teran-Escandon et al. 1999). Unlike several other infectious diseases, including leprosy, limited attempts to find linkage or association to *TNF* in the class III region have often proved negative (Shaw et al. 1997; Goldfeld et al. 1998; Selvaraj et al. 2001). As linkage disequilibrium between class II and III regions is rarely considered, and rarely formally tested, the relative contributions of distinct loci are sometimes hard to unravel. At a minimum, *TNF* is not a major contributor to disease susceptibility.

A few studies have tested the effect of HLA on immune response phenotypes. Most, but not all, concern cellular responsiveness (Bothamley et al. 1989; Jepson et al. 1997b). Selection of active, cured, or relapsed patients may account for some of the discrepancies in the literature. In a small study of Mexican–Americans, where *HLA-DR3* was at a reduced frequency in TB patients, there was an indication of an HLA effect in patients for in vitro proliferation to purified protein derivative (PPD) (*HLA-A9-B40* in strong responders and *HLA-B14-DR1* in weak responders), but not skin test responsiveness (Cox et al. 1988). These findings contrasted with several earlier studies. In a much later study, patients carrying *HLA-DR2* showed an increased proliferative response to *M. tuberculosis* culture filtrate antigens to patients who were *HLA-DR2* negative, whereas low responsiveness to antigen and mitogens was seen in *HLA-DR3*-positive compared with negative healthy individuals (Uma et al. 1999). However, a twin study in The Gambia suggested that non-HLA genes were more important than HLA in determining immune responses to PPD (Jepson et al. 1997b).

Studies in Gambian TB patients showed weak association with the *IL1* gene cluster, *IL1RN*2* of the 86-bp repeat in intron 2 being less common in Gambian TB patients than in controls (Bellamy et al. 1998a). A study on Gujarati TB patients in the UK also implicated the *IL1* gene cluster; however, evidence remains weak (Wilkinson et al. 1999). IL-1Ra and IL-1β induced by *M. tuberculosis* varied according to *IL1RN* and *IL1B* genotype. Although there was no association between genotype and disease, haplotypic associations were seen with tuberculous pleurisy and Mantoux responsiveness.

Polymorphisms in *NRAMP1* were originally identified with a view to ascertaining the role of this macrophage function gene in susceptibility to TB (Liu et al. 1995; Searle and Blackwell 1998). Linkage to the 2q35 region was weak (Shaw et al. 1997), but some association studies have been positive in African, Asian, and later Caucasian populations (Bellamy et al. 1998c; Cervino et al. 2000; Gao et al. 2000; Ryu et al. 2000; Ma et al. 2002). Polymorphic sites, the extent of linkage disequilibrium between these sites, and the degree of association with disease appear to vary between populations. As for HLA, associations may be influenced by fine differences in disease status such as HIV co-infection (Ma et al. 2002) and disease severity (Soborg et al. 2002). A 5′ repeat polymorphism has received attention as a result of its proposed functionality (Gao et al. 2000; Awomoyi et al. 2002), with allele 2 associated with TB and LPS-induced IL-10 production by macrophages (Awomoyi et al. 2002). Monocytes from TB patients also showed high IL-10 production, suggesting that the influence of *NRAMP1* on susceptibility to TB is, at least in part, mediated by IL-10. Roles for TNF-α and IL-1β, regulated by *NRAMP1*, were not shown in this instance. One of the most interesting recent papers shows linkage of TB to 2q35, but not to the MHC, in an extended Aboriginal Canadian family (Greenwood et al. 2000). This was unusual because linkage was conducted using a

parametric approach with an autosomal dominant mode of inheritance. Individuals were assigned to liability classes, including information on tuberculin skin test responsiveness, yielding a LOD score of 3.81 with a close marker, D2S424. This emphasized both the power of parametric approaches and the need to be mindful of confounding factors such as immunization status. Despite the identification of *NRAMP1* as a candidate gene through murine studies, and apparent major gene control in this family, differences in the role of *NRAMP1* in TB between mice and humans are evident. Studies of *MBL* have also been mixed with little association seen with TB in Gambians (Bellamy et al. 1998b), but stronger evidence for a role in Indians, independent of association of pulmonary TB with *HLA-DR2* (Selvaraj et al. 1999).

A *VDR* codon 352 genotype, associated with osteoporosis and high levels of circulating 1,25-dihydroxyvitamin D_3 (1,25-D_3), which has widespread effects on cellular immunity, showed a slightly reduced frequency in TB patients (Bellamy et al. 1999). This supports an old observation that vitamin D may be beneficial in treatment of TB, and in vitro observations that 1,25-D_3 enhance the ability of monocytes to restrict growth of *M. tuberculosis* (Bellamy and Hill 1998). More recently three *VDR* polymorphisms were studied in UK Gujaratis, in whom phenotypic vitamin D deficiency was associated with active TB. Although there was no clear association between *VDR* genotype and TB, combination of particular genotypes and vitamin D status is consistent with a contributory role of host genotype (Wilkinson et al. 2000).

Exposure to TB has been suggested to reduce the risk of developing asthma (von Mutius et al. 2000). It is highly likely that such effects are in part influenced by human genotype, particularly through cytokine genes. However, studies are limited on the role of genes such as those in the chromosome 5 cluster and susceptibility to TB. The largely non-MHC control for cellular and humoral responses to PPD also remains to be elucidated (Jepson et al. 1997b), although candidate genes already implicated in susceptibility to TB are implicated in control of lymphocyte responsiveness and tuberculin reactivity (Selvaraj et al. 2000). Overall, TB is a well-studied disease; however, candidate gene associations to date have been weak and inconsistent, with substantial differences between populations (reviewed by Delgado et al. 2002), leaving much scope for identification of further genetic effects.

Salmonellosis

Infection with *Salmonella* spp. is interesting for geneticists on a number of counts. As cited above, rare deleterious mutations in genes such as *IL12B* and *IL12RB1* result in failure to produce IFN-γ, hence the failure in granuloma formation and susceptibility to non-typhi *Salmonella* spp. (Jouanguy et al. 1999a; Lammas et al. 2002). The tiny number of studies in human populations doubtless reflects the difficulties of collecting good patient groups for an acute disease. In contrast, work on mice and chickens spans several decades. Murine infection with *Salmonella typhimurium* is controlled by *Ity* (*NRAMP1*) and *Lps*, involved in innate immunity and macrophage function, and *xid* which modulates humoral immune responses by preventing the production of antibodies against polysaccharides. *Xid* is homologous with the human gene *XLA*, which causes X-linked agammaglobulinemia, and results from mutations in Bruton's tyrosine kinase (Malo and Skamene 1994). A recent genome scan for clearance of *S. enteritidis* in mice identified three regions; one was likely to be *Nramp1* and two were previously unknown (Caron et al. 2002). However, no common variants of human *NRAMP1* were associated with susceptibility to typhoid fever in Vietnamese patients (Dunstan et al. 2001a). In the same Vietnamese population, HLA class II and *TNF* associations were identified (Dunstan et al. 2001b): haplotypes that were protective (*TNF_{-308}*1-DRB1*04*) or predisposed individuals to typhoid fever (*$TNF-_{-308}$*2-DRB1*0301*) were determined.

The second most common single gene disorder prevalent in western Caucasian populations is cystic fibrosis. Homozygosity for mutant alleles has had a massive impact on reproductive fitness. The gene determining cystic fibrosis, *CFTR*, the cystic fibrosis transmembrane conductance regulator, codes for a transmembrane Cl^- channel. About 1 in 23 people are heterozygous carriers for a mutant allele, about 70 percent of which are the ΔF508 mutation, resulting in loss of a single phenylalanine and no protein expression at the cell surface. It has long been supposed that there must be heterozygote advantage in order to maintain mutant alleles at such high frequency. One suggestion is that such an advantage might be an increased resistance of heterozygotes to fluid loss caused by response to toxins from enteric pathogens such as *Vibrio cholerae*. Alternatively it has been proposed that *S. typhi*, but not *S. typhimurium*, uses the *CFTR* to enter intestinal epithelial cells, with ΔF508 cells being partially refractory, potentially decreasing the susceptibility of heterozygous carriers to typhoid fever (Pier et al. 1998). At present there is insufficient evidence for firm conclusions (Guggino 1999).

Immunogenetic studies concerning a number of other human bacterial pathogens appear in the literature. Many are in the context of mouse model systems, where variation in disease outcome between inbred strains, associated with variation in genetic background, is suggestive of genetic control. Use of transgenic and knockout mice has given clues as to immunological mechanisms and potential candidate genes. In some instances mouse susceptibility loci have been mapped, but potentially difficult studies in humans have not

followed, e.g. the *Lgn1* gene on mouse chromosome 13 controls susceptibility to *Legionella pneumophila* (Beckers et al. 1997; Diez et al. 2003) and for *Rickettsia tsutsugamushi* a single murine gene has been mapped (Malo and Skamene 1994). In some instances there are HLA association studies, although these are looking for an effect of HLA on immune environment rather than specific phenotypes. *Pseudomonas aeruginosa* susceptibility genes are *Pscr1* and *Pscr2* in mice (listed in McLeod et al. 1995); however, in humans, the effect of HLA on pulmonary phenotype as the site of infection for *P. aeruginosa* in cystic fibrosis patients has been examined (Aron et al. 1999). Genetic studies in humans are found mainly for pathogens associated with particular pathologies of interest. These pathologies may be associated with more than a single pathogen. In some cases the bacteria are intracellular and consequent inflammatory responses are involved.

Meningococcal meningitis

The best-known infection associated with deficiency of the terminal complement components C6–C9 is *Neisseria meningitides*. Although such deficiencies are not necessarily polymorphic, but strictly speaking are rare variants, and studies are usually on individual families, they are still relatively common. Deficiencies of particular components may be the result of a variety of mutations and certain deficiencies are prevalent in certain communities (O'Hara et al. 1998). The genes *C6*, *C7*, and *C9* form a gene cluster on chromosome 5 (Alvarez et al. 1995), and linkage disequilibrium occurs between common variants and the deficiencies (Wurzner et al. 1992).

There have also been multiple studies on the role of FcγR genes in susceptibility to infection and severity of meningococcal meningitis. FcγR polymorphic forms are associated with different functional activities (van der Pol et al. 2001). The FcγRIIA (CD32) R/R131 allotype, which binds IgG2 poorly, was more common in children who had survived fulminant meningococcal septic shock than healthy controls (Bredius et al. 1994). In some cases allotypes have been reported to be similar in patients versus controls; however, the R/R131 allotype is at an increased frequency in the more severe forms of disease such as fulminant meningococcal disease and meningococcemia without meningitis (Domingo et al. 2002). R/R131 homozygous polymorphonuclear leukocytes (PMN) have reduced ability to internalize IgG2 opsonized meningococci (Fijen et al. 2000). Some of the studies have considered extended haplotypes in the FcγR gene cluster on chromosome 1.

Further genetic observations concern molecules involved in inflammation. Homozygosity for a deletion in the gene coding for angiotensin-converting enzyme (ACE), associated with higher tissue ACE, is correlated with increased disease severity (Harding et al. 2002). However, findings on *TNF* and *IL1* cluster genes have been variable. Although some resistance to a fatal outcome was found for $IL1B_{-511}$ heterozygotes, the same survey found no effect of variation in *TNF* (Read et al. 2000). In other studies, the frequency of *IL1RN*A2*, probably in linkage disequilibrium with the *IL1B* locus, and associated with increased IL-1Ra, was the same in cases and controls (Carrol et al. 2002), and the TNF_{-308} polymorphism influenced mortality as a result of meningococcal meningitis (Nadel et al. 1996). Studying first-degree relatives of meningococcal disease patients, families with low TNF-α and high IL-10 production were associated with increased risk of fatal outcome. Whereas TNF-α and IL-10 production were shown to be highly heritable, there was no difference in TNF_{-308} and TNF_{-238} genotype in relatives of survivors versus nonsurvivors (Westendorp et al. 1997).

Helicobacter pylori and gastric cancer

A twin study for infection with *H. pylori* demonstrated a heritability of 57 percent (Malatay et al. 1994). However, the greatest interest in *H. pylori* is a result of infection being a risk factor for gastric cancer. The extent of gastritis in response to *H. pylori* influences risk of cancer, with hypochlorhydria being a precursor. Gastric cancer and duodenal ulceration are mutually exclusive outcomes. Although *HLA-DQB1*0602* is increased and *HLA-DQB1*0301* decreased with different gastric cancer-associated phenotypes in Taiwanese (Wu et al. 2002a), and *HLA-DQA1*0102* associated with resistance in Japanese (Azuma et al. 1998), more complete study of the HLA class II region suggests that the limited associations observed to date are not strong enough to stand correction for multiple comparisons (Yoshitake et al. 1999). A study of three polymorphisms within *IL1B* and the $IL1RNV_{NTR}$ polymorphism demonstrated that haplotypes putatively associated with upregulation of IL-1β had an increased frequency in cases of hypochlorhydria induced by *H. pylori* and of gastric cancer (El-Omar et al. 2000). Most recently, a genome scan of Senegalese siblings used the quantitative trait of specific IgG for *H. pylori*. Linkage analysis followed by transmission disequilibrium testing pinpointed a single gene: *IFNGR1* (Thye et al. 2003). Three variants at positions −56, 318, and 450 were associated with high antibody concentrations, and were more common in Africans than Europeans, perhaps explaining the relatively low pathogenicity in Africans.

Spondyloarthropathies

Both genetic predisposition and a number of bacterial pathogens are implicated in spondyloarthropathies; in particular bacteria are triggers for reactive arthritis,

e.g. *Salmonella enteritidis* (see above), *Listeria monocytogenes*, *Yersinia enterocolitica*, *Chlamydia trachomatis*, and *Borrelia burgdorferi* (Lyme disease). A well-known HLA association is that of *HLA-B27* with ankylosing spondylitis. In fact the *HLA-B27* association is with a broad range of spondyloarthropathies, for both development and chronicity of disease. The mechanism of predisposition is unclear but could involve use of HLA-B27 as an antigen-presenting molecule or, alternatively, class II-mediated presentation of B27-derived peptides (Lopez de Castro 1998). Undoubtedly, other molecules involved in immune responsiveness for pathogens will prove to be involved (Pacheco-Tena et al. 2002). For some of the pathogens implicated in reactive arthritis, genetic studies are largely confined to mouse models, e.g. *L. monocytogenes* with a single gene identified, *Lsr1* (Malo and Skamene 1994). For others there are a handful of human studies. For *C. trachomatis* infection, which stimulates an inflammatory response, often resulting in tissue scarring, the less common alleles TNF_{-308}*2 and TNF_{-238}*2 are associated with scarring trachoma (Conway et al. 1997). TNF-α was detected more frequently in tear fluid in patients than in controls, in line with indications that the less common alleles determine higher levels of TNF-α. This is independent of an *HLA-A*6802* association with scarring trachoma. *IL4*, *IL10*, and *MBL* are not strongly implicated (Mozzato-Chamay et al. 2000). In humans, *C. trachomatis* is also known to be a cause of pelvic inflammatory disease and infertility, but there have been no genetic studies to date. In mice, susceptibility to chlamydial salpingitis and subsequent infertility is under genetic control, with both H-2 and non-H-2 genes involved (Tuffrey et al. 1992).

Periodontitis

Oral gingivitis and the more severe periodontitis are the result of mixed microbial infections. Colonizing nonpathogenic bacteria generate the conditions required for secondary species, which tend to be gram-negative anaerobes. The outcome depends both on the species of bacteria and on the human inflammatory response. Hence the multitude of genetic studies often concern the inflammatory responses and consequent pathology, not necessarily susceptibility to bacterial species as such. Bacteria associated with gingivitis include *Streptococcus*, *Actinomyces*, *Haemophilus*, and *Capnocytophaga* spp. whereas tissue-damaging species associated with chronic periodontitis include *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Bacteroides forsythus*, and with aggressive periodontitis *Actinobacillus actinomycetemcomitans*. There are case–control studies for candidate genes. In many instances the studies are small and cross-sectional, and use few polymorphic sites. One problem of drawing together findings is the range of phenotypes and severity criteria used for analysis. Bacterial species are not usually identified. Candidate genes have included *IL2*, *IL4*, *IL10*, *TGFB1*, *TNF*, *LTA*, *ACE*, and *VDR*. Although many polymorphisms show no association, some are weakly associated, and further analysis suggests both gene–gene and gene–environment interactions (Holla et al. 2001), e.g. the gene for plasminogen activator-inhibitor I is a risk factor for periodontitis, particularly in nonsmokers (Holla et al. 2002). Perhaps the most surprising result is a consistent failure to show an effect of variation in *TNF* on susceptibility or severity, even when several polymorphisms are employed (Craandijk et al. 2002).

However, the vast majority of studies concern the *IL1* and *FCGR* gene clusters. A number of investigations have highlighted association between allele 2 of $IL1A_{+4845}$ and $IL1B_{+3954}$ and disease, which may relate to the role of IL-1 as a stimulator of bone resorption. Based on the two *IL1* associations, a predictive test has been developed. However, the usefulness of this test has been questioned (Greenstein and Hart 2002), and population differences in allele frequencies may render the test inappropriate outside western Europeans (Armitage et al. 2000). In the context of IL-1, smoking is an important risk factor, although the nature of this interaction is unclear (Lang et al. 2000; Parkhill et al. 2000; Cullinan et al. 2001; Laine et al. 2001; Meisel et al. 2002). Only a few studies have attempted to correlate *IL1* genotype with protein production in vivo or in vitro (Mark et al. 2000; Shirodaria et al. 2000). Similarly, only a few studies have sought to identify the bacterial species present (Laine et al. 2001; Cullinan et al. 2001), although *IL1* genotype may well influence bacterial flora (Socransky et al. 2000).

The genes *FCGR2A*, *FCGR3A*, and *FCGR3B* have also been widely studied. A *FCGR3B* allele has been reported to influence disease in diverse population groups: elevated in African–Americans with aggressive periodontitis (Fu et al. 2002) and, together with an *FCGR3A* allele, associated with severity of chronic periodontitis in Japanese (Kobayashi et al. 2001). *FCGR3A* is in linkage disequilibrium with *FCGR3B*. In general, allotypes with low activity have been shown to be risk factors for disease. Cells carrying the periodontitis-associated *FCGR3B* allele are less efficient at both phagocytosing and inducing an oxidative burst, in response to IgG1- and IgG3-opsonized *P. gingivalis* (Kobayashi et al. 2000). However, in a group of Caucasian patients there was no significant difference in allele frequencies between mild and severe cases of periodontitis, although the severity of bone loss was correlated with the *FCGR3A* genotype coding for a high affinity receptor (Meisel et al. 2001).

FUNGAL INFECTIONS

There have been surprisingly few studies of genetic susceptibility to fungal infections, because there are

many parallels with nonfungal infections. In several respects they provide interesting features. In many cases exposure is widespread with only a few individuals contracting clinical disease. In some instances the fungi are opportunistic infections on backgrounds as diverse as the single gene disorders, such as cystic fibrosis, to acquired immunodeficiency. Immune responses may involve macrophages (*Pneumocystis carinii* – Ezekowitz et al. 1991), *Candida albicans* – Ashman and Papadimitriou 1995), allergic immediate hypersensitivity (aspergillosis – Chauhan et al. 2000), and granuloma formation (blastomycosis, coccidioidomycosis, and histoplasmosis), granulomas themselves being a potential source of pathology. These often chronic infections are likely to be influenced by MHC genes and other genes associated with macrophage function and allergic responses (Taylor et al. 1997; Chauhan et al. 2000).

PROTOZOAL INFECTIONS

Leishmaniasis

There are a number of species of Old and New World *Leishmania*, which are transmitted by sandflies and cause visceral leishmaniasis (VL) or cutaneous leishmaniasis (CL). All are obligate intracellular macrophage pathogens. There is also intraspecific pathogen diversity, although, in a single study, *L. aethiopica* variation was not associated with clinical phenotype in humans (Schonian et al. 2000). However, the *L. infantum* zymodeme MON-1 has been associated with both VL and CL, whereas MON-24 is a common agent of CL (Belhadj et al. 2003). As for mycobacterial disease, infection can be subclinical. In some individuals with the visceral parasite *L. donovani*, post kala-azar dermal leishmaniasis (PKDL) may occur after cure of the initial disease. Similarly, in a proportion of individuals, localized cutaneous lesions are followed by severe and disfiguring mucocutaneous lesions (MCL), sometimes many years after the initial lesion. MCL is economically the most important form of CL, requiring prolonged and expensive antimonial therapy. Particular zymodemes of *L. braziliensis* may have long evolution times and high frequency of involvement in mucosal disease (Saravia et al. 1998). Other species can cause diffuse CL in rare instances. In most instances, cure is dependent on macrophage activation by IFN-γ and production of IL-4, IL-5, and IL-10 is detrimental, although roles vary according to infecting species. The proinflammatory cytokines have protective roles, with NO as a mediator of parasite killing. MCL is characterized by a hyperreactive cellular immune responses with high production of both IFN-γ and IL-4/IL-5. Rare diffuse CL patients have poor cellular immunity and produce ineffectual antibodies in a situation analogous to lepromatous leprosy. The use of *Leishmania* spp. in murine models of immune responsiveness has provided a plethora of candidate genes for study, although there is no good mouse model for MCL. However, the paucity of studies in humans is noticeable.

It is clear from murine studies that different genes may control susceptibility to different *Leishmania* spp. In some murine strains, susceptibility to the visceral parasite *L. donovani* is under single gene control, with susceptibility being recessive, caused by *Nramp1* (see earlier). *Nramp1* also affects visceralization and metastasis of the cutaneous parasite *L. mexicana* (Roberts et al. 1989), but is not involved in *L. major* infection. Findings for cutaneous parasites have been more diverse. A gene controlling chronic *L. major* lesion expansion has been mapped to a region homologous with the human 5q cluster (Roberts et al. 1993; Guler et al. 1996). As well as many candidate cytokine genes, this region is also rich in receptor genes such as that coding for the glucocorticoid receptor, which has been shown to play a role in response to *M. bovis* BCG infection in congenic mouse strains (Brown et al. 1993). Another gene, *Scl-1*, which controls healing of *L. major* lesions, has been mapped to a region of conserved synteny with human chromosome 17 (Mock et al. 1993; Roberts et al. 1993), encoding members of the cc intercrine family as inflammatory agents produced by macrophages and *NOS2A* encoding inducible NO synthase (iNOS). *Scl-2* controlling a sex-related no-lesion growth phenotype for *L. mexicana* is provisionally mapped to a region encoding IFN-α and IFN-β and Janus kinases (Roberts et al. 1990). In 1997, two murine genome scans for susceptibility to *L. major* found H-2 (chromosome 17) and a new region on mouse chromosome 9 (Roberts et al. 1997), and six regions on chromosomes 6, 7, 10, 11, 15, and 16, to be involved in susceptibility (Beebe et al. 1997). Analysis indicates some epistatic interactions between these murine loci (Roberts et al. 1999). Further studies using immunological phenotypes have identified more loci (Lipoldova et al. 2000). Many of these murine loci are unconfirmed and nomenclature is becoming a problem.

Observations on human populations have shown that the proportion of subclinical infections, the proportion of localized CL cases progressing to MCL, and the severity of MCL, vary markedly between racial groups and families (Walton and Valverde 1979). This is likely to reflect genetic variation between populations of both host and parasite. Segregation analysis and initial examination of candidate immune response genes support the role of host genetics in control of human CL. Segregation analysis of more than 2000 individuals, from a total cross-sectional survey of LCL caused by *L. peruviana* in six Andean valleys, supported a two-locus model with a major gene plus a 'modifier' locus (Shaw et al. 1995). A later study of LCL caused by *L. braziliensis* in Bolivia concluded that, in some families, a recessive major gene controlled susceptibility to the primary cutaneous lesion,

but did not consider MCL (Alcais et al. 1997). High levels of serum TNF-α are found in MCL patients, and in a small Venezuelan case–control study, a high risk of mucocutaneous disease was observed in individuals carrying *TNF$_{-308}$*2* or *LTA*2*, particularly for females (Cabrera et al. 1995). The only other studies of American CL were three studies, which found variable associations with HLA (Barbier et al. 1987; Lara et al. 1991; Petzl-Erler et al. 1991). These associations may not be primary risk factors for disease but reflect strong linkage disequilibrium.

There have been very few studies of human genetics in VL. Segregation analysis has provided support for genetic control of susceptibility, although differentiation between genetic models was poor (Peacock et al. 2001). Confounding factors, such as nutritional status, are difficult to account for in genetic studies. HLA associations with VL are weak or negative (Blackwell 1998; Meddeb-Garnaoui et al. 2001; Peacock et al. 2002). Most notably, Faghiri et al. (1995) reported a 13-fold increase in risk for VL in Iranian *HLA-A26* carriers. There is an effect of variation in *TNF* in some populations (Meddeb-Garnaoui et al. 2001; Karplus et al. 2002), with haplotypes containing *TNF$_{-308}$*2 2* associated with symptomatic VL by transmission disequilibrium testing in a Brazilian population. Despite absence of a role for *NRAMP1* in a Brazilian population (Blackwell 1998), in a Sudanese population *NRAMP1* influences susceptibility to VL/PKDL (Blackwell et al. 2003).

Toxoplasmosis

The prevalence of *Toxoplasma gondii* infection is high compared with many other pathogens, with seroprevalence varying from 20 percent to over 80 percent. The pathogenesis of disease varies: acute infection causes transient flu-like symptoms, whereas reactivation can lead to serious eye disease in immunocompetent individuals and fatal toxoplasmic encephalitis (TE) in immunosuppressed individuals. Up to 50 percent of AIDS patients who are seropositive for *Toxoplasma* spp. eventually develop TE. Progressive ocular toxoplasmosis, leading to blindness, has a sporadic distribution in most populations; however, clusters of cases occur in South America. *T. gondii* is also a major cause of congenital disease, and twin studies of human congenital infections suggest a genetic component for transplacental transmission (McLeod et al. 1995). There is relatively little variation in *T. gondii*, with a clear distinction between a subset of strains (type 1), which are regarded as acute virulent, and remaining isolates (type 2 and 3) (Howe and Sibley 1995). The majority of clinical disease in humans is caused by type 2 isolates. Limited information is available on human genetic susceptibility to phenotypes caused by *T. gondii*; however, macrophage-mediated mechanisms are of key importance in disease limitation and prevention of reactivation. There are studies of murine toxoplasmosis of phenotypes including cyst numbers in the brain and resistance to, and survival of, encephalitis. Effects of MHC class I and II genes are well established (Brown et al. 1995; Johnson et al. 2002). Other genes implicated will have substantial overlap with loci implicated in control of murine leishmaniasis, with different genes operating in acute versus chronic disease.

Trypanosomiasis

There is very little known about genetic susceptibility to African or American trypanosomes in humans. African trypanosomiasis, caused by *Trypanosoma brucei* subspecies, causes fatal sleeping sickness in humans, and is also an important cause of disease in domestic livestock such as cattle. Resistant cattle breeds are known and genome scans are being performed. Control of susceptibility to *T. congolense* in mice was largely accounted for by genes on chromosomes 1, 5, and 17 (Kemp et al. 1997). Although *H-2* on chromosome 17 is implicated, responses to challenge with trypanosomes other than *T. congolense* suggest that the MHC is not involved in murine resistance. American trypanosomiasis (Chagas' disease) is caused by *T. cruzi*, with cardiomyopathy seen in approximately 30 percent of patients. *T. cruzi* seropositivity showed a heritability of 56 percent, with an additional 23 percent of variation caused by shared household environment, in a Brazilian population (Williams-Blangero et al. 1997a). Whereas an *HLA-DRB1*14-DQB1*0301* haplotype was correlated with protection against *T. cruzi* infection in an endemic area of Peru, no HLA associations were detected for disease severity (Nieto et al. 2000). Similarly, *HLA-A30* conferred susceptibility to Chagas' disease, irrespective of the clinical presentation in Brazilian patients (Deghaide et al. 1998). As for *Leishmania* and *Toxoplasma* spp., *NRAMP1* is a good candidate gene for resistance to *T. cruzi*; however, variation in *NRAMP1* has not been shown to influence *T. cruzi* infection or cardiomyopathy to date (Calzada et al. 2001).

Malaria

There has been a large amount of work on genetic susceptibility to malaria. Aside from the long history of research on the Duffy blood group, which determines resistance to *Plasmodium vivax*, the majority of studies concern *P. falciparum* infection. Genetic factors clearly play a role in infection, development of clinical disease, and prognosis. The single gene variation associated with the red cell hemoglobinopathies is not covered in this text, nor the red cell enzyme glucose-6-phosphate dehydrogenase, or the roles of the glycophorins or band 3. This work stimulated major interest in malaria as a selective force. Malaria remains the only human

infectious disease with strong evidence for selective effects, most commonly by selection of otherwise deleterious alleles through heterozygote advantage. Perhaps because of the long history of research into genetic susceptibility and malaria, there have been few formal segregation analyses. Three west African ethnic groups living under similar conditions showed differences in a number of malarial parameters including antibody response, suggestive of genetic involvement (Modiano et al. 1996). A twin study in The Gambia suggested that risk of disease rather than infection was under genetic control (Jepson et al. 1995). Three segregation analyses are all consistent with genetic control of *P. falciparum* parasitemia in west African populations (Abel et al. 1992a; Garcia et al. 1998a; Rihet et al. 1998a) and of incidence and clinical infection in a Sri Lankan mixed *P. falciparum* and *P. vivax* area (Mackinnon et al. 2000). There are also familial correlations for some *P. falciparum*-specific cellular and antibody responses (Stirnadel et al. 1999b; Aucan et al. 2001).

It has been known for a number of years that resistance to *P. chabaudi* in mice, primarily looking at levels of parasitemia and survival, is controlled by multiple genes (Malo and Skamene 1994). More recently genome-wide approaches have identified several quantitative trait loci, named '*Char*' for chabaudi resistance (reviewed in Fortin et al. 2002). *Char1* on mouse chromosome 9 and *Char2* on chromosome 8 (Foote et al. 1997) were described at the same time as the same chromosome 8 locus and a chromosome 5 locus by a second group (Fortin et al. 1997). A third H-2-linked locus on chromosome 17 controlled parasite clearance in females (*Char3* – Burt et al. 1999). Use of recombinant congenic strains, created from strains with susceptibility alleles at *Char1* and *Char2*, identified a fourth locus on chromosome 4 (*Char4* – Fortin et al. 2001a). The genes involved have not yet been identified.

Recent human studies have concentrated on candidate genes involved in immune responsiveness and particularly severe pathology. Surprisingly, there have been few linkage studies and no genome scans.

HLA-B5301, common in sub-Saharan African populations, has been associated with resistance to severe malaria (cerebral malaria and severe malarial anemia) in Gambians (Hill et al. 1991). This observation was followed by elution of peptides from B53 and screening of candidate *P. falciparum* epitopes. HLA-B53-restricted CTLs recognized a conserved nonamer from liver stage-specific antigen 1 (LSA-1) (Hill et al. 1992). Such observations have clear implications for vaccine design and, subsequent to identification of restricted epitopes in LSA-1 for $CD8^+$ cytotoxic T cells, IFN-γ production by CTLs in response to LSA-1 peptide was correlated with protection in a Papua New Guinea (PNG) population (Bucci et al. 2000). Furthermore, the *HLA-DRB1*1302-HLA-DQB1*0501* haplotype was associated with resistance to severe malarial anemia (Hill et al. 1991). This haplotype was more common than the anticipated haplotypic association of *HLA-DRB1*1302* with *HLA-DQB1*0604* seen in Caucasian populations. In Kenyans, however, different associations were seen, with a protective effect for *HLA-DRB1*0101*, but not *HLA-B5301* or *HLA-DRB1*1302*, suggesting significant heterogeneity (Hill 1998, 1999). In a study of variants of circumsporozoite protein CTL epitopes for *HLA-B*3501*, recognition differed significantly between individuals with and those without *B*3501*, with reciprocal antagonism between variants (Gilbert et al. 1998; Hill 1998). This is one of very few studies attempting to identify directly associations between parasite and host genetics reflecting co-evolution.

However, comparison of three ethnic groups in Burkina Faso suggests that susceptibility to malaria may be correlated with immunogenetic differences other than HLA-B53 or hemoglobin S (HbS) (Modiano et al. 2001). This appears particularly true for T-cell help and antibody phenotypes (Riley 1996). Jepson found linkage of mild malaria to HLA using Gambian dizygotic twins as affected sib-pairs (Jepson et al. 1997b), and also looked at twin effects for immune responses, including those to malarial antigens and PPD. Control appeared to be largely non-MHC for cellular and humoral responses (Jepson et al. 1997a). Observation of concordance of antibodies produced in response to Pf155/RESA in monozygotic and dizygotic twins has provided evidence of genetic control, but not within the HLA class II region (Sjoberg et al. 1992). In a PNG population there were no class I or II associations for immune responsiveness to the antigens RESA and MSA-2 (Stirnadel et al. 1999a), although IgG2 against MSP2 was partly explained by class II haplotype sharing (Stirnadel et al. 2000). A strong negative association was found between antibody responses to the synthetic malaria vaccine SPf66 and *DRB1*15* (Beck et al. 1995).

The observation published in 1994 by McGuire et al. that Gambian children homozygous for TNF_{-308}*2 have a seven fold relative risk for death or severe neurological sequelae from cerebral malaria was the first of many studies of this polymorphism. This association was independent of HLA class I and II effects; however, association was with the uncommon homozygous genotype. This was followed some years later by the observation that the less common allele of another *TNF* promoter polymorphism (−238) was associated with severe malarial anemia (McGuire et al. 1999). Strangely this allele was in positive linkage disequilibrium with the protective *HLA-B53*, although in negative linkage disequilibrium with the protective *HLA-DRB1*1302*. Logistic regression analysis indicated another independent genetic effect on susceptibility to cerebral malaria caused by a polymorphism determining the transcription factor OCT-1-binding site at position −376 (Knight et al. 1999). This allele was not in linkage disequilibrium with *HLA-B53* but was in negative linkage disequilibrium

with TNF_{-308}*2, and positive linkage disequilibrium with TNF_{-238}*2, in Gambians. A contrasting Kenyan dataset showed similar findings for *TNF−376* and *TNF−308*, but a decreased susceptibility to cerebral malaria associated with TNF_{-238}*2 (Knight et al. 1999). TNF_{-376}*2 occurs only in individuals carrying TNF_{-238}*2, hence this haplotype is associated with susceptibility to cerebral malaria in Gambians, but has no effect in Kenyans. A haplotypic analysis of five *TNF* promoter SNPs in patients with cerebral malaria from Myanmar showed an effect of *TNF*-promoter diversity but not at positions −308 or −238 (Ubalee et al. 2001). Preliminary analyses of *TNF*-promoter polymorphism in other African populations have not always indicated an obvious effect (Stirnadel et al. 1999b), and in other instances only relatively mild effects on reinfection patterns (Meyer et al. 2002) and antibody responsiveness (Migot-Nabais et al. 2000). The unraveling of the complex evolutionary history of the *TNF*-promoter region has undoubtedly some way to go.

Two studies on west Africans have shown some evidence for linkage of blood parasitemia to the chromosome 5q31–q33 cluster (Garcia et al. 1998b; Rihet et al. 1998b). Although this trait remains relatively unexplored, the difficulties of getting good quantitative phenotypic information and the limited number of loci selected for testing to date leave many possibilities for further investigation. Investigation of this region in relation to anti-malarial antibody responsiveness is perhaps more tractable (Luoni et al. 2001).

NO is important in malarial pathology. In Gambians the short allele of the *NOS2A* microsatellite was associated with fatal cerebral malaria (Burgner et al. 1998). In contrast, in a longitudinal Gabonese study, the *NOS2A-954* polymorphism protected against severe malaria in the heterozygous state, and stimulated cells from heterozygous carriers showed sevenfold enhancement of iNOS activity (Kun et al. 2001). Whereas an early study suggested an inverse relationship between disease severity and NO production in Tanzanian children, these polymorphisms were not associated with disease severity, *NOS2A* expression, or NO production (Levesque et al. 1999). However, a recent study identified a new polymorphism at position −1173 (Hobbs et al. 2002). The less common allele was significantly associated with protection from symptomatic malaria in Tanzanian children and severe malarial anemia in Kenyan children. The protective allele was associated with increased fasting urine and plasma NO metabolite concentrations, in support of a protective role for NO.

There are few studies on other genes relating to IFN-γ and NO production. Consistent with a potential role of IFN-γ in susceptibility to malaria (Gourley et al. 2002), a study of *IFNGR1*-promoter polymorphisms in Gambian children showed preliminary evidence of associations with cerebral malaria in Mandinkas (Koch et al. 2002). In addition, homozygotes for an *IL12B*-promoter polymorphism showed decreased production of iNOS as reflected in urinary NO, and homozygosity was associated with fatal cerebral malaria in Tanzanian, but not severe malaria in Kenyan, children (Morohan et al. 2002).

The Duffy blood group gene (*FY*) was one of the first loci mapped on human chromosome 1, and was one of the first identified blood group systems (reviewed in Hadley and Peiper 1997). Red cells of most white Europeans are recognized by one or both anti-Fya and anti-Fyb, according to the presence of *FY*A* and *FY*B* allelic specificities. However, the null phenotype Fy(a^-b^-) is common in Africa, particularly west Africa where it is close to fixation. Fy(a^-b^-) red cells fail to be recognized by the rare anti-Fy3 which recognizes a specificity common to both Fy(a^+b^-) and Fy(a^-b^+) individuals. Fy(a^-b^-) red cells are totally refractory to invasion by *P. vivax*, making Duffy the most common immunogenetic variation resulting in an absolute effect on infectious disease susceptibility. However, the molecular basis of the Duffy system was not unraveled until the 1990s. *FY* codes for the Duffy antigen receptor for chemokines (DARC), present on the red cell surface and elsewhere, which binds to molecules such as IL-8 and RANTES. A Duffy binding ligand has been identified in the parasite. The Fy(a^-b^-) phenotype in west Africans arises from a T-to-C substitution at position −46 of an *FY*B* allele (Tournamille et al. 1995). This mutation disrupts the binding of the GATA1 erythroid transcription factor and reduces expression. More recently this same mutation has been discovered on a *FY*A* background in a PNG population, showing it to have arisen independently in at least two places, and more recently in PNG (Zimmerman et al. 1999).

ICAM-1 is thought to be involved in sequestration of parasitized red cells to the vascular endothelium. A series of observations suggests some role for ICAM-1 in malaria pathology, although, at the time of writing, the story is both complex and incomplete. A base change at position 179 results in a lysine-for-methionine substitution at amino acid 29 ($ICAM1_{Kilifi}$) (Fernandez-Reyes et al. 1997). Homozygotes had a relative risk of 2 for cerebral malaria versus nonsevere malaria in a Kenyan study, although this effect was not seen in a Gambian population (Hill 1999). The mutant allele is at a frequency of about 0.3 in Africa, which would be surprisingly high for an allele predisposing to cerebral malaria. However, comparing severe and mild malaria in a Gabonese population, $ICAM1_{Kilifi}$ appeared to be associated with protection against severe malaria (Kun et al. 1999). Functional studies have shown that $ICAM\text{-}1_{Kilifi}$ causes loss of fibrinogen binding and reduction of LFA-1 binding, suggesting a complex scenario with respect to effects of selection (Craig et al. 2000).

Another molecule thought to be involved in sequestration of parasitized red cells is CD36. *CD36* is highly polymorphic, with a number of diverse mutations resulting in CD36 deficiency. Such mutations are relatively

common in Africa. However, different mutations are present in Asian populations, and as, unexpectedly, African mutations were initially shown to be associated with susceptibility to severe malaria, it was suggested that selection pressures other than malaria were operating (Aitman et al. 2000). However, a later study of a single polymorphism in African children showed the rare allele to be associated with protection from severe malaria (Pain et al. 2001). A detailed investigation, considering common haplotypes and patterns of linkage disequilibrium, of multiple *CD36* polymorphisms in Thais concluded some protection from cerebral malaria, for mutations coding for lack of production of CD36 (Omi et al. 2003). The above investigations on *ICAM1* and *CD36*, with conflicting results, have stimulated some discussion on the importance of these molecules for malarial infection, the complexity of selection pressures operating on different populations, and whether significance levels from multiple statistical comparisons are valid (Craig et al. 2001). Although it seems likely that diversity in these two genes will prove relevant, the detail is likely to prove as complex as the story developing for the *TNF*-promoter region.

Many other genes have been investigated. Some are adhesion molecules such as *CD31* (Casals-Pascual et al. 2001; Kikuchi et al. 2001); others are receptors such as Ig receptors (Shi et al. 2001; Omi et al. 2002). However, there is no large body of work on such genes and findings are variable in different populations. There is scope for invoking malaria as a selective force in multiple populations, but it is unfortunate that other major diseases have received less consideration from evolutionary biologists.

HELMINTH INFECTION

Immune responses in helminth infection are characterized by high levels of total and specific IgE and eosinophilia, upregulated by Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13. Th2 responses are protective in murine models, and there is accumulating evidence for Th2-mediated protection in human infection. Similar Th2 upregulation is seen in asthma and atopy, which are under strong genetic control. Obvious candidate genes for susceptibility to helminth infection and disease are thus those thought to be involved in the control of Th2 responses in asthma, such as the Th2 cytokine cluster on 5q, *FCER1B*, and *IL4RA*. There is evidence of genetic control of helminth infection from mouse model infections, the most commonly used being *Heligmosomoides polygyrus* and *Trichuris muris* (Wakelin and Blackwell 1988). The first genome scan for a murine parasitic nematode has just been completed for *Angiostrongylus costaricensis* (Ohno et al. 2002), with another for *H. polygyrus* in progress (Behnke et al. 2000). There is also increasing interest in sheep genes controlling gastrointestinal nematode infection.

Gastrointestinal and filarial nematodes

There is evidence for genetic control of worm burden in human populations, with heritabilities of 23–44 percent for *Ascaris lumbricoides*, *Necator americanus*, and *Trichuris trichuria* loads reported (Williams-Blangero et al. 1997b, 1999, 2002a). In contrast, studies of *A. lumbricoides* together with *T. trichuria* in Malaysians and *Strongyloides fuelleborni kellyi* in PNG did not support genetic control of worm burdens (Chan et al. 1994; Smith et al. 1991). Partitioning variance for helminth infections can be problematic, particularly partitioning genetic and household effects, and some differences may be accounted for by study design. Consideration of eosinophil rates among Brazilians infected with intestinal worms with an extra-digestive cycle, supported genetic susceptibility to helminths through influence on eosinophils (Conti et al. 1999). This finding fits well with current views on genetic control of eosinophilic inflammation (Brodie et al. 1999), and with the chromosome 5q cluster implicated in control of asthma/atopy and known to determine autosomal dominant familial eosinophilia (Rioux et al. 1998). There is less information on filarial nematode infection. A study of *Loa loa* in The Cameroons was consistent with some genetic control of microfilaremia, and a dominant major gene model was suggested (Garcia et al. 1999). Similarly genetic effects were compatible with distribution of *Brugia malayi* microfilariae, and possibly anti-filarial IgG4, in an Indonesian population (Terhell et al. 2000). However, maternal infection with *Wuchereria bancrofti* is known to be a risk factor for infection in children (Lammie et al. 1991), perhaps through early induction of immunological tolerance, which highlights potential confounding factors to genetic control. *Onchocerca volvulus*, one of the better-studied parasites for candidate genes, has not been the subject of a heritability study.

There are few small case–control studies suggesting a role for HLA in susceptibility and responsiveness to gastrointestinal nematodes (Holland et al. 1992; Satoh et al. 1999). In Venezuelan children associations were found between homozygosity for Arg16 at *ADRB2* and *A. lumbricoides* specific phenotypes, although other candidate genes on 5q33 were not investigated (Ramsay et al. 1999). This was followed by the only human genome scan for a nematode infection. Williams-Blangero et al. (2002b) genotyped 444 Nepalese, with ascaris worm burdens assessed by egg count. Quantitative trait nonparametric linkage was carried out by variance components methods and significant linkage was found to regions on chromosomes 1 and 13. The chromosome 13 region contains a candidate gene *TNFSF13B* (*BlyS*), and was also found to influence serum IgE levels. There are no obvious effects of HLA on lymphatic filariasis, either Indonesian brugian

filariasis by association (Yazdanbakhsh et al. 1995, 1997) or Polynesian bancroftian filariasis by linkage (Ottesen et al. 1981). In contrast, a small study in India suggested associations with *CHIT1* (coding for a phagocyte-specific chitotriosidase) and *MBL2* (Choi et al. 2001). It is notable that the choice of candidate genes in this study varied from those typically used for other infectious diseases. For *O. volvulus* *HLA-DQA1*0501-DQB1*0301* occurred at an increased frequency in putatively immune versus diseased individuals (Meyer et al. 1994). *HLA-DQA1*0101-DQB1*0501*, and independently *HLA-DQB1*0201*, occurred at increased frequency in individuals with generalized disease compared with localized disease and putatively immune individuals (Meyer and Kremsner 1996). In general, findings for localized disease lay between those for putatively immune and generalized disease. A follow-up study also implicated *HLA-DPA1*, with *HLA-DPA1*02011/2* having increased frequency among the putatively immune and *HLA-DPA1*0301* being associated with localized disease (Meyer et al. 1996). A methionine at position 11 of DPα1 conferred a relative risk of disease of 3.3. A later study comparing patients who had cutaneous depigmentation with patients with high microfilarial loads broadly implicated the same haplotypes (Murdoch et al. 1997). A comparison of generalized onchocercal disease versus patients with low microfilarial numbers and strong IgE responsiveness suggested some association with the Gln110Arg *IL13* polymorphism, which has also been associated with several asthma/atopy phenotypes (Hoerauf et al. 2002).

Schistosomiasis

In 1991, Abel et al. reported a segregation analysis of Brazilian families with major gene (*SM1*) control of intensity of infection by *Schistosoma mansoni*, although there has been debate over the relative role of genetics and exposure (Woolhouse 1992). The same group also suggested major gene control of IL-5 production in the Brazilian *S. mansoni*-infected population (Rodrigues et al. 1996), although whether this is the same gene as that controlling infection intensity remains unclear. A later proposal of a polygenic model would seem more realistic (Bethony et al. 2002). The first genome scan for a human parasitic disease was carried out on the intensity of *S. mansoni* infection using parametric linkage analysis with the model of inheritance defined by segregation analysis of the same population (Marquet et al. 1996). There was strong evidence for linkage to the chromosome cluster at 5q31–q33, with a multipoint LOD score of 5.45. The nearest locus was *CSF1R*. Finding a single region in a genome scan indicates the limitations of this parametric approach and re-analysis of these data using model-free linkage analysis found suggestive evidence for linkage to two further regions on 1p21–q23 and 6p21–q21 (Zinn-Justin et al. 2001). Confirmation of the importance of 5q31–q33 has come from another linkage study of a Senegalese population (Muller-Myhsok et al. 1997). There is less evidence of genetic control of hepatic morbidity resulting from infection with *S. mansoni* (Kariuki et al. 2001); however, this may partly result from variable definitions of severe disease, and pedigree clustering has been observed (Mohamed-Ali et al. 1999). Segregation analysis indicated a co-dominant major gene (*SM2*) controlling advanced hepatic periportal fibrosis in Sudanese *S. mansoni*-infected patients (Dessein et al. 1999). Examination of four candidate loci found evidence of linkage (multipoint LOD score 3.12) to *IFNGR1* at 6q22–q23 (Dessein et al. 1999).

A number of studies have been carried out on HLA control of infection or disease in schistosomiasis. Many of the studies are small and there is some lack of consistency in allelic associations. Early studies concerned HLA control of proliferative responses to *S. japonicum* antigens (Ohta et al. 1982; Hirayama et al. 1987). These were accompanied by an observed increase in *HLA-B40* in high responders to schistosomal antigens and hepatosplenic patients (Ohta et al. 1987). Recent studies on *S. japonicum* are devoted to HLA class II associations and advanced disease/fibrosis (Hirayama et al. 1998, 1999; Waine et al. 1998; McManus et al. 2001). Despite some inconsistencies, one interesting suggestion, from an examination of class II haplotypic associations, is that *HLA-DR* and *HLA-DQ* loci may influence early liver changes, whereas the *HLA-DP* locus may be more important in the later phase (Hirayama et al. 1999). HLA class I associations for hepatosplenic disease, caused by *S. mansoni*, in Egyptian populations vary (Abdel-Salam et al. 1986; Hafez et al. 1991). *HLA-DQB1*0201* was positively associated with hepatosplenic disease in Brazilian patients aged over 15 years (Secor et al. 1996), but not in an Egyptian population (Hafez et al. 1991). A study specifically examining the role of *HLA-DP* showed *HLA-DPA1*0301* to be associated with schistosomiasis and a high re-infection rate after treatment (May et al. 1998).

CONCLUSION

At the time of writing, there is good evidence for genetic control of major viral, bacterial, protozoal, and helminth infections. There are indications of some of the regions involved from linkage studies, in particular from genome scans for several major infections. There are many more association studies for candidate genes, a good proportion of which concern the MHC. The scope of studies is often limited, with insufficient sample sizes and limited polymorphisms investigated. Many findings remain to be replicated, which suggests that there may be many false positives or considerable population heterogeneity. It will be interesting to include information on pathogen diversity in future studies. Moreover

there is little understanding of the impact of genetic diversity on immune function. In this respect, knowledge of complex haplotypes in relation to disease susceptibility and immune function is an immediate goal.

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Viral interference with the host immune response

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INTRODUCTION

The clearance of viruses from infected tissues requires a coordinated scenario of multiple effector functions. The immune response with its two arms of innate and adaptive immune responses represents the system by which the organism controls, preserves, and restores integrity. Clearing of the infection can often cause a substantial loss of cells with little functional impairment as a result of cellular regeneration. In the absence of cellular regeneration, e.g. in the central nervous system (CNS), the same loss may already result in considerable functional defects. Whether or not the immunological clearing of a virus infection protects tissues or causes pathology is defined by the speed and amount of virus replication, the strength of the immune response, and the properties of the infected tissues. Therefore, the outcome of either immunoprotection or immunopathology is defined by properties of the virus, the strength of the immune response and by the regenerative activity of the tissues (Levine 2002). Remarkably, viral immune evasion mechanisms target and modulate all aspects of the innate and the adaptive immune response. To discuss the functional relevance of the different viral function it should be recollected that host defense against viruses occurs in a series of waves.

Cells monitor their normal state and many of the effects of viruses on the intracellular milieu tend to activate the process of apoptosis or programmed cell death. This 'suicide' response can be considered a form of host defense, and most viruses seem to need to interfere with this response in order to be able to replicate. The innate immune response starts with the release of soluble mediators of inflammation. These mediators comprise, among others, the interferons, cytokines, chemokines, complement fragments, prostaglandins, and nitric oxide. These key regulators control cell trafficking and cell recruitment to inflamed sites, cell adhesion, and cell activation, but also induce apoptosis. Different cells produce different mediators. Usually, the organism seeks to eliminate the virus completely and the antiviral activities of some soluble mediators are very potent. However, the life cycle of some viruses, in particular those that establish persistent infections, is more complex and for them the evasion of innate immunity is important to reach the target tissues from which the virus cannot be cleared. Among other viruses the well-known hepatitis viruses B and C, human immunodeficiency virus (HIV), and herpesviruses share this property.

In the temporal scenario of the immune response natural killer (NK) cells form the second line of defense. NK cells play a major role in the early defense to viruses. They do not require prior sensitization to recognize and to kill virus-infected cells. The response is induced within hours of infection and usually peaks around 3 days. Herpesviruses, for example, are very

sensitive to the control by NK cells and the molecules by which these viruses defend themselves against this strong effector mechanism of innate immunity today are the subject of active research.

The third wave includes adaptive immune responses which comprise cellular and humoral effector arms. Important cellular effectors are CD8 and CD4 T cells: these are first detectable at days 3–4, and usually peak at about 7 days. CD8 T cells play the critical role and in most cases are able to eliminate the virus. It was noticed first that viruses manage to escape the control of T cells by various molecular principles and this area of research has brought several interesting findings that reshape our understanding of the virus–host balance.

In the antibody response initially immunoglobulin IgM appears about 10 days postinfection, followed by IgG. Antibody plays some role in controlling primary infection but its main role is to prevent reinfection (by neutralization). Antibody effects can be mediated by complement activation. Even the antibody response and complement are diverted by some viruses to their profit.

EFFECTS ON MOLECULES THAT SIGNAL BETWEEN CELLS

Modulation of interferon functions

Interferons (IFN) were discovered because of their ability to protect cells from viral infection. The key role of both type I (α and β) and type II (γ) IFN as one of the first antiviral defense mechanisms is highlighted by the fact that anti-IFN strategies are present in most viruses (Figure 1).

Interferons are produced in response to viral infection, secreted from cells, and bind specific receptors that trigger intracellular events through Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathways (Goodbourn et al. 2000). These signaling events result in the upregulation of cellular proteins that limit viral replication in several ways. Some of the genes upregulated by IFN are the low-molecular-weight protein (LMP)-2 and LMP-7 subunits of the proteasome, involved in the generation of viral peptides

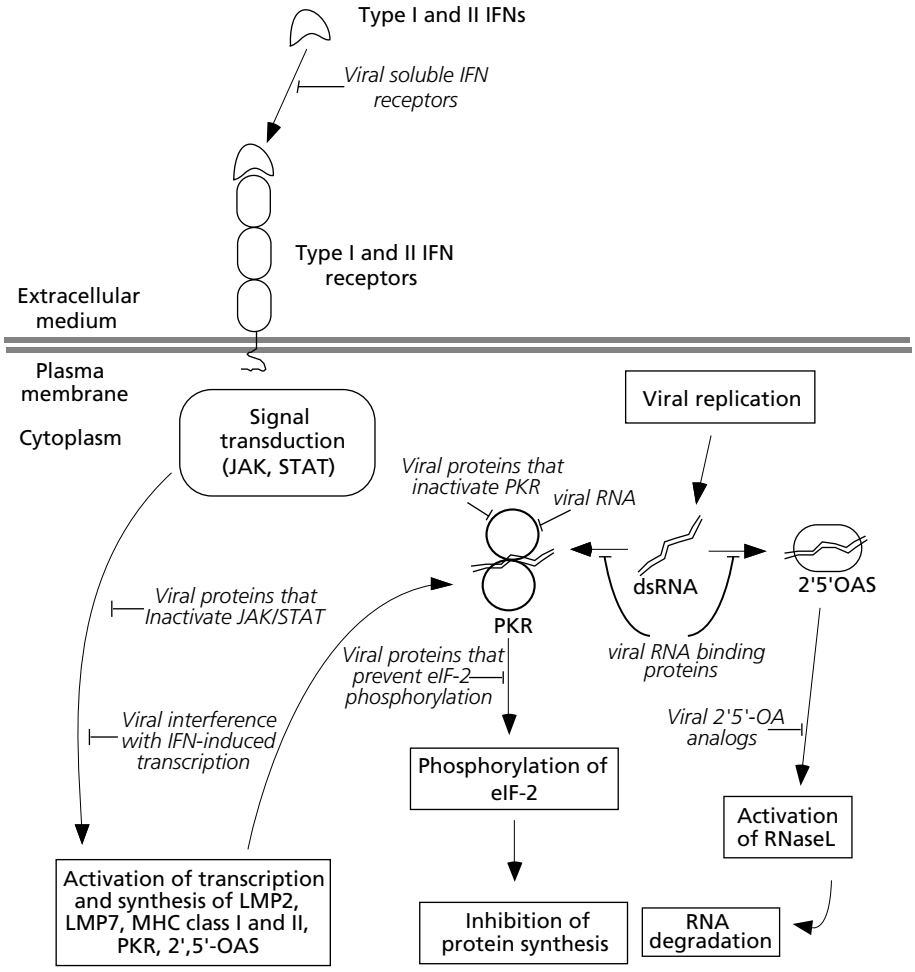

Figure 1 *Viral inhibition of interferon activity. See text for abbreviations.*

for the major histocompatibility complex (MHC) class I and class II molecules, the molecules that present viral peptides to T cells. Two major pathways that induce an antiviral state in cells are also upregulated by IFN: the double-stranded ribonucleic acid (RNA) (dsRNA)-dependent protein kinase (PKR) and the 2′,5′-oligoadenylate system (2′,5′-OAS) that activates RNase L. In addition, IFNs have immunomodulatory activity that can influence the type of immune response mounted as a reaction to infection.

Viruses block the activity of IFNs at three different levels: (1) blockade of IFN binding to specific receptors; (2) inhibition of signal transduction pathways induced by IFNs; and (3) inhibition of antiviral effector functions induced by IFNs (see Figure 1).

SECRETED VIRAL IFN RECEPTORS AND IFN-BINDING PROTEINS

Poxviruses encode proteins that are secreted from infected cells and bind with high affinity type I or II IFN (McFadden and Murphy 2000; Alcami 2003). These proteins prevent the interaction of IFNs with their receptors and the initiation of biological effects that lead to an antiviral state in the cell. Soluble cytokine receptors or binding proteins for other cytokines have been identified, mainly in the poxvirus family, and examples are discussed below.

The viral IFN-γ receptor (vIFN-γR) was first identified in myxoma virus (MV) and later in other members of the poxvirus family including vaccinia virus (VV) and cowpox virus (CV) (Upton et al. 1992; Alcami and Smith 1995; Mossman et al. 1995). The vIFN-γR has sequence similarity to the IFN-binding domain of the cellular IFN-γ receptor. In addition, VV and other poxviruses also encode a secreted IFN-α/β receptor or binding protein (vIFN-α/βBP) that has very limited sequence similarity to the cellular counterparts (Colamonici et al. 1995; Symons et al. 1995). The vIFN-α/βR has the unique property of binding to the cell surface after secretion (Alcami et al. 2000). This property allows the vIFN-α/βR to cover infected tissues with decoy receptors that prevent in a very efficient way the activation of IFN-mediated pathways. The role of vIFN-γR and vIFN-α/βBP during viral infection has been demonstrated by the attenuated phenotype of VV and MV mutants lacking these IFN inhibitors (Symons et al. 1995; Mossman et al. 1996).

INTERFERENCE WITH IFN SIGNALING

Some viral proteins interfere with the signaling cascade initiated in cells by the interaction of IFN with their receptors, and prevent the activation of antiviral mechanisms that limit viral replication (Goodbourn et al. 2000), e.g. the T antigen of murine polyoma virus binds to and inactivates JAK1 (Weihua et al. 1998), whereas protein V from simian virus 5 interacts with STAT1 and induces its degradation by the proteasome (Didcock et al. 1999). A different mechanism is used by Kaposi's sarcoma-associated herpesvirus (KSHV), a virus that encodes a homolog of the IFN-regulatory factor (IRF) which represses the IFN-mediated transcriptional activation of host genes (Zimring et al. 1998).

VIRAL MODULATION OF IFN EFFECTOR FUNCTIONS

One of the major antiviral effects of IFNs is the induction of the dsRNA-dependent PKR and 2′,5′-OAS pathways which lead to the blockade of gene expression in the cell and prevent the formation of infectious virus particles (Goodbourn et al. 2000). The replication and transcription of viral genomes can lead to the formation of double-stranded RNA (dsRNA) that activates PKR, which phosphorylates the translation initiation factor 2α (eIF-2α), causing its inactivation and the subsequent arrest of protein synthesis in infected cells. Viruses use several strategies to inhibit the activity of PKR (Gale and Katze 1998; Katze et al. 2002). Some viruses encode proteins that bind dsRNA and prevent activation of PKR, such as the VV E3L and reovirus σ3 proteins. Adenovirus and HIV encode RNAs that bind PKR but do not activate the enzyme, whereas herpes simplex virus (HSV) US11 and HIV Tat bind directly to PKR and inactivate the enzyme. An alternate strategy to block the PKR pathway is to prevent the phosphorylation of eIF-2α. This can be achieved by the expression of an eIF-2α homolog, the K3L protein of VV, which acts as a substrate of PKR, or by HSV IC34.5 protein-activating protein phosphatase 1α which removes the phosphate groups from eIF-2α. The formation of dsRNA during viral replication also activates the enzyme 2′,5′-OAS, producing 2′,5′-OA which in turn activates RNase L and the degradation of RNA within the infected cell, causing the blockade of viral replication. The synthesis of analogs of 2′,5′-OA by HSV, which bind RNase L, inhibits this pathway (Goodbourn et al. 2000). The proteins σ3 from reovirus and E3L from VV bind dsRNA and thus inhibit the activation of 2′,5′-OAS as well as PKR (see above).

Modulation of cytokine networks

Cytokines constitute a numerous family of molecules that play an important role in the initiation and regulation of the immune response. Cytokines are normally secreted from cells, although some are expressed at the cell surface, and bind to specific receptors on other cells. In this way, cytokines are used by the immune system to communicate among cells and to send signals that drive the immune response in a particular direction. In addition, some cytokines such as IFN and tumor necrosis factor (TNF) may act directly on infected cells and restrict virus replication.

The importance of cytokines in antiviral defense is highlighted by the number of mechanisms that viruses encode to block the activity of cytokines. The viral anticytokine strategies will normally result in down-regulation of immune responses. However, there are examples in which these viral mechanisms increase viral replication. Large DNA viruses such as poxviruses and herpesviruses have captured in their genomes genes that encode host cytokines and cytokine receptors (McFadden and Murphy 2000; Alcami 2003). This strategy enables these viruses to interfere with or to exploit cytokine pathways for their own benefit (Figure 2).

VIRAL CYTOKINES

The first viral cytokine identified was the VV epidermal growth factor homologue (VGF). This viral protein activates cell growth and favors viral replication, but an immune-related function has not been ascribed to it (McFadden and Murphy 2000). The first cytokine with immunomodulatory activity found in viruses is the IL-10 homolog (vIL-10) encoded by Epstein–Barr virus (EBV) (Hsu et al. 1990). Genes encoding IL-10 homologs have also been identified in other viruses. It has been shown that the EBV vIL-10 suppresses cellular immunity by inhibiting the production of IFN-γ, but has lost the immunostimulatory properties of the host counterpart. Other viral cytokines are the IL-6 homolog (vIL-6) encoded by KSHV (Moore et al. 1996) and the IL-17 homolog (vIL-17) encoded by herpesvirus Saimiri (HVS) (Yao et al. 1995). These cytokines may have immunomodulatory activity but vIL-6 and vIL-17 may also promote the proliferation of B and T cells, respectively, which represent the major host cell types for these viruses.

The identification of semaphorin homologs (vSEMA) in poxvirus and herpesvirus genomes was somehow surprising because semaphorins were described as chemoattractants and chemorepellents involved in axonal guidance during nervous system development, and only CD100 had been identified as a semaphorin involved in the immune system (Spriggs 1999). The characterization of vSEMA encoded by VV and ectromelia virus (EV) uncovered a specific receptor in macrophages and its proinflammatory activity, which was observed in mice infected with a VV expressing an active vSEMA protein. The precise role of vSEMA in immune modulation during viral infection remains to be determined.

SECRETED VIRAL CYTOKINE RECEPTORS

Viral genes encoding soluble cytokine receptors have been identified mainly in the poxvirus family. These cytokine receptors lack the transmembrane and cytoplasmic domains of the host counterparts, are secreted in large quantities from infected cells, and have the ability to bind with high affinity the cognate cytokine and block its activity. The poxvirus soluble cytokine receptors were initially identified because of their sequence similarity to the extracellular binding domain of their host counterparts. A second class of cytokine receptors, known as binding proteins, have very limited sequence similarity to the host cytokine receptors and were identified in cytokine binding or activity assays.

The first viral cytokine receptor identified was the viral TNF receptor (vTNFR) M-T2 encoded by MV (Smith et al. 1991). Subsequent studies identified four different genes encoding vTNFR in poxviruses such as CPV, VV, and EV, and were designated cytokine response modifier B (CrmB), CrmC, CrmD, and CrmE (Hu et al. 1994; Smith et al. 1996; Loparev et al. 1998; Saraiva and Alcami 2001). CrmB is equivalent to MV M-T2 but the other proteins are distinct with respect to binding properties and molecular size, and are produced at different times during viral replication. The reasons for such a variety of proteins apparently targeting the

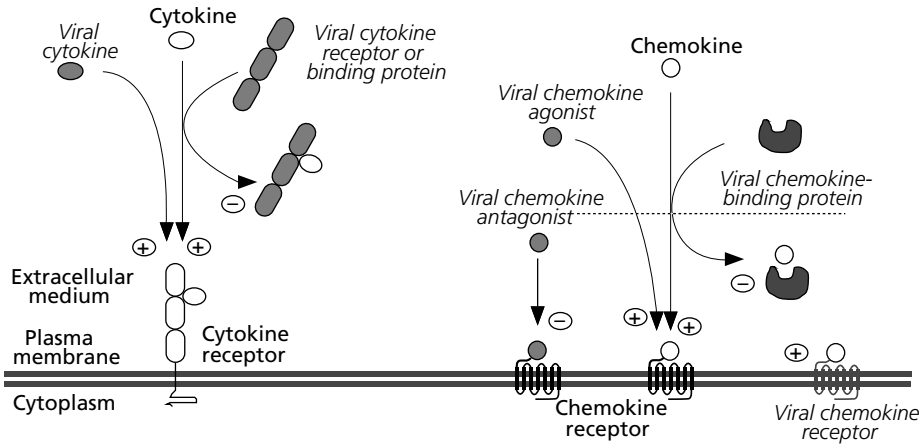

Figure 2 *Viral mimicry of cytokines, chemokines, and their receptors.*

same ligand are unknown. Clinical isolates of human cytomegalovirus (HCMV), but not laboratory strains, encode a membrane TNFR homolog related to herpesvirus entry mediator (HVEM) which is retained intracellularly (Benedict et al. 1999). No ligand has been identified for this protein and its function remains to be determined. More recently, another member of the TNFR superfamily has been identified in CPV and EV. In this case, the viral protein is a secreted and shorter version of human and mouse CD30 (Panus et al. 2002; Saraiva et al. 2002). Viral cytokine receptors function as decoy receptors neutralizing the activity of cytokines and, accordingly, vCD30 blocks the interaction of CD30 to CD30 ligand (CD30L). However, in this case the ligand is expressed only at the cell surface and vCD30 induces reverse signaling in CD30L-expressing cells. The function of vCD30 in the context of infection has not been elucidated, but administration of vCD30 to mice has been shown to downregulate type 1 cytokine-mediated inflammatory responses, a property that may help the virus to escape an efficient antiviral response (Saraiva et al. 2002).

The viral IL-1β receptor (vIL-1βR) is related to the human type II IL-1R and also acts as a decoy receptor (Alcami and Smith 1992; Spriggs et al. 1992). In contrast to the membrane IL-1Rs encoded by the host, the viral protein is specific for IL-1β and does not bind IL-1α or IL-1 receptor antagonist. Deletion of this gene in VV causes attenuation when the virus is administered intracranially (Spriggs et al. 1992), but leads to enhanced virulence associated with fever and accelerated death in mice infected through the respiratory route, a natural route of poxvirus transmission (Alcami and Smith 1992, 1996). The enhanced virulence observed after deletion of the vIL-1βR from VV illustrates that the role of some viral immunomodulatory proteins may be to reduce pathology induced by excess production of cytokines, in this case IL-1β, during infection rather than to cause immunosuppression.

VIRAL CYTOKINE-BINDING PROTEINS

IL-18 is a proinflammatory cytokine that is required, together with IL-12, for induction of IFN-γ and the generation of an efficient cellular response against viral infection. The poxvirus-encoded IL-18 binding protein (vIL-18BP) is a homolog of the human IL-18BP but has no sequence similarity to membrane IL-18 receptors. The vIL-18BP is an effective scavenger of IL-18, and downregulates IFN-γ production and NK responses in EV-infected mice (Born et al. 2000; Smith et al. 2000). Interestingly, the vIL-18BP is also expressed by molluscum contagiosum virus (MCV), a poxvirus that causes benign skin tumors and persists in the skin for months without inducing an inflammatory response (Xiang and Moss 1999; Smith et al. 2000). The vIL-18BP is the only cytokine receptor encoded by MCV and may be responsible in part for the lack of inflammation in MCV lesions.

The only soluble cytokine-binding protein identified in herpesvirus is encoded by EBV and binds colony-stimulating factor (vCSF-1BP) (Strockbine et al. 1998). This protein has limited sequence similarity to the cellular receptor and may modulate the response of macrophages during infection. Another example of a viral cytokine-binding protein that binds ligands using different host receptors is a secreted protein that binds granulocyte–macrophage-CSF and IL-2 encoded by orf virus, a virus that causes skin lesions in sheep, goats, and humans (Deane et al. 2000).

Modulation of chemokine networks

Chemokines are a family of small-molecular-size cytokines that induce cell migration and other biological effects, such as cell differentiation and angiogenesis, and play a critical role in the regulation of cells of the immune system throughout the body. There are more than 40 chemokines identified that can be structurally classified as CC, CXC, C, or CX3C, and mediate their biological response by binding and signaling through G-protein-coupled receptors, proteins that have seven-transmembrane domains. More than 20 chemokine receptors have been identified and their distribution in immune cells determines which type of cell migrates in response to infection or inflammation. There are three structural concepts used by viruses, mainly herpesviruses and poxviruses, to modulate chemokine activity: expression of chemokine homologs, chemokine receptor homologs, and secreted chemokine-binding proteins unrelated to the sequence of known host receptor proteins (McFadden and Murphy 2000; Murphy 2001; Alcami 2003)

VIRAL CHEMOKINE HOMOLOGS

The virus-encoded chemokine homologs are secreted from infected cells and function as agonists or antagonists. Chemokine antagonists such as vMIP-II from KSHV and MC148 from MCV, bind to chemokine receptors but do not transduce signals (Boshoff et al. 1997; Kledal et al. 1997; Krathwohl et al. 1997; Damon et al. 1998). These antagonists function as inhibitors of specific chemokine pathways and influence the migration of immune cells into infected tissues. By contrast, the viral chemokine agonists induce cell migration into areas of infection, and thus their function may be unrelated to immune evasion. The current thinking is that viral chemokine agonists recruit to areas of infection immune cells that represent good targets for viral replication and enhance viral dissemination. The chemokine encoded by the gene *UL146* from HCMV, known as vCXC-1, induces neutrophil chemotaxis by binding to CXCR2

and may explain the association of neutrophils with HCMV infections (Penfold et al. 1999). Human herpesvirus 6 (HHV-6) U83 induces monocyte migration that may favor the establishment of viral latency and persistence in the infected host (Zou et al. 1999). Another example of chemokine mimetics is the protein Tat from HIV which shows limited sequence similarity to chemokines. HIV Tat is a chemoattractant for monocytes and may enhance replication and spread of HIV in infected individuals (Albini et al. 1998). Direct evidence for a role of viral chemokine agonists in vivo comes from studies with murine cytomegalovirus (MCMV) mutants lacking the chemokine genes *M131/129*. The MCMV chemokine is encoded by the *m131orf* and is expressed as a fusion protein including the *m129 orf*. The isolated synthetic *m131* product attracts activated macrophages by engaging a receptor with structural properties related to human CCR3 (Saederup et al. 1999). The mutant MCMV lacking the function shows reduced spread to salivary glands, indicating that monocyte attraction serves for virus trafficking.

The role of the viral chemokines encoded by KSHV in viral dissemination has not been defined but they may play a role in pathogenesis as a result of their angiogenic properties which may cause the enhanced vascularization observed in Kaposi's sarcoma lesions (Boshoff et al. 1997). In addition, the KSHV chemokines are chemoattractants for Th2-polarized T cells and may influence the type of immune response initiated after viral infection (Sozzani et al. 1998; Dairaghi et al. 1999; Endres et al. 1999; Stine et al. 2000).

An interesting and recent addition to chemokine mimicry by viruses is the finding that glycoprotein G (gG) of respiratory syncytial virus has partial sequence similarity to fractalkine and has chemokine-like activity (Tripp et al. 2001). This property of gG may be used to facilitate virus attachment to infected cells and to modulate the host immune response.

VIRAL CHEMOKINE RECEPTOR HOMOLOGS

There are several examples of chemokine receptor homologs in members of the herpesvirus family and some in the poxvirus family. The function of these receptors in the viral infectious cycle is varied and difficult to demonstrate. Some viral chemokine receptors bind chemokines and transduce signals inside the cell. The ORF74 protein from KSHV is constitutively active and binding of chemokines can modulate the activation status of the receptor (Arvanitakis et al. 1997; Rosenkilde et al. 1999). The receptor has been shown to induce cell proliferation when expressed in cells in culture and its expression in transgenic mice causes the development of Kaposi's sarcoma-like lesions (Arvanitakis et al. 1997; Yang et al. 2000). The possibility that expression of KSHV ORF74 may contribute to neoplasia through a paracrine effect has been proposed, but may be difficult to demonstrate in KSHV-infected individuals. In any case, ORF74 illustrates how such proteins encoded by viruses may have a direct and profound effect on the pathology caused by viral infection. In vivo studies on the homologous ORF 74 encoded by murine γ-herpesvirus 68 (MHV-68) have revealed that the virus uses this receptor for virus reactivation from latently infected mouse splenocytes (Lee et al. 2003). Of the four chemokine receptor homologs encoded by HCMV, UL78, UL33, US27, and US28, US28 is most extensively studied. MCMV encodes the viral chemokine receptor (vCKR) M33 and M78, which are homologous to their counterparts in HCMV. HCMV US28 can modify the chemokine environment of infected cells by sequestering and internalizing chemokines, and depleting them from the medium (Bodaghi et al. 1998). Similarly, HHV-6 UL51 reduces extracellular accumulation of chemokines by sequestration, but also by a novel mechanism of downregulation of CCL5 (RANTES) transcription (Milne et al. 2000). The potential contribution of viral chemokine receptors to pathology is also illustrated by the fact that HCMV US28 expression on the cell surface mediates cell adhesion and vascular smooth muscle cell migration, and it is thought to be related to vascular disease (Streblow et al. 1999). Evidence for a role for viral chemokine receptors in virus replication in vivo was demonstrated first for MCMV M33 (Davis-Poynter et al. 1997).

VIRAL CHEMOKINE-BINDING PROTEINS

The third class of viral chemokine inhibitors are the viral chemokine-binding proteins (vCKBP) that sequester chemokines in solution without apparent sequence similarity to host chemokine receptors. These proteins were initially described in poxviruses, but an example has been identified recently in the herpesvirus family.

The MV M-T7 (vCKBP-1) is a soluble vIFN-γR with sequence similarity to human IFN receptors but was later found to interact with chemokines. MV M-T7 binds a broad spectrum of chemokines and it has been proposed that it prevents the interaction of chemokines with glycosaminoglycans (GAG) and the correct presentation of chemokines to leukocytes (Lalani et al. 1997). There is evidence that the interaction of chemokines with cell-surface GAGs is important for their function in vivo. The protein vCKBP-2 was identified in the medium from cultures infected with MV, VV, or CPV (Graham et al. 1997; Smith et al. 1997; Alcami et al. 1998). In this case, the protein binds CC chemokines with high affinity and neutralizes their effect by preventing the binding of chemokines to specific receptors in leukocytes. The protein M3 (vCKBP-3) encoded by murine MHV-68 was found to bind a broad range of chemokines, including C, CC, CXC, and CX3C chemokines, and to

block their interaction with cellular receptors (Parry et al. 2000; van Berkel et al. 2000).

The generation of relevant virus mutants lacking specific vCKBPs and the infection of rabbits or mice suggest that these proteins may block chemokine-mediated leukocyte infiltration into infected areas in vivo (Mossman et al. 1996; Graham et al. 1997; Lalani et al. 1997). In addition, MHV-68 M3 has been proposed to be required by the virus to establish virus latency and to enable persistence of the virus in the host (Bridgeman et al. 2001). Sequestration of chemokines can also prevent the process of dendritic cell (DC) maturation and trafficking (see below).

VIRAL MODULATION OF CYTOKINE EXPRESSION AND ACTIVATION

Other mechanisms that modulate the synthesis of cytokines and the signaling cascade triggered by them have been described. The expression of cytokines mediated by the nuclear factor κB (NF-κB) and nuclear factor-activated T cell (NFAT) transcription factors in macrophages is blocked by an IκB homolog encoded by African swine fever virus (Miskin et al. 1998). The CPV protein CrmA was found to inhibit the IL-1β-converting enzyme (ICE) (or caspase-1), which proteolytically cleaves the precursor form of proinflammatory cytokine IL-1β (pro-IL-1β) to generate mature, active IL-1β (Ray et al. 1992). CPV CrmA may also prevent the maturation of IL-18, which is also proteolytically cleaved by ICE, and is an inhibitor of apoptosis (see below under Viral inhibitors of caspases).

There are several examples of viruses that inhibit cytokine-mediated signaling. Adenoviruses interfere with signaling induced by TNF at different levels (Mahr and Gooding 1999), and some herpesviruses and poxviruses inhibit signaling through death domains that lead to apoptosis (see below under Viral inhibitors of death receptor-mediated apoptosis). The poxvirus VV encodes two intracellular proteins that block signaling mediated by Toll-like and IL-1 receptors by mimicking intracellular regulatory domains of these receptors (Bowie et al. 2000). The Toll-like receptor pathway is important in NF-κB activation and amplification of inflammatory signals.

Lastly, some viruses may subvert cytokine-mediated signaling for their own benefit. The latent membrane protein 1 (LMP1) of EBV recruits components of the TNFR and CD40 transduction machinery and induce biological responses such as cell proliferation that may enhance virus replication (Farrell 1998).

APOPTOSIS

Programmed cell death (apoptosis) is a ubiquitous and evolutionarily conserved mode of cell death. Individual cells are eliminated or removed from the organism in a temporal manner or in response to specific signals without affecting neighboring cells. Apoptosis plays a central role in tissue homeostasis throughout life (Rathmell and Thompson 2002). Apoptosis is characterized by loss of cell volume or cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, phosphatidylserine exposure, membrane blebbing, and the formation of apoptotic bodies. These stereotypical morphological changes, associated with natural death, are based on a highly regulated genetic program. The activation of a class of cysteine proteases, termed 'caspases', as the central effectors plays an essential role. Caspases are synthesized initially as single polypeptide chains representing latent precursors (zymogens) which undergo proteolytic processing at specific aspartic acid residues to produce two distinct subunits that assemble to the active heterotetrameric protease. In mammalian cells, activation of the caspase zymogens has been reported to occur through at least three independent mechanisms: cleavage by upstream active caspases; cleavage by granzyme B, an aspartate-specific serine protease found in the granules of cytolytic T cells; and autoprocessing of zymogens with assistance from other caspase-interacting proteins that can occur in either a *cis*- or *trans*-acting manner (for reviews, see Salvesen and Dixit 1997; Benedict et al. 2002; Bortner and Cidlowski 2002; Trapani and Smyth 2002)

There are several caspases that are functionally redundant. Once activated, one caspase can activate other caspases in the proteolytic cascade (Stennicke et al. 1998). The trigger of the proteolytic cascade involves the physical association of zymogens via adaptor proteins with protein interaction modules that assemble the receptor signaling complex. The apoptotic program in cells includes mechanisms that activate and those that inhibit apoptosis. There are two main pathways of apoptosis.

Death receptor-initiated activation of caspases

Cells can receive extrinsic signals that instruct them to die. The primary receptor through which death signals are signaled are members of the TNFR family, such as Fas and TNFR-1(19,24). Binding of receptor and ligand on the cell surface results in the formation of the hetero-oligomeric death-inducing signal complex (DISC). The apoptosis-inducing quality of a death receptor ligand system is defined by the death domain (DD) located in the intracellular portion of the death receptor. The DISC is assembled by complex protein interactions. The DDs and death effector domains (DED) carried by different proteins, e.g. caspases, are essential components. DISC proteins interact with other proteins through homotypic interactions of DD and DED contacts. The basis of the interaction is structural motifs

of the DD which form six antiparallel helical bundles. Adaptor proteins such as FADD contain both a DD and a DED motif in one polypeptide and provide the bridging between DD domains of receptors with a DED-containing caspase. Among the caspases, mainly the initiator caspases-8 and -10 are recruited to the DISC. It is thought that the conformational properties and local concentrations of different proteins in the DISC complex govern caspase activation by autocatalytic processing (Schmitz et al. 2000; Walczak and Sprick 2001).

Mitochondrial pathway of caspase activation

Independent of the extrinsic death receptor pathway, apoptosis is also controlled intrinsically at the level of mitochondria. Mitochondrial death can result from the loss of extracellular vital signals (death by neglect), and by signals affecting the cell cycle, through activation of p53 and the proapoptotic p53 target genes (Rathmell and Thompson 2002). Changes in nutrient uptake, serum withdrawal, the deprivation of cytokines, and other metabolic events that affect the cellular redox potential, or the ability properly to regulate active oxygen species, lead to apoptosis. An important molecule in mitochondrial apoptosis is cytochrome *c*. Cytochrome *c* resides in the space between the inner and outer membranes of the mitochondria. The release of cytochrome *c* initiates mitochondrial apoptosis at the apoptosome, a protein complex called Apaf-1. It consists of the caspase-activating molecules, which can bind to caspase-9 through homotypic interaction via a domain defined as caspase-recruitment domain (CARD). For activation of caspases, Apaf-1 requires cytochrome *c*. A number of signals control the oligomerization of Apaf-1–caspase-9 complexes. Additional proteins that have CARD domains can have opposing activities on apoptosome formation, either inhibition or enhancement.

Procaspase-8 activation can also occur at sites other than the cell surface. BAP-31, an integral protein of the endoplasmic reticulum, can recruit an isoform of procaspase-8, procaspase-8L, containing an N-terminal extension in response to apoptotic signals. This suggests an additional organelle-specific pathway of cell death (Ng et al. 1997; Nguyen et al. 2000; Breckenridge et al. 2002). This pathway is also regulated by the Bcl-2 proteins discussed below (Figure 3).

Cellular regulation of extrinsic apoptosis

The cellular homolog of the viral caspase-8 (FLICE) inhibitory protein (cFLIP) consists of two DED domains and a caspase domain that lacks the enzymatic active site. Therefore, cFLIP can bind but not cleave cellular zymogen substrates. It is spliced in two forms; one form contains only the two DED domains; the other, long form includes the caspase-like domain. In addition, cFLIP may block caspase-8 recruitment to the DISC or may inhibit proteolytic processing of the proenzyme. However, cFLIP can also promote recruitment of caspase-8, which can stimulate autoprocessing to the active form.

REGULATION OF CASPASE ACTIVITY

Regulation of caspase activity is central to the death process (Bortner and Cidlowski 2002). The inhibitor of apoptosis (IAP) family of proteins regulates caspase activation and activity. The first member of the IAP family was found in baculovirus. The cellular proteins with antiapoptotic function share, with the baculovirus protein, the baculoviral IAP repeat (BIR), a domain that consists of about 70 amino acids with conserved spacing of cysteine and histidine residues, which fold into a zinc-binding structure. Through the BIR domain, IAP molecules bind and directly inhibit caspase activation. Several IAPs also contain a ring-finger domain located near the C termini. Through the activity of the ring-finger domains, IAPs can target caspases for ubiquitinylation and degradation. IAPs themselves are again subject to inhibition by other proteins that activate caspases. IAPs can bind to caspases in either the active configuration or the inactive proform state.

REGULATION OF MITOCHONDRIAL APOPTOSIS

The Bcl-2 family of proteins controls the release of proapoptotic proteins from mitochondria. Bcl-2 is present in the outer mitochondrial membrane and Bcl-2-like proteins have pore-forming activities and prevent the release of cytochrome *c*. The Bcl-2 family proteins are characterized by protein domains defined as Bcl-2 homology (BH). Four such domains, BH1–4, have been identified. BCL-2 proteins have up to four BH domains. The α-helical BH3 domain of one protein interacts with a receptor formed by a hydrophobic pocket created by BH1–3 of the other protein. Through BH domains BH2 family members interact with each other and form homo- and heterodimers, which results in the promotion of abrogation of the function of the binding partner. Bcl-2 family members have either proapoptotic or antiapoptotic properties. Many antiapoptotic Bcl-2 family members contain domains BH1, -2, and -4. Proteins that carry only the BH3 domain define a family of proapoptotic proteins. Many death-signaling pathways require one BH3-only protein to activate the Bcl-2 family members BAX and BAK in order to activate the caspase. BAX and BAK are functionally redundant and their overexpression leads to mitochondrial accumulation of the protein and initiation of apoptosis. BAK-type proteins make the mitochondrial membrane permeable so that it releases intermembrane apoptogenic factors.

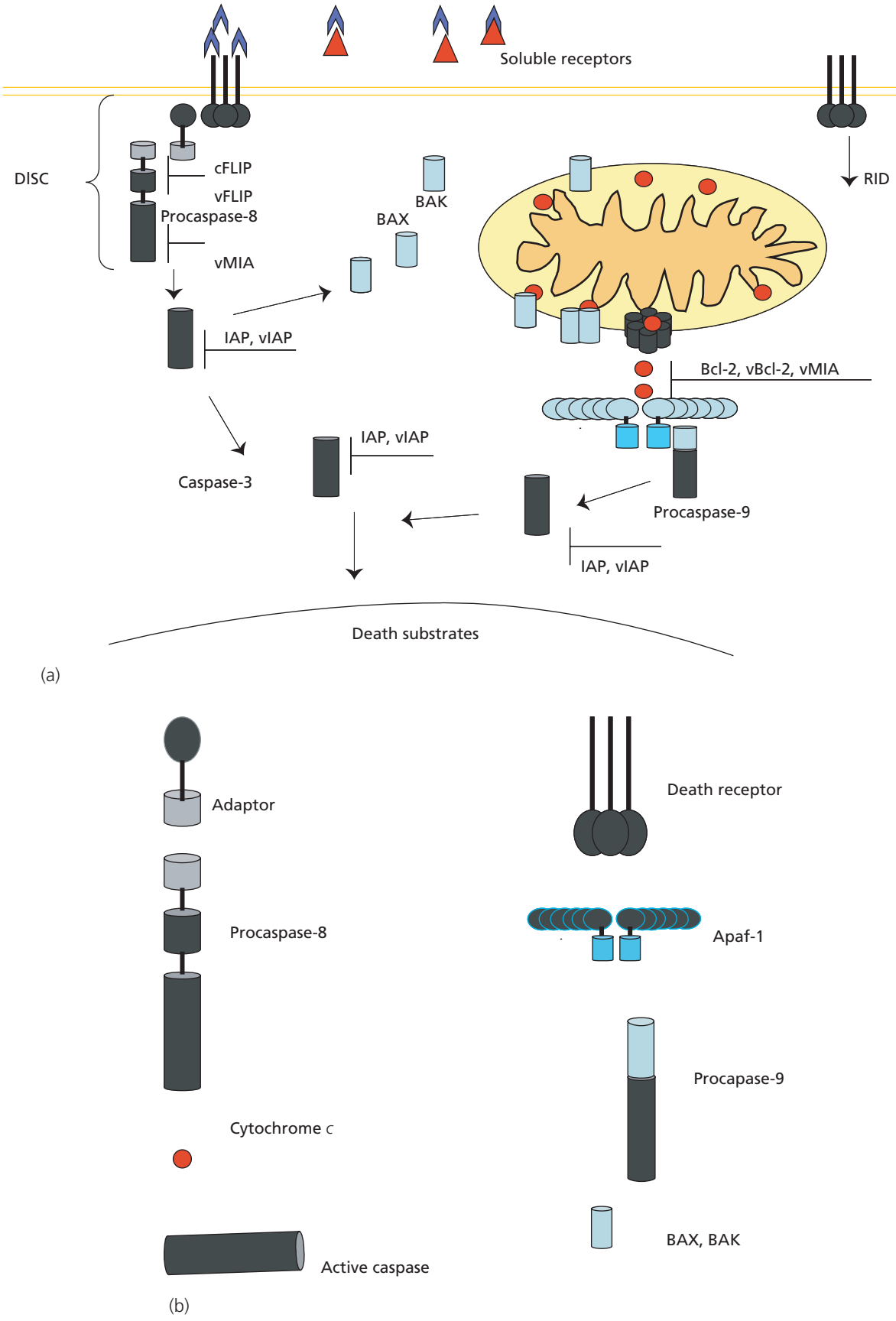

Figure 3 *Viral regulation of apoptosis. See text for abbreviations.*

BAX is primarily cytoplasmic but translocates to mitochondria on apoptotic stimuli. Specific conformational alterations of BAX allow mitochondrial docking. Like BAX, BAK has a transmembrane domain and is targeted to mitochondria where it exists as a monomer or in a complex with other Bcl-2 family members. Antiapoptotic Bcl-2 family proteins bind and inhibit the proapoptotic proteins.

CELLS DIFFER IN APOPTOSIS REGULATION

Cell lines differ with respect to the preferential usage of the two signaling pathways leading to apoptosis. Type 1 cells constitutively contain a large amount of caspases at the DISC. Fas triggering results in caspase-8 activation, followed by cleavage of caspase-3 and loss of the mitochondrial transmembrane potential. In type 2 cells, there is little DISC formation and the intrinsic mitochondrial pathway is required as an amplifier to initiate the full apoptosis cascade after Fas activation. Only in type 2 cells can apoptosis triggered by extrinsic signals be blocked by BCL-2 overexpression. Apoptosis in type 2 cells is dependent on the activities of mitochondria, whereas apoptosis in type 1 cells is not. T cells have type 1 and liver cells have type 2 characteristics (Scaffidi et al. 1998).

Chaperones and chaperone-like proteins that support the folding of newly synthesized proteins can also have pro- and antiapoptotic functions. Heat shock protein (HSP) 70-like chaperones inhibit apoptosis, whereas HSP-60 and HSP-10 have proapoptotic functions. Likewise, protein kinases modulate apoptosis. Mitogen-activated kinases (MAPK) control apoptosis through transcription, e.g. the upregulation of cFLIP, and also through transcription-independent mechanisms.

Viral control of apoptosis

Replication for viruses, as obligatory intracellular parasites, is dependent on cellular function. Therefore, for virus replication it is important that apoptosis triggered by soluble mediators of innate immunity via death receptors and caspase cascades is inhibited. The viral replication machinery diverts and exhausts cellular functions. Thus, viral protein synthesis, viral morphogenesis, and viral release must destabilize the homeostasis and integrity of the cell. This results in the activation of the intrinsic pathway of apoptosis. For effective viral progeny generation, it is important to delay this process. Some viruses also have proapoptotic properties, which may contribute to the release of virus particles from the cell that is already depleted from its resources. The execution of a proapoptotic function may also be advantageous, if apoptosis of an infected cell causes phagocytosis by another cell and enables the virus transfer to cells that the virus cannot infect directly. Therefore, it is not surprising that viruses may contain both antiapoptotic and proapoptotic functions. Some of the viral genes controlling apoptosis are derived from the host cell genome; for others there are no known homologs. NK and T-effector cells have two important functions: they kill infected cells through the perforin/granzyme pathway or by engaging fas, and they secrete several cytokines, among which IFN-γ is known to be essential for control of virus in some situations. Both pathways of cytolysis induce apoptosis of targets. The viral antiapoptotic functions may play a role in diminishing effector cell efficacy, but caution is warranted before making this assumption. Virus-infected cells are generally readily lysed by T cells, so long as the T-cell receptor (TCR) is triggered, in spite of the presence of antiapoptotic mechanisms. It is likely that the strength of the death signal imposed, particularly by the perforin/granzyme pathway, is able to overcome most antiapoptotic mechanisms.

VIRAL INHIBITORS OF DEATH RECEPTOR-MEDIATED APOPTOSIS

Poxviruses secrete proteins that bind to cytokines, which in turn activate death receptor-mediated apoptosis. This probably represents the earliest possible defense against apoptosis signals. The expression of the death receptor CD95 is inhibited by viral IFN regulatory factors from HHV-8 that inhibit the binding of IFN-γ induced IFN regulatory factor 1 (IRF-1) to specific domains in the CD95L promoter (Kirchhoff et al. 2002). The functions of adenovirus interfere with the apoptosis mediated by the TNF receptor superfamily at a very early stage and at various levels (Burgert et al. 2002; McNees and Gooding 2002). Post-translational downregulation of FAS is carried out by the E3/10.4K–E3/14.5K protein complex, also named receptor internalization (RID) and degradation, together with the E3/6.7K protein. The RID complex contains transport motifs in the cytoplasmic tail of the proteins which are recognized by the intracellular transport machinery that directs the CD 95/RID complex to lysosomes. Apart from CD95, the TNF-related apoptosis-inducing ligand (TRAIL) receptor can form a death-inducing signaling complex. In addition TRAIL receptors are downregulated by the E3 proteins of adenovirus.

The DISC is the next target for modulatory viral proteins. It is assembled by protein interaction between DDs and DEDs. Search for proteins carrying homology to DEDs led to the identification of viral proteins with two DEDs (Derfuss and Meinl 2002). These viral proteins block the activation of caspase-8 (FLICE) and were therefore named viral FLICE inhibitor proteins (vFLIPs). These vFLIPs have been detected in poxvirus and several herpesvirus genomes. In addition, vFLIPs regulate the expression of transcription factors for proteins involved in apoptosis regulation. The product of

the HCMV gene, *UL36 vICA*, binds procaspase-8 but lacks homology to cFLIP (Goldmacher 2002).

VIRAL INHIBITORS OF CASPASES

The archetypal member of the cellular IAP family was found in baculovirus. Through the BIR, a motif that can be present up to three times, IAP molecules bind and directly inhibit caspase activation. Other caspase inhibitors are the poxvirus serine protease inhibitor CrmA (which targets a number of caspases) and related proteins from other poxviruses and African swine fever virus, and p35 of baculovirus. Interestingly, viral proteins can combine IAP properties with properties of BCL-2 protein functions (Wang et al. 2002b).

VIRAL INHIBITORS OF MITOCHONDRIAL APOPTOSIS

Prevention of mitochondrial permeabilization is essential for the replication of many viruses. Many γ-herpesviruses encode one or more molecular homologs of BCL-2, the cellular antiapoptotic protein family (Cuconati and White 2002). Viral BCL-2 homologs are also found in adenovirus and poxvirus genomes. Viral BCL-2 proteins act as apoptosis inhibitors and their structure is similar to that of the cellular BLC-2 proteins. However, despite the preservation of the overall structure the unstructured loops that encode sites for phosphorylation and proteolysis, and which may be required for regulation, the function of the cellular proteins differs between vBCL-2 and cellular BCL-2 proteins. All vBCL-2s have a BH1 and at least one other BH2 or BH3 region. Mitochondrial apoptosis is a limiting factor in the life cycle of DNA viruses, because in cells deficient in BAX and BAK the onset of cytopathic effects is delayed and more viral replication can occur. In adenovirus the E1-B19K inhibits the formation of the highly oligomeric BAX/BAK complex at the mitochondrial membrane.

The viral mitochondrial localized inhibitor of apoptosis encoded by HCMV (vMIA) also targets mitochondria but acts differently from the viral BCL-2 homologs (Goldmacher 2002). It has no BCL-2 homology domains and has no affinity to BAX. However, vMIA forms a complex with adenine nucleotide translocator (ANT), a component of the mitochondrial transition pore complex, which cooperates with BAX in the mitochondrial control of apoptosis (Marzo et al. 1998).

CELLULAR EFFECTOR CELLS OF INNATE IMMUNITY

Natural killer cells, effector cells of innate immunity

Natural killer cells are large granular lymphocytes that have potent antiviral effector mechanisms: they kill infected cells using the perforin/granzyme pathway, and release copious amounts of IFN-γ. In many virus infections, viral titers start to decline as a result of NK function well before the adaptive CD8 immune response has any effect. Unlike T cells, NK cells do not have clonally expressed antigen receptors with specificity for viral peptides. Instead, they use a series of germline-encoded receptors to detect the consequences of virus infection on cells, irrespective of the antigenic composition of the infecting virus. Some of these receptors are activating, and some are inhibitory. Signals from these receptors are integrated, leading either to killing and release of IFN, or no action.

For NK cells to control virus, they need (1) to be induced, (2) to traffic to the site of virus infection, (3) to be able to detect virus-infected cells, and (4) to perform their effector functions (killing cells and releasing IFN-γ). Discussion of interference with NK cells is usually limited to the third step – NK recognition of infected cells. NK trafficking and effector mechanisms are very similar to those of CD8 T cells, and are discussed below. NK cells are induced by cytokines – particularly type I interferons and IL-12, which are produced by infected tissues, and viral interference with production of those cytokines affects the NK response.

Natural killer cells integrate signals from activating and inhibitory receptors to achieve activation threshold (Colonna et al. 1999; Fong and Cambier 1999; Long 1999; Ravetch and Lanier 2000; Lanier 2001). It is likely that not all of these receptors and their ligands have been identified. Two important activating receptors are known: CD16 is an Fc receptor that enables NK cells to lyse cells with bound antibody, a process known as antibody-dependent cell-mediated cytotoxicity (ADCC) (see below under Inhibition of humoral immunity). ADCC is not generally considered to play a major role in antiviral immunity, and obviously plays no role in the NK control of the first few days of infection.

A second major activating receptor – NKG2D – has recently been described (Bauer et al. 1999; Wu et al. 1999). NKG2D is a C-type lectin that homodimerizes to generate a functional receptor. Most NK cells express NKG2D, making it an important activating receptor. Through a positively charged residue in its transmembrane domain, it interacts with the immunoreceptor tyrosine based activation motif (ITAM)-containing DAP10, to deliver a strong activating signal to the cell. In addition to NK cells, NKG2D is expressed on activated classic CD8 T cells and on γδ T cells: for these cells, it acts as a costimulatory molecule, i.e. signaling through NKG2D is not enough to activate the cell. For NK cells, however, NKG2D signaling is sufficient for activation. The ligands for NKG2D show loose homology to MHC class I. It is not yet clear whether all ligands have been identified, and the main ligands are different for humans and mice. In humans, MICA and MICB are stress-inducible molecules

expressed mainly in epithelial tissues, and, interestingly, by most carcinomas (Bauer et al. 1999). MIC expression is induced by HCMV infection (Groh et al. 2001). A second set of receptors are the UL16-binding proteins (ULBP), discovered as the targets for HCMV *UL16* (see below) (McNees and Gooding 2002). The ULBP are homologous to one set of murine ligands for NKG2D – the retinoic acid early inducibles (RAE). The other known murine ligand is H60, a minor histocompatibility antigen expressed in BALB/c mice (Diefenbach et al. 2000)

There are two families of inhibitory receptors for human NK cells: the killer immunoglobulin-like receptors (KIR) and the C-type lectin NKG2A/CD94 paired heterodimer. The KIR are members of the immunoglobulin superfamily, and are encoded in a locus on chromosome 19 (Vilches and Parham 2002). They interact with classical MHC class 1 molecules, different members having different specificities. In particular, important inhibitory KIR recognize a limited polymorphism on HLA-C and HLA-B molecules. KIR are expressed on subsets of NK cells; one theory of NK cell development suggests that during their education NK cells must express at least one inhibitory receptor capable of recognizing self-MHC. NKG2A/CD94 interacts with HLA-E, a nonpolymorphic class I molecule that binds peptides derived from the leader sequence of classic MHCI molecules (Braud et al. 1998a). Murine NK cells also have two families of inhibitory receptors. They share NKG2A/CD94 with humans and the murine homolog of HLA-E is Qa1. The second inhibitory receptor family is Ly49, a family of C-type lectins that also recognize polymorphic class I molecules. All of these inhibitory receptors recognize MHCI and they thus enable the NK cells to monitor the function of the class I pathway. Viruses that interfere with the class I antigen-presenting pathway run the risk of rendering infected cells susceptible to NK lysis.

It is important to note that not all KIR and Ly49 molecules are inhibitory. Some lack cytoplasmic tails with immunoreceptor tyrosine-inhibition motifs (ITIM) and instead pair with adaptor molecules to activate cells. Furthermore, some NKG2 family members that recognize HLA-E molecules have an activating function. The function of the inhibitory members of these families in the control of NK lysis appears plausible; the function of the activating members is less clear (with the exception of Ly49H, discussed below).

VIRAL INTERFERENCE WITH NK RECOGNITION OF INFECTED CELLS

Several viruses have been described to interfere with the ability of NK cells to detect infection; this is summarized in Figure 4.

Human cytomegalovirus UL18 is a class I homolog – it shares 25 percent amino acid homology with classic class I, and pairs with host β_2-microglobulin for expression on the surface of infected cells. When it became appreciated that loss of cell surface class I, induced by HCMV infection, would make infected cells vulnerable to NK lysis, the idea that UL18 would engage NK inhibitory receptors and prevent NK lysis became an attractive hypothesis. Indeed Reyburn et al. (1997) used transfected cells expressing UL18 to demonstrate protection from NK lysis in vitro. However, it is controversial whether UL18 functions in this way in vivo. First, cell surface expression of UL18 in infected cells is very low. Second, none of the well-characterized NK inhibitory receptors have been shown to interact with UL18. Third, when a ligand for UL18 was identified (see section The leukocyte immunoglobulin-like receptors below), it was found to be mostly expressed on macrophages, B cells, and DCs, although it is also expressed on some NK cells (Cosman et al. 1997). The ligand, leukocyte immunoglobulin-like receptor 1 (LIR-1), is inhibitory, so it may indeed function to inhibit NK lysis.

Murine CMV also encodes a gene – *m144* – with homology to classic MHCI. The gene *m144* also pairs with β_2-microglobulin to produce a class I-like structure. Mice infected with a virus lacking *m144* control it better than they control wild-type virus (Farrell et al. 1997). The difference in titers is particularly evident 3 days after infection, when NK control is most important. Furthermore, mice depleted of NK cells show no difference in their ability to control wild-type virus or the *m144* deletion mutant. All this suggests that *m144* would inhibit NK recognition of infected cells. However, a direct demonstration that *m144* interacts with NK cells to inhibit their activation is lacking. Although it is clear that *m144* acts somewhere in the NK pathway, the exact site of action remains to be demonstrated. As with UL18, the discovery of other class I-interacting molecules (see The leukocyte immunoglobulin-like receptors below) raises possibilities other than direct interaction with NK inhibitory receptors.

Molluscum contagiosum virus is a poxvirus that establishes persistent infection, unlike other poxviruses. It also encodes an MHCI homolog of unknown function, which complexes with β_2-microglobulin (Senkevich and Moss 1998).

Another MCMV gene – *m152* – has been clearly shown to interfere with NK lysis. The gene *m152* is also loosely related to class I by secondary structure. Its best understood function is interference with antigen presentation to $CD8^+$ T cells (see below under Retention of class I molecules in pre-golgi compartments). However, Krmpotic et al. (2002) found a that a virus lacking *m152* had much lower titers 3 days after infection – a time at which NK cells are the main antiviral mechanism – than wild-type virus. This was surprising because *m152* is known to decrease cell surface expression of classical MHC1, and it was thought that it should therefore make the virus more (not less) vulnerable to

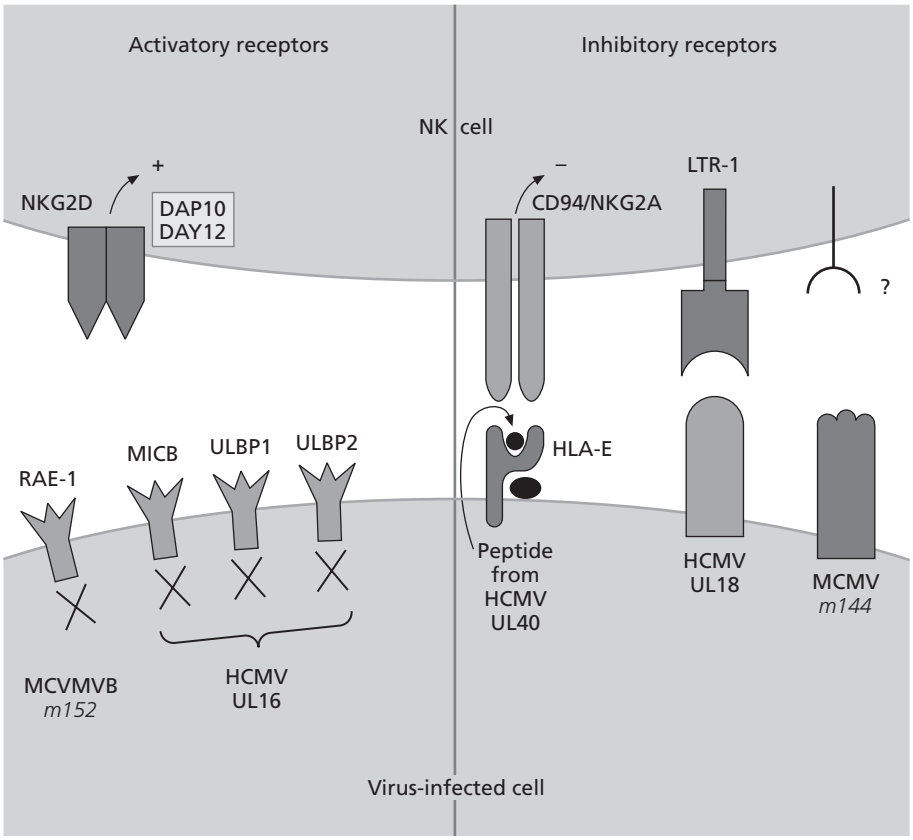

Figure 4 *Viral interference with natural killer (NK)-activating receptors and provision of 'decoys' for inhibitory receptors. See text for abbreviations.*

NK control. The riddle was solved when Krmpotic et al. found that *m152* also reduced the level of an NK ligand on the surface of infected cells. As described above, the RAEs and H60 are loosely class I-like molecules, and serve as a ligand for the important NK-activating receptor NKG2D. Cells infected with a virus lacking *m152* had high ligand levels and were more susceptible to NK lysis than those infected with wild-type virus.

In HCMV a soluble form of the *UL16* gene product was found to interact with human ligands for NKG2D: MICB, ULBP1, and ULBP2 (Cosman et al. 2001). The ULBPs were actually identified by their interaction with UL16. UL16 retains MICB, ULBP1, and ULBP2 inside the cell. In fact, HCMV increases the transcription of all NKG2D ligands, and cell surface expression of MICA and ULBP3, the two ligands not targeted by *UL16*, is increased by HCMV infection. How these contradictory effects translate into susceptibility to or protection from NK lysis is still an open question.

Another NK inhibitory effect has been described for HCMV. As described above, NKG2A/CD94 is an important inhibitory receptor on NK cells. NKG2A recognizes the nonpolymorphic HLA class I molecule HLA-E (Braud et al. 1998a). HLA-E binds peptides derived from the leader sequence of classical class I molecules, which must be transported back into the endoplasmic reticulum by transporter associated with antigen processing (TAP) (Braud et al. 1998b). HLA-E cell surface expression is reduced if classical class I is absent or if TAP is impaired. It thus serves to monitor the integrity of the class I pathway. As the HCMV genes *US2* and *US11* cause rapid destruction of newly synthesized class I (see below under Degradation of newly synthesized MHCI molecules) and *US6* blocks the function of TAP, HCMV infection should render cells vulnerable to NK lysis through silencing of NKG2A/CD94 signaling. However, another HCMV protein, UL40, has in its leader sequence a peptide capable of binding to HLA-E (Tomasec et al. 2000). Furthermore, this peptide remains in the endoplasmic reticulum after signal sequence cleavage and is able to bind to HLA-E in the absence of functional TAP. Thus HCMV is able to circumvent the HLA-E pathway. It is still controversial whether the relative effects of either UL40 or class I downregulation dominate the susceptibility of HCMV-infected cells to NK recognition, i.e. whether the final effect is susceptibility or protection (Falk et al. 2002; Wang et al. 2002a). Again, the final outcome may be different in different cell types, times after infection, etc.

HIV does not directly interfere with NK recognition. However, investigators have noted that HIV-nef interference with class I cell surface expression affects HLA-A and -B molecules but not HLA-C (Collins et al.

1998). As HLA-C isoforms are the most important ligands for KIRs, this may prevent HIV-infected cells becoming vulnerable to NK attack as a result of loss of KIR inhibition.

KSHV-K5 is known to downregulate cell surface classic class I molecules. However, it also downregulates intercellular adhesion molecule 1 (ICAM-1) and B7-1, known to be important for NK cell lysis (Ishido et al. 2000). In consequence, K5 expression protects transfected cells from NK lysis.

A viral gene has been reported which, instead of protecting from NK lysis, actually renders infected cells susceptible to a protective NK response. It was known that a gene in the NK locus has a major effect on the susceptibility of different mouse strains to MCMV. Mice from the resistant strains have a more effective NK cell response. It was recently discovered that the gene controlling resistance is *Ly49H* – an activating receptor found on a subset of NK cells. The surprising finding was that the ligand for *Ly49H* is not, as had been presumed, a host-encoded molecule upregulated by infection, but is actually a viral gene product encoded by the gene *m157* (Arase et al. 2002; Smith et al. 2002). The gene *m157* has no known other function and is dispensable for virus replication in vitro and in vivo. It also interacts with an inhibitory receptor, *Ly49C*, and investigators have postulated that this is its true evolutionary function.

THE LEUKOCYTE IMMUNOGLOBULIN-LIKE RECEPTORS

Human CMV encodes a molecule – UL18 – with 25 percent homology to MHCI heavy chain. As described above, it was assumed that UL18 would interact with KIRs and prevent NK lysis of HCMV-infected cells. This idea was supported when it was shown that cells transfected with UL18 were resistant to lysis by some NK cells (Reyburn et al. 1997). However, identification of the ligand for UL18 led to the discovery of an entirely new gene family – the leukocyte immunoglobulin-like receptors (LIR) (Cosman et al. 2001). The LIRs are structurally related to the KIRs and human IgA Fc-receptor, and are encoded in close proximity to those genes on chromosome 19. There are eight LIRs – all but one are membrane glycoproteins; some contain ITIMs, whereas others have an arginine in their transmembrane region which suggests that they pair with another chain and have an activating function (Fanger et al. 1999). The ligand identified for UL18 is LIR-1; its cytoplasmic tail contains ITIMs. It is expressed on most B cells and monocytes and a variable number of T cells and NK cells. In addition to UL18, LIR-1 binds to most HLA-A, -B, and -C molecules. The physiological function of this molecule, and hence what HCMV achieves by encoding a ligand for it, is currently unknown.

Most of the other LIRs are also expressed on monocyte and DCs but not on lymphocytes. LIR-2 also binds class I molecules, but the ligands for the other LIRs are not yet identified. The discovery of this family indicates that the role of MHCI in immunobiology is not yet fully understood, and this should be taken into account when considering the function of viral genes that inhibit class I expression.

EFFECTS ON THE ADAPTIVE IMMUNE RESPONSE

Viral interference with DC functions

The cellular arm of the adaptive immune response consists of CD4 and CD8 T cells. CD8 T cells are the most important effector arm of adaptive antiviral immunity. The effective antiviral T-cell response requires orchestration. Antigen-presenting cells (APC), usually DCs, capture antigen and travel from the site of infection to the draining lymph nodes, where they activate naïve T cells. Naïve T cells that recognize antigen on DCs remain trapped in the lymph node. They start to proliferate for several days, and acquire the effector phenotype. Activated effector T cells can enter tissues. This is essential for CD8 T cells to interact directly with and to lyse virus-infected cells. Infected tissues provide chemokine and cytokine signals that enable T cells to extravasate and direct them to the site of infection. In the tissues, CD8 T cells have two main effector mechanisms that are antiviral: cytolysis of infected cells and release of antiviral cytokines, especially IFN-γ. CD4 T cells can make antiviral cytokines, but also provide critical help for CD8 T cells and for antibody production. Viruses have found several points of intervention within this process. These will now be dealt with individually: first, processes that would affect both CD4 and CD8 T cells, followed by mechanisms specific for each cell type.

Among APCs, mainly DCs are able to activate naïve T cells. DCs are generated in the bone marrow, circulate in the blood, and enter tissues in an immature state. Immature DCs are active in phagocytosis, but are poor APCs as a result of intracellular retention of class I and class II molecules and low expression of costimulatory molecules such as CD80 and CD86. Immature DCs can phagocytose dying cells, cellular debris, immune complexes, and proteins. They can also be infected by many viruses. In the context of adequate mediators produced during infection, they differentiate to mature DCs that are extremely potent APCs and stimulators of T cells. Maturation also triggers their migration into the lymphatics and to the T-cell areas of draining lymph nodes. Maturation signals produced during virus infection include type I IFN from infected cells and TNF-α from tissue macrophages. DCs have two main ways of

activating CD8 T cells to initiate the T-cell response to viruses. First, infected DCs can present antigen through the classic endogenous pathway of MHCI-restricted antigen presentation. Second, they can 'crosspresent' antigens that they acquire by phagocytosis of proteins, which are degraded to peptides for loading on to their class I molecules. Sources of material that can be 'cross-presented' include cells dying by either apoptosis or necrosis; cellular debris, immune complexes, and perhaps heat shock proteins from dying cells that have bound viral peptides. Crosspresentation is described in Figure 5. The relative importance of the two DC presentation pathways depends on (1) the ability of viruses to directly infect DCs and (2) the extent to which viruses interfere with the direct presentation pathway (Figure 5).

For CD4 T cells, crosspresentation is the classical pathway of antigen presentation and is called exogenous presentation. Still controversial is the role of loading of class II molecules with endogenously synthesized viral proteins in infected class II-positive cells. This is discussed further below. Like CD8 T cells, naïve CD4 T cells are found only in blood and secondary lymphoid tissue, and they depend on trafficking of DCs to lymph nodes to be activated. Mature DCs that present viral antigen to naïve T cells are critical for the initiation of a T-cell response. There are three aspects of this with which viruses can interfere:

1 Interference with DC trafficking: DCs enter uninfected tissue, but they enter sites of infection in much greater numbers. Interference with chemokines and cytokines could affect migration.
2 Interference with the ability of DCs to present viral antigen: many viruses interfere with the class I antigen processing and presentation pathway. This would interfere with and perhaps prevent directly infected DCs from activating T cells, but would not interfere with crosspresentation. Although viruses might also be able to interfere with crosspresentation (perhaps via a secreted product), so far no such mechanism has been described.
3 Interference with the ability of immature DCs to mature, which is essential for APC function. The normal response of a DC to virus infection is to mature into an APC. Type I IFNs, induced by most virus infections, are powerful DC maturation stimuli. However, several viruses have been described that do not initiate maturation of DCs, and in fact interfere with the ability of the DCs to mature in response to other stimuli (Figure 6).

A well-studied viral interference with DC function occurs during measles virus infection; this infection elicits good protective immunity, including CD8 T cells, and yet is associated with a profound immunosuppression which supports additional infection by other infectious agents. This superinfection contributes to the morbidity and mortality associated with measles. The interaction of measles virus and DCs is complex. Measles virus infects DCs and induces their maturation and IL-12 production (Schnorr et al. 1997). This stimulates virus replication in the infected DCs. However, measles virus-infected DCs inhibit rather than promote proliferation of T cells. A few infected DCs are enough to inhibit the ability of a large number of uninfected

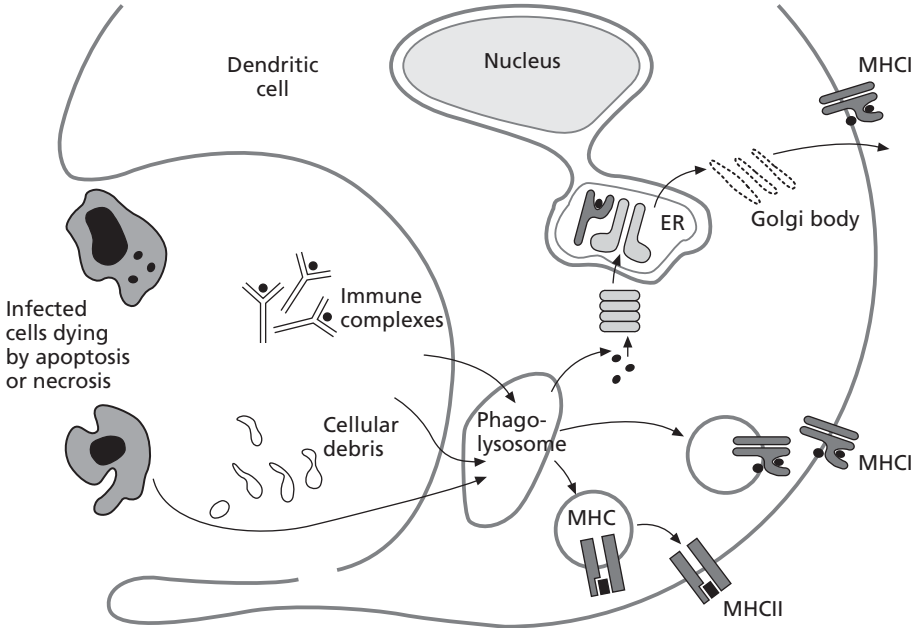

Figure 5 *Crosspresentation of viral antigens to CD8 T cells: activation of antiviral T cells despite immune evasion genes. Phagocytosomal material is re-presented on the dendritic cell's MHC molecules. The pathways for crosspresentation via MHCI are still poorly defined.*

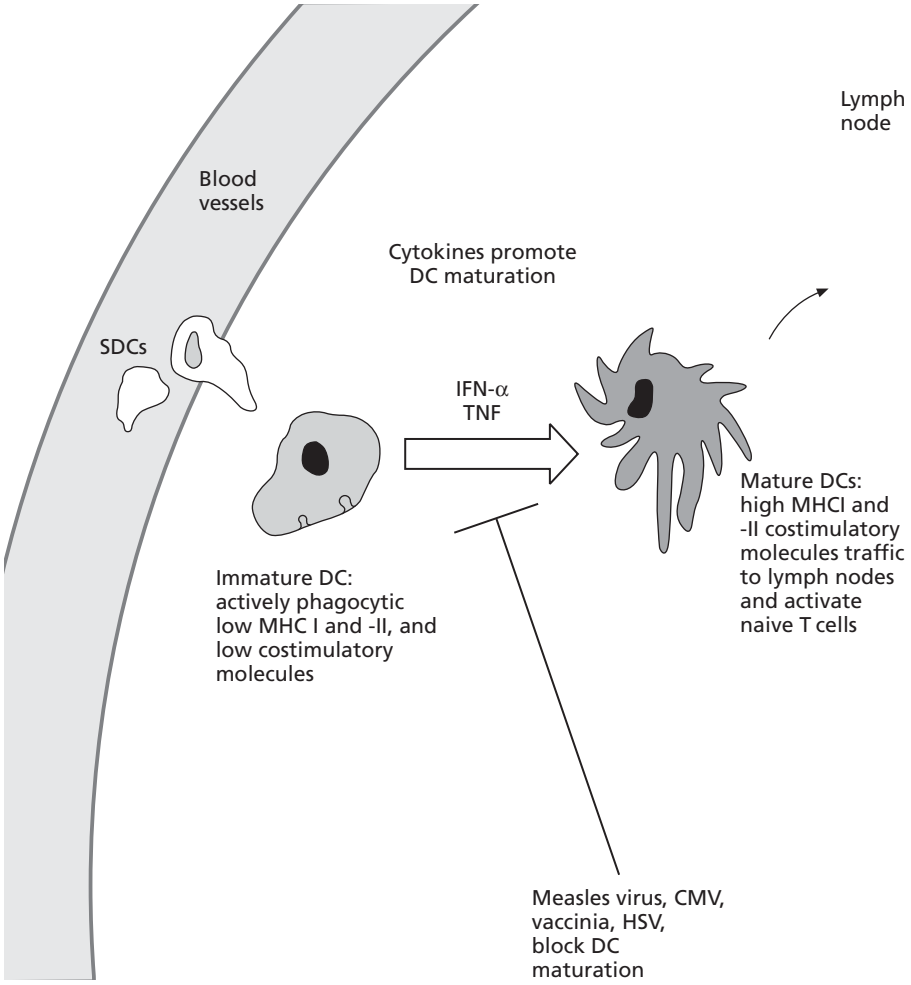

Figure 6 *Viral interference with dendritic cell (DC) maturation or function. CMV, cytomegalovirus; IFN-α, interferon α; TNF, tumor necrosis factor.*

DCs to promote T-cell proliferation (Grosjean et al. 1997; Schnorr et al. 1997). Measles virus induces the expression of the apoptosis-inducing ligand TRAIL on DCs (Vidalain et al. 2000); T cells and DCs cocultured with measles virus both undergo apoptosis (Fugier-Vivier et al. 1997; Servet-Delprat et al. 2000). Measles virus interference with DC function is thought to be the major mechanism of the generalized immunosuppression associated with MV infection.

Engelmayer et al. (1999) reported that VV impaired maturation of human DCs, and concluded that this would impair immunity. Drillien et al. (2000) also noted that VV inhibited maturation of human monocyte-derived DCs. However, VV-infected DCs efficiently stimulate memory T cells from infected individuals. In fact, infected DCs have been observed interacting with T cells in vivo (Norbury et al. 2002). Another study in mice concluded that both direct presentation by virus-infected DCs and crosspresentation contribute to CD8 T-cell priming in VV-infected mice (Basta et al. 2002). VV, the vaccine for smallpox, is the poster child of good viral immunogens. If VV does impair DC function, this effect is presumably mild and does not prevent an immune response.

Inhibition of DC maturation has also been reported for HSV (Salio et al. 1999) and MCMV (Andrews et al. 2001). The CD8 T-cell response to MCMV is thought to be largely achieved by crosspresentation (Gold et al. 2002), which suggests that functional DC impairment could play a role. HCMV is also thought to affect DC function. Moutaftsi et al. (2002) reported that HCMV impaired DC maturation. Mature DCs, on the other hand, were reported to increase costimulatory molecules after HCMV infection (Raftery et al. 2001). They also expressed the apoptosis-inducing ligands, FasL and TRAIL, suggesting that they might induce a generalized immunosuppression similar to measles.

Interference with antigen presentation in the MHC class I pathway

The most widely studied and best-appreciated viral immune evasion mechanisms have been found to affect

CD8 T-cell recognition of virus-infected cells. There are two basic strategies: viruses can interfere with the MHCI pathway of antigen presentation, or they can mutate the epitopes that CD8 T cells recognize, forcing the immune system to generate new effectors from naïve precursors.

Several viruses, mostly herpesviruses, have evolved genes that interfere with the MHCI pathway. Immune evasion genes of this type have been called viral genes that interfere with antigen presentation (VIPR) (Yewdell and Hill 2002). The pathway of MHCI-mediated antigen presentation is quite complex, and the past few years have seen a flurry of discoveries of viral gene products that interfere, to varying degrees of specificity, with almost every aspect of this pathway. Some viruses – notably CMV – actually encode several genes that all target this pathway. We begin this section by following the class I antigen presentation pathway and describing the various mechanisms that viruses use to attack it.

ANTIGEN DEGRADATION

The first step in antigen processing for the MHCI pathway is degradation of viral proteins in the cytosol, mostly by the proteasome. Proteasome function is vital for the health of the cells, and few viruses target this aspect. However, the EBNA-1 protein of EBV contains a long segment of repeated glycine and alanine residues, which renders the protein resistant to proteasomal degradation, and peptides for cytotoxic T lymphocyte (CTL) recognition are not generated (Levitskaya et al. 1995). EBNA-1 is the only protein that EBV needs to be expressed during latency, and this mechanism may help to prevent eradication of the latent virus pool.

When HCMV infects a cell the phosphoprotein pp65, a structural protein that has kinase activity, is also introduced. This protein is apparently able to prevent presentation of the immediate early (IE) peptide of the immunodominant IE1 protein, perhaps by phosphorylating IE1 and altering its proteolysis (Gilbert et al. 1996) (Figure 7).

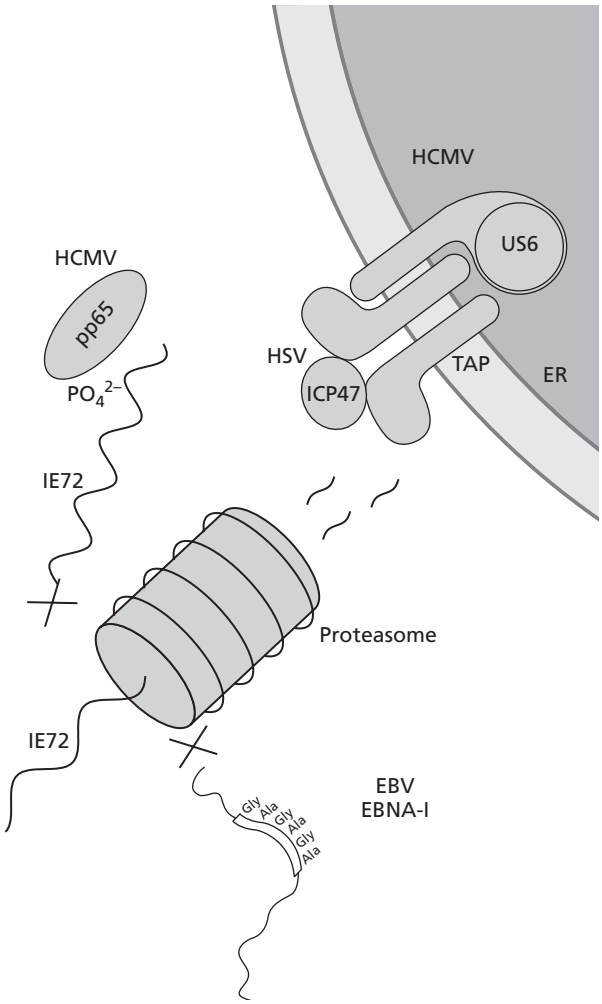

Figure 7 *Interference with production of peptides and transport into the endoplasmic reticulum (ER). See text for abbreviations.*

TRANSPORT OF PEPTIDES BY TAP

Peptides are transported by the TAP from the cytosol into the endoplasmic reticulum where they can bind to class I molecules. TAP is a member of the seven-transmembrane ATP-binding cassette family of transporters, and appears to have no other function. It can thus be incapacitated by viruses without impairing other cellular functions that the virus may need. TAP is specifically targeted by at least two VIPRs. The HSV IE gene *US12* encodes a polypeptide of 87 amino acids, ICP47, which is abundantly expressed in the cytosol (York et al. 1994). It binds to TAP and inhibits peptide transport, with the result that newly synthesized class I molecules are unable to leave the endoplasmic reticulum, and antigen presentation is prevented (Fruh et al. 1995; Hill et al. 1995). Both HSV-1 and HSV-2 encode ICP47; as a result of a frame shift mutation, they are homologous only in their first 35 amino acids, and this region of the molecule is all that is required for inhibition of TAP (Galocha et al. 1997).

The HCMV protein US6 also binds to TAP and inhibits peptide transport, although not as potently as ICP47 (Ahn et al. 1997; Hengel et al. 1997; Lehner et al. 1997). US6 is a transmembrane glycoprotein that binds TAP on the luminal side of the endoplasmic reticulum.

THE PEPTIDE-LOADING COMPLEX

In the endoplasmic reticulum, MHCI molecules associate with TAP as part of a peptide loading complex that also includes tapasin, calreticulin, and a thiol oxidoreductase. This complex not only ensures that class I is in the optimal place to acquire peptides, but also orchestrates peptide loading and provides poorly understood quality control functions. Several VIPRs associate with this complex, but it is not understood whether this is a coincidental consequence of binding their

main targets or whether it plays a role in their interference with the pathway. The adenovirus protein Ad5E3 19K binds TAP and interferes with the ability of tapasin to bring class I and TAP together in the peptide-loading complex (Bennett et al. 1999). This function may supplement its ability to retain class I (see below under Retention of class I molecules in pre-golgi compartments).

SYNTHESIS OF MHCI MOLECULES

Many viruses interfere with host gene expression, either globally or selectively. As newly synthesized class I molecules are required for peptide loading, interference with synthesis of MHCI affects antigen presentation. However, the extent to which this mechanism would affect the antiviral efficacy of CTL depends on a number of timing issues: the half-life of class I RNA (if transcription but not RNA stability is affected); the average residence time of MHCI in the endoplasmic reticulum (which varies considerably between class I isoforms); the timing in the viral replication cycle of antigen expression (the earliest expressed proteins and structural viral proteins that can be processed immediately upon viral entry are less affected); and the length of the viral replication cycle (viruses with long replication cycles can gain more from mechanisms that would only impact CTL recognition after a lag time of many hours or days). One virus for which interference with MHCI synthesis has a clear impact on CTL efficacy is HSV. The viral host protein shut off (vhs) factor encoded by HSV UL40 is a virion structural protein. Upon virus entry it strips RNAs from ribosomes, allowing viral transcripts to be translated at the expense of host transcripts. The function is especially potent in HSV-2: within half an hour of virus infection synthesis of host proteins, including MHCI, essentially ceases. HSV UL40 has been shown to affect CTL recognition of infected cells (Tigges et al. 1996).

DEGRADATION OF NEWLY SYNTHESIZED MHCI MOLECULES

One of the most effective VIPR mechanisms is that employed by the HCMV proteins US2 and US11. These proteins bind MHCI in the endoplasmic reticulum and cause it to be transported back through the translocon. In the cytosol, MHCI is ubiquitinylated and destroyed by the proteasome (Wiertz et al. 1996a,b). The process is very potent and in HCMV-infected fibroblasts metabolic labeling reveals very little class I (Beersma et al. 1993). These particular genes have been intensely studied, not just because of their immune evasion function, but also because they have helped to elucidate an important cell biological pathway – the degradation of endoplasmic reticulum proteins. US2 and US11 are both type I membrane glycoproteins, and both bind MHCI via their luminal domains. However, they are not structurally homologous, and show some differences in the way that they achieve MHCI destruction, e.g. the carboxyl end of US2, but not US11, is required for MHCI degradation (Furman et al. 2002). The cytoplasmic tail of MHCI is, however, required (Story et al. 1999). These molecules show a specificity for class I isoforms and degrade HLA-A and -B, but not HLA-C, molecules (Schust et al. 1998). HLA-C can present peptide antigens to CTL, but it also has an important role as ligand for inhibitory KIR receptors on NK cells (see above).

The mouse γ2 herpesvirus 68 also causes destruction of class I molecules. The K3 protein of MHV-68 is a ubiquitin ligase: it ubiquitinylates the cytoplasmic tail of endoplasmic reticulum-resident MHCI, targeting it for destruction in the cytosol (Boname and Stevenson 2001). Interestingly, the highly homologous K3 and K5 proteins of KSHV also ubiquitinylate MHCI, but this serves to target cell surface MHCI for degradation (Figure 8).

RETENTION OF CLASS I MOLECULES IN PRE-GOLGI COMPARTMENTS

Several VIPRs prevent MHCI from trafficking to the cell surface without causing their destruction. The adenovirus 5 protein E3 19K contains a di-lysine motif in its cytoplasmic tail; this motif binds to coat protein 1 (COP-1), which retrieves vesicles from the Golgi and returns them to the endoplasmic reticulum (Jefferies and Burgert 1990). In fact, this pathway was identified because of the E3 19K protein. The result is that MHC I is retained in the endoplasmic reticulum. Ad5E3 19K does not interact with all MHCI molecules equally, and the result is that some MHCI isoforms are more efficiently retained than others.

The HCMV protein US3 also causes MHCI to be retained in the endoplasmic reticulum. US3 binds to peptide-loaded MHCI molecules. The mechanism of retention is not clear, because US3 does not contain an endoplasmic reticulum retention motif, and is in fact exported (Ahn et al. 1996; Jones et al. 1996). It is thought that the mechanism of retention involves repeated release and rebinding by newly synthesized US3 (Gruhler et al. 2000). As with most of the HCMV VIPRs, US3 has been studied primarily in transfected cells. In infected cells, the destruction of newly synthesized MHCI as a result of US2 and US11 is so rapid that the role of US3 is hard to appreciate. US3 is synthesized earlier in the viral replication cycle than US2 and US11, and it has been hypothesized that it prepares MHCI for destruction by US2 and US11 (Ahn et al. 1996).

Murine CMV also encodes a VIPR that retains class I molecules. The *m152/gp40* causes MHCI to be retained in the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and, as with US3, the mechanism is unclear (Ziegler et al. 1997). It has quite different efficacy against different MHCI isoforms (Kavanagh et al. 2001). Yet even for isoforms in which retention is not especially impressive (e.g. Kb, which is only 50 percent retained by *m152*), *m152* has a profound

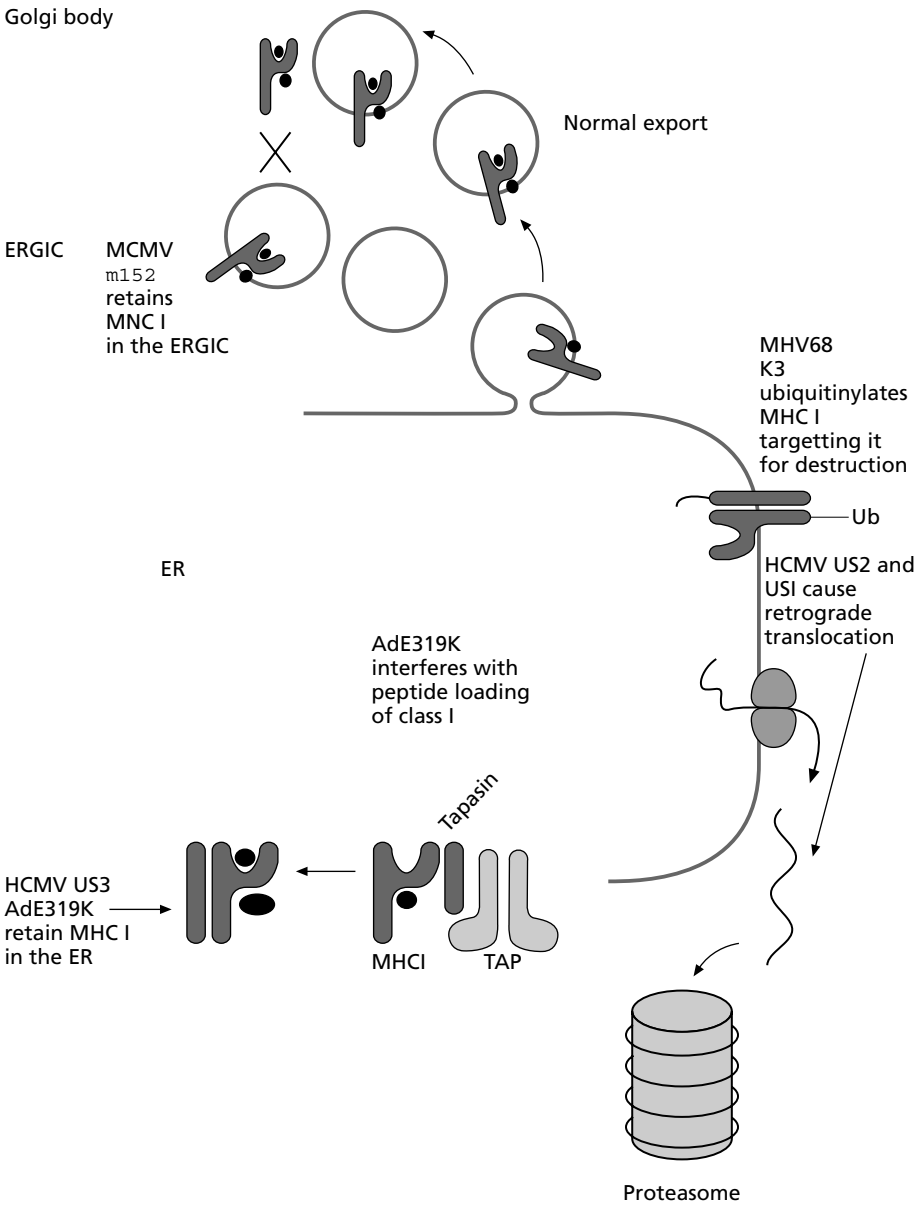

Figure 8 *Interference with MHC class I in the endoplasmic reticulum (ER) and endoplasmic reticulum Golgi intermediate compartment (ERGIC).*

effect on antigen presentation. Cells infected with wild-type virus are not detected by CTL, but cells infected with a virus lacking *m152* are readily detected. To prevent recognition of wild-type-infected cells by Kb-restricted CTL, the virus needs the two other MCMV VIPRs – *m4* and *m6* – the mechanisms of which are discussed below. Even so, the importance of *m152* remains enigmatic, and it may indicate that there is more profound synergism in the functions of these genes than is appreciated. Something similar may explain why HCMV needs its four VIPRs. As discussed above, in addition to retaining classical MHCI molecules, *m152* also prevents surface expression of molecules, which activates NK cells (Krmpotic et al. 2002).

REMOVAL OF MHCI MOLECULES FROM THE GOLGI APPARATUS OR THE CELL SURFACE

Several VIPRs remove class I from the cell surface. The KSHV genes *K3* and *K5* both reduce cell-surface MHCI expression. K3 ubiquitinylates MHCI after endoplasmic reticulum export. This signals MHCI internalization from the plasma membrane for endosomal degradation (Hewitt et al. 2002). The MCMV protein m6 contains a di-leucine motif in its cytoplasmic tail, which sorts it to lysosomes. It binds MHCI in the endoplasmic reticulum, and targets MHCI to the lysosome for destruction (Reusch et al. 1999). Of the three MCMV VIPRs, m6 has by far the most profound effect on cell-surface class I; from about 6 h after infection MHCI is rapidly lost

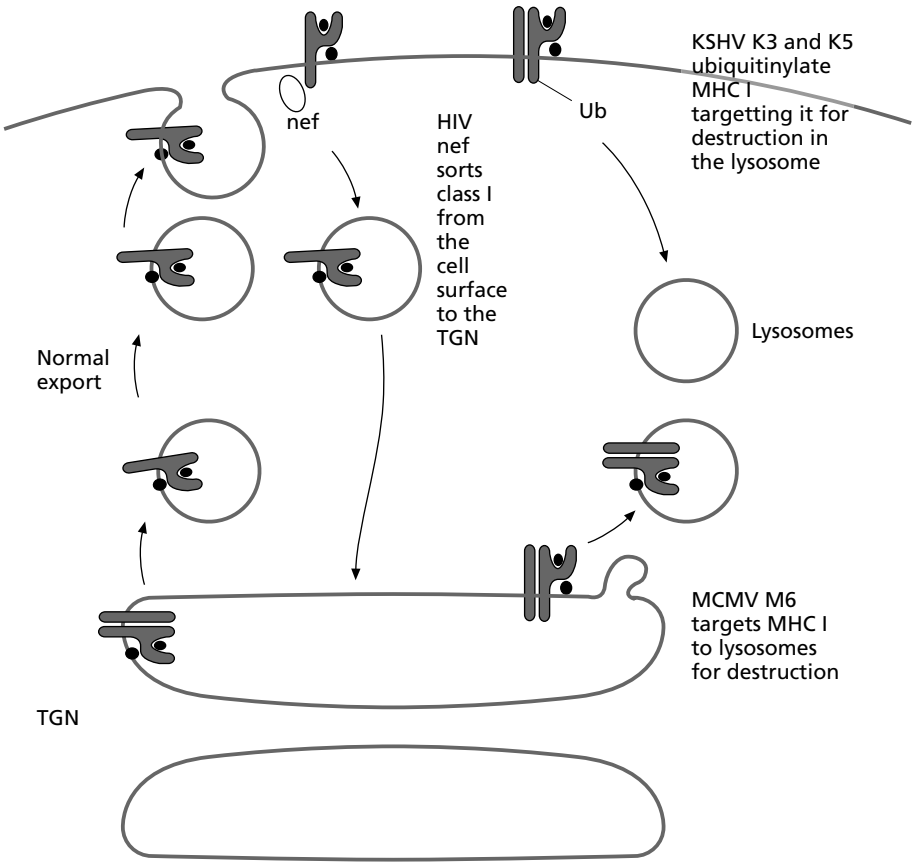

Figure 9 *Interference with MHC class I at the Golgi body and cell surface.*

from the cell surface if m6 is present (Wagner et al. 2002).

The HIV (and simian immunodeficiency virus (SIV)) nef protein serves a number of functions. One of them is to remove MHCI from the cell surface and take it to the trans Golgi network (TGN) – an effect that has been shown to impair CTL destruction of HIV-infected cells. The mechanism of action of HIV nef is quite different from that of KSHV-K3. Nef binds to the PACS-1 protein that sorts molecules to the TGN: presumably it acts as a bridge between class I and PACS-1 (Piguet et al. 2000) (Figure 9).

The MCMV protein m4/gp34 binds to MHCI both in the endoplasmic reticulum and at the cell surface. m4 is necessary to prevent lysis of infected cells by K^b-restricted CTLs (Kavanagh et al. 2001). The mechanism of action of m4 is not understood, but it is likely that its association with class I at the cell surface prevents the productive engagement of the TCR.

CD8 T-cell epitope variation

With the exception of nef, all the VIPRs described above are encoded by large DNA viruses, the coding capacity of which gives them the luxury of such highly specialized immune evasion devices. Small RNA viruses have also other means of avoiding CTLs. As a result of their rapid mutation rate, they exist in nature as a diverse genetic mix, often referred to as a 'viral swarm'. Mutations in CTL epitopes should allow the selection of escape variants, unless the exact epitope sequence is crucial for viral replicative function. This means that, as CTL clones develop and become immunodominant, virus variants arise that escape detection. Whether CTL escape mutants help the virus to persist in a host has been a matter of debate for some time. However, recent evidence in both monkeys and humans demonstrates that mutants of a particular epitope have a selective advantage in hosts that possess the MHCI molecule capable of binding the epitope, but not in hosts that do not possess that allele (Allen et al. 2000; Goulder et al. 2001).

Significance of viral interference with CD8 function in vivo

Most of the mechanisms reported above have been identified in vitro, and many of them have been described only using cells transfected with an isolated viral gene driven by a strong promoter. Their significance for infected cell recognition by CD8 T cells in vivo remains to be demonstrated. Obviously, true in vivo studies can be carried out only in animal models. The mouse is by far the most useful model for this purpose, although the importance of the HIV epidemic has led to studies of

SIV in infected macaques. As a result of the high degree of variation between the MHCs of different species, and the specificity of VIPRs for MHCI isofoms, studies of immune evasion need to be conducted in the natural virus host. For mice, MCMV is the most useful model, although the vole virus MHV-68 infects laboratory mice well and has provided important insights.

The first VIPR to be studied in vivo was the MCMV gene *m152*. Although wild-type MCMV-infected cells often escape detection by CTLs in vitro, cells infected with viruses lacking *m152* are always readily detected by CTLs. Krmpotic et al. showed that a virus lacking *m152* grew to about a log lower titer at day 7 (the time of peak CTL activity) than wild-type virus, but grew to the same titers in CD8 T-cell-deficient animals (Krmpotic et al. 1999). This effect was modest given the impact that *m152* has on CTL recognition in vitro, and challenges our concept of CD8 T-cell effector function in vivo. It has further been noted that a viral epitope whose presentation is completely abolished by *m152* in vitro is nevertheless equally immunodominant in mice infected with wild-type virus and with virus lacking *m152*, at least during the acute response. This suggests that cross-presentation is the main mode of antigen presentation driving the acute CTL response (Gold et al. 2002). However, viruses lacking *m152*, or indeed all three VIPRs, continue to provoke a very strong CTL response throughout the life of the animal. Therefore, it is unclear how the VIPRs of MCMV modulate the immunobiology of the virus.

MHV-68 K3 downregulates MHCI and prevents CTL recognition of infected cells. MHV-68 has a complex pattern of infection, analogous to EBV (Doherty et al. 2001). After initial replication in the lungs, which is controlled by IFNs and to some extent by CD4 T cells and CD8 T cells, MHV-68 seeds the splenic B cells. These undergo a massive CD4-dependent proliferation, with concomitant amplification of the latent virus load. This situation is brought under control by the immune response, and a truly latent infection is established. Although expression of viral genes is limited during the splenic amplification phase, K3 is expressed. A virus lacking K3 grew to normal titers in the initial lung infection, but did not undergo splenic amplification unless CD8 T cells were removed (Stevenson et al. 2002). Thus this VIPR function is thought to be to prevent CD8 T-cell control of the splenic amplification stage.

The selective advantage of CTL escape mutants in SIV and HIV infection in vivo has been clearly established, as described above under CD8 T-cell epitope variation. There is also evidence that the MHCI downregulating ability of nef is important. As nef has multiple functions, a specific mutant that affected its ability to downregulate MHCI, but not CD4, molecules was made. The mutant and wild-type viruses grew equally well in vitro. However, because the mutation affected a single codon, a mutation back to the wild-type sequence occurred in all four animals infected with the mutant virus within a few weeks (Munch et al. 2001). This suggested a strong selective pressure for the ability to downregulate MHCI.

Modulation of MHCII expression

CD4 T cells play a role in antiviral immunity, but the exact role is controversial. They provide essential help for B cells, and effective CD8 memory also depends on CD4 T cells. Two mechanisms may be involved in CD4 T-cell help for CD8 T cells: secretion of growth-supporting cytokines such as IL-2 and activation of DCs for activation of CD8 T cells. This so-called 'licensing' role requires direct recognition of the DCs by the CD4 T cell. CD4 T cells may also be direct antiviral effectors in their own right. They synthesize antiviral cytokines, especially IFN-γ, and if stimulated by professional APCs in the vicinity of the infection their role in secreting IFN-γ may be significant. Also, some viruses replicate in professional APCs that express MHCII, and many somatic cells are induced to express MHCII by IFN-γ. If these cells express class II loaded with viral antigen, CD4 T cells may have a direct antiviral effect by either killing the infected cell or secretion of cytokines.

Several viruses decrease the cell surface expression of class II by interfering with its synthesis. Most non-hemopoietic cells do not express MHCII, but (at least in humans) can be induced to do so by IFN-γ. Viruses such as herpesviruses and adenoviruses that interfere with IFN-γ signaling pathways will therefore interfere with induction of class II on these cells. MCMV induces the secretion of IL-10 by macrophages, and this leads to a decrease in macrophage MHCII expression (Redpath et al. 1999). Cells infected with HCMV also have decreased cell surface MHCII as a result of transcriptional inhibition of MHCII (Sedmak et al. 1994).

Some of the same HCMV proteins that interfere with MHCI expression have also been shown – in transfected cells – to interfere with MHCII in a similar manner. US2 causes HLA-DRα chains to be re-transported into the cytosol and degraded by the proteasome. Interestingly, it has the same effect on the α chain of HLA-DM, the essential chaperone for peptide loading on to class II (Tomazin et al. 1999). US3 has also been shown to cause intracellular MHCII retention. (Hegde et al. 2002). From the above, it is clear that there may be some advantages to viruses in interfering with MHCII molecules, but in general the functional evidence for interference with presentation of endogenous viral antigens is lacking, and there are no in vivo data. The real significance of these phenomena remains to be demonstrated.

Superantigens

Some viruses manipulate the immune system by stimulating T cells of irrelevant specificity. The B-type

retrovirus, mouse mammary tumor virus (MMTV), encodes a superantigen that binds to certain TCR β chains and to MHCII, causing a massive proliferation of all CD4 T cells with the appropriate TCR. These activated T cells provide the necessary stimulation to drive proliferation of virus-infected B cells, which is essential for the infective process (Huber et al. 1994). EBV also has superantigen-like activity. Proliferation of CD4 T cells expressing the V-β13 TCR chain is a feature of EBV infection (Sutkowski et al. 1996). It has recently been shown that EBV activates an endogenous human retrovirus, HERV-K18, the envelope glycoprotein of which has superantigen activity (Sutkowski et al. 2001). Whether this activity plays a role in EBV pathogenesis is unclear but, as EBV undergoes latency in B cells, concomitant B-cell proliferation could aid the establishment of a latent virus pool.

Inhibition of humoral immunity

The production of antibodies specific to viral antigens may neutralize virus particles and thus prevent the infection of susceptible cells. Antibodies bound to viral antigens of cells can be detected by Fc-receptor-carrying effector cells. Viral antigens can activate the complement cascade, directly or in association with antibodies, leading to the destruction of infected cells or enveloped virus particles. Viruses with an RNA genome accumulate mutations caused by the low fidelity of the RNA polymerase. Virus particles with different antigenic properties may evade recognition by specific antibodies. Genetic variability may also generate variant peptide sequences representing new antigens or losing the ability to bind to MHC molecules, thus evading recognition by the cellular immune system as well.

Herpes simples virus and other viruses encode receptors for the Fc domain of IgG to evade the action of antibodies (Johnson and Hill 1998) (Figure 10).

In the case of HSV, glycoproteins gE and gI form a heterodimeric Fc receptor that participates in a process termed 'bipolar bridging', in which antibodies bind to their specific HSV antigen and simultaneously interact with gE–gI (Lehner et al. 1975; Frank and Friedman 1989). In this way, the viral Fc receptors inhibit complement-mediated antibody neutralization and antibody-dependent cellular cytotoxicity by cells carrying Fc receptors. HSV gE–gI may also bind other ligands and it has been suggested that it acts as a receptor-binding glycoprotein in the process of cell-to-cell spread.

Several members of the herpesvirus family encode homologs of complement regulatory proteins that block the complement cascade in several ways (Johnson and Hill 1998; Favoreel et al. 2003).

The HSV glycoprotein gC binds the complement factor C3; it protects cell-free virus from complement-mediated neutralization and inhibits the lysis of HSV-infected cells by antibody and complement (Lubinski et al. 1999). HSV gC also functions in virus attachment to cell-surface glycosaminoglycans, especially on the basolateral surfaces of epithelial cells (Sears et al. 1991); thus it has been difficult to interpret the attenuated phenotype of gC virus mutants in vivo (Lubinski et al. 1999). However, in C3 knock-out mice, the disease caused by the gC virus mutant was similar to that of wild-type HSV, suggesting a major role of gC in resistance to complement (Lubinski et al. 1999). HVS, a virus that infects monkeys, encodes a homolog of the

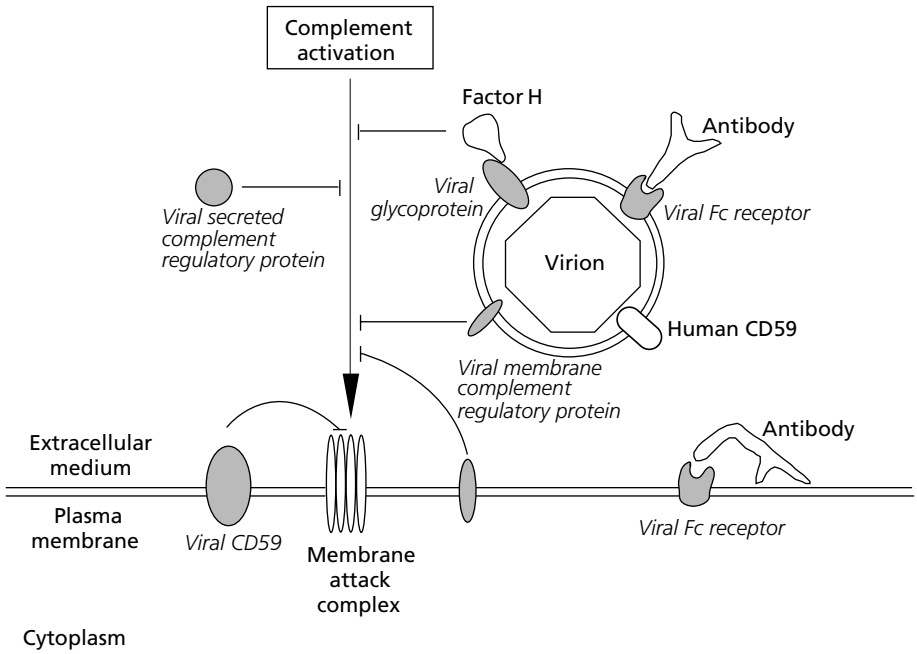

Figure 10 *Viral modulation of complement and antibody responses.*

inhibitor of the membrane attack complex CD59, which protects the infected cells from complement-mediated lysis (Rother et al. 1994).

A strategy used by HIV is to recruit the soluble factor H that inhibits complement activation by interacting with the gp120–gp41 complex (Stoiber et al. 1996). Removal of factor H from human serum augments the effect of complement on HIV-infected cells. The poxviruses variola virus (VaV), VV, and CPV encode a secreted protein, known as viral complement control protein (VCP), which blocks the classic and alternate pathways of complement activation (Kotwal et al. 1990; Rosengard et al. 2002). Inactivation of the VCP gene in VV causes virus attenuation in animal models, demonstrating an important role of this protein in preventing host defense mechanisms and a role for complement in antiviral immunity (Isaacs et al. 1992). Interestingly, inactivation of VCP from CPV caused a substantial increase in the inflammatory response in infected mice compared with wild-type CPV infections (Miller et al. 1997). This illustrated that inhibition of the inflammatory response by viral proteins may reduce tissue damage and allow virus replication in host cells that would otherwise be eliminated by the immune response.

Instead of encoding viral homologs of host proteins, HIV, VV, and HCMV, incorporate into the virion envelope complement regulatory proteins, such as CD56 and CD59, which confer complement resistance to the virus particle (Spear et al. 1995; Vanderplasschen et al. 1998)

CONCLUSIONS

A simplistic view of a virus–host interaction is that of a fierce competition between the virus infecting and replicating in a cell until its death, and then spreading to uninfected cells and tissues, whereas the immune system of the host seeks to eliminate all infected cells. This may be true for viruses that are restricted in their growth to mucosal surfaces, those that replicate fast, and those that are directly transmitted. Many viruses replicate slowly and have a tropism only for specific tissues in certain organs to which they have to be transported or disseminated, and often the sites of entry and the sites of release differ. Cell tropism is defined not only by the virus' inability to infect other tissues, but also as a result of the stringent immune control of the host which limits viral spread. Under conditions of immune suppression herpesviruses can infect many tissues, showing that the restriction of the infection to certain sites reflects the virus–host balance of the immunocompetent host. Therefore, the diversity in the biology of closely related viruses has been shaped by evolution, and the biological diversity reflects the function of viral proteins that have been selected to modulate cellular functions.

To describe viral functions as immunoevasive may have been correct for those that directly interfered with antigen processing in the MHCI and MHCII pathways. The reader is cautioned to treat the growing list of viral immune evasion strategies with some skepticism. These strategies are best regarded as modifiers of the immune response, not as immune evasion, e.g. despite the mechanisms described for herpesviruses, they are highly susceptible to NK control in vivo. In fact, recurrent herpesvirus infection is the clinical hallmark of NK deficiency. Later, a host of viral effects on cell functions were also listed as immunoevasive. However, many of these functions affect not only cells of the immune system, e.g. viral functions that affect apoptosis may serve to inhibit proapoptotic molecules released from NK and T cells. However, in the absence of viral anti-apoptotic functions some herpesviruses completely fail to replicate in a number of cell types. Other herpesviruses use functions to keep their host cells in a state of continuous division and to prevent cell death at the same time. Thus, these functions select the viral host cell range and define the biology of a virus.

In a similar vein, the research on viral modulation of chemokine networks has led to hypotheses on Janus-like effects of these viral proteins. A nonfunctional receptor can neutralize the cell-attractive function of a chemokine for cells of the immune system, and thus truly serve an immunoevasive function. On the other hand, there are virus-encoded chemokines that attract host cells. It is thought that these cells serve the virus for reaching target tissues. A remarkable aspect is that some of the cells that provide the 'taxi' service are those that normally would fight the viral infection. Therefore, there is certainly a score that orchestrates the roles of viral chemokines and chemokine receptors in viral dissemination and persistence, but it will take years to understand the music.

Examples of viral proteins that modulate host immune functions presented in this section were mainly taken from viruses with large DNA genomes. In part this is the result of the limited expertise of the authors. Second, many of these functions were first detected in DNA viruses. There is no doubt that several RNA viruses that were not been listed also encode such functions. In general, it appears that viruses need functions to dampen the function of innate immunity and the powerful antiviral functions of the soluble mediators such as the interferons. The analysis of viral functions that affect soluble mediators and/or their subsequent signaling cascades is an area of active research. Another reason for reporting on DNA virus functions is the working hypothesis that in a large virus genome a protein would perhaps serve only one function. There is the tacit belief that the function seen for the isolated gene in vitro also represents the functions that it has in the viral context in vivo. This may probably turn out to be not entirely correct and examples have been mentioned where one protein affects different antiviral principles. If this is true, the pressure for multi-

functionality of proteins is even higher in RNA viruses with their smaller genomes.

In apparent contrast to the multifunctionality of proteins is the fact that some viruses encode multiple genes for similar functions, all of which attack the class I pathway. It is not always clear what the purpose of this multiplicity is. One purpose appears to be to deal with MHC polymorphism. MHCI molecules belong to the most polymorphic proteins, as a result of the three class loci – HLA-A, -B, and -C in humans – and to extensive polymorphism at each locus. The length of coevolution of class I and viruses – particularly herpesviruses – makes it likely that some of this polymorphism has evolved to counteract VIPRs. In fact, many VIPRs deal with different class I isoforms with different degrees of efficiency. Thus, the function of some viral proteins are considered complementary. Another function for multiple genes is cooperativity: neither gene is capable of inhibiting antigen presentation alone, but together they function effectively to prevent CTLs recognizing infected cells. A similar multiplicity of genes with related function is also seen with respect to genes controlling apoptosis, and genes controlling chemokine and cytokine networks.

The analysis of the principles evolved by the viruses to target the same mechanism, e.g. the peptide transport or MHCI maturation, indicates that the biological problem – defense against CD8 T-cell control – has been independently addressed by the α and β herpesviruses. Analogous but mechanistically completely different solutions were found. This indicates that the virus subfamilies were already separated before the acquisition of these functions. The primordial immune system fighting the archetype herpesvirus progenitor lacking this function was different as well. There is a strong belief that the constant attack by viruses has shaped both the virus and the mammalian immune system that we know today. Some viral immunomodulatory functions, e.g. some antiapoptotic genes, operate across species barriers, whereas others, e.g. MHCI modulators, often do not. This is explained by the coevolution of viruses with their hosts and could mean that the host-specific functions were acquired at a later stage.

The true function of the immunomodulatory genes can be delineated only by in vivo studies using appropriate combinations of virus mutants and host mutants. Given the plethora of genes, the redundancy of related functions, the multifunctionality and cooperativity of proteins, and the intricate interplay of functions that act differently on different cells at different states of activation and differentiation, and with a different efficacy with respect to the genetic background of the host, it is clear that it will take a long time to test present concepts and to develop a conceptual network of hierarchical functions. This may be of use for modern medicine with its new concepts of treatment that affect immune functions and thus disturb the virus–host balance so carefully tuned by evolution.

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Evasion of immune responses by bacteria

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INTRODUCTION

The success of pathogenic microorganisms is dependent on their survival and replication in the face of antimicrobial defense mechanisms, mediated by innate and adaptive responses of the host's immune system. Although the immune system is capable of preventing infection, and eliminating or containing most bacteria, many, if not all, bacterial pathogens have evolved strategies to evade or suppress immune responses to prolong their survival in the host. Ideally, the bacteria should be capable of avoiding or modulating innate and adaptive immunity to such an extent that the host does not succumb to a lethal infection but survives to pass on the organisms to secondary hosts.

Innate immunity

Following infection of host tissue with pathogenic bacteria, tissue macrophages quickly phagocytose and attempt to kill the bacteria (Figure 1). This initiates an inflammatory response with the release of cytokines and chemokines and the recruitment of neutrophils, monocytes, and lymphocytes to the site of infection. Binding of conserved secreted or cell-surface bacterial products to molecular structures, called pathogen recognition receptors (PRR), on cells of the innate immune system activates immune response genes, leading to inflammatory cytokine and chemokine production, and enhanced cell surface expression of molecules involved in antigen presentation (Janeway and Medzhitov 2002).

Activation of the alternate complement pathway by bacterial cell wall components results in the production of C3b, which opsonizes bacteria and, together with antibodies, facilitates their phagocytosis by neutrophils and macrophages. Bacteria also activate the classic pathway of complement. The complement membrane attack complex can directly kill certain bacteria and participate in the inflammatory response.

Once inside the phagocytic cells, the bacteria-containing phagosome undergoes a series of fusions with endosomes and lysosomes to form a phagolysosome in which the bacteria are killed by a variety of mechanisms, including reactive oxygen- and nitrogen-dependent mechanisms. However, phagocytic and killing mechanisms of host cells can be overcome by various physical and biochemical evasion strategies evolved by the bacteria. The composition of the bacterial cell wall has a major influence on the ability of phagocytic cells to take up and destroy the bacteria. All bacteria have an inner cell membrane and peptidoglycan layer, which can be attacked by lysozymes and enzymes in the lysosomal vesicles of phagocytic cells. Gram-negative bacteria have an outer lipid bilayer (with lipopolysaccharide), which can be attacked by cationic lipids and complement. Certain bacteria have capsules that prevent phagocytosis or have an extremely complex cell wall (e.g. mycobacteria), which is highly resistant to killing. Bacteria

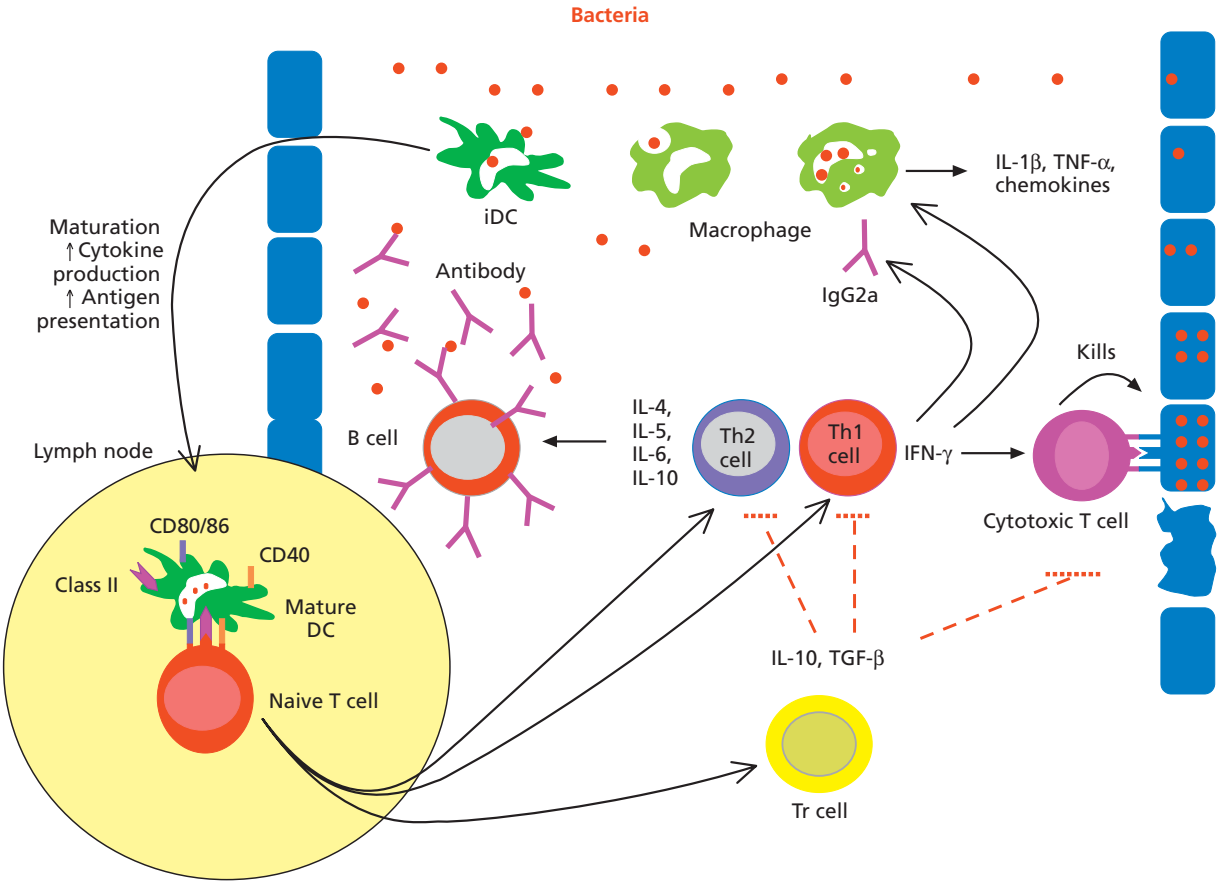

Figure 1 *Innate and adaptive immunity to bacteria. Bacteria that enter mucosal tissue, such as the lungs or gastrointestinal tract, are phagocytosed by macrophages and dendritic cells (DCs) of the innate immune system. Immature DCs (iDC) are activated and migrate to the draining lymph node, where they present processed bacterial antigens to naïve T cells. Effector T cells migrate back to the tissues and mediate and regulate the adaptive immune response to the bacteria. T-helper (Th1) cells secrete interferon γ (IFN-γ), which activates macrophages to kill the intracellular bacteria and cytotoxic T lymphocytes (CTL) to kill host cells infected with intracellular bacteria. Th2 and Th1 cells secrete cytokines that activate B-cell antibody production, which prevents bacteria binding to host cells, promotes opsonization, or neutralizes toxins. Finally, Tr cells turn off the adaptive immune response and limit collateral damage to host tissues.*

can also inhibit killing by preventing phagosome–lysosome fusion or by inducing lysosomal discharge into the cytoplasm. Induction of apoptosis in neutrophils, macrophages, and dendritic cells (DC), critical cells in antibacterial host defense and in activating adaptive immunity, is the ultimate subversion strategy evolved by a number of bacterial species to protective immune responses of the host.

Adaptive immunity

Adaptive immune responses are mediated by lymphocytes, called T and B cells. B cells produce antibodies, whereas $CD8^+$ T cells kill host cells infected with intracellular microorganisms and $CD4^+$ T cells provide help for B-cell production of antibody and regulate the immune response to infection (see Figure 1). In addition to $CD4^+$ and $CD8^+$ T cells, unconventional T cells, including γδ T cells which recognize phospholigands and CD1-restricted αβ T cells or T cells that express natural killer (NK) markers, termed NKT cells, which recognize glycolipids, also play a role in immunity to intracellular bacteria (Schaible and Kaufmann 2000). Although it takes days rather than hours before the adaptive immune response is effective, unlike the innate immune system, it is able to recall previous encounters with antigen, through the activation of memory T and B cells. Therefore a second or subsequent infection with the bacteria is dealt with more effectively.

After uptake of the bacteria by a macrophage or DC, the professional antigen-presenting cells (APC), process and present peptides of the bacterial antigens to T cells in association with major histocompatibility complex (MHC) molecules on their cell surface. All bacteria, whether they survive intracellularly or extracellularly in the host, can activate MHC class II (MHCII)-restricted $CD4^+$ T-helper (Th) cells. In addition, certain intracellular bacteria can also activate MHCI-restricted $CD8^+$

cytotoxic T lymphocytes (CTL). Induction of MHCII-restricted T cells requires processing by an exogenous route. After binding to specific cell-surface receptors on macrophages and DCs, and uptake into APCs via opsonization, phagocytosis, or receptor-mediated endocytosis, the bacterial antigens are degraded in endosomes or early lysosomes, and digested peptides, 7–20 amino acids long, associate with newly formed or recycled MHCII molecules before being exported to the cell surface. Induction of class I-restricted CTLs is dependent on bacterial antigens entering the endogenous route of antigen processing, usually through their escape from vacuoles/endosomes into the cytoplasm. Many of the intracellular steps of antigen processing are targets of immune evasion by pathogenic bacteria.

Class I-restricted CTLs kill host cells infected with intracellular bacteria, whereas the main function of class II-restricted $CD4^+$ T cells is to release cytokines that activate phagocytosis and killing of bacteria by macrophages, and to provide helper function for antibody production. $CD4^+$ T cells can be divided into a number of functionally distinct subtypes discriminated on the basis of cytokine secretion. Th1 cells secrete interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β), activate phagocytosis and killing by macrophages, and provide help for the production of opsonizing (murine IgG2a) antibodies. Th2 cells secrete interleukins IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and are considered to be the main helper cells, especially for immunoglobulin (Ig) IgG1, IgA, and IgE. A further subtype of $CD4^+$ T cells, which secrete high levels of IL-10 and/or transforming growth factor (TGF)-β but not IL-4 or IFN-γ, termed regulatory T (Tr) cells, have recently been described during infection with certain pathogens, including bacteria (McGuirk and Mills 2002). Tr cells have suppressive function and may be induced by the host to turn off immune responses, especially Th1 responses, once the bacteria are eliminated and thereby limit immunopathology. Alternately their induction may serve as an evasion strategy by the bacteria to suppress protective Th1 responses.

Although cell-mediated immunity is important in controlling intracellular bacteria, humoral immunity plays a major role in protection against extracellular bacteria. Antibodies help to prevent infection or to eliminate bacteria in a number of ways, and antibodies of different classes and subclasses perform different functions. Extracellular bacteria often bind to epithelial cells in the respiratory or gastrointestinal (GI) tract via adhesins or pili; antibodies against these bacterial virulence factors can help to prevent colonization. The diseases caused by many bacteria are mediated by toxins; antibodies, especially those of the murine IgG1 subclass, can neutralize these toxins and prevent their binding to host target cells, and thereby reduce the severity of disease. Antibodies of the murine IgG2a subclass can promote phagocytosis by opsonizing the bacteria and stimulating complement components C3b and iC3b, which bind complement receptors and further promote phagocytosis. IgA antibodies, induced after bacterial infection of the respiratory tract, GI tract, or other mucosal tissues, function to limit the infection to mucosal surfaces. In an immune individual primed by a selflimiting infection or immunization, antibodies probably play the major role in conferring protective immunity. Almost all bacterial vaccines in use today confer protection by the generation of circulating IgG or memory B cells, which produce an anamnestic antibody response after re-exposure to the bacteria. However, the generation of antibody responses is also dependent on priming of helper T cells, so both T and B cells are critical in the primary as well as the secondary response to bacterial infection. Bacteria have also evolved strategies to circumvent humoral immunity, either by directly cleaving the antibody molecule or by interfering with the functions of B or Th cells.

EVASION OF PHAGOCYTIC KILLING

Phagocytosis provides a specialized mechanism for ingestion and intracellular destruction of microbial pathogens by neutrophils and macrophages (Aderem and Underhill 1999). These include receptors for the opsonic immunoglobulin Fc domain (e.g. FcγR) and the C3b and C3bi complement fragments (e.g. CR3). FcγRs cluster when bound to an IgG-coated bacterium and become phosphorylated by Src tyrosine kinases. Downstream events include activation of the small GTPases, Rac, and Cdc42, which results in polymerization of actin to form an actin cup at the site of bacterial binding to the plasma membrane, followed by the appearance of F-actin-rich membrane ruffles and internalization of the bound particle surrounded by a lipid bilayer membrane and a halo of actin. CR3-mediated phagocytosis is similar to FcγR-mediated phagocytosis in many ways, but uses Rho GTPases instead of Rac and Cdc42. In addition, CR3-mediated phagocytosis does not induce inflammatory mediators.

Together with internalization of the target, intracellular signaling stimulates interaction of the phagosome with the endocytic network, activates the production of reactive oxygen intermediates (ROI) and enhances the expression of specific genes, especially those encoding cytokines (Duclos and Desjardins 2000; Tjelle et al. 2000). The newly formed phagosomes are immature organelles unable to kill or degrade microorganisms and, in order to acquire and exert their microbicidal function, phagosomes must engage in a maturation process referred to as phagolysosome biogenesis. Phagosomes fuse transiently and sequentially with endosomes of increasing age or of increasing maturation level (early endosomes, late endosomes, and lysosomes). The Rab GTPases are associated with different stages in the fusion pathway: Rab5 enables fusion of the phagosome

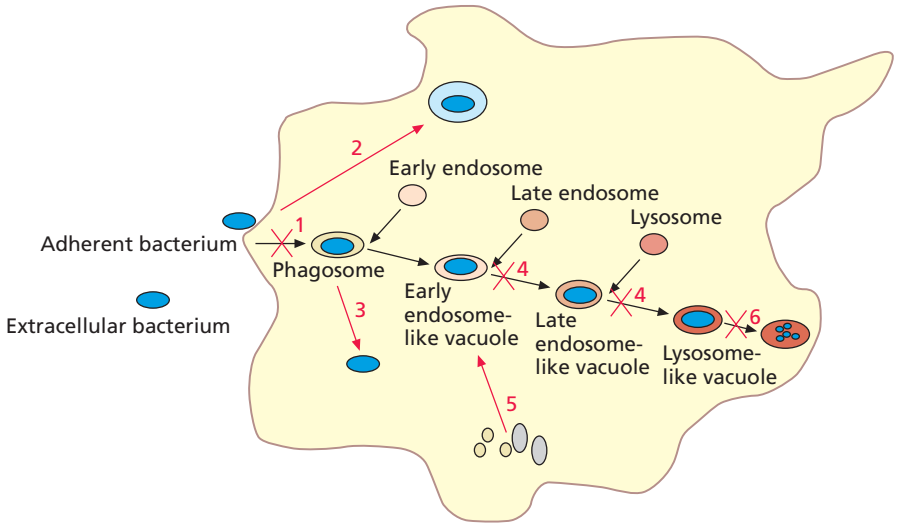

Figure 2 *Evasion of phagocytic killing. Phagocytic cell receptors recognize and bind microorganisms on the cell surface. The bacteria are taken up into phagosome vesicles originating from the plasma membrane. Phagosomes mature by fusing transiently and sequentially with endosomes of increasing age and maturation (early endosomes, late endosomes, and lysosomes). Eventually a phagolysosome is formed with an acidic and lytic compartment to kill, degrade, and further process microorganism antigens for presentation at the cell surface. Bacteria have developed a number of strategies to evade killing by phagocytic cells including: (1) inhibition of phagocytic uptake, (2) bacteria-mediated invasion into a nonlysosomal pathway, (3) escape to cytosol, (4) inhibition of endosomal and lysosomal fusion, (5) fusion with nonlysosomal organelles, and (6) resistance to lysosomal killing.*

with early endosomes, whereas Rab7 allows phagosome fusion with late endosomes. During the maturation process phagosomes acquire markers of late endosomes and lysosomes including the proton pump adenosine triphosphatase (ATPase), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, MHCII molecules, various hydrolases including several cathepsins, and specific antimicrobial pore-forming peptides. In addition, maturing phagosomes acquire Nramp1, which transports divalent cations and modulates intraphagosomal iron content. The combination of reactive oxygen and nitrogen, acidic pH, pore-forming peptides, iron imbalance, and hydrolytic enzymes in the final phagolysosome provides a highly effective antimicrobial combination that is capable of killing a wide variety of bacteria, and further degrading and processing the microorganism antigens for presentation at the cell surface. Bacteria have developed a number of strategies to evade killing by phagocytic cells (Figure 2).

Inhibition of chemotaxis

Pathogens can prevent killing by neutrophils and macrophages through the inhibition of the migration of these phagocytic cells to the site of infection. Cell chemotaxis is controlled by inflammatory cytokines and chemokines, and complement components, and through the expression of adhesion molecules on the cell surface; each of these regulatory mechanisms can be targeted by a number of pathogens. Pneumolysin, a sulfhydryl-activated toxin produced by *Streptococcus pneumoniae*, can inhibit chemotaxis of human polymorphonuclear cells (Paton and Ferrante 1983). The peptidoglycan fraction of staphylococci induces production of an inhibitory protein by mononuclear cells that inhibits neutrophil chemotaxis and the bacterial peptide *N*-formyl-Met-Leu-Phe inhibits neutrophil migration and killing of *Staphylococcus epidermidis* (Donabedian 1985; Li et al. 2002b).

The AB toxin, pertussis toxin (PT), plays a major role in *Bordetella pertussis* pathogenesis. Binding of the B-oligomer to host cells allows the A subunit to enter the cell, where adenosine diphosphate (ADP)-ribosylation of G_i proteins that transmit inhibitory signals to the adenylyl cyclase complex occurs, resulting in elevated intracellular cyclic adenosine monophosphate (cAMP), which affects signaling pathways in many cell types, including cells of the immune system; this contributes to immune dysfunction. PT has been shown to inhibit macrophage chemotaxis in vivo (Meade et al. 1985) and neutrophil and lymphocyte chemotaxis in vitro by altering intracellular calcium levels (Spangrude et al. 1985). Recent evidence has shown that the leukocytosis induced by PT may result from G-protein-dependent, chemokine-regulated extravasion of leukocytes. PT inhibits the increase in intracellular Ca^{2+} levels in $CD4^+$ and $CD8^+$ T cells, induced by the CC chemokines, macrophage chemoattractant protein 1 (MCP-1), MCP-2, MCP-3, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES (Loetscher et al. 1994), Ca^{2+}-mediated activation of neutrophils induced by IL-8 (Schorr et al. 1999), and MCP-1-induced chemotaxis of NK cells (Allavena et al. 1994).

Inhibition of phagocytic uptake

Successful extracellular bacteria have the ability to inhibit uptake by phagocytic cells (Ernst 2000). *Yersinia* spp. share a common mechanism to inhibit phagocytosis by macrophages (Cornelis et al. 1998). These bacteria and a number of other virulent gram-negative animal and plant pathogens possess a dedicated pathogenicity secretion system called a type III secretion system (TTSS), which is responsible for directly delivering effector molecules from the bacterium into the eukaryotic cytosol. These effectors interfere with normal immune cell function, primarily disrupting signal transduction pathways and cytoskeleton arrangements. *Yersinia* spp. express a number of TTSS effectors that inhibit phagocytic uptake, one of which is YopH which after entry into the cell cytoplasm rapidly blocks phagocytosis through Fcγ receptors (Grosdent et al. 2002). YopH is a tyrosine phosphatase that dephosphorylates focal adhesion kinase (FAK) and $p130^{Cas}$. These proteins are found at sites of focal adhesions and are thought to play an important role as adaptor proteins during phagocytosis (Black and Bliska 1997; Persson et al. 1997). A second *yersinia* TTSS effector, YopE, acts together with YopH to inhibit phagocytosis (Rosqvist et al. 1988). YopE is a GTPase-activating protein that selectively increases GTP hydrolysis by RhoA, Rac, and Cdc42, key regulators of actin cytoskeleton dynamics, thereby inactivating them (Von Pawel-Rammingen et al. 2000). The TTSS effector ExoS of *Pseudomonas aeruginosa* acts in a similar manner to disrupt actin filaments and inhibit phagocytosis (Pederson et al. 1999).

Enteropathogenic *Escherichia coli* (EPEC) inhibits FcγR-mediated macrophage phagocytosis but not CR3-mediated phagocytosis via its TTSS (Celli et al. 2001). This is the result of inactivation of phosphatidyl inositol (PI)-3-kinase, leading to inhibition of pseudopod extension and complete phagocytic cup formation. *Helicobacter pylori* expresses another kind of secretion system associated with pathogenic processes – a type IV secretion system. This secretion system also delivers effector molecules into the eukaryotic cell and is responsible for inhibition of bacterial uptake by phagocytic cells (Ramarao et al. 2000). *Mycobacterium tuberculosis* and/or its lipoarabinomannan downregulates macrophage activation, and thereby phagocytosis, by stimulation of SHP-1, a cytosolic protein tyrosine phosphatase (Nandan et al. 2000).

Bacteria-mediated invasion into phagocytic and nonphagocytic cells

Some pathogens survive by living intracellularly in eukaryotic cells. In this way the bacteria avoid recognition by soluble factors, such as complement and antibody, and by immune cells. The bacteria direct their own uptake into nonphagocytic cells and/or phagocytic cells via receptors other than Fc receptors, and in this way bacteria avoid triggering activation of the intracellular bacterial killing mechanisms, so that the bacteria can survive and replicate safely within the cell.

Invasion is initiated by binding of the bacteria to the eukaryotic cell surface. This adhesion is mediated by bacterial adhesins and/or invasins binding to specific receptors on the cell surface. The invasin–receptor interaction then triggers uptake of the bacteria. One set of receptors on eukaryotic cells that is often used by bacteria to enter cells is the integrins (Isberg et al. 2000), which are large heterodimeric transmembrane proteins that are capable of transmitting signals to the cell cytoskeleton after adhesion to substrates. The invasin protein of *Yersinia enterocolitica* uses this receptor to pass through M cells from the gut to the underlying tissue. Bacterial attachment initiates receptor clustering to direct signaling for cytoskeleton rearrangements and entry, a process in which FAK and Rho GTPases are involved. The internalin protein of *Listeria monocytogenes* binds E-cadherin, a cell surface protein involved in cell–cell interactions, and the *Neisseria* family of Opa proteins bind the CD66 family of receptors (Mengaud et al. 1996). Receptors can also be glycosaminoglycans and extracellular matrix proteins such as fibronectin (Joh et al. 1999).

Bacteria can subvert complement for invasion of eukaryotic cells. In contrast to FcγR-induced phagocytosis, complement–induced phagocytosis does not provoke a respiratory burst, nor does it induce inflammatory mediators (Caron and Hall 1998), so the bacteria bypass powerful cellular killing mechanisms, such as H_2O_2 and oxygen free radicals. Deposition of complement on the cell surface allows bacteria, such as *Legionella pneumophila*, to enter host cells via C receptors (Cooper 1991). Alternately, bacteria can enter cells via complement receptors independently of complement itself, such as *E. coli* via Dr adhesins (Pham et al. 1997). Several bacteria use CR3 for intracellular entry, e.g. filamentous hemagglutinin (FHA) of *B. pertussis* (Ishibashi et al. 1994). *M. tuberculosis* binds CR1 and CR3 to enhance its uptake into cells and InlB of *L. monocytogenes* mediates bacterial entry via gC1q-R/p32, a C1q-binding protein (Braun et al. 2000; Schlesinger et al. 1990).

Bacteria can induce their internalization via subversion of actin skeleton arrangements, a process that is often dependent on TTSS effectors (Steele-Mortimer et al. 2000). Entry of *Salmonella* and *Shigella* spp. into eukaryotic cells is determined by actin polymerization at the site of bacterial contact with the cell membrane and, at the peak of extension formation, numerous cytoskeleton proteins are recruited which form the entry foci. The TTSS effectors of *Salmonella* and *Shigella* spp. first interact with specific cytoskeleton proteins to depolymerize actin filaments and microtubules to allow

rearrangement of the cytoskeleton components for mediating bacterial entry. The effectors then activate Cdc42 and Rac1 GTPases to induce actin polymerization and stabilize the newly formed cytoskeleton structure to engulf the bacteria.

The mode of entry of the bacteria into the eukaryotic cell will influence their trafficking along the phagolysosomal pathway and the signaling cascades that are activated. Thus, the receptor that mediates particle internalization and the nature of the internalized particle play an important role in phagosome maturation and phagosome–lysomal fusion.

Escape into cytoplasm

Bacteria such as *Listeria* spp., *Rickettsia* spp., enteroinvasive *E. coli*, and *Shigella* spp. lyse the primary phagosome and enter the cytoplasm where they can safely replicate (Goebel and Kuhn 2000). Entry of bacteria into the cytosol is achieved by the action of phospholipases and pore-forming toxins; the mechanism employed by *Listeria* spp. has been studied best. Escape from the phagosome is necessary for intracellular survival and proliferation of *L. monocytogenes*, a process in which the pore-forming cytolysin listeriolysin O (LLO) plays an essential role (Vazquez-Boland et al. 2001). LLO is active only at low pH and thus it specifically lyses the phagosomal membrane when it is released by the bacteria in the phagosome, and not the plasma membrane of the cell, because after vacuole lysis the pH equilibrates and LLO is deactivated (Beauregard et al. 1997). LLO forms holes in the phagosomal membrane allowing access of the bacteria's own phospholipases, or even cellular lipases, to the phagosomal membrane, which result in the subsequent membrane disruption (Smith et al. 1995).

Inhibition of lysosomal fusion

After uptake into the phagosome several bacterial pathogens have the ability to halt maturation of the vacuole, and trafficking to late endosomal and lysosomal compartments by inhibiting endosome–lysome fusion. *Salmonella typhimurium* traffics to an early endosome-like vacuole that does not mature into a lysosome-like vacuole, where the bacteria grow and multiply (Gorvel and Meresse 2001). Direct interaction of the vacuole containing the *Salmonella* with late endosomal compartments is inhibited (Meresse et al. 1999) and TTSS effectors of *Salmonella* are involved in this inhibition of phagosome–lysosome fusion (Uchiya et al. 1999). These effectors are also necessary to prevent the trafficking of the NADPH oxidase complex to the *Salmonella*-containing vacuole (Vazquez-Torres et al. 2000).

Mycobacterium tuberculosis also inhibits lysosomal delivery. The vacuole containing *Mycobacterium* lacks late endosomal markers and retains Rab5, but not Rab7, thus implicating a block between early and late endosomal fusion (Via et al. 1997). A molecule called TACO (coronin 1) is actively retained at the mycobacterial phagosome membrane limiting interaction with endocytic organelles, and allowing *Mycobacterium* to survive within the phagosomes (Ferrari et al. 1999). Phagosomes containing *Mycobacterium* also lack V-ATPase, thereby preventing acidification (Sturgill-Koszycki et al. 1994).

Chlamydia spp. express proteins that modify the vacuole to ensure that trafficking of bacteria is diverted away from the endocytic pathway to the exocytic pathway (Hackstadt et al. 1997; Wyrick 2000). The vacuole interacts with vesicles derived from the trans-Golgi protein trafficking network en route to the cell surface and thus acquires sphingomyelin. It also acquires phosphatidylcholine and PI from the endoplasmic reticulum (ER) and cardiolipin from the mitochondria, so that the vacuole is thus camouflaged and does not interact with lysosomal compartments (Wylie et al. 1997). Chlamydial proteins IncA–G are also incorporated into the vacuole membrane and may be involved in inhibition of vacuole maturation (Scidmore-Carlson et al. 1999).

Transport to nonlysosomal organelles

Instead of trafficking to the endosomal pathway, virulent *Brucella abortus*, *L. pneumophila*, and *Porphymonas gingivalis* traffic to autophagosome-like vacuoles (Dorn et al. 2002). Autophagy is a cellular process for the degradation of organelles and cellular compartments, resulting in breakdown products that are recycled for the synthesis of new components. The nascent autophagosome is a vacuole derived from the rough ER, and contains organelles and undegraded cytoplasmic substances that had been selectively or nonselectively sequestered within the autophagosome. This early autophagosome matures to an acidic late autophagosome lacking hydrolytic enzymes, which then docks and fuses with a lysosome-containing acid proteases. The sequestered cellular components are then degraded and recycled.

Early after infection *Brucella*-containing phagosomes transiently fuse with early endosomes; however, they do not display Rab7 and do not fuse with lysosomes or late endosomal compartments (Pizarro-Cerda et al. 1998). Instead they acquire ER and autophagosomal characteristics, perhaps by fusion with an early autophagosome. Eventually *Brucella* transits from the autophagosome to the ER, where bacterial replication occurs. In this niche the bacteria not only avoid degradation but also obtain a supply of metabolites. The type IV secretion system of *Brucella* sp. is essential for the biogenesis of the autophagosome-like vacuole and for intracellular survival and replication (Comerci et al. 2001). In the absence of a

functional type IV secretion system *Brucella* fails to evade the endocytic pathway and is instead targeted to lysosomes for subsequent degradation.

On entry into cells, vacuoles containing *Legionella* sequentially associate with smooth vesicles, mitochondria, and the ER (Horwitz 1983). The vacuoles fail to acquire late endosomal/lysosomal markers, fail to acidify, and are competent for bacterial replication. They are similar to autophagic vacuoles. This process is also controlled by a type IV secretion system, called dot/icm (Roy et al. 1998).

Resistance to lysosomal killing

Pathogenic bacteria have the ability to survive within phagolysosomes. The acidity of these vacuoles is a major obstacle for the bacteria to overcome. Some pathogens can survive in this acidic environment, whereas others attenuate the acidic pH. *Coxiella* spp. survive and multiply in mature lysosomes where they avoid digestion by lysosomal enzymes and actually require the acidic environment to stimulate metabolism and growth (Heinzen et al. 1996). *Bordetella bronchiseptica* can survive the acidic environment in the lysosome and replicate intracellularly, whereas *B. pertussis* cannot (Schneider et al. 2000). In contrast *Mycobacterium* maintain a neutral pH by retaining endosomal Na^+/K^+ ATPase, as do *Chlamydia* (Sturgill-Koszycki et al. 1994; Schramm et al. 1996).

Reactive oxygen species are generated by the respiratory burst in the macrophage vacuoles to kill intracellular bacteria (Hampton et al. 1998; Zahrt and Deretic 2002). The respiratory burst is caused by the NADPH oxidase complex, which assembles at the phagolysosomal membrane. Electrons are transferred from cytoplasmic NADPH to oxygen on the phagosomal side of the membrane, generating superoxide plus a range of other ROIs with toxic bactericidal activity.

Bacteria have many mechanisms for protection against ROIs. Some involve the production of enzymes to inactivate these molecules. *S. typhimurium* expresses superoxide dismutases, enzymes that catalyze the formation of hydrogen peroxide from superoxide anion and which contribute significantly to *Salmonella* virulence (De Groote et al. 1997; Farrant et al. 1997). Superoxide dismutase also protects *M. tuberculosis* from respiratory burst killing (Piddington et al. 2001). Catalases in turn detoxify hydrogen peroxide to water. The *Salmonella* catalase SodC and the *Campylobacter jejuni* KatA enhance survival toward the respiratory burst (De Groote et al. 1997; Day et al. 2000). In addition, several scavenging enzymes including glutathione reductase and thioredoxin reductase use reducing equivalents from NADPH either to generate low-molecular-weight antioxidant species or to repair oxidative lesions. DNA repair enzymes are required for *Salmonella* survival in mice and for resistance to the respiratory burst, because DNA is a critical bacterial target for reactive oxygen (Buchmeier et al. 1993). The production of ROIs can be inhibited by preventing NADPH oxidase complex formation in the vacuole membrane. *Ehrlichia* sp. inhibit superoxide production by downregulating expression of the NADPH oxidase complex component $gp91^{phox}$ (Banerjee et al. 2000) and *L. pneumophila* inhibits protein kinase C which is necessary for the mobilization of cytosolic NADPH phagocyte oxidase components to the membrane (Jacob et al. 1994).

Reactive nitrogen intermediates (RNI) are also formed in the lysosome by inducible nitric oxide synthase (iNOS). Resistance to RNIs can be mediated by the scavenger enzymes that also confer resistance to ROIs (Zahrt and Deretic 2002). It remains unclear whether bacteria have a dedicated RNI resistance mechanism, or whether resistance is the result of coordinated expression of multiple survival-related pathways.

MODULATION OF PRO- AND ANTI-INFLAMMATORY CYTOKINE PRODUCTION

Bacterial activation of cells of the innate immune system

Macrophages, DCs, NK cells, and nonconventional T cells, including NKT cells and γδ T cells, all function in immunity to bacterial infections, either by uptake and killing of the bacteria, lysis of infected host cells, presentation of antigens to T cells, or release of cytokines that activate antibacterial activity of other cells, or by regulating the adaptive immune responses to the bacteria. The immediate inflammatory response to bacterial infection involves the production of proinflammatory cytokines, including TNF-α, IL-1β, IL-12, and IL-18, and chemokines, including MIP-1α MIP-1β, MIP-2, and RANTES, largely by cells of the innate immune system. These cytokines and chemokines recruit leukocytes to the site of infection, activate their antimicrobial function, and in addition regulate the induction of the adaptive response to the bacteria. IL-12 and IL-27 promote the differentiation of Th1 cells, which mediate cellular immunity against intracellular bacteria and also stimulate the production of opsonizing and complement-fixing antibodies. This proinflammatory response is beneficial to the host in eliminating the bacteria, but is also responsible for many undesirable systemic and central effects, including fever and sepsis, which if uncontrolled can cause immunopathological damage to host tissues. The induction of anti-inflammatory cytokines by the bacteria, especially IL-10 and TGF-β, helps to suppress inflammation, but can also be exploited by the bacteria to turn off protective immune responses.

Bacteria stimulate cells of the innate immune system through interaction of conserved bacterial molecules with receptors on the host cells. Whole bacteria and specific bacterial products, termed pathogen-associated molecular patterns (PAMP), including lipopolysaccharide (LPS), CpG motifs in bacterial DNA, and peptidoglycan, bind to PRRs, which include but are not exclusively Toll-like receptors (TLR), expressed on a variety of cells, e.g. macrophages and DCs (Guermonprez et al. 2002). Differential expression of TLRs and other PRRs on distinct DC subtypes determine the response to different PAMPs (Janeway and Medzhitov 2002). Plasmacytoid DCs selectively expressed TLR-7 and TLR-9 and respond to bacterial CpG motifs, whereas myeloid DCs express all TLRs except TLR-7 and TLR-9, and do not respond to CpG, but are stimulated by LPS and peptidoglycan, which bind to TLR-4 and TLR-2, respectively (Jarrossay et al. 2001). Binding to TLRs activates signaling through NF-κB, leading to the production of inflammatory cytokines including IL-12, TNF-α, IL-1β, IL-6, and IL-18, and the increased surface expression of maturation markers on DCs. The same bacteria can also stimulate the production of anti-inflammatory cytokines, IL-10, TGF-β, and IL-1 receptor antagonist (IL-1ra), either using the same or distinct conserved structures, which bind to and activate distinct signaling receptors and pathways.

Suppression of IL-12 and TNF-α production

Suppression of proinflammatory cytokine production, especially IL-12 and TNF-α, either through induction of IL-10 or through interference with steps in the NFκB signaling pathways is a common strategy employed by bacteria to subvert macrophage activation and induction of Th1 responses. *B. pertussis* FHA suppresses IL-12 by macrophages and DCs and the suppression is mediated by the induction of IL-10 production (McGuirk and Mills 2000; McGuirk et al. 2002). Furthermore FHA suppressed IL-12 and IFN-γ production in vivo in response to LPS in a model of septic shock and suppressed influenza virus-specific IFN-γ production when coadministered to mice with influenza virus by the nasal route (McGuirk et al. 2002).

The AB-type toxins cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT), also modulate the production of regulatory cytokines and chemokines by cells of the innate immune system. CT, LT and the partially toxic mutant LTR72 suppress production of IL-12 and TNF-α and of the inflammatory chemokines, MIP-1α and MIP-1β (Lavelle et al. 2003; Ryan et al. 2000). Furthermore, CT inhibits the production of bioactive IL-12 (p70) and the expression of the β1 and β2 chains of the IL-12 receptor on human monocytes and DCs, leading to a suppression of Th1 cell differentiation and a predominantly Th2-type response (Braun et al. 1999b). Similarly, exposure of human DCs to CT inhibits IL-12p70, TNF-α, but not IL-6, IL-8, or IL-10 secretion in response to LPS or CD40 ligand (Gagliardi et al. 2000). A mutant of LT devoid of ADP-ribosylating activity does not inhibit IL-12 or TNF-α, suggesting that the enzyme activity is responsible for the suppression. Intracellular cAMP induced by active CT and LT appears to affect signaling pathways within the cells involved with IL-12 and TNF-α production. Furthermore, accumulation of intracellular cAMP directly inhibits cytokine production by Th1 cells and enhances Th2 cells (Ryan et al. 2000).

Another ADP-ribosylating toxin, anthrax edema toxin from *Bacillus anthracis*, inhibits LPS-induced TNF-α, and IL-6 production in human monocytes by increasing intracellular cAMP levels (Hoover et al. 1994). Furthermore, exotoxin A from *P. aeruginosa*, which also has ADP-ribosylating activity, inhibits release of TNF-α, TNF-β, IFN-γ, IL-1β, and IL-1α from both macrophages and lymphocytes (Staugas et al. 1992; Braun et al. 1999b). TNF-α production is also suppressed at the site of infection and in Peyer's patches of mice infected with *Y. enterocolitica*; the YopB protein from this bacteria was found to inhibit TNF-α production, but not other inflammatory cytokines, such as IL-1β and IL-6, by murine macrophages in vitro (Beuscher et al. 1995). Suppression of TNF-α production by *Y. enterocolitica* was associated with inhibition of NF-κB activation (Ruckdeschel et al. 1998). High-molecular-weight proteins released by *Brucella suis* inhibit TNF-α production by human macrophages infected with *E. coli* (Caron et al. 1996). Other bacteria, including *M. tuberculosis*, *Lactobacillus reuteri*, and *Histoplasma capsulatum*, suppress macrophage IL-12 production, but the bacterial molecules involved have not been identified (McGuirk and Mills 2002).

Induction of IL-10 and TGF-β production

A number of bacteria, including *Mycobacterium* spp., *L. pneumophilia*, *S. typhimurium*, *L. monocytogenes*, and *Y. enterocolitica*, stimulate IL-10 production by macrophages (Redpath et al. 2001). In a limited number of cases the bacterial molecule responsible has been identified. FHA, an adhesin from *B. pertussis* that binds to CR3 (Ishibashi et al. 1994), induces IL-10 and IL-6 production by macrophages and DCs (McGuirk and Mills 2000; McGuirk et al. 2002). The induction of IL-10 production was linked to suppression of IL-12, induction of Tr cells and suppression of Th1 responses in the lungs and local lymph nodes of *B. pertussis*-infected mice.

The immunosuppression associated with *M. tuberculosis* and the suppressed IFN-γ production in TB-infected individuals has been attributed to IL-10 and TGF-β production by mononuclear cells, including

macrophages and DCs. Purified protein derivative (PPD) of *Mycobacterium* induces IL-10 and TGF-β production by human mononuclear cells (Toossi et al. 1995; Othieno et al. 1999) and suppresses the production of IFN-γ, whereas neutralization of IL-10 and TGF-β enhances IFN-γ production (Othieno et al. 1999). Naturally occurring inhibitors of TGF-β (latency-associated peptide and decorin) restored in vitro T-cell responses of patients with TB and decreased growth of the bacteria in vitro and in vivo (Hirsch et al. 1997; Wilkinson et al. 2000). However, in a longitudinal study of TB patients, PPD-specific IL-10 and TGF-β production by peripheral blood mononuclear cells (PBMC) returned to baseline levels after 3 months, although PPD-specific IFN-γ production remained depressed for up to 12 months (Hirsch et al. 1999), suggesting that PPD-specific IL-10 and TGF-β are not the only factors involved in the immunosuppression.

Lipoarabinomannan (LAM), a cell wall component of *M. tuberculosis*, induces IL-10 production from human mononuclear cells (Barnes et al. 1992) and TGF-β from human monocytes (Dahl et al. 1996), and this has also been provided as an explanation for the immunosuppressive effects of the bacteria on antigen-induced T-cell proliferation. However, LAM also stimulated production of granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-1, IL-6. and IL-8 (Barnes et al. 1992), suggesting that it had pro- as well as anti-inflammatory effects. Infection of macrophages with *M. tuberculosis* induces the production of IL-10, IL-1, IL-6, IL-18, and TNF-α, but not IL-12 except in the presence of anti-IL-10 antibody. In contrast, infected DCs secrete IL-12, IFN-α, and TNF-α, but not IL-10 (Giacomini et al. 2001). It was suggested that the differential responses of the cells related to their distinct functions in infection, macrophages establishing inflammation inside granulomas and DCs directing T-cell responses in the draining lymph nodes. It has also been demonstrated that infection of DCs with *M. bovis* BCG induces release of IL-10 and IL-12, and that infected DCs from IL-10-deficient (IL-$10^{-/-}$) mice are more effective at trafficking to the lymph nodes, secrete higher levels of IL-12, and induce higher IFN-γ levels in response to mycobacterial antigens than wild-type DC (Demangel et al. 2002). An analogous finding has been reported for *Chlamydia trachomatis*, where modulation of endogenous IL-10 production by using IL-$10^{-/-}$ mice or antisense IL-10 enhances the ability of APCs to induce *Chlamydia*-specific Th1 responses (Igietseme et al. 2000). This suggests that upregulation of IL-10 production by DCs may serve as a strategy by the pathogen to turn off protective Th1 responses, and that inhibition of IL-10 production by APCs may provide a strategy for therapy or immunization against these diseases.

The virulence of *M. tuberculosis* strains has been linked to their ability to induce IL-10 production, with certain clinical isolates stimulating the highest level of this cytokine from human macrophages (Barnes et al. 1992). Another consequence of monocyte/macrophage IL-10 production by *M. avium* is the induction of soluble TNF receptor 2 (sTNFR2), which can inactivate TNF-α and block TNF-α-induced apoptosis (Balcewicz-Sablinska et al. 1998). Apoptosis of infected cells is a host defense measure to prevent intracellular multiplication of bacteria and this can be circumvented by *M. tuberculosis* and other bacteria (see below under Apoptosis).

There is also evidence from other bacterial species linking virulence to the ability to stimulate IL-10 or TGF-β production. The virulence-associated V antigen (LcrV) of pathogenic *Yersinia* spp. induces IL-10 release from mouse and human macrophages and as a consequence inhibits TNF-α production (Sing et al. 2002). It has been suggested that replication of *Coxiella burnetii* and the development of chronic Q fever may be facilitated by the induction of IL-10 production in macrophages; *C. burnetii* stimulates IL-10 and inhibits TNF-α production by monocytes and bacterial replication is inhibited by neutralizing anti-IL-10 antibodies (Ghigo et al. 2001). Lipidated outer surface protein A (L-OasA) from *Borrelia burgdorferi*, the etiological agent of Lyme disease, induces IL-10 production by human monocytes (Giambartolomei et al. 1998). Inhibition of endogenous IL-10 production with anti-IL-10 inhibits IL-12 and IL-6 antibodies but not IL-1β or TNF-α; this has been explained by a difference in the kinetics of cytokine production by monocytes in response to *B. burgdorferi*, with TNF-α production peaking within 1 h, followed by IL-10 and IL-1β, whereas IL-6 and IL-12 were produced later (Murthy et al. 2000). Inhibition of endogenous or bacteria-stimulated TGF-β2 production in mice infected with *L. monocytogenes* enhances IFN-γ, TNF-α, and IL-12 production and increases survival of the mice (Szalay et al. 1999). Although the source of the TGF-β has not been identified, it has been suggested that it may be derived from γδ T cells, NKT cells, or other cells of the innate immune system. In contrast, injection of anti-TGF-β1 diminishes resistance to *L. monocytogenes*, whereas administration of TGF-β1 protected mice from the infection (Nakane et al. 1996)

Studies in cytokine knock-out mice have provided more definitive evidence of a role for endogenous or bacteria-stimulated IL-10 production on the course of bacterial infections. In certain cases infection is less severe in IL-$10^{-/-}$ mice, confirming that this cytokine may help to subvert protective immunity by suppressing innate and adaptive immune responses, e.g. IL-$10^{-/-}$ mice are highly resistant against infection with *Yersinia* spp. and the bacteria-dependent suppression of TNF-α, mediated by IL-10, was absent in the knock-out mice (Sing et al. 2002). Cell-mediated immunity to *M. bovis* BCG is enhanced in IL-$10^{-/-}$ mice and the knock-out mice eliminate the bacteria faster than wild-type (Jacobs et al. 2000).

However, in other infections, the severity of disease is exacerbated in IL-$10^{-/-}$ mice, suggesting that IL-10 has a protective role, possibly by limiting immune-mediated pathology. IL-$10^{-/-}$ mice succumb to primary and secondary infection with *L. monocytogenes*; the number of cells in the inflammatory infiltrate, the inflammatory cytokine production in the brain and the severity of brain lesions is enhanced in the knock-out mice (Deckert et al. 2001). Although the numbers of bacteria were not significantly different between wild-type and IL-$10^{-/-}$ mice infected with *L. monocytogenes*, there are also examples of bacterial infection in IL-$10^{-/-}$ mice where disease pathology is enhanced or where bacterial clearance is accelerated. Peritonitis and mortality from *E. coli* infection is enhanced in IL-$10^{-/-}$ mice, despite accelerated clearance of the bacteria in knock-out compared with wild-type mice (Sewnath et al. 2001). The colonization of the gastric mucosa by *H. pylori* is reduced in IL-$10^{-/-}$ mice, but the severity of chronic active gastritis is significantly higher than in the wild-type mice (Chen et al. 2001). Similarly, IL-$10^{-/-}$ mice infected with *Helicobacter hepaticus* develop severe inflammation associated with IL-12 production and Th1 responses (Kullberg et al. 2001).

CYTOLYSIS AND APOPTOSIS OF MACROPHAGES, DCS, AND T CELLS

Apoptosis

Apoptosis or programmed cell death is a normal mechanism to remove unwanted cells from the body and plays a critical role in homeostasis in the immune system. After infection, apoptosis of lymphocytes reduces the pool of effector T and B cells, which have recently expanded through stimulation with foreign antigens. The process is carried out and controlled within the cell by a family of cysteine proteases, called caspases, which cleave cellular proteins and activate other caspases, eventually leading to the dismantling of cellular components. Apoptosis is considered to be a mechanism for removing unwanted cells without inducing an inflammatory response. Phagocytic cells bind dying and apoptotic cells, phagocytose them, and prevent release of their intracellular contents.

Induction or inhibition of apoptosis can influence the outcome of infection. Induction of programmed cell death in cells of the immune system, including T and B lymphocytes, macrophages, and DCs, is a common feature of many bacterial infections and serves as a strategy to eliminate cells directly involved in protective immunity. Induction of apoptosis of macrophages infected with intracellular bacteria serves to prolong infection by hindering bacterial killing and promoting the spread of the bacteria. It has also been proposed that bacteria-induced apoptosis may be a mechanism to initiate tissue damage, and evidence has been reported to link apoptosis to acute colitis associated with shigellosis (Zychlinsky and Sansonetti 1997).

Elimination of immune cells can also be achieved via cytolysis. Cytolysins are pore-forming bacterial toxins that lyse cells by cell membrane disruption. As with apoptosis induction, stimulation of this cell death mechanism enhances bacterial survival by killing cells such as leukocytes, monocytes, and neutrophils. These toxins play a crucial role in virulence as judged by in vivo studies and are important components contributing to severe inflammatory disease in response to bacterial infection. In some instances (often dependent on toxin concentration and target cell type) these toxins induce apoptosis.

Bacterial virulence factors can modulate cell death pathways in a variety of ways including:

1. disruption of membrane integrity and leakage of cellular components by pore-forming bacterial toxins
2. inhibition of host cell protein synthesis by bacterial toxins
3. delivery of effector proteins into host cells by bacterial TTSS, some of which can directly interact with caspases
4. deletion of T cells by bacterial superantigens
5. activation of second messengers by a bacterial effector.

Pore-forming toxins

Pore-forming toxins are released from bacteria as monomers, which then assemble into oligomeric pores in the target eukaryotic cell membrane. The pores lead to enhanced membrane permeability to monovalent ions and ATP release and hence cytolysis. Repeat-in-toxin (RTX) toxins (also called α-hemolysins) are one class of pore-forming toxins that are produced by a number of bacterial species and include Lkt of *Pasteurella haemolytica* and α-hemolysin of *E. coli* (Weinrauch and Zychlinsky 1999). Binding of RTX toxins to β_2-integrins results in pore formation, increases in intracellular calcium, and cytolysis (Jeyaseelan et al. 2000; Lally et al. 1997). Both α- and β-hemolysins of *Staphylococcus aureus* and *Clostridium perfringens* β-toxin are β-barrel pore-forming toxins (Menestrina et al. 2001). There are no known receptors for these toxins, which cause cell lysis via release of K^+ and also cause rapid calcium influx by opening of endogenous channels. Thiol-activated cholesterol-binding cytolysins are produced by gram-positive bacteria and include streptolysin O produced by *Streptococcus* spp. and pneumolysin produced by *S. pneumoniae* (Palmer 2001). These toxins are important for virulence of the invasive diseases caused by *Streptococcus* spp. They specifically require cholesterol for membrane insertion and act on any cell

type displaying this molecule (Shany et al. 1974; Ohno-Iwashita et al. 1992). The pores formed by these toxins are larger than those formed by hemolysis and permit flow of ions, small metabolites, and macromolecules. Even bacterially secreted proteins such as proteases and nucleases can enter the eukaryotic cells via the pores.

Nucleated animal cells may recover from toxin attack by membrane repair mechanisms, and thus low doses of toxin will affect but not kill target cells. Exposure of immune cells to sublethal doses of pore-forming toxins triggers a variety of proinflammatory reactions such as enhanced cytokine release, cytokine receptor shedding, and nitric oxide generation, as a result of stress activation of NF-κB (Hackett and Stevens 1992; Walev et al. 1996; Braun et al. 1999a).

Pore-forming toxins can also induce apoptosis depending on the toxin structure, toxin concentration, and target cell type. The RTX toxins induce apoptosis by perturbation of mitochondrial structure and function, and by activating caspase-3 and caspase-9 (Guzman et al. 1996; Lally et al. 1997; Korostoff et al. 2000). *L. monocytogenes* kills infected DCs by apoptosis thorough release of listeriolysin O (Guzman et al. 1996). In contrast, *Listeria* spp. do not induce apoptosis in macrophages (Zychlinsky et al. 1992). *B. pertussis* induces apoptosis of macrophages via the activity of adenylyl cyclase hemolysin toxin (AC-Hly) (Khelef et al. 1993; Gueirard et al. 1998). The toxin is a bifunctional protein, with a pore-forming hemolysin domain and an adenylyl cyclase domain that elevates intracellular cAMP. Elevated intracellular cAMP interferes with intracellular signaling in cells of the immune system and modulates a variety of immune effector functions, and also induces apoptosis in macrophages and DCs (Khelef et al. 1993; Gueirard et al. 1998).

Induction of DNA damage and inhibition of host-cell protein synthesis by bacterial toxins

Cytolethal distending toxins (CDT) produced by a variety of bacteria induce apoptosis through DNA damage and it has recently been reported that CDTs from *Haemophilus ducreyi* induce apoptosis in immature, but not mature, DCs (Li et al. 2002a). Apoptosis of HeLa cells by CDTs is associated with phosphorylation of histone H2AX and relocation of the DNA repair Mre11 complex (Li et al. 2002a). Diphtheria toxin (DT) from *Cornyebacterium diphtheria* induces macrophage apoptosis by binding to elongation factor 2 and inhibiting translation; mutant toxins deficient in ADP-ribosylating activity are not cytotoxic (Chang et al. 1989; Morimoto and Bonavida 1992). It has also been suggested that DT has nuclease activity, which may contribute to apoptosis by causing DNA fragmentation (Chang et al. 1989).

CT and LT induce apoptosis in T cells, especially $CD8^+$ T cells. Some reports have suggested that the proapoptotic effect of CT is linked to the ADP-ribosyltransferase activity of the S-1 subunit and increased intracellular cAMP (Yamamoto et al. 1999). However, others have shown that the recombinant B subunit of LT can also induce apoptosis in $CD8^+$ T cells by activating the transcriptional factor c-Myc and caspase-3 via a pathway involving NF-κB (Soriani et al. 2001; Salmond et al. 2002).

Products of type III secretion system interacting with caspases

Shigella spp. induces apoptosis of macrophages but not of other cell types. After phagocytosis of *Shigella* by macrophages it escapes from the phagosome into the cytoplasm, inducing apoptosis (Zychlinsky and Sansonetti 1997). The TTSS effector IpaB of *Shigella* sp. induces apoptosis by binding to and activating caspase-1 (Hilbi et al. 1998). Macrophage apoptosis induced by *Shigella flexneri* is associated with release of IL-1 and IL-18, which require caspase-1 for their activation. Caspase-1-deficient mice do not develop acute inflammation that is normally associated with *Shigella* infection, but they are unable to resolve the infection (Sansonetti et al. 2000). *Salmonella* spp. also induce apoptosis in infected macrophages and the mechanism appears to involve the TTSS and is similar to that described for *Shigella*. Although *Salmonella* sp. does not escape the phagolysosome, the SipB effector protein of *Salmonella* like IpaB of *Shigella*, associates with and activates caspase-1 (Hersh et al. 1999).

The establishment of infection by *Yersinia* spp. is dependent on the TTSS which encodes several Yop effectors; these interfere with the function of cells of the immune system. It has been reported that YopJ/YopP is essential for inducing apoptosis in vivo and in cultured macrophages (Monack et al. 1997, 1998). Induction of apoptosis, together with suppression of TNF-α production, facilitates the establishment of *Yersinia* infection in mice (Beuscher et al. 1995). YopP/YopJ induces apoptosis by impairing NF-κB signaling via binding and inhibiting IKKβ, the NFκB-controlling protein (Ruckdeschel et al. 1998). The inhibition of NF-κB and induction of apoptosis are enhanced by LPS (Ruckdeschel et al. 2001).

Fas-mediated apoptosis

Infection of human alveolar macrophages with *M. tuberculosis* causes apoptosis (Keane et al. 1997). Increased expression of FasL has been shown in macrophages within multibacillary leprosy and tuberculosis granulomas (Mustafa et al. 2001). Fas is a member of the TNF receptor family, which plays an important role in main-

taining lymphocyte homeostasis, and activation of Fas in the target cells by FasL is a key mechanism of $CD8^+$ CTL and Th1-mediated killing of target cells. The apoptosis can be rescued by TNF-α, IL-1β, or LPS (Mangan et al. 1991; Mustafa et al. 2001). However, it has also been shown that virulent strains of *M. tuberculosis* induce less macrophage apoptosis than attenuated strains; it was demonstrated that *M. tuberculosis* may evade apoptosis of macrophages through inhibition of TNF-α, and by stimulating release of IL-10 and soluble TNFR2 (Balcewicz-Sablinska et al. 1998).

Survival of *H. pylori* and the manifestation of peptic gastrointestinal diseases has been attributed to the *cag* pathogenicity island (PAI). Products of the *cag* PAI have been linked to the induction of apoptosis in epithelial cells (Wang et al. 2001; Jones et al. 2002). Mice deficient in Fas signaling have reduced epithelial cell apoptosis, impaired Th1 responses, and enhanced disease following *H. pylori* infection (Jones et al. 2002). Furthermore, antibodies to Fas or a caspase-8 inhibitor block the induction of apoptosis by *H. pylori*, whereas inhibition of FasL cleavage with a metalloprotease inhibitor increases apoptosis and this has been linked to products of the *cag* PAI (Wang et al. 2001). Persistent infection with strains expressing the *cag* PAI may be facilitated through negative selection of T cells encountering *H. pylori* antigens. *H. pylori* also induces apoptosis of macrophages and this has been linked to NF-κB-dependent induction of arginase (Gobert et al. 2002).

MODULATION OF DC MATURATION

Dendritic cells are considered to be the primary APCs for driving the differentiation of naïve T cells into distinct T-cell subtypes; therefore they play a key role in establishing immunological memory and in directing different arms of the T-cell response (Guermonprez et al. 2002). Immature DCs (iDC) are found in all lymphoid and many nonlymphoid organs, including mucosal tissues, where they exert a sentinel function, with high phagocytic activity. These iDCs endocytose antigens through a variety of receptors and have high capacity to process antigen for presentation by MHC molecules. Interaction with pathogens, including bacteria or their products, causes maturation of the DCs and migration to the T-cell areas of secondary lymphoid organs. The matured DCs have enhanced surface expression of MHCII costimulatory molecules, CD80 and CD86, and are highly efficient at presenting the processed MHC-bound peptides to T cells.

Infection of DCs with many bacterial species, including *L. monocytogenes*, *Salmonella* spp., *M. tuberculosis*, *M. bovis* BCG, *Treponema pallidum*, *Lactobacillus casei*, *B. pertussis*, *E. coli*, *S. aureus*, *Streptococcus gordonii*, *Mycoplasma catarrhalis*, and *C. trachomatis*, has been shown to activate DC maturation (Reis e Sousa et al. 1999). Maturation with most of these bacteria was associated with enhanced IL-12 secretion. In certain cases the bacterial molecules responsible for DC activation have been identified and many of these bind to TLRs and other PRRs. CpG motifs in bacterial DNA bind to TLR-9, flagellin from *L. monocytogenes* and *S. typhimurium* binds to TLR-5, LPS from gram-negative bacteria binds to CD14 and transduces signals through TLR-4, whereas peptidoglycan and lipoteichoic acid of gram-positive bacteria signal through TLR-2 (Ozinsky et al. 2000; Michelsen et al. 2001; Janeway and Medzhitov 2002). TLR-2 also recognizes lipoproteins derived from *M. tuberculosis*, *B. burgdorferi*, *T. pallidium*, and *Mycoplasma fermantes*, LAM from mycobacteria, glycolipid from *Treponema* spp., and LPS from *Leptospira* and *Porphyromonas* spp. (Akira et al. 2001).

Activation of DCs by different pathogen-derived molecules can influence the differentiation of naïve $CD4^+$ T cells into distinct T-cell subtypes, including Th1, Th2, or Tr cells (Figure 3). Binding of microbial molecules to TLRs activates a signaling pathway through NF-κB and MAP kinases, leading to activation of genes coding for inflammatory cytokines, including IL-12. CpG, LPS alone or with PT, can stimulate IL-12 production and activate iDCs into DC1 cells that drive the differentiation of naïve T cells into Th1 cells (Ausiello et al. 2002; de Jong et al. 2002). Stimulation of iDCs with CT or LT activates DC2 cells, which drive the differentiation of Th2 cells (Gagliardi et al. 2000). Finally, CT and *B. pertussis* FHA have been shown to activate DCs into a phenotype, termed DCr, which drives the induction of type 1 Tr cells (Lavelle et al. 2003; McGuirk and Mills 2002).

The induction of Th1 cells in response to infection with intracellular bacteria or the induction of Th2 cells by bacterial toxins or extracellular bacteria is central to the host antibacterial immunological defense mechanism. However, diverting the T-cell response to another nonprotective arm, such as Th2 or Tr1 responses during infection with intracellular bacteria, may be a strategy evolved by the bacteria to subvert protective immunity (see below under Modulation of cellular immunity). Alternately inhibition of maturation of the DCs by bacteria or their products can suppress the development of adaptive immunity to the pathogen. *L. casei* induces IL-12 production and maturation of DCs, whereas *L. reuteri* reduces *L. casei*-induced expression of IL-12 and CD86 (a cell surface costimulatory molecule) (Christensen et al. 2002), and induces a DC phenotype that may favor the induction of Th2 or Tr cells. Stimulation of murine DCs with FHA from *B. pertussis*, as well as activating DCs that drive the induction of Tr1 cells, inhibits the iDCs from maturing into DC1 cells, an effect mediated in part through the induction of IL-10 production (McGuirk et al. 2002).

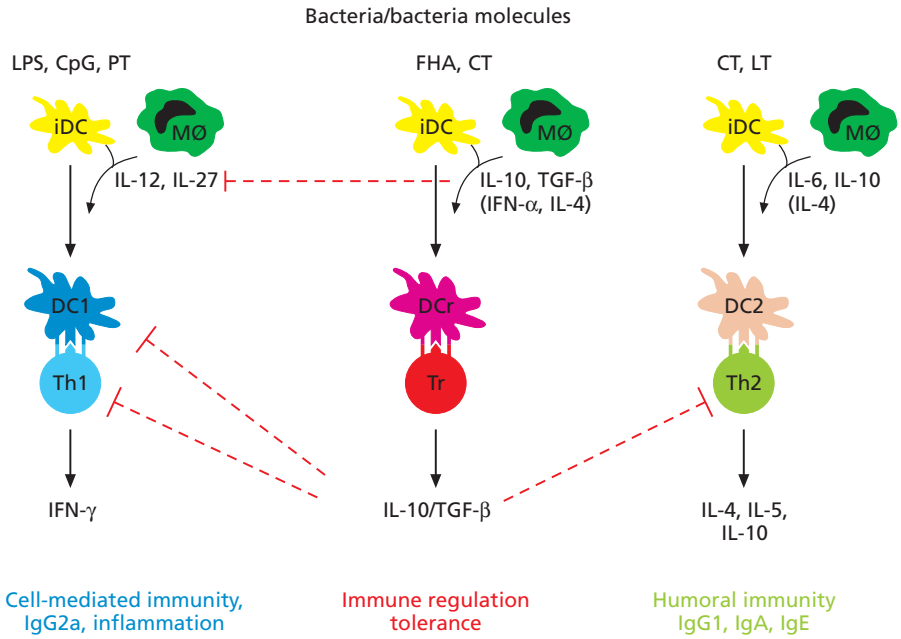

Figure 3 *Th cell subtypes in immunity to bacteria. Conserved bacterial structures interact with pathogen recognition receptors (PRRs) and activate maturation of the immature DCs (iDCs) into DC1, DC2, and DCr, which drive the differentiation of naive T-helper cells into Th1, Th2, or Tr cells respectively. Induction of regulatory cytokines by macrophages (Mϕs) and DCs in response to conserved bacterial molecules or virulence factors also plays a critical role in the selective development of distinct Th cell subtypes. Protective Th1 or Th2 cells are suppressed either directly or at the level of the antigen-presenting cells, through inhibitory cytokines, IL-10 and TGF-β, secreted by Tr cells. See text for abbreviations.*

MODULATION OF ANTIGEN PROCESSING AND PRESENTATION

Following bacterial uptake, antigens from the bacteria are processed by APCs and degraded peptides are presented in association with MHC molecules to T cells. $CD4^+$ T cells recognize processed antigens in association with MHCII molecules, which have been processed by an exogenous route of antigen processing. In contrast, antigens that are synthesized in host cells or escape from endosomes into the cytoplasm can enter the endogenous route of antigen processing for presentation by MHCI molecules to $CD8^+$ T cells. Finally, glycolipid antigens from bacterial cell walls can be presented by nonpolymorphic class I-like CD1 molecules to $CD8^+$ T cells or $CD4^-CD8^-$ NKT cells. Interference with the intracellular pathways that lead to the production of processed peptides or with the expression of MHC molecules on the surface of the APCs is a common strategy adopted by bacteria to subvert adaptive immune responses (Figure 4).

Interference with MHCII processing

Professional APCs, including DCs and macrophages, take up bacteria or their secreted soluble antigens by phagocytosis, macropinocytosis, or receptor-mediated endocytosis. The last occurs through interaction between antibody-coated, or antibody- and complement-coated, bacteria and Fc or complement receptors, or through direct binding of bacterial virulence factors to host cell surface ligands expressed on APCs and exploited by the bacteria for their uptake. Once inside an endosome the bacteria are transferred to late endosomes where they are degraded by lysosomal enzymes. Processed bacterial peptides associate with MHCII molecules after degradation of the invariant chain. Newly synthesized MHCII molecules with associated invariant chain are transported to the endocytic pathway, where an invariant chain-derived peptide class II-associated invariant chain peptide (CLIP) occupies the peptide-binding site in the class II molecule. In the class II-loading compartment CLIP is removed by interaction with HLA-DM and the bacterially-derived peptide is loaded. The stable MHCII-exogenous peptide complex is released to the cell surface where it interacts with the T cell receptor (TCR) of a $CD4^+$ T cell specific for that peptide.

The VacA toxin produced by *H. pylori* interferes with endocytosis in a process that requires Rab7 (Papini et al. 1997) and inhibits antigen processing by interfering with late endocytic membrane trafficking by APCs (Molinari et al. 1998). By lowering the amount of T-cell epitopes generated in the antigen-processing compartments, the toxin is capable of suppressing T-cell activation induced by APCs. Other pathogens have targeted the function of the class II-loading compartment. Patients with Chédiak–Higashai syndrome have a defect in a lyso-

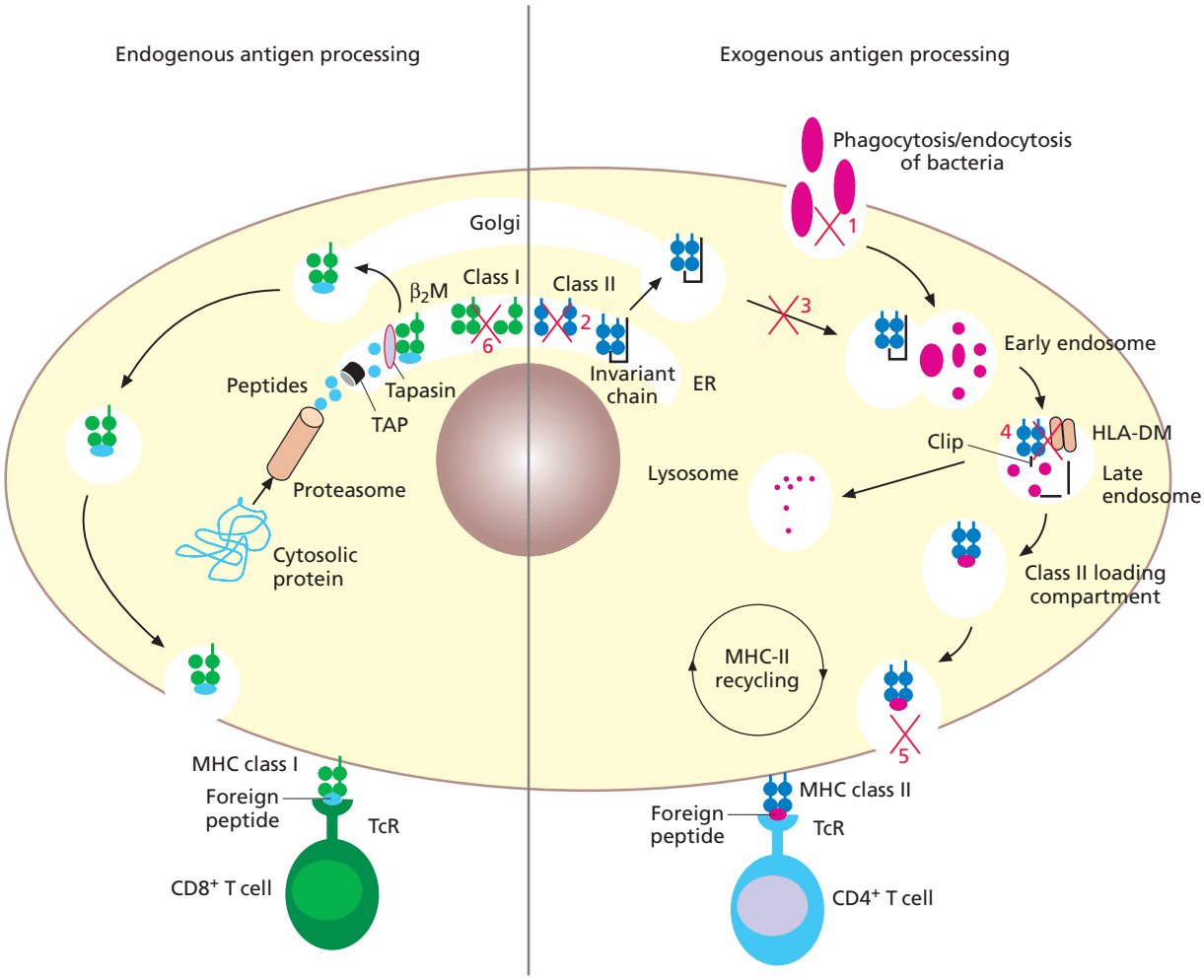

Figure 4 *Evasion of antigen processing and presentation. Exogenous antigens, including whole bacteria or bacterial proteins, are taken up by phagocytosis, endocytosis, or pinocytosis, and then degraded by proteolytic enzymes. In the endosome the processed antigen fragments or peptides associate with newly synthesized MHC class II molecules after removal of the invariant chain and the CLIP peptide by HLA-DM. The loaded MHCII molecule is exocytosed and presents the foreign peptide to CD4+ T cells on the surface of the APCs. Endogenous antigens that are synthesized in the host cells, or are released from endosomes into the cytosol, are processed by the proteasomes and TAP, before being loaded onto newly synthesized MHCI molecules in the endoplasmic reticulum. The loaded MHCI–peptide complex is exported to the cells surface, where it activates CD8+ cytoxic T lymphocytes. Bacteria can interfere with class II or I antigen processing and presentation by inhibiting: (1) phagocytosis/endocytosis, (2) class II biosynthesis, (3) export of class II molecules from the Golgi body to endosomes, (4) class II peptide loading, 5) export of the MHCII–peptide complex, or (6) class I biosynthesis. See text for abbreviations.*

somal-trafficking regulator and have enlarged lysosomes; B cells from these patients have altered peptide repertoires and delayed antigen presentation to T cells (Faigle et al. 1998). Enhanced interaction of human leukocyte antigen (HLA)-DR and HLA-DM, and more efficient removal of the CLIP peptide, may explain alterations in the dynamics of peptide loading observed in Chédiak–Higashai syndrome and in cells infected with *C. burnetii*, the rickettsial organism that causes Q fever (Lem et al. 1999).

Intracellular survival of *S. typhimurium* is facilitated by the *phoP* virulence locus, which controls the synthesis of many *Salmonella* proteins required for survival. The products of this locus also appear to affect antigen processing. Macrophages process model antigens expressed by *Salmonella* spp. more efficiently in *phoP-null* than wild-type bacteria (Wick et al. 1995). Furthermore, antigens in bacteria constitutively expressing PhoP are processed less efficiently by macrophages than wild-type bacteria. The *Salmonella phoP* virulence locus has also been found to be responsible for inhibiting antigen presentation in DCs. Although *S. typhimurium* expressing an ovalbumin (OVA) peptide induces maturation of the DCs, the *Salmonella phoP* virulence locus interfered with presentation of the OVA peptide to an OVA-specific T-cell hybridoma (Svensson et al. 2000).

Hemolytic strains of *L. monocytogenes* inhibit MHCII-restricted presentation of antigens to T cells. It has been suggested that listeriolysin, the hemolysin secreted by *L. monocytogenes*, inhibits antigen proces-

sing (Cluff et al. 1990). It was later reported that listeriolysin inhibits presentation of native antigens, but not peptides, to T cells; it was suggested that the defect was not caused by inhibition of antigen processing, but may result from irreversible inactivation of T cells that recognize antigen on listeriolysin-treated APCs (Darji et al. 1997).

Antigen processing can be inhibited by the ADP-ribosyltransferase activity of AB toxins; *E. coli*: heat-labile enterotoxin (LT) and cholera toxin (CT), but not the LT B subunit, CT B subunit, or nontoxic LT mutant LT-E112D, suppress intracellular antigen processing in APCs (Matousek et al. 1998). CT and LT inhibited processing of hen egg lysozyme (HEL) expressed in *E. coli* as a HEL fusion protein (containing the HEL 48–61 epitope), but not presentation of pre-existing peptide MHCII complexes to T-cell hybridomas (Matousek et al. 1996, 1998). In contrast, the B subunits of CT or LT or a mutant of LT with reduced enzyme activity do not affect antigen processing, but do enhance antigen presentation (Matousek et al. 1996, 1998). This is consistent with the demonstration that pre-treatment of APCs with wild-type LT or partial inactive mutant LTR72, but not the nontoxic mutant LTK63, suppresses IFN-γ production by a Th1 clone (Ryan et al. 2000). However, APC treatment with LT or LTR72 enhances IL-5 production by a Th2 clone (Ryan et al. 2000).

Yersinia YopH can inhibit activation of B cells by interfering with B-cell antigen-receptor adhesion. Following coincubation with *Yersinia*, B cells are unable to upregulate surface expression of B7-2 (CD86) in response to antigen stimulation (Yao et al. 1999). Downregulation of B7 expression on APCs may also contribute to defective antigen-induced T-cell activation after infection of mice with *M. tuberculosis* (Saha et al. 1994). Delayed-type hypersensitivity responses to crude soluble mycobacterial antigen were suppressed in infected susceptible BALB/c mice, but not in resistant C3H/HeJ mice. The defect appeared to lie in the inability of infected macrophages to provide a costimulatory signal for T cells (Saha et al. 1994).

Modulation of MHCII biosynthesis, intracellular trafficking, and cell surface expression

Different strains of mycobacteria have been shown to have varying abilities to inhibit antigen presentation to T cells by downregulating MHCI and MHCII expression on APCs, and the virulence of the organism has been related to these properties (Weiss et al. 2001). Diminished IFN-γ-induced cell-surface expression of HLA-DR on cells infected with *M. tuberculosis* results from defective transport and processing of class II molecules through the endosomal/lysosomal system (Hmama et al. 1998). Infection of macrophages with *M. bovis* has also been shown to downregulate IFN-γ-induced expression of class II transactivator (CIITA) mRNA, leading to inhibition of induction of MHCII molecules (Wojciechowski et al. 1999). Infection of macrophages with *M. avium* has been shown to arrest phagosome maturation, which limits the intersection of mycobacteria-containing phagosomes with the intracellular trafficking pathways of antigen presentation and results in sequestration of intracellular pools of MHCII and H-2 M molecules (Ullrich et al. 2000).

Phagocytosis of *S. aureus*, *E. coli*, *P. aeruginosa*, or *Salmonella enteritidis* by human monocytes reduces their ability to present an unrelated antigen, purified protein derivative (PPD) to T cells (Pryjma et al. 1994). Although antigen processing is not affected, the infected cells have reduced expression of MHC and accessory molecules, suggesting that the defect is caused by interference with antigen presentation. Phagocytosis of mycobacteria or *E. coli* by monocytes resulted in a failure to process and present antigens to MHCII-restricted T cells as a result of downregulation of MHCII expression, caused by a transient block in the transport of mature class II heterodimers to the surface of the APCs (De Lerma Barbaro et al. 1999). *Chlamydia* spp. can inhibit IFN-γ-induced MHCII expression on epithelial cells, but not IFN-γ-induced expression of the adhesion molecule ICAM-1 or IFN regulatory factor-1 (IRF-1), suggesting that it specifically targets signaling pathways required for MHCII expression (Zhong et al. 1999). The mechanism appears to involve diminished expression of IFN-γ-induced CIITA resulting from degradation of the transcriptional factor, upstream stimulatory factor-1 (USF-1), required for IFN-γ induction of CIITA.

Interference with MHC class I processing and presentation

Unlike viruses, where $CD8^+$ T cells play a major role in limiting infection by killing virally infected cells, MHCI-restricted T cells are induced only against certain intracellular bacteria. Extracellular bacteria do not enter the endogenous route of antigen processing and many intracellular bacteria can survive in intracellular locations distinct from class I processing sites. Therefore strategies for bacterial evasion of class I processing and presentation pathways are more limited. Nevertheless there are a number of reports that demonstrate that certain intracellular bacteria can inhibit expression of MHCI molecules or can bring about changes in the peptide repertoire bound to MHCI molecules.

Infection of murine macrophages with *L. monocytogenes* suppresses constitutive and IFN-γ-induced MHCI gene expression (Schuller et al. 1998). *Chlamydia* spp. can also suppress IFN-γ-induced MHCI expression in infected cells and the mechanism appears to involve degradation of USF-1 and RFX5, an essential down-

stream transcription factor required for constitutive and IFN-γ-induced MHCI expression (Zhong et al. 2000). *Chlamydia pneumonia* infection of monocytes down-regulates expression of MHCI molecules by inducing the production of IL-10 (Caspar-Bauguil et al. 2000). Thus *C. pneumonia* may suppress $CD8^+$ T-cell responses by inhibiting presentation of bacterial epitopes by MHCI molecules.

Expression of the nonclassic class I molecule, CD1, which presents mycobacterial glycolipids to unconventional T cells, is inhibited after infection of APCs with *M. tuberculosis* (Stenger et al. 1998). The loss of CD1 from the cell surface was associated with complete inhibition of antigen presentation to CD1-restricted T cells.

Invasion of HLA-B27-expressing HeLa cells by *S. typhimurium* enhances expression of IFN-γ-inducible proteasome genes, *LMP*, *MECL*, and *PA28*, and alters the peptide repertoire of HLA-B27 in the infected cell (Maksymowych et al. 1998). The altered peptide repertoire appeared to include peptides of host origin, suggesting that the infection may lead to the generation of autoreactive $CD8^+$ CTLs.

EVASION OF THE HUMORAL RESPONSE

Following activation of the adaptive immune response, B cells produce high concentrations of high-affinity pathogen-specific antibodies, which play a major role in protection against extracellular bacteria. Antibodies directed against adhesins and invasins limit colonization, whereas those directed against bacterial toxins prevent their binding to host target cells and thereby reduce the severity of disease. Murine IgG2a antibodies promote phagocytosis by opsonizing the bacteria and stimulating the complement components C3b and iC3b, which bind complement receptors and further promote phagocytosis. IgA antibodies, induced after bacterial infection of mucosal tissues, function to limit the infection at these sites. In an immune individual primed by a self-limiting infection or immunization, antibodies probably play the major role in conferring protective immunity. Therefore bacteria have evolved strategies to circumvent humoral immunity by evading both complement- and antibody-mediated immune responses (Figure 5).

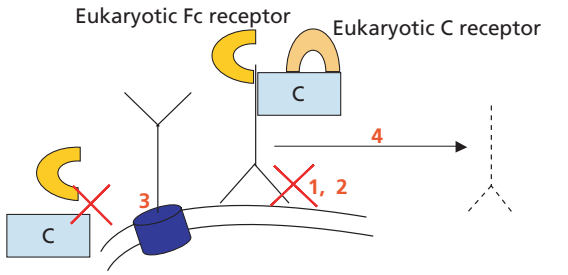

Figure 5 *Evasion of antibodies. Specific antibodies bind their cognate ligands on the bacterial surface, recruiting complement factors to the site. The antibodies and complement are recognized by Fc and C receptors on phagocytic cells, which mediate uptake into these cells. The complement cascade is also activated to form membrane attack complexes (MAC) and lyse the bacteria. Evasion of antibodies by bacteria can occur in a number of ways: (1) masking of the bacterial surface from antibody recognition, (2) avoidance of antibody recognition by antigenic and phase variation, (3) binding of antibodies by antibody-binding proteins, and (4) degradation and modification of antibodies.*

Antigenic variation and phase variation

Most bacteria can vary their surface composition to some extent (Henderson et al. 1999; Hallet 2001). The capacity to vary surface antigens can aid in evasion of complement-mediated killing and opsonophagocytosis, as new antigens are not recognized by existing antibodies and the classic pathway of complement does not become activated. Many of the complement-binding molecules of bacteria undergo phase and/or antigenic variation, giving rise to differences in the levels of serum resistance in different isolates and strains of the same bacterial species.

Antigenic variation of proteins occurs mainly by spontaneous point mutations in the genes encoding the protein antigen, such that the physical topography of the molecule is altered and antibodies directed against the original antigen do not recognize the altered one. Using phase variation, bacteria frequently and reversibly undergo altered expression of surface molecules resulting from genetic alterations in specific loci of their genomes. These two types of genetic alterations are particularly dominant in two classes of loci: (1) genes encoding surface structures such as LPS, flagella, and fimbriae, and (2) genes encoding virulence factors that modify host cell physiology or protect the bacteria from antimicrobial systems in the host. These surface structures not only affect bacterial virulence but are also key targets of the host immune system, resulting in selective pressure to generate polymorphism in genes coding for antigenic diversity. Varying expression and composition of these molecules allow bacteria not only to counteract the host immune defenses but also to colonize new ecological niches. These variations can be very extensive. In *S. enterica* there are more than 2000 separate serotypes based on differences in carbohydrate structures, such as LPS, and also flagella antigens (Fierer and Guiney 2001). There is enormous variation in the virulence and epidemiology of these serotypes.

CAPSULES

Phase variation can control gene expression in an 'on–off' manner. Genes for the biosynthesis of capsules in *Neisseria meningitidis* and *Haemophilus influenza* undergo this type of regulation (Gilsdorf 1998). The presence of a capsule may be beneficial in some circum-

stances, e.g. in the formation of biofilms, and a hindrance in others, e.g. for the motile stages of bacterial life. Bacteria growing in biofilms are highly resistant to the immune system of the host. Soluble factors and immune cells have limited access to bacteria in these circumstances as a result of the network of extracellular material surrounding the bacteria. Chronic bacterial infections, such as *P. aeruginosa* infections in the lungs of cystic fibrosis patients and *S. aureus* infections of prostheses, are difficult to treat because of the biofilm nature of growth of the bacteria. However, spread of the bacteria is enhanced when they are released from the biofilm capsule.

LPS AND LIPOOLIGOSACCHARIDE

Bacteria can alter surface molecule composition by controlling expression of genes for LPS and lipooligosaccharide (LOS) biosynthesis and modification (Lerouge and Vanderleyden 2002). LPS variations are mostly caused by differences in the polysaccharide side chains resulting from polymorphisms in the genes that encode the machinery to synthesize these polysaccharides (Liu et al. 1991). Furthermore, the regulation of lipid A modifications by *Salmonella* spp. renders these bacteria more resistant to antimicrobial peptides and renders the LPS less stimulatory for macrophages (Guo et al. 1997). LPS interacts with both antibodies and complement, and much of antibody response to *Salmonella* infection is directed against LPS O-antigens. LPS-specific antibodies play a major role in protecting against *Salmonella* infection and LPS variation allows *Salmonella* spp. to infect hosts that already have antibody directed against a different O-antigen serotype. Variation of LPS during infection may also be advantageous. Changes in *Salmonella* LPS structure result in changes in opsonin binding, in phagocytosis and killing by neutrophils, and in virulence (Jimenez-Lucho et al. 1987; Johnson et al. 1992). Other bacteria that alter their LPS composition include *H. pylori* and *L. pneumophila* (Appelmelk et al. 2000). During the course of an infection, antigens may vary or change with the host response and in *H. pylori* certain LPS structures mimic the eukaryotic Lewis antigens, allowing the bacteria to evade recognition and elimination. *C. jejuni*, *Neisseria gonorrhoeae*, and *H. influenzae* are all capable of altering their LOS structure (Gilsdorf 1998; Harvey et al. 2000; Linton et al. 2000). Acquisition of new LOS epitopes by *H. influenzae* is associated with increased resistance to complement-mediated serum and occurs rapidly among isolates (Kimura and Hansen 1986).

PROTEINS

Outer membrane proteins (OMP) frequently undergo phase and antigenic variation. *N. gonorrhoeae* strains may carry up to 12 phase-variable *opa* genes, encoding OMPs mediating bacterial adhesion and invasion. The expression of these functionally redundant genes is variable, thereby allowing the generation of antigen variants while maintaining and diversifying essential activities. The surface layer protein of *Campylobacter fetus* and OspC of *Borrelia* spp. also undergo antigenic variation (Dworkin and Blaser 1997). Pili and flagella are subject to variation, e.g. *N. gonorrhoea* has 18–19 pilin cassettes with which to make variable type IV pili. *Salmonella* flagella stimulate and activate macrophages and different flagella have varying potencies, suggesting that this flagellar phase variation may be a mechanism to downregulate inflammatory responses (Ciacci-Woolwine et al. 1998).

The *H. influenzae* IgA protease undergoes antigenic variation with over 30 serotypes and, in some cases, these variants result in changes in IgA protease specificity and its antibody inhibition pattern. (Lomholt et al. 1993). A global effect on gene expression can be achieved by variation in expression of a global regulator such as BvgAS of *Bordetella* spp. The acquisition or loss of unstable genetic elements (e.g. in *L. pneumophila*) or pathogenicity islands (e.g. SPI-1 and SPI-2 of *Salmonella* spp.) or pathogenicity plasmids (e.g. pYV plasmids of *Yersinia* spp.) in virulent bacteria can lead to significant changes in the protein composition of the bacterial membrane.

Antigenic variation contributes to the adaptive capacity of the bacterium for new environments and for survival. The ability to rapidly produce and modify specific structures on the cell surface is crucial in the establishment of infection, particularly with respect to the variety of biological niches within the host. Phase variation results in a more versatile and heterogeneous population that can cope better with a variety of different environments. It may result in better colonization as changes arise in structures mediating attachment and invasion of eukaryotic cells. It may lead to better evasion of host defenses as variation allows the bacteria to escape the neutralizing activities of antibodies and thus evade pre-existing host immunity.

Antibody-binding proteins

Several bacteria express immunoglobulin Fc-domain binding proteins, which are associated with the cell surface or released in soluble form from the surface. Binding of immunoglobulins via their Fc portion on the bacterial surface is thought to form a protective covering on the bacteria so that the bacteria are no longer recognized as foreign, and access of complement and specific antibodies to the bacteria is obstructed. Binding of IgG to Fc receptors on bacteria (e.g. SfbI on *Streptococcus pyogenes*) inhibits Fc-mediated phagocytosis by macrophages and antibody-dependent cell-mediated cytotoxicity (Medina et al. 1999). The best-known Ig-binding proteins are protein A of *Staphylococcus* spp. and protein

G of *Streptococcus* spp. (Kronvall 1973; Reis et al. 1984). One of the key events in the elimination of antigen or antigen–antibody complexes (immune complexes) is the covalent binding of C3b. This binding promotes uptake by phagocytic cells via C3 receptors. The two C3b-binding sites on IgG overlap the two regions that are bound by bacterial proteins such as protein A and protein G. Thus, these bacterial proteins directly interfere with C3b binding to the Fc region of IgG (Munoz et al. 1998). The surface-bound, M-protein family of *Streptococcus* spp. is made up of highly characterized, immunoglobulin-binding proteins and in most cases they bind only a single antibody class (Navarre and Schneewind 1999). There is also species specificity in the antibody binding. Binding of both IgG and IgA or IgG alone is found in all-invasive disease, group A streptococcus clinical isolates, whereas it is not so common in septicemia and noninvasive throat isolates (Raeder and Boyle 1995).

Human IgA is abundant in the secretions at mucosal surfaces such as those lining the lungs, gut, and genitourinary tract. These surfaces represent major potential sites of invasion and the immune protection offered by secretory IgA, as the predominant antibody at these sites, serves as a critical initial defense against pathogens. IgA recognizes and binds bacteria and triggers their elimination by interaction of its Fc domain with an Fcα receptor (FcαRI) expressed on neutrophils, eosinophils and macrophages. This interaction stimulates phagocytosis, superoxide generation and release of enzymes and inflammatory mediators. Two *S. pyogenes* IgA Fc-binding proteins, Arp4 and Sir22, both of the M-protein family, are important for virulence (Stenberg et al. 1994; Thern et al. 1998). Arp4 has been shown to inhibit a respiratory burst triggered by the binding of IgA-Fc to FcαRI on neutrophils. B protein of group B *Streptococcus* spp. also binds IgA (Jerlstrom et al. 1991). These three proteins all bind the Cα2-Cα3 interdomain of the IgA Fc domain. This domain is responsible for binding FcαRI and the streptococcal IgA-binding proteins interfere with this interaction (Pleass et al. 2001).

Antibody degradation and modification

Bacteria can degrade immunoglobulins to inactivate them. The degrading proteins are enzymes of broad proteolytic activity, which may also degrade complement and other host factors. IgG is degraded by the *P. gingivalis* PrtH protease, which in addition degrades the complement component C3 (Fletcher et al. 1995). Similar cell-associated proteases are present in *Lactobacillus* and *Streptococcus* spp. (Siezen 1999). IgA1 is degraded by the three leading causes of bacterial meningitis, *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*, important urogenital pathogens, and some members of the oropharyngeal flora. These proteases are important for the ability of bacteria to colonize mucosal membranes in the presence of secretory IgA antibodies and result in a local functional IgA deficiency.

IgA can be inactivated by modification of its immunoglobulin structure. *Streptococcus* and *Veillonella* spp. can remove terminally positioned sialic acid from IgA1. This may have an immune evasion function, because sialic acid protects glycoproteins, including immunoglobulins, from proteolytic enzymes. Deglycosylation of antibodies increases their sensitivity to proteolytic degradation and inhibits the Fc-mediated effector functions that mediate antigen disposal.

EVASION OF COMPLEMENT

The complement system plays a major role in resistance against microbial infections participating in both specific and nonspecific immunity (Rautemaa and Meri 1999). The complement activation pathways comprise a complex set of serum proteins that are activated by antigen–antibody complexes (classic pathway), certain carbohydrates (lectin pathway), or a variety of pathogen surface molecules (alternate pathway). Activation of complement has a number of consequences. Some complement activation products such as C3b, iC3b, C4b, and C1q are opsonins, inducing recognition and phagocytosis of the bacteria by macrophages and polymorphonuclear granulocytes. C3a, C4a, and C5a increase vascular permeability and induce inflammation. The chemotactic C5a also attracts leukocytes to the site of infection and activates them. Membrane attack complexes (MAC), composed of multiple complement factors, form on the surfaces of gram-negative bacteria and cause the collapse of membrane potential, leading to lytic cell death. Microbial activation of the complement system eventually leads to phagocytosis of the target and/or lysis of the bacteria by MACs. Given the dramatic effects of complement activation in the host, the complement system is tightly controlled to protect host cells from complement attack. Both soluble inhibitors, such as factor H, and cell membrane glycoproteins, such as protectin, are critical for regulating complement activation. Protectin regulates MAC insertion into membranes. Factor H inhibits the alternate pathway and C4bp inhibits the classic pathway, by both inhibiting interactions between the complement components and enhancing complement component degradation. Pathogenic bacteria have evolved strategies to evade and inhibit the complement system in order to resist opsonophagocytosis and/or complement-mediated cytolytic damage (Figure 6) (Rautemaa and Meri 1999; Wurzner 1999).

Masking the bacterial surface

Pathogens can mask their membrane surfaces to avoid recognition by complement. Prevention of access of

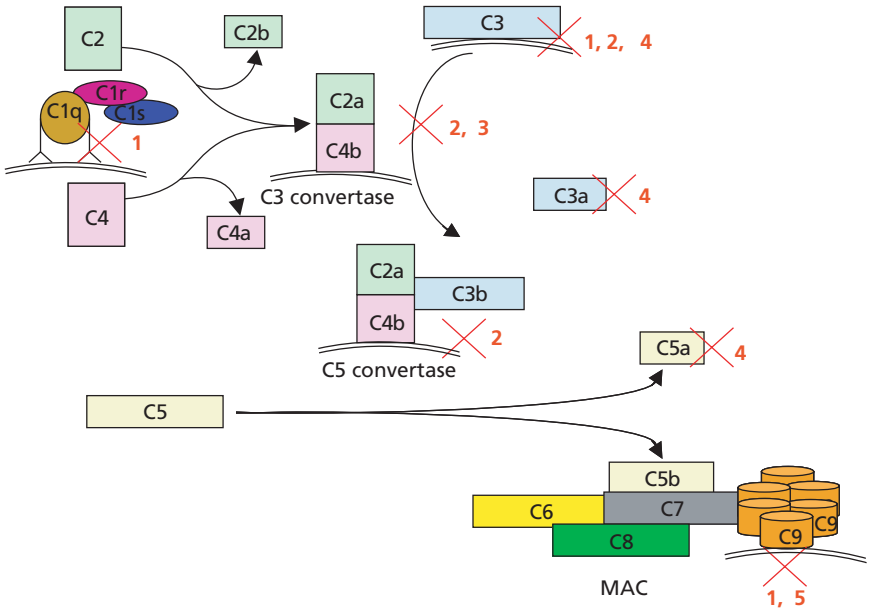

Figure 6 *Evasion of complement. In the classic complement cascade antibodies bound to the surface of bacteria recruit C1q, C1r, and C1s. This binding stimulates the cleavage of C2 and C4, releasing C2b and C4a. C2a and C4b associate to form the C3 convertase, which induces cleavage of C3 to C3a and C3b, thus forming a C5 convertase. C1q, C3b, and C4b are opsonins inducing recognition and phagocytosis of the bacteria by macrophages and polymorphonuclear granulocytes, whereas C4a and C5a are anaphylatoxins increasing vascular permeability and inducing inflammation. C5b and the chemotactic C5a are formed from C5, and C5b together with C6, C7, and C8 recruits the assembly of C9 components to form a lytic membrane attack complex (MAC). C3 and C5 convertases and MACs are also generated by the alternate complement pathway. Bacteria use a number of mechanisms to evade complement activation and killing: (1) masking of the bacterial surface, (2) shedding of complement complexes from the bacterial surface, (3) binding of complement inhibition factor H, (4) cleavage of complement components, and (5) inhibition of MAC insertion.*

complement to the cell membrane can by achieved by expression of polysaccharide capsules and/or long and dense LPS by gram-negative bacteria. These structures hinder access of both the initial complement factors and the lytic MACs. The insertion of MACs into the bacterial membrane is inhibited in gram-positive bacteria as a result of the presence of the surrounding peptidoglycan. *S. aureus* capsule expression leads to reduced C3 deposition on the bacterial surface, in turn leading to decreased phagocytic killing in vitro and increased lethality in vivo (Thakker et al. 1998; Cunnion et al. 2001). *S. pneumoniae* capsule expression also leads to decreased C3 deposition and the *H. influenzae* capsule is required for serum resistance and virulence of invasive strains (Brown et al. 1983; Noel et al. 1996). Group B meningococci and group B streptococci express capsular polysaccharides that are rich in sialic acid, which is relatively nonimmunogenic and prevents complement activation (Edwards et al. 1982). LPS composition influences recognition by complement, as in the case of some *Salmonella* spp. which possess LPS with long *O*-polysaccharide side chains that sterically hinder access of complement components such as C1q and MACs to the bacterial membrane and bacterial OMPs (Joiner et al. 1982a, 1982b; Liang-Takasaki et al. 1983). The LPS structure of *Klebsiella pneumoniae*, *C. burnetti*, and *B. abortus* also renders these bacteria more resistant to complement (Vishwanath et al. 1988; Merino et al. 1992; Eisenschenk et al. 1999).

Shedding

Bacterial pathogens can shed strong complement-activating surface molecules and immune complexes from their cell surfaces. Shedding prevents the accumulation of complement factors on the bacterial membrane and also diverts antibodies away from the bacterial surface. *S. aureus* sheds up to 30 percent of the C3 deposited on its surface (Cunnion et al. 2001) and *E. coli* and *S. minnesota* also shed complement factors and/or assembled complement complexes from their cell surfaces (Joiner et al. 1982a; Joiner 1988). Shedding of complement complexes from the surface of *S. pneumoniae* involves the action of the host complement inhibitory factor, protein H (Berge et al. 1997).

Cleavage of complement

Just as bacteria degrade antibodies to inactivate them, bacteria also degrade complement factors to inhibit activation of the complement pathway. *P. aeruginosa* cleaves complement components by extracellular secreted proteases, as do *H. pylori* and *P. gingivalis* (Hong and

Ghebrehiwet 1992; Schenkein et al. 1995; Rokita et al. 1998). *P. aeruginosa* elastase inactivates the anaphylatoxins C3a and C5a, resulting in a reduced inflammatory response. The PrtH protease of *P. gingivalis* is an important virulence factor for the bacterium because it degrades C3, resulting in a reduction of C3b and C3bi on the bacterial surface and inhibition of phagocytosis by neutrophils. *S. pneumonia* degrades C3 by a cell-associated activity that leads to inactivation of C3 and release of C3 from the bacterial surface whereas group A *S. pyogenes* possesses a cell wall-anchored C5a peptidase to inactivate C5a (Wexler et al. 1985; Angel et al. 1994).

Binding complement inhibition proteins

To avoid elimination by complement, microbes can exploit complement regulatory factors of the host. Some bacteria bind proteins, such as factor H or C4bp, that negatively regulate complement activation. The binding of factor H or C4bp confers serum resistance to bacteria and prevents their phagocytosis. Many of these binding molecules undergo phase and/or antigenic variation, giving rise to differences in the levels of serum resistance in different isolates and strains of the same bacterial species. Serum resistance is a major determinant of *N. gonorrhoeae* virulence, because serum-sensitive gonococci predominantly result in symptomatic local inflammation, whereas serum-resistant strains are associated with disseminated infection. The source of complement resistance in these bacteria is sialyation of low-molecular-weight LOS (Ram et al. 1998). Sialic acid-containing structures enhance the interaction between C3b and factor H. Binding of factor H to sialyated LOS leads to rapid conversion of surface-bound C3 to iC3b and to elimination of complement activation components from the cell surface. OMPs are also involved in binding factor H with similar consequences, e.g. *N. gonorrhoeae* Por1A which also binds C4bp and the expression of which correlates with serum resistance and disseminated gonococcal disease (Ram et al. 1998, 2001). Other factor H- and/or C4bp-binding proteins include the majority of the streptococcal M protein family, a family of more than 80 antigenically distinct proteins, most of which have antiphagocytic activity (Horstmann et al. 1988; Johnsson et al. 1996). The M-protein–C4bp interaction is important for serum resistance in these bacteria. In addition, several M-proteins bind immunoglobulins IgG and/or IgA through the constant Fc portion.

Inhibition of MAC insertion

Several gram-negative bacteria can interfere with the assembly of terminal MACs and thereby prevent lethal outer cell membrane damage. The related OMPs, Rck of *S. typhimurium* and Ail of *Y. enterocolitica*, cause a failure in the formation of fully polymerized tubular MACs (Heffernan et al. 1994). The TraT lipoproteins of *Salmonella* spp. and *E. coli* increase resistance to complement killing by interference with the generation of complement factors, and the correct assembly and membrane insertion of MACs (Pramoonjago et al. 1992). The Sic protein of *S. pyogenes* is important for mouse mucosal colonization and inhibits the normal cytolytic effect of MACs (Akesson et al. 1996).

MODULATION OF CELLULAR IMMUNITY

Inhibition of T-cell activation and proliferation

T cells recognize antigens through engagement of the TCR with processed antigenic peptide associated with an MHC molecule expressed on the surface of an APC. However, activation of the T cell is dependent on a second signal provided by interaction of CD28 on the T-cell surface with costimulatory molecules CD80 or CD86, expressed on the surface of activated APCs. In the absence of the second signal, the T cells become anergic and are no longer able to divide or respond to antigen. Engagement of CD3 and CD28 results in redistribution of lipid rafts to the interface between the T cells and APCs. Clustering of TCRs activates the T cell by bringing together tyrosine kinases and permitting their phosphorylation, which initiates a signaling cascade through the CD3 complex and leads to activation of genes required for proliferation and cytokine secretion. Proliferation of T cells with APCs or unpurified PBMCs in vitro, in response to foreign antigen or mitogens, which crosslink surface receptors on the T cells, is often used as a measure of T-cell activation. Alternately, the cytokine secretion pattern is used as an index of activation (or suppression) of distinct T-cell subtypes. Although IL-2 has been used as a marker of Th1 cells, it is now evident that this cytokine can be secreted by precursor T cells as well as by activated Th1 cells. IFN-γ production, without IL-4, IL-5, or IL-10 is a useful marker of Th1 responses, whereas IL-4 and IL-5 without IFN-γ can be used to define Th2 cells. The recently described Tr cells can also be discriminated from Th1 or Th2 cells on the basis of anti-inflammatory cytokines: IL-10 with or without TGF-β but no IL-4 or IFN-γ for Tr1 cells, and TGF-β, with or without IL-10 but no IL-4 or IFN-γ for Th3 cells.

As T-cell activation is an essential step in cellular and humoral immune responses to infectious pathogens, many bacteria have evolved strategies to evade adaptive immunity by suppressing T-cell responses. In a limited number of cases the bacterial molecule and its host cell target have been identified, but most studies on

suppressed T-cell responses during bacterial infection have not established the mechanism. Interference with any aspect of antigen uptake processing or presentation by the APCs or signaling in the T cell can inhibit T-cell activation. Furthermore, as activation of distinct T-cell subtypes is regulated by the reciprocal cell populations, factors that strongly promote the induction of one subtype may inhibit the activation of another. Consequently, suppression of T-cell responses or specific T-cell subtypes may reflect subversion of many different aspects of innate as well as adaptive immunity dealt with in other parts in this chapter.

Infection of mice with an attenuated *aroA* strain of *S. typhimurium* results in profound immunosuppression, with inhibition of T- and B-cell proliferation to foreign antigen (al-Ramadi et al. 1991a) and of the T cell's production of IL-2 in response to concanavalin A (Con A) (al-Ramadi et al. 1991b). *H. pylori* suppresses in vitro proliferative responses of human PBMCs to mitogens and antigens and a soluble cytoplasmic fraction of *H. pylori* mediates the suppression by acting on monocytes and directly on the T cells (Knipp et al. 1994). Nitric oxide (NO) was later identified as a soluble macrophage-derived factor capable of mediating *Salmonella*-induced immunosuppression (Gregory et al. 1993; MacFarlane et al. 1999). Treatment of mice with the NO inhibitor, aminoguanidine hemisulfate, blocks the suppressive effect of *Salmonella* infection on T- and B-cell responses, but it also blocks influx of neutrophils and macrophages into the spleen, which was associated with enhanced bacterial load and higher mortality of the mice (MacFarlane et al. 1999), suggesting that NO is involved in host defense as well as immunosuppression. Suppression of T-cell responses by *S. typhimurium* was also found to be mediated by soluble factors released by monocytes/macrophages and was reversed by the addition of IL-4 (al-Ramadi et al. 1991b).

In a limited number of bacterial infections where suppression of T-cell responses has been observed, the immunomodulatory molecules involved have been identified. *Yersinia pseudotuberculosis* can inhibit T- and B-cell activation by interference with T- and B-cell antigen-receptor activation; the effect is mediated by YopH, which has tyrosine phosphatase activity (Yao et al. 1999). Patients with lepromatous leprosy are highly immunosuppressed; their T cells do not respond to *Mycobacterium leprae* antigens and macrophages and $CD8^+$ T cells have been implicated in the suppression (Mehra et al. 1984; Salgame et al. 1984). The *M. leprae* antigen LAM-B, can suppress proliferative responses of PBMCs from lepromatous leprosy patients, tuberculoid leprosy patients, and normal individuals (Kaplan et al. 1987). Lipoglycans from *M. leprae* have also been shown to induce immune suppression in mice, including inhibition of delayed type hypersensitivity (DTH) responses and proliferation of lymph node cells to mitogens (Moura and Mariano 1997).

The OspA protein of *B. burgdorferi* inhibits proliferative responses of human PBMCs to phyohemagglutinin and Con A (Chiao et al. 2000). *B. burgdorferi* infection of disease-susceptible (C3H/HeJ) and disease-resistant (BALB/c) mice results in impaired proliferation of IL-2 and IL-4 production to mitogens (de Souza et al. 1993). More recently it has been suggested that Th1 responses are suppressed whereas Th2 responses are enhanced; *B. burgdorferi* transmission by *Ixodes scapularis* suppressed IL-2 and IFN-γ and enhanced IL-4 production in mice (Zeidner et al. 1997). Similarly, the inhibitory effect of *E. coli* LT on T-cell responses was shown to be specific for Th1 cells and was mediated by the effect of ADP-ribosyltransferase enzyme activity on APCs as well as on T cells (Ryan et al. 2000). Furthermore, it has been reported that *E. coli* LT-treated epithelial cells release soluble factors, probably prostaglandins, that inhibit proliferation of a T-cell hybridoma in vitro (Lopes et al. 2000).

Upsetting Th1/Th2 balance

The demonstration that T cells could be discriminated on the basis of cytokine secretion and function had a considerable impact on our understanding of immunity to infectious diseases. Following the discovery of Th1 and Th2 subtypes of $CD4^+$ T cells in the 1980s, a dogma emerged which stated that Th1 cells secreted IFN-γ and TNF-β and mediated cellular immunity against intracellular pathogens, including viruses and intracellular bacteria, whereas Th2 cells were the true helper cells and mediated humoral immunity against extracellular bacteria and parasites. Furthermore, as Th1 and Th2 responses are crossregulated through IL-4/IL-10 and IFN-γ secreted by the reciprocal subtypes, any upset in the balance of Th1 or Th2 cells could prolong the course of different infections and this could be used as a strategy for pathogens to prolong their survival in the host (see Figure 3, p. 13). To a certain extent that still holds; however, it is now full recognized that Th1 cells also provide help for B-cell production of antibody subclasses involved in virus neutralization, opsonization of extracellular bacteria, and complement fixation. Furthermore, other populations of $CD4^+$ T cells with suppressive function, termed 'Tr cells' may also inhibit protective Th1 and possibly Th2 responses to infectious agents.

In the field of bacterial immunity, *M. leprae* provides one of the best examples of the influence of Th1/Th2 responses on the outcome of infection. In patients with lepromatous leprosy, cellular immune responses to many antigens are profoundly depressed and T cells in these patients are also anergized to unrelated antigens. These patients make potent antibody responses to the bacteria but are not protected. In contrast, patients with tuberculoid leprosy have potent cellular immune responses and

their macrophages can kill the bacteria. The important distinction between the two diseases states is that the patients with lepromatous leprosy mount Th2 responses, which are not protective, whereas in the tuberculoid leprosy patients Th1 cells are dominant and IFN-γ secreted by these cells activates infected macrophages to kill the bacteria (Sieling and Modlin 1994).

A failure of Th1-cell induction or function has also been shown to result in increased susceptibility to other bacterial infections. Development of *B. burgdorferi* infection after transmission by *I. scapularis*, in disease-susceptible C3H/HeJ mice, is associated with the development of Th2 responses, but not in disease-resistant BALB/c mice (Zeidner et al. 1997). Th1 cells have been implicated in clearance of primary infection and preventing reinfection with *B. pertussis* and mice develop disseminating disease in the absence of functional IFN-γ (Mahon et al. 1997). However, Th1 responses are suppressed in the lungs and draining lymph nodes during acute infection (McGuirk et al. 1998), and this appears to result from a variety of immune subversion strategies by the bacteria, including the induction of *B. pertussis*-specific Tr cells (McGuirk et al. 2002).

Pathogen-specific IL-10/TGF-β secreting Tr cells

$CD4^+$ T cells that constitutively express the IL-2α receptor (CD25) in vivo have been shown to have immunosuppressive properties and to be capable of preventing autoimmune diseases mediated by Th1 cells. Suppressor $CD4^+$ T cells that secrete TGF-β were termed 'Th3 cells' (Chen et al. 1994) and suppressor T-cell clones that secreted high levels of IL-10 and IL-5, but not IFN-γ or IL-4, were termed 'Tr1 cells' (Groux et al. 1997). It was suggested that the primary function of these Tr cells is to maintain immunological homeostasis and tolerance to selfantigens. However, recent evidence suggests that these cells can also be generated against foreign antigen on infectious pathogens.

The first report on Tr cell clones specific for a bacterial antigen were murine Tr1 cells specific for the *B. pertussis* antigens FHA and pertactin from the lungs of *B. pertussis*-infected mice (McGuirk et al. 2002). Human Tr1 or Th3 clones have also been generated against the filarial parasite *Onchocerca volvulus* (Doetze et al. 2000) and the hepatitis C virus core protein (MacDonald et al. 2002). Functional studies on the *B. pertussis*-specific Tr1 clones demonstrated that they were capable of suppressing IFN-γ production by Th1 responses against the same or an unrelated influenza virus antigen in vitro, and also suppressed protective Th1 responses against the bacteria in vivo (McGuirk et al. 1998). The induction of Tr cells against a pathogen may be a means for the host to control or terminate Th1 responses in vivo and thereby limit immunopathology and collateral damage to host tissue. However, it may represent a novel evasion strategy adopted by bacteria to subvert protective cell-mediated immunity. It is interesting that local Th1 responses are suppressed in the lungs and draining lymph nodes during acute infection of mice with *B. pertussis* (McGuirk et al. 1998). Furthermore, in infants and young children the disease is very protracted, with secondary infection in the lung.

In addition to the chronic viral and parasitic diseases, there are a number of other bacterial infections that are either persistent or associated with immunosuppression in vivo. Although there are a variety of explanations, including bacterial interference with antigen processing and presentation or production of anti-inflammatory cytokines by innate cells, the induction of Tr cells by the pathogen may also contribute to this immunosuppression. With the exception of the *B. pertussis* study, bacteria-specific Tr cells have not been cloned or specifically identified at the polyclonal level. However, there is indirect evidence of suppressor, anergic, or IL-10- or TGF-β-secreting T cells in a number of bacterial infections, including infections with *Mycobacteria*, *Pneumocystis*, *Helicobacter*, and *Lactobacillus* spp.

A proportion of patients with active pulmonary TB have defective DTH responses to mycobacterial antigens (i.e. they do not mount a skin test response to PPD) and have a poor clinical outcome. T cells from these anergic patients secreted IL-10, but not IFN-γ, after in vitro stimulation with PPD, whereas PPD skin test-reactive patients secreted IL-10 and IFN-γ; IFN-γ production is augmented in T cells from anergic patients by the addition of anti-IL-10 (Boussiotis et al. 2000). Defective phosphorylation of TCRζ and defective activation of ζ-associated protein (ZAP)-70 and the mitogen-activated protein (MAP) kinase extracellular regulated kinase (ERK) was also demonstrated in the anergic T cells stimulated with anti-CD3 and anti-CD28 (Boussiotis et al. 2000). Furthermore treatment of mice with killed *Mycobacterium vaccae* induces allergen-specific $CD4^+CD45RB^{Lo}$ Tr cells which are capable of protecting against airways inflammation, through an IL-10- and TGF-β-dependent mechanism (Zuany-Amorim et al. 2002). In a study of *Pneumocystis carinii*-infected mice, it was demonstrated that Tr cells may function to control the Th1 response, which are responsible for eliminating the bacteria and mediating lung immunopathology. Adoptive transfer of $CD4^+CD25^+$ T cells delays bacterial clearance, but also prevents development of lethal pneumonia by $CD4^+CD25^-$ T cells (Hori et al. 2002). $CD4^+$ T cells from *Helicobacter hepaticus*-infected mice block colitis in recombinant activation genes $RAG^{-/-}$ of mice induced by transfer of *H. hepaticus*-specific T cells from IL-$10^{-/-}$ mice; suppression was mediated by IL-10 and has been attributed to *H. hepaticus*-induced Tr cells (Kullberg et al. 2002). *Lactobacillus paracasei* has been shown to induce IL-10 and

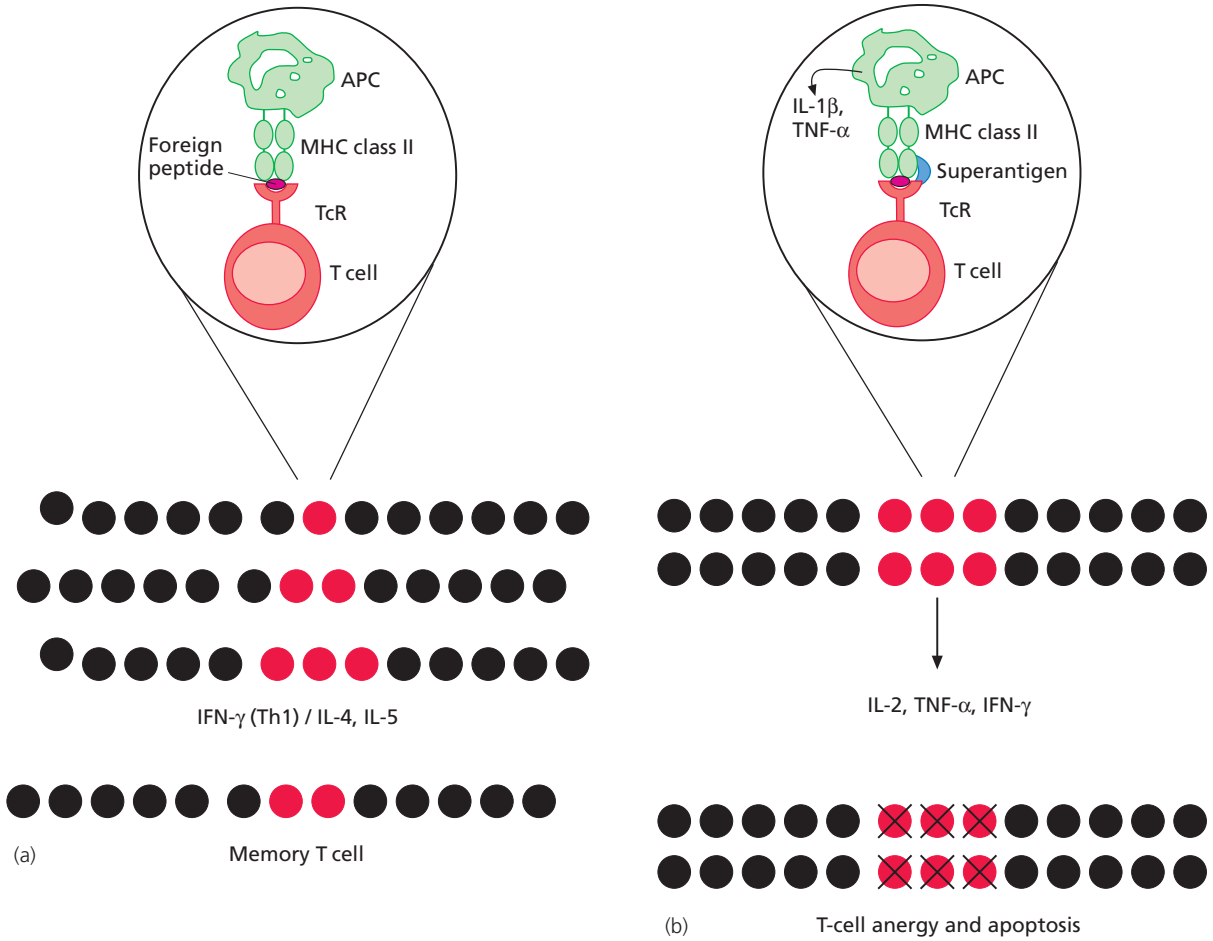

Figure 7 *Superantigen versus conventional antigen activation of T cells.* **(a)** *Conventional foreign antigens are processed by antigen-presenting cells (APCs) and peptide fragments associate with variable regions in the groove of the MHC class II molecule, and interact with variable regions of the T-cell receptor (TCR). Only a low frequency of T cells specific for the peptide MHC complex are activated. Activated T cells proliferate and secrete cytokines, and then a proportion goes on to become memory T cells.* **(b)** *Bacterial superantigens bind as intact proteins to MHCII molecules outside the foreign peptide binding groove and crosslink with the variable region of the TCR β chain on T cells. Following this encounter the APC produces proinflammatory cytokines, including interleukin IL-1β and tumor necrosis factor α (TNF-α) and all T cells bearing that Vβ element are activated to produce Th1-type cytokines IL-2, TNF-α, and interferon γ (IFN-γ), and then undergo apoptosis. This results in massive secretion of proinflammatory cytokines and deletion of the T cells expressing that particular TCR Vβ chain.*

IL-12 production by murine spleen cells, and addition of bacteria to cultures of $CD4^+$ T cells stimulated with alloantigens suppresses IFN-γ, IL-4, and IL-5 production and expands a population of Tr-type cells that secreted IL-10 and TGF-β (von der Weid et al. 2001).

Although there is evidence that iDCs drive the induction of anergic or Tr cells, there is growing evidence that differentiation of Tr cell from naïve precursor T cells appears to require DCs in a particular activation status distinct from that required to drive Th1 or Th2 cells. A number of bacterial products that bind to PRRs on macrophages and DCs have been shown to activate DC1 and DC2 in subtypes that direct naïve T cells into Th1 or Th2 respectively (see Figure 3, p. 13). It has now been reported that FHA from *B. pertussis* may drive a third population of DCs, termed 'DCr', that drive the induction of Tr1 cells (McGuirk and Mills 2002). IL-10 and IFN-α have been shown to function in differentiation of Tr1 cells, whereas TGF-β and IL-4 have been implicated in the differentiation of Th3 cells. FHA stimulates macrophages and DCs to secrete IL-10 and experiments with TCR transgenic mice demonstrated that FHA-stimulated DCs drove induction of Tr1 cells and this was switched to a Th1 phenotype in the presence of anti-IL-10 antibody. A number of bacteria have been shown to stimulate IL-10 production by macrophages and DCs and, although this may directly contribute to their anti-inflammatory and immunosuppressive effects, it may also create the appropriate cytokine milieu for the induction of Tr cells in vivo. There is also evidence that DCs or other innate cells in the lungs (Akbari et al. 2001), Peyer's patch (Iwasaki and Kelsall 1999), and liver (Khanna et al. 2000) have a propensity either to secrete IL-10 or to promote the induction of

anergic T cells. This may facilitate the induction of Tr cells at mucosal surface, where they may play major roles in maintaining tolerance against inhaled antigen, food antigens, and commensal. However, these DCs may be exploited for the induction of antigen-specific Tr cells by many pathogenic bacteria, viruses, and parasites that invade the body through the respiratory tract, gastrointestinal tract, or other mucosal surfaces.

Superantigens

Superantigens are microbial toxins with potent immunomodulatory properties, altering normal immune responses to foreign antigens and promoting autoimmune diseases. Unlike conventional T cells where the TCR binds to processed peptide antigens in the groove of an MHC molecule, superantigens bind outside the grove of MHC and crosslink MHCII molecules with a variable region of the β chain of the TCR, forming a trimolecular complex that activates a much larger number of resting T cells than does conventional antigen (Figure 7). Furthermore, superantigens are presented to T cells by MHC molecules usually without the need for antigen processing. Superantigens activate proliferation, secretion of cytokines, including TNF-α, IFN-γ, and IL-2 by 20–25 percent of resting T cells, eventually resulting in anergy, apoptosis, and depletion of peripheral T cells expressing specific TCR Vβ chain, with a resultant immunosuppression. A number of pathogens including *S. aureus*, *S. pyogenes*, *Mycoplasma arthritidis*, *Y. enterocolitica*, *C. perfringens*, and *M. tuberculosis* secrete toxins, which act as superantigens (Fraser et al. 2000; McKay 2001). The best-studied superantigens include the families of staphylococcal enterotoxins and streptococcal pyrogenic exotoxins. These toxins cause food poisoning and toxic shock-like syndrome, and may contribute to autoimmune diseases by breaking tolerance to selfantigens (Fraser et al. 2000).

The enhancement of autoimmune diseases by superantigens appears to be related to enhancement of proinflammatory cytokine production. *M. arthritidis* mitogen (MAM), a superantigen secreted by *M. arthritidis*, induces type 1 cytokine secretion (IL-12, IFN-γ, and TNF-γ, but no IL-4 or IL-10) in arthritis-susceptible C3H/HeJ mice, but Th2 responses in arthritis-resistant BALB/c mice (Mu et al. 2000). In contrast, it has also been demonstrated that repetitive injection of staphylococcus enterotoxin induces a state of unresponsiveness characterized by lack of secretion of the type 1 cytokines IL-2 and IFN-γ. Unresponsive animals were found to have IL-10-secreting $CD4^+$ Tr cells capable of suppressing bacterial superantigen-reactive T cells (Noel et al. 2001). Conversely, it has been demonstrated that natural $CD4^+$ $CD25^+$ Tr cells (Pontoux et al. 2002) can control superantigen-induced proinflammatory cytokine production. Activation of T cells with superantigens in vivo can indirectly induce maturation of DCs into a phenotype distinct from LPS-matured DCs, with high MHCII and CD40 expression but low-to-moderate expression of CD80 and CD86 (Muraille et al. 2002). This DC phenotype is distinct from DC1 cells, suggesting that superantigens may not necessarily favor the induction of Th1 cells.

CONCLUSIONS

The immune system has developed highly sophisticated mechanisms for combating infectious diseases and is capable of eliminating most pathogenic bacteria that invade the host. However, many of these bacteria can cause severe disease, especially in naïve and immunocompromised individuals. This is largely a reflection of the success of pathogenic bacteria in evading or subverting protective immune responses. This chapter has detailed some, but by no means all, of the strategies adapted by bacteria to target different aspects of innate and adaptive immunity. Indeed this may be just the tip of the iceberg and, as a periodic review of the current literature on the immunology of infectious diseases will reveal, there is rapidly expanding knowledge of the interplay between pathogens and their host. We still have a great deal to learn about our own immune system by studying how pathogens have evolved to evade it.

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Parasite evasion

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Parasites (Greek: pará-sitos, i.e. 'eat together') are unicellular (protozoan) or multicellular (metazoan) organisms that live in a close relationship on or within their hosts and are able to affect them adversely by acquisition of nutrients, damage to body surfaces, or penetration into tissues. They continue to be an increasing health problem and create a substantial economic burden, especially in less developed areas within the tropics.

Compared with the 5000 genes found in *Escherichia coli* bacteria, parasites have of the order of 7 000–20 000 protein-encoding genes. This genetic complexity may be required for the organization of the eukaryotic cell apparatus, but it is likely that a substantial number of genes contribute to the maintenance of the very diverse lifestyles that are perfectly adapted to survival in intermediate and definitive hosts with extremely different environmental conditions. Many parasites are more nuisance than danger to humans; some can, however, cause severe disease.

It is a characteristic hallmark of parasitic diseases that they are chronic in nature, with or without an acute onset. Chronicity of disease clearly indicates that either the immune system is unable to eradicate the invading pathogen or the continuous struggle of the immune system to combat the invading organisms leads to a state of immunopathology. Chronicity of disease also implies that the parasites have the ability to subvert or evade the immune response. Protozoan parasites with their smaller genomes usually need a relatively short time to replicate, and therefore evade host immune responses, often through mechanisms based on quick mutational or adaptive changes such as variation of their surface antigenic coat. In contrast, helminths have much longer generation times and therefore employ an array of other evasion mechanisms. For some organisms, such as *Trypanosoma brucei*, and, more recently, for *Plasmodium* spp. the molecular basis for immune evasion is quite well understood, whereas for others, especially helminth infections, the underlying mechanisms are much less clear.

The morphological and genetic complexity and the remarkably divergent lifestyles of parasites make them extremely interesting and, at the same time, difficult to approach for immunological studies. Nevertheless, considerable achievements have been made in the last decade and some of the parasites, e.g. *Leishmania* spp., have become paradigmatic to the understanding of basic immunological principles. This knowledge has helped to disclose some of the many determinants that regulate the type, the course, and the intensity of immune responses. It is also a prerequisite to establish a rational basis for the development of prophylactic and therapeutic immune intervention measures.

PROTOZOA AND THE DISEASES THAT THEY CAUSE IN HUMANS

Among the parasitic protozoa there are five major groups: Apicomplexa, Kinetoplastida, Trichomonadida,

Diplomomnadida, and Amebida. Apicomplexans include *Plasmodium* spp., *Toxoplasma gondii*, and *Cryptosporidium parvum*, *Leishmania* spp., and *Trypanosoma* spp. belong to the kinetoplastid protozoa. *Trichomonas vaginalis* is grouped in the order Trichomonadida, and *Giardia lamblia* belongs to the order Diplomonadida. Pseudopodia-forming organisms such as *Entamboeba histolytica* are allocated to the order Amebida. During evolution apicomplexan parasites have adapted to a life within host cells and have distinct organelles that are important for the invasion process. They infect different but distinct cell types depending on the stage of development. Flagellated organisms live outside (*T. vaginalis*, *G. lamblia*) or inside the body, either extracellularly within the bloodstream or the brain (*T. brucei*) or intracellularly in many different cell types (*T. cruzi*) or restricted to a narrow spectrum of host cells such as granulocytes, macrophages, dendritic cells, or fibroblasts in the case of *Leishmania* spp.

Plasmodium spp.

Four different species of *Plasmodium* are responsible for malaria in humans, i.e. *P. falciparum* (for genome sequence, see Gardner et al. 2002), *P. vivax*, *P. malariae*, and *P. ovale*. During a blood meal of an infected female anopheles mosquito (for genome sequence of *A. gambiae*, see Holt et al. 2002) they enter the bloodstream directly as sporozoites. Within less than an hour the sporozoites invade hepatocytes by recognizing glycosaminoglycans on the liver cells, through binding via a set of surface proteins called circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) (Muller et al. 1993; Frevert 1994). Inside the hepatocyte, the parasites divide and release merozoites that bind to different glycoproteins on erythrocytes, e.g. to a distinct region of erythrocytic glycophorin A via surface proteins called erythrocyte-binding antigen (EBA) (175) and merozoite surface proteins (MSP) (Orlandi et al. 1992; Perkins and Rocco 1988) or by ligation of the parasite protein P. falciparum erythrocyte membrane protein 1 (PfEMP1) to the complement receptor CR1 (CD35). Interestingly, the CR1 receptor is increased 40- to 70-fold in malaria-exposed African populations (Krych-Goldberg et al. 2002). Inside the erythrocyte, the parasites start to differentiate and multiply to produce more merozoites, which, after rupture of the red blood cell, are released to infect new erythrocytes. Some parasites differentiate into gametocytes which, after ingestion by a blood-feeding mosquito, fuse to form a zygote; this subsequently gives rise to the development of sporozoites that can infect additional humans.

After a typical incubation time of 1–2 weeks, the key features of acute plasmodial disease comprise sudden fever attacks caused by abrupt release of merozoites together with ruptured erythrocytes and its contents and, more seriously, cerebral coma and multi-organ failure (kidney, liver, lung). The latter results from reduced microcirculatory blood flow caused by structural and functional changes of infected (and uninfected) erythrocytes (Dondorp et al. 2000). Parasitized red blood cells adhere to endothelial cells primarily via PfEMP1 proteins, which are expressed on modified membrane regions called 'knobs' and bind to endothelial CD36, intercellular adhesion molecule (ICAM), thrombospondin, platelet endothelial cell adhesion molecule-1 (PECAM-1), and, especially in the placenta, hyaluronic acid and chondroitin sulfate A. In later stages of the disease, anemia develops together with splenomegaly accompanied by leukopenia and thrombocytopenia.

Protective immunity against *Plasmodium* spp. develops slowly and it may be necessary for a child born in a malaria-endemic area to be exposed to many successive infections for a number of years before developing any resistance; complete protection may never be achieved. The apparent inefficacy of antimalarial immunity may be explained by the parasite subverting or evading the immunological armamentarium by sequestration, poor immunogenicity of its antigens, or antigenic diversity and variation. The recent deciphering of the genome of *P. falciparum* (Gardner et al. 2002) has shown that many genes at the end of the parasite's chromosomes code for the expression of different versions of merozoite proteins on the surface of infected erythrocytes, thus confounding the immune responses that aim to destroy the infected cells.

Toxoplasma gondii

Infection with *T. gondii* is one of the most common parasitic infections of humans, other mammalians, birds, and other vertebrates. It is found worldwide with nearly one-third of the world population being exposed. In immunocompetent people, it does not cause serious illness but can lead to devastating disease in those with immunoincompetence, including fetuses and children born to mothers with primary infection during pregnancy as well as in patients with AIDS.

The definite host for the parasite is primarily the young cat (Dubey et al. 1970; Sibley et al. 1998; Carruthers et al. 2000; Brecht et al. 2001). In cats, the parasite's sexual stage occurs intracellularly in intestinal epithelia, leading to non-infective oocysts that are passed with the cat's feces in feed, water, or soil. After sporulation ('ripening') infective oocysts develop. When ingested by intermediate hosts (humans, livestock animals), the oocysts release tachyzoites. Ubiquitously expressed glycosaminoglycans on the host cells recognize secreted toxoplasmal proteins (MIC1 and MIC2), leading to adherence. Invasion occurs actively through an actin–myosin-based process with which the parasites glide, rotate, and undulate into a vacuole that is created mostly from material secreted by apical organelles

(Sibley et al. 1998; Mordue et al. 1999b; Brecht et al. 2001). Through formation of the vacuolar membrane, it selectively excludes host cell plasma membrane proteins (Mordue et al. 1999a) and thus may fail to induce host cell signaling. Within the cell, *Toxoplasma* spp. remain inside the parasitophorous vacuole by inserting their own proteins into the membrane. This prevents acidification and fusion with the lysosomal contents (Carruthers et al. 2000). The parasites divide until the host cell is filled with parasites and transform into bradyzoites. Neighboring cells may be infected or – especially after the host has acquired immunity – the bradyzoites may build up a cystic membrane with hundreds of parasites that can persist for prolonged periods of time, possibly life-long. When cysts, e.g. in undercooked or raw meat, are eaten by another mammal, the bradyzoites can continue the life cycle. After ingestion by a cat, they can initiate the sexual cycle.

In immunocompetent people, most infections are asymptomatic or show mild clinical signs such as low-grade fever, fatigue, headache, and muscle and joint pain. More common is painful swelling of cervical lymph nodes. In people with impaired cellular immunity, as occurs after HIV infection with CD4 T lymphocytes of less than 50/μl peripheral blood, *Toxoplasma* spp. can cause a life-threatening disease. On liberation of bradyzoites from the cysts, about one-third of AIDS patients develop a necrotizing focal encephalitis with dissemination in various organs, often with lethal consequences. Another extremely susceptible population are unborn children who acquire *T. gondii* from women who experience a primary infection during pregnancy. Depending on the intensity and the time point of infection, stillbirth, hydrocephalus, or intracerebral calcification including the retina can occur.

Encystment of toxoplasma parasites is dependent on a robust acquired immunity involving dendritic cells, CD4 and CD8 lymphocytes, as well as interleukin-12 (IL-12) and interferon-γ (IFN-γ) as the central protective elements (Yap and Sher 1999; Scanga et al. 2002).

Cryptosporidium parvum

Cryptosporidia are apicomplexan parasites that have long been known as pathogens for livestock animals, especially calves. The first case of human cryptosporidiasis was reported only in 1976 (Meisel et al. 1976; Nime et al. 1976). The organism is spread from animals to humans but also from person to person, and is often transmitted by contaminated water. It is distributed throughout the world. Its risk for human health is exemplified by a recent outbreak as a result of failure of appropriate water purification processes in the USA (MacKenzie et al. 1994).

The life cycle of *C. parvum* is confined to the microvillous region of the intestinal epithelia within one definitive host. After ingestion of infective oocytes, sporozoites are released which invade enterocytes and form an intracellular but extracytoplasmic parasitophorous vacuole, which is limited by a four-layer membrane. After multiplication and differentiation, merozoites are released which can infect other enterocytes through autoinfection. Some merozoites develop into sexual stages that produce a thick-walled oocyst after fusion in the parasitophorous vacuole, which is shed through the feces and can infect other individuals.

Infection with *C. parvum* may be without any symptoms or may lead to a self-limiting disease with watery diarrhea, nausea, and abdominal cramping for a few days. In patients with underlying illnesses affecting the adaptive immune system such as those with AIDS or on immunosuppressive therapy, the intensity and duration of gastrointestinal symptoms can be life threatening. In addition to the gastrointestinal tract other epithelia such as those of the respiratory system or the gallbladder can be infected (Chen et al. 2002).

Despite substantial knowledge of the life cycle and excellent morphological evidence, comparatively little is known about the immunobiology of *C. parvum* infection as a result of the lack of an appropriate animal model and the inability to culture sufficient numbers of parasites in vitro. Existing evidence suggests, however, that, very similar to Toxoplasma infection, the adaptive cellular immune system is dominant over the humoral immune system to provide protection. The increasing number of genes or genomic DNA fragments registered in GenBank (www.ncbi.nlm.nih.gov/cgi-bin/genbank?Cryptosporidium) will greatly facilitate our understanding of immunological and basic biology aspects in the near future.

Trypanosoma spp.

TRYPANOSOMA BRUCEI COMPLEX (AFRICAN TRYPANOSOMES)

Based on similarities in geographical distribution, infectivity to humans, life cycle, morphology, and major biochemical features, the *Trypanosoma brucei* complex comprises three subspecies: *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense*. In this chapter they are referred to as *T. brucei*. The parasites are endemic in many countries of sub-Saharan Africa.

Metacyclic forms of *T. brucei* are transmitted by the bite of an infected tsetse fly (*Glossina* spp.) through contaminating saliva. After inoculation, they remain at the infectious site for a short time and induce a local inflammatory response of the subdermal tissue (‘chancre’). Subsequently, they are disseminated throughout the body once they have gained access to lymph and blood vessels. They multiply rapidly extracellularly and lead to different waves of parasitemia, which are accompanied by systemic inflammation with perivascular infiltration of leukocytes. In later stages of the disease, they enter the central nervous system (CNS), the heart, and endocrine

organs. CNS involvement is associated with a wide array of behavioral changes, ranging from aggressiveness to lethargy, somnolence ('sleeping sickness'), or death.

The way in which *T. brucei* causes disease is unclear, but the success of infection is linked to the parasite's ability to select for the expression of one out of hundreds of genes that code for a major surface coat antigen, the variant surface glycoprotein (VSG) (Donelson et al. 1998; Andrews 2002; Berriman et al. 2002). Each VSG induces a strong and specific antibody response, which clears up to 99 percent of the parasites bearing it during a given wave of parasitemia. A minor population of pre-existing parasites (between 0.000001 and 1 percent) expresses a different VSG and thus is not recognized by the specific antibodies. This population then can outgrow and give rise to the next wave of parasitemia, until the new immune response leads to their demise; this scenario is repeated several times.

TRYPANOSOMA CRUZI (NEW WORLD TRYPANOSOMES)

T. cruzi parasites are endemic in several Central and South American countries and they are the cause of Chagas' disease. Metacyclic trypomastigotes are deposited on the skin through the feces of blood-feeding triatomine bugs. They are motile and have the capacity to penetrate abraded skin or the wound caused by the bite of the bug. They can also cross the conjunctiva or nasal and oral mucosa, if bug feces reach these surfaces. Once inside the host, they enter phagocytic or nonphagocytic cells and convert into replicating amastigotes. The responsible attachment and invasion receptors and ligands are not well defined. Contact of trypomastigotes with the host cells leads to the recruitment of host cell lysosomes to the plasma membrane, where both coalesce and fuse, forming a compartment in which the parasites enter to form a phagosome (Andrews 2002) (www.archive.bmn.com/supp/part/andrews.html). This process is initiated by a parasite oligopeptidase B, which triggers transient elevation of the intracellular calcium level in the host cell (Burleigh 2000) and is dependent on the activation of the transforming growth factor β (TGF-β) signaling pathway. *T. cruzi* resides only transiently within the phagosome, because it releases membrane-dissolving molecules that allow the parasite to enter the cytoplasm. After nine cycles of binary replication, the amastigotes differentiate into trypomastigotes and are released in large numbers into the bloodstream and can infect other cells or a triatomine vector.

T. cruzi leads to a number of clinical manifestations, which include lymphadenopathy, generalized edema, enlargement of the heart with arrhythmias, dysfunctions in the alimentary tract with loss of peristalsis, severe obstipation, and progressive dilation of the gut. These signs result from chronic infection of the respective cells, although chronic autoimmune inflammation has been suggested as an underlying pathogenic principle. In patients with AIDS the organism commonly crosses the blood–brain barrier with fatal meningoencephalitis. Both antibodies and T cells are important antiparasitic effector mechanisms necessary to combat the trypanosomal infection at intra- and extracellular locations (Ouaissi et al. 2002).

Leishmania spp.

Leishmania spp. are parasites that infect humans in all continents except Australia. Eighteen species are known to cause human disease. Some lead to localized cutaneous disease, whereas others cause systemic disease.

Leishmania spp. are transmitted by the bite of sand flies (*Phlebotomus* and *Lutzomyia* spp.). In the gut of the insect they develop as flagellated motile promastigotes, which are highly infective in their metacyclic stage – found in the salivary glands (Sacks 2001). During blood feeding they are deposited, together with the sand fly's saliva, in the epidermal and dermal tissue. Saliva components greatly facilitate the establishment of an infection and reduce the infectious dose required to establish a successful infection (Belkaid et al. 2001; Zer et al. 2001). Promastigotes have a dense layer of a lipophosphoglycan (LPG) and a 63-kDa glycoprotein (gp63) zinc metalloprotease, which both serve as virulence factors (Sacks and Kamhawi 2001; Joshi et al. 2002). The outer portion of the LPG extends from the surface of the parasite to form a glycocalix, which activates host complement and leads to deposition of C3 on the parasite's surface. At the same time the glycocalix and gp63 prevent membrane attack complex (C5–C9) being inserted into the parasite's membrane. The C3-coated promastigotes attach to complement receptors 1 and 3 (CR1, CR3) on macrophages. On reorganization of the actin–myosin cytoskeletal apparatus (May and Machesky 2001) of the host cell membrane, components of the endoplasmic reticulum (ER) are recruited close to the surface of the cell, where they fuse with the plasma membrane, which opens up at the site of parasite contact. The parasite then slides into the open ER, whereas the plasma membrane is resealed and the formation of the phagosomal vacuole is initiated (Gagnon et al. 2002).

At very early stages of infection, *Leishmania* spp. are phagocytosed by neutrophilic polymorphonuclear granulocytes (PNG), which are attracted to the site of infection through a parasite-derived chemotactic factor (Van Zandbergen et al. 2002). Inside the PNGs, they delay the usually rapidly occurring granulocytic apoptosis through inhibition of a caspase 3-related pathway (Aga et al. 2002). Thereby they are shielded from the hostile extracellular host milieu until ingestion by mononuclear cells. Once inside the definitive host cells, *Leishmania* are directed to the lysosomal compartment, where they transform into nonflagellated amastigotes, persist, and replicate. Through various mechanisms they inhibit

signaling events that would normally lead to macrophage activation and parasite killing. However, interferon-γ (IFN-γ) is able to activate macrophages to overcome this parasite-induced block and is able to promote parasite killing by inducing inducible nitric oxide (NO) synthase expression and NO production (Bogdan 2001). The murine mouse model of subcutaneous *L. major* infection has become paradigmatic for studying the adaptive immune response with respect to the roles of T-helper 1 (Th1) and T-helper 2 (Th2) lymphocytes in vivo.

In humans, disease develops as a result of incomplete parasite killing. This in turn results in chronic lesions at skin or mucosal sites (*L. major*, *L. amazonensis*, *L. braziliensis*, and *L. chagasi*) or in internal organs rich in macrophages such as the liver, spleen, or bone marrow (*L. donovani*, *L. infantum*, and *L. braziliensis*). In addition, excessive and damaging immune responses to the parasite may cause serious and debilitating disease (mucocutaneous leishmaniasis caused by *L. amazonenis*).

Entamoeba histolytica

Six species of amoebae are commonly found in the human gastrointestinal tract, of which *E. histolytica* is of medical importance. The organism is taken up with contaminated drinking water as an infectious cyst. Intraintestinally, the cyst wall is dissolved and gives rise to trophozoites, which can either form new cysts or occasionally penetrate the mucosa and disseminate to other organs, with serious implications such as amoebic colitis or liver abscesses. *E. histolytica* trophozoites are able to kill epithelial and immune cells rapidly. The killing is contact dependent and mediated by the trophozoite, Gal/GalNac lectin, which recognizes and binds host cell glycoconjugates and is associated with the release of pore-forming proteins (Leippe et al. 1994; Petri et al. 2002). Once lectin-mediated host–parasite contact occurs, the parasite activates host cell caspase 3, resulting in immediate cell death by apoptosis (Huston et al. 2000). Both local and systemic humoral and cellular immunity appear to play a role in protection, which may open up a window for the development of a vaccine (Miller-Sims and Petri 2002).

IMMUNITY, PERSISTENCE, AND EVASION IN INFECTIONS WITH PROTOZOAN PARASITES

As has been outlined so far, protozoan parasites have an extremely diverse array of lifestyles. This poses a challenge to the immune system which goes far beyond what is well understood by using experimental model antigens such as bovine serum albumin or relatively simple organisms such as viruses. Instead, the innate immune response has to recognize, at the same time, a vast variety of molecular patterns at one or different sites in the body. In addition, host defense has to be extremely flexible in tailoring the appropriate adaptive immune response as a result of the developmental stages through which many parasites go. The site of infection (skin, mucosa, conjunctiva, direct inoculation by blood-feeding arthropods with saliva), the number of infecting organisms, the parasitic lifestyle (intracellular or extracellular, within a vacuole or in the cytoplasm, replicative or not, production of antigenically distinct progeny), the overall constitution of the host's immune system (influenced by genetic susceptibility/resistance, age, nutritional status, previous experience with parasitic or other infections) are all determinants for what constitutes a protective immune response.

Paradigmatic experimental work on the analysis of the immune response to *L. major* after experimental subcutaneous syringe infection of different inbred mouse strains has shown the importance of the development of a polarized Th1 or Th2 answer. Animals that are able to mount an adaptive response dominated by Th1 lymphocytes survive and are resistant to re-infection, whereas those with a Th2-biased response are highly susceptible and succumb after exposure (for review, see Solbach and Laskay 2000). From these findings it was expected that, in all parasitic infections, a Th1 response would have protective effects, whereas Th2 responses would be associated with a failure to resist infection. It appeared, however, that this perception was not true. Work on immunity to intestinal helminths showed an exactly opposite pattern of severity of disease and the type of adaptive immune response: Th2-type responses in many cases allowed the resolution of infection, whereas Th1-type responses promoted chronic and nonprotective courses of the disease (MacDonald et al. 2002). This led, for a while, to the simplistic concept that a Th1 response alone might be sufficient for immune control of intracellular protozoa whereas a Th2 response, being responsible for the development of antibodies and the dampening of a potentially overreacting Th1 cellular immune response, was meant to keep extracellular parasites restricted. It soon turned out, however, that a delicate balance between Th1 and Th2 immune responses is responsible for shaping the outcome of most, if not all, parasitic infections. It also became clear that this balance varies considerably over time and controls the production of cytokines such as IFN-γ, the interleukins IL-4, IL-10, and transforming growth factor β (TGF-β). At the same time, T cells by themselves are under the tight control of cytokines which constitute the complex mixture of humoral factors at the infectious site. The result of all these determinants is responsible for activation of the protective antiparasitic effector arms.

The kinetics and magnitude of cytokines produced by T cells is determined by the initial interactions of the parasites with early inflammatory cells such as granulocytes and dendritic cells. They are equipped with an array of pathogen recognition receptors (PRR) on their surface. PRRs are activated by the parasite's pathogen-

associated molecular patterns (PAMP) and thus serve as innate 'master regulators' of the ensuing adaptive immune response, e.g. the PAMP-protein Tc52 from *T. cruzi* (which is also crucial for the parasite's survival) binds to the PRR Toll-like receptor 2 (TLR-2) on dendritic cells. This interaction induces host cell maturation and production of inflammatory chemokines, and protects mice from an otherwise lethal infection (Ouaissi et al. 2002).

In contrast to prokaryotes, many infecting eukaryotic parasites undergo a series of developmental changes during their establishment, development, or proliferation within the host. In consequence, although infected with only one given parasite species, the host is exposed to numerous sets of PAMPs as the infectious organism proceeds through its developmental pathway, e.g. infection with *Plasmodium* spp. exposes the host to four different extracellular life-cycle stages – the sporozoite, the merozoite, and the male and female gametocytes – plus the additional forms that live within the hepatocytes or erythrocytes. Although all these life-cycle stages share the expression of a basic set of genes, they also express stage-specific genes that code for the proteins required for successful completion of the life cycle. Thus, primary exposure to malaria leads to a series of immune responses directed to related, but antigenically distinct, organisms. The situation is even more complex in helminthic infections. Here, the larval form is often completely different from the adult worm or its progeny, and thus a whole series of different immune responses are initiated and maintained, e.g. eggs produced by adult schistosomes produce a Th2 response, whereas the worms themselves do not (Pearce and MacDonald 2002).

As a response to the antigenic complexity of parasites, the host creates a similarly complex set of immune responses. The relative contribution of the various immune mechanisms in operation throughout the course of infection is far from being understood, e.g. the early CD8 T-cell response is important in dealing with the hepatocytic stage of the infection with *Plasmodium* spp., but is largely irrelevant once the parasites have entered erythrocytes (Good and Doolan 1999). In contrast, in leishmania infections, CD4 T cells of the Th1 type mediate a protective immune response during the acute stage, whereas, for the induction of immunity with low-dose infections and its maintenance in chronic stages, CD8 T cells may be important (for review, see Solbach and Laskay 2000; Belkaid et al. 2002). Finally, maintenance of multiple effector responses, including antibodies, CD4 Th1 cells, and CD8 T cells, is required for the control of *T. cruzi* infections in both the acute and the chronic stages (Tarleton 2001).

In most parasitic infections the immune system sees and responds to a complex array of antigens, possibly with some exceptions, where a restricted number of antigens is dominant, e.g. *T. brucei* populations express for a brief period a given set of variant surface glycoprotein acronym>VSG, and in *L. major* infection of BALB/c mice the leishmania homolog of the receptor for activated c kinase (LACK) antigen is immunodominant (Schilling and Glaichenhaus 2001). The complexity in the immune responses makes it a challenge to develop an appropriate set of vaccine antigens that will elicit the appropriate protective effector mechanisms at relevant sites of infection, i.e. either in the tissues or at mucosal surfaces.

Most parasites survive within most of their hosts for long periods, thereby causing chronic infection (and in some cases chronic disease) and long-term stimulation of the immune system. From an evolutionary point of view, chronicity of parasitic infections indicates success for both the parasite and the host, because both are able to survive with each other for a long time. Recent experimental and clinical evidence suggests that immune responses induced by chronic helminthic infections during early life via the anti-inflammatory activity of IL-10 may shape the development of the immune system towards a Th1 bias such that it prevents immune dysfunctions that result in diseases such as allergy (van den Biggelaar et al. 2000; Bashir et al. 2002; Yazdanbakhsh et al. 2002).

Antigen variation

Chronicity of infection requires that the parasite must in some way be able to evade the immune attack to avoid its own elimination. Some protozoan parasites evade the immune response by sequential variation of their antigenic coat. African trypanosomes live extracellularly in an environment with high concentrations of antibodies which they induce themselves. They evade immune responses by selecting for the expression of one gene from a repertoire of up to 1000 variant genes through transcriptional activation. These genes encode the monomolecular VSG. VSG is composed of glycosylphosphatidylinositol (GPI)-anchored homodimers that are identically oriented and densely packed (10^7 molecules/cells) on the membrane surface. The repetitive VSG elements are scanned by B cells, which become activated without the help of T lymphocytes to produce a rapid IgM response. Within the parasite population, however, a spontaneous VSG gene switching occurs with one switch in 10^2–10^6 cells (Turner 1997). In consequence, the immune response occurring at a given time is appropriate and successful at killing trypanosomes through antibodies directed against the dominant surface antigens. However, the switch in antigen phenotype of an initially small population of parasites allows their expansion until sufficient antibodies are produced that recognize the new VSG variant. In this situation, the host eventually dies without ever completely eliminating the organisms from the body.

Although VSG-specific antibodies provide an important mechanism for controlling trypanosome numbers in the vasculature, antibodies alone are not sufficient to provide a significant level of host resistance. For control of parasites residing in the tissues, an early Th1 response with IL-12-driven and IFN-γ-dependent activation of macrophages is indispensable. This T-cell response is mainly induced by antigens that stem from an internal subregion of the VSG molecules which are liberated from the surface coat.

Antigenic variation is also employed by malaria parasites to avoid destruction by immune mechanisms (Kyes et al. 2001; Beeson and Brown 2002). Once the parasites have gained entry into the erythrocytes, they modify the infected cells with several proteins, which make them 'sticky' to the endothelium (Smith et al. 2002). One of the principal parasite-induced antigen on the red blood cell (RBC) surface is the PfEMP1 which is encoded by a clonally variant, multicopy, nonallelic family termed '*var*'. Modification of the erythrocyte's surface can be regarded as an important adaptation because it prevents the parasitized RBCs from being swept to the spleen, where they would be removed as a result of the aberrant shape that they acquire when parasitized. This advantage of course bears the risk of being identified by the immune system. In fact, antibodies specific for PfEMP1 can prevent the adhesion of parasitized cells to endothelial cells, thereby facilitating sequestration to the spleen. In response, the parasite selects for expression of one out of about seventy of PfEMP1-encoding genes at a given time, thus confounding the immune responses that aim to destroy the infected cells. Genome-wide analysis has identified more than 200 plasmodial genes which are likely to be involved in immune evasion, most of which are found near the ends of the chromosomes (Gardner et al. 2002).

Immediately after inoculation, plasmodium sporozoites circulate for a short time as extracellular organisms rich in surface circumporozoite protein (CSP). CSP is an antigen with multiple tandem repeats which induces a polyclonal Th cell-independent B-cell response. Plasmodia overcome this response by sloughing off their surface CSP coat, thus withdrawing the targets from the attack of antibodies (Ramasamy 1998).

Antigenic variation has also been observed in infections with the intestinal flagellated parasite *G. lamblia*. During infection, the parasite survives the host's reactions by undergoing continuous antigenic variation of its major variant surface protein (VSP). VSPs from *G. lamblia* are cysteine-rich proteins with considerable heterogeneity in size. In vivo, the VSPs expressed by the parasite population continuously diversify under the selection pressure of IgA antibodies and proteases secreted by intestinal cells (Muller and Gottstein 1998).

Intracellular refuge and its consequences

Intracellular residency of protozoans such as *Trypanosoma cruzi*, *Toxoplasma gondii*, or *Leishmania* spp. protects them from detection by defense systems. This advantage, however, requires mechanisms that assure survival and replication within that shelter. In the initial phase of the infection the host cells in many cases are from the macrophage lineage or granulocytes (Aga et al. 2002). They have the potential to inhibit the replication or to kill the microbes, once they become activated to do so. Many parasites have developed strategies to subvert this cellular activation. Metacyclic forms of *Leishmania* spp., for example, bind different sets of entry ligands that contribute to silent entry. These include binding and uptake via CR1 and CR3 receptors, scavenger receptors, the mannose fucose receptor (Solbach and Laskay 2000), or receptors that recognize phosphatidylserine. The last strategy, which is also employed by helminthic schistosomes, fakes the host cell because it signals (falsely) apoptotic structures that are considered to be dealt with without induction of inflammation (Freire-de-Lima et al. 2000; Freitas Balanco et al. 2001; Luder et al. 2001; Van Der Kleij et al. 2002). Other mechanisms include the generation of a 'safe' compartment inside the cell, e.g. *T. cruzi* parasites secrete a lytic protein that releases the parasites into the cytoplasm, thus escaping fusion of the phagosome with the lysosome and its toxic components. *L. donovani* and *T. gondii* create a parasitophorous vacuole that does not fuse with lysosomes. They can also inhibit the acidification of the vacuole or inhibit toxic proteases within the phagolysosome (Mauel 1996).

Once inside the cell, the parasites can suppress the triggering of the respiratory burst and the production of NO or activating cytokines such as IL-1β or IL-12 (Alexander et al. 1999; Bogdan and Rollinghoff 1999; Bogdan 2001). On the other hand, infected macrophages may be induced to overproduce cytokines such as IL-10 or TGF-β which dampen antimicrobial effector mechanisms exerted by IFN-γ-producing natural killer (NK) cells or T lymphocytes.

Activation of the adaptive immune system requires that the infected cells are ready for peptide processing, for antigen presentation via the class I or class II major histocompatibility complex (MHC) pathway and for expression of co-stimulatory molecules. All of these components are inhibited or deviated in parasitized cells depending on the intracellular lifestyle and the direction of the protein release into the different antigen presentation pathways (reviewed in Bogdan and Rollinghoff 1999). An alternate strategy is employed by parasites such as *T. cruzi* which release many different or related proteins of the *trans*-sialidase family into the class I MHC-processing pathway. Immunogenic peptide frag-

ments from these proteins could lead to suboptimal adaptive immune responses either by competing with each other for MHC binding or by acting as inhibitory altered peptide ligands for each other (Vidal and Allen 1996; Plebanski and Hill 2000).

Intracellular life is not restricted to macrophages or erythrocytes but also includes cells found in muscular tissue (chronically infected with *T. cruzi*) or in the CNS (chronically infected with *T. gondii*). Very little is known about the reasons for this tissue propensity but it is tempting to speculate that factors such as anatomical barriers for immunological scanning, poor antigen-presentation capacity of the host cells, and an 'anti-inflammatory' environment all play a role.

HELMINTHS AND THE DISEASES THEY CAUSE IN HUMANS

Helminths are a major public health problem in economically disadvantaged populations and over 2 billion people are affected worldwide (Crompton 1999).There are three major groups of helminth parasites: trematodes, cestodes, and nematodes. Most helminths dwell in the lumen of the gut, and many live in accessory organs of the gastrointestinal tract. Others are adapted to a life within host tissues such as the blood or connective tissue.

Furthermore, helminths have complex life cycles including adult worms, as well as several larval stages that may penetrate the host skin, migrate through tissues, or have phases of arrested development. Except in very few cases, helminths do not replicate within their hosts, but produce offspring in the form of eggs or larvae. These have to reach a new host either by fecal–oral transmission of eggs, penetration of the skin by larvae, ingestion of larvae with contaminated meat of intermediate hosts, or by transmission through vector arthropods. In many species, adult helminths reach life spans of more than 10 years and thus establish long-lasting chronic infections. The worms are therefore exposed to very different environments in their hosts and have to cope with different local immune responses.

Trematodes

The human pathogenic trematodes can be grouped according to their localization: intestinal flukes (many species), liver flukes (*Opisthorchis*, *Clonorchis*, and *Fasciola* spp. as a potentially human pathogenic parasite), lung flukes (*Paragonimus* spp.), and blood flukes (*Schistosoma* spp.). The most important trematode helminths for human are *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. Intermediate hosts are freshwater snails, which release cercariae that, by secreting a broad-spectrum elastase and other proteases, actively penetrate through the exposed skin of humans and migrate via the arterial system to the portal vein of the liver (*S. mansoni* and *S. japonicum*) or the blood vessels around the urinary bladder (*S. haematobium*). At these sites, the parasites mature and mate, and female worms produce hundreds of eggs every day. These traverse the wall of the gut or the bladder, and are released to the exterior, where they can infect the intermediate snail host. As the eggs are deposited in the vasculature, the bloodstream may also carry them into the liver or venous capillaries of the bladder, where they are trapped. Antigens released from eggs induce a vigorous Th2 response that orchestrates the formation of a granuloma, which separates them from the host tissue. The granulomatous lesions resolve after death of the encapsulated eggs, but they leave behind fibrotic scars with IL-13 being of central importance in the development of fibrosis. These pathological changes can give rise to portal hypertension or, in the bladder, they can significantly increase the risk of malignant cancer (Pearce and MacDonald 2002).

Cestodes

The most important cestodes (tapeworms) that infect humans are *Taenia saginata*, *T. solium*, and *Echinococcus* spp. The first two worms lodge in the human intestine in their adult stage and are transmitted through intermediate bovine (*T. saginata*) or porcine (*T. solium*) hosts, respectively. The adults produce eggs that, after release with human feces, are ingested by the intermediate hosts, where the larva (oncosphere) hatches and penetrates the intestinal barrier. It then migrates to muscles and other organs where the metacestode, often called a cysticercus, develops. Transmission to humans occurs by ingestion of undercooked meat; inside the gut; the metacestodes are activated and give rise to the adult tapeworms. If a person swallows eggs of *T. solium*, the larvae will hatch and go on to produce cysticerci in many tissues including the brain, eye, heart, and striated muscles.

The adults of *Echinococcus* spp. live in the intestine of canides such as foxes or dogs. The eggs are passed with the feces and have to infect an intermediate host. Eggs of *E. granulosus*, the dog tapeworm, usually infect ungulates such as sheep or cows, where the metacestode forms large liquid-filled cysts (hydatids), mostly in the liver. The eggs of the fox tapeworm *E. multilocularis* develop in small rodents, where the metacestodes proliferate in the liver in a tumor-like manner. After accidental ingestion of eggs by humans, metacestodes of both species may develop in the liver, lung, or brain, and cause severe disease.

Nematodes

Intestinal nematodes are the most prevalent human parasites on earth. Species such as *Ascaris lumbricoides*,

the hookworms *Necator* and *Ancylostoma*, the whipworm *Trichuris*, and, to a lesser extent, parasites such as *Trichinella* and *Strongyloides* are found in more than half of the world's population. Infection mostly occurs through the fecal–oral route, with the exception of hookworms and *Strongyloides* spp. that actively penetrate the host's skin. Most nematodes live exclusively in the intestinal lumen and only have a subtle pathogenicity. However, in association with malnutrition and undernutrition they contribute significantly to poor health in many parts of the world, because malnutrition promotes the establishment, survival, and fecundity of these parasites (Koski and Scott 2001).

More severe pathological changes are caused by filarial nematodes that are transmitted by blood-feeding insects. The adult worms live within the lymphatics or in the skin, and produce thousands of first-stage larvae (microfilariae) per day. *Wuchereria bancrofti* and *Brugia malayi*, the causative agents of lymphatic filariasis, are transmitted by mosquitoes such as *Culex*, *Anopheles*, or *Aedes* spp. The filariae cause severe pathological changes through inflammatory obstruction of the lymphatics, which can lead to swellings of the affected limbs ('elephantiasis'). *Onchocerca volvulus* is transmitted by the bite of black flies (*Simulium* spp.). The adult nematodes live within subcutaneous nodules and inflammatory reactions induced by the skin-dwelling microfilariae lead to alterations of the skin and ocular damage ('river blindness'). Recent research has deciphered that intracellular bacterial endosymbionts (*Wolbachia* spp.) of *Onchocerca* may be a valuable target for antibiotic therapy of human filariasis with doxycycline. This therapy lead to a block in microfilarial embryogenesis and, possibly, to a significant reduction in filarial pathology (Hoerauf and Brattig 2002).

IMMUNITY AND PERSISTENCE IN INFECTIONS WITH HELMINTHIC PARASITES

Most helminthic parasites survive within most of their hosts for long periods, thereby causing chronic infection (and in some cases chronic disease) and long-term stimulation of the immune system. From an evolutionary point of view, chronicity of parasitic infections indicates success for both the parasite and the host, because both are able to survive with each other for long periods. Recent experimental and clinical evidence suggests that immune responses induced by chronic helminthic infections during early life via the anti-inflammatory activity of IL-10 may shape the development of the immune system toward a type of response that prevents immune dysfunctions such as allergy and chronic inflammatory diseases (van den Biggelaar et al. 2000; Bashir et al. 2002; Yazdanbakhsh et al. 2002). In this sense helminth infections were even discussed to have beneficial effects in some circumstances (Pritchard and Brown 2001).

One of the hallmarks of helminths is their longevity, with life spans of more than 10 years not being exceptional. This persistence implies that helminths are able to evade the immune effector mechanisms of their hosts, e.g. by skewing the cytokine network toward a predominance of Th2-like responses. Patients with helminthic infections show abundant circulating T cells with a Th2-like response to parasite antigen, with concurrent pronounced IgG4 and IgE antibodies and a high eosinophilia (Mahanty et al. 1992; Williams et al. 1994). T-cell proliferation in patients is severely downregulated, probably as a consequence of a selective anergy or suppression of the Th1-type cells (Allen and Maizels 1996). Even light infections might have such effects, because, for example, a single experimentally implanted *Brugia malayi* filaria is sufficient to inhibit the proliferation of T lymphocytes (Loke et al. 2000). This type of setting favors the survival of established worms, without preventing the host from defending itself against other pathogens.

The induced changes allow not only the tolerance of established worms, but at the same time also protective immune responses against newly intruding larvae of the same species. The main effector mechanism against the larvae is antibody-dependent cellular cytotoxicity (ADCC) with IgE as the most important antibody and eosinophils as effector cells. This 'concomitant immunity' can be considered as a mechanism for the regulation of parasite density (Cox 2001). Therefore, worm burdens in balanced host–parasite relationships are typically low and are usually tolerated without inducing severe pathological effects. Helminthic diseases are usually associated with high worm burdens and in most cases are a consequence of host immune reactions, rather than being the result of direct effects of the worms.

As a cautionary remark, our understanding of immune responses against helminths and their evasion mechanisms is largely based on studies from animal models that investigate the reactions against human pathogenic parasites. Not all of these data have been substantiated by studies in humans. Unfortunately, animal models for human disease are lacking in many cases and, therefore, one has to resort to the study of animal parasites in their natural hosts and draw indirect conclusions.

With regard to the trematodes, the most complete picture of immune reactions and evasion mechanisms comes from the schistosomes. The invasive stages of schistosomes (schistosomula) activate complement and are the target of ADCC reactions as well as of activated macrophages. Efficient killing of the IgE-opsonized schistosomula seems to be a predominant mechanism of protective immunity, whereas adult schistosomes are resistant to immune attack (Butterworth 1998; Pearce and MacDonald 2002).

Adult cestodes affect their hosts mainly as competitors for nutrients and cause little pathogenicity. As the worms dwell in the gut lumen and the only direct contact with the host is through the attachment of the scolex, immune reactions are of limited importance. Established metacestodes are not eliminated by host immune responses, but their growth is influenced by host responses, as shown by differences of parasite proliferation in various inbred strains of mice (Gottstein and Hemphill 1997). The metacestodes develop from larvae ('oncospheres') which are released from ingested tapeworm eggs. They cross the intestinal wall and migrate through blood vessels to their final location. These oncospheres are highly susceptible to lysis by complement-fixing antibodies, which leads to a limitation of superinfections (Lightowlers and Rickard 1988).

Gut-dwelling nematodes reach the intestines either directly or by migration of the larvae through various host tissues. On their way they can be killed by ADCC and inflammatory reactions, particularly in the lungs. The adult stages in the gut are mainly affected by IgE-sensitized mucosal mast cells. After antigen contact, they release mediators that lead to an immediate overproduction of mucus by goblet cells and to an increase in intestinal motility. In consequence, the worms are rapidly expelled (Ahmad et al. 1991). Protective intestinal immune responses against helminths are entirely dependent on Th2 responses and can be ablated by a reversion of the immune response towards a Th1 type (Else and Finkelman 1998; Finkelman et al. 1999). It is currently not clear whether and to what extent reactions of this type occur in humans.

In infections with filarial nematodes, the immune response and the resulting immunopathology depend on largely unknown factors. A minority of exposed persons is able to prevent infections with as yet unknown mechanisms ('putatively immunes'), whereas another subset of individuals is susceptible to infection. Patients who develop pronounced Th2 responses tolerate microfilariae, whereas others develop ADCC reactions and are able to clear the microfilariae.

Immune evasion mechanisms of helminths

Evolution has matched the host's immune armamentarium with a multitude of strategies by which helminths evade and subvert the immune responses (Maizels et al. 1993). As a result of their large genome (about 10^8 base-pairs) and relative long replication times, helminths cannot outpace the immune system by mechanisms involving mutations and gene rearrangements, such as the antigen variation of protozoan parasites. However, it is conceivable that they invest a similar proportion of genes into other types of immune evasion strategies. As ADCC and inflammatory reactions are the main immune effector mechanisms against helminths, their evasion mechanisms are tailored towards specifically coping with:

- complement activation
- the effect of antibodies
- the cytotoxic effect of phagocytes
- antigen presentation
- skewing of the cytokine network.

The strategies are of different complexity and can be grouped into passive approaches such as immune avoidance and blocking of effector mechanisms, and diversion strategies targeting the balance of the immune system by specifically acting on the cytokine network.

Avoidance strategies

Many immune effector mechanisms, similar to complement activation and ADCC, are directed against the surface of helminths. As has been shown for schistosomes, their surface is perfectly adapted to evade immune effector mechanisms. They are covered with a syncytial tegument that consists of an unusual double membrane complex with an apical plasma membrane and an overlying envelope (Hockley and McLaren 1973). The outer membrane is relatively fluid, ensuring a high lateral mobility of membrane lipids which favors repair after immune attack (Foley et al. 1986). The outer membrane of some stages can be replaced by shedding and incorporation of lipid compounds from tegumental storage vesicles (Kusel et al. 1975). Similar processes have also been observed for the infective larvae and adult worms of *Trichinella spiralis* (Philipp and Rumjaneck 1984) and other helminths (for a review, see Lightowlers and Rickard 1988).

Shortly after infection, the surface of schistosoma larvae is recognized by antiparasite antibodies and activates complement, although it gradually loses this antigenicity, acquiring a state of relative inertness. A part of this effect is ascribed to the incorporation of host molecules into the outer membrane of schistosomes, as a result of 'antigen disguise' (Terry and Smithers 1975). It was shown that schistosomes also incorporate membrane elements of the attacking neutrophils and other cells as well as lipids from host serum into their outer membrane (Pearce and Sher 1987). Indeed, MHC molecules, blood group glycolipids, fibronectin, and antibodies bound to the parasite surface by their Fc portion of host origin have all been detected. It was proposed that the outer schistosome membrane contains more host than parasite antigens (Saunders et al. 1987). This mechanism of antigen disguise seems to be employed also by other helminths, e.g. the surface of microfilariae of *W. bancrofti* contains host albumin as a major component (Maizels et al. 1984) and exposes blood group substances A and B (Ridley and Hedge 1977), whereas metacestodes of *E. granulosus* capture complement proteins from their host (Ferreira et al. 2000).

The fact that nematodes exhibit stage-specific patterns of surface antigens has also been interpreted in terms of immune evasion, e.g. the various larval stages of *Trichinella spiralis* have distinct sets of surface antigens (Philipp et al. 1980). It was suggested that antibodies induced by stage-specific surface antigens of an early larval stage do not recognize the surface of later stages. Each molt should thus provide an advantage, forcing the host to build up new and effective anti-surface antibodies. However, this concept was challenged by the finding that surface antigens crossreact between different stages and species of filariae, in spite of an apparent stage specificity as determined by molecular mass analysis (Maizels et al. 1983). Such shared antigenic determinants dominated the immune response, rendering the role of stage-specific antigens uncertain.

A further avoidance strategy consists of the production of a 'mucosal' glycocalix which serves as a surface coat to build a physical barrier between the worm surface and host effector mechanisms. Most probably, all helminths produce such mucous layers. Larvae of the nematode *Toxocara canis* produce a surface coat of about 10-nm thickness which surrounds the worm like a fuzzy envelope and is composed of mucins of different chain lengths that are secreted by the esophageal and secretory glands (Maizels et al. 2000). In vitro incubation of such larvae in immune sera leads to binding of antibodies that mediate the attachment of eosinophils. Subsequent shedding of the surface coat together with the bound eosinophils prevents intimate contact, and thus reduces the damage to the larvae (Badley et al. 1987). A similar shedding was also observed for juvenile stages of *Fasciola hepatica*. Larvae of this fluke are covered by a continuous layer of IgG after incubation in immune sera, and dispose of it by shedding and then replacing the glycocalix. This active process removes effector cells such as eosinophils and neutrophils and reduces the damaging effect of degranulation (Glauert et al. 1985).

Some helminths stimulate the host to produce layers of connective tissue, serving as a barrier that limits the access of immune cells, e.g. adult *Onchocerca volvulus* nematodes induce fibrous nodules in the skin (onchocercomas). Although the females are firmly embedded in these onchocercomas, the males probably move between nodules to inseminate the female worms (Schulz-Key and Karam 1986), and the microfilariae released by the female worms are also capable of leaving the nodule. The connective tissue of the nodule is infiltrated by neutrophils and eosinophils (Buttner et al. 1988). The influx of eosinophils seems to be triggered by the production of microfilariae by female worms (Wildenburg et al. 1996), whereas neutrophils are attracted by products of endobacteria living in symbiosis with the filarial nematodes (Brattig et al. 2001). In a comparable manner, the larval stages of some gut-dwelling nematodes induce the formation of tissues cysts, which may function to create a niche protected from immune effector mechanism. In infections with *Taenia solium* and *Echinococcus granulosus*, a connective tissue capsule with interspersed inflammatory cells surrounds the metacestodes, and the adults of *Paragonimus westermani* are located in cysts connected to the bronchial airways.

Blocking of complement and antibodies

Helminths can evade the consequences of complement-mediated damage through inhibition of complement action and through increased local consumption of complement factors. Moreover, they are able to inactivate antibodies and to induce the production of blocking antibodies.

Although antigens of many helminths activate the complement system, complement-dependent cytotoxicity is apparently restricted to the attrition of cestode oncospheres, which are destroyed by complement-fixing antibodies (Lightowlers and Gauci 2001). The activation of complement components by helminths without subsequent killing suggests the occurrence of mechanisms that inhibit the complement cascade. Indeed, a number of helminth complement inhibitors have been identified.

Young schistosomal larvae are highly susceptible to complement attack, but acquire resistance by several mechanisms within a few hours of penetrating their host. Shortly after infection, they express and probably secrete paracrystalline 'elongate bodies', which consist of the muscle protein paramyosin (Matsumoto et al. 1988). Through binding of C1q and inhibition of the binding of C4, paramyosin can prevent complement activation and thus protect the parasites (Laclette et al. 1992). In addition, a lipid-anchored 28-kDa protease on the surface of schistosome larvae can cleave C3 and C9 complement components and inhibit complement-mediated, neutrophil-dependent killing (Ghendler et al. 1996). Schistosomes also have a protease inhibitor with similarity to human CD59, which blocks the assembly of the membrane attack complex (Parizade et al. 1994).

Schistosomes and *Echinococcus granulosus* can use host-derived molecules for inactivating complement components. In the case of schistosomes, they incorporate the decay-accelerating factor (DAF) in their outer surface membrane or, in the latter case, 'factor H' in the wall of the cysts (Pearce et al. 1990; Ferreira et al. 2000). Both DAF and factor H inhibit the activation of the complement cascade downstream of the formation of the C3b–Bb complex.

Another way of knocking out disadvantageous complement effects is to 'stick' early complement factors to helminthic mucopolysaccharides. Such factors are found in the cystic bladder fluids of the tapeworm *Taenia taeniaeformis* (Hammerberg and Williams 1978).

Antibodies against surface proteins of helminths opsonize the parasites for recognition and effector activation by eosoinophils, neutrophils, and macrophages. Anti-

body degradation can therefore lead to the inhibition of these functions. Larvae of schistosomes and newly excysted juveniles of *Fasciola hepatica* secrete proteases that cleave IgG or IgE antibodies. As a consequence, the attachment of eosinophils to the worms is inhibited. The degradation products of the antibodies may serve for camouflage. Moreover, the peptides released during this process may downregulate macrophage activation (Auriault et al. 1981; Carmona et al. 1993; Pleass et al. 2000).

The skewing of immune responses after helminthic infections results in a shift of antibody classes and subclasses, which has profound effects on antibody-dependent effector mechanisms. Many helminths induce the production of high levels of IgG4 and IgE, parasite-specific IgG4 being present in much higher quantities compared with IgE, e.g. in filarial infections (Hussain and Ottesen 1986). The massive amounts of IgG4 are supposed to compete with IgE for epitopes, and may thus block otherwise occurring IgE-mediated cytotoxicity and allergic responses. In a similar way, IgG2 and IgM antibodies block anti-schistosomula granulocyte responses that depend on recognition of other isotypes for ADCC reactions. Furthermore, parasite-specific anti-helminth IgE antibodies occur together with unspecific IgE responses arising from the general elevation of IgE production. This effect would dilute parasite-specific IgEs on mast cells, and thus reduce allergic responses.

Inhibition of effector cell functions

Host effector cells are attracted to the site of infection by a variety of chemoattractants. To reach the parasites, the cells have to adhere to the vascular endothelial lining and to cross it by diapedesis. Helminths interfere with this process by various means. The hookworm *Necator americanus* secretes a protease which rapidly degrades the eosinophil-attracting chemokine eotaxin (Culley et al. 2000), and *Brugia malayi* microfilariae inhibit attraction of mononuclear cells and granulocytes by secretion of a serine protease inhibitor. In addition, this protease can inhibit granulocytic enzymes such as elastase and cathepsin G (Zang and Maizels 2001). The adhesion of granulocytes to vascular endothelial cells is inhibited by a glycoprotein from *Ancylostoma* and by eicosanoids from schistosomes (Nevhutalu et al. 1993; Moyle et al. 1994). Tissue-invading larvae of the lung fluke *Paragonimus westermani* inhibit eosinophils to degranulate and produce reactive oxygen species (ROS) through release of a cysteine protease (Shin et al. 2001) and mast cell degranulation is dampened by excretory/secretory (ES) products from adult *Schistosoma mansoni* worms (Mazingue et al. 1980).

Several species of intestinal nematodes secrete acetylcholinesterase, which can hydrolyze acetylcholine. In consequence, many proinflammatory functions of acetylcholine, such as release of granulocytic lysosomal enzymes, release of histamine, or other anaphylactogenic molecules by mast cells, are reduced and thereby may serve the 'well-being' of the nematodes (Lee 1996).

Defense against reactive oxygen species and detoxification

Activated macrophages, neutrophils, and eosinophils produce a variety of toxic products such as hydrogen peroxide, superoxide anion, and hydroxyl radicals, which are collectively termed ROS. ROS lead to protein oxidation, lipid peroxidation, depolymerization of polysaccharides, and DNA modifications, and thus has important antimicrobial functions. All aerobic organisms require mechanisms that protect cells against ROS produced during their own metabolism. Therefore, parasites have antioxidant enzymes such as superoxide dismutases, glutathione peroxidases, catalases, and peroxiredoxins, as components of their normal metabolism. Secretion of antioxidants by nematodes and their presence on or directly beneath the surface suggest, however, that helminths use antioxidants to block the products of the respiratory burst produced by immune cells (Henkle-Dührsen and Kampkotter 2001). Peroxiredoxins have been described from several filarial species and *Ascaris* spp., and it was shown that these proteins remove H_2O_2 and protect DNA from nicking by hydroxyl radicals in vitro. Glutathione peroxidases were identified as major soluble surface proteins of lymphatic filarial species and may function in the repair of oxidatively damaged membranes and the removal of H_2O_2 (Cookson et al. 1992). Catalases and superoxide dismutases have been described in a large number of nematodes and, although parasite-specific functions have been postulated, they have not yet been conclusively demonstrated (Henkle-Dührsen and Kampkotter 2001).

The glutathione *S*-transferases (GST) are a family of detoxification enzymes that catalyze the conjugation of reduced glutathione to xenobiotic and endogenous compounds. They are involved in the protection of tissues against oxidative damage by detoxification of lipid peroxidation products. Schistosomes have several isoforms of GSTs which are abundant in larval and adult worms. The enzymes are located in the subtegument, and at least transiently at or near the worm surface. Enzyme-blocking antibodies induced by experimental animal immunization with recombinant GSTs affect worm viability and fecundity after infection with *Schistosoma mansoni* or *S. japonicum* (Boulanger et al. 1991; Shuxian et al. 1997; Remoué et al. 2000). Studies in *Onchocerca volvulus* showed that this nematode also possesses and releases GSTs (Liebau et al. 1994) and immunization of cattle with GSTs from *Fasciola hepatica* induced protection against metacercarial infection (Morrison et al. 1996).

Inhibition of lymphocyte proliferation

Helminth infections often lead to diminished proliferation of antigen- or mitogen-stimulated lymphocytes. This reaction is the result of secreted products that can directly inhibit T or B cells, act indirectly through the induction of cytokine responses, or have combined activity.

Phosphorylcholine (PC) is a small component of phospholipids that occurs in a multitude of pathogens such as bacteria, trematodes, cestodes, and nematodes, but it is also present in the inner leaflet of vertebrate membranes. Nematodes are very rich in PC; part of the inhibitory activities of helminth culture supernatants is caused by this component. In-depth structural and functional studies on PC were performed with the rodent filaria *Acanthocheilonema viteae*. In vitro studies using the secreted PC-containing protein ES-62 revealed that the proliferation of activated B and T lymphocytes could be inhibited significantly (Harnett and Harnett 2001). The unresponsiveness of B lymphocytes is caused by PC-mediated uncoupling of the antigen receptor from intracellular proliferation signals such as the phosphoinositide-3-kinase and the Ras mitogen-activating protein kinase pathways (Deehan et al. 1998).

Another modulator of innate immune defenses found in many nematodes is the cysteine protease inhibitor cystatin. Recombinant cystatin from *Onchocerca volvulus* (onchocystatin) suppressed the proliferation of human T cells and the expression of MHC class II and CD86 molecules on monocytes. These findings are in line with studies on cystatins of *Brugia malayi* and of a rodent intestinal nematode that revealed an inhibition of class II MHC-restricted antigen processing (Dainichi et al. 2001; Manoury et al. 2001). At the same time, cystatin induced monocytes to produce enhanced levels of IL-10 and tumor necrosis factor α (TNF-α) and reduced amounts of IL-12 (Schonemeyer et al. 2001). These observations suggest that filarial cystatins shift the activities of macrophages toward an anti-inflammatory phenotype. In vivo studies in a rodent model confirmed the relevance of these observations, showing that application of filarial cystatin via osmotic pumps diminished the antigen-specific proliferation of spleen cells, whereas the antibody production was not suppressed (Pfaff et al. 2002). Not only are protease inhibitors found in nematodes; taenia cestodes secrete an inhibitor (taeniastatin) that is able to inhibit the production of IL-2 by T lymphocytes (Leid et al. 1986).

Immunomodulation by cytokine homologs

Helminths not only use molecules such as PC or protease inhibitors to alter the balance of the cytokine network, but also produce functional cytokine homologs that interact with the immune system. This information was initially gained by data mining of genome projects and represents a new aspect of the relationship between helminths and their hosts. For viruses, the strategy to produce mammalian cytokine or cytokine receptor homologs has long been known. The Epstein–Barr virus releases IL-10 homologs that account for a skewing of the cytokine balance towards Th2 responses (Liu et al. 1997) and the myxoma virus and other poxviruses encode for soluble IFN-γ receptors that bind and inactivate host IFN-γ (Upton et al. 1992; Alcami and Smith 1995).

To date there are two examples of helminth-produced cytokine-like molecules, but more of these proteins are likely to be discovered. A comparison of expressed sequence tags revealed that *Brugia malayi* has two genes with homology to macrophage migration inhibitory factor (MIF) (Pastrana et al. 1998). The gene of *B. malayi* MIF has an identity of 40–42 percent with vertebrate MIFs, and genomic homologs have been identified in *Onchocerca volvulus*, *Wuchereria bancrofti*, and also the free-living nematode *Caenorhabditis elegans*. In mammals this cytokine, secreted by T cells, macrophages, and eosinophils, induces macrophage kinesis and is associated with proinflammatory activities. Functionally, recombinant *B. malayi* MIF led to an upregulation of mediator production of macrophages with an ensuing marked eosinophil recruitment (Falcone et al. 2001). Thus, filarial MIF reveals an important link between macrophages and eosinophils during helminth infections. The MIF gene of *B. malayi* is transcribed in microfilariae and adult worms and is secreted in the vicinity of the worms, but it is also present in the hypodermis of adult worms and in the reproductive organs of females. Possibly, the continuous secretion of MIF induces a counterinflammatory phenotype of macrophages and contributes to the generation of 'alternately activated macrophages' that exert counterinflammatory activities (Loke et al. 2000).

In addition to MIF *B. malayi*, microfilariae and adult worms have genes to produce and secrete TGF-β-like proteins that bind to mammalian TGF-β receptors (Maizels et al. 2001). As TGF-β is involved in many downregulatory mechanisms, it is conceivable that this protein has a role in reducing inflammatory host responses.

OUTLOOK

In many areas of the world, individuals experience one or more parasitic infections. Despite considerable progress in controlling parasitic diseases, the threat to human health is increasing. Drug-resistant malaria has re-emerged as one of the most prevalent and dangerous diseases. Sleeping sickness is again epidemic in several countries in central and north-eastern Africa. Schistosomiasis cases do not decline despite available drug therapy.

Pathogens such as cryptosporidia or cyclospora are recognized increasingly as risk factors in food or water.

Understanding of the multiple effects of parasitic infections on the immune system is of increasing interest. Today there is no licensed vaccine for any human parasitic infection. This reflects the complexity of defining and directing of the correct protective immune response. Recent full-sequencing of the *P. falciparum* and *P. yoelii* genome, as well as ongoing efforts to sequence the genomes of *Trypanosoma brucei*, *T. cruzi*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Entamoeba histolytica*, *Schistosoma mansoni*, or *Brugia malayi*, provide the library from which vaccine candidates will be selected and creates considerable expectations for successful new vaccines. New technologies such as DNA immunization, expression library immunization, prime-boost regimens, or recombinant viruses will aid in the development and delivery of new vaccines. DNA-microarray technology will help to identify genes that are expressed at particular stages of the infectious process, at the site of both the parasite and the host. In vivo assays to induce or block the organ-specific expression of proteins will help to predict their contribution to immune control. In this context, it will be of prime importance to consider the immune escape mechanisms of protozoan and helminth parasites. Furthermore, parasites are a source of molecules with immunomodulatory activity, because evolution forged these parasites to become specialists in immunomodulation. Knowledge of the mechanisms of parasite immune evasion will thus add to our understanding of immune regulation in general and might at the same time provide concepts and molecules for the management of undesired immune responses.

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Shock/sepsis

DANIELA N. MÄNNEL

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INTRODUCTION

Despite considerable advances in treating the primary causes of sepsis and shock, high rates of morbidity and mortality still remain associated with these conditions. The eradication of the septic focus, killing of invading microorganisms, and the support of blood circulation should allow a full recovery of the body's homeostasis. Unfortunately, this is not always achieved. Microorganisms and their products activate systemic host defense systems, which comprise both humoral (complement and coagulation cascade) and cellular components (monocyte/macrophage, neutrophils, and endothelial cells). Activated cells, in turn, release a vast array of mediators (cytokines, such as tumor necrosis factor (TNF) and interleukin 1 (IL-1), arachidonic acid metabolites, and nitric oxide) that amplify the inflammatory response. Circulatory failure, leading to inadequate oxygen supply to tissues, and direct cytotoxic effects of various mediators released in the course of the generalized inflammatory process may result. As a result of the extremely complex biochemical events that are involved in the pathophysiology of shock after severe sepsis, endogenous factors may contribute to prolongation and aggravation of the exogenous insult leading to a life-threatening autodestructive and selfsustaining perpetuation of inflammation.

DEFINITIONS

Many bacterial and nonbacterial factors may cause inflammation. Sepsis describes a systemic inflammatory response to a documented infection (Balk 2000). The local inflammatory process can cause an exaggerated systemic response, with inflammatory damage to otherwise healthy cells and organs distant to the primary site of the insult. For patients with such a deleterious systemic response without any detectable bacterial focus, the term systemic inflammatory response syndrome (SIRS) is used (Bone et al. 1992). Severe sepsis is sepsis associated with organ dysfunction, hypoperfusion, and hypotension. Multiple organ failure (MOF) has been defined as sequential failure of two or more organ systems in patients with clinical signs of sepsis (Baue 1975). Patients with severe sepsis and sepsis-induced hypotension despite adequate fluid resuscitation, along with the presence of perfusion abnormalities such as lactic acidosis, oligurea, or an acute alteration in mental status, are considered to be in septic shock. Sepsis and organ failure accompanied by (micro)circulatory failure can lead to impaired tissue oxygen supply and refractory shock. Septic shock refers to an acute event induced by bacteria and/or their products and is the most severe case of severe sepsis (Figure 1).

MEDIATORS OF IMMUNE REACTIONS

Sensing of pathogen-specific molecular patterns

In addition to microbial toxins and superantigens (which directly affect and modulate the hosts' immune response), a variety of constitutive microbial cell compo-

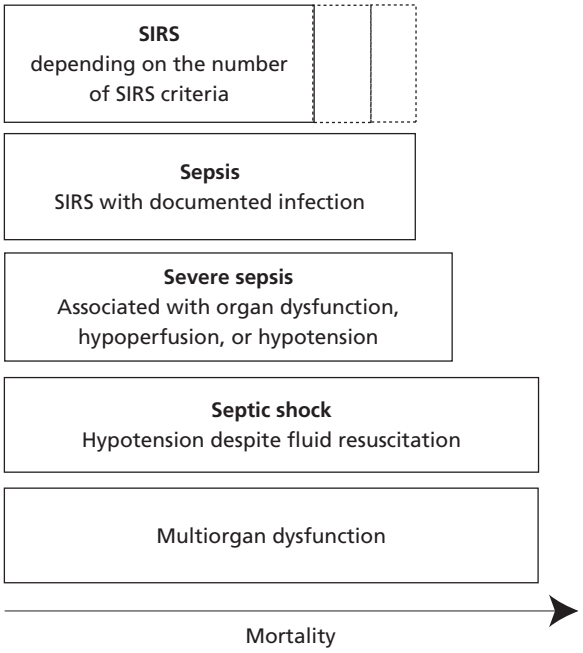

Figure 1 *Mortality in the systemic inflammatory response syndrome (SIRS). A progressively higher mortality of patients admitted to intensive care units can be observed as more severe inflammatory response criteria are met.*

nents induce inflammatory reactions. Although, for decades, studies concerning sepsis and shock dealt with gram-negative bacteria, infections with gram-positive bacteria, e.g. *Staphyloccus aureus* and enterococci, are recognized more and more to be of similar clinical relevance. The pathophysiological consequences of the systemic effects of infection are determined by a combination of factors derived from the infecting agent and responses of the host organism to the infection. Invading microorganisms such as bacteria, mycoplasmas, fungi, protozoa, or viruses and products thereof are immediately sensed by either serum proteins or membrane-bound proteins with predefined specificities. In recent years, a large number of so-called 'pathogen-specific pattern recognition molecules' have been described both as plasma components and as cell-surface receptors.

Characteristically, such pathogen-specific pattern recognition molecules specifically bind to repetitive carbohydrate structures on the surface of microorganisms or to an increasing number of defined molecules of microbial origin such as bacterial lipopolysaccharide (LPS), lipoteichoic acid (LTA), or bacterial DNA with cytidine phosphate-guanosine dinucleotide (CpG) motifs, now often referred to as pathogen-associated molecular patterns (PAMP) (Medzhitov and Janeway 1997).

Bacterial LPS, a constituent of the outer cell membrane of gram-negative bacteria, has been extensively studied for many years and represents the endotoxic principle of gram-negative bacteria (Rietschel et al. 1996). In the infected host, LPS is sensed by a specific LPS-binding protein (LBP) which in turn supports the interaction of LPS with either soluble or membrane-associated CD14. LPS-mediated cellular signaling, however, is dependent on a specific cell-surface receptor for LPS, now known as Toll-like receptor 4 (TLR-4) which cooperates with MD-2 (Beutler 2000; Alexander and Rietschel 2001; Heinrich et al. 2001) (Figure 2). TLR-4 signaling induces the release of proinflammatory mediators such as TNF (Jack et al. 1997) and initiates a pleiotropic cascade of events, including activation of the coagulation system, recruitment of phagocytes, and localization of the septic focus (Echtenacher et al. 1996). Similarly, LTA (which could be considered as a marker molecule of gram-positive bacteria) triggers the innate immune response using another member of the Toll-like receptor family, i.e. TLR-2, as a signal-transducing membrane receptor (Morath et al. 2002). Likewise, bacterial DNA containing immune stimulatory CpG motifs binds and signals through another member of the Toll-like receptor (TLR) family, i.e. TLR-9 (Krieg 2002).

ACTIVATION OF THE CELLULAR SYSTEM

Toll-like receptors

On the cell surface the Toll-like receptors (TLR) are crucial in transmitting the signal from structures commonly found on or in microorganisms (nonself) but absent on eukaryotic cells (self) into cells (Janeway 1992). A plethora of structures additional to LPS, LTA, and CpG are recognized by members of the TLR family including peptidoglycan (PGN), bacterial or mycobacterial lipoproteins, lipoarabinomannan, yeast cell wall components, or bacterial flagellin. By recognition of and response to a broad spectrum of diverse microbial patterns, the innate immune system orchestrates an inflammatory response to microbial invasion and triggers secretion and increased biosynthesis of key components in the inflammatory process. These genes include those encoding cytokines (TNF, interleukins IL-1, IL-6, and IL-12), chemokines (MIP-1α, IL-8), adhesion molecules, intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM), inducible nitric oxide synthase (iNOS), and antimicrobial peptides, all of which contribute to the early host response against the invading pathogen (Jefferies and O'Neill 2002). The intracellular signaling cascade after activation of TLR-4 and TLR-2 is well characterized and uses common intracellular signaling intermediates MyD88, IRAK, and TRAF-6, resulting in activation of the nuclear factor κ B (NF-κB), mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) pathway activation which initiates the transcription of proinflammatory cytokines (Takeuchi and Akira 2001) (see Figure 2).

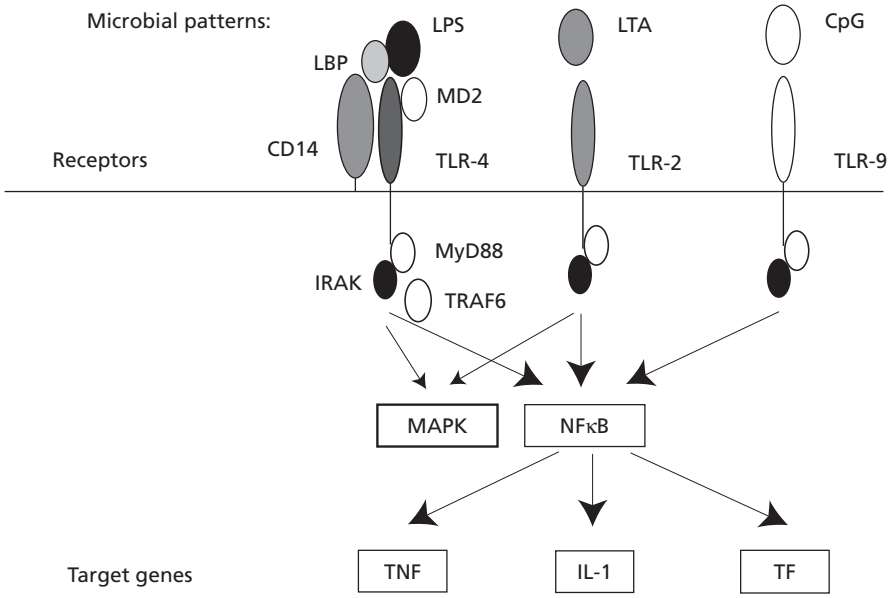

Figure 2 *The signaling pathway of Toll-like receptor 4 (TLR-4) after recognition of bacterial lipopolysaccharide (LPS) is mediated by LPS-binding protein (LBP), and CD14, TLR-4, and MD2. IRAK, IL-1 receptor-associated kinase; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κ B; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factor 6; TF, tissue factor.*

Inflammatory cytokines

Among the proinflammatory cytokines, TNF and IL-1, are regarded as key players because their biosynthesis is quick and immensely efficient and their biological activities very powerful in starting off a cascade of additional proinflammatory mediators, e.g. via activation of NF-κB. TNF in particular appears to be the most potent mediator of the pathophysiology of the sepsis syndrome (Männel and Echtenacher 2000; Beutler 2002; Knuefermann et al. 2002). In combination with bacterial products or with IL-1, TNF rapidly leads to death with all symptoms of severe sepsis and shock in experimental settings (Okusawa et al. 1988a; Rothstein and Schreiber 1988; Waage and Espevik 1988). Effects on tissue metabolism, cardiac function, and vascular tone are three major mechanisms for the damage exerted by a concerted action of TNF with other mediators through the induction of effector molecules such as NO (Parrillo 1993). Serum TNF as well as IL-1 levels positively correlate with severity of infection (Calandra et al. 1990; Marks et al. 1990). Nuclear extracts from peripheral blood mononuclear cells from patients who succumbed to sepsis showed a significant increase in the nuclear binding activity of NF-κB compared with those of survivors. This supports the view that inflammatory cytokines such as TNF and IL-1 are central regulators of the inflammatory process in sepsis (Bohrer et al. 1997). Elevated levels of macrophage migration inhibitory factor (MIF) – yet another proinflammatory mediator that overrides the anti-inflammatory and immunosuppressive effects of glucocorticoids – can be detected in patients with severe sepsis and septic shock and enhanced survival is achieved by blocking MIF in experimental models of sepsis (Calandra et al. 2000). This gives further emphasis to the causal relationship of an exaggerated inflammatory immune status with sepsis and shock.

Although activation of cells of the innate immune system, e.g. macrophages for production of proinflammatory cytokines and antimicrobial molecules, is essential during the early phase of the infection, upregulation of costimulatory molecules and activation of dendritic cells leads to the instruction of the adaptive immune system. As professional antigen-presenting cells, dendritic cells bridge the gap between the innate and the adaptive immune response. Thus, TLRs can be considered as adjuvant receptors linking innate and adaptive immunity throughout the entire course of the host response to the infection (Kaisho and Akira 2002).

ACTIVATION OF HUMORAL SYSTEMS

Complement

The complement system is an integral part of the innate defense against infection and mediates many cellular and humoral interactions within the immune response, including chemotaxis, phagocytosis, and cell adhesion. Complement may be activated via three different routes: the classical pathway, the lectin pathway, and the alternative pathway (Figure 3). The classic activation pathway is initiated by immune complexes, whereas the lectin pathway and the alternative pathway activation

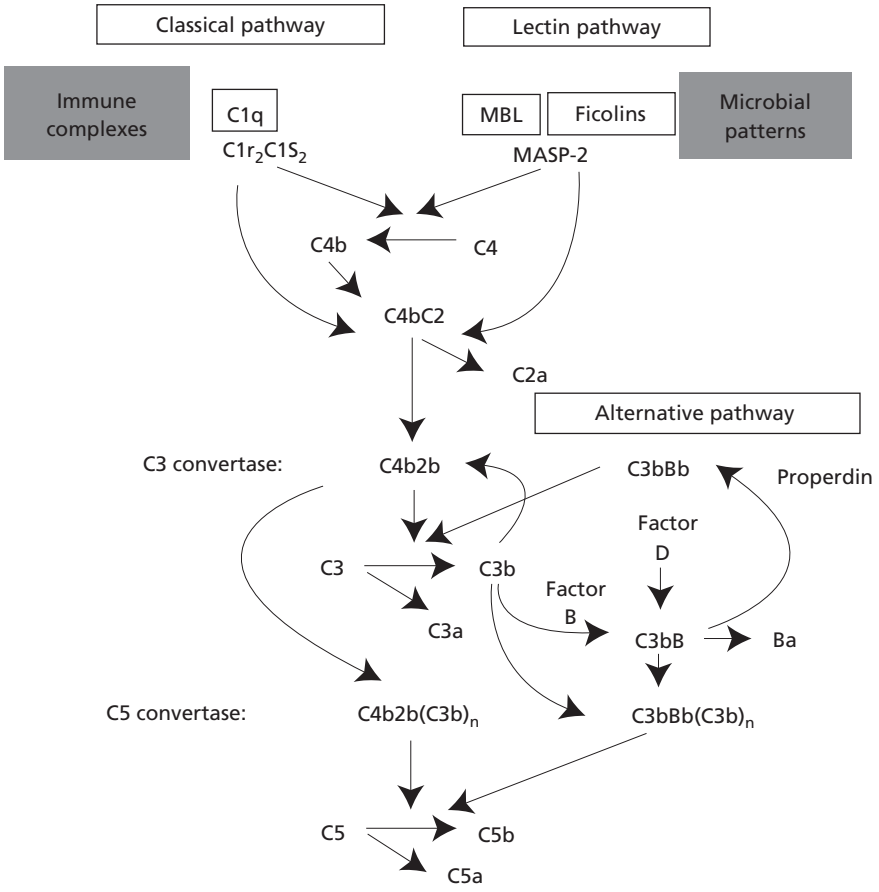

Figure 3 *The complement system is activated via the classic pathway initiated by immune complexes or via the lectin pathway initiated by pathogen-specific pattern recognition molecules such as the serum mannan-binding lectin (MBL) or ficolins leading to the activation of MBL-associated serine proteases (MASP). The alternate complement pathway stabilizes the C3 and C5 convertases, leading to generation of the anaphylatoxins C3a and C5a.*

can be initiated in the absence of immunoglobulins. Lectin pathway specific recognition molecules such as mannan-binding lectin (MBL) and the ficolins typically bind repetitive carbohydrate structures present on the surface of microbial organisms and translate this recognition into enzymatic activation of serine proteases associated with these recognition molecules (Neth et al. 2000; Petersen et al. 2001). The first PAMP recognition molecule identified to initiate the lectin pathway activation route of complement was the serum lectin MBL. Three lectin pathway-specific serine proteases have been identified so far and were termed MBL-associated serine proteases (MASP) (Thiel et al. 1997; Vorup-Jensen et al. 2000). As with MBL, ficolins were shown to form multimolecular serum complexes capable of activating complement after binding to microbial carbohydrate structures using MASP. MBL and the ficolins differ in their binding affinity to certain surface carbohydrate residues and thereby broaden the spectrum of potential triggers for lectin pathway activation.

Initiation of the lectin pathway activates the complement factors C4 and C2 to form C4b2b, a central enzymatic complex, which is able to cleave the abundant serum protein C3, thus generating C3 convertase which is common to both the classic and the lectin activation pathways of complement. As a result of complement activation pathway formation of the C3 and C5 convertases, the anaphylatoxins C3a and C5a are generated which increase plasma exudation and polymorphonuclear neutrophil chemotaxis.

The inherently labile C3 and C5 convertase complexes are stabilized by an amplification loop provided by the activation of the alternative complement pathway. A central component of this pathway is properdin; this is a positive regulator of complement activation and is rapidly released from polymorphonuclear neutrophils (PMN) in response to a variety of inflammatory stimuli such as LPS, TNF, or the anaphylatoxin C5a (Wirthmueller et al. 1997; Schwaeble and Reid 1999). Thus, in sites of inflammation where cytokines are secreted and complement is activated, secretion of properdin will amplify the complement cascade. A detailed discussion of the role of complement in infections is given in
Complement).

Inappropriate or excessive complement activation has been associated with septic shock. In plasma of patients

with sepsis, complement activation products, especially anaphylatoxins, can be found (Nakae et al. 1996). High concentrations of the anaphylatoxins C3a and C5a and terminal C5b–9 complement complexes seem to correlate with development of MOF and circulatory shock (Hack et al. 1989; Smedegard et al. 1989). Complement will directly promote procoagulant activity and indirectly induce cytokine production (Okusawa et al. 1988b; Scholz et al. 1990). The membrane-bound C5b–9 membrane attack complex and the anaphylatoxins induce tissue factor expression on endothelial cells and monocytes, thereby contributing to increased vascular permeability (Carson and Johnson 1990). Of the anaphylatoxins, C5a in particular is highly chemotactic for PMNs and induces the secretion of lysosomal enzymes from neutrophils and macrophages (Bengtsson et al. 1993). C5a seems to be a key mediator in inflammatory reactions, leading to vascular leakage by inducing TNF and endothelial P-selectin expression, and adhesion, migration, and aggregation of PMNs (Strachan et al. 2000). The receptor for C5a (C5aR) is found to be upregulated in sepsis on a variety of cells such as neutrophils, phagocytes, endothelial cells, and mast cells (Riedemann et al. 2002). Improved survival rates as well as attenuated inflammatory cytokine serum levels after blockade of the C5aR in sepsis models demonstrates the importance of the C5a receptor activation.

Coagulation

Activated humoral protease systems, i.e. coagulation, fibrinolysis, and the plasma kallikrein–kinin system, characterize severe infection and sepsis. Systemic activation of coagulation generates the deposition of fibrin in small blood vessels and microvascular thrombosis in critical organs, leading to organ failure. This occurs as consequence of the cellular activation during infection and of the release of inflammatory cytokines such as TNF and IL-1 (van der Poll et al. 1990). In addition, release of proteases from circulating cells has been suggested as an important factor in developing endothelial damage, leading to extensive permeability changes in various organs (Aasen and Buo 1993).

Tissue factor

A variety of mediators such as LPS and other bacterial products, various cytokines, among others particularly TNF, antigen–antibody complexes, and activated complement components are inducers of monocyte tissue factor (TF). Such mediators of inflammation as LPS, TNF, and IL-1 elicit concurrent changes in expression of leukocyte adhesion molecules on endothelial cells. Endothelial activation can be considered as a coordinated pathophysiological response to inflammation (Pober and Cotran 1990). The adherence of monocytes to P-selectin, an adhesion molecule expressed on the surface of activated platelets and endothelial cells, also induces TF expression. The transcription of the TF gene is mediated by both NF-κB and AP-1 sites located within the TF gene promoter (Mackman et al. 1991). During gram-negative sepsis circulating monocytes express significant levels of TF activity. TF is considered to be the primary triggering agent for the coagulation cascade and by far the most potent procoagulant substance known. Therefore, pathological expression of TF in monocytes and endothelial cells certainly contributes to the enhanced thrombogenesis in sepsis patients.

Platelets

Activated platelets that adhere and aggregate in large numbers at sites of vascular injury rapidly generate high local concentrations of thrombin (Morrissey and Drake 2002). Activation of platelets can also occur by complement components such as C5a and the C5b–9 complex (Rinder et al. 1995). Local activation of the coagulation cascade is immediately countered by anticoagulant systems such as the expression of thrombomodulin, antithrombin, production of TF pathway inhibitor, and protein C. Considering the prominent role of microvascular coagulation in sepsis and of activated protein C in hemostasis, antithrombotic and anticoagulant therapy seemed a reasonable attempt to prevent multiple organ dysfunction syndrome in severe sepsis (Aasen and Buo 1993).

Platelet-activating factor

Platelet-activating factor (PAF), a potent mediator implicated particularly in inflammatory conditions, is an alkylphospholipid produced by, and acting on, a variety of different cell types including PMNs, eosinophils, monocytes, macrophages, platelets, and endothelial cells (Ayala and Chaudry 1996). PAF is a potent chemotactic agent for neutrophils, inducing superoxide release, aggregation, and degranulation. Selfgenerating positive feedback cycles of PAF and various cytokines have become established. Synergistic effects of LPS and TNF with PAF on neutrophils may be crucial in initiating vascular damage in the very early phases of sepsis and shock. Impairment and retraction of endothelial cells by PAF has been observed in experimental systems in vitro.

CIRCULATORY FAILURE AND DIRECT CYTOTOXIC EFFECTS

A new understanding of sepsis pathophysiology has emerged, focused on the tight interplay and coupling of inflammation, microvascular coagulation, and endothelial cell dysfunction (Joyce and Grinnell 2002). In the early events leading to severe sepsis, the host response results in the activation of a number of systems aimed at

ridding the host of the infection. After activation of the humoral systems, i.e. the complement cascades, coagulation, fibrinolysis, and the kallikrein–kinin system, cells such as neutrophils, platelets, and mast cells also become involved. Circulating neutrophilic leukocytes are activated and adhere to the endothelium of the blood vessels. Such margination of PMNs and leukostasis provoke massive release of proteases, radicals, and phospholipid derivatives. Infiltrating neutrophils trigger the release of a second wave of chemoattractants/cytokines which amplify the recruitment of additional phagocytes. Margination and activation of neutrophils as a result of the action of inflammatory cytokines can trigger pulmonary vascular endothelial injury and lead to the adult respiratory distress syndrome (ARDS). Sequestration of activated leukocytes may also affect the intestine, leading to translocation of bacteria, and their toxins, leading to further recruitment and activation of monocytes/macrophages, neutrophils, and endothelial cells, respectively.

Microcirculation

The activation of the humoral and cellular inflammatory systems affects the endothelial barrier function and vasoregulation and leads to sepsis-induced dysregulation of the microcirculation at the level of resistance arterioles, nutritional capillary perfusion, and/or postcapillary venules (Lehr et al. 2000). The microcirculation constitutes a functionally highly active system which dynamically interacts with circulatory and tissue-associated cells such as leukocytes, platelets, neutrophils, and mast cells, produces mediators, and contributes to the regulation of the vascular tone. Consequences of microcirculatory dysfunction are the breakdown of endothelial and epithelial barrier function, vasodysregulation, and disturbance of oxygen transport.

A characteristic of septic (hyperdynamic) shock is the increased cardiac output and the loss of peripheral resistance resulting in increased transport of blood into the periphery (Lehr et al. 2000). With the inadequate peripheral resistance which is probably caused by sepsis-induced overproduction of NO and the resultant massive peripheral vasodilatation, the cardiac output is redistributed with compensatory hypoperfusion of visceral organs.

As a consequence of leukocyte activation in sepsis and disseminated intravascular coagulation (DIC) the microcirculation becomes overburdened with inflammatory cells. Endothelial cell swelling, leukocyte sequestration, and microthrombus formation are signs of endothelial cell activation and characterize the state of microcirculation in sepsis. In agreement with clinical observations in septic patients, sequestration of activated leukocytes has been shown to contribute to subsequent tissue injury in animal models.

Neutrophils, macrophages, and other phagocytic cells are capable of generating large amounts of highly toxic molecules such as reactive oxygen intermediates and reactive nitrogen intermediates (Bogdan et al. 2000). Besides their antimicrobial activity, these reactive intermediates are powerful immunoregulators. By inhibition of lymphocyte proliferation they may also serve to control inflammatory processes and to account for the immunosuppressed state in certain infections. Important signaling pathways regulating cytokine production and responses, cell survival, and tissue damage are influenced by reactive oxygen and nitrogen intermediates.

Tissue damage, and in particular endothelial cell damage mediated by free radical production, seems to be of considerable importance in sepsis and shock. The crucial cells for free radical production are PMNs (Nussler et al. 1999). In the inflammatory environment these cells become recruited and activated, and they adhere to the endothelial surface and release lysosomal proteases as described above. Activated neutrophils also produce superoxide by undergoing a respiratory burst. Superoxide is rapidly converted to hydrogen peroxide and toxic free radicals which can damage the endothelium (Ayala and Chaudry 1996). Endothelial membrane injury then leads to increased vascular permeability.

Septic shock is characterized by hypotension, vascular collapse, and multiorgan dysfunction. In sepsis, cardiac dysfunction and altered vascular tone, the loss of vascular homeostasis, and diffuse endothelial cell injury all increase microvascular permeability and favor the development of zones of ischemia. NO released by endothelial cells is a major endogenous arteriolar vasodilator. Thus, the large amounts of NO produced in the vascular wall by the cytokine-inducible NO synthase is considered to play an important role in septic shock (Li and Forstermann 2000). As with many important mediators of the host response to infection, the multifaceted actions of NO generate a picture of beneficial as well as detrimental effects. NO has both anti-inflammatory and proinflammatory properties depending on the type and phase of the inflammatory reaction. NO has a vasoprotective function by inhibiting platelet aggregation and adhesion to the cell wall, thus activating antithrombotic and anti-inflammatory effects by downregulation of NF-κB activation. However, NO is proadhesive for PMNs and can also be seen as an indirect toxic effector substance via the formation of reactive nitrogen species or as a mitochondrial inhibitor (Feihl et al. 2001). A correlation between NO-induced mitochondrial dysfunction and the severity and outcome of septic shock suggested that bioenergetic failure could be an important pathophysiological mechanism for MOF (Brealey et al. 2002).

Endothelial cell damage

The endothelial interface plays a central role in coordinating the microcirculatory system and promoting tissue perfusion and oxygen supply. Endothelial cells serve a

number of metabolic and regulatory functions, e.g. production of vasoactive compounds such as prostacyclin, thromboxane, and NO, control of hemostasis, blood coagulation, and fibrinolysis, platelet and leukocyte interactions with the vessel wall, and regulation of the vascular tone and blood pressure. The release of inflammatory mediators such as TNF in the early phase of sepsis initiates endothelial cell-surface activation affecting a number of pathways, e.g. oxidation, adhesion, cytokine release, apoptosis, and NO production. In addition, TNF production contributes to further inflammation through thrombin-induced activation of endothelial cells, platelets, and vascular smooth muscle. These responses can directly damage the vascular endothelium by inducing enhanced microvascular coagulation, endothelial dysfunction, and apoptosis of endothelial cells. However, so far there are no data documenting any role for endothelial cell apoptosis in the pathogenesis of sepsis (Hotchkiss et al. 2002).

As a result of DIC and microvascular thrombosis, decreased tissue perfusion, and hypoxemia organ dysfunction, organ failure, and shock can ensue (Figure 4). Therefore, endothelial dysfunction resulting from the cascades of successive activation events is considered to play a central role in modulating the tightly linked and interdependent inflammatory and thrombotic responses that characterize the pathophysiology of systemic inflammation. In patients, various markers for an activated endothelium such as ICAM, vascular cell adhesion molecule (VCAM), and E-selectin are found to correlate with the severity of sepsis (Reinhart et al. 2002). The breakdown of normal vascular homeostasis, combined with depressed myocardial function, alterations in peripheral vasculature, and failure of oxygen exchange, will finally lead to tissue hypoxia and organ failure.

IMPLICATIONS FOR THERAPIES

An infectious insult results in stimulation of the immune system. This response is normally advantageous, but, when uncontrolled or excessive, it becomes deleterious for the host. This is the situation in septic shock.

Management of coagulopathy

The importance of the coagulation system resulting in, and exacerbating, the blockade of microvascular flow, and thus tissue oxygenation in sepsis, has suggested antithrombotic strategies as promising therapeutic approaches. A number of clinical sepsis trials with antithrombin or tissue factor pathway inhibitor have, however, shown that correcting the coagulation defect alone in sepsis was not sufficient to prevent mortality. Besides prevention of DIC by antithrombotic treatment, e.g. with activated protein C, it also seemed important to inhibit the thrombin-induced proinflammatory effects such as activation of platelets and endothelial cells,

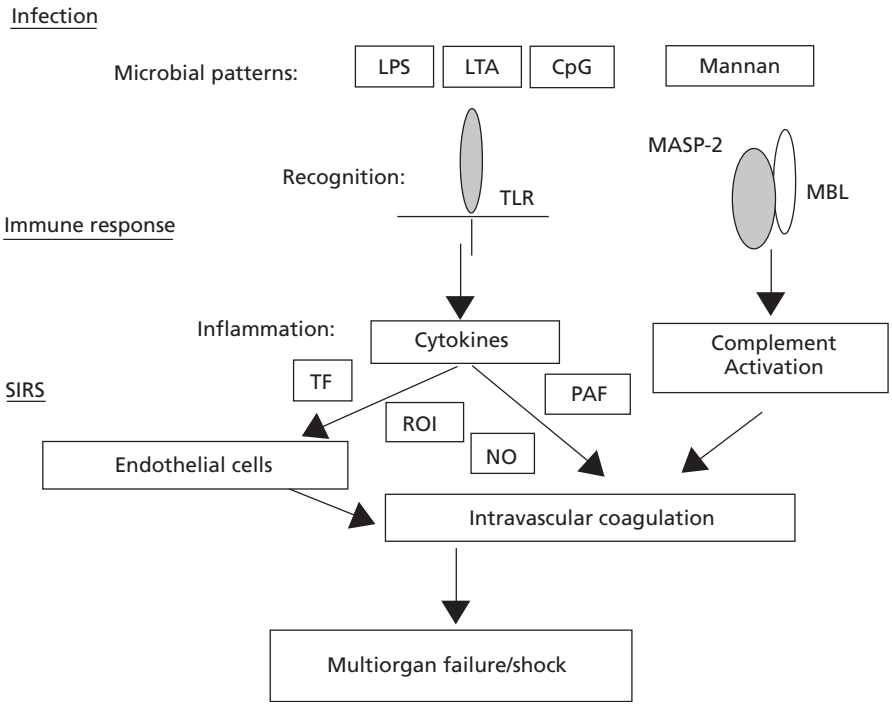

Figure 4 *Schematic overview of the pathogenesis of the systemic inflammatory response syndrome (SIRS) developing after infection and leading to severe sepsis and shock. LPS, lipopolysaccharide; LTA, lipoteichoic acid; MASP, MBL-associated serine protease; MBL, mannan-binding lectin; NO, nitric oxide; PAF, platelet-activating factor; ROI, reactive oxygen intermediates; TLR, Toll-like receptor; TF, tissue factor.*

NF-κB-dependent cytokine production, and adhesion molecule expression (Bernard et al. 2001). In addition, direct thrombin-independent anti-inflammatory effects of activated protein C, such as reduced NF-κB-dependent gene activation, may also result in protection of endothelial cells from apoptosis and promote cell survival and therefore may be important in restoring endothelial and mononuclear cell function.

Counterregulation of inflammation

The immune system has evolved potent anti-inflammatory programs to terminate and counterbalance inflammation because homeostasis requires compensatory mechanisms to maintain the internal milieu. A number of anti-inflammatory mediators such as IL-4, IL-10, IL-13, TGF-β, soluble TNF receptors, and IL-1 receptor antagonist are released for downregulating the immune response. In addition to these extracellular molecules, the integral nature of apoptosis provides a means of regulation of immune responses at the cellular level (Mahidhara and Billiar 2000). Delayed apoptosis of neutrophils (correlating with an upregulated inflammatory response), as well as increased lymphocyte apoptosis (possibly implicating immunosuppression), have been observed in patients with severe sepsis.

Endogenous corticosteroids downregulating the inflammatory response play a very important role in infections in general and are essential for the host to survive septic shock. Apart from modulating the immune system, corticosteroids have an important role in modulation of blood pressure and expression of adrenergic receptors. Steroid use in septic shock makes some sense but has conflicting support in the literature (Ritacca et al. 2002). A beneficial effect of corticosteroids in the treatment of sepsis may be a matter of dose, timing, duration of treatment, and patient selection (Spijkstra and Girbes 2000). The observations of beneficial effects of corticosteroids at doses that are close to the levels measured in stress situations support the idea of endocrine effects influencing the inflammatory responses in sepsis.

The theory of uncontrolled inflammation ultimately causing system-wide effects, shock, and end-organ failure, and the understanding of the importance of counterregulation lead to many therapeutic strategies aimed at blocking the early mediators of the proinflammatory response in sepsis. Among others, neutralization of bacterial products (LPS), inhibition of neutrophil recruitment, priming and activation by inhibition of chemoattractants (C3a, C5a), neutralization of chemokines, inhibition of rolling (selectin mediated), sticking (integrin mediated), and adherence, modulation of inflammatory mediators (C5a, TNF, IL-1, MIF, PAF), blockade of NO, arachidonic metabolites, reactive oxygen metabolites, and interference with signal transduction (NF-κB) activity have been tried in many experimental as well as clinical settings. Even though data from good animal models have conclusively demonstrated the importance of these mediators in the pathogenesis of the dysregulation in septic shock, the results of anti-inflammatory therapeutic strategies in the clinic were disappointing.

However, one has to keep in mind that the imminent problem in sepsis therapy is based on the fact that as yet no good parameters are available to evaluate the immunological status of the patients. After infection, patients go through an early inflammatory phase characterized by immediate responses of the innate immune system. In addition to the innate immune response, the adaptive immune response is recruited via, among others, activation of dendritic cells. This activation of the immune system provokes a counterregulatory anti-inflammatory or hypoinflammatory phase. For some time an overlap of the hyperinflammatory and hypoinflammatory phases may be present in the same infected organism, depending on the type of infection and the cell type, organ, or location under investigation. Although at the same time the presence of inflammatory mediators is required for antimicrobial action, anti-inflammatory mechanisms are essential for protection of tissue and organs. In such situations, the application of granulocyte colony-stimulating factor (G-CSF) might be a solution because transiently activated PMNs will be recruited with potent antimicrobial activity and – by an unknown mechanism – G-CSF acts in an anti-inflammatory manner (Boneberg and Hartung 2002).

There are some indications that careful patient stratification based on immunological characteristics that determine the timing for intervention might improve the outcome, as illustrated in the sepsis trial with TNF inhibition (Abraham et al. 1997; Grau and Männel 1997). As global tissue hypoxia is a key development preceding MOF and death, early goal-oriented manipulations of cardiac overload, afterload, and contractility to achieve balance between systemic oxygen delivery and oxygen demand seems to be a more definitive and effective resuscitation strategy (Rivers et al. 2001).

At least of equal importance is the notion that microcirculatory dysfunction during sepsis is not the consequence of one single mediator but of complex cascades of mediator systems, with these mediators having a striking redundancy in their modes of action. Evidently, inhibition of one mediator, even if it is an important one, might not be sufficient to get the dysregulated system back to homeostasis (Lehr et al. 2000). This is suggested by the relatively successful clinical trial with activated protein C where antithrombotic and anti-inflammatory effects are combined (Bernard et al. 2001). The combination of the divergent as well as the redundant effects of the different mediators, the difficulty of obtaining clear parameters to determine the immune status of the patient, as well as the detrimental synergistic positive feedback actions of the inflammatory

pathways involved, will continue to make it extremely difficult to find a successful and safe therapeutic strategy for severe sepsis and shock.

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DTH-associated pathology

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INTRODUCTION

A number of microorganisms have the capacity to remain viable within the host although the latter mounts an adequate immune response against them. This gives rise to a T-cell-mediated chronic inflammatory response aimed at restraining the harmful effects of the persisting microorganism. This response follows a characteristic pathophysiological pattern (granuloma formation) and often entails significant tissue damage in the form of necrosis or fibrosis. A major driving force in current inflammation research is the molecular dissociation of protection and pathology associated with this type of chronic inflammatory response. It remains a challenging therapeutic goal to manipulate, at will, the pathological sequelae of inflammation without impairing antimicrobially active immune defenses expressed concomitantly. This chapter outlines the salient features of immunopathology associated with T-cell-mediated delayed-type hypersensitivity (DTH) and discusses some strategies to prevent them.

DELAYED-TYPE HYPERSENSITIVITY

Definition

Delayed-type hypersensitivity is a highly dynamic, T-cell-mediated inflammatory response to persisting antigen. It is a typical immunological reaction to a variety of facultative intracellular bacteria, fungi, and parasites (Table 1) (Zumla and James 1996). Contact sensitivity to some chemical agents, experimental allergic encephalomyelitis, and graft rejection are other prototypes of DTH reactions induced by nonmicrobial stimuli.

The best known example of a DTH reaction is the response of a sensitized individual to intradermal injection of a purified protein derivative (PPD) of the causative agent of tuberculosis, *Mycobacterium tuberculosis*. The DTH reaction to PPD starts with reddening and induration at the site of antigen deposition after about 12 h. This delayed reaction peaks after 48–72 h and slowly wanes thereafter (Figure 1). Morphologically, the DTH skin reaction is characterized by the perivascular accumulation of predominantly mononuclear cells in the subcutaneous tissue and dermis. The majority of infiltrating lymphocytes are of the $CD4^+$ subtype. Over time, activated monocytes differentiate into epithelioid macrophages. As microvascular permeability is also increased, there is dermal edema and deposition of fibrin in the interstitium. The induration characteristic of a skin DTH reaction is a consequence of both the persistent infiltration of mononuclear cells and a limited amount of inflammatory fibrosis.

DTH and granuloma formation

Granulomas are defined as focal accumulations of mononuclear cells in response to a chronic antigenic

Table 1 *Selected bacteria, fungi and parasites causing DTH reactions and granulomatous disease*

Gram-positive bacteria	Gram-negative bacteria	Fungi	Parasites
Listeria monocytogenes	*Yersinia enterocolitica/ pseudotuberculosis/pestis*	*Cryptococcus neoformans*	*Toxocara canis/cati*
Rhodococcus equi	*Salmonella typhi/paratyphi*	*Blastomyces dermatitidis*	*Leishmania major/donovani*
Nocardia asteroides	*Francisella tularensis*	*Sporothrix schenckii*	*Trypanosoma cruzi*
Treponema pallidum	*Chlamydia trachomatis*	*Coccidioides immitis*	*Schistosoma mansoni/ japonicum*
Mycobacterium tuberculosis	*Bartonella henselae/bacilliformis*	*Paracoccidioides brasiliensis*	
Mycobacterium avium/ leprae	*Rickettsia prowazeki/conori*	*Histoplasma capsulatum*	
	Coxiella burnetii		
	Tropheryma whippelii		
	Calymmatobacterium granulomatis		
	Burkholderia pseudomallei		
	Brucella abortus		

stimulus. Thus, the skin DTH reaction may alternatively be termed a 'mini-granuloma' of the skin. Vice versa, antigen-specific granulomas in visceral organs in most instances fulfill all criteria of a DTH reaction and may therefore be viewed simply as tissue correlates of a DTH reaction.

Histopathologically, granulomas are characterized by aggregates of epithelioid cells with indistinct cell boundaries, which often seem to fuse into one another. The nucleus of activated macrophages is less dense than that of the surrounding lymphocytes ($CD4^+$, $CD8^+$ T cells and B cells), is oval or elongate ('shoe imprint' morphology), and has prominent nucleoli (Figure 2). Older granulomas are surrounded by a collar of fibroblasts and connective tissue, e.g. collagen fibers. Depending on the eliciting microorganism, neutrophil and eosinophil granulocytes are also present. Frequently, epithelioid cells fuse to form multinucleated giant cells, which may become as large as 50 μm in diameter, and show either an ordered peripheral arrangement of nuclei (Langhans-type giant cell; Figure 2) or a haphazard distribution of nuclei (foreign body-type giant cell).

In contrast to foreign body granulomas, which occur independently of T cells and are characterized by little cellular turnover, T-cell-mediated (DTH-type) granulomas are highly dynamic: in addition to local proliferation of T cells, new mononuclear cells are constantly recruited into the lesion by virtue of mediators secreted by activated, antigen-specific T cells.

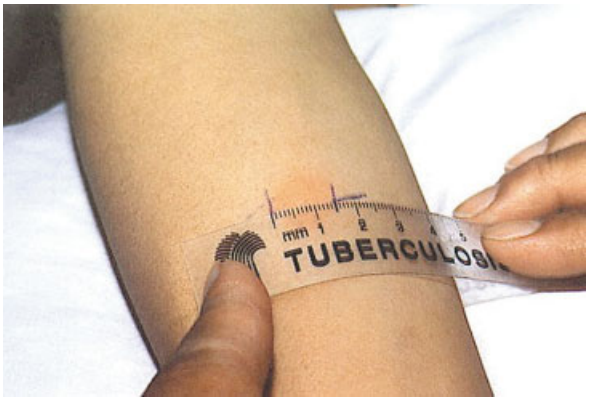

Figure 1 *Delayed-type hypersensitivity skin reaction to intracutaneous injection of purified protein derivative of* Mycobacterium tuberculosis*. Within 48–72 h, a positive tuberculosis (TB) skin test is marked by an area of reddish induration greater than 10 mm. It is the palpable induration that determines the size, not the area of redness. (Photograph reproduced with permission from the Saskatchewan Tuberculosis Control Guide (http:\\www.lung.ca/tb/images/full_archive/ 102_tuberculin_wheal.jpg) copyright 2002.)*

Mechanisms of granulomatous inflammation

In the course of a primary immune response, $CD4^+$ cells are sensitized to digested antigen presented in the context of major histocompatibility complex (MHC) class II and appropriate costimulatory molecules, such as CD80 or CD86 on the surface of antigen-presenting cells, e.g. dendritic cells (Jankovic et al. 2001). The nature of the cytokine environment present during this priming phase ultimately determines the differentiation of the $CD4^+$ effector cell subtype: the presence of interleukin (IL) 12 and interferon γ (IFN-γ) will promote the development of T-helper (Th) 1 cells, which are capable of secreting IL-2, IFN-γ, and lymphotoxin α (LT-α), whereas the predominance of IL-4 and IL-10 will favor the differentiation of Th2 cells which preferentially produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Chtanova and Mackay 2001). Some of these differentiated cells will become long-lived memory cells. Antigen-specific effector and memory T cells recirculate in the bloodstream and can enter sites of antigen deposition where they subsequently produce cytokines for further amplification of the type 1 and 2 immune reaction patterns, respectively.

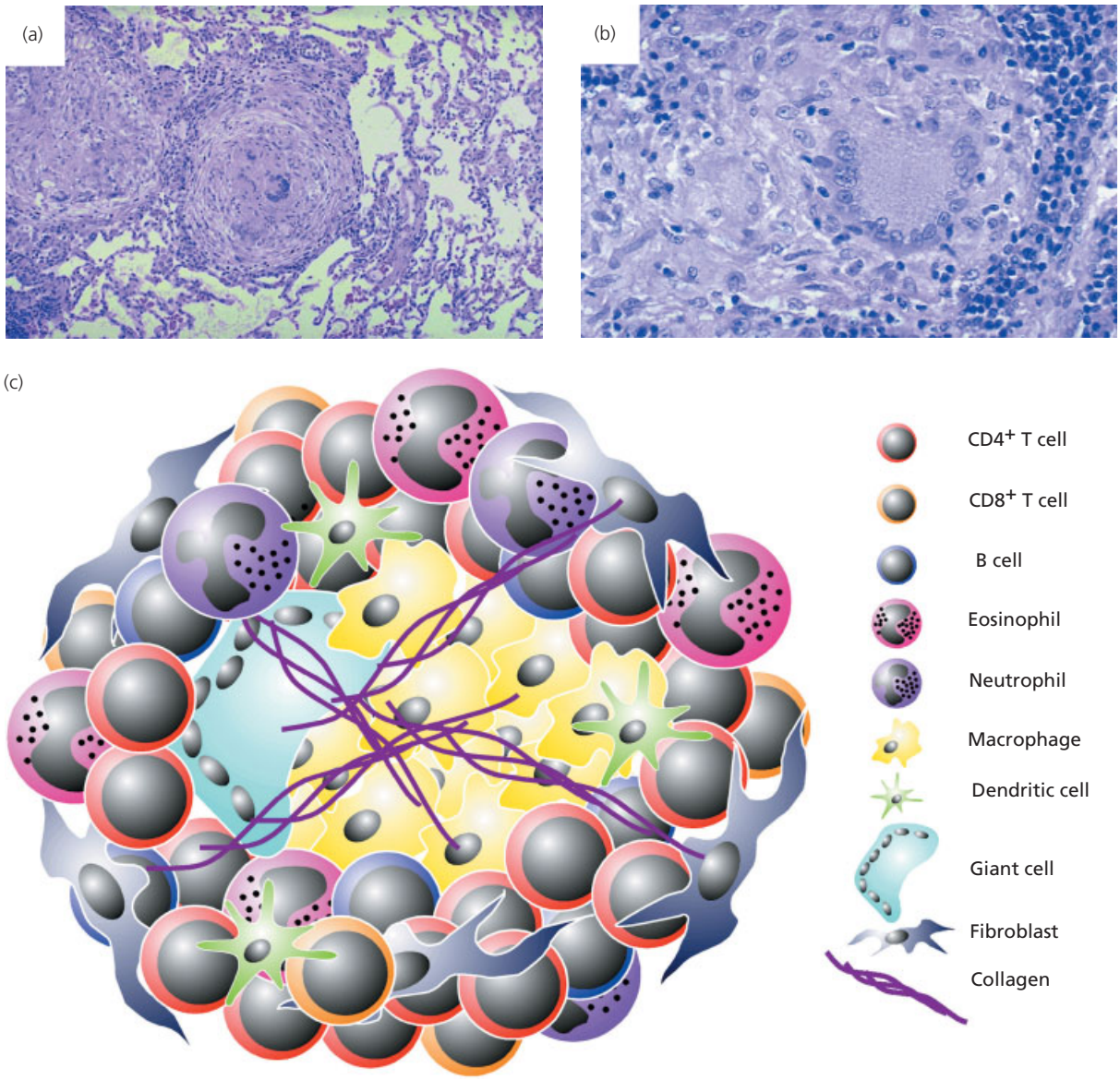

Figure 2 *Granulomatous inflammation:* **(a)** *medium power microscopic view of two pulmonary granulomas with giant cells;* **(b)** *high power microscopic view of well-structured granuloma with epithelioid macrophages and Langhans giant cell; and* **(c)** *schematic drawing of cells organized within the granuloma structure. (Photographs* **(a)** *and* **(b)** *reproduced with permission from Webpath, Version 7, 14 March, 2002, copyright 2002, Edward C. Klatt, MD.)*

The recruitment of effector $CD4^+$ cells into inflammatory lesions requires interaction with adhesion molecules as well as the sensing of a gradient of chemoattractant molecules by specific receptors. The course of events is initiated by activated complement components (e.g. C5a), vasoactive amines, bradykinin, leukotriene B_4, platelet-activating factor, and small amounts of IL-1 and tumor necrosis factor (TNF) produced at the site of insult. The last two cytokines upregulate the expression and increase the avidity of selectin and integrin receptors on the surface of the endothelium, thereby facilitating extravasation of leukocytes from the bloodstream (Shikama et al. 1989; Issekutz 1993a,b; Lukacs et al. 1994b).

To produce a manifest DTH, further amplification of this initial inflammatory reaction is necessary. The subsequent quality of the DTH response therefore largely depends on the cytokines produced by the $CD4^+$ effector cells of either Th1 or Th2 subtype initially recruited to the lesion, and the chemokine cascade that they initiate. It is convenient at this point to distinguish between type 1 and type 2 DTH and granuloma formation, depending on the T-helper cell subset mediating the monocytic inflammation. Although for the most part this chapter summarizes data obtained from in vivo infection models, many of the results reviewed in this section are based on an in vivo model of hypersensitivity granuloma elicitation in which presensitized mice are

challenged with beads coated with PPD of *Mycobacterium bovis* (Th1-induced type 1 granulomas) or soluble *Schistosoma mansoni* egg antigens (Th2-induced type 2 granulomas) (Kunkel et al. 1996, 1998).

T-helper 1 cells secrete copious quantities of TNF and IFN-γ capable of attracting additional monocytes and T cells into the lesions, mostly by increasing the binding of these cells to inflamed endothelium (Springer 1994). In particular, TNF and IFN-γ were shown to upregulate intercellular adhesion molecule (ICAM) expression on endothelial cells (Lukacs et al. 1994b), a molecule expressing binding sites for lymphocyte function-associated antigen 1 (LFA-1) (present on lymphocytes) and for complement receptor 3 (present on monocytes). The essential role of TNF and IFN-γ for granuloma initiation and maintenance has been amply demonstrated in many models of infection in which neutralization of TNF and IFN-γ effectively abrogates granuloma formation, or converts it to a Th2 pattern with increased eosinophil infiltration and fibrotic degeneration (Kindler et al. 1989; Amiri et al. 1992; Mastroeni et al. 1992; Joseph and Boros 1993; Chensue et al. 1997b; Smith et al. 1997). The injection of IFN-γ alone is able to induce a lymphocytic and monocytic infiltration (Nathan et al. 1986; Issekutz and Issekutz 1993), and TNF and IFN-γ act synergistically in this respect.

Lymphotoxin (LT), another member of the TNF superfamily synthesized by activated lymphocytes, occurs in a homotrimeric form ($LT\alpha_3$) or a heterotrimeric form ($LT\alpha_1\beta_2$), the latter being membrane bound. While the heterotrimer is probably involved in the direct activation of macrophages (Ehlers et al. 2003), $LT\alpha_3$ is necessary for establishing the proper architecture of the granuloma, because, in the absence of $LT\alpha_3$, T cells do not migrate into the granulomatous lesion, but only accumulate in perivascular cuffs (Roach et al. 2001).

The structural organization of type 1 granulomas involves elaboration of specific chemokines and induction of specific chemokine receptors on target cells. TNF and $LT\alpha_3$ orchestrate the early induction of chemokines necessary for initial leukocyte recruitment and thus facilitate granuloma initiation (Roach et al. 2001, 2002), e.g. TNF and lymphotoxins are major inducers of the chemokine CCL5, and IFN-γ and TNF synergize in eliciting CCL5 from endothelial cells (Marfaing-Koka et al. 1995). CCL5 attracts activated or memory Th1 cells, bearing the cognate receptor CCR5, into inflammatory lesions. IFN-γ induces the chemokines CXCL9, CXCL10, and CXCL11, which in turn attract effector Th1 cells bearing the common receptor for these chemokines, CXCR3 (Matsukawa et al. 2001). When newly recruited monocytes become activated and differentiate into epithelioid cells, they are also capable of secreting chemokines, e.g. CCL2, that further attract and activate monocytes (Matsushima et al. 1988; Zachariae et al. 1990). Therefore, when the receptor for CCL2 (CCR2) is absent, type-1 granuloma formation is impaired (Boring et al. 1997). Multinucleated giant cells probably also participate in amplifying the type 1 granulomatous response, because transcripts for TNF, IL-1β, IL-6 were detected in single cells generated in vitro (Seitzer et al. 1997) or analyzed in vivo (Myatt et al. 1994).

Nitric oxide (NO) is probably involved in the termination of the Th1 granulomatous response: NO, produced by the IFN-γ-inducible enzyme nitric oxide synthase (2NOS-2), inhibits type 1 granuloma size and cellularity, particularly with respect to eosinophils and neutrophils, by modulating chemokine (CCL2, CCL3, CXCL10) as well as TNF and IFN-γ expression (Hogaboam et al. 1997; Ehlers et al. 1999).

T-helper 2 cells orchestrate the development of a different kind of granuloma. IL-4 leads to a significant increase in cellularity, size, procollagen type III expression, and accumulation of eosinophils (even in preexisting Th1 granulomas). In addition, endothelial cells may become activated by IL-4 and secrete CCL7 which also induces the recruitment of eosinophils (Shang et al. 2002). IL-4, in the presence of TNF, can induce CCL2 and CCR2 expression in fibroblasts. CCL2 is involved in fibrosis through the regulation of profibrotic cytokines, e.g. transforming growth factor β (TGF-β), and the generation and deposition of a collagen matrix (Hogaboam et al. 1999). Fibroblasts from type 2 granulomas generate more CCL2 than Th1 fibroblasts. Generally speaking, CCL2, CCL7, and CCL8 are more involved in type 2 than in type 1 inflammation (Chensue et al. 1996; Qiu et al. 2001). Likewise, CCL11 (eotaxin) and CCR3 (eotaxin receptor) levels are higher in Th2 than in Th1 granulomas. CCR8 (the receptor for CCL1, CCL4, and CCL17 also found predominantly in type 2 granulomas) is exclusively expressed on Th2 cells and contributes to a functional Th2 granuloma, because, in its absence, there is impaired IL-5 production and eosinophil recruitment (Chensue et al. 2001).

Whereas NOS-2 dominates Th1 immune reactions, arginase is predominantly expressed in Th2 responses and is responsible for fibrosis in type 2 granulomas (Hesse et al. 2001). Arginase production is mediated by IL-13 and regulated by substrate depletion through Th1-induced NOS-2 activity (Rutschman et al. 2001).

It is already obvious from the above that there is extensive opportunity for crossregulation between these two pathways of granuloma formation (Matsukawa et al. 2001), e.g. Th2 cells may – via the secretion of IL-10, IL-4, and IL-13 – effectively diminish a Th1 response by downregulating IL-12, IFN-γ, TNF, and chemokine production in Th1 cells (Ruth et al. 2000). In contrast, the type 1 cytokine IL-12 dramatically reduces type 2 lesion size, primarily curtailing eosinophil recruitment (Chensue et al. 1997a). In the absence of IL-4, type 1 inflammation is exacerbated, but an established Th2 granuloma will not convert to a Th1 granuloma, because persistent expression of IL-13, IL-5, and CCL7 can compensate for the defect (Chensue et al. 1997a). CCL5 promotes the Th1

response and mediates crossregulatory inhibition of type 2 granulomas (Chensue et al. 1999). However, chemokines influence granuloma formation not only through a direct effect on leukocyte chemotaxis, but also through altering the Th1/Th2 cytokine balance, e.g. CCL2 promotes IL-4 production by T cells and inhibits IFN-γ production by Th1 cells (Hogaboam et al. 1998, 1999), and CCL5 downregulates IL-4, IL-5, IL-10, and IL-13 production by Th2 cells (Chensue et al. 1999).

It is a hallmark of the T-cell-mediated granulomatous inflammation that it is qualitatively different from an acute inflammatory reaction, particularly in terms of its dependence on adhesion co-receptors, for example, although the acute myelomonocytic infiltrate that occurs in response to infection with *Listeria monocytogenes* (an intracellular bacterium causing granulomatosis infantiseptica) can be experimentally abrogated by administration of monoclonal antibodies to CD11b (an integrin, part of the complement receptor 3 complex), T-cell-mediated granuloma formation is completely independent of this molecule (Mielke et al. 1989, 1997). Certain tissue selectivities also exist, e.g. recruitment of Th1 cells into the skin is dependent on E- and P-selectin ligands on T cells, whereas this probably does not hold true for homing into other organs (Smithson et al. 2001; Erdmann et al. 2002).

Some textbooks include, among DTH reactions, a form that is mediated by $CD8^+$ cytotoxic cells. These cells are induced during a primary immune response by MHCI presentation of proteasome-processed antigens derived from the cytosol. During the effector phase, these specific $CD8^+$ cells are capable of lysing infected cells, thereby causing tissue damage. As a result of this, and because these cells produce limited amounts of IFN-γ in response to antigen stimulation, they have also been defined as TC1 $CD8^+$ cells (Sad et al. 1995). Although these cells may contribute to the extent of DTH, the overwhelming evidence suggests that no skin DTH, and indeed no T-cell-dependent granuloma formation can occur without the active participation of $CD4^+$ cells, and that specific $CD8^+$ cells alone are incapable of initiating a granulomatous response (Mielke et al. 1988, 1992), at least in response to bacterial infections. TC1 cells clearly represent an important mechanism in combating viral infections and can be associated with significant T-cell-mediated tissue damage, but this takes the form of direct tissue cytotoxicity with subsequent extravasation of granulocytes to clear the debris, rather than the form of classic DTH described above with predominantly mononuclear cell infiltration and fibrin deposition.

Functions of granulomatous inflammation

Extracellular bacteria are ingested and killed by polymorphonuclear leukocytes, leading to abscess formation. Microorganisms that can survive intracellularly require a different strategy for their elimination or containment. The structure of a granuloma, with microbe-bearing macrophages at the center surrounded by T cells, affords a close cooperation of these specialized cells. T cells, by secreting macrophage-activating molecules such as IFN-γ or TNF, or via contact-dependent mechanisms such as granule exocytosis, reprogram the macrophage for bactericidal and bacteriostatic functions (Stenger and Modlin 1999; Ehrt et al. 2001). Conversely, macrophages and dendritic cells secreting cytokines and presenting antigens stimulate and select the type of T cell that ultimately decides the fate of the infectious lesion. Microorganisms may cause macrophage death by apoptosis or necrosis, and the removal of dead and dying cells and their replacement with freshly activated macrophages is a dynamic process guaranteed by the antigen-stimulated T cells in close proximity (Adams 1976).

In addition, the concept of physically walling off the offending organisms within a granuloma appears attractive, particularly when large parasites are involved. According to this view, granulomas would serve to contain microorganisms within a separate compartment, the perigranulomatous fibrotic reaction sealing off indigestible bacteria or parasites and preventing dissemination when macrophages burst or decay. In this secluded compartment, cytotoxic T cells may even lyse exhausted or ineffective macrophages in order to release bacteria that may subsequently be engulfed by newly recruited, activated macrophages, the granulomatous capsule preventing leakage into lymphatic or blood vessels (Kaufmann 1988; Saunders and Cooper 2000; Collins and Kaufmann 2001b). The granuloma is thus viewed as a local inflammatory reaction to prevent systemic sequelae of immune activation. Although this hypothesis has its merits, granulomas – like other tissues – are vascularized and may therefore not have the formidable barrier function ascribed to them. However, when a granuloma becomes entirely fibrotic or calcifies, complete sequestration of enclosed microorganisms is probably achieved.

DTH and pathology

Granulomatous inflammation, although necessary for containment of the infectious burden, displaces parenchymal tissue, thereby impairing organ function, such as pulmonary compliance and hepatic blood circulation. The extent of this damage depends on the size and exact location of the granulomas, and may remain undetected for long periods of time, e.g. when present in the mesenteric lymph nodes, yet cause rapid neurological defects when affecting the spinal cord.

One of the most severe forms of pathology associated with DTH is known as the Koch phenomenon, i.e. T-cell-mediated granuloma necrosis in response to mycobacterial antigens. When Robert Koch injected *M. tuberculosis*-infected guinea-pigs with a crude extract of *M.*

tuberculosis (old tuberculin) intradermally, he noted sloughing of the skin at the injection site as well as necrosis at original sites of *M. tuberculosis* implantation in the lung (Koch 1890). Tuberculin in non-sensitized guinea-pigs did not cause this pathology. Koch immediately proposed that this finding be put to clinical use in the diagnosis and therapy of tuberculosis (TB). The former is still in use today in the form of the diagnostic DTH reaction with appropriate doses of more purified protein extracts of *M. tuberculosis* cultures (see Figure 1, p. 706); the latter dismally failed when inappropriately high amounts were applied in severe cases of pulmonary TB, effectively killing patients because of the hyperinflammatory tissue necrosis that was induced. As pulmonary granuloma caseation and cavitation represent the detrimental outcome of a type 1 DTH reaction, we have termed this 'type 1 DTH-associated pathology' (Figure 3).

Another typical form of T-cell-mediated tissue damage caused by DTH is granuloma fibrosis. In chronic schistosomiasis, it is the vigorous granulomatous response, aggravated by fibrosis, rather than any toxicity of the parasite itself, that is responsible for the severe hepatic scarification and liver malfunction characteristic of this disease. As unrestrained granuloma fibrosis represents the detrimental outcome of a type 2 DTH reaction, we have termed this 'type 2 DTH-associated-pathology' (Figure 3). These two prototype pathological sequelae of a specific T-cell response are now described in more detail in terms of the cells and mediators involved. It should be noted, however, that these two types of pathology can overlap to a considerable extent, and that the complete dissociation as described below is merely an idealization for didactic purposes.

TYPE 1 DTH-ASSOCIATED PATHOLOGY: GRANULOMA NECROSIS DURING TUBERCULOSIS

Clinical manifestations of tuberculosis

Approximately one-third of the world population is infected with the causative agent of TB, *Mycobacterium tuberculosis*. Of infected individuals 90 percent will not develop any overt signs of disease throughout their lifetime, attesting to the potency of innate resistance and acquired immunity against TB. Five percent of those infected will come down with primary progressive TB within months of infection, and another 5 percent of those infected will develop post-primary, reactivating disease after a long period of 'latency', i.e. disease-free

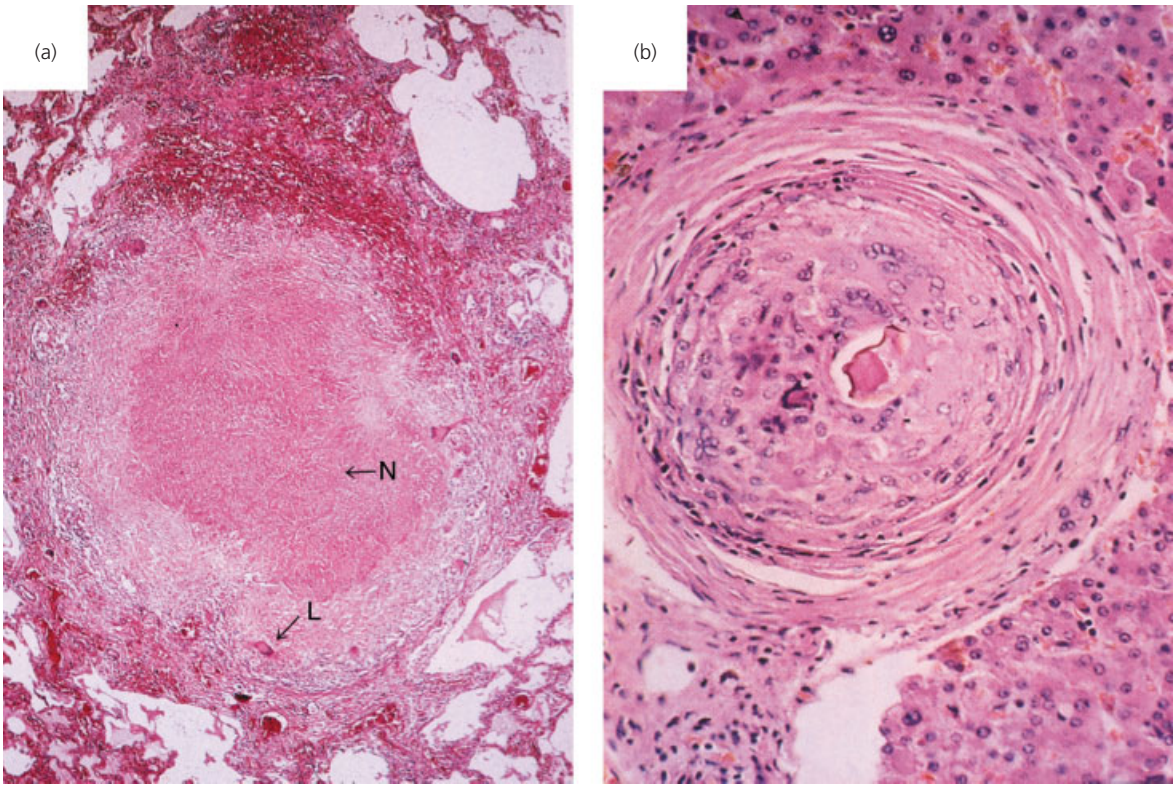

Figure 3 *Type 1 and type 2 DTH-associated pathology:* **(a)** *necrotizing granuloma in the lung of* Mycobacterium tuberculosis-*infected patient (type 1 pathology); N, necrosis; L, Langhans' giant cell;* **(b)** *fibrotic granuloma surrounding parasite egg in the liver of* Schistosoma mansoni-*infected patient (type 2 pathology). (Photographs reproduced with permission from C. Thomas et al. (eds),* Infektionskrankheiten, *copyright 1991, Schattauer Verlag.)*

persistence of primary foci containing viable, nonreplicating mycobacteria. Each year, there are approximately 8 million new cases of TB world wide, and 1.8 million individuals will succumb to the disease, making TB the most deadly bacterial infection (World Health Organization 2002).

Tuberculosis is a systemic disease that becomes manifest most prominently in the lung. Prolonged episodes of fever, night sweats, chest or pleuritic pain, and pleural effusions are the most common initial symptoms of disease. Polymorphous pulmonary infiltrates appear soon after infection, are frequently restricted to one lung lobe, and are often associated with hilar lymphadenopathy. Progressive primary TB occurs when the initial inflammatory focus is unable to contain mycobacterial replication and the disease spreads to other parts of the lung, most often to the lower lung fields. Cough with variable amounts of sputum is the typical symptom, but dyspnea, chest discomfort, fever, chills, and weight loss also occur. Lung consolidation on chest radiographs is confluent and extensive, and single cavities may develop.

Post-primary disease develops when a lesion containing a previously 'dormant' organism reactivates. This organism may have been implanted years before during a primary infection that was successfully contained by the host's immune defenses. Reactivation is a consequence of a perturbed immune status of the host, i.e. old age, suboptimal nutrition, or therapy with immunosuppressive drugs. Disease onset is insidious and accompanied by cough and purulent sputum. Symptoms may range from weight loss, fatigue, and low-grade temperature to acute onset with fever, night sweats, and productive cough. Purulent-appearing sputum is produced whenever granuloma necrosis occurs. Some patients may present with hemoptysis, i.e. coughing of blood as a result of ulceration of granulation tissue or cavity vessels.

During primary infection, small numbers of *M. tuberculosis* invariably gain access to the circulation via the lymphatics and embolize to the capillary beds of most organ systems, e.g. liver, spleen, marrow, and brain. Almost always these metastatic foci heal by granulomatous encapsulation, sometimes with calcification, and with little tissue necrosis. Reactivation later in life may lead to localized extrapulmonary TB.

Miliary TB (so termed because of the gross appearance of the affected organ, 'covered with firm small white corpuscles, of the size of millet seeds' – Manget 1700) represents the unchecked hematogenous dissemination, either during primary infection or after reactivation of a latent focus of *M. tuberculosis*. This is most often seen in hosts with highly impaired cellular immunity.

Immunology and pathogenesis of disease

Mycobacterium tuberculosis is a slowly replicating acid-fast rod. Infection occurs after inhalation of droplet nuclei of 5 μm or less in diameter coughed up by an infected individual. These nuclei, containing 1–10 bacteria, are deposited in the alveoli where they are ingested by alveolar macrophages. Depending on the genetic resistance of an individual, either no further replication occurs or unchecked growth ensues until the incompetent macrophage is lysed, seeding bacteria to secondary sites and leading to local mediator release (TNF, IL-1, prostaglandins, vasoactive amines) and development of a small inflammatory focus. Digested fragments of mycobacteria are taken up by dendritic cells and transported to the local lymph node, where a specific immune response is initiated (Figure 4). TNF, IL-1, IL-6, IL-12, IL-18, and IL-27 are important soluble cofactors in the priming of naïve T cells, and costimulatory accessory molecules on the surface of antigen-presenting cells, such as CD80, direct the development of Th1 effector cells (Swain 2001; Colombo and Trinchieri 2002; Heath et al. 2002; Pflanz et al. 2002; Robinson and O'Garra 2002). Proliferation of T cells is dependent on IL-2, and further maintenance of long-lived memory cells requires IL-15 and IL-23, particularly in the case of $CD8^+$ cells (Umemura et al. 2001; Frucht 2002).

Circulating antigen-specific T cells home to sites of antigen deposition where $CD4^+$ cells cause granuloma formation. These 'tubercles' (literally: little hard nodes) are the hallmark of all mycobacterial infections (Figure 5). At this stage, depending on the bacterial load and the magnitude of the acquired immune response, either infection may be contained or growth and spreading of mycobacteria continue. In most cases, macrophages become sufficiently activated to kill mycobacteria, and tubercles undergo fibrosis and calcification, effectively sealing off the few remaining viable mycobacteria, usually for the lifetime of the host.

Protective immunity involves the secretion of IFN-γ by effector T cells which activates macrophages for bacteriostatic and bactericidal functions (Flynn and Ernst 2000; Shtrichman and Samuel 2001). These include the maturation and acidification of the phagolysosome, the deprivation of nutrients, as well as the generation of oxygen and nitrogen radicals, but the ultimate mechanisms of killing mycobacteria are poorly understood. This is particularly true for human macrophages in which even activation by TNF and IFN-γ results, at best, in bacteriostasis. Alternate mechanisms have therefore been invoked, such as T-cell-induced apoptosis of macrophages, the granule-mediated lysis of macrophages by T cells and killing of mycobacteria by granulysin contained in the granules, and the requirement for additional costimuli, such as CD40 ligand engagement or vitamin D_3 (Mazzaccaro et al. 1998; Stenger et al. 1999; Cho et al. 2000; Collins and Kaufmann 2001b; Hogan et al. 2001). On the other hand, TGF-β (produced in significant quantities within tuberculous lesions) undermines host immune responses, because it promotes T-cell suppression, macrophage deactivation, and tissue

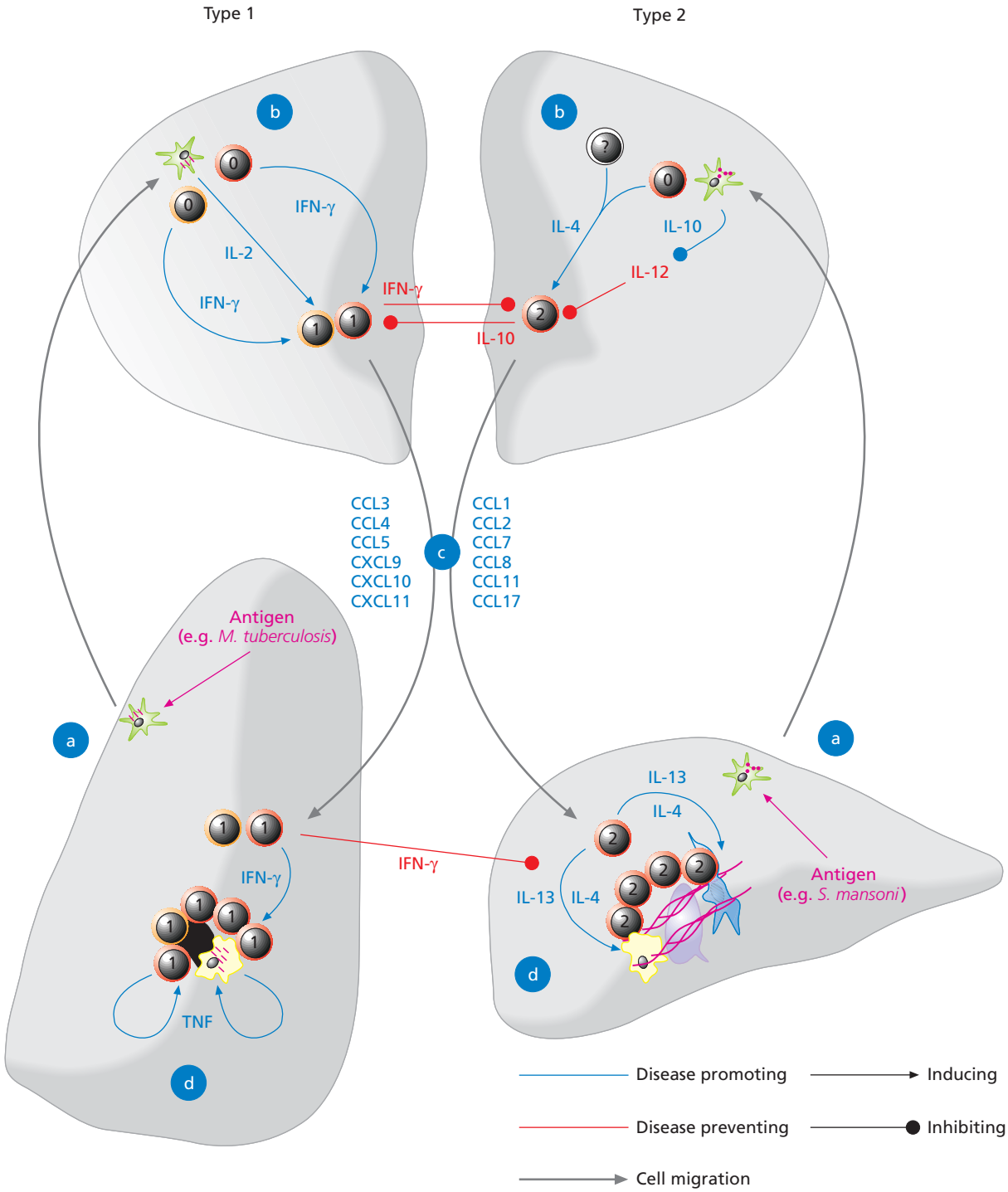

Figure 4 *Mechanisms of granuloma formation:* **(a)** *antigen uptake and activation of dendritic cells in infected tissue, e.g. lung or liver;* **(b)** *priming of specific T cells in draining lymph node;* **(c)** *cell recruitment of specific T cells into infected tissue by chemokines; and* **(d)** *granuloma formation and effector response of T cells in infected tissue. 0=Th0, 1=Th1, 2=Th2. For details, see text.*

destruction (Toossi and Ellner 1998). In the absence of significant immunity, i.e. in infants and immunodeficient adults, there is progressive spread with tuberculous pneumonia or miliary TB.

In the face of developing immunity and high antigenic load, there is frequently necrosis of macrophages at the center of the granulomas. Within these necrotic areas, mycobacteria are located extracellularly and do not replicate well. When the necrotic center of a granuloma liquefies, a cavity is formed and multiplication of *M. tuberculosis* resumes. When bronchial erosion occurs, the infective material may disseminate through the

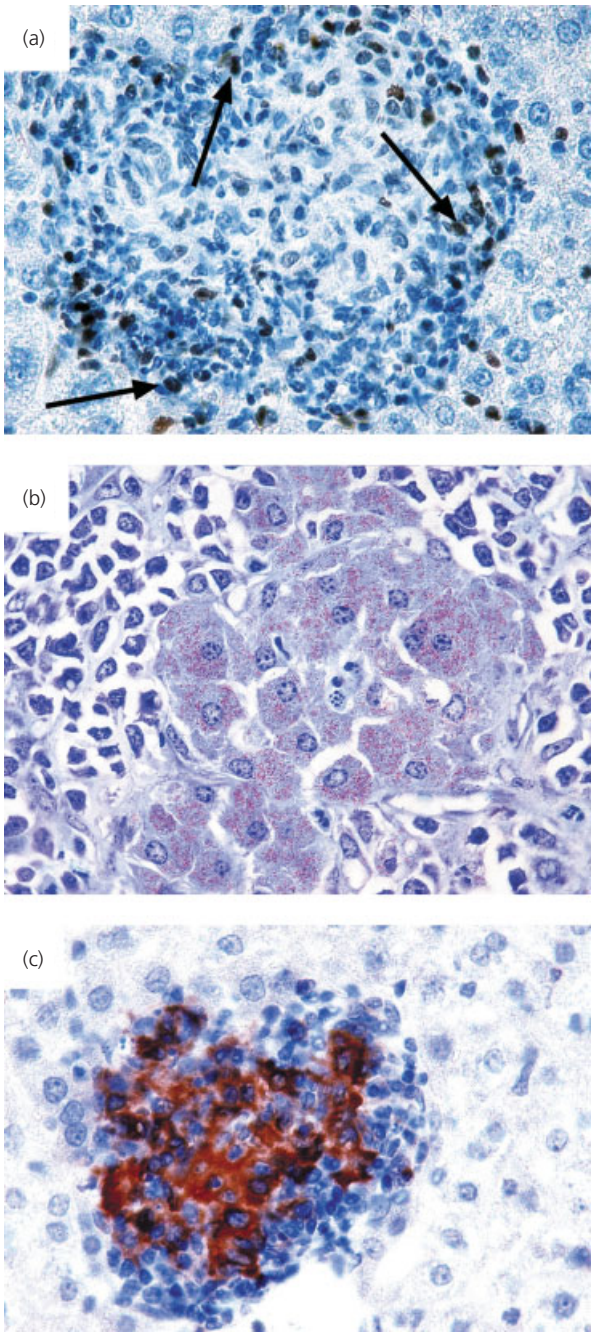

Figure 5 *Immunohistopathology of a* mycobacteria*-induced granuloma.* **(a)** *$CD3^+$ (T) cells (in dark brown, arrows) at the periphery of an epithelioid granuloma;* **(b)** *acid-fast bacteria (red rods) located exclusively within macrophages at the center of a granuloma, surrounded by lymphocytes with a dense nucleus; and* **(c)** *NOS-2^+ cells (macrophages, in brown) at the center of an epithelioid granuloma.*

airways to other sites in the lung or may be coughed up, creating infectious aerosols.

In those cases in which T-cell immunity is grossly impaired (e.g. in patients with AIDS), rapid growth of mycobacteria results in widespread tissue necrosis without granulomatous encapsulation. This form of nondemarcated tissue necrosis is probably a consequence of the inherent cytotoxic nature of *M. tuberculosis* and must be clearly differentiated from the typical form of granuloma caseation brought about by specific T cells.

Morphology of type 1 DTH-associated pathology

A typical tuberculous lesion is composed of coalescent granulomas, containing epithelioid cells surrounded by a zone of fibroblasts and lymphocytes. In mice, most of the T lymphocytes are of the $CD4^+$ subtype, but in the periphery $CD8^+$ cells also exist (Gonzalez-Juarrero et al. 2001). There is a considerable number of B cells, often arranged as lymphoid follicles, present in mature granulomas. A study using in situ hybridization and immunohistology to analyze cytokines present in human tuberculous lesions showed that granulomas usually stain positive for IL-12p40, IFN-γ, and TNF transcripts and protein, with a minority also showing IL-4 expression (Fenhalls et al. 2000, 2002). IL-4 positive granulomas were always concomitantly positive for IFN-γ, and necrotic granulomas were always positive for TNF.

As a result of macrophage necrosis within the granuloma, caseation is usually present in the centers of these tubercles – so called because of the cheesy appearance when cut by a pathologist's knife (Figure 6). Adjacent to the caseous center, a large number of neutrophils are often found. In addition to 'necrosis' as judged by light microscopic evaluation, there is significant apoptosis of both T cells and macrophages in mature granulomas (Fayyazi et al. 2000).

As the lesions progress, more tubercles coalesce to create a confluent area of consolidation. At this stage, the entire area may be converted to a fibrocalcific scar, or the residual caseous debris may become walled off by collagenous connective tissue. However, when the tubercle erodes into a bronchus, drainage transforms it into a cavity (Figure 6). Cavitary fibrocaseous TB may affect one, many, or all lobes of both lungs in the form of isolated small tubercles, confluent caseous foci, or large areas of caseation necrosis (Kobzik and Schoen 1994).

Delayed-type hypersensitivity-associated pathology in the form of granuloma necrosis is therefore not only potentially hazardous to the diseased individual, aggravating tissue damage and organ malfunction, but also epidemiologically relevant, because necrosis, liquefaction, and bronchial erosion ultimately favor the spread of viable mycobacteria into the environment in an aerosolized form.

Mechanisms of granuloma necrosis

In order to dissect a process as complex as T-cell-mediated tissue injury, it is imperative to have a reproducible animal model that mimics the pathology occurring in

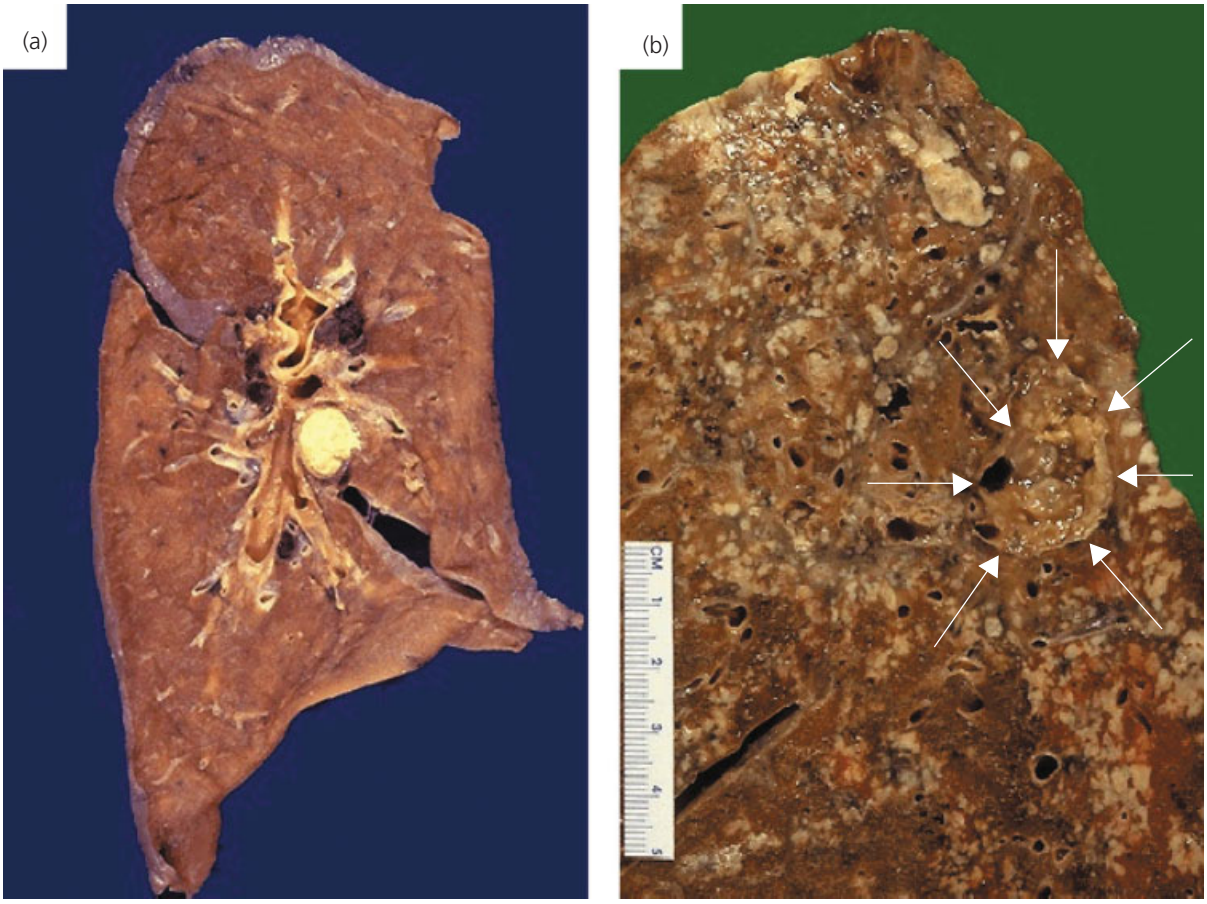

Figure 6 *Type 1 pathology: caseous necrosis and cavity formation in the lungs of tuberculosis patients.* **(a)** *Caseating granulomatous lesion in a hilar lymph node.* **(b)** *Extensive caseous necrosis throughout the lung with prominent cavitation (area demarcated by arrows). (Photographs reproduced with permission from Webpath, Version 7, 14 March, 2002, copyright 2002, Edward C. Klatt, MD.)*

humans and allows for a mechanistic study of the factors involved (McMurray et al. 1996).

Aerogenic infections in rabbits faithfully reproduce the pathology present in humans, including liquefaction of caseous necrosis and subsequent cavity formation (Figure 7). However, most studies have remained purely descriptive of this phenomenon because immunological reagents are missing to dissect, at the molecular level, the processes underlying granuloma necrosis. A few studies demonstrated that activated macrophages surrounding the caseous center stain positive for β-galactosidase, acid phosphatase, and cathepsin D which may implicate lysosomal enzymes such as proteases, lipases, or nucleases in the liquefaction process (Dannenberg 1968; Dannenberg and Sugimoto 1976; Suga et al. 1980; Converse et al. 1996). Moreover, the endothelial adhesion molecules ICAM-1 and VCAM were found to be preferentially expressed during the chronic stage of infection suggesting their involvement in the granulomatous, rather than acute inflammatory, response (Abe et al. 1996). Aerogenic infections in guinea-pigs also induce caseous necrosis in developing pulmonary granulomas. This is associated with a marked DTH skin response. Again, mechanistic studies on the molecular mediators of tissue necrosis have been lacking because of a paucity of immunological reagents to measure them.

Aerogenic infection in laboratory mice with *M. tuberculosis* is not followed by granuloma necrosis, but is rather associated with chronic interstitial fibrosis (North 1995; Rhoades et al. 1997). However, when mice deficient in the γδ T-cell-receptor (TCR)-positive cell subset are infected, they develop a purulent form of granuloma with many neutrophils infiltrating necrotic areas at the center of the granuloma (D'Souza et al. 1997). Infection with *Listeria monocytogenes* also results in centrally necrotic and purulent granulomas (Mombaerts et al. 1993). These data suggest that γδ T cells perhaps play an important role by influencing local cellular traffic, promoting the influx of lymphocytes and monocytes, and limiting the access of inflammatory cells that do not contribute to protection but may cause tissue damage. Alternately, γδ T cells may produce cytokines and/or chemokines that regulate the potentially harmful effect

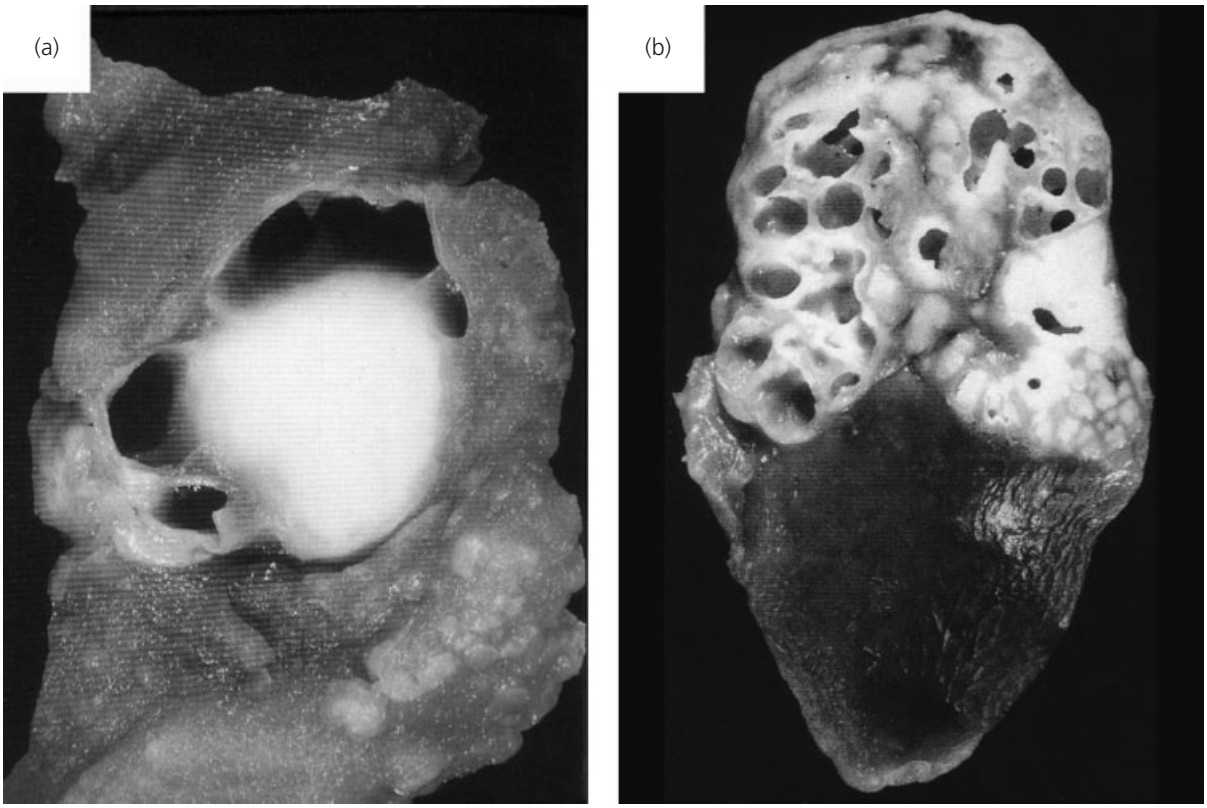

Figure 7 *Type 1 pathology in rabbits.* **(a)** *Caseous tubercle at the bifurcation of a bronchus near the hilus in a rabbit after low-dose aerosol infection with* Mycobacterium bovis. **(b)** *Multiloculated cavities in the lungs of aerosol-infected rabbits. (Photographs reproduced with permission from Converse et al. 1996; copyright 1996, The American Society for Microbiology.)*

of a hyperinflammatory Th1 reaction initiated by αβ-TCR$^+$ CD4$^+$ T cells.

Fortuitously, aerogenic infection in mice with a highly virulent strain of *M. avium* (the causative organism of avian TB) results in progressive granuloma caseation with perigranulomatous fibrosis, strongly resembling the pathology occurring in human TB patients (Benini et al. 1999). This model is therefore perfectly suited for the molecular analysis of granuloma necrosis, because immunological tools, such as knock-out mice, may be applied. Using this model, αβ-TCR$^+$ T cells of the CD4$^+$ subset are found to be necessary for granuloma necrosis, whereas γδ-TCR$^+$ cells and CD8$^+$ cells do not contribute (Figure 8) (Ehlers et al. 2001). Perforin and apoptotic mechanisms are not required for necrosis in this model (Florido et al. 2002). However, IL-12 and IFN-γ are essential for granuloma necrosis, and in the absence of IL-10 granuloma necrosis is slightly accelerated (Ehlers et al. 2001; Florido et al. 2002). Most interestingly, the absence of NOS-2 does not impact on granuloma necrosis, revealing that IFN-γ-induced mechanisms mostly associated with antibacterial protection (such as induction of NOS-2) need not necessarily coincide with mechanisms that lead to tissue destruction (Ehlers et al. 2001). It was hypothesized that IFN-γ may preferentially induce a variety of chemokines with anti-angiogenic effects, and that reduced vascularization may then lead to granuloma hypoxia and subsequent necrosis, similar to a scenario previously demonstrated in infection-associated tumor regression (Hunter et al. 2001).

Tumor necrosis factor has long been postulated to be directly involved in caseation necrosis, because infection of cells with *M. tuberculosis* rendered them highly sensitive to killing by TNF in vitro (Filley et al. 1992). Moreover, DTH sites were particularly necrosis prone after TNF injection, when small amounts of IL-4 were also present (Hernandez-Pando and Rook 1994; Rook and Zumla 2001). Finally, treatment of *M. tuberculosis*-infected mice with recombinant TNF resulted in increased inflammation in the lungs and accelerated mortality (Moreira et al. 2002).

Studies in *M. tuberculosis*-infected TNF- and TNFRp55-deficient mice are more difficult to interpret, because TNF is necessary for granuloma initiation and maintenance as well as antibacterial protection. TNF-deficient mice will therefore succumb to excessive bacterial loads in a completely necrotic lung, a scenario more reflective of primary progressive TB in immuno-

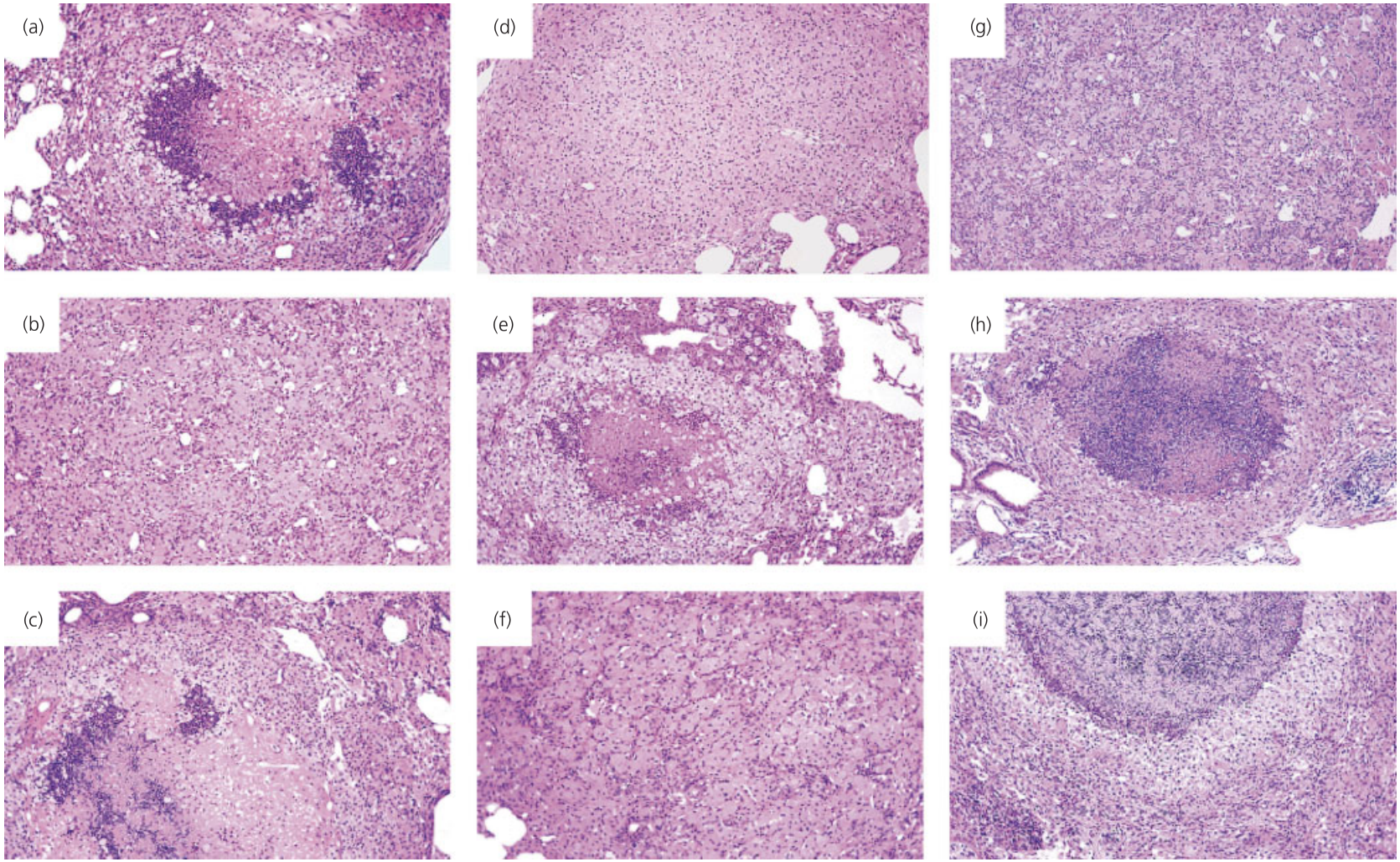

Figure 8 *Mechanisms of granuloma necrosis in a mouse model of type 1 pathology. Mice were infected aerogenically with* Mycobacterium avium *and sacrificed 16 weeks after infection.* **(a)** *Centrally necrotizing granulomatous lesion in wild-type mice;* **(b)** *absence of granuloma necrosis in αβTCR-deficient mice;* **(c)** *centrally necrotizing granuloma in γδTCR -deficient mice;* **(d)** *absence of granuloma necrosis in MHC class II-deficient mice;* **(e)** *central granuloma necrosis in β_2-microglobulin-deficient mice;* **(f)** *absence of granuloma necrosis in IL-12p40-deficient mice;* **(g)** *absence of centrally necrotizing lesions in IFN-γ-deficient mice;* **(h)** *central granuloma necrosis in IL-10-deficient mice; and* **(i)** *central granuloma necrosis in NOS-2-deficient mice. (Low power micrographs reproduced with permission from Ehlers et al. 2001; copyright 2001, The Rockefeller University Press.)*

deficient individuals (Flynn et al. 1995). Further studies using different microbial challenges, such as *M. avium* and *Corynebacterium parvum*, established that the absence of TNF signaling is associated with an excessive inflammatory response, and (as in the aerosol model of necrosis) is dependent on T cells and IL-12, probably reflecting dysregulated inflammation (Marino et al. 1997; Benini et al. 1999; Ehlers et al. 2000). TNF (and also IFN-γ), possibly via the induction of NOS-2, therefore also serve to downregulate an exacerbated inflammatory response, in part by inducing apoptosis of effector T cells (Kanaly et al. 1999; Chu et al. 2000; Dalton et al. 2000; Cooper et al. 2002).

The frequent presence of granulocytes close to the necrotic center of granulomas is intriguing. It is possible that they act merely as scavengers clearing the debris, but they may also be the source of cytokines and chemokines (such as CXCL9 and CXCL10), amplifying the tissue-damaging loop by recruiting more T cells (Gasperini et al. 1999). Granulocytes may also degranulate, contributing to tissue destruction by a similar mechanism to that postulated for activated macrophages, i.e. the release of aggressive lysosomal proteases and other hydrolyzing enzymes (Dannenberg and Sugimoto 1976).

TGF-β has been implicated in the pathogenesis of many diseases that are associated with tissue destruction and fibrosis (Border and Noble 1994). It promotes the production and deposition of a collagen matrix, but it also increases the production of macrophage collagenases (Wahl et al. 1993). As it is a strong chemotactic molecule for monocytes and polymorphonuclear cells, both of which are rich sources of metalloproteases, TGF-β may be particularly involved in tissue remodeling, with a potential bias towards tissue destruction and scarring (Wahl et al. 1987; Toossi and Ellner 1998).

Finally, degradation of collagen and other matrix proteins is achieved by a family of metalloproteases, which are dependent on zinc ions for their activity (Parks and Shapiro 2001; Brinckerhoff and Matrisian 2002). Metalloproteases comprise: (1) interstitial collagenases, which cleave the fibrillar collagen types I, II, and III; (2) gelatinases, which degrade amorphous collagen as well as fibronectin; (3) stromelysins, which act on a variety of matrix components, including proteoglycans, laminin, fibronectin, and amorphous collagens; and (4) matrilysin, which plays a role in innate immunity of epithelial barriers. These enzymes are produced by several cell types (fibroblasts, macrophages, neutrophils, epithelial cells), and their secretion is induced by many stimuli, including growth factors platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)), cytokines (IL-1, TNF, TGF-β), and phagocytosis. They are elaborated in a latent form that can be activated by oxygen radicals and proteases produced during inflammation, and can be inhibited by specific tissue inhibitors of metalloproteases (TIMP). Degrading collagen is essential in wound repair, and metalloproteases may therefore not only promote, but also alleviate tissue destruction in DTH-associated pathology, depending on the stage of disease (Parks and Shapiro 2001; Winkler and Fowlkes 2002). Macrophage metalloelastase, by cleaving elastin, generates fragments that are chemotactic for monocytes, and may perpetuate macrophage accumulation, leading to progressive and chronic lung destruction (Senior et al. 1980). Table 2 summarizes some of the mediators and mechanisms that contribute to granuloma necrosis.

Strategies to reduce granuloma necrosis: Implications for vaccine development

The importance of defining the molecular pathways ultimately causing type 1 DTH-associated pathology is highlighted by the results of recent immunization studies in guinea-pigs. Animals that received a DNA vaccine encoding heat shock protein 60 of *M. tuberculosis* – published as highly efficacious as a therapeutic vaccine (Lowrie et al. 1999) – succumbed to infection significantly earlier than controls showing widespread caseation necrosis in their lungs (Turner et al. 2000). Thus, more immunogenic vaccines simply triggering more of the mediators common to antibacterial protection and granuloma necrosis will inevitably result in more tissue damage, effectively rendering them useless. The trick will be to inhibit, or modulate the pathways involved in DTH-associated pathology while, at the same time, boosting the mechanisms involved in antibacterial protection.

Some investigators have suggested that this may be possible with a careful selection of the antigens in vaccine preparations, i.e. the use of antigens common to all mycobacterial species and avoidance of the species-specific *M. tuberculosis* antigens (Rook and Hernandez-Pando 1996; Rook et al. 2001). Thus far, vaccine and immunotherapy trials have not produced convincing proof of this hypothesis (Durban Immunotherapy Trial Group 1999).

A different strategy is specifically to target $CD8^+$ cells in a vaccine protocol, reducing $CD4^+$ T cell-mediated DTH while possibly exploiting granule-mediated pathways of antibacterial protection (Kaufmann 2000; Stenger and Modlin 1998; Stenger et al. 1998; Collins and Kaufmann 2001a). Given the uncertainty concerning the true level of protection $CD8^+$ T cells afford in vivo against *M. tuberculosis* challenge, this approach may be only part of the solution (Sousa et al. 2000; Mogues et al. 2001). If it turns out that the distinction between antibacterial protection and tissue damage (in terms of mediators required) is more of a quantitative rather than of a qualitative nature, 'differential vaccination' may be altogether out of reach.

Table 2 *Some cellular and molecular mediators of type 1 DTH-associated pathology in tuberculosis*

Cells and molecules	Effect on granuloma necrosis	Putative mechanisms regulating necrosis	Reference
$CD4^+$ T cells	+	Production of IFN-γ	Ehlers et al. (2001); Florido et al. (2002)
$CD8^+$ T cells	?/+	Lysis of infected macrophages	Kaufmann (1988); Stenger and Modlin (1999)
$\gamma\delta$-TCR^+ cells	–	Promoting cellular influx preventing necrosis	D'Souza et al. (1997)
	–	Harnessing $\alpha\beta$-TCR^+ cells	Mombaerts et al. (1993)
CD40	+	Costimulation of T cells	Florido et al. (2002)
IL-12	+	inducing IFN-γ production	Ehlers et al. (2001); Florido et al. (2002)
IL-10	0/–	Inhibiting IFN-γ production	Ehlers et al. (2001); Florido et al. (2002)
IFN-γ	+	Chemokine induction	Ehlers et al. (2001)
	–	Limiting T-cell responses	Dalton et al. (2000)
TNF	+	Apoptosis of sensitized cells	Filley et al. (1992); Rook et al. (2001)
	–	Regulating T-cell responses	Kanaly et al. (1999); Ehlers et al. (2000)
IP-10, MIG	?/+	Antiangiogenic effect	Hunter et al. (2001)
MCP-1	?/+	Recruitment of monocytes	Orme and Cooper (1999)
Nitric oxide	0	No direct effect	Ehlers et al. (2001)
	–	Regulating T-cell responses	Dalton et al. (2000); Cooper et al. (2002)
Lysosomal enzymes	+	Tissue digestion	Dannenberg and Sugimoto (1976)
Metalloproteases	+	Breakdown of connective matrix	Matyszak and Perry (1996)
	–	Repair and fibrosis	Parks and Shapiro (2001)

+, inducing; –, limiting; 0, no effect, ?, effect unknown.
For abbreviations see the text.

TYPE 2 DTH-ASSOCIATED PATHOLOGY: GRANULOMA FIBROSIS IN SCHISTOSOMIASIS

Manifestations of clinical schistosomiasis

Schistosomiasis is a disease of the tropical world. According to the World Health Organization, the disease is endemic in 74 countries, affecting about 200 million people (Pearce and MacDonald 2002). The causative agents are digenic trematode worms, also called blood flukes. Three species are important causes of human infection: *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. The adult forms of *S. mansoni* and *S. japonicum* live in the portal and mesenteric veins; *S. haematobium* inhabits the vesical plexus of the bladder. Humans are the principal definitive host during the life cycle of these three species of schistosomes and adult worms live in the venous systems of the intestine. The female worm inhabits a groove in the lateral edge of the male's body and produces from 300 (*S. mansoni*) to 3000 (*S. japonicum*) eggs every day. These eggs pass through the venule walls, cross the intestinal mucosa, find their way into the lumen of the bowel, and are excreted with the feces (Figure 9). In fresh water, the eggs hatch and release motile ciliated miracidia which penetrate the body of the intermediate host, usually a snail of the genus *Biomphalaria* (*S. mansoni*) or *Oncomelania* (*S. japonicum*). The miracidia multiply asexually inside the snail; within a few weeks, hundreds of motile fork-tailed cercariae emerge. When the cercariae encounter human skin they penetrate it, lose their tails, and change into schistosomula forms, which migrate to the lungs and liver. In approximately 6 weeks they mature into adult worms and pass through the venous system to their final habitat. The life span of the worm is 5–10 years, and by a variety of mechanisms it is capable of evading the host immune response. The worms feed on host erythrocytes and body solutes, and their presence is innocuous to the host. Some of the eggs deposited by the females into the bloodstream are excreted with the feces. However, eggs that do not reach the intestinal lumen are swept into the portal circulation and are trapped in the intestinal wall, the liver, or the lung. This continuous egg deposition induces a chronic granulomatous response in the host that is responsible for the major disease syndrome of chronic schistosomiasis.

The two main clinical conditions, acute and chronic schistosomiasis, are associated with different immune responses. Acute schistosomiasis in humans is a debilitating febrile illness that can occur before the appearance of schistosoma eggs in the feces and which peaks

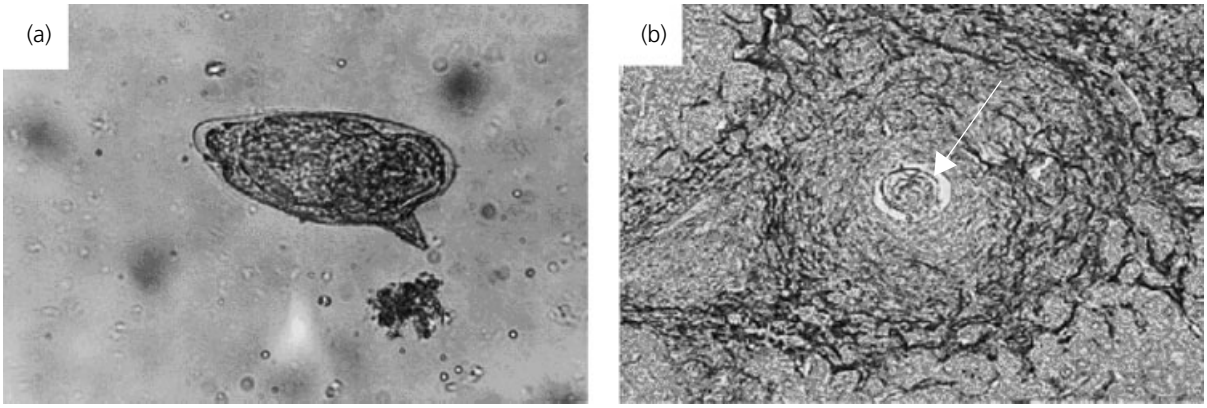

Figure 9 *Type 2 pathology:* Schistosoma mansoni*-induced hepatic fibrosis.* **(a)** S. mansoni *egg isolated from hepatic granulomas.* **(b)** *Hepatic granuloma surrounding deposited egg (arrow). Fibrosis in liver section is detected by aniline blue staining. (Micrograph in* **(a)** *reproduced with permission from the Microbiology Laboratory Reference Center Homepage, UT Southwestern Medical Center Dallas, Texas, USA, copyright 2002, Jerry Y. Niederkorn, PhD; micrograph in* **(b)** *reproduced with permission from the Laboratory of Parasitic Diseases Homepage, NIAID, Bethesda, Maryland, USA, copyright 2002, Alan Sher, PhD.)*

generally between 6 and 8 weeks after infection (Rabello 1995). In chronic schistosomiasis, there is a progressive increase in egg burden which affects primarily the intestines and liver. Antigenic properties of the parasite induce a strong immune response resulting in granuloma formation and fibrosis around the eggs in these organs (Figure 9). This may result in portal hypertension, massive splenomegaly, and gastrointestinal bleeding from esophageal varices. Patients experience fatigue, abdominal pain, and diarrhea, and in the most severe cases jaundice and liver failure. Occasionally eggs may bypass the liver via the portosystemic collateral circulation and give rise to granulomas in the lung, with pulmonary hypertension and cor pulmonale, or lesions in the brain or spinal cord, which may present as space-occupying lesions, generalized encephalopathy, or transverse myelitis. The severity of the disease directly correlates with the number of eggs present in the tissues. The liver sustains the most severe damage in chronic schistosomiasis, because the chronic granulomatous inflammation results in severe fibrosis which accumulates over the long period of the disease.

Immunology and pathogenesis of schistosomiasis

Schistosomiasis causes a range of illnesses, the development of which is influenced to a large extent by the nature of the induced immune response and its effects on granuloma formation and associated pathologies in target organs (Boros 1999; Dunne and Pearce 1999; Cheever et al. 2000). Host genetics, parasite burden, and sensitization to schistosoma antigens all influence the development of the immune response and, thus, disease severity.

In contrast to early studies showing the participation of eosinophils and IgE in in vitro killing of *S. mansoni* larvae in antibody-dependent cellular cytotoxicity (ADCC) reactions (Romagnani 1996), in vivo studies employing mouse models argue against a function of IgE and eosinophils and support a role for Th1 responses in protection against *S. mansoni* (de Andres et al. 1997; King et al. 1997). During the larval migration and maturation period, resistance is dependent on $CD4^+$ T cells and is associated with a Th1 cytokine response (Vignali et al. 1989; Sher et al. 1990). In particular, IL-12-induced IFN-γ is mandatory for resistance after immunization with irradiated parasites (Smythies et al. 1992; Wilson et al. 1996; Anderson et al. 1998). IFN-γ-activated macrophages and endothelial cells kill larval schistosomes in vitro via an arginine-dependent mechanism involving NO production (James and Glaven 1989; Oswald et al. 1994). In vivo, most parasites are eliminated as they traverse the lungs of immunized mice (Dean and Mangold 1992; Wilson et al. 1996) and NOS-2 can be identified in the pulmonary inflammatory foci around the migrating larvae (Wynn et al. 1994). Moreover, larvacidal properties of NO represent an important effector mechanism because NOS-2-deficient mice display a reduced resistance to *S. mansoni* challenge (James et al. 1998).

Some Th2 effector mechanisms, however, also contribute to protection against *S. mansoni*. In particular, IL-4Rα-mediated signaling by IL-4 and IL-13 participates in the development of immunity. In immunization studies, the defective Th2-type antibody response (i.e. no IgG1 or IgE) in IL-4Rα-deficient mice was associated with increased parasite loads which could be partially prevented by serum transfer (Mountford et al. 2001). As IgE has no protective role against challenge with *S. mansoni* (King et al. 1997; de Andres et al. 1997), protection is probably associated with the IgG1 antibody subclass.

In chronic schistosomiasis, the pathology is predominantly caused by the host reaction to parasite eggs, which are laid in the portal venous system and subsequently trapped in the liver and intestine (Cheever

1972). Although Th2 responses have a beneficial role in modulating potentially life-threatening disease during the initial stages of schistosomiasis, prolonged Th2 responses are responsible for the development of egg-induced hepatic fibrosis and chronic morbidity (Cheever et al. 2000). Even though IL-4 is the primary cytokine driving the differentiation of $CD4^+$ T cells into the Th2 subset, fibrotic pathology in schistosomiasis is not ameliorated in IL-4-deficient mice (Wynn et al. 1993; Cheever et al. 1994; Metwali et al. 1996; Pearce et al. 1996; Chensue et al. 1997b). However, granuloma formation and fibrosis do depend on IL-4Rα-mediated signaling (Kaplan et al. 1996, 1998). As IL-4 and IL-13 share the IL-4Rα as a common receptor component and both activate the Stat6 signaling pathway (Brombacher 2000; Hölscher 2003), pathology in chronic schistosomiasis is a result of IL-13-mediated mechanisms. *Schistosoma*-infected mice in which IL-13 is either neutralized by treatment with soluble IL-13Rα_2-Fc (Chiaramonte et al. 1999a) or absent (IL-13-deficient mice) (Fallon et al. 2000) fail to develop the severe hepatic fibrosis – marked by extensive collagen deposition – that normally occurs during infection, and survive longer (Figure 10). Th2 responses are maintained in IL-13-deficient animals, and blocking IL-13 in IL-4-deficient mice does not perturb the Th1/Th2 cytokine balance (Chiaramonte et al. 1999a; Fallon et al. 2000). However, fibrosis is maximally reduced in these mice, again emphasizing the important role played by IL-13 in fibrogenesis.

Given its critical role in fibrosis, differences in the expression of IL-13 could provide a simple explanation for the different degrees of fibrosis that different inbred mouse strains develop after infection with *S. mansoni* (Cheever et al. 1987). However, there is no correlation between the expression of IL-13 and the severity of fibrosis in these strains (Chiaramonte et al. 2001). On the other hand, IFN-γ and IL-10 are key regulatory cytokines in fibrosis (Czaja et al. 1989; Hoffmann et al. 2000; Louis et al. 2000; Nelson et al. 2000; Chiaramonte et al. 2001). In schistosomiasis, the antifibrotic effects of the Th1 cytokine IFN-γ are well documented (Czaja et al. 1989; Wynn et al. 1995). IFN-γ directly downregulates the synthesis of extracellular matrix components (Stephenson et al. 1985), and the IL-13:IFN-γ ratio is predictive of the magnitude of fibrosis (Chiaramonte et al. 2001). In humans, severe hepatic fibrosis correlates with a mutation in the IFN-γR1 (Dessein et al. 1999), and can be explained by the lack of effective suppression of fibrogenesis by IFN-γ. High IL-13 and low IL-10 levels are also predictive of severe fibrosis in inbred mice, whereas inbred mice displaying a low IL-13:IL-10 ratio tend to develop a more modest degree of fibrosis after infection with *S. mansoni* (Chiaramonte et al. 2001). In summary, the magnitude of fibrosis is controlled by the profibrogenic activities of IL-13 and antifibrotic effects of IFN-γ and IL-10.

Morphology of type 2 pathology

Histological examination of lesions from chronically infected individuals with type 2 pathology shows granulomas to be composed mainly of lymphocytes, macrophages, and eosinophils, exhibiting a wide range of granuloma size around deposited eggs from *S. mansoni* (Boros 1989). The cellular constituents of murine liver granulomas also comprise lymphocytes, plasma cells, macrophages, epithelioid and giant cells, eosinophils, neutrophils, mast cells, and fibroblasts (Stenger et al. 1967; Smith 1977). In particular eosinophils and mast cells produce several Th2-associated cytokines (Sabin et al. 1996; Woerly et al. 1999) and are often described as important effector cells in a variety of Th2-mediated pathological disorders. However, there is no correlation between the magnitude of hepatic fibrosis and the cellular composition of the lesions (Chiaramonte et al. 2001).

In general, the development of fibrous tissue is part of the normal process of healing after injury. Nevertheless, in schistosomiasis, fibrosis enhances the disease pathology and greatly contributes to the mortality after infection with *S. mansoni* (Friedman et al. 2000). Fibrotic changes are ranked on the basis of ultrasound patterns that provide a quantitative tool for assessing the severity of disease (Hatz 2001). After infection with *S. mansoni*, the fibrotic reaction is characterized by collagen deposition and collagen synthesis in fibrotic livers increases up to 40-fold over that of normal livers (Dunn and Kamel 1981). Consequently, type I, type III, and B collagens are found in portal spaces, fibrous septa, and granulomas (Biempica et al. 1983). Hepatic fibrosis is mostly associated with circumoval granulomas (see Figure 3b, p. 710, and 35.9) and follows the granulomatous inflammatory response occurring at the site of egg deposition (Dunn et al. 1977; Dunn and Kamel 1981). Total liver collagen production positively correlates with egg load (Cheever et al. 1983), but there is no direct relationship between granuloma size and the degree of fibrosis (Hood and Boros 1980).

In *S. mansoni* infection, cells within hepatic granulomas are potentially harmful to adjacent parenchymal cells, because they are in a metabolically activated state, capable of secreting a number of tissue-destroying substances. During phagocytosis, cell activation, or cell death, these mediators diffuse from the granulomas and damage tissue parenchymal cells. Potential tissue-damaging agents that are released in granulomas are lysozyme, β-glucuronidase, collagenase, elastase, proteases, prostaglandins, leukotrienes, superoxide anions, IL-1, and type 1 interferons (James and Glaven 1989). Indeed, healed granulomas usually leave behind scar tissue in their vicinity, indicating focal damage and repair (Von Lichtenberg 1975).

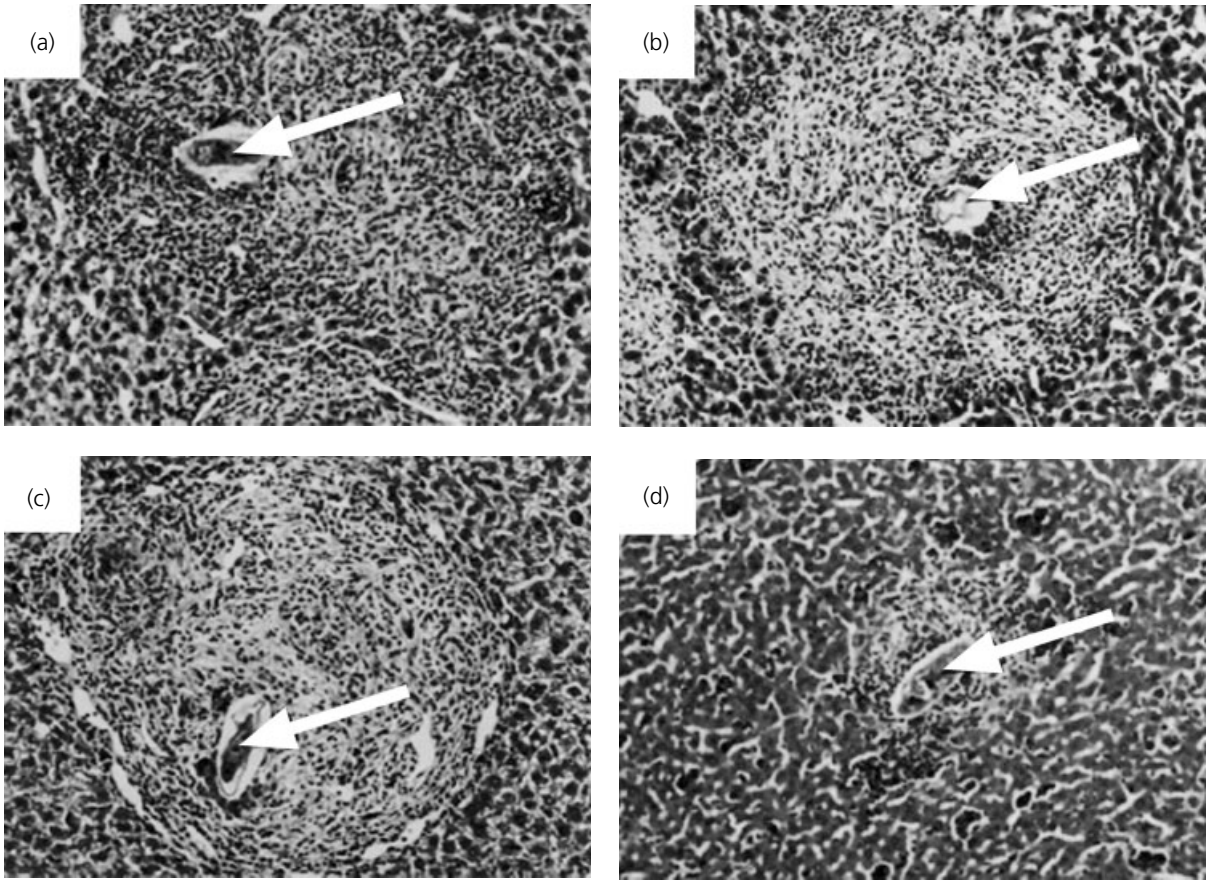

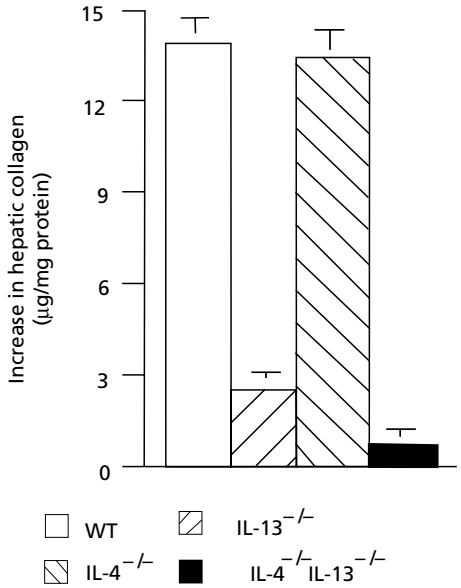

(e)

Figure 10 *IL-13-mediated type 2 pathology. Mice were infected with* Schistosoma mansoni *and analyzed for hepatic pathology induced by deposited egg (arrow).* **(a)** *Hepatic granuloma in wild-type mice;* **(b)** *hepatic granuloma in IL-4-deficient mice;* **(c)** *hepatic granuloma in IL-13-deficient mice;* **(d)** *reduced granuloma formation in IL-4/IL-13-deficient mice; and* **(e)** *decreased collagen deposition in the absence of IL-13 (tissue collagen in the livers of uninfected and infected mice was quantified and expressed as the increase in hepatic collagen in micrograms of collagen per milligram of protein). (High power micrographs reproduced with permission from Fallon et al. 2000; copyright 2000, The American Association of Immunologists.)*

Mechanisms of granuloma fibrosis in schistosomiasis

The effects of IL-13 on fibrosis are direct and are not dependent on induction of other profibrotic mediators (Lee et al. 2001) or perturbations in the Th1/Th2 cytokine response (Wynn et al. 1995). IL-13 receptors are expressed on fibroblasts (Murata et al. 1998) and IL-13 increases adhesion molecule and inflammatory cytokine expression (Doucet et al. 1998). Moreover, fibroblasts of different origin are able to respond to IL-13 with collagen production (Chiaramonte et al. 1999a; Oriente et al. 2000). Although IL-13 and IL-4 are both capable of promoting collagen production in fibroblasts (Oriente et al. 2000) after infection with *S. mansoni*, expression of IL-13 largely exceeds that of IL-4 (Chiaramonte et al. 1999a). In the pulmonary granuloma model IL-4 is more transiently expressed after egg exposure, whereas expression of IL-13 is much more sustained over time and is observed at greater levels than IL-4 in the granulomatous tissues (Chiaramonte et al. 1999b).

Hepatic fibrosis can be reduced when the *S. mansoni* egg-specific response is directed into a Th1- rather than a Th2-dominated reaction. This is accomplished by sensitization to schistosome egg antigens in the presence of IL-12 or CpG oligonucleotides (Wynn et al. 1995). Several Th1-associated mediators are believed to be involved in preventing the fibrotic response (Hoffmann et al. 1998), particularly NOS-2 (Hesse et al. 2000). NOS-2-deficient mice that have been sensitized with schistosome egg antigen and IL-12 develop a Th1-dominated response, but fail to control the granulomatous inflammation and display a marked exacerbation of hepatic fibrosis. Therefore, the critical downstream component that controls fibrosis in a Th1-dominated response is the functional activity of NOS-2-expressing cells.

As NOS-2 shares L-arginine as a substrate with arginase, substrate depletion by either enzyme is a key regulatory mechanism (Rutschman et al. 2001) and the differential expression of NOS-2 and the hepatic isoform of arginase (Arg-1) is important for regulating or promoting fibrosis. Macrophages are a prominent participant in *S. mansoni* egg-induced granuloma and are the primary producers of NOS-2 and Arg-1. Although it is not yet known which cells, besides macrophages, express Arg-1 after infection with *S. mansoni*, granuloma formation correlates with high arginase activity (Hesse et al. 2001). The Th1 cytokines IFN-γ and TNF activate NOS-2 production in 'classically activated' macrophages, thus regulating hepatic fibrosis by substrate depletion for Arg-1. On the other hand, the profibrotic Th2 cytokine IL-13 induces Arg-1 activity in 'alternately activated' macrophages (Modolell et al. 1995), and Th2-driven arginase expression and activity are the critical component in the development of type 2 pathology (Hesse et al. 2001) (Figure 11).

Arg-1 is an essential enzyme of the urea cycle and is expressed at high levels in hepatocytes. The enzyme hydrolyses L-arginine to urea and L-ornithine; therefore, its main function in the liver is the detoxification of ammonia. As L-ornithine, a product of arginase activity, is a necessary metabolite for the production of prolines which in turn control collagen production, arginase activity is linked to hepatic fibrosis after *S. mansoni* infection. The direct involvement of the arginase biosynthetic pathway in type 2 pathology was tested by blocking the L-ornithine-dependent synthesis of polyamines. After blocking ornithine carbamoyltransferase (ODC), competition between ODC and ornithine aminotransferase (OAT) for L-ornithine is eliminated and hepatic fibrosis after infection with *S. mansoni* increases (Hesse et al. 2001). Thus, the utilization of L-ornithine by OAT, which increases the synthesis of prolines (the basic building block of collagen), could explain the enhanced collagen deposition. An earlier study of schistosomiasis had already shown that fibrosis is directly controlled by the availability of proline (Dunn et al. 1981). As IL-4/IL-13-activated macrophages are an important source of proline (Hesse et al. 2001), macrophages act as important controllers of type 2 pathology. However, recently established macrophage-specific IL-4R-α-deficient mice (Herbert et al. 2004), carrying a defective receptor for IL-4 and IL-13 only on macrophages revealed that other cell types such as fibroblast are involved in the development of type 2 pathology.

Biochemically, collagen synthesis occurs by conversion of proline into hydroxyproline. The endogenous proline used for collagen synthesis diffuses into hepatic granulomas and is hydroxylated by prolylhydroxylase, an enzyme that is greatly elevated within granulomatous livers (Dunn et al. 1979). At the peak of granuloma formation, vigorous collagen production is coincident with maximal lesion size after which synthesis declines. However, at this time the collagen content remains maximal because enzymatic degradation of the connective tissue matrix is inhibited and collagen accumulates in hepatic lesions (Dunn et al. 1979). Intragranulomatous protease inhibitors were identified as α_2-macroglobulin and α_1-antiprotease (Truden and Boros 1988). The net effect of synthesis versus degradation results in remodeling of the affected tissues.

Fibroblasts, macrophages, and smooth-muscle cells have all been identified as initiators of hepatic granuloma fibrosis (Boros 1989; Hesse et al. 2001). Granuloma fibroblasts produce CCL2 and CCL3 and are implicated in promoting leukocyte recruitment, granuloma growth, and maintenance of the granulomatous lesion (Lukacs et al. 1994a). Migration of fibroblasts to the site of injury and their subsequent proliferation are triggered by growth factors, such as PDGF, epidermal growth factor (EGF), FGF, and TGF-β, and fibrogenic cytokines derived in part from inflammatory macrophages (Mornex et al. 1994). Some of these growth factors also stimulate synth-

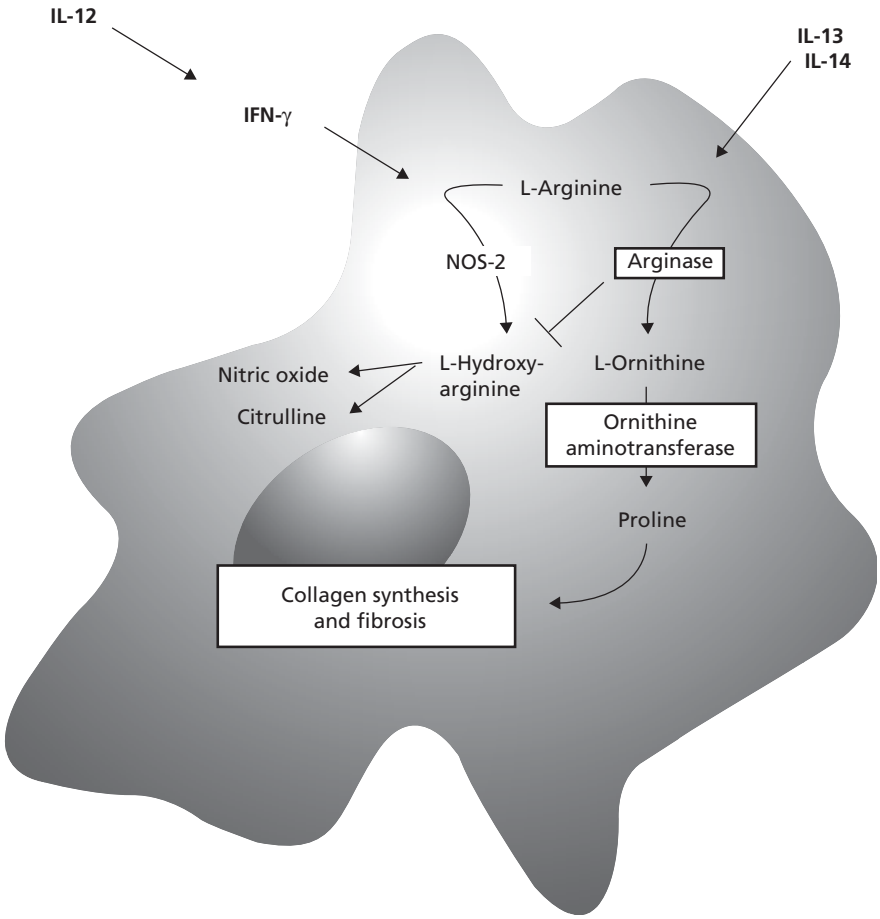

Figure 11 *Mechanisms of granuloma fibrosis in type 2 pathology. For details, see the text.*

esis of collagen and other connective tissue molecules and modulate the synthesis and activation of metalloproteases, enzymes that serve to degrade these extracellular matrix components (Winkler and Fowlkes 2002). Mice overexpressing TGF-β in the liver develop multiple tissue lesions including hepatic fibrosis and hepatocyte apoptosis (Sanderson et al. 1995). The inhibition of TGF-β action by the administration of agents that bind TGF-β or block TGF-β expression is effective in experimental models, but its efficacy and safety in human liver fibrosis remain unknown (Gressner et al. 2002).

Strategies to prevent granuloma fibrosis: implications for immunotherapy

Findings from experimental schistosomiasis clearly point to IL-13 as a central and indispensable mediator of fibrosis. Therefore, development of an IL-13 antagonist might be a strategy to treat established and progressive disease. Treatment with soluble IL-13Rα_2 has no influence on egg deposition and granuloma formation, but prevents progression of fibrosis (Chiaramonte et al. 2001). In future studies, it will be interesting to combine the beneficial action of IL-13 antagonists with the chemotherapeutic elimination of the parasite for further acceleration of the healing process.

In this context, molecules that inhibit Th2 responses by favoring Th1 development and modulating IL-13 production might also impact on strategies to treat hepatic fibrosis, for example, the chemokine CCL5 has been implicated in contributing to Th1 immune responses while limiting Th2 responses (Chensue et al. 1999). Treatment with recombinant CCL5 reduced type 2 granuloma size by one-third. Therefore, CCL5 may have therapeutic potential in the treatment of established type 2 hypersensitivity.

Another approach focuses on preventing granuloma fibrosis by immunization. Immunization with irradiated larval stages is followed by a protective Th1 immune response that disturbs the migration of the larvae through the lung (Wilson et al. 1999). Sensitization with eggs in the presence of IL-12 dramatically reduces the tissue fibrosis induced by subsequent infection with *S. mansoni* without affecting protective responses (Wynn et al. 1995). This strategy is an example of an immunization that acts by preventing type 2 pathology rather than infection itself.

THE RELATIONSHIP BETWEEN CELL-MEDIATED IMMUNITY AND DELAYED-TYPE HYPERSENSITIVITY

When Mackaness first introduced the concept of the activated macrophage, the temporal coincidence of its appearance with that of the DTH reaction was taken to imply that both were merely two manifestations of the same cellular and molecular processes (Mackaness 1962, 1964) – hence the frequent description of protective immunity as a 'double-edged sword'. Later, it became apparent that, in some instances, acquired resistance could be maintained even in the absence of granulomatous inflammation and that, in other instances, severe DTH-mediated tissue damage could occur in the virtual absence of antibacterial protection. It thus became necessary conceptually to distinguish between protective cell-mediated immunity (CMI) and tissue-damaging DTH. Today, most investigators hold that separate entities (cells and/or mediators) are responsible for macrophage activation versus tissue damage, although the level at which the respective mechanisms are thought to dissociate is open to debate (Table 3).

An opposing opinion argues that the cellular and molecular bases of both CMI and DTH are mostly identical, but are regulated differentially in space and time, particularly in terms of the quantities of cytokines/chemokines secreted and of homing capacities of the cells involved, for example, mice lacking the signature structure on ICAM-1 that allows monocytes to migrate into inflamed lesions were still able to contain mycobacterial growth, but showed significantly reduced granulomatous inflammation early during infection (Johnson et al. 1998). However, when the long-term outcome of infection in ICAM-deficient mice was analyzed, they were found to succumb to elevated bacterial loads because they lacked organized granulomas (Saunders et al. 1999).

In experimental infections with *L. monocytogenes*, it was clearly demonstrated that granuloma formation depends entirely on the $CD4^+$ T-cell subset, whereas the most potent antibacterial protection is conferred by $CD8^+$ T cells, revealing that pathology and protection could be completely dissociated at the level of effector T cells (Mielke et al. 1988, 1992).

In schistosomiasis, the course of immune responses can be separated into a protective Th1 response early after infection and a pathological Th2 immune response characterized by chronic hepatic fibrosis. With respect to crossregulatory mechanisms, promotion of protective Th1 development results in preventing pathological Th2 responses as demonstrated in IL-12-modulated immunization studies (Lukacs et al. 2001). Treatment of an already established Th2 immune reaction reduces granuloma fibrosis without negatively affecting protection. Therefore, IL-13 antagonists represent a promising strategy to treat already established and progressive type 2 DTH-associated pathology.

In *M. tuberculosis* infection, no equally discriminatory basis for macrophage-activating and tissue-damaging pathways has been identified (Ehlers 1999). Rather, the most powerful antibacterially active T cells are of the $CD4^+$ subtype and secrete IFN-γ, as are the most potent DTH-conferring T cells. Similarly, TNF is involved in both antibacterial defense mechanisms and is pivotal during the inflammatory response. It has even been argued that type 1 DTH-associated pathology can have beneficial effects, because it deprives mycobacteria of their intramacrophage habitat and exposes them to life in a hostile environment, i.e. hypoxia, acid pH, and coagulation necrosis (Dannenberg 1968). According to this view, granuloma necrosis would be the ultimate form of antibacterial protection if macrophage activation fails (Dannenberg 1991).

In an attempt at revitalizing the conceptual dissociation of CMI and DTH in TB, it has been proposed that cytokines, such as IFN-γ, might be viewed as mostly involved in protection, whereas chemokines, such as CCL2 or CXCL10, might be considered as mostly associated with the DTH response (Orme and Cooper 1999). Although it is true that chemokines, in their majority, are not known directly to contribute to antibacterial effector mechanisms, cytokines are clearly major players in granuloma formation (TNF, IFN-γ), somewhat invalidating this hypothesis. It is, however, possible that the amplification

Table 3 *Mediators of protection and pathology in Th1 and Th2 responses*

Granuloma type	Cells and mediators	Protection	Pathology
Necrotic (type 1)	$CD4^+$ T cells	+	+
	$CD8^+$ T cells	+	?/+
	γδ-TCR^+ cells	?/+	?/–
	TNF	+	?/+
	IFN-γ	+	+
	IL-10	–	–
	NOS-2	+	0/–
	MCP-1	0	?/+
	RANTES	?/0	?/+
	MIG	0	?/+
Fibrotic (type 2)	Th1 $CD4^+$	+	–
	Th2 $CD4^+$	0/–	+
	IFN-γ	+	–
	TNF	+	?/0
	IL-4	0/-	0
	IL-10	–	–
	IL-12	+	–
	IL-13	0	+
	NOS-2	+	–
	Arginase	?/0	+
	RANTES	+	–

+, inducing; –, limiting; 0, no effect; ?, effect uncertain.
For abbreviations see the text.

of the inflammatory response, and therefore the excess of inflammation leading to tissue damage, is mostly the result of chemokine secretion. As outlined above, it is becoming increasingly apparent that dissociation between macrophage activation and DTH-associated pathology may exist beyond the level of IFN-γ production. Protective mechanisms induced by IFN-γ (e.g. NOS-2) can clearly be dissociated from necrosis-inducing processes that are IFN-γ dependent, but NOS-2 independent, and that may involve certain chemokines. The latter therefore represent attractive targets for therapeutic immunointervention aimed at harnessing tissue-damaging pathways without compromising antibacterial protection.

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Airway hypersensitivity

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INTRODUCTION

Airway hyperresponsiveness is a prominent feature of a variety of pulmonary diseases, although it is most often associated with asthma. Asthma is a complex disease of the airways associated with bronchial inflammation, reversible airway obstruction, mucus hypersecretion, and hypersensitivity (increased bronchial reactivity to nonspecific spasmogenic stimuli), defined as airway hyperresponsiveness (AHR). In people with chronic asthma these processes culminate in irreversible changes in airway physiology. There has been a marked increase in the prevalence of asthma in the last few decades. Although there is undoubtedly a genetic component underlying the predisposition toward onset of disease, the scale of the increase in the developed world suggests that environmental factors associated with changes in lifestyle and an improvement in the standard of living must also play a role. Asthma is polygenic and multifactoral in origin and is often classified as intrinsic (antigen independent) or extrinsic (allergic) asthma. Allergic asthma is triggered by antigen-specific exposure to common environmental aeroallergens, such as those present in grass or tree pollen, animal dander, or house dust mites. The aim of this chapter is to review our current understanding of the immunological mechanisms underlying asthma and AHR in the light of recent studies that suggest that the increasing prevalence of asthma may be the result of a loss of inherent immunoregulatory mechanisms.

Airway hyperreactivity

The mechanisms underlying the development of AHR are not fully understood. It is thought to arise as a result of successive inflammatory processes in the airway mucosa triggered by respiratory infections or repeated exposure of susceptible individuals to aeroallergens, resulting in the induction of T-helper (Th)2 cytokines, which play both a direct role in the induction of AHR, as well as an indirect role associated with tissue damage caused by the release of inflammatory mediators from effector cells such as eosinophils recruited into the airways after allergen exposure (Holt et al. 1999). Pathophysiological changes in the airways resulting from chronic inflammation, compounded by altered neural regulation of airway tone, are thought to contribute toward AHR. Increased deposition of extracellular matrix (ECM) proteins in the airway submucosa, increased vascularity, and tissue edema, as well as an increase in airway smooth muscle mass (ASM), lead to thickening of the airway wall and exaggerated airway narrowing in response to various stimuli. A number of mediators released during the inflammatory response are though to contribute toward increased ASM. Histamine, platelet-activating factor (PAF), and the leukotrienes (LT), released from mast cells and eosinophils, are potent inducers of increased bronchial permeability, thereby contributing directly to bronchial edema. The injured airway epithelial cell (AEC) is also a potent source of a number of cytokines, chemokines, and

inflammatory mediators, thereby contributing toward AHR and airway remodeling (Holgate 1998).

Early acute phase response

Mast cells are essential for the IgE-dependent, immediate hypersensitivity acute phase reaction to aeroallergens. Within minutes of allergen exposure, cross-linking by multivalent allergen of preformed IgE bound to the high-affinity IgE receptor (FcεRI) on mast cells results in an immediate hypersensitivity reaction, characterized by the release of the preformed mediators histamine and the proteases (tryptase and chymase), cytokine transcription, and the de novo synthesis and secretion of the cyclooxygenase and lipoxygenase metabolites of arachidonic acid, prostaglandin D_2 (PGD_2), and leukotriene C_4 (LTC_4). These inflammatory mediators activate inflammatory effector cells, and cause mucus secretion, smooth muscle constriction, and increased vascular permeability, thereby potentiating airway inflammation and AHR.

Late phase response

The late phase response (LHR) is associated with the eosinophil recruitment, activation and release of the eosinophil-derived inflammatory mediators, eosinophil cationic protein (ECP), major basic protein (MBP), and eosinophil derived neurotoxin (EDN), in addition to leukotrienes, prostaglandins, particularly PGD_2, and PAF. Eosinophils are also a source of a number of cytokines, including the eosinophil activation and survival factor, IL-5, as well as the profibrotic factor transforming growth factor β (TGF-β). There is a close association between pulmonary eosinophilia and AHR in both extrinsic and intrinsic (nonatopic) asthma. Elevated levels of ECP and MBP have been detected in the bronchoalveolar lavage (BAL) of patients with asthma. These proteins can induce AHR as a direct consequence of their damaging effect on the airway epithelium (Wardlow et al. 1988).

Airway remodeling

Airway remodeling and the resulting structural changes are features of chronic asthma, observed in both atopic and nonatopic individuals. Although the inflammatory response associated with both forms of asthma is characterized by the presence of Th2 cytokines, the existence of a specific IgE is not a prerequisite for the development of asthma, which suggests that abnormalities in the airway epithelium, which forms a barrier to the external environment, results in sensitization to allergens and subsequent development of the asthmatic phenotype. This process may be facilitated by exposure to pathogens such as respiratory viruses, or irritants, which further damage the epithelium. In chronic asthma, structural changes in the bronchial epithelium arise as a result of increased collagen deposition beneath the basal lamina with thickening of the basement membrane, goblet cell hyperplasia, and increased mucin production. These changes eventually lead to irreversible airway obstruction, and a reduction in airway function associated with increased bronchial hyperresponsiveness. Increases in mucus production and goblet cell hyperplasia are common features in cases of fatal asthma (Aikawa et al. 1992).

In asthma, products released by mast cells and eosinophils are directly responsible for damage to the epithelium. The interleukin cytokines IL-4, IL-5, IL-9, and IL-13 released by Th2 effector cells are responsible for many of the features of airway remodeling. Goblet cell metaplasia and mucus production are associated with IL-4- and IL-13-induced increases in expression of the genes *muc5ac*, *muc2*, and *muc4* which are detected in bronchial biopsies obtained from people with asthma. Overproduction of IL-4 in the lung induces *muc5ac* gene expression and mucus secretion by airway epithelial cells expressing the common IL-4Rα chain (Temann et al. 1997), and is dependent on STAT6 signaling (Kuperman et al. 1998). The immunoregulatory molecules TGF-β, and more recently IL-10, have been shown in independent studies to induce subepithelial fibrosis, suggesting that certain aspects of airway remodeling may be an undesirable consequence of an attempt to downregulate chronic inflammatory responses (Elias et al. 1999; Lee et al. 2002)

IMMUNOLOGICAL MECHANISMS UNDERLYING THE ASTHMATIC PHENOTYPE

IgE-mediated pathology in asthma and AHR

Genetic linkage studies have identified an association between chromosome 5q and two of the principal features of allergic asthma: total serum IgE levels and bronchial hyperresponsiveness (Marsh et al. 1994; Postma et al. 1995). A number of clinical studies have demonstrated that elevated specific IgE levels in sera are associated with asthma. Whether this reflects the location on chromosome 5q of the cytokines IL-4 and IL-13, which are directly responsible for increased airway hyperresponsiveness, as well as the switch to ϵ germline transcription and IgE synthesis by B cells (Oettgen and Geha 2001), or an essential role for IgE in the initiation of asthmatic responses is disputed. A recent study carried out on a cohort of Australian families concluded that, of the multiple genetic traits that were found to contribute toward the pathophysiology of asthma, the two dominant traits, namely increased serum IgE levels

and AHR, were clearly independently inherited (Palmer et al. 2000). Numerous clinical studies have demonstrated that increased serum IgE, although associated with allergic disease, does not necessarily predispose toward development of asthma and AHR (Sunyer et al. 1995). Most studies in murine models of allergic asthma have shown that AHR can be induced in the absence of B cells or specific IgE antibodies (Mehlhop et al. 1997; Hogan et al. 1997; Hamelmann et al. 1997, 1999).

Nevertheless, allergic sensitization to allergens and IgE-mediated responses play a significant role in the development of asthma in a large number of patients. Specific IgE contributes towards the clinical phenotype by inducing mast cell degranulation and release of inflammatory mediators, following crosslinking of the high-affinity FcεR. Mediators released from mast cells trigger a cascade of events which culminate in asthma and increased AHR. Recently, completed phase II clinical trials have shown that patients with severe asthma, who are known to respond poorly to steroid treatment, demonstrated a marked improvement in symptoms after treatment with a humanized anti-IgE monoclonal antibodies (McAbs). Anti-IgE McAbs are thought to act by binding to and neutralizing circulating IgE antibodies, thereby preventing them from binding to FcεRs (Bush 2002; Lemanske et al. 2002).

Mechanisms of $CD4^+$ Th2 cytokine-induced pathology in asthma and AHR

Asthma is a chronic inflammatory disorder that can eventually lead to structural changes in the airways, arising as a result of increased collagen deposition, smooth muscle proliferation, and mucus hyperplasia which cause bronchial hyperresponsiveness and variable airflow obstruction. In both allergic and intrinsic asthma, the inflammatory response is associated with the recruitment of activated $CD4^+$ T lymphocytes expressing a Th2 cytokine profile (Ying et al. 1997a), and an influx of eosinophils. The interleukins, IL-4, IL-5, IL-9, and IL-13, expressed by activated $CD4^+$ Th2 cells which are the dominant cell type in bronchoscopic biopsies and BAL samples obtained from patients with asthma, are thought to play a central role in the pathophysiology of asthma (Robinson et al. 1992).

Genetic linkage studies identified an association with asthma and related traits in the region of chromosome 5q, in which the IL-4, IL-5, IL-9, and IL-13 genes are clustered (Marsh et al. 1994; Postma et al. 1995), and a region of chromosome 16p12 in which the gene encoding the IL-4Rα chain is located (Hershey et al. 1997). Multiple polymorphisms have been identified within the IL-13 gene and IL-4R gene complex, which reassociated with asthma (Heinzmann et al. 2000; Ober et al. 2000).

There is considerable functional redundancy among these pleiotropic Th2-derived cytokines in asthma. For this reason, their relative importance in the development of AHR is still not fully defined. Temporal differences in the kinetics of cytokine expression and in the distribution of their receptor molecules will determine the contribution of these cytokines toward the asthmatic response. It has proved difficult to establish the respective roles of the Th2-derived cytokines IL-4 and IL-13. These cytokines exhibit partially overlapping effector profiles as a result of their common use of the IL-4 receptor α chain (IL-4Rα) as the signaling component within the multimeric IL-4 and IL-13 receptor complexes. The IL-4 R consists of the IL-4Rα chain and the common γ chain (of the IL-2 receptor). The functional IL-13 receptor complex is a heterodimer consisting of the IL-13Rα1 chain which interacts with either the IL-4Rα chain or the IL-13Rα2 chain. Signaling through the IL-4Rα chain leads to activation of the Janus kinase family of tyrosine kinases, resulting in the recruitment, phosphorylation, and nuclear translocation of the transcription factor signal transducer and activator of transcription 6 (STAT6) (Shirakawa et al. 2000). The importance of the STAT6 signaling pathway in the induction of airway eosinophilia and increased AHR was demonstrated in STAT6-deficient mice. These mice failed to develop specific IgE antibodies, airway eosinophilia, or AHR on exposure to methylcholine, after sensitization and challenge of the airways with allergens. Mucus production was significantly reduced compared with wild-type animals (Akimoto et al. 1998; Kuperman et al. 1998).

Using selected lines of Th2 cytokine-deficient mice, deficient in single or multiple cytokine genes, it has been possible to demonstrate that, although IL-5, IL-9, and IL-13 all contribute toward the magnitude of the inflammatory response, IL-4 is essential for initial activation of Th2 effector functions (Fallon et al. 2002). IL-4 is critical for the induction of Th2 responses to allergen, but is not essential for the development of late-phase airway inflammation and AHR. AHR can be induced in naïve mice after adaptive transfer of antigen-specific $CD4^+$ Th2 cells obtained from IL-4-deficient mice (Coyle et al. 1995; Cohn et al. 1998). Although not essential, IL-4 enhances a number of STAT6-dependent responses in the airways which contribute toward AHR. These include enhanced mucus production through the induction of *muc5ac* gene expression by epithelial cells in the airways, release of the eosinophil chemoattractant eotaxin, and upregulation of VCAM-1 expression on endothelial cells, thereby promoting increased recruitment of lymphocytes and eosinophils (Nakajima et al. 1994; Rankin et al. 1996; Temann et al. 1997; Mocjizuki et al. 1998).

The pleiotrophic Th2-derived interleukin IL-13 is now recognized as being largely responsible for initiating and maintaining many of the functions associated with the late-phase inflammatory response, which result in altered airway physiology and remodeling, including

goblet cell hyperplasia with mucus hypersecretion and AHR. Notably, both these features of the asthmatic response are commonly associated with asphyxiation (Aikawa et al. 1992). IL-13 also exhibits a number of effects that are responsible for enhancing the IgE-mediated acute phase response, including induction of IgE production by B cells and upregulation of CD23 (FcεRII) expression on B cells, and the upregulation of the expression of the integrin vascular adhesion molecule VCAM-1 on endothelial cells (Zurawski and de Vries 1994; Bochner et al. 1995; Emson et al. 1998). Instillation of IL-13 into the lungs of naïve mice was sufficient to induce many of the features of asthma, including elevated serum IgE, airway eosinophilia, increased mucus production, and AHR. The central role of IL-13 as a mediator in asthma was demonstrated by inhibiting its activity through the administration of the soluble IL-13Rα_2-IgGFc fusion protein, to sensitized and challenged mice, before secondary exposure to allergen via the airways. These mice exhibited attenuated goblet cell hyperplasia, mucus production, and AHR on challenge with a spasmogenic agent, despite detectable airway eosinophilia and normal IgE levels (Wills-Karp et al. 1998).

The independence of IL-13-mediated AHR in the airways on T-cell-driven airway inflammation and IgE production was demonstrated in RAG-1$^{-/-}$-deficient mice, which lack T and B lymphocytes, indicating that IL-13 mediates its effects though direct activation of cells in the airways (Grunig et al. 1998). This view was confirmed in subsequent studies, which demonstrated that IL-13 is not only necessary but also sufficient for the induction of AHR. The failure of mice deficient in either IL-13 or IL-4 and IL-13 to develop AHR in spite of increases in IL-5-mediated airway eosinophilia, after sensitization and challenge with allergen, could be reversed by the instillation of recombinant IL-13 alone into the airways (Walter et al. 2001). Chronic expression of IL-13 in the airways was sufficient to cause enhanced recruitment of lymphocytes, eosinophils, and macrophages into the airways, together with subepithelial fibrosis, upregulation of mucin (*Muc5ac*) gene expression, and mucus hypersecretion and increased AHR (Zhu et al. 1999). Increases in both mucus hypersecretion and AHR were mediated by IL-13 through STAT6-dependent mechanisms (Kuperman et al. 2002). However, increases in AHR were also observed in IL-4Rα chain-deficient mice constitutively expressing IL-13 in the airways, or after transfer of IL-13-producing, antigen-specific, CD4$^+$ T cells, indicating that separate components of the STAT6-dependent signaling pathway were involved (Mattes et al. 2001). In addition to its direct effect on airway epithelial cells, IL-13 may influence AHR through direct effects on airway smooth muscle and activation of fibroblasts, resulting in altered deposition of matrix proteins.

IL-13-mediated recruitment and activation of CD4$^+$ Th2 lymphocyte and eosinophils in the airways during the inflammatory response also contribute toward AHR. The coordinated temporal and site-directed expression of chemokines and chemokine receptors is central to the complex pathophysiology of asthma. Elevated levels of the CC chemokines, eotaxin, eotaxin-2, RANTES (monocyte chemotactic protein (MCP)-1, -3. and -4 and the chemokine receptor CCR3 expressed on mast cells, basophils, and eosinophilis, as well as CD4$^+$ Th2 lymphocytes and myeloid dendritic cells (DC), have been observed in the lungs of patients with allergic asthma (Ying et al. 1999). IL-13 is a potent inducer of a number of chemokines involved in the development of the asthmatic phenotype. Levels of mRNA for these chemokines were increased in the lungs of CC10-IL-13 transgenic mice, which selectively overexpress the IL-13 transgene in the airways. Many of the inflammatory and profibrotic effects of IL-13 are mediated through the induction of chemokines responsible for the recruitment of inflammatory cells into the airways. IL-13 is a potent inducer of eotaxin expression by airway epithelial cells (Li et al. 1999), which is a principal chemoattractant for eosinophils, and CCR3 expressing CD4$^+$ Th2 lymphocytes during the initiation of airway inflammation. IL-13-induced increases in production of monocyte-derived chemokine (MDC) and MCP-1, the respective ligands for CCR4 expressed on activated CD4$^+$ Th2 cells and CCR2 expressed on macrophages, are thought to enhance recruitment of these cells into the airways. The importance of the coordinated expression of these chemokines in the induction of airway inflammation and remodeling that results in AHR has been demonstrated in murine models of asthma. Neutralization of MDC activity by the use of specific antibodies lead to reductions in CD4$^+$ T lymphocytes recruited from the airway lumen into the lung, which correlated with attenuated AHR (Gonzalo et al. 1999). The enhanced airway inflammation and tissue fibrosis, observed in CC10-IL-13 transgenic mice, were greatly reduced in mice deficient in CCR2 (Zhou et al. 2002). By inducing increased production of MCP-1, the ligand for CCR2, IL-13 drives the recruitment of macrophages into the airways, where they are though to initiate subepithelial fibrosis, through production of the profibrotic factor TGF-β_1 (Lee et al. 2001). These studies indicate that IL-13, through the induction of eotaxin, MDC, and MCP-1, indirectly regulate Th2-mediated airway inflammation, eosinophilia, and tissue fibrosis, respectively.

The central role played by IL-13 in the pathology associated with pulmonary inflammation and fibrosis is not restricted to asthma, and has been observed in a number of respiratory diseases including respiratory syncytial virus infection, fungal pneumonitis, and chronic obstructive pulmonary disease (Lukacs et al. 2001; Blease et al. 2000). Alveolar macrophages in the airways may be the initial source of IL-13 in pulmonary inflam-

matory disease (Prieto et al. 2000), with relative levels of IL-12 and IL-13 production by macrophages in the airways during primary antigen challenge influencing the phenotype of effector T cells.

Levels of the Th2-derived cytokine IL-9 and expression of the IL-9R are elevated in the lungs of patients with allergic asthma (Shimbara et al. 2000). IL-9 stimulates a number of different cell types, including B cells, mast cells, eosinophils, and airway epithelial cells, leading to the development of a number of features that are characteristic of asthma and AHR (Petit-Frere et al. 1993; Longphre et al. 1999; Gounni et al. 2000; Louahed et al. 2001). Transgenic mice exhibiting either constitutive or inducible IL-9 expression in the lung have many of the features associated with asthma, including eosinophilic and lymphocytic infiltration of the airways, mucus hypersecretion, subepithelial fibrosis, mast cell hyperplasia, and AHR (Tenmann et al. 1998). The effects mediated by IL-9 may be the result of increased expression of the Th2 cytokines IL-4, IL-5, and IL-13 in the lungs of IL-9 transgenic mice. Neutralization of IL-13 completely abolished both lung inflammation and mucus production (Tenmann et al. 2002), indicating that expression of IL-9 alone is insufficient to induce pathological changes associated with asthma and that these cytokines are required to act in combination in order to achieve optimal stimulation. The unique role of IL-13 in the induction of AHR is most probably the result of its direct effects on airway epithelial cells, mediated independently of the common IL-4Rα chain.

The role of mast cells in the induction of AHR

Mast cells are important in initiating and amplifying the IgE-mediated acute hypersensitivity response to allergens. Crosslinking of FcεRs by IgE–allergen complexes causes degranulation and release of inflammatory mediators such as histamine, prostaglandin (PGD_2) and the leukotrienes (LTC_4) which trigger bronchoconstriction in the asthmatic lung (Metcalf et al. 1997; Matsuoka et al. 2000). As a major primary source of IL-4 in the lungs, mast cells and tissue basophils may also play an important role in priming Th2 responses in the airways (Nouri et al. 2001). A number of cytokines (TNF-γ, IL-1, IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-6, and IL-8) and chemokines (MCP-1, macrophage inflammatory protein 1α [MIP-1α], MIP-1β, and eotaxin), expressed by mast cells are important for the recruitment and activation of effector cells, including eosinophilis, which are involved in the late phase response (Das et al. 1998). Nevertheless, studies in mast cell-deficient $Kit^{w}/Kit^{w\text{-}v}$ mice indicate that many features of the late phase response, including eosinophilia and AHR, can occur in the absence of mast cells, suggesting that there is considerable redundancy in the mechanisms contributing toward the allergic response (Takeda et al. 1997). Studies in animal models of asthma indicate that, at low concentrations of allergen exposure, mast cells play a prominent role in the induction of IgE-mediated AHR (Kobayashi et al. 2000). Binding of IgE to the FcεRI on mast cells induces the upregulation of FcεRI, thereby enhancing IgE-dependent release of inflammatory mediators even at low levels of allergen exposure (Yamaguchi et al. 1999). Furthermore, binding of monomeric IgE to the FcεRI on mast cells inhibits apoptotic signals, thereby enhancing survival of mast cells in the airways and leading to further amplification of the asthmatic response (Asai et al. 2001).

The role of eosinophils in the induction of AHR

Eosinophils are considered to be the major effector cells in the pathogenesis of the late phase asthmatic response and consequently of AHR. Inflammatory mediators released from their granules, in particular the leukotrienes, can lead to damage of the airway epithelium resulting in AHR (Gleich et al. 1993). Some, but not all clinical studies (McFadden 1994) have observed a correlation between eosinophil levels in BAL and bronchial biopsies obtained from allergic patients, and disease severity as assessed by AHR (Wardlow et al. 1988). There are considerable clinical data that support the view that disease severity in asthma directly correlates with IL-5 and eosinophil levels (Bentley et al. 1993; Sur et al. 1995). The Th2-derived cytokine IL-5 is a key regulator of airway eosinophilia, acting as both a growth factor and a chemoattractant to induce differentiation, activation, recruitment, and survival at sites of allergen exposure (Lopez et al. 1988). Nevertheless, the role of IL-5-dependent airway eosinophilia in the induction of AHR has not been fully clarified. A recent clinical trial performed using a humanized McAb raised against IL-5 revealed that, despite attenuating eosinophil recruitment into the airways, there was no resolution of AHR in patients (Leckie et al. 2000).

Animal models of pulmonary inflammation have provided some insight into the role of eosinophils in the induction of AHR. Increased levels of eosinophils in the airways are associated with AHR (Tomkinson et al. 1999) and AHR can occur independently of the presence of both IL-5 and eosinophils (Corry et al. 1996). However, treatment of sensitized mice with anti-IL-5-neutralizing antibodies before allergen challenge of the airways, while having no effect on early acute phase responses, abolished late-phase inflammatory responses, with a loss of both airway inflammation and AHR (Foster et al. 1996; Hamelmann et al. 1999). STAT6 is essential for many IL-4- and IL-13-mediated responses which impact on the levels of AHR (Kaplan et al. 1996).

Therefore, the ability to induce not only airway eosinophilia, but also AHR on exposure of STAT6-deficient mice to allergen by reconstitution with IL-5 indicates that eosinophilis are sufficient to induce AHR in the absence of specific IgE, mucus production, or Th2 effector cells (Tomkinson et al. 1999). These discrepancies in the data may partially reflect genetic differences between the inbred strains of mice used, which could influence susceptibility to AHR (Wilder et al. 1999; Ewart et al. 2000).

Data obtained from animal models of disease have been interpreted on the assumption that IL-5 is principally responsible for airway eosinophilia. For this reason, studies investigating the role of eosinophils in the induction of AHR have focused primarily on the use of IL-5-deficient mice or IL-5-neutralizing antibodies and have indicated that neutralization of IL-5 is insufficient to abrogate airway eosinophilia. In addition to IL-5, the chemokine eotaxin acts as a major chemoattractant not only for eosinophils, but also for activated $CD4^+$ Th2 cells expressing the CCR3 chemokine receptor (Sallusto et al. 1997). CCR3 is the receptor for a number of chemokines associated with Th2-mediated airway inflammation in asthma, including eotaxin, and appears to be essential for the recruitment of both eosinophils and $CD4^+$ Th2 cells and in the induction of AHR (Lloyd et al. 2000; Ma et al. 2002). Analysis of bronchial biopsies from patients with asthma revealed that the intensity of eotaxin and CCR3 mRNA expression correlated with increased AHR (Ying et al. 1997b). Interestingly, the Th2-derived cytokine IL-13 can also upregulate eotaxin production by residual cells in the airways, including smooth muscle, fibroblasts, and airway epithelial cells, thereby contributing indirectly to tissue eosinophilia (Matsukura et al. 2001; Hirst et al. 2002; Wenzel et al. 2002). Binding of the very-late-activating antigen 4 (VLA-4), expressed on both eosinophils and T cells, to VCAM-1 expressed on the surface of endothelial cells is essential for cellular recruitment into the airways. Attenuation of both airway eosinophilia and AHR, by administration of neutralizing antibodies raised against IL-5 or VLA-4 to sensitized mice immediately before challenge of the airways with allergen, suggests a potentially independent role for $CD4^+$ Th2 effector cells and eosinophils in the induction of AHR (Tomkinson et al. 2001).

Although eosinophils undoubtedly play a prominent role in contributing toward bronchial hyperreactivity, there is considerable evidence that AHR can be induced in their absence. Th2 cells are not only responsible for initiating airway inflammation and eosinophilia but are also directly responsible for inducing AHR through production of cytokines, notably IL-13. This has been clearly demonstrated in STAT6-deficient mice in which the transfer of $CD4^+$ T cells from sensitized mice was sufficient to induce AHR on challenge of the airways with allergen, even in the absence of eosinophilia (Tomkinson et al. 2002). Nevertheless, it is likely that eosinophils contribute toward the induction of AHR, not only as effector cells through their ability to release inflammatory mediators, but also through the induction of Th2 responses. Studies by Mattes et al. (2002) have demonstrated that mice deficient in both IL-5 and eotaxin failed to develop airway eosinophilia and AHR on allergen exposure, and furthermore that $CD4^+$ Th2 cells isolated from these mice were impaired in their ability to produce IL-13, which is directly responsible for the induction of AHR. Transfer of eosinophils from wild-type mice was sufficient to re-establish IL-13 production by $CD4^+$ Th2 effector cells, supporting evidence that eosinophils, acting as antigen-presenting cells, have the potential to promote Th2-dependent responses in the asthmatic airways. Eosinophils isolated from the airways of patients with allergic asthma exhibited elevated levels of MHC class II and the costimulatory molecules CD80 and CD86 (MacKenzie et al. 2001). Furthermore, on exposure to allergen, eosinophils migrate from the airways to the T-cell-rich paracortical zones of draining lymph nodes, where antigen presentation takes place (Shi et al. 2000). It is likely that multiple factors acting either independently or in concert contribute toward AHR. $CD4^+$ Th2 cells are central for the induction of airway inflammation and eosinophilia, and it is therefore likely that they contribute, through the cytokines that they release, both directly and indirectly to AHR. As a major source of both IL-5 and IL-13 (Schmid-Grendelmeier et al. 2002), eosinophils may play a role in amplifying the inflammatory response, leading to chronic airway inflammation, enhanced bronchoconstriction, and AHR.

Antigen-presenting cells in the mucosal surfaces of the airways and their role in the induction of asthma

Dendritic cells are professional antigen-presenting cells that form a dense network in the airways lining the nasal and bronchial mucosa and lung interstitium (Holt et al. 1989). These cells are, therefore, ideally placed to sample antigen, which is then processed and presented to naïve T cells, following migration of DCs to the lymph nodes draining the airways (Vermaelen et al. 2001). Studies in animal models demonstrate that DCs play a critical role not only in the induction of $CD4^+$ Th2 cell-mediated responses and IgE synthesis, but also in maintaining late phase responses in sensitized animals, including airway eosinophilia and goblet cell hyperplasia (Lambrecht et al. 1988). Studies in patients with allergic asthma showed that $CD11c^+$ $HLA\text{-}DR^+$ DCs are rapidly recruited to the airways within hours of allergen exposure (Jahnsen et al. 2001). Independent studies have reported that the number of circulating $CD33^+$ DCs in the peripheral blood of patients with

asthma is reduced after allergen exposure (Upham et al. 2002). Evidence for expression of the receptor for the chemokine eotaxin, CCR3, by $CD34^+$ human DCs of the myeloid lineage suggests that these cells are recruited into the airways of people with asthma after upregulation of eotaxin, where they may play a role in maintaining Th2-mediated inflammatory responses (Beaulieu et al. 2002). Recent studies demonstrated that $CD11c^+$ $CD11b^+$ myeloid DCs in the airways are capable of retaining antigen for prolonged periods of time and, furthermore, that these cells are primarily responsible for presenting antigen to effector T cells in the lung (Julia et al. 2002). The presence of specific IgE bound to the high-affinity FcεRI on DCs in the periphery and the bronchial mucosa could potentially lower the activation threshold, thereby increasing responsiveness at low levels of allergen exposure (Tunon de Lara et al. 1996; Holloway et al. 2001). These data suggest that DCs are essential not only for initiating the T-cell-mediated, late phase response to allergens, but also for maintaining low levels of chronic inflammation, which could ultimately lead to airway remodeling.

The airway mucosa is continually exposed to common nonpathogenic environmental antigens. It is, therefore, likely that mechanisms have evolved for maintaining homeostasis, thereby preventing the induction of inappropriate inflammatory responses in the airways. The dramatic increase in the incidence of atopic asthma in the developed world, which has coincided with an increase in auotoimmune diseases, may reflect a loss of inherent immunoregulatory mechanisms. IL-10 is an immunoregulatory cytokine that is produced by a number of cell types and has a wide range of immunosuppressive effects including inhibition of DC maturation and macrophage activation, downregulation of leukocyte adhesion molecule expression, and suppression of T-cell proliferation and Th1 cytokine production (Moore et al. 2001). IL-10 has recently been described as a growth factor for a subset of $CD4^+$ $CD25^+$ T-regulatory cells, which are thought to be central to the induction and maintenance of immunological tolerance to selfantigens, thereby preventing the onset of autoimmune disease (Groux et al. 1997).

Dendritic cells in the airways, through their role in sampling antigen from the environment and their unique ability to prime naïve T-cell responses, may play a determining role in the induction of tolerance and immune regulation on exposure to allergens. In the absence of infection or inflammatory stimuli, DCs retain an immature phenotype. The presentation of antigen to naïve T cells by immature DCs would hypothetically result in T-cell-receptor ligation in the absence of costimulation, leading to the induction of a state of tolerance (Mueller et al. 1989). A direct role for pulmonary DCs in the induction of mucosal tolerance to inhaled antigen has been demonstrated in vivo. DCs isolated from the bronchial lymph nodes draining the lungs of mice exposed via the nasal mucosa to soluble antigen, in the absence of inflammatory stimuli, were able to adoptively transfer a state of antigen-specific tolerance to naïve mice. By repeating these studies in IL-10 knock-out mice, the authors were able to demonstrate that the expression of a tolerogenic pheonotype by DCs was dependent on the presence of IL-10. Furthermore, they found that, by culturing antigen-specific $CD4^+$ T cells with these IL-10-producing pulmonary DCs, they were able to induce a $CD4^+$ T regulatory cell-like phenotype as associated with IL-10 production (Akbari et al. 2001).

In the presence of pathogen-derived inflammatory stimuli, DCs undergo maturation and upregulation of the chemokine receptor CCR7, which facilitates migration to lymph nodes where DCs, which have acquired potent antigen-presenting capacity afer upregulation of MHCII molecules and the costimulatory molecules CD80 and CD86, prime T-cell responses. In vitro studies have demonstrated that autocrine IL-10 can suppress DC maturation in response to stimulation with lipopolysaccharide (LPS), or soluble CD40 ligand, thereby inducing a tolerogenic phenotype (Corinti et al. 2001; Steinbrink et al. 1997). Furthermore, the ability of IL-10 to inhibit upregulation of the chemokine receptor CCR7 on stimulated DCs could prevent DCs from migrating to the draining lymph nodes, where antigen presentation and T-cell priming would normally take place (D'Amico et al. 2000). Studies using labeled T cells demonstrated that primed effector T cells that had migrated to the airways, although being capable of producing cytokines and inducing eosinophilia, failed to proliferate on exposure to antigen (Harris et al. 2002). As effector $CD4^+$ T cells have lost their capacity to migrate out of peripheral tissues, continued priming of effector memory T-cell populations by DCs in the lymph nodes may be necessary in order to maintain a chronic asthmatic state. Production of IL-10 within the airways at the time of allergen challenge may play a role in limiting the inflammatory asthmatic response.

ASSOCIATION BETWEEN INNATE AND ADAPTIVE IMMUNITY IN THE INDUCTION OF AHR

Immunoregulatory mechanisms of γδ T cells in maintaining homeostasis in the airways

Intraepithelial γδ T cells, which are located in the mucosal surfaces of the airways, may be involved in maintaining homeostasis on exposure to inflammatory stimuli, thus protecting against pathological processes culminating in bronchoconstriction and AHR. Mice genetically deficient in γδ T cells (T-cell receptor (TCR)-$\delta^{-/-}$) exhibited elevated levels of AHR on exposure to methylcholine following sensitization and chal-

lenge with allergen. No increases in immunoglobulins, Th2-derived cytokines, or eosinophil levels were observed, indicating that γδ T cells exert their regulatory function through antigen-independent mechanisms, most probably involving a direct interaction with neighboring airway epithelial cells, thereby inhibiting the release of proinflammatory cytokines and chemokines (Lahn et al. 1999). By promoting clearance of necrotic tissue in the airways, γδ T cells may also play a role in limiting pulmonary injury (King et al. 1999).

Complement factors

Epidemiological studies indicate an association between exposure to particulate matter in air pollutants and exacerbation of bronchoconstriction, leading to increased AHR (Dockery and Pope 1994). This view is supported by data obtained from animal studies, demonstrating an increase in AHR on exposure to particulate matter in diesel fumes (Ohta et al. 1999). Activation of the complement pathway, resulting in cleavage of complement factors C3 and C5 and release of the anaphylotoxins C3a and C5a, is an integral part of the innate immune response to mucosal infection. Their receptors, C5aR and C3aR, are constitutively expressed on bronchial epithelial cells, AECs, smooth muscle cells, and vascular endothelial cells (Drouin et al. 2001). Many of the features of asthma, including smooth muscle contraction, mucus secretion, increased vascular permeability, as well as the recruitment and activation of inflammatory cells, can be mediated by the actions of these anaphylotoxins. C3a is a chemoattractant and activation factor for mast cells and eosinophils. Patients with allergic asthma exhibit increased levels of C3 in the BAL fluid and upregulated expression of C3aR on airway smooth muscle cells shortly after exposure to allergen. Furthermore, mice deficient in C3 or the C3aR fail to develop AHR on sensitization and exposure to allergen, despite exhibiting Th2-mediated airway eosinophilia and increased IgE production (Humbles et al. 2000). Exposure to particulate matter was sufficient to induce AHR in wild-type, but not C3-deficient, mice (Walters et al. 2002). Increases in air pollution may, therefore, account for the rise in both extrinsic and intrinsic asthma, assuming that the complement pathway acts as an antigen-independent link between the innate and adaptive immune response. Interestingly, the gene-encoding complement factor 5 (C5) has been identified as a susceptibility locus for allergen-induced AHR in mice. Studies suggest that C5-mediated responses may protect against the development of Th2-dependent increases in AHR. Blockage of C5a receptor-mediated signaling using a C5aR antagonist inhibited the production of the Th1-promoting cytokine IL-12 by human monocytes stimulated with *Staphylococcus aureus* and furthermore reversed IFN-γ-mediated suppression of IL-10 production (Karp et al. 2000).

IMMUNOREGULATORY NETWORKS

The immunological basis for the hygiene hypothesis

There is increasing evidence that the development of allergic asthma after sensitization to allergen is a consequence of the interaction between environmental factors and the genetic background of that individual. The prevalence of asthma has increased most markedly in the developed world. Although increased pollution and exposure to indoor allergens were originally proposed to account for this increase, epidemiological studies found that asthma prevalence was lower in former East Germany, in spite of high levels of pollution, than in the cleaner more affluent former West Germany (Nicolai and von Mutius 1997). In contrast, epidemiological and immunological data appear to support the 'hygiene hypothesis', which proposes that the increase in the prevalence of asthma is the result of a reduction in childhood infections. The study performed by von Mutius is of particular interest, because it draws attention to the possible role of the bacterial product endotoxin, a LPS found in the outer wall of gram-negative bacteria, in shaping the immune response. This study demonstrated that children raised in rural areas and heavily exposed to farm animals were less likely to develop allergy and asthma than children who had grown up in the same environment but were not exposed to farm animals. Natural exposure to environmental endotoxins in the stables appeared to protect children from developing allergies (Von Mutius et al. 2000). Epidemiological studies provide some support for this hypothesis. An inverse correlation was observed between positive DTH responses to tuberculin and the development of atopy (Shirakawa et al. 1997). Separate studies indicate that early immunization with mycobacteria BCG protects against the development of allergic disease (Aaby et al. 2000).

Studies in animal models of allergic asthma have demonstrated that pretreatment with the BCG before sensitization protects against the subsequent induction of Th2-mediated IgE production, airway eosinophilia, and increased AHR. Protection correlated with increased antigen-specific Th1 immunity and IFN-γ production (Erb et al. 1998; Hertz et al. 1998). Furthermore, administration of mycobacterial antigens to previously sensitized animals attenuated the late phase inflammatory responses and AHR, although IgE levels in serum and early acute phase reactions were not affected (Hopfenspirger and Agrawal 2002). The protective effect of mycobacteria is thought to arise through the induction of IL-12 and IFN-γ resulting in enhanced Th1 immunity,

which counterbalances the Th2 response to allergens. Mucosal gene transfer of IFN-γ directly into the airways of sensitized mice significantly inhibits the induction of Th2-mediated airway eosinophilia and AHR on exposure to allergen (Li et al. 1996). Nevertheless, data obtained from a number of independent studies indicates that IFN-γ-dependent Th1 responses may not only fail to counterbalance Th2-mediated immunity, but may also actually exacerbate the ongoing inflammatory response (Hansen et al. 1999).

Mycobacteria may protect against the induction of the Th2-mediated inflammatory response to allergens, by initiating a number of immunoregulatory mechanisms. Mycobacteria are known to stimulate the innate immune response, leading to enhanced levels of IL-12 and IFN-γ production by antigen-presenting cells including DCs, macrophages, and natural killer (NK) cells. Mycobacterially derived heat shock proteins have been shown to protect against the induction of allergen-induced AHR by triggering increased production of both IL-10 and the Th1-derived cytokine, IFN-γ. Recent studies indicate that this protective effect is dependent on γδ T cells, possibly activated through antigen-independent mechanisms such as increases in TNF-α production by DCs and macrophages, after uptake of mycobacteria. γδ T cells may exert their immunoregulatory effects by acting directly on airway epithelial cells to inhibit the release of factors that, either directly or indirectly through stimulation of Th2-mediated inflammatory responses, result in AHR (Kanehiro et al. 2001; Rha et al. 2002).

Lipopolysaccharide/endotoxin, a major constituent of bacterial cells walls, also appears to downregulate Th2 responses by stimulating production of the immunosuppressive and anti-inflammatory cytokine IL-10. Studies in rodent models have shown that exposure to LPS either before or during allergen provocation can protect against the induction of Th2-mediated airway inflammatory responses and eosinophilia, but failed to protect against the development of AHR (Gerhold et al. 2002). Furthermore, once airway inflammation has been established, exposure to LPS exacerbates the inflammatory response (Tulic et al. 2000).

LPS also induces secretion of IL-10 by a number of cell types, including DCs, which are critical for priming T-cell responses to allergens. This has led to speculation that LPS may protect against the development of asthma by the induction of immunoregulatory mechanisms rather than, or in addition to, protecting against Th2 immunity. A number of pathogens appear to trigger IL-10 production. Epidemiological studies have identified a disassociation between helminth infection and allergic disease. The induction of IL-10 production by parasites was associated with reduced skin test reactivity to aeroallergens (van den Biggelaar et al. 2000). This has led to the hypothesis that the immune system may have evolved mechanisms, such as the production of IL-10, for limiting the extent of the inflammatory response to microbial pathogens. The decrease in exposure to parasites or infectious agents, which has occurred in the western world over the last few decades, may account for a reduction in inherent immunoregulatory mechanisms. This may also explain the increasing incidence of autoimmune diseases, which have paralleled the rise in incidents of allergic asthma. Notably, animal studies have demonstrated that IL-10 knock-out mice are highly susceptible to both Th1- and Th2-mediated inflammatory diseases (Hoffmann et al. 2000). Furthermore, mucosal tolerance, which is thought to depend on IL-10 production, cannot be readily induced in animals kept in germ-free conditions, suggesting a minimal requirement for nonpathogenic gut microflora (Sudo et al. 1997). Epidemiological studies have found a significant correlation between the use of antibiotics during the first year of life and the subsequent development of allergic diseases (Wickens et al. 1999). The frequent use of antibiotics may have resulted in a reduction in the gut microflora.

The potential importance of IL-10 in regulating the asthmatic phenotype was revealed in comparative studies, which showed a marked reduction in IL-10 protein levels in BAL fluid of patients with allergic asthma and in cultures of peripheral blood cells on stimulation with LPS, suggestive of an inherent defect in IL-10 production (Borish et al. 1996). Nevertheless, it is highly likely that, in addition to IL-10, a number of other factors, including TGF-β and the activity of $CD4^+CD25^+$ regulatory T cells, contribute to the regulation of immunopathological processes that are involved in bronchoconstriction and AHR. Furthermore IL-10, although exhibiting protective properties during the induction and effector phase of asthma, can also under certain circumstances contribute toward increased AHR.

Immunoregulatory and pathogenic roles of IL-10 in AHR

The role of IL-10 in the development of AHR remains disputed. Evidence for an association between elevated IgE levels and polymorphisms in the promoter region of the IL-10 gene (Hobbs et al. 1998) is backed by clinical studies that have revealed reduced levels of IL-10 transcripts and protein levels in BAL fluid obtained from patients with allergic asthma (Borish et al. 1996). Furthermore, successful standard immunotherapy to insect venom, as assessed by reductions in clinical symptoms on allergen exposure, correlated with an increase in IL-10 production by allergen-reactive T cells in the periphery (Akdis et al. 1998). Animal models have been used in an attempt to evaluate the role of IL-10 in the pathogenesis of asthma and AHR. These studies suggest that, although generally considered as a regulatory and anti-inflammatory cytokine, IL-10 can also contribute toward the pathophysiology of AHR in asthma. Zuany-

Amorim demonstrated that administration of IL-10 into the airways of mice at the time of antigen challenge, but not later, caused a marked reduction in IL-5 production and airway eosinophilia, suggesting that IL-10 may inhibit localized priming of Th2 responses (Zuany-Amorim et al. 1995; Yang et al. 2000). A role for endogenous IL-10 in regulating T-cell-mediated inflammatory responses was confirmed using IL-10 gene knock-out mice (Grunig et al. 1997). An independent study revealed that, in spite of developing pronounced airway inflammation associated with increased levels of the Th2 cytokines IL-5 and IL-13 in BAL, eosinophilia, and mucus production, IL-10-deficient mice failed to develop AHR. Transfer of the IL-10 gene before allergen challenge of the airways was sufficient to reconstitute AHR in sensitized mice, indicating that IL-10 is an important cofactor for induction of AHR (Makela et al. 2000; Justice et al. 2001). However, in the absence of prior sensitization and challenge of the airways with allergen, IL-10 alone is insufficient to induce AHR (Van Scott et al. 2000). In view of the various studies performed to date, it is likely that the role played by IL-10 in the induction of AHR is indirect and not unique. Studies have shown that AHR could be induced after respiratory syncytial virus (RSV) infection of IL-10-deficient mice exhibiting allergen-induced airway inflammation. Furthermore, reductions in airway eosinophilia following treatment of RSV-infected mice with anti-IL-5 neutralizing antibody significantly reduced AHR following allergen exposure, indicating a potential role for eosinophil-derived factors in AHR (Makela et al. 2002). Previous studies indicated that the Th2 cytokines, IL-5 and IL-13, contribute directly toward AHR, suggesting that factors released during airway inflammation are also required for induction of AHR (Foster et al. 1996; Garlisi et al. 1997; Grunig et al. 1998; Hogan et al. 1998; Wills-Karp et al. 1998). Recent studies have shown that eosinophils derived from patients with asthma express IL-13, and furthermore that IL-13. Furthermore, following stimulation with IL-5, IL-13 can be induced in eosophinols obtained from non-asthmatic controls. These data suggest that in an allergic Th2 cytokine-driven asthmatic response, eosinophils, by acting as a source of IL-13, may contribute indirectly towards the induction of AHR (Schmid-Grendelmeier et al. 2002). Most probably elevated levels of IL-13, arising as a result of IL-10-mediated suppression of Th1 cytokine responses, may be a compounding factor in the onset of AHR, because many of the factors known to contribute toward AHR, including mucus metaplasia, are IL-13 dependent.

Immunoregulatory role of IL-12

Allergic asthma and AHR is associated with skewed Th2 cytokine production. It has been proposed that the balance between IL-12-induced Th1 responses and levels of Th2 cytokines, notably IL-4 and IL-13 in the airways, or responsiveness to these cytokines could influence development of an allergic asthmatic phenotype. Interestingly, the genes encoding the Th2 cytokines (IL-4, IL-5, IL-9, and IL-13), as well as the p40 subunit of IL-12, are located within chromosome 5q (Marsh et al. 1994). Identification of rare polymorphisms in the promoter region of the IL-12 subunit genes (Pravica et al. 2000) and their association with disease severity (Morahan et al. 2002) has led to speculation that a failure to suppress aberrant Th2-mediated responses to common aeroallergens in genetically predisposed individuals results in the development of allergic diseases. IL-12, produced by antigen-presenting cells including DCs and macrophages after stimulation with microbial pathogens, is critical for the development of a Th1 phenotype (Trinchieri 1994). Signaling through the IL-12 $R\beta_2$ subunit is required for Th1 differentiation after STAT4 nuclear translocation and IFN-γ gene transcription. Notably, cells expressing a Th2 phenotype have lost IL-$12R\beta_2$ expression and therefore their ability to respond to IL-12 (Szabo et al. 1995).

There is increasing evidence from clinical studies that a deregulated IL-12 response may contribute toward the development of an asthmatic phenotype. Bronchial biopsy specimens taken from patients with allergic asthma were found to contain lower levels of IL-12 p40 mRNA than healthy controls (Naseer et al. 1997). The immunoregulatory cytokines TGF-β and IL-10 as well as a number of factors associated with the allergic inflammatory response, in particular IL-4, IL-13, and the inflammatory mediators PGE_2 and histamine, can down-regulate IL-12 production, thereby further amplifying the allergic response (Van der Pouw Kraan et al. 1995; Meyaard et al. 1996; Elenkov et al. 1998). An increase in IL-12 and decrease in IL-13 levels after steroid treatment were observed only in those patients who responded to therapy. Failure to respond to steroid treatment may be linked to reduced levels of IL-$12R\beta_2$ chain expression (Matsui et al. 1999; Wright et al. 1999).

Studies in murine models of allergic disease have demonstrated the importance of IL-12 in regulating pathogenic Th2 responses. IL-12 appears to play a critical role in protecting against the initial induction of allergic responses. Blockage of endogenous IL-12 in the unresponsive C3H/HeJ strain resulted in development of Th2-mediated responses on allergen exposure with elevated serum IgE levels, eosinophilic infiltration of the airways, and enhanced AHR (Keane-Myers et al. 1998). IL-12 knock-out mice showed enhanced levels of eosinophils in bone marrow and BAL following antigen challenge, compared with wild-type controls (Zhao et al. 2000). Administration of IL-12 during either sensitization or allergen challenge markedly reduced AHR (Kips et al. 1996; Sur et al. 1996; Schwarze et al. 1998). Suppression of Th2-mediated airway responses was asso-

ciated with a Th1 shift in cytokine responses. Nevertheless, studies in IFN-γR knock-out mice revealed that, although the initial inhibition of IgE production by IL-12 was IFN-γ dependent, suppression of airway eosinophilia and AHR was only partially dependent on IFN-γ, suggesting that other mechanisms are involved in mediating late phase inflammatory responses and AHR (Brusselle et al. 1997). A clinical study demonstrated that subcutaneous administration of recombinant IL-12 had no effect on the late phase airway hyperresponsiveness to allergen challenge, despite a significant reduction in eosinophil numbers and an improvement in the immediate acute hypersensitivity response (Bryan et al. 2000).

CONCLUSIONS

Asthma is a disease of the airways associated with pulmonary inflammation, eosinophilia, mucus hypersecretion, and increased AHR. Although both polygenic and multifactoral in origin, the recent rise in prevalence of asthma in the developed world has increased interest in the immunological mechanisms underlying the pathophysiology of disease. Asthma is associated with recruitment of activated $CD4^+$ Th2 cells producing IL-4, IL-5, IL-9, and IL-13. These cytokines exhibit considerable functional redundancy and only recently, through the use of animal models, has it been possible to establish their role in the asthmatic response. Whereas IL-4 is essential for the induction of Th2 responses, IL-13 appears to be essential for the late phase response associated with AHR. Through the induction of chemokines, IL-13 mediates the recruitment and activation of $CD4^+$ Th2 lymphocytes and eosinophils involved in airway inflammation. In addition, IL-13, through its direct effects on resident airway epithelial cells, fibroblasts, and smooth muscle cells, mediates mucus hypersecretion, tissue fibrosis, and AHR.

The correlation between the rise in incidence of asthma and reduced exposure to bacterial and parasitic infections, which has arisen as a result of improved living standards, has led to speculation that anti-inflammatory responses induced by these agents may protect against the development of aberrant Th2 immune responses to environmental allergens. Immunoregulatory mechanisms, which include IL-10 production by DCs, are also involved in mucosal tolerance induction on exposure of the airways to allergen.

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Autoimmunity

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DEFINING AUTOIMMUNITY AND AUTOIMMUNE DISEASE

Autoimmunity is defined as activation of an immune response against self-tissue (Ermann and Fathman 2001). A degree of autoimmunity, or self-reactivity, is a phenomenon that occurs even in healthy individuals. Alternatively, autoimmune disease is an 'attack', which may afflict patients with conditions that are debilitating, even life threatening. Autoimmune disease is marked by the presence of pathogenic antibodies and/or lymphocytes that target self-antigens and mediate tissue destruction. A multitude of factors influence one's susceptibility to autoimmune disease, including host genetics, environmental factors, and immune disregulation (Ermann and Fathman 2001) (Figure 1).

The subject of autoimmune disease encompasses a broad spectrum of illnesses of ambiguous etiology and pathogenesis. Despite the uncertainty underscoring any discussion of autoimmune disease, unambiguous evidence demonstrates that self-tolerance has been corrupted. The main function of the immune system is to protect self from nonself. For this purpose, it must operate by its ability to discriminate between what is self and what is not, a paradigm first described by Burnet in

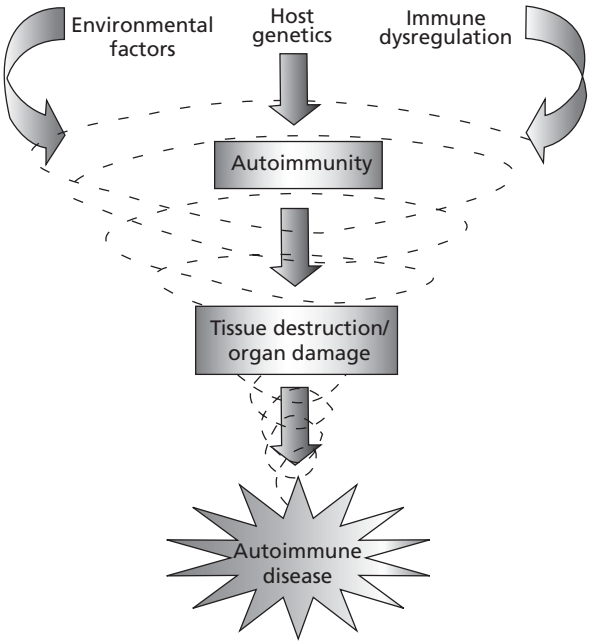

Figure 1 *Etiology of autoimmune disease: with several factors underlying its etiology, the development of an autoimmune response is a downward spiral, resulting in tissue destruction, organ damage, and autoimmune disease.*

1959. 'Survival requires both the ability to mount a destructive immune response against non-self and the inability to mount a destructive response against self' (Huston 1997). With a break in self-tolerance, the immune system proceeds to attack itself with the same vigor as it would to eliminate an infectious organism. Ultimately, the dangerous cascade of an autoimmune disease may compromise survival.

Autoimmune diseases affect approximately 5 percent of the global population (Jacobson et al. 1997). In the USA, roughly 14 million people are afflicted with various autoimmune diseases. Women are disproportionately affected, because they represent 75 percent of autoimmune disease patients. To date, over 80 distinct types of autoimmune diseases have been identified. Diseases may be categorized into one of two groups: organ-specific or systemic autoimmune disease (Table 1). In organ-specific autoimmune diseases, including Graves' disease (Weetman 2001) and type 1 diabetes (Wucherpfennig and Eisenbarth 2001), the targeted self-antigen is expressed only in the affected organ, whereas, in systemic autoimmune diseases, the self-antigen is expressed throughout the host eliciting tissue damage systemically. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are two systemic autoimmune diseases with a high prevalence in the USA (Jacobson et al. 1997).

THE DISCOVERY AND REDISCOVERY OF AUTOIMMUNITY

The study of autoimmunity has a controversial, albeit intriguing, history (Silverstein 2001). In 1901, Paul Ehrlich, famed immunologist, adamantly denounced the existence of autoimmune disease or what he dubbed as *horror autotoxicus*. Ehrlich declared that it would be against the design of nature for an organism to jeopardize itself by producing pathogenic autoantibodies. Shortly thereafter, investigators Julius Donath and Karl Landsteiner published findings regarding the pathogenesis of paroxysmal cold hemoglobinuria (PKH). PKH is an unusual disease whereby patients develop acute intravascular red blood cell destruction and subsequent hemoglobinuria following exposure to the cold. In 1904, they proposed that this rare disease is autoimmune in origin, citing their demonstration of a pathogenic autoantibody that reacts with its target cell in the cold. Suffice it to say, Ehrlich and his colleagues disputed these findings and maintained their stance that autoimmune disease did not exist, thereby influencing generations of immunologists to come.

The concept of autoimmunity as a disease model reappeared in the 1950s. Ernest Witebsky published a report demonstrating the induction of autoimmunity in an animal model of chronic thyroiditis (Witebsky and Rose 1956). Indeed, Witebsky was an unlikely candidate to reintroduce the concept of autoimmunity, because he was a faithful disciple of Ehrlich and his campaign to refute autoimmunity. Despite the initial resistance of Ehrlich, Witebsky, and other investigators to acknowledge the existence of autoimmunity, today it is recognized as a major societal burden. Intensive investigative efforts are focused on uncovering the etiology, mechanisms, and treatments for many autoimmune diseases.

MECHANISMS OF TOLERANCE

The ability of the immune system to protect the body against challenge from innumerable exogenous pathogens is conferred in part by a diverse lymphocyte repertoire. In fact, the total diversity of the αβ T-cell receptor (TCR) and B-cell immunoglobulin (Ig) repertoires is estimated at 10^{18} and 10^{14}, respectively (Janeway et al. 2001). This diverse repertoire supports B- and T-cell recognition of a broad range of antigens. The tremendous degree of diversity is further enhanced by the 'plasticity' or cross-reactivity of the receptors (Mason 1998). This flexibility has been attributed to conformational changes in the TCR that occur during engagement of the major histocompatiblity complex (MHC) (Garcia et al. 1998). Given the capacity to recognize countless

Table 1 *Prevalence of autoimmune diseases: top ten autoimmune diseases in order of decreasing prevalence*

Disease	Prevalence (%)	Category	Target
Graves' disease	1.1	Organ specific	Thyroid
Rheumatoid arthritis	0.9	Systemic	Joints, lungs, heart, etc.
Hashimoto's thyroiditis	0.8	Organ specific	Thyroid
Vitiligo	0.4	Organ specific	Melanocytes
Type 1 diabetes	0.2	Organ specific	Pancreatic β cells
Pernicious anemia	0.15	Organ specific	Gastric mucosa
Multiple sclerosis	<0.1	Organ specific	Brain (spinal cord)
Glomerulonephritis	<0.1	Organ specific	Kidney
Systemic lupus	<0.1	Systemic	Skin, joints, kidney, brain, blood, lungs, etc.
Sjögren's syndrome	<0.1	Organ specific	Lacrimal and salivary glands

(Jacobson et al. 1997)

antigens, it is not surprising that lymphocytes may inadvertently recognize self-antigens and mount an autoimmune response.

Two criteria underlie the mechanisms by which the immune system protects the body against autoimmunity: self-recognition and self-tolerance. At the initiation of an immune response, self-recognition, or the ability to discriminate between what is self and what is nonself or foreign, is intrinsic to the goal of targeting only foreign antigens. As an immune response is maintained, self-tolerance, or the ability to silence self-reactivity, is an essential component for halting autoimmunity. One of the most effective mechanisms to ensure both self-recognition and self-tolerance is positive and negative selection during T-cell maturation. This occurs centrally in the thymus from the perinatal period until puberty. Later in development, other critical mechanisms of tolerance induction occur in the periphery, including the suppression or elimination of autoreactive lymphocytes.

Establishing self-tolerance in the central lymphoid organs

The first and most effective route for eliminating autoreactive lymphocytes occurs during central tolerance induction in the bone marrow and thymus. After the rearrangement and expression of Ig and TCR genes, B and T cells express antigen-specific receptors. These receptors may have specificity for either self or nonself antigens. Although T cells with high avidity for self-determinants are clonally deleted in the thymus on encounter of self-antigens in association with self-MHC, self-reactive B cells in the bone marrow may escape deletion and modify their Ig by receptor editing (Tiegs et al. 1993).

Central tolerance is an insufficient route for eliminating the entire autoreactive T- and B-lymphocyte population. An analysis of multiple sclerosis (MS) patients demonstrated that autoreactive T cells specific for myelin basic protein (MBP) escape deletion during thymic selection (Ota et al. 1990). Upon activation in the periphery, perhaps during a viral infection, these cells may initiate the destruction of the central nervous system. Self-reactive lymphocytes may escape deletion for several reasons. In the thymus, some self-antigens may not be expressed at sufficient levels to mediate deletion or, perhaps, some self-antigens are not present at all.

Maintaining self-tolerance in the periphery

In the periphery, autoreactive T cells are held in check by tolerance mechanisms that may be characterized as T-cell intrinsic or T-cell extrinsic mechanisms (Walker and Abbas 2002). T-cell intrinsic mechanisms act directly on the T cell, whereas T-cell extrinsic mechanisms involve the tolerogenic functions of additional cells.

T-CELL INTRINSIC MECHANISMS OF PERIPHERAL TOLERANCE

Intrinsic mechanisms include the following: ignorance, anergy, phenotypic skewing, and apoptosis (Walker and Abbas 2002).

T-cell ignorance

This is the process by which autoreactive T cells (or B cells) are unresponsive to antigen. The T cells may ignore the self-antigen because either it binds the TCR with low affinity or it is present at a low concentration (Janeway et al. 2001). Kurts et al. (1999) experimentally documented this state by transferring ovalbumin-specific lymphocytes to mice expressing low levels of pancreatic ovalbumin. The T cells remained ignorant of the ovalbumin antigen at low doses, but were responsive to the antigen at high doses. Interestingly, tissue-specific antigens, such as insulin in the pancreas, are expressed at high concentrations at specific sites. These antigens often have a higher association with autoreactivity (Janeway et al. 2001).

Some T cells remain ignorant because they are never exposed to inaccessible self-antigens, e.g. intraocular proteins are sequestered in the eye, an immunologically privileged site. Unless these proteins are released, autoreactive T cells specific for these proteins are never activated.

T-cell anergy

The state of anergy occurs when lymphocytes do not respond to antigen stimulation. In vivo, T-cell inactivation resulting from anergy induction is dependent on the recognition of the B7 molecule by CTLA-4 (Perez et al. 1997). In mice, the significance of anergy induction is demonstrated by CTLA-4 deficiency that results in severe lymphoproliferation and early death (Waterhouse et al. 1995). The association between CTLA-4 and autoimmune diseases is discussed.

Phenotypic skewing

The induction of tolerance via phenotypic skewing is dependent on which cytokine and chemokine profile is elicited after T-cell activation by a self-antigen. A self-antigen alone may only be able to induce a T-helper type 2 (Th2) response, which is associated with dampening autoimmunity. A self-antigen in association with additional adjuvants, such as the case with bacterial lipopolysaccharide (LPS), may induce a shift towards a T-helper type 1 (Th1) response. The proinflammatory Th1 response is associated with perpetuating an autoimmune response.

Apoptosis

Apoptosis via activation-induced cell death (AICD) is a mechanism for eliminating self-reactive B and T cells in the periphery. The process of apoptotic cell death promotes an anti-inflammatory environment via two routes: (1) inhibition of proinflammatory cytokines by monocytes and macrophages and (2) inducing the release of immunosuppressive cytokines, including IL-10 and transforming growth factor β (TGF-β) (Savill et al. 2002).

Self-reactive, immature B cells expressing surface IgM are eliminated by apoptosis (Janeway et al. 2001). In the case of self-reactive T cells, Walker and Abbas (2002) postulate that the repetitive engagement of TCR by ubiquitous self-antigens may induce the upregulation of the cell-death Fas ligand, thereby initiating AICD. The significance of Fas-mediated apoptosis as an immunoregulatory mechanism to eliminate autoreactive lymphocytes is demonstrated in humans with autoimmune lymphoproliferative syndrome (ALPS). These patients have deleterious defects in the Fas pathway that result in expanded lymphocyte populations and compromised peripheral self-tolerance (Fisher et al. 1995).

T-CELL EXTRINSIC MECHANISMS OF PERIPHERAL TOLERANCE

Extrinsic tolerance mechanisms evoke the functions of dendritic cells (DC) and regulatory T cells (Walker and Abbas 2002). Although it is apparent that both DCs and regulatory T cells play a role in determining whether an immune response is enhanced or suppressed, the precise mechanism of action of these cell types remains to be determined.

Dendritic cells

The DC is a powerful antigen-presenting cell (APC) located in the skin, blood, and lymphatic network (Steinman and Nussenzweig 2002). Immature DCs capture and process antigens, whereas mature DCs, in the presence of infection, present these antigens in association with MHC to T cells (Yewdell and Tscharke 2002) (Figure 2). Immature DCs are associated with T-cell tolerance, whereas mature ones are associated with T-cell activation. As DC maturation plays a crucial role in initiating and modulating the immune response, it is subject to regulation, e.g. the maturation and presentation of antigen by DCs is inhibited upon the uptake of dying, apoptotic cells in an effort to maintain tolerance and suppress autoimmunity (Savill et al. 2002). In addition, the maturation of DCs is inhibited by the anti-inflammatory cytokine, interleukin (IL)-10 (Haase et al. 2002).

Dendritic cells also play a role in providing signals to distinguish between an appropriate antigen, as in the case of a pathogen, or an inappropriate antigen, such as a self-antigen. In 1992, Janeway postulated that DCs have pattern-recognition receptors that are specific for motifs associated with microorganisms. The engagement of these receptors triggers an immune response. This

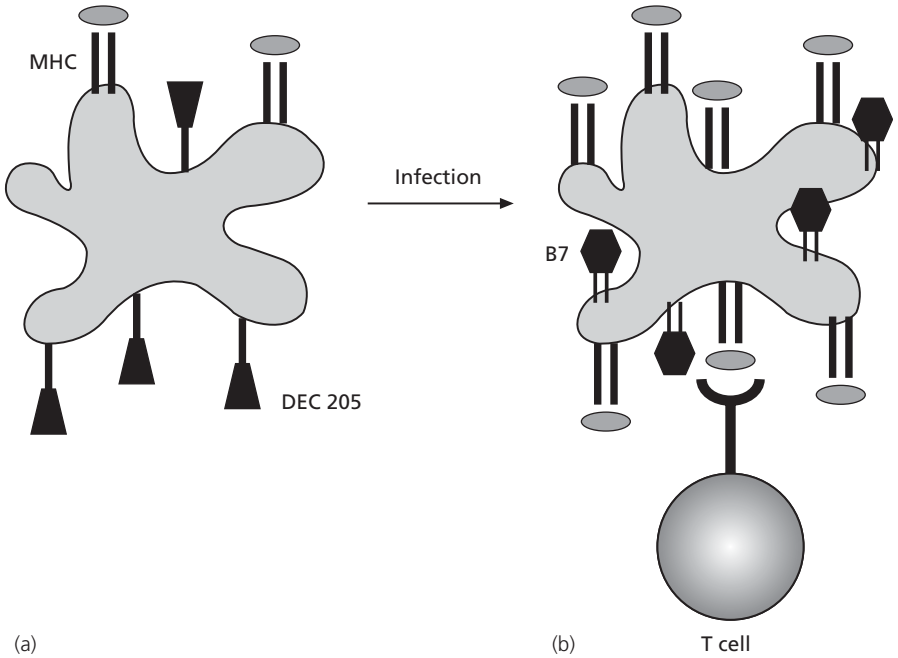

Figure 2 (a) *Immature and* **(b)** *mature dendritic cell phenotypes: immature dendritic cells are located in the surface epithelia and solid organs. They express low levels of major histocompatiblity complex (MHC) molecules, as well as the DEC 205 molecule, an antigen-phagocytosing receptor. During infection, immature dendritic cells migrate to lymphoid tissue. These mature dendritic cells increase expression of MHC and B7, a co-stimulatory molecule (Janeway et al. 2001).*

theory was substantiated in 1997 with the identification of Toll-like receptors (TLR) (Medzhitov et al. 1997). TLRs, on the recognition of molecular patterns present on microorganisms, induce an immune response that includes the expression of co-stimulatory molecules and the release of cytokines (Gordon 2002).

Regulatory T cells

Regulatory or suppressor T cells that co-express CD4 and CD25 (the interleukin IL-2 receptor α chain) represent less than 5 percent of the T cells in the blood. These 'sentinel' T cells have the capacity to monitor immune responses by suppressing both $CD4^+$ and $CD8^+$ T cells (Steinman and Nussenzweig 2002). The mechanism of suppression has not yet been elucidated, although it is associated with decreased levels of IL-2 transcription (Thornton and Shevach 1998). The critical role of regulatory T cells in tolerance is demonstrated in a mouse model where a reduced level of regulatory T cells is associated with autoimmune disease (Asano et al. 1996).

Regulatory cells are also subject to immunoregulation. IL-10, a suppressor cytokine, may trigger the generation of regulatory cells. In addition, the presence of immature DCs may generate regulatory T cells (Shevach 2002). DCs mature in the presence of TLR signaling; the absence of mature DCs may be a marker for the absence of an invading pathogen, favoring the suppression of a potential autoimmune response.

GENETICS AS A FACTOR IN AUTOIMMUNE DISEASE SUSCEPTIBILITY

A role for genetic predisposition in autoimmune disease development was suspected, given the propensity for familial aggregation of certain autoimmune diseases. The association was positively identified by a study of the disease concordance rates in monozygotic twins. The disease concordance rates for certain autoimmune diseases, such as type 1 diabetes and SLE, were quite high, 50 and 57 percent, respectively (Jarvinen et al. 1992; Kumar et al. 1993; Redondo et al. 1999). By comparison, the incidence for diabetes and SLE in the general population is significantly less, 0.4 and 0.2 percent, respectively, suggesting an underlying genetic component to disease development.

Today, at the forefront of the search for autoimmune disease genes, there are powerful tools, including linkage analysis, animal models, and gene chip microarrays (Wanstrat and Wakeland 2001). Despite these advantages, the search for genes affecting autoimmune disease is complicated by several factors, including genetic heterogeneity. Genetic heterogeneity, or the various combinations of genes that contribute to a certain phenotype, underlies the genetic predisposition to most autoimmune diseases (Wanstrat and Wakeland 2001). A clear characterization of the role of genetic predisposition in autoimmune disease development is further obscured by epistatic interactions between 'susceptibility genes' and 'resistance genes' (Ermann and Fathman 2001). Fortunately, completion of the Human and Murine Genome Projects offer valuable maps of the mammalian genome that will probably facilitate groundbreaking discoveries in the search for autoimmune disease genes.

The effects of MHC genes on autoimmune disease development

The most intensely studied genes associated with autoimmune disease development are the alleles of the MHC. These genes encode cell-surface molecules on APCs that bind peptides for presentation to antigen-specific T lymphocytes. Some hypothesize that the association between MHC and autoimmune disease susceptibility originates during T-cell development. During this period, the signals resulting from the TCR and MHC molecule–peptide complexes shape the thymic T-cell repertoire and may potentiate the escape of autoreactive T cells. Particular class II MHC molecules may select for autoreactive T cells by increasing positive selection or decreasing negative selection (Marrack et al. 2001), e.g. the MHC II molecule, $I\text{-}A^{g7}$, in the nonobese diabetic (NOD) mouse model, binds peptides very poorly (Carrasco-Marin et al. 1996). If self-peptides are inadequately presented by $I\text{-}A^{g7}$ during selection, self-reactive T cells will not be eliminated. Others speculate that the linkage between MHC and autoimmunity is a simple scenario whereby disease susceptibility is determined by the varying degree to which different MHC alleles present self-peptides to autoreactive T cells (Janeway et al. 2001). In the periphery, certain disease-associated MHC II molecules predispose to autoimmunity by presenting self-peptides to autoreactive $CD4^+$ T cells.

The MHC and autoimmune disease linkages are identified by comparing the frequency of certain alleles in patients to the frequency of those alleles in the normal population (Janeway et al. 2001). One of the earliest links between autoimmunity and MHC was noted in the 1970s (Feldmann 2001). HLA-DR (human leukocyte antigen DR) allele subtypes were associated with RA via a 'shared epitope', a six amino acid sequence occurring in the peptide-binding groove of the DR alleles of 70–80 percent of RA patients. Currently, the HLA-DR alleles, HLA-DRB1*0401 and -*0404, are recognized as the most significant contributors to genetic susceptibility to RA (Nepom 1998).

Multiple sclerosis is also associated with particular alleles of HLA-DR, specifically HLA-DR2, as well as alleles of HLA-DQ. However, a comprehensive linkage

Table 2 *Examples of some MHC/autoimmune disease associations*

Disease	Association	
	Class I MHC	Class II MHC
Reactive arthritis	HLA-B27	
Ankylosing spondylitis	HLA-B27	
Multiple sclerosis		HLA-DR2
Treatment-resistant Lyme arthritis		HLA-DR4, HLA-DR1
Rheumatoid arthritis		HLA-DR1, HLA-DR4
Type 1 diabetes		HLA-DQ8, HLA-DQ2

(Wucherpfennig, 2001)

analysis determined that disease development was probably the result of multiple environmental and genetic factors, as opposed to a single disease gene (Haines et al. 1996).

The HLA-DR and HLA-DQ alleles are also strongly associated with type 1 diabetes (Wucherpfennig and Eisenbarth 2001). Interestingly, the complexity arising from the interaction between 'disease-causing genes' and 'disease-modifying genes' is evident in a transgenic mouse model of type 1 diabetes. Mice transgenic for HLA-DQ8 develop spontaneous disease, but the frequency of disease is lower when the mice co-express HLA-DR4 and HLA-DQ8, suggesting a protective role for HLA-DR4 allele (Wucherpfennig and Eisenbarth 2001). In humans, the HLA-DR2–DQ6 haplotype, representing a linkage disequilibrium, is associated with dominant protection from type 1 diabetes (Nepom and Erlich 1991).

The vast majority of MHC and autoimmune disease associations are linked to class II molecules (Table 2), although there are exceptions. Ankylosing spondylitis and reactive arthritis are two examples; both diseases are strongly associated with the MHC I molecule, HLA-B27 (Levitin et al. 1976; Pasternack and Tiilikainen 1977).

The effects of non-MHC genes on autoimmune disease development

Genetic mapping studies have identified individual genes on various chromosomes associated with susceptibility towards certain autoimmune diseases. These non-MHC genes are involved in regulating the recognition, presentation, and concentration of autoantigens (Marrack et al. 2001). A polymorphism in the gene that encodes the CTLA-4 is linked to the development of type 1 diabetes, celiac disease, Graves' disease, and autoimmune hypothyroidism (Nistico et al. 1996; Djilali-Saiah et al. 1998). CTLA-4 is a surface T-cell molecule that negatively controls T-cell activation (Linsley et al. 1992). Another polymorphism in the gene encoding IL-2 is associated with NOD mice and experimental autoimmune encephalomyelitis (EAE) animal models (Encinas et al. 1999). IL-2 stimulates the proliferation of T lymphocytes *and* keeps lymphoid growth in check; this example underscores the significance of IL-2 in maintaining peripheral tolerance. A deficiency of C1q in certain strains of mice, as well as in humans, results in a lupus-like disease characterized by autoantibodies to double-stranded DNA or nuclear components (Slingsby et al. 1996; Botto et al. 1998). C1q, a component of the classic complement pathway, aids in the clearance of apoptotic cellular debris including potential nuclear autoantigens. Two mutations that encode transcription factors of unknown function have been implicated in type 1 diabetes, one on chromosome 21 and another on the X chromosome. The former, an autosomal recessive mutation, results in autoimmune polyendocrine syndrome type 1 (APS-1) (Nagamine et al. 1997), and the latter causes X-linked autoimmunity-allergic disregulation syndrome (XLAAD) (Chatila et al. 2000). Mice deficient in IL-10 develop chronic enterocolitis, a disease that is pathologically similar to inflammatory bowel disease (IBD) in humans (Kuhn et al. 1993). Clearly, many non-MHC genetic abnormalities have been associated with autoimmunity.

ENVIRONMENTAL FACTORS AND AUTOIMMUNE DISEASE

An individual's predisposition towards acquiring an autoimmune disease is influenced not only by genetic susceptibility, but also by the environment. In humans, incomplete autoimmune disease concordance rates in monozygotic twins suggest an underlying environmental component to autoimmune disease development. In identical twin pairs, less than 50 percent of the siblings of affected twins will acquire an autoimmune disease (Ermann and Fathman 2001). An intriguing epidemiological study of the incidence of MS in a migrant population revealed that migrants acquired the MS risk of the region to which they relocated (Weinshenker 1996). In animals, inbred strains susceptible to the spontaneous development of SLE and diabetes display incomplete disease penetrance (Wanstrat and Wakeland 2001). In NOD mice, the incidence of diabetes drops dramatically with bacterial infection (Todd 1991).

The association between infectious agents and autoimmune disease has been the subject of intense investigation over the course of the last two decades. Even since its earliest description, autoimmune disease has been linked with infection. In the aforementioned research on PKH, the collaborators Donath and Landsteiner were struck by the correlation between PKH and a pre-existing syphilis infection (Silverstein 2001). To date, contemporary researchers have added considerably to the number of autoimmune diseases linked with pathogens (Table 3). The classic examples include: β-hemolytic streptococci and rheumatic fever (Benoist and Mathis 1998); *Trypanosoma cruzi* and Chagas' disease

Table 3 *Pathogens and autoimmunity: some common examples of autoimmune diseases linked with pathogens*

Disease	Pathogen	Host self-antigen
Rheumatic fever	Group A streptococci	Cardiac myosin
Guillain–Barré syndrome	*Campylobacter jejuni*	Peripheral nerve ganglioside proteins
Treatment-resistant Lyme arthritis	*Borrelia burgdorferi*	LFA-1
Myocarditis	Group B Coxsackie virus	Cardiac antigens
Chagas' disease	*Trypanosoma cruzi*	Cardiac myosin heavy chain
Herpetic stromal keratitis	Herpes simplex virus	Cornal antigen and IgG2a
Type 1 diabetes	Group B Coxsackie virus	Glutamate decarboxylase
Myasthenia gravis	Herpes simplex virus	Acetylcholine receptor
Multiple sclerosis	Epstein Barr virus	Myelin basic protein

Benoist and Mathis (2001), Regner and Lambert (2001), Marrack et al. (2001).

(Khoury and Fields 1980); and *Borrelia burgdorferi* and Lyme arthritis (Gross et al. 1998). Animal models in which microbial infection directly triggers certain autoimmune diseases further support this link between infection and autoimmunity. In one such model, particular strains of mice develop an autoimmune response against a corneal antigen after ocular infection with herpes simplex virus 1 (Panoutsakopoulou et al. 2001). The mice develop herpetic stromal keratitis (HSK), a leading cause of blindness in humans (Streilein 1997).

Despite the pathogen and autoimmune disease correlations in both humans and mice, the exact mechanisms whereby an infection leads to autoimmune disease have not yet been resolved. In fact, it remains unclear as to whether the pathogen directly or indirectly contributes to autoimmune disease development. Possibly, cross-reactive immune responses against the pathogen and self-molecules closely resembling the pathogen produce disease. Or, perhaps, the inflammation provoked by the pathogen at the site of infection causes a disregulated immune response that disturbs self-tolerance. The etiology of an autoimmune disease triggered by infection may be multifactorial, implicating several mechanisms in pathogenesis that may be relevant at different disease stages (Wucherpfennig 2001).

Mechanisms for pathogen-induced autoimmunity

Several mechanisms have been proposed to account for the post-infectious activation of the immune response against self-targets (Wucherpfennig 2001). Mechanisms may be categorized into two groups: antigen-specific and antigen nonspecific. In the first group, the pathogen directly induces autoimmunity through an antigen-specific response involving a microbial product; molecular mimicry is a well-characterized example. In molecular mimicry, the immune response to a microbial antigen cross-reacts with a host antigen. In the second group, the response is antigen nonspecific; autoimmunity is a product of the pathogen-induced inflammatory environment, e.g. inflammation might prompt the expansion of previously activated T cells, a mechanism known as bystander activation (Wucherpfennig 2001).

ANTIGEN-SPECIFIC MECHANISMS FOR PATHOGEN-INDUCED AUTOIMMUNITY

Molecular mimicry

The theory of molecular mimicry argues that a viral or bacterial pathogen expresses an antigenic determinant on one of its proteins that is sufficiently similar in sequence or structure to a self-peptide (Fujinami and Oldstone 1985). During an infection, the microbial determinant, a linear sequence of 8–15 amino acids, may be presented in the context of a particular MHC molecule to the TCR of an autoreactive T cell. As a result, naïve autoreactive T cells are activated and expanded.

An animal model of EAE provided early evidence for molecular mimicry. Rabbits were immunized with a hepatitis B virus polymerase peptide which shared a six amino acid sequence similarity with the encephalitogenic region of rabbit MBP. After immunization with the peptide, T-cell reactivity to MBP could be demonstrated. Some of the animals also developed EAE as determined by histology (Fujinami and Oldstone 1985).

Autoimmune disease that is MHC I dependent was demonstrated in a mouse model of IBD. Intestinal inflammation was induced by transferring $CD8^+$ T cells, which recognize both mycobacterial and murine heat shock proteins (hsp) in TCR $\beta^{-/-}$ mice. The T-cell infiltrates into the small intestine and the liver resulted in lesions. The results could be reproduced in germ-free mice, implicating host hsp as a target for the $CD8^+$ T cells (Steinhoff et al. 1999).

A highly cited case for the role of molecular mimicry as a mechanism of autoimmune disease is in the pathogenesis of treatment-resistant Lyme arthritis (TRLA). Lyme disease is a multisystem illness caused by the tick-borne spirochete *Borrelia burgdorferi* (Steere et al. 1983). The Lyme disease pathogen expresses numerous antigens, including outer surface protein A (OspA) both on its surface and in its periplasmic space (Barbour and Hayes 1986). The clinical course of the disease begins

within days of the blood meal of the *Ixodes scapularis* tick. The characteristic rash, erythema migrans, occurs initially and is followed by cardiac, neurological, and musculoskeletal impairments, including joint inflammation (Steere 1989). A course of antibiotics typically resolves the illness. However, the arthritis never resolves in approximately 10 percent of patients despite extensive antibiotic therapy (Steere et al. 1984). These TRLA patients, who have no detectable spirochetal DNA in joint fluid or synovial tissue, are often HLA-DRB1*0401$^+$ and HLA-DRB1*0101$^+$, the same alleles associated with RA. Furthermore, during arthritic episodes, spiking titers of anti-OspA IgG are detectable in the patient serum (Kalish et al. 1993). These immune responses correlate with the severity and duration of the arthritis. Collectively, this evidence supports the hypothesis that TRLA is an autoimmune disease (Gross et al. 1998).

With molecular mimicry implicated as a mechanism for TRLA, the identification of the immunodominant epitope of OspA, as well as the search for a potential autoantigen, followed suit (Gross et al. 1998). Using an algorithm developed for the identification of peptides binding to HLA-DRB1*0401 (Hammer et al. 1994), $OspA_{165–173}$ was calculated to have the highest score for possible association with this class II molecule. To confirm that this was indeed the immunodominant epitope, HLA-DRB1*0401 transgenic mice were immunized with OspA and their immune T cells were tested in vitro for reactivity with an overlapping OspA peptide library. $OspA_{165–173}$ elicited the strongest recall response in these cells, a result that was confirmed by the response pattern of human T cells isolated from the synovial fluid of DRB1*0401$^+$ TRLA patients. A Genbank Database search for a human autoantigen with a similar amino acid sequence as seen in $OspA_{165–173}$ led to the identification of a stretch of amino acids from 332 to 340 in human leukocyte function antigen-1α (LFA-1α). T cells from the synovial fluid of TRLA patients were also responsive to human LFA-1α (Gross et al. 1998). According to the algorithm of Hammer et al. (1994), $LFA\text{-}1\alpha_{332–340}$ was predicted to be effectively bound by HLA-DRB1*0401.

With the identification of the candidate autoantigen, human LFA-1α, a model for autoimmunity as a mechanism for TRLA may be proposed. Following infection with *Borrelia burgdorferi*, the spirochete disseminates systemically to many locations, including the joint. After a period of varying duration, perhaps several months, the inflammatory immune response begins in the joint. The nine-residue peptide $OspA_{165–173}$ is presented in the context of HLA-DRB1*0401, resulting in activation and expansion of $OspA_{165–173}$-specific T cells and an inflammatory Th1 response, characterized by secretion of interferon γ (IFN-γ). The inflammatory milieu in the joint results in the upregulation of MHC II on APCs, as well as the upregulation of LFA-1α on leukocytes and synoviocytes. Despite the eradication of the spirochete by antibiotic treatment, the $OspA_{165–173}$-specific Th1 cells in the joint continue to be activated via cross-reaction with the self-peptide $LFA\text{-}1\alpha_{332–340}$, thus propagating the joint arthritis (Figure 3).

Superantigen activation

Viral or bacterial superantigens activate T cells expressing particular Vβ genes segments. Superantigens have

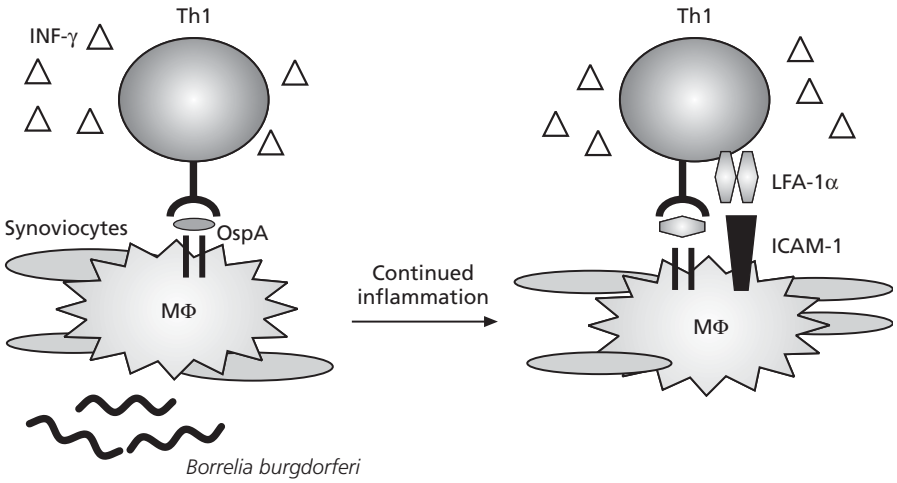

Figure 3 *Model for induction of autoimmunity in treatment-resistant Lyme arthritis (TRLA). Following infection with* Borrelia burgdorferi*, the spirochete disseminates systemically to many locations, including the joint. Macrophages (Mϕs) and dendritic cells present the outer surface protein* $OspA_{165–173}$ *to T cells. These activated T cells secrete interferon γ (INF-γ). The inflammatory milieu in the joint results in the upregulation of MHC II on antigen-presenting cells (APCs), as well as the upregulation of human leukocyte function antigen-1α (LFA-1α) on leukocytes and synoviocytes. Despite the eradication of the spirochete by antibiotic treatment, the cross-reactive T cells in the joint continue to be activated via the presentation of self-peptides derived from LFA-1α, propagating the joint arthritis. ICAM, intercellular adhesion molecule.*

the capacity to cause aberrant activation of large numbers of T cells, regardless of their peptide specificity. A subset of these superantigen T cells may be specific for a self-antigen and, thus, could mount an autoimmune response (Wucherpfennig 2001).

Superantigens may also exacerbate an autoimmune process or induce a relapse. In EAE, a murine model of MS, episodes of relapsing paralysis can be induced in PL/J mice with the bacterial superantigen staphylococcal enterotoxin B (SEB). In these mice, EAE is elicited by immunization with the amino-terminal peptide of MBP, Ac_{1-11}. Following an initial paralysis and clinical remission, administration of SEB can induce another episode of paralysis in these mice. The autoreactive T cells specific for the Ac_{1-11} epitope express Vβ8 and are reactivated by the SEB superantigen (Brocke et al. 1993).

ANTIGEN-NONSPECIFIC MECHANISMS FOR PATHOGEN-INDUCED AUTOIMMUNITY

Bystander activation

The inflammatory milieu resulting from a microbial infection may result in the expansion of autoreactive T cells in the absence of antigenic specificity. Activated T cells have a lower threshold for activation than resting T cells and, thus, may target self-antigens (Benoist and Mathis 1998). Microbial products, including LPS, can be potent adjuvants activating the innate immune response and eliciting an increase in proinflammatory cytokines. In this inflammatory environment, APCs enhance antigen processing, increase cell surface MHC II expression and upregulate co-stimulatory molecules such as B7-2 (Vella et al. 1997). Adjuvants may also sustain the antigen-specific T-cell population by inhibiting the AICD death of antigen-specific T cells (Kearney et al. 1994; Vella et al. 1997).

Another possible effect elicited by an inflammatory environment is a shift towards a Th1 T-cell population (Marrack et al. 2001). $CD4^+$ helper T cells differentiate into either Th1 or Th2 cells (Figure 4). Typically, microbial infections are associated with a Th-cell commitment to a Th1 cell (Yazdanbakhsh et al. 2002). This subset of cells is associated with the proinflammatory cytokine, INF-γ, and cell-mediated immunity. Th1 cells are also associated with many autoimmune diseases, including RA, Crohn's disease, type 1 diabetes, and MS. Comparatively, Th2 cells produce IL-4 and IL-5 and are associated with allergic and anti-helminth responses. After a microbial infection, the T-cell population may shift towards the Th1 subset, thereby priming an individual for an autoimmune disease. This theory remains hotly contested for a

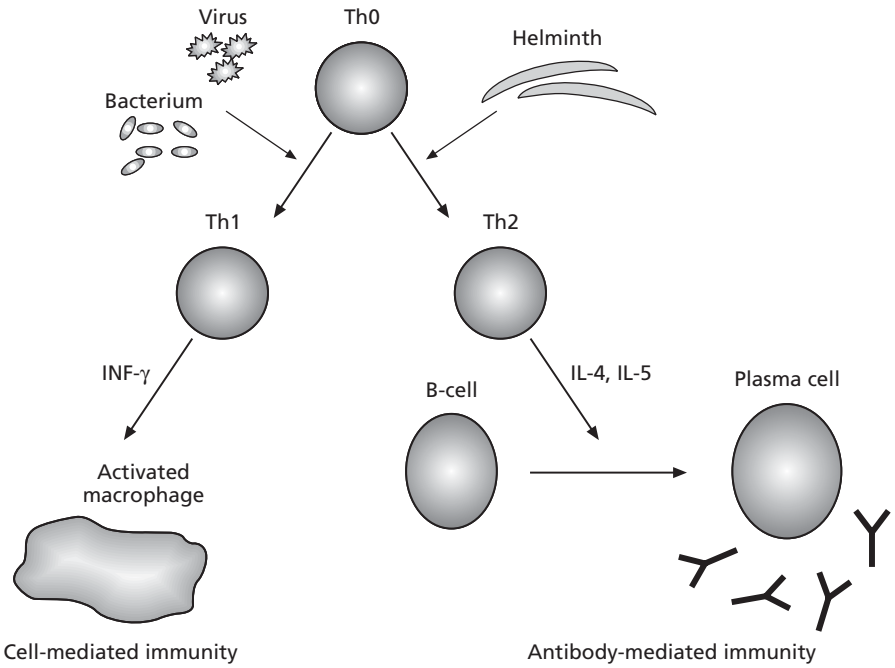

Figure 4 *T-helper 1 or 2 (Th1/Th2) cell phenotypes. $CD4^+$ Th cells differentiate into either Th1 cells or Th2 cells. The commitment to a Th1 or a Th2 cell fate is largely determined by transcriptional mechanisms induced by the interleukins IL-12 and IL-4, respectively (Murphy and Reiner 2002). In Th1 cell development, the T-box transcription factor T-bet is responsive to IL-12 and responsible for inducing the production of interferon α (INF-γ). During the commitment of Th cells to Th2 cells, IL-4 promotes the increased expression of the zinc finger transcription factor GATA3 which, in turn, enables the remodeling of the IL-4 locus. Typically, microbial and viral infections are associated with $CD4^+$ T-helper cell differentiation into Th1 cells (Lucey et al. 1996). This subset of cells is associated with activated macrophages secreting the proinflammatory cytokine, INF-γ, as well as cell-mediated immunity. Alternatively, Th2 cells produce IL-4 and IL-5 and are associated with allergic and anti-helminth responses. Th2 cells are associated with antibody-mediated immunity driven by plasma cells. (Adapted from Murphy and Reiner 2002 and Janeway et al. 2001.)*

variety of reasons, including the argument that cytokines have the dual capacity both to heighten and to constrain autoimmune responses. Furthermore, two common autoimmune diseases, ulcerative colitis and SLE, are associated with Th2 responses (O'Shea et al. 2002).

Epitope spreading

During chronic autoimmune disease, the T-cell response may acquire additional epitope specificities, shifting from a dominant epitope to subdominant epitopes on the self or foreign protein (Lehmann et al. 1992). 'Intramolecular spreading' refers to new epitopes on the same protein and 'intermolecular spreading' refers to epitopes on other proteins, perhaps other antigens in the same tissue (Vanderlugt and Miller 2002).

The earliest demonstration of this diversification of the T-cell response was shown in the murine model of EAE (Lehmann et al. 1992). After immunization with either whole MBP or only the N-terminal peptide of MBP ($Ac_{1–11}$), the T-cell response undergoes intramolecular epitope spreading, shifting reactivity from the original immunodominant peptide to different epitopes of the MBP protein. Evidence of intermolecular epitope spreading is depicted in a murine model of a chronic demyelinating disease mediated by $CD4^+$ T cells. Following infection with Theiler's picornavirus, mice develop a chronic progressive course of encephalomyelitis that resembles MS. The course of the disease appears biphasic. At the onset of clinical symptoms, T-cell responses to the virus are detected. Then, later in the course of the disease, responses to central nervous system antigens are seen, including determinants on the encephalitogenic proteolipid protein (PLP), MBP, and myelin oligodendrocyte glycoprotein (MOG).

Nonmicrobial environmental factors and autoimmunity

The evidence supporting a role for nonmicrobial environmental factors in the development of autoimmune disease is often based on epidemiological studies. Excessive intake of dietary iodide, a component of thyroid hormone, is a risk factor for autoimmune thyroid disease (Bagchi et al. 1985). Other studies correlate stressful life events with the development of Graves' disease (Matos-Santos et al. 2001). A recent study demonstrates that women who breast-feed have a decreased risk of developing SLE, a trend that is correlated with the number of babies breast-fed and the duration of breast-feeding (Cooper et al. 2002). Some literature cites occupational exposure to silica dust as a risk factor for the development of RA, lupus, scleroderma, and glomerulonephritis (Parks et al. 2002). In most cases, further studies are necessary to substantiate a role for nonmicrobial environmental factors in autoimmunity. However, in the case of sympathetic ophthalmia, there is unequivocal proof that trauma to one eye leads to an autoimmune-mediated attack of the other (Jakobiec et al. 1984). The eye is a well-defined immunoprivileged site, complete with T-cell infiltration barriers, and immunosuppressive cytokines, including TGF-β. When injury at this isolated site occurs, previously sequestered self-antigens are available for immunoreactivity.

THE EFFECTS OF IMMUNE DISREGULATION ON AUTOIMMUNE DISEASE DEVELOPMENT

Many of the immune regulation abnormalities identified in autoimmune disease are inextricably linked to genetic mutations, e.g. severe impairment of the AICD pathway results from mutations in the death receptor Fas (CD95) or Fas ligand (FasL or CD178). This pathway plays a critical role in maintaining peripheral tolerance via elimination of autoreactive cells. The absence of Fas or FasL in either *lpr* or *gld* mice, respectively, results in a severe autoimmune phenotype, an SLE-like disorder, marked by lymphadenopathy, autoantibody production, and nephritis (O'Shea et al. 2002). In humans, mutations in Fas result in ALPS. These patients present with lymphadenopathy, hepatosplenomegaly, and hypersplenism (Straus et al. 1999).

Other aberrant immunoregulatory mechanisms have not yet been linked to a single genetic defect, e.g. the T-cell response in active RA patients is dampened, perhaps as a result of aberrant calcium signaling and abnormal ξ chain expression by the TCR. These abnormalities may be a result of the overexpression of tumor necrosis factor α (TNF-α) in RA synovial tissue. These same abnormalities can be reproduced in vitro by chronic stimulation of T cells with TNF-α (Feldmann 2001).

Systemic lupus erythematosus may be the archetypal disease of immune disregulation. SLE, a systemic autoimmune disease, is defined by the production of autoantibodies. The disease has an enormous range of clinical manifestations that may involve the skin, joints, kidneys, and heart. SLE is associated with various abnormalities in lymphocytes at the cellular and molecular level that include antigen-receptor signaling events and cytokine production. In humans, abnormal, hyperreactive B-cell responses have been characterized. They include an increased expression of the CD40 ligand and a co-stimulatory molecule, as well as an increased production of the B-cell-stimulatory cytokines, IL-6 and IL-10 (Lipsky 2001). Altered T-cell signaling may result from the abnormal expression of the TCR ξ chain as well as decreased levels of p65-Rel A protein (Tsokos et al. 2000). The nuclear translocation of the p65-Rel A protein is associated with the activity of the transcription factor, nuclear factor-κB (NF-κB), which in turn is responsible for the activation of IL-2.

MECHANISMS OF TISSUE DESTRUCTION AND ORGAN DAMAGE IN AUTOIMMUNE DISEASES

The 'initiation phase' of an autoimmune disease is mediated by several factors: genetic predisposition, environmental influence, and immune disregulation (Ermann and Fathman 2001). Over time, a chronic autoimmune response invariably results in an 'effector phase' of the disease. During the effector phase, tissues and/or organs sustain damage imparted by destructive cells and cellular products. The effector phase of certain autoimmune diseases may be the product of both immune and nonimmune mechanisms (Ermann and Fathman 2001).

Demyelination and axonal loss in MS

Multiple sclerosis occurs in early adulthood and is the most common demyelinating disease in adults. The disease is characterized by relapsing and remitting episodes of neurological impairment. MS appears to be an autoimmune response targeting various proteins of the myelin sheath, including MBP, MOG, PLP, and αβ crystallin. Lymphocytes are initially activated by a microbial infection or a self-antigen, and then they are essentially 'pulled through' the blood–brain barrier by integrin and adhesion molecule interactions. After the degradation of the extracellular matrix by matrix metalloproteases (MMP), lymphocytes can access the white matter of the central nervous system, resulting in autoantibody production to myelin proteins by B cells and lymphotoxin-α (LT-α) and TNF-α cytokine secretion by T cells. These responses prompt the release of the free radical nitric oxide (NO) and the T-cell stimulator osteopontin (OPN) by macrophages, microglial cells, and astrocytes. The end result of this effector phase cascade is multifocal demyelination and axonal loss in the spinal cord, ultimately resulting in paralysis (Steinman 2001).

Immune complex deposition in SLE

Systemic lupus erythematosus is a systemic autoimmune disease that frequently strikes women during their reproductive years. In humans, this disease has an enormous range of clinical presentations which may include skin lesions, arthritis, serositis, nephritis, and hematological disorders. In fact, renal involvement is the most life-threatening complication of SLE. The immediate precursor to renal tissue destruction and kidney damage in SLE is the IgG autoantibodies to double-stranded DNA. Serum titers of these antibodies correlate with glomerulonephritis in humans and mice (Pearson and Lightfoot 1981). Other autoantibodies are also associated with the tissue destruction found in SLE, including anticardiolipin antibodies and thrombosis, as well as anti-Ro antibodies and congenital heart block (Lipsky 2001). Autoantibodies are the direct mediators of tissue injury in SLE. After the deposition of antigen–antibody immune complexes and the fixation of complement, tissue damage occurs in the manner of a type III hypersensitivity reaction.

Cartilage destruction and bone loss in RA

Another autoimmune disease, RA, afflicts about 1 in 100 people with chronic inflammation of the synovial joints. This chronic immune response ultimately destroys cartilage and bone, rendering the affected joints immobile. Although activated T cells specific for a joint antigen appear to be critical in the initiation of the disease process; cartilage and bone loss are also the products of nonimmune mediators. Enzymes play a critical role in cartilage destruction. Cartilage, an intricate matrix of type II collagen and aggrecan, is degraded by the activity of MMPs, as well as two aggrecanases or ADAMTS (a disintegrin or metalloproteinase with thrombospondin type I motif) (Feldmann 2001). Bone lesions are the product of the destructive osteoclast cell. Inflammation favors bone loss because osteoclastic precursors are induced to differentiate into osteoclasts by proinflammatory cytokines TNF-α and IL-1.

Thyroid-associated ophthalmopathy in autoimmune thyroid disease

Thyroid-associated ophthalmopathy (TAO) is a serious complication of autoimmune thyroid disease. The pathogenesis of TAO includes the deposit of excess connective tissue and fat in the orbital cavity, as well as edema. The clinical outcome is exophthalmos, corneal damage, and, on a rare occasion, loss of sight. The effector phase of TAO begins when the inflammatory cell infiltrate penetrates the extraocular muscles. These T cells mount a response against an orbital autoantigen that is similar enough to a thyroid autoantigen, inciting a cross-reaction. With the release of cytokines, including IL-1, TNF, and INF-γ, and the concomitant activation of fibroblasts, two mediators of edema are released by the fibroblasts: hydrophilic glycosaminoglycan hyaluronate and chondroitin sulfate (Weetman 2001). Interestingly, a subset of these orbital fibroblasts may differentiate into fat cells, explaining the excess orbital fat in TAO. Although the pathology of TAO is unraveling, the identity of the cross-reactive autoantigen remains at large.

EXPERIMENTAL ANIMAL MODELS OF AUTOIMMUNITY

The use of animal models has played a pivotal role in furthering our understanding of autoimmunity. Animal

models are implemented to investigate the etiology, pathogenesis, and therapeutic strategies associated with many of the 80 autoimmune diseases that collectively affect roughly 5 percent of the world's population. An animal model offers a wealth of information, far exceeding what can be gleaned from most in vitro experiments; however, it is never an exact replica of a human autoimmune disease. First, studying a human disease in a highly inbred mouse model is not an ideal scenario because humans are outbred relative to mice. Thus, the study of an inbred mouse strain is roughly the equivalent of a 'single case study' in humans (Atkinson and Leiter 1999). Second, and to an even greater extent, extrapolating from an animal model to a human disease is dubious because of the genetic differences between species. There are inherent limitations in extracting pathogenic mechanisms from an animal model of disease as a result of the underlying genus- and species-specific differences that occur between an animal and a human. Under circumstances of critical importance, such as the testing of gene therapy, a species closer to humans, such as primates, may be a better candidate to serve as an experimental surrogate.

The NOD mouse as a model of human type 1 diabetes mellitus

The NOD mouse was first identified in 1980 as a useful model for investigating insulin-dependent human diabetes (Makino et al. 1980). Although the predecessor to the NOD mouse, the biobreeding (BB) rat, was identified several years earlier (Nakhooda et al. 1978), the NOD mouse gained favor and is currently the model of choice for type 1 diabetes researchers. In fact, mice are generally preferable to rats, because their genome is more familiar and analytical reagents, such as monoclonal antibodies, are more readily available (Atkinson and Leiter 1999).

Human type 1 or insulin-dependent diabetes mellitus is a $CD4^+$ and $CD8^+$ T-cell-mediated autoimmune disease that results in the targeted destruction of the insulin-secreting β cells of the pancreatic islets. Diabetic NOD mice display the biochemical and pathological characteristics of diabetes, including a lymphocyte infiltration into the pancreatic islets. Diabetes in NOD mice is heterogeneous, because its development is determined by more than 19 loci (Wucherpfennig and Eisenbarth 2001). In humans, many genetic loci are also responsible for the development of diabetes. Both NOD mice and humans have autoimmune responses to glutamic acid decarboxylase (GAD), insulin, and insulinoma-associated protein 2 (Ia2) (Taneja and David 2001).

A recent breakthrough in the study of the etiology of type 1 diabetes occurred from a comparative analysis between humans and NOD mice. Lee et al. (2001) determined that susceptibility to diabetes is conferred by a mutation in the P9 peptide-binding pocket of the MHC II molecules HLA-DQ8 and $I\text{-}A^{g7}$ in humans and NOD mice, respectively. Using X-ray crystallography, this group was able to characterize the three-dimensional structures of these molecules and determine that they lacked an aspartic acid residue at position 57 of the β chain, resulting in a net positive charge for these molecules. Functionally, the mutation in these MHC II molecules enables them to bind a different repertoire of peptides, including the immunodominant T-cell epitopes of islet antigen peptides in NOD mice (Yu et al. 2000). These results infer that type 1 diabetes in mice and humans may stem from the same antigen-presenting events. They further demonstrate the significance of the NOD mouse as an animal model of type 1 diabetes.

THE NEW ZEALAND BLACK/WHITE MOUSE AS A MODEL OF HUMAN SLE

Systemic lupus erythematosus is a systemic autoimmune disease associated with pathogenic autoantibodies. Some of these pathogenic autoantibodies include anti-DNA in glomerulonephritis, anticardiolipin in thrombosis, and anti-Ro in heart block (Lipsky 2001). The disease has been linked with several MHC genes. In fact, certain MHC alleles have been associated with the occurrence of particular autoantibodies, including HLA-DR2 and HLA-DR3 with anti-Ro and anti-La antibodies, as well as HLA-DR4 and HLA-DQ3 with anti-Sm and anti-RNP antibodies (Chernajovsky et al. 2000). Complement deficiencies, involving the complement molecules C1q, C2, and C4, have also been sited in SLE, as well as a host of other immunoregulatory defects.

The F1 hybrid cross between New Zealand black (NZB) and New Zealand white (NZW) mice generates the NZB/W mouse that is recognized as the best murine model of SLE (Drake et al. 1995). In these mice, the disease develops spontaneously and is characterized by high titers of IgG anti-DNA antibodies. Death in these animals occurs by a progressive, severe glomerulonephritis, similar to the renal destruction seen in human SLE. The development of both human and murine SLE is strongly linked with particular MHC alleles.

The NZB/W mouse model has been used to identify a myriad of abnormalities associated with the autoimmune disease SLE. This animal model was used to identify B-cell hyperreactivity as an immunoregulatory defect in SLE. On T-cell stimulation, resting B cells from these mice display increased proliferation, IgM secretion, and upregulation of co-stimulatory molecules (Jongstra-Bilen et al. 1997). Genetic loci on chromosome 1 in both parental strains NZB and NZW have been implicated in this B-cell hyperreactivity (Vyse et al. 1997; Sobel et al. 1999). This model was also used to delineate a role for cytokines in SLE pathogenesis. The F1 hybrid mouse inherits a dominant gene from the NZW parent that correlates with reduced levels of TNF-α production. The

administration of replacement TNF-α delayed the onset of lupus nephritis (Jacob and McDevitt 1988).

This model has also been used to identify multiple genetic susceptibility loci for SLE. Breeding of NZW and NZB strains has yielded a variety of strains called NZM. Using genetic mapping techniques, the analysis of these NZM strains has enabled researchers to identify susceptibility loci for SLE in this model. The analysis of congenic strains with these loci is enabling researchers to determine the functional role that these loci may have in SLE disease pathogenesis.

Strain K/BxN: a spontaneous mouse model of rheumatoid arthritis

In autoimmune disease research, an animal model can often be the framework supporting a new theory in the pathogenesis of an autoimmune disease. Occasionally, an animal model provides evidence to challenge an existing theory, such as in the case of rheumatoid arthritis (RA). RA is a chronic, inflammatory joint disease, characterized by synovial thickening and lymphocyte infiltration, as well as cartilage and bone destruction in the joint. Given the association of this disease with certain MHC II alleles, contemporary researchers believe that $CD4^+$ T cells play a crucial role in the pathogenesis of RA. Therapeutic trials were conducted with antibodies to CD4, but they were not very successful (Feldmann 2001).

Kouskoff et al. (1996) uncovered an intriguing new animal model of arthritis. This group crossed the TCR transgenic line KRNxC57BL/6 (the TCR recognizes a bovine ribonuclease peptide in the context of $I\text{-}A^k$), with the NOD strain. The resulting strain, K/BxN, has a TCR that recognizes a peptide of glucose-6-phosphate isomerase (GPI) in the context of the NOD-derived $I\text{-}A^{g7}$ (Matsumoto et al. 1999). GPI is a ubiquitously expressed protein that catalyzes reactions in glycolysis and gluconeogenesis. All of the K/BxN offspring develop a spontaneous arthritis that is strikingly similar to human RA. Although arthritis is initiated by T-cell activation in this model, joint-specific pathology is mediated by anti-GPI antibodies. In fact, transfer of antibodies can reproduce disease. This model suggests that antibodies may not merely be a phenomenon in RA; they may in fact be the primary mediators of disease.

Rodent EAE as a model of human MS

In the aforementioned animal models, disease develops spontaneously. In contrast, the rodent model of EAE is an induced model of disease. Induced models are advantageous because the onset and progression of the disease can be carefully monitored (Taneja and David 2001). The EAE rodent model is used extensively as a model for human MS. EAE can be induced with a variety of autoantigens, including MBP, PLP, and MOG, e.g. in PL/J mice, EAE is induced with the N-terminal peptide of MBP (Ac1-11). Histologically, the EAE pathology is similar to MS, although the degree of demyelination and the distribution of the lesions are different (Taneja and David 2001). In rodents, predisposition to EAE is also associated with II MHC alleles, similar to MS in humans.

The rodent model of EAE has been a powerful tool for examining important mechanisms in autoimmune disease pathology. Experiments in this model revealed that superantigens could induce relapses or exacerbate autoimmune disease (Brocke et al. 1993). This model was also used to demonstrate the concept of 'epitope spreading', as previously discussed.

Furthermore, the EAE model will invariably play an instrumental role in identifying candidate therapies for human MS, e.g. the injection of a herpes simplex virus vector driving the human fibroblast growth factor gene is reversing the pathological signs of EAE in C57BL/6 mice (Ruffini et al. 2001). Recently, Youssef et al. (2002) have identified a role for atorvastatin, a drug currently given to reduce high cholesterol, in ameliorating the disease severity in three different mouse models of EAE. On repeated administration of atorvastatin, investigators noted increased levels of anti-inflammatory cytokines, including IL-4, IL-10, and TGF-β. The EAE mouse model has also been used to test a therapeutic strategy involving the blockade of very late activated antigen-4 (VLA-4), a T-cell antigen that aids T cells in penetrating the blood–brain barrier. The results of the trial were promising because they demonstrated a reversal of paralysis and eliminated relapses in treated mice. Clinical trials are currently under way with a human antibody to VLA-4 (Steinman 2001).

AUTOIMMUNITY THROUGH IMMUNIZATION

The associations between infection and autoimmunity have accumulated rapidly in the scientific literature since the initial connection between the two was observed in the mid-1960s (Puxeddu et al. 1965). Precisely how an immune response to a pathogen evolves into an autoimmune disease remains unknown. Nevertheless, if an immune response to a pathogen may incite an autoimmune disease, perhaps an immune response to a vaccine may also elicit an autoimmune disease. Given the potential cross-reactivity between an autoantigen and a microbial antigen, could a vaccine pose the threat of priming for autoimmune disease in genetically susceptible individuals? Or could a potent vaccine adjuvant alter immunoreactivity, favoring a break in self-tolerance?

At a recent conference organized by the Fondation Merieux, Schoenfeld and Pless reported their conclusions with hesitancy (Regner and Lambert 2001). They

concluded that, despite the lack of data to make a careful assessment, it appears that the connection between immunization and autoimmune disease is weak. Furthermore, they stated that the benefits of immunization far exceed the risks (Regner and Lambert 2001). The initial assessments regarding the safety of the Lyme disease vaccine demonstrated that there were no long-term adverse effects from immunization (Poland and Jacobson 2001; Sikand et al. 2001). In another investigation, immunizing infants with a recombinant hepatitis B virus vaccine did not result in an increase in autoantibody production at a 6-year follow-up study (Belloni et al. 2002). Given the slight possibility for vaccine-induced immunization, the implications for future vaccine design are evident, e.g. a modified vaccine design may include mutating a cross-reactive epitope to preclude molecular mimicry-based autoimmunity.

TREATMENT STRATEGIES FOR AUTOIMMUNE DISEASE

Treating a patient with an autoimmune disease is an inordinately complex task. Treatment is complicated because of the heterogeneous nature of these diseases. At the cellular and molecular level, neither the etiology nor the pathogenesis of autoimmune disease is well defined. Furthermore, for a physician, autoimmune diseases may be vexing for the following reasons: they are chronic; the course of the disease may vacillate between periods of relapse and periods of remission; patients may be affected differently by the same disease; and patients often present when the disease has already progressed to tissue and organ damage (Chernajovsky et al. 2000). Treatment is initiated to reinstate 'immune homeostasis' in the autoimmune disease patient.

Autoimmune diseases are wildly diverse. Although they span the gamut of clinical manifestations, the current management of most autoimmune diseases relies on immunosuppression, including the use of corticosteroids such as prednisone, e.g. patients with RA are administered methotrexate which acts via adenosine to suppress inflammation (Chan and Cronstein 2002). The current treatment strategy for MS includes synthetic glatiramer acetate, an agent that reportedly acts by shifting the T-cell phenotype to Th2 cells (Gran et al. 2000). Generally, immunosuppression has had limited success and is often used for lack of a better alternative (Persidis 1999).

In the case of some autoimmune diseases, recent approaches to modifying the immune system have demonstrated promising clinical improvements. Sustained therapy with TNF-α blockers has been successful in arresting RA (Pisetsky 2000) and Crohn's disease (Podolsky 2002). Patients with relapsing–remitting MS have been responsive to INF-β treatment, a drug administered to inhibit lymphocyte migration to the brain (Mehindate et al. 2001; Steinman 2001). These therapeutic strategies involve immunomodulation with cytokines and cytokine antagonists. Anti-T-cell monoclonal antibodies, signal transduction blockers, and pharmacological agents have also been implemented (Tsokos and Nepom 2000).

Gene therapy is employed by researchers to develop revolutionary treatments for autoimmune disease. This cutting-edge technology is the process of inserting and expressing foreign genes into the host cell genome. Many systems utilize viral vectors as a vehicle to insert genes by transduction. Successful animal trials with gene therapy have included the treatment of RA, MS, diabetes, and lupus (Tarner and Fathman 2001). These successes have supported the clinical trial of gene therapy in humans. With an estimated disease burden of $US65 billion (Persidis 1999), arthritis has featured prominently in human gene therapy investigations. In a recent study, a gene encoding the IL-1 receptor antagonist was transferred to the metacarpophalangeal joints of patients with advanced RA (Evans et al. 1996). Synoviocytes were injected ex vivo with a retroviral vector and reimplanted into the joint. The successful completion of this trial shows promise for the further application of gene therapy (Gouze et al. 2001; Horwood et al. 2002).

CONCLUSION

Autoimmunity constitutes the sustained 'attack' of self-tissue mediated by pathogenic, self-reactive antibodies and/or lymphocytes. Autoimmune diseases are a heterogeneous collection of about 80 distinct illnesses representing a broad range of pathology. The enormous complexity associated with the etiology, mechanisms, and pathogenesis of autoimmune disease is not rivaled by any other human disease.

Autoimmunity and autoimmune disease make up a topic teeming with discrepancies: decades ago, scientists refuted the existence of a disease that is today recognized as a serious societal burden. Autoimmune disease is the erroneous attempt of an organism to protect itself as it attacks itself. The present treatment of autoimmune disease relies on immunosuppression, rendering the patient susceptible to infection and illness.

Despite the complicated, and often contradictory, nature of autoimmune disease, scientists are deftly using valuable tools to unravel autoimmunity, including experimental animal models and gene therapy. With these efforts, scientists seek to understand, as well as to reinstate, the 'immune homeostasis' that has been lost in the autoimmune response.

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The immunocompromised host

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There are more than 100 recognized diseases that are genetically determined and compromise immune defenses against microorganism invasions. Mutations of 86 genes (Table 1) have so far been identified in association with as many immunodeficient conditions. Advances in genomics have helped enormously in identifying these genes. Today, the understanding of how their products contribute to the many complex reactions of different cells involved in immune defense (both innate and adaptive) contributes to a better knowledge of our immune system. This chapter reviews what we know about these conditions.

GENETIC DISORDERS OF INNATE IMMUNITY

Deficiency in granulocyte production

Inherited severe congenital neutropenia (SCN) was reported more than 40 years ago as Kostmann's syndrome (Kostmann 1956). Since then, various forms of inherited neutropenias have been described (Figure 1). When neutrophil counts are <200/mm^3 of blood, neutropenia exposes the patient to severe bacterial and fungal infections, which can spread throughout the bloodstream and cause death in the absence of appropriate treatment. The usual sites of infections are located at the interface between body cavities and the outside environment. In addition, chronic severe periodontitis is a hallmark of severe neutropenia. In most cases, Kostmann's syndrome is associated with a block in granulocyte differentiation at the promyelocyte stage. Both autosomal dominant (with variable penetrance) and autosomal recessive patterns of inheritance have been described. In recent years, it has been found that most patients with SCN carry mutations of the neutrophil elastase-2 gene (*ELA-2*) (Dale et al. 2000). The physiopathology of defective neutrophil production is still unclear, although it is thought that mutations of the elastase gene result in a premature apoptosis of neutrophil precursors (Aprikyan et al. 2001). Interestingly, cyclic neutropenia, a condition characterized by regular 21-day cycles of neutrophil counts oscillating between 0 and normal values, is also caused by mutations of ELA-2 with an autosomal dominant inheritance (Horwitz et al. 1999; Dale et al. 2000). The mechanism underlying cyclic hemopoiesis is not understood.

Recently it has been proposed that susceptibility to infections in SCN is the consequence not only of the absence of mobile phagocytic cells migrating to infected tissues, but also of defective release of antibacterial peptides such as α defensins (Putsep et al. 2002).

Other complex conditions can cause severe congenital neutropenia such as Shwachman's syndrome, which is characterized by exocrine pancreas insufficiency, and metaphyseal dysplasia with an autosomal recessive inheritance. The molecular mechanism is presently unknown, although it has recently been shown that Shwachman's syndrome is caused by mutations of the *SBDS* gene (Kyono and Coates 2002; Boocock et al. 2003). Neutropenia is also a feature of glycogenosis Ib (Kyono and Coates 2002). A gain of a function mutation

Table 1 *Genetically characterized primary immunodeficiencies*

Gene/product	Expression	Disease
Immunodeficiencies of neutrophils		
Elastase	Neutrophils	Agranulocytosis cyclic neutropenia
SBDS	Neutrophils	Shwachmann's syndrome
WASP (activating mutation)	Leukocytes	Agranulocytosis
β_2-Integrin	Leukocytes	LAD-I
GDP fucose transporter	Leukocytes/endothelial cells	LAD-II
β-A	Neutrophils	Neutrophil actin dysfunction
Rac-2	Neutrophils	LAD variant
C/EBPϵ	Neutrophils	Specific granule deficiency
CyBB (gp91 cytochrome *b*)	Phagocytic cells	XL CGD
CyBA (gp22 cytochrome *b*)	Phagocytic cells	AR CGD
NFC1 $p47^{phox}$	Phagocytic cells	AR CGD
NFC2 $p67^{phox}$	Phagocytic cells	AR CGD
G6PD	Phagocytic cells	CGD-like syndrome
Cathepsin C	Neutrophils (?)	Papillon–Lefèvre syndrome
Plasma proteins		
Classic pathway, of complement C1q, -r, -s, C4, C2	Plasma	Infections by encapsulated bacteria
Alternate pathway: B, D,	Plasma	Infections by encapsulated bacteria
Alternate pathway: properdin	Plasma	Neisseria infections
Lectin pathway, MBL	Plasma	?
C3	Plasma	Infections by encapsulated bacteria
C3 regulators: H, I	Plasma	Infections by encapsulated bacteria
Membrane attack complex C5, -6, -7, -8, -9?	Plasma	Neisseria infections
Spleen		
Connexin 43	Spleen	Situs abnormality (asplenia)
ZIC3	Spleen	Situs abnormality (asplenia)
Interface innate/adaptive immunity		
NEMO (IKKγ)	Leukocytes	Anhidrotic ectodermic dysplasia with immunodeficiency
EVER-1, -2	Keratinocytes?	Epidermodysplasia verruciformis
IL-12B (p40)	T, NK cells	
IL-12RB1	T, NK cells	
IFNγR1	Dendritic cells/macrophages	Mendelian susceptibility to mycobacterial infections
IFNγR2	Dendritic cells/macrophages	
STAT-1	Dendritic cells/macrophages	
T lymphocytes	Lymphocyte precursors	
Adenosine deaminase		SCID
γc	T, NK lymphocyte precursors	XL SCID
JAK-3	T, NK lymphocyte precursors	AR SCID
Rag-1	T-, B-lymphocyte precursors V(D)J	AR SCID
Rag-2	T-, B-lymphocyte precursors V(D)J	AR SCID
Artemis	T-, B-lymphocyte precursors V(D)J	AR SCID
IL-7Rα	T-lymphocyte precursors	SCID
CD45	T-, (B)-lymphocyte precursors	SCID
WHN	Epithelial cells	Nude phenotype
Purine nucleotide phosphorylase	T lymphocytes	T-cell deficiency
CD3γ, ϵ	T lymphocytes	T-cell deficiency
ZAP-70 kinase	T lymphocytes	T-cell deficiency
IL-2Rα	T lymphocytes	T-cell deficiency
Caspase 8	Lymphocytes	Partial mixed deficiency
CIITA	Antigen-presenting cells	

(*Continued over*)

Table 1 *Genetically characterized primary immunodeficiencies (Continued)*

Gene/product	Expression	Disease
RFXANK/B	Antigen-presenting cells	HLA-II deficiency
RFX 5	Antigen-presenting cells	
RFX AP	Antigen-presenting cells	
TAP-1	Antigen-presenting cells	HLA-I deficiency
TAP-2	Antigen-presenting cells	
Immunodeficiencies with genomic instability		
DNA ligase IV	Lymphocytes	Combined immunodeficiency
DNA ligase I	Lymphocytes	Combined immunodeficiency
ATM	Lymphocytes	Ataxia telangiectasia (AT)
MRE-11	Lymphocytes	AT-like disorder
NBS I/Nibrin	Lymphocytes	Nijmegen breakage syndrome
DNMT 3B	Lymphocytes	ICF syndrome
BLM	Ubiquitous	Bloom syndrome
Immunodeficiencies with bone disease		
RMRP	?	Cartilage hair hypoplasia
SMARCAL	?	Schimke immuno-osseous dysplasia
Other immunodeficiencies		
WASP	Hemopoietic cells	Wiskott–Aldrich syndrome
CD40L	T lymphocytes	Hyper-IgM syndrome type 1
CD40	B lymphocytes, dendritic cells	Hyper-IgM syndrome type 3
T/NK cytolytic deficiencies		
Perforin	T/NK cytolytic cells	Lymphohistiocytosis
LYST	Ubiquitous	Chédiak–Higashi syndrome
RAB27a	Cytolytic cells, melanocytes	Griscelli's syndrome
SAP/SH2DIA	Lymphocytes	XLP–Purtilo syndrome
Miscellaneous		
AIRE	Thymic epithelial cells	APECED (candidiasis/autoimmunity)
B-cell immunodeficiencies		
μ Heavy chain	B-cell precursors	
Igα	B-cell precursors	
λ5	B-cell precursors	AR agammaglobulinemia
BLNK	B-cell precursors	
BTK	B-cell precursors	XL agammaglobulinemia
AID	B cells (germinal centers)	Hyper-IgM syndrome type 2
IgHγ1	B cells	IgG1 deficiency
IgLK	B cells	IgK deficiency

APECED, polyendocrinopathy–candidiasis–ectodermal dystrophy; AR, autosomal recessive; AT, ataxia telangiectasia; CGD, chronic granulomatosis disease; G6PD, glucose-6-phosphate dehydrogenase; ICF, immunodeficiency centromeric facial; IL, interleukin; LAD, leukocyte adhesion deficiency; MBL, mannose-binding lectin; NK, natural killer; SCID, severe combined immunodeficiency; XL, X-linked.

of the *WASP* gene is causing an X-linked form of SCN (Devriendt et al. 2001). Therapy by administration of granulocyte colony-stimulating factor (G-CSF) is efficient in most forms of SCN (Devriendt et al. 2001). Kostmann's syndrome as well as Schwachman's syndrome predispose to myeloid leukemia. In the former setting, leukemia is often associated with the acquisition of somatic mutations of the intracellular region of the G-CSF receptor (Dale et al. 1993; Tidow et al. 1997; Devriendt et al. 2001). The mechanism of this predisposition is unknown. It is, however, suspected that enhanced precursor proliferation caused by premature daughter cell death could favor secondary genetic alterations.

Disorders of neutrophil function

MIGRATION: LEUKOCYTE ADHESION DEFICIENCY

Neutrophils migrate to tissues from blood vessels upon appropriate inflammatory signaling. Molecules involved in the process of adhesion and migration have now been characterized in part because natural mutants of neutrophil adhesion/migration have been described. These are called leukocyte adhesion deficiency (LAD).

LAD-I, a rare autosomal recessive disorder, is characterized by accumulation of neutrophils in the bloodstream whereas infection sites are devoid of pus. Severe,

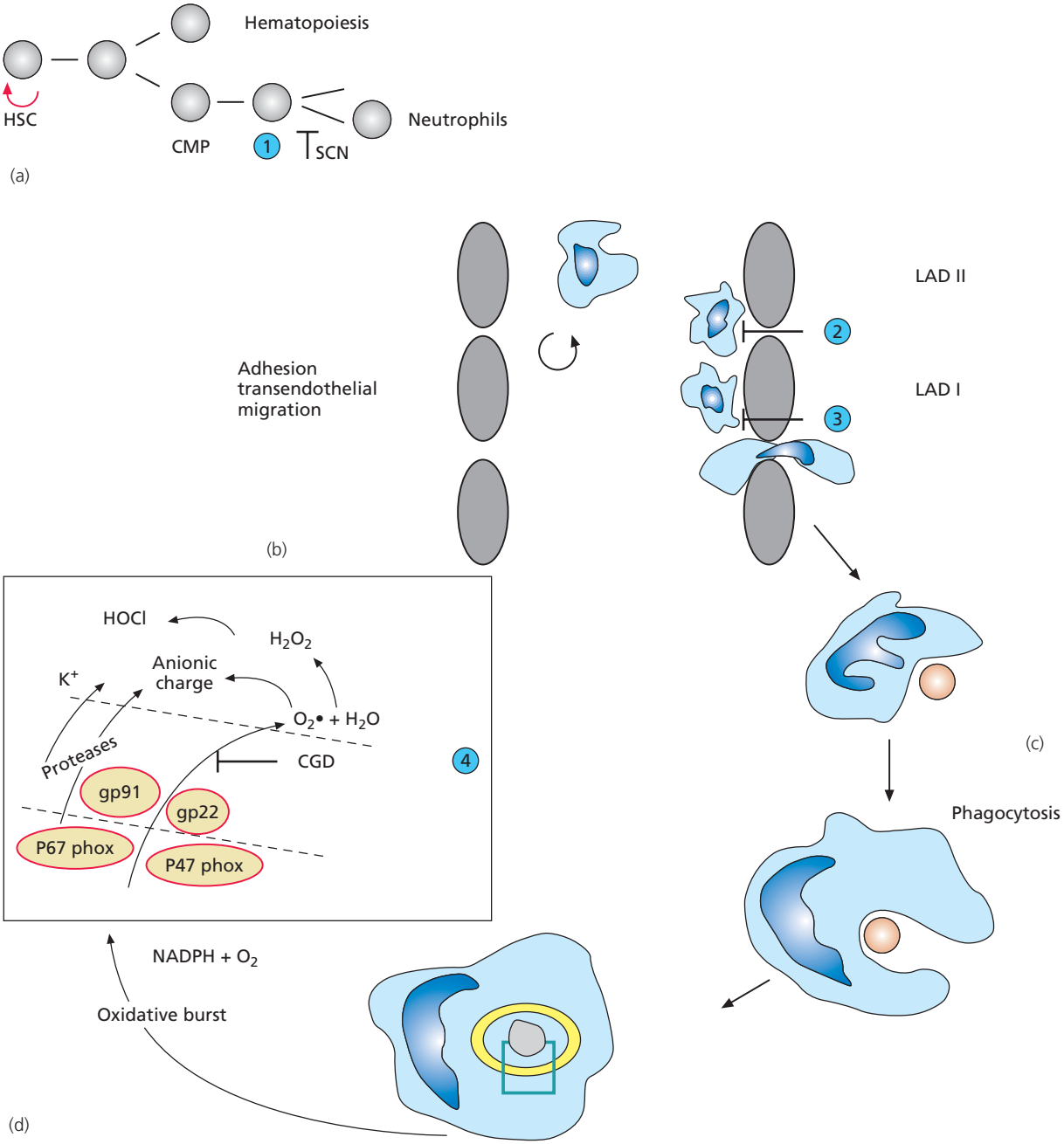

Figure 1 *Main phagocytic cell disorders.* **(a)** *Hemopoiesis. HSC, hemopoietic stem cells with self-renewal capacity; common myeloid progenitors. Neutrophil development from myeloid progenitors is impaired in severe congenital neutropenia (SCN) and cyclic neutropenia (CN) caused by elastase mutations (1).* **(b)** *Adhesion to endothelial cells and transendothelial migration of neutrophils. Rolling of neutrophils is abrogated in leukocyte adhesion deficiency II (LAD-II) (2). β_2-integrin-mediated adhesion and transendothelial migrations are blocked in LAD-I (3).* **(c)** *Phagocytosis.* **(d)** *Oxidative burst (magnification in inset). Once microorganisms are phagocytosed, activation of membrane NADPH oxidase composed of four subunits leads to superoxide production, then H_2O_2 after superdismutase action and HOCl (chlorination). In addition, the induced anionic charge triggers a K^+ influx and bacteriolytic protease release into the phagolysosome. Deficiency in any component of NADPH oxidase causes a chronic granulomatous disease (CGD) (4). CMP, common myeloid progenitor.*

unabated, bacterial infections, mostly of soft tissues, ensue, lead to extensive necrosis (Anderson et al. 1985; Fischer et al. 1988). LAD-I is also often associated at birth with delayed loss of the umbilical cord and omphalitis. The absence of pus and leukocytosis led to the suspicion that neutrophils were unable to adhere to and/or migrate along a chemotactic gradient. This was indeed proved to be the case in vitro and in vivo. It was recognized that expression of heterodimeric molecule complexes was defective at leukocyte membranes, i.e.

β_2-integrins including $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (MAC-1), and $\alpha_x\beta_2$ (p150, 95) molecules. Mutations of the gene encoding the β_2-integrin subunit cause this disorder (Kishimoto et al. 1987). Null mutations result in severe life-threatening leukocyte adhesion deficiency whereas missense mutations enabling residual β_2-integrin expression and function are compatible with life, although severe bacterial infections can occur. β_2-integrins are involved in leukocyte adhesion to the endothelial wall and then transendothelial migration after activation of β_2-integrins by chemokines. β_2-integrins bind to intercellular adhesion molecule 1 (ICAM-1) at endothelial surfaces and also iC3b on bacterial surfaces. It is thought that this condition is primarily a neutrophil dysfunction because other leukocytes such as monocytes can use the $\alpha_4\beta_1$-integrin not expressed by neutrophils to compensate for the β_2-integrin deficiency.

Variants characterized by the presence of a nonfunctional β_2-integrin have been described. In these cases, inside-out signaling leading to β_2-integrin activation was shown to be impaired (Kuijpers et al. 1997; Harris et al. 2001). More recently, a third similar case has been reported (Mcdowall et al. 2003). In the latter not only β_2- but also β_1- and β_3-integrins were disabled despite normal structure and expression of these molecules. A bleeding disorder was therefore also present because β_3-integrin is involved in platelet aggregation. The molecular basis of this impaired β-integrin activation is unknown. However, these findings point remarkably to the role of inside-out signaling in driving neutrophil activation to adhere to and migrate toward infectious zones.

LAD-II

Etzioni et al. (1992) have reported a similar condition associated with a dysmorphic syndrome, failure to thrive, and learning disability. This autosomal recessive condition has been found in a very small number of patients so far. There is a lack of expression of the H antigen by erythrocytes (Bombay phenotype) and a defect of leukocytes to migrate from blood. This condition is the consequence of a fucosylation defect (Luhn et al. 2001), which leads to a defective formation of the sialyl Lewis X (sLe^x) carbohydrate, the selectin ligand (L-, E-, and P-selectins). Therefore, the rolling process mediated by repeated short interactions between leukocytes and endothelial cells does not occur properly, impairing the further step of adhesion.

NEUTROPHIL ACTIN DYSFUNCTION

A small number of patients have been reported with a condition characterized by recurrent bacterial and fungal infections associated with abnormal neutrophil movement, whereas β_2-integrins are normally expressed (Luhn et al. 2001). There is decreased actin polymerization in neutrophils in one case, associated with a decrease in a 89-kDa protein expression and an increased expression in leufactin, a protein interacting with F-actin (Coates et al. 1991). A dominant β-actin mutant has been found in another child with a similar syndrome (Nunoi et al. 1999).

RAC-2 DEFICIENCY

In two patients with severe bacterial infections, poor wound healing, and leukocytosis, neutrophils were found to be unable to move along a chemotactic gradient, and also unable to produce superoxide anion when stimulated with the bacterial peptide flmp. These patients were shown to exhibit Rac-2 gene mutation. Rac-2 is a Rho guanosine triphosphatase (GTPase) involved in induction of actin polymerization, integrin complex formation, and activation (Ambruso et al. 2000; Williams et al. 2000).

SPECIFIC GRANULE DEFICIENCY

This rare condition results from the combination of defective chemotaxis with a deficiency in lysosomal proteins such as defensins and lactoferrin. Clinical phenotype is characterized by recurrent bacterial and fungal infections. Specific granules are lacking in neutrophils. Mutations of the transcription factor (CCAAT) enhancer-binding protein (C/EBPϵ), which controls granule protein synthesis, are causing this condition (Lekstrom-Himes and Xanthopoulos 1999).

OXIDATIVE BURST: CHRONIC GRANULOMATOUS DISEASE

Phagocytic cells generate reactive oxygen species by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The enzyme catalyzes the oxygen reduction to superoxide which can be further transformed to H_2O_2 and, by the myeloperoxidase, to HOCl. These reactive oxygen species (ROS) generated in the membrane of phagolysosomes on activation are thought to play a major role in the killing of engulfed bacteria and fungi by neutrophils and macrophages. This is best demonstrated by chronic granulomatous disease (CGD) in which this generation of ROS is defective. Its incidence is estimated at 1:250 000 live births (Winkelstein et al. 2000). Four molecular defects have been characterized (Segal et al. 2000):

1. deficiency in gp91, a membrane cytochrome *b* component encoded by an X-linked gene (*CYBB*) which is defective in 70 percent of cases
2. deficiency in gp22, a second subunit of cytochrome (CYBA) accounting for 5 percent of CGD
3. $p47^{phox}$ (*NFC1* gene), a cytosolic protein that associates to the cytochrome on neutrophil stimulation; its deficiency accounts for about 20 percent of CGD
4. the $p67^{phox}$ (*NFC2* gene), another cytosolic component binding the cytochrome on activation (5 percent of CGD).

Patients with autosomal recessive CGD tend to have a slightly milder phenotype. CGD is characterized by bacterial infections, mostly as abscesses located in lymph nodes, liver, lung, and bones – all tissues or organs rich in macrophages. Typically, catalase-positive bacteria (which destroy endogenously produced H_2O_2), *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia*, and fungi (*Aspergillus* species) are the most frequently encountered pathogens. Of note, non-infectious granuloma can develop in various sites associated with varyingly severe consequences, such as bladder obstruction, pyloric stenosis, colitis, or chronic interstitial pneumonitis. Infections result in a 2.5–5 percent death toll/year of affected patients (autosomal recessive and X-linked form, respectively) (Winkelstein et al. 2000).

The physiopathology concept of this condition has been challenged by Segal and coworkers who proposed, as based on mouse models, that ROS generation is creating an increased anionic charge compensated by entry in a pH-dependent manner of K^+ ions. The rise in ionic strength is stimulating release of proteases, which are possibly responsible for the killing of microorganisms and degradation of debris (Reeves et al. 2002). The same authors have recently shown that catalase-positive and -negative strains of *S. aureus* are equally virulent in CGD mice models, further suggesting that H_2O_2 and chlorinated reagents might not be the primary killer effectors (Messina et al. 2002), thus challenging previous views.

Myeloperoxidase deficiency is a fairly frequent condition, which results in impaired production of chlorinated reagents within phagolysosomes (Nauseef 1990). Interestingly enough, it is mostly an asymptomatic setting except in individuals with diabetes who are prone to candida abscesses (Cech et al. 1979), an observation perhaps further reinforcing Segal's view on how phagocytes kill microorganisms.

NEUTROPHIL GLUCOSE-6-PHOSPHATASE DEHYDROGENASE DEFICIENCY

In very rare patients, the most severe glucose-6-phosphatase dehydrogenase (G6PD) deficiencies lead not only to hemolytic anemia but also to a CGD-like phenotype because G6PD (Mamlok et al. 1987) catalyses the production of NADPH, the required substrate of NADPH oxidase.

The above-mentioned Rac-2 deficiency can also be regarded as an oxidative burst deficiency because Rac-2 is involved in the activation of oxidase activity.

PAPILLON–LEFÈVRE SYNDROME

Papillon–Lefèvre syndrome is characterized by a palmoplantar keratosis associated with a severe periodontopathy and, sometimes, pyogenic liver abscesses. This autosomal recessive disorder is caused by mutations in the cathepsin C gene (Toomes et al. 1999). Cathepsin C processes other proteases, such as granzymes, and may be required in the maturation of proteases necessary to bacterial killing.

A DEFICIENCY OF UNKNOWN MECHANISM: THE HYPER-IGE SYNDROME

The hyper-IgE syndrome (HIES) or Job's syndrome is a rare condition with autosomal dominant inheritance characterized by the association of a dysmorphic syndrome (coarse facies), tooth abnormalities, osteoporosis, and a unique phenotype of susceptibility to infections (Grimbacher et al. 1999a).

These patients suffer from childhood with lung abscesses, and skin, soft tissue, and bone infections associated with very mild inflammatory reaction ('cold abscesses'). *S. aureus* and fungi are the most frequently encountered microorganisms. In the lungs, pneumatoceles are formed as both sequelae and sites of new infections. In most textbooks, HIES is classified as a neutrophil disorder because it has been shown that there is a mild neutrophil chemotaxis disorder in this condition. It is far from clear that this poorly reliable finding contributes to the understanding of HIES physiopathology. There is an HIES locus on chromosome 4 and, so far, almost nothing is known about disease mechanisms (Grimbacher et al. 1999b). The biological hallmark of HIES is an elevated IgE serum level which can be very high in young children (>40 000 IU/ml) and tend to diminish with age, although susceptibility to infections persists. It is thus not known whether hyper-IgE is involved in pathogenesis or is just a surrogate marker. Mystery has been further reinforced by the observation that allogeneic hemopoietic stem-cell transplantation does not cure the disease (Gennery et al. 2000), a finding that suggests that abnormal cells are not of hemopoietic origin. It is thus highly likely that HIES should not in the future be classified within the neutrophil dysfunction chapter!

Disorders of plasmatic proteins

The complement cascade (Figure 2) plays an important role in the early steps of defense against pathogens (preimmune step) as well as in immunized individuals. Actually, most of our understanding of the role of the complement system relies on the identification of the natural defects of almost every one of its components (Walport 2001a, 2001b). The complement system can be activated by three mechanisms, i.e. the classic pathway usually triggered by antigen–antibody complexes, mediated by C1q, -r, -s, and C4 and C2, the alternate pathway triggered by microbial surfaces, mediated by C3, B, and D factors and properdin, and the lectin pathway induced by the mannose-binding lectin (MBL). All three result in C3 activation by cleavage into the C3b opsonin. Also, C3b triggers the formation of the membrane attack complex (MAC) formed by C5–9 components. Deficiencies of C3 activation inhibitors (H and I, which cause a C3 depletion), as well as of the classic and the alternate pathway,

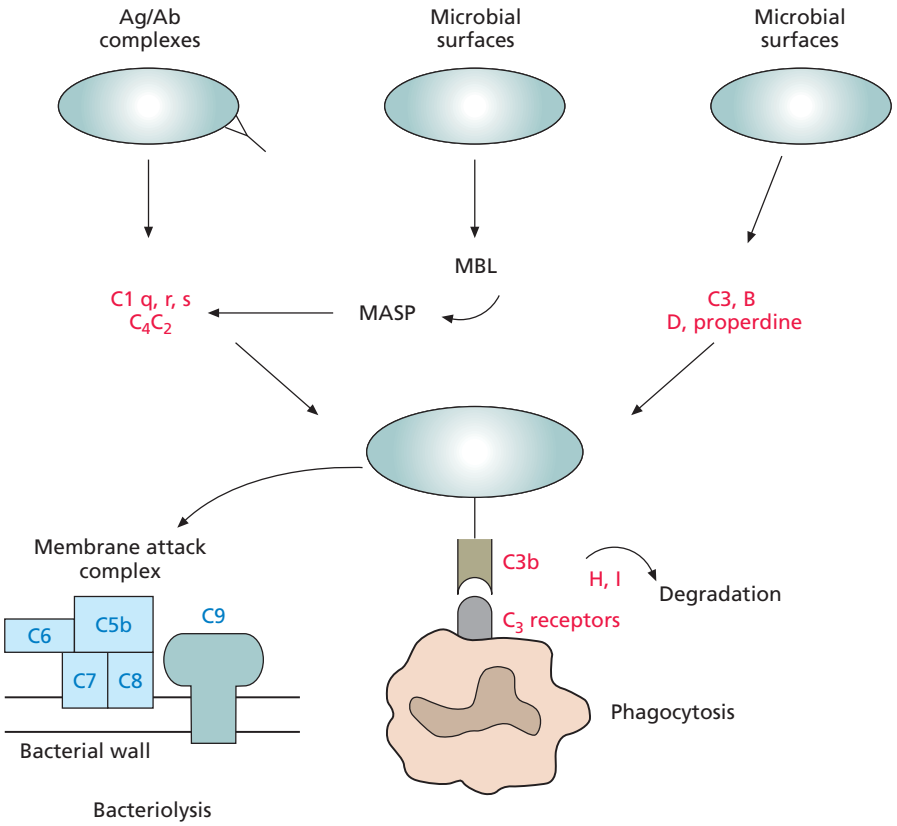

Figure 2 *The complement system. C1q, -r, -s and C4, C2, H, I, C3, B, D: encapsulated bacteria; C5, C6, C7, C8, C9: gram-negative bacteria,* Neisseria meningitis.

except for properdin deficiency, all result in infections by encapsulated bacteria such as *Streptococcus pneumoniae*, *S. pyogenes*, and *Haemophilus influenzae*. Infection can be blood borne or localized to the respiratory tract. These findings underline the role of C3b in facilitating opsonization of encapsulated bacteria. C3 deficiency tends to have more severe consequences given its central role. X-linked properdin deficiency is a very rare condition leading in most cases to fulminant meningitis caused by *Neiserria meningitidis* from the unusual strains y and w135. MBL deficiency may be a susceptibility factor for bacterial and candida infections, although this is still debated (Summerfield et al. 1995). Deficiencies from factors of the MAC lead to susceptibility to infection by *Neisseria* spp., particularly *N. meningitidis*, emphasizing the role of MAC-mediated bacteriolysis in the control of this class of bacteria.

Complement deficiencies are also associated with autoimmune manifestations (systemic lupus erythematosus, vasculitis, glomerulonephritis) because of the role of the complement system in the clearance of antigen–antibody complexes and apoptotic bodies.

Congenital asplenia

Congenital absence of spleen can be rarely observed either as one of the manifestations of situs abnormalities – Ivemark syndrome caused by mutations of the Connexin 43 gene (Britz-Cunningham et al. 1995) or the X-linked *ZIC3* gene (Gebbia et al. 1997) – or as an isolated condition with an autosomal dominant inheritance and variable penetrance. Its cause is still unknown. This condition exposes the individual to fulminant, blood-borne infections caused by encapsulated bacteria, mostly *S. pneumoniae*, thought to be the consequence of the defective splenic macrophage filter.

Undescribed deficiencies of innate immunity

It has now been well established that receptors for structures not expressed by eukaryotes are present in phagocytic and other cells. Toll-like receptors are the most salient example. They serve to signal dangers, induce innate immune response mostly through activation of the factor NFκB, and link innate to adaptive immunity (Barton and Medzhitov 2002). A mouse natural mutant of Toll-R4 has been described and characterized by an absence of response to lipopolysaccharide (LPS), its main ligand (Poltorak et al. 1998). A number of knockout (KO) mice in which a given Toll receptor or associated signaling molecule is absent have been engineered and found to be susceptible to different classes of pathogens. Surprisingly, no human counterpart has yet been

described. It is not known whether this can be explained by unexpected redundancy between receptors and/or associated signaling pathways, or whether they are very rare conditions.

Nevertheless, Galin et al. reported a single case of a patient with defective cytokine production on bacterial challenge associated with bacterial infections and limited inflammatory response (Kuhns et al. 1997). This syndrome, not yet molecularly characterized to our knowledge, is a good candidate to represent a first example of a Toll receptor pathway deficiency.

NEMO/IKKγ deficiency

NEMO deficiency results in a complex syndrome in which both innate and adaptive immune responses are defective (Zonana et al. 2000; Doffinger et al. 2001; Jain et al. 2001). NEMO is a regulatory element, which forms, together with IKKα and -β, the kinase required to phosphorylate and therefore induced ubiquitinylation of NFκB inhibitors (IκBα), thus enabling NFκB complexes to migrate to the nucleus and exert their transcriptional activity (Courtois et al. 2001). NEMO deficiency can impair responses to cytokines such as interleukin (IL) 1 and tumor necrosis factor (TNF) and also potentially some of the Toll-like receptors. This is, however, a more complex syndrome because NFκB is, for instance, also involved in mediating CD40-induced signals in B lymphocytes; thus NEMO deficiency can result in impaired antibody production. These patients present predisposition to infections with encapsulated bacteria but also mycobacteria in more severe cases. It is thus difficult to distinguish which pathway perturbation is responsible for a given type of infection. There is heterogeneity in the consequences of mutations of the NEMO gene (knowing that null mutations are lethal), which adds to the complexity of the analysis.

As NFκB is also involved in signaling from cells outside the immune system, patients may variably present with anhidrotic ectodermal dysplasia, lymphedema, and osteopetrosis.

Natural killer cells

Natural killer (NK) cell function lies at the border of innate and adaptive immunity. The role of NK cell-mediated defense against infections is not yet well known although recent evidence for a role in resistance to cytomegalovirus (CMV) associated with the Ly-49H molecule in mice has been provided (Brown et al. 2001). No such information has yet been obtained from studies on human immunodeficiencies. A single case of NK cell deficiency has been reported. It has been found associated with recurrent infections of the herpes virus group (Biron et al. 1989). However, no molecular mechanism has been described. In addition, recurrent infections, notably with herpes simplex virus, have been described associated with decreased blood NK cell counts and a modified FcγRIIIA (CD16) receptor. Relationship to pathogenesis has not, however, been firmly established (de Vries et al. 1996; Jawahar et al. 1996). Of note, NK cell cytotoxicity impairment, found in patients with perforin deficiency (together with cytolytic T-cell function impairment), is associated with an entirely distinct phenotype (see below).

Susceptibility to human papilloma virusinfection

In rare families, mutations of either of two genes, *EVER-1* and *EVER-2*, have been reported to predispose to infection, with an autosomal recessive inheritance to epidermodysplasia verruciformis (EV). EV consists of chronic skin infection caused by certain strains of human papilloma virus (HPV), e.g. HPV-5, and predisposing to squamous cell carcinomas (Ramoz et al. 1999, 2002). This is a rather unique setting, because these conditions do not create a susceptibility status for other microorganisms. *EVER-1* and *EVER-2* gene products are expressed among other cells in keratinocytes – the target cell of HPV – and could thus probably interfere locally with viral persistence. This is a privileged setting to analyze further local immunity to HPV. It stresses that the genetic susceptibility factor for infections may not be associated with the classic arms of the immune system.

GENETIC DISORDERS AT THE INTERFACE BETWEEN INNATE AND ADAPTIVE IMMUNITY

Susceptibility to mycobacterial infections

In recent years, it has been determined that several conditions are associated with a Mendelian susceptibility to nonpathogenic mycobacterial infection and to some extent to salmonella infections (Casanova and Abel 2002; Ottenhoff et al. 2002). It turned out that all of them impair the IL-12/interferon-γ (IFN-γ) pathway known to be a major inducer and effector of so-called T-helper 1 responses (Figure 3). In about 100 such patients, it was determined that deleterious mutations of IL-12B (IL-12 p40 subunit) (Altare et al. 1998b), IL-12RB1 (IL-12 receptor β subunit) (Altare et al. 1998a), interferon γ receptor β_1 subunit (IFNGR1) (Jouanguy et al. 1996; Newport et al. 1996), IFNGR2 (interferon γ receptor β_2 subunit) (Dorman and Holland 1998), or STAT-1 (transcription factor mediating interferon-induced signaling) (Dupuis et al. 2001) were found.

Clinical severity varies according to several parameters, i.e. type of mutated genes, type of mutations

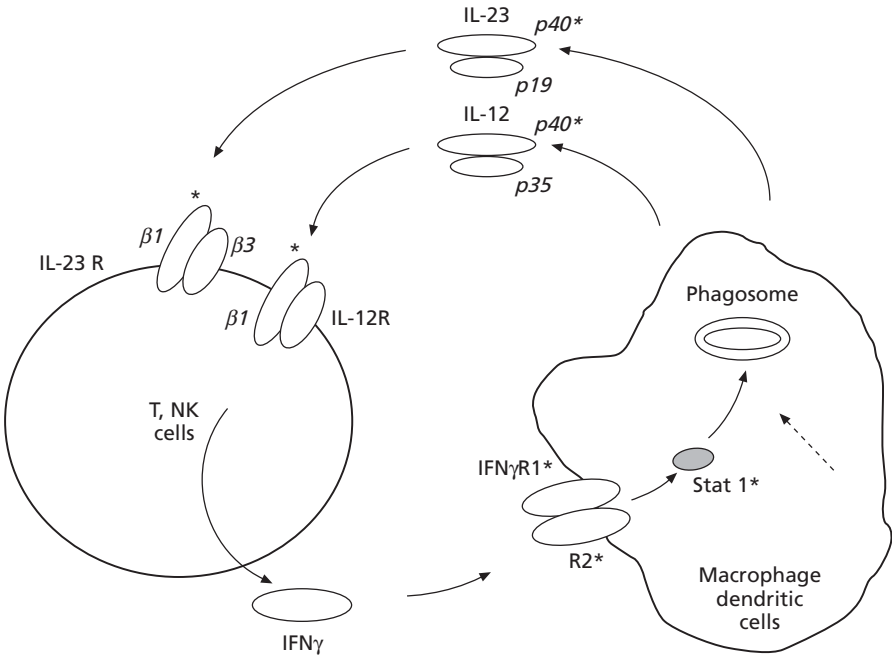

Figure 3 *The interleukin 12/interferon γ (IL-12/IFNγ) axis and its defects. *Described deficiencies. Other elements of the pathway are not shown for sake of simplification. The dotted arrow in the macrophage indicates other components of antimycobacterial immunity unrelated to IFNγ.*

from null to missense mutations, and probability to be infected with nonpathogenic mycobacteria. Associated genetic susceptibility factors could potentially also be involved. Both autosomal recessive and dominant (IFNGR1, STAT-1) inheritance patterns have been described. There are some differences in clinical presentation according to the affected genes. Complete IL-12 and IL-12RB1 mutations tend to lead to less severe mycobacterial infections with a reduced lethality compared with complete IFNGR1 deficiency. In the latter case, there is a heavy toll of early mortality caused by disseminated nonpathogenic mycobacterial infections (BCG or environmental) with a characteristic feature of the absence of typical granulomas around infected macrophages (Pierre-Audigier et al. 1997). It is possible that in the IL-12 axis deficiency, a partial IFN-γ production is preserved, accounting for a milder phenotype. In contrast, deficiency of the IL-12RB1 predisposes not only to mycobacterial infections but also to infection with *Salmonella* spp., a rather uncommon finding in IFNγR1/2 deficiencies. This suggests an additional role of IL-12 in immunity toward *Salmonella*.

A surprising hallmark of these syndromes is the rarity of infections caused by other opportunistic infections such as *Pneumocystis carinii* or *Toxoplasma gondii* and of viral infections, which were observed in only two cases. This contrasts sharply with the known susceptibility of IFNγ-deficient mice to virus challenges. No good explanation can as yet be proposed to account for this apparent discrepancy. Study of Mendelian susceptibility to mycobacterial infections has been determinant in pointing to the major and somewhat selective role of the IL-12/IFNγ pathway in the control of mycobacterial infections in humans. As this condition has also been found in a number of patients without defects in IL-12/IFNγ cytokine/receptor, more remains to be learnt about it. It is also not known whether mutations of these genes with milder consequences, could represent susceptibility elements in multifactorial risk factors for *M. tuberculosis* or *M. leprae*, in addition to the few described such as HLA-II, NRAMP1, and as yet undefined loci (Cooke and Hill 2001; Casanova and Abel 2002; Ottenhoff et al. 2002). In other words, do these Mendelian conditions represent the extreme of a more common spectrum of susceptibility?

GENETIC DISORDERS OF ADAPTIVE IMMUNITY

T lymphocytes

Since the 1960s, the central role of T lymphocytes in adaptive immunity has been recognized. The observation of patients in whom no thymus could be detected (Good 2002) brought a significant contribution to that understanding. Since then, a number of rare conditions characterized by defective T-cell development have been described, including intrinsic abnormalities defined as severe combined immunodeficiency (SCID) and environmental abnormalities related to thymic defects.

SEVERE COMBINED IMMUNODEFICIENCY

Strictly defined, SCID represents a group of rare conditions (about $1{:}10^5$ births) characterized by a complete absence of mature T cells. The presence of circulating maternal T cells, which cannot be rejected, or the presence of autologous T cells in 'leaky' phenotypes can sometimes be misleading (Buckley 2000; Fischer 2000). Absence of functional T cells is incompatible with life beyond the first year, emphasizing the major role of adaptive immune response in humans living in a normal environment.

Patients, after a 2- to 3-month latency, become prone to fungal infections, multiple viral infections in particular causing protracted diarrhea and infections resulting from opportunistic agents such as *Pneumocystis carinii*. Interstitial pneumonitis caused by *P. carinii* is actually the most frequent infection leading to diagnosis (Buckley et al. 1997). Also, live vaccines such as BCG can lead to an overwhelming infection with lethal consequences (Stephan et al. 1993). As a result of the absence of T cells, B cells, when present, are also nonfunctional. Multiple SCID phenotypes have been described according to associated lymphocytic (NK, B lymphocytes) or myeloid defects, inheritance pattern and, more recently, molecular analysis (see Figure 4). They result either from defective signaling through γc-dependent cytokine receptors (γc, JAK-3, and IL-7Rα specific for the IL-7 receptor) blocking T-cell development before T-cell receptor β (TCRβ) gene rearrangement (Sugamura et al. 1995). A purine metabolism pathway deficiency, i.e. adenosine deaminase deficiency (ADA), which leads to dATP accumulation in lymphocyte precursors and premature cell apoptosis (Hershfield and Mitchell 1995), or from defective V(D)J rearrangements of T- and B-cell antigen receptor genes. The lymphoid-specific *Rag-1* or *Rag-2* genes can be mutated as well as the Artemis gene, which encodes a member of the nonhomologous end-joining repair pathway as the DNA-dependent protein kinase, the abnormal element of severe combined immunodeficient mice (Schwarz et al. 1996; Moshous et al. 2001). The other forms of SCID (reticular dysgenesis and CD45 deficiency) are extremely rare. There are no major differences in predisposition to infections according to the different phenotypes.

Atypical forms of SCID have been described as a cause of leaky phenotype related to mutations with milder consequences or, in a few cases of ADA and γc deficiencies, to a spontaneous reverse mutation in a T-cell precursor. Some mutations of the γc gene enable T-cell development whereas T-cell functions are impaired (but not absent). In some cases, responses to certain γc-dependent cytokines are more affected than others. It results in prolonged survival with occurrence of infections usually after 2–5 years of life. Missense mutations of *Rag-1* and *Rag-2* enabling residual recombinatorial activity can result in the emergence of expanded T-cell clones found in the so-called Omenn syndrome (Villa et al. 1998). Although these patients exhibit the same susceptibility pattern to infections as typical SCID, they, in addition, suffer from immunopathological lesions caused by T-cell infiltration of the skin, gut, and other organs. Recently, hypomorphic Artemis mutations were also shown to cause genomic instability predisposing to lymphomas, although the severity of the immunodeficiency is relatively milder compared with typical SCID (Moshous et al. 2003).

THYMIC DEFECTS

DiGeorge syndrome

DiGeorge syndrome consists of an embryopathy variably associating with conotruncal heart malformation, hypoparathyroidy, a dysmorphic syndrome, and an immunodeficiency. The last results from a defective migration of the

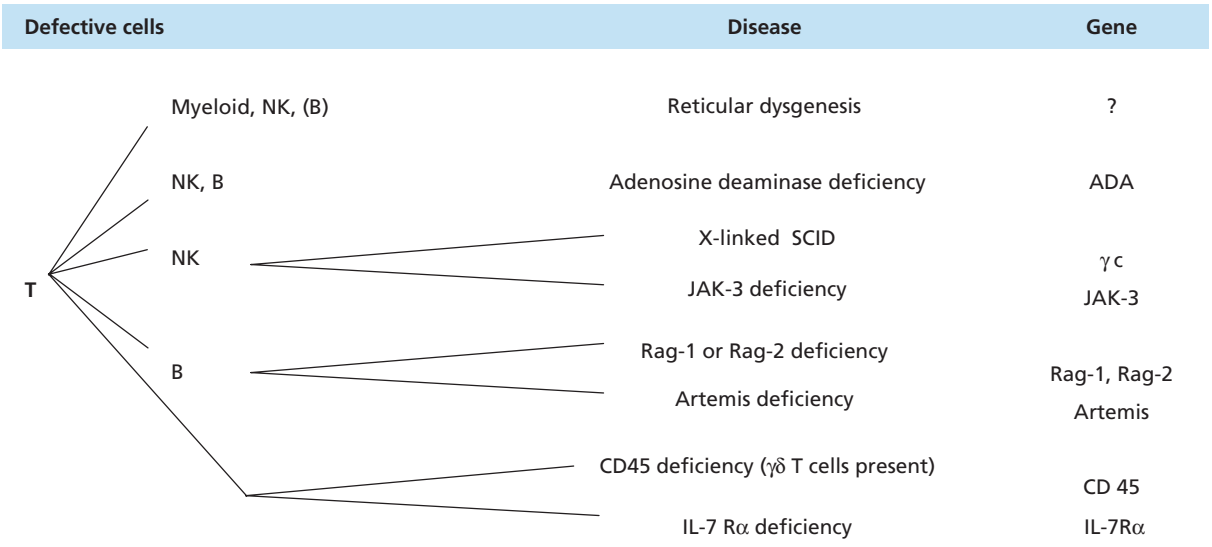

Figure 4 *Severe combined immunodeficiencies (SCID). IL, interleukin; NK, natural killer*

third and fourth embryonic branchial arches, which initiate the formation of the thymic gland (Perez and Sullivan 2002). In the rare cases where the thymic defect is complete (about 1 percent of patients with DiGeorge syndrome), patients have virtually no mature T cells in the periphery, showing that extrathymic T-cell differentiation is very limited in humans. Some patients can nevertheless develop oligoclonal T-cell populations in the periphery. In this setting, clinical consequences are as severe as those of SCID conditions. More frequently, T-cell counts are >500/mm^3 of blood and patients are not prone to infections, although late B-cell immunodeficiency and autoimmune manifestations have been occasionally reported (Perez and Sullivan 2002). The most frequent genetic defect found in patients with DiGeorge syndrome consists of hemizygous interstitial deletions of chromosome 22q11 (Carey et al. 1992; Wilson et al. 1992). There is no correlation between phenotype and genotype because some carriers of a same deletion can be very mildly symptomatic whereas others exhibit a full-blown DiGeorge syndrome including profound T-cell lymphocytopenia. There are several candidate genes located within the deletion which usually spans a 250-kb segment of DNA, TBX1, encoding a transcription factor, and *CRKOL* and *UFD1L* genes are expressed in the branchial arches during embryogenesis (Schinke and Izumo 2001). It is nevertheless unclear whether the phenotype can result from deletion of a single gene. Interstitial chromosome 10q deletion has also been rarely reported with DiGeorge syndrome. Interestingly, it has been recently shown that human leukocyte antigen (HLA)-incompatible, newborn thymic transplantation can lead to the development of functional T cells (Markert et al. 1999). These results imply that both positive and negative selection of thymocytes can be mediated by (recipient) bone marrow-derived cells. Also, expansion of HLA-identical peripheral T cells can be of benefit to DiGeorge syndrome patients with severe immune deficiency.

Nude phenotype

In one Italian kindred, a severe T-cell immunodeficiency has been found in association with baldness. This phenotype, highly reminiscent of nude mice, led to the finding of mutations in the *WHN* (winged-helix-nude gene) (Frank et al. 1999). The *WHN* gene is also mutated in the murine counterpart. It encodes a transcription factor expressed in epithelial cells of skin and thymus. Its defect impairs formation of the thymic microenvironment required for T-cell development because *WHN* induces expression of important genes in the program of thymic epithelial cell differentiation.

OTHER T-CELL IMMUNODEFICIENCIES

There are a number of primary immunodeficiencies associated with the detection of T cells in the periphery with variable functional defects. They all create a severe predisposition to infections, particularly to viruses, often associated with inflammatory bowel disease and autoimmune manifestations (Berthet et al. 1994).

Purine nucleoside phosphorylase

Purine nucleoside phosphorylase (PNP) deficiency is a rare cause of immunodeficiency with autosomal recessive inheritance. It is a purine metabolism error preventing conversion of guanine and leading to the toxic accumulation of dGTP in T-cell precursors. The immunodeficiency is often detectable after several years of life. Autoimmunity is a very frequent associated finding, together with a primary neurodevelopmental delay (Aust et al. 1992).

CD3 deficiencies

Four cases from three families with an immunodeficiency related to defect in CD3 components have been described. Although T-cell counts are within normal range, reduced expression of the TCR/CD3 complex was found associated with low CD8 T cell counts in two patients. Proliferation capacity of T cells driven by anti-CD3 antibody was low whereas antigen-induced responses were partially preserved. In one family, heterozygous mutations of the *CD3ϵ* gene were found whereas, in the other two, distinct mutations of the *CD3γ* gene were identified (Perez-Aciego et al. 1991; Soudais et al. 1993). Patients suffer from variable manifestations from bacterial lung infections to severe autoimmunity.

ZAP-70 deficiency

T-cell activation via the TCR leads to src kinase activation immediately followed by recruitment and activation of the ZAP-70 kinase, which binds to the immunoreceptor tyrosine-based activation (ITAM) motifs of CD3ζ.

A small number of patients with a complete ZAP-70 deficiency have been described (Arpaia et al. 1994; Elder et al. 1994). The clinical phenotype is severe, characterized by early onset of severe opportunistic infections, almost as in SCID patients. Surprisingly, the immunological phenotype is characterized by a selective CD8 T-cell lymphocytopenia. Nevertheless, ZAP-70-deficient CD4 T cells cannot be activated either in vitro or in vivo by antigens.

Other ill-defined severe T-cell deficiencies have been reported, including phenotypes of calcium influx deficiency (Le Deist et al. 1995), functional p56 lck deficiency (Goldman et al. 1998), and defects in cytokine production (Castigli et al. 1993).

IL-2 receptor α chain deficiency

In a single patient, a partial T-cell immunodeficiency has been found in association with lymphoid infiltration in

various organs. This immunodeficiency is caused by defective expression of the IL2-Rα chain (Sharfe et al. 1997). It combines inappropriate T-cell response to infectious agents as well as the lymphoproliferative syndrome, possibly related to defective T-lymphocyte apoptosis.

Caspase 8 deficiency

A homozygous caspase 8 deficiency has recently been described in two patients from a single kindred. There is, as expected, a defect in lymphocyte apoptosis and homeostasis, but, surprisingly, there is also an activation deficiency of T, B, and NK lymphocytes (Chun et al. 2002). It resulted in the occurrence of infections, i.e. recurrent herpes simplex virus infections, pneumonia, and chronic diarrhea. This observation reveals an unexpected, yet poorly understood, role of caspase 8 in the early steps of lymphocyte activation.

HLA class II expression deficiencies

These are a rare primary immunodeficiency with autosomal recessive inheritance characterized by defective expression of the HLA-II molecules. It is associated with a severe immunodeficiency. About 100 cases have been reported so far. Infection onset occurs within the first years of life, usually with recurrent pulmonary infections and chronic diarrhea. Chronic cholangitis caused by cryptosporidia is a frequent complication. Viral meningoencephalitis can also occur. Various autoimmune manifestations can be associated. The immunological phenotype is characterized by a CD4 T lymphocytopenia, defective, antigen-specific, HLA-II-restricted, T-cell responses, and defective antibody production (Klein et al. 1993). Detectable CD4 T cells are thought to be the consequence of residual HLA-II expression by thymic epithelial cells. The disease is genetically heterogeneous and four complementation groups have been described. Corresponding genes have been cloned. Their products are all involved in the transcriptional control of HLA-II genes. In group A, a class II transactivator (CIITA), a protein inducible by IFNγ, is defective. It acts as a master control of the binding of proteins to the promoter (Steimle et al. 1993). In groups B, C, and D, respectively, RFX-ANK/RFX-B, RFX5, and RFX-AP were found to be the deficient factors. They are all transcription factors binding to the so-called X box present in HLA-II gene promoters (Steimle et al. 1995; Durand et al. 1997; Masternak et al. 1998; Nagarajan et al. 1999). HLA-II expression cannot occur in the absence of one of them. A variant of the disease with a milder deficiency was recently shown to be a group B variant (Nekrep et al. 2002). There are, however, some variations in phenotype because milder clinical and immunological expressions were described in a small number of kindreds without clear evidence of a leakiness of HLA-II expression (Wiszniewski et al. 2001). HLA-II expression deficiency is instrumental in demonstrating the in vivo central role of CD4 T cells in the generation of efficient immune responses in humans (Villard et al. 2001).

TAP deficiencies

About 10 cases of either TAP-1 or TAP-2 deficiencies have been reported (de la Salle et al. 1994, 1998). TAP-1/TAP-2 make up the HLA-I-associated peptide transporter that goes from the cytosol into the endoplasmic reticulum. Their deficiencies lead to reduced HLA-I expression, inconstantly associated with CD8 T and NK cell lymphopenia.

Clinical consequences are rather unexpected and consist of chronic bronchopneumonitis and in some patients severe vasculitis possibly caused by NK cell aggression to endothelial cells that weakly express HLA-I (Moins-Teisserenc et al. 1999). No clear susceptibility to viral infections has been shown.

Immunodeficiencies with genomic instability

A number of primary immunodeficiencies have been found to be associated with lymphocytic cytogenetic abnormalities, which were later found to correspond to a genomic instability syndrome. They usually provoke a combined T- and B-cell immunodeficiency of increasing severity with age as a result of progressive lymphocytopenia. They are variably associated with a risk of malignancies, particularly lymphomas.

DNA LIGASE 4 DEFICIENCY

DNA ligase 4 deficiency has first been retrospectively found in a child who developed unexpected severe complications of chemotherapy received as treatment for leukemia. It was then found that missense mutations of DNA ligase 4 cause a complex syndrome associating microcephaly, mild learning disability, dysmorphic syndrome, and a combined T- and B-cell immunodeficiency of variable intensity (O'Driscoll et al. 2001). DNA ligase 4 is involved in the ligation of coding ends in the nonhomologous end-joining (NHEJ) process used for V(D)J rearrangements of TCR and B-cell receptor (BCR) genes. It is thus understandable that hypomorphic mutations lead to a partial T- and B-cell immunodeficiency as also found for Artemis deficiency (see above). A case of DNA ligase 1 deficiency has been described in a patient with growth failure, sensitivity to ultraviolet light, severe combined immunodeficiency, and lymphoma (Barnes et al. 1992).

ATAXIA TELANGIECTASIA

Ataxia telangiectasia (AT) is a condition with autosomal recessive inheritance and a 1:40 000 incidence, associating progressive cerebellar ataxia with degeneration

of Purkinje cells, oculocutaneous telangiectasias, and an immunodeficiency. The last, in most cases, consists of severe IgA, IgG2, and IgE deficiencies causing recurrent sinopulmonary infections. A progressive reduction in T-cell counts is also observed, although usually there is no susceptibility to opportunistic infections. A hallmark of the disease is illegitimate recombination of BCR and TCR genes inducing chromosomal translocations or inversions. It is the consequence of a genomic instability syndrome marked by a loss in the cell cycle checkpoints because AT cells exhibit radio-resistant DNA synthesis (Gatti et al. 1991; Shiloh 1997). These patients are at very high risk for lymphomas and also mucosal carcinomas. The AT gene has been cloned by Shiloh's group. The *ATM* gene encodes a 350-kDa protein of the phosphatidylinositol-3-phosphate kinase family (Savitsky et al. 1995). It acts as a sensor of DNA lesions together with the MRE 11/Nbs-1/Rad50 complex (see below) and activates a number of metabolic pathways involved in DNA repair or inducing cell apoptosis (Shiloh and Kastan 2001).

A minority of patients with a similar phenotype (AT-like disorder or ATLD) carries mutations of the *MRE-11* gene, part of a complex with Nbs-1 and Rad-50 involved in DNA break sensing and repair (Stewart et al. 1999).

NIGMEGEN BREAKAGE SYNDROME

Nigmegen breakage syndrome (NBS) shares some common findings with AT, i.e. abnormal chromosomal rearrangements, immunodeficiency (although more severe in NBS), and predisposition to lymphomas (Weemaes et al. 1981). There are, however, neither ataxia nor telangiectasias. In contrast, NBS patients present with microcephaly and learning disability. There is a profound T-cell lymphocytopenia in NBS with very poor survival. The NBS gene encodes nibrin or nbs-1, a member of the MRE-11/Rad-50/NBS-1 complex, which is activated by *ATM*, binds to DNA double-stranded breaks, and is involved in DNA repair (Carney et al. 1998; Varon et al. 1998).

IMMUNODEFICIENCY, CENTROMERIC INSTABILITY, AND FACIAL ANOMALIES

This rare syndrome with autosomal recessive inheritance associates a facial dysmorphic syndrome and an immunodeficiency. The latter varies from mild hypogammaglobulinemia to severe T-cell immunodeficiency with severe chronic diarrhea (Brown et al. 1995). There is a typical cytogenetic feature consisting of multi-branched chromosomal configurations caused by elongation of secondary constrictions. This abnormality results from defective DNA methylation caused by recessive mutations of the DNA methyl transferase *DNMT3B* gene (Ellis et al. 1995; Xu et al. 1999). The relationship between DNA methylation and immunodeficiency is presently unclear.

BLOOM SYNDROME

This is a rare entity characterized by short stature, light-induced skin lesions, and predisposition to cancer. An immunodeficiency, mostly hypogammaglobulinemia, is often found. This condition is caused by deficiency in the Bloom mutant BLM, a DNA helicase (Xu et al. 1999).

Immunodeficiency with bone abnormalities

Short limb dwarfism or cartilage hair hypoplasia (CHH) is an autosomal recessive disorder associating a short stature with metaphyseal chondrodysplasia, hypoplastic hair, and a T-cell immunodeficiency. This condition is rare and has been mostly described in Finnish and Amish populations, although cases have been found worldwide. The T-cell immunodeficiency varies from a severe T lymphopenia with predispositions to classic infections to a mild, almost asymptomatic immunodeficiency (Makitie and Kaitila 1993). Associated features such as erythroblastopenia, cancer, and Hirschsprung's disease are not uncommon. The CHH-related gene has been recently identified. It encodes an untranslated RNA molecule, which forms part of an RNase ribonuclease mitochondrial RNA processing gene (RMRP) gene complex with endoribonuclease activity. Multiple mutations have been found (Ridanpaa et al. 2001). The physiopathology so far remains obscure.

A severe T-cell immunodeficiency has also been described in association with spondyloepiphyseal dysplasia, nephropathy, cortical blindness, and pigmentary skin changes. This very rare 'Schimke immuno-osseous dysplasia' has recently been shown to be caused by mutations of the *Smarcal* gene (Boerkoel et al. 2002).

Wiskott–Aldrich syndrome

Wiskott–Aldrich syndrome (WAS) is a well-known X-linked inherited condition characterized by the association of a complex immunodeficiency with a bleeding disorder and eczema (Ochs and Rosen 1998). Its incidence is around 1:250 000 live births. There is a spectrum of severity of the disease from isolated thrombocytopenia to a severe immunodeficiency with autoimmune manifestations, which include diffuse vasculitis (McCluggage et al. 1999) and a risk of lymphomas. Patients are prone to infections by encapsulated bacteria, essentially because IgM production is reduced. There is also a susceptibility to viral infections (herpes group particularly) possibly because of a gradual loss in T lymphocytes with age (Ochs and Rosen 1998). The *WASP* gene encodes the *WASP* protein, which is expressed by hemopoietic cells (Derry et al. 1994). *WASP* has multiple interaction sites for protein–protein interactions. In T lymphocytes, *WASP* is involved in rearrangement of the actin cytoskeleton on activation.

WASP-deficient lymphocytes lack surface microvilli and do not normally form filopodal and lamellipodal extensions (Remold-O'Donnell et al. 1996). Polarization of TCR-activated T cells is abnormal (Dupre et al. 2002). It is likely that *WASP* defects impair complex processes involved in activation, migration (in particular response to chemokines), and possibly survival of lymphocytes. There is apparently some degree of correlation between *WASP* genotypes and severity of the phenotypes (Ochs and Rosen 1998). Of note, female cases with WAS have been described in relation either to an abnormal X-chromosome inactivation pattern (predominant inactivation of the X chromosome carrying a normal *WASP* allele) (Parolini et al. 1998) or possibly to an unrelated disease.

Hyper-IgM syndrome: conditions predisposing to opportunistic infections

A 'classic' form of B-cell immunodeficiency is characterized by defective immunoglobulin class switch recombination (CSR) resulting in normal to high levels of serum IgM with very low production of IgG, IgA, and IgE. It is termed the hyper-IgM syndrome (HIgM). Genetic and clinical heterogeneities were noticed a long time ago because there are both X-linked and autosomal recessive forms of HIgM and some patients are prone to opportunistic infections not seen in B-cell immunodeficiencies. These observations led to a progressive delineation of very distinct conditions within the HIgM entity. In this section, only HIgM conditions leading to opportunistic infections are described.

X-LINKED HIGM-CD40L DEFICIENCY

B lymphocytes from X-linked HIgM patients can be induced to switch in vitro to the production of the different Ig isotypes, provided appropriate stimuli are present. This X-linked condition is caused by mutations of the gene encoding the CD40 ligand (CD40L) protein (Notarangelo and Hayward 2000). The latter is expressed at the surface of activated T cells; it is a member of the TNF family. By interacting with CD40 at the B-cell surface, CD40L delivers an essential (but not sufficient) signal for inducing Ig CSR and germinal center formation. However, several patients with CD40L deficiency not only are at risk for bacterial infections of the respiratory tract, as observed in other B-cell immunodeficiencies (see below), but also develop infections related to other mechanisms (Levy et al. 1997). Infected oral ulcerations can be a consequence of neutropenia, frequently found during the course of an infection and probably explained by a CD40-mediated, stress-induced granulopoiesis. More striking is the high risk of CD40L-deficient patients developing infections caused by pathogens that can invaginate in the membrane of epithelial cells, i.e. *P. carinii* and *Cryptosporidium parvum*, causing respectively interstitial pneumonitis and chronic diarrhea. In addition, chronic infections by *Toxoplasma gondii* have been reported. These infections are life-threatening in this setting, e.g. chronic *C. parvum* infection can lead to cholangitis and eventually liver cirrhosis caused by chronic inefficient TNF-mediated inflammation (Ponnuraj and Hayward 2002). These observations stress the role of CD40L in immunity to such pathogens. Mechanism(s) by which CD40L mediates effector functions is not precisely known. It has been shown in mice that CD40-expressing cells of hemopoietic origin are required for efficient immunity to *C. parvum* (Hayward et al. 2001). It could be that CD40L on activated T cells triggers cytokine production, such as IL-12, by CD40-expressing macrophages and/or that CD40-expressing cells trigger an unknown T-cell function. The former mechanism cannot be considered as sufficient because IL-12-deficient patients have a different spectrum of predisposition to infections (see above).

CD40 DEFICIENCY

A small number of patients with autosomal recessive HIgM exhibit a similar spectrum of infections as a consequence of recessive mutations of the CD40 gene, impairing its expression at the surface of B and other cells (Ferrari et al. 2001).

Deficiencies of the perforin-dependent cytolytic pathway

The study of the so-called hemophagocytic syndrome (HPS), which can be a genetically determined condition, has led to the understanding that impairment of the T- and NK cell cytolytic pathway carries rather unexpected consequences. HPS is characterized by polyvisceral infiltration by activated polyclonal T lymphocytes, mostly CD8, associated with macrophage activation (with hemophagocytosis) and massive release of proinflammatory cytokines (IFNγ, IL-1, IL-6, TNFα) (Henter et al. 1991; de Saint Basile and Fischer 2001). HPS occurrence is usually driven by an infection, very frequently by a herpes group infection, among which Epstein–Barr virus plays an important role. There are four known genetic conditions that can induce this HPS. Three of these conditions clearly share an identical immunological phenotype, i.e. defective T and NK cytotoxic activities. These are familial hemophagocytic lymphohistiocytosis (FHL) caused by perforin deficiency (Stepp et al. 1999), Chédiak–Higashi syndrome (CHS) caused by deficiency in the cytolytic lyst protein (Barbosa et al. 1996), and Griscelli's syndrome caused by a deficiency in the small G protein Rab27a (Menasche et al. 2000).

In the first condition, a major cytolytic component – perforin – is missing. In the next two, the intracellular transport of lysosomal secretory granules is defective

leading to impaired exocytosis of cytolytic granules (Stinchcombe et al. 2001). It is striking to note that cytolytic T-/NK cell deficiency is not primarily a condition of high susceptibility to viral infections (as severe T-cell deficiencies are) but rather a disease of faulty contraction of expanded CD8 T cells after antigen stimulation (viral infection). This is a similar picture to the one observed in otherwise phenotype-free perforin$^{-/-}$ mice, once infected with the lymphocytic choriomeningitis virus (Matloubian et al. 1999).

A fourth condition is inherited with an X-linked pattern – X-linked proliferative syndrome (XLP) or Purtilo's syndrome. Affected males can develop a similar HPS syndrome usually once infected by Epstein–Barr virus (EBV), although some others can develop lymphomas and/or hypogammaglobulinemia (Morra et al. 2001). XLP is the consequence of mutations of the *SAP/SH2DIA* gene, which is expressed by T and NK cells and is involved in signaling by membrane molecules of the SLAM family (SLAM and 2B4 particularly) (Latour et al. 2001). SLAM–SLAM homologous interaction, and possibly 2B4 interaction with CD48 at the surface of EBV-infected B cells triggers fyn tyrosine kinase activation (Morra et al. 2001). It is unclear yet what the roles of these signaling pathways are. They have been shown to control T-helper 2 T-cell immune response. Interestingly, in the absence of SLAM-associated protein (SAP), the 2B4 NK cell co-receptor triggers inhibitory signals for cytotoxicity (Parolini et al. 1998). It is thus plausible that HPS as observed in XLP usually driven by an EBV infection is also the consequence of defective cytotoxic T lymphocyte (CTL)/NK cytolytic activity. Much remains to be done to understand its mechanism fully. Of note, there are more genetically determined HPS conditions that have not yet been molecularly characterized.

Other T-cell immunodeficiencies

CHRONIC MUCOCUTANEOUS CANDIDIASIS

Some patients exhibit an exquisite sensitivity to infections by candida species. This condition is known as chronic mucocutaneous candidiasis (Kirkpatrick 1994; Lilic 2002). It can persist as a strictly specific predisposition to candida infections of nails, skin, and mucosae associated with a specific deficiency in T-cell response to *Candida*, or become associated later in life with variable Ig isotype deficiencies. Several patterns of inheritance have been described. Cerebral aneurysms can complicate this condition, possibly as a consequence of a vascular fungal infection (Loeys et al. 1999). A locus has been mapped to chromosome 2 (Atkinson et al. 2001), but so far there is no hint about the molecular mechanism underlying this unique infection predisposition. In some cases, chronic candida infection is associated with autoimmune manifestations particularly targeting endocrine glands. This syndrome is known as autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) Ahonen et al., 1990; Finnish–German APECED Consortium 1997). This rare condition is caused by mutations of the gene encoding the AIRE protein, a transcription factor expressed by thymic medullary epithelial cells (TEC) (Nagamine et al. 1997). It has been recently shown that in AIRE$^{-/-}$ mice, which also exhibit autoimmune polyendocrinopathy, AIRE-deficient TEC cells do not express proteins from mature organs, thus preventing a negative selection of autoimmune T-cell clones (Anderson et al. 2002). This work does not, however, provide an explanation on the mechanism of susceptibility to candida infections.

IDIOPATHIC CD4 LYMPHOCYTOPENIA

This condition resembles AIDS, because it is marked by a profound (<300/mm^3) CD4 T-cell lymphocytopenia, but is not caused by HIV, or by any other known environmental trigger (Smith et al. 1993). Opportunistic infections occur as a consequence of this syndrome frequently associated with inflammatory or autoimmune diseases. No etiology is known. The mechanism of CD4 T-cell lymphocytopenia (premature apoptosis?) remains elusive.

B lymphocytes

Genetic disorders of B lymphocytes predominate by far, because they represent 70 percent of primary immunodeficiency disorders (Figure 5). Bruton (1952) made the very first description of an immunodeficiency in 1952, X-linked agammaglobulinemia. Since then, several syndromes have been described and, accordingly, the role of the different Ig isotypes in infectious immunity has been better understood. Still, the molecular mechanism of the most frequent form(s) of B-cell immunodeficiency, i.e. common variable immunodeficiency and IgA deficiency, remains elusive (see below).

AGAMMAGLOBULINEMIA

Prototypic B-cell immunodeficiency consists of genetic defects impairing B-cell development. Four such conditions have been described leading to the autosomal recessive form of agammaglobulinemia (Gaspar and Conley 2000). An early block in B-cell development results in a complete absence of mature B cells and thus of Ig production whereas the other cells of the immune system are spared. They thus do provide a 'pure' model to study consequences in humans of an absence of B-cell-mediated immunity. Onset of infections occurs in the first year of life after a few months' delay because of the transient protection provided by maternally transmitted IgG. The most frequent sites of infection are the upper respiratory tract, the lower respiratory tract, the

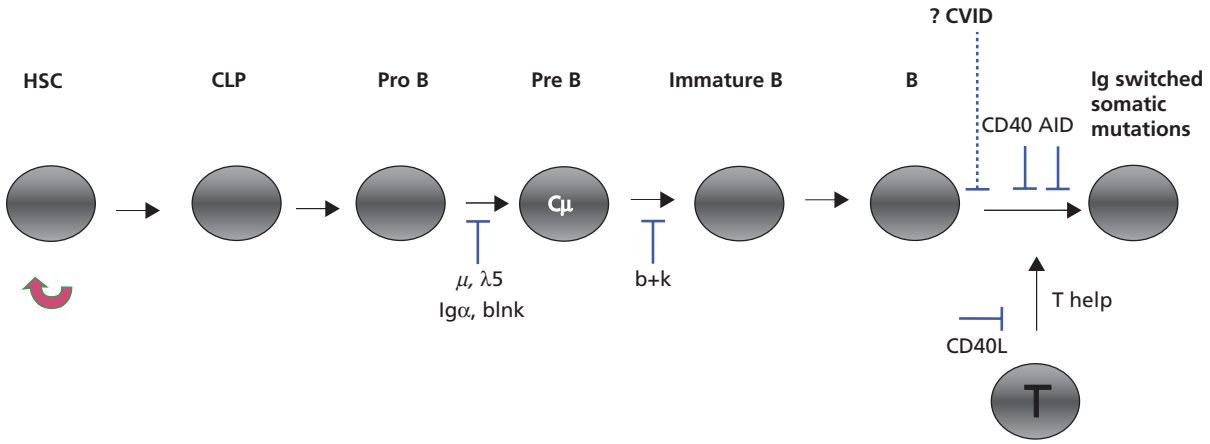

Figure 5 *B-cell immunodeficiencies. HSCs (hemopoietic stem cells) differentiate into putative CLPs (common lymphocyte progenitors), giving rise among other lineages to the B-lymphocyte lineage. There is a block in B-cell development (see text for explanation). CVID, common variable immunodeficiency.*

gut, the skin joints, the eyes, and the central nervous system, and sepsis also occurs (Smith and Witte 1998).

Upper and lower respiratory tract infections are recurrent, progressively leading to chronic infectious lesions of the sinuses and bronchi. Bronchiectasis develops which can lead in the absence of treatment to pulmonary insufficiency (Quartier et al. 1999; Conley and Howard 2002; Lopez Granados et al. 2002). The most frequently encountered microorganisms are *H. influenzae*, *S. pneumoniae*, gram-negative bacilli such as *Pseudomonas aeruginosa*, i.e. encapsulated bacteria. In the gut, *Giardia lamblia* causing a chronic parasitic infection can occur. Chronic mycoplasma infections of the joints have also been described. Agammaglobulinemic patients are not prone to viral infections with a single important exception. Chronic enteroviral infections are indeed not uncommon in this setting. Live attenuated polioviruses can induce a severe chronic neurological disease. Chronic enteroviral infections have a large spectrum of expression from chronic diarrhea, failure to thrive, arthritis, hepatitis, pseudo-dermatomyositis osteomyeolitis, to more commonly meningoencephalitis (Quartier et al. 1999; Conley and Howard 2002; Lopez Granados et al. 2002). It remains by far the most severe infectious complication in B-cell-deficient patients. Enteroviral infection can occur in an otherwise well-treated patient. It is difficult to control. It is still unknown how agammaglobulinemic patients exhibit such a high susceptibility to enteroviral infections despite the normality of their T-cell compartments. Some specificity of enterovirus–host interaction remains obviously unknown.

It is interesting to note that IgG substitution by intravenous or subcutaneous route is highly efficient in preventing most of bacterial infections (unless bronchiectasis or a chronic sinusitis is present) suggesting that IgG can essentially substitute the IgA function at mucosal surfaces. Although it is unclear how IgG diffuses at mucosal surface, this observation may help to understand that many IgA-deficient patients are not particularly susceptible to infections.

AUTOSOMAL RECESSIVE AGAMMMAGLOBULINEMIA

There are four conditions characterized by a complete absence of mature B cells because of a block in B-cell differentiation at the pro-B-cell level (Yel et al. 1996; Minegishi et al. 1998, 1999a, 1999b). Mutations in the μ heavy chain gene, the λ5 surrogate light chain gene, the Igα, or the BLNK genes all result in the same phenotype. The faulty expression of a pre-B-cell receptor (Ig μ heavy chain/surrogate light chain) or of the associated signaling apparatus (Igα/CD89a or blnk) prevents further progression in the B-cell development pathway. These are rare conditions, but other autosomal recessive inherited agammaglobulinemias have not yet reached molecular understanding.

X-LINKED AGAMMAGLOBULINEMIA (BRUTON'S DISEASE)

X-linked agammaglobulinemia (XLA) accounts for about 80 percent of the cases of 'agammaglobulinemia'. It is caused by mutations of the Bruton tyrosine kinase gene (*btk*) (Vetrie et al. 1993; Tsukada et al. 1993). However, in most cases, B-cell development block is not as complete as it is in autosomal recessive agammaglobulinemia. Patients tend to have low but variably detectable serum concentrations of the different Ig isotypes. Peripheral B cells can in some cases be detected in the periphery. Typically, bone marrow B-cell differentiation is blocked at the pre-B-cell level (B cells expressing the μ/pseudo light chain complex at the surface). Therefore, the clinical consequence, although very similar to those of autosomal recessive agammaglobulinemia, tends to have a slightly later onset and be slightly less severe (Lopez Granados et al. 2002).

Phenotypic variability is unexpectedly high because several patients with *btk* mutations exhibit a phenotypic picture reminiscent of common variable immunodeficiency (CVID) (see below), i.e. B cells are detectable in periphery (up to 5 percent lymphocytes) with serum IgG concentration up to 2–3 g/l. This variability is independent of the genotype as observed within affected kindreds (Smith and Witte 1998). The extreme of the spectrum has been described as a pure deficiency in antibody response to polysaccharide antigens (Wood et al. 2001). This is actually reminiscent of the *btk*-deficient murine model, either natural (CBA/N-XID strain) or obtained by *btk* gene inactivation, which essentially exhibits a defect in antibody response toward type 2 T-independent antigens (Rawlings et al. 1993). It is possible that modifier genes (with or without environmental factors) modulate the phenotypic expression of XLA.

The btk kinase belongs to the Itk/Tec tyrosine kinase family. It is expressed by hemopoietic cells but seems essential in B-cell and possibly mastocyte development/function. Mutations affecting essentially all domains of the molecules (PH, TH, SH_3, SH_2, and kinase) have been described; *btk* appears as an essential component for signal transduction from several B-cell receptors (BCR) including surface Ig (antigen-specific BCRs), CD40, and cytokine receptors for IL-5, -6 and -10 (Smith and Witte 1998). Calcium flux after phospholipase C γ (PLCγ) phosphorylation, an essential event in BCR-triggered B-cell activation, is dependent on *btk* integrity.

HYPER-IGM SYNDROMES

As described above, HIgM syndromes are the in vivo consequences of defective Ig class switch recombinant (CSR). Besides CD40L–CD40 deficiencies, there are pure HIgM B-cell immunodeficiency conditions. Defective IgG and IgA production is associated with typical susceptibility to bacterial infections in the respiratory and gastrointestinal tracts (Conley et al. 1994). This is most often accompanied by lymph node enlargement as a consequence of hypertrophic germinal centers, full of proliferating B cells. Further phenotypic characterization of this syndrome has revealed that there is also a defect in IgV region somatic hypermutations (SHM). This condition, called HIgM2 syndrome, has been ascribed to activation-induced deaminase (AID) gene recessive mutations (Revy et al. 2000). AID is selectively expressed in germinal centers. It belongs to an RNA-editing enzyme family. AID is thus necessary for both events of somatic Ig gene rearrangements and somatic mutations required for antigen-driven antibody maturation. It is still not known whether AID indeed edits mARN-encoding protein(s) involved in CSR and SHMs or directly modifies DNA as recently suggested (Petersen-Mahrt et al. 2002). There are still HIgM syndromes that are not accounted for by AID gene mutations. Their study might contribute to a further description of the molecular pathways involved in CSR and SHMs.

COMMON VARIABLE IMMUNODEFICIENCY AND IGA DEFICIENCY

IgA deficiency is the most common form of primary immunodeficiencies but it is frequently an asymptomatic condition. Common variable immunodeficiency (CVID) occurs in 1:25 000 individuals, with onset of symptoms usually during the second or the third decade of life (Hammarstrom et al. 2000). It is characterized by a variably decreased concentration of all Ig isotypes. Patients with CVID and symptomatic IgA deficiency are prone to infections similar to the ones encountered in agammaglobulinemic patients. In addition, however, these patients, during adulthood, become at risk of developing granulomatous lesions, inflammatory bowel disease, autoimmune diseases, and cancer. Inheritance pattern of CVID and IgA deficiency is not entirely clear but it is often compatible with an autosomal dominant pattern with variable penetrance. Both conditions can be found in the same kindred or subsequently in the same individual, suggesting that these two conditions are allelic.

Despite intensive research, there is still very little known about the physiopathology of CVID and IgA deficiency. B-cell counts in blood can be diminished (with a trend to decline). There are variable B-cell phenotype abnormalities, which have led to CVID subclassification (Warnatz et al. 2002). It is not known, however, whether this corresponds to potentially distinct disease mechanisms. In some of the patients, functional T-cell defects have also been observed. It is also not known whether there are primary or secondary T-cell abnormalities. Patients with IgA deficiency very often have anti-IgA antibodies. It has been suggested that anti-IgA antibodies could play a causative role because women transmitting the disease have a higher prevalence of anti-IgA antibodies that could induce IgA deficiencies in offspring (Vorechovsky et al. 1999, 2000). Genetic linkage between CVID and IgA deficiency and the HLA locus has been demonstrated. There is a debate about whether the HLA-III region (between the C4b and the C2 genes) and/or the HLA-II region (HLA-DQ locus) is involved (Schroeder et al. 1998; Vorechovsky et al. 1999).

Of note, drug-induced Ig deficiencies (IgA deficiency or CVID) have been described without known mechanisms. IgA deficiency has been also described in patients with aberrations of chromosome 18.

IGG ISOTYPES DEFICIENCIES

Variable IgG isotypes deficiencies have been reported, as either, very rarely, the consequence of autosomal recessive deletions of IgH genes (IgG1 deficiency) or selective IgG2 and -4 (often with IgA) deficiency

without abnormality of IgH genes (Oxelius et al. 1981). In the last case, there is a selective antibody production deficiency toward the polysaccharide wall of *S. pneumoniae*, *H. influenzae*, and *P. aeruginosa*, which make these patients very susceptible to infections by these bacteria. The relationship between the γ2 gene and specificity of IgG2 antibodies remains puzzling. The physiopathology is unknown. There are family cases suggestive of autosomal recessive inheritance.

Selective antibody production deficiency to polysaccharides without diminution in serum Ig isotypes levels has also been described in a few cases. It has been proposed that such deficiency could reflect a defect in T-independent B1 cell function, but this remains highly hypothetical.

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Acquired immunodeficiencies

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INTRODUCTION

Acquired immunodeficiency represents an emerging medical challenge, the major medical causes of which are summarized in Table 1. In contrast to primary immune deficiencies, which are rare and at constant numbers over the last decades, the numbers of patients with acquired immunodeficiency are dramatically increasing. This is the result not only of the pandemic spread of the human immunodeficiency virus (HIV) (Clinton 2003) but also of rising numbers of patients on immunosuppressive therapy or who have diabetes, leukemia, Hodgkin's disease, or rheumatic disorders, or who are alcohol abusers (Murray and Lopez 1997; Singer and McCune 1999; Mossad et al. 2001; Simon and Fleischhack 2001; Messingham et al. 2002). In addition, older age is a well-known condition of acquired immunodeficiency (immunosenescence), and the numbers of older individuals have been continuously increasing in developed countries (Gavazzi and Krause 2002). In some regions of the world, e.g. Australia, the increasing ultraviolet radiation may also exert immunosuppressive effects (Staples et al. 2003).

Importantly, malnutrition of children in developing countries is still an unsolved problem, leading in these children to immunodeficiency, which considerably increases their susceptibility to the leading infectious causes of death world wide: enteric and pulmonary infections (reviewed by Bhaskaram 2002). It may come as a surprise to many that malnutrition in childhood is less often the result of lack of food than of the enormous burden of infectious diseases, notably diarrheal diseases, that commonly afflicts children in the developing world. Infective diarrhea reduces food intake and increases the loss of important nutrients from the body. After a single infection the child can recover rapidly and may suffer from only a brief period of accelerated height and weight velocity called 'catch-up' growth. In the developing world, however, repetitive episodes of diarrhea are often seen in children, thus greatly impairing their chances of regaining lost growth. The accumulative effect of such episodes greatly influences the growth and mental development of these children, and at the same time also has a profound impact on their immunological defense mechanisms (reviewed by Keusch 1982, 2003).

In addition to the aforementioned causes for acquired immunodeficiency, phases of immunosuppression may also occur in the context of various infectious diseases. Infections with HIV, human T-cell lymphotropic virus 1 (HTLV-1), and measles virus all induce suppression of cellular immune responses, leading to clinically apparent secondary infections and/or tumors. These diseases are of varying epidemiological importance. The horrendous spread of the HIV-1 pandemic is well recognized and the World Health Organization (WHO) has estimated that there were 40 million infections worldwide at the end of 2001, whereas HTLV-1 infection possesses rather regional interest. Sadly, despite the opportunity to prevent measles through effective immunization, about 1.1 million children died in 1990, mostly in developing countries, from measles-related events (Murray and Lopez 1997), and in 1999 the number of deaths caused by measles was estimated as 700 000 (Duke and Mgone 2003). In most cases, secondary infections, such as bacterial pneumonia, often combined with malnutrition, but not measles itself, were the cause of death (reviewed in Schneider-Schaulies and ter Meulen 2002).

Table 1 *Causes of acquired immunodeficiency*

Disease	Suspected immune defect	Frequent infectious diseases
Infection		
HIV	Loss of $CD4^+$ T cells, MHC I downregulation	PCP, toxoplasmosis, CMV infections, microsporidiosis, and others
HTLV-1	Impaired T- and B-cell function	Strongyloidiasis, cryptococcal meningitis, PCP
Measles	Transient lymphopenia, transient reduced Th1 response	Bacterial pneumonia, otitis media, sinusitis
Neoplasms		
Hodgkin's disease	Impaired T-cell function	Infections with intracellular bacteria: TBC, listeriosis, salmonellosis
Multiple myeloma	Hypogammaglobulinemia	Bacterial pneumonia and meningitis
Chronic lymphocytic leukemia	Hypogammaglobulinemia	Bacterial pneumonia and meningitis
Acute myelocytic or lymphocytic leukemia	Granulocytopenia	Infections with extracellular gram-positive and gram-negative bacteria, fungal infections
Other conditions		
Diabetes	Impaired cell-mediated immunity and phagocyte function	Fungal infections (candidiasis, mucormycosis), urinary tract and soft tissue infections
Alcoholism	Impaired cell-mediated immunity and phagocyte function	TBC, soft tissue infections, osteomyelitis
Old age (elderly)	Impaired T- and B-cell function	Bacterial pneumonia, TBC, urinary tract and soft tissue infections, viral diarrhea, *Clostridium difficile* toxin-induced colitis
Splenectomy[a]	Reduced immune response against bacteria	Bacterial pneumonia, bacterial meningitis, babesiosis
Iatrogen (therapy-induced immune suppression)		
Drugs frequently used in autoimmune diseases		
Glucocorticoids	Impaired monocyte and T-cell activation	Fungal infection, bacterial pneumonia
Cyclophosphamide[b]	Reduces antigen driven T- and B-cell response	Bacterial and fungal infection, PCP, CMV infections
Methotrexate[b]	Inhibition of antibody production, lymphopenia, monocytopenia	Bacterial and fungal infection, PCP, CMV infections
Anti-TNF-α[c]	Suppression of inflammatory response, reduces granulocyte and macrophage activation	Reactivation of TBC, bacterial and fungal infections
Drugs frequently used in organ transplant recipients		
Ciclosporine, FK-506 (tacrolimus), rapamycin	Inhibition of T-cell activation (inhibition of IL-2 gene activation)	Reactivated viral infections (especially herpesvirus group), infections with respiratory syncytial virus, adenovirus, and parvovirus B19
Azathioprine	Inhibition of antibody production, lymphopenia, monocytopenia	Bacterial and fungal infections
Anti-CD3, anti-CD4, anti-IL2	Inhibition of T-cell activation	PCP, reactivated viral infections (especially herpesvirus group), infections with respiratory syncytial virus, adenovirus, and parvovirus B19
Drugs and methods frequently used in tumor therapy		
Cytostatic therapy	Reducing the number of B cells, T cells, macrophages, and granulocytes	CMV infections, progressive multifocal leukencephalitis (JC virus infection), PCP
Radiation	Suppression of number and function of T and B cells	CMV infections, progressive multifocal leukencephalitis (JC virus infection), PCP

CMV, cytomegalovirus; IL, interleukin; MHC, major histocompatibility complex; PCP, *Pneumocystis carinii* pneumonia; TBC, tuberculosis; TNF, tumor necrosis factor.

a) If splenectomy can be planned patients should receive haemophilus, meningococcal, and pneumococcal immunization before.

b) Patients who are treated with this immunosuppressive drug (either cyclophosphamide or methotrexate) in combination with glucocorticoids should receive trimethoprim–sulfamethoxazole as prophylaxis against PCP.

c) Patients with positive tuberculin test or TBC in their history treated with anti- TNF-α should receive isoniazid as prophylaxis against TBC reactivation.

The scope of this chapter is to concentrate on these three viral infections which have various similarities but also significant differences with respect to their effects on cellular immune functions. Importantly, HIV and HTLV-1 are retroviruses and integrate via a provirus in the human genome; thereby they can persist and potentially cause life-long damage to the immune system. In contrast, effective immune responses are usually induced against measles virus; these lead to viral clearance, and immunosuppression lasts for only a limited period of time.

HIV INFECTION

The virus

In 1983 scientists from the Pasteur Institute in Paris recovered the causal agent of acquired immune deficiency syndrome (AIDS) from lymph nodes of an asymptomatic individual with generalized lymphadenopathy (Barré-Sinoussi et al. 1983). This new retrovirus was finally named HIV-1. HIV-1 belongs to the lentiviruses in the taxonomic classification of retroviruses (Gelderblom et al. 1985). The lentiviruses are complex spherical viruses characterized by a unique virion morphology with cylindrical or conical cores (Gelderblom et al. 1987). The proviral genome of HIV-1 encodes *gag*, *pol*, and *env* genes characteristic of all known retroviruses. Both ends of the proviral genome are flanked by noncoding direct-repeat sequences called long terminal repeats (LTR). LTRs mediate proviral integration and contain *cis*-acting regulatory elements important for viral transcription, viral mRNA processing, and reverse transcription. The LTRs of HIV-1 also contain the binding site for NF-κB, which is present in the nuclei of activated human T lymphocytes and can stimulate both basal and Tat-induced levels of HIV-1 replication (Nabel and Baltimore 1987). The genome structure of human pathogenic retroviruses is more complex than that of other retroviruses, containing several additional open reading frames encoding for nonstructural regulatory or accessory proteins. Some of these viral proteins may contribute to immunosuppression.

Tat is an indispensable viral protein, which increases the steady-state levels of viral RNA by a multiplication factor of around several hundred (Dayton et al. 1986). Tat seems to be involved in T-cell activation that results in an increased viral transcription in two ways: increasing the concentration of cellular factors CDK9 and cyclin T1 for Tat activation and increasing the activation of NF-κB. Tat may also inhibit antigen-induced, but not mitogen-induced, proliferation of peripheral blood mononuclear cells (PBMC) and therefore play a role in immunosuppression (Viscidi et al. 1989).

Nef seems to have a positive influence on the progression to AIDS in vivo (Kestler et al. 1991). It may ensure survival of HIV-1 through distinct mechanisms of immune evasion and antiapoptosis (for a review, see Fackler and Baur 2002). In vitro, several functions of Nef have been described:

- Nef downregulates the cell surface expression of CD4 (Garcia and Miller 1991). Studies with HIV-Nef mutants in severe combined immunodeficiency (SCID) mice implanted with human fetal thymus revealed that the functional domain that is required for CD4 downregulation correlates with Nef-mediated enhancement of viral pathogenesis (Stoddart et al. 2003). The ability of Nef to downregulate CD4 may influence the function of T-helper cells and contribute to acquired immunosuppression of HIV-infected patients.
- Nef downregulates the cell surface expression of major histocompatibility complex (MHC) class I molecules (Schwartz et al. 1996). This MHC I downregulation may limit the ability of cytotoxic T lymphocytes (CTL) to recognize and eliminate HIV-infected cells and may be one way that the virus escapes the host's immune response (Collins et al. 1998). More sophisticated Nef selectively downregulates certain MHC I molecules, avoiding downregulation of those (HLA-C and HLA-E), that would induce attack by natural killer (NK) cells (Cohen et al. 1999).
- One important function of Nef may be the activation of lymphocytes (Luo and Peterlin 1997). A natural occurring mutant simian immunodeficiency virus (SIV) nef allele induces extensive T-cell activation and proliferation (Du et al. 1995). This mutant is only an arginine-to-tyrosine mutation in the *nef* gene, which results in a sequence reminiscent of an immunoreceptor tyrosine-based activation motif (ITAM). Such motifs in the cytoplasmic tails of T- and B-cell receptors are essential for lymphocyte activation. The SIV *nef* mutant seems to activate T-cell signaling (Luo and Peterlin 1997). Macaques infected with this SIV strain rapidly develop a fatal disease, leading to the death of the animals because of severe gastrointestinal symptoms (Du et al. 1995). Nef expression in macrophages induces expression and secretion of the chemokines macrophage inflammatory protein (MIP) MIP-1α and MIP-1β, which are capable of activating lymphocytes (Swingler et al. 1999).

Natural course of HIV infection

The course of HIV-1 infection is usually divided into three phases: (1) the primary HIV-1 infection, (2) the chronic asymptomatic phase and (3) AIDS; the individual pattern and course of the disease may, however, be highly variable.

THE PRIMARY HIV-1 INFECTION

Primary HIV-1 infection (PHI) is rarely diagnosed and represents a self-limited disease. However, recognition

of this early phase of HIV-1 infection is important for two major reasons. First, the severity of PHI predicts progression to AIDS; it is therefore thought that treatment of PHI may prevent or retard progression to AIDS. Second, from the public health perspective the diagnosis of PHI is important because these patients are highly infectious and early diagnosis will prevent the spread of disease to the patients' sexual partners. The severity of symptoms during PHI is highly variable; about 12 percent of seroconverting patients are hospitalized (for a review, see Apoola et al. 2002).

At the beginning of PHI, patients are usually seronegative for anti-HIV antibodies; the p24 antigen-capture assay and polymerase chain reaction (PCR) for the HIV genome are positive. Viremia levels reach extremely high values, and high titers of infectious virus can be isolated from the blood. During the first 5–10 days of PHI, the patients show a characteristic lymphopenia which affects both $CD4^+$ and $CD8^+$ T cells. In this phase, $CD4^+$ T cells may drop to levels as low as those observed in AIDS, and in vitro tests of both B and T cells show impairment. Nevertheless opportunistic infections in this short period are rare (for a review, see Apoola et al. 2002).

During PHI the virus spreads to several organs of the body including the lymph nodes, central nervous system, skin, and mucosa. Therefore, patients with PHI may present variable symptoms. Cutaneous manifestations are frequent and include the classic maculopapular rash or ulcers, which may occur at genital, oral, and esophageal mucosae (Cooper et al. 1985; Schacker et al. 1996). Ulcerations in other parts of the gastrointestinal mucosa are more rarely observed. A common feature of PHI is the so-called 'mononucleosis-like illness', which is characterized by fever, pharyngitis, arthralgia, myalgia, and lymphadenopathy (for a review, see Apoola et al. 2002).

THE CHRONIC ASYMPTOMATIC PHASE

The primary HIV-1 infection is followed by a long-lasting phase of clinical latency of about 8–12 years with a median time of 9.8 years (Bacchetti and Moss 1989). However, 10–20 percent of HIV-infected individuals progress more rapidly to the final stage of the disease in about 5 years (Phair et al. 1992). This group is called 'rapid progressors'. At the other extreme, 5–15 percent of HIV-infected people, called 'slow progressors', will remain in this stage of the disease for about 15 years (Sheppard et al. 1993). This wide variability of the natural course of the disease implicates different driving forces, including genetic factors of the host as well as virological factors (Fauci 1996). In this chronic phase of HIV infection no symptoms are present. Virus replication and $CD4^+$ T cells remain relatively stable during this time. Virus replication and the accumulation of virus trapped by follicular dendritic cells occur in lymph nodes, however, where a progressive structural and functional deterioration takes place, resulting in an impairment of specific immune response over time (Pantaleo et al. 1993). Finally, there is steady decrease of $CD4^+$ T cells with an increasing risk of developing infections (Figure 1). Acquiring an AIDS-defining opportunistic infection leads to the transfer to the end-stage of the disease.

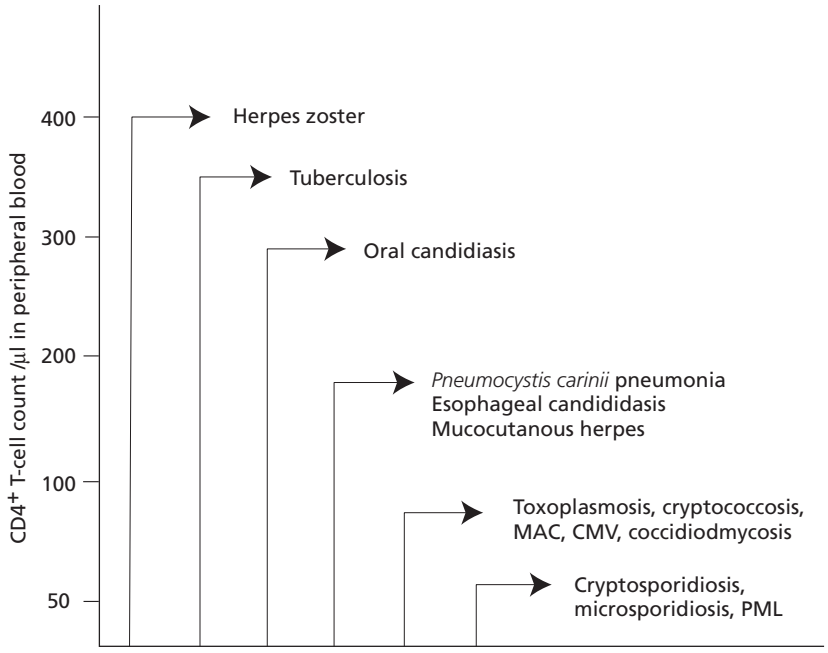

Figure 1 *Association between opportunistic infections and CD4 $^+$ T-cell count.* CMV, *cytomegalovirus; MAC,* Mycobacterium avium complex; *PML, progressive multifocal leukoencephalopathy.*

AIDS

AIDS defines the end-stage of HIV-1 infection. Before the introduction of highly active antiretroviral therapy (HAART), this phase of the disease leads to death within 1–2 years (Bacchetti et al. 1988). The major causes of death are opportunistic infections resulting from a breakdown of the immune system. The hallmark of the disease is a steady decline in the number of $CD4^+$ T cells, which correlates with an increasing number of certain opportunistic infections (Figure 1). Fortunately, the introduction of HAART has significantly decreased morbidity and mortality of HIV-1 infection (Palella et al. 1998).

Immune dysfunctions

$CD4^+$ T LYMPHOCYTES

The progressive loss of peripheral $CD4^+$ T lymphocytes is a hallmark of HIV infection, coinciding with the failure of the immune control toward a variety of infectious pathogens including HIV-1 itself. The underlying mechanisms, however, are still a matter of controversial debate. The increased proliferation and loss of CD4 cells were interpreted as the death of virally infected cells being succeeded by a homeostatic proliferation of new T cells (Ho et al. 1995; Wei et al. 1995). Nevertheless, these conclusions have been questioned based on the observations that (1) relatively small numbers of T cells are actually productively or latently infected in vivo (Haase 1999), (2) significant numbers of uninfected cells apparently die (Finkel et al. 1995), and (3) the decline of CD4 cells in the peripheral blood is not necessarily paralleled by similar changes in the lymphoid tissues (Rosok et al. 1996). As a result, a different scenario was proposed where a general activation of the immune system leads to dysregulated homeostatic processes.

Different T-cell labeling techniques in vivo and mathematical models have been applied to investigate whether the T-cell proliferation is the immunological response to previous T-cell death, or whether cell death is the result of previous activation and proliferation, and T-cell loss is exclusively caused by continuous destruction or additional to impaired production (Douek et al. 2003). The data, however, are inconclusive, indicating complex processes influenced by various host and viral factors, e.g. CD4 T-cell dysfunctions, viral cytopathicity, cytotoxicity, apoptosis, activation-induced cell death (AICD), altered T-cell migration patterns, and impaired T-cell production.

$CD4^+$ T-cell dysfunction

Cytokine production by $CD4^+$ T cells is profoundly perturbed in HIV-1 infection leading to qualitatively and quantitatively different cytokine secretion patterns (see section on Cytokines). In addition, proliferation to alloantigens, e.g. tetanus toxoid and purified protein derivative, and importantly also to HIV antigens is decreased, which is the result of a progressive loss of memory CD4 T cells (Miedema et al. 1988; Clerici et al. 1989; Meyaard et al. 1994). In fact, HIV-specific $CD4^+$ T cells in the peripheral blood are easier to detect by intracellular cytokine staining than by proliferation (Pitcher et al. 1999). The reduced responsiveness of $CD4^+$ T cells in HIV-1 infection correlates with a decreased expression of CD28 and CD40 ligand (Borthwick et al. 1994; Chirmule et al. 1995), as well as changes in the $CD4^+$ T-cell receptor repertoire (Connors et al. 1997; Gea-Banacloche et al. 1998). Notably, patients with long-term non-progressing (LTNP) disease exhibit strong antigen-specific proliferative responses (Rosenberg et al. 1997).

Cytopathicity

HIV-1 preferentially infects rapidly expanding, activated, naïve (HIV-1 specific) CD4 T cells (Douek et al. 2002), and kills the cells by apoptosis independently of the expression of Fas (Gandhi et al. 1998), although necrotic cell death of HIV-1-infected T cells has recently been reported (Lenardo et al. 2002). Additional cytopathic effects involving viral gp120 and CD4 can lead to giant cells (syncytia) that can contain uninfected T cells (Lifson et al. 1986; Sodroski et al. 1986). In vivo this mechanism may be of less importance except for the central nervous system where giant cell formation is more frequently seen in the context of HIV-1 infection (Williams and Hickey 2002). Although viral burden in lymphoid tissue is greater than in peripheral blood where only small numbers of infected cells are usually seen, virus-mediated killing alone most probably does not account for the decline of peripheral CD4 T cells and the development of AIDS.

Cytotoxicity

Death of HIV-infected cells may also be caused by cytotoxic immune responses. There is an early and strong induction of HIV-1-specific CTL responses, and antibody-dependent cellular cytotoxicity (ADCC) may contribute to the reduction of HIV-1 infected cells. Nevertheless, as most of these immune responses are also, at least to some extent, impaired in chronic HIV-1 infection, they most probably do not primarily drive the depletion of $CD4^+$ T cells. Interestingly, lysis of uninfected $CD4^+$ T cells by $\gamma\delta$ T cells has recently been described, thus suggesting a role for this subset in the CD4 T-cell decline in HIV-1 patients (Sindhu et al. 2003).

Apoptosis

In vitro, T cells from HIV-1 infected individuals die of spontaneous apoptosis, which is further enhanced on

stimulation (Groux et al. 1992; Meyaard et al. 1992), whereas spontaneous T-cell apoptosis is reduced in cells from LTNPs (Liegler et al. 1998). In vivo, apoptosis of infected, and to a greater extent of uninfected, bystander CD4 T cells has been noted (Finkel et al. 1995) and attributed to direct, e.g. virus-induced apoptosis of infected cells, and indirect, e.g. macrophage-mediated, apoptosis of uninfected CD4 T cells (Herbein et al. 1998).

Fas (CD95) expression on T cells from HIV-1-infected individuals is increased and its ligation induces apoptosis (Kobayashi et al. 1990; Katsikis et al. 1995). After the interaction of Nef with the T-cell receptor ζ chain, HIV also induces Fas ligand expression on T cells (Xu et al. 1999), whereas Fas ligand expression is increased on macrophages in the lymphoid tissue from HIV patients (Dockrell et al. 1998). Apoptosis of uninfected T cells can occur on interaction with Fas and secretion of tumor necrosis factor (TNF) α (Badley et al. 1997).

Moreover, cross-linking of CD4 with gp120 efficiently primes CD4 T cells for Fas-mediated apoptosis (Banda et al. 1992). Monocytes can bind immune-complexed gp120 via CD16, and thereby interact with CD4 on T cells and induce Fas-dependent apoptosis (Orlikowsky et al. 1997; Oyaizu et al. 1997). Importantly, various HIV proteins, e.g. Nef and Vpu, can downregulate CD4, thus avoiding death of infected $CD4^+$ T cells through this mechanism (Willey et al. 1992; Salghetti et al. 1995). Fas-independent mechanisms might contribute to accelerated T-cell apoptosis (Badley et al. 2000; Gougeon 2003). Thus, increased apoptosis of uninfected (and infected) T cells may critically add to the HIV-induced immunopathogenesis. Regulation of apoptosis in HIV-1 infection seems, however, to be complex, because various HIV gene products have been attributed to pro- as well as antiapoptotic functions (Badley et al. 2000; Gougeon 2003).

Activation-induced cell death

There is increasing evidence for a critical role of activation of the immune system and the induction of T-cell proliferation and, as its physiological consequence, AICD of uninfected cells in the immunopathogenesis of HIV infection. A prerequisite for AICD is the interaction of Fas and its ligand, and the expression of both molecules is significantly increased in HIV-1 infection, as discussed above.

Studies in HIV-1 patients before and after antiretroviral therapy suggested that T-cell proliferation is mainly driven by immune activation, suggesting AICD as an additional mechanism of CD4 cell depletion besides virus-mediated killing (Hazenberg et al. 2000). This immune activation is primarily the result of antigenic stimulation by HIV-1 itself, as well as opportunistic infections (Orendi et al. 1998; Cohen Stuart et al. 2000), correlating both with apoptosis and better with disease progression than plasma viral burden (Gougeon et al. 1996; Giorgi et al. 1999). Consistent with these findings are data obtained from HIV-2 infected patients. Here, lower levels of T-cell activation and apoptosis correlate with a better clinical prognosis, whereas HIV-2 patients with a similar level of immune activation as seen in HIV-1 infection also suffer from CD4 T-cell depletion despite significantly lower viral loads (Michel et al. 2000; Sousa et al. 2002). Similarly, both rhesus macaques and sooty mangabeys develop high viremia after SIV infection, but, although the macaques ultimately succumb to simian AIDS, mangabeys do not develop immunodeficiency correlating with differences in cellular and humoral immune responses (Kaur et al. 1998). It is interesting that the numbers of $CD25^+CD4^+$ T lymphocytes (regulatory T cells?) increase in infected mangabeys, whereas this cell population declines in macaques. Finally, although chimpanzees can be infected with HIV-1 but rarely develop immunodeficiency, increased levels of apoptosis and immune hyperactivation were seen in two animals who did develop a progressive loss of $CD4^+$ T cells after HIV-1 infection (Davis et al. 1998). The critical impact of immune activation on T-cell homeostasis was recently demonstrated in a murine study using CD70 transgenic mice where severe T-cell depletion, induced by chronic activation of CD27, led to lethal *Pneumocystis carinii* infections (Tesselaar et al. 2003).

Reciprocally, in chronic HIV-1 infection, the activation of latently infected cells favors the local transmission of virus in the lymphoid tissue, thereby supporting the viral life cycle (Grossman et al. 1998).

Altered T-cell migration patterns

Exposure of resting $CD4^+$ T lymphocytes to HIV-1 upregulated the homing receptor CD62L (L-selectin) on the cellular surface, thereby supporting the migration of these cells to the lymphoid tissue of SCID mice (Wang et al. 1997). Interestingly, signaling through these homing receptors (but not through other surface molecules) led to apoptotic cell death of immigrated CD4 T cells (Wang et al. 1999). Likewise, reinfused $CD4^+$ blood T lymphocytes of HIV-1 patients rapidly migrated and homed to lymph nodes and bone marrow at increased levels (Chen et al. 2002). Therefore, HIV-induced alterations of T-cell migration and homing may add to the decline of peripheral CD4 T cells in HIV-1 infection.

Impaired T-cell production

Regenerative dysfunction or failure of the bone marrow is commonly seen in late-stage HIV-1 patients, leading to suppressed hemopoiesis. Its origin, however, is often multifactorial, i.e. besides HIV-1 infection itself, opportunistic infections and the application of myelosuppressive drugs may considerably contribute to this development (Moses et al. 1998). As infection of $CD34^+$ hemopoietic progenitor cells is apparently rather low,

HIV-1-induced alterations of bone marrow functions are mostly the result of infected auxiliary cells and perturbations of cytokine secretion. In addition, dysfunction of the thymus caused by HIV-1 infection has been attributed to impaired T-cell production (McCune 2001).

In summary, HIV-1-induced immune activation and changes in T-cell homeostasis most probably lead to increased T-cell proliferation to maintain sufficient numbers of infectable CD4 T cells at the principal sites of viral replication, i.e. the lymphoid tissues, followed by cell death of infected and uninfected cells. Various mechanisms appear to contribute synergistically to the development of $CD4^+$ T-cell immunodeficiency that is paralleled by the decline of these cells in the periphery. The impact of these mechanisms on HIV-1 immunopathogenesis may vary in different populations of infected individuals, e.g. neonates and adults.

$CD8^+$ T LYMPHOCYTES

Specific $CD8^+$ CTLs combat HIV-1 infection by both lysis of target cells via the granule exocytosis pathway and the secretion of β-chemokines, i.e. RANTES (regulated on activation, normal T cell expressed and secreted, CCL5), MIP-1α (CCL3), and MIP-1β (CCL4), which block viral entry at the site of the co-receptor (Gandhi and Walker 2002). Other crucial soluble mediators secreted by CTLs and involved in inhibition of viral replication include the interleukin (IL)-16 (Baier et al. 1995) (see section on Cytokines) and α-defensins (Zhang et al. 2002).

An effective viral strategy to avoid lysis of infected cells is the emergence of escape mutants where changes in the epitopes interfere with peptide binding to the MHC molecule, leading to unrecognized target cells (Johnson and Desrosiers 2002). In addition, the phenotype and function of $CD8^+$ T cells can be altered and impaired at multiple levels. More sensitive in vitro assays have been developed for better detection of antigen-specific interferon (IFN)-γ-secreting cells in the blood or lymphoid tissues, because monitoring of tetramer-positive, HIV-specific cells does not necessarily provide information on the functional status of the cells. On application of these methods, significant numbers of HIV-specific CTLs unable to produce IFN-γ indicated impaired cellular functions (Goepfert et al. 2000; Shankar et al. 2000), whereas opposing results were found by others (Appay et al. 2000; Goulder et al. 2000). Recent data suggest a better correlation between tetramer staining and IFN-γ secretion early after infection, compared with later stages of the infection (Kostense et al. 2002). In addition, the impaired cytolytic capacity of $CD8^+$ T cells has been related to significantly lower levels of perforin in CTLs in the blood and lymphoid tissues from HIV-1 patients (Andersson et al. 1999; Appay et al. 2000).

Defects in $CD8^+$ T-cell-mediated immune responses in HIV-1 infection have also been detected at the level of antigen presentation. The viral protein Nef can downmodulate MHC I molecules on infected cells, resulting in reduced peptide presentation and thereby protection against killing by CTLs (Schwartz et al. 1996; Collins et al. 1998). In addition, downmodulation of the CD3 ζ chain expression and decreased expression of the protein tyrosine kinases and the signal-transducing molecules Lck, Fyn, and ZAP-70 in peripheral CD8 cells from HIV-infected individuals contributes to impaired T-cell receptor (TCR) signaling and is associated with disease progression (Stefanova et al. 1996; Trimble and Lieberman 1998). At the same time, the co-stimulatory molecule CD28 is downregulated on these cells (Vingerhoets et al. 1995; Trimble et al. 2000).

Other phenotypic distinctions of HIV-1-specific CD8 T cells are a persistent CD27 expression suggesting impaired maturation of the cells (Appay et al. 2000), and an enhanced expression of the activation markers HLA-DR and CD38 (Giorgi et al. 1994). The expression of CD38 correlates with rapid CD4 depletion whereas the presence of $HLA\text{-}DR^+CD38^-$ cells is associated with a better disease prognosis. Of additional importance may be the expression of HLA-class I-specific inhibitory receptors (iNKR) (De Maria and Moretta 2000). Similar to NK cells, $CD8^+$ T lymphocytes can express molecules from different families of receptors that can downmodulate T-cell functions, e.g. cytolytic activity, upon MHC I binding. In HIV-1-infection, increased expression of killer cell Ig-like receptors (KIR), as well as receptors from the lectin-type CD94/NKG2 family, has been shown for $CD8^+$ T cells. These $CD8^+iNKR^+$ T cells may further contribute to the HIV-1-induced impairment of cellular immune functions by enhanced secretion of IL-4 and IL-5 (De Maria et al. 2000). Notably, $CD8^+$ T cells from LTNPs express similar levels of iNKR as $CD8^+$ T cells from uninfected controls (Costa et al. 2003).

$CD8^+$ T cell homeostatic defects in HIV-1 infection comprise alterations in migration, proliferation, and cell death. Effector CTLs accumulate at considerably lower numbers in lymphoid tissues, the sites of the predominant viral replication (Pantaleo et al. 1997), and these migratory defects could be caused by a reduced expression of homing receptors to lymphoid tissue, e.g. CCR7 (Chen et al. 2001). A high turnover of CTLs has been observed in HIV-1-infected individuals, ultimately leading to the decline of HIV-1-specific and -unspecific CD8 cells (Sachsenberg et al. 1998; Kovacs et al. 2001). Death of CTLs has been attributed to lymphocyte-reactive autoantibodies or apoptosis induced by FasL-expressing HIV-infected cells (Wang et al. 1998; Mueller et al. 2001). Remarkably, HIV-1-specific $CD57^+CD8^+$ T lymphocytes were unable to proliferate on antigenic stimulation, but died of activation-induced apoptotic death (Brenchley et al. 2003).

The loss of CD4 help caused by the decline of CD4 T cells in the course of the disease might have a significant

impact on the failure of CD8-mediated HIV-1 control (Altfeld and Rosenberg 2000). HIV-1-specific proliferative responses correlate with levels of CTLs and inversely correlate with plasma viral load. In late stages of HIV-1 infection and in the absence of HIV-1-specific CD4 T cells, despite larger numbers of HIV-1-specific CTLs, the effector activity of these CTLs is markedly reduced (Spiegel et al. 2000). In vertically infected children, impaired CTL functions were seen in those with low CD4 counts but also in children younger than 3 years, indicating that the development of effective CD8 T-cell responses may take years (Sandberg et al. 2003). In summary, the data suggest considerably impaired (mainly HIV-1-specific) effector functions of $CD8^+$ T lymphocytes in HIV-1-infected individuals.

DENDRITIC CELLS

Dendritic cells (DC) can be subdivided into two phenotypically and functionally distinct populations, i.e. myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC). Myeloid DCs comprise most of the DC subsets characterized to date; potent antigen-presenting cells (APC) in the blood and peripheral tissues from where they migrate via the afferent lymphatics to the lymph nodes. In contrast, pDCs represent a unique cell population, able to secrete large amounts of type I interferons on stimulation and found only in the blood and lymphoid tissue, to which they migrate via the high endothelial venules. Both DC populations are of particular interest in the context of HIV-1 infection for their potent immunostimulatory capacities, although direct effects of HIV-1 on both subsets might significantly promote HIV-1-induced immunosuppression (Weissman and Fauci 1997; Siegal and Spear 2001; Frank and Pope 2002).

Myeloid DCs are probably the first cells that encounter HIV-1 during mucosal transmission of the virus (Steinman et al. 2003). Immature mDCs, such as Langerhans' cells (LC) in the epidermis, are located in the periphery directly under the epithelial layers and express the receptors required for viral entry, i.e. CD4 and chemokine receptors, at an immature state of differentiation, mainly CCR5 (Zaitseva et al. 1997). In addition, DC-SIGN (CD209), a recently identified C-type lectin receptor on the cell surface, can bind HIV-1 envelope glycoprotein gp120 and transfer the virus to T cells (Geijtenbeek et al. 2000). Its in vivo relevance remains controversial, however, because vaginal, but not rectal, epithelial mDCs are $CD209^-$ (Soilleux and Coleman 2001; Jameson et al. 2002), and, at least in the SIV macaque model, monocyte-derived DCs can transmit the virus independently of CD209 (Wu et al. 1999). Other lectins, such as langerin, might therefore primarily serve as HIV-1-binding sites on epithelial mDCs whereas emigrated DCs and pDCs may predominantly bind gp120 via CD4 (Turville et al. 2002). After binding and/or internalization of HIV-1, mDCs can hand over the virus to the $CD4^+$ T-lymphocyte compartment, thereby readily initiating viral replication in these cells either at the site of infection or in the draining lymph nodes. Viral replication in mDCs themselves is inhibited on maturation of the cells (Bakri et al. 2001). After the establishment of infection, only low numbers of infected mDCs, compared with $CD4^+$ T cells, can be found in the tissue and lymphoid organs (Steinman et al. 2003).

Although pDCs might significantly contribute to the early immune activation in HIV-1 infection, particularly through the secretion of IFN-α (Ferbas et al. 1994), they also co-express CD4 and various chemokine receptors (Penna et al. 2001), can be infected with HIV-1 in vitro (Patterson et al. 2001), and are therefore also possible targets of direct HIV-1 infection. Although the literature is controversial on this subject, both mDCs and pDCs may be impaired in numbers and/or functions in chronically HIV-1-infected individuals (Chehimi et al. 2002; Barron et al. 2003), and proviral DNA was recently detected in both mDC and pDC subsets in vivo (Donaghy et al. 2003). Thus, although both antigen-presenting mDCs and IFN-α-secreting pDCs are most probably involved in the innate and adaptive immune responses against HIV, dysfunctions of these cells may contribute to the immune suppression during HIV-1 infection, in particular in later stages of the disease.

MONOCYTES AND MACROPHAGES

Monocytes and macrophages express CD4 and, depending on their stage of differentiation, various chemokine receptors, and therefore serve as potential targets for HIV-1. Only low numbers (< 1 percent) of HIV-1-infected monocytes can be detected in the peripheral blood and bone marrow of HIV-1 patients (Kedzierska et al. 2003). Likewise, the in vitro infectibility of freshly isolated monocytes is limited and considerably less than in macrophages. In vitro culture and activation/differentiation of monocytes, however, allow a productive, yet noncytopathic, infection. There are conflicting data about whether macrophages contain increased amounts of HIV-1 as such compared with monocytes in HIV-1-infected patients. However, tissue macrophages can become a source of vigorous viral production in the context of opportunistic infections (Orenstein et al. 1997). Moreover, monocytes from HIV-1-infected patients produce high amounts of various proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, which have been shown to promote replication of HIV-1 in vitro (Poli et al. 1990a, b).

A decline in chemotaxis and the in vitro survival of monocytes isolated from the peripheral blood of HIV-1 patients has been noted early (Smith et al. 1984; Muller et al. 1990b). Similarly, a decreased oxidative burst activity of monocytes and monocyte-derived macrophages was found (Muller et al. 1990a, b; Spear et al. 1990). Monocytes from HIV-1 patients also express fewer

co-stimulatory molecules than cells from uninfected controls, coinciding with impaired T-cell-stimulatory capacity (Miedema et al. 1988; Dudhane et al. 1996). Recent flow cytometry studies from whole blood obtained from HIV-1-infected patients indicated an activated phenotype of monocytes in vivo (increased CD11b and decreased CD62L, L-selectin, expression, as well as increased actin polymerization), enhanced H_2O_2 production, and decreased expression of the adhesion molecules sialyl-Lewis X and CD31 related to the disease stage of the patients (Elbim et al. 1999; Pillet et al. 1999).

Phagocytosis of *Staphylococcus aureus* by monocytes from AIDS patients was decreased (Pos et al. 1992), and ADCC and cytotoxic activity, via CD4 against gp120/41-expressing cells by HIV-1-infected macrophages, and monocytes from HIV-1-infected individuals, were reduced (Baldwin et al. 1990; Ahmad and Menezes 1996). How ever, normal or increased chemotaxis, phagocytosis, and superoxide anion release by monocytes from HIV-1 patients (Nielsen et al. 1986; Bandres et al. 1993), as well as normal or increased phagocytosis by alveolar macrophages, have also been reported (Musher et al. 1990; Gordon et al. 2001).

The macrophage mannose receptor (MMR), a pattern recognition molecule and C-type lectin, is involved in recognition and binding of *Pneumocystis carinii* (now known as *P. jiroveci*), an opportunistic pathogen frequently seen in AIDS patients. Consequently, the downregulation of the MMR in alveolar macrophages isolated from HIV-1-infected patients, which has been related to a Tat-mediated reduction of the receptor promoter activity, interferes with these functions (Wehle et al. 1993; Koziel et al. 1998; Caldwell et al. 2000). The additionally impaired oxidative burst response and an altered cytokine secretion by these cells in response to *P. carinii* may critically contribute to patients' increased susceptibility to *P. carinii* pneumonia (Kandil et al. 1994; Koziel et al. 2000). Further defects of macrophage antifungal activity may exist against *Histoplasma capsulatum*, *Aspergillus fumigatus*, and *Candida* spp. (Baldwin et al. 1990; Kedzierska et al. 2003).

Similarly, HIV-1-infected macrophages and monocyte(s)-derived macrophages isolated from AIDS patients displayed defects in phagocytosis and intracellular killing, together with reduced H_2O_2 production, of opsonized *Toxoplasma gondii*, another opportunistic infectious agent in HIV-1 patients (Delemarre et al. 1994; Biggs et al. 1995). Phagocytosis of opsonized *T. gondii* has been shown to be Fc receptor (FcR) dependent, and FcRs as well as complement receptors are actually decreased on macrophages on in vitro infection with HIV-1 and on monocytes from HIV-1-infected individuals, possibly related to changes in intracellular tyrosine phosphorylation (Kedzierska et al. 2003).

In summary, various HIV-1-related alterations of monocyte/macrophage phenotype and functions have been reported. Methodological problems may arise, however, from the evaluation of functions of primary tissue cells, such as macrophages, after in vitro generation, differentiation, and infection. These problems, as well as different experimental approaches and additional effects on monocyte/macrophage functions, e.g. by drug treatment, may account for some conflicting results.

B LYMPHOCYTES

HIV-1 infection exerts profound effects on the B-lymphocyte compartment, i.e. polyclonal B-cell activation with spontaneous secretion of HIV-1-specific as well as HIV-1-nonspecific antibodies, leading to hypergammaglobulinemia, accompanied by decreased B-cell responses to mitogens in vitro (Lane et al. 1983; Shirai et al. 1992). These in vivo and in vitro effects have been attributed to a newly appearing B-cell subpopulation expressing reduced levels of CD21 (Moir et al. 2001).

Viral gp120 has been shown to bind to B cells expressing immunoglobulin VH3 gene products and activate these as a superantigen (Berberian et al. 1993). This is followed by a decrease of this cell subset with changes being more prominent in activated 'memory' rather than resting B cells. It may contribute to impaired responses to bacterial infections and partly explain the frequency of pneumococcal infections in which B cells expressing VH3 genes are important for the antibody repertoire against polysaccharides (Janoff et al. 1992; Scamurra et al. 2000). In addition to viral components, membrane-bound TNF-α on HIV-1-infected $CD4^+$ T lymphocytes may critically be involved in these B-cell perturbations (Macchia et al. 1993).

Immaturity and activation of peripheral B cells in HIV-1 infection coincide with a loss of $CD27^+$ memory B cells (Martinez-Maza et al. 1987; De Milito et al. 2001) and elevated serum levels of soluble CD27 (Widney et al. 1999). This could be the result of an enhanced expression of the CD27 ligand CD70 by activated T lymphocytes, thereby stimulating memory B cells and driving their differentiation to Ig-secreting plasma cells resulting in hypergammaglobulinemia (Nagase et al. 2001). Virions bound to follicular DCs in lymph nodes might significantly contribute to the observed B-cell activation (Zamarchi et al. 2002), whereas activated B cells themselves can promote viral expression in T cells by TNF-α and IL-6 secretion (Rieckmann et al. 1991). Further, alterations in the B-cell compartment of HIV-1-infected individuals comprise significantly less IgA secretion at mucosal sites (Kotler et al. 1987), elevated IgE levels (Wright et al. 1990), and reduced HLA-DR expression by B cells (Ginaldi et al. 1998).

The chronic B-cell activation and concomitant loss of T-cell function promote the genesis of B-cell lymphoma in AIDS patients, and cytokine homologues encoded by additional viral pathogens, such as vIL-10 by Epstein–Barr virus and vIL-6 by the human herpesvirus 8 might significantly contribute to this development

(Martinez-Maza and Breen 2002). Thus, in HIV-1-infected patients several functional abnormalities of B lymphocytes and Ig production are seen that often precede the characteristic decline of peripheral $CD4^+$ T cells.

NEUTROPHIL GRANULOCYTES

Several studies have demonstrated altered neutrophil functions in HIV-1 patients. Increased oxidative burst activity was detected in neutrophils from HIV-1 patients (Murphy et al. 1988; Bandres et al. 1993), whereas others reported on unchanged or even decreased production of reactive oxygen intermediates, possibly resulting from previous activation in vivo (Nielsen et al. 1986; Roilides et al. 1990; Chen et al. 1993; Pitrak et al. 1993; Flo et al. 1994; Wenisch et al. 1996). Similarly, increased phagocytosis of *Staphylococcus aureus*, *Escherichia coli*, and *Candida* spp. (Bandres et al. 1993; Wenisch et al. 1996), but also a reduction in phagocytosis of bacterial and fungal pathogens, were reported (Pos et al. 1992; Gabrilovich et al. 1994). Several studies suggested considerably decreased intracellular killing of bacteria or fungi by neutrophils from HIV-1-infected individuals (Ellis et al. 1988; Murphy et al. 1988; Roilides et al. 1993; Wenisch et al. 1996), whereas two groups reported on unaltered killing of *S. aureus* and *Candida* spp. (Pos et al. 1992; Cassone et al. 1997). Further defects in neutrophils from HIV-1-infected individuals include reduced chemotaxis (Valone et al. 1984), accelerated neutrophil apoptosis (Pitrak et al. 1996), and a reduced 5-lipoxygenase metabolism, leading to lower leukotriene production (Coffey et al. 1998). Remarkably, a soluble factor in the serum of HIV-1 patients reduced the ability of neutrophils to opsonize bacterial pathogens (Ellis et al. 1988). Beside some neutrophil dysfunctions, HIV-1-patients may have neutropenia which occurs either induced by infection or, more often, as a result from myelosuppressive therapies.

In conclusion, the literature on potential dysfunctions of neutrophil granulocytes in HIV-1 patients is inconclusive, most probably the result of methodological differences. In addition, the patients included in these studies may not have been comparable over the stages of disease and pharmacological treatments with possible side effects on neutrophil functions. Nevertheless, non-neutropenic HIV-1-infected individuals apparently do not have invasive mycoses, such as aspergillosis or candidemia, to the same extent as patients who are neutropenic as a result of chemotherapy.

INNATE CYTOTOXIC CELLS (NK CELLS AND γδ T CELLS)

Natural killer cells are critically important in the early combat of viral infections. In HIV-1 infection NK cell-mediated direct lysis of infected target cells, ADCC, and cytokine secretion (especially of IFN-γ and TNF-α) represent important mechanisms that interfere with viral replication. Similar to NK cells, T lymphocytes bearing the γδ TCR are cytotoxic and can release proinflammatory cytokines. These cells constitute a small proportion (up to 10 percent) of peripheral $CD3^+$ T cells, and, in contrast to other immune cells, they recognize phosphorylated nonpeptidic microbial metabolites and alkylamines. Both ID="12332"NK and γδ T cells can additionally interfere with HIV-1 replication by the secretion of chemokines; in the case of NK cells an inverse relationship between this function and the level of viremia has been described (Malkovsky et al. 2000; Levy 2001; Kottilil et al. 2003).

There are various studies demonstrating increasingly impaired NK cell functions in HIV-1-infected individuals correlating with disease progression. Qualitative defects in cytotoxicity of NK and lymphokine-activated killer (LAK) cells in HIV-1-infected patients appear early during HIV-1 infection and are accompanied by a low NK cell responsiveness to IFN-α (Brenner et al. 1993; Ullum et al. 1995, 1999). A deficiency of tubulin rearrangement may significantly contribute to the reduced potency of the cells to kill target cells (Sirianni et al. 1988).

Binding of Ig to the FcγRIII (CD16) on NK cells initiates ADCC. A loss of cytolytic $CD16^+$ NK cells in the peripheral blood was described early, however (Brenner et al. 1989), and explains the profound defects of ADCC found in HIV-1-infected individuals (Brenner et al. 1991; Ahmad et al. 1994). Essential signaling molecules involved in the CD16-dependent cytolysis, i.e. ζ molecules, were found to be decreased in NK cells from HIV-1 patients (Geertsma et al. 1999). Moreover, a selective depletion of primarily cytotoxic, mature, $CD56^{dim}$ NK cells without major changes in the cytokine-secreting $CD56^{bright}$ population correlates with reduced NK cell activity (Hu et al. 1995; Tarazona et al. 2002). Simultaneously increased numbers of a more immature $CD16^+CD56^-$ NK cell subset may indicate a defective differentiation of NK cells in HIV-1 infection.

An important strategy of HIV-1 to escape NK cell-mediated lysis is the selective downregulation of HLA-A and -B, leaving HLA-C and -E on the cellular surface, thereby avoiding NK cell cytotoxicity (Cohen et al. 1999). Further phenotypic changes involve the expression of inhibitory cellular receptors on NK cells which can dampen their function on binding to MHC I molecules. C-type lectin receptors of the CD94/NKG2 family are upregulated on NK cells in HIV-1 infection whereas the results regarding KIR expression are controversial (Andre et al. 1999; Galiani et al. 1999).

Alterations also occur on the level of γδ T cells in HIV-1 infection. The majority (60–95 percent) of peripheral γδ T cells in healthy individuals express the Vγ9 and Vδ2 TCR chains and inhibitory NK cell receptors, mostly of the CD94/NKG2 complex, but lack both CD4 and CD8 molecules. In HIV-1 infection, this subset is considerably diminished and displays serious functional defects, including reduced secretion of IFN-γ

and TNF-α, which may contribute to the increased susceptibility of HIV-1 patients to opportunistic infections (Poccia et al. 1996; Wallace et al. 1997). At the same time, $V\delta1^+$ cells are significantly expanded in both bone marrow and peripheral blood of HIV-1-infected individuals (De Paoli et al. 1991; De Maria et al. 1992; Rossol et al. 1998). These cells represent an activated, potentially cytotoxic population, fully capable of proinflammatory cytokine secretion (Boullier et al. 1997), and a recent study suggests that these cells may contribute to HIV-1-associated immunopathogenesis through depletion of uninfected $CD4^+$ T lymphocytes (Sindhu et al. 2003). An inverse correlation of cytotoxicity with CD4 counts has also been reported for NK cells (Ahmad et al. 2001). Thus, alterations of subpopulations and/or functions of NK and γδ T cells may contribute to the overall immunosuppression observed in HIV-1 patients.

CYTOKINES

The secretion of IL-2, IL-12, and IFN-γ has been associated with protective immune responses to HIV-1; however, secretion of IL-2 is significantly reduced in HIV-1 patients, possibly as a result of the increased susceptibility of IL-2-producing Th1 cells to apoptosis (Clerici and Shearer 1994; Ledru et al. 1998). Similarly, IL-12 is produced in PBMCs significantly less from HIV-1 patients (Chehimi et al. 1994). The percentages and absolute numbers of Gag-specific $IFN\text{-}\gamma^+IL\text{-}2^+CD4^+$ T lymphocytes in the blood correlate inversely with viral loads in LTNPs (Boaz et al. 2002), and low IFN-γ secretion (by $CD8^+$ T cells and others) was related to disease progression (Ullum et al. 1997). Restoration of immune functions under antiretroviral therapy of HIV-1-infected infants is associated with increased IFN-γ and IL-2 secretion (Reuben et al. 2002).

The loss of the previously discussed cytokines and/or increased levels of IL-4 and/or IL-10 may favor disease progression in both HIV-1-infected infants and adults. In particular, IL-10 secretion has been attributed to T-cell dysfunction, disease progression, and poor prognosis in HIV-1 infection (Clerici and Shearer 1994). Notably, whole blood cultures recently revealed a reduced secretion of IL-10 as well as of TNF-α, IFN-γ, IL-1, IL-12, and IL-2 on stimulation with lipopolysaccharide (LPS) and phytohaemagglutinin (PHA), correlating with progression of HIV-1 (Ostrowski et al. 2003).

In HIV-1 infection altered secretion is also seen for other cytokines. Levels of IL-7 are increased, most probably as part of a homeostatic mechanism in response to T-cell depletion, and a reduced expression of its receptor (CD127) was found on $CD8^+$ T lymphocytes from HIV-1-infected individuals (Fry et al. 2001; MacPherson et al. 2001; Napolitano et al. 2001). Although IL-15 could potentially exert some beneficial effects on functions of various immune cells in HIV-1 infection (Waldmann and Tagaya 1999), it also stimulated the expression of inhibitory NK cell receptors on $CD8^+$ T and NK cells (Mingari et al. 1997, 1998). In addition, high IL-15 serum levels in HIV patients, which correlate with the extent of hypergammaglobulinemia, point towards a role for IL-15 in the pathogenesis of HIV infection (Waldmann and Tagaya 1999).

Levels of IL-16 are increased during the asymptomatic phase of HIV-1 infection, followed by a significant decline of IL-16 serum concentrations in disease progression (Amiel et al. 1999). IL-16 is secreted by CD8 cells and capable of inhibiting HIV-1 infection in CD4 T cells by repressing the HIV-1 promoter activity (Baier et al. 1995; Maciaszek et al. 1997). The reduction of activation-induced cell death of lymphocytes by IL-16 may contribute to a suggested protective role in HIV infection (Idziorek et al. 1998). T-cell clones from LTNPs produce significantly larger amounts of IL-16 than cell clones from asymptomatic or AIDS patients (Scala et al. 1997).

Both IFN-α and IFN-β possess similar in vitro activity against HIV-1. Paradoxically, high levels of IFN-α and IFN-inducible protein 10 are detected in HIV patients, associated with advanced disease (Stylianou et al. 2000). In addition, an acid-labile form of IFN-α with reduced antiviral activity in vitro and associated with poor disease prognosis has been described (DeStefano et al. 1982; Gendelman et al. 1992). Therefore, these data indicate that the altered production of various cytokines may add to the observed defects in cellular immune responses in HIV-1 patients.

Particular immune dysfunctions in distinct immune compartments

LYMPH NODES

One of the characteristic clinical signs of HIV infection is the development of generalized lymphadenopathy. Studies on lymph node biopsies revealed at least three distinct histological patterns: follicular hyperplasia, follicular involution, and lymphocyte depletion (Tenner-Racz et al. 1985; Biberfeld et al. 1987; Racz et al. 1991). Similar changes were found in the spleen (Falk et al. 1988). It must be emphasized that the changes described in lymph nodes from HIV-infected patients are not specific because follicular hyperplasia and follicular involution, in particular, can also be found in other pathological conditions such as bacterial infections, and in rheumatoid arthritis (Ewing et al. 1985; Stanley and Frizzera 1986). Changes in lymph nodes, however, mirror the progression of the disease, with implications for the pathogenesis of AIDS (Pantaleo et al. 1993).

Immunpathogenesis

HIV reaches the lymph nodes during PHI. The virus is trapped by follicular DCs and replicates in $CD4^+$ T cells.

HIV particles can be demonstrated in the germinal centers of lymph nodes by electron microscopy and immunohistology (for a review, see Pantaleo et al. 1993). Adhesion of HIV virions to the follicular DC network in lymph nodes and germinal centers may contribute to the B-cell activation observed during HIV infection. It could be further demonstrated that lymph nodes are the major reservoir for HIV in the human body (for a review, see Pantaleo et al. 1993). Indeed, in the early stage of HIV infection, levels of viral burden and replication are far greater in lymph nodes compared with peripheral blood. During this time about 25 percent of $CD4^+$ T cells in germinal centers are infected with HIV (for a review, see Pantaleo et al. 1993). In the early and intermediate stages of the disease viral particles in lymph nodes are restricted to germinal centers with follicular DCs. HIV accumulates in germinal centers complexed with Ig and complement trapped by the follicular DCs, in agreement with their role in antigen clearance and presentation (for a review, see Pantaleo et al. 1993). Follicular DCs in germinal centers induce increased expression of the HIV-1 co-receptor CXCR4 on surrounding $CD4^+$ T cells, thereby facilitating infection of these cells by HIV. In contrast to their central role in induction of protective immune response in other infections, follicular DCs in HIV infection promote and enhance the spread and replication of HIV by presenting the replication-competent virus to susceptible cells such as $CD4^+$ T cells and macrophages (for a review, see Pantaleo et al. 1993).

As antigen-activated lymphocytes migrate into and through lymph nodes, they may come into contact with HIV immune complexes on the surface of follicular DCs and infect themselves. This may explain the slow but steady decrease of $CD4^+$ T cells in the peripheral blood (for a review, see Pantaleo et al. 1993). In late stages of the disease germinal centers involute and the network of follicular DCs is destroyed. Thus, the virus is no longer trapped in the lymph nodes and can quickly spread unchecked, resulting in viremia, characteristic of late-stage disease – AIDS. Furthermore, the absence of an intact network of follicular DCs in the germinal centers of lymph nodes results in an impaired immune response toward other infections by an inadequate antigen presentation necessary for the maintenance of T- and B-memory responses (for a review, see Pantaleo et al. 1993). Besides the direct reduction in the number of follicular DCs in the germinal centers of lymph nodes during infection with HIV/SIV, a reduced chemokine production and a reduced expression of markers that are involved in the trafficking of follicular DCs may further contribute to progressing systemic immunodeficiency in the course of HIV/SIV infection (Choi et al. 2003).

THYMUS

The thymus is not necessary for normal T-lymphocyte function in adults because a subset of mature post-thymic peripheral T lymphocytes is self-renewing (Stutman 1978). However, when the normal T-lymphocyte pool is destroyed by infection with HIV, the thymus may be necessary for complete regeneration of T lymphocytes. In late stages of HIV-1 infection, pronounced lymphocyte depletion and destruction of the thymus are observed (Schnittmann et al. 1990). Work on CD45 isoform expression by the T-cell subset has provided evidence for defective thymopoiesis in AIDS because peripheral $CD4^+$ T cells, which appear after HAART in AIDS patients, are derived from a regeneration of peripheral, post-thymic, $CD45RO^+$, memory T lymphocytes and not from $CD45RA^+$, naïve T cells produced in the thymus (Ho et al. 1995).

As in lymph nodes, three different histopathological patterns were determined.

Thymitis

HIV-induced thymitis is characterized by irregularly shaped follicles, $CD8^+$ T-cell infiltration of the follicles, and the start of follicular DC network fragmentation (Prevot et al. 1992). Thymic epithelial cells are injured early in infection as seen in HIV-infected humans (Seemayer et al. 1984). The degree of thymic hyperplasia in early HIV-1 infection may be variable, but can lead to an abnormally enlarged thymus (Prevot et al. 1992).

Precocious involution

As with lymph nodes, the thymus of patients in the intermediate and late stages of HIV infection shows a thymic involution (Seemayer et al. 1984). This stage of the disease may be accompanied by the infiltration of plasma cells (Seemayer et al. 1984) and macrophages (Papiernik et al. 1992) in the fat adjacent to the cord of the involved thymus.

Thymic dysplasia

This is the final stage of thymus changes during HIV infection, characterized by epithelial rosette formation (Seemayer et al. 1984) and calcification or loss of Hassall's bodies (Seemayer et al. 1984).

HIV target cells in the thymus

Not only $CD4^+$ thymocytes but also immature triple-negative thymocytes, which express CD4 but at very low levels, are susceptible to HIV-1 infection (Schnittmann et al. 1990). A further separation of these triple-negative cells revealed that only $CD1a^+$, but not $CD1a^-$, triple negative thymocytes (presumably the most immature subset) can be infected by HIV-1 (Valentin et al. 1994). Interestingly, protease inhibitor-resistant HIV-1 strains, which develop in some patients treated with protease inhibitors, are impaired in their capability to replicate in thymus tissue (Stoddart et al. 2001). This would explain why patients

with increasing viral load resulting from protease-resistance do not have a fall in their $CD4^+$ T-cell count.

Immune reconstitution of the thymus in HIV-infected patients

Neither thymic transplantation (Ciobanu et al. 1985; Dwyer et al. 1987) nor substitution of thymus-derived hormones (Hermans and Clumeck 1989) in HIV-infected patients leads to convincing evidence of immune reconstitution. The introduction of an effective antiretroviral therapy – HAART – clearly has a beneficial effect on the immune reconstitution of the thymus, however (Sempowski and Haynes 2002). The restoration of thymic function during HAART could be better monitored by the development and introduction of a new assay of thymus function in adults (the measurement of T-cell receptor excition circles (TREC)) (Ye et al. 2003). Measurement of TRECs revealed a loss of TREC levels within naïve $CD4^+$ T cells, indicating reduced thymic output during HIV infection (Douek et al. 2001). Poor $CD4^+$ T-cell increase in patients with good virological response to HAART seems to be caused by low thymic output (Teixeira et al. 2001). Fortunately, most HIV-1 infected patients, adults (Franco et al. 2002) and children (Correa and Munoz-Fernandez 2002), show an increased thymic output of $CD4^+$ T cells, which seems to correlate with an increase in thymic size in adults during the treatment with HAART (Franco et al. 2002).

HIV AND THE GASTROINTESTINAL MUCOSA

The gastrointestinal tract is not only a potential portal for HIV entry, but also an organ that plays a central role in pathogenesis and secondary opportunistic infections (for review see Schneider et al. 1997a). During the course of HIV infection, most of the patients develop gastrointestinal symptoms. The intestinal immune system, represented by specialized local immune system termed gut-associated lymphoid tissue (GALT), has special features which seem to be involved during HIV/SIV infection (for a review, see Schneider et al. 1997a).

HIV infection of the gastrointestinal tract

As lamina propria lymphocytes (LPL) belong to the effector compartment, most of them are already activated and represent a memory phenotype which makes these cells susceptible to HIV infection without prior stimulation (Lapenta et al. 1999). The increased state of activation seems to be the major factor in increased HIV-1 replication in lamina propria $CD4^+$ T lymphocytes (Sheriff et al. 2004). The increased susceptibility to HIV infection of intestinal $CD4^+$ T cells may also result from the expression of a higher amount of the chemokine receptors CCR5 and CXCR4 (Anton et al. 2000). Indeed, independent of the route of infection, HIV/SIV can even be found early after infection in the lamina propria of the intestinal tract (Veazey et al. 1998; Kewenig et al. 1999). The virus content seems to be higher in the intestinal mucosa compared with the peripheral blood (Kotler et al. 1991; Fackler et al. 1998; Veazey et al. 1998; Kewenig et al. 1999), and this higher mucosal virus load seems to result from an increased replication or translation (Fackler et al. 1998). The major target cells in the lamina propria of the intestine are $CD4^+$ T cells and not macrophages (Meng et al. 2000). Experimental infection of rhesus macaques by atraumatic application of pathogenic SIV to the tonsils, a mucosa-associated lymphoid organ, leads to primary infection of $CD4^+$ T lymphocytes and not to infection of the DC-rich squamous epithelium (Stahl-Hennig et al. 1999). The epithelial cells of the intestinal mucosa are not productively infected but serve as virus transmitters (Bomsel 1997). The transepithelial transport of HIV-1 by epithelial and M cells is mediated by the same receptors: the galactosyl cerebroside molecule and the chemokine receptors CCR5 and CXCR4 (Yahi et al. 1992; Fotopoulos et al. 2002). There is in vitro evidence that HIV can be transported via M cells (Amerongen et al. 1991). It is interesting that primary intestinal epithelial cells selectively transfer R5 HIV-1 to $CCR5^+$ $CD4^+$ T cells of the lamina propria (Meng et al. 2002).

HIV-induced immune activation and the consequences for the gastrointestinal mucosa

The mucosal infection with HIV-1 induces a primary T-cell activation which may lead to ulcers – the so-called 'giant ulcer' – in some patients (R. Ignatius and T. Schneider unpublished data). An initial mucosal immune activation with high production of cytokines (R. Ignatius and T. Schneider unpublished data) may induce a hyperproliferative villus atrophy in the small intestine, as seen in HIV-infected patients and even in very early stages of SIV infection (Kotler et al. 1984; Kewenig et al. 1999). The massive immune activation with secretion of proinflammatory cytokines such as TNF-α will affect the barrier function, inducing a leak flux that may further contribute to diarrhea without the presence of secondary opportunistic enteric pathogens (Schafer et al. 2002; Schmitz et al. 2002). Mucosal inflammation in HIV-infected patients may reach levels comparable to those of patients with inflammatory bowel disease (Olsson et al. 2000).

Mucosal immunodeficiency

- $CD4^+$ T cells: the course of mucosal HIV/SIV infection is further characterized by a rapid and nearly complete loss of $CD4^+$ T LPLs (Lim et al. 1993a; Schneider et al. 1995), which occurs in the SIV model within 2 weeks after infection (Veazey et al. 1998; Kewenig et al. 1999). The decline of $CD4^+$ T cells in the intestinal mucosa is more pronounced, especially

in the early stages of the disease than in the peripheral blood (Figure 2) (Schneider et al. 1995). As measurements of $CD4^+$ T LPLs in this compartment are thus far based on percentages, it is not yet completely clear whether this reflects a real loss of $CD4^+$ T LPLs or is the result of a massive increase of $CD8^+$ T LPLs with a relative decrease of $CD4^+$ T lymphocytes, as is the case for other body compartments (Sopper et al. 2003). There is a preferential decrease of $CCR5^+CD4^+$ T cells, which express the mucosa-homing receptor α4β7 (CD103) in the peripheral blood of HIV-infected patients (Krzysiek et al. 2001). However, a selective reduction of $CD103^+CD4^+$ LPLs could not be found in late stages of the disease (Schneider et al. 1994).

- $CD8^+$ T cells: in the lamina propria, the percentage of $CD8^+$ LPLs is increased, which seems to be caused by an increase of activated $HLADR^+CD8^+$ LPLs and cytotoxic $CD11a^+CD8^+$ LPLs (Schneider et al. 1994). These activated cytotoxic $CD8^+$ LPLs may recognize HIV antigens and play a role in the control of HIV infection (Couedel-Courteille et al. 1997), but they could also be harmful by damaging the intestinal epithelium as in HIV-associated alveolitis (Autran et al. 1988).
- Natural killer cells: NK cells that can eliminate virus-infected and tumor cells were found to be reduced in the intestinal mucosa of AIDS patients, which may further facilitate infections and malignancies in the intestine (Schneider et al. 1994).
- Intraepithelial lymphocytes: the percentage of γδ receptor-positive intraepithelial lymphocytes (IEL) in the intestine of AIDS patients seems to be altered. Interestingly, reduced numbers of γδ IELs in the duodenum in late stages of the disease were associated with short survival expectancy (Nilssen et al. 1996).
- Macrophages: macrophages of the intestinal mucosa in HIV-infected patients are also impaired because the antigen-presenting subset is reduced (Lim et al. 1993b).
- B cells and mucosal humoral immunity: insufficient helper function of $CD4^+$ T LPLs is also indicated by the reduced proportion of IgA plasma cells in the large intestine of AIDS patients because terminal IgA B-cell differentiation is $CD4^+$ T-cell dependent (for a review, see Schneider et al. 1997b). Most investigators found a reduced IgA production in the intestine of HIV-infected patients and SIV-infected macaques (Kotler et al. 1987; Schafer et al. 2002). More impressive, however, is an increase in the production of the normally not predominant intestinal IgG (Janoff et al. 1994; Schneider et al. 1998; Schafer et al. 2002). As only secretory IgA exhibits the full protective immune function at mucosal surfaces (for a review, see Brandtzaeg et al. 1985; Mazanec et al. 1993) by immunoexclusion (Stokes et al. 1975), and even intracellular neutralization of viruses within epithelial cells (Bomsel et al. 1998), a predominance of IgG and a lack or decrease of IgA may reflect a breakdown of the mucosal immune effector arm. Indeed, a lack of or restricted secretory HIV/SIV-specific IgA production is found in HIV-infected humans and SIV-infected macaques (Janoff et al. 1994; Schneider et al. 1997a; Schafer et al. 2002). This local immune deficiency may further contribute to a rapid and massive spread of HIV/SIV in the intestinal mucosa (Kotler et al. 1991; Fackler et al. 1998; Veazey et al. 1998; Kewenig et al. 1999).

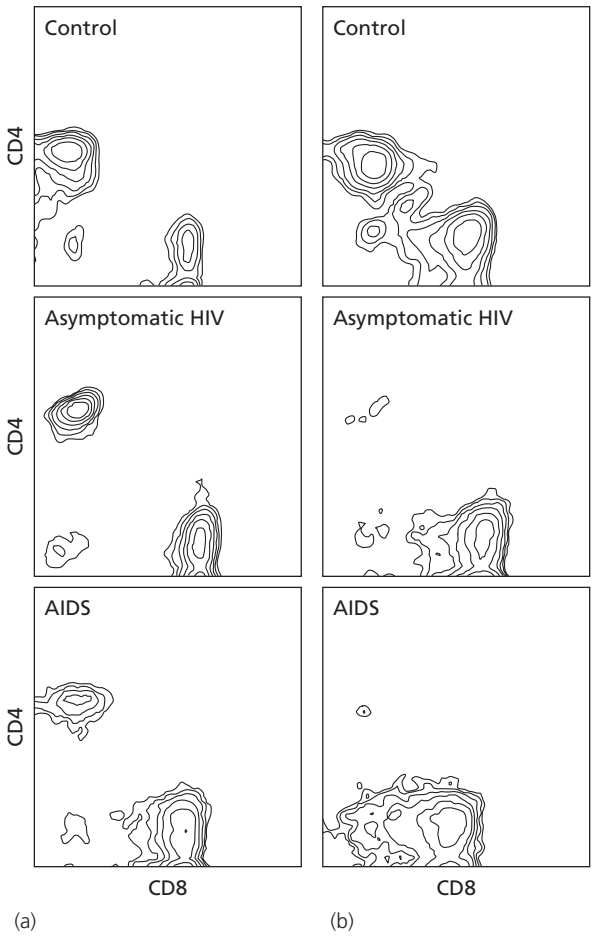

Figure 2 *Pronounced loss of CD4 $^+$ T cells in duodenal mucosa compared with the peripheral blood. Contour blots of two-color immunofluorescence profile of gated $CD3^+$ T cells isolated from* **(a)** *the peripheral blood and* **(b)** *the duodenum of a control patient, of an asymptomatic HIV-1-infected patient, and an AIDS patient. Logarithmic intensities for red fluorescence (CD4) and green fluorescence (CD8) are shown on the* y *axis and* x *axis, respectively (Schneider et al. 1995).*

Immune reconstitution under HAART

The immune reconstitution currently measured by the increase of $CD4^+$ T cells in the peripheral blood after installation of HAART is, beside the HIV RNA levels,

the central parameter in determining the success or failure of treatment (O'Brien et al. 1997). It still remains unclear, however, if all functions and the entire repertoire of $CD4^+$ T cells can be restored after prolonged infection with HIV (Connors et al. 1997). A three-phase T-cell reconstitution is generally seen after initiating HAART: (1) an early rise of memory $CD4^+$ cells, (2) a reduction in T-cell activation correlated to the decreasing retroviral activity, together with an improved $CD4^+$ T-cell reactivity to recall antigens, and (3) a late rise of naïve $CD4^+$ lymphocytes while $CD8^+$ T cells decline (Autran et al. 1997). In one study 20 patients treated with HAART were followed up for 12 months, and CD4 cell proliferative responses to cytomegalovirus and tuberculin antigens were monitored. The investigators were able to demonstrate that HAART can induce sustained recovery of CD4 T-cell reactivity against opportunistic pathogens in severely immunosuppressed patients (Li et al. 1998). The restoration of cytomegalovirus-specific $CD4^+$ T-cell response in HIV-infected patients treated with HAART could be further confirmed by more specific techniques (Komanduri et al. 1998). Immune recovery under HAART could also be observed for other opportunistic pathogens such as *Toxoplasma gondii* (Fournier et al. 2001). Nevertheless, there is lack of functional reconstitution of HIV-specific, CD4-mediated immune response after HAART. Only in some patients can an increase in HIV-specific neutralizing antibodies be detected (Kim et al. 2001). A stringent consequence of the immune recovery of HIV-infected patients under HAART is the impressive decrease in the incidence of opportunistic infections and Kaposi's sarcoma in HIV-infected patients since the introduction of HAART (Palella et al. 1998). Fortunately, immune reconstitution can also be achieved in HIV-infected children, even in those with late stage disease, with HAART (Ometto et al. 2002). One problem of the immune reconstitution under HAART is, in rare cases, the immune reconstitution inflammatory syndrome, which is characterized by an exuberant inflammatory response towards previously present opportunistic pathogens (Shelburne and Hamill 2003).

HTLV-1 INFECTION

HTLV-1 and disease

Human T-cell lymphotropic virus type 1 was the first recognized human retrovirus (Poiesz et al. 1980). The best-known disease linked to HTLV-1 infection is the adult T-cell leukemia/lymphoma (ATL) (Uchiyama et al. 1977). In 1985 a group of patients with a neurological disease known as tropical spastic paraparesis (TSP) was found to be seropositive for HTLV-1 (Gessain et al. 1985). Subsequently, a report from Japan described a neurological disorder as HTLV-1-associated myelopathy, now considered to be identical to TSP (Osame et al. 1986).

Chronic inflammatory arthropathy and uveitis seems to be a rare complication in HTLV-1-infected individuals (Sato et al. 1991; Mochizuki et al. 1996). Beside these diseases patients with ATL or carriers of HTLV-1 show impairment of cellular immune responses (Cappell and Chow 1987).

Epidemiology

The three major reported routes of transmission are sexual intercourse, administration of blood products, and mother-to-child transfer. In endemic areas seropositive persons are clustered around the families, reflecting the predominance of mother-to-child transmission. It is estimated that approximately 10–20 million people are infected by HTLV-1 (Franchini 1995).

HTLV-1 infection is present in widely scattered populations in the world. The best-studied areas are the islands of southwestern Japan, where approximately 20 percent of the adults are seropositive (Hinuma et al. 1982), and the Caribbean basin, which includes the West Indies, northern South America, and the southeastern USA, where 2–5 percent of black adults are seropositive (Schupbach et al. 1983). Other hot spots of HTLV-1 infections have also been reported from west and central Africa, Melanesia, India, the Middle East, and parts of South America (Ifthikharuddin and Rosenblatt 2000).

The virus

HTLV-1 virions are spherical, 100 nm in diameter, and composed of an internal core of structural proteins (p15, p24, and p19) surrounding the viral RNA and polymerase, and an outer layer of viral envelope glycoproteins (gp46 and gp21) anchored in a lipid membrane (Wong-Staal and Gallo 1985). The proviral genome of HTLV-1 encodes *gag*, *pol*, and *env* genes characteristic of all known retroviruses. Both ends of the proviral genome are flanked by noncoding direct-repeat sequences called LTRs. LTRs mediate proviral integration and contain *cis*-acting regulatory elements important for viral transcription, viral mRNA processing, and reverse transcription. Beside the genes for the structural proteins, HTLV-1 has additional open reading frames, which encode for the important regulatory proteins such as Tax and Rex (Franchini 1995).

HTLV-1 Tax is a 40-kDa protein essential for the viral life cycle and transformation of human T lymphocytes (Slamon et al. 1984). It usually localizes in the nucleus of infected cells, here it is capable of activating several cellular transcription factors resulting in an enhanced trancription of cellular genes such as the interleukins IL-1, IL-2, IL-3, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), c-*fos*, c-*cis*, c-*myc*,

vimetin, parathyroid hormone-related protein (PTHrP), transforming growth factor β1 (TGF-β1), MHC I, NF-κB, nerve growth factor, and TNF-β (Franchini 1995). Tax is also capable of transactivating the adhesion molecule ICAM-1 (Tanaka et al. 1996). Such promiscuous transactivation of numerous genes by Tax is thought to play a crucial role in neoplastic transformation of T cells and may also be of central importance for the development of T-cell deficiency in HTLV-I infection.

The 27-kDa regulatory protein Rex plays an important role in regulating viral mRNA processing and is essential for export of full-length *gag*/*pol* and single-spliced *env* mRNA from nucleus to cytoplasm (Hanly et al. 1989). Furthermore, Rex enhances the expression of the IL-2Rα-chain (Kanamori et al. 1990), whereas p12, another regulatory protein of HTLV-1, seems to downregulate the β and γ_c chains of IL-2R (Mulloy et al. 1996). The modification of the IL-2 receptor of HTLV-1-infected T cells by regulatory proteins may influence T-cell function and T-cell transformation in HTLV-1 infection.

HTLV usually infects T cells. However, the cellular receptor for this virus is unknown so far. Experiments with the HTLV-1 surface glycoprotein revealed binding on T and B cells, NK cells, and macrophages (Nath et al. 2003). The putative HTLV-1 receptor seems to be upregulated by T-cell activation (Manel et al. 2003; Nath et al. 2003), especially during early activation (Manel et al. 2003). Moreover, the amount of HTLV-1-infected cells in HTLV-1 carriers seems to be increased during inflammation of infectious diseases (Nishimura et al. 2003). For the spread of HTLV-1 between cells and between individuals, cell contact is required because naturally infected lymphocytes produce virtually no cell-free infectious HTLV-1 particles. After contact between an infected and uninfected T cell, a polarization of the cytoskeleton of the infected cell to the cell–cell junction is induced (Igakura et al. 2003). HTLV-1 core complexes and the HTLV-1 genome accumulate at the cell–cell junction and are then transferred to the uninfected cell (Igakura et al. 2003).

Natural course of HTLV-1 infection

ADULT T-CELL LEUKEMIA/LYMPHOMA

Adult T-cell leukemia/lymphoma is a proliferative disorder of T cells characterized by lymphadenopathy, hypercalcemia, lytic bone lesions, skin involvement, and hepatosplenomegaly (Takatsuki et al. 1985). The skin lesions of HTLV-1 infection are manifold, including diffuse papules, nodules, plaques, erythematous patches, and diffuse erythroderma. In the blood abnormal lymphocytes with convoluted nuclei – so-called flower cells – are found. The malignant T cells in ATL are mature $CD4^+/CD8^-$ and have increased IL-2R α chain (CD25) expression (Hattori et al. 1981). The lifetime risk of developing ATL in HTLV-1 patients is estimated at 1–4 percent (Tajima and Kuroishi 1985). The latent phase from infection to actual development of disease is estimated to be 30–50 years (Tajima and Kuroishi 1985; Murphy et al. 1989). This long incubation period implies that most adult persons developing ATL were infected during early childhood. The most frequent mode of HTLV-1 transmission during this time is breast-feeding by HTLV-1-positive mothers. After manifestation of ATL the prognosis is poor and depends on the disease subtype, which can be distinguished based on clinical criteria (Shimoyama 1991). One of the major reasons for death in ATL patients is the development of opportunistic infections because of the acquired immunodeficiency.

TROPICAL SPASTIC PARAPARESIS

Tropical spastic paraparesis is also is a chronic progressive demyelinating disease affecting the spinal cord and the white matter of the central nervous system (CNS) (Bhigjee et al. 1991). The lifetime incidence of TSP in HTLV-1-infected patients is less than 5 percent (Kaplan et al. 1990). The disease appears usually in the fourth decade of life and women are about twice as likely to be affected as men (Vernant et al. 1987). TSP and ATL rarely occur in the same patient (Kawai et al. 1989). Similar to acute disseminated encephalomyelitis in measles, an autoimmune process is thought to play a key role in the pathogenesis of TSP. Myelin and axon destruction is accompanied by infiltrating $CD8^+$ T cells (Bhigjee et al. 1991), and a high frequency of cytolytic T cells with specificity against the HTLV-1 regulatory protein Tax was found in TSP patients (Koenig et al. 1993). Recently, a cross-reactive epitope between Tax and human neurons was described (Levin et al. 2002). The progression from asymptomatic to symptomatic HTLV-1 infection seems to be triggered by the increasing production of proinflammatory cytokines such as IFN-γ and TNF-α by Tax-expressing cells (Furukawa et al. 2003). For both diseases, ATL and TSP, until now no effective treatment exists.

Immunosuppression

Patients with ATL are immunocompromised and at increased risk of dying as a result of opportunistic infections. The median survival time of patients with acute ATL is only 10 months (Shimoyama et al. 1991). Most deaths in patients with symptomatic HTLV-1 disease are caused by opportunistic infections (White et al. 1995). As in HIV-infected patients, in HTLV-1-infected ATL patients *P. carinii* pneumonia, cryptococcal meningitis, and other disseminated fungal infections are seen (Bunn et al. 1983), which indicate an impaired Th-cell function. In addition, herpesvirus co-infection such as cytomegalovirus, herpes zoster,

and generalized herpes simplex virus infection is frequent in ATL patients (Bunn et al. 1983).

In particular, hyperinfection with *Strongyloides stercoralis* is a common problem in ATL patients (Nakada et al. 1987; Sato and Shiroma 1989; Newton et al. 1992; Plumelle et al. 1997). This phenomenon may indicate a selective immune defect (Newton et al. 1992). A Japanese study showed that 58 percent of the patients with *S. stercoralis* infection are HTLV-1 positive and, of these, 67 percent had a monoclonal integration of HTLV-1 proviral DNA in their blood lymphocytes (Nakada et al. 1987). This leads to speculation that strongyloides infection may be a co-factor for the development of ATL. Leukemic cells of ATL patients suppress B-cell immunoglobulin secretion by a complex mechanism involving induction of suppressor cells after activation of normal suppressor cell precursors (Waldmann et al. 1984).

Immunity in HTLV-1-infected patients seems to be impaired even before diseases like ATL or TSP become apparent. Fungal and bacterial skin infections occur frequently (Kawano et al. 1985; LaGrenade et al. 1990) and may be a marker for the HTLV-1 infection in endemic regions (LaGrenade et al. 1990). Delayed-type hypersensitivity is suppressed in HTLV-1 carriers (Murai et al. 1990).

Double infections with HIV-1 and HTLV-1 have been reported, but it is not clear so far if this accelerates progression to AIDS (Bartholomew et al. 1987; Cleghorn and Blattner 1992; Gotuzzo et al. 1992).

MEASLES

Measles is a highly contagious viral disease. After a prodromal stage with fever, coryza, cough, and conjunctivitis, a generalized maculopapular rash appears. Introduction of measles into virgin populations, as well as endemic transmission in populations lacking access to adequate medical care, is associated with high mortality (Griffin 2001; Duke and Mgone 2003). Despite the successful development of a live attenuated vaccine, measles remains a major cause of mortality in children of developing countries, accounting for 1 100 000 deaths annually (Murray and Lopez 1997; Duke and Mgone 2003).

The virus

Morbilliviruses are distinct from other paramyxoviruses in that they form intranuclear inclusion bodies. Virions are pleomorphic and range in size from 100 to 300 nm. The envelope carries two viral proteins: transmembrane hemagglutinin and fusion glycoproteins. The nucleocapsid is packed within the envelope in the form of an asymmetrical coil. Nonstructural proteins encoded by the measles virus include the C and the V proteins. Both are dispensable for viral replication in vitro, but may play a role in viral pathogenesis in vivo (for a review, see Griffin 2001).

RECEPTOR USAGE

CD46 (membrane co-factor protein (MCP)) is expressed on human nucleated cells and was the first cellular receptor identified for the measles virus. An extended binding site within CD46, spanning the two short conserved domains, SCR1 and SCR2, most distal to the membrane, interacts with the measles virus transmembrane hemagglutinin protein; the binding sites for the natural ligands, the C3b/C4b complement components, map in SCR3 and SCR4. However, more recent studies have questioned the role that CD46 plays for measles virus not passaged in vitro. These and all other measles virus strains tested so far use CD150 (signaling lymphocyte activation molecule (SLAM)) as an entry receptor. The expression of CD150 on activated T and B cells, memory cells, and immature thymocytes seems to be compatible with the well-recognized tropism of measles virus for the cells of the lymphoid compartment (for a review, see Schneider-Schaulies and Ter Meulen 2002).

NATURAL COURSE OF MEASLES INFECTION

Immunopathology

Measles is typically a childhood infection spread by the respiratory route. Virus replication takes place first in tracheal and bronchial epithelial cells (Sherman and Ruckle 1958). There is evidence that the virus is acquired from the basolateral side of epithelial cells by tissue-resident macrophages and DCs (Mrkic et al. 2000). The virus is probably transported by these cells to local lymph nodes, where the measles virus replicates and spreads by cell-associated viremia to a variety of organs (Ruckle and Rogers 1957; Forthal et al. 1992). The primary target cell in the blood is the monocyte (Esolen et al. 1993), and the viremia is accompanied by leukopenia (Sergiev et al. 1960). Lymphoid organs and tissues such as the thymus, spleen, lymph nodes, appendix, and tonsils are prominent sites of virus replication (Hall et al. 1971). Infection of thymocytes with the measles virus leads to apoptosis of these cells and a prolonged decrease in the size of the thymic cortex (White and Boyd 1973). Measles virus spreads to numerous other organs, including the skin, kidney, lung, gastrointestinal tract, genital mucosa, and liver. In these organs the virus replicates primarily in endothelial and epithelial cells. Endothelial cell infection is accompanied by vascular dilatation, increased vascular permeability, mononuclear cell infiltration, and infection of surrounding tissue (Denton 1925). Bright-red spots with a bluish center, the so-called Koplik's spots, appear on the oral mucosa 1–3 days before the skin rash, are pathologically similar to it, and involve the submucous glands (Denton 1925).

Clinical course

The clinical picture of measles develops after an incubation period of 10–14 days. The first prodromal symptoms are fever, malaise, cough, and coryza. This prodrome usually lasts 2–3 days during which the Koplik's spots appear on the oral mucosa. The typical maculopapular rash then appears, first on the face and behind the ears, from where it spreads centrifugally to the trunk and extremities. The rash fades 3–4 days from the start. During this time lymphadenopathy, mild splenomegaly, and appendicitis may be present. Clinical recovery begins soon after the appearance of the rash (Figure 3).

COMPLICATIONS

Predictors of poor outcome are severe lymphopenia (Coovadia et al. 1978) and poor antibody response (Wesley et al. 1982). Mortality in infection with measles is more frequent at the extremes of age. Most of the fatal outcomes observed in measles occur in children in developing countries. Malnutrition increases mortality caused by measles, and measles in turn worsens malnutrition (Dossetor et al. 1977). Death is most often the consequence of secondary infections or the reactivation of persistent infections such as tuberculosis (Duke and Mgone 2003). Most of the severe life-threatening pneumonia observed during measles is the result of secondary bacterial and viral infections (Beckford et al. 1985; Quiambao et al. 1998). The reactivation and exacerbation of tuberculosis were already mentioned in medical textbooks of the nineteenth century, and in 1908 von Pirquet reported the disappearance of delayed-type hypersensitivity skin responses to tuberculin during a measles outbreak. More recently, the reactivation and spread of herpesvirus infections, especially in the respiratory tract, has been reported (Orren et al. 1981; Suga et al. 1992). Importantly, young children in developing countries frequently develop diarrhea as a result of secondary bacterial and protozoal infections, worsening the precarious nutritional status of these children during infection with measles (Greenberg et al. 1991). Secondary and reactivated infections complicate the natural course of measles and are often the cause of fatal outcomes, underlining the importance of immune suppression during infection with the measles virus.

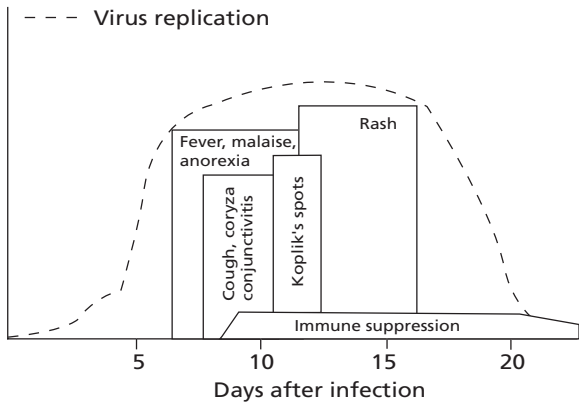

Figure 3 *Natural course of* measles virus *infection. Note that immune suppression begins usually 8–10 days after infection and before Koplik's spots and the skin rash appear. The duration of immune suppression exceeds that of clinical symptoms and may last up to 4 weeks after the rash has disappeared.*

Immunosuppression

Although the measles virus was the first pathogen recognized to cause immunosuppression, it was only recently that the underlying mechanisms were partially revealed. In vivo, delayed-type hypersensitivity skin test responses to recall antigens, such as tuberculin, are suppressed before the rash and for several weeks after apparent clinical recovery from measles infection (Figure 4) (Von Pirquet 1908; Tamashiro et al. 1987). Furthermore, cellular and humoral immune responses to new antigens during infection with measles are impaired (Coovadia et al. 1974). Isolated peripheral blood lymphocytes from patients with measles respond with reduced proliferation (Figure 5) and impaired cytokine production after stimulation with mitogens (Hirsch et al. 1984; Ward et al. 1991).

MONOCYTES AND DCS

Monocyte function is impaired by several mechanisms during measles. Both measles virus receptors, CD46 and CD150, are downregulated on the cell surface after contact with measles virus (for a review, see Schneider-Schaulies and ter Meulen 2002). The downregulation of CD46 may trigger lysis of uninfected lymphocytes by activating complement, as shown in vitro, and so may contribute to enhanced immune suppression. There is some evidence that CD46 can function, together with CD3, as a co-stimulatory molecule in the induction of T-cell proliferation and T-cell activation, which could as a consequence be blocked by measles-induced

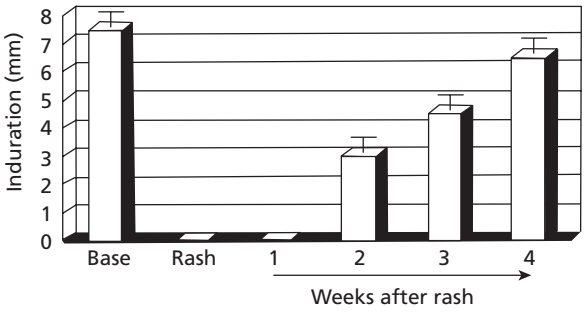

Figure 4 *Immune suppression during measles infection: changes in tuberculin-induced delayed-type hypersensitivity (DTH) skin test responses in children with measles who had previously been immunized against tuberculosis. (Modified after Tamashiro et al. 1987 and Griffin 2001, with permission.)*

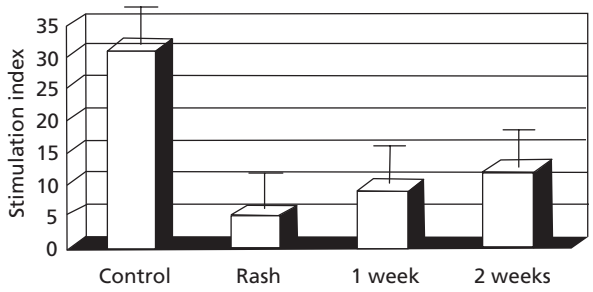

Figure 5 *Immune suppression during measles infection. Changes in proliferation of peripheral blood mononuclear cells to the mitogen phytohemagglutinin. (Modified after Hirsch et al. 1984, and Griffin 2001).*

downregulation. The downregulation of CD46 on monocytes infected with the measles virus results in a decreased IL-12 production (for a review, see Schneider-Schaulies and Ter Meulen 2002). The measles virus nucleocapsid protein inhibits IL-12 release from mouse monocytes. These observations are further supported by in vivo data showing a prolonged impairment of IL-12 production by monocytes from patients after natural measles infection. A decrease of IL-12 production by macrophages and monocytes may result in a decreased IFN-γ production by T cells. Furthermore, as stimulation of macrophages via CD46 seems to be involved in the response of macrophages to IFN-γ which stimulates the production of nitric oxide (NO) (for a review, see Schneider-Schaulies and Ter Meulen 2002). The downregulation of CD46 may result in a reduced production of NO and an altered immune response to intracellular pathogens such as mycobacteria.

The measles virus hemagglutinin seems to be responsible for CD150 downregulation (Tanaka et al. 2002). Recently, it could be demonstrated in an animal model that wild-type measles virus strains, which in contrast to vaccine virus induce strong immune suppression, exclusively used the CD150 and not the CD46 receptor (Pfeuffer et al. 2003). After infection and downregulation of CD105 on T cells, cell division and proliferation of these cells are inhibited (Hahm et al. 2003). Signaling events triggered after binding to this receptor are complex and can result not only in activation but also in cellular apoptosis (Sidorenko and Clark 1993). The IFN-γ production of $CD4^+$ T cells can be upregulated by ligation to the CD150 receptor (Aversa et al. 1997). Thus, downregulation of this molecule after infection with measles virus may impair Th1 responses toward intracellular pathogens, as seen in humans with measles (Griffin 1995). This hypothesis is further supported by recent experiments in transgenic mice expressing the measles virus receptor CD46. After infection with measles virus these animals were more susceptible to infection with *Listeria monocytogenes* as a result of the reduced number of macrophages and substantial defects in IFN-γ-producing $CD4^+$ T cells (Slifka et al. 2003). IFN-γ production is important for the defense of intracellular bacteria (Kaufmann 1992) and impairment in this pathway explains the observed increased bacterial infections during and after infection with measles. TNF-α production by monocytes is reduced during acute infection and recovery (Griffin et al. 1987). In vitro experiments with monocytic cell lines show a decrease in TNF-α production after infection with the measles virus (Leopardi et al. 1992).

The effect of the measles virus on DCs has not been clear until now. DCs isolated from peripheral blood showed normal release of IL-12 after stimulation with bacterial cell wall components; DCs generated in vitro, however, showed impaired production of IL-12 (for a review, see Schneider-Schaulies and Ter Meulen 2002). The reduced IL-12 production by DCs and monocytes may have consequences affecting the quality of T-cell responses and could explain an inadequate Th1 response during measles infection (Griffin et al. 1989; Ward and Griffin 1993). In fact measles virus-infected DCs are unable to stimulate allogenic T-cell proliferation, and even actively suppress in vitro-stimulation of T-cell proliferation (for a review, see Schneider-Schaulies and Ter Meulen 2002).

LYMPHOCYTES

A lymphopenia affecting both CD4 and CD8 cells is observed during infection with measles (Okada et al. 2000). Although the CD4:CD8 T-cell ratio normally remains unaltered (Arneborn and Biberfeld 1983), a greater decrease of $CD4^+$ T cells with decreased CD4:CD8 ratios has been found in children with malnutrition (Ryon et al. 2002). The mechanism responsible for the lymphopenia is thought to be measles-induced apoptosis of uninfected lymphocytes (Okada et al. 2000; Ryon et al. 2002). A selective loss of peripheral lymphocyte function-associated antigen ($LFA\text{-}1^{high}$)-expressing T cells was observed in the course of measles virus infection, and this could reflect aberrant lymphocyte trafficking by random homing to tissues (Nanan et al. 1999). This may also contribute to leukopenia. The role of leukopenia for measles virus-induced immunosuppression is unclear, because the duration of immune function impairment clearly exceeds the panlymphopenic phase of the disease (Okada et al. 2000).

One reason for the reduced T-cell proliferation in vitro to mitogens during measles infection and during the first weeks of convalescence is inadequate IL-2 production. Addition of recombinant IL-2 to lymphocyte cultures from patients with measles restores lymphocyte proliferation ability (Griffin et al. 1986; Ward and Griffin 1993). Investigation of other T-cell cytokines revealed low IFN-γ release and high IL-4 production compared with controls (Ward et al. 1991; Griffin and Ward 1993). In vitro neutralization of IL-4 with antibodies also improves lymphocyte proliferation (Griffin

and Ward 1993). This T-cell cytokine profile suggests a shift toward a Th2-type response and may explain the suppression of delayed-type hypersensitivity during and after the first weeks of infection.

In contrast to natural infection with measles virus, measles virus immunization does not result in a reduced IL-12 and IFN-γ production. Measles virus glycoprotein seems directly to block the proliferative response of lymphocytes to mitogens in vitro, whereas anti-measles virus antibodies and genetic exchange of measles glycoprotein abolish such inhibition of proliferation (for a review, see Schneider-Schaulies and Ter Meulen 2002). Direct in vitro infection of T cells results in a reduced proliferative response to mitogens and soluble antigens as well as in an impaired cytotoxic function (for a review, see Schneider-Schaulies and Ter Meulen 2002). IFN-α production is important to limit viral infections and infections with secondary viral infections, especially herpesvirus infections, which are frequently observed during and after measles virus infection (for a review, see Schneider-Schaulies and Ter Meulen 2002). In agreement with this clinical observation is the experimental finding that, after in vitro infection with measles virus, a suppression in the IFN-α-signaling pathway was documented (Yokota et al. 2003). Infection with the measles virus resulted in a suppression of Jak1 phosphorylation and association of the viral accessory proteins C and V with the IFN-α receptor complex (Yokota et al. 2003).

Infection of B cells in vitro with the measles virus reduces Ig production (Casali et al. 1984; Tishon et al. 1996). Measles virus nucleocapsid protein binds to activated B cells and reduces antibody production in vitro (Ravanel et al. 1997). However, in vivo experimental measles virus infection revealed an impaired T-cell function but no B-cell defect (Niewiesk et al. 2000).

Soluble factors such as cytokines and viral proteins account for the immunosuppression of infected and uninfected lymphocytes during and for several weeks after measles infection and seem to be more important than mechanisms directly induced by infection of T and B cells. However, the in vitro effects of infection of leukocytes, macrophages, and B and T cells by measles virus cannot be ruled out, and may also be relevant to the immune suppression observed during natural measles infection.

Impaired function of immunocompetent cells such as macrophages, DCs, and B and T cells can be found at different time points after measles virus infection. There seems to be a dichotomy between the immune response toward measles virus and other pathogens. This dichotomy may be resolved by investigations of a newly discovered regulatory $CD4^+CD25^+$ T-cell subpopulation. These cells are present after several infections in order to restrict and limit immune responses to certain pathogens, thus avoiding the induction of autoimmune diseases (Belkaid et al. 2002). The following scenario is therefore possible: after measles virus infection, a strong measles virus-specific T-cell and B-cell response is induced, which is capable of clearing the measles virus; after several days this immune response has to be restricted, and regulatory $CD4^+CD25^+$ T cells appear, which suppress and limit ongoing or newly induced immune responses; these cells may be active for several weeks, during which time the host is vulnerable to secondary infections.

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New approaches to vaccine delivery

CHARALAMBOS D. PARTIDOS

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THE RATIONALE FOR NEEDLE-FREE DELIVERY OF VACCINES

Vaccination has arguably had the greatest impact of any medical intervention on human health. Worldwide immunization/vaccination programs have been successful in significantly reducing the incidence of serious infectious diseases, such as poliomyelitis and measles, and eradicating smallpox. In many industrialized countries, effective immunization campaigns have resulted in a drop in incidence rates of the target diseases to such low levels that adverse reactions to vaccines rather than the diseases themselves have become a new area of awareness for the public (Andre 2001). To this end, public confidence and media coverage play a significant role in vaccine compliance. Participation rates in immunization programs drop rapidly after negative publicity about adverse effects of injecting vaccines (Miller and Pisani 1999). In addition, children normally associate the sight of a needle with pain which could result in a drop of the rate of compliance (Cohen et al. 2001). As an average, an infant receives 20–25 injections for immunization against different childhood infections by 18 months of age, there is considerable pressure to minimize vaccine-related visits by combining antigens and simplifying immunization procedures (Ward 2000; Jacobson et al. 2001). Needle-free immunization practices hold a great deal of promise in changing the public's attitude of 'being forced to get a shot' to seeking out vaccines.

Most vaccines are injectable preparations administered via parenteral routes. As a result, immunization requires needles and syringes and trained medical personnel, is expensive, normally does not induce mucosal immunity, and may lead to injection site reactions. In addition, in many instances, particularly in less developed countries, immunization may lead to infections by blood-borne pathogens as a result of the use of contaminated needles (Kane et al. 1999; Simonsen et al. 1999). Routine immunization programs account for about a billion injections per year (Kane et al. 1999) and such injections are believed to be safer than many nonimmunization injections (therapeutic injections) in most countries. However, the World Health Organization (WHO) has recently estimated that up to one-third of immunization injections are unsafe in four of six regions of the world (Miller and Pisani 1999). Several reports from countries in sub-Saharan Africa, Asia, and the Middle East have estimated that from 31 percent to more than 90 percent of childhood immunizations are unsafe (Simonsen et al. 1999). The main reasons for unsafe injections are: lack of sufficient quantities of syringes and needles, lack of operational sterilization equipment or fuel to operate such equipment, and patient demand for injections (Kane et al. 1999). Furthermore, there is a common misconception that it is safe to reuse syringes between patients if the needle is changed. In some countries, four of five disposable syringes, many of them supplied for immunization, are reused.

Population-based studies from China, India, and former Soviet states have shown unsafe injections to be a major source of hepatitis B virus (HBV) infection (Simonsen et al. 1999). In Egypt, nationwide injection campaigns against schistosomiasis between 1920 and 1980 played a major role in the extensive spread of hepatitis C virus (HCV) leading to the high current prevalence (18 percent) of the virus in the population

(Simonsen et al. 1999). It has been estimated that unsafe injections may cause 8–16 million HBV infections every year, 2.3–4.7 million HCV infections, and 80 000–160 000 HIV/AIDS infections (Kane et al. 1999). The transmission of hepatitis viruses and HIV are by no means the only risks involved with unsafe injections; many outbreaks involving other pathogens, such as Ebola and Lassa virus infections, and malaria have been reported (Simonsen et al. 1999).

With the availability of whole genome sequences, the potential number of candidate vaccine antigens has increased, and it is anticipated that the number of injections will increase further. Therefore, it is not surprising that the development of new approaches for their effective and safer delivery to the immune system ranks high in the WHO's priority list (Jodar et al. 2001). Safety is a major issue because vaccines are administered to prevent disease in healthy people (Clements et al. 1999). To increase compliance, as well as to make the vaccine more economical, vaccines should be effective after a single immunization using needle-free or noninvasive delivery systems.

Ideally, future vaccines should:

- be administered via noninvasive routes, such as the mucosae (436 m^2) and the skin (2 m^2) which cover a large surface area of the human body
- require only a single administration, or at the most two, to elicit protective immune responses
- be capable of immunizing at a very early age
- be available in formulations already combined with either multiple other vaccines or other vaccines at the moment of administration
- exhibit temperature stability to minimize the stringency of cold chain requirements.

New skin and mucosal vaccine delivery options (vaccine patches, nasal sprays, or edible vaccines) are promising to revolutionize the way that vaccines will be delivered in the future. Needle-free vaccine delivery systems will make immunization simpler, more economical, practical, and safer.

SKIN DELIVERY OF VACCINES

The skin barrier

The skin is the largest organ of the body (10 percent of body mass) and represents a readily accessible surface area for absorption (2 m^2 in adult humans). This offers a distinct advantage for exploiting the skin immune system for delivery of vaccines. The skin is made up of the epidermis and the dermis. The dermis (1–2 mm) contains fibroblasts, nerves, and blood vessels. The outermost layer of the epidermis, which constitutes an effective physical and chemical barrier, in the stratum corneum (10–20 μm thick in humans), is composed of keratinocytes anchored in a lipophilic matrix. Keratinocytes undergo terminal differentiation, a specialized form of programmed cell death. As a result, they lose their nuclei and crosslink, becoming resilient rigid corneocytes, and eventually they complex with lipids (Fuchs 1990). This is a well-regulated process required for the maintenance of the physiological thickness of the skin and the preservation of its barrier functions. Ceramides, cholesterol, and free fatty acids seem to play an important role in the corneocyte cohesion, contributing also to the permeability barrier of the skin (Morganti et al. 2001). Thus, the function of the skin is to preclude appreciable exchange of materials between the skin surface and the deep layers, and to protect against ultraviolet light.

Below the stratum corneum are layers of epidermis (50–100 μm) which are made up of keratinocytes at various stages of differentiation (Morganti et al. 2001). The stratum basale, just above the dermis, is the most metabolically active layer of the epidermis with large columnar cells. Interspersed among the basal keratinocytes are other cells, such as melanocytes, Langerhans' cells (LC), and sensory Merkel cells. Once keratinocytes leave the basal layer, they migrate upward via the stratum spinosum and stratum granulosum to the stratum corneum, acquiring the characteristics of fully differentiated corneocytes which are eventually sloughed at the skin surface. During this migration process, keratinocytes undergo morphological and biochemical changes necessary to discard their proliferative functions and adopt new protective roles.

The skin as a site of immune surveillance

As the skin is the principal interface with the external environment, it acts as an immune barrier protecting the host from invading pathogens. For this purpose, it is equipped with a unique set of immunocompetent cells, i.e. keratinocytes, and LCs, strategically located lymph nodes, and subsets of T lymphocytes that constitute the skin-associated lymphoid tissue (SALT) (Streilein 1983; Bos and Kapsenberg 1993). The intraepidermal lymphocytes constitute only about 2 percent of SALT and the majority are $CD8^+$ T cells (De Panfilis 1998; Debenedictis et al. 2001). The predominance of $CD8^+$ epidermal T cells is in contrast to the normal 2:1 ratio of $CD4^+$:$CD8^+$ T cells seen in peripheral blood; therefore, it is apparent that the population of T cells found in the epidermis is a special population of memory cells, which have selectively extravasated into the epidermis (Debenedictis et al. 2001). By contrast, the dermis contains both populations of $CD4^+$ and $CD8^+$ T cells in equivalent ratios (Debenedictis et al. 2001).

Keratinocytes, apart of being responsible for establishing the physical barrier of the skin (the viable

epidermis is composed of about 90–95 percent keratinocytes) and guaranteeing the structural integrity of the epidermis, produce a wide range of cytokines on activation by various stimuli. These include interleukins (IL), interferons (IFN), colony-stimulating factor (CSF), tumor necrosis factor (TNF), growth factors, and chemokines (Uchi et al. 2000). Resting keratinocytes synthesize and secrete low levels of cytokines. However, stimuli such as allergens, irritants, ultraviolet light, bacterial products, and physical trauma induce keratinocytes to produce significant amounts of cytokines (Uchi et al. 2000). These cytokines shape the local microenvironment of the skin to help maintain the appropriate balance of skin immune responses, and stimulate the maturation and migration of LCs. In particular, IL-1β and low levels of TNF-α are critically involved in the induction of LC migration from the skin (Wang et al. 1999). Keratinocytes do not just secrete cytokines; they also perform a scavenger function, eliminating foreign material from the intercellular spaces of the epidermis (Wolff and Honigsmann 1971) and responding to cytokines secreted by neighboring cells (Debenedictis et al. 2001). When they are exposed to IFN-γ produced by T cells, keratinocytes upregulate intercellular adhesion molecule 1 (ICAM-1) and class II major histocompatibility complex (MHC) antigens (keratinocytes do not constitutively express MHC class II molecules). This enables them to serve as accessory cells by providing co-stimulatory signals to support a T-cell-mediated response to bacterially derived superantigens, phytohemagglutinin, and anti-CD3 monoclonal antibody (Nickoloff et al. 1993).

LCs are powerful antigen-presenting cells (APC) (up to 1 000 cells/mm^2) that cover almost 20 percent of the surface area through their horizontal orientation and long protrusions (Debenedictis et al. 2001). They are of myeloid lineage, originally migrating to the skin during fetal development. LCs are defined by their dendritic morphology and the presence of a unique intracytoplasmic organelle, the Birbeck granule (Lappin et al. 1996). They are located at the basal layer of the epidermis as immature cells playing a sentinel role in the epidermis. LCs capture and process antigens and, during their migration via the efferent lymphatics to the paracortical T-cell areas of the draining lymph nodes, they mature and present antigenic peptides to naïve T cells. At this stage, they express co-stimulatory molecules of the B7 family, upregulate the surface expression of MHC class I and II molecules bound to peptides, and secrete high levels of proinflammatory cytokines, such as IL-12 and IL-1 (Lappin et al. 1996; Debenedictis et al. 2001).

Percutaneous absorption of antigens

Antigens applied on to bare skin penetrate across the continuous stratum corneum, mainly via the intracellular or intercellular routes (Barry 2001). However, appendages including hair follicles, or sebaceous or sweat glands can also serve as portals of antigen entry (Figure 1). The specific pathway that an antigen will follow depends on several parameters, such as its physicochemical characteristics and the method of antigen delivery. As the stratum corneum is lipophilic, antigens of a lipophilic nature would be better accepted (Naik et al. 2000; Barry 2001). Moreover, an antigen must be sufficiently 'mobile' to diffuse across the stratum corneum. Solute diffusivity has been shown to decrease exponentially as molecular volume (and hence molecular weight) increases, imposing a size restriction on favorable transport across the skin (Naik et al. 2000). After antigen diffusion through the stratum corneum, its re-partition into the more aqueous viable epidermis beneath would facilitate its move toward the basal layer, where LCs reside. Therefore, ideally, the antigen should possess both lipoidal and aqueous solubility for effective skin penetration (Naik et al. 2000; Barry 2001).

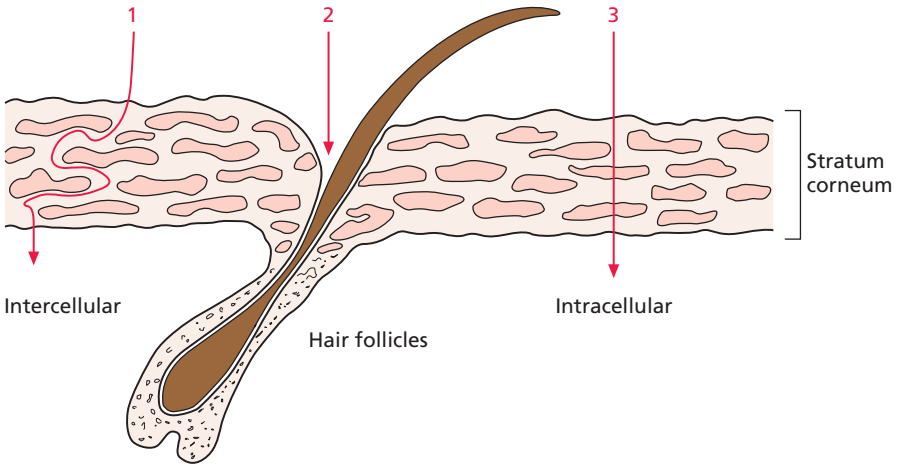

Figure 1 *Percutaneous absorption of antigens. Routes that an antigen can follow after application on to bare skin.*

However, when the skin is hydrated, its barrier functions are less pronounced and large molecules can pass through.

Studies on percutaneous absorption of various molecules have shown that the intercellular pathway is mainly favored by liposomes or low-molecular-mass uncharged molecules, whereas molecules delivered by electroporation or gene gun penetrate via the intracellular space (Morganti et al. 2001). Hair follicles could provide a shunt route through which large molecules, such as oligonucleotides, liposome-complexed DNA, or naked plasmid DNA (pDNA), can cross the stratum corneum (Prausnitz 1997; Fan et al. 1999; Morganti et al. 2001).

As stratum corneum lipids limit the transport of most compounds, efforts to increase percutaneous absorption have often focused on altering the lipid bilayer structure. This can be achieved by chemical or physical approaches. The most effective chemical enhancers act by disrupting or fluidizing lipid bilayer structures within the stratum corneum, thereby increasing the diffusion of molecules through the skin (Prausnitz 1997; Faldvari 2000; Barry 2001). Chemical enhancers such as dimethyl sulfoxide, unsaturated fatty acids, or surfactants have been shown to increase percutaneous absorption of small molecules (Prausnitz 1997). However, they frequently cause significant skin irritation and may affect the stability of co-administered molecules. For these reasons they have little practical impact beyond preclinical studies.

Liposomes have been shown to increase topical and transdermal delivery of various low-molecular-mass molecules. In particular, lipid-based biphasic delivery systems are capable of encapsulating compounds such as proteins and DNA and delivering them effectively through the skin, e.g. formulations with recombinant *Pasteurella haemolytica* leukotoxin and hen egg lysozyme can induce strongly polarized, T-helper 2 (Th2)-type, antigen-specific, immune responses after topical application (Baca-Estrada et al. 2000a). Transdermal delivery of lipid-based DNA vaccines can induce both antibody and cytokine responses in mice, although the responses are weak (Baca-Estrada et al. 2000b). When formulating vaccine antigens with liposomes, it is critical to determine the compatibility of the antigen with the liposome because different antigens can have different chemical properties that influence their formulation. Furthermore, the composition of the lipid-based delivery systems can influence their percutaneous absorption (Babiuk et al. 2000). To improve the absorption of topically applied liposomes, ultradeformable liposomes termed 'transferosomes' have been developed (Cevc et al. 1998). These carriers can cross the intact mammalian skin with an efficacy close to 100 percent, and ensure an efficiency of delivery greater than 50 percent. After topical application, they have been shown to increase the immunogenicity of incorporated Gap junctional proteins (Paul et al. 1998). Their mechanism of skin penetration appears to be related to their ability to squeeze through polar channels on hydration gradients (Cevc et al. 1998).

One of the physical enhancement technologies that has been tested for transdermal vaccine delivery is electroporation (Vanbever and Préat 1999). It uses high-voltage, short-duration pulses that induce: (1) a disorganization of the stratum corneum lipid bilayers; (2) an increase of skin hydration; and (3) a decrease of skin resistance. It permeabilizes keratinocytes without affecting skin viability in vivo (Dujardin et al. 2002). Skin electroporation was shown to increase immune responses to a myristilated peptide derived from hepatitis B surface antigen (HBsAg) when administered without adjuvant but not to a larger size antigen, such as diphtheria toxoid (DT) (Misra et al. 1999). This suggests that small antigens can be targeted in sufficient amounts to the LC-rich area of the epidermis, to mount a good immune response. However, there are still limited in vivo data concerning immunogenicity and skin toxicological studies for establishing the clinical value of this method.

A novel technology known as transdermal powder delivery (TPD) represents an alternate physical method for vaccine delivery. This method uses a supersonic flow of gas to accelerate vaccine-coated particles to a sufficiently high speed to enable them to penetrate the outer layers of the skin. The particles are sufficiently hard and robust not to deform significantly or shatter on impact, and penetrate the epidermis and dermis proportional to the product of their velocity, diameter, and density. A single administration of influenza vaccine using a helium-powered device elicited serum antivirus IgG antibody titers that were significantly higher than those induced by either intramuscular or subcutaneous injection. A booster administration resulted in a further increase of antibody titers and 100 percent protection against homologous virus challenge (Chen et al. 2000). Similarly, powderject delivery of DT was increased by 25- and 250-fold when 1 μg DT was co-administered with alum or a CpG motif, respectively. The induced antibodies neutralized the toxin and were long lasting, and their isotype was dependent on the adjuvant used (Chen et al. 2001a). Epidermal powderject immunization was also effective in eliciting mucosal immunity after administration of an inactivated influenza virus (Chen et al. 2001b), and cytotoxic T-cell responses after delivery of HBsAg or a synthetic peptide from influenza virus nucleoprotein (Chen et al. 2001c). The efficacy of this system can be attributed to the fact that it delivers vaccine antigens to LCs.

Another physical approach to vaccine delivery is the use of needle-free jet injectors (JI) that deliver the dose of vaccine at high velocity into dermal and subcutaneous layers with high-pressure fluid. Needle-free injectors have the potential to eliminate the risk of

accidental needlestick injuries after injections and during discard of waste. However, vaccine administration with JIs has been associated with higher levels of pain in female recipients, reported at the time of immunization, as well as with the occurrence of local injection site reactions in comparison to vaccine administration with needles and syringes (Jackson et al. 2001). When four types of injectors were tested for their safety, all were found to transmit significant (>10 pl) volumes of blood, although the volumes and frequency of contamination varied with the injector (Hoffman et al. 2001). This raises the risk of transmission of infectious diseases, such as HBV. Also a problem associated with the use of needle-free jet injection devices is that currently they cost several times what less developed countries now pay for conventional syringes and needles. In addition, a healthcare worker is required to administer the vaccine.

Immunization on to bare skin

For a long time it was thought that the skin barrier was impermeable to large molecules, but studies by Glenn et al. (1998a) have demonstrated that topical application of cholera toxin (CT) on to hydrated skin can induce strong systemic and mucosal immune responses and confer protection against lethal mucosal toxin challenge (Glenn et al. 1998b). CT is an exotoxin secreted by *Vibrio cholerae*. It is made up of five identical B subunits that bind with high affinity to G_{M1} gangliosides at cell surfaces and a single A subunit. The latter, after proteolytic cleavage, generates the enzymatically active A1 peptide, which activates adenylyl cyclase (Spangler 1992). A similar effect was demonstrated with another adenosine diphosphate (ADP)-ribosylating exotoxin, the heat-labile enterotoxin (LT) of *Escherichia coli* (Glenn et al. 1999; Beignon et al. 2001). Parallel to their immunogenic potential, these molecules act as adjuvants, potentiating immune responses to topically co-applied antigens, including toxoids (Scharton-Kersten et al. 1999; Hammond et al. 2000), proteins (Glenn et al. 1998a; Scharton-Kersten et al. 1999; Partidos et al. 2001), peptides (Beignon et al. 2001, 2002a), and viruses (El-Ghorr et al. 2000; Hammond et al. 2001a). It is the combination of their binding activity and built-in adjuvanticity that make these molecules powerful immunogens and adjuvants. When recombinant CTB, which is devoid of ADP-ribosylating activity, was used as an adjuvant with DT, anti-DT antibody responses were of a lower magnitude than those of the group in which CT was used. However, when a small amount of CT was added to the recombinant CTB, the adjuvant activity was restored to levels comparable to that of CT (Scharton-Kersten et al. 2000). In another study, preincubation of CT with G_{M1} gangliosides before topical application significantly reduced the serum and mucosal anti-CT antibody responses (Beignon et al. 2001).

Following these initial observations, the potential of skin as a noninvasive route for vaccine delivery has been demonstrated with several types of antigens:

- viruses: herpes simplex virus (HSV), adenovirus, inactivated rabies virus, recombinant mengo virus
- parasites: *Dirofilaria immitis*
- plasmid DNA: HBsAg, influenza antigens
- bacterial toxins (CT, LT), toxoids (tetanus toxoid (TT), DT)
- proteins: bovine serum albumin (BSA), β-galactosidase
- peptides: Th, B, cytoxic lymphocyte (CTL) epitopes.

Also, preliminary results from a phase 1 trial conducted in human volunteers (Glenn et al. 2000) have shown that topical application of LT with a patch: (1) does not induce local or systemic adverse reactions – only one volunteer developed a mild dermatitis at the adhesive site; (2) elicits anti-LT IgG responses that were boosted after the second and third topical application of LT; (3) induces long-lasting immunity and detectable anti-LT IgG or IgA antibodies in urine and stools; and (4) visualizes enlarged and rounded LCs using anti-CD1a staining at the site of immunization after 24 and 48 h.

Overall, these findings suggest that the skin barrier is not as highly resistant to permeation by antigenic molecules as previously thought, but rather has variable resistance. However, it should be noted that, in certain instances of skin disease, i.e. atopic dermatitis or psoriasis, as well as in skin wounds and burns, the permeability of the skin is increased as a result of alterations of its lipid content (Morganti et al. 2001).

CHARACTERISTICS AND REQUIREMENTS FOR APPLYING VACCINES ON TO BARE SKIN

Immunization on to bare skin is a needle-free and pain-free immunization procedure, which delivers antigens into the epidermis by simple topical application (Figure 2). With the experimental evidence that has been accumulated so far, some general rules can be drawn for vaccine application on to bare skin.

Skin hydration

Hydration of the skin before vaccine application appears to disrupt the barrier function of the stratum corneum to absorption. An increase in hydration of the skin is associated with a swelling of the stratum corneum and a softening of its texture (Barry 2001). Water is the most natural penetration enhancer that strongly binds to the keratin of corneocytes and increases the 'hydrophilic' character of the stratum corneum (Barry 2001). Glycerol has also been evaluated for its potentiating effects on the immune responses to CTB after topical application. Combination of hydration with glycerol enhanced the anti-CTB antibody responses approximately two- to

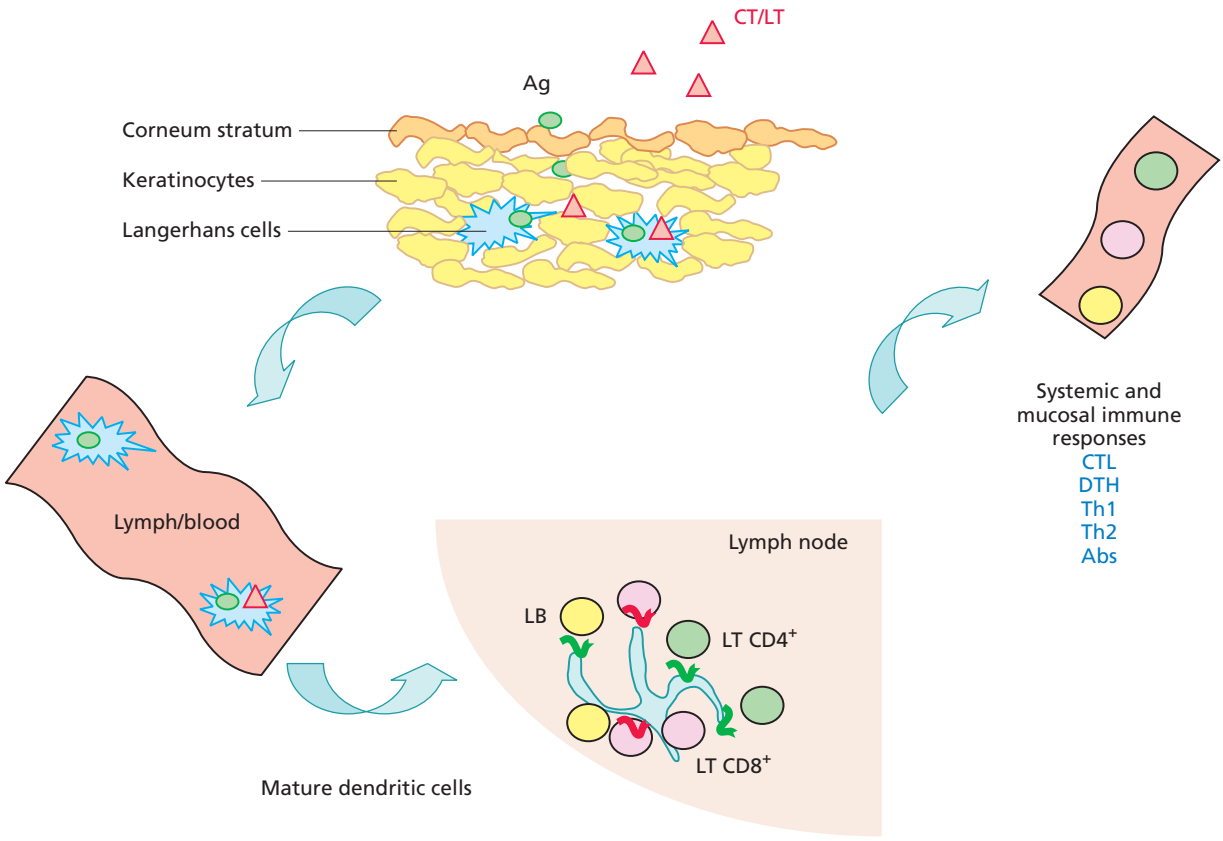

Figure 2 *Immunization on to bare skin. After penetration of vaccine formulation across the stratum corneum, the antigen is taken up by Langerhans' cells (LCs) and, in the presence of the co-administered adjuvant, they mature and migrate to the regional lymph nodes, where they present antigenic peptides to naïve T cells. This results in the induction of antigen-specific humoral and cellular responses.*

fourfold in comparison to the responses measured in mice with skin that was not hydrated (Hammond et al. 2001b).

The requirement of an adjuvant

Adjuvants are important to potentiate immune responses to antigens and ADP-ribosylating exotoxins, such as CT and LT, are suitable adjuvants for skin immunization. They enhance the antibody and T-cell responses to the co-administered antigen without signs of toxicity, normally associated with their mucosal delivery (when high doses are used). Furthermore, the anti-toxin antibody response does not interfere with the immune responses to co-administered antigen. Therefore, the same adjuvant can be used for multiple applications. The selection of adjuvant is also critical for the induction of long-lasting immunity and type of Th response. Several other adjuvants have been tested for skin immunization, such as synthetic oligodeoxynucleotides (ODN) containing CpG motifs, lipopolysaccharide (LPS), muramyl dipeptide (MDP), alum, IL-2, and IL-12 (Scharton-Kersten et al. 2000):

- ADP-ribosylating exotoxins (CT, pertussis toxin (PT), LT)
- mutants of ADP-ribosylating toxins (LTK63, LTR72, LTR192G)
- dangerous signals (LPS, CpG motifs, MDP)
- cytokines (IL-1b, IL-2, IL-12, TNF-α, GM-CSF).

Although they enhanced the antibody titers to co-applied antigen, the responses were short-lived and weaker than those induced in the presence of CT or LT.

Both CT and LT elicit predominantly Th2-type responses that might have a detrimental effect in individuals sensitive to allergic reactions. Normally, some adverse reactions to immunization are common and tolerated for the benefit of immunity. In several instances high levels of IgE responses have been noticed with DT vaccines (Mark et al. 1995). Although the exact role of IgE responses remains to be elucidated, there is always the possibility that the presence of high levels of IgE might be associated with high risk of anaphylaxis, particularly in individuals with atopic predisposition (Sakaguchi and Inouye 2000). On the other hand, antigen-specific IgE appears to correlate with protection in diseases such as schistosomiasis (Khalife et al. 2000). Therefore, successful immunization protocols will be needed to induce the appropriate types of immune responses in a selective and reliable way.

Beignon et al. (2002a) have demonstrated the possibility of modulating immune responses to topically applied antigens. Co-application of a promiscuous (non-MHC-restricted) Th epitope from influenza virus hemagglutinin (amino acids 307–319) with CT and an ODN containing a CpG motif on to hydrated bare skin of mice elicited peptide- and virus-specific $CD4^+$ T cells, increased the levels of IFN-γ and IL-6 secretion, and downregulated the total serum IgE antibody levels. CpG motifs have been shown to induce activation and maturation of LCs (Hartmann et al. 1999). Furthermore, their injection into the dermis enhances the expression of MHC and co-stimulatory molecules by LCs, and promotes their migration (Ban et al. 2000). Thus, it appears that CpG motifs can act synergistically with CT to promote a Th1-type of response. The exact series of events that lead to this synergistic effect is not yet clear. Similarly, mutants of LT with no or residual toxicity have been shown to enhance antigen-specific antibody and T-cell responses after topical immunization (Scharton-Kersten et al. 2000; Beignon et al. 2002b). Furthermore, the mutant LTR72, which retains some residual toxicity, was shown preferentially to stimulate the secretion of high levels of IFN-γ whereas the mutant LTK63, which is devoid of toxicity, did not (Beignon et al. 2002b).

Topical application of DNA vaccines

The success of topical application of DNA vaccines on to untreated skin depends on the ability to transfect effectively cells of the epidermis that will subsequently express the encoded antigen in sufficient quantities to elicit an immune response (Shi et al. 1999). It appears that the production of very small amounts of protein in the skin is sufficient to elicit an immune response. LCs and dermal dendritic cells (DC), which are specialized in initiating immune responses, can acquire antigen through direct transfection or through uptake and presentation of antigen synthesized by transfected keratinocytes or other skin cells (Pfutzner and Vogel 2000). The application of pDNA expressing the lacZ and HBsAg on to intact skin resulted in the induction of anti-HBsAg antibody and cellular responses (Fan et al. 1999). The predominant isotype of antibodies was IgG1, suggesting a Th2-type of response. For the HBsAg, the antibody and cellular responses were of the same order of magnitude of those induced after intramuscular injection with the commercially available recombinant HBsAg vaccine (Fan et al. 1999). Finally, in the same study it was demonstrated that efficient uptake of pDNA required the presence of intact hair follicles. Thus, the distal part of the human hair follicle immune system appears to represent a specialized compartment, where interacting intraepithelial T cells and LCs can generate effective immune responses (Christoph et al. 2000). The feasibility of topical genetic immunization has also been demonstrated with chitosan-based nanoparticles containing plasmid DNA (Cui and Mumper 2001). Two types of nanoparticles were investigated: (1) pDNA-condensed chitosan nanoparticles and (2) pDNA coated on preformed cationic chitosan/carboxymethylcellulose (CMC) nanoparticles. After their application on to bare skin, antigen expression was detectable at quantifiable levels in mouse skin after 24 h, and antigen-specific antibodies were measured in serum samples.

Induction of mucosal immunity

Immunization on to bare skin of mice with ADP-ribosylating exotoxins mixed with proteins elicits not only serum antibody responses but also significant levels of IgG and more modest levels of IgA antibodies in serum and multiple mucosal secretions (Glenn et al. 1999; Gockel et al. 2000; Beignon et al. 2001; Yu et al. 2002). Secretory IgA antibodies can decrease the colonization of mucosal surfaces by a number of microorganisms, and can effectively neutralize viruses and bacterial toxins generated within the lumen of the gut. In addition IgG antibodies present in mucosal fluids have recently been demonstrated to neutralize viruses (Baba et al. 2000). Thus, topical immunization provides an effective means to induce both systemic and mucosal immunity.

The exact mechanisms involved in the induction of mucosal immunity are still not clear. Studies by Enioutina et al. (2000) have provided evidence that 1,25-dihydroxy-vitamin D_3 [$1,25(OH)_2D_3$], which is derived from normal circulating 25-hydroxy-vitamin D_3 ($25(OH)D_3$) after its exposure to the enzymatic activity of 1α-hydroxylase, redirects the migration of dendritic cells from the skin into lymphoid organs committed to initiating mucosal immune responses. CT and LT, as a result of their ability to activate adenylyl cyclase, share the capacity to elevate intracellular levels of cAMP (Spangler 1992). Taking into account that the vitamin D_3–1α-hydroxylase gene, encoding the cellular enzyme that converts $25(OH)D_3$ to $1,25(OH)_2D_3$ is under cAMP control (Kong et al. 1999), it could be argued that ADP-ribosylating exotoxins stimulate the induction of mucosal immunity through the stimulation of $25(OH)D_3$–1α-hydroxylase expression (Enioutina et al. 2000).

Variability of immune responses and potential of peptides as immunogens

Data from immunogenicity studies using proteins, toxoids, and viruses suggest that large molecules can penetrate the skin barrier and with the help of an adjuvant elicit immune responses (Hammond et al. 2001b). However, antibody responses to co-applied antigens are normally variable, significantly lower, and with slower onset compared with those induced by co-application

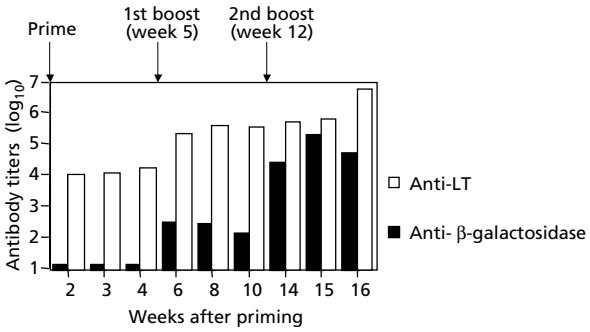

Figure 3 *Kinetics of serum anti-heat-labile enterotoxin (LT) and anti-β-galactosidase antibody responses after co-immunization on to bare skin of BALB/C mice (n = 6) with 100 μg LT and 100 μg β-galactosidase. Data represent mean of antibody titers.*

with CT or LT (Figure 3). Moreover, in a recent study where the safety and immunogenicity of a prototype enterotoxigenic *E. coli* vaccine was assessed in adult volunteers after topical application, only 68 and 53 percent were found to have serum anti-colonizing factor CS6 IgG and IgA antibodies, respectively (Guerena-Burgueno et al. 2002).

The use of synthetic peptides that normally have a small molecular size could potentially overcome this problem. This view is supported by the observation that most of the commonly used and effective drugs applied to bare skin have a molecular mass of <500 Da. In addition, chemicals that cause allergic contact dermatitis are <700 Da (Bos and Meinardi 2000). Peptide antigens have indeed been reported to induce protective anti-tumor CTL responses after application on to disrupted (by stripping) skin (Seo et al. 2000). This mechanical intervention removes the stratum corneum and activates LCs (through the stimulation of cytokine secretion by keratinocytes). Therefore, immune responses can be induced without the need of an adjuvant (Katoh et al. 1997); however, this procedure is painful and poorly reproducible. Recent studies have demonstrated that peptides are effective immunogens when co-applied with an ADP-ribosylating exotoxin or mutants of LT on to bare skin, eliciting peptide- and antigen-specific $CD4^+$ T cells (Beignon et al. 2001, 2002a, 2002b). This is particularly important in the context of vaccine design and delivery, because $CD4^+$ T cells produce cytokines that have a direct effect on viruses, help B cells to produce neutralizing and protective antivirus antibodies, and enhance the magnitude of cytotoxic cell responses to clear virus-infected cells.

FUTURE PROSPECTS AND CHALLENGES FOR DELIVERING VACCINES ON TO BARE SKIN

Over the last few years there has been remarkable progress in the field of immunodermatology. More recently, the realization that the skin has a number of characteristic features, such as easy accessibility, the presence of powerful APCs, and cells such as keratinocytes that secrete a wide range of cytokines, makes it an attractive route for vaccine delivery. However, for effective delivery, several variables related to the nature of the antigen and characteristics of the skin barrier must first be overcome, e.g. the diffusion of an antigen through the stratum corneum is dependent on its physicochemical properties and its molecular interactions with skin constituents (Morganti et al. 2001). These variables are:

- variability of percutaneous absorption as a result of the site, disease, age, species differences, and physicochemical characteristics of the vaccine formulation
- dermal metabolism of topically applied antigens
- incomplete understanding of the technologies that may be used to facilitate absorption and mechanisms of immune induction.

This could explain the differences in immunogenicity of several antigens after their application to bare skin. It is also important to stress the fact that the increased immunogenicity of ADP-ribosylating exotoxins depends mainly on their inherent adjuvanticity and ability to bind to cell surface receptors. Furthermore, the skin of humans and animals both pose unique barriers resulting from differences in anatomy and physiology between species, e.g. small laboratory animals always present a skin barrier that is more permeable than that of larger animals, including humans (Wester and Maibach 1987). The primary difference between rodent and human skin is the lipid composition and organization of the stratum corneum (Bond and Barry 1988). For these reasons, all these variables make the task of extrapolating the findings of studies performed in different animal species to the target species difficult. Percutaneous absorption through porcine and monkey skin (squirrel and rhesus) is more closely related to that through human skin (Wester and Maibach 1987). Porcine skin has been shown to be histologically and biochemically similar to human skin and, in addition, has permeability characteristics similar to that of human skin (Wester and Maibach 1987; Dick and Scott 1992); as such, it provides the most suitable experimental model for dermatological research on humans.

In situations of skin disease, eczematous reactions can develop after topical application of protein allergens on the nonlesional skin of atopic dermatitis patients, demonstrating that high-molecular-mass proteins could penetrate through this type of skin and induce an allergen-specific, delayed-type hypersensitivity response (Castelain 1995). This suggests that the skin can be a site of sensitization to environmental protein allergens. Therefore, topical application of vaccines might be an unsuitable option for immunization of individuals with skin disease. The main challenge ahead is the development of approaches to increase the permeability of the skin barrier to various vaccine antigen formulations, and

to optimize the induction of long-term mucosal and systemic immune responses.

MUCOSAL DELIVERY OF VACCINES

The mucosal routes offer an attractive alternative to currently used parenteral immunization. This is mainly because vaccines can be easily administered via the nose or orally, and mucosal immunization stimulates the induction of both systemic and local immunity. The latter is of paramount importance because most invading pathogens enter the body via mucosal surfaces. Over the last few years significant progress has been made in understanding the immunological mechanisms involved for maintaining the balance between immunity and tolerance. This has helped in the design and development of new immunization strategies and technologies, promising to deliver future vaccines via the mucosal routes using edible preparations or nasal sprays.

Antigen uptake by the mucosal surfaces

The mucosal surfaces constitute a vast surface area (about 300–400 m^2 in an adult), permanently challenged by numerous exogenous microorganisms associated with infectious diseases. As a consequence, the mucosal epithelium has evolved into a sophisticated physical and immunological barrier, protecting the host from environmental pathogens. Its cellular organization (stratified epithelium versus simple epithelium) and antigen-sampling mechanisms differ, depending on the mucosal site (Table 1).

The stratified epithelium is composed of multiple layers of cells that lack tight junctions. However, its barrier function is provided by a glycoprotein that they secrete, which seals the intercellular space and excludes the entry of most infectious agents and vaccines (Neutra et al. 1996). In stratified epithelia, DCs migrate to the outer limit of the epithelium, where they sample antigens for subsequent presentation in local or distant organized lymphoid tissues (McWilliam et al. 1994). Simple mucosal epithelia are made up of a layer of epithelial cells and their barrier function is provided by tight junctions that seal the intercellular space (Neutra et al. 1996). Antigens are sampled by microfold (M) cells that transport them from the lumen to the organized lymphoid tissue within the underlying mucosa. These are specialized epithelial cells located in the dome region of the epithelial layer, known as follicle-associated epithelium (Neutra 1999). Parallel to their sampling function, M cells can also serve as a portal of entry for several microorganisms, including bacteria, viruses, and protozoa (Siebers and Finlay 1996). Adherence to M cells has also been shown greatly to facilitate antigen transport and enhance immunogenicity (Frey and Neutra 1997; Wu et al. 2001).

Mucosa-associated lymphoid tissue and induction of immune responses

The mammalian mucosal immune system has evolved to fulfill several functions, including: (1) the production of secretory IgA (sIgA) antibody responses; (2) the distribution of effector B and T cells to local and distant mucosal sites; and (3) the induction of tolerance. The mucosa-associated lymphoid tissue (MALT) can be divided into two anatomically and functionally distinct compartments or sites: (1) the inductive sites, which are defined lymphoid microcompartments, and (2) the effector sites, which contain diffuse accumulations of large numbers of lymphoid cells that do not associate into apparently organized structures (McGhee et al. 1992). In the upper respiratory tract of rodents and humans, the major inductive sites are the nasal associated lymphoid tissue (NALT), which is organized lymphoid tissue at the base of the nasal cavity and the tonsils, respectively (Kuper et al. 1992). Mucosal inductive sites of the gastrointestinal tract include the Peyer's patches (PP), appendix, and solitary lymphoid nodules, which collectively comprise gut-associated lymphoid tissue (GALT).

The antigen is first encountered in the inductive sites, which contain B and T lymphocytes and APCs, and initial responses are elicited. After sensitization, antigen-specific B and T lymphocytes migrate from the inductive sites to regional lymph nodes and proceed via the lymph into the circulation, from where they 'home' to distant mucosal effector sites, such as the lamina propria of the respiratory, gastrointestinal, and reproductive tracts and

Table 1 *Types of mucosal epithelia and antigen-sampling mechanisms*

Type of epithelium	Mucosal site	Antigen-sampling
Stratified epithelia	Lower genital tract Upper digestive tract	APCs migrate to the outermost limit of the epithelium where they sample antigens
Simple epithelia	Lower digestive tract Airways	Migrating APCs can sample antigen
	Upper genital tract	Resident M cells transport antigens across the epithelial barrier

APCs, antigen-presenting cells; M, microfold.

the lacrimal, salivary, and mammary glands where immune responses are expressed. This cell distribution from the inductive sites to the effector sites has been termed the 'common mucosal immune system' (McDermott and Bienenstock 1979) and ensures that all mucosal surfaces are furnished with antigen-specific immunocompetent cells for protection.

Following mucosal immunization, humoral and cellular immune responses can be developed. After the antigen is sampled and passed through endocytic vesicles to the underlying lymphoid cells in the submucosa, antigen processing and presentation occur. This results in the activation of T cells which in turn provide help to B cells to develop into IgA plasma cells. Secretory IgA plays an important role in mucosal defense by providing protection against viruses or bacteria, and neutralizing microbial toxins (McGhee et al. 1992). These functions appear to be facilitated, at least in part, by the high-affinity binding of sIgA to mucus. Intranasal immunization induces sIgA responses to a wider range of mucosal tissues than does oral immunization. This is probably caused by the more promiscuous profile of homing receptors possessed by the circulating IgA-secreting cells that is induced after intranasal immunization (Quiding-Jarbrink et al. 1995).

For the induction of an effective mucosal immune response, the activation of T cells ($CD4^+$ and $CD8^+$) is of paramount importance because they provide help to B cells to become IgA-producing cells. These cells also release cytokines and chemokines that are involved in all aspects of mucosal immunity and, finally, they contribute to clearing viral infections. As $CD4^+$ T cells mature in response to foreign antigens, they take on unique characteristics normally manifested by production of a distinct set of cytokines. The Th1 cells, which result after infection with viruses or intracellular bacteria, produce IL-2, IFN-γ, and IL-12, and the Th2 cells, which are triggered by exogenous antigens, produce IL-4, IL-5, and IL-10 (Mosmann and Coffman 1989). Some of these cytokines regulate the expression of B-cell Ig isotypes and subclasses as well as influence the maturation of B-cell responses through induction of terminal differentiation into plasma cells.

Edible vaccines

The idea of using genetically modified plants to produce and deliver protective antigens via the oral route has several attractive features:

- Edible vaccines can be easily produced at low cost. The cultivation, harvesting, storage, and processing of transgenic crops could use the existing infrastructure and therefore would require relatively little capital investment. In this way, the purchase price of a dose of vaccine can be significantly reduced, increasing the chances of sustainable immunization strategies, particularly in less developed countries where vaccines are still expensive. It has been estimated that the cost of producing recombinant proteins in plants could be 10- to 50-fold lower than producing the same protein in *E. coli* (Kusnadi et al. 1997).
- There is a reduced health risk from pathogen contamination because plants do not harbor human infectious pathogens.
- Edible vaccines have the added advantage of providing natural bioencapsulation to the expressed antigens, protecting them from degradation in the harsh environment of the stomach.
- Edible vaccines induce mucosal immunity that can be advantageous for the control of pathogens entering via the mucosal surfaces and causing enteric, respiratory, and sexually transmitted infections.
- They can be administered easily and have the potential to deliver multiple immunogens, so increasing vaccine compliance.
- They can deliver multiple antigens.

EDIBLE VACCINES EXPRESSING VIRAL ANTIGENS

In 1992, Mason and co-workers successfully integrated the DNA-encoding HBsAg into tobacco plants. The expressed HBsAg retained its capacity for self-association and, more importantly, was structurally and antigenically similar to the HBsAg derived from human serum and recombinant yeast. Subsequent studies have demonstrated that oral immunization of mice with HBsAg expressed in potato tubers with CT as an adjuvant elicited strong serum antibody responses that reached a peak of 103 mIU/ml 4 weeks after the third dose and >10 mIU/ml (level of protective serum anti-HBsAg antibodies) at week 11 (Kong et al. 2001). Oral feeding of potato tubers expressing HBsAg has also resulted in strong memory responses, as assessed by the rise of antibody titers to 3 300 mIU/ml 3 weeks after parenteral immunization with a subimmunogenic dose of alum-absorbed recombinant HBsAg (Kong et al. 2001). Thus, the strategy of combining the oral and parenteral routes of immunization can be advantageous in less developed countries where the logistics of multiple delivery doses of parenteral vaccines have hampered global HBV immunization efforts. In the same study it was demonstrated that cooking the transgenic potatoes (boiling) before feeding mice significantly reduced the immunogenicity of the edible vaccine. An alternate approach to overcome this problem could be the use of transgenic bananas, which are grown extensively throughout the less developed world and, in contrast to potatoes, can be eaten raw.

As degradation of proteins in the stomach limits their bioavailability, much higher concentrations of antigens are normally required for effective oral immunization compared with parenteral immunization. Therefore,

increasing the levels of antigen expression in plant tissues would enhance the utility of transgenic plants as a source of recombinant antigen. A systematic study of factors influencing the accumulation of HBsAg in transgenic potatoes has demonstrated a striking improvement of antigen expression. This resulted from alternate polyadenylation signals and fusion protein-targeting signals designed to enhance integration or retention of HBsAg in the ER of plant cells (Richter et al. 2000). However, there is always the risk that a single high dose or repeated low doses of a protein antigen can induce oral tolerance. To overcome this potential problem, mucosal adjuvants that enhance antigen-specific mucosal and systemic immune responses have been used (Mason et al. 1996).

Another example of edible vaccine is the expression of Norwalk virus capsid protein (NVCP) in transgenic plants (Mason et al. 1996). The virus is responsible for an acute gastroenteritis in humans. The expression of recombinant NVCP in transgenic tobacco leaves resulted in self-assembled virus-like particles that were morphologically and physically similar to the recombinant protein produced in insect cells. When extracts of tobacco leaves expressing the NVCP were given orally (50 μg protein with or without 10 μg CT on days 1, 2, 11, and 28) by gavage to mice, both IgG and secretory IgA anti-NVCP antibodies were detected in serum and fecal samples. The presence of CT enhanced antibody levels during the early phases of immunization but, at the final time point (40 days after the first immunization), the titers in mice fed with transgenic tobacco with or without CT were very similar (Mason et al. 1996). In a human clinical trial, ingestion of raw transgenic potatoes constitutively expressing the NVCP induced immune responses in the absence of adjuvant (Tacket et al. 2000). The majority (95 percent) of volunteers had a significant increase in the number of antigen-specific IgA antibody-secreting cells, 20 percent developed antigen-specific serum IgG antibodies, whereas, in 30 percent of the volunteers, antigen-specific IgA antibodies were detected in their stools. In terms of safety, there were few symptoms other than nausea and cramps. More importantly, none of the volunteers had a change in serum anti-potatin (the major protein contained in potatoes) IgG antibodies after ingesting the transgenic potato vaccine (Tacket et al. 2000).

EDIBLE VACCINES EXPRESSING BACTERIAL ANTIGENS

The feasibility of using transgenic plant expression systems for vaccine use has also been documented with bacterial antigens. Mason et al. (1998) have designed and constructed a plant-optimized synthetic gene encoding the *Escherichia coli* heat-labile enterotoxin B subunit (LTB). Expression of LTB gene in potatoes increased antigen expression in leaves and tubers, and LTB molecules were assembled into native pentameric structures that were able to bind to their ganglioside receptors. When mice were fed with raw tuber slices they developed serum and fecal IgA anti-LTB antibodies, and were partially protected against challenge to orally delivered LT (Mason et al. 1998). This edible vaccine was also tested in human volunteers who received 50–100 g raw transgenic potato. Most of the participants (10 of 11 tested) developed serum anti-LTB antibodies (Tacket et al. 1998). Also transgenic potatoes engineered to synthesize CTB pentamers with affinity to G_{M1} gangliosides were immunogenic (inducing serum and intestinal CTB-specific antibodies) when fed to mice (Arakawa et al. 1998a). The serum samples neutralized the activity of CT on Vero cells and the immunized mice were partially protected (60 percent reduction in diarrheal fluid accumulation in the small intestine).

A plant-based multicomponent vaccine has been demonstrated to protect mice from enteric disease (Yu and Langridge 2001). This edible vaccine consisted of two CT fusion genes, one expressing a 22-amino acid immunodominant epitope of the murine rotavirus enterotoxin NSP4 to the CTB subunit (C-terminal fusion) and the other expressing the *E. coli* fimbrial colonization factor CFA/I to the CTA2 subunit (N-terminal fusion). When mice were fed with transgenic potato tuber tissues expressing the CT fusion proteins, antibody responses (serum IgG and intestinal IgG and IgA) against the NSP4, CTB, and CFA/I were detected. Cultured splenocytes from immune mice secreted IL-2 and IFN-γ, and diarrheal symptoms were reduced in severity and duration in passively immunized neonatal mice following rotavirus challenge.

FUTURE PROSPECTS AND CHALLENGES FOR THE DEVELOPMENT OF EDIBLE VACCINES

For almost a decade, research on the development of edible vaccines has focused on viral and bacterial antigens. These studies helped to establish the proof of the concept and the identification of appropriate methods for enhancing protein expression, as well as providing answers to fundamental questions such as the selection of an appropriate crop, the quantity of plant tissue constituting a vaccine dose, and the importance of structural integrity of expressed antigen. Moreover, human clinical trials have demonstrated that edible vaccines are safe and can induce systemic and mucosal immune responses without the aid of an adjuvant. Despite all these positive aspects, the immune responses elicited in this way are still modest and this remains one of the limiting factors for the development of an effective edible vaccine for human use. Furthermore, there is always the risk that induction of immunity after oral immunization may lead to inflammatory or allergic reactions as a result of the breakdown of the physiological tolerance toward food antigens.

By improving our understanding of the basic mechanisms involved in induction of immunity or tolerance, it

should be possible to manipulate the GALT. This will allow the preferential stimulation of protective systemic and mucosal immune responses against infectious pathogens or antigen-specific tolerance, which could be advantageous for treating autoimmune diseases or allergic disorders (Arakawa et al. 1998b). Furthermore, the determination of the molecular requirements for directing mucosally primed immunocompetent cells from the inductive sites to the effector sites will help in the design of more effective mucosal vaccines.

Nasal vaccines

Vaccines that can be administered via the nose appear to hold more promise than oral vaccines, because intranasal immunization generally requires a much lower dose of antigen, and elicits more potent immune responses (Table 2). There are several advantages to using the nose as a route of immunization and an intense search to develop safe and immunogenic nasal vaccines is currently under way:

- It is easily accessible.
- It is highly vascularized.
- The presence of numerous microvilli covering the nasal epithelium generates a large absorption surface.
- After intranasal immunization both systemic and mucosal immune responses are induced.
- Immune responses can be induced at distant mucosal sites.
- Its a noninvasive route and therefore it does not require needles and syringes.

After intranasal immunization, the balance between active immunity and tolerance would depend greatly on the nature of the antigen and its interaction with the NALT, as well as on the dose, adjuvant, frequency of antigen administration, and genetic background of the host (Kuper et al. 1992). Particulate antigens can be either removed by the mucociliary clearance system or sampled by M cells (which overlay the NALT); they are then transported to the posterior cervical lymph nodes (Kuper et al. 1992). In the case of soluble antigens, these molecules can penetrate the mucosal epithelium and reach the superficial cervical lymph nodes (Kuper et al. 1992). High doses of antigen administered intranasally are likely to reach the intestinal tract (Willoughby and Willoughby 1977) or be taken up directly by the posterior cervical lymph nodes instead of the superficial ones (Kuper et al. 1992). The superficial cervical lymph nodes, which drain the nasal mucosa, appear to be instrumental in the induction of mucosal tolerance (Kuper et al. 1992). Posterior cervical lymph nodes are involved in the generation of sIgA responses (Kuper et al. 1992). This suggests that the lymphocyte composition differs in different lymph nodes as a result of their 'homing' behavior, which underlies the observed divergence in the induction of either tolerance or immunity.

Table 2 *Differences between oral and nasal routes of immunization*

	Oral route	Nasal route
Proteolytic activity	High	Moderate
Distance to transport	Long	Small
Dose of antigen	High	Medium
Requirement of adjuvant	Yes	Yes
Antigen uptake	Poor	Good
Targeting requirement	Yes	Not necessary
Humoral responses	Low	High
Cellular responses	Low	High

VACCINE DELIVERY VIA THE NOSE

For the development of safer and better vaccines, much effort has been devoted to defining and producing protective antigens or epitopes from the pathogen of concern. When these antigens are given intranasally, they are weakly immunogenic or induce tolerance. To circumvent these problems, delivery systems and mucosal adjuvants have been developed:

- Use of replicating vectors expressing the antigen (live attenuated viruses or bacteria).
- Use of nonreplicating vectors (liposomes, microparticles, immune-stimulating complexes (ISCOM)).
- Use of recombinant plant viruses expressing antigens (cowpea mosaic virus).
- Use of cold-adapted influenza virus (FluMist vaccine).
- Antigen co-administered with an adjuvant (CT, LT, CpG, cytokines, etc.).
- Antigen adsorbed on bioadhesive polymers (chitosan, microparticles, etc.).

Vectors for delivering antigens via the nose

Replicating delivery systems present the antigen (protein or epitopes) in the context of a genetically modified live attenuated bacterium or virus, which provides a continuous antigenic stimulation and elicits broad humoral or cellular immune responses. It is important that these vectors express adequate amounts of antigen, have a tropism for the mucosal epithelium, and do not revert to the virulent form. As vectored vaccines are live, they are likely to share the same antigen-processing and presentation pathway as the pathogen, and therefore elicit the desired type of immune responses. Among the recombinant vectors that have been widely tested are: *Mycobacterium bovis* (Langermann et al. 1994), *Vibrio cholerae* (Chen et al. 1998), *E. coli* (Turner et al. 2001), *Shigella flexneri* (Anderson et al. 2000), *Salmonella typhi* (Galen et al. 1997), vaccinia virus (Bernstein 2000), adenovirus

(Gallichan and Rosenthal 1996), and poliovirus (Crotty et al. 1999). However, for live attenuated microorganisms, the induction of immunity to the organism used as a carrier could preclude their use for booster immunizations, and reversion to virulence may be a potential problem. As an alternative, plant viruses, which are not infectious to mammals, have been tested with antigenic peptides (Dalsgaard et al. 1997; Durrani et al. 1998; Olszewska and Steward 2001), and commensal lactobacilli (*Lactobacillus plantarum*) have begun to receive attention as safe noninvasive vectors (Shaw et al. 2000).

The intranasal delivery of vaccines using nonreplicating vectors has the advantage that it eliminates one of the major concerns, that of reversion to virulence. For successful intranasal immunization, the nonreplicating vector should: (1) protect the antigen from degradation; (2) allow its transport via the M cells; and (3) promote the appropriate type of immune responses required for protection. Among the various nonreplicating vector systems, particles such as liposomes (Mader et al. 2000), virosomes (Cusi et al. 2000), ISCOMs (Hu et al. 2001), biodegradable microparticles (Vajdy and O'Hagan 2001), biovector systems (Von Hoegen 2001), and proteosomes (Levi et al. 1995) have been extensively studied. The availability of so many different formulations allows the selection of the most appropriate to address the clinical needs, the nature of the antigen, and the type of desired immune responses. However, for some of these vectors there are certain limitations for human use, e.g. toxicity is a major drawback of using ISCOMs as a vaccine delivery system. This is because of the presence of saponins that have strong hemolytic and cytolytic activity even at low doses. For biodegradable microparticles, there are several concerns about the stability of the entrapped antigen during storage, the efficacy of microparticle uptake by the mucosal epithelium, and problems in the production of sterile preparations for human use.

An alternate approach to encapsulation is to adsorb antigens on to the surface of biodegradable polymeric lamellar substrate particles (PLSP). This is a simple procedure that avoids pH changes caused by bulk polymer degradation and the use of solvents and, therefore, should be less damaging to the vaccine. Several antigens such as proteins, peptides, and viral particles have been adsorbed on to PLSPs and tested for immunogenicity after nasal administration (Jabbal-Gill et al. 2001), e.g. adsorbed influenza vaccine induced strong secretory and systemic antibody responses (Jabbal-Gill et al. 2000). Carbohydrate biopolymers have also been tested for their ability to enhance the immunogenicity of adsorbed antigens. In particular, chitosan, which is a cationic polysaccharide and binds strongly to negatively charged cell surfaces and mucus, greatly improves the absorption of antigens from the nasal cavity and augments antigen-specific immune responses (Bacon et al. 2000; Illum et al. 2001).

Adjuvants for intranasal immunization

Administration of antigens via the nose has the potential to induce a wide range of immune responses; the types of response induced in this way can be significantly influenced by the use of adjuvants. In experimental animal models, the most potent mucosal adjuvants are CT and LT. Both are strong mucosal immunogens in their own right and can act as effective adjuvants to intranasally co-administered antigens by enhancing serum and secretory antibody responses and cytotoxic T-cell responses (Imaoka et al. 1988; Rappuoli et al. 1999; Kurono et al. 1999; Partidos et al. 1999). As their enzymatic activity precludes their use in humans, much effort has been devoted to attempting to dissociate their adjuvant properties from their toxicity. Using site-directed mutagenesis, several mutants have been generated with significantly reduced ADP-ribosylating activity and toxicity compared with the holotoxin (Rappuoli et al. 1999; Pizza et al. 2001). These molecules have been shown to exert adjuvant activity to co-administered antigens by increasing serum and secretory antibody responses (Pizza et al. 2001), modulating immune responses as a consequence of their ADP-ribosylating activity (Ryan et al. 2000), and potentiating CTL responses (Partidos et al. 1996). However, there are several concerns about the role of G_{M1}-binding molecules that target neuronal tissues in mucosal immunity. Intranasal delivery of CT targets the main olfactory nerves and epithelium, which is directly connected with the olfactory bulbs, and raises the possibility of the induction of neuronal damage (Van Ginkel et al. 2000). Furthermore, it has been suggested that the use of CT as intranasal adjuvant results in the redirection of vaccine proteins into the CNS (Van Ginkel et al. 2000). However, recent studies in mice have shown that LT mutants are safe and that they do not induce any detectable inflammatory response in any anatomical site (Peppoloni et al. 2003).

Bacterial DNA has potent immunostimulatory properties caused by the presence of unmethylated CpG motifs, which are far more common in bacterial than in vertebrate DNA (Krieg 2002). A similar immunostimulatory effect can be seen with synthetic ODNs containing CpG motifs (ODN-CpG) (Roman et al. 1997). In studies where vaccine antigens were co-delivered with an ODN-CpG via the mucosal routes, strong systemic and mucosal immune responses were detected (McCluskie et al. 2001a). Furthermore, in some instances protective immune responses were enhanced, as demonstrated in mice immunized intranasally with an ODN-CpG and a recombinant glycoprotein of HSV1 (Gallichan et al. 2001) or with an influenza virus peptide representing a mimic of the antigenic site A of hemagglutinin (Partidos et al. 2001). CpG motifs can also modulate the adjuvant effect of CT or mutants of LT when they are co-administered with recombinant proteins or peptides, promoting a Th1-type immune responses (Olszewska

et al. 2000; McCluskie et al. 2001b). Therefore, CpG-ODNs are novel mucosal adjuvants, which either alone or in combination with other adjuvants can modulate and enhance humoral and cellular immunity.

An alternate approach to the potentiation of immune responses to intranasally administered antigens is to use cytokines as adjuvants. The cytokine microenvironment at the site of antigen delivery plays a critical role in the induction and regulation of immune responses. Boyaka et al. (1999) and Boyaka and McGhee (2001) have demonstrated that intranasally delivered IL-6 and IL-12 can enhance antigen-specific serum antibody responses to co-administered protein antigen, but only IL-12 is able to trigger antigen-specific secretory IgA responses. Also it has been reported that IL-1α and IL-1β exhibit mucosal adjuvant activity for the induction of serum IgG and mucosal IgA antibody responses when they are nasally co-administered with soluble protein antigens (Staats and Ennis 1999). In addition, it was found that the combination of IL-1α + IL-18, IL-1α + IL-12, and IL-1α + IL-12 + GM-CSF each induced optimal splenocyte anti-HIV CTL responses in mice immunized intranasally (Staats et al. 2001). Despite the mucosal adjuvanticity of certain cytokines it should be noted that they are pleotropic molecules and, therefore, they have complex effects that depend on the state of activation of different immunocompetent cells and the balance of other cytokines.

CONCLUDING REMARKS

This is an exciting time in the field of vaccinology. Thanks to the technological advances in biomedical sciences we now have a wide range of new immunization strategies and vaccine delivery options. More importantly, the widespread administration of vaccines without the use of needles is now a distinct possibility and this is a significant advantage for immunization programs in less developed countries, because such administration would be simple, painless, and economical.

An intranasal vaccine against influenza virus (NasalFlu, containing inactivated virus and LT as an adjuvant) has already been approved in Switzerland. In addition, another influenza vaccine – the FluMist, a live cold-adapted virus – has been developed for delivery in the form of a spray and is close to being licensed for human use. It is certain that more 'needle-free' vaccines will follow.

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New vaccine technologies

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Immunization/vaccination remains the most efficient intervention against infectious diseases. However, for many infections the traditional approaches have not yielded safe, effective, convenient or affordable vaccines. For these applications and in the interest of safer and more practical interventions for diseases already covered by traditional vaccines, there remains a need for improved vaccine technologies. In this chapter, we summarize some of the most promising vaccine delivery technologies and evaluate their future promise.

TRANSCUTANEOUS IMMUNIZATION

Transcutaneous immunization (TCI) is the direct application of antigen and adjuvant to intact or slightly abraded skin. Once through the skin, an antigen encounters a dense population of efficient antigen-presenting cells (APC), the Langerhans' cells, which trigger innate and adaptive immune responses to antigens surmounting the integument (Hammond et al. 2001a). The potential advantages of TCI are many. Foremost is the ease of vaccine administration, avoiding the expense, logistics, and risk of needle-based injection. TCI is presumably safer than other parenteral routes, although information about this is still preliminary.

Successful TCI requires delivery of the immunogen through the stratum corneum (SC), the skin's principal barrier to the passage of molecules (Scharton-Kersten et al. 1999). Several strategies have been devised to permit facile penetration of the SC by molecular immunogens. Simple hydration results in significant, but transient, changes in the SC, and may be sufficient to allow penetration of some immunogens (Kaiserlian and Etchart 1999).

Preclinical experience with TCI appears very promising, and has illuminated several prerequisites of this approach. Foremost among these is the need for adjuvant co-administration to enhance immune responsiveness. The adjuvants most frequently employed are the cholera enterotoxin (CT) and the related *E. coli* heat-labile toxin (LT) (Glenn et al. 1998, 1999; Scharton-Kersten et al. 2000; Chen et al. 2002; John et al. 2002; Yu et al. 2002), although other adjuvants may also be effective (Scharton-Kersten et al. 2000). The mixture of CT or LT with tetanus toxoid dramatically increases antibody responses when compared with the administration of the antigens alone (Hammond et al. 2001b). Mice immunized with diphtheria toxoid (DTx) and CT developed excellent immune responses which persisted for over 9 months. Using tetanus toxoid as a model antigen, Hammond et al. (2001b) found that TCI induced both T-helper (Th) 1 and 2 (Th1 and Th2) responses (with a Th2 bias) partly dependent on choice of adjuvant. Several studies have also suggested that TCI can elicit mucosal immune responses (John et al. 2002; Yu et al. 2002) as well as cell-mediated responses (Yu et al. 2002). John et al. (2002) found that TCI of mice with CT resulted in mucosal responses similar to those engendered by a live attenuated cholera vaccine administered orally.

The adjuvant-dependent immunogenicity of a TCI vaccine was demonstrated in volunteers receiving CS6 colonization factor of enterotoxigenic *E. coli* (ETEC). Participants received patches containing 250, 500, 1000, or 2000 μg CS6 alone or with 500 μg LT, dosed at 0, 1, and 3 months (Guerena-Burgueno et al. 2002). In the absence of LT, there were no demonstrable immune

responses to the CS6 antigen. In contrast, when CS6 was given with LT, 68 percent and 53 percent had anti-CS6 IgG and IgA, respectively, and 100 percent and 90 percent had anti-LT IgG and IgA, respectively. Fourteen (74 percent) of 19 volunteers developed mild delayed-type hypersensitivity (DTH) skin reactions after the second or third dose; no other adverse events were reported.

Questions that remain to be answered about TCI include the precision of dosing, as well as efficacy and safety in infants and young children, whose skin may be more porous than adults and in whom hypersensitivity may be a concern. In addition, the efficacy of LT/CT as an adjuvant may be limited in immune populations, perhaps precluding its use more than once (see

New approaches to vaccine delivery for further discussion of this aspect of vaccinology).

VIROSOMES

Liposomes, vesicles comprised of membrane-type phospholipids, can act as effective adjuvants for protein antigens that are incorporated into the vesicle membrane (Gregoriadis and Allison 1974; Allison and Gregoriadis 1976, 1990; Tan and Gregoriadis 1990) (Figure 1). Liposomes are efficiently taken up by APCs and their contents are processed largely via major histocompatibility complex (MHC) class II-dependent pathways. Liposome uptake can be further facilitated in a number of ways. Coating liposomes with antibodies, for example, can target them to Fcγ receptors on APCs. Liposomes produced by reconstitution of viral glycoproteins and lipids (virosomes) have many of the properties of the parent viruses. Virosomes have been adapted as delivery vehicles for antigens, antigen DNA, drugs, proteins, and water-soluble materials (Bron et al. 1993, 1994).

Virosomes initiate an immune response by binding and fusing with the eukaryotic plasma membrane, thereby discharging their cargo into the cytoplasm. As would therefore be predicted, antigens are chiefly presented in the context of MHC-I molecules. Effective $CD8^+$ cytotoxic T-cell (CTL) responses to virosome-encapsulated antigens are reported (Gregoriadis and Allison 1974; Gregoriadis 1990; Nerome et al. 1990; Miller et al. 1992; Arkema et al. 2000; Gluck et al. 2000). Bungener et al. (2002a, 2002b) have shown that membrane fusion-active virosomes, but not fusion-inactive virosomes, are able to deliver a test antigen to dendritic cells for MHC-I presentation; fusion activity of virosomes was not required for MHC-II presentation of the same antigen. Mucosal application of virosomes can elicit systemic and secretory IgA (sIgA) responses (Cusi et al. 2000, 2002).

Virosome-based influenza vaccines

Influenza virosomes are prepared by detergent purification of influenza surface glycoproteins and reconstitution with a mixture of phosphatidylcholine (70 percent), phosphatidylethanolamine (20 percent), and influenza virus envelope phospholipids (10 percent). This mixture was carefully chosen: egg yolk phosphatidylcholine has an excellent safety record of administration to humans; phosphatidylethanolamine has been shown to stimulate B cells to secrete antibody in the absence of T-cell help (Garcon and Six 1991); and the influenza virus glycoproteins haemagglutinin (HA) and neuraminidase (NA) play key roles in virosome function. HA is the major fusion-promoting protein of the virosome, and also directly mediates binding to APCs.

The influenza virosome vaccine has been tested in humans of all ages and appears to be safe and immunogenic when administered intranasally (Kaji et al. 1992;

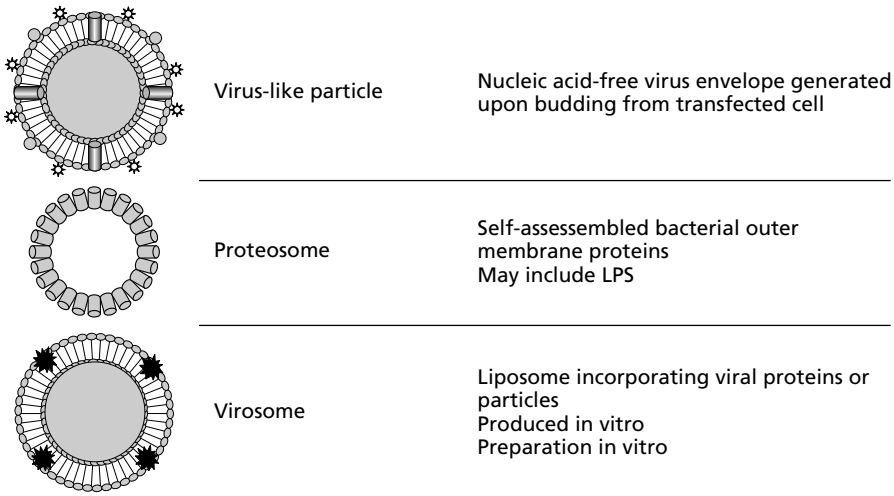

Figure 1 *Virus-like particles, proteosomes, and virosomes are artificial, membrane-bound bodies that are used to deliver antigens to the immune system.*

Gluck et al. 1994, 2000; Conne et al. 1997; Schaad et al. 2000; Herzog et al. 2002). Following the excellent performance of the influenza prototype and the natural versatility of the virosome approach, antigens from other viruses have been incorporated into influenza virosomes. These notably include the F antigen from respiratory syncytial virus (Cusi et al. 2002), the protective core peptide of hepatitis C virus (Hunziker et al. 2002), and the hepatitis A virus vaccine, considered in greater detail below.

Hepatitis virosome vaccines

The first breakthrough in the use of virosomes for delivery of foreign antigen was the generation of a hepatitis A vaccine comprising hepatitis A virus (HAV) strain RG-SB integrated into influenza-derived virosomal particles (Loutan et al. 1994). In phase 3 clinical studies, seroconversion was documented in more than 98 percent of volunteers within 4 weeks of immunization (Argentini et al. 1995; Gluck et al. 1992). Booster injections of the virosomal vaccine 12 months after the initial dose resulted in 100 percent seroconversion, increasing anti-HAV antibody titers by 11- to 40-fold (Loutan et al. 1994).

The nature of virosomes makes them particularly amenable to the integration and presentation of multiple antigens. Hepatitis B surface antigen (HBsAg) has been successfully integrated into HAV-containing virosomes (Mengiardi et al. 1995). In further steps, the diphtheria toxoid, the α-tetanus toxoid, the β-tetanus toxoid, and the inactivated hepatitis A virion were additionally crosslinked to the virosome surface (Gluck 1995). Several such combination vaccines are licensed in Europe and Asia.

Other virosome applications

As virosomes efficiently fuse their contents with the cell cytoplasm, virosomes may be effective vectors for nucleic acid vaccine delivery (Cusi and Gluck 2000; Cusi 2001). They may also increase the uptake of antisense oligonucleotides (ODN), considered as promising therapeutic agents against viral diseases and cancer, or as immunomodulators.

Virosomes were used to deliver synthetic peptide-based malaria vaccine SPf66 (Poltl-Frank et al. 1999). Immunization of mice with two intramuscular doses of the virosome vaccine engendered higher antibody titers to the malaria antigen than when the antigen was administered with alum. An HIV vaccine, based on genetically modified glycoprotein and nonstructural peptides, is also under development (Kaneda 2000).

PROTEOSOMES

The proteosome nomenclature emphasizes the distinctive physical and biological properties of preparations of the outer membrane proteins (OMP) of gram-negative bacteria (Lowell et al. 1988a, 1988b). As membrane proteins, OMPs are highly hydrophobic; when isolated by detergent extraction, hydrophobic protein–protein interactions cause them to self-assemble into multimolecular particles with morphological characteristics of vesicles up to several hundred nanometers in diameter. The typical method of creating a proteosome-based vaccine is to process a mixture of detergent-solubilized OMPs with amphiphilic antigens, in a manner that facilitates noncovalent association of the antigens with the proteins and excludes the detergent (Wetzler et al. 1989, 1992; Wetzler 1994). Proteosomes therefore comprise noncovalent complexes of bacterial OMPs studded with amphiphilic antigen. A nasal proteosome influenza vaccine constructed in this way is currently in clinical trials. A second approach to deriving proteosome vaccines is to formulate a mixture of vaccine antigen with a soluble pre-formed proteosome adjuvant preparation made up of proteosome particles complexed with lipopolysaccharide (LPS). This system (termed 'IVX-908') may either incorporate amphiphilic or entirely hydrophilic molecules, or result from mixing of antigens with the pre-formed LPS-solubilized proteosome particles. IVX-908 possesses immunostimulatory properties in part resulting from its LPS. A proteosome shigella LPS vaccine has been shown to be promising in phase 1 and 2 clinical trials (see below) (Fries et al. 2001).

Proteosome vaccine particles exhibit certain characteristics that improve their safety and immunogenicity. Their surface hydrophobicity may improve uptake and processing by APCs, in particular promoting Th1-type responses. It has also been suggested that the immunogenicity of proteosomes is improved by the interaction of bacterial OMPs (Massari et al. 2002) and LPS (Chow et al. 1999) with the Toll-like receptors (TLR) of APCs. Although interaction with TLRs could cause some concern with inflammatory side effects, experience with the gram-negative OMPs (from *Neisseria meningitidis*) serving as carriers in *Haemophilus influenzae* vaccines has been positive (Heath 1998).

FluINsure, the nasal proteosome-based vaccine for influenza under development by ID Biomedical Corp., has undergone extensive clinical testing, with generally positive results (Plante et al. 2001). Phase 1 or 2 clinical trials of FluINsure using single-dose (15, 30, or 45 μg HA) and two-dose (7.5, 15, or 30 μg HA per dose) regimens in a total of 330 healthy adults have documented a high degree of safety and tolerance, in the presence of serum IgG responses similar to intramuscular vaccines (Treanor et al. 2001; Halperin et al. 2002). In addition, FluINsure recipients exhibited rises in sIgA titers to levels thought to be protective against influenza infection (Plante et al. 2001).

The proteosome shigella vaccine candidate comprises LPS and OMPs of *Shigella sonnei* and *S. flexneri* 2a (Fries et al. 2001). In dose-escalating phase 1 and 2 clinical

studies, 111 healthy adults received two intranasal doses of the vaccine 2 weeks apart (Fries et al. 2001; McKenzie et al. 2001). Adverse effects were limited to mild nasal congestion and rhinorrhea in half to two-thirds of vaccinees. More than 90 percent of vaccinees demonstrated significant antibody-secreting cell responses (ASC); 60–90 percent responded with serum antibodies (four-fold geometric mean titer or GMT increases in IgG and IgA were typical at 1-mg doses).

In a challenge study using live *S. flexneri* given less than 48 days after immunization, vaccine efficacy was 36 percent against any diarrhea ($p = 0.04$), 78 percent against fever ($p = 0.003$), and 56 percent against severe symptoms including dysentery ($p = 0.03$) (Durbin et al. 2001). Although this vaccine appears to be well tolerated and fairly immunogenic, the brief duration of immune response after two doses limits its appeal. Nevertheless, the vaccine provides an interesting proof of principle that could be improved on in further iterations of this strategy. The most practical target for intranasal proteosome vaccines is likely to be respiratory viruses, for which a brief period of protection during the peak season would be beneficial. Parenterally administered proteosome vaccines could have broader application, but require additional clinical development.

VIRUS-LIKE PARTICLES

Virus-like particles (VLP) are nonreplicating, recombinant, viral capsids, with a structure similar to that of the virions from which they are derived. VLPs are formed by self-assembly of viral structural protein(s) on synthesis in eukaryotic or prokaryotic expression systems; they therefore present the native conformation of viral antigens but lack viral nucleic acid (Kruger et al. 1999). VLPs are particularly useful when native virions cannot be readily isolated or produced or when a very high degree of vaccine safety is desired (e.g. immunization of immunocompromised patients). They may be made up of single viral capsid proteins (e.g. papillomavirus and hepatitis B core protein) or multiple proteins contributing to a more complex structure (e.g. herpesviruses, rotavirus, and Ebola virus).

The immune response to VLPs is typically balanced to include neutralizing antibodies and cytotoxic T-cell responses, presumably as a result of the presentation of multiple native antigens in each particle (Sedlik et al. 1997, 1999, 2000). The balance of systemic vs mucosal immune responses is determined by the route of administration, as well as by the antigenic characteristics of the VLP itself. Papillomavirus VLPs have been shown to induce strong peripheral IgG responses on parenteral administration, and good sIgA responses when given by the oral route (Gerber et al. 2001). Unlike many other new vaccine delivery approaches, immunogenicity is strong without the need for an additional adjuvant (see below). However, although cytotoxic (Sedlik et al. 1997) and Th1 responses (Lo-Man et al. 1998) are typically robust on VLP administration, responses can be further augmented or modified by co-administration of adjuvant (Siadat-Pajouh and Cai 2001), often approaching that derived from native viral infection.

Manufacture of VLPs has been made possible only by the advent of molecular biotechnology. The fundamental approach entails the cloning of genes encoding viral capsid proteins into a mammalian expression vector or other expression system. Among the many systems successfully adapted are baculovirus (Garnier et al. 1995), *Pichia pastoris* (yeast cells) (Sasnauskas et al. 1999), plant systems (e.g. *Nicotiana*) (O'Brien et al. 2000), and bacterial expression systems (Chen et al. 2001). The expression vector and host cell system typically must be optimized. On protein expression, some VLPs bud from the plasma membrane and self-assemble, whereas others must be post-translationally modified or otherwise manipulated.

The versatility of the VLP approach has proved to be formidable. A partial list of vaccines in various stages of testing include those against rotavirus (O'Neal et al. 1998; Jiang et al. 1999; Coste et al. 2000, 2001; Siadat-Pajouh and Cai 2001), Norwalk virus (Ball et al. 1999), parvovirus (Sedlik et al. 1997, 1999), hepatitis viruses (Li et al. 1997; Lorenzo et al. 2001), papillomavirus (Pumpens et al. 2002; Schiller and Lowy 2001b), calicivirus (Nagesha et al. 1999), HIV (Garnier et al. 1995; Weber et al. 1995; Peters et al. 1997), hantavirus (Koletzki et al. 2000), polyoma virus (Goldmann et al. 1999), and influenza virus (Latham and Galarza 2001; Watanabe et al. 2002).

Some VLPs have progressed to phase 1 or 2 clinical trials (Ball et al. 1999; Zhang et al. 2000; Evans et al. 2001; Schiller and Lowy 2001a). In nearly all such studies, VLPs have fulfilled their promise in providing an excellent safety profile, coupled with good immunogenicity. Evans et al. (2001) reported on a phase 1 study of VLPs comprising the L1 protein of human papilloma viruses (HPV). The vaccine was well tolerated and induced high levels of both binding and neutralizing antibodies. The vaccine induced strong B- and T-cell responses, and T-helper epitopes appear to be conserved across HPV types. Harro et al. (2001) reported the results of a double-masked, placebo-controlled, dose-escalation trial to evaluate the safety and immunogenicity of an HPV type 16 (HPV16) L1 VLP vaccine in healthy adults. Volunteers were given intramuscular injections with placebo or with 10- or 50-μg doses of HPV16 L1 VLP vaccine without adjuvant, or with alum or MF59 as adjuvants at 0, 1, and 4 months. All vaccine formulations were well tolerated, and all participants receiving vaccine seroconverted. With the higher dose, most of the recipients achieved serum antibody titers that were approximately 40-fold higher than what is observed in natural infection. Zhang et al. (2000) administered multiple doses of HPV VLPs (HPV6b) without

adjuvant to 33 participants with genital warts. All subjects mounted DTH responses to the vaccine. Complete regression of genital warts was observed in 25 of 33 evaluable participants over the 20-week observation period. The vaccine was well tolerated.

Ball et al. (1999) have conducted trials of a Norwalk virus (NV) VLP made up of a single capsid protein. Twenty adults received two doses of vaccine 3 weeks apart. No side effects were observed or reported by the volunteers. All vaccinees given the highest dose responded with fourfold or more increases in serum IgG antibody titers. Most of the volunteers (83 percent; 15 of 18) responded after the first rNV VLP dose and showed no increase in serum IgG antibody titer after the second dose. Tacket et al. (2003) fed Norwalk VLPs without adjuvant to 36 healthy adult volunteers at doses of 250 μg (n = 10), 500 μg (n = 10), and 2000 μg (n = 10); six volunteers received a single dose of placebo. Of those who received 250 μg 90 percent developed rises in serum anti-VLP IgG antibodies. Higher doses did not produce higher responses. All vaccinees developed significant rises in IgA anti-VLP ASCs, although only 30–40 percent of volunteers developed mucosal anti-VLP IgA antibodies. Lymphoproliferative responses and interferon γ (IFN-γ) production were observed transiently among those who received 250 μg or 500 μg, but not 2000 μg of VLP.

Peters et al. (1997) reported the results of a phase 2 study of the yeast-derived VLP HIV vaccine Ty.p24.VLP (p24-VLP), in HIV-antibody-positive asymptomatic volunteers. Fifteen volunteers, with p24 antibody titers >1/100, p24 antigen <20 pg/l, and CD4 $>350 \times 10^9$/l, were enrolled. Five were immunized with aluminum hydroxide placebo, five with 25 μg, and five with 100 μg p24-VLP in alum adjuvant at weeks 0 and 4 by the intramuscular route. No serious adverse events were observed in any of the groups, although efficacy data have not been provided.

VLPs represent a highly versatile strategy for vaccine delivery. Human trials have thus far borne out the safety and immunogenicity of this immunization strategy, although no VLP vaccine has yet been licensed. One important question about this class of vaccines is the duration and breadth of protection, and the possible requirement for adjuvant has not yet been determined for mucosally administered VLP vaccines.

LIVE ATTENUATED VECTOR VACCINES

The use of attenuated bacterial strains as mucosal live vectors to deliver antigens of unrelated pathogens is a highly promising vaccine strategy for several reasons (Medina and Guzman 2001):

- The mucosal route of administration efficiently induces both systemic and local protective immunity, which is favorable for pathogens and toxins that enter through mucosal surfaces.
- Mucosal delivery is usually associated with low reactogenicity.
- Logistics of vaccine administration are typically easier for mucosal vaccines, especially in the context of mass immunization campaigns.
- As they are perceived as less invasive than parenteral vaccines, oral vaccines have a generally high level of public acceptance and increased compliance.
- Genetic engineering strategies used to construct mucosal vaccines confer a high degree of versatility. Live attenuated enteric vaccines would be expected to have relatively low manufacturing costs compared with many other vaccines.

Live attenuated salmonella vaccines

Attenuated *Salmonella enterica* serovar Typhi vaccines were among the first oral bacterial vaccines, and they remain among the most promising. The great promise of *S. typhi* as a live attenuated vaccine derives from its pathogenic strategy (Kingsley and Baumler 2000; Santos et al. 2003; Zhang et al. 2003). *S. typhi* briefly colonizes the small intestinal mucosa, and invades at the level of the Peyer's patch epithelium (Figure 2). On entry into the lymphoid follicles, the organisms are taken up into macrophages, which transport the bacteria throughout the body. Attenuation strategies have been devised that permit the organism to enter the lymphoid tissue but not to persist or circulate via the bloodstream. This modified pathway is ideal for presentation of antigens to the gut-associated lymphoid tissue.

Germanier and Fuer (1975) derived the first attenuated *S. typhi* vaccine (designated Ty21a) by nonspecific chemical mutagenesis of parent strain Ty2. Ty21a harbors a galactose epimerase mutation, does not express the Vi (virulence) capsular polysaccharide, and exhibits many additional mutations that collectively contribute to its attenuation. Although Ty21a has served as a safe and effective typhoid fever vaccine for more than a decade, it has certain shortcomings, chief among these being the requirement for multiple doses to achieve protective efficacy (Levine et al. 1990). The advent of genetic engineering provides an opportunity to exploit specific attenuation strategies, because this organism is genetically tractable.

Several groups have prepared recombinant *S. typhi* vaccine candidates and tested them in phase 1 studies in human volunteers. Four strains proved to be sufficiently well tolerated and immunogenic to stimulate interest in additional clinical trials. These include: Ty800 (attenuated by virtue of mutation in the two-component regulatory system PhoPQ) (Hohmann et al. 1996), χ4372 (inactivated in the CRP transcriptional regulator) (Tacket et al. 1992a), CVD 908-*htrA* (inactivated in aromatic biosynthesis pathway and production of a stress protease) (Tacket et al. 2000b), and ZH9 (inactivated in

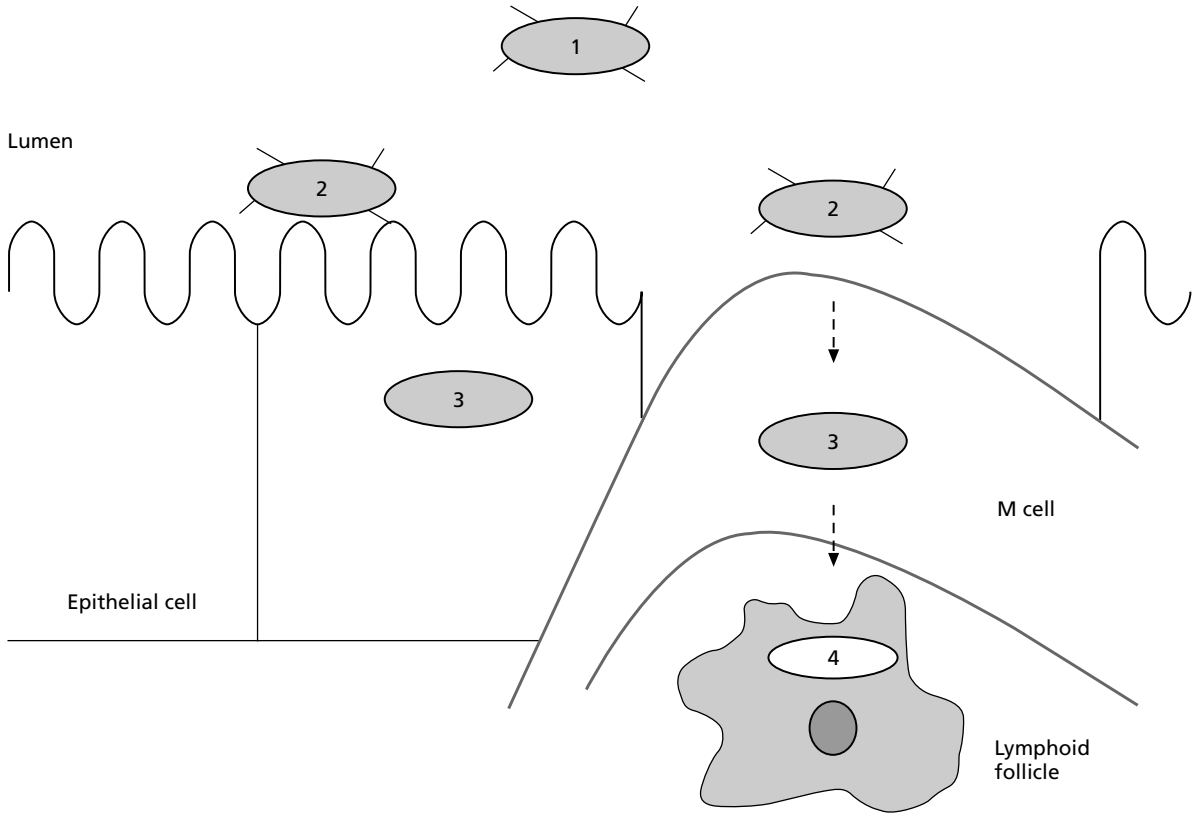

Figure 2 *Microanatomical locations of live attenuated vaccine vectors. Nonliving vaccines remain in the lumen of the gastrointestinal tract (1) and can be sampled passively by the gut-associated lymphoid tissue. Some live vaccine vectors, e.g. those derived from* Vibrio cholerae*, actively adhere to the surface of the absorptive epithelium (2) or to the M cells overlying the lymphoid follicles. Invasive bacterial vectors, e.g. strains of* Shigella *and* Listeria *spp., invade the epithelial cells but remain in the cytoplasm (3), whereas vectors such as* Salmonella *translocate through the epithelial layer and access the underlying antigen-presenting cells (4). The focus of live attenuated vector technology is to construct mutants that target the immunogen to one or more of these compartments.*

the aromatic biosynthesis pathway and the salmonella pathogenicity island-2 type III secretion system secreton) (Hindle et al. 2002; Khan et al. 2003).

The immediate predecessor of CVD 908-*htrA* was CVD 908 (Hone et al. 1991), attenuated by virtue of two nonreverting deletion mutations within the chromosomal *aroC* and *aroD* genes. These two genes encode enzymes critical in the biosynthetic pathway leading to synthesis of chorismate, the key precursor required for synthesis of the aromatic amino acids (Hoiseth and Stocker 1981); chorismate is further required for the synthesis of *p*-aminobenzoic acid, which is ultimately converted to the purine nucleotides ATP and GTP. Although immunization of volunteers with CVD 908 is well tolerated and highly immunogenic (Tacket et al. 1992a, p. 216), a silent vaccinemia was detected in 100 percent of volunteers who ingested 5×10^8 colony-forming units (cfu), wherein vaccine organisms were recovered from blood cultures collected between days 4 and 8 after immunization (Levine et al. 1996, 1997). This silent bacteremia was not associated with adverse clinical symptoms and was self-limiting. Nevertheless, further attenuation to obviate vaccinemias was pursued.

CVD 908 was attenuated further by a defined deletion mutation in the *htrA* gene, which encodes a heat shock-induced serine protease responsible for degradation of misfolded periplasmic proteins (Pallen and Wren 1997). As predicted, CVD 908-*htrA* was well tolerated at doses up to 5×10^9 without vaccinemia (Tacket et al. 1997). Volunteers ingesting CVD 908-*htrA* manifested a broad immune response to *S. typhi* antigens, including intestinal sIgA antibodies, serum IgG antibodies, and cellular immune responses.

The versatility of *S. typhi* as a vector to deliver foreign antigens has been demonstrated in both CVD 908 and CVD 908-*htrA* backgrounds. Volunteers receiving two doses of *S. typhi* CVD 908 expressing the *Plasmodium falciparum* circumsporozoite protein (CSP) developed cytotoxic T lymphocytes (CTL) to CSP-transfected target cells (Gonzalez et al. 1994). In a second study, 21 healthy adult volunteers received a single dose of CVD 908-*htrA*-carrying, plasmid-encoded, tetanus toxin fragment C under the control of one or two different promoters induced in vivo (*nirB* and *lpp*) (Tacket et al. 2000a). Neither vaccine induced fever or vaccinemia. Of three seronegative volunteers who received 10^9 cfu CVD

908-*htrA* (pTET*lpp*), a single oral dose of the live vector expressing fragment C seroconverted one of the three and elicited a rise in serum tetanus anti-toxin 43 times above the protective level.

McKenzie et al. (2002) fed CVD 908-*htrA*-expressing *Helicobacter pylori* urease to healthy volunteers at doses up to about 5×10^9 cfu; the vaccine was well tolerated and induced urease-specific IgA and IgG ASCs in 25 percent and 88 percent of the immunized individuals.

With the establishment of the ability of *S. typhi* to deliver foreign antigens, attention has turned to improving the technical aspects of heterologous gene expression (Galen and Levine 2001). Accordingly, genetic tools have been developed to express heterologous antigens from strong in vivo-induced promoters (see above); assure recombinant plasmid replication in the absence of antibiotic selection (Galen et al. 1999); and enable secretion of foreign antigens to the extracellular milieu. With experience and expertise accumulating rapidly, the future of *Salmonella* spp. as a live attenuated vector vaccine appears bright.

Live attenuated shigella vaccines

Similar to *Salmonella*, shigella strains invade the intestinal mucosa and enter the gut-associated lymphoid tissue. Unlike *Salmonella*, *Shigella* spp. are not adapted for long-term persistence in macrophages, and bacteremias are rare. *Shigella* spp. therefore offer some advantages and some disadvantages to use as a live vector. Similar to *Salmonella*, advances in the understanding of shigella pathogenesis allow precise disruption of virulence genes without alteration in the invasive phenotype, which is believed to be required for a protective response. Recently identified attenuating strategies are finally yielding vaccine candidate strains that are immunogenic in humans, yet are well tolerated clinically.

Sansonetti and co-workers (1991) constructed a series of *Shigella flexneri* 5a strains with mutations in the *virG* (*icsA*) gene, which is involved in cell-to-cell spread (Bernardini et al. 1989) but which is not required for invasiveness. Additional mutations were constructed in *ompB* or *iuc*, the latter encoding the siderophore aerobactin (Sansonetti et al. 1991). In animal studies, the *ompB/virG* strain was well tolerated but not fully protective against shigella challenge. In contrast, the *virG*/*iuc* strain caused mild adverse effects in some animals, but 100 percent protection after challenge (Sansonetti and Arondel 1989). Similarly, a *S. flexneri* 2a strain with the *virG* and *iuc* mutations (SC602) caused symptoms of mild shigellosis in healthy adult volunteers at doses of 10^6 and 10^8 cfu, whereas a dose of 10^4 caused only mild symptoms in a few volunteers (Coster et al. 1999). Importantly, volunteers ingesting a single dose of 10^4 SC602 were protected against challenge with wild-type *S. flexneri* 2a. This experience underscores the dose-related relationship between immune response and reactogenicity; however, doses tolerated by healthy adults may not be so for children or other more vulnerable hosts, and thus a higher level of safety is desirable.

A *virG*-deleted strain of *S. sonnei*, strain WRSS1, has been evaluated in a phase 1 clinical study. A single dose of 10^3, 10^4, 10^5, or 10^6 cfu caused low-grade fever or mild diarrhea in 22 percent of recipients (Kotloff et al. 2002). However, the vaccine was highly immunogenic, generating impressive levels of serum anti-shigella LPS and anti-LPS IgA ASCs (Kotloff et al. 2002).

Work at the CVD has yielded similar experience. Noriega and co-workers (1994) generated *S. flexneri* 2a vaccine candidate CVD 1203, with mutations in *aroA* and *virG*. A single dose of 10^6 CVD 1203 was well tolerated and immunogenic. However, at doses of 10^8 or 10^9 cfu, short-lived adverse reactions, including fever, diarrhea, and/or mild dysentery, were observed (Kotloff et al. 1996). This experience again illustrates the dose-dependent nature of adverse effects and calls for a higher degree of safety in attenuated shigella constructs.

In progress toward achieving the correct balance of immunogenicity and safety, Noriega and co-workers (1994) generate *S. flexneri* 2a strain CVD 1204, harboring a deletion in the *guaBA* operon (Anderson et al. 2000). This mutation, which renders the bacteria auxotrophic for guanine, is more attenuating and virtually nonreactogenic in the guinea-pig model of keratoconjunctivitis. Two further derivatives of CVD 1204 were constructed: CVD 1208 carries additional mutations in the genes encoding shigella enterotoxins 1 and 2 (ShET1 and ShET2, encoded by *set* and *sen*, respectively), and CVD 1207, which harbors a deletion in *virG* in addition to the mutations in *guaBA*, *set*, and *sen* (Kotloff et al. 2000; Levine 2000). Human studies on this series of candidates are under way. Both CVD 1207 and CVD 1208 have proved to be well tolerated but the latter appears to be more immunogenic (Kotloff et al. 2000).

Live attenuated *Vibrio cholerae* vaccines

Vibrio cholerae has many attributes that make it an attractive vaccine delivery vehicle. It is a noninvasive organism which colonizes the human intestinal mucosa and induces potent, long-lived mucosal and systemic humoral responses (Kaper et al. 1995). The lack of invasiveness of *V. cholerae* reduces the likelihood of systemic reactions and theoretically reduces the need for global attenuation of the pathogen. *V. cholerae* has been intensively studied at the molecular level and is genetically tractable.

Several live attenuated *V. cholerae* vaccine constructs are safe and immunogenic in humans (Sack and Albert 1994; Liu et al. 1995; Cohen 1997; Herzog et al. 1997;

Tacket et al. 1999; Yamamoto 2000). CVD 103HgR (*ctxA*−, *hly*−, *recA*, classic Inaba strain 569B) is the first recombinant live attenuated cholera vaccine, and it continues to set the standard for safety and efficacy for cholera vaccines (Levine et al. 1988; Migasena et al. 1989; Cryz et al. 1990; Kotloff et al. 1992; Su-Arehawaratana et al. 1992; Tacket et al. 1992b, 1999; Gotuzzo et al. 1993; Simanjuntak et al. 1993; Sack and Albert 1994; Lagos et al. 1995; Losonsky et al. 1997; Perry et al. 1998; Yamamoto 2000). The organism is principally attenuated via deletion of the CTA subunit, but its parent strain also shows a mild defect in its ability to colonize the human intestine (Levine et al. 1988). CVD 103-HgR has been administered to more than 6000 individuals in placebo-controlled phase 1 and 2 trials, including infants and HIV-infected individuals (Levine et al. 1988; Migasena et al. 1989; Cryz et al. 1990, 1992; Kotloff et al. 1992; Su-Arehawaratana et al. 1992; Suharyono et al. 1992; Tacket et al. 1992b, 1999; Gotuzzo et al. 1993; Losonsky et al. 1993, 1997; Simanjuntak et al. 1993; Lagos et al. 1995, 1999a, b; Perry et al. 1998; Taylor et al. 1999; Richie et al. 2000). In all studies, neither diarrhea nor any other adverse reaction occurred significantly more often in vaccinees than in placebo recipients. CVD 103HgR conferred strong protective immunity in North American volunteers against experimental challenge with wild-type *V. cholerae* strains. In one study, a single dose conferred 100 percent protection against experimental challenge with a toxigenic classic strain when volunteers were challenged only 8 days after immunization; protective immunity was also documented to extend to at least 6 months after immunization, the longest interval tested (Tacket et al. 1992b). In a randomized, double-masked, placebo-controlled, multicenter trial, a single dose of this strain conferred 91 percent protective efficacy against moderate or severe diarrhea after challenge with toxigenic El Tor *V. cholerae* (Tacket et al. 1999). The ability of CVD 103-HgR to provide long-term protection against natural challenge in cholera-endemic countries has not been established. Over 4 years of surveillance during a field trial in Indonesia, only 14 percent efficacy was observed overall (Richie et al. 2000). During the first 6 months of this trial (when protection might be expected to b maximal), few cases of cholera occurred. However, during a post-licensure intervention with CVD 103-HgR carried out under the auspices of the World Health Organization during a cholera outbreak in Micronesia, it was calculated that the vaccine provided 79 percent effectiveness in preventing cholera (Claire-Lise Chaignat, WHO, personal communication). In view of its consistent efficacy in multiple experimental challenge studies in conferring a high level of protection in North American volunteers against both classic and El Tor cholera caused by either Inaba or Ogawa serotypes, and its ability to elicit strong vibriocidal antibody responses, CVD 103-HgR has been licensed in many countries (including Canada, Australia, and Switzerland).

Several other attenuated cholera vaccines have been tested in humans (Levine et al. 1988; Taylor et al. 1994; Kaper et al. 1995; Liu et al. 1995; Sack et al. 1997; Benitez et al. 1999; Valle et al. 2000). A series of nonmotile cholera variants is well tolerated and immunogenic in healthy adults and is undergoing further clinical trials (Taylor et al. 1994).

V. cholerae vector strains have been used to deliver heterologous antigens (Butterton et al. 1995) such as *Shigella sonnei* LPS (Favre et al. 1996), *E. coli* Shiga toxin B subunit (Acheson et al. 1996), enterohemorrhagic *E. coli* intimin (Butterton et al. 1997), tetanus toxin fragment C (Chen et al. 1998), *B. pertussis* tracheal colonization factor (Chen et al. 1998), *Entamoeba histolytica* surface protein (Ryan et al. 1997b), and *Clostridium difficile* toxin A (Ryan et al. 1997a).

BCG-based vector vaccines

The currently licensed bacille Calmette–Guérin (BCG) vaccine is a multiply passaged *Mycobacterium bovis* strain, originally developed in 1908 (Fine 1989). The bacterium is highly attenuated and acceptably safe, but it is only partially protective against tuberculous disease, principally the extrapulmonary forms (Fine 2001). By virtue of its derivation from *M. bovis* and its history of multiple passage, BCG is lacking 16 discrete regions of the *M. tuberculosis* genome. There is therefore no risk of reversion to virulent *M. tuberculosis*. The efficacy of BCG varies among countries, and indeed the BCG vaccines used throughout the world display substantial genetic diversity attributed to its handling over the course of the twentieth century (Fine 1995, 2001; Behr and Small 1997, 1999; Behr et al. 1999; Fine and Vynnycky 1998).

Similar to mycobacteria that naturally infect humans, BCG is taken up into macrophage and dendritic cells and undergo limited replication within the phagolysosome (Inaba et al. 1993). As expected, therefore, BCG presents antigens predominantly in the context of MHC-II (Kaufmann and Ladel 1994; Kaufmann et al. 1995; Ladel et al. 1995a, b); BCG elicits predominantly a CD4-mediated Th1-type response. However, modifications of the route, timing, and dose of administration can elicit more balanced Th1/Th2 immunity (Bretscher 1992, 1994; Power et al. 1998).

BCG has been considered as a vector for delivery of antigens when a Th1 response is desired. As *Listeria monocytogenes* is an intracellular pathogen normally controlled by Th1 responses, several investigators have adapted BCG for use in listeria vaccine development (Hess et al. 1998; Conradt et al. 1999). Hess and colleagues expressed the p60 protective antigen of *L. monocytogenes* in various cellular compartments of the BCG bacterium (Hess 2002). Not unexpectedly, surface

display or secretion of p60 provided the strongest responses. The market for a human listeria vaccine is doubtful, but progress in a BCG-derived listeria vaccine development may constitute an important proof of principle.

BCG-derived vaccine candidates have been constructed against various pathogens, including bacteria (e.g. *Borrelia burgdorferi* (Langermann et al. 1994a), *Bordetella pertussis* (Nascimento et al. 2000), and *Streptococcus pneumoniae* (Langermann et al. 1994b; Miyaji et al. 2001)); parasites (*Leishmania* (Connell et al. 1993; Abdelhak et al. 1995) and *Schistosoma* (Kremer et al. 1996)); and viral pathogens (HIV (Aldovini and Young 1990, 1991) and papillomavirus (Jabbar et al. 2000)). Edelman et al. (1999) at the University of Maryland tested a recombinant BCG (rBCG) vaccine expressing the outer surface protein A (OspA) of *B. burgdorferi*. Before human studies, the vaccine had proven highly immunogenic and protective in mice (Stover et al. 1993). Twenty-four adults received one intradermal injection of the rBCG-OspA vaccine containing escalating vaccine doses ranging from 2×10^4 to 2×10^7 cfu (Edelman et al. 1999). Local ulceration was the most common adverse effect; however, 83 percent of recipients of the highest dose developed BCG-positive draining lesions at the immunization site, persisting for up to 70 days (Figure 3). Despite these reactions, none of the volunteers at any dose manifested detectable post-immunization OspA antibody titers, although over half of vaccine recipients developed positive purified protein derivative (PPD) responses. These data illustrate the limitations of BCG as a vaccine vector, including limited antibody responses; more importantly, however, the threat of persistent shedding of BCG from cutaneous sites would constitute a potential risk to immunocompromised contacts.

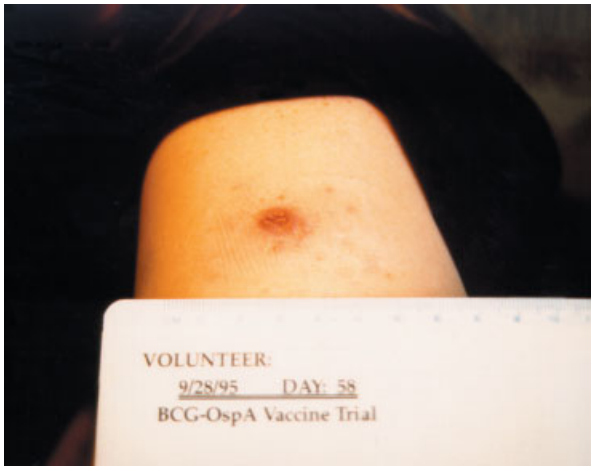

Figure 3 *Eschar caused by recombinant BCG vaccine. Large ulcerating eschar on the arm of a patient receiving one dose of BCG–outer surface protein A (OspA) vaccine 58 days previously. (Courtesy of R. Edelman, University of Maryland Center for Vaccine Development.)*

Live attenuated listeria vaccines

As a prototypical intracellular pathogen amenable to genetic manipulation, *L. monocytogenes* has been proposed as a vaccine vector against infections controlled by CTL and CD8 responses. *L. monocytogenes* pursues a pathogenic strategy similar to that of *Shigella*, in which the organism is engulfed by the eukaryotic cell, lyses the phagosome, and takes up residence in the cytoplasm (Portnoy et al. 2002). From this privileged site, the bacterium is protected from antibody responses, but can be eradicated by strong, cell-mediated, IFN-γ-driven responses (Portnoy et al. 1989; Finelli et al. 1999; Kerksiek and Pamer 1999; O'Riordan et al. 2002).

L. monocytogenes has been exploited for MHC-I presentation of a large number of viral, parasite, and tumor antigens, in either protein or nucleic acid form (Schafer et al. 1992; Dietrich et al. 2000, 2002; Gentschev et al. 2000, 2001, 2002; Spreng et al. 2000). Several of these constructs have proved to be safe and effective vaccines in mouse models (Ikonomidis et al. 1994; Ochsenbein et al. 1999; Dietrich et al. 2002; Soussi et al. 2002). However, as *L. monocytogenes* is a human pathogen, there is justified concern over possible adverse effects, especially in immunocompromised vaccinees. Friedman et al. (2000) have constructed a highly attenuated strain of *L. monocytogenes* that requires D-alanine, which is not available in human tissues. These investigators engineered the bacteria to express the HIV-1 Gag protein, and showed that the recombinant vaccine was as efficient at stimulating Gag-specific human CTLs in vitro as wild-type recombinants. This vaccine is considered to be a candidate for human trials and could serve as an important test case for *Listeria*-based vaccine strategies.

Other vector vaccines

Several other bacteria have been advanced as vector vaccines. Among these are several commensals, which imply a high degree of safety. Medaglini and colleagues (1995) engineered *Streptococcus gordonii*, a normal inhabitant of the human mouth, to express antigens of the M protein of *S. pyogenes*. In preparation for clinical trials of the vectored vaccine, K.L. Kotloff (personal communication) and co-workers evaluated the ability to implant the carrier strain (*S. gordonii*) resistant to streptomycin and 5-fluoro-2-deoxyuridine) into the nose and mouth of volunteers, and then delineated the time to eradication, both spontaneous and antibiotic induced. Inoculation was generally well tolerated. Symptoms reported most often within 4 days of inoculation were stuffy nose (36 percent), headache (30 percent), and sore throat (19 percent). The strain was detected by culture in the mouths of 97 percent of participants. A 5-day

course of azithromycin eliminated the strain in all participants. Without antibiotics, 82 percent spontaneously eradicated the implanted strain within 1 week, and all cleared by 1 month. Results of these studies will be used to design future phase I trials to evaluate a *S. gordonii* live vector vaccine expressing, on its surface, the conserved region of *S. pyogenes* M protein.

PRIME-BOOST IMMUNIZATION

Despite the success of vaccine technologies against a plethora of infectious diseases, certain infections have resisted vaccine strategies. Chief among these are HIV, malaria, and to a large extent tuberculosis. These infections have in common a requirement for strong cell-mediated responses, which are difficult to generate using traditional parenteral vaccine delivery strategies. In response to this problem, various innovative strategies have been devised, including some already discussed in this chapter. An additional, highly innovative immunization approach comprises a multiple-dose schedule in which the initial dose(s) prime the immune response, and subsequent doses deliver the same target antigens in the context of a different delivery vehicle, thereby engendering strong responses. This strategy, termed 'heterologous prime boost', has led to vigorous cell-mediated responses not as yet achieved by any other approach.

One of the earliest illustrations of the value of prime boost for generation of protective cell-mediated immunity (CMI) responses was provided by Li and coworkers in 1993. These investigators constructed a recombinant influenza virus expressing a CD8 T-cell epitope from the CSP of *Plasmodium yoelii*. Immunization of mice with the recombinant influenza virus, followed by a recombinant vaccinia virus expressing the entire CSP, induced protective immunity against a live sporozoite challenge. However, immunization with recombinant vaccinia virus first, followed by a booster injection with recombinant influenza virus, failed to induce protection. Subsequent to this promising start, several other groups corroborated the findings in malaria using several different heterologous prime-boost regimens (Hill et al. 2000; Bruna-Romero et al. 2001; Jones et al. 2001; Rogers et al. 2001; Schneider et al. 2001), and the utility of this approach for malaria immunization is being hotly pursued.

Also, in 1993, two separate groups reported highly promising results employing a heterologous prime-boost approach to HIV immunization. Graham et al. (1993) inoculated 12 volunteers with recombinant vaccinia virus expressing HIV gp160 glycoprotein, and then boosted them with recombinant gp160 protein. After booster immunization, the sera of all vaccinees showed strong antibody responses detected by western blot and enzyme-linked immunosorbent assay (ELISA); five blocked binding of CD4 cells to gp120. The prime-boost strategy was more immunogenic than either product alone. The same month, Cooney et al. (1993) independently reported the results of a similar phase 1 study in which 13 participants immunized with recombinant vaccinia virus expressing gp160 were boosted with recombinant gp160. T cell and antibody responses detected after immunization with either vaccine alone were of low magnitude and transient. In contrast, recipients of the combination regimen developed higher, more prolonged, T-cell proliferative responses to gp160.

Since these pioneering studies, the power of the prime-boost phenomenon in HIV immunization has been extended to other targets as well. Evans et al. (1999) have shown that a canarypox vaccine expressing multiple HIV antigens yielded stronger responses in volunteers when accompanied by injection of recombinant gp120. An interesting twist to this study was that the gp120 inoculation produced the same boost when administered simultaneously with the canarypox vaccine.

A great deal of attention has surrounded the phenomenon of priming with a nucleic acid vaccine, followed by parenteral boost with the protein antigen, usually delivered in a viral vector. Kent et al. (1998) evaluated a consecutive immunization strategy in mice involving priming with DNA and boosting with recombinant fowlpox virus vaccines, each encoding Gag and Pol HIV-1 antigens. The heterologous prime-boost approach induced greater HIV-1-specific immunity than either vaccine given alone; mice given the prime-boost regimen were protected from challenge with a recombinant vaccinia virus expressing HIV-1 antigens. In a subsequent experiment using rhesus macaques, a dramatic boosting effect on DNA vaccine-primed HIV-1-specific helper and cytotoxic T-lymphocyte responses was observed, although accompanied by a decline in HIV-1 antibody titers. The prime-boost regimen protected macaques from an intravenous HIV-1 challenge.

Robinson et al. (1999, 2000) compared eight different immunization protocols for their abilities to induce responses to nonpathogenic immunodeficiency virus challenge in rhesus macaque monkeys. The best containment response was induced by a regimen comprising intradermal DNA priming followed by fowlpox virus booster immunization; responses were characterized by strong T-cell responses which were over ten-fold higher than homologous prime-boost regimens. These investigators subsequently delivered Gag, Pol, and Env antigens in the context of two DNA injections (intramuscular or intradermal) given 8 weeks apart. They then boosted the monkeys with the same proteins expressed in the vaccinia virus Ankara vector (MVA), given intramuscularly and intradermally at 24 weeks. This regimen elicited high levels of antigen-specific T cells, of both CD4 and CD8 type. The monkeys were protected from intrarectal viral challenge with the highly pathogenic simian–human immunodeficiency virus 89.6P given 7 months after immunization. Curiously, however, these

investigators were later to show that two doses of the MVA vaccine, without DNA vaccine boost, were able to induce significant protection.

Since the initial success with prime boost in malaria and HIV vaccine efforts, this approach has been extended to other vaccines. In sum, this is substantial evidence that mucosal priming followed by parenteral boosting yields augmented, and broadened, immune responses, including TH1, TH2, and SIGA components. The concept of enteral prime/parenteral boost was first suggested by human studies using oral polio vaccine (OPV) and parenteral inactivated polio vaccine (IPV) recipients, boosted with either OPV or IPV (Herremans et al. 1999). In this study, parenteral IPV immunization boosted systemic and mucosal IgA antibody responses in previously OPV-immunized patients, but not in IPV-immunized individuals. Interestingly, IPV immunization of OPV-primed patients, although immunized decades earlier, mounted rapid (by day 7) and vigorous IgG as well as IgA responses in the periphery as well as at the mucosal level. IPV boosting of OPV-primed individuals resulted in broader immune responses which were at least as vigorous in all aspects as those seen in individuals primed and boosted with the parenteral vaccine.

These human data have subsequently been reproduced in mouse models. Using the pertactin protein of *Bordetella pertussis* expressed cytoplasmically in an attenuated salmonella vector, Anderson et al. (1996) demonstrated that effective boosting can occur in the setting of very weak responses to the initial prime (i.e. the mucosa may be primed without exhibiting demonstrable antibody response). Neither mice immunized with pertactin alone nor those immunized just with two doses of recombinant *S. typhimurium* pertactin exhibited anamnestic responses. The investigators also measured anti-pertactin IgG subclasses and found that mice immunized orally with *S. typhimurium* pertactin and boosted with 10 μg purified antigen produced IgG2a and IgG2b, whereas purified pertactin alone induced primarily IgG1. Splenic T-cell proliferation was observed in mice immunized orally with *S. typhimurium* pertactin and boosted with only 0.1 μg pertactin, thus demonstrating not only antibody but also T-cell priming by *S. typhimurium* pertactin. Interestingly, the 0.1 μg parenteral boosting dose of pertactin did not generate an immune response in the absence of priming. Ward et al. (1999) have documented similar findings using *S. typhimurium* expressing the C-terminal domain of *Clostridium difficile* toxin A (cytoplasmically). Using *Salmonella*-expressing antigens from *Streptococcus mutans* (Jespersgaard et al. 2001) and *Helicobacter pylori* (Londono-Arcila et al. 2002), other investigators have demonstrated that mucosal prime/parenteral boost results in boosting of systemic responses with good TH1/TH2 balance and boosting of SIGA. The limitations of enteral prime-parenteral boost are yet to be fully delineated, although the data generated thus far suggest that this is a very promising approach to the development of a strong, balanced response to protein antigens.

The immunological basis of the prime-boost phenomenon remains incompletely understood. The priming antigen apparently induces the maturation of antigen-specific T and B cells, some of which will persist and circulate widely as memory cells when the antigen is no longer present. These memory cells will rapidly expand on re-exposure to the same antigen, even if the antigen is given at a site very distant from the initial exposure. The mechanism behind the apparent synergy of prime-boost responses is harder to explain. One hypothesis states that, as prime-boost presents the dominant epitopes in different backgrounds, the immune response is spared the 'noise' of a large array of low-affinity epitopes. This focused response may be optimally vigorous. A second hypothesis suggests that the heterologous prime-boost approach avoids a secondary response to the vector, which some feel may be inhibitory to maximal antigen-specific boosting. Complete understanding of this phenomenon will be a significant contribution to future vaccine development strategies.

CONCLUSIONS

The science of vaccinology has produced some of the most important breakthroughs in the history of medicine. Yet despite our ability to control and even eradicate infections using immunization, limitations and weaknesses remain. Among these are infections so far recalcitrant to immunization strategies, our inability to engender the most appropriate class of immune response, and the need for ever-increasing levels of vaccine safety. In this chapter, we have highlighted just a few of the many new applications of biotechnology to address these problems. These and many other technologies offer hope in providing economically feasible barriers to control some of our most dangerous enemies.

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Peptide vaccines

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INTRODUCTION

For several decades synthetic peptides have been extremely valuable tools for dissecting the immune response to complex antigens and pathogens on an epitope level. Peptides as antigens have been extensively reviewed (Van Regenmortel and Muller 1999). Vaccines based on synthetic peptides or defined epitopes have met considerable interest and high expectations. A large number of vaccines have been tested in clinical trials and even more in animal models, but so far none has been licensed. This chapter is an attempt to present the current status of the vast field of experimental or candidate vaccines based on synthetic peptides. Much of what can be said about peptide vaccines applies also to other epitope-based strategies, but these are beyond the scope of this review. We acknowledge that such a review can never be complete and is necessarily biased toward the authors' experience – or the lack of it.

T- AND B-CELL EPITOPES

Sequence and post-translational modifications provide a protein with a unique three-dimensional surface architecture, which is the basis of cognate interactions with its molecular partners. The unique surface structure is exploited by the immune system to recognize antigens and to discriminate between self and nonself. Complementary domains, epitopes, and paratopes of antigens and antibodies are the minimal cognate structures (Jerne 1960), and the basis of the primordial intelligence that drives the teleological choices of the immune system, first at the induction stage and again at the effector stage of the immune response (Muller 2001b). T and B cells recognize epitopes and antigens in fundamentally different ways. Although B cells bind to intact antigens, T cells recognize only processed antigens presented as peptides by molecules of the major histocompatibility complex (MHC) of antigen presenting cells. Over the past years, both T-cell epitopes (TCE) and B-cell epitopes (BCE) have been identified in most antigens. Peptides have been used as minimal surrogate antigens to induce a prophylactic or therapeutic T- and/or B-cell response against pathogens, tumors, and other diseases.

PEPTIDE VACCINES BASED ON B-CELL EPITOPES

Conformational and sequential BCEs

The antibody paratope recognizes the congruent and complementary epitope of the antigen in its native, unprocessed, three-dimensional conformation. Complementary features of epitope and paratope converge to form relatively flat, bumpy surfaces of several square nanometers in size covering only a fraction of the solvent accessible surface of the antigen. Seminal radiological studies with hen egg lysozyme revealed, for instance, that 16 residues interact with 17 amino acids of

a monoclonal antibody (reviewed by Bentley 1996). When analyzed by amino acid replacement techniques, antibody binding appears to be mediated by a smaller set of well-defined 'contact' residues plus additional weaker interactions, which are usually more difficult to define but add to the affinity of binding. Mutation of a critical contact residue may abrogate antibody binding, and allow a pathogen to escape neutralization. Usually such contact residues are on distant sequence domains, brought together by protein folding to form conformational, discontinuous, or assembled epitopes. Most neutralizing and protective BCEs are conformational antigenic sites. Occasionally most elements of an epitope are contributed by amino acid residues, which are all within a short stretch of sequence. These continuous, segmental, linear, or sequential epitopes, in contrast to conformational epitopes, can be operationally defined by their ability to be mimicked by synthetic peptides of the same sequence. Despite sometimes-complex conformations such as helices, β sheets, or loops, these can be mimicked by sequence-homologous synthetic peptides (Table 1).

Identification of sequential BCEs

C- and N-terminal ends of proteins are frequently surface exposed. As a result of lower structural constraints, termini tend to be more flexible and are more easily mimicked by peptides. Peptides of many antigenic N- or C-terminal epitopes have been used to obtain antibodies against proteins. In some cases the N-terminus carried a protective epitope (Langeveld et al. 1994a). Loops protruding from the antigen surface and surface-accessible helices are also frequent targets of antibodies and sometimes they can be mimicked by short synthetic peptides. In the absence of structural data, the prediction of sequential epitopes is guesswork. Among the numerous strategies that have been utilized to identify *protective* sequential BCEs (Morris 1996), the most straightforward is to screen overlapping peptides covering the whole sequence or at least predicted surface-exposed domains with protective monoclonal antibodies (mAb) by pepscan, multispin assays, or SPOT assays. Larger (e.g. recombinant, tryptic, or other) fragments or peptide libraries (e.g. synthetic or phage displayed) and polyclonal antibodies can serve as second-rate alternatives to define sequential epitopes. Substitution analogs of synthetic peptides (e.g. alanine scan) combined with mAbs are powerful tools to identify contact residues of sequential epitopes. As a result of the many degrees of rotational freedom, the conformation of most peptides is ill-defined. For these reasons, only a limited number of neutralizing or protective sequential epitopes have been identified for some pathogens and none for others (Table 1).

Mimotopes mimic conformational BCEs

Conformational epitopes are even more difficult to mimic with small synthetic molecules, referred to as mimotopes. Mimotopes may have no sequence homology with the protein, but the spatial arrangement of their residues provides an array of atoms that resembles those contacted by the paratope in the protein epitope. Among a variety of approaches the most powerful are based on synthetic peptide libraries or (random) peptide libraries expressed by phages. Peptides as mimotopes have been used as surrogate antigens of experimental vaccines against proteins (Steward 2001) and even carbohydrates (reviewed by Monzavi-Karbassi et al. 2002). Many (antigenic) mimotopes that react with (preformed) antibodies have been described, but only a fraction of them also induced antibodies that crossreacted in vivo. When peptide mimotopes are used for immunization, usually few of the highly antigenic peptides induce virus-crossreactive antibodies, reflecting a severe disparity among antigenicity, immunogenicity, and crossreactive immunogenicity (El Kasmi et al. 1999b) with or without neutralizing or protective activity (reviewed by Deroo and Muller 2001) (Table 2).
Steward and colleagues (Steward et al. 1995; Olszewska et al. 2000) described a remarkable peptide mimotope, which was selected against an mAb of the measles virus fusion protein. Although the mAb did not neutralize the virus in vitro, the mimotope induced antibodies, which protected in a rodent model against a viral challenge. In another study, the same group described a peptide mimotope selected with a combinatorial solid-phase peptide library against a protective mAb of the fusion protein of the respiratory syncytial virus. These mimotopes, presented as multiple antigenic peptides (MAP), induced antibodies, which neutralized the virus in vitro, and reduced the virus load in immunized mice to 1 percent (Chargelegue et al. 1997, 1998).

Peptide vaccines based on BCEs: general features

THE BEGINNING

Chemical synthesis of peptides was developed during the 1960s (Marglin and Merrifield 1970), and thanks to numerous technological advances peptides became increasingly popular in immunology. In 1963, Anderer and colleagues showed for the first time that a C-terminal peptide of the coat protein of tobacco mosaic virus induced virus-neutralizing antibodies. More than 10 years later, Langbeheim et al. (1976) inactivated a bacteriophage by immunizing with a synthetic polymer of its coat protein made of 20 peptides. In the early 1980s protective immunity in the host animal was obtained with a peptide corresponding to the GH loop of the VP1 capsid protein of food-and-mouth disease

Table 1 *B-cell immunity peptide vaccines*[a]

Pathogen	Antigen	Delivery system	Host[b]	Activity	References
Viruses					
MV	F protein	Peptide/CFA	Mice	N, P	Obeid et al. (1995); Atabani et al. (1997); Partidos et al. (1997)
		Peptide/CT	Mice	N, P	Hathaway et al. (1995, 1998)
	H protein NE	Peptide/CFA	Mice	N, P	El Kasmi et al. (1998, 1999a)
	H protein HNE	Peptide/CFA	Mice	N, P	El Kasmi et al. (2000)
		DT, TT/alum	Mice	P	Putz et al. (2003b, 2004)
		Recombinant polyepitope/CFA	Mice	N	Bouche et al. (2003)
CDV	F protein	Peptide/CFA	Mice	P	Obeid et al. (1995)
RSV	G protein	MVA	Mice	P	Simard et al. (1995)
		KLH/CFA	Mice	P	Simard et al. (1997)
		Recombinant fd/CFA	Mice	P	Bastien et al. (1997)
		Peptide/CT	Mice	P	Bastien et al. (1999)
Influenza virus	HA	TT/CFA	Mice	N, P	Muller et al. (1982)
		TT/CFA	Mice		Muller et al. (1990)
		BSA, OVA/CFA liposomes	Mice	P	Friede et al. (1994)
FMDV	Site A VP1	KLH/CFA	Mice	N	Strohmaier et al. (1982)
		KLH/alum, CFA	Guinea-pigs	N, P	Bittle et al. (1982); Pfaff et al. (1982)
		KLH, TT/alum liposomes	Guinea-pigs	N	Francis et al. (1985)
		Peptide, KLH/CFA	Guinea-pigs, *cattle*	N, P	DiMarchi et al. (1986)
		Peptide-P3C	Guinea-pigs, *cattle*	P	Wiesmüller et al. (1989); Hohlich et al. (2003)
		MAP/IFA, alum	Guinea-pigs	N	Francis et al. (1991)
		Retro-all-d/KLH/alum	Guinea-pigs	N, P	Briand et al. (1997)
		Peptide/oil adjuvant	*Cattle*	N, P	Taboga et al. (1997)
		Peptide/Montanide	Guinea-pigs, *swine*	N, P	Wang et al. (2001, 2002a)
Poliovirus	VP1	BSA, KLH/CFA	Rabbits	N	Emini et al. (1983); Wimmer et al. (1984); Jameson et al. (1985)
	VP2	BSA/CFA	Rabbits	N	Emini et al. (1984b)
HAV	VP1	BSA/CFA	Rabbits, guinea-pigs	N	Emini et al. (1985)
		KLH, MAP/liposomes	Mice	N	Haro et al. (1995, 2003)
	VP1, VP2, VP3	CFA + liposomes	Mice	N	Bosch et al. (1998)
HCV	HVR1	Recombinant HVR1-CTB via TMV	Mice		Nemchinov et al. (2000)
		KLH/CFA	Mice		Li et al. (2001)
	Multivalent	Recombinant polyepitope/alum	Mice, monkeys		Li et al. (2003)
HIV-1	gp120 V3 loop	MAP/Alum	Guinea-pigs	N	Wang et al. (1991)

(*Continued over*)

Table 1 *B-cell immunity peptide vaccines* [a] *(Continued)*

Pathogen	Antigen	Delivery system	Host[b]	Activity	References
		MAP-P3C/Liposomes	Mice, guinea-pigs, rabbits	N	Defoort et al. (1992a); Nardelli et al. (1992)
		Peptide/CFA	Mice	N	Ahlers et al. (1993)
		fil HA, PPD, toxin A, TT	Guinea-pigs	N	Cryz et al. (1995); Rubinstein et al. (1999)
		Peptide/CT	Mice	N	Staats et al. (1996); Bradney et al. (2002)
		Recombinant rhinovirus	Guinea-pigs	N	Zhang et al. (1999)
		BSA/CFA	Mice	N	Xiao et al. (2000)
		Branched peptide/Alum	*Humans*	N	Gorse et al. (1996); Keefer et al. (1997)
		PPD	*Humans*	N	Rubinstein et al. (1995, 1999)
		MAP/alum	*Humans*	N	Kelleher et al. (1997); Phanuphak et al. (1997); Li et al. (1997)
		MAP/Alum	*Humans*	N	Lambert et al. (2001a)
	gp160	Peptide/Montanide	*Humans*	N	Pinto et al. (1999)
	Multivalent	MAP/CT	Mice	N	Bukawa et al. (1995); Kato et al. (2000)
		BSA/CFA	Rabbits	N	Lu et al. (2000)
HIV-2	gp 125	ISCOMs	Macaques	P	Putkonen et al. (1994)
SIV	gp 120, gp32	Recombinant β-Gal/CFA	Macaques	N, P	Shafferman et al. (1991)
CPV	VP2	KLH/alum + Quil A	*Dogs*	P	Langeveld et al. (1994a)
		KLH/alum + Quil A,CFA	Rabbits	N	Langeveld et al. (1994b)
		CPMV VLP/CFA	*Dogs*	P	Dalsgaard et al. (1997)
		KLH,OVA/CFA	Mice, rabbits	N	Casal et al. (1995); Langeveld et al. (2001a)
MEV	VP2	KLH/ISCOMs	Mink	P	Langeveld et al. (1995)
HBV	HBsAg S region	KLH/CFA	Chimpanzees	P	Gerin et al. (1983)
		KLH/CFA	Chimpanzees	P	Itoh et al. (1986)
		Peptide/CFA	Chimpanzees	P	Emini et al. (1989)
		MAP/CFA	Rabbits		Tam and Lu (1989)
HPV	E7 protein	Polyepitope/CFA, ISCOMs	Mice		Fernando et al. (1995)
		ISCAR/CFA	Mice	P	Tindle et al. (1995)
	L2 capsid protein	BSA/CFA	Mice	N	Kawana et al. (1999)
		Peptide/CT	Mice	N	Kawana et al. (2001)
		Peptide envelope	*Humans*	N	Kawana et al. (2003)
Japanese encephalitis virus	Glycoprotein	OVA/CFA	Mice	N	Dewasthaly et al. (2001)
Bacteria					
Neisseria meningitidis	Class 1 OMP	TT/Alum, Quil A	Mice	B	Hoogerhout et al. (1995)
	PorA	Peptide/CFA	Mice	P	Koroev et al. (2000)
	OpaB	Peptide/CFA	Mice	P	Koroev et al. (2001)

(*Continued over*)

Table 1 *B-cell immunity peptide vaccines*[a] *(Continued)*

Pathogen	Antigen	Delivery system	Host[b]	Activity	References
Streptococcus pyogenes	M protein	Peptide/CFA	Mice, rabbits	P	Beachey et al. (1981, 1986)
	TT/CFA		Mice, rabbits	P	Dale et al. (1983); Beachey et al. (1984)
Group A streptococcus	M protein C region	CTB	Mice	P	Bessen and Fischetti (1988, 1990)
		KLH/CFA or CTB	Rabbits, mice	P	Bronze et al. (1992)
		Peptide/CFA	Mice	B	Pruksakorn et al. (1992)
		LCP	Mice	P	Olive et al. (2002a)
		DT/CTB	Mice	P	Olive et al. (2002b)
		DT/Alum, SBAS2	Mice	B, P	Batzloff et al. (2003)
		Peptide/alum	Mice	P	Bruner et al. (2003)
	Streptolysin S	KLH/CFA	Rabbits	B	Dale et al. (2002)
Porphyromonas gingivalis	Fimbrillin	Peptide/IFA	Guinea-pigs	P	Ogawa (1994)
		BSA/CFA	Mice	P	Deslauriers et al. (1996)
	RgpA-Kgp	DT/IFA	Mice	P	O'Brien-Simpson et al. (2000)
Bacillus anthracis	*B. anthracis* capsule	BSA, rPA, rEPA	Mice		Schneerson et al. (2003)
		KLH/CFA	Mice		Wang et al. (2004)
Staphylococcus aureus	FnBP	CPMV VLP/ISCOM, QS-21	Mice		Brennan et al. (1999a)
Pseudomonas aeruginosa	OMP F	CPMV VLP/alum, CFA	Mice	P	Brennan et al. (1999b)
		Chimeric influenza virus	Mice	P	Staczek et al. (1998)
		TMV VLP/Alum, CFA	Mice	P	Staczek et al. (2000)
	Pilin	TT/Adjuvax	Mice	P	Cachia et al. (1998); Cachia and Hodges (2003)
Nontypable *Haemophilus influenzae*	P5	Peptide/CFA	Chinchilla	P	Bakaletz et al. (1997, 1999)
	Peptide/CFA		Rats	P	Webb and Cripps (2000)
	BSA, KLH/CFA		Chinchilla, rat	P	Kyd et al. (2003)
Vibrio cholerae	TcpA pilin	KLH/CFA	Rabbits, mice	P	Sun et al. (1997)
		Peptides/PCPP, CRL-1005	Mice	P	Wu et al. (2001)
Parasites					
Schistosoma mansoni	9B-antigen	BSA/CFA	Mice	P	Tarrab-Hazdai et al. (1998)
		Recombinant salmonella flagellin	Mice	P	Ben-Yedidia et al. (1999)
	Sm14	MPL-TDL	Mice	P	Vilar et al. (2003)
	Multivalent	Recombinant polyepitope or DNA	Mice		Yang et al. (2000)
	Glyceraldehyde-3-phosphate dehydrogenase	MAP/CFA	Mice	P	Tallima et al. (2003)

(*Continued over*)

Table 1 *B-cell immunity peptide vaccines*[a] *(Continued)*

Pathogen	Antigen	Delivery system	Host[b]	Activity	References
Plasmodium berghei	Pre-erythrocytic stage CSP	TT/CFA	*Mice*, rats	P	Zavala et al. (1987)
		MAP/CFA	*Mice*	P	Tam et al. (1990); Zavala and Chai (1990)
		BSA/RaLPS	*Mice*	P	Reed et al. (1996)
		MAC/RaLPS	*Mice*	P	Reed et al. (1997)
Plasmodium yoelii	Pre-erythrocytic stage CSP	MAC/RaLPS	*Mice*		Reed et al. (1997)
		Recombinant HBcAg	*Mice*	P	Schodel et al. (1997)
Plasmodium vivax	Pre-erythrocytic stage CSP	MAC/alum, RaLPS	Monkeys	P	Yang et al. (1997)
Plasmodium falciparum Pre-erythrocytic stage	CSP	TT/CFA	Rabbits	N	Zavala et al. (1985)
		TT/Alum	*Humans*	P	Herrington et al. (1987); Etlinger et al. (1988)
		MAP/CFA, alum	Mice, monkeys		Moreno et al. (1999)
		MAP/Alum,QS21	*Humans*		Nardin et al. (2000)
		Polyoxime MAP-P3C	*Humans*		Nardin et al. (2001)
		MAP/CFA	Mice	N	Joshi et al. (2001)
		Recombinant HBc/CFA, alum	Mice, monkeys		Birkett et al. (2002)
	LSA-3	Palmitoyl-lipopeptide	Chimpanzees	P	BenMohamed et al. (1997); Daubersies et al. (2000)
Asexual blood stage	MSP-1	BSA, TT/CFA	Rabbits, monkeys	N, P	Cheung et al. (1986)
		MAP/CFA	Monkeys	P	Rivera et al. (2002)
	RESA	Recombinant polyepitope/CFA	Monkeys	P	Collins et al. (1986)
	KLH, TT/CFA		Rabbits	N	Berzins et al. (1986)
	RESA, Pf55, Pf35	BSA/CFA	Monkeys	P	Patarroyo et al. (1987); Rodriguez et al. (1990)
Multistage multivalent	'SPf66'	Polyepitope/CFA	Monkeys	P	Rodriguez et al. (1990); Ruebush et al. (1990)
		Polyepitope/alum	*Humans*	P	Patarroyo et al. (1988); Amador et al. (1992a); Valero et al. (1993)
		Polyepitope/alum	*Humans*	N	Salcedo et al. (1991)
	CDC/NIIMALVAC-1	Recombinant polyepitope/CFA, P1005	Rabbits, Mice	N	Shi et al. (1999, 2000)
				N	Shi et al. (2000)
Taenia solium	KETc7	Peptide, MAP, OVA/CFA	Mice	P	Toledo et al. (1999)
	KETc1, KETc12	Peptide/Saponin	Mice	P	Toledo et al. (2001)
	KETc1, KETc7, KETc12	Peptide/Saponin	*Swine*	P	Huerta et al. (2001)
Toxins					
Vibrio cholerae	Cholera toxin B chain	TT/CFA	Rabbits	P	Jacob et al. (1983, 1986a)
		Recombinant β-Gal/CFA	Rabbits	P	Jacob et al. (1985)
		Peptide-MDP	Rabbits	P	Jacob et al. (1986b)

(*Continued over*)

Table 1 *B-cell immunity peptide vaccines* [a] *(Continued)*

Pathogen	Antigen	Delivery system	Host[b]	Activity	References
Escherichia coli	Heat-labile enterotoxin	TT/CFA	Rabbits	P	Jacob et al. (1986a)
Corynebacterium diphtheriae	Diphtheria toxin	BSA/CFA	Guinea-pigs	P	Audibert et al. (1981)
		Peptide-MDP	Guinea-pigs	P	Audibert et al. (1982)
Shigella dysenteriae	Shiga toxin B chain	pDGDL, TT/CFA	Mice, rabbits, rats	N, P	Harari et al. (1988); Harari and Arnon (1990)
Bungarus multicinctus (Taiwan banded krait)	β_1-Bungarotoxin	Peptide, OVA/CFA	Mice	P	Dolimbek and Atassi (1996)
		BSA/CFA	Mice	P	Yang and Chan (1998)
Contraceptive vaccines					
	hCG	TT/CFA, CGP-11637	*Mice, rabbits,*	C	Stevens et al. (1981a, b)
		TT/CGP-11637	*Baboons, humans*		Jones et al. (1988)
	ZP3	KLH/CFA	*Mice, macaques*	C	Millar et al. (1989); Mahi-Brown and Moran (1995)
		Peptide/CFA	*Mice, macaques*	C	Lou et al. (1995); Bagavant et al. (1997)
		DT/CFA	*Rabbits*		Afzalpurkar et al. (1997a, b)
		Peptide, TT/MDP + Morris	*Marmosets, mice,* primates	C	Paterson et al. (1999, 2002)
	GnRH	KLH/CFA	*Pigs*	C	Meloen et al. (1994); Beekman et al. (1999)
		KLH, OVA/CFA, Specol	*Pigs*	C	Oonk et al. (1998), Zeng et al. (2001)
	LHRH	Peptide/CFA	*Mice*	C	Ghosh and Jackson (1999)
	Sperm antigen LDH-C_4	DT/CGP-11637	*Baboons*	C	O'Hern et al. (1995)
		Peptide/CFA, CGP-11637	*Rabbits, baboons*	C	O'Hern et al. (1997)
	Sperm antigen YLP12	CTB	*Mice*	C	Naz and Chauhan (2002); Naz (2004)
	ZP3, LDH-C_4	Peptide/CFA	*Mice*	C	Sadler et al. (1999)
	RCP	Peptide/CFA	*Rats*	C	Subramanian et al. (2000)
Therapeutic vaccines					
Alzheimer's disease	Amyloid-β peptide	Peptide/CFA	Mice	P	Schenk et al. (1999)
		Peptide/LT	Mice	P	Weiner et al. (2000); Lemere et al. (2001)
		Recombinant fd	Mice	N, P	Frenkel et al. (2001, 2003)
		Polylysine peptide/CFA	Mice	P	Sigurdsson et al. (2001)
		Palmitoylpeptide/liposomes	Mice	P	Nicolau et al. (2002)
		BSA/CFA	Mice		Monsonego et al. (2001)
		Peptide oligomers/CFA	Rabbits	N	Lambert et al. (2001b)
		Peptide/alum	*Humans*		Schenk (2002); Nicoll et al. (2003)
Myasthenia gravis	AChR B-cell ARM	KLH/CFA	Rat	P (EAMG)	Araga et al. (1993)
	AChR T-cell ARM	MAP/CFA	Rat	P (EAMG)	Araga et al. (2000)

(*Continued over*)

Table 1 *B-cell immunity peptide vaccines*[a] *(Continued)*

Pathogen	Antigen	Delivery system	Host[b]	Activity	References
Multiple sclerosis	MBP T-cell ARM	KLH/CFA	Mice	P (EAE)	Zhou and Whitaker (1996)
Guillain–Barré syndrome	Bovine P2 T-cell ARM	KLH/AjuPrime	Mice	P (EAN)	Araga et al. (1999)
AIDS	CD4–CDR2 effector site	Peptide/CFA	Guinea-pigs	N	Wang et al. (2002b)
Tumour vaccines (BCE)					
Adenocarcinoma (breast, ovary)	HER-2	Universal ThB cell epitope-peptide conjugate (44-mer) + squalene/Arlacel A + CRL 1005	Antibodies in 3/5 killing human breast cancer cells in vitro		Dakappagari et al. (2000)
Breast cancer (metastatic)	MUC1	16-mer conjugated to KLH + Detox	Antibody in 3/16. $CD8^+$ in 7/11. No clinical response reported		Reddish et al. (1998)
Adenocarcinoma (various tumors)	MUC1	VNTR (5 repeats, 100-mer) conjugated to mannan	DTH. Antibody in 60% of intraperitoneal injected patients. T-cell responses in 28%. No clinical response		Karanikas et al. (2001)
Adenocarcinoma (breast, colon, stomach)	MUC1	VNTR (5 repeats) conjugated to mannan	MUC1 antibodies in 13/25. CTLs in 2/10. Th in 4/15. No clinical response reported		Karanikas et al. (1997)
Breast cancer	MUC1	VNTR (1.5 repeats, 30-mer) conjugated to KLH +QS-21	Antibody (9/9), no T-cell response. Recurrence of disease in only 2/9 high-risk patients		Gilewski et al. (2000)
Follicular B lymphoma	Idiotype	Peptide-KLH conjugate + DC	2/4 CRs, 1 PR		Hsu et al. (1996)
B lymphoma	Idiotype	Peptide-KLH conjugate + GM-CSF	Tumor-specific T cells in 19/20; 8/11 molecular remission		Bendandi et al. (1999)

a) Abbreviations: AChR, nicotinic acetylcholine receptor; ARM, antigen receptor mimetic; B, bactericidal activity; BCE, B-cell epitope; β-Gal, β-galactosidase fusion protein; BSA, bovine serum albumin conjugate; C, contraception or castration; CDV, canine distemper virus; CFA, complete Freund's adjuvant; CPMV, cowpea mosaic virus; CPV, canine parvovirus; CR, complete remission; CSP, circumsporozoite protein; CT, cholera toxin; CTB, cholera toxin B subunit; CTLE, cytotoxic T lymphocyte epitope; DT, diphtheria toxoid conjugate; EAE, experimental allergic encephalomyelitis; EAMG, experimental autoimmune myasthenia gravis; F, fusion protein; fil HA, filamentous hemagglutinin of *Bordatella pertussis*; FMDV, foot-and-mouth disease virus; FnBP, fibronectin-binding protein; H, measles virus hemagglutinin protein; HA, influenza hemagglutinin protein; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HVR, hypervariable region; IFA, incomplete Freund's adjuvant; ISCARs, immunostimulatory carrier; ISCOMs, immunostimulating complexes; gp, glycoprotein; KLH, keyhole limpet hemocyanin conjugate; LCP, lipid core peptide construct; LDH, lactate dehydrogenase; LOS, lipo-oligosaccharide; LT, heat-labile enterotoxin; MAC, multiple-antigen construct; MAP, multiple-antigen peptide; MBP, myelin basic protein; MDP, *N*-acetylmuramyl-l-alanyl-d-isoglutamine; MEV, mink enteritis virus; MPL-TDL, monophosphoryl lipid A/trehalose dicorynomycolate; MSA, merozoite major surface antigen; MV, measles virus; MVA, recombinant modified vaccinia virus Ankara; N, in vitro neutralization/growth inhibition; OMP, outer membrane protein; OVA, ovalbumin conjugate; P, in vivo protection or reduction of viral load; P3C, tripalmitoyl-*S*-glyceryl-cysteinylserylserine; PCPP, poly-di(carboxyatophenoxy)phosphazene; pDGDL, poly-d-glutamic-acid-d-lysine conjugate; peptide, free synthetic linear or chimeric TCE-BCE peptide; PPD, purified protein derivative of *Mycobacterium tuberculosis*; PR, partial remission; RaLPS, detoxified lipopolysaccharide; RCP, riboflavin carrier protein; rec fd, recombinant filamentous bacteriophage; rEPA, recombinant *Pseudomonas aeruginosa* exotoxin conjugate; RESA, ring-infected erythrocyte surface antigen; rPA, recombinant *B. anthracis* protective antigen conjugate; SIV, simian immunodeficiency virus; TCE, T-cell epitope; ThE, T-helper cell epitope; toxin A, *Pseudomonas aeruginosa* toxin A conjugate; TPI, triose phosphate isomerase; TMV, tobacco mosaic virus; TT, tetanus toxoid conjugate; VLP, virus-like particles.

b) Natural hosts in italic.

Table 2 *Examples of neutralizing and protective mimotopes*[a]

Pathogen	BCE	Delivery system	Host	Activity	References
Viruses					
MV	F protein mimotope	Peptide/CFA	Mice	P	Steward et al. (1995); Olszewska et al. (2000)
RSV	F protein mimotope	MAP/CFA	Mice	N, P	Chargelegue et al. (1997, 1998)
	F protein mimotope	MAP, CTLE, ThE	Mice	P	Hsu et al. (1998)
	F protein mimotope	MAP, CTLE, ThE/IFA	Mice	P	Hsu et al. (1999)
Hepatitis C	HVR1 mimotopes	MAPs/CFA	Rabbits		Roccasecca et al. (2001)
Hepatitis B	HbsAg mimotope	Recombinant phage/CFA	Mice		Motti et al. (1994)
Bacteria					
Neisseria meningitidis	Mimotope	Proteosome complex	Mice	P	Westerink et al. (1995); Prinz et al. (2004)
	Polymimotopes	DNA/Alum	Mice	N, P	Westerink et al. (2001); Prinz et al. (2003)
	Mimotope	DT/CFA	Mice		Charalambous and Feavers (2000)
Nontypable *Haemophilus influenzae*	LOS mimotope	KLH/CFA	Rabbits, mice	P	Hou and Gu (2003)
Parasites					
Schistosoma mansoni	9B-antigen mimotope	BSA/CFA	Mice	P	Arnon et al. (2000)
Cryptococcus neoformans glucuronoxylomannan		P13-BSA P13-TT	Mice Mice	Prolonged Survival	Fleuridor et al. 2001
Other					
	IgE mimotope	Peptide	Rabbits	P, N	Rudolf et al. (1998); Stadler et al. (1999)
Fibrosarcoma	Sialyl Lewis X	MAP mimotope	Mice	Inhibition of tumor growth	Kieber-Emmons et al. (1999)
Lymphoma (RAW117-HA)	EDA-alpha		Mice	Inhibits metastasis	Ishikawa et al. (1998)

a) Abbreviations: see Table 1.

(DiMarchi et al. 1986). These early studies encouraged many authors to explore immunization strategies based on sequential BCE and synthetic peptides. Peptides corresponding to neutralizing epitopes have been identified in a number of surface proteins of viruses, bacteria, and parasites (see Table 1). However, after the initial enthusiasm, it became clear that peptides are far from being ideal antigens. Our own experience taught us that it is a long way from the identification of a neutralizing linear epitope to a protective vaccine (Ziegler et al. 1996; Putz et al. 2004).

CHEMICALLY DEFINED VACCINES

A major advantage of chemically synthesized peptides in comparison to biological products is at the level of production, quality, and safety. Peptides are relatively simple to synthesize in large quantities. They are chemically fully defined, and can be obtained with high purity and minimal batch variations. They are thermostable and have long shelf-lives at room temperature. They do not require cold chains, which are expensive and difficult to maintain in developing countries. In comparison to recombinant products, peptides do not require complex fermenting and downstream-processing capacity. Unlike live vectors, peptides have no infectious potential. Containment requirements are minimal in comparison to those for the production of large quantities of infectious microorganisms. There is no risk of reversion of attenuation.

CONFORMATIONAL STABILITY

Most neutralizing and protective BCEs are conformational and difficult to mimic with linear peptides (see earlier section 'Mimotopes mimic conformational BCEs'). In the case of a sequential epitope, linear peptides may miss some structural features of the native epitope involved in antibody interaction. Furthermore, peptides are highly flexible structures, presenting to the

B-cell repertoire a multitude of different conformations, only some of which may resemble the native epitope and induce antibodies crossreacting with the parent protein (crossreactive immunogenicity).

To overcome problems with peptide flexibility, different strategies have been explored including constraining peptides by chemical or peptidic scaffolds (Valero et al. 2000; Hudecz 2001), e.g. the M protein of the group A streptococci (GAS) contains a sequential epitope targeted by opsonizing and protective antibodies (reviewed by Olive et al. 2004). However, synthetic peptides representing this epitope alone did not display the helical structure of the native protein. Only when flanked by irrelevant yeast-derived GCN4 sequences did the epitope peptide fold into the helical structure and induce protective antibodies (Relf et al. 1996; Hayman et al. 1997). Such strategies reduce conformational diversity but structural similarity may suffer. A poor balance between conformational constraints and conformational similarity of the peptide with the native protein critically determines crossreactive immunogenicity.

IMMUNOGENICITY

Live-attenuated viruses induce a strong immune response, protecting sometimes for decades after a single injection without the need of adjuvants. Recombinant proteins formulated with adjuvants protect after several boosts for 5–10 years. In comparison, synthetic peptides have a weak inherent immunogenicity; normally they are much less immunogenic than their parent proteins even after repeated injections with relatively high doses. They depend on potent adjuvants (Van Regenmortel and Muller 1999), which are not licensed for human use. Most experimental vaccines have been tested by invasive routes using a variety of formulations, adjuvants, and cytokines (see Table 1). Adjuvants have been reviewed by Del Giudice and Rappuoli (see Adjuvants and subunit vaccines). The low immunogenicity, combined with conformational diversity of a peptide antigen, are the major obstacles to the induction of high levels of crossreactive or neutralizing antibodies.

ENZYMATIC DEGRADATION IN VIVO

In vivo, peptides are rapidly degraded by proteases further limiting their immunogenicity. To overcome this drawback numerous attempts have been made to develop more stable peptide derivatives using non-natural amino acids (Chorev and Goodman 1995; Briand et al. 1997; Nargi et al. 1999), analogs with modified peptide bonds (pseudopeptides) (Bianco 1999) and other so-called peptidomimetics (Benkirane et al. 1996a; Stemmer et al. 1999), e.g. a retro-inverso peptide induced protective antibodies against foot-and-mouth disease (Briand et al. 1997) and a pseudopeptide inhibited experimental allergic encephalitis (Jameson et al. 1994).

Requirement for TCEs

A CHOICE OF HELPER TCE

To be immunogenic in the context of a natural population, the BCEs must be combined with a peptide containing a potent T-helper cell epitope, which can be presented by most MHC class II haplotypes or an immunogenic carrier protein. Broadly, MHC II-reactive TCEs have been proposed for this purpose (Table 3), including non-natural pan-DR-binding peptides, engineered to bind to a wide range of DR molecules (Panina-Bordignon et al. 1989; Alexander et al. 1994, 1998). The carrier proteins most frequently used in experimental conjugate vaccines include tetanus toxoid (TT), diphtheria toxoid (DT), keyhole limpet hemocyanin, ovalbumin (OVA), and bovine serum albumin

Table 3 *Promiscuous T-helper cell epitopes*[a]

Antigen	Sequence	Position	Reference
Tetanus toxoid	NSVDDALINSTKIYSYFPSV	580–599	Ho et al. (1990)
Tetanus toxoid	QYIKANSKFIGITEL	830–844	Demotz et al. (1989)
Tetanus toxoid	PGINGKAIHLVNNESSE	916–932	Ho et al. (1990)
Tetanus toxoid	FNNFTVSFWLRVPKVSASHLE	947–967	Panina-Bordignon et al. (1989)
Tetanus toxoid	GQIGNDPNRDIL	1273–1284	Demotz et al. (1989)
P. falciparum CSP	EKKIAKMEKASSVFNV	380–395	Sinigaglia et al. (1988)
MV F protein	LSEIKGVIVHRLEGV	288–302	Partidos and Steward (1990)
FMDV VP1 protein	ETQVQRRHHTDVGFIMDRFY	21–40	Collen et al. (1991)
HIV gp160	KQIIMNWQEVGKAMYAPPISGQIR	428–451	Berzofsky et al. (1991)
Pan-DR epitope	aK(X)VAAWTLKAa	Non-natural	Alexander et al. (1994)
HAV	NVPDPQVGITTMRDLKG	17–33	Ivanov et al. (1994)
HAV	MSRIAAGDLESSVDDPRSEEDRR	276–298	Ivanov et al. (1994)
HBV envelope protein	WPEANQVGAGAFGPGF	52–67	Doh et al. (2003)
HPV16	DRAHYNI	48–54	Tindle et al. (1991)

a) Abbreviations: see Table 1.

(BSA). TT and DT, two highly immunogenic carriers both licensed for human use, have been successfully utilized in experimental peptide conjugate vaccines (Muller et al. 1982; Herrington et al. 1987; Putz et al. 2004). Despite difficulties to obtain efficient loading and homogeneous conjugates, this technology is commonly used in licensed polysaccharide conjugate vaccines against bacteria. Coupling the BCE to a TCE or carrier protein, however, undermines some advantages of peptides including chemical purity, homogeneity, and stability.

DESIGN OF PEPTIDES CONTAINING BOTH T- AND B-CELL EPITOPES

Although in some cases simple mixing of a TCE peptide with a BCE peptide has given satisfactory results (Partidos et al. 1992a; Brons et al. 1996), covalent coupling is generally believed to be required. Co-linear synthesis of the BCE with a peptide containing a (promiscuous) TCE is one option (Partidos et al. 1991). Although the technology to produce peptides of increasing length has made considerable progress (reviewed by Demotz et al. 2001), yield, purity, and production costs are likely to limit peptide length. The longer the peptide, the less competitive the chemical synthesis will be in comparison to production of recombinants.

The need for a conjugated TCE adds several levels of complexity to the design of peptide vaccines: the antigenic peptide may be conjugated C or N terminally to one or several TCE peptides (or a carrier protein). Orientation, number, and structural propensity of the TCE critically determine the conformation of the BCE, and crossreactive immunogenicity with the native protein as well as neutralizing activity of antibodies (Dyrberg and Oldstone 1986; Lipkin et al. 1988; Lu et al. 1991; Partidos et al. 1992b, c; Fernandez et al. 1993; Sharma et al. 1993; El Kasmi et al. 1998, 2000). Although some permutational T–B constructs produced low levels of antibodies without crossreactivity, others containing the same BCE framed differently with the same TCE, or flanked in the same way with different TCE, induced crossreactive antibodies. Although inactive and cross-neutralizing antibody-inducing constructs can be very similar, iterative optimization can be tedious (El Kasmi et al. 1999a). Systematic studies seem to suggest that TCE–TCE–BCE ('TTB') constructs may be the most efficient (Obeid et al. 1995; El Kasmi et al. 1999a). The orientation of co-linear peptides may also influence the immunogenicity of the TCE by activating T cells against neoepitopes which have no resemblance to pathogen-specific TCEs. TCEs and BCEs may also be combined using different chemical conjugation strategies including the formation of thioesters (Defoort et al. 1992b), oxime bonds (Rose 1994), disulfide bonds (Drijfhout and Bloemhoff 1991), thioether bonds (Schnolzer and Kent 1992), thiazolidine bonds (Rao and Tam 1994), and others (Spetzler and Tam 1995; Purcell et al. 2003; reviewed by Jackson et al. 2002). These coupling chemistries differed in conjugation yield, in vivo stability, and immunogenicity (Powell et al. 1993; Fitzmaurice et al. 2000; W. Zeng et al. 2001). In addition to linear coupling strategies, peptides can also be assembled in branched geometries as in MAPs on lysine scaffolds (Tam 1988; Tam et al. 1990; Lu et al. 1991; Ahlborg 1995; Fitzmaurice et al. 1996; reviewed by Nardelli and Tam 1995). In MAPs, the orientation appears to be less critical for immunogenicity, and optimal MAP constructs were shown to contain equimolar amounts of BCEs and TCEs (Tam et al. 1990). Enhanced immunogenicity of branched peptides seems to be the result of a higher resistance of MAPs to enzymatic degradation and more efficient antigen presentation (Fitzmaurice et al. 2000).

IMMUNE RESPONSE TO PEPTIDE-CARRIER CONJUGATES

Conjugation of a (small) BCE to a (bulky) carrier protein tends to divert the immune response to the larger immunogenic carrier protein and away from the (peptide) epitope (Herzenberg and Tokuhisa 1980; Schutze et al. 1985; Etlinger et al. 1988). Pre-existing antibodies against the carrier protein may influence the immunogenicity of the conjugate, although this does not seem to be a major obstacle at least in the case of polysaccharide conjugate vaccines (Eskola et al. 1990; Kurikka et al. 1996). In the case of an experimental peptide-conjugate vaccine considerable differences between the resistance to pre-existing antibodies of TT and DT were observed (Putz et al. 2004). Peptide-specific epitopic suppression was stronger after passive priming with carrier or conjugate antibodies. DT as a carrier was less susceptible to suppression than TT and suppression could be overcome by boosting (Putz et al. 2004), as also shown for polysaccharide conjugates (Sarvas et al. 1992). The ability to provide T-cell help without diverting the immune response is perhaps the major advantage of using a defined T-helper epitope.

ONE OR MORE EPITOPES

Important mutations in the targeted epitopes of pathogens may require the combination of peptides corresponding to several nonoverlapping neutralizing epitopes or a mix of mutated peptides of the same epitope. Vaccines based on single epitopes may favor the development of escape mutants. In this case, the epitope vaccines should include variants of a given epitope (Wang et al. 2001) or more than one BCE. This can be produced by chemical polymerization (Patarroyo et al. 1988), as MAPs (Tam et al. 1990), multiple antigen constructs (Yang et al. 1997), or polyoximes (Nardin et al. 1998), or by genetic fusion (Shi et al. 1999; Theisen et al. 2000; Bouche et al. 2003).

Noninvasive routes of delivery

Some experimental vaccines have also been tested by nasal delivery using cholera toxin (CT) from *Vibrio cholerae* (Bergquist et al. 1998) and heat-labile enterotoxin from *Escherichia coli* (LT, Hathaway et al. 1995; Partidos et al. 1996), or nontoxic mutants as mucosal adjuvants (Di Tommaso et al. 1996; Yamamoto et al. 1998). CpG (reviewed by Krieg 2002) and immunostimulating complexes (ISCOM) (Sjolander et al. 2001) have been proposed as mucosal adjuvants and proteosomes as carrier proteins (Levi et al. 1995; Mallett et al. 1995) for intranasal immunization in humans (Haneberg et al. 1998; Oftung et al. 1999). Studies also seem to suggest that peptides can be delivered through the bare skin when mixed with LT, although the efficiency of this route remains to be demonstrated (Partidos et al. 2001; MM Putz and CP Muller, unpublished data).

Targeting selected epitopes

PROTEINS: A CHOICE OF EPITOPES

All currently licensed vaccines and most of those that are in clinical trials are meant to induce a strong neutralizing and protective immune response against pathogens prone to mutations in genetically diverse human or animal populations. The efficacy of these vaccines, irrespective of genetic variability of the host or pathogen, relies on the ability of the immune system to select from a large number of epitopes (TCEs or BCEs). In prophylactic vaccines, the exact epitopes involved in the response are normally of little clinical interest. With the advent of recombinant technology, we have learned to appreciate the advantages of guiding immune reactions against defined proteins of a pathogen and this is now being exploited in numerous experimental and a few licensed recombinant vaccines. In contrast to protein subunit vaccines, peptides rely on only a few epitopes of an antigen. Relative advantages such as chemical stability, biological safety, and low production costs normally do not outweigh the low (crossreactive) immunogenicity of peptide vaccines in different genetic contexts of hosts and pathogens.

PEPTIDES: AN EPITOPE OF CHOICE

Immunologically, the most important feature of peptide vaccines or other epitope vaccines is their ability to target defined epitopes. It has been proposed, for instance, to use peptides complementary to major antigenic regions of autoantigens to induce anti-idiotype antibodies for the treatment of autoimmune diseases (reviewed by Weathington and Blalock 2003). Peptide-induced anti-idiotype antibodies have also been successfully used to treat B-cell lymphomas (Hsu et al. 1996, Bendandi et al. 1999; Table 4). Peptides are also useful to avoid undesired immune responses, such as non-neutralizing antibodies which block neutralizing antibodies, enhancing or tumor-stimulating antibodies, autoimmune responses, or the induction of tolerization or suppressor cells (Aichele et al. 1995; Toes et al. 1996a, b). When the use of whole pathogens or proteins is limited by important constraints, epitope-based vaccines are unrivalled by other approaches. Although peptides may outrun other epitope immunization strategies, alternatives such as DNA minigenes (see

Naked DNA vaccines) or recombinant polyepitopes (reviewed by Suhrbier 2002) should also be considered. Essentially every feature of peptide vaccines has to be evaluated against competing vaccine technologies. There are still only few examples where a vaccine would clearly benefit from selected beneficial BCEs or from excluding detrimental BCEs, but applications are emerging for the treatment of autoimmunity (Weathington and Blalock 2003), allergies, and malignancies (Table 4), as well as infectious diseases (see below).

An example of a prophylactic epitope vaccine

The following describes one of the few examples of a prophylactic vaccine strategy against a pathogen where an epitope approach may be specifically justified. Infants are partially protected against measles by transplacentally acquired maternal antibodies. However, many children lose these antibodies within the first months of birth. As a result they become susceptible to measles before they can be immunized with the current live-attenuated vaccine recommended only at 9–15 months of age (Muller 2001a; Putz and Muller 2003). At this age, measles is associated with many complications and a high mortality rate. To close this susceptibility gap in infants, we developed an immunization strategy based on two subdominant sequential epitopes of the measles virus (MV) hemagglutinin protein (Ziegler et al. 1996; Fournier et al. 1997). Peptides corresponding to these sequential epitopes induce neutralizing and protective antibodies against the virus, but are not neutralized themselves by passively acquired antibodies induced by natural measles infection or after immunization with whole virus (El Kasmi et al. 1999a, 2000). Antibodies against measles surface glycoproteins provide full protection even in the absence of a T-cell response. In particular, newborns are solely protected by passively acquired maternal antibodies. Despite immaturity of their immune system, newborns and infants are capable of developing vigorous and protective antibody responses against proteins (e.g. DT and TT). In fact, during their early lives, antibodies are the main mechanisms of vaccine-induced protection of infants.

The generation of protective antibodies against the peptides requires a vigorous T-helper (Th) 2 response.

Table 4 *Experimental peptide vaccines against tumor antigens tested in cancer patients*

Tumor	Tumor antigen	Peptide vaccine formulation	Immunological outcome/clinical outcome	Reference
Adenocarcinoma (breast, ovary, colon)	HER-2	CTL epitope (9-mer) + IFA	Peptide-specific CTL in 3/4. No clinical response	Zaks and Rosenberg (1998)
Adenocarcinoma (breast, ovary, lung)	HER-2	3 peptides (15–18-mers) each with overlapping Th and CTL epitopes + GM-CSF	CTLs in 86% of patients. Peptide-specific CTLs kill HER-2 expressing tumor cells. T-helper response against peptide (14/18) and against HER-2 protein (up to 50%). Immune response lasted > 1 year. Tumor not evaluable	Knutson et al. (2001)
Adenocarcinoma (breast, ovary, lung)	HER-2	2 mixtures of 3 Th epitopes (15–18-mers, ICD, ECD) + GM-CSF	T-cell proliferation against peptides (8/8) and protein (6/8). Tumor not evaluable	Disis et al. (1999, 2000); Disis and Cheever (1998)
Adenocarcinoma (breast, ovary, lung)	HER-2/neu	3 mixtures of 3 T-helper epitopes + GM-CSF	T cells to peptides in 92%, 68% to protein	Disis et al. (2002)
Adenocarcinoma (breast, ovary)	HER-2/neu	9-mer CTL epitope + GM-CSF	CTLs in 2/4	Knutson et al. (2002)
Adenocarcinoma (breast, lung)	HER-2/neu	Mixtures of 4 peptide T-helper epitopes	T-cell response in 25% of patients	Salazar et al. (2003)
Adenocarcinoma (breast, ovary, pancreas)	MUC-1	Synthetic mucin polypeptide core (105-mer) with 5 repeated epitopes + BCG	Infiltration of T cells at immunization site in 37/55. Increased CTLs in 7/22. DTH in 3/63. No clinical response.	Goydos et al. (1996)
Adenocarcinoma expressing CEA	CEA	CTL epitope (9-mer) + Detox PC	CTLs secreting low levels of IFN-α in 1/5	Arlen et al. (2000)
Colon, breast, ovary, pancreas	CEA	CAP-1 (9-mer + DC)	No DTH. 1/19 minor clinical response.	Morse et al. (1999)
Thyroid carcinoma	CEA	CAP-1 (9-mer + DC)	1/7 PR	Schott et al. (2001)
Pancreas carcinoma	K-ras	Mononuclear cells pulsed with peptide (17-mer)	$CD4^+$ in 2/5. No clinical response	Gjertsen et al. (1995, 1996a, b)
	K-ras	ras (13-mer) + Detox	$CD4^+$ in 3/10, $CD8^+$ in 3/10 against peptide. No clinical response	Khleif et al. (1999)
	K-Ras	4 peptides (17-mers) with mutated TCE + GM-CSF	Peptide-specific T cells or DTH of 25/43. Significant increase in survival	Gjertsen et al. (2001)
Prostate carcinoma	Prostate acid phosphatase	CTL peptide + GM-CSF	> 50% reduction of PSA in 3/31.	Small et al. (2000)
	PSMA	CTL peptides + DC (PSM-P1, PSM-P2)	DTH, $CD8^+$ response. 19/62 reduction in PSA. 2/25 CRs, 6 PRs	Murphy et al. (1999a); Tjoa et al. (1999); Lodge et al. (2000); Salgaller et al. (1998)
	PSMA	PSM-P1 (LLHETDSAV) $\pm$ DC	Specific CTLs in few patients	Murphy et al. (1996)
		PSM-P2 (ALFDIESKV) $\pm$ DC	7/51 reduction in PSA	Tjoa et al. (1997)
	PSMA	PSM-P1 peptide, PSM-P2 peptide	DTH, $CD8^+$. No clinical response	Murphy et al. (2000)

(*Continued over*)

Table 4 *Experimental peptide vaccines against tumor antigens tested in cancer patients (Continued)*

Tumor	Tumor antigen	Peptide vaccine formulation	Immunological outcome/clinical outcome	Reference
Bladder cancer	MAGE-3	CTL peptide + DC	1/4 PR	Nishiyama et al. (2001)
HPV-associated carcinomas	E7 of HPV16	86-mer CTL peptide + PADRE + Montanide ISA51	Induction of CTLs. No clinical response	Steller et al. (1998)
	E7 of HPV16	10-mer + 8-mer + PADRE	Response to PADRE (4/12). No CTL response. No clinical response	Van Driel et al. (1999); Ressing et al. (2000)
E7 protein of HPV16		CTL epitope (9-mer) plus a CTL-pan Th (PADRE) lipopeptide conjugate + IFA	Epitope-specific CTL activity in 10/16. No DTH. 3/17 CRs, 9/17 PRs	Muderspach et al. (2000)
Chronic myelogenous leukemia	Bcr-abl fusion protein, p210	5 peptides (9–25-mers) with Th and CTL epitopes + QS21	Peptide-specific T-cell proliferation in 50% of patients. No CTL activity	Pinilla-Ibarz et al. (2000)
Acute myeloid leukemia	WT1	9-mer CTL peptide + KLH + GM-CSF	CR in 1/1	Mailander et al. (2004)
Melanoma (metastatic)	gp100	Modified CTL epitope (9-mer), + IFA + either IL-2, GM-CSF, or IL-12	Tumor regressions in 6/16 patients tested. IFA + IL-2. IFA + GM-CSF: 0/13. IFA + IL-12: 0/14	Rosenberg et al. (1999)
Melanoma	MAGE-3	CTL peptide (9-mer), no adjuvant	No peptide-specific CTLs. 3/25 CRs, 4/25 PRs. 2/25 disease free for 2 years	Marchand et al. (1999)
Melanoma	MAGE-3	CTL peptide (9-mer) with pan class II peptide + IFA	Tumor response not evaluable (n = 18)	Weber et al. (1999)
Melanoma	MAGE-3	CTL peptides + DC	8/11 peptide-specific CTLs. 54% tumor response (single metastases)	Thurner et al. (1999)
Melanoma	MAGE-3	CTL peptide	Peptide-specific CTLs in 4/9 responding; in 1/14 non-responding patients. 39% tumor response	Coulie et al. (2002)
Melanoma	MAGE-3	DC pulsed with Th epitope	Majority of patients developed peptide-specific Th cells.	Schuler-Thurner et al. (2002)
Melanoma and others	NY-ESO-1	3 CTL peptides (9–11-mers) ± GM-CSF	4/12 peptide-specific CTLs, 4/12 DTH. 7/12 SD	Jager et al. (2000a)
Melanoma	Tyrosinase	4 CTL peptides (9-mers) + GM-CSF	4/18 peptide-specific CTLs. 1/5 mxR, 2/5 SD	Scheibenbogen et al. (2000)
Melanoma	Tyrosinase	CTL peptides (9-mer) + QS21	0/7 tumor response	Lewis et al. (2000)
Melanoma	gp100	3 CTL peptides (9-mer) + IFA	1/20 tumor response	Salgaller et al. (1996)
Melanoma	$gp100_{209–217}$ 210M	Modified CTL epitope (9-mer) + IFA or +IFA + IL-2	Peptide-specific CTLs in 91%. IFA: 0/11 tumor responses. IFA + IL-2; 1/31 CRs. 12/31 PRs	Rosenberg et al. (1998)
Melanoma	gp100	CTL epitope (9-mer) + 1. QS21 or Montanide ISA-5I2. Tetanus helper peptide + QS21 or Montanide ISA-5I3. Gp100-tetanus 24-mer + QS21 or Montanide ISA-5I	Peptide-specific Th and CTL response in 79% and 14% of patients respectively. Tumor not evaluable (n = 20). 75% survival after 4.7 years	Slingluff et al. (2001)
Melanoma	gp100 (210M), tyrosinase	Modified CTL epitope (9-mer) + IFA ± IL-12	37/42 peptide-specific CTLs. Tumor not evaluable. Increased disease-free survival	Lee et al. (2001)
Melanoma	gp100, tyrosinase	4 CTL epitopes (9-mers) + GM-CSF + Montanide ISA-51	CTL responses to peptides in 2–5/5 tumor-specific CTLs (5/5) in vitro	Yamshchikov et al. (2001)
Melanoma	gp100, tyrosinase	CTL peptides ± GM-CSF	37/42 peptide-specific CTLs; improved immunization. Tumor not evaluable	Weber et al. (2003)

(*Continued over*)

Table 4 *Experimental peptide vaccines against tumor antigens tested in cancer patients (Continued)*

Tumor	Tumor antigen	Peptide vaccine formulation	Immunological outcome/clinical outcome	Reference
Melanoma	Melan-A/MART-$1_{27–35}$	CTL epitope (9-mer) + IFA	7/8 peptide-specific CTLs. 1/18 PRs	Cormier et al. (1997)
Melanoma (resected)	Melan-A/MART-$1_{27–35}$	CTL epitope (9-mer) + IFA + CRL 1005	IFN-γ-secreting peptide-specific T cells in 12/20. Tumors not evaluable because of resection. Increased disease-free survival correlated with immune response	Wang et al. (1999a)
Melanoma	MART-1	CTL peptide (10-mer) + SB-AS 2 adjuvant/Montanide	Peptide-specific CTLs	Pittet et al. (2001)
Melanoma	MART-1, gp100	CTL peptides + DCs + no adjuvant	1/5 developed peptide-specific CTLs. Tumor response 1/7	Panelli et al. (2000)
Melanoma	Melan-A/MART-1, tyrosinase, gp100/Pmel17	6 CTL epitope (9, 10 mers)	5/6 DTH, 2/6 SD	Jaeger et al. (1996)
Melanoma	MART-1, tyrosinase, gp_{100}	CTL peptides + DC	2/28 CRs, 1/28 PRs	Lotze et al. (2000)
Melanoma	Melan-A/MART-1, tyrosinase, gp100, MAGE-3	CTL peptides + DC	16/18 peptide-specific CTLs. Immunity to peptides correlated with delayed tumor progression. Regression of some metastasis	Banchereau et al. (2001)
Melanoma	Tumor lysate	Peptide cocktail or tumor lysate + DC	DTH reaction to peptide-pulsed DC in 11/16. 31% tumor response	Nestle et al. (1998)
Melanoma	$gp100_{209–217}$210M0	Modified CTL peptide alone or modified CTL peptide + IL-2	Peptide-specific CTLs in 7/7. 0/7 tumor response	Lee et al. (1999)
			Peptide-specific CTLs in 1/11. 5/11 tumor response	
		Modified CTL peptide + IL-12	Peptide-specific CTLs in 4/5. 0% tumor response	
Melanoma	N-ras	ras (25-mer) + GM-CSF	DTH in 8/10, $CD4^+$ in 2/10. Clinical response, not examined	Hunger et al. (2001)
Melanoma	gp100, tyrosinase	4 CTL epitopes + GM-CSF + universal T-helper epitope + Montanide ISA-51	T-cell response in 42% patients.	Slingluff et al. (2003)
Melanoma	gp100 (210 M), MART-1	2 CTL epitopes	T-cell response in 95%. Clinical response in 0/22	Phan et al. (2003)
	gp100 (210 M), MART-1	2 CTL epitopes + 1 helper epitope	T-cell response in 50%. Clinical response in 1/19	
Melanoma	gp100, MART-1, tyrosinase	3 CTL epitopes + IFA + SD-9427	7/10 in ELISPOT essay. 11/12 in MHC-pep-tetramer essay	Pullarkat et al. (2003)
Melanoma	Melan-A	10-mer CTL peptide + rhIL-12	1/24 CRs, 1/24 PRs, 6/24 SD	Cebon et al. (2003)
Melanoma	Melanoma tyrosinase	9-mer CTL peptide + KLH + GM-CSF	T-cell response in 5/9	Scheibenbogen et al. (2003)
Melanoma	Tyrosinase	9-mer CTL peptide + KLH + GM-CSF	T-cell response in 2/2. 2 years disease-free recurred	Scheibenbogen et al. (2002)
Melanoma	MAGE-3	9-mer CTL peptide + KLH + GM-CSF	T-cell response in 2/9. Regression in 5/9	Coulie et al. (2002)

BCG, Bacillus Calmette–Guérin; CEA, carcinoembryonic antigen; IFA, incomplete Freund's adjuvant; MUC, mucin; PSA, peptide-specific antigen; PSMA, peptide-specific membrane antigen; rhIL-12, recombinant interleukin 12; SD, stable disease. See Table 1 for other abbreviations.

The induction of MV antibodies is complicated by observations of enhanced atypical disease after immunization with inactivated MV vaccine during the 1960s. This complication is now attributed to priming of nonprotective MV-specific Th2-biased cells (Polack et al. 1999). Thus, it is critical that the Th cells are unrelated to MV. Therefore, the peptides corresponding to the above sequential epitopes were conjugated to (unrelated) carrier proteins such as DT or TT. These peptide conjugates induced high levels of anti-peptide antibodies, which protected against MV utilizing TCEs of the carrier protein (Putz et al. 2004). Thus the risk of priming for measles-specific T cells was avoided and the main cause of atypical measles, i.e. an aberrant measles-specific Th2 priming, was precluded.

In this example the advantage of using a peptide as a protective BCE was a three-fold:

1. The peptide, in contrast to whole virus or viral proteins, was not susceptible to neutralization by pre-existing anti-whole virus antibodies.
2. By limiting the MV sequence to 20 amino acids priming by pathogenic TCEs was essentially avoided.
3. The relatively low immunogenicity and the short duration of an anti-peptide response may prove to be an asset here because protection of limited duration, at least against severe disease (to reduce complication rates and mortality, but not necessarily infection) is required, until the live-attenuated vaccine can be administered.

We have shown that this experimental vaccine is compatible both with pre-existing antibodies against the carrier protein DT and against whole MV, and that subsequent boosting with whole virus is possible (Putz et al. 2004).

CANDIDATE PEPTIDE VACCINES AGAINST BCES

Paramyxoviruses

MEASLES VIRUS

This single-strand, negative-sense RNA virus is the prototype morbillivirus (family of Paramyxoviridae). The current live-attenuated vaccine is poorly immunogenic in early infancy, as a result of the presence of neutralizing maternal antibodies passively acquired through the placenta and the immaturity of the infant immune system. Therefore a number of experimental vaccines based, for example, on recombinant MV proteins, viral and bacterial vectors, or naked DNA, have been developed (reviewed by Putz et al. 2003a). Among these numerous immunization strategies some are based on peptides of the hemagglutinin (H) and fusion (F) protein. MV-neutralizing antibodies are directed against both the F and the H glycoproteins, and sequential epitopes have been identified for both proteins. The rationale of peptides as a vaccine to protect during early infancy against measles is discussed in the previous section.

Steward and colleagues described two adjacent sequential epitopes contained within a short immunodominant region (F388–420) of the F protein (Partidos et al. 1991), crossreacting with sera from African children with acute measles (Atabani et al. 1997). Co-linearly synthesized with one or two copies of a promiscuous TCE (Partidos and Steward 1990), one of these peptides was immunogenic in mice and induced MV-crossreactive and -neutralizing antibodies (Atabani et al. 1997). Passive transfer of anti-peptide serum partially protected mice against a challenge with a neuroadapted MV strain (Obeid et al. 1995; Atabani et al. 1997) and cross-protected against canine distemper virus, a related morbillivirus (Obeid et al. 1995). In addition, intranasal co-immunization with a synthetic peptide containing two copies of a TCE and one copy of a BCE together with cholera toxin B (CTB) induced MV-neutralizing antibodies and conferred protection in the mouse model (Hathaway et al. 1995, 1998). When copies of the BCE (Obeid et al. 1995) and an N protein TCE were fused C terminally to the secretion signal of *E. coli* hemolysin and expressed by the attenuated *S. typhimurium* aroA strain, an MV-specific humoral and cellular immune response was induced after oral immunization which partially protected susceptible mice (Verjans et al. 1995; Spreng et al. 2000).

Two sequential BCEs have been identified on the H protein by screening a panel of neutralizing and protective mAbs against a complete set of overlapping synthetic peptides (Ziegler et al. 1996; Fournier et al. 1997). Several mAbs reacted with peptides defining the neutralizing epitope (NE) (H236–256) and blocked hemolysis of monkey erythrocytes without inhibiting hemagglutination, suggesting that they acted at the functional interface of the H and F protein and did not inhibit virus binding to host cells (Fournier et al. 1997). Co-linear synthesis of this BCE in different orientations and copy numbers with various TCE-generated peptides was capable of inducing antibodies that neutralized MV in vitro (El Kasmi et al. 1998). After passive transfer, these sera were shown to protect young mice against a lethal challenge with the neuroadapted MV strain (El Kasmi et al. 1999a). Interestingly, NE peptides were not recognized by mouse sera generated by immunization with whole virus, and protective levels of passively transferred anti-MV antibodies did not suppress a humoral response against the NE peptide (El Kasmi et al. 1999a). Mimotopes generated against mAbs of this epitope were highly antigenic but induced no neutralizing antibodies (Deroo et al. 1998; El Kasmi et al. 1998, 2000).

Another set of mAbs identified a second sequential epitope of the H protein, the hemagglutinin noose epitope (HNE) (H381–400). This sequence contains

three cysteine residues (in positions 381, 386, and 394) which are conserved in all morbilliviruses (Ziegler et al. 1996), and HNE peptides formulated in Complete Freund's adjuvant (CFA) induced MV-crossreactive and -neutralizing sera even in the presence of protective levels of whole virus-specific antibodies (El Kasmi et al. 2000). The HNE domain is a highly conserved, subdominant, antigenic determinant (Putz et al. 2003b) and it was shown that sera of measles in late-convalescent women of child-bearing age do not recognize this peptide (Ziegler et al. 1996). These results suggest that passively acquired maternal antibodies in infants would not interfere with the immune response to this peptide. The protective binding motif of this peptide was revealed using surface plasmon resonance and a shortened, optimized sequence of the HNE, conjugated to immunogenic carrier proteins (DT or TT) and an adjuvant licensed for human use (alum) induced MV-crossreactive and protective antibodies (Putz et al. 2003b, 2004). Co-administration of the HNE conjugates with cholera toxin induced MV-crossreactive and -protective antibodies after intranasal and lower levels of peptide-specific antibodies following transcutaneous immunization (MM Putz and CP Muller, unpublished results).

Transgenic edible plants have been used to express viral antigens and chimeric high-molecular-weight polyepitopes. Such constructs (24.5–45.5 kDa), based on the permutational recombination of multiple paired copies of the HNE and NE sequences with a promiscuous, TT-derived TCE were developed and expressed in transgenic carrots (Bouche et al. 2003). One of these polyepitope proteins was shown to induce high levels of neutralizing antibodies in mice. These sera neutralized a wide range of wild-type MV isolates of different genotypes and from geographic origins (Bouche et al. 2003). Moreover, these sera neutralized all wild-type isolates tested irrespective of mutations in the HNE epitope (unpublished results).

RESPIRATORY SYNCYTIAL VIRUS

This single-strand, negative-sense RNA virus (pneumovirus of the Paramyxoviridae family) causes a severe lower respiratory tract infection in infants and immunosuppressed individuals. Despite decades of research, no vaccine has been licensed to protect against this disease. The numerous experimental vaccines also include a strategy based on synthetic peptides.

Residues 124–203 of the G glycoprotein represent a major immunogenic domain. When mice were immunized with a recombinant vaccinia virus expressing the corresponding peptide, protein G-crossreactive antibodies were induced and viral load in the lungs was significantly reduced following intranasal challenge with live respiratory syncytial virus (RSV) (Simard et al. 1995). Interestingly a short polymer of 14 peptides (amino acids 174–187), derived from this domain and coupled to keyhole limpet hemocyanin (KLH) conjugate, was sufficient to reduce viral load in the lungs of challenged animals (Simard et al. 1997). The same peptide epitope, inserted into the pIII coat protein of a recombinant bacteriophage, also elicited high levels of RSV-specific antibodies which conferred complete resistance to an RSV challenge (Bastien et al. 1997). Intranasal delivery of this peptide epitope mixed with CT also gave complete protection (Bastien et al. 1999).

By screening a random combinatorial octameric peptide library, a number of peptide mimotopes were identified against a neutralizing and protective mAb specific for the RSV F protein (Chargelegue et al. 1997). When mice were immunized with MAP constructs carrying these peptide mimotopes mixed with a promiscuous Th epitope peptide and CFA, virus-crossreactive antibodies were elicited which neutralized RSV in vitro. In vivo a significant decrease of infectious particles was found in the lungs of immunized mice (Chargelegue et al. 1997, 1998). A significant reduction of viral load was also achieved in mice immunized intranasally with cytotoxic T lymphocytes (CTL) epitope peptides (Hsu et al. 1998). When mice were immunized subcutaneously with a cocktail containing the MAP-mimotope construct, and Th and CTL peptides emulsified in incomplete Freund's adjuvant (IFA), an almost 200-fold reduction in the viral load was detected after challenge in comparison to the mice immunized with either of the peptides alone (Hsu et al. 1999). The RSV infection was more effectively cleared by a combined humoral and cellular response induced by the peptide cocktail than either peptide-induced humoral or cellular immunity alone. With the difficulties involved in developing an RSV vaccine by any strategy, the peptide approach is worth pursuing, although it is not clear why an approach based on defined epitopes would be an advantage.

Orthomyxovirus

INFLUENZA A VIRUS

The development of efficient influenza vaccines is complicated by the many subtypes and high mutation rates and of the virus and its reassortment. Thus, this virus is less than ideal for the development of an epitope-based vaccine. None the less, various hemagglutinin-derived peptides, conjugated to TT and immunized with CFA, induced antibodies inhibiting hemagglutination and virus growth in vitro and partially protecting mice against challenge with different influenza strains (Muller et al. 1982; Shapira et al. 1984). Peptides may thus induce a broader crossreactive and protective humoral response than expected. A similar observation was made with a polyepitope consisting of repeats of the MV HNE epitope, which induced antibodies neutralizing several MV strains with mutated critical contact residues in their HNE domain (Bouche et al. 2004).

Protective antibodies against influenza were also elicited using liposome-conjugated peptides even without CFA (Friede et al. 1994), and protective immunogenicity was further improved with cyclic peptides, assuring a better structural mimicry of the viral antigenic determinant (Muller et al. 1990).

Picornaviruses

FOOT-AND-MOUTH DISEASE VIRUS

This member of the aphthoviruses is one of the most infectious veterinary viruses, causing a devastating disease of livestock. Immunization with an inactivated whole virus precludes the possibility of distinguishing between immunized and infected animals. A safe, nonreplicating, subunit vaccine inducing a protective response in cattle and swine, against both disease and infection, and which could be differentiated from natural infection, would be an asset. Among the four structural proteins VP1–VP4, VP1 induces neutralizing antibodies (Pfaff et al. 1982), targeting two dominant antigenic sites (A and C). Synthetic peptides, corresponding to these domains, conjugated to KLH-induced antibodies that crossreacted with foot-and-mouth disease virus (FMDV) particles (Parry et al. 1988), neutralized virus infectivity in vitro, and protected in vivo (Bittle et al. 1982). Protective immunity was achieved in animal models, as well as in the natural host species, i.e. swine and cattle (DiMarchi et al. 1986; Morgan and Moore 1990). Chimeric peptides including epitopes of two different serotypes protected guinea-pigs against both serotypes (Doel et al. 1992). Serotype variability and possibly escape by mutation of this RNA virus may be overcome by the combination of peptides. In larger immunization trials of cattle with peptides derived from site A and C, coupled to a VP1-derived immunodominant TCE (Collen et al. 1991), only 40 percent protection was achieved (Taboga et al. 1997). Protection was undermined by the rapid generation of escape mutants in immunized animals (Taboga et al. 1997). In a more recent study with a vaccine consisting of unconjugated peptides with the built-in adjuvant tripalmitoyl-*S*-glyceryl-cysteinylserylserine (P3C) (Wiesmüller et al. 1989; Hohlich et al. 2003), protection did not correlate with the detection of high levels of antibody. Instead, strong peptide-specific T-cell responses were detected, (Hohlich et al. 2003). Retro-all-d-peptide analogs of site A (Muller et al. 1995; Benkirane et al. 1996b) further improved the immunogenicity and induced high levels of neutralizing and protective antibodies (Briand et al. 1997).

Various different peptide formulations were used. Neutralizing and protective antibodies were obtained by peptides either conjugated to an immunogenic carrier protein (e.g. KLH, TT; Bittle et al. 1982), unconjugated P3C peptides (Wiesmüller et al. 1989; Hohlich et al. 2003) or delivered in liposomes (Francis et al. 1985), as MAP constructs (Francis et al. 1991) or emulsified in oil adjuvants (Taboga et al. 1997; Wang et al. 2001, 2002a). Site A epitope is a highly disordered loop within the VP1 (Acharya et al. 1989). Crossreactive immunogenicity of a synthetic peptide epitope results from the right balance between rigidity and flexibility, and cyclization of site A peptide was shown to produce a more stable immunogenic structure (Camarero et al. 1993; Valero et al. 2000). The peptide was also constrained in recombinant proteins (Benito and Villaverde 1994; Benito and Van Regenmortel 1998) and, if one gave a better mimic of the viral epitope than conjugated synthetic peptides, others did not (Benito and Van Regenmortel 1998).

POLIOVIRUS

At the present, late stage of polio eradication, the development of an effective nonreplicating vaccine becomes an important issue. Although the inactivated Salk vaccine would be suitable, current production capacities are not sufficient. The development of a peptide vaccine is complicated by the three serotypes, each requiring its own neutralizing antibodies. Several VP1- and VP2-derived peptide epitopes were identified and shown to induce virus-neutralizing antibodies in various animal models, including guinea-pigs and rabbits when conjugated to carrier proteins (Emini et al. 1983, 1984a; Jameson et al. 1985). However, crossreactive immunogenicity was modest, as less than 30 percent of the animals developed neutralizing antibodies.

HEPATITIS A VIRUS

A sequential neutralizing epitope has been identified on the VP1 protein by comparison of surface probability profiles between hepatitis A virus (HAV) and poliovirus (Emini et al. 1985), despite minimal homology between their primary and secondary structures. Virus cross-reacting and in vitro neutralizing antibodies were induced with a VP1-peptide BSA (Emini et al. 1985), KLH conjugate, or MAP constructs (Haro et al. 1995). Two other sequential BCEs were reported on the VP2 (Robertson et al. 1989) and VP3 surface proteins (Wheeler et al. 1986). Each of the three VP1, VP2, and VP3 peptides, encapsulated in multilamellar liposomes, induced virus-neutralizing antibodies (Bosch et al. 1998). The best virus-crossreactive/neutralizing antibodies were obtained with VP3-derived peptide (Bosch et al. 1998). However, this strategy would offer no obvious advantage over current vaccines.

Flavivirus

HEPATITIS C VIRUS

This is a major cause of acute and chronic hepatitis with 180 million cases worldwide. The development of an effective vaccine against hepatitis C virus (HCV) has been so far unsuccessful. The hypervariable region 1 (HVR1) of the main E2 envelope protein has been identified as a target of neutralizing antibodies (Farci et al.

1996). A consensus sequence of HVR1 was genetically fused to the C terminus of the B subunit of CTB and expressed in plants using a tobamoviral vector (Nemchinov et al. 2000). Intranasal immunization of mice with a crude plant extract containing the recombinant chimeric HVR1-CTB protein induced antibodies crossreacting with HCV-like particles (Nemchinov et al. 2000). HCV-crossreacting antibodies were also elicited in mice using HVR1-derived peptides conjugated to KLH (Li et al. 2001) or recombinantly expressed polyepitope constructs (Li et al. 2003), and in rabbits with MAPs containing mimotope peptides (Roccasecca et al. 2001).

Retrovirus

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Despite worldwide efforts, there is no vaccine available against human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2). The characteristic viral envelope spike is formed by monomers of the two surface glycoproteins gp41 and gp120 and carries several well-characterized BCEs targeted by broadly reactive human neutralizing mAbs (Burton et al. 2004). For safety reasons, vaccine development has principally focused on subunits rather than live attenuated or chemically inactivated virus. The development of an effective vaccine is particularly difficult because there is no natural recovery from infection, which could serve as a model for a protective immune response, but neutralizing antibodies are (again) thought to be important for protection. The situation is further complicated of course by antigenic variability.

Most of the BCE experimental vaccines against HIV are based on the 'principal neutralizing domain', i.e. the third variable domain (V3) of the gp120 glycoprotein of HIV-1. MAPs, derived from this sequence, coupled to the 'built-in' adjuvant tripalmitoyl-*S*-glycerylcysteine (Deres et al. 1989) and formulated in liposomes, induced in laboratory animals gp120-specific antibodies which neutralized virus infectivity in vitro (Defoort et al. 1992b; Nardelli et al. 1992). However, neutralizing antibodies were type specific and crossreactivity in enzyme-linked immunosorbent assay (ELISA) with other strains was weak (Nardelli et al. 1992). 'Cluster peptides', consisting of synthetic peptide constructs of the V3 loop and overlapping multideterminant Th epitopes of gp160, induced V3-specific neutralizing antibodies in several strains of mice (Berzofsky et al. 1991; Ahlers et al. 1993). Intranasal immunization with free V3 loop peptides and the mucosal adjuvant CT generated HIV-1-neutralizing antibody responses in two strains of mice (Staats et al. 1996). In addition to high serum anti-peptide responses, significant vaginal anti-peptide IgG and IgA levels were obtained (Staats et al. 1996). Neutralizing secretory IgA antibodies were also induced after oral delivery of multivalent MAPs mixed with CT (Bukawa et al. 1995; Kato et al. 2000). Systemic and mucosal antibody responses were further improved by co-administration of proinflammatory cytokines, such as the interleukins IL-1α, IL-12, and IL-18, instead of CT (Bradney et al. 2002).

Synthetic peptide vaccines have also been tested in clinical trials with encouraging long-term safety profiles when adjuvants other than IFA were used. Long-lasting, peptide-specific, neutralizing humoral responses were obtained with a purified protein derivative (PPD) conjugate vaccine without the use of an adjuvant (Rubinstein et al. 1995, 1999). HIV-seropositive vaccinees over time exhibited a uniform increase in antibodies capable of neutralizing laboratory-adapted and primary isolates of HIV-1. Highest titers were observed after 12 months in a regimen where the peptide conjugate was inoculated intradermally, monthly for the first 3 months and then at 3-month intervals to 18 months. In three of seven patients, a progressive decline in viral loads occurred (Rubinstein et al. 1999). Although viral mutants with amino acid substitutions within the V3 loop sequence appeared in these three patients during the immunization program, virus loads remained at low levels for several months (Lenz et al. 2001). However, about a year after discontinuation of immunization, in six patients viremia was at preimmunization levels or higher and was paralleled by a loss in V3 loop-specific antibodies (Rubinstein et al. 1999). An unconjugated, octameric, branching, V3 loop peptide adsorbed on alum induced in vitro neutralizing antibodies in eight of ten HIV-1-uninfected, healthy adults after intramuscular administration (Gorse et al. 1996). Whereas the same vaccine formulation displayed a similar high neutralizing efficacy (19 of 20 vaccinees) in a trial in Bangkok, Thailand (Phanuphak et al. 1997), lower seroconversion rates (3 of 13 vaccinees) were observed in a trial in Longchuan County, China (Li et al. 2001). This octameric peptide vaccine was weakly immunogenic (4 of 24 vaccinees seroconverted) when delivered subcutaneously in combination with alum (Kelleher et al. 1997), and after oral administration of peptides encapsulated in biodegradable microspheres no significant humoral, cellular, or mucosal immune responses were found (Lambert et al. 2001a).

However, sterilizing immunity to HIV-1 will most probably be achieved only through a combination of protective antibody and CTL responses. Promising results have been obtained with peptide vaccines eliciting cellular immune responses (Letvin 1998; Suhrbier 2002).

Parvoviruses

CANINE PARVOVIRUS

The use of a peptide vaccine against canine parvovirus (CPV) was among the first to demonstrate the induction

of protection in the natural host (Langeveld et al. 1994b). Among the three structural proteins VP1–VP3 of the virus capsid, VP2 is the major immunogen. Immunization with two overlapping peptides of the N-terminal domain of VP2 conjugated to KLH protected dogs against a lethal challenge with CPV, despite the intriguing observation that virus-neutralizing antibodies were not detected in any of the immunized and protected animals before challenge (Langeveld et al. 1994a, b). Protection was attributed to the high flexibility of the epitope and its functional importance for viral replication (Langeveld et al. 1994a). Efficacy of the peptide vaccine was shown to result from two distinct but overlapping epitopes, each of which induced neutralizing antibodies in rabbits (Langeveld et al. 1994b; Casal et al. 1995). Mutation rates are low in this DNA virus (in comparison to RNA viruses) and this N-terminal epitope is conserved in different virus isolates (Parrish et al. 1988), including the three closely related virus species, mink enteritis virus (MEV), feline panleukopenia virus (FPLV), and raccoon parvovirus. Thus, the same epitope could be used in three important animal species: dogs (Langeveld et al. 1994a), cats (Langeveld et al. 2001a), and band minks (Langeveld et al. 1995). Immunization with the CPV-derived peptide conjugated to KLH protected minks against MEV (Langeveld et al. 1995). When the corresponding MEV oligonucleotide coding sequence was inserted into the plant virus, cowpea mosaic virus, the epitope was expressed on the surface of the chimeric virus particles propagated in the black-eyed bean, an edible plant. Subcutaneous injections of minks with recombinant virus-like particles protected against clinical disease and virtually abolished shedding of virus after challenge with virulent MEV (Dalsgaard et al. 1997). Chimeric virus-like particles also protected dogs from a lethal challenge with CPV (Langeveld et al. 2001b). A multivalent vaccine based on peptides of CPV, canine distemper virus (CDV), and *Pseudomonas aeruginosa* could be a commercially viable strategy because they would protect dogs against two important canine diseases and minks against the three major diseases in mink farming.

Hepadnavirus

HEPATITIS B VIRUS

It is estimated that 400 million people are chronically infected worldwide with hepatitis B virus (HBV). Protective antibodies in humans mostly target the hepatitis B virus surface antigen (HBsAG). Several continuous BCEs have been identified on HBsAg (Gerin et al. 1983). When coupled to a carrier protein, such as KLH, some of these peptides induced a weak, but partially protective, humoral response in chimpanzees (Gerin et al. 1983; Itoh et al. 1986). Protective immune responses were also obtained with unconjugated, longer polymers of 55 peptides (Emini et al. 1989). MAPs induced increased antibody responses in rabbits (Tam and Lu 1989). A peptide-based approach using CTL epitopes has also been explored (reviewed by Engler et al. 2001). After initial encouraging results (Vitiello et al. 1995; Livingston et al. 1997, 1999) CTL responses were weak in later human trials (Heathcote et al. 1999).

Papovavirus

HUMAN PAPILLOMAVIRUS

Human papillomavirus (HPV) is a double-stranded, circular DNA virus with more than 100 genotypes. In particular the high-risk genotypes (HPV16, -18, -31, -33, and -45) are found in almost all cervical carcinomas. Experimental preventive and therapeutic vaccines against HPV-mediated cervical cancer were recently reviewed (Fausch et al. 2003; Roden and Wu 2003). A peptide from the E7 protein of HPV16 containing a BCE, a promiscuous T helper, and a CTL epitope were coupled to immunostimulatory carriers (ISCAR) (Tindle et al. 1995) or combined as polymeric conjugates (Fernando et al. 1995) with either CFA or ISCOMs. These constructs induced E7-crossreactive antibodies and E7-specific CTLs with in vitro killing activity (Tindle et al. 1995), and conferred in vivo protection against an E7 gene-transfected tumor cell inoculum (Tindle et al. 1995). Sequential neutralizing BCEs have also been identified of the L1 (major) and L2 (minor) structural proteins using mAbs (Kawana et al. 1998). A BSA-conjugated L2-derived peptide induced antibodies that crossneutralized HPV16 and -6 (Kawana et al. 1999). Intranasal immunization with the same epitope in TCE-containing chimeric peptides, mixed to CT, induced antibodies cross-binding to HPV6, -16 ,and -18, and neutralizing HPV11 authentic virions and HPV16 pseudovirions in vitro (Kawana et al. 1999, 2001). These results suggest that this BCE is shared by other genital HPVs and may induce broadly reactive, cross-neutralizing antibodies. Early safety and immunogenicity trials in humans were promising. Intranasal delivery of a linear polymer of 13 peptides without adjuvant induced antibodies neutralizing HPV15 and -52 (Kawana et al. 2003). Peptide vaccines targeting tumor-specific CTL responses are discussed later in the chapter.

Bacteria

NEISSERIA MENINGITIDIS SEROGROUP C

Most of the infections by *Neisseria meningitidis*, gram-negative encapsulated bacteria, are caused by any of the four prevalent serogroups: A, B, C, and Y. Polysaccharide-conjugate vaccines induce neutralizing antibodies against surface loops of class 1 outer membrane proteins (OMP),

which play a major role in protection. TT-based conjugates loaded with cyclic peptides derived from OMP induced mouse antibodies with bactericidal activity in vitro (Hoogerhout et al. 1995). Protective immune responses against experimental challenge were seen in mice with unconjugated, free peptides of the PorA and the OpaB outer membrane proteins (Koroev et al. 2000, 2001). Polysaccharides can be mimicked using peptides or anti-idiotype antibodies as surrogate antigens (reviewed by Monzavi-Karbassi et al. 2002). Immunization with mimotopes conjugated to DT induced antibodies crossreacting with the meningococcal outer-membrane lipo-oligosaccharide (Charalambous and Feavers 2000). Short peptides derived from the CDR sequences of protective anti-idiotype antibodies, and conjugated to proteosomes, induced antibodies protecting mice against a lethal dose of *N. meningitidis* (Westerink et al. 1995; Prinz et al. 2004). Mimotope peptides were also incorporated into DNA constructs. After DNA immunization, significant anti-polysaccharide antibody responses were induced, neutralizing bacterial activity in vitro and protecting against a challenge in vivo (Westerink et al. 2001; Prinz et al. 2003).

Several other peptide-based vaccines have been developed to protect against *Pseudomonas aeruginosa* (reviewed by Cachia and Hodges 2003) and group A streptococcus (reviewed by Olive et al. 2004) and others (see Table 1).

Parasites

MALARIA

This is by far the most important parasitic disease in humans, caused by four *Plasmodium* species (*P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*) and spread by female anopheles mosquitoes. More than 200 species are known to parasitize mammals (e.g. *P. berghei* and *P. yoelii* in mice, *P. knowlesi* in monkeys), birds, and reptiles. The development of a malaria vaccine is a difficult task for various reasons: the life cycle of malaria parasites in humans is complex with morphologically and antigenically distinct stages. There is a high degree of antigenic variation among *Plasmodium* species. Immunity is stage specific. An important part of the population does not respond to particular parasite antigens. Nevertheless, several peptide epitopes inducing neutralizing and protective antibodies have been identified on antigens of the pre-erythrocytic (e.g. circumsporozoite protein (CSP); thrombospondin-related anonymous protein (TRAP); liver stage antigen 3 (LSA-3)) and the asexual blood stage (e.g. major merozoite surface protein (MSP-1); ring-infected erythrocyte surface antigen (RESA)). Malaria vaccine developments have been reviewed by Tsuji and Zavala (2001) and Moorthy et al. (2004).

A synthetic immunodominant repeat sequence, derived from the *P. falciparum* CSP, conjugated to TT, induced antibodies crossreacting with the parasite surface and neutralizing its infectivity in vitro (Zavala et al. 1985). This peptide conjugate proved its safety and immunogenicity in phase I and II clinical trials where the majority of recipients seroconverted. One of three challenged vaccinees was totally protected and in the others the infection was delayed and/or reduced (Herrington et al. 1987). Significant T-cell responses to the coupled peptide were reported which may play a role in priming for protection (Etlinger et al. 1988). In another trial, however, the clinical efficacy of the conjugate was substantially lower and only one of seven vaccinees was protected against a sporozoite challenge (Herrington et al. 1990). MAPs, containing BCEs and TCEs, significantly improved immunogenicity (Tam et al. 1990). MAPs induced higher titers of neutralizing antibodies in mice (Joshi et al. 2001) and, in combination with alum and saponin QS21, in monkeys (Moreno et al. 1999) and humans (Nardin et al. 2000). Novel multiepitope polyoxime constructs containing the CSP repeat epitope, a CSP-derived universal TCE and the built-in adjuvant P3C, induced high antibody levels in human volunteers of diverse HLA types (Nardin et al. 2001).

A repeat domain epitope derived from *P. berghei* CSP induced high serum titers of protective antibody responses in mice when administered as TT conjugates. In these constructs, the orientation of the conjugated peptide, as well as the peptide:carrier ratio, was critical for protection (Zavala et al. 1987). Only BSA conjugates with a high enough peptide load achieved total protection in animals (Reed et al. 1996). Multiple antigen delivery systems containing *P. berghei* CSP-derived BCEs and TCEs induced protective immune responses in mice when combined with CFA (Tam et al. 1990) or detoxified lipopolysaccharide (RaLPS) (Reed et al. 1997). Despite the presence of a *P. yoelii* CSP-derived BCE in the multiple antigen constructs of Reed et al. (1997), protection was weak against *P. berghei* and negative against *P. yoelii*. However, when delivered in a recombinant HBV nucleocapsid antigen (HBcAg), the *P. yoelii* CSP-derived BCE induced protective immune responses in mice (Schodel et al. 1997) and results were promising in preclinical trials in primates (Birkett et al. 2002).

Multiple antigen constructs (MAC) containing a BCE from the *P. vivax* CSP repeat region and a promiscuous TCE from TT induced a protective immune response in monkeys when administered with alum or RaLPS (Yang et al. 1997).

LSA-3 is a highly conserved pre-erythrocytic antigen and a promising vaccine candidate (Perlaza et al. 2001). A 26-residue sequence of this protein induced strong B, Th cell, and CTL responses in chimpanzees when delivered as palmitoyl-conjugated lipopeptides (BenMohamed et al. 1997). Protective immune responses were achieved with a combination of lipopeptides (Daubersies et al. 2000).

Several peptides derived from the merozoite MSP-1 antigen were included in conjugates using BSA or TT as carriers (Berzins et al. 1986) or, more recently, in 'double dimer constructs' (Rivera et al. 2002). These peptides induce antibodies that partially protected monkeys against a challenge infection. Peptides of another well-characterized asexual blood stage antigen, the RESA protein, coupled to TT or KLH, inhibited merozoite reinvasion of erythrocytes in vitro (Cheung et al. 1986) and gave partial protection in monkeys (Patarroyo et al. 1987). Similarly, peptide conjugates from other merozoite stage antigens, such as Pf55 and Pf35, provided partial protection in monkeys and even complete protection when three conjugates were combined (Patarroyo et al. 1987).

Multicomponent and multistage strategies have been considered in order to induce several lines of defense against the different stages of its life cycle. Patarroyo and colleagues developed a synthetic hybrid polymer, containing the peptide sequences derived from MSP-1, Pf55, and Pf35, and repeat sequences from CSP, which was further polymerized using cysteine residues at its N- and C-terminal ends to result in a high-molecular-weight synthetic protein (150 000) inducing protective immune responses in monkeys (Patarroyo et al. 1988; Rodriguez et al. 1990; Ruebush et al. 1990). Very promising results were initially obtained with this fully synthetic protein termed 'SPf66', in early clinical trials, when administered together with alum as adjuvant: delay or even complete suppression of parasitemia was observed in immunized human volunteers (Patarroyo et al. 1988; Amador et al. 1992a, b; Valero et al. 1993). Antibodies induced in humans markedly inhibited parasite growth in vitro (Salcedo et al. 1991). The vaccine was shown to be highly immunogenic and safe in children aged 1–14 years (Patarroyo et al. 1992). Efficacy of up to 80 percent was observed (Amador et al. 1992b). However, the efficacies reported subsequently in larger field trials were consistently below 40 percent (Valero et al. 1993, 1996) and in a study in northern Thailand, an area with a high *P. falciparum* attack rate, no protection was observed (Nosten et al. 1996). In a different approach Shi and colleagues used a recombinant polyepitope containing twelve BCE, six T-helper epitopes, and three CTL epitopes derived from nine stage-specific *P. falciparum* antigens expressed in the baculovirus system (Shi et al. 1999). This polyepitope induced antibodies cross-reacting with the different stages of the parasite and neutralizing parasite activity in vitro (Shi et al. 1999). Recombinant polyepitope constructs based on CTL epitopes are being further explored with encouraging results (Tsuji and Zavala 2001).

SCHISTOSOMIASIS

Schistosomes are parasitic trematodes causing chronic disease in an estimated 200 million people worldwide. Infections in humans are mostly caused by one of the three helminth species *Schistosoma manson i*, *S. haematobium*, and *S. japonicum*, and candidate vaccines are currently being investigated in clinical trials (Capron et al. 2002). Several antigens or fragments derived from them, e.g. 9B (Tarrab-Hazdai et al. 1998), P28-1 (Wolowczuk et al. 1989), Sm23 (Reynolds et al. 1992), triose phosphate isomerase (TPI) (Reynolds et al. 1994), Sm14 (Tendler et al. 1996), and glutathione-*S*-transferase (Sm28-GST) (Ferru et al. 1997) have been identified as targets of specific B-cell and Th-cell responses. Early work on synthetic peptide vaccines against schistosomiasis has been discussed by Harn et al. (1995). In more recent studies a polymer of 14 peptides derived from the 9B antigen, conjugated to BSA, induced an immune response that was able to reduce significantly the worm burden after challenge in a murine model (Tarrab-Hazdai et al. 1998). Protective immune responses were also observed after intranasal immunization with a recombinant salmonella flagellin protein carrying this 9B epitope sequence (Ben-Yedidia et al. 1999). By screening a solid-phase octamer library against a 9B-specific mAb, four peptide mimotopes were identified, one of which induced a protective immune response (Arnon et al. 2000). It is noteworthy that protection against *S. mansoni* challenge was also obtained after immunization with two unconjugated peptides (derived from the Sm14 antigen) in the presence of the adjuvant system monophosphoryl lipid A/trehalose dicorynomycolate (MPL-TDM) (Vilar et al. 2003). Interestingly, these Sm14-derived epitopes cross-protected mice against a challenge with *Fasciola hepatica*, a helminth affecting sheep and cattle (Vilar et al. 2003). A multiepitope approach, combining 10 different epitopes of various *S. mansoni* antigens, was investigated using DNA and recombinant protein immunization. However, none of the tested constructs induced a protective response (Yang et al. 2000).

CYSTICERCOSIS

Taenia solium, a parasitic tapeworm causing cysticercosis, is highly prevalent in humans and pigs in Latin America, Asia, and Africa, and has serious health and economic consequences. Experimental vaccines for use in pigs, the obligatory intermediate host, are currently under development and include a peptide-based approach (reviewed by Sciutto et al. 2002).

Four recombinant antigens (KETc1, -4, -7, and -12) expressed during the cysticercus stage of both *T. solium* and *T. crassiceps*, a cestode causing murine cysticercosis, confer different levels of protection in immunized mice (Manoutcharian et al. 1996). A synthetic 18-residue peptide GK-1, derived from the KETc7 protein and emulsified in saponin, induces antibodies crossreacting with the cognate antigen without the use of a carrier protein (Toledo et al. 1999). It is noteworthy that this

GK-1 peptide contains at least one TCE, which was demonstrated by its ability to stimulate proliferation of $CD8^+$ and to a lesser extent $CD4^+$ T cells. GK-1-induced immunity only partially protected against a challenge infection with *T. crassiceps* and this did not improve when the peptide was delivered as MAP or coupled to BSA (Toledo et al. 1999). Similarly, two polymeric peptides of 13 and 9 residues, derived from the KETc1 and -12 antigens, induced B-cell and T-cell responses, partially protecting mice against cysticercosis caused by *T. crassiceps* (Toledo et al. 2001). In a controlled field trial, immunization of piglets with a combination of the unconjugated peptides KETc1, -7, and -12 with saponin decreased the total number of viable *T. solium* cysticerci by almost 98 percent and the prevalence of cysticercosis among immunized animals by more than half (Huerta et al. 2001).

Contraception

A vaccine providing safe, inexpensive, and reversible contraception would greatly help to regulate the world's growing population (reviewed by Aitken 2002). A peptide approach would at least be inexpensive, reversible after 1–2 years, and would not require a cold chain.

A C-terminal peptide of human chorionic hormone (hCG), an essential hormone during pregnancy, coupled to TT, induced high titers of antibodies, successfully reducing fertility in female baboons (Stevens et al. 1981a). Further studies in mice, rabbits, and baboons led to the formulation of a vaccine based on the water-soluble synthetic adjuvant, *N*-acetylglucosamine-3-yl-acetyl-l-alanyl-d-isoglutamine (CGP-11637) suitable for use in humans (Stevens et al. 1981b). In clinical trials, hCG-crossreactive antibodies were observed, which were thought to neutralize the biological activity of hCG, but definitive proof of a contraception was not obtained in this study (Jones et al. 1988). The low immunogenicity of this hCG peptide was overcome by immunizing with the entire β-subunit of hCG and prevention of pregnancy was shown in clinical trials (Talwar 1999). The luteinizing hormone-releasing hormone (LHRH) is a short decapeptide hormone secreted by the hypothalamus. Branched peptide constructs containing the LHRH peptide and a T-helper cell epitope (ThCE) induced sterilizing antibodies in female mice (Ghosh and Jackson 1999).

A synthetic BCE of the zona pellucida glycoprotein ZP3, either conjugated to KLH (Millar et al. 1989) or delivered as a chimeric peptide containing a foreign promiscuous TCE (Lou et al. 1995; Sadler et al. 1999), induced antibodies in mice crossreacting with the cognate protein, providing a long-term, but reversible, contraception without impairment of normal ovarian functions. Immunocontraception was also produced in female macaques after administration of conjugates with coupled human and macaque ZP3 peptides (Mahi-Brown and Moran 1995; Mahi-Brown 1996). Antibodies inhibiting human sperm binding to oocytes in vitro were obtained using chimeric TCE–BCE peptides in macaques (Bagavant et al. 1997) and common marmosets, but the antibodies did not reduce fertility in a primate model (Paterson et al. 1999, 2002). Antibodies targeting an epitope located on the human sperm antigen lactate dehydrogenase (LDH)-C_4 have been induced in baboons using peptide conjugates and chimeric peptides (O'Hern et al. 1995, 1997). Fertility in immunized animals was reduced by 70 percent and the contraceptive effect lasted for almost a year. The same epitope in chimeric peptides induced significant contraception in mice (Sadler et al. 1999). Recently, a novel peptide epitope (YLP12) was identified on human sperm, eliciting long-lasting sperm-specific antibodies that assured contraception in female mice when conjugated to CTB and administered intranasally (Naz and Chauhan 2002; Naz 2004). Fertility was fully restored after 10 months when the antibody titers had fallen below a certain threshold.

Immunocastration has been developed as an economical alternative to surgical castration of male pigs to prevent the growth of testes and the production of male sexual steroid hormones, which is associated with an unappealing odor of the meat. Immunocastration was obtained in male pigs after CFA-adjuvanted immunization with peptides derived from gonadotropin-releasing hormone (GnRH) and conjugated to KLH or OVA (Meloen et al. 1994) or ISCOMs (Beekman et al. 1999). Similarly high responding rates were obtained with low doses of conjugated double-dimer peptides mixed with a mild oil adjuvant (Specol; Oonk et al. 1998; X.Y. Zeng et al. 2001). With the encouraging results and the specific advantages of a peptide vaccine, this approach may become a truly inexpensive alternative to current contraceptive drugs.

Alzheimer's disease

Alzheimer's disease, the most common cause of dementing disorders in elderly people, causes progressive loss of central nervous functions. Pathogenesis involves the overproduction of the 42-residue amyloid-β peptide ($A\beta_{42}$) derived from the amyloid precursor protein (APP), progressive accumulation of $A\beta_{42}$ in extracellular plaques within the cerebral cortex and the hippocampus (Reilly et al. 2003), followed by neurodegeneration (Selkoe 1999). The progress of and drawbacks in antibodies as therapeutic agents in Alzheimer's disease have been extensively discussed by Morgan (2003) and Solomon (2002).

Transgenic mouse models have been used to investigate the possibility of therapeutic immunization against Alzheimer's disease (Games et al. 1995). Immunization with the synthetic $A\beta_{42}$-induced $A\beta_{42}$-specific antibodies,

which prevented the development of β-amyloid plaque and Alzheimer's disease-type neuropathologies in animals immunized at a young age (Schenk et al. 1999). Moreover, these antibodies greatly reduced progressive degeneration and even cleared pre-existing plaques of $A\beta_{42}$ deposits in older animals (Schenk et al. 1999). Furthermore, $A\beta_{42}$ immunization prevented learning and age-related memory defects normally developing in transgenic mice (Janus et al. 2000; Morgan et al. 2000). Significant decrease in the cerebral β-amyloid plaque burden was also obtained in mice after intranasal administration of synthetic $A\beta_{42}$ using LT as adjuvant (Weiner et al. 2000; Lemere et al. 2001). In vitro studies revealed that mAbs specific for a short $A\beta_{42}$ N-terminal fragment prevented fibrillar aggregation and $A\beta_{42}$-mediated neurotoxicity in a concentration-dependent manner (Solomon et al. 1997). Immunization of mice using a recombinant filamentous phage displaying an N-terminal derived tetramer peptide sequence induced antibodies that exhibited in vitro anti-aggregating properties and neutralizing $A\beta_{42}$-related toxicity (Frenkel et al. 2001, 2003). This phage also markedly reduced the number of plaques in the brain of transgenic mice (Frenkel et al. 2003). Similar protective in vivo effects were obtained after immunization with a modified $A\beta_{42}$-derived, polylysine peptide (Sigurdsson et al. 2001). Furthermore, low antibody titers and self-tolerance were proposed to result primarily from inadequate T-cell help, which could be overcome by using palmitoylated $A\beta_{42}$ reconstituted in liposomes–lipid A (Nicolau et al. 2002) or by conjugating a short $A\beta_{42}$-derived polymer of 15 residues to BSA (Monsonego et al. 2001). However, immunization with conjugated, short, serum amyloid protein-derived peptides did not protect mice against β-amyloid plaque deposits (Schenk et al. 1999). Antibodies against plaque components themselves may not be sufficient to prevent plaque aggregation and antibodies targeting specifically the $A\beta_{42}$ seem to be required for preventing disease.

A phase I study showed that immunization with the synthetic $A\beta_{42}$ was well tolerated and some patients developed $A\beta_{42}$-specific antibodies (Schenk 2002). However, phase IIa trials had to be discontinued because of several reports of inflammatory symptoms of the central nervous system which resembled encephalitis or meningitis (Check 2002). Autopsy of a brain from a treated patient provided astonishing evidence of how anti-$A\beta_{42}$ antibodies efficiently cleared $A\beta_{42}$ deposits in the cerebral cortex (Nicoll et al. 2003). Intriguing parallels between the rare cerebral amyloid angiopathy-associated inflammation and the vaccine-related encephalitis suggests that the vaccine-mediated inflammatory response was directed not only against the $A\beta_{42}$ plaques but also against vascular amyloid (Greenberg et al. 2003; Nicoll et al. 2003; Robinson et al. 2003). With more refined immunotherapeutic approaches, which will avoid autoimmune and acute neuroinflammatory responses (Robinson et al. 2003; Weller and Nicoll 2003), peptide-based immunotherapy against Alzheimer's disease may become a realistic option.

BCE peptide vaccines against cancer

Although most studies with peptide vaccines against tumors target CTLs (see later), interesting results have also been obtained with peptides eliciting anti-tumor antibodies, e.g. Kaumaya and colleagues (Dakappagari et al. 2000) developed a chimeric HER-2 BCE vaccine in mice transgenic for a homolog of human HER-2. These mice developed mammary tumors that resembled human breast cancer. Peptides corresponding to predicted BCEs were co-linearly synthesized with a promiscuous TCE of MV, induced antibodies in rabbits that crossreacted and interrupted the development of mammary tumors in the above transgenic mouse model, and mediated killing of human tumor cells in vitro (Dakappagari et al. 2000). Table 1 shows that such antibodies against breast cancer and others have also been elicited in humans by immunization with peptides derived from different tumor-associated antigens (TAA). In the case of B-cell lymphomas, peptides inducing anti-idiotype responses resulted in a clinical response in most patients (Hsu et al. 1996; Bendandi et al. 1999).

PEPTIDE-BASED VACCINES AGAINST TCES

Although peptides have also been used to induce protective CTLs against infectious and other disease, the focus of this section is on CTLs of malignant tumors.

Tumor immunology and peptides

A number of reviews have been published on different aspects of synthetic peptides as cancer vaccines (Mayordomo et al. 1996; Slingluff 1996; Olive et al. 2001; Buteau et al. 2002; Machiels et al. 2002; Parmiani et al. 2002a, b; Sundaram et al. 2002). Some authors have concentrated on certain tumors, or tumor antigens including melanoma (Mocellin et al. 2003), HPV-associated malignancies (Fausch et al. 2003), and Her-2/neu (Correa and Plunkett 2001; Disis et al. 2001; Knutson and Disis 2001; Salit et al. 2002).

Early studies showed that the injection of tumor-derived material in animals or even cancer patients limited tumor growth. With the discovery of TAAs it became possible to investigate the immune response against cancer (reviewed by Herlyn and Koprowski 1988; Schreiber et al. 1988; Urban and Schreiber 1992; Boon et al. 1994). These studies showed that T cells mediate the principal effector mechanisms against tumors, although the humoral response as demonstrated

by therapeutic monoclonal antibodies such as trastuzumab (Herceptin, Baselga 2001), rituximab (Rituxan, Davis et al. 1999; Huhn et al. 2001), and peptide-specific antibodies (see Table 1) must not be underestimated.

Unlike B cells (see earlier), T cells do not recognize intact proteins but rather MHC-bound antigen-derived peptides. These peptides are generated during antigen processing and are presented by MHC class I or II molecules to $CD8^+$ or $CD4^+$ T cells. Normally CTL epitopes originate from intracellular proteins degraded in the cytosol by the proteasome and carried by specialized transporters (TAPs) into the endoplasmic reticulum, where they bind to MHCI to be taxied to the cell surface for a date with $CD8^+$ T cells. In contrast, $CD4^+$-restricted epitopes originate from exogenous proteins taken up and degraded in phagolysosomes into peptides that co-localize with MHCII, which shuttles them back to the cell surface, where they meet with $CD4^+$ T cells. MHC molecules fix peptides in an extended conformation and synthetic peptides are undistinguishable from natural peptides.

T cells specific for TAAs were found in many cancer patients but also in healthy donors. In healthy individuals, frequencies of such CTLs seem to be lower (Marincola et al. 1996; Altieri et al. 1999; Nagorsen et al. 2000; Andersen et al. 2001) and of a different phenotype. In healthy donors these were of the $CD45RO^+$ naive T-cell subset, whereas in melanoma patients peptide-specific CTL precursors were of the functionally distinct $CD45RO^+$, $CCR7^+$ memory phenotype (D'Souza et al. 1998; Anichini et al. 1999; Dunbar et al. 2000).

In clinical trials in tumor patients, promising results such as full or partial remission have been largely attributed to the induction of $CD8^+$ MHCI-restricted CTLs. Many TAA-derived MHCI-restricted peptides are recognized by tumor-specific CTLs at both the induction and the effector stage (see Table 4; Jager et al. 2001).
In addition, MHCII-restricted ThCEs of a number of TAAs have been identified (Topalian et al. 1996; Manici et al. 1999; Pieper et al. 1999; Wang et al. 1999b; Chiari et al. 2000; Renkvist et al. 2001). In the first application of a peptide vaccine in humans, HBV-negative volunteers were immunized with a lipopeptide construct containing an HLA-A*0201-restricted peptide, a pan-class II-binding peptide, and palmitic acid tails. CTLs were induced that lysed syngeneic HBV-infected target cells in vitro (Vitiello et al. 1995). Cytotoxic TCEs restricted by some of the more frequent MHCI alleles have been identified for an increasing number of TAAs (reviewed by Maeurer et al. 1996). Peptides derived from a variety of TAAs have sometimes induced vigorous responses against tumors. Regression of primary tumors and metastasis were seen using a variety of different immunization protocols.

However, to measure the success of tumor immunization, the identification of surrogate in vitro read-outs, which correlate and may even predict clinical outcome, is of paramount importance. Besides conventional assays such as CTL and cytokine assays, increasingly sophisticated reagents based on synthetic peptides such as a MHC–tetramer (www.niaid.nih.gov/reposit/tetramer/index.html) and MHC–Streptamer complexes (Knabel et al. 2002, www.iba-go.com) become commercially available to study and even isolate peptide-specific T cells for functional assays.

Identification of CTL epitopes

Synthetic peptides have been powerful tools to identify class II- and I-restricted TCEs by systematic screening in functional assays. MHC-bound peptides eluted directly from tumor material of patients were identified on the basis of their mass and partial sequence by highly sensitive tandem mass spectrometry (Hunt et al. 1992). Increasingly refined prediction algorithms support these studies since the structural basis of MHC-peptide binding became understood (Pamer et al. 1991). Pool sequencing of MHC-eluted peptides (Rotzschke et al. 1990; Falk et al. 1991) has revealed that peptides bind to MHCI via an allele-specific consensus motif of anchor residues. Thus, MHC molecules encoded by different gene loci and different haplotypes bind their own distinct (but sometimes overlapping) family of peptides, usually with eight to ten amino acids (for a review, see Rammensee 1995; Rammensee et al. 1995, 1997). Interactive websites (www.syfpeithi.bmi-heidelberg.com, www.bimas.dcrt.nih.gov/molbio/index.html) identify peptides within a submitted antigen sequence that bind to a given MHC. Other consensus motifs have been identified to predict peptides surviving antigen processing (Nussbaum et al. 1998, 2001; Kuttler et al. 2000) and contributed to interactive websites (www.paproc.de, www.mpiib-berlin.mpg.de/MAPPP) to predict TCEs. In the light of these developments, prediction algorithms based on structural considerations such as amphipathic helices (DeLisi and Berzofsky 1985) must now be considered obsolete.

The rationale of peptide-based vaccines against cancer TCEs

Cancer vaccines can be based on whole cancer cells, TAAs, or peptide fragments of TAAs. Whole tumor cells of a good quality and quantity can be obtained only from large surgical tumors. Early detection of small tumors and tumor regression under conventional therapy severely limits the availability of tumor material. Immunization as an adjuvant therapy against (minimal) residual disease would also suffer from lack of tumor material. Furthermore, the preparation of a vaccine from an ex vivo tumor is laborious and expensive. Although recombinant TAAs may seem like obvious

antigens of tumor vaccines, most TAAs are not tumor specific. TAAs often resemble antigen with limited expression in healthy tissues and may succumb to self/nonself discrimination. In some cases, such as Her-2/neu antigen, peptides elicited immunity when whole proteins were unable to do so (Disis et al. 1996). In humans, delivery systems to induce CTL responses such as replicating or nonreplicating viral vectors, DNA, or liphophilic adjuvants are often only poorly immunogenic or are not safe in immunosuppressed tumor patients. Peptides, which can be produced easily and in large quantities, are more than an alternative of convenience; in fact, peptides derived from TAAs are the true antigens recognized by the T cells. In contrast to proteins, peptides can deliver epitopes in high molar concentrations (directly in vivo, or by dendritic cell (DC) pulsing in vitro) to improve MHC loading, although high concentrations have sometimes led to tolerization (Aichele et al. 1995; Toes et al. 1996a, b).

Increasing the immunogenicity of peptide tumor vaccines

Although clinical trials demonstrated that peptides induce CTLs, their immunogenicity in cancer patients is often too low to clear the tumor and even partial tumor regression is unreliable (Marchand et al. 1999). To enhance the immunogenicity of TAA-derived peptides numerous strategies have been developed.

CD4 T-CELL HELP

Activated $CD4^+$ T cells help to initiate and amplify the CTL response. Class II-restricted TCEs have been identified from a number of tumor antigens (Jager et al. 2000b; Zarour et al. 2000; G. Zeng et al. 2001). The identification of class II-restricted epitopes is complicated by the open-ended binding groove of MHCII which accommodates peptides that are much less well defined in terms of length (Chicz et al. 1992; Rammensee et al. 1995), anchor positions (Rammensee et al. 1997), and processing (Robinson and Delvig 2002). Some studies have included TAA-derived class II-restricted TCEs in peptide vaccine trials (see Table 4).
If these may be more efficient, they need to be defined for each TAA and the MHC context of every individual patient. Alternately, it has been proposed to include promiscuous helper TCEs that bind to many class II molecules (see earlier). These TCEs are normally derived from tumor-unrelated antigens such as TT (Kaumaya et al. 1993; Dakappagari et al. 2000; Slingluff et al. 2001) or they are totally artificial such as the broadly reactive PADRE peptide (Weber et al. 1999) (see Table 3). Despite some exceptions (Phan et al. 2003), clinical studies seem to suggest that the inclusion of a class II-restricted helper TCE may improve the level and duration of anti-tumor CTL activity, although few systematic studies have been reported.

MODIFIED PEPTIDES

HLA–peptide–T-cell receptor (TCR) complexes can be optimized for improved T-cell activation by peptide modifications (Chen et al. 1996), e.g. modification of MHC-binding anchor residues of peptides of the Melan-A/MART-1(27–35), the gp100 (209–217), Her2/neu, or folate-binding protein resulted in improved peptide–HLA binding and stability (Fisk et al. 1995; Parkhurst et al. 1996; Valmori et al. 1998; Tourdot et al. 2000). By mutating peptide residues of epitopes of Melan-A/MART-1(27–35), carcinoembryonic antigen (CEA) and NY-ESO-1 signaling of TCR and T-cell stimulation was enhanced (Rivoltini et al. 1999; Salazar et al. 2000). Systematic positional scanning with sets of peptide libraries, each containing in each position every amino acid flanked by undefined amino acids, has been performed to improve peptide interactions within the MHC–TCR complex (Fleckenstein et al. 1996; Wiesmüller et al. 2001). Peptide analogs with improved properties have been defined for Melan-A-specific CTL clones (Pinilla et al. 1999, 2001). Other modifications including terminal acetylation or amidation may protect against premature proteolytic degradation in vivo (Brinckerhoff et al. 1999).

PEPTIDE FORMULATIONS

Alum is the only adjuvant licensed for human use, with decades of experience. However, this adjuvant is well known to induce a Th2-biased response, which elicits a humoral rather than a CTL response. More recently virosomes (influenza virus-like particles) have been licensed (Moser et al. 2003) but so far this has not been included in cancer peptide vaccine trials. Thus, peptide vaccine trials used either no adjuvants (see Table 4) or experimental adjuvants or cytokines (reviewed by Machiels et al. 2002). Although, in early studies, peptides without adjuvant have led to complete remission of metastatic tumors (Marchand et al. 1995), immunization regimens reinforced with adjuvants and cytokines tend to improve the immune response and, sometimes, the clinical outcome. Some adjuvants (Montanide ISA-51, QS-21) induced specific CTLs in a few high-risk resected melanoma patients associated with a 75 percent 5-year survival rate (Slingluff et al. 2001). The combination of different adjuvant strategies may have some benefit, e.g. low-dose IL-12 improved the immune response of IFA-emulsified peptides, but without clinical improvement (Lee et al. 2001). The modified gp100 peptide g209-2M, combined with IFA and high doses of IL-2 (but not without IFA or with granulocyte–macrophage colony-stimulating factor (GM-CSF)), showed a good clinical response (Rosenberg et al. 1999). Besides soluble or lipophilic adjuvants, other

formulations include biodegradable beads targeting peptides to antigen-presenting cells (Frangione-Beebe et al. 2000, 2001). Table 4 shows many examples of adjuvants used in clinical trials, but patient groups and interventions are difficult to compare in a systematic way. In these trials side effects were attributed mostly to the adjuvants used (Goydos et al. 1996), rather than the peptides that normally show no or only mild toxicities.

DENDRITIC CELLS-PRESENTED PEPTIDES

Dendritic cells (DC) are the most potent antigen-presenting cells and they effectively induce CTL responses (reviewed by Banchereau et al. 2000; Guermonprez et al. 2002). They can be obtained from the patient's blood or bone marrow and expanded to large numbers in vitro (reviewed by Mayordomo et al. 1997). Peptides can be loaded on to autologous differentiated DCs, which are returned to the patient to induce tumor-specific T cells (for a review, see Parmiani et al. 2002a, b). The route of administration (e.g. intravenously or subcutaneously) of peptide-loaded DCs seemed to be important, perhaps because of differential homing of the DCs into regional lymph nodes or the spleen (Eggert et al. 1999). Co-administration of cytokines such as IL-12 (Grohmann et al. 1997a, b) or pulsing DCs with mixtures of CD4- and CD8-restricted TCEs improved results. Overall, this very fastidious procedure gave mixed and sometimes very encouraging results (Banchereau et al. 2001; Thurner et al. 1999; Nestle et al. 1998; Murphy et al. 1999b; Kugler et al. 2000). TAA-peptide-loaded DC vaccines seem to generate CTL responses, more often and more efficiently, than peptide with or without IFA, but clinical responses remained low. A notable exception was the anti-idiotype vaccine against follicular B-cell lymphoma which gave, in two of four patients, a complete and, in one, a partial clinical response (Hsu et al. 1996). Follow-up studies with GM-CSF as an adjuvant instead of DCs gave a tumor-specific T-cell response and a complete and durable molecular remission in eight of eleven patients with minimal residual disease (Bendandi et al. 1999).

Alternate delivery of peptide vaccines

To improve the immune response in the cancer patient, multiple CTL epitopes of one or several TAAs combined with or without BCEs and aided by vigorous T-helper epitopes may be required. A number of strategies have been proposed to bring together peptides of different epitopes covalently. The synthesis of constructs consisting of longer or multiple peptides rivals recombinant strategies. Numerous strategies based on recombinant viruses, DNA, or RNA have been developed to deliver oligonucleotides encoding peptides directly into host cells via different invasive or noninvasive routes (reviewed by Arnon and Ben-Yedidia 2003). The use of synthetic TCE vaccines consisting of one or a few peptides is limited by MHC polymorphism. To overcome these limitations peptides have been fused genetically to form polyepitopes (reviewed by Suhrbier 2002). These were either produced as recombinant proteins fusing, e.g. a large number of CTL epitopes of Epstein–Barr virus, or as recombinant viral vectors (modified vaccinia Ankara (MVA)), or DNA in the case of HIV (Hanke and McMichael 2000; Wee et al. 2002) and *Plasmodium falciparum* epitopes (Gilbert et al. 1997; Richie and Saul 2002). Polyepitopes were also used to pulse DCs to immunize against malignant melanoma (Smith et al. 2001) and breast cancer (Scardino et al. 2001). A detailed discussion of these recombinant polyepitope strategies is beyond the scope of this review.

Synthetic TCE peptide vaccines against tumor viruses

Prophylactic immunization against viruses with MHC-restricted peptides is not practical for obvious reasons. However, some viruses are responsible for the induction of human cancers, including hepatocellular carcinoma, cervical carcinoma, and others. With the long tradition of immunization against viruses it was logical that the first peptide immunization against cancer was a viral peptide derived from HBV (Vitiello et al. 1995), although most progress has been reported with peptide immunization against HPV (reviewed by Fausch et al. 2003). Early animal studies showed that peptides derived from HPV protected mice against a HPV protein-expressing tumor both prophylactically and curatively (Feltkamp et al. 1993; Zwaveling et al. 2002). With the identification of HLA-A2-restricted HPV16 and HPV18 peptides, which induced CTLs, clinical trials became possible (Feltkamp et al. 1995). These were further encouraged by observations that class II-restricted memory responses against E6 and E7, the only HPV proteins expressed in cervical carcinoma, were found in patients but not in healthy volunteers (Da Silva et al. 2001). In a clinical trial with advanced cancer patients, two HPV-E7 peptides and a T-helper peptide in Montanide ISA51 induced a T-helper response in four of twelve patients but no E7-specific response (Ressing et al. 2000). In another trial with late-stage tumors, a lipidated E7 peptide induced a specific CTL response but no clinical response (Steller et al. 1998). Ten of sixteen patients with high-grade tumors showed CTL responses and partial or complete remission (Muderspach et al. 2000).

Peptides as cancer vaccines: Concluding remarks

The use of synthetic peptides has resulted in significant advances in the understanding of the immune response

at the epitope level. They have also been shown to induce vigorous CTL responses, which sometimes correlated with partial or even complete tumor regression. In some cases this response was long lasting. However, a number of challenges must be tackled to turn peptides into effective anti-cancer vaccines (Buteau et al. 2002). Heterogeneity and small numbers of patients, immunization regimens that are difficult to compare, and variability in monitoring clinical and immunological outcome are only some of the factors that complicate comparisons between different clinical trials. Clinical trials comparing different parameters are therefore warranted, each with sufficient numbers of patients. Some authors believe that, before any further progress can be made, the understanding of the mechanisms involved in tumor killing needs to improve (Mocellin et al. 2003), e.g. Rosenberg et al. (1998) observed that co-administration of IL-2 with gp100 peptides dramatically enhanced tumor response but reduced peptide-specific CTL response from 10 of 11 to 3 of 19 (see Table 4). This sheds serious doubts on the utility of CTL assays as an objective parameter to monitor tumor cytotoxicity and more appropriate assays may be required for immune monitoring (Machiels et al. 2002). It also seems important to complement in vitro cytotoxicity with a detailed phenotyping of CTLs. More molecular studies are also necessary to understand the mechanism by which the tumor undermines the development of an efficient immune response and escapes effector mechanisms (Marincola et al. 2000).

Mechanisms that lead to epitope-specific T-cell tolerization, instead of T-cell activation, need to be explored (Aichele et al. 1995; Toes et al. 1996a, b). Immunization with certain peptides can lead to tolerance in protocols that induce protection with other peptides (Feltkamp et al. 1993; Mandelboim et al. 1994; Melief and Kast 1994, 1995; Toes et al. 1996b). CTL epitopes have so far been identified for only a few HLA molecules. Although it can be expected that, after proof of the concept, peptides restricted by most MHC molecules would be identified, MHC polymorphism severely limits peptide immunization studies in humans. MHC transgenic mice could help but experimental tumors poorly reflect human tumors. On the other hand, polyepitope vaccines based on (genetically) fused peptides could overcome some of these problems, although some regulatory issues remain unsolved (Suhrbier 2002). With few exceptions clinical response was below 30 percent. Although this seems low, many of the studies were done in patients with advanced stage disease and this response has been obtained with minimal side effects. Peptide immunization is probably more efficient in low-grade tumor patients with an intact immune system. Thus, peptide immunization could be a treatment of choice in the case of minimal residual disease when the immune system has recovered from suppression caused by the tumor and aggressive therapy and when tolerance for side effects is low (Bendandi et al. 1999; Jager et al. 1999). The demonstration of a benefit would, however, take many years of observation. Immunization protocols are far from being optimal and most results need to be confirmed in large prospective clinical trials, to systematically compare different TAAs, epitopes, peptide formulations, adjuvants, and routes.

FINAL COMMENTS: DIFFICULTIES AND MISCONCEPTIONS

The main difficulty in developing a peptide-vaccine based on a BCE is its conformational diversity. Even in the case of a peptide inducing (low) neutralizing antibodies, chemical stabilization of the proper conformation should be considered. In this respect, most peptides are perhaps only lead structures that guide the development of structurally stabilized antigens, rather than being the antigens themselves.

Many of the obstacles to the development of TCE-based peptide vaccines against tumors are inherent to tumor immunology and are not particular to peptide vaccines. In this respect, peptides as tumor vaccines have done very well. With the ease of immunization with peptides and the low levels of side effects, their potential should be more systematically explored in minimal residual disease or in patients in remission with a high risk of relapse.

Peptide vaccines tend to suffer from an inherent contradiction: low cost, biological safety, and chemical and thermal stability are mostly features of prophylactic vaccines, meant to protect healthy vaccinees against pathogens. Such prophylactic vaccines, however, do not normally require an epitope-based approach. In contrast, in therapeutic vaccines, in particular against neoplasms, allergies, and autoimmune disease where epitope-based strategies may be important, features such as low cost, safety, and stability are much less relevant.

To judge the success or failure of experimental peptide vaccines, a comparison with other vaccine strategies is warranted. No peptide vaccine is licensed so far, but also only a few recombinant vaccines had reached the (American) market by 2001 (HBV, cholera, pertussis) without being subsequently withdrawn (*Borrelia*, rotavirus). The US Institute of Medicine estimated that, of the more than 30 new vaccines that will reach the market by 2010, about 25 percent are obtained by recombinant technology, but none will be peptide based (Landry 2002).

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Live vaccine carriers

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Since the first recognized vaccinations performed by Edward Jenner the use of live attenuated microorganisms as vaccine components has been a practical option. Live vaccines often offer the advantage over nonliving vaccines of enhanced immunogenicity. In the early days of vaccine development when investigators used essentially empirical approaches, live vaccines were often a favored practical route because there is no requirement for the identification of component protective antigens. Also, dead whole cells were often either reactogenic or nonprotective. Although live approaches were often useful for inducing immunity, old-style live vaccines ran the risk of reversion to virulence to the extent that, over the years, there were many accidents with their use. Over the past few decades our ability to manipulate microorganisms using genetic approaches has opened up the option of rational attenuation. This is the so-called designer approach to live vaccine development. Rational attenuation involves the identification and stable inactivation of genes in pathogens that normally contribute to pathogenesis in the host. By knocking out genes that are essential for virulence, but not for life, it is possible selectively to disable pathogens in terms of their ability to cause disease. Theoretically, combinations of attenuating mutations can be employed to optimize both immunogenicity and safety simultaneously. In practice this can be a difficult goal to achieve, particularly if optimization is first attempted in a model rather than the target host. An alternative to rational attenuation is the complete characterization (total sequence) of the genetic content of well-characterized attenuated vaccine strains. Examples here would include various attenuated viruses such as those derived from polio virus and vaccinia or even bacterial vaccine strains. This sequence information can be used as a platform to quality control vaccine lots or provide a basis for further genetic modifications of the vaccine strain.

It is clear from the above that we are now entering a new era of rational live vaccine development. The aim is to develop live vaccines that are safe (nonreverting and nonreactogenic) in both normal and compromised hosts and which are protective following the administration of single vaccine doses. A further attraction of live vaccination is that with some attenuated microorganisms there is the option of immunizing using alternative (intranasal, oral, rectal, etc.) routes of immunization that could avoid the use of needles while offering the potential of inducing local alongside systemic immune responses (see New approaches to vaccine delivery).

Our enhanced ability to manipulate microorganisms genetically has opened up further attractive options for vaccine development. Using recombinant DNA approaches, it is now possible to introduce the genes for heterologous protective vaccine antigens into attenuated microorganisms where the foreign antigens can potentially be expressed as components of the normal proteosome of the host vector. The challenge here is to express the foreign antigen in a stable manner that does not compromise the inherent immunogenicity of the vaccine carrier. Taking this approach it is enticing to imagine the

development of live vaccine strains which, in a single dose, can be used to immunize simultaneously against more than one pathogen. It might be feasible, for example, to immunize in a single vaccine shot against bacterial and viral infectious agents. In fact this goal has now been achieved on many occasions in model hosts such as the mouse (Chatfield et al. 1989), but this technology is proving more difficult to translate into practical immunization formulations for use in humans or target veterinary species (Mastroeni et al. 2001). Part of the problem is the major differences in the way that the immune systems of different hosts respond to vaccines. In addition, individual pathogen strains are often adapted to favor growth in a particular host. Thus, a formulation can be highly immunogenic in one host but poorly immunogenic even in a closely related species. This means that clinical studies can often be disappointing and we need to learn more about how to transfer technology from one species to another. In other words we need better immune correlates. Nevertheless, despite some early problems there is still a great deal of interest and effort in this area, which is the subject of this chapter.

LIVE VACCINES ARE INHERENTLY IMMUNOGENIC

By learning more about the way that the immune system recognizes infectious agents and discriminates between antigens from dangerous sources, such as pathogens from self, it should be possible to understand why live vaccines are immunogenic. Pathogens harbor or synthesize particular degenerate structures that are distinct to themselves and often not natural components of higher eukaryotic hosts. Examples of such molecules include lipopolysaccharide (LPS), flagellins, and double-stranded RNA (dsRNA). Mammalian cells have evolved a series of ligands and receptors that target these pathogen-specific structures. Perhaps the best characterized of these are the Toll receptors which recognize pathogen-associated molecular patterns (PAMP). Binding of the Toll pathway by pathogen components can lead to the activation of the innate immune system and, ultimately, to the amplification of acquired immunity. In fact we now know that many adjuvants act by activating the innate immune system via these types of pathways and that live vectors have inherent immunogenicity because they incorporate PAMPs.

As we move towards pure nonliving vaccines there is a tendency to remove PAMPs from vaccine formulations and consequently to reduce inherent immunogenicity. Adjuvants then have to be added back. We can take advantage of PAMPs for improving vaccine formulations. Even bacterial DNA has a signature that is distinct from eukaryotic DNA. Consequently, bacterial DNA is targeted by Toll-like receptor 9 (TLR9) in both mice (Hemmi et al. 2000) and humans (Takeshita et al. 2001). The technique of so-called DNA immunization (see Naked DNA vaccines) has now been extensively studied since the first demonstration that intramuscular injection of bacterially derived plasmid DNA could result in the expression of an encoded protein (Wolff et al. 1990) capable of eliciting humoral immune responses (Tang et al. 1992), as well as priming antigen-specific cytotoxic T lymphocytes (CTL) (Ulmer et al. 1993). DNA vaccines are effective partly because of the immunostimulatory properties of unmethylated cytidine phosphate–guanosine (CpG) dinucleotide sequences that can upregulate expression of interleukins IL-12, IL-6, and interferon γ (IFN-γ) by macrophages and B cells as well as activating dendritic cells (DC) (Jakob et al. 1998). Immunostimulatory CpG motifs are either absent from eukaryotic DNA or modified. Simply cloning an active CpG site in the correct context into an existing DNA vaccine construct can markedly enhance its efficacy (Sato et al. 1996).

Clearly the combination of PAMPs associated with live vectors enhances immunogenicity but live vaccines are normally much more immunogenic than the equivalent dead microbes. Hence, the fact that live organisms colonize the host using the steps normally associated with infection also clearly enhances their immunogenic potential. Live microorganisms will target and colonize host cells, including immune cells, and antigens will be processed along natural intracellular pathways. It is extremely difficult to reproduce such patterns of immunostimulation and antigen processing using dead cell vaccines. Adjuvants are substances incorporated into vaccine formulations to accelerate, enhance, or prolong the specific immune response. Mechanisms of adjuvant action can include increasing the half-life of vaccine antigens in vivo, inducing the production of immunomodulatory cytokines or other immunomodulatory molecules, or improving vaccine delivery to antigen-presenting cells (APC) and enhancing antigen processing or presentation (Vogel 2000). In a sense, live immunization has the potential to meet all of these needs although microorganisms that target APCs and modify their activity could be particularly attractive.

EXAMPLES OF VIRAL AND BACTERIAL VECTORS

Various attenuated vaccine strains have been modified to present heterologous antigens to the immune system. These combined vaccines can be based on attenuated strains, which in their own right can be used as vaccines against a particular disease, e.g. *Salmonella enterica* serovar Typhi (*S. typhi*) strains that have been developed as human oral typhoid vaccines and have been exploited as carriers with the aim of extending the range of protection (Tacket et al. 2000b). In contrast vaccinia viruses, which are the basis of smallpox vaccines, have been exploited with more focus on their simple use as

vectors, although the emphasis may change in the future now that smallpox vaccines are back on the agenda (Fauci 2002). Many different pathogens have been engineered as vectors for expression of polypeptides from other pathogens. A list of some of these is provided in Table 1 which is not comprehensive but includes some selected examples. The goal of the use of these vectors is to present foreign antigen to the immune system in the context of live vector and thereby develop broad immunity (humoral and cellular) to the corresponding pathogen from which the antigen is derived. The recombinant polypeptide is expressed and either transported to the host cell surface to stimulate antibody production or is broken down into peptides that are transported to the cell surface in the context of major histocompatibility complex (MHC) molecules, where they elicit cellular responses such as CTL responses. In other words, immunity to the heterologous antigen is enhanced through co-delivery with the vector. Clearly, the type of immunity induced will be greatly influenced by the choice of vector for delivery. Also, different antigens may be more suitable for delivery by one vector compared with another, e.g. many viral proteins cannot be easily expressed in a conformationally correct form in bacteria so viral vector delivery might be the best option in these cases. Alternately, *Salmonella* may be more attractive for delivery of an antigen from another enteric bacterium, particularly if mucosal targeting is desired. In this chapter, examples of representative vectored delivery systems are described but there is no attempt to cover all vectors.

Attenuated viral vectors

Attenuated viruses can be modified for use as vaccine carriers by inserting sequences encoding target antigens into their genome. A number of different viruses have been engineered as vectors for expression of 'foreign'

Table 1 *Examples of host/vector system*

Vector	Antigen	Reference
BCG	*Borrelia burgdorferi* OspA	Langermann et al. (1994a, 1994b)
	Toxoplasma gondii GRA1	Supply et al. (1999)
	Plasmodium yoelii (MSP-1)	Bruna-Romero et al. (2000)
	SIV Gag, Pol, Env, and Nef	Leung et al. (2000)
	Measles virus N protein	Zhu et al. (1997)
	Schistosoma mansoni Sm28GST	Kremer et al. (1996)
	HIV-1 V3	Kawahara et al. (2002)
	Schistosoma haematobium Sh28GST	Kremer et al. (1998)
	Pertussis toxin subunit S1	Abomoelak et al. (1999)
	Leishmania surface protease gp63	Connell et al. (1993)
Salmonella	*Helicobacter pylori* urease	Londono-Arcila et al. (2002)
	L1 papillomavirus	Revaz et al. (2001)
	Hepatitis B virus core	Schodel et al. (1996)
	Toxin hybrid	Barry et al. (1996)
	Plasmodium falciparum (NANP)	Ruiz-Perez et al. (2002)
	Pneumococcal PspA	Kang et al. (2002)
	Lassa virus nucleocapsid protein	Djavani et al. (2001)
	Tuberculosis ESAT-6	Mollenkopf et al. (2001)
	Leishmania mexicana gp63	Gonzalez et al. (1998)
	Pneumococcal surface protein A	Nayak et al. (1998)
Shigella	(HIV-1) gp120 DNA	Shata and Hone (2001)
	Measles virus DNA	Fennelly et al. (1999)
	HIV DNA	Vecino et al. (2002)
Yersinia	*Brucella abortus* P39 DNA	Al-Mariri et al. (2002)
Bordetella pertusis	*Schistosoma mansoni* Sm28GST	Mielcarek et al. (1998)
	Neiserria meningitidis TbpB	Coppens et al. (2001)
MVA/Vaccinia	SHIV-89.6P	Barouch et al. (2001)
	Herpes simplex virus-2 glycoprotein D	Meseda et al. (2002)
	Leishmania infantum P36/LACK	Gonzalo et al. (2001)
	Plasmodium yoelii (AdPyCS)	Bruna-Romero et al. (2000)
	Mycobacterium tuberculosis Ag85	Malin et al. (2000)
Adenovirus	*Mycobacterium tuberculosis* Mtb39	Lewinsohn et al. (2002)
	Foot-and-mouth disease virus	Grubman and Mason (2002)

For abbreviations see the text.

polypeptides from other pathogens. The goal is to present foreign antigen to the immune system in the context of the live vector, so that the immune system responds to the antigen as a live immunogen and thereby develop broader immunity (humoral and cellular) to the corresponding pathogen. One advantage of this approach is that it is possible to avoid administering viruses such as HIV which could be considered unsafe even in an attenuated form. Recombinant vaccinia and related poxviruses have attracted particular attention as possible vectors for immunization against both infectious disease agents and cancers (Wang et al. 1995). Other viral vectors that have received considerable attention are the adenoviruses, which have also been used extensively for delivering genes as gene therapy vectors (Chen et al. 1996). The advantages of viral vectors include the production of the inevitable cellular damage that occurs during viral infection. Cellular damage could generate danger signals that could be a basis for activating the innate immune system. Viruses also have the potential to directly infect APCs with subsequent processing of viral antigens via the class I pathway.

However, there are obviously potential disadvantages with the use of viruses, particularly with the use of vaccinia vectors where the presence of pre-existing immunity can dampen responses to the heterologous antigen, compromising homologous boosting approaches. In addition, because of our limited repertoire of antiviral agents, there are additional risks from potential enhanced vaccine-associated infection in the immunocompromised.

The prototype viral vaccine vector is vaccinia virus, an animal-derived poxvirus that was used in the immunization program that successfully eradicated smallpox. Poxviruses are among the largest viruses and are almost visible to the naked eye using conventional microscopy. They harbor relatively large genomes and encode a variety of immunomodulatory proteins that can regulate the immune response during infection and potentially immunization. Different vaccinia derivatives harbor distinct repertoires of immunomodulatory genes and differ in their potential to replicate in different mammalian hosts. Hence, it is possible to have both replication-proficient and replication-defective forms of poxvirus vectors. Fowlpoxvirus and canarypox viruses are also being developed as live vectors that can infect human cells, but not produce infectious viral progeny (Moss 1992), thus providing a mechanism for natural attenuation (Fries et al. 1996). This clearly has implications for both immunogenicity and safety.

Many different recombinant polypeptides have been expressed in vaccinia virus, and immunization of animals with such vectors can protect against the pathogen encoding the recombinant polypeptide (Moss 1992; see Table 1). This approach has become routine as an experimental system. Some of the recombinant vaccinia viruses, e.g. those expressing influenza virus hemagglutinin and type I herpesvirus glycoprotein D have been shown to be protective in mice against subsequent challenge with the corresponding virulent heterologous viruses (Bennink et al. 1984). Recombinant vaccinia virus immunization has also now been extended to several veterinary target species. However, live recombinant vectors that can replicate in vivo may not be considered sufficiently safe for widespread use as prophylactic vaccines in humans.

To increase the safety of the poxviruses as vaccine vectors, the replication-incompetent strains have been exploited. Modified vaccinia virus Ankara (MVA) is replication impaired in primary human cells (Blanchard et al. 1998) and has been proposed as an alternate safe vaccine for use in smallpox eradication programs. The genome of MVA has also been sequenced and harbors six large deletions resulting in a loss of 31 kb of the genome. Lost genes include those encoding receptors for IFN-γ, tumor necrosis factor α (TNFα), type I IFN, and CC chemokines (Blanchard et al. 1998). Although delivery by MVA can be used to enhanced immunogenicity often this approach alone is not sufficient to induce protection. The potency of MVA/vaccinia immunization can be further enhanced by adopting 'prime-boost' strategies. A particularly fruitful approach has been to use an initial DNA vaccine as a priming agent followed by a recombinant viral vector such as modified vaccinia as a boost (Ramshaw and Ramsay 2000). The ability to induce $CD8^+$ CTL responses can be greatly increased by combining DNA vaccines with attenuated live viral vectors encoding the same or similar antigens (Sutter and Moss 1992; Ramshaw and Ramsay 2000). This prime-boost strategy has been further enhanced by co-administering cytokines such as IL-12, IFN-γ, or granulocyte–macrophage colony-stimulating factor (GM-CSF) (Chow et al. 1998). In the murine malaria model, immunization with DNA or MVA vaccines alone did not induce complete protection against virulent challenge, whereas a heterologous prime-boost strategy of DNA priming followed by MVA boosting was highly effective at inducing $CD8^+$ T cells and conferred complete protection (Schneider et al. 1998). Similar success in enhancing immunogenicity has been obtained with immunization exploiting viral antigens (Degano et al. 1999) and strongly reactive antigen-specific $CD8^+$ CTL responses have been demonstrated in primate models of HIV infection (Allen et al. 2000). Alternate prime-boost strategies, involving initial immunization with DNA or viruses and boosting with the relevant heterologous protein, have generally been poor inducers of CTLs, with specific humoral responses being stronger (Excler and Plotkin 1997).

Clinical trials using the MVA-based approach are under way for several infectious diseases including malaria (Seder and Hill 2000) and there is much interest in determining whether prime-boost methods can

enhance the cellular responses to tumor-associated antigens. Animal studies have already shown that heterologous boosting strategies can be useful in increasing the efficacy of recombinant DNA anti-cancer vaccines (Irvine et al. 1997) and early trials in humans appear promising (Bonnet et al. 2000; Oertli et al. 2002).

Many other viruses have been used as carriers of heterologous antigens in immunization experiments. One of the most spectacular projects has been the exploitation of the components of the yellow fever flavivirus genome as a platform for vaccines against several diseases including dengue (Guirakhoo et al. 2001). A yellow fever–dengue live chimeric vaccine has been taking into the clinic with excellent safety and immunogenicity profiles (Monath et al. 2002). This vaccine has the potential to be the first licensed recombinant viral carrier for humans. The use of some viruses such as polio virus as carriers is compromised to some extent by the relatively small capacity of the genomes to incorporate the genes for heterologous antigens but nevertheless some interesting progress has been made (Mandle et al. 2001). Adenoviruses have been extensively exploited as vectors for gene therapy and have also received attention as live vaccine components, particularly in prime-boost immunization (Gilbert et al. 2002). Adenoviruses have the potential to be used as mucosal targeting agents increasing their potential. Other viruses such as herpesviruses (Varghese and Rabkin 2002) have also been tested as experimental live carriers, resulting partly from their ability to infect nondividing cells, potential high expression levels and broad host-range.

Live attenuated bacteria as vaccine vectors

Various bacterial species have been studied for their ability to deliver heterologous antigens, including *Salmonella*, *Shigella* spp., *Escherichia coli*, and *Listeria* spp. (Shata et al. 2000). Bacteria as live vectors have the potential advantage over viruses in that their genomes are theoretically able to harbor many foreign genes, in contrast to viruses, which offer limited capacity to encapsulate foreign DNA. Recent advances in our understanding of the molecular pathogenesis of bacterial infections, particularly the roles of virulence genes and pathogenicity islands (Jones and Falkow 1996; Groisman and Ochman 1996) have encouraged the rational development of attenuated strains for use as vaccines (Levine et al. 1996). The ideal bacterial vaccine vector should retain enough infectivity to invade and target APCs, while eliciting an effective immune response with a minimum of reactogenicity (Raupach and Kaufmann 2001). The most common bacteria used as live vectors are members of the Enterobacteriaceae and the mycobacteria, particularly BCG. *Salmonella* Typhi has been the focus of the many efforts in terms of live vaccine development and clinical testing (Tacket et al. 2000a). *Shigella flexneri* has also been engineered to deliver different heterologous antigens. Interestingly, attenuated *S. flexneri* was used as a prototype strain to delivery antigens encoded on recombinant plasmids capable of driving antigen expression from eukaryotic promoters (as a combined live vector/nucleic acid vaccine) (Sizemore et al. 1995). *S. flexneri* are known to readily escape from intracellular vacuoles into the cell cytoplasm in vivo, possibly facilitating the transfer of DNA from the bacteria to the eukaryotic nucleus or transcription apparatus.

Salmonella as live vectors

Attenuated mutants of *S. enterica* have been extensively exploited for the delivery of heterologous antigens for immunization against bacteria, parasites, and viruses (Chatfield et al. 1989). Also, in recent years, these bacteria have been increasingly used in experimental models of anticancer immunization (Chabalgoity et al. 2002). In contrast to killed bacterial preparations, live attenuated *S. enterica* can induce cellular immune responses to bacterial and heterologous recombinant antigens (Hormaeche et al. 1995). *Salmonella* Typhi also offers the attraction of being deliverable via the mucosal route. After oral ingestion, attenuated *Salmonella* Typhi has the potential to invade or interact with Peyer's patch-associated M cells, enterocytes, macrophages, and DCs. Hence, the combination of mucosal delivery and immune targeting has encouraged research in this area.

Salmonella Typhi is a serovar of *S. enterica*. As *Salmonella* Typhi is human restricted much of the work associated with salmonella vaccine development has focused on *S. enterica* serovar Typhimurium (*Salmonella* Typhimurium) which is adapted to the mouse and consequently is much more convenient to study. By selecting the combination of attenuating mutations carefully, it is possible to design *S. enterica* derivatives with differing potential to replicate to some extent intracellularly or to disseminate to reticuloendothlial tissues in different hosts (Sirard et al. 1999). Thus, not only can the immunogenicity of the strain be modified, but the degree of dissemination in normal or immunocompromised individuals can theoretically be controlled. This has even been shown to be the case in humans where *Salmonella* Typhi *aro* mutants were able to disseminate, whereas similar *Salmonella* Typhi *aro*, *htrA* mutants were not detected in blood of human volunteers (Tacket et al. 1997). Many different attenuated derivatives of *S. enterica* have been constructed and characterized. Among the first defined mutant strains were the *aroA* auxotrophic derivatives of *Salmonella* Typhimurium which are defective in aromatic amino acid biosynthesis and dependent for growth on supplementation of aromatic compounds into their culture medium (Hoiseth and Stocker 1981). *Salmonella* Typhimurium *aroA* (and other single or

combination *aro* mutants) retain immunogenicity and favor stimulation of a Th1 response in mice (Dunstan et al. 1998). After oral administration to mice, these salmonella derivatives invade APCs in the gut mucosa and are retained within endocytic vacuoles (Figure 1).

The subsequent lack of their essential metabolic supplements induces many of the administered bacteria to die within 24–48 h, releasing their contents into the APCs. Compared with extracellular bacteria, antigens carried by *Salmonella* have the enhanced potential to be presented in association with MHC class I molecules by infected macrophages and DCs, clearly a desirable mechanism if protective $CD8^+$ T-cell responses are sought (Wick and Ljunggren 1999). However, it should be noted that repeated experiments have shown that *Salmonella* Typhimurium derivatives are less potent inducers of CTLs than many other adjuvants and delivery systems (Allsop et al. 1996). Nevertheless, the subsequent location of the recombinant antigens within the APCs may influence subsequent immunity. Interestingly some mutations, such as those in the *sifA* gene (Beuzon et al. 2000), increase the level of release of *Salmonella* Typhimurium from the vacuole in APCs and these mutations may have some potential for enhancing or modulating *Salmonella* Typhimurium-associated immune responses (Figure 1).

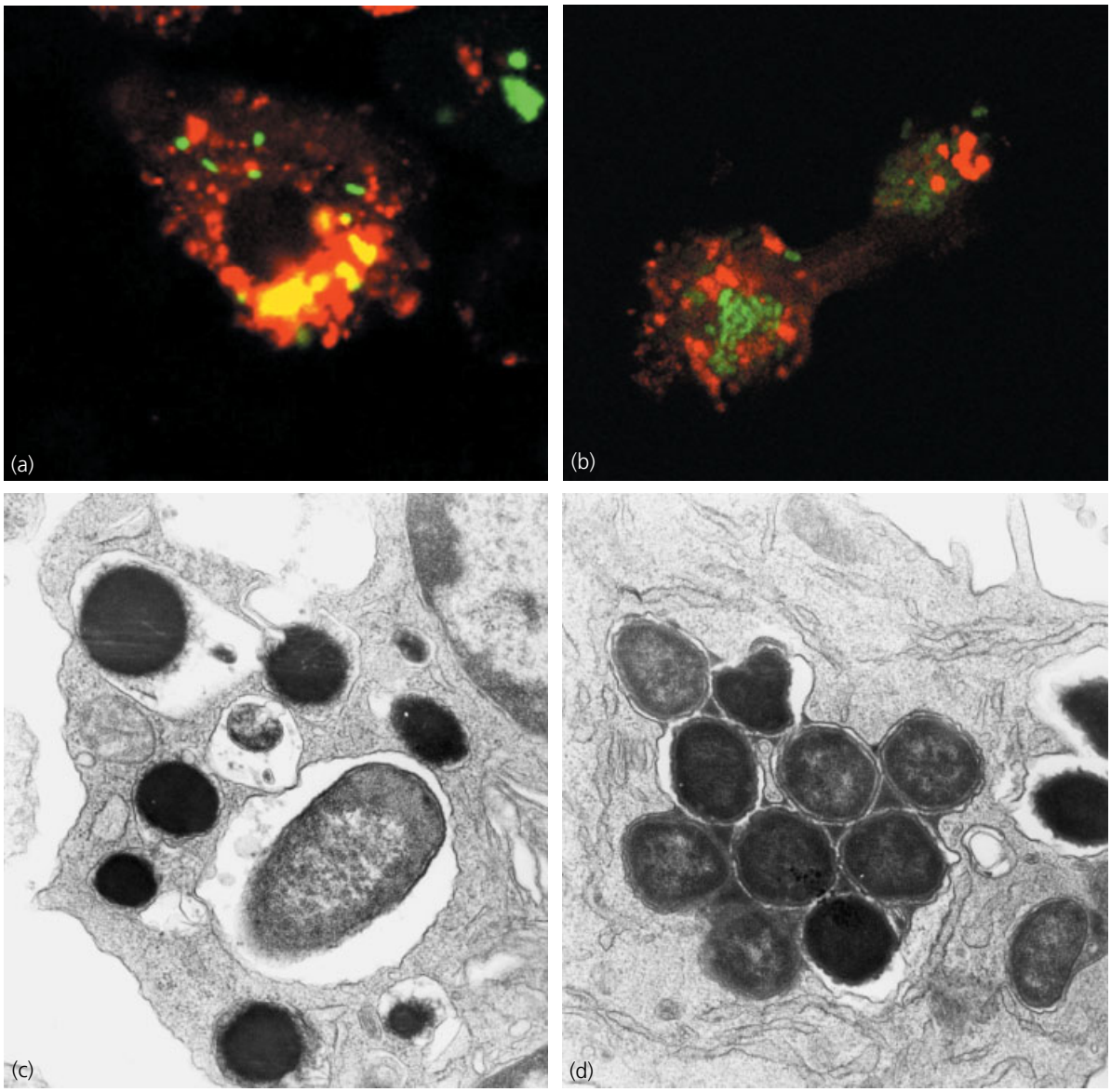

Figure 1 *Interaction of green fluorescent protein-positive* Salmonella *with dendritic cells cultured from the bone marrow of a mouse. Note that normal bacteria* **(a)** *interact with MHC molecules (yellow color) whereas sifA mutants* **(b)** *which escape the vacuole do so less well. The equivalent electron micrographs showing sifA mutants escaping the vacuole are shown in* **(c)** *(wild-type* Salmonella*) and* **(d)** *(sifA mutant* Salmonella*).*

The location or distribution of the expressed heterologous antigen within the *Salmonella* Typhimurium carrier cell may influence the subsequent immune response to the antigen. Although antigens expressed in the bacterial cytoplasm can be used to immunize and protect effectively, surface expression or secretion has the potential to enhance immunogenicity to some antigens (Strugnell et al. 1992). Antigen location within the bacterial cell may be critical for the induction of CTL responses which are often poor and efforts have been made to target antigen to or beyond the surface of the bacterial cell (Hess et al. 1996). The subsequent route for processing and presentation of MHC class I-associated salmonella antigens may also differ between APCs (Yrlid et al. 2000). Furthermore, because *Salmonella* Typhimurium invasion of macrophages can induce apoptosis (Monack et al. 1996), membrane 'blebs' containing bacterial antigens (and potentially plasmid DNA) may be taken up and processed by DCs via the MHC-I and -II pathways, resulting in activation of $CD4^+$ and $CD8^+$ T cells (Yrlid and Wick 2000).

The genes that encode heterologous antigens are often introduced into salmonella vaccine strains on plasmids. Plasmids that direct the expression of potentially toxic foreign proteins under the control of unregulated promoters can be rapidly lost from attenuated bacterial vector strains in vivo (Maskell et al. 1987). This gene loss can significantly reduce the level of the immune response to the heterologous antigen. To maintain stable antigen expression and thus enhance vaccine immunogenicity, growth-phase inducible promoters may be required (Dunstan et al. 1999; Marshall et al. 2000). Alternately, the stability of heterologous antigen expression may be enhanced by placing genes encoding heterologous antigens on the bacterial chromosome (Strugnell et al. 1990).

The intracellular life cycle of *Salmonella* also appears well suited to delivering expression plasmids as DNA vaccines. Indeed, many studies of bacterial to mammalian gene transfer have been carried out using *Salmonella* Typhimurium (Weiss and Chakraborty 2001). *Salmonella*-mediated mucosal DNA immunization was effectively demonstrated in mice using *Salmonella* Typhimurium to elicit the expression of ActA and listeriolysin from eukaryotic promoters, encoded on plasmids delivered to eukaryotic cells via these bacterial vectors. The development of protective CTL and antibody responses against the listeria-derived antigens was demonstrated after immunization with live *Salmonella*Typhimurium vectors (Darji et al. 1997). *Salmonella* Typhimurium was also successfully exploited as a vector to induce protection against a murine fibrosarcoma expressing β-galactosidase as a model tumor antigen using both eukaryotic and prokaryotic promoters to drive antigen expression (Paglia et al. 1998; Medina et al. 1999). Interestingly, in a mouse model of melanoma, attenuated *Salmonella* Typhimurium was used to deliver a DNA vaccine construct harboring the murine ubiquitin gene fused to minigenes encoding peptide epitopes gp100(25–33) and TRP-2(181–188). Immunized mice displayed protective anti-tumor immunity (Xiang et al. 2000). Similarly, transgenic mice expressing the tumor antigen carcinoembryonic antigen (CEA) had their peripheral tolerance broken by oral DNA immunization, with *Salmonella* Typhimurium, generating protection against CEA-expressing lung carcinoma cells (Niethammer et al. 2001).

Shigella

A great deal of effort has been made to generate different shigella derivatives for use as live oral vaccines against *Shigella*-associated dysentery. Despite many promising starts many candidate vaccines have floundered because of reactogenicity or poor immunogenicity in volunteers. Consequently, there are no licensed live shigella vaccines. *Shigella* has long been considered as a potential carrier of heterologous antigens. Early nonrecombinant experiments used hybrid genetic crosses between *Shigella* and the genomes of other enteric bacteria as possible extended-spectrum diarrhea vaccines. However, this approach ultimately proved unfruitful. As with *Salmonella*, *Shigella* spp. are also facultative intracellular bacteria but in comparison they have an enhanced ability to escape from the vacuole, become motile within the cytoplasm, and spread directly to adjacent cells (Sansonetti et al. 1986). A number of attenuated shigella mutants are available as experimental vaccines and these have been exploited (Karnell et al. 1995) to carry heterologous antigens (Noriega et al. 1996). An auxotrophic shigella mutant, dependent on supplementation with diaminopimelate (*dap*) for cell wall synthesis, was used for the first demonstration of functional DNA transfer from bacteria to mammalian cells (Sizemore et al. 1995). Since then, several studies have shown the induction of strong $CD8^+$ immune responses against viral antigens delivered as DNA vaccines by *Shigella* (Fennelly et al. 1999; Shata and Hone 2001). In a murine model of DNA immunization against HIV antigens, a *dap*-dependent shigella vector stimulated higher levels of IFN-γ-secreting $CD8^+$ cells compared with either *Salmonella* Typhimurium or *Salmonella* Typhi Ty21a (Vecino et al. 2002).

BCG

BCG is an attenuated live bacteria derived from a virulent *Mycobacterium bovis* by extensive laboratory propagation and is widely used for human and animal immunization. *Mycobacterium* spp. have the capacity to establish persistent infections and in vivo recombinant BCG may provide a continuous and prolonged stimulation of the cellular immune system. Both cellular and humoral immune responses against a number of foreign antigens delivered by BCG have been observed in mice and other species including primates such as the rhesus

monkey (Leung et al. 2000). Some of the BCG-based heterologous vaccines have been demonstrated to elicit protection against the corresponding pathogen. BCG expressing the outer surface protein A (OspA) from *Borrelia bugdorferi* induced a long-term protective response after intranasal immunization. Highly sustained levels of anti-OspA and BCG-specific secretory IgA and serum IgG responses were observed (Langermann et al. 1994a, b). As BCG vaccine can be given at birth, without interfering with maternal neutralizing antibodies, the possibility of administering recombinant antigens against childhood diseases such as measles has also been explored in rhesus monkeys. Intranasal administration of recombinant BCG producing the measles virus N nucleoprotein provided protection against subsequent challenge with measles virus. Even though immunization did not prevent systemic infection, immunized monkeys showed a significant reduction of lung inflammation and reduced virus titers in lymph nodes. Nucleoprotein specific T-cell proliferative responses to the measles virus were detected in the absence of anti-N antibody response, suggesting T-cell prevention of virus-induced lung pathology (Zhu et al. 1997). Recombinant BCG vectors can induce humoral and cellular immune response to the major proteins of simian immunodeficiency virus (SIV) polypeptides Gag, Pol, Env, and Nef in rhesus monkeys. A single simultaneous inoculation of all four recombinants elicited SIV-specific IgA and IgG antibodies, as well as cellular immune responses, including CTL and helper T-cell proliferation (Leung et al. 2000). Live BCG secreting a carboxyl terminus of MSP-1 from *Plasmodium yoelii* induced more efficient protective immunity against plasmodium challenge, inducing MSP-1-specific antibodies and IFN-γ (Bruna-Romero et al. 2000).

Other bacterial vectors

Vaccines based on *Escherichia coli* vectors are at a relatively early stage of development. Investigators have concentrated on trying to maximize the targeting of antigens to macrophages and DCs. One approach has been to link heterologous protein expression to the α-haemolysin (HlyA) type I secretory system of uropathogenic *E. coli* to release antigens into the phagosome or cytosol of infected cells (Gentschev et al. 2002). An alternate method of increasing cytosolic antigen delivery (and therefore MHC-I presentation of antigen) is to enable *E. coli* itself to escape from the phagosome. This was demonstrated by transforming an attenuated auxotrophic *E. coli* mutant with a plasmid encoding the *hly* gene from *Listeria monocytogenes*, thereby producing the pore-forming listeriolysin and releasing bacteria and their carried antigens into the cytosol (Higgins et al. 1999). One potential problem with the use of *E. coli* for delivery is that these bacteria normally reside in the lumen of the intestine and consequently may be inherently less immunogenic than the invasive enteric bacteria. DNA delivery using modified *E. coli* strains has been achieved by adapting *E. coli* to express the *invasin* gene from *Yersinia pseudotuberculosis* to enhance the invasion rate of nonphagocytic cells and enhance vacuole escape through the hemolysin (Grillot-Courvalin et al. 1998). The bacterium *L. monocytogenes* can also efficiently escape from intracellular vacuoles, replicate in the cytosol, and spread directly to adjacent cells, albeit at a lower efficiency than *Shigella* (Tilney and Portnoy 1989; Chastellier and Berche 1994). *Listeria* has a powerful ability to stimulate MHC-I-restricted $CD8^+$ cytotoxic immunity to itself and, potentially, carried antigens (Schafer et al. 1992). A number of attenuated listeria mutants have been adapted for heterologous antigen delivery (Dietrich et al. 1998; Thompson et al. 1998). An attenuated listeria mutant was used to induce immune responses against influenza nucleoprotein serving as a model antigen in tumor cells, and protection was induced against challenge with tumor cells expressing this antigen (Pan et al. 1995). The anti-tumor response involved the stimulation of multiple effector pathways, including $CD4^+$ and $CD8^+$ T cells, DCs, NK cells, and several key cytokines including IL-2, IL-4, IL-12, IFN-γ, and TNF-α (Weiskirch et al. 2001). Attenuated listeria mutants have also been used in several other murine tumor models including immunization against a β-galactosidase-expressing fibrosarcoma (Paglia et al. 1997), E7 protein from the cervical cancer-associated human papillomavirus 16 (HPV16), and a lymphocytic choriomeningitis virus (LCMV) nucleoprotein-expressing glioma (Liau et al. 2002).

ENHANCED TARGETING OF THE IMMUNE SYSTEM WITH LIVE VECTORS

The mechanisms by which the immune system protects against disease are diverse and include the induction of neutralizing antibodies and the generation of T-cell responses, including T-helper cells and CTLs. Antibodies provide protection against some bacteria by recruiting bactericidal mechanisms and neutralizing bacterial toxins. Antibodies also play a pivotal role in protection against some viral diseases, such as polio, by virus neutralization mechanisms and preventing viruses from infecting cells. T cells contribute to resistance to bacterial and viral diseases by producing cytokines and inflammatory molecules, which can stimulate antibody, macrophage activation, and CTL responses. Several strategies have been developed specifically to enhance CTL immune responses and improve antigen presentation on MHC-I molecules. Viral and bacterial vectors that can actively invade cytoplasm of host cells have been introduced into APCs for targeted delivery to the immune system. Live vectors that have been used for this purpose include vaccinia, canarypox virus, adenoviruses, *L. monocytogenes*, *Salmonella*, and BCG.

Based on their biology and ability to present antigens, DCs have been described as 'nature's adjuvant' (Banchereau and Steinman 1998) and this is one of the key reasons why some recent therapeutic strategies have incorporated DCs as an effective way to target carrier vectors to immune sites (Banchereau et al. 2001). While in an immature, sentinel state, DCs effectively take up antigens, and subsequently process them through both the class I and II MHC pathways, a step required for the efficient activation of $CD8^+$ CTL and $CD4^+$ T-helper cells. After exposure to certain infectious agents, inflammatory cytokines, or activation of their CD40 receptors, DCs are mobilized from the site of antigen uptake to the peripheral lymphoid organs, where encounter with antigen-specific T cells can take place. During this migration, DCs mature into cells that are specialized in presenting high numbers of peptide/MHC complexes in a rich co-stimulatory context (Banchereau et al. 2000), necessary for activating T-cell responses. The level of maturation of the DCs may be of critical importance in the nature of immune response to DC-delivered antigen. Recent studies suggested that, in some cases, antigen-loaded mature DCs induce a protective (Th1) immune response, whereas antigen-loaded immature DCs may 'silence' the immune response via expansion of IL-10 producing T cells (Jonuleit et al. 2000; Dhodapkar et al. 2001). The expression pattern of different chemokines and chemokine receptors involved in homing dendritic cells to the lymphoid organs also varies throughout the dendritic cell maturation process (Sallusto et al. 1999). DCs have also been shown efficiently to take up and crosspresent antigens from apoptotic or necrotic cells in their vicinity (Albert et al. 1998). This hallmark has being used as a means to target antigens directly to DCs in vivo. Fas-mediated apoptotic cell death can lead to more efficient delivery of HIV-1 antigens to professional APCs, enhancing the priming of antigen-specific CTLs (Chattergoon et al. 2000).

As DCs are critical for the initiation of a cellular immune response, they have become a focus for many researchers to examine the possibility of inducing protective immunity against intracellular pathogens. DCs can efficiently internalize and process live intact bacteria for presentation by both MHC-I and -II molecules (Yrlid and Wick 2000). The bacterial carriers may function as a natural adjuvant, because they can induce the release of proinflammatory mediators such as TNFα, IFN-γ, and IL-12 and enhance early innate immunity, which creates an inflammatory context that probably induces dendritic maturation and antigen-presenting function (Banchereau and Steinman 1998). Bone marrow-derived DCs pulsed ex vivo with BCG and administered intratracheally can generate activated T cells and IFN-γ-secreting cells in the draining lymph nodes, leading to significant protection against aerosol *M. tuberculosis* infection (Demangel et al. 1999) in mice. Likewise, DCs pulsed ex vivo with killed *Chlamydia* and administered intravenously to mice-primed $CD4^+$ T cells provided protection against genital chlamydial infection equivalent to that obtained after immunization with live organisms. Besides intracellular pathogens, DCs have been successfully used as a vaccine against noninvasive, extracellular bacteria. DCs pulsed ex vivo with *Pseudomonas aeruginosa* and administered intravenously to mice induced a $CD4^+$ T-cell proliferative response and prolonged survival after a lethal pulmonary challenge with *P. aeruginosa* (Worgall et al. 2001). Homologous or even heterologous DCs pulsed with heat-killed *Bordetella pertussis* administered intravenously or intranasally to mice induced protective immune responses associated with strong *B. pertussis*-specific IgG and IgA responses in the lung (George-Chandy et al. 2001).

Monocyte-derived DCs infected with live viral replicative or nonreplicative vectors are being used as an effective means of delivery in HIV-1 vaccine development. Nonreplicative viruses are considered as a safer approach for immunizing human volunteers, but are typically weak immunogens, leading to efforts to target the vaccine to DCs for eliciting effective antivaccine immunity such as expanded CTL production. Immature monocytic DCs infected with vesicular stomatitis virus–pseudotyped HIV-1 presented antigens to $CD4^+$ and $CD8^+$ T cells from HIV-infected individuals as effectively as replicative pox vectors (Granelli-Piperno et al. 2000). Replicative recombinant canarypox (ALVAC) HIV-1 vaccines have been administered to >200 HIV-seronegative individuals with a good safety profile. However, this vaccine elicited antigen-specific CTLs in only a third of the volunteers, and therefore, in an attempt to engineer a better vaccine, monocytic DCs pulsed ex vivo with the vaccine are being considered as a possible delivery route for targeting immune cells. Preclinical data showing that the infected DCs are functional in T-cell stimulation assays performed using cells from HIV-1-infected individuals support the ongoing HIV vaccine trial that is comparing conventional vaccine delivery routes with ex vivo vaccine-loaded autologous DCs.

Adenoviruses are ubiquitous human pathogens and can be used efficiently to transduce a wide range of human cells and tissues, including DCs. With this property in mind, adenoviruses have been considered as a vaccine delivery vector, but a recent report by Roth et al. (2002) suggested that pre-existing antiviral immunity may limit the possibility of their clinical application.

INFLUENCE OF ROUTE OF DELIVERY

The route of delivery of a vaccine can have a major influence on the type of immunity induced. The mucosal route has been an attractive option for avoiding the use of needles and stimulating local mucosal immunity along with systemic responses. The oral route of vaccine administration is particularly attractive, but the ability to elicit mucosal immunity using nonliving antigens

involves the use of high doses of antigens and repeated administrations. This results partly from the instability of the antigens in the proteolytic and acidic environment of the stomach, but is also caused by the poor natural mucosal immunogenicity of many antigens at mucosal surfaces. Clearly, live delivery offers potential advantages for oral immunization. However, there are still potential problems. There is potential strong ecological competition for live vectors with resident commensal flora in the gastrointestinal tract. Already problems have been encountered in moving some live vaccines from western volunteers into the field in less developed countries and the normal flora, together with immunological barriers, may be responsible. However, such barriers may not exist for all vaccine vectors.

An attractive alternative to oral delivery is potentially the intranasal route of immunization. Intranasal immunization has the advantage that the respiratory tract is less acidic and also less heavily colonized by microorganisms than the gastrointestinal tract. Less antigen is also needed to induce mucosal immunity via the intranasal compared with the oral route (Douce et al. 1999). Several vectors, including BCG and *Salmonella* Typhi, have been successfully used to deliver heterologous antigens via both the oral and intranasal routes (Langermann et al. 1994a; Hopkins et al. 1995; Corthesy-Theulaz et al. 1998; Kremer et al. 1998). Specific antigen presentation by the resident APCs in the respiratory and gastrointestinal tracts can differ compared with the systemic immune response. When recombinant BCG expressing an intracellular 28-kDa glutathione *S*-transferase from *Schistostoma haematobium* (Sh28GST) was administered intranasally to mice, the vaccine induced high levels of humoral systemic immune responses to Sh28GST, levels similar to responses after intraperitoneal administration of the recombinant antigen. Intranasal delivery also induced a mucosal IgA response against Sh28GST, which was not elicited after intraperitoneal administration. This anti-Sh28GST response was greatly enhanced after a second intranasal immunization that remained high for at least 12 weeks after the boost (Langermann et al. 1994b). When the related 28-kDa glutathione *S*-transferase from *Schistostoma mansoni* (Sm28GST) was administered intraperitoneally, this antigen also induced equivalent levels of anti-glutathione *S*-transferase (anti-GST) antibodies, but, surprisingly, intranasal administration of Sm28GST induced lower levels of anti-GST antibodies than Sh28GST (Kremer et al. 1996).

As transmission of HIV occurs predominantly via mucosal surfaces, immunization targeted to the induction of HIV-specific immune responses at mucosal sites could contain viral replication in the early stages of infection. Many studies have investigated the effect of the route of immunization with recombinant modified vaccinia viruses on eliciting mucosal immune responses. Mucosal immunization of macaques, with MVA-based vectors expressing the simian–human immunodeficiency virus (SHIV) strain SHIV89.6gp160, provided better protection than subcutaneous immunization, after intrarectal exposure to SHIV. These studies suggest that cutaneous or intramuscular immunization generated antigen-specific cells that may not travel to mucosal sites (Kantele et al. 1999; Cromwell et al. 2000). When highly attenuated poxvirus vectors (NYVAC) encoding the SIV_{makow} Gag, Pol, and Env products were introduced intramuscularly into macaques, they provided long-term virus containment after both intravenous or intracellular challenge with SIV_{mac251} (Benson et al. 1998). The results suggested that, even in the absence of priming at mucosal sites, systemic immunization can lead to virus control after mucosal challenge (Benson et al. 1998; Hel et al. 2001). However, a recent study, designed to explore the extent of the mucosal immune responses to a Gag-immunodominant SIV_{mac251} epitope after intramuscular, intranasal, or intracellular route of immunization with highly attenuated poxvirus NYVAC/SIV_{gpe} in macaques, concluded that immunization with live vector vaccine results in the elicitation of $CD8^+$ T-cell responses at mucosal sites even when the vaccine is delivered by nonmucosal routes (Stevceva et al. 2002).

CONCLUSIONS

Live vectors are receiving considerable attention as vehicles for the efficient delivery of antigens via several different routes of immunization and in a number of different target species including humans. In humans, some vectored delivery systems have proved to be less immunogenic than in model animals, e.g. *Salmonella* Typhimurium vectors in mice have consistently proved more efficient at heterologous antigen delivery compared with *Salmonella* Typhi in humans. This could be the result of inherent differences in the immunogenicity of *Salmonella* Typhimurium compared with *Salmonella* Typhi vectors (Hindle et al. 2002) but is equally likely to be the result of the use of poorly optimized expression systems used to drive the expression of the heterologous antigens in the clinical studies. The application of optimized delivery systems together with prime-boost approaches may prove fruitful for the development of a new generation of live human vaccines.

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Naked DNA vaccines

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Over the last decade, there has been considerable excitement about the potential of a radically new approach to infectious disease prophylaxis stemming from the development of 'naked' DNA vaccine technology. Although previous vaccine strategies have relied on using a live replicating vector, purified protein, or an attenuated version of the pathogen, DNA vaccines consist of a plasmid DNA expression vector encoding the gene of interest for targeting the immune response. When administered to an animal, cellular uptake of the plasmid DNA results in expression of the encoded antigen in situ, leading to the induction of antigen-specific immunity.

In many ways, DNA vaccines share attributes of live attenuated vaccines, as exemplified by their ability to synthesize native protein for B-cell recognition and generate antigens endogenously, to enter the major histocompatibility complex (MHC) class I processing pathway and stimulate induction of $CD8^+$ cytotoxic T lymphocytes (CTL). However, unlike live vaccine vectors, DNA vaccines are noninfectious (therefore considered safe to administer in immunocompromised individuals), relatively easy to engineer and manufacture, and heat stable, and can easily be lyophilized (therefore without the need for cold storage). Despite the elegance and simplicity of DNA vaccines, the generation of a strong humoral immune response has not been as universally successful as immunization with protein combined with an adjuvant. This may be the result of the small amount of antigen that is produced. Studies with plasmids encoding reporter proteins indicate that only picogram to nanogram amounts of protein are expressed (Wolff et al. 1990; Klavinskis et al. 1999).

This chapter discusses the background to this technology, the architecture of the vectors used for immunization, modes of administration, the immunological basis for the responses elicited by DNA vaccines, current approaches to augment vaccine potency, and considers safety for human use, including findings from early human clinical trials.

HISTORICAL BACKGROUND

The inception of DNA vaccines goes back to a largely unnoticed observation by Ito (1960) who demonstrated the induction of papillomas in rabbit skin by injection of purified nucleic acid extracted from the Shope rabbit papilloma virus. It was not until Wolff et al. (1990) reported that intramuscular injection of plasmid DNA in a simple saline solution could transfect muscle cells in vivo and persist in an episomal form that the potential to induce an immune response against the expressed proteins became appreciated. Tang et al. (1992) were the first to demonstrate induction of antigen-specific antibody from plasmid DNA injected via a 'gene gun' into mice. This was rapidly followed by a consortium from Vical and Merck (Ulmer et al. 1993) who demonstrated that DNA vaccines could protect mice from influenza virus challenge by induction of

antibody and $CD8^+$ CTL responses. Since then, the immunogenicity or protective efficacy of DNA vaccines in preclinical models of infectious disease, allergy, and cancer has been established in several animal species, and attests to the simplicity and robustness of the technology.

DESCRIPTION OF DNA EXPRESSION VECTORS

DNA vaccines include the basic elements of a bacterial plasmid (the vector: Figure 1): a promoter and enhancer (to enable transcription of the inserted foreign gene), an mRNA transcription termination/polyadenylation signal (for directing expression in mammalian cells), an antibiotic resistance gene (to confer an antibiotic selectable marker for growth in *Escherichia coli*), an origin of replication for growth in *E. coli*, and specific nucleotide sequences, cytidine phosphate–guanosine (referred to as CpG motifs) that are stimulatory for lymphocytes and act as an adjuvant.

Plasmid vector

The essential feature of a plasmid DNA vector for optimal expression in mammalian cells is a strong promoter/enhancer and transcriptional/termination sequence. Most DNA vaccines contain the human cytomegalovirus (CMV) immediate/early (IE) promoter (Boshart et al. 1985), the SV40 early promoter (Moreau et al. 1981), or the Rous sarcoma virus long terminal repeat (LTR) (Gorman et al. 1982) in conjunction with polyadenylation sequences from bovine growth hormone (BGH) or SV40 to stabilize the mRNA (Pfarr et al. 1986). There is some evidence that interferon-γ (IFN-γ), released in situ during the priming of an immune response (as a result of the inflammatory cytokine-inducing properties of bacterial DNA), can suppress transcription from many viral promoters (Harms and Splinter 1995; Romero and Lavine 1996). This in turn can affect the magnitude of the immune response, by limiting antigen production for B- and T-cell priming. Alternatives to virus-derived control elements, based on promoter/enhancer sequences driving expression of MHC class I and II (Xiang et al. 1997; Gebhard et al. 2000), dectin (in Langerhans' cells: Bonkobara et al. 2001) and muscle-specific genes (Barnhart et al. 1998; Kwissa et al. 2000), have been used successfully in DNA vaccines. Tissue and/or cell-specific expression has been achieved by these promoter elements but levels of expression do not exceed those achieved from the human cytomegalovirus (HCMV) immediate early gene (IE) promoter combined with the CMV intron A. Further optimization of expression has been reported by the inclusion of a 'Kozak' translational initiation sequence 5′ to the start codon of the inserted 'foreign' gene.

The ColE1 origin of replication (ori) derived from bacterial pUC plasmids is generally included in DNA vaccine vectors, because it provides an increased copy number in bacteria and high yields of plasmid DNA on purification. Early DNA vaccines studied in mice contained the ampicillin resistance gene (for plasmid selection during bacterial culture); however, this is precluded for use in humans and has been replaced by

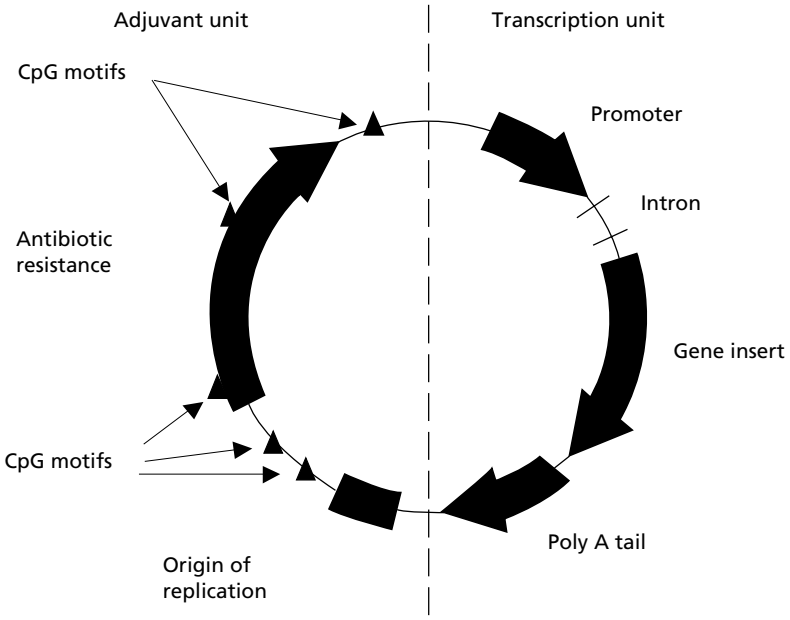

Figure 1 *Schematic representation of the basic elements of a plasmid DNA vector. The transcriptional unit consists of a viral promoter (e.g. human cytomegalovirus immediate early promoter), the gene of interest for targeting the immune response and transcription/termination sequences (poly-A tail). The backbone of the vector contains a bacterial origin of replication and an antibiotic resistance gene, which allow for growth and selection in bacteria. These elements of the vector also contain a number of CpG motifs and represent the adjuvant unit of the vector.*

kanamcyin or antibiotic-free approaches for selection of plasmid DNA (Cranenburgh et al. 2001).

CpG motifs of plasmid DNA

Aside from the regulatory elements of the vector concerned with transcription, bacterial plasmid DNA also contains immunostimulatory sequences (ISS) consisting of CpG dinucleotide sequences, usually flanked by two 5′ purines and two 3′ pyrimidines (Sato et al. 1996). Stimulatory bacterial CpGs differ from vertebrate DNA in that they are unmethylated and expressed at the expected frequency (predicted from random base utilization), whereas this frequency is suppressed 20-fold in vertebrate genomes (Bird 1986). These structural differences within bacterial DNA are recognized as 'foreign' by vertebrate immune systems and directly activate the innate immune system to produce multiple proinflammatory cytokines (including tumor necrosis factor α (TNF-α), IL-6, IL-12, IL-18, and IFN-α and -γ (Klinman et al. 1996)) which enhance the ability of antigen-presenting cells (APC) to present antigen and stimulate T-cell activation. This property of bacterial DNA has been described as the 'natural' adjuvant of DNA vaccines. Direct proof of the contribution of CpG DNA to the immunogenicity of DNA vaccines was provided by substitution of a CpG-containing ampR gene for a kanR-selectable marker (containing additional CpG motifs). The modified plasmid DNA was found to induce an enhanced CTL and IgG response compared with the original vector (Sato et al. 1996). This ability of CpG sequences in DNA vaccines to stimulate an immune response is abrogated by in vitro methylation (Pasquini et al. 1999). Co-administration of an empty vector (i.e. without any antigen-coding sequence) may boost the immune response to a DNA vaccine; however, there appears to be an upper limit to the ability of additional CpG motifs to improve the immune response (Krieg et al. 1998). This may reflect saturation of the innate cell-signaling pathway (see discussion below).

IMMUNOSTIMULATORY EFFECTS AND MECHANISMS OF CpG

Recognition of bacterial CpG is mediated by a conserved receptor, Toll-like receptor 9 (TLR9) expressed by macrophages and dendritic cells (DC) (Bauer et al. 2001). Current data suggest that CpG oligonucleotides cross the cell membrane via sequence nonspecific receptor-mediated endocytosis and are recruited to an endosomal compartment where TLR9 co-localizes (Ahmad-Nejad et al. 2002). One possibility is that plasmid-derived CpG oligonucleotides are liberated after degradation of injected plasmid DNA by extracellular nucleases. It appears that endosomal acidification of CpG DNA is required to initiate signaling via the Toll/interleukin 1 receptor (IL-1R) pathway, because specific inhibitors such as chloroquine block the immunostimulatory activity of CpG DNA (Hacker et al. 1998). In macrophages and DCs, CpG-DNA-mediated TLR9 signaling is transduced by a common adapter protein, myeloid differentiation factor 88 (MyD88), which results in the downstream generation of intracellular reactive oxygen species (Yi et al. 1998), and activation of the mitogen-activated kinases (MAPK) and nuclear factor κB (NFκB: Yi and Krieg 1998; Yi et al. 1998). The biological outcome of CpG-driven TLR9 ligation in DCs is secretion of T-helper 1 (Th1) polarizing cytokines (IL-12 and IL-18), upregulation of co-stimulatory molecules (CD40, CD80, and CD86) and functional maturation for antigen presentation (Sparwasser et al. 1998). These observations indicate that the plasmid backbone functions as a Th1-polarizing adjuvant in DNA vaccines and explains the IgG2a antibody and CTL responses seen after intramuscular immunization. CpG DNA also delivers T-independent survival signals to DCs, which are mediated by upregulation of the cellular inhibitor of active caspase (cIAP: Park et al. 2002). This might be a mechanism by which CpG DNA may prolong DC presentation of naked DNA-encoded antigen and compensate for the relatively low levels of antigen produced (compared with replicating vectors). CpG DNA also directly activates monocytes and macrophages to secrete Th1-like cytokines and express increased levels of cell surface co-stimulatory molecules (Schattenberg et al. 2000b). The signaling pathways activated by CpG DNA in B cells stimulate secretion of IL-6, IL-10, and IL-12 and also immunoglobulin in a polyclonal T-independent manner (Krieg et al. 1995). These CpG-mediated effects synergize with signaling through the B-cell antigen receptor by antigen secreted in situ from naked DNA.

Immune recognition of CpG DNA varies between species, with mouse cells expressing a high-responder phenotype and human cells a low-responder phenotype (Bauer et al. 2001). The CpG motifs optimally active in mice are poor stimulators of innate immunity in human peripheral blood mononuclear cells (PBMC: Bauer et al. 2001). Extensive screening assays have identified structurally distinct CpG oligodeoxynucleotide (ODN) sequences, with enhanced stimulation for human cells. K-type CpG ODNs stimulate IL-6 production by monocytes/DCs and B-cell proliferation and IgM production, whereas D-type CpG ODNs preferentially stimulate IFN-γ production by natural killer (NK) cells (Verthelyi et al. 2001). Thus, it may be possible to improve DNA vaccines for human use by the mutagenesis of endogenous plasmid CpG sequences. However, as discussed above, a relative increase in the levels of IFN-γ induced by CpG-DNA will be compensated by IFN-γ-mediated suppression of viral promoter activity, which drives antigen expression. A further level of complexity that is being unraveled is the differential responsiveness of human DC subsets. Human plasmacytoid DCs ($CD11c^-$ $CD123^+$) are responsive to CpG DNA, but monocyte-derived $CD11^+$ DCs lack TLR9 and do not appear to be

stimulated (Bauer et al. 2001). Currently, this difference is difficult to rationalize, in view of the front-line defensive location of $CD11c^+$ DCs at body surfaces.

IMMUNOSUPPRESSIVE CpG Motifs

Certain CpG motifs can be inhibitory and abrogate immune activation and cytokine production (Hacker et al. 1998). These motifs termed 'neutralizing' CpG (CpG-N) contain a dinucleotide motif preceded by a cytidine (i.e. CCG) followed by a guanosine (i.e. CGG) or contain CG repeats (CGCGCG). The immunostimulatory (s) activity of a CpG-S oligonucleotide can be blocked by certain suppressive motifs. Removal of the suppressive motifs from the plasmid DNA backbone improved the immunogenicity of the plasmid DNA (Krieg et al. 1998). These observations suggest that the presence or absence of these inhibitory sequences in DNA vaccines may profoundly affect the immunogenicity of the vaccine.

ROUTES AND MODE OF DNA VACCINE DELIVERY

A variety of routes have been used to deliver DNA vaccines. Systemic routes of delivery include intramuscular or intradermal needle injection, transdermal application via occluded patches, or ballistic delivery into the skin. For needle injection, naked plasmid DNA is administered in water, saline, phosphate-buffered saline, or sucrose solution. Ballistic delivery of DNA vaccines involves bombardment of cells with plasmid DNA precipitated onto gold or tungsten microparticles. This is usually achieved by a blast of pressurized gas (generally helium) delivering DNA-coated particles from a gene gun (Feltquate et al. 1997). Topical or aerosol delivery of naked DNA vaccines to mucosal surfaces has also been achieved and is highly desirable, because this provides the possibility of generating immunity both at the sites of pathogen entry and in the systemic compartment. Moreover, needle-free delivery increases patient compliance.

Intramuscular delivery of naked DNA

One of the simplest modes of administering naked DNA is in saline to the quadriceps or tibialis anterior muscle in mice or to the axillary or gluteal muscle in nonhuman primates. Typically 10–50 μg plasmid DNA is required to elicit a response in mice but substantially higher doses (1–5 mg) are required to elicit detectable responses in nonhuman and human primates. Some investigators have reported improved immune responses induced by pretreatment of the muscle tissue with agents including cardiotoxin, local anesthetics such as bupivacaine, or hypertonic solutions (Davis et al. 1993; Coney et al. 1994). Whether or not this effect reflects an increase in plasmid-encoded antigen expression in regenerating muscle cells or an increase in recruitment of APCs to the site of tissue damage is contentious. There is agreement that, after intramuscular injection, plasmid DNA rapidly disseminates throughout the body and persists briefly in highly vascular tissues and in muscle at the site of injection for at least 8 weeks (Parker et al. 1999). The persistence of plasmid DNA, principally in muscle cells, may provide a reservoir of antigen production to sustain (via crosspriming, see 'Processing and presentation of plasmid DNA-encoded antigen' below) the strong CTL responses reported in mice.

The characteristics and quantity of an antigen are known to be important in defining the type of immune response elicited. However, intramuscularly injected DNA vaccines typically, but not exclusively, elicit a Th1 immune response, characterized by IFN-γ production by antigen-restimulated $CD4^+$ T cells, and the predominance of serum IgG2a antibody to the antigen. This profile is attributed to the adjuvant effect of the CpG motifs within the plasmid backbone, which induce IL-12 secretion by APCs.

Intradermal or ballistic delivery of naked DNA

Intradermal or ballistically delivered DNA vaccines target the dermal and epidermal tissues of the skin. Small amounts of DNA, typically 1–10 μg (for needle injection) or sub-microgram amounts (for ballistic delivery), are required to elicit immune responses in mice that are often superior to those elicited at higher concentrations by the intramuscular route. This most probably reflects the higher concentration of APCs, Langerhans' cells, and dermal DCs within the skin and also the cascade of cytokines (IL-1α, IL-1β, TNFα, IL-6, IL-7, IL-10, IL-12, IL-15) and chemokines (macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein 2 (MCP-2), RANTES, IP-10) that are liberated after needle or ballistic trauma. Initial reports suggested that gene-gun- (or ballistically-) delivered DNA vaccines elicited a predominantly Th2 response and serum IgG1 antibody, whereas intramuscular injection with the same plasmid DNA resulted in Th1 priming for CTLs and predominantly serum IgG2a antibody (Feltquate et al. 1997). Other reports indicate induction of potent Th1 responses after intradermal injection of plasmid DNA (Raz et al. 1994). These differences may be reconciled with the type of plasmid DNA-encoded antigen, the age and type of mouse strain used, and the dose of plasmid DNA (low doses contributing to exclusive Th2 priming), rather than a correlation between injection route and polarization of the immune response. It has been suggested that the adjuvant effect of plasmid DNA operates in priming Th1 responses at a dose range of 20–100 μg/vaccine (Reimann and Schirmbeck 2000), accounting for the preferential priming of Th2 responses by the intradermal or ballistic route. However, priming for Th1 responses

can be established at low plasmid DNA doses by co-injection of low concentrations of CpG oligonucleotide at the intradermal site of plasmid DNA delivery (Reimann and Schirmbeck 2000). Other reagents (IFNs, IL-12, IL-18, or poly[I/C]) can also deviate a plasmid DNA-elicited Th2 response to a Th1 type in this system.

Mucosal delivery

Protection against infection at the mucosal epithelia generally requires induction of local immunity, which is optimally elicited by vaccines delivered to mucosal inductive sites (Czerkinsky et al. 1999). To date, the vast majority of DNA vaccines have been delivered by parenteral routes, although data indicating induction of secretory IgA or IgG antibodies by intramuscularly or intradermally administered plasmid DNA have not been compelling (Fynan et al. 1993). A number of studies have reported the induction of weak immune responses by direct topical delivery of naked plasmid DNA to various mucosal surfaces, including the nasal, oral, and vaginal epithelia (Etchart et al. 1997; Wang et al. 1997; Klavinskis et al. 1999). These observations may be the result of the low efficiency of transgene expression achieved at nasal and intestinal epithelia by naked plasmid DNA (Klavinskis et al. 1999; L.S. Klavinskis, C. Barnfield, and P. Hobson, 2000, unpublished observations). This may arise from the presence of physical and chemical barriers impeding transfection in the respiratory and gastrointestinal tract, including endonucleases, mucolytic enzymes, low pH, and turbulence. To increase plasmid DNA uptake and transfection efficiency, several approaches have focused on condensing (or encapsulating) the naked plasmid DNA with either cationic liposomes (Perrie et al. 2001), cytofectins based on cationic lipids (Klavinskis et al. 1999), cationic block co-polymers (Laus et al. 2001), or biodegradable microspheres such as poly (dl-lactide-co-glycolide: Herrmann et al. 1999; Kaneko et al. 2000). Bacterial systems including attenuated *Shigellla flexneri* and *Salmonella typhimurium*, which invade mucosal epithelial cells, have also been developed for oral delivery of DNA vaccines (Darji et al. 2000; Shata and Hone 2001). Detailed discussion of these systems lies outside the focus of this chapter.

GENERATION OF IMMUNE RESPONSES BY NAKED DNA VACCINES

Plasmid DNA uptake

The precise mechanism by which naked plasmid DNA enters cells and is translocated to the nucleus to initiate transcription is not fully understood. Evidence from electron micrographs suggests that plasmid DNA is internalized by electrostatic binding to the cell membrane and endocytosed via clathrin-coated pits (Figure 2) (Zabner et al. 1995; Friend et al. 1996). Direct fusion with the cell membrane and/or fluid phase endocytosis (macropinocytosis: Apodaca 2001) may also contribute to cellular uptake of plasmid DNA (Figure 2). Endosomal uptake of plasmid DNA can be increased by complexing the DNA with cationic liposomes, cytofectins, or ligands to cell surface receptors (Fasbender et al. 1997; Harbottle et al. 1998; Klavinskis et al. 1999; Perrie et al. 2001). Once plasmid DNA is internalized, rapid escape from the endosome is critical to the efficiency of plasmid-encoded antigen expression. Endosomes mature through various stages of acidification and eventually fuse with lysosomes containing enzymes, which degrade DNA. Release of plasmid DNA from the endolysosome is thought to be a consequence of membrane disruption. Cationic lipids or peptides (e.g. from the hemagglutinin of influenza virus) that fuse with the endosomal membrane have been reported to increase transfection efficiency (Fasbender et al. 1997; Zhang et al. 2001). Despite these modifications, only a fraction of internalized plasmid DNA penetrates the cytoplasm. Plasmid DNA is prone to further degradation by cytosolic nucleases. Thus, only a very small proportion of transfected DNA is available for transport across the nuclear membrane (reviewed by Johnson-Saliba and Jans 2001). The size of plasmid DNA vectors (2–10 MDa) is generally assumed to preclude efficient diffusion across the nuclear pore complex, thus compounding the inefficiency of plasmid DNA transfection. After transcription, mRNA is translated and plasmid-encoded protein within the cytoplasm is accessible to the TAP-dependent MHC-I-processing pathway. The induction of immune responses by DNA vaccines is all the more remarkable in view of the multiple cellular barriers that impede efficient transfection of plasmid DNA and attests to the importance of the adjuvant activity of CpG DNA.

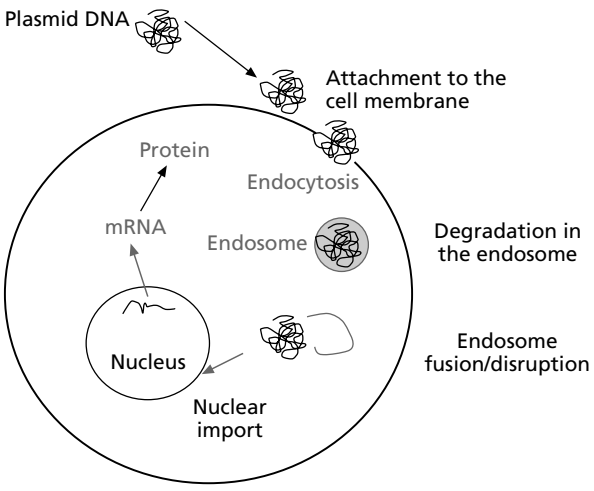

Figure 2 *Schematic representation of critical steps in the cytosolic synthesis of plasmid encoded antigen after membrane binding of plasmid DNA.*

Processing and presentation of plasmid DNA-encoded antigen

For the presentation of plasmid DNA-encoded antigen to B cells, it appears that, as long as antigen is accessible to the B-cell receptor, the nature of the transfected cell exporting the plasmid DNA-encoded protein is immaterial (Loirat et al. 1999; Armand et al. 2000). For DNA vaccines expressing purely cytosolic proteins, it had been suggested that antigen release to the extracellular milieu might be effected by CTL lysis of the transfected cell. However, recent data indicated that neither perforin nor $CD8^+$ T-cell-mediated lysis is required for priming $CD4^+$ T-cell or antibody responses (Hassett et al. 1999).

For the priming of $CD8^+$ T-cell responses there are at least three mechanisms by which a plasmid DNA-encoded antigen may be processed and presented. These include: (1) direct transfection and priming by somatic cells (such as myocytes, fibroblast, and keratinocytes); (2) crosspriming, whereby antigen is transferred from transfected somatic cells to professional APCs; or (3) direct transfection of APCs.

DIRECT PRIMING BY SOMATIC CELLS

The seminal observation that intramuscular injection of plasmid DNA-induced protein expression in muscle (Wolff et al. 1990) and elicited strong $CD8^+$ CTL responses in mice (Ulmer et al. 1993) suggested that muscle cells were critically involved in the initiation of CTL responses. In support of this premise, it was shown that adoptive transfer of stably transfected myoblasts expressing influenza nucleoprotein into mice was sufficient to induce $CD8^+$ CTLs and protect from virus challenge (Ulmer et al. 1996). This provided compelling evidence that antigen expression by muscle cells was sufficient to prime for CTL-mediated protection. It was unresolved whether CTL responses were directly induced by myocytes expressing the encoded antigen or they were indirectly induced through antigen transfer from myocytes to professional APCs. A conceptual difficulty with direct priming was that muscle cells do not express co-stimulatory molecules (CD80, CD86) essential to initiate T-cell responses and were therefore unlikely to act as APCs. To resolve the contribution of somatic versus bone-marrow derived APCs in CTL priming, bone-marrow-derived cells were adoptively transferred to irradiated F1 hybrid mice, to create bone marrow chimeras with somatic cells of both F1 MHC haplotypes and APCs of a single MHC haplotype of the donor bone marrow. Peptide-specific CTLs were induced only by immunization of mice with plasmid DNA encoding the peptide presented by the MHC-I molecules found on the donor bone marrow (Fu et al. 1997; Iwasaki et al. 1997). These studies demonstrated that bone-marrow-derived APCs play a dominant role in CTL priming and suggested that either APCs were directly transfected or antigens (or their epitopes) synthesized in somatic cells could be transferred to APCs (by crosspriming, see below) for presentation by MHC-I molecules.

CROSSPRIMING BY DNA VACCINES

Crosspresentation of antigenic material from apoptotic cells to DCs has been established as an important mechanism for the induction of $CD8^+$ CTL responses against tumor or pathogen-derived antigens (Bevan 1976). In vitro studies have established that this mechanism is distinct from the classic endogenous TAP-dependent pathway of MHC class I restricted peptide presentation. Several lines of evidence indicate that crosspriming also occurs after DNA immunization. Loirat et al. (1999) have shown that immunization with a plasmid DNA vector driven by a muscle-specific promoter elicited CTLs, despite expression of antigen restricted to muscle cells, suggesting that crosspresentation of antigen to APCs was involved. Second Corr et al. (1999) demonstrated, using a transactivating plasmid system and bone marrow chimeras, that CTL induction by plasmid DNA was critically dependent on antigen expression by somatic cells and transfer to APCs. However, a complementary mechanism involving direct transfection of APCs could not be excluded (see below). More recently, cross-presentation of antigen to DCs has been facilitated by constructing DNA vaccines, which induce apoptotic death of the antigen-expressing cell. Co-immunization with DNA vaccines that express Fas or a mutated version of a caspase gene that induces the creation of apoptotic bodies has been reported to enhance cell-mediated immunity (Chattergoon et al. 2000; Sasaki et al. 2001). The underlying mechanism by which antigen is transferred from somatic cells to APCs is not fully understood. Chaperones, including heat shock proteins 70 and 73 (hsp70, hsp73), have been implicated in the transfer of immunogenic material from plasmid DNA-transfected macrophages or somatic cells to DCs (Kumaraguru et al. 2000; Kammerer et al. 2002). The precise mechanism by which chaperone-bound peptides are released and cross-presented by DCs remains to be resolved. Candidate mechanisms include either a facilitated exchange or delivery system, whereby chaperone-bound peptide is internalized after receptor-mediated DC binding with peptide exchange between nascent MHC class I molecules and the chaperone (Figure 3a). Alternately, chaperone-bound peptide may be directly exchanged with cell-surface MHC class I bound peptide (Figure 3a).

DIRECT TRANSFECTION OF APCS BY DNA VACCINES

Although current data provide a compelling case for indirect CTL priming through crosspresentation, other studies provide evidence that APCs are directly transfected after DNA immunization. Removal of muscle (within 10 min) from the injection site of a DNA

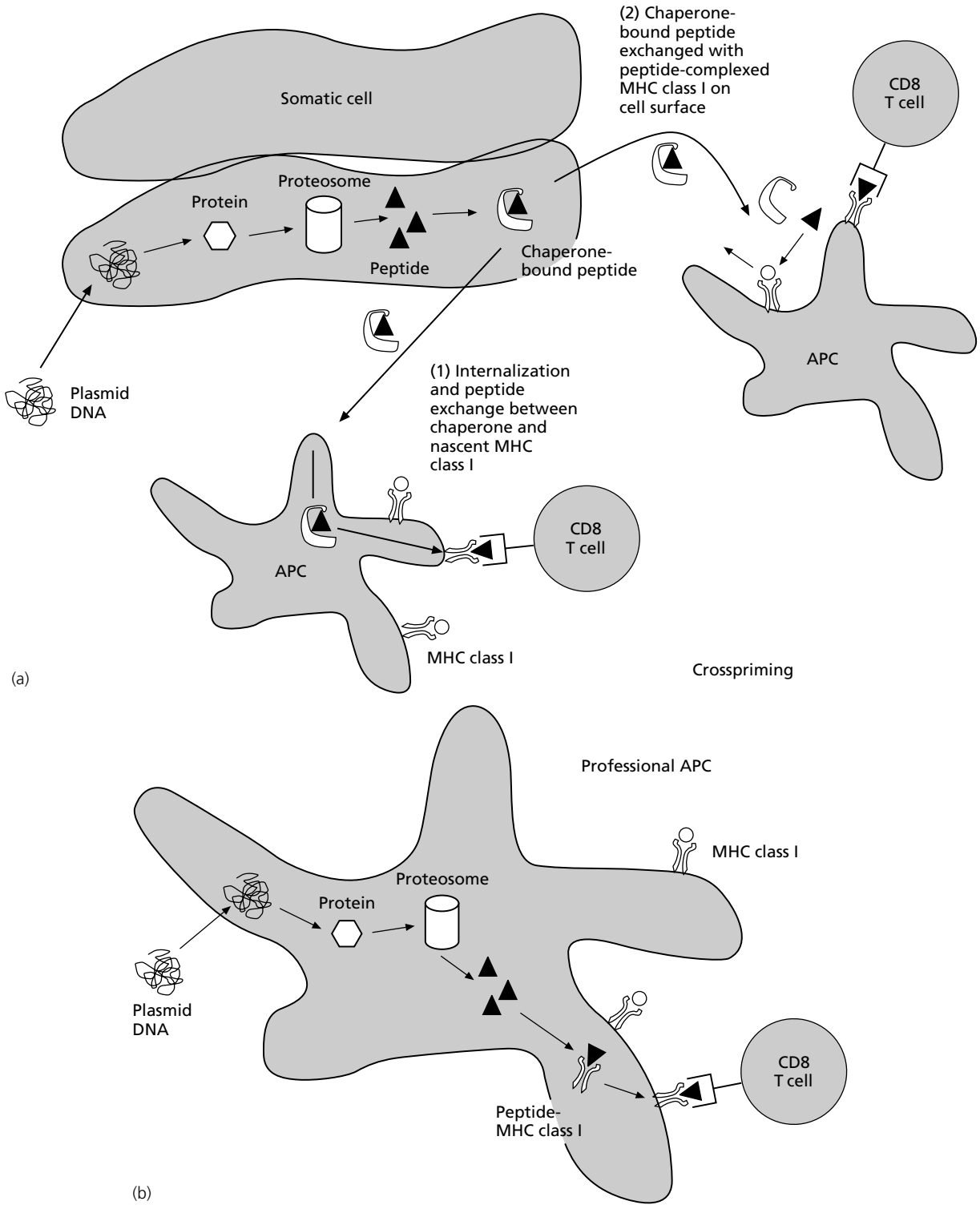

Figure 3 *Mechanisms of antigen presentation after DNA immunization.* **(a)** *Crosspresentation of antigenic material from plasmid DNA-transfected somatic cells to antigen-presenting cells (APCs) in vivo. Injection of plasmid DNA leads to transfection of somatic cells (muscle cells, keratinocytes or any MHC class II negative cells). Newly synthesized protein is targeted to proteosomes for proteolytic cleavage and peptide transport across the endoplasmic reticulum (ER). Chaperones, such as heat shock proteins, bind these peptides, which are crosspresented by MHC-I molecules on professional APCs to CD8$^+$ T cells. The precise mechanism is unclear but may involve either (1) receptor-mediated internalization of peptide bound chaperone and peptide exchange between the chaperone and newly synthesized MHC class I or (2) peptide exchange between cell surface MHC class I and chaperone-bound peptide.* **(b)** *Direct transfection of bone marrow-derived APCs. Injection of plasmid DNA leads to direct transfection of a small number of dendritic cells. Peptides generated* in situ *are presented by MHC class I molecules to CD8$^+$ T cells*

vaccine did not alter the magnitude or quality of the subsequent immune response, suggesting that transfection of cells (possibly APCs) distal to the injection site is sufficient to prime for CTLs (Torres et al. 1997). Antigen and reporter genes have been demonstrated in macrophages, Langerhans' cells, and DCs present both at the site of DNA vaccine delivery and in draining lymph nodes (Chattergoon et al. 1998; Akbari et al. 1999; Barnfield et al. 2000). This suggests that APCs are transfected in situ and migrate to draining lymph nodes or alternately are transfected within lymph nodes after lymphatic drainage of plasmid DNA (Figure 3b). Conclusively, plasmid-encoded mRNA has been demonstrated in isolated DCs after either intradermal or mucosal DNA vaccine delivery (Bouloc et al. 1999; Barnfield et al. 2000). Functional studies have demonstrated that DCs isolated from plasmid DNA injected skin or nasal tissue were able to stimulate the proliferation of T-cell responses in vitro (Bouloc et al. 1999; Barnfield et al. 2000). Second, DCs isolated after DNA immunization were shown to prime for CTL responses in naïve mice following adoptive transfer (Bouloc et al. 1999). These reports indicate that different routes of DNA vaccine delivery result in direct transfection of APCs and lead to induction of CTL responses. Current evidence suggests that the efficiency of DC transfection in vivo is very low (Barnfield et al. 2000). However, considering the potent stimulatory activity of DCs (reviewed by Steinman and Poe 2002), large numbers of transfected DCs may not actually be essential to elicit CTL responses. In support of this hypothesis, adoptive transfer of as few as 500–1000 in vitro transfected DCs to mice induced a comparable cellular and humoral immune response to that elicited by gene-gun immunization with the same antigen-encoding plasmid DNA (Timares et al. 1998).

In summary, two major pathways for CTL priming by DNA vaccines have been established. Enhancement of crosspriming has been established by co-delivery of plasmid DNA-encoding apoptotic proteins. Formulations or delivery systems that target APCs directly may also increase plasmid DNA uptake and CTL priming.

AUGMENTING THE IMMUNOGENICITY OF DNA VACCINES

Preclinical studies in several murine models of infectious disease have shown that DNA vaccines effectively elicit cellular and humoral immune responses, which confer protection from pathogen challenge (e.g. Ulmer et al. 1993; Li et al. 1998; Gebhard et al. 2000). Although these observations have been very encouraging, the magnitude of the immune responses induced in human and nonhuman primates by the first generation of naked DNA vaccines has proved lower than that achieved by attenuated or protein adjuvanted vaccines (see MacGregor et al. 1998; Amara et al. 2001). This difference in efficacy of DNA vaccines between species results partly from the higher level of protein expression achieved in mice after intramuscular injection than in other species (Jiao et al. 1992). Second, the dose of plasmid DNA injected in mice per kilogram body weight significantly exceeds the per kilogram dose administered to human and nonhuman primates, so comparisons of immunogenicity are not appropriate. Furthermore, murine cells reportedly demonstrate greater sensitivity than human cells to the adjuvant activity of CpG DNA (Bauer et al. 2001). Thus although naked DNA vaccines on their own may not be adequate to protect against disease in humans, technological developments in vector construction, incorporation of genetic or conventional adjuvants, deployment of novel delivery vehicles, or poxvirus booster inoculations (see below) may lead to the development of effective DNA vaccines.

Modification of the vector

PROMOTER

It is generally believed that the level of gene expression attained in vivo after DNA immunization correlates with the immune response generated. Therefore, approaches to improve gene expression may enhance DNA vaccine potency. Virally-derived promoters, including the most frequently used CMV immediate early enhancer–promoter, provide high levels of gene expression within mammalian cells (Manthorpe et al. 1993). However, these promoters are subject to transcriptional silencing, after CpG DNA-mediated activation of proinflammatory cytokines (Romero and Lavine 1996). Elimination or methylation of CpG motifs within the plasmid backbone reportedly increases the duration of antigen expression (e.g. Yew et al. 2001; Reyes-Sandoral and Ertl 2004). Although this modification may provide the longer-term expression required for gene replacement therapy, it needs to be balanced with the loss of adjuvant activity required for DC maturation and to initiate T-cell priming. Alternate promoters, including cell-specific, tissue-specific, and hybrid CMV promoters, have been evaluated (Xiang et al. 1997; Barnhart et al. 1998; Gebhard et al. 2000; Kwissa et al. 2000; Bonkobara et al. 2001). Plasmid vectors containing the human ubiquitin promoter could be particularly useful. Sustained, high-level gene expression has been achieved with this promoter in the presence of plasmid backbones containing CpG motifs (Yew et al. 2001).

CODON OPTIMIZATION

Optimizing codon usage for eukaryotic cells can also enhance expression of antigens. Codon bias has been observed in several species and may differ from the codons most frequently used by pathogens. Changes in codon usage, which modify RNA structure, have been shown to enhance the expression of HIV *env* and *gag* genes. In

mammalian cells the *env* and *gag* genes are made as long unspliced transcripts that contain overlapping reading frames with the small regulatory genes of HIV *rev* and *tat*. These transcripts are retained in the nucleus and are rapidly spliced in the absence of *rev*, thus preventing the transport of full-length message to the endoplasmic reticulum (ER). The transcripts are inhibited from transport to the ER by *rev*-dependent sequences that are themselves part of the structural gene sequence. Structural modification of the RNA through changing codon usage has led to an increase in HIV *envelope* and *gag* expression with an increase in anti-HIV gp160 CTLs and antibody reported (Andre et al. 1998; Vinner et al. 1999).

Modification of the inserted gene

By altering the cellular location where antigen is expressed or by the inclusion of protein sorting or targeting sequences, DNA vaccines have been modified either to increase priming for $CD8^+$ T-cell responses or to enhance B-cell activation. The addition of N-terminal ubiquitin sequences has been reported to lead to an increase in CTL precursor frequency and a reduction in antibody responses (Wu and Kipps 1997; Rodriguez et al. 1998). This is most probably the result of enhanced targeting of *de novo* synthesized cytosolic proteins to the proteosome (an organelle in which the protein is hydrolyzed to short peptides for TAP-dependent entry into the ER and MHC-I presentation). Proteins are thus rapidly processed to short peptides, which precludes or reduces the release of native protein for efficient antibody production. Another approach has been to design vectors that incorporate the E3 leader sequence from adenovirus, which facilitates transport of antigens from the cytosol directly into the ER for binding to MHC-I molecules. The addition of the E3 leader sequence has been reported to increase CTL responses to certain (Ciernik et al. 1996; Iwasaki et al. 1999), but not all (Ciernik et al. 1996), plasmid DNA-encoded antigens. Similarly, immunization with plasmid DNA encoding an antigen fused C terminally to a truncated cytosolic chaperone (heat shock protein 73 or hsp73) has been reported to increase CTL induction by bypassing the normal proteosome/TAP route of peptide entry into the ER (Kammerer et al. 2002). The stable *de novo* expression of antigen with hsp73 in the cytosol from a DNA vector may facilitate crosspresentation of antigen to DCs in vivo.

$CD4^+$ T-cell responses can be augmented by addition of sorting signals from membrane proteins, which are normally targeted to the lysosomal compartment, thereby directing plasmid-encoded proteins toward the MHC-II antigen-presentation pathway (Langlade-Demoyen et al. 2003). The immunological consequence of lysosomal targeting appears variable and depends on the primary sequence of the encoded antigen (Rodriguez et al. 2001). Plasmid DNA-encoded $CD4^+$ T-cell epitopes, which contain cleavage sites for the lysosomal endopeptidase (cathepsin D), were destroyed when targeted to lysosomes.

Humoral immune responses can be enhanced by the addition of a signal sequence fused to the antigen-coding sequence to direct protein transport through the ER and export from the cell surface (Lewis et al. 1999). Thus, synthesized protein bypasses the normal proteosome/TAP route of peptide delivery into the ER and is secreted for B-cell binding. Immune responses in mice immunized with a secreted form of antigen demonstrate an enhanced serum IgG1 response and increased levels of IL-4 by antigen-restimulated lymph node T cells in vitro (Lewis et al. 1999).

DNA delivery

The distribution of plasmid DNA from the site of vaccine delivery and efficiency of cellular uptake are key factors limiting the potency of DNA vaccines. One approach to increase DNA delivery is in vivo electroporation. A low intensity of electric pulses (100–200 V/cm) delivered from electrodes applied to the muscle or skin has resulted in significant enhancement in the cellular and humoral immune responses generated to injected DNA vaccines (Kadowaki et al. 2000; Widera et al. 2000). The wide-scale feasibility, user friendliness, and safety of this immunization approach remains to be established. The potential for DNA integration into host genomes is a concern, because electroporation results in a much larger copy number of copies of plasmid DNA per cell than achieved by injected naked plasmid DNA (Dupuis et al. 2000).

An alternate approach to increase DNA vaccine delivery is through formulations designed to protect plasmid DNA from degradation and to increase the release of plasmid DNA from the endosomal compartment and entry into the nucleus. Various liposomes, cationic lipids, and cationic block polymers (polycations combined with hydrophilic nonionic polymers) were initially developed to enhance therapeutic gene therapy (reviewed by Lechardeur and Lukas 2002). Several groups have since demonstrated that cationic lipid or polymer-complexed plasmid DNA also enhanced the induction of cellular and humoral immune responses (Klavinskis et al. 1999; Hartikka et al. 2001; D'Souza et al. 2002). There is some evidence to indicate that these formulations (or cytofectins) may not only increase plasmid DNA uptake but also synergize with bacterial CpG to activate the innate response and augment T-cell priming by DNA vaccines. Lipid-complexed plasmid DNA has been reported to increase DC activation and increase proinflammatory cytokine (IL-12 and IFN-γ) production at the site of plasmid DNA delivery (Freimark et al. 1998; Barnfield et al. 2000). Lipid-complexed plasmid DNA may (like liposome-complexed antigens) demonstrate preferential uptake by APCs.

Another approach to augmenting the immunogenicity of plasmid DNA-encoded antigens has been the use of biodegradable polymeric microparticles. Plasmid DNA encapsulated by the polymer poly(lactide-co-glycolide) (PLG) can be delivered by systemic and mucosal routes and has been reported to elicit immune responses (Chen et al. 1998; Kaneko et al. 2000) that confer protection in a murine rotavirus challenge model (Chen et al. 1998). Adsorption of plasmid DNA on to pre-formed PLG microparticles has also been shown to prime for both antibody and cellular immune responses (Singh et al. 2000). PLG-mediated vaccine delivery appears to act, in part, by targeting PLG-containing microspheres to APCs, rather than achieving an increase in gene expression (Singh et al. 2000).

Genetic adjuvants for DNA vaccines

Several groups have used plasmid DNA encoding various cytokine, chemokine, or co-stimulatory molecules to enhance or polarize the type of immune response generated by DNA immunization (summarized in Tables 1 and 2). In some instances the outcome attributed to a particular immunomodulator has been inconclusive or variable depending on the antigen, mouse strain, or plasmid vector used. The variability in response may also reflect the complexity of effects attributed to an immunomodulator and those elicited in response to bacterial components of the vaccine vector (described earlier). Co-immunization with plasmid DNA encoding Th1 cytokines, IL-12, IL-18, IFN-γ, and TNFα or the chemokines granulocyte–macrophage colony-stimulating factor (GM-CSF) or RANTES with antigen-coding DNA vaccine constructs has resulted in enhanced CTL and/or Th-cell proliferative responses (summarized in Tables 1 and 2). Co-immunization with plasmid DNA encoding IL-12 also appears to prolong the duration of the CTL response (Chattergoon et al. 2004). Conversely, co-immunization with plasmid DNA encoding certain Th2 cytokines (e.g. IL-4, IL-5, IL-6), or IL-2, IL-15, IL-18, or GM-CSF, has been reported to augment humoral immune responses (Table 1).

Other investigators have targeted transgene products to lymph nodes or APCs by constructing DNA vaccines encoding secreted fusion proteins that contain CTLA-4, L-selectin, or CD40L, the cognate ligands of which are present on APCs. By targeting secreted antigens for APC uptake, stronger immune responses have been reported (Mendoza et al. 1997; Boyle et al. 1998; Sin et al. 2000). Similarly, expression of plasmid DNA-encoded co-stimulatory molecules (CD80 or CD86) on transfected somatic or APCs can provide critical secondary signals for T-cell activation, through ligation of cognate receptors (CD28/CTLA-4). A number of studies have reported that co-administration of plasmid DNA-encoding antigen and CD80 or CD86 increased T-cell responses without any change in the humoral immune response (Iwasaki et al. 1997; Kim et al. 1997).

Table 1 *Modulation of DNA vaccines by co-delivery of cytokines and chemokines*

Cytokine	Antibody	Cellular response	CTLs	Reference
Chemokine				
IL-1	↑ IgG	↑ proliferation	↑ CTLs	Hakim et al. (1996); Kim et al. (1998)
IL-2	↑ IgG	↑ proliferation	↑ CTLs	Kim et al. (1998)
			↑CTLs	Barouch et al. (2000)
IL-4	↑ IgG1	↓ DTH, ↑ IL-4		Geissler et al. (1997)
Il-5	↑ IgG1	± proliferation		Kim et al. (1998)
IL-6	↑ IgG, ↑ IgA	↑ proliferation		Barnfield (2000)
IL-7	↑ IgG2a	↑ IFN-γ		Prayaga et al. (1997)
IL-8	↑ IgG	↑ proliferation		Kim et al. (2000)
	↑ neutrophils			Hengge et al. (1995)
IL-10	↓ IgG2a	↓ proliferation		Daheshia et al. (1997)
IL-12	↑ IgG2a	↑ DTH	↑ CTLs (and memory)	Iwasaki et al. (1997); Okada et al. (1997); Chattergoon et al. (2004)
IL-15	↑ IgG	± ↑ proliferation	↑ CTLs	Kim et al. (1998)
IL-18	↑ IgG	↑ proliferation	↑ CTLs	Kim et al. (1998)
TNF-α	↑ IgG	↑ proliferation	↑ CTLs	Kim et al. (1998)
GM-CSF	↑ IgG	↑ proliferation	↑ CTLs	Iwasaki et al. (1997)
	↑ IgG1	↑ IL-4		Xiang and Ertl (1995)
IFN-γ	↑ IgG	↑ IFN-γ, ↓ IL-5		Xiang and Ertl (1995)
MIP-1α	↑ IgG	↑ proliferation		Kim et al. (2000)
γIP-10	± ↑ IgG	± no effect		Kim et al. (2000)
RANTES	± no effect	↑ proliferation		Sin et al. (2000)
		↑ proliferation	↑ CTLs	Kim et al. (2000)

See text for abbreviations.

Table 2 *Modulation of DNA vaccines by co-delivery of co-stimulatory genes*

Co-stimulatory gene	Antibody	Cellular response	CTLs	Reference
CD80			↑ CTLs	Iwasaki et al. (1997) Kim et al. (1997)
CD86		↑ DTH	↑ CTLs	Iwasaki et al. (1997) Kim et al. (1997)
CD40L	↑ IgG2a	↑ IFN-γ	↑ CTLs	Mendoza et al. (1997)
	↑ IgG2a	↑ proliferation, ↑ IFN-γ		Sin et al. (2001)
LFA-3		↑ proliferation, ↑ IFN-γ	↑ CTLs	Kim et al. (1999)
L-Selectin	↑ IgG	↑ proliferation		Boyle et al. (1998)
CTLA-4	↑ IgG (IgG1 > IgG2a)	↑ proliferation		Boyle et al. (1998)

See text for abbreviations.

PRIME-BOOST APPROACHES

The relative lack of effectiveness of DNA vaccines in nonhuman primates has led to the development of heterologous immunization regimens, where the immune response induced by plasmid DNA is enhanced by a boost from a heterologous vector or protein. Strategies have included priming with a DNA vaccine and boosting with a recombinant protein-adjuvanted vaccine. Enhancement in the humoral immune response has been demonstrated with variable protection of nonhuman primates from pathogen challenge (Letvin et al. 1997; Putkonen et al. 1998; Cherpelis et al. 2001). Superior CTL responses in nonhuman primates against antigens from HIV, simian immunodeficiency virus (SIV), Ebola virus, and malaria have been demonstrated where DNA vaccines were used as priming agents and augmented by various viral vectors, such as fowlpox viruses (Kent et al. 1998; Robinson et al. 1999), vaccinia virus (Sedegah et al. 2000), modified vaccinia Ankara (MVA: Scheider et al. 1998; Hanke et al. 1999; Allen et al. 2000; Mwau et al. 2004), or adenovirus (Sullivan et al. 2000; Shiver et al. 2002). The superiority of the DNA prime-viral vector boost approach may reside in the ability of the DNA vaccine component to focus the immune response on to a single antigen, without competition from the myriad of viral vector-encoded antigens. The relative inefficiency of DNA vaccines to boost immune responses primed by viral vectors is most probably the result of the small amount of antigen generated and the weak induction of immunomodulatory cytokines and chemokines required to trigger expansion of antigen-specific memory cells.

SAFETY CONSIDERATIONS FOR VACCINE ADMINISTRATION IN HUMANS

To date, DNA vaccines have been shown to be well tolerated in both preclinical and phase 1 clinical trials (see below). Immunopathological reactions such as inflammation and general immunosuppression have not been observed, although a number of safety concerns remain. These include: (1) the possibility that DNA vaccines could integrate into the host genome, which could result in the activation of oncogenes or the inactivation of tumor suppressor genes; (2) the induction of responses against self-antigens expressed on transfected cells and that initiate autoimmune disease; (3) the induction of tolerance rather than active immunity to plasmid DNA-encoded antigens; and (4) the stimulation and production of antibodies to the injected DNA.

Potential for integration

The primary safety concern of plasmid DNA vaccines is their potential to integrate into the genome of host cells. This could increase the risk of malignancy by causing either the activation of oncogenes or inactivation of tumor suppressor genes. If integration occurs in germline cells, there is also a potential for germline transmission. These concerns stem from the knowledge that plasmids can integrate at a very low level into cellular DNA when transfected into actively dividing cells. However, it is believed that only a small fraction of integration events would alter a gene and prove harmful to a host cell. The probability of integration in vivo is likely to be further reduced by physical–chemical barriers that impede efficient uptake of plasmid DNA in vivo, including exonucleases, interstitial DNA-binding proteins, and a lower rate of cellular proliferation than cells in vitro synchronized for transfection under S-phase conditions. Despite concerns raised, there is no clear evidence to date that naked DNA vaccines can integrate. Gel-purified genomic DNA isolated from many tissues (including germline) following intramuscular injection of plasmid DNA and assayed by polymerase chain reaction (PCR) has produced no evidence of integration to a detection sensitivity of one copy plasmid per microgram of DNA (Ledwith et al. 2000; Schattenberg et al. 2000a). This rate is at least three orders of magnitude lower than

the theoretical frequency of spontaneous gene-inactivating mutations (Nicols et al. 1995). Thus, for plasmid DNA vaccines delivered by intramuscular injection, the risk of mutation caused by integration appears to be negligible. Other methods of plasmid DNA delivery (including gene gun or electroporation), or use of novel formulations or adjuvants that increase the efficiency of cell transfection, could potentially influence the integration frequency. Indeed compelling evidence for integration of plasmid DNA following electroporation has recently been reported (Wang et al. 2004).

Potential for induction of immunological tolerance or autoimmunity

Most vaccines intended for human use are administered to infants and young children. As a result of the immaturity of their immune system, there is a potential risk that exposure to DNA vaccines during the neonatal period may induce tolerance rather than immunity. As protein encoded by a DNA vaccine is produced endogenously and expressed in the context of self-MHC, the potential exists for the neonatal immune system to recognize the encoded antigen as 'self', resulting in tolerance rather than immunity. Consistent with this possibility, in one report a plasmid DNA vaccine encoding the circumsporozoite protein (CSP) of malaria induced long-lasting tolerance when administered to newborn but not adult mice (Ichino et al. 1999). Co-administration of plasmid DNA encoding GM-CSF prevented the development of neonatal tolerance in this model (Ishii et al. 1999). The simplest explanation appears to be that, although CpG motifs associated with plasmid DNA are responsible for the activation of DC, additional maturational signals may be required to optimize the response to certain DNA vaccines in neonates. Nevertheless, most studies performed to date in rodents and nonhuman primates have demonstrated induction of significant cellular and humoral immunity in neonates (Manickan et al. 1997; Prince et al. 1997; Sarzotti et al. 1997; Bot et al. 1998). Increasing the content of CpG oligonucleotides or co-administration of plasmid DNA-encoding, Th1-driving cytokines (IL-12 or IFN-γ) has augmented Th1-type responses where additional DC maturational signals were found to be required in neonates (Kovarik et al. 1998). Overall, neonatal DNA vaccines, rather than proving tolerogenic, are substantially more effective in inducing Th1 responses than conventional vaccines.

Another theoretical concern is that autoimmune responses could occur as a result of immune-mediated destruction of plasmid DNA-transfected cells expressing antigen. Cytolysis of antigen-expressing cells would release self-antigens for uptake and presentation by DCs, theoretically capable of initiating autoreactive T- and B-cell responses. However, presentation of self-antigen occurs during normal cell turnover and in the course of viral and bacterial infections, without pathological sequelae. In this respect, it appears unlikely that DNA vaccines would pose any greater risk in initiating organ-specific autoimmunity than conventional viral or bacterial vaccines. A theoretical concern that has not been addressed to date is the ability of DNA vaccines to induce responses to cryptic or subdominant epitopes, which theoretically may crossreact with 'self' and trigger potentially autoreactive T cells.

Potential for induction of anti-DNA antibodies

Concerns that DNA vaccines may promote the development of autoantibodies to double-stranded DNA (dsDNA) (believed to be the hallmark of autoimmune disease such as systemic lupus erythematosus (SLE)) arise from a series of observations. Bacterial DNA has been reported to induce anti-dsDNA antibodies in normal mice and to accelerate the development of autoimmunity in strains genetically predisposed to lupus disease (Gilkeson et al. 1993). Bacterial CpG can also stimulate the production of IL-6, block apoptosis of activated lymphocytes, and block the suppressive effect of regulatory T cells (Krieg et al. 1995; Klinman et al. 1996; Pasare and Medzhitov 2003), mechanisms that may facilitate the persistent activation of B cells and predisposition to the development of SLE. Although an increase in IgG anti-DNA-secreting cells has been reported immediately after repeated immunization of normal mice with plasmid DNA, the increases were small when compared with the spontaneous production of autoantibodies reported in lupus-prone mice (Mor et al. 1997). Furthermore, the transient increase in serum IgG anti-DNA reported did not result in either the development of disease in normal mice or acceleration of disease in lupus-prone strains of mice (Mor et al. 1997). Although the possibility remains that a subset of DNA vaccines encoding determinants crossreactive with self-antigens may stimulate an autoimmue response, current data suggest that the level of anti-DNA antibody induced by plasmid DNA is unlikely to induce systemic autoimmune disease.

CLINICAL TRIALS

The first clinical trials were designed to determine whether DNA vaccines were safe, well tolerated, and immunogenic in normal healthy adults. A naked anti-malaria DNA vaccine administered by the intramuscular route was well tolerated up to a 2.5 mg dose and induced CTLs in 11 of 17 individuals, in the absence of detectable antibody (Wang et al. 1997). Pilot studies with an HIV *env/rev* and an HIV *gag/pol* DNA vaccine administered by the intramuscular route also demonstrated that weak T-cell responses were induced in the absence of adverse

local or systemic effects at doses as high as 3 mg (Boyer et al. 2000; Tellez et al. 2000). A candidate HIV-1 clade A (HIVA) DNA vaccine (containing more than 25 HIV CTL epitopes fused in tandem to the HIV *p17* and *p24* gene) has undergone phase 1 clinical trials in the UK and Kenya. The vaccine appears to be well tolerated and CTL responses have been detected in a number of vaccinees (McMichael et al. 2002). Ongoing studies, sponsored by the UK Medical Research Council and the International AIDS Vaccine Initiative (IAVI) are testing an HIVA DNA vaccine augmented by an MVA boost (coding the same HIV epitopes). Data reported from individuals receiving the combination vaccine are encouraging for the prime-boost approach and indicate that eight out of nine volunteers elicited CTL responses after the boost (McMichael et al. 2002; Mwau et al. 2004). Similar findings have been reported from a phase 1 trial of an HIV clade B *gag* DNA vaccine boosted with an adenoviral vector encoding the cognate HIV antigens (Emini 2002).

CONCLUSIONS

DNA vaccines have advanced rapidly in the past 10 years from laboratory phenomena into clinical trials. We now have a broader understanding of how they work and the limitations of the first generation of naked DNA vaccines in nonhuman primates, and have an appreciation of their safety profile. Several promising technologies are under development for increasing DNA vaccine potency. Data from the preliminary phase 1 clinical trials are encouraging and indicate that DNA priming followed by recombinant virus boost works significantly better than naked DNA alone. As newer technologies proceed into the clinic, the true potential of this vaccine technology will be established.

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Adjuvants and subunit vaccines

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Adjuvants are substances that, when used in combination with vaccine antigens, induce a stronger and more effective response to the vaccine compared with that induced by the vaccine alone. Most vaccines currently used, especially those consisting of inactivated (killed) microorganisms and those containing purified molecules, are given together with adjuvants. Despite the major efforts made in the past decades to develop new vaccine adjuvants (Edelman 2000), the first adjuvants ever reported in the scientific literature, i.e. aluminum salts, still remain the standard adjuvants licensed for use in human beings.

The development of aluminum salts as the first adjuvants and the development of the first subunit vaccines are strictly linked together. An ideal subunit vaccine is a vaccine that contains the microbial antigen(s) critical in the pathogenesis of the infection, against which we want to induce a protective immune response. Thus, historically bacterial toxins in their inactivated forms remain the first subunit vaccines ever developed. This was made possible by the demonstration of the antitetanus immunity conferred to rabbits by immunization with attenuated tetanus toxin (von Behring and Kitasato 1890), and by the demonstration that formalin treatment fully inactivated the diphtheria toxin and guinea-pigs immunized with the formalin-treated toxin were protected against a lethal challenge (Glenny and Hopkins 1923; Ramon 1923). The first attempts to enhance the immunogenicity of these toxoids in animals were reported by Ramon (1925), who observed that the highest antibody titers were obtained in animals in which local inflammatory reactions were induced by the concomitant injection of other substances, such as sterilized tapioca, aluminum salts, lanolin, tannin, kaolin, etc. The use of aluminum hydroxide as an adjuvant to increase the immunogenicity of the diphtheria toxoid was introduced the following year by Glenny et al. (1926).

The approaches toward the development of new vaccines foresee, more and more, the use of purified components of the microorganisms, as native or recombinant proteins, or as synthetic peptides, either alone or conjugated to bacterial poly- or oligosaccharides. These highly purified proteins are less immunogenic than the traditional toxoids and inactivated vaccines, which contain impurities and are much less characterized at the molecular level. Thus, for new generation vaccines adjuvants stronger than aluminum salts are required to induce appropriate effective protective immunity. The correct construction of the vaccine molecule and the use of an appropriate adjuvant system will also be crucial for the induction of the most suitable immune effector mechanisms necessary to confer protection. We had to wait until the second half of the 1990s to see new vaccine adjuvants being approved for use in human beings. Several reasons may have limited the adoption of new vaccine adjuvants (Del Giudice et al. 2001b), among which are safety issues, and the appropriateness of a given adjuvant for the particular vaccine in terms of physical interaction, quality of the effector immune response to induce, and route of immunization that one wants to use. There is, however, hope that research now in progress will result in the development of new adjuvants to be used with existing vaccines, or with newly developed subunit vaccines.

ADJUVANTS

General considerations on mechanisms of action and routes of delivery

Despite the fact that vaccine adjuvants have been in use for more than 70 years, very little is known about the exact mechanisms involved in their biological effects. The depot effect postulated for aluminum salts and other adjuvants cannot by itself explain all the immunological phenomena that are triggered by vaccine adjuvants and bring about the activation and recruitment of professional antigen-presenting cells (APC) which, in turn, induce activation and expansion of antigen-specific T- and then of B-cell populations. The depot effect of aluminum salts, resulting in a reduced rate of antigen clearance from the site of injection, was first suggested by Glenny et al. (1931). However, a large series of experimental data has clearly shown that a slow release of the vaccine over time is very unlikely to contribute to the adjuvanticity of aluminum compounds (Gupta et al. 1996). Very many adjuvants, including aluminum salts, have been postulated to exert their adjuvanticity through their ability to activate APCs and increase the expression of major histocompatibility complex (MHC) class II molecules and several co-stimulatory molecules (Ulanova et al. 2001); however, the exact mechanisms leading to these phenomena are still unclear.

Over the past few years, there have been major advances in the field of innate immunity (Janeway and Medzhitov 2002) which have led to the recognition of several molecules and their relevant ligands as important elements in first-line defense against invading microorganisms. These findings are turning out to be instrumental in understanding the mechanisms of adjuvanticity of several substances, mainly those derived from bacterial components. The family of Toll-like receptors (TLR), first described in *Drosophila*, is emerging as a key player in the delivery of initial signals not only by invading pathogens, but also by some of their components, which may eventually be used as vaccine adjuvants.

Activation of cells by bacterial lipopolysaccharide (LPS) requires the presence on APCs of an intact TLR4: this will induce the expression of inflammatory cytokines and co-stimulatory molecules (Medzhitov et al. 1997). In addition to LPS, TLR4 is also implicated in the recognition of the heat shock protein (HSP) of 60 kDa (hsp60) (Ohashi et al. 2000) and lipoteichoic acid (Takeuchi et al. 1999), which have been known for some time to have adjuvant activity (Edelman 2000).

By forming heterodimers with TLR1 and TLR6, TLR2 recognizes several microbial components, such as peptidoglycan, bacterial lipoproteins, and zymosan (Aliprantis et al. 1999; Brightbill et al. 1999). It is likely that the strong adjuvant activity of complete Freund's adjuvant, which contains mycobacteria, and of monophosphoryl lipid A (MPL), derived from the bacterial cell wall, may be at least partly explained by the interaction with TLR2.

Finally, the strong immunostimulatory effect of unmethylated CpG DNA oligodeoxynucleotides (see below and see Naked DNA vaccines), which leads to strong induction of a T-helper (Th) 1)-type response, has been shown to be mediated by the specific recognition of TLR9 by these motifs (Hemmi et al. 2000). This leads to activation of macrophages and dendritic cells (DC), upregulation of MHC class I molecules and co-stimulatory molecules, and the production of proinflammatory (Th1-type) cytokines.

Several adjuvants do not, however, contain any microbial components and, thus, there is no indication that their mechanism(s) of action is mediated by interactions with TLR, e.g. it is very unlikely that aluminum salts exert their adjuvanticity through a TLR, because aluminum hydroxide behaves as a good adjuvant in genetic knockout mice lacking MyD-88, an adaptor molecule in the TLR-signaling pathway (Schnare et al. 2001).

Another point deserves comment. It is now very well known that adjuvants can profoundly affect the polarization of the T-cell response (and thereby of the effector – and possibly protective – immune response) induced by vaccines. Just to give a few examples, it is known that aluminum adjuvants can induce, in animals and humans, the production of antigen-specific IgE antibody (Nagel et al. 1979; Gupta and Relyveld 1991). This is very probably mediated by the high propensity of aluminum salts to polarize the $CD4^+$ T-cell response toward a Th2-type response, through an increased expression of IL-4 (Ulanova et al. 2001). At the other extreme, there is the example of the unmethylated CpG DNA oligodeoxynucleotides (see below), which lead to an exquisite, strongly polarized $CD4^+$ Th1-type response. In addition, activation of antigen-specific cytolytic $CD8^+$ cells may require the use of other adjuvants and/or delivery systems, such as bacterial or viral delivery systems, immunization with naked DNA, lipidation of the antigen, etc., most of which are discussed in other chapters of this book (see The role of CD8 T cells in the control of infectious disease and malignancies; New vaccine technologies, and Live vaccine carriers), and will not be specifically covered here.

Finally, because of their intrinsic anatomical characteristics and being the portal of entry of most pathogens, mucosal surfaces represent ideal sites for vaccine delivery. Live-attenuated vaccines (e.g. polio and salmonella vaccines) are given orally. However, nonreplicating, purified antigens are poorly immunogenic when delivered mucosally and may even induce a state of tolerance. The best mucosal immunogens are those with an inherent ability to attach to epithelial cells, e.g. the *Escherichia coli* heat-labile enterotoxin (LT), cholera

toxin (CT), bacterial fimbriae, some lectins, etc. (De-Aizpurua and Russell-Jones 1988). The existing adjuvants and the vast majority of those under investigation are specifically designed for parenteral administration. It is thus clear that strong and safe adjuvants specifically designed for delivery of vaccines at the mucosal surface are strongly needed. A lot of exciting research is currently being devoted to this issue, and it is briefly summarized in this chapter.

New vaccine adjuvants

It goes without saying that vaccine adjuvants can be evaluated as such only when they are associated with a vaccine. Consequently, in the following sections, adjuvants are always discussed in the context of their use in association with specific subunit vaccines. Space limitations preclude detailed discussion of all adjuvants under experimental and clinical evaluation (Table 1 gives an overview). Experimental adjuvants, such as complete and incomplete Freund's adjuvants (Freund et al. 1937), are not considered because of the major safety issues linked to them, although vaccines adjuvanted with incomplete Freund's adjuvant have been developed for veterinary use (McKercher and Graves 1977), and some have even been employed in humans in the past (Salk et al. 1952). Major emphasis will be given to those innovative adjuvants that in recent years have successfully become a reality in the vaccine field and to those that are receiving more attention within the scientific community. Readers interested in more detailed, technical information on specific adjuvants not covered here are referred to the literature published in the past few years (Edelman 1997; O'Hagan 2000; O'Hagan et al. 2001; Kenney et al. 2002).

ADJUVANTS LICENSED FOR USE IN HUMANS

MF59

MF59 is an oil-in-water emulsion consisting of uniformly small (<250 nm in diameter) and stable droplets of the natural and fully metabolizable oil squalene (a natural metabolite of cholesterol and a normal component of cell membranes), which is stabilized by the addition of two emulsifiers (Ott et al. 2000). The adjuvant is very stable over several years at 4°C and exhibits high versatility because it can be successfully formulated with fully soluble antigens (e.g. viral recombinant antigens), hydrophobic proteins (e.g. the influenza virus hemagglutinin (HA)), and particulate antigens (e.g. the hepatitis B virus (HBV) surface antigen (HbsAg) containing the pre-S2 region).

The fine mechanisms of the adjuvanticity of MF59 are not fully understood yet. The depot effect does not appear to play a major role because the adjuvant does not affect the distribution of the co-injected vaccine antigen (Dupuis et al. 2000). Two days after intramuscular injection, MF59 localizes in cells with the characteristics of mature macrophages. This adjuvant clearly induces an influx of macrophages at the site of injection, which appears to depend on the presence of chemokine receptor 2 (Dupuis et al. 2001). Irrespective of the mechanism(s) of adjuvanticity, from the data available it is clear that MF59 preferentially polarizes the immune response toward a Th2-type functional phenotype, as evidenced by the preferential induction of antigen-specific IgG1 antibody and Th2-type cytokines such as interleukin 4 and 5 (IL-4 and IL-5), using a variety of model vaccines (Valensi et al. 1994; Singh et al. 1998).

The successful results obtained with this adjuvant in the preclinical setting were instrumental in the move toward clinical studies in humans which, as in the case of influenza, have led to the licensing of the MF59-adjuvanted vaccine. Indeed, MF59 strongly enhanced (from 5 to >100 times) the immunogenicity of flu vaccines in various animal species (Ott et al. 1995). It fully restored the ability of old mice to mount a strong antibody response to flu vaccine equal to that observed in young, fully immunologically competent mice (Higgins et al. 1996), and increased the protective efficacy of the vaccine, even at lower doses (Cataldo and Van Nest 1997). In addition, this enhanced response was not affected by pre-existing immunity against the influenza virus (Higgins et al. 1996). This is particularly important because flu immunization is carried out every year and pre-existing immunity can negatively influence the efficacy of subsequent immunizations. The very strong adjuvanticity of MF59 has also been demonstrated with several other viral and bacterial vaccines, not only in mice, but also in monkeys, e.g. MF59 induced up to a 127-fold increase of the immunogenicity of a Chinese hamster ovary (CHO) cell-derived HBV vaccine containing the pre-S2 region compared with conventional, alum-adjuvanted vaccine, with protective antibody titers persisting for several months (Traquina et al. 1996). Similarly, in baby baboons it dramatically enhanced the protective (bactericidal) antibody response after immunization of conjugate vaccines against *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis* group C (MenC) (Granoff et al. 1997). Finally, MF59 induced a strong and persisting antibody response against recombinant FimH from uropathogenic *E. coli* in cynomologus monkeys, which were protected against bacteriuria and pyuria induced by an infectious challenge (Langermann et al. 2000).

Conventional, nonadjuvanted influenza vaccines exhibit a 70–90 percent efficacy in young adults (Advisory Committee on Immunization Practices 2001). However, this efficacy dramatically drops in older individuals, with a prevention of the clinical illness in only 5–33 percent of the immunized patients (Strassburg et al. 1986), probably as a result of reduced immunogenicity of influenza vaccines in older individuals (Keren et al. 1988). It is thus clear that more efficacious, improved

Table 1 *Vaccine adjuvants under investigation: state of advancement*

Adjuvant	Clinical experience	Licensure
Mineral salts		
Aluminum hydroxide	✓	✓
Aluminum phosphate	✓	✓
Calcium phosphate	✓	✓
Bacterial products		
Cell wall skeleton of *Mycobacterium phlei* (Detox)	✓	
Muramyl dipeptides and tripeptides:		
Threonyl MDP (SAF-1)	✓	
Butyl-ester MDP (Murabutide)	✓	
Dipalmitoyl phosphatidylethanolamine MTP	✓	
Monophosphoryl lipid A (MPL)	✓	
MPL + alum (AS04)	✓	
Lipid A derivatives (e.g. OM 174)	✓	
CpG oligonucleotides	✓	
E. coli heat-labile enterotoxin (LT), LT mutants, LTB	✓	
Cholera toxin B subunit (CTB), CT mutants	✓	
Trehalose dimycolate		
Emulsions		
Water-in-oil emulsions:		
Mineral oil (Freund's incomplete)	✓	
Vegetable oil (peanut oil)	✓	
Squalene and squalane	✓	
Montanide ISA 720, ISA 51	✓	
Oil-in-water:		
MF59 (squalene + Tween80 + Span 85)	✓	✓
Surface-active agents		
Nonionic block polymer surfactants	✓	
Saponin (QS-21)	✓	
QS-21 + MPL (AS02)	✓	
Dimethyl dioctadecyl ammonium bromide (DDA)		
Microparticles		
Virosomes	✓	✓
Liposomes	✓	
Immunostimulating complexes (ISCOMs and Iscomatrix)	✓	
Meningococcal outer membrane proteins (proteosomes)	✓	
Biodegradable polymer microspheres:		
Polylactide and polyglycolide	✓	
Polyphosphazenes	✓	
Chitosan and derivatives	✓	
Polyanions		
Dextran		
Double-stranded polynucleotides		
Polyacrylics		
Polymethylmethacrylate		
Acrylic acid cross-linked with allyl sucrose (Carbopol)		
Cytokines and complement factors		
IL-2	✓	
IFN-α	✓	
IFN-γ	✓	
GM-CSF	✓	
IL-12	✓	
IL-6		
C3d		

vaccines against influenza are needed. When given to older people, the MF59 trivalent subunit vaccine against influenza exhibited a very good safety profile, with local reactions that were mild and of short duration and, importantly, did not increase after repeated immunizations in the following influenza seasons (Minutello et al. 1999; Podda 2001). A consistent finding of several clinical trials carried out was increased immunogenicity of the MF59-adjuvanted vaccine compared with the conventional vaccine, which was observed even after repeated immunizations (Minutello et al. 1999; Podda 2001). This confirmed the lack of interference by preexisting immunity previously observed in animals (Higgins et al. 1996). Two observations are of particular interest. First, a significant increased immunogenicity of the MF59-adjuvanted vaccine was observed in older participants with chronic diseases, such as respiratory and cardiovascular diseases, and diabetes mellitus. Second, the use of MF59 increased the immunogenicity to heterovariant strains of the influenza virus (Podda 2001). More recently, MF59 was shown to enhance dramatically the immunogenicity to an A/H5N3 antigen, from a potentially pandemic strain of influenza virus (Nicholson et al. 2001). This study clearly showed that protective antibody titers were reached with reduced amounts of flu antigens per dose if the adjuvant was used. In addition, the induced antibody persisted for a much longer time and was significantly boosted by a secondary immunization 16 months after the first (Stephenson et al. 2003).

The MF59-adjuvanted subunit vaccine against influenza is now licensed and available in most European countries. The clinical experience with the MF59 adjuvant is much wider than its use with the influenza vaccine. MF59 has been tested in formulation of vaccines against HBV, hepatitis C virus (HCV), herpes simplex virus (HSV), human papillomavirus (HPV), cytomegalovirus (CMV), human immunodeficiency virus-1 (HIV-1), and is being also tested in humans with bacterial vaccines (Podda and Del Giudice 2003). Two major findings from these studies should be highlighted. First, MF59 was shown to enhance strongly the immunogenicity of an HBV vaccine containing the pre-S2 region, conferring seroprotection to 89 percent of immunized patients already after the first dose of the vaccine (Heineman et al. 1999). Second, recombinant proteins from CMV and HIV-1 formulated with MF59 and given to toddlers (second year of life) and newborns (within the first 3 days of life) were more immunogenic and safer than the same vaccines given to adults (Borkowsky et al. 2000; Cunningham et al. 2001; McFarland et al. 2001; Mitchell et al. 2002). Furthermore, as in the case of flu vaccine, the use of the MF59 adjuvant allowed the use of a significantly reduced amount of vaccine to induce high antibody and cellular immune responses, compared with the same vaccine given with alum as adjuvant (Borkowsky et al. 2000).

Virosomes

Virosomes are spherical, unilamellar vesicles with a mean diameter of 150 nm containing 70 percent egg yolk phosphatidylcholine and 20 percent synthetic phosphoethanolamine. The influenza virus hemagglutinin (HA) and trace quantities of viral neuraminidase (NA) from the H1N1 A/Singapore 6/86 virus strain are intercalated within the phospholipid bilayer. The role of the HA in the preparation of these vesicles appears to be that of binding to sialic residues on the cells to enhance endocytosis and fusion of the virosome with endosomes, with the final objective of enhancing the uptake of the associated vaccine and its resulting immunogenicity (Gluck 1999).

Several vaccines have been formulated with virosomes and tested for their safety and immunogenicity in clinical trials in humans, among which are the inactivated hepatitis A virus (HAV) vaccine (Holzer et al. 1996), the influenza subunit vaccine (Conne et al. 1997), diphtheria and tetanus toxoids (Zurbriggen and Gluck 1999), and vaccine combinations (Mengiardi et al. 1995). Virosome-adjuvanted vaccines against HAV and influenza virus have been licensed in several countries. More recently this technology has been applied to the delivery of synthetic peptides from malaria parasites (Poeltl-Frank et al. 1999; Moreno et al. 2001) and from HCV (Hunziker et al. 2002), showing the possibility of inducing priming of peptide-specific cytotoxic T lymphocytes (CTL).

ADJUVANTS AT AN ADVANCED STAGE OF DEVELOPMENT

MPL, QS-21, and their association (AS02)

Monophosphoryl lipid (MPL) A is derived from the LPS of *Salmonella minnesota*. Its adjuvant activity seems to be mediated by its interaction with the TLR2. MPL appears to enhance the migration and maturation of DCs (De Becker et al. 2000) and to promote the generation of Th1-type responses, with enhanced expression of the B7.1 co-stimulatory molecule, and the production of IL-2, IFN-γ, and IL-12 (Moore et al. 1999). The preferential activation of Th1-type cells was also demonstrated in vivo using a 3-deacylated form of MPL. Indeed, immunization of cotton rats with a recombinant respiratory syncytial virus (RSV) chimeric glycoprotein (Prince et al. 2000) or a formalin-inactivated RSV vaccine together with MPL (Prince et al. 2001) significantly reduced the degree of Th2-mediated alveolitis and interstitial pneumonitis after challenge with infectious virus.

MPL has been tested in humans using vaccines against different pathogens, cancer, and allergies. However, probably as a result of not having a very strong intrinsic adjuvanticity and/or an appropriate safety profile, MPL is now being used mainly in association with other adjuvants. One of the best associations studied is with

aluminium salts (referred to as AS04, formerly SBAS4). This adjuvant combination enhanced the immunogenicity of HBV vaccine given to volunteers with different immunization schedules (Thoelen et al. 1998; Ambrosch et al. 2000). This vaccine, however, induced a higher local reactogenicity than the conventional alum-adjuvanted vaccine, and did not enhance the immune response in HLA-DQ2-positive participants, who are poor responders to the HBV vaccine (Desombere et al. 2002).

The other well-investigated association is the one with QS-21, referred to as AS02 and formerly SBAS2. QS-21 is an acylated 3,28-*o*-bisdesmodic triterpene saponin (M_r = 1990 Da) derived from the bark of the South American tree *Quillaja saponaria* Molina (Jacobsen et al. 1996). Tested in different animal models and also in several human trials with different vaccine constructs (against HIV-1, influenza, HSV, HBV, malaria, cancer, etc.), QS-21 appears to augment both Th1- and Th2-type responses (Moore et al. 1999) and to favor the in vivo priming of antigen-specific $CD8^+$ cytotoxic cells (Newman et al. 1997), although this last activity still remains controversial (Evans et al. 2001). In HIV clinical trials comparing different adjuvants, QS-21 was associated with moderate-to-severe local reactions (Keefer et al. 1997); it appears, however, that these can be reduced by decreasing the amount of adjuvant used.

The combination of MPL and QS-21 adjuvants in an oil-in-water formulation (AS02) has been clinically tested in tuberculosis, HBV, HPV, HIV-1, cancer, etc. (Kenney et al. 2002). The best experience of this adjuvant system is, however, with a malaria vaccine both in challenge trials and in efficacy trials in endemic areas. A recombinant *Plasmodium falciparum* circumsporozoite vaccine fused with HBsAg plus AS02 (referred to as RTS,S/AS02) protected six of seven immunized volunteers against a challenge with infectious sporozoites. Interestingly, in this study protection correlated well with anti-sporozoite antibody titers, and was not observed in volunteers immunized with the same vaccine formulated with the AS04 (MPL + alum) adjuvant system (Stoute et al. 1997). Six months after the last immunization specific antibody titers decreased to a half/third of the peak titers. After a new challenge with sporozoites, only two of the previously protected participants were still protected, and no correlation was observed between antibody titers and protection (Stoute et al. 1998). The immune response to the vaccine was clearly polarized toward a Th1-type functional phenotype (Stoute et al. 1998; Lalvani et al. 1999). In a subsequent challenge study in a larger number of participants receiving different immunization schedules, this vaccine exhibited an overall protective efficacy of 41 percent (Kester et al. 2001). When tested in malaria-endemic areas to evaluate its protective efficacy against natural *P. falciparum* infection, the RTS,S/AS02 vaccine showed a 34 percent efficacy (71 percent in the first week of follow-up, 0 percent in the last 6 weeks). After a fourth dose of the vaccine 1 year later, the efficacy was 41 percent (Bojang et al. 2001).

This adjuvant system is now under evaluation using other plasmodial antigens as vaccine candidates. An AS02-adjuvanted LSA-3-based vaccine conferred protection to chimpanzees against a sporozoite challenge (Daubersiers et al. 2000). However, an AS02-adjuvanted RTS,S vaccine containing the sporozoite protein TRAP was not efficacious in the human challenge model (Kenney et al. 2002).

ISCOMs and Iscomatrix

Immunostimulating complexes (ISCOM) are cage-like structures of about 40 nm diameter containing the saponin Quil A, cholesterol, and phospholipids. Antigens are incorporated within the ISCOM structure. However, not all antigens incorporate spontaneously into ISCOMs, unless extensive antigen modifications are introduced. Iscomatrix is totally identical to ISCOMs except for the fact that antigen is not incorporated, but simply mixed with the particles or chemically coupled to them (Barr et al. 1998; Sjolander et al. 1998; Sjolander et al. 2001). Interestingly, ISCOMs have been shown in different models to exert their adjuvanticity not only after parenteral, but also after mucosal, administration (Hu et al. 2001). The adjuvant effect of ISCOMs both parenterally and mucosally requires the induction of the production of IL-12. Indeed, their adjuvanticity is significantly reduced in IL-12 knock-out mice, but not in mice lacking the IL-6, IL-4, or IFN-γ receptors, or the inducible nitric oxide synthase (Grdic et al. 1999; Mowat et al. 1999; Smith et al. 1999). It has been proposed that ISCOMs act by specifically targeting adjuvant and antigen to APCs, such as macrophages and/or DCs (Mowat et al. 1999). This peculiar targeting property may allow the antigen to enter the route of antigen processing that will result in the presentation of relevant epitopes within the context of MHC-I antigens, thus leading to the priming of antigen-specific MHC-I-restricted $CD8^+$ CTLs. Indeed, this is certainly the case in mice (Sambhara et al. 1998) and priming of CTL responses has also been shown in monkeys (Sjolander et al. 2001). The induction of a strong and long-lasting CTL response has been shown in rhesus monkeys immunized with the core protein of HBV adsorbed on to Iscomatrix (Polakos et al. 2001). In this and other experiments in rhesus monkeys, ISCOM-adjuvanted vaccine induced strong proliferative responses of $CD4^+$ cells able to produce both Th1- and Th2-type cytokines (Verschoor et al. 1999).

ISCOMs have been tested in a wide variety of animal species (Barr et al. 1998; Sjolander et al. 1998). In addition, ISCOM-adjuvanted vaccines have been clearly shown to confer protection against infectious challenge in relevant animal models, e.g. against genital infection

with *Chlamydia trachomatis* in mice (Igietseme and Murdin 2000) and HIV-1 in rhesus monkeys (Verschoor et al. 1999). It must also be mentioned that immunization of macaques with ISCOMs containing measles virus HA and fusion proteins induced high titers of neutralizing antibodies even in the presence of maternal antibodies, which, in monkeys receiving conventional attenuated measles vaccine, were able to abrogate the induction of protective antibodies (Van Binnendijk et al. 1997).

The results of the first trials in humans with Iscomatrix-adjuvanted influenza vaccines have been reported recently. In one study, the ISCOM-adjuvanted influenza vaccine induced $CD8^+$ CTL responses in 50–60 percent of participants compared with only 5 percent of those receiving the conventional vaccine (Ennis et al. 1999). In a subsequent study, the adjuvanted vaccine accelerated the appearance of anti-influenza virus antibody response against the three strains present in the vaccine, induced strong antigen-specific proliferative responses, and induced CTL responses in a higher proportion of vaccinees (Rimmelzwaan et al. 2001).

CpG

The immunostimulatory properties of bacterial DNA have been known for some time (see Naked DNA vaccines). However, only relatively recently this property was recognized to be linked to the presence of unmethylated CpG motifs in bacterial DNA, which are absent in vertebrate DNA. CpG motifs are C-G deoxynucleotides linked by a phosphodiester bond, and flanked by appropriate bases, which can vary depending on the animal species in which they will be used (Krieg 2002). The immune modulatory effects of CpG motifs depend on their interaction with the TLR9. Mice lacking the TLR9 do not exhibit the CpG-driven effects (Hemmi et al. 2000). In addition, cells transfected with the murine or human receptor acquire the ability to respond to the more appropriate CpG motifs (Bauer et al. 2001). In humans the expression of TLR9 is highest in those cells that are more consistently directly activated by CpG motifs, such as B cells and DCs (Bauer et al. 2001; Krug et al. 2001). CpG motifs induce strong activation of B cells with production of IL-6 (Yi et al. 1996a); they also increased expression of MHC-II molecules and co-stimulatory molecules such as CD80 and CD86 (Davis et al. 1998), and an overall anti-apoptopic effect associated with increased NFκB activity and c-*myc* expression (Yi et al. 1996b). CpG motifs strongly activate plasmacytoid DCs inducing increased expression of MHC, adhesion, and co-stimulatory molecules, and enhanced ability to produce cytokines such as TNFα, IFN-α, and IL-12 (Hartmann and Weiner 1999; Kadowaki et al. 2001; Krug et al. 2001). The outcome of these activation phenomena will be the polarization of the immune response toward a strong Th1-type functional phenotype. This polarization is so prominent that it also takes place when CpG oligonucleotides are administered in association with other adjuvants, such as aluminum salts, known specifically to induce Th2-type responses, and when they are given neonatally in individuals who are prone to develop Th2-type immune responses (Brazolot Millan et al. 1998). These characteristics are now being exploited for the development of vaccines against cancer and allergies (Krieg 2002). CpG has also been shown to act as a strong mucosal adjuvant (McCluskie et al. 2001).

CpG motifs are now under investigation as vaccine adjuvants in different models, e.g. they have shown optimal adjuvanticity in rhesus monkeys immunized with a heat-killed vaccine candidate against *Leishmania* (Verthelyi et al. 2002) and in orangutans, in which they overcome hyporesponsiveness to an HBV vaccine (Davis et al. 2000). In human phase 1 studies with HBV vaccine, CpG motifs were well tolerated, except for an increased frequency of local pain and flu-like symptoms compared with the currently available vaccine, and induced protective levels of antibodies in 75 percent of vaccinees after the first dose and 100 percent after the second dose (Kenney et al. 2002).

CpG DNA is among the strongest adjuvant systems available to date for the induction of Th1-type immune responses. However, some concerns have been raised about the risk of inducing or reactivating autoimmune phenomena in susceptible individuals, because of their strong immunomodulatory activity mainly at the level of B cells and DCs (Bachmaier et al. 1999; Tsunoda et al. 1999; Miyata et al. 2000) and their ability to activate microglial cells in vitro and in vivo (Dalpke et al. 2002).

Mutants of bacterial toxins as mucosal adjuvants

The ADP-ribosylating toxins LT and CT remain the strongest mucosal adjuvants known so far. Their toxicity, however, seriously limits their use in humans because very limited amounts cause diarrhea in recipients (Levine et al. 1983). These toxins consist of two subunits: the A subunit, which contains the enzymatic activity, and the B subunit, which binds to the G_{M1} ganglioside and other glycolipids. After internalization into the cell, the A subunit of the toxin is proteolytically cleaved, binds to NAD, and transfers the ADP-ribose group to the α subunit of Gs, a GTP-binding protein that regulates the activity of adenylyl cyclase (Holmes 1997). This enzyme becomes permanently activated and causes accumulation of cAMP and secretion of electrolytes and water (Field et al. 1989).

The first approach to develop mucosal adjuvants derived from these toxins, but devoid of their toxic activity, was to use their B subunit (Holmgren et al. 1997). It is now clear, however, that the B subunit alone is a very poor adjuvant. The activity originally reported

was very probably the result of traces of wild-type toxin still contaminating the preparation of the B subunit. The most successful approach to develop nontoxic derivatives of the toxins while still retaining their strong mucosal adjuvanticity has been to produce mutants by site-directed mutagenesis (Freytag and Clements 1999; Rappuoli et al. 1999; Pizza et al. 2001). Some mutations at the active enzymatic site, such as at position 63 in the A subunit (e.g. LTK63 and CTK63 (S → K substitution)), results in total loss of enzymatic activity and toxic properties in vitro and in vivo, even at doses up to 1 mg. Other mutants, such as the LTR72 (in which there is an A → R substitution), retain some residual enzymatic and toxic activity, although at levels several orders of magnitude lower that those observed with the wild-type toxin (Giuliani et al. 1998). Mutants have also been developed to make the toxin resistant to the proteolytic cleavage of the A subunit. The prototype of these mutants, referred to as LTG192 (R → G substitution), however, retains most of the toxic properties of the wild-type parent molecule both in vitro (cytotoxicity on Y1 cells) and in vivo (rabbit ileal loop) (Giannelli et al. 1997).

All these mutants (and others not reported here because of space limitation) behave as strong mucosal adjuvants in mice and other animal models, when co-administered with recombinant proteins, synthetic peptides, and other vaccine constructs, and to favor the development of protective immunity in appropriate challenge models (Pizza et al. 2001). Interestingly, LTK63 and LTR72 mutants also exhibited a strong adjuvanticity when delivered systemically (Jakobsen et al. 2002) and transdermally (Scharton-Kersten et al. 2000). The mucosal adjuvanticity of these mutants was potentiated when the vaccine and the adjuvant were delivered intranasally together with nanoparticles, favoring the uptake of the vaccine at the mucosal level (Baudner et al. 2002). Furthermore, not only did these mutants prime $CD4^+$ T cells; they also primed $CD8^+$ CTLs specific for the co-administered antigen (Simmons et al. 1999), irrespective of the route used for immunization. If CT and CT mutants appear to activate predominantly Th2-type responses (Braun et al. 1999), LT mutants induce more widespread responses, with a preferential induction of Th1-type responses (Takahashi et al. 1996), even in newborn mice that are genetically prone to mount Th2 responses.

From the data available so far, it is clear that the holotoxin structure of the mutants is important for a strong adjuvanticity; indeed, as mentioned above, the B subunit alone is a very poor adjuvant. The enzymatic activity is not necessary itself, because the LTK63 mutant exhibits an important adjuvanticity. A residual enzymatic activity (as in the case of the LTR72 mutant) enhances the adjuvanticity of the molecule in a dose-independent manner.

Clinical experience with these molecules is still limited. The LT or CT B subunits have been extensively tested and shown to be safe after oral delivery together with an inactivated cholera vaccine (Holmgren et al. 1997) and with a vaccine against enterotoxigenic *E. coli* (ETEC) (Hall et al. 2001). The safety of this subunit has also been shown in humans after intranasal immunization (Bergquist et al. 1997). The LTG192 mutant at the level of the protease-sensitive loop has been tested in humans with a whole-cell *Helicobacter pylori* candidate vaccine (Kotloff et al. 2001). In agreement with the animal studies, it caused unwanted effects, such as diarrhea, in about a third of the recipients at doses as small as 25 μg. The LTK63 mutant is now being tested as an intranasal adjuvant in a phase 1 clinical trial together with a subunit influenza vaccine.

SUBUNIT VACCINES

Subunit vaccines consist of one or more antigens, purified from the microorganism or produced by recombinant DNA technology or chemical synthesis, which are able to protect against disease. It is clear that the development of subunit vaccines requires knowledge of the protective antigen(s), the ability to produce and purify them on a large scale, and the ability to prove their protective efficacy in appropriate animal models in vivo and/or in relevant in vitro assays suited to quantify immune responses that correlate with protection. The knowledge of the effector immune response necessary to confer protection in vivo is also very important in order to construct the vaccine with the most suitable formulation (e.g. the most appropriate adjuvant system) and to deliver it via the most effective route. Subunit vaccines against a wide panoply of pathogenic microorganisms are now under development: some are still at the experimental, preclinical stage; others are being tested in clinical trials in humans (at different phase of development); others, finally, have been licensed for human use (Table 2).

The identification of protective antigens is a complex problem, which involves approaches that can differ for viruses, bacteria, and parasites. Viruses have generally small genomes encoding a few proteins, the selection of which for vaccine development is relatively easier than with larger microorganisms. Envelope proteins and glycoproteins are the primary candidates for the induction of neutralizing antibodies, whereas core antigens are usually good candidates for CTL responses. For bacteria and parasites, which have complex genomes, there can be several hundred potential candidate antigens. Therefore, the identification of the potential candidates for the development of subunit vaccines has to be based on a rational approach combining genetic, biochemical, and immunological studies. Until very recently, the starting point was often represented by studies on the pathogenic mechanisms of disease, with the definition of molecules critical in the induction of pathology in the host and/or in the survival of the microorganisms. This approach has been particularly

Table 2 *Status of development of subunit vaccines*[a]

Pathogen	Vaccine construct	Preclinical	Phase of clinical development			Licensure
			I	II	III	
Viruses						
Cytomegalovirus	Recombinant glycoproteins			✓		
Dengue virus	Recombinant envelope	✓				
Ebola virus	Recombinant subunit	✓				
Epstein–Barr virus	Recombinant glycoprotein			✓		
Hantaan virus	Recombinant subunit	✓				
Hepatitis A virus	Proteins in vectors	✓				
Hepatitis B virus	Yeast-derived HBsAg					✓
Hepatitis C virus	Recombinant envelope proteins		✓			
Hepatitis E virus	Recombinant proteins			✓		
Herpes simplex viruses 1 and 2	Recombinant glycoproteins				✓	
HIV-1	Recombinant glycoproteins				✓	
HIV-2	Recombinant glycoproteins	✓				
Human papillomavirus	Recombinant proteins/VLPs				✓	
Influenza virus	Subunit plus MF59					✓
	Recombinant HA			✓		
Measles virus	Recombinant HA and fusion proteins	✓				
Norwalk virus	Recombinant proteins		✓			
Parainfluenza virus	Purified HN and F proteins	✓				
Respiratory syncytial virus	Purified proteins and fusions			✓		
Rotavirus	Recombinant VPs	✓				
Varicella-zoster virus	Recombinant glycoproteins	✓				
West Nile Virus	Recombinant envelope protein	✓				
Bacteria						
Bacillus anthracis	Recombinant subunit	✓				
Bordetella pertussis	Purified/Recombinant antigens					✓
Borrelia burgdorferi	Recombinant OspA					✓[b]
Chlamydia pneumoniae	Purified MOMP, hsp	✓				
Chlamydia trachomatis	Purified major outer membrane protein (MOMP)	✓				
Clostridium botulinum	Toxoid			✓		
Clostridium difficile	Toxoids			✓		
Enterohemorrhagic *E. coli*	Nontoxic mutant toxins/conjugates	✓				
Enterotoxigenic *E. coli*	LTB		✓			
Uropathogenic *E. coli*	Recombinant FimH		✓			
Group A streptococci	Conjugates/M protein			✓		
Group B streptococci	Conjugates			✓		
Haemophilus ducreyi	Various proteins	✓				
H. influenzae nontypeable	Recombinant proteins/Conjugates			✓		
H. influenzae type b	Conjugates					✓
Helicobacter pylori	Recombinant antigens		✓			
Legionella pneumophila	Purified proteins	✓				
Leptospira spp.	Recombinant protein	✓				
Listeria monocitogenes	Recombinant protein	✓				
Moraxella catarrhalis	Various proteins in conjugates	✓				
Mycobacterium tuberculosis	Recombinant proteins	✓				
Mycoplasma pneumoniae	Purified/Recombinant proteins	✓				
Neisseria gonorrheae	Recombinant proteins		✓			
N. meningitidis group A	Conjugates			✓		
N. meningitidis group B	Recombinant proteins		✓			
N. meningitidis group C	Conjugates					✓

(*Continued over*)

Table 2 *Status of development of subunit vaccines*[a] *(Continued)*

Pathogen	Vaccine construct	Preclinical	Phase of clinical development			Licensure
			I	II	III	
N. meningitidis group W135	Conjugates		✓			
N. meningitidis group Y	Conjugates		✓			
Porphyromonas gingivalis	Recombinant proteins	✓				
Pseudomonas aeruginosa	Purified proteins/Conjugates				✓	
Rickettsia rickettsii	Recombinant surface proteins	✓				
Salmonella typhi	Purified Vi					✓
	Conjugated Vi				✓	
Shigella dysenteriae	Conjugate			✓		
Shigella flexneri	Conjugate			✓		
Shigella sonnei	Conjugate				✓	
Staphylococcus aureus	Conjugate				✓	
Staphylococcal enterotoxin B	Recombinant toxin	✓				
Streptococcus pneumoniae	Conjugate					✓
	Recombinant proteins		✓			
Treponema pallidum	Surface lipoproteins	✓				
Vibrio cholerae	Conjugate LPS	✓				
Yersinia pestis	Recombinant subunit	✓				
Fungi						
Blastomyces dermatitidis	Purified/Recombinant proteins	✓				
Candida albicans	Surface oligomannosyl epitope	✓				
Coccidioides immitis	Purified/Recombinant proteins	✓				
Cryptococcus neoformans	Conjugate		✓			
Histoplasma capsulatum	Purified/Recombinant proteins	✓				
Paracoccidioides brasiliensis	Purified/Recombinant proteins	✓				
Pythium insidiosum	Purified proteins	✓				
Parasites						
Ancylostoma duodenale	Recombinant protein	✓				
Brugia malayi	Purified antigens	✓				
Entamoeba histolytica	Recombinant antigens	✓				
Leishmania spp.	Recombinant antigens	✓				
Onchocerca volvulus	Recombinant antigens	✓				
Plasmodium falciparum	Recombinant CSP				✓	
	Recombinant asexual stage antigens			✓		
	Recombinant sexual stage antigens	✓				
Plasmodium vivax	Recombinant cirumsporozoite protein		✓			
	Recombinant asexual stage antigens	✓				
	Recombinant sexual stage antigens	✓				
Schistosoma spp.	Purified/Recombinant larval antigens	✓				
Toxoplasma gondii	Recombinant surface proteins	✓				
Trichomonas vaginalis	Recombinant protein	✓				
Trypanosoma cruzi	Recombinant antigen	✓				

a) Only purified antigens, recombinant antigens, and conjugated constructs are included. Conventional vaccines (e.g. tetanus and diphtheria toxoids), naked DNA constructs, and synthetic peptides are not included.
b) This vaccine is not commercially available any longer.

successful for those molecules produced only by pathogenic bacteria, such as bacterial toxins and capsular polysaccharides (a few examples follow). However, in several instances the situation may be more complex and the approach to vaccine development may lead to the selection of antigens which, after being tested in animals, are discarded because they do not induce protective responses in humans. The selection then starts again. More recently another way of selecting potentially protective antigens has been applied to vaccine development. Thanks to the progress in computer science and molecular biology, it is now possible to sequence the entire bacterial genome in a short period of time. This permits the comprehensive testing of all the potentially protective antigens in appropriate animal models, and to select vaccine candidates that would not

have been selected based on conventional approaches. Examples of the application of both the conventional and genomic approaches to vaccine development follow.

Past and present: from toxoids and capsular polysaccharides to conjugate vaccines

TOXOIDS AND POLYSACCHARIDES

The first subunit vaccines developed have been the diphtheria and tetanus toxoids (Rappuoli 1997a; Relyveld 1996). As already mentioned at the beginning, the seminal observation leading to the development of these vaccines was that both diseases were caused by a toxin that was produced by the bacterium, and that serum antibodies able to neutralize the toxin were sufficient to protect from disease. The semipurified toxins are inactivated essentially by chemical (formaldehyde) treatment and used as vaccines. For these two vaccines, the titers of serum antitoxin antibodies correlate well with protection. Tetanus and diphtheria toxoids are among the most used vaccines worldwide, because they are part of the pediatric immunizations, which are done within the frame of the Expanded Programme of Immunization of the WHO and Unicef.

In the case of encapsulated bacteria, the choice of the capsular polysaccharide as the antigen of choice for the development of subunit vaccines was based on the observation that mutants without the capsule are nonpathogenic because they are highly sensitive to serum complement. In the case of meningococci, group-specific immunity against disease is mediated by serum antibodies directed against the group-specific capsular polysaccharide (Goldschneider et al. 1969a), through a complement-mediated bactericidal activity (Goldschneider et al. 1969b) and opsonophagocytosis (Vioarsson et al. 1994).

Purified capsular polysaccharides have been used to develop vaccines against MenC, *N. meningitidis* group A (MenA), *N. meningitidis* group Y (MenY), and *N. meningitidis* group MenW135 (MenW135) (Jodar et al. 2002; Morley and Pollard 2002), against 23 serotypes of *S. pneumoniae* (Wuorimaa and Kayhty 2002), against Hib (Ward and Zangwill 1999), and against *Salmonella typhi* using the Vi polysaccharide (Plotkin and Bouveret-Le Cam 1995). All these vaccines have several drawbacks. Capsular polysaccharides are T-independent antigens and they induce transient antibody responses (mostly of IgM and IgG2 isotypes) mainly in individuals aged over 18 years. Their immunogenicity and efficacy are very poor or absent in infants. Furthermore, polysaccharides do not induce immunological memory and repeated immunization not only fails to induce any increase in specific antibody titers, but can also in some cases even induce tolerance in adults (Granoff et al. 1998a). In the case of meningococcal meningitis, polysaccharide vaccines have been proved efficacious in adults and in controlling a MenA epidemic in Africa (Hassan-King et al. 1988). However, the vaccine had limited effects on bacterial colonization and transmission (Hassan-King et al. 1988; Wenger et al. 1997). In the case of pneumococci, the efficacy of the 23-valent polysaccharide vaccine against pneumonia and invasive diseases still remains controversial (Wuorimaa and Kayhty 2002). There is agreement, however, on the fact that this vaccine does not have any effect on carriage rates in children and adults (Dagan et al. 1996).

CONJUGATE VACCINES

The solution to these drawbacks was found by putting together the first and the second subunit vaccines, i.e. the toxoids and the polysaccharides. During the 1920s it was formally demonstrated that the immunogenicity of saccharides was significantly increased when animals were immunized with the sugar covalently linked to a protein, which thus behaved as a carrier molecule (Avery and Goebel 1929). It is now clear that this procedure converts T-independent antigens into T-dependent antigens by providing a source of appropriate T-cell epitopes (present in the carrier protein) that are able to prime carrier-specific T cells; these will provide help to B cells for the production of sugar-specific antibodies. This technology has now been successfully applied to the development of conjugate vaccines replacing simple polysaccharide vaccines. The carrier proteins used in the development of conjugate vaccines are essentially tetanus toxoid, diphtheria toxoid, and the nontoxic mutant of diphtheria toxin, the crossreacting material (CRM) 197 (see below). The only limitation that this technology faces may relate to the overload of carrier protein that patients would receive in the situation where all conjugated vaccines used the same carrier. Indeed, carriers not only help the production of anti-oligosaccharide antibodies, they also induce specific antibodies against themselves. Anti-carrier antibodies present at the moment of immunization with a given conjugate vaccine may suppress the induction of anti-oligosaccharide antibodies. This has been well documented in animals (Barrios et al. 1992), although conflicting observations have been reported so far in humans. Nevertheless, active research is now in progress to select new carrier molecules that would avoid the potential drawbacks of epitope suppression, e.g. synthetic peptides containing selected $CD4^+$ epitopes have been used (Alexander et al. 2000). Alternately, recombinant proteins corresponding to strings of several universal $CD4^+$ T-cell epitopes have been successfully tested as carrier proteins in mice (Falugi et al. 2001).

Hib

The first conjugate vaccine developed has been the vaccine against Hib (Ward and Zangwill 1999). Conjugated Hib vaccines have been shown to be highly

immunogenic and efficacious, and to induce immune memory, Ig isotype switching, and antibody affinity maturation in children aged less than 18 months. All these responses could not be induced after the use of unconjugated polysaccharide vaccine. These vaccines have now been introduced in several countries worldwide (often in association with diphtheria, tetanus, and pertussis vaccines), and their use is being implemented in less developed countries. In the countries where it is used to immunize infants, often starting at the age of 2 months, the conjugated Hib vaccine has contributed to the dramatic reduction in the incidence of invasive Hib diseases (Peltola 2000), e.g. in the USA, from its introduction to 1996, the incidence of Hib invasive disease (i.e. meningitis, sepsis) among children aged less than 5 years had declined by more than 99 percent (Anonymous 1998), a decline that has been maintained also in the following years (Anonymous 2002). It is interesting that among the same pediatric population the incidence of invasive disease caused by nontype b *H. influenzae* remained unchanged (>1 per 100 000 children) since the introduction of the Hib vaccine (Anonymous 2002), suggesting that other serotypes are not overtaking, at least up to now, the ecological niche vacated by Hib. The same dramatic drop of incidence of invasive Hib disease has been reported from several countries (Ward and Zangwill 1999), and more recently from a regional study in Italy (Gallo et al. 2002). Immunization clearly also reduces the carriage of Hib, leading to a reduced transmission of the bacteria to unimmunized patients (Murphy et al. 1993).

Meningococci

The successful approach of conjugate vaccine development against Hib prompted several groups and governmental health authorities to consider the development of a conjugate vaccine against MenC. MenC and MenA vaccines had been already developed and tested favorably in humans for their safety and immunogenicity (Lieberman et al. 1996). However, until very recently the development of a MenA vaccine was almost discontinued by major vaccine companies because of the high prevalence of this group of neisseriae in poor countries, such as those in sub-Saharan Africa (Anonymous 1997), whereas the MenC vaccine was fully developed and finally licensed in several countries. The MenC vaccine, containing as carrier protein the CRM197, was strongly immunogenic (Richmond et al. 2001), and in the UK it exhibited an efficacy of 97 percent among adolescents (15–19 year olds) and 92 percent among toddlers (2–3 year olds) (Ramsay et al. 2001). Longer follow-ups will be required to evaluate whether group C meningococci may be replaced by other strains not contained in the vaccine, as observed with the multivalent pneumococcal vaccine (Eskola et al. 2001), or may undergo C-to-B capsule switching as already documented in neisseriae (Vogel et al. 2000). For the time being, vaccine manufacturers are actively working to develop conjugated vaccines against MenA, MenY, and MenW135 to be incorporated into a tetravalent anti-neisserial vaccine. No major technical problems are foreseen for these vaccines. The real obstacle preventing the development of a definitive anti-neisserial vaccine still remains the problem in producing a vaccine against group B meningococci (MenB) (see below).

Pneumococci

Over the past 16 years, since the first report of a monovalent penumococcal conjugate vaccine (Schneerson et al. 1986), mono-, bi-, tetra-, penta-, hepta-, octa-, nona-, and undecavalent conjugated vaccines against pneumococci have been developed using various carrier proteins, and tested in a large number of clinical trials (Wuorimaa and Kayhty 2002). The conjugate vaccine that has been licensed for human use in Europe and the USA contains polysaccharides from seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) conjugated to the CRM197 as carrier protein. This vaccine has been shown to be safe and highly immunogenic in all age groups (Eskola 2000) and to prime memory responses in early life (Ahman et al. 1998). Importantly, it exhibited 56 percent efficacy in preventing acute otitis media caused by the *S. pneumoniae* strains covered by the vaccine (although protection varied widely among serotypes) (Eskola et al. 2001), and 73 percent efficacy in preventing clinically diagnosed and radiologically confirmed pneumonia in infants (Black et al. 2000). Experiments in mice have recently shown that these vaccines can also be delivered mucosally (intranasally) at birth with nontoxic mutants of LT as mucosal adjuvants and can protect animals against a lethal challenge with pneumococci (Jakobsen et al. 2002), suggesting the possibility of immunizing against these pathogens very early in life by alternate routes.

Other conjugate vaccines

A similar approach is now under consideration for the development of subunit vaccines against other pathogens, e.g. a conjugate vaccine consisting of the capsular polysaccharide Vi of *S. enterica* serovar Typhi conjugated to a nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A, was more than 90 percent efficacious against typhoid fever after two immunizations, 6 weeks apart, in 2- to 5-year-old children in a highly endemic area (Lin et al. 2001). Other conjugate vaccines are under development against various serotypes of group B streptococci using the type-specific capsular polysaccharides (Paoletti et al. 2000; Paoletti and Kasper 2002), and against *Shigella sonnei* and *S. flexneri* 2a using the *O*-specific polysaccharide of LPS

(Cohen et al. 1997; Ashkenazi et al. 1999; Passwell et al. 2001).

Recombinant DNA approach for subunit vaccines

It is not always easy to develop subunit vaccines using molecules directly purified from a given microorganism. In several instances, for many reasons, it is necessary to produce the appropriate antigens by genetic engineering. The issue remains, both for native antigens and for recombinant proteins, of how to select the vaccine candidates. The conventional biotechnological approach to identify microbial components suitable for vaccine development generally starts with the pathogens being cultivated in the laboratory. (This implies that this approach can be very difficult or impossible to apply to microorganisms that *cannot* be grown in the laboratory.) The components of the pathogens are then identified sequentially using biochemical, immunological, and genetic methodologies. The identification of potential vaccine candidates requires the separation of each single microbial constituent. This approach takes time, allows only one protein to be taken into consideration each time, and, importantly, permits the identification of only those antigens that are expressed or can be purified in large quantities. In many cases the most abundant proteins are not the most suitable vaccine candidates, and the genetic tools to identify less abundant candidates may be inadequate or not available. It is also possible that antigens expressed during the infection in vivo are not equally well expressed in vitro during cultivation. This approach can take several years to be successful, and in some cases it can totally fail to identify the relevant vaccine candidates. Once a suitable antigen is identified, most often it is expressed in genetically engineered prokaryotic or eukaryotic vectors, and then purified as a recombinant protein. This conventional approach to vaccine development has been successful in some instances, although it took a long time to provide vaccines against pathogens for which the solution was easy. However, this approach has failed (at least up to now) for those complex pathogens, such as bacteria and parasites, for which obvious major protective antigens were not readily available. Some examples of the successes and the failures of the conventional recombinant DNA approach to the development of subunit vaccines follow.

SUCCESSES IN THE DEVELOPMENT OF SUBUNIT VACCINES

HBV vaccine

The first recombinant subunit vaccine developed was the one against HBV (Valenzuela et al. 1982). This development was particularly fortunate because it was already known that the surface antigen of the virus (the HBsAg) circulated in the bloodstream in large quantities, and that the antibody response to the HBsAg was responsible for protection (Mahoney and Kane 1999). The yeast- and mammalian cell-derived vaccine has replaced the first-generation, plasma-derived vaccine. This vaccine is highly effective: it induces seroprotective levels of antibodies (i.e. >10 mIU/ml) in >95 percent of individuals and prevents the development of chronic hepatitis in at least 75 percent of immunized infants born to hepatitis Be antigen (HBeAg)-positive mothers (Poovorawan et al. 1992). This vaccine has now been incorporated into the routine infant or adolescent immunization programs of 129 countries worldwide (Van Damme and Vorsters 2002). In addition to preventing both acute and chronic diseases, mass immunization against HBV has clearly reduced the incidence of hepatocellular carcinoma in children from Taiwan, where it dropped from 0.70/100 000 children in 1981–86 to 0.57 in 1986–90, and further to 0.36 in 1991–94 (Chang et al. 1997, 2000). The HBV vaccine can therefore be considered as the first successful anti-cancer vaccine.

Recent work is now concentrated on improving the efficacy of this vaccine by, for example, using novel adjuvants such as MF59 (Heineman et al. 1999), the AS04 (MPL + aluminum hydroxide) adjuvant system (Desombere et al. 2002), or the CpG oligonucleotides (Davis et al. 2000). The final objective of this work is to reduce the number of nonresponders to the currently available vaccine, and to attempt the development of a therapeutic vaccine for individuals with chronic hepatitis using either available recombinant vaccines or synthetic constructs containing selected HBcAg CTL epitopes (Engler et al. 2001).

Acellular vaccine against pertussis

A genetically detoxified pertussis toxin was the first bacterial recombinant subunit vaccine developed (Rappuoli 1997b; Del Giudice et al. 1998). Instead of preparing toxoids through chemical treatment, as in the case of diphtheria and tetanus toxins, the approach consisted of knocking out the enzymatic (toxic) activity of the pertussis toxin by substituting in the S1 subunit of the amino acids that were critical for enzymatic activity. The mutant at the level of amino acid positions 2 and 179 (Pizza et al. 1989) was further characterized and associated with other *Bordetella pertussis* antigens such as filamentous HA (FHA) and pertactin for the development of an acellular pertussis vaccine (Rappuoli 1997b; Del Giudice et al. 1998). The vaccine containing the genetically detoxified toxin was shown to be safe and efficacious in various trials. It is interesting that, 5–6 years after the primary immunization schedule, this vaccine still exhibited an efficacy of about 80 percent (Salmaso et al. 2001).

The genetic detoxification of ADP-ribosylating toxins was then extended to LT and CT to develop safe and

effective mucosal adjuvants. These molecules have already been discussed above. The genetic detoxification will now also be possible for the anthrax toxin, after the elucidation of its fine structure (Lacy and Collier 2002), for the development of a new generation of safer and more effective anthrax vaccines.

Lyme disease

The search for antigens suitable for the development of protective vaccines against Lyme disease, a tick-borne bacterial infection caused by *Borrelia burgdorferi*, has essentially concentrated on outer surface proteins (Osp). In preclinical studies, the recombinant OspA, OspB, and OspC gave the best levels of protection against infectious challenge (Fikrig et al. 1992). The vaccine developed consisted, however, of one single recombinant – lipidated protein, OspA – which in 1999 was recommended for use in 15- to 70-year-old people living or visiting areas at risk of tick bites (ACIP 1999). In large human trials, this vaccine exhibited an overall efficacy of 80–90 percent after three doses of vaccine (Sigal et al. 1998; Steere et al. 1998). As OspA is expressed by *B. burgdorferi* when it develops in the tick, protection conferred by this vaccine is mediated by a transmission-blocking immunity, through anti-OspA antibodies that are able to destroy and eliminate the spirochetes in the midgut of the ticks (De Silva et al. 1996).

Based on observations made in mice (Feng et al. 1995), some concerns were raised on the risk that Lyme arthritis could be induced by OspA-based vaccines. Human DRB1*0401-positive synovial fluid T cells can respond to OspA; this is most probably the result of the recognition of the OspA amino acid sequence 165–173 which is homologous to a sequence present within the α_L chain of human LFA-1 (amino acids 332–340) (Gross et al. 1998). It is not clear yet whether this vaccine can really induce Lyme arthritis via antigenic mimicry, at least in susceptible individuals. A recent comprehensive post-licensure study on serious adverse events after the administration of over 1.4 million doses of the Lyme disease vaccine did not detect unexpected or unusual patterns. Importantly, the number of reported arthritis-related events were much lower than those expected by the safety data reported in the efficacy trial (Lathrop et al. 2002).

FAILURES IN THE DEVELOPMENT OF SUBUNIT VACCINES

Among the examples of failures of the conventional approach to development of subunit vaccines are three diseases, which account for a heavy burden of morbidity and mortality: AIDS, tuberculosis, and malaria. In these three cases, the complexity of the pathogens, the effector immune mechanisms to be induced, and the systems required for this induction are such that multifaceted approaches are required in order to achieve adequate protective efficacy.

In the case of AIDS, the conventional approaches to vaccine development have essentially focused onto the proteins of the HIV-1 envelope, mainly the gp120, facing the serious problem of antigen variability and, as a consequence, the poor ability of these vaccine constructs to induce antibodies that are able also to neutralize heterologous viral strains. More recent approaches are based on the selection of more conserved antigen candidates, such as regulatory proteins of HIV-1 (see below). On the other hand, observations coming from both experimentally infected animals and naturally infected individuals strongly suggest a central role for CTLs, together with antibodies, in conferring sustained immunity against HIV-1. This has implied the development of a panoply of approaches aimed at inducing both antibody and CTL responses through the use of various delivery systems for both DNA and protein constructs. Many of these constructs have been tested, or are being tested in phase 1 and 2 clinical trials. One of them is in phase 3 (efficacy) trial. All these 'trials and tribulations' in the field of HIV-1 vaccines have been extensively reviewed in the past few years in excellent reviews (Johnston and Flores 2001; Weiss 2001; Graham 2002; Letvin et al. 2002; Lifson and Martin 2002; McMichael and Hanke 2002).

A live-attenuated vaccine against tuberculosis (the so-called bacillus Calmette–Guérin (BCG)) has been available for more than 70 years. Despite the fact that it is one of the most used vaccines in the world, its efficacy remains controversial, e.g. it protects infants against disseminated tuberculosis, but not adults against the pulmonary disease (Fine 1995). In consideration of the increasing burden of this disease, the development of new efficacious vaccines against tuberculosis is highly desirable. Several factors, however, limit its development, including the intracellular location of *Mycobacterium tuberculosis*, which leads to the need for the development of Th1-type and MHC class I-restricted CTL responses for efficacious clearance of the bacteria. A further problem is the lack of ideal experimental animal models of infection. Surface antigens, such as the antigen 85, hsp60, ESAT-6, etc. have been tested in the preclinical setting, mainly in the form of DNA vaccines, but also as recombinant antigens. However, most of the approaches rely on the development of viable mycobacterial vaccines and on mixed approaches (Ridzon and Hannan 1999; Kaufmann 2000, 2001).

The situation is even more complex for malaria, the burden of which is increasing as a result of drug resistance and worsening of environmental and social conditions, mainly in Africa (Greenwood and Mutabingwa 2002). Immunity against malaria parasites is not only species specific (thus requiring specific constructs for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*), but also specific for each stage of development. In addition,

antigenic variability has been widely described in malaria parasites. Antigens specific for the pre-erythrocytic forms (sporozoites and liver stages), several antigens of asexual forms, and other sexual forms of the parasites have been characterized and produced under different forms by recombinant DNA technology. Furthermore, the effector mechanisms required for protection are different for pre-erythrocytic vaccines (Th1-type, CTLs) and for erythrocytic (both sexual and asexual) vaccines (mainly, but not exclusively, antibodies). Some of the recombinant antigens have also been tested in still nonideal animal models (primates) and some also in humans, as in the case of the circumsporozoite protein fused to the HBsAg particle (referred to as RTS,S) and formulated with the so-called AS02 adjuvant system (MPL + QS-21 (Stoute et al. 1997; Bojang et al. 2001; see also above)). Despite the low levels of transient protection reported, these constructs must be considered as first-generation candidates that still need optimization for their application in malaria-endemic areas (Good 2001; Richie and Saul 2002). It is likely that an ideal malaria vaccine in the future will consist of a mixture of antigens from the different stages of development of the parasite.

There is hope that the comprehensive genomic approach will provide the solution in the selection of the most appropriate antigens in the development of effective vaccines against malaria and tuberculosis.

Genomics applied to vaccine development

The possibility of access to the whole genome sequences of microorganisms, even the most complex, has entirely changed the perspectives and ways of thinking in the process toward vaccine development. By completely reverting the conventional approach (hence the definition of 'reverse vaccinology' coined by one of us (R.R.) (Rappuoli 2000)), the genomic approach does not start from microbiology, but from the comprehensive genomic sequence of the pathogen and, by strict computer analysis, it can predict those antigens that are most likely to be vaccine candidates. One of the novelties of this approach is that cultivation of the pathogen is not required. This avoids handling of dangerous pathogens and also offers the possibility to conceive the development of vaccines for pathogens that cannot be cultured. In addition, the analysis of the potential vaccine candidates takes into consideration virtually all the proteins of the microorganism, irrespective of whether they are expressed in vitro and/or in vivo, the abundance of the protein, whether it is immunogenic in vivo during natural infection, etc. In this respect, this approach is totally unbiased, because all proteins are equally screened as potential vaccine candidates. The feasibility of the genomic approach relies heavily on the availability of a high-throughput system allowing the screening for the development of protective immunity of a very large number of potential candidates reproducibly and in a relatively short period of time. The availability of good animal models of infection and protection, and the availability of immunological correlates of protection, can enormously help the identification of good vaccine candidates. Unfortunately, good correlates of protection are rarely available and this limits the effectiveness of the approach, e.g. despite the fact that the genome of *Helicobacter pylori* has been available for a few years, the development of a vaccine against *H. pylori* has been based up to now essentially on the conventional approaches detailed above (Del Giudice et al. 2001a, b). The other limit of the genomic approach is inherent to molecular biology, and refers to its inability to identify nonprotein antigens, such as polysaccharides, which are components of successful vaccines (see above), and to identify CD1-restricted antigens such as glycolipids, which represent new promising vaccine candidates, as in the case of tuberculosis (Schaible and Kaufmann 2000).

THE GENOMIC APPROACH TO BACTERIAL VACCINES

Group B meningococci. The first bacterial pathogen to be tackled using the genomic approach was the MenB, for which all the conventional approaches used so far have consistently failed. This is even more dramatic because MenB still remains the most prevalent cause of meningococcal meningitis in a very large portion of the world (Rosenstein et al. 2001).

Unlike MenC, for which a conjugate vaccine now exists and for MenA, MenW135, and MenY, for which conjugate vaccines are under development, the use of capsular polysaccharide as the basis of a vaccine against MenB is highly problematic. The MenB capsular polysaccharide is identical to a carbohydrate ($\alpha(2\rightarrow8)$*N*-acetyl neuraminic acid or polysialic acid), which is widely expressed by human cells, e.g. neural cells during ontogenesis (Finne et al. 1987). This renders this structure very poorly immunogenic (Zollinger et al. 1997). Moreover, the administration of this polysaccharide as a conjugate may elicit antibodies crossreacting against the self-antigen (Hayrinen et al. 1995; Granoff et al. 1998b).

Another approach to vaccine development against MenB is based on surface-exposed proteins contained in outer membrane vesicles (OMV). These vesicles contain the major porin proteins, other proteins of molecular masses between 60 and 100 kDa, and LPS. In different efficacy trials, OMV-based MenB vaccines have been shown to elicit serum bactericidal antibody responses and to protect against the development of meningococcal disease (Bjune et al. 1991; de Moraes et al. 1992; Boslego et al. 1995; Tappero et al. 1999). On the one hand, these trials demonstrated that the development of efficacious vaccines against MenB was feasible, but, on the other, they clearly showed the intrinsic limitations of

the OMV-based approach, mainly as a result of the high degree of sequence and antigenic variability of the major proteins that they contain (mainly class 1 porin protein, PorA). Indeed, OMV vaccines induced protective (bactericidal) antibodies at all ages only against the homologous strain of bacteria; they failed, however, to induce protection against heterologous strains, mainly among the youngest children (Tappero et al. 1999), i.e. those more susceptible to meningococcal diseases. To enhance the number of potential strains covered by the vaccine, OMVs were prepared from two strains, each expressing three different PorA proteins. This hexavalent PorA vaccine exhibited poor activity in infants in which a fourth dose was required to induce substantial bactericidal antibody titers (Cartwright et al. 1999). Furthermore, as a result of the extremely high variability of the PorA proteins, such a vaccine would have the potential to cover less than 50 percent of the circulating MenB strains (Tondella et al. 2000), without considering the risk of antigenic drift or the emergence of pathogenic PorA-negative strains.

The attempts to develop noncapsular, non-OMV vaccines using potentially conserved proteins have also failed so far. The proteins that have mostly been investigated are either variable at the level of amino acid sequence, such as the transferrin-binding protein B (TbpB) (Rokbi et al. 2000), or expressed by only about 50 percent of the MenB strains circulating, such as the neisserial surface protein A (NspA) (Moe et al. 1999).

To identify novel potential vaccine candidates able to induce protective immunity against all MenB strains, the genomic approach was applied by sequencing and deciphering the entire genome of the virulent strain MC58 (Tettelin et al. 2000). While the work of nucleotide sequencing was still in progress, unassembled DNA fragments were investigated to identify open reading frames that potentially coded for novel surface-exposed or exported proteins. Computer analysis predicted more than 600 novel antigens, 350 of which were successfully expressed in prokaryotic cells, purified and used to immunize mice. The immune sera were then tested to confirm to surface location of the antigens by fluorescence activated cell sorter (FACS) analysis and to evaluate the presence of antibodies with bactericidal activity against a broad range of MenB strains, the titers of which are known to correlate with protective immunity against meningococci. Of the 85 novel surface-exposed antigens, 25 identified induced bactericidal antibodies at titers, for some of these antigens, similar to those induced by efficacious OMV vaccines. By analyzing in detail the nucleotide and amino acid sequences in large numbers of strains, it was evident that most of these newly described antigens were very well conserved in strains representative of the circulating meningococcal population, thus suggesting the potential of their use to induce protective immunity against a broad range of strains (Pizza et al. 2000). Some of these conserved antigens that could induce bactericidal antibodies in mice against large panel of MenB strains are now being developed for testing in phase 1 trials in humans.

The information derived from the genome is also being exploited to identify those bacterial antigens that are selectively expressed in vivo at the moment of interaction with the endothelial cells. Using DNA microarrays of MenB it has been shown that the contact of the bacterium with the cell induces upregulation of 189 genes, 40 percent of which are located at the bacterial surface. Five of these surface antigens, not originally retained by the first screening of potential candidates, induce bactericidal antibodies in mice and may represent additional promising vaccine candidates against MenB (Grifantini et al. 2002).

Other bacteria

The genomic, reverse vaccinology approach is now being exploited for the identification of vaccine candidates for the development of vaccines against tuberculosis (Cole et al. 1998), syphilis (Norris and Weinstock 2000), group B streptococci (Tettelin et al. 2002), pneumococci (Wizemann et al. 2001), *Chlamydia pneumoniae* (Montigiani et al. 2002), and others. The entire genomes of many bacteria have been published or are going to be published. The reader is referred to the website www.tigr.org for detailed and fast-growing information. Again, the availability of high-throughput screening systems will enormously help in accelerating the selection of vaccine candidates to move from experimental laboratory models to clinical testing.

THE GENOMIC APPROACH TO PARASITE VACCINES

The attempts made to develop a vaccine against malaria have already been mentioned above. Despite the preliminary results obtained in the field with a recombinant pre-erythrocytic vaccine construct given with a particular adjuvant system (see above), there is still a long way to go before the successful development of an efficacious vaccine able to combat this disease. Malaria, together with AIDS and tuberculosis, remains one of the most dangerous diseases threatening human health. The increase of resistance to anti-malarial drugs and anti-vector insecticides makes the development of an efficacious vaccine of paramount priority. A panoply of plasmodial antigens has been identified as potential vaccine candidates; some have been tested in clinical trials in humans, but none has so far provided the desired breakthrough (Richie and Saul 2002). The final solution may derive from the genomic approach. Of the 14 chromosomes of *P. falciparum*, two have been fully sequenced (Gardner et al. 1998; Bowman et al. 1999), whereas the annotation for others is in progress (website www.tigr.org). The analysis of the whole genome will show which genes are expressed by the parasite at its

different developmental stages (sporozoite, live stages, and asexual and sexual erythrocytic stages). Expression of genes predicted to be immunogenic as recombinant proteins or as DNA vaccines will eventually provide the effective vaccine against malaria. The task is not easy, because malaria species that infect humans can infect only particular monkey species, thus limiting the possibilities of high-throughput screening of the candidate antigens, and requiring more and more clinical trials with new candidate vaccine constructs. However, it is encouraging that in recent years there has been an increasing involvement of governments and nonprofit foundations, which has significantly increased the amount of funds allocated for malaria vaccine research and development (Nossal 2000).

Active work is now in progress toward the sequencing of the genome of the other human malaria parasite, *P. vivax*, trypanosomes, *Schistosoma mansoni*, *Brugia malayi*, and other parasites (website www.tigr.org).

THE GENOMIC APPROACH TO VIRAL VACCINES

Despite their small size and the fact that viral genomes have been available for a long time, the information derived from the genomic approach has been poorly exploited for vaccine development. The best example, and also the first, of reverse vaccinology applied to virus discovery and the development of vaccines against viral infections is given by the HCV. The virus cannot be cultured and it has never been observed by electron microscopy. Identification of the etiological agent was only possible through cloning and sequencing of the HCV genome (Choo et al. 1989), which allowed the prediction of the envelope proteins; these were then shown to induce protective immune responses in chimpanzees (Choo et al. 1994).

Until very recently, most of the attention has been focused on proteins from the viral particle, such as those of the envelope and the core of the virus particle. The best example is provided by the long-term efforts to develop HIV-1 vaccines based on different forms of the major envelope protein gp120 and/or on the other structural proteins encoded by the *gag* gene. It is now evident that other proteins, which are expressed in too limited amounts to be purified from the virus or which are not structural proteins (e.g. Tat, Nef, Rev, and Pol), can be protective in relevant experimental animal models and may be exploited for the development of vaccines in humans (Cafaro et al. 1999, 2001; Osterhaus et al. 1999; Pauza et al. 2000; Ensoli and Cafaro 2002). The promising results obtained so far with these 'unusual' antigens strongly suggest that we have to change our way of thinking about the way of inducing protective immune responses and to consider that even nonstructural, nonexposed proteins, and even naturally poorly immunogenic antigens, can represent potential candidate vaccines when they are correctly expressed and appropriately delivered to the host, e.g. it has been shown that the HIV-1 Tat protein is able to target to DCs and to favor their maturation and function, thereby enhancing antigen-specific T-cell responses (Fanales-Belasio et al. 2002), a finding totally unexpected based on the information available from the conventional approaches to vaccine development.

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Mathematical models of vaccination

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INTRODUCTION

The purpose of this chapter is to introduce the use of mathematical models in the design of vaccination programs. Like any other healthcare interventions, the aim is to reduce disease, and the success of the program can be measured in terms of the amount of disease prevented, i.e. a quantity. Whenever a problem can be expressed in quantitative terms (i.e. numbers), it lends itself to mathematical formulation, and a whole host of techniques can be used to help solve it. Many people have an aversion to mathematics, but this should not prevent its use when its use is clearly indicated. In this context mathematics is acting as a supporting subject. Just as the development of diagnostics often requires support and insight from chemistry, so the control of infectious disease can make use of mathematics. However, we are not attempting to produce a manual for mathematical models, or indeed convert those with an aversion to equations; rather we seek to explain the content of models and the conclusions that flow from them.

Models (mathematical or otherwise) are conceptual simplifications of complex biological problems. As such, they are always open to the criticism that some relevant and important process or factor is missing. The adage that 'all models are wrong, but some are useful' is true. The decisions taken about which processes are to be included (and which are to be omitted) are critical to determining the usefulness of a model. This is true of mathematical models of problems in other subjects, e.g. engineering. It is clear that judicious use of mathematics can make bridges safer, even when many of the potential complexities cannot be modeled. The definitive test is always when the bridge is actually built and, indeed, there are good examples of bridges failing when the models clearly missed out important processes. However, the next time you are passing over a bridge, it is of some comfort to think that somebody has performed some calculations to demonstrate that it can take your weight.

The principal reason for using mathematical models in the design of vaccination programs is that appropriate experiments are impossible, usually on logistic grounds. If somebody develops a vaccine that they believe induces a protective immune response, there is an agreed route through which they can demonstrate efficacy and safety. It might then be suggested that this vaccine, if given to 75 percent of the population, will prevent 1 500 deaths per year. The only way of demonstrating the potential impact of the vaccine at the population level without actually introducing the program is to model it, i.e. use quantitative frameworks to predict the potential outcome. Note that this is true of the majority of public health interventions – designating 'treatment' and 'control' populations is usually not possible. Clearly, to predict the impact of a vaccine even reasonably accurately requires that we can initially predict the outcome of infection without any intervention.

Just as mathematics has become a mainstay of engineering, so mathematical or quantitative descriptions of pathogen transmission dynamics are increasingly used to support decision-making in design of control programs against infectious disease. Our emphasis in this chapter

is on the insights that modeling provides and in what ways these might be instructive in vaccine program design. This chapter concentrates on vaccination, and the central questions of vaccination program design is: 'Who should be vaccinated, what with, and when?'

These are nontrivial questions, and the literature addressing them is substantial and growing. An introduction is given by Anderson and May (1991), which provides a (rather technical) base for more recent advances. More accessible reviews include Nokes and Anderson (1988) and Fine (1993). Consequently, in this chapter, we concentrate on the situation for which the results are best-established: viruses with single serotypes, where immunity after infection is long lasting. The classic examples (archetypal viruses) are measles, mumps, and rubella (MMR). It is no coincidence that this is the simplest circumstance – a consensus on the population biology and epidemiology of more complicated infections is yet to be found.

BASIC CONCEPTS

In this section, we introduce the central idea of infectious disease epidemiology: it is a dynamic process driven proximately by the rate of infection and, ultimately, the availability of people to be infected.

Individual vs population

The population level patterns (epidemiology) of infectious disease are very different from noninfectious disease. An infected individual acts as a potential source of infection to others in a population – an individual with a noninfectious condition poses no such risk. With infectious disease there is a necessary relationship between the *incidence* and *prevalence*: if the prevalence of infection is zero, then there is no possibility of incidence being anything other than zero (Figure 1), i.e. if nobody is infected with smallpox, nobody can become infected with smallpox. This is not the case for noninfectious or noncommunicable disease, where the individual risk of developing the disease is little influenced by the amount of disease in the population.

It follows from this simple observation that infectious disease has to be studied at a population or community level if it is to be controlled. The risk of disease to an individual depends on the health (infection) status of all the other individuals with whom they come into contact. Likewise, an intervention that acts on an individual will have consequences for everybody else in the population. If you successfully vaccinate 20 percent of a population, the remaining 80 percent who were unvaccinated will still receive some protection from infection because they have fewer people from whom to acquire infection.

An important distinction must be drawn between infection and disease. Infected individuals are not guaranteed to develop disease and, for most pathogens, most infections are relatively asymptomatic. However, an individual infected with influenza but without clinical symptoms can transmit the virus to another individual who subsequently dies. It follows that control of infectious disease at the population level requires that it is infection that is considered rather than disease. Unfortunately, most data available are highly biased towards cases of clinical disease, and uncomplicated infection is usually unobserved unless special effort is made.

Transmission dynamics and susceptibility

It is also clear that incidence is dependent on susceptibility to infection: if there are no susceptible individuals to infect, the incidence is, again, necessarily zero. If it were possible to make everybody immune to measles tomorrow, nobody would become infected. This relationship between prevalence and incidence gives an inherent nonlinearity in patterns of infectious disease. Under simple assumptions of random transmission (see below), we can write the incidence of infection as the product of three numbers. Suppose that individuals in a population contact each other at some rate, c, and there are X susceptible and Y infected individuals, making a total population size of N ($=X + Y$). Then the rate at which each susceptible contacts an infected individual is:

$$c\frac{Y}{N} \qquad (1)$$

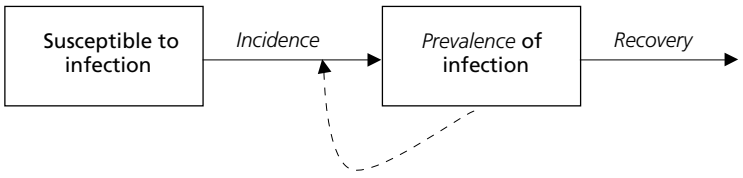

Figure 1 *Relationship between incidence and prevalence for an infectious disease. The boxes represent two sorts of individuals: those who might be infected (i.e. are susceptible to infection) and those who are infected. The prevalence of infection is the number of individuals currently infected, and is expressed as a proportion or percentage. The solid arrows represent time-dependent rates of movement of individuals. Incidence is a measure of the rate at which individuals become infected, and is often expressed as a proportion of the population per unit time (the attack rate). Recovery from infection can generally be the result of immunological clearance of infection or death of the infected individual. The dotted line represents the dependence of incidence on prevalence.*

For example, suppose that the contact rate is 10 individuals per week, and half of the population is infected, then the average susceptible will contact 5 infected people per week. Now suppose that on each contact between a susceptible and infected individual there is a probability, p, that transmission occurs (i.e. the susceptible becomes infected). Consequently, the rate of infection per susceptible is:

$$pc\frac{Y}{N} \tag{2}$$

This quantity is also known as the 'force of infection'. Continuing our numerical example, if the probability of transmission is 0.1, the rate of infection per susceptible is 0.5 per week. This can be translated into an average time before infection of 1/0.5 = 2 weeks. The unsurprising result is that the more infected individuals there are in a population, the more likely that each susceptible becomes infected, and the faster they become infected.

The total rate of infection in the population (i.e. taking into account all the susceptibles) is the incidence (e.g. the number of cases of infection per week):

$$pc\frac{Y}{N}X = \frac{pcY(N-Y)}{N} \tag{3}$$

This relationship is drawn in Figure 2. The implicit assumption for this simple example is that individuals are either susceptible or infected. Lack of either susceptible or infected individuals reduces incidence. Incidence is greatest when there is a plentiful supply of both. This nonlinear relationship between incidence and susceptibles and infecteds is what creates 'transmission dynamics'. During an epidemic, the number of infected individuals starts small, but a positive incidence means that infection increases, increasing incidence further, and so on. Eventually, the numbers of susceptibles will be depleted, and incidence will start to fall. Thus, changes in incidence at any point in time have important consequences in the future, making prediction of the dynamic (through time) changes intrinsically complicated.

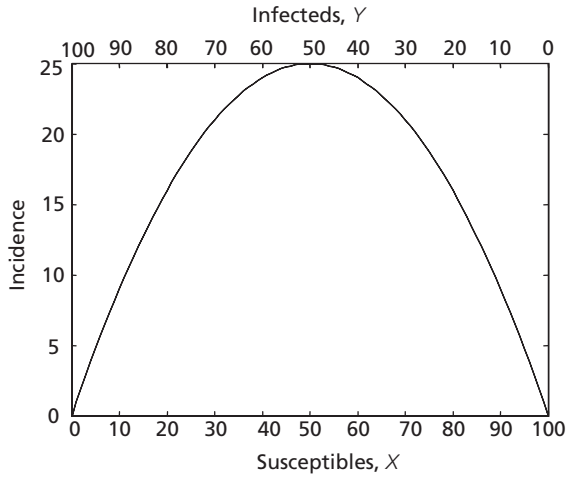

Figure 2 *The relationship between incidence (number of new cases per unit time), and the numbers of susceptibles in a population of 100 individuals. The incidence is zero when either there is nobody to infect (X = 0, Y = 100) or there are no infectious individuals (X = 100, Y = 0). See text (Equation 3).*

The simplest dynamic model

The preceding section makes it clear that there are intimate links between rates of infection, prevalence of infection, and proportion susceptible. To understand these links and the dynamic consequence of changing any of these quantities (e.g. reducing susceptibility by vaccinating), it is necessary to consider them together in a single model framework. Mathematics provides the tools to do this.

Imagine a closed population such as the passengers and crew on a cruise liner, i.e. a population in which birth, death, immigration, and emigration do not usually occur. This implies that we are thinking of a relatively short time period. Let us consider the simplest situation (i.e. the MMR paradigm). We can divide the population into three mutually exclusive groups or classes: susceptible, infected (and infectious), and recovered (and immune).

In this case, the only process occurring as far as the susceptible population is concerned is infection, which occurs at a rate given in Equation 3. The number of infected individuals is being increased by infection (at the same rate as susceptibles are lost), but also decreased by recovery. The immune population is increased in the same manner, i.e. as each person recovers and leaves the infected class, he or she enters the immune class. We can tie these processes together by considering the rate at which the populations are changing. Figure 3 sketches a 'flow diagram' indicating the three classes of individual and the rates of flow between them.

As before, we define X and Y to be the numbers susceptible and infected respectively, and introduce Z as the numbers immune. We can then say that the rate of change of X is written as dX/dt. This quantity has units of 'people per unit time': if it is zero, the susceptible population is not changing; if it is greater than zero the susceptible population is increasing; if it is less than zero (negative) the susceptible population is decreasing. Taking all the assumptions together, the equations for this simplest model are:

$$\frac{dX}{dt} = -pc\frac{Y}{N}X \tag{4}$$

$$\frac{dY}{dt} = +pc\frac{Y}{N}X - \frac{1}{D}Y \tag{5}$$

$$\frac{dZ}{dt} = \frac{1}{D}Y \tag{6}$$

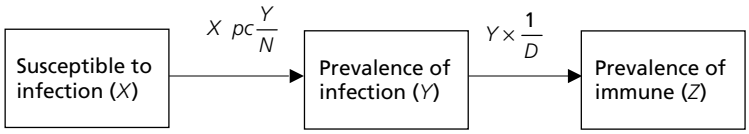

Figure 3 *A flow diagram for the simplest possible model of a microparasitic infection that produces a solid, life-long immunity (e.g. mumps, measles, and rubella or MMR). In comparison with Figure 1, we have now introduced an explicit immune class of individuals, and added variables (*X, Y, *and* Z*), which are the numbers of individuals within each class. The total population is* N = X + Y + Z*. Each individual in the population falls into one, and only one, class. The arrows between the classes represent the flow of individuals, and the rate of flow is given as an equation. See text (Equations 4–6).*

The incidence rates are as previously (see Equation 3). The recovery rate is written as $1/D$ where D is the duration of infection. Note that the parameter c is a rate and D is a duration, and must be expressed in the same time units, i.e. it is wrong to include c as five contacts per week and D as a duration of 14 days. The only other information necessary to solve these equations is the 'initial conditions', i.e. the numbers in each class at the start.

We are not going to enter into a mathematical discussion of these equations, but rather simply state that they are easily solved in many computer packages. Figure 4 gives an example of such a solution. The epidemic is shown as the bold line, reaching a peak prevalence of 40 people in 100 infected after 4 weeks. The susceptible population falls continuously, although note that a small proportion of people escape infection. The immune population (dashed line) rises throughout as people pass from susceptible through infected and recover.

One thing that should be clear from this model is that the shape of the epidemic is determined by both the availability of infection and the susceptibility. The reason that everybody does not become infected on the first day is that infection is rare, but the prevalence of infection increases because the rate of infection is greater than the rate of recovery. The reason that infection prevalence falls after 4 weeks is that only about 30 people are susceptible, so the rate of recovery is greater than the rate of infection.

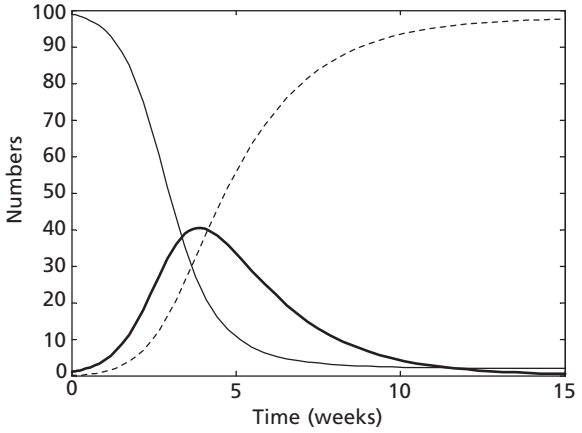

Figure 4 *An example outcome from the simplest model (see text, Equations 4–6). The lines show the numbers in each class: susceptible (*X*), infectious (*Y*, bold) and immune (*Z*, dashed). The parameters are:* c *= 20 per week,* p *= 0.1, and* D *= 2 weeks. The initial conditions are* X *= 99,* Y *= 1, and* Z *= 0.*

Statistical and political complications

Generally, for infectious disease studies, individuals cannot be considered as statistically independent observations. For infectious disease, the incidence is a (nonlinear, complex) function of the prevalence. Noninfectious disease epidemiology is essentially a statistical study of the relationship between risks and disease where individuals can be treated as heterogeneous, but independent, replicates. This makes observational research into infectious disease more difficult and, to some extent, the appropriate methods are still under development (e.g. see Becker 1989).

Infectious disease also introduces conflicts between populations and individuals, because of the interdependence. Some of these conflicts are discussed in Medley (1996), but a couple of examples will demonstrate. No vaccine is completely free from risk of adverse reactions, so each parent would like their children to avoid both infection and vaccine. This is possible only if all other parents have their children vaccinated, i.e. everybody would like everybody else to use the vaccine, because then they would not have to (see also Nokes and Anderson 1988, 1991).

Another conflict arises if chemotherapy is available to prolong the infectious life of infected individuals. Clearly, such a therapy is advantageous to the individual, but will almost certainly increase the total number of people infected, which is disadvantageous to the population (e.g. Anderson et al. 1991). These conflicts are occasionally seen when those responsible for the health of populations (e.g. government departments) enforce control measures that are apparently detrimental to individuals. A conflict arises because the best policy depends on the perspective taken: what is best for an individual might not be the best for the population, and vice versa.

PROCESSES OF INFECTIOUS DISEASE EPIDEMIOLOGY

Having introduced the basic concepts, in this section we go through the processes that drive infectious disease

epidemiology, and hence form the components of mathematical descriptions of infectious disease dynamics.

Routes of transmission

Pathogens must be passed from one individual to another if they are going to survive, even if only because no host is immortal. From an evolutionary point of view, the pathogen that transmits the most is the most successful (the 'fittest'), and will out-compete the less transmissible. From an epidemiological viewpoint, if the transmission dynamics of infection, and hence the impact of vaccination, are to be predicted, a critical knowledge is how many and which individuals are infected by an infectious case.

Transmission can be classified in a number of ways. In Table 1 we give a classification system based on three criteria. First, infections can be considered in terms of their ultimate source: other people, other animal species (zoonotic), or the environment. Second, within each of these categories, the route of transmission essentially defines who is likely to be exposed, e.g. an infection that can use the maternal route will potentially infect children; a sexually transmitted pathogen will potentially infect sexual partners, etc. Vectors can transmit both human and zoonotic pathogens. Third, the mode of infection essentially defines the physical manner in which the pathogen transfers from one individual to another, so that children can be infected maternally via a number of modes (e.g. across the placenta or via breast milk). Thus, knowing the route of transmission helps define the at-risk contacts of an infected individual, and knowing the mode of transmission helps define control strategies to reduce the risk of those individuals. Distinction should also be made between transmission modes that are contaminative (i.e. there is no multiplication of the pathogen) and reservoir; multiplication of malaria within the vector reservoir is different from the contamination of shared drug-injecting equipment with HIV.

Quantification of transmission probability

Contact between an infectious and a susceptible individual does not guarantee transmission. To estimate a probability of transmission given a contact, the contacts have to be defined and countable. This is not straightforward, e.g. for sexually transmitted infections (STI) there is the possibility of measuring the contact rates and transmission probability in terms of both sexual partnerships and sexual acts (within a partnership) (see Blower and Boe 1993).

Suppose that we are interested in transmission of HIV from intravenous drug-using women. There are three routes through which infection can pass, and hence three groups who might be infected: children, equipment-sharing partners, and sexual partners. Suppose that these women have, on average, one child every 4 years; then the contact rate, c_1, for this route is 0.25 different children per year. Likewise we can define c_2 as the average number of different injecting partners per year,

Table 1 *Classification of transmission*

Source	Route	Mode	Examples (organism)
Human	Maternal	Transplacental	*Toxoplasma gondii*, rubella, CMV, *Treponema pallidum*
		Transmammary	HIV, CMV
	Environmental contamination	Food borne	HAV, *Vibrio cholerae*
		Other (fomite) ingestion	*Shigella* spp.
	'Close contact'	Respiratory (airborne)	Influenza 2, RSV, *Bordetella pertussis*, MMR
		Oral–fecal	*V. cholerae*, rotavirus
		Physical (nonsexual)	HBV, HSV1, EBV
	Sexual	Genital–genital, anogenital, etc.	HIV, HBV, HSV2 *Neisseria gonorrhoeae*, *Treponema pallidum*
	Vector-borne	Vector-borne	*Plasmodium* spp., urban YFV, dengue
	Blood-borne	Needle sharing/transfusion	HBV, HIV, HCV
Zoonotic	Physical contact	Various	Rabies, Q-fever, brucellosis, *Campylobacter* spp.
	Vector-borne	Vector-borne	*Yersinia pestis*, jungle YFV, *Borrelia burgdorferi*
Environmental	Environment	Respiratory (airborne)	*Legionella* spp.
		Oral/Mechanical	*Clostridium tetani*, *Listeria* spp., *Mycobacterium bovis*

CMV, cytomegalovirus; EBV, Epstein–Barr virus; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; MMR, measles, mumps, and rubella; RSV, respiratory syncytial virus; YFV, yellow fever virus.

and c_3 as the average number of different sexual partners per year. If the probability of a child becoming infected from its infectious mother is p_1, and similarly for p_2 and p_3, the total average rate of people infected by each infected woman is:

$$c_1p_1 + c_2p_2 + c_3p_3 \quad (7)$$

There is considerable blurring across boundaries in Table 1, as with any attempt to impose categories on to continuous scales, e.g. when does close contact, oral–fecal transmission become food-borne environmental contamination? One can see the difficulty in defining the nonsexual 'close contact' rates (although see Edmunds et al. 1997). This is also complicated by the fact that close contact modes and sexual modes are not mutually exclusive – if two individuals are close enough to touch, they can transmit via the respiratory mode. In this sense, the sexual route is a 'very close contact'. Figure 5 schematically demonstrates that, within the close contact/sexual routes, there is a relationship between the number of contacts and intimacy of contact (which is very probably correlated to probability of transmission). Thus, we speak to more people than we touch, and touch more people than we have sex with.

By defining a transmission mode as 'respiratory', we are defining the lower limit of its transmission modes, because it will also be transmitted during more intimate contacts. Figure 6 schematically illustrates that this lower limit will not always coincide with the maximum transmission route: how much influenza is sexually transmitted (that is, transmitted during sexual contact)?

Social and environmental setting

Having defined and quantified transmission in terms of contact rates, the next level of complexity is determining with whom the contact occurs. The simplest assumption is that all individuals within the population are equally likely to contact all other individuals. This is in fact the underlying rationale behind Equations 1–3, and the effect is that doubling the number of infectious individuals doubles the rate at which each (and every) susceptible contacts an infectious individual. In reality this is rarely the case – we do not have an equal chance of meeting everybody. Social systems effectively mean that we have a group of people we meet much more often (e.g. family, close friends, and work colleagues), and have a very low chance of meeting most other people.

There are a large number of variables that determine who our contacts are. These include age, gender, occupation, and geography. Taking age as an example, people tend to mix with individuals of the same age, although parents and teachers with children are examples of mixing across age groups. Geography is somewhat spurious, because it does determine much of the contact, but behavior tends to be overriding, so that it is possible to have more regular and closer contact with family members in North America, than with next-door neighbors in the UK.

There is also considerable heterogeneity in the amount of contact, so that some individuals have much higher contact rates than others. The individuals with the highest contact rates are simultaneously at greatest risk of becoming infected, and have the greatest opportunity for infecting others. In the context of STIs, these high-contact individuals are termed the 'core group', but all infections have a 'core group', and the same phenomenon is probably universal for infectious agents (Woolhouse et al. 1997).

In much work in the literature, there is an implicit assumption that the rate of infection is determined by the density (or size) of the population, rather than being

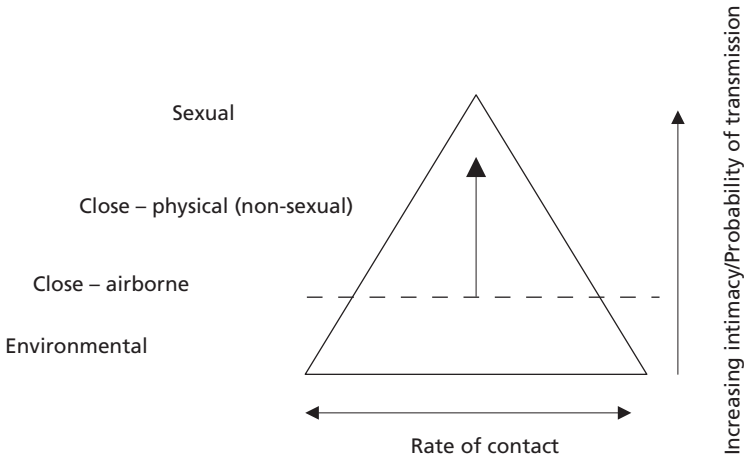

Figure 5 *Schematic diagram representing the relationship between the frequency and intimacy of contact. The higher up the triangle, the greater the probability of transmission, but the lower the rate of that contact. The horizontal line represents a hypothetical infection which is transmissible via direct airborne spread, but shows that it is also transmissible by other routes (see text).*

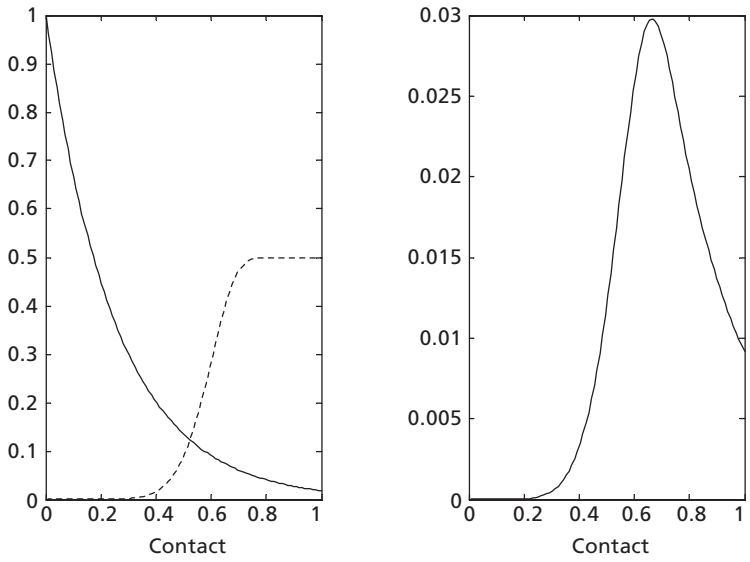

Figure 6 *Putative relationships between (relative) contact rate,* c *(solid line left panel), and probability of transmission,* p *(dashed line left panel), varying with increased intimacy of contact. The right-hand panel shows the product* c^*p *over the same scale, i.e. the effective contact rate. Note that if the contact rate falls and probability of transmission reaches a plateau, this latter relationship will always have a single maximum, and the maximum will be at or before the maximum transmission probability is reached.*

determined by contact explicitly. In this case, Equation 2 is rewritten as:

$$\beta Y \tag{8}$$

where β is a 'transmission coefficient'. This formulation is known as the 'mass action' assumption: the name derives from similar models (and assumptions) in chemical and physical processes. If the population size (N) is constant, the two assumptions (contact-driven and mass action – Equations 2 and 8, respectively) are equivalent, and the transmission coefficient subsumes the population size. If the population size is changing, the two are equivalent only if the contact rate is a linear function of the population size (i.e. if doubling the number of people doubles the average rate of contact). This might be a fair approximation for transmission via more environmental routes (such as airborne), but is unlikely to be true for more sociological routes (such as sexual contact).

It is clear that much of this discussion about social and environmental setting and its relationship to transmission rates depends on the population under consideration. For any model, defining the population is a critical step, because it effectively defines the scope of the model, which then determines what is outside the model, e.g. producing a model of pneumococcal transmission in school might be erroneous if most of the transmission occurs in households. The other important feature to come out of this discussion is that heterogeneities (i.e. differences) are critically important. The simplest model formulations have everything identical. In biology, this is very rarely the case – most hosts and most pathogens are distinct from each other. This theme is picked up continuously through the remainder of the chapter.

Natural history

For the purposes of describing transmission dynamics, the natural history of infection charts the progress of *an individual* through time, starting with exposure and successful infection. Within each infected individual, there is a dynamic duel fought between pathogen and immunity – vaccines are designed to give immunity the upper hand (Nowak and May 2000). But, at a population level, there are four characteristics of relevance: (1) the time course of infectiousness; (2) the immunity to subsequent infection (i.e. susceptibility); (3) the time course of immune markers of (past) infection; and (4) the time course of disease. Figure 7 shows schematic pictures of these four aspects for three diseases. Measles, mumps, and rubella are archetypal viral infections, where the infection, disease, and immune response to infection all occur over a matter of days. The infection (and infectiousness) is curtailed by the immune response that persists for life, and antibody markers of previous infection can be found in the serum and saliva of most individuals, although their concentration may fall over a period of years without re-exposure.

For bacterial infections (e.g. *N. gonorrhoea*, *Streptococcus pneumoniae*), the timescale of infection is months, there is no solid immunity, and immune markers are generally not persistent.

For hepatitis B virus (HBV), there are two possible outcomes of infection. After initial exposure, and a risk of acute hepatitis, the majority of individuals recover and become immune to re-infection, i.e. the natural history is as for measles, but with a timescale of months rather than days. However, as illustrated, some

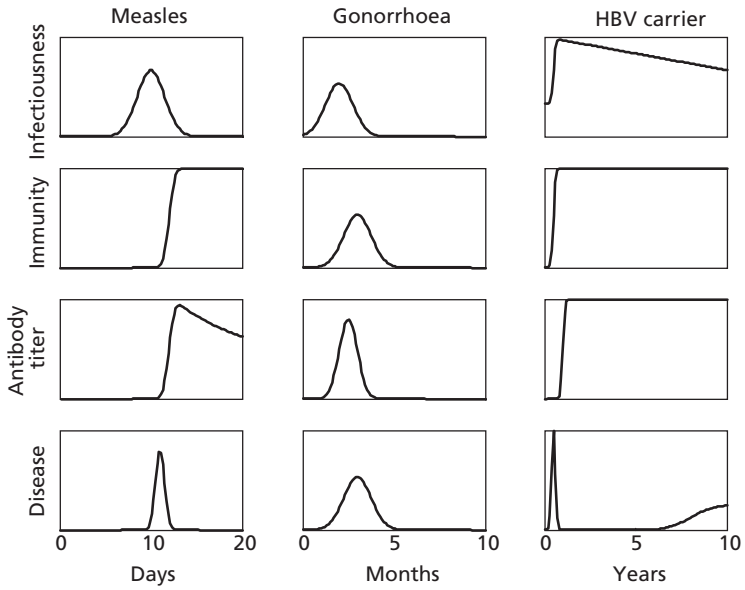

Figure 7 *Schematic representation of three different natural histories without intervention (treatment). The three columns show (left to right) measles, gonorrhea, and* hepatitis B virus *carriage. Note the different time scales (i.e. the time since infection). The four rows show (top to bottom) the infectiousness, the immunity to reinfection, the titer of antibodies (markers of previous infection), and the risk of disease and/or symptoms.*

individuals develop a carrier state, and remain more or less continually infectious, although they are probably immune to further infection, and maintain some antibody markers of infection. The risk of cancer and cirrhosis increases with duration of carriage.

To develop a mathematical description of the natural history, the most common (and common-sense) approach is to divide the population into a series of mutually exclusive categories or classes of individuals (as was done in Figure 3). Figure 8 illustrates such compartments for the three infections in Figure 7. The arrows denote the flow or movement of individuals between categories. For measles, there are only three states: susceptible, infectious, resistant (SIR), and individuals move from susceptible to infectious (when infected) and from infectious to immune as they recover. This is the classic 'SIR' model. Frequently, an exposed category is added between susceptible and infectious to include those individuals who are infected but not yet infectious: this is the SEIR model. For most bacterial infections there is no effective solid immunity, so individuals return to susceptible after resolution of infection:

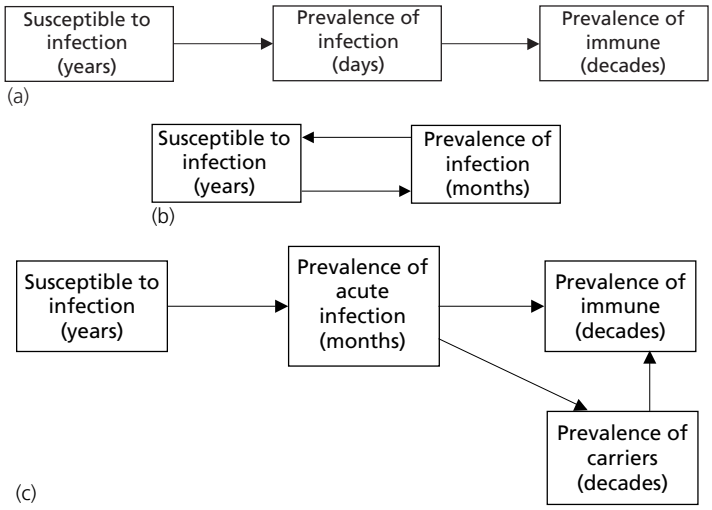

Figure 8 *Compartmental model structures for the natural histories of three infections illustrated in Figure 7.* **(a)** *Measles,* **(b)** *bacterial sexually transmitted disease, and* **(c)** hepatitis B virus*. The duration of stay within each compartment is written in braces. The first two model structures are SIR and SIS respectively.*

the SIS model. Again, if there is a period of immunity after infection, before recovery of susceptibility, an SIRS framework is possible, as seems likely for respiratory syncytial virus (RSV) and rotavirus. For HBV, the compartmental model should include both consequences of initial (acute) infection: immunity and carriage. Note that carriage can resolve, so that there is a flow from carriers to immune. Figure 8 is not exhaustive: indeed there are a great many possibilities for sewing together compartments representing the natural histories of different pathogens (which might be different in different host populations). But note that the compartments are based on what happens within individuals.

Figure 8 also shows the duration of stay within each compartment. This is important, because it largely determines the prevalence of each compartment, i.e. what percentage of individuals are in which state. A short duration stage has a lower prevalence than a long duration stage, e.g. the prevalence of people whose 40th birthday is today is much lower than the prevalence of people in their 40s: because it takes only one day to have a 40th birthday, but 10 years to move between 40 and 50.

As discussed below, for the purposes of understanding transmission, the duration of infectiousness (D) is the most important parameter of the natural history.

Pathogen heterogeneities

> Measles occupies a rather unique position in epidemiological theory, as it is largely an infection of childhood with very clear-cut immunizing characteristics, so that the susceptible population (and its new 'recruits') are fairly well defined. (Bartlett 1960, p. 73)

We can perhaps add mumps and rubella, but most other pathogens display variation, particularly with respect to immunity determinants. Agents with antigenic diversity (e.g. influenza virus, rhinoviruses, rotavirus) appear to have natural histories like the second type in Figures 7 and 8, i.e. every infection is like a new one. The typical scenario might be that of a strain of influenza, which follows a similar life history to that of, say, measles, but, unlike measles, the solid immunity generated to the specific strain is not solid across strains. Thus an individual's immunity to 'influenza' as a whole is compromised by the antigenic diversity (existing or immune selected), such that in a subsequent season previously infected individuals succumb to a new (drifted) variant. From a host viewpoint, this looks like loss of immunity to influenza. From the virus's viewpoint, all those hosts that were previously infected and immune are now susceptible and ripe for re-infection.

This picture is complicated by the concepts of partial and temporary immunity. If immunity is partial, it is effective against a proportion of the pathogen population, whereas, if it is temporary, it is effective against the whole pathogen population, but this immunity wanes. Further complication arises if the immunity is to disease rather than infection: for RSV and rotavirus disease is usually expressed or most severe on first infection.

Basic reproduction number

The processes outlined above (transmission, contact, and natural history) are the determinants of the epidemiology of an infectious disease in a given host population. That HBV has a different epidemiology in developed versus developing countries, or that STIs are different from childhood viral infections, is the result of differences in these three processes.

To understand the population dynamics of infection, we should capture the processes as they determine transmission from person to person. This can be done in terms of a single number: the basic reproduction number, R_0 (pronounced R-zero or R-nought). This turns out to be the central concept in infectious disease epidemiology.

Consider a completely susceptible population (i.e. without any immunity), with a single infected case introduced. In this situation, the transmission that derives from the infected case will be maximal – it is the ideal situation for the pathogen. The infected case will be infectious for a period determined by the natural history, and in the simplest circumstance we can represent this by the parameter D. This determines the time window or duration over which infection can occur.

During this period, the case will make contact with susceptible people at a rate c, which is determined by the transmission route and the population characteristics (environment and behavior). So, overall, the number of potential infectees (secondary cases) will be cD – the number of contacts during the infectious period. But not all will become infected, and we can write p as a transmission probability, i.e. given that a susceptible contacts an infectious person, p is the probability that transmission will occur. So the basic reproduction number can be defined as:

$$R_0 = pcD \tag{9}$$

Note that this is a single number and is defined as the average number of secondary cases that are derived from one primary case in a completely susceptible population. As previous discussion on heterogeneity in transmission and natural history illustrates, the word 'average' in this definition glosses over much complexity, but the concept conveys the essential idea that the maximal expected transmission in a given setting can be quantified in terms of route of transmission, environmental/behavioral setting, and natural history.

The most important concept resulting from R_0 is that of a threshold value for an epidemic to occur. If an infection is to be able to propagate through a population, i.e. to cause an epidemic, each new infection will have to (on average) infect at least one other person. So for an epidemic to occur:

$$R_0 > 1 \tag{10}$$

Imagine, if $R_0 = 2$, each infectious case will create a chain of new cases, so that one case becomes 2, which beget 4 cases, which beget 8 cases, and so forth.

Effective reproduction number

Given that susceptibility is the key quantity – the food on which infection feeds – there ought to be some way of determining a sufficient supply of susceptibles required to maintain infection. This idea is captured in the *effective reproduction number*, R. This is defined as the average number of secondary cases from each primary case. Note the similarity with the definition of the basic reproduction number, which differs only by having the extra condition of a completely susceptible population. The effective reproduction number does not have this condition – it is more general and applies regardless of the state of the population.

There is a simple relationship between these two reproduction numbers. The assumption previously was that each contact (potential transmission) that the infectious case had was with a susceptible person. However, if only a proportion of the population is susceptible, x, then the number of contacts each infectious case has during the infectious period is xcD, and consequently:

$$R = pxcD = xR_0 \tag{11}$$

When everybody is susceptible, $x = 1$, and $R = R_0$. If there are no susceptibles, $x = 0$, and $R = 0$, i.e. there can be no transmission.

Host population effects

Viruses survive in host populations, so, not surprisingly, the population dynamics of hosts is an important factor in determining the epidemiology of a virus. There are two aspects that we concentrate on here: the availability of susceptible hosts and the variability of the effect of infection on different hosts. A key concept is that hosts are not identical, and we emphasize the role of host age.

In Figure 4 the epidemic ends and the virus that caused it will become eliminated from the host population. This is because all (but a small fraction of) hosts have been infected and are immune, and there is no susceptibility available for the virus to exploit. Pathogens need susceptibility to survive. A pathogen with a different natural history, e.g. one with sufficient antigenic diversity, might be able to re-infect hosts, thus creating susceptibility. The other principal source of susceptibles is the arrival of new hosts through birth or immigration. An alternative strategy is for the pathogen to find a site in which it can survive the longer term and wait for susceptibility to return. A carrier state and survival in the environment are both examples of such strategies; vertical transmission is also a good adaptation for a virus to survive in a small population of hosts.

We can adapt the simplest model in Equations 4–6 to include a supply of susceptibles. Imagine that, in our population, individuals leave the population after 20 weeks on average, and they are immediately replaced by susceptibles, in order to keep the population constant. This can be thought of as birth/death or immigration/emigration, and is captured in a parameter, μ. We also include partial immunity by adding a parameter, π, which we define as the proportion of recovering hosts that are immediately susceptible (so that $1 - \pi$ become immune). The equations for this system are:

$$\frac{dX}{dt} = \mu N - pc\frac{Y}{N}X + \pi\frac{1}{D}Y - \mu X \tag{12}$$

$$\frac{dY}{dt} = +pc\frac{Y}{N}X - \frac{1}{D}Y - \mu Y \tag{13}$$

$$\frac{dZ}{dt} = (1-\pi)\frac{1}{D}Y - \mu Z \tag{14}$$

Four examples of the results are shown in Figure 9. Introduction of a trickle of susceptibility that does not depend on infection (through birth/death) results in the infection being maintained in the population – the supply of susceptibles is enough to keep all the chains of transmission from going extinct. Note that this introduces 'damped oscillations', as the infection reaches its endemic, equilibrium prevalence. Decreasing partial immunity increases the prevalence of infection and reduces the amplitude of the oscillations.

This model (Equations 12–14) is an example of a 'toy model' or 'thought experiment' in which the underlying effect of explicitly stated assumptions can be investigated. Nobody would pretend that a framework such as this be used for quantitative prediction or designing control programs. More complicated models, e.g. introducing explicit host age, gender, and pathogen diversity, would be required for these purposes. However, these more complex models tend to echo the important results from the simpler models, which are consequently often more useful for developing understanding. A fuller review of the dynamics of SIR and SIS models is given by Gomes et al. (2004).

Dynamic equilibria

Implicit in Figure 9 is that if there are sufficient susceptibles to maintain an infection in a host population, i.e. it becomes endemic; then the prevalence of infection tends toward a constant equilibrium. This can

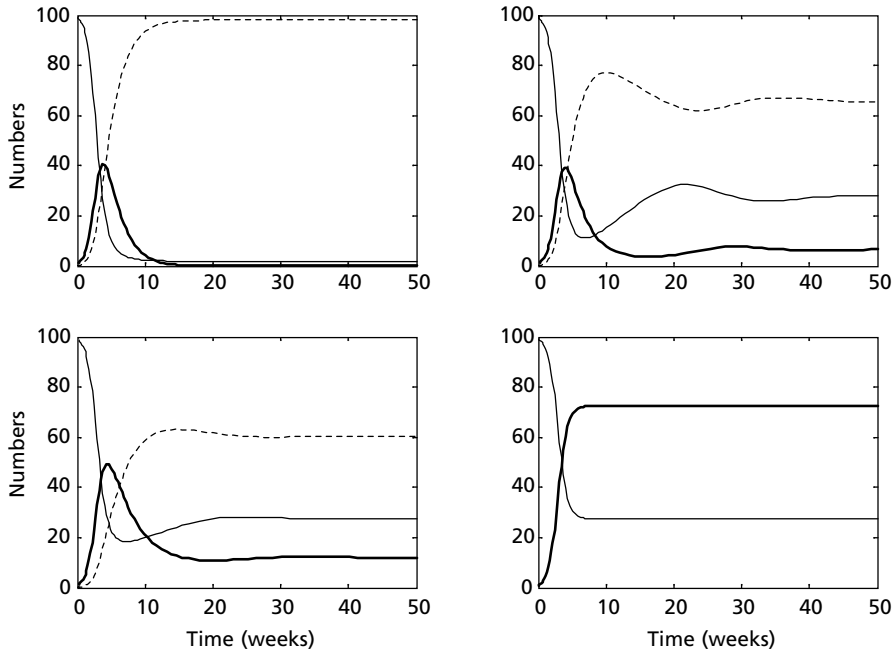

Figure 9 *The effect of supplying susceptibles on epidemic propagation (see text and Equations 12–14). The first panel shows the epidemic with no susceptible supply (SIR – as Figure 4). The second panel introduces birth/death (with $\mu = 0.05$ so that each individual has an expectancy of staying 20 weeks in the population). The third panel introduces partial immunity ($\mu = 0.05$, $\pi = 0.5$). The fourth panel shows the SIS situation, with no immunity ($\mu = 0.05$, $\pi = 1.0$). The remaining parameters are as Figure 4.*

be related back to the effective reproduction number. At endemicity, on average, each case will give rise to one other case, i.e. $R = 1$, and prevalence is constant.

This equilibrium is also dynamically stable. Figure 10 shows the situation where additional susceptibles are either added or removed from a population at endemic equilibrium. The removal of susceptibles (e.g. by a one-time vaccination campaign – left column in Figure 10) results in a decrease in transmission ($R < 1$) and consequently the number of infectious individuals. Susceptibles entering the population are less likely to get infected than at equilibrium, so their prevalence rises, as does R. When $R = 1$ the number of infectious individuals bottoms out, and starts to rise as the numbers of susceptibles increases and $R > 1$. A small epidemic ensues. The addition of susceptibles (right column in Figure 10) immediately results in an epidemic ($R > 1$) during which the additional susceptibles are 'consumed'. In both cases susceptibility and infection return to equilibrium where $R = 1$.

The value $R = 1$ is a special situation, and decreasing or increasing the proportion susceptible moves R away from this value, although infection dynamics will act to increase or decrease the proportion susceptible and bring R = 1. The value $R = 1$ occurs when the proportion susceptible is at a *critical value*:

$$x^* = \frac{1}{R_0} \qquad (15)$$

Transmission dynamics acts to keep the proportion susceptible at this critical value.

BASIC PRINCIPLES OF VACCINATION

Having discussed some of the basic concepts and potential complexities, we turn, at last, to the effects of vaccination. For each individual host, successful vaccination is a short cut in the natural history that results in moving from susceptible to immune without the infection. For the pathogen, vaccination means removing susceptibility, i.e. potential hosts. As we have seen, susceptibility is a key quantity in determining the population level dynamics of infection, so vaccination will have dynamic consequences. In particular, because individuals are not independent, vaccinating some hosts will have consequences for those hosts who are not vaccinated.

Herd immunity and reproduction numbers

An important concept in infectious disease is that of herd immunity, which is often confused so we adopt a particular definition. A population (herd) can be said to be immune to a specific pathogen when that infection is not endemic and cannot be introduced. In particular, the in-migration of infected people will not result in continuous chains of transmission and an epidemic. A more poetic term is 'community immunity'.

For an epidemic to occur, and consequently the infection has the potential to become endemic, the effective reproduction number must be greater than one. In a

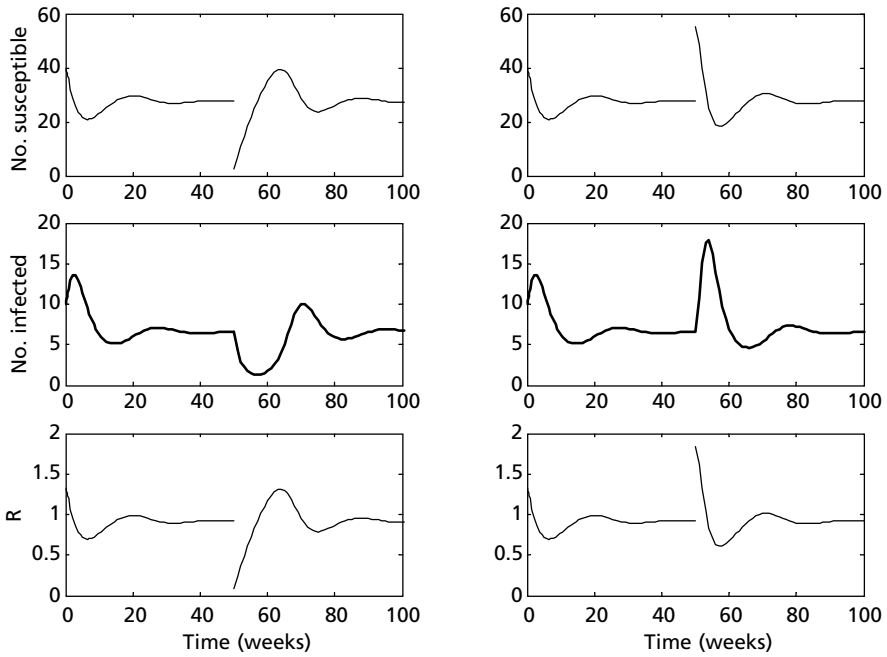

Figure 10 *Dynamic effect of increasing or decreasing the proportion susceptible at one point in time. The model (Equations 12–14); $\mu = 0.05$, $\pi = 0.0$) is run for 50 weeks, then either 90 percent of susceptibles are moved to immune class (left panels) or the number of susceptibles is doubled by movement from the immune class (right panels). The rows show the effect on susceptible class (top row), infected class (middle) and effective reproduction number (bottom) (immune population not shown). Note that susceptible numbers and effective reproduction numbers are linear transformations of each other (Equation 11).*

completely susceptible population, the basic and effective reproduction numbers are equal, but reduction in susceptibility directly (and linearly) reduces the effective reproduction number (R_0 remains unchanged by susceptibility). Without any natural infection in the population, we can divide the population into two categories: the proportion susceptible, x, and the proportion who have been successfully made immune to infection through vaccination, p_v. Figure 11 shows the relationships between these quantities. If the proportion susceptible is forced below its critical value, x^*, by immunization, there are insufficient susceptibles to permit an epidemic. The proportion of the population that must be immunized to achieve this is:

$$p_v^* = 1 - x^* = 1 - \frac{1}{R_0} \qquad (16)$$

Thus, if $R_0 = 2$ then $p_v{}^* = 0.5$ so that immunizing more than 50 percent of the population will mean that, if the infection is introduced, $R < 1$ and an epidemic will not occur.

The tantalizing upshot is that a population is immune to an epidemic even though a proportion of the individuals remains susceptible. The unvaccinated individuals derive their protection from the vaccinated. Vaccination of individuals not only protects them directly, but also protects the unvaccinated. Much of the theory of immunization is about working out how large the proportion susceptible can be without leaving the community vulnerable to pathogen invasion.

Vaccination and susceptibility

In the previous section, we considered the introduction of a pathogen *after* the introduction of vaccination. However, the more usual situation is introduction of the vaccine into a population in which the pathogen is already endemic.

The fundamental concept is that, while an infection remains endemic within a population, vaccination does not reduce the proportion susceptible in the population. Instead it replaces those with a previous history of infection (and now immune) with those vaccinated. This is illustrated in Figure 12, using the simple model of Equations 12–14, but allowing a proportion, v, of births to enter the immune class directly (i.e. they are vaccinated before any exposure). As susceptible individuals are removed by vaccination into the immune class, the proportion susceptible *temporarily* declines. The dynamic effect is very similar to that shown in Figure 10. Eventually, for a given vaccination rate (which does not eliminate the infection, $v < 0.7$) there is a balance achieved between input to the susceptible pool (from births) and output from the susceptible pool, which is now divided up by the processes of infection *and* vaccination. The upshot is that the proportion susceptible regains its pre-vaccine level, but not so the proportion infected, because removal of susceptibles is now a process shared between infection and immunization.

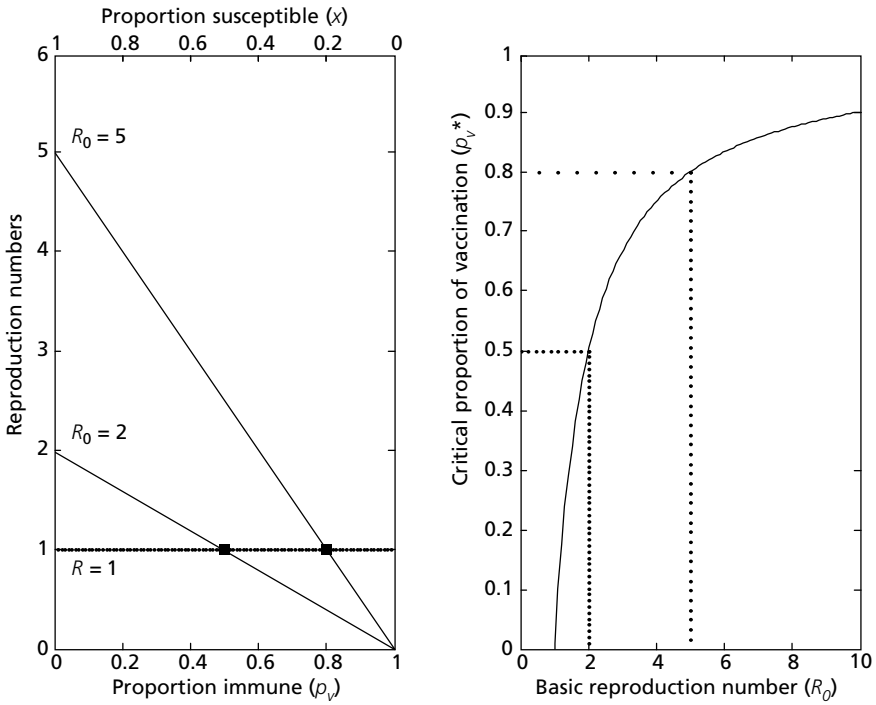

Figure 11 *Community immunity: the relationships between the proportion vaccinated,* p *, the proportion susceptible,* x*, and the reproduction numbers,* R_0 *and* R*. The left panel gives two examples, for* $R_0 = 5$ *and* $R_0 = 2$*. As the proportion immunized increases, so the effective reproduction number (solid lines) in each case decreases linearly. The critical value, i.e.* R *= 1, is marked. The right panel shows the relationship between the basic reproduction number and the immunization proportion required for* R *= 1. The two examples (*$R_0 = 5$ *and* $R_0 = 2$*) are marked as dotted lines.*

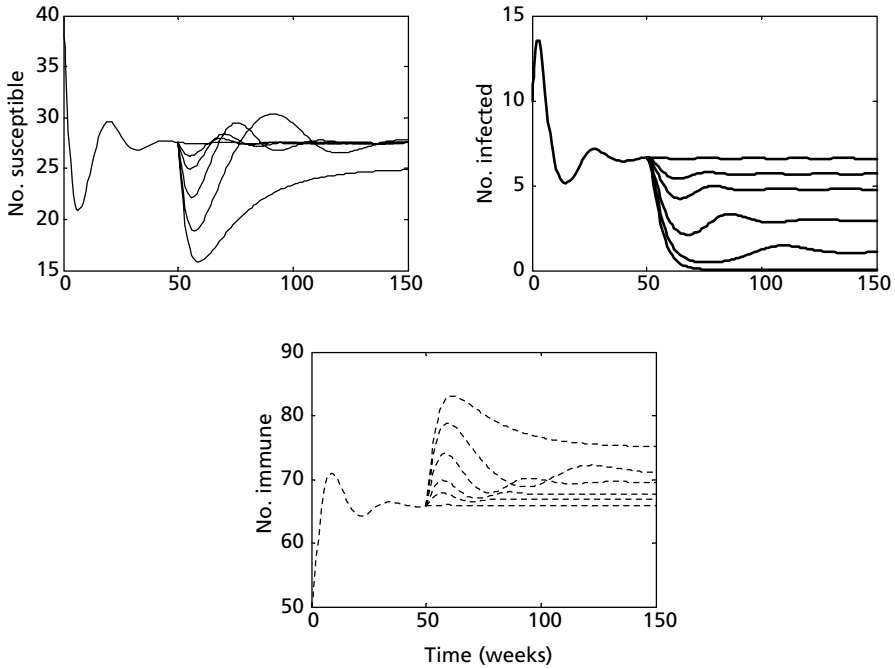

Figure 12 *Illustration of the dynamic effects of immunization using the model defined in Equations 12–14, modified to include vaccination at birth (see text). The panels show the numbers of susceptible, infected, and immune respectively. The model is run to equilibrium for 50 weeks, and immunization introduced at six levels (*v *= 0, 0.1, 0.2, 0.4, 0.6, and 0.75 where* v *= 0 is no vaccination). Here* $R_0 = 3.33$ *and* $p_v^* = 0.7$*.*

Only when immunization forces the proportion susceptible below the critical proportion, x^*, does the pathogen become extinct. For this example, when $v = 0.75$, which is greater than 0.7, there are not sufficient susceptibles entering the population for $R = 1$ to be maintained. In this case the infection becomes extinct, but a proportion of the population (25 percent) remains susceptible and 75 percent are immune. Note that the threshold required to eliminate infection from a population is the same as that required to protect a population from invasion (i.e. herd immunity). Again, it is not necessary to immunize all the members of a population to eliminate the infection.

Time (dynamic) effects of vaccination

Following the introduction of a vaccine, given a high enough coverage to appreciably reduce the proportion susceptible, cases of infection will decrease, often markedly so. This is particularly the case with infections that have short latent and infectious periods. A period of low incidence occurs, which is frequently ended by an alarming rebound in cases. This rebound, after what has been termed a 'honeymoon period' (McLean 1992), arises because of a rebound in the proportion susceptible well above the epidemic threshold, while incidence is low (see above and Figure 10). The rebound is an expected epidemiological consequence of vaccination and not a failure of vaccination policy as such, although they are frequently ascribed to a failure in vaccine or vaccine delivery. In the longer term, although vaccination coverage remains below that required for elimination, epidemics are likely to continue but less frequently than was the case before vaccination. This is because after each epidemic the rate of replenishment of the susceptible pool to epidemic proportions is reduced compared with pre-vaccination in proportion to level of vaccine coverage.

In general the impact of vaccination programs may not be immediate, because the targeting of single age classes generates only a gradually increasing proportion of the population with vaccine-induced immunity. The extreme of this would be a program of universal infant vaccination for an infection transmitted by sexual contact, such that a lag of a decade would arise before those protected by vaccination aged into the at-risk sexually active groups. In response to this it is now common to introduce routine immunization procedures with an initial one-off catch-up program of vaccination to a wide segment of the risk population.

Age effects of vaccination

Given that vaccination will tend to result in a reduction in the proportion infected, and hence lower the force of infection (see above and Figures 10 and 12), the average time for an individual, following birth, to be exposed and infected will increase. Thus the average age at infection will increase as a result of vaccination. The relationship between the transmission potential of an infection (R_0), the proportion vaccinated, v, and the long-term (equilibrium) average age at infection, A_v, is:

$$A_v = \frac{L}{R_0(1-v)} \qquad (17)$$

where L is the life expectancy at birth. This is shown in Figure 13 for an archetypal virus (also assuming one survival type where all individuals born live until the average life expectancy from birth). Clearly, the effect on A_v is greater for higher levels of coverage, for less transmissible infections (lower R_0), the younger the age at delivery (i.e. where the proportion susceptible is higher), and for nonselective rather than selective vaccination.

The implications of increasing the average age at infection are various (Nokes and Anderson 1988; Anderson and May 1991), dependent, for example, on the age-related nature of disease caused by infection (see also Coleman et al. 2001). Where, like measles, the risk of mortality declines with age, a rise in age at infection in those who missed vaccination will translate into an additional indirect benefit of the vaccine program.

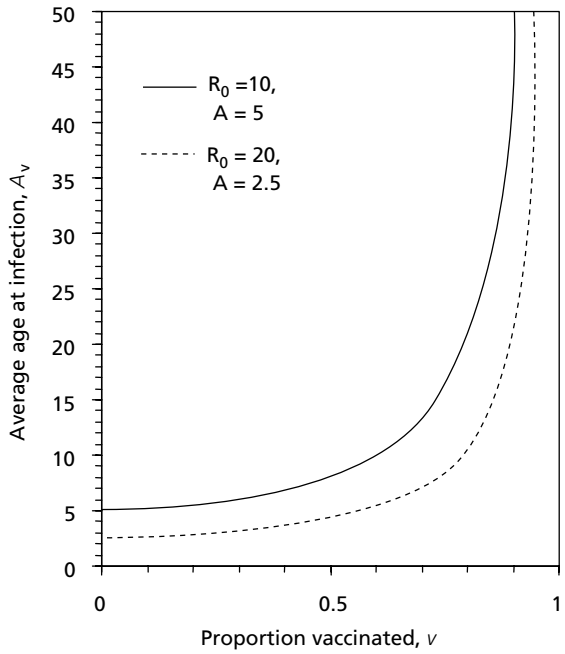

Figure 13 *Vaccination increases the average age at infection. The relationship between the proportion vaccinated at birth, v, and the new average age at infection under such vaccination pressure, A_v, is shown for two infections with different reproductive capacities. Where the value of R_0 (20) is highest, and thus the average age at infection, A, in the absence of vaccination is lowest, the impact of a specific level of vaccine coverage on A_v is, in absolute terms, less than for the infection with lower R_0 (higher A) – although the proportional change is equal. (Adapted from McLean 1992.)*

This would also apply to HBV infection and the risk of developing long-term carriage (Medley et al. 2001). In contrast, where the risk of disease increases appreciably with age, e.g. the risk of intrauterine infection from rubella which increases nonlinearly in relation to fertility, the beneficial effect of a particular vaccine program in reducing cases of infection may be severely undermined by the increased risk of severe disease arising from those cases that remain and occur at an older age (Anderson and Grenfell 1986). Observational evidence that supports this theoretical prediction was a recent epidemic of rubella among women of childbearing age in Greece (Panagiotopoulos et al. 1999).

In parallel with the increase in average age at infection resulting from vaccination, the average age of the proportion susceptible will increase (Nokes and Anderson 1988). Again, this is supported by data (Ukkonen and Von Bonsdorf 1988; Ukkonen 1996; Bahri et al. 2003). As different age groups show differing behavior, e.g. mixing patterns, changes in the age distribution of susceptibles may appreciably alter the observed epidemiology of the infection where the behavior in question is related to the route of transmission. For example, studies demonstrate that the susceptible risk of exposure to a variety of viral infections (e.g. MMR, HBV, pertussis) changes with age, presumably because of age-related changes in mixing, and other behavioral characteristics (Anderson and May 1985; Nokes et al. 1986; Edmunds et al. 1997). A general trend appears to be for a decrease in the force of infection with increasing age. Thus, vaccination tends to move susceptibles into older age groups where they are both less likely to acquire infection and less likely to transmit it. One outcome of this phenomenon is that the predicted vaccination coverage for herd immunity is diminished.

Vaccine characteristics

Vaccines are usually imperfect. The immunogenic and reactogenic characteristics of vaccines are central to predictions of their impact at a population level. Most successful vaccines have been developed against infectious agents possessing a single serotype, i.e. all variants are antigenically similar, and the host immune system sees them as the same entity with equal variant specific to variant cross-immunity. The immunogenic properties have been classified into three parameters: take, degree, and duration (Halloran et al. 1992; McLean and Blower 1993): 'take' is the proportion of vaccinated individuals who produce an immunological response to the vaccine; 'degree' is the degree of protection provided by the immunological response generated by the vaccine; and 'duration' is the time span of the immunological response.

These three attributes capture both variability between individuals (take) and the average response within those individuals that respond (degree and duration). The efficacy of a vaccine is the combined effect of a vaccine within an individual (and is a combination of all three characteristics). The effectiveness of a vaccine is a measure of the combined effect of a vaccine within a population. The effectiveness thus includes the results of herd immunity, and includes the protection given to the nonvaccinated individuals.

Delivering the vaccine

Mechanisms for delivering vaccine to the target population vary considerably. The reasons for these differences arise from (1) the need to target individuals most at risk, e.g. *Haemophilus influenzae* in infants versus hepatitis B in drug-using adults; (2) existence of mechanisms by which to deliver vaccines efficiently, e.g. maternal–child health services or general practice clinics; and (3) different transmission routes.

From the perspective of representing the delivery mechanisms accurately in models, the key characteristics are (1) age at delivery, (2) proportion receiving vaccine, (3) vaccine failure rate, and (4) selectivity of vaccination based on past disease, risk group, or vaccine history. Model structures may need to alter to reflect the particular mechanisms of delivery, e.g. universal infant vaccination might be modeled most simply as the redirection of a proportion (v) of all individuals born into the population into a vaccine-immunized class whereas the remainder ($1 - v$) enter other population classes – usually the susceptible class (see Figure 12). This assumes 0 percent primary vaccine failure, no influence of maternal-derived specific antibody, and random delivery of vaccine, i.e. no overt selection procedure for vaccination. However, this simple model might not adequately describe the situation if the vaccine is neutralized by the presence of maternal antibody (e.g. live attenuated vaccines), or must be delivered across a range of age classes as in campaign or catch-up programs. In these instances, it will be more appropriate to consider age classes within the model structure.

The assumption of random delivery is implicit within most models, and in the estimation of summary parameters such as the herd immunity threshold. The basic assumption is that all susceptibles are equal, insofar as prevention of infection in any individual will prevent an equal number of future infections that would have arisen had that individual not been vaccinated. However, not all individuals are equal with respect to transmission: certain age groups may have higher rates of contact within their own and with other age groups (e.g. important in transmission of aerosol-transmitted infection); core groups exist in relation to the transmission of infections by sexual contact; super-spreaders have been suspected in recent severe acute respiratory syndrome (SARS) outbreaks in east Asia (Riley et al. 2003). So, targeting such groups with vaccine has a

disproportionately large, indirect, protective effect at the community level. In a different way, the control of smallpox through targeted vaccination of contacts to suspected cases illustrates a nonrandom delivery mechanism. In either case, the level of effective coverage required to develop herd immunity will be significantly less than that predicted through random delivery of vaccination, provided, of course, that the identification of 'super-spreaders' or contacts is accurate.

From an economic perspective, selectivity in delivery of vaccine may have cost–benefit implications, e.g. the benefits of selectively targeting highest risk groups (such as individuals attending STI clinics receiving HBV vaccine), with reduced numbers of vaccines delivered, may be outweighed by the costs associated with delivering the vaccine, which might involve prior screening for susceptibility and counseling (Williams et al. 1996).

Vaccination by repeated campaigns

The usual notion of a vaccination program is to vaccinate individuals as they pass some age gateway, e.g. infant vaccination schedules for diphtheria/tetanus/polio (DTP), HBV, *Haemophilus influenzae* b (Hib). However, vaccination across an age segment of a population has gained prominence in the last two decades either as one-off campaigns for the prevention of suspected epidemics or for 'catch-up' purposes, or repeated campaigns or pulses of vaccine aimed toward elimination. The idea is not new, and indeed, in the late 1950s, modeling studies of measles campaign interventions helped select the time interval between measles campaigns in west Africa, and led to temporary elimination of measles circulation for 3 years. More recently, repeated national vaccination days through social mobilization significantly contributed to the success in Latin America and the Caribbean in controlling measles and polio in the early to mid-1990s. This strategy has subsequently been extended globally for polio with remarkable success, and increasingly is part of accelerated measles control programs.

The principle underlying the idea of pulse vaccination is the targeting and removal of a large proportion of susceptible individuals in a short space of time through vaccination, thus suppressing the proportion susceptible below the threshold required to maintain chains of transmission, i.e. $R < 1$. This leads to a rapid decline in incident cases, particularly if the infectious agent in question has a short infectious period. If this process is repeated at a frequency that prevents the susceptible proportion ever rising to epidemic proportions, inevitably the infection must be eliminated.

This principle is schematically presented in the Figure 14. Based on this understanding it is possible to establish some simple expressions characterizing the frequency of campaigns given a particular level of campaign vaccine coverage. At its simplest, if a proportion v' of the population (i.e. of susceptibles if applied at random) is effectively vaccinated in a single pulse, then the time taken until the effective reproductive number attains unity ($R = 1$), is the pulse interval, given by:

$$T_v = \frac{v' x^*}{f} = v' A \qquad (18)$$

where f is the proportion of the population born each unit of time (e.g. year) and A is the average age at infection (before vaccination) (Nokes and Swinton 1997). Furthermore, if routine universal immunization (at

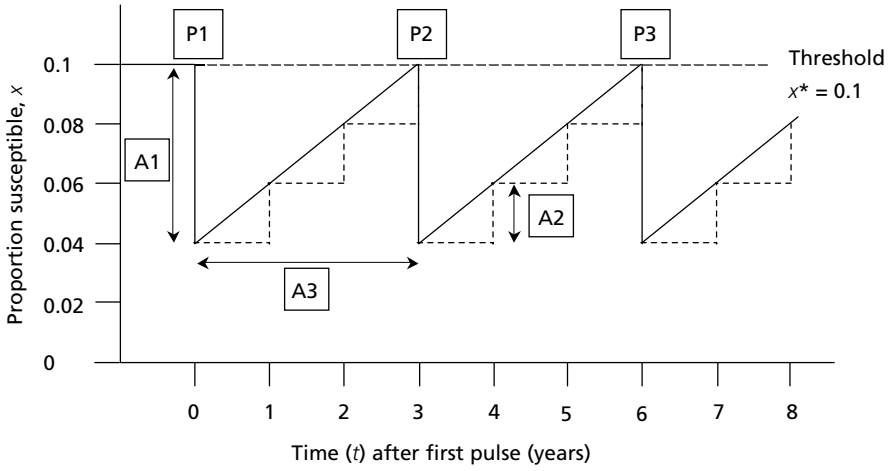

Figure 14 *Principle of pulse vaccination. A proportion, v′ = 0.6 (i.e. 60 percent) of all individuals are vaccinated in pulses P1–P3 at times 0, 3, 6, . . . et seq., when the proportion of the population susceptible is at its epidemic threshold (i.e. x = x* = 0.1 or 10 percent, arrow A1). Thus the proportion susceptible is reduced by 6 percent (i.e. v′x* = 0.06). Subsequent to each pulse, susceptibles increase at a maximum rate of f = 0.02 per year (A2) as a result of births (i.e. 2 percent of the population are born – and die – each year), assuming no continued infection (which would otherwise reduce the rate of replenishment of susceptibles). Thus, in 3 years (A3) the deficit in susceptibles caused by the vaccine pulse has been recruited by births, regaining epidemic proportion (x = x*) whence a further pulse is due. (Taken from Nokes and Swinton 1997.)*

around birth) is in place, at an effective coverage of v, then this slows the rate of replenishment of the susceptible pool, and extends the pulse interval:

$$T_v = \frac{v'A}{1-v} \tag{19}$$

Thus we obtain some general concepts about what is important in determining the program requirements for control through pulse vaccination, namely epidemiology and demography (i.e. x^* and f, which define A) and programmatics (i.e. v' and v). So that, given two infections that differ in their transmissibility such that one has a lower average age at infection than the other, then, within the same demographic context, control of infection 1 will either require more frequent pulses, or higher level campaign coverage, than infection 2. This is of some practical value in informing policy makers. In particular, the timing of pulses should be determined by the infection.

More generally vaccination is applied to specific age groups, e.g. under 5 year olds, where it is assumed most susceptibles are, and thus vaccine (which is usually delivered irrespective of past history of disease or vaccination) will be most efficiently used. Consequently, if effective vaccine coverage is not 100 percent, susceptible individuals may accumulate in older age groups, beyond the reach of childhood vaccination, which may alter the predictions of when subsequent pulses are required. This strategy may also accumulate numbers of at-risk individuals, e.g. women of childbearing age susceptible to rubella.

Again, this model deals with the issues very simplistically. More useful models for predictive purposes require explicit descriptions of these features, i.e. age structure, age-related mixing rates, accurate demographic schedules (Nokes and Swinton 1997). Such models thus become more specific to particular locations and setting and less general, i.e. they lose the quality of general insights to be gained from the 'too simple' models.

MODEL APPLICATION

In this section, we discuss some examples where models have been used to address specific problems. There are many types of model, some of which we have referenced here: none of them is 'right' in the sense that it captures reality; many of them will be 'useful' in the sense that they provide insight, understanding, and some quantitative predictions.

The question remains, for the non-modeler, of how to judge the quality of a model. There are two aspects. First, the assumptions used to design the model should be clearly and explicitly stated. You, the reader, should decide what processes you think are important (e.g. host age and gender) and determine if they are included. To simplify, models usually have to 'average' over heterogeneities, e.g. if the modeler does not believe that host gender has any effect, the model will have 'people' as its fundamental unit, implicitly taking an average of male and female. If there are significant differences between genders, the average will not be sensible and the model results are less likely to be useful.

Second, and more difficult for the nonspecialist, is that the model defined should be analyzed and solved properly. The best advice is to treat the model as any other technical, laboratory procedure. In that case you will be looking for some demonstration that the results are reliable, and that, in defined and known circumstances, the model produces the expected results.

Design of vaccination programmes: MMR

Rubella occupies a unique position in viral infections. A solid, life-long immunity develops after either infection or vaccination, and the disease caused by infection is solely related to infection during pregnancy. The objective of a vaccination program is clear – to reduce the incidence of congenital rubella syndrome (CRS). Given the relative simplicity, there have been several models developed that address this problem (e.g. Anderson and May 1983; Anderson and Grenfell 1986; Massad et al. 1994, 1995; van der Heijden et al. 1998; Edmunds et al. 2000).

The results of these models (borne out by observation) demonstrate the dangers of vaccinating at coverages well below that required for elimination. Although those individuals vaccinated are protected, those who are not vaccinated experience an increased average age at infection and hence a greater risk of disease. The population level benefit derived from the vaccinated does not necessarily outweigh the worsened situation in the unvaccinated. This situation is complicated when vaccine is available through private purchase, because the distribution of benefit and detriment might be expected to exacerbate inequalities (Vynnycky et al. 2003).

To prevent recurrent epidemics in a vaccinated population, it has become increasingly common to recommend multiple schedules, i.e. vaccine is offered twice at different ages (Babad et al. 1995). The two-dose MMR vaccine program in the UK was supported by modeling, as was the MR 'catch-up' campaign (Gay et al. 1995).

Design of vaccination programmes: hepatitis B virus

The disease caused by HBV infection can be divided into acute and chronic. Acute disease occurs during the weeks and months after infection, and, like many other viral infections (e.g. rubella), the risk of disease probably increases with age at infection. If the immune response is able to clear the infection, the individual is

left solidly immune to further infection. The alternative outcome is that a chronic infection develops and individuals become infectious carriers, and can remain so for many years. The risk of developing chronic carriage is highly age dependent: over 90 percent for infected infants and perhaps 5 percent for adults. The sequelae of chronic carriage include cirrhosis and primary hepatic cancer. There are safe, cheap, and effective vaccines to prevent acute infection. How should they be used?

The answer to this question will depend on the epidemiology of the infection, which varies dramatically across the globe. In The Gambia (west Africa) HBV is a childhood infection, with over 80 percent of children infected by the age of 10 years. The exact route of transmission is essentially unknown. As the risk of developing carriage is higher for those infected as children, the proportion of the population with chronic infection in areas of high endemicity is also high (perhaps as high as 10 percent). In complete contrast, in countries such as the UK, HBV is essentially an adult infection, with the majority of transmission associated with sexual contact or intravenous drug use. Consequently, carriage is relatively rare, and certainly less than 1 percent in the general population (Hahne et al. 2004). Between these two extremes, there is a whole range of endemicities (e.g. Coursaget et al. 1994).

There is little doubt that immunization is indicated in areas of high endemicity (e.g. Edmunds et al. 1996), but in low endemic populations the risk of infection is so low that there is some debate as to whether mass immunization is required, and is essentially a cost-effectiveness issue (see below). However, the relationship between average age at infection and probability of developing carriage provides a possible explanation for the global variation in HBV endemicity (Medley et al. 2001). If this process is important, the effect of vaccination is further enhanced.

Cost-effectiveness and health economics

Decisions concerning the implementation of any public health intervention should involve a consideration of the economic costs and expected health gains. Models of the type discussed here are capable of providing predictions of the latter, but care needs to be taken to ensure that the two frameworks are compatible in terms of scope (or perspective) and discounting. In particular, the prediction of the health impact of introducing a vaccine should include consideration of the effect on unprotected individuals (Edmunds et al. 1999).

Quantitative prediction

As mathematical models deal with numbers, for most non-modelers it would seem that the principal use of models should be to provide quantitative predictions. However, we hope that this chapter has demonstrated that accurate quantitative prediction is very difficult, and that qualitative prediction and increased understanding are the best output. Even in situations where the only value of the model is numerical prediction (e.g. Ghani et al. 2003), the predictions provide a test of our understanding; if the predictions are wrong, clearly there are processes that are not included or fully understood – the model is wrong. Note, however, that, if predictions prove correct, this is not proof of model correctness. In situations where models are intended to guide policy, testing them against data through prediction does provide some validation. When models are not validated against data, they have no such empirical support.

Evolution

Another unique feature of infectious disease (in contrast to noninfectious) is that the organisms involved (hosts and pathogens) are subject to evolutionary change. As pathogens usually have a much shorter generation time than their hosts, they are expected to evolve faster. In terms of vaccination, a pathogen strain that is able to circumvent the immunity provided by a vaccine has a large population of 'susceptibles' to infect. However, McLean (1995) argues persuasively that, even if such an escape mutant occurs, it will be less infectious and therefore easier to control than the original pathogen. Until such an event arises and is well documented, these predictions remain untested.

CONCLUSIONS

Given a reasonable understanding of the natural history of an infection within the typical individual and the key factors leading to transmission between individuals, mathematical models may be developed that capture the essence of infection dynamics within a population of hosts. Such models can be put to various uses in relation to vaccine intervention, ranging from developing our understanding of underlying principles to detailed cost-effectiveness analyses.

To date, most modeling work has been undertaken retrospectively to understand why things have or have not happened as a result of vaccine intervention. However, the understanding that arises from using quantitative methods to study the transmission dynamics of infectious disease is becoming widespread. Fine (1994) suggests that it is as much the conceptual issues that modeling has developed which has been instructive to those on the policy-making committees, rather than a targeted modeling study that has led the specific decision being made.

Our own perspective, of course, is that modeling should be a requirement for the assessment of any public health intervention, including vaccination. But that this modeling

should take its place as a source of information and a vehicle for testing knowledge and understanding, rather than as a definitive prediction of outcome. To return to our bridge analogy – building a bridge without predicting its effect on traffic flow or probability of collapse is irresponsible, so why implement a vaccination program without calculating its expected effects?

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Historical introduction: evolution of knowledge of the fungi and mycoses from Hippocrates to the twenty-first century

EDOUARD DROUHET

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Since the beginnings of medical mycology at the turn of the century, with the discovery of the principal agents of human and animal mycoses (ringworm, aspergillosis, candidosis, cryptococcosis, histoplasmosis, etc.), there has been continual progress in our knowledge of pathogenic fungi that cause diseases and their role in the pathology of infectious diseases.

The pathogenic fungi and the mycoses, which have emerged in recent years in the immunocompromised host, have emphasized the importance of medical mycology, within the discipline of medical microbiology. This has been made possible by the pioneers in medical mycology, and by enthusiastic medical mycologists who founded the International Society of Human and Animal Mycology (ISHAM) in 1954. This now has more than 1 000 members and a score of affiliated medical mycological societies throughout the world. The expansion, over the past 50 years, of the spectrum of fungal infections, the clinical and pathological aspects of the mycoses, the ecology and epidemiology of the classical pathogenic and opportunistic fungi, as defined in 1962 at the International Symposium on Opportunistic Infections, has been accompanied by an increased understanding of pathogenic fungi and the mycoses. The physiology, biochemistry, immunology, morphogenesis (studied by transmission and scanning electron microscopy), sexuality, genetics, and molecular biology of fungi pathogenic to humans and animals have been studied in depth.

Many of the systemic mycoses have been known since the end of the nineteenth century, but they were considered to be extremely rare, usually discovered postmortem, and observed only in compromised patients. In recent decades, since the introduction of antimicrobials in 1945, of corticosteroids in 1950, of immunosuppressive therapy

in 1960, of catheters, prosthetic devices, and organ transplantation in the 1960s and 1970s, and with the emergence of the acquired immunodeficiency syndrome (AIDS) and the increasing use of bone marrow grafting in the 1980s, these deep mycoses have started to develop new clinical aspects, occurring with high frequency and severity. Many of the opportunistic mycoses are now nosocomial, hospital-acquired, invasive infections caused by a new spectrum of opportunistic pathogenic fungi (Bodey 1988; Weber and Rutala 1988; Jarvis 1995).

FUNGI IN NATURE AND PATHOLOGY: CLASSIFICATION

Among the more than 72 000 described species of fungi that are widespread in nature (soil, plants, water, air), more than 300 are now recognized as true pathogens or potential pathogens responsible for mycoses in humans and animals. The word fungus is derived from the Latin *fungus*, which came into use in the fifteenth century. The Latin was derived from the Greek *sphongirs* or sponge. The term 'mycosis' was derived from the Greek word *mykes* for mushroom.

The Fungi represent one of the five kingdoms of organisms classified, according to Whittaker (1969) and Margulis and Schwartz (1988), into three levels of organization:

1. the prokaryotes (kingdom Monera, including bacteria, actinomycetes, blue–green bacteria)
2. the unicellular eukaryotes (kingdom Protoctista), protozoa, nucleated algae, oomycetes)
3. the eukaryotes (kingdom Fungi, Plantae or Animalia).

The actinomycetes, which are true bacteria, have traditionally been studied by medical mycologists because, like fungi, they have mycelial forms and cause similar diseases.

The boundaries of these kingdoms and their components are still not well delineated. In the past, the causative agents for key mycoses, such as histoplasmosis (*Histoplasma capsulatum* var. *capsulatum*), which was discovered in 1906 by Darling, and coccidioidomycosis (*Coccidioides immitis*), which has been known since 1892 (Posadas 1892; Wernicke 1892; Rixford and Gilchrist 1896), were described by protozoologists as 'protozoans' because of their invasive unicellular tissue forms. They were recognized as fungi many years later – *H. capsulatum* var. *capsulatum* in 1934 by DeMonbreun, and *C. immitis* in 1900 by Ophüls and Moffitt – when they were isolated and cultured in their mycelial, saprophytic form.

Even now, revolutionary changes may occur, as with *Pneumocystic carinii*, which was reclassified as a fungus by molecular taxonomists because of its ribosomal RNA (rRNA) sequences (Edman et al. 1988). The study of the 5S rRNA of *P. carinii* showed that its phylogenetic position is closely associated with the Rhizopoda (ameba), Myxomycota, and Zygomycota (Watanabe et al. 1989); the most recent genetic studies in biomolecular taxonomy reinforced the close relationship of this organism to fungi rather than protozoa (Cushion et al. 1994) (see also *Pneumocystis* pneumonia).

Two algae that lack chlorophyll and have long been included in the kingdom Protoctista are dealt with by medical mycologists. The first of these species was described as *Prototheca zopfii* by Krüger in 1894 and the second as *Prototheca wickerhamii* by Tubaki and Sonida in 1954; they have been recognized, since the first proven infection in humans found by Davies et al. in 1964, as new pathogens (Ravisse et al. 1993).

Oomycetes are also within the province of medical mycologists. *Pythium insidiosum* (de Cock et al. 1987) was first described by Bridges and Emmons (1961) as *Hyphomyces destruens* in infected horses; it is now an emerging agent of animal (cattle, dogs, and cats) and of human infections in Australia, Japan, Latin America, Thailand, and the USA (Mendoza et al. 1996).

The principal fungi causing the mycoses are responsible for:

- superficial and cutaneous mycoses
- deep, systemic mycoses caused by the opportunistic yeasts or filamentous fungi or highly pathogenic dimorphic fungi that are introduced primarily into the respiratory tract (Figures 1 and 2) and
- subcutaneous mycoses.

This clinical classification is not rigorous because superficial fungal infections may become systemic mycoses that present as mucocutaneous manifestations.

MILESTONES OF MEDICAL MYCOLOGY

In two fascinating books, one devoted to the history of mycology (Ainsworth 1976) and the other to the history of medical and veterinary mycology (Ainsworth 1986), Ainsworth, the author and former Director of the Commonwealth Mycological Institute (at Kew in the UK), gives a straightforward account of the main views held about fungi in the past three millennia and the development of the study of fungi as a branch of the science of medical microbiology over the last 250 years.

As emphasized by Ajello (1975a), the evolution of medical mycology can be compared to the great network of roads that linked the farthest reaches of the Roman empire to the Eternal City, where at designated distances between cities, stone pillars were erected to mark the miles. The milestones of medical mycology were designated by Ainsworth (Ainsworth 1976, 1986; Ainsworth and Stockdale 1984), Ajello (1975a), Emmons et al. (1977), Seeliger (1985, 1988), Rippon (1988), and numerous other authors in their introductions to books and articles (Baldwin 1981; Huntington 1985; Drouhet 1991; Kwon-Chung and Bennett 1992).

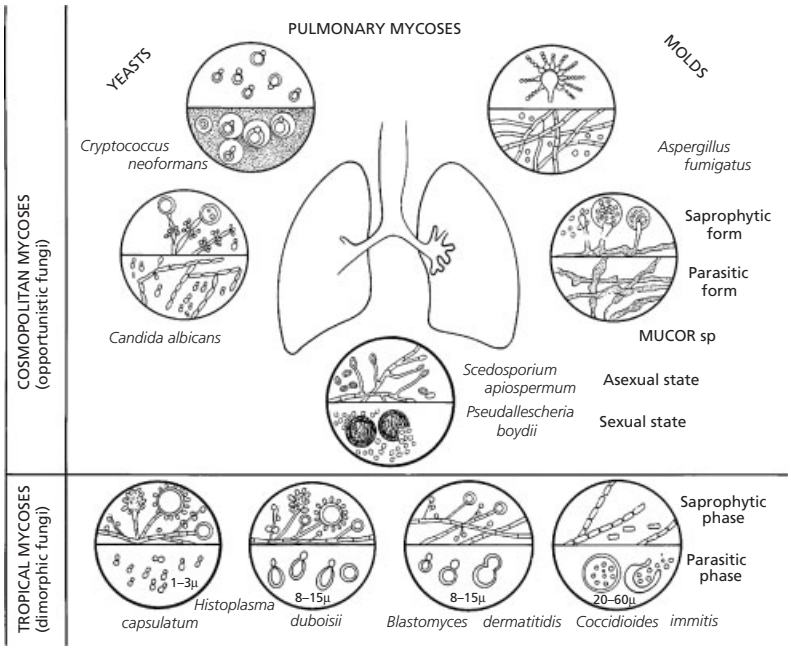

Figure 1 *Principal fungi responsible for pulmonary and deep mycoses: saprophytic form (upper hemisphere) and parasitic form (lower hemisphere). For* Scedosporium apiospermum *(asexual state), the sexual stage is in the lower hemisphere (*Pseudallescheria boydii*).*

The earliest known record of a mycotic infection is that in the Atharva Veda in India (about 2000–1000 BC), of mycetoma of the foot which was described under the name 'foot anthill.'

As a result of their visibility, the superficial mycoses of the mucosa or tegument have been known since antiquity. Hippocrates (460–377 BC) and Gallen (AD 130–200) described *aphthae albae* (in English called thrush, and in French *muguet* because of the whiteness of the infected tongue), whereas in 1847, Robin identified the yeast *Oidium (Candida) albicans* as the agent of thrush in infants (Robin 1853).

Celsus (first century AD), following the nomenclature of Hippocrates, dealt, in *De re medicina*, not only with thrush but described favus, the inflammatory lesions of some ringworm infections – kerion of Celsus. The first dermatophytic fungal infections were recognized only about 1840 by Remak, Schoenlein, and Gruby (Seeliger 1985, 1988).

The centrifugal growth of fungi in a circular form on the skin led to various names such as herpes (Greek: dandruff), now known as *herpes circinatus*. The Romans associated the lesions with insects and named the disease tinea, meaning any small insect larva. This name has remained in English clinical terminology (tinea capitis, tinea corporis, tinea pedis, etc.), and is translated in French as *teigne* and in popular English as ringworm. *Favus* (Latin: honeycomb), a specific chronic scalp

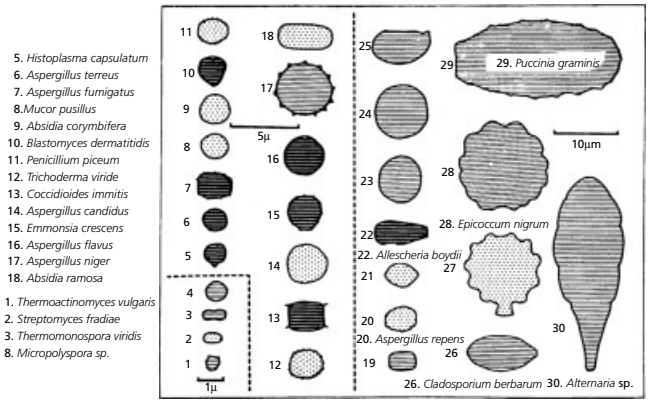

Figure 2 *Spores in allergies and mycoses of humans and other animals. Outlines of the airborne spores of Fungi and Actinomycetes causing allergies and mycoses. Directly pathogenic species – dark shading; allergenic species – light shading; species isolated from animal lungs – dotted. (Redrawn from Austwick 1966)*

mycosis, characterized by yellowish cup-shaped crusts (scutula) was the first human mycosis to be recognized as caused by a dermatophytic fungus by Remak and Schoenlein (Schoenlein 1839; Remak 1845); this discovery has been described by Seeliger (1985). Tinea imbricata, a tropical dermatophyte disease, easily recognized by the polycyclic, concentrically arranged rings of scales which are scattered over most of the body, was described by travellers in the Pacific Islands of Oceania. Dampier (1729) named it 'Tokelau disease' after the Islands where the disorder was prevalent. This disease was recognized also in southeast Asia, and it has been suggested that it may be of anthropological interest, as it was possibly introduced to the western coasts of South and Central America by pre-Colombian voyagers from Polynesia (Rippon 1988). Only in 1896 did Blanchard describe the agent as *Trichophyton concentricum* and later isolated cultures with difficulty.

The invention of the microscope during the last decade of the sixteenth century enabled seventeenth century microscopists, such as Hooke of England with his *Micrographia* London (1665), who presented the first illustrations of microfungi such as *Mucor*, followed by the Dutchman van Leeuwenhoek (1632–1723) and the Italian Malpighi (1628–94), in *Anatomia plantarum pars altera*, London, 1679, to give the first descriptions of some common microfungi. van Leeuwenhoek is credited with being the first person to have observed yeasts microscopically. In 1680 he sent descriptions and drawings of yeast cells to the Royal Society of London, 4 years after making public his observations on bacteria.

Aided by the binomial system of the Swedish physician and botanist Linnaeus (1707–78), descriptive mycology grew gradually, but steadily. Micheli (1679–1737), a Florentine botanist of the Public Gardens in Florence, was the first to give generic names to fungi that are now classified among the Hyphomycetes. His monograph *Nova plantarum genera* (published in Florence in 1729) was not only a historical milestone but also the starting point for mycology as a science. Familiar genera such as *Botrytis* and *Mucor* were described for the first time.

CONCEPT OF PATHOGENICITY

It required more than 200 years for a proposal of the concept of infectivity to be made. Fracastora of Verona (1546) postulated the existence of a *contagium vivum* as the cause of infectious diseases and foresaw a relationship between microfungi in nature and fungal diseases in humans and animals.

The first milestone in the medical mycological highway is attributed by most historians of medical mycology to the Italian, A. Bassi (1773–1856) (Ajello 1975a, 1993; Ainsworth 1976, 1986). In 1807, after he had studied 'physics, chemistry, natural history, some branches of medicine and the principles of mathematics,' Bassi began to study a devastating disease of the silkworm (*Bombyx mori*), the muscardine, and proved that it was caused by a fungus subsequently named, in his honor, *Beauveria bassiana*.

Bassi's revelation of the role played by a fungus in an animal disease inspired others to investigate the etiology of human diseases. Schoenlein and his assistant Remak succeeded in discovering, in 1837, the agent of the human favus, which was named *Achorion (Trichophyton) schoenleinii* by Remak (1845) in honor of his mentor Schoenlein (1793–1864), but the studies of Gruby (1810–98) proved to have a greater impact among the physicians at that time through his communications to the Academy of Sciences of Paris (1841–44) about the fungal origin of favus. For the first time, Gruby tried to reproduce a human infection by inoculating a colleague in the arm and also his own body with the fungus. He fulfilled Koch's postulates for the criteria of the etiology of infection 40 years before Koch had formulated them. In 1841, Gruby also described the in vivo appearance, in thrush, of the yeast named *Oidium (Candida) albicans* by Robin (Figure 3) in his doctoral thesis 6 years later (Robin 1847). Robin (1821–85), elected to the chair of histology at the University of Paris, made other important contributions to mycology, describing for the first time the dermatophyte *Trichophyton ('Microsporon') mentagrophytes* in his book published in 1853: *Histoire naturelle des végétaux parasites qui croissent sur l'homme et des animaux*. Gruby described an important agent of tinea capitis which he named *Microsporum audouinii* in honor of the great Parisian naturalist, V. Audouin (1797–1841), of the Paris Natural History Museum, known for his work on muscaridine disease. This is considered the second important historical milestone of medical mycology (Zakon and Benedek 1944). His microscopic descriptions (after his death more than 15 000 microscopic preparations and 2 000 photomicrographs were found among his possessions), interpreting host–parasite relationships, prepared the work of Sabouraud (Rook 1978). The role of fungi in these early concepts was preceded by the work of dermatologists such as W. Wilson (1817–84), J. Hoog (1817–99), T. Fox (1836–79), F. van Hebra (1816–80) of Vienna, and E. Bazin (1807–78); the last named wrote a thesis entitled 'La contagion et l'infection' (1839) and became a physician at the St Louis Hospital in Paris, publishing a monograph on ringworm in 1853.

Over several decades, pathogenicity was associated with the concept of spontaneous generation, and finally Pasteur succeeded in winning the fight against this concept of spontaneous generation of microorganisms – a theory that had been well established since antiquity. Pasteur (1822–95; Figure 4), unanimously recognized as the founder of microbiology, in the famous memoirs about the 'organised corpuscles existing in the atmo-

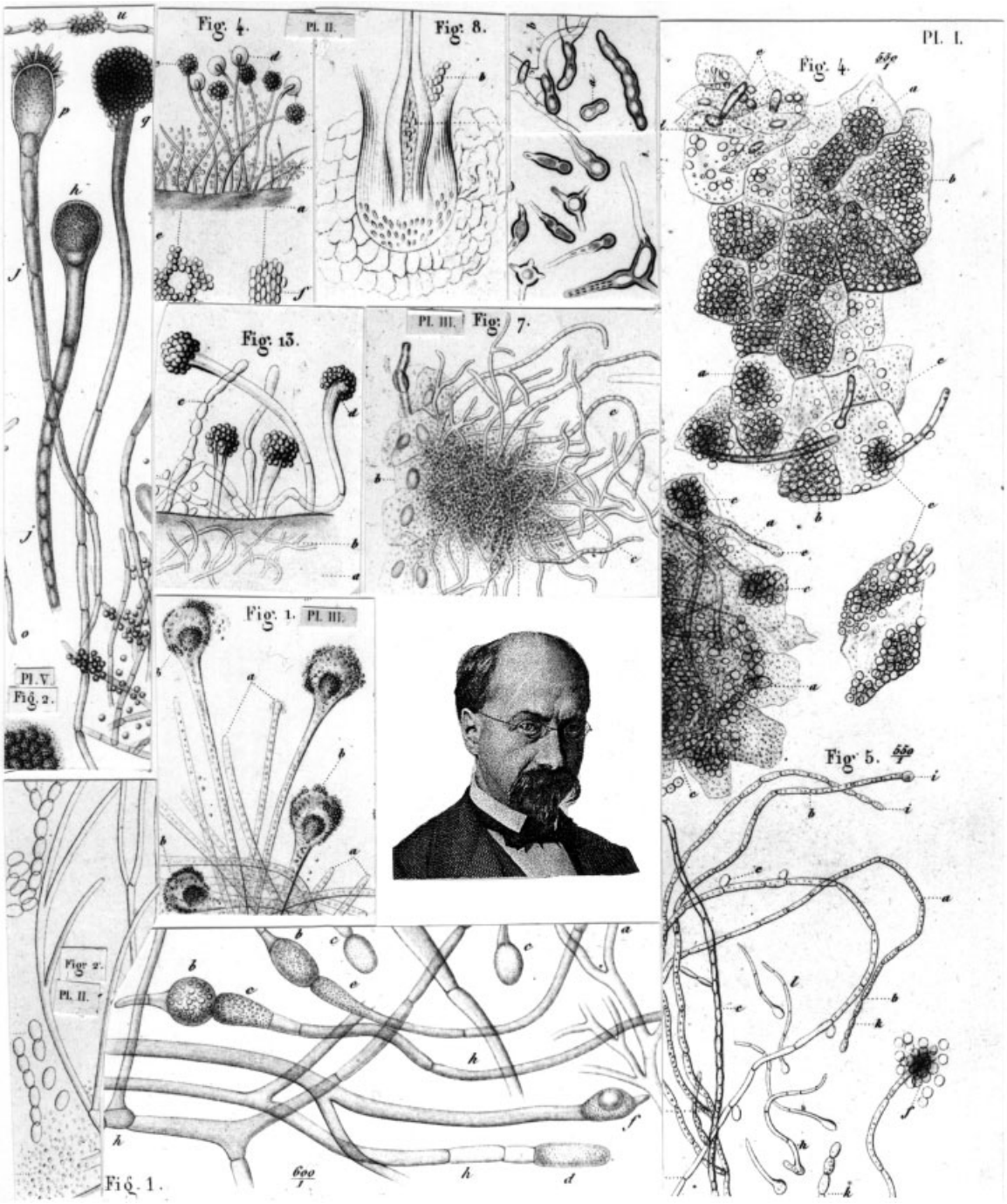

Figure 3 *Robin (1821–85), center, described and drew with tremendous detail the human and animal pathogenic fungi, observed in vivo ('d'après nature') on skin, oral, genital, digestive mucosae, ears, lungs, etc., in his book/atlas* Natural history of Plant's parasites of living humans and animals *(Robin 1853: 15 plates, 100 figures all detailed).* Oidium albicans *(for the first time the name of the thrush agent is specified) is represented as: clusters of yeast cells on epithelial cells and filaments which become entangled in depth; dermatophytes of tinea capitis with follicle invaded by spores on the inside and outside of hair; molds such as aspergilli in the auditory canal, lungs of ducks, or aerial sacs of other birds; and even mucorales in a bovine uterus.*

sphere,' reported his studies of air microorganisms collected in Paris and at 2 000 metres in Chamonix, near Mont Blanc. From his drawings, it is easy to recognize yeasts, yeast-like fungi (*Trichosporon*, *Geotrichum*), and filamentous fungi (*Penicillium*, *Mucor*, etc.) in the aerial biota, which later led to the science of aeromycology. His student, Miguel, made detailed studies for a doctoral thesis in 1883 about microorganisms present in the air, not only in the Paris Observatory, but also in dwellings, houses, and hospitals. The interest

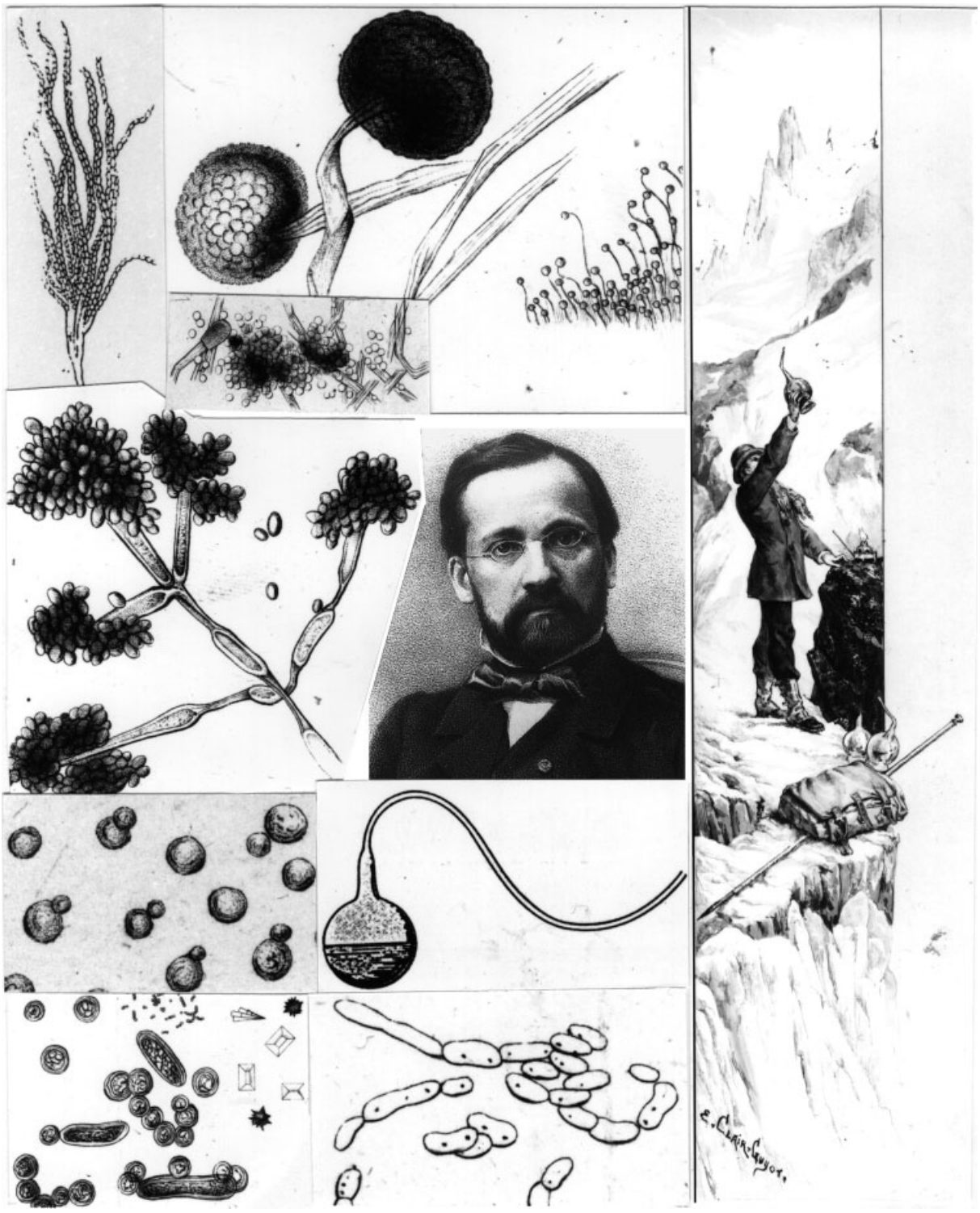

Figure 4 *Pasteur (1822–95), center, has drawn the 'organized corpuscles from the atmosphere' in the studies which came out against the theory of spontaneous generation (Pasteur 1861), illustrating the 'air spora' formed by filamentous and yeast-like fungi collected from the air of Paris and Chamonix in swan-neck flasks (at 2000 metres; aquarelle by Clair-Guyot, on the right).*

of Pasteur and his disciples in mycology was considerable. He revealed the role of yeasts in enzymatic fermentation, and he was the first to describe dimorphism in such filamentous fungi as *Mucor* spp., which transformed into a yeast-like form, and to improve culture media.

Jules Raulin (1836–96), van Tieghem (1839–1914), and Duclaux (1840–1904) were the first three students

to work with Pasteur in his laboratory at the Ecole Normale in Paris in the early 1860s. Raulin's study was designed to elucidate the mineral nutrition of *Aspergillus niger* by quantitative measurement of the growth obtained in recognition of the importance of trace elements for the culture of fungi. The controversy between Pasteur and Leibig, about the size of inoculum necessary for the growth of yeasts, led to the bios of Wildiers, and the growth factors known as vitamins.

van Tieghem and Le Monnier (1873) introduced the microscopic examination of hanging drop cultures of fungi (Figure 5) – the 'van Tieghem cell;' they described the genus *Absidia* in 1876 and the species *Mucor circinelloides* in 1875. In 1887, the German Petri invented the 'Petri dish' – the covered glass plate in which the solid support of gelatine or agar allowed the culture of bacteria and fungi; this led to the greater development of bacteriology and mycology (Petri 1887).

Sabouraud (1864–1938), a young dermatologist at the St Louis Hospital, started his studies on dermatophytes in 1890 with the famous 'Great Course of Microbiology' inaugurated by Roux 2 years after the foundation of the Pasteur Institute (1888), at the end of Pasteur's historical career. This explains Sabouraud's studies on the culture of dermatophytes. Duclaux (1886) described an enzyme (zymase) in a dermatophyte (*Trichophyton tonsurans*) and discredited the opinion held by some of his predecessors and contemporaries that all dermatophytoses were caused by a single fungal species, a mutable *Aspergillus* or *Penicillium* species. Pure cultures were needed for the identification and definition of fungus species on solid taxonomic grounds, and to reveal the existence of a multiplicity of pathogenic species; this was realized by Sabouraud.

Other milestones in the history of medical mycology continued in the 1880s, 1890s, and 1900s, with the discovery of the principal agents of systemic and subcutaneous mycoses and with the evolution of the diseases that they provoked.

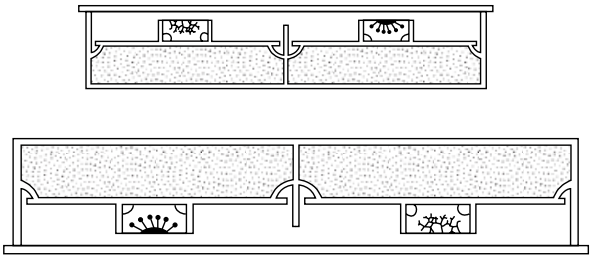

Figure 5 *The 'van Tieghem cell.' (From van Tieghem and Le Monnier 1873)*

HISTORY OF THE PRINCIPAL FUNGI RESPONSIBLE FOR MYCOSES

Dermatophytes and dermatophytoses: essential milestones in the history of medical mycology

The history of the tineas and their agents, the dermatophytes, started with Sabouraud in his mongraph, *Les Teignes* in 1910. The history was continued by Ainsworth (1976, 1986), Ajello (1974, 1975a), Emmons et al. (1963, 1970, 1977), Seeliger (1985, 1988) and later Rippon (1988). They outlined the evolution of our knowledge of this important group of mycoses and their etiological agents.

The work of Sabouraud is one of the most important milestones in the history of medical mycology. He truly revolutionized our concepts about dermatophytes and significantly contributed to the development of medical mycology. He studied medicine in Paris, was a man of multiple talents and an excellent dermatologist. He was also a talented sculptor. He trained in microbiology at the Pasteur Institute under Roux and so knew pure culture techniques. His studies, which started in 1892, culminated in 1910 in the publication of his classic book *Les teignes* (Sabouraud 1910), the most comprehensive account of all the then-known dermatophytes, to which mycologists still refer. He published 1 000 scientific papers and 12 huge volumes. His work was acknowledged at the dermatology hospital, the St Louis Hospital, by the creation of a laboratory for dermatophyte infections and a Children's School for the treatment and education of children suffering from tinea capitis, a severe, chronic disease that was difficult to treat at that period. He used pure culture techniques and new media to determine the physiological and morphological characteristics and parasitological aspects of the development of dermatophytes in vivo, and observed variations and pleomorphism in dermatophytes. Sabouraud laid down a solid foundation for modern medical mycology, and consequently the journal of the ISHAM was initially named *Sabouraudia* (1961). His medium for the identification of the dermatophytes still bears his name and is universally used.

Sabouraud developed a classification system, based on the in vitro and in vivo characteristics of dermatophytes, which remains valid for the three genera *Epidermophyton*, *Microsporum*, and *Trichophyton*, but not for the genus *Achorion*. The last was invalidated by Emmons (1934; Figure 6), based on the botanical rules of nomenclature and taxonomy. Many of the common dermatophytoses and dermatophytes in humans were first differentiated in Sabouraud's time: in 1902 Bodin described *T. violaceum*; Castellani and Chalmers (1910) described *T. rubrum* (as *E. rubrum*); Sabouraud (1910) proposed the name *E. inguinale* for the fungus respon-

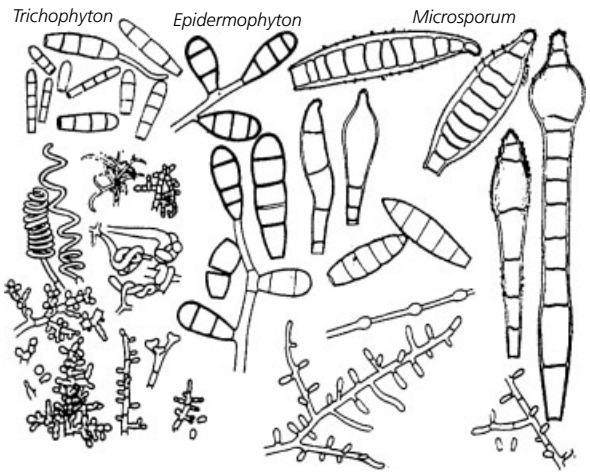

Figure 6 *Drawings from Emmons (1934): dermatophytes. Natural groupings based on the form of conidia and accessory organs.*

sible for tinea cruris (now *E. floccosum*); the Hebra's eczema marginatum described in 1860; and also *T. persicolor* (1902) now classified in the genus *Microsporum*. Animal dermatophyte infections included ringworm of cattle (*T. verrucosum* Bodin 1902), of horses (*T. equinum* Matruchot and Dassonville 1898), of cats, dogs, and humans (*Microsporum canis* Bodin 1902), and of fowl (*Microsporum gallinae* as *E. gallinae* by Megnin in 1881). Other dermatophytes were discovered, such as *M. ferrugineum* Ota 1921, long considered to be a *Trichophyton* species, until typical echinulate macroconidia were obtained by Vanbreuseghem et al. (1970). This mold is found in Japan and the Far East and is responsible for tinea capitis.

In the years after Sabouraud, various tentative modifications of his generic classification were suggested, especially by Langeron (1874–1950) of the Faculty of Medicine of Paris, Vanbreuseghem (1909–93), who developed the field of medical mycology in Belgium after studying with Langeron, and Rivalier (1892–1979), who became the Director of Sabouraud's Laboratory at the St Louis Hospital. Many mycologists adopted Sabouraud's classification, which was simplified by Emmons (1934; see Figure 6). In 1954, Conant who had worked with Sabouraud (1936–37) also modified his classification (Conant 1936a, b, 1937a).

Langeron and Milochevitch (1930) emphasized the importance of natural media to induce the characteristic sporulation of dermatophytes. Studies on the nutrition of dermatophytes, as related to morphogenesis in chemically defined synthetic or semisynthetic media, were carried out after 1950, essentially by Georg (Georg 1950; Georg and Camp 1957), Drouhet and Mariat (1952a, 1953), Drouhet (1952), and Silva and Benham (1952). The need was demonstrated for vitamins and amino acids as essential or complementary growth factors for certain groups of dermatophytes which were strictly adapted to human or animal hosts. The capacity for sporulation was lost in media without the addition of exogenous growth factors. These biochemical and physiological studies led to routine nutritional tests for the specific identification of dermatophytes (Georg and Camp 1957).

The introduction by Vanbreuseghem (1952) of the hair-baiting technique, for the specific purpose of isolating dermatophytes from soil, revealed the worldwide occurrence of dermatophyte-like fungi and geophilic dermatophytes, as studied and reviewed by Ajello (1974, 1975a) and Rippon (1988).

The next major development in the taxonomy of dermatophytes came in 1959 with the description of the teleomorph, the perfect or sexual stage of *Trichophyton (Keratinomyces) ajelloi* by Dawson and Gentles (1959) under the name *Arthroderma uncinatum*.

Nannizzi (1877–1961) of the University of Sienna (Italy) had, in 1927, described the sexual state of *Microsporum gypseum* as *Gymnoascus gypseum* (Figure 7). In 1960, just before his death, Griffin reisolated the perfect stage of *M. gypseum* from Australian soil. Independently, in 1961, Stockdale (1927–89), of England's Commonwealth Mycological Institute, discovered several teleomorphic stages of this dermatophyte and proposed the new genus *Nannizzia* to accommodate the teleomorphs of the *Microsporum* spp. (Stockdale 1961, 1963, 1967; Hasegawa and Usui 1975). Nannizzi's remarkable breakthrough was not recognized by some medical mycologists at that time because the substrates used were erroneously considered not to be sterile. Since then, numerous imperfect or anamorphic species have been found to have one or more ascigerous states obtained in pure culture with cycloheximide [the introduction of cycloheximide (Acti-Dione) in the selective isolation of pathogenic fungi to humans by Georg et al. (1954) contributed to the discovery of numerous dermatophytes in their sexual states]. The seminal work of

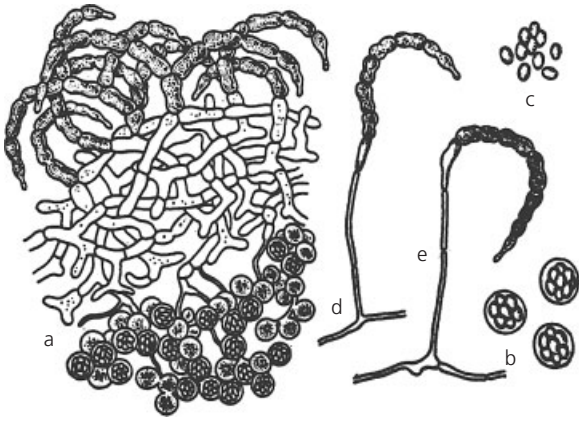

Figure 7 Gymnoascus gypseus Nannizzi, *teleomorphic state of* Microsporum gypseum *(Nannizzi 1927). Stockdale described several teleomorph states for* Microsporum gypseum *and created the genus* Nannizzia *in honor of Nannizzi (1877–1961).*

Nannizzi was unanimously recognized (Rippon 1985; Ajello 1993). The perfect state was discovered for common dermatophytes such as *Microsporum canis*, named *Nannizzia otae* in honor of the famous Japanese mycologist Ota (Hasegawa and Usui 1975). All of the teleomorphs of dermatophytes and dermatophyte-like fungi were classified into two genera until 1986, when Weitzman et al. proposed treating *Nannizzia* as a later synonym of the genus *Arthroderma*. Currently, the use of physiological characteristics, mating types, mitochondrial DNA, scanning and transmission electron microscopy, and antigenic analysis, based on monoclonal antibodies, in addition to classic methods of descriptive mycology, has given the taxonomy of dermatophytes and other pathogenic fungi a firm scientific basis (Tanaka et al. 1992) (see also Fungal structure and morphology).

The combination of improved hygiene and the revolutionary application of griseofulvin in 1958 in the treatment of dermatophytoses decreased the prevalence of tinea capitis in occidental regions, although this disease remains prevalent in some tropical areas. On the other hand, although unknown in Sabouraud's era, tinea pedis caused by *Trichophyton rubrum* of Asian origin has now spread worldwide.

Dermatophyte-like infections

Ringworm-like nondermatophyte infections (skin, nails) caused by pheoid (black) fungi of tropical origin, such as *Hendersonula (Nattrassia) toruloidea* Nattrass 1930, *Scytalidium hyalinum* Campbell and Mulder 1977, have been described in Europe (Gentles and Evans 1970; Hay and Moore 1984), as well as some tropical superficial mycoses known for numerous years as hair colonizers: white piedra caused by *Trichosporon beigelii* (1887), black piedra caused by *Piedraia hortai* (1913) and the skin lesion, tinea nigra, caused by *Exophiala (Cladosporium) werneckii* (1921). Since their discovery, these superficial dermatomycoses represent a rare group of cutaneous mycoses.

OPPORTUNISTIC YEASTS AND FILAMENTOUS FUNGI AS AGENTS OF DEEP SYSTEMIC OR VISCERAL MYCOSES

Opportunistic fungi and opportunistic fungal infections: predisposing factors

These new terms have been introduced into medical mycology since the International Symposium on opportunistic infections in 1962, as emphasized by Ajello, Baker, Emmons, Segretain, Utz, and other participants at that historical meeting. These mycoses have increased progressively and intensively as a result of the favorable factors for opportunistic fungal infections which have evolved over the past decades (Emmons 1962).

INTRINSIC FACTORS DEPENDENT ON THE HOST

Apart from physiological factors resulting from age, pregnancy, immunological and/or endocrinological conditions, the pathological factors are most important. For a long time it has been known that normally saprophytic fungi may invade patients with severe, debilitating, primary diseases such as diabetes, malignant blood diseases, Hodgkin's disease, and various neoplasms. However, before the advent of antibiotic and corticosteroid treatments, opportunistic mycoses were found only in patients in the final stages of serious microbial infections, severe diabetes, other endocrinopathies or malignant diseases (Emmons et al. 1963, 1970, 1977; Drouhet 1972; Warnock and Richardson 1982, 1991; Armstrong 1981). At present, these secondary opportunistic fungal infections are extremely common in immunocompromised hosts, particularly those with granulocytopenia below 1 000 cells/ml. *Candida* spp. infections are seen as sequels to antibiotic treatment. Sometimes fungal infections are diagnosed before the primary disorder and the mycoses become indicative 'signal' diseases such as cryptococcosis. The occurrence of opportunistic fungal infections has risen progressively (Drouhet 1972; Fraser et al. 1979; Bullock and Deepe 1989). Invasive fungal infections have been reported in recent years in 26 percent of patients who are chronically and intensively immunosuppressed as a result of underlying disease or drug therapy. The incidence of aspergillosis increased from 1.91 to 4.8 per million persons (+ 158 percent). *Cryptococcus neoformans* infections increased from 1.3 per million to 2.3 per million (+ 78 percent) between 1970 and 1976 in the USA. However, the incidence rate became substantially higher after 1980 as a result of the opportunistic infections, cryptococcosis, candidiasis, and histoplasmosis, which occur in AIDS (Graybill 1988; Dupont et al. 1994; Stevens et al. 1994).

Alterations of the immune system

The pathogenesis of fungal infections in the immunocompromised host has recently been reviewed and discussed in the general context of microbial infections. Nonspecific immune mechanisms, including cellular and humoral ones, and specific cell-mediated and humoral immunity play important but unequal roles in host resistance to fungal infections (Warnock and Richardson 1982, 1991; Murphy et al. 1994).

Functional defects in chemotaxis

Adherence, or killing (degranulation), phagocytic disorders caused by low cell numbers, granulopenia, and

disorders of leukocyte metabolism are often correlated with fungal infections such as aspergillosis, candidiasis, and other opportunistic mycoses (Waldorf 1986; Walsh et al. 1994).

Acquired immunodeficiency syndrome

Severe fungal opportunistic infections have been reported in the USA, Europe, Africa, and other countries (Chandler 1985; Drouhet and Dupont 1990; Dupont et al. 1994). Usually candidiasis, particularly thrush and esophagitis (Gottlieb et al. 1981; Klein et al. 1984; Tavitian et al. 1986; Dupont and Drouhet 1988), cryptococcosis (Kovacs et al. 1985; Dupont 1989; Drouhet and Dupont 1990) and histoplasmosis (Graybill 1988; Mackenzie 1992), are observed because natural resistance to these infections relies on cell-mediated immunity. Other opportunistic infections in the immunocompromised host, such as disseminated aspergillosis, were very rare in acquired immunodeficiency syndrome (AIDS) in the 1980s, because their development correlates with the absence or reduced function of polymorphonuclear leukocytes. The mycosis with the highest incidence in AIDS, between 50 and 90 percent, is oral and esophageal candidiasis. In high-risk patients, the presence of unexplained oral candidiasis predicts the development of serious opportunistic infections more than 50 percent of the time. Cryptococcosis is the most dramatic fungal infection in AIDS. In the USA, out of 13 834 AIDS patients registered by the Centers for Disease Control (CDC) in October 1985, 904 cases of cryptococcosis (6.5 percent) were diagnosed. In France during the years 1985–95, most of the 1 300 diagnosed cases of cryptococcosis developed in AIDS patients. Although histoplasmosis may occur in immunocompetent patients, we wonder, as do other authors, whether it should not be considered one of the major deep infections in those patients with AIDS who have resided in an endemic area.

A new candidate for systemic mycosis has appeared in AIDS – infection caused by *Penicillium marneffei*, an Asian tropical fungus described in 1956 by Capponi et al. which was responsible for a mycosis of the reticuloendothelial system in Vietnamese bamboo rats, and for an accidental human infection (Capponi et al. 1956; Segretain 1959). Since 1987, human cases of disseminated, pulmonary, cutaneous penicilliosis caused by *P. marneffei* have been observed in England, France, Italy, and the USA, in AIDS patients who travelled in southeast Asia or autochthonous patients with AIDS living in China, Hong Kong, and Thailand. The 44 cases reported in patients positive for the human immunodeficiency virus (HIV) traveling or living in southeast Asia during 1985–93 increased to 117 cases in the following 2 years and to more than 400 cases in 1995 (Drouhet 1993a; Supparatpinyo et al. 1994; Drouhet and Dupont 1995) (see also Penicilliosis).

The pattern of mycotic infection in AIDS patients has been radically altered by the introduction of combination antiretroviral chemotherapy.

EXTRINSIC, IATROGENIC FACTORS

These include effects caused by either drug treatment or breach of the integument resulting from medical (vascular catheters for drug administration or parenteral nutrition and other catheters) and surgical (organ transplants, abdominal surgery, etc.) devices or interventions, which allow the penetration of opportunistic fungi.

Antibiotics

The role of antibiotics in the pathogenesis of infections with *Candida* spp. is based not only on yeast selection in the alimentary canal and mucous membranes (imbalance between the bacterial and yeast biota), but also on the action of antibiotics on the immunological defence systems (Drouhet 1972). The latter includes decreased antibody production, suppression of phagocytosis, and metabolic alteration of the host cells. Recent data indicate that antibiotics acting against bacteria augment the adhesion of *C. albicans* to the surface of epithelial cells by mannan receptors taking the unoccupied place of the bacteria that disappeared, thereby facilitating the transition of *C. albicans* from the yeast form to its filamentous aggressive form (Kennedy 1988; Kennedy et al. 1992). This was demonstrated both in vivo and in vitro in a few hours and may explain the remarkable in vivo activity of azoles, such as ketoconazole, which arrest mycelial but not yeast cell development at very low concentrations (Borgers and Vanden Bossche 1982).

Immunosuppressants

Corticosteroids, immunosuppressants (such as azathioprine, cyclophosphamide, cyclosporin, etc.) and cytostatic agents favor the development of opportunistic fungal infections. The granulopenia induced by these drugs is responsible for susceptibility to the invasive forms of deep mycoses (Odds 1988). In the 1960s and 1970s, major renal transplant units reported fungal fatalities of up to 15 percent and up to 30 percent in heart transplant recipients (Warnock and Richardson 1991).

Intravenous drug abuse

Heroin addicts are a population at risk for certain infectious diseases, such as bacterial and fungal endocarditis, and hepatitis B. Since 1980, a new pathological septicemic syndrome of *C. albicans* infection, characterized by fever followed immediately by cutaneous disseminated lesions of folliculitis, pustulosis, subcutaneous nodules, and ocular metastasis, and several weeks later by osteoarticular involvement, was observed in the brown heroin addict population. This was observed not

only in France (Drouhet et al. 1981; Drouhet and Dupont 1983; Dupont and Drouhet 1985) where more than 200 cases occurred, but also in Italy and principally in Spain (> 890 cases) and even in Australia. Although crude heroin or Iranian or Asian brown heroin does not contain *C. albicans*, the yeast is introduced by the syringes used by the addicts, which are contaminated from the spoon in which the heroin is dissolved in lemon juice. All patients have oral and digestive tract candidiasis as the primary source of this contamination. Alterations of T- and null-lymphocyte frequencies in the peripheral blood of opiate addicts were observed; these result from the opiate receptor sites on T lymphocytes. An immunosuppressive of the cellular immunity contaminant of brown heroin may also have been the origin of this new pathology, which is unknown in the USA where white or purified heroin is used.

Desferrioxamine

Therapy for iron or aluminum overload in dialyzed patients, with desferrioxamine, has become an important predisposing factor, since 1986, for disseminated severe zygomycosis caused by *Rhizopus* spp. (Kwon-Chung and Bennett 1992).

Candida spp. and candidoses

What is extraordinary in the history of *C. albicans*, and the 10 major pathogenic thermophilic *Candida* spp. adapted to warm-blooded animals including humans, is that these unicellular fungi, which have been recognized in the past 130 years, are capable of showing up multiple new conditions in all branches of medicine. The notion of the 'compromised' patient was developed by the famous French pediatricians Trousseau (1868) and Parrot (1869a, b) who revealed that athrepsy in newborn infants may lead to disseminated thrush of the digestive and pulmonary tracts (Figure 8a). The first description by Parrot (1869a, b, 1877) of esophageal, gastric, intestinal, laryngeal, tracheal, and pulmonary thrush, initiated by oropharyngeal thrush, was histologically proven to be a clinical entity (Figure 8b). In the first edition of his monumental book *Candida and candidosis* published in 1979, Odds analyzed 2 265 references, but in the second edition (1988) no fewer than 5 796 references were cited. This emphasized the many developments that have occurred in the molecular, medical, and genetic aspects of *C. albicans* and candidiasis (Kwon-Chung et al. 1985; Whelan 1987; Lott et al. 1992; Odds 1994).

Thrush, an important opportunistic marker in AIDS patients (Gottlieb et al. 1981; Klein et al. 1984; Tavitian et al. 1986), is a clinical manifestation caused exclusively by *C. albicans*. The other forms of candidiasis can be produced by various other *Candida* spp. The superficial manifestations of candidiases are conventionally separated from deep-seated, disseminated or systemic candidiasis, which now principally appears in iatrogenic conditions, but the boundary is difficult to establish between the two forms (Bodey and Fainstein 1988, 1993).

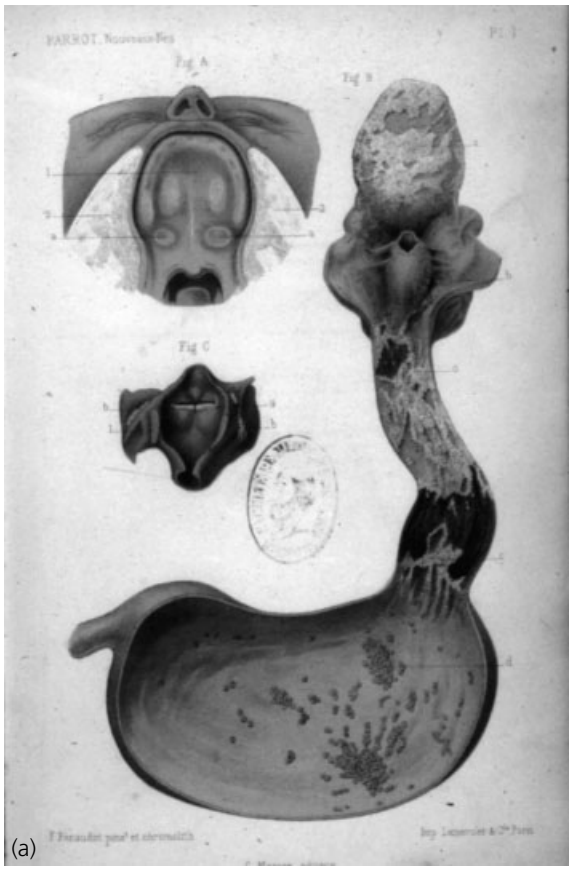

Figure 8 *Drawings of Parrot (1869a) showing* **(a)** *the clinical aspects of generalised thrush in sick newborn infants with white patches on the tongue, esophagus, gastric mucosa, larynx (even bronchi and lungs, not shown here). (Continued over)*

Chronic mucocutaneous candidiasis (CMC), characterized by recurrent and persistent infections of the mucosa, nails, and skin by *C. albicans,* can be observed in patients with cellular immune abnormalities (Kirkpatrick et al. 1971). It has been shown that some of these abnormalities may be primarily induced by the mannan antigen of *C. albicans* (Durandy et al. 1987). The elimination of this immunosuppressive antigen by intensive antifungal therapy (amphotericin B, ketoconazole) restores the impaired cell-mediated immunity to *C. albicans* and leads to the disappearance of severe, chronic skin lesions (Drouhet and Dupont 1980, 1983; Drouhet et al. 1985).

The incidence of candidiasis has increased annually since the introduction of antibiotic therapy, immunosuppressive therapy, and other conditions. Odds (1988), basing estimates on official statistics and publications in four countries over a period of 30–35 years starting in

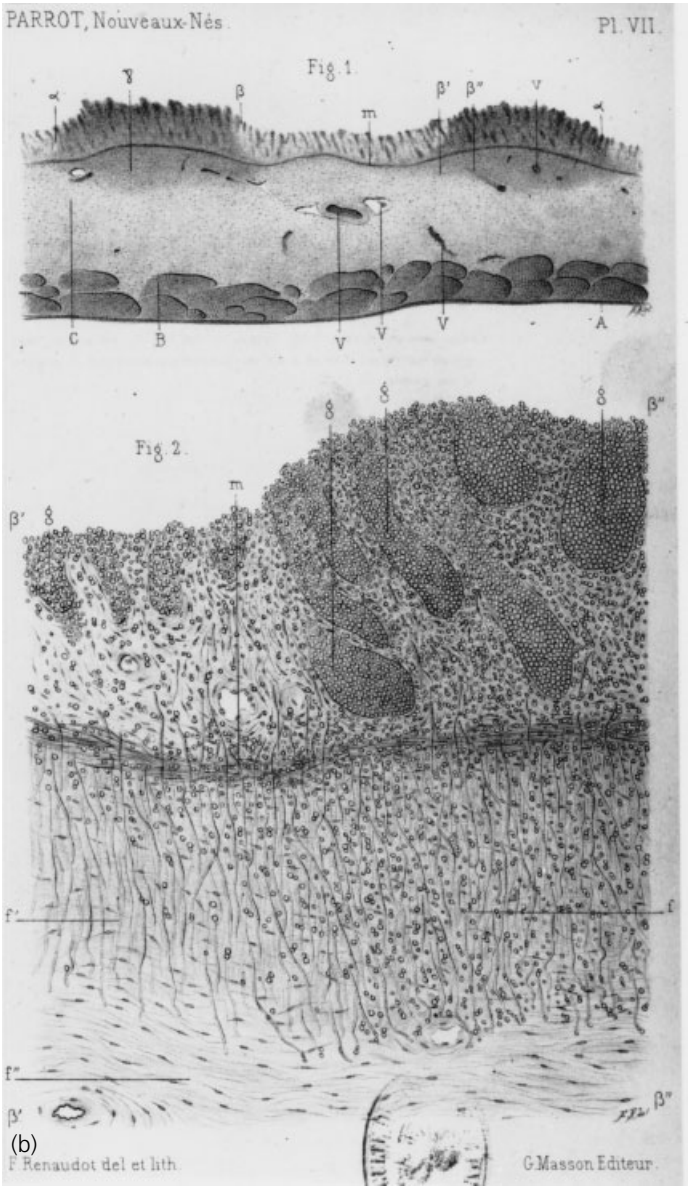

Figure 8 *Drawings of Parrot (1869a) showing* **(b)** *Histological aspect of* Candida albicans *yeast cells on the epithelial digestive mucosa with deep penetration by filaments ('aggressive form') into submucosal and muscular strata (explaining septicemia). The famous pediatricians Trousseau and Parrot at that time had described systemic candidiasis (Continued).*

1950, showed that, in the USA, the number of deaths attributed to candidiasis has increased roughly threefold between the late 1960s and the late 1970s; in France there has been a twofold increase between 1973 and 1982. During the 1980s and 1990s, the incidence of fungemia caused by *Candida* spp. acquired in hospitals increased more than threefold. Finally, adherence of *Candida* spp. to host surfaces was shown to be an important feature in the pathogenesis of candidiasis, and biomolecular studies about adherence accordingly developed rapidly (Kennedy et al. 1992; Robert et al. 1992) (see also Candidiasis).

Lipophilic yeasts and malasseziasis

The history of lipophilic yeasts grouped in the genera *Malassezia* Baillon 1889, and *Pityrosporum* Sabouraud 1904 are now unified in a single genus *Malassezia* (Guého and Meyer 1988). Their clinical manifestations – pityriasis versicolor, pityriasis capitis, and seborrheic dermatitis – are among the most interesting in medical mycology. Eichstedt (1846) and Sluyter (1856) termed the disease pityriasis versicolor as a result of the yellow–brown or achromic macules (*pityron* = bran or scale forming in Greek). In 1853, Robin observed the

spherical budding cells grouped in clusters associated with hyphae and named the agent *Microsporon furfur*. Rivolta (1873) considered it to belong to *Cryptococcus*, but Malassez (1974) clearly described the yeast-like cells from scalp scales and dandruff (pityriasis simplex capitis) as spores. Bizzozero, in 1884, observed the morphological diversity in the epidermal scales, and named the organisms with spherical cells *Saccharomyces sphaericus* and those with oval cells *S. ovalis*. In 1889, Baillon in his *Traité de botanique* created the generic name *Malassezia* and named the agent of pityriasis versicolor *Malassezia furfur*. Matakieff (1899) drew the sculpting of the cell wall, showing spiral grooves and branching hyphae in the scales of pityriasis versicolor (Figure 9a). These aspects were confirmed more recently using electron microscopy (Guillot et al. 1995) (Figure 9b). Sabouraud (1904) individualized clearly the 'scaling dermatoses' such as pityriasis capitis (dandruff) and seborrheic dermatitis (Figure 10) – a dermatologically complex subject of controversy (see Hay and Midgley 1988). He also created the genus *Pityrosporum*, with *P. malassezi* being distinct for him from the dimorphic agent of pityriasis versicolor. Attempts to elucidate the role of yeasts in these scaling dermatoses were hindered by difficulties in culturing the lipophilic organism and determining its exact growth requirements, until studies by Benham (1939) and Gordon (1951). The first culture of this yeast was obtained in the presence of lipids by Ota and Huang (1933). The polymorphism of *M. furfur* led Gordon to separate *P. orbiculare*, considered to be the etiologic agent of pityriasis versicolor, and *P. ovale* related to pityriasis capitis and seborrheic dermatitis as Sabouraud had claimed in 1904. There followed a long series of studies favoring this proposition, but these were contradicted by others (reviewed by Midgley 1989). In 1925, Weidman isolated a yeast from a severe exfoliative dermatitis of an Indian rhinoceros (*Rhinoceros unicornis*), which he cultured without the addition of lipids; he named it *P. pachydermatis*. Later, it was described as *P. canis*, by Gustafson in 1955. This species is largely observed in dogs. Studies on the ultrastructure and genomic structure confirm the validity of three species: *M. furfur* (*M. furfur* biovar. *orbiculare* and *M. furfur* biovar. *ovale*), *M. pachydermatis*, and *M. sympodialis* (Guého et al. 1987, 1994; Guého and Meyer 1988; Simmons and Guého 1990). This new classification has recently been extended further. An experimental model of Malassezia infection reproduced seborrheic dermatitis (Drouhet et al. 1980, 1988b) with follicular and cutaneous invasion in guinea pigs and mice, as shown by Sabouraud in 1904 (Figure 10). The therapeutic activity of topical azoles, and particularly of oral ketoconazole, led to the cure of pityriasis capitis, seborrheic dermatitis, and pityriasis versicolor. Clinical studies have shown the important role of lipophilic yeasts in folliculitis after antibacterial and corticosteroid therapy, and in AIDS (Drouhet and Dupont 1990; Hay 1991) as well as in septicemia and systemic malassezia infection in preterm newborns receiving parenteral lipid hyperalimentation (Dankner et al. 1987; Guého et al. 1987) (see also Superficial diseases caused by *Malassezia* species).

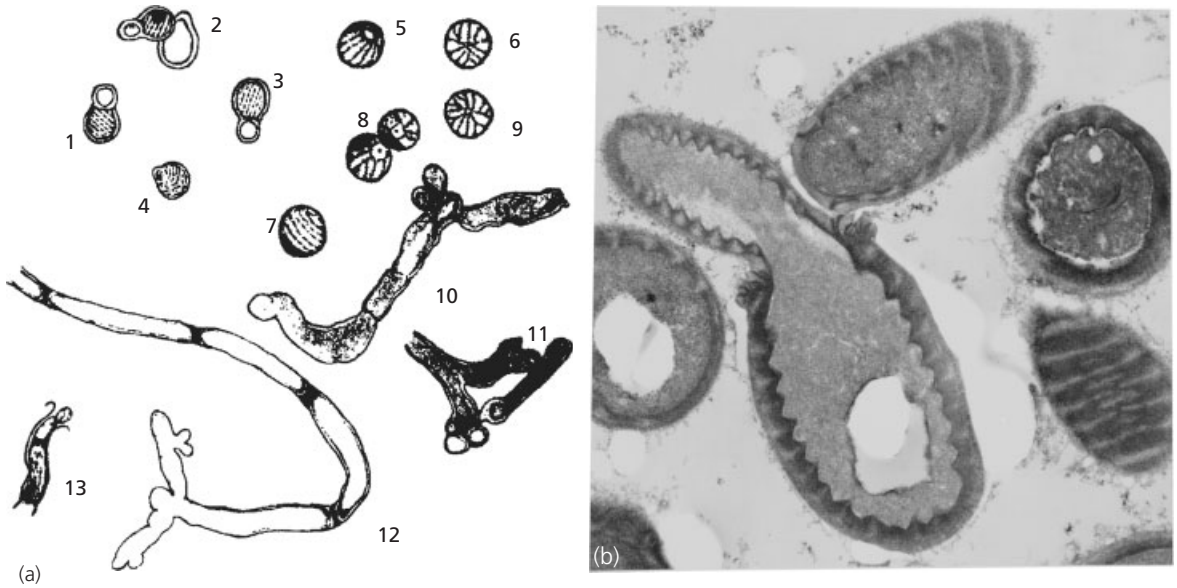

Figure 9 (a) *From Matakieff (1899): sketches 1–9 took into account the sculpturing of the innermost layer of the yeast's cell wall; sketches 10–12 illustrated budding and branching hyphae in scales of pityriasis versicolor; sketch 13 showed the purported formation of endospores.* **(b)** *Ultrastructure of the serrated cell wall of the yeast* Malassezia *showing growths on the plasma membrane as Matakieff had observed almost 100 years ago with an ordinary microscope. (From Guillot et al. 1995.)*

Figure 10 *Sabouraud's drawings of the follicular lesions with Malassez's 'spores' in the center of the follicle in pityriasis of the medial thoracic region (Sabouraud 1904).*

Cryptococcus neoformans and cryptococcosis

In 1894, the Italian microbiologist Sanfelice isolated an encapsulated yeast-like fungus, from peach juice, which he named *Saccharomyces neoformans* because of the pseudotumoral, sarcoma-like, lymphadenitic lesions observed in animals after intraperitoneal inoculation (Sanfelice 1895). At the same time, in Germany, a pathologist, Busse (1894, 1895), and a surgeon, Buschke (1895), separately reported the same fungus from sarcoma-like lesions of the tibia, and cutaneous and other lesions in a 31-year-old woman; they drew different aspects of the capsulated yeast including pseudomycelial or moniliform cells (Figure 11) in addition to histopathological cellular reactions (Figure 12). In 1896, in France, Curtis described a similar case using the term human saccharomycosis for the observed disease and he called the organism *Saccharomyces subcutaneous tumefaciens*. This second human case of cryptococcosis was retrospectively considered to be *Cryptococcus neoformans* var *gattii*, serotype B. When, in 1901, the eminent French mycologist Vuillemin (1861–1932) from Nancy examined several cultures, he did not find ascospores characteristic of the genus *Saccharomyces* and named it *Cryptococcus* – a name used earlier in 1833 by Kutzing. Therefore, he reclassified the yeast isolate of Busse and of Curtis into the genus *Cryptococcus* and the isolate of Sanfelice (1894, 1895) as *C. neoformans*. Subsequently, several cases were reported, especially in Europe, but the disease was confused with blastomycosis, so it was known as European blastomycosis. In 1905 Von Hansemann was the first to observe this fungus in meningitis. In 1916, Stoddard and Cutler described the clinical aspects of the disease. Ignoring the European literature, the authors mistook the mucoid capsule of the yeast for histolysis of host tissue, and therefore named the fungus *Torula histolytica*. Unfortunately, this name became popular in the Anglo-Saxon literature, and the disease was known for a long time as torulosis or torula meningitis, until the terms of Vuillemin (1901) – *C. neoformans* and cryptococcosis – became widely used in medical mycology. In 1935, Benham clearly differentiated cryptococcosis from the 'blastomycoses,' especially from blastomycosis (caused by *Blastomyces dermatitidis*), by studying numerous strains of cryptococci. She showed that only one species was responsible for cryptococcosis, although serological differences existed among the strains (Benham 1935, 1950). Benham is considered to be one of the most remarkable women pioneer scientists in medical mycology (O'Hearn 1985).

An important step in the history of this fungus was the discovery in 1951 by Emmons of *C. neoformans* in barnyard soil and in 1955 the frequent saprobic association with nests and droppings of pigeons (Emmons 1955). Although it was known that *C. neoformans* was isolated from a peach by Sanfelice in 1894, during the following 55 years isolation of this fungus was only obtained from humans and lower animals, so an endogenous source of infection was erroneously assumed. Since these discoveries, the epidemiology of cryptococcosis has been intensively studied and the role of other plant sources such as *Eucalyptus* species acknowledged.

Another important step originated from the studies of the capsular polysaccharides of *C. neoformans*. In 1950, Drouhet et al. showed that the principal capsular polysaccharide of *C. neoformans* was a glucuronoxylomannan, a virulence factor that inhibits the migration of leukocytes (Drouhet and Segretain 1951). Since then numerous studies, particularly by Bulmer and Sans (1967, 1968), showed the interrelationships of the polysaccharide capsule, phagocytosis and the mechanism of host defence against *C. neoformans*. The quantity of capsular polysaccharide in the host may be so great that it can be found as a free, circulating antigen in body fluids and easily detected by a latex agglutination test (Bloomfield et al. 1961). Evans, in 1950, identified three serotypes (A, B, and C), and in 1951 confirmed that glucuronomannan is the predominant capsular polysaccharide, but also found the presence of galactose (Evans and Kessel 1951), to which a fourth serotype D was added by Wilson et al. (1968), on the basis of antigenic differences in capsular polysaccharides. The historical Sanfelice's strain (CBS 132 = ATCC 32045) belongs to serotype D, Busse's strain (CBS 879 = ATCC 4189) to serotype A, and Curtis' strain (CBS 1622 = ATCC 2344) to serotype B.

The observation that pigeon droppings were a nutrient source led Staib (1963), from the Koch Institute in

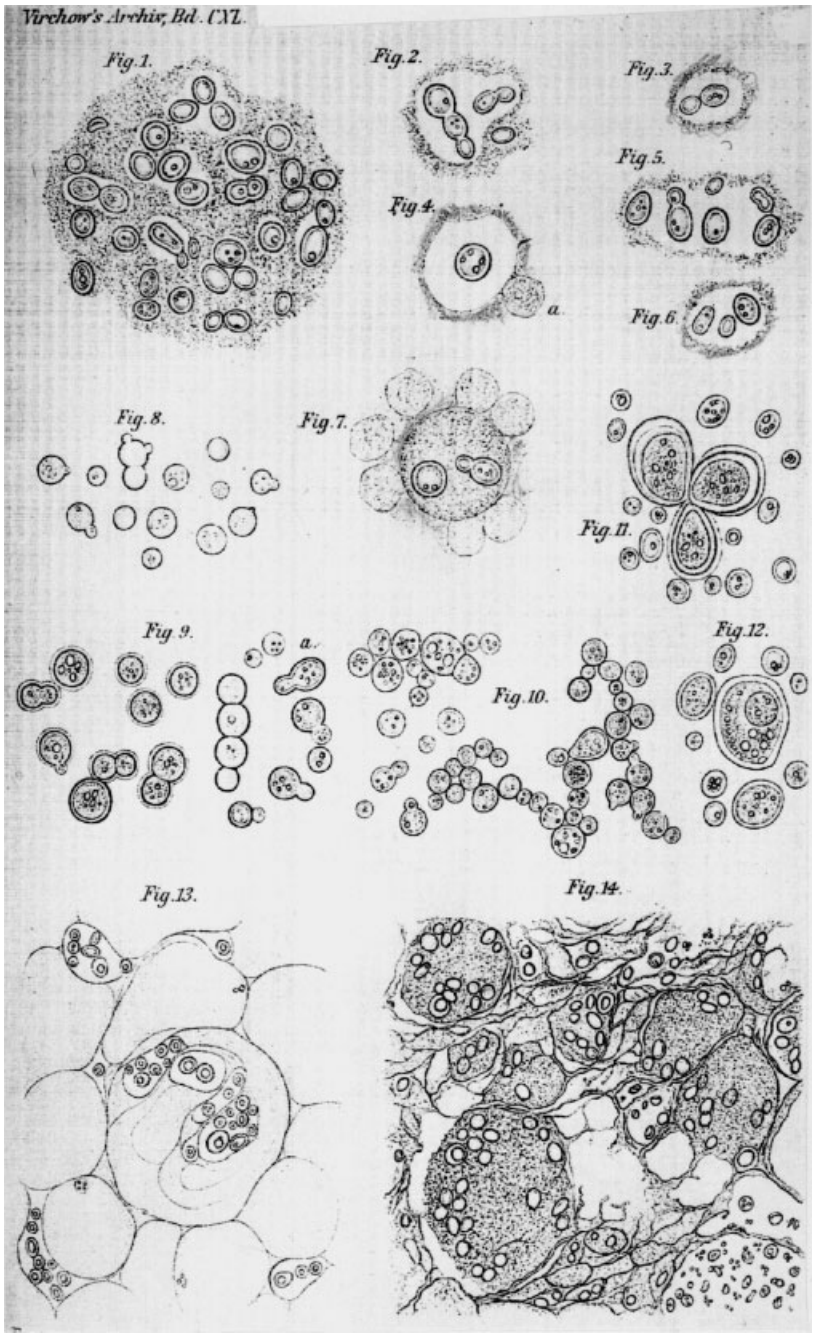

Figure 11 *Busse and Buschke's drawings of capsulated cell of the* Cryptococcus neoformans *(called* Saccharomyces *and the disease saccharomycosis hominis in their publication), showing a moniliform yeast form converted into a 'gelatinous cyst' (Busse 1895).*

Berlin, to new concepts for the identification of the fungus. Staib reported the brown color effect specific for *C. neoformans* produced by nigerseed of the composite *Guizotia abyssinica*. The enzyme responsible for the pigment formed was a phenoloxidase involved in virulence, according to the studies of Kwon-Chung, Polacheck and Rhodes in the 1970s. This work has now been extended with the recognition of melanin as a key virulence determinant.

Another valuable addition to the knowledge about this pathogenic yeast was the discovery of its sexual reproduction by Kwon-Chung (1975), who proposed the genus *Filobasidiella* to accommodate this basidiomycetous yeast species. Kwon-Chung (1976) showed that there are two varieties of *F. neoformans*: *F. neoformans* var. *neoformans*, corresponding to the asexual state *C. neoformans* var. *neoformans*, and *F. neoformans* var. *bacillispora*, corresponding to the asexual state *C. neoformans* var. *gattii.* This last variety was isolated by Gatti (Gatti and Eeckels 1970) in Zaire from an African child with meningitis. It was described as *C. neoformans* var. *gattii* by Vanbreuse-

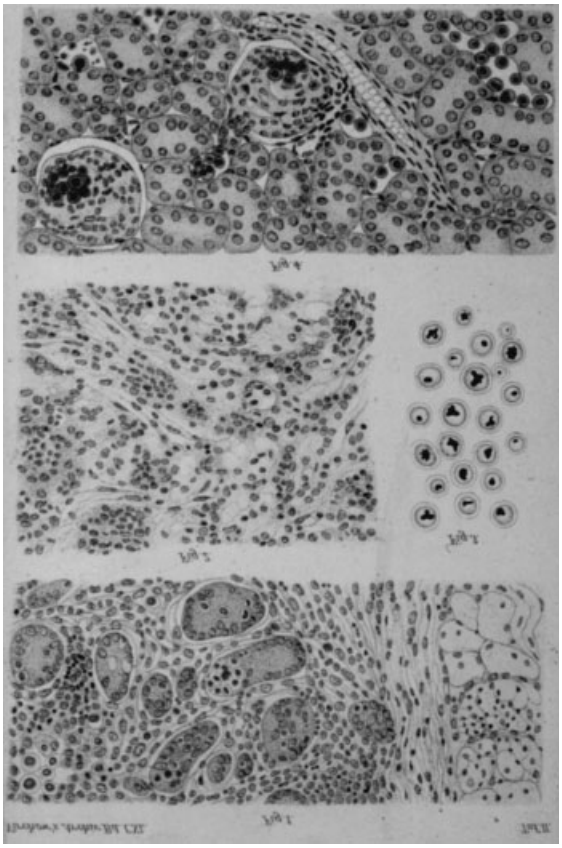

Figure 12 *Histopathological lesions of cryptococcosis with stained yeasts undergoing granulomatous cellular reactions and yeast cells infiltrating the tissue with no cellular reaction. Drawings by Busse (1894).*

ghem and Takashio (1970). Further studies showed that important biochemical, epidemiologic, and pathological differences existed among the four serotypes and the two sexual forms of this basidiomycete fungus (Bennett et al. 1977, 1978; Kwon-Chung et al. 1992, 1994).

In order to evaluate antifungal compounds in a reproducible way, Van Cutsem et al. (1986) established a model in the guinea pig of progressive, disseminated, cryptococcal infections including skin manifestations, as observed in patients with systemic cryptococcosis, particularly in AIDS patients showing a dermotropism of *C. neoformans*.

Another important event in the history of cryptococcosis is that this disease, which was very rare in the past, essentially developed only in people with low host resistance, malignant diseases of the reticuloendothelial system (Hodgkin's disease, lymphoma), or endocrine problems, and was named the 'sleeping giant'by Ajello in 1970. Within a few years cryptococcosis became the 'awakening giant' (Kaufman and Blumer 1978), probably as a result of the intensive immunosuppressive treatments of patients with leukaemia and those undergoing organ transplantations. The incidence in 1978 ranged from 200 to 400 cases of cerebral meningitis per year in the USA, to 15 000 subclinical respiratory infections in New York City alone. A few years after the emergence of AIDS, the frequency and the severity of cryptococcosis increased considerably. In addition, the disease responded poorly to the classic treatment (amphotericin B + 5-fluorocytosine), which necessitated the introduction of new triazole molecules (fluconazole, itraconazole). Cryptococcosis, now a principal life-threatening opportunistic mycosis, was considered to be the 'mycosis of the future' (Drouhet and Dupont 1990). In the USA, among 13 834 AIDS patients, registered by the CDC in October 1985, 904 cases of cryptococcosis (6.5 percent) were diagnosed. In France during the period 1985–88, among 283 diagnosed cases of cryptococcosis, 204 (72.08 percent) had developed in AIDS patients (Drouhet and Dupont 1990) and in 1994 more than 1 300 cases were reported (see also Cryptococcosis).

Aspergillus spp. and aspergillosis

Micheli (1729) described the genus *Aspergillus* and derived its name from the Latin word *aspergillum* (to sprinkle) which referred to the perforated globe used to sprinkle holy water during Catholic religious ceremonies '*aspergus te.*' Link delineated several distinct species in 1809 such as *A. candidus*, *A. flavus*, and *A. glaucus*, which he had isolated from decaying vegetation.

The first reports of infections with *Aspergillus* spp. were those of Mayer and Emmert in 1815, who described an infection in the lung of a European jay (*Garrulus glandarius*), but principally those of the German Fresenius, in 1850, who observed an air sac infection in a captive bird, the Great bustard (*Otis tarda*) at the Frankfurt Zoo. Fresenius named the isolate *Aspergillus fumigatus* and first used the term aspergillosis.

The first cases in humans with detailed microscopic drawings of an *Aspergillus* sp. in vivo were described by Virchow in 1856 (Figure 13). Virchow noted that the organism he had observed (probably *A. fumigatus*) in human disease was closely related to the 'parasitic' vegetable structures growing in living animals reported by Bennett in 1844 and by Sluyter in 1847, except for the association of the pulmonary form with 'pigeon feeder's' disease by Virchow and later, in 1890, by Dieulafoy, Chantemesse, and Widal. In the following years, very few cases of the disease were reported. In 1897, Renon published his thesis showing the association of aspergillosis with certain occupations including pigeon stuffers ('*gaveurs de pigeons*') and wig cleaners. They concluded that moldy grain was the source of the infectious conidia. Renon also showed that some patients had tuberculosis because their primary disease antedated the aspergillosis. Between 1897 and 1938, the aspergillosis mentioned was not the localized fungus ball ('bronch-

Figure 13 *Virchow's drawings of the first case of human pulmonary aspergillosis.* Aspergillus fumigatus *can be recognized from the drawings.*

iectosiant aspergilloma'), which was defined by the French surgeon Devé only in 1938 as a rare colonization of congenital bronchial cysts (see also Aspergillosis).

The growing importance of aspergillosis was recognized only after the remarkable description, by Monod (surgeon), Pesle (physician) and Segretain (mycologist) (Monod et al. 1954), of the development of bronchopulmonary aspergilloma (fungus ball) in the pulmonary cavities of tuberculous patients who had been treated with powerful antituberculous medications. Radiological evidence demonstrated that *Aspergillus* spp., most frequently *A. fumigatus*, colonized preformed cavities in 12 percent of healed tuberculous patients along the radiolucent crest. Aspergilloma is accompanied by the appearance of anti-*A. fumigatus* precipitating antibodies, studied particularly since the 1960s in the UK (Pepys 1969; Longbottom 1986; Longbottom and Austwick 1986), and in France (Biguet et al. 1964; Drouhet et al. 1972). This localized form was observed particularly in Europe (Segretain 1962) where the incidence of tuberculosis was high, but it is now observed worldwide. At the same time, allergic bronchopulmonary aspergillosis was described by Hinson et al. (1952). In addition, progressive invasive aspergillosis in the immunocompromised host has became more and more frequent and severe. Invasive aspergillosis has become a major problem in immunosuppressed or myelosuppressed patients, or recipients of organ transplants. It has raised new, epidemiologic, clinical, immunological, and therapeutic problems. The First International Symposium on *Aspergillus* spp. and aspergillosis (Vanden Bossche et al. 1988) presented a summation of the then current knowledge of this mycosis which has become one of the most important in fungal diseases.

Scedosporium spp. and pseudallescheriasis

The history of the taxonomy of the fungi and of the clinical and epidemiologic aspects of the mycosis, known under various names such as monosporiosis, allescheriosis, petriellidiosis, pseudallescheriasis, or scedosporiosis, is fascinating.

The history of *Monosporium apiospermum,* described in 1911 by the famous Italian taxonomic mycologist Saccardo (1845–1920), started with a white grain mycetoma of the foot of an Italian patient. In 1922, Shear (1865–1956) in Texas (USA) described and named an ascospore-producing fungus isolated from a mycetoma of the foot as *Allescheria boydii*, in honor of the clinician studying this American case. For 30 years, the two fungi were accepted as different agents of mycetoma, until 1944 when Emmons reported that *M. aspiospermum* was the anamorph or asexual state of *A. boydii*. The names of both the teleomorphic and anamorphic states have undergone several successive changes: the genus *Allescheria* was reclassified, in 1970, by Malloch as *Petriellidium*. McGinnis et al., in 1982, recognized the priority of the genus *Pseudallescheria* Negroni et Fisher 1944 and the sexual telemorph became *Pseudallescheria boydii* (Shear) McGinnis, Padhye et Ajello 1972. The anamorph *Monosporium apiospermum* was invalidated by Hughes in 1958 and *Scedosporium apiospermum* (Saccardo) Castellani et Chambers 1919 is now the valid name of the anamorph.

After the first isolation from the soil by Ajello and Zeidberg (1951), numerous studies established its presence as a saprophyte in nature, explaining the source of post-traumatic infections and bronchopneumopathies.

The first clinical infection, other than a mycetoma, was observed by Benham and Georg (1948), who described granulomatous meningitis caused by *P. boydii* in a patient with no underlying disease, after spinal anesthesia a month before the onset of symptoms. The first reported cases of pulmonary infection, resulting from *P. boydii* infections, were those of Creitz and Harris (1955) and Drouhet (1955), followed by numerous reports carefully reviewed by Rippon (1988) and Dupont et al. (1991).

In 1984, Malloch and Salkin described a new species of *Scedosporium, S. inflatum*. The fungus was isolated from a bone biopsy from a 6-year-old boy. Previously, a fungal osteoarthritis of the knee with severe joint destruction, caused by an atypical *S. apiospermum* that was highly virulent for laboratory mice, had later been identified as *S. inflatum* (Drouhet et al. 1991). Since then, more than a dozen cases caused by this species have been described in young boys as a result of trauma to the knee or the foot, in patients who have undergone renal transplantation or in drug abusers (endocarditis and hip arthritis). In 1991, Guého and de Hoog, based on genomic taxonomy (DNA/DNA reassociation, G + C percentage) and the cultural aspects of numerous strains of *Sepedonium* and *Pseudallescheria*, found that *S. inflatum* was identical to *Lomentospora prolificans* Hennebert et Desai 1974, a soil fungus. They proposed the name, *Scedosporium prolificans* (Malloch et Salkin) Guého et de Hoog 1991 (Figure 14).

Mucor spp., Mucorales and zygomycosis (in part)

The nonseptate filamentous fungi with sporangia and sexual reproduction by zygospores, now belonging to the class Zygomycetes and the order Mucorales, were recognized by the first microscopists, namely Hooke (1636–1703), Malpighi (1628–84) and van Leewenhoek (1637–1723). They illustrated these fungi in 1665, 1669 and 1680, respectively, but the genera related to pathogenic fungi were defined and described later as *Mucor* Micheli 1729, *Rhizopus* Corda 1838, *Absidia* van Tieghem 1876, *Rhizomucor* Lucet et Costantin 1899, *Cunninghamella* Matruchot 1903 and, more recently, *Saksenaea* Saksena 1953 and *Apophysomyces* (Misra et al., 1979), which are all new emerging opportunistic pathogens in medical mycology.

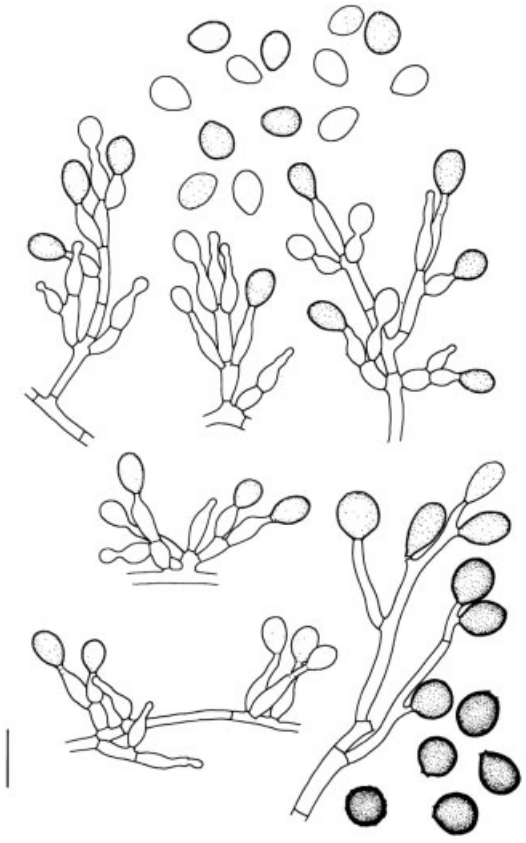

Figure 14 Scedosporium prolificans *(Hennebert and Desai) Guého and de Hoog comb. nov. (1991): a new emerging fungus.*

The earliest reports of authentic zygomycosis were described 150 years ago, but the clinical descriptions were often inaccurate and incomplete as was the identification of the isolates, necessitating accommodation in genera other than those indicated in the old literature. The first authentic human pulmonary case of zygomycosis was recognized by the German Kurchenmeister in 1855, in a cancerous lung. Based on the drawings showing sporangia and nonseptate hyphae, the isolated fungus was called a *Mucor*. After two other doubtful pulmonary cases reported by Fürbinger in 1876, Paltauf published, in 1885 in *Virchow's Arch Pathol Anat*, the first observation of disseminated infection that had started in the larynx and pharynx. The classic primary localizations of rhinocerebral zygomycosis were first observed more than 50 years later (Gregory et al. 1943). Paltauf called the etiologic agent *Mucor corymbifera*, in spite of the absence of cultures for this case. In 1884, Lichtheim isolated from nature two species of *Mucor*, which caused fatal mycoses in rabbits: *Mucor corymbifera* Cohn 1884 and *M. rhizopodiformis* Cohn apud Lichtheim 1884. After a new case of pulmonary mucormycosis described by the Belgian Herla in 1895, Lindt described and found *M. pusillus*, in 1896, as well as *M. racemosus* Fresenius 1850 in animal mycoses.

Since 1943, when Gregory et al. described three cases of rhinocerebral zygomycosis and delineated this syndrome, 40 cases of rhinocerebral zygomycosis are noted per year in the USA. Around 1960, zygomycosis became a progressively increasing mycosis in compromised hosts with ketoacidotic diabetes mellitus (the rhinocerebral severe form) and in immunocompromised hosts; since 1984, the pulmonary form has been observed in 18 percent of patients with malignant leukemias, lymphoma, and severe neutropenia treated with corticosteroids. Gastrointestinal and esophageal zygomycosis is observed in 20 percent of kwashiorkor, malnourished children eating moldy food, and cutaneous or subcutaneous forms in 3 percent of cases from various origins (burns, local trauma, etc.). Zygomycosis became the fourth most common opportunistic mycosis in the compromised host after candidiasis, aspergillosis, and cryptococcosis. Since 1960, organ transplant recipients, particularly recipients of renal transplants who have been treated with stronger and stronger immunosuppres-

sive drugs and who do or do not have associated diabetes, have caused a considerable increase in the number of cases of severe zygomycosis.

Since the 1980s, nosocomial zygomycosis, presenting as primary skin and wound infections caused by *Rhizopus* spp., *Cunninghamella bertholletiae*, *Rhizomucor pusillus*, *Absidia corymbifera*, and now *Saksenaea vasiformis* and *Apophysomyces elegans*, is often reported. Infections have been contracted from contaminated elastic bandages, infected burns, intravenous catheters, surgical wounds, ostomy sites or intravenous drug abuse whether or nor associated with AIDS. Another important condition is desferrioxamine therapy for iron or aluminum overload, which was responsible, between 1986 and 1990, for 24 lethal disseminated cases of zygomycosis caused particularly by *R. arrhizus (oryzae)*, *R. rhizopodiformis*, and *C. bertholletiae*. (For further information on zygomycosis, see also Systemic zygomycosis.)

McGinnis et al. (1993) reviewed 10 cases of rapidly fatal necrotizing fasciitis (Figure 15) caused by *Apophysomyces elegans* after trauma or burns in healthy patients or from various other predisposing conditions.

Figure 15 *Necrotizing fasciitis caused by* Apophysomyces elegans. *Back of patient showing extensive deep débridement in the exposed back muscles and necrotic margins. (From McGinnis et al. 1993)*

Another emerging organism with coenocytic hyphae and asexual spores produced in a sporangium is the oomycete *Pythium insidiosum* (de Cock et al. 1987) (synonyms *P. destruens* Shipton 1987, *Hyphomyces destruens* Bridges et Emmons 1961); this has been responsible for animal diseases and, since 1987, for severe human orbitofacial infections and arteritis among patients in Thailand and the USA with α- or β-thalassemia (Rinaldi et al. 1989). (For further information about *Pythium insidiosum*, see also Pythiosis.)

SYSTEMIC MYCOSES CAUSED BY DIMORPHIC FUNGI

Fungal dimorphism

In medical mycology the term dimorphism has been employed for the pathogenic, highly virulent, dimorphic fungi with a saprophytic, vegetative, mycelial form in nature or under ordinary laboratory conditions, and a pathogenic, unicellular, yeast-like, or spherule form in human and animal tissues. Under this medical definition, three important groups of dimorphic fungi are included, based on their morphology in vivo (see Figure 1).

The first group consists of yeast-like dimorphic fungi (Figures 16 and 17) such as:

- *Histoplasma capsulatum* var. *capsulatum* (dimorphism proven by DeMonbreun 1934) and *H. capsulatum* var. *duboisii*, respective agents of small-form or classic histoplasmosis and African or large-form histoplasmosis
- *Blastomyces dermatitidis*, agent of blastomycosis
- *Paracoccidioides brasiliensis*, agent of paracoccidioidomycosis
- *Sporothrix schenckii*, agent of sporotrichosis.

To this group *Penicillium marneffei* has been added. It is a redoubtable fungus emerging since 1988 in AIDS patients in southeast Asia which has been reviewed (Drouhet 1993a; Drouhet and Dupont 1995). It is the only known species, among more than 200 *Penicillium* spp., that is transformed in tissues into a unicellular form which multiplies not by budding, but by schizogeny. (For further information about *Penicillium marneffei*, see also Penicilliosis).

The second group consists of dimorphic fungi forming spherules in vivo:

- the agent of coccidioidomycosis, *Coccidioides immitis*, was first considered to be a protozoan but proved to be a dimorphic mold only in 1900 (Ophüls and Moffitt 1900) (see also Coccidioidomycosis)
- the agent of rhinosporidiosis thought to be a mycosis by Seeber (1900), but the agent *Rhinosporidium seeberi* has yet to be cultured (see also Rhinosporidiosis).

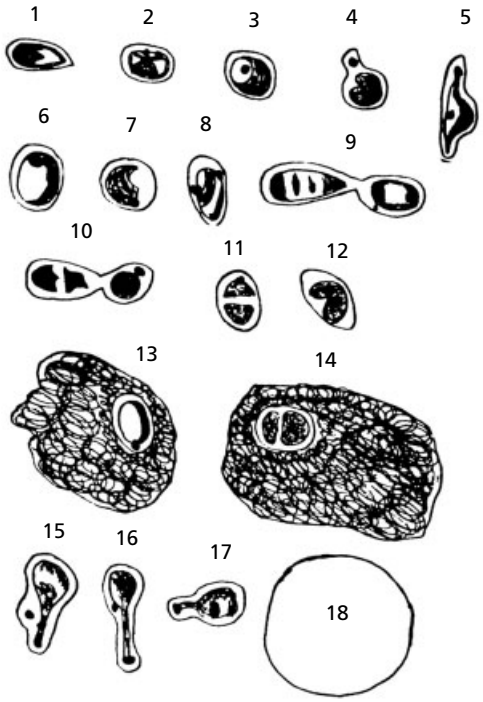

Figure 16 *Darling's drawing of intrahistiocytic aspects of* Histoplasma capsulatum *var* capsulatum *which he considered to be a protozoan, in spite of visible budding yeast cells.*

- the agents of adiaspiromycosis, a rare pulmonary human mycosis, but a frequent pulmonary mycosis in rodents caused by *Emmonsia crescens* and *E. parva* (see also Adiaspiromycosis and other infections caused by *Emmonsia* species).

Besides these pathogenic dimorphic fungi with yeast-like or spherical unicellular forms in vivo, there are other dimorphic or rather polymorphic fungi such as *Candida albicans* with unicellular budding cells and hyphae in tissues or as *Exophiala (Wangiella) dermatitidis*, *Phialophora verrucosa, Fonsecaea pedrosoi*, which are agents of chromoblastomycosis when, in vivo, they form muriform cells. When this parasitic form is absent and subcutaneous, even deep organs such as the brain are invaded by hyphae; the disease is then referred to as a pheohyphomycosis (Ajello 1975b). Their mycological, clinical, and epidemiologic aspects are different from the systemic mycoses caused by the dimorphic fungi.

Pasteur's experimental conditions for M → Y transformation of *Mucor* spp., such as CO_2 high tension, were found for most pathogenic thermal dimorphic fungi for in vitro transformation. The Y form of *Sporothrix schenckii* is obtained at 37°C in a 5 percent CO_2 atmosphere in a simple chemical defined medium with an inorganic $[(NH_4)_2SO_4]$ or organic (arginine) nitrogen source, glucose, biotin (role in CO_2 fixation into the cell)

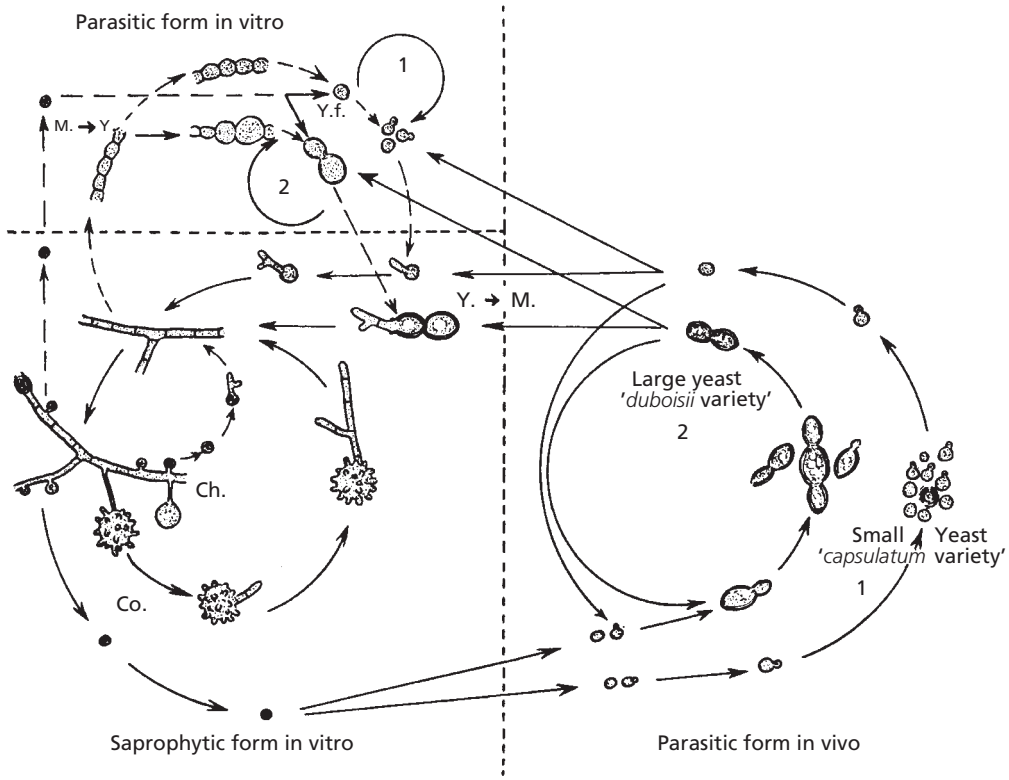

Figure 17 *Diagrammatic representation of the cycle in vitro (saprophytic and parasitic forms) and in vivo of* Histoplasma capsulatum *var* capsulatum *(1) and the* duboisii *var of* H. capsulatum *(2). (Ch., chlamydoconidia; Co. conidium; M.→Y., mycelial–yeast conversion; Y., yeast; Y.f., yeast form; Y.→M., yeast–mycelial conversion.) (Redrawn from Mariat 1964)*

and pyrimidine as growth factors, minerals, and oligoelements (Drouhet and Mariat 1952b). Conant (1941) obtained the difficult transformation of *H. capsulatum* var. *capsulatum* on a complex blood agar medium in sealed paraffinized tubes which provided an increased CO_2 tension. A higher content of CO_2 (15–20 percent) was necessary for *H. capsulatum* var. *farciminosum* on blood agar at pH 7.4 by Bullen (1949). Pine (1954) also found a stimulating effect of CO_2 and, in a more complex, chemically defined, nutritional agar medium in sealed tubes, obtained the transformation and conservation of cultures of the *capsulatum* and *duboisii* varieties of *H. capsulatum* for the production of antisera and vaccines (Pine and Drouhet 1963). Spherule formation in *Coccidioides immitis* is stimulated by 10 percent CO_2 in a synthetic medium.

This category of mycoses includes infections caused by species of *Blastomyces, Coccidioides, Histoplasma*, and *Paracoccidioides*. Their fascinating history began at the turn of the century and is still evolving today.

Histoplasmosis

Histoplasmosis was discovered in 1905 by Darling (1872–1925), an American pathologist and parasitologist working at the Canal Zone Hospital in Panama. There he observed three fatal cases of an intracellular infection of the reticuloendothelial system involving lymphatic tissues and all the principal organs of the body. Darling considered the observed microorganism to be a protozoan and named it *Histoplasma capsulatum* (Figure 16). However, da Rocha-Lima, a Brazilian studying in Hamburg, suggested that *H. capsulatum* was a fungus in 1912–13, because of its similarity to the tissue forms of the agent of epizootic lymphangitis of horses, an intracellular yeast then known as *Cryptococcus farciminosum* (Rivolta 1873) but now known as *H. capsulatum* var. *farciminosum*.

In 1934, DeMonbreun, a pathologist at Vanderbilt University in Nashville, TN, obtained a culture of the etiologic agent of histoplasmosis from a child diagnosed antemortem by Dodd and Tompkins (DeMonbreun 1934). He established the dimorphic nature of the fungus (Figure 17) by studying its in vitro characteristics at 25 and 37°C, and reproduced the disease in experimental animals. Independently, in 1934, Hausman and Schenken also obtained the mycelial form of *H. capsulatum* but erroneously considered it to belong to the genus *Sepedonium*. The culture of *H. capsulatum*, originally grown by DeMonbreun, served as the source of histoplasmin (comparable to coccidioidin from *C. immitis* used by Smith since 1943 to detect coccidioidomycosis), which was used by Christie and Peterson in 1945 in a skin-test survey. They showed that histoplasmosis capsulati was responsible for pulmonary calcifications in infants, young World War II recruits and young control subjects, who were negative for both tuberculin and coccidioidin. Parsons and Zarafonetis reviewed 71 cases of histoplasmosis, in 1945, collected during 1905–45, and found that the disease was rare but uniformly fatal. However, since the work of Christie, Peterson, Palmer, and others, benign asymptomatic histoplasmosis has been considered a widely prevalent disease.

Emmons (1949) first isolated the *capsulatum* variety of *H. capsulatum* from soil around a rodent burrow and under a chicken coop. Positive cultures were obtained only after 156 negative specimens – a feat that demonstrated that perseverance is necessary for successful completion of such a study. Hundreds more isolations from suspected sites and patients in many parts of the world have supplied information of epidemiologic importance. Soil contaminated by the excreta of chickens, pigeons, and bats in the USA, Central and South America, and South Africa, and by the nocturnal fruit-eating oil bird (*Steatornis caripensis*) in caves in Colombia, Peru, Trinidad, and Venezuela, as shown by Ajello and Zeidberg (1951) and Emmons (1958), and air and water, as shown by Furcolow (1958), have proved to be sources of benign pulmonary outbreaks of histoplamosis. The first epidemiologic study on a large scale (since the study in 1945 by Furcolow) led to the rapid development of investigations of all phases of the disease. Since the first complement fixation serological test by Tenenberg for diagnosis in 1947, numerous other tests have been developed for antigen and antibody detection (Wheat et al. 1986). Clinical aspects of histoplasmosis have been described, ranging from pulmonary and splenic calcifications (Schwarz et al. 1955) to the disseminated form, which is now observed in immunosuppressed patients and particularly in AIDS patients (Graybill 1988). Epidemiologic and clinical studies have revealed that histoplasmosis has a worldwide distribution with the densest endemic areas chiefly in the USA, where 40 000 000 people have had the disease and 500 000 new infections occur every year (Rippon 1988). Parallel with these studies, there has been intensive study of the biological aspects of dimorphism (Rippon 1980; Szaniszlo et al. 1983), the regulation of the metabolism of the yeast (Y) and mycelial (M) forms at a molecular level, differences in cell wall composition accompanying morphogenesis (San-Blas and San-Blas 1985, 1993) and the role of temperature for the regulation of the Y′M form (Howard 1967). Another important contribution was the discovery by Kwon-Chung, in 1972, of the sexual state of *H. capsulatum*: *Emmonsiella capsulata* (1972), now known as *Ajellomyces capsulatus* (Kwon-Chung 1972) McGinnis et Katz 1979.

More historical details on this fascinating disease were discussed at two national conferences held in the USA in 1952 and 1962, organized by Furcolow and Ajello, and in books such as *Histoplasmosis* by Sweany in 1960.

A new form of histoplasmosis was discovered as a geographically localized form in central Africa, particularly Senegal, Nigeria, and Zaire. It is characterized by a parasitic tissue-form comprising large, oval yeast cells in giant cells (Figure 17) and by a distinct clinical disease. The cutaneous, subcutaneous and bone lesions predominate over pulmonary and visceral disease. The first case was reported by Catanei and Kervran in 1945 at the Pasteur Institute in Algiers. Other fatal cases were observed by Kervran and Aretas in 1947 and by Duncan, who suggested that the agent was either a species distinct from *H. capsulatum* or a variant (Duncan 1947, 1958). This agent was considered to be a distinct species by Vanbreuseghem in 1952, who named it *H. duboisii* in honor of Dubois, Director of the Institute of Tropical Medicine (Antwerp), where the strain from a Belgian patient infected in Africa was studied (Dubois et al. 1952; Vanbreuseghem 1953). The disease was called African or large-form histoplasmosis, although histoplasmosis is also endemic in Africa. For that reason an alternative term, histoplasmosis duboisii, was proposed (Ajello 1983) to describe the disease caused by *H. capsulatum* var. *duboisii* (Drouhet 1957). Subsequently, this organism was confirmed by Kwon-Chung (1972, 1975) who showed that the sexual states of both varieties are identical. This new form of histoplasmosis, also endemic in Madagascar, now has a small but important place in the pathology of Africa and is an imported mycosis in Europe, particularly in Belgium and France (Drouhet 1989).

More than 200 published cases have been reported among Africans and Europeans, with 30 percent developing severe systemic infections. In animals, the spontaneous infection has been observed only in monkeys imported from Africa into France, England, and the USA. The natural history of this particular form is not well known, but, recently, Guignani et al. (1994) reported the isolation of *H. capsulatum* var. *duboisii* from soil admixed with bat guano from a sandstone bat cave in a rural area of Nigeria.

The third form of histoplasmosis (see also Histoplasmosis), epizootic lymphangitis of horses and mules, was first described by Rivolta (1873), who named the agent *Cryptococcus farciminosum*. This fungus was renamed *H. farciminosum* by Redaelli and Ciferri (1934). It is now considered to be a variety of *H. capsulatum*: *H. capsulatum* var. *farciminosum* (Rivolta) Weeks, Padhye et Ajello 1985, responsible for the disease. It is particularly prevalent among equines in Egypt, India, and Russia.

Blastomycosis

In 1894, Gilchrist observed the first case of a verrucous, granulomatous dermatitis produced by a yeast-like fungus. In 1896, it was named *Blastomyces dermatitidis*, when together with Stokes he published the second case (Gilchrist and Stokes 1896). This disease, once called Gilchrist's disease and the Chicago disease (15 cases were described there in 1901), was considered to be endemic in North America until 1953, when cases were observed in Tunisia (Vermeil et al. 1954), and later in Algeria, Central Africa, Saudi Arabia, India, Israel, Morocco, Mozambique, and South Africa, indicating extensive geographical distribution. The natural habitat of *B. dermatitidis* remains an enigma in spite of various isolates from nature. The discovery by McDonough and Lewis, in 1967, of the sexual state of *B. dermatitidis*, named *Ajellomyces dermatitidis*, is an important event in the history of this disease. (See also Blastomycosis.)

Paracoccidioidomycosis

This Latin American mycosis was first described by Lutz (1908), in a Brazilian patient with severe granulomatous naso-oropharyngeal lesions and cervical adenopathy. Lutz described and cultured this dimorphic fungus which he considered, in tissue, to resemble the parasitic form of coccidioidomycosis, and called the disease a pseudococcidioidal mycosis. The Italian microbiologist, Splendore, described the fungus and its disease in several studies between 1908 and 1912, and Almeida named the fungus *Paracoccidioides brasiliensis* in 1930. Consequently, the disease was called the Lutz–Splendore–Almeida disease. In 1942, Conant and Howell considered this fungus to be similar to *B. dermatitidis* and named it *B. brasiliensis*, and the disease South American blastomycosis. However, later investigators restored the names *P. brasiliensis* and paracoccidioidomycosis (Figure 18). The ecology of *P. brasiliensis* remains an unsolved puzzle (Restrepo 1985), but the disease responds well to azole antifungals (Restrepo 1990). (See also Paracoccidioidomycosis.)

Coccidioidomycosis

Now recognized as a disease of major public health importance in the USA and especially Mexico, coccidioidomycosis was first described, in 1892, by Posadas in Argentina. Posadas was a student of the famous pathologist Wernicke. He considered this granulomatous disease to be a neoplasia. Two years later, Rixford and Gilchrist (1896) observed a new case in California, considered that the agent was a protozoan, and named it *Coccidioides immitis*. It was later identified by Ophüls and Moffitt (1900) as a fungus with a dimorphic life cycle (Figure 19). The dimorphism of *C. immitis* was critically studied and reviewed by Cole et al. (1993). The disease is considered to be rare in Argentina (only a few cases in 1929, 27 in 1967); however, it is most common in southern California. In 1915, Dickson of Stanford University reported 40 cases of a primary pulmonary infection with mild and

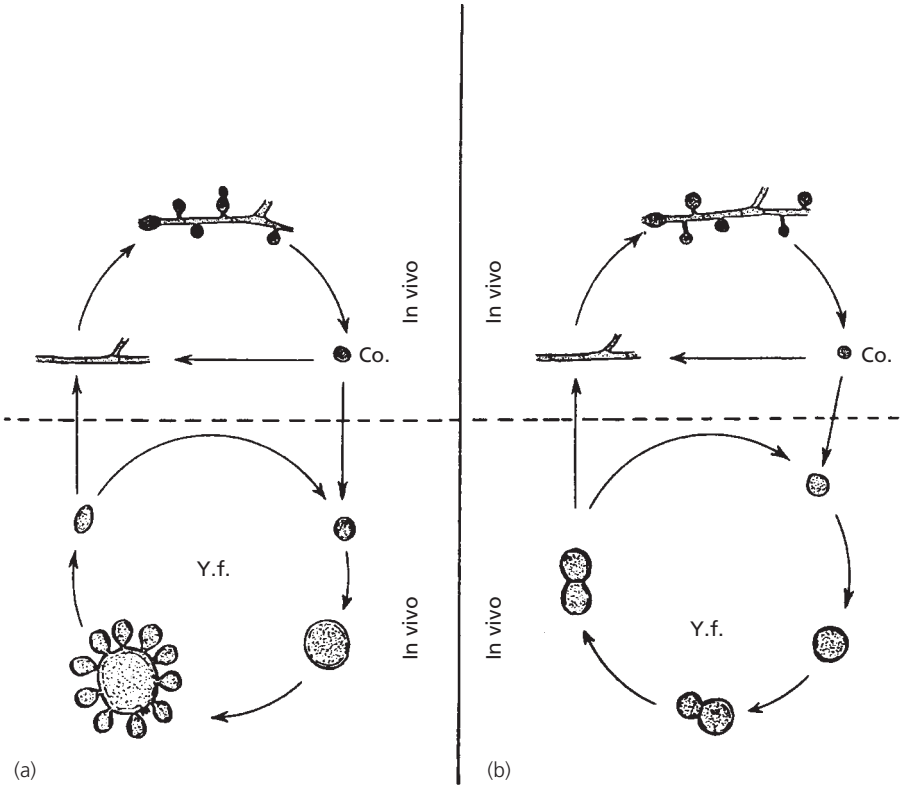

Figure 18 *Diagrammatic representation of cycles in vitro and in vivo of* **(a)** Paracoccidioides brasiliensis *and* **(b)** B. dermatitidis *(Co., conidium; Y.f., yeast form).*

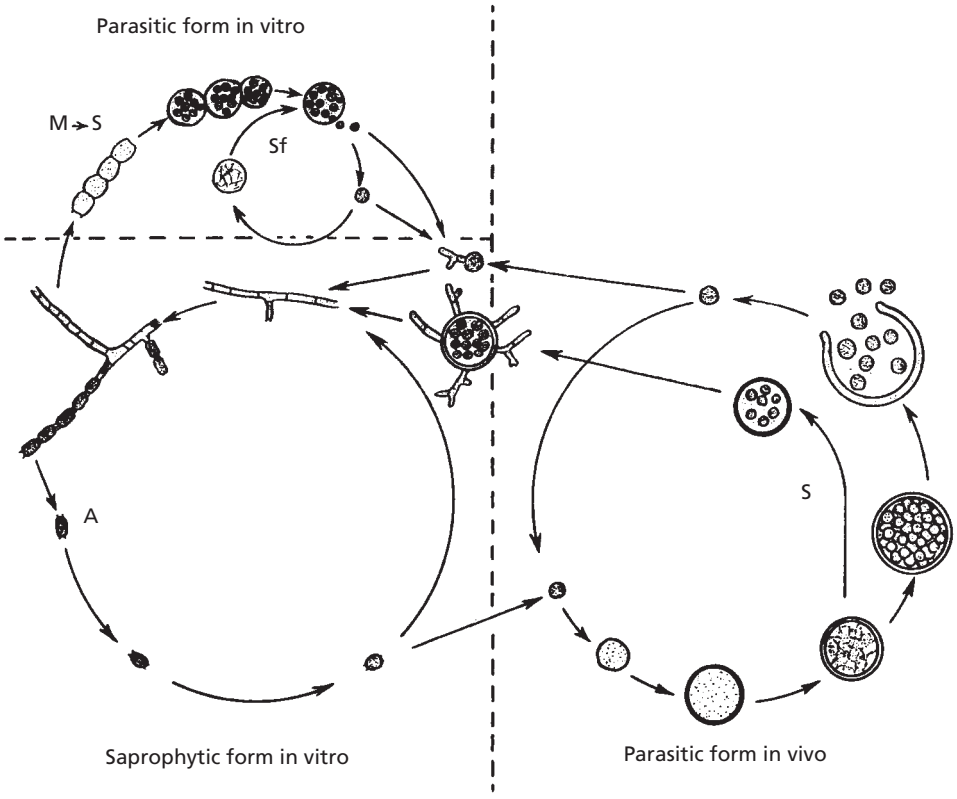

Figure 19 *Diagrammatic representation of cycle in vitro (saprophytic and parasitic forms) and in vivo of* Coccidioides immitis *(A, arthroconidias; M→S, mycelium–spherule conversion; S, spherule; Sf, spherule form). (From Mariat 1964)*

fatal disseminated evolution. In 1932, Stewart and Meyer isolated *C. immitis* from soil; in 1942 Emmons isolated it from soil as well as from rodents, and as a result of the extensive studies of Dickson, Gifford, and Smith, it was established that San Joaquin Valley fever was coccidioidomycosis. The work of C.E. Smith (1904–67) on the epidemiology, diagnosis, and prophylaxis of coccidioidomycosis was considerable and has been the subject of a voluminous literature. The work of Smith, Huppert, Cole, Pappagianis, Levine, and others contributed largely to the understanding of the immunology of this disease. Ajello edited the proceedings of the second symposium on coccidioidomycosis in 1967, and of the third symposium in 1977, followed by the fourth symposium in 1984 and several books (Fiese 1958; Ajello 1977). (See also Coccidioidomycosis.)

Emmonsia spp. and adiaspiromycosis

Studying the ecology and natural history of *C. immitis* in the desert of southern Arizona, Emmons and Ashburn (1942) revealed that many rodents had mycotic pulmonary lesions and described the mycelial fungus obtained in culture as *Haplosporangium parvum* because of its resemblance to the phycomycete *H. bisporale*. Emmons described the adiaspores in the tissues and the later work showed that the conidia of the new filamentous fungus grew in vivo to form uninucleate spherules (< 40 mm), with thick laminated walls and no endospores. this cystic pulmonary disease had been described earlier by Kirschenblatt in 1939. He had observed adiaspores in the lungs of rats from Transcaucasia (Russia), and named the organism *Rhinosporidium pulmonale.* The organism was not isolated and was therefore not given a Latin name. In 1947, Dowding reported a fungus similar in culture to *H. parvum*, but with adiaspores attaining a diameter of 270 mm in rodent lungs from western Canada and the northern USA. Jellison, in numerous publications between 1950 and 1959, reported the widespread distribution of this mycosis in rodent lungs and other small mammals in Africa, Ecuador, Finland, Japan, Korea, Norway, Sweden, the USA, and Yugoslavia, in animals preserved in formalin since 1845, and in France, in certain specimens of animals preserved for 60 years in the Museum of Natural History of Paris (Jellison et al. 1961; Jellison 1969).

The natural habitat of the *Emmonsia (Haplosporangium)* spp. is soil. In 1954, Menges and Haberman reported the isolation of *H. parvum* from soil in Missouri, but Emmons found the strain to be different from isolates of this species. In 1959, Ciferri and Montemartini isolated *E. parva* from soil in Italy. They renamed this fungus and placed it in the new genus *Emmonsia*. In 1960, Emmons and Jellison, after studying several hundred dimorphic fungi in cultures and in pulmonary tissues of naturally, or experimentally, infected animals, described a new species *E. crescens* which could be segregated from *E. parva* on the basis of its larger adiaspores, which also enlarge without multiplication in host tissue. Since the first human case of pulmonary adiaspiromycosis described in France by Doby-Dubois (1964), numerous other chronic benign pulmonary cases have been reported in Brazil, Czechoslovakia, France, India, Russia, and Venezuela. All these cases were caused by *E. crescens*, except for a case in 1993 in an AIDS patient observed in Colombia which was caused by *E. parva* (Echavarria et al. 1993). (See also Adiaspiromycosis and other infections caused by *Emmonsia* species.)

Infection caused by *Penicillium marneffei* – the new emerging systemic mycosis in AIDS

Penicillium marneffei (Capponi et al. 1956; Segretain 1959), the only dimorphic pathogenic *Penicillium* sp. among 223 described species of *Penicillium*, was first isolated from bamboo rats (*Rhizomys sinensis*), native to Burma, China, and Vietnam (Capponi et al. 1956). These rats had been kept in captivity for experimental infections at the Pasteur Institute of Indochina at Dalat, and they developed a fatal reticuloendothelial mycosis. The fungus isolated from a rat was used to infect a laboratory mouse and both fungus and mouse were sent to the Pasteur Institute in Paris to be studied. The fungus was identified by Segretain as a new species, *P. marneffei*, in honor of Marneffe, Director of the Pasteur Institute of Indochina.

The typical feature of this pathogenic fungus is its thermal dimorphism: unicellular, oval or elongated cells, which multiply by schizogeny in vivo in histiocytes and macrophages or in vitro at 37°C. At 25°C, *P. marneffei* grows as a mold with hyphae and conidia, producing a characteristic diffusible red pigment.

Segretain (1959) pricked his finger accidentally, during experimental studies, with a needle used to inoculate hamsters with *P. marneffei*. He developed a small nodule at the site of inoculation 9 days later, followed by lymphangitis and axillary lymph node hypertrophy. Antifungal sensitivity studies by Drouhet (in Segretain 1959) demonstrated high in vitro sensitivity of this fungus to nystatin. An intensive treatment with oral nystatin (20 million units – 10–13 times the usual daily dose) for 30 days cleared the infection. This accidental infection emphasized the possibility of human pathogenicity.

The first natural human infection was described only 17 years later by DiSalvo et al. (1973) in the USA, in a 64-year-old minister who had traveled in southeast Asia. *P. marneffei* infection was diagnosed when the patient underwent a splenectomy for management of Hodgkin's disease. A second imported pulmonary case was reported in the USA by Pautler et al. (1984), in a

59-year-old man who had travelled extensively in the Far East.

Since 1984, cases of *Penicillium* infection have been reported in southern China by Deng and Connor in 1985, and in Thailand by Jaynetra et al. in 1984, among healthy or immunocompromised patients, as a systemic mycosis involving the entire reticuloendothelial system, and mimicking histoplasmosis. Since 1988, numerous cases have been reported among AIDS patients from Australia, France, Italy, the Netherlands, the UK, and the USA, who had travelled in southeast Asia, and in an African patient diagnosed in France who had acquired a laboratory infection in Paris. The clinical, epidemiologic, mycological and biological aspects of 44 cases in patients negative for the human immunodeficiency virus (HIV) and 44 cases in HIV-positive patients were analysed and compared (Drouhet 1993a). In the following 2 years an increasing number of AIDS patients from Thailand and Hong Kong (Supparatpinyo et al. 1994) have been infected with *P. marneffei*. The number of cases reported is now close to 400 (Drouhet and Dupont 1995).

Epidemiologic studies since 1986 have shown that wild bamboo rats in China, Thailand and Vietnam – *Cannomys badius*, *R. pruinosus*, *R. sinensis*, and *R. sumatrensis* – are commonly infected by *P. marneffei* (Deng et al. 1988; Ajello et al. 1995). Penicilliosis can be considered to be a 'new emerging systemic mycosis' for individuals with HIV infection living or traveling in southeast Asia. This constitutes a dangerous risk for contracting penicilliosis. This disease must be included in the clinical definition of AIDS as other systemic mycoses already are (candidiasis, coccidioidomycosis, cryptococcosis, and histoplasmosis) in defined endemic areas (Drouhet 1993a). (See also Penicilliosis.)

SUBCUTANEOUS MYCOSES AND OTHER DEEP MYCOSES

Mycetoma

The term mycetoma encompasses all pseudotumors produced by fungi. It was first used by Vandyke Carter in 1874 after he had established the fungal etiology of this disease. The division of this disease into separate categories dependent on a fungal or actinomycotic etiology was first suggested by Pinoy (1913). At present, according to the literature, including comprehensive and extensive reviews by Mariat et al. (1977), who surveyed 854 cases identified between 1940 and 1960, and the classic manuals of medical mycology by Emmons et al. (1977) and Rippon (1988), the single clinical entity mycetoma may be evoked by 33 well-recognized agents, 23 species of true fungi (i.e. eukaryotic microorganisms) and 10 actinomycetes (i.e. prokaryotic organisms). These figures, as well as the number of cases, have increased according to the last review of Kwon-Chung and Bennett (1992).

Many authors from all over the world have studied this disease. In particular several groups of medical mycologists have contributed: Abbott in 1956 in clinical studies of 1 231 Sudanese mycetomas, Murray on the immunology of mycetoma, Hay and Mackenzie on ultrastructure and histopathology, Mahgoub in 1976 in Sudan (epidemiology, immunology, and therapy of mycetoma), Latapi and Lavalle in Mexico, Mackinnon in Uruguay, Borelli in Venezuela, da Silva Lacaz in Brazil (da Silva Lacaz 1983), Ajello, Conant, Emmons, Gordon, and Rippon in the USA, Avram et al. in Romania, Vanbreuseghem in Belgium, and many others. In France, the first historical studies on actinomycotic and fungal agents of mycetoma by Vincent in 1894, Brumpt in 1906, Laveran in 1906, Pinoy in 1913, and Langeron in 1928, have been developed intensively at the Pasteur Institute since 1956 by Segretain and Mariat, in association with the histopathologists Destombes, Camain, and Ravisse; there was also help from French and African physicians, surgeons, and hygienists. All these data have been reviewed (Mariat et al. 1977). Several discoveries have helped to clarify the extremely confusing host–parasite relationship of this syndrome with its 23 fungal agents. In addition to the discovery of new agents of mycetoma (Figure 20), i.e. *Leptosphaeria senegalensis* (Segretain et al. 1959), *Leptosphaeria tompkinsii* (Segretain et al. 1974), *Neotestudina rosatii* (Segretain and Destombes 1961), there have been new data on the epidemiology of mycetomas, such as the discovery by Segretain of these two fungi in African soil and on the thorns of acacia trees, the discovery of the sulfurophilic fungus *Madurella mycetomatis* in termitaria (Segretain and Destombes 1969), the classification of mycetomas according to the microscopic aspects of actinomycotic and fungal 'grains' in tissues (Figure 20). There are many questions to be answered and work to be done on the eumycetomas, particularly on their treatment which is ineffective in most cases, and which necessitates amputation of legs and feet. Two International Symposia (Venezuela 1977; Mexico 1987) have been dedicated to the mycetomas. (See also Eumycetomas.)

Pheohyphomycoses

As of 1994, 101 species of pheoid fungi had been isolated from human and animal infections and grouped by Ajello (1975b) under the umbrella term pheohyphomycoses. Taxonomically, the fungi responsible for pheohyphomycosis include hyphomycetes in the Dematiaceae, Spheropsidales and various Ascomycetes. Clinically, there is a wide spectrum of infections which vary from superficial colonization of the skin and hair or the cornea of the eye, to cyst formation, to cutaneous, subcutaneous, cerebral, and systemic opportunistic infec-

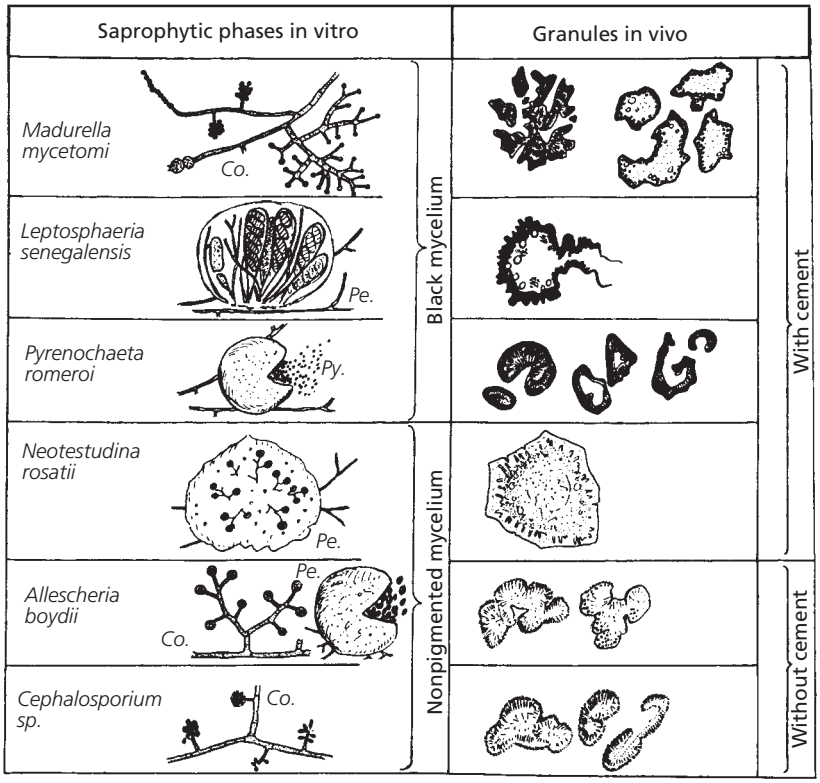

Figure 20 *Diagrammatic representation of the principal agents of fungal mycetomas in vitro and in vivo (Co., conidium; Pe., perithecium; Py., pycnidium). (Redrawn from Mariat 1964)*

tions; these have all been well detailed by Ajello (1975b, 1981); McGinnis (1983); Rippon (1988); and Matsumoto et al. (1994). (See also Adiospiromycosis and other infections caused by *Emmonsia* species.)

Sporotrichosis

As pointed out by Mariat (1968), sporotrichosis can be considered a historical 'model of medical mycology.' The first case of sporotrichosis was reported by Schenck, from the Johns Hopkins Hospital in Baltimore in 1898. The fungus was isolated by Schenck and classified in the genus *Sporotrichum* by the plant mycologist E.F. Smith. The second case, also reported from the USA (Chicago) by Hektoen and Perkins in 1900, was observed in a boy who had struck his finger with a hammer. He developed an inoculation chancre with lymphocutaneous lesions. The authors named the fungus *Sporothrix schenckii*. It is a dimorphic fungus (Figure 21) with a yeast form obtained in a chemically defined medium under 5 percent carbon dioxide (Drouhet and Mariat 1952b). The lesions cured spontaneously and 65 years later the patient still had a positive sporotrichin skin test. In Europe, the disease was initially described in 1903 by de Beurmann and Ramond. The fungus isolated was studied in 1910 by the French botanist, Matruchot, who considered it to be a species different from *S. schenckii* because of the presence of a black pigment. It was named *Sporotrichum beurmannii*. The identity of the American and French species was established in 1921 by Davis and, in 1962, Carmichael determined that *Sporothrix schenckii* is the correct binomial for the fungus. Between 1906 and 1912, de Beurmann and Gougerot identified at least 10 more cases and tabulated more than 200 additional cases in their famous book, *Les sporotrichoses* (de Beurmann and Gougerot 1912). They were the first to suggest that sporotrichosis could be considered as an opportunistic infection, which has recently been confirmed by the occurrence of sporotrichosis in AIDS. Following the suggestion of Sabouraud, in 1903 de Beurmann and Ramond were the first to employ iodides in the chemotherapy of sporotrichosis with remarkable success (de Beurmann and Gougerot 1912). This therapy still remains the treatment of choice for sporotrichosis. Over a period of 50 years, potassium iodide remains the only oral chemotherapeutic agent for deep mycoses. To date several hundred cases have been reported all over the world, particularly in Europe. Curiously, since 1940, sporotrichosis has virtually disappeared from Europe. (See also Sporotrichosis.)

Intensive studies on the ecology and epidemiology of sporotrichosis, reviewed by Mariat (1968) and Rippon (1988), established that *S. schenckii* can be isolated from soil, timber, house plants, straw, bird nests, and animal burrows, etc. The most endemic areas are Latin America

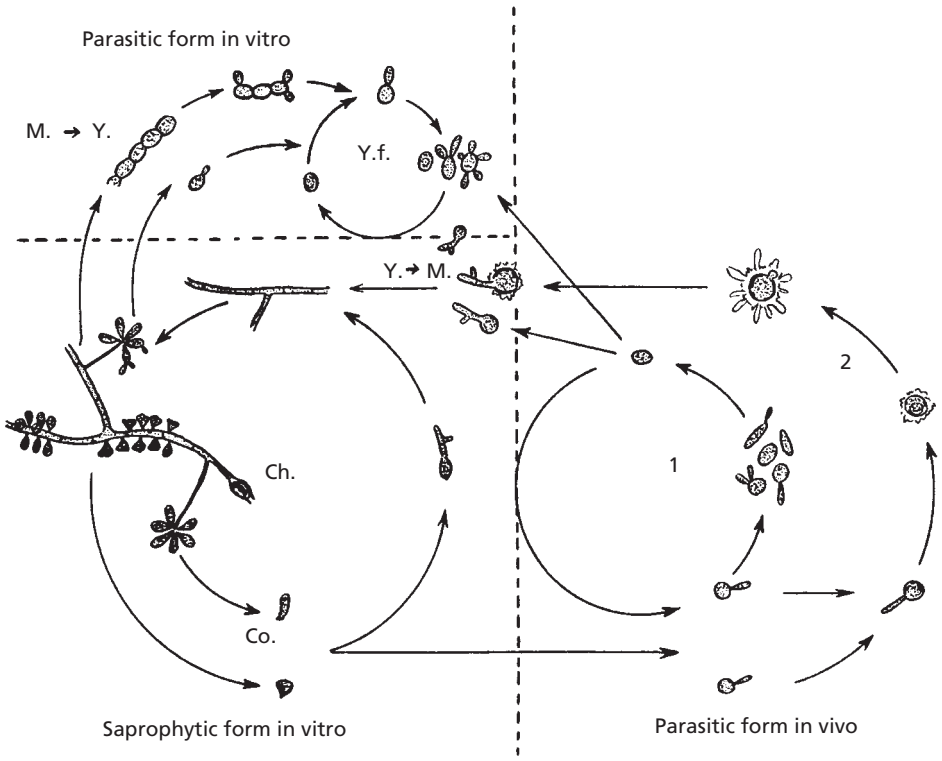

Figure 21 *Diagrammatic representation of the cycle of* Sporothrix schenckii *in vitro (mycelial and yeast forms) and in vivo (Ch., chlamydoconidium; Co., conidium; M.→Y., mycelial–yeast conversion; Y.f., yeast form; Y.→M., yeast–mycelial conversion; 1, 'cigar bodies;' 2, asteroid bodies). (From Mariat 1964)*

and South Africa. *S. schenckii* is phylogenetically related to the plant fungus *Ceratocystis stenoceras* (Mariat 1977).

Chromoblastomycosis and its agents

The term chromoblastomycosis was coined by the Brazilian mycologists and dermatologists Terra, Torres, Fonseca, and Area Leao in 1922, to define a clinical entity presenting with verrucous dermatitis caused by various pheoid fungi. The attempt by Moore and Almeida, in 1935, to replace the term chromoblastomycosis with chromomycosis, including cases of similarly pigmented hyphal fungi, was disputed by Ajello (1974) in favor of the term pheohyphomycosis for cutaneous, subcutaneous, cerebral, and systemic opportunistic infections, caused by a larger spectrum of pheoid fungi. Historically, although chromoblastomycosis is essentially a tropical and subtropical disease, the first authentic case was reported in a patient from Boston (USA) by Lane and Medlar in 1915. The fungus was named *Phialophora verrucosa* Medlar 1915. Pedroso and Gomes (1920) reported four cases from Brazil of the disease called locally 'formiguiero.' One of the patients had been observed since 1911 and is considered to be the first known case of chromoblastomycosis. The etiologic agent was considered to be different from *P. verrucosa* and was named *Hormodendron pedrosoi* by Brumpt in 1920. The valid name is *Fonsecaea pedrosoi* (Brumpt) Negroni 1936. This species is the most frequent agent of chromoblastomycosis in Latin America. The first case outside the Americas was described in 1927 by Montpellier and Catanei from the Pasteur Institute of Algiers. The responsible fungus was described as *Hormodendron algeriensis* (now *Fonsecaea pedrosoi*). In Madagascar, Brygoo and Segretain (1960), during the 4-year period 1955–59, reported a high prevalence of 129 cases (a case rate of one per 32 500 population). Numerous other cases were found in Central and South America, Africa, Asia, and Australia, where intensive mycological studies were performed for identification and taxonomic classification of the dimorphic agents (Figure 22) of chromoblastomycosis. (See also Chromoblastomycosis.)

Five principal agents of chromoblastomycosis have been recognized since 1915:

1. *Phialophora verrucosa* Medlar 1915
2. *Fonsecaea pedrosoi* (Brumpt) Negroni 1936
3. *Fonsecaea compacta* Carrion 1940
4. *Cladophialophora carrionii* Trejos 1954, genetically related to *P. verrucosa* and *F. pedrosoi*
5. *Rhinocladiella aquaspersa* (Borelli) Schnell, McGinnis et Borelli (1983).

Among numerous historical, mycological, and epidemiologic studies on chromoblastomycosis and its responsible fungi, special mention is necessary for the

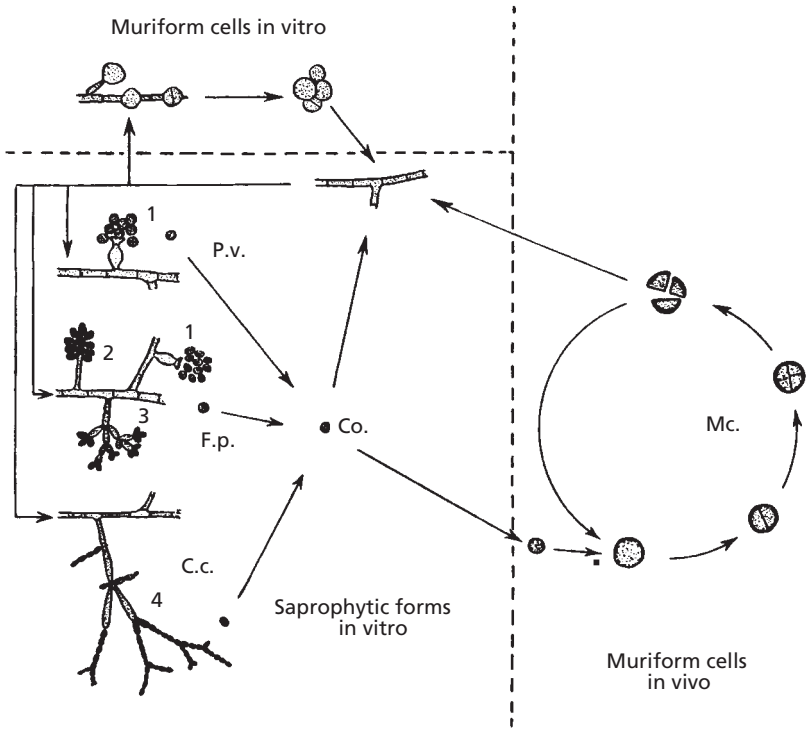

Figure 22 *Diagrammatic representation of the cycles of three agents of chromoblastomycosis in vitro (saprophytic and tissue forms) and in vivo (C.c.,* Cladophialophora carrionii*; Co., conidium; F.p.,* Fonsecae pedrosoi*; Mc., muriform cells; P.v.,* Phialophora verrucosa*; 1, phialide form; 2, acrotheca form; 3, short cladosporium form; 4, long cladosporium form). (Redrawn from Mariat 1964)*

studies of Carrion between 1935 and 1971 and Silva between 1947 and 1971 at the School of Tropical Medicine, San Juan, Puerto Rico and Columbia University, New York, where new species were identified and the parasitic form obtained in vitro. The first evidence that the etiologic agents exist in nature was obtained by Conant (1937b) at Duke University (Durham, NC). He demonstrated that *P. verrucosa* was a cause of 'blueing' of lumber, followed by the discovery of the presence of *F. pedrosoi* and *C. carrionii* in timber, soil, wood or pulps, and air, and in numerous infections in animals with characteristic muriform cells in tissues. The first immunological studies in chromoblastomycosis were performed by Conant and Martin (1937). More recently, in Venezuela, Zeppenfeldt et al. (1994) isolated *C. carrionii* from the medullary tissue of two members of the family *Cactaceae* with 'muriform cells' similar to those in patients with chromoblastomycosis.

HISTORICAL DEVELOPMENTS IN MEDICAL MYCOLOGY

The International Society for Human and Animal Mycology

Medical mycology was recognized as an individual full branch of microbiology only in 1954 with the creation of the International Society for Human and Animal Mycology (ISHAM), at the Thirteenth International Botanical Congress in Paris. Until that time, sessions on the mycoses were admitted (sometimes with difficulty) at dermatological and botanical congresses. The idea had long been encouraged by some mycopathologists, such as those participating in the Fifth Congress on Microbiology in Rio de Janeiro (1950), but nothing was decided until the Sixth International Congress on Microbiology in Rome in 1953, when Redaelli and Ciferri invited a small group of mycologists composed of Vanbreuseghem (Belgium), Ainsworth (the UK), Segretain, Drouhet, Mariat (France), Lodder (the Netherlands), Seeliger (Germany), Paldrok (Sweden), and several others to found the International Society for Human and Animal Mycology (ISHAM), which was formalized in 1954, in Paris. A president was elected (Redaelli, Milan), as well as a very active general secretary (Vanbreuseghem, Anvers) and four vice-presidents: Segretain (Paris), Emmons (Bethesda), Ainsworth (Exeter), and Negroni (Buenos Aires). Mycologists from a host of other countries have joined ISHAM, which, as a result of the value of scientific contributions from its members, has encouraged the steady development of medical mycology as an individual branch of medical microbiology, alongside bacteriology, parasitology, and virology.

Once ISHAM was established, some founders and charter members claimed the right to form National Societies of Medical Mycology for intensive education,

training, and development of research in this field in their own countries. Foundation of the Société Française de Mycologie Médicale in 1956 was followed by the Japanese Society for Medical Mycology (in 1957), the Deutschsprachige Mykologische Gesellschaft (in 1960), the British Society for Mycopathology (in 1961), the Société Belge de Mycologie Humaine et Animale, the Medical Society of the Americas (in 1961) and another 18 other national societies all affiliated to ISHAM. The ISHAM congresses to date have been held in Paris (1954), Lisbon (1958), Montréal (1962), New Orleans (1967), Paris (1971), Tokyo (1975), Tel Aviv (1979), Palmerston (1982), Atlanta (1985), Barcelona (1988), Montréal (1991), Adelaide (1994), Salsomaggiore, Italy (1997), Buenos Aires (2000), and San Antonio (2003).

International and National Societies have contributed through their symposia, workshops, reference committees, and publications to the development of basic and applied research, education, and training. Their historical evolution is recorded in several publications (Ainsworth 1986; Drouhet 1991; Espinel-Ingroff 1996).

Literature on medical mycology

The literature of medical and veterinary mycology directed towards teaching and research has increased considerably since 1945, at a rate estimated to be about equal to that of scientific literature in general. The *Review of Medical and Veterinary Mycology* (*RMVM*), the quarterly journal of abstracts published by the Commonwealth Mycological Institute, presents complete information on this literature. In 1966, Ainsworth, analyzing the pattern of the medical and veterinary literature reviewed since the foundation in 1943 of the *RMVM*, estimated that the increase in the number of abstracted papers was linear and had doubled over a 12-year period. Since these data, the increasing number of papers published concern principally the immunology and chemotherapy of the mycoses. The presidential address of Mackenzie (1992) at the Eleventh Congress of ISHAM held in Montréal (Canada) gave a full analysis of the evolution over the last five decades, with topics of interest ranging from laboratory studies largely directed at fungal biology (the 1960s), pathology, etiology/epidemiology (up to the 1970s) to antifungal/chemotherapy (dominant since the 1980s), immunology, host–parasite relations, and, more recently, molecular biology.

Several publications are devoted exclusively, or in large part, to medical mycology:

- The *Journal of Medical and Veterinary Mycology* (*JMVM*), formerly named *Sabouraudia*, in 1961, when this journal was founded as the official journal of ISHAM. The four annual numbers of *Sabouraudia* were increased to six in 1985 when the journal changed its name to *JMVM* with nearly 150 papers annually. Two large volumes were published of the Proceedings of the last Congresses of ISHAM (Montréal, 1992; Adelaide, 1994) [*J Med Vet Mycol* 1992, **30** (Suppl 1): 1–331; *J Med Vet Mycol* 1994, **32** (Suppl 1): 1–420].
- *Mycopathologia*, founded in 1938 by Ciferri and Redaelli under the name *Mycopathologia et Mycopathologia Applicata*, one of the oldest journals of mycology, devoted to the fungi of plant pathology, mycotoxicoses, applied industrial mycology, and human and animal mycoses.
- *Mycoses*, a bimonthly journal since 1988, formerly named *Mykosen*, official publication of the German-speaking Mycological Society.
- The *Journal de Mycologie Médicale (Journal of Medical Mycology)*, founded in 1991 (formerly, since 1956, the *Bulletin de la Société Française de Mycologie Médicale*), an international journal (in English and French) for mycological studies related exclusively to human and animal mycology.
- The *Japanese Journal of Medical Mycology*, founded in 1956.

Besides these publications devoted to medical mycology, there are other general medical journals that publish, only occasionally, articles on various aspects of human mycoses, such as *Journal of Infectious Diseases, Reviews of Infectious Diseases, Infection and Immunity, Clinical Infectious Diseases, American Journal of Medicine,* etc., or about fungi of medical interest, such as *Antonie van Leewenhoek, Mycotaxon, Experimental Mycology*, and national or regional journals of some Societies of Medical Mycology.

During the period up to World War I (1914), the representative mycopathological textbooks and monographs included those on dermatophytes, such as Morris's *Ringworm in the light of recent research* (London), published in 1898, although the main texts were the monumental book of Sabouraud, *Les teignes,* published in 1910, and the book on sporotrichoses by de Beurmann and Gougerot (1912). Robin's *The vegetals* (1853), in which the mycoses in human and lower animals are developed, and two French monographs on aspergillosis by the veterinarian Lucet and the medical pathologist Renon, published in 1897, complete this pioneering period. The truly educational period for mycopathologists began with the famous *Manual of tropical medicine* (first published in 1910; subsequent editions in 1913 and 1919) by Castellani, considered to be one of the first modern medical mycologists, and Chalmers, and the *Précis de parasitologie* by Brumpt (first edition in 1910, sixth edition in 1949). These handbooks were followed by Dodge's *Medical mycology* (1935), an extensively referenced book with 900 pages and 4 000 references. The breakthrough in medical mycology teaching occurred in 1944, when Conant and his colleagues Martin, Smith, Baker, and

Callaway at Duke University, Durham, NC, published the *Manual of medical mycology* (Conant et al., 1944, 1954, 1971). This book set the pattern for subsequent textbooks such as those of Emmons, Kwon-Chung, and Rippon, the latter from the Pritzker School of Medicine, University of Chicago, entitled *Medical mycology*. From the first edition of 1974 to the third edition of 1988, this book gave the most significant changes occurring in recent years, but it also gives historical data for all pathogenic fungi and actinomycetes. This book still represents a powerful tool which will inspire researchers and clinicians for many years to come.

Other general textbooks, manuals, monographs and proceedings (such as Langeron 1945, 1952; Nickerson 1947; Lodder and Kreger-van Rij 1952; Littman and Zimmerman 1956; Baker 1971; McGinnis 1980; Howard 1983; Rose and Harrison 1987; etc.) have been published and the reader can also refer to Ainsworth (1986) and Drouhet (1991).

Education in medical mycology

In medicine and health sciences, medical mycological education was carried out with minimal mycological input at the start; but, progressively, postgraduate courses have been developed by well-trained mycologists. The training of mycopathologists and education in medical mycology started in the second half of the nineteenth century in Europe (mainly in France, Great Britain, Italy, Germany, Belgium, the Netherlands), North America (the USA, Canada), Latin America (Argentina, Brazil, Uruguay, Colombia, Venezuela, Mexico) and in the second half of the twentieth century in Asia, Africa, and Australia. This evolution has largely been described and analyzed for all countries by Ainsworth (1986); Drouhet (1991) and more recently for the USA by Espinel-Ingroff (1996). Ajello (1975a) pointed out that the history of medical mycology can well be divided into the periods BC and AC – before Conant and after Conant. He cited the work of Bill Conant and his colleagues at Duke University as a milestone in ending the previous perfunctory treatment of medical mycology which had prevailed before his era.

Another important course on medical mycology was initiated in 1947 by Ajello, Director of the Division of Mycotic Diseases at the Centers for Disease Control in Atlanta, Georgia. This month-long course was directed primarily at staff members of state and city health departments and federal agencies, and later also to international institutions (Ajello et al. 1975). In addition to the 4-week course with Georg, Kaplan, Padhye, Kaufman, Standard, and Ajello, courses in veterinary and medical mycology, mycoserology, exoantigen procedures for pathogenic fungi, and public health mycology were organized.

Other federal mycological laboratories, including the National Institutes of Health (Bethesda, MD), where Emmons (1900–85) was appointed in 1936, contributed to the development of modern medical mycology. Emmons' book, *Medical mycology* (Emmons et al. 1963, 1970, 1977), followed by that of Kwon-Chung and Bennett (1992), contributed significantly to the education of mycopathologists. A great number of other medical mycological courses are now available in the USA, Salkin (1988). Twenty-four universities or colleges in the USA provide graduate research programs in medical mycology leading to a doctoral degree.

In the UK, since the end of the nineteenth century, research in medical mycology was dominated by interest in the dermatophytes (Fox 1863; Adamson 1895). New developments were realized after World War II. Duncan (1884–1958), at the London School of Hygiene and Tropical Medicine, was the first Reader in Medical Mycology at London University. Ainsworth, who was interested in medical and veterinary mycology, was one of the founding members of ISHAM (1953–54). He contributed to the creation of the Medical Mycology Committee of the Medical Research Council (1943–69) and developed, with the late Phyllis Stockdale, the *Review of Medical and Veterinary Mycology* (*RMVM*), a joint publication of the Bureau of Hygiene and Tropical Medicine, the Imperial Bureau of Animal Health at Weybridge, and the Imperial Mycological Institute. Important centers for medical mycology at Leeds, Glasgow, and Bristol were developed. A history of important developments in medical mycology in the UK was outlined by Ainsworth, who was author of numerous books dedicated to fungi and to the history of medical and veterinary medicine (1986), general mycology (1976) and animal mycoses (Ainsworth and Austwick 1973). Consequently, Ainsworth made a substantial contribution to bettering knowledge of this branch of microbiology. At the Commonwealth Mycological Institute, Kew, Stockdale rediscovered the sexual state of *Microsporum gypseum*, a starting point for significant further research in this field. In 1958 at Glasgow University, Gentles discovered the oral activity of griseofulvin on tinea. Stockdale, editor of the *RMVM* for many years, and Gentles, Odds and Evans, editors of *Sabouraudia* (later *JMVM*), contributed a great deal to education in medical mycology. A Mycological Diagnostic Laboratory was set up in 1959 in the Department of Microbiology, Queens University, Belfast.

In France, Sabouraud (1864–1938), trained at the Pasteur Institute under Roux (1891) and at the Saint Louis Hospital, the center of French dermatology at that time, was the father of modern medical mycology even though he did not do much university teaching. His 'Laboratoire des Teignes' became a shrine for dermatologists and mycopathologists from all over the world. The first regular university course on medical mycology was organized, at the Faculty of Medicine in Paris, by

Langeron (1874–1950); it took place in the Department of Parasitology and was directed by Brumpt (1877–1951), who had been interested over many years in the human mycoses (particularly mycetomas and black piedra). Numerous French and other distinguished mycologists such as Talice (Uruguay), Negroni (Argentina), Ota (Japan), and Milochevitch (Yugoslavia), followed his teaching that was devoted, particularly, to yeasts and dermatophytes. In 1887, Pasteur (1822–95), at the end of his long career as father of modern microbiology, founded the Institute that bears his name, independent of university and state institutions. He assigned Roux to organize a course for the study of all microorganisms responsible for infections in humans and animals, which became the well-known annual Great Course of the Pasteur Institute. Each microorganism was treated by a specialized microbiologist. The pathogenic fungi of humans and animals were well covered in this course. Four lectures were given by Magrou (1883–1951), botanist and physician, head of the Department of Mycology and Plant Physiology (created by Pinoy in 1912), and a fifth lecture was given by Sabouraud every year until his death in 1938. Three mycologists of the Pasteur Institute, trained as microbiologists – Segretain, Mariat, and Drouhet – but oriented their research work and teaching principally towards medical mycology after 1946. After the death of Magrou in 1951, an annual postgraduate course in medical mycology was set up in 1953. Twenty-four participants – graduates in medicine, veterinary sciences, pharmacy, and science – particularly interested in teaching and research are enrolled annually: two-thirds from France and one-third from other countries, principally Europe, Latin America, and Africa. The course covers all aspects of fungi and actinomycetes that cause diseases in humans and animals and provides extensive practical laboratory work full time. After theoretical and practical examinations, a diploma is awarded by the Institute Pasteur. This course has trained more than 1 000 medical mycologists. There are now more than 15 university centers in the principal cities of France, in which advanced medical mycological teaching is given (Drouhet 1991).

At the Centraalbureau voor Schimmelcultures, Baarn, in the Netherlands, courses on medical and veterinary mycology were organized by de Vries since 1953; now these are organized by de Hoog and provide for an international group of students. Another important course in tropical mycology was given every year at the Institut de Médecine Tropicale Prince Leopold, Antwerp; it was started by Vanbreuseghem.

HISTORICAL EVOLUTION OF THE DIAGNOSTIC MYCOLOGY LABORATORY

The evolution of laboratory diagnoses from the classic conventional methods to the modern, rapid techniques in general followed a similar course to that in microbiology and other new and developing disciplines (immunology, electron microscopy, molecular biology). Their progress contributed, to a great extent, to the development of medical mycology.

The discovery of the microscope (first mentioned by John Faber around 1625) was followed by its application to the study of fungi in nature (Hooke 1665; van Leeuwenhoek 1673; Malpighi 1679; Micheli 1729; Pasteur 1861) and only later, at the turn of the century, to the study of fungi involved in human and animal infections. Phase contrast microscopy, invented by the Dutch physicist F. Zernicke (recipient of a Nobel Prize in 1932), prepared the way for the introduction in the 1950s of transmission electron microscopy (TEM). This was introduced in medical mycology only in the 1960s (Garrisson 1983). It was followed by the apperance of scanning electron microscopy (SEM); both electron microscopic techniques have had a broad application to fungal studies since the 1970s (Cole 1986). Fluorescent microscopy using nonspecific staining procedures (calcofluor white) has been used since the 1980s; specific immunolabeling, which has developed since 1956 as a result of Coon's labeling of protein with fluorescent substances, has become an important tool for the immunological diagnosis of mycoses. Fluorescent microscopy was successfully applied to medical mycology by Kaplan and Kaufman (1961), Drouhet et al. (1972), and others, and was reviewed by Kaufman and Reiss (1986), Longbottom (1986), and Drouhet (1988). Immunoperoxidase (IPO), using an anti-IgG serum label attached to the peroxidase, rather than to fluorescein isothiocyanate, has proved to be as valuable as the immunofluorescent method.

Microscopic detection of fungi in clinical preparations (KOH/NaOH, lactophenol cotton blue, India ink) is made on smears or histological sections stained first with classic stains used in bacteriology and parasitology, such as Gram's stain (1884), May–Grünwald–Giemsa (Wright's) stain, which are based on methylene blue eosinates (1902), hematoxylin–eosin–saffran (HES) (1911) and mucicarmine. Later, two fungus-specific stains superseded all others, namely:

1. the periodic acid–Schiff (PAS) stain based on the same Schiff reagent as that used in Gram's stain, introduced in 1947 by Hotchkiss–MacMannus with Kligman and Mescon's modification (1950), and
2. Gomori's (1946) and Grocott's (1955) silver methenamine stain (GMS).

These two stains that color the cell wall distinctly red (PAS) or black (GMS), are used universally by histopathologists (Baker 1957, 1971; Chandler et al. 1980).

Culture of fungi evolved after the creation of Raulin's first chemically defined liquid medium under standardized conditions, from cultures on shallow porcelain dishes to glass flasks or tubes sealed or plugged with

cotton wool to prevent contamination (Pasteur 1861; Roberts 1874). The use of solid media, such as gelatin, used in 1852 by Vittadini for culture of *Botrytis (Beauveria) bassiana*, was followed by use of agar poured onto glass dishes – a simple technique described by the German bacteriologist Petri (1887), one of Koch's assistants. The agar Petri dish technique, which allowed isolation and identification by morphogenesis, was adapted for bacteriology and mycology. In his early work, Sabouraud (1896, 1910) grew dermatophytes on a variety of solid media and noted that their colony characteristics and microscopic features differed considerably on the various media. Sabouraud's glucose medium became the most widely used in medical mycology, not only for dermatophytes, but also for the isolation and routine subculture of most fungal pathogens (Odds 1991). Later, it was modified by the addition of thermostable antibacterial antibiotics (chloramphenicol, 1947; gentamicin, 1969) and cycloheximide (Acti-Dione) for the selective isolation and identification of pathogenic fungi (Georg et al. 1954). A new culture medium called the dermatophyte test medium (DTM), based on a modification of Sabouraud's medium by the addition of a pH indicator and cycloheximide which inhibits saprophytic mold, led to a simplified and rapid detection of dermatophytes. Many natural media (potato dextrose, potato–carrot–bile, and malt extract agars) or chemically defined media with essential carbon and nitrogen compounds, vitamins or growth factors such as oleic acid for lipophilic yeasts have been described for particular groups or single species of yeasts and molds (Saint-Germain and Summerbell 1996).

Important improvements allowed for the rapid isolation of fungi from blood (lysis–centrifugation system, non-radiometric CO_2 detection, after the 1980s), rapid yeast identification procedures, germ tubes for *Candida albicans*, assimilation tests using C and N compounds (up to 32 carbon sources) by a scaling down of old techniques (described by Beijering in the 1800s, by Wickerham in 1948, and transformed and developed as commercial kits on microplate systems, including rapid enzyme identification, e.g. phenol oxidase developed by Staib 1962, urease, etc.). All these new procedures have been reviewed (Pincus et al. 1988; Roberts et al. 1992; Salkin et al. 1994).

The immunoidentification of fungal pathogens by exoantigens, soluble immunogenic macromolecules produced by fungi, has been intensively developed and standardized by Kaufman and Standard since 1978. The earliest attempt to identify fungi by their soluble antigens was made by Manyck and Sourek in 1966, when they used a single-diffusion tube precipitin test and inoculated cultures of *B. dermatitidis*, *C. immitis*, *H. capsulatum*, and *P. brasiliensis* on the surface of serum agar; their test required up to 21 days to complete, however. Extensive studies carried out since 1983, by Kaufman et al., using standardized rapid procedures with specific reference antigens, allow the identification of most dimorphic fungi, hyaline and pheoid fungi in 2–5 days.

Since 1990, DNA probes have been developed for the rapid identification of those medically important fungi (*H. capsulatum*, *B. dermatitidis*, *C. immitis*), for which conventional techniques are time-consuming. Probe assay results are available within 2 hours compared with the 72 hours for exoantigen tests (Roberts et al. 1992; Mitchell et al. 1994).

IMMUNODIAGNOSIS OF INVASIVE, DEEP MYCOSES

The need for sensitive, rapid, routine, standardized tests for the immunodiagnosis of these mycoses has become increasingly important, and the detection of circulating fungal antigens and serum antibodies has been emphasized in recent reviews (de Repentigny and Reiss 1984; Longbottom and Austwick 1986; Drouhet 1988, 1993b; Buckley et al. 1992; de Repentigny et al. 1994). A new discipline, molecular immunology in medical mycology, was developed (Reiss 1986).

Fungal antibodies

Over the last decades, important progress has been made in the detection of specific serum antibodies as a result of improved antigen preparation and the techniques of immunodiffusion in gels and immunoelectroprecipitation.

These methods were developed at the Pasteur Institute: in 1946, Oudin's gel precipitation method using simple and double diffusion (DD) in tubes, followed in 1948 by Ouchterlony's modification of his practical DD plate technique carried out in Petri dishes. Important improvements were achieved by Grabar and Williams in 1952 with the introduction of immunoelectrophoresis, which combined gel electrophoresis with immunodiffusion. In 1959, Bussard described counter-immunoelectrophoresis (CIE), a highly sensitive and rapid method based on the migration of antigens towards the anode and antibodies towards the cathode in a gel (agarose) at pH 8.2. The two-dimensional immunoelectrophoresis (2DIE) technique was described by Ressler in 1960 and developed by Laurell in 1965 under the name crossed immunoelectrophoresis. In mycology this method made a significant contribution to the identification and characterization of the complex antigens of the fungi and to the antigenic interrelationships of antigenic preparations of species and genera. In 1973, Axelsen first applied 2DIE to the antigenic mosaic of *C. albicans* which is made up of at least 78 antigens, including several major ones. Numerous variants have been reviewed (Axelsen 1973; Longbottom 1986).

Specific enzyme staining techniques applied to immunodiffusion and immunoelectrophoresis of pathogenic fungi started with the work of Uriel in 1963 at the Pasteur Institute. The French school of Biguet showed that the principal precipitating antigen–antibody complexes have enzymatic activities of diagnostic interest, such as the activities of chymotrypsin and catalase for *A. fumigatus,* of chymotrypsin and malic dehydrogenase for *A. flavus*, of malic dehydrogenase activity for *Candida* spp., and of catalase and glucuronidase activities for *H. capsulatum.*

The first enzyme immunoassay (EIA) techniques were described independently by Avrameas and Guilbert in 1971 at the Pasteur Institute and by Engvall and Perlman who gave the name ELISA to enzyme-linked immunosorbent assay. Subsequently, this test was rapidly and extensively applied to the immunology of pathogenic fungi and their respective mycoses. These tests provide an objective end point; they can be automated and are simple to carry out. In addition, they have a sensitivity and specificity comparable to radioimmunoassay (RIA), which is capable of detecting nanogram amounts of antigens and antibodies. Various adaptations of EIA, particularly the enzyme-linked immunoelectrotransfer blot or Western blotting technique developed by Towbin in 1979 and Burnett in 1981, have been reported and compared with other immunological tests (Mackenzie 1983, 1996; de Camargo et al. 1984; de Repentigny and Reiss 1984; Longbottom 1986; Drouhet 1988; Guesdon et al. 1988; de Repentigny et al. 1994).

CIRCULATING FUNGAL ANTIGENS

Research on fungal circulating antigens received considerable attention, principally because of the evolution of systemic opportunistic mycoses in immunocompromised or AIDS patients.

Since the First International Symposium on Fungal Antigens held in 1986 at the Pasteur Institute in Paris, during which many problems in isolation, purification and detection of principal fungal antigens were analysed, new tests for the detection of circulating antigens have been developed for aspergillosis, candidiasis and cryptococcosis; these tests are rapid, routine and commercially available, compared with other reference tests (Drouhet et al. 1988a; Drouhet 1989, 1993b; Buckley et al. 1992; de Repentigny et al. 1994).

Historically, the first circulating antigen was the polysaccharide galactoxylomannan from *C. neoformans*, which was detected by Neil and colleagues in infected animals (Neil and Kapros 1950) and later in humans (Neil et al. 1951). In 1961, Bloomfield et al. developed a simple rapid reactive test for antigen detection in body fluids by latex particle agglutination (LAT), using 0.8 mm particles coated with a polyclonal rabbit anti-*C. neoformans* IgG. This was an extremely sensitive and reliable test when standard conditions were used. The monoclonal antibodies, first reported in 1975 by Kohler and Mildstein, were successfully applied by Dromer et al. (1987) for *C. neoformans* galactoxylomannan antigen detection. Monoclonal antibodies against the mannan of *C. albicans* and galactomannan of *A. fumigatus*, prepared by Stynen (Diagnostics Pasteur 1989), were successfully used in invasive candidiasis (Georges et al. 1991) and aspergillosis (Dupont et al. 1990), respectively; these have high specificity but less sensitivity than the tests for cryptococcosis.

Numerous circulating antigen tests have been performed not only by LAT, but also by EIA and RIA, using monoclonal and polyclonal antisera from cell wall polysaccharides, heat labile glycoproteins (*C. albicans*), cytoplasmic thermostable glycoprotein and 48 kDa protein (*C. albicans*), 18, 32, 92 kDa proteins (*A. fumigatus*) or yeast or spherule forms of dimorphic fungi (*H. capsulatum*, *C. immitis*). Most of these rapid tests are now commercially available (Drouhet 1993b; Dromer 1993).

SENSITIVITY TESTING OF ANTIFUNGAL AGENTS

Although in bacteriology the necessity of laboratory sensitivity followed soon after the discovery of antibiotics, in 1945–50, by disk sensitivity and minimum inhibitory concentration (MIC) testing under standardized conditions (microdilution techniques), in medical mycology the use of in vitro tests and the assay of drugs in body fluids only started in the 1970s with flucytosine (5-FC); this resulted from the emergence of primary or secondary resistant strains and a need to monitor body fluid levels. The tests have also become available for the azoles since the 1980s. Disk as well as diffusion and microplate automated techniques became easier to carry out and were standardized for the in vitro evaluation of antifungal agents against yeasts and sporulating pathogenic fungi (Drouhet et al. 1986), but not for filamentous nonsporulating fungi or dimorphic fungi (Galgiani et al. 1992). Physicochemical methods were used mainly for assays of drugs in body fluids. The methodology, development, and standardization for these tests have been reviewed (Speller 1980; Drouhet and Dupont 1986; Drouhet et al. 1986; Iwata and Vanden Bossche 1986; Galgiani et al. 1992; Van Cutsem et al. 1994; Vanden Bossche et al. 1994).

FINAL CONCLUSIONS

Since the beginnings of the concerted interest in medical mycology which began at the turn of the last century, with the discovery of the principal agents of human and animal mycoses, there has been constant progress in our knowledge of the pathogenic fungi that cause mycotic diseases.

The opportunistic pathogenic fungi and their resultant mycoses, which have emerged in the last few decades under new environmental and host conditions, have reinforced the place of medical mycology as a key component of microbiology. This became possible as a result of the work of the pioneers of medical mycology: enthusiastic medical mycologists, who founded the ISHAM in Paris in 1954, which, with its affiliates, now has more than 1 000 members in medical mycology throughout the world.

The evolution over the last 50 years in the spectrum of fungal infections, their clinical and pathological features, and the ecology and epidemiology of pathogenic and opportunistic fungi has been accompanied by an increased understanding of the fungal cell. Our knowledge of the physiology, biochemistry, immunology, morphogenesis, ultrastructure, sexuality, genetics, and molecular biology of many pathogenic fungi in humans and animals has advanced enormously, but it still needs further intensive investigation.

The need for education and training in medical mycology, as with all closely related basic and applied scientific disciplines, requires further development, with the objective of resolving the burning issues of fungal disease, such as mycoses in high-risk individuals and immunocompromised patients, immunological defence, the cost-effective management of life-threatening mycoses, and the rapid and early detection of infection.

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Kingdom Fungi: fungal phylogeny and systematics

THOMAS G. MITCHELL

Origin of the Fungi	1	Phylum Chytridiomycota	9
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> Taxonomy is often regarded as the dullest of subjects, fit only for mindless ordering and sometimes denigrated within science as mere 'stamp collecting.' If systems of classification were neutral hat racks for hanging the facts of the world, this disdain might be justified. But classifications both reflect and direct our thinking. The way we order represents the ways we think. Historical changes in classification are the fossilized indicators of conceptual revolutions. (Gould 1996)

ORIGIN OF THE FUNGI

The evolutionary origins of biology began with the prokaryotes, the Archaea, and Eubacteria. The Eukaryota, or eukaryotes, are believed to have evolved from prokaryotes about 1.6–2.1 billion years ago with the emergence of protists (Javaux et al. 2001). The protists include protozoa, certain algae, and fungal-like organisms, the Oomycota. Eukaryotic cells are distinguished by a cytoskeleton and intracellular organelles, which are usually enclosed by membranes. The endomembranes of eukaryotic cells include the nuclear membrane, endoplasmic reticulum, Golgi apparatus, peroxisomes, and the plasma membrane. Most of the DNA is organized into chromosomes and contained with the nucleus, but DNA is also found in the mitochondria (in fungi and animals) as well as in extrachromosomal forms. The cytoskeleton includes major proteins that evolved early and have been conserved throughout the evolution of eukaryotes, such as tubulin (which forms microtubules) and actin (microfilaments).

Two major lineages arose early, one led to the algae and terrestrial plants, and the other gave rise to fungi and animals (Figure 1). The common origins of animals and fungi are supported by phylogenetic analyses of the nucleotide sequences of several conserved genes, such as the small subunit (SSU) 18S and large subunit (LSU) 26S of ribosomal DNA (rDNA), elongation factor, tubulin, and actin (Bruns et al. 1991; Baldauf and Palmer 1993; Wainright et al. 1993), although not by sequences of the RNA polymerase II gene (Sidow and Thomas 1994). Based on phylogenetic studies, molecular clock estimations of the rate of mutation, and limited fossil data, the time when animals and fungi diverged has been estimated at approximately 1.5 billion years ago (Heckman et al. 2001; Hedges et al. 2004).

Most fungi grow vegetatively as yeasts or molds, and fungal cells may have more than one nucleus. Similar to other eukaryotes, fungi typically contain mitochondria, a Golgi apparatus, and endoplasmic reticulum. They also contain vacuoles, but lack chloroplasts and pseudopodia. Most have cell walls composed of chitin and β-glucans, as well as other polysaccharides, proteins, and lipids.

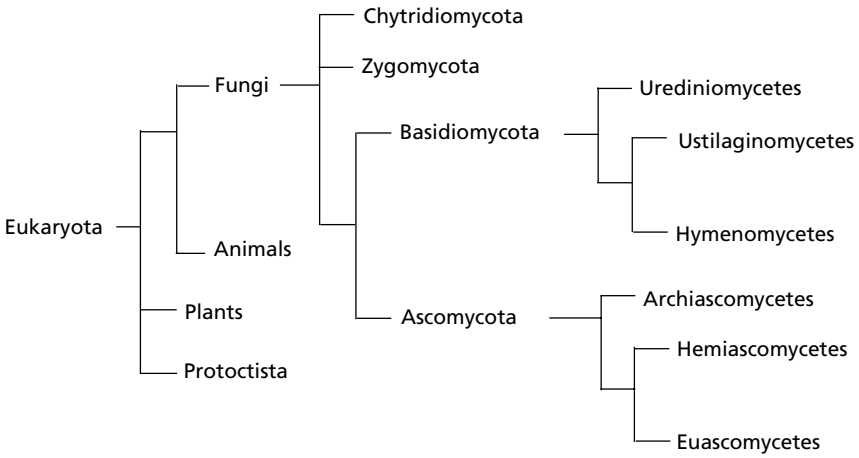

Figure 1 *Major kingdoms of the Eukaryota, the four phyla of the Fungi, and the three classes of the Basidiomycota and the Ascomycota.*

Collectively, fungi exhibit a rich diversity, inhabit every ecological niche on the planet, and manifest a variety of lifestyles. Most fungi are chemotrophic and secrete catabolic enzymes that digest vicinal substrates, which are absorbed, as are minerals, salts, and other small molecules, by passive or active transport mechanisms. Fungi distinctively utilize α-aminoadipic acid in the biosynthesis of lysine. As saprobic fungi proliferate in aquatic and terrestrial locales, they degrade organic substrates for their nutrition, and in the process recycle the organic detritus of plants and animals. Other fungi are exploited by industry for the production of secondary metabolites or pharmacological compounds. Some serve science as model eukaryotes for the study of cellular, molecular, and genetic processes (e.g. *Saccharomyces cerevisiae*, *Neurospora crassa*). Many provide gustatory pleasure, either directly (edible mushrooms, truffles, morels) or indirectly (bread, cheese, alcoholic beverages). Certain groups of fungi are intriguingly specialized, and have the ability to entrap nematodes, form symbiotic relationships (lichens), nourish or protect plants (mycorrhizae and endophytes, respectively), or produce disease in plants or animals. More than 80 000 species of fungi have been described, but the total number of extant fungal species has been estimated to be about 1 500 000 (de Hoog et al. 2000; Kirk et al. 2001). Compared to the enormous kingdom of fungi, the number of infectious, or mycotic, species is minuscule. Nevertheless, the medical fungi exert a profound, global impact on human health. Their numbers continue to increase, but no one knows how many human pathogenic fungi exist. To date, approximately 400 clinically relevant species have been described (de Hoog et al. 2000; Kirk et al. 2001; Howard 2003).

CONCEPTS AND TERMS

Systematics aims to describe and organize biological diversity, usually by determining the phylogenetic relationships among organisms. Systematicists study the relationships of organisms and the processes by which they evolved and are maintained. Classification is the assignment of organisms to defined groups. Systematics entails nomenclature and taxonomy, the science of naming and classifying organisms. Because of their historical association with plants, the fungi are named according to the rules established by the International Code of Botanical Nomenclature. However, reflecting their affinity with animals, the higher fungal groups are called Phyla, not Divisions. Major taxonomic groups of fungi are denoted by the following suffixes: Phylum, -mycota; Subphylum, -mycotina; Class, -mycetes; Subclass, -mycetidae; Order, -ales; Suborder, -ineae; Family, -aceae; and Subfamily, -oideae. The basic taxon is the species. However, the species name is often the antepenultimate rank of classification, just above the variety and individual (Kirk et al. 2001).

Each species is identified by its genus and species name, followed if necessary by a varietal or subspecies designation. A full descriptor of a given species will include the binomial in Italic font followed in non-Italic font by the surname(s) of the author(s) who first described and named the species and the year of its publication, for example, *Cryptococcus neoformans* (Sanfelice) Vuillemin (1901). If a species name is modified, the names of subsequent authors may be added. Whenever possible, the original isolate from which the species was described becomes its permanent reference or the type strain for that species, and it is deposited in an established culture collection (e.g. American Type Culture Collection, Centraalbureau voor Schimmelcultures). If a species is inadvertently described more than once, its name reverts to the original description (with sanctioned exceptions for names with longstanding familiarity).

There are several definitions of a fungal species (Taylor et al. 2000; Burnett 2003). Members of a species are quite similar, if not identical, in their morphology,

mode of reproduction, physiology and temperature range, biochemical structure, ecology, and genomic sequence. Species have been historically demarcated on the basis of morphological and other phenotypic similarities. Biological species are defined by mating compatibility, the abilities to engage in sexual reproduction and successfully interbreed, thus sharing a common gene pool with other members of the species (Burnett 2003). Other criteria have been used to define certain species, including both ecological isolation in nature and the host specificity of pathogenic fungi. Species may also be identified by phylogenetic analyses, using molecular markers as characters to determine evolutionary relationships. Phylogenetic criteria define a species as the smallest recognizable population of individuals or lineage united by shared derived characters (synapomorphy) (Burnett 2003; Vilgalys 2004). Molecular studies have discovered situations where organisms were morphologically indistinguishable, similar in numerous other phenotypes, and occupied the same habitat (sympatric), but they were not members of the same species. One such cryptic species is *Candida dubliniensis*, which was long misidentified as *Candida albicans* (Sullivan and Coleman 1998; Sullivan et al. 2004). Another example is the discovery that the common cause of coccidioidomycosis, *Coccidioides posadasii*, was for decades subsumed by the phenotypically similar species, *Coccidioides immitis* (Fisher et al. 2002).

An important route to speciation occurs when organisms become geographically and reproductively isolated (allopatry). Over time, mutations accrue, selective pressure or genetic drift may impact the organisms, and they become sufficiently divergent that mating with the ancestral population is no longer possible. Advances in fungal genomics are beginning to discover some of the genetic mechanisms that may drive speciation, including mitotic recombination (parasexuality), gene duplications, and chromosomal rearrangements and translocations (Lynch 2002; Wong et al. 2002; Dujon et al. 2004).

The sexually reproductive life cycle of a fungal species is its teleomorph, which indicates that the species has the ability to undergo meiosis and produce meiospores. The anamorph refers to mitotic or asexual reproduction. If a teleomorphic species produces multiple anamorphs, they may be called synanamorphs. Since many fungal species lack a known teleomorph, or were described before their sexual reproductive mode was recognized, their names were based on their anamorphic or asexual reproductive morphology and ontogeny. This method of classification relies on morphology and other phenotypes, and it may or may not reflect evolutionary relationships. Many fungi have both a teleomorphic and one or more anamorphic species names, reflecting their separate description at different times and places. The teleomorph, anamorph, and any synanamorphs of a fungal species comprise its holomorph.

Furthermore, the reproductive structures of many fungal species are indistinguishable. Mating between fungal isolates usually constitutes evidence of species identity. However, isolates with identical anamorphs may not be recognized as different species if they are incapable of sexual reproduction. Consequently, an anamorphic 'species' may represent more than one teleomorphic species, and vice versa. The dermatophytes offer good examples: the geophilic anamorph *Trichophyton terrestre* may represent three different teleomorphs, *Arthroderma quadrifidum*, *Arthroderma insingulare*, or *Arthroderma lenticulare*. Similarly, the teleomorphic species *Arthroderma uncinatum* has two anamorphs, *Trichophyton ajelloi* and *Trichophyton phaseoliforme* (Summerbell and Kane 1997).

The apparent predominance of anamorphy among mycotic fungi may be advantageous for pathogenicity. Asexual fungal species tend to consist of one to several distinct clones or lineages, and sexual reproduction is limited or absent. Evolution occurs by various genomic mechanisms, as noted above, and the gradual accumulation of spontaneous mutations (Taylor et al. 1999b; Taylor et al. 2000; Xu and Mitchell 2002). The environmental growth conditions will subsequently promote the positive selection, survival, and proliferation of clones with favorable mutations. By contrast, sexual reproduction offers the opportunity to more rapidly exchange genetic information. However, in sexually reproducing populations, favorable combinations of mutations that arise in some lineages may be lost through subsequent recombination. Many fungi exhibit a mixture of both clonal reproduction, which can be favored under uniform growth conditions, and sexual recombination, which can be advantageous in changing environments. Perhaps as commensal and pathogenic fungi become more entrenched on the mammalian host, their need for genetic variation is minimized, and anamorphy becomes an important survival mechanism. The dermatophytes exemplify support for this concept. Except for *Trichophyton mentagrophytes* and *Microsporum canis*, the anthropophilic and zoophilic species exist only as anamorphs (e.g. *Trichophyton rubrum*, *Trichophyton tonsurans*, *Trichophyton verrucosum*, *Trichophyton equinum*, *Microsporum ferrugineum*, *Epidermophyton floccosum*), but most of the saprobic, geophilic species have teleomorphic states (e.g. *Microsporum cookei*, *Microsporum gypseum*, *Microsporum persicolor*) (Howard et al. 2003). The evolution to anthropophilism can also be associated with the loss of asexual sporulation. Routine cultures of most geophilic species regularly manifest both macroconidia and microconidia, but many human pathogens tend to produce only, or predominantly, microconidia (*Trichophyton soudanense*, *T. rubrum*, *T. tonsurans*, *Trichophyton yaoundei*), macroconidia (*E. floccosum*), or neither (*Microsporum*

audouinii, *M. ferrugineum*, *Trichophyton concentricum*, *Trichophyton schoenleinii*, *T. verrucosum*, *Trichophyton violaceum*).

Phylogenetics is the taxonomical classification of organisms that reflects their evolutionary relationships. Using molecular or phenotypic characters, a phylogenetic tree depicts the ancestor–descendant relationships among organisms. Morphology – macroscopic, microscopic and ultrastructural – provided the original basis for classifying fungi, as well as other organisms, and proposing phylogenetic relationships. Molecular methods include comparative analyses of the nucleotide sequences of RNA and DNA and the amino acid sequences of proteins (Hillis et al. 1996a). The most comprehensive phylogenies have been constructed from comparisons of the highly conserved nucleotide sequences of ribosomal RNA, especially alignable regions of the small (18S) and large (26S) subunits (SSU and LSU) of rDNA.

A cluster of taxa on a branch of a phylogenetic tree or clade is monophyletic if all organisms in that group are known to have developed from a common ancestor, and all descendants of that ancestor are included in the group. A group is paraphyletic if all the members have a common ancestor but the group does not include all the descendants of the common ancestor. Taxonomic groups that contain organisms but not their common ancestor are called polyphyletic. Phylogenetic classifications aim to group species such that every group is descended from a single common ancestor, and the elimination of groups that are found to be polyphyletic often stimulates major revisions of the classification.

PHENOTYPES AND MOLECULAR MARKERS TO IDENTIFY FUNGI

As summarized in Table 1, traditional methods of classifying fungi utilize phenotypic differences in vegetative and reproductive morphology (e.g. colonial and micromorphology), physiology (e.g. patterns of assimilation and fermentation, temperature, and pH range of growth), the presence of structural macromolecules (e.g. cell wall polysaccharides, isoprene units of coenzyme Q), and sexual mating. Since most medical fungi do not evince a teleomorph, their identification and classification have rested on phenotypic characteristics. Definitive phenotypic features are often difficult to discern or highly variable. Thus, for many pathogens, the species boundaries are loosely defined. For example, any elliptical, fermentative, nonpigmented, asexual yeast producing multilateral buds and pseudohyphae is likely to be placed in the genus *Candida*, which is large, multifarious, and polyphyletic (Meyer et al. 1998; Diezmann et al. 2004).

Molecular markers are definitive and more stable than most phenotypic attributes, and they are helping to resolve these issues. There is continuing progress in the biotechnology of rapid, high-throughput methods to genotype strains and the development of innovative statistical approaches and software to analyze the data. Some of the more common DNA-based methods are listed in Table 2, discussed in Laboratory diagnosis, and detailed in recent reviews (Bruns et al. 1991; Taylor et al. 1999a; Mitchell and Xu 2003; Taylor and Fisher 2003).

Table 1 *General phenotypic approaches to identify and characterize fungi*

Phenotype category	Observations (examples)
Morphology	Teleomorph: Sexual reproduction (zygospore, ascocarp, basidiocarp) Anamorph: Mitospore ontogeny and morphology (sporangia, conidiogenesis) Vegetative yeast or hyphal morphology (septate, dematiaceous, Diazonium Blue B) Colony texture, pigment, size Septal pore type Dimorphism
Structural macromolecules	Cell wall polysaccharides (chitin, glucans, mannans) Coenzyme Q isoprene units Isozymes Antigens
Metabolism	Assimilation of particular compounds as sole nitrogen or carbon sources Fermentation of various carbon compounds Metabolic pathways Vitamin requirements (biotin, thiamin) Production of enzymes (proteases, phospholipases, urease, catalase, etc.) Secondary metabolites (mycotoxins)
Growth parameters	Optimum and tolerable range of temperature, pH, concentration of salts, oxygen requirements, etc.
Susceptibility to inhibitors	Inorganic compounds (chlorine salts) Fungicides, detergents, organic compounds (canavanine, benomyl, etc.) Drugs (antifungal antibiotics, cycloheximide) Killer yeasts
Pathogenicity	Host range Virulence factors (adhesins, secretory enzymes, etc.)

Table 2 *General DNA-based methods in medical mycology*

Method	Application		
	Strain identification, molecular epidemiology, population genetics	Species identification	Phylogenetics and systematics
Electrophoretic karyotype	✓		
RFLP	✓	✓	
Southern hybridization	✓	✓	
RAPD, PCR fingerprint, AFLP	✓	✓	
Microsatellites	✓	✓	
Microarrays	✓	✓	
SNP, MLST, DNA sequencing	✓	✓	✓

Abbreviations: AFLP, amplified fragment length polymorphism; MLST, multilocus sequence typing; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism, detected by high-throughput, automated system. Microarray technology is currently only theoretical.
Other methods: mol% G+C; DNA–DNA hybridization.
References: Bruns et al. 1991; Mitchell and Xu 2003; Taylor and Fisher 2003.

A molecular marker is defined as any identifiable region of the genome. Some markers are better at discriminating individual strains, discrete species or higher taxonomic groups. For certain studies, it is desirable to use markers in specific genes, and for others, markers in noncoding, usually anonymous portions of the genome are preferable because they are assumed to be neutral or unaffected by selective pressure. Molecular markers for fingerprinting medical fungi include allozymes, electrophoretic karyotypes, DNA hybridization probes, PCR-based genotypes, endonuclease restriction fragment length polymorphisms (RFLP), and DNA sequencing (Table 2).

The comparison of nucleotide sequences among species has revolutionized phylogenetic studies. The most reliable DNA markers identify nucleotide sequence polymorphisms, such as single nucleotide polymorphisms (SNP), insertions or deletions (indels), and length variations in tandem oligonucleotide repeats (microsatellites). Such markers can be detected by an array of molecular techniques, such as PCR amplicon length polymorphisms, differences in the lengths of RFLP, single-strand conformational polymorphisms (SSCP), or direct DNA sequencing of homologous genomic regions of the strains or species under comparison [multilocus sequence typing (MLST)]. These methods and their applications to fungal phylogenetics have been described in several reviews (Berbee and Taylor 1999; Taylor et al. 2000; Mitchell and Xu 2003; Taylor and Fisher 2003). Some methods are more suitable for population and phylogenetic studies of closely related taxa and strains of a species, such as isozymes (see below), amplified fragment length polymorphisms (AFLP), randomly amplified polymorphism DNA (RAPD), and hybridization of nucleotide probes to electrophoretic blots of digested genomic DNA (Bakkeren et al. 2000; Soll 2000; Mitchell and Xu 2003).

Protein polymorphisms, such as isozymes, provide excellent markers for population genetics, but they are not widely applicable for large phylogenetic investigations. They usually reflect nucleotide sequence differences resulting in the substitution of one or more amino acids, which alter the chromatographic mobility of the protein to enable detection (Brandt et al. 1993; Murphy et al. 1996; Mitchell and Xu 2003).

In summary, these markers and the attendant analytical software have greatly innovated the methods to:

- resolve taxonomic uncertainties
- investigate the transmission of strains in local outbreaks
- accurately identify fungi in clinical specimens
- recognize strains with clinically important phenotypes (e.g. virulence factors, resistance to antifungal drugs)
- analyze the genetic structure of populations of a pathogen
- trace the evolution of mycotic species
- validate the medical relevance of strains used in basic research, comparative genomics, and the development of new antifungal antibiotics and diagnostic tests.

PHYLOGENETIC METHODS

The sequences of any two or more related taxa can be compared to detect phylogenetic relationships. The underlying assumptions are that with the passage of time, there is an increase in the number of mutations, divergence, and phylogenetic distance between organisms and their genomic sequences. The ‘molecular clock’ hypothesis assumes that molecules evolve in direct proportion to time; consequently, the differences between homologous DNA sequences (or proteins) can be used to estimate the elapsed time since the two DNA sequences (or their taxa) last shared a common ancestor (Hillis et al. 1996b; Hedges and Kumar 2004). However, the observed rate at which nucleotides change is not constant. Some sequences exhibit less variation over

time than expected because their functionality is essential and lethal mutations are lost. This problem is usually obviated by analyzing sequences of noncoding DNA. However, after sufficient time, some nucleotides may undergo further mutation(s) and revert to the ancestral sequence. Most algorithms to construct phylogenetic trees consider such variables and produce a consensus or single 'best' tree with branches of optimal length and likelihood. The common approaches of phylogenetic analysis include Neighbor-Joining (NJ), parsimony, Maximum Likelihood (ML), and Bayesian methods (Xu and Mitchell 2002; Baldauf 2003b; Felsenstein 2003; Holder and Lewis 2003).

The usual phylogenetic methods use algorithms that are based on genetic distances, optimality criteria, such as parsimony or ML, or Bayesian probabilities (Swofford et al. 1996). The first step involves the alignment of nucleotide (or protein) sequences of the taxa being compared. The most popular distance methods are NJ and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA), which estimate phylogenies based on the nucleotide differences among the taxa. Maximum Parsimony (MP) or ML apply optimality criteria to infer phylogenies that minimize the number of evolutionary steps required to explain the data. The selection of one or more methods is governed by the number and diversity of the taxa, as well as the sequences that are compared. Other considerations are the types of assumptions associated with a particular data set (e.g. constant or varying rate) and the required computational power, as algorithms such as ML are more demanding than others.

The products of these methods are phylogenetic trees, which estimate the evolutionary history of a group of sequences. A phylogenetic tree is often rooted with an outgroup of some evolutionary distance from the taxa being analyzed, which anchors the study taxa and provides structure for the tree. Two kinds of diagrams are commonly generated with rooted trees – cladograms and phylograms. A cladogram presents only the nodes and branches. In a phylogram, branch length is proportional to the evolutionary distance, providing a schematic depiction of the number of character state differences (e.g. nucleotide substitutions) between the taxa on that branch. There are several excellent reviews of phylogenetic methods and theory (Hillis et al. 1996a; Swofford et al. 1996; Felsenstein 2003; Holder and Lewis 2003; Hall 2004).

Distance methods approximate a phylogenetic tree by estimating the relative number of nucleotide differences between each pair of taxa in a multiple alignment. This method usually assumes that the rate of mutation for the aligned sequences is neutral, independent, and constant, and that the occurrence of multiple changes of the same nucleotide(s) can be estimated. However, corrections for unequal substitution rates and other variables are commonly introduced. UPGMA is the simplest clustering algorithm, which starts with the two most similar sequences, and then sequentially adds taxa of increasing genetic distance. UPGMA also assumes that all taxa are equally distant from the root (i.e. assumes a molecular clock) and thus yields symmetrical trees. NJ is a similar distance method but does not assume a molecular clock. NJ initially calculates the net divergence of each taxon from all the others, which is the sum of the individual differences from the taxon. Next, the program selects the pair of taxa that are least diverged and calculates their distances from the node that connects them. This node is substituted for the pair, and the process continues, reducing the matrix with each step. One of the accessible programs for constructing phylogenies is PAUP* (Swofford 2002; Hall 2004). UPGMA and NJ programs are the least computationally demanding of the common methods and run very quickly on most computers. However, their speed sacrifices accuracy, as neither method attempts to optimize a tree to determine the best estimate. By contrast, MP, ML, and Bayesian inferences perform iterative rounds of optimization to find the best tree.

An excellent test of the statistical support for each branch of the tree is bootstrapping, which can be appliedto all methods (Felsenstein 1985). There is some debate about the interpretation of bootstrap values in phylogenetic analyses. For parsimony analysis, bootstrap values >70 percent are usually considered acceptable, but for the other methods, values >90 percent are considered significant support for a clade. Bayesian probabilities often achieve >95 percent support for branches.

Unlike distance methods, MP seeks a tree(s) that requires the minimum number of nucleotide (or other characters) changes. Therefore, MP only needs to analyze informative sites, namely, nucleotide changes that occur in two or more sequences. The algorithm then determines which one or more of the possible tree(s) requires the fewest number of changes.

The ML approach is usually applied to a particular tree or evolutionary model and computes the probability of generating that tree from the dataset. ML seeks to infer the tree with highest probability from the observed sequence alignment.

A Bayesian analysis (BA) undertakes a succession of iterative phylogenetic analyses, each of which employs the optimized parameters of the previous analysis, until a best tree or set of trees is obtained. BA resembles ML, but ML searches for the best tree that maximizes the probability of observing the data for that tree, and BA seeks the tree(s) that maximizes the posterior probability of the tree for that data. BA, which is commonly employed with the MrBayes program, using the Metropolis-coupled Markov Chain Monte Carlo sampling method, is more robust and exhaustive and will determine the best set of trees (Huelsenbeck and Ronquist 2001). These programs do their work by analyzing the alignments, sequentially and repetitively comparing tree configurations to identify the tree or trees with the

highest probability (Huelsenbeck et al. 2001; Holder and Lewis 2003; Hall 2004).

In addition to genomic sequences, other molecular characters and phenotypes are helpful in analyzing phylogenies. Certain cell wall polysaccharides are associated with specific fungi, and the chemical or immunological detection of signature motifs can be used to identify fungi within those groups. Similarly, the capacity to produce secondary metabolites and metabolic byproducts (e.g. arabinitol) under the appropriate conditions have sufficient specificity in clinical settings to be useful in diagnostic and prognostic tests, and these properties may be significantly correlated with certain taxonomic lineages. These structural and metabolic compounds include coenzyme Q, which is a mitochondrial electron carrier with a varying number of isoprene units. The length of the isoprene chain is often the same within a monophyletic group (Yamada et al. 1977). As noted below, pathogenicity is a polyphyletic trait, as it evolved, and was lost, multiple times throughout the history of fungi. Many of the attributes required for virulence by certain fungal species are absent in other pathogens, as well as present in nonpathogens. There are many examples of such properties, including dimorphism, secretory enzymes that catabolize host substrates, and cell wall ligands that promote adhesion to host cell receptors. At least one phenotype seems to be universally required if not necessarily sufficient for systemic fungal infection, growth at 37°C.

GENOMICS

The new disciplines of genomics and bioinformatics offer panoptic approaches to integrate molecular and phenotypic data to elucidate fungal classification and evolution. The number of complete genome sequences of mycotic, phytopathogenic, and model fungi is increasing (www.genome.gov/11008243). These databases permit the comparison of not only genes but entire genomes, and there are multiple ways to exploit these resources. Following completion of the genome sequence of *S. cerevisiae*, sequences of 13 related hemiascomycetous yeasts were compared to evaluate the conservation of chromosome maps and the similarity and synteny among genes (Gaillardin et al. 2000; Malpertuy et al. 2000; Souciet et al. 2000). Data from this ongoing multilaboratory project are regularly updated at the Génolevures ('yeast gene') web site: cbi.labri.fr/Genolevures/. A subsequent comparison of the genomic sequences of *Candida glabrata*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *S. cerevisiae*, and *Yarrowia lipolytica*, which manifest different reproductive modes, led to the discovery of many new genes and gene families and specific mechanisms whereby these yeast lineages have evolved (Dujon et al. 2004). Comparative genomics approaches are being used to identify phyla and other taxon- or pathogen-specific genes, as well as test the validity of phylogenetic methods (Hardison 2003).

CLASSIFICATION

It has been difficult to resolve the ancestry of fungi and other eukaryotes, and analyses of various taxa and genes have generated inconsistent phylogenies. Several issues contribute to the problem of parsing these ancient lineages:

- there is a dearth of fossil data
- extant ancestral species have saturated their available mutable nucleotides, even back-mutating to their original sequences
- the results vary depending upon the selected taxa, genes, methods, and outgroups (Baldauf 2003a).

Eventually these problems will be surmounted by increasing the number of taxa, using multigene datasets, and refining the phylogenetic methods.

Lacking a consensus about the classification of the deep branches of the eukaryotic domain, a neutral approach has been to place the diverse ancestral phyla within the Kingdom Protoctista, which contains early eukaryotes that do not qualify as plants, animals, or fungi (Figure 1). Some of the 18–20 protoctistan phyla were previously thought to be fungi, such as the Acrasiomycota (cellular slime molds), Myxomycota (plasmodial slime molds), and Oomycota (water molds). The Protoctista also includes the protozoa and algae. Table 3 lists the higher taxa containing pathogenic fungi, including for comparison a few prominent taxa that do not contain mammalian pathogens. At least two human pathogens are included among these 'pseudofungi,' *Rhinosporidium seeberi* in the Mesomycetozoa (Herr et al. 1999; Mendoza et al. 2002) and *Pythium insidiosum* in the Oomycota (Cooke et al. 2000; Tyler 2001).

The true fungi are contained within the Kingdom Eumycota, which is comprised of four phyla, the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota (Figure 1). Multiple phylogenetic analyses of molecular, morphological and other phenotypic data support the view that the Kingdom Fungi is monophyletic and originated with the Chytridiomycota (Sugiyama 1998). Fungi were classically defined by the limits of mating, and three phyla – Zygomycota, Ascomycota, and Basidiomycota – are named according to the product of sexual reproduction. For asexual fungi, the morphology and ontogeny of their mitosporic reproductive structures became the basis for the artificial form-taxa within the Class Deuteromycetes or Fungi Imperfecti. This alternative approach groups anamorphs according to their morphology, such as yeasts (blastomycetes) and molds (hyphomycetes). However, these morphology-based schemata do not represent phylogenetic relationships and thus do not reflect reliable biological or evolutionary information. Using available SSU rDNA sequences, the phylogram in Figure 2

Table 3 *Higher taxa of mycotic fungi and selected pseudofungi*

Kingdom	Phylum	Class	Order	Mycotic family or species
Protoctista	Acrasiomycota			
	Hyphocytriomycota			
	Labyrinthulomycota			
	Myxomycota	Dictyosteliomycetes		
		Myxomycetes	Physarales	
	Mesomycetozoa	Mesomycetozoea	Dermocystida	*Rhinosporidium seeberi*
			Ichthyophonida	
	Oomycota	Peronosporomycetes	Peronosporales	
			Pythiales	*Pythium insidiosum*
			Saprolegniales	
Eumycota	Chytridiomycota			*Basidiobolus ranarum*
	Zygomycota	Trichomyetes		
		Zygomycetes	Entomophthorales	*Ancylistaceae*
				Basidiobolaceae
			Mortierellales	*Mortierellaceae*
			Mucorales	*Cunninghamellaceae*
				Mucoraceae
				Saksenaeaceae
				Syncephalastraceae
				Thamnidiaceae
	Ascomycota	Archiascomycetes	Neolectomycetes	*Neolecta*
			Pneumocystidales	*Pneumocystidaceae*
			Taphrinales	*Taphrinaceae*
		Schizosaccharomycetes	Schizosaccharomycetales	*Schizosaccharomycetaeae*
		Hemiascomycetes	Saccharomycetales	*Ascoideaceae*
				Candidaceae
				Dipodascaceae
				Endomycetaceae
				Eremotheciaceae
				Lipomycetaceae
				Metschnikowiaceae
				Saccharomycetaceae
		Euascomycetes	Chaetothyriales	*Herpotrichiellaceae*
			Clavicipitales	*Clavicipitaceae*
			Dothideales	*Botryosphaeriaceae*
				Didymosphaeriaceae
				Dothioraceae
				Lophiostomataceae
				Mycosphaerellaceae
				Piedraiaceae
			Eurotiales	*Eremomycetaceae*
				Monascaceae
				Pseudeurotiaceae
				Thermoascaceae
				Trichocomaceae
			Hypocreales	*Hypocreaceae*
			Lectiales	*Leotiaceae*
			Microascales	*Microascaceae*
			Onygenales	*Arthrodermataceae*
				Gymnoascaceae
				Onygenaceae
			Ophiostomatales	*Ophiostomataceae*
			Pezizales	*Ascodesmiaceae*
			Phyllachorales	*Phyllachoraceae*
			Pleosporales	*Leptosphaeriaceae*
				Pleosporaceae
			Polystigmatales	*Polystigmataceae*
			Sordariales	*Chaetomiaceae*
				Coniochaetaceae
				Lasiosphaeriaceae
				Magnaporthaceae
				Myxotrichiaceae
				Sordariaceae

(*Continued over*)

Table 3 *Higher taxa of mycotic fungi and selected pseudofungi (Continued)*

Kingdom	Phylum	Class	Order	Mycotic family or species
	Basidiomycota	Hymenomycetes	Agaricales	*Coprinaceae*
			Stereales	*Bjerkanderaceae*
				Corticiaceae
				Schizophyllaceae
			Tremellales	*Filobasidiaceae*
		Urediniomycetes	Sporidiales	*Sporidiobolaceae*
		Ustilaginomycetes	Malasseziales	
			Microstromatales	
			Tilletiales	
			Trichosporonales	
			Ustilaginales	

References: de Hoog et al. 2000; Mendoza et al. 2002.

illustrates the relationships among many of the medical fungi.

As noted above, traditional classifications of the fungi relied on morphological characters and sexual reproduction (Guarro et al. 1999; de Hoog et al. 2000). These approaches continue to serve well, especially for the identification of taxa. Of course, clinical mycology has more pragmatic goals: to obtain a rapid and accurate identification of a fungal isolate from a clinical specimen. Most human pathogenic fungi are anamorphic, and with rare exceptions (the homothallic ascomycete, *Piedraia hortai*), they are asexual when causing an infection. Therefore, traditional and commercial methods have combined morphological and physiological phenotypic characters to develop keys for the timely identification of fungal isolates from clinical specimens. Expedient shortcuts utilizing physiological tests are especially useful for the rapid identification of yeasts (Freydiere et al. 2001).

Beyond the bedside, clinical mycology benefits from an authentic phylogeny of pathogenic fungi. Knowledge of the lineages and nearest relatives of the mycotic fungi is useful because closely related species share similar genes, macromolecules, and biochemical processes. This information is invaluable in selecting comparative taxa to investigate the specificity of new diagnostic tests and antifungal antibiotics, as well as directing rational studies of pathogenesis, virulence factors, and mechanisms of drug resistance.

Modern systematics uses molecular data to assign asexual species to their likely positions within the taxonomic framework based on their most closely related taxa, as determined by molecular and phenotypic similarities. The ultimate goal – to create a unified taxonomy that reflects the phylogenetic evolution of the fungi – has been facilitated by advances in biotechnology, sequencing, and computer programs to analyze the data. As molecular evolution is the foundation of biology, the aim is to assign asexual species to their proper phylogenetic place in the classification, and it is feasible with sufficient molecular data and analyses. However, molecular data are lacking for many pathogenic anamorphic fungi (and countless saprobic anamorphs). Such species are usually classified with phenotypically similar fungi (Seifert and Gams 2001).

The classification of fungi will continue to be a work in progress for many years. New species are constantly being discovered. The phenotypic and genotypic characterization of each new species allows it to be placed on the mycological tree, which will often lead to refinement and adjustment of various branches of the phylogeny. This ongoing process will further elucidate the relationships among fungi and their evolution. Species, families, and orders will continue to be created, conflated, and reorganized. Thus revision is constant and inexorable, understandably but unfortunately causing some confusion, as familiar fungal names and taxonomic associations are periodically supplanted. This perpetual change is often accompanied by controversy and reasoned disagreement based on conflicting data or differing interpretations of the data.

The protocols for naming new species and higher taxa are well established, and mycologists follow the rules of Botanical Nomenclature. However, there is not a recognized mycological governing body to arbitrate controversies and decide controversial issues. Lacking an official consensus, the classification presented here is an amalgam of several respected sources, including recent publications (de Hoog et al. 2000; Kirk et al. 2001; Howard 2003) and useful web sites, such as Myconet (www.umu.se/myconet/Myconet.html), the Fungal Tree of Life (ocid.nacse.org/research/aftol/), the Index Fungorum (www.indexfungorum.org/Names/Names.asp), Deep Hypha (ocid.nacse.org/research/deephyphae/index.php?id=projects), and the Tree of Life Web Project (tolweb.org/tree?group=Fungi&contgroup=Eukaryotes).

PHYLUM CHYTRIDIOMYCOTA

Members of this phylum are organized into a single class with five orders. The Chytridiomycetes are characterized by motile asexual zoospores, and more than 900 species have been described (de Hoog et al. 2000; Kirk et al. 2001). The Orders Neocallimasticales and Blastocladiales

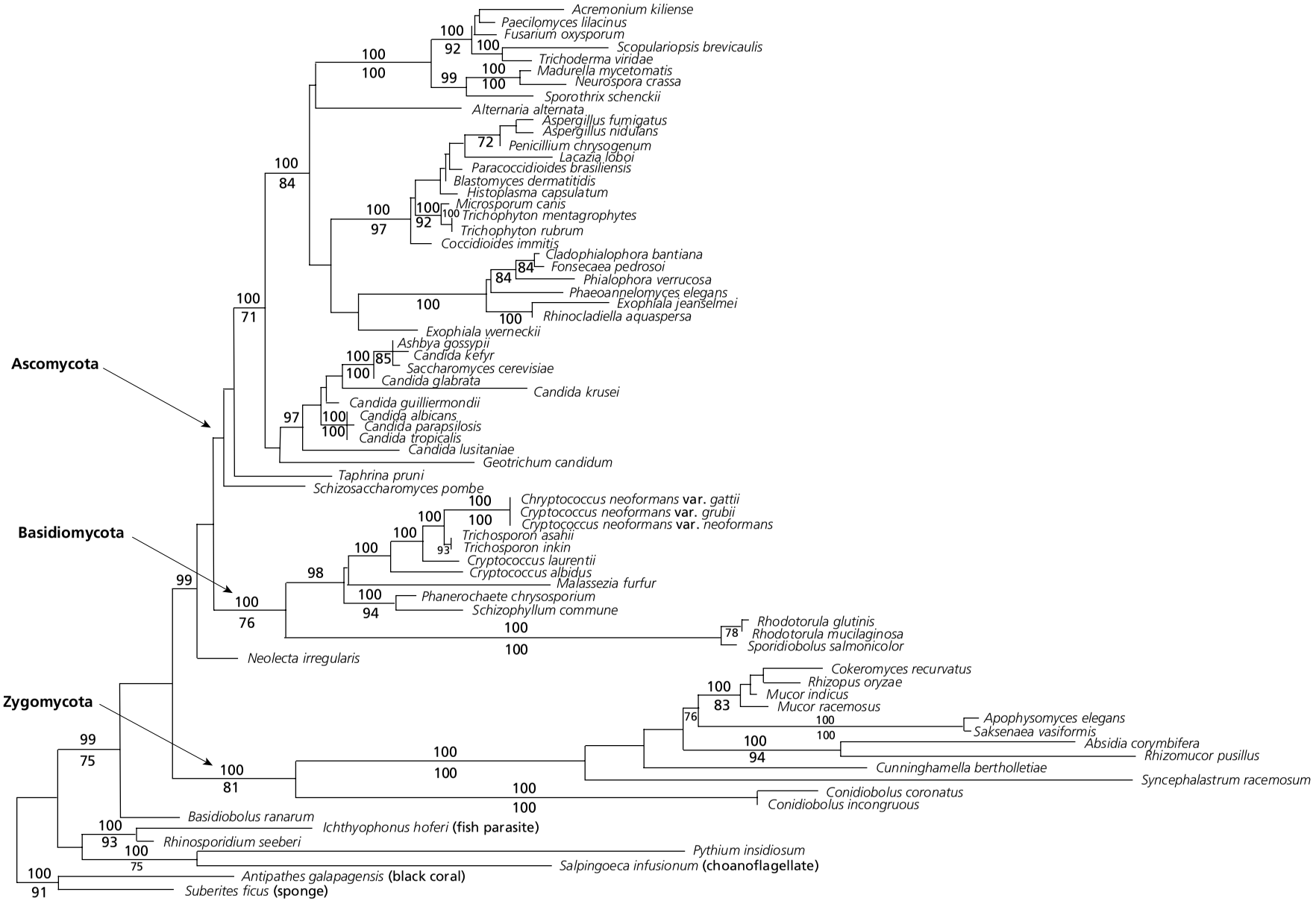

Figure 2 *Phylogram of representative mycotic fungi. Single most likely tree based on a ML analysis of the nuclear SSU rDNA sequences. Nodes with statistically significant support by bootstrap (⩾70 percent) or Bayesian (⩾95 percent) analysis are indicated by the values below or above the nodes, respectively.*

contain intestinal anaerobes and invertebrate pathogens, respectively. The Order Chytridiales has pathogens of nematodes, rotifers, and amphibians. Phylogenetic evidence suggests that both the Chytridiomycota and the Zygomycota are polyphyletic (Sugiyama 1998).

Basidiobolus ranarum may be the sole human pathogen among the chytridiomycetes (Table 3). *B. ranarum* is now recognized as the only pathogenic species of *Basidiobolus*, as previously described mycotic species (viz. *Basidiobolus haptosporus* and *Basidiobolus meristosporus*) are considered synonymous (Ribes et al. 2000). Based on several SSU rDNA phylogenies, *B. ranarum* has been relocated from the Zygomycota to the Chytridiomycota (Nagahama et al. 1995; Jensen et al. 1998; James et al. 2000). As James et al. observed, *Basidiobolus* spp. are the only known nonzoosporic fungi that possess a nucleus-associated organelle (NAO) containing microtubules, although the number and arrangement of the microtubules differ from those of the centriolar microtubules of the chytrids (McKerracher and Heath 1985; James et al. 2000). However, phylogenetic analyses of the α-tubulin and β-tubulin genes provided strong support for classifying *B. ranarum* within the Zygomycetes (Keeling 2003). These discrepant results may be explained by the apparently faster rate of evolution of the SSU rDNA of the chytrids and zygomycetes compared with the tubulin genes (James et al. 2000; Keeling 2003). Another phylogeny of these basal fungi analyzed sequences of the largest subunit of the gene encoding the DNA-dependent RNA polymerase II (*RPB1*), and these results indicated that *B. ranarum* is distantly related to the zygomycetes (Tanabe et al. 2004).

PHYLUM ZYGOMYCOTA

The Phylum Zygomycota has two classes, the Zygomycetes and the Trichomycetes, and together they contain about 1 100 described species (de Hoog et al. 2000; Kirket al. 2001). The Trichomycetes are obligate parasites of arthropods, but two orders of the Class Zygomycetes, Entomophthorales and Mucorales, have important mammalian pathogens. The vegetative hyphae of zygomycetous molds are coenocytic or sparsely septated. The zygomycetes are defined by the mode of sexual reproduction, which is initiated by hyphal anastomosis between compatible mating types of the same species. Mating compatibility is determined by opposite alleles at the mating type locus. Following fusion of the haploid nuclei, meiosis, and one mitotic division, one haploid nucleus usually survives to develop into a zygospore. When exposed to the appropriate growth conditions, the zygospore of Mucorales species will produce a sporangiophore and a sporangium, yielding asexual sporangiospores that germinate to form vegetative haploid hyphae. Most members of the Order Entomophthorales reproduce asexually with forcibly discharged conidia.

The zygomycetous pathogens are listed in Table 4. Within the Entomophthorales, the two classically recognized pathogenic genera, *Basidiobolus* and *Conidiobolus*, cause primary subcutaneous infections and are responsible for subcutaneous phycomycosis and rhinoentomophthoromycosis, respectively (see Subcutaneous zygomycoses). However, as noted above, molecular phylogenies have indicated that *B. ranarum* may be more closely related to nonflagellate chytridiomycetes (Nagahama et al. 1995; Jensen et al. 1998; James et al. 2000).

Most of the pathogens in this phylum are members of the Order Mucorales and agents of the more invasive infection, mucormycosis, whose name refers to the order and not the genus, *Mucor*, which is a rare cause of disease. However, as elaborated in Systemic zygomycosis, most clinical mycologists favor the term 'zygomycosis,' referring to an infection caused by any member of the Class Zygomycetes. The most common agent of invasive zygomycosis in humans is *Rhizopus arrhizus* (*Rhizopus oryzae*) (Ribes et al. 2000).

PHYLUM ASCOMYCOTA

The Ascomycota is the largest fungal phylum, and it embraces 90 percent of the nearly 400 pathogenic fungi (Table 5). Their evolution has been estimated to date from 900 to 500 million years ago (Berbee and Taylor 1993; Taylor et al. 1999; Hedges et al. 2004). Approximately 33 000 ascomycetous species have been described, and they are unified by sexual reproduction that culminates in the production of asci and ascospores (de Hoog et al. 2000; Kirk et al. 2001). In addition, most of the ≈16 000 anamorphic species of fungi, which were first identified according to their conidial mitospores, have been placed within the existing ascomycetous classification based on their molecular and phenotypic affinities. The availability of nucleotide sequences has enabled asexual fungi to be placed with confidence among their closest relatives. The Ascomycota is also home to most of the lichenized fungi.

During vegetative growth, ascomycetous fungi produce either budding or fission yeast cells or branching substrate (vegetative) and aerial (asexually reproductive) hyphae. Hyphal septa possess simple pores that permit cytoplasmic continuity between cells. Some species, including many important human pathogens, are dimorphic, and the morphogenetic conversion to hyphae or yeasts (or spherules) is triggered by environmental signals such as temperature, available nutrients, or carbon dioxide concentration. The Phylum Ascomycota has three classes, the Archiascomycetes, the Hemiascomycetes (yeasts), and the large Euascomycetes (molds). According to SSU rDNA sequence analyses, the Archiascomycetes contains taxa that arose before the latter two classes, which are monophyletic lineages (Bruns et al. 1992; Berbee and Taylor 1993; Nishida and

Sugiyama 1994). Members of the Archiascomycetes are highly diverse, encompassing mycelial species, fission yeasts, and unicellular forms. The genera include many nonpathogens, such as *Protomyces* and *Saitoella*, as well as *Taphrina*, *Schizosaccharomyces*, and *Pneumocystis* (Table 5). Evidence from molecular and biochemical studies indicates that some of these taxa are monophyletic, but the status of others is questionable (Sjamsuridzal et al. 1997). Figure 3 depicts an rDNA phylogeny of representative ascomycetes.

Class Hemiascomycetes

The Hemiascomycetes (yeasts) comprise one large order, the Saccharomycetales, which was established in 1960 by V.I. Kudrjavzev (Kurtzman and Fell 1998; Kirk et al. 2001). The monumental compendium of yeasts, edited by Kurtzman and Fell, lists 42 teleomorphic and 15 anamorphic genera of ascomycetous yeasts (including several genera of Archiascomycetes). The other indispensable resource, written by Barnett et al. (2000), describes 678 species of ascomycetous and basidiomycetous yeasts. Members of the Saccharomycetes are characterized by:

- absent or only rudimentary hyphae
- vegetative cells that reproduce by budding or fission
- cell walls that lack chitin
- asci that are formed singly or in chains (Kurtzman and Fell 1998).

Phylogenetic analyses of SSU rDNA and RNA polymerase II (*RPB2)* gene sequences support a single evolutionary origin of the Saccharomycetales (Berbee and Taylor 1992; Liu et al. 1999). Perhaps the largest phylogenetic analysis of the Saccharomycetes entailed more than 500 species and a comparison of LSU rDNA sequences (Kurtzman and Robnett 1998).

The Saccharomycetales contains many yeasts of medical importance, such as species of *Candida*, which are the most prevalent pathogenic fungi (Table 5; Management of superficial infections; 15, Superficial candidiasis; and 30, Candidiasis) (Calderone 2002). As mentioned earlier, *Candida* is a large and broadly defined anamorphic genus with 163 species (Meyer et al. 1998). They include *C. albicans*, *C. glabrata*, *Candida parapsilosis*, and *Candida tropicalis*. In a recent analysis of the medical yeasts and related hemiascomycetes, DNA sequences of six nuclear genes were analyzed using ML and Bayesian phylogenetic methods to produce the phylogram in Figure 4, which depicts the relationships of the most common mycotic yeasts with rare opportunists and strict saprobes (Diezmann et al. 2004). During the translation of mRNA to polypeptides, several species of *Candida* exhibit alternative codon usage; the codon CUG is translated as serine instead of leucine, as in most other

Table 4 *Mycotic taxa of Zygomycota*

Class	Order	Family	Species
Zygomycetes	Entomophthorales	*Ancylistaceae*	*Conidiobolus coronatus*
			Conidiobolus incongruus
			Conidiobolus lamprauges
		Basidiobolaceae	*Basidiobolus*
	Mortierellales	*Mortierellaceae*	*Mortierella polycephala*
			Mortierella wolfii
	Mucorales	*Cunninghamellaceae*	*Cunninghamella bertholletiae*
		Mucoraceae	*Absidia coerulea*
			Absidia corymbifera
			Apophysomyces elegans
			Chlamydoabsidia padenii
			Mucor amphibiorum
			Mucor circinelloides
			Mucor hiemalis
			Mucor indicus
			Mucor racemosus
			Mucor ramosissimus
			Rhizomucor miehei
			Rhizomucor pusillus
			Rhizomucor variabilis
			Rhizopus arrhizus (*oryzae*)
			Rhizopus azygosporus
			Rhizopus microsporus
			Rhizopus schipperae
			Rhizopus stolonifer
		Saksenaeaceae	*Saksenaea vasiformis*
		Syncephalastraceae	*Syncephalastrum racemosum*
		Thamnidiaceae	*Cokeromyces recuvatus*

References: Kurtzman and Fell 1998; Guarro et al. 1999; de Hoog et al. 2000.

Table 5 *Mycotic taxa of the Phylum Ascomycota*

Class	Order	Family	Teleomorph	Anamorph
Archiascomycetes	Pneumocystidales	*Pneumocystidaceae*	*Pneumocystis jiroveci*	
	Schizosaccharomycetales	*Schizosaccharomycetaeae*	*Schizosaccharomyces pombe*[a]	
	Taphrinales	*Taphrinaceae*	*Taphrina*[a]	
Hemiascomycetes	Saccharomycetales	*Ascoideaceae*	*Yarrowia lipolytica*	*Candida lipolytica*
		Candidaceae		*Arxula*
				Blastobotrys
				Candida albicans
				Candida castelli
				Candida glabrata
				Candida haemulonii
				Candida inconspicua
				Candida intermedia
				Candida maltosa
				Candida norvegica
				Candida parapsilosis
				Candida rugosa
				Candida tropicalis
				Candida viswanathii
				Candida zeylanoides
		Dipodascaceae		*Geotrichum*
			Galactomyces geotrichum	*Geotrichum candidum*
			Dipodascus (Blastoschizomyces) capitatus	*Geotrichum capitatum*
				Geotrichum clavatum
		Endomycetaceae	*Stephanoascus ciferrii*	*Candida ciferrii*
			Debaryomyces hansenii	*Candida famata*
			Pichia guilliermondii	*Candida guilliermondii*
			Pichia fermentans	*Candida lambica*
			Pichia norvegensis	*Candida norvegensis*
			Pichia (Hansenula) anomala	*Candida pelliculosa*
			Pichia jadinii	*Candida utilis*
		Eremotheciaceae	*Eremothecium gossypii*	*Ashbya gossypii*[a]
		Lipomycetaceae	*Kluyveromyces marxianus*	*Candida kefyr*
		Metschnikowiaceae	*Clavispora lusitaniae*	*Candida lusitaniae*
			Clavispora opuntiae[a]	
			Metschnikowia pulcherrima	*Candida pulcherrima*
		Saccharomycetaceae	*Dekkera*	*Brettanomyces*
			Debaryomyces	
			Zygoascus hellenicus	*Candida hellenica*
			Issatchenkia orientalis	*Candida krusei*
			Arxiozyma telluris	*Candida pintolopesii*
			Saccharomyces cerevisiae	
Euascomycetes	Chaetothyriales	*Herpotrichiellaceae*		*Anthopsis deltoidea*
				Cladophialophora arxii
				Cladophialophora bantiana
				Cladophialophora boppii
				Cladophialophora carrionii
				Cladophialophora devriesii
				Cladophialophora modesta
				Exophiala bergeri
				Exophiala castellanii (*Exophiala mansonii*)
				Wangiella dermatitidis (*Exophiala dermatitidis*)
				Exophiala jeanselmei
				Exophiala lecanii-corni
				Exophiala moniliae
				Exophiala pisciphila
				Exophiala salmonis
				Exophiala spinifera
				Fonsecaea compacta
				Fonsecaea pedrosoi
				Phaeoannellomyces (Exophiala) elegans
			Capronia semiimmersa	*Phialophora americana*

(*Continued over*)

Table 5 *Mycotic taxa of the Phylum Ascomycota (Continued)*

Class	Order	Family	Teleomorph	Anamorph
				Phialophora bubakii
				Phialophora europaea
				Phialophora reptans
				Phialophora repens
				Phialophora richardsiae
				Phialophora verrucosa
				Ramichloridium obovoideum (*R. mackenziei*)
				Ramichloridium schulzeri
				Rhinocladiella aquaspersa
				Rhinocladiella atrovirens
				Sarcinomyces phaeomuriformis
				Veronaea botryosa
				Xylohypha (Cladophialophora) emmonsii
	Clavicipitales			*Beauveria bassiana*
				Engyodontium album
				Paecilomyces fumosoroseus
				Paecilomyces javanicus
	Dothideales	*Botryosphaeriaceae*	*Botryosphaeria rhodina*	*Lasiodiplodia theobromae*
			Botryosphaeria subglobosa	*Sphaeropsis subglobosa*
		Didymosphaeriaceae	*Neotestudina rosatii*	
		Dothioraceae	*Discosphaerina fulvida*	*Aureobasidium pullulans*
				Cyphellophora laciniata
				Cyphellophora pluriseptata
			Sydowia polyspora	*Hormonema dematioides*
				Hortaea (Phaeoannellomyces) werneckii
				Scytalidium dimidiatum (*Nattrassia mangiferae*)
				Scytalidium hyalinum
				Pseudomicrodochium suttonii
		Lophiostomataceae		*Madurella grisea*
				Madurella mycetomatis
				Pseudochaetosphaeronema larense
				Pyrenochaeta mackinnonii
				Pyrenochaeta romeroi
				Pyrenochaeta unguis-hominis
				Tetraploa aristata
		Mycosphaerellaceae		*Cladosporium cladosporioides*
				Cladosporium elatum
			Mycosphaerella tassiana	*Cladosporium herbarum*
				Cladosporium oxysporum
				Cladosporium sphaerospermum
		Mytilinidiaceae		*Taeniolella exilis*
				Taeniolella stilbaspora
		Piedraiaceae	*Piedraia hortai*	
	Eurotiales	*Eremomycetaceae*	*Eremomyces langeronii*	*Arthrographis kalrae*
		Monascaceae	*Monascus ruber*	*Basipetospora rubra*
		Pseudeurotiaceae	*Pseudeurotium ovale*	*Sporothrix*
		Thermoascaceae	*Byssochlamys*	
			Thermoascus crustaceus	*Paecilomyces crustaceus*
		Trichocomaceae	*Petromyces alliaceus*	*Aspergillus alliaceus*
				Aspergillus avenaceus
				Aspergillus caesiellus
				Aspergillus candidus
				Aspergillus carneus
			Eurotium chevalieri	*Aspergillus chevalieri*
				Aspergillus clavato-nanicus
				Aspergillus clavatus
				Aspergillus conicus
				Aspergillus deflectus
			Neosartorya fischeri	*Aspergillus fischerianus*
			Fennellia flavipes	*Aspergillus flavipes*
				Aspergillus flavus

(*Continued over*)

Table 5 *Mycotic taxa of the Phylum Ascomycota (Continued)*

Class	Order	Family	Teleomorph	Anamorph
				Aspergillus fumigatus
				Aspergillus granulosus
			Eurotium herbariorum	*Aspergillus glaucus*
				Aspergillus janus
				Aspergillus japonicus
			Emericella nidulans	*Aspergillus nidulans*
			Emericella echinulata	*Aspergillus nidulans*
				Aspergillus niger
			Fennellia nivea	*Aspergillus niveus*
				Aspergillus ochraceus
				Aspergillus oryzae
			Eurotium repens	*Aspergillus reptans*
				Aspergillus restrictus
				Aspergillus sclerotiorum
				Aspergillus sydowii
				Aspergillus tamarii
				Aspergillus terreus
			Emericella quadrilineata	*Aspergillus tetrazonus*
			Neosartorya pseudofischeri	*Aspergillus thermomutatus*
			Neosartorya spinosa	*Aspergillus spinosus*
			Emericella unguis	*Aspergillus unguis*
				Aspergillus ustus
				Aspergillus varians
				Aspergillus versicolor
			Eurotium amstelodami	*Aspergillus vitis* (*Aspergillus hollandicus*)
				Paecilomyces lilacinus
				Paecilomyces marquandii
				Paecilomyces puntonii
				Paecilomyces variotii
				Paecilomyces viridis
				Penicillium aurantiogriseum
				Penicillium brevicompactum
				Penicillium chrysogenum
				Penicillium citrinum
				Penicillium commune
				Penicillium decumbens
				Penicillium expansum
				Penicillium griseofulvum
				Penicillium marneffei
				Penicillium piceum
				Penicillium purpurogenum
				Penicillium rugulosum
				Penicillium spinulosum
				Penicillium verruculosum
				Polypaecilum insolutum
	Hypocreales	*Hypocreaceae*	*Neocosmospora vasinfecta*	*Acremonium*
				Acremonium atrogriseum
				Acremonium blochii
				Acremonium curvulum
				Acremonium falciforme
				Acremonium hyalinulum
				Acremonium kiliense
				Acremonium potronii
				Acremonium recifei
				Acremonium roseogriseum
				Acremonium strictum
				Acremonium spinosum
				Cylindrocarpon cyanescens
			Nectria radicicola	*Cylindrocarpon destructans*
				Cylindrocarpon lichenicola
				Fusarium antophilum
			Cosmospora episphaerica	*Fusarium aquaeductuum*
				Fusarium chlamydosporum
				Fusarium dimerum
				Fusarium incarnatum

(*Continued over*)

Table 5 *Mycotic taxa of the Phylum Ascomycota (Continued)*

Class	Order	Family	Teleomorph	Anamorph
				Fusarium napiforme
			Gibberella nygamai	*Fusarium nygamai*
				Fusarium oxysporum
			Gibberella fujikuroi	*Fusarium proliferatum*
			Gibberella fujikuroi var. subglutinans	*Fusarium subglutinans*
			Nectria haematococca var. breviconia	*Fusarium solani*
			Gibberella moniliformis	*Fusarium verticillioides*
				Metarhizium anisopliae
				Trichoderma harzianum
			Hypocrea koningii	*Trichoderma konigii*
				Trichoderma longibrachiatum
			Hypocrea pseudokoningii	*Trichoderma pseudokoningii*
			Hypocrea rufa	*Trichoderma viride*
	Leotiales	*Leotiaceae*		*Ochroconis constricta*
				Ochroconis gallopavum
				Ochroconis humicola
				Ochroconis tshawytschae
				Pleurophoma cava
				Pleurophomopsis lignicola
				Pleurophoma pleurospora
	Microascales	*Microascaceae*	*Petriella setifera*	*Graphium*
			Pseudallescheria boydii	*Graphium eumorphum*
				Graphium putredinis
			Petriella setifera	*Scedosporium*
			Pseudallescheria boydii	*Scedosporium apiospermum*
				Scedosporium prolificans (S. inflatum)
			Microascus cinereus	*Scopulariopsis cinereus*
			Microascus cirrosus	*Scopulariopsis paisii*
			Microascus manginii	*Scopulariopsis candida*
				Scopulariopsis acremonium
				Scopulariopsis asperula
			Microascus brevicaulis	*Scopulariopsis brevicaulis (S. koningii)*
				Scopulariopsis brumptii
				Scopulariopsis candida
				Scopulariopsis flava
				Scopulariopsis fusca
	Onygenales	*Arthrodermataceae*		*Epidermophyton floccosum*
				Keratinomyces ceretanicus
			Arthroderma corniculatum	*Microsporum*
			Arthroderma borellii	*Microsporum amazonicum*[a]
				Microsporum audouinii
			Arthroderma otae	*Microsporum canis*
			Arthroderma cajetani	*Microsporum cookei*
				Microsporum equinum
				Microsporum ferrugineum
			Arthroderma fulvum	*Microsporum fulvum*
			Arthroderma grubyi	*Microsporum gallinae*
			Arthroderma gypseum	*Microsporum gypseum*
			Arthroderma incurvatum	*Microsporum gypseum*
			Arthroderma obtusum	*Microsporum nanum*
			Arthroderma persicolor	*Microsporum persicolor*
				Microsporum praecox
			Arthroderma racemosum	*Microsporum racemosum*
			Arthroderma grubyi	*Microsporum vanbreuseghemii*
			Arthroderma uncinatum	*Trichophyton ajelloi*
				Trichophyton concentricum
				Trichophyton duboisii
				Trichophyton equinum
			Arthroderma gloriae	*Trichophyton gloriae*[a]
				Trichophyton gourvilii
			Arthroderma vanbreuseghemii	*Trichophyton mentagrophytes*

(*Continued over*)

Table 5 *Mycotic taxa of the Phylum Ascomycota (Continued)*

Class	Order	Family	Teleomorph	Anamorph
				var. *interdigitale*
				Trichophyton kanei
				Trichophyton krajdenii
				Trichophyton megninii
			Arthroderma benhamiae	*Trichophyton mentagrophytes*
				Trichophyton phaseoliforme[a]
				Trichophyton raubitschekii
				Trichophyton rubrum
				Trichophyton schoenleinii
			Arthroderma simii	*Trichophyton simii*
				Trichophyton soudanense
			Arthroderma insingulare	*Trichophyton terrestre*[a]
			Arthroderma lenticulare	*Trichophyton terrestre*[a]
			Arthroderma quadrifidum	*Trichophyton terrestre*[a]
				Trichophyton thuringiense
				Trichophyton tonsurans
			Arthroderma gertleri	*Trichophyton vanbreuseghemii*
				Trichophyton verrucosum
				Trichophyton violaceum
				Trichophyton yaoundei
		Gymnoascaceae	*Gymnoascella dankaliensis*	
			Gymnoascella hyalinospora	
			Narasimhella hyalinospora	
				Malbranchea gypsea
				Malbranchea pulchella
			Arachnomyces nodosetosus	*Onychocola canadensis*
		Onygenaceae	*Ajellomyces dermatitidis*	*Blastomyces dermatitidis*
			Aphanoascus fulvescens	*Chrysosporium*
			Arthroderma tuberculatum	*Chrysosporium*
			Nannizziopsis vriesii	*Chrysosporium*
				Chrysosporium inops
			Aphanoascus keratinophilus	*Chrysosporium keratinophilum*[a]
				Chrysosporium pannicola
			Uncinocarpus queenslandicus	*Chrysosporium queenslandicum*
				Chrysosporium tropicum
			Uncinocarpus orissi	*Chrysosporium zonatum*
				Coccidioides immitis
				Coccidioides posadasii
			Ajellomyces crescens	*Emmonsia crescens*
				Emmonsia parva
				Emmonsia pasteuriana
			Ajellomyces capsulatus	*Histoplasma capsulatum*
			Ajellomyces capsulatus	*Histoplasma capsulatum* var. *duboisii*
				Histoplasma capsulatum var. *farciminosum*
				Lacazia loboi
			Uncinocarpus reesii	*Malbranchea*[a]
			Neoarachnotheca keratinophila	*Myriodontium keratinophilum*
				Paracoccidioides brasiliensis
	Ophiostomatales	*Ophiostomataceae*	*Ophiostoma stenoceras*	*Sporothrix*
				Sporothrix schenckii
	Pezizales	*Ascodesmiaceae*		*Cephaliophora irregularis*
	Phyllachorales	*Phyllachoraceae*	*Plectosphaerella cucumerina*	*Plectosporium tabacinum*
	Pleosporales	*Leptosphaeriaceae*	*Leptosphaeria coniothyrium*	*Coniothyrium fuckelii*
			Leptosphaeria senegalensis	
			Leptosphaeria thompkinsii	
				Microsphaeropsis olivacea
		Pleosporaceae		*Alternaria alternata*
				Alternaria chartarum
				Alternaria chlamydospora
				Alternaria dianthicola
			Lewia infectoria	*Alternaria infectoria*
				Alternaria longipes
				Alternaria tenuissima

(*Continued over*)

Table 5 *Mycotic taxa of the Phylum Ascomycota (Continued)*

Class	Order	Family	Teleomorph	Anamorph
			Cochliobolus australiensis	*Bipolaris australiensis*
			Cochliobolus hawaiiensis	*Bipolaris hawaiiensis*
				Bipolaris papendorfii
			Cochliobolus spiciferus	*Bipolaris spicifera*
				Botryomyces caespitosus
				Corynespora cassiicola
				Curvularia brachyspora
				Curvularia clavata
			Cochliobolus geniculatus	*Curvularia geniculata*
			Cochliobolus lunata	*Curvularia lunata*
			Cochliobolus pallescens	*Curvularia pallescens*
				Curvularia senegalensis
			Cochliobolus verruculosus	*Curvularia verruculosa*
				Dichotomophthoropsis nymphaearum
				Dissitimurus exedrus
				Drechslera biseptata
				Exserohilum longirostratum
				Exserohilum mcginnisii
			Setosphaeria rostrata	*Exserohilum rostratum*
				Mycocentrospora acerina
				Papulaspora equi
				Phaeosclera dematioides
				Phaeotrichoconis crotalariae
				Phoma dennissiii var. oculo-hominis
				Phoma eupyrena
				Phoma glomerata
				Phoma herbarum
				Phoma hibernica
				Phoma minutella
				Phoma minutispora
				Phoma sorghina
				Polycytella hominis
				Ulocladium botrytis
				Ulocladium chartarum
	Polystigmatales	*Polystigmataceae*		*Colletotrichum coccodes*
				Colletotrichum crassipes
				Colletotrichum dematium
			Glomerella cingulata	*Colletotrichum gloeosporioides*
			Glomerella tucumanensis	*Colletotrichum graminicola*
	Sordariales	*Chaetomiaceae*	*Thielavia terrestris*	*Acremonium alabamense*
			Ascotricha chartarum	*Dicyma ampullifera*
			Chaetomium atrobrunneum	
			Chaetomium funicola	
			Chaetomium globosum	
			Chaetomium murorum	
			Chaetomium perlucidum	
			Chaetomium (Achaetomium) strumarium	
			Corynascus heterothallicus	*Myceliophthora thermophila*
				Myceliophthora lutea
				Phaeoisaria dematidis
				Staphylotrichum coccosporum
		Coniochaetaceae		*Acrophialophora fusispora*
			Coniochaeta ligniaria	*Lecythophora hoffmannii*
				Lecythophora mutabilis
				Phaeoacremonium inflatipes
				Phaeoacremonium parasiticum
				Phaeoacremonium rubrigenum
				Phialemonium curvatum
				Phialemonium obovatum
		Lasiosphaeriaceae	*Arnium leporinum*	
				Arthrinium phaeospermum
				Nigrospora sphaerica
		Magnaporthaceae	*Omnidemptus* sp.	*Mycoleptodiscus indicus*
		Myxotrichiaceae	*Myxotrichum deflexum*[a]	

(*Continued over*)

Table 5 *Mycotic taxa of the Phylum Ascomycota (Continued)*

Class	Order	Family	Teleomorph	Anamorph
				Geomyces pannorum
				Oidiodendron cerealis
				Ovadendron sulphureo-ochraceum
		Sordariaceae	*Neurospora sitophila*	*Chrysonilia sitophila*
				Thermomyces lanuginosus (*Humicola lanuginosa*)
		Xylariaceae		*Nodulisporium*

References: Summerbell & Kane 1997; Kurtzman & Fell, 1998; Guarro et al. 1999; de Hoog et al. 2000; Machouart-Dubach et al. 2002; Revankar et al. 2002; Barron et al. 2003; Schell 2003; Sigler 2003a, b; Summerbell 2003; Cano et al. 2004; Sutton et al. 2004.
a) Questionable human pathogen.

eukaryotes (Massey et al. 2003). However, *Candida* is not a monophyletic genus, and species vary in codon usage as well as the number of isoprene units in coenzyme Q and other characters (Diezmann et al. 2004).

Unlike molds, which are identified primarily by observing their colonial and microscopic morphology, yeasts vary little in morphology and are routinely speciated according to a battery of physiological criteria (Freydiere et al. 2001; Hazen and Howell 2003). However, a commercial method has been recently developed for the automated sequencing of the D2 region of LSU rDNA to identify both yeast and mold cultures within 24 hours (Hall et al. 2003, 2004).

Class Euascomycetes

This large class of molds holds most of the Ascomycota, including most of the anamorphic taxa (see Table 5). The phylogenies of numerous groups of Euascomycetes have been analyzed using a number of gene sequences (e.g. SSU and LSU rDNA, *RPB1*, *EF2*) and phylogenetic approaches (e.g. MP, NJ, Bayesian) (Prillinger et al. 2002; Reeb et al. 2004). The number of orders and families remains in flux, and the classification of the Euascomycetes will likely be mutable for many years as there is a steady and natural accrual of new information (Kirk et al. 2001; Prillinger et al. 2002; Eriksson et al. 2004). Some of the more medically relevant groups are described below.

ORDER ONYGENALES

Many notable pathogens are members of the Order Onygenales, which was originally defined using detailed morphological observations and other phenotypic data (Currah 1985). This order is noted for the production of enclosed ascomata, either cleistothecia or gymnothecia. The anamorphs produce distinctive thallic conidia, such as macroconidia, microconidia, chlamydospores, and arthroconidia. Two medically prominent families are the *Arthrodermataceae*, which contains the dermatophytes and related keratinophilic saprobes, and the *Onygenaceae*, which includes the endemic, dimorphic pathogens (Table 5). The more famous onygenalean genera are *Blastomyces*, *Coccidioides*, *Epidermophyton*, *Histoplasma*, *Microsporum*, *Paracoccidioides*, and *Trichophyton*. The *Arthrodermataceae*, *Onygenaceae*, and *Gymnoascaceae* were subjected to a masterful phylogenetic investigation that involved a comparison of sequences of both the SSU and LSU rDNA using NJ and ML methods (Sugiyama et al. 2002). From this analysis of 80 taxa, four major clades emerged. One clade included saprobes and systemic pathogens, while the *Arthrodermataceae* were ensconced in a different clade. Two pathobiological phenotypes, keratinolysis and dimorphism, are likely apomorphic, deriving independently by convergent evolution, as they occurred in taxa in different clades. For example, since *C. immitis* was in a different clade from the *Ajellomyces* spp., pathogenicity and dimorphism are apparently apomorphic (Sugiyama et al. 2002).

Initially described in the 1840s, the dermatophytes are among the earliest documented human pathogens (Howard et al. 2003). The many species of *Microsporum* and *Trichophyton* are closely related and similar in their morphology, physiology, degradative enzymes (e.g. keratinases, elastases), and antigens (Kane et al. 1997). However, some species exhibit marked differences in ecology and host range. As mentioned above, the evolution of dermatophytes toward zoophilism and anthropophilism correlates with the loss of both sexual and asexual sporulation. Most of the clinical species are resolutely anamorphic and defined by subtle phenotypic features. After decades of taxonomic revisions, several molecular phylogenies are finally resolving the phylogeny of the dermatophytes, but classifying dermatophytes remains a dynamic enterprise (Gräser et al. 1999; Makimura et al. 1999).

ORDER EUROTIALES

In the Order Eurotiales, sexual reproduction results in perithecia or cleistothecia, and the anamorphs produce a variety of morphologically distinct conidiophores and phialoconidia. As indicated in Table 5, the family *Trichocomaceae* includes *Penicillium* and *Aspergillus* (Berbee et al. 1995; LoBuglio and Taylor 1995; Summerbell 2003). The Order Ophiostomatales contains the dimorphic *Sporothrix schenckii* and a number of closely related phytopathogens (de Beer et al. 2003). Numerous dematiaceous pathogens are

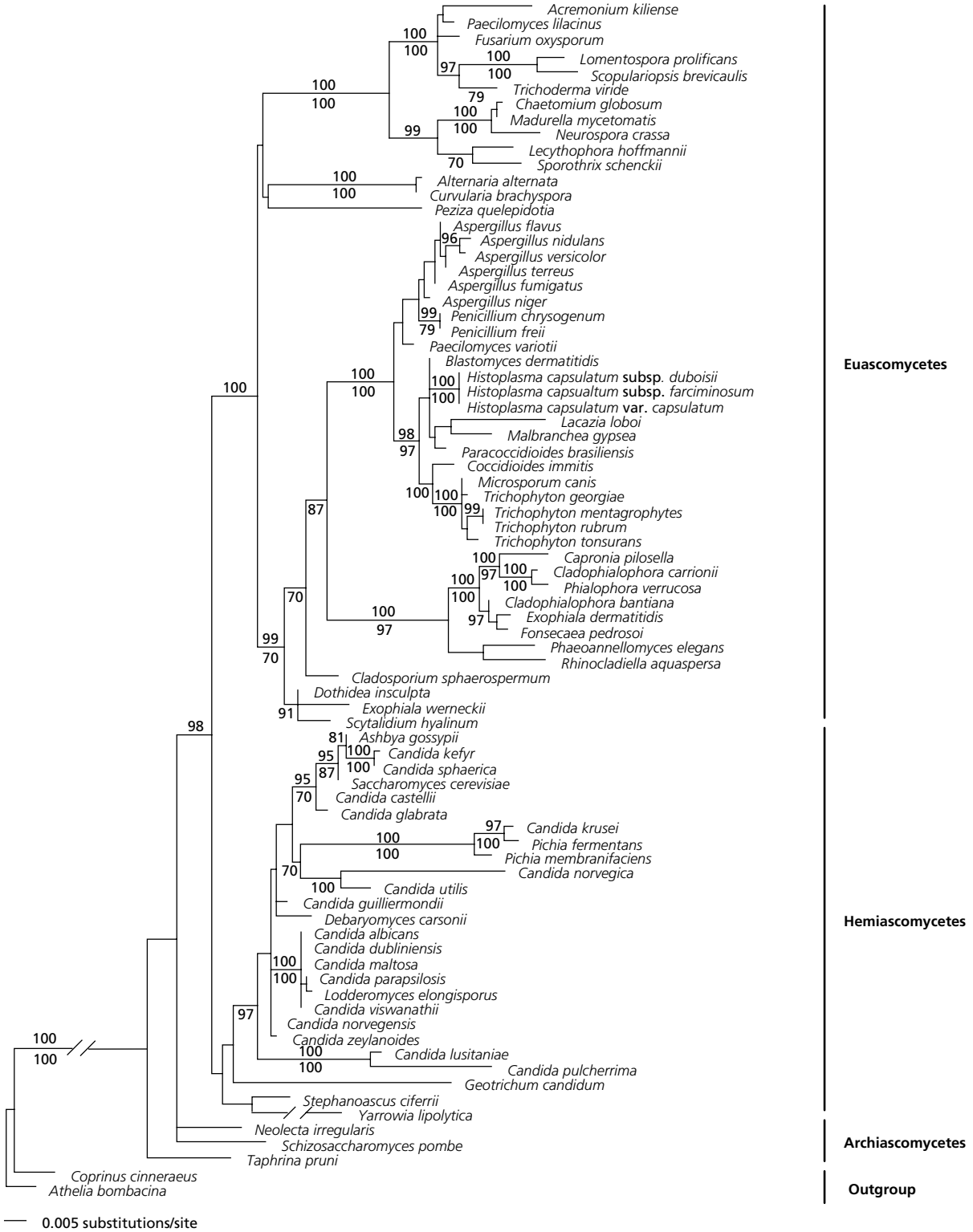

Figure 3 *Phylogram of representative and related mycotic ascomycetes. Single most likely tree based on a ML analysis of the nuclear SSU rDNA sequences. Nodes with statistically significant support by bootstrap (⩾70 percent) or Bayesian (⩾95 percent) analysis are indicated by the values below or above the nodes, respectively. Vertical lines on the right denote the three classes of the Ascomycota.*

classified in the Order Dothideales (Spatafora et al. 1995; de Hoog et al. 1999; Sterflinger et al. 1999). Although the exact phylogenetic placement of these taxa is the subject of ongoing investigations, many familial relationships have been resolved; for example, the family *Herpotrichiellaceae* contains species of

Cladophialophora, *Exophila*, *Fonsecaea*, and *Phialophora* (Haase et al. 1999).

In a superb review of the Eurotiales, which was published in 2003 but apparently written in 2000, Richard Summerbell questioned the validity of many case reports attributable to species of *Aspergillus* (Summerbell 2003). The following species are listed as pathogens in Table 5, but their status as agents of aspergillosis is questionable because of the lack of definitive documentation: *Aspergillus glaucus* group, *Aspergillus reptans*, *Aspergillus restrictus*, *Aspergillus unguis*, *Aspergillus janus*. Of course, many other species of *Aspergillus* have been recovered from patients but are not listed because they were isolated only as contaminants from nonsterile clinical specimens (e.g. skin, sputa, sinuses). Other *Aspergillus* spp. are listed even though they have to date only caused infection in nonhuman mammals, such as *Aspergillus rugulovalvus* (*Emericella rugulosa*) in cattle and *Aspergillus deflectus* in dogs. Nevertheless, they have been cited in numerous texts and reviews. Exclusively nonmammalian pathogens are not listed.

PHYLUM BASIDIOMYCOTA

Each of the three classes of the Phylum Basidiomycota – Urediniomycetes, Ustilaginomycetes, and Hymenomycetes – contains species of yeasts and molds, as well as species that are capable of infecting mammalian hosts. Similar to the Ascomycota, the basidiomycetes are ubiquitous. Many are phytopathogens, such as the rusts and smuts, which are found in the Orders Uredinales and Ustilaginales, respectively. Approximately 30 000 basidiomycetous species have been described, yet only a dozen or so cause human infection (Table 6). (Toxigenic fungi, such as poisonous mushrooms, are not included in Table 6.)

The basidiomycetes are characterized by the sexual production of basidia and basidiospores. Sexual reproduction is initiated by the fusion of compatible mating partners, and a dikaryon is often formed in which each cell contains two haploid nuclei, one from each partner. This dikaryophase may be protracted, and the hyphae develop clamp connections to ensure that each cell maintains the requisite two nuclei. The clamp connections are accompanied by complex septal pores. Clamp connections are only produced by basidiomycetes, but they are not made by every member of the Basidiomycota. The signature feature of basidiomycetes is the production of basidia, which culminates the sexual cycle. The basidium is the site of karyogamy, which is followed by meiosis and mitosis, typically yielding four haploid basidiospores. The basidiospores germinate to form vegetative septate hyphae or budding yeast cells. Similar to the Ascomycota, much phenotypic and molecular evidence suggests that the Basidiomycota is monophyletic (Oberwinkler 1987; Swann and Taylor 1993, 1995b; McLaughlin et al. 1995; Prillinger et al. 2002).

Most described basidiomycetes are members of the Class Hymenomycetes, and they share the feature of distinctive, perforated, dolipore septa (Moore 1978). The largest subdivision of the Hymenomycetes is the Homobasidiomycetes, which houses mushrooms, puffballs, and other higher fungi. The classification of the higher taxa of Hymenomycetes is not completely resolved (Binder and Hibbett 2002). Another significant clade of the Hymenomycetes is the Tremellales, which contains many of the basidiomycetous yeasts (Swann and Taylor 1995a, c; Fell et al. 2000). The genera of greatest clinical importance are *Filobasidiella* (*Cryptococcus*) (Sivakumaran et al. 2003), *Malassezia* (Kano et al. 1999) *Rhodosporidium* (*Rhodotorula*), *Sporidiobolus* (*Sporobolomyces*), and *Trichosporon* (Guého et al. 1992; Sugita et al. 2002).

CLINICAL APPLICATIONS

As mentioned earlier, DNA sequences have been exploited not only to determine phylogenetic relationships but to develop molecular methods to identify cultures and detect fungal DNA in clinical material, to genotype species and strains for molecular epidemiology, and to analyze the population structure of pathogens (Taylor et al. 1999a; Burnett 2003; Xu and Mitchell 2003).

Phylogenetic studies have greatly abetted efforts to develop more accurate methods to identify species (and strains) of many pathogenic fungi. Signature DNA sequences of the target species are identified, often within the rDNA gene cluster, and specificity can be evaluated by including the closest taxa based on phylogenies of the target species (Iwen et al. 2002). PCR methods are rapid, definitive, specific, and considerably more accurate and reliable than conventional methods of identification. Increasing reports confirm the utility of PCR methods to identify isolates of dermatophytes (Kanbe et al. 2003; Shin et al. 2003), dimorphic pathogens (Walsh et al. 2003), and opportunistic molds (Kanbe et al. 2002; de Aguirre et al. 2004) and yeasts (Elie et al. 1998; Ahmad et al. 2002; Luo and Mitchell 2002; Selvarangan et al. 2003). A new commercial method, originally developed for the identification of bacteria, has proven successful in identifying fungal cultures (Hall et al. 2003, 2004). After amplifying the D2 region of the LSU rDNA of the isolate, the amplicon is then purified, cycle-sequenced, and the nucleotide sequence is compared with a database of D2 sequences of >1 000 mycotic fungi to identify the closest taxon. Multiple isolates of the same species usually exhibit <1.00 percent variation (Hall et al. 2003, 2004).

The natural next step is to develop methods to recognize and diagnose mycotic infections directly from clinical specimens, and many DNA probes and PCR

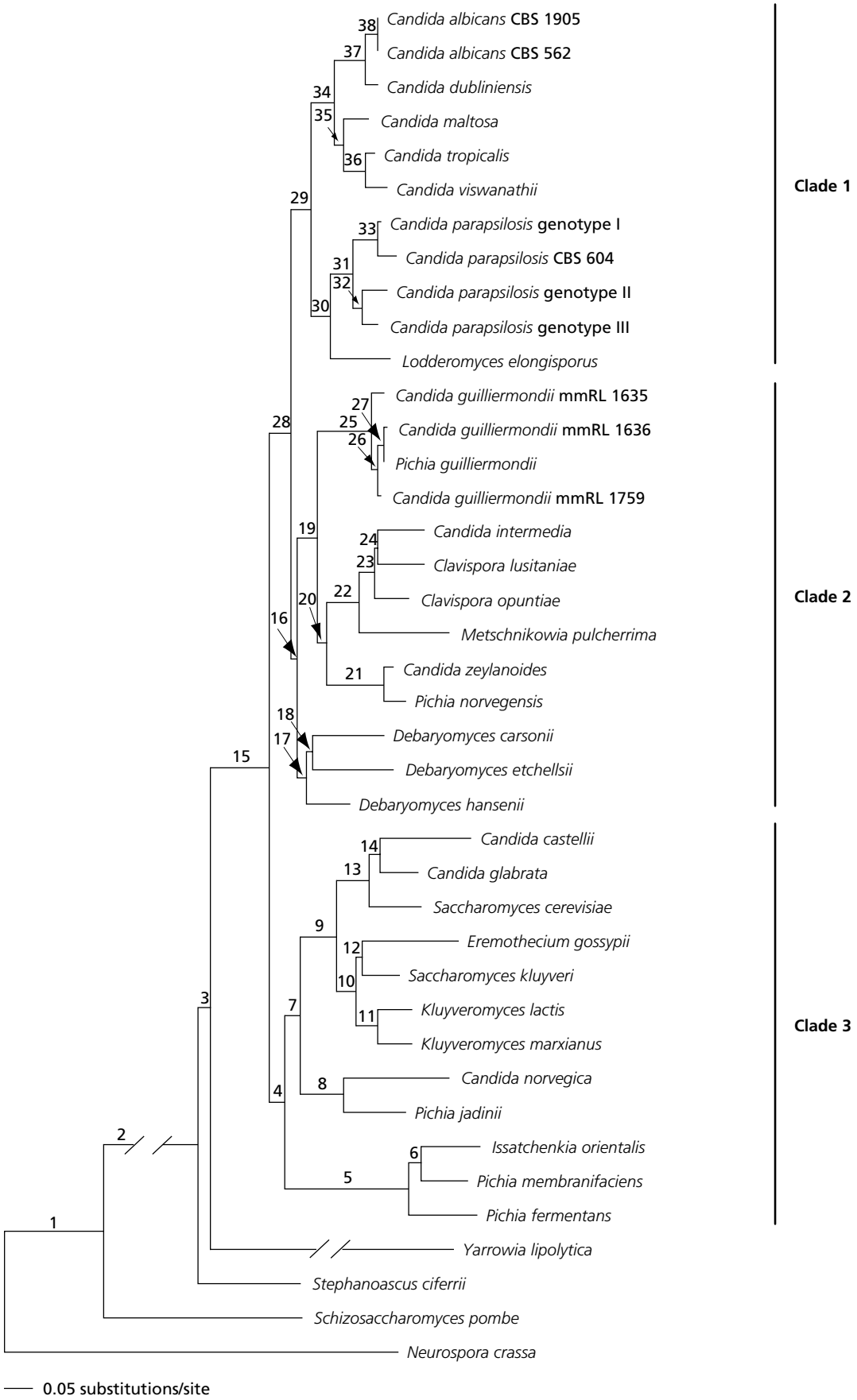
Candida albicans CBS 1905
Candida albicans CBS 562
Candida dubliniensis
Candida maltosa
Candida tropicalis
Candida viswanathii
Candida parapsilosis genotype I
Candida parapsilosis CBS 604
Candida parapsilosis genotype II
Candida parapsilosis genotype III
Lodderomyces elongisporus
Candida guilliermondii mmRL 1635
Candida guilliermondii mmRL 1636
Pichia guilliermondii
Candida guilliermondii mmRL 1759
Candida intermedia
Clavispora lusitaniae
Clavispora opuntiae
Metschnikowia pulcherrima
Candida zeylanoides
Pichia norvegensis
Debaryomyces carsonii
Debaryomyces etchellsii
Debaryomyces hansenii
Candida castellii
Candida glabrata
Saccharomyces cerevisiae
Eremothecium gossypii
Saccharomyces kluyveri
Kluyveromyces lactis
Kluyveromyces marxianus
Candida norvegica
Pichia jadinii
Issatchenkia orientalis
Pichia membranifaciens
Pichia fermentans
Yarrowia lipolytica
Stephanoascus ciferrii
Schizosaccharomyces pombe
Neurospora crassa
Clade 1
Clade 2
Clade 3
0.05 substitutions/site

Table 6 *Mycotic taxa of the Phylum Basidiomycota*

Class	Order	Family	Teleomorph	Anamorph
Hymenomycetes	Agaricales	*Coprinaceae*	*Coprinus cinereus*	*Hormographiella aspergillata*
				Hormographiella verticillata
	Stereales	*Bjerkanderaceae*	*Bjerkandera adusta*	
		Corticiaceae	*Phanerochaete chrysosporium*	*Sporotrichum pruinosum*
		Schizophyllaceae	*Schizophyllum commune*	
	Tremellales	*Filobasidiaceae*		*Cryptococcus albidus*
				Cryptococcus ater
				Cryptococcus curvatus
				Cryptococcus laurentii
				Cryptococcus macerans
			Filobasidiella bacillispora	*Cryptococcus neoformans var. gattii*
			Filobasidiella neoformans	*Cryptococcus neoformans var. grubii*
			Filobasidiella neoformans	*Cryptococcus neoformans var. neoformans*
			Filobasidium uniguttulatum	*Cryptococcus uniguttulatus*
	Trichosporonales			*Cryptococcus humicola* complex
				Trichosporon asahii
				Trichosporon asteroides
				Trichosporon cutaneum
				Trichosporon inkin
				Trichosporon loubieri
				Trichosporon mucoides
				Trichosporon ovoides
Urediniomycetes	Sporidiales	*Sporidiobolaceae*	*Rhodosporidium diobovatum*	*Rhodotorula glutinis*
			Rhodosporidium sphaerocarpum	*Rhodotorula glutinis*
			Rhodosporidium toruloides	*Rhodotorula glutinis*
				Rhodotorula minuta
				Rhodotorula mucilaginosa (*R. rubra*)
			Sporidiobolus salmonicolor	*Sporobolomyces salmonicolor*
			Sporidiobolus johnstonii	*Sporobolomyces holsaticus*
				Sporobolomyces roseus
Ustilaginomycetes	Malasseziales			*Malassezia furfur*
				Malassezia globosa
				Malassezia obtusa
				Malassezia pachydermatis
				Malassezia restricta
				Malassezia slooffiae
				Malassezia sympodialis
				Moniliella suaveolens[a]
	Microstromatales			*Fugomyces (Cerinosterus) cyanescens*
	Tilletiales			*Tilletiopsis minor*

References: Kurtzman and Fell 1998; Guarro et al. 1999; de Hoog et al. 2000; Fell et al. 2000; Takashima et al. 2001.
a) Questionable human pathogen.

Figure 4 *Combined ML analysis of six genes (*ACT1, EF2, RPB1, RPB2, *SSU and LSU rDNA) for 38 taxa of Hemiascomycetes and two outgroup species, an Archiascomycetes (*Schizosaccharomyces pombe*) and a Euascomycetes (*Neurospora crassa*). The following nodes are supported by heterogeneous Bayesian posterior probabilities ⩾95 percent as calculated in the combined analysis: 2, 4, 5, 7–11, 13, 15, 16, 21–23, 25–38. Branch lengths leading to* Yarrowia lipolytica *(0.19157), and the outgroup taxa (0.31967) were shortened to fit the figure. Vertical lines on right indicate the three major clades (Diezmann et al. 2004).*

tests have been developed to identify specific fungal DNA in clinical specimens (Yeo and Wong 2002). These topics are amplified in Laboratory diagnosis; and 5, Mycoserology and molecular diagnosis.

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Fungal structure and morphology

PAUL F. LEHMANN

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The species of the fungi that are associated with infections in humans represent a number of very different groups within the Kingdom Fungi. Three phyla of the true fungi, the Ascomycota, Basidiomycota, and Zygomycota, include infectious fungi. The remaining phylum, the Chytridiomycota, includes the order Neocallimastigales within which several genera of anaerobic fungi have been described. Anaerobic fungi have been found in the gut lumen of several herbivores but they have not been associated with human disease.

Additional organisms resemble fungi and are studied by medical mycologists. These include a pathogenic member of the genus *Pythium* (*Pythium insidiosum*), but this is a genus that is no longer included within the Kingdom Fungi being assigned, with other oomycetes, to the Stramenopiles or Kingdom Chromista (Hawksworth et al. 1995; Van de Peer et al. 2000). Another group of species, those in the *Pneumocystis carinii* complex, have traditionally been studied by parasitologists on account of their morphological similarity to some of the parasitic protozoa; however, comparative analysis of the sequences of the ribosomal RNA (rRNA) and of a number of different genes has placed this complex of species among the fungi (Cailliez et al. 1996). It is likely that several other organisms that have been treated as parasitic protozoa will be found to be fairly close relatives of fungi. Such appears to be the case for *Encephalitozoon caniculi*, a microsporidian that has emerged as a problem in immunosuppressed patients including those with the acquired immunodeficiency syndrome (AIDS) (Vivarès et al. 2002).

Most recently, the class Mesomycetozoea has been defined within the Kingdom Protozoa. This includes a number of fungus-like organisms which, based on rRNA sequences, have evolved as a lineage which is distinct from the eukaryotic kingdoms represented by fungi, animals, and plants (Mendoza et al. 2002). The Mesomycetozoea include a number of fish parasites and the human pathogen, *Rhinosporidium seeberi*, the etiologic agent of rhinosporidiosis. Relative to the fungal pathogens from the Kingdom Fungi that are the traditional study of medical mycologists, these organisms are poorly understood and, unless directly noted, will not be the subject for further discussion within this chapter.

In spite of their diversity, fungi share many features. At the simplest level, they are nonphotosynthetic, eukaryotic organisms that possess a cell wall lying external to the plasma membrane. This membrane, also known as the plasmalemma, represents the outermost cellular membrane. It is the cell wall that imparts a shape to the fungal cell and prevents it from bursting when the fungus is placed in environmental settings with low osmotic pressure. If the wall is removed, by enzymatic digestion, the cell takes on a spherical form and becomes osmotically fragile. Differential patterns of cell wall synthesis play a central role in determining both the shape of the fungal cell and the patterns of fungal growth that will be discussed herein.

INTRACELLULAR COMPONENTS

Being eukaryotic cells, all true fungi possess a nuclear pore complex within a nuclear membrane. Within the nucleus there is a nucleolus and linear chromosomes. The cytoplasm contains a cytoskeleton in which the microfilaments, which are composed largely of actin, and the tubulin-containing microtubules provide the major components (Gow and Gadd 1995). The cytoplasm also contains organelles including mitochondria, although these are not found in the anaerobic fungi, the Golgi apparatus, vacuoles, often several types of vesicle and 80S ribosomes. The plasmalemma typically contains large quantities of sterols, usually ergosterol. It is their preferential binding to ergosterol rather than cholesterol, the sterol that is dominant in human cell membranes, which explains the selectivity of the polyene antifungal agents, nystatin and amphotericin B. The pathway for synthesis of ergosterol is the target of two groups of antifungal agents. The azole antifungals, which include fluconazole, itraconazole, ketoconazole, miconazole, and voriconazole, inhibit the cytochrome P450-dependent lanosterol demethylase, while the allylamines, which include terbinafine and naftidine, inhibit squalene expoxidase.

BASIC GROWTH PATTERNS: MOLDS AND YEASTS

Fungi, not being photosynthetic, require environmental sources of fixed carbon for their growth, and two major strategies for exploiting substrata have been developed. Filamentous growth and apical extension of the filament, in association with the formation of lateral branches, are exemplified by hyphae and mycelium, while single cell division is associated with yeasts (Figure 1). Apical growth, in association with a somewhat rigid wall, allows a hypha to generate forward pressure at its tip and this, combined with the release of degradative enzymes, enables it to force its way through tissues. Branching of the hyphae leads to the production of mycelium, a large structure composed of interconnected hyphal filaments.

In contrast to the filamentous form of growth that leads to the production of a mycelium, the yeast form of growth results in the physical separation of parental and progeny cells. To exploit new sources of nutrients, yeast cells spread to more distant sites by being carried passively in moving fluids, such as in plasma, or in fluid films such as those that bathe tissue surfaces.

In addition to the basic pattern of growth by molds and yeasts, several fungi or fungus-like organisms have unusual morphologies and methods of growth. These include *Pneumocystis* where clusters of multinucleate cysts and trophozoite (single-celled) stages are seen in infected persons. In both *Coccidioides* and *Rhinosporidium*, parasitic development is characterized by the production of spherical bodies that undergo endosporulation and with the subsequently-released endospores swelling to form new spherical structures. In *Coccidioides* spp., the spherical structure (= a spherule) undergoes segmentation and fungal cell wall material divides the contents into sections in which the endospores mature. Rupture of the spherule leads to release of endospores. By contrast, the fungus-like *R. seeberi* produces a large spherical sporangium in which there is no segmentation and the endospores appear to be released through a pore in the sporangial wall (Mendoza et al. 1999). A further form of growth is seen for the parasitic stage of some etiologic agents of chromoblastomycosis: these produce sclerotic bodies showing planar division. Figures showing these forms can be seen in the appropriate chapters covering these diseases.

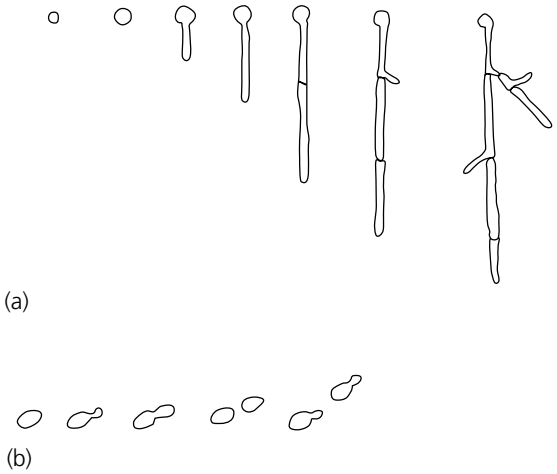

Figure 1 *Growth forms of fungi:* **(a)** *molds – the ungerminated conidium swells, then germinates to produce an apically growing hypha which develops septa and branches. Growth continues at the apices of the original hypha and branches.* **(b)** *Budding yeast cell: a common growth pattern where reproduction occurs by separation of the emerging bud from the mother cell. Both mother and daughter cells continue to bud.*

Cell wall synthesis

The actual shape of the fungal cell is determined by the cell wall. This lies exterior to the cell membrane and is composed primarily of carbohydrates, some of which are covalently linked to proteins. The cell walls of distantly related fungi can show substantial differences in their chemical composition, and so it is remarkable that hyphae of quite distantly related species may appear very similar both in morphology and in their pattern of growth. The carbohydrate components appear to be synthesized at the surface of the plasmalemma and include materials that are crystalline, such as α-glucans and chitin, which is the β(1,4)-linked polymer of *N*-acetyl-D-glucosamine. The crystalline materials are thought to provide strength and rigidity to the wall.

Other polysaccharidic components include matrix materials such as mannoproteins that may allow the wall to have some degree of flexibility. Evidence for covalent crosslinking of cell components has been found and this would be expected to strengthen the walls. Those glucan synthetases that generate β-glucans within the cell wall provide the target for the echinocandin antifungal agents such as caspofungin acetate.

The expanding hyphal tip has, at its apex, a cell wall that shows significant plasticity. Here the wall is quite thin and also is thought to be composed of materials that are more loosely associated with each other than that seen in the more mature hyphal walls where the crosslinking of the various materials appears to have been completed. For example, in contrast to the situation with mature walls, the newly synthesized chitin chains found in the tip appear not to be fully crystallized. Models for fungal cell wall synthesis have been reviewed (Sentandreu et al. 1994; Sietsma and Wessels 1994; Wessels 1994).

Hydrophobic proteins (hydrophobins) are often produced by aerial hyphae and conidia. The assembly of these molecules results in a superficial hydrophobic rodlet layer. Our understanding of these hydrophobic molecules is far from complete (Wessels 1994).

Mold growth pattern

APICAL EXTENSION OF HYPHAE

During hyphal extension, there is directed flow of precursors into the hyphal tip. Many vesicles are detectable at hyphal tips and these are considered to be the vehicles for both the enzymes and possibly some of the precursors that are required for cell wall synthesis (Heath 1994; Sentandreu et al. 1994; Sietsma and Wessels 1994; Wessels 1994; Gow and Gadd 1995). For example, some of the vesicles, termed 'chitosomes,' may carry chitin synthase in its zymogenic form. Chitosomes, when activated and incubated with UDP-*N*-acetyl-D-glucosamine, have been shown to cause the synthesis of chitin microfibrils (Bracker et al. 1976). A cluster of vesicles near growing hyphal tips can be observed by light microscopy and it appears as a distinct structure that has been named Spitzenkörper. This structure has been suggested to represent a distribution center for the vesicles migrating to the growing tips of hyphae. The Spitzenkörper is not observed when hyphal growth ceases and experimentally induced changes to its position relative to the growing tip have been linked to changes in the shape of the hyphal wall (Bartnicki-Garcia et al. 1995).

An understanding of the basic mechanisms that are involved both in maintaining the uniform cylindrical shape of a growing hypha and in producing apical extension of the hyphal tip is beginning to emerge. Multiple gene products are involved: indeed a large-scale genetic screen for mutations in *Neurospora crassa* that were associated with defects in polarized growth and hyphal morphogenesis obtained >100 complementation groups and 21 different morphological phenotypes (Seiler and Plamann 2003). Among the genes involved were those regulating the formation of the cytoskeleton or were part of the secretory pathway. Genes encoding members of the cell wall and outer membrane biosynthetic pathways and also encoding components of the signal transduction pathways, including interactive kinases, were also identified, indicating that the process of morphogenesis is complex.

The large number of morphological phenotypes and the abundance of complementation groups involved in morphogenesis enable the creation of many possible models for the control of hyphal growth. Clearly the three-dimensional form of the cytoskeleton is important; indeed, the shape may be the predominant factor that controls the shape of the cell (Heath 1994; Wessels 1994). However, a simple model is inadequate. For example, Heath has pointed out that apical growth cannot simply be explained by use of a model where turgor pressure causes tip expansion by pushing the cell contents outwards at the site where the hyphal cell wall is weakest, i.e. at the hyphal tip (Heath 1994). Among the phenomena that are not explained by that model are: the capability of cell wall mutants, for example, mutants of *Aspergillus niger* that lack chitin, to produce relatively normal looking hyphae in media of high osmotic strength, and the poor correlation between changes in internal turgor pressure and the hyphal growth rate.

As the hypha grows forward, materials are incorporated into the wall at the apex of its hyphal tip. The newly synthesized wall pushes aside the previously formed wall components that soon become part of the mature walls. In actively growing fungi, the hyphal contents do not have a uniform metabolic activity throughout the length of the hypha. Maximal activity is found at the growing tip and it is in this region that most of the absorption of nutrients appears to occur and where hydrolytic enzymes, such as lipases, polysaccharidases and proteases, are secreted. These enzymes may remain in the hyphal wall or they can be released into the surrounding substrate.

The factors controlling polar growth exhibited by hyphae have been examined for a number of different species and the subject has been reviewed fairly recently (Momany 2002). The reader is invited to obtain references to several important papers in the original literature from that review as they are beyond the scope of this introductory chapter. In spite of the difference in typical growth habit of yeasts and molds, studies have demonstrated a similarity between the central protein machinery required for the polarized growth exhibited by hyphae of filamentous fungi and the protein machinery used in maintaining polarized growth

associated with the more filamentous growth morphology of baker's yeast, *Saccharomyces cerevisiae*. This form of growth can be seen when yeast is grown on certain media. For example, a Rho-GTPase, Cdc42, drives polar growth in yeast and the homologous protein appears to perform a similar function in the ascomycetes, *Ashbya gossypii* and *Penicillium marneffei*. The function of Cdc42 appears to be to act as a target for additional proteins that themselves act as a scaffold for the cytoskeletal components (Johnson 1999). Polar growth appears dependent on the state of the plasma membrane where it has been suggested that rafts of polarity-inducing proteins may be localized. Post-translational lipid modification of some of these proteins via *N*-myristoylation (*Aspergillus nidulans*) or geranylgeranylation (*S. cerevisiae*) has been suggested as a mechanism whereby the polarity proteins develop affinity for the plasma membrane but this alone does not explain the control and maintenance of a polarized form of growth. Here, it is likely that vesicle trafficking is organized so that vesicles containing wall precursors are targeted to a single area, a process that is complex and certainly involves the correct deposition of both formins and septins. This is discussed further in the next section.

DEVELOPMENT OF MYCELIUM

Although the mature hyphal wall becomes thickened and rigid, it may still undergo modifications at a later stage. When, at some distance behind the growing hyphal apex, a small area of wall is rendered elastic, a lateral hypha can emerge as a branch (Figure 2). Typically, this new hypha grows apically and itself may give rise to lateral branches. The whole pattern leads to the development of an interconnected mycelium.

Cross-walls, known as septa, are often formed. Septa have a variety of structures with certain types being restricted to specific major divisions of the fungi, for example, septa containing dolipores are restricted to the Basidiomycota (Figure 3). The different types of septa have different effectiveness in providing barriers to the movement of nutrients, vesicles, organelles, and nuclei. Those with large pores appear to provide few restrictions. This contrasts with the control provided by septa with small or complex pores.

Septum production has been studied fairly extensively for *Aspergillus nidulans* and requires, after a conidium has germinated, that the hypha reaches a certain minimum size before a primary septum is formed. The septum develops from a cylindrical band of material that includes actin. This ring constricts so that fungal components become separated after mitosis has occurred. Septal formation is linked to mitotic events: the linkage retained as the hypha continues to grow and form new septa being at some distance behind the hyphal tip. Several proteins are involved in septal formation:

- the actins that form a ring appear to interact with the microtubules of the mitotic spindle

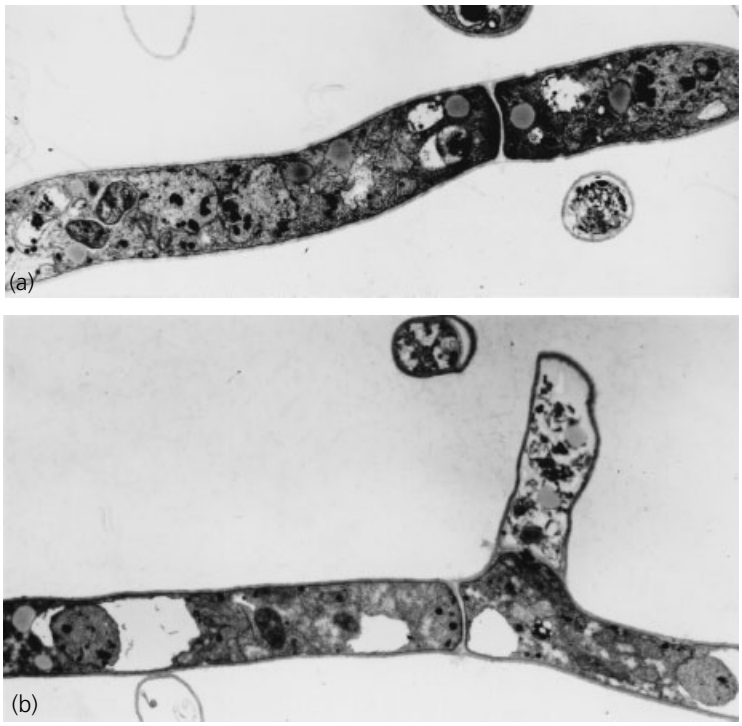

Figure 2 Coccidioides immitis *hyphae:* **(a)** *showing septum and intracellular organelles (magnification ×4 765);* **(b)** *showing formation of a branch (magnification ×6 800). Courtesy of K.R. Seshan*

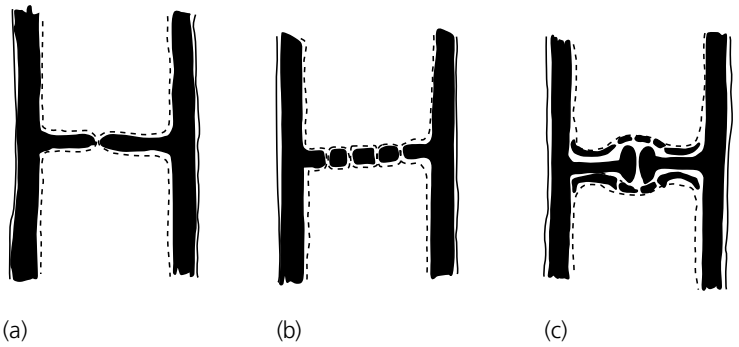

Figure 3 *Septa: there are several types. The diagramatic representations show a hyphal cross-wall developing from inward growth of the inner cell wall layers with* **(a)** *a simple pore,* **(b)** *multiple pores, and* **(c)** *a complex dolipore. In the last, the doughnut-shaped septal pore can be covered by a perforated cap.*

- the septins that belong to a conserved family of proteins found in eukaryotes where they act as morphogenetic scaffolds important in polarized growth and cell division
- the formins that seem to be needed for the correct organization of the actin cytoskeleton during cell division and the concurrent constriction of the ring of septal material.

One formin, SepA, has been detected both in the hyphal tip and at sites where septation is occurring; it appears to be required for proper deposition of the septin, AspB (Sharpless and Harris 2002). Though deposited on both sides of the constricting septum, the AspB deposited on the basal surface is not retained and disappears. By contrast, AspB is retained on the apical side of a septum and it may act as an signal or marker for cell polarity that can be exploited by the fungal cell (Harris 2001).

Some fungi, notably many species within the Zygomycota, produce few or no septa within a mycelium. Such a mycelium is essentially a coenocytic organism. There is little understanding of the molecular mechanisms accounting for polarized growth and branching of hyphae in these organisms.

The yeast pattern of growth

The pattern of growth that follows cell divisions, which result in the physical separation of daughter and parental cells, is quite different from that found for mycelial growth. Most yeasts of medical importance produce daughter cells by budding (see Figure 1). These species include *Cryptococcus neoformans*, the species of *Candida*, and the parasitic forms of several dimorphic pathogens including *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Sporothrix schenckii*. Typically, in a budding yeast, cell wall synthesis occurs over the whole surface of the bud and is not directed at a single pole. After mitosis and the migration of a nucleus into the bud, the separation of the daughter cell from the parent cell involves both the formation of a cross-wall at the site of the bud and the subsequent cleavage of this wall as the daughter cell dissociates. The wall formed at the bud site has a different structure from the rest of the yeast's cell wall and is termed the 'bud scar.' A great deal is known about the control of the growth cycle and budding in brewer's yeast, *S. cerevisiae*, which therefore acts as a model for budding yeast growth. Many of the features are conserved among species, for example, the production of a chitin-rich ring in the bud scar representing the site on the mother cell from which the daughter yeasts are cleft. But there can be significant differences in the complement of enzymes expressed by different species. Given the differences in cell wall composition associated with different species, and especially when basidiomycetous yeast species such as *Cryptococcus* are compared to ascomycetous species, it is clear that a thorough understanding of the mechanism of budding growth will require its investigation in several species.

A second form of cell division is characterized by the fission yeasts. Here the parental cell elongates and, after mitosis has occurred, the nuclei become separated by a septum that divides the elongated cell. The two daughter yeasts then dissociate after cleavage of the septum. This pattern of division is seen among few species of infectious fungi. It can be observed in the parasitic form of *Penicillium marneffei*, and also occurs in an incomplete fashion during the development of the muriform cells that are formed in vivo by the agents of chromoblastomycosis. The muriform cells are composed of cells that have reproduced by elongation and have formed a dividing septum. However, fission is delayed and further septal formation in the progeny cells can lead to somewhat irregular multicellular structures.

The basis of maintaining the growth patterns seen for budding and fission yeasts have been subject to extensive research and detailed discussion is beyond the scope of this introduction. While signal transduction via MAP kinases and other pathways such as TOR, which acts as a nutrient sensor and controls translation, are essential for allowing of growth to proceed (Crespo and

Hall 2002), it is clear that there also have to be intracellular structures that direct synthesis in an ordered manner. These structures appear to be supplied in part by polarized actin cables that, as is also seen in mycelium, are produced in a process that is dependent of formins. The actin cables act as tracks along which myosin V motors transport vesicles, organelles, and vacuolar components into the bud. The subject has been reviewed for *S. cerevisiae* by Bretscher (2003).

DIMORPHISM

Though a certain plasticity in morphology is often seen for fungi grown under different environmental conditions, several fungi associated with infections of humans can switch between the two basic growth patterns which are typified by molds and yeasts (Table 1). Indeed, the basic growth pattern of a fungus found during an infection can be quite different from that seen when it grows as a saprophyte in an external habitat such as soil. For example, *H. capsulatum* produces mycelium at environmental temperatures of less than 30°C, but reproduces as a budding yeast when growing intracellularly in patients with histoplasmosis. The capacity to switch back and forth between very distinct forms of growth is probably quite usual for fungi. Several organisms show unusual morphologies when placed at high temperature, for example, the conidia of *Aspergillus niger* can form spherical swollen cells in vitro (Anderson and Smith 1972). Such swelling is also seen in lung tissues containing the adiaspores of *Emmonsia parva*.

Table 1 *Dimorphism among selected infectious fungi*

Species	Parasitic form[a]
Agents of chromoblastomycosis[b]	Muriform cells
Blastomyces dermatitidis	Yeasts
Coccidioides immitis	Spherules
Emmonsia parva	Adiaspores[c]
Histoplasma capsulatum	Yeasts
Paracoccidioides brasiliensis	Yeasts
Penicillium marneffei	Yeasts
Sporothrix schenckii	Yeasts
Mixed growth patterns in vivo	
Malassezia furfur	Yeasts and short hyphae[d]
Candida spp.[e]	Yeasts, pseudohyphae, hyphae and chlamydoconidia[f]

a) Characteristic form seen during active infection.
b) In several examples of chromoblastomycosis a mixture of structures may be seen. In some cases, the dominant morphology may depend in part on the host immune reactivity.
c) These represent swollen spores and do not replicate.
d) This yeast species, which requires lipid for growth, generally does not produce filaments when cultured on conventional media.
e) There is substantial variability among the species. Some *Candida* spp., for example *C. (Torulopsis) glabrata*, grow only in the yeast form. Some others produce a mixture of yeasts and pseudohyphae, yet do not appear to produce true hyphae.
f) Among *Candida* spp., only *C. albicans* is known to produce chlamydoconidia in vivo. Structures that probably represent these conidia have been detected in experimentally infected mice (see text).

The reversible modification of growth pattern in response to environmental changes results from modifications affecting both the components of the cell wall and the pattern in which cellular components are deposited. This capacity to switch the pattern of growth in response to an environmental change has been termed 'dimorphism.' The same term has also been applied to describe situations where a multiplicity of growth forms can be produced by making adjustments to the conditions under which a species is cultivated. The signal for a switch in morphology from the saprophytic form to the parasitic form can, in many cases, be shown to follow an elevation in environmental temperature. However, adequate nutrients must also be present: for example, the switch to the parasitic yeast form by *H. capsulatum*, which is achieved by transferring a culture from room temperature to 37°C, is inhibited if cysteine is not included within the culture medium. Nutritional changes and elevations in P_{CO_2} can be required to induce changes in the growth patterns of *Coccidioides immitis*. For *Candida albicans*, several seemingly independent environmental stimuli may enhance a switch between yeast and filamentous growth patterns (Berman and Sudbery 2002).

A number of species are known that produce a variety of growth patterns even while causing infection. *Malassezia furfur*, an obligatorily lipophilic yeast, produces both budding yeast and short hyphae on the skin surface. *C. albicans* and a number of other *Candida* spp. grow both as budding yeasts and in filamentous forms. Indeed, in a single lesion caused by *C. albicans*, one may see histological evidence for yeast cells, hyphae, and pseudohyphae (Figure 4). In addition to its growth in filamentous forms and as a yeast, *C. albicans* is also known to produce chlamydoconidia. These spherical conidia, having thick cell walls, are easily seen when the species is cultivated on yeast morphology agars such as rice extract agar. Although similar conidia have been seen in sputum from an AIDS patient, and can be detected by use of differential cell wall staining and electron microscopy in submucosal tissues of immunosuppressed mice after experimental intragastric infection with *C. albicans* (Chabasse et al. 1988; Cole et al. 1991), it is remarkable that chlamydospores are hardly ever reported from clinical specimens. The *C. albicans* chlamydoconidium may be a resting structure and may not actually represent a replicative form.

With the recent sequencing of the *C. albicans* genome (www-sequence.stanford.edu/group/candida/search.html), it has been possible to identify many genes that appear to be homologous to ones previously investigated in *S. cerevisiae*. The expression of the genes involved in morphogenesis can change quite substantially during dimorphic growth and this is discussed in a recent review

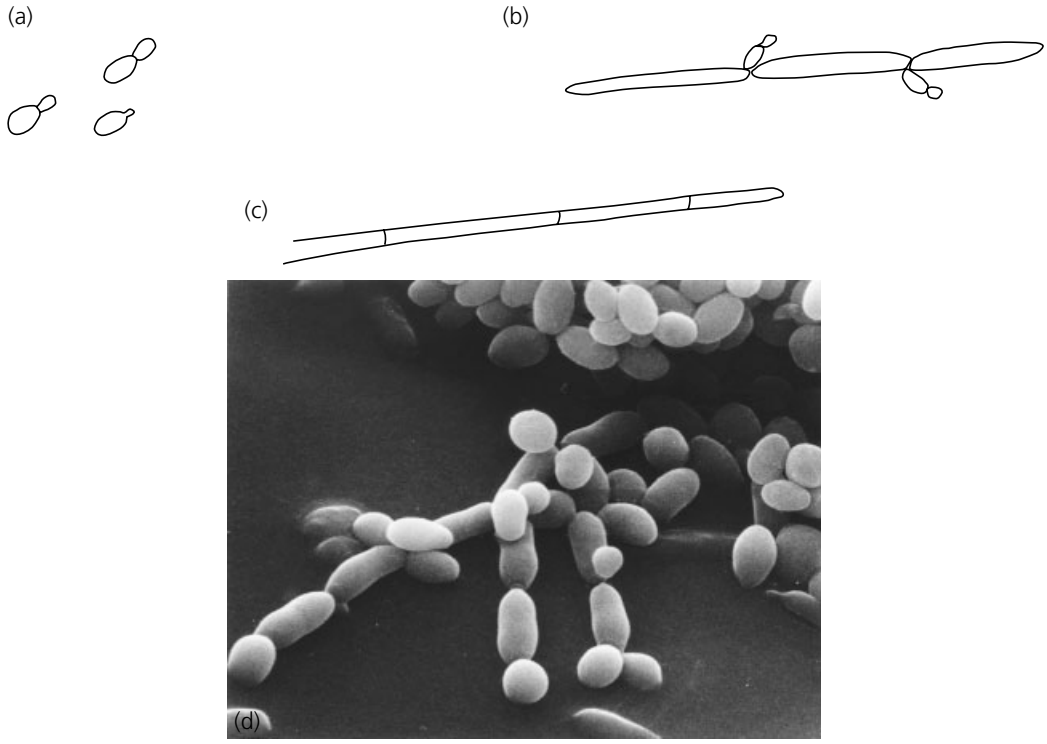

Figure 4 *The diagramatic representation shows morphological types of the yeast* Candida albicans. **(a)** *Yeast cells;* **(b)** *pseudohyphae and yeast cells;* **(c)** *a hypha;* **(d)** *budding yeast cells and pseudohyphae of* Candida kefyr *(magnification ×2 520). Courtesy of G.T. Cole*

which includes photomicrographs indicating how septin is deposited in a different fashion in the various morphological forms of *C. albicans*, i.e. budding yeast, hyphae, and pseudohyphae (Berman and Sudbery 2002). The pseudohyphal rather than the hyphal form of *C. albicans* appears to show most similarity with the 'filamentous' form of *S. cerevisiae*. At least four major signal transduction pathways appear involved in controlling the switch from yeast to filamentous forms of *C. albicans*.

Typically, *Candida* spp. grow as yeasts in media containing abundant glucose and oxygen, and isolates of many of these species can produce filamentous extensions when placed in conditions where the PCO_2 is raised and the glucose concentration is reduced. Such conditions are associated with growth under coverslips on cornmeal agar and in media used to test for germ-tube production by *C. albicans*. Other yeast species, such as *Candida glabrata*, a species which is more closely related to *S. cerevisiae* than to *C. albicans*, show no evidence for this capacity and, until recently, were widely considered to be species having no capacity to form filaments. However, as already alluded to above, research with *S. cerevisiae* has elucidated nutrient conditions that can induce polar cell growth causing production of pseudohyphae (Gimeno et al. 1993). Thus, even in species where there has been little evidence of dimorphism, there may exist an unexpected flexibility in growth pattern. Indeed, the haploid pathogenic basidiomycetous yeast species, *Cryptococcus neoformans*, can be induced to grow in a filamentous fashion when cultivated under conditions that differ from the standard glucose-rich media used in Sabouraud's agar or other media that are typically used when cultivating fungi in the clinical laboratory (Wickes et al. 1996). Before this discovery, a filamentous morphology had only been known for the teleomorph produced when two haploid strains mate.

Although it seems likely that a capacity to produce filamentous growth patterns will be found for many other yeasts that previously have not been considered dimorphic, it remains to be proven that the filamentous growth, seen only on certain media, plays any significant role in the establishment and pathogenesis of infections caused by either *Cryptococcus neoformans* or *S. cerevisiae*. By contrast, the yeast and filamentous forms of *C. albicans* behave so differently that it is likely that dimorphism is essential for maximal virulence. For example, they show different capacities for binding to epithelial cells and to several host proteins including complement components: such variability likely enables the fungus to colonize, grow, and become released from a variety of niches. The report that a mutant lacking mitochondrial NADH dehydrogenase complex I fails to develop filaments, though it grows normally as a yeast, suggests that energy demands for yeast and filamentous growth could be rather different with the hyphal form requiring relatively high production of ATP and

reducing power (McDonough et al. 2002). While differences in the metabolic state of cells might contribute to the induction of morphological variability, including white–opaque switching (next section), environmental cues are important for inducing the morphological switch as culture media containing very similar nutrients can support both types of growth. The morphological flexibility of *C. albicans* is so notable in that it is extremely rare to find clinical lesions where only one morphological form exists and, indeed in biofilms formed on plastic appliances, both forms are observed (Chandra et al. 2001).

INSTABILITY IN GROWTH PATTERN: 'PHENOTYPIC SWITCHING'

In addition to the changes in growth patterns associated with dimorphism, an additional form of morphological flexibility has been described whereby, at least in culture, certain species undergo 'phenotypic switching.' This process, first described for certain strains of *C. albicans*, differs from dimorphism in that the changes in growth form appear spontaneously, usually at a rate of between 1:1 000 and 1:10 000 progeny cells, (Slutsky et al. 1985). The different patterns of growth result in the generation of differently shaped colonies when these are cultivated on agar media. Most progeny colonies from a single colony type retain the growth pattern of the parental colony; however, some colonies develop where the pattern appears to have switched to one of a number of alternative phenotypes (Figure 5). It appears that phenotypic switching increases the capability of *C. albicans* to adapt to different environments and environmental stresses for switches of metabolic activity may also be found. Features of the switching process, including its relevance to pathogenesis, have been reviewed (Soll 1993).

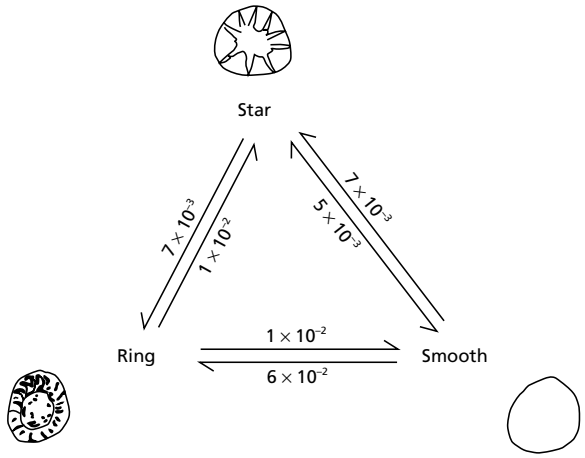

Figure 5 *Phenotypic switching in* Candida albicans. *Switching rates from one colony phenotype to another are examples taken from data showing switching between several distinctive colony phenotypes that were reported by Slutsky et al. (1985).*

A second phenotypic switching system, the white–opaque system, has been described and the regulation of genes expressed in each form has been investigated by using a microarray of *C. albicans* genes (Lan et al. 2002). There were substantial changes not only in the expression of genes that are likely to interact directly with the host, but also in the general metabolic activities of the yeasts in these two phases. In addition, there was an upregulation of the expression of mating type-associated genes in the opaque phenotype. This latter property correlates well with the recent discovery that the opaque cell type can undergo efficient mating (Miller and Johnson 2002), a feature that is not seen for the regular 'white' phenotype which is characterized by having a heterozygous mating type locus (*MTL***a**/**α**). In contrast, the mating type locus found in the opaque phenotype is homozygous (*MTL***a**/**a** or *MTL*α/α). This novel finding suggests a process by which gene flow can occur fairly easily in populations of *C. albicans*.

Phenotypic switching is now reported for several other fungal pathogens of humans including *Candida parapsilosis* and *Candida tropicalis*, which are both closely related to *C. albicans*, and for more distant species including *C. glabrata*, a species that is more closely related to *Saccharomyces*, and the basidiomycetous yeast *Cryptococcus neoformans* (cited in Soll et al. 2003). It has been clearly linked to pathogenicity in *C. albicans* and it is very likely to found in several other organisms, including plant pathogens and non-pathogens, which can exist in highly variable environmental niches where the switching may be coordinated by changes in the activity of global regulators controlling the expression of families of genes. Further insights into the signaling process leading to phenotypic switching could identify useful targets of antifungal chemotherapy.

STRUCTURES USED IN IDENTIFICATION OF FUNGI

Although there have been substantial advances in the use of molecular characteristics, such as antigens and nucleic acid sequences, for the identification of fungal pathogens, the morphological features of fungi still play a dominant role in allowing microbiologists to identify filamentous fungi. By far the most important taxonomic characters are those associated with the morphology of spores and fungal structures on or within which the spores are produced. With molds, spore-bearing hyphae typically grow aerially in a manner that raises the spores away from the vegetative hyphae. A substantial number of differential characteristics are associated with these specialized hyphae and the spores they bear, and these allow isolates to be identified both to genus and, very often, to species level. The vegetative hyphae, by contrast, tend to be fairly simple and show few characteristics that allow different species to be discriminated. However, certain hyphal features, including

pigmentation, size, and the type and abundance of the septa, may be helpful in eliminating particular species as choices in the identification of an unknown mold.

Many fungal propagules may be produced in an asexual process whereby their production does not require prior fusion of nuclei and a subsequent meiosis. Sporulating colonies that bear such conidia are known as anamorphs and, because more than one form of conidium may be produced by some fungi, there are examples where a single species can produce more than one anamorph. The sporulating structures that are associated with sexual spores that are produced after meiosis has occurred are named teleomorphs. The terms anamorph and teleomorph are also known as the imperfect and perfect states, and both are separately classified and named for a genus and species. As a single organism may produce both anamorphs and teleomorphs, many fungi have more than one name. It is important to realize that the different names do not describe different organisms; rather they describe different reproductive types. One sees a parallel among humans where a change of name does not alter the identity of the person.

The reason that more than one name is used for a fungus is largely based on practicality. Most fungi are heterothallic, i.e. they have mating types that ensure outbreeding, and for these species the production of teleomorphs requires the mating of different strains. As most clinical isolates involve a single strain, it would be necessary to employ a battery of tester strains were we to try to identify each anamorph via the production of its teleomorph. Not only is this inconvenient compared with naming a fungus on the basis of its anamorph, but conditions for obtaining teleomorphs have not been found for many important pathogenic species, for example *Aspergillus fumigatus* and *Coccidioides immitis*. Even when a teleomorph is known, the anamorphic form may have been studied for many decades before the discovery of the perfect state. Such situations exist for many important pathogens that are primarily known by the name given to their anamorphs. Such species include *B. dermatitidis*, *H. capsulatum*, *Cryptococcus neoformans*, and many species of dermatophytes within the genera *Trichophyton* and *Microsporum*. Imposition of nomenclature based on teleomorphs, for these examples *Ajellomyces dermatitidis*, *Ajellomyces capsulatus*, *Filobasidiella neoformans*, and species of *Arthroderma*, would be very confusing for most physicians and clinical microbiologists.

The major groups of anamorphic sporulation structures associated with infectious fungi are illustrated in Figures 6, 7 and 8. There are two major groups of asexual propagules. Those propagules that are produced as naked, asexual, nonmotile propagules, and which usually fall away from the parent hypha or yeast, are known as conidia. The specialized hypha on which they are formed is termed a 'conidiophore.' There are two basic ways that conidia are produced. Blastoconidia differentiate after ballooning out of a fertile hypha and are characterized by developing a conidial wall that is produced *de novo* (Figure 6). By contrast, those conidia that are produced via thallic conidiogenesis (Figure 7) develop from a preexisting hypha where modification of the cell wall and the cell contents leads to the development of the conidium (Cole and Samson 1979).

The second major form of asexual sporulation involves the production of sporangiospores (Figure 8). These asexual spores are only produced by fungi within the phylum Zygomycota. The most common form of sporangiospore production is associated with the multispored sporangium which has the appearance of a swollen vesicle produced on the end of a specialized hypha, the sporangiophore. Within the sporangium the spores are formed after nuclear divisions and cytoplasmic cleavage have taken place. After this, new spore walls are synthesized and these surround the spore initials (Cole and Samson 1979). Once the sporangiospores are mature, the sporangial wall breaks open to allow their release. In species that produce sporangiola, such as those within the genus *Cunninghamella*, the spores are pushed out of the swollen ends of the branches produced by the sporangiophore and the spore wall becomes fused to the sporangial wall. At first sight the sporangiola can resemble conidiophores that bear blastoconidia, but their pattern of spore development is quite different (Cole and Samson 1979).

Current taxonomic studies are increasingly becoming dependent on molecular genetic analysis, especially with the introduction of several allied organisms including agents of the Mesomycetozoea that were discussed in the introduction to this chapter. However, within the true fungi, the study of teleomorphs has been most useful in clarifying the systematic position of many anamorphic fungi and yeasts. For any one species, one type of spore is produced by the teleomorph. Species from within the phyla Ascomycota, Basidiomycota, and Zygomycota produce, respectively, ascospores, basidiospores, and zygospores.

Spores produced by teleomorphs appear rarely to be involved with human infections although their infectious potential has seldom been studied. However, there are certain situations where the sexually derived spores appear important. In black piedra, the asci and ascospores of *Piedraia hortae* are found in the nodules that develop on infected hairs. Also, basidiospores are the most likely source of infection in the few cases of invasive disease that have been reported for the mushrooms, *Schizophyllum commune* and *Coprinus cinereus*.

CONIDIAL STRUCTURE AND GERMINATION

Asexual propagules produced by the infectious fungi are the form of the fungus responsible for establishing most human infections, especially those infections that follow pulmonary exposure. Fungal conidia also play an

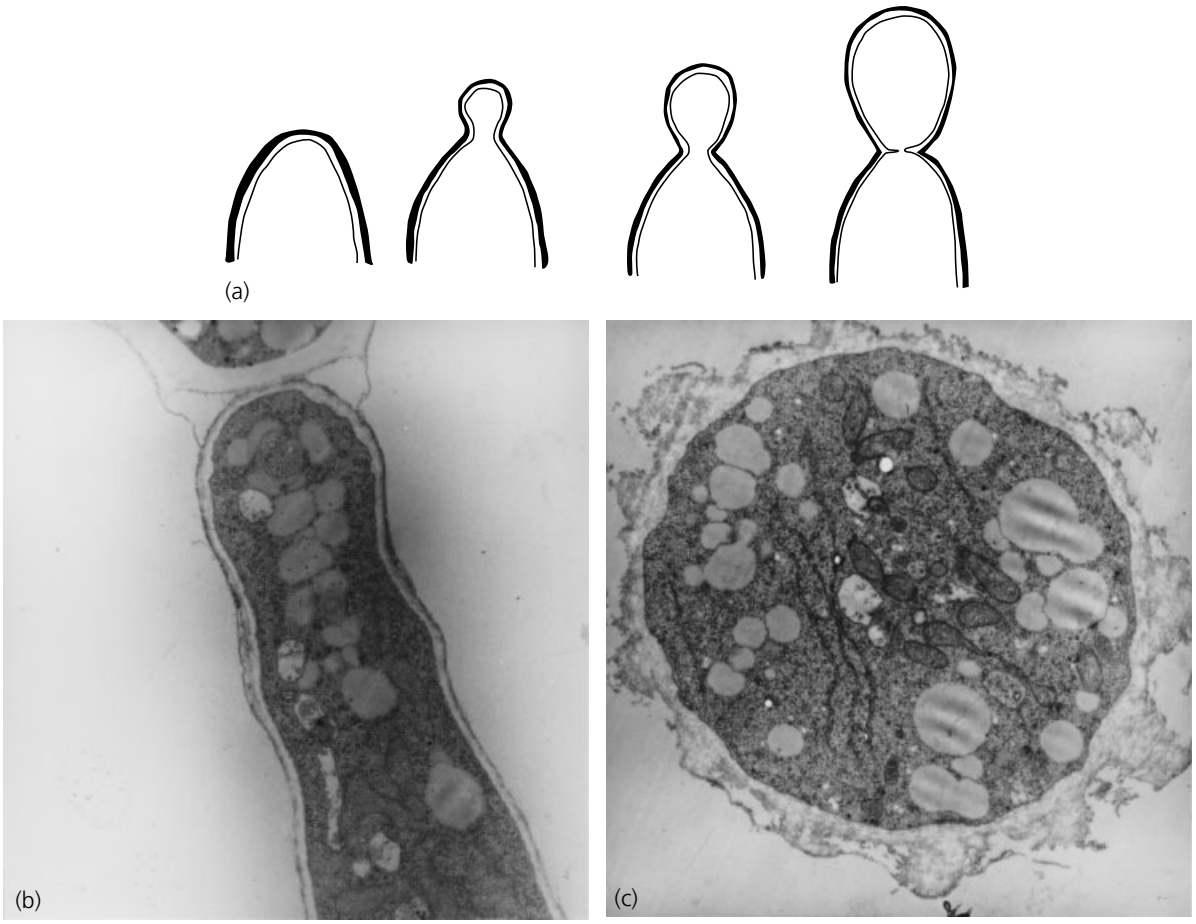

Figure 6 (a) *Conidiospore development by blastic conidiogenesis where the conidium is pushed out of a conidiophore and its cell forms newly synthesized cell wall material. The inward growth of the inner wall seals the area of emergence.* **(b)** Scopulariopsis brevicaulis *conidiophore (magnification ×11 000) and* **(c)** *mature conidium (magnification ×14 350). Courtesy of G.T. Cole*

important role as allergens and may carry mycotoxins. Although the sexual reproductive process has been studied extensively because of its importance in identification, the biological activities of the fungal propagules produced by pathogens of humans have not received the same scrutiny.

It is clear that the conidium's cell wall can show quite different surface features to the walls of vegetative cells. In many cases it can be quite hydrophobic. The components responsible for the hydrophobicity appear in the form of sheets of rodlets that have been named 'rodlet fascicles.' These, depending on the species and strain, can be extracted from the conidial surface by use of water and detergents (Cole and Samson 1984; Sietsma and Wessels 1994). The hydrophobic rodlet material resembles the hydrophobins that are found in mushroom fruiting bodies, but the latter require more powerful solubilization techniques, such as the use of trifluoroacetic acid (Lugones et al. 1996).

Conidia germinate by producing germ tubes that represent hyphal outgrowths. Regardless of conidium type, the germination process usually requires the presence of CO_2, oxygen, and water for activation to occur. In many cases, exogenous low molecular weight nutrients may also be required. When conidial germination inhibitors are present in the conidia, they can be leached into the surrounding liquid and their dilution allows the conidium to begin to germinate. Germination is associated with the swelling of the conidium where water is taken up and the wall begins to expand. At the same time, there is a marked increase in metabolic activity that is observable before the emergence of the germ tube. When cell wall deposition becomes polarized, the germ tube forms. Though much research has been done on factors influencing the germination of spores, there have been relatively few studies on the molecular controls of the process in different fungi. Spores often contain large quantities of trehalose, a disaccharide that is hydrolyzed to glucose by spore-associated trehalases. These may require activation during germination or, if in a separate cellular compartment such as the cell wall, may be brought into contact with trehalose when the spore swells (D'Enfert 1997).

Germination signals can vary for different species and for different conidial types produced by a single species. For example, the arthroconidia of *Trichophyton*

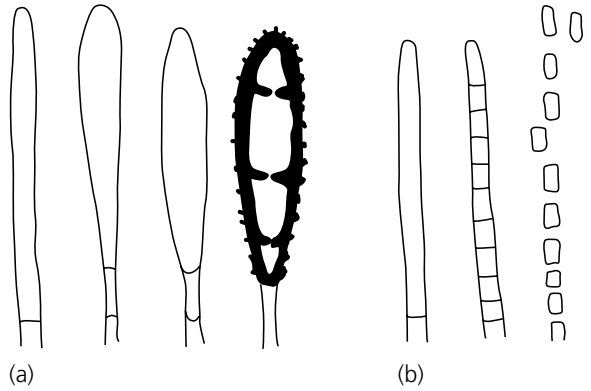

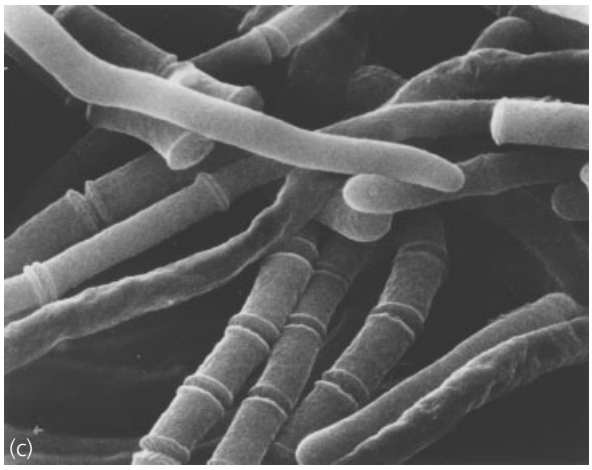

Figure 7 *Conidiospore development by thallic conidiogenesis where a conidium forms after remodeling of existing hyphal walls.* **(a)** *Development of an aleuriconidium after swelling, septation, and ornamentation of a hypha.* **(b)** *Development of arthroconidia after formation of cross-walls that allow disarticulation of the hypha.* **(c)** *Hyphae and arthroconidia formed by a species of* Trichosporon *(magnification ×1 680). Courtesy of G.T. Cole*

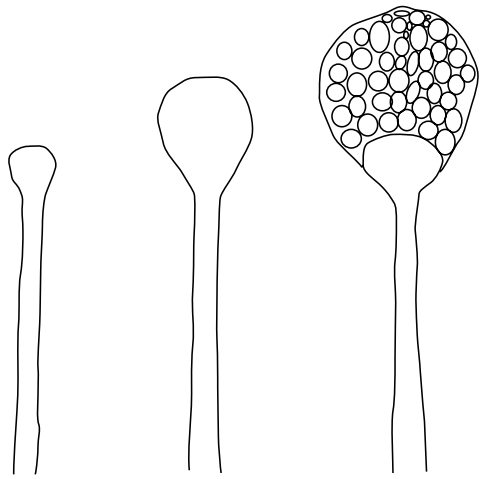

Figure 8 *Sporangiospore development: spores form after division of nuclei and cleavage of the cytoplasm which accumulates within the swollen tip of the sporangiophore. Mature sporangiospores are formed within a sac (the sporangium) and are released when this ruptures.*

mentagrophytes germinate after being 'activated' by either a short exposure to elevated temperature (45°C, 10 minutes), or by soaking in water at room temperature for 24 hours. After this pretreatment, the conidia germinate in the absence of exogenous nutrients, producing germ tubes when held in water at 37°C. By contrast, the microconidia of *T. mentagrophytes* do not germinate in the absence of exogenous amino acids or dipeptides. Such differences in germination requirements may well explain the poor infectivity of microconidia when compared to arthroconidia produced by the same dermatophyte (Hashimoto 1991).

FEATURES OF FUNGI ASSOCIATED WITH PATHOGENICITY AND VIRULENCE

Much has been written concerning those characteristics of pathogens that contribute to their virulence. Certain properties are necessary for effective pathogenicity, for example, tolerance of body temperatures and ability to grow using the nutrients available in tissues. But a successful pathogen must also be able to establish an infection, a process that not only involves ensuring that the host is exposed to infectious propagules but also that, once exposed, the propagules can survive and grow in the face of a mobilized host defense. In the case of the pathogens that infect via the lungs, spore size can be of great importance. For example, the conidia of *Aspergillus fumigatus* are small enough to enter the alveoli, a feature that is not the case for all aspergilli.

There is a significant body of work demonstrating the importance of the antiphagocytic capsule to the virulence of *Cryptococcus neoformans*. But other features, such as the production of phenoloxidase (a laccase), can play a role in virulence when this species invades the central nervous system. The features associated with virulence are considered in the chapters concerning the different fungi.

Normal cellular processes such as the signal-transduction cascades, exemplified by the MAP kinases, have been found to be necessary for normal development and virulence in *C. albicans* and *Cryptococcus neoformans*. The specificity of pathway selection and activation during host–parasite interactions is an area of active research and it seems likely that at least some of these signals induce the production of virulence factors by pathogens (Lengeler et al. 2000). However, this may not turn out to be the case for many of those opportunistic fungi that are saprophytes and are very unusually seen as pathogens in humans or animals. In a similar fashion to the situation with signal transduction pathways, certain steps in metabolic pathways may need to be present for effective pathogenicity. Auxotrophic mutants can have greatly diminished virulence, a feature reported for *Aspergillus nidulans* (Purnell 1973), *Cryptococcus neoformans* (Perfect et al. 1993), and *C. albicans* (Guo and Bhattacharjee 2003). While such 'normal'

processes may not play a central role in host–parasite interactions, they might well provide useful targets for antifungal drugs.

Evidence of fungal components being important in virulence has been sought by using molecular genetic methods. Typically, a protein that is thought to be important in virulence may be inactivated, usually by generating a mutant. The most efficient way to generate targeted mutations where reversion does not occur has been to disrupt the gene encoding the target protein. After gene disruption and demonstration that the protein is no longer expressed, the significance of the protein may be estimated by comparing the virulence of the mutant to the original wild-type. Should the mutant show reduced virulence, it is then essential to show that it regains virulence when a functional target gene is reinserted. What has become clear is that most fungi appear to exploit numerous components for virulence and that simple disruption of one gene may not have much obvious effect when there are several genes encoding a particular activity, for example with serine proteinases or chitin synthetases. In some cases, gene disruption shows significant effects on certain stages in the infectious process: for example phospholipase B1 mutants of *C. albicans* retain some pathogenicity but have reduced invasive capacity (Ghannoum 2000). Details of current knowledge on fungal virulence factors are beyond the scope of this introductory chapter.

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Laboratory diagnosis

GLENN D. ROBERTS AND NORMAN L. GOODMAN

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GENERAL STATEMENT

The diagnosis of a fungal disease is a complicated event requiring the cooperation and collaboration of many people with diverse expertise. Most fungal diseases require a laboratory diagnosis, so it is imperative that the laboratory is in close communication with the physician. This is especially important with the dramatic changes in potential hosts for fungal diseases in this era of immunosuppression and transplantation. As the host has changed, so has the range of potentially 'pathogenic' fungi. It is now well documented that many fungi in the environment may cause disease in severely compromised human hosts. The physician and laboratorian must thus be prepared to consider any fungal isolate as being etiologically significant and take appropriate measures to incriminate or disprove its role in each patient's case. The most commonly used methods for the laboratory diagnosis of fungal infections are, in general, old and time-honored. The recovery of the etiologic agent from clinical specimens is still considered to be the 'gold standard.' Methods used for identification of the fungi are a mixture of traditional and newer commercially available systems, e.g. yeast identification kits. Molecular methods are being developed for the detection and identification of fungi at a very fast rate; however, no comparative studies have been done to determine which are optimal and no standards for testing have been developed to date. Molecular testing is the future for many areas of clinical mycology but it will be some time before routine testing will be standard practice within the laboratory. A section at the end of this chapter will present a preview of what has been done and some insight in to what will be useful in the future.

SPECIMEN SELECTION

The laboratory diagnosis of a fungal disease starts with careful collection of the appropriate clinical specimen. The specimen must contain the viable etiologic agent, if it is to be recovered and identified. The anatomic site in which the organism is present must be carefully selected and the specimen collected in such a manner that it will allow the fungus to remain viable in its 'natural' state, without contamination (Merz and Roberts 2003). Sites from which to collect specimens for the most common mycoses are given in Table 1.

When the appropriate site is selected, the specimen should be placed in a suitable container and promptly sent to the laboratory.

SPECIMEN COLLECTION

Specimens that are inappropriately collected, stored, or not processed on time may cause misdiagnoses, delays in appropriate therapy and increased costs for the patient.

The following are guidelines for specimen collection:

- Collect the specimen from an active lesion; old 'burned-out' lesions often do not contain viable organisms.

Table 1 *Selection of clinical specimens for fungal culture: order of preference for site selection*[a]

Specimen	Blastomycosis	Coccidioidomycosis	Histoplasmosis	Paracoccidioidomycosis	Candidiasis	Cryptococcosis	Aspergillosis	Zygomycosis	Hyalohyphomycosis	Dermatophytosis	Chromoblastomycosis	Pheohyphomycosis	Sporotrichosis	Mycetoma
Lower respiratory tract	1	1	1	2	x	1	1	1	3	–	–	–	3	–
Blood	–	6	2	–	1	2	–	–	2	–	–	–	x	–
Bone	4	x	–	x	–	x	x	x	–	–	–	–	x	–
Bone marrow		x	3	x	x	x	–	–	x	–	–	–	–	–
Brain	x	x	–	x	x	x	x	3	x	–	x	2	–	–
Cerebrospinal fluid	x	x	x	–	x	5	–	–	–	–	–	–	x	–
Eye	–	–	x	–	x	–	x	x	5	–	–	–	–	–
Nose–nasal sinus	x	x	–	x	x	–	2	2	4	–	–	–	4	–
Prostate	x	x	–	–	x	–	–	–	–	–	–	–	–	–
Mucous membrane	3	2	5	3	4	x	x	x	x	–	x	–	x	–
Subcutaneous tissue	–	x	–	–	x	–	–	x	–	–	2	1	2	1
Joints	x	x	–	–	x	–	x	–	x	–	–	–	–	–
Urine	5	5	4	–	2	3	x	–	–	–	–	–	–	–
Skin	2	3	x	1	x	x	x	4	1	1	1	–	1	2
Hair and nails	–	–	–	–	x	–	x	–	x	2	–	–	–	–
Multiple systemic sites during disseminated infection	6	4	6	4	3	4	3	5	6	–	x	–	x	–

a) Predominant sites for recovery of organism are ranked in order of importance (based on most common clinical presentations). x indicates other sites from which organisms have been recovered.

- Collect the specimen under aseptic conditions.
- Collect a sufficient amount of the specimen.
- Collect specimens before instituting therapy.
- Use sterile collection devices and containers.
- Label the specimens appropriately; all clinical specimens should be considered as potential biohazards and should be handled with care using universal precautions.

Respiratory tract specimens

The most common specimen for the diagnosis of fungal pneumonia and chronic pulmonary disease is sputum. The best specimen can be obtained when the patient first awakens from sleep, after the oral cavity has been cleansed. Bronchial alveolar lavage (BAL) and bronchial brushings are usually superior to sputum, provided that double- or triple-lumen bronchoscopes are used to prevent upper respiratory microbial contamination. Well-collected lower respiratory specimens are very useful for direct microscopic observation and culture for fungi.

Sterile body fluids (cerebrospinal, peritoneal, pericardial, synovial and vitreous humor)

These provide excellent specimens because there are usually no contaminating organisms present. Sterile fluids should be obtained under aseptic conditions using a sterile syringe. The specimen should be transferred to a sterile, closed tube for transport to the laboratory. Small amounts of fluid can be transported in the syringe, after the needle has been removed and the syringe re-capped.

Tissue

Tissue from the site of active disease process is the ideal specimen for diagnosing a mycosis and it should be collected under strict aseptic conditions. It should be placed into a sterile, closed container of the appropriate size. An adequate volume of sterile saline, without preservative, should be added to the container to keep the tissue moist. If the tissue cannot be processed immediately, it may be refrigerated, but not frozen, before it is examined microscopically and cultured.

Exudates/pus

Specimens from closed lesions and abscesses are usually of high quality. The outer surface of the site should be thoroughly cleansed and decontaminated. The specimen should then be aspirated with a syringe and needle and transferred to a sterile, closed container. Swabs should never be used to collect a specimen from a closed lesion that has been surgically opened. Drainage from open lesions should be collected only after the lesion has been thoroughly cleansed. Care should be taken to collect any granules that may be present in specimens from open or closed lesions. If swabs must be used to collect a specimen from an open lesion, a maximum amount of material should be collected on the swab and kept moist *en route* to the laboratory.

External eye

Corneal scrapings should ideally be placed directly on to the surface of fungal culture media and on to the center of a clean, glass microscope slide to be used for direct microscopic examination. Fluid from an eye should be aspirated with a sterile syringe and needle and, if only a small quantity is present, transported to the laboratory in the syringe. Quantities greater than 0.5 ml should be transferred to a sterile tube for transport to the laboratory (Jones et al. 1981).

Urine

Urine specimens (50–200 ml) should be placed in a sterile, screw-capped container. Urine specimens should be freshly collected. Specimens from catheter bags are unacceptable. Urine may be refrigerated if it cannot be cultured promptly.

Blood

Blood should be collected under strict aseptic conditions. The specimen may be collected directly into a culture system or with a syringe, and then transferred to an appropriate culture medium. Strict aseptic conditions must be maintained in all situations (see section on Blood cultures below for more details).

Stool

Under very special circumstances, fecal cultures for fungi are appropriate, e.g. profoundly immunosuppressed bone marrow transplant recipients. The specimen should be collected in a sterile, screw-capped container or on two rectal swabs.

Hair

Infected hair should be drawn from the scalp with forceps and placed into a clean, dry container. Clipped hair is not acceptable because the root is the prime specimen. It is not necessary to use a sterile container. Hair for culture should be free of topical medications, conditioners or dressings.

Skin

Specimens of skin may be obtained by scraping or biopsy. A site on the active border of the lesions should be used. As skin is usually contaminated with other microorganisms, the surface of the selected site should be cleansed with 70 percent isopropyl alcohol. The epidermis should be scraped with a scalpel or other instrument and the scrapings placed in a clean, dry container, such as a Petri dish, specimen cup or envelope. Biopsied specimens should be placed in a sterile container.

Nails

Nails should be cleansed with 70 percent alcohol. A portion of the infected nail should be clipped and the excess keratin scraped from the nail bed. The clippings and scrapings should then be placed in a clean, dry container.

SPECIMEN TRANSPORT AND STORAGE

For best results, all clinical specimens should be microscopically examined and cultured as soon as possible. Except for blood and corneal scrapings, it is not necessary to transport the specimen in a transport medium. Blood should be placed in the preferred culture system or the lysis centrifugation system (Isolator, Carter Wallace Wampole Laboratories, Cranbury, NJ, USA). Corneal scrapings should be inoculated onto the medium when collected. Specimens should not be frozen before culture. Specimens not likely to contain contaminating microorganisms, e.g. spinal fluid, and those that could contain dermatophytes should not be refrigerated. Transported specimens should never be allowed to dry out. Sputum specimens may be transported without preservation if only dimorphic fungi are to be recovered (Hariri et al. 1982).

SPECIMEN PROCESSING

When specimens reach the laboratory, they must be appropriately processed to ensure viability of the etiologic agent and to minimize the chances of contamination. Specimens from normally sterile sites, e.g. spinal and peritoneal fluids, need no special processing, but may be cultured directly onto primary recovery media. If the volume is adequate, specimens should be concentrated by centrifugation membrane filtration, and the concentrated specimen aseptically transferred to primary recovery media for culture and on to a clean microscope slide for direct microscopic examination. Aseptically collected tissue should be homogenized in a tissue grinder, mortar pestle or 'stomacher.' A portion of tissue suspected to contain a zygomycete should be finely minced with a pair of scissors or scalpel.

A highly viscous specimen, such as sputum, should be liquefied before culture, but it should not be excessively diluted. It should be concentrated by centrifugation if it is too dilute.

CULTURE

Most of the mycotic diseases are diagnosed by recovering and identifying the etiologic agent in the clinical laboratory. Few fungal diseases can be diagnosed clinically. The recovery of fungi from a clinical specimen begins with the selection of appropriate primary culture media that must include one that will support the growth of any potentially pathogenic fungus in the specimen and others that will selectively inhibit contaminating microorganisms. These media must be selected with great care; however, a number of options are available.

Media

Fungi may be recovered on most bacteriological media if given enough time to grow. There are, however, well-established fungal culture media, that are optimal for the recovery of fungi from clinical specimens. The following are some of the most commonly used media for recovering fungi from clinical specimens: Sabouraud's dextrose agar; Emmons' modified Sabouraud's agar; brain–heart infusion (BHI) agar (with or without 5 percent sheep red blood cells), Sabouraud's brain–heart infusion (SBHI) agar; inhibitory mold agar (IMA); mycosel or mycobiotic agars; potato flake agar; and yeast extract phosphate agar.

Combinations of antibacterial antibiotics and cycloheximide may be added to these media to make them selective. Suggested uses for these media are presented in Table 2 and, as indicated, a battery of different media should be used to culture specimens when more than one organism is expected to be recovered.

As in all areas of microbiology, the key to rapid and accurate identification of an organism is a pure culture. Isolated colonies are needed for morphological or biochemical identification. It is, therefore, necessary to spread specimens suspected of containing multiple organisms on to a large surface area. Petri dishes are generally optimal but others, such as tissue culture flasks or large bottles, are adequate. Standard, microbiological, culture tubes should not be used because colonies cannot be easily isolated from fluid specimens such as sputum and urine.

Incubation conditions

Fungi commonly associated with disease will grow well at temperatures between 25°C and 30°C. It is generally accepted that 30°C is the optimal temperature for

Table 2 *Media useful for the recovery of fungi from clinical specimens*

Specimen	Inhibitory media (bacteria and/or saprobic fungi)[a] with 5% sheep blood	Inhibitory media (bacteria and/or saprobic fungi)[b]	Yeast extract–phosphate agar
Lower respiratory tract	x	x	x
Secretions (e.g. sputum)	x	x	x
Tissue (lung or pleura)	x	x	–
Bone marrow	x	x	–
CSF	x	x	–
Eye	–	x	–
Nares/nasal sinuses	x	x	–
Skin/mucous membrane lesions	x	x	–
Tissue biopsy (not contaminated)	x	x	–
Contamination suspected	x	x	–
Urine	x	x	–

a) Includes brain–heart infusion (BHI) agar.
b) Includes Sabouraud's dextrose agar; Emmons' modification of Sabouraud's dextrose agar; BHI agar; Sabouraud's brain–heart infusion (SBHI) agar; inhibitory mold agar (IMA); mycosel or mycobiotic agars and potato flake agar.

recovery of fungi from clinical specimens, although 'room' temperature (25–30°C) is adequate. Care should be taken to provide a humid environment, primarily to prevent plated media from drying during the incubation period of 4–6 weeks. Adequate moisture can usually be achieved by placing containers of water within the incubator or placing plates in a sealed oxygen-permeable plastic bag. Other specific incubator environments are necessary for special procedures; e.g. conversion of dimorphic fungi from the mold to the yeast form requires a temperature of 35–37°C and a moist environment. Higher temperatures may be needed to test for temperature-tolerance studies, which will differentiate some fungi. It should be noted that growth at 37°C is not a requirement for pathogenic fungi that may infect anatomic sites having a lower temperature.

Blood cultures

Fungemia has become a more common event, particularly in immunosuppressed patients and those who receive broad-spectrum antibiotics, or in those with intravascular devices in place. It is common for nosocomial fungal infections to develop in these groups of patients. Further, fungemia caused by dimorphic fungi has increased over the past few years. Laboratories must be sufficiently proficient to offer a reliable blood culture system that will recover the etiologic agent in the shortest possible time.

A number of variables must be carefully considered in the provision of optimal recovery of fungi from blood. Blood should be collected at intervals as described for the detection of bacteremia (Geha and Roberts 1994). The volume of blood cultured is critical in ensuring optimal recovery with, in general, the larger the volume collected, the greater the chance for recovery. It is necessary to obtain at least 10 ml of blood in a 1:10 ratio of blood to broth in bottles, or 10 ml inoculated on to the surface of several different media after lysis-centrifugation. Automated systems may allow for use of a larger amount of blood for inoculation.

Blood culture bottles must be vented, either transiently or permanently, and the temperature of incubation should range from 25 to 30°C, with the higher temperature being preferable. The duration of incubation for most isolates of yeasts is one week, although the range of time for recovery of dimorphic fungi may extend to 4–6 weeks. Using lysis-centrifugation (with an Isolator), 3 weeks of incubation seems to be adequate. The average recovery time for yeasts and molds using the lysis-centrifugation method is 3.8 and 10.5 days, respectively (Telenti and Roberts 1989).

Media used for the recovery of fungi from blood vary with the system used in the laboratory. For the most part, media such as those used in commercially available bottles will recover yeast species adequately. The lysis-centrifugation method allows the user to select the solid media used. Yeasts are often recovered by employing sheep blood or chocolate agars. The addition of BHI, IMA and Sabouraud's dextrose agars allows for the recovery of all fungal pathogens within 2 weeks of incubation.

As a rule, broth cultures require about twice as long for recovery as the lysis-centrifugation method. *Cryptococcus neoformans* may not be recovered or the time for its recovery is significantly delayed by commercially available systems. Furthermore, the dimorphic fungi are frequently not recovered using broth media or commercial systems. However, a Myco/F/Lytic culture medium is reported potentially to offer an alternative method for the recovery of dimorphic fungi from blood (Waite and Woods 1998; Vetter et al. 2001). Other studies have not shown the Myco/F/Lytic culture medium to be superior to previously reported methods (Fuller et al. 2001).

The detection of fungemia has improved remarkably over the years and recovery time is clinically relevant. The lysis-centrifugation system is optimal when considering the recovery of all agents of fungemia; when only yeasts are considered, however, broth media or commercially available systems are probably satisfactory. The lysis-centrifugation method is preferred for the recovery of *Histoplasma capsulatum* and *Cryptococcus neoformans*; the system also has the added benefit of providing quantitative cultures.

DIRECT MICROSCOPIC EXAMINATION

A definitive diagnosis of most fungal diseases requires recovery and identification of the fungus from cultures. This often requires a period of several days to weeks for the organisms to produce enough growth and sporulation for identification. A tentative or preliminary diagnosis can often be made by microscopically detecting fungal elements present in the clinical specimen. A well-trained person, with knowledge of fungal morphology, can often provide a definitive diagnosis by this method or at least provide a tentative diagnosis days or weeks before culture confirmation.

The direct microscopic examination of clinical specimens may be as simple as placing a drop of liquid specimen on to a clean microscope slide and examining it with a light microscope, or it may involve more complex procedures, including staining of tissue. The sensitivity of direct examination depends on several factors, including quality of specimen, specimen type, quality of the microscope and, not least, the expertise of the microscopist in recognizing fungal elements and their diagnostic morphology.

If the specimen is transparent, it may be examined without treatment. If it is opaque, it must be cleared to reveal fungal elements, or the elements must be differentially stained within the specimen.

Treatment with potassium hydroxide (KOH) will clear the specimen of cells or debris Calcofluor white (Hageage and Harrington 1984); Gram's, Giemsa, Wright, periodic acid–Schiff (PAS), methenamine silver, and Papanicolaou's stains (Chandler et al. 1989; Conner et al. 1997) are satisfactory for use in detecting fungi in clinical specimens. These procedures may be performed in different areas of a laboratory, e.g. cytology, surgical pathology, hematology or microbiology; but it must be remembered that the above factors affecting sensitivity and specificity apply to any laboratory. The morphology of fungi appears essentially to be the same in other clinical specimens as it does in tissue, so it is possible to become very proficient in reading and interpreting results from a variety of specimens. Methods commonly used for detecting fungal elements in clinical specimens are presented in Table 3.

Fungi demonstrate enormous diversity in morphological features. Environmental factors play a significant role in determining how a fungus grows, so morphological diversity of fungi therefore should be expected in diseased tissue. Despite this, there is reasonable stability in the general morphology of the groups of fungi found in clinical specimens. Yeast forms are readily identifiable, but hyphae may be pleomorphic, depending on the source and host response. The microscopist must view a sufficient number of fields to ensure observation of the true morphological form of a fungus. Direct microscopic examination of clinical specimens can be very helpful in providing an early diagnosis for the clinician. The characteristics of the fungal elements commonly found in tissue and other clinical specimens are given in Table 4.

Examples of yeasts, pseudohyphae, septate and non-septate hyphae in tissue are shown in Figures 1, 2, 3 and 4.

METHODS OF IDENTIFICATION

When a fungus is recovered from a clinical specimen, a decision must be made as to its importance as a cause of disease. It is very helpful to know if the isolate is a 'classic pathogen' or one of the opportunistic fungi asso-

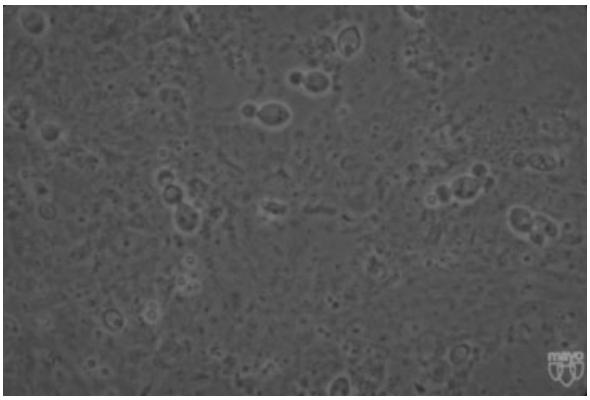

Figure 1 *Wet preparation of* Histoplasma capsulatum *var.* capsulatum *yeasts in bronchial lavage.*

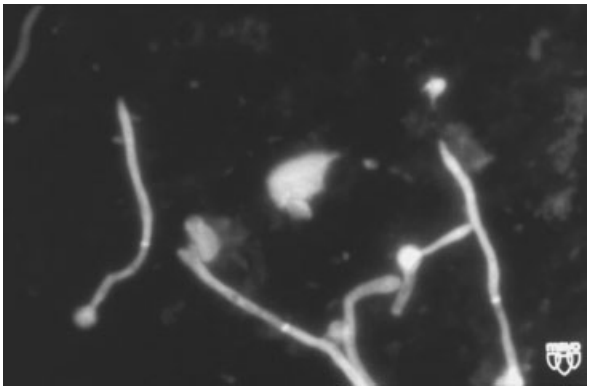

Figure 2 *Wet preparation of pseudohyphae of* Candida albicans *in scraping of oral lesion.*

Table 3 *Selected methods useful for direct microscopic detection of fungal elements in clinical specimens*

Method	Use	Time required	Advantages	Disadvantages
Calcofluor white	Detecting fungi	1 min	Can be mixed with KOH; fungi brightly fluoresce	Requires fluorescence microscope and special filters. Background sometimes fluoresces but fungi exhibit more intense fluorescence
Giemsa stain	Examining bone marrow and peripheral blood smears	15 min	Detects intracellular *H. capsulatum*	Detection is usually limited to *H. capsulatum*
Gram's stain	Detecting bacteria	3 min	Commonly performed on most clinical specimens submitted for bacteriology and detects most fungi present	Some fungi stain well; however, others (e.g. *Cryptococcus* spp.) stain weakly in some instances and exhibit only stippling. Common GS artifacts appear as yeast cells
India ink	Detecting *C. neoformans* in CSF and other body fluids	1 min	When positive in CSF, diagnostic of cryptococcosis	Negative in many cases of meningitis (non-AIDS)
Potassium hydroxide (KOH)	Clearing of specimens to make fungi more readily visible	Variable, depending on specimen	Rapid detection of fungal elements	Experience required; background artifacts are often confusing
Methylene blue	Detecting fungi in skin scrapings	2 min	Usually added to KOH; provides contrast for detection of fungal elements	Background staining of cells makes reading difficult
Methenamine silver stain	Detecting fungi in histological sections	1 hour	Best stain for detecting fungal elements	Requires specialized staining method not usually available in microbiology laboratories
Papanicolaou stain	Examining secretions for presence of malignant cells	30 min	Cytotechnologist can detect fungal elements	Requires specialized staining and a reader familiar with this stain
PAS stain	Detecting fungi	20 min; 5 min additional if counterstain is employed	Stains fungal elements well; hyphae and yeasts can be readily distinguished	*B. dermatitidis* appears pleomorphic. PAS-positive artifacts can appear as yeast cells
Wright stain	Examining bone marrow and peripheral blood smears	7 min	Detects intracellular *H. capsulatum*	Detection is usually limited to *H. capsulatum*

PAS, periodic acid–Schiff.

Figure 3 *Preparation of non-septate hyphae in bronchial washings stained with calcofluor white.*

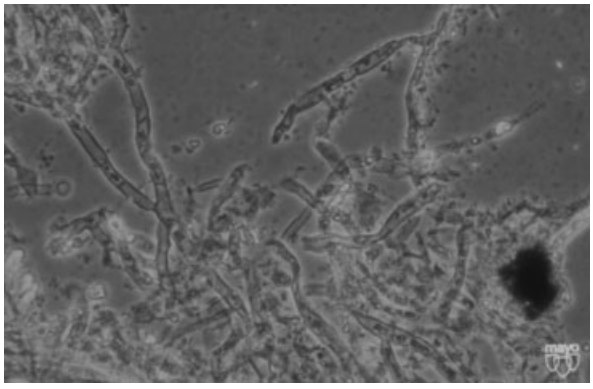

Figure 4 *Wet preparation of septate hyaline hyphae in bronchial washings.*

Table 4 *Characteristics of fungal elements seen in direct examination of clinical specimens*

Morphology of fungal elements	Organism(s)	Diameter range (μm)	Characteristic features
Yeast form	*Blastomyces dermatitidis*	8–15	Cells usually large and spherical, doubly refractile; buds usually single and attached by broad base, small forms of 2–5 μm may be seen
	Cryptococcus neoformans	2–15	Cells vary in size; usually spherical but may be football shaped; buds usually single and 'pinched off'; capsule may or may not be evident; rarely, pseudohyphae forms with or without a capsule may be seen
	Histoplasma capsulatum var. *capsulatum*	2–5	Small; oval to round budding cells; often found clustered within histocytes; difficult to detect when present in small numbers
	Paracoccidioides brasiliensis	5–60	Cells usually large and surrounded by smaller buds around periphery ('mariner's wheel appearance'); smaller cells (2–5 μm) that resemble *H. capsulatum* may be present; buds have 'pinched-off' appearance
	Sporothrix schenckii	2–6	Small; oval to round to cigar shaped; single or multiple buds present; often not seen in clinical specimens
Yeast form and pseudohyphae or true hyphae	*Candida* spp.	3–4 (yeast forms); 5–10 (pseudohyphae)	Cells usually exhibit single budding; pseudohyphae, when present, are constricted at ends and remain attached like links of sausage; true hyphae, when present, have parallel walls and are septate
Yeast form and hyphae in ski Yeast form in tissues	*Malassezia furfur*	3–8 (yeast forms); 2.5–4 (hyphae) 2.5–4.0 (yeast)	Short, curved hyphal elements usually present along with round yeast cells that retain their spherical shapes in compacted clusters; when found in skin exhibit monopolar budding, small broad base at point of attachment of buds
Spherules	*Coccidioides immitis*	10–200	Spherules vary in size; some contain endospores, others are empty. Adjacent spherules may resemble *B. dermatitidis*; endospores may resemble *H. capsulatum* but show no evidence of budding. Spherules may produce multiple germ tubes if direct preparation is kept in moist chamber for ⩾24 hours; hyphae may be found in cavitary lesions
Sporangia	*Rhinosporidium seeberi*	6–300	Large, thick-walled sporangia containing sporangiospores; mature sporangia are larger than spherules of *C. immitis*
Wide, non-septate hyphae	Zygomycetes: *Mucor* spp; *Rhizopus* spp. and other genera	10–30	Hyphae are large, ribbon-like, often fractured or twisted. Occasionally, septae may be present, branching usually at right angles. Smaller hyphae overlap those of *Aspergillus* spp., particularly *A. flavus*
Hyaline septate hyphae	Dermatophytes: skin, hair and nails	3–15	Hyaline septate hyphae commonly seen; chains of arthroconidia may be present
	Aspergillus spp.	3–12	Hyphae are septate and exhibit dichotomous, 45° angle branching; cannot be distinguished from other hyaline molds; larger hyphae may resemble those of zygomycetes
	Geotrichum spp.	4–12	Hyphae and rectangular arthroconidia are present and are sometimes rounded. Irregular forms may be present
	Trichosporon spp.	2–4 by 8	Hyphae and rectangular arthroconidia are present and are sometimes rounded. Blastoconidia may be produced, but are difficult to observe
	Scedosporium aprospermum (cases other than mycetoma)	3–12	Hyphae are septate and are impossible to distinguish from those of other hyaline molds, e.g. *Aspergillus* spp.
	Fusarium spp.	3–12	Hyphae are septate and are impossible to distinguish from those of other hyaline molds, e.g. *Aspergillus* spp.

(*Continued over*)

Table 4 *Characteristics of fungal elements seen in direct examination of clinical specimens (Continued)*

Morphology of fungal elements	Organism(s)	Diameter range (μm)	Characteristic features
Pheoid septate hyphae	*Bipolaris* spp. *Curvularia* spp., *Exserohilum* spp., *Exophiala* spp., *Phialophora* spp., *Wangiella dermatitidis*, *Cladophialophora bantiana*	2–6	Pheoid polymorphous hyphae are seen; budding cells with single septa and chains of swollen rounded, sometimes budding, cells may be present. Occasionally, aggregates may be present when infection is caused by *Phialophora* and *Exophiala* spp.
	Phaeoannellomyces elegans	1.5–5	Usually, large numbers of frequently branched hyphae are present along with budding cells
Muriform cells	*Cladophialophora carrionii*, *Fonsecaea compacta*, *Fonsecaea pedrosoi*, *Phialophora verrucosa*, *Rhinocladiella aquaspersa*	5–20	Pheoid, round to pleomorphic, thick-walled cells with transverse septa. Commonly, cells contain two fission plates that form a tetrad of cells. Occasionally, branched septate hyphae may be found in addition to muriform cells
Granules	*Acremonium falciforme*, *A. kiliense*, *A. recifei*	200–300	White, soft granules without cement-like matrix
	Curvularia geniculata, *C. lunata*	500–1 000	Black, hard grains with cement-like matrix at periphery
	Aspergillus nidulans	65–160	White, soft granules without cement-like matrix
	Exophiala jeanselmei	200–300	Black, soft granules, vacuolated, without cement-like matrix, made of dark hyphae and swollen cells
	Fusarium spp.	200–500	White, soft granules without cement-like matrix
	F. moniliforme, *F. solani*	300–600	
	Leptosphaeria spp.	400–600	Black, hard granules; cement-like matrix; periphery composed of polygonal swollen cells and center composed of hyphal network
	L. senegalensis, *L. tompkinsii*	500–1 000	
	Madurella grisea	350–500	Black, soft granules without cement-like matrix; periphery composed of polygonal swollen cells and center composed of hyphal network
	M. mycetomatis	200–900	Black to brown hard granules of two types: (1) rust brown, compact, and filled with cement-like matrix; (2) deep brown, filled with numerous vesicles, 6–14 μm in diameter, cement-like matrix in periphery and central area of light-colored hyphae
	Neotestudina rosatti	300–600	White, soft granules with cement-like matrix at periphery
	Scedosporium aprospermum	200–300	White, soft granules composed of hyphae and swollen cells at periphery in cement-like matrix
	Pyrenochaeta romeroi	300–600	Black, soft granules composed of polygonal swollen cells at periphery; center is network of hyphae; no cement-like matrix

ciated with infection of immunocompromised hosts. A rapid identification of the fungus is thus very important for diagnosis and early management of fungal diseases (Kwon-Chung and Bennett 1992).

Morphology

The standard method for identifying moulds is by observing their colonial and microscopic morphological features and following taxonomic keys to make an identification. This usually entails observing, on standardized media, the rate of growth, colonial morphology and color of the colony, and microscopic morphology including: formation of conidia; conidial arrangement; conidial morphology and pigmentation; and hyphal morphology and pigmentation. There is considerable variation in the characteristics of molds and their identification is difficult for an untrained laboratory worker.

Yeasts can be identified by morphological and biochemical methods. Details on the identification of yeasts can be found in Candidiasis and Cryptococcosis. There are many commercial systems available for identifying yeasts on the basis of biochemical reactions. Most of these systems are very good for the identification of the clinically important yeasts, provided that their morphology is considered when interpreting the biochemical results. Most systems,

Table 5 *Commonly used media for determining morphology of fungi*

Organism	Media
Aspergillus spp.	Czapek's Dox agar
Pigmented molds	Cornmeal agar, Sabouraud's dextrose agar, Lactrimel agar
Dermatophytes	Cornmeal agar, potato dextrose agar
Hyaline molds	Cornmeal agar, Sabouraud's dextrose agar (Emmons' modification), potato flake agar, inhibitory mold agar
Yeasts	Cornmeal agar
Zygomycetes	Cornmeal agar, potato flake agar, Water agar, Sabouraud's dextrose agar

however, have serious flaws in identifying some yeasts without morphological information. It is necessary to use standardized media to be able to observe consistent morphological features that are stable enough to be used in taxonomic keys. Some of the more commonly used media are listed in Table 5.

Incubation requirements for morphological media are generally the same as for primary isolation. When a fungus appears mature enough to have formed conidia, it is necessary to examine the microscopic structures. Looking at colonies under a stereoscopic microscope is sometimes helpful for observing large fruiting structures and conidial arrangements. Culture dishes should not be opened during observation.

More detailed observation requires removal of a small representative sample of a portion of the colony, and its transfer to a drop of lactophenol cotton blue or aniline blue placed on a clean microscope slide.

The colony segment is carefully torn apart, using inoculation wires or picks; a clean coverslip is then added and the specimen is observed under the low- and high-power objectives of a microscope. If the fungal colony is soft, a portion is placed in the dye on a microscope slide and the specimen is covered with a glass coverslip, which is pressed down with a pencil eraser to flatten the specimen. An alternative and often-superior method for observing the morphology of molds is the 'Scotch tape' technique. A strip of clear Scotch tape is pressed firmly on the colony; it is then placed in a drop of lactophenol cotton blue or aniline blue dye on a clean microscope slide. The tape holds hyphae and reproductive structures in place and serves as a coverslip.

Most morphological structures can be observed adequately by transmitted light microscopy, but some unusual structures, e.g. annellides, may require more sophisticated microscopy, such as phase contrast or a high-resolution digital photograph that can be enlarged. A detailed description of the morphological characteristics of fungi is given in Fungal structure and morphology.

Identification of dimorphic fungi using nucleic acid probes

The definitive identification of the dimorphic fungi *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides immitis* has traditionally been made on the basis of the microscopic morphology of both mold and parasitic (yeast or spherule) forms. In vitro conversion of the mould form to its corresponding yeast form for *H. capsulatum* and *B. dermatiditis* is often difficult and requires an extended period of time. Conversion of *C. immitis* requires specialized media and conditions; clinical laboratories do not undertake it. Laboratories with little experience have found that it is impractical to use the in vitro conversion process.

The exoantigen test (Kaufman and Standard 1978) was considered for many years as the method of choice for the definitive identification of dimorphic fungi. The method relied on the principle that soluble antigens are produced by fungi when grown in a broth medium and that they can be extracted and concentrated and subsequently reacted with serum known to contain antibodies specific for a particular species. This immunodiagnostic method utilized the immunodiffusion test as a means of detecting the species-specific antigens produced by these dimorphic fungi.

The exoantigen test has been replaced by specific nucleic acid probes that provide a more rapid and specific identification. The identification of dimorphic fungi using nucleic acid probes has changed the laboratory aspects of clinical mycology more than any other single contribution made during the past few years. This technology allows for the rapid (one hour) identification of the organisms, whether found in mixed culture or not. The probes are commercially available from Gen-Probe Incorporated (San Diego, CA) and are genus and species specific.

Fungal cells are lysed and the ribosomal RNA is hybridized to a DNA probe which has been labeled with an acridinium ester (Stockman et al. 1993). In short, the hybrids are detected by the chemiluminescence of the attached probe. Chemiluminescence is quantitated using a luminometer and the limits for positivity are well defined. The method is simple and rapid. It is recommended for laboratories that deal with dimorphic fungi with some frequency. Financial considerations must be made before incorporating these probes into a clinical laboratory's diagnostic routine; however, the time saved for patients awaiting a diagnosis is dramatically shortened and appropriate therapy can be instituted promptly.

FUNGAL SEROLOGICAL TESTING

Serological procedures, together with clinical and patient history data, are often useful in obtaining an early diagnosis of the life-threatening systemic fungal diseases (Kaufman et al. 1997). Most of the currently available, standardized, serological procedures detect antibodies to specified fungi; they are, therefore, of limited value in diagnosing disease in immunocompromised patients. This deficiency is being addressed by the rapid development of tests for fungal antigens.

The use of serological tests to measure antibodies is most useful in the diagnosis of acute, systemic, fungal diseases. Agglutination, precipitation (immunodiffusion), complement fixation, and enzyme immunosorbent assays are widely available commercially for use in the diagnosis of aspergillosis, blastomycosis, coccidioidomycosis, histoplasmosis and paracoccidioidomycosis. The most widely used is the assay for the detection of *Histoplasma* antigen (Durkin et al. 1997). This test has proven useful for the detection of antigenemia in patients having disseminated disease (Wheat et al. 1986), particularly those with acquired immunodeficiency syndrome (AIDS) (Wheat et al. 1992). Serological tests have been developed commercially for detecting cryptococcal and candida antigens. The latex agglutination test for cryptococcal capsular antigen is one of the most reliable fungal serological tests for use in diagnosing cryptococcal meningitis. The role of tests for antigens of *Candida* spp. in diagnosing candidemia is unclear. Considerable effort is being directed towards developing tests to detect fungal antigens, in the serum and other body fluids, as a means of diagnosing the increasing number of opportunistic fungal diseases in immunocompromised patients (Yeo and Wong 2002). The galactomannan EIA assay offers promise of being an important test for invasive aspergillosis (Wheat 2003). A listing of the currently commercially available serological tests and their uses can be found in Table 6. A more detailed description of fungal immunoserology can be found in Mycoserology and molecular diagnosis.

LABORATORY SAFETY

Biosafety in the clinical laboratory is of the utmost importance to the laboratory worker and to those who work in adjacent areas. The fungi produce conidia that are easily aerosolized and may contaminate the laboratory, air-handling systems, and, in some instances, other cultures and specimens. Comprehensive safety guidelines should be available to laboratory workers to minimize the risk of acquiring infection in this setting. The following are guiding principles which should be remembered and practised by laboratory personnel:

- All clinical specimens should be considered as infectious and universal precautions must be used to protect laboratory personnel.
- All fungi should be considered as potentially pathogenic. Some laboratory personnel may be immunocompromised and highly susceptible to infection.
- Even fungi that are not endemic to a certain locale must be taken into consideration when making a diagnosis. Cultures of *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Penicillium marneffei* may be encountered in any laboratory as a result of the ease of travel today.
- All molds must be handled within a biological safety cabinet to prevent aerosolization of their conidia within the laboratory.
- When laboratory-teaching rounds are done, cultures of molds should not be passed around from person to person; they should remain on the bench to prevent inadvertent breakage of a culture dish or tube.
- When flaming loops or wires, excess inoculum should be removed before flaming. Flaming should be used to decontaminate the entire wire or loop, not only the tip.
- Culture dishes can be used safely in the laboratory; however, lids should be removed only inside a biological safety cabinet. Lids should be taped entirely or in two places to prevent inadvertent opening.
- Biological safety cabinets must be certified annually to ensure proper protection to the laboratory worker.
- Work surfaces must be wiped with a disinfectant after a spill and before leaving for the day. A number of suitable disinfectants are available; however, hypochlorite is cost-effective and is suitable.
- A laboratory safety manual must be prepared that includes procedures for treatment of laboratory spills, an evacuation plan, and overall laboratory decontamination in the event of biohazardous spills.

MOLECULAR TESTING

When one reviews the literature relating to molecular methods used in fungal diagnosis, a plethora of reports are found. The number of reports is so numerous that it is difficult to determine which are of practical value in the clinical laboratory (Sandhu et al. 1995; Chen et al. 2002; Erjavec and Verweij 2002; Stevens 2002; Yeo and Wong 2002). Many of the studies have been validated only by the reporting investigator; many used only a small number of samples for the validation, and most lacked strong clinical correlation. However, these studies represent the beginning of a molecular era that will evolve over the next several years and these tests will find their particular niches within the laboratory. Most clinical mycology laboratories will not replace all traditional testing with molecular methods due to the extremely large number of fungi that are capable of causing human disease. To develop testing that will detect each etiologic agent would be neither practical nor cost-

Table 6 *Commonly used fungal serological tests*

Disease	Test antigen(s)	Interpretation
Aspergillosis	CF (aspergillin)	Titer ⩾1:32 suggestive of infection
	Aspergillus fumigatus, *A. niger* and *A. flavus*	Higher titers have a stronger correlation with disease
	ID, aspergillin EIA, galactomannan assay uses a monoclonal antibody	Multiple precipitin bands increase the suspicion of active disease. Positive in 95% of cases with fungus ball; 50% with bronchopulmonary disease. May be positive in invasive disease, rarely. Galactomannan antigen is detected in some patients, more evaluations are needed to determine efficacy. Piperacillin-tazo bactam cause false positive reactions
Blastomycosis	CF, yeast form of *Blastomyces dermatitidis*	Titers of ⩾1:8 are significant. Higher titers may be more indicative of disease. Cross-reactions occur in patients with histoplasmosis and coccidioidomycosis may occur. High percentage of cases are negative
	ID, yeast culture filtrate	Specific band for A antigen. Many culture-proved cases are negative
Candidiasis	ID, *Candida albicans* sonicate	Most cases have bands; however, high percentage of normal populations have antibody to *C. albicans*
	LA, yeast cytosol or mannan	⩾1:4 or 1:8 are considered positive; difficult to interpret; must have clinical correlation
Coccidioidomycosis	CF, coccidioidin	Titer ⩾1:2 significant. Higher titer parallels severity of disease. May cross-react with patients with histoplasmosis and blastomycosis. May not be positive early in disease
	ID, coccidioidin	Specific F antigen; correlates with CF results
	TP, coccidioidin	Detects IgM during first 3 weeks of disease
	LA, coccidioidin	Correlates well with CF
	EIA, coccidioidin	A positive relative EIA value indicates disease. Separate test for IgG and IgM. Positive IgM indicates early disease
Cryptococcosis	LA, anticryptococcal capsular polysaccharide	Detects cryptococcal capsular polysaccharide in cerebrospinal fluid or serum. Any titer significant. Rheumatoid factor causes false positive; 95% of *Cryptococcus* meningitis cases are positive *neoformans*
	EIA, antibody to cryptococcal capsular polysaccharide	Appears to be more sensitive than LA test. Use as quantitative test not confirmed
Histoplasmosis	CF, histoplasmin (mycelial form)	⩾1:8 significant. Higher titers parallel more severe disease. May be influenced by skin test
	CF, yeast cells (yeast form)	⩾1:8 significant. Higher titers parallel more severe disease. Usually higher titer than mycelial form. May cross-react with blastomycosis and coccidioidomycosis
	ID Histoplasmin	Specific bands to H and M antigens. 'M' band usually appears first in disease and persists. 'H' band is associated with active disease and often disappears during therapy. 1.0–2.0 indicates a weak positive; suggest repeat; 2.1–4.0, positive; 4.1–10.0, moderate positive; >10.0, highly positive (all values are EIA units).
	Histoplasma Immunosorbent Assay[a]	–
Sporotrichosis	LA, Yeasts of *Sporothrix schenckii*	Titer ⩾1:80 usually indicates active disease. Usually negative with systemic sporotrichosis

CF, complement fixation; EIA, enzyme immunoassay; ID, immunodiffusion; LA, latex agglutination; TP, tube precipitin.
a) Testing performed only by MiraVista Laboratories, Indianiapolis, IN.

effective; but, testing for selected fungi is certainly possible and work is already well underway to provide this to the clinical laboratory. However, amplification with universal fungal primers followed by sequencing of the amplicon offers the promise of being able to detect a variety of fungi in clinical specimens.

Molecular assays will allow the laboratory to detect and/or identify the fungi present in clinical specimens whether it is done by nucleic acid amplification technology or by specific probes or nucleic acid sequencing. This is an exciting time since molecular technology is in its infancy in routine clinical laboratories and many new developments lie ahead. A brief summary of what has been done to date will be presented; however, it will not be exhaustive. In this way, the reader will have a general appreciation for what has been currently accomplished

and, perhaps, what might find its way into the routine clinical laboratory. Before choosing a specific molecular testing method, a thorough comparison of useful methods should be completed so that the best can be verified and validated prior to being used. Further, standards must be set for test performance analytical and clinical sensitivity; specificity, and reproducibility within the laboratory. We must develop a focused and standard approach that is optimal for patient diagnosis. Hopefully this will occur, but it may not be realistic with the financial and personnel constraints imposed on clinical laboratories currently. Molecular testing should be embraced with great enthusiasm and excitement since it has the potential to provide for a more accurate and sensitive diagnosis of fungal infections in patients whom we serve.

Direct detection of the nucleic acid of fungi in clinical specimens

Immunocompromised patients and fungal infections are being seen at a much higher rate than ever before. Traditional cultures often require an extended time before the etiologic agent can be recovered and identified. During this, the disease may have progressed to a point where therapy may be ineffective; molecular detection should allow for monitoring patients on a periodic basis for the presence of circulating nucleic acid of the infecting organism. Further, if nucleic acid can be detected in blood or urine, this would dramatically reduce the need for invasive procedures such as biopsy or bronchoalveolar lavage. Being able to start therapy at a much earlier time during the clinical course should significantly affect the survival rate of the patient with a life-threatening fungal infection.

One of the simplest approaches used has been in situ hybridization using specific nucleic acid probes for the identification of organisms in patient specimens. This method lacks amplification and is less sensitive than other assays but is useful for identifying fungi that can be seen in tissue and other clinical specimens. Assays for *Aspergillus*, *Candida* and many other fungi are used primarily in pathology laboratories and are helpful when a morphological identification cannot be made (Hayden et al. 2001, 2002, 2003; Procop 2002).

Amplification assays using the polymerase chain reaction (PCR) or similar methods allow for the detection of small amounts of target DNA in clinical specimens. Specific primers with or without specific probes have been used with some success. Recent reviews by Yeo and Wong (2002) and by Chen et al. (2002), summarize specific targets used and detection methods. Assays have been developed to detect DNA of *Candida* (Chryssanthou et al. 1999; Loeffler et al. 2000a; Wahyiningshi et al. 2000; Ahmad et al. 2002), *Aspergillus* (Lass-Florl et al. 2000; Buchheidt et al. 2001, 2002; Ferns et al. 2002), *Fusarium* (Hue et al. 1999), *Cryptococcus* (Sandhu et al. 1995; Hendolin et al. 2000), *Histoplasma* (Bialek et al. 2002; Rickerts et al. 2002), *Blastomyces* (Sandhu et al. 1995; Bialek et al. 2003), *Coccidioides* (Sandhu et al. 1995), *Paracoccidioides* (Gomes et al. 2000), *Pneumocystis jirovecii* (Lindsley et al. 2001; Nevez et al. 2002), *Penicillium marneffei* (Vanittanakom et al. 2002), and dermatophytes (Turin et al. 2000).

More recent technology has utilized real time PCR using the Light Cycler offered by Roche Molecular Systems (Mannheim, Germany). Fluorescent resonance energy transfer is used to detect amplified products and the system allows for the quantification of the target DNA. Results are available within one hour of testing and sensitivity is exquisite. Loeffler has used this technology to develop assays for *Aspergillus* (Loeffler et al. 2000b), *Candida* (Loeffler et al. 2000a), and the zygomycetes (Loeffler et al. 2001). A recent report described the use of real time PCR for the identification of culture isolates of *Histoplasma capsulatum* (Martagon-Villamil et al. 2003). This study also reported the detection of *H. capsulatum* DNA in a few clinical specimens; however, these are preliminary data and clinical trials will be needed to confirm the utility of the test as a diagnostic tool. Further, the MagNApure (Roche Molecular Systems, Mannheim, Germany) automated DNA extraction system was evaluated for use with the fungi (Loeffler et al. 2002). A combination of the Light Cycler and MagNApure appears to offer great promise for the detection of fungal DNA in clinical specimens in a very rapid manner. The Light Cycler may also be used to identify organisms to the species level using specific primers and probes.

Traditionally, the identification of clinically important fungi has been made based on macroscopic and microscopic morphological features. Considering the large number of fungi in the environment that are capable of causing human disease, it is difficult to believe that molecular methods will replace conventional methods anytime soon. However, a limited number of fungi may be identified to genus and species using PCR and specific probes. Yeasts in blood cultures have been identified and most were species of *Candida* (Chang et al. 2001). *Aspergillus* (Henry et al. 2000), zygomycetes (Voigt et al. 1999), dermatophytes (Mochizuki et al. 2001; Ninet et al. 2003), and several filamentous fungi may be identified using amplification and probes. Nucleic acid sequencing has been use with great success for the identification of fungi in culture (Turenne et al. 1999). Currently, a comprehensive database of fungal sequences is available in the MicroSeq 500 microbial identification system (Applied Biosystems, Foster City, CA). This database is not yet complete, but when used with GenBank and other public databases, many fungi may be more easily be identified when compared to traditional identification methods.

The use of molecular methods is important to investigate the epidemiology and environmental sources of fungi that infect immunocompromised patients and, in some instances, immunocompetent patients. Epidemiologic typing can determine whether or not organisms share the same DNA profile and this can be related to environmental isolates to determine the point source. Most of the studies have been related to isolates of *Candida* (Stephan et al. 2002), *Cryptococcus* (Anonymous 2002), *Aspergillus* and *Fusarium* (Raad et al. 2002). The use of molecular tools has allowed for the reduction of hospital-acquired infections and their spread.

Overall, molecular methods have and will continue to have a major impact on the diagnosis and appropriate treatment of fungal infection. Analytical parameters of these methods need to be standardized to optimize sensitivity and specificity and comparative studies need to be performed to determine which are best to use in the laboratory. Ideally, tests should be as simple as possible to perform so that most clinical laboratories can use them. The most important element, clinical correlation, must be established for methods. If these criteria are met, most of the newly developed molecular-based tests will be available to all of the patients whom we serve. One needs to remember that all of us should be in the field to improve patient care and that the needs of the patient come first.

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Mycoserology and molecular diagnosis

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This chapter describes the application of serology and the polymerase chain reaction (PCR) to the diagnosis of systemic fungal infections. Culture-confirmation is still the cornerstone of diagnosis, and has become even more desirable as new treatment options and antifungal sensitivity testing become available. But the problem remains that positive cultures can be difficult to obtain – the yield being low, growth often slow, and biopsying a deep infected site may be difficult or impossible. Therefore, new, nonculture-based tests have been developed, usually based on detection of circulating fungal antigens or DNA, in an attempt to make diagnosis more rapid and reliable.

Traditionally the rationale behind this strategy has been that by confirming the diagnosis more quickly, antifungal treatment will be initiated earlier thereby reducing mortality (Horn et al. 1985) – confirmation being particularly important when the only treatment option was conventional amphotericin B. Now that safer antifungals are available, there is greater use of antifungal prophylaxis in high-risk groups and of empiric therapy in symptomatic patients – but these approaches are not without problems. Paralleling the increased use of fluconazole in the early 1990s, fluconazole-resistant non-*albicans* species of *Candida* have become commoner (Kontoyiannis and Lewis 2002). Amphotericin B resistance is much rarer but well documented. Greater empiric use of antifungal drugs inevitably exposes a larger number of patients, some inappropriately, to the risk of drug-induced toxicity and substantially increases pharmaceutical costs. Therefore the goal should remain to develop reliable diagnostic tests to provide an early diagnosis, preferably before beginning treatment.

Some of these assays are commercially available, whereas others are still under research and development. Inevitably the latter have not been widely assessed outside the originating research laboratory, although they may take advantage of the latest technical advances. Assessing the diagnostic value of a serology test raises questions such as those given in Table 1. For the referring clinician to get the most out of serodiagnostic assays, it is important that they provide a full clinical history, ascertain the frequency with which sera should be examined, and, where possible, avoid repeated freeze-thawing of samples.

CANDIDIASIS

Clinical diagnosis of invasive (systemic and disseminated) candidiasis is hampered by a lack of specific features in most cases. Confirmation of the diagnosis from blood cultures tends to be slow (growth taking 2–5 days) and insensitive. Blood cultures are negative in about half of necropsy-proven cases (de Repentigny and Reiss 1984). Recovery from blood cultures can be improved using lysis-centrifugation or the BACTEC high-blood-volume fungal medium (Telenti and Roberts 1989; Wilson et al. 1993), but the sensitivity of even the most advanced blood culture systems becomes progressively poorer when fewer than three deep-tissue sites are infected (Berenguer et al. 1993). In chronic, hepatosplenic candidiasis, only five out of 60 cases had positive blood cultures (Thaler et al. 1988; Kauffman et al. 1991).

A single positive blood culture or culture from a normally sterile organ, body cavity, or fluid, in a

Table 1 *Assessment of serodiagnostic tests for systemic fungal infections*

Question	Example
How frequently should sera be examined?	Mannanemia is transient so frequent sampling is essential for detecting DC
How sensitive is the assay in different forms of the infection?	Acute DC versus hepatosplenic candidiasis; ABPA versus aspergillosis
How sensitive is the assay at different stages of the infection?	ID tests for dimorphic fungi usually miss early primary infections when IgM predominates
Is serology likely to make the diagnosis before other diagnostic procedures?	Cryptococcal RPLA tests, when direct microscopy is negative
Is sensitivity affected by the underlying condition?	Neutropenia; AIDS
Can it be used to distinguish between systemic infection and colonization?	Generally true of candidal antigen detection, but not candidal antibody assays
Can it be used prognostically?	Cryptococcal RPLA tests; serial measurement of candidal antibodies
Can it be used to monitor response to treatment and to detect relapses?	Cryptococcal RPLA tests; *Aspergillus* galactomannan tests; some candidal antigen detection systems; *Histoplasma* antigen detection in AIDS
Is the antigen detected stable to heat or protease treatment or freeze-thawing?	Protease pretreatment is essential for immune complex dissociation before mannan detection
Is the assay species specific?	Assays for *C. albicans* mannan miss *C. krusei*
Can the assay determine intrinsic (species dependent) or acquired antifungal resistance?	Potentially PCR versus resistance genes; species-specific targets, e.g. *Candida* mannan
Known causes of false positives?	*T. beigelii* with cryptococcal RPLA tests
Known causes of false negatives?	Prozone effect with cryptococcal RPLA tests
Reagent costs and hands-on time	Relatively high for PCR
Trained staff, special equipment, and facilities	GLC; becoming less of a problem with PCR

ABPA, allergic bronchopulmonary aspergillosis; AIDS, acquired immune deficiency syndrome; DC, disseminated candidiasis; EIA, enzyme immunoassay; GLC, gas–liquid chromatography; ID, immunodiffusion; IgM, immunoglobulin M; PCR, polymerase chain reaction; RPLA, reverse passive latex agglutination.

septic patient, is in practice enough to start therapy, but care should be taken to avoid contamination. For example, preferably, blood cultures should be taken percutaneously rather than through an intravenous line – which may have become colonized. In the latter case, simply removing the infected line may resolve the infection, provided the *Candida* has not already spread, via the bloodstream, to set up a second focus of infection.

Serology and PCR are particularly useful in helping to support the diagnosis in suspected cases in whom culture-confirmation cannot be obtained. They can help distinguish disseminated candidiasis (DC) from transient candidemia or heavy colonization. They can also be used to screen high-risk patients or monitor response to treatment in known cases. Development of these assays is complicated by the diversity of candidiasis: a test designed to detect circulating candidal antigen in acute DC will not necessarily detect heptosplenic candidiasis or localized infections such as candidal peritonitis or endophthalmitis. Sensitivity may also vary between different patient populations (such as neutropenic compared to non-neutropenic). Another increasingly important challenge is the detection of a wide range of species, not just the commonest, *Candida albicans*, and ideally their speciation, as an indication of their probable antifungal sensitivity pattern.

Candida antibodies

Several kits for measuring antibodies to *Candida* spp. are commercially available (Gutierrez and Maroto 1993; Ruchel 1993), but these are intrinsically flawed because antibody often occurs in superficially infected individuals, antibody takes time to develop, and patients with worsening candidiasis often fail to make or sustain a detectable antibody response (Matthews et al. 1984, 1987). An important exception is endocarditis caused by *Candida* spp. Here the antibody is raised and this may be of diagnostic value (Dee and Rytel 1977). In invasive candidiasis, the development of a good antibody response has been used as a marker of recovery, whereas lack of antibody is strongly indicative of a bad prognosis. This lack of antibody may reflect antibody consumption or host immunosuppression.

Cand-Tec latex agglutination assay

The first of the commercially available antigen-based diagnostics was the Cand-Tec test developed by Gentry et al. (1983) and marketed by Ramco Laboratories Inc., Houston, TX. It is an example of reverse passive latex agglutination (RPLA), latex particles being sensitized with rabbit antibodies raised against heat-killed blastoconidia.

The target antigen has not been fully characterized but is proteinaceous, clearly distinct from mannan, and could not be found in the culture supernatant of *Candida* spp. by Ruchel (1989a). It is heat labile at 57°C.

Considerable variation in the success rate of this particular test is reported in the literature, with sensitivities ranging from 33 percent (Burnie and Williams 1985) to 71 percent (Fung et al. 1986), but more commonly around 44 to 48 percent (Kahn and Jones 1986; Lemieux et al. 1990; Herent et al. 1992). This probably reflects differences in the patient population being examined and the threshold titer regarded as significant. Burnie (1991a) found that the test gave a better bimodal distribution between colonized and systemically infected patients when assessed using postoperative patients with DC rather than neutropenic patients. This could possibly be related to the higher frequency with which neutropenic patients produce the immunoglobulin IgM over IgG, which may lead to less free antigen being present (Matthews et al. 1987) or to a need for the antigen being measured to be processed by leukocytes (Ruchel 1993). In the instructions that come with the kit, a titer of 1:4 is regarded as positive. Both Burnie and Williams (1985) and Ruchel (1989b) commonly observed weakly positive titers (1:4) in neutropenic patients with DC. Although a low threshold is necessary in neutropenic patients, in order to retain sensitivity, a higher cutoff point of 1:8 is probably more accurate in non-neutropenic patients to raise specificity.

Candidiasis caused by species such as *Candida krusei*, *Candida lusitaniae*, and *Candida parapsilosis* is not readily detected by Cand-Tec (Ness et al. 1989; Ruchel 1993). Fungal infections caused by *Aspergillus* spp. (Ness et al. 1989; Phillips et al. 1990; Ruchel 1993) or *Cryptococcus neoformans* (Lemieux et al. 1990) can give a positive Cand-Tec result. False positives occur as a result of rheumatoid factor and this needs to be excluded. Burnie and Williams (1985) found 12 percent of rheumatoid factor-positive sera gave false-positive reaction with titers as high as 1:8. The importance of frequent, preferably daily, serum sampling to raise sensitivity was also emphasized. The test proved helpful in monitoring response to therapy, titers falling as the patient recovered and rising during relapse.

In conclusion, Cand-Tec does have a limited, but sometimes useful, role to play in the diagnosis and management of DC, particularly if care is taken to exclude rheumatoid factor-positive patients, daily sera are available, and titers are interpreted in the context of whether or not the patient is neutropenic. Its greatest asset is that it is quick and easy to perform.

Mannan

The first of the circulating antigens for *Candida* spp. to be fully characterized was the cell wall mannoprotein or mannan, consisting of the carbohydrate homopolymer mannose plus small amounts of protein and phosphate. This antigen is stable and resistant to boiling, protease treatment, and acidic pH. In the serum, mannan is found in immune complexes and the first step in its detection is the dissociation of these complexes by protease digestion, exposure to extremes of pH (such as NaOH at 56°C for 2 hours), boiling, or heat extraction.

Some of the first assays developed for its detection were enzyme-linked immunoassays (EIA) based on indirect inhibition (Segal et al. 1979; Meckstroth et al. 1981). These were superseded by the double antibody sandwich EIAs, developed by several different groups, reviewed by Reiss and Morrison (1993). These had the advantage that absorbance units could be directly converted into concentration (ng/ml). Specificity was consistently high, probably helped by the fact that sera were first heat treated to dissociate immune complexes, but sensitivity varied from 23 percent (Lemieux et al. 1990) to 90 percent (Fujita et al. 1986). Factors that probably influenced sensitivity included the quality of the antibody probe, the infecting species of *Candida*, the patient's underlying condition, frequency of serum sampling and time of collection relative to disease severity.

The relative species specificity of mannan antibodies is well documented. Nakamura et al. (1991), using an avidin-biotin-amplified EIA with an overall sensitivity of 84 percent for DC, found the lower limits of mannan detection were 1.0–2.8 ng/ml for *C. albicans*, *Candida tropicalis*, and *C. parapsilosis*, 6.7 ng/ml for *Candida guilliermondii*, 20 ng/ml for *Candida glabrata*, and more than 50 ng/ml for *C. krusei*. By including antibodies to the mannan of *C. krusei* as well as *C. albicans*, Fujita and Hashimoto (1992) identified a case of DC caused by *C. krusei* which was only detectable with the homologous antibody. They noted a further six cases of *C. glabrata* DC in whom serum mannan could not be detected by either antibody.

Therefore, incorporation of antibodies to the mannan of several species of *Candida* improves the performance of these assays. The greatest limitation, however, is probably that the circulation of mannan is only transient (Kahn and Jones 1986; Herent et al. 1992). In the rabbit, the serum half-life of mannan was only 2 hours (Kappe and Muller 1991). As a result of the fast clearance of mannan from the blood, tests for mannan must be performed frequently. Studies in which a large number of sera have been examined per patient, or samples have been collected when DC was most severe, are likely to give higher sensitivities.

Sensitivities reported for RPLA tests for mannan have varied from 0 to 81 percent. The highest sensitivity was obtained by Bailey et al. (1985) who used their own RPLA assay with polyvalent rabbit antisera raised against heat-killed *Candida* spp. and dissociated immune complexes with protease and heat. Sensitivity was 81 percent (17 of 21 patients) for DC, 60 percent (three of

five) for candidemia and 40 percent (four of 10) for esophagitis. Five of six cases of DC caused by *C. tropicalis* were also detected. It was negative in patients with endophthalmitis or superficial candidiasis. One false positive occurred in 86 controls. Mannan was, however, reliably detected only late on in the course of infection. Sera examined during the penultimate week before death were less than 50 percent likely to be positive. Another disappointing feature was the lack of correlation between antigen titer and severity of infection.

There are two commercially available kits for the detection of mannan by RPLA: one uses a polyclonal antibody probe (the LA-Candida Antigen Detection System, Immuno-Mycologics, Inc., Norman, OK) and the other a monoclonal antibody (Pastorex *Candida*, BioRad, Marnes-la-Coquette, France). Using the former, Fung et al. (1986) failed to detect mannan in 24 patients who had positive cultures for *C. albicans*, six of whom had DC. These results were inferior to Cand-Tec which had a sensitivity of 71 percent and a specificity of 98 percent, using a titer of 1:8 or greater as the criterion for dissemination. Disappointing results were also obtained by Phillips et al. (1990). With the Pastorex test, Herent et al. (1992) detected mannan in 10 of 19 patients with DC (52.6 percent) compared with nine positives using the Cand-Tec test. Specificity was 100 percent for Pastorex and 98 percent for Cand-Tec. Ruchel (1993) detected mannan in about half the patients with DC tested with Pastorex.

The RPLA test may be intrinsically less sensitive than EIA. Fujita and Hashimoto (1992) compared RPLA and a sandwich EIA for *C. albicans* and *C. krusei* mannan in parallel with the same antibody reagents and clinical specimens. The sensitivity of the EIA was 74 percent whereas that of the RPLA was only 38 percent. A sandwich EIA is commercially available (ICON *Candida*, Hybritech Inc., Torrey Pines, CA) and has been assessed by Pfaller et al. (1993). Of 14 patients developing DC, the test was positive in 12 (86 percent) but, notably, antigenemia preceded diagnosis by culture or biopsy in just five cases. Only a single serum was available from the two patients who gave false negatives. The authors found the test easy to perform and interpret. Girmenia et al. (1997), using dot immunobinding, found that 7.1 percent of patients with transient or catheter-related candidemia had detectable mannan compared with 76.5 percent (13 of 17) patients with persistent candidemia.

In conclusion, the commercially available RPLA tests do not, on the basis of currently published reports, significantly outperform Cand-Tec, although the sensitivity of EIA may be better, and these are becoming commercially available (Erjavec and Verweij 2002). They may miss cases of DC caused by *C. glabrata* and *C. krusei*. Use of a combination of tests to detect both mannan antigen and anti-mannan antibody may improve sensitivity, since Sendib et al. (1999) found the presence of serum mannan to be inversely related to the presence of anti-mannan antibody – detecting both raised sensitivity to 80 percent, with specificity being 93 percent.

Another cell wall component, glucan, has been found circulating in patients with candidemia or invasive aspergillosis (Obayashi et al. 1995; Mori and Matsumura 1999). It is a characteristic component of fungal cell walls (except zygomycetes) but lacking in human cells, prokaryotes, and viruses. An assay for its detection is available commercially (Fungitec G-test, Seikagaku Corp., Tokyo, Japan). The test depends on the activation, by glucan, of factor G, a horseshoe-crab coagulation factor. A broad range of fungi induce reactivity, with high sensitivity (90 percent) and specificity (100 percent) being reported in patients undergoing treatment for hematological malignancies (Obayashi et al. 1995), but negative results have been observed in pulmonary cryptococcosis.

Protease

Both a circulating secreted acid protease antigen and specific antibodies have been described in patients infected with *C. albicans* (MacDonald and Odds 1980; Ruchel et al. 1988). The protease is thought to facilitate epidermal invasion. An assay detecting urinary protease in a rabbit model has been reviewed by Reiss and Morrison (1993), but there are few human data (Ruchel et al. 1988).

It is now known that there are at least seven genes encoding a family of secreted proteases in *C. albicans*, of which six are differentially expressed and one has not yet been successfully expressed (Hube et al. 1994). Different species of *Candida* secrete antigenically distinct proteases and some species, such as *C. krusei*, do not appear to secrete proteases. Na and Song (1999) found that an inhibition enzyme-linked immunosorbent assay (ELISA) for the detection of secretory aspartyl proteinase, using a specific monoclonal antibody, gave a sensitivity of 94 percent and specificity of 96 percent when applied to retrospectively selected sera from patients with invasive candidiasis.

Enolase and heat-shock protein 90

The application of immunoblotting to the serology of invasive candidiasis created the opportunity to examine the antibody responses of infected patients to a range of *Candida* antigens in one assay. This led to the identification of two immunodominant antigens which have been used as diagnostic markers: the 47 kDa antigen (Matthews et al. 1984), now identified as the carboxy fragment of heat shock protein 90 (Hsp90) (Matthews and Burnie 1989), and the 48 kDa antigen (Strockbine et al. 1984), subsequently identified as enolase (Sundstrom and Aliaga 1992; Franklyn and Warmington 1993).

Enolase is an abundant cytoplasmic enzyme involved in the glycolytic pathway of yeasts. As it is produced by all *Candida* spp., species other than *C. albicans* should also be detected by an enolase assay, although *C. glabrata* may be an exception (Walsh et al. 1991). To detect serum enolase, a liposomal sandwich-type immunoassay was developed and made available commercially (Directigen, Becton Dickinson Inc., Philadelphia, PA). Evaluating the assay prospectively in oncology patients, Walsh et al. (1991) obtained an overall sensitivity of 75 percent and specificity of 96 percent. Many patients who were positive on almost daily sampling would have been negative if sampling had occurred weekly. In a prospective comparative assessment with Pastorex, Gutierrex and Maroto (1993) found the Directigen test to be positive in eight of 10 patients (13 of 20 sera) with candidemia, with a specificity of 97.1 percent, whereas the Pastorex test remained negative. The Directigen test never became widely used and it was subsequently withdrawn by the manufacturer.

Heat-shock proteins, also known as stress proteins, are highly conserved, ubiquitous proteins which have been categorized into families, such as Hsp90, on the basis of their molecular mass and sequence homologies. They act as immunodominant antigens in a range of bacterial, parasitic, and fungal infections. Both murine monoclonal (Matthews et al. 1991a) and human recombinant antibodies (Matthews et al. 1995) to Hsp90 were protective in mouse models of the infection and an antibody to Hsp90 is currently being assessed as a therapeutic agent. The 47 kDa antigen can be isolated from the sera of systemically infected patients by affinity chromatography (Matthews et al. 1987) and was used as a diagnostic marker in a dot immunobinding assay (Matthews and Burnie 1988).

Once *C. albicans* Hsp90 had been sequenced it was possible to map the epitopes recognized by infected patients' sera. Both a conserved immunodominant epitope, LKVIRK, and a species-specific epitope recognized by about half the patients, DEPAGE, were identified (Matthews et al. 1991b). A rabbit antiserum raised against LKVIRK, again in the dot immunobinding assay, picked up 70 percent of patients with DC caused by *C. albicans*, with a further 18 percent giving weak positives and 12 percent false negatives. Two patients with DC caused by *C. glabrata* gave negative results but a patient with *C. parapsilosis* was positive. For 12 patients, multiple sera were available and here there was a good correlation with clinical outcome, titers falling in those who recovered and rising in those who died. Each of four blood culture-negative patients with severe focal infections requiring amphotericin B were positive. Colonized patients were negative or only weakly positive. All 404 uninfected control patients were negative in this series, but false positives have been observed with some other infections, possibly as a result of circulating microbial Hsp90. One possibility would be to use antibody against the conserved LKVIRK epitope to capture serum Hsp90 and a range of species-specific epitopes, such as DEPAGE in the case of *C. albicans*, for speciation.

Metabolites

Most species of *Candida*, with the exception of *C. glabrata* and *C. krusei*, produce the metabolite D-arabinitol (Bernard et al. 1981). It can be measured by gas–liquid chromatography (GLC) but, as this is a cumbersome technique, its availability as a diagnostic procedure is limited. As arabinitol is excreted by the kidney, it accumulates in patients with renal insufficiency, so an arabinitol/creatinine ratio is calculated to be independent of renal function.

An enzymatic fluorometric method has been developed as an alternative to GLC (Soyama and Ono 1985). It depends upon the oxidation of arabinitol by arabinitol dehydrogenase, with concomitant reduction of NAD, generating NADH. The initial rate of NAD reduction is proportional to the serum arabinitol content and can be measured by a recording spectrofluorometer. A potential problem is that mannitol is also reduced by arabinitol dehydrogenase producing NADH.

Several preliminary assessments have been published of the kit made commercially available by Nacalai Tesque Co. Ltd (Kyoto, Japan). Fujita and Hashimoto (1992), in a series of 58 patients with DC, reported a sensitivity of 50 percent and a specificity of 91 percent. This compared with a sensitivity of 74 percent for an EIA test for mannan. The false positives obtained (13 of 109 patients) were attributed to heavy colonization with *Candida* spp. causing a rise in serum arabinitol or interference with the fluorometric assay by mannitol. De Repentigny et al. (1985) likewise found that an EIA for mannan, combined with blood cultures, gave superior results to GLC measurement of arabinitol. In that series the low sensitivity, 13 percent, of arabinitol/creatinine ratios was associated with a significant increase in arabinitol concentrations in high-risk patients without candidiasis compared with normal blood donors. One cause of these false positives could be steroid therapy, which has been shown to raise arabinitol levels in rabbits.

Urinary samples can be used to monitor the ratio of D-arabinitol (fungal origin) to L-arabinitol (human origin) and this was found to be positive 3–31 days (mean 12 days) before blood cultures become positive or empiric therapy initiated, in immunocompromised children with systemic candidosis (Christensson et al. 1997).

PCR

The application of PCR to fungal diagnosis has been reviewed by Walsh and Chanock (1998), Buchheidt et al. (2000), and Erjavec and Verweij (2002). Early assays

were directed against a defined target gene present in a single species or genus, but now panfungal PCR primers are being used increasingly to detect a broad range of fungi, including yeasts and molds. Many of these assays are now achieving very high levels of sensitivity in-house but head-to-head comparisons and standardized, inter-laboratory assessments are as yet rare.

The first target for the development of PCR-based diagnostics for invasive candidiasis was the gene *L1A1* encoding cytochrome P450 lanosterol 14α-demethylase which catalyzes an essential step in the conversion of lanosterol to ergosterol. As ergosterol is not a component of human or bacterial cells, it is believed to be fungus specific. The enzyme is inhibited by the imidazoles. Therefore, antifungal resistance related to alteration in the sequence of this gene might potentially be used to pick up resistant yeasts. Buchman et al. (1990) carried out an assessment with 13 clinical specimens, including urine, sputa, wound fluid, and two whole bloods, all from sites that were culture positive for *C. albicans*, and in each case they were PCR positive. Among 17 additional culture-negative specimens, PCR suggested the presence of a yeast in one blood and one urine specimen. Results were available within 6 hours and it was estimated, by serial dilution of *C. albicans* mixed with fresh blood, that as few as 12 yeast cells per 0.1 ml could be detected.

Chryssanthou et al. (1994) developed a nested PCR against the same target to increase sensitivity. They also avoided the cumbersome sample preparation required for whole blood by detecting naked DNA in serum. The primers used by Buchman et al. (1990) were used for the outer pair, and an inner pair of primers was selected which gave a PCR product of the expected size. This fragment was only amplified for *C. albicans*, other faint bands appearing with *C. lusitaniae* and *C. tropicalis*. They applied it to serum and tissue samples from infected mice and the sera of patients with DC and candidemia. The nested PCR detected as little as 1 pg *C. albicans* DNA, serially diluted in water, compared with 10 pg using the outer primer pair alone. It was more sensitive than culture in mouse experiments and in seven blood culture-negative, necropsy-positive patients, five of whom gave PCR-positive sera before death (usually taken within a week of death but in one case 84 days before and therefore of doubtful significance). However, in 11 patients with proven or, in one case, suspected candidemia, 15 of 17 blood cultures were positive, compared with only 11 of 17 sera positive by PCR. These discrepancies might result partly because blood cultures and sera for PCR were not taken simultaneously.

Buchman's group followed up their earlier work on *C. albicans* by sequencing homologous genes from *C. glabrata* and *C. krusei*, and using a published sequence for *C. tropicalis* to develop a nested PCR (Burgener-Kairuz et al. 1994). The first PCR, with genus-specific primers from a conserved region, detected a wide range of yeast species when tested with purified DNA, including *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *Cryptococcus neoformans*. The second nested PCR with species-specific primers had a detection level 10 times higher for the homologous species. Southern blots were used to confirm the nature of the PCR product and the sensitivity was increased slightly further. The sensitivity and specificity of the nested PCR on 80 culture-positive clinical specimens were 71 percent and 95 percent for *C. albicans* and 100 percent and 97 percent for *C. glabrata*, respectively. Clinical specimens often contained large quantities of leukocytes, cell debris, and bacteria, all potential sources of strong inhibitors of the PCR which could be causes of false negatives.

In a prospective study, by Morace et al. (1999), in febrile patients with hematological malignancies, the P450 lanosterol 14α-demethylase gene was amplified and the resulting amplicon digested with restriction enzymes (RE) in order to speciate the *Candida*. The polymerase chain reaction-restriction enzyme amplification (PCR-REA) was much more sensitive (92.8 percent) and faster (24–36 hours) than blood culture systems, but there was some question over the method's specificity. Posteraro et al. (2000) used the same primers to develop a PCR-reverse crossblot hybridization assay. Chryssanthou et al. (1999) undertook a prospective study in high-risk patients comparing a nested PCR against lanosterol 14α-demethylase, with urinary D/L-arabinitol ratios and three commercially available tests for the detection, respectively, of serum glucan, mannan, and the Cand-Tec assay. No single test was sufficient for diagnosis and the correlation between some of the tests was poor. This study illustrated the difficulty in evaluating these diagnostic tests because of the lack of a definitive culture-confirmed diagnosis in many cases. In a comparison of a PCR assay (targeting a rRNA gene), the β-glucan and the Cand-Tec test, Sakai et al. (2000) showed a significant correlation between the PCR and β-glucan assays and that PCR was significantly more sensitive than the β-glucan test.

Hsp90 is an alternative target, a potential source of both species-specific and conserved DNA sequences. By selecting a species-specific primer, Crampin and Matthews (1993) obtained the expected 317 bp product only with *C. albicans* and, less intensely, *C. parapsilosis*. In a prospective study on a 100 clinical specimens, 23 percent were positive by routine culture, 31 percent by extended culture, and 37 percent by PCR. The identity of the PCR product was confirmed by hybridization with an internal probe and also by obtaining the predicted restriction enzyme digest pattern. This assay was sensitive down to 50 pg of DNA (5 pg after Southern blotting) and 100 yeast cells. Generally, specimens that were culture negative or grew bacteria or germ-tube-negative yeasts gave negative PCR results. The exceptions were

four urine samples from female patients and seven high vaginal swabs from women with vaginal discharge. Five culture-positive swabs gave negative PCR results, possibly as a result of a sampling error because plates for culture were inoculated first. Whole blood seeded with *C. albicans* gave positive results only when inhibitors were removed by the glass adhesion method. Some of the other yeast species, particularly *C. glabrata* and *C. parapsilosis*, gave bands about the same size as *C. albicans*, but in each case these were accompanied by ectopic bands not seen with *C. albicans*.

Another target is the actin gene (Kan 1993). Primers were selected which amplified a 158 bp fragment and the product was confirmed by hybridization against an internal probe. This gave a lower limit of detection estimated as 10 yeast cells for clinical specimens. The assay was assessed in both experimentally infected mice and patients with candidemia. In the mouse model of candidemia, pooled plasma samples were culture and PCR positive, whereas in the thigh-abscess model they were culture and PCR negative. In the 14 blood culture-positive patients, six of whom had *C. albicans*, 11 (79 percent) gave a positive PCR. This was performed on sera rather than on whole blood. The greatest difficulty was experienced in detecting *C. krusei* and *C. parapsilosis*. Sera from 12 patients with superficial candidiasis were PCR negative as were 17 normal volunteers.

Flahaut et al. (1998) targeted the *C. albicans* secreted aspartic proteinase (*SAP*) genes, a multigene family. A single primer pair was designed to detect six of these *SAP* genes, thereby increasing the sensitivity of the test. Detection of the PCR product by EIA was found to be as sensitive as Southern blotting with a *SAP*-labeled probe, and the sensitivity and specificity, when applied to clinical samples, were 100 percent and 98 percent, respectively.

Others have deliberately selected multicopy targets such as mitochondrial or ribosomal DNA in an attempt to maximize sensitivity. Miyakawa et al. (1992), targeting a species-specific mitochondrial DNA fragment 1.8 kbp long, could detect as few as 10 yeast cells in human urine but as many as 105 cells were required in human blood before detection. They attributed this to the loss of yeast cells during precipitation from the blood and the use of too long a target. They then developed a means of coprecipitating *C. albicans* with carrier *C. tropicalis* cells, aided by a monoclonal antibody which recognized both species, and selected a shortened sequence of 120 bp. This raised the sensitivity of detection of *C. albicans* seeded into blood to 3 cells/0.1 ml (Miyakawa et al. 1993). No clinical specimens were examined.

Panfungal PCR assays directed against rDNA are increasingly being used because not only are they present in multiple copies but, as a result of slow divergence of rDNA over evolutionary time, regions of sequence conservation across entire kingdoms can be identified. Separating the multicopy rRNA gene subunits are noncoding intergenic transcriber spacers (ITS). While rRNA genes are highly conserved, the ITS regions are divergent both in sequence and size. Consequently, panfungal primers can be used to amplify the fungal DNA and then species-specific ITS DNA probes can be used to speciate the amplicon.

Using such panfungal primers, directed against a region conserved throughout the kingdom Fungi, Hopfer et al. (1993) amplified a 310 bp product from fungi, while obtaining negative results from human and bacterial DNA. This PCR detected 10–15 yeast cells, but sensitivity and specificity with clinical samples were not determined. *Hae*III digests were used to characterize the fungus into one of five groups: *Aspergillus*, *Candida*, *Cryptococcus*, and *Trichosporon*, zygomycetes, and the dimorphic fungi. Holmes et al. (1994), also targeting rDNA, co-amplified using both conserved primers, suitable for the detection of many fungal species, and *C. albicans*-specific primers, facilitating rapid speciation. A 105 bp fragment was amplified from all the yeast species tested, whereas a second 684 bp fragment was amplified only from the *C. albicans* DNA. The level of sensitivity was 15 yeast cells/ml using spiked blood specimens. Makimura et al. (1994), using primers from conserved fungal rDNA sequences, applied this to a limited number of clinical specimens, including blood and cerebrospinal fluid, and also blood samples from mice systemically infected with *C. albicans*. Their preliminary results were encouraging, suggesting PCR to be more sensitive than conventional blood cultures.

Again targeting fungal rDNA, Fujita et al. (1995) developed a microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* spp. Species-specific probes for *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* were labeled with the digoxigenin, avoiding the use of radioisotopes. The PCR product was hybridized to both digoxigenin- and biotin-labeled probes and detected in an EIA by capture on streptavidin-coated microtiter plates. The assay detected as few as 2 cells/0.2 ml of blood seeded with *C. albicans*. Although no clinical samples were examined, this approach holds great promise. It combines the use of universal fungal primers against a multicopy target to broaden detection capabilities with identification of the fungus, using species-specific probes, in an EIA format that is particularly suitable to clinical laboratories; this has the potential for automation unlike Southern blotting. Ahmad et al. (2002) applied a seminested PCR, using panfungal primers against rDNA followed by the same species-specific primers from these four *Candida* spp., to detect and speciate from the sera of patients with candidemia: the results were in full agreement with blood culture results with respect to both positivity and species identity, and more sensitive than culture. Others have targeted ITS regions using species-specific primers in a multiplex PCR to speciate multiple fungal

pathogens in a single PCR reaction, from either fungal colonies (Fujita et al. 2001; Luo and Mitchell 2002) or positive blood culture broths (Chang et al. 2001). Luo and Mitchell (2002) found that DNA from only about half the molds (*Aspergillus fumigatus*) examined could be amplified directly from the mycelial fragments whereas DNA from every yeast colony (*Candida* spp. and *C. neoformans*) could be readily amplified. Rigby et al. (2002) have applied fluorescence in situ hybridization with peptide nucleic acid probes (DNA probe mimics) to rapid identification of *C. albicans* directly from positive blood culture bottles.

Shin et al. (1997) successfully applied PCR-EIA to identify *Candida* spp. in BacT/Alert blood culture bottles in 7 hours – 2.5 days sooner than by conventional culture. Alternatively, the variability in the length of the ITS2 region can be used to speciate fungal isolates. These size differences cannot be reliably detected by agarose gel electrophoresis and instead automated capillary electrophoresis has been used (Turenne et al. 1999; Chen et al. 2000).

Burnie et al. (1997) applied a similar semiquantitative PCR-EIA to sera from infected patients, this time digoxigenylating the amplicon during the PCR process. The assay was positive (optical density >0.1) in 28 cases of culture-confirmed DC. It was negative in 15 control patients and 13 of 16 patients with local infections. The remaining three, each of whom received amphotericin B, were positive. A universal, rather than yeast-specific, method of extracting the DNA was applied to the sera.

Einsele et al. (1997), targeting a consensus sequence from a variety of fungal pathogens present in 18S rRNA, achieved 100 percent sensitivity when two blood samples were examined in 21 bone marrow transplant patients with documented invasive fungal infections. Specificity was 98 percent. PCR remained persistently positive in patients failing to respond to antifungal therapy but cleared in those who did respond. Van Burik et al. (1998) developed a panfungal PCR assay optimized to detect *C. albicans* and *A. fumigatus* from whole-blood specimens, and obtained positive results in four patients with candidemia and three with pulmonary aspergillosis (one of these occurring a week prior to culture-confirmation by bronchoalveolar lavage). Wahyuningsih et al. (2000) successfully used the QIAamp blood kit (Qiagen, Hilden, Germany) to extract *C. albicans* DNA from human serum samples. They used fungus-specific universal primers against ITS regions corresponding to the 5.8S and 28S rRNA genes for amplification and then hybridized this to a biotinylated probe directed against a *C. albicans*-specific ITS2 region located between these two genes, on a streptavidin-coated plate. All seven patients with culture-confirmed invasive candidiasis were positive by PCR as were a further three high-risk patients.

PCR can be performed with either serum or whole blood. Bougnoux et al. (1999) formally compared serum with whole blood samples, in rabbits with candidemia, applying a nested PCR with five species-specific inner pairs of primers directed against ITS. Their results suggested that serum was the sample of choice.

Hendolin et al. (2000) applied a panfungal PCR to the detection of fungi in tissue specimens. Using broad-range fungal primers, they PCR amplified the fungal ITS region (ITS1-5.8SrRNA-ITS2) and then captured this with species-specific probes against eight fungal pathogens (*Candida* species, *Cryptococcus neoformans*, and *Aspergillus* spp.), species identification therefore relying on both specific hybridization and an assessment of PCR product length. A detection level of 0.1–1 pg of purified DNA was achieved. Of the 20 specimens, PCR was positive for 19 (95 percent), of which 10 (53 percent) were hybridization positive – compared to 12 (60 percent) positive by direct microscopy and seven (35 percent) by culture.

Reiss et al. (1998) applied TaqMan (Perkin-Elmer, Foster City, CA) PCR directed against the ITS2 region to blood cultures, to obtain species identification in 5 hours. TaqMan combines, in one step, PCR, hybridization with a fluorogenic probe, and fluorescent signal generation. Guiver et al. (2001) applied TaqMan PCR to the rapid identification of *Candida* species within 4 hours, compared to a mean of 3.5 days for conventional phenotypic tests. Alternatively, real-time species identification and quantification of fungal load in clinical specimens can be achieved with a LightCycler (Roche Diagnostics, Mannheim, Germany), which combines rapid thermocycling with glass capillaries with online fluorescence detection of the PCR amplicon (Loeffler et al. 2000).

In conclusion, PCR has the potential to offer a high level of sensitivity and specificity within hours of specimen collection. Care must be taken to avoid laboratory contamination, but automated sample preparation and use of real-time PCR is helping to standardize these procedures and reduce the risk of false positives. PCR is likely to be used increasingly, not only to confirm the diagnosis but to speciate the fungus.

ASPERGILLOSIS

The key to understanding the serodiagnosis of infection with *Aspergillus* spp. is an appreciation of the heterogeneity of the diseases that these fungi can cause and the resultant variations in host response. The three main conditions are:

1. an aspergilloma occurring in a pre-existing lung lesion
2. allergic bronchopulmonary aspergillosis (ABPA)
3. invasive aspergillosis.

In all three conditions, the prime organ involved is the lung, which means that diagnosis based on culture is dependent on obtaining adequate bronchial secretions. For aspergilloma, recovery rates vary from 31 to 91

percent (Schonheyder 1987). In ABPA, a positive sputum and the demonstration of hyphae of *Aspergillus* spp. are some of the features necessary to prove the diagnosis; detection of antibody is also essential (Wardlaw and Geddes 1992).

In invasive aspergillosis, lung involvement occurs in 90 percent of cases. The classic clinical presentation is that of unremitting fever, usually higher than 38°C, in a neutropenic patient, but 10 percent of cases occur in non-neutropenic patients in whom steroid therapy is often a complicating factor (Young et al. 1970). Dyspnea and a nonproductive cough are often absent during the early stage of the disease, but pleuritic chest pain and a pleural rub occur in 15–61 percent of patients (Young et al. 1970; Meyer et al. 1973). No sputum is produced by 50 percent of patients and two-thirds of those who do produce sputum are culture negative (Fisher et al. 1981). Positive cultures can occur in the absence of significant clinical disease and this may reflect the aspiration of conidia or possibly hyphal fragments, which transiently contaminate respiratory secretions. Tregar et al. (1985) reviewed 89 patients with positive sputum cultures. In nine cases there were two or more positives and eight of these patients were subsequently proved by histology to have invasive disease. Histological evidence of mycelial invasion from tissue biopsy is often not possible because of the clinical condition of the patient and often the presence of thrombocytopenia. Computed tomography can be used to detect lesions typical of invasive aspergillosis and has a significant input on management (Caillot et al. 1997). Blood cultures are occasionally of value in endocarditis caused by *Aspergillus* spp., but in invasive aspergillosis a positive blood culture is normally the result of contamination (Pennington 1980).

The failure of conventional techniques based on culture has led to numerous attempts to develop alternative assays. These can be grouped into antibody detection, antigen detection, and the detection of the DNA of *Aspergillus* spp. by PCR. *A. fumigatus* is the dominant species, but clinically significant numbers of infections can be caused by other species such as *Aspergillus niger* and *Aspergillus flavus*. Tests which specifically target one species may miss infection caused by a different species. Furthermore, *A. fumigatus* is a diverse microorganism phenotypically (Burnie et al. 1989); therefore strain-specific antigens must be avoided in developing antigen-based diagnostics. *Aspergillus* spp. can colonize patients without causing disease, so very sensitive tests may need to be quantified.

Allergic bronchopulmonary aspergillosis

Clinical diagnosis is dependent on the typical chest x-ray changes of fleeting alveolar infiltrates caused by eosinophilic pneumonia, or a combination of mucus plugging with segmental, lobar, or whole lung collapse. Detection of antibody is essential to the diagnosis of the disease. This includes IgE-mediated immediate-type hypersensitivity to *A. fumigatus* as defined by a positive immediate skin-prick test, a positive radioallergosorbent test (RAST), a high total IgE level (i.e. >2 500 ng/ml), and a raised IgG (Wardlaw and Geddes 1992). These can be demonstrated by radioimmunoassay and EIA, although the latter has been criticized for its lack of reproducibility (Richardson et al. 1982; Richardson and Warnock 1984). The antibody response has been further characterized by self-crossed radioimmunoelectrophoresis (Longbottom 1986). Antigens were subdivided into those with strong precipitin reactions and weak IgE and those that were poorly precipitating but showed strong IgE binding. Of these last antigens, three reacted with more than 50 percent of sera and were regarded as the major allergens.

Piechura et al. (1983) analyzed the cell sap and culture filtrate from three strains of *A. fumigatus*. Two-dimensional electrophoresis followed by gel filtration identified a major component of apparent molecular mass 150 kDa (CS2). On immunoelectrophoresis this produced a major precipitin arc with aspergilloma sera or sera of ABPA patients. On SDS-PAGE, following reduction, it produced three major bands at molecular masses of 41, 53, and 81 kDa. Kurup et al. (1986) prepared culture filtrate antigens from *A. fumigatus* by isoelectric focusing with a pH gradient of 4–6.5. A fraction with an isoelectric point of 6.5 had three components of apparent molecular masses 20, 40, and 80 kDa on two-dimensional electrophoresis. This fraction reacted in an EIA, showing high levels of IgG and IgE reactivity in patients with ABPA and of IgG in those with aspergilloma.

The advent of immunoblotting has helped with detailed dissection of the antibody response, but many anomalies arose regarding the apparent molecular masses of immunodominant antigens. These may reflect, in part, the different conditions under which the gels were run. With a gradient gel of 11–13 percent acrylamide, Brouwer (1988) identified antigens at molecular masses of 36, 50, and 100–120 kDa, the last being estimated by extrapolation. Baur et al. (1989), analyzing 28 patients at varying stages in the disease, found that IgE levels increased as the disease progressed. Immunodominant bands at molecular masses of 40, 51, and 80 kDa were observed, similar in size to the same antigens described by Piechura et al. (1983). Immunodominant antigens at molecular masses of 23 kDa and 17 kDa were thought to be identical to Ag3 (Longbottom 1986) and Ag40 (Wallenbeck et al. 1984). Ag40, along with Ag10, was isolated from a complex mixture of more than 50 precipitating antigens and identified as the major allergens in six patients with ABPA and in 80 percent of 25 patients with asthma caused by *Aspergillus* spp. (Wallenbeck 1991). Samuelsen et al. (1991), looking

at the IgE of patients exposed to *A. fumigatus*, found that bands at molecular masses of 20, 31, 44, 50, 53, 77, and 92 kDa reacted with more than 50 percent of sera. Others have described immunodominant antigens at 92 kDa, a 55 kDa glycoprotein (Teshima et al. 1993), and a protease at 32 kDa which was subsequently cloned and identified as a monoglycosylated alkaline protease with elastase activity (Moser et al. 1993, 1994). Leser et al. (1992) examined sera from 43 cases of ABPA and showed specific IgG and IgE against 12 different bands varying from 15 to 97 kDa. The IgG response broadly reflected the IgE with strong antibody responses to high-molecular-mass bands (between 66 and 97 kDa) as well as bands at 15, 35, and 40 kDa. Arruda et al. (1992) identified major antigens of 18 and 45 kDa. Of the 24 patients with ABPA, 11 had IgE to the 18 kDa antigen. Kobayashi and Miyoshi (1993), examining 10 aspergilloma patients and three ABPA patients, demonstrated IgG binding to antigens of greater than 30 kDa in all sera tested, and IgE binding to the 18 kDa antigen in seven of eight sera with a positive precipitin reaction by counterimmunoelectrophoresis (CIE). When immunoblotting sera from patients with cystic fibrosis and ABPA, Knutsen et al. (1994) observed heterogeneous IgE, IgG, and IgA responses to 11 major bands at 14, 18, 20, 34, 47, 50, 55, 61, 70, 81, and 93 kDa. IgE was more frequently detected against the bands at 47, 50, 61, 70, and 81 kDa.

Some of these bands are likely to be heat-shock proteins. Immunodominant antigens at 88, 84, 51, and 40 kDa were demonstrated by immunoblotting sera from 35 patients with ABPA (Burnie and Matthews 1991). The antigens at 88, 51, and 40 kDa were then shown to react with monoclonal antibodies against Hsp90 of *C. albicans* and the oomycete *Achlya ambisexualis*. Screening an *A. fumigatus* cDNA library with pooled sera from ABPA patients produced a clone which, on sequencing, was homologous with *Candida*, *Saccharomyces*, and human Hsp90 (Kumar et al. 1992). Expressed as a fusion protein, it reacted with the IgE and IgG of patients with ABPA.

Aspergilloma

A pulmonary aspergilloma can often be diagnosed on chest x-ray, or by computed tomography, by the characteristic sign of an opacity surrounded by a crescent-shaped translucent area. Levels of antibody are high and multiple lines can be detected by immunodiffusion (ID) or CIE against either mycelial or culture filtrates (Kurup and Kumar 1991). In patients with ABPA, the same levels of *A. fumigatus*-specific antibodies occur in both subclasses IgG1 and IgG2. The sera of patients with aspergilloma react to the carbohydrate and glycoprotein antigens of *A. fumigatus* with more IgG1 than IgG2 (Kurup et al. 1990).

Immunoblotting has been used to analyze the antibody response. Matthews et al. (1985) demonstrated bands ranging from 33 to 88 kDa with an immunodominant antigen at 40 kDa. Latgè et al. (1991) identified immunodominant antigens of 18, 28, 30, 38, 45–48, 94, 110, and 125 kDa with sera from 75 patients, but only a 35 kDa antigen reacted specifically with sera from the aspergilloma patients. The 18 kDa antigen was purified to homogeneity and related to a similar antigen found in the urine of patients with invasive aspergillosis (Haynes et al. 1990). It was subsequently shown to have ribonuclease activity (Latgè et al. 1993). Burnie and Matthews (1991) demonstrated immunodominant antigens at 40, 84, and 88 kDa. The 88 kDa antigen reacted with monoclonal antibodies against Hsp90 of *Candida* and *Achlya* spp., as did the extract from an aspergilloma removed from a patient. This gave a prominent band at 50–52 kDa. Kobayashi et al. (1993) confirmed the immunodominance of a band at 88 kDa but suggested that it was not Hsp90. Moutaouakil et al. (1993) biochemically characterized a 33 kDa serine protease but failed to demonstrate specific antibodies against it in aspergilloma patients.

Increasingly, recombinant proteins are being used as the basis of assays for the detection of specific, circulating antibodies to *A. fumigatus*. These are often highly specific and, in the case of aspergilloma, highly sensitive: 100 percent sensitivity was obtained by Holdom et al. (2000) using recombinant Cu, Zn superoxide dismutase, by Weig et al. (2001) using recombinant mitogillin protein, and by Chan et al. (2002) using recombinant galactomannoprotein.

Invasive aspergillosis

ANTIBODY DETECTION

Early investigations into the serodiagnosis of invasive aspergillosis concentrated on antibody detection. Coleman and Kaufman (1972) found precipitins in 82 percent of proven cases of invasive aspergillosis and in 83 percent of suspected cases. Positive precipitins in invasive disease were also observed by Meyer et al. (1973), Warnock (1977), and Kurup and Fink (1978).

Several groups working independently with different assays have found a correlation between rising antibody titers and recovery. Gold et al. (1980) developed a passive hemagglutination test based on sheep red cells treated with concanavalin A and sensitized with a partially purified antigen to *Aspergillus* spp. Sequential testing in 55 cancer patients, with sera available in the 2 weeks before diagnosis of invasive aspergillosis was made, demonstrated that 15 seroconverted. Recovery was associated with an increased antibody titer. Holmberg et al. (1980) developed an EIA with a concentrated culture filtrate precipitated by 75 percent saturated

ammonium sulfate. Examining material from 10 cases of invasive aspergillosis, they correlated a serial rise in EIA titer with recovery. Matthews et al. (1985) found, by immunoblotting serial sera, that antibody to an immunodominant 40 kDa antigen was notably absent or fell in titer in 12 fatal cases of invasive aspergillosis but persisted in two survivors. A solid-phase radioimmunoassay has also been developed for measurement of antibody (Marier et al. 1979).

There have been a number of studies describing immunodominant antigens. Antigens at 41, 54, and 71 kDa were described by de Repentigny et al. (1991) using a rabbit model. Fratamico and Buckley (1991), examining 172 sera from 38 patients with invasive aspergillosis, found 90 percent of sera detected a 58 kDa antigen on immunoblots. This antigen was abundant in mycelial extracts and comprised about 50 percent of the Coomassie blue-stained protein. It contained carbohydrate, staining with periodic acid–Schiff agent and binding to the lectin concanavalin A. Monoclonal antibodies raised against it reacted with a similar antigen in *C. albicans*, shown by localized cell surface immunofluorescence. They also recognized an *Aspergillus* antigen with a molecular mass of more than 89 kDa (Fratamico et al. 1991). Burnie (1995) immunoblotted sera from 55 cases of proven and 20 cases of suspected invasive aspergillosis and demonstrated immunodominant bands at 88, 84, 63, and 40 kDa. Possession of either IgM or IgG against the bands at 88, 84, and 40 kDa correlated with survival. The band at 88 kDa was heat inducible and reacted with monoclonal antibodies specific to the Hsp90 homologs of *A. ambisexualis* and *C. albicans* (Burnie and Matthews 1991). Reactivity was neutralized by cross-absorption with Hsp90.

Hamilton et al. (1995) identified a Cu, Zn superoxide dismutase which, by immunoblot, gave a sensitivity of 25 percent in cases of invasive aspergillosis (Holdom et al. 2000). Using recombinant mitogillin protein, in an ELISA, Weig et al. (2001) obtained a sensitivity of 62.2 percent and specificity of 95.4 percent (in normal blood donors), with a cutoff value of mean ±2 SD. Increasing the stringency by using a cutoff value of mean ±5 SD improved specificity by lowered sensitivity. Using recombinant galactomannoprotein, Chan et al. (2002), with a cutoff value of mean ±5 SD, obtained a sensitivity of 33.3 percent (five of 15 sera from 15 patients with invasive aspergillosis positive) and specificity of 100 percent.

ANTIGEN DETECTION

In cases of suspected invasive aspergillosis, antigen detection is generally preferred because the at-risk groups are likely to be immunocompromised and poor antibody producers. Antigen detection stemmed from the initial description of circulating galactomannan in the serum of a patient with invasive aspergillosis (Reiss and Lehmann 1979). The importance of this antigen has been confirmed in a rabbit model (Dupont et al. 1987) and in patients using radioimmunnoassay, EIA, and latex agglutination (LA) (Shaffer et al. 1979; Dupont et al. 1990).

In clinical studies, the radioimmunoassay had a sensitivity of 70–80 percent, a specificity of 90 percent, a positive predictive value of 82 percent, and a negative predictive value of 85 percent (Talbot et al. 1987). Antigen was detected before invasive aspergillosis was suspected in 30 percent and before laboratory confirmation in 46 percent of patients. The main limitations were the use of radioactive material and the absence of antigen in about 25 percent of cases.

Sabetta et al. (1985) devised a competitive EIA that detected an undefined carbohydrate antigen in five of six immunosuppressed, infected rabbits and 11 of 19 patients with invasive aspergillosis. In experimentally infected rabbits, de Repentigny et al. (1987) detected circulating antigen which bound to concanavalin A with an apparent molecular mass of 50–100 kDa. The size of the galactomannan antigen was then substantiated by a monoclonal antibody which reacted with the immunodominant oligogalactoside side chains of *A. fumigatus* galactomannan recognizing 80, 62, and 49 kDa components of a hyphal homogenate (Ste-Marie et al. 1990).

Rogers et al. (1990) described two inhibition EIAs using a high-titer human serum to *Aspergillus* and a rat IgM monoclonal antibody to galactomannan, respectively. The results were encouraging in that 15 of 16 patients with invasive disease had detectable antigen at some point during their illness, but only 13 percent of sera and 20 percent of urine specimens were positive with the human serum. This improved to 18 percent for sera and 44 percent for urine samples when the monoclonal antibody against galactomannan was employed.

A commercially available RPLA test, Pastorex *Aspergillus* (BioRad, Marnes-la-Coquette, France) utilized a rat monoclonal EB-A2 specific to the galactomannan of *Aspergillus*. It reliably detected galactomannan in the sera of experimentally infected guinea pigs and showed no crossreactivity in animals with invasive candidiasis or cryptococcosis (Van Cutsem et al. 1990). Sensitivities of up to 95 percent were reported in patients with proven invasive aspergillosis (Dupont et al. 1990; Haynes and Rogers 1994), though stored sera were liable to become negative (Warnock et al. 1991; Knight and Mackenzie 1992). Hopwood et al. (1995), examining patients undergoing liver and bone marrow transplants, found that four of eight patients subsequently diagnosed as having invasive aspergillosis were Pastorex positive, but only for eight of the 187 sera examined from these four patients. In a prospective study, Verweij et al. (1995b) obtained a positive Pastorex test in eight patients who were subsequently shown to have invasive aspergillosis. The

maximum titer was 1 in 8. The initial serum sample was negative in five of the cases. Storage at −20°C for longer than 6 months resulted in a loss of reactivity in six of the 13 sera tested. A further problem was the crossreactions observed, with no defined cause, reported in five patients by Hopwood et al. (1995) and also in a serum contaminated by *Penicillium chrysogenum* (Kappe and Schulze-Berge 1993).

Styner et al. (1995) demonstrated that the limit of detection was lowered 10-fold in an EIA employing the galactomannan monoclonal antibody as both captor and detector. Comparison with LA demonstrated improvement in both sensitivity and specificity (Verweij et al. 1995c). There is now a commercially available sandwich ELISA (Platalia Aspergillus, Bio-Rad Laboratories, Marnes La Coquette, France) with a reported sensitivity of 67–100 percent and specificity of 81–98 percent. Most importantly, this assay is sensitive enough to give positives at an early stage of the infection. Antigen titers increase in the terminal stages of the disease and serial assessment of serum titers is useful in monitoring response to treatment (Verweij et al. 1996, 1997). Prospective studies in patients with hematological malignancies have shown high sensitivity (>89 percent) and specificity (>92 percent) (Maertens et al. 1999, 2001; Sulahian et al. 2001). Sera were collected twice a week and a positive result reported when galactomannan was detected in two consecutive samples: increasing antigen titers in consecutive samples was a particularly strong indication of infection. These studies suggested that the cutoff value recommended by the manufacturer could be lowered. There is evidence that the test can be reliably applied to bronchoalveolar lavage and CSF specimens (Verweij et al. 1995a; Viscoli et al. 2002). The great majority of galactomannan in the CSF appeared to be produced intrathecally. The usefulness of monitoring response to treatment may vary with the drug used, since there is some data from a rabbit model of pulmonary aspergillosis to suggest that during treatment with the echinocandin caspofungin circulating antigen failed to decrease despite clinical improvement (Petraitiene et al. 2002).

Urine from recipients of bone marrow transplants with invasive aspergillosis contained not only galactomannan with a molecular weight of over 45 kDa but also several protein antigens between 11 and 44 kDa (Haynes et al. 1990). Other antigens described include an 80 kDa antigen (Phillips and Radigan 1989) and antigens at around 88, 40, 27, and 20 kDa (Yu et al. 1990). Burnie (1991b) developed a RPLA test detecting *Aspergillus* antigen with a rabbit polyclonal antiserum raised against a crushed extract of *A. fumigatus*. Antigen extracted from high-titer patient sera, by affinity chromatography using the same rabbit antiserum, reacted with monoclonal antibodies to *A. ambisexualis* and *C. albicans* Hsp90, showing that *Aspergillus* Hsp90 circulated during infection (Burnie and Matthews 1991).

PCR

In invasive aspergillosis, targets for PCR have been limited copy genes, for example the 18 kDa IgE-binding protein (Reddy et al. 1993) and alkaline protease (Tang et al. 1993), or multicopy genes such as the 26S intergenic spacer gene of rDNA (Spreadbury et al. 1993), the 18S rRNA genes (Yamakami et al. 1996), mitochondrial DNA (Jones et al. 1998), or panfungal targets such as the small subunit rRNA gene (van Burik et al. 1998). Specimens obtained at bronchoscopy have been tried, but the sensitivity can be low and false positives have been a persistent problem (Brètagne et al. 1995; Verweij et al. 1995a; Bart-Delabesse et al. 1996). This problem may be independent of the target sequence; it may be caused by colonization of the patient's respiratory tract or by contamination during processing.

Yamakami et al. (1996) developed an aspergillus-specific nested PCR, based on sequences derived from the 18S rRNA gene, which detected *Aspergillus* DNA in the sera of experimentally infected mice (sensitivity 71 percent) and 20 patients with invasive aspergillosis (sensitivity 70 percent). The same gene was targeted by Einsele et al. (1997), who detected circulating DNA in all 13 patients with invasive aspergillosis. Golbang et al. (1999) modified this assay to convert it from a nested PCR to a semiquantitative PCR-EIA, incorporating the digoxigenin label during the PCR and binding the amplicon to a streptavidin plate with a biotinylated capture probe. This assay was used to quantify the level of circulating DNA in invasive aspergillosis. The sensitivity was 91 percent, 30 of the 33 culture-confirmed cases of invasive aspergillosis giving positive optical densities (>0.1). It was negative in all 20 control sera. Where sequential samples were available, levels fell in survivors and gradually rose in fatal cases. PCR has also been successfully applied to cerebrospinal fluid specimens in cases of aspergillus meningitis (Kami et al. 1999; Verweij et al. 1999) and to ocular samples in fungal endophthalmitis (Jaeger et al. 2000). Comparative studies of the galactomannan ELISA with PCR have yielded conflicting results as to which is superior (Bretagne et al. 1998; Kawamura et al. 1999; Becker et al. 2000). Both systems have shown the ability to detect the infection early (Verweij et al. 1997; Hebart et al. 2000), opening up the possibility of prospective screening of high-risk patients and prompter treatment.

Real-time PCR greatly reduces the risk of false-positive results because the reaction tubes do not need to be opened following amplification, thus avoiding environmental contamination with the amplicon, the main source of contamination. Using the LightCycler, and a fully automated nucleic acid extraction technique to further reduce the risk of cross-contamination, Costa et al. (2002) compared the performance of real-time PCR with an ELISA for detecting galactomannan. They found serum to be preferable to white cells as a source

of *A. fumigatus* DNA. The galactomannan assay was more frequently positive than the PCR assay (52 percent versus 45 percent). It may be that *Aspergillus* releases large amounts of galactomannan, which is relatively easily detected compared to the tiny quantities of circulating fungal DNA, despite amplification by PCR. Even before death, the DNA concentration was never greater than 30 fg/ml of serum. More studies on the origins and kinetics of fungal DNA release would help resolve these issues.

CRYPTOCOCCOSIS

This systemic infection is caused by the encapsulated basidiomycetous yeast *Cryptococcus neoformans*. It can cause meningitis in non-immunocompromised patients, but the great majority of cases now occur in patients with the acquired immunodeficiency syndrome (AIDS), in whom it may present as meningitis, pneumonia, or fever resulting from an underlying cryptococcemia. Although the principles of laboratory diagnosis are the same, the lack of sufficient capsular material produced by some isolates from AIDS patients occasionally causes problems for standard LA tests.

Latex agglutination

Several latex agglutination tests (RPLA) are commercially available for the rapid detection of the capsular polysaccaride antigen. The capsular antigen is produced in large quantities, has a serum half-life of 24 hours (Kappe and Muller 1991), and is stable against heat or pronase treatment, allowing dissociation from bound antibodies. These RPLA tests, which use antibody-coated latex particles, are easy to perform and highly sensitive, and specific for the diagnosis of meningeal and disseminated cryptococcosis (Prevost and Newell 1978; Kauffman et al. 1981; Bhattacharjee et al. 1984). Although *C. neoformans* is usually easy to isolate from clinical material, it may take up to 10 days to appear. The RPLA test can give the diagnosis in 30 minutes. Titres of 1:100 or more can be demonstrated in the CSF and, particularly in AIDS patients, in serum. Antigen titers can help monitor response to therapy and give an indication of the extent of infection and, indirectly, of the prognosis. Antibody detection adds little (Hay 1992).

The sensitivity of RPLA tests is high, well above 90 percent. Reports as to the lower limits of detection with the Cryptococcal Antigen Latex Agglutination System (CALAS, Meridian Diagnostics Inc., Cincinnati, OH) and the Crypto-LA test (International Biological Labs Inc., Cranbury, NJ) are in the range 2.5–10 ng/ml of capsular polysaccharide for serotypes A–D (Temstet et al. 1992) and 7.6–61 ng/ml, the highest level being for serotype D (Gade et al. 1991). False positives can occur. A heat-stable cell wall component of *Trichosporon beigelii* (syn. *Trichosporon cutaneum*) crossreacts with cryptococcal polysaccharide (Melcher et al. 1991). Disseminated infections caused by *T. beigelii* can occur in immunosuppressed patients and they are a well-documented cause of positive results with cryptococcal RPLA tests (McManus et al. 1985). Other rare causes of false positives are malignancy (Hopfer et al. 1982) and septicemia with DF-2 (Westerink et al. 1987). Technical false positives have been reported as a result of the introduction of talc from latex gloves into CSF samples, following immersion of a platinum wire inoculating loop into a CSF sample for culture before testing, and after repeated washing of ring slides with certain disinfectants and soaps (Heelan et al. 1991; Blevins et al. 1995).

A prozone-like effect, probably the result of immune complex formation, can give rise to false negatives. This can be avoided by diluting out the specimen (Stamm and Polt 1980) or treating with pronase. Pronase considerably reduces the occurrence of false reactions by cleaving antibodies present in immune complexes, which can otherwise mask the detection of antigen, and destroying rheumatoid factor (Sadamoto et al. 1993). Pronase pretreatment is a recommended modification of many of the commercially available RPLA tests; it increases sensitivity for most sera and some (but not all) CSF samples, and improves the end-point readings by making the agglutination reactions stronger (Gray and Roberts 1988; J.R. Hamilton et al. 1991). Kohno et al. (1993) obtained significantly higher rates of detection of serum cryptococcal antigen using the Eiken test (Eiken Co., Tokyo) in conjunction with pronase pretreatment.

One of the commercially available LA tests (Pastorex *Cryptococcus*, BioRad, Marnes-La-Coquette, France) has a murine monoclonal antibody to cryptococcal polysaccharide (Dromer et al. 1987), sensitizing the latex particles instead of the more commonly used rabbit polyclonal antisera. Temstet et al. (1992) compared this with two commercially available RPLA tests using polyclonal immune sera, CALAS and Crypto-LA. Clinical specimens from 87 AIDS patients, 40 of whom had culture-proven cryptococcosis, were retrospectively tested in a blind study. Pastorex performed well during the initial screening, concordance among the three tests being 97 percent. The predominant serotypes were A and D, only one patient having serotype B and none having serotype C, which reflected the prevalent serotypes. However, the threshold for detection of capsular polysaccharide with Pastorex was at least a log poorer for serotypes B, C, and D, compared with CALAS and Crypto-LA. Pronase treatment enhanced both the sensitivity and specificity of Pastorex and it was recommended for all samples except urine. Interestingly pronase treatment did not resolve the prozone effect in one case which was corrected by sample dilution. The antigen titers obtained with Pastorex and CALAS after pronase treatment agreed within one dilution for 67 percent of CSF samples and 57 percent of sera. It was

emphasized, as previously observed (J.R. Hamilton et al. 1991), that kits for the detection of cryptococcal antigen cannot be used interchangeably to monitor antigen titres in patients.

Occasionally false negatives occur in AIDS patients as a result of a capsular-deficient variant of *C. neoformans*, resulting in low concentrations of the antigen. Both serum and CSF specimens are usually positive in AIDS patients with cryptococcal meningitis, definitive diagnosis then being made by culture of the CSF (Panther and Sande 1990). Routine screening for serum cryptococcal antigen does not, however, appear to predict patients who subsequently develop cryptococcal meningitis (Nelson et al. 1990). Localized pulmonary cryptococcosisis is likely to give negative RPLA results with serum samples (Prevost and Newell 1978; Kauffman et al. 1981), although Baughman et al. (1992) successfully screened specimens of bronchoalveolar lavage fluid with the CALAS test to diagnose cryptococcal pneumonia.

EIA

An EIA (Premier, Meridian Diagnostics), using a polyclonal capture system to bind capsular polysaccharide and visualized with a peroxidase-labelled monoclonal antibody, was compared with the CALAS RPLA by Gade et al. (1991). They obtained close agreement (98 percent). Overall sensitivity for the EIA was 99 percent and specificity 97 percent, there being 120 EIA and latex agglutination (LA) positives out of the 475 specimens screened. Detection limits for serotypes A–D were 0.63, 0.63, 7.8, and 62 ng/ml, respectively. Knight (1992) compared this with the RPLA test of Immuno-Mycologics and reported 92 percent agreement, after examination of 54 samples, mainly sera and CSF, from patients with and without cryptococcosis. There was some indication, but only in a couple of patients, that the EIA gave fewer false positives and improved the diagnosis of genuine early cases. The EIA test was easy to perform and had a similar cost. As a screening test it took no longer than the RPLA, although determining the titer of first time positives took longer.

Sekhon et al. (1993) compared the Premier EIA with two commercially available RPLA tests, Immuno-Mycologics and Crypto-LA, and a non-commercial latex agglutination test. Of 143 sera and CSF from proven and suspected cases, 115 were negative by EIA and RPLA. The sensitivity of the EIA test was 85.2 percent and specificity 97 percent, compared with 100 percent sensitivity and specificity for the LA tests. Out of 26 true-positive specimens, 23 were EIA positive but three RPLA positive specimens were negative by EIA. In the great majority of cases, however, the EIA yielded higher titers than the RPLA, especially for specimens from AIDS patients, observations similar to those made by Gade et al. (1991). They found the EIA, unlike RPLA, did not require pretreatment of specimens to prevent false-positive reactions.

An EIA has also been described by Casadevall et al. (1992), who used monoclonal antibodies, of different isotypes, both to capture and to detect cryptococcal polysaccharide. Immuno-Mycologics have produced a biotin-amplified sandwich EIA.

Noncapsular antigens

Chaturvedi et al. (2001) have cloned and compared the Cu, Zn superoxide dismutase gene from three varieties of *C. neoformans*, with variable host predilection, in order to provide insight into its role in pathogenesis. A.J. Hamilton et al. (1991) have produced a range of murine monoclonal antibodies which recognized culture filtrate antigens (exoantigens) from both encapsulated and non-encapsulated isolates of *C. neoformans*. This suggests that they did not recognize capsular polysaccharide material, and immunofluorescence data showed reactivity with the cytoplasm and cell membranes only. Some of these monoclonal antibodies crossreacted with *T. beigelii*. One monoclonal, which recognized neither *T. beigelii* nor *C. neoformans* var. *gattii*, recognized a species-specific epitope on a 34–38 kDa glycoprotein produced as an exoantigen by *C. neoformans* var. *neoformans* (Hamilton et al. 1992). Another monoclonal antibody recognized a 115 kDa exoantigen which has been shown to react strongly with the sera of patients with cryptococcosis (Hamilton and Goodley 1993). Monoclonal antibodies such as these may prove useful in the serodiagnosis of cryptococcosis in AIDS patients infected with isolates lacking polysaccharide capsules.

PCR

PCR has been successfully applied to the detection of *C. neoformans* in clinical specimens from confirmed cases (Rappelli et al. 1998; Posteraro et al. 2000), but whether it can outperform the exisiting, highly successful assays for this infection remains to be determined. Bialek et al. (2002c) have developed nested and real-time PCR assays, both targeting the 18S rRNA gene, to a murine model of cryptococcal meningitis.

HISTOPLASMOSIS

This is a systemic fungal infection caused by the dimorphic fungus *Histoplasma capsulatum*. The fungus is found in the soil and endemic to extensive parts of the Americas, Africa, and Asia. There are two varieties: the small-celled form (var. *capsulatum*), which occurs globally, and the large-celled form (var. *duboisii*), confined to Africa and Madagascar. Histoplasmosis is a common

and serious complication of AIDS, occurring with highest frequency in the endemic areas of the USA (Wheat et al. 1991). Infection usually begins in the lungs, but a progressive disseminated form of the disease often develops in AIDS patients. Clinical diagnosis may be hampered because the severity of the systemic disease overshadows the respiratory component and radiologically the chest may appear normal initially. Most of these patients present with fever and weight loss.

In exposed communities, up to 70 percent of the population have positive histoplasmin skin tests. The histoplasmin skin test is similar to the Mantoux test. It indicates exposure to the organism, and is used mainly for epidemiologic purposes. Many patients with disseminated infections are anergic and give negative skin tests whereas, in a sensitized individual, skin testing can induce antibodies which then complicate the serological diagnosis (Hay 1992; Kaufman 1992).

Laboratory diagnosis is required to confirm clinical and radiological signs of a systemic mycosis and to establish the etiology. Histopathological examination and culture of bone marrow biopsies and blood cultures are common means of obtaining a definitive diagnosis. But isolation and identification of the *capsulatum* variety of *H. capsulatum* from clinical material can take several weeks and requires conversion of the mycelial isolate to the yeast form or identification by commercially available exoantigen tests or DNA probes (Kaufman 1992; Huffnagle and Gander 1993). Therefore, serology is still the best method available for screening suspected cases of histoplasmosis.

Antibody assays

Proper interpretation of serology is helped by taking a full clinical history, including occupation, travel, residence, and whether the patient has undergone histoplasmin skin testing. The laboratory, which must be familiar with the tests' capabilities and limitations, can then select the appropriate battery of tests. Complement fixation (CF), ID, and LA tests for the detection of antibody are available commercially.

The histoplasmin LA test (available commercially from Immuno-Mycologics) detects IgM, which is produced in the early stages of infection. It is therefore only positive early in the course of a primary infection, becoming negative within the first few months of a chronic infection.

The ID test is based on the principles of double diffusion. The patient's serum and soluble antigen are placed in separate wells cut in a suitable diffusion medium (such as agarose or Cleargel) and allowed to diffuse outward. Visible lines of precipitate form where the antigen and antibody have combined in the proper relative concentrations (equivalence). Using the mycelial-form culture filtrate of *H. capsulatum* var. *capsulatum* as a source of H and M antigens ('histoplasmin'), both H and M precipitins are detected, the M precipitin band appearing nearer the antigen well. Positive control sera, containing specific antibodies against H and M antigens, are placed adjacent to each patient serum in order to determine the specificity of any patient reactions through the formation of an 'identity' or 'partial identity' reaction where the two precipitin bands interact.

The ID test detects IgG antibodies and is negative in the first 3–6 weeks of a primary infection when IgM predominates. It usually becomes positive within about 1 month of infection, but can remain completely negative in patients with confirmed histoplasmosis (Helner 1958). The M band is present in 70 percent of active cases (Kaufman 1992). Provided a skin test has not been performed recently (Kaufman 1973), it is an indicator of active infection or recent past infection, because M precipitins persist some months after recovery. The H band occurs less frequently than the M band, and the absence of an H band does not exclude active histoplasmosis. In active cases, 2 percent are H+ and 10 percent are H+ and M+ (Kaufman 1992). An H band usually indicates active clinical disease and, if both M and H precipitins are present, it is highly suggestive of active histoplasmosis regardless of other serological results. Demonstration of M and H precipitins in the CSF is indicative of meningeal histoplasmosis (Kaufman 1992).

The CF test is based on the principle that complement (C′) is irreversibly taken up by certain classes of antibody when they react with antigen, the presence of free C′ being indicated by lysis of sheep red blood cells sensitized with antibody because C′ is required for this process. If complement-fixing antibodies are present, there is no hemolysis. It is essential to determine serum anticomplementary activity. This is when the patient's serum fixes or destroys C′ in the absence of added antigen and can be caused by a number of factors. Other essential prerequisites include determination of the optimal antigen dilution with known case sera (not hyperimmune animal control sera), standardization of all reagents, and inclusion of positive and negative control sera.

The CF test is more sensitive than ID. Both histoplasmin (H and M), derived from the mycelial form of *H. capsulatum* var. *capsulatum*, and yeast form antigens are available commercially for use in CF tests. Leland et al. (1991) compared reagents from Immuno-Mycologics with those obtained from Meridian Diagnostics and found the former to be more reactive. Measurement of yeast antibody with the CF test has a higher sensitivity (90 percent) than histoplasmin antibody (80 percent) (Kaufman 1992). CF titers of more than 32, or a fourfold rise in titer, give good presumptive evidence of histoplasmosis. Lower titers are nonspecific, particularly with the yeast antibody. Skin testing may interfere with histoplasmin antibody, which can be induced or significantly

raised in histoplasmin-sensitized individuals after one histoplasmin skin test. Crossreactions can occur with sera from patients with blastomycosis, coccidioidomycosis, and other mycoses, and, with yeast-form antigen, they can occur in patients with leishmaniasis (Kaufman 1992). Lack of an antibody response should not exclude the diagnosis, particularly if only one specimen has been tested and the clinical picture is highly suggestive. CSF titers are usually 1:8 or greater in cases of meningeal histoplasmosis (Plouffe and Fass 1980).

Antibody assays are less reliable in immunocompromised patients and disseminated histoplasmosis. Torres et al. (1993) found that neither CF nor an EIA was useful for the diagnosis of disseminated histoplasmosis in AIDS patients. Kaufman (1992) found that the histoplasmin antigens used in CF and ID tests more readily detected histoplasmosis in AIDS patients than yeast form antigens.

Detection of antigens

A radioimmunoassay for detection of *H. capsulatum* var. *capsulatum* polysaccharide in urine or serum has been used successfully in the rapid diagnosis of disseminated (Wheat et al. 1989) and pulmonary (Wheat et al. 1992) histoplasmosis in AIDS patients. The antigen was found in the urine of 92 percent and serum of 88 percent of such AIDS patients. It could also be detected in the bronchoalveolar lavage fluid of 70 percent of cases, and was not detectable in negative controls. Wheat et al. (1991) found the assay particularly useful in the detection of relapses in AIDS patients with histoplasmosis. Antigen levels fell in response to antifungal therapy and an increase of more than 2 units in antigen levels during maintenance therapy was strongly indicative of relapse. They suggested routine measurement of antigen levels in both serum and urine every 1–3 months and in the event of clinical evidence of relapse. But in a comparative assessment of itraconazole versus fluconazole for the treatment of disseminated histoplasmosis in patients with AIDS, they found clearance of fungal blood cultures a better measure of antifungal effect than clearance of antigen (Wheat et al. 2002).

Other histoplasma antigens are now being characterized. Hamilton et al. (1990a) have immunological data suggesting that the M antigen of *H. capsulatum* var. *capsulatum* is a catalase. They have also produced monoclonal antibodies that distinguish between the varieties *capsulatum* and *duboisii*. These could be useful for epidemiology and serodiagnosis in areas where both these forms of histoplasmosis occur (Hamilton et al. 1990b). Torres et al. (1993) identified antibodies against four different antigens of *Histoplasma* spp. (91, 83, 70, and 38 kDa) by immunoblotting sera from patients with histoplasmosis. Gomez et al. (1992, 1995) have identified two heat-shock proteins, Hsp60 (HIS-62) and Hsp70 (HIS-80), mediating protective immunity against murine histoplasmosis through induction of cellular immunity. Maresca et al. (1994) have correlated the virulence of a strain with the time required to transform to the yeast form and to express heat shock genes. Detection of Hsp60 or Hsp70 in serum or urine could have diagnostic value for histoplasmosis analogous to the detection of Hsp90 in DC.

PCR

The detection of *H. capsulatum* in the soil currently involves a slow (6–8 weeks) and expensive assay requiring inoculation of mice. Therefore the development by Reid and Schafer (1999) of a 2-day PCR method, using fungal-specific primers followed by nested primers against an ITS region of a rRNA gene, should be a significant advantage. Bialek et al. (2002a) have developed a nested PCR assay to unambiguously identify *H. capsulatum* in tissue sections.

BLASTOMYCOSIS

This is caused by the dimorphic fungus, *Blastomyces dermatitidis*, endemic to parts of North America, Africa, India, and the Middle East. Diagnosis is usually made by the identification of the yeast in body fluids or tissues (such as sputa, bronchial lavage, or biopsy specimens) by potassium hydroxide smears, cytology, histology, or culture (Lemos et al. 2000; Martynowicz and Prakash 2002). As for histoplasmosis, serology can provide an indication of the likely diagnosis earlier than culture or histology. Serodiagnosis has improved following better purification of the A antigen of *B. dermatitidis*. Sensitivity varies, depending on the assay format, from 40 percent to 88 percent, but specificity is consistently very high (98–100 percent) (Kaufman 1992). Commercial sources include Immuno-Mycologics, Meridian Diagnostics, and Gibson Laboratories, Lexington, KY (formerly Nolan Scott Biological Laboratories, Atlanta, GA).

The widely used CF and ID tests, with purified A antigen, are only positive, respectively, in 40 percent and 65 percent of confirmed cases (Turner et al. 1986). Therefore a negative result in no way excludes active blastomycosis. The ID test is totally specific, an A band being presumptive evidence of active or recent infection. Sera from patients with other infections, such as histoplasmosis and coccidioidomycosis, may produce bands against the antigen to *B. dermatitidis*, but these do not form identity reactions with the A bands produced by the positive control serum.

More sensitive assay systems are now being developed. An indirect EIA, using purified A antigen, gave a sensitivity of 80 percent and a specificity of 98 percent (Turner et al. 1986). Titers of 1:32 or greater were indicative of

active blastomycosis while lower titers were considered suggestive but could be the result of crossreactivity with other dimorphic fungi, particularly the *capsulatum* variety of *H. capsulatum*. Lo and Notenboom (1990), using commercial A antigen captured with rabbit antibody to A antigen with a sandwich EIA, succeeded in avoiding crossreactivity with sera from patients with histoplasmosis or coccidioidomycosis. They detected seven of eight active cases of blastomycosis, compared with three positive cases by ID or CF. The false-negative result was from a patient with AIDS. A cell wall-derived radiolabelled 120 kDa protein has been used to detect antibody in infected patients by Klein and Jones (1990).

COCCIDIOIDOMYCOSIS

This systemic mycosis, caused by the dimorphic fungus *Coccidioides immitis*, generally takes the form of an acute, mild respiratory disease but can become chronic or disseminated, and occurs as an opportunistic infection in AIDS patients. The fungus is endemic only to certain semidesert-like regions between southwest USA and Argentina. Both clinical diagnosis and laboratory diagnosis, by demonstration of the organism histologically or by culture, are sometimes difficult. Often positive serology is the first evidence of the diagnosis, indicating the need for more definitive techniques which may require biopsy.

Serodiagnosis is generally based on the detection of antibodies to two main antigens to *C. immitis*: the tube precipitin (TP) and CF antigens found in mycelial-form culture filtrate (coccidioidin). Antibody to the former was thought to be mainly IgM, although recent data suggest that IgG responses are often largely directed against TP (Gade et al. 1992). Antibody to CF antigens is mainly IgG (Pappagianis and Zimmer 1990). The early IgM response is useful in the diagnosis of acute primary coccidioidomycosis. Later, IgG is produced, usually outlasting the IgM, and persisting in chronic coccidioidomycosis.

Partial characterization of both TP and CF antigens has occurred. Both 120 kDa and 110 kDa components of TP have been identified as a glycoprotein co-localized within cytoplasmic vesicles and the wall of the spherule (Cole et al. 1991). Deglycosylation of these antigens resulted in significant loss of reactivity with patient IgM. Although mannose and glucose were the predominant monosaccharides, a 3-*O*-methylmannose sugar is also present which appears to be unique to this systemic fungal pathogen and at least partially responsible for the reactivity of IgM with the 120 kDa antigen. One of the CF antigens, which participates in the ID reaction, appears to be a chitinase (Johnson and Pappagianis 1992).

A LA test is commercially available (Immuno-Mycologics). It uses TP-coated latex particles to detect agglutinating antibodies. These are primarily IgM, but high titers of IgG can also cause agglutination (Martins et al. 1995a). It is a sensitive and simple rapid screening test, useful in detecting early acute disease, but it has a high rate of false positives so confirmation by ID is essential (Drutz and Cantanzaro 1978). Rheumatoid factor is one cause of false positives and should be excluded. It can be removed by IgG-absorbent diluents and columns that leave the IgM intact (Martins et al. 1995b).

The ID test can be used to detect antibodies to TP or CF antigens. The immunodiffusion tube precipitin (IDTP) test, thought to measure IgM, is a sensitive and specific indicator of early acute disease (Pappagianis and Zimmer 1990; Kaufman 1992). IgG is detectable by immunodiffusion complement fixation (IDCF) or CF tests and is useful in diagnosing later stages of infection. It can also be used to detect past infection. Quantification of antibody titers provides an indicator of disease progression, falling titers being a measure of response to therapy. Changing or high CF titers are diagnostic (Kaufman 1992). Lower CF titers may reflect crossreactivity and such results should be confirmed by IDCF. The IDCF test can be used as a qualitative screen or it may be quantified. CF and IDCF can be applied to serum or CSF samples.

An EIA for the detection of IgM and IgG against a purified mixture of TP and CF is now commercially available from Meridian Diagnostics (Gade et al. 1992). In assessing this assay, Martins et al. (1995a) compared the IgM EIA with LA, and the IgG EIA with ID and CF tests. It proved to be a reliable assay for the detection of antibodies in serum or CSF against both TP and CF antigens. The best overall correlation was achieved when EIA was compared with the combined results of the traditional assays; there was an agreement of 96.7 percent, a specificity of 98.5 percent and a sensitivity of 94.8 percent. EIA had several advantages. It used specific anti-IgG and anti-IgM conjugates to give a definitive IgG or IgM result. Results were determined objectively with a spectrophotometer. Unlike the CF test, indeterminate results caused by anticomplementary activity were not a problem, and there was no need to heat inactivate the specimen or for an overnight incubation. The EIA could be run in less than 2 hours, allowing results to be reported the same day the sample was received. A disadvantage of the EIA was that because the same serum dilution was used for both IgG and IgM testing, there was no IgG absorbent in the diluent; samples positive for specific IgM must therefore be tested for rheumatoid factor.

PARACOCCIDIOIDOMYCOSIS

This systemic mycosis, caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is endemic to Latin America. It primarily involves the lungs but may disseminate, causing secondary lesions. Definitive diagnosis

can only be achieved using laboratory procedures such as microscopy, histology, and culture.

Many different tests have been used for the serodiagnosis of paracoccidioidomycosis. A comparative assessment including ID, CIE, and CF tests was reported by Del Negro et al. (1991). The ID and CIE tests with yeast culture filtrate were the most sensitive (91.3 percent and 95.6 percent, respectively) and highly specific (100 percent), significantly outperforming the CF test. A positive ID result is, therefore, taken as presumptive evidence of current or recent infection. The ID test is commercially available for research use from Immuno-Mycologics. Most frequently a single E band (also called band 1) is produced, but sometimes two additional bands are present. The greatest limitation of the test is seen with specimens from patients with early primary infections. During the first 4–8 weeks, IgM antibodies predominate and these react poorly in gel diffusion tests, because of their large size. In disseminated disease, CF antibody titers are high, usually falling with successful therapy and rising with relapses, but not uncommonly titers of 8–32 persist even after successful therapy (Restrepo et al. 1978). Do Valle et al. (2001) found immunoblotting gave better results than double immunodiffusion, raising sensitivity to 100 percent. Costa et al. (2000) improved the diagnostic coverage of ID from 93–95 percent, using crude antigens, to 100 percent in an enzyme-linked ID assay capture test using the gp43 antigen described below. Mamoni et al. (2001) developed a capture ELISA to detect specific IgE, the levels of which correlated with clinical severity, falling with clinical improvement. In conclusion, measurement of antibody is useful for both diagnosis and prognosis.

Detection of circulating *P. brasiliensis* antigens could facilitate early diagnosis or confirm a preliminary diagnosis when antibody assays are inconclusive. Several techniques with different sensitivities have been used (Brummer et al. 1993; Mendes-Giannini et al. 1993). Mendes-Giannini et al. (1989) identified a circulating 43 kDa glycoprotein, gp43, in the sera of both acute and chronic cases. This shared epitopes with Fava-Netto's (1990) cytoplasmic polysaccharide antigen (FNPA), another antigen widely used diagnostically (Rodrigues and Travassos 1994). The gp43 antigen has now been cloned and expressed in *Escherichia coli* (Diniz et al. 2002). Taborda and Camargo (1993, 1994) have developed both a passive hemagglutination assay and a dot immunobinding assay, based on detection of antibody to gp43, for the diagnosis of paracoccidioidomycosis.

Two heat-shock proteins, Hsp60 and Hsp87, have been characterized. The 87 kDa antigen was detected in infected patients' sera, its levels correlating well with response to treatment and clinical cure. Direct amino acid sequencing revealed substantial homology with heat-shock proteins and immunological crossreactivity with a monoclonal antibody against *H. capsulatum* Hsp80 (Diez et al. 2002). A monoclonal antibody to this 87 kDa antigen demonstrated that the level of expression was higher in the yeast than the mycelial phase and that the yeasts expressed the antigen in tissues from infected patients. The Hsp60 gene from *P. brasiliensis* has been cloned and characterized; both the recombinant protein and Hsp60 extracted from the yeast reacted with sera from infected patients (Izacc et al. 2001). Immunoblots of the recombinant protein indicated reactivity with 72 of 75 sera from paracoccidioidomycosis patients (Cunha et al. 2002); there was also some reactivity with sera from healthy controls (9.5 percent) and patients with histoplasmosis (11.5 percent).

Bialek et al. (2000b) developed a nested PCR directed against an outer-membrane protein gene unique to *P. brasiliensis*, and successfully used it to detect the fungus in tissue samples from a mouse model of the infection. Gomes et al. (2000) have a PCR assay based on the gene sequence of the gp43 antigen. Others have sequenced rDNA genes and intergenic spacers to derive primers capable of discriminating *P. brasiliensis* from other human fungal pathogens by PCR (Motoyama et al. 2000). Sano et al. (2001) have applied nested PCRs based on both the gp43 gene and the 5.8S ribosomal RNA gene to infected murine tissues. These early developments may well evolve into PCR-based assays useful in the diagnosis of this infection.

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Histopathological diagnosis of mycotic diseases

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INTRODUCTION

Ideally, diagnosis of a fungal pathogen is made by observation of typical clinical symptoms, by demonstration of the presence of a fungus within lesions accompanied by a host reaction, and by subsequent isolation in culture of the infectious agent. Unfortunately, this has been achieved comparatively rarely in certain deep mycoses. Moreover, patients with fungal infections are frequently also suffering from other diseases, which may mask the fungal-related symptoms. As a result of the nonspecific nature of the clinical observations including radiological findings in fungal infections, a diagnosis usually depends on several laboratory approaches apart from mycological culture and histopathology. These include detection of:

- fungal antigens in serum, spinal fluid, bronchoalveolar lavage specimens, and urine as, for example, *Histoplasma* antigen, cryptococcal antigen, and galactomannan in cases of aspergillosis
- antifungal antibodies useful in, for example, histoplasmosis
- fungal metabolites such as D-arabinitol in *Candida* infections
- cell wall markers like (1→3)-β-D-glucan which is present in the wall of almost all fungi with the exception of zygomycetes (Reiss et al. 2000).

In recent years, molecular-based techniques such as the polymerase chain reaction (PCR) have been developed for the non-culture based diagnosis of fungal infections in blood and other clinical specimens. Due to the risk of false-negative and false-positive results in both culture- and nonculture-based diagnostic tests for mycotic infections, clinical features should always complement histopathology in the diagnosis of fungal infections.

Many of the nonculture-based techniques are not available for many of the unusual mycotic pathogens. Isolation of fungi from tissues is often also problematic. Culture of fungi is often a slow process, and results may not be available for several days or weeks. Moreover, when a mycosis is not suspected (often they are mistaken for neoplasms or inflammation of unknown etiology), the whole biopsy specimen is often fixed for histopathological examination, and thus portions are not available for culture (Jensen 1994). When this occurs, histopathological and immunohistological techniques may be the only means of establishing an etiologic diagnosis short of repeating the biopsy procedure. A systematic approach must then be taken to arrive at the most accurate diagnosis possible by examining fixed, paraffin-embedded tissue sections. Although the morphology of fungal elements within tissues may provide a tentative diagnosis, it is not always possible to establish a clearcut diagnosis based on morphological details because of morphological similarities among tissue forms of several fungal genera and the presence of sparse or atypical fungal elements (Chandler et al. 1980; Jensen et al. 1996).

Histopathological studies can contribute in a variety of ways to the diagnosis of diseases caused by fungi and related pathogens. A generic or specific etiologic

diagnosis may be made in 24 hours or less by the unequivocal demonstration and identification of characteristic fungal elements in tissue sections (Baker 1971; Anthony 1973; Schwarz 1982). Direct microscopic examination is the only reliable way of diagnosing certain fungal diseases, such as lobomycosis, rhinosporidiosis, and *Pneumocystis* infections, for which isolation techniques do not currently exist. The etiologic significance of a culture isolate can usually be determined by careful histopathological evaluation (Chandler and Watts 1987). Microscopic demonstration of tissue invasion and host reaction resolve the clinical dilemma of whether a fungal isolate is truly pathogenic, or merely a superficial colonizer, a component of the normal mycobiota (e.g. *Candida albicans*), or an environmental contaminant (e.g. a species of *Aspergillus* or a member of the class Zygomycetes) (Hoog and de Guého 1985). Microscopic evaluation of the inflammatory reaction and the distribution of fungal elements in a tissue section can also help to determine whether the disease is an invasive infection or a purely allergic reaction (e.g. invasive versus allergic bronchopulmonary aspergillosis); it can sometimes be used to assess the efficacy of antifungal therapy in pre- and post-treatment biopsy specimens (Chandler and Watts 1987). Finally, histopathological studies may sometimes confirm the presence of multiple infections, e.g. in acquired immunodeficiency syndrome (AIDS), or exclude a fungal disease from the clinical differential diagnosis altogether by revealing another process that accounts for the clinical and radiological findings. Accurate diagnosis may not only give the benefit of optimal therapy, but is also essential for the study of the pathological and epidemiologic aspects of different mycoses (Jensen 1994).

HISTOLOGICAL STAINS FOR DEMONSTRATING FUNGI

A number of traditional histochemical stains can be used to detect fungi and related pathogens in tissue sections. These stains, along with their applications and limitations, are summarized in Table 1. Details of the staining procedures are not included here, but can be found in standard manuals and textbooks (Elias 1982; Bancroft and Stevens 1996) of histochemistry and histological techniques.

Hematoxylin and eosin (H&E) is a versatile stain which enables pathologists to evaluate the host's inflammatory response, including the Splendore–Hoeppli (asteroid bodies) reaction, and to determine whether a fungus is hyaline (colorless) or pheoid (naturally pigmented–dematiaceous) (Matsumoto et al. 1994). It is the histological stain of choice to demonstrate the hematoxylinophilic or amphophilic nuclei of yeast-form cells that are multinucleated, particularly those of *Blastomyces dermatitidis*, *Loboa loboi*, and *Paracoccidioides brasiliensis*. Some fungi, e.g. the aspergilli and zygomycetes, are hematoxylinophilic and readily delineated with H&E, but many fungal agents are not stained or stain poorly. Even in the instances of poor staining, careful examination often reveals the outlines of unstained fungal elements, which suggest the existence of a fungal infection. Moreover, pathologists should always pay special attention to and carefully search for fungal elements when a granulomatous or pyogranulomatous inflammation is present (Jensen 1994). The H&E procedure has limitations, however. With this stain, it is sometimes difficult to distinguish fungal elements from tissue components, e.g. thin blood vessels within brain and lung tissues, and, when fungi are sparse, they are easily overlooked (Jensen et al. 1997). As a result of these limitations, special stains for fungi are usually needed to adequately reveal the presence and morphology of fungal pathogens in tissue sections (Chandler and Watts 1987).

Special histological stains such as Gomori's methenamine silver (GMS), the periodic acid–Schiff (PAS) reaction, and Gridley's fungus (GF) procedures are useful to demonstrate fungi in tissue sections. The staining reactions of these procedures are based on the principle that adjacent hydroxyl groups of complex polysaccharides in fungal cell walls are oxidized to aldehydes by chromic acid or periodic acid. In the GMS procedure, the aldehydes reduce the methenamine silver nitrate complex; fungal cell walls stain brownish–black because reduced silver is deposited wherever aldehydes are located. In the PAS and GF procedures, the aldehydes react with Schiff's reagent, coloring fungi magenta. All three procedures stain the cell walls of fungal elements, but tend to obscure the internal details of these cells. Moreover, hydroxyl groups in tissue elements such as basement membranes will also be stained by these techniques. The GMS stain is the best procedure for detecting fungi in tissue sections because it provides better contrast for screening and, in most instances, it will stain old and nonviable organisms that may be refractory to the PAS and GF procedures. Fungi stained with the last-mentioned procedures also tend to fade with prolonged storage. When properly done, the GMS procedure usually eliminates nonspecific background staining of normal tissue components and necrotic debris, whereas the PAS and GF procedures do not. The GMS stain is also more sensitive because it demonstrates certain nonfungal pathogens, e.g. *Actinomyces israelii* and related species, *Nocardia* spp., *Mycobacterium* spp., nonfilamentous bacteria with polysaccharide capsules, and free-living soil amoebae, algal cells, spores of microsporidians, and cytoplasmic granular inclusion bodies of cytomegalovirus (Chandler and Watts 1987). Such an all-purpose, low-specificity stain is extremely helpful for the rapid screening of specimens from immunocompromised patients, who are often infected by multiple pathogens.

When performing the GMS stain, the histotechnologist should vary the staining time according to the etiologic agent under consideration, guided by a

Table 1 *Stains for demonstrating fungi and related pathogens in tissue sections*

Stains	Applications	Limitations
Hematoxylin and eosin (H&E)	Demonstrates inflammatory response Stains some fungi Allows determination of innate color of pheoid (pigmented) fungi Demonstrates Splendore–Hoeppli material Hematoxylin stains nuclei of most yeast-like cells	Does not stain many fungi Inadequate to screen for sparse fungal elements Does not stain individual filaments of *Actinomyces*, *Nocardia* and *Streptomyces* spp.
Special stains for fungi	All three stains are excellent for detecting fungi	Do not allow determination of the innate color of a fungus (GMS stains fungi black–brown; GF and PAS stain fungi red–purple, which mask innate color)
Gomori's methenamine silver (GMS)	The GMS is better than GF or PAS for screening; it stains old and nonviable fungal elements better than the other two stains, and it also demonstrates filaments of *Actinomyces* spp. and *Nocardia* spp. and elements of *Pneumocystis carinii*	GMS may overstain fungi and obscure internal details
Gridley's fungus (GF)		GMS and GF do not allow proper study of host response
Periodic acid–Schiff (PAS)		GF and PAS do not stain filaments of the actinomycetes
GMS with H&E counterstain	Permits study of host response and is excellent for detecting fungi and most actinomycetes The stain of choice, if only one slide is available for histopathological evaluation Excellent for photomicrography	Does not allow determination of the innate color of phaeoid (pigmented) fungi
Mucin stains		
Mayer's mucicarmine	Stain mucopolysaccharide capsular material of *Cryptococcus neoformans*	Not specific for *Cryptococcus neoformans* (may stain *Rhinosporidium seeberi* and some cells of *Blastomyces dermatitidis*)
Southgate's mucicarmine Alcian blue	Permit differentiation of *C. neoformans*, from other fungi of similar size and form	
Modified Gram's stains		
Brown and Brenn	Demonstrate gram-positive filaments of *Actinomyces* spp., *Nocardia* spp. and other actinomycetes	Do not selectively stain most fungi (those that do stain are gram positive)
Brown–Hopps	Useful for detecting bacterial copathogens	
MacCallum–Goodpasture	Demonstrate gram-positive and gram-negative bacteria of botryomycosis	
Modified acid-fast stains		
Modified Ziehl–Neelsen	Useful to detect *Nocardia asteroides*, *N. brasiliensis*, and *N. otitidiscaviarum*	*Nocardia* spp. are weakly acid-fast and non-alcohol-fast in tissue sections
Fite–Faraco Kinyoun's	Valuable to detect *Mycobacterium tuberculosis* and other mycobacteria	Fungal cell walls and the agents of actinomycosis are not acid-fast
Melanin stain		
Modified Fontana–Masson	Stains cell walls of *Cryptococcus neoformans* and other *Cryptococcus* spp. Accentuates melanin or melanin-like substances in weakly pigmented agents of pheohyphomycosis	May stain cell walls of *Sporothrix schenckii* and immature spherules of *Coccidioides immitis*
Whitening agents		
Calcofluor White M2R, Blankophor, Uvitex 2B and others	Stain cell walls of most fungi	Require a fluorescence microscope Do not consistently stain fungal elements May not stain degenerated fungi

concurrently stained positive control slide. To accomplish this, the control slide should be periodically removed from the silver nitrate bath and examined under a light microscope for optimal staining. Optimal staining time in the silver nitrate solution is usually increased when old, degenerated, and nonviable fungal elements are suspected, such as those of *Coccidioides immitis* and *Histoplasma capsulatum* var. *capsulatum* in the caseated centers of residual granulomas. The depth of staining should never be so intense that it obscures the morphological details of fungal elements; preferably, some internal (cytoplasmic and nuclear) details should remain visible. When a tissue section is overstained with GMS, the contrast between fungal cells and background tissue elements is obscured. Blood vessels with darkened basement membranes can mimic fungal hyphae, particularly if the vessels are empty, branched, and of approximately the same caliber as the suspected fungus. Erythrocytes, leukocytes, and naked nuclei can be overstained and mimic yeast cells, whereas darkened reticular and elastic fibers can be mistaken for filamentous fungi or bacteria. In caseated granulomas, calcific bodies that stain with H&E and PAS procedures often simulate yeast cells, particularly those of *Cryptococcus neoformans* and *H. capsulatum* var. *capsulatum*. As the chromic acid used as an oxidant in the GMS and GF procedures dissolves calcium, calcific bodies are unstained when these procedures are used to confirm the presence of a fungal pathogen (Chandler and Watts 1987).

A major disadvantage of special stains is that they mask the innate color of fungal elements, making it impossible to determine whether a fungus is hyaline or pheoid. Such a determination may be crucial to the diagnosis of a mycosis caused by pigmented fungi, e.g. pheohyphomycosis, chromoblastomycosis, and black-grain eumycotic mycetomas (Chandler and Watts 1987). As a result of masking, duplicate H&E-stained or unstained tissue sections should always be examined to look for brown pigmentation of fungal cell walls. Another limitation of the special stains for fungi is that they do not allow adequate study of the host reaction to fungal invasion. To avoid this limitation, H&E can be used as the counterstain for the GMS procedure. This combination of stains (GMS–H&E) readily colors fungal elements brownish–black while staining background tissue components as expected. Thus, it is possible simultaneously to detect the fungus and to evaluate the host's inflammatory response and its relationship to the fungus. Tissue sections stained with GMS–H&E are also optimal for photomicrography.

When only a single unstained section from a suspected lesion is available for examination, GMS–H&E is the best stain combination for attempting to make a diagnosis. If desired, the section can be stained initially with H&E, checked for the presence of pheoid fungi, and then restained with GMS–H&E. For retrospective studies of unexplained lesions, tissue sections previously stained with Giemsa, modified Gram's, PAS, and GF procedures can be decolorized in acid alcohol after the coverslip has been removed and then restained with GMS.

Stains for mucin, such as Alcian blue and Mayer's or Southgate's mucicarmine procedures, readily demonstrate the mucopolysaccharide capsule of *Cryptococcus neoformans*. This staining reaction is a convenient diagnostic marker and usually differentiates typical cryptococci from nonencapsulated yeast-form pathogens of similar size and appearance. In rare cases, however, capsule-deficient cryptococci may not stain positively for mucin. In addition, mucin stains are not specific for *C. neoformans*, because the cell walls of *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Rhinosporidium seeberi* are also variably colored with these stains. As the last three fungi are nonencapsulated and morphologically distinctive, ordinarily they would not be mistaken for *C. neoformans*. Mucin stains are not required for their identification.

The cell walls of cryptococci contain silver-reducing substances (melanin-like substances derived from dihydroxyphenylalanine), which react positively with a modified Fontana–Masson stain for melanin (Kwon-Chung et al. 1981; Wheeler and Bell 1987; Dixon and Polak 1991). As this stain reaction does not depend on the presence of a mucinous capsule, it can be used routinely to demonstrate capsule-deficient cryptococci (so-called 'dry' variants) (Ro et al. 1987). Capsule-deficient cryptococci stained with conventional procedures can easily be confused with nonencapsulated yeasts of similar size and morphology. When the agents of pheohyphomycosis are either nonpigmented or lightly pigmented in tissue sections, stains for melanin can also be used to accentuate and confirm the presence of melanin in their cell walls (Wood and Russel-Bell 1983; Chandler and Watts 1987). Studies have shown that the pigment in many of these fungi is a particular type of melanin derived from dihydroxynaphthalene, which differs from the melanin produced by *C. neoformans* (Dixon et al. 1991).

Modified Gram's stains, such as the Brown and Brenn and MacCallum–Goodpasture procedures, demonstrate the delicate, branched, gram-positive filaments of the agents of actinomycosis, nocardiosis, streptomycosis, dermatophilosis, and actinomycotic mycetomas. The GMS procedure can also be used to demonstrate these agents, but they often do not stain intensely or uniformly. The H&E, PAS, and Gridley procedures do not stain the actinomycetes (although entire granules in actinomycosis and actinomycotic mycetomas stain well, individual filaments within the granules are unstained). Modified Gram's stains are also needed to detect bacteria other than actinomycetes that may complicate a mycotic or actinomycotic infection, or that might be the primary cause of disease, e.g. botryomycosis (Chandler and Watts 1987). Some fungi, especially the yeast forms of *Candida* spp. and the conidia of *Aspergillus* spp. are usually gram-positive. Gram's stains are not, however,

recommended for screening or for definitive diagnosis of mycoses caused by these agents.

As the *Nocardia* spp. are weakly acid-fast and non-alcohol-fast in tissue sections, these filamentous bacteria can be distinguished from the agents of actinomycosis with modified acid-fast stains that use a weak aqueous solution consisting of 0.5 or 1.0 percent sulfuric acid for decolorization; the nocardiae are weakly acid-fast, whereas the agents of actinomycosis are not acid-fast. The cytoplasm of certain fungi with yeast-like tissue forms, especially *B. dermatitidis* and *H. capsulatum* var. *capsulatum*, is also variably acid-fast (Wages and Wear 1982). The acid-fast properties of fungi are, however, inconsistent and should not be depended on for diagnosis. The cell walls of fungi are not acid-fast.

When H&E-stained tissue sections that contain fungi are examined under ultraviolet light, some of the fungal elements usually autofluoresce (Graham 1983). Although this property may help delineate sparse or poorly stained fungi, fungal autofluorescence is inconsistent and should not be used as a substitute for the more reliable special stains such as GMS or for immunohistological procedures. Some fungi in paraffin-embedded or fresh-frozen tissue sections also stain nonspecifically with optical brightening histochemistry, i.e. whitening agents that have a diaminostilbene backbone structure, such as Calcofluor White M2R, Blankophor, and Uvitex 2B, which fluoresce under ultraviolet light (Monheit et al. 1984, 1986; Jensen 1994). Most of these fluorescent brighteners have an affinity for certain glycosidic linkages of the polysaccharides of the fungal cell wall. Tissue sections are usually stained for one minute with a 0.1 percent solution of the whitening agent, counterstained with Evan's blue, and examined with a fluorescence microscope with an excitation band of 500 nm. This rapid and simple procedure has been used in the intraoperative examination of fresh-frozen tissues for fungi, scrapings, swabs, and body fluids. Whitening agents, however, do not always highlight fungi, and these compounds should only be used for definitive diagnosis when positive. There is no advantage in using whitening agents compared with the rapid methenamine silver techniques for the detection of fungi in tissue sections (Shimono and Harman 1986).

HISTOPATHOLOGICAL IDENTIFICATION OF FUNGI

As a result of their large size, polysaccharide content, and morphological diversity, fungi can be detected readily and may also to a certain level be identified in histological sections by conventional light microscopy (Schwarz 1982; Salfelder 1990). Fungal elements in tissue sections are either hyaline or pheoid, and usually appear as yeast-like cells, hyphae, endosporulating spherules, granules, or a combination of these forms. These four broad morphological categories form a useful nontaxonomic system to approach the histopathological differential diagnosis of fungal diseases. Once a pathogen is correctly recognized as belonging to one of the four categories, it can in many cases be accurately identified to the level of genus or even to the species level by further consideration of its defining morphological and tinctorial features. For example, within the broad category of yeast-like pathogens, further defining features would include:

- size and shape of individual cells
- thickness of the cell wall
- number and shape of blastoconidia (buds)
- type of attachment of buds to parent cells
- presence or absence of septations
- pigmentation
- encapsulation
- number of nuclei
- presence of pseudohyphae, hyphae or arthroconidia.

Thus, a yeast-like pathogen with a thick cell wall, single broad-based bud, and multiple nuclei can only be *B. dermatitidis*. An encapsulated yeast-like pathogen can only be a *Cryptococcus* sp. A pathogen that forms budding yeast-like cells, pseudohyphae, and hyphae, but not arthroconidia, and is not pheoid (naturally pigmented), can only be a *Candida* sp. The system is less reliable for the category of pathogens with hyphal tissue forms, many of which appear quite similar. Moreover, the appearances of hyphae in sections are often affected by steric orientation, age of the hyphae, type of infected tissue, and the host response (Jensen and Schønheyder 1989). The morphological and tinctorial features of fungi that occur in tissue as yeast-like cells, large spherules, and nonpigmented hyphae are summarized in Tables 2, 3 and 4.

In the practice of surgical pathology, a specific etiologic or generic diagnosis of a fungal infection often has important prognostic and therapeutic implications. Certain fungal diseases are caused by agents that can be specifically identified in tissue sections because they are morphologically distinctive. In this group, a single species or variety of fungi is usually responsible for each particular disease, and if sufficient numbers of typical organisms are demonstrated, an etiologic diagnosis can be made. This group of diseases includes adiaspiromycosis, blastomycosis, coccidioidomycosis, cryptococcosis, small and large form histoplasmosis, lobomycosis, paracoccidioidomycosis, infection due to *Penicillium marneffei*, rhinosporidiosis, sporotrichosis, and pneumocystosis. Other fungal diseases are caused by any of several members of a genus that, in tissues, are all morphologically similar to each other. Typical forms of these fungi also have a distinctive morphology but can only be identified to the genus level. Diseases caused by fungi in this group, which can be diagnosed generically, include candidosis and trichosporonosis. Table 4 clearly shows that agents of aspergillosis, fusariosis and scedosporiosis most often cannot be separated in tissue

Table 2 *Morphological features of fungi that occur as yeast-like cells in tissue*

Feature	*Histoplasma capsulatum* var. *capsulatum*	*Histoplasma capsulatum* var. *duboisii*	*Blastomyces dermatitidis*	*Cryptococcus neoformans*	*Penicillium marneffei*	*Paracoccidioides brasiliensis*	*Loboa loboi*	*Sporothrix schenckii* var. *schenckii*[d]	*Candida* spp.	*Candida glabrata*	*Pneumocystis carinii*
Size (μm)	2–4	6–12	7–15[a]	2–20	2.5–5	5–60	5–12	2–10	3–6	2–5	2-10
Shape	Spherical or oval	Oval	Spherical	Pleomorphic	Spherical, oval or elongated	Spherical	Spherical	Pleomorphic[e]	Spherical or oval	Spherical or oval	Spherical, oval or crescentic
Number of buds	Single	Single	Single	Single and rarely multiple	None	Multiple; 'steering wheel' forms	Multiple; chains	Single and rarely multiple	Single; chains	Single	None
Attachment of buds	Narrow	Narrow	Very broad	Narrow	NA[c]	Narrow	Narrow; tubular	Narrow	Narrow	Narrow	NA[c]
Schizogony	–	–	–	–	+	–	–	–	–	–	+
Thickness of cell wall	Thin	Thick	Thick	Thin	Thin	Variable	Thick	Thin	Thin	Thin	Thick in 'cysts'; thin in 'trophozoites'
Pseudohyphae and/or hyphae	Rare	Absent	Rare	Rare	Absent	Rare	Absent	Rare	Characteristic	Absent	Absent
Number of nuclei	Single	Single	Multiple	Single	Single	Multiple	Multiple	Single	Single	Single	Multiple in 'cysts'; single in 'trophozoites'
Mucicarmine reaction	–	–	±	+[b]	–	±	–	–	–	–	–

a) Microforms, 2–4 μm in diameter, also may occur in tissue.
b) Some strains may be capsule deficient and noncarminophilic.
c) NA, not applicable.
d) The cells of *S. schenckii* var. *luriei* are larger (10–20 μm) compared with the var. *schenckii*, and typical 'eyeglass' forms are often seen.
e) Elongated, cigar-shaped cells characteristic but infrequent.

Table 3 *Morphological features of fungi that occur as large spherules in tissue*

Feature	*Coccidioides immitis*	*Rhinosporidium seeberi*	*Chrysosporium parvum* var. *crescens*
External diameter of spherule (μm)	20–200	100–350	200–400
Thickness of spherule wall (μm)	1–2	3–5	20–70
Diameter of endospores	2–5	6–10	None
Hyphae or arthroconidia	Rare	None	None
Special stain reactions			
GMS	+	+	+
Mucicarmine	–	+	–

sections (Jensen et al. 1996). Moreover, aspergillosis, fusariosis and scedosporiosis may in some cases also be confused with zygomycosis and candidosis, respectively (Jensen et al. 1997). In especially chronic lesions with atypical fungal forms, similar problems do also apply to a number of other fungi that belong to different genera but appear morphologically similar to each other in tissue sections. Although the etiologic agent in some lesions cannot be specifically or generically identified, the disease can still be named, i.e. chromoblastomycosis, dermatophytosis, hyalohyphomycosis, pheohyphomycosis, and zygomycosis.

In organs with access to air, especially within the upper parts of the respiratory tract, but also within the lungs, conidial heads are sometimes formed. The morphology of such frutification bodies may sometimes allow an identification of the infecting agent to the species level (Salfelder 1990). Heavy deposits of birefringent calcium oxalate crystals in tissues may also be used to sustain the identification of *Aspergillus niger* (Salfelder 1990). In other cases, *A. niger* infections may result in the developing of generalized oxalosis (Salfelder 1990).

Mycetomas constitute special cases. By studying the granules – the diagnostic hallmark of the mycetomas – in appropriately stained sections of infected tissue, one can easily determine whether the etiologic agent is an actinomycete (branched, filamentous bacterium) or a eumycete (true fungus), and, if a eumycete, whether it is hyaline (white grained) or pheoid (black grained). The size, shape, color, and microscopic architecture of the granules are correlated with the etiologic agent, and each species, with some exceptions, forms its own distinctive type of granule (Chandler and Watts 1987). Thus, the gross and microscopic appearances of the granules provide insight into the identity of the organisms involved. Definitive identification of the etiologic agents should, however, be based, whenever possible, on microbiological culture.

Finally, in some cases, intact fungal elements or fragments may be detected in tissue sections but cannot be definitively or even presumptively identified. In other cases, the morphology of fungi may be atypical or bizarre as a result of changes induced by hypoxia, necrosis or antifungal therapy. Although the disease cannot be named in such instances, the pathologist can nevertheless often conclude that a fungal infection is present and apply a panel of specific antibodies using different immunohistochemical techniques (Jensen et al. 1996).

The histomorphological features of the common pathogenic fungi and related microorganisms are summarized in Table 5 and illustrated in Figures 1–62 at the end of the chapter. To facilitate differential diagnosis, the diseases cited in the table are grouped according to the morphological similarities of their etiologic agents in tissue. As diseases caused by the actinomycetes and the protothecae traditionally fall within the province of medical mycology, they are included in Table 5. Fungal infections that involve the hair exclusively, e.g. black piedra and white piedra, are not included in Table 5 because histological methods are not required for their diagnosis.

Although certain inflammatory patterns in an otherwise healthy person may suggest that a fungal infection

Table 4 *Morphological features of fungi that occur as hyaline hyphae in tissue*

Feature	*Aspergillus* spp.	*Fusarium* spp.	*Scedosporium* spp.; *Pseudallescheria boydii*	*Rhizopus* spp. and other zygomycetes
Width (μm)	3–6	3–8	2–5	6–25
Contours	Parallel	Parallel	Parallel	Irregular
Pattern of branching	Dichotomous	Dichotomous or right angle	Dichotomous and/or haphazard	Haphazard
Orientation of branches	Parallel or radial	Random and parallel	Random and parallel	Random
Frequency of septation	Frequent	Frequent	Frequent	Absent or infrequent
Angioinvasiveness	Yes	Yes	Yes	Yes

Table 5 *Histomorphological features of the common pathogenic fungi and related pathogens*

Etiologic agent(s)	Mycosis	Typical morphology
Pathogens that occur in tissue as yeast-like cells		
Blastomyces dermatitidis	Blastomycosis	Spherical, multinucleate, yeast-like cells, 7–15 μm diameter, with thick walls and single, broad-based buds
Cryptococcus neoformans	Cryptococcosis	Pleomorphic yeast-like cells, 2–20 μm diameter, with mucin-positive capsules and single or, rarely, multiple narrow-based buds; some strains are capsule-deficient and their capsular material may not be detected with mucin stain
Histoplasma capsulatum var. *capsulatum*	Histoplasmosis (classic, small form, histoplasmosis capsulati)	Spherical to oval, uninucleate, yeast-like cells, 2–4 μm diameter; often clustered because of growth within mononuclear phagocytes
Histoplasma capsulatum var. *duboisii*	African histoplasmosis (large form, histoplasmosis duboisii)	Spherical to oval uninucleate, yeast-like cells, 6–12 μm diameter, which have thick walls and bud by a narrow base, creating typical 'hour-glass' or 'figure-of-eight' forms
Loboa loboi	Lobomycosis	Spherical, budding, yeast-like cells, 5–12 μm diameter, that form chains of cells, each connected by a narrow tube-like isthmus; secondary budding sometimes present
Paracoccidioides brasiliensis	Paracoccidioidomycosis	Large, spherical, yeast-like cells 5–60 μm diameter, with multiple buds attached by narrow necks ('steering wheel' forms)
Penicillium marneffei	Infection due to *Penicillium marneffei* (penicilliosis marneffei)	Spherical to oval yeast-like cells, 2.5–5 μm diameter, with a single transverse septum; short hyphal forms and elongated, curved 'sausage' forms with one or more septa may be formed in necrotic and cavitary lesions
Sporothrix schenckii var. *schenckii*	Sporotrichosis	Pleomorphic, spherical to oval and, at times, 'cigar'-shaped yeast forms, 2–10 μm diameter, which produce single and, rarely, multiple buds
Sporothrix schenckii var. *luriei*	Sporotrichosis due to *S. schenckii* var. *luriei*	Hyaline, large (10–20 μm) thick-walled yeast forms together with the typical 'eyeglass' configuration of incompletely separated cells
Candida glabrata	Candidosis	Spherical to oval yeast-like cells 2–5 μm diameter
Pneumocystis carinii	Pneumocystosis	Thin-walled spherical, oval and crescentic trophozoites, 2–10 μm diameter; cysts are thick-walled, 4–6 μm diameter
Pathogens that occur in tissue as yeast-like cells and mycelial elements		
Candida albicans, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. tropicalis* and others	Candidosis	Oval, budding, yeast-like cells (blastoconidia), 2–6 μm diameter; pseudohyphae; and septate hyphae
Geotrichum candidum	Geotrichosis	Septate, infrequently branched hyphae, 3–6 μm wide; spherical yeast-like cells; and rectangular or oval arthroconidia, 4–10 μm wide, with rounded or squared ends
Trichosporon beigelii, *T. capitatum* (*Blastoschizomyces capitatus*)	Trichosporonosis	Pleomorphic yeast-like cells, 3–8 μm diameter; septate hyphae; and, rarely, arthroconidia
Pathogens that occur in tissue as large spherules		
Chrysosporium parvum var. *crescens*	Adiaspiromycosis	Large adiaconidia, 200–400 μm diameter, with thick (20–70 μm) walls; budding and endosporulation do not occur
Coccidioides immitis	Coccidioidomycosis	Spherical, thick-walled, endosporulating spherules, 20–200 μm diameter; mature spherules contain small, 2–5 μm diameter, uninucleate endospores; septate hyphae and chains of arthroconidia sometimes occur in necrotic nodules

(*Continued over*)

Table 5 *Histomorphological features of the common pathogenic fungi and related pathogens (Continued)*

Etiologic agent(s)	Mycosis	Typical morphology
Prototheca wickerhamii, *P. zopfii*	Protothecosis	Spherical, oval or polyhedral sporangia, 2–25 μm diameter, that, when mature, contain 2–20 sporangiospores
Rhinosporidium seeberi	Rhinosporidiosis	Large sporangia, 100–350 μm diameter, with thin walls (3–5 μm) that enclose numerous sporangiospores, 6–10 μm diameter
Pathogens that occur in tissue as hyaline hyphae		
Absidia corymbifera, *Basidiobolus ranarum*, *Conidiobolus coronatus*, *Mucor ramosissimus*, *Rhizomucor pusillus*, *Rhizopus arrhizus*, and others	Zygomycosis	Broad, thin-walled, aseptate or infrequently septate hyphae, 6–25 μm wide, with non-parallel contours and random branches. Sporangia sometimes formed in cavitary lesions with access to air
Aspergillus fumigatus, *A. flavus*, *A. niger*, and other *Aspergillus* spp.	Aspergillosis	Septate, dichotomously branched hyphae of uniform width (3–6 μm); conidial heads sometimes formed in cavitary lesions with access to air. Calcium oxalate crystals may be present in case of *A. niger*
Pathogenic members of the genera *Acremonium*, *Beauvaria*, *Cylindrocarpon*, *Fusarium*, *Paecilomyces*, *Phialomonium*, *Scedosporium*, *Scopulariopsis*, and others	Hyalohyphomycosis	Septate, branched, hyaline hyphae, 2–8 μm wide, which usually have regular contours and dichotomous random branches
Malassezia furfur	Pityriasis versicolor	Short, curved, and bent hyaline hyphae, 2–4 μm wide; and clusters of spherical to oval, thick-walled phialoconidia, 3–8 μm diameter
Pathogenic members of the genera *Epidermophyton*, *Microsporum*, and *Trichophyton*	Dermatophytosis	Branched, septate hyphae that break up into chains of arthroconidia
Pseudallescheria boydii (anamorph of *Scedosporium apiospermum*)	Pseudallescheriosis (scedosporidiosis)	Septate, randomly branched hyphae, 2–5 μm wide; conidia of scedosporium type may be formed in cavitary lesions
Pigmented (pheoid) nonmycetomatous pathogens		
Phaeoannellomyces werneckii, *Stenella araguata*	Tinea nigra	Pheoid, branched, septate hyphae, 1–3 μm wide; and elongated budding cells, 1–5 μm diameter
Cladophialophora carrionii, *Fonsecaea compacta*, *F. pedrosoi*, *Phialophora verrucosa*, and *Rhinocladiella aquaspersa*	Chromoblastomycosis	Large, 6–12 μm diameter, spherical to polyhedral, thick-walled, dark-brown, muriform cells (sclerotic bodies) with septations along one or two planes; pheoid hyphae sometimes present
Bipolaris spicifera, *Cladophialophora bantiana*, *Exophiala jeanselmei*, *Phialophora parasitica*, and other pheoid fungi	Pheohyphomycosis	Pheoid (brown) hyphae, 2–6 μm wide, which may be branched and are often constricted at their frequent and prominent septations; pheoid yeast forms, and chlamydoconidia sometimes present
Mycetomatous pathogens		
Acremonium spp., *Aspergillus nidulans*, *Curvularia geniculata*, *Exophiala jeanselmei*, *Leptosphaeria senegalensis*, *Madurella grisea*, *M. mycetomatis*, *Neotestudina rosatii*, *Pseudallescheria boydii*, *Pyrenochaeta romeroi*, and others	Mycetoma (eumycotic) or eumycetoma	Granules, 0.2 mm to several millimeters in diameter, composed of broad (2–6 μm), hyaline or phaeoid, septate hyphae that often branch, and form chlamydoconidia
Actinomadura madurae, *A. pelletieri*, *Nocardia* spp., *Nocardiopsis dassonvillei*, *Streptomyces somaliensis*, and others	Mycetoma (actinomycotic) or actinomycetoma	Granules, 0.1 mm to several millimeters diameter, composed of delicate gram-positive filaments, 1 μm wide, which are often branched, and beaded
Filamentous bacterial pathogens		
Actinomyces israelii, *A. meyeri*, *A. naeslundii*, *A. odontolyticus*, *A. viscosus*, *Arachnia propionica*, *Rothia dentocariosa*, and others	Actinomycosis	Organized aggregates (granules) composed of delicate, branched, gram-positive filaments, 1 μm wide, that are often beaded; entire granules are 30–3000 μm diameter
Nocardia asteroides, *N. brasiliensis*, and *N. otitidiscaviarum*	Nocardiosis	Delicate, branched, individual filaments, 1 μm wide, that are gram-positive, weakly acid-fast, and often beaded

exists, there are no absolute histological criteria that permit an etiologic diagnosis based only on host response. In addition, inflammatory patterns are often atypical and unreliable in immunodeficient patients. A fungus must first be detected and then identified in tissue sections before a definitive histopathological diagnosis can be made. Knowledge of the patient's travel history may help resolve differential diagnostic problems because the geographic distribution of some pathogenic fungi is limited (Warnock et al. 1998).

DIAGNOSTIC APPLICATION OF IMMUNOHISTOCHEMICAL TECHNIQUES

The prerequisite for all immunohistochemical staining systems is a primary antibody properly characterized especially in terms of specificity under optimal conditions (Jensen 1994). The technique used for obtaining an immunohistochemical diagnosis of mycoses may be either direct (conjugated primary antibodies) or indirect (conjugated secondary antibodies or tertiary enzyme complexes). In accordance with other immunohistochemical staining systems, the reaction complexes may be vizualized with the help of fluorochromes [e.g. fluorescein isothiocyanate (FITC) and tetra-methylrhodamine isothiocyanate isomer R (TRITC)], gold-silver complexes, or complexes of enzymes [e.g. peroxidase–antiperoxidase (PAP) techniques, and alkaline phosphatase-antialkaline phosphatase (APAAP) techniques] (El Nageeb and Hay 1981; Kaufman 1992; Jensen 1994). Also, avidin–biotin enzyme complex (ABC) methods may be used with horseradish peroxidase (HRP) and galactosidase (Jensen et al. 1996). The major advantage of using enzyme immunohistochemistry compared with immunofluorescence techniques is that permanent sections are provided, specialized microscopic equipment is not needed, and pathological reactions may be assessed simultaneously during the evaluation of the immunoreactivity (Jensen 1994; Jensen et al. 1996). Application of immunohistochemical techniques may, in several cases, be the only means of establishing an accurate etiologic diagnosis in fixed tissue sections because of morphological similarities among the tissue forms of several fungal genera (e.g. aspergillosis, fusariosis, and scedosporiosis), when atypical forms of a fungus are present, or when fungal elements are sparse (Jensen et al. 1996). A range of different forms of fungal elements are frequently observed within lesions, especially when more than one organ is studied, and it must be ascertained whether these elements belong to a single or more taxa. In such cases, dual immunostaining techniques are useful tools for obtaining a reliable diagnosis (Jensen et al. 1996). In the case of *Cryptococcus neoformans*, the combination of reactions obtained by different primary antibodies is used to differentiate between serotypes (Krockenberger et al. 2001)

Apart from tissue sections, immunohistochemical techniques can also be used to identify fungi in smears of lesional exudates, bronchial washings, bronchoalveolar lavage fluid, blood, bone marrow, cerebrospinal fluid, and in sputum specimens that have been enzymatically or chemically digested.

An important limitation to the widespread application of immunohistochemical techniques and their use in the routine diagnosis of mycoses lies in the fact that sensitive and specific reagents are usually derived from multiple heterologously adsorbed polyclonal antisera, which are not commercially available (Jensen et al. 1996). However, in recent years more specific monoclonal antibodies have been developed, some of which can be obtained through companies offering diagnostic reagents (Jensen et al. 1997). Moreover, in addition the development of specific DNA probes for in situ hybridization techniques may facilitate the access to specific fungal identification (Jensen et al. 1996). In Table 6, fungi and related pathogens, which can be identified immunohistochemically, are listed.

Before immunostaining, localization and presumptive identification of fungal elements in GMS-stained tissue sections enable the pathologist to narrow the etiologic

Table 6 *Fungal and related pathogens that can be identified immunohistochemically in deparaffinized sections of formalin-fixed tissue*

Fungal and related pathogens
Actinomycetes
Actinomyces israelii
Actinomyces naeslundii
Actinomyces viscosus
Arachnia propionica
Fungi
Aspergillus to the genus level or to the species level, i.e. *A.fumigatus*, *A. flavus*, and *A. niger*
Blastomyces dermatitidis
Candida to the genus level or to the species level, i.e. *C. albicans*, *C. glabrata*, *C. tropicalis*
Coccidioides immitis
Cryptococcus neoformans to the genus level or to the level of varieties and serotypes, i.e. *C. neoformans* var. *neoformans* (A, D, and AD) and *C. neoformans* var. *gatti* (B and C), respectively
Fusarium spp.
Geotrichum candidum
Histoplasma capsulatum (tissue forms of capsulatum and duboisii varieties)
Paracoccidioides brasiliensis
Penicillium marneffei
Pneumocystis carinii
Pseudallescheria boydii and *Scedosporium* spp.
Sporothrix schenckii var. *schenckii*
Trichosporon beigelii and *Trichosporon capitatum* (*Blastoschizomyces capitatus*)
Zygomycotic fungi (Order Mucorales) to the level of genus, i.e. *Absidia*, *Rhizopus*, and *Rhizomucor*
Algae
Prototheca wickerhamii
Prototheca zopfii

Table 7 *Special terminology*

Term	Definition
Anamorph	A somatic or reproductive structure of the asexual cycle
Adiaconidium	Asexual conidium (spore) that enlarges after formation in vitro or implantation in vivo; adiaconidia do not reproduce in vivo
Arthroconidium	Asexual conidium (spore) formed by mycelial disarticulation
Asteroid body	Radially oriented Splendore–Hoeppli material that sometimes surrounds the yeast-like cells of *Sporothrix schenckii* or other fungi in tissue
Bud (blastoconidium)	Variety of conidium produced by lateral outgrowth from a parent cell; buds may be single or multiple
Budding	Asexual reproductive process characteristic of unicellular fungi or conidia in which a lateral outgrowth from the parent cell is pinched off to form a new cell
Capsule	Hyaline (colorless) mucopolysaccharide coat external to the wall of a fungal cell or conidium
Chlamydoconidium	Thick-walled, rounded, resistant conidium formed by direct differentiation of the mycelium
Coenocytic	Nonseptate
Conidiophore	Specialized hypha that produces and bears conidia
Conidium (plural, conidia)	Asexual propagule formed on, but easily detached from, a hypha, conidiophore, or other parent cell
Dematiaceous	Naturally pigmented, usually brown or black; term considered to be obsolete; replacement by the term pheoid advocated
Dichotomous	Equal branching of hyphae
Dimorphic	Having two forms; growth as hyphae in vitro at 25°C and as budding yeasts, muriform cells, or spherules in infected tissues or in vitro at 37°C on special media
Endospore	Asexual spore formed within a closed structure such as a spherule
Endosporulation	Process of producing endospores
'Fruiting body'	Imprecise term for conidia-bearing organs produced by fungi
Germ tube	Tube-like process, produced by a germinating conidium, which eventually develops into a hypha
Granule (grain)	Mass of organized mycelium that may be embedded in a cement-like matrix; formed in actinomycosis and in mycetomas caused by fungi and aerobic actinomycetes
Hyaline	Colorless
Hypha (plural, hyphae)	Filament that forms the thallus or body of most fungi
Intercalary	Between two fungal cells
Muriform	Having horizontal and vertical septations or cross-walls
Mycelium (pl. mycelia)	Mass of intertwined and branched hyphae
Perfect state	Developmental state of a fungus when sexual reproduction takes place and sexual spores are produced
Pheoid	Dark, dusky; term applied to fungal cell walls darkened by melanins
Phialide	A conidiogenous cell that successively produces and extrudes conidia from within
Pseudohypha	Short hyphal-like filament produced by successive yeast buds that elongate and fail to separate
Schizogony	Process of multiplication of yeasts by the splitting and separation of a septum that permits adjacent cells to be set free
Septate	Having cross-walls
Septum (pl. septa)	Cross-wall of a mycelial filament or conidium
Spherule	Closed, thick-walled, spherical structure within which asexual endospores are produced by progressive cytoplasmic cleavage
Splendore–Hoeppli material	Eosinophilic, refractile, homogeneous and often radially oriented material sometimes found around fungi in tissue sections; represents a localized antigen–antibody reaction in a hypersensitized host
Sporangium (pl. sporangia)	A closed structure within which asexual spores (sporangiospores) are produced by cytoplasmic cleavage
Sterigma (pl. sterigmata)	Short or elongate specialized projection of a conidiophore on which conidia are developed (see Phialide)
Yeast	Spherical to oval unicellular fungus that reproduces by a budding or fission process

possibilities, so that the most appropriate panel of immunoreagents can be employed. Depending on the nature of reactive fungal epitopes, pretreatment with enzymes (e.g. trypsin and pepsin) and addition of a substance such as dimethyl sulfoxide may enhance the reactivity of tests on formalin-fixed tissues. A number of appropriate control procedures are essential in the evaluation of immunohistochemical diagnostic assays, e.g. replacement of antifungal antibodies by antibodies raised against irrelevant antigens and including both positive and negative control sections with each batch of slides (Jensen et al. 1996).

As the somatic and cell wall antigens of most pathogenic fungi survive formalin fixation and processing into paraffin quite well, immunohistochemical and in situ techniques can be performed retrospectively on archival material. Unstained sections, paraffin blocks, or formalin-fixed 'wet' tissue can be sent to specialized reference centers for immunohistopathology. If the number of sections available is limited, those already stained with H&E, Giesma or tissue Gram's stains can be decolorized in acid–alcohol and reused for immunohistochemistry. If immunofluorescence is used, as much as possible of the eosin should be removed because this stain autofluoresces. In cases where the antigenic epitope is a polysaccharide, sections previously stained with GMS, PAS, or GF procedures are not suitable for immunohistochemistry, because oxidation of the cell wall polysaccharides irreparably alters the antigenicity of the fungal cells.

SPECIAL TERMINOLOGY

As medical mycology and the histopathology of mycotic diseases are highly specialized disciplines, the following selected terms, used to describe the appearance of fungi and related pathogens in tissue sections, are defined as shown in Table 7.

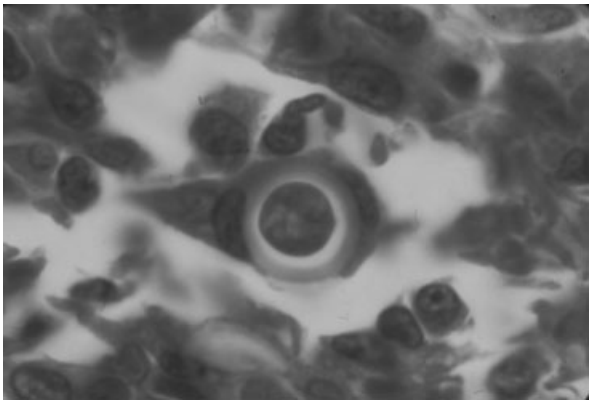

Figure 1 *Pulmonary blastomycosis: a multinucleated giant cell contains a single yeast form of* Blastomyces dermatitidis. *In tissue, the cells of this fungus are yeast-like, spherical to oval, 8–15 μm in diameter, and have thick 'doubly contoured' walls and single buds attached to parent cells by a broad base. Note that the fungal cell is multinucleate. H&E; magnification × 1 125*

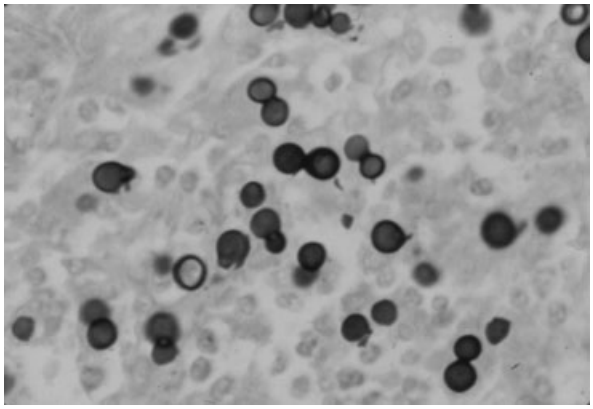

Figure 2 *Pulmonary blastomycosis in an immunocompromised patient. Numerous yeast-like cells of* Blastomyces dermatitidis *fill alveolar spaces. Note the diagnostic broad-based budding, thick cell walls, and variation in size of fungal cells. GMS; magnification × 360*

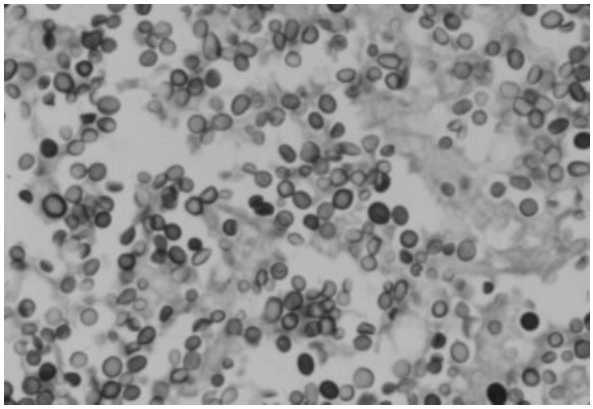

Figure 3 *Disseminated cryptococcosis: pleomorphic yeast forms of* Cryptococcus neoformans *are predominantly extracellular, bud by a relatively narrow base, range from 2 to 20 μm in diameter, and are surrounded by unstained (optically clear) capsules. A multiple-budding cell is seen at left center. GMS; magnification × 225*

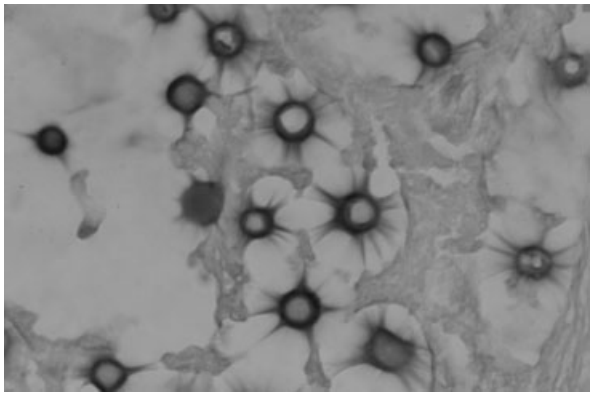

Figure 4 *Cerebromeningeal cryptococcosis: yeast-like cells of* Cryptococcus neoformans *distend the leptomeninges, elicit little or no inflammation, and are surrounded by wide, carminophilic capsules that have a crenated or spinous appearance because of uneven shrinkage during tissue processing. Mayer's mucicarmine; magnification × 563*

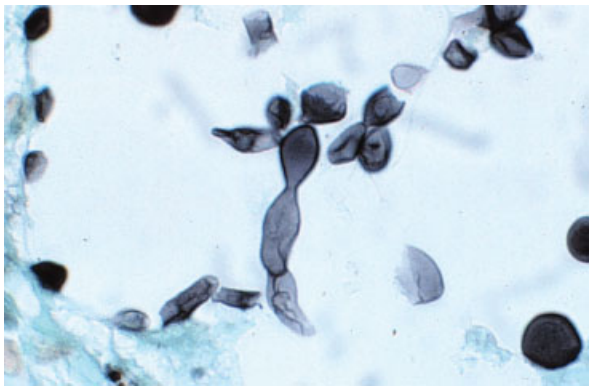

Figure 5 *Hepatic cryptococcosis: the cells of* Cryptococcus neoformans *may sometimes form hyphae and pseudohyphae that may be confused with elements of candidosis. GMS; magnification* × *360*

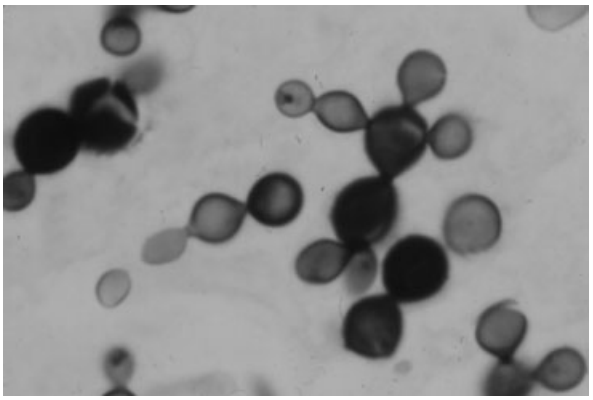

Figure 8 *Lobomycosis: spherical to oval and budding yeast-like cells of* Loboa loboi, *5–12 μm in diameter, are attached in chains. Budding cells are often connected by a narrow, tube-like isthmus. GMS; magnification* × *1 125*

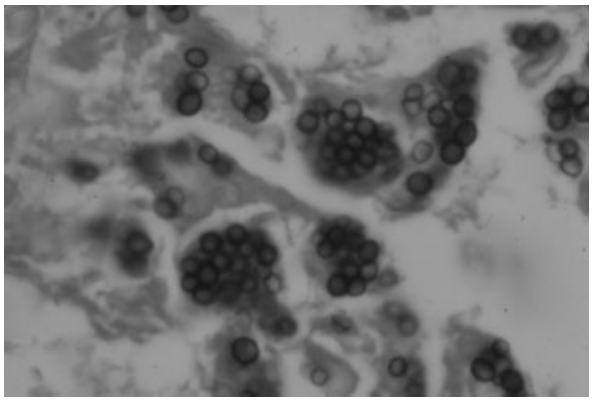

Figure 6 *Disseminated histoplasmosis involving the adrenal gland. Numerous spherical to oval yeast forms of* Histoplasma capsulatum *var.* capsulatum, *2–4 μm in diameter, have single buds attached to parent cells by a relatively narrow base, and are clustered within histiocytes. GMS; magnification* × *1 125*

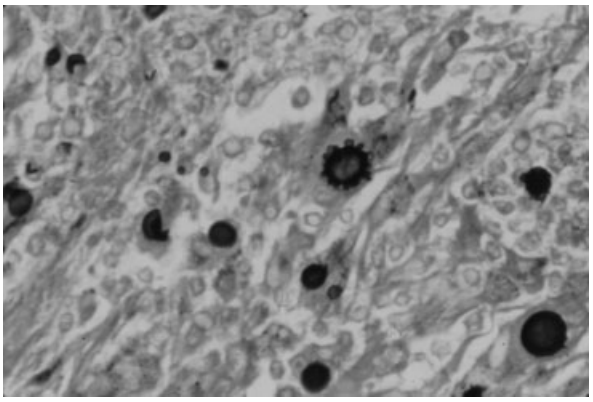

Figure 9 *Pulmonary paracoccidioidomycosis. A granuloma contains diagnostic, multiple-budding ('steering wheel') yeast forms of* Paracoccidioides brasiliensis *(right center), as well as nonbudding and single-budding cells that range from 10 to 60 μm in diameter. Note the small detached blastoconidia. GMS; magnification* × *225*

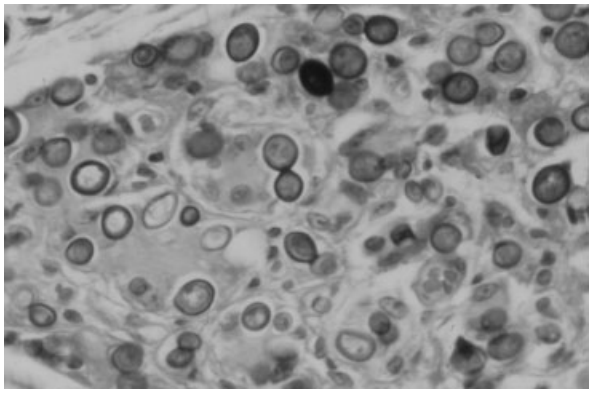

Figure 7 *Cutaneous lesion of African histoplasmosis. Histiocytes and multinucleated giant cells contain numerous yeast-like cells of* Histoplasma capsulatum *var.* duboisii, *8–15 μm in diameter. Note the classic 'double-cell' yeast form (top center) with narrow-based bud and thick cell walls. GMS–H&E; magnification* × *360*

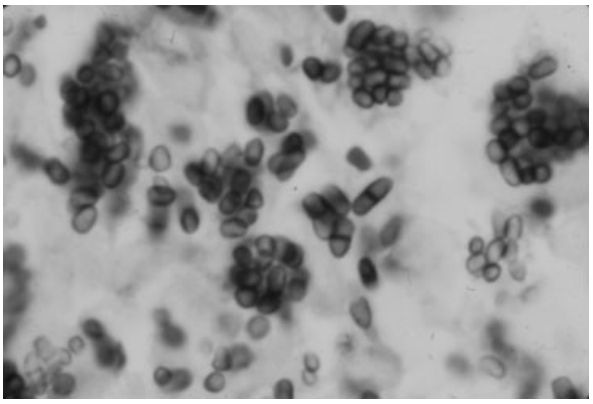

Figure 10 *Disseminated* Penicillium marneffei*: hepatic granuloma contains individual and clustered, spherical to oval yeast-like cells of* Penicillium marneffei, *2.5–5.0 μm in maximum diameter. Note the diagnostic yeast forms with single, wide, transverse septa. GMS; magnification* × *1 125*

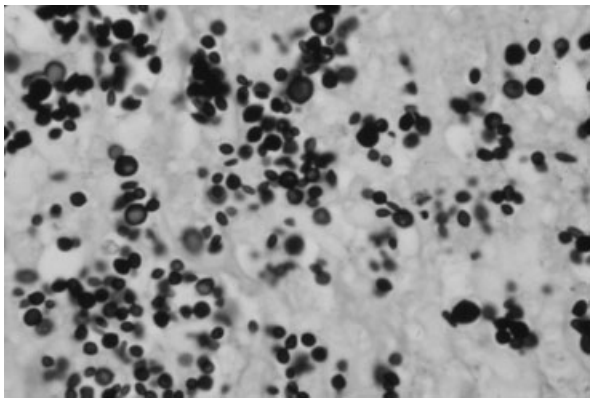

Figure 11 *Disseminated sporotrichosis: pleomorphic spherical and elongated (cigar-shaped) yeast-like cells of* Sporothrix schenckii *are located in a testicular granuloma. GMS; magnification* × *1 125*

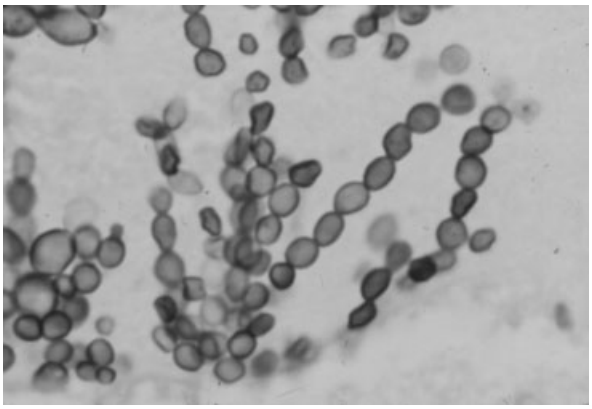

Figure 14 *Disseminated candidosis: numerous yeast-like cells and mycelial elements of* Candida albicans *occupy a nodular infarct in the lung of a granulocytopenic patient. GMS; magnification* × *563*

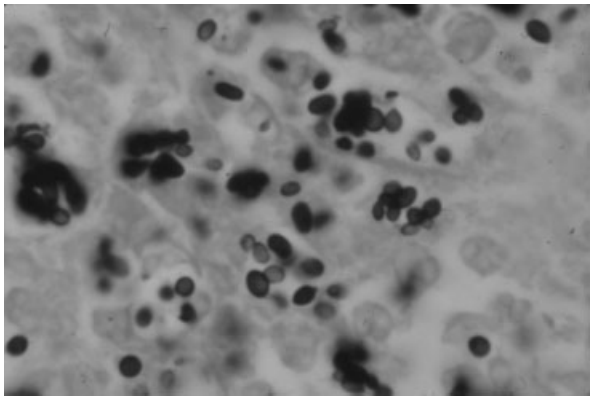

Figure 12 *Disseminated candidosis. Individual and clustered yeast-like cells of* Candida glabrata *are spherical to oval, 2–5 μm in diameter, have single buds attached to parent cells by a relatively narrow base, and resemble those of* Histoplasma capsulatum *var.* capsulatum. *GMS; magnification* × *1 125*

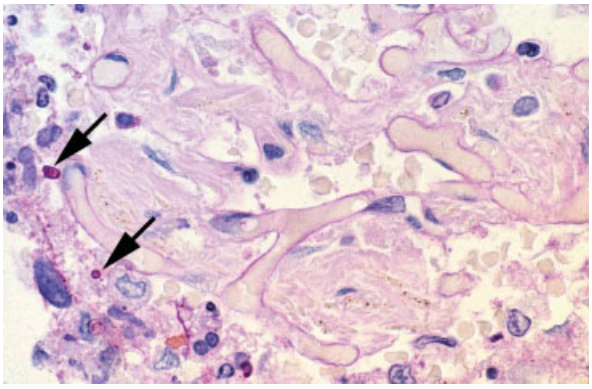

Figure 15 *Pulmonary candidosis: capillaries may sometimes be mistaken for fungal hyphae. However, by paying special attention, endothelial cells usually can be identified. In the present case, only a few PAS-positive* Candida glabrata *cells were demonstrated (arrows). PAS; magnification* × *360*

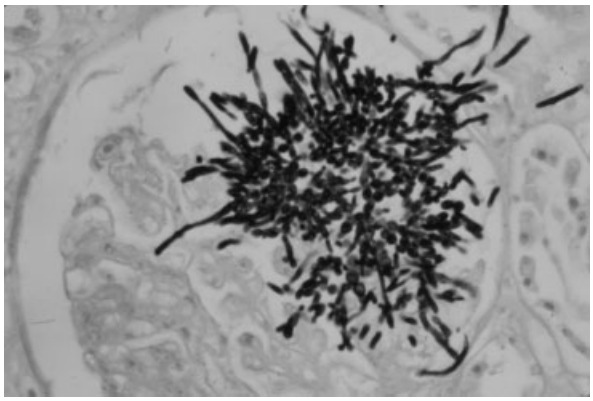

Figure 13 *Renal candidosis caused by* Candida albicans. *Blastoconidia (budding yeast-like cells), pseudohyphae and true hyphae proliferate in a radial pattern, and efface the normal renal architecture. GMS; magnification* × *225*

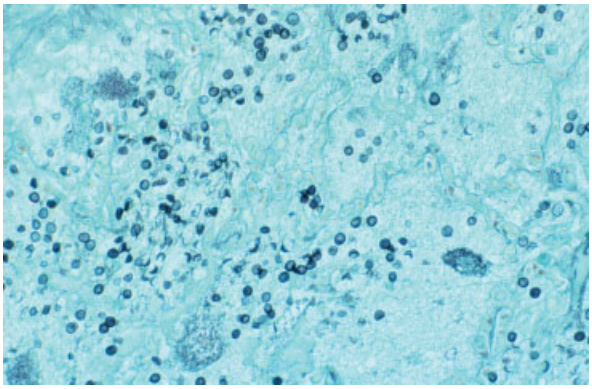

Figure 16 *Pulmonary pneumocystosis.* Pneumocystis carinii – *'cysts' and 'trophozoites' are seen as round, distorted, or crescent structures from 3 to 8 μm in diameter. GMS; magnification* × *360*

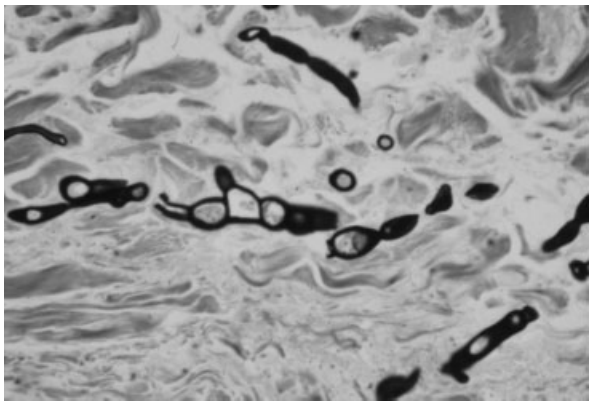

Figure 17 *Cutaneous geotrichosis: individual hyphae of* Geotrichum candidum *have non-parallel contours and prominent septa, and branch at acute angles. Spherical yeast-like cells and rectangular arthroconidia, 4–10 μm wide, with rounded or squared ends are also produced by this fungus. GMS; magnification × 360*

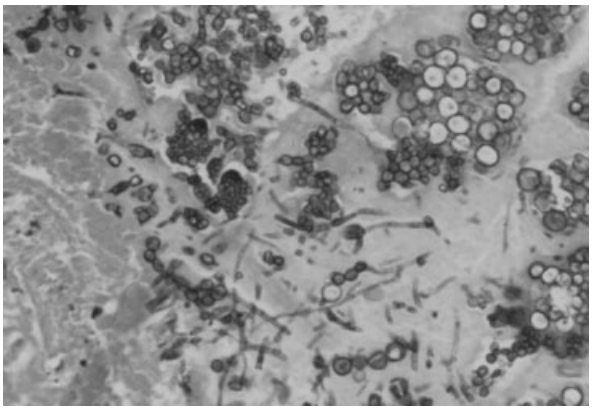

Figure 20 *Miliary pulmonary coccidioidomycosis: necrotic nodule contains characteristic endosporulating spherules, immature spherules, hyphae and arthroconidia (lower centre) of* Coccidioides immitis. *GMS–H&E; magnification × 113*

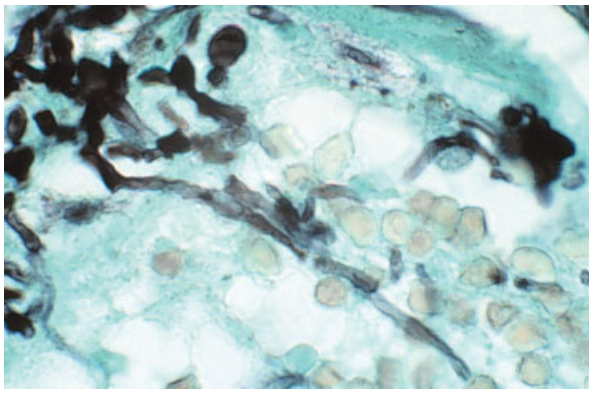

Figure 18 *Disseminated trichosporonosis: a splenic infarct contains radiating, septate hyphae and pleomorphic yeast-like cells of* Trichosporon beigelii. *GMS; magnification × 360*

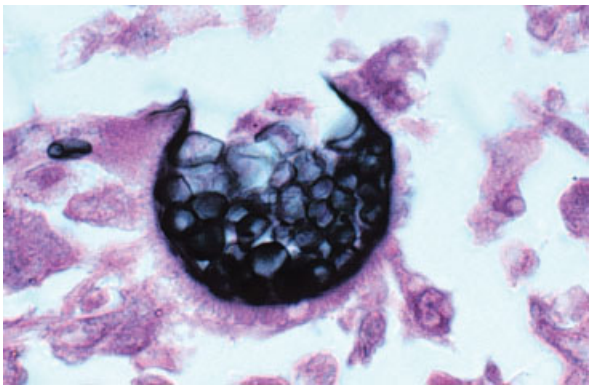

Figure 21 *Coccidioidomycosis: an intact endosporulating spherule of* Coccidioides immitis *is located in a pulmonary granuloma. GMS–H&E; magnification × 563*

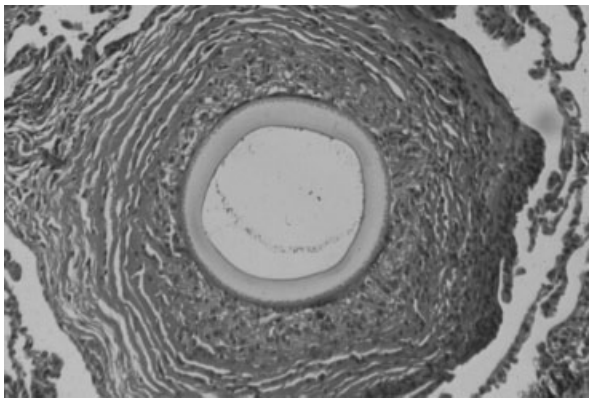

Figure 19 *Adiaspiromycosis: a large, spherical, thick-walled adiaconidium of* Chrysosporium parvum *var.* crescens *is surrounded by a fibrogranulomatous reaction in a lung biopsy specimen. The interior of the conidium is empty. Budding and endosporulation do not occur. H&E; magnification × 56*

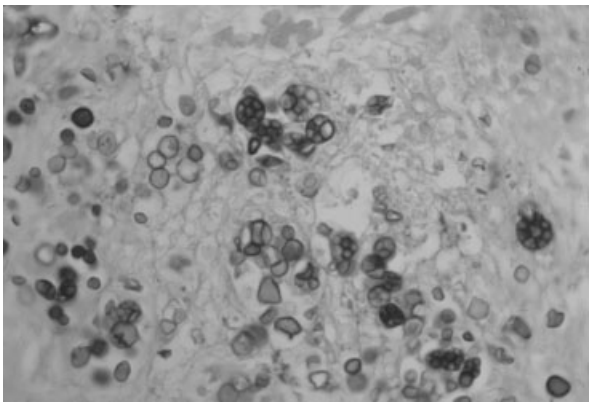

Figure 22 *Prototheca l olecranon bursitis: a necrotizing granuloma contains numerous single and endosporulating sporangia of* Prototheca wickerhamii. *Note the characteristic 'morula' forms. Gridley; magnification × 225*

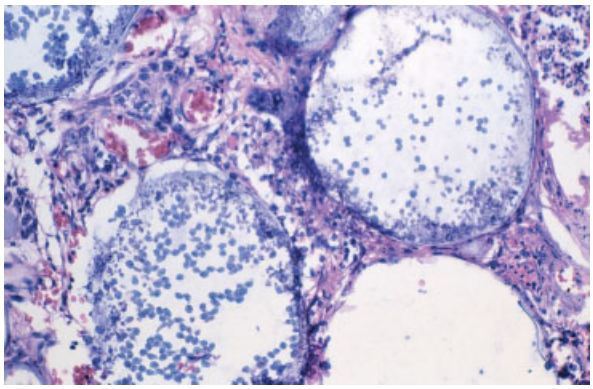

Figure 23 *Rhinosporidiosis: two mature sporangia of* Rhinosporidium seeberi *with thin walls are located in the inflamed stroma of a nasal polyp. Note typical maturation and zonation of sporangiospores. H&E; magnification × 113*

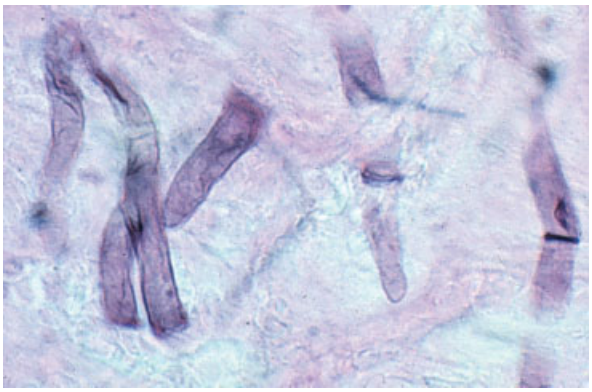

Figure 26 *Disseminated zygomycosis involving the myocardium. When hyphae with uniform wide, parallel walls, and dichotomous branching are formed, the identification may be confused with aspergillosis. PAS; magnification × 563*

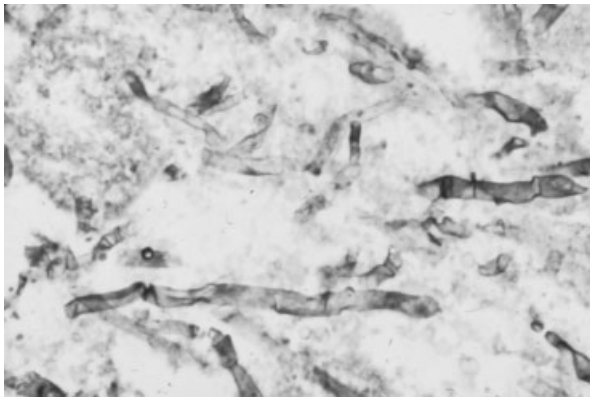

Figure 24 *Disseminated zygomycosis involving the myocardium. Typical hyphae of the mucoraceous zygomycetes are 6–25 μm wide, thin walled, infrequently septate, and have nonparallel contours and random branches. Hyphae of the zygomycetes are weakly stained with GMS procedure. GMS; magnification × 360*

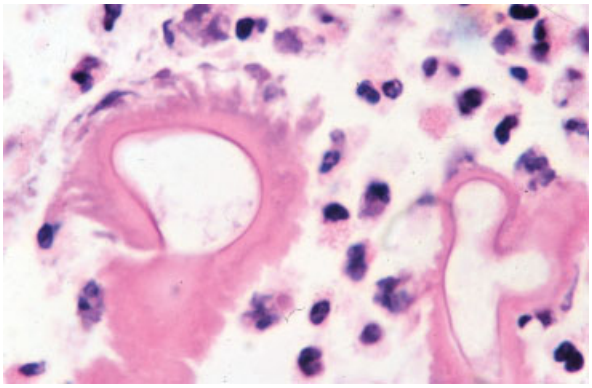

Figure 27 *Zygomycotic enteritis. Around hyphae, huge amounts of eosinophilic, homogenous and radially radiating Splendore-Hoeppli material has formed. H&E; magnification × 1 125*

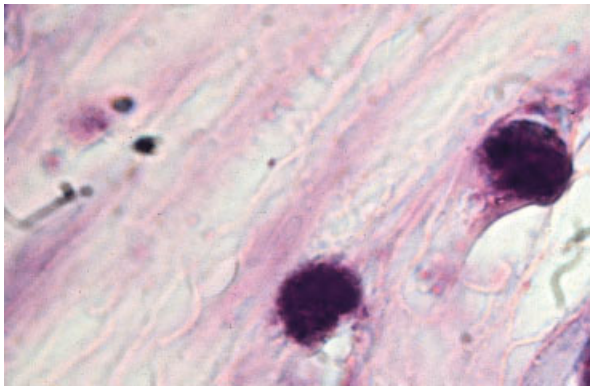

Figure 25 *Pulmonary zygomycosis caused by* Absidia corymbifera. *In tissues with access to air, sporangia are sometimes produced. If sectioned optimally, the sporangia may be useful for the identification of the fungus. H&E; magnification × 360*

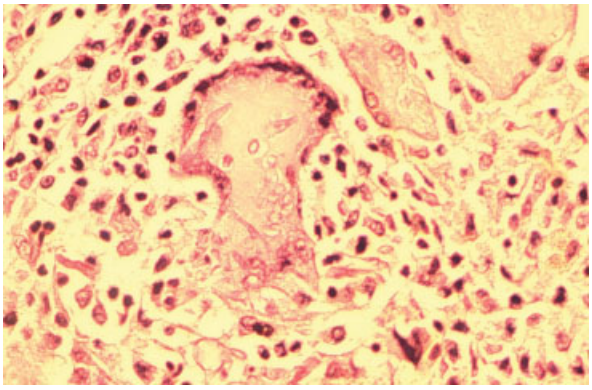

Figure 28 *Chronic zygomycotic lymphadenitis. Within the giant cells only sparse fungal elements are seen. The identification of elements as belonging to the zygomycetes was confirmed by immunohistochemistry. (Compare with Figure 59.) H&E; magnification × 360*

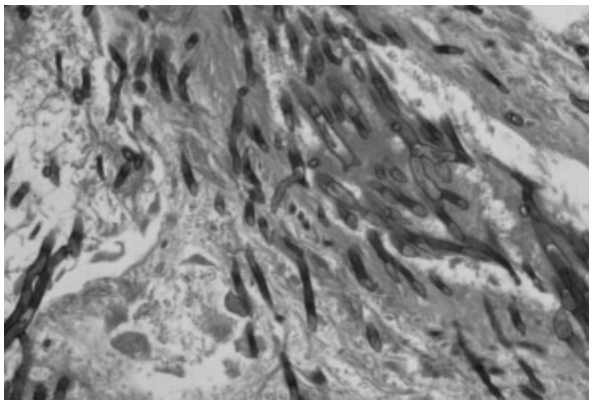

Figure 29 *Invasive pulmonary aspergillosis: hyphae of the Aspergillus spp. are septate, uniform in width (3–6 μm), have parallel contours, and branch progressively and dichotomously. The aspergilli are readily demonstrated with GMS and other special histological stains for fungi. GMS; magnification × 360*

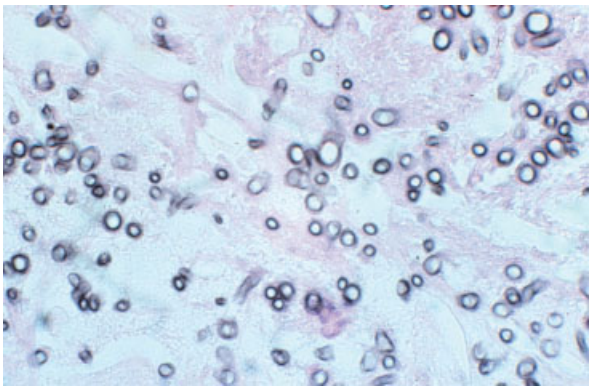

Figure 32 *Pulmonary aspergillosis. In cross-section, hyphae of* Aspergillus *spp. may be confused with yeast cells. However, the elements may easily be identified as* Aspergillus *spp. by immunohistochemistry. (Compare with Figure 55.) GMS; magnification × 563*

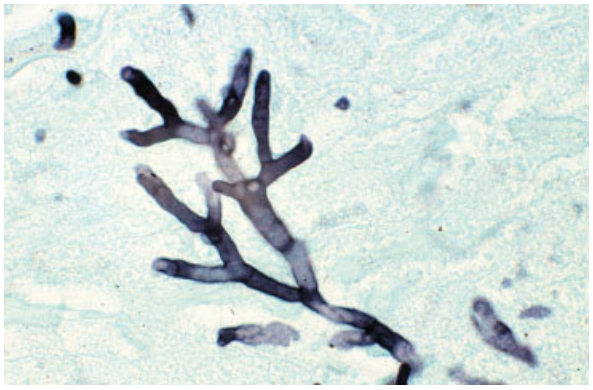

Figure 30 *Disseminated aspergillosis involving the myocardium. The hyphae present are typical of* Aspergillus *spp. They are frequently septate, have uniform widths (3-6 μm), have parallel contours, and branch dichotomously. GMS; magnification × 563*

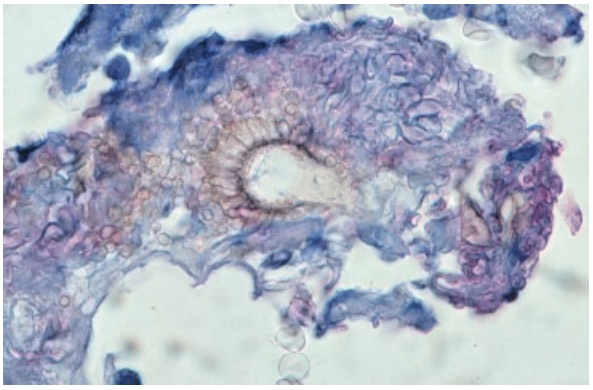

Figure 33 *Pulmonary aspergillosis caused by* Aspergillus fumigatus. *In tissues with access to air, conidial heads are sometimes produced. If the conidial heads are cut optimally they are useful for the identification of the fungus. H&E; magnification × 563*

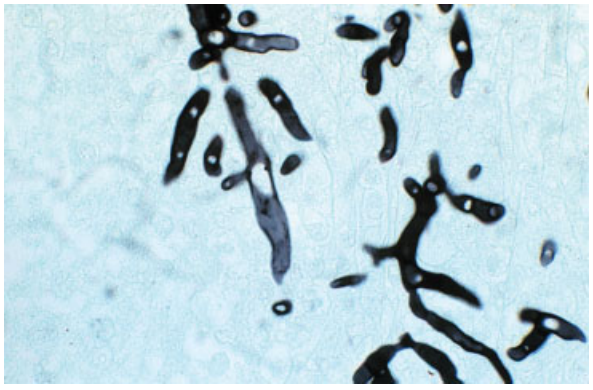

Figure 31 *Cerebral aspergillosis caused by* Aspergillus fumigatus. *Hyphae of* Aspergillus *spp. may adapt to highly bizarre forms in chronic lesions, which may be confused with hyphae of zygomycotic fungi. GMS; magnification × 360*

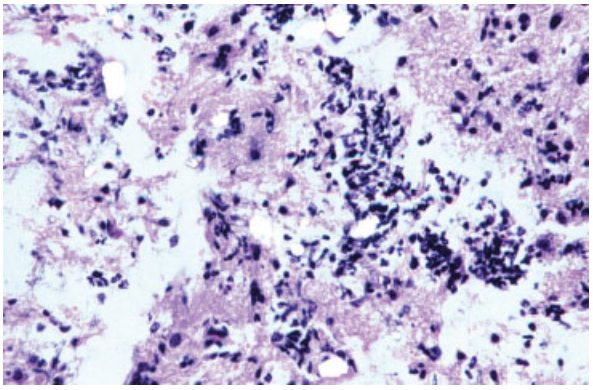

Figure 34 *Cerebral aspergillosis caused by* Aspergillus niger. *Deposits of birefringent calcium oxalate crystals are sometimes observed in lesions caused by* Aspergillus niger. *H&E and polarized light; magnification × 225*

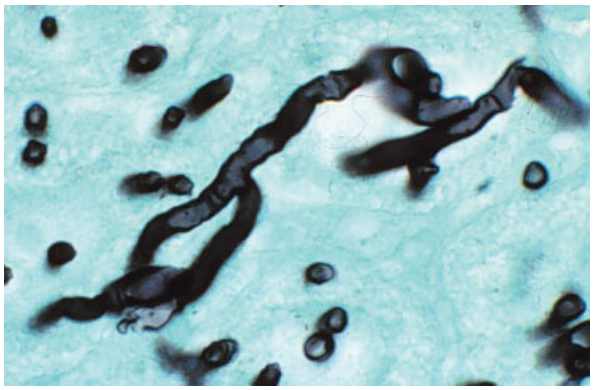

Figure 35 *Renal fusariosis caused by* Fusarium solani. *Hyphae in cross-sections and longitudinal sections are seen. Hyphae are septate, 3–8 μm wide and branch at dichotomous angles. Often fusariosis cannot be differentiated from aspergillosis and scedosporiosis. GMS; magnification × 563*

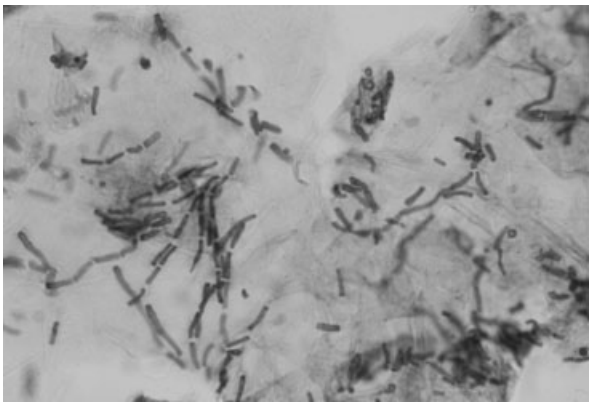

Figure 38 *Pityriasis versicolor: in skin scrapings,* Malassezia spp. *appear as clusters of single and budding yeast-like cells, 3–8 μm in diameter, mixed with short, narrow and sometimes curved hyphal fragments, 2.5–4 μm wide, that are oriented end to end. PAS; magnification × 225*

Figure 36 *Cerebral scedosporiosis caused by* Scedosporium apiospermum. *Hyphae of* Scedosporium *spp. are septate, 3–8 μm wide and branch at dichotomous angles. Often scedosporiosis cannot be differentiated from aspergillosis and fusariosis. GMS; magnification × 563*

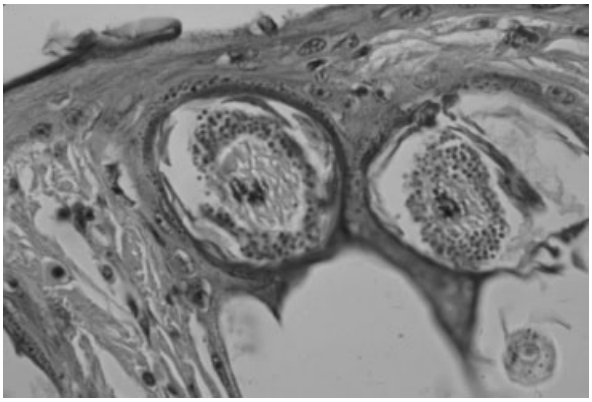

Figure 39 *Dermatophytosis caused by* Microsporum canis. *Hematoxylinophilic arthroconidia within two hair follicles surround partially degenerated hairs. H&E; magnification × 360*

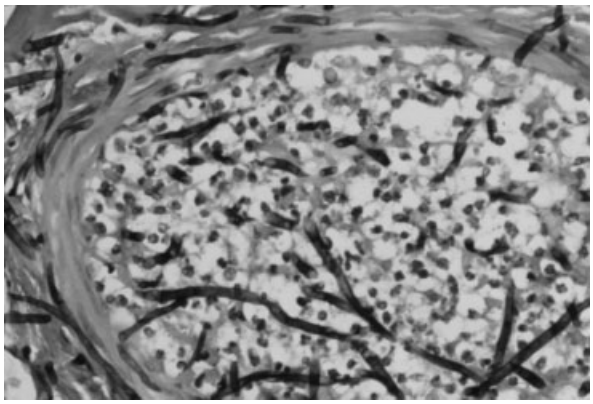

Figure 37 *Cutaneous hyalohyphomycosis caused by* Fusarium moniliforme *in a burned patient. Hyphae of the* Fusarium *spp. are septate, 3–8 μm wide and usually branch at right angles. Hyphal branches are often constricted at their origins from parent hyphae. GMS–H&E; magnification × 225*

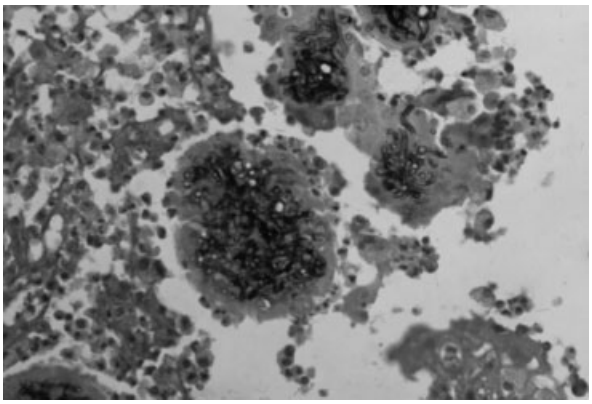

Figure 40 *Deep dermatophytic granuloma (pseudo-mycetoma) caused by* Microsporum canis. *The deep dermis contains two compact aggregates (pseudogranules) of distorted dermatophytic hyphae ensheathed by eosinophilic, Splendore–Hoeppli material. GMS–H&E; magnification × 225*

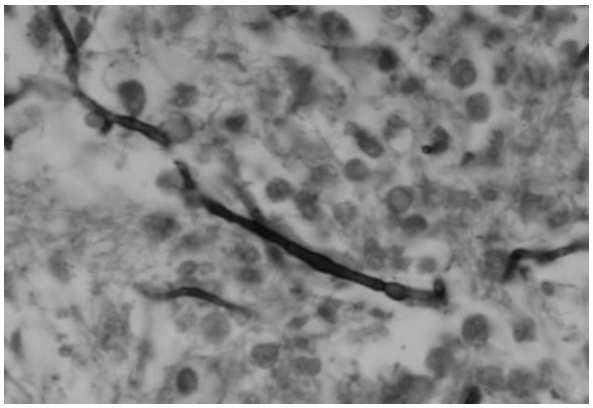

Figure 41 *Disseminated hyalohyphomycosis: a cerebral infarct contains narrow (2–5 μm), septate, randomly branched hyphae of* Pseudallescheria boydii. *GMS; magnification × 563*

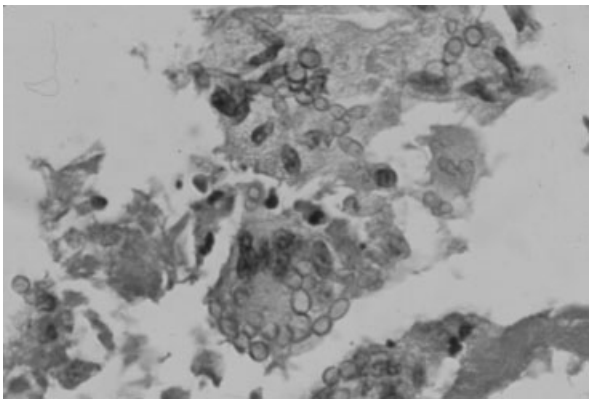

Figure 44 *Systemic (cerebral) pheohyphomycosis caused by* Cladophialophora bantiana. *Multinucleate giant cells in the wall of a cystic granuloma contain numerous pheoid yeast forms and hyphae that are constricted at their prominent septations. H&E; magnification × 563*

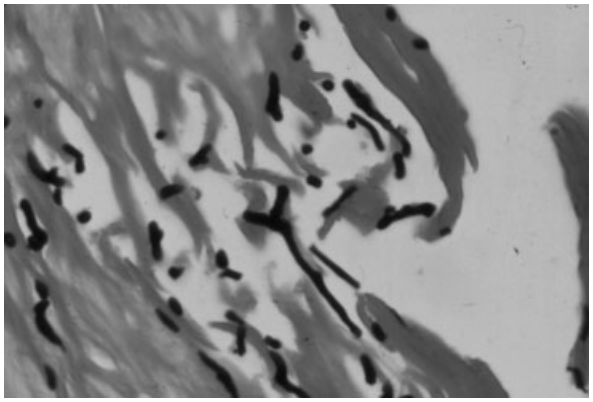

Figure 42 *Tinea nigra: branched, septate hyphae of* Phaeoannellomyces werneckii, *1–3 μm wide, are present in the hyperkeratotic layer of the epidermis. In H&E-stained tissue sections, the fungal elements are distinctly pheoid (naturally pigmented). GMS–H&E; magnification × 563*

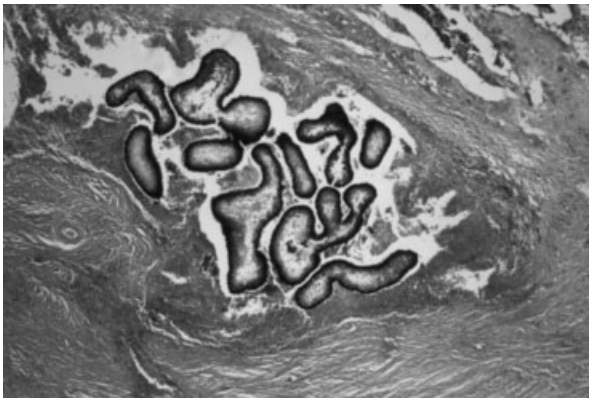

Figure 45 *Eumycotic black grain mycetoma caused by* Curvularia geniculata. *Several pheoid, lobulated grains (granules) are embedded in an abscess which is encapsulated by dense fibrous connective tissue. H&E; magnification × 45*

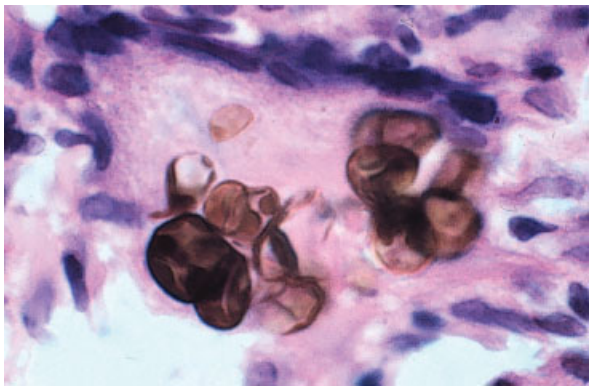

Figure 43 *Chromoblastomycosis caused by* Phialophora verrucosa. *Phaeoid muriform cells (sclerotic bodies) that divide by septation in one or more planes are located within multinucleated giant cells in a dermal granuloma. H&E; magnification × 1125*

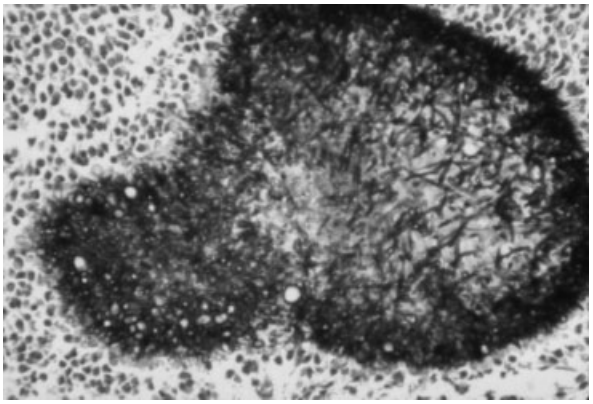

Figure 46 *Eumycotic white grain mycetoma caused by* Aspergillus nidulans. *The grain (granule) is composed of compact, radially oriented hyphae and vesicular chlamydoconidia. GMS–H&E; magnification × 113*

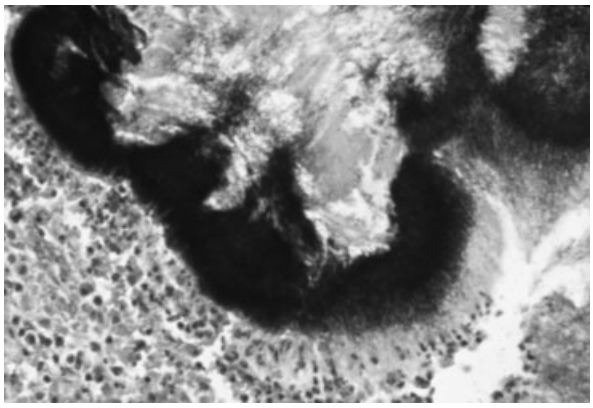

Figure 47 *Actinomycotic mycetoma caused by* Actinomadura pelletieri. *A deeply hematoxylinophilic, lobulated granule is embedded in a subcutaneous abscess. The granule is composed of delicate, compact, wavy and randomly oriented bacterial filaments, about 1 μm wide, that are often branched and beaded. H&E; magnification × 50*

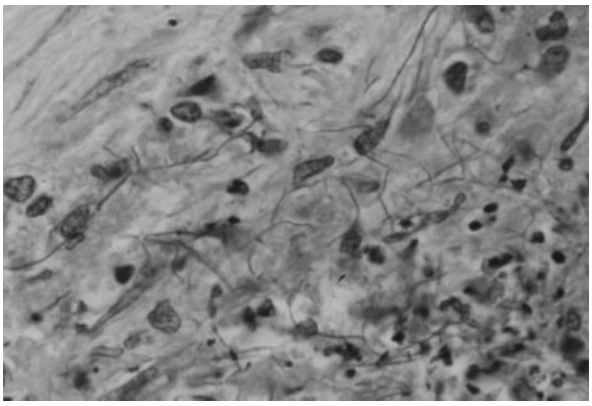

Figure 50 *Disseminated nocardiosis involving the cerebrum. Delicate, weakly acid-fast filaments of* Nocardia asteroides, *about 1 μm in width, branch at predominantly right angles. Modified Ziehl–Neelsen; magnification × 563*

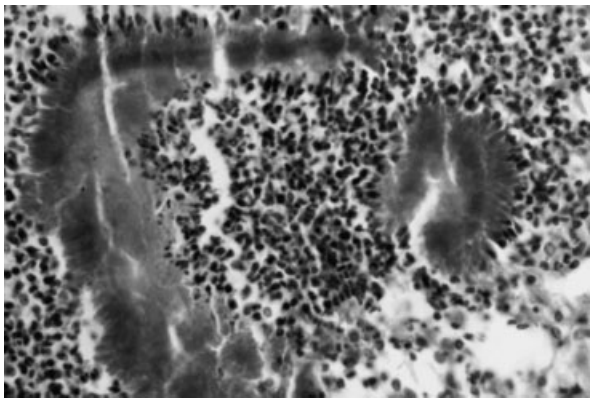

Figure 48 *Thoracic actinomycosis caused by* Actinomyces israelii. *A pulmonary abscess contains two granules that are bordered by intensely eosinophilic, club-like, Splendore–Hoeppli material. Individual filaments within the granules are not visible with the H&E stain. H&E; magnification × 225*

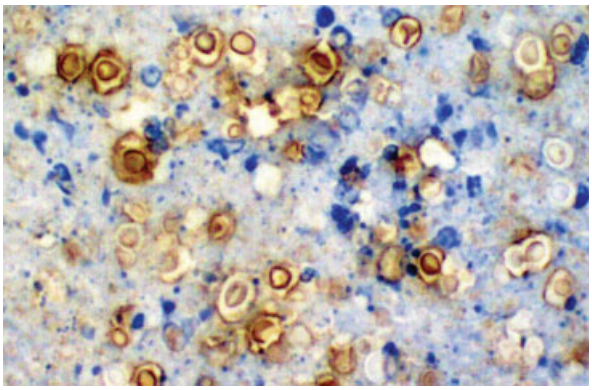

Figure 51 *Cerebral cryptococcosis. By the application of a serotype (A + D) specific primary monoclonal antibody in an avidin–biotin–horseradish peroxidase (HRP) technique, the etiology was found to be* Cryptococcus neoformans *var.* gatti. *HRP; magnification × 563*

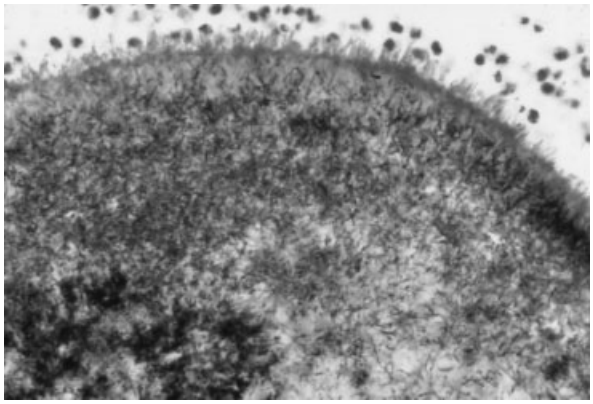

Figure 49 *Abdominal actinomycosis: the granule is composed of randomly oriented, gram-positive, branched and beaded bacterial filaments, about 1 μm in width, embedded in a matrix or ground substance of uncertain composition. The peripheral Splendore–Hoeppli material is gram negative. Brown and Brenn; magnification × 225*

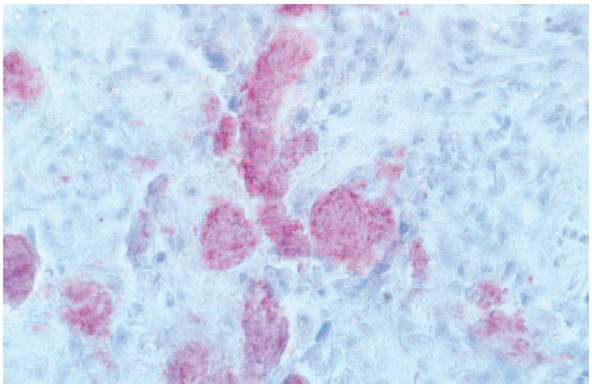

Figure 52 *Pulmonary pneumocystosis.* Pneumocystis carinii *within alveolar space was demonstrated immunohistochemically by the use of a specific monoclonal antibody in an APAAP technique. APAAP; magnification × 563*

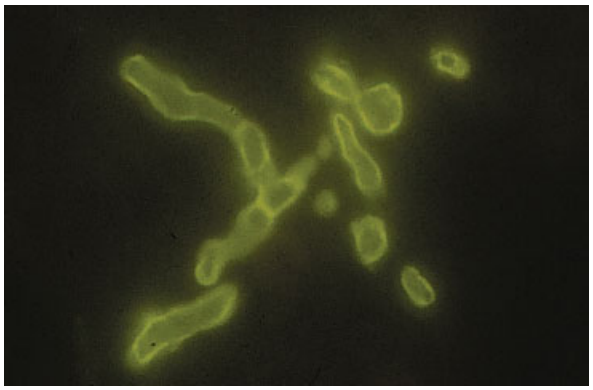

Figure 53 *Pulmonary aspergillosis caused by* Aspergillus fumigatus. *The hyphae were stained by an indirect immunofluorescence (IIF) technique using FITC as fluorochrome. IIF; magnification × 1 125*

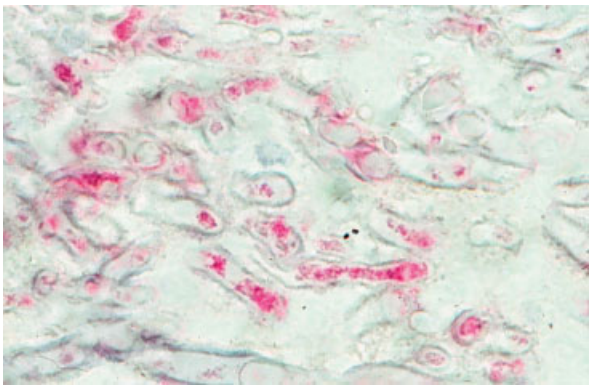

Figure 56 *Pulmonary aspergillosis. Hyphae of* Aspergillus fumigatus *may also be identified by in situ hybridization. In the present case, an alkaline phosphatase-conjugated DNA probe hybridizing with genomic DNA and mRNA responsible for the 33 kDa protease was used. In the technique, Fast red was the substrate. Magnification × 1 125*

Figure 54 *Myocardial aspergillosis. The hyphae were identified immunohistochemically by using a primary genus specific monoclonal antibody raised towards galactomannan. APAAP technique; magnification × 1 125*

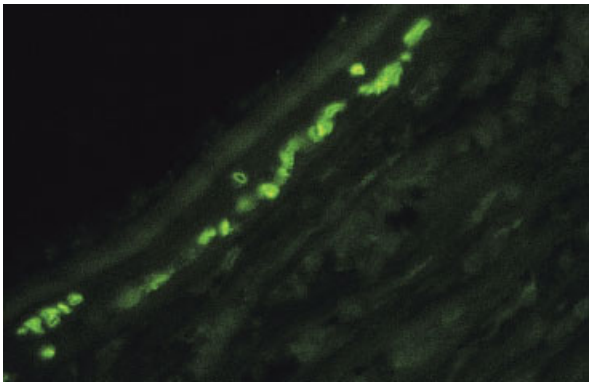

Figure 57 *Corneal fusariosis. The hyphae within a corneal biopsy were identified by the application of heterologously adsorbed polyclonal antibodies in an IIF technique using FITC as fluorochrome. The primary antibody was raised against somatic antigens of* Fusarium solani. *IIF; magnification × 1 125*

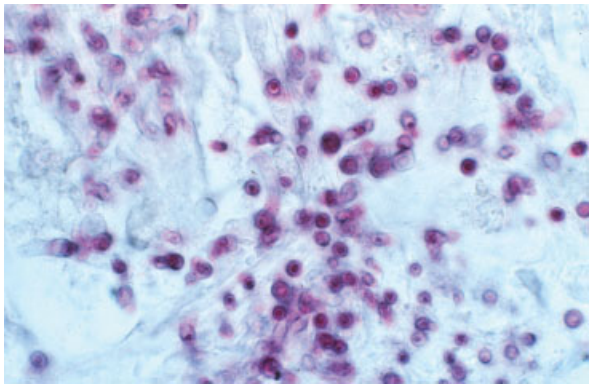

Figure 55 *Pulmonary aspergillosis. Immunohistochemically, cross-sectioned hyphae of* Aspergillus *spp. may be identified by using a genus specific primary monoclonal antibody in an APAAP technique. (Compare with Figure 32.) APAAP; magnification × 563*

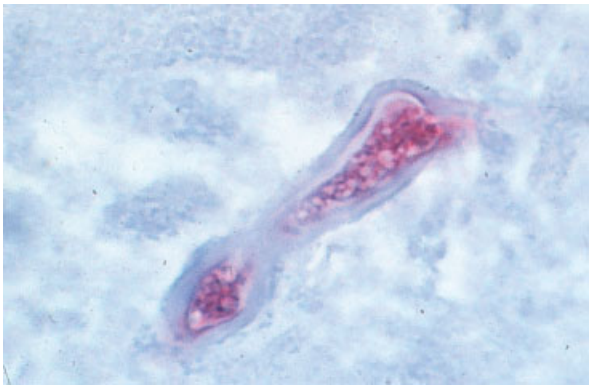

Figure 58 *Zygomycotic lymphadenitis caused by* Rhizopus arrhizus. *When using a monoclonal antibody raised somatic antigens as the primary antibody in an APAAP technique, the hyphal walls remain unstained. APAAP; magnification × 1 125*

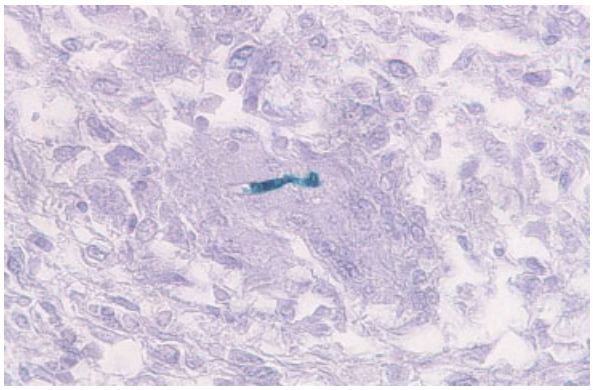

Figure 59 *Chronic zygomycotic lymphadenitis. A single fungal fragment within a giant cell may be identified immunohistochemically by using a panel of specific primary antibodies. (Compare with Figure 28.) In the present case the agent was identified as a zygomycotic fungus due to the reactivity with a monoclonal antibody raised towards somatic antigens of* Rhizopus arrhizus*. The primary monoclonal antibody was used in a PAP technique. PAP; magnification × 360*

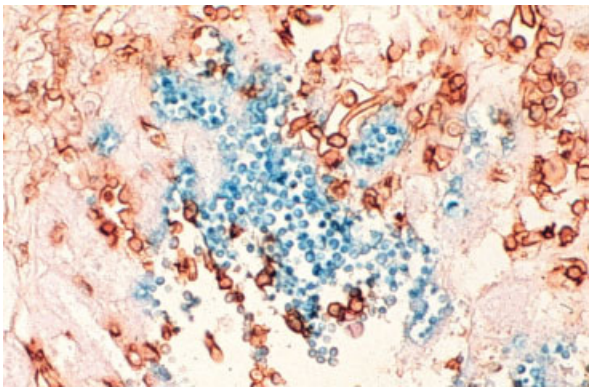

Figure 61 *Concomitant intestinal infection due to* Candida albicans *and* Candida glabrata*. The elements of* Candida glabrata *were immunostained bluish by an indirect ABC method using galactosidase as an enzyme and 5-bromo-4-chloro-indolyl-galactosidase as a substrate. The* Candida albicans *elements were stained brownish by a PAP technique using 3,3́-diaminobenzidine tetrahydrochloride as substrate. ABC and PAP; magnification × 360*

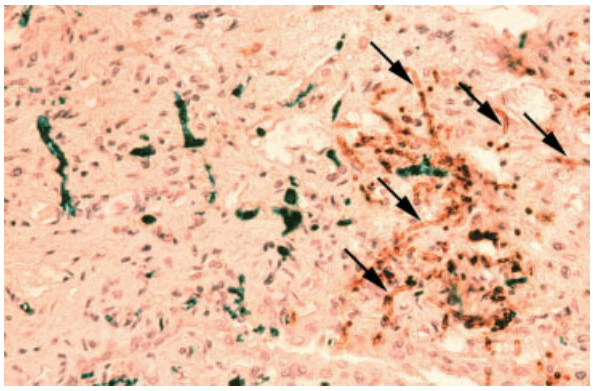

Figure 60 *Concomitant enteral candidosis and zygomycosis. The* Candida *hyphae (arrows) were immunostained brown (substrate = 3-amino-9-ethylcarbazole) by a genus-specific polyclonal rabbit antibody, whereas the zygomycotic hyphae were immunostained green (substrate = dioctyl sulfosuccinate sodium and 3,3́,5,5́-tetramethylbenzidine) by a murine monoclonal antibody. Both antibodies were used in a PAP technique. PAP; magnification × 360*

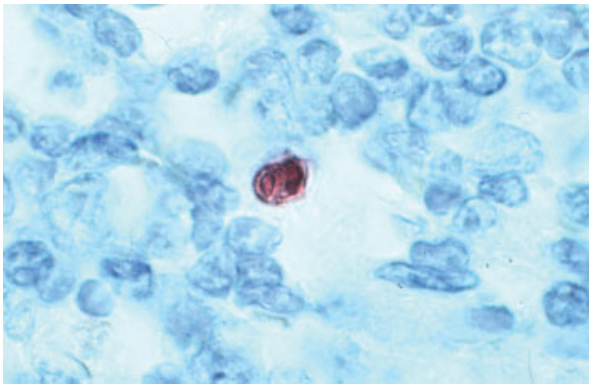

Figure 62 *Protothecal lymphadenitis. In tissues the cells of* Prototheca zopfii *can be identified immunohistochemically by using primary heterologously adsorbed polyclonal antibodies in a PAP technique. PAP; magnification × 563*

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Antifungal agents and antifungal susceptibility testing

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In the late nineteenth century, the appreciation of systemic infections by *Coccidioides immitis* and *Histoplasma capsulatum* launched an effort to find effective treatment for these rare, debilitating, and lethal diseases. Unfortunately, broad-spectrum agents were not discovered until the 1950s, in the forms of amphotericin B, hamycin, filipin, and nystatin. As a result of their great toxicity, only hamycin and nystatin survived as topical drugs, and amphotericin B as a systemic drug. As fungal infections were rather esoteric, relatively few physicians used amphotericin B. In more recent decades, the increasing use of antibacterial drugs, immunosuppressive agents, and prosthetic implanted devices, and the appearance of the acquired immunodeficiency syndrome (AIDS) provided settings for a dramatic increase in fungal infections, and a subsequent increase in the development of systemic antifungal drugs.

Flucytosine was introduced in the 1960s. Although rapidly active as an antimetabolite, the spectrum was limited to *Candida* spp. and *Cryptococcus neoformans*, and the drug now is used only in combination with amphotericin B (Francis and Walsh 1992).

A new era in antifungal therapy was introduced with the azole antifungals, developed with the aim of reducing toxicity and improving efficacy against fungal pathogens. The first of these drugs was clotrimazole. When given parenterally, clotrimazole was soon found to activate the hepatic cytochrome enzymes responsible for its degradation, a sort of suicide effect, which limited its use as a systemic antifungal agent. Presently, clotrimazole is used only for topical therapy of mucosal candidiasis and for dermatophyte infections. The second of the imidazole drugs was miconazole, which was used intravenously for several years. Unfortunately, the carrier vehicle caused severe histamine-mediated reactions, and miconazole today is used almost exclusively topically (Stevens 1977; Heel et al. 1980).

The major breakthrough in antifungal drug development was ketoconazole, which was released in the late 1970s. Ketoconazole has a broad antifungal spectrum, is absorbed when given orally, and is much better tolerated than amphotericin B or flucytosine (Dismukes et al. 1983; Saag et al. 1985; Sugar et al. 1987; Graybill 1989, 1990; Bodey 1992; Como and Dismukes 1994). However, its lack of oral absorption in seriously ill patients, its poor central nervous system penetration, and its lack of activity against molds limited its clinical use. Thus, in the late 1980s, ketoconazole was supplemented and, in some countries supplanted by, the more recently developed azoles fluconazole and itraconazole (Como and Dismukes 1994). These triazole antifungals are very well tolerated and achieve significant activity in systemic disease. Modifications of these molecules are aimed at further improvement of their clinical properties.

During this period of azole expansion, multiple efforts to modify the amphotericin B molecule to improve tolerance were initiated, but they all failed because of toxicity. The most recent efforts involved incorporating amphotericin B or other toxic antifungals such as nystatin into lipid vehicles, which reduce the dose-limiting toxicity of those compounds. Despite continuing advances, none of the drugs presently available is

absolutely fungicidal, either in vivo or in vitro, so that further advances in antifungal drugs are still needed.

SYSTEMIC ANTIFUNGAL DRUGS IN CURRENT USE

Polyenes

MECHANISM OF ACTION

The polyenes are macrolide antibiotics with unsaturated diene bonds. All the polyenes rapidly bind to sterols, preferentially to ergosterol, the primary fungal cell membrane sterol, and less avidly to cholesterol, the primary mammalian cell membrane sterol (Table 1). The consequences of this binding include disruption of osmotic integrity of the membrane, with leakage of intracellular potassium and magnesium (Hamilton-Miller 1974; Hammond 1977; Kuroda et al. 1978; Brajtburg et al. 1980, 1986; Vertut-Doi et al. 1994; Wasan et al. 1994). The polyenes also disrupt the function of oxidative enzymes in target cells (Wilson et al. 1991). Of the polyenes in use, amphotericin B is the only one with a toxicity that is sufficiently limited to permit intravenous administration. However, tolerance is also limited by toxic effects on renal cells, with secondary renal tubular acidosis and hypokalemia. Ultimately there is glomerular failure, suppression of erythropoietin synthesis, and anemia (MacGregor et al. 1978). Other systemic symptoms include chills, fever, and nausea. The spectrum of antifungal activity of polyenes is similar and broad; few fungal species are resistant to these compounds. Resistance is most commonly mediated by substitution of ergosterol by other sterols in fungal cell membranes (Woods et al. 1974). Resistance tends to be species dependent and emerges uncommonly and slowly in isolates from patients treated with amphotericin B (Woods et al. 1974; Pappagianis et al. 1979; Dick et al. 1980; Guého et al. 1994; Matsumoto et al. 1994). These include *Candida lusitaniae*, *Fusarium* spp., *Trichosporon beigellii*, *Pseudallescheria boydii*, and *Scedosporium prolificans*. Some other species of *Candida* may develop lesser degrees of resistance.

Despite the very broad range of amphotericin B susceptibility, there has been a study suggesting that higher minimum inhibitory concentration (MIC) of *Candida* species are correlated with clinical failure (Vanden Bossche et al. 1994b). Nguyen et al. (1998) have reviewed in vitro responses and outcome in 105 patients treated with amphotericin B. They found that isolates with an MIC at 24 hours $\geqslant$1 μg/ml correlated with more clinical failures than those with MIC $<$1 μg/ml. They also found that the minimum lethal concentration (MLC) was even more useful, correlating well with failure ($P<0.03$). In their study an MLC $>$1 μg/ml was associated with 78 percent failure in 18 patients, versus only 22 percent failures in 87 patients with isolates having MLC $\leqslant$1 μg/ml. Their method utilized antibiotic medium 3, which is now recommended for amphotericin B testing.

NYSTATIN

This drug is used topically, as an oral suspension at a concentration of 100 000 units/ml for mucosal candidiasis, where it is marginally effective. It is also used in ophthalmic preparations and other ointments.

AMPHOTERICIN B

The most widely used form of amphotericin B is commercially available as a deoxycholate and is infused

Table 1 *Antifungal mechanisms of action*

Target	Chemical class	Antifungal agent
DNA/RNA synthesis	Pyrimidine	Flucytosine
Membrane barrier function	Polyenes	Amphotericin B, nystatin
Ergosterol synthesis		
Squalene epoxidase	Allylamines	Terbinafine, naftifine
14α-Demethylase	Azoles	
	Imidazoles	Clotrimazole, econazole, ketoconazole, miconazole
	Triazoles	Fluconazole, itraconazole, voriconazole, posaconazole
Δ^{14}-Reductase/Δ^{7}–Δ^{8}-isomerase	Morpholines	Amorolfine
Mitosis (sliding of microtubules)		Griseofulvin
1,3-β-Glucan synthesis	Echinocandins	Caspofungin, anidulafungin, micafungin
Metabolite uptake	Pyridone	Ciclopiroxolamine
Chitin synthase	Nikkomycin	Nikkomycins K, Z, T
Cell wall	Pradimicin	BMS-181184
Protein synthesis (elongation factor 2)	Sordarins	GM-237354

Adapted from Vanden Bossche et al. (1994a).

intravenously at doses ranging from 0.3 to 1.5 mg/kg over 1–4 hours (Gallis et al. 1990). The optimal time of infusion has been controversial. Two studies have addressed this, with conflicting conclusions. Oldfield et al. (1990) examined 128 infusions in 12 patients. They were randomized to 1 versus 4 h. Fever, rigors, and need for meperidine (pethidine) were similar in both groups, though the onset of infusion-related toxicity occurred more quickly in 1-hour infusion recipients. Conversely, Ellis et al. (1992) found that chills, meperidine dose, and maximum 7-day total pulse rise were all worse in patients receiving 1-hour infusions. Infusion-related intolerance includes fever, chills, rigors, and thrombophlebitis, which may occur in more than 70 percent of patients; hypotension occurs rarely. These side effects may be blocked by premedication with antihistamines, corticosteroids, and acetaminophen (paracetamol) (Goodwin et al. 1995). However, the efficacy of these measures remains controversial (Goodwin et al. 1995). Nephrotoxicity is dose limiting for patients who have received in excess of 5–10 g. To minimize nephrotoxicity, 1 liter of physiological saline is usually infused before amphotericin B is given (Heidemann et al. 1983). Dosing is often interrupted if serum creatinine rises above 2.5 mg/dl. Potassium wasting occurs initially as kidney tubule function is disrupted. Hypokalemia is a common toxicity of amphotericin B administration, which occurs before the development of renal insufficiency. As glomerular function later declines, patients develop typical metabolic acidosis of uremia, with potassium retention. Rapid administration to these patients may cause leaking of potassium into the plasma and, with poor clearance from the kidneys, acute hyperkalemia may develop. Although amphotericin B is highly nephrotoxic, only 10–20 percent of the dose is excreted by the kidneys. Therefore, anephric patients may receive identical doses to individuals with normal renal function.

LIPID VEHICLES FOR POLYENES

Three licensed lipid preparations are available to encase amphotericin B in lipid vehicles that bypass the kidneys, the primary site of nephrotoxicity, but permit access to phagocytic cells (Table 2). Nephrotoxicity is markedly reduced with each of these lipid preparations, but patients still encounter infusion-related side effects and hypokalemia (Chopra et al. 1991; Collette et al. 1991; de Marie et al. 1994; Jangnegt et al. 1995). Acute dyspnea has also been reported in a few patients (Arning et al. 1995). Although these lipid preparations are less toxic than amphotericin B deoxycholate, it appears that on a milligram per milligram basis they are less potent (Collette et al. 1991; Meunier et al. 1991; Schmitt 1993; de Marie et al. 1994; Jangnegt et al. 1995). Doses of 3–5 mg/kg of liposomal forms of amphotericin B are well tolerated and commonly used (Collette et al. 1991; Meunier et al. 1991; Schmitt 1993; de Marie et al. 1994; Jangnegt et al. 1995). These preparations are all efficacious (Chopra et al. 1991; Ringden et al. 1991; Mills et al. 1994; Viviani et al. 1994). Comparative studies with amphotericin B deoxycholate are now emerging. The first such report, comparing amphotericin B lipid complex (ABLC) 5 mg/kg/day with amphotericin B 0.6 mg/kg/day for candidiasis, found comparable responses, with less renal toxicity in the ABLC-treated patients (Anaissie et al. 1995b).

All are effective antifungal agents for a variety of mycoses, including *Candida* species, *Aspergillus*, *Fusarium*, *Cryptococcus neoformans*, and others (Anaissie and Ramphal 1992; Jangnegt et al. 1995; Tollemar and Ringden 1995; Walsh et al. 1997; White et al. 1997; Graybill et al. 1998b). These agents have also been used for empirical therapy for potential mycoses in the febrile neutropenic patient. Here it appears that AmBisome is superior to amphotericin B, whereas Amphotec is only equal (Walsh et al. 1998; White et al. 1998). Anecdotal data are accumulating that high doses ($\geqslant$5 mg/kg/day) of lipid amphotericin B preparations may be more efficacious than amphotericin B deoxycholate for the treatment of invasive zygomycosis, and some would suggest that these agents be considered as first line therapy for these refractory infections (Gonzalez et al. 2002; Mondy et al. 2002). The very high costs of lipid preparations (up to US$1 000 per day) may preclude their widespread use for indications such as empirical therapy, though pharmacoeconomic analyses point out that this may be offset by reducing the costs associated with renal dysfunction and other toxicities attributed to standard amphotericin B (Graybill 1996; Hann and Prentice 2001; Ostrosky-Zeichner et al. 2003).

Flucytosine

This is the only antifungal compound of its kind. Flucytosine is activated by deamination within fungal cells to

Table 2 *Characteristics of lipid-associated amphotericin B*

Drug	Form	Percentage amphotericin	Dose (mg/kg)
Amphotericin B lipid complex	Ribbons	33	Up to 5
Amphotericin B colloidal dispersion	Disks	50 (molar)	Up to 7
Amphotericin B liposomes	Small unilamellar vesicles (SUV)	10	Up to 5
Amphotericin B + Intralipid	Micelles	30	Up to 1.5

the antimetabolite 5-fluorouracil. Flucytosine is highly water soluble and can be given orally or intravenously, although the intravenous preparation is not widely available (Francis and Walsh 1992). Flucytosine is nontoxic in in vitro assays to mammalian cells, but more than one-third of patients treated with this drug experience bone marrow depression and gastrointestinal intolerance. The toxicity of flucytosine in vivo is thought to be mediated by bacterial conversion intraluminally, in the gut, of flucytosine to 5-fluorouracil, a compound with significant human toxicity (Harris et al. 1986). Flucytosine has a narrow spectrum aimed largely at yeasts, including *Candida* spp. and *Cryptococcus neoformans* (Tassel and Madoff 1968). Some activity has been reported against *Aspergillus* spp. and some agents of chromoblastomycosis (Vandevelde et al. 1972). Flucytosine has been used as monotherapy for treatment of cryptococcosis, but resistance emerged rapidly and only 10 (43 percent) of 23 evaluable patients had a response to therapy (Hospenthal and Bennett 1998). Flucytosine is subject to development of resistance by mutation of cytosine permease, deaminase, and other mechanisms (Francis and Walsh 1992). Thus, flucytosine is used clinically only in combination with amphotericin B and, more recently, fluconazole (Larsen et al. 1994). Flucytosine is excreted largely unchanged by the kidneys. Renal failure, commonly induced by amphotericin B, causes an increase in plasma concentrations of flucytosine which potentiates its toxicity. Some clinicians have argued that serum concentrations should be measured on all patients receiving flucytosine, but others have found that monitoring leukocyte counts and creatinine, and adjusting doses based on these parameters, is sufficient to minimize intolerance. This has best been demonstrated in a study of cryptococcosis, wherein 2 weeks of amphotericin B was compared with and without flucytosine. If dosing was adjusted for renal and bone marrow function, toxicity was not sufficient to remove or exclude many patients from the study (Van der Horst et al. 1997).

Azoles

All of the azole antifungal agents act by inhibiting fungal cytochrome 14α-demethylase (Table 1) (Vanden Bossche 1985). Inhibition of this enzyme blocks synthesis of ergosterol for fungal cell membranes. Consequently, the integrity of membrane-associated oxidative enzymes is impaired, with accumulation of phospholipids within the cell and ultimately cell death. The action of antifungal azoles takes several generations of fungi to become apparent and is consequently much slower than that of the polyenes.

Differences among antifungal azoles are primarily in their pharmacokinetics, and in the relative avidity of the drug for fungal versus mammalian enzymes (Tables 3, 4, 5, and 6). In distinction to the drugs developed initially, fluconazole and itraconazole have a vastly increased affinity for fungal membranes over mammalian cell membranes, and are better tolerated than ketoconazole (Table 5) (Como and Dismukes 1994). Pharmacokinetics distinguish two general classes of azole antifungals (Table 3). Fluconazole is the only representative of the first group available for therapeutic use at the present time. It is relatively water soluble, is absorbed extremely well, distributed to all tissues, and is excreted largely unchanged by the kidneys (Humphrey et al. 1985). There are relatively few side effects with fluconazole as compared with ketoconazole and itraconazole, the other azoles commonly used (Table 6) (Lazar and Wilner 1990; Como and Dismukes 1994). However, fluconazole is less potent than other azoles against many fungi and its spectrum of activity is confined largely to yeasts (Table 4). Fluconazole is 10–100 times less potent than itraconazole against many species, including *Penicillium marneffei*, *Aspergillus* spp., and some *Candida* spp. (Drouhet 1993; Supparatpinyo et al. 1993). Fluconazole is remarkably well tolerated even when given at doses as high as 2000 mg/day (Anaissie et al. 1995a).

Table 3 *Azole antifungals*

Parameter	Ketoconazole	Itraconazole	Fluconazole	Voriconazole	Posaconazole
C_{max} (μg/ml) (200 mg orally)	2–4	0.2–0.4	8–12	2.7	0.3
Intravenous	No	Yes	Yes	Yes	No
Oral absorption					
Acid	+++	++	0	0	–[a]
Lipid meal	+	+++	0	–	++
Clearance	Hepatic	Hepatic	Renal	Hepatic	Hepatic
Linear	Yes	No	Yes	No	Yes
$t_{1/2}$ (h)	7–10	34–42	22–31	6	24
Penetration (% serum)					
Urine	<5	<1	>70	<2	–
Cerebrospinal fluid	3–7	<1	>70	38–68	–

Graybill (1994); Hoffman et al. (2000).
a) – indicates information is not known.

Table 4 *Spectrum of activity of azole compounds*

Organism	Ketoconazole	Itraconazole	Fluconazole	Voriconazole	Posaconazole
Opportunistic yeasts					
C. albicans	++	+++	++++	++++	++++
C. tropicalis	++	++	++	+++	+++
C. krusei	+	++	0	+++	+++
C. glabrata	+	++	+	+++	+++
C. parapsilosis	++	+++	++++	++++	++++
Cryptococcus neoformans	+	++	+++	+++	+++
Opportunistic molds					
Aspergillus spp.	0	+++	0	++++	++++
Fusarium spp.	0	±	0	++	+
Pseudallescheria boydii	+	++	0	+++	++
Zygomycetes	0	0	0	0	++
Agents of phaeohyphomycosis	+	+++	+	+++	+++
Dimorphic molds					
H. capsulatum	++	++++	+++	++++	++++
Blastomyces dermatitidis	++	+++	+	++++	++++
Coccidioides immitis	++	+++	+++	++++	++++
Sporothrix schenckii	+	++++	++	++	+++
Paracoccidioides brasiliensis	+++	++++	++	+++	
Penicillium marneffei	+	++++	+	+++	

Ketoconazole and itraconazole share several pharmacokinetic parameters (Brass et al. 1982; Heykants et al. 1982, 1989; Daneshmend et al. 1984; Hardin et al. 1988; Barone et al. 1993) (Tables 3, 4, and 5). They are both absorbed optimally in the gastric acid environment, and are cleared by the liver via cytochrome P450-mediated degradation (Table 3). In achlorhydric patients, absorption of these drugs is markedly decreased (Chin et al. 1995). Itraconazole has recently been reformulated in a solution with β-hydroxy-cyclodextrin. This allows intravenous administration and also improves oral absorption of the drug by >60 percent. Further, the pH-associated absorption is no longer an effect, and the drug is best absorbed when taken in a fasting state (Reynes et al. 1997; Vandewoude et al. 1997; Barone et al. 1998). Itraconazole solution is as effective as fluconazole for treatment of esophagitis and thrush, and topical effect may allow treatment of mucosal candidiasis even when patients are taking rifampin. Coadministration of drugs such as rifampin (rifampicin), rifabutin, or carbamazepine, which induce hepatic drug-metabolizing enzymes, causes itraconazole or ketoconazole serum concentrations to fall, and may be associated with treatment failure (Tucker et al. 1992; Como and Dismukes 1994). Alternatively, itraconazole and ketoconazole may cause elevations of serum concentrations of other drugs by competing for the same site that is degraded by the same hepatic enzymes. Such

Table 5 *Adverse reactions to azole compounds*

Intolerance	Ketoconazole	Itraconazole	Fluconazole	Voriconazole	Posaconazole
Nausea, vomiting	++++	+	+	+	+
Rash	±	±	+	++	±
Hepatic	+++	+	+	++	+
Endocrine					
Decreased testosterone	+++	0	0	0	±
Decreased cortisol	++	±	0	0	0
Miscellaneous					
Hypokalemia	0	+	0	0	0
Hypertension	0	+	0	0	0
Edema	0	+	0	0	0
Headache	+	+	+	+	+
Altered mentation	+	+	+	0	0
Dizziness	+	+	+	0	+
Visual disturbances	0	0	0	+++	0

Table 6 *Important drug interactions with azole compounds*

Interaction	Ketoconazole	Itraconazole	Fluconazole	Voriconazole
Increased azole clearance				
Rifampin	++++	++++	++	++++
Rifabutin		+++	+	++++
Phenytoin	+++	+++	0	++
Isoniazid	+++	0	0	
Increased levels of other drug				
Phenytoin	++	++	+	++
Carbamazepine	++	++	+	+++
Warfarin	++	++	+	++
Ciclosporin	+++	+++	+	+++
Terfenadine	+++	++	+	+++
Astemizole	++	++	?	+++
Sulfonylureas	+	+	+	+
Digoxin	+	+		0
Increased level of azole				
Clarithromycin			+	0

drugs include ciclosporin, digoxin, coumadin (warfarin), terfenadine, and astemizole, and may include oral sulfonylureas (Kramer et al. 1990; Crane and Shih 1993; Pohjola et al. 1993; Sachs et al. 1993; Como and Dismukes 1994) (Table 6).

Itraconazole is in general more potent than ketoconazole or fluconazole, has a broader spectrum, and may be used to treat infections with *Aspergillus* spp. (Table 4) (Denning et al. 1989). Itraconazole is much better tolerated than ketoconazole. Ketoconazole binds more avidly to mammalian cells than itraconazole, and causes hepatotoxicity in up to 5 percent of patients (Sugar et al. 1987), whereas hepatotoxicity is rare with itraconazole. Ketoconazole also suppresses synthesis of testosterone and, at high concentrations, cortisol (Table 5) (Pont et al. 1982, 1984). Itraconazole in high doses causes edema, hypokalemia, and hypertension (Sharkey et al. 1991). All three azole compounds, when given at high doses for a prolonged time, may cause alopecia.

The doses of all three azole drugs are 200–400 mg/day, although higher doses of fluconazole appear well tolerated and may be more effective. The half-life of ketoconazole is less than 12 hours, and only at doses above 400 mg/day is there an accumulation from one day to the next. As a result of slow clearance, with more than a 24-hour half-life, there is accumulation over 2 weeks for itraconazole and fluconazole to reach a steady state (Hardin et al. 1988; Como and Dismukes 1994). Some recommend using 300 mg twice daily for 3 days as a 'loading dose'(for itraconazole, and an initial dose twice that of a daily dose for fluconazole)(Wheat et al. 1995). As yet, there is no clear correlation between clinical response and serum concentration, but serum concentration of itraconazole should be measured several days after starting treatment, especially if the patient is seriously ill; this indicates that the drug has been absorbed.

In neutropenic patients fluconazole has been used at 400 mg/day for empirical therapy or prophylaxis of fungal infections. Inherently resistant species that are not *Candida albicans*, such as *Candida glabrata* and *Candida krusei*, may emerge in that setting (Goodman et al. 1992; Rex et al. 1995b). In patients infected with the human immunodeficiency virus (HIV) with severe immune deficiency, fluconazole is the mainstay of treatment for thrush. However, long courses and low-dose exposure to fluconazole are associated with emergence of resistance to *C. albicans* (Heinic et al. 1993; Redding et al. 1994; Rex et al. 1995b). The antifungal spectrum of the antifungal azoles is broadest for itraconazole, which is effective against *Aspergillus* spp., *P. marneffei*, and many *Candida* spp. resistant to fluconazole (Denning et al. 1992; Supparatpinyo et al. 1992, 1993; Barchiesi et al. 1994a). In non-HIV-infected patients with candidemia, there has been a change from *C. albicans* to non-*albicans Candida* spp., such as *C. glabrata* and *Candida tropicalis*. These are more resistant to fluconazole. However, against fluconazole-resistant *Candida* spp. isolates, the potency of itraconazole is somewhat less than against fluconazole-susceptible isolates (Barchiesi et al. 1994a).

At present there are no indications that azoles have cumulative toxicity. Fluconazole has been used at 400–800 mg/day for treatment of fungal meningitis for years, and for shorter times up to 2 000 mg/day have been used with no major adverse sequelae (Berry et al. 1992; Galgiani et al. 1993). As fluconazole is excreted largely unchanged by the kidneys, it accumulates in renal failure. Other antifungal azoles are degraded by hepatic cytochrome P450 enzymes and have a number of important drug interactions (Table 6). Maximal chronic dosing of itraconazole tolerance is probably in the range of 600–800 mg/day, and is mediated by gastrointestinal intolerance and a curious phenomenon of hypotension,

edema, and hypokalemia (Sharkey et al. 1991). No endocrine or renal cause for this phenomenon has been identified. Ketoconazole toxicity is mediated by gastrointestinal intolerance, cortisol suppression, and testosterone suppression at levels of 1 200 mg/day or more (Pont et al. 1984).

Voriconazole is the first of the second generation triazole antifungals, which includes posaconazole and ravuconazole (Johnson and Kauffman 2003). These agents are potent, broad-spectrum agents which were developed specifically to target molds, especially *Aspergillus*, and yeasts other than *C. albicans*, that may exhibit resistance to the earlier triazoles, fluconazole, and itraconazole (Sheehan et al. 1999).

Voriconazole is active in both oral and parenteral formulations and has been extensively evaluated in clinical trials for a wide range of fungal pathogens. In vitro, voriconazole demonstrates fungicidal activity against many strains of *Aspergillus*, including *Aspergillus fumigatus* (Espinel-Ingroff et al. 2001). It also demonstrates activity against *Aspergillus terreus*, which may be resistant to amphotericin B, although MICs to voriconazole may be increased (Walsh et al. 2003). The broad spectrum of activity extends to other molds such as *Fusarium* and *Scedosporium*, and includes agents of pheohyphyomycosis, such as *Bipolaris*, *Scedosporium*, *Alterneria*, and others, but not the zygomycetes (Pfaller et al. 2002). Notably, excellent in vitro and animal model data support its potent activity against *C. krusei* as well as *C. glabrata*, although susceptibility may be less for the latter, particularly when the strains are fluconazole resistant (Pfaller et al. 2003a). Except for *C. krusei*, generally a linear relationship exists between fluconazole and voriconazole, with MICs being approximately 100-fold lower for voriconazole, although breakpoints have yet to be established.

Clinical studies have documented superior efficacy and better tolerance of voriconazole as compared to standard amphotericin B for the treatment of invasive aspergillosis so that voriconazole should be considered for primary therapy for most patients with invasive aspergillosis (Denning et al. 2002; Herbrecht et al. 2002). Improved outcomes were also demonstrated in patients with extensive infection, including central nervous system infection (Troke et al. 2003). For rare and refractory molds, such as *Fusarium* and *Scedosporium*, results have also been favorable with successful outcomes reported in 30–40 percent of patients failing other antifungal therapies (Walsh et al. 2002a; Perfect et al. 2003). Clinical data are less extensive for serious *Candida* infection, although good efficacy and safety was demonstrated in patients with *Candida* esophagitis (Ally et al. 2001). Additional clinical data for candidemia are in progress. In an open labeled trial comparing voriconazole to liposomal amphotericin B for fever in patients with neutropenia, voriconazole was less successful at achieving overall success, but importantly was more effective at reducing the clinically relevant endpoint of breakthrough fungal infections, particularly *Aspergillus* (Walsh et al. 2002b).

The safety of voriconazole has been favorable in clinical trials of patients with serious fungal infections (Herbrecht et al. 2002; Walsh et al. 2002a). The most common serious adverse event has been a transient and reversible visual toxicity that has not been associated with pathology and is rarely dose limiting (Johnson and Kauffman 2003). This effect occurs after oral or intravenous administration and likely correlates with higher serum levels and occurs in approximately 30 percent of patients, generally 30 minutes after administration, and lasts for a mean of 30 minutes. Other less common events include liver toxicity in 10–15 percent, skin rash, nausea, and vomiting.

Voriconazole achieves CSF levels of approximately 50 percent that of serum but little (<5 percent) active drug is excreted in the urine. A loading dose of 6 mg/kg every 12 hours for two doses is recommended for initial therapy intravenously, followed by 4 mg/kg twice daily for invasive aspergillosis and molds for which the drug is indicated in the USA. Oral therapy is recommended at 200 mg twice daily. The drug is metabolized through the cytochrome P450 enzyme system so that drug interactions and variability in patient metabolism can occur (Groll et al. 2003). Metabolism occurs most extensively through CYP2C19, which demonstrates genetic variability, so that some patients such as non-Indian Asians may have higher drug levels. CYP3A4 and other isoenzymes generally contribute less to its metabolism. Drug interactions are common including those with immunosuppressive agents. Dose reductions of ciclosporin by half and tacrolimus by one-third are advised along with close follow-up of the immunosuppressive drug level and for clinical toxicity. Sirolimus is contraindicated because of the dramatic increase in those levels that can occur. Importantly, the immunosuppressive drug dosage will need to be increased after voriconazole is discontinued.

A major issue is whether the azoles and amphotericin B are antagonistic when used together. There is some suggestion, in animal models, that this may be the case in aspergillosis and candidiasis (Polak et al. 1982; Schaffner and Frick 1985). Sugar argues that fluconazole is water soluble, can pass readily into the fungal cell, and may act at a site distant from the cell membrane site of amphotericin B (Sugar 1995). Thus there is no antagonism, and there may even be an additive effect of fluconazole and amphotericin B. In a large study comparing fluconazole alone versus fluconazole with amphotericin B for treatment of candidemia, no clinical antagonism was observed, and there was a trend actually favoring the combination (Rex et al. 2003). Conversely, the lipid-soluble triazoles may concentrate in the cell membrane and act at a site or in a way that antagonizes polyenes, though clinical data are sparse.

Echinocandins

A unique mechanism of action sets the echinocandins apart from other antifungals. Echinocandins are semisynthetic cyclic glycopeptides that inhibit fungal 1,3-β-glucan synthase, which is necessary for the production of 1,3-β-glucan, a major component in many fungal cell walls (Hector 1993; Kurtz et al. 1994a; Abruzzo et al. 1995; Bartizal et al. 1995). Along with chitin and mannans and mannoproteins, glucans make up the major components of the fungal cell wall. The cell wall is responsible for maintaining integrity of the cell shape, for ion and small molecule exchange between the cell and its milieu, and for presentation of surface molecules. 1,3-β-Glucan synthase is an attractive target because it is not present in mammalian cells. This class of drugs appears to have no nephrotoxicity and minimal hepatotoxicity. However, oral absorption is poor and parenteral administration is required. In vitro studies and animal model studies have confirmed great potency in candidiasis and aspergillosis (Abruzzo et al. 1995). *Cryptococcus neoformans*, which contains mostly 1,6-β-glucans in its cell walls, is resistant.

A stop-and-start phase of initial development ultimately led to the first derivative, cilofungin (Lilly). Cilofungin failed in phase I clinical trials because it required a vehicle for aqueous solubilization which turned out to be nephrotoxic. Also limiting cilofungin was the perceived narrow antifungal spectrum, which was largely limited to *Candida*. This class of drugs was at one time close to being discarded because of narrow spectrum and very poor oral absorption. However, persistent efforts on the part of several pharmaceutical companies have led to the licensing of caspofungin (Merck), and late phase II trials with two other contenders, micafungin (Fujisawa) and anidulafungin (Vicuron). The rapid development of echinocandins and their introduction into clinical practice has been nothing short of spectacular. Indeed, the advent of echinocandins is one major reason why Ostrosky-Zeichner et al. were recently able to sound the death knell for amphotericin B desoxycholate (Ostrosky-Zeichner et al. 2003).

The action of echinocandins is very rapid, occurring in minutes, and is noncompetitive (Uzun et al. 1997; Karlowsky et al. 2001). Echinocandins bind to the fungal cell sites where cell wall synthesis is most active. In yeast cells, such as *Candida* spp., echinocandins bind to the cell in many places, and diffusely affect cell wall synthesis. As a result, the fungal cell collapses, resembling an erythrocyte, and may die. Against *Candida*, echinocandins have a very prolonged post-antifungal effect, and are considered fungicidal in vitro (Ernst et al. 2000). In contrast to yeasts, the activity of echinocandins in filamentous fungi is concentrated at the hyphal tip and the budding sites (Kurtz and Rex 2001; Bowman et al. 2002). Supravital stains have shown that echinocandins are more or less fungistatic against *Aspergillus* (Kurtz et al. 1994b). Part of the fungal mycelium is destroyed, but not all of it.

The antifungal spectrum of echinocandins appears to be similar for caspofungin, anidulafungin, and micafungin. These agents are broadly effective in vitro and in animal models against *Candida* spp., including fluconazole-susceptible or resistant isolates (Nelson et al. 1996; Graybill et al. 1997; Zhanel et al. 1997; Petraitiene et al. 1999; Abruzzo et al. 2000). It does not appear at present that the higher MIC values reported for caspofungin against *Candida parapsilosis* are reflected in clinical failures (Mora-Duarte et al. 2002). There is more varying activity against molds, where a new test, the minimal effective concentration (MEC), appears more useful (Oakley et al. 1998). Most *Aspergillus* spp. and some other molds, such as certain species of phaeohyphomycetes, appear to be susceptible, while zygomycetes and *Trichosporon beigelii* are resistant (Espinel-Ingroff 1998; Del Poeta et al. 1996). *Coccidioides immitis* is susceptible, while there are conflicting reports of *H. capsulatum* (Graybill et al. 1998a; Kohler et al. 2000; Gonzalez et al. 2001). The in vitro antifungal spectrum has been most widely explored for caspofungin, and we are assuming that the other echinocandins are similar. The limited data in vitro and in animals have acted to delay clinical exploration of these compounds in mycoses other than *Candida* and *Aspergillus* infections. There is a recent report of a patient with breakthrough *T. beigelii* infection while receiving caspofungin, which suggests resistance (Goodman et al. 2002). The full range of activity of echinocandins has yet to be elucidated. Because the target is unique to fungi (with little toxicity to mammalian cells), the echinocandins are thought to be excellent candidates to replace the more toxic conventional amphotericin B – at least against a few major pathogens.

All three echinocandins now in clinical use or trials share characteristics of water solubility and poor oral absorption. Thus all drugs must be administered intravenously. Although there have been concerns of histamine-like reactions following intravenous infusion, these have been rare. There are some differences among the drugs, but it is not yet clear whether these will impact on clinical use (Table 7). The slower clearance of anidulafungin, as well as its clearance entirely by chemical, not metabolic, degradation, may allow for less frequent dosing. The lower protein binding of anidulafungin (86 versus >95 percent for caspofungin and micafungin) may also influence the ultimate determination of in vitro breakpoints.

It is thought that the peak serum concentration/MIC ratio determines effect of echinocandins. This is similar to amphotericin B, but in contrast to fluconazole, where the AUC/MIC ratio appears more important (Andes 2003). With very few serious adverse reactions to the echinocandins (though data are much more extensive with caspofungin), the maximum tolerated dose and the

Table 7 *Characteristics of echinocandins*

Property	Caspofungin 40 mg dose	Micafungin 50 mg dose	Anidulafungin 50 mg dose
Protein binding (%)	97	>99	86
AUC (μg/h/ml)	56		102
C_{max} (μg/ml)	6		4.1
Terminal $t_{1/2}$ (h)	11	15	28
Clearance	Hepatic, metabolism	Hepatic, metabolism	Chemical degradation
Drug interaction	? Ciclosporin	None	None
Urine excretion (%)	<5	<5	<5

Azuma et al. (1998); Stone et al. (2002); Dowell et al. (2003).

therapeutic ratio have not been determined (Arathoon et al. 2002; Mora-Duarte et al. 2002; Letscher-Bru and Herbrecht 2003). Caspofungin and anidulafungin have adverse effects similar to fluconazole (Villanueva et al. 2002; Krause et al. 2003).

The major clinical uses of echinocandins are being defined. All three drugs are effective against *Candida* mucosal infection, with responses similar to fluconazole. A dose of 50 mg/day appears to be effective, given for up to 2 weeks for patients with esophagitis (Kohno et al. 2001; Arathoon et al. 2002; Krause et al. 2003). The only large study with systemic candidiasis was caspofungin versus amphotericin B, and found that both drugs were similarly effective, between 65 and 75 percent, but that caspofungin had far fewer adverse reactions (Mora-Duarte et al. 2002). Caspofungin is also effective in mucosal infection caused by fluconazole-resistant *Candida* (Kartsonis et al. 2003). For fluconazole-resistant *Candida* in the bloodstream, caspofungin is now considered the drug of choice (Walsh 2002).

Studies of aspergillosis are generally unpublished and open label, salvage trials (Maertens et al. 2000). Caspofungin is as effective as a variety of other alternatives. Data with micafungin and anidulafungin are spotty. Micafungin has been compared with fluconazole for antifungal prophylaxis. Results are equivalent to fluconazole, but unfortunately not quite superior to fluconazole against *Aspergillus*. Animal data suggest that there may be additive effects of micafungin and a triazole against *Aspergillus* (Petraitis et al. 2003). Clinically, there are only a few collections of anecdotes, and these are not helpful.

Overall, clinical experience has been restricted to some comparative trials with mucosal candidiasis, one large study with disseminated *Candida*, and unpublished data on prophylaxis and aspergillosis. Beyond candidemia, the roles of the echinocandins have yet to be defined.

FUTURE ANTIFUNGAL DRUGS

New triazoles are under consideration, including posaconazole (Schering, NJ, USA). These agents are much more lipid soluble than fluconazole, have an increased fungal spectrum against fluconazole-resistant *C. albicans*, other *Candida* spp., and molds (Atkinson et al. 1994; Hoffman et al. 2000), and are excreted primarily by hepatic degradation. Hepatic metabolism places them at potentially similar risks of drug reaction as ketoconazole and fluconazole. Although these drugs have increased activity against fluconazole-resistant *Candida* spp., the MIC is higher than for fluconazole-sensitive isolates.

Other antifungal targets are being explored. These include elongation factor 2, chitin synthases, topoisomerase, translation, myristoylation, and steps specific to certain fungi, such as phenyloxidase and capsule synthesis for *Cryptococcus neoformans*. Agents aimed at these new targets have not yet reached clinical trials. Finally, there are numerous in vivo animal studies examining combined antifungal drugs and immune modulators, such as phagocyte colony-stimulating factors, interferons, and interleukins (Rex et al. 1991; Uchida et al. 1992; Ausiello et al. 1993; Brummer and Stevens 1994; Brummer et al. 1994; Joly et al. 1994; Nassar et al. 1994; Roilides et al. 1994, 1995; Sewell et al. 1994; Vitt et al. 1994). The potential clinical value of these cytokine/antifungal combinations has not been clearly defined.

ANTIFUNGAL SUSCEPTIBILITY TESTING

The combined effects of increasing numbers of fungal infections, growing numbers of patients at risk, the increasing rate of fungal resistance, and the expanded antifungal armamentarium have led to an increased recognition of the need for standardized laboratory testing for antifungal drug susceptibility. However, numerous problems have been encountered in the quest for a simple, reproducible, and inexpensive testing system applicable to all clinically important fungi. These include multiple variables affecting outcome, such as pH, inoculum size, medium, and time and temperature of incubation, which produce varying results; unclear in vitro to in vivo correlation; and differences in testing yeasts versus filamentous fungi (Rex et al. 1993). Several excellent reviews have been written on the subject, which detail the difficulties in developing in vitro

antifungal susceptibility testing and insights into controversial issues (Stevens 1984; Drutz 1987; Galgiani 1987, 1993; Espinel-Ingroff and Shadomy 1989; Pfaller and Rinaldi 1993; Rex et al. 1993, 2001; Sheehan et al. 1993; Pfaller et al. 1997; Pfaller and Yu 2001; Rex and Pfaller 2002).

Early studies

Only recently has antifungal drug susceptibility testing become an important issue. Initially, amphotericin B was the only agent available for systemic infection, so testing did not appear to have practical clinical significance. Even after several antifungal agents became available, only a few laboratories were performing antifungal testing, usually less than 10–20 isolates per year (Rex et al. 1993). Studies in the 1980s revealed that between-laboratory susceptibility results varied by as much as 512-fold, and standard methods were not available for susceptibility testing (Rex et al. 1993). It was noted, however, that the rank order of isolates was relatively consistent among the different laboratories, suggesting a definite need for standardized methods.

Many techniques have been used to determine antifungal susceptibility, including measurement of germ tubes, uptake of metabolites, flow cytometry, agar-based methods, and broth dilution (Rex et al. 1993). Most are considered impractical for large-scale use. Agar techniques are appealing because of their ease and low cost, but suffer from a wide variation of results, including factors such as inoculum size, temperature, time of incubation, and ability of poorly soluble drugs, such as ketoconazole and itraconazole, to diffuse through agar (Rex et al. 1993). Broth dilution methods are widely used, and have been standardized by the National Committee for Clinical Laboratory Standards (NCCLS) for testing of *Candida*, *Cryptococcus* (document M-27A), and a variety of clinically important filamentous fungi (document M-38A) (National Committee for Clinical Laboratory Standards 1997, 2002).

Factors affecting antifungal susceptibility testing

END-POINT DETERMINATION

This aspect of antifungal testing is an important source of variability among laboratories in the testing of azoles and occasionally 5-flucytosine. These agents are generally fungistatic and usually tend not to have distinct end points on MIC testing, and thereby introduce subjective interpretations of susceptibility results. By contrast, amphotericin B, which is fungicidal, generally produces distinct end points. Azoles in particular may also produce 'trailing end points,' with growth evident at all concentrations tested (Rex et al. 1993). A solution to quantify end-point determination has been proposed by the NCCLS method, which uses an 80 percent decrease in turbidity as compared with control to establish an end point. A study by Pfaller et al. (1995) demonstrated that agitation of a microbroth well or spectrophotometric measurement led to a more definitive end point. More recently, studies have suggested that a 50 percent end point may lead to fewer trailing end points and may better predict clinical outcome (St Germain 2001; Arthington-Skaggs et al. 2002). Isolates that are susceptible at 24 hours but resistant at 48 hours due to trailing appear to revert to susceptible at lower media pH, and actually behave as if they are susceptible in vivo (Revankar et al. 1998b; Rex et al. 1998; Marr et al. 1999). Further clinical studies are needed with these isolates.

For testing echinocandins, a unique end point termed the MEC has been proposed that addresses the difficulties in assessing response to the drug, particularly for filamentous fungi (Oakley et al. 1998).

INOCULUM SIZE

It has been shown that increasing the size of inoculum can drastically increase MICs for most drugs tested, and interlaboratory consistency is improved by using smaller inocula (Rex et al. 1993). The NCCLS method for yeasts suggests an inoculum of $0.5–2.5\times10^3$ c.f.u./ml, prepared by matching turbidity at 530 nm of a 0.5 McFarland standard (National Committee for Clinical Laboratory Standards 1997). For filamentous fungi, NCCLS recommends an inoculum of $0.4–5\times10^4$ c.f.u./ml, by spectrophotometric measurement, with varying optical densities depending on the species tested (National Committee for Clinical Laboratory Standards 2002).

Hemocytometer count is also a reasonable technique for preparing an inoculum, although with results that are less consistent in between-laboratory tests (Rex et al. 1993). In comparing a wide variety of filamentous fungi, this method may be more reliable, though it is unclear if this would significantly affect the results of susceptibility testing compared with the standardized method (Aberkane et al. 2002).

INCUBATION

Both time and temperature can have significant effects on MIC determinations. MICs generally tend to increase with longer incubation periods (Rex et al. 1993). Temperature variations are not as predictable, but are felt to be least apparent at 35°C (Rex et al. 1993). The NCCLS methods recommend incubation at 35°C for 48 hours for *Candida* spp., 72 hours for *Cryptococcus* spp., and 24–72 hours for molds, depending on the species (National Committee for Laboratory Standards 1997, 2002).

MEDIA

Different media can give rise to widely varying results of antifungal testing, due to interactions between the drugs and the testing media. This can be especially seen with relatively undefined media. The pH can also be important, with a lower pH of the test medium associated with higher MICs of most antifungal agents. For this reason, the use of a defined, synthetic medium, RPMI-1640 (Sigma Chemical Company, St Louis, MO, USA), pH 7.0 with 3-[*N*-morpholino]-propanesulfonic acid (MOPS) (Sigma) is advocated by NCCLS for both yeasts and filamentous fungi (National Committee for Clinical Laboratory Standards 1997, 2002).

Testing of filamentous fungi

Until recently, no standardized method for antifungal susceptibility testing of filamentous fungi was available. This area of susceptibility testing presents its own, unique problems, namely how to quantify inhibition of growth for organisms that change morphological forms. For example, *Aspergillus* spp. have conidia, which are small sphere-like forms that are easily quantifiable, but germinate to hyphal forms that are not. Many studies have used germination of hyphal growth as an indicator in susceptibility testing. Denning et al. (1992) found a wide range of in vitro susceptibility results while reviewing studies by different investigators, although within their own laboratory they achieved consistent results. In response to these issues, the NCCLS developed M-38A, a standardized broth dilution method for susceptibility testing of filamentous fungi (National Committee for Clinical Laboratory Standards 2002). The primary differences with the yeast testing method are variable incubation times for different species and a no growth or 100 percent inhibition end point for amphotericin B, itraconazole, and the newer azoles. Other methods have also been studied, including E-test, automated blood culture systems, and colorimetric methods, which in general have had good correlation with the broth method, particularly the E-test (Espinel-Ingroff 2001; Meletiadis et al. 2001; Rath et al. 2002; Pfaller et al. 2003b). In addition, the concept of minimum fungicidal concentration (MFC) may be important for in vitro–in vivo correlation, and a proposed definition is the lowest dilution with <3 c.f.u. on subculture, corresponding to 99–99.5 percent killing based on the NCCLS standardized inoculum (Espinel-Ingroff et al. 2002).

METHODS OF SUSCEPTIBILITY TESTING

Macrobroth dilution

This is the currently approved reference method for antifungal susceptibility testing of yeasts and filamentous fungi. Numerous studies have shown good correlation between laboratories using this methodology: amphotericin B, 90 percent; ketoconazole, 75 percent, 5-flucytosine, 85 percent; fluconazole, 88 percent (Rex et al. 1993). However, the NCCLS broth technique is insensitive for detecting resistance to amphotericin B (Rex et al. 1993). The current recommendation uses a macrobroth technique with 1 ml of medium, but microbroth dilution using 0.2 ml in a 96-well microtiter plate may be more practical for rapid, widespread use, and appears to produce similar results (Barchiesi et al. 1994b; Pfaller et al. 1994a; Sewell et al. 1994; Hacek et al. 1995). It is considered an acceptable alternative to the standardized method by the NCCLS.

Colorimetric

ALAMAR BLUE

Alamar Blue (Alamar Biosciences, Inc., Sacramento, CA) is a novel colorimetric indicator that changes color from blue to red when reduced in the presence of microbial growth (Pfaller and Barry 1994). It has shown excellent correlation with bacterial reference methods and several studies have shown promise in antifungal susceptibility testing (Pfaller and Barry 1994; Pfaller et al. 1994a, b; Tiballi et al. 1995; To et al. 1995). In general, studies have confirmed that results agree closely with the NCCLS macrobroth method at 24 hours and less so at 48 hours (Pfaller and Barry 1994; Pfaller et al. 1994b; To et al. 1995). Some combinations, such as fluconazole with *C. albicans*, *C. glabrata*, and *C. tropicalis*, had relatively poor correlations (11–65 percent) in one study (To et al. 1995). A commercial system, Sensititre YeastOne (TREK Diagnostics Systems Inc., Westlake, OH) which uses Alamar Blue, was recently approved by the FDA. Studies using this system have shown good correlation with the NCCLS method, though some drug-species combinations have less correlation than others (Espinel-Ingroff et al. 1999; Morace et al. 2002).

TETRAZOLIUM SALTS

A tetrazolium salt, 2,3-bis-2-methoxy-4-nitro-5-sulphophenyl-5-[phenylaminocarbonyl]-2*H*-tetrazo-lium hydroxide (XTT), has been used in eukaryotic cell drug assays (Tellier et al. 1992). It is a member of a class of compounds that produce a colored formazan crystal when reduced, which can be detected using a spectrophotometer (Tellier et al. 1992). A study by Tellier et al. (1992) used this agent to test various yeast species, and distinct MICs were observed, although this method was not directly compared to the NCCLS reference method.

Another tetrazolium derivative, 3-4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), was used to determine antifungal susceptibility in a study by Jahn et al. (1995). It demonstrated reasonable

correlation to NCCLS macrobroth methods, but few isolates were tested.

E-test

This method of susceptibility testing has been used successfully with bacteria. It involves use of a plastic strip impregnated with a defined gradient of the antimicrobial agent to be tested. The strip is then placed on agar seeded with the test organism. The graded zone of inhibition, if present, is read from a scale printed on the strip, corresponding to the particular concentration of drug. Multiple studies have shown that good correlation is achieved with the NCCLS method with amphotericin B, azoles, and caspofungin, though occasional problems were noted. A study by Colombo et al. (1995) comparing the E-test and NCCLS macrobroth methods showed reasonable MIC correlations, with 71 percent for ketoconazole, 80 percent for fluconazole, and 84 percent for itraconazole. However, the E-test results were usually one- to twofold less than for the NCCLS method, with *C. tropicalis* showing the least concordance (Colombo et al. 1995). Another study by Sewell et al. (1994) demonstrated similar findings, with poor correlations against NCCLS methodology for *C. tropicalis* and *C. glabrata* at 24 and 48 hours. *C. albicans* testing was in good agreement with NCCLS at 24 hours, but not at 48 hours (Sewell et al. 1994).

Other methods

Alternative testing methods that may be easier to perform and interpret than the NCCLS method continue to be evaluated and show promise. These include use of flow cytometry (results within 6 hours), automated blood culture systems, disk diffusion, and sterol quantitation (Ramani and Chaturvedi 2000; Arthington-Skaggs et al. 2002; Rath et al. 2002; Matar et al. 2003). In addition, testing of *Candida* spp. in the presence of biofilms has been studied to better evaluate the effect of antifungal drugs in this setting (Ramage et al. 2001). Agar dilution screening for *Candida* and *Cryptococcus* has also shown promising results compared with the NCCLS macrobroth method, and may be useful to rapidly detect clinical resistant isolates (Patterson et al. 1996; Kirkpatrick et al. 1998; Nelson and Cartwright 2003).

IN VIVO–IN VITRO CORRELATION

Many studies have shown a disparity between in vitro MICs and in vivo efficacy for antifungal agents tested. However, standardized methods have improved correlation in more recent studies. There are interpretive breakpoint guidelines for antifungal susceptibility testing of fluconazole and itraconazole to *Candida* spp. (Rex et al. 1997). These were primarily based on studies of the correlation between in vitro testing and clinical response in oropharyngeal candidiasis in HIV-infected patients (Laguna et al. 1997; Revankar et al. 1998a). Correlations between increased MICs of *C. albicans* to fluconazole and poor clinical response in oropharyngeal candidiasis have been shown (Redding et al. 1994; Revankar et al. 1998a). Studies of systemic fungal infections have also shown correlation with in vitro testing (Aller et al. 2000; Kovacicova et al. 2000; Lee et al. 2000). There are also clinical responses when in vitro testing reveals the organism to be resistant. In a study by Rex et al. (1995a), bloodstream isolates from *Candida* spp. revealed an inverse correlation between in vitro resistance to fluconazole at MIC of 32 μg/ml or more and response to therapy at 400 mg/day, suggesting that in this setting other factors may be more important in determining clinical outcome than in vitro susceptibility testing. However, the number of patients with resistant organisms was very small. Increased MICs to amphotericin B to over 0.8 μg/ml were associated with higher mortality in oncology patients in a study by Powderly et al. (1988). In a study by Denning et al., in vitro testing conditions for itraconazole were adjusted to produce good correlation to clinical response with six isolates of *A. fumigatus*, of which two were resistant (Denning et al. 1997). When it occurs, a lack of correlation between in vitro susceptibility and in vivo efficacy may result from host factors or differences in drug pharmacokinetics that alter its availability at the site of infection. In summary, clinical correlation of in vitro activity of antifungal agents is a complex issue that will require ongoing study. There is emerging evidence, however, that in vitro testing will be useful to the clinician.

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Principles of antifungal therapy

PETER G. PAPPAS

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Any discussion of antifungal therapy must begin with a clear understanding of the terminology that describes the strategies for prevention and treatment of invasive fungal infections. Moreover, a basic understanding of the problems inherent to clinical trial design and the challenges in interpretation of clinical trial data involving antifungals is essential for the appropriate clinical application of these therapies. This chapter provides a brief overview of these two important areas.

PROPHYLACTIC AND PREEMPTIVE THERAPY

The terms prophylaxis, targeted prophylaxis, and preemptive are often used interchangeably in conjunction with antifungal therapy. While there is some overlap in meaning, these terms represent different approaches to therapy. Prophylaxis generally refers to the broad use of antifungal therapy in a heterogeneous group of patients who are at variable risk of developing superficial or invasive fungal infection. By definition, prophylactic antifungal therapy is administered to patients who are considered to be at risk for fungal infection, but who have no symptoms of infection at the time that the antifungal agent is initiated. Prophylaxis may be systemic (e.g. oral or parenteral fluconazole) or topical (e.g. oral nystatin). Virtually any population can be given prophylactic antifungal therapy, but it is typically administered to high-risk patient populations including selected medical and surgical intensive care unit (ICU) patients (Eggimann et al. 1999; Garbino et al. 2002), allogeneic hematopoietic stem cell transplant recipients (Winston et al. 2003), and selected solid organ transplant recipients (Winston et al. 1999). The chief aim of antifungal prophylaxis is the prevention of colonization and disease due to target organisms.

Targeted prophylaxis implies a measure of selectivity, that is, utilizing specific risk factors or patient characteristics in determining the population to whom prophylaxis is administered. It is assumed that the target population is uniformly at higher risk of developing fungal infection than patients with similar underlying conditions or severity of illness, and this high-risk population is selected on the basis of certain known risk factors. For example, targeted prophylaxis using fluconazole in the ICU is often administered to patients who have multiple risk factors for invasive candidiasis such as the presence of a central venous catheter, systemic antibacterial therapy, endotracheal intubation, renal failure, and recent abdominal surgery (Garbino et al. 2002). Targeted antifungal prophylaxis with voriconazole is often given to prevent invasive aspergillosis and other mold infections among allogeneic stem cell transplant recipients at risk for graft-versus-host disease.

Preemptive antifungal therapy is administered to persons who are not only at risk but also have markers of early infection, for example, colonization with a fungal pathogen. The group that has been best described in conjunction with preemptive therapy is the liver transplant population who meet criteria for very high risk of invasive fungal infection including prolonged intraoperative time, preexisting renal failure, early colonization with *Candida* spp., retransplant for early graft failure, and choledochojejunostomy anastomosis (Collins et al. 1994).

The optimal use of any form of antifungal prophylaxis or preemptive therapy must utilize a practical and validated assessment of risk. Studies to better define specific risk factors for invasive fungal infection in a variety of clinical settings are essential, recognizing that risk varies substantially between study populations, and that each patient group is characterized by unique conditions that place them at a greater or lesser risk for fungal colonization and disease.

EMPIRIC THERAPY

Empiric antifungal therapy refers to the use of these agents among patients with findings and/or symptoms of suspected invasive fungal disease. The use of empiric antifungal therapy has been studied most extensively in persistently febrile and neutropenic patients (Walsh et al. 1999, 2002; Wingard et al. 2000). The primary goal of empiric antifungal therapy in this setting is to prevent breakthrough fungal infections and to treat baseline infections due to molds and other important fungi in this uniquely susceptible host. However, all neutropenic patients are not alike, and the risk of invasive fungal infection is directly related to the underlying condition(s) and the duration and depth of neutropenia. For instance, induction therapy for acute myelogenous leukemia is associated with a very high rate of invasive fungal infection and associated high mortality, especially due to invasive mold infections. By contrast, myeloablative therapy for most solid tumors usually leads to shorter periods of neutropenia and far less risk of invasive fungal infection. The appropriate use of empiric antifungal therapy in the setting of persistent fever and neutropenia requires that the patient have persistent fever despite a reasonable course (usually 96 hours or more) of broad-spectrum antibacterial therapy, and that there is no other obvious explanation for the clinical deterioration of the patient.

The administration of empiric antifungal therapy to persistently febrile non-neutropenic ICU patients with presumed invasive candidiasis has become common practice in developed countries throughout the world, yet studies that address the appropriateness of this practice and the best approach to therapy are lacking. Several epidemiologic studies have identified multiple risk factors associated with nosocomial candidiasis (Blumberg et al. 2001; Trick et al. 2002; Pappas et al. 2003). Despite the recognition of these risk factors, this group of patients has proven particularly difficult to enroll into clinical trials, and thus, the use of empiric antifungal therapy in the ICU patients with unexplained fever, leukocytosis, hypotension, or other evidence of clinical deterioration is the most common reason to prescribe parenteral antifungal therapy. It remains one of the most poorly understood and understudied areas in the discipline of antimicrobial therapy.

SPECIFIC THERAPY

Specific therapy refers to therapy directed at a specific pathogen which has been detected by culture, histopathology, and/or serology, or in the absence of laboratory evidence, then clinical/radiographic evidence strongly suggestive of invasive fungal disease. These include such findings as hepatosplenic bull's eye lesions suggesting chronic disseminated candidiasis, the halo or air crescent sign on chest CT suggesting invasive aspergillosis, and disseminated cutaneous lesions consistent with invasive candidiasis. Specific therapy is often based on presumptive evidence of infection, but it is nonetheless targeted towards the organism(s) most likely responsible for the clinical picture.

In practice and clinical trials, few infections have received as much attention as candidemia in the non-neutropenic host because of its prevalence and the general familiarity of most clinicians with this entity. Our current practices towards the treatment of candidemia generally reflect the results of large and well-done published comparative clinical trials in this arena (Pappas et al. 2004). Similarly, the treatment of central nervous system (CNS) cryptococcosis has become more uniform because of data generated from therapeutic trials among patients with and without the acquired immunodeficiency syndrome (AIDS) who have CNS cryptococcosis (Saag et al. 2000). By contrast, very few large clinical trials have been done among patients with less common invasive mycoses such as aspergillosis, histoplasmosis, blastomycosis, coccidioidomycosis, and sporotrichosis. Thus, the 'gold standard' of treatment for these mycoses is often based upon data generated from smaller comparative studies without significant power to discern differences in therapeutic outcome.

CLINICAL TRIAL DESIGN FOR ANTIFUNGAL COMPOUNDS

The design and implementation of the clinical trials involving antimycotic agents has been a challenge to clinicians since the availability of amphotericin B in 1958. The key problems encountered in conducting clinical trials of antifungal agents are not dissimilar to those facing the development of other antimicrobial agents; however, these issues have been recently examined in the context of systemic antifungals because of the availability of a growing number of these agents, and the growing difficulties encountered in studying them in a randomized and controlled fashion. The key challenges to the clinical investigator include:

- slow patient accrual because of restrictive eligibility criteria for uncommon diseases
- establishment of meaningful clinical end points
- the absence of validated surrogate markers of success or failure (e.g. serological studies)

- the unwillingness of many investigators to perform double-blinded clinical trials
- the need for large numbers of study centers to facilitate completion of these trials in a reasonable timeframe (Rex et al. 2001).

Patient accrual is an obstacle faced by most clinical trials of anti-infective agents, thus it is not unique to trials of antifungal agents. With the exception of candidemia and possibly CNS cryptococcosis, invasive mycoses are relatively uncommon. Yet, despite the low frequency of most invasive mycoses, the relative lack of effective agents has served as an incentive to place patients into some of these trials for less common mycoses such as histoplasmosis and blastomycosis. By contrast, randomized trials for the treatment of candidemia in non-neutropenic patients have enrolled slowly despite the relatively high incidence of candidemia in the nosocomial setting, reflecting, in part, fairly restrictive entry criteria and an inability to establish a diagnosis consistently within a short timeframe. For example, a recently published candidemia treatment trial enrolled only about 10 percent of all patients with candidemia at study sites (Pappas et al. 2003; Rex et al. 2003). Most of these potentially eligible patients were excluded due to prior antifungal therapy, abnormal laboratory values, age, comorbid conditions, and other considerations. Patient accrual into studies for therapy of invasive aspergillosis has been even more challenging. In a recent open-label trial comparing voriconazole to amphotericin B and other licensed antifungal therapy for the primary treatment of invasive aspergillosis, it required more than 5 years and almost 100 centers to enroll almost 300 eligible patients, again reflecting the effect of restrictive inclusion criteria and the difficulty in establishing a firm diagnosis of an uncommon disorder on patient accrual (Herbrecht et al. 2002).

The reluctance of many investigators to perform nonrandomized trials and the unwillingness of regulatory agencies to accept data from uncontrolled trials for purposes of licensing are other important considerations in the conduct of these trials. Double-blinded studies are difficult to perform, particularly when one of the study arms includes an amphotericin B (AmB) formulation. However, several recent studies have been successfully completed that have successfully blinded both conventional AmB and lipid formulations of AmB (Mora-Duarte et al. 2002; Rex et al. 2003). A randomized and double-blind study comparing AmB in combination with high-dose fluconazole (800 mg/day) to high-dose fluconazole alone for non-neutropenic patients with candidemia, demonstrated that a double-blinded placebo-controlled trial involving AmB can be performed successfully and without undue patient risk (Rex et al. 2003). Similarly, a randomized and double-blinded study comparing AmB to liposomal AmB for empiric antifungal therapy in persistently febrile and neutropenic patients demonstrated the ability to perform a double-blind trial with these agents (Walsh et al. 1999).

Study end points have generated considerable controversy in antifungal trials, relying almost exclusively on mycological (culture), clinical and/or radiographic parameters. Among trials comparing agents for treatment of candidemia, rendering the blood culture negative in conjunction with resolution of clinical signs and symptoms has become a standard end point for a successful outcome. However, clinical end points, including resolution of fever, hypotension, and/or other clinical signs of candidemia, are often difficult to discern due to concomitant illness and other comorbidities that are common among these patients. Thus, in most candidemia studies, an element of investigator bias is introduced in what at first glance might appear to be a fairly 'hard' end point. Similarly, end points for studies of CNS cryptococcosis have utilized a composite end point of cerebrospinal fluid culture negativity at important time intervals such as 2 and 10 weeks, together with the somewhat subjective measurement of clinical improvement based on neurological status and other clinical parameters subject to investigator bias. In both of these examples, the ability to use a surrogate marker of efficacy, such as a serological assay, might have helped improve objectivity and lessen reliance on subjective clinical measurements.

Controversy has surrounded the use of the five-component composite end point utilized in several of the comparative trials of empiric antifungal therapy in persistently febrile and neutropenic patients (Walsh et al. 1999, 2002). The five components include:

1. survival at 7 days after study drug completion
2. no discontinuation of study drug due to toxicity or lack of efficacy
3. prevention of emergence of incident fungal infection
4. successful treatment of baseline fungal infection
5. resolution of fever during antifungal therapy.

To be considered a success in these studies, all five end points must be met. Not surprisingly, these studies have had rates of success that vary between 26 and 50 percent when these criteria are applied stringently. Again, the use of a validated surrogate marker might obviate the need for such complex end points, simplifying outcome assessment by removing such vague and questionable end points as 'fever resolution,' which is prone to much subjective interpretation by investigators.

By necessity, most large antifungal trials require large numbers of participating sites to provide adequate patient accrual, adding another degree of complexity in terms of quality assurance, consistent practice patterns, and variations in interpretation of the protocol.

Despite these important considerations, much progress has been made in the design and implementation of clinical trials of antifungal agents in the last two decades. It will be important in the design and

implementation of future studies to consider each of the challenges, and to strive for more efficient patient selection and more reproducible and timely results.

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Resistance to antifungal agents

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INTRODUCTION

The field of antifungal therapy is now enjoying its most dynamic era. The increased interest in antifungal agents is attributable to several factors. First, the fungi, which were once known only as the common causes of superficial infections, now have a well-established role in opportunistic invasive infections. The increase in the incidence of invasive mycoses originates from the increase in the number of patients who are at high risk of developing these infections. The use of more intensive chemotherapeutic regimens in patients with cancer, the appearance of acquired immunodeficiency syndrome (AIDS), and the increasing practice of organ and bone marrow transplantation all resulted in emergence of a particular host population who are highly immunocompromised and predisposed to develop invasive mycoses. The most significant aspects of these infections are that they are difficult to treat and associated with high rates of mortality (Anaissie 1992; McNeil et al. 2001; Kullberg and Lashof 2002). Second, more efficacious and less toxic antifungal agents appeared in clinical practice. Novel compounds with distinctive modes of antifungal activity and novel derivatives of the currently available agents were developed. As a consequence, we now have a diverse spectrum of antifungal agents in contrast to the limited number of such compounds available in the past (Table 1) (Arikan and Rex 2001, 2002). Third, due both to the increase in invasive infections and the related more common use of antifungal therapy, resistance to antifungal agents has become an issue, with both intrinsic (primary) and acquired (secondary) resistance being realized (Alexander and Perfect 1997; Bille 2000; Canuto and Rodero 2002; Sanglard and Odds 2002).

This chapter reviews:

- the definitions of resistance
- methods for in vitro antifungal susceptibility testing
- the correlation between in vitro resistance and clinical response in fungal infections
- the modes of action and mechanisms of resistance for currently available and novel antifungal drugs.

DEFINITIONS OF RESISTANCE

Meaning of resistance

Resistance, by definition, denotes the absence of activity of a drug against a particular microorganism. However, due to the multifactorial feature of the issue, the term is imprecise when used alone. It is important to distinguish in vitro, molecular, and clinical resistance (White et al. 1998). In vitro resistance is a minimum inhibitory concentration (MIC) value (μg/ml) of a drug against a particular strain that is above a predefined limit for that drug. Molecular resistance, on the other hand, is demonstration of resistance at genetic level and may include detection of point mutations, gene conversions, gene amplifications leading to overexpression, or mitotic recombinations. The best way to explore molecular mechanisms of resistance is to use matched sets of susceptible and resistant clinical isolates from the same strain. Clinical resistance is the ultimate issue and means

Table 1 *Classification of antifungal agents according to their chemical structure and modes of action*

Chemical structure	Antifungal agent(s)	Mode of action
Allylamines	Terbinafine, naftifine, butenafine	Inhibition of ergosterol synthesis
Azoles	Imidazoles: ketoconazole, clotrimazole, econazole, miconazole, oxiconazole, sulconazole Trizoles: fluconazole, itraconazole, voriconazole, posaconazole, ravuconazole	Inhibition of ergosterol synthesis
Echinocandins	Caspofungin, anidulafungin, micafungin	Inhibition of glucan synthesis
Fluorinated pyrimidine	Flucytosine	Nucleic acid inhibitor
Morpholine	Amorolfine	Inhibition of ergosterol synthesis
Peptide-nucleoside	Nikkomycin	Inhibition of chitin synthesis
Polyenes	Amphotericin B Lipid amphotericin B formulations Amphotericin B lipid complex (ABLC) Amphotericin B colloidal dispersion (ABCD) Liposomal amphotericin B (L-AMB) Nystatin Liposomal nystatin Pimaricin Filipin	Disruption of fungal cytoplasmic membrane
Tetrahydrofuran derivatives	Sordarins Azasordarins	Inhibition of fungal protein synthesis
Other	Griseofulvin	Antimitotic activity (spindle disruption)

clinical failure in response to treatment with an antifungal agent. The most striking point is that detection of resistance in vitro does not always mean clinical resistance. This follows from the multifactorial nature of the clinical response. Not only the in vitro activity of the drug but also its pharmacokinetic and pharmacodynamic properties, the immune status of the host, virulence factors of the infecting fungus, and existence of indwelling catheters determine the ultimate clinical outcome (Ghannoum and Rice 1999; Rex and Pfaller 2002). The major topic of interest is, of course, clinical resistance and we use in vitro susceptibility tests and molecular methods in an effort to predict clinical response.

Origin of resistance

The sources for the emergence of resistant fungal strains are diverse. Primary (intrinsic) resistance denotes natural resistance of a particular genus or species. The most typical example for this type of resistance is the resistance of *Candida krusei* (Collin et al. 1999) and *Aspergillus* to fluconazole (Edlind et al. 2001). Secondary (acquired) resistance, on the other hand, is acquisition of resistance or selection of a resistant strain following the pressure of antifungal therapy. Secondary resistance may emerge in various ways. One of the commonly observed types of secondary resistance is replacement with a more resistant species. Fluconazole prophylaxis or therapy, for instance, may lead to selection of a fluconazole-resistant *Candida glabrata* (Collin et al. 1999) or *C. krusei* strain (Bignardi et al. 1991; Casanovas et al. 1992). Antifungal therapy may also result in replacement with a more resistant strain. For example, following prolonged fluconazole therapy, a previously fluconazole-susceptible strain of *Candida albicans* may become fluconazole-resistant (Revankar et al. 1996; Marr et al. 1997; Franz et al. 1999). Temporary (epigenetic) resistance is another type of secondary resistance. This mostly originates from transient gene expressions under the pressure of antifungal therapy. Such a strain quickly reverts to a susceptible phenotype once the drug pressure is eliminated (Calvet et al. 1997). Finally, a single strain may include a resistant subpopulation which may again be selected under the pressure of antifungal therapy. This entity originates from genomic instability within a single strain and is known as 'population bottleneck' (Schoofs et al. 1997; White et al. 1998). Beyond these forms of resistance, alterations in cell type, such as yeast–hypha transitions or phenotypic switches (Ha and White 1999), as well as existence of putative virulence factors (Fekete-Forgacs et al. 2000) may be associated with resistance or decreased susceptibility. However, related data are limited.

DETECTION OF RESISTANCE IN VITRO

In vitro antifungal susceptibility testing emerged as an area of very active research since the 1990s. The increase in the invasive fungal infections and availability of an increased number of antifungal agents than ever before led to a great demand for standardization of antifungal susceptibility testing methods. The first standardized procedure for *Candida* and *Cryptococcus neoformans* was proposed in 1992 as NCCLS M27-P, which was followed by M27-T in 1995, M27-A in 1997, and finally M27-A2 in 2002 (National Committee for

Clinical Laboratory Standards 2002b). This remarkable progress led to the development of a reference method for some filamentous fungi as well; the NCCLS M38-P document was published in 1998 for testing *Aspergillus*, *Fusarium*, *Rhizopus*, *Pseudallescheria*, and mycelial phase of *Sporothrix schenckii*. The M38-P document has also been revised recently and the M38-A document is now also available (National Committee for Clinical Laboratory Standards 2002a). These testing procedures are based on a microdilution format procedure and have proven valuable in many settings. Some uncertainties still remain and clinical data are required for clarification of some in vitro findings. Some modifications of the reference microdilution method and assays other than microdilution (e.g. disk diffusion, Etest, and flow cytometry) have also been investigated and appear useful (Ramani and Chaturvedi 2000; Cuenca-Estrella and Rodriguez-Tudela 2001; Rex et al. 2001; Vandenbossche et al. 2002). Nevertheless, the reference microdilution method remains as the standard procedure and in vitro–in vivo correlation studies are mostly based on microdilution data.

The challenges for the reference method in terms of detection of resistance

Despite its being a great step in the field of mycology, the reference susceptibility testing method still provides only limited data. For yeasts, the MIC breakpoints to be used in detection of resistant isolates have as yet been defined only for fluconazole, itraconazole, and flucytosine and only against *Candida* (Table 2). For molds, an MIC breakpoint was proposed for detection of itraconazole resistance in *Aspergillus* (>8 μg/ml) (Espinel-Ingroff et al. 2001). The situation is also quite limited for amphotericin B against both yeasts and molds. The method frequently fails to detect amphotericin B-resistant isolates. Although some modifications of the reference method [antibiotic medium 3 instead of RPMI 1640 medium (Rex et al. 1995; Lozano-Chiu et al. 1998a, b); Etest instead of microdilution (Wanger et al. 1995)] have led to easier discrimination of the resistant isolates in some investigators' hands, work by others has failed to support the finding, particularly about the use of antibiotic medium 3 (Nguyen et al. 1998) and significant variability is the rule rather than the exception (Lozano-Chiu et al. 1997). Detection of unusually high MICs, however, may suggest putative resistance for any fungus–drug combination with undefined MIC breakpoints.

Resistant fungi

The overall data obtained by the reference method suggest that resistance is more likely for particular fungal genera or species against specific antifungal agents. This finding emphasizes the significance of identification of a fungal isolate to species level. The fungi which have been observed to display significant rates of antifungal resistance are summarized in Table 3. For some of these, resistance is primary and includes all strains classified in that genus (e.g. fluconazole resistance in *C. krusei*). For others, resistance is detected at variable rates for the strains of a species. Apart from those noted in Table 3, strain-to-strain variations in antifungal susceptibility profiles are always possible even for a genus known to be susceptible to an antifungal agent. The typical example for this entity is *C. albicans*, a species well known to be susceptible to azoles. As noted previously, fluconazole resistance may develop in a previously susceptible *C. albicans* strain following antifungal prophylaxis or therapy with fluconazole (Marr et al. 1997).

Another topic that merits discussion under this heading is cross-resistance. Cross-resistance is now more significant than ever before due to the availability of novel derivatives of the azoles. Does, for instance, a fluconazole-resistant *C. glabrata* isolate remain resistant to itraconazole or voriconazole as well? Available data suggest that cross-resistance between old and novel azoles is likely but not universal (Cartledge et al. 1997; Nguyen and Yu 1998; Goldman et al. 2000; Muller et al. 2000; Mosquera and Denning 2002). The same is true for polyenes. Some amphotericin B-resistant *Candida* isolates remain susceptible to nystatin and liposomal nystatin (Arikan et al. 2002a). These data indicate that

Table 2 *MIC interpretive guidelines for susceptibility testing of* Candida *(National Committee for Clinical Laboratory Standards 2002b)*

Antifungal agent	Susceptible (S)	Susceptible-dose dependent (S-DD)	Intermediate (I)	Resistant (R)
Fluconazole[a]	⩽8	16-32[b]	–	⩾64
Itraconazole	⩽0.125	0.25-0.5[c]	–	⩾1
Flucytosine	⩽4	–	8-16[d]	⩾32

a) *C. krusei* is intrinsically resistant to fluconazole and these guidelines should not be used for assessment of fluconazole susceptibility of *C. krusei*.
b) Fluconazole doses of at least 400 mg/day may be required in adults with normal renal function to achieve adequate blood levels for treating infections due to these isolates.
c) Plasma itraconazole concentrations of >0.5 μg/ml may be required for optimal response in treating infections due to these isolates.
d) Isolates with uncertain susceptibility profile.

Table 3 *Fungal genera/species for which a significant incidence of resistance to antifungal agents has been observed*[a]

Fungus	Antifungal agent	Type of resistance	Reference(s)
Yeasts			
Candida glabrata	Azoles	Primary/secondary	Yamaguchi et al. (1989); Collin et al. (1999); Arikan et al. (2002b); Pfaller et al. (2002)
Candida guilliermondii	Amphotericin B	Primary	Dick et al. (1985); Collin et al. (1999); Krcmery and Barnes (2002)
Candida krusei	Azoles	Primary	Rex et al. (1998); Collin et al. (1999); Pfaller et al. (2001)
	Amphotericin B	Primary	Rex et al. (1998); Arikan et al. (2002a); Krcmery and Barnes (2002)
Candida lusitaniae	Amphotericin B	Primary/secondary	Pietrucha-Dilanchian et al. (2001); Arikan et al. (2002a); Ellis (2002); McClenny et al. (2002)
Cryptococcus neoformans	Flucytosine	Primary/secondary	Block et al. (1973); Brandt et al. (2001)
Trichosporon spp.	Amphotericin B	Primary	Walsh et al. (1990); Arikan and Hascelik (2002); Toriumi et al. (2002)
Molds			
Aspergillus spp.	Fluconazole	Primary	Abrahamsen et al. (1992); Edlind et al. (2001)
Aspergillus terreus	Amphotericin B	Primary	Lass-Florl et al. (1998); Sutton et al. (1999)
Fusarium solani	Amphotericin B	Primary	Anaissie et al. (1992); Arikan et al. (1999); Ellis (2002)
Scedosporium apiospermum	Amphotericin B	Primary	Walsh et al. (1995); Espinel-Ingroff (1998); Ellis (2002)
Scedosporium prolificans	Amphotericin B	Primary	Berenguer et al. (1997); Maertens et al. (2000); Ellis (2002)

a) This table covers the fungi that are relatively commonly reported to be resistant to antifungal agents. Fungi for which resistance was detected less frequently and/or in a few strains were not included. It should be remembered that strain-to-strain variations in susceptibility are possible.

class-based in vitro susceptibility tests are not adequate for definitive determination of the susceptibility profile of an isolate.

CORRELATION BETWEEN IN VITRO AND CLINICAL RESISTANCE

A recent meta-analysis of the available in vitro–in vivo correlation studies found that the percentage of clinical success was 91 percent for infections due to isolates susceptible to the corresponding antifungal agent while it was 48 percent for those where the isolate were resistant (Rex and Pfaller 2002). These success rates were shown to be similar to the success rates for therapy of susceptible/resistant bacterial isolates, and the general concept of a 90/60 rule was proposed in which susceptible isolates responded about 90 percent of the time and resistant isolates responded about 60 percent of the time. This analysis makes it clear that in vitro susceptibility tests can predict clinical resistance. However, several other factors, such as the immune status of the host and severity of the infection in the interim, influence the clinical outcome and should be considered.

Noteworthy, in vitro–in vivo correlation studies for amphotericin B are not included in this meta-analysis. Available data are limited and remain ambiguous in this respect. Although a correlation between in vitro and clinical resistance could be demonstrated in some *Candida* (Nguyen et al. 1998) and *Aspergillus* (Lass-Florl et al. 1998) infections by using a proposed MIC breakpoint value, further data to support these findings, unfortunately, are not available.

MECHANISMS OF ANTIFUNGAL RESISTANCE

The cellular and molecular bases of resistance are tightly related to the modes of antifungal activity. The modes of action of currently available and novel antifungal agents are summarized in Table 1.

Polyenes

This group of antifungal agents includes amphotericin B, nystatin, lipid formulations of amphotericin B and nystatin, pimaricin, and filipin (Table 1). The most significant agent in terms of clinical implications of resistance is amphotericin B and most of our knowledge about polyene resistance is provided by clinical and investigational data for amphotericin B.

Amphotericin B has been in clinical use since the 1960s and remains a mainstay of systemic antifungal therapy. The use of amphotericin B was further augmented by the development of its lipid formulations [amphotericin B lipid complex (ABLC), amphotericin B

colloidal dispersion (ABCD), and liposomal amphotericin B (L-AMB)] in the 1990s. These lipid formulations are at least as efficacious as the parent drug and definitely less nephrotoxic than the conventional formulation, amphotericin B deoxycholate. Their acquisition cost, however, is remarkably high (Arikan and Rex 2001, 2002).

As noted previously, the major challenge with in vitro amphotericin B susceptibility testing is the frequent inability of the reference method in detection of resistant isolates. Unusually high MICs of amphotericin B have been remarkable for isolates of *Candida guilliermondii*, *C. krusei*, *Candida lusitaniae*, *Trichosporon* spp., *Aspergillus terreus*, *Scedosporium apiospermum*, *Scedosporium prolificans*, and *Fusarium solani* (Table 3). Although some differences have been detected between the in vitro activity of amphotericin B deoxycholate and its lipid formulations (Pahls and Schaffner 1994; Carrillo-Munoz et al. 1999; Oakley et al. 1999), based on the fact that the compound responsible for the antifungal activity is identical in all formulations, most authorities suggest that testing the in vitro activity of lipid formulations is not required. Importantly, in vivo resistance to amphotericin B is not common and, when present, usually originates from the impaired immune status of the host. Development of secondary resistance to amphotericin B is also not a major problem, despite its being in clinical use for more than 40 years. This is probably due to the fungicidal nature of the agent.

The antifungal activity of amphotericin B and other polyenes is via a specific interaction between the agent and ergosterol, the bulk sterol found in fungal cell membrane. Ergosterol is an essential element to maintain fluidity and integrity of the membrane and proper functioning of the membrane-bound enzymes. Amphotericin B intercalates into the membrane and generates channels and pores. Through these pores, several cellular components, particularly potassium and magnesium ions, leak and destroy the proton gradient within the membrane, leading to death of the fungal cell (Vanden Bossche et al. 1994a; Ellis 2002). In addition, it has previously been reported that amphotericin B may be cytotoxic to *Candida* via its prooxidative activity (Sokol-Anderson et al. 1986). However, and contrary to this finding, more recent data suggest that amphotericin B may act as an antioxidant (Osaka et al. 1997) and this question remains unresolved. The affinity of amphotericin B is high for ergosterol and less for 3-hydroxy or 3-oxo sterols. This low affinity for sterols such as fecosterol and episterol may be significant in emergence of resistance to amphotericin B (Ghannoum and Rice 1999).

Any quantitative or qualitative change in sterol content of the fungal cell influencing the amount or the availability of ergosterol may result in resistance to amphotericin B (Table 4). The three mechanisms of quantitative ergosterol changes are decrease in amount of ergosterol due to inhibition of its synthesis (Merz and Sanford 1979; Dick et al. 1980; Peyron et al. 2002), replacement of ergosterol with episterol, fecosterol or other sterols (Woods et al. 1974; Kelly et al. 1994; Peyron et al. 2002), and alterations in the ratio of sterols to phospholipids. A fourth mechanism that may lead to resistance is a qualitative change in ergosterol; its reorientation or masking in the membrane. This in turn results in sterically or thermodynamically less favored binding with amphotericin B (Ghannoum and Rice 1999). A fifth mechanism related to amphotericin B resistance is related to the growth phase of the fungal cell. As expected, during exponential growth, breakdown and resynthesis of the cell wall occurs at a high rate, providing enhanced access of amphotericin B to the cell membrane. By contrast, at stationary growth phase, breakdown and synthesis of the cell wall occurs at a much lower rate, leading to relative resistance to amphotericin B (Gale et al. 1975).

Table 4 *Possible mechanisms of amphotericin B resistance*

Mechanism
Reduced ergosterol content of the fungal cytoplasmic membrane (defective *ERG2* or *ERG3* genes)
Alterations in sterol content of the membrane (predominancy of sterols with reduced affinity, such as fecosterol and episterol)
Alterations in sterol to phospholipid ratio in the membrane
Reorientation or masking of ergosterol in the membrane
Stationary growth phase of the fungus
Increase in the levels of alkali-soluble and -insoluble glucans in the cell wall
Production of melanin
Previous exposure to azoles

There are two newly proposed mechanisms associated with amphotericin B resistance. The first is related to the cell wall. Alterations in the cell wall components of mycelia, specifically an increase in the levels of alkali-soluble and insoluble glucans, have been postulated to lead to amphotericin B resistance in *Aspergillus flavus* (Seo et al. 1999). The second is related to the production of melanin. Killing assays demonstrated that melanized isolates of *Cryptococcus neoformans* and *Histoplasma capsulatum* were less susceptible to amphotericin B compared to the nonmelanized strains. Of interest, the reference M27-A method failed to detect this melanin-associated difference in amphotericin B susceptibility (van Duin et al. 2002).

Previous exposure to an azole compound may also be responsible for amphotericin B resistance. The key issue here is the depletion of ergosterol, the target molecule of amphotericin B, by the previous antifungal activity of the azole. This kind of interaction of an azole with amphotericin B is one of the major examples for antag-

onism of the action of one antifungal by that of another (Schaffner and Böhler 1993; Martin et al. 1994; Sugar and Liu 1998). However, the related clinical implications are complex and the details are beyond the scope of this chapter.

MOLECULAR ASPECTS OF AMPHOTERICIN B RESISTANCE

Only a few studies have focused on the genetic basis of amphotericin B resistance in clinical isolates. Several enzymes take part in the chain of reactions starting from squalene and ending up with synthesis of ergosterol. In this ergosterol synthesis pathway, the mutations in two genes encoding two enzymes have been found to be associated with amphotericin B resistance. C-8 sterol isomerase catalyzes the production of episterol from fecosterol and is regulated by the *ERG2* gene. C-5 sterol desaturase, on the other hand, is one of the enzymes in conversion of episterol to ergosterol and is encoded by the *ERG3* gene. Clinical strains of amphotericin B-resistant *C. albicans* with reduced ergosterol content and defective *ERG2* or *ERG3* genes have been reported (Broughton et al. 1991; Haynes et al. 1996; Nolte et al. 1997). The stability of these mutations remains unknown. Sterol analysis of an amphotericin B-resistant mutant isolate of *C. neoformans* has also suggested an alteration in the *ERG2* gene (Kelly et al. 1994).

Azoles

This group of antifungal agents includes imidazoles and triazoles (Table 1). Fluconazole is the most widely used azole compound. Its favorable clinical efficacy and safety, as well its availability in both oral and parenteral formulations, led to widespread use of fluconazole for both treatment and prophylaxis. With such broad usage, it is perhaps not surprising that fluconazole-resistant isolates were soon noted and this led to efforts to develop novel compounds with favorable activity against fluconazole-resistant organisms.

The antifungal activity of the azoles is via inhibition of lanosterol (14-alpha) demethylase, an enzyme which functions in synthesis of ergosterol from lanosterol (Sanati et al. 1997). There are data suggesting that the azoles primarily target the heme protein which cocatalyzes cytochrome P450-dependent 14-alpha-demethylation of lanosterol (Hitchcock et al. 1990). The inhibition of demethylation results in depletion of ergosterol and accumulation of 14-alpha-methylated sterols such as lanosterol, 4,14-dimethylzymosterol, and 24-methylene-dihydrolanosterol. Finally, the integrity of the membrane and its functions, such as nutrient transport and chitin synthesis, are lost, leading to inhibition of fungal growth. The mechanism of antifungal activity may vary depending on the genus of the fungus. For example, fluconazole and itraconazole affect the reduction of obtusifolione to obtusifoliol as well as inhibiting 14-alpha-demethylase in *C. neoformans* and both mechanisms result in accumulation of methylated sterol precursors (Vanden Bossche et al. 1993; Ghannoum et al. 1994).

The clinical effect of inhibition of sterol biosynthesis on human enzymes merits discussion. This adverse effect is mostly observed for ketoconazole. Azoles block the synthesis of mammalian cholesterol as well at the stage of 14-alpha demethylation. However, it has been demonstrated by Hitchcock et al. (1995) that the 50 percent inhibitory concentration of voriconazole needed to inhibit 14-alpha-demethylase of rat liver cholesterol is much higher than that required to inhibit fungal demethylase (7.4 μM versus 0.03 μM).

As shown in Table 5, azole resistance may originate from qualitative or quantitative changes in the target enzyme lanosterol demethylase, reduction in the intercellular concentration of the drug or a combination of these mechanisms (Lupetti et al. 2002; Sanglard and Odds 2002). Qualitative alterations in the enzyme frequently originate from the mutations in the encoding gene and result in reduced binding affinity of the enzyme for azoles. Interestingly, species-dependent intrinsic qualitative differences may also be observed. Compared to that of *C. albicans*, lanosterol demethylase of *C. krusei* displays reduced susceptibility to inhibition by fluconazole (Orozco et al. 1998). Quantitative modifications, on the other hand, are due to increased copy number of the enzyme and lead to increased ergosterol synthesis due to its overexpression. Azole resistance may sometimes develop due to its unfavorably low intercellular concentration. This may be observed for two reasons. First, the azole may penetrate poorly across the membrane due to the alterations in sterol and/or phospholipid composition of the membrane and related reduced permeability. Second, and more importantly, the azole can readily get access to its target initially but is then pumped out by overexpressed efflux systems (Ghannoum and Rice 1999). There are no reports suggesting the relevance of modification of azoles as a mechanism of resistance.

Table 5 *Possible mechanisms of azole resistance*

Mechanism
Alteration in lanosterol demethylase
Overexpression of lanosterol demethylase
Reduction in intercellular concentration of the azole
1 Overexpression of energy-dependent efflux systems
i ATP-binding cassette (ABC) superfamily proteins
ii Major facilitator superfamily proteins
2 Changes in sterol and/or phospholipid composition of fungal cell membrane leading to decreased permeability

MOLECULAR ASPECTS OF AZOLE RESISTANCE

Lanosterol demethylase is encoded by the *ERG11* gene and the active site pocket of the enzyme is located on top of the heme cofactor. This gene has previously been referred to as *ERG16* and *CYP51A1* in *C. albicans*. Various genetic alterations in *ERG11*, including point mutations, overexpression, gene amplification leading to overexpression, gene conversion, and mitotic recombination have been observed in *C. albicans* (White et al. 1998) and appear related to azole resistance. In addition, alterations in other *ERG* genes encoding enzymes that function in ergosterol biosynthetic pathway may also be associated with azole resistance. Among these are *ERG3* (Kelly et al. 1997) and *ERG5* (White et al. 1998).

Point mutations in *ERG11*

Point mutations in *ERG11* associated with azole resistance have been reported. While some of them have been identified in clinical isolates, others were developed in laboratory strains. Seven different point mutations associated with azole resistance have so far been defined. However, since matched sets of susceptible and resistant isolates from the same strain have not been tested, it is hard to know whether these mutations are the actual causes of resistance (Loeffler et al. 1997). Moreover, recent data suggest that the resistance mechanisms identified in matched sets of susceptible and resistant isolates frequently remain insufficient to explain resistance in unmatched clinical isolates (White et al. 2002). A point mutation leading to replacement of arginine with lysine at amino acid 467 (abbreviated as *R467K*) has been found to be associated with azole resistance in a clinical strain of *C. albicans* when matched strains were tested (White 1997b). Another point mutation was constructed in the laboratory in a *C. albicans* strain, leading to the replacement of threonine with alanine at position 315 (*T315A*) and this mutation has also been studied in *Saccharomyces cerevisiae*. Importantly, the *T315A* mutation was located in the active site pocket of the enzyme and led to reduction in azole binding and fluconazole resistance (Lamb et al. 1997). *D116E* and *E266D* are the other two of the most common point mutations in *ERG11* of *C. albicans*. However, these mutations are frequently observed and are not necessarily associated with resistance (White et al. 2002). Geber et al. (1995) demonstrated that the *ERG11* deletion mutant of *C. glabrata* was resistant to fluconazole (at 100 μg/ml) and itraconazole (at 16 μg/ml) as well as amphotericin B (at 2 μg/ml).

Overexpression of *ERG11*

Although overexpression of the *ERG11* gene has been shown in clinical isolates of *C. albicans* resistant to azoles, the contribution of this phenomenon alone to development of resistance remains unknown (Sanglard et al. 1995; Albertson et al. 1996; White 1997a; Sanglard et al. 1998). Other alterations, such as the *R467K* mutation and the overexpression of the genes encoding efflux pump systems, have always been present in the resistant isolates with an overexpressed *ERG11* gene. Importantly, recent data from a collection of unmatched clinical isolates of *C. albicans* suggest that overexpression of *ERG11* is not correlated with azole resistance (White et al. 2002). Overexpression of *ERG11* may originate from an increase in the number of gene copies and *ERG11* gene amplification is another postulated mechanism for azole resistance. In a clinical isolate of resistant *C. glabrata*, *ERG11* gene amplification, increase in *ERG11* mRNA levels, and an associated increase in lanosterol demethylase have been detected. The gene amplification observed in this isolate was linked to a chromosome duplication. In the same isolate, increased drug efflux and change in *ERG7* activity was also present (Vanden Bossche et al. 1992, 1994a, b; Marichal et al. 1997). Overexpression of *ERG11* in *C. albicans*, however, could not be linked to *ERG11* amplification by some authorities (Sanglard et al. 1995; White 1997a).

Efflux pumps

There are two types of efflux pumps that contribute to azole resistance; ATP binding cassette (ABC) transporters and major facilitator superfamily (MFS) proteins. The ABC system uses ATP as the energy source while the MFS proteins use proton motive force of the membrane (the H^+ gradient across the membrane). Efflux pump systems have been found to be associated with fluconazole and itraconazole resistance in *C. krusei* (Marichal et al. 1995; Venkateswarlu et al. 1996) and *C. glabrata* (Sanglard et al. 1999), itraconazole resistance in *Aspergillus fumigatus* (Denning et al. 1997), and secondary fluconazole resistance in *C. albicans* (Marr et al. 1997).

The ABC transporters are encoded by several genes. The major ABC genes proven to be correlated with azole resistance are the *CDR* genes. *CDR* genes appear to function in efflux of various azoles. Overexpression of *CDR1* (Sanglard et al. 1995; White 1997a; Maebashi et al. 2001; White et al. 2002) and *CDR2* (Maebashi et al. 2001; White et al. 2002) have been correlated with azole resistance in *C. albicans* strains. Similar to the data demonstrated for *C. albicans*, the *CgCDR1* gene was found to be involved in azole resistance in *C. glabrata* (Sanglard et al. 1999). The *ABC1* gene, on the other hand, appears to be potentially involved in azole resistance of *C. krusei* (Katiyar and Edlind 2001).

The data concerning the genetic regulation of MFS proteins are more limited than those for the ABC system. *MDR1* (*BEN-R*) is the major MFS gene cloned from *C. albicans* and appears to be specific for fluconazole. Current data suggest a correlation between *MDR1*

overexpression and azole resistance in *C. albicans* (Sanglard et al. 1995; Albertson et al. 1996; White 1997a) and *C. dublininensis* (Wirsching et al. 2001). No correlation could be found between overexpression of *FLU1*, which is an efflux pump gene related to *MDR1*, and azole resistance (White et al. 2002).

The overexpression of *CDR* and *MDR1* genes has so far been demonstrated by the increase in their mRNA levels. Increased mRNA levels of CDR were found to be associated with development of rapid, transient fluconazole resistance in *C. albicans* (Marr et al. 1998). The definitive causes of the increase in mRNA levels, however, remain yet unknown (Lyons and White 2000). It may result from gene amplification, increased transcription from endogenous copies of the gene, or increase in half-life of mRNA and its slower degradation (Borst 1991).

Decreased membrane permeability

Changes in sterol and/or phospholipid composition of the fungal cell membrane have been postulated by some investigators to lead to decreased membrane permeability and a reduction in the amount of the drug taken up by the fungal cell. The most significant alteration in sterol composition was found to be the depletion of ergosterol and its replacement with methlyfecosterol and other methylated sterols in *C. albicans* (Kohli et al. 2002). However, other workers reported that no correlation between the sterol composition and azole resistance could be demonstrated in *C. albicans* (Ghannoum and Rice 1999). A recent study by Kohli et al. (2002) demonstrated that changes in status of the membrane lipid phase, asymmetrical distribution of phosphatidylethanolamine and its increased exposition to the outer monolayer could contribute to azole resistance in *C. albicans*.

Cross-resistance in azoles

CDR overexpression and the *R467K* point mutation in *ERG11* appear to be associated with azole cross-resistance. Due to its specificity for fluconazole, *MDR1* overexpression does not lead to cross-resistance to other azoles (Sanglard et al. 1995, 1996). Whether other point mutations and mechanisms result in cross-resistance awaits further analysis.

Current status

Azole resistance is a gradual process and involves several alterations, particularly in strains under continuous antifungal drug pressure (Marr et al. 1997; Franz et al. 1998; Lopez-Ribot et al. 1998). The data obtained by analysis of ergosterol biosynthetic pathway in a particular genus or species may not be relevant to others, including the closely related ones. The contribution of some mechanisms alone remains unknown and available data suggest that further mechanisms may have a significant role in development of resistance. One of the novel mechanisms reported to be associated with fluconazole resistance in *C. albicans* is sequestration of fluconazole in vesicular vacuoles (Maebashi et al. 2002). Importantly, simultaneous contribution of several mechanisms to fluconazole resistance in *C. albicans* strains was verified by Perea et al. (2001). In this study, overexpression of efflux pump genes, amino acid substitutions in lanosterol demethylase due to mutations in *ERG11*genes, and overexpression of *ERG11* were detected in 85, 65, and 35 percent, respectively. Overall, multiple mechanisms of resistance were found to be active in 75 percent of the studied isolates.

Allylamines

This group of antifungal agents (Table 1) yield antifungal activity via inhibition of squalene epoxidase enzyme (encoded by the *ERG1* gene) which converts squalene to 2,3-oxidosqualene in the ergosterol biosynthetic pathway. This in turn leads to accumulation of squalene and a resulting increase in membrane permeability and disruption of cellular organization. Terbinafine is the most commonly used allylamine derivative in clinical practice. While it has proven to be efficacious in cutaneous mycoses, subcutaneous mycoses (including sporotrichosis, chromoblastomycosis, and maduromycosis), refractory and disseminated aspergillosis, infections due to some azole-resistant *Candida* strains, pheohyphomycosis, and cutaneous leishmaniasis (Ghannoum and Elewski 1999; Perez 1999), dermatophytosis is the most common clinical indication for terbinafine use (Gupta and Shear 1997).

Cases of onychomycosis that failed to respond to terbinafine have been seen particularly with *Trichophyton rubrum* infection. However, in vitro susceptibility data suggested that clinical failure in these cases was not due to development of resistance to terbinafine and host-related factors might have contributed (Bradley et al. 1999). More recently, primary resistance to terbinafine was observed in a clinical isolate of *T. rubrum* from a patient with onychomycosis, suggesting that terbinafine-resistant dermatophytes are rare, but do exist (Leidich et al. 2001).

MOLECULAR ASPECTS OF ALLYLAMINE RESISTANCE

Due to the very rare detection of terbinafine-resistant dermatophytes, no data are available on genetic analysis of terbinafine resistance in this particular group of fungi. However, parallel data for other fungal genera suggest that efflux pumps may play a role in terbinafine resistance. Vanden Bossche et al. (1998) reported that overexpression of *MDR1* may be related to resistance to fluconazole and terbinafine while that of *CDR1* may lead to cross-resistance to fluconazole, itraconazole,

ketoconazole, and terbinafine in *S. cerevisiae*. Also, *ERG* upregulation, particularly of *ERG11*, has been observed in vitro following treatment of *C. albicans* cultures with azoles and terbinafine (Henry et al. 2000).

Flucytosine

Flucytosine (5-fluorocytosine) is a fluorinated pyrimidine that exerts antifungal activity via inhibition of nucleic acid and protein synthesis. Flucytosine is taken up into the cell by fungal cytosine permease and then is deaminated to 5-fluorouracil (5-FU) via action of cytosine deaminase. 5-FU is initially converted to 5-fluorodeoxyuridine monophosphate and 5-fluorouridylic acid. The latter reaction is catalyzed by uracil phosphoribosyltransferase (UPRTase). Further phosphorylation results in production of 5-fluorouracil triphosphate (5-FUTP). There are two key metabolites of flucytosine that provide antifungal activity. The first, 5-fluorodeoxyuridine monophosphate, inhibits DNA synthesis via inhibition of thymidylate synthase. The second, 5-FUTP, is incorporated into RNA and disrupts protein synthesis (Polak 1977).

Flucytosine is primarily indicated for treatment of cryptococcal meningitis in combination with amphotericin B and/or fluconazole due to its favorable interaction with amphotericin B in this particular setting (Dismukes et al. 1987; Rodero et al. 2000). Primary resistance to flucytosine has been reported in *C. albicans*, non-*albicans Candida*, *C. neoformans*, and *Aspergillus*, while secondary resistance has been observed in *C. albicans* and *C. neoformans* (Vanden Bossche et al. 1994b, 1998; Barchiesi et al. 2000; Cuenca-Estrella et al. 2001). The key precaution to prevent development of secondary resistance to flucytosine is to avoid flucytosine monotherapy (Vermes et al. 2000). Resistance to flucytosine may develop due to loss of permease activity, a defect in cytosine deaminase activity or decrease in the activity of UPRTase enzyme. The defect in cytosine deaminase usually results in primary resistance, while a decrease in UPRTase activity leads to secondary resistance in *C. albicans* and *C. neoformans* (Whelan 1987; Vanden Bossche et al. 1994b). Loss of permease activity, on the other hand, has been found to be associated with flucytosine resistance in *C. glabrata* and *S. cerevisiae*, but does not seem to be significant in *C. albicans* and *C. neoformans* (Jund and Lacroute 1970; Whelan 1987).

MOLECULAR ASPECTS OF FLUCYTOSINE RESISTANCE

Genetic basis of flucytosine resistance has been explored in several fungi, particularly in *C. albicans* and *C. neoformans*. Two resistance genes, *FCY1* and *FCY2*, have been defined for flucytosine (Alexander and Perfect 1997). Since the fungus is diploid, it has two alleles for each gene and the sequential differences between the two alleles lead to *FCY* homozygotes and heterozygotes. Accordingly, a strain may be homozygous sensitive (*FCY*/*FCY*), homozygous resistant (*fcy*/*fcy*), or heterozygous (*fcy*/*FCY*). The heterozygotes are slightly resistant to flucytosine but they occur at significantly high ratio. Also, they carry preexisting resistance determinants which may lead to clinical treatment failures following development of homozygosity. Resistance in *fcy1*/*fcy1* strains was found to be associated with decreased UPRTase activity, whereas resistance in *fcy2*/*fcy2* strains was associated with decreased cytosine deaminase activity (Whelan 1987).

Echinocandins

Echinocandins (Table 1) are lipopeptides which exert antifungal activity via noncompetitive inhibition of synthesis of 1,3-beta-D-glucan, the essential cell wall homopolysaccharide in several pathogenic fungi. Glucan synthase is a heteromeric enzyme complex composed of two subunits; the large integral membrane protein referred to as FKS1 or FKS2 (encoded by *FKS1* and *FKS2* genes which are highly homologous), and the small subunit more loosely associated with the membrane and referred to as *RHO1*. *FKS1p* constitutes the majority of the activity in vegetatively growing cells while the *FKS2* gene product is required for sporulation. Although some investigators have postulated that inhibition of glucan synthesis by echinocandins is via inhibition of glucan synthase enzyme, definitive evidence to support this theory is lacking. According to these authorities, echinocandins affect the function of the Fksp component from either *FKS* gene (Kurtz and Douglas 1997).

Echinocandins are active against *Candida* and *Aspergillus* spp. Based on their distinct mode of action, activity of echinocandins against azole-resistant strains is one of their most significant features (Cuenca-Estrella et al. 2000). Caspofungin is as yet the only licensed novel echinocandin in the USA and is indicated for salvage therapy in refractory invasive aspergillosis (Groll and Walsh 2001). *C. neoformans* and *Fusarium* spp. are intrinsically resistant to echinocandins. The data obtained by Feldmesser et al. (2000) suggest that the limited action of the echinocandins against *C. neoformans* may originate from their reduced activity against glucan synthase of this particular organism or from yet undefined mechanisms. Interestingly, the production of melanin has recently been demonstrated to lead to reduced susceptibility to caspofungin in *C. neoformans* and *H. capsulatum* (van Duin et al. 2002). Echinocandin-resistant mutants of *S. cerevisiae* and *C. albicans* have been studied. The results obtained by Kurtz et al. (1996) suggested that these mutants may have alterations in glucan synthase activity. The use of caspofungin has been limited to date and reports of secondary resistance are not available.

MOLECULAR ASPECTS OF ECHINOCANDIN RESISTANCE

Although genetic studies in *S. cerevisiae* have shown that mutations in the *FKS1* and *FKS2* genes result in caspofungin resistance, no data have been available about the influence of gene overexpression on caspofungin resistance. *FKS1* mutations conferred high-level resistance to echinocandins. Mutations in another cell wall synthesis gene, *GNS1* (encodes an enzyme that functions in fatty acid elongation), on the other hand, were shown to result in low level resistance (Kurtz and Douglas 1997). In addition and recently, a novel mechanism of echinocandin resistance has been proposed. Osherov et al. (2002) demonstrated that overexpression of Sbe2p (encodes a Golgi protein involved in the transport of cell wall components) under the regulated control of the *GAL1* promoter results in caspofungin resistance in *S. cerevisiae*.

Griseofulvin

Griseofulvin has remained a first-line drug for treatment of dermatophytosis for may years. However, following the emergence of more effective and less toxic alternatives, itaconazole and terbinafine, the clinical use of griseofulvin is now limited (Zaias et al. 1996). Griseofulvin exerts antifungal activity via inhibition of fungal mitosis by disrupting the mitotic spindle through interaction with polymerized microtubules (Gull and Trinci 1973). Cases refractory to griseofulvin therapy have been reported (Lukacs et al. 1994). The clinical success rate in dermatophytic onychomycosis may be as low as 30 percent (Roberts 1994). The lack of a standardized method for susceptibility testing of dermatophytes and the absence of a defined griseofulvin MIC breakpoint limit our ability to estimate the rate of in vitro resistance to griseofulvin. Griseofulvin yielded higher MICs compared to terbinafine and itraconazole when tested against clinical *Trichophyton rubrum* isolates (Korting et al. 1995; Jessup et al. 2000) and was found to be less active than voriconazole against most dermatophytes (Wildfeuer et al. 1998). The in vitro activity of griseofulvin against *Trichophyton mentagrophytes* was less when compared to that against *T. rubrum* (Korting et al. 1995). Laboratory mutants resistant to griseofulvin have been observed following exposure to increasing gradients of the drug, mutagenic treatment or UV radiation (Aytoun et al. 1960; Iwata 1991; Fachin et al. 1996). Data on griseofulvin resistance are thus limited and the molecular basis of resistance mechanisms remains uncertain.

CONCLUSION AND FUTURE DIRECTIONS

Antifungal resistance is a complex, gradual, and multifactorial issue. Areas of significant uncertainty persist and molecular assays to detect antifungal resistance are not simple. Based on the dynamic interaction between the host, the infecting fungus, and the antifungal agent, antifungal resistance is not the only factor influencing clinical outcome. The immune status of the host plays a very important role in this regard. Proper dosing strategies and restricted and well-defined indications for antifungal prophylaxis appear as the future directions to avoid development of secondary resistance.

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Management of superficial infections

JEFF WEEKS AND BONI ELEWSKI

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Successful treatment of superficial fungal infections requires knowledge of the various antifungal agents and familiarity with measures that can be taken to reduce the chance of spread or reinfection. There are multiple topical and systemic antifungal agents available to treat these infections. In order to choose the most effective medication, the clinician needs a basic understanding of the drugs available as well as their mechanisms of action, indications, dosages, interactions, and adverse effects. Many conditions, such as tinea corporis and tinea cruris, usually respond well to topical agents. Others, such as tinea capitis and onychomycosis, generally require systemic therapy. The newer systemic agents including fluconazole, itraconazole, and terbinafine offer greater efficacy, broader spectrums of activity and shortened treatment duration. However, they are not without potential adverse effects and drug interactions and their use requires careful patient selection and monitoring.

This chapter discusses the management of a variety of superficial cutaneous infections including:

- superficial mycoses
- dermatophytoses
- disease caused by nondermatophyte molds
- cutaneous candidiasis.

A summary of the topical and systemic pharmaceutical agents commonly used for these conditions is presented. This includes a discussion of the indications, dosages, mechanisms of action, drug interactions, and adverse effects of selected antimycotics. Also discussed are measures that should be taken to reduce the chance of spread or recurrence of the infection.

ANTIFUNGAL AGENTS

There are multiple topical and systemic antimycotics that are effective in the management of superficial fungal infections. The most widely used today fall into three main classes: the polyenes, azoles, and allylamines/benzylamine. Others that are not included in these classes include griseofulvin, ciclopirox, tolnaftate, and selenium sulfide.

Polyenes

The polyenes were the first specific antifungals (Phillips and Rosen 2001). Members of this group include topical/oral nystatin and topical/intravenous (IV) amphotericin B (see Table 1). Although nystatin can be orally dosed, it is not absorbed. The polyenes bind fungal cell wall sterols, thus altering membrane permeability and allowing leakage of intracellular contents. Both agents are active against *Candida* species when topically applied but are ineffective against dermatophytes. Although still available from the manufacturer upon special request, topical amphotericin is rarely used today (Phillips and Rosen 2001). The usage of IV amphotericin is generally limited to systemic mycoses, and is beyond the scope of this chapter.

Azoles

The azole family can be subdivided into the topical/oral imidazoles (see Table 1) and the newer oral/IV triazoles (fluconazole, itraconazole, and voriconazole). Compared to the polyenes, both subsets have extended

Table 1 *Selected topical antifungals*

Class	MOA	Active against	Dosing frequency
Polyenes			
Nystatin	Bind to fungal cell wall sterols, increasing permeability	*Candida*	b.i.d.–t.i.d.
Amphotericin B			b.i.d.–q.i.d.
Imidazoles			
Clotrimazole	Block lanosterol 14α-methylase thus inhibiting fungal cell wall ergosterol synthesis	*Candida*, dermatophytes, *Malassezia*; econazole has some antibacterial activity	b.i.d.
Econazole			q.d.–b.i.d.
Ketoconazole			q.d.–b.i.d.
Miconazole			b.i.d.
Oxiconazole			q.d.–b.i.d.
Sulconazole			q.d.–b.i.d.
Allylamines			
Butenafine	Inhibit squalene epoxidase thus inhibiting fungal cell wall ergosterol synthesis. Squalene accumulation within cell is toxic	*Candida*, dermatophytes	q.d.
Naftifine			q.d.
Terbinafine			q.d.–b.i.d.
Substituted pyridone			
Ciclopirox	Alters active membrane transport, cell membrane integrity and cell respiratory processes	*Candida*, dermatophytes, *Malassezia*, and some bacteria. Some anti-inflammatory activity	b.i.d.
Others			
Tolnaftate	Inhibits squalene epoxidase	Dermatophytes, *Malassezia*	b.i.d.
Selenium sulfide	Cytostatic effect on keratinocytes Fungicidal against *Malassezia*	*Malassezia*	q.d.

spectrums of antifungal activity. Not only effective against *Candida*, they also inhibit dermatophytes and *Malassezia* (De Doncker et al. 1996; Odom et al. 1996; Elewski et al. 1997; Havu et al. 1997; Scher 1999). Additionally, topical econazole appears to exhibit some antibacterial activity (Brooks 1996; Rupke 2000). Their fungistatic properties are due to inhibition of the cytochrome P450-dependent enzyme, lanosterol 14α-demethylase, an important step in the synthesis of ergosterol. Ergosterol is vital for fungal cell membrane integrity.

The inhibition of the cytochrome P450 enzyme system can lead to significant drug interactions when the systemic azoles are taken with other drugs metabolized by these enzymes, such as astemizole, some benzodiazepines, cisapride, cyclosporine, warfarin, and the statins. Medications such as antacids, proton-pump inhibitors, and H_2-blockers may decrease the systemic absorption of ketoconazole and itraconazole by raising the gastric pH. These drugs are better absorbed when taken with food or an acidic beverage. By contrast, fluconazole can be taken without regard to food.

Following oral administration and absorption, both fluconazole and itraconazole achieve excellent tissue distribution. Fluconazole is hydrophilic and is widely distributed throughout the body. It has been detected in the skin and nails within 2–3 hours of oral administration (Haneke 1990, 1992; Hay 1993; Scher 1999; Moossavi et al. 2001). Therapeutic levels remain in the stratum corneum for at least one week after treatment (Wildfeuer et al. 1994; Lesher 1999). In contrast to fluconazole, itraconazole is highly lipophilic. It also has a strong affinity for keratin, resulting in persistently high concentrations is the stratum corneum and in nails. Significant drug levels have been found in the stratum corneum from the back, palms and face for 2, 3, and 4 weeks, respectively, after stopping therapy (Cauwenbergh et al. 1988; Lesher 1999). A level far exceeding the minimum inhibitory concentration (MIC) of common dermatophytes was detected in nails 90 days after completing a 7-day course (Meinhof 1993; Scher 1999). This preferential persistence in keratin permits dosage flexibility such as once weekly administration of fluconazole as well as 'pulse' dosing of itraconazole.

Adverse effects of the topical azole antifungal agents are generally limited to local irritation, stinging, burning, and contact dermatitis. However, gastrointestinal adverse events are not uncommon with the oral agents. Indeed, approximately 10 percent of patients taking itraconazole or ketoconazole may report nausea or vomiting (Moossavi et al. 2001), which can be improved by dosing with meals. Gastrointestinal symptoms are also seen with fluconazole, although less frequently. Six percent of the patients taking fluconazole in a study reported in 1998 experienced headache, which was the most frequently reported adverse effect (Scher et al. 1998). Itraconazole has recently been associated with a nega-

tive inotropic effect on the heart and congestive heart failure. It is contraindicated in patients with known ventricular dysfunction (Ahmad et al. 2001). The most worrisome adverse effect seen with systemic azole therapy is hepatotoxicity. The incidence of ketoconazole-induced hepatotoxicity ranges from 1:10 000 to 1:15 000 and has been fatal in at least seven people. The hepatotoxicity seems to be idiosyncratic and is not related to the dose or length of therapy (Knight et al. 1991; Moossavi et al. 2001). This, combined with the significant drug interactions, has effectively ended frequent systemic use of ketoconazole for cutaneous fungal infections. Serum transaminases may also be elevated with itraconazole (Tucker et al. 1990) and less frequently with fluconazole (Scher et al. 1998). Monitoring of hepatic enzymes is recommended in patients taking itraconazole continuously for longer than one month and in those with preexisting hepatic function abnormalities (Mehta 2001; Moossavi et al. 2001) but routine monitoring is generally not required with fluconazole.

Allylamines/butenafine

A third group of antifungals is composed of the allylamines (topical naftifine and topical/oral terbinafine) and a single topical benzylamine (butenafine) (see Table 1).
Like the azoles, these agents have broad antifungal spectrums, and they have the additional advantage of fungicidal activity. This activity is due to their inhibition of squalene epoxidase, an enzyme needed for the production of ergosterol. As a result, squalene accumulates, producing a toxic effect on the organism and eventual cell death (Ryder 1992). It is still unclear if the in vitro fungicidal activity is clinically relevant.

Unlike the azole family, oral terbinafine does not affect the cytochrome P450 3A4 isoenzyme, so drug interactions are less problematic. Cimetidine may increase the levels of terbinafine by decreasing its metabolism and clearance. Conversely, rifampin induces hepatic metabolism of terbinafine and causes decreased blood levels. The combination of cyclosporine and terbinafine may increase the clearance of cyclosporine. Terbinafine does inhibit cytochrome P450 2D6, which may lead to toxic levels of tricyclic antidepressants and thioridazine.

Terbinafine is well absorbed after oral administration and absorption is not affected by administration with food. Delivery to the stratum corneum occurs rapidly. Levels were detected in peripheral nail clippings of healthy volunteers after 7 days of 250 mg/day dosing (Gupta et al. 1997; Scher 1999). The concentrations in the stratum corneum of patients taking the 250 mg/day dose for 1 or 2 weeks were far greater than the MICs of most dermatophytes (10–100 and 100–1 000, respectively) (Faergemann et al. 1994; Lesher 1999). Similarly, the drug level in nails 90 days after completing one week of therapy was much higher than the MICs (Gupta et al. 1997; Scher 1999).

As with other oral antifungals, gastrointestinal side effects are among the most commonly reported with terbinafine. Hepatic adverse effects range from transient elevations of serum transaminases in 3.3–7 percent of patients to severe cholestatic or hepatocellular hepatotoxicity (Boldewijn et al. 1996; Moossavi et al. 2001). This is thought to be an idiosyncratic, nondose-related reaction. Rarely, severe cutaneous events have been reported with terbinafine including toxic epidermal necrolysis, erythema multiforme, Stevens–Johnson syndrome, and acute generalized exanthematous pustulosis (Moossavi et al. 2001). The topical agents are well tolerated. Adverse effects such as allergic or irritant contact dermatitis, itching, burning or stinging have rarely been reported (Phillips and Rosen 2001).

Griseofulvin

Griseofulvin was the first widely used systemic antifungal medication. Its spectrum of activity is limited to dermatophytes and it is ineffective against *Candida*, *Malassezia*, bacteria, deep fungal infections, and nondermatophyte molds such as *Scopulariopsis* and *Scytalidium* (Moossavi et al. 2001). Griseofulvin's fungistatic activity is due to interference in microtubule spindle formation in actively growing fungi. Poor absorption from the gastrointestinal tract is counteracted by microsizing or ultramicrosizing griseofulvin particles. The ultramicrosized particles are better absorbed and require lower dosages. Absorption is also enhanced by administration with a fatty meal. Drug interactions are generally due to griseofulvin's induction of the cytochrome P450 enzyme system with resultant decreased blood levels of other drugs metabolized by this system. Examples include warfarin, oral contraceptives, and salicylates. Headache is the most common adverse effect and this usually resolves with continued therapy. Although rare, hepatotoxicity, neutropenia, and leukopenia have been reported.

Miscellaneous antimycotic agents

CICLOPIROX

Ciclopirox is a broad-spectrum topical antifungal which is effective against dermatophytes, yeasts, fungal saprophytes, and some gram-positive and gram-negative bacteria (Jue et al. 1985) (see Table 1). In addition, it has some anti-inflammatory activity and may be especially useful in treating superficial inflammatory mycoses (Hanel et al. 1991). It acts by altering active membrane transport, cell membrane integrity, and cell respiratory processes (Abrams et al. 1991; Phillips and Rosen 2001).

It is available as a cream, gel, lotion, nail lacquer, and shampoo.

TOLNAFTATE

Tolnaftate is a topical antifungal with a narrow spectrum of activity (see Table 1). It is effective in superficial infections caused by dermatophytes and *Malassezia*, but is not active against *Candida* or bacteria (Gupta et al. 1998; Weinstein and Berman 2002). It inhibits squalene epoxidase, thus interfering with fungal cell wall sterol synthesis (Phillips and Rosen 2001).

SELENIUM SULFIDE

Selenium sulfide is a liquid topical antifungal agent used in the treatment of tinea versicolor and seborrheic dermatitis (Sanchez and Torres 1984; Faergemann 2000) (see Table 1). It has some in vivo fungicidal activity against *Malassezia* (Phillips and Rosen 2001). It also appears to have a cytostatic effect on keratinocytes, which may explain its effectiveness as an adjutant in the treatment of tinea capitis (Allen et al. 1982; Givens et al. 1995).

Choice of agent

Topical antifungal agents can be used as sole therapy in most superficial fungal infections. They offer the advantages of very few adverse effects and negligible systemic absorption. For these reasons, they are generally considered first-line therapy except in tinea capitis and onychomycosis, which do not respond well to topical therapy and require treatment with an oral agent. Other instances in which systemic antifungal therapy may be required include:

- failure of topical agents
- patients immunocompromised by disease or therapy
- extensive fungal disease.

THERAPY OF SELECTED SUPERFICIAL INFECTIONS

Malassezioses

TINEA VERSICOLOR

Tinea versicolor usually responds well to topical therapy. Selenium sulfide 2.5 percent, ketoconazole 2 percent, or zinc pyrithione 1 percent shampoos/lotions can be applied to the affected areas and left in place for 5–10 minutes once a day for 2 weeks. Alternatively, the azole antifungal creams or ciclopirox 0.1 percent solution are also generally effective when used twice daily (Corte et al. 1989).

Failure of the above regimens or extensive disease may force trial of systemic medications (see Table 2).

Table 2 *Systemic antifungals for tinea versicolor*

Drug	Dosage
Fluconazole	400 mg once 400 mg once and repeat in 7 days
Itraconazole	200 mg/day for 7 days
Ketoconazole	200 mg/day for 5–7 days 400 mg once and repeat in 7 days

Ketoconazole 200 mg/day for 5–7 days has been proven effective. One study showed 17 of 23 patients treated with fluconazole in a 400 mg one-time dose were clear of lesions 3 weeks after treatment (Faergemann 1992). Another study found itraconazole 200 mg/day for 7 days to be safe and effective (Hickman 1996). Oral terbinafine does not appear to be effective in treating tinea versicolor (Lesher 1999).

As many as 60–80 percent of patients successfully treated with oral or topical therapy will have a recurrence of tinea versicolor within one year (Rupke 2000). To reduce this rate, appropriate prophylaxis may be necessary. A single dose of oral ketoconazole, fluconazole, or itraconazole could be dosed once or twice a month. Topical antifungal agents may also be used once or twice monthly. In either case, however, compliance may be an issue.

SEBORRHEIC DERMATITIS

Although the role of *Malassezia* in the etiology of seborrheic dermatitis is unclear, agents effective against these organisms are also generally effective in controlling this condition. Ketoconazole 2 percent shampoo has been successful when applied at least twice a week (Peter and Richarz-Barthauer 1995). Ciclopirox 1 percent, selenium sulfide 2.5 percent shampoo (Shuster 1984) and zinc pyrithione shampoo (Marks et al. 1985) have also worked well. Antifungal creams offer another therapeutic option. Due to its anti-inflammatory activity, ciclopirox may be particularly useful in seborrheic dermatitis (Hanel et al. 1991). Nonantifungal treatments for seborrheic dermatitis on the face include low-potency topical corticosteroids such as desonide, hydrocortisone, or alclometasone. More potent corticosteroid lotions, solutions, or foams may be used on the scalp. The topical immunomodulating agent, pimecrolimus may prove to be effective (Ling 2001; Crutchfield 2002).

PITYROSPORAL FOLLICULITIS

Pityrosporal folliculitis usually responds well to topical treatment with an azole cream or shampoo. Selenium sulfide shampoo/lotion may also be used (Ford et al. 1982). When patients do not respond to topical therapy, oral fluconazole (100–200 mg/day for up to 3 weeks), ketoconazole (200 mg/day for 7–10 days), or itraconazole (200 mg/day for 5–7days) may prove effective (Ford et al. 1982; Rhie et al. 2000). Itraconazole may be the

most effective option because its lipophilic properties permit penetration into the pilosebaceous unit.

Other superficial mycoses

TINEA NIGRA

Topical azole antifungals such as ketoconazole, econazole, or miconazole are normally sufficient for clearing tinea nigra (Marks et al. 1980; Burke 1993; Hughes et al. 1993). Ciclopirox and terbinafine have also been effective when used topically (Sayegh-Carreno et al. 1989; Shannon et al. 1999). Whitfield's ointment is an old medication that has also been used successfully. Some authors have suggested scraping away the visibly affected area before topical application (Sayegh-Carreno 1989). Oral therapy is rarely required.

PIEDRAS

Both black and white piedra may be treated by shaving or clipping off the infected hairs. This is not always a cosmetically acceptable therapy, however. Ketoconazole 2 percent shampoo, ciclopirox solution, selenium sulfide, and zinc pyrithione have been used with varying degrees of success. A small study reported complete cure in 11 of 12 patients with white piedra treated with 100 mg of itraconazole taken by mouth once daily for 8 weeks (Khandpur and Reddy 2002).

Dermatophytoses

TINEA CORPORIS AND TINEA CRURIS

Most topical antimycotics are effective in treating uncomplicated cases of tinea corporis and tinea cruris when used as directed by the manufacturer (Drake et al. 1996a; Brodell and Elewski 1997). See Table 1 for a list of these medications, as well as dosing frequencies. Nystatin is ineffective against dermatophytes and would therefore not be a good choice in treating tinea corporis (Lesher 1996). In intertriginous areas, tolnaftate should not be used, as it is not effective against *Candida* (Demis 1999). When bacterial superinfection is also suspected, topical antifungals with some antibacterial activity, such as econazole or ciclopirox, may be particularly useful (Drake et al. 1996a). All topical agents should be applied to the lesion and an approximately 2.5 cm border of surrounding normal skin. Ideally, application should be continued for 1–2 weeks after all signs of infection have cleared in order to minimize recurrence. More specific dosing suggestions can be found in the manufacturer's recommendations.

Indications for systemic therapy include failure of topical antimycotic agents, extensive disease, primary or secondary immune deficiency, and inflammatory infections such as Majocchi's granuloma. In addition, severely obese persons with tinea cruris may require systemic therapy. Suitable oral medications include griseofulvin, fluconazole, itraconazole, and terbinafine (Lambert et al. 1989; Lesher 1999) (see Table 3).

Due to the ubiquity of the pathogens, reinfection and recurrence are common. Simple strategies to minimize recurrence in patients with intertriginous dermatophytoses such as tinea cruris include keeping the area dry in order to make the environment less conducive to fungal growth. In addition, patients should avoid tight-fitting, nonabsorbent clothing and minimize exposure to wet clothing such as bathing suits. Affected areas should be dried thoroughly after bathing and hand-held hair dryers work well for this purpose. Other suggestions might include application of absorbent antifungal powders and weight reduction.

TINEA BARBAE

Although some cases of inflammatory tinea barbae may resolve spontaneously, alopecia and scar formation can occur. The less common, noninflammatory sycosiform variant rarely heals without therapy. Since the hair follicles are infected, systemic therapy is required (Drake et al. 1996a). Griseofulvin, fluconazole, itraconazole, and oral terbinafine have all been used successfully (see Table 4). In most cases, therapy should be continued until all lesions have cleared and new hair growth is occurring.

Adjunctive therapies may be of some benefit. Warm compresses may soften crusts and help in the removal of debris. Ketoconazole 2 percent or selenium sulfide 2.5 percent shampoos also help remove debris and may provide some antifungal activity.

Since most cases of tinea barbae are acquired from zoophilic sources, avoidance, identification, and treatment of infected animals are important.

TINEA PEDIS AND MANUUM

Interdigital tinea pedis and tinea manuum with no associated onychomycosis in immune competent patients generally respond well to topical antifungals. See Table 1 for a list of suitable agents along with their dosing frequencies. Antifungals with antibacterial

Table 3 *Systemic antifungals for tinea corporis and tinea cruris*

Medication	Dose
Fluconazole	50–100 mg daily or 150 mg once weekly for 2–3 weeks[a]
Itraconazole	100 mg daily for 2 weeks or 200 mg daily for 7 days[a]
Terbinafine	250 mg daily for 1–2 weeks[a]
Griseofulvin	Microsize 20 mg/kg/day for 2–4 weeks Ultramicrosize 15 mg/kg/day for 2–4 weeks

a) Lesher (1999)

Table 4 *Systemic antifungals for tinea barbae*

Drug	Dose
Griseofulvin	Microsize 20 mg/kg/day Ultramicrosize 15 mg/kg/day Continue for at least 2 weeks after resolution. Six weeks or longer are generally required
Fluconazole	150–300 mg once a week for 4–6 weeks or 200–400 mg daily for up to 4 weeks
Itraconazole	400 mg/day for 1 week. May require a second 1-week pulse 3 weeks after the first pulse is completed or 200 mg/day for 2–4 weeks
Terbinafine	250 mg/day for 2–4 weeks

activity, such as econazole and ciclopirox, are good initial choices as interdigital disease is often complicated by secondary bacterial infections (Rupke 2000). Activity against *Candida* may also be important and drugs such as tolnaftate, which lack this attribute, are not first-line (Drake et al. 1996b). Generally, topical antifungal agents should be used for about 4 weeks or as directed by the manufacturer. Topical terbinafine and butenafine may be effective in as little as one week of therapy (Villars and Jones 1989; Bergstresser et al. 1993; Savin et al. 1994, 1997; Elewski et al. 1995). Emollients containing lactic acid or urea may be useful adjuncts to the antifungals when extensive scaling or hyperkeratosis is present.

Systemic antifungals are generally required in moccasin and inflammatory tinea pedis. Other patients who may require oral therapy include those with concomitant onychomycosis, peripheral vascular disease, or compromised immune status. Griseofulvin may be ineffective in tinea pedis (Moossavi et al. 2001) but terbinafine, itraconazole, and fluconazole have all been used successfully (Lesher 1999) (see Table 5). When

Table 5 *Systemic antifungals for tinea pedis*

Drug	Dosage
Fluconazole	150–300 mg once a week for up to 8 weeks (Lesher 1999)
Itraconazole	100 mg/day for 2 weeks or 400 mg/day for 1 week
Terbinafine	250 mg/day for 1–2 weeks

onychomycosis is present, the nail infection should be treated to prevent recurrence of tinea pedis or tinea manuum. See Table 6 for medications and dosages used in treating onychomycosis.

As with other dermatophytoses, efforts to minimize exposure to the infective fungi help to prevent initial or recurrent infections. Protective footwear should be worn in public facilities such as locker rooms and hotel bathrooms. Taking measures to eliminate the warm, moist conditions in which dermatophytes thrive can also reduce the recurrence rate. For example, patients should be instructed to dry feet thoroughly after bathing, and to apply an antifungal powder to both the feet and inside shoes. The use of cotton socks wicks moisture away from the feet. Prevention and effective treatment of tinea pedis is likely to prevent tinea manuum and tinea corporis.

TINEA UNGUIUM (ONYCHOMYCOSIS)

Topical therapy of onychomycosis is often unsuccessful. Ciclopirox 8 percent in nail lacquer applied daily to infected nails for 48 weeks produced a complete cure in only 5.5 percent of patients in one study and 8.5 percent in another (Dermik Laboratories 2000; Mehta 2001). Evaluations of other topical agents are ongoing.

Due to the low efficacy of topical medications, oral antifungals are generally required (Drake et al. 1996b). Fluconazole, itraconazole, and terbinafine are options. See Table 6 for dosage and monitoring guidelines. Continuous terbinafine therapy has been shown to be more effective when compared to both pulsed and continuous itraconazole (Brautigam et al. 1995; De

Table 6 *Systemic antifungals for onychomycosis*

Drug	Dosage	Duration	Monitoring
Fluconazole	150–300 mg once a week	3 months for fingernails, 6 months for toenails. Some authors recommend 6–9 months	Generally none recommended
Itraconazole	Intermittent (pulse) dosing – 200 mg b.i.d.	One week per month for 3 consecutive months for toenails, 2 months for fingernails	Baseline LFTs. Monitor LFTs every 4–6 weeks for continuous dosing of more than 1-month duration (Rodgers and Bassler 2001)
	Continuous dosing – 200 mg q.d.	8 weeks for fingernails, 12 weeks for toenails	
Terbinafine	250 mg/day	6 weeks for fingernails, 12 weeks for toenails	Baseline liver function tests (LFTs) and complete blood count (CBC). Repeat monthly (Rodgers and Bassler 2001)

Backer et al. 1998; Evans and Sigurgeirsson 1999). A large North American study showed terbinafine to have the greatest in vitro activity against dermatophytes and nondermatophyte molds when compared to fluconazole and itraconazole. Itraconazole had the greatest activity against *Aspergillus* spp. and *Candida parapsilosis*. Fluconazole was the most active of the three against *Candida albicans* (Ghannoum et al. 2000). Although its use appears to be growing, there are few data available comparing the clinical efficacy of fluconazole to the other agents. Griseofulvin is used infrequently due to its low cure rates, high recurrence rates, and the need for protracted treatment courses. Likewise, oral ketoconazole is rarely used due to its hepatotoxicity.

In addition to systemic antifungal therapy, certain adjunct measures may provide additional benefits and help recurrent infections. Occasionally, a nidus of infection (dermatophytoma) may form under the nail, which responds poorly to current therapy and may require nail avulsion. Topical antifungals may also be useful, especially when patients have severe tinea pedis. The patient should be instructed to wear protective footwear in public showers and facilities and to avoid sharing manicure equipment and other fomites. Feet should be kept as dry as possible. Patients should be advised to wear cotton socks and to change them regularly. Antifungal powders help keep feet dry and may provide some therapeutic benefit. Finally, early recognition and effective treatment of tinea pedis will help prevent tinea unguium.

TINEA CAPITIS

Topical treatment of tinea capitis is considered ineffective as sole therapy and systemic therapy is required for penetration of hair follicles (Elewski 1999). See Table 7 for medications, dosages, and duration of therapy. Griseofulvin has been the gold standard and remains an effective alternative. In the USA, the microsized preparation is available in a 125 mg/5 ml suspension, 250 mg tablets and 500 mg tablets. The ultramicrosized product has enhanced absorption and is available in tablets ranging from 125 mg to 330 mg in strength. Availability may be limited in some countries. Griseofulvin's disadvantages include its prolonged treatment course and relatively high dosage when compared to the newer antifungals. The bitter taste of the suspension makes compliance a problem. Nausea, vomiting, and headache are not uncommon adverse effects that may reduce compliance, as does the bitter taste of the liquid formulation. Perhaps as a result of these disadvantages, fluconazole, itraconazole, and terbinafine have become more commonly used to treat tinea capitis. The pleasant-tasting fluconazole suspension is especially appealing for children. It is available in 10 mg/ml and 40 mg/ml strengths. In addition, tablet strengths range from 50 to 200 mg. Precise dosing of itraconazole is more difficult. The liquid preparation should not be considered a substitute for the capsule due to the adverse effects associated with its cyclodextrin-containing vehicle. The inability to divide the 100 mg capsule makes accurate pediatric dosing problematic. Unlike fluconazole, itraconazole needs to be taken with food or a cola beverage to enhance absorption. Terbinafine may be taken with or without food. It is available only in 250 mg tablets that can be divided for administration to children. In tinea capitis caused by *Microsporum canis*, terbinafine may prove less effective than the other systemic alternatives (Mock et al. 1998). Tinea capitis caused by this organism generally requires longer treatment courses with any selected antimycotic (Hamm et al. 1999).

In addition to systemic therapy, use of topical medications may help minimize spread of the infection. Selenium sulfide 1 and 2.5 percent (Allen et al. 1982; Givens et al. 1995) and ketoconazole 2 percent shampoos (al-Fouzan et al. 1993; Greer 2000) are thought to reduce shedding of infected hairs and to stabilize infectious particles. Although there are fewer data on ciclopirox shampoo, it is likely to be equally efficacious. Patients should be instructed to use one of these several times a week. When feasible, family members should also use

Table 7 *Systemic antifungals for tinea capitis*

Drug	Pediatric dose	Adult dose
Griseofulvin	Microsized 20–25 mg/kg/day in single or divided doses for 8–12 weeks Ultramicrosized 15 mg/kg/day for 8–12 weeks	Same
Fluconazole	6 mg/kg/day for 3–6 weeks	200 mg/day for 3–6 weeks
Itraconazole	5 mg/kg/day for 4 weeks Most require 100 mg/day Very small children: 100 mg every other day *M. canis* may require 6–8 weeks	5 mg/kg/day Usually 200 mg/day for 4 weeks
Terbinafine	Weight 10–20 kg: 62.5 mg/day (1/4 tablet) Weight 20–40 kg: 125 mg/day (1/2 tablet) Weight > 40 kg: 250 mg/day (1 tablet) For 2–4 weeks *M. canis* may require 6–8 weeks	250 mg/day for 2–4 weeks

these shampoos, because they may be asymptomatic carriers (Elewski 2000). Other preventative measures include discarding fomites such as combs, brushes, and other hair accessories. When a zoophilic pathogen such as *M. canis* is isolated, an attempt to identify and treat the infected animal could prove beneficial.

Nondermatophyte molds

Treatment of onychomycosis caused by nondermatophyte molds such as *Scytalidium* spp., *Acremonium* spp., *Fusarium* spp. and *Scopulariopis* spp. is difficult. Topical antifungals are ineffective, as are systemic ketoconazole and griseofulvin (Gupta and Elewski 1996). A study published in 2000 showed that terbinafine had the highest in vitro antifungal activity against *Acremonium*, *Fusarium*, *Scopulariopsis*, and *Scytalidium* spp. when compared to fluconazole, itraconazole, and griseofulvin (Ghannoum et al. 2000). However, the clinical relevance is uncertain. Terbinafine and itraconazole have also shown some clinical efficacy against *Scopulariopsis brevicaulis* (Gupta et al. 2001). Avulsion may be the best option.

Superficial candidiasis

CANDIDAL PARONYCHIA

Chronic paronychia may be associated with *Candida* infection in some patients. However, one study has shown that treatment of chronic paronychia with a topical steroid (methylprednisolone aceponate) was more effective than systemic antifungal therapy with itraconazole or terbinafine (Tosti et al. 2002). Nevertheless, both topical and oral antifungals continue to be used with varying degrees of success. Topical imidazoles and polyene antifungal solutions, gels, or lotions are applied frequently over a prolonged course of therapy. Thymol 4 percent solution in acetone, isopropyl alcohol, or chloroform and sulfacetamide lotion have also been used (Hay 1999). Oral fluconazole and itraconazole are alternatives, but their effectiveness has not yet been established (Hay 1999). Patients should also be instructed to keep their fingers warm and dry.

CANDIDAL ONYCHOMYCOSIS

Systemic therapy is often required for microscopy, biopsy or culture-proven candidal onychomycosis. Itraconazole has been used successfully in three pulsed doses for toenails and two pulses for fingernails (Gupta et al. 2000). Weekly dosing of 150–300 mg of fluconazole should also be effective although few trials have been conducted. Terbinafine has unpredictable in vitro activity against *C. albicans* (Ryder et al. 1998) and has not shown consistently good results clinically (Rex et al. 2000).

CANDIDAL INTERTRIGO AND INTERDIGITAL CANDIDIASIS

In contrast to onychomycosis, topical therapy is usually sufficient for candidal intertrigo (including diaper dermatitis) and interdigital candidiasis. Topical allylamines, butenafine, or imidazoles are applied once or twice daily (Drake et al. 1996c). As in any intertriginous condition, it is important for patients to keep the area dry. This can be done by using a hair dryer and by applying an antifungal powder.

OROPHARYNGEAL CANDIDIASIS (THRUSH)

For immunocompetent patients, topical treatment of oropharyngeal candidiasis with nystatin or clotrimazole is generally effective. Nystatin suspension or lozenges are given four to five times a day in doses ranging from 100 000 to 500 000 units. Clotrimazole 10 mg troches are given five times a day. Immunocompromised patients may require systemic antifungals. Fluconazole or itraconazole are given in 100–200 mg daily doses for 5–10 days. The development of resistance to these drugs can be a problem, especially in patients with acquired immunodeficiency syndrome (AIDS). Generally, when patients fail to respond clinically, the medication dose is doubled. If they still do not respond, it is necessary to check the identity and MIC value of the organism and to change drugs (Hay 1999).

VAGINAL CANDIDIASIS

Topical antifungals are usually effective in treating vaginal candidiasis. Imidazole (miconazole, terconazole, etc.) or nystatin vaginal creams, tablets or suppositories are used for from 1 to 7 days. Alternatively, oral therapy with fluconazole (100 mg/day for 5–7 days or 150 mg once) (Hay 1999), or itraconazole (200 mg/day for 3 days or 200 mg b.i.d. for 1 day) (Sobel et al. 1998) is also effective.

CHRONIC MUCOCUTANEOUS CANDIDIASIS

Systemic therapy with fluconazole, ketoconazole or itraconazole is usually necessary for patients with chronic mucocutaneous candidiasis. Dosing strategies similar to those used for oropharyngeal candidiasis in immune suppressed patients are employed. Once remissions are induced, maintenance therapy is not used in order to reduce the development of drug resistance (Hay 1999).

CONCLUSION

Successful management of superficial fungal infections requires not only knowledge of the various antimycotic agents but also familiarity with adjunctive measures that can help reduce the chance of spread or reinfection. This chapter has been a discussion of these measures along with a summary of the indications, dosages, mechanisms of action, adverse effects, and drug interactions of the

most commonly used topical and systemic antifungal drugs. Some superficial infections respond well to therapy with topical medications alone. Others such as tinea capitis require systemic therapy. The newer oral agents such as fluconazole, itraconazole, and terbinafine offer greater efficacy, broader spectrums of activity and shortened treatment duration. Awareness of their limitations, potential adverse effects and drug interactions is essential for proper patient selection and monitoring.

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White piedra, black piedra, and tinea nigra

G. SYBREN DE HOOG AND EVELINE GUÉHO

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Superficial mycoses are defined as the development of fungal growth on epithelial tissues such as human hair, skin, or nails, without noticeable invasion of living tissue and without apparently provoking an immune response by the host. (The term 'pityriasis' is often preferred rather than 'tinea' because the latter term should properly be reserved for dermatophyte infections.) Ecologically, such mycoses are comparable to epiphytic or epilithic growth, i.e. superficial colonization of plants, wood, or stones, almost without making use of the supporting substratum. In the case of colonizers of human skin, utilization of products excreted by the host seems likely.

Two ecological categories can be distinguished in superficial fungi. Growth on the human body may be entirely coincidental, the fungus occupying a natural niche outside the human body. This is the case with *Hortaea werneckii* (*Phaeoannellomyces werneckii*), the causative agent of human tinea nigra, which is a halophilic fungus living in salt pans at subtropical coasts (Zalar et al. 1999). Alternatively, the mammalian body may be the natural habitat of the fungus. This is the case, for example, with members of the ascomycete genus *Piedraia*, which are unique in the fungal kingdom in that they complete their entire lifecycle, including production of ascospores, on hairs of living humans.

ETIOLOGIC AGENTS

White piedra is caused by species of *Trichosporon* Behrend, a genus of basidiomycetous arthroconidial yeasts. *Trichosporon* spp. are also known as agents of cutaneous and of systemic infections. Black piedra is caused by members of the ascomycete genus *Piedraia* da Fonseca and de Area Leão, which are exclusively known as agents of this particular disorder. Tinea nigra is caused by the single species, *Hortaea werneckii* (Horta) Nishimura and Miyaji [*Phaeoannellomyces werneckii* (Horta) McGinnis and Schell].

ECOLOGY

Commensal fungi assimilate compounds that are produced by the human body, without invasion of living tissue (de Hoog et al. 2000). They may also decompose dying or dead host remains without the need for the host itself to be present. As they are frequently found on humans and may form macroscopically visible colonies, the fungi under consideration have frequently been referred to as opportunistic pathogens. However, infections caused by most of the species treated in this chapter are rare, even in immunocompromised patients.

When the target compound is an integral part of the host, e.g. keratin assimilated by dermatophytes, adjacent living tissue can also be affected, and a gradual evolution towards true pathogenicity may be expected (Rippon 1985). In the presence of excreted target compounds, the fungi remain strictly superficial. This is the case with the agents of black piedra and of tinea nigra, and with members of the lipophilic genus *Malassezia* Baillon.

WHITE PIEDRA

Clinical presentation

White piedra is a non-inflammatory, non-invasive fungal growth on the outside of the hair shaft. Soft nodules are formed, which can easily be pulled off the hair. The nodules are white, pale greenish, or yellowish and are composed of compacted fungal elements (Figure 1). At the outside the elements produce rounded arthroconidia-like cells. The hairs are not invaded, but they may break if the fungi have been present for long periods.

White piedra is caused by several *Trichosporon* species. The infections may affect hairs on the head, in the axilla, or on the crural area. Human white piedra of the scalp occurs with a low incidence in temperate and subtropical climates (Kaplan 1959a; Gold et al. 1984). Hairs of the beard, eyelashes, eyebrows, and scalp may be affected. In the nineteenth century, the disorder may have been quite common in temperate climates, judging from the fact that patients harbored such infections for several years (Guého et al. 1992a). White piedra has also been observed on the fur of other mammals such as the horse and lower primates worldwide (Kaplan 1959a).

White piedra of the groin does not depend on personal hygiene (Gold et al. 1984; Kalter et al. 1986). At present, genital white piedra is surprisingly common in the Americas (Fischman et al. 1980; Benson et al. 1983; Kalter et al. 1986), Africa (Thérizol-Ferly et al. 1994a) and Europe (Stenderup et al. 1986). Smith et al. (1973) suspected that the disorder might be underdiagnosed, as a result of the site of infection. It is frequently accompanied by intertrigo (Thérizol-Ferly et al. 1994a), but it may also be strictly harmless (Benson et al. 1983; Ellner et al. 1991). Prevalence varies with socioeconomic background (Ellner et al. 1991), gender (Thérizol-Ferly et al. 1994a), sexual behavior (Torssander et al. 1985; Stenderup et al. 1986), and with race (Avram et al. 1987), although marked regional differences can be noted. The fungus may also colonize adjacent cotton fibers in underwear (de Almeida et al. 1990). Perhaps the warm and moist microclimate of groin and axilla is a major predisposing factor. Axillary white piedra has not been investigated in detail; it probably occurs with a comparable frequency.

Microbiology and identification

TRICHOSPORON ASAHII AKAGI

Colonies on Sabouraud's glucose agar (SGA) are dry, pustular, with a broad, deeply fissured, marginal zone and with a white, farinose covering at the center. Budding cells and lateral conidia are absent. Arthroconidia are regular and barrel shaped (Figure 2). Appressoria are absent. Guého et al. (1992a) found that documented strains from superficial mycoses mainly originated from animal white piedra; numerous other strains came from human cutaneous and deep infections.

TRICHOSPORON CUTANEUM (DE BEURMANN ET AL.) OTA

Colonies on SGA are moist and shiny, with a broad, fissured, marginal zone without farinose covering. Budding cells are abundant in primary cultures, but hyphae predominate after repeated transfer. Arthroconidia are cylindrical to ellipsoidal, and lateral conidia are present. The species is known only from a small collection of strains, among which is one originating from axillary white piedra.

TRICHOSPORON INKIN (OHO EX OTA) DO CARMO SOUSA AND VAN UDEN

Colonies on SGA are restricted, cerebriform, with no marginal zone, often cracking the agar medium. Budding cells and lateral conidia are absent. Arthroconidia are long and cylindrical, and appressoria are present (Figure 3). Sarcinae may be present in tissue; in vitro this is reproduced on media with high sugar content. The species is limited to hairs in the groin area.

Figure 1 *Nodules of white piedra caused by* Trichosporon inkin *on pubic hair. Magnification* × *940*

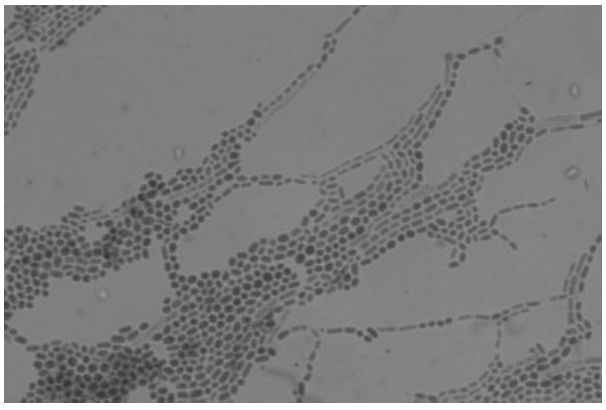

Figure 2 *Regular arthroconidia of* Trichosporon asahii. *Magnification* × *940*

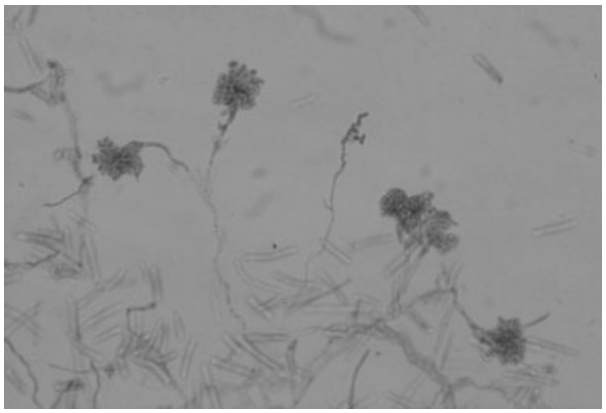

Figure 3 *Arthroconidia and appressoria of* Trichosporon inkin. *Magnification* × *940*

TRICHOSPORON MUCOIDES GUÉHO AND M.TH. SMITH

Colonies on SGA are moist and shiny, and elevated with deep, narrow, radial fissures. Arthroconidia are rectangular. Short lateral branches terminating with clavate conidia are present (Figure 4). The taxon is the prevalent species in groin infections of humans in Africa and is frequently isolated from other superficial locations in Europe.

TRICHOSPORON OVOIDES BEHREND

Colonies are white, farinose, and irregularly wrinkled at the center, with a flat marginal zone. Budding cells and lateral conidia are absent. Arthroconidia are cylindrical and appressoria are present. The species is physiologically indistinguishable from *T. asahii*. A strain originating from scalp white piedra, CBS 7556, was used as the neotype strain for the type species of the genus *Trichosporon* (Guého et al. 1992a).

DISCUSSION OF THE GENUS *TRICHOSPORON*

The genus *Trichosporon* has been revised using molecular characteristics (Guého et al. 1992b). A large number of species was recognized, several of which may be encountered as causative agents of opportunistic mycoses. Since that time several new species have been introduced (Sugita et al. 1997, 2001; Sugita and Nakase 1998; Middelhoven et al. 1999, 2000, 2001), but none of these is an agent of white piedra. Overviews of clinically relevant species were published by Guého et al. (1994) and Middelhoven (2002). It should be noted that the species involved in animal mycoses are different to those found in the environment. Thus, infections by *Trichosporon* spp. are probably transmitted through human or animal vectors.

Members of the genus have multilamellar cell walls in common and contain more or less developed dolipore septa, with or without vesicular or tubular parenthesomes (Guého et al. 1992b). They are therefore classified in the Basidiomycetes and are related to *Filobasidiella* (*Cryptococcus*), a position that has been confirmed by partial 26S rRNA sequencing (Guého et al. 1993) and antigenic similarity (Seeliger and Schröter 1963; McManus and Jones 1985). Nevertheless, the phylogenetic distance to remaining heterobasidiomycetes has been judged large enough to erect a separate order, the Trichosporonales (Fell et al. 2000).

Trichosporon spp. do not generally form budding cells but cubic or rectangular arthroconidia. Identification of the five species has been summarized by Guého et al. (1994) (Table 1; see Figure 1) and Therizol-Ferly et al. (1994b). Serological recognition of the three most prevalent species was demonstrated by Douchet et al. (1994).

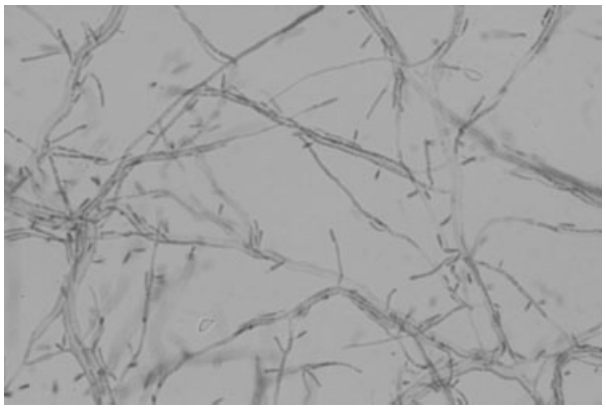

Figure 4 *Arthroconidia and lateral blastoconidia of* Trichosporon mucoides. *Magnification* × *940*

Therapy

The disorder can be controlled by shaving and local application of clotrimazole cream or 5 percent ammoniated mercury ointment (Kwon-Chung and Bennett 1992). All clinically significant *Trichosporon* spp. show high in vitro susceptibility to amphotericin B, clotrimazole, ketoconazole, and itraconazole (Kalter et al. 1986; Guého et al. 1994). Remissions of the disorder are, however, frequent (Walzman and Leeming 1989).

BLACK PIEDRA

Clinical presentation

Black piedra is an asymptomatic but macroscopically visible colonization of the shaft of scalp hair, caused by species of the ascomycete genus *Piedraia*. The species form hard, black nodules, consisting of dense fungal stromata, which are thicker (up to 150 μm) at one end. The nodules cannot be pulled off the hair shaft. The hairs may break after prolonged fungal growth. The disorder is restricted to humid tropical areas (Fischman

Table 1 *Key characteristics of* Trichosporon *spp. isolated from white piedra nodules using the API 20C Aux Identification System (bioMérieux)*[a]

Species	Con	Glu	Gly	2-Ket	Ara	Oxy	Ado	Xyl	Gal	Ino	Sor	α-Met	*N*-Ace	Cel	Lac	Mal	Suc	Tre	Mel	Raf
T. ovoides[b]	–	+	–	+	–	+	–	–	+	+	–	+	+	+	+	+	+	–	v	–
T. inkin	–	+	–	+	–	+	–	–	+	+	–	+	+	+	+	+	+	+	v	–
T. asahii	–	+	–	+	+	+	–	–	+	+	–	+	+	+	+	+	+	s	–	–
T. cutaneum	–	+	v	+	+	+	–	–	+	+	–	–	+	v	+	+	+	v	v	v
T. mucoides	–	+	+	+	+	+	s	s	+	+	+	+	+	+	+	+	+	s	v	+

Ado, adonitol; Ara, L-arabinose; Cel, cellobiose; Con, control; Gal, galactose; Glu, glucose; Gly, glycerol; Ino, inositol; Lac, lactose; Mal, maltose; Mel, melezitose; *N*-Ace, *N*-acetyl-D-glucosamine; Oxy, D-oxylose; Raf, raffinose; Sor, sorbitol; Suc, sucrose; Tre, trehalose; Xyl, xylitol; α-Met, α-methyl-D-glucoside; 2-Ket, 2-keto-D-gluconate.

a) After a 4-day incubation at 25°C: –, no growth; +, growth in 24–48 h; s, growth in 72–96 h; v, variable growth. The five species are further separated by absence of growth at 37°C for *T. cutaneum* and no colonial margin for *T. inkin* (see Guého et al. 1994).

b) *T. ovoides*, responsible for the scalp hair white piedra, is the type species of the genus (Guého et al. 1992a).

et al. 1980), particularly South America (Coimbra and Santos 1989), Asia (Adam et al. 1977) and Africa, and may be associated with hair care involving the application of lipid-containing substances (Simons 1954). Black piedra commonly occurs on the pelts of primates living in tropical forests (Kaplan 1959b; Ajello 1964).

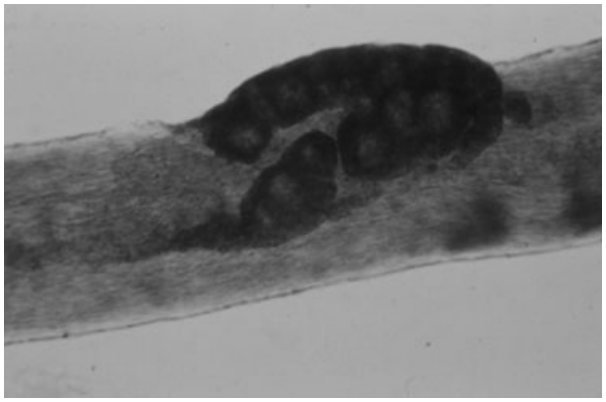

Figure 5 *Black piedra caused by* Piedraia hortae *on Asian scalp hair. The nodule corresponds to pseudoparenchymatous ascomata. Magnification × 940*

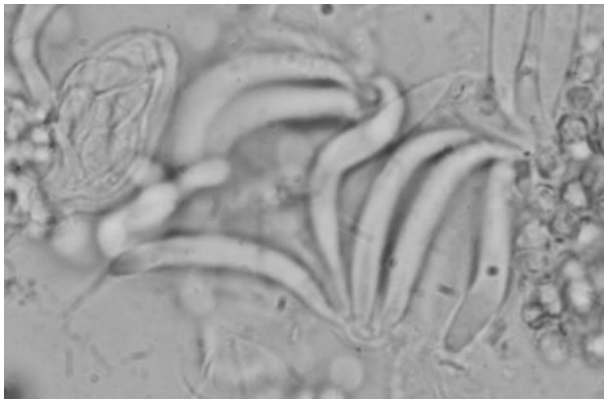

Figure 6 *Spindle-shaped ascospores of* Piedraia hortae *tapering towards both ends to form whip-like extensions. Magnification × 2 350*

Microbiology and identification

PIEDRAIA HORTAE (BRUMPT) DA FONSECA AND DE AREA LEÃO

Colonies on SGA containing glycerin grow slowly and are folded, velvety, and dark-brown to black; most remain sterile. Ascostromata on hairs are compact, elongated, up to 1 mm long and 0.3 mm wide. Black, spherical cavities (Figure 5) are formed by lysis, each containing a few broadly ellipsoidal asci, usually with eight ascospores (Figure 6). Ascospores are hyaline, one-celled, reniform (30–45 × 5.5–10 μm) with whip-like appendages at both ends.

DISCUSSION OF THE GENUS *PIEDRAIA*

A related species which does not produce ascospore appendages is *Piedraia quintanilhae* van Uden, reported from chimpanzees in Central Africa (Takashio and de Vroey 1975). It is not known to occur on humans.

Therapy

Treatment of black piedra is notoriously difficult, although topical application of azole antifungals or selenium sulfide has been used with variable degrees of success.

TINEA NIGRA

Clinical presentation

Brown to black lesions are formed, mainly on the skin of the palm (Severo et al. 1994), but occasionally on the sole of the foot. Pigmentation is more intense near the border. Only the outer, dead layers of the skin (stratum corneum) are affected; no invasion of living tissue occurs. The fungus is present in the form of melanized, ellipsoidal cells, which often have a thick median cross-wall.

Microbiology and identification

HORTAEA WERNECKII (HORTA) NISHIMURA AND MIYAJI

One of the synonyms is *Phaeoannellomyces werneckii* (Horta) McGinnis and Schell. Colonies on oatmeal agar are restricted, smooth, and slimy with an oily, glistening, olivaceous–black color. After several transfers the colonies may become velvety as a result of the production of aerial hyphae. Budding cells are subhyaline, 0–1 septate, and produce daughter cells by annellidic conidiogenesis from their poles. Hyphae are up to 6 μm wide, becoming thick-walled, olivaceous–black, and densely septate at maturation. Conidia are produced laterally from hyphae or from the poles of budding cells by annellidic conidiogenesis. Annellated zones are conspicuous and 1–2 μm wide, with clearly visible annellations. Conidia are initially hyaline and one-celled but soon become olivaceous and measure 7–9.5 × 3.5–4.5 μm. After liberation they inflate and develop transverse and occasionally oblique septa; liberated cells are converted into budding cells or chlamydospore-like cells (Figure 7).

DISCUSSION OF THE GENUS *HORTAEA*

The genus *Hortaea* contains two species. In addition to *H. werneckii*, which is halophilic (Zalar et al. 1999), *H. acidophila* has been introduced (Hölker et al. 2004), which is an extremophile able to grow in very acidic substrates. *H. werneckii* is recognized by resistance to 10 percent sodium chloride, production of extracellular DNAase (de Hoog and Gerrits van den Ende 1992), and by the presence of over 1 μm wide annellated zones with relatively widely spaced annellations (Miyaji and Nishimura 1985). Conidia soon become dark, swell, and develop transverse and oblique septa. Hyphae become dark and thick-walled with age and develop further transverse septa. The old hyphal cells are frequently wider than long, and finally produce extracellular granular material. Yeast-like strains without hyphae are recognized morphologically by bipolar annellated zones.

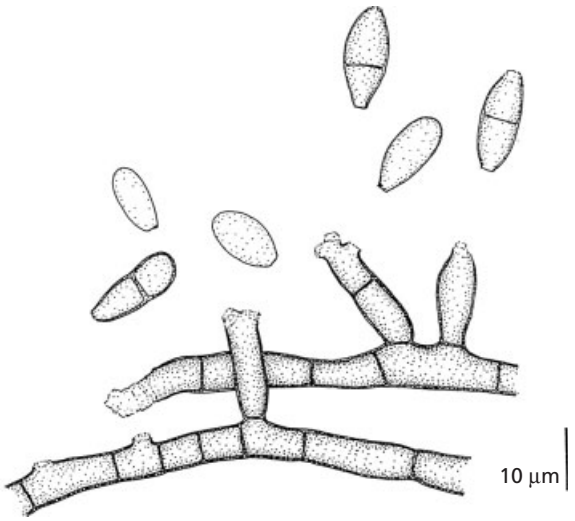

Figure 7 Hortaea werneckii*: morphology of 1-month-old culture on oatmeal agar.*

Phylogenetic position

The species was found to be phylogenetically related to *Aureobasidium pullulans* (De Bary) Arnaud (Haase et al. 1995; Spatafora et al. 1995), which may be an anamorph member of the ascomycete family *Dothioraceae* (order Dothideales). These findings are consistent with the similarities in karyology and thallus maturation (Takeo and de Hoog 1991), coenzyme Q systems (Yamada et al. 1989) and the absence of Woronin bodies at septal pores (Mittag 1993). The family contains plant-parasitic fungi and osmo- and acidophilic species living in the phyllosphere; numerous species occur on inert surfaces such as stone (Sterflinger et al. 1999). No human pathogenic species are known. Phylogenetically they are not related to the human pathogenic black yeasts classified in *Exophiala* Carmichael (Masclaux et al. 1995), which are anamorph members of the ascomycete family *Herpotrichiellaceae* (order Chaetothyriales).

Ecology

Hortaea werneckii is halophilic, its natural niche containing significantly elevated concentrations of salt, e.g. beach soil, salt pans, sea water, or salted fish (Zalar et al. 1999). The finding of this habitat confirms speculations of de Cock (1994) and Uijthof et al. (1994) based on mitochondrial DNA and molecular fingerprinting, respectively. Human cases are primarily reported from coastal areas in the subtropics (Rippon 1988). Hyperhydrotic humans may acquire the colonization of their skin. Primary adhesion may be explained by hydrophobic interaction of fungal cells with human skin; the fungus may subsequently thrive on the human body by the production of extracellular polysaccharides and feed by the assimilation of lipidic excretion products (Göttlich et al. 1995).

Similar behaviour may be adduced for the equally saprophytic fungus, *Stenella araguata* Sydow (*Cladosporium castellanii* Borelli and Marcano). This species was reported as an etiologic agent of cases of tinea nigra in Venezuela (di Prisco and Borelli 1973; Marcano and Hutton 1973; Reyes and Borelli 1974).

Therapy

The fungus can easily be removed by keratinolytic compounds such as Whitfield's ointment (Rippon 1988).

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Superficial diseases caused by *Malassezia* species

VICENTE CRESPO ERCHIGA AND EVELINE GUÉHO

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Characteristics of *Malassezia* spp.	2	Molecular methods of identification	13
Ecology of *Malassezia* spp.	8	References	15

INTRODUCTION

Definition and history

The genus *Malassezia* comprises opportunistic lipophilic yeasts with a natural habitat on the skin of humans and warm-blooded animals. Their ability to cause diseases in humans was recognized more than 150 years ago, when Eichsted in 1846 stated that a fungus was responsible for the lesions of pityriasis versicolor (PV). However, the impossibility at this early stage of isolating and culturing the pathogenic agent brought a long-lasting nomenclatural and clinical controversy that was detailed by Slooff in 1970. As a short introductory history, Baillon created the genus *Malassezia* in 1889, with the species *Malassezia furfur* as the agent of PV. In the lesions, yeasts and filaments (= mycelium or hyphae) were observed, but in the absence of growth on isolation there was no certainty that both features were produced by a single microorganism. The genus *Pityrosporum* was thus created to accommodate similar yeasts observed, without any filaments, in the scales of the human scalp (*Pityrosporum ovale* Castellani and Chalmers 1913), rhinoceros skin (*Pityrosporum pachydermatis* Weidman 1925), and dog-ear (*Pityrosporum canis* Gustafson 1955). The species *Pityrosporum orbiculare* (Gordon 1951b) had been also described because of the morphological similarity of yeasts present in PV scales with those of the two former *Pityrosporum* species. The two species described in humans were found to require complex media to grow whereas the two zoophilic species were less demanding for lipids, growing on enriched media such as Sabouraud dextrose agar (also mycosel agar). The lipophilicity, which excluded the use of any yeast identification system, either conventional or commercial, led mycologists, in the absence of clear key characteristics, to limit this group of fungi to two species (Yarrow and Ahearn 1984): a human species *M. furfur*, and an animal species *Malassezia pachydermatis*, the generic name *Malassezia* having a nomenclatural priority to *Pityrosporum*. In 1990, Simmons and Guého described a third species *Malassezia sympodialis* on the basis of genome differences, but in the absence of well-defined differences, mycologists, and dermatologists as well, up to 1996 ignored this new species.

Phylogeny and taxonomy

The genus *Malassezia* was recognized as having basidiomycete characteristics, even although no teleomorph could be found for any of the species. These features included a multilayered cell wall, repetitive monopolar budding, and a capacity to hydrolyze urea and to give red staining with diazonium Blue B. These characteristics, combined with the lipophilicity, were the basis for a study involving the collection of as many fresh strains as possible from humans and animals, coupled with the

various isolates still available in international fungal collections, and for performing a taxonomic revision of the genus. The access to new molecular techniques, such as ribosomal RNA sequencing, allowed investigators to identify seven genetic species (Guillot and Guého 1995). In addition to the already known species *M. furfur*, *M. pachydermatis*, and *M. sympodialis*, the species *Malassezia globosa*, *Malassezia obtusa*, *Malassezia restricta*, and *Malassezia slooffiae* were thus added to the genus, all species having morphological and physiological key characteristics (Guého et al. 1996).

Then, the genus *Malassezia* with its seven species was confirmed as belonging phylogenetically to the Basidiomycota. These species form a branch, individualized as the order of Malasseziales, in the class of Ustilaginomycetes. However, and surprisingly, this branch is more closely related to plant pathogens such as *Exobasidium* spp. and *Tilletia* spp. (Begerow et al. 2000; Fell et al. 2000) than to the human and animal pathogens *Cryptococcus neoformans* and *Trichosporon* spp. classified as Tremellales and Trichosporonales, respectively (Sugita et al. 2002; Boekhout and Guého 2003).

The seven species can be easily identified in routine of medical laboratories, by combining their morphological and physiological characteristics (Guillot et al. 1996; Mayser et al. 1997; Guého et al. 1998). In cases of doubt, sequencing removes any ambiguity (Duarte et al. 2002). Furthermore, owing to the great reliability of sequences, several techniques for molecular identification of *Malassezia* yeasts can be developed (see section on Molecular methods of identification below).

These different techniques of identification have led already to a number of studies that have investigated the relationships of different species with both diseased and healthy skin. From some of these works (Crespo Erchiga et al. 1999a, b, 2000, 2002; Nakabayashi et al. 2000; Aspiroz et al. 2002), it now appears clear that *M. globosa* is the main species associated with PV. This is the only cutaneous disease in which the involvement of *Malassezia* is undisputed. Nevertheless, this species can also be found in normal skin, alone, or mixed with *M. sympodialis*, *M. restricta*, or *M. furfur* (Sugita et al. 2001). In the remaining dermatological disorders associated with *Malassezia* yeasts, their role is controversial. In seborrheic dermatitis, atopic dermatitis, and folliculitis, several studies have focused on the immunological aspects that might explain the pathogenic mechanisms (Ashbee et al. 1994a, b; Devos and van der Valk 2000; Kesavan et al. 2000; Koyama et al. 2000; Tengvall Linder et al. 2000; Faergemann et al. 2001; Ashbee and Evans 2002; Faergemann 2002). In other diseases, such as confluent and reticulate papillomatosis, neonatal pustulosis, psoriasis, blepharitis, otitis, and onychomycosis, the presence and/or significance of *Malassezia* is still a matter of discussion.

CHARACTERISTICS OF *MALASSEZIA* SPP.

Identification in vivo and isolation

The cutaneous pathologies caused by *Malassezia* yeasts are sufficiently characteristic to be identified readily by dermatologists. Usually, because they are not equipped to perform cultures, dermatologists do not isolate yeasts from lesions, but often they confirm their diagnosis with a direct microscopic examination of material from lesions. Indeed, the yeasts are among the smallest seen in tissues, but the repetitive unipolar budding leaves a very typical prominent bud scar on the mother cells. The most satisfactory method for visualizing this phenomenon can be performed when ink (Parker Permanent Blue/Black Quink) is incorporated with the classical 20–30 percent potassium hydroxide in equal proportions (Cohen 1954). *Malassezia* elements (yeasts and/or filaments) take up the stain immediately and appear blue against a clear background (Figure 1). A more selective staining method is the calcofluor white stain which can only be visualized if the laboratory has access to a fluorescence microscope. This technique provides an excellent view of seborrheic dermatitis and healthy skin samples (Figure 2). Indeed, the appearance of the fungus in tissues, including the yeast shape, the presence or absence of filaments, and the frequency of the two features, provides information about the disease itself. When just one species *M. furfur* (Yarrow and Ahearn 1984) was recognized in humans (alias *P. ovale* or *P. orbiculare*), and another, *M. pachydermatis* (alias *P. pachydermatis* and *P. canis*), in animals, further identification from cultures was not necessary. The interest in *Malassezia*-related pathology has resurrected recognition that several lipid-dependent *Malassezia* species are present in humans (and animals), and those that can be isolated from healthy or diseased skin.

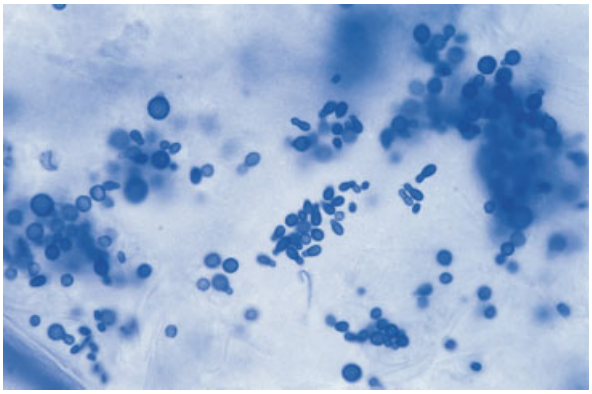

Figure 1 *Oval and globose yeasts in skin scales in a case of Gougerot–Carteaud disease. KOH+Parker ink, ×1 000*

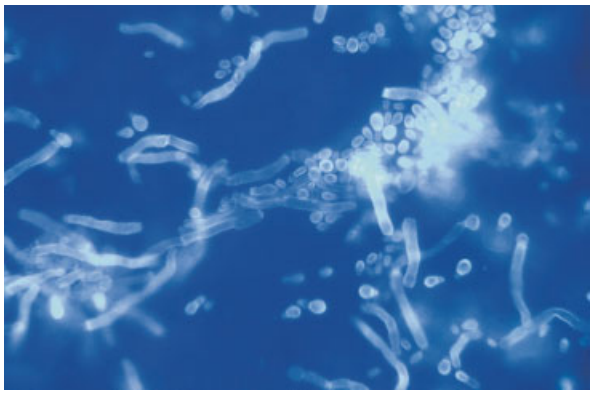

Figure 2 Malassezia *oval yeasts and pseudo-hyphae in healthy human skin from the paranasal area. Calcofluor white, ×1 000*

For years the lipophilic fungus *Malassezia* was isolated from skin scales, nails, or other infected materials on regular Sabouraud agar (2 percent glucose, 1 percent peptone, 2 percent agar) or 1 percent olive oil Sabouraud agar, both media supplemented with 0.5 percent chloramphenicol and 0.5 percent cycloheximide to eliminate, as much as possible, contamination by bacteria and molds, respectively. The regular Sabouraud medium was sufficient to isolate the lipophilic, but nonlipid-dependent, species *M. pachydermatis*, whereas the same medium enriched with 1 percent emulsioned olive oil seemed to be very convenient for isolation of the lipid-dependent species *M. furfur* (Guého and Meyer 1989). The taxonomic revision showed that olive oil could be toxic for a few species, so this medium, which is too selective, is no longer used. Two media are now widely employed to isolate and grow all *Malassezia* species: the Dixon medium described by van Abbe in 1964 or its modified formula (mDixon): 3.6 percent malt extract, 0.6 percent peptone, 2.0 percent desiccated ox-bile (Oxoid), 1.0 percent Tween 40, 0.2 percent glycerol, 0.2 percent oleic acid, and 1.2 percent agar, and the medium described by Leeming and Notman in 1987. Because *Malassezia* yeasts are a part of the cutaneous flora of warm-blooded animals, the addition of chloramphenicol and cycloheximide is recommended for all media. The advantage of the former medium (mDixon) over the latter (Leeming and Notman) is that its dark coloration makes easier counting of colonies, particularly when several species are mixed, and a better recognition of their morphologies. This is the reason that the present authors favor the mDixon rather the Leeming and Notman formula in their work. Moreover, before the taxonomic revision, cultures were almost systematically incubated at 37°C. As for the olive oil, the taxonomic revision has shown that several species are inhibited above 37°C. So all cultures are now performed between 30 and 35°C, according to the facilities of the laboratory, but never at 37°C or above, except to characterize the maximum temperature of growth.

Identification in vitro

The seven species of *Malassezia* were clearly separated genetically (Guillot and Guého 1995). However, they are easily identified with routine techniques in any microbiological laboratory on the basis of their characteristics, including their location on the body, colony and yeast morphology, maximum growth temperatures, β-glucosidase (splitting of esculin), and catalase activities, and requirements for growth, in other words growth with Tweens 20, 40, 60, 80, and cremophor EL as sole source of complex lipid. In the lead taxonomic paper (Guého et al. 1996), growth with Tweens at various concentrations was evaluated in liquid medium. Maximum temperatures of growth and catalase activity were fixed for all strains included in the study. Then a simplified method of identification was deduced from these basic results with the diffusion of Tweens in Sabouraud agar plates (Guillot et al. 1996). The identification scheme was improved by Mayser et al. (1997) who suggested the addition of cremophor EL to the diffusion plates and the characterization of the β-glucosidase activity using the esculin agar tubes. The complete identification system was finally summarized by Guého et al. in 1998 (see also Figure 3).

M. furfur forms thick, convex or umbonate colonies, 4–5 mm in diameter, with a surface that is smooth to rough and a cream color (Figure 4). The texture is soft and the cells are easy to emulsify. This is not the case for all species and, for eliciting this characteristic, it is important to prepare suspensions for all experiments. The species is morphologically heterogeneous with globose, oval, or cylindrical yeast cells (usually not in the same culture), 4.0–5.0 μm and up to 6.0 μm long, some strains being able to produce filaments, spontaneously or under particular culture conditions (Figures 2 and 5). These are now considered to be pseudohyphae since they have no dolipores, which interrupt the true filaments in Basidiomycetes (Guillot et al. 1995). This species is genetically heterogeneous with high ribosomal RNA similarity, but with two karyotypes (Boekhout et al. 1998). By contrast, *M. furfur* is physiologically homogeneous. It can be routinely identified by combining all its characteristics: ability to grow above 37°C, a strong catalase reaction, an absence or very weak β-glucosidase, and an equal growth in the presence of Tweens 20, 40, 60, 80, and cremophor EL as sole lipid (Figure 3). In fact, *M. furfur* is weakly lipid dependent since any lipid supplement is sufficient for its growth. Mayser et al. (1997) demonstrated this as castor oil and ricinoleic acid were sufficient for growth of *M. furfur*. Murai et al. (2002) showed also that *M. furfur* was the least dependent species with a unique positive effect on the growth with glycine. Specific characteristics are quite stable for all the species. However, atypical variants may occur, such as the *M. furfur* dog isolate,

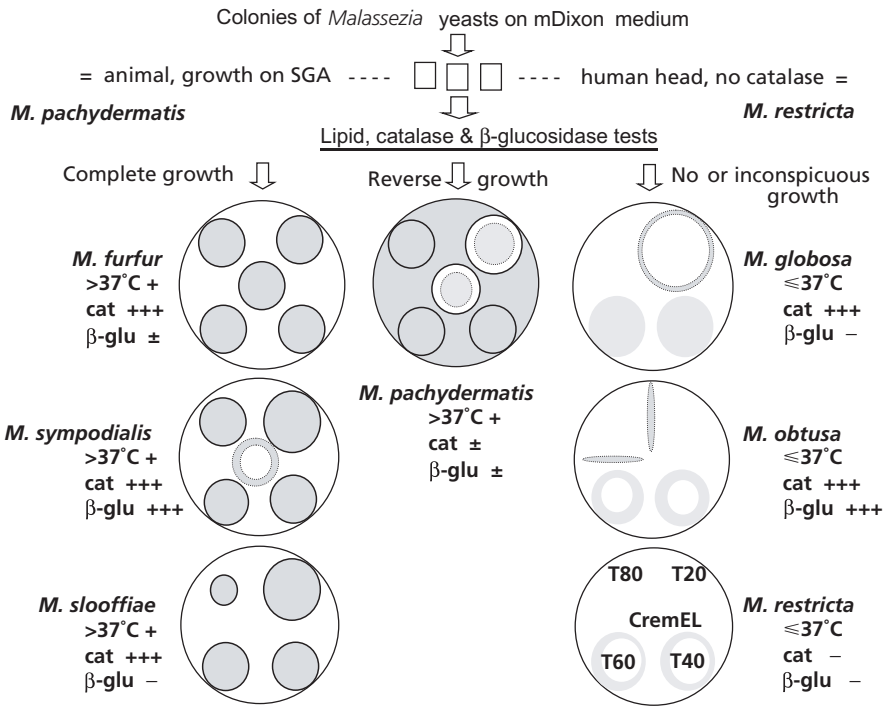

Figure 3 *Scheme of* Malassezia *spp. identification. Each culture isolated on mDixon agar is tested for its catalase (cat) and β-glucosidase (β-glu) activities; its growth at 37°C is given; then its capacity to grow with Tweens 20, 40, 60, 80, and Cremophor EL is evaluated using Sabouraud agar plates: complete growth (circled gray color); inconspicuous growth (dotted-circled gray color); only precipitate (not circled light gray color).*

confirmed specifically by rDNA sequencing, but unable to grow with cremophor EL (Duarte et al. 2002). Normal isolates of the species are also regularly, but not abundantly, isolated from various animals (Crespo et al. 1999, 2002).

M. pachydermatis is historically the second species described in the genus. It is different from all the other species since it is able to grow on rich conventional laboratory media (Sabouraud or mycosel agars). The species is lipophilic but can grow in the presence of simple lipids present in the peptone of such media. *M. pachydermatis* grows also on mDixon agar and generates thick, cream-colored, and convex colonies with a mat surface and usually a brittle texture which makes it difficult to emulsify (Figure 6). The cells are small, 2.5–4.0 μm, oval to short, and cylindrical with a very broad base to the bud (Figure 7). Pink isolates (Midgley 1989) have been reported and a genotype, isolated from dogs, and characterized by smaller colonies, was shown to be lipid dependent in primary culture (Bond and Anthony 1995; Guillot et al. 1997). Duarte et al. (2002) isolated also an atypical lipid-dependent strain of *M. pachydermatis* that remained dependent on lipids even after transfers. Therefore, all epidemiologic surveys of

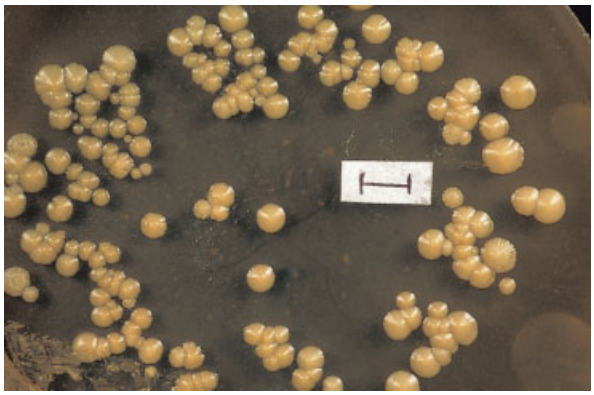

Figure 4 *Colonies of* M. furfur, *7 days old, on mDixon medium*

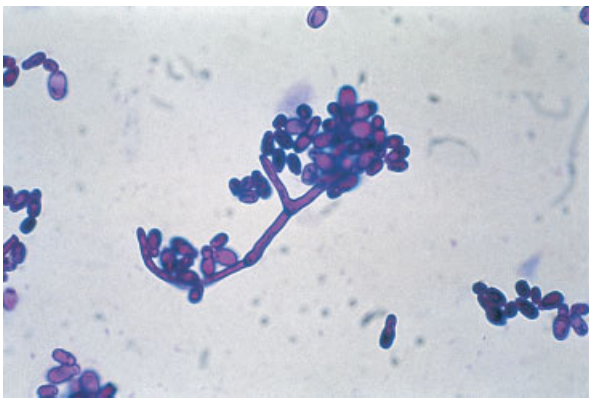

Figure 5 *Yeast cells and pseudohyphae of* M. furfur. *Periodic acid Schiff stain, × 1 000*

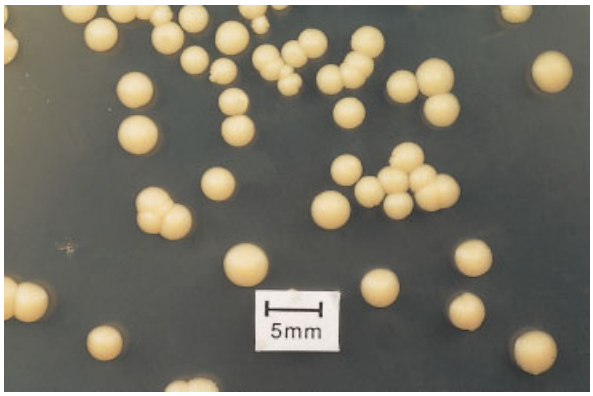

Figure 6 *Colonies of* M. pachydermatis*, 7 days old, on mDixon medium*

Figure 8 *Mixed colonies of* M. sympodialis *(a few) and* M. restricta *(numerous), 7 days old, on mDixon medium*

Malassezia yeasts, from animal or human sources, should utilize directly lipid-supplemented media. The taxonomic revision maintained a single genetic species (Guillot and Guého 1995) but many of its characteristics show that it may be in the process of speciation, a possible adaptation in host specificity (Midreuil et al. 1999). All isolates grow at 37°C, but differences occur in catalase and β-glucosidase expression, and the Tweens 20, 40, 60, 80, and cremophor EL growth tests. These different compounds, particularly Tween 20 and cremophor EL, may be more or less inhibitory, a secondary growth appearing around the wells after dilution of the substrate by diffusion (Guého and Guillot 1999, and Figure 3). This species is rare in humans, although it has been found to cause epidemics of sepsis, usually in infants as a complication of prematurity (Mickelsen et al. 1988). In humans its isolation from skin may be considered as accidental. By contrast, the species is likely to be responsible for seborrheic dermatitis and otitis in warm-blooded animals, especially dogs (Guillot and Bond 1999).

M. sympodialis is an easy species to identify. It forms extended, up to 5–6 μm in diameter, flat colonies, often with a slight central elevation. These are cream to buff in color with a smooth, shiny surface, and a homogeneous texture, and are very easy to emulsify (Figure 8). The yeasts are small, 2.5–5.0 μm, and ovoid with a narrow bud base (Figure 9). As a result of sympodial budding at the same site, yeasts may remain somewhat grouped as 'clover leaves'. The species correlates with the former *M. furfur* serovar A (Cunningham et al. 1990). It grows at 37°C and it is easily characterized in routine by a strong β-glucosidase activity, which deeply darkens the esculin medium in 24 hours at 37°C, and the assimilation of the four Tweens, but only very weakly of cremophor EL (Figures 3 and 12.10). Primary isolates may develop a ring of tiny colonies at some distance from the cremophor source, as also seen in a few *M. slooffiae* isolates, but this latter species does not split esculin.

Sugita et al. in 2002 isolated and described a new species, *Malassezia dermatis*, from patients with atopic dermatitis. Analysis of ribosomal DNA 26S and internal transcribed spacers (ITS)s sequences showed this species to be closely related to *M. sympodialis*. The two species differ by only 1.2 percent rDNA base divergence, a difference acceptable as occurring within a single species (Kurtzman and Blanz 1998), and their colony

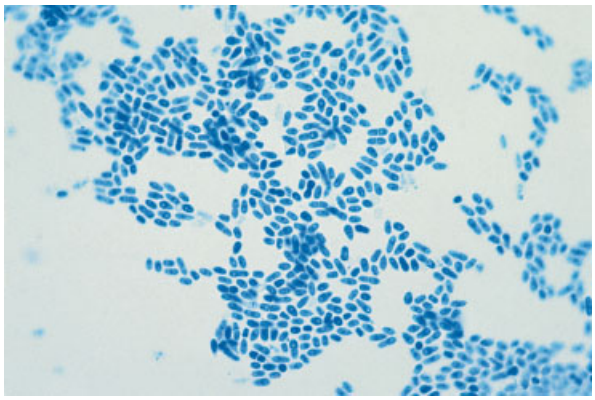

Figure 7 *Yeast cells of* M. pachydermatis*. Lactophenol Cotton Blue, ×1 000*

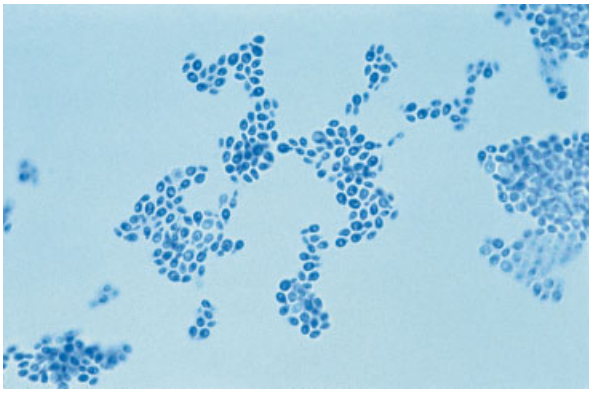

Figure 9 *Yeast cells of* M. sympodialis*. Lactophenol cotton blue, ×1 000*

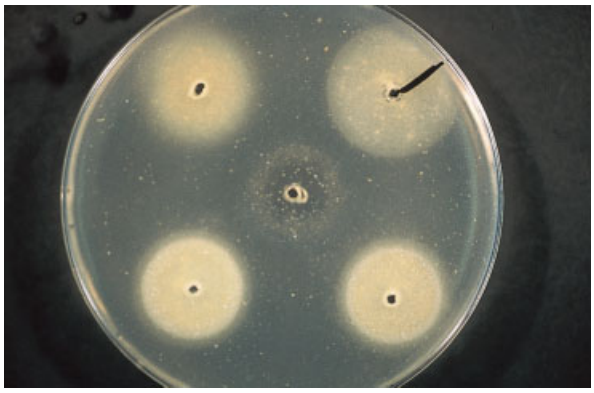

Figure 10 M. sympodialis *showing assimilation of the four Tweens (right top, Tween 20 with a black mark, and clockwise, Tweens 40, 60, and 80), and in the center, a ring of tiny colonies around Cremophor EL*

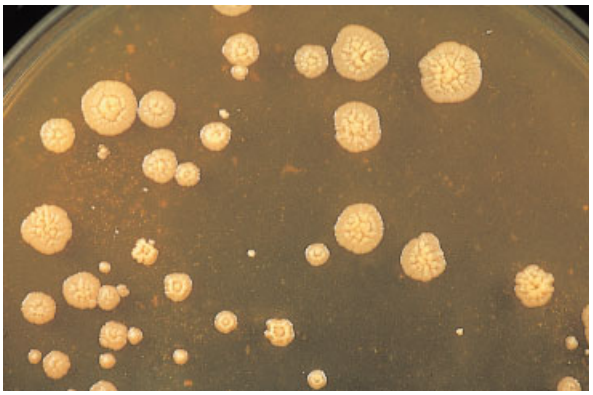

Figure 11 *Typical wrinkled colonies of* M. globosa*, 7 days old, on mDixon medium*

characteristics, but they cannot be separated with the basic laboratory tests (Guillot et al. 1996). *M. dermatis*, as with *M. sympodialis*, has strong catalase activity and assimilates the four Tweens. However, esculin and cremophor EL are missing in the description of this new species. Similarly, several horse *Malassezia* isolates were found with a rDNA sequence analysis that would suggest that they represent two new species (Nell et al. 2002). No description was given in the paper, although *Malassezia equi* was suggested as the name of one of these new species. As with *M. dermatis*, these zoophilic entities are very closely related to *M. sympodialis* phylogenetically. More studies are in process to better characterize and possibly validate these different entities. *M. nana*, another new species isolated from animals, also resembles *M. sympodialis* but does not split esculin (Hirai et al. 2004). The species *sensu stricto* was found to be associated with otitis externa in two cats (Crespo et al. 2000a).

M. globosa corresponds morphologically to the original description of *P. orbiculare* given by Gordon (1951a). This specific name was not preserved, because it was obvious that most data published in the past as *P. orbiculare* corresponded to one or the other lipid-dependent species. Immunologically, *M. globosa* correlates with the former serovar B (Cunningham et al. 1990). The species produces recognizable colonies, 4 mm in diameter, rough with a deeply folded surface, cream to buff in color, and a very brittle texture; they are particularly difficult to emulsify (Figure 11). The cells are spherical, reaching in some strains 6–8 μm in diameter with buds formed on a narrow base (Figure 12). In PV skin scales, these yeasts are regularly mixed with pseudofilaments, which are usually still present in primary cultures as germinative tubes, but unfortunately they disappear after several transfers. The species does not grow at 37°C, or very poorly, does not split esculin, and does not grow with any individual lipid supplements (Figure 3). Fresh isolates may produce tiny colonies in the presence of Tweens, in particular Tween 20. Whitish disks, frequently present with the different species, around Tweens 40 and 60 correspond to precipitates (Guillot et al. 1996). *M. globosa* is very often associated with another species, especially *M. sympodialis*, but also *M. furfur*, *M. slooffiae*, or *M. restricta* (Crespo Erchiga et al. 1999b). It has been isolated occasionally from horses and domestic ruminants (Crespo et al. 2002).

M. obtusa is a species that may be confused with *M. furfur* morphologically but not physiologically. The species grows slowly, so colonies remain small in diameter with a sticky texture. By contrast, cylindrical cells are among the biggest in the genus, up to 10 μm long when the bud is still attached to the mother cell. The species does not grow at 37°C, or with any of the five lipids used as identification criteria as with *M. globosa* and *M. restricta*, but expresses strong β-glucosidase activity (Figure 3). It is a very rare species that so far is known to occur only in healthy skin. In animals it has been isolated, with *M. furfur*, from a case of canine otitis (Crespo et al. 2000b), and from healthy horses and goats (Crespo et al. 2002).

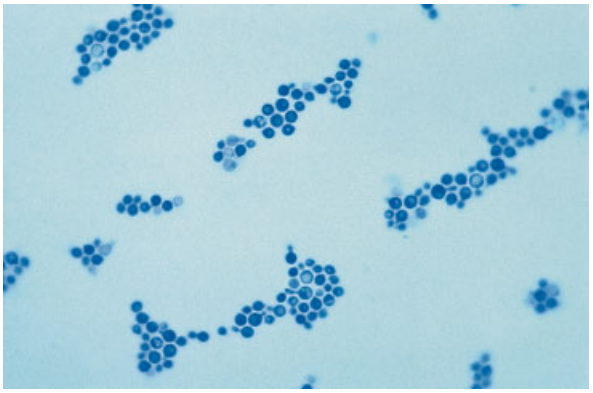

Figure 12 *Typical spherical yeast cells of* M. globosa*. Lactophenol Cotton Blue, ×1 000*

M. restricta might correspond to the former *P. ovale*, but it is difficult to correlate with the old literature, a task that is even greater than for *M. globosa*. The species was named 'restricta' because of its very poor performances. It grows very slowly, giving irregular, small (2 mm average in diameter) and cream-colored colonies with a hard texture; these are very difficult to emulsify (see Figure 8). Cells are spherical to oval, 2.0–4.0 μm, with buds formed on a relatively narrow base (Figure 13). Its micromorphology resembles that of *M. sympodialis*, but hopefully not its colonial macromorphology. This new species could be correlated with serovar C, the third serotype characterized by Cunningham et al. (1990) among lipid-dependent *Malassezia* yeasts. At this point it is important to point out that serotyping, as did biotyping proposed by Aspiroz et al. (1999), recognized only the three species *M. sympodialis*, *M. globosa*, and *M. restricta*, and ignored the others, in particular *M. furfur* and *M. slooffiae*. Hopefully for its identification, *M. restricta* is the only lipid-dependent species lacking catalase activity. This test is thus recommended as a first step, particularly when very restricted numbers of *Malassezia* colonies are obtained from the head. Furthermore, this species does not grow at 37°C, does not split esculin, and does not grow in the presence of different Tweens or cremophor EL (Figure 3). The species, as with *M. obtusa*, has also been isolated for the first time from domestic animals (Crespo et al. 2002). Because of the great difficulty encountered with this species, its presence in humans, and possibly in animals, is likely to have been underestimated. Furthermore, such difficulties in isolation, obtaining sufficient cultures, and maintaining *M. restricta* may be a barrier to understanding the dermatological disorders such as seborrheic dermatitis and atopic dermatitis, and consequently their treatment.

M. slooffiae, one of the four new species, is less frequently isolated from humans but appears to be regularly associated with the skin of pigs. It may be morphologically misidentified as *M. furfur*. It has colonies with finely folded margins, that are 3 mm in diameter, cream to buff in color, and have a brittle texture (Figure 14). The cells are small and shortly cylindrical, 1.5–3.5 μm long, but never globose or elongate as are some strains of *M. furfur* (Figure 15). However, physiologically, there is no ambiguity in clearly identifying organisms as *M. slooffiae*. The species has catalase activity and grows at up to 40°C as does *M. furfur*, but it does not split esculin at all, grows weakly with Tween 80, and does not grow with cremophor EL (Figure 3). The species is found principally on healthy skin, or mixed with other species such as *M. furfur*, *M. sympodialis*, *M. globosa*, or *M. restricta*, but always at a lower frequency. It is unlikely to be a major pathogen, at least on humans.

Figure 14 *Typical colonies of* M. slooffiae *with folded margins, 7 days old, on mDixon medium*

Maintenance of *Malassezia* in collections

Several reasons explain why the taxonomy of the genus *Malassezia* remained for so long a matter of controversy. Primarily, the lipophilicity was not understood and for years the different entities were known only from the literature, without any material deposited in fungal collections. When cultural isolation became possible,

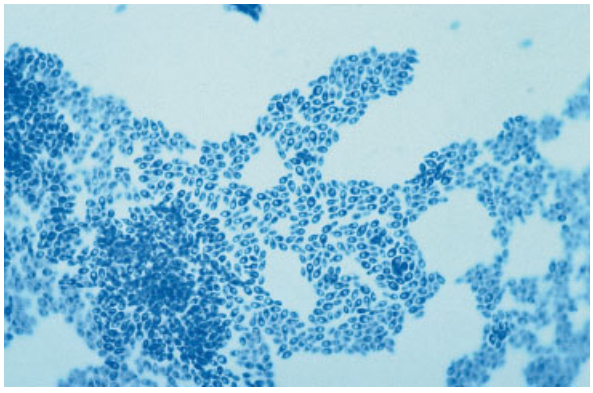

Figure 13 *Yeast cells of* M. restricta. *Lactophenol Cotton Blue, ×1 000*

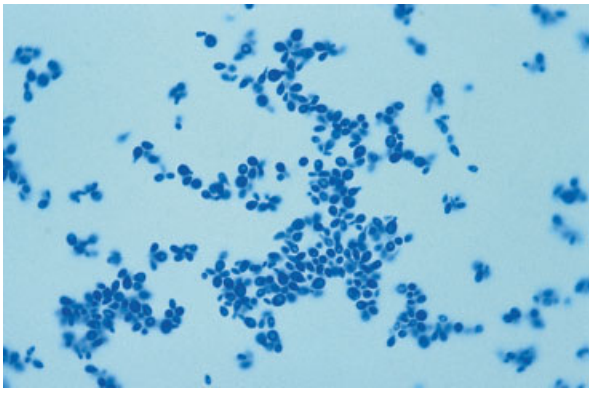

Figure 15 *Cylindrical yeast cells of* M. slooffiae. *Lactophenol cotton blue, ×1 000*

isolates started to be deposited. However, with time, only the less demanding species, essentially *M. furfur* and *M. pachydermatis*, have survived. In 1989, Guého and Meyer published the reevaluation of the genus *Malassezia*, based for the first time on genome comparisons but using lyophilized strains or strains maintained for years in the Dutch CBS fungal collection (Centralbureau voor Schimmelcultures). Furthermore, the lipid-dependent strains were grown on olive-oil-supplemented medium. The authors limited, as described by Yarrow and Ahearn (1984), the genus to only two species. In fact, only *M. furfur*, *M. pachydermatis*, *M. sympodialis*, and *M. slooffiae* survive lyophilization. For successful taxonomic revision (Guého et al. 1996), the work compared strains maintained in collections housing a large number of fresh isolates. All cultures were performed on mDixon which allows a better maintenance of all species. They are also never incubated or preserved at room temperature, but at 34°C with transfers every few months. Indeed, at room temperature, even with *M. pachydermatis*, losses are significant. The collection was then preserved in parallel with mDixon freeze-dried cultures, stored at –80°C. Crespo et al. (2000c) confirmed the importance of storage conditions used to preserve *Malassezia* yeasts.

ECOLOGY OF *MALASSEZIA* SPP.

The *Malassezia* yeasts have an absolute requirement for lipids to grow and survive. Furthermore, they are mesophilic, in other words their optimal temperature of life is between 30 and 35°C. These two physiological peculiarities explain why they are known only from the skin of warm-blooded animals. In the laboratory they can be obtained and maintained in culture only if specific nutrients and growth temperature are provided. Veterinarians have investigated the role of *M. pachydermatis* in causing ear infections in dogs for a long time (Guillot et al. 1998; Guillot and Bond 1999). Recently, some cases have been reported where *M. furfur* and *M. obtusa* were isolated as agents of otitis externa in dogs, and *M. sympodialis* in a similar condition in cats (Crespo et al. 1999, 2000a, b). Many studies had already demonstrated that *Malassezia* lipid-dependent spp. also colonize the skin of many other animals: monkeys, pigs, rhinoceros, bears, and birds (Midgley and Clayton 1969; Dufait 1985; Guillot et al. 1994, 1998).

The presence of *Malassezia* spp. in healthy human skin had been detected in earlier work during the second half of the nineteenth century. The frequency and density of colonization are related to the subject age and to sebaceous gland activity in the studied area (Marcon and Powell 1992).

Since the new descriptions, some studies have focused on their precise distribution in normal human skin. Crespo Erchiga et al. (1999a, b, 2002) with three successive studies made up of nearly 300 adults, showed that *M. sympodialis* appears as the predominant species in healthy skin, especially of the trunk, where it can be recovered in great numbers from more than 50 percent of individuals. It can also be found on the face, though at a lower percentage, together with *M. globosa*. This latter species can be found alone in about 10 percent of subjects. In a new ongoing study by the same authors (unpublished data), *M. restricta* seems to be predominant in the scalp, where it is isolated in 20 percent of adults, though always associated with a certain degree of scaling. Aspiroz et al. (1999) also found *M. restricta* associated particularly with the scalp skin, *M. sympodialis* with the back, while *M. globosa* was evenly distributed on scalp, forehead, and trunk. These data are similar to those published by Midgley in the UK in 2000, although this author reports a higher percentage of *M. globosa* in the scalp (45–50 percent). In these different surveys, the number of *M. furfur sensu stricto* isolates is very low (Midgley 2000; Crespo Erchiga et al. 2002), and even absent (Aspiroz et al. 1999).

In a Japanese study of 35 normal subjects, only *M. globosa* (51 percent) and *M. sympodialis* (26 percent) were recovered from the trunk. Sixty percent of scalp and face samples remained negative in culture, whereas *M. globosa*, *M. furfur*, and *M. sympodialis* were isolated from a few cases (Nakabayashi et al. 2000). In Russia, *M. sympodialis* was found to be the most common species in the skin of 32 healthy individuals, whilst *M. globosa* was encountered much less frequently (Arzumanyan 2001). In Canada, *M. sympodialis* was also the commonest species in 20 healthy control subjects (Gupta et al. 2001a). In this study, *M. globosa* was equally recovered from the scalp, forehead, and trunk, but less from the arms and legs, whereas *M. restricta* and *M. slooffiae* were recovered more frequently from the scalp and forehead than from the lower body. Differences encountered among these various studies may be explained by the different sampling techniques, culture media (Leeming-Notman by Gupta et al. 2001a, and mDixon by Midgley 2000, Nakabayashi 2002 and Crespo Erchiga et al. 2002), and possibly also by ethnic and geographical factors.

Finally, Sugita et al. in 2001, using a nested polymerase chain reaction (PCR) technique, detected *Malassezia* DNA in 78 percent of the scalp and nape samples of 18 healthy subjects. Among these isolates, *M. restricta*, *M. sympodialis*, *M. globosa*, and *M. furfur* were found at frequencies ranging from 61 to 11 percent. These results confirm that at least four *Malassezia* species, *M. sympodialis*, *M. restricta*, *M. globosa*, and *M. furfur*, are common inhabitants of the human skin.

SUPERFICIAL DISEASES ASSOCIATED WITH *MALASSEZIA* SPP.

The yeasts of the genus *Malassezia* are associated in a variable way with a number of human diseases, basically

affecting the skin, although in the last two decades a number of opportunistic systemic infections, most in premature neonates and related to the administration of lipids through intravenous catheters, have been described (Mickelsen et al. 1988; Midgley 2000; Crespo Erchiga and Delgado 2002).

Pityriasis versicolor

Pityriasis versicolor (PV) is a chronic, benign cutaneous disorder, usually asymptomatic, and characterized by slightly scaly patches of variable color (either pink, brown, or white) localized in the upper trunk. It has a worldwide distribution, though it is more frequent in tropical climates, where it has been reported in 30–40 percent of the population. The incidence in temperate climates is much lower (1–4 percent). It affects mainly young adults of both sexes although in tropical zones it is also very common in infancy and even in babies. The onset of the disease seems to be related mainly to local factors such as high temperature and humidity. The appearance on the trunk in the classic form of PV is probably related to occlusion produced by clothing. The condition appears generally in otherwise healthy people, and its incidence is not higher in those with acquired immunodeficiency syndrome (AIDS).

The diagnosis is usually performed by direct observation of the typical yeast and pseudo-hyphae features present in PV skin scales, using the reactive KOH-Parker ink (Figure 16). The yeasts are spherical, as are those of *M. globosa*, in the majority of cases, and occasionally oval, as are those of *M. furfur*. Indeed, in the taxonomic revision, it appeared that only *M. globosa* and *M. furfur* were able to filament more or less spontaneously (Guého et al. 1996).

Historically, Gordon was the first to assume that the rounded forms of *Malassezia* yeasts, he referred to *P. orbiculare*, were the etiologic agents of PV (Gordon 1951a, b). Following the description of new taxa, a few epidemiologic studies have dealt with this question.

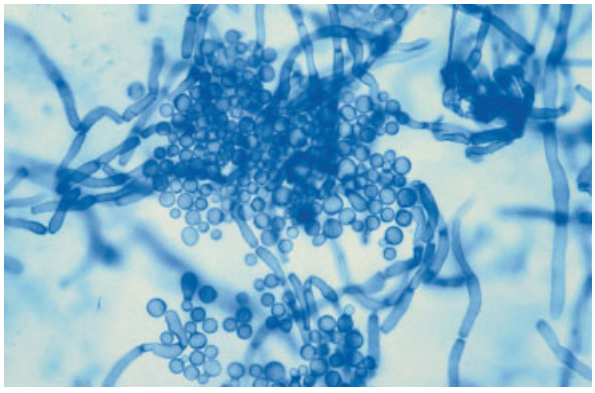

Figure 16 *Typical globose yeasts and pseudohyphae in PV skin scales. KOH+Parker ink, ×1000*

In Japan, Nakabayashi et al. (2000) found *M. globosa* present in 55 percent of PV scale samples of 22 patients, whilst all other species were below 10 percent. They suggested that this species plays a pathogenic role in the disease, as proposed previously by Crespo Erchiga et al. (1999a).

In a study carried out in Canada, Gupta et al. (2001b) found *M. sympodialis* (59 percent), *M. globosa* (25 percent), and *M. furfur* (11 percent) in 111 PV cases. In this study, a high number of cases with positive microscopy failed to grow in culture, and the authors suggested that this was consistent with the standard sampling practice of scraping the older (central) parts of PV lesions rather than the margins.

In Southern Spain, Crespo Erchiga et al. (2000) performed three successive epidemiologic studies, comparing the isolation of *Malassezia* spp. from PV, seborrheic dermatitis, and healthy skin from the same patients, with normal subjects. In the lesions, direct microscopy always showed the typical mixture of globose yeasts and filaments. As a result of this study, the authors did not find that the central parts of clinical lesion were less suitable for culture than margins. In contrast to other dermatomycoses (such as tinea), *Malassezia* yeasts and pseudomycelium can be distributed in similar amounts all over the affected skin, while scraping margins could lead to a higher recovery of surrounding commensal species, such as *M. sympodialis*. In the last group of this survey, consisting of 96 adult patients, *M. globosa* was isolated from 97 percent of cases, alone in 60 percent of them, and associated with *M. sympodialis* in 29 percent, or *M. slooffiae* in 7 percent. These two species were also found in similar percentages in the clinically uninvolved skin of the trunk, whereas *M. globosa* was not isolated at these sites. However, on the forehead, a small number of *M. globosa* colonies were recovered in 12 percent of cases. The presence of this species, in its yeast phase, in seborrheic dermatitis and healthy skin, indicates that local factors (humidity, sweat, heat, and seborrhea), together with some degree of idiosyncratic individual predisposition are responsible for the transformation to the mycelial form and the development of clinical lesions. In 2002, Crespo Erchiga et al. presented new results obtained with a case study of 210 PV patients. These new results were consistent with previously published data that thus confirmed the role of *M. globosa* in the etiology of PV, at least in the clinical form seen in temperate climates.

Three other studies support this conclusion. In Spain (Zaragoza), Aspiroz et al. (2002), with a survey of 79 PV patients, recovered *M. globosa* in 90% and *M. sympodialis* in 41 percent. In Japan, Nakabayashi (2002), using PCR identification of *Malassezia* species involved in PV, found *M. globosa* (97 percent) as the most commonly detected species, followed by *M. restricta* (79 percent) and *M. sympodialis* (68 percent). Finally, in Greece,

Gaitanis et al. (2002), using a DNA-PCR procedure, directly applicable to pathological skin scales, detected and identified only *M. globosa* from PV scales, an identification also confirmed by positive culture and biochemical tests.

An interesting point, already discussed by earlier authors, is whether the species associated with PV can vary depending on clinical and geographical distribution. Ingham and Cunningham in 1993 affirmed that this discussion had started at the beginning of the century. Castellani (1925), for instance, thought that pityriasis seen in tropical zones was a separate entity, and Panja (1927) divided the disorder into two diseases, named pityriasis versicolor (due to *M. furfur*) and pityriasis flava (due to *M. tropica*). In temperate zones, lesions affect the upper trunk principally and, although there are variations under direct microscopy, they regularly show globose cells. In tropical regions, by contrast, lesions preferentially affect the face and limbs and oval or cylindrical cells are found. Midgley (2000) concluded that the species responsible for PV could vary with the body site and different regions of the globe. According to Crespo Erchiga's experience, only 1 percent of PV cases displays, under direct scale examination, oval or cylindrical yeasts (Figure 17). The lesions showing this kind of picture are usually of an atypical distribution, affecting the face or limbs, and seem to be found mostly in patients from tropical regions. In Guého's experience, the number of PV cases for which the responsibility of *M. furfur* can be proved increases significantly when material taken from lesions of PV is incubated at 37°C (Guého and Meyer 1989). Indeed, *M. furfur* is a regular skin inhabitant and this species is responsible for all deep infections due to a lipid-dependent *Malassezia* species whatever the geographical origin of the patient (Boekhout et al. 1998; Theelen et al. 2001). However, this species has been unexpectedly rare in most recent investigations using either the Dixon or Leeming and Notman media. *M. furfur* may also be considered to be a regular but sparsely distributed inhabitant of human skin, taking advantage of an ecological niche only when the other species are eliminated or not present.

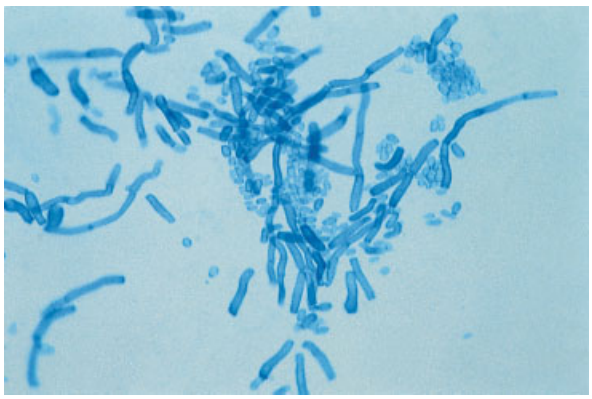

Figure 17 *Atypical oval yeasts and pseudohyphae in PV scales. The patient was a child with hypochromic lesions on the face. KOH+Parker ink, ×1 000*

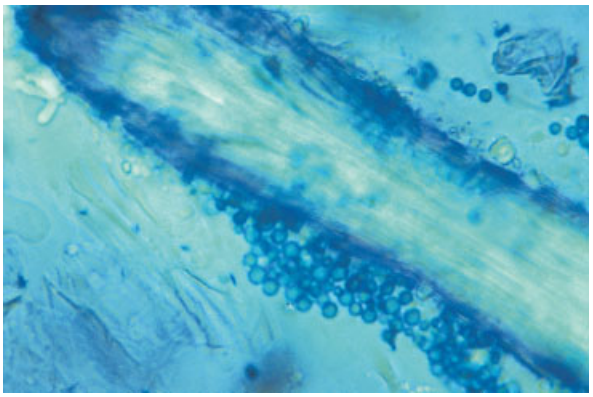

Figure 18 *Direct microscopy of PV scales, with* M. globosa *yeasts surrounding a villous hair and penetrating the follicle. KOH+Parker ink, ×1 000*

The immunological responses of PV patients to *Malassezia* yeasts have been investigated with equivocal results. Wu and Chen (1985) found an increase in humoral *Malassezia* antibodies in patients, compared to control subjects, while other studies did not find any difference between the two groups (Faergemann 1983; Midgley and Hay 1988; Ashbee et al. 1994a). Conflicting results have also been documented in investigations of cellular immunity.

The treatment of PV is simple, with rapid disappearance of lesions after treatment with different antifungal drugs, given either orally or topically, although the achromic areas can persist for weeks and even months. Topically, the most common substances used are selenium sulfide, propyleneglycol, and antifungal azoles. In extensive cases, the short-term use of systemic drugs, such as ketoconazole or itraconazole, is recommended. However, in spite of good initial results, the rates of recurrence after treatment are very high, about 60 percent after 1 year and 80 percent after 2 years, probably because underlying conditions and local factors remain unmodified. The maintenance of yeasts in the deep portions of the pilosebaceous follicles, frequently observed in KOH (Figure 18), may also explain such a high rate of recurrence. Prophylactic itraconazole treatment, 200 mg 1 day per month for 6 consecutive months, showed mycological cures in 88 percent of cases at the end point (Faergemann et al. 2002).

Seborrheic dermatitis

Seborrheic dermatitis (SD) is a chronic, relapsing skin disorder, characterized by the appearance of greasy, scaly, reddish patches localized in sebum-rich areas, such as the scalp, eyebrows, paranasal and middle thoracic

regions. Dandruff, which affects 5–10 percent of the adult population, is considered to be the mildest or initial form of the disease. AIDS patients have an increased incidence of SD, suggesting that the immune system plays an important role in the pathogenesis of the disease. The incidence is also higher in patients with neurological disorders such as Parkinson's disease, multiple sclerosis, and mood depression. The rare cases of *Malassezia* blepharitis can also be related to this skin disorder (Thygeson and Vaughan 1954; Ninomiya et al. 2002).

The relationship between *Malassezia* yeasts and SD is still a matter of controversy. Sabouraud (1932), during the first half of the past century, considered the yeast *Pityrosporum malassezii* to be the causative agent of the disease. In a critical review of the literature, Shuster (1984) described *P. ovale* as the etiologic agent. Since then, numerous studies have shown that a relationship exists between the severity of SD and the density of *Malassezia* cells present in dandruff. The taxonomic revision has generated several studies, aimed at tracing the different distribution of species in SD lesions. In Japan, Nakabayashi et al. (2000) isolated *M. furfur* (35 percent) and *M. globosa* (22 percent) from SD facial lesions of 42 patients, and less from normal subjects. In Canada, during a study using contact plates from 28 SD patients, Gupta et al. (2001a), found decreasing proportions of *M. globosa*, *M. sympodialis*, and *M. slooffiae*. In Spain, Crespo Erchiga et al. (1999a) concluded, in a survey of 75 SD patients, that *M. restricta* (43 percent) and *M. globosa* (34 percent) were the predominant species on the scalp and face of SD patients. Indeed, *M. sympodialis* (19 percent), *M. slooffiae* (2.5 percent), and *M. furfur* (0.8 percent) were much less prominent in this clinical feature. In more than 50 percent of lesional skin specimens, more than one species was obtained in culture. By contrast, healthy skin of the same area (forehead) remained sterile in 90 percent of samples, whereas 62 percent of the healthy trunk skin (shoulders) of the same patients yielded *M. sympodialis* predominantly. This striking difference might, of course, reflect the use of shampoos containing antifungal additives.

Crespo Erchiga and Delgado Florencio (2002) in a new study of 100 SD patients confirmed that *M. restricta* (65 percent) and *M. globosa* (64 percent) were the predominant species in SD lesions, followed by *M. sympodialis* (27 percent), mainly in lesions located on the trunk, and *M. furfur* (4 percent). In the healthy skin of the forehead, *M. sympodialis* was found in 20 percent of patients and *M. globosa* in 10 percent, whilst on the shoulders *M. sympodialis* was present in 58 percent of individuals and *M. globosa* in 7 percent. The same year, Gemmer et al. in the USA (Cincinnati), in a survey of 70 subjects with SD, using a direct fluorescent nested rDNA PCR of scalp samples, demonstrated that *M. restricta* (50–72 percent, depending on the flaking levels) and *M. globosa* (33–45 percent) were the predominant species in dandruff, whilst *M. sympodialis* was detected in a very small percentage (7–8 percent), and *M. furfur* not at all.

The increased incidence of SD in inmunosuppressed patients suggests that immune mechanisms are important in the pathogenesis of this disease. Both cellular and humoral immune responses have been investigated with conflicting results. Some studies of humoral *Malassezia* antibodies, compared with normal subjects, have shown increased titres in patients (Ashbee et al. 1994b; Silva et al. 1997a), whereas another did not show significant differences (Parry and Sharpe 1998). Lately, Midgley (2000) has shown that 72.5 percent of SD patients and 32 percent of PV patients had precipitating antibodies against *M. globosa*, in contrast to only 20 percent of normal control subjects. The results of testing cellular immunity are also contradictory. Meuber et al. (1996) found a reduced lymphocyte stimulation reaction when SD patients' cells were stimulated with *P. ovale* extract. By contrast, Ashbee et al. (1994b) showed an increase in both lymphocyte transformation responses and leukocyte migration inhibition. Faergemann et al. (2001) have reported increased numbers of NK1+ and CD16+ cells and in complement staining that suggest that an irritant nonimmunogenic stimulation of the immune system is important in the pathogenesis of the disease. The results of interleukin (IL) assays (increase in the production of inflammatory IL and in the regulatory IL for both Th1 and Th2 cells) showed similarities to the immune response described for *Candida* infections.

Kesavan et al. (2000) compared the inmunomodulatory capacity of normal and lipid-depleted *Malassezia* yeasts (using *M. sympodialis*, *M. globosa*, and *M. restricta*). They demonstrated that the extraction of lipids reversed their capacity to reduce the levels of proinflammatory cytokines. The lipid microfibrillar layer of *Malassezia* cells may prevent them from inducing inflammation and explain their normal commensal status on human skin. The absence or alteration of this lipid layer may provide an explanation for the inflammatory nature of the lesions.

Treatment options include selenium sulfide-, pyrithione zinc-, piroctone olamine-, or ketoconazole-containing shampoos, topical terbinafine solution or ketoconazole cream as well as topical corticosteroids. The corticosteroids, even the mildest ones such as hydrocortisone acetate, provide a rapid improvement of skin and scalp lesions, and relapse may be delayed by combining corticosteroids with ketoconazole. These favorable treatment responses suggest that *Malassezia* yeasts, especially *M. restricta* and *M. globosa*, are important in the etiology of SD and dandruff.

This skin disorder is also known from animals. Historically, the species *M. pachydermatis* was isolated from an exfoliative dermatitis of an Indian rhinoceros (Weidman 1925), and now the species is regularly

reported to cause SD in various animals, dogs and cats particularly, possibly because pets are more often investigated than other animals (Guillot et al. 1998; Guillot and Bond 1999). Bond and Lloyd (2002) compared IgG responses of various healthy and infected dogs, using Western immunoblotting. They found differences in the immunoreactivity according to the type of dog.

Atopic dermatitis

Atopic dermatitis (AD), or atopic eczema, is a common chronic inflammatory skin disease of unknown etiology. Clemmensen and Hjorth suggested for the first time in 1983 a role for *Malassezia* yeasts in this disease. Furthermore, they found a significant effect of oral ketoconazole in the treatment of adult patients with eczematous lesions on the head and neck. Since then, many reports highlighted the importance of such yeasts in AD. Faergemann has published comprehensive reviews of this disorder (Faergemann 1999, 2002). He concluded that *Malassezia* yeasts may play a major role as allergens in some patients, especially those with a head and neck eczema distribution, and suggested that treatment of such cases should include antifungal therapy. Tengvall Linder et al. (2000) found *P. orbiculare*-specific IgE in serum from 13/15 AD patients, and a positive patch test reaction in 8 of them, whereas SD patients and healthy controls were radioallergoabsorbent test (RAST) and patch test negative. They concluded that *Malassezia* yeasts can trigger an eczematous reaction in sensitized patients. Devos and van der Valk in 2000 have also shown that *P. ovale*-specific IgE is strongly related to a head and neck AD localization. Koyama et al. (2000) have described a *M. globosa* glycoprotein, named Malg46b, as a possible major antigen for IgE antibodies in these patients. Using a nested PCR technique, Sugita et al. (2001) compared the cutaneous colonization by *Malassezia* species in AD patients and healthy subjects. *M. restricta* and *M. globosa* were detected in approximately 90 percent of the patients, and *M. sympodialis* and *M. furfur* in about 40 percent (species mixture in a certain number of cases). In control subjects, *M. restricta*, *M. sympodialis*, *M. globosa*, and *M. furfur* were isolated at a frequency ranging from 61 to 11 percent. They concluded that the four species are common inhabitants of the skin of both AD patients and healthy subjects, but with an increased prevalence of *M. restricta* and *M. globosa* in AD patients. A new species, closely related to *M. sympodialis*, was isolated during this AD study from three patients, and was described as *Malassezia dermatis* by Sugita et al. (2002). However, its rarity excludes this species as being solely responsible for the disease. In 2003, Sugita et al., using the rRNA intergenic spacer (IGS) 1 region, divided *M. globosa* into four major groups and suggested that one of these genotypes may play a significant role in AD. Just after that, the same team (Sugita et al., 2003b) described *Malassezia japonica*, another new species that may be implicated in AD. This species is able to assimilate Tween 40 and Tween 60, but not Tween 20 and Tween 80; however, this is not clearly defined as yet.

Further studies are needed to clarify the inmunological as well as the mycological aspects of this dermatological condition, including a clear identification of etiologic species and of their antigens. Indeed, studies using *M. restricta* and *M. globosa*, the two major species recognized in AD, are still very rare (Koyama et al. 2000). When old names are cited, the connection of these organisms to the present species is not evident. *P. orbiculare* and *P. ovale* in such studies probably correspond to *M. sympodialis* in most cases (Mayser and Gross 2000), or to *M. furfur*.

Malassezia folliculitis

Mallassezia folliculitis (MF) is a benign disorder, characterized by follicular papules and pustules, localized predominantly on the back and chest. The condition is probably common, but often misdiagnosed as acne. The pruritus, associated with the absence of comedones and facial lesions, may facilitate the distinction between the two diseases. MF is more frequent in tropical countries, or during the summer in temperate regions. Occlusion seems to play an important role in this disorder, which has also been associated with antibiotic treatment (tetracyclines), corticosteroids, and immunosuppression (Alves et al. 2000). A minor epidemic outbreak has been reported in an intensive care unit (Archer-Dubon et al. 1999), and, during a 4-month period, 11 cases were described (out of 198 patients) in heart transplant recipients receiving immunosuppressive treatment (Rhie et al. 2000).

Direct microscopy and histopathology of pustules show the presence of abundant *Malassezia* yeasts in the pilosebaceous follicles. Back et al. (1985) observed round yeasts in most cases. At that time they identified yeasts obtained in culture as *P. orbiculare*. Such results suggest that *M. globosa*, as in PV, could be the predominant MF causative agent. Rhie et al. (2000) referred some of their cases as either being due to *M. furfur* or *M. pachydermatis*, but these authors did not apply the new *Malassezia* taxonomy. However, if hair follicle colonization by *Malassezia* yeasts is not abnormal, the diagnosis of MF is confirmed indirectly by the positive response to the antifungal therapy. Topical treatment may be effective in most cases, whilst others may respond to oral ketoconazole, itraconazole, or fluconazole.

Confluent and reticulate papillomatosis of Gougerot–Carteaud

The role of *Malassezia* yeasts in this rare cutaneous disorder, characterized by confluent hyperkeratotic papulae, grayish-brown pigmented, and located prefer-

entially on the trunk, is controversial. The diagnosis of confluent and reticulate papillomatosis of Gougerot–Carteaud (CRP), as that of MF, is based both on the observation (direct microscopy and histopathology) of lipophilic yeasts in the stratum corneum, and on a favorable response to topical or systemic antifungal treatments. In 1969, Roberts and Lachapelle suggested that CRP could represent a peculiar host reaction to heavy colonization by *P. orbiculare*. However, in the series revised by Nordby and Mitchell in 1986, only 5 of 19 patients responded to topical treatment with selenium sulfide. Other reports described complete recovery of CRP using retinoids (Baalbaki et al. 1993), minocycline (Shimizu and Han-Yaku 1997) or azithromycin (Raja Babu et al. 2000). Furthermore, two cases were also successfully treated with topical vitamin D analogs, either calcipotriol (Gülec and Seçkin 1999) or tacalcitol (Ginarte et al. 2002).

It is possible that this rare dermatological disorder represents more than one pathological condition, with *Malassezia* yeasts involved in only a few cases, perhaps those with a clinical pattern similar to that of PV. It could also be a basic disorder of keratinization, determined by an abnormal host response to *Malassezia* yeasts, or to bacteria or their products.

Neonatal *Malassezia* pustulosis

Since 1991, a few cases, characterized by non-follicular papulopustules located on face and neck of newborn infants, have been reported (Aractingi et al. 1991; Rapelanoro et al. 1996; Pont et al. 1998). The diagnosis, made in most of these cases by direct microscopy and histopathology, shows typical *Malassezia* yeasts. Moreover, the lesions cleared up after topical ketoconazole therapy. Rapelanoro et al. assumed that this condition could have been misdiagnosed as neonatal acne. Two more studies (Niamba et al. 1998; Bernier et al. 2002) have suggested that *M. sympodialis* could be the triggering agent for this rash.

Otitis

In 2000, a case of malignant otitis externa caused by *M. sympodialis* has been described in Australia (Chai et al. 2000). The patient, a man with a poorly controlled type II diabetes mellitus, had developed a severe infection, which started around the external auditory canal, and progressed with marked destruction of the temporal bone and an adjacent large soft tissue mass, below the skull base. The infection showed a total resolution after therapy with intravenous amphotericin B and fluconazole.

In animals, especially dogs, the role of the species *M. pachydermatis* in otitis is now well recognized by veterinary teams (Guillot et al. 1998; Guillot and Bond 1999; Crespo et al. 2000a, b).

Onychomycosis

A few onychomycosis cases, involving *Malassezia* spp., have been reported (Crozier and Wise 1993; Silva et al. 1997b; Escobar et al. 1999). However, the presence of *Malassezia* yeasts in nail material, a well-known though infrequent finding, is probably caused by subungual colonization by members of the cutaneous flora which makes the relevance of such cases uncertain. *Malassezia* yeasts have been observed occasionally in patients with distal fingernail onycholysis, associated with *Candida* spp. (Crespo Erchiga, personal observation). *M. furfur* was identified from an AIDS patient with an infected nail (Guého, unpublished result), and in a series of patients with onychomycosis in Brazil (Silva et al. 1997b). This species, which is also able to form hair nodules (Lopes et al. 1994, with identification confirmed afterwards by E. Guého), seems to be the only *Malassezia* species able to survive in such unusual conditions. The role of *Malassezia* yeasts in psoriasis (nails or scalp) is similarly questionable (Elewski 1990).

MOLECULAR METHODS OF IDENTIFICATION

The lipophilic yeasts of the genus *Malassezia* remained for years a complex and indeterminate group with a taxonomy based more on clinical and epidemiologic features than mycological characteristics, as demonstrated by the four former species *P. ovale*, *P. pachydermatis*, *P. orbiculare* and *P. canis*. The absence of a sexual cycle, appropriate classification of yeast morphology, and the absolute growth requirements for complex media were the reasons for this situation. Access to molecular methodology, in particular large subunit (LSU) ribosomal RNA sequencing, has led to a solution of these problems (Guillot and Guého 1995; Guého et al. 1996). Once the taxa were defined genetically, it was possible to define key characteristics applicable for routine use in any microbiological laboratory (Guého et al. 1998). However, such methodologies are time consuming (minimum 4 days after growth of cultures), and demand a high standard of practice in laboratory work. More and more laboratories are now equipped with PCR and are able to develop reproducible, precise and, above all, rapid molecular methods of identification. However, the molecular method must be chosen depending on the purpose of the study, either identification of species, typing of strains for epidemiologic surveys, or in vivo diagnosis from hosts. Indeed, the unique and ideal technique for every application does not exist as yet.

Species identification

RIBOSOMAL DNA (OR RNA) SEQUENCING

The taxonomic revision of the genus *Malassezia* was based on sequencing of the large subunit of ribosomal RNA D1/D2 domains which allowed investigators to define the seven species (Guillot and Guého 1995; Guého et al. 1996). These papers showed that this variable rRNA was a good alternative to nuclear DNA/DNA reassociation for yeast taxonomic work. Indeed, the former conventional characters of the nDNA guanine+cytosine content (GC %) and nDNA/DNA reassociation have now been used to validate each specific sequence. The DNA methodology, which is time consuming and fastidious yet still does not provide sufficient information, has now been abandoned in favor of rRNA or rDNA sequencing. Because most yeast mycologists have adopted the same rDNA region, recognition of an unusual yeast is becoming comparatively easy. Furthermore, with a sequence library extending day after day, a new species may be instantly positioned among the other related species (Nell et al. 2002), and in the classification of the Fungi (Sugita et al. 2002). In common with these latter authors, other mycologists have completed LSU rDNA sequencing with the use of rDNA ITSs. However, at the species level, one must keep in mind that strains of the same species may display about a 1.0 percent sequence divergence in the rRNA or rDNA D1/D2 regions (Kurtzman and Blanz 1998), and even more with other regions (Makimura et al. 2000). However, in 1997, Guillot et al., using the former rRNA D1/D2 sequences, recognized within the sole species *M. pachydermatis* seven sequence types (sequevars), with up to a 2 percent base substitution, but they did not divide the species. By contrast Sugita et al. (2002) described a new species *M. dermatis* which differs from *M. sympodialis* by only a 1.2 percent base substitution in their 26S rDNA D1/D2 and internal transcribed spacer 1 (ITS1)/internal transcribed spacer 2 (ITS2) region sequences.

PCR-RESTRICTION ENDONUCLEASE ANALYSIS

PCR-restriction endonuclease analysis (REA) with PCR of the 26S rRDA sequence allows one to separate the seven species (Guillot et al. 2000). The PCR products are digested with only three restriction endonucleases, *Ban*I, *Hae*II, and *Msp*I, which generate a unique pattern for each species. Moreover, this technique is very cheap and rapid, and detects and characterizes mixtures of several species. Gupta et al. (2000) have also assayed a similar analysis but the patterns generated were useful in the identification of only five species out of seven.

PULSED FIELD GEL ELECTROPHORESIS

Pulsed field gel electrophoresis (PFGE) shows that all *Malassezia* species display different karyotypes that do not vary intraspecifically, except in *M. furfur* (Boekhout et al. 1998). Senczek et al. (1999) applied PFGE, coupled to phenotypic characteristics, successfully to identify 220 *Malassezia* isolates, mainly from dogs. However, this technique, which has confirmed the seven genetic *Malassezia* species, does not appear to be the best choice for rapid species identification.

Molecular typing of *Malassezia* species

RNA AND DNA SEQUENCES OF IGS1 AND ITS1 REGIONS

rRNA sequences of the IGS1 region allowed the characterization of four genotypes within the species *M. globosa* with short sequence repeats CT(n) and GT(n). The authors (Sugita et al. 2003a) suggested also that one of these genotypes could be related to AD.

DNA sequences of ITS1 region may be used to identify (see above) or type species. Makimura et al. in 2000 using ITS1 sequencing were able to separate the seven *Malassezia* species and to recognize 22 ITS1-identical, individual groups. Among the 46 clinical isolates, they identified only *M. furfur* (11 strains from PV, four from SD, and four from AD), *M. sympodialis* (seven from PV, three from SD, one from AD and 11 from healthy controls), and *M. slooffiae* (three from chronic otitis and two from healthy controls).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Restriction fragment length polymorphism (RFLP) of nDNA detects more or less specific variability according to the choice of the region amplified and the restriction enzyme battery. This technique allows investigators to trace sources of *M. pachydermatis* in bloodstream infections. One isolate was correlated in this way to a nurse's pet dog (Chang et al. 1998).

RANDOM AMPLIFIED POLYMORPHIC DNA

Random amplified polymorphic DNA (RAPD) implies a genomic DNA PCR using oligonucleotides as primers. In contrast to specific sequences of an rRNA- or rDNA-determined region, the RAPD markers are amplified by using arbitrary sequences. Boekhout et al. (1998) applied this methodology to the genus *Malassezia*, and found genetic variations in all species, particularly with four out of the 20 decamer primers tested.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM

Amplified fragment length polymorphism (AFLP) is a powerful method for fingerprinting genomic DNA, as well as generating a large number of dominant markers for genotypic analysis (Mitchell and Xu 2003). The

technique, which combines the strategy of enzymatic digestion and, usually, two PCR steps, is able to generate numerous fragments, when varying the restriction enzymes and the selective primers. In addition, the fragments are stable and highly reproducible. Like RAPD markers, AFLP markers are amplified partly by arbitrary primers, but with greater reproducibility and fidelity. However, this sophisticated technique, which generates complex patterns, is more appropriate for solving an unusual epidemiologic question. Theelen et al. (2001) found better typing resolution of *Malassezia* yeasts, in particular within *M. furfur*, with AFLP than with RAPD. Using AFLP, the seven species were separated and four genotypes could be distinguished within *M. furfur*, the genotype four containing only isolates from deep human sources.

MULTILOCUS ENZYME ELECTROPHORESIS

Multilocus enzyme electrophoresis does not use PCR technology but is among the most cost-effective methods for investigating genetic variations within a species at the molecular level. It was applied to *M. pachydermatis* (Midreuil et al. 1999), which was confirmed to be genetically heterogeneous. A total of 27 electrophoretic types were identified that could be divided into five groups with different host specificity. Unfortunately, traces of lipids used to grow lipid-dependent species were present, a problem that could not be resolved in this study.

In vivo identification of *Malassezia* species

Malassezia yeasts, particularly *M. globosa* and *M. restricta*, have fastidious culture conditions and exceedingly different growth rates. These difficulties have stimulated investigators to devise molecular techniques that are able to recognize *Malassezia* species directly from hosts. In 2001, Sugita et al. proposed the first method. They examined variations in cutaneous colonization by *Malassezia* species in AD patients and healthy subjects. DNA was extracted directly from dressings and amplified in a nested PCR assay. The diversity of *Malassezia* species found in AD was greater than in healthy subjects. In 2002, two teams described similar methods. Gaitanis et al. extracted *Malassezia* DNA directly from pathological skin scales by single and nested PCR. These authors amplified the rDNA ITS genes and then analyzed the PCR products by the technique of RFLP. The same analyses were obtained from positive cultures obtained from the same site and identified using basic biochemical tests. They obtained a good correlation with the two approaches and recognized the seven species, which were present as single or multiple isolates in the same specimen. The second paper (Gemmer et al.) investigated only the scales of dandruff from two groups of subjects, differentiated by their rate of composite adherent scalp flaking scores. Their technique involved fluorescent nested PCR of ITSs, coupled to a terminal fragment length polymorphism analysis. In this work, *M. restricta* and *M. globosa* were found to be the predominant species present in both groups, while the authors eliminated *M. furfur* as the causal organism for dandruff in their patients.

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The dermatophytes

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HISTORY

Historically, Agostino Bassi (1835–36) was the first to elucidate the microbial nature of a deadly disease of silkworms (*Bombyx mori*). Through meticulous studies and animal experiments carried out over a period of 25 years, he established unequivocally that a mold, now known as *Beauveria bassiana*, was the cause of a devastating disease of the commercially valuable silkworm. This was the first microorganism to be recognized as causing disease, long before the classic work of Robert Koch and Louis Pasteur. The discovery that a fungus could cause dermatophytosis was probably made by Robert Remak, who observed unusual microscopic structures in favic lesions. According to Seeliger's (1985) historical account, Remak claimed that he did not at first recognize the structures he saw as being fungal. Instead, he credited this recognition to his mentor, Professor Johann L. Schönlein, who described the fungal etiology in 1839. Remak (1845) later, however, cultured the causal fungus, established that it was infectious, and described the etiologic agent, in the context of its attendant disease symptoms, as *Achorion schoenleinii*.

Although Remak has priority for the discovery of the first fungus causing human disease, the real founder of medical mycology, based on his discoveries from 1841 to 1844, was the Parisian physician, David Gruby (Gruby 1841a, b, 1843, 1844; Zakon and Benedek 1944). Unaware of the observations of Remak and Schönlein, Gruby described the clinical and microscopic features of the causal agent of favus, and established the contagious nature of the disease (Gruby 1841a, b). He also described ectothrix hair invasion in beards and scalp hair of other patients, and named the etiologic agent involved *Microsporum audouinii*. The generic name he established referred in its etymology to the small spores that surrounded the hair shaft (Gruby 1843). Gruby (1844) also described a fungus he saw causing endothrix hair invasion as *Herpes (Trichophyton) tonsurans*.

In the early 1890s, Raimond Sabouraud, a French dermatologist, convincingly established the 'plurality' of the ringworm fungi and integrated the mycological and clinical aspects of ringworm. Based on the advances made by his contemporaries in medicine and veterinary science, as well as on his personal observations, Sabouraud wrote and published his monumental *Les teignes* in 1910. He classified dermatophytes into four genera: *Achorion*, *Epidermophyton*, *Microsporum*, and *Trichophyton*. This taxonomy was based on the clinical aspects of the disease that they caused, combined with their cultural and microscopic characters.

Emmons (1934) modernized the taxonomic scheme of Sabouraud. He critically reviewed the myriad dermatophyte species that had been described by Sabouraud and later workers in the European school, and discarded genera and species based on extrinsic factors such as their clinical manifestations and the host from which they were isolated. In addition, he rejected the

attribution of undue taxonomic import to trivial and highly variable characters, such as colony texture, chlamydospores, nodular organs, pigmentation, racquet hyphae, and spiral appendages. He discarded the genus *Achorion* and redefined the remaining three anamorphic genera – *Epidermophyton*, *Microsporum*, and *Trichophyton* – based on conidial morphology and other relatively reliably formed microscopic structures. Two decades later, nutritional, physiological, and morphological studies (Georg 1952; Silva and Benham 1952; Benham 1953; Swartz and Georg 1955; Ajello and Georg 1957; Georg and Camp 1957; Padhye et al. 1980) simplified the identification of the dermatophytes and led to a reduction in the number of species recognized.

The discovery of the teleomorphs (sexual or perfect states) of *Trichophyton ajelloi*, *Trichophyton terrestre*, *Microsporum gypseum*, and *Microsporum nanum* (Dawson and Gentles 1961; Stockdale 1961), using the hair-bait technique (Vanbreuseghem 1952), led to rapid discoveries of the teleomorphs of several other dermatophyte species and related keratinophilic taxa. The successful oral treatment of experimental *Microsporum canis* infections in guinea pigs with griseofulvin, by Gentles in 1958, opened new doors to the therapy of the dermatophytoses, especially ringworm of the scalp.

ETIOLOGIC AGENTS

The dermatophytes are closely-related keratinophilic fungi causing dermatophytosis (ringworm or tinea) by virtue, in part, of their ability to degrade keratin and thus to invade the skin, hair, and nails. The three anamorphs (asexual, conidial or imperfect state) of genera *Epidermophyton*, *Microsporum*, and *Trichophyton* are recognized as belonging to the family *Arthrodermataceae* of the order Onygenales, a group also containing other human pathogens such as *Histoplasma capsulatum* and *Coccidioides immitis*. Formerly, these asexual states were placed in a separate group known as the Deuteromycota or Fungi Imperfecti, but molecular studies and official changes in the guidelines for fungal nomenclature have allowed the abandonment of this artificial taxonomic category.

The dermatophytes capable of reproducing sexually are considered to be members of the genus *Arthroderma* (Weitzman et al. 1986) in the family *Arthrodermataceae*. The currently recognized dermatophytes and related nonpathogenic dermatophytoids are listed in Table 1 along with the names of their corresponding teleomorphs.

Physiologically, the dermatophytes facilitate distinction with special growth media by virtue of their high tolerance for cycloheximide and their ability to use proteins as a sole carbon source, releasing excess ammonium ion in a way that causes the pH of protein-containing media to rise into the alkaline range.

Table 1 *Dermatophytes and their nonpathogenic relatives: teleomorph and anamorph states*

Teleomorph (sexual or perfect state)	Anamorph (asexual or imperfect state)
Arthroderma spp.	*Microsporum* and *Trichophyton* spp.
A. benhamiae	*T. mentagrophytes* complex in part
A. cajetani	*M. cookei*
A. fulvum	*M. fulvum*
A. grubyi	*M. vanbreuseghemii*
A. gypseum	*M. gypseum* complex in part
A. incurvatum	*M. gypseum* complex in part
A. otae	*M. canis*
A. obtusum	*M. nanum*
A. persicolor	*M. persicolor*
A. racemosum	*M. racemosum*
A. simii	*T. simii*
A. vanbreuseghemii	*T. mentagrophytes* complex in part

Relatively few other fungi except some closely-related nonpathogenic members of the *Arthrodermataceae* combine these attributes, and among those that do, e.g. some *Exophiala* species in the 'black yeast' family *Herpotrichiellaceae*, most do not resemble dermatophytes in culture.

Morphological identification of the dermatophytes today essentially follows the classification scheme of Emmons: it is predominantly based on the microscopic characters of the anamorph and on the macromorphology of the colony on Sabouraud's glucose (dextrose) agar (SGA). The addition of 1 percent yeast extract to SGA, or use of other media such as cornmeal glucose agar or potato glucose agar may be necessary to stimulate conidiation. In addition, nutritional and other physiological tests may be required for species identification, especially of the *Trichophyton* spp.

Colonies should be examined for the color of the surface and underside, presence of diffusible pigment, surface texture, topography, and rate of growth. With regard to microscopic morphology, the types of conidia present, and their shapes and sizes, are essential criteria for identification; the presence of other structures such as pectinate (comb-like), coiled or antler-like hyphae, reflexive branches, chlamydospores or nodular organs may be helpful.

Many dermatophytes, especially those primarily associated with nonhuman hosts, regularly produce two kinds of conidia, large multicellular macroconidia and smaller unicellular microconidia. The presence or absence of these conidial types and the appearance of the walls of macroconidia (rough or smooth) are of generic significance. Species identification is largely based on the morphology and arrangement of the conidia.

Epidermophyton (Sabouraud 1907)

This genus is characterized by the presence of numerous, broadly clavate, nearly smooth-walled macroconidia; microconidia are absent. There is only a single recognized species, *E. floccosum*. It is anthropophilic, that is, specifically adapted to cause ongoing human infection. This species attacks skin, nails, and, very rarely, hair (Sherna et al. 1993).

Microsporum (Gruby 1843)

Members of this genus usually produce both macroconidia and microconidia. The essential distinguishing feature is the presence of macroconidia that have rough walls, with textures ranging from spiny to warty. The macroconidia vary in shape from egg-shaped (obovate) to cylindrofusiform; they may have thin to thick cell walls and 1–15 septa depending upon the species. The roughness of the cell walls may not be readily apparent in some isolates or species, and may require special media to induce its formation (Kane et al. 1997). Similarly, special media may be required for conidial production. Microconidia are typically clavate (club-shaped). In microscopic examination, one should also note the formation and arrangement of both types of conidia. Members of this genus regularly attack skin and hair but only occasionally nails.

Trichophyton (Malmsten 1845)

Smooth-walled macroconidia and microconidia are produced by typical members of this genus. The macroconidia may range in shape from elongate to pencil-shaped, clavate, fusiform, or cylindrofusiform. They are multiseptate and may be thin or thick walled. Microconidia are usually produced in greater abundance than macroconidia and their shape varies from pyriform, clavate, spherical to elongate. They are borne along the hyphae singly or in clusters and are sessile or borne on short stalks. Their arrangement on fertile hyphae is one of the important factors in their identification. Members of this genus attack skin, hair, and nails. For many *Trichophyton* spp., special media may be required to stimulate conidial formation. Some species produce few or no conidia regardless of the growth conditions used.

Table 2 summarizes the essential features of the dermatophyte genera; Table 3 summarizes the recognized species, along with some nonpathogenic relatives, and Tables 4 and 5, (p. 7) present short synopses of the characteristic features of the species.

ECOLOGY

Dermatophytes have been grouped into geophilic, zoophilic and anthropophilic species based on the ecological niches in which they naturally occur. For pathogens, this classification is based on their host preferences as manifested at the population level (Georg 1960; Ajello 1962). That is to say, sporadic occurrences of pathogenic species on atypical hosts are ignored. Table 6, (p. 8) summarizes classification of dermatophyte species according to ecology and host preference.

Geophilic species

Geophilic species are considered ancestral to the pathogenic dermatophytes (Chemel 1980; Ozegovic 1980; Rippon 1988; Weitzman and Summerbell 1995). The natural habitat of these species is the soil, where they are associated with decomposing keratinous material, e.g. hair, feathers, horns, hooves, nails, etc. Carnivore dung predominantly containing animal hair is another prominent habitat; the inoculum presumably comes from underlying soil. Exposure to the soil is the main source of infection for humans and lower animals (Alsop and Prior 1961; Chemel and Buchvald 1970; Ajello 1974; Rippon 1985). Transmission of geophilic species from lower animals to humans or from human to human is rare. *M. gypseum*, one of the best examples of a geophilic dermatophyte, was reported as the causal agent of two unusual outbreaks in association with soil

Table 2 *Characteristics of the genera of the dermatophytes*

Genus	Macroconidia	Microconidia	Clinical
Microsporum	Rough walls; egg-shaped, fusiform; usually present and more numerous than microconidia	Usually present; pyriform or clavate; typically borne singly along the hypha	Members may attack skin, hair, rarely nails. *M. persicolor* does not invade hair
Trichophyton	Smooth-walled; pencil-shaped, clavate, fusiform to cylindrofusiform; often absent or less numerous than microconidia	Usually more numerous than macroconidia but may also be scarce or absent; clavate, pyriform or spherical, in clusters or singly along the hypha	Members may attack skin, hair or nails. *T. concentricum* does not attack hair
Epidermophyton	Smooth-walled; broadly clavate; singly or in banana-like clusters	Absent	Attacks skin and nails; generally does not invade hair

Table 3 *Dermatophytes and their nonpathogenic relatives: species of the genera* Epidermophyton, Microsporum, *and* Trichophyton

Epidermophyton	*Microsporum*	*Trichophyton*
E. floccosum	*M. audouinii*	*T. concentricum*
	M. canis	*T. equinum*
	M. cookei	*T. erinacei*
	M. ferrugineum	*T. interdigitale*
	M. fulvum	*T. megninii*
	M. gypseum	*T. mentagrophytes*
	M. gallinae	*T. rubrum*
	M. nanum	*T. schoenleinii*
	M. persicolor	*T. simii*
	M. praecox	*T. soudanense*
	M. racemosum	*T. tonsurans*
	M. vanbreuseghemii	*T. verrucosum*
		T. violaceum

(Alsop and Prior 1961; Rippon 1985). One such outbreak occurred in a cucumber greenhouse among workers handling soil enriched with horn and hoof fertilizer. Secondary infections occurred in children and adults in close contact with the infected greenhouse workers (Alsop and Prior 1961).

Zoophilic species

Zoophilic species have gradually evolved from soil to parasitize animals (Chemel 1980; Ozegovic 1980; Rippon 1988). They have lost their ability to survive over the long term in nonsterile soil, though their mating to form teleomorphic structures cannot occur on the host and must therefore occur on shed hairs of the host, along with other such keratinous materials, on soil. These fungi are primarily animal parasites or may be carried by apparently healthy, wild animals (Feurman et al. 1975; Chemel 1980; Ozegovic 1980; Pier et al. 1994). Human infections are acquired either by direct contact with an infected animal or indirectly by contact with fomites or other inanimate objects associated with keratinous material from the animal (Pier et al. 1994; Thomas et al. 1994; Weitzman and Summerbell 1995). Human infections by *M. canis*, an important zoophile, are usually acquired from cats and dogs (Rippon 1985; Pier et al. 1994).

Anthropophilic species

Anthropophilic dermatophyte species appear generally to have evolved from zoophilic species, although recent molecular studies indicate that *E. floccosum*, possibly derived from within the *M. gypseum* complex, may be an exception (Gräser et al. 2000a). Humans are the normal hosts for this group of species and transmission may occur by direct contact or indirectly by fomites (Weitzman and Summerbell 1995). Human-to-animal transmission of infection by an anthropophilic species is rare but has been documented in the literature (Kaplan and Gump 1958; Mayr 1989).

The evolution from saprobic life in soil to an almost exclusive existence as a colonizer of keratinized tissue of humans generally corresponds to a decrease or loss of conidial formation (Rippon 1985; Rippon 1988), as well as an inability to reproduce sexually (Rippon 1988; Tanaka et al. 1992; Summerbell 2002). This loss of sexual reproduction (Kwon-Chung and Bennett 1992) is observed in almost all of the anthropophilic dermatophytes, although a small number of apparently anthropophilic members of the *Trichophyton mentagrophytes* complex (isolates with velutinous morphology traditionally associated with the name *Trichophyton interdigitale*) retain the ability to mate as *Arthroderma benhamiae* or *Arthroderma vanbreuseghemii* in laboratory tests (Watanabe and Hironaga, 1981; Vasilyev and Bogomolova, 1985; Hejtmánek and Hejtmánkova 1989).

Certain zoophilic and anthropophilic species may represent an intermediate stage in the evolution of dermatophytes towards loss of the ability to reproduce sexually. *Arthroderma otae* (anamorph *M. canis*) exists predominantly as one mating type but is still capable of reproducing sexually when paired with the rare + mating type (Weitzman and Padhye 1978). Many anthropophilic and a few zoophilic species are apparently incapable of reproducing sexually but appear to exist exclusively as one mating type; they may be induced to form rudimentary sexual organs (abortive gymnothecia) when paired with appropriate tester strains of *Arthroderma simii* (Stockdale 1968; Young 1968; Kwon-Chung and Bennett 1992). An example of such a species is *Trichophyton rubrum*, which consists entirely of strains of (−) mating type.

In addition to a decrease or loss of conidial production and the ability to reproduce sexually, anthropophilic fungi generally tend to produce chronic infections that are less likely to resolve spontaneously (Rippon 1988). The distinction between a geophilic and zoophilic species may not always be obvious and may even be controversial. A typical geophilic species is *M. gypseum*. Its natural habitat is soil and it is not always associated with animals (Weitzman and Summerbell 1995). *M. gypseum* can be isolated readily from garden soil by using Vanbreuseghem's hair-baiting technique (Vanbreuseghem 1952). In addition, it can not only survive and flourish in the soil environment, but also propagates by producing numerous macroconidia in nature (Gordon 1953). It is also a common sporadic animal pathogen, and may cause limited outbreaks, but it is not known to cause enduring, chronic, directly transmissible infections in populations of any animal species. Although *M. nanum* causes ringworm mainly in pigs, its partially geophilic nature has been well established by its isolation from soil as well as by the demonstration that its characteristic macroconidia could be seen directly in soil suspensions (Ajello et al. 1964). If it

Table 4 *Characteristic of pathogenic* Epidermophyton *and* Microsporum *species in culture*

Species	Colony on SGA	Microscopic morphology	Key tests, features or comments
Epidermophyton floccosum	Surface olive, khaki or yellow-brown, flat or radially folded, white tufts with age; suede-like texture; reverse yellow-brown, slow growth rate	Numerous macroconidia, 20–40 × 6–8 μm; broadly clavate, 0–4 septa, single or in clusters	No microconidia
Microsporum audouinii	Surface white, gray or tan; flat or velvety; reverse salmon-pink or rust; moderate growth	Conidia few or absent; macroconidia bizarre, fusiform, elongated, thick, rough walled, septa irregular in intervals; microconidia clavate, borne singly along sides of hyphae, pectinate hyphae, terminal or intercalary chlamydospores	Brownish discoloration of rice grains and poor growth; does not perforate hair in vitro
M. canis (= *M. canis* var. *distortum*)	Surface white to buff, woolly, reverse yellow, orange to orange-brown; nonpigmented and dysgonic variants occur; growth rapid	Numerous macroconidia, fusiform with thick, rough walls, 18–125 × 5–25 μm, knobbed apex, up to 15 septa; few microconidia; in *M. canis* var. *distortum* many distorted thick, rough-walled macroconidia	Heavy sporulation on rice grains; perforates hair in vitro
M. cookei	Surface yellowish, reddish tan or lavender, powdery or granular; reverse deep wine-red; moderate growth	Numerous macroconidia, mostly ellipsoid, thick walled, some moderately thin walled resembling *M. gypseum*; abundant microconidia	Must be distinguished from *M. gypseum* and *M. racemosum*
M. ferrugineum	Surface yellowish to rust; heaped, folded, waxy; very slow growth	Irregular hyphae lacking conidia; coarse hyphae with prominent septa ('bamboo hyphae')	Does not perforate hair in vitro
M. gallinae	Surface white to pinkish, satiny, slightly folded; reverse strawberry red diffusible pigment; rapid growth	Macroconidia elongate, curved with blunt tips, smooth to slightly roughened at tips, 2–10 cells, 6–8 × 15–50 μm; microconidia present	Characteristic diffusible red pigment; does not perforate hair in vitro
M. gypseum complex (*M. gypseum*, *M. fulvum*)	Surface tan, rosy-buff, cinnamon, powdery to floccose; reverse buff or reddish brown; rapid growth	Numerous ellipsoidal to fusiform macroconidia, 25–60 × 7–15 μm, thin, rough walls; up to 6 septa; microconidia abundant	Differentiation of *M. gypseum* from *M. fulvum* by inducing the teleomorph on special media
M. nanum	Surface cream to tan; powdery; reverse reddish brown; moderate growth	Abundant ovoid or egg-shaped 1–3 celled macroconidia, 10–30 × 6–13 μm; microconidia rare to moderate	Colony resembles *M. gypseum* but with slower growth rate; macroconidia slightly resemble conidia of *Trichothecium roseum*
M. persicolor	Surface yellowish buff to peach; powdery or downy; reverse pink to reddish brown	Abundant spherical microconidia; usually in clusters, some clavate to pear-shaped; macroconidia clavate, smooth, thin walls, rough walls with age or on special media; spiral hyphae often present	Uncommon pathogen; perforates hair in vitro, growth at 37°C minimal; rosy colonies on peptone agar; no alkalinity on BCP milk solids glucose agar in contrast to similar-looking *T. mentagrophytes*
M. praecox	Surface pale yellow to yellow, powdery to velvety; pale yellow to orange on reverse; moderate growth	Abundant long, fusiform macroconidia, 40–90 × 7–17 μm, thin, smooth to spiny macroconidia, 2–8 septa; microconidia absent	Rare pathogen; does not perforate hair in vitro

(*Continued over*)

Table 4 *Characteristic of pathogenic* Epidermophyton and Microsporum species in culture (*Continued*)

Species	Colony on SGA	Microscopic morphology	Key tests, features or comments
M. racemosum	Surface beige-cream, granular; flat; wine red reverse; rapid growth	Abundant macroconidia, fusiform to ellipsoidal, 41–77 × 9–10 μm; resembles *M. gypseum*; numerous clavate, stalked microconidia in grape-like clusters (racemes)	Must be differentiated from *M. gypseum*, *M. cookei*; rare pathogen; perforates hair in vitro
M. vanbreuseghemii	Surface yellowish, cream or pink; powdery to downy; buff to pale yellow reverse	Numerous cylindrofusiform macroconidia, 44–87 × 11 μm; up to 12 septa, thick walls smooth to spiny; numerous microconidia	Rare pathogen; perforates hair in vitro

were a true geophile, however, it would be distributed in many environments lacking its specific animal population host, but this has never been demonstrated for *M. nanum*. Visualization of the characteristic macroconidia in the environment offers proof that a capability for saprobic existence has been retained, in that these characteristic conidia are not formed in infected animals or humans (Ajello 1974; De Vroey 1985). Summerbell (2002) has pointed out that the ability for sexual reproduction, a process that can only occur in the context of saprobic growth, can be correlated with numerous other characters indicating an alternatingly soil-borne and host-borne existence for most zoophilic dermatophytes.

Some mycologists consider *Microsporum persicolor* and *T. simii* to be geophilic (Padhye and Thirumalachar 1967; Matsumoto and Ajello 1987; Kwon-Chung and Bennett 1992), whereas others have treated them as zoophilic (Rippon 1988; Tanaka et al. 1992). Both species have been isolated repeatedly from soil and from the hair of apparently healthy animals harboring these fungi without any ringworm lesions (Gugnani et al. 1967; Contet-Audonneau and Percebois 1986). *M. persicolor* is most frequently isolated from rodents, notably bank voles (Ozegovic 1980) and can cause severe human infections (Rippon 1988). The isolation of *M. persicolor* from the cutaneous lesion of a woman and from her garden soil (both isolates being of the same mating type) (Contet-Audonneau and Percebois 1986) could be taken to suggest geophilism, though recent contact of infected voles with the garden in question can by no means be excluded. Similarly, *A. simii* (anamorph *T. simii*) causes sporadic ringworm in dogs, monkeys, poultry, and humans in India (Gugnani et al. 1967). Macroconidia associated with *M. persicolor* and *T. simii* have not been observed in the direct examination of soil samples.

EPIDEMIOLOGY

Species identification of the dermatophytes and knowledge of their host preference and ecology play an important role in epidemiology, public health issues, and infection control. Of special concern are those fungi capable of producing family or institutional outbreaks. Knowledge of the geographically endemic dermatophytes such as the African *Trichophyton soudanense* is also relevant to epidemiology, as it allows pinpointing exposure during travel, residence, or contact with a person from an endemic area. Geographical distribution will be covered elsewhere in this chapter (see Geographical distribution).

Geophilic fungi such as *M. gypseum* are usually transmitted from a soil source and can be secondarily transmitted by animals to humans (Kaplan et al. 1957).

Infections caused by *M. canis* can involve a variety of animals as hosts, but cats and dogs are the principal carriers (Rebell and Taplin 1974; De Vroey 1985; Rippon 1985, 1988; Kwon-Chung and Bennett 1992). Fomites play an important role in the acquisition of infections (De Vroey 1985; Thomas et al. 1994). Wakimoto et al. (1985) reported an outbreak of ringworm caused by *M. canis* in Japan involving a kitten, fomites, and person-to-person spread of the infection. However, institutional outbreaks of infections in adults in a chronic healthcare facility (Shah et al. 1988), and in neonates (Mossovitch et al. 1986; Snider et al. 1993), have been reported with no clearly demonstrated link to an infected animal. Transmission in these cases resulted from person-to-person spread and fomites. *Trichophyton verrucosum* and zoophilic forms of *T. mentagrophytes* are typically encountered in rural areas and are acquired by humans in these locations mostly from herd animals, usually cattle (Georg 1956, 1960; Rippon 1985). Tinea capitis and tinea corporis are the most common manifestations in children and tinea corporis, while tinea barbae occurs in adult males (Rippon 1985). Fomites play an important role in zoophile infections, as noted in an outbreak caused by the mouse dermatophyte, traditionally called *T. mentagrophytes* var. *quinckeanum* (Blank 1957; Georg 1960) but recently renamed *T. mentagrophytes sensu stricto* (i.e. 'in the strict sense of the name') (Gräser et al. 1999c). Granular varieties within the *T. mentagrophytes* complex, formerly referred to as *T. mentagrophytes* var. *mentagrophytes*, have rodent

Table 5 *Characteristics of common pathogenic* Trichophyton *species in culture*

Species	Colony on SGA	Microscopic morphology	Key tests, features, or comments
T. concentricum	Surface cream, amber or brown; heaped, folded; glabrous to velvety; very slow growth	Conidia absent, only sterile hyphae, antler-like hyphae lacking terminal swelling may be present	50% isolates stimulated by thiamine; geographically limited
T. equinum	Surface white, fluffy, dome shaped; becoming buff and folded; reverse lemon yellow to reddish brown, growth moderate	Abundant microconidia, variable, spherical, pyriform to elongated clavate forms, borne usually singly along hyphae, occasionally in small clumps; macroconidia rare, clavate, smooth walled	Requires nicotinic acid; autotrophic strains only known from Australia and New Zealand; some isolates perforate hair in vitro
T. erinacei	Surface white, powdery, flat; yellow pigment on reverse; rapid growth	Numerous clavate to pyriform microconidia borne singly along the hyphae and in regular clusters; macroconidia clavate to cylindrical, smooth, thin walled, rare	Perforates hair in vitro; urease test often slow
T. interdigitale anthropophilic subtype	White to cream-colored surface, downy to fluffy, reverse tan	Microconidia few, moderate or heavy, pyriform, singly along hyphae	Perforates hair in vitro
T. megninii	Surface pinkish, velvety; radial folding, reverse wine red; moderate growth rate	Abundant pyriform to clavate microconidia; macroconidia pencil-shaped, smooth, rare	Requires L-histidine; urease positive; in urea indole broth, may be negative in Christensen urea broth in 7 days
T. mentagrophytes complex, zoophilic types	Surface creamy tan to pink; powdery to granular, flat; reverse buff to red or reddish- brown; rapid growth	Abundant spherical microconidia in grape-like clusters; macroconidia smooth walled, clavate to pencil-shaped, spirals often present	No nutritional requirement; urease positive; perforates hair in vitro
T. rubrum	Surface typically white, velvety to fluffy, occasionally powdery to granular; reverse wine red, rarely yellow or brown diffusible pigment; moderate to slow growth; dysgenic, lavender heaped varieties rare	Microconidia scanty to numerous, pyriform borne singly along the hyphae; macroconidia rare in fluffy isolates, numerous pencil-shaped to clavate macroconidia in granular to powdery colonies, 15–30 × 4–6 μm	Urease negative except in some flat, heavily conidial isolates of the afro-asiatic variant, in vitro hair perforation negative
T. schoenleinii	Surface glabrous, waxy, heaped, cerebriform becoming velvety, white, gray to tan; very slow growth, cracks agar	Conidia usually absent; microconidia in old, velvety subcultures; antler-like hyphae (favic chandeliers) with swollen nail-head tips; chlamydospores and hyphal swellings frequent	Autotrophic for vitamins, equal growth at 25–37°C differentiates it from *T. verrucosum*
T. simii	Surface white, cream or buff; powdery, granular, flat; reverse straw to salmon; rapid growth	Abundant clavate to fusiform to cylindrical, thin-walled macroconidia, 30–80 × 6–11 μm, fragmenting or with intercalary chlamydospores; microconidia pyriform, clavate, singly along the hyphae, spirals may be present	Resembles *T. mentagrophytes* but produces an unusually high number of macroconidia in isolates that have been transferred several times
T. soudanense	Surface flat, yellow to wine red, with star-like margin in primary isolates	Macroconidia absent, microconidia clavate, scarce or absent; reflexive branches are seen along larger hyphae within radial striations	May or may not appear to have vitamin requirements

(*Continued over*)

Table 5 *Characteristics of common pathogenic* Trichophyton species in culture (*Continued*)

Species	Colony on SGA	Microscopic morphology	Key tests, features, or comments
T. tonsurans	Surface white, cream, pale yellow to sulphur yellow, tan, pink; powder, suede-like or velvety; flat or raised and folded; mahogany reddish-brown reverse; slow growth	Microconidia numerous, clavate or elongate, some swollen, variable in size and shape, borne singly along hyphae, often on relatively long, empty 'matchstick' stalks and often attached to open branched clusters of hyphae; macroconidia rare, cylindrical, smooth, thin walled, tips often bent on one side	Growth stimulated by thiamine; urease positive; in vitro hair perforation variable; major incitant of endothrix type of ringworm
T. verrucosum	Surface cream to tan, flat, velvety, discoid; white, heaped glabrous to downy; yellow-ochre, glabrous, convoluted; very slow growth	Conidia usually absent; microconidia clavate; macroconidia 'rat-tail', irregular; chlamydospores in chains characteristic ('string of pearls') best at 37°C	About 16% of isolates require thiamine and inositol; growth stimulated at 37°C (faster than at 25°C)
T. violaceum	Surface lavender to deep purple, glabrous, heaped, very slow growth; white glabrous variants may occur	Conidia typically absent except after extended cultivation on thiamine-rich medium; irregular hyphae and chlamydospores present	Growth and sporulation stimulated by thiamine

Gräser et al. (2000c) concluded that *T. megninii* is synonymous with *T. rubrum* and that *T. gourvilii*, *T. soudanense*, and *T. yaoundei* were synonymous with *T. violaceum*; Summerbell (2003), however, concluded that *T. megninii* was a distinct species and that *T. gourvilii* was a synonym of *T. soudanense*, while *T. yaoundei* was a synonym of *T. violaceum*.

or rabbit-family (lagomorph) population hosts, and inoculum from such sources have caused infections in numerous laboratory workers (Georg 1960; Hironaga et al. 1981; Sewell 1995). Zoophilic members of the *T. mentagrophytes* complex (including isolates mating as *A. benhamiae* as well as isolates mating as *A. vanbreuseghemii*) have been isolated from many mammalian species (Ozegovic 1980; Rippon 1985), but chronic population carriage is only known from rodents, lagomorphs, and hedgehogs to date.

Table 6 *Classification of dermatophytes based on ecology and host preference*

Geophilic	Zoophilic	Anthropophilic
M. cookei	*M. canis*	*E. floccosum*
M. gypseum complex	*M. gallinae*	*M. audouinii*
M. nanum	*T. equinum*	*M. ferrugineum*
M. persicolor[a]	*T. erinacei*	*T. concentricum*
M. praecox	*T. mentagrophytes* complex, in part	*T. interdigitale*
M. racemosum	*T. verrucosum*	*T. megninii*
M. vanbreuseghemii		*T. rubrum*
T. simii[a]		*T. schoenleinii*
		T. soudanense
		T. tonsurans
		T. violaceum

a) Considered as zoophilic by Rippon (1988) and Weitzman and Summerbell (1995)

Some examples of less common infections contracted from other animals include *Microsporum gallinae* from fowl (Rippon 1985), *M. nanum* from pigs (Ajello et al. 1964; Morganti et al. 1976), *Trichophyton equinum* from horses (Georg et al. 1957; Zuckerman et al. 1992), *T. mentagrophytes* var. *quinckeanum* from mouse (Blank et al. 1961), *Trichophyton erinacei* (= *T. mentagrophytes* var. *erinacei*) from hedgehogs (Morris and English 1973; Philpot and Bowen 1992), *M. persicolor* from field voles (English et al. 1978; Onsberg 1978), and *T. simii* from poultry, dogs, and monkeys (Gugnani et al. 1967; Tewari 1969). Infections acquired from animals are usually inflammatory, but are more likely to resolve spontaneously than are infections caused by anthropophilic dermatophyte species (Rippon 1988; Kwon-Chung and Bennett 1992).

Infections caused by anthropophilic fungi are mostly acquired by direct contact with infected humans; fomites also play an important role and infection may even be acquired after aerosolization of infectious arthroconidia. *M. audouinii* and *T. tonsurans* have been isolated from the air (Friedman et al. 1960; Arnow et al. 1991) and several other dermatophyte species belonging to all three ecological groupings have been reported from house dust (Shimmura 1985). *M. audouinii* is an example of the extent of contagion and the potentially rapid spread of ectothrix tinea capitis. It was responsible for an epidemic in Europe in the nineteenth century that was later exported to North America and ended in the

mid-1950s (Rippon 1985). It was essentially eliminated by griseofulvin, but was replaced by a *T. tonsurans* epidemic that swept into the south and southwest of the USA and Canada, apparently derived from an ongoing epidemic in Puerto Rico and other Hispanic countries (Rippon 1985). Tinea capitis, in the form caused by *T. tonsurans*, is an endothrix type of scalp infection. It may persist subclinically in the scalp, resulting in long-term carriers who may shed viable propagules for decades (Weitzman and Summerbell 1995). In adults, it is more frequently manifested as tinea corporis and uncommonly as tinea manuum and tinea unguium (Weitzman and Summerbell 1995).

T. tonsurans has also been responsible for several institutional outbreaks (Mackenzie 1961; Kane et al. 1988; Arnow et al. 1991). One such outbreak of nosocomial tinea corporis occurred in a pediatric ward. The infection was spread by the index patient to the staff and a visitor. *T. tonsurans* was isolated from the air, soiled linen and the backs of chairs (Arnow et al. 1991). Another nosocomial outbreak was reported in a nursing home for elderly people and persisted for 9 months despite remedial and sanitary measures (Kane et al. 1988). The index patient, who was immobile, infected a staff member who, in turn, may have spread the infection to other immobile patients who in turn infected other staff. *T. tonsurans* was found in several environmental samples. A third outbreak occurred in a residential school for girls where clinical infections were found in 21 children, mostly as tinea capitis and less frequently as tinea corporis or tinea unguium (Mackenzie 1961). The fungus was isolated from such inanimate sites as the air, combs, brushes, bedding, floors, and a curtain. Tinea corporis among adolescents and young adults, caused by *T. tonsurans*, has been reported from contact sports such as wrestling (Stiller et al. 1992; Beller and Gessner 1994).

Tinea capitis is most commonly a dermatophytosis of childhood, as is tinea corporis caused by *M. canis*. Other forms of tinea are seen most commonly in adolescents and adults, and are most commonly caused by anthropophilic dermatophytes. Tinea cruris (groin), tinea pedis (feet), and tinea unguium (nails) are frequently caused by *T. rubrum*, *T. interdigitale* (a name recently redefined after molecular studies by Gräser et al. (1999c) and used here in its modern sense), and *E. floccosum*, although this may vary with geographical location. Fomites play an important role in transmission, but host factors such as immunological status and local factors such as trauma, excessive moisture or occlusive clothing may constitute risk factors when combined with exposure to the fungus.

Outbreaks of tinea cruris involving *T. rubrum* and *E. floccosum* have been attributed to sources of contagion such as toenail clippings, bedding, toilet seats, and saunas, as well as to exchange of clothing (Neves and Xavier 1964; Lundell 1974; Rippon 1988). Ringworm caused by other anthropophilic species such as *T. violaceum* and *T. schoenleinii* can be transmitted from person to person by clothing, cotton caps, pillows, combs, and towels (Padhye 1962; Kwon-Chung and Bennett 1992). Infection by *Trichophyton concentricum* is transmitted from mother to child after birth by contact (Rippon 1988), but fomites may also play a role in transmission (De Vroey 1985). Contagion either by direct contact or from fomites has been essentially established in tinea capitis, tinea corporis, and tinea barbae. With regard to tinea pedis, exogenous exposure to the fungus in showers, swimming pools, locker rooms, etc., plays an important role in the acquisition of clinical disease, though host susceptibility factors may also be involved (Gentles 1957; Gentles and Holmes 1957; Gip 1967; Zaias et al. 1996). Viable propagules of dermatophytes are shed from the feet of people with tinea pedis and these fungi have been isolated from areas where people walk bare footed and from towels, shoes, and socks (Ajello and Getz 1954; Gentles 1957; Gip 1967; Fujuhiro 1994). Epidemiological study showed a threefold increase of tinea pedis when communal showers were installed in a coal mine (Gentles and Holmes 1957).

The dermatophyte propagules most commonly associated with contagion in all anthropophilic dermatophyte infections and most zoophile infections are the substrate arthroconidia found within desquamated epithelium and hairs; these conidia can survive for an extended period of time in the environment (McPherson 1957; Mackenzie 1961; Kane et al. 1988; Rippon 1988; Kwon-Chung and Bennett 1992).

GEOGRAPHICAL DISTRIBUTION

The geographical distribution of the anthropophilic and zoophilic dermatophytes, rather than being static within a city, state, or country, is dynamic. It tends to be affected by periods of social change featuring changing immigration patterns, health habits, standards of living, or propensities for travel. For example, before 1900, tinea capitis was rare in North America, and was caused mostly by *M. canis* transmitted by animal contact (Rippon 1985). From 1900 to the mid-1950s a 'gray patch' ectothrix type of ringworm in children, caused by *M. audouinii*, spread over the USA and Canada. As mentioned above, this epidemic was in turn displaced by 'black dot' endothrix ringworm caused by *T. tonsurans* (Rippon 1985, 1992; Babel 1990), which was for the most part imported from Latin America and the Caribbean but went on to become the current predominant cause of tinea capitis in North America (Rippon 1985; Gupta and Summerbell 2000). *T. tonsurans* is now the second most commonly isolated dermatophyte in the USA; only *T. rubrum* is more common. *M. audouinii*, although still cultured sporadically, usually in connection with recent migrants from Africa, has virtually disappeared in the

USA (Sinski and Kelley 1991). In some southern European and middle eastern countries, *M. canis* is the most frequently isolated dermatophyte and threatens to become a serious epidemiological problem as a result of carriage by feral cats (Lunder and Lunder 1992). The geographical distribution of the geophilic, zoophilic, and anthropophilic dermatophytes is shown in Tables 7, 13.8 and 13.9. Dermatophytes endemic in certain geographical areas may be isolated outside such locations as a result of travel and immigration. *Trichophyton violaceum* (= *T. soudanense*), for example, which is widespread in Africa, has been reported among African immigrants in several European countries and in North America (Kwon-Chung and Bennett 1992; Viguie et al. 1992). For a more comprehensive review of the geographical distribution of the dermatophytes, readers should refer to publications by Ajello (1960); Sinski and Flouras (1984); Rippon (1988) and Kwon-Chung and Bennett (1992).

MOLECULAR TAXONOMY AND PHYLOGENETIC RELATIONSHIPS

In recent years, dermatophytes have been subject to extensive molecular systematic study. It has been confirmed that all sexual and asexual dermatophytes are, indeed, closely related to one another, and that all are members of the family *Arthrodermataceae*. (Anamorphic species and genera are now correctly said to belong to teleomorphic families in mycology when this information is unambiguously known, e.g. through molecular study.) Though further study is needed before firm conclusions can be drawn, it appears there may be four genera rather than three within the traditional grouping of dermatophytes and dermatophytoids. (The latter term refers to nonpathogenic species in genera containing dermatophytes.) The studies of Y. Gräser and colleagues (Gräser et al. 1999b, 2000a) have found that most of the nonpathogenic *Trichophyton* spp., e.g. *T. ajelloi* and *T. terrestre*, form a clade separate from the pathogenic species. *Microsporum* appears to be a distinct genus, but *Epidermophyton*, while supported as separate by some studies (Kano et al. 1999, 2002), is apparently contained within *Microsporum* in other studies (Gräser et al. 1999b, 2000a, b). Molecular mycologists tend to find it inconsistent to recognize the anamorphs in a clade as a separate genus, but not at the same time to recognize the generic status of the corresponding teleomorphs. Application of this viewpoint in the dermatophytes would tend to revive the generic name *Nannizzia* for teleomorphs in the *Microsporum* clade. No formal proposal to this effect, however, has yet been made.

At the species level, the separate status of all known sexual species within the dermatophytes has been upheld wherever studied. It has been found, however, that many asexual species appear to have arisen relatively recently in evolutionary time, and each of them except *E. floccosum* is closely related to a known sexual species. This has caused the validity of some species to be questioned. Numerous taxonomic challenges are posed by unusual population genetics patterns that can be seen within the asexual dermatophytes and their

Table 7 *Geographical distribution of geophilic dermatophytes*

Geophilic	Distribution
M. cookei	Cosmopolitan
M. gypseum and *M. fulvum*	Cosmopolitan
M. nanum	Cosmopolitan
M. persicolor	Cosmopolitan
M. praecox	Western Europe, North America
M. racemosum	Romania, Venezuela, USA
M. vanbreuseghemii	Russia, Africa, India, USA
T. simii	India

Table 8 *Geographical distribution of zoophilic dermatophytes*

Species	Geographical distribution in major animal hosts
M. canis	Cosmopolitan
M. canis var. *distortum*	Australia, New Zealand, USA
M. canis var. *equinum*	Africa, Australia, Europe, New Zealand, North and South America
M. gallinae	Cosmopolitan
T. equinum	Cosmopolitan
T. erinacei	Europe, New Zealand
T. mentagrophytes complex (zoophilic types)	Cosmopolitan; Australia, Canada, Eastern Europe, Italy
T. verrucosum	Cosmopolitan

Table 9 *Endemic geographical distribution of anthropophilic dermatophytes, excluding isolates in travelers and recent immigrants*

Species	Major geographical regions
E. floccosum	Cosmopolitan
M. audouinii	Parts of Africa
M. ferrugineum	Far East, West Africa, Eastern Europe
T. concentricum	Parts of Asia, Oceania, isolated areas inhabited by indigenous peoples in parts of Latin America
T. megninii	Portugal, Sardinia, Corsica, and parts of Africa
T. interdigitale	Cosmopolitan
T. rubrum	Cosmopolitan
T. schoenleinii	Parts of Africa and Asia
T. tonsurans	Cosmopolitan
T. violaceum	North and east Africa, west Asia

immediate sexual relatives. In some cases, sequencing studies have found interbreeding strains within a sexual species to be apparently more distantly related to one another than some of them are to certain morphologically and physiologically distinct asexual species. Moreover, in such cases, the genetically varied individual sexual species and the genetically restricted asexual species to which they appear to have given rise tend to infect different population hosts. For example, based on ribosomal internal transcribed spacer (ITS) sequencing, oligonucleotide polymerase chain reaction (PCR) typing, and amplified fragment length polymorphism (AFLP) typing, CBS 495.86, the feline-derived, (+) mating type, taxonomic cotype strain of *Arthroderma otae*, the teleomorph of *M. canis*, is more closely related to the anthropophilic, asexual species *M. audouinii* than it is to the (–) *A. otae* mating type strain, CBS 496.86, that serves as its complementary cotype (Gräser et al. 1999c). While it is not difficult to conceive that one clone of the (+) mating type of the cat dermatophyte may have transformed itself within a moderately short evolutionary time frame into a morphologically and physiologically distinct organism with a uniquely strong genetic adaptation for causing ectothrix infection of human preadolescent scalps, it is somewhat controversial to interpret this event as a speciation process. Sequences of noncoding and housekeeping parts of the genome, e.g. the ribosomal sequence and associated spacer regions, tend to have changed only slightly between at least some *A. otae*/*M. canis* strains and *M. audouinii*. At the same time, it is clear that in both interbreeding status and general population genetics, *M. canis* has a strong potential unity as a 'biological species,' and also has a common habitat leading to stabilizing selection of pathogenicity factors, morphology, and other aspects of phenotype. None of these factors is shared by *M. audouinii*, the connection of which to *M. canis* is purely historical. In view of this population genetics divergence, Summerbell (2002) has recently proposed recognizing *M. audouinii* and all other 'unifactorial asexual radiate lineages' within the dermatophytes, that is, all single-mating-type, asexual dermatophyte lineages that have undergone a population-host shift in respect to their most closely related sexual relative, as being distinct at the species level.

Some long-recognized asexual dermatophyte species have been formally proposed as synonymous with closely related species as a result of molecular phylogenetic studies. For example, *T. equinum* has been proposed as a synonym of *T. tonsurans* by Gräser et al. (1999c) based on ITS sequence and other molecular similarities. The rejection of this synonymy has subsequently been suggested, however, since the former dermatophyte, with equine population hosts, is of the (+) mating type, while the latter, with human hosts, is of the (–) mating type (Summerbell 2003). This evidence of long-term evolutionary separation was not acknowledged in the study originally proposing the synonymy of these species. Existing data suggest separate, though relatively evolutionarily recent, origins from a common ancestor rather than conspecificity for *T. equinum* and *T. tonsurans*.

Trichophyton gourvilii, *T. soudanense*, and *T. yaoundei* have been suggested to be synonyms of *T. violaceum* (Gräser et al. 2000c). Summerbell (2003), citing the apparently premature synonymization of *T. tonsurans* and *T. equinum* based on similar techniques, has called for additional confirmatory molecular genetic study before the morphologically and geographically distinct cluster containing *T. soudanense* and *T. gourvilii* is synonymized with *T. violaceum*.

Population genetics studies within other traditionally defined dermatophyte species have revealed highly divergent patterns. *T. rubrum*, for example, has been found to be characterized by a high degree of overall genetic uniformity suggestive of recent subclonal derivation from a preexisting clonal lineage that also gave rise to *T. violaceum* and *T. soudanense* (Gräser et al. 1999b, 2000c; Summerbell et al. 1999). *T. mentagrophytes*, on the other hand, has been found to subtend an unexpected degree of biodiversity. While it was long known to contain anamorphs of two biological species, *A. benhamiae* and *A. vanbreuseghemii*, and to be closely related to the *T. simii* anamorphs of *A. simii*, *T. mentagrophytes* as traditionally conceived by Emmons (1934) has recently been found also to phylogenetically encompass asexual radiates such as *T. schoenleinii*, *T. concentricum*, *T. verrucosum*, and the lineages giving rise to *T. equinum*, *T. tonsurans*, *T. soudanense*, *T. violaceum*, *T. megninii*, and *T. rubrum*. Gräser et al. (1999c) have proposed a formal nomenclatural neotypification that limits application of the name *T. mentagrophytes* to the group of isolates formerly referred to as *T. mentagrophytes* var. *quinckeanum*, the mouse favus dermatophyte. These isolates are capable of mating successfully with mating type testers of *A. benhamiae* (Weitzman and Padhye 1976); this teleomorphic species, however, is conceived as sufficiently phylogenetically distinct to be accepted as a taxon separate from *T. mentagrophytes sensu stricto* (Probst et al. 2002). This viewpoint is based on the common knowledge that in many biological groups, the ability to manifest interfertility under artificial conditions may be retained as a symplesiomorphic (inherited ancestral-type) character among closely related species that never interbreed in nature. The apparently clonal (Mochizuki et al. 1990, 1996) *T. mentagrophytes*-like lineage that is contagious among humans has been referred to a neotypified *T. interdigitale* (Gräser et al. 1999c). A few isolates compatible with this species retain an ability to mate with testers of the normally zoophilic *A. vanbreuseghemii*; the exact status of *A. vanbreuseghemii vis-à-vis T. interdigitale* is not yet settled. Certain other *T. mentagrophytes*-like lineages

that are capable of interbreeding, at least in vitro, with members of the established biological species, have also been conceived as separate species, e.g. the hedgehog dermatophyte, *T. erinacei*. In general, unraveling the complexity of these phylogenetic and tokogenetic relationships among dermatophyte lineages is a process that will require additional study.

CLINICAL FORMS

Infections caused by dermatophytes (ringworm, tineas) are clinically classified on the basis of the location of the lesions on the body. Although different body sites may be affected, each focus of infection is generally the result of local inoculation. The invading dermatophyte grows in a centrifugal manner, forming irregular rings with inflammatory borders with some clearing in the central area of the lesion. The name ringworm was based on the worm-like appearance of the lesions with irregular, inflammatory borders. The infection is named according to body site after the Latin word *tinea*. The word tinea itself comes from the clothes moth and the similarity of its effects on wool garments to the shape of the dermatophyte skin lesions (Ajello 1974). The clinical manifestations are classified as follows: tinea pedis (feet); tinea cruris (groin); tinea corporis (glabrous skin); tinea barbae (ringworm of the beard and moustache); tinea capitis (scalp, eyebrows, eyelashes); tinea manuum (hand); tinea unguium (nails); tinea favosa (favus); and tinea imbricate (ringworm caused by *T. concentricum*). The clinical manifestations and their major etiologic agents are described here briefly. More detailed information may be obtained by referring to texts by Rippon (1988); Kwon-Chung and Bennett (1992) and Kane et al. (1997).

Tinea pedis ('athlete's foot')

Ringworm of the feet involves infection of the interdigital webs and soles. The most common clinical manifestation is the intertriginous form associated with maceration, scaling, fissuring, and erythema, which presents with itching and burning. The other clinical form, which is commonly noted, involves a chronic, squamous, hyperkeratotic type of infection of the soles and heels extending up the sides of the foot (moccasin foot). This is an acute condition characterized by the formation of vesicles, and inflammation plus occasional pustules and bullae are most commonly caused by granular, zoophilic members of the *T. mentagrophytes* complex. The common agents of tinea pedis in cases of chronic infection are *E. floccosum*, *T. interdigitale*, and *T. rubrum*.

Tinea cruris ('jock itch')

This infection of the inguinal area involves the groin, perianal, and perineal areas, often involving the upper thighs. It is frequently caused in adults by *T. rubrum* and *E. floccosum*. Lesions are erythematous and scaly, exhibiting raised, inflamed borders often with vesicles. They are symptomatically associated with itching and burning. They are usually bilateral, extending down the inner thighs and over the proximal waist area and buttocks.

Tinea corporis

This ringworm of the upper parts of the body usually involves the shoulders, axilla, chest, and back. It may also involve the face, the legs, or the dorsa of hands or feet. The lesions are well marginated with raised erythematous, vesicular borders. The infection may be mild to severe. The annular, scaly patches may coalesce to form large areas of chronic infection when *T. rubrum* is the etiologic agent. Zoophilic dermatophytes frequently cause this infection.

Tinea barbae

This infection may be mild and superficial but, more often, the lesions are inflammatory and pustular, especially when caused by zoophilic dermatophytes such as *T. mentagrophytes* and *T. erinacei*. Erythematous patches are often scaly, and show lusterless hairs and folliculitis.

Tinea capitis

This ringworm involves infection of the scalp hairs and intervening skin. Infection may range from mild, with slight erythema and a few, patchy scaly areas, to severe, with inflammatory lesions involving folliculitis or kerion formation, often with secondary bacterial infection resulting in scarring and alopecia. Hair invasion occurs in two patterns, ectothrix and endothrix. In the ectothrix type of invasion, the invading fungus infects the hair shaft at mid-follicle and forms a sheath of hyphae and arthroconidia 2–3 μm in diameter surrounding the hair shaft. The infected hair become lusterless and brittle, and the hair filaments break off at the level of the scalp to give an appearance of partial alopecia. The best known agents of ectothrix scalp ringworm are *M. audouinii* (seen in Africa as a variant classically indicated by the synonymous name *M. langeronii*), *M. canis*, *M. ferrugineum*, and *M. gypseum*. *T. verrucosum* and *T.megninii* also occasionally cause ectothrix colonization of the hair shaft. In endothrix hair invasion, the hyphae invade the hair follicle and then grow into the hair shaft, forming numerous arthroconidia within it and severely damaging the hair in the process. The infected hairs become grayish-white and break off easily at the level of the scalp to give a 'black dot' appearance. The predominant cause of endothrix tinea capitis in the USA at

present is *T. tonsurans*. *T. violaceum* and *T. soudanense* are prevalent in other parts of the world. Lesions may coalesce to form large patches of alopecia.

Tinea manuum

This infection involves the palmar and interdigital areas of the hands. Lesions present as diffuse hyperkeratotic areas. Most infections are caused by *T. rubrum*.

Tinea unguium

Invasion of the nail plate by a dermatophyte is referred to as tinea unguium. Distal subungual infection is one of the common patterns of nail invasion and generally involves the nail bed and the underside of the distal portion of the nail. A mild inflammation produces hyperkeratosis and focal parakeratosis (Elewski et al. 1995). Subungual hyperkeratosis causes lifting of the nail plate and its detachment from the nail bed. *T. rubrum*, *T. interdigitale*, and *E. floccosum* are the main causes of tinea unguium in the USA (Elewski et al. 1995) as well as most other parts of the world.

Tinea favosa

Favus (meaning honeycomb) is now an exceedingly rare disease except in a few redoubts in central Africa and central Asia. It is usually caused by *T. schoenleinii*. Infections can be severe and chronic and involve pus formation in the hair follicles, a process producing cup-shaped crusts called scutula. Longstanding favus infections may result in alopecia with scarring.

Tinea imbricata

This chronic infection is caused by *T. concentricum*. Seven different clinical patterns of infection are distinguished: imbricate, featuring concentric rings of overlapping scales, lamellar, lichenified, plaque-like, annular, palmar/plantar, and ungual (Hay et al. 1984; Rippon 1988).

Dermatophyte pseudomycetoma

A distinct but uncommon dermatophyte infection is the invasion of subcutaneous tissue. In such infections, fungal elements form yellowish-white pseudogranules lacking any of the cementing material found in some true mycetomas. Granulomatous dermatitis is rare and is generally observed when a hair follicle ruptures and individual fungal hyphal elements escape into the dermis. This condition, also known as Majocchi's dermatophyte granuloma, results from instances of trauma to the infected skin. For example, a puncture wound may introduce the dermatophyte into deep tissue and facilitate invasion. Loose or compactly arranged hyphal aggregates of varying sizes embedded in eosinophil-rich Splendore–Hoeppli material may be erroneously referred to as granules or grains, by analogy with structures seen in mycetoma, and the lesions may indeed be referred to as mycetoma. However, the fungal aggregates may result from hair follicles with mycelial growth rupturing, permitting the mycelial filaments and fungal cells formed therein to escape into the surrounding dermis and continue to develop, eliciting a strong Splendore–Hoeppli reaction and granulomatous tissue response. Based on their loosely arranged mycelial aggregation surrounded by striking and abundant Splendore–Hoeppli reaction material, they should more appropriately be called pseudogranules and the lesion should be called a dermatophytic pseudomycetoma (Ajello et al. 1980; Chandler and Watts 1987). The most common cause of dermatophytic pseudomycetoma is *M. canis*. Other species that have been reported are *M. ferrugineum* and *M. audouinii* (Kwon-Chung and Bennett 1992).

IMMUNOLOGY

Dermatophytes colonize the keratinized tissue of the stratum corneum. The degree of inflammation produced in the lesions depends primarily on the species of the causal agent and, to some extent, on the immunological competence of the host. Infections by anthropophilic fungi, such as *T. rubrum*, often elicit less inflammatory response than is seen with infections caused by zoophilic and geophilic dermatophytes such as *T. verrucosum* or *T. mentagrophytes*.

Invasion by dermatophytes may be favored by environmental conditions. Warm, moist conditions are known to favor infection, as was seen in swampy areas of Vietnam featuring a high incidence of dermatophytosis in combat troops (Blank et al. 1969). Occlusion of sites exposed to dermatophyte inoculum appears to increase susceptibility. In experimental infections, this factor played a major role, apparently because it increased hydration of the underlying skin and retention of CO_2, helping dermatophyte growth (King et al. 1978).

Medical conditions such as collagen vascular disease, Cushing's disease, diabetes mellitus, hematological malignancy, atopy, and old age may play a significant role in predisposing patients to chronic dermatophytic infections, as may systemic corticosteroid therapy. Susceptibility to chronic dermatophytosis by *T. concentricum* may occur as an inherited trait (Hay 1992). An inherited autosomal recessive trait has been shown to predispose to infection by *T. concentricum* (Serjeantson and Lawrence 1977; Ravine et al. 1980). Similarly, susceptibility to chronic *T. rubrum* infection is likely to be mediated by inheritance of an autosomal dominant allele (Zaias et al. 1996).

Although a host develops a variety of antibodies as a response to a dermatophyte infection, including antibodies derived from immunoglobulins IgM, IgG, IgA, and IgE, it has been accepted that IgE plays a key role in the suppression of cell-mediated immunity (CMI), perhaps through modulating histamine activity. The variability seen among patients in humoral immunity responses to dermatophytosis has been attributed to a lack of standardized antigens and to differences in the sensitivity and specificity of the methods used (Matsumoto et al. 1996). It is known that CMI is the cornerstone of host defense in regard to dermatophytosis. The development of CMI, which is correlated with delayed-type hypersensitivity, is usually associated with clinical cure and with exclusion of the dermatophyte elements from the stratum corneum (Dahl 1993; Jones 1993). Delayed hypersensitivity responses to intradermal injections of trichophytin are commonly observed in the normal population (Wood and Cruickshank 1962; Palmer and Reed 1974; Grossman et al. 1975). These responses are probably caused by earlier exposures to dermatophytoses, or by crossreactivity with one or more related environmental organisms.

The immune response to dermatophytic infections has been investigated in human infections as well as in various experimental studies using calves, guinea pigs, rabbits, and rats. Experimental dermatophyte infections in animals appear to result in CMI to the antigens of the infecting dermatophyte (Cruickshank et al. 1960; Krebs et al. 1977; Green and Balish 1980). In guinea pigs with experimental infections caused by a member of the *T. mentagrophytes* complex, maximal erythema in the infected skin occurs at the time the infected animals developed CMI to trichophytin (Krebs et al. 1977). Similar findings have been noted in cattle experimentally infected with *T. verrucosum*. The delayed hypersensitivity reactions in the skin corresponded to clearance of infection by means of an increase in the desquamation rate of the stratum corneum (Lepper 1974; Lepper and Anger 1976; Wagner and Sohnle 1995). A live vaccine (LTF 130) against *T. verrucosum* ringworm in cattle was developed and successfully used to reduce infections in cattle herds in the former Soviet Union and some countries in eastern Europe (Segal 1989). The use of vaccine in humans, however, has not been effective.

TREATMENT

The increase in mycotic infections stimulated the pharmaceutical industry to develop new drugs to meet this challenge. The newer antifungal agents available to treat the dermatophytoses include orally active triazoles (fluconazole and itraconazole), the allylamines (naftifine and terbinafine), and the morpholines (amorolfine). These antifungals can be added to others currently in use, such as orally administered griseofulvin and itraconazole as well as topical antifungals such as haloprogin, the thiocarbonates (tolnaftate), hydroxypyridones (ciclopirox olamine), and numerous other imidazoles (Smith 1993; Gupta et al. 1994a).

Decisions regarding the best regimen for therapy must consider the extent, location, and clinical type of infection, the etiologic agent, the spectrum of activity and pharmacokinetics of the antifungal, any pharmacodynamic drug–drug interactions that may occur (Schafer-Korting 1993; Bickers 1994; Brodell and Elewski 1995), side effects (Hay 1993), cost (Einarson et al. 1994), and the overall risk/benefit analysis. The interested reader is referred to several reviews in which antifungal agents for the dermatophytoses are discussed along with their modes of action, dosages, therapeutic indications and contraindications, and drug–drug reactions (Elewski 1993; Stiller et al. 1993; Degreef and DeDoncker 1994; Gupta et al. 1994a, b; Brodell and Elewski 1995).

Oral antifungals are usually prescribed for tinea capitis, tinea unguium, chronic dry forms of tinea cruris, invasive infections, and infections resistant to topical therapy or with extensive involvement. These require extensive treatment before a clinical cure can be achieved. Generally, successful therapy necessitates compliance and good personal hygiene.

Tinea capitis

Oral griseofulvin has been the mainstay of therapy for tinea capitis since the 1950s. It is the most frequently prescribed therapy, usually requiring a 6-week course (Frieden and Howard 1994). No topical therapy has been shown to be as effective as oral griseofulvin, although topical applications of various preparations may be used as adjuncts to impede shedding of viable fungi from infected scalps (Del Palacio et al. 2000). Ectothrix infections caused by *M. canis* and other species of *Microsporum* usually respond best to griseofulvin (Jacobs 1990; Degreef and DeDoncker 1994); however, Jacobs (1990) considered ketoconazole as the first choice for treating endothrix infections caused by *T. tonsurans*.

Other studies indicate better results with oral ketoconazole (Gan et al. 1987; Tanz et al. 1988). Generally, oral ketoconazole is considered as an effective alternative when there is resistance, intolerance, or nonresponse to griseofulvin (Elewski 1994; Frieden and Howard 1994). Rare side effects, such as hepatotoxicity with long-term use of ketoconazole, limit its application to second-line treatment. Legendre and Escola-Macre (1990) and Elewski (1994) found itraconazole to be an effective alternative to either griseofulvin or ketoconazole. Gupta and colleagues (Gupta et al. 1997, 1998; Gupta and Adam 1998) investigated the efficacy of pulse therapy with itraconazole and terbinafine in children with *T. tonsurans* infections reporting 100 percent cure

rate when one to three pulses of itraconazole were used and 92 percent cure rate with terbinafine. Gupta et al. stated that, given the pharmacokinetics of both drugs, they found the pulse-dosing format to be a reasonable option. The pulse regimens allowed the physician to tailor therapy to the patient's response. Terbinafine, given orally, was found to be a safe and effective drug for treating tinea capitis (Haroon et al. 1992; Villars and Jones 1992).

Adjunct therapy may include selenium sulfide shampoos (Frieden and Howard 1994) or ketoconazole shampoo and topical econazole nitrate (Elewski 1994) to decrease inoculum shedding, as well as oral antibiotics to treat secondary infection and prednisone to diminish severe kerion (Frieden and Howard 1994). In addition to treatment with antifungals, general sanitation measures are necessary to prevent spread and recurrence of infection (Weitzman and Summerbell 1995). However, fomites and asymptomatic carriage contribute to spread and reinfection (Frieden and Howard 1994; Howard and Frieden 1995). Hebert et al. (1985) recommended treating not only the patient, but also all family members including adults, and sanitizing the home environment.

Tinea corporis

Tinea corporis, when caused by geophilic or zoophilic dermatophyte, usually resolves spontaneously within a few months. A variety of topical agents are usually applied to speed up healing of local and uncomplicated lesions (Smith 1993; Gupta et al. 1994a; Weitzman and Summerbell 1995). Topical imidazoles appeared to be highly effective with cure rates of up to 80 percent (Del Palacio et al. 2000). Amorolfine and allylamines are another alternative (Del Palacio-Hernanz et al. 1989; Del Palacio et al. 1991). Topical therapy is preferable because there is less potential for side effects. However, systemic therapy is also indicated for extensive lesions resulting from the anthropophilic dermatophyte species, for invasive lesions, and for chronic dry manifestations of tinea corporis caused by *T. rubrum*. For many years, oral griseofulvin and ketoconazole have been used as alternatives to treat recalcitrant or widespread lesions. However, griseofulvin-resistant mutants have rarely been reported (Artis et al. 1981). It has also been noted that one-third of patients do not respond to treatment with griseofulvin (Jacobs 1990). The therapeutic use of ketoconazole is limited by the rare side effects of this drug, such as liver toxicity and depressed adrenal activity and testosterone secretion (Kwon-Chung and Bennett 1992). Ketoconazole also has drug–drug interactions that may be problematical (Bickers 1994; Brodell and Elewski 1995). Itraconazole and terbinafine, taken orally, were reported to be generally safe and effective therapies against most of the common dermatophytes (Degreef and DeDoncker 1994; Gupta et al. 1994b; Weitzman and Summerbell 1995; Del Palacio-Hernanz et al. 1990; Del Palacio et al. 1993; Faergemann et al. 1997; Nozickova et al. 1998).

Tinea cruris

A variety of topical medications, applied as creams, lotions, or powders, may be used for uncomplicated tinea cruris (Smith 1993; Gupta et al. 1994a; Weitzman and Summerbell 1995). However, the dry recalcitrant type of infections caused by *T. rubrum* may require systemic therapy such as griseofulvin, itraconazole, fluconazole, or terbinafine (Gupta et al. 1994b). Relapses are more common with griseofulvin. Butenafine is effective in tinea cruris with 2 weeks of treatment, with a cure rate around 70 percent (Lesher et al. 1997). Fluconazole (150 mg once weekly) for 4–5 weeks has been effective in the management of tinea cruris and tinea corporis with a 74 percent clinical cure rate (Faergemann et al. 1997). Terbinafine was reported to be superior to griseofulvin in several studies (Degreef and DeDoncker 1994). Currently, itraconazole may be given as a dose of 400 mg/day given as two daily doses of 200 mg for 1 week as an effective treatment for tinea cruris infection (Del Palacio et al. 2000).

Tinea unguium

Tinea unguium, or dermatophyte infection of the nails, has generally been found to be difficult to cure. This has especially been noted with infections of the toenails, except in cases of superficial white onychomycosis. Tinea unguium was generally regarded as an incurable disease until the advent of systemic oral antifungal drugs. Previous treatment consisted of mechanical or chemical avulsion of the nail followed by topical therapy. Recurrence was, however, common. The use of older topical antifungal agents applied directly to the nail has given variable and disappointing results (Hay 1993; Hay et al. 1994) as a result of the inability of these agents to penetrate through the nail plate down to the nail bed where the fungus resides. Newer topically active antifungals, such as 28 percent tioconazole, amorolfine, and 8 percent ciclopirox, have produced higher remission rates than did older topical formulations, but overall cure rates have also been disappointing (Hay 1993). The use of the newer agents as an adjunct to oral antifungal therapy with griseofulvin improved recovery rates significantly (Hay 1993).

Griseofulvin was the first orally active, systemic antifungal agent applicable for the treatment of onychomycosis (fungal infection of the nail), but its use was limited to dermatophytes and required long-term therapy of 6–9 months for fingernails and 12–18 months for toenails (Roberts 1994). This was because it did not

diffuse into the nail bed but only reached the site of infection via uptake of newly produced nail keratin. In addition, without removal of the nail, overall cure rates of toenail infections were low, around 30–50 percent, and relapse was common (Roberts 1994).

Newer, orally active systemic medications have recently been shown to be effective when administered for short periods (up to 3 months) or when used intermittently. Both itraconazole and terbinafine reach the nail via incorporation into the matrix and remain unchanged in the nails at a therapeutic level for at least 6 months after termination of therapy in patients treated for 12 weeks (Hay 1993; Hay et al. 1994; Del Palacio et al. 1994, 1999). Either continuous therapy with itraconazole (200 mg/day) for 3 months or pulse therapy (200 mg twice a month) for 3–4 months may be used (Heikkila and Stubb 1997). The pharmacokinetic comparison of continuous and intermittent itraconazole pulse therapy showed that intermittent therapy resulted in relatively high itraconazole plasma concentrations but relatively low drug exposure. This resulted in a lower nail concentration of itraconazole in intermittent therapy than that obtained with continuous itraconazole therapy (Havu et al. 1999). However, this characteristic of intermittent therapy did not result in a reduced cure rate, as the concentration of itraconazole in the nail remained within the therapeutic range. The total itraconazole administered in pulse therapy was about half of what was given in a continuous therapy. This reduction in the amount of itraconazole administered reduced side effects and improved cost-effectiveness, in addition to providing increased convenience for the patient (Van Doorslaer et al. 1996; Nolting et al. 1998).

Nail removal plays a limited role in the therapy of onychomycosis (Hay 1993). It may be accomplished by surgery combined with the use of systemic antifungals before avulsion. Alternatively, it may be done chemically. The best known chemical procedure entails the use of 40 percent urea paste formulation under occlusion, sometimes with the incorporation of 1 percent bifonazole into the paste.

Although dermatophytes play a major role as causative agents of onychomycosis, nondermatophytic molds and yeasts account for a significant number of cases. Laboratory tests are needed to confirm the species involved and to rule out bacterial or nonfungal etiology; this will also ensure selection of the most effective treatment for each patient, and increase the probability of a complete cure (Elewski et al. 1995).

LABORATORY DIAGNOSIS

Direct microscopic examination

The direct microscopic examination of a properly collected specimen is one of the most rapid and effective methods of detecting a fungal infection. This highly effective screening technique will provide useful information regarding the etiologic agent, e.g. whether it is a mold or a yeast, and whether it causes ectothrix, endothrix, or favic hair invasion. In a few cases, direct specimen microscopy may suggest the genus of the causative agent.

The choice of method for the direct examination will depend upon the type of specimen screened and the laboratory's resources. The typical specimens submitted for the diagnosis of a dermatophyte infection are skin scrapings, hair stubs, and nail clippings or scrapings. The solutions usually chosen for the examination of these specimens are potassium or sodium hydroxide preparations, most commonly potassium hydroxide (10–20 percent KOH), often in combination with the fluorescent stain calcofluor white. In some laboratories and procedures, the periodic acid–Schiff (PAS) stain is used.

POTASSIUM HYDROXIDE

Potassium hydroxide is the most widely used preparation for the direct examination of clinical specimens for the presence of fungi. Basically, a drop of 10–20 percent KOH is placed on a slide, a small amount of specimen is added to the drop, a coverslip is placed over it and the preparation is gently heated (short of boiling). The KOH softens and clears the specimen for easier detection of hyphae by digesting any proteinaceous debris and disrupting the keratin's cellular sheets, thereby rendering the more biochemically resistant fungus more visible as highly refractile, hyaline, septate, branched, or unbranched hyphae and arthroconidia. In hairs, fungal elements may appear as arthroconidia on the outside (ectothrix invasion) of the hair shaft, or on the inside (endothrix), or they may appear as hyphae co-occurring with bubbles and tunnels (favic invasion). The reader is referred to several texts for descriptions and photographs of dermatophytes in invaded keratinized tissue (Fragner 1987; Rippon 1988; Weitzman et al. 1988; Kwon-Chung and Bennett 1992; Weitzman et al. 1995; Weitzman and Summerbell 1995; Kane et al. 1997).

Through the years, several modifications of the basic 10 percent KOH preparation have been made for more rapid detection. One modification is the incorporation of Parker superchrome blue-black ink in the KOH solution for selective staining of the fungus (Rippon 1988). Other modifications of the basic method include the addition of 36 percent dimethyl sulfoxide (DMSO) to 20 percent KOH to aid in the preparation and clearing of the specimen without heating (Rebell and Taplin 1974; Rippon 1988), and the addition of 5–10 percent glycerine to the KOH preparation (usually 10–25 percent for nails) to delay dehydration, crystallization of the KOH, and concomitant degradation of the fungal structures (Rebell and Taplin 1974; Weitzman and

Summerbell 1995). The slides are examined under the microscope with reduced light by lowering the condenser and adjusting the condenser's diaphragm.

Fungal hyphae must be differentiated from a variety of hypha-like artifacts such as cotton wool and synthetic fibers and from the so-called 'mosaic fungus.' This last artifact, which is more difficult to distinguish, consists of cholesterol crystals deposited around the periphery of the epidermal cells. It can be recognized by its regularity of outline, abrupt changes in width, presence of re-entrant angles in the flat crystalline structures, and lack of internal organelles (Rippon 1988). In experienced hands, the KOH preparation is one of the most useful and inexpensive diagnostic procedures in medical mycology.

CALCOFLUOR WHITE

Calcofluor white is a whitening agent used in the textile and paper industry. It binds to chitin and cellulose in fungus cell walls and fluoresces on excitation by long-wave ultraviolet rays or short-wave visible light. The use of this technique requires a fluorescent microscope with a proper ultraviolet source and a filter combination that may be too expensive for many laboratories. This method has, however, the advantage of allowing relatively easy fungal detection with reduced search time and a lower degree of technical experience (Elder and Roberts 1986). Calcofluor white can be combined with KOH for rapid clearance of the specimens. Fungal hyphae must be differentiated from textile fibers that may also fluoresce. Several publications are available describing calcofluor white application and technique (Hageage and Harrington 1984; Elder and Roberts 1986; Aslanzadeh and Roberts 1991; Harrington and Hageage 1991). Although background elements may also fluoresce, the fungal components are generally brighter and readily recognizable.

PERIODIC ACID–SCHIFF (PAS) STAIN

The PAS stain is based on the reaction of fungal cell wall polysaccharide with the PAS reagents, resulting in the fungus developing a red-violet fuchsin color in the tissue. This stain has also been applied successfully to histopathological analysis of nail plate clippings where onychomycosis was suspected (Suarez et al. 1991). One must be aware that a negative direct microscopic examination does not rule out the existence of a fungal infection.

Culture

ISOLATION MEDIA

Culture is a necessary adjunct to direct microscopic examination. It is only by culture that we can achieve definitive identification of the etiologic agent. In many instances, the choice of therapy may depend upon the specific identification of the invasive mold. This is especially important in nail and skin infections caused by nondermatophytic filamentous fungi, which are often resistant to the therapy used for dermatophyte infections (Summerbell et al. 1989).

A primary medium for the isolation of dermatophytes should be selective against bacteria and non-dermatophyte saprobic molds. Two such media that are commonly used are Sabouraud glucose agar (SGA) (a glucose peptone agar) with cycloheximide and chloramphenicol, and the dermatophyte test medium (DTM). Both media are available commercially and contain cycloheximide to inhibit saprobic contaminating molds. DTM incorporates gentamicin and chloramphenicol to inhibit bacteria and a phenol red indicator that changes color from yellow to red when the medium becomes alkaline as the result of growth of dermatophytes (Rebell and Taplin 1974). However, nonpathogenic fungi can also turn this medium red (Merz et al. 1970). Some systemically infecting fungi such as *Coccidioides immitis* (Salkin 1973) can resemble a dermatophyte on this medium, causing a serious biohazard situation. In addition, some dermatophytes, for example, *M. canis*, may give a false-negative reaction (Moriello and Deboer 1991). Therefore, DTM is a good screening medium for the isolation of dermatophytes but not a specific indicator of a dermatophyte. It also has the disadvantage of not allowing visualization of pigmentation on the reverse of the colony – a characteristic often used in identification. Clearly, for maximum yield of pathogenic fungi, it is important also to use a medium devoid of cycloheximide, especially for the culture of specimens from nails, soles and palms where nondermatophytic fungi sensitive to cycloheximide may be the causal agents (Summerbell et al. 1989; Weitzman and Kane 1991; Kane et al. 1997).

Nondermatophytic fungal elements may often resemble dermatophyte elements in direct microscopic examination, and hence culture on cycloheximide-free medium is necessary for their isolation and identification. The simple, commercially available, but effective, cycloheximide-free medium is SGA. If used without antibacterial agents, it allows bacterial growth from skin and nail specimens, and this may prevent the outgrowth of the fungi causing the infection. If used with chloramphenicol or with combined chloramphenicol and gentamicin, it can be very effective in the isolation of causal nondermatophytes; incidental contaminating fungi, of course, will also grow, and dermatophytes may do so as well. Other media containing antibacterial agents and lacking cycloheximide are inhibitory mold agar (BBL Microbiology Systems, Cockeysville, MD, USA) and Littman oxgall agar (Difco Laboratories Detroit, MI, USA) with added gentamicin and chloramphenicol (Summerbell et al. 1989; Weitzman and Kane 1991). Most of these media are available commercially. Specialized isolation media include: a Casamino acids/erythritol/albumin medium for the isolation of

dermatophytes from lesions heavily contaminated with bacteria or with cycloheximide-tolerant *Candida albicans* (Fischer and Kane 1974); and a bromcresol purple (BCP)/casein yeast extract agar designed for the isolation and recognition of *T. verrucosum* in specimens from rural areas (Kane and Smitka 1978). The addition of 0.1 percent yeast extract or thiamine to SGA may increase the isolation rate of dermatophytes, especially of *T. verrucosum* (Carmichael and Kraus 1959; Aly 1994).

Identification

Identification of a dermatophyte is based on its gross colonial morphology on SGA and on its microscopic morphology. These criteria, however, may not be sufficient, especially for *Trichophyton* spp. that resemble each other or are variable in appearance or atypical. In addition, some isolates do not sporulate readily and special sporulation media that may not be readily available are necessary to obtain sporulation. Thus, additional physiological and biochemical tests may be necessary in conjunction with gross and microscopic morphology for species identification.

COLONY CHARACTERISTICS

Gross colony characters observed on SGA include:

- color of the surface, i.e. of the aerial mycelium
- color of the reverse or obverse faces of the colony
- production of a diffusible pigment
- texture of the surface (glabrous or waxy, powdery, granular, suede-like, velvety, downy, or fluffy)
- topography (flat, cerebriform, crateriform)
- growth rate.

These features are best noted when cultures are grown on Petri dishes rather than in tubes.

MICROSCOPIC MORPHOLOGY

Microscopic morphology may be studied in teased mounts, slide cultures (Riddell 1950; Larone 1995), or pressure-sensitive tape preparations (Rebell and Taplin 1974; St Germain and Summerbell 1996); they are examined for micro- and macroconidia and the other structures described in Tables 4 and 5. These preparations may be mounted in lactophenol cotton blue (Poirrer's blue) or lactophenol aniline blue (St Germain and Summerbell 1996). Specialized media that may be necessary to stimulate sporulation include corn meal, potato flake, potato glucose, Sabouraud's glucose + 3–5 percent NaCl (Kane and Fischer 1975), lactrimel (Kaminski 1985) and autoclaved polished rice grains (McGinnis 1980). The formulas for these media are found in several textbooks (McGinnis 1980; Rippon 1988; Kwon-Chung and Bennett 1992; de Hoog et al. 2000).

PHYSIOLOGICAL TESTS

In vitro hair perforation test

This test (Ajello and Georg 1957; Padhye et al. 1980; Clayton and Midgley 1989) originally devised to distinguish atypical isolates of *T. mentagrophytes* and *T. rubrum* (Ajello and Georg 1957), may also be used to distinguish *M. equinum* from *M. canis* (Padhye et al. 1980). *T. mentagrophytes* and *M. canis* perforate hair whereas *T. rubrum* and *M. equinum* do not. This test is also helpful in identifying other dermatophyte species (Padhye et al. 1980).

Production of urease or urea hydrolysis

The ability to hydrolyze urea (Philpot 1967; Clayton and Midgley 1989), in either an agar or a broth medium, aids in the distinction of *T. rubrum* (urease-negative) and *T. mentagrophytes* (urea-positive). The dermatophyte *Trichophyton raubitschekii*, considered by some mycologists to be a variant of *T. rubrum* (Kwon-Chung and Bennett 1992; Summerbell et al. 1999; Gräser et al. 2000a, c) or as a distinct species by others, is urease-positive (Kane et al. 1981). *T. megninii* [considered by some (Gräser et al. 1999a, 2000c) to be synonymous with *T. rubrum*] is urease positive in a urea-indole broth (Sequeira et al. 1991), but may be negative on Christensen's urea medium, whereas *T. rubrum*, apart from the relatively uncommon afro-asiatic isolates sometimes separated as *T. raubitschekii*, is urease-negative in both media (Rosenthal and Sokolsky 1965; Sequeira et al. 1991). Both Christensen's urea agar and broth media may be used. A broth medium is, however, preferred over the agar medium because it is more sensitive (Kane and Fischer 1971). The test is considered negative if there is no color change from straw to reddish purple within 7 days at 23–30°C. Cultures to be tested must be pure, because the presence of bacteria can produce a false-positive reaction. When in doubt about the purity of a culture, incubate the test culture on a blood agar medium to detect the presence of bacteria, before carrying out the urease test.

Nutritional requirements

A series of vitamin and amino acid test agars that is available commercially as trichophyton agars 1–7 (Difco, Remel, Lenexa, KA, USA) is used to distinguish some *Trichophyton* spp. by demonstrating their requirements for special growth factors (Georg and Camp 1957; Clayton and Midgley 1989). Trichophyton agar no. 1 is a vitamin-free casein basal medium to which various growth factors have been added, namely, inositol for Trichophyton agar no. 2, inositol and thiamine for no. 3, thiamine for no. 4, and nicotinic acid for no. 5. Ammonium nitrate basal medium makes up Trichophyton no. 6, and the same medium with added histidine makes up

medium no. 7. The special nutritional requirements are indicated in descriptions of the *Trichophyton* spp. in Table 5. Care must be taken to avoid vitamin and amino acid carryover from the transferring agar and to avoid use of large inocula.

Additional useful tests for identification may include examination of the type of growth and change of indicator color occurring on bromcresol milk solids glucose agar (Padhye et al. 1973; Kane et al. 1987; Summerbell et al. 1988; Weitzman et al. 1995), a test for growth on autoclaved polished rice grains, and tests for temperature tolerance and high-temperature enhancement of growth (Clayton and Midgley 1989; Weitzman et al. 1995). Details regarding the preparation of these media, performance of these tests, and their results are discussed by Kane et al. 1997, Clayton and Midgley 1989, Ellis et al. 1992, St Germain and Summerbell 1996, and Weitzman et al. 1995.

Histopathology

Dermatophyte infections are generally restricted to the horny epidermal layers of the skin. A toxic reaction in the epidermis is often the first response to the presence of the fungus in the stratum corneum. It presents as an eczemiform pattern or as a nonspecific, subacute, chronic dermatitis. If the biopsy tissue is from a dry scaly area, hyperkeratosis and parakeratosis are seen. Special fungal stains demonstrate mycelium in the horny layer of the skin. In tinea capitis, there is no specific histological picture for dermatophytic infections. Fungal hyphal elements are seen sparsely in the stratum corneum penetrating through the squames. Hyphae extend down into the hair shaft and penetrate hair lying parallel to them. Hyphal tips growing down within the shaft reach to the edge of living keratinizing cells and form 'Adamson's fringe.' The overall pattern in ringworm of the scalp is that of a subacute or chronic dermatitis. The newly keratinized material of the growing hair extending through the aperture of the follicle carries, either within it (endothrix) or surrounding the cuticular surface (ectothrix), hyphal elements of the invading fungus which break up into arthroconidia. In tinea favosa, mycelium is present in the horny layer of the scalp, within and around the hairs, and in the scutulum. The scutula consist of mycelial masses, scales, sebum, and other debris, cementing together to form a cup-shaped crust (Rippon 1988; Weitzman and Summerbell 1995). Development of a hypersensitive kerion reaction on the scalp is accompanied by infiltration of lymphocytes, plasma cells, eosinophils, and neutrophils into the dermis (Kwon-Chung and Bennett 1992).

In the most common, distal-lateral subungual type of tinea unguium, fungi are readily seen in infected nail sections stained with PAS. Filamentous hyphae and arthroconidia are generally aligned horizontally between lamellae of the nail and restricted to the lowermost portion of the nail. There is little or no inflammation in the underlying tissue. The etiologic agent is usually *T. rubrum*, though *E. floccosum* can also be involved. By contrast, in superficial white onychomycosis, infection is restricted to the surface of the nail and is characterized by irregular hyphae that are flattened with frond-like branches that spread out between keratin lamellae. The etiologic agent is generally *T. interdigitale*, but *Fusarium oxysporum*, *Acremonium* spp., and *Scopulariopsis* spp. are among numerous other organisms that may also be involved.

A rare but distinctive clinical manifestation of dermatophytosis is invasion of the subcutaneous tissue with formation of yellowish-white pseudogranules. These structures measure 80–500 μm in diameter and consist of septate hyphae, frequently vesiculate, surrounded by a thick, radiating coat of Splendore–Hoeppli eosinophilic material. Most of the causal agents are *Microsporum* spp. or *T. rubrum* and patients are often north Africans, Australian aboriginals, and African-Americans (Chandler et al. 1980; Kwon-Chung and Bennett 1992).

PREVENTION AND CONTROL

The prevention and control of dermatophytic infection depend on the area of the body involved, the causal agent, and the source of infection. In connection with scalp ringworm caused by *M. audouinii*, all contacts with infected individuals should be carefully examined using Wood's light for fluorescent hair. In *M. canis* infections, likely pet animal sources should similarly be examined along with other persons who may have been in contact with them. In chronic care and scholastic residential institutions where *M. canis* may spread widely from a single index patient who has contacted an infected animal, all inmates (e.g. patients, students) and staff should be examined. In case of nonfluorescent tinea capitis caused by *T. tonsurans* and *T. violaceum*, scalp examination should be done more carefully for spotty alopecia and scaly lesions. Hair from suspicious areas should be cultured regularly. The hairbrush method (Mackenzie 1963) may be helpful in collecting scalp hair from children. In populations and geographical areas with an ongoing tinea capitis problem, routine scalp examinations of young school children should be done on a regular basis to avoid large-scale outbreaks. Good hygiene and avoidance of sharing headgear, combs, and hairbrushes should be stressed to students. Barbershop instruments should be disinfected after use. All children or adults infected with ringworm of the scalp should be treated promptly to prevent further transmission of their infections.

Nosocomial spread of dermatophytosis, although rare, has been reported in recent years (Mossovitch et al. 1986; Kane et al. 1988; Shah et al. 1988; Arnow et al.

1991; Snider et al. 1993). In one such outbreak, caused by *T. tonsurans*, the infection was transmitted from an infected child to hospital personnel (Arnow et al. 1991). Two other outbreaks resulted from transmission of *M. canis* to neonates by nursing personnel (Mossovitch et al. 1986; Snider et al. 1993). Such nosocomial outbreaks should be investigated promptly to avoid further dissemination. Involved personnel and direct contacts should be screened using Wood's light, and scrapings from suspected areas of skin and scalp should be cultured. Until the source of infection in a nursery is identified and treated, protective clothing should be worn by healthcare workers to avoid direct contact.

Tinea corporis and tinea cruris caused by anthropophilic species can be transmitted by contaminated clothing, bedding, towels, etc., and these should be washed and disinfected before being used by other people. Individuals with tinea infection of the glabrous skin should also avoid contact sports such as wrestling. A number of recent dermatophytic outbreaks among high school and college wrestlers have been reported in recent years (Stiller et al. 1992; Beller and Gessner 1994).

When zoophilic species such as *M. canis*, *T. mentagrophytes*, and *T. verrucosum* are detected as etiologic agents, infected animal reservoir sources should be sought out and treated. Infections caused by *T. verrucosum* are difficult to detect with the use of Wood's light because infected hairs do not fluoresce, and the shed, infected scales have been shown to survive for years on fomites. Good hygiene and sanitation, and use of fungicidal sprays and washes, are effective methods for prevention of such infections (Weitzman and Summerbell 1995).

The spread of tinea pedis infections may be controlled by good foot hygiene, use of antifungal powders, avoiding excessive moisture, and occlusion of feet by wearing sandals or ventilated footwear, and avoiding walking barefooted in swimming pool areas, community baths, and shower areas. Prompt treatment of individuals with tinea pedis should be undertaken to avoid spread of infection.

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Dermatomycotic molds

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Although the dermatophytes show considerable adaptation to their life as epidermal parasites, it is clear that most other fungi are unable to invade keratinized tissue. In those cases where nondermatophyte molds are recognized as superficial pathogens, the affected site is remote from living tissue, as in the extremities of the nails, or there is structural and presumably biochemical abnormality of the keratin as a result of trauma or preexisting disease. Very few molds have been able to invade the epidermis of unaltered skin in the way dermatophytes can, and only the tropical ascomycete, *Piedraia hortai*, invades hair. This chapter describes the characteristics of mold infections of skin and nail, and of otitis externa, together with the distinguishing features of species commonly involved.

SKIN AND NAIL INFECTIONS

Clinical features

The main clinical features, etiological aspects, and therapy of nail infections have been reviewed (Midgley et al. 1994; Denning et al. 1995). Estimates of the importance of molds, compared with dermatophyte fungi in skin and nail infections, vary considerably. Summerbell et al. (1989) reported that less than 10 percent of isolations from infected nails were nondermatophytes. Of these, half were yeasts (mostly *Candida* spp.) and half were various mold species. Certain classes of patient show a raised incidence of nondermatophyte molds in nails. In particular, elderly or immunocompromised individuals and patients with keratin abnormalities may be affected. Such infections are more often reported from toenails than fingernails. English and Atkinson (1972), using the efficient sampling method of pulverized nails, estimated that 18 percent of elderly chiropody patients seen and 68 percent of those with positive direct microscopy and culture, were infected by molds.

Two main types of nail infection have been recognized: the common distal and lateral subungual onychomycosis and the less common superficial white onychomycosis. Both are typically caused by dermatophyte fungi such as *Trichophyton rubrum* and *Trichophyton mentagrophytes* var. *interdigitale* in addition to a number of mold species. In the case of the molds, infection is often confined to a single nail. In the distal subungual form, the fungus invades underneath the free end of the nail plate, eliciting a hyperkeratotic response in the nailbed epithelium. This results in a build-up of amorphous keratinized tissue, greatly thickening the nail and, in time, lifting the nail plate to an angle of 30° or more from its original position.

Although the hard nail plate may be invaded, the softer amorphous material is the major site of invasion by the fungal mycelium. A variety of yeasts and bacteria may also grow in this material and it is difficult to attribute sole pathogenic role to the presence of the mold. Many affected nails become discolored, possibly resulting from the mixture of organisms present and from the opacity of the amorphous keratin itself.

In superficial white onychomycosis, the fungus infects the top surface of the hard nail plate, and slowly erodes the keratin to give a white powdery area. The disease seldom progresses beyond the superficial layers of the nail plate and there is no response by the underlying nail bed epithelium.

Diagnosis

As most nondermatophyte species invading keratinized tissue are also common saprophytes (a few are plant parasites), some authors have stressed the importance of assessing the significance of isolation from diseased sites. This is done by the demonstration of hyphae in tissue and by growth of a mold in pure culture from most tissue samples. Growth of one or only a few colonies from perhaps 10 or 20 samples cannot be interpreted, especially in the absence of direct observation of hyphae in the tissue, because they may have grown from dormant spores contaminating the skin or nail. Unfortunately not all reports of mold infection have made this distinction. Zaias et al. (1969) reported isolation of mold species from 183 abnormal toenails and demonstrated that KOH-negative nails could yield a profusion of saprophytic molds in culture.

If there is ready access to a fluorescence microscope, then use of the optical brightener calcofluor white can enhance the detection of fungal elements in skin, nail, and hair specimens. For skin and hair specimens, calcofluor white can be used in equal proportion to KOH and placed over the specimen on a microscope slide, covered with a coverslip and left to digest for at least 20 minutes. During this time the slides should be protected from light. It is important that nail samples are presoftened before the addition of calcofluor white or it is unable to penetrate the tissue.

Microscopic examination will reveal the presence of any fungal hyphae. It is a feature of dermatophyte infections that the production of the asexual conidia is suppressed in the infectious state, with direct microscopy of the tissue revealing only vegetative hyphae, some of which may show fragmentation into arthroconidia. Thus, chains of rectangular spores are typical of dermatophyte infection but are not usually seen in mold-infected nails; this is therefore an important feature to note as it may help in the assessment of significance of a subsequent mold isolate. Some molds, such as *Scopulariopsis brevicaulis* and *Scytalidium dimidiatum*, may be distinguished from dermatophytes on direct examination. Mold hyphae may produce fronding which is not usually seen with dermatophyte hyphae and occasionally, when there is an air pocket in the nail, sporing structures may be visible within the nail preparation (Figure 1). Molds usually infect nails damaged either due to trauma, disease, or underlying dermatophyte infection and sometimes form fungal balls within the nail plate.

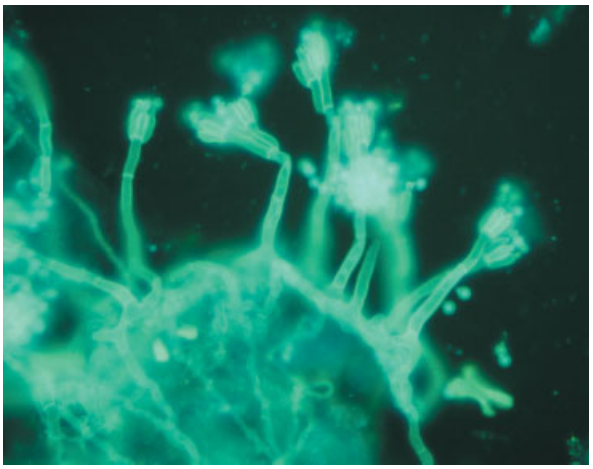

Figure 1 *Calcofluor staining of a nail specimen infected with a* Penicillium *sp. demonstrating the production of sporing structures produced in an air pocket within the nail*

The common use of cycloheximide in agar culture media for selection of dermatophyte fungi may mask the presence of infectious molds in skin and nail because this drug is inhibitory to most saprophytic molds. Many laboratories now use a cycloheximide-free medium for isolation from nails in addition to the routine medium. It should be remembered, however, that mold infections of the skin do occur (see sections on Infections by *Scytalidium* spp., Infections by *Aphanoascus fulvescens* (*Anixiopsis stercoraria*) , and Infections by *Onychocola canadensis*), and these will also require a less selective medium.

Isolation of a mold is not considered significant if direct microscopy was negative; a repeated attempt at isolation of a dermatophyte should also be considered if chains of arthroconidia were observed on direct microscopy as these are more indicative of a dermatophyte infection. If a mold is isolated from a specimen from which a dermatophyte is recovered, the mold is likely to be an incidental contaminant. In a study of onychomycosis, Ellis et al. (1997) reported the presence of a mold or yeast in addition to a dermatophyte in 64 percent of 118 patients. Significantly, only three of these patients (2.5 percent) yielded the same yeast or mold from two or more consecutive specimens.

Etiology

The relative incidence of the mold species reported in nails varies from one study to another, but it is clear that *Scopulariopsis brevicaulis* (see section on *Scopulariopsis brevicaulis* Bainier below) is the mold most readily acknowledged as capable of causing nail dystrophy. It is one of the few molds that appears to be able to infect otherwise healthy nails and can sometimes be identified on microscopy of an infected nail because of its propensity to produce conidia in vivo. Microscopy reveals broad, septate hyphae, which are easily distinguished from those

Table 1 *Other molds rarely isolated from cases of onychomycosis*

Species	References
Aspergillus sclerotiorum	Feuilharde de Chauvin and de Bièvre 1985; Garcia-Martos et al. 2001
Aspergillus ustus	Walshe and English 1966
Aspergillus unguis	Schönborn and Schmoranzer 1970
Chaetomium globosum	Costa et al. 1988; Naidu et al. 1991; Hattori et al. 2000
Curvularia lunata	Barde and Singh 1983
Exophiala jeanselmei	Boisseau-Garsaud et al. 2002
Geomyces pannorum	Schönborn and Schmoranzer 1970
Geotrichum candidum	Restrepo and de Uribe 1976
Gymnascella dankaliensis	Summerbell et al. 1989
Lasiodiplodia theobromae	Restrepo et al. 1976; Vélez and Díaz 1985
Myxotrichum deflexum	de Vroey 1976
Ophiostoma stenoceras	Summerbell et al. 1993
Penicillium spp.	Walshe and English 1966; McAleer 1981; Vélez and Díaz 1985
Pyrenochaeta unguis-hominis	Punithalingham and English 1975
Polypaecilum insolitum	Piontelli and Toro 1988

of dermatophytes. A few reports exist of onychomycoses caused by other *Scopulariopsis* spp., including *Scopulariopsis acremonium*, *Scopulariopsis asperula*, *Scopulariopsis fusca*, and *Scopulariopsis koningii* (Krempl-Lamprecht 1970; Schönborn and Schmoranzer 1970), and *Scopulariopsis flava* (Piontelli and Toro 1988).

Next in order of frequency to *S. brevicaulis* are species of *Aspergillus*, *Acremonium*, and *Fusarium*. The species causing deep aspergillosis have all been described from nails, although the *Aspergillus versicolor* group (including *Aspergillus sydowii*), the *Aspergillus glaucus* group, and *Aspergillus candidus* (see sections on *Aspergillus versicolor* (Vuill.) Tiraboschi, *Aspergillus glaucus* group, and *Aspergillus candidus*) are probably more frequent (Walshe and English 1966; Zaias 1972). As with onychomycosis caused by *Scopulariopsis* spp., a small number of reports involve well-documented cases of infection by related but little-known species. These include *Aspergillus hollandicus*, a member of the *A. glaucus* group (Grigoriu and Grigoriu 1975), *Aspergillus restrictus*, *Aspergillus unguis* (Schönborn and Schmoranzer 1970), and *Aspergillus sclerotiorum* (Feuilharde de Chauvin and de Bièvre 1985; Garcia-Martos et al. 2001). Reports of onychomycosis caused by *Fusarium* and *Acremonium* (*Cephalosporium*) spp. usually have not carried the identification beyond genus level (Walshe and English 1966; Zaias 1972; McAleer 1981; Vélez and Díaz 1985), although experience suggests that *Fusarium solani*, *Fusarium oxysporum*, and *Acremonium strictum* are the most common (see sections on *Fusarium solani* (Mart.) Sacc., *Fusarium oxysporum* Schlecht, and *Acremonium strictum* W. Gams, respectively). There have been reports in neutropenic patients of disseminated infections with *Fusarium* spp. arising from cutaneous lesions associated with infected nails (Merz et al. 1988; Girmenia et al. 1992). *Paecilomyces variotii* and *Paecilomyces lilacinus* are occasionally implicated in nail infection (see Arenas et al. 1998 for a review of onychomycosis due to *P. variotii*). Onychomycosis due to *Alternaria* spp., in particular *Alternaria alternata*, is rare, but there are several reports suggesting their involvement (Wadhwani and Srivastava 1985; Singh et al. 1990; Glowacka et al. 1998; Romano et al. 2001)

In patients of tropical origin, onychomycoses can result from *Scytalidium hyalinum* and *Scytalidium dimidiatum*. These, together with the mold recently described from nails as a new genus and species, *Onychocola canadensis*, are discussed in detail below. Other molds are occasionally reported, some of which may have been implicated without a careful appraisal of their true significance. Those with good evidence of infection are summarized in Table 1.

INFECTIONS BY *SCYTALIDIUM* SPP.

Gentles and Evans (1970) were the first to report human infection by *S. dimidiatum* (under its earlier name, *Hendersonula toruloidea*; see section on *Scytalidium dimidiatum* (Penz.) Sutton and Dyko (pycnidial synanamorph *Nattrassia mangiferae* (Sydow and Sydow) Sutton and Dyko)). First described in 1933 as a cause of die-back and wilt disease in stone-fruit trees in Egypt, it had become well recognized as an important pathogen of a wide range of tropical crop plants. Over a period of 7 years, Gentles and Evans collected eight cases of a condition clinically indistinguishable from tinea pedis, in which this species was the only isolate recovered, sometimes repeatedly. In four cases, the skin of the palms or fingernails was also involved. Most patients were middle-aged male immigrants to the UK from tropical countries. Subsequent reports from other UK laboratories (Campbell et al. 1973; Moore 1978) followed and established this fungus as a frequent cause of infection of both hands and feet in people who originated from tropical countries. More recently, reports have appeared of its occurrence as a human pathogen in Canada

(Summerbell et al. 1989), Nigeria (Gugnani and Oyeka 1989), Sweden (Rollman and Johansson 1987), Spain (Revilla et al. 1992), Thailand (Kotrajaras et al. 1988), and the USA (Greer and Gutierrez 1987; Frankel and Rippon 1989; Elewski and Greer 1991).

Unusually for nondermatophyte pathogens, *S. dimidiatum* and *S. hyalinum* (see section on *Scytalidium hyalinum* Campbell and Mulder below) are able to invade the skin as well as nails, mimicking tinea manuum and tinea pedis caused by the dermatophyte *T. rubrum* (Hay and Moore 1984). The microscopic appearance of the infectious hyphae in skin shows, however, great variation in width along any one length and some hyphae are twisted and contorted, unlike the appearance of typical dermatophyte hyphae (Campbell et al. 1973).

During an investigation into the occurrence of *S. dimidiatum* infections, several cases of essentially the same disease entity were encountered in which the isolated fungus was a white arthroconidiate mold. Campbell and Mulder (1977) described eight such cases and named the organism *Scytalidium hyalinum*. All occurred in immigrants from the Caribbean area or west Africa. Moore (1986) later described the same species in 11 other British immigrants, also originating from these areas. Pointing to the many parallels in the clinical presentation, conidiogenesis, and antigenic composition of these two species, Moore suggested that *S. hyalinum* could merely be a nonpigmented variant of *S. dimidiatum*. This is now supported by DNA comparisons (Roeijmans et al. 1997).

A small survey carried out on hospital attendees in Tobago indicated that infections by this fungus may be common in some tropical areas (Allison et al. 1984). Of 45 patient samples, seven showed microscopic and/or cultural evidence of *S. hyalinum*, and three others yielded *S. dimidiatum*. With the figures added for positive microscopy but failed culture, almost half the total population studied would appear to have had some form of *Scytalidium* spp. infection. None of these people was aware of the infection. Possibly patients referred to clinics in the UK have been motivated by severity of the lesions. Reports of *S. hyalinum* infections have also come from Canada (Summerbell et al. 1989), the USA (Elewski and Greer 1991), Australia (Maslen and Hogg 1992), and Nigeria (Gugnani and Oyeka 1989).

INFECTIONS BY *APHANOASCUS FULVESCENS* (*ANIXIOPSIS STERCORARIA*)

Although rare (it is the subject of only a handful of case reports), this keratinophilic soil fungus (see section on *Aphanoascus fulvescens* (Cooke) Apinis below) stands out as unusual among nondermatophyte molds in that it apparently invades skin rather than nail; it is, however, unwise to rule out the possibility of nail infection. Ringworm-like lesions with epidermal scaling have been described on thighs (Rippon et al. 1970), arms (Albala et al. 1982), neck (Marín and Campos 1984), and scalp (Guého et al. 1985), and similar infections have occurred in animals (Rippon et al. 1970; Vanbreuseghem and de Vroey 1980). In most of these cases, the anamorphic state – a *Chrysosporium* sp. close to *Chrysosporium keratinophilum* – was isolated, and the *Aphanoascus* state developed on prolonged incubation. Other *Chrysosporium* spp. have also been associated with skin lesions (de Hoog et al. 2000).

INFECTIONS BY *ONYCHOCOLA CANADENSIS*

Onychocola canadensis (see section on *Onychocola canadensis* Sigler below) is a slow-growing, arthroconidiate hyphomycete which was first reported in Canada from nail and skin infections (Sigler and Congly 1990), although evidence of skin infection was not supported by positive microscopy. Since then the organism has been reported from a number of other countries, including France (Contet-Audonneau et al. 1997), Spain (Llovo et al. 2002), New Zealand (Sigler et al. 1994), and the UK (Campbell et al. 1997) where it is referred to the Mycology Reference Laboratory for identification several times a year. There has also been a further more extensive report of cases in Canada and a review of the literature (Gupta et al. 1998).

Microscopy of the subungual keratin from infected nails shows a picture distinct from that seen with dermatophytes. There are septate, hyaline, branching hyphae of varying widths reminiscent of those seen from infections with *S. dimidiatum* and *S. hyalinum*. The round-to-barrel-shaped arthroconidia are more rounded than the cylindrical arthroconidia seen in dermatophyte infections. There are also unusual thick-walled, light-brown hyphae which may be a feature of chronic infections as similar hyphae are only produced on prolonged culture in vitro.

The environmental source of *O. canadensis* has not been established, but it is likely to be a soil saprophyte in common with other nondermatophyte agents of onychomycosis. In vitro studies have shown that it is unable to break down the keratin of hair, but weak cellulolytic activity has been demonstrated. As elderly individuals are most often affected, it appears that altered keratin may be necessary for infection. A description of its teleomorph, *Arachnomyces nodosetosus* sp. nov., was published by Sigler et al. in 1994.

OTITIS EXTERNA

Fungal infections of the ear are common conditions in which there is infection, usually of the external auditory canal, with fungi that are either spread by airborne conidia or derived from direct invasion of the epithelium

through the external canal (Kaur et al. 2000; Ozcan et al. 2003). Examples of the former include *Aspergillus* spp., and of the latter, dermatophytes. Sometimes cutaneous pathogens cause this condition, but their presence is likely to have followed airborne contamination by spores, e.g. *S. brevicaulis* (Besbes 2002). Rarely, otitis externa may follow dissemination of a systemic fungal infection such as coccidioidomycosis (Harvey et al. 1978). As such, the pathogenetic role of each organism has to be carefully weighed against the pathological response, as in some cases it is likely that the isolation of a fungus is merely a reflection of temporary carriage of the organism in the external canal. Growth of the fungus by itself can cause loss of hearing due simply to obstruction of the lumen. *Aspergillus niger*, for instance, is a common cause of this form of hearing loss (Loh et al. 1998). The main organisms isolated are *Aspergillus* and *Candida* spp., a whole range of different environmental fungi including *Scedosporium apiospermum* have been implicated (Milne et al. 1986). Rarely, infection may result in an acute and severe destructive process with subsequent invasion of adjacent tissue, malignant otitis externa (Bellini et al. 2003). This is a rare event and mainly associated with profound immunosuppression; the commonest cause is bacterial infection with *Pseudomonas*, but fungi such as *Aspergillus* spp. are occasional causes (Bellini et al. 2003). Other even rarer causes have included *Malassezia pachydermatis* (Chai et al. 2000) and *Absidia corymbifera* (Paterson et al. 2000).

Factors predisposing to infection of the external auditory canal range from existing disease of the external canal such as seborrhoeic dermatitis, eczema, or psoriasis, to trauma and climatic conditions. The latter seem to be particularly important as overgrowth of organisms such as *A. niger* is more common in tropical and humid conditions (Schuster et al. 2002). It is also apparent that in many cases there is mixed growth of different organisms from the canal.

The pathogenesis of fungal otitis externa is not always clear. As stated previously, growth of fungi may simply reflect colonization with growth exacerbated by climatic conditions or other factors such as the administration of antibiotics. However, the excessive growth of fungi may physically obstruct the narrow lumen. Otherwise, invasion is almost always confined to the epithelium except in malignant otitis externa (Vennewald et al. 2003). In addition, in some cases of *A. niger* infection local deposition of oxalate crystals may contribute to underlying tissue damage (Landry and Parkins 1993).

The main clinical features of fungal otitis externa are irritation and discharge from the external auditory canal (Kurnatowski and Filipiak 2001). There may also be hearing impairment due to the production of wax, scaling, or fungal growth. On inspection, the ear canal is usually reddened and may have an inflammatory exudate. If *A. niger* is present, the fungal growth may appear as a dark mat and black fruiting heads are seen together with accompanying oozing and scaling. The malignant form of otitis externa shows ulceration and necrosis with bleeding. There may be pain, often indicating deeper extension into the mastoid ear cells.

Fungi may also be found deeper in the middle ear in association with chronic otitis media. Once again their pathogenic role is not clear and many are contaminants. The organisms isolated from this site are similar to those found in otitis externa (Vennewald et al. 2003).

These infections are managed through a variety of different techniques such as local toilet including debridement and intensive cleansing (Kurnatowski and Filipiak 2001). Topical antifungals such as ciclopirox olamine (del Palacio et al. 2002), 1 percent bifonazole (Falser 1984), clotrimazole, nystatin, or the application of gauze packs soaked in a 1 percent azole solution have also been used. These are repeatedly changed. Oral or intravenous therapy is indicated for necrotizing infections.

The external ear itself may also be affected by facial infection, the commonest of which is *T. rubrum*. However, rarer infections such as chromoblastomycosis and lobomycosis may also affect the external ear. *Rhinosporidium* may also involve this area.

DESCRIPTIONS OF MOLD FUNGI CAUSING ONYCHOMYCOSIS AND DERMATOMYCOSIS

Acremonium strictum W. Gams

Colonies are moderately fast growing, attaining a diameter of 50 mm in 1 week at 30°C. The surface is flat to slightly folded and may be covered in a loose network of aerial hyphae, or may be wet and almost glabrous. The predominant color of the aerial growth is pale salmon pink, although the more floccose strains may be white. Microscopically there is an abundance of small (3.5–5×1–2 μm) conidia, cylindrical to ovoid in shape, which are produced sequentially from the phialidic tips of long, slender, lateral hyphae (Figure 2). These arise from bundles of aerial hyphae. The conidiophores can be viewed in situ on the colony using a binocular dissecting microscope, when each phialide can be seen to carry a droplet-like mass of conidia, held together by surface tension.

Alternaria alternata (Fr.) Keissl

The genus *Alternaria* is characterized by producing branching chains of multicellular conidia with dark pigmented walls and septa arranged longitudinally, transversely, and obliquely. The group are mostly pathogens of plant leaves, but *A. alternata* seems to be mainly saprophytic and is a common laboratory contaminant.

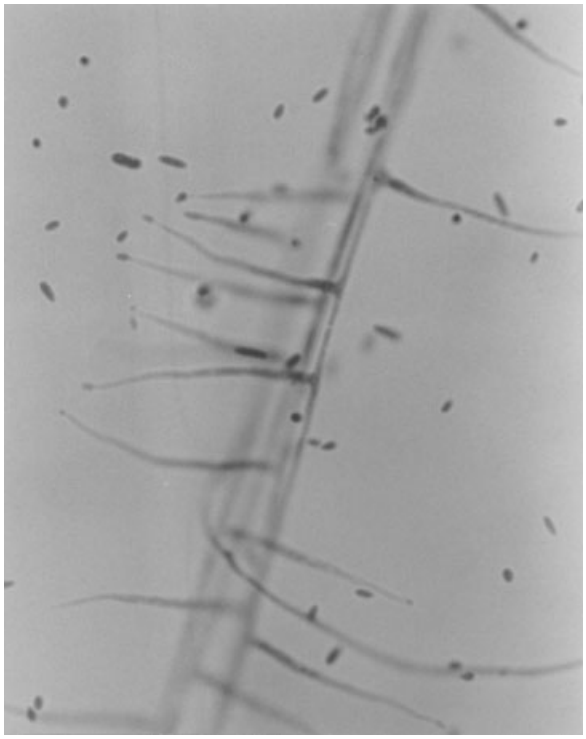

Figure 2 Acremonium strictum*: slide culture preparation showing delicate lateral phialides with terminal conidia. In this mount all but the last-formed conidia have dispersed into the mounting fluid. In life they are aggregated in round heads on the phialide apices.*

On normal media it grows as a cottony white colony which slowly develops black pigment in the center. On less rich media the colonies are black to dark brown, suede-like or powdery, and abundent conidia are produced. Conidia are typically clavate, about 25–50 μm long, with a short apical beak which produces the successive conidium in the chain.

Aphanoascus fulvescens (Cooke) Apinis

Colonies are of medium growth rate, flat and felt-like, white to cream in color. On prolonged incubation, a ring of pale purple–brown ascocarps develops near the center. On microscopy there are many club-shaped aleurioconidia, of similar shape to the microconidia of dermatophytes but much larger (mostly 10–15×4–6 μm) and frequently slightly curved. This is the conidial state (*Chrysosporium* spp.). The *Aphanoascus* teleomorph is a smooth-walled ascocarp, up to 500 μm diameter, with pale-brown, rough-walled, ovoid ascospores in round asci.

Aspergillus versicolor (Vuill.) Tiraboschi

This is an *Aspergillus* sp. of moderate growth rate, reaching 10–20 mm diameter in 1 week. *A. versicolor* is so called because it can appear in a variety of colonial colors, ranging from shades of green, light brown, yellow, and grayish pink. The conidial color is, however, dull gray–green and this dominates in cultures that spore well. Microscopically, the distinguishing features are the small head, consisting of an ovoid vesicle that bears supporting cells (metulae) below the layer of phialides (i.e. biseriate heads). There may also be reduced heads resembling those of *Penicillium* spp. *A. sydowii*, often reported from nails, is microscopically identical but has a blue–green colony color. Some mycologists place it as a variant of *A. versicolor* (Figure 3).

Aspergillus glaucus group

The name *A. glaucus* is no longer used as a single species but represents an assemblage of closely related aspergilli separable mostly on the basis of their sexual ascosporic states. The colony is generally slow growing on normal media, but this can often be accelerated on special media with low water activity. On glucose peptone agar, the surface is granular and colored mid-green (conidia), with areas of bright-yellow ascocarps. Microscopically, the heads are small relative to the wide stalks, and are composed of small phialides mounted directly upon the subspherical vesicle. In many strains aberrant heads, consisting of secondary conidiophores radiating out from the vesicle, may be seen.

Aspergillus candidus Link

Colonies are of moderate growth rate. Young colonies often appear wet or glabrous but develop into dry colonies, varying from dense and granular to loose and floccose. The conidia are unpigmented so even heavily sporing cultures are white or pale cream. Microscopically, there may be large spherical heads, but reduced heads reminiscent of *Penicillium* spp. may be present. Unlike the dry conidia of most *Aspergillus* spp., the conidia of *A. candidus* readily become wetted when touched with a needle.

Fusarium solani (Mart.) Sacc.

The colony is fast growing, reaching a diameter of 30–40 mm in a week, and producing a uniform, densely floccose, aerial growth. Many strains remain white or dull cream–buff, although others develop shades of bluish–purple. Microscopically, the conidia are ovoid to kidney-shaped, 4–8×3–4 μm and are produced on long hypha-like phialides.

Fusarium oxysporum Schlecht

This species is similar to *F. solani*, but colonies are salmon pink to violet, and the conidia are produced on

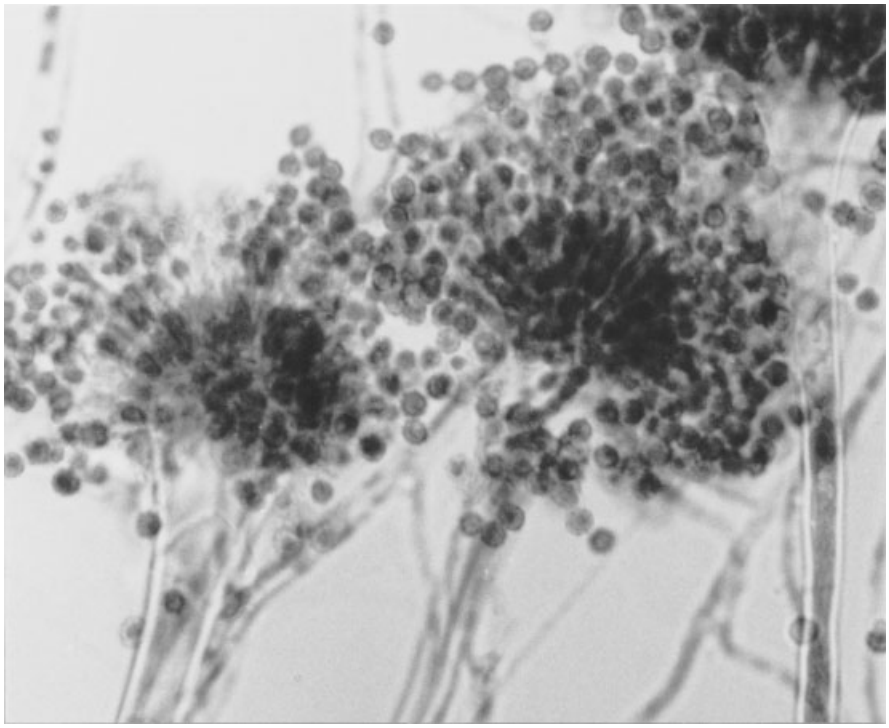

Figure 3 Aspergillus versicolor*: sporing heads showing the typical small vesicles with 2 rows of cells below the conidia*

short lateral phialides. Both species may produce smaller numbers of sickle-shaped multicellular macroconidia.

Onychocola canadensis Sigler

Unlike species of *Scytalidium*, *O. canadensis* is resistant to cycloheximide at 0.4 mg/l so it will grow on routine isolation media but shows enhanced growth on carbohydrate-rich medium. Colonies are restricted, attaining a diameter of 14–20 mm in 6 weeks at 25°C on glucose peptone agar, and may cause cracking of the agar. Typically, after 2 weeks at 25°C, colonies are more than 1 cm in diameter, yellowish–white to pale gray with a darker brownish–gray reverse. The surface is domed with abundant aerial mycelium and a cottony, velvety, or woolly texture. *O. canadensis* will grow at 37°C but is less vigorous.

Microscopy of older cultures may reveal abundant broad, pheoid hyphae up to 6.5 μm in width with few septa and with darker-brown wall thickenings; these are similar to the hyphae observed in infected nail specimens. Sporulation is slow, conidia are formed in chains from poorly differentiated, septate, hyaline hyphae 1.5–3 μm wide. Formation of conidia may be either intercalary along the length of the hyphae or in basipetal succession on lateral chains. Conidia (arthroconidia) are broadly ellipsoidal, cylindrical, or irregular in shape, and either unicellular or with a single septum, often persisting in long, branched chains. Release of arthroconidia is by dissolution (rhexolysis) of adjacent thin-walled cells or by schizolysis of adjacent conidia; the remnants of wall material may be visible on the end walls of the detached conidium (Figure 4).

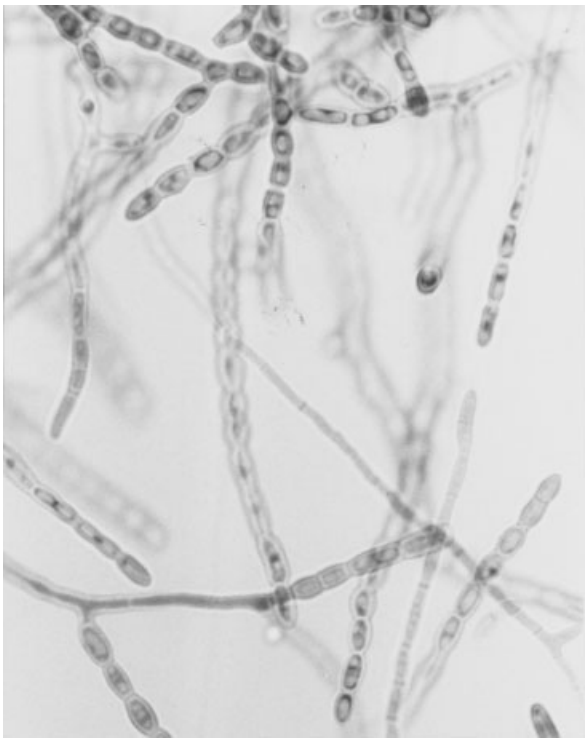

Figure 4 Onychocola canadensis*: thick-walled arthroconidia in sparsely branching chains removed from a 4-week-old culture*

Paecilomyces variotii and *P. lilacinus*

The genus is a group of saprophytes and insect parasites closely related to *Aspergillus* and *Penicillium*. The conidia are produced in unbranching chains from phialides. In some species the phialides are grouped in whorls as in *Penicillium*, but single phialides are common, as are irregular branching systems of phialides. In contrast to many *Aspergillus* spp. and all *Penicillium* spp., the conidia are not colored green. *P. variotii* produces colonies which are flat, fast growing, and with a finely powdery surface. The conidia are variable in size as they continue to grow in size as the chain of spores develops, but are generally ovoid to lozenge-shaped, 3–7 μm long (Figure 5). The other species in nails, *P. lilacinus*, produces domed, cottony colonies, which become flattened with age. The aerial growth is a pale lilac purple color. Microscopically the phialides are narrow and tightly compacted together in irregular *Penicillium* like clusters. Conidia are smaller than in *P. variotii*, mostly about 3 μm long.

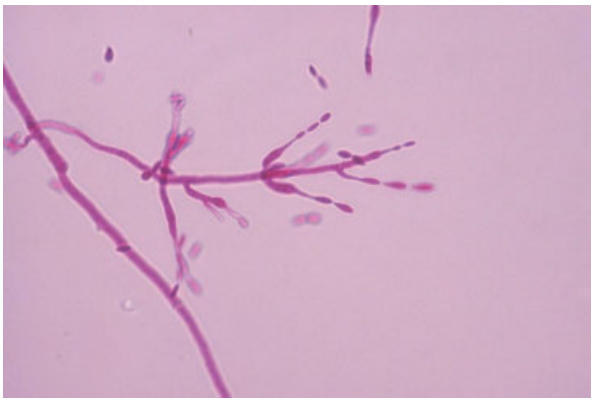

Figure 5 Paecilomyces variotii *showing slender phialides producing unbranched chains of oat-shaped cells*

Scopulariopsis brevicaulis Bainier

S. brevicaulis is an annelidic hyphomycete which will grow on cycloheximide-containing medium and forms spreading colonies, often with a raised and furrowed central area. It has a powdery surface as a result of the production of abundant conidia, and it is grayish–white at first, developing a cinnamon-colored central area on production of conidia.

Microscopy reveals an abundance of lemon-shaped, roughened conidia with truncated bases, which are produced in basipetal chains from the tips of annellidic conidiogenous cells. The annellides, which increase in length with age as each successive spore leaves a collar of wall material, are produced singly or in penicillate heads (Figure 6).

Scytalidium dimidiatum (Penz.) Sutton and Dyko (pycnidial synanamorph *Nattrassia mangiferae* (Sydow and Sydow) Sutton and Dyko)

In cultures of *S. dimidiatum*, two types of growth may be produced. These were described in detail by Campbell

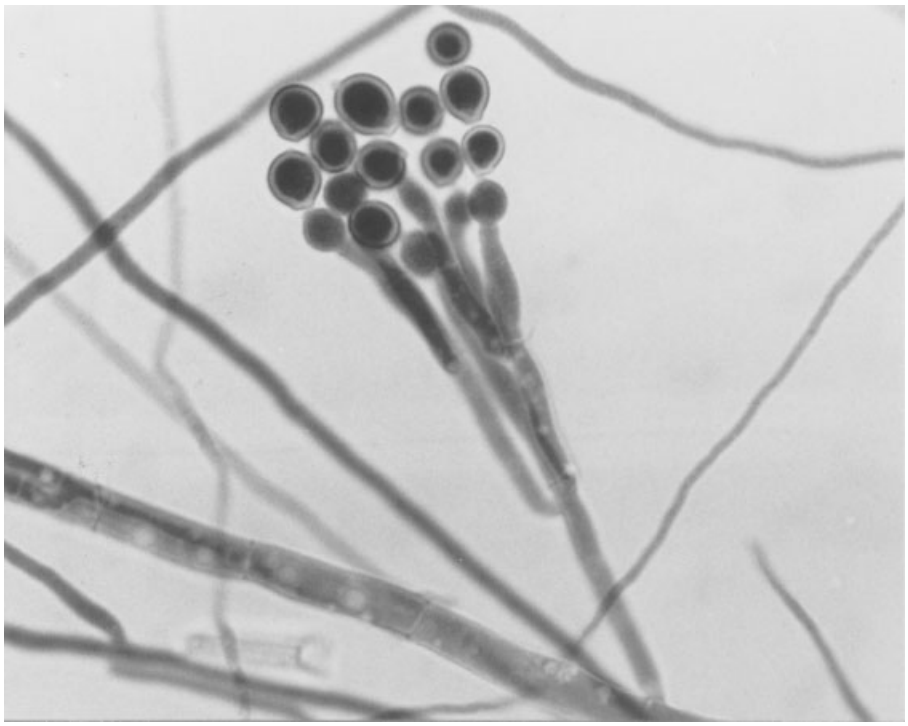

Figure 6 Scopulariopsis brevicaulis*: unbranched conidial chains, each with the youngest conidium at the base*

(1974). Type A is fast growing, filling a 9 cm Petri dish in 2–4 days with a dark-gray, floccose, aerial mycelium which becomes brownish–black after a week. A few strains show concentric zones of growth. Microscopy of this type shows a mixture of wide, brown-pigmented hyphae and narrow hyaline hyphae. Both hyphal forms are much fragmented into long-cylindrical to barrel-shaped arthroconidia, 2.5–10×2.5–7 μm, many with two cells (Figure 7). Type B is much slower growing, reaching a diameter of 2 cm in 1 week, with little aerial mycelium; the colour is gray–olive, with an irregular submerged margin. Scanty brown arthroconidia are seen on microscopy. In addition to the spores, this type also features coiled hyphae, and older hyphae may bear irregular thickenings on the outer surface of the walls. Type A was associated with immigrants from the Caribbean area, and type B with patients of Indo-Pakistani origin. In a larger series of cases, Moore (1988) found the same geographical split between the fast- (type 1) and slow- (type 3) growing strains. In this study several intermediate colonial forms (type 2) were also found.

On prolonged incubation, strains of type A may develop the pycnidial state, referred to the taxon *N. mangiferae* (Sydow and Sydow) Dyko and Sutton or its earlier synonym *H. toruloidea* Nattrass. This takes the form of hard, carbonaceous stromata, each with several multilocular pycnidia embedded inside. Each connects to the surface via an ostiole through which the pycnidioconidia are released. The pycnidioconidia are ovoid with pointed ends, 10–14×4–6 μm, and become biseptate, with the central cell darker than the end cells.

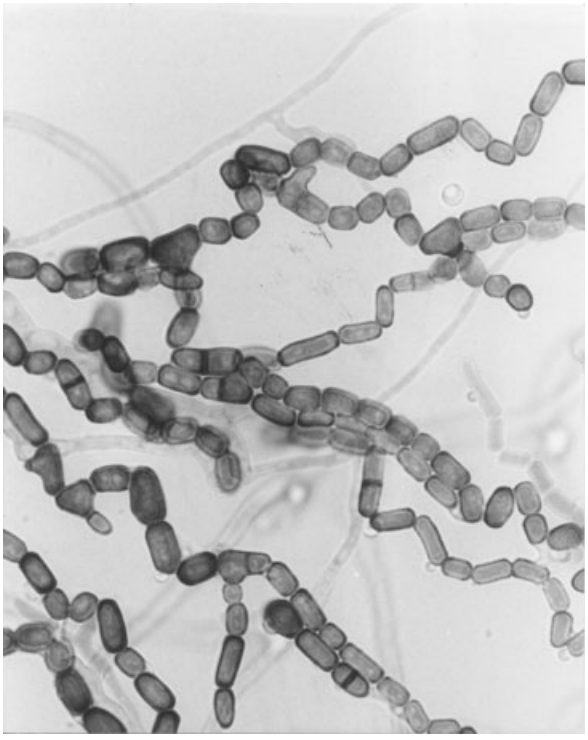

Figure 7 Scytalidium dimidiatum*: brown-walled arthroconidia formed by the irregularly timed formation of cross-walls results in some spores with two cells*

Scytalidium hyalinum Campbell and Mulder

S. hyalinum grows rapidly on glucose peptone agar or other common media at 28–32°C to produce a white, effuse, cottony, aerial mycelium. With age, a yellowish color may develop and the mycelium collapses somewhat. Microscopy shows an abundance of arthroconidia formed in chains by the disintegration of the aerial hyphae. The spores are cylindrical to ellipsoidal, mostly aseptate but some with two cells, thin-walled, smooth and hyaline, separated by septal splitting. Attempts to induce pycnidia or other conidial states have failed.

TREATMENT OF ONYCHOMYCOSIS AND DERMATOMYCOSIS CAUSED BY MOLDS

Treatment of onychomycosis due to molds is difficult and there is no consistent treatment of proven efficacy. Organisms such as *S. brevicaulis* are not susceptible in vitro to any of the agents licensed for superficial fungal infections. However, in vivo–in vitro correlation has not been established for molds and, despite high MICs in vitro, there are reports of successful treatment. It is possible that a significant number of mold infections only arise due to trauma to the nail by an underlying dermatophyte infection. In these situations it may be that the presence of the dermatophyte is masked by the overgrowth of mold. However, treatment of the underlying dermatophyte can lead to successful resolution of the mold infection. It was reported by Ellis et al. (1997) in their study on onychomycosis that the presence of a mold had no influence on the outcome of treatment with terbinafine.

Treatment of nail infections usually entails oral treatment with a systemically active agent such as terbinafine or itraconazole. Topical application of an antifungal agent, such as terbinafine, tioconazole, or amorolfine, as a paint or lacquer directly onto the nail surface, is unlikely to have the penetrative power to reach all infected tissue, although it may be useful in cases of superficial white onychomycosis. There has also been a report of successful treatment of a *S. hyalinum* onychomycosis with frequent application of amorolfine nail lacquer (Downs et al. 1999), and it may lead to higher rates of resolution when used in combination with an oral agent or following nail avulsion.

Another factor that may have some bearing on outcome of treatment of a mold infection is that oral treatment with itraconazole can lead to high tissue concentrations, particularly in keratinized tissue. Levels in skin and nail tissue may far exceed serum levels; thus

the drug may have activity against molds demonstrating relative insusceptibility in vitro. The accumulation and persistence of this drug in keratinized tissue also allows for the use of this agent as pulse therapy. The regimen suggested for pulse therapy is 200 mg twice daily for 1 week followed by a 3-week break. Two pulses may be sufficient for fingernail infections whereas three pulses are usually required to eradicate toenail infection.

A prospective, comparative parallel group, single-blind, randomized study investigated the efficacy of systemic fluconazole, griseofulvin, itraconazole, ketoconazole, and terbinafine in the treatment of 59 patients with toenail onychomycosis due to *S. brevicaulis*. Standard dosage regimens were employed and patients were evaluated for clinical and mycological cure at 12 months after the start of treatment. Terbinafine and itraconazole produced the best response rates with high levels of both clinical and mycological cure (Gupta and Gregurek-Novak 2001). A second group evaluated the efficacy of systemic itraconazole or terbinafine, topical terbinafine following nail plate avulsion, and ciclopirox lacquer in the treatment of patients with onychomycosis due to a variety of molds. Response rates were highest with *Aspergillus* spp. infections (100 percent), *Acremonium* spp. (71.4 percent), *S. brevicaulis* (69.2 percent), but only 40 percent of 26 patients with onychomycosis due to *Fusarium* spp. responded (Tosti et al. 2000).

An initial treatment with any of the systemically active antifungal agents licensed for dermatophyte infections may be beneficial in secondary mold infections, but itraconazole and terbinafine appear to be associated with the most consistent success rates. In extreme cases, it may be necessary to perform surgical removal of the nail plate or avulse the nail with a chemical application such as urease paste. However, following such treatment the nail tissue regrowth may not appear entirely normal.

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Superficial candidiasis

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C. albicans as a commensal of the gastrointestinal and genitourinary tracts	3	Host defense against superficial candidiasis at anatomic sites	7
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Superficial candidiasis includes infections of cutaneous or mucocutaneous tissues. *Candida albicans* is the causative agent in the majority of infections. Other *Candida* species that can cause the superficial infections include *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. stellatoidea*, *C. dubliniensis*, and *C. krusei* (Cann 1992). Superficial candidiasis can be acute, chronic, or recurrent. *Candida* spp. and *C. albicans*, in particular, are ubiquitous dimorphic fungal organisms that are part of the normal microflora of healthy individuals. However, they are also opportunistic pathogens that can quickly transform from harmless mucosal commensals to a highly pathogenic organism of the same tissues with significant morbidity and even mortality under the appropriate conditions (Fidel 1999).

Superficial candidiasis can include skin, oropharyngeal, gastrointestinal, vaginal, and conjunctival tissue infections. Cutaneous candidiasis usually occurs as erythematous patches on the thigh and buttocks, or as superficial infections of the hands in individuals who routinely wear plastic gloves. Mucocutaneous infections may be chronic and/or recurrent and one particular form is classified as the disorder known as chronic mucocutaneous candidiasis (CMC). These infections affect a variety of mucosal tissues, skin, and nails. Oropharyngeal candidiasis (OPC) involves infections of the buccal mucosa, gingiva, and tongue. The infections can be atrophic with erythematous lesions or pseudomembranous (thrush) with characteristic white lesions. Esophagitis is the most common form of gastrointestinal candidiasis. The second most common site of gastrointestinal candidiasis is the stomach. Vaginitis involves infections of the vaginal lumen and often the vulva as well. An estimated 75 percent of all women will experience at least one episode of acute vulvovaginal candidiasis (VVC). A separate population of women (5–10 percent) will experience recurrent VVC (RVVC) (Sobel 1988). Conjunctivitis caused by *C. albicans* infects the mucous membranes of the eye, while keratitis affects the cornea. The common superficial *Candida* infections are summarized below:

- Cutaneous
- Mucocutaneous
  - Oropharyngeal
  - Gasatrointestinal
    - Stomach
    - Esophageal
  - Vaginal
  - Conjunctival/keratitis

Host defense mechanisms that protect against *C. albicans* infections include innate resistance and acquired immunity. The relative contributions of these two systems, however, differ depending upon the site at which *C. albicans* enters the host. Innate host defenses (i.e. polymorphonuclear leukocytes, macrophages) are heavily involved in protecting the systemic circulation, antibodies (i.e. IgA, IgG) appear to play a role at both

systemic and mucosal sites, and cell-mediated immunity (CMI) (i.e. T cells, relevant cytokines) protects primarily mucosal tissues. With regard to mucosal tissues, while historically there was no distinction between different anatomic sites, there is now increasing evidence that 'not all mucosal tissues are created equal' relative to protective host defenses. Accordingly, current data show roles for several types of host responses at any one site, albeit at different levels of priority with respect to their protective capacity. This review covers the epidemiology of each superficial infection, properties of *C. albicans* as a commensal of the gastrointestinal and genitourinary tracts, properties of *C. albicans* as a pathogen causing superficial infections, and host defenses critical to protection against and susceptibility to superficial candidiasis at the various anatomic sites.

EPIDEMIOLOGY

Cutaneous/mucocutaneous candidiasis

Cutaneous infections affect the skin, whereas mucocutaneous infections affect the mucous membranes. Cutaneous candidiasis can be acute or chronic (reviewed in Odds 1988a) (Kirkpatrick 1989) and are characterized by erythema, edema, and a creamy exudate. Acute infections usually occur as a diaper rash in infants or between folds of skin in obese individuals or on the hands of individuals who routinely wear plastic gloves. The major symptoms are burning and itching. If left untreated, infections usually persist as long as the tissue remains moist. Chronic infections usually involve the feet and present with a thick layer of infected stratum corneum overlaying the epidermis.

Chronic mucocutaneous candidiasis is a heterogenous description that applies to infections involving chronic or recurrent infections of the skin, nails, and mucous membranes. The disease usually appears in the first 3 years of life, and affects males and females equally (Kirkpatrick 1984). Four types of CMC exist, including familial CMC, CMC with endocrinopathy, CMC with familial susceptibility and endocrinopathy, and CMC with onset after 10 years of age (reviewed in Ruhnke 2002). Patients usually develop oropharyngeal candidiasis followed by perianal candidiasis. Additionally, lesions appear on the scalp, torso, and extremities, and nail infections occur (Kirkpatrick 1984). Several disorders are associated with CMC, including hypoendrocrine function, autoimmune diseases, immunosuppression, hepatitis, diabetes, thymoma, dental enamel dysplasia, vitiligo, alopecia, and infectious diseases (Filler and Edwards 1993; Kirkpatrick 1994). Symptoms of CMC include disfiguring lesions on the skin and nails and dysphagia, due to ulcerative lesions on the buccal mucosa and esophagus (Kirkpatrick 1984).

Oropharyngeal candidiasis

Oropharyngeal candidiasis (OPC) involves infections of the hard and soft palate, tongue, buccal mucosa, and floor of the mouth. It presents as reddened patches (erythematous) or white curd-like lesions (pseudomembranous). Chewing and swallowing can be difficult under these conditions. Infections can be acute or recurrent, and are common in immunocompromised patients, especially those infected with the human immunodeficiency virus (HIV). OPC is also a common manifestation of CMC (reviewed in Odds 1988a) and occurs in patients with lymphoma, those undergoing steroid therapy, and in transplant recipients. Although OPC will occur under several immunocompromising conditions, it appears to be much more common in HIV-infected persons than under any other condition. In fact, OPC is often one of the first clinical signs of underlying HIV infection and will occur in 50–95 percent of all HIV-positive persons sometime during their progression to full-blown acquired immunodeficiency syndrome (AIDS) (Rabeneck et al. 1993). Thus, it is possible that a link between HIV and OPC is present that enhances susceptibility to OPC. Interestingly, highly active antiretroviral therapy (HAART) has reduced the incidence of OPC (Palella et al. 1998). This is postulated to be due both to increased immune responsiveness as well as the action of the protease inhibitors in HAART on the secretory aspartyl proteases (SAP), important virulence factors of *C. albicans* (Cassone et al. 1999; Gruber et al. 1999; Calderone and Fonzi 2001).

Gastrointestinal candidiasis

For the purposes of the discussion at hand, gastrointestinal (GI) candidiasis will be defined as infections of the esophagus, stomach, and small and large intestines. GI candidiasis is not easily recognized clinically but does occur in cancer patients who are heavily immunosuppressed and patients receiving antibiotic prophylaxis (Anaissie and Bodey 1990; Samonis et al. 1994). Esophagitis is the most common form of gastrointestinal candidiasis that occurs in later stages of immunosuppression and is considered an AIDS-defining illness (Macher 1988). It is extremely painful and can promote wasting because of the difficulty with swallowing. White plaques are usually observed similar to thrush. Complications include bleeding and perforation. Diagnosis is made on appearance of ulcers and erosions on the esophagus, and may need to be confirmed by endoscopy and biopsy of the affected site (Wheeler et al. 1987). The second most common site of GI candidiasis is the stomach where symptoms of gastritis occur. GI candidiasis is sometimes difficult to diagnose due to the lack of specific symptoms and the lack of the ability to differentiate between infectious and commensal *C. albicans*.

Vaginal candidiasis

Vulvovaginal candidiasis (VVC) affects a significant number of women predominantly in their reproductive years (Sobel 1988, 1992; Kent 1991). An estimated 75 percent of all women will experience an episode of acute VVC in their lifetime with another 5–10 percent succumbing to RVVC (Sobel 1988, 1992). Vaginitis involves infections of the vaginal lumen and often the vulva as well. Symptoms include burning, itching, soreness, an abnormal discharge, and dyspareunia. Signs include vaginal and vulvar erythema and edema. Acute VVC has several known predisposing factors including antibiotic and oral contraceptive usage, hormone replacement therapy, pregnancy, and uncontrolled diabetes mellitus (Sobel 1988, 1992; Kent 1991). RVVC is multifactorial in etiology, but is usually defined as idiopathic with no known predisposing factors in the majority of those affected (Sobel 1988, 1992). In women with RVVC, antifungal therapy is highly effective for individual symptomatic attacks, but does not prevent subsequent recurrence. There is little evidence that resistance to antifungal drugs plays a role in the pathogenesis of RVVC (Lynch et al. 1996). Instead, susceptibility to RVVC is postulated to be immune-based in that these otherwise healthy women experience repeated symptomatic episodes as a result of some immunological dysfunction or deficiency. Furthermore, it is postulated that recurrences are a result of relapse rather than reinfection since strain types of *C. albicans* tend to remain the same in women for multiple recurrences over several years (Vazquez et al. 1994). This is consistent with the fact that antifungal drugs are static rather than cidal and, as such, do not completely eliminate the organisms.

Conjunctivitis and keratitis

Conjunctivitis caused by *C. albicans* infects the mucous membranes of the eye. The eyelid and surrounding soft tissue can be swollen by adjacent tissue involved in the mycotic infection (Klotz et al. 2000). *Candida* spp. have been found in cases of ulcerative blepharitis in patients with skin atopy (Huber-Spitzy et al. 1992). Symptoms of *Candida* conjunctivitis include conjunctival erythema, cheesy discharge, and corneal ulceration. However, *Candida* can be isolated from the conjunctiva and eyelids of normal healthy individuals and remain asymptomatic (commensal) (reviewed in Klotz et al. 2000).

Keratitis caused by *Candida* spp. affects the cornea and causes ulcerative lesions on the eye (Klotz et al. 2000). The predisposing factor most often associated with *Candida* keratitis is chronic dry eye, but it can also be found in cases of trauma to the eye, abuse of corneal anesthetics, and following corneal transplant (reviewed in Klotz et al. 2000). Additionally, keratitis can be caused by wearing hard and soft extended-wear contact lenses, due to the fact that *Candida* has the ability to adhere to contact lenses and form a biofilm (Butrus and Klotz 1986; Elder et al. 1995). *Candida* keratitis presents as a small, demarcated ulcer with underlying opacity, and a granular infiltrate in the corneal epithelium and stroma can be observed. Diagnosis can be made upon microscopic examination of corneal scrapings.

General diagnosis

Together with compatible signs and symptoms, a definitive diagnosis of cutaneous and mucocutaneous *C. albicans* infection involves the demonstration of hyphae detected by microscopic analysis of tissue scraping (KOH wet mount preparation) or tissue invasion by histopathological examination. A swab culture is used to isolate the organism. Colonies are speciated in the laboratory using a variety of tests, such as germ tube formation, appearance (color) of colonies on CHROMagar Candida (Odds and Bernaert 1994; Sullivan and Coleman 1998), commercial API ID 32C or API 20C AUX (Richardson and Carlson 2002), polymerase chain reaction (PCR) (Janusz et al. 1988; Okhravi et al. 1998; Guiver et al. 2001), contour-clamped homogeneous electric field electrophoresis (CHEF) (Vazquez et al. 1992), DNA fingerprinting (Lockhart et al. 1997, 2001; Steffan et al. 1997), as well as other biochemical tests (Sood et al. 2000; Freydiere et al. 2002; Richardson and Carlson 2002).

Treatment

Nystatin, a topical polyene antifungal related chemically to amphotericin B, can be used for treatment of cutaneous candidiasis. Azoles (i.e. fluconazole, ketoconazole, butaconazole, itraconazole, etc.) are recommended for all other forms of superficial candidiasis. Antifungal drugs are highly effective for individual attacks or acute infections, but recurrence remains a problem. In fact, recurrent attacks can occur within days of cessation of treatment. Thus, in the appropriate clinical setting, maintenance therapy is encouraged. Table 1 summarizes the symptoms, diagnosis, and treatment for the various forms of superficial candidiasis.

C. ALBICANS AS A COMMENSAL OF THE GASTROINTESTINAL AND GENITOURINARY TRACTS

As stated previously, *C. albicans* is a commensal organism of the gastrointestinal and reproductive tracts. In this colonized state, individuals are considered asymptomatic carriers. It is widely recognized that as a commensal, *C. albicans* exists exclusively in the blastospore form, although it is rarely detectable in wet

Table 1 *Symptoms, diagnosis, and treatment of superficial candidiasis*

Infection	Signs/symptoms	Diagnosis	Treatment
Cutaneous	Erythema, edema, creamy exudates, burning, itching	Erythematous patches in folds of skin, KOH stain showing hyphae	Nystatin
Oropharyngeal	Reddened erythematous or white curd-like lesions, difficulty swallowing	Erythematous or pseudomembranous lesions (can be scraped), KOH stain showing hyphae	Azoles
Gastrointestinal–esophageal–stomach	Difficulty swallowing, bleeding, perforation, gastritis	Ulcers and erosions on esophagus, biopsy of lesion, KOH stain showing hyphae	Azoles
Vaginal	Burning, itching, soreness, white cottage cheese-like discharge, vuvlar edema, pruritis	KOH stain showing hyphae, culture positive, signs and symptoms	Azoles
Conjunctival/keratitis	Conjunctival edema, cheesy discharge, corneal ulceration	Corneal scraping, KOH stain showing hyphae	Topical/injected azoles

mount preparations of tissue scrapings. Instead, asymptomatic colonization is usually detected by swab culture. The most common sites showing detectable asymptomatic colonization are the rectum, oral cavity, and vagina. Although many *Candida* spp. are considered normal flora, *C. albicans* is the most common species recovered. In the oral cavity, the approximate rate of yeast carriage is 50–60 percent (Glick and Siegel 1999), but it has been reported to be as low as 2 percent and as high as 69 percent (Odds 1988b). In recent studies from our laboratory, we found that HIV-negative individuals had an oral colonization rate between 45 and 50 percent, with *C. albicans* present in 85 percent of isolates recovered. In contrast, HIV-positive individuals had an asymptomatic colonization rate in the oral cavity as high as 76 percent, with *C. albicans* present in 83 percent of isolates recovered (Leigh et al. 1998; Wozniak et al. 2002a). The distribution of *C. albicans* in the oral cavity includes the tongue, palate, and buccal mucosa. It can also be recovered from diverse areas of the gastrointestinal tract, including oropharynx, stomach, jejunum, illeum, and colon. In the stomach, *Candida* colonization ranges from 47 percent (when stomach pH is less than 3) to 70 percent (when pH is greater than 3), and species found include *C. albicans*, *C. krusei*, and *C. tropicalis* (Bernhardt and Knoke 1997). Additionally, at pH greater than 3, *C. glabrata* has been isolated from the stomach (Bernhardt and Knoke 1997). Rectal swabs were positive for *C. albicans* in 20–25 percent of healthy individuals (Odds 1988b; Soll et al. 1991). A similar proportion of normal healthy women (i.e. 20–25 percent) carry *C. albicans* in the vagina (Sobel 1988), although one study reported carriage in 40 percent of women (Soll et al. 1991), and two recent studies reported asymptomatic colonization of 13 percent (Fidel et al. 2003) and 11 percent (Bauters et al. 2002), respectively. A study of HIV-positive women reported that 37 percent were asymptomatically colonized at the vaginal mucosa, compared to 21 percent of HIV-negative high-risk women from the same study (Schuman et al. 1998b). The recovery of yeast increases during pregnancy and during select stages of the menstrual cycle, in particular when reproductive hormones are elevated (luteal phase). In contrast to colonization with *C. albicans* at mucosal sites, *C. albicans* is not considered normal flora of the skin. *C. albicans* is also infrequently isolated from water sources, soil, air, and plants, and those isolates recovered from food and beverages are considered contaminants from human and animal sources (Hasenclever et al. 1961).

Colonization with *C. albicans* at mucosal tissues is considered to occur at an early age, the organism having been acquired either by traveling through the birth canal, nursing, or from food sources. Aside from direct culture of mucosal surfaces, long-term colonization with *C. albicans* is assumed to be responsible for acquired immune responsiveness. Anti-*Candida* antibodies, circulating IgG, and mucosal IgA and IgG, can be detected in most healthy individuals (Witkin et al. 1988, 1989; Regulez et al. 1994; Wozniak et al. 2002a). Moreover, greater than 80 percent of healthy persons have positive cutaneous skin test reactivity to *Candida* antigen, and peripheral blood lymphocytes from more than 90 percent of healthy individuals proliferate in vitro to *Candida* antigen (Kirkpatrick et al. 1971; Mathur et al. 1977; Odds 1988a; Fidel et al. 1993). It is presumed that these acquired host responses in conjunction with innate resistance (i.e. polymorphonuclear leukocytes and macrophages) play a significant role in restricting *C. albicans* to mucosal surfaces in an asymptomatic commensal state.

C. ALBICANS AS A PATHOGEN OF THE GASTROINTESTINAL AND GENITOURINARY TRACTS

There are several scenarios in which *C. albicans* can convert from the harmless commensal to a serious pathogen. These include a lack of protective host defenses, expression of virulence factors by the organism, or environmental conditions that allow the yeast to overwhelm the protective host defenses.

C. albicans has several virulence factors that promote adherence and ultimately disease. Dimorphism itself is considered a virulence factor of *C. albicans* (reviewed in Calderone and Fonzi 2001). There are several environmental factors that allow *C. albicans* blastospores to transform into the more pathogenic hyphal form, the most common of which are temperature and pH. Acidic pH and temperatures below 35°C usually favor the blastospore form, whereas alkaline pH (> 6.5) and temperatures 37°C or higher favor hyphal formation. Although both forms can exist at any mucosal site, it is the hyphal form of *C. albicans* that is often observed in infected tissue. Hyphae appear to adhere and invade tissue by elaborating proteolytic enzymes. A secondary effect of dimorphism that can be associated with virulence is antigenic variation (De Bernardis et al. 1994). Antigens present on the blastospore and hyphal surface may be different and thus one or the other form may evade immune surveillance.

Aside from dimorphism, there are other components of *Candida* that are considered to be virulence factors. One virulence factor is the presence of integrin analogs on *C. albicans* that enhance adhesion to epithelial cells (Cann 1992). Interestingly, compared to non-*albicans* species, *C. albicans* has been reported to express the highest levels of the integrin analog. Another virulence factor is hyphal wall protein-1 (HWP-1), which is an adhesin that is important in attachment to human buccal epithelial cells through the action of transglutaminase (Staab et al. 2000). Other adhesins that are considered to be virulence factors include agglutin-like sequence-1 (Als1p), Als5p, and integrin-like protein-1 (Int1p) (reviewed in Calderone and Fonzi 2001). Phenotype switching, an in vitro phenomenon which involves phenotypic changes in the absence of genotypic changes, represents yet another possible virulence factor (Soll 1992, 2002). If it occurs in vivo, as predicted, it would affect both adherence and immunological escape. Also, SAPs, which are known to be involved in invasion of tissues and tissue damage, are thought to be virulence factors for *Candida* (reviewed in Calderone and Fonzi 2001; Hube and Naglik 2002) (De Bernardis et al. 1990, 1999). This seems especially true at the oral and vaginal mucosa where isolates recovered from infections have been shown to express high levels of specific SAPs (Hube et al. 1997; De Bernardis et al. 1999; Schaller et al. 1999). Additional virulence factors are the secretion of hydrolases which enhance adherence to mucosal tissues, and heat-shock proteins that bind to serum proteins and inhibit proper folding of the serum proteins as well as interactions with other proteins (reviewed in Ross et al. 1990; Matthews et al. 1998; Calderone and Fonzi 2001). More recently, other components, such as spindle assembly checkpoint component CaMad2p (Bai et al. 2002), copper and zinc-containing superoxide dismutase (Cu/Zn SOD) (Hwang et al. 2002), the transcription factor Spt3 (Laprade et al. 2002), and the two-component response regulator SSK1 (Calera et al. 2000) have been suggested as possible virulence factors for *Candida*. Finally, antimycotic drug resistance of some *Candida* spp. allows them to escape therapeutic treatment regimens.

Regardless of the level of tissue penetration that is usually quite shallow as a superficial infection, there can be large numbers of organisms associated with the tissue. It is generally considered that as a result of some change in the host, the organism grows in high enough numbers to overcome the host defenses, penetrates the tissue and begins to cause the symptoms associated with the infection. It is unclear, however, at what point in the pathway the organism converts into the hyphal form. It is postulated that the conversion begins prior to penetration and that the extension on the germ tube initiates the penetration into the tissue. Other proponents suggest that the yeast initially invades the tissue and the hyphal form occurs as a consequence of exposure to serum components as the immune cells infiltrate the tissue in an attempt to eliminate the organism. The latter is supported by the high incidence of mucosal infections caused by *C. glabrata*, an organism that does not form hyphae (Fidel et al. 1999b). Nevertheless, it is clear that both the host and the organism contribute significantly to *C. albicans* as a pathogen.

HOST RESPONSE AGAINST *C. ALBICANS*

Innate resistance

The polymorphonuclear leukocyte (PMNL) (neutrophil) is the first line of defense against *C. albicans*. The role of macrophages in defense is less clear although they readily phagocytize the yeast form of *Candida*. Anti-*Candida* activities by PMNL or macrophages can be oxidative (i.e. hydrogen peroxide, nitric oxide, superoxide dismutase) or non-oxidative (i.e. lactoferrin, lysozyme, calprotectin, defensins) (reviewed in Fidel 1999). Phagocytosis is usually required for oxidative mechanisms although there is some evidence that oxidative products can be secreted by PMNL that affect nearby fungal hyphal forms too large to be phagocytosed (Levitz and Farrell 1990).

PMNL are relatively effective as a first line of defense, providing that organisms gain access to the vasculature or that the PMNL attach to hyphae. However, PMNL function can be greatly enhanced by cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-2, IL-8, and colony-stimulating factors (CSF) (Djeu 1993). Oxidative mechanisms are enhanced in the presence of serum as well. There are also both classical and alternate complement pathways that become activated by *C. albicans* which promote increased adherence to phagocytes

(Heidenreich and Dierich 1985; Edwards et al. 1986; Kozel et al. 1987).

The functional mechanisms by which monocytes and macrophages affect *C. albicans* are less well defined (reviewed in Fidel 1999). In general, once phagocytosed in vitro, killing is at least as effective as PMNLs and involves oxidative mechanisms and candidicidal molecules. Activation of macrophages with cytokines appears to increase the killing activity, although the effects appear short-lived at best. Killing of both blastospores and hyphae have been demonstrated, but the rate and efficiency depends on the source of the cells. For example, pulmonary macrophages appear to be particularly efficient against *C. albicans*.

A role for dendritic cells against fungal infections has also been suggested. Immature myeloid dendritic cells can phagocytose both yeast and hyphae of *C. albicans* (Romani et al. 2002). Recognition of yeast forms is through the mannose-fucose and complement receptor 3 (CR3) receptors, while recognition of hyphae is through the FcγR and CR3 receptors (Romani et al. 2002). Mature dendritic cells are less phagocytic than immature cells, but they have increased expression of major histocompatibility complex (MHC) class II, which leads to more efficient antigen presentation (Banchereau and Steinman 1998; Pulendran et al. 2001; Reis e Sousa 2001). Dendritic cells are also capable of directing the acquired immune response to *Candida*, depending on the form phagocytosed and the receptors used, and the type of response induced can affect whether a protective or nonprotective response is generated (Romani et al. 2002).

The activity of natural killer (NK) cells against *C. albicans* has been controversial. While a limited number of studies showed inhibition of *C. albicans* growth by murine leukocyte suspensions containing NK cells (Brummer et al. 1986; Morrison et al. 1987; Steele et al. 1999a), the general consensus is that NK cells are not effective against *Candida* (Djeu and Blanchard 1987; Arancia et al. 1995). Suffice it to say that, although inhibition of *C. albicans* can be mediated by cells from lymphoid tissue, the primary and secondary effector cells are innate, not specific, and consist of PMNLs and macrophages, respectively.

A final type of cellular innate resistance mechanism has only recently been discovered and involves epithelial cells lining the surface of mucosal tissues. Oral and vaginal epithelial cells from mice, macaques, and humans have the ability to inhibit the growth of *Candida* in vitro (Steele et al. 1999a, b; Barousse et al. 2001). The inhibition requires contact between viable epithelial cells and *Candida*, with no role for soluble factors and does not involve phagocytosis (Steele et al. 1999a, 2000). While the mechanism is not fully elucidated, the inhibition appears to be mediated by a carbohydrate moiety on viable epithelial cells (Steele et al. 2001) and is static rather than cidal (Nomanbhoy et al. 2002).

Humoral immunity

C. albicans antigens readily induce the production of immunoglobulins as evidenced by the presence of *Candida*-specific IgG in serum and IgA in mucosal secretions of healthy individuals. Thus, it might be presumed that humoral immune mechanisms are important host defenses in both the systemic circulation and at mucosal sites. However, a role for antibody-mediated immunity at mucosal sites has been controversial. A major argument against a role for humoral immunity comes from clinical observations wherein individuals with congenital or acquired B-cell abnormalities are not more susceptible to mucosal (or systemic) candidiasis than immunocompetent individuals (Rogers and Balish 1980). Other observations suggestive of a lack of a role for antibody include the following:

- levels of *Candida*-specific IgA were higher in the saliva of HIV-positive patients than in HIV-negative controls (Coogan et al. 1994; Challacombe and Sweet 1997; Millon et al. 2001)
- levels of *Candida*-specific IgA, sIgA, IgG, or subclasses did not differ in saliva of HIV-positive OPC-positive patients compared to HIV-negative OPC-negative controls (Wozniak et al. 2002a)
- women with RVVC had normal levels of *Candida*-specific IgG in serum and IgA in vaginal secretions (Kirkpatrick et al. 1971; Mathur et al. 1977; Fidel et al. 1997)
- mice protected from a secondary vaginal challenge did not have detectable levels of *Candida*-specific antibodies in vaginal secretions (Wozniak et al. 2002b)
- in vitro studies have provided little evidence that serum antibody and complement can kill *C. albicans* (Rogers and Balish 1980) despite increased phagocytosis of the organism in the presence of complement.

Despite the vast information showing little role for *Candida*-specific antibodies or at least little evidence of any deficiency in such naturally induced antibodies, there are considerable data derived from animals that support a role for *Candida*-specific IgA and IgG antibodies in protection (Cassone et al. 1995; Polonelli et al. 1996; De Bernardis et al. 1997; Han et al. 1998, 2000). These contradictory findings may be reconciled by taking into consideration a theory proposed by Casadevall (1995). He suggested that a pool of natural antibodies may consist of a mixture of protective, nonprotective, and indifferent antibodies. The type that predominates in the pool of antibodies dictates the outcome (protection versus infection). If this theory is correct, then 'protective' antibodies should be present under conditions of 'immunity' and should be identifiable and able to be isolated. Unfortunately, the lack of protection observed in the presence of normal levels of

Candida-specific antibodies suggests that 'protective' antibodies, if they exist, are in the minority of most pools examined. On the other hand, protection in animal models using 'protective antibodies' with known specificities and of a specific class and subclass, provides considerable hope for the use of these antibodies for vaccine development.

Cell-mediated immunity (CMI)

In contrast to the controversial issues regarding the role of humoral immunity against mucosal candidiasis, the role of CMI is somewhat better defined, at least as far as clinical correlations are concerned. Clinical observations support an important role for CMI in protection of mucosal tissues against invasion by *C. albicans*. The strongest evidence is that individuals with reduced or absent CMI (AIDS patients, transplantation recipients, lymphoma patients treated with T-cell immunosuppressive therapy, and patients on corticosteroid therapy) have a high incidence of mucosal candidiasis (i.e. oropharyngeal, esophageal, mucocutaneous) (reviewed in Fidel and Sobel 1994). In addition, there is a direct causal relationship between reduced *Candida*-specific CMI in the peripheral circulation and the incidence of individuals with CMC (Kirkpatrick et al. 1970, 1971). The role of T cells, however, at each respective mucosal site (i.e. oral mucosa, vaginal mucosa, GI tract) is poorly understood as are the relative roles of cells migrating from the systemic circulation into the mucosal sites versus those resident in the mucosal tissue.

The mechanisms associated with CMI against *C. albicans* appear to be dominated by $CD4^+$ T cells. Protection often correlates with delayed-type hypersensitivity (DTH), but a direct role for DTH has been difficult to assess. There have been reports where the presence of DTH did not correlate with protection (Fidel et al. 1994; Garner and Domer 1994). Nevertheless, *Candida*-specific DTH develops in vivo in the immunocompetent individual. DTH is demonstrable at an early age and likely results from early exposure to *C. albicans*. Thus, a pool of *Candida*-specific memory T cells continues to circulate in the peripheral circulation. The response is presumably initiated at the mucosal site where antigen-presenting cells such as Langerhans cells or dendritic cells process and present the antigen to locally associated T cells or transport it to the draining lymph nodes where T cells can be primed. Upon migration of the *Candida*-specific T cells to the site of antigen deposition and contact with additional processed antigen, cytokine secretion by the T cells activates tissue macrophages and signals other phagocytic cells to infiltrate the site of the insult (mononuclear infiltrate), in an attempt to eliminate or reduce the organism. The current dogma is that Th1-type responses are associated with resistance against intracellular pathogens, while Th2-type responses are associated with resistance to extracellular pathogens. However, for *C. albicans*, and in fact most medically important fungi, Th1-type responses have been associated with resistance to infection, with Th2-type responses showing an association with susceptibility to infection (Magee and Cox 1995; Romani et al. 1996; Cenci et al. 1999; Tachibana et al. 1999; Cain and Deepe 2000; Wuthrich et al. 2000).

In contrast to the role $CD4^+$ cells play in protection from *C. albicans* infections at mucosal tissues, a role for $CD8^+$ cells as an acquired host defense is unknown. To date, there has been no evidence that $CD8^+$ cytotoxic T lymphocytes (CTL) are directly involved in host defense against *C. albicans*. However, gnotobiotic mice deficient in $CD8^+$ T cells are susceptible to gastric candidiasis (Balish et al. 1996; Jones-Carson et al. 2000). Additionally, $CD8^+$ T cells have a role in suppression of CMI in candidiasis. There are numerous reports showing that $CD8^+$ T cells specific for *C. albicans* cell wall antigens, both glycoprotein and mannan, suppress DTH and lymphocyte proliferation in infected mice (Domer et al. 1989; Garner et al. 1990; Garner and Domer 1994). Suppressor or regulatory cells also appear to hinder the ability to clear the infection (Garner and Domer 1994). It appears that the downregulation of DTH includes an antagonistic function of IL-12 p40, and that the production of $CD8^+$ downregulatory T cells is dependent on the presence of IL-10, IL-4, and IFN-γ in the absence of biologically active IL-12 (Li et al. 1998; Wang et al. 1998). Additionally, naïve $CD8^+$ T cells stimulated with IL-2 inhibit the growth of *C. albicans* (Beno et al. 1995). This was first shown in cells from uninfected mice, but more recently has also been suggested clinically in persons who have had a recent episode of OPC (Colon et al. 1998). The mechanism appears to be non-MHC restricted through the action of excreted enzymes (Mathews and Beno 1993; Beno et al. 1995) and may in fact be a form of innate immunity.

HOST DEFENSE AGAINST SUPERFICIAL CANDIDIASIS AT ANATOMIC SITES

Cutaneous/mucocutaneous candidiasis

Host defense against cutaneous or mucocutaneous candidiasis involves both innate and adaptive host defenses. Innate defenses involve PMNL and monocytes/macrophages. These cells can be resident in the respective tissues or recruited to the site by chemotactic factors. The adaptive host defenses are predominantly T cells, most probably of the $CD4^+$ Th1-type cells. In cases of CMC, in vitro *Candida*-specific responses by peripheral blood mononuclear cells (proliferation and cytokine production) are reduced compared to those without CMC, suggesting some immunological defect in CMI (Kirkpatrick et al. 1971; Ruhnke 2002). In addition,

delayed skin test reactivity to *Candida* antigen is usually absent (Kirkpatrick et al. 1970; Ruhnke 2002). These reduced responses can be restricted to *Candida* or can be part of an overall reduced responsiveness to several antigens (Kirkpatrick et al. 1970, 1971). The results of studies involving experimental cutaneous disease also suggest a protective role for CMI (Wilson and Sohnle 1986). In a more recent report, in fact, the authors have described the costimulatory isoform of B7 (B7-2) as being required on antigen-presenting cells to induce a protective Th1-type response (Gaspari et al. 1998). By contrast, there is little evidence that humoral immunity has a role in protection in CMC. *Candida*-specific antibodies are present in sera and mucosal secretions of those with or without CMC (Lehner et al. 1972; Ruhnke 2002). However, aside from immunoreactivity for detection of antibodies, no studies have assessed the actual function of the antibodies present. Serum factors have been suggested as a possible source of the immunological defect(s) in patients with CMC (Valdimarsson et al. 1973; Twomey et al. 1975), in that it has been reported that *Candida*-specific carbohydrate antigens can be present and persist in the serum of those with CMC that inhibit peripheral blood lymphocyte responses (Fischer et al. 1978).

Interestingly, the immune responses against cutaneous and mucocutaneous candidiasis may involve an immune network in the epidermis termed skin-associated lymphoid tissue (SALT) as well as those infiltrating from the periphery (Singl et al. 1999). SALT includes epidermal Langerhan's cells, dermal dendritic cells, dermal T lymphocytes consisting primarily of α/β T-cell receptor (TCR)-positive cells and small numbers of γ/δ TCR^+ cells, epidermal T cells with considerably more γ/δ TCR^+ cells, keratinocytes, and microvascular endothelial cells (Bos et al. 1990).

Gastrointestinal candidiasis

Host defense against GI candidiasis has been studied primarily through the use of immunodeficient mice. Studies using both congenitally deficient and severe combined immunodeficient (SCID) mice have shown that T cells are critical for effective protection against GI candidiasis (Cantorna and Balish 1990). SCID mice were shown to be more susceptible to GI tract colonization, but were not susceptible to intravenous challenge or dissemination from the GI tract. Mice with congenital T-cell (*nude/nude*) and phagoctyic cell (*beige/beige*) deficiencies (*nu/nu, bg/bg)* developed persistent GI candidiasis, while those with only phagocytic cell deficiencies (*nu/+*, *bg/bg*) cleared *C. albicans* from the GI tract effectively. The *nu/+* mice (wild-type) responded to *Candida* antigens with IL-2 production and DTH, while *nu/nu* mice did not, suggesting the correlation between a Th1-type response and clearance of the organism. An important question, however, is what role circulating and mucosal T cells actually play in the protection. It has been shown that the mucosal tissue of the GI tract has intraepithelial lymphocytes (IEL), Peyer's patches (organized lymphoid tissue), T cells, and antigen-presenting cells that function in many types of GI infections. Much less is known, however, about the function of any of these cell populations against *C. albicans*, although Bistoni and colleagues have begun to address this issue. It was shown that Peyer's patch lymphocytes from colonized mice which went on to clear the fungus from the GI tract produced Th1, but not Th2-type cytokines in response to *Candida* antigen (Bistoni et al. 1993). Additionally, GI candidiasis appears to conform to the Th1/Th2-type immune response pattern seen in cutaneous infection and experimental systemic infections, namely that Th1-type responses are associated with resistance against infection, while Th2-type responses promote susceptibility (Magee and Cox 1995; Romani et al. 1996; Cenci et al. 1999; Tachibana et al. 1999; Cain and Deepe 2000; Wuthrich et al. 2000). The role of antibodies against GI candidiasis has not been evaluated experimentally except through early studies that showed no protection from *Candida* antibody-containing sera (Balish and Filutowicz 1991; Greenfield 1992).

Host response to esophageal candidiasis has not been studied clinically or in animal models. However, clinical observations show susceptibility to infection almost exclusively during severe T cell immunocompromised conditions (i.e. late-stage AIDS) (Macher 1988; Hoover et al. 1993; Martinez et al. 2000), suggesting that protective host defenses are intact for longer periods of time in the esophagus compared to the oral cavity, although this could also be due to less of a commensal presence of *Candida* in the esophagus. Additionally, animal models have been developed for study of esophageal candidiasis (De Repentigny et al. 2000; Walsh et al. 2000), but have been used for evaluation of chemotherapeutic treatment or fungal pathogenesis, and studies on host defense against esophageal candidiasis are still sorely needed.

Oropharyngeal candidiasis

Oropharyngeal candidiasis (OPC) occurs primarily in individuals with HIV (Nielsen et al. 1994; Sangeorzan et al. 1994; Schuman et al. 1998a; Greenspan et al. 2000). Host defense against OPC has only recently begun to be studied in greater detail due primarily to the HIV epidemic. Animal models have not been of great value in understanding the natural host defense, as the disease resolves too quickly with variable and inconsistent fungal titers in immunocompetent animals (reviewed in Samaranayake and Samaranayake 2001). On the other hand, information has accumulated from humans who

are immunocompromised. In general, most data to date suggest that $CD4^+$ Th1-type cells are critical for host defense against infection. Clinically, OPC is most common in HIV-positive persons when $CD4^+$ cell numbers drop below 200 cells/μl (Rabeneck et al. 1993; Nielsen et al. 1994; Schuman et al. 1998a; Greenspan et al. 2000). In vitro immune analyses using peripheral blood mononuclear cells (PBMC) show that cells from most individuals respond to *Candida* antigens with Th1-type cytokines. Thus, it is generally considered that susceptibility to OPC is enhanced under reduced $CD4^+$ T cells due to either a lack of Th1-type responses and/or a shift to Th2-type responses. Some early studies in HIV-positive individuals suggested that reduced Th1-type cytokine responses were responsible for OPC (Quinti et al. 1991). However, a study evaluating PBMC reactivity in HIV-negative and HIV-positive persons with or without symptomatic OPC stratified by $CD4^+$ T-cell numbers showed little to no appreciable differences in *Candida*-specific proliferation or cytokine production between the two groups (Leigh et al. 2001). In another study, no demonstrable deficiencies in clonal responses of the PBMC from such patients to a variety of antigenic peptides were shown (Kunkl et al. 1998). These results suggested that the *Candida*-specific T cells themselves were not becoming defective with immunosuppression, but that a threshold number of $CD4^+$ T cells was required to protect the oral cavity against infection by this commensal organism. Below this threshold number of cells, local immune mechanisms must function exclusively for protection. The prevalence of OPC may then depend on the status of the local immune mechanisms. Indeed, some individuals with < 200 CD4 cells/μl never have OPC while others have recurrent bouts of OPC.

Local immunity has only now begun to be studied clinically. In support of the Th1/Th2 dichotomy concept, it was recently reported that HIV-negative individuals had Th1/Th0 cytokines in their saliva, whereas HIV-positive individuals had primarily Th2-type cytokines, which were in higher concentration in those patients with OPC (Leigh et al. 1998). Interestingly, the Th2-type profile was the result of reduced Th1-type cytokines rather than increased Th2-type cytokines. Lymphocytes have also been examined in the OPC lesions and both $CD4^+$ and $CD8^+$ cells have been identified (Romagnoli et al. 1997). Other investigators have suggested that only $CD8^+$ cells were present. Data from our laboratory supports the latter observation, and, in fact, we have shown an accumulation of $CD8^+$ T cells at a considerable distance from the *Candida* located superficially at the outer epithelium (Myers et al. 2003). This cellular accumulation is not seen in those without OPC. This may suggest a role for $CD8^+$ T cells against infection, with a potential problem in cell trafficking leading to OPC. Interestingly, a murine AIDS model (MAIDS) showed a rate of 30 percent recurrent OPC in inoculated mice with a predominance of $CD8^+$ T cells recruited into the tissues (Deslauriers et al. 1997).

Studies using experimental models support a role for a combination of T cells and innate cells against OPC. Both $CD4^+$ and $CD8^+$ T cells were shown to be recruited along with macrophages into the mucosal tissue, and intraepithelial $CD4^+$ T cells persisted after resolution of the infection (Allen et al. 1994; Chakir et al. 1994). Moreover, there was a time-dependent recruitment of γ/δ TCR^+ cells that correlated with the resolution of the disease (Chakir et al. 1994; Elahi et al. 2000). Analysis of cytokines showed a role for both Th1- and Th2-type cytokines in resistance to infection depending on the strain of mice used (Elahi et al. 2000). A recent study examined cytokines in oral tissues from infected mice and found increases in IL-6, IFN-γ, and TNF-α in mice recovering from oral infection (Farah et al. 2002). However, no IL-2, 4, or 10 was detected during infection (Farah et al. 2002). As with GI candidiasis, however, little is known concerning the specific roles of mucosal versus systemic T cells in the mucosa versus circulating T cells in protection against infection. In a recent study in vivo cellular depletions were used to create immunocompromised mice. $CD4^+$ T cells, PMNL, and macrophages all appeared to be important in resistance to disease (Farah et al. 2001).

Humoral immunity, on the other hand, again does not appear to play a role in protection against or susceptibility to OPC. Early studies of *Candida*-specific antibodies in saliva of HIV-positive persons with or without OPC yielded similar or elevated levels of IgA or IgG, although in one study a reduced affinity of *Candida*-specific IgA antibodies was shown in AIDS patients (Coogan et al. 1994). There is no evidence to date, however, that a deficiency in *Candida*-specific antibodies is present in HIV-positive persons that could account for the increased prevalence of OPC (Wray et al. 1990; Millon et al. 2001). A recent comprehensive analysis of *Candida*-specific IgA and IgG in the saliva of a large number of HIV-negative and HIV-positive persons stratified by OPC status as well as $CD4^+$ T-cell numbers, which included subclass analysis, supports the earlier findings (Wozniak et al. 2002a).

Other anti-OPC mechanisms involve innate cellular defenses. PMNL appear to play a role, as neutropenic patients are susceptible to OPC (Anaissie and Bodey 1990). However, in-depth studies with PMNL in the oral cavity have not been conducted. Epithelial cells represent another cell type with potential innate function. As stated earlier, epithelial cells have been shown to inhibit the growth of *Candida* spp. in vitro by a static mechanism (Steele et al. 1999a, 2000; Nomanbhoy et al. 2002). While both oral and vaginal epithelial cells have anti-*Candida* activity via cell contact through a putative carbohydrate moiety (Steele et al. 2001), oral epithelial cells have a much increased capacity to inhibit the growth of *Candida* (up to 80 percent versus 40 percent

for vaginal cells) (Steele et al. 2000). Analysis of oral epithelial cells in HIV-positive persons showed significantly reduced activity when the cells were derived from patients with OPC compared to that from patients without OPC, potentially contributing to the susceptibility to infection (Steele et al. 2000). Additionally, epithelial cells produce both cytokines and chemokines in response to *Candida*, which may contribute to the innate and/or adaptive immune response (Steele and Fidel 2002). Thus, several lines of defense may be important for protection against OPC, many of which do not become evident until $CD4^+$ cells are reduced below the protective threshold.

Vaginal candidiasis

In contrast to the fairly well documented role for CMI by $CD4^+$ T cells against oral, gastrointestinal, and cutaneous candidiasis, the role for CMI against vaginal candidiasis has not been clear and more recently has been challenged. Thus, despite intense efforts to date, the natural protective host defenses against VVC remain poorly understood.

Studies of host defense against VVC began with clinical observations in which women with RVVC were examined for systemic *Candida*-specific responsiveness by culture of their PBMC in vitro or through skin-testing in search of DTH responses. The results over time conflicted with the results of some studies wherein it was suggested that there was a deficiency in *Candida*-specific CMI, or an antigen nonspecific deficiency, or no deficiency at all (reviewed in Fidel and Sobel 1996). Thus, these studies did little to identify an immunological deficiency, if any, in women with RVVC.

In subsequent studies, animal models have been employed to attempt to understand the natural host defense mechanisms against vaginal *C. albicans* infection. Such studies have included rat, mouse, and macaque models of vaginal candidiasis (reviewed in Fidel and Sobel 1999). All are dependent on a state of pseudoestrus for the infection to become established. The rat model has been particularly helpful in suggesting a potential role of antibodies against *C. albicans* at the vaginal mucosa. The mouse model, on the other hand, has been used primarily to study the role of CMI (reviewed in Sobel et al. 1998). The macaque model is relatively new and to date has been described with only limited immunological data (Steele et al. 1999b). Mice and rats, in contrast to macaques and humans, are not normally colonized with *C. albicans* and do not have any preexisting *Candida*-specific immunity.

Mice inoculated under pseudoestrus conditions acquire a persistent vaginal infection together with systemic Th1-type responses, as evidenced by Th1-type cytokine production in response to *Candida* antigen by lymph node cells draining the vaginal tissue, and *Candida*-specific DTH. However, this systemic Th1-type CMI or that preinduced by systemic immunization with *Candida* antigen in adjuvant did not protect mice against vaginal candidiasis (Fidel et al. 1994). Partial protection against vaginitis was achieved, however, in animals given a second inoculation following the spontaneous resolution of a primary infection in the absence of estrogen (Fidel et al. 1995b). Interestingly, suppression of *Candida*-specific systemic CMI or systemic T-cell depletion had no effect on the protection, suggesting that systemic CMI had a limited role at the vaginal mucosa in protection against vaginal candidiasis. In support of this suggestion as well, mice, either resistant or susceptible to systemic *C. albicans* infection (Hector et al. 1982), were shown to be equally susceptible to vaginitis and could all be partially protected against a second vaginal infection (Fidel et al. 1995a). These data were supported by those gathered in another study in which six different strains of mice representing four haplotypes were used (Black et al. 1999a). Finally, adoptive transfer of *Candida*-sensitized T cells into T-cell deficient (nude) mice had no effect on the course of vaginitis (Black et al. 1999b). Based on these results, it was postulated that some form of locally acquired mucosal immunity, T-cell and/or antibody-mediated, was responsible for protecting mice against vaginal *C. albicans* infection and that the vaginal mucosa had a significant level of immunological independence with little influence from systemic sources.

The independent compartmentalization concept correlates well with a clinical study showing that RVVC patients had normal levels of *Candida*-specific Th1-type CMI in the peripheral circulation (Fidel et al. 1993). It should be mentioned, however, that others have obtained data to the contrary (Corrigan et al. 1998; Carvalho et al. 2002). Nevertheless, the lack of identifiable deficiencies in systemic CMI in women with RVVC correlates with two relevant clinical observations; women with RVVC are not susceptible to CMC or other forms of cutaneous candidiasis (Sobel 1988), and women with CMC are generally not susceptible to RVVC (Odds 1988a). Thus, the immune deficiency, if any, in RVVC patients is presumed to be localized to the vagina and does not occur at the systemic level. These data were the first evidence to suggest some level of immunological independence regarding CMI host defense mechanisms at the vaginal mucosa against *C. albicans*. Further support for these observations comes from studies with HIV-infected women, who, despite the higher incidence of OPC under conditions of immunosuppression, do not get VVC any more frequently than HIV-negative women (Clark et al. 1995; White 1996; Schuman et al. 1998b; Leigh et al. 2001). Furthermore, peripheral blood lymphocyte proliferation and Th1/Th2 cytokine production in response to *Candida* antigens was relatively similar in HIV-positive women with or without symptomatic VVC (Leigh et al. 2001), similar to RVVC patients.

Subsequent studies focused on the presence of T-cell subpopulations in the vaginal mucosa. It had been established that the vaginal mucosa, while not composed of organized lymphoid tissues as the Peyer's patches in the GI tract, is immunologically competent for both CMI and T-dependent antibody production with the presence of both T cells and MHC class II^+ cells. To this end, we and others reported that vaginal lymphocytes are phenotypically distinct from those in the peripheral circulation (Nandi and Allison 1991; Fidel et al. 1996). Despite the fact that $CD4^+$ α/β TCR^+ cells predominate in the vaginal tissue, a higher percentage of γ/δ TCR^+ cells are present with few if any $CD8^+$ cells, compared to the systemic circulation. Interestingly, human vaginal lymphocytes are also phenotypically distinct in comparison to those in the peripheral circulation, but differ from mice in the types and relative proportions of cells; the human vagina has increased $CD4^+$ and γ/δ T cells but also equal numbers of $CD8^+$ T cells (Hladik et al. 1999; Fidel et al. 2001).

Studies to examine changes in vaginal T cells during experimental vaginal *Candida* infection have all shown little evidence for modulation of α/β or γ/δ vaginal T cells during either primary or secondary experimental vaginal *C. albicans* infections, and no evidence for systemic T-cell infiltration during a vaginal *Candida* infection (Fidel et al. 1999a; Wormley et al. 2001a). These results are consistent with those from immunodeficient or knockout mice (Cantorna et al. 1990; Black et al. 1999b), and correlate with the systemic immune studies in immunocompetent mice (Fidel et al. 1994, 1995a, b, c). This lack of responsiveness to *Candida* infection in mice is in direct contrast, however, to a murine model of a genital *Chlamydia trachomatis* or herpes simplex virus-2 (HSV-2) infection where $CD4^+$ T cells were shown to infiltrate readily into the vaginal mucosa in response to infection (Kelly and Rank 1997; Parr and Parr 1997). Taken as a whole, these data emphasize the uniqueness of the host vaginal response(s) against *C. albicans*. Interestingly, a study in which a dual infection with *Candida* and *Chlamydia* was studied, showed a complete independence of 'responsiveness' in the upper genital tract (*Chlamydia*) and 'lack of responsiveness' in the lower tract (*Candida*) (Kelly et al. 2001), illustrating further the complexity of immunity in the genital tract. Together these data suggested that instead of a simple lack of a role for systemic or local CMI against *C. albicans* at the vaginal mucosa, there may be some form of immunoregulation or tolerance that prohibits a more profound CMI response.

There are several pieces of evidence, both experimentally and clinically, that support immunoregulation against CMI at the vaginal mucosa. First, experimental vaginitis in δ-chain TCR knockout mice has been found to be less severe, suggestive of a tolerance role for vaginal γ/δ T cells (Wormley et al. 2001b). Evaluation of Th cytokines in the vaginal tissue homogenates from mice showed high constitutive concentrations of transforming growth factor-beta (TGF-β) in naïve mice that were increased further in estrogen-treated and/or vaginally infected mice; these same mice had low levels of other Th1- or Th2-type cytokines (Taylor et al. 2000). Clinically, TGF-β was found in fairly high concentrations in human vaginal secretions with modulations upward over the course of the menstrual cycle (Fidel et al. 2003). In addition, although Th1/Th2 cytokines are detectable in vaginal secretions of women with RVVC, there are no discernible patterns that would account for susceptibility to RVVC when comparisons were made to control women (Fidel et al. 1997). In the mouse model of infection, T cells with appropriate homing receptors for infiltration into the vaginal mucosa are reduced/lost during a vaginal *Candida* infection despite the upregulation of reciprocal adhesion molecules on the vaginal endothelium (Wormley et al. 2001b). Further supporting immunoregulation, a T-cell-derived antigen-binding molecule (TABM) has been identified in serum of RVVC patients, and to a lesser extent in control women, that can become associated with TGF-β and inhibit *Candida*-specific peripheral blood leukocyte (PBL) proliferation and cytokine production (Little et al. 2000). Finally, despite some preliminary evidence that the intravaginal challenge of normal healthy women with *Candida* antigen stimulated Th1-type cytokine secretion in vaginal lavages 16–18 hours post-challenge (Fidel et al. 1997), the results of a more recent study in which responsiveness to the antigen in a large number of women was tested during different stages of the menstrual cycle showed no evidence of immune responsiveness (Fidel et al. 2003). Thus, there is convincing evidence that immunoregulation or tolerance is functioning at the vaginal mucosa and that it inhibits the ability of *Candida*-specific systemic CMI to reach the vaginal mucosa.

The rat model, used primarily to study humoral immunity, was also used to evalulate CMI (De Bernardis et al. 2000). The progression of infection, however, is quite different in the rat than the mouse, in that spontaneous resolution occurs within a 3-week period in the rat. Consistent with this property is the fact that T cells could be demonstrated infiltrating the vaginal mucosa following infection, and Th1-type cytokines were produced and secreted into vaginal secretions. In addition, in contrast to data obtained from the mouse model of vaginitis, a recent study in the rat model showed that passive transfer of both $CD4^+$ and $CD8^+$ T cells from previously infected rats accelerated clearance of *Candida* in the vagina of naïve animals (Santoni et al. 2002). Also, the $CD4^+$ T cells were more effective at clearance than the $CD8^+$ T cells, suggesting that, in the rat model, vaginal lymphocytes, especially $CD4^+$ T cells, are important in clearance of *Candida* from the vagina (Santoni et al. 2002). Thus, the rat, in contrast to the mouse and human, may exemplify what

would occur in response to infection in the absence of immunoregulation.

The role of humoral immunity in recovery from vaginitis is equally controversial. Immunoglobulins are clearly present in vaginal secretions, including *Candida*-specific antibodies (IgG and IgA) (Witkin et al. 1988, 1989; Regulez et al. 1994). Interestingly, though, B cells are a minor constituent of the mouse, rat, or human vaginal mucosa (Parr and Parr 1991). In contrast to other mucosal sites, IgG antibodies predominate over IgA antibodies, at least on the basis of total immunoglobulin (Fidel et al. 1997; Kutteh et al. 1998). Despite that, clinical studies show that serum IgG and vaginal IgG and IgA are similar in women with RVVC (symptomatic or in remission) and healthy control women with no history of RVVC (Kirkpatrick et al. 1971; Mathur et al. 1977; Fidel et al. 1997). Experimentally, similar results were observed in infected and uninfected rhesus macaques, including the predominance of vaginal IgG over IgA immunoglobulins (Steele et al. 1999b). In addition, in a mouse model of vaginal infection, *Candida*-specific IgA and IgG were not detectable in lavage fluids of animals protected against secondary challenge, suggesting little or no protective role for *Candida*-specific antibodies against the murine vaginal infection (Wozniak et al. 2002b). While these experimental and clinical observations largely fail to support a role for antibody against candidiasis, there are studies in rodents that indicate a protective role for humoral immunity in experimental vaginal candidiasis. Pollonelli and colleagues showed that rats given an intravaginal immunization using antibodies that neutralized the anti-*Candida* activity of a yeast killer toxin were protected against a vaginal infection (Polonelli et al. 1994). The protection was found to be associated with rising vaginal titers of anti-idiotypic IgA antibodies that could transfer protection passively to unimmunized rats. Interestingly, killer toxin anti-idiotypic-like antibodies were also detected in women with symptomatic vaginitis (Polonelli et al. 1996). Similarly, Cassone and colleagues (Cassone et al. 1995; De Bernardis et al. 1997) have shown that a primary *C. albicans* vaginal infection in estrogen-treated rats induced anti-mannan and anti-aspartyl IgA antibodies in vaginal secretions, and that those antibodies appeared to protect the same rats against a secondary vaginal infection. Moreover, recipient rats treated intravaginally with vaginal fluids from primary-infected rats were protected. In a follow-up study, vaginal lymphocyte analysis during primary or secondary vaginal *C. albicans* infections in rats revealed an increase in B cells in the tissue (De Bernardis et al. 2000). Successful protection against vaginitis in the presence of these IgA antibodies could reflect 'protective' antibodies based on the Casadevall concept of nonprotective, protective, and indifferent antibodies (Casadevall 1995). In support of this, Cutler and colleagues have shown that two different 'protective' antibodies specific for mannan (IgM and IgG3) protect mice against *Candida* vaginitis when given either locally or systemically (Han et al. 1998, 2000). Taking this into account, one might speculate that when antibodies are detected clinically that do not appear to have a protective effect, indifferent or unprotective antibodies may predominate in the antibody pool. If, indeed, there is some precedent for this, it appears to be the norm rather than the exception.

Innate immunity appears to play a significant role in protection against vaginitis as well. This was initially predicted based on a leukocytic infiltrate of predominantly PMNL that is often observed in vaginal lavage fluid of infected animals. These leukocytic cells are usually associated with or attached to the hyphae and/or sheets of epithelial cells. On the other hand, when this infiltrate is present during infection it does not correlate with lower vaginal fungal burden (Fidel et al. 1999a; Saavedra et al. 1999). Furthermore, this leukocytic infiltrate is generally not observed in clinical cases of *Candida* vaginitis. Although PMNL and macrophages are potential candidates for anti-*Candida* innate resistance and are present at or near the vaginal mucosa, Balish and colleagues (Cantorna et al. 1990) showed that animals with the beige mutation, which are immunodeficient in phagocytic cells (*bg/bg*), were not more susceptible to a natural *C. albicans* infection under non-estrogenized conditions. Two more recent studies addressed the specific role of PMNL during experimental vaginitis. The results indicated that depletion of PMNL under estrogen- or non-estrogen-treated conditions had no effect on vaginal fungal burden (Black et al. 1998; Fidel et al. 1999a), suggesting that PMNL do not play a significant role against *C. albicans* in the vagina despite their presence in the vaginal lumen during some experimental infections. Perhaps this reflects their inability to function against *C. albicans* in the vaginal microenvironment compared to their ability to function well in blood or tissue culture. It might reflect immunoregulatory activity as well. Alternatively, the presence of PMNL during an infection might not represent a response to the yeast, but simply reflect their normal presence during the diestrus stage of the menstrual cycle (every 2 days in mice) when they are deployed to phagocytize the apoptotic squamous epithelial cells from the estrus (2-day cycle) stage.

As mentioned above, vaginal epithelial cells represent a potential innate anti-*Candida* host defense mechanism. This has been shown for both mouse, macaque, and human cells (Steele et al. 1999b, 2000; Barousse et al. 2001). As described earlier, the anti-*Candida* activity mediated by a putative carbohydrate moiety inhibits rather than kills the *Candida* (Nomanbhoy et al. 2002), but vaginal cells are not as effective as oral epithelial cells (Steele et al. 2000). If these cells are important as an innate mechanism, less activity by vaginal cells may reflect a higher prevalence of vaginal versus oral candidiasis when both tissues are colonized

Table 2 *Host defenses against superficial* Candida *infections*

Host defense mechanism	Site of infection			
	Cutaneous	GI[a]	Oral	Vaginal
Innate				
PMNL[b]	+[d]	– –	+/?	– –/?
Macrophage	+	– –	– –	– –
NK cells[c]	– –	– –	– –	– –
Epithelial cells	?	?	++	+
Humoral				
IgM	– –	– –	– –	+/–?
IgG	– –	– –	– –	+/–?
IgA	– –	– –	– –/?	+/–?
Cell-mediated				
$CD4^+$ T cells (Th1)	+++	+++	+++	–/?
$CD8^+$ T cells	– –	+/?	++	– –

a) Gastrointestinal.
b) Polymorphonuclear leukocytes.
c) Natural killer cells.
d) Symbols: + to +++ indicates level of activity from weakly positive (+) to strongly positive (+++); +/–? indicates variable activity, if any; +/? indicates probaby activity, but unknown at present; – –/? indicates probably negative activity, but unknown at present; – – indicates negative activity; ? indicates unknown.

(Steele et al. 2000). Interestingly, a recent study in humans showed that while the vaginal epithelial cell-mediated anti-*Candida* activity was not different at the various stages of the menstrual cycle, it was significantly reduced in women with a history of RVVC (Barousse et al. 2001). Thus, reduced epithelial cell anti-*Candida* activity may represent, in part, a local immune deficiency associated with RVVC.

Table 2 summarizes the various host defenses against each form of superficial candidiasis with what is known and what is still questionable.

IMMUNOTHERAPY

In addition to antifungal drugs available, new treatment strategies are being explored, especially in the case of azole-resistance (Walker et al. 2000; Mathema et al. 2001; Ferris et al. 2002) and the appearance of infections by non-*albicans* species that are intrinsically azole resistant (Sobel 1998; Fidel et al. 1999b; Abu-Elteen 2001). One goal of studying host defense is to design possible immunotherapeutic strategies to treat or prevent infection. For example, antibodies directed at SAPs, mannoproteins, mannan, and mannoprotein extract have been proposed as potential treatments and vaccine candidates (reviewed in Magliani et al. 2002). Intravaginal and intranasal immunization using mannoprotein MP65 and Sap2 have both proved to be protective in a rat model of vaginal candidiasis (De Bernardis et al. 2002) and a mannan-specific antibody was protective against a murine vaginal infection (Han et al. 2000). Additionally, anti-idiotypic (Id) vaccination and anti-Id therapy against the yeast killer toxin receptor (KTR) have been described for both systemic and mucosal *Candida* infection (reviewed in Magliani et al. 2002). Monoclonal and recombinant rat and mouse antibodies to the KTR (mKAb and rKAb) have been produced and can kill *C. albicans* in vitro (Magliani et al. 1997; Polonelli et al. 1997). In humans, *Streptococcus gordonii*, which is a commensal organism of the vaginal mucosa, has been engineered to secrete rKAb, and this has been shown to be as effective as fluconazole treatment against vaginitis in the rat model (Beninati et al. 2000). Recently, protease inhibitors used for treatment of HIV have been shown to reduce the incidence/occurrence of OPC in HIV-positive individuals (Cassone and Cauda 2002). This is thought to be mediated by inhibiting SAPs, which are a major virulence factor and are important in invasion and tissue damage (reviewed in Hube and Naglik 2002) (De Bernardis et al. 1990, 1999). In a clinical study that examined OPC following treatment with protease inhibitors, a reduction in OPC cases as well as a reduction of the presence of SAPs in the saliva of treated patients was observed (Cassone et al. 2002). Furthermore, in the case of vaginitis, overcoming immunoregulation by gene therapy, administration of cytokines, or use of antibodies against immunoregulatory cytokines may be potential methods of immunotherapy. Alternative treatments for candidiasis are promising and may be used to treat antifungal drug-resistant infections.

CONCLUSION

In summary, superficial candidiasis includes several types of infections, including cutaneous, oropharyngeal, gastrointestinal, vaginal, and ocular. Infections at each site are accompanied by unique signs and symptoms and affect different groups of individuals. Protective host defenses against infection at each anatomic site are independent/compartmentalized, and quite distinct, with

some sites revealing mechanisms consistent with the dogma of $CD4^+$ Th1-type CMI as the main protective mechanism, and other sites, particularly the vaginal mucosa, challenging this dogma. In addition, innate immunity appears to be playing more of a role than previously thought, while humoral immunity is still considered controversial. Finally, immunotherapeutic strategies developed as a result of the research efforts appear promising, with more strategies predicted as additional immune mechanisms against superficial candidiasis are uncovered.

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Oculomycosis

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Opportunistic fungal infections that occur in the eye and its associated structures are collectively termed 'oculomycoses.' Until about 30 years ago, instances of oculomycosis were sufficiently infrequent to justify single case reports in the ophthalmic literature if the etiology of the disorder could be conclusively established by culture (Wilson and Ajello 1998). However, such infections are being increasingly recognized as important causes of morbidity and blindness; certain types of oculomycoses may even be life threatening (Yohai et al. 1994; Levin et al. 1996). The cornea is the site most frequently affected (Srinivasan et al. 1991), but the orbit, lids, lacrimal apparatus, conjunctiva, sclera, and intraocular structures may also be involved.

Fungal infections of the eye affect the orbit and the eyeball (globe) itself; infections of the globe manifest as external ocular or intraocular infections.

ETIOLOGIC AGENTS OF OCULOMYCOSES

By 1998, some 105 species in 56 genera of fungi had been reported as causes of mycotic keratitis and other oculomycoses (Wilson and Ajello 1998). Since the publication of the previous edition of this book, other species and genera of fungi have been reported as ophthalmic pathogens, including *Chrysosporium parvum* (Wagoner et al. 1999), *Metarhizium anisopliae* var. *anisopliae* (de Garcia et al. 1997), *Phaeoisaria clematidis* (Guarro et al. 2000), and *Sarcopodium oculorum* (Guarro et al. 2002). Unfortunately, it may be difficult to distinguish between true ophthalmic pathogens and organisms from the environment that are inadvertently introduced into specimens during or after collection (D'Mellow et al. 1991). Hence, certain criteria need to be satisfied before a fungal strain isolated from an ocular specimen can be considered significant; this is especially important when reporting fungal genera or species that have not been previously implicated in ocular infections.

McGinnis (1980) applied strict criteria to evaluate more than 300 reports pertaining to human fungal infections published in the literature from the late 1940s to the beginning of 1979. A report was considered to be acceptable when:

- an adequate clinical history was presented that suggested a mycotic infection
- the fungus was seen in the clinical specimens
- the morphology of the fungus in the clinical specimens was compatible with the reported etiologic agent
- there was adequate evidence that the fungus was identified properly.

The assessment included reports on 60 species in 30 genera of fungi isolated from ophthalmic infections, principally keratitis, but only reports pertaining to 32 species in 19 genera of fungi satisfied the strict criteria to be considered acceptable. In this chapter, an attempt has been made to apply these and similar criteria (Thomas 2003) to assess the significance of the fungal genera and species that have been reported as pathogens in different parts of the eye (Tables 1, 2 p. 6, 3, p. 8, and 4, p. 9). In vitro susceptibility data pertaining to various species of fungi that have been implicated as causes of ocular infection, and derived from studies performed by a method approved by the National Committee for Clinical Laboratory Standards (NCCLS) are also presented (Sutton et al. 1998). It should be noted that the clinical relevance of these data to the therapy of ocular fungal infections, especially when topical antifungals are used, is uncertain. It is also not clear whether these data can be used as a guide to therapy.

There appear to be variations in the genera and species of fungi reported as causes of oculomycoses, depending on the geographical location and the time period. Up to 1962, most published reports on mycotic keratitis had cited species of *Aspergillus* as the causative organisms (Gingrich 1962); since that time, however, species of *Fusarium* have been reported as frequent causes of mycotic keratitis (Nelson et al. 1994; Thomas 1994, 2003). In the first half of a 9-year study on microbial keratitis in South Florida, nine strains of *Candida albicans* were isolated, but only one in the second half (Liesegang and Forster 1980). In the period from 1969 to 1977, *Fusarium solani* was the commonest fungal isolate from patients with keratitis in South Florida (Liesegang and Forster 1980) whereas from 1982 to 1992, *Fusarium oxysporum* was the commonest isolate (Rosa et al. 1994). Although *Fusarium solani* is apparently the commonest cause of mycotic keratitis in many parts of the world (Rosa et al. 1994; Srinivasan et al. 1997), species of *Aspergillus* have predominated in many reports from the Indian subcontinent (Srinivasan et al. 1991; Upadhyay et al. 1991; Dunlop et al. 1994; Thomas 1994). *Candida albicans* is a frequent cause of keratitis in temperate regions, including the northern United States (Thygeson and Okumoto 1974; Chin et al. 1975; Tanure et al. 2000), but is infrequently reported from tropical and subtropical regions (Rosa et al. 1994; Thomas 1994; Srinivasan et al. 1997). The reasons for these variations are not clear.

Hyaline filamentous fungi

Species of *Fusarium*, widespread saprobic or parasitic organisms that grow on decaying vegetation and various parts of wild and cultivated plants, also cause important plant diseases, particularly in major crops (Cuero 1980). These fungi are believed to account for at least one-third of all reported cases of mycotic keratitis in tropical and subtropical zones (Srinivasan et al. 1997; Gopinathan et al. 2002; Leck et al. 2002); they have also been reported to cause intraocular infections (Pflugfelder et al. 1988; Patel et al. 1994; Glasgow et al. 1996; Goldblum et al. 2000; Sponsel et al. 2002; Verma and Tuft 2002) and scleritis (Moriarty et al. 1993). *Fusarium solani* is the species most frequently isolated from ocular infections, other species reported being *Fusarium dimerum*, *Fusarium episphaeria*, *Fusarium moniliforme*, *Fusarium nivale*, and *Fusarium oxysporum* (Rebell and Forster 1980; Gonawardena et al. 1994; Rosa et al. 1994; Gopinathan et al. 2002; Vismer et al. 2002). In ocular samples, *Fusarium* spp. appear as septate, hyaline, branching hyphae (2–4 μm in diameter). Adventitious sporulation (the formation of conidia in tissues) may also occur; the conidia of *Fusarium* spp. are generally seen to be larger than those of *Paecilomyces* spp. (Liu et al. 1998). In culture, species of *Fusarium* exhibit distinctive, septate, large, banana-shaped macroconidia, with apical and basal cells (Figure 1, p. 10), occurring on sporodochia. This sporodochial type often mutates, with an associated loss of virulence, giving rise to either a mycelial type or a pionnotal type (Toussoun and Nelson 1976). Species of *Fusarium* are notoriously resistant to antifungal agents in vitro. At one reference laboratory, only 25 percent of isolates of *Fusarium solani* were found susceptible to amphotericin B in vitro, while none of the isolates tested was susceptible to flucytosine, fluconazole, itraconazole, ketoconazole, or miconazole, when tested by the NCCLS method (Sutton et al. 1998).

Fungi of the genus *Aspergillus* abound in the environment worldwide, thriving on various substrates, including corn, decaying vegetation, and soil. These fungi are also found commonly in hospital air (Leenders et al. 1999); an outbreak of ocular aspergillosis following cataract surgery was traced to ongoing hospital construction, with probable wide dispersal of conidia of *Aspergillus* spp. (Tabbara and al-Jabarti 1998). *Aspergillus* spp. are important causes of keratitis following occupational trauma (Upadhyay et al. 1991; Dunlop et al. 1994; Gopinathan et al. 2002) or surgery (Heidemann et al. 1995; Sridhar et al. 2000a) in both tropical and temperate zones of the world. Species of *Aspergillus* have also been implicated in dacryocystitis (Levin et al. 1996; Kristinsson and Sigurdsson 1998), exogenous endophthalmitis (Tabbara and al-Jabarti 1998), endogenous endophthalmitis (Weishaar et al. 1998), orbital lesions (Kronish et al. 1996; Levin et al. 1996; Johnson et al. 1999), and scleritis (Bernauer et al. 1998). While *Aspergillus fumigatus* is the most commonly isolated species, *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus sydowii* have also been reported as etiologic agents in ocular infections (Rippon 1988; Sutton et al. 1998). In ocular samples, species of *Aspergillus* manifest as

Table 1 *Etiologic agents of and types of ocular mycoses: hyaline molds*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
Acremonium				
A. atrogriseum	CO	Read et al. 2000	a	. . .
A. curvum	CO	Wilson and Ajello 1998	. . .	1
	E	Wilson and Ajello 1998	. . .	1
A. kiliense	CO	Weissgold et al. 1998	a	. . .
	E	Paiva et al. 1960	b	. . .
A. potronii	CO	Forster et al. 1975a	b	. . .
A. recifei	CO	Simonsz 1983	. . .	3
Acremonium sp.	CO	Cho and Lee 2002	. . .	3
Arthrographis				
A. kalrae	CO	Perlman and Binns 1997	a	. . .
Aspergillus				
A. candidus	E, optic nerve	McCormick et al. 1975	. . .	2
A. clavatus	CO	Wilson and Ajello 1998	. . .	1
A. fischerianus	C, CO, E	Coriglione et al. 1990	a	. . .
A. flavipes	CO	Jones 1975	. . .	2
A. flavus	CO	Albesi and Zapater 1975	b	. . .
	E	Lance et al. 1988	a	. . .
	O	Miloshev et al. 1966	a	. . .
	S	Carlson et al. 1992	a	. . .
A. fumigatus	C	Perry et al. 1998	. . .	3
	CO	Singh et al. 1989	a	. . .
	S	Rodriguez-Arres et al. 1995	. . .	3
	E	Tabbara and al-Jabarti 1998	a	. . .
	O	Kronish et al. 1996	a	. . .
A. janus	CO	Neuhann 1976	. . .	2
A. glaucus	CO, E	Wilson and Ajello 1998	. . .	1
A. nidulans	CO	Koul and Pratap 1975	. . .	3
	E	Wilson and Ajello 1998	. . .	1
A. niger	CO	Abboud and Hanna 1970	b	. . .
	E	Brar et al. 2002	a	. . .
A. oryzae	CO	Wilson and Ajello 1998	. . .	1
	E	Wilson and Ajello 1998	. . .	1
	O	Miloshev et al. 1966	b	. . .
	S	Stenson et al. 1982	. . .	3
A. terreus	CO	Singh et al. 1990	a	. . .
	E	Kalina and Campbell 1991	a	. . .
A. wentii	CO	Jones 1975	. . .	2
Beauveria				
B. bassiana	CO	Kisla et al. 2000	a	. . .
Blastomyces				
B. dermatitidis	CO	Rodrigues and Laibson 1973	a	. . .
	E	Li et al. 1998	a	. . .
	EL	Bartley 1995	a	. . .
	O	Li et al. 1998	a	. . .
	U	Gottlieb et al. 1995	a	. . .
Cephaliophora				
C. irregularis	CO	Mathews and Kuriakose 1995	a	. . .
Chrysonilia				
C. sitophila	CO	Rosa et al. 1994	. . .	3
	E	Theodore et al. 1962	. . .	2
Chrysosporium				
C. parvum	CO	Wagoner et al. 1999	. . .	4

(*Continued over*)

Table 1 *Etiologic agents of and types of ocular mycoses: hyaline molds (Continued)*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
Coccidioides				
C. immitis	C	Maguire et al. 1994	a	. . .
	CO	Wilson and Ajello 1998	. . .	1
	E	Cutler et al. 1978	a	. . .
	EL	Irvine 1968	a	. . .
	O	Wilson and Ajello 1998	. . .	1
	S	Wilson and Ajello 1998	. . .	1
	U	Bell and Font 1972	a	. . .
Cylindrocarpon				
C. lichenicola (*C. tonkinense*)	CO	Laverde et al. 1973	b	. . .
Engyodontium				
E. alba	CO	McDonnell et al. 1984–85	a	. . .
Fonsecaea				
F. pedrosoi	C	Wilson and Ajello 1998	. . .	1
	CO	Barton et al. 1997	a	. . .
Fusarium				
F. aquaeductum	E	Wilson and Ajello 1998	. . .	1
F. dimerum	CO	Zapater et al. 1972, 1976	b	. . .
	CO	Greer et al. 1973	b	. . .
	E	Wilson and Ajello 1998	. . .	1
F. verticilloides (*F. moniliforme*)	CO	Anderson et al. 1959	b	. . .
	CO	Polenghi and Lasagni 1976	b	. . .
	E	Wilson and Ajello 1998	. . .	1
F. nivale	C	Wilson and Ajello 1998	. . .	1
	CO	Perz 1966	. . .	3
F. oxysporum	CO	Mikami and Stemmermann 1958	b	. . .
F. solani	CO	Gugnani et al. 1976	b	. . .
	E	Goldblum et al. 2000	a	. . .
F. subglutinans	CO	Wilson and Ajello 1998	. . .	1
F. ventricosum	CO	Wilson and Ajello 1998	. . .	1
Histoplasma				
H. capsulatum var. *capsulatum*	E	Goldstein and Buettner 1983; Pulido et al. 1990	a	. . .
	O	Wilson and Ajello 1998	. . .	1
	U	Macher et al. 1985	a	. . .
H. capsulatum var. *duboisii*	O	Bansal et al. 1977	a	. . .
	O	Ajayi et al. 1986	. . .	3
Metarhizium				
M. anisopliae	CO	de Garcia et al. 1997	a	. . .
Microsporum				
Microsporum sp.	CO	Cho and Lee 2002	. . .	3
M. canis	EL	Wilson and Ajello 1998	. . .	1
Ovadendron				
O. ochraceum	E	Wilson and Ajello 1998	. . .	1
O. sulphureo-ochraceum	CO	Wilson and Ajello 1998	. . .	1
	E	Lee et al. 1995	a	. . .
	S	Wilson and Ajello 1998	. . .	1
Paecilomyces				
P. farinosus	CO	Gonawardena et al. 1994	a	. . .
P. lilacinus	CO	Gordon and Norton 1985	a	. . .
	E	Mosier et al. 1977	b	. . .
	LS	Henig et al. 1973	. . .	2
	O	Agrawal et al. 1979	a	. . .

(*Continued over*)

Table 1 *Etiologic agents of and types of ocular mycoses: hyaline molds (Continued)*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
P. variotii+[d]	CO	Wilson and Ajello 1998	. . .	1
P. viridis	E	Rodrigues and MacLeod 1975	b	. . .
Paracoccidioides				
P. brasiliensis	CO	Silva et al. 1988	a	. . .
	EL	Belfort et al. 1975	a	. . .
	U	Silva et al. 1988	a	. . .
Penicillium				
P. citrinum	CO	Gugnani et al. 1976	b	. . .
P. expansum	CO	Gugnani et al. 1976	b	. . .
Pythium				
P. insidiosum	CO	Virgile et al. 1993	a	. . .
	O	Mendonza et al. 1996	a	. . .
Rhinosporidium				
R. seeberi	C	Engzell and Jones, 1973; Rolon 1974	a	. . .
	EL	Kapoor et al. 1976	a	. . .
	LS	Sengupta et al. 1975	a	. . .
	S	Wilson and Ajello 1998		1
Rhizoctonia				
Rhizoctonia sp.	CO	Srivastava et al. 1977	b	. . .
Sarcopodium				
S. oculorum	CO	Guarro et al. 2002	a	. . .
Scedosporium				
S. apiospermum (reported as *Pseudallescheria boydii*)	CO	Ernest and Rippon 1966	b	. . .
	O	Anderson et al. 1984	a	. . .
	S	Taravella et al. 1997	a	. . .
	E	Ksiazek et al. 1994	a	. . .
S. prolificans	C	Arthur et al. 2001	. . .	3[e]
	S	Moriarty et al. 1993	. . .	3
Scopulariopsis				
S. brevicaulis	CO	Ragge et al. 1990	a	. . .
Sporothrix				
S. schenckii	C	Wilson and Ajello 1998	. . .	1
	CO	Wilson and Ajello 1998	. . .	1
	E	Kurosawa et al. 1988	a	. . .
	EL	Wilson and Ajello 1998	. . .	3
	O	Streeten et al. 1974	a	. . .
	S	Wilson and Ajello 1998	. . .	1
	U	Font and Jakobiec 1976	a	. . .
Trichophyton				
Trichophyton sp.	EL	Velazquez et al. 2002	. . .	3
Tritirachium				
T. oryzae	CO	Rodrigues et al. 1975	b	. . .
Tubercularia				
T. vulgaris	E	Wilson and Ajello 1998	. . .	1
Verticillium				
Verticillium sp.	CO	Shin et al. 2002	. . .	3
V. searrae	CO	Wilson and Ajello 1998	. . .	1
Volutella				
V. cinerescens	E	Foster et al. 1958	b	. . .

a) C, conjunctiva; CO, cornea; E, endophthalmitis; EL, eyelid; LS, lacrimal system; O, orbit and optic nerve; S, sclera; U, uvea.
b) a, microscopy and culture positive, reliable identification; b, deemed accepted by McGinnis (1980).
c) 1, not listed in Medline; 2, deemed questionable by McGinnis (1980); 3, details of identification inadequate; 4, morphology in tissue not consistent with the fungus isolated.
d) *Paecilomyces variotii*+, initially identified as *Paecilomyces viridis*.
e) Subsequently identified as *Acrophialophora fusispora*.

Table 2 *Etiologic agents and types of ocular mycoses: pheoid (dematiaceous) molds*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
Alternaria				
A. alternata	CO	Ando and Takatori 1987	a	. . .
	E	Wilson and Ajello 1998	. . .	1
A. infectoria	CO	Forster et al. 1975b	. . .	2
Alternaria sp.		Azar et al. 1975	. . .	2
Aureobasidium				
A. pullulans	CO	Jones and Christensen 1974	. . .	2
Bipolaris				
B. hawaiiensis	CO	Anandi et al. 1988	a	. . .
	E	Pavan and Margo 1993	. . .	3
	O	Maskin et al. 1989	a	. . .
B. spicifera[d]	CO	Zapater et al. 1975	b	. . .
Cladorrhinum				
C. bulbillosum	CO[e]	Chopin et al. 1997	a	. . .
Cladosporium				
C. cladosporioides	CO	Polack et al. 1976	. . .	2
Colletotrichum				
C. capsici	CO	Upadhyay et al. 1991	. . .	3
C. coccodes	CO	Liesegang and Forster 1980	. . .	3
C. dematium	CO	Liao et al. 1983; Fernandez et al. 2002	. . .	3
C. graminicola	CO	Ritterband et al. 1997	a	. . .
C. gloeosporioides	CO	Yamamoto et al. 2001	a	. . .
Colletotrichum state of *Glomerella cingulata*	CO	Shukla et al. 1983	a	. . .
Curvularia				
C. brachyspora	CO	Marcus et al. 1992	a	. . .
C. geniculata	CO	Nityananda et al. 1964; Warren 1964	b	. . .
C. lunata	CO	Nityananda et al. 1962	b	. . .
	E	Kaushik et al. 2001	a	. . .
C. pallescens	CO	Wilson and Ajello 1998	. . .	1
C. senegalensis	CO	Forster et al. 1975b	b	. . .
C. verruculosa	CO	Forster et al. 1975b	. . .	2
Dichotomophthoropsis				
D. nymphearum	CO	Wright et al. 1990	a	. . .
D. portulacae	CO	Wilson and Ajello 1998	. . .	1
Doratomyces				
D. stemonitis	CO	Wilson and Ajello 1998	. . .	1
Exophiala				
E. dermatitidis	CO, E	Levenson et al. 1984[f]	a	. . .
	CO, E	Benaoudia et al. 1999	. . .	3
E. jeanselmei	CO	Laverde et al. 1973	b	. . .
	E	Hammer et al. 1983	. . .	3
Exserohilum				
E. rostratum[g]	CO	Jones 1975	b	. . .
E. longirostratum	CO	Bouchon et al. 1994	a	. . .
Fonsecaea				
F. pedrosoi	C	Wilson and Ajello 1998	. . .	1
	CO	Barton et al. 1997	a	. . .
Lasiodiplodia				
L. theobromae	CO	Rebell and Forster 1976	b	. . .
	E	Borderie et al. 1997	a	. . .
Lecythophora				
L. mutabilis	CO	Ho et al. 1991	. . .	3

(*Continued over*)

Table 2 *Etiologic agents and types of ocular mycoses: pheoid (dematiaceous) molds (Continued)*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
Microsphaeropsis				
M. olivacea	CO, E	Shah et al. 2001	. . .	3
Phaeoisaria				
P.clematidis	CO	Guarro et al. 2000	a	. . .
Phaeotrichoconis				
P. crotalariae	CO	Shukla et al. 1989	a	. . .
Phialophora				
P. bubakii	CO	Wilson and Ajello 1998	. . .	1
P. richardsiae	LS	Pitrak et al. 1988	a	. . .
P. verrucosa	CO	Wilson et al. 1966	b	. . .
Phoma				
P. oculo-hominis	CO	Punithalingam 1976	. . .	2
Phoma sp.	CO	Rishi and Font 2003	a	. . .
Sphaeropsis				
S. subglobosa	CO, E	Kirkness et al. 1991	a	. . .
Tetraploa				
T. aristata	CO	Newmark and Polack 1970	b	. . .

a) C, conjunctiva; CO, cornea; E, endophthalmitis; EL, eyelid; LS, lacrimal system; O, orbit and optic nerve; S, sclera; U, uvea.
b) a, microscopy and culture positive, reliable identification; b, deemed accepted by McGinnis (1980).
c) 1, not listed in Medline; 2, deemed questionable by McGinnis (1980); 3, details of identification inadequate.
d) Identified as *Drechslera spicifera*.
e) Isolated from a Percheron cross horse.
f) Identified as *Wangiella dermatitidis*; inoculation into animal cornea reproduced disease.
g) Identified as *Drechslera rostrata*.

septate, hyaline, branching hyphae, 3–6 μm wide, which exhibit parallel walls and radiate from a single point (Figure 2, p. 10), and which are smaller than the hyphae of zygomycetes (Liu et al. 1998); dichotomous (45°) branching may occur (Figure 2), and although widely believed to be pathognomonic of *Aspergillus* spp., may be mimicked by *Pseudallescheria boydii* (anamorph *Scedosporium apiospermum*) in ocular samples (McGuire et al. 1991). Identification of the genus *Aspergillus* in culture depends on the morphology of its asexual reproductive structures; the conidiophore, with its swollen terminal end, is surrounded by flask-shaped sterigmata, each of which produces long chains of coccoid conidia that radiate out from the terminal end. Identification of the species involved is important since different species may exhibit differing susceptibilities to antifungals. For example, in vitro, 95 percent of isolates of *Aspergillus fumigatus* were found susceptible to amphotericin B, 97 percent to itraconazole, 92 percent to ketoconazole, 50 percent to miconazole, and none to fluconazole or flucytosine; by contrast, 67 percent of isolates of *Aspergillus flavus* were found to be susceptible to amphotericin B, 98 percent to itraconazole, 100 percent to ketoconazole and miconazole and none to fluconazole or flucytosine, while only 13 percent of strains of *Aspergillus terreus* were reported susceptible to amphotericin B (Sutton et al. 1998). These data suggest that different species of *Aspergillus* show varying degrees of susceptibility to amphotericin B but are generally susceptible to the imidazoles and triazoles (except fluconazole).

Scedosporium apiospermum (teleomorph *Pseudallescheria boydii*), which is found in soil, sewage, and polluted water, has been reported to cause severe ocular infection following trauma during agricultural work or by polluted water, or following immunosuppression (Taravella et al. 1997). There is unequivocal evidence to implicate *Scedosporium apiospermum* as a cause of endogenous or exogenous endophthalmitis (McGuire et al. 1991; Pfeifer et al. 1991; McKelvie et al. 2001), keratitis (Bloom et al. 1992; Sridhar et al. 2000b; Tanure et al. 2000; Diaz-Valle et al. 2002; Wu et al. 2002; Saracli et al. 2003), orbital infections (Anderson et al. 1984; Nunery et al. 1985; Jones et al. 1999), and scleritis (Moriarty et al. 1993; Taravella et al. 1997). Another species, *Scedosporium prolificans*, which was first described as a human pathogen in 1984, has been reported to cause sclerokeratitis (Sullivan et al. 1994; Kumar et al. 1997). In infected tissue, *Scedosporium apiospermum* and *Scedosporium prolificans* resemble other hyaline filamentous fungi in having septate, hyaline, branching hyphae, 2–4 μm wide. In culture, *Scedosporium apiospermum* forms white to pale or dark gray, floccose colonies on glucose peptone agar after one week (Figure 3a, p. 10). Microscopic morphology is characterized by abundant yellow to pale brown oval conidia (with a scar at the base) formed from single or branched long slender annellides (Figure 3b, p. 10),

Table 3 *Etiologic agents of, and types of, ocular mycoses: yeasts*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
Candida				
C. albicans	C	Gumbel et al. 1990	. . .	3
	CO	Wilhelmus and Robinson 1991	a	. . .
	E	Stone et al. 1975	a	. . .
	LS	Purgason et al. 1992	a	. . .
	S	Garg et al. 2003	a	. . .
	U	Elliott et al. 1979	a	. . .
C. famata	CO	Wilson and Ajello 1998	. . .	1
	E	Rao et al. 1991	a	. . .
C. glabrata	CO	Djalilian et al. 2001	. . .	3
	E	Chapman et al. 1998	a	. . .
	O	Wilson and Ajello 1998	. . .	1
	U	Larsen 1973	. . .	3
C. guilliermondii	C	Wilson and Ajello 1998	. . .	1
	CO	Ainbinder et al. 1998	a	. . .
C. krusei	CO	Tandon et al. 1984	a	. . .
	E	McQuillen et al. 1992	. . .	3
C. lusitaniae	U	Wilson and Ajello 1998	. . .	1
C. parapsilosis	CO	Rhem et al. 1996	a	. . .
	E	Fekrat et al. 1995	a	. . .
	L	Wilson and Ajello 1998	. . .	1
	S	Wilson and Ajello 1998	. . .	1
	U	Wilson and Ajello 1998	. . .	1
C. tropicalis	CO	Rosa et al. 1994	. . .	3
	E	Behrens-Baumann et al. 1991	. . .	3
	U	Wilson and Ajello 1998	. . .	1
Cryptococcus				
C. laurentii	CO	Ritterband et al. 1998	a	. . .
	E	Custis et al. 1995	. . .	3
C. neoformans	C	Waddell et al. 2000	a	. . .
	CO	Tanure et al. 2000	. . .	3
	E	Charles et al. 1992	a	. . .
	EL	Coccia et al. 1999	. . .	3
	O	Cohen and Glasgow 1993	a	. . .
	S	Limathe et al. 2002	. . .	3
	U	Muccioli et al. 1995	a	. . .
Geotrichum				
G. candidum	C	Wilson and Ajello 1998	. . .	1
	CO	Wilson and Ajello 1998	. . .	1
Malassezia				
M. furfur	LS	Wilson and Ajello 1998	. . .	. . .
	EL	Toth et al. 1996	. . .	3
	CO	Roodhooft et al. 1998	. . .	3

(*Continued over*)

Table 3 *Etiologic agents of, and types of, ocular mycoses: yeasts (Continued)*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
M. pachydermatis	LS	Wilson and Ajello 1998	. . .	. . .
Rhodotorula				
R. glutinis	CO	Guerra et al. 1992	. . .	3
R. minuta	E	Pinna et al. 2001	. . .	3
R. rubra	C	Segal et al. 1975	. . .	2
	CO	Romano et al. 1973	. . .	2
	E	Merkur and Hodge 2002	. . .	3
	LS	Muralidhar and Sulthana 1995	a	. . .
Rhodotorula sp.	CO	Panda et al. 1999	a	. . .
Torulopsis				
T. magnoliae	E	Rosenfeld et al. 1994	. . .	3
	O	Wilson and Ajello 1998	. . .	1
Trichosporon				
T. asahii	U	Wilson and Ajello 1998	. . .	1
T. beigelii	E	Sheikh et al. 1974	b	. . .

a) C, conjunctiva; CO, cornea; E, endophthalmitis; EL, eyelid; LS, lacrimal system; O, orbit and optic nerve; S, sclera; U, uvea.
b) a, microscopy and culture positive, reliable identification; b, deemed accepted by McGinnis (1980).
c) 1, not listed in Medline; 2, deemed questionable by McGinnis (1980); 3, details of identification inadequate.

and sometimes aggregated into bundles (*Graphium* state); ascocarps are sometimes present. *Scedosporium prolificans* grows more slowly, forming oval conidia (frequently in groups) from short annellides, with inflated bases and narrow tapering tips. The correct identification of the species, with confirmation by DNA sequencing if warranted (Guarro and Gené 2002), is important in view of differing susceptibilities to different antifungals. In vitro susceptibility testing by the NCCLS method revealed that all isolates of *Scedosporium apiospermum* were susceptible to miconazole, 95 percent to ketoconazole, 77 percent to fluconazole, and 58 percent to amphotericin B; by contrast, two of nine isolates of *Scedosporium prolificans* were found to be susceptible in vitro to miconazole, whereas all isolates tested were resistant to amphotericin B, flucytosine, itraconazole,

Table 4 *Etiologic agents of and types of ocular mycoses: zygomycetes*

Genera and species	Types of ocular lesions[a]	Articles in which cited	Acceptable[b]	Uncertain[c]
Absidia				
A. corymbifera	CO	Marshall et al. 1997	a	. . .
	O	Gebhard et al. 1995	a	. . .
Apophysomyces				
A. elegans	O	Fairley et al. 2000	a	. . .
Chlamydoabsidia				
C. padenii	CO	Wilson and Ajello 1998	. . .	1
Mucor				
M. ramosissimus	O	Bullock et al. 1974	b	. . .
Rhizopus				
R. arrhizus (oryzae)	O	Kronish et al. 1996	a	. . .
R. stolonifer	O	Wilson and Ajello 1998	. . .	1
	EL	Champion and Johnson 1969	. . .	2
	EL	Coetzee and de Bruin 1974	. . .	2

a) CO, cornea; EL, eyelid; O, orbit and optic nerve.
b) a, microscopy and culture positive, reliable identification; b, deemed accepted by McGinnis (1980).
c) 1, not listed in Medline; 2, deemed questionable by McGinnis (1980); 3, details of identification inadequate.

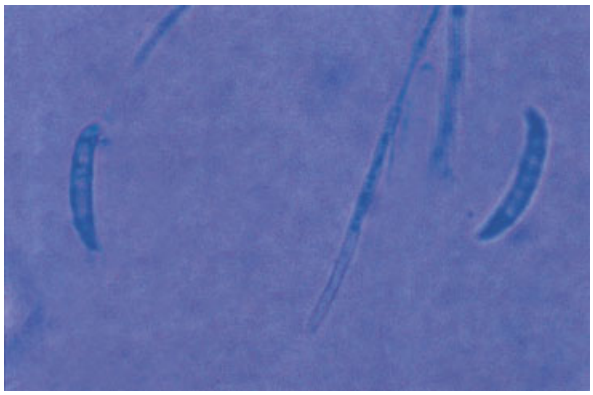

Figure 1 *Photomicrograph of large, multicelled, banana-shaped macroconidia of* Fusarium solani *in culture. Lactophenol cotton blue stain; magnification ×400*

ketoconazole, and fluconazole (Sutton et al. 1998). Again, the relevance of these data to the therapy of oculomycoses is uncertain.

Species of *Paecilomyces* are found worldwide in soil and decaying vegetation. They may also contaminate sterile solutions and culture media, since they are resistant to most of the common sterilizing procedures (Castro et al. 1990). Many of the documented infections by *Paecilomyces* spp. in the eye and associated structures have followed surgical procedures (Castro et al. 1990). The species implicated in human infections are *Paecilomyces lilacinus*, *Paecilomyces variotii*, and *Paecilomyces marquandii* (Castro et al. 1990; Sutton et al. 1998). Documented ocular infections include endophthalmitis (Pettit et al. 1980; Okhravi et al. 1997), intralenticular infection (D'Mellow et al. 1991), keratitis (Kozarsky et al. 1984; Gordon and Norton 1985; Starr 1987; Legeais et al. 1994), and orbital infection (Agrawal et al. 1979). Keratitis due to *Paecilomyces lilacinus* frequently fails to respond to medical therapy, necessitating surgery, and endophthalmitis due to this fungus

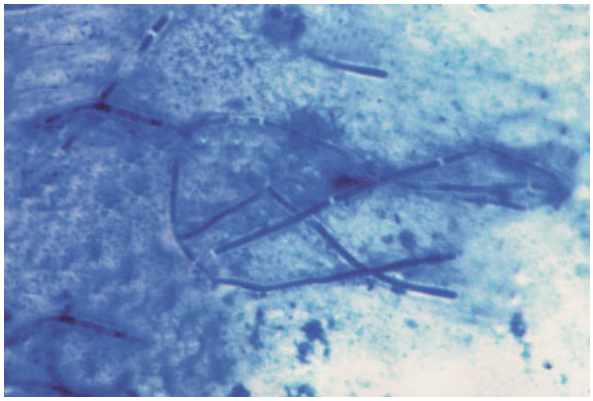

Figure 2 *Photomicrograph of septate, regular hyphae of* Aspergillus flavus *in corneal scrape material; dichotomous branching seen. Lactophenol cotton blue stain; magnification ×400*

Figure 3 (a) *Macroscopic appearance of* Scedosporium apiospermum *(7-day-old growth) on Sabouraud glucose neopeptone agar.* **(b)** *Microscopic morphology of* Scedosporium apiospermum *(7-day-old culture, Sabouraud glucose neopeptone agar). Abundant pale-brown, oval conidia (with scar at the base) formed from long slender annellides. Lactophenol cotton blue stain; magnification ×400*

does not respond to amphotericin B (Scott et al. 2002). In infected tissue, species of *Paecilomyces* exhibit septate, hyaline branching hyphae (2–4 μm wide), which are indistinguishable from those of other agents of hyalohyphomycosis. However, abundant adventitious sporulation frequently occurs, and has been reported from samples of patients with keratitis; adventitious conidia are subglobose to very short ellipsoidal, and this feature may help in identification and selection of appropriate antifungal therapy (Liu et al. 1998). Culture is essential to identify the causal agent. In culture at 25–30°C, *Paecilomyces lilacinus* grows rapidly; the colonies are initially white in color but gradually take on a lilac–lavender hue. Culture mounts reveal flask-shaped phialides with a swollen basal portion tapering in a long distinct neck; the conidiogenous cells are borne singly, in whorls or in penicillate heads. Accurate species identification is important when dealing with *Paecilomyces*

infection since *Paecilomyces lilacinus* and *Paecilomyces marquandii* are highly resistant to polyene antifungals (amphotericin B, natamycin) and to flucytosine and susceptible to imidazoles (clotrimazole, ketoconazole, econazole, miconazole), whereas *Paecilomyces variotii* is almost universally susceptible to amphotericin B and flucytosine (Castro et al. 1990; Sutton et al. 1998).

Species of *Acremonium* are ubiquitous, being found in abundance in the soil and air (Guarro et al. 1997). *Acremonium atrogriseum*, *Acremonium kiliense*, and *Acremonium potronii* have been reported as causes of endophthalmitis (Weissgold et al. 1996; Guarro et al. 1997) and keratitis (Fincher et al. 1991; Mino de Kasper et al. 1991; Vajpayee et al. 1993; Chander and Sharma 1994; Read et al. 2000). In infected tissue, septate, hyaline, branching hyphae (2–4 μm wide) are seen; adventitious sporulation may occur (Liu et al. 1998). In culture on glucose peptone agar at 30°C, colonies are flat, smooth, gray to orange, and rapidly growing, attaining a diameter of 50 mm in 1 week; conidiophores are long, straight and slightly tapering, while conidia are ellipsoidal and accumulated in slime balls (Campbell et al. 1996).

Arthrographis kalrae is an ascomycetous filamentous fungus that is widespread in decaying plant material and in the soil. McAleer et al. (1988) reported the isolation of this fungus in two corneal scrapings taken from a patient with a corneal ulcer. Perlman and Binns (1997) reported the isolation of this fungus from corneal lesions in a contact lens wearer, although the patient was believed to have acquired the infection while not wearing the lens; although there was no response to topical natamycin 5 percent, the keratitis ultimately resolved with hourly topical 0.15 percent amphotericin B and daily oral ketoconazole 100 mg. Interestingly, this isolate of *Arthrographis kalrae* was reported to be susceptible to ketoconazole, itraconazole, and amphotericin B, but resistant to natamycin. Reported minimal inhibitory concentrations for *Arthrographis kalrae* are 0.5 μg/ml amphotericin B, 0.06 μg/ml itraconazole, and 0.5 μg/ml ketoconazole (Sutton et al. 1998). Colonies of *Arthrographis kalrae* on potato flakes agar at 25°C are rather slow-growing. Microscopy reveals septate hyaline hyphae; arthroconidia formed from short, branched or unbranched conidiophores are one-celled, hyaline, and rectangular (Sutton et al. 1998).

Fungi of the genus *Cylindrocarpon* are soil fungi and are rarely associated with human disease. *Cylindrocarpon lichenicola*, previously known as *Cylindrocarpon tonkinensis* or *Cylindrocarpon tonkinense*, has a wide geographical distribution, but occurs frequently in tropical climates, where it has a wide host range for woody post-harvest fruit invasion (de Hoog and Guarro 1995). In experimental rabbit eyes, *Cylindrocarpon tonkinense* was found to produce as severe a corneal infection as did *Fusarium solani* (Ishibashi et al. 1986). A literature survey on human keratitis due to *Cylindrocarpon lichenicola* revealed only three cases (Laverde et al. 1973; Matsumoto et al. 1979; Mangiaterra et al. 2001). The histological appearance of the fungus in corneal tissue is very similar to that seen in infection by species of *Aspergillus* or *Fusarium*, with septate branching hyphae and globular structures associated with the hyphae being noted. In fact, *Cylindrocarpon* spp. appear closely related both morphologically and taxonomically to *Fusarium* spp., with both sharing teleomorphs in the genus *Nectria* (Samuels and Brayford 1993; James et al. 1997). Because of morphological similarities, separation of the genus *Cylindrocarpon* from the genus *Fusarium* by cultural characteristics may become problematic at times (Guarro and Gené 1992). *Cylindrocarpon lichenicola* closely resembles *Fusarium solani*, but differs from it microscopically by forming macroconidia which are predominantly straight rather than curved, by having apical cells that are rounded rather than tapering, by having basal cells with truncate and offset rather than attenuated pedicels (foot cells) (Figure 4), by lacking microconidia, by having pigmented chlamydoconidia, and by the formation of a brown color rather than cream color on reverse of the Sabouraud glucose neopeptone agar (Iwen et al. 2000).

Dematiaceous fungi

The primary factor unifying the dematiaceous fungi is the dark pigmentation of the hyphae (McGinnis et al. 1986). This cell coloring results in colonies that are olive to black and in a brown-to-olive-to-black color in the cell walls of their vegetative cells, conidia or both. The dematiaceous fungi reported as causes of ocular infections, especially keratitis, include *Alternaria alternata*, *Aureobasidium pullulans*, *Bipolaris spicifera*, and *Bipolaris hawaiiensis*, *Curvularia lunata*, *Curvularia geniculata*, *Curvularia brachyspora*, *Curvularia senegalensis*, *Dichotomophthoropsis nymphaearum*, *Exophiala*

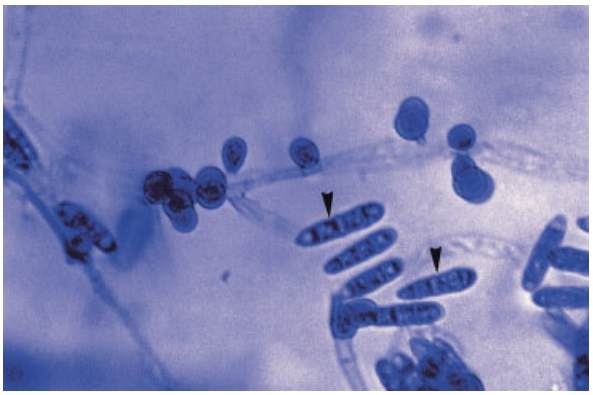

Figure 4 *Microscopic morphology of* Cylindrocarpon lichenicola *(7-day-old culture, Sabouraud glucose neopeptone agar). Multicelled macroconidia are predominantly straight, with rounded apical cells and truncate basal cells. Lactophenol cotton blue stain; magnification ×400*

jeanselmei var. *jeanselmei*, *Exophiala* (formerly *Wangiella*) *dermatitidis*, *Exserohilum rostratum*, *Exserohilum longirostratum*, *Lecytophora* (formerly *Phialophora*) *mutabilis*, *Lecytophora hoffmannii*, *Phaeoisaria clematidis*, *Phialophora verrucosa*, *Phoma oculo-hominis*, *Phoma* spp., and *Tetraploa aristata* (Newmark and Polack 1970; Jones and Christensen 1974; Forster et al. 1975b; Zapater et al. 1975; Punithalingam 1976; Liesegang and Forster 1980; Levenson et al. 1984; McGinnis et al. 1986; Douer et al. 1987; Wright et al. 1990; Ho et al. 1991; Stern and Buttross 1991; Marcus et al. 1992; Bouchon et al. 1994; Chang et al. 1994; Sutton et al. 1998; Guarro et al. 1999, 2000; Garg et al. 2000; Kaushik et al. 2001; Rishi and Font 2003). However, the validity of some of the earlier reports has been questioned (McGinnis 1980). In various studies, dematiaceous fungi are reported to be the third most important cause of fungal keratitis (behind *Aspergillus* and *Fusarium*) (Sundaram et al. 1989; Panda et al. 1997; Srinivasan et al. 1997; Garg et al. 2000; Gopinathan et al. 2002; Leck et al. 2002). Dematiaceous fungi may also cause infections of the orbit (Jay et al. 1988; Maskin et al. 1989), or intraocular infections (Kaushik et al. 2001). For diagnosis, direct microscopy of wet preparations of clinical material in 10 percent potassium hydroxide wet mounts permits the demonstration of the characteristic brown-pigmented septate fungal hyphae (Garg et al. 2000); lactophenol cotton blue stain can also be used (Figure 5) (Thomas et al. 1991b). Histology is also helpful in the diagnosis, although these fungi may be difficult to identify in hematoxylin and eosin-stained sections due to variability in the amount of pigment in the walls; to overcome this difficulty, the microscope condenser is lowered to make the hyphae more refractile, or the sections are stained by melanin-specific stains such as Fontana–Masson (Chandler 1991). Culture is required to confirm the etiologic diagnosis and to initiate appropriate treatment. Plates of Sabouraud glucose neopeptone agar or other appropriate media are inoculated with the material and incubated at 25–30°C for up to 3 weeks. In general, the colonies tend to be light-brown to olive-gray to black in color. The different genera and species are identified by the microscopic morphology of the conidia (McGinnis et al. 1986; Sutton et al. 1998).

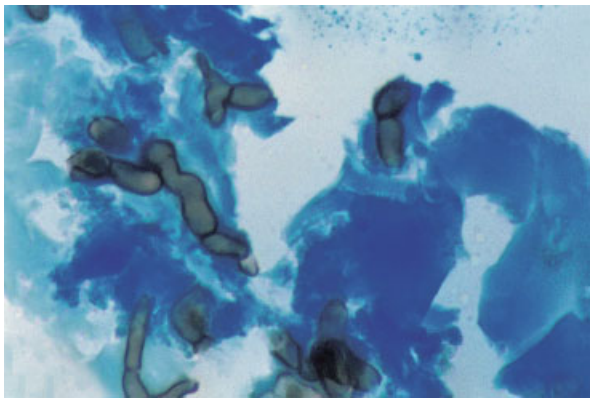

Figure 5 *Photomicrograph of pigmented fungal hyphae seen in the corneal scrape material of a patient with keratitis due to* Curvularia geniculata. *Lactophenol cotton blue stain; magnification ×400*

The form class Coelomycetes comprises asexual fungi that produce conidia within fruiting bodies named conidiomata. These structures may be spherical (pycnidia), with conidiogenous cells lining the inner cavity wall, or cup-shaped (acervuli), with the conidiogenous cells forming a palisade on the surface of the conidiomata. Coelomycetous fungi implicated in ocular infections include *Lasiodiplodia theobromae*, species of *Colletotrichum*, and *Phoma* spp.

Lasiodiplodia theobromae is an important cause of rot in corn, yams, citrus, bananas, and other plants, mainly in tropical regions (Adisa and Fajola 1983; Nwufo and Fajola 1988; Sutton et al. 1998). This fungus was initially implicated as an etiologic agent of human keratitis in two patients in India (Puttana 1967). Subsequently, reports from Colombia, France, Ghana, other parts of India, the Philippines, the southern USA, and Sri Lanka have confirmed the pathogenic potential of this fungus for the human cornea (Laverde et al. 1973; Valenton et al. 1975; Rebell and Forster 1976; Liesegang and Forster 1980; Slomovic et al. 1985; Thomas et al. 1991a, 1995; Gonawardena et al. 1994; Borderie et al. 1997; Garg et al. 2000; Gopinathan et al. 2002; Leck et al. 2002). This fungus causes severe keratitis in experimental animals and in humans (Puttana 1967; Rebell and Forster 1976; Thomas et al. 1991a, 1995; Borderie et al. 1997), perhaps due to its ability to secrete collagenases (Rebell and Forster 1976) and amylolytic and cellulolytic enzymes (Adisa and Fajola 1983; Nwufo and Fajola 1988). This fungus has been found to form intrahyphal hyphae and thickened cell walls in parasitized human corneal tissue (Figure 6a), and also in vitro, in the presence of an antifungal (Thomas et al. 1991a, 1995). Such intrahyphal hyphae have been demonstrated in experimental *Fusarium solani* keratitis in rabbits (Kiryu et al. 1991), and may represent a virulence factor for these fungi. In infected corneal tissues, *Lasiodiplodia theobromae* exhibits brown, highly bulged, septate hyphae. In culture on glucose peptone agar, the colony is rapidly growing (90 mm in one week), floccose, and gray to brown–black in color, the reverse being black (Figure 6b). Macroscopically visible fruiting bodies (pycnidia) are formed in culture. The pycnidial stromata, which are only formed on cornmeal or other nutritionally deficient media, are up to 5 mm in diameter, consisting of several pycnidia, each with a wide ostiole to permit release of the conidia; the conidia (Figure 6c) are 20–30 μm × 10–15 μm, initially colorless, ellipsoidal, and nonseptate, later becoming dark brown and septate, with longitudinal striations and

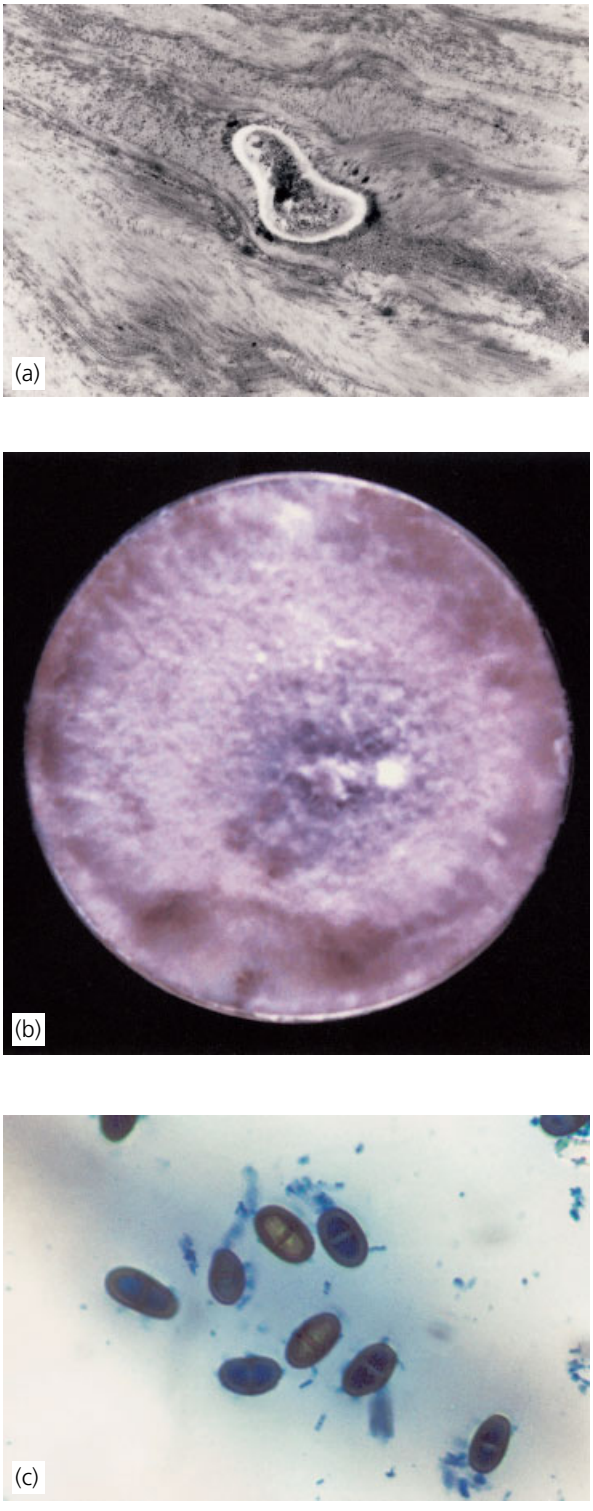

Figure 6 (a) *Electron micrograph of* Lasiodiplodia theobromae *fungal cell showing thickened cell wall in corneal scrape material (×6 050).* **(b)** *Macroscopic appearance of* Lasiodiplodia theobromae *(7-day-old growth) on Sabouraud glucose neopeptone agar.* **(c)** *Microscopic morphology of* Lasiodiplodia theobromae *(10-day-old culture, Sabouraud glucose neopeptone agar). Oval conidia, dark-brown, septate with longitudinal striations, truncate bases. Lactophenol cotton blue stain; magnification ×400*

truncate bases (Campbell et al. 1996). In vitro, *Lasiodiplodia theobromae* is resistant to many antifungals; minimal inhibitory concentrations exceeding 100 μg/ml for miconazole, ketoconazole, itraconazole, and natamycin, and ⩽10 μg/ml for econazole against ocular isolates of this fungus have been reported (Thomas et al. 1995).

The complex form genus *Colletotrichum* Corda comprises several hundred species, which are anamorphs of the genus *Glomerella* Spauld & H. Schrenk; they are placed in the order Phyllachorales, although there is evidence linking them to the Sordariales (Uecker 1994). *Colletotrichum* spp. are usually pathogenic on plants, in which they cause anthracnosis, necrosis, leaf spot, and fruit rot, and are believed to be only rarely pathogenic to humans. Although a search of the Medline database through PubMed yielded four reports describing 12 patients with *Colletotrichum* keratitis (Shukla et al. 1983; Ritterband et al. 1997; Yamamoto et al. 2001; Fernandez et al. 2002), a more detailed manual search through the results of various studies on mycotic keratitis reveals that species of *Colletotrichum* have been previously implicated as etiologic agents in as many as 20 patients with keratitis (Kaliamurthy et al. 2004). The species reported include *Colletotrichum gloeosporioides* in seven patients (Shukla et al. 1983; Matsuzaki et al. 1988; Yamamoto et al. 2001; Fernandez et al. 2002), *Colletotrichum coccodes* (reported as *Colletotrichum atramentarium*) in four (Liesegang and Forster 1980; Rosa et al. 1994), *Colletotrichum dematium* in three (Liao et al. 1983; Fernandez et al. 2002), *Colletotrichum graminicola* in one (Ritterband et al. 1997), *Colletotrichum capsici* in one (Upadhyay et al. 1991), and unidentified species of *Colletotrichum* in four (Fernandez et al. 2002). Antecedent ocular trauma appears to have been the principal risk factor for keratitis in most of these patients, but insulin-dependent diabetes mellitus, prolonged use of topical corticosteroids, and prior use of antivirals were also reported as risk factors. These patients received different antifungal drugs, principally topical natamycin alone (seven patients), amphotericin B alone or in combination with an azole or flucytosine (four patients), combined therapy with natamycin, intraocular amphotericin B, and oral azole (two patients), and natamycin followed by topical amphotericin B therapy. In most of the patients, the corneal lesions resolved completely with good visual recovery following initiation of medical therapy, possibly because the lesions were relatively superficial in nature. Microbiological investigations such as culture (Figure 7a) are essential to confirm the diagnosis of *Colletotrichum* keratitis and to ensure prompt initiation of specific antifungal therapy.

Colletotrichum dematium can be clearly distinguished from other species of *Colletotrichum* by the occurrence of falcate macroconidia (Figure 7b) (de Hoog and Guarro 1995). The gross appearance of these

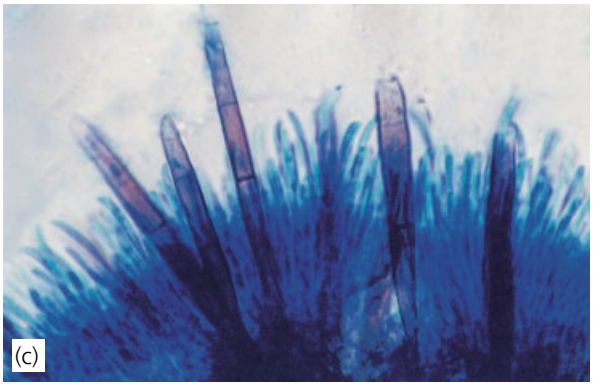

Figure 7 (a) *Macroscopic appearance of* Colletotrichum dematium *(7-day-old culture, Sabouraud glucose neopeptone agar).* **(b)** *Microscopic morphology of a* Colletotrichum *sp. (7-day-old culture, Sabouraud glucose neopeptone agar). Falcate macroconidia. Lactophenol cotton blue stain; magnification ×400.* **(c)** *Microscopic morphology of a* Colletotrichum *sp. (14-day-old culture, Sabouraud glucose-neopeptone agar). Brown-colored setae and sclerotia. Lactophenol cotton blue stain; magnification ×400*

macroconidia is very similar to that of the macroconidia of species of *Fusarium*. This may lead an inexperienced observer to mistakenly identify an isolate of *Colletotrichum dematium* as a *Fusarium* sp., particularly if the characteristic sclerotia and setae are not visible when the young culture is examined. To overcome this drawback, it is advisable to incubate all mycology culture plates for an additional 2 weeks after growth occurs, particularly if such falcate macroconidia have been observed (Kaliamurthy et al. 2004). It is important to remember that the macroconidia of *Colletotrichum dematium* are aseptate (*Fusarium* macroconidia are septate), and appear thinner than the macroconidia of species of *Fusarium*. Sclerotia and setae are found in abundance in older cultures of *Colletotrichum dematium* (Figure 7c); such structures are not observed in cultures of *Fusarium*.

Yeast and yeast-like fungi

Most yeast infections in corneal ulcers and other ocular infections are due to various *Candida* spp., predominantly *Candida albicans* and *Candida parapsilosis*. There is conclusive evidence to implicate species of *Candida* in dacryocystitis (Purgason et al. 1992), infectious crystalline keratopathy (Wilhelmus and Robinson 1991), intraocular lesions (Okhravi et al. 1998; Hidalgo et al. 2000; Jaeger et al. 2000), and keratitis (Rosa et al. 1994; Tanure et al. 2000); there is less convincing evidence for their implication in blepharitis, conjunctivitis, dacryocanaliculitis, and orbital lesions (McGinnis 1980; Behrens-Baumann 1999). The presence of small budding yeast cells (3–4 μm in size) and pseudohyphae in a corneal scraping is almost diagnostic for *Candida*. On microscopic examination of samples collected from the conjunctiva and lid margins, species of *Candida* can be easily distinguished from *Malassezia* spp. by the narrow attached base of the bud, the lack of symmetry of the yeast, and the off-axis position of the bud in *Candida*. Although both the yeast and the hyphal forms may be seen in corneal scrapings from *Candida* infections, the mycelial form is considered to be the invasive form (O'Day and Burd 1994). In culture, species of *Candida* form smooth, creamy-white colonies, resembling staphylococci in the very early stages of growth. The organisms grow well on blood agar, and do not require special conditions for isolation. It is important to identify isolates of *Candida* down to the species level, since different species exhibit varying susceptibilities to antifungal agents (Sutton et al. 1998). For example, 96–100 percent of isolates of *Candida parapsilosis* and 81–100 percent of isolates of *Candida albicans*, when tested by the recently standardized NCCLS broth microdilution method, were found susceptible to amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, and miconazole; by contrast, only 59 and 77 percent of

isolates of *Candida tropicalis*, respectively, and 85 and 52 percent strains of *Candida glabrata*, respectively, were found to be susceptible to fluconazole and itraconazole (Sutton et al. 1998). However, the relevance of such in vitro susceptibility test results to the clinical outcome of ocular *Candida* infections remains unclear.

The yeast *Cryptococcus neoformans* var. *neoformans* is typically 5–10 μm in diameter (range 2–20 μm). Acute inflammation and necrosis may obscure the organism, but a useful cytological feature is the appearance of teardrop-shaped, narrow-based budding. The identification of this yeast as the etiologic agent in ocular infections still rests primarily on its cytomorphology (Klotz et al. 2000), with confirmation, as necessary, by special stains such as methenamine silver, which stain the organisms, and mucicarmine, periodic acid–Schiff, and alcian blue, which stain the capsule. These yeasts may be mistaken for *Histoplasma capsulatum* var. *capsulatum* when engulfed by histiocytes or when nonencapsulated (Powers 1998). There is clear evidence to show that *Cryptococcus neoformans* var. *neoformans* can cause blepharitis (Doorenbos-Bot et al. 1990; Coccia et al. 1999), chorioretinitis (Morinelli et al. 1993), endophthalmitis (Morinelli et al. 1993), keratitis (Liesegang and Forster 1980; Tanure et al. 2000), and solitary subretinal lesions (Hester et al. 1992). Recently, *Cryptococcus laurentii* was reported as the etiologic agent of keratitis, in conjunction with *Fusarium solani*, in one eye of a person wearing gas-permeable contact lenses (Ritterband et al. 1998). In vitro, 94–100 percent of strains have been found susceptible to fluconazole, itraconazole, amphotericin B, and ketoconazole (Sutton et al. 1998).

Zygomycetous fungi

Zygomycetes are filamentous fungi which exhibit aseptate or sparsely septate, hyaline hyphae. There are two orders, the Mucorales and the Entomophthorales, containing organisms causing human disease, although most human infections are caused by the Mucorales (Ribes et al. 2000). Ocular zygomycosis is commonly linked to *Rhizopus* spp., particularly *Rhizopus arrhizus*, but species of *Absidia*, *Apophysomyces*, *Cunninghamella*, *Mucor*, *Rhizomucor*, *Saksenaea*, and *Syncephalastrum* have also been implicated (Kaufman et al. 1988; Yohai et al. 1994; Marshall et al. 1997; Fairley et al. 2000; Ribes et al. 2000). Ocular infections caused by this group of fungi include rhino-orbital-cerebral zygomycosis (Anand et al. 1992; Yohai et al. 1994), where the spores are inhaled, and keratitis (Marshall et al. 1997), where the infection manifests after trauma. A *Rhizopus* sp. has also been reported as a cause of scleritis (Locher et al. 1998), but the evidence is not convincing. The detection of fungi belonging to the Mucorales in clinical material by direct microscopy is more significant than their isolation in culture since, even if cultured, contamination always has to be excluded (Richardson and Shankland 1999; Ribes et al. 2000). In clinical material, the hyphae are broad and aseptate or sparsely septate with right-angled (90°) branching (Figure 8); they do not possess parallel walls, nor do they radiate from a single point in tissues (Liu et al. 1998). Although the hyphae stain poorly with periodic acid–Schiff, they stain well with hematoxylin–eosin and Gomori methenamine silver stains. Cresyl-fast violet stains zygomycete walls brick red, and other fungi blue or purple (Richardson and Shankland 1999). In tissue sections, the hyphae are seen in the midst of associated prominent inflammation, necrosis, and invasion of blood vessels (Ribes et al. 2000). In vitro, 91 percent of strains of *R. arrhizus*, 100 percent of strains of *Absidia corymbifera*, and 50 percent of strains of *Apophysomyces elegans* were found susceptible to amphotericin B, while most strains were resistant to flucytosine, fluconazole, and itraconazole (Sutton et al. 1998). In practice also, amphotericin B is the drug of choice in the treatment of ocular zygomycosis.

Thermally dimorphic fungi

Blastomycosis, a granulomatous inflammatory disease caused by *Blastomyces dermatitidis*, usually occurs following inhalation of airborne spores that arise from the soil, and thus the primary infection is usually pulmonary; however, characteristic cutaneous lesions are often the presenting symptom. Eyelid lesions are believed to be the most common ocular manifestation of disseminated infection due to *Blastomyces dermatitidis* (Barr and Gamel 1986; Slack et al. 1992); however, a recent survey of 79 patients revealed just one with eyelid lesions (Bartley 1995). Other reported ocular lesions due to *Blastomyces dermatitidis* include conjunctivitis (Slack et al. 1992), orbital cellulitis (Vida and Moel 1974; Li et al. 1998), keratitis (Rodrigues and Laibson 1973),

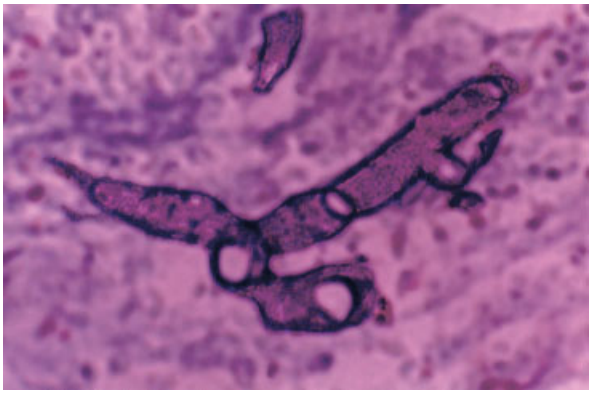

Figure 8 *Large, aseptate, irregular, bizzare hyphae of a zygomycetous fungus in tissue section of necrotic orbital material from a patient with rhino-orbital zygomycosis. Hematoxylin and eosin stain; magnification × 1 200*

and intraocular lesions such as anterior uveitis, iris nodules, or yellow-white posterior fundus lesions (Safneck et al. 1990), or endophthalmitis (Safneck et al. 1990; Li et al. 1998). Potassium hydroxide mounts of clinical material reveal characteristic yeasts with broad-based buds. The tissue reaction is a combination of suppurative and epithelioid cell granulomas with giant cells. Yeast-like spherical cells of *Blastomyces dermatitidis* are generally larger (8–20 μm) than those of cryptococci, and have refractile, double-contoured walls; broad-based budding is a useful criterion to differentiate *Blastomyces dermatitidis* from other dimorphic yeasts (Powers 1998). In culture at 26°C, the mycelial phase is noted (round or oval microconidia borne on conidiophores along sides of hyphae or terminally on hyphal branches), while at 37°C, on enriched media, the yeast phase is produced (large, oval, multinucleate yeasts, budding on a broad base, often in chains, with refractile cell walls). In vitro, most strains of *Blastomyces dermatitidis* are reported to be susceptible to amphotericin B, fluconazole, itraconazole, and ketoconazole (Sutton et al. 1998).

Coccidioidomycosis is caused by the thermally dimorphic fungal pathogen *Coccidioides immitis*. The occurrence of ocular lesions in coccidioidomycosis has been reported infrequently (Rodenbiker and Ganley 1980). Anterior segment manifestations are usually a mild hypersensitivity response, occurring as a chronic granulomatous iridocyclitis (Moorthy et al. 1994; Cunningham et al. 1998); however, phlyctenular conjunctivitis, episcleritis, scleritis, and keratoconjunctivitis may occur in association with underlying pulmonary infection, and lid granulomata and inflammation in disseminated disease. The manifestations in the posterior part of the eye range from asymptomatic focal chorioretinitis to a fulminating granulomatous process involving the entire eye. In potassium hydroxide mounts of pus or necrotic material from infected ocular tissue, large multinucleate thick-walled cells, the spherules, are seen, usually within giant cells (Rippon 1988); at maturity, these spherules are filled with spores, which escape by rupture of the cell wall. In vitro, more than 90 percent of strains of *Coccioides immitis* have been found susceptible to amphotericin B, miconazole, ketoconazole, and itraconazole, and 86 percent to fluconazole (Sutton et al. 1998).

The classical form of histoplasmosis is caused by the dimorphic fungus *Histoplasma capsulatum* var. *capsulatum*, while a variant form, known as African or large-celled histoplasmosis, is caused by *Histoplasma capsulatum* var. *duboisii*. The mode of infection is presumably by inhalation or entry through broken skin surfaces, manifesting as acute pulmonary infections or cutaneous and subcutaneous disease; dissemination may occur in immunocompromised patients (Gonzales et al. 2000). *Histoplasma capsulatum* var. *capsulatum* has been isolated from the eyes of patients suffering from the 'presumed histoplasmosis syndrome,' suggesting that this fungus is the etiologic agent (Roth 1977; Khalil 1982). *Histoplasma capsulatum* var. *capsulatum* has been reported to cause endogenous endophthalmitis (Carroll and Franklin 1981; Goldstein and Buettner 1983; Gonzales et al. 2000), exogenous endophthalmitis after cataract extraction (Pulido et al. 1990), and panophthalmitis (Schwarz et al. 1977). Anterior segment lesions due to *Histoplasma capsulatum* var. *capsulatum* are less common but have been reported (Feman and Tilford 1985; Font et al. 1995). In patients with the acquired immunodeficiency syndrome (AIDS), *Histoplasma capsulatum* var. *capsulatum* has been reported to cause endophthalmitis (Gonzales et al. 2000) and lesions of the choroid, retina, and optic nerve (Specht et al. 1991; Yau et al. 1996). *Histoplasma capsulatum* var. *duboisii* has been reported to cause orbital lesions (Bansal et al. 1977; Ajayi et al. 1986). Organisms may be missed by direct microscopic examination of wet preparations of necrotic tissue, hence all material should be examined as stained smears (Powers 1998). Culture is done on media at 26°C (for mycelial phase) and enriched media at 37°C (for yeast phase). Conversion of the mycelial phase to the yeast phase must be demonstrated by culturing the mycelial phase on appropriate media at 37°C, or by inoculating the organism into a mouse, and reisolating it from the liver and spleen. In vitro, 100 percent of strains are susceptible to amphotericin B, itraconazole, and ketoconazole, 82 percent to fluconazole, and 50 percent to flucytosine (Sutton et al. 1998).

Paracoccidioides brasiliensis causes a severe, usually chronic disease involving the skin, lungs, and lymphoid organs. Ocular involvement usually represents reactivated disease, and commonly presents as a chronic papular or ulcerating lesion of the eyelid in a male over 30 years of age engaged in agriculture, and coming from a region endemic for paracoccidioidomycosis (Silva et al. 1988). Although ocular paracoccidioidomycosis, which is usually unilateral, rarely occurs in the absence of lesions in other parts of the body, palpebral lesions (Burnier and Sant'Anna 1997) and granulomatous uveitis (Dantas et al. 1990) have sometimes been reported as the first indications of the disseminated disease. In an experimental model of ocular paracoccidioidomycosis in guinea pigs obtained by the intracardiac inoculation of yeast forms of *Paracoccidioides brasiliensis*, ocular involvement was observed in 80 percent of the infected animals; the uvea, ciliary body, choroid, iris, lids, and the conjunctiva were the structures most commonly affected (Kamegasawa et al. 1988). In the initial stages, ocular paracoccidioidomycosis needs to be differentiated from hordeolum, bacterial blepharitis, trachoma, leishmaniasis, sporotrichosis, lupus erythematosus, tuberculosis, and secondary syphilis (Silva et al. 1988). Spherical yeast-like cells with multiple buds attached by narrow necks ('steering wheel forms') can be seen in 10 percent potassium hydroxide mounts of necrotic tissue material or in tissue sections. In culture, *Paracoccidioides*

brasiliensis exhibits dimorphism, which is temperature- and media-dependent.

Sporotrichosis, which is caused by the thermally dimorphic fungus *Sporothrix schenckii*, occurs worldwide in both tropical and temperate regions, and manifests as nodular lesions in the cutaneous and subcutaneous tissues that suppurate, ulcerate, and drain. Up to 1990, 17 episodes of endophthalmitis due to this fungus (Witherspoon et al. 1990) and orbital sporotrichosis (Streeten et al. 1974) had been reported. Since then, granulomatous uveitis progressing to scleral perforation and endophthalmitis due to use of corticosteroids (Cartwright et al. 1993), scleritis (Brunette and Stulting 1992), uveitis (Vieira-Dias et al. 1997), and a retinal granuloma associated with disseminated lesions (Curi et al. 2003) have been reported. In tissues or in culture at 37°C, the fungus appears as small, spherical, oval or elongated budding yeast cells with irregular-stained cytoplasm, or is 'cigar-shaped.' In culture at 26°C, the mycelial phase is recovered. In tissues, 'asteroid bodies' may occur, consisting of a central spherical or oval basophilic cell (3–5 μm diameter), surrounded by a thick, radiate, eosinophilic substance (which is believed to be a mass of antigen–antibody complexes); the asteroid body is characteristic of sporotrichosis only if the fungal cell within is typical of *Sporothrix schenckii*, since similar bodies may be observed in other mycoses (Rippon 1988).

Organisms of uncertain taxonomic classification

Rhinosporidiosis is caused by *Rhinosporidium seeberi*, an endosporulating microorganism traditionally considered to be a fungus but now of uncertain taxonomic classification (Pe'er et al. 1996). Rhinosporidiosis appears to be endemic in the Indian subcontinent (Chitravel et al. 1980; Krishnan et al. 1986; Moses et al. 1990; Shrestha et al. 1998); the prevalence of the condition in a south Indian village was reported to be 470/100 000 population (Chitravel et al. 1980). However, a significant number of cases has also been reported from other countries (Jimenez et al. 1984; Reidy et al. 1997). Farmers or their families, particularly children or young (up to 30 years of age) adults, and males, appear to be the most frequently affected; however, a female predominance is sometimes noted (Chitravel et al. 1980). The mode of transmission, while not definitely known, is believed to be by frequent exposure to water contaminated with spores of *Rhinosporidium seeberi* (Moses et al. 1990); in conjunctival rhinosporidiosis, the infection may arise by accidental injury to the eye by soil dust containing the spores (Jimenez et al. 1984). Lesions of rhinosporidiosis manifest as polypoid or papillomatous, very friable, proliferative outgrowths principally in the nasal cavity; ocular lesions may account for 13 percent of all lesions, the ratio of nasal to ocular lesions being 1.4:1 (Owor and Wamukota 1978). The principal ocular lesion in rhinosporidiosis is conjunctival, but involvement of the limbus, sclera, lacrimal sac (Krishnan et al. 1986; Kalavathy et al. 1998), and peripheral cornea (Bhomaj et al. 2001) have also been reported. Two distinct phases, namely, trophic and endosporulating, have been noted in the life cycle of this organism in tissues (Savino and Margo 1983), and the diagnosis of rhinosporidiosis is based on detecting these cyst-like structures in the affected tissues (Figure 9). The formation of the cyst wall appears to be a continuous morphological and biochemical spectrum throughout the cytological maturation of the organism (Sunba and al-Ali 1989); variations in this pattern have been noted to occur, probably as a protective mechanism, in concurrent rhinosporidiosis and *Papillomavirus* infection.

Pythium insidiosum, a cosmopolitan fungus-like aquatic organism, is found predominantly in swampy environments, where water lilies, various vegetables, and especially certain grasses support the asexual phase of its life cycle; motile zoospores, which appear to be chemotactically attracted to plant leaves or human/horse hairs, are the likely infective particles (Sutton et al. 1998). Originally considered to be an oomycetous member of the kingdom Fungi and later reclassified in the kingdom Protoctista (Imwidthaya 1994; Sutton et al. 1998), *Pythium insidiosum* is now considered to belong to the kingdom Stramenopila, organisms that are related to algae (Sutton et al. 1998). This organism causes diseases in plants and animals (horses, cattle, dogs, cats, or fishes), particularly in tropical and subtropical parts of the world (Imwidthaya 1994; Thianprasit et al. 1996). Virgile et al. (1993) presented what they believed to be the first case of a human corneal ulcer due to *Pythium insidiosum*. Subsequently, *Pythium insidiosum* has been reported as a cause of keratitis in Thailand (Imwidthaya 1994, 1995), where it has also been reported to cause subcutaneous lesions in thalassemic patients (Thianprasit

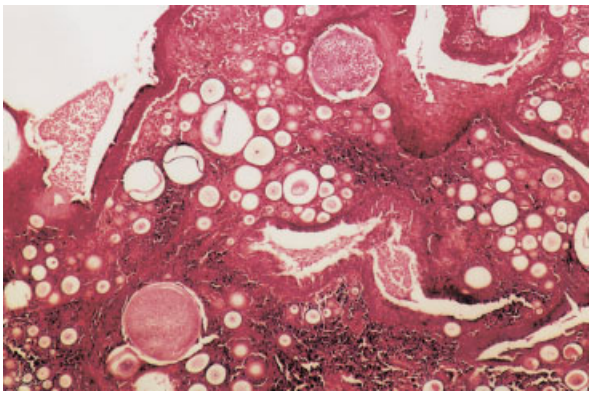

Figure 9 *Photomicrograph showing presence of sporangia (cysts) of* Rhinosporidium seeberi *in stroma of lacrimal sac. The cysts possess a sharply defined wall, with the largest sporangium revealing maturing endospores. Hematoxylin and eosin stain; magnification ×400*

et al. 1996), and in the temperate climate of New Zealand (Murdoch and Parr 1997), endophthalmitis (Srimuang et al. 1996), and orbital cellulitis with deep facial tissue involvement (Mendonza et al. 1996). Colonies on potato flakes agar at 25°C (optimal growth is at 33–36°C) are white to yellowish-white with short aerial hyphae; these are rapid-growing, and addition of human blood appears to stimulate colonial growth (Sutton et al. 1998). The most significant diagnostic microscopic morphological feature, biflagellate/motile asexual zoospores, may be induced by placing small pieces of *Pythium insidiosum* grown on agar into water and incubating at 37°C in the dark; after 1 hour incubation, encysted zoospores may be seen and after 24 hours, numerous zoosporangia may be observed (Sutton et al. 1998). In vitro susceptibility data are currently not available. Serological diagnosis of infection due to *Pythium insidiosum* by using sera from patients with keratoconjunctivitis or corneal ulcers with endophthalmitis has been reported (Srimuang et al. 1996). Badenoch et al. (2001) described a patient with keratitis in which DNA-based technology yielded a diagnosis of *Pythium insidiosum* keratitis.

Pneumocystis carinii was originally considered to be a protozoon, based on its morphology and response to antifungal drugs (Cailliez et al. 1996). However, subsequent to analysis of its nucleic acids, it has been reclassified as a member of the kingdom Fungi (Cailliez et al. 1996). It has been implicated as a cause of choroiditis (Dugel et al. 1990; Foster et al. 1991; Shami et al. 1991) and orbital infection (Friedberg et al. 1992) in patients with AIDS. In tissues, granulomatous inflammation mixed with foamy material containing *Pneumocystis carinii* is seen; round cysts with thickened walls, containing the crescent-shaped trophozoites, are demonstrated by the methenamine silver or Giemsa stains (Friedberg et al. 1992). No continuous in vitro culture system has been described for this organism. However, animals can be infected.

FUNGAL INFECTIONS OF THE ORBIT

Fungal infections of the orbit rarely occur spontaneously. Fungi usually gain access to the orbital space by direct extension from adjacent tissues (sinuses, teeth, lacrimal sac, lids); traumatic implantation of foreign bodies contaminated with fungi, or hematogenous seeding of fungi from a distant focus may also occur. Spread of infection from the sinuses to the orbit is believed to occur in 67–85 percent of orbital infections (Goodwin 1985; Parfrey 1986). In children, orbital complications usually accompany concurrent maxillary and ethmoid sinus infection, whereas in adults, frontal, maxillary, and ethmoidal sinus disease are about equally responsible for orbital complications (Hornblass et al. 1984). Due to the intimate relationship between the orbit and paranasal sinuses, virulent fungal pathogens causing sinusitis can devastate orbital structures by contiguous spread through the wafer-thin walls of the sinuses (Maskin et al. 1989). In fact, the variable presentations of orbital fungal infections parallel the presentations of paranasal sinus mycoses (Maskin et al. 1989; Klapper et al. 1997). Just as the distinctions between the different types of paranasal sinus mycoses are not clear because of closely associated patterns of clinical behaviour and pathological reactions (Levin et al. 1996; de Shazo et al. 1997), so also, the distinctions between the different types of orbital fungal infections, especially the chronic varieties, may not always be clear-cut.

Orbital fungal infections are usually demarcated into the invasive category, comprising the acute/fulminant (typified by rhinocerebral zygomycosis) and chronic/indolent (granulomatous) varieties (typified by orbital aspergillosis), and the noninvasive category, comprising the 'fungus ball' and orbital manifestations of allergic fungal rhinosinusitis (Klapper et al. 1997).

Since rhinocerebral zygomycosis and sino-orbital aspergillosis appear to be the principal types of orbital fungal infections, these are dealt with in greater detail.

Acute/fulminant orbital infections

RHINO-ORBITAL-CEREBRAL ZYGOMYCOSIS (RHINOCEREBRAL MUCORMYCOSIS)

Rhino-orbital-cerebral zygomycosis is the prototype of the acute/fulminant variety of invasive fungal orbital infection, usually running an acute course in an immunocompromised host (rarely in non-immunocompromised individuals), with angioinvasion and marked tissue necrosis being key features. The infection usually begins in the paranasal sinuses and then spreads to involve the orbit, face, palate, or brain (Anand et al. 1992). *Rhizopus* spp., especially *Rhizopus arrhizus*, are the most frequent causes (Ribes et al. 2000), less common causes being *Absidia corymbifera*, *Apophysomyces elegans* (Radner et al. 1995; Brown et al. 1998; Fairley et al. 2000), *Cunninghamella* spp. (Chetchotisakd et al. 1991), and *Saksenaea vasiformis* (Ajello et al. 1976; Kaufman et al. 1988). The major mode of disease transmission for the zygomycetes is presumed to be by inhalation of fungal conidia from environmental sources (Yohai et al. 1994; Ribes et al. 2000).

Being opportunistic pathogens, the Mucorales require a breakdown in immune defense mechanisms, especially those disease processes leading to neutropenia or neutrophil dysfunction, to cause disease. Neutrophil dysfunction induced by ketoacidosis underlies the majority of cases of human zygomycosis (Ribes et al. 2000), and this occurs even in juvenile diabetics. Although neutropenia induced by bone marrow suppression during chemotherapy, or immunosuppression induced following transplantation, is also believed to contribute to this type of pathology, zygomycosis was

observed in only 13 (0.9 percent) of 1 500 consecutive patients who underwent bone marrow transplantation (Morrison and McGlave 1993). Immune system compromise has also been linked to the development of zygomycosis. Corticosteroid use, in particular, may increase susceptibility to the development of zygomycosis by suppressing the normal inflammatory cell response, and also by inducing a diabetic state. An association has been noted between the occurrence of zygomycosis in patients on dialysis who were receiving deferoxamine/desferrioxamine for iron or aluminum overload (Daly et al. 1989; Boelaert et al. 1991; Slade and McNab 1991). Rhinocerebral manifestations were reported in 31 percent of 59 dialysis patients who developed zygomycosis due to *Rhizopus* spp. (Boelaert et al. 1991). In an alloxan-induced immunocompromised model of zygomycosis in mice, desferrioxamine iron chelation produced rhinocerebral zygomycosis in those animals that were challenged intrethmoidally with *Rhizopus* spores (Anand et al. 1992), presumably because desferrioxamine, in the iron-chelate form, feroxamine, provides iron to fungi belonging to the Mucorales. Rhino-orbital-cerebral zygomycosis may also supervene in the presence of predisposing factors such as protein-calorie malnutrition and iron overload, intravenous drug abuse, leukemia, aplastic anemia, myelodysplastic syndrome, concurrent diabetes mellitus, and adrenogenital syndrome, and treatment with the immunosuppressive medications necessary to maintain solid organ transplants, such as liver transplants, and in patients receiving donor leukocyte infusions (Boelaert et al. 1991; Weinberg et al. 1993; Nussbaum and Hall 1994; Penalver et al. 1998; Raj et al. 1998; Webb et al. 1998; Stern and Kagan 1999; Ribes et al. 2000). The disease may rarely occur in non-immunocompromised individuals if there is some associated antibiotic use or a breakdown in the mucocutaneous barrier (Bhattacharya et al. 1992; Parthiban et al. 1998; Saltoglu et al. 1998); such patients may respond to treatment better than immunocompromised patients.

In recent years, *Apophysomyces elegans* has been reported as a cause of rhino-orbital-cerebral zygomycosis in immunocompetent hosts (Radner et al. 1995; Brown et al. 1998; Fairley et al. 2000). The disease entity caused by *Apophysomyces elegans* appears to differ from that caused by the more common genera of zygomycetes in that the disease frequently follows traumatic inoculation and/or soil contamination, and tends to occur in warm climates in patients who do not exhibit well-recognized immunological or metabolic abnormalities (the patients are frequently immunocompetent) (Fairley et al. 2000).

Fever has been reported to occur in 44 percent of patients with rhinocerebral zygomycosis, nasal ulceration, or actual necrosis in 38 percent and periorbital or facial edema in 34 percent; other findings (in descending order of frequency of occurrence) include decreased vision, ophthalmoplegia, sinusitis, headache, facial pain, decreased mental status, leukocytosis, nasal discharge, nasal stuffiness, corneal anesthesia, orbital cellulitis, and proptosis (Yohai et al. 1994). Necrosis of the facial skin and bone with typical black eschar formation, which is usually considered to be pathognomonic of rhinocerebral zygomycosis, is reported by some to be a late manifestation (Ferry and Abedi 1983). Others believe that the classic presentation in a susceptible patient is with unilateral severe headache and facial pain, nasal stuffiness with granular or purulent discharge, facial or eyelid edema, fever, and leukocytosis (Nussbaum and Hall 1994). Bacterial cavernous sinus thrombosis may sometimes mimic rhino-orbital-cerebral zygomycosis, but early visual loss and retinal artery occlusion are believed to suggest a diagnosis of the latter condition, since blindness is a much later finding in the former condition (Fairley et al. 2000).

Orbital findings result from ischemic necrosis of the intraorbital contents and cranial nerves; however, bony involvement is uncommon because of the angioinvasive nature of the fungus. Fungal invasion of the eyeball in patients with rhino-orbital-cerebral zygomycosis is considered to be a poor prognostic index, since all six patients reported up to 1992 with this condition succumbed to the disease (Sponsler et al. 1992). Rhino-orbital-cerebral zygomycosis has also been reported to manifest as a painless orbital apex syndrome without any sign of orbital cellulitis or acute systemic disease (Balch et al. 1997), which may have a good outcome with medical therapy, orbital infarction syndrome (Borruat et al. 1993), bilateral cavernous sinus thrombosis (Van Johnson et al. 1988; Alleyne et al. 1999), isolated pontine infarction (Calli et al. 1999), palatal ulcer (Van der Westhuijzen et al. 1989), chiasmal infarction and sudden blindness (Lee et al. 1996), fever with right-sided hemiparesis, and dysarthria in juvenile diabetics (Adler et al. 1998) and numbness, loss of sensation over the temporal region, loss of vision, and proptosis (Attapattu 1995).

Magnetic resonance imaging (MRI) and computerized tomography (CT) are important aids in establishing an anatomic diagnosis in suspected rhinocerebral fungal infections (Moll et al. 1994; Levin et al. 1996). Reported MRI manifestations of rhino-orbital-cerebral zygomycosis include (Press et al. 1988): sinus and orbital disease followed by deep facial extension, involvement of the basal portions of the cerebral hemispheres, brainstem, and hypothalamus and hyperintense regions (signifying intracerebral inflammation). Limitations in establishing the diagnosis of both cerebral zygomycosis and cavernous sinus thrombosis (Van Johnson et al. 1988) may be overcome by performing sequential CT and MRI studies in a patient suspected of having rhino-orbital-cerebral zygomycosis (Boelaert 1994; Moll et al. 1994). It is also not clear whether there are any specific radiological findings for rhinocerebral zygomycosis. CT nonenhancement of the superior ophthalmic artery and vein may

represent a specific sign of orbital zygomycosis (Kilpatrick et al. 1984; Gamba et al. 1986). McLean et al. (1996) documented, by MRI and pathology, the perineural spread of rhinocerebral zygomycosis, following the trigeminal nerve to the pons. While CT and MRI scans help to define the extent of bone and soft-tissue destruction, they are probably more useful in planning surgical intervention than in establishing a diagnosis. MRI scanning may be preferred for the diabetic patient in whom CT contrast agents may be contraindicated.

A prompt and accurate diagnosis of orbital zygomycosis necessitates a high level of clinical suspicion, and good coordination between the clinical and laboratory staff. Multiple biopsies should be taken from necrotic tissue and, if possible, from the nose, paranasal sinuses, and oropharynx as well; abscesses should be aspirated, and lesions on the mucous membranes should be irrigated or scraped (Richardson and Shankland 1999). Swabs are not satisfactory. Once collected, samples should be transported at once to the laboratory since immediate information is required and because zygomycetes are rather fragile organisms that do not survive more than a few hours at refrigerator temperature; if overnight storage is required, samples may be kept in Stuart's transport medium and left at room temperature. The tissue should be minced and not ground, to ensure that any viable fungal elements are not destroyed. Zygomycetes may not be found at the center of the necrotic tissue but, rather, at the edge or proximal to the necrotic material (Richardson and Shankland 1999).

The microscopic demonstration of zygomycetes in clinical material taken from necrotic lesions is more significant than their isolation in culture (Figure 10a), since zygomycetous fungi are frequent contaminants in the clinical microbiology laboratory (Ribes et al. 2000). In tissue material stained by hematoxylin-eosin, abundant, large, often bizzare, aseptate or sparsely septate, irregularly branching hyphal elements (Figure 8) can be seen invading intact tissue; however, failure to observe such elements does not exclude the diagnosis of such an infection. Although nasal, palatal, and sputum cultures seldom contribute to the diagnosis, isolation of Mucorales from sputum, aspirated material from sinuses, or bronchial washings taken from diabetic or immunosuppressed patients should not be ignored (Richardson and Shankland 1999). The confirmatory identification of the genus or species requires culture of the specimen. Although zygomycetes are not especially fastidious, cultures of necrotic tissue frequently fail to yield fungi even when direct microscopy is positive; therefore, culture media should be heavily inoculated with as much material as possible. Sabouraud glucose neopeptone agar (with an antibacterial such as chloramphenicol or polymyxin B, but no cycloheximide) is adequate; squares of sterile home-made bread (without preservatives) have also been suggested. Mucorales usually grow within 2–5 days (Figure 10a), and rapidly fill the petri plate or tube.

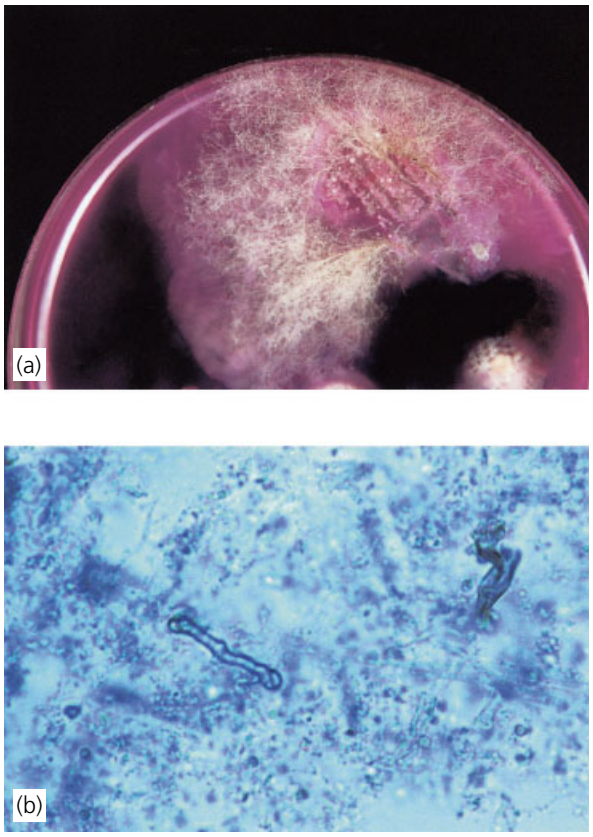

Figure 10 (a) *Macroscopic appearance of a* Mucor *sp. (isolated from rhino-orbital zygomycosis) on rose bengal agar.* **(b)** *Photomicrograph of nasal material from a patient with allergic fungal rhinosinusitis, exhibiting sparse fungal hyphae (*Aspergillus fumigatus *recovered in culture). Lactophenol cotton blue stain; magnification ×400*

In addition to the conventional methods of diagnosis, Kaufman et al. (1988) were able to demonstrate antibodies to *Saksenaea vasiformis* by enzyme-linked immunosorbent assay in a patient with rhinocerebral zygomycosis due to this fungus. However, demonstration of antibodies to Mucorales by this serological technique may not be possible if the disease progresses so rapidly that the patient is dead before specific antibodies can be formed.

General principles in the treatment of acute invasive rhinocerebral zygomycosis and other acute invasive orbital mycoses include:

- control of diabetic ketoacidosis or other systemic underlying diseases, and elimination of predisposing factors
- surgical debridement and restoration of sinus drainage
- intravenous amphotericin B (conventional or lipid associated).

In the diabetic patient, there should be prompt correction of acidosis and other metabolic abnormalities, and elimination of predisposing factors.

Surgery consists of wide local excision and debridement of all involved and devitalized oral, nasal, sinus, and orbital tissue while establishing adequate sinus and orbital drainage (Kohn and Hepler 1985); this may be quite mutilating and multiple operations are often required. Infected tissue typically bleeds little due to the vaso-occlusion caused by the Mucorales; therefore, necrotic tissue can be removed until normal bleeding is encountered. However, this may not be possible with extensive infections which extend to the dura or beyond (Yohai et al. 1994). Since rhinocerebral zygomycosis spreads by direct contiguous extension to surrounding tissues, and because the zygomycete hyphae are easily recognized on frozen section biopsies, this disease entity is potentially amenable to complete resection by frozen section guidance; one such technique of frozen section-guided surgical debridement for biopsy-proven rhino-orbital-cerebral zygomycosis, in conjunction with local and systemic amphotericin B, has recently been described (Langford et al. 1997). Serial radiological imaging may also help to identify the extent of disease and response to treatment. In recent years, endoscopic sinus surgery has been tried, alone or in combination with the traditional surgical procedures, to attain the goal of radical resection with less operative morbidity and greater operative accuracy (Jiang and Hsu 1999).

The interval between onset of symptoms and surgery was found to play a key role in the ultimate survival of evaluable patients with rhino-orbital-cerebral zygomycosis reported in the literature between 1970 and 1994; when the interval was 1–6 days, as many as 81 percent survived compared to 42 percent when the interval was 13–30 days (Yohai et al. 1994). In extreme cases, for example in an acutely infected orbit with a blind immobile eye, orbital exenteration, which entails removal of the eyeball together with its extraocular muscles and other soft tissues of the orbit, has to be performed.

Although treatment modalities have not undergone clinical trials, and in spite of the emergence of new antifungals in the therapy of invasive fungal disease, the combination of aggressive surgical debridement and intravenous amphotericin B therapy continues to be the treatment of choice in rhino-orbital-cerebral zygomycosis (O'Keefe et al. 1986; Yohai et al. 1994). An analysis of the case records of 145 patients with rhino-orbital-cerebral zygomycosis reported between 1970 and 1994 revealed that when the interval from onset of symptoms to initiation of amphotericin B therapy was 6 days or less, 76 percent of patients survived, but when the interval was 7–30 days, only 36 percent survived (Yohai et al. 1994). Amphotericin B has been administered by various routes in an effort to improve the outcome of this condition, which normally results in extreme morbidity. Kohn and Hepler (1985) were among the first to use this approach by supplementing the standard therapeutic regimen for rhino-cerebral zygomycosis with daily amphotericin B (1 mg/ml) irrigation and packing of the involved orbit and sinuses; this is likely to have aided the delivery of amphotericin B to poorly perfused infected and/or necrotic tissue, so that excellent results were obtained in their small series of patients, even though orbital exenteration was not performed. The use of a nasal-sinus catheter (Cohen and Greenberg 1980) or percutaneous catheter (Nussbaum and Hall 1994) for local irrigation, the use of adjunctive local nebulized amphotericin B (Raj et al. 1998) as well as administration of amphotericin B by using the intracavitary/interstitial and cerebrospinal fluid (intraventricular) routes (Adler et al. 1998), in addition to conventional intravenous administration of amphotericin B, have also been tried.

Since the use of intravenous amphotericin B is fraught with numerous side effects, attempts have been made in recent years to prepare new formulations of amphotericin B which maintain the efficacy of the antifungal without its toxicity. One such formulation is amphotericin B encapsulated in liposomes (liposomal amphotericin B). Lipid association significantly decreases the toxicity of amphotericin B, enabling higher dosages to be administered, and appears to enhance delivery to fungi, infected organs, and phagocytes. Treatment comprising liposomal amphotericin B and surgical debridement has been reported useful for a small number of patients with rhinocerebral zygomycosis (Penalver et al. 1998; Saltoglu et al. 1998). Strasser et al. (1996) documented the medical cure of a 60-year-old diabetic woman with rhinocerebral zygomycosis involving the cavernous sinus whose infection responded to medical therapy with another modification of this drug, namely, amphotericin B lipid complex. Yet another modification is amphotericin B colloidal dispersion, a 1:1 complex of cholesteryl sulfate and amphotericin B; administration of this preparation, in combination with surgical debridement, resulted in cure of life-threatening rhinocerebral zygomycosis in two patients, and in a cure of the fungal infection in a third patient (Moses et al. 1998).

Additional therapeutic modalities, including the triazoles itraconazole and fluconazole, have been reported useful in therapy of a small number of patients with rhinocerebral zygomycosis (Sica et al. 1993; Parthiban et al. 1998); however, further clinical studies are necessary to substantiate these preliminary results. The efficacy of granulocyte colony-stimulating factor, in conjunction with fluconazole therapy, was evaluated in the treatment of four patients with histopathologically proven rhinocerebral zygomycosis (Sahin et al. 1996); two patients refractory to fluconazole therapy were treated with liposomal amphotericin B. The improvement in clinical manifestations was closely related to neutrophil recovery, and all patients were alive at the end of therapy; in addition to surgical debridement and antifungal therapy, granulocyte colony-stimulating factor seems to have played a role in their survival. Hyperbaric oxygen has also been described as an addi-

tional therapeutic option. Yohai et al. (1994) evaluated the effect of hyperbaric oxygen therapy in patients with a low survival rate, that is, in those with bilateral rhinocerebral zygomycosis; 22 percent of patients who received standard therapy survived while 83 percent of patients who received standard therapy plus adjunctive hyperbaric oxygen survived. Although the possible adverse effects of hyperbaric oxygen therapy, such as decompression sickness and aeroembolism, need to be kept in mind (Maskin et al. 1989), it has been suggested that hyperbaric oxygen be considered as part of the initial therapy and should be persisted with until evidence of disease regression is observed (Yohai et al. 1994).

In a review of the published case histories of 145 patients who presented with rhinocerebral zygomycosis from 1970 to 1994, delayed diagnosis and treatment, hemiparesis or hemiplegia, bilateral sinus involvement, leukemia, renal disease, and treatment with desferrioxamine were identified as significant factors related to a lower survival rate; the association of facial necrosis with a poor prognosis also appeared to be clinically important (Yohai et al. 1994). The presence of ocular infiltration by fungus may indicate a poor prognosis in rhinocerebral zygomycosis (Sponsler et al. 1992). The mortality of rhinocerebral zygomycosis caused specifically by *Apophysomyces elegans* is currently unknown because of the rarity of diagnosed cases, but it would seem to fall at the more favorable end of the spectrum (Fairley et al. 2000).

ACUTE ORBITAL INFECTIONS DUE TO OTHER FUNGI

Fungi belonging to the order Mucorales are so frequently the causes of acute orbital mycoses that the term 'mucormycosis' has often been used as being synonymous with a fulminant orbital fungal infection. However, species of *Aspergillus* (Levin et al. 1996), *Scedosporium apiospermum* (*Pseudallescheria boydii*) (Anderson et al. 1984), and different genera of dematiaceous filamentous fungi (Jay et al. 1988; Maskin et al. 1989) may also cause this disease entity.

Although orbital aspergillosis usually manifests as a chronic condition, it may sometimes present as acute fulminant disease, where rapid, uncontrollable progression leads to intracranial involvement and sometimes death (Mauriello et al. 1995; Kronish et al. 1996; Johnson et al. 1999; Kusaka et al. 2003); in such cases, the key presenting complaints include abrupt onset of proptosis, ophthalmoplegia, blepharoptosis with precipitous visual loss, debilitating periorbital pain or headache, without inflammatory signs, and sometimes a temporal arteritis (Mauriello et al. 1995; Kronish et al. 1996; Hutnik et al. 1997; Johnson et al. 1999). Acute orbital infections caused by *Scedosporium apiospermum* and dematiaceous fungi are clinically characterized by rapid acute onset, orbital pain, fever, rhinorrhea, and signs of orbital inflammation, such as acute visual loss and total internal and external ophthalmoplegia.

In the treatment of fulminant rhino-orbital infections caused by fungi other than the Mucorales, treatment is essentially as described for orbital zygomycosis; however, antifungals other than amphotericin B may play a prominent role. The dematiaceous fungus *Bipolaris hawaiiensis* was identified as the cause of a devastating bilateral optic neuropathy and orbitopathy following contiguous fungal sinusitis in an apparently immunocompetent young man. Therapy with 3 700 mg of amphotericin B failed, but itraconazole therapy was initiated because of in vitro susceptibility of the fungus to itraconazole, and the detection of fungicidal levels of itraconazole in the sinus mucosa after oral therapy; at the time of reporting, the patient had been on itraconazole for 16 months and appeared stable (Maskin et al. 1989). An acute orbital infection and brain abscess due to *Scedosporium apiospermum* (*Pseudallescheria boydii*) in a 4-year-old boy failed to respond to 6 days of intravenous amphotericin B but was successfully treated with intravenous miconazole and multiple surgical debridements (Anderson et al. 1984). Surgical debridement of the orbit and a 6-week course of intravenous miconazole resulted in reduction of *Scedosporium apiospermum* orbital infection in another patient as well (Nunery et al. 1985).

Chronic invasive orbital infections

The chronic invasive form of fungal sinusitis, which slowly results in rhino-orbital-cerebral disease, manifests as a slowly destructive disease process. It may occur in immunocompromised (Levin et al. 1996) or non-immunocompromised patients (Klapper et al. 1997).

CHRONIC ORBITAL ASPERGILLOSIS

It is not clear whether the entity commonly referred to as 'chronic orbital aspergillosis,' which is described as being more common in hot, humid climates (Deans et al. 1996; Krause and Bullock 1996), refers to the chronic invasive disease (associated with granulomatous inflammation and fibrosis) occurring in immunocompromised persons (Levin et al. 1996), or to chronic invasive disease that occurs in non-immunocompromised patients (Klapper et al. 1997). *Aspergillus* spp. have been implicated in a wide variety of primary ocular orbital conditions. Predisposing factors include alcoholism, high-dose corticosteroid therapy, insulin-dependent diabetes mellitus and AIDS (Mauriello et al. 1995; Kronish et al. 1996; Johnson et al. 1999). Chronic presentations of orbital aspergillosis, which are more common than the acute variety, tend to be more localized and include an aspergilloma extending from one of the periorbital sinuses into the orbit, an aspergilloma in an exenteration socket, a complex dacryocystitis, a nerve tumor, and

postoperative periorbital swelling (Swoboda and Ullrich 1992; Levin et al. 1996). The presenting complaints in chronic disease may be nonspecific. Chronic sino-orbital aspergillosis may present subacutely in older patients with diabetes mellitus or pulmonary diseases, and in those receiving immunosuppressive drugs. Some presentations of orbital aspergillosis, such as optic nerve involvement, may lead to the use of systemic corticosteroids, delaying diagnosis and, possibly, potentiating the infectious process. In neutropenic, or otherwise immunocompromised patients, a high index of suspicion should be maintained to forestall the emergence of fulminant aspergillosis (Levin et al. 1996). For diagnosis of orbital aspergilloma, aspiration cytology and immunohistochemistry have been described (Oneson et al. 1988).

Management of invasive sino-orbital aspergillosis, as has been reported in patients with AIDS (Vitale et al. 1992; Meyer et al. 1994; Kronish et al. 1996; Johnson et al. 1999), revolves around aggressive surgical debridement and the use of intravenous amphotericin B. In spite of these measures, the prognosis is very poor, and mortality may be as high as 75–80 percent (Kronish et al. 1996; Johnson et al. 1999), primarily due to intracranial extension of the disease process. Most patients succumb to their illness within a few months. However, the survival of a patient with invasive aspergillosis involving the orbit, paranasal sinus, and central nervous system for more than 9 years has recently been reported (Kusaka et al. 2003). The prognosis of this disease depends on the location and duration of the infection and the patient's immunological status, with sphenoidal aspergillosis being particularly aggressive in nature due to its close relation to the skull base. The use of protease inhibitors may prolong survival in some of these patients (Johnson et al. 1999). Fortunately, therapy of the more localized forms of orbital aspergillosis carries a better prognosis. In such cases, conservative orbital debridement (for example, resection of an orbital abscess) with amphotericin B administered intravenously and locally (by means of an indwelling cathether or intraorbital injection), may be effective, especially in patients with reversible immunosuppression and good preoperative visual acuities (Harris and Will 1989; Cahill et al. 1994; Seiff et al. 1999). Rieske et al. (1998) described the use of amphotericin B colloidal dispersion in a child who had sino-orbital aspergillosis. Massry et al. (1996) reported successful resolution of sino-orbital aspergillosis following initiation of itraconazole treatment, without recurrence at 10 months follow-up, in an immunocompetent patient in whom traditional therapeutic modalities (surgical debridement and amphotericin B therapy) had not resulted in resolution; thus, oral itraconazole could be considered as a treatment option in orbital aspergillosis occurring in immunocompetent patients who have recurrent or recalcitrant disease, or in those who cannot tolerate amphotericin B.

CHRONIC RHINOCEREBRAL ZYGOMYCOSIS

In the chronic presentation of rhinocerebral zygomycosis, which is less common than the acute form, the disease is indolent and slowly progressive over weeks to months. Harril et al. (1996), who described two patients with chronic rhinocerebral zygomycosis treated at their institution and reviewed 16 other cases reported in the English-language literature, observed that the median time from onset of symptoms to diagnosis was 7 months; the most common presenting features of chronic rhinocerebral zygomycosis were ophthalmological and included ptosis, proptosis, visual loss, and ophthalmoplegia. This entity was found to occur predominantly in patients with diabetes and ketoacidosis. The incidence of internal carotid artery and cavernous sinus thrombosis was higher in patients with chronic rhinocerebral zygomycosis than in those with the acute disease, although the overall survival rate for the former was 83 percent.

CHRONIC ORBITAL INFECTIONS DUE TO OTHER FUNGI

Other fungi implicated as causes of chronic invasive orbital infection include *Paecilomyces lilacinus*, *Scedosporium apiospermum*, *Bipolaris spicifera*, and *Bipolaris hawaiiensis*, other genera and species of hyaline and dematiaceous fungi, and dimorphic fungi, such as *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum* var. *duboisii* (Olurin et al. 1969; Streeten et al. 1974; Vida and Moel 1974; Agrawal et al. 1979; Nunery et al. 1985; Jay et al. 1988; Maskin et al. 1989; McGuire et al. 1991; Jacobson et al. 1992). The patients may present with nasal congestion, postnasal drip, and chronic sinus pain, citing frequent bouts of acute sinusitis (Washburn et al. 1988). There may be slowly progressive unilateral proptosis with decreased vision. In *Bipolaris* infections, impairment of extraocular motility and proptosis appear to occur more frequently than visual loss (Jacobson et al. 1992). A mass may be seen originating from an adjacent paranasal sinus, with sclerotic margins, and producing an inflammatory reaction in the orbit (Washburn et al. 1988). Other orbital inflammatory signs may be absent. Histologically, this form is characterized by granulomatous inflammation and tissue invasion. Although the prognosis in this variety of disease is certainly much better than that in acute fulminant disease, intraorbital and intracranial extension may occur, leading to significant morbidity (Klapper et al. 1997). Treatment is surgical debridement and systemic antifungal therapy; antifungals other than amphotericin B may play an important role in such infections.

Chronic localized noninvasive fungal infections

Chronic, localized noninvasive fungal infections or 'fungus balls' tend to occur in normal anatomic cavities

(e.g. paranasal sinuses) or cavitations induced by disease (pulmonary cavities due to tuberculosis). An example of such 'fungus balls' is the aspergilloma, which may occur in a paranasal sinus or in a lung cavity (Klapper et al. 1997; Latgé 1999). Histologically, it manifests as a non-invasive, tightly packed fungal mycelium or 'fungus ball' (mycetoma) (Latgé 1999). The normal orbital cavity is filled with the eyeball and ocular adnexa, and surrounding orbital fat. Hence, a fungus ball can occur only where orbital exenteration (removal of the entire contents of the orbit) has been performed. Levin et al. (1996) described the occurrence of *Aspergillus* infection in an exenterated socket. This type of 'fungus ball' may also occur in the orbital prosthesis used to fill an exenterated socket. Oestreicher et al. (1999) described the occurrence of a fungal abscess (subsequently shown by histopathology to be filled with fungal hyphae having the morphology of *Aspergillus* spp.) within an hydroxyapatite orbital implant 58 months after uncomplicated implant surgery; the patient's symptoms resolved following removal of the implant.

Allergic fungal rhinosinusitis with orbital involvement

The name 'allergic *Aspergillus* sinusitis' was proposed in 1983 (Katzenstein et al. 1983) to describe a newly recognized form of chronic sinusitis, but this entity is now called 'allergic fungal sinusitis' or 'allergic fungal rhinosinusitis' since many genera of dematiaceous fungi (*Bipolaris*, *Exserohilum*, *Curvularia*, and *Alternaria*) are now believed to be responsible for many cases (Adam et al. 1986; Brummund et al. 1986; Klapper et al. 1997; Ferguson 1998). This condition occurs in immunocompetent, atopic individuals. Here, the fungus does not invade the surrounding tissue, but serves as an 'allergen,' resulting in the formation of a thick ('peanut butter-like') tenacious, green or brown 'allergic' mucin. The patients may give a history of nasal polyposis or recurrent chronic sinusitis. Symptoms described include nasal obstruction, local pain, rhinorrhea, visual loss, diplopia, proptosis, epiphora, cranial nerve palsies, and facial deformity. Up to 17 percent of patients with allergic fungal rhinosinusitis may experience orbital symptoms (Klapper et al. 1997). Allergic fungal rhinosinusitis is a unique subset of fungal sino-orbital disease which differs from invasive sino-orbital disease in many respects. Key elements to the diagnosis of this condition include a predominantly eosinophilic response of the sinus mucosa, the presence of the characteristic mucoid exudate containing fungal hyphae (Figure 10b), peripheral blood eosinophilia, serum precipitins against the fungus involved, elevated serum total and fungus-specific IgE and IgG concentrations, and an immediate hypersensitivity response to the concerned fungal antigen (Brummund et al. 1986). Patients with allergic fungal rhinosinusitis generally have high levels of total IgE and IgE-mediated atopy to multiple fungal and nonfungal antigens; hence, immunotherapy has now been advised for such patients since it appears to significantly alter the clinical course of these patients, sparing them from repeated surgery and the use of systemic corticosteroids (Mabry and Mabry 1998). It has been advised that patients with allergic fungal rhinosinusitis should receive:

- conservative yet thorough surgical exenteration of the lesion by endoscopic techniques
- adjuvant therapy using perioperative systemic corticosteroids and topical corticosteroids as the patients heal and before beginning immunotherapy
- irrigation and debridement as necessary after surgery
- immunotherapy with all relevant fungal and nonfungal antigens to which they are found to be allergic about a month or so following surgery (Ferguson 1998; Mabry and Mabry 1998).

Allergic fungal rhinosinusitis does not require aggressive surgical debridement or intravenous amphotericin B.

MYCOTIC INFECTIONS OF EYELIDS

Although infections of the eyelids are caused most frequently by bacteria (particularly *Staphylococcus* species), fungi may also cause superficial or deep eyelid lesions.

Types

Necrotizing fasciitis of the eyelids and periorbital area due to *Cryptococcus neoformans* was reported in a young man after a trivial trauma by a wooden splinter (Doorenbos-Bot et al. 1990), while an eyelid nodule and ulcerative lesions have been reported to constitute a sentinel lesion of disseminated cryptococcosis in a patient with AIDS (Coccia et al. 1999).

Use of broad-spectrum antibacterials or immunosuppressive agents may predispose to spread of infection from a focus, resulting in the occurrence of an eyelid lesion due to a *Candida* sp. (Alvarez and Tabbara 1996). Ulceration begins at the base of an eyelash; small granulomata appear at its edge, and vesicles and pustules may also be present. In a study of 407 patients with chronic severe ulcerative blepharitis, Huber-Spitzy et al. (1991) noted the occurrence of positive cultures for *Candida* spp. in 47 (12 percent); most of these patients also had atopic dermatitis.

Malassezia spp. (formerly *Pityrosporum orbiculare* and *Pityrosporum ovale*) (Ashbee and Evans 2002) are superficial fungi that may be associated with seborrheic blepharitis and pityriasis versicolor, a chronic mild skin infection sometimes found around the eyebrows and eyelids. When lid scrapings from 40 patients with active seborrheic or mixed seborrheic/staphylococcal

blepharitis were subjected to microbiological investigations, yeasts with the morphology of *Malassezia* spp. were detected by direct microscopic examination in the scrapings of 39 of the 40 patients, while fungi were isolated from those of about half the patients (Nelson et al. 1990).

Lesions caused by the dermatophytes begin as erythematous scaly papules that slowly enlarge; healing simultaneously occurs in the central, paler area. Infections caused by species of *Microsporum* and *Trichophyton* result in the hair breaking at the level of the skin surface.

Although the eyelid is generally considered to be the most common site of ocular infection due to *Blastomyces dermatitidis* (Safneck et al. 1990; Slack et al. 1992), Bartley (1995) reported that only one of 79 patients with systemic blastomycosis seen by him had such lesions. The lesions may arise due to contiguous spread from facial lesions, or due to hematogenous dissemination from a pulmonary lesion. Small abscesses may be visible around the eyelashes. These later form granulomatous ulcers with thick crusts and an underlying purplish discoloration of the skin. Healing of the lesions may lead to severe cicatrization and ectropion formation (Slack et al. 1992). Conjunctival lesions generally occur due to contiguous spread from eyelid lesions, but may also occur as separate entities (Slack et al. 1992).

Eyelid lesions, alone or in association with corneal and conjunctival lesions, have been found to occur in more than 50 percent of reported cases of ocular paracoccidioidomycosis (Belfort et al. 1975; Silva et al. 1988; Burnier and Sant' Anna 1997). The palpebral lesion starts as a papule, usually close to the lid border, and grows and ulcerates in the center. The base of the ulceration reveals fine hemorrhagic punctate and elevated, thickened and hardened borders. The lesions evolve toward palpebral coloboma, with loss of the eyelashes.

Rhinosporidiosis of the lid margins is a rare occurrence (Reidy et al. 1997).

Diagnosis

Infections due to dermatophytes may exhibit fluorescence when exposed to a Wood's lamp. The hyphae or yeast cells of fungi causing eyelid lesions can be demonstrated by examination of a 10 percent potassium hydroxide mount or a Gram-stained smear of scrapes from the eyelids.

Therapy

Several therapeutic regimens have been recommended for mycoses of the eyelids (Alvarez and Tabbara 1996; Behrens-Baumann 1999), but the basis on which these suggestions have been made is not clear.

For infections due to the dermatophytes, terbinafine, itraconazole, or local antifungal drugs (active ingredients being combinations of fatty acids and salicylic acid) probably suffice (Ellis 1985). In one patient, lesions of the eyebrow due to *Trichophyton rubrum* were found to have disappeared almost entirely after 3 weeks of oral itraconazole therapy (Hiruma et al. 1991).

Nelson et al. (1990) reported that when topical 2 percent ketoconazole cream with lid hygiene was used to treat seborrheic blepharitis (due to *Malassezia* spp.) and mixed seborrheic/staphylococcal blepharitis, patients receiving ketoconazole more frequently had normal or markedly improved lids after treatment than did a control group of patients receiving only placebo and lid hygiene.

For infections due to *Candida* spp., topical agents, including ointments of nystatin (100 000 units/g), ketoconazole (2 percent cream), miconazole (2 percent), clotrimazole (1 percent) or econazole (1 percent), and oral ketoconazole (200–400 mg/day) are reportedly effective (Ellis 1985), while oral fluconazole (150 mg) once or twice weekly for 2–3 months has also been reported to be useful for such infections(Alvarez and Tabbara 1996). Oral ketoconazole twice daily (100 mg/day) with topical miconazole ointment for 6 weeks has been recommended for treatment of blepharitis due to *Candida* spp. (Huber-Spitzy et al. 1991).

Lesions of the eyelid in a patient with disseminated infection due to *Cryptococcus neoformans* var. *neoformans* was controlled (although there were occasional recurrences) by a combination of surgical excision and intravenous amphotericin B (Coccia et al. 1999).

Verrucous lesions of the eyelid due to *Blastomyces dermatitidis* were reported to resolve with a combination of antifungals (potassium iodide, intravenous amphotericin B) and surgery (Barr and Gamel 1986); oral fluconazole (150 mg) once or twice weekly for 2–3 months has also been reported to be useful (Alvarez and Tabbara 1996). Itraconazole is an alternative.

Papular lesions of the eyelids due to *Paracoccidioides brasiliensis* were reported to respond to intravenous amphotericin B, alone or in combination with oral ketoconazole, without any surgery being required (Silva et al. 1988).

Lesions of the eyelid due to *Rhinosporidium seeberi*, which are rare, require excision.

Deep infections of the eyelids, which may occur in infections due to the thermally dimorphic fungi or in infections due to species of *Aspergillus*, *Candida*, or *Cryptococcus*, must be treated with drugs administered systemically; intravenous amphotericin B is usually the treatment of choice, although oral ketoconazole, itraconazole, or fluconazole may be considered in milder infections (Ellis 1985; Grant and Clissold 1989; Alvarez and Tabbara 1996). In one patient, preseptal cellulitis

due to a *Trichophyton* spp. was described to have completely resolved following two courses of oral itraconazole (100 mg/day) (Velazquez et al. 2002).

MYCOTIC DACRYOCANALICULITIS

Fungi, such as *Alternaria* spp., *Aspergillus fumigatus* and other *Aspergillus* spp., *Candida* spp., dermatophytes, *Fusarium* spp., *Penicillium* spp., *Scopulariopsis* spp., and *Sporothrix schenckii*, have been reported as causes of canaliculitis (Levin et al. 1996; Behrens-Baumann 1999), although the significance of some of these isolates is doubtful since they do not appear to satisfy the criteria described earlier (McGinnis 1980). Most of the clinical features described, as well as the surgical procedures recommended, are for dacryocanaliculitis in general, and are not necessarily specific for fungal infection.

Dacryocanaliculitis usually manifests as persistent unilateral epiphora (watering), with an itching sensation; there may be associated unilateral mucopurulent conjunctivitis. The eyelid is usually red and swollen in the area of the affected canaliculus, with associated unilateral conjunctivitis (conjunctival follicles may be present); reddening and swelling of the canaliculus itself (the opening is dilated and the edges are elevated and inflamed); a mucopurulent discharge and white, yellow, or brown concretions (dacryoliths) in the lacrimal punctum may also be observed (Vecsei et al. 1994). The remainder of the lacrimal passages is patent, and there is no preauricular lymphadenopathy. Local environmental factors in the canaliculus, such as stasis arising out of congenital diverticula, may predispose to these infections.

Pavilack and Frueh (1992) underscored the importance of thorough curettage as the most effective treatment for chronic canaliculitis, although their recommendations were not based on the study of specific actinomycotic or fungal causes of canaliculitis. Essentially, following topical and local anesthesia, the punctum is dilated and a small curette is introduced. If there is extensive ectasia and retention of concretions in diverticuli, a canaliculotomy may be necessary (Behrens-Baumann 1999). Following curettage (Pavilack and Frueh 1992), the canaliculus is thoroughly irrigated to remove any remaining fragments, identify unrecognized pockets of retained debris, and ensure that the distal part of the drainage system is patent.

Mycotic dacryocanaliculitis is reported to be usually satisfactorily treated by topical administration of 5 percent natamycin or by topical application and local syringing of the canaliculi and sac with amphotericin B solution (1.5–8 mg/ml) or nystatin solution (25 000–100 000 units/ml) (Wilson and Ajello 1998; Behrens-Baumann 1999); however, the basis for these recommendations is not clear. If medical therapy fails, surgery (canaliculotomy) is required. All material removed is stained and cultured, and the canaliculus is then syringed with the medications. Silicone intubation may be required for reconstruction of the canaliculus.

In a study on 40 patients with canaliculitis, Vecsei et al. (1994) reported that only 10 percent were cured by medical treatment alone and that 40 percent showed a recurrence; by contrast, 80 percent of patients who underwent canaliculotomy were cured. These results suggest that surgical treatment of canaliculitis, in combination with medical therapy, may yield better results than medical therapy alone.

MYCOTIC DACRYOCYSTITIS

Dacryocystitis, which is an infection of the lacrimal sac (Brook and Frazier 1998), is the most common infection of the entire lacrimal apparatus, and generally arises due to the stasis accompanying obstruction of the nasolacrimal duct; there are several possible causes (Behrens-Baumann 1999). Dacryocystitis may be acute or chronic, but fungi usually do not cause acute dacryocystitis. Chronic dacryocystitis usually arises following partial or complete obstruction at a single site within the lacrimal sac or within the nasolacrimal duct; infection generally follows obstruction, and does not cause it (Kalavathy et al. 1986).

Bacteria (especially aerobic and facultative anaerobic bacteria) may be the etiologic agents of infection in more than 90 percent of patients with acquired dacryocystitis, fungi accounting for only about 5 percent of infections (Kalavathy et al. 1986; Brook and Frazier 1998). However, in congenital dacryocystitis, fungi may account for almost 14 percent of infections (Ghose and Mahajan 1990). The genera of fungi that have been implicated as causes of dacryocystitis include *Acremonium* spp., *Aspergillus* spp., *Candida* sp., *Paecilomyces* spp., *Rhinosporidium seeberi*, dermatophytes, and *Sporothrix schenckii* (Henig et al. 1973; Kalavathy et al. 1986; Ghose and Mahajan 1990; Purgason et al. 1992; Levin et al. 1996; Reidy et al. 1997; Kristinsson and Sigurdsson 1998; Wilson and Ajello 1998; Behrens-Baumann 1999); however, the significance of some of these species as actual causes of dacryocystitis is uncertain, if one analyzes the descriptions per the criteria described earlier (McGinnis 1980). Infections due to *Sporothrix schenckii* and *Acremonium* spp. are reported to manifest as chronic suppurative dacryocystitis, with preauricular and submaxillary lymphadenitis and possible abscess formation. On the other hand, species of *Aspergillus*, *Candida* and *Paecilomyces*, *Rhinosporidium seeberi*, and the dermatophytes may cause chronic granulomatous dacryocystitis (Purgason et al. 1992; Levin et al. 1996; Kalavathy et al. 1998; Behrens-Baumann 1999). With partial or complete obstruction of the nasolacrimal duct, a laminated concretion (dacryolith) may develop in the lacrimal sac.

While epiphora is frequently the only clinical finding in patients with chronic dacryocystitis, there may also be

lid edema, conjunctival infection, and a swelling in the medial canthus; pressure over the area usually results in a purulent discharge through the lower punctum (Kalavathy et al. 1986). Kristinsson and Sigurdsson (1998) described a patient in whom *Aspergillus fumigatus* caused plugging of the lacrimal sac, leading to extreme tenderness of the lacrimal sac, epiphora, and discharge from the lacrimal punctum. Krishnan et al. (1986) described the occurrence of a diverticulum of the lacrimal sac in association with rhinosporidiosis while, more recently, Kalavathy et al. (1998) described rhinosporidiosis of the lacrimal sac in two patients in whom bloodstained epiphora (due to the extreme fragility of the lesion) was the presenting complaint.

Management of this condition is by dacryocystectomy (for rhinosporidiosis), where the lacrimal sac is removed in toto, or dacryocystorhinostomy, where the patency of the nasolacrimal duct is restored; dacryoliths, if present, should be surgically removed and dacryocystorhinostomy performed. Chronic mycotic dacryocystitis in six patients was found to resolve completely following dacryocystectomy (Kalavathy et al. 1986), while some patients with congenital mycotic dacryocystitis were reported to respond satisfactorily to topical antifungals, probing, and syringing (Ghose and Mahajan 1990). Dacryocystitis due to *Candida albicans* was reported to resolve completely following surgery, alone or in combination with topical miconazole and natamycin therapy (Purgason et al. 1992). Dacryocystitis following lacrimal sac plugging due to *Aspergillus fumigatus* was relieved by the simple procedure of removing the plug after opening the lacrimal sac; dacryocystorhinostomy was not performed, and the patient was symptom-free one year after the procedure (Kristinsson and Sigurdsson 1998). Recently, minimally invasive, transnasal dacryocystorhinostomy surgery employing endoscopic and laser technologies has been introduced. Postoperative infection could probably be reduced by intraoperative or postoperative antifungal therapy.

MYCOTIC DACRYOADENITIS

Acute fungal infections of the lacrimal gland are rare; zygomycetes can cause a necrotizing dacryoadenitis in the setting of contiguous sino-orbital disease(Behrens-Baumann 1999). Chronic granulomatous dacryoadenitis can be caused by certain filamentous fungi (Wilson and Ajello 1998; Behrens-Baumann 1999). Such an infection is treated by using systemic antifungal agents.

MYCOTIC CONJUNCTIVITIS

Under normal circumstances, fungi are transient inhabitants of the normal conjunctival sac (Williamson et al. 1968; Brinser and Burd 1986). However, the unrestricted use of topical antibacterials or corticosteroids may predispose to fungal infection (Williamson et al. 1968; Ben Ezra 1994). Acute conjunctivitis due to fungi appears to be uncommon. In a microbiological study of 102 patients with clinically diagnosed acute conjunctivitis (Boralkar et al. 1989), fungi were isolated from only 14 samples, and here also the criteria used to define significant growth of fungi from these 14 samples were not stated. Severe necrotizing granulomatous conjunctivitis due to *Coccidioides immitis* has been described in a patient who had received aggressive treatment with corticosteroids by various routes (Maguire et al. 1994).

The clinical manifestations of mycotic conjunctivitis are described as being dependent on the fungi involved (Ben Ezra 1994; Behrens-Baumann 1999), but the basis for these observations is unclear. The manifestations described include:

- simple acute or subacute superficial epithelial conjunctivitis due to *Candida* spp. (purulent infection), dermatophytic fungi (chronic lesions) and *Malassezia* spp. (catarrhal conjunctivitis)
- nodular conjunctivitis, with associated deep lesions and local lymphadenopathy due to *Sporothrix schenckii*
- follicular conjunctivitis due to *Blastomyces dermatitidis*, *Coccidioides immitis*, or *Paracoccidioides brasiliensis*, or chronic conjunctivitis with black conjunctival secretions due to *Aspergillus niger*.

Treatment of this condition can be difficult. Local therapy with topical antifungals may suffice if only the conjunctiva is involved, but systemic therapy is also needed if the eyelids are involved (Ellis 1985). In one patient, severe necrotizing granulomatous conjunctivitis due to *Coccidioides immitis* was finally controlled only after aggressive debridement of the affected area and months of topical amphotericin B and oral fluconazole therapy (Maguire et al. 1994).

Conjunctival rhinosporidiosis

Most reported ocular lesions due to rhinosporidiosis have occurred in hot, dry, climatic regions, with the occasional case from temperate zones (Reidy et al. 1997). While nasal lesions predominate in endemic areas, a predominance of ocular lesions is believed to indicate an epidemic (Vukovic et al. 1995). Ocular rhinosporidiosis most frequently manifests as single or multiple polypoidal outgrowths of the palpebral conjunctiva; these are pink or red, granular or lobulated (occasionally flattened), sessile or stalked, and attached to the upper or lower fornix or the tarsal conjunctiva (Ukety et al. 1992; Gaines et al. 1996; Pe'er et al. 1996; Reidy et al. 1997; Shrestha et al. 1998). Conjunctival rhinosporidiosis with associated scleral melting and staphyloma formation, a rare occurrence, was recently reported in three patients in India, with the lesions manifesting as gray–white spherules without polyps (Castelino et al.

2000). The occurrence of rhinosporidiosis of the lid margins, canaliculus, and lacrimal sac has been described earlier.

Rhinosporidial lesions of the eye are usually unilateral and solitary, and cause no discomfort to the patient; however, there may be increased lacrimation, discharge, tenderness of the lids, and photophobia. A clinical diagnosis of ocular rhinosporidiosis is justified if there are similar, extremely friable lesions elsewhere in the body presenting as small, white dot-like structures against a red background (the cysts embedded in the vascular tissue bed). However, a focal lesion on the conjunctiva, eyelid, or sclera needs to be differentiated from a cystic inclusion or adenoma of the various glandular structures, pterygium, pedunculated granuloma due to retained foreign body or end-stage chalazion (Prevost et al. 1980).

Since all attempts to cultivate *Rhinosporidium seeberi* in artificial culture media have been unsuccessful, histopathology is required to establish the diagnosis. The typical histological picture is that of a granuloma with marked inflammatory cell infiltrate (Rippon 1988), but a chronic nongranulomatous type of inflammation has also been described (Pe'er et al. 1996). All stages of the life cycle can be seen in excised tissue, from small trophocytes to large sporangia-containing sporoblasts. Hematoxylin and eosin staining is sufficient to demonstrate the distinctive spherical bodies (spherules or sporangia), which vary in size from 6 to 30 μm (Figure 9). The presence of such well-defined spherical bodies of varying size in a rather dense stroma covered by hyperplastic epithelium is a distinctive feature (Rippon 1988); however, these structures need to be differentiated from the spherules of *Coccidioides immitis*. Serological tests have not been found useful for the diagnosis of the condition.

No drug treatment has proven effective for ocular rhinosporidiosis. This condition is treated by surgical excision of the lesions.

MYCOTIC KERATITIS (KERATOMYCOSIS)

Frequency of occurrence

Mycotic keratitis, a suppurative, usually ulcerative, fungal infection of the cornea, may be responsible for more than 50 percent of all cases of ocular mycoses (Srinivasan et al. 1991) and of all patients with culture-proven microbial keratitis (Hagan et al. 1995), especially in tropical and subtropical environments. This condition apparently occurs more frequently in developing countries (e.g. India) than in the developed world (e.g. the USA). In one study in southern India, 139 patients with culture-proven mycotic keratitis were observed in a period of just 3 months (Srinivasan et al. 1997), whereas in the USA, 125 patients with culture-proven mycotic keratitis were seen over a 10-year period in southern Florida (Rosa et al. 1994) and just 24 such patients over a 9-year period at Philadelphia (Tanure et al. 2000). Although a high incidence of mycotic keratitis can be expected in countries with similar annual rainfall and temperature range, this is not always so and also appears to depend on the extent of urbanization (Houang et al. 2001).

Types

In terms of occurrence, risk factors, and therapeutic approaches, two basic types of this condition are commonly seen, namely, keratitis due to filamentous fungi and keratitis due to yeast-like and related fungi (Jones 1980); a third type, keratitis due to thermally dimorphic fungi such as *Blastomyces dermatitidis* (Rodrigues and Laibson 1973) and *Paracoccidioides brasiliensis* (Silva et al. 1988) occurs rarely. There appears to be a strong geographical influence on the occurrence of the different forms of mycotic keratitis. A review of the data from 39 studies on microbial keratitis or mycotic keratitis reported in the literature between 1976 and 2001 (23 of the studies had been performed in Asia, six in North America and five in Africa) revealed that the proportion of corneal ulcers caused by filamentous fungi tends to increase towards tropical latitudes, whereas in more temperate climates, fungal ulcers appear to be uncommon and to be more frequently associated with *Candida* spp. than filamentous fungi (Leck et al. 2002).

Filamentous fungi are the principal causes of mycotic keratitis in most parts of the world. In 29 of the 39 studies reported between 1976 and 2001, and in another two studies published in 2002 (Gopinathan et al. 2002; Leck et al. 2002), either *Aspergillus* spp. or *Fusarium* spp. were the commonest isolates. The high proportion of corneal infections caused by *Aspergillus* spp. in drier climates may be because spores of *Aspergillus* spp. can tolerate hot, dry weather conditions (Khairallah et al. 1992); however, *Aspergillus* spp. may also predominate in more temperate latitudes (Leck et al. 2002). Dematiaceous fungi, such as *Curvularia* spp., have been reported to be the third most important cause of keratitis in a number of studies (Srinivasan et al. 1997; Garg et al. 2000; Gopinathan et al. 2002; Leck et al. 2002).

Keratitis due to yeasts and yeast-like fungi is most frequently caused by *Candida albicans* (Liesegang and Forster 1980; Tanure et al. 2000); less frequent causes include *Candida parapsilosis*, *Candida tropicalis*, and *Cryptococcus neoformans* var. *neoformans* (see Table 3). Since *Candida albicans* is an ubiquitous commensal of mucous membranes in humans with no geographical dominance, keratitis due to this organism tends to occur more frequently in areas where traumatic keratitis is uncommon, but where other predisposing factors are important (Jones 1980). *Candida albicans* was

reported to be the commonest fungal species isolated from culture-proven mycotic keratitis in southern California (Ormerod et al. 1987) and in Philadelphia (Tanure et al. 2000), but species of *Candida* accounted for only 12.5 percent of isolates in culture-proven mycotic keratitis in Miami (Rosa et al. 1994). Species of *Candida* and related fungi have been infrequent isolates in most recent studies performed in tropical countries (Dunlop et al. 1994; Gonawardena et al. 1994; Hagan et al. 1995; Srinivasan et al. 1997; Gopinathan et al. 2002; Leck et al. 2002), possibly due to the predominance of livelihoods, such as agriculture, which carry a higher risk for occurrence of trauma-related keratitis caused by filamentous fungi than for keratitis due to *Candida albicans*.

Risk factors

Although the risk factors for microbial keratitis, in general, are well-known (Rosa et al. 1994; Tanure et al. 2000), there have been few attempts to identify specific risk factors for mycotic, as opposed to bacterial, keratitis. In one study in Bangladesh, 35 percent of patients with mycotic keratitis and 52 percent of patients with bacterial keratitis reported antecedent ocular trauma, while dacryocystitis was noted in 12 percent of bacterial and 4 percent of mycotic keratitis (Dunlop et al. 1994). The data derived from a retrospective case–control study in Singapore suggested that mycotic keratitis (principally due to *Fusarium* spp. and *Aspergillus* spp.) was frequently associated with mechanical ocular trauma, whereas bacterial keratitis (principally due to *Pseudomonas aeruginosa*) was more likely to be related to contact lens wear and preexisting ocular diseases; also, antecedent topical corticosteroid therapy (which is traditionally believed to be a specific risk factor for mycotic keratitis) appeared to predispose more frequently to bacterial (38 percent), than to fungal (25 percent), keratitis (Wong et al. 1997).

Certain conditions may predispose more frequently to filamentous fungal keratitis than to keratitis caused by yeast-like fungi. Filamentous fungal keratitis, especially that due to *Fusarium* spp. or *Aspergillus* spp., appears to occur most commonly in healthy young men engaged in agricultural work or outdoor occupations. Trauma is the principal risk factor in 44–55 percent of such patients; various traumatizing agents have been reported, including vegetable matter, mud or dust particles, paddy grain, the swish of a cow's tail, tree branches, and metallic foreign bodies. Prolonged use of topical corticosteroids or antibacterials, diabetes mellitus, preexisting ocular diseases, and contact lens wear, preexisting allergic conjunctivitis or vernal keratoconjunctivitis, and the occupation of onion harvesting, are less frequently encountered factors in filamentous fungal keratitis (Vajpayee et al. 1990; Rosa et al. 1994; Panda et al. 1997; Gupta et al. 1999; Lin et al. 1999; Xie et al. 2001; Gopinathan et al. 2002). Trauma and prior use of corticosteroids have also been noted to be the most frequent risk factors in keratitis due to *Curvularia* spp. (Wilhelmus and Jones 2001). Seasonal variations that have been observed in the incidence of filamentous fungal keratitis, and in the predominant genera of fungi isolated from such cases, have been linked to environmental factors, such as humidity, rainfall, and wind, and also to the harvest (Cuero 1980; Liesegang and Forster 1980; Thomas 1990; Rosa et al. 1994). By contrast, in a study at Philadephia (Tanure et al. 2000) in which *Candida albicans* was the most common isolate (46 percent), the three most common risk factors were found to be chronic ocular surface disease, contact lens wear, and use of topical corticosteroids. A case–control study to compare the relative contribution of different risk factors in determining whether a patient develops keratitis due to filamentous fungi, or that due to yeast or yeast-like fungi, would be of great value.

Diagnosis

A rapid and accurate diagnosis of mycotic keratitis improves the chances of a complete recovery. A detailed clinical history should first be obtained. This should include details of possible predisposing factors (trauma, use of contact lenses), prior therapy with antibacterials, corticosteroids or other compounds, and preexisting ocular disease (allergic conjunctivitis, lagophthalmos). The clinician should then look for ocular or systemic defects that may have predisposed to the keratitis, since these require correction. Symptoms are usually similar to those seen in other types of keratitis, but, perhaps, more prolonged in duration (5–10 days).

Filamentous fungal keratitis, which may involve any area of the cornea, usually presents with firm (sometimes dry) elevated necrotic slough (Figure 11a), 'hyphate' lines extending beyond the ulcer edge into the normal cornea, and multifocal granular (or feathery) gray-white 'satellite' stromal infiltrates (Figure 11b); an 'immune ring,' minimal cellular infiltration in the adjacent stroma, mild iritis, and an endothelial plaque and hypopyon may also occur. Rosa et al. (1994) observed irregular feathery margins in 62.4 percent, elevated borders (52 percent), dry rough texture (47 percent), satellite lesions (41 percent), and Descemet's folds in 40 percent of 125 patients with culture-proven mycotic keratitis. There may be variations from this pattern depending on the etiologic agent (Wilhelmus and Jones 2001). *Fusarium solani* usually causes a severe infection (Figure 11c), with perforation, deep extension, and malignant glaucoma supervening (Verma and Tuft 2002). Keratitis due to *Aspergillus* spp. is generally believed to be less severe than keratitis due to *Fusarium* (Thomas et al. 1988; Thomas and Rajasekaran 1988),

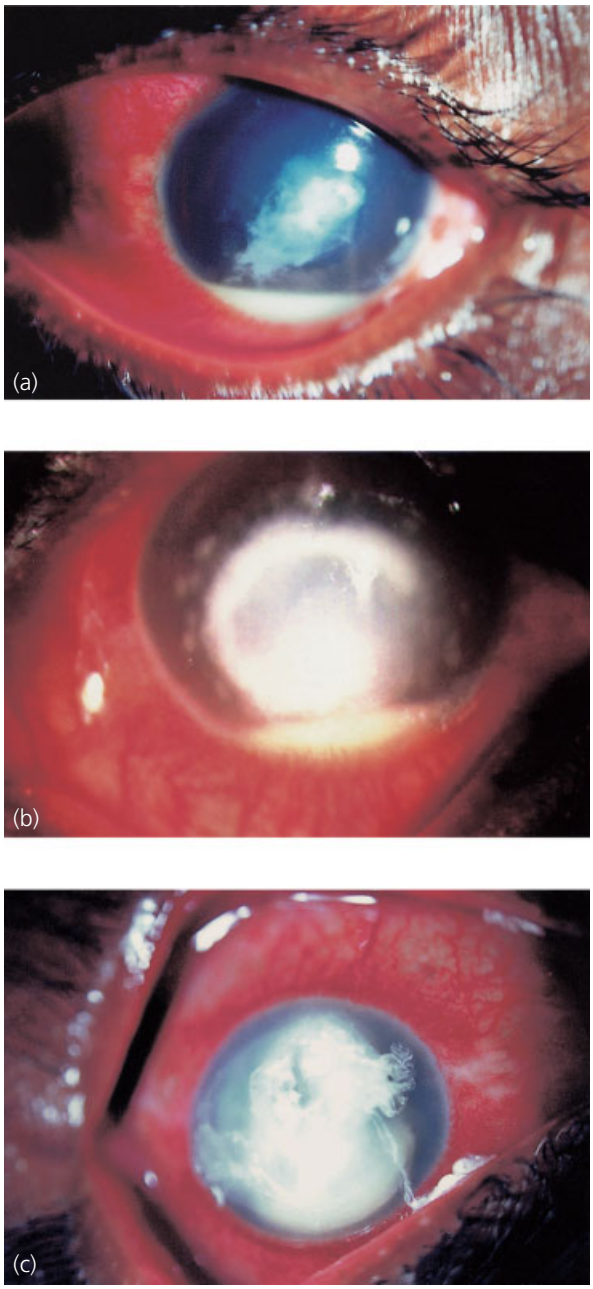

Figure 11 (a) *Mycotic keratitis due to* Fusarium solani *exhibiting elevated necrotic slough.* **(b)** *Mycotic keratitis due to* Aspergillus flavus *exhibiting 'satellite' lesions.* **(c)** *Severe mycotic keratitis due to a* Fusarium *sp.*

although one group of workers (Vemuganti et al. 2002) noted that a high percentage of patients with *Aspergillus* keratitis ultimately required therapeutic penetrating keratoplasty due to nonresponsiveness to medical therapy. In keratitis due to certain dematiaceous hyphomycetes (species of *Curvularia*, *Bipolaris*, or *Exserohilum*), a persistent, low-grade, smoldering keratitis, with minimal structural alteration and pigmentation, may occur (Garg et al. 2000). Fungi that have been found to cause severe forms of keratitis clinically, which respond poorly to medical therapy, include *Lasiodiplodia theobromae* (Borderie et al. 1997; Thomas et al. 1998), *Pythium insidiosum* (Virgile et al. 1993; Thianprasit et al. 1996), and *Scedosporium apiospermum* (*Pseudallescheria boydii*) (Zapater and Albesi 1979; Wu et al. 2002). Chronic, severe, filamentous fungal keratitis may resemble bacterial suppuration, and may involve the entire cornea (Klotz et al. 2000). The stromal keratitis caused by *Candida albicans* and related fungi resembles bacterial keratitis, with an overlying epithelial defect, a more discrete infiltrate, and slow progression; such ulcers frequently occur in eyes with preexisting corneal disease, and in areas of exposure, such as inferocentrally, at the junction of the superior two-thirds and inferior one-third of the cornea (O'Day and Burd 1994). Keratitis due to a zygomycete, such as *Rhizopus* spp. (Rosa et al. 1994), or *Absidia corymbifera* (Marshall et al. 1997), which is a rare occurrence, runs a very fulminant course and is unresponsive to medical therapy, so that penetrating keratoplasty is required within a few days of presentation.

The clinical suspicion of mycotic keratitis can sometimes be confirmed by noninvasive means using confocal microscopy, an imaging technique that allows optical sectioning of almost any material, with increased axial and lateral spatial resolution, and better image contrast than conventional slit-lamp microscopy. In clinical keratitis due to an *Aspergillus* sp., fungal hyphae were imaged as high-contrast filaments, 60–400 μm long, and 6 μm in width (Winchester et al. 1997), while in another patient with keratitis due to *Fusarium solani* (Florakis et al. 1997), in vivo scanning slit confocal microscopy helped to first establish the diagnosis, then to demonstrate progression of the disease, and finally to confirm the success of penetrating keratoplasty. This technique has been used, in conjunction with culture, in establishing a diagnosis of mycotic keratitis (Xie et al. 2001; Verma and Tuft 2002). The potential benefits of confocal microscopy in demonstrating the presence of fungal hyphae in vivo within the human cornea by noninvasive means need to be weighed against limitations such as limited resolution of the microscope, difficulty in performing serial examinations, and the lack of a distinctive morphology of some pathogens.

For the microbiological diagnosis of mycotic keratitis, corneal material (as scrapings or biopsy material) is the specimen of choice. Prior to performing a corneal scraping, it is generally recommended that material be collected from the ipsilateral and contralateral lid and conjunctiva (by sterile cotton-tipped or calcium alginate swabs) to ensure that the organisms isolated on the corneal media have not come from the transient commensal fungal flora of the lid margins or conjunctival sac (Jones et al. 1981). However, Sharma et al. (1994) have questioned the rationale for performing such cultures of swabs from the lids and conjunctivae, stating that in their experience, a correlation between

the corneal and conjunctival isolate occurred in only 3 percent of cases; these workers opined that processing of such samples might, in fact, provide misleading data.

Corneal scrapings are obtained by using an instrument (platinum spatula, Beaver blade, Bard-Parker knife no. 15, blunt cataract knife) to debride material from the base and edges of the ulcerated part of the cornea (Agrawal et al. 1994); this should be done several times to obtain as much material as possible. The blade or spatula may be reused if a sterile medium has been streaked, but must be changed (the spatula can be flamed) if the instrument has made contact with an unsterile slide (Agrawal et al. 1994). Although cotton-tipped swabs do not seem to be a useful means of debriding the necrotic corneal slough, calcium alginate swabs (premoistened with tryptone soy broth), when used for the debridement, may facilitate recovery of fungi in culture (Jacob et al. 1995).

If corneal scrapings do not yield positive results, a corneal biopsy may aid diagnosis since a greater quantum of tissue can be obtained from a greater depth of the cornea. A simple method is to perform free dissection of the corneal lamellae by a sharp surgical knife; here, corneal perforation is a distinct risk. Another method involves removal of the epithelium and necrotic debris overlying the suppurated area and incision of the corneal stroma by a Bard-Parker no. 15 blade and corneal forceps to about one half the corneal thickness (Ishibashi et al. 1987). A corneal trephine may be used to define the precise diameter and depth (0.2–0.3 mm) of corneal tissue that is to be removed (Lee and Green 1990; Kompa et al. 1999). The biopsies may be relatively superficial (keratectomy) or deep. The biopsied tissue may be stained by ink-potassium hydroxide (Ishibashi et al. 1987) or lactophenol cotton blue (Kompa et al. 1999). Several experimental studies have unequivocally highlighted the value of corneal biopsy samples in diagnosis of keratitis due to *Aspergillus fumigatus*, *Candida albicans*, or *Fusarium solani* (Ishibashi and Kaufman 1986b; Ishibashi et al. 1987). The results obtained in a clinical setting are less clearcut. There is anecdotal evidence, in small numbers of patients, to support the use of corneal biopsy samples in diagnosis of mycotic keratitis (Ishibashi and Kaufman 1986b; Brooks and Coster 1993; Rosa et al. 1994; Kompa et al. 1999). However, Lee and Green (1990) reported that only nine of 42 samples submitted from patients with clinically evident infectious ulcerative keratitis were positive for infectious agents (including the presence of fungi in three of the nine positive samples).

While direct microscopic examination of corneal scrapes or corneal biopsy samples permits a rapid presumptive diagnosis of mycotic keratitis, it is generally not considered possible to identify the fungal genus involved. However, the occurrence of adventitious sporulation (presence of conidial structures) in tissue samples, including corneal material, may aid in the differentiation of genera of hyaline filamentous fungi, such as *Acremonium*, *Fusarium*, and *Paecilomyces* (Liu et al. 1998). Examination of a wet preparation (using 10 percent potassium hydroxide, ink-potassium hydroxide, or lactophenol cotton blue), a smear stained by the Gram method (Figure 12a) or Giemsa method, and a smear stained by special fungal stains, such as methenamine silver, periodic acid–Schiff, calcofluor white (Figure 12b), may yield valuable results. The corneal material should be spread out as thinly as possible on the microscope slides to facilitate easy visualization of the fungal structures.

In culture-proven mycotic keratitis, sensitivities of 72.2 percent (Xie et al. 2001) to 91 percent (Gopinathan et al. 2002) for potassium hydroxide mounts, 31.6 percent (Panda et al. 1997) to 98 percent (Dunlop et al. 1994) for Gram-stained smears, 27 percent (Rosa et al. 1994) to 85 percent (Gopinathan et al. 2002) and 56 percent (Upadhyay et al. 1991) to 86 percent (Forster et al. 1976) for smears stained by Gomori methenamine silver, and 91.4 percent for calcofluor white-stained smears (Gopinathan et al. 2002) have been reported.

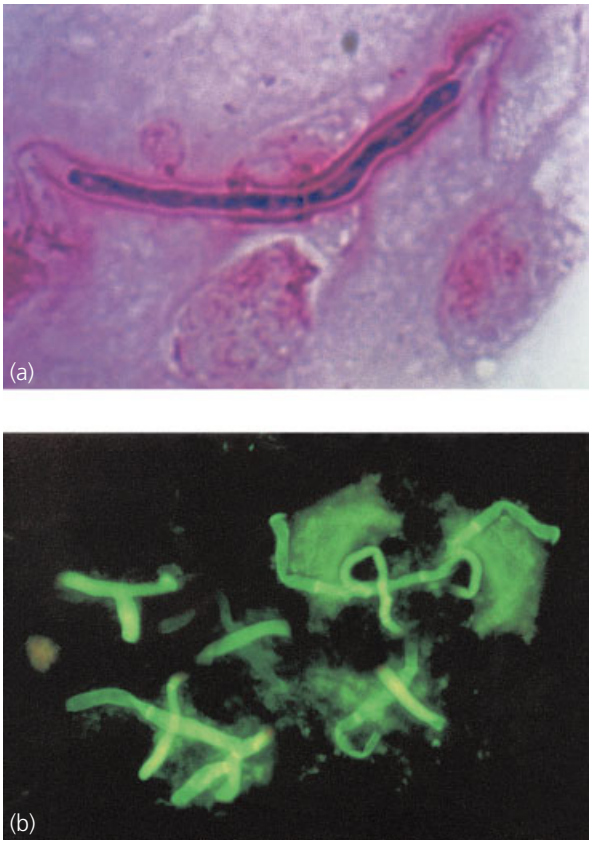

Figure 12 (a) *Photomicrograph of fungal hyphae in corneal scrape material. Cytoplasm is stained, cell wall and cross-walls are unstained. Gram stain; magnification ×1 200.*
(b) *Photomicrograph of fungal hyphae in corneal scrape material. Fungal hyphae fluoresce greenish-yellow against a dark background. Calcofluor white stain; magnification ×400*

Lactophenol cotton blue-stained mounts of corneal scrapes (Figure 5) have been found to yield positive results in about 78 percent of culture-proven mycotic keratitis (Thomas et al. 1991b; Sharma et al. 1998), while acridine orange-stained smears yielded positive results in 76 percent of clinically suspected cases of mycotic keratitis (Kanungo et al. 1991). A fluorescent Gram-stain technique permitted a rapid presumptive diagnosis of mycotic keratitis in five patients, with the diagnosis being confirmed by culture in all five (Roychoudhury et al. 1997); this stain also detected fungi in the vitreous biopsy of one patient with culture-proven *Aspergillus flavus* endophthalmitis. However, this staining technique has not acquired widespread use. When combined potassium hydroxide–calcofluor white staining was used to detect fungi in corneal scrapings from 114 patients with early keratitis, and 363 patients with advanced keratitis, a sensitivity of 61.1 percent and specificity of 99.0 percent in early keratitis, and a sensitivity of 87.7 percent and specificity of 83.7 percent in advanced keratitis was achieved; while the predictive values were high for potassium hydroxide–calcofluor white in the detection of fungus, this technique yielded false positives in 16.3 percent of patients with advanced keratitis (Sharma et al. 2002). All these results indicate that direct microscopic examination of corneal material permits a rapid presumptive diagnosis of mycotic keratitis, and correlates well with positive results in culture.

CULTURE

A diagnosis of mycotic keratitis is confirmed by isolation of fungi from corneal scrapes or biopsies which are inoculated onto the surface of solid media by making rows of 'C' streaks (two rows from each scraping), with only growth on the 'C' streaks being deemed significant (Figure 13), or by immersing the tip of the spatula, loop, or swab into broth (liquid) media. Commonly used culture media include Sabouraud glucose neopeptone agar (Emmons' modification, neutral pH), which is incubated at 25°C, 5 percent blood (preferably sheep blood) agar (25°C and 37°C), brain heart infusion broth (25°C), and thioglycollate broth (25–30°C) (O'Day and Burd 1994). Using these different media, fungi grew out in culture within 2 days in 54 percent, 3 days in 83 percent, and 1 week in 97 percent of patients with mycotic keratitis; 90 percent of scrapings yielded a positive initial culture (Rosa et al. 1994). Chocolate agar (Rosa et al. 1994), cystine tryptone agar (Thomas 1994), and rose bengal agar (P.A. Thomas, unpublished observations) are other media that can be used. Antibacterial compounds, such as chloramphenicol (40 μg/ml) or a penicillin–streptomycin combination, are usually incorporated in the media to suppress bacterial growth, but cycloheximide must never be used since it may suppress most ocular fungal pathogens (O'Day and Burd 1994). While some investigators(Upadhyay et al. 1991) advocate the use of liquid-shake cultures to facilitate recovery of ocular fungi, others (Srinivasan et al. 1997) discourage their use since equivocal results may occur. Although fungi are usually recovered in culture within 3–4 days, culture media may need to be kept for up to 4–6 weeks. 'Sham cultures' are maintained to ensure that there is no contamination, either from the environment or media, during sample collection. A fungal strain recovered in culture from corneal material is considered significant if isolated more than once, or in two or more media, or on multiple 'C' streaks of one medium with fungal hyphae or yeast cells detected by direct microscopic examination of the corneal material (Liesegang and Forster 1980; Agrawal et al. 1994).

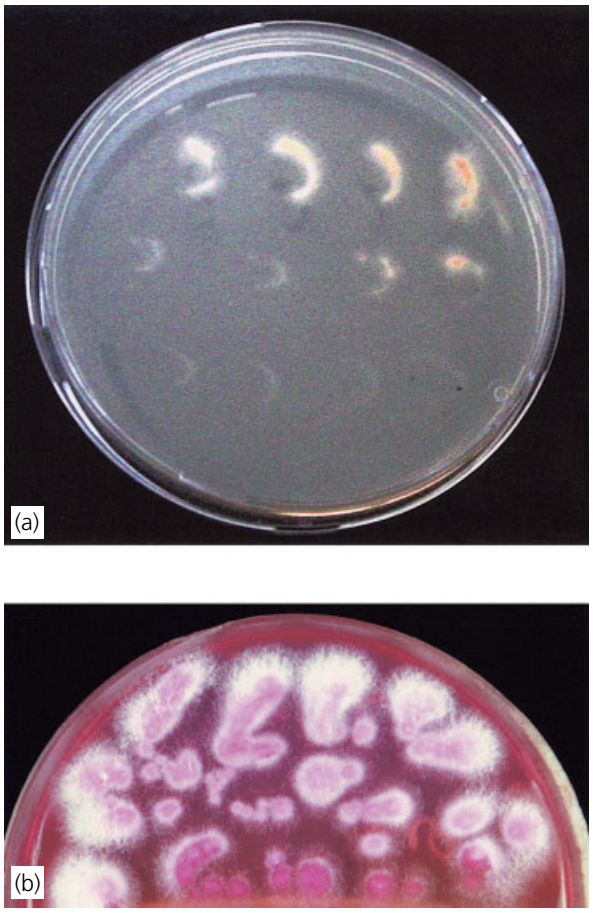

Figure 13 (a) *Twenty-four-hour growth of* Fusarium solani *on 'C' streaks of corneal scrape material on Sabouraud glucose neopeptone agar.* **(b)** *Forty-eight-hour growth of* Fusarium solani *on 'C' streaks of corneal scrape material on rose bengal agar.*

HISTOPATHOLOGICAL STUDIES

These offer certain advantages over culture, when used in the diagnosis of mycotic keratitis, since contamination is avoided, tissue penetration by fungi can be gauged, and the outcome of surgical procedures can be anticipated (Vemuganti et al. 2002). Some workers (Ishibashi et al. 1987; Rosa et al. 1994) believe that direct examination

of corneal biopsies or corneal buttons yields positive results more frequently than fungal cultures of the same specimens; others (Alexandrakis et al. 2000) believe the opposite. Corneal material for histopathology is obtained as a biopsy or button following penetrating keratoplasty. Most fungi can be satisfactorily stained and studied in tissue sections by light microscopy. Sections stained by hematoxylin and eosin demonstrate many salient details, but species of *Fusarium* or *Candida* may not be stained at all. Conversely, while fungi can be easily detected in sections of corneal tissue stained by the Gomori methenamine silver or periodic acid–Schiff stains (Vemuganti et al. 2002), little else can be visualized. Hence, a replicate tissue section stained with hematoxylin and eosin should always be examined before special stains for fungi are used; alternatively, a section stained by Gomori methenamine silver can be counterstained by hematoxylin and eosin to simultaneously demonstrate a mycotic agent and the evoked tissue response (Chandler 1991). The purulent inflammatory cellular reaction (mostly of lymphocytes and plasma cells, with variable involvement of polymorphonuclear leukocytes) is usually less intense in fungal than in bacterial keratitis; filamentous fungi are usually found deep in, and arranged parallel to, the corneal stromal lamellae, while being absent on the surface (Figure 14). Stromal abscesses arising from coagulative necrosis, 'satellite' microabscesses, focal necrosis of the corneal stroma, and clusters of acute inflammatory cells may also be seen (Agrawal et al. 1994). At this stage, healing of the epithelium is seen with a coexisting active proliferation of the fungus in the deeper stroma, which may explain why culture of corneal biopsies, where material is obtained from the depth of the stroma, yields results superior to culture of corneal scrapings (Ishibashi and Kaufman 1986b; Brooks and Coster 1993; Rosa et al. 1994; Kompa et al. 1999). Invasion and penetration of an apparently intact Descemet's membrane may occur rarely (Vemuganti et al. 2002), particularly when *Fusarium solani* or *Fusarium* spp. are the infecting organisms (Jones 1975; Kuriakose and Thomas 1991).

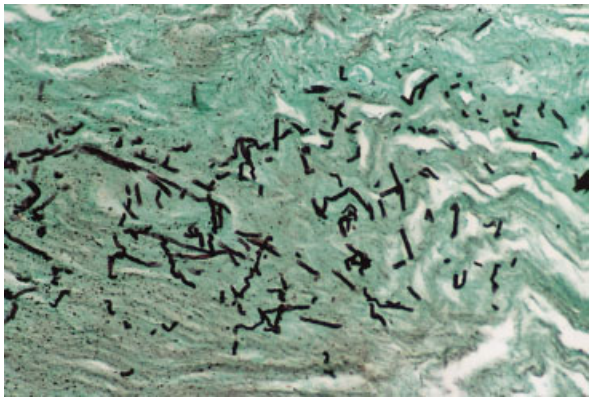

Figure 14 *Photomicrograph of a tissue section from a corneal button removed at the time of therapeutic penetrating keratoplasty. Fungal hyphae are seen in the middle and deep layers of the corneal stroma. The hyphae are oriented mostly in a direction parallel to that of the corneal collagen bundles. Gomori methenamine silver stain; magnification ×400*

Lectins, such as concanavalin A and wheat germ agglutinin, are ubiquitous proteins, found especially in plant seeds that bind specifically to carbohydrates. Fluorescein-conjugated concanavalin A provided consistently bright staining of fungal structures in corneal scrapes from 18 patients with culture-proven mycotic keratitis (Robin et al. 1989). A peroxidase-labeled wheat germ agglutinin staining technique for diagnosis of experimental keratitis due to *Candida albicans*, *Aspergillus fumigatus*, and *Fusarium solani* has recently been described (Garcia et al. 2002); this technique exhibited excellent sensitivities and specificities for all three infections, with a high degree of test–retest and inter-rater concordance between two independent observers. Such a sensitive and specific technique could prove useful in the clinical setting without the need for microscopes with attachments for fluorescence microscopy.

Fluorescence microscopy of a tissue section stained by hematoxylin and eosin revealed the presence of yeast cells of *Blastomyces dermatitidis* in periocular cutaneous lesions that had initially been misdiagnosed as squamous cell carcinoma (Margo and Bombardier 1985). Detection of fungi by direct immunofluorescence of formalin-fixed, paraffin-embedded ocular tissue sections has helped to confirm a presumptive histological diagnosis of ocular infection due to *Blastomyces dermatitidis* (Vida and Moel 1974), *Histoplasma capsulatum* var. *capsulatum* (Macher et al. 1985), *Sporothrix schenckii* (Font and Jakobiec 1976), *Pythium insidiosum* (Mendonza et al. 1996), and a zygomycete (Orgel and Cohen 1989). This technique can be used to detect a variety of dimorphic and hyaline filamentous fungi, even when these are present in small numbers or as atypical forms (Chandler 1991). Moreover, for retrospective studies, tissue sections previously stained by the hematoxylin and eosin, Giemsa, and modified Gram procedures can be decolorized in acid-alcohol and then restained with the specific reagents used for immunofluorescence (Chandler 1991). Performing immunofluorescence on a routine basis requires a microscope with attachments for fluorescence microscopy, antibodies of good quality, and standardization of the reagents and procedures used.

An impression debridement procedure has been described for corneal ulcers, although not specifically for mycotic keratitis (Arora and Singhvi 1994), where cellulose acetate filter paper (of the type used for conjunctival impression cytology) is applied with gentle pressure to the ulcerated part of the cornea; debridement is achieved with no trauma to the cornea since the necrotic material sticks to the filter paper, and this material can then be removed and stained. Degenerated epithelial and inflammatory cells and microorganisms can be seen

by microscopic examination of the necrotic material (Arora and Singhvi 1994).

Despite a clear clinical presentation of suppurative keratitis, traditional diagnostic laboratory methods, including microscopy and culture, may yield negative results in 31–50 percent of patients, possibly due to difficulties in obtaining sufficient corneal material from large, late-stage ulcers, due to the risk of perforation, and from early stage, small ulcers from which little material is available (Leck et al. 2002). Self-administration of antimicrobials by patients before seeking medical attention may compound the problem (Dunlop et al. 1994). This has led to an evaluation of the polymerase chain reaction (PCR) as an aid to the diagnosis of mycotic keratitis.

POLYMERASE CHAIN REACTION

This technique, which achieves enzymatic amplification of a specific sequence of DNA, can be used to detect the DNA of fastidious microorganisms which cannot be cultured easily or for which culture is prolonged (for example, in patients who have been on antimicrobial therapy). DNA can be extracted from minute quantities (1 μl) of intraocular fluid (aqueous or vitreous), tears, any fresh ocular tissue, formalin-fixed or paraffin-embedded tissue, and even stained or unstained cytology slides or tissue sections for analysis by the PCR (Rajeev and Biswas 1998). However, this technique can neither be used to monitor a patient's response to treatment, nor to distinguish viable from nonviable organisms. It may thus be difficult to assess the relevance of a positive PCR, especially in locations such as the conjunctival sac, where bacteria, and even fungi, may normally be found; similarly, PCR cannot differentiate between active and latent viral infection (Rajeev and Biswas 1998). A few culture media will suffice to detect and grow the common ocular pathogens, but PCR must be multiplexed for each microorganism that is suspected (the use of panfungal primers may alleviate this problem). PCR can detect only those organisms for which the DNA sequence and primers are known, and cannot provide details of cellular morphology or localization (Rajeev and Biswas 1998).

In ocular fungal infections, PCR has principally been used in the diagnosis of endophthalmitis due to *Candida* spp. (Okhravi et al. 1998; Hidalgo et al. 2000; Jaeger et al. 2000). However, this technique permitted a postmortem diagnosis of *Fusarium* panophthalmitis in one patient (Alexandrakis et al. 1996). When the PCR was applied to the diagnosis of experimental keratitis due to *Fusarium solani*, using primers directed against one part of the *Fusarium* cutinase gene, amplified target DNA sequences were noted in 89 percent of 28 samples from *Fusarium*-infected corneas, whereas only 21 percent of 14 samples from infected corneas were positive by culture. However, one of eight negative control samples was also positive by PCR (specificity of 88 percent for PCR) while none of the negative controls yielded growth in culture (100 percent specificity for culture) (Alexandrakis et al. 1998). When PCR was applied to the study of corneal scrapes from 30 patients with presumed microbial keratitis, using primers to target the 18S ribosome of fungi, PCR and culture yielded positive results for fungi in samples from 15 patients, and both techniques were negative in seven samples; however, PCR was positive for fungi with a negative culture in seven patients, while PCR was negative for fungi with a positive culture in one patient. Taking a positive fungus culture as the 'gold standard,' the PCR technique had a sensitivity of 94 percent and a specificity of 50 percent in this study (Gaudio et al. 2002). The specificity of this technique for diagnosis of mycotic keratitis requires further evaluation.

ANTIFUNGAL SUSCEPTIBILITY TESTING

Some investigators have reported that antifungal susceptibility testing aids the selection of the appropriate antifungal for successful treatment of mycotic keratitis (Jones 1975; Ishibashi et al. 1984) or orbital infections (Maskin et al. 1989; Massry et al. 1996). Unfortunately, many of these reports have not provided details of the test procedures used, nor have standardized techniques been followed. The future use of reproducible tests conforming to rigorous standards, such as the approved document (M27A) of the National Committee for Clinical Laboratory Standards (1997) for sensitivity testing of yeasts, and a standard method for susceptibility testing of filamentous fungi, especially *Aspergillus* spp., may clarify whether antifungal susceptibility testing is at all useful in guiding therapy of ophthalmic mycoses. Above all, the relationship between in vitro susceptibility data and clinical response to topical antifungal medication needs to be clarified; hitherto, no studies have been performed in this important area.

Management of mycotic keratitis

Mycotic keratitis is managed by medical or surgical means. Medical therapy consists of nonspecific measures, and the use of specific antifungal agents. Cycloplegics are used to relieve the iridocyclitis (anterior uveitis) that usually accompanies mycotic keratitis while broad-spectrum antibacterials may be needed to combat secondary bacterial infection (Rosa et al. 1994; Wong et al. 1997). Where access to specific antifungal drugs is limited, efforts have also been made to evaluate various antiseptics, such as polyhexamethylene biguanide (PHMB), chlorhexidine, and silver sulfadiazine in therapy of mycotic keratitis.

ANTISEPTICS FOR MYCOTIC KERATITIS

PHMB, which exhibits good in vitro activity against bacteria, fungi, and *Acanthamoeba*, has been used as a swimming pool disinfectant, sanitizer, and preservative in

topical ophthalmic preparations (Imayasu et al. 1992), and for treatment of *Acanthamoeba* keratitis at concentrations of 0.02–0.053 percent with no adverse effects (Duguid et al. 1997). PHMB 0.02 percent was found to significantly reduce fungal growth in a New Zealand white rabbit model of *Fusarium solani* keratitis, with 58 percent of PHMB-treated eyes exhibiting no growth compared to only 17 percent of placebo-treated eyes (Fiscella et al. 1997).

Chlorhexidine, a bisbiguanide antiseptic which inhibits microbial function by affecting the functioning of the cell membrane, therein leading to a leak of cell electrolytes, has notable bactericidal and amebicidal effects (Seal et al. 1996). When corneal isolates of fungi were tested against chlorhexidine, povidone-iodine, propamidine, PHMB, and econazole by a crude, nonstandardized in vitro method, chlorhexidine exhibited antifungal activity at high concentrations while povidone-iodine was active at all concentrations tested and econazole was the most effective of all (Martin et al. 1995–96). However, in an agar-dilution method of susceptibility testing, chlorhexidine gluconate did not exhibit notable in vitro activity against ocular isolates of *Aspergillus* and *Fusarium* even at concentrations of 64 μg/ml (P. A. Thomas, unpublished observations). In a masked, randomized study on consecutive patients with microscopy-positive (later proven to be culture-positive) mycotic keratitis, 0.2 percent chlorhexidine appeared to be more effective than 5 percent natamycin in patients who did not have prior antifungal treatment (Rehman et al. 1997). In another study on 71 patients in Bangladesh (Rehman et al. 1998), neither 0.2 percent chlorhexidine gluconate nor 2.5 percent natamycin proved effective for healing of severe ulcers at 21 days, while 66.7 percent of nonsevere ulcers treated with chlorhexidine, and 36 percent of those treated with natamycin, had healed at 21 days, a relative efficacy of 1.85 (Rehman et al. 1998). However, since only a 2.5 percent strength natamycin suspension was used, and not the conventional 5 percent concentration, the relative efficacy of chlorhexidine *vis-à-vis* natamycin was probably overestimated.

Silver sulfadiazine functions as an organic base-heavy metal release system by liberating silver. The silver component binds to microbial DNA, preventing unzipping of the helix and therein inhibiting the replication of microorganisms without interfering with epithelial cell regeneration. In a prospective, controlled, randomized, double-masked trial on therapy of mycotic keratitis (Mohan et al. 1988), a higher success rate was achieved with the use of 1 percent silver sulfadiazine ointment (80 percent), without significant ocular or systemic adverse effects, than with 1 percent miconazole ointment (55 percent); the response of *Aspergillus* keratitis was comparable in both groups but silver sulfadiazine was superior to miconazole in patients with *Fusarium* keratitis. However, these excellent results have not been corroborated by other workers.

SPECIFIC ANTIFUNGAL AGENTS

Medical therapy of mycotic keratitis is usually protracted since the effective concentrations achieved by most specific antifungal agents in the cornea, with the possible exception of amphotericin B, only inhibit growth of the fungus, and host defense mechanisms must eradicate the organism (Stern and Buttross 1991). The antifungal agents used in clinical practice can be classed as polyenes, pyrimidines, and azoles.

Polyenes

Amphotericin B exerts its antifungal effect by directly bonding to ergosterol, a sterol unique to fungal cell membranes, therein disrupting the structural integrity of the membrane (O' Brien 1999). This antifungal penetrates the deep corneal stroma after topical application, and its bioavailability is sufficient for susceptible fungi; it also exhibits immunoadjuvant properties (Johns and O'Day 1988). In a model of deep stromal infection due to *Candida albicans* in pigmented rabbits, amphotericin B 0.15 percent and 0.075 percent and natamycin 5 percent exhibited a significant antifungal effect when the cornea was debrided every day; when the epithelium was left intact, the efficacy was much reduced or negligible (O'Day et al. 1984a). In the same model, the efficacy of amphotericin B (0.15 percent and 0.5 percent) appeared unaffected when the antifungal was given with 1 percent prednisolone acetate (O'Day et al. 1984b). Amphotericin B methyl ester was also found to be very efficacious in this model (O'Day et al. 1984c). Since the initial report of Wood and Williford (1976), varying degrees of success have been reported when amphotericin B (0.15 to 0.3 percent) is used to treat clinical mycotic keratitis. Topical hourly applications of amphotericin B (0.15 percent) resulted in resolution of lesions in 56 percent of keratitis due to *Fusarium* and 27 percent of keratitis due to *Aspergillus* in Indian patients (Thomas and Rajasekaran 1988), while use of amphotericin B ointment resulted in resolution of keratitis due to *Aspergillus* spp. and *Fusarium* spp. (Hirose et al. 1997). Keratitis due to *Curvularia brachyspora* resolved with topical amphotericin B therapy alone (Marcus et al. 1992). An analysis of several reports (Wilhelmus and Robinson 1991; Rosa et al. 1994; Hemady 1995; Ainbinder et al. 1998; Lin et al. 1999; Tanure et al. 2000; Djalilian et al. 2001) reveals that 24 of 28 patients with keratitis due to *Candida albicans* and other *Candida* spp. responded to amphotericin B (15 patients to topical amphotericin B alone, one to intravenous amphotericin B alone, and eight to topical amphotericin B in combination with natamycin or systemic azoles). When collagen shields that had been soaked in 0.5 percent amphotericin B for 2 hours at 25°C before application were used in conjunction with amphotericin B 0.25 percent eye drops (applied every 2 hours), cultures from the eyes of three patients with *Aspergillus* keratitis

became negative within 15 days of treatment, although two patients subsequently required keratoplasty (Mendicute et al. 1995). Collagen shields, thus prepared and replaced daily, may deliver adequate concentrations of amphotericin B to the cornea with better tolerance than when conventional drops are used, therein improving the prognosis of *Aspergillus* keratitis. Although topical application of amphotericin B (in a desoxycholate vehicle) was found to prevent healing of epithelial defects in rabbit eyes, and to cause severe stromal edema and worsening iridocyclitis (Foster et al. 1981), the 0.15 percent solution prepared from the commercially available intravenous preparation appears to be well-tolerated (Tanure et al. 2000). Peripheral keratitis accompanying nasal rhinosporidiosis was found to resolve following topical administration of amphotericin B, prompting the suggestion that amphotericin B is an effective drug in this condition (Bhomaj et al. 2001). However, the peripheral keratitis in this patient is likely to have resolved due to the diagnostic corneal scraping that was performed, as well as the nasal polypectomy.

Natamycin (pimaricin), the first antifungal specifically developed for topical ophthalmic use, also binds to ergosterol to induce fungal cell membrane damage (O'Brien 1999). This drug is heat stable, permitting heat sterilization to be used, and the 5 percent topical ophthalmic suspension is well-tolerated, effective, and safe, although occasional punctate keratitis has been reported (Johns and O'Day 1988). Subconjunctival injection leads to conjunctival necrosis, and natamycin is not available for systemic administration due to its low aqueous solubility (O'Brien 1999). It has been suggested that natamycin does not penetrate into the deep corneal stroma after topical application. However, radiolabeling studies revealed that 13 topical applications of natamycin (one every 5 minutes) resulted in a drug concentration of approximately 2.5 mg/g of cornea, levels that were substantially higher than those following application of amphotericin B, although the levels achieved were far less in nondebrided corneas with intact epithelium (O'Day et al. 1986). About 2 percent of the total drug in the corneal tissue is bioavailable. Where commercially available, natamycin 5 percent is used as primary therapy for mycotic keratitis, particularly that due to filamentous fungi (Agrawal et al. 1994; Rosa et al. 1994; Panda et al. 1997), but interpretation of data relating to response to therapy has been difficult. In one series in Miami, Florida (Rosa et al. 1994), patients with presumably superficial keratitis due to *Fusarium* spp. received topical natamycin alone (average duration of treatment was 38 days), while those with presumably deep lesions received topical natamycin and systemic antifungals. Details of response to therapy were not provided, but 22 (28 percent) of 79 eyes with *Fusarium* keratitis ultimately required penetrating keratoplasty, and one eye required enucleation. It is uncertain whether the patients who did not require surgery ultimately responded to therapy with natamycin (with or without systemic antifungals). A recent report from the same institution (Dursun et al. 2003) provided data about 10 cases (out of a total of 159 patients with *Fusarium* keratitis) that progressed to endophthalmitis; combination therapy with oral azoles (fluconazole or ketoconazole) and topical natamycin appeared to be inadequate in severe *Fusarium* keratitis with intraocular spread. In the same center, four patients with keratitis due to *Candida* spp. were reported to have responded to topical natamycin therapy alone (Rosa et al. 1994). In a review of patients with *Curvularia* keratitis in Houston, Texas, it was noted that the corneal lesions resolved with topical natamycin therapy alone in 16 patients; in an additional seven patients, the lesions resolved following medical therapy with topical natamycin and other antifungals, and in another six patients, the keratitis resolved with topical natamycin, other antifungals, and surgery (Wilhelmus and Jones 2001). Variable results have been reported when topical natamycin has been used to treat keratitis due to *Scedosporium apiospermum* (reviewed by Wu et al. 2002).

Pyrimidines

Flucytosine (5-fluorocytosine), a low molecular weight, fluorinated cytosine analog that was first synthesized as a potential antineoplastic agent, exhibits moderate aqueous solubility, a high bioavailability, and achieves anterior chamber levels of 10–40 μg/ml after oral administration of 200 mg/kg body weight/day (Jones 1975). In spite of these advantages, flucytosine has limited use in ocular fungal infections. Since many strains of fungi have rapidly developed resistance to this drug, flucytosine should not be administered alone to treat mycotic keratitis or intraocular infections, even when these are caused by susceptible fungi (O'Brien 1999). Flucytosine also has a limited spectrum of activity against the commonest filamentous fungi implicated in ocular disease (Sutton et al. 1998). Since low intraocular levels of the drug are achieved after topical and subconjunctival administration, the use of oral doses of 50–150 mg/kg body weight/day or a topical 1 percent solution is recommended (O'Brien 1999).

Azoles

As a group, the azoles are potentially as effective as amphotericin B since they have a broad spectrum of activity, are generally less toxic, and can be administered by different routes. Since azoles bind to a cytochrome P450 fungal enzyme involved in the 14α-demethylation of either lanosterol or 25-methylene-dihydrolanosterol, ergosterol synthesis decreases and 14-methylated sterols accumulate, leading to increased membrane permeability, inhibition of growth, and alteration of membrane

enzymes, ultimately causing cell death, and also rapid accumulation of chitin over the entire cell wall (Vanden Bossche et al. 1993). All azoles, except for fluconazole, appear to decrease immune cell function, especially that of lymphocytes, thus affecting the degree of tissue damage occurring with the inflammatory reaction, but also affecting the efficacy of the azoles in vivo (Yamaguchi et al. 1993). In view of the limited concentrations of azoles that can be achieved in the eye, these drugs are to be considered as fungistatic in ocular fungal infections (Johns and O'Day 1988).

Clotrimazole is a trityl imidazole of low human toxicity with in vitro activity against species of *Aspergillus*, *Alternaria*, and *Candida*, but not against species of *Fusarium* or *Paecilomyces* (Jones 1975). Clotrimazole is poorly soluble in water, hence parenteral administration is not feasible. Following oral administration, satisfactory blood levels are reached during the first 2 weeks of therapy, but subsequently fall due to rapid induction of hepatic drug-metabolizing enzymes. Hence, clotrimazole is no longer recommended for oral administration (O'Brien 1999). Successful therapy of 16 patients with fungal keratitis (including eight due to various species of *Aspergillus*, and three due to *Candida albicans* and other *Candida* spp.) by using a commercial preparation of clotrimazole (Canesten, Bayer, Germany) was reported in 1976 (Jones et al. 1976). A topical 1 percent solution of clotrimazole in arachis oil was also found useful in therapy of superficial corneal infections (Jones et al. 1979). There are no recent reports of the use of clotrimazole in therapy of mycotic keratitis.

Econazole, a dichloroimidazole that is similar to miconazole in structure and mode of action, has a wide spectrum of activity against filamentous fungi in vitro, but is less effective than miconazole against *Candida* spp. These data may explain why econazole was found effective in treatment of keratitis due to various fungi, including species of *Fusarium* (Jones 1975; Jones et al. 1979; Arora et al. 1983). However, topical administration of a 1 percent econazole solution in polyethylene glycol 400 was not efficacious in experimental deep stromal *Candida albicans* keratitis in pigmented rabbits (O'Day et al. 1984a), irrespective of whether the corneal epithelium was intact or debrided. A recent study in India suggested that 2 percent econazole was as effective as 5 percent natamycin in therapy of filamentous fungal keratitis (Prajna et al. 2003); however, the results were not stratified as to severity of keratitis or the fungus involved.

Miconazole, a phenylethyl imidazole which exhibits a broad spectrum of activity against yeasts and filamentous fungi, is soluble in organic solvents, and may be administered intravenously in normal saline after dissolving in cremophor EL; this vehicle is believed to cause the toxic reactions observed with intravenous miconazole administration (Anderson et al. 1984). Following subconjunctival and topical administration, miconazole was found to penetrate the corneas of rabbit eyes, resulting in high tissue concentrations that were enhanced by epithelial debridement (Foster and Stefanyszyn 1979). In a rabbit model, topical 1 percent miconazole nitrate did not retard the closure of 8.5 mm corneal epithelial defects (Foster et al. 1981). Topical administration of 1 percent miconazole nitrate in polyethylene glycol 400 was not effective in a rabbit model of deep stromal *Candida albicans* keratitis (O'Day et al. 1984a), irrespective of whether the corneal epithelium was intact or debrided. In another study (O'Day et al. 1984b) topical 1 percent prednisolone acetate was found to adversely influence the efficacy of 1 percent miconazole and other antifungals when given in combination (O'Day et al. 1984b). Although intravenous administration of miconazole resulted in undetectable drug concentrations in the cornea and vitreous of rabbit eyes (Foster and Stefanyszyn 1979), intravenous administration in a dose of 600–3 600 mg/day was reported to be effective in the treatment of clinical mycotic keratitis due to *Phialophora gougerotii* (Jones 1975), *Lasiodiplodia theobromae* (Ishibashi and Matsumoto 1984b), *Beauveria bassiana* and *Aspergillus fumigatus* (Ishibashi et al. 1984). Topical and subconjunctival miconazole therapy (Foster 1981) resulted in resolution of all lesions, including endothelial plaques and a descemetocele, in seven patients with keratitis due to *Candida albicans* or *Aspergillus* species; all seven fungal strains were reportedly susceptible in vitro to miconazole. Topical (1 percent) and subconjunctival miconazole therapy, combined with oral ketoconazole, evoked a favorable response in 13 of 20 patients with keratitis due to species of *Fusarium*, *Curvularia*, or *Candida*; a modification of the regimen produced healing in three more patients, while the lesions progressed in four patients (Fitzsimons and Peters 1986). Since many strains of *Scedosporium apiospermum* (*Pseudallescheria boydii*) are reported to be susceptible to miconazole in vitro (Sutton et al. 1998), miconazole is believed to be an important drug in treatment of clinical keratitis due to this fungus. A recent review of 15 patients with this condition (Wu et al. 2002) appears to endorse this view (only reports where details of treatment regimen and visual outcome were provided were included in the review, and patients with initial scleral involvement were excluded). Four (67 percent) of six individuals who had received miconazole and three (33 percent) of nine persons who had not received miconazole retained form vision (counting fingers or better). It is important to remember that the severity of the keratitis at presentation could be an important determinant of outcome of medical therapy. Topical miconazole therapy is sometimes associated with superficial punctate keratitis which resolves when treatment is withdrawn (Foster 1981).

Ketoconazole, the first successful orally absorbable, broad-spectrum antifungal azole, is a substituted imidazole compound that is currently available as an oral preparation (200 mg) worldwide and as a 1 percent topical ocular medication in India. Following subconjunctival, topical, or oral administration of a 1 percent ketoconazole solution to rabbits with undebrided corneas and an intact epithelium, relatively high concentrations of ketoconazole were achieved in the cornea (44.0±10.1 μg/g) but relatively nondetectable levels in the vitreous; greatly increased corneal drug levels (1 391.5±130.0 μg/g) were achieved, particularly after topical administration and, to a lesser extent, after subconjunctival injection, after debridement of the corneal epithelium (Hemady et al. 1992). Following systemic administration, ketoconazole is highly bound to serum protein, but also has a high tissue distribution (O'Brien 1999). Transscleral iontophoresis (4–6 mA for 15 minutes) resulted in peak ketoconazole concentrations of 10.2 μg/ml in the aqueous after 1 hour (which persisted for about 8 hours) and 0.1 μg/ml in the vitreous; transcorneal iontophoresis (1.5 mA for 15 minutes) resulted in peak corneal concentrations (27.6 μg/ml) and aqueous concentrations (1.4 μg/ml) after 1 hour, which were sustained for 2 hours. These concentrations were significantly higher than those achieved by subconjunctival injections (Grossman and Lee 1989).

Oral doses of ketoconazole greater than 400 mg/day may cause nausea, vomiting, and transient elevations in liver enzymes, which resolve upon withdrawal of the drug; sexual impotence, hair loss, gynecomastia, and oligozoospermia may also occur, due to decreased steroid synthesis (O'Brien 1999). While a 1 percent ketoconazole suspension (prepared in polyethoxylated castor oil) was reported to produce modest pathological changes in the regenerating corneal epithelium in a rabbit model (Foster et al. 1981), topical administration of 1 percent, 2 percent, and 5 percent solutions of ketoconazole in arachis oil did not cause corneal toxicity in rabbit eyes (Oji 1982b). When a topical 2 percent ketoconazole suspension (prepared in a 4.5 percent sterile boric acid solution with hydroxypropylmethyl cellulose added to increase viscosity) was used to treat clinical mycotic keratitis, it did not evoke significant ocular toxicity (Torres et al. 1985).

The results of experimental studies suggest that the response of ocular fungal infections to ketoconazole is species-specific. Topical 1 percent ketoconazole (in arachis oil) applied every hour for 10 hours for 16 days was effective in clearing *Aspergillus flavus* keratitis in a rabbit model (Oji 1982a) whereas oral or topical ketoconazole therapy was not successful in experimental *Aspergillus fumigatus* keratitis (Komadina et al. 1985). Topical 1 percent ketoconazole solution was ineffective in one experimental model of *Candida albicans* keratitis (O'Day et al. 1983), but topical 2 percent ketoconazole ointment was effective in another model (Ishibashi and Kaufman 1986a). Oral ketoconazole therapy was also effective in experimental *Candida albicans* keratitis (Ishibashi and Matsumoto 1984a).

In a clinical setting, oral ketoconazole therapy was not found effective in one patient with *Aspergillus fumigatus* keratitis, perhaps because of intraocular invasion (Searl et al. 1981), but was effective in a dose of 300 mg/day in two patients with mycotic keratitis, one due to *Fusarium solani* (therapy for 3 weeks), and the other due to an unidentified fungus (therapy for 8 weeks) (Ishibashi 1983). Topical 1 percent ketoconazole, given for 7 weeks, was reported to be effective in the treatment of six patients with mycotic keratitis, principally caused by *Aspergillus* and *Fusarium* (Torres et al. 1985). In these reports, the efficacy of ketoconazole therapy in severe mycotic keratitis caused by different fungi could not be assessed. When 30 patients suffering from mycotic keratitis were treated with oral ketoconazole (200 mg three times per day), there was complete resolution of lesions in all 14 patients with superficial lesions, and six of 16 patients who had deep lesions; none of the patients reported adverse effects to the drug, nor were marked elevations in serum enzyme levels observed (Thomas et al. 1987). In an extension of this study, 10 of 15 patients treated only with a topical 1 percent ketoconazole suspension in distilled water responded, while in 25 of 35 patients treated with topical and oral therapy, a complete or partial response of the keratitis was noted (Rajasekaran et al. 1987a). A comprehensive assessment of the data from both studies indicates that oral therapy alone was effective in 37 percent of patients with deep lesions, topical therapy alone was effective in 20 percent, and combined oral and topical therapy effected complete or partial resolution of the keratitis in 60 percent of patients, including 10 of 30 patients with *Fusarium* keratitis, 11 of 22 patients with *Aspergillus* keratitis, three of five patients with *Penicillium* keratitis, three of four patients with *Candida albicans* keratitis and in all six patients with *Curvularia* keratitis (Rajasekaran et al. 1987a; Thomas et al. 1987). A combination of ketoconazole and amphotericin B therapy and keratoplasty resulted in a favorable outcome of post-traumatic keratitis due to *Scopulariopsis brevicaulis* in a patient (Ragge et al. 1990), whereas treatment with oral ketoconazole could not halt progressive infection due to the thermotolerant mold *Neosartorya fischeri* var. *fischeri*, the teleomorph of *Aspergillus fischerianus* (Coriglione et al. 1990). In south Florida, 25 of 125 patients with mycotic keratitis, seen over a 10-year period, received oral ketoconazole (400 mg/day for a median duration of 2 weeks), in addition to topical 5 percent natamycin, due to severe keratitis, scleritis, and endophthalmitis; nine (36 percent) of these 25 patients did not require a therapeutic penetrating keratoplasty or other major surgical

intervention to manage their keratitis (Rosa et al. 1994). All these results suggest that while oral ketoconazole therapy is undoubtedly efficacious in nonsevere mycotic keratitis, its efficacy when deep lesions are present is variable, and also depends on the fungal species involved.

Whether the results of oral ketoconazole therapy for mycotic keratitis have any relevance at all to topical therapy is debatable. Triturated (crushed and suspended) ketoconazole has been recommended for the treatment of mycotic keratitis when commercial antifungal eye drops are not obtainable (Guzek et al. 1998). Ketoconazole and itraconazole tablets were triturated to 20 mg/ml in polyvinyl alcohol, boric acid, olive oil, or balanced salt solution (BSS) and applied topically to de-epithelialized rabbit corneas (one drop/15 minutes for 2 hours). The concentrations of ketoconazole in corneal tissue treated with the drug in BSS, olive oil, polyvinyl alcohol, and boric acid were calculated to be 512, 773, 1 221, and 1 492 μg/g, respectively. Since the vehicle used to triturate antifungals may affect the tissue concentration, the development of effective vehicles may have an impact on the therapy of mycotic keratitis.

Itraconazole, an orally absorbable synthetic dioxolane triazole with a broad spectrum of antifungal activity after oral administration, is larger than fluconazole, very hydrophobic, and poorly soluble in aqueous solution. It is well absorbed orally, especially when given with a meal or formulated in polyethylene glycol, with more than 90 percent being bound to protein in serum, and it is highly concentrated in lipid-rich tissue (Van Cauteren et al. 1987). Although a single 200 mg oral dose results in a peak serum level of about 0.3 μg/ml, which is far below that of ketoconazole or fluconazole (Van Cauteren et al. 1987), the serum level can be increased to 3.5 μg/ml by multiple long-term dosing (200 mg/day orally for 2 weeks) (Carlson et al. 1992). The major drawback of using itraconazole by the oral route for therapy of ocular fungal infections is its poor penetration into the cornea, aqueous humor, and vitreous, when compared to fluconazole and ketoconazole. This was clearly demonstrated in a rabbit model of *Candida* endophthalmitis, even when itraconazole was given in a dose of 80 mg/kg orally (Savani et al. 1987). The ocular pharmacokinetics of a newly developed itraconazole oral solution need to be defined (Harrousseau et al. 2000). Itraconazole is generally well-tolerated after oral administration, the most common complaint being gastrointestinal upset; less frequently observed side effects include hypertriglyceridemia, hypokalemia, edema, decreased libido, and gynecomastia (O'Brien 1999).

Oral itraconazole therapy was found to be effective in the therapy of experimental keratitis due to *Aspergillus* spp. (Van Cutsem and van Gerven 1991). In India (Thomas et al. 1988), 22 of 40 patients with mycotic keratitis (15 with nonsevere and seven with severe keratitis) responded to oral itraconazole (200 mg once daily for a median duration of 17 days); serious adverse reactions did not occur, and all hematological, urine, and biochemical parameters remained normal during and after itraconazole therapy. In a related study (Rajasekaran et al. 1987b), seven of eight nonsevere ulcers and one of eight severe ulcers healed with topical therapy alone, while 17 of 21 nonsevere ulcers and 14 of 33 severe ulcers responded to a combination of oral itraconazole (200 mg once daily) with hourly topical applications of 1 percent itraconazole. To summarize these data, 84 percent of nonsevere ulcers and 57 percent of severe ulcers showed excellent or moderate responses to some form of itraconazole therapy; 76 percent of patients with *Aspergillus* keratitis, 70 percent of those with dematiaceous filamentous fungal keratitis, and 60 percent of patients with *Fusarium* keratitis exhibited excellent or moderate responses to some form of itraconazole therapy (Rajasekaran et al. 1987b; Thomas et al. 1988). In another study, a recurring corneal infection due to *Fonsecaea pedrosoi* was treated by a large penetrating keratoplasty, removal of the involved part of the iris and the entire lens, and a 5-month course of oral itraconazole; this resulted in no recurrence of the infection (Barton et al. 1997).

Bioassay of corneas treated with itraconazole in different vehicles (BSS, polyvinyl alcohol, boric acid, olive oil) demonstrated approximate itraconazole concentrations of 200–250 μg/g tissue (Guzek et al. 1998). Another avenue to pursue is the possible use of itraconazole subconjunctivally. In a novel method for the qualitative evaluation of the pharmacokinetics of subconjunctivally injected antifungals in rabbits, itraconazole (2.5 mg/ml) was found to persist for at least 24 hours in normal and debrided corneas, in contrast to amphotericin B, miconazole, fluconazole, and ketoconazole, which did not persist beyond 4–8 hours (Klippenstein et al. 1993).

Fluconazole, a synthetic bistriazole antifungal compound, is a smaller molecule than itraconazole, soluble in water, and only 10–20 percent protein bound in serum; it has a long half-life and is excreted renally (Savani et al. 1987). In one study (O'Day et al. 1990), oral fluconazole was found to readily penetrate all ocular tissues and fluids of Dutch-belted rabbits, with no difference observed between phakic and aphakic eyes; after a single oral dose of 20 mg/kg, the levels achieved were 13.3±1.4 μg/g (cornea), 7.4±0.3 mg/l (aqueous), 9.8±0.9 mg/l (vitreous), and 5.2±0.4 μg/g (choroid/retina), with the corneal concentrations being highly correlated with serum concentrations. A steady accumulation in both normal corneas, as well as those infected with *Candida albicans*, was noted when given in a twice-daily divided dose; the presence of inflammation induced by fungal infection did not influence corneal uptake (O'Day et al. 1990). In a related experimental study in

Dutch-belted rabbits (O'Day 1990), oral fluconazole therapy resulted in a significant therapeutic effect in keratitis due to *Aspergillus fumigatus* keratitis and that due to *Candida albicans*; pretreatment for 1 day followed by 5 days post-inoculation treatment, led to a significant decrease in isolate recovery and in clinical disease.

Since fluconazole is a stable, water-soluble, low molecular weight bistriazole with high bioavailability and low toxicity, it is potentially useful as a topical ocular agent. In New Zealand white rabbits, topical application of 0.2 percent fluconazole to debrided and nondebrided corneas resulted in peak corneal levels of 8.2±1.2 μg/g (debrided corneas) and 1.6±0.6 μg/g (nondebrided) being noted after 5 minutes, and aqueous humor levels of 9.4±2.3 μg/ml and 1.6±0.6 μg/ml after 15 minutes; the half-life of fluconazole in debrided eyes was 15 minutes and that in nondebrided eyes was 30 minutes (Yee et al. 1997).

In experimental *Candida albicans* keratitis, topical (2 mg/ml) fluconazole, applied to debrided and nondebrided corneas of rabbits, was significantly better than control saline therapy in effecting clinical resolution of corneal lesions and hypopyon, nonprogression to corneal perforation and descemetocele, and eradication of the infecting fungus from the cornea (Behrens-Baumann et al. 1990). In a clinical setting, six consecutive eyes of patients with microbiologically proven *Candida* keratitis with abscess formation, which had failed to respond to topical miconazole or natamycin therapy, responded well to topical fluconazole (20 mg/ml) given for a mean duration of treatment of 22.6±2.3 days (Panda et al. 1996), while oral fluconazole therapy was reported to be useful in therapy of keratitis due to an unspecified fungus (Thakar 1994), and, in association with topical natamycin and intracameral amphotericin B and various surgical measures, to effect the eradication of corneal infection due to *Colletotrichum graminicola* (Ritterband et al. 1997). Although *Candida guilliermondii* is usually susceptible to fluconazole in vitro (Sutton et al. 1998), infectious crystalline keratopathy due to this yeast in a corneal transplant progressed, in spite of 6 weeks of topical amphotericin B, and an additional 6-week course of topical and oral fluconazole (Ainbinder et al. 1998).

SELECTION OF ANTIFUNGAL THERAPY

The selection of an antifungal agent for therapy necessarily depends on its easy availability and on other criteria. If direct microscopic examination of corneal scrapes or corneal biopsies yields definite results that are consistent with the clinical picture, treatment may be initiated; otherwise, therapy may need to be withheld until culture reports become available (Johns and O'Day 1988). Topical natamycin (5 percent), if commercially available, or amphotericin B (0.15 percent) is usually selected as first-line therapy for superficial keratitis (topical econazole is used in many parts of the UK) whether or not septate hyphae or yeast cells have been seen by direct microscopy; if deep lesions are present, intravenous amphotericin B, subconjunctival or intravenous miconazole, oral ketoconazole, oral itraconazole or oral fluconazole is added to the therapeutic regimen (Rosa et al. 1994; Wong et al. 1997; Tanure et al. 2000). If hyphae have been seen by microscopy, and a filamentous fungus is isolated in culture, natamycin appears to be the treatment of choice when available (Rosa et al. 1994; Tanure et al. 2000); topical 0.15 percent amphotericin B (Johns and O'Day 1988; Wong et al. 1997) is an alternative. If yeasts or pseudohyphae are seen by microscopy, and species of *Candida* or *Cryptococcus* are isolated in culture, topical 0.15 percent amphotericin B appears to be preferred when available (Rosa et al. 1994; Tanure et al. 2000), although natamycin (Rosa et al. 1994; Panda et al. 1996) and topical (1 percent) miconazole (Foster 1981; Panda et al. 1996) are the choices of some other clinicians. In the absence of controlled clinical trials and since the number of patients involved may be very small, the validity of these patterns of prescribing is difficult to assess. However, it may be possible to discern patterns by a detailed analysis of the available literature.

Thomas (2003) performed an analysis of 85 patients with keratitis due to *Fusarium* spp. reported in 15 studies in the literature where details of outcome of therapy were provided. The analysis revealed that more than 70 percent of patients with superficial keratitis due to *Fusarium solani* and other *Fusarium* spp. apparently responded to medical therapy alone (topical amphotericin B alone or in combination with topical natamycin, oral ketoconazole and/or topical ketoconazole, oral itraconazole); although several antifungals were found effective, administration of natamycin may have forestalled surgical intervention. In striking contrast, almost 70 percent of patients with *Fusarium* keratitis with deep lesions did not respond to medical therapy alone, particularly if natamycin was not used, and some form of surgical intervention was necessary. The data pertaining to outcome of therapy was analyzed (Thomas 2003) for 61 patients with *Aspergillus* keratitis (Figure 15) reported in 13 studies in the literature. The data suggested that more than 80 percent of patients with superficial keratitis due to *Aspergillus flavus*, *Aspergillus fumigatus*, and other *Aspergillus* spp. responded to medical therapy with a variety of topical (amphotericin B, natamycin, ketoconazole, itraconazole) or systemic (oral ketoconazole, oral itraconazole) antifungals, with surgery not being required; however, in the presence of deep corneal lesions, almost 60 percent of patients did not respond to medical therapy alone, particularly if natamycin was not used, and surgery was required to control the infection.

Details of reponse of keratitis due to *Candida* spp. to therapy were analyzed (Thomas 2003) for 38 patients reported in eight studies. The medical therapy of kera-

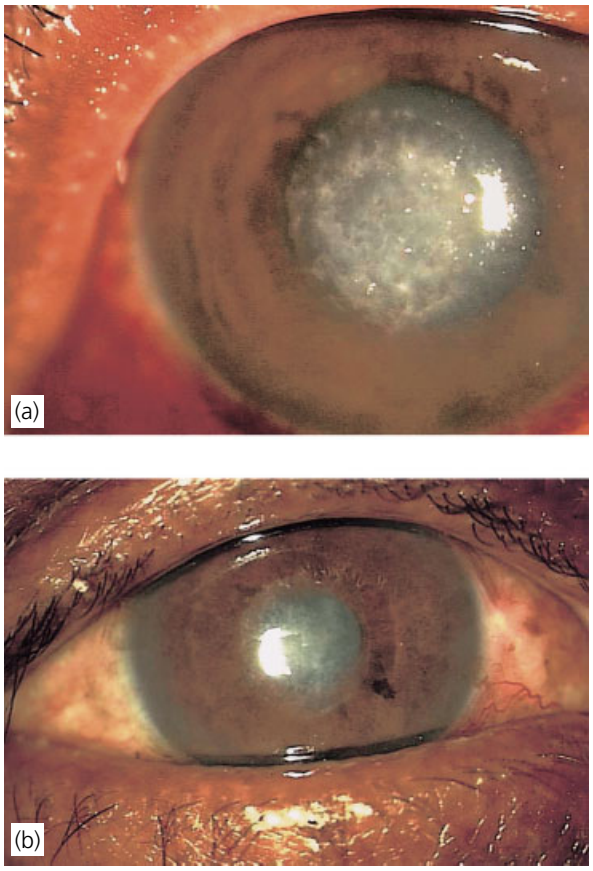

Figure 15 (a) *Keratitis due to* Aspergillus flavus *before treatment.* **(b)** *Keratitis due to* Aspergillus flavus*, 1 month after treatment with topical 5 percent natamycin. Lesions have resolved, leaving a faint scar.*

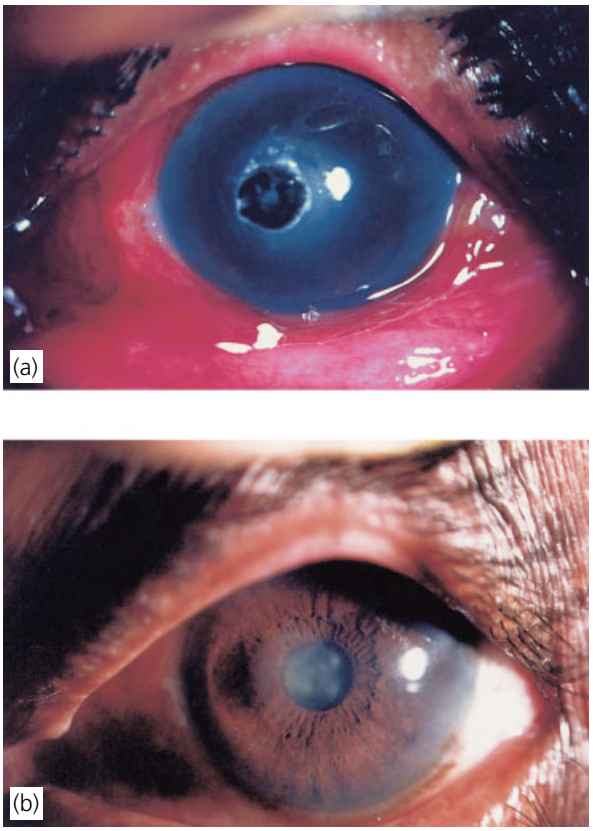

Figure 16 (a) *Keratitis due to a* Curvularia *sp. before treatment.* **(b)** *Keratitis due to a* Curvularia *sp. 1 month after treatment with topical 5 percent natamycin. Lesions have resolved, leaving a faint scar.*

titis due to *Candida albicans* and other *Candida* spp. generally had a favorable prognosis, particularly when topical amphotericin B was used alone or in combination with systemic azoles; topical natamycin and topical fluconazole alone also sufficed in some instances, and the presence of deep lesions appeared not to be a major hurdle.

Data pertaining to 42 patients with keratitis due to *Curvularia* spp. (Figure 16) reported in seven studies in the literature were analyzed (Thomas 2003). In 35 (83 percent) of the 42 individuals, the corneal lesions responded to antifungals alone (19 to topical natamycin alone, eight to natamycin and other antifungals, six to oral ketoconazole, and one each to topical miconazole and topical amphotericin B). In an additional three patients, the keratitis resolved with keratectomy and antifungal therapy, while penetrating keratoplasty was required in four patients who did not respond to medical therapy alone. Most of the papers analyzed, however, did not provide details about the severity of the corneal lesions in the patients, which is an important aspect that requires consideration. In one study on dematiaceous fungal keratitis (Garg et al. 2000), antifungal therapy alone (principally natamycin, alone or in combination with topical azoles) sufficed for resolution of lesions in 88 percent of patients with superficial lesions; however, only 46 percent of patients with deep keratitis responded to antifungal therapy alone (topical antifungals combined with oral ketoconazole), and surgery was required for the other patients. Keratitis due to dematiaceous fungi other than *Curvularia* spp. appears to respond to primary therapy with topical natamycin, oral and/or topical ketoconazole, oral ketoconazole with topical miconazole, topical amphotericin B or oral itraconazole (Thomas et al. 1987, 1988; Rosa et al. 1994; Wong et al. 1997; Garg et al. 2000). However, therapy of keratitis due to *Lasiodiplodia theobromae* is often difficult. A successful outcome of this type of dematiaceous fungal keratitis was reported in patients receiving natamycin ointment and topical or subconjunctival amphotericin B (Rebell and Forster 1976). Intravenous miconazole was also reported to be useful, but this was based on the study of a single patient (Ishibashi and Matsumoto 1984b). Poor results have been reported in patients receiving azoles (Thomas et al. 1991a, 1998; Borderie et al. 1997).

The outcome of therapy of keratitis due to *Scedosporium apiospermum* is varied. A review of 13 cases reported up to 1979 revealed a generally poor outcome,

with six of the cases eventually requiring enucleation or evisceration (Zapater and Albesi 1979). At least 14 patients with keratitis due to *Scedosporium apiospermum* have been reported in the literature since 1991 (reviewed by Wu et al. 2002). An analysis of the data pertaining to these patients (Thomas 2003) revealed that medical therapy alone sufficed for resolution of lesions in eight (57 percent) of these 14 patients (three of the 'responders' had keratitis with deep lesions), penetrating keratoplasty was needed in three patients (all had deep keratitis), and evisceration or enucleation was needed for three patients (two of whom had deep corneal lesions). Eight patients (five with deep keratitis and three with keratitis of undetermined severity) received natamycin at some time; corneal lesions resolved with medical therapy alone in three, while penetrating keratoplasty was required in the eyes of three patients, and enucleation had to be done for two patients. Six individuals (two with superficial keratitis, three with deep keratitis and one with keratitis of unknown severity) received miconazole; four resolved with medical therapy alone (two had superficial keratitis), while evisceration/enucleation was needed for two eyes (both with deep keratitis). These data suggest that, currently, keratitis due to *Scedosporium apiospermum* more frequently has a favorable outcome than that reported in the past, with evisceration or enucleation being the final result in 21 percent (compared to 54 percent in patients reported up to 1979). However, if severe keratitis is present, penetrating keratoplasty is required in addition to medical therapy. It is difficult to assess the relative efficacy of miconazole versus natamycin in view of the small numbers of patients involved.

A combination of topical antifungal therapy and keratoplasty appears to provide the most adequate treatment for keratitis due to *Acremonium* spp. (Fincher et al. 1991). A prospective evaluation of the comparative safety and efficacy of topical natamycin and 0.2 percent fluconazole was made in eight patients with filamentous fungal keratitis, including five due to *Acremonium* spp. and two due to *Curvularia* spp. (Rao et al. 1997). Corneal lesions resolved in three of four patients receiving primary natamycin treatment for a mean duration of 20 days (the keratitis worsened in the fourth patient) whereas corneal lesions failed to resolve in all four patients who received topical fluconazole as primary treatment (two subsequently responded to natamycin therapy). This study is important in providing evidence of the efficacy of topical natamycin, and the relative inefficacy of topical fluconazole, in therapy of keratitis due to filamentous fungi.

A common problem reported by all those who have had to treat keratitis due to *Pythium insidiosum* is that it is not sensitive to any of the currently available antifungals; wide surgical excision including penetrating keratoplasty has been advised for such patients, with enucleation or evisceration being required in patients who fail to respond to these measures (Virgile et al. 1993; Imwidthaya 1995; Thianprasit et al. 1996).

Fungal keratitis usually responds slowly over a period of weeks to antifungal therapy. Clinical signs of improvement of a fungal corneal ulcer include:

- diminution of pain
- decrease in size of infiltrate
- disappearance of satellite lesions
- rounding out of the feathery margins of the ulcer
- hyperplastic masses or fibrous sheets in the region of healing fungal lesions (Jones 1975; Johns and O'Day 1988).

Conjunctival chemosis and injection and punctate epithelial keratopathy may indicate toxicity of the antifungal agent being used. Negative scrapings during treatment do not always indicate that the fungus has been eradicated since it may have become deep-seated; hence therapy should be maintained for at least six weeks (Johns and O'Day 1988).

Although a combination of natamycin and ketoconazole was found to be beneficial in an animal model of *Aspergillus fumigatus* keratitis (Komadina et al. 1985), there are believed to be risks of antagonistic effects developing by the combination of certain antifungals such as amphotericin B and miconazole (Johns and O'Day 1988). Thus, methods to enhance the efficacy of existing antifungal agents require careful study.

MEASURES TO SUPPRESS CORNEAL DAMAGE DUE TO MICROBE- OR HOST TISSUE-DERIVED FACTORS

The inflammatory reaction directed against an infecting microorganism may sometimes be so severe as to damage adjacent tissues. To reduce these effects, therapy with corticosteroids and specific antimicrobials has sometimes been contemplated, since this regimen has proven satisfactory in disciform keratitis and central stromal keratitis due to the herpes simplex virus (O'Day 1991). In a recent study, Schreiber et al. (2003) tried to determine the most efficient time point and concentration of topical corticosteroids in *Candida albicans* keratitis treated with fluconazole. Their results suggested that fluconazole plus adjunctive high-dose prednisolone treatment was most effective when administered 9 days after infection. These workers concluded that the delayed application of corticosteroids after treatment with antifungals in patients with mycotic keratitis need not be a contraindication and, in fact, could be beneficial in some instances. However, most available evidence appears to weigh against the use of corticosteroids in the therapy of mycotic keratitis. In an experimental model of keratitis due to *Candida albicans*, the use of a topical corticosteroid was found to reverse the therapeutic effect of all the topical antifungals used, except for amphotericin B (0.5 or 0.15 percent) and to permit a greater replication of organisms, in comparison to

untreated controls (O'Day et al. 1984b). Moreover, corticosteroids appear to be necessary to create experimental models of mycotic keratitis (Ishibashi and Kaufman 1986a; O'Day et al. 1991), and have been found to worsen the course of existing, but unrecognized, fungal infection in the cornea (Stern and Buttross 1991; Rosa et al. 1994). This may explain why currently, the use of corticosteroids is definitely contraindicated when a specific microbicidal therapy is not used concurrently, and especially when a fungal pathogen is present. Possible 'inflammatory rebound,' a potentially devastating complication that occurs when corticosteroid therapy is abruptly terminated, also needs to considered, since this could be confused with a worsening of infection (O'Day 1991).

Since corticosteroids cannot be used to decrease the unwanted side effects of the inflammatory response in mycotic keratitis, other molecules are being evaluated. In an animal model of keratitis due to *Candida albicans*, ketorolac (a nonsteroidal anti-inflammatory compound) was found to satisfactorily reduce the tissue necrosis occurring as a result of inflammatory mechanisms, without, however, permitting progression of the infection (Fraser-Smith and Matthews 1987); there do not appear to be published reports regarding the use of this compound in clinical mycotic keratitis. A variety of mechanisms have been found to contribute to corneal damage in sterile (nonmicrobial) corneal ulceration, including:

- activation of corneal collagenase, infiltration by polymorphonuclear leukocytes, and release of free radicals in keratitis due to alkali burns (Burns et al. 1990; Alio et al. 1993, 1995)
- release of lipid mediators (Tjebbes et al. 1993)
- activation of platelet-activating factor (Tao et al. 1995).

Whether such mechanisms are relevant in pathogenesis of mycotic keratitis requires further study.

Therapeutic surgery

In the management of mycotic keratitis, surgery may be indicated in those patients who respond poorly, or not at all, to medical therapy, or where perforation or descemetocele formation is likely to occur. Prior to surgery, medical therapy should be administered for as long as possible to render the infecting fungus nonviable and therein to improve the outcome of surgery. The modalities of surgical management include removal of the corneal epithelium and anterior lamellar keratectomy, conjunctival flaps, tissue adhesives, and penetrating keratoplasty. These techniques aid medical therapy by removing infected corneal tissue (therein reducing or eliminating the microbial load) and increasing drug penetration, by bringing in blood vessels (when conjunctival flaps are formed), by stabilizing the corneal epithelial surface itself or by providing tectonic support to the entire globe when its integrity is threatened by thinning or perforation of the cornea (Agrawal et al. 1994).

In mycotic keratitis, regular debridement of the base of the ulcer helps in elimination of fungi and necrotic material and also facilitates penetration of antifungal drugs such as amphotericin B 0.15 percent and 0.075 percent and natamycin into the corneal stroma (O'Day et al. 1984c). Debridement is usually performed, under topical anesthesia, with a Bard-Parker blade no. 15, ensuring that a margin of 1–2 mm is left at the limbus (Agrawal et al. 1994). Debridement of necrotic material may suffice to induce resolution of dematiaceous fungal keratitis. The procedure of anterior lamellar keratectomy helps to remove the thick mat of fungal filaments on the cornea, therein facilitating increased drug penetration and resolution of the corneal lesions in patients with dematiaceous fungal keratitis (Jones 1975; Garg et al. 2000). Anterior (superficial) stromal corneal infiltrates can also be ablated by the excimer laser for therapeutic purposes. In an experimental model of corneal infection due to a *Fusarium* spp., photoablation by the 193 nm excimer was found useful to eradicate early, localized microbial infections, but advanced infections, with deep stromal involvement and suppuration, could not be eradicated by this technique (Gottsch et al. 1991).

Conjunctival flaps help in achieving a stable conjunctival surface in cases of persistent or recurrent epithelial defects and progressive ulceration; such flaps are especially helpful in chronic peripheral disease, where the flap does not encroach onto the visual axis (Portnoy et al. 1989; Agrawal et al. 1994). Blood vessels present in the flap brought in to cover the ulcerated area help in healing of peripheral fungal corneal ulcers; a superficial lamellar keratectomy should first be done to remove the necrotic stroma, and then a thin conjunctival flap should be anchored over the ulcerated site (Alino et al. 1998). There have been no studies evaluating the specific use of conjunctival flaps in central fungal corneal ulcers.

Permanent or temporary amniotic membrane transplantation may be a useful adjunctive surgical procedure for the management of microbial (including mycotic) keratitis by promoting wound healing and reducing inflammation (Kim et al. 2001). However, a larger number of patients needs to be studied to confirm its use in mycotic keratitis. It is also not clear whether this technique will be useful in patients who have extensive corneal epithelial ulceration and stromal infiltration, and whether the infecting fungus is completely eradicated in this procedure, or whether foci of viable fungi persist in the corneal tissue.

Tissue adhesives (cyanoacrylate 'glue') provide support to a thinned-out cornea, can seal a corneal perforation that is 2 mm or less in size (Forster 1994), and are also bacteriostatic for gram-positive bacteria

(Agrawal et al. 1994). Necrotic stromal and epithelial debris is first removed from the base of the ulcer, following which the adhesive is applied and a bandage contact lens fitted. The adhesive is left in place until it loosens spontaneously, or the bed becomes vascularized, or keratoplasty is performed.

Although penetrating keratoplasty appears to be rarely required for the treatment of active bacterial keratitis, probably due to the availability of specific antibacterial drugs, it may be required in about 15.3–27 percent of cases with mycotic keratitis (Rosa et al. 1994; Garg et al. 2000), particularly when the keratitis is caused by species of *Aspergillus* (Rosa et al. 1994; Vemuganti et al. 2002), *Fusarium* (Vemuganti et al. 2002), *Lasiodiplodia theobromae* (Borderie et al. 1997; Thomas et al. 1998), *Pythium insidiosum* (Imwidthaya 1994), and *Paecilomyces lilacinus* (Gordon and Norton 1985; Okhravi et al. 1997). Mycotic keratitis is reported to be associated with a five- to sixfold higher risk of subsequent perforation and need for penetrating keratoplasty than bacterial keratitis (Wong et al. 1997). The outcome of penetrating keratoplasty (Figure 17) also depends on whether the surgery has been performed for a bacterial or fungal corneal ulcer. In one study (Cristol et al. 1996), it was found that the graft tended to opacify much earlier in mycotic keratitis (median duration 4.0 weeks) than in bacterial keratitis (median duration 12.9 weeks). Similarly, the reported success rate for grafts in mycotic keratitis (20–60 percent) appears to be much lower than that in bacterial keratitis (70–75 percent) (Panda et al. 1991; Killingsworth et al. 1993). In one study, 25 percent of grafts performed for mycotic keratitis showed reinfection (Rosa et al. 1994). To decrease the incidence of recurrence, at least 0.5 mm of clear tissue all around the infected area should be excised. Although antifungal therapy should be continued postoperatively, topical corticosteroids should be used with caution (Stern and Buttross 1991). When donor grafts 8 mm or less in diameter were used for penetrating keratoplasty in fungal corneal ulcers, the outcome was better than when larger grafts were used (Killingsworth et al. 1993). Topical cyclosporin A (5 mg/ml) was reported to be useful as a primary or an adjunctive therapy for prevention of allograft rejection in a small number of patients with culture-proven mycotic keratitis (Perry et al. 2002).

In a recently published review of the results obtained in 45 patients with severe mycotic keratitis, in whom therapeutic penetrating keratoplasty was performed using cryopreserved donor corneas, with additional measures being irrigation of the anterior chamber with fluconazole (2 mg/ml), dissection of fibrinoid membrane on the iris and iridectomy, it was noted that the fungal infection was eradicated, and the anatomic integrity was maintained, in 39 eyes (Yao et al. 2003). Thus, cryopreserved donor corneas may be effective substitutes in therapeutic penetrating keratoplasty in severe mycotic keratitis.

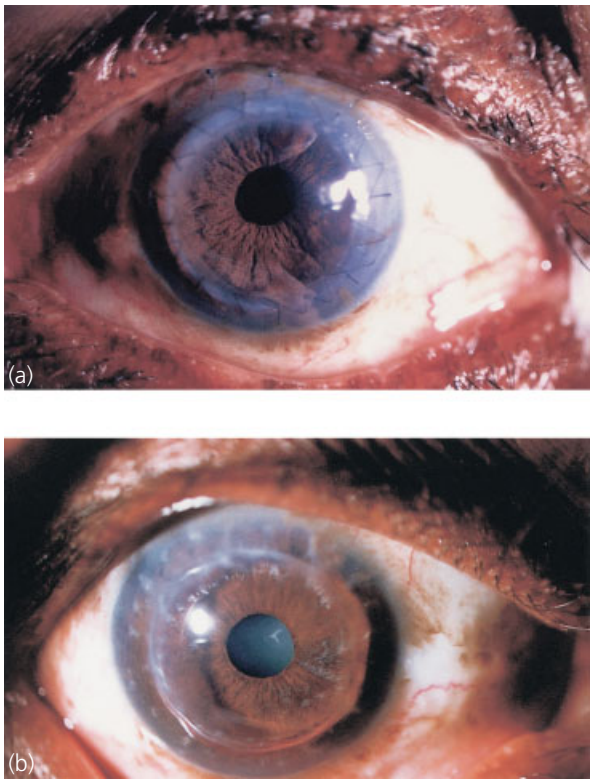

Figure 17 (a) *Keratitis due to* Fusarium solani*, 1 month after therapeutic penetrating keratoplasty. The corneal graft is clear.* **(b)** *Keratitis due to* Fusarium solani*, 4 months after therapeutic penetrating keratoplasty. The corneal graft remains clear.*

MYCOTIC SCLERITIS

Although uncommon, fungal lesions of the sclera are of great importance since they may spread from fungal infections of contiguous structures, such as the cornea or conjunctiva, or may occur secondary to trauma or surgery. Endogenous infections have also been reported. Scleritis arising due to spread of infection from keratitis due to *Absidia corymbifera* (Marshall et al. 1997), *Acremonium* spp., and *Lasiodiplodia theobromae* (Borderie et al. 1997) has been reported. Similarly, scleritis due to fungi (*Aspergillus* spp. or *Sporothrix schenckii*) may follow ocular trauma (Rodriguez-Arres et al. 1995). A unique subset of microbial scleritis following ocular surgical procedures is being increasingly reported; this is dealt with in another section (see later under Fungal ocular infections after ophthalmic surgical procedures).

One patient with *Scedosporium prolificans* corneoscleritis responded to intensive antifungal therapy and aggressive scleral debridement (Kumar et al. 1997), whereas another patient responded poorly to medical therapy (topical natamycin and amphotericin B, oral itraconazole and ketoconazole), necessitating eventual enucleation (Sullivan et al. 1994).

The outcome of scleritis due to *Scedosporium apiospermum* is also reported to be varied, with good results being obtained in some patients (Moriarty et al. 1993), and poor results in others (Taravella et al. 1997). Mycotic scleritis due to species of *Candida* or filamentous fungi (except *Fusarium*) may be treated by subconjunctival miconazole (and intravenous miconazole, if necessary). Additional remedies include oral flucytosine, oral ketoconazole, oral itraconazole, oral fluconazole, intravenous amphotericin B, topical miconazole, or topical natamycin. *Sporothrix schenckii* infection has been successfully treated with oral potassium iodide (50 mg/drop), 10 drops thrice daily, slowly increasing to 24 drops thrice daily (Brunette and Stulting 1992). Posttraumatic scleritis due to *Aspergillus fumigatus* was found to worsen in spite of oral fluconazole and topical amphotericin B therapy; cryotherapy and dura mater grafting were then performed, which appeared to control the infection (Rodriguez-Arres et al. 1995). Oral itraconazole therapy brought about resolution of inflammation in scleritis due to *Aspergillus flavus*; the patient's condition had worsened during therapy with oral ketoconazole and topical amphotericin B (Carlson et al. 1992).

INTRAOCULAR MYCOSES

Definitions

Uveitis may be defined as intraocular inflammation (Weinberg 1999). There are several systems classifying the many uveitis entities, one of which is uveitis arising due to infections caused by various microorganisms, including fungi. The intraocular inflammation caused by fungal infection usually develops slowly, is spread to the eye through the bloodstream, and consists of focal or multifocal lesions in the choroid and retina (chorioretinitis). Anterior segment inflammation is variable, but can be severe with hypopyon (Weinberg 1999). When the initial focus of intraocular fungal infection in the choroid and retina extends into the vitreous to produce inflammation, which may involve the entire internal structure of the eye, endophthalmitis results. It may be difficult, from a practical viewpoint, to discuss fungal retinitis separately from fungal endophthalmitis (Hamza et al. 1999). However, intraocular findings have been classified, based on ophthalmoscopic examination in patients with suspected ocular involvement in candidemia, into endophthalmitis (focal chorioretinitis with extension into the vitreous, intravitreal fluff balls, or vitreous haze associated with typical chorioretinal lesions), chorioretinitis (focal, deep, white chorioretinal lesions with no evidence of direct vitreous involvement), and 'nonspecific' fundus lesions (clear vitreous and no focal chorioretinitis, including cotton wool spots, retinal hemorrhages, and Roth spots) (Donahue et al. 1994). Endophthalmitis, in turn, can be divided into endogenous endophthalmitis, which arises from hematogenous spread from a focus of infection elsewhere in the body, and exogenous endophthalmitis, resulting from primary inoculation of the eye following surgery or penetrating trauma.

Etiologic agents

The major causes of intraocular mycoses are species of *Candida* (especially *Candida albicans*), *Aspergillus* (especially *Aspergillus fumigatus*), *Blastomyces dermatitidis*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Scedosporium apiospermum* (*Pseudallescheria boydii*), and *Sporothrix schenckii* (Hamza et al. 1999). Species of *Fusarium* (Patel et al. 1994; Goldblum et al. 2000), *Acremonium* (Weissgold et al. 1996), *Paecilomyces* (Pettit et al. 1980; Kozarsky et al. 1984; Scott et al. 2002), zygomycetes (Orgel and Cohen 1989), and *Pneumocystis carinii* (Dugel et al. 1990; Foster et al. 1991) have also been reported to cause such lesions. Although most of these organisms produce endophthalmitis, they may also cause focal (localized) chorioretinitis or a granulomatous lesion in the iris or ciliary body (Weinberg 1999).

Frequency of occurrence

In the 1970s and 1980s, the incidence of endogenous fungal endophthalmitis in patients with candidemia or candidiasis, or both, was reported to range from approximately 10 to 40 percent. A study in the late 1980s reported that ocular lesions developed within 72 hours of the suspected onset of fungemia in approximately 90 percent of nontreated, hospitalized patients with candidemia (Brooks 1989). However, a more recent study (Scherer and Lee 1997) estimated the incidence of chorioretinal findings consistent with early endogenous fungal endophthalmitis to be 2.8 percent. This low incidence was attributed to the fact that 90 percent of the patients were being treated with systemic antifungal medication (mostly amphotericin B and/or oral fluconazole) at the time of the ophthalmological consultation. *Candida* endophthalmitis now also appears to be rare in patients with candidemia, with none of 118 adults with candidemia showing evidence of endophthalmitis, only 9.3 percent exhibiting chorioretinitis, and 20.3 percent nonspecific fundus lesions (Donahue et al. 1994). In a study of 30 hospitalized children with suspected or known systemic infections caused by *Candida* spp., all of whom had risk factors for disseminated candidiasis (broad-spectrum intravenous antibiotics, chronic debilitation with indwelling catheterization, total parenteral nutrition, immunocompromised state), none was found to have either endophthalmitis or chorioretinitis (Donahue et al. 2003). The low rate of ocular involvement in both series, compared with earlier reports, was believed to be due either to earlier treatment with antifungal agents or

to the lack of rigorous criteria for endophthalmitis in previous studies.

Predisposing factors

Intravenous drug abusers are at great risk to develop endogenous fungal endophthalmitis, probably because they may use contaminated drugs, syringes, needles, or cotton (Elliott et al. 1979). Contaminated preserved lemon juice used to dissolve heroin was found to be the cause of *Candida albicans* endophthalmitis in intravenous drug abusers in Glasgow (Shankland and Richardson 1989). Vitreous opacities may be a prominent manifestation in such individuals (Aguilar et al. 1979). In a retrospective analysis of clinical and histopathological features in 25 patients who underwent enucleation (13 with morphological features and/or positive culture for *Aspergillus* and 12 with histological evidence and/or positive culture for *Candida*), Rao and Hidayat (2001) noted a history of gastrointestinal surgery, hyperalimentation, or diabetes mellitus in nine of 12 patients with *Candida* endophthalmitis (and none of the patients with aspergillosis), whereas 12 of 13 patients with *Aspergillus* endophthalmitis, and only one of 12 patients with *Candida* endophthalmitis, had received immunosuppressive agents or had undergone organ transplants or valvular cardiac surgery. Thus, it might be possible clinically to suspect *Candida* endophthalmitis in patients who have undergone gastrointestinal surgery, and *Aspergillus* infection in those with drug-induced immune suppression or in patients who have had cardiac surgery. In another retrospective analysis of endogenous fungal endophthalmitis (where *Candida* species were the major causes), in 79 eyes of 46 patients the principal predisposing factors noted were the presence of β-D-glucan in concentrations of at least 20 pg (in 90 percent of patients), intravenous hyperalimentation (87 percent), fever of unknown origin (76 percent), male gender (74 percent), cancer (72 percent), and neutrophils in a count of at least 500/ml (67 percent) (Tanaka et al. 2001). Recent prolonged broad-spectrum antibacterials, indwelling intravenous catheters, pressure-monitoring devices, organ transplantation, and generalized immunosuppression associated with malnutrition, debilitating diseases, iatrogenic neutropenia, or corticosteroid use (Hamza et al. 1999) are other reported predisposing factors.

Diagnosis

Since anterior chamber and/or vitreous inflammation may obscure visualization of the retina, echography may help to determine the anatomic status of the retina, the extent of inflammation, the presence of choroidal detachment, and the presence and location of intraocular foreign bodies. Apart from localizing such foreign bodies, computed tomography may not be very useful, nor are electrophysiological studies (Boldt and Mieler 1996).

Since the diagnosis of exogenous fungal endophthalmitis is ultimately established by demonstrating infectious organisms in the eye, samples of aqueous and vitreous should be obtained prior to instituting therapy. A vitrectomy specimen (where as much as necessary of the vitreous is cut and removed, usually by a vitreous cutter) is preferred to a vitreous aspirate (vitreous tap) sample since the latter may fail to sample the locus of infection (Hamza et al. 1999). Anterior chamber aspirates are also a poor diagnostic technique. Although cultures of vitreous samples are more likely to yield positive results than cultures of aqueous samples, it is recommended that both samples be obtained. A conjunctival swab is of relevance only if there is a leaking filtering bleb. Fungal endophthalmitis can be confirmed by direct microscopic demonstration of fungal hyphae or yeast cells in 10 percent KOH wet mounts, or smears stained by calcofluor white and the Gram method (Brar et al. 2002). Culture is performed on appropriate media. Direct inoculation of vitreous and aqueous samples on the culture media immediately after collection is recommended. To increase the chances of recovering fungi, vitrectomy samples can be concentrated in an ultracentrifuge and then inoculated, or can be passed through cellulose membrane filters which are then applied to the culture media plates.

Fungal chorioretinitis and endogenous fungal endophthalmitis represent exceptions to the rule requiring isolation of the fungus from ocular tissue in order to establish an ocular fungal infection. For example, the presence of a typical fundus lesion, as well as a history of *Candida* spp. being cultured from blood, urine, intravascular cathethers, or other body sources days or weeks prior to the onset of symptoms, permits a presumptive diagnosis of *Candida* retinitis (Hamza et al. 1999). If nonspecific findings are present, or if there is no positive culture from an extraocular site, a diagnostic vitrectomy can be performed (Hamza et al. 1999). Similarly, in intraocular histoplasmosis and coccidioidomycosis, which are commonly associated with characteristic chorioretinal lesions, isolation of the fungus from another anatomic site, or measurement of antibody titers to the fungus, is usually deemed sufficient evidence to establish one of these fungi as the cause of the eye disease (Klotz et al. 2000). Since ophthalmic infections due to *Cryptococcus neoformans* usually occur in conjunction with meningoencephalitis, isolation of cryptococci from blood and/or cerebrospinal fluid is usually sufficient explanation for the associated eye findings (Klotz et al. 2000).

In recent years, the value of DNA-based technology in the diagnosis of fungal endophthalmitis has been evaluated. PCR assay was evaluated in the diagnosis of *Candida* endophthalmitis in four patients (Hidalgo et al. 2000); vitreous cultures were negative in two of the four

patients, but characteristic PCR products were generated in all four patient specimens, permitting the diagnosis of *Candida* endophthalmitis in all four. A protocol employing two novel panfungal primers complementary to 18S rRNA sequences of *Candida albicans*, *Aspergillus fumigatus*, and *Fusarium solani*, followed by three nested PCRs utilizing species-specific primers, was applied to ocular material from three patients with suspected endophthalmitis (Jaeger et al. 2000); a positive result for *Candida albicans* DNA and isolation of *Candida albicans* in culture was obtained from one sample and a positive result for *Candida albicans* DNA with a negative culture result was obtained in another sample (the third sample was negative for fungal DNA and was also culture negative). The two patients whose samples were positive for *Candida albicans* DNA were reported to have had clinical signs typical of fungal endophthalmitis and both patients responded to treatment with antifungal agents (Jaeger et al. 2000). Further reports are necessary before conclusions can be drawn.

Treatment

Intravitreal injections are performed in order to improve the intraocular concentration of antifungal drugs, to concentrations greater than can be achieved by systemic preparations, but toxicity may result if the dilutions are not prepared properly, or if the dilutions are injected into air-filled eyes. In the local treatment of endogenous fungal endophthalmitis, intravitreal amphotericin B is the usual recommended initial treatment, while intravitreal miconazole can be considered for those rare fungal cases resistant to amphotericin B (Flynn 2001; Song et al. 2002). Periocular amphotericin B is not commonly used because it has poor intravitreal penetration and usually causes marked conjunctival necrosis. Systemic (intravenous) amphotericin B causes serious and sometimes irreversible toxicity, hence its use is limited to very advanced endogenous fungal endophthalmitis, especially when other nonocular sites are involved (Weishaar et al. 1998; Flynn 2001).

Less toxic azole compounds, for example, fluconazole for *Candida*, can supplement intravitreal therapy and may contribute to a favorable clinical response without the risk of unwanted side effects of systemically administered amphotericin B (Borne et al. 1993; Luttrull et al. 1995; Christmas and Smiddy 1996; Flynn 2001). The possible use of intravitreal itraconazole also requires clarification. Ocular toxicity studies performed in New Zealand rabbits 5 weeks after intravitreal administration of itraconazole 10–100 μg (dissolved in 100 percent DMSO) showed no substantial retinal or histopathological changes in eyes that had been injected with 100 percent DMSO or 10 μg itraconazole, although higher doses caused focal areas of retinal necrosis (Schulman et al. 1991). Based on the results of an experimental study, Yoshizumi and Banihashemi (1988) suggested that a single intravitreal dose of ketoconazole ($\leqslant$540 μg) in DMSO could be safely used for fungal endophthalmitis. Intravenous administration of fluconazole (5 or 25 mg/kg) in albino rats resulted in aqueous, vitreous, and serum levels (1 hour after administration) of 2.87, 1.72, and 4.6 μg/ml (5 mg/kg) and 14.9, 7.05, and 20.6 μg/ml (25 mg/kg), respectively; the intraocular penetration was moderately enhanced by vitrectomy (Mochizuki et al. 1992). In vitro electroretinograms (ERG) remained unchanged after perfusion with fluconazole (20 μg/ml) while the in vivo ERG and visual evoked potentials were unchanged after daily fluconazole (25 mg/kg) for 8 days, suggesting good safety profile. Following intravenous inoculation of fluconazole 20 mg/kg as a single dose or 20 mg/kg every 12 hours for four doses in nonpigmented rabbits, fluconazole concentrations in the aqueous, vitreous, CSF, and serum were determined by a microbiological assay; the penetration of fluconazole in all the anatomic compartments was found to be >70 percent of that in serum (Mian et al. 1998). Since the CSF and ocular pharmacokinetic parameters closely resemble each other, either could be used as a surrogate for the other (Mian et al. 1998). A biodegradable polymeric scleral implant containing fluconazole was reported to be a promising intravitreal drug delivery system to treat fungal endophthalmitis (Miyamoto et al. 1997). Scleral implants loaded with 10, 20, and 30 percent doses gradually released fluconazole over 4 weeks in vitro while those with 50 percent doses released most of the drug in one week; implants with 30 percent fluconazole that were studied in pigmented rabbits resulted in vitreous concentrations of fluconazole (sustained for 3 weeks) sufficient to inhibit *Candida albicans*. Intravitreal injection of up to 100 μg fluconazole per 0.1 ml of vitreous did not produce biomicroscopic, ophthalmoscopic, electroretinographic, or light microscopic evidence of intraocular toxicity, even 8 days after inoculation (Schulman et al. 1987).

The typically slow replication of fungal elements may contribute to a less favorable response to intravitreal antifungal therapy alone; therefore, pars plana vitrectomy is often selected for initial therapy to obtain a satisfactory specimen for culture and to remove vitreous infiltrates. Vitrectomy for the treatment of fungal endophthalmitis has been advocated in all but very mild or exceptional cases. The indications for vitrectomy in patients with fungal chorioretinitis and endophthalmitis are advanced cases with extensive vitreous involvement and poor response to systemic antifungal therapy (Pettit et al. 1996; Smiddy 1998). The potential benefits of vitrectomy include:

- debulking of inflammatory and infectious material from the vitreous
- acquisition of a larger sample for laboratory study
- potential for concentrating the sample by centrifugation or filtration to give a better yield on culture

- an opportunity for intravitreal injection of antifungals
- removal of the scaffolding for vitreoretinal traction bands and epiretinal membranes that can contribute to late-developing macular pucker and retinal detachment (Pettit et al. 1996).

However, vitrectomy and intravitreal injections are better avoided in cases of neonatal endophthalmitis.

Essman et al. (1997) performed a retrospective analysis of treatment outcomes of 20 eyes (18 patients) with culture-proven endogenous fungal endophthalmitis (17 due to *Candida* species, three due to *Aspergillus* spp.) seen over a 10-year period. After initial examination, 17 of 20 eyes underwent pars plana vitrectomy and 19 of 20 received intravitreal amphotericin B; 16 patients received systemic antifungals (oral ketoconazole, oral fluconazole). Thirteen (76 percent) of 17 eyes with *Candida* infection achieved visual acuities equal to or better than 20/400, while none of the three eyes with *Aspergillus* infection achieved such a visual acuity. Based on these results, these authors recommended pars plana vitrectomy, intravitreal amphotericin B, and administration of appropriate systemic antifungals (oral fluconazole for *Candida* infections) in managing patients with marked vitreous infiltrates due to endogenous fungal endophthalmitis.

Recommendations for postoperative and post-traumatic exogenous fungal endophthalmitis (Pflugfelder et al. 1988) include surgical measures (excision of clinically involved tissue, vitrectomy if there is visible vitreous involvement, retention of the intraocular lens unless there is extensive infiltration around the lens, or recurrent infection) and medical therapy:

- intraocular amphotericin B 5–10 μg in regions of maximal involvement, repeatable
- miconazole 25–50 μg in patients with *Paecilomyces lilacinus* infection or in treatment failure with amphotericin B
- topical (hourly) 5 percent natamycin, or topical (hourly) 0.15 percent and subconjunctival 500–1000 μg amphotericin B or topical (hourly) 1 percent and subconjunctival 5–10 mg miconazole, if there is corneal, scleral, or anterior chamber involvement
- systemic (oral) ketoconazole 400–600 mg/day.

Recommendations for exogenous fungal endophthalmitis developing from fungal keratitis include surgery (penetrating keratoplasty for deep keratitis with retrocorneal or anterior chamber involvement unresponsive to medical therapy, retention of the iris and lens if possible, or iridectomy, lensectomy, and vitrectomy if there is clinical evidence of infiltration of these structures, or if there is recurrence of the endophthalmitis) and medical therapy:

- intravitreal amphotericin B 5–10 μg if the anterior chamber or vitreous is involved
- topical hourly natamycin 5 percent, topical 0.15 percent amphotericin B or topical 1 percent miconazole
- subconjunctival miconazole or amphotericin B
- oral ketoconazole.

The role of intravitreal corticosteroids remains controversial, but they can be considered if appropriate antimicrobial coverage of the causative organism can be assured (Flynn 2001). Interestingly, Majji et al. (1999) reported on the role of intravitreal dexamethasone in the management of 20 patients with exogenous fungal endophthalmitis; all 20 patients had undergone pars plana vitrectomy, and had received intravitreal amphotericin B and oral ketoconazole, and some patients had also received intravitreal dexamethasone. Overall, nine of the 20 patients achieved a final visual acuity of better than counting fingers at 3 metres. The rate of clearance of inflammation was better in eyes that had received intravitreal dexamethasone. Also, a favorable visual outcome was achieved in a higher number of eyes that had received dexamethasone, compared to eyes not receiving dexamethasone; however, this difference was not statistically significant. These authors cautioned that while the results of their retrospective study suggested that corticosteroids may be beneficial in promoting faster clearance of inflammation in fungal endophthalmitis, the sensitivity of the fungi to antifungals, the dose and timing of steroid, and institution of effective antifungal medication prior to the use of corticosteroids were all essential factors which needed to be examined further in a prospective manner.

Specific types of intraocular mycoses due to different etiologic agents

CANDIDA SPECIES

Candida spp. are common causes of disseminated disease in drug abusers, in severely ill hospitalized patients, and in those patients who are immunocompromised (Donahue et al. 1994). Hence, it is not surprising that in patients with endogenous fungal endophthalmitis, the most common causative organism is *Candida* spp. (Flynn 2001). Endogenous *Candida* endophthalmitis differs from endogenous bacterial endophthalmitis in being more often slowly progressive, sometimes capable of successful management by systemic therapy alone, and in having a more favorable visual prognosis (Brod et al. 1990; Christmas and Smiddy 1996; Essman et al. 1997; Flynn 2001). *Candida albicans* is the most common fungal pathogen causing chorioretinitis or endophthalmitis, while other *Candida* spp., including *Candida glabrata* and *Candida parapsilosis*, are less commonly implicated (Parke et al. 1982; Pettit et al. 1996). This greater tendency of *Candida albicans*, compared to other *Candida* spp., to produce chorioretinitis is thought to be because *Candida albicans* rapidly forms germ tubes in serum (whereas other *Candida* spp. do not), which lodge in the choriocapillaries more easily and more frequently

than other species, or due to differences in the patterns of phospholipase and protease production among different *Candida* spp. (Moyer and Edwards 1993).

Although a number of factors (mentioned above) may predispose to endogenous fungal endophthalmitis, specific risk factors for endophthalmitis due to *Candida* spp. appear to be major surgery involving the gastrointestinal system, parenteral hyperalimentation, and diabetes mellitus (Rao and Hidayat 2001). *Candida* endophthalmitis has also been reported after induced abortion (Chen et al. 1998) and in association with retinopathy in premature infants (Gago et al. 2002). Cataract and intraocular inflammation, and progressive retinopathy of prematurity and tractional retinal detachment, secondary to *Candida* septicemia were also recently reported in a markedly premature infant (Shah et al. 2000); pars plana vitrectomy and lensectomy revealed lens material infiltrated by *Candida*-like yeast forms and pseudohyphae. Song et al. (2002) reported the occurrence of endogenous fungal retinitis, presumed to be due to a *Candida* sp., in a 3-year-old boy who had acute lymphocytic leukemia and a skin rash proven by culture to be due to a *Candida* sp.

The most characteristic ophthalmoscopic sign of *Candida* chorioretinitis is a creamy-white, well-circumscribed lesion involving the retina and choroid in the posterior pole, which may be single or multiple, unilateral or bilateral; the lesions may exist in a satellite pattern, and retinitis may progress to vitreous involvement. Vitreous opacities are typically yellow–white, may be connected by strands, giving a 'string of pearls' appearance, and may be so severe as to obscure the view of the fundus, thereby making the clinical diagnosis difficult (Aguilar et al. 1979; Hamza et al. 1999). In a study of 12 eyes of 12 patients that had been enucleated due to *Candida* endophthalmitis, it was noted that the initial presentation of *Candida* infection was as a vitritis or vitreoretinitis in seven, panuveitis in three, and endophthalmitis in two patients (Rao and Hidayat 2001). Intraretinal hemorrhages may also occur; in fact, focal retinal necrosis and scarring combined with vitreoretinal membrane formation and contraction are the major causes of permanent visual loss (Aguilar et al. 1979; Barrie 1987; Brod et al. 1990). Choroidal neovascularization is a potential cause of late visual loss in patients who have had *Candida albicans* sepsis and endogenous *Candida albicans* chorioretinitis (Jampol et al. 1996). *Candida parapsilosis* may cause a low-grade postcataract extraction endophthalmitis (Hamza et al. 1999), while multifocal endophthalmitis due to *Candida tropicalis* has been reported as the only initial manifestation of pacemaker endocarditis (Shmuely et al. 1997).

Candida retinitis or endogenous endophthalmitis should be suspected in any patient with one of the known predisposing conditions and who presents with progressive chorioretinitis or posterior uveitis. Criteria for the diagnosis of such an infection are discussed above. In addition, detection of anti-*Candida* antibodies in aspirates of anterior chamber fluid may be useful in establishing a diagnosis (Mathis et al. 1988). In histopathological studies on 12 eyes of *Candida* endophthalmitis, fungi were located in the vitreous alone in seven patients, in the vitreous and retina in two, in the vitreous, retina, subretina, and choroid in two, and in the iris and ciliary body in one (Rao and Hidayat, 2001).

Based on a retrospective analysis of treatment outcomes in 20 eyes of 18 patients, Essman et al. (1997) recommended pars plana vitrectomy, intravitreal amphotericin B, and administration of appropriate systemic antifungals (oral fluconazole for *Candida* infections) in managing patients with marked vitreous infiltrates due to endogenous fungal endophthalmitis. However, other therapeutic approaches have been described for chorioretinitis and endophthalmitis due to *Candida* spp. Laatikainen et al. (1992) described a patient with bilateral *Candida* endophthalmitis who was treated with oral fluconazole, and in whom vitrectomy and intravitreal amphotericin B was given for one eye only; both eyes healed equally well. Borne et al. (1993) described a patient with postoperative endophthalmitis due to *Candida parapsilosis* in whom vitrectomy and intravitreal amphotericin B had failed, whereas oral fluconazole resulted in a successful outcome. Luttrull et al. (1995) described rapid (within 24 hours of initiation of therapy) and dramatic responses to oral fluconazole therapy only (400 mg/day) in one patient with culture-proven and in three patients with presumed endogenous *Candida* endophthalmitis. Christmas and Smiddy (1996) reported that pars plana vitrectomy, oral fluconazole therapy (100 mg once or twice daily for 3–6 months), and subconjunctival gentamicin and dexamethasone were sufficient to bring about clinical resolution of lesions and visual improvement in six eyes of five patients with *Candida* endophthalmitis (four eyes culture-positive for *Candida albicans*, one eye culture-positive for *Candida tropicalis*, and one eye direct microscopy positive but culture-negative) without the need for intravitreal amphotericin B or fluconazole therapy. In one patient, oral antifungals alone had been unsuccessful before pars plana vitrectomy, while in two other patients with asymmetric bilateral vitritis, the second eye had not improved despite 11 days of oral fluconazole therapy. These authors concluded that vitrectomy and oral fluconazole without intravitreals could be recommended for successful management of endogenous *Candida* endophthalmitis. More recently, Mora et al. (2002), based on their experience of management and therapy of fungal endophthalmitis in 17 patients (27 eyes) seen over a 9-year period, concluded that while oral fluconazole was successful in managing mild fungal endophthalmitis, additional vitrectomy (sometimes more than once) and intraocular amphotericin B were necessary for more severe cases. Presumed bilateral endophthalmitis due to *Candida* spp. that developed in an infant with bilateral

posterior stage 3 retinopathy of prematurity was successfully eradicated from each eye by vitrectomy with instillation of 5 μg amphotericin B (Gago et al. 2002). In patients who have developed choroidal neovascularization secondary to *Candida* chorioretinitis, laser photocoagulation or surgical excision of the neovascular complex may be of benefit in selected cases (Jampol et al. 1996).

ASPERGILLUS SPECIES

Aspergillus spp. may cause exogenous or endogenous endophthalmitis. Narang et al. (2001), who reviewed data pertaining to 27 patients with exogenous fungal endophthalmitis, principally due to *Aspergillus* spp., noted substantial corneal involvement in 14 eyes; multivariate analysis revealed that this was the single most important risk factor in determining final visual outcome. Tabbara and al-Jabarti (1998) reported an outbreak of ocular aspergillosis (*Aspergillus fumigatus*) after cataract surgery; the outbreak was finally found to be associated with ongoing hospital construction. All five patients developed endophthalmitis, while two patients also had superior limbal, scleral, and corneal infiltration. Endogenous *Aspergillus* endophthalmitis has been reported in patients who have received immunosuppressive agents or who have undergone organ (heart, lung, or liver) transplants or valvular cardiac surgery (Weishaar et al. 1998; Rao and Hidayat 2001) and intravenous drug abusers (Weishaar et al. 1998). Kalina and Campbell (1991) reported endophthalmitis in a patient suffering from chronic lymphocytic leukemia who had presented with acute visual loss and redness in one eye. Although results of stains and cultures of anterior chamber fluid were negative, cultures from antemortem sputum and skin samples were positive for *Aspergillus terreus* while postmortem histological results showed extensive invasion of the posterior vitreous, retina, choroid, and anterior optic nerve by filamentous fungi presumed to be *Aspergillus terreus*. Predisposing systemic conditions may modify the probability of dissemination, risk of mortality, and role for systemic treatment.

The clinical features of endogenous *Aspergillus* endophthalmitis, the ocular presentation, and findings often are characteristic and provide possible diagnostic utility. In a retrospective analysis of ten patients (12 eyes) with culture-proven endogenous *Aspergillus* endophthalmitis, Weishaar et al. (1998) reported that all patients had a 1–3-day history of pain and marked loss of visual acuity in the involved eyes and that varying degrees of vitritis were present in all 12 eyes; in eight of 12 eyes, a central macular chorioretinal inflammatory lesion was present, and an inferior gravitational layering of inflammatory exudate in either or both the subhyaloid and subretinal space. In another retrospective analysis of 13 patients with histopathologically proven *Aspergillus* intraocular infection, the findings at presentation were endophthalmitis in four, panuveitis, acute retinal necrosis, chorioretinitis, or vitritis/vitreoretinitis in two each, and as granulomatous anterior uveitis in one patient (Rao and Hidayat 2001). The severity of retinal involvement in endogenous *Aspergillus* endophthalmitis may range from subretinal or subhyaloid infiltrates to vascular occlusion and full-thickness retinal necrosis; intraretinal hemorrhages are frequent. Rao and Hidayat (2001) reported that in 13 eyes with *Aspergillus* endophthalmitis, fungi were seen in the vitreous, retina, subretina, and choroid in eight, in the vitreous alone in three, and in the vitreous and retina in two. These authors concluded that, histopathologically, the vitreous is the primary focus of infection for *Candida*, whereas it appears that *Aspergillus* grows preferentially along the subretinal pigment epithelium and subretinal space.

Compared with *Candida* spp., *Aspergillus* endophthalmitis is typically more difficult to diagnose because:

- skin test and serology are unreliable
- the diagnostic yield of blood cultures, pulmonary radiographic studies, and echocardiograms is low
- isolation from blood cultures is difficult even in patients who are immunosuppressed with definite dissemination
- systemic manifestations are often lacking in intravenous drug abusers with this infection (Lance et al. 1988; Weishaar et al. 1998).

A diagnostic vitreous aspirate for cytology and culture isolation is usually necessary to identify this etiologic agent (Lance et al. 1988). However, diagnosis of endogenous *Aspergillus* endophthalmitis by anterior chamber or vitreous aspirates alone may be unreliable (Weishaar et al. 1998). According to Rao and Hidayat (2001), since aspergillosis clinically presents with extensive areas of deep retinitis/choroiditis, vitreous biopsy might not always yield positive results. Pars plana vitrectomy specimens assisted by Gram or Giemsa stains appear to have the highest yield of positive cultures for *Aspergillus*; in the series of Weishaar et al. (1998), eight of nine previously untreated eyes had a positive culture from the vitrectomy specimen. Anand et al. (2001) compared PCR against the conventional mycological methods of microscopy and culture for growth of fungi in the diagnosis of *Aspergillus* endophthalmitis in 27 intraocular specimens from 22 patients with suspected fungal endophthalmitis (which were proven to be nonbacterial in origin); 10 patients with non-infective intraocular disorders were the controls. None of the controls was positive by microscopy, culture, or PCR. Among the 27 test samples, four were positive by culture for *Aspergillus* spp. and were also positive by PCR. In addition, PCR detected and identified *Aspergillus* spp. in two culture-negative specimens. The average time required for culture and identification of *Aspergillus* was 10 days, whereas PCR was able to yield results in just 24 hours.

The optimal treatment for endogenous *Aspergillus* endophthalmitis remains controversial. This is in part due to the fact that, in contrast to the outcome of endophthalmitis due to *Candida* spp., the mortality is high in patients with *Aspergillus* endophthalmitis, particularly those who have received organ transplants or who have undergone cardiac surgery (Rao and Hidayat 2001). Moreover, endogenous *Aspergillus* endophthalmitis during systemic aspergillosis usually requires more than systemic treatment to achieve resolution of the ocular infection. The visual outcome in these patients is influenced primarily by the propensity of *Aspergillus* for initial macular choroidal involvement. Based on their results in treatment of endogenous *Aspergillus* endophthalmitis, Weishaar et al. (1998) recommended the following:

- pars plana vitrectomy when vitreous seeding is present to remove the focus of infection and to improve the likelihood of a positive culture, but not necessarily in fellow eyes in which only isolated chorioretinal lesions are present and when the diagnosis can be made from evaluation of the first eye
- amphotericin B (5–10 μg) as intravitreal antifungal treatment
- intravitreal dexamethasone 400 μg (although controversial) to reduce the marked intraocular inflammation in many of these eyes
- intravenous amphotericin B to supplement the effectiveness of intravitreal amphotericin B therapy and to treat proven or clinically suspected systemic aspergillosis
- a well-tolerated and less toxic systemic medication (example, itraconazole) to supplement intravitreal amphotericin B in patients with only endogenous *Aspergillus* endophthalmitis and no systemic manifestations
- repeat intravitreal amphotericin B (5–10 μg) and possibly repeat vitrectomy in patients with persistent vitreous infiltrates and suspected recurrent disease after initial treatment.

Using this regimen to treat 12 eyes of 10 patients with endogenous *Aspergillus* endophthalmitis, final visual acuities of 20/25 to 20/200 were attained in three eyes without central macular involvement; in eight eyes with initial central macular involvement, final visual acuities were 20/400 in three eyes and 5/200 or less in four eyes. Two painful eyes with marked inflammation, hypotony, and retinal detachment were enucleated (Weishaar et al. 1998).

Lance et al. (1988) reported a distinctive case of endogenous endophthalmitis due to *Aspergillus flavus* in an intravenous drug abuser in which the patient recovered useful vision after resolution of the endophthalmitis, which did not recur in spite of extensive retinal and vitreous involvement, since vitrectomy and intravitreal injection of amphotericin B were performed within 48 hours of presentation, followed by intravenous administration of amphotericin B several days later. Unfortunately, others have reported less favorable results. Coskuncan et al. (1994) reported on two cases of *Aspergillus flavus* retinitis (proven by recovery of the fungi in culture of vitreous samples) in patients who had undergone bone marrow transplantation 120 days before; the eyes responded poorly to antifungals. Tabbara and al-Jabarti (1998) reported an outbreak of culture-proven exogenous endophthalmitis due to *Aspergillus fumigatus* in five patients who had undergone cataract extraction during hospital construction; severe postoperative uveitis occurred in all five patients 4–15 days postsurgery and failed to subside with topical corticosteroid therapy. One patient received only antifungals (intravitreal and subconjunctival miconazole, intravenous and intravitreal amphotericin B), two patients had pars plana vitrectomy and intravitreal amphotericin B, while one patient had only pars plana vitrectomy. Unfortunately, evisceration or enucleation had to be ultimately performed for all five patients. Brar et al. (2002) reported the occurrence of *Aspergillus niger* endophthalmitis 9 weeks after cataract surgery, with extension to the cornea; the outcome of the infection was unknown since the patients did not return for follow-up evaluation.

DEMATIACEOUS FUNGI

Fungal endophthalmitis has been reported to be caused by dematiaceous fungi such as *Alternaria alternata* (Rummelt et al. 1991), *Curvularia lunata* (Kaushik et al. 2001), *Exophiala jeanselmei* var. *jeanselmei* (Hofling-Lima et al. 1999) following cataract surgery, and by *Lasiodiplodia theobromae* (Borderie et al. 1997) and *Microsphaeropsis olivacea* (Shah et al. 2001) following keratitis. The prognosis in such infections is unsatisfactory. In the cases reported by Kaushik et al. (2001) and Shah et al. (2001), the intraocular part of the infection resolved quickly after intraocular debridement, and intravitreal, topical, and systemic antifungals, but the corneal infection persisted.

Hofling-Lima et al. (1999) reported on two cases of late endophthalmitis caused by *Exophiala jeanselmei* var. *jeanselmei* after cataract surgery. Although intravitreal and anterior chamber amphotericin B were used in both cases, with initial resolution of the lesions, recurrence occurred in both instances, leading to endophthalmitis.

SCEDOSPORIUM APIOSPERMUM (PSEUDALLESCHERIA BOYDII)

Endogenous endophthalmitis due to *Scedosporium apiospermum* (teleomorph *Pseudallescheria boydii*) also appears to carry a poor prognosis. McGuire et al. (1991) reviewed the clinical and other features of 17 patients with ocular infections due to this fungus reported up to 1991. Numerous predisposing factors in these patients included trauma (penetrating injury, burn, or foreign body), a chronic underlying disease (diabetes mellitus or systemic lupus erythematosus), drowning with aspira-

tion, immunosuppression (for renal transplantation), multiple surgical procedures, and cataract surgery. The clinical findings of the 17 infected eyes included ten endophthalmitides, five corneal ulcers, and two orbital cellulitides. *Scedosporium apiospermum* (reported as *Pseudallescheria boydii*)was cultured in 17 of the 17 reported cases; ocular histopathological descriptions reported hyphae and/or conidia in 13 of the 17 cases. Therapeutic surgical procedures included enucleation for five of the 17 eyes, with one instance each of lysis of adhesions and resection of a mycetoma, orbital debridement, evisceration, lensectomy, and keratoplasty; one patient required a sclerotomy, iridectomy, lensectomy, and synechiectomy; two patients underwent a vitrectomy; and two underwent orbital debridement. Antifungal agents were used in 14 of the 17 patients; however, the etiologic diagnosis was delayed in all 17 patients. Seven patients were treated with only amphotericin B; one required enucleation to eradicate the infection, but there were no deaths in this treatment group. Four patients were treated with amphotericin B initially, then treated with miconazole; two underwent enucleation and two died. One patient had partial treatment with amphotericin B, then miconazole, and finally fluconazole; this patient underwent enucleation before death. In this series of ocular infections, four patients (all disseminated disease) died. In an experimental study by the same group of workers, the fungus was found to produce a severe endogenous endophthalmitis in nine of 17 immunosuppressed rabbits following its injection into the carotid artery; three instances of lens/lens capsule destruction were seen in this group (McGuire et al. 1991). These authors concluded that ocular infections due to *Pseudallescheria boydii* (*Scedosporium apiospermum*) mimic both clinically and histologically those caused by *Aspergillus fumigatus*. More recent reports confirm the severity of endogenous endophthalmitis due to *Scedosporium apiospermum*. McKelvie et al. (2001) reported about two patients with acute myeloid leukemia and neutropenia who developed endophthalmitis due to *Scedosporium apiospermum* and who ultimately succumbed to overwhelming fungal septicemia in spite of treatment with amphotericin B alone in one patient, and combined amphotericin B and fluconazole in the other. Luu et al. (2001) reported the development of endophthalmitis due to this fungus (in spite of concurrent intravenous amphotericin B therapy) in a patient who had undergone liver transplantation, and who had a ring-enhancing brain lesion; the inflammation progressed in spite of pars plana vitrectomy and intravitreal amphotericin B, and the patient died of complications due to brain abscess.

COCCIDIOIDES IMMITIS

Intraocular coccidioidomycosis, which may occur in otherwise healthy individuals, usually presents with multiple, yellow–white, juxtapapillary chorioretinal lesions with pigmented borders, but retinal exudates or serous retinal detachment may also occur (Rodenbiker and Ganley 1980). Fluconazole appears to be useful in the treatment of this condition. Luttrull et al. (1995) reported successful treatment of bilateral multifocal choroiditis and unilateral granulomatous iridocyclitis due to *Coccidioides immitis* in a patient who received 600 mg fluconazole daily. Cunningham et al. (1998) described two patients with intraocular coccidioidomycosis, one with a unilateral, granulomatous iridocyclitis with multiple iris nodules and a large vascularized anterior chamber, and the other with papilledema and multifocal choroiditis, in whom the diagnosis was established by detection of *Coccidioides immitis* spherules in skin biopsy samples; the patients were successfully treated with local and systemic amphotericin B and oral fluconazole.

BLASTOMYCES DERMATITIDIS

Intraocular blastomycosis is an unusual occurrence. Choroidal involvement (manifesting as bilateral, multiple, yellow–white posterior fundus lesions) has been reported to occur in association with miliary or disseminated blastomycosis (Sinskey and Anderson 1955; Lewis et al. 1988), while isolated endophthalmitis (Safneck et al. 1990) and panophthalmitis (Font et al. 1967) due to *Blastomyces dermatitidis*, without any systemic manifestations, has also been reported. In a case described by Bond et al. (1982), a patient with biopsy-proven sarcoidosis developed fever, skin lesions and anterior uveitis, vitritis, and a large posterior yellow, elevated choroidal lesion in one eye and three white posterior small choroidal lesions in the other eye. Although a diagnosis of blastomycosis was established by the biopsy of the skin lesion, it was unclear whether the ocular manifestations in this patient were due to blastomycosis or sarcoidosis. Li et al. (1998) reported a patient with concurrent unilateral endophthalmitis and orbital cellulitis proven by histological findings and by culture to be due to *Blastomyces dermatitidis*. Amphotericin B therapy was shown to produce a rapid resolution of choroidal and extraocular lesions in one patient (Lewis et al. 1988), without any recurrence over a 6-month follow-up period. However, in general, the therapy of intraocular blastomycosis appears to be unrewarding, with enucleation of the affected eye having to be performed to control the infection in most instances (Sinskey and Anderson 1955; Font et al. 1967; Safneck et al. 1990; Li et al. 1998). Intraocular dissemination of blastomycosis should be suspected in the differential diagnosis of endophthalmitis in patients with previous or active pulmonary lesions of equivocal nature. Early diagnosis and prompt treatment with antifungal medications are essential.

CRYPTOCOCCUS NEOFORMANS

Intraocular cryptococcosis usually results from cryptococcal septicemia with severe meningeal infection; such

sequelae may be seen in patients with AIDS. Cohen and Glasgow (1993) reported the occurrence of sudden, simultaneously bilateral blindness in a patient with AIDS who had cryptococcal meningitis; although the sudden, bilateral visual loss in this patient was ultimately found to have been caused by focal but fulminant necrosis of both optic nerves, the authors believed that the presence of cryptococcal organisms throughout the basal meninges and in the sheaths of both optic nerves suggests that cryptococcosis may produce visual loss by damaging multiple areas of the anterior visual pathway. Agarwal et al. (1991) reported the occurrence of retinitis (presumably due to cryptococcal infection) following disseminated cryptococcosis in a patient who had received a renal allograft. However, Hester et al. (1992) described isolated ocular cryptococcosis in a 62-year-old female immunocompetent patient. Hence, ocular cryptococcal infection must be suspected, even in the absence of predisposing factors or systemic findings. The diagnosis of cryptococcal chorioretinitis is a presumptive one in a patient with characteristic fundus lesions, with or without vitritis, and documented cryptococcal meningitis or disseminated cryptococcosis (Klotz et al. 2000). In one patient, transscleral needle biopsy of a subretinal mass was used to establish the diagnosis of subretinal cryptococcosis (Hester et al. 1992). A vitreous tap or biopsy may be done if vitritis is present. Cryptococcal chorioretinitis can be treated with intravenous amphotericin B (Hester et al. 1992) or oral fluconazole (Agarwal et al. 1991). Vitrectomy may be needed if chorioretinitis progresses to endophthalmitis.

PARACOCCIDIOIDES BRASILIENSIS

Ocular paracoccidioidomycosis usually manifests as lesions in the eyelid and conjunctiva (Silva et al. 1988). Cases of choroidal granuloma and endophthalmitis have been described, but few have been histopathologically proven (Bonomo et al. 1982). Finamor et al. (2002) recently described the occurrence of ocular and central nervous system paracoccidioidomycosis in a pregnant woman with AIDS. The patient presented with an acute and painful eye, and ophthalmological examination revealed unilateral severe iridocyclitis, severe vitritis, and a granulomatous chorioretinal lesion adjacent to and involving the optic nerve; the lesions evolved after a week to iris neovascularization, neovascular glaucoma, and retinal detachment. The eye was enucleated due to intense pain. Macroscopic study of the enucleated eye showed a total retinal detachment with subretinal exudate and a white mass in the vitreous cavity displacing the lens to the anterior chamber. Histopathological examination of the enucleated eye, as well as a biopsy of an oral lesion, revealed a chronic granulomatous inflammation with epithelioid cells, multinucleated giant cells, and several round yeasts exhibiting 'ship's wheel' external budding in sections stained by periodic acid–Schiff and Grocott methenamine silver stains, an appearance typical of *Paracoccidioides brasiliensis*. With the diagnosis of disseminated ocular paracoccidioidomycosis, the patient was treated with trimethoprim-sulfamethoxazole with a satisfactory outcome and reduction in the size of the brain lesion (Finamor et al. 2002).

HISTOPLASMA CAPSULATUM

The 'presumed ocular histoplasmosis syndrome' is characterized by the presence of multifocal choroiditis scattered throughout the fundus, the peripapillary area, and sometimes in the macular area, with some lesions exhibiting healing with variable chorioretinal scarring (Katz et al. 1997). This syndrome is not associated with intraocular inflammation, and is well tolerated by the eye, unless complications of subretinal neovascularization, such as progressive visual loss, arise (Gonzales et al. 2000). In such instances, vitreoretinal surgical techniques have been used to remove the subfoveal neovascular membranes while preserving the overlying neurosensory retina, and thus preserving central visual acuity (Thomas and Kaplan 1991). *Histoplasma capsulatum* var. *capsulatum* has been isolated from the eyes of patients suffering from this syndrome, suggesting that this fungus is the etiologic agent (Katz et al. 1997), although this is a contentious point. However, *Histoplasma capsulatum* var. *capsulatum* has been implicated as the etiologic agent in several other intraocular lesions. Specht et al. (1991) reported on ocular histoplasmosis with retinitis in a patient with AIDS and disseminated histoplasmosis, who had complained of a hazy spot in the vision of his left eye; distinct creamy-white intraretinal and subretinal infiltrates were visualized in both eyes. After the patient succumbed to his illness, light microscopy of his eyes removed at autopsy revealed that the most prominent colonization of ocular tissue by yeasts was in the retinae and optic nerves, with focal choroiditis near the retinal lesions. These lesions showed variable numbers of lymphocytes and histiocytes, and yeasts with a morphology suggestive of *Histoplasma capsulatum* var. *capsulatum*, free or phagocytized within cells, in all layers of the retinal lesions, sometimes with extension to the subretinal and subhyaloid spaces. One eye also exhibited necrotizing granulomatous cyclitis with aggregates of yeasts. Histoplasmic optic neuritis was reported by both Macher et al. (1985) and Specht et al. (1991), while histoplasmic cyclitis was also noted by Specht et al. (1991). Endophthalmitis due to *Histoplasma capsulatum* var. *capsulatum* in a patient with AIDS has also been reported (Gonzales et al. 2000).

SPOROTHRIX SCHENCKII

Risk factors for endophthalmitis due to *Sporothrix schenckii* include AIDS (Kurosawa et al. 1988) and trauma (Witherspoon et al. 1990); however, this ocular

infection may occur even without trauma or systemic infection (Cartwright et al. 1993). Vieira-Dias et al. (1997) reported the occurrence of concomitant ocular and cutaneous sporotrichosis, where the fungus was isolated from skin lesions and the aqueous humor. Endophthalmitis due to *Sporothrix schenckii* usually presents initially as a granulomatous uveitis, which may be treated with corticosteroids, therein leading to progression of the lesion and resulting in frank endophthalmitis (Kurosawa et al. 1988) or scleral perforation (Cartwright et al. 1993). Up to 1990, 17 episodes of endophthalmitis due to *Sporothrix schenckii* had been reported (Witherspoon et al. 1990). Curi et al. (2003) described the occurrence of fluffy vitreous opacities and a retinal granuloma in a young man who presented with disseminated ulcerated skin lesions that were proven by biopsy and by culture to be due to *Sporothrix schenckii*. It is important to remember that although intraocular infection due to *Sporothrix schenckii* is uncommon, it can occur in cases of disseminated sporotrichosis. Systemic therapy with potassium iodide or itraconazole is a successful means to control skin and ocular sporotrichosis.

ACREMONIUM SPECIES

Exogenous endophthalmitis due to *Acremonium* spp. may arise due to contamination from a common environmental source. Weissgold et al. (1996) described four patients who presented after cataract surgery with delayed-onset endophthalmitis caused by *Acremonium kiliense* with in vitro sensitivity to amphotericin B. In all patients, ocular infection was recalcitrant to single-dose intravitreous amphotericin B injection (5 μg) and systemic administration of fluconazole. These patients subsequently responded to vitrectomy followed by additional intravitreous amphotericin B injection. These authors concluded that single-dose administration of intravitreous amphotericin B may be inadequate treatment for fungal endophthalmitis caused by *Acremonium kiliense*, and that vitrectomy with repeated intravitreous administration of amphotericin B may be necessary to eradicate intraocular infection caused by this organism.

ZYGOMYCETES

Orgel and Cohen (1989) described the first case of postoperative zygomycete endophthalmitis that occurred after uncomplicated phacoemulsification and the insertion of a posterior chamber intraocular lens. Diagnosis, in the presence of negative aqueous and vitreous cultures, was confirmed by immunofluorescence staining of an anterior chamber inflammatory mass. Successful treatment of the eye with 20 μg intraocular amphotericin B may have been made possible in part by the fact that the posterior capsule remained intact, keeping the eye bicompartmental. The evolution of fundus changes from edema of the optic disk and peripapillary retina to optic atrophy and chorioretinal pigmentary changes has been rarely reported in rhino-orbital zygomycosis (Newman and Kline 1997; Kadayifcilar et al. 2001). Unique chorioretinal changes caused by choroidal ischemia secondary to rhino-orbital zygomycosis have also been reported (Kadayifcilar et al. 2001).

FUSARIUM SPECIES

Intraocular lesions due to *Fusarium* spp. may occur in patients with leukemia or as a consequence of the progression of corneal lesions. Patel et al. (1994) reported on a patient with acute lymphocytic leukemia with both endogenous *Fusarium* endophthalmitis and leukemic ocular infiltrates who presented with sudden visual loss in one eye, which exhibited a dense white placoid infiltrate in the macula extending into the vitreous, and with an iris nodule and hypopyon in the other eye. Histopathological studies performed after the patient's death from multisystem failure disclosed panophthalmitis with *Fusarium* invading all the ocular coats in the right eye, while in the left eye there were leukemic infiltrates in the iris, anterior chamber, and trabecular meshwork. These authors postulated that the ocular destructiveness of *Fusarium* spp. may be caused by marked mycotic vascular invasion and occlusion with consequent infarction and necrosis of ocular tissues. Dursun et al. (2003) reported that 10 out of a total of 159 patients with keratitis due to *Fusarium* spp. (seven due to *Fusarium oxysporum*, two to *Fusarium solani*, and one to a *Fusarium* sp.) progressed to endophthalmitis. Cultures of aqueous and intraocular tissues grew *Fusarium* in eight cases, whereas vitreous cultures were positive in two. Nine cases had preexisting risk factors. All patients received oral ketoconazole or fluconazole and topical natamycin 5 percent, and intravitreal amphotericin B injections were also given in two patients. Four patients required a penetrating keratoplasty, enucleation was performed in two patients, two patients required a combination of a penetrating keratoplasty and pars plana vitrectomy, and one patient developed phthisis bulbi.

PAECILOMYCES LILACINUS

Paecilomyces lilacinus is an uncommon cause of exogenous endophthalmitis. However, this form of endophthalmitis has generally been associated with poor visual outcome. In a series of 13 cases of *Paecilomyces lilacinus* endophthalmitis following implantation of contaminated intraocular lenses, eight eyes were enucleated, two had a final vision of no light perception, and one had only light perception; retention of useful vision in the remaining two eyes was attributed to early removal of the lens and inflammatory material in one case and prompt use of intraocular miconazole in the other (Pettit et al. 1980). In another series of patients with postoperative *Paecilomyces lilacinus* endophthal-

mitis, enucleation was performed in two of the three cases; the salvaged eye was treated with miconazole early in the course of the disease (Kozarsky et al. 1984). In a case report of successful treatment of *Paecilomyces lilacinus* endophthalmitis following cataract extraction, treatment consisted of early vitrectomy, multiple intravitreal injections of amphotericin B and miconazole, intravenous miconazole, and later, oral ketoconazole; the intraocular lens removed 6 weeks after initiation of therapy was found to contain *Paecilomyces lilacinus*. After 8 months, excellent recovery of visual acuity was noted (Levin et al. 1987). Current management of ocular infections due to *Paecilomyces lilacinus* continues to be challenging because of the organism's resistance to amphotericin B, natamycin, and, in some cases, fluconazole (Aguilar et al. 1998; Sutton et al. 1998) and because miconazole and ketoconazole, agents to which *Paecilomyces lilacinus* is usually susceptible, are not usually included in the initial management of suspected fungal endophthalmitis (Scott et al. 2002). The sensitivity profile of *Paecilomyces lilacinus* also differs significantly from that of *Paecilomyces variotii* (Sutton et al. 1998). Therefore, initial treatment with miconazole (intravitreal, 25 μg/0.1 ml) has been suggested as a good option since, to date, miconazole is the only known medication to which *Paecilomyces lilacinus* and *Paecilomyces variotii* are consistently sensitive. Miconazole is currently available in powder form, which can be used to prepare topical and intravitreal preparations. If, however, miconazole is not readily available, a combination of intravitreal amphotericin B and oral ketoconazole (200–400 mg four times daily) has been suggested since *Paecilomyces variotii* is sensitive to amphotericin B and *Paecilomyces lilacinus* is sensitive to ketoconazole (Scott et al. 2002). However, while intravitreal amphotericin B is readily available from most pharmacies, ketoconazole is available only in an oral preparation. Intravitreal administration of fluconazole may be useful since approximately 10 percent of *Paecilomyces lilacinus* organisms are sensitive to fluconazole, which can be prepared in an intravitreal form. In the absence of miconazole, oral ketoconazole and intravitreal fluconazole may be considered (Scott et al. 2002).

PNEUMOCYSTIS CARINII

Patients with AIDS are at risk to develop pulmonary disease due to *Pneumocystis carinii*. Although aerosolized pentamidine may be given as prophylaxis in such patients, this does not protect against extrapulmonary disease. Dugel et al. (1990) and Foster et al. (1991) described the occurrence of choroidal lesions which appeared to be typical of *Pneumocystis carinii* in two and three patients, respectively, who were receiving prophylactic aerosolized pentamidine therapy. The lesions resolved with intravenous pentamidine therapy in four of the patients, while the lesions in the remaining patient resolved with intravenous trimethoprim and sulfamethoxazole. All these patients did not have clinical or laboratory evidence of *Pneumocystis carinii* infection other than in the eye. The choroidal lesions of *Pneumocystis carinii* manifest as yellow–white to orange spots without vitreous inflammation (Morinelli et al. 1993). Early ophthalmological examination may detect these lesions before they are threatening to sight, and allows systemic therapy to be instituted before widely disseminated infection due to *Pneumocystis carinii* results in a fatal outcome. Intravenous pentamidine therapy appears to control *Pneumocystis carinii* choroiditis but may need to be continued to prevent exacerbation. Presumed *Pneumocystis carinii* choroiditis may serve as a marker for disseminated infection.

FUNGAL OCULAR INFECTIONS AFTER OPHTHALMIC SURGICAL PROCEDURES

Certain surgical procedures are unique to ophthalmology. The most important ophthalmic surgical procedure is cataract extraction, and the most important fungal infection following cataract extraction is fungal endophthalmitis (see earlier under Intraocular mycoses). Fungal infections, such as keratitis and scleritis, have been reported following corneal refractive surgical procedures [radial keratotomy, photorefractive keratectomy, laser-in-situ keratomileusis (LASIK)], keratoplasty (corneal transplantation), pterygium excision, and cataract extraction.

Postoperative infectious keratitis is an uncommon, but serious, complication of radial keratotomy; the use of topical corticosteroids and the presence of corneal incisions are probably risk factors. There have been at least five reported cases of mycotic keratitis following radial keratotomy, two each due to species of *Fusarium* and *Aspergillus*, and one due to *Candida parapsilosis* (Maskin and Alfonso 1992; Gussler et al. 1995; Heidemann et al. 1995; Panda et al. 1998). Three of these responded to antifungals alone; the other two required therapeutic penetrating keratoplasty, wherein the lesions resolved.

Since LASIK combines the precision of excimer laser photoablation with the advantages of an intrastromal procedure that maintains the integrity of Bowman's layer and the overlying corneal epithelium, the risk of infectious keratitis following this procedure should be, theoretically speaking, minimal. However, microbial contamination of the corneal stromal bed may occur during surgery due to the proximity of the eyelids, eyelashes, conjunctiva, and microkeratome. This, combined with the use of topical corticosteroids, unstable epithelium at the edge of the lamellar flap, reduced corneal sensitivity and use of contact lenses, all render these eyes susceptible to infection. Read et al. (2000) reported the first case of mycotic keratitis due to *Acremonium atrogriseum* following LASIK; since then, there have been reports of at least five other patients afflicted with

this condition, in which six different species of fungi from five genera (*Aspergillus flavus*, *Aspergillus fumigatus*, *Curvularia* spp., *Fusarium solani*, *Scedosporium apiospermum*) have been implicated (Chung et al. 2000; Sridhar et al. 2000a, b; Kuo et al. 2001; Verma and Tuft 2002). Only two of these patients responded to medical therapy alone, and therapeutic penetrating keratoplasty was ultimately required for the other four. To achieve a favorable outcome in such infections, the fungus involved should first be identified as soon as possible; sampling at the site of infection (from underneath the flap) provides the best chance of obtaining a positive culture. This is followed by irrigation of the stromal bed with antifungal agents, and intensive treatment with specific systemic antifungals.

Mycotic keratitis has been reported to occur following superficial (lamellar) (Panda et al. 1999) or deep (penetrating) keratoplasty (Levenson et al. 1984; Wilhelmus and Robinson 1991; Ainbinder et al. 1998; Benaoudia et al. 1999), following repair of a dehiscent keratoplasty wound (Kisla et al. 2000), and secondary to endophthalmitis occurring after phacoemulsification and intraocular lens implantation surgery (Fekrat et al. 1995; Weissgold et al. 1998). The nine patients involved required surgery (therapeutic penetrating keratoplasty in seven, optical keratoplasty in one, and debridement in one). Four different yeast species (*Candida albicans*, *Candida guilliermondii*, *Candida parapsilosis*, and a *Rhodotorula* sp.) were isolated from the lesions of four patients, and four different species of filamentous fungi (*Exophiala dermatitidis* in two patients, *Acremonium kiliense*, *Beauveria bassiana*, and a presumed *Fusarium* sp. in one patient each) from the others.

Fungal scleritis has been reported to occur in at least 13 patients following various ophthalmic surgical procedures, including excision of pterygia (fleshy conjunctival growths) without or with β-irradiation (Margo et al. 1988; Moriarty et al. 1993; Sullivan et al. 1994; Kumar et al. 1997; Taravella et al. 1997), trabeculectomy (filtering surgery for glaucoma), and cataract extraction (Carlson et al. 1992; Bernauer et al. 1998; Locher et al. 1998). Various modalities of antifungal therapy, as well as surgical debridement, were not found useful in five of these 13 patients, with enucleation eventually having to be performed; *Scedosporium apiospermum* (*Pseudallescheria boydii*) was identified as the cause in two of these patients, *Scedosporium prolificans* in one, and an *Aspergillus* sp. in one. The identification of a *Rhizopus* sp. in the remaining patient is fraught with uncertainty since fungal hyphae were not visualized in the samples collected, and just one colony of the fungus was recovered in culture (Locher et al. 1998). Eight of 13 patients required some form of surgical intervention such as scleral debridement or resection, or removal of plaque, to ensure resolution of the infection; the fungi involved in these eight patients were *Aspergillus flavus* and *Aspergillus* spp. (in five patients), and *Scedosporium apiospermum*, *Scedosporium prolificans*, and a *Fusarium* sp. (in one patient each) (Carlson et al. 1992; Moriarty et al. 1993; Kumar et al. 1997; Bernauer et al. 1998).

Fungal infection of the self-sealing incision used for cataract surgery was reported in seven patients from different locales in India (Garg et al. 2003). The initial diagnoses at the time of onset of symptoms were keratitis, scleritis, and excessive anterior chamber reaction, but all cases subsequently developed deep keratitis. The diagnosis of fungal infection was established by microscopy and culture of corneal scrapings, corneoscleral biopsies, and anterior chamber aspirates, and the fungi isolated were species of *Aspergillus* in six patients and *Candida albicans* in one patient. The infection resolved with good visual recovery by medical therapy alone in two patients, but progressed to endophthalmitis and complete visual loss in five eyes.

OPHTHALMIC MYCOSES ASSOCIATED WITH AIDS

Infections by opportunistic microorganisms constitute an important ocular manifestation of AIDS, although ocular findings are infrequent in human immunodeficiency virus (HIV)-infected, asymptomatic individuals. Cytomegalovirus retinitis is reported to be the most common intraocular infection in AIDS patients while other opportunistic ocular infections are considerably less common (Jabs et al. 1989; Seal et al. 1998). Various types of ophthalmic mycoses, principally affecting the orbit and intraocular structures, have been reported in association with AIDS.

Frequency

Autopsy findings in 25 patients who died of AIDS revealed opportunistic ocular infections in eight patients, including retinitis due to a *Candida* spp. in one patient and choroiditis due to *Cryptococcus neoformans* var. *neoformans* in one patient (Jabs et al. 1989). Earlier, Schuman and Friedman (1983) had reported the occurrence of retinitis due to *Candida albicans* and *Cryptococcus neoformans* in two of 34 patients with AIDS; bacterial corneal ulceration was noted in 2 percent, but fungal corneal ulceration was not noted at all.

Orbital infections

Opportunistic infections of the orbit due to bacteria, fungi, and protozoa constitute serious complications of HIV infection and frequently lead to ocular morbidity and mortality. Orbital mycoses associated with AIDS have been reported in 15 patients since 1991 (Blatt et al. 1991; Friedberg et al. 1992; Vitale et al. 1992; Meyer et al. 1994; Kronish et al. 1996; Lee et al. 1996; Johnson et al. 1999; Hejny et al. 2001). The orbital mycotic infec-

tion resolved or improved in just six of these patients. Surprisingly, there was resolution or improvement following debridement and intravenous amphotericin B therapy in two of the three patients with rhino-orbital zygomycosis, perhaps because these had relatively focal lesions (Blatt et al. 1991; Hejny et al. 2001). There was complete resolution of lesions in the one patient with *Pneumocystis carinii* infection after treatment with trimethoprim and sulfamethoxazole (Friedberg et al. 1992). Eleven patients had infections due to *Aspergillus fumigatus*, and 10 of these were treated with surgery and intravenous amphotericin B. The patient survived and the mycotic infection resolved in only three of these patients who appeared to have relatively focal orbital lesions without intracranial disease; two of the survivors received surgery and amphotericin B (intravenous and local irrigation), while the third was treated by debridement, amphotericin B lipid complex, liposomal amphotericin B, and orbital exenteration (Vitale et al. 1992; Kronish et al. 1996; Johnson et al. 1999). Overall, it appears that orbital aspergillosis in patients with AIDS has a poor outcome.

Optic neuritis

Although there are many causes of optic neutritis in AIDS patients, a fungal etiology has been reported in two patients, the pathogen being *Histoplasma capsulatum* var. *capsulatum* (Specht et al. 1991; Yau et al. 1996); in one of these, the optic neuritis was associated with retinitis and uveitis.

Corneal infections

Fungal corneal infections appear to be infrequent in HIV-infected patients, but tend to be severe and prone to corneal perforation when they do occur and to manifest even when known risk factors for ulcerative keratitis are absent (Hemady 1995). There have been reports of six patients with mycotic keratitis associated with AIDS (in one patient, the diagnosis of AIDS was made postmortem); *Candida albicans* was the fungus implicated in all six patients (Parrish et al. 1987; Hemady 1995). The keratitis resolved in all six with topical amphotericin B (0.15 percent) therapy.

Other mycotic infections of the anterior segment reported in AIDS include limbal nodules (and multifocal choroiditis) in one patient (Muccioli et al. 1995) and an iris inflammatory mass in another (Charles et al. 1992); histopathological studies permitted a presumptive diagnosis of *Cryptococcus neformans* var. *neoformans* infection.

Presumed mycoses of the posterior segment in patients with AIDS include multifocal choroiditis (choroidopathy) and retinitis due to cryptococcosis, histoplasmosis, candidiasis, and *Pneumocystis carinii* infection (Macher et al. 1985; Jabs et al. 1989; Shami et al. 1991; Morinelli et al. 1993; Muccioli et al. 1995; Verma and Graham 1995). The choroidal lesions of *Pneumocystis carinii* manifest as yellow–white to orange spots without vitreous inflammation (Morinelli et al. 1993). Culture-proven endogenous endophthalmitis due to *Bipolaris hawaiiensis*, a *Fusarium* sp., *Sporothrix schenckii*, and *Histoplasma capsulatum* var. *capsulatum* has also been reported (Kurosawa et al. 1988; Pavan and Margo 1993; Glasgow et al. 1996; Gonzales et al. 2000); complete resolution of lesions was achieved by surgery and amphotericin B and fluconazole therapy only in the patient with the *Bipolaris* infection. Although central nervous system infection with *Cryptococcus neoformans* var. *neoformans* is common in patients with AIDS, actual invasion of the intraocular structures by the fungus appears relatively uncommon. In one study of 80 HIV-seropositive patients with cryptococcal infections, papilledema, visual loss, abducens nerve palsy, and optic atrophy were the important ophthalmic aberrations noted; however, actual invasion of the intraocular structures was an uncommon complication (Kestelyn et al. 1993).

OPHTHALMIC MYCOSES ASSOCIATED WITH OCULAR BIOMATERIALS

Microbial colonization of indwelling and implanted biomedical devices constructed of polymers, silicones, and metals, such as shunts or catheters, can lead to serious, often lethal, infection (Wilson 1996). The organisms responsible for such biomaterial-related infections are usually part of the resident microbial flora at a particular area of the body, therein posing a constant threat. Factors believed to contribute to the mechanisms of infection associated with biomedical devices include:

- intraoperative contamination during surgical implantation or extraluminal migration of organisms, which permits potential pathogens to transcend normal protective barriers
- production of mucoid substances by microorganisms, which facilitates adhesion of colonizing microorganisms, and also protects them from various host defense mechanisms
- the presence of plasma proteins (especially fibronectin) on the surface of the biopolymer, which may promote attachment of staphylococci and *Candida* spp. to the surface (Wilson 1996).

Contact lens plastics and their storage cases, and intraocular lens implants, are the two most important categories of biomaterials used in ophthalmology.

Fungal contamination of intraocular lenses

The occurrence of postoperative inflammation following implantation of an intraocular lens depends on various

factors, such as the type and surface quality of the lens, the fixation of the posterior chamber lens, the surgical technique adopted, the use of topical corticosteroids, systemic antibacterials, and nonsteroidal anti-inflammatory drugs as a prophylactic measure, as well as the experience of the surgeon. The use of polymethylmethacrylate single-block lenses in eyes with uveitis is advantageous since the complement cascade is not activated, while surface-modified lenses, such as heparin-coated models, may confer some protection against reactivation of inflammation (Biswas and Kumar 2002). Although improvements in the quality of the lenses and microsurgical techniques have led to a decrease in the frequency of complications from intraocular lenses, lenses that have been implanted intraocularly may still need to be explanted for various reasons. In a cytopathological study of five intraocular lenses that had to be removed due to endophthalmitis, fibrinous material was noted on three, granulomatous inflammation on one, and nongranulomatous inflammation with fungal filaments on the surface on one (Biswas and Kumar 2002); thus, careful examination of the intraocular lens surface in such cases would help in establishing a diagnosis of fungal endophthalmitis (Figure 18). In instances where the explantation of a contaminated intraocular lens is contraindicated, drug therapy may be the only alternative. Penk and Pittrow (1999) recently reviewed the role of fluconazole in the long-term suppression of fungal infections (mainly due to *Candida* spp.) in patients with artificial implants; their review included intraocular lenses in nine patients. These authors concluded that in such cases, fluconazole exhibited an excellent safety profile and therapeutic efficacy in doses of 750 mg/day; however, they cautioned that higher doses and lifelong treatment might be indicated in certain instances.

An outbreak of *Candida parapsilosis* endophthalmitis was reported in 13 patients from three states of the USA who had had an intraocular lens implantation between November 1983 and January 1984 (McCray et al. 1986). This outbreak was traced to the introduction in July 1983 of a new brand of BSS as an intraoperative ophthalmic irrigation solution that was subsequently recalled because of intrinsic fungal contamination. A retrospective cohort study revealed that definite exposure to the product was a significant risk factor for *Candida parapsilosis* endophthalmitis, while a retrospective case–control study, including 203 control patients with definite exposure to BSS, suggested that exposure to systemic steroids was an additional risk factor for this type of infection. All 13 patients received topical, intraocular, or systemic antifungal therapy (or a combination of these modalities), and 10 also underwent vitrectomy. No patients had systemic symptoms or complete visual loss. Laboratory investigations showed a 6.7 percent overall contamination of the product with *Candida parapsilosis*. The problem did not recur following recall of the product by the manufacturer.

Thirteen cases of fungal endophthalmitis caused by *Paecilomyces lilacinus* were attributed to implantation of intraocular lenses that had been sterilized in sodium hydroxide and neutralized in sodium bicarbonate (Pettit et al. 1980). Twelve of the 13 patients had received neutralizing solution from one particular lot, and the same fungus that caused the endophthalmitis was cultured from several of the neutralizing solutions from this particular lot. Eight of the 13 eyes eventually required enucleation. Of the remaining five eyes, only two recovered useful vision. This surgically induced epidemic of fungal endophthalmitis clearly shows the major consequences of a breakdown in quality control for any substance or material used intraocularly. Tabbara and al-Jabarti (1998) reported on the occurrence of exogenous endophthalmitis due to *Aspergillus fumigatus* in five patients who had undergone extracapsular cataract surgery with posterior chamber intraocular lens implantation. In these patients, however, the intraocular lens was not at fault but, rather, ongoing hospital construction work.

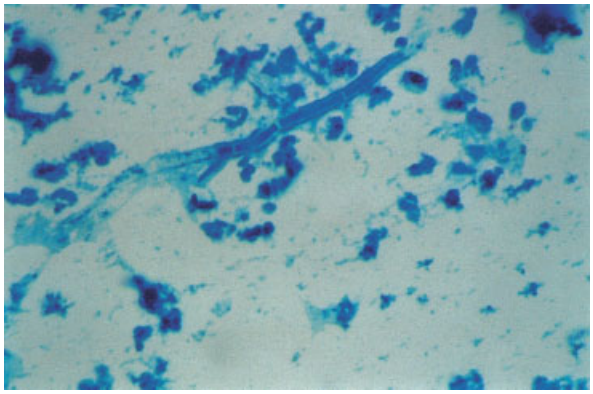

Figure 18 *Photomicrograph of fungal hyphae (*Aspergillus fumigatus*) on an intraocular lens that was removed due to endophthalmitis. Lactophenol cotton blue stain; magnification ×400*

Fungal contamination of contact lenses

Contact lens-associated mycotic keratitis appears to be relatively uncommon, when compared to contact lens-associated bacterial or *Acanthamoeba* keratitis, because fungi isolated from the healthy outer eye only transiently colonize this area, and are not normally resident in the outer eye (Srinivasan et al. 1991; Liesegang 1997). When soft lenses are worn continuously, fungal conidia adhere to the lens surface and, under favorable conditions, germinate; fungal hyphae are able to enter the matrix of the soft lens, project through the posterior surface and then penetrate the corneal epithelium, resulting in fungal infection (Simmons et al. 1986). Factors that possibly contribute to such infiltration

include fungal enzymatic activity and properties of the lens material, which provide a matrix as well as a source of nutrients for growth of the fungi (Yamamoto et al. 1979). Fungi have been found to penetrate the matrix of extended-wear soft contact lenses, both during normal use (Yamamoto et al. 1979; Berger and Streeten 1981; Wilson and Ahearn 1986) and in laboratory studies (Yamaguchi et al. 1984). When contact lenses with a water content of 45 or 73.5 percent were challenged with *Fusarium solani* or *Aspergillus flavus* on Sabouraud glucose neopeptone agar, it was found that the fungi penetrated both lenses but appeared to grow more vigorously into the lenses with the higher water content, resulting in physical and metabolic degradation of these lenses (Yamaguchi et al. 1984). In another study, it was thought that contamination of 11 extended-wear soft contact lenses by fungi occurred while the lenses were on the eye (Wilson and Ahearn 1986); in two cases, the same fungi isolated from the contaminated lenses were also obtained from corneal ulcers immediately underlying the fungal contamination on the soft lens. Simmons et al. (1986) reported that filamentous fungi of the genera *Acremonium*, *Aspergillus*, *Alternaria*, *Cladosporium*, *Curvularia*, and *Fusarium* penetrated the matrix of soft contact lenses during normal use and in laboratory studies. The species of *Acremonium*, *Aspergillus*, and *Fusarium* readily penetrated lenses having a water content of 55 percent or greater following incubation for 120–168 hours; when germinating on lenses, the conidia of these fungi appeared to be often associated with small salt deposits. Dematiaceous fungi appeared to penetrate intraocular lenses very rapidly. Conidia of *Cladosporium cladosporioides* were found to germinate on a new 55 percent water-content lens, either producing flattened appressorium-like cells on the lens surface or penetrating the lens with a finely coiled and tapered hyphal element within the first 12 hours of incubation; conidia of *Curvularia lunata* were found to germinate and penetrate into and through a new 55 percent water-content lens within 96 hours. Some hyphae adhered primarily to ridges or crevices or occasionally to small salt deposits on the lenses, but adherence to smooth surfaces was also observed. Growth of the fungal hyphae (which were coiled within the lens matrix) increased with increasing water content of the lens (Simmons et al. 1986). Based on these findings, these authors agreed with the general recommendation that lenses with fungal deposits be replaced, and also advised frequent and regular cleaning and disinfection of extended-wear lenses. They also suggested that activities in moldy habitats, such as raking leaves, cleaning dusty areas, and showering in rooms with moldy plastic curtains or tiles, be completely avoided while wearing extended-wear lenses, or that the lenses be cleaned and disinfected after such activities.

If fungal conidia alight on the surface of a contact lens, they are normally removed by surface cleaning of the lens. If such lenses are worn without proper cleaning for an extended duration, fungi may adhere, and penetrate the contact lens (Wilson 1996). This may explain why soft lenses for aphakia and therapeutic extended wear have been most frequently implicated in such infections (Wilson and Ahearn 1986; Starr 1987; Wilhelmus et al. 1988).

Fungal infection was reported in four (4 percent) of 90 contact lens wearers, and in four (27 percent) of patients who wore therapeutic bandage-contact lenses (Wilhelmus et al. 1988). Filamentous fungi (*Aspergillus flavus*, *Fusarium dimerum*, *Fusarium* sp.) were also reported to have been isolated from the corneal scrapes of several other patients in South Florida who had contact lens-associated fungal keratitis (Liesegang and Forster 1980; Alfonso et al. 1986). Interestingly, Wilhelmus et al. (1988) observed that filamentous fungi are more likely to be associated with keratitis in persons wearing cosmetic or aphakic lenses, whereas yeasts were more frequently found with therapeutic lens use. Rosa et al. (1994) reported that six of their 125 patients with mycotic keratitis in south Florida wore extended-wear contact lenses; *Fusarium oxysporum* was isolated from four patients and *Candida albicans* and a *Paecilomyces* sp. from one patient each. In one patient who wore a bandage contact lens, keratitis due to *Candida parapsilosis* developed. Liesegang and Forster (1980) had earlier reported the occurrence of fungal keratitis in three patients who wore soft contact lenses, the fungi isolated being *Aspergillus flavus* and *Fusarium dimerum*. *Candida parapsilosis* keratitis, associated with contact lens wear, was reported in an elderly Israeli patient who developed stromal infiltration at the donor/recipient interface 2 years after penetrating keratoplasty, while wearing a 'piggyback' type of contact lens; the infection resolved with amphotericin B and flucytosine (Kremer et al. 1991). Perry et al. (1998) reported the occurrence of a conjunctival mass and keratoconjunctivitis in an immunocompetent patient; detailed examination revealed the presence of a soft contact lens, covered by mucoid material, at the posterior aspect of this mass, and the simple removal of the lens resulted in a resolution of all signs and symptoms, with the contact lens growing *Aspergillus fumigatus*. Keratouveitis associated with the intraocular long-term retention of a contact lens was reported in a 76-year-old female patient (Arthur et al. 2001); a fungus was found to be involved, and this was identified as *Scedosporium prolificans*. However, this identification was found to be erroneous, and the isolate was later reidentified as *Acrophialophora fusispora* (Guarro and Gené 2002).

While the occurrence of contact lens-associated bacterial and *Acanthamoeba* keratitis has been correlated with the presence of bacteria and *Acanthamoeba* in contact lens cases (Seal et al. 1992; McLaughlin-Borlace et al. 1998), it is unclear whether such a relationship occurs in contact lens-associated fungal keratitis. However, Wilson et al. (1991) demonstrated the

adherence of *Candida albicans* within a biofilm to polyethylene contact lens case plastic; this was found to be more resistant to the action of contact lens disinfectants than bacteria. A survey of contact lens cases from 101 asymptomatic daily-wear, cosmetic contact lens wearers in a domiciliary contact lens practice revealed contamination in 82 (81 percent) cases; 77 percent grew bacteria, 24 percent fungi, and 20 percent protozoa (Gray et al. 1995). Keratitis due to *Cryptococcus laurentii* and *Fusarium solani* was recently reported in a diabetic male patient who wore a gas-permeable contact lens (Ritterband et al. 1998); both fungi were isolated from the patient's corneal button, infected toe nails, and contact lens storage case, and enucleation eventually had to be performed.

Overnight soaking of soft lenses in 3 percent hydrogen peroxide (longer than 4 hours), with neutralization in the morning by thiosulfate solution, catalase solution, or catalase tablets is perhaps the safest way to ensure killing of bacteria, *Acanthamoeba*, and fungi (Chandler 1990; Wilson 1996). However, in one study, as many as 75 percent of individuals who used hydrogen peroxide disinfection for their contact lenses were found to have contact lens cases that were contaminated with microbes (Gray et al. 1995); all the contaminating microorganisms were found to possess the enzyme catalase (which breaks down hydrogen peroxide to water and oxygen). Recommendations for contact lens wearers to prevent microbial contamination of the lens and case include regular scrubbing of the interior of contact lens cases to disrupt biofilms, exposure of the contact lens case to very hot water ($\geqslant$70°C), air-drying of the contact lens case between use, use of a two-step system for hydrogen peroxide disinfection, and regular replacement of the contact lens case (Gray et al. 1995; Seal et al. 1998).

Fungal contamination of other ocular biomaterials

Punctal occluders or plugs aid the management of dry eye syndrome. However, since these devices are left in situ for a long duration, nonspecific microbial attachment, surface colonization, and biofilm formation may occur. Fungi such as *Curvularia lunata* may sometimes be implicated (Wilson 1996).

Several alloplastic biomaterials have been used for repair of the orbital floor and to restore an anophthalmic socket. Oestreicher et al. (1999) described a patient who developed an *Aspergillus* abscess within a hydroxyapatite orbital implant 58 months following uncomplicated implant surgery; the symptoms resolved following removal of the implant.

Patients with corneal or scleral defects have been treated with Gore-Tex grafting; Huang et al. (1994) reported the occurrence of fungal contamination in one such graft, which ultimately led some months after graft removal to fungal endophthalmitis and penetrating keratoplasty.

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Subcutaneous zygomycosis

DAVID H. ELLIS

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The Entomophthorales are a group of primitive fungi belonging to the Zygomycetes, with infrequently septate (coenocytic) hyphae in which the sporangium has been reduced to function as a single conidium that is forcibly discharged at maturity. If the conidium does not land on a favorable substrate, it in turn produces a second and smaller conidium, which is again shot away. This cycle may be repeated several times. Members of the Entomophthorales are often parasitic on insects, and other animals and some species have developed very potent proteolytic enzyme systems. Although they are capable of growing saprobically in pure culture, many species require a complex nutrient medium to stimulate sporulation (King 1983). Currently the Entomophthorales are classified into six families with 22 genera containing some 132 species (Humber 1989). However, the order may be heterogeneous as recent molecular data has shown *Basidiobolus* to cluster with some of the Chytridiales, and *Conidiobolus*, *Entomophthora*, and *Zoopthora* with the Mucorales (Nagahama et al. 1995).

Basidiobolus ranarum (*Basidiobolaceae*), *Conidiobolus coronatus* (*Ancylistaceae*), and *Conidiobolus incongruus* are the only species that are known to cause human disease (Table 1). *Conidiobolus lamprauges* has also been reported once from a horse (Humber et al. 1989). Infections caused by *B. ranarum* usually present as chronic, painless, indurated subcutaneous masses and have been referred to by a variety of names, such as basidiobolomycosis (Kwon-Chung and Bennett 1992), entomophthoramycosis basidiobolae (Emmons et al. 1977), and subcutaneous zygomycosis (Rippon 1988; Goodman and Rinaldi 1991). *C. coronatus* infections usually occur as chronic granulomatous lesions of the nasal mucosa, often spreading to the contiguous facial skin and have also been called a variety of names, such as conidiobolomycosis, entomophthoramycosis conidiobolae, and rhinoentomophthoromycosis (Emmons et al. 1977; Kwon-Chung and Bennett 1992).

The term 'zygomycosis' has often been used to describe in the broadest sense any infection caused by a member of the Zygomycetes. However, many authors still prefer to use the term 'mucormycosis' to describe mycoses caused by members of the Mucorales and entomophthoromycosis (or entomophthoramycosis – the spelling used seems to depend upon the author) to describe mycoses caused by species belonging to the Entomophthorales. In general, fungi in the order Mucorales cause the more severe forms of disease (see

Systemic zygomycosis), whereas those in the order Entomophthorales cause a more chronic disease of the nasal mucosa and subcutaneous tissue (Goodman and Rinaldi 1991).

ETIOLOGIC AGENTS OF MEDICAL IMPORTANCE

Basidiobolus ranarum Eidem

The genus *Basidiobolus* is distinguished primarily by the morphology and development of forcibly discharged conidia and 'beaked' zygospores, both of which must be observed for reliable identification to genus and species level (King 1983). As all species of *Basidiobolus* lose their ability to sporulate after being maintained in culture for a relatively short time, it is very important to examine freshly isolated strains as soon as possible (Drechsler 1955; King 1979, 1983). This lack of

Table 1 *Families and genera of the Entomophthorales of medical importance*

Family	Genus	Key identification features
Ancylistaceae	*Conidiobolus*	Sporophores without subsporangial vesicle; nucleus difficult to observe during mitosis; nucleolus prominent Forcibly discharged multireplicative conidia with prominent papillae and villi
Basidiobolaceae	*Basidiobolus*	Sporophores with subsporangial vesicle; all cells uninucleate Forcibly discharged conidia and zygospore morphology

sporulation in culture, combined with other unsatisfactory criteria for separating the species, such as optimum growth temperature, *Streptomyces*-like odor, and zygospore wall undulation, has led to some confusion in the identification of the pathogenic isolates (Hutchison et al. 1972). In the past, pathogenic isolates have been classified as *B. ranarum* (Emmons et al. 1977), *Basidiobolus meristosporus* (Drechsler 1956; Greer and Friedman 1966; Clark 1968; Coremans-Pelseneer 1973), and *Basidiobolus haptosporus* (Srinivasan and Thirumalachar 1965, 1967; Miller and Pott 1980; Miller and Turnwald 1984). However, recent advances in taxonomy, including antigenic studies (Yangco et al. 1986), restriction analysis of rDNA (Nelson 1989), and isoenzyme banding (Cochrane et al. 1989), indicate that all human pathogenic isolates of *Basidiobolus* belong to *B. ranarum* (McGinnis 1980b; Kwon-Chung and Bennett 1992). *B. ranarum* is also known to produce extracellular protease and lipase, which can be detected in agar media (Okafor et al. 1987).

B. ranarum is commonly present as a saprophyte in soil, decaying fruit and vegetable matter, and in the dung of amphibians and reptiles. The animals show no intestinal lesions (Clark 1968; Nickerson and Hutchinson 1971; Tills 1977; Gugnani and Okafor 1980; Okafor et al. 1984; Zahari and Shipton 1988; Nelson et al. 2002), although cutaneous lesions have been reported in amphibians (Groff et al. 1991; Taylor et al. 1999). Less commonly, it has been recovered from the intestinal tract of insectivorous bats, the dung of macropod species, and from wood lice (Chaturvedi et al. 1984; Speare and Thomas 1985; Zahari and Shipton 1988). Subcutaneous infections have been well documented in horses (Miller and Pott 1980; Miller and Campbell 1982, 1984; Miller 1985), dogs (Greene et al. 2002), and in humans (Joe and Njo-Imjo 1956; Dasgupta et al. 1976; Mugerwa 1976; Bittencourt et al. 1979, 1991; Davis et al. 1994); gastrointestinal lesions have also been noted in dogs (Miller 1985), and with increasing occurrence in humans (de Agular et al. 1980; Schmidt et al. 1986; Yousef et al. 1999; Khan et al. 2001; Lyon et al. 2001). *B. ranarum* has been reported from tropical Africa, India, Indonesia, and southeast Asia, including northern Australia (Zahari et al. 1990), with human infections being limited to the warmer regions of the world (King 1979, 1983; Gugnani 1999).

Colonies of *B. ranarum* are moderately fast growing at 30°C, flat, yellowish-gray to creamy-gray, glabrous, becoming radially folded and covered by a fine, powdery, white surface mycelium. Note that conidia ejected from the primary colony often germinate to form satellite colonies. Microscopic examination usually shows the presence of large vegetative hyphae (8–20 μm in diameter) forming numerous round (20–50 μm in diameter), smooth, thick-walled zygospores that have two closely appressed beak-like appendages (Figure 1). The production of 'beaked' zygospores is diagnostic for the genus. Two types of asexual conidia are formed: primary and secondary. Primary conidia are globose, one celled, solitary, and forcibly discharged from a sporophore. The sporophore has a distinct swollen area just below the spore, which actively participates in the discharge of the spore (Figure 1). Secondary (replicative) conidia are clavate, one celled, and passively

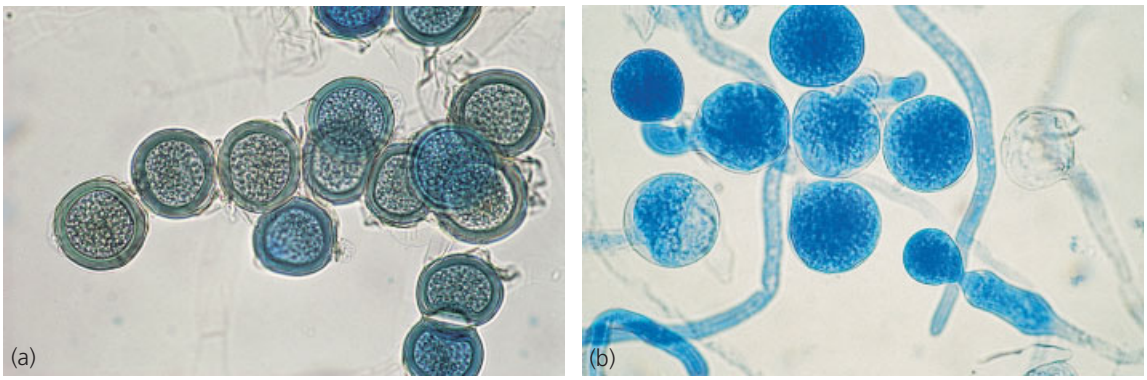

Figure 1 Basidiobolus ranarum*:* **(a)** *zygospores;* **(b)** *conidia and sporophores. Magnification ×495.*

released from a sporophore. These sporophores are not swollen at their bases. The apex of the passively released spore has a knob-like adhesive tip. These spores may function as sporangia, producing several sporangiospores. Note that isolates often lose their sporulating ability with subculture and special media, incorporating glucosamine hydrochloride and casein hydrolysate, may need to be used to stimulate sporulation (Shipton and Zahari 1987). Key features include forcibly discharged conidia and the presence of smooth-walled zygospores with prominent beak-like appendages. (For additional information, see Srinivasan and Thirumalachar 1965, 1967; Greer and Friedman 1966; Dworzack et al. 1978; McGinnis 1980a; King 1983; Rippon 1988; Drouhet and Ravisse 1993; Jong and Dugan 2003.)

Conidiobolus species

The species of the genus *Conidiobolus* produce characteristic multinucleate primary and secondary (replicative) conidia on top of unbranched conidiophores. Each subspherical conidium is discharged as a result of the pressure developed within the conidium, and each bears a more or less prominent papilla after discharge (King 1983). The genus contains 27 species; it has recently undergone taxonomic revision and is now classified in the family *Ancylistaceae* (see King 1976a, b, 1977, 1979; Humber 1989). However, isolates of *C. coronatus* and *C. incongruus* are the only species that are known to cause human disease, although *C. lamprauges* has also been reported once from a horse (Humber et al. 1989). Isolates of the *Conidiobolus* species do not tend to lose the ability to sporulate as readily as isolates of *B. ranarum*. Most strains of *C. coronatus* readily produce villous conidia.

CONIDIOBOLUS CORONATUS (COSTANTIN) BATKO

C. coronatus, synonym *Entomophthora coronata* (Costantin) Kevorkian, has a worldwide, mainly tropical distribution and is commonly present as a saprophyte in soil and on decaying vegetation (King 1983). It has been most frequently isolated in the tropical rain forests of Africa (Fromentin and Ravisse 1977). It is an occasional pathogen of insects worldwide, and has been isolated from dolphins (Sweeney and Migaki 1976), chimpanzees (Roy and Cameron 1972), sheep (Carrigan et al. 1992; Ketterer et al. 1992), llama (Moll et al. 1992; French and Ashworth 1994), and from horses as far north as southern Texas (Emmons and Bridges 1961; French et al. 1985) and as far south as Australia (Hutchins and Johnson 1972; Miller and Campbell 1982). Human infections are usually restricted to the rhinofacial area (Rippon 1988; Costa et al. 1991; Ng et al. 1991; Gugnani 1992; Kwon-Chung and Bennett 1992). However, there are occasional reports of dissemination to other sites (Rinaldi 1989; Jaffey et al. 1990; Walker et al. 1992). All human infections have been confined to the tropics (King 1983).

There has been much confusion as to the correct name of this species, and it is often listed under an older synonym *Delacoixia coronata* (Cost.) Sacc. & P. Syd. (de Hoog and Guarro 1995). This has been further compounded by recent investigations of sterol content, which suggest separation of *C. coronatus* (as *Delacoixia coronata*) from other fungi in *Entomophthora*, *Basidiobolus*, and *Conidiobolus* (Weete and Gandhi 1997). However, the name *C. coronatus* is now generally accepted and is retained for the purposes of this chapter. With the possible exception of a few human isolates, all strains of *C. coronatus* produce villous conidia, and about 75 percent of the strains isolated from plant debris produce multireplicative conidia (King 1976a, 1979, 1983). Some strains also produce abundant spherical chlamydoconidia submerged in the agar, but zygospores are not known to be formed by this species (King 1983).

Colonies grow rapidly and are flat, cream colored, glabrous, becoming radially folded and covered by a fine, powdery, white surface mycelium and conidiophores. The lid of the Petri dish soon becomes covered with conidia that were forcibly discharged by the conidiophores. The color of the colony may become tan to brown with age. Conidiophores are simple, forming solitary, terminal conidia, which are spherical, 25–45 μm in diameter, multinucleate, single celled, and have a prominent papilla (Figure 2). Conidia may also produce hair-like appendages called villi. Conidia germinate to produce either:

- single or multiple hyphal tubes which may also become conidiophores that bear secondary conidia, or
- multiple short conidiophores, each bearing a small secondary conidium.

Key features include forcibly discharged conidia with conspicuous basal papillae and villous hair-like projections that readily distinguish *C. coronatus* from other species. (For additional information see Emmons and Bridges 1961; King 1976a, 1983; McGinnis 1980a; Rippon 1988; Kwon-Chung and Bennett 1992; de Hoog et al. 2000.)

CONIDIOBOLUS INCONGRUUS DRECHSLER

C. incongruus is only rarely isolated as a saprophyte from plant debris or as a pathogen causing human or animal infection. Animal infections have been reported once from a deer (Stephens and Gibson 1997) and from five cases of rhinocerebral and nasal zygomycosis in sheep (Ketterer et al. 1992). Human infections are also infrequent. The first case report was of a 15-month-old boy with indolent pneumonia, mediastinitis, and pericarditis who was successfully treated with amphotericin B (Gilbert et al. 1970; Eckert et al. 1972; King and Jong 1976). A fatal disseminated infection was then reported by Busapakum et al. (1983). Since then a case of

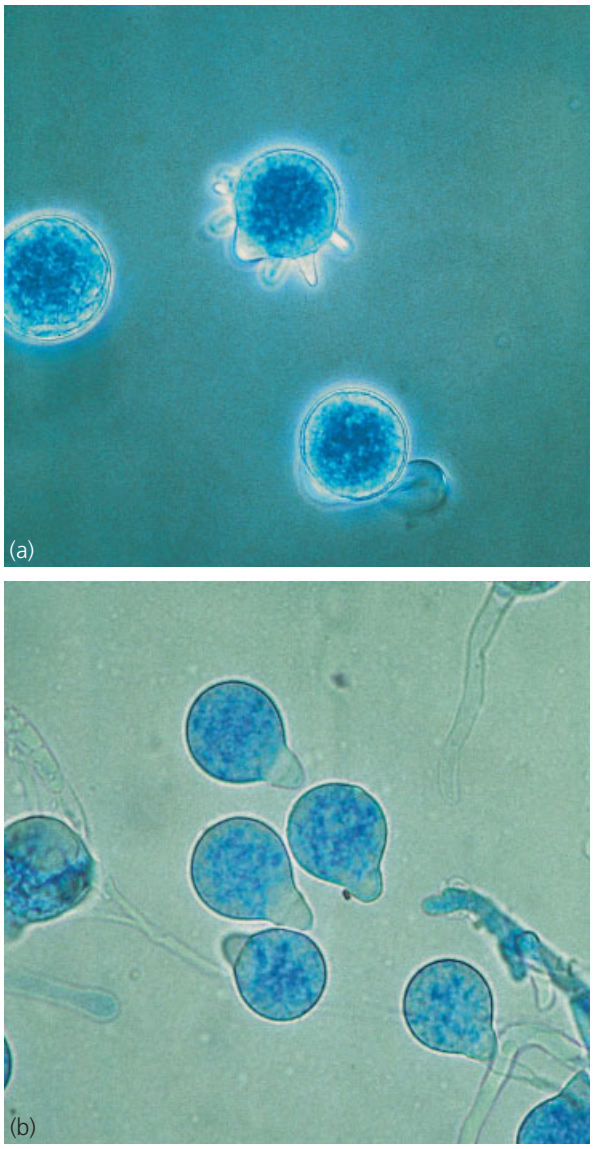

Figure 2 Coniodobolus coronatus: **(a, b)** *spherical conidia with hair-like appendages (villi) and prominent papillae. Magnification ×495.*

endocarditis in a cocaine abuser (Jaffey et al. 1990), a fatal disseminated case in a renal transplant patient (Walker et al. 1992), and an orbitofascial infection in a child (al-Hajjar et al. 1996) have also been reported. Another recent human case report described a granulocytic patient with pulmonary and pericardial infection and concluded that *C. incongruus* was an uncommon but highly invasive fungal pathogen that may be resistant to amphotericin B (Walsh et al. 1994). Colony characteristics are similar to *C. coronatus* and are flat, white becoming brown with age, and glabrous becoming radially folded with minimal surface mycelium. Zygospores are yellowish, spherical to elongate (15–25 μm in diameter), thick walled and without beaks. Primary conidia are forcibly discharged, spherical to pyriform (11–37×12–42 μm in diameter) with a tapering, often sharply pointed, basal papilla. Zygospores and multiplicative conidia must be observed to identify *C. incongruus*. Key features include forcibly discharged conidia with pronounced elongated basal papilla but without the hair-like villous projections that distinguish this species from *C. coronatus*. (For additional information, see King and Jong 1976; King 1977, 1979, 1983; Busapakum et al. 1983; Rippon 1988; Kwon-Chung and Bennett 1992.)

CONIDIOBOLUS LAMPRAUGES DRECHSLER

C. lamprauges is also rare, having been isolated from plant debris and occasionally from insects, and from a granulomatous nasopharyngeal nodule of a horse (Humber et al. 1989). No human infections have been reported. *C. lamprauges*, like *C. coronatus*, produces zygospores but not villose conidia. Colony characteristics are similar to *C. coronatus* and are flat, whitish becoming radially folded with minimal surface mycelium. Zygospores are spherical (12–18 μm in diameter), thick walled and with two protruding lateral appendages or beaks. Conidiophores are hypha-like, irregular, and are not swollen at their apices. Primary conidia are forcibly discharged, densely granular when immature, spherical (13–22 μm in diameter), thin walled, with one to several flat papilla after liberation. Key features include zygospores with two 'beaks,' forcibly discharged conidia with one or more papilla but without the hair-like villous projections that also distinguish this species from *C. coronatus*. (For additional information, see Kwon-Chung and Bennett 1992; de Hoog et al. 2000; Jong and Dugan 2003.)

HOST RESPONSES

Infections caused by entomophthoraceous fungi result from species of 2 genera: *Basidiobolus* and *Conidiobolus*. Infections are chronic, slowly progressive, and generally restricted to the subcutaneous tissue in otherwise healthy individuals. Other characteristics that separate these infections from those caused by mucoraceous fungi are a lack of vascular invasion or infarction and the production of a prolific chronic inflammatory response, often with eosinophils and the Splendore–Hoeppli phenomenon around the hyphae (Kwon-Chung and Bennett 1992).

Entomophthoromycosis caused by *B. ranarum* is a chronic inflammatory or granulomatous disease generally restricted to the subcutaneous tissue of the limbs, chest, back, or buttocks, primarily occurring in children and with a predominance in males (Martinson 1972; Clark 1975; Bittencourt et al. 1979; Rippon 1988; Rinaldi 1989; Kwon-Chung and Bennett 1992; Gugnani 1999). Initially, lesions appear as subcutaneous nodules which develop into massive, firm, indurated, painless swellings which are freely movable over the underlying muscle, but attached to the skin, and which may become hyperpigmented although not ulcerated (Kwon-Chung and

Bennett 1992). The first human case caused by *B. ranarum* was reported in 1956 in Indonesia (Joe and Njo-Imjo 1956) and subsequently cases have been reported in India (Dasgupta et al. 1976), Africa (Mugerwa 1976), South America (Bittencourt et al. 1979), and northern Australia (Davis et al. 1994). The first culture-proven invasive infection by *B. ranarum* (syn. *B. haptosporus*) was reported in the USA (Dworzack et al. 1978). This was an unusual case, with a description of an ulcerative lesion perforating the hard palate of an adult patient. Gastrointestinal infection appears to be an emerging entity (de Agular et al. 1980; Schmidt et al. 1986; Yousef et al. 1999; Khan et al. 2001; Lyon et al. 2001) and poses several diagnostic difficulties. Its clinical presentation is nonspecific, there are no identifiable risk factors, and all age groups are susceptible; as a result many cases have been confused with Crohn's disease (Zavasky et al. 1999). A case of muscle invasion by *B. ranarum* resulting from deep penetration of this fungus has also been described (Kamalam and Thambiah 1984). *B. ranarum* most probably gains entrance to human tissue following traumatic implantation by insect bites, scratches, and minor trauma (Kwon-Chung and Bennett 1992). This may also explain why it is most commonly encountered in children, involving the thighs or buttocks.

Entomophthoromycosis caused by *Conidiobolus* spp. is a chronic inflammatory or granulomatous disease, which is typically restricted to the nasal submucosa and characterized by polyps or palpable restricted subcutaneous masses (Martinson 1972; Herstoff et al. 1978; Winter and Carme 1984; Rippon 1988). Clinical variants, including pulmonary and systemic infections, have also been described (Busapakum et al. 1983). Human infections occur mainly in adults with a predominance in males (80 percent of cases). Most cases have been reported from the tropical rainforest areas of central and west Africa (Okafor et al. 1983; Winter and Carme 1984) and South and Central America (Rippon 1988). Infections usually begin with unilateral involvement of the nasal mucosa. Symptoms include nasal obstruction, drainage, and sinus pain. Subcutaneous nodules develop in the nasal and perinasal regions and progressive generalized facial swelling may occur (Richardson and Warnock 1993). Infections also occur in horses, usually producing extensive nasal polyps (Emmons and Bridges 1961; Miller and Campbell 1982; Miller 1983; French et al. 1985), and other animals (Miller 1985). *C. coronatus* is also a recognized pathogen of termites, other insects, and spiders (King 1979). *Conidiobolus* infections are believed to occur following inhalation of conidia and subsequent tissue invasion of traumatized nasal mucosa (Kwon-Chung and Bennett 1992).

LABORATORY DIAGNOSIS

A tissue biopsy is essential, and swabs are not recommended. Tissue samples should be kept moist with saline or brain–heart infusion broth and be transported to the laboratory as soon as possible. It is essential that both direct microscopy and culture be performed on all specimens. It should also be noted that zygomycetous fungi have coenocytic hyphae, which will often be damaged and become nonviable during the biopsy procedure or by the chopping up or tissue grinding processes in the laboratory. This is why zygomycetous fungi that are clearly visible in direct microscopic or histopathological mounts are often difficult to grow in culture from clinical specimens. Therefore it is important to avoid excessive tissue damage when collecting the specimen and in the laboratory to gently tease the tissue apart and inoculate it directly onto the isolation media.

Direct microscopy and histopathology

Histopathology is the most rapid diagnostic method and the presence in clinical specimens of broad, 5–15 μm wide, infrequently septate, thin-walled hyphae that are surrounded by an eosinophilic sheath, known as the Splendore–Hoeppli phenomenon, is characteristic (Figure 3). There are two major histological differences between subcutaneous zygomycosis caused by *Basidiobolus* and *Conidiobolus* (entomophthoromycosis) and zygomycosis caused by members of the Mucorales (mucormycosis). First, in entomophthoromycosis, an eosinophilic sheath surrounds the hyphae and there is a lack of vascular invasion, which is so characteristic of infections caused by the Mucorales. Second, the hyphal elements of Mucorales are sparsely septate in tissue, whereas frequent septation is seen in tissue hyphae of *Basidiobolus* or *Conidiobolus*. Direct microscopy of biopsy tissue may be performed using 10 percent KOH ink or calcofluor mounts. A small piece of tissue may be mounted in KOH on a glass slide, teased apart, and gently heated to dissolve the tissue prior to microscopic

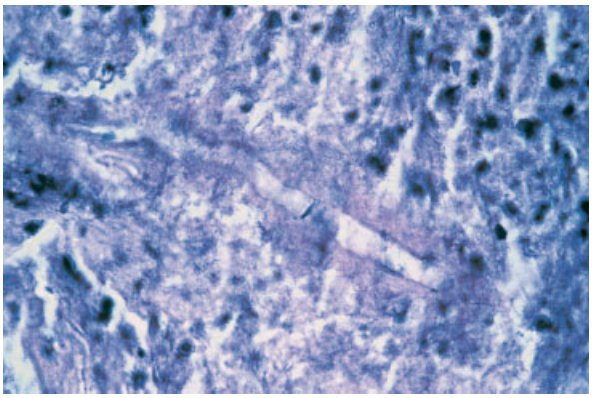

Figure 3 *Tissue morphology in entomophthoromycosis: H&E stained section of infected tissue showing broad, infrequently septate, hyphae surrounded by an eosinophilic sheath (Splenodore–Hoeppli phenomenon), typical of* Basidiobolus ranarum.

examination for the presence of zygomycetous hyphae. Tissue sections should be stained with hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Gomori's methenamine silver (GMS) (Grocott 1955) stains. H&E-stained tissue sections are best to observe the distinctive eosinophilic sheath (Splendore–Hoeppli phenomenon) that typically surrounds the hyphae. However, GMS-stained sections are still necessary as often the hyphae stain poorly (Chandler et al. 1980; Rippon 1988; Salfelder 1990).

Culture

Biopsy tissue should be inoculated onto culture media without delay; it should not be kept for an extended time in a refrigerator (Burkitt et al. 1964). Sabouraud's dextrose agar containing antibacterial antibiotics, but without cycloheximide (actidione), is usually used as the primary isolation medium. Strains of *Basidiobolus* also rapidly lose their ability to sporulate after relatively short periods of time in culture. This problem has been partially overcome by the use of media that incorporate glucosamine hydrochloride and casein hydrolysate (Shipton and Zahari 1987). Nevertheless, isolates should be examined and preserved in liquid nitrogen as quickly as possible after isolation (Jong and Dugan 2003). Most isolates of *Basidiobolus* and *Conidiobolus* are fast growing, 2–5 days at 26–28°C, initially whitish, later becoming buff or gray, with a glabrous to waxy or powdery radiate surface. For isolation from environmental sources such as soil, decaying leaves, or the intestinal contents of reptiles, specimens can be shaken in sterile water and the supernatant then pipetted onto a sterile Whatman no. 1 filter paper. The filter paper is then placed on the inside of a petri dish lid covering an agar plate. The petri dish is then incubated upside down to allow the forcible discharged conidia to land on the agar surface. Colonies take about 3 days to appear in positive samples (Rippon 1988).

Immunology and serology

Serological testing for antibodies by immunodiffusion against both *Basidiobolus* and *Conidiobolus* spp. has been developed (Kaufman et al. 1990; Imwidthaya and Srimuang 1992) and has been used on a limited basis for diagnostic purposes, especially when the culture result is negative (Lyon et al. 2001). Exoantigen tests have also been developed for the identification of cultures by immunodiffusion (Yangco et al. 1986; Toriello et al. 1989).

TREATMENT (MANAGEMENT/THERAPY)

In subcutaneous infections caused by *B. ranarum*, the therapy of choice still appears to be saturated potassium iodide solution. The usual dose has been about 30 mg/kg/day, given either as a single dose or divided into three daily doses, which should be given for 6–12 months (Koshi et al. 1972; Mugerwa 1976; Vismer et al. 1980; Kamalam and Thambiah 1982; Drouhet and Ravisse 1993; Nazir et al. 1997). In a few patients, oral ketoconazole (Piens et al. 1985), itraconazole (Gugnani 1999), and fluconazole (Davis et al. 1994; Gugnani et al. 1995) have sometimes been successful, but amphotericin B has seldom been helpful (Khan et al. 2001). Some more recent reports have demonstrated the successful use of combination potassium iodide and ketoconazole (Bittencourt et al. 1991) and itraconazole and fluconazole (Valle et al. 2001). Surgical resection alone is not curative (Kwon-Chung and Bennett 1992). In one series of gastrointestinal infection, all patients were successfully treated by the combination of surgical resection of the infected portions of the gastrointestinal tracts and itraconazole therapy, and this may be the treatment of choice for these patients (Lyon et al. 2001). In vitro antifungal susceptibility testing of nine isolates of *B. ranarum* reported low minimum inhibitory concentrations (MIC) to the azoles fluconazole, itraconazole, ketoconazole, and miconazole (Guarro et al. 1999).

In patients with submucosal infections caused by *Conidiobolus* spp., treatment options have so far been disappointing. Surgical resection of infected tissue is seldom successful and may even hasten spread of infection. Potassium iodide solution, amphotericin B, and trimethoprim–sulfamethoxazole have all been used with mixed success (Martinson 1971; Herstoff et al. 1978; Kwon-Chung and Bennett 1992). Some more recent reports of successful treatment include using fluconazole (Gugnani et al. 1995), or combination therapy with amphotericin B and terbinafine (Foss et al. 1996) and ketoconazole plus potassium iodide (Mukhopadhyay et al. 1995). In vitro resistance to amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, ketoconazole, and miconazole has been reported for seven isolates of *Conidiobolus* (Guarro et al. 1999). In the rare cases of disseminated infections caused by *C. incongruus*, only one of three patients has survived following surgical debridement and amphotericin B treatment (Busapakum et al. 1983; Walsh et al. 1994; Dromer and McGinnis 2003). There is a single patient report of an 18-month-old girl with periorbital cellulites secondary to *C. incongruus* who initially failed amphotericin B therapy was successfully treated by radical resection with antifungal chemotherapy and hyperbaric oxygen (Temple et al. 2001).

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Chromoblastomycosis

PHILIPPE ESTERRE

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Chromoblastomycosis is a non-contagious, chronic, localized fungal infection of cutaneous and subcutaneous tissues caused by several species of pheoid (dematiaceous) fungi. The disease is characterized by the presence in infected tissues of brown, thick-walled, multiseptate, fungal forms known as muriform bodies (synonym: sclerotic cells, Medlar bodies) (Figure 1). The etiologic agents of chromoblastomycosis are black molds that are found world-wide growing as saprophytes of wood, other vegetation and soil (Gezuele et al. 1972; Dixon et al. 1980; Iwatsu et al. 1981; Okeke and Gugnani 1986) (Table 1). Infection follows traumatic implantation of the etiologic agent beneath the epidermis via penetration by foreign bodies such as wood splinters and tree thorns, or other minor wounds or abrasion. After implantation, the pseudo-yeast cells undergo a morphological change into the distinctive muriform bodies, and the fungus persists in this form within the infected host tissue. Nearly all cases of chromoblastomycosis are accounted for by the species *Fonsecaea pedrosoi*, *Cladophialophora carrionii* (*Cladosporium carrionii*) and *Phialophora verrucosa*. The disease is reported most frequently in individuals living in tropical and subtropical regions, notably the West Indies, Brazil, Northern Venezuela, and Madagascar, but it is cosmopolitan in distribution. The actual prevalence and incidence of chromoblastomycosis are unknown because of sporadic reporting, but in areas where interest and endemicity are high, rates of infection have ranged from 1 per 32 500 population to one per 7 000 population (Ajello 1970). In some villages in Madagascar and Northern Venezuela, the prevalence is as high as 1 per 480 inhabitants (Esterre et al. 1996) or even higher (Rodrigues et al. 1992). Historically, chromoblastomycosis has usually been refractory to treatment, but two recently developed antifungal compounds, itraconazole and terbinafine, appear to hold promise for a greatly improved prognosis in many cases.

HISTORY

The earliest known discovery of the fungal etiology of chromoblastomycosis was made by Alexandrino Pedroso in 1911 in São Paulo, Brazil, when he observed muriform bodies in nodular and ulcerated foot and leg lesions of a patient, and recovered the pheoid mold known today as *Fonsecaea pedrosoi.* Pedroso did not publish his observations, however, until 1920 (Carrion and Silva 1947). In the meantime, Max Rudolph had published, in 1914, his clinical observations from cases encountered in the Brazilian states of Minas Gerais and Goiás. In 1915, Medlar and Lane of Boston became the first investigators to publish clinical observations that were supported by positive culture results. The etiology in their case was *Phialophora verrucosa*.

Subsequently, the nomenclature for infections caused by these fungi became confused. Following the initial clinical reports, chromoblastomycosis was known by several names (McGinnis 1983). The name chromoblastomycosis (*chromo* = coloured; *blasto* = budding)

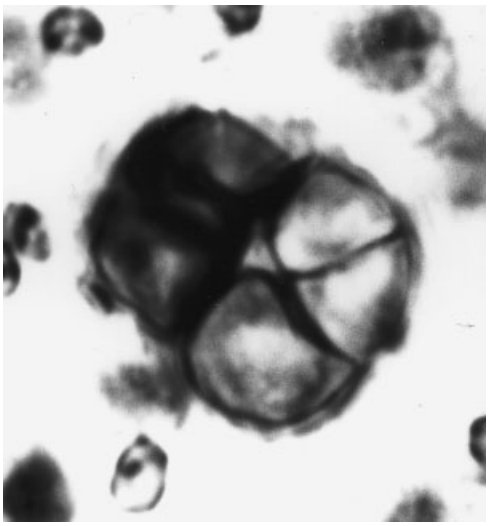

Figure 1 *Muriform bodies, pathognomonic for chromoblastomycosis, are subglobose, 5–13 μm in diameter, multiseptate, and dark brown in color as a result of the synthesis of melanin within the cell walls.*

was first proposed in 1922 (Terra et al. 1922). A later proposal, however, reasoned that the term 'chromoblastomycosis' is a misnomer because muriform bodies multiply by splitting apart rather than by budding, and therefore a more appropriate name would be chromomycosis. Further confusion arose from the occasionally indiscriminate use of both these terms to refer to an assortment of infections caused by pheoid fungi, irrespective of whether or not the characteristic muriform bodies were formed in tissue.

To eliminate ambiguity caused by misapplication of these two terms, the name chromoblastomycosis was retained in its original sense, chromomycosis was rejected, and the name pheohyphomycosis was coined to encompass infections caused by pheoid fungi that are clinically and pathologically different from chromoblastomycosis (Ajello et al. 1974; McGinnis 1983). Pheohyphomycosis is characterized by pheoid fungi that grow in infected tissue as pseudohyphae, hyphae, or any combination of these forms, but not as muriform bodies. It is caused by at least 100 fungal species, and has clinical features that generally differ from chromoblastomycosis. Today, the distinctions embodied by the terms 'chromoblastomycosis' and 'pheohyphomycosis' have become widely accepted. As for most biological phenomena, however, efforts to categorize the manifestations of pathogenic fungi have seldom been entirely satisfactory. For example, the two leading agents of chromoblastomycosis, *P. verrucosa* and *F. pedrosoi*, have been encountered in rare cases that can best be characterized as mycetoma and pulmonary pheohyphomycosis, respectively (Morris et al. 1995; Turiansky et al. 1995). Although there is some overlap, there is diagnostic utility in maintaining the two clinical terms, stemming from the strong predictive correlation of morphology in tissue with etiology, clinical course, and prognosis.

ECOLOGY AND EPIDEMIOLOGY

Virtually all cases of chromoblastomycosis are caused by the species *F. pedrosoi*, *C. carrionii*, and *P. verrucosa*. Additional fungi, including *Fonsecaea compacta* (McGinnis and Schell 1980), *Rhinocladiella aquaspersa* (Borelli 1972; Schell et al. 1983), *Exophiala spinifera* (Barba-Gomez et al. 1992; Padhye et al. 1996), *Cladophialophora arxii* (Tintelnot et al. 1995), *Phaeosclera dematioides* (McGinnis et al. 1985), *Botryomyces caespitosus* (de Hoog and Rubio 1982), *Sporothrix schenckii* var. *luriei* (Padhye et al. 1992) and *Wangiella (Exophiala) dermatitidis* are also capable of forming muriform bodies in infected tissue but, with the exception of *W. dermatitidis*, these species are exceedingly rare as etiologic agents of infection in humans. Infections resulting from *W. dermatitidis* occur occasionally and are typified in tissue by short chains of spherical cells that have 0–1 septa; only very rarely do muriform bodies form, and for this reason most mycologists consider the species to be an agent not of chromoblastomycosis but of pheohyphomycosis.

Worldwide, *F. pedrosoi* causes the great majority of infections. *C. carrionii*, in addition to *F. pedrosoi*, is particularly common in Australia, Southern Madagascar and South Africa. *P. verrucosa* is reported in smaller numbers from various temperate to subtropical climates. The reasons for regional variation in the relative etiologies seemed to be linked to local ecological conditions. For example, investigation of natural and clinical isolates in Australia between 1957 and 1961 has suggested that *C. carrionii* is particularly likely to occur in drier regions that have as little as 500 mm of annual rainfall (Ridley 1961). In 1960, Brygoo and Segretain reached a similar conclusion for Madagascar, finding that *C. carrionii* was predominant in a spiny desert region of 500–600 mm of annual rainfall, whereas *F. pedrosoi* infections predominated in regions having annual rainfall of 2200–3200 mm. These observations were later extended by a retrospective study of 170 clinical isolates in Madagascar from 1970 to 1994 (Esterre et al. 1996). This study found that in the southern dry region, all cases of chromoblastomycosis (representing more than 38 percent of the total cases in Madagascar) were caused by *C. carrionii*, whereas in the evergreen forest region of much greater rainfall only *F. pedrosoi* (62 percent of the total sample) was encountered. Sources of saprobic strains of *C. carrionii* provide additional support for the concept that this species can exist in dry substrata, such as soil in Arizona (USA) (Padhye 1986), fence posts in Australia and Nigeria made from *Eucalyptus cebra* (Ridley 1961) and a *Pinus* sp. (Okeke and Gugnani 1986) and cactacae in northern Venezuela (Richard-Yegres and Yegres 1987). Morphological

Table 1 *Representative environmental samples for* Fonsecaea pedrosoi, Phialophora verrucosa, *and* Cladophialophora carrionii

Environmental sample	Isolates/samples (no.)	Reference
F. pedrosoi		
Plant debris, soil	34/328	–
Animal feces, nests, burrows, moss, water, palm fruit, invertebrates, epiphytes	0/294	Gezuele et al. 1972
Rotting wood	1/78	–
Lumber, logs, bark, soil, sawdust, nests	0/99	Iwatsu et al. 1981
Wood piles	2/42	–
Tree stumps, tree trunks, tree bases	6/76	–
Sawdust dump	1/76	–
Pine fenceposts	1/6	–
Beneath trees	2/10	–
Soil, stacked planks	0/16	Okeke and Gugnani 1986
Tree branch bark	1/1	Rubin et al. 1991
Rotten plants, soil	2/98	Montenegro et al. 1996
P. verrucosa		
Plant debris, soil, wasps nests, epiphytes, bird droppings	31/328	–
Animal feces, ant nests, burrows, moss, water, palm fruit, invertebrates	0/297	–
Rotting wood, lumber, sauna bath wood	38/78	Gezuele et al. 1972
Lumber, logs, pine bark, rotting wood, soil	17/157	–
Other bark, sawdust, nests	0/20	Iwatsu et al. 1981
Wooden outdoor furniture, lumber, toolshed *Pinus* sp.	5/10	–
Soil, various woody materials	0/28	Dixon et al. 1980
Wood piles	2/42	–
Stacked planks	2/11	–
Tree trunks, soil, base of tree, fencepost, sawdust dump	0/173	Okeke and Gugnani 1986
Pulp	3/84	Wang 1965
Rotten plants, soil	6/98	Montenegro et al. 1996
C. carrionii		
Soil	NS	Padhye 1986
Pine fenceposts	2/6	–
Stacked planks	3/11	–
Soil	1/5	Okeke and Gugnani 1986
Fenceposts of *Eucalyptus* spp.	2/NS	Ridley 1961
Xerophilous *Prosopis juliflora*	1/25	Richard-Yegres and Yegres 1987
Cactus *Opuntia caribea*	1/7	–

NS, not specified.

identification of muriform-like cells was even made in the medulla of two cactus species (Zeppenfeldt et al. 1994) (see Table 1). *P. verrucosa* seems to be the third most common etiologic agent, after *F. pedrosoi* and *C. carrionii*, and has been reported from North, Central, and South America, Asia, and Africa. In one study, it was concluded that isolates of *P. verrucosa* were more likely to be encountered in relatively cooler climates than warmer climates; five of six isolates of *P. verrucosa* among 90 cases of chromoblastomycosis came from temperate regions as opposed to subtropical and tropical regions (Carrion and Silva 1947). It is of interest that the relative proportions of species among clinical isolates do not necessarily agree with relative proportions among environmental isolates. For example, Fukushiro (1983) found 86 and 1.6 percent of chromoblastomycosis in Japan to be caused by *F. pedrosoi* and *P. verrucosa*, respectively, whereas Iwatsu et al. (1981) were able to culture only one isolate of *F. pedrosoi*, in contrast to 17 isolates of *P. verrucosa* from environmental sources in Japan.

Cutaneous and subcutaneous infections begin when the fungus is introduced by a break in the dermal barrier. In rare instances, splinters that presumably harbored the fungus and initiated the infection were found within infected tissue (Mead and Ridley 1957; Tschen et al. 1984; Connor and Gibson 1985). In one case of chromoblastomycosis that developed following

abrasion by the branch of a tree, investigators were able to return to the site 2 months later and isolate the etiologic agent by culturing portions of the branch (Rubin et al. 1991). Although some patients are able to recount specific trauma to the affected site as a possible initiating event, some as recently as 3 weeks before (Gruber et al. 1988), most patients cannot specifically recall any precipitating injury. Presumably, this is because the initiating event was often trivial or unnoticed, and symptoms, which may not occur until much later, can be mild and seemingly of little consequence initially. It is also typical that there is a considerable delay in the medical diagnosis of chromoblastomycosis. For example, in Madagascar, where there is both a relatively high prevalence of infection and a high awareness on the part of local healthcare providers, the average time of diagnosis is 1–4 years after initial symptoms arise (Esterre et al. 1996). Probable factors for delay in medical diagnosis include the gradual and indolent nature of the infection and, in some localities, a limited availability of easily accessible healthcare.

Chromoblastomycosis has been reported in most regions of the world, but is more common in tropical and subtropical settings, as evidenced by Carrion who found that 79 percent of reviewed cases occurred in patients living in such climates (Carrion and Silva 1947). Furthermore, most of these individuals resided in rural areas and were involved in agricultural or related activities. In one rural tropical region of Brazil where mycoses are a relatively frequent finding, chromoblastomycosis has remained one of the most common of the serious mycoses (Talhari et al. 1988). Most patients were between 30 and 55 years of age, and there seems to be no predilection for any particular race, because the regions of high endemicity have included populations of European, African, or Asian origin (Carrion and Silva 1947; Al-Doory 1972; Lavalle 1980; Tsuneto et al. 1989). The role of gender is unclear; mostlarge reports have concluded that infections are more likely to occur in men than in women, but othershave found female patients in equal or greater numbers (Carrion and Silva 1947; Campins and Scharyj 1953; Al-Doory 1972; Bansal and Prabhakar 1989; Tsuneto et al. 1989; Hiruma et al. 1993; Esterre et al. 1996). It is probable that these contradictory findings merely reflect the difference in sex ratios within various occupations in different cultures and geographical regions.

Chromoblastomycosis occurs most often in individuals who have no apparent underlying illness or debility. However, some preliminary data suggest that a genetic susceptibility to chromoblastomycosis might exist. In a study of 32 infected people and 77 control individuals, the presence of HLA antigen A29 was linked to a 10-fold increase in susceptibility to chromoblastomycosis (Tsuneto et al. 1989). Other people who are at risk of developing infections are those whose immune system is impaired as a result of organ transplantation or corticosteroid use (Nishimoto et al. 1984; Morales et al. 1985; Wackym et al. 1985; Perry and Sheretz 1987; Greene et al. 1990). In countries where these medical modalities are widely used, such patient populations may constitute potential for an increasing prevalence of chromoblastomycosis. Infections have also been noted in conjunction with malignancy (Foster and Harris 1987; Hiruma et al. 1992) and leprosy (Pavithran 1988; Lacaz et al. 1994), although the significance of these observations is unknown. Chromoblastomycosis has been rarely reported in animals including dogs, horses (Abid et al. 1987), toads (Bube et al. 1992) and frogs, although only some of the reports are well documented. The infection is not known to be transmissible from animals or between humans.

MYCOLOGY

In terms of mycological classification, the phylogeny of the suspected fungi has not been conclusively established and the species are currently placed into form-taxa within a parallel system of classification known as the Deuteromycota. It is suspected that the fungi, which cause chromoblastomycosis, may be related to the family *Herpotrichiellaceae*, order Ophiostomatales, phylum Ascomycota (de Hoog et al. 1995), but no sexual stage has yet been discovered in the lifecycle of any of the causative species. As this classification necessarily employs highly artificial and potentially unreliable criteria, attempts at classifying species usually do not extend above the genus level. However, those species classified in the Deuteromycota and which contain large amounts of melanin in their cell walls, causing the colony color of these species to range from pale gray or brown to black, are often collectively referred to as the black or pheoid fungi. Based on this common feature, they are sometimes ascribed the status of a form-family, the *Dematiaceae* (Matsumoto et al. 1994; Pappagianis and Ajello 1994).

Individual species of these pheoid opportunistic pathogens are identified primarily based upon various features of their microscopic morphology, including characteristics of the conidia (spores) and the cells from which the conidia arise. Some of these species are pleomorphic, meaning that they have the ability to form more than one asexual reproductive morphology in culture. Each of these different morphs is sometimes given a separate latinized name for technical reasons, as permitted by the International Code of Botanical Nomenclature. Thus, the identification process for pleomorphic isolates can be problematic. In such instances, the accepted approach has been to choose the most distinctive, conspicuous and stable morph as the basis of the binomial name (identification) that will be applied to the isolate. Any accompanying morph that is present may be referred to using a separate genus level name.

However, the Code permits taxonomists to disagree about which morph should be chosen as the basis of the binomial. For example, in the case of *F. pedrosoi*, the *Fonsecaea* morph usually predominates, and a *Rhinocladiella* morph is often well represented, whereas a very few examples of a *Phialophora* morph can sometimes be seen as well. For this reason, some taxonomists have chosen to name the fungus *F. pedrosoi* whereas others prefer *Rhinocladiella pedrosoi*. The reader is referred for further discussion of this matter to the literature (McGinnis and Salkin 1993; Schell et al. 1995).

Newer methods of identification are less practical for clinical work, but are considered to be more reflective of phylogeny, and thus have had impact upon the taxonomy of these organisms (Honbo et al. 1984a; de Hoog et al. 1995). For example, results from restriction fragment length polymorphism (RFLP) patterns of mitochondrial DNA (Kawasaki et al. 1999) or large subunit RNA analysis (de Hoog et al. 1995) have led to the confirmation of *F. pedrosoi* as an homogeneous species or the reclassification of *Cladosporium carrionii* as *Cladophialophora carrionii*.

IDENTIFICATION

Fonsecaea pedrosoi

Colonies are olivaceous–black, slow in growth rate, and velvety in texture. Conidiogenous cells are cylindrical, usually with an irregularly swollen apex that is studded with scars from conidial attachment. Conidia measure approximately (1.5–2.5) × (3.5–5) μm, are one celled and broadly clavate with a flat base. Each conidium usually gives rise to several additional conidia in a repeating process that ultimately forms a complexly branched head consisting of three to four levels of conidia (Figure 2). A *Rhinocladiella* morph (Figure 3) is usually present, and a *Phialophora* morph (Figure 4) can also be present (McGinnis and Schell 1980). *F. pedrosoi* can easily be confused with *Cladosporium* spp. and *Rhinocladiella* spp. (Schell et al. 1995).

Cladophialophora carrionii (*Cladosporium carrionii*)

Colonies are olivaceous–black, slow in growth rate, and velvety in texture. Conidiophores bear outwardly spreading, sparsely branching, long chains of ellipsoidal, symmetrical, one-celled conidia that measure approximately (2.2–2.6) × (4.5–6.0) μm (Figure 5). A *Phialophora* morph can also be present (Trejos 1954; Honbo et al. 1984a, b). This species can easily be confused with *Cladosporium arxii*, *Cladosporium* spp., *F. pedrosoi*, and *Cladophialophora bantiana* (Schell et al. 1995).

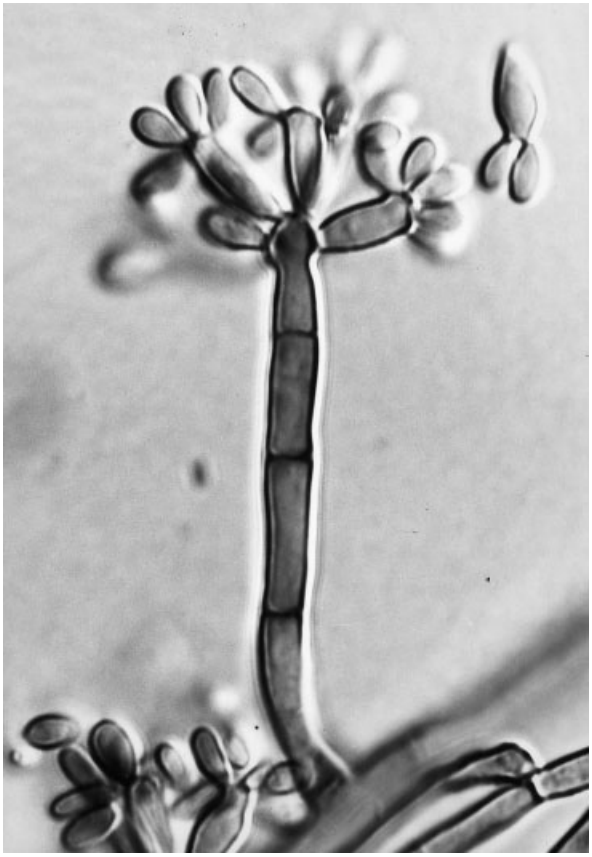

Figure 2 Fonsecaea pedrosoi *can produce conidia by multiple mechanisms. Usually, conidia arise from the swollen apex of the conidiophore, and then give rise to additional conidia, resulting in two to four levels of conidia formation. This form is termed the 'Fonsecaea synanamorph,' and is generally considered to be the most distinctive.*

Phialophora verrucosa

Colonies are olivaceous–black, slow in rate of growth, and velvety in texture. Conidiogenous cells are phialides, vase-like in shape, with a prominent collarette (Figure 6). Conidia measure approximately (1.5–2.5) × (2.5–4.5) μm, are one-celled, ellipsoidal, and emerge sequentially from phialides to accumulate in subglobose masses. The species can be confused with other species of *Phialophora* and *Fonsecaea* (Schell et al. 1995).

CLINICAL PRESENTATIONS OF CHROMOBLASTOMYCOSIS

In its various stages, chromoblastomycosis can mimic the following:

- a foreign body granuloma (Wackym et al. 1985)
- a ganglion cyst (Jayalakshmi et al. 1990)
- lupus (Molina Lequizamon et al. 1984)
- leprosy (Banks et al. 1985; Pavithran 1992)
- leishmaniasis or cutaneous tuberculosis (Boudghene-Stambouli and Merad-Boudia 1994)

Figure 3 Fonsecaea pedrosoi *also often sporulates by forming each conidium sequentially at the continually elongating tip of the conidiophore. This form is termed the '*Rhinocladiella *synanamorph' based upon its resemblance to fungi in the genus* Rhinocladiella.

Figure 4 Fonsecaea pedrosoi *can sometimes sporulate by forming phialides, where conidia emerge from a cup-like orifice in a sequential process. This is termed the '*Phialophora *synanamorph' because of its resemblance to the fungus* Phialophora verrucosa. *In this figure, a portion of the* Fonsecaea *synanamorph is also visible.*

- keratoacanthoma (Wiss et al. 1986)
- cutaneous sarcoidosis (Perry and Sheretz 1987)
- common wart (Jayalakshmi et al. 1990)
- malignancy (Pavlidakey et al. 1986; Jayalakshmi et al. 1990; Hiruma et al. 1992).

Lesions of chromoblastomycosis most often arise on an extremity and usually begin as small scaly papules or nodules which are painless but may be itchy (Fukushiro 1983). Satellite lesions may gradually arise from auto-inoculation as a result of scratching the lesions, or by spread of the fungus through the lymphatic system (Carrion and Silva 1947; Takase et al. 1988; Hiruma et al. 1992). Rash-like areas enlarge and become raised irregular plaques, often scaly or verrucose (Figure 7). Lesions in cases of long duration can become large, tumorous and even cauliflower-like in appearance (Figure 8). Flat lesions that exhibit central healing and scarring can sometimes be seen. Blood, serous, or caseous material can often be expressed from lesions (Carrion and Silva 1947). Damaged tissue, clotted blood, and fungal cells are expelled through the epidermis by the process of transepithelial elimination and are deposited in the form of small dark aggregations at the surface of the lesion (Uribe et al. 1989). These are commonly known as black dots, and can be selectively removed and examined microscopically for the diagnostic presence of muriform bodies.

Other prominent features of chromoblastomycosis are epithelial hyperplasia, microabscess formation in the epidermis, and dermal fibrosis. There are data to suggest that intense extracellular matrix cross-linking, associated with the sustained contribution of transforming growth factor beta (TGF-β)-activated fibroblasts, exists in tissues of chromoblastomycosis compared with healthy tissue and tissue from other chronic skin infections. Keloid-like irreversible fibrosis seems to result from lysyl oxidase-mediated pyridinoline (Esterre et al. 1992) and transglutaminase-mediated glutamyl-lysine (Esterre et al. 1998b) collagen cross-linking.

Chromoblastomycosis is slowly progressive and can persist for decades if neglected or not successfully treated. An analysis of cases for a 20-year period in Jamaica found a mean duration of 8 years, and a range of 3 months to 27 years (Bansal and Prabhakar 1989). A persistence up to 30 years has been recorded in the Malagassy focus (Esterre et al. 1996). Untreated lesions can progress to involve an entire limb, although muscle and bone do not become infected. In the absence of adequate management, chromoblastomycosis is a

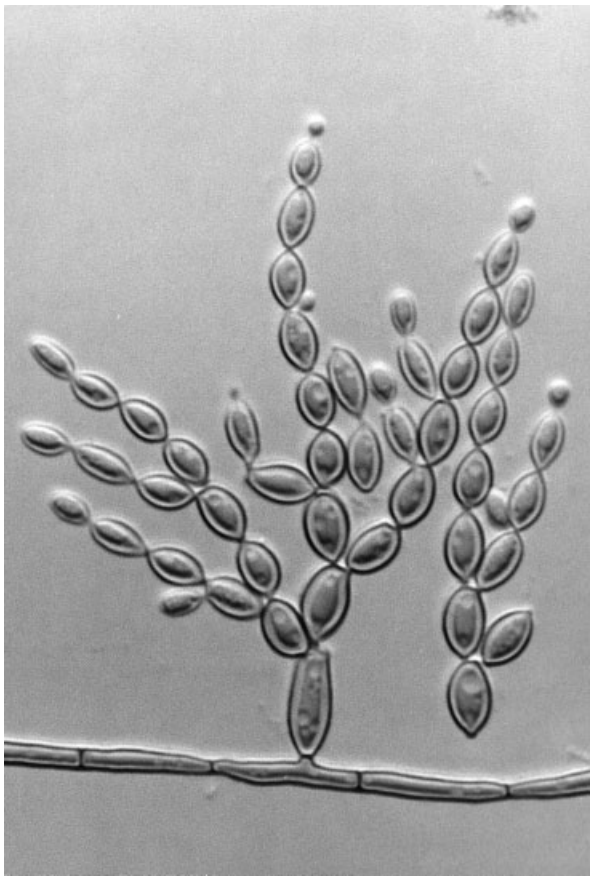

Figure 5 Cladophialophora carrionii *forms conidia in acropetal, occasionally branched, chains.*

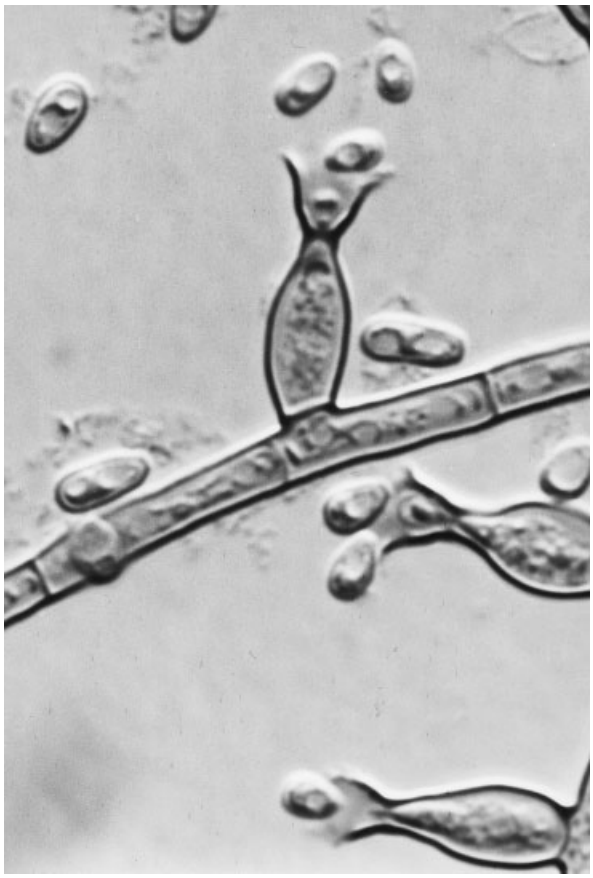

Figure 6 Phialophora verrucosa *sequentially forms conidia at the orifice of phialides which bear distinctive cup-like collarettes.*

chronic, progressive, and recalcitrant disease that may eventually become incapacitating. Complications include secondary bacterial infections, lymphostasis, swelling and contraction of the affected limb. For those living in tropical climates, feet and legs are the parts of the body that are most frequently subjected to minor wounds involving soil and vegetation; as a result, the disease most often involves these sites, although any exposed part of the body is vulnerable (Nakamura et al. 1972; Iwatsu et al. 1983; Zaror et al. 1987; Gruber et al. 1988; Gross and Schosser 1991; Hiruma et al. 1992; Esterre et al. 1996 and Figure 9). Hematogenous spread and cerebral infections are rare complications, and seem to occur only with *F. pedrosoi* in Japan (Fukushiro 1983). Primary infection of the lung has only been reported twice for *F. pedrosoi* (Zaharopoulos et al. 1988; Morris et al. 1995).

HISTOPATHOLOGY

Muriform bodies are the characteristic tissue form of all agents of chromoblastomycosis. They are pheoid, thick-walled, multiseptate bodies, 5–13 μm in diameter and are typically found at the surface of the lesions (see Figure 1). The host tissue response is a granulomatous reaction with polymorphonuclear neutrophils, sometimes showing ultrastructurally a frustrated phagocytosis pattern (Esterre et al. 1993), and macrophages infiltrate and formation of numerous microabscesses. Muriform bodies are found singly or in clusters within the microabscesses, and sometimes within giant cells

Figure 7 *Chromoblastomycosis lesions are often scaly or crusty, and frequently feature small, dark aggregations of clotted blood and fungal cells (right). A portion of the lesion (left) had been biopsied the preceding day.*

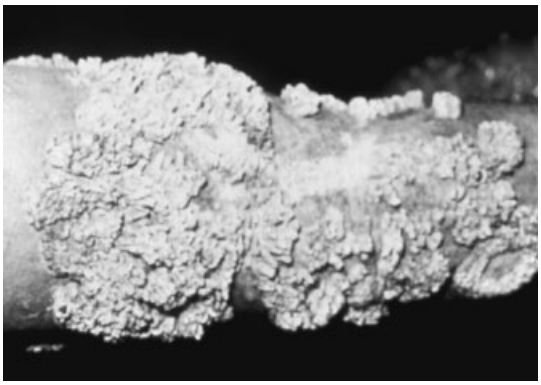

Figure 8 *Chromoblastomycosis of many years' duration can result in large, verrucous or cauliflower-like lesions. (Courtesy T.G. Mitchell.)*

(Fukushiro 1983; Uribe et al. 1989). Epitheliomatous hyperplasia is generally present. Melanin is produced in the cell walls of the muriform bodies, which results in a dark-brown coloration that can easily be seen in histological sections stained with hematoxylin and eosin. They routinely divide, not by budding as seen in yeasts, but by separation along the interface of double septa. Budding of the muriform bodies is sometimes seen, but is not believed to contribute significantly to multiplication. Muriform bodies can be induced in vitro under defined conditions, and experimental data suggest that the concentration of calcium ions within infected tissue may play a role in regulating in vivo dimorphism (Mendoza et al. 1993). Detecting the presence of muriform bodies establishes a diagnosis, but as all etiologic agents of chromoblastomycosis have the same features in tissue, the particular species involved in a case can be determined only by recovery of the fungus in culture.

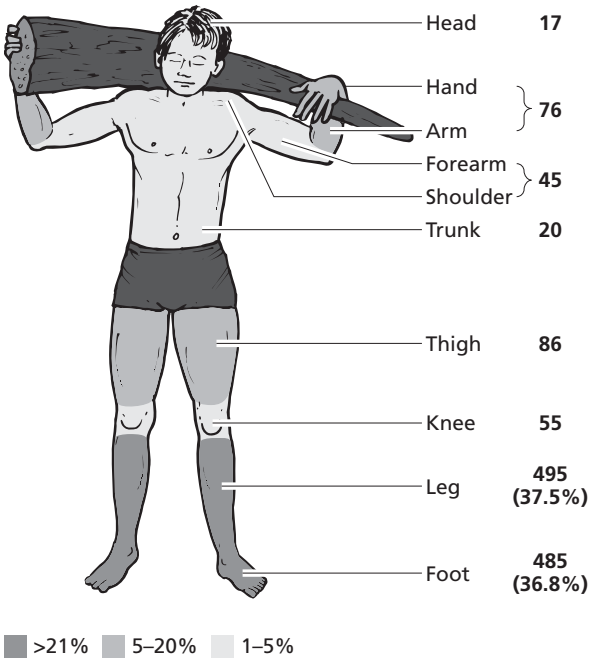

Figure 9 *Distribution of skin lesions observed on 1 316 cases, during a 40-year (1955–94) epidemiologic survey in Madagascar.*

IMMUNOLOGY AND SEROLOGY

Relatively little is known about the immunological aspects of chromoblastomycosis, but evidence so far demonstrates that host responses to fungal antigens occur at both cellular and humoral levels. Antibodies against the causative fungi have been detected by various methodologies in a high percentage of patients (Villalba 1988; Villalba and Yegres 1988; Esterre et al. 1997a, 2000), including some asymptomatic cases in endemic areas (Esterre et al. 1997a). Experimentation using animals has suggested that purified rabbit IgG can substantially inhibit growth of *F. pedrosoi* in culture (Ibrahim-Granet et al. 1988), but whether these findings reflect a role for protective immunity in humans against this mycosis is not yet certain. Host cellular response has been experimentally demonstrated in mice. Fungal cell wall lipids coated on to charcoal particles induced significant granulomatous reactions in mice for agents of chromoblastomycosis, as compared with control charcoal particles (Lacaz and Ekizlerian 1985).

In addition, a preparation of fractionated non-lipid cell wall material common to four etiologic agents has been found to induce a granulomatous reaction and death in mice, thereby showing that nonlipid cell wall components, as well as lipids, are capable of causing a cell-mediated host reaction (Lacaz and Fazioli 1985). Data from a recent clinical study of eight patients with chromoblastomycosis, compared with healthy control patients, suggest that individuals with chromoblastomycosis exhibit a normal cellular response to bacterial antigens, but that a partial suppression of cell-mediated immune response to fungal antigens arises at some point during the infection. The authors have suggested that such suppression may play a role in the pathogenicity of these fungi (Fuchs and Pecher 1992). Skin-test antigens have been prepared in research settings, and limited testing has found reactivity among patients diagnosed with chromoblastomycosis. Skin testing of larger population subsets to determine the extent of exposure to the etiologic fungi has not been attempted, but an enzyme-linked immunosorbent assay (ELISA)-based survey has been organized in the endemic eastern region of Madagascar, revealing a 6 percent level of seropositivity in the whole population (Esterre et al. 1997a).

LABORATORY DIAGNOSIS

As chromoblastomycosis can be confused with many other afflictions, it is necessary to establish the diagnosis through laboratory testing before proceeding with specific treatment. Serological testing for chromo-

blastomycosis is reportedly very sensitive (Esterre et al. 2000), but is not widely used because the necessary specialized reagents are not available commercially. By contrast, microscopy and culture provide highly sensitive means of diagnosis that are both simple and inexpensive. The most rapid diagnostic technique is collection of skin scrapings or a biopsy specimen from the active margin of the lesion and examination microscopically in 10–20 percent KOH for the presence of muriform bodies. So-called black dots, which are often present on the lesion's surface, are most likely to contain the muriform bodies and are excellent specimens (Zaias and Rebell 1973). In unusual cases, superficial crusts from lesions or specimens from pulmonary infection may show septate, pheoid hyphae instead of muriform bodies (Carrion and Silva 1947; Lavalle 1980; Fukushiro 1983; Morris et al. 1995). The addition of the fluorescent reagent calcofluor white to the KOH procedure has greatly increased the sensitivity of microscopic examination for most mycotic infections. However, it is not reliable for detecting heavily melanized fungal cells, such as muriform bodies, and therefore should not be the only method of microscopic examination used in suspected cases of chromoblastomycosis.

When microscopy is negative, culture can establish the diagnosis because of its superior sensitivity as a clinical procedure. As all agents of chromoblastomycosis have the same morphology in tissue, culturing the fungus is the only means of establishing the etiology in a given case, and is a prerequisite if antifungal susceptibility testing is to be performed. Furthermore, because of the current proliferation of newly developed antifungal compounds, it is important to culture and correctly identify causative fungi before instituting therapy so that potential patterns of clinical resistance or susceptibility to individual drugs can eventually become recognized and utilized by the medical community.

TREATMENT

Historically, successful treatment of chromoblastomycosis has been exceedingly difficult. Surgical excision of small areas of infection has provided the most reliable means of cure. Successful surgery necessarily requires removal of a margin of uninfected tissue. In addition to traditional surgical management, success has been reported with cryosurgery using liquid nitrogen in 11 patients infected with *F. pedrosoi* (Pimentel et al. 1989), with CO_2 laser surgery in one case (Kuttner and Siegle 1986) and with Mohs' micrographic surgery in another (Pavlidakey et al. 1986). Both thiabendazole and flucytosine (5-fluorocytosine), an orally administered drug, have led to improvement in several cases where excision was not feasible. As a result of toxicity from metabolites of flucytosine, however, it is necessary to monitor the level of this drug in the patient's serum during the course of therapy.

The first consistently effective antifungal compound for infections caused by pheoid fungi appears to be the new triazole itraconazole. No prospective, controlled trials have yet been conducted for this compound but evidence so far indicates that cures and significant improvements can be expected for many patients, even for some who have failed to respond to other therapies. In one series of 19 patients with inoperable lesions caused by *F. pedrosoi*, duration of their infections ranged from 6 to 36 years, with 14 patients having had previous therapy. All the patients responded to itraconazole therapy, with eight patients achieving clinical and biological cure (Queiroz-Telles et al. 1992). From Natal, South Africa, a report suggests that itraconazole is especially effective for infections with *C. carrionii* (Bayles 1992).

Another new oral compound, terbinafine, has been recently investigated for infections caused by pheoid fungi. An open pilot study in two hospitals in Madagascar, using 500 mg/day during 6–12 months, reported a high clinical and mycological efficiency (about 85 percent) without significant adverse reactions nor relapse during a 2-year follow-up (Esterre et al. 1997b, 1998a). Short treatments (3–4 months) or 250 mg/day dose have been tested with success on recent lesions (Esterre et al. 1997b) and even poorly responsive patients could be cured by a combination therapy with oral itraconazole (200–400 mg/day) and terbinafine (250–750 mg/day) (Gupta et al. 2002). In addition to its fungicidal properties, terbinafine revealed two unexpected and interesting bypass effects at the skin level: an antifibrotic activity (Esterre et al. 1998a; Esterre and Ricard-Blum 2002) and a reversal of immunological anergy (Elewski et al. 2002).

Unfortunately, both itraconazole and terbinafine are expensive at present and therefore of limited affordability. An inexpensive approach that has yielded good results in several cases is treatment with topical heat. Chemical and electric pocket warmers and heat bulbs resulted in cures for 15 of 19 *F. pedrosoi* infections in one reported series (Hiruma et al. 1993). Spontaneous resolution of untreated lesions has been reported but appears to be a rare occurrence (Nishimoto et al. 1984).

ACKNOWLEDGMENTS

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Sporotrichosis

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INTRODUCTION

Sporotrichosis is a varied disease caused by a single dimorphic fungal species, *Sporothrix schenckii*. It occurs worldwide, growing as a mold in association with dead or senescent plant material. When cells of the mold gain entry to a susceptible animal host, they transform into budding yeast cells and persist as such within the infected tissue. Sporotrichosis most commonly presents as a chronic ulcerative infection of cutaneous and subcutaneous tissue, and tends to spread along channels of the lymphatic system. These cutaneous and lymphocutaneous infections typically are initiated by trauma or minor abrasion from plant material harboring cells of the fungus. Lesions normally develop within 3–12 weeks following inoculation. In unusual instances, the fungus can spread from lesions in subcutaneous tissue to nearby joints, or more rarely to other organs. Pulmonary sporotrichosis is an infrequent syndrome which starts when airborne conidia from the mold, growing as a saprobe upon natural substrata, are inhaled into the alveoli of the lungs. In such cases, an infection can develop that resembles other chronic lung infections such as tuberculosis and histoplasmosis.

Sporotrichosis is a cosmopolitan disease and is probably the most frequently encountered subcutaneous mycosis in the world. Most cases occur among people living in tropical and subtropical regions, and the disease is relatively common in Mexico, Uruguay, and Brazil. In Brazil, the national incidence of sporotrichosis is second only to paracoccidioidomycosis among the deep mycoses (Costa et al. 1994). In Sao Paulo, sporotrichosis has accounted for as much as 0.5 percent of all mycoses for which medical attention was sought (Sampaio et al. 1954). The number of cases in North America has been estimated at 2.4 per million population per year, but because the mycoses have never been designated as notifiable (reportable) diseases, the true incidence of sporotrichosis remains unknown (Ajello 1970; Reingold et al. 1986).

HISTORY

The first case report and documentation of the fungal etiology of sporotrichosis appeared in 1898 in the USA (Schenck 1898). By 1932 about 200 infections had been reported in the USA (Jacobson 1932), and there was subsequently a rapid increase in reported cases in this and other countries (Mackinnon 1948; Goncalves and Peryassu 1954; Sampaio et al. 1954; Mayorga et al. 1978; Lavalle and Mariat 1983; Reingold et al. 1986; Rippon 1988; Talhari et al. 1988). In Japan, for example, 13 cases had been reported as of 1945, but since that time more than 2 500 cases have been dealt with by the Japanese healthcare system (Fukushiro 1984). A retrospective study at one dermatology clinic in Japan found that 0.17 percent (150 patients) of all patients entering the clinic were diagnosed with sporotrichosis (Kusuhara et al. 1988), and similar findings were reported from a second clinic (ltoh et al. 1986; Eisfelder et al. 1993). By

contrast. there has been some recent evidence to suggest an overall decline in the prevalence of sporotrichosis in parts of Japan from 1983 onwards, as well as a decrease in the number of infections in Europe, possibly resulting partly from a decreasing number of people being engaged in agriculture (Alberici et al. 1989; Barile et al. 1993; Eisfelder et al. 1993). Epidemiologic data from about 822 cases were recorded in a 37-year period in Jalisco, Mexico (Mayorga et al. 1997) and a review, mainly about epidemiology, microbiology, and treatment, was published in 2001 (De Araujo et al. 2001).

Sporotrichosis continues to be a chronic problem for public health programs in certain rural areas of the world; in addition, there are newer aspects of the disease that are particularly relevant in urban and suburban settings. One of these is the emergence of sporotrichosis within the subpopulation of people who are seropositive for the human immunodeficiency virus (HIV). A second recently recognized aspect is the potential for zoonotic infections, particularly those contracted from the domestic cat *Felis catus*. Several medical therapies are available for the treatment of sporotrichosis, and cure of the lymphocutaneous form of infection can be achieved with relative ease. By contrast, pulmonary, skeletal, and disseminated infections can be very difficult to manage and are life-threatening conditions.

ECOLOGY AND EPIDEMIOLOGY

Sporothrix schenckii is widespread in nature and can be isolated as a saprobe from dead or senescent vegetation, such as thorns, hay, straw, sphagnum moss, and wood. For this reason, sporotrichosis is a recognized occupational hazard for nursery and forestry workers, and is also often associated with farmers, florists, leisure gardeners, and others whose occupation or avocation brings them into frequent and sometimes traumatic contact with plant material or soil. The great majority of infections arise under such circumstances, as reflected by one of the common names for sporotrichosis – 'rose gardener's disease.' Also at risk are people who work as manual laborers in rural areas and who wear little protective clothing or footwear, and thus have increased potential for exposure to the fungus (Forester 1924; Lavalle and Mariat 1983; Itoh et al. 1986).

Children comprise a significant portion of sporotrichosis patients, and presumably acquire most of these infections while playing or helping with chores out of doors (Rudolph 1984; Prose et al. 1986; Frumkin and Tisserand 1989; Yamada et al. 1990). Two reports from Mexico have shown that as many as 10–25 percent of sporotrichosis patients in that country are children (Leon 1964; Solano 1966). Retrospective examination of records at a university in Japan has shown that children under the age of 12 have a particularly high rate of infection. The face is a frequent site of infection in children, as evidenced by a finding that 92 percent of 529 pediatric cases of sporotrichosis included lesions of the face (Fukushiro 1984; Eisfelder et al. 1993).

A report of 238 cases occurring in a relatively remote area of the south central highlands of Peru showed 60 percent in children and the most commonly affected anatomic site was the face. The incidence of sporotrichosis in this region ranged from 48 to 60 cases per 100 000 persons and was highest among children aged 7–14 years, approaching one case per 1 000 persons (Pappas et al. 2000). In Mexico, 10–34 percent of cases are children, the youngest patient has been a child, 2 days old, who acquired sporotrichosis after a rat bite and the oldest a 117-year-old man. Lymphocutaneous forms are observed in 65–82 percent, fixed forms in 20–30 percent and the systemic ones in 2–5 percent (Mayorga et al. 1997; Arenas 2003).

Cluster infections can occur when people are exposed to a common source of inocula. Past examples of small outbreaks included 12 workers in two geographical regions who were infected by hay mulch (Cook et al. 1984), six students who became infected by packing straw (Sanders 1971), four workers who were infected by sphagnum moss at a garden center (Remington et al. 1982), four people who contracted infections from a contaminated camping tent (Campos et al. 1994), and two employees of a meat packing plant who developed pulmonary infection, presumably from a common source (Dewan et al. 1986).

A 32-year-old white man developed lymphocutaneous sporotrichosis at the same site as a self-administered tattoo on the dorsal aspect of the left foot, but he stated that he had mowed the lawn wearing only sandals on the same day that he had tattooed his foot (Bary et al. 1999). Cutaneous sporotrichosis evolved in a 20-year-old woman who underwent electrolysis on the anterior aspect of her neck (Ditmars and Maguina 1998) and a case of *Sporothrix* conjunctivitis was described as an atraumatic infection (Hampton et al. 2002).

ln addition, there are numerous reports of multiple people contracting sporotrichosis from an infected cat (Table 1).

More rarely, sporotrichosis can occur in epidemic proportions. The largest reported outbreak of sporotrichosis occurred during 1941–44 when at least 2 899 cases were seen among gold miners in the Transvaal region of South Africa (du Toit 1942; Helm and Berman 1947). Of these, 2 441 occurred in several levels of a single mine, although a total of at least nine mines was involved in the outbreak. The fungus was found to be growing upon timbers used to shore the mine shafts, and infections would begin when bare skin was abraded by the colonized timbers. The epidemic was brought under control by application of a fungicidal compound to the timbers (Brown and Weintroub 1947), and formaldehyde fumigation of clothing and living quarters (du Toit 1942). All infections were cured using oral potassium

Table 1 *Zoonotic sporotrichosis transmitted by* Felis catus

Vector	No. of infected humans	Associated trauma	Reference
1 cat	3	3	Larsson et al. 1989
5 cats	7	3 of 7 (2 bitten; 1 preexisting scratch)	Dunstan et al. 1986a, b
1 cat	6	None	Read and Sperling 1982
1 cat	2	1 (scratched)	Nusbaum et al. 1983
1 cat	3	1 (scratched)	Schiappacasse et al. 1985
1 cat	1	Scratched	Samorodin and Sina 1984
1 cat	1	None	Reed et al. 1993
1 cat	1	NS	Dunstan et al. 1986a, b
1 cat	3	2 of 3 (bitten, scratched)	Naqvi et al. 1993
1 cat	1	None	Travassos and Lloyd 1980
3 cats	7	None	Samorodin and Sina 1984
5 cats	5	4 of 5 (bitten, scratched)	Zamri-Saad et al. 1990
4 cats	3	1 of 3 (scratched)	Marques et al. 1993
4 cats	4	4 scratched	Fleury et al. 2001
2 cats	13	2 of 13 scratched	Kauffman 1999
117 cats	66	52 contact, 31 (scratched or bitten)	Bastos de Lima-Barros et al. 2001; Schubach et al. 2001
Totals: 149 cats	126[a]	53 of 125	–

a) 89 of 42 people denied incurring trauma from the cats.

iodide daily for 4–5 weeks in most cases (Helm and Berman 1947; Quintal 2000).

The largest single outbreak in the USA occurred in 1988, infecting 84 people in 15 states and spanning a distance of hundreds of kilometers. These individuals, most of whom were forestry workers, became infected from handling sphagnum moss that had been applied to the roots of tree seedlings in order to humidify and protect them during shipment from central nurseries (Coles et al. 1992). Sphagnum is also used by nurseries to protect seedling roots from cold storage conditions during the winter season. Contaminated sphagnum moss, used in horticulture or forestry activities, has caused additional outbreaks (Gastineau et al. 1941; Crevasse and Ellner 1960; Hayes 1960; D'Alessio et al. 1965; Storrs et al. 1969; Powell et al. 1978; Grotte and Younger 1981; Remington et al. 1982; Cote et al. 1988). Nearly all of the sphagnum harvested in the USA grows in bogs in central Wisconsin.

Although sporotrichosis has long been linked in North America to the handling of sphagnum moss, it is still unknown at what point the fungus comes into association with the moss. Environmental sampling from earlier moss-related outbreaks suggested that *S. schenckii* is likely to be found in conjunction with harvested moss that has become wet during storage, rather than with moss that has been continually stored under dry conditions (McDonough et al. 1970; Powell et al. 1978). During investigation of the 1988 outbreak, samples of moss taken at the point of harvest were found to be culture negative for *S. schenckii*. Pond water used at one of the nurseries to dampen the moss before its use as packing material was also culture negative. Additional testing under laboratory conditions showed that *S. schenckii* did not grow on living sphagnum, but would grow very quickly on dead sphagnum in the presence of adequate humidity and warmth. Thus, it is suspected that *S. schenckii* can be introduced into sphagnum during or after harvest, but that the moss does not pose an infectious hazard as such, until it becomes moistened and warmed to an extent that allows the fungus to multiply (Zhang and Andrews 1993). Similar findings have been reported with regard to hay (Cook et al. 1984).

An outbreak was associated with stored hay or hay bales harvested in a military installation in southwestern Oklahoma. Four patients had maintained hay bales in a house and the fifth patient had visited the house once. As in three previous reports, this contact with hay should be recognized as a risk factor for infection with *S. schenckii* (Dooley et al. 1997).

Although most infections originate when the fungus is introduced by splinters, thorns, abrasions, and other minor wounds, there is evidence to suggest that a wound of the cutaneous barrier is not essential for infection to occur. Experimental results obtained with human subjects in conjunction with the African epidemic showed that one in 10 developed sporotrichosis when a thick suspension of viable conidia was placed onto the skin, covered with gauze, and allowed to incubate (Simson et al. 1947). A 1924 report attributed the source of an infection to the handling of contaminated bandages from lesions of a patient who was being treated for sporotrichosis (Forester 1924). In 1992, there was a compelling incident further suggesting that a wound is not a prerequisite for initiation of cutaneous

sporotrichosis. In this instance, infection developed on the finger of a researcher who had used a mortar and pestle to homogenize a strain of *S. schenckii* (Cooper et al. 1992). Molecular typing of both the isolate cultured from the lesion and the stock culture that the researcher had worked with showed that they were the same strain of *S. schenckii*. Molecular typing has been used in additional cases to establish the source of infection (Reed et al. 1993), and to suggest that a person can be simultaneously infected with more than one strain of *S. schenckii* (Kobayashi et al. 1990).

As these accounts imply that sporotrichosis can sometimes develop without a preceding breach of the dermal barrier, the question of contagion warrants consideration. Historically, sporotrichosis has not been considered a transmissible infection even though rare instances of person-to-person spread had been suggested long ago (Forester 1924; Smith 1945). Recent reports, such as that of an infection apparently passed from the arm of a woman to the face of her 3-year-old child, give cause to reconsider this potential aspect of sporotrichosis (Jin et al. 1990). It was a series of animal-acquired infections, however, that provided some of the strongest evidence that, in some circumstances, infections can be contagious and possibly occur through the invasion of apparently intact dermis. The infections in these cases were acquired from domestic cats. In one example, all four family members developed sporotrichosis from their infected pet. When the cat was presented for veterinary care, a veterinary technician and a veterinary student also contracted sporotrichosis soon after handling the animal. Significantly, none of the infected individuals could recall being scratched or bitten by the cat (Read and Sperling 1982).

In another example, a veterinary technician wore gloves and had no wounds while examining an infected cat but 3 weeks later developed lesions of sporotrichosis at the cuff line where glove protection ended (Dunstan et al. 1986b). Infections attributed to cats are known from at least four continents. A recurring feature of interest in these reports is that many patients were certain that they had not been wounded during handling of the infected animals. At a minimum, 43 infections in humans have been attributed to 26 cats, and 26 of 42 people for whom information was available denied incurring trauma from the animal (see Table 1).

It has long been known that cats are quite susceptible to sporotrichosis (Barbee et al. 1977). It is not clear how cats become infected, but in one instance eight people contracted infections from two cats that frequented a site where sphagnum moss had been used in landscaping. One possibility is that the fungus might adhere to the feet and claws of the cats, and lesions could arise as the inocula are implanted during fights between animals (Dunstan et al. 1986b). Another possibility is the inclination of cats to rub repeatedly against rough or thorny vegetation, which could initiate lesions of sporotrichosis. A related observation is that lesions in cats can be teeming with cells of the fungus (Werner et al. 1971; Nusbaum et al. 1983; Dunstan et al. 1986b; Marques et al. 1993; Reed et al. 1993). This is in stark contrast to lesions in humans where cells of *S. schenckii* are typically very sparse. It has been suggested that a potentially heavy load of organisms in the lesions of cats may be related to the high number of transmissions to humans and to the lack of evident trauma in some of these cases. In view of these reports, it should become accepted medical practice that patients with sporotrichosis be questioned to ascertain whether pets, particularly cats, may have been the source of infection, and that such animals be investigated as a potential threat to the health of other people.

In southeastern Brazil, four cases of human sporotrichosis transmitted by domestic cats were described. There was a previous history of a cat scratch before the development of the lymphocutaneous lesions. In cats, the lesions were multiple, extensive, necrotic, exudative, and ulcerated. In two necropsied cats, disseminated lymphatic and visceral mycotic infection was observed and histopathology revealed widespread histiocytic reaction with a large number of fungal organisms (Fleury et al. 2001).

In Rio de Janeiro over a period of 12 years (1987–98), 13 cases of human sporotrichosis were recorded. Two of them were associated with the scratch of a sick cat. During the subsequent two years (1998–2000), 66 human, 117 cats and seven dogs with sporotrichosis were diagnosed. Fifty-two human (78.8 percent) reported contact with cats with sporotrichosis, and 31 (47 percent) reported a history of a scratch or bite. This epidemic involving cats, dogs, and human beings is unprecedented in the literature, and may have started insidiously before 1998 (Kauffman 1999; Bastos de Lima-Barros et al. 2001; Schubach et al. 2001). In the study in Brazil, 72 of the cats (61.5 percent) were male with ages between 3 months and 18 years (median 2 years). The majority of the cutaneous lesions were located on head (46.6 percent) and limbs (35.9 percent). Most of the cases were from the underprivileged areas of the city. Nowadays, veterinarians, technicians, and cat owners or caregivers should be considered to be new categories at risk of acquiring the mycosis. The zoonotic potential of feline sporotrichosis is possibly due to the large number of organisms detected in feline tissues (Bastos de Lima-Barros et al. 2001).

Sporotrichosis has also been attributed to wounds inflicted by various other animals. Two well-documented cases occurred in young boys, each of whom was bitten by a captured field mouse (Moore and Davis 1918; Frean et al. 1991). In another rodent-related report, a rancher developed sporotrichosis after killing and handling gophers that had numerous cutaneous lesions (Olson 1912). Sporotrichosis has also been documented in a number of other animals including the armadillo,

boar, camel, cattle, chimpanzee, dog, dolphin, donkey, fowl, fox, horse, mule, and rat (Kaplan et al. 1982).

The relationship of sporotrichosis to the armadillo is remarkable. It has been established that *S. schenckii* can be found on vegetation lining the burrows of armadillos (Mackinnon et al. 1969). In Uruguay, two armadillo species, *Dasypus novemcinctus* and *Dasypus septemcinctus*, are hunted as game. For a 16-year period, of 138 cases for which the source of infection could be discerned, 81 percent were attributed to contact with armadillos, their burrows or equipment used in hunting them (Conti-Diaz 1980). Of the remaining infections, wounds from plants or other objects caused 15 percent and 4 percent, respectively. Furthermore, it was shown that infections were most numerous during the peak hunting months, and that very few cases of sporotrichosis were seen during the armadillo breeding season when hunting is traditionally suspended. The indigenous population has long been aware of this association and refers to sporotrichosis as *mulita* (armadillo) sickness (Conti-Diaz 1980). Sporotrichosis has also been found in two wild armadillos (*D. novemcinctus*) captured in the USA for purposes of leprosy research (Kaplan et al. 1982). The widespread exposure of various animals to *S. schenckii* has been demonstrated experimentally by skin-testing surveys. In Sao Paulo, testing of 96 zoo animals with sporotrichin, a skin-test antigen derived from *S. schenckii*, showed a positivity rate of 57 percent among terrestrial animals and 4 percent among arboreal animals (Costa et al. 1994).

Sporotrichosis has also been reported in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*), and there has been suggestion that it could possibly be associated with fish as well (Migaki et al. 1978). In Guatemala, 53 cases of sporotrichosis were observed during a 3-year period among people living in the Lake Ayarza District (Mayorga et al. 1978). Of 30 patients able to recall injury, six infections were attributed to trauma by woody material, but 24 individuals recounted trauma associated with handling fish taken from the lake. A report from South America relates an infection attributed by the patient to a knife wound sustained while cleaning fish (Beer-Romero et al. 1989).

Based on these types of reports, it seems that the role of animal vectors should probably be given greater attention in the consideration of the epidemiology of sporotrichosis. Outbreaks of the kind associated with sphagnum moss are dramatic, but may prove to be the easiest to prevent or control (Coles et al. 1992). By contrast, the potential for zoonotic infections remains high, and in some settings does not readily lend itself to prevention strategies.

S. schenckii has been also been encountered as a saprobe from a variety of unexpected substrates. It has been recovered from commercial potting soil (Kenyon et al. 1984), found as a food contaminant of meat sausages (Ahearn and Kaplan 1969) and culinary mushrooms (Kazanas and Jackson 1983), and found growing in a container of intravenous fluid at a medical center (Matlow et al. 1985). *S. schenckii* has been recovered from samples taken for routine microbiological monitoring of various surfaces within an indoor swimming pool complex. Although the fungus was detected in moderate numbers from swabs of wall and floor surfaces, no plants or wooden materials, which could have provided a substrate for amplification of the organism, were found in the building (Staib and Grosse 1983). No infections were associated in any of these instances.

The pandemic spread of HIV has brought particular attention to the mycoses, in that most of the acquired immune deficiency syndrome (AIDS)-defining illnesses are fungal infections. Sporotrichosis is now one of the opportunistic infections encountered in very small numbers among AIDS patients (Lipstein-Kresch et al. 1985; Shaw et al. 1989; Heller and Fuhrer 1991; Perfect et al. 1993; Bolao et al. 1994; Donabedian et al. 1994). Only about a dozen cases have been reported to date, but sporotrichosis in HIV-seropositive patients is extremely serious. In patients whose conditions have deteriorated to clinical AIDS, sporotrichosis becomes disseminated and the prognosis is grave. Disseminated infection can involve one or more joints or organ systems, including the central nervous system (CNS). Such infections are problematic not only from the standpoint of therapy but also from the basis of diagnosis. As the AIDS epidemic spreads, there are certain to be more cases of disseminated sporotrichosis. Whether the prevalence of sporotrichosis in this group of people can be expected to increase is unknown.

There appears to be no relationship of gender to sporotrichosis. Although some reports have suggested that patients are more likely to be men, others have shown nearly equal ratios and a few have noted a preponderance of female patients (Conti-Diaz 1980, 1989; Fukushiro 1984; Itoh et al. 1986; Kusuhara et al. 1988). Differences among these reports may result from varying sex distributions for occupations and tasks among the differing regions, and thus might correlate with frequency of exposure to the fungus rather than a gender-based predilection for infection. Statistics from the recent USA outbreak suggest that gender was not a risk factor, and that the greatest predictor of infection in this particular series was the duration of exposure to the fungal-ridden moss (Coles et al. 1992).

In a recent report in Mexico, the frequency was higher in women (62 percent), children, and adolescents under 20 years of age (34 percent) and adults older than 50 years of age (28 percent) (Espinosa-Texis et al. 2001).

MYCOLOGY

It has long been recognized that the genus *Sporothrix* has accommodated a heterogeneous assemblage of

morphologically similar species. One species, *Sporothrix cyanescens*, has been found to possess a septal ultrastructure that clearly shows affinity to the basidiomycetes. and so has been transferred to the genus *Cerinosterus* as *Cerinosterus cyanescens* (Smith and Batenburg-van der Vegte 1985). A teleomorph of *S. schenckii* has not been conclusively demonstrated in spite of many attempts at mating various clinical and environmental isolates. Nevertheless, as a result of similarities in morphology of the conidial morph formed by the ascomycete *Ophiostoma (Ceratocystis) stenocera* as compared with conidial formation in clinical isolates of *S. schenckii,* a phylogenetic relationship was surmised (Mariat 1971; de Hoog 1974). By contrast, studies of the polysaccharides and DNA base composition in these two taxa did not support this conclusion (Travassos and Lloyd 1980). Similarly, subsequent mitochondrial DNA typing by restriction fragment length polymorphism (RFLP) analysis showed a great deal of variation among isolates of *S. schenckii*, and this led to the conclusion that *S. schenckii* and *O. stenocera* may not be of the same holomorph (Suzuki et al. 1988). However, recent comparison of 18S rRNA gene sequence characters found that *S. schenckii* (two strains) and *O. stenocera* (one strain) differed at only three sites of the 1 700 nucleotides sequenced (Berbee and Taylor 1992). These findings strongly support the conclusion that *S. schenckii* is, or was at one time, the anamorph of an *Ophiostoma* spp. As a result of the wide variation in morphological and molecular characteristics reported thus far in these fungi, testing of additional strains is warranted. Presently, *S. schenckii* is classified in the mitosporic fungi (deuteromycetes).

S. schenckii synthesizes melanin via the 1,8-dihydoxynaphthalene pentaketide pathway, and this dark pigment has been found exclusively in the conidia, which are the infecting structures of this organism. Cultures of a reddish-brown mutant were identified by thin-layer chromatography, high-performance liquid chromatography, and ultraviolet (UV) spectra and material believed to be melanin was absent in conidial walls of the albino mutant. These results demonstrate that melanin may protect *S. schenckii* against certain oxidative antimicrobial compounds and against attack by macrophages, because melanized cells are less susceptible to oxidative killing in vitro and to phagocytosis by human monocytes and murine macrophages (Romero-Martinez et al. 2000).

IDENTIFICATION

Growth of *S. schenckii* can usually be observed within 3–5 days on culture media incubated at 30°C. Colonies typically are cream-colored initially, smooth and moist, gradually becoming dark brown and filamentous in texture. There is, however, much variation among isolates and even among subcultures of the same isolate. Some remain smooth and exhibit few or no aerial hyphae; others remain white to cream-colored with little or no brown pigmentation, or are brown during all stages of growth. Texture and coloration are also influenced by various culture media. Microscopic features include elongated conidiophores with a swollen, scarred apex which bears one-celled, obovoid (egg-shaped), hyaline to subhyaline conidia that measure approximately (1.4–2.5) × (2–4) μm; these are sometimes in radial 'flower-like' clusters (Figure 1). Isolates also form thick-walled conidia borne laterally along the hyphae, with each conidium being individually attached to the hypha (Figure 2).

These conidia are usually subglobose but can be triangular in some isolates. They can be colorless, but usually are dark brown and are chiefly responsible for the dark color of the colony. The various species in the genus *Sporothrix* are similar in appearance, but only *S. schenckii* is known to be pathogenic for humans and other animals. In addition, several other genera bear morphological resemblance to the genus *Sporothrix* (de Hoog 1974; de Hoog et al. 1985). For these reasons, in the absence of positive histology, it is necessary to confirm the identity of a suspected isolate of *S. schenckii*. This is accomplished by proving that, in addition to having both conidial forms as described above, the isolate will grow in a stable yeast-Iike form at 35–37°C (Figure 3). It should be noted that environmental, nonpathogenic isolates of *Sporothrix* spp. can grow as a yeast at 35–37°C, but lack the thick-walled, pheoid conidia that are attached individually along the hyphae (Dixon et al. 1991). When cultured at 35–37°C on chocolate agar or other enriched media, most isolates of *S. schenckii* will quickly show partial or complete conversion to a stable yeast-Iike form. Occasional isolates are more difficult to convert and may require extended incubation (up to 3 weeks) or frequent subculturing of the isolate (Kwon-Chung 1979). Some isolates from fixed cutaneous lesions reportedly will grow at 35°C but not at 37°C, and this thermal limitation was also shown through experimentation with mice (Kwon-Chung 1979). Other investigators have been unable to demonstrate this difference in isolates from fixed cutaneous lesions (de Albornoz et al. 1995).

A murine model suggest that the virulence of *S. schenckii* conidia may be determined by their cell wall composition. The more virulent forms of the fungus showed differences in cell wall sugar composition with rhamnose:mannose molar ratios of 1.7:1.0 for cells cultured for 4 days and 1.0:1.7 for conidia cultured for 12 days (Fernandes et al. 1999).

The interaction between *S. schenckii* and several extracellular matrix (ECM) proteins have been studied since adhesion is the first step involved with the dissemination of pathogens in the host. *S. schenckii* can bind to fibronectin, laminin, and type II collagen and also show

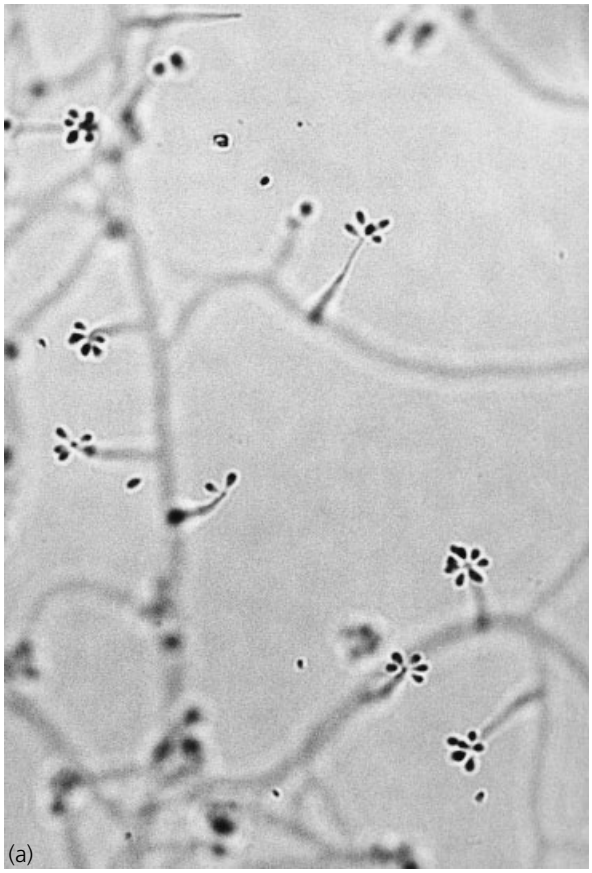

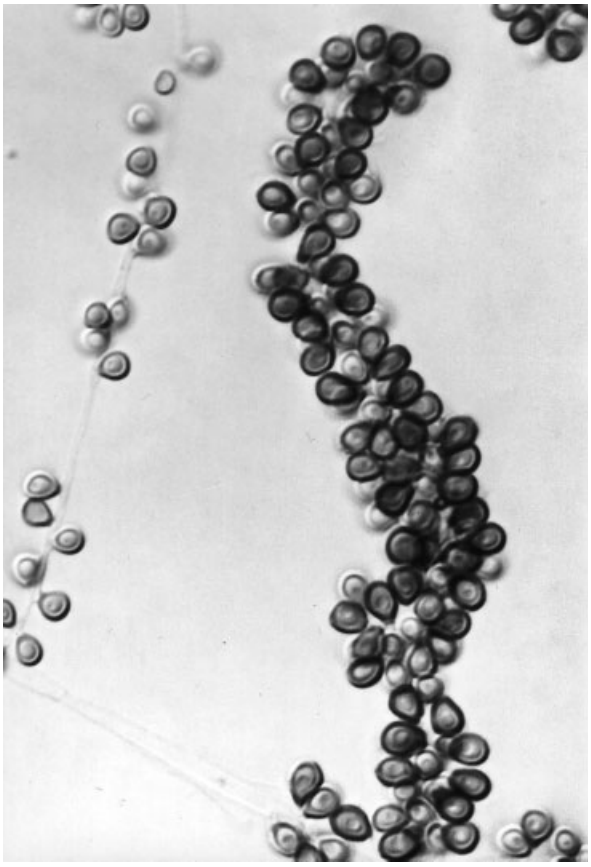

Figure 2 S. schenckii *also forms conidia that are thick-walled, dark brown as a result of melanin formation and attached directly to the hyphae, often in a dense sleeve-like manner.*

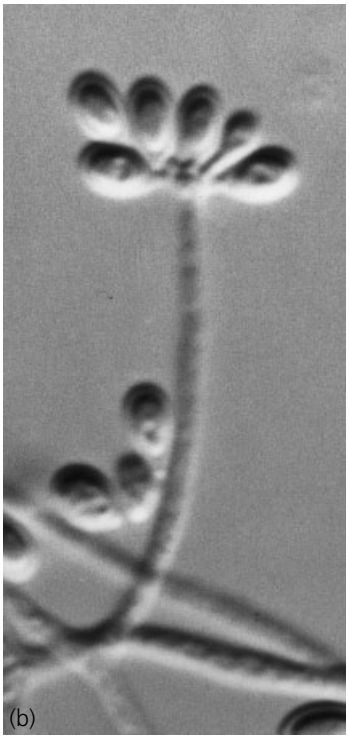

Figure 1 S. schenckii *forms conidia in a radial manner, each being attached to the apex of the conidiophore, resulting in a flower-like appearance.*

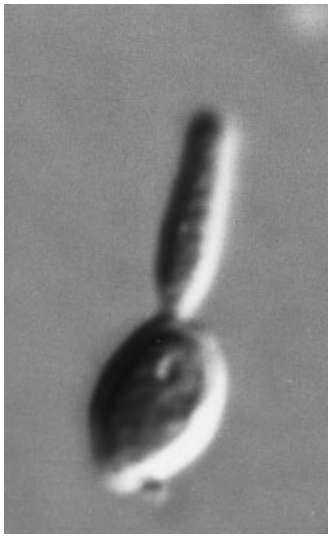

Figure 3 *In vitro conversion of* S. schenckii *from the mold to the yeast phase can be induced using a rich medium incubated at 35–37°C. Buds will often exhibit a characteristic cylindrical or 'cigar' shape.*

differences in binding capacity according to the morphological form of the fungus (Lima et al. 1999).

A new variety, *S. schenckii* var. *luriei*, was described in 1969 based on a case of sporotrichosis in which the fungal morphology manifested in host tissue was significantly different from that of typical sporotrichosis. Three cases have now been reported. In these infections, the fungus exhibited large (10–30 μm), subglobose to elongated, thick-walled (up to 2 μm) cells (Ajello and Kaplan 1969; Alberici et al. 1989; Padhye et al. 1992). These cells each formed a single septum that allowed separation into two separate cells. During separation, a fragment of the cell wall previously shared by the two cells can temporarily remain attached in a bridge-Iike manner, giving the fungus the distinctive appearance of spectacles. In some instances, a second septum would form, giving the fungus a muriform appearance. In the absence of culture, it is possible that the histological appearance of this new variety could be mistaken for chromoblastomycosis, although its cells do not contain visible amounts of melanin as seen in chromoblastomycosis. Mitochondrial DNA typing has also confirmed that *S. schenckii* var. *luriei* is markedly different from *S. schenckii* var. *schenckii* (Suzuki et al. 1988). In culture, whether as a mold or a yeast, *S. schenckii* var. *luriei* is morphologically indistinguishable from *S. schenckii* var. *schenckii.* There is insufficient experience with this variety to know if there are associated serological, therapeutic, or prognostic differences.

UV exposure of *Sporothrix* strains produces a high frequency of morphological variants and one common feature among them was that they were smaller in size compared to the wild type. These findings suggest that there is a highly sensitive chromatin in the conidial genome that is the target of UV light (Torres-Guerrero and Arenas-López 1998).

CLINICAL PRESENTATIONS OF SPOROTRICHOSIS

Sporotrichosis can be mistaken for numerous skin diseases including pyoderma gangrenosum (Spiers et al. 1986; Liao et al. 1991), rosaceae (Day et al. 1984), sarcoma (Sen et al. 1986) and leishmaniasis (Talhari et al. 1988). A recent field study in Brazil found that most patients with an initial diagnosis of leishmaniasis were infected instead with *S. schenckii* (Dr Aloisio Falqueto, personal communication).

Three cases of coinfection of leishmaniasis with *Sporothrix* spp. in the same lesion have been described in rural areas where people frequently try to remove the scabs using thorns or wooden splinters, thereby facilitating the entry of *S. schenckii*, which is present in the same geographic areas as *Leishmania* spp. (Agudelo et al. 1999)

Confusion with other diseases can lead not only to delay of suitable therapy, but also to the use of anti-inflammatory corticosteroids which can cause marked proliferation of the fungus within the lesions and a worsening of the infection (Bickley et al. 1985; Padhye et al. 1992). In a recent outbreak involving 84 patients, only 11 were correctly diagnosed at their first medical evaluation; 48 cases were misdiagnosed as bacterial infections, six as spider bites and two as cancer (Coles et al. 1992). In another series, the average delay in correct diagnosis was 4 months overall and 25 months for cases of extracutaneous sporotrichosis (Rowe et al. 1989).

The typical clinical presentation of sporotrichosis is that of a chronic cutaneous and subcutaneous infection. In these cases, lesions begin as a firm nodule, which initially is fluctuant but which gradually becomes immovable. The nodule becomes softened and in most cases eventually ulcerates to discharge pus or serous material (Figure 4). Satellite lesions will sometimes arise. A review of 235 cases showed that 23 percent were characterized by such fixed cutaneous lesions (Sampaio et al. 1954). Furthermore, facial lesions in children are often of this type (Fukushiro 1984; Eisfelder et al. 1993). In most cases, however, yeast cells from the initial lesion are phagocytosed and carried into the lymphatic system where a line of cutaneous ulcerative lesions then develops in an ascending fashion along lymph channels (Figure 5). *S. schenckii* has been isolated from blood, but such a finding is rare (Morgan et al. 1984; Kosinski et al. 1992).

In Mexico, upper limbs are affected in 45–53 percent, the face in 14–21 percent, lower limbs in 18–23 percent, and it is exceptional in the trunk (Arenas 2003). Lymphangitic forms account for 82 percent (Espinosa-Texis et al. 2001).

Complications in sporotrichosis are unusual, but the disease can be very refractory to medical management. Skeletal involvement (Atdjian et al. 1980; Arenas and Latapi 1984; Chang et al. 1984; Kumar et al. 1984; Yacobucci and Santilli 1986; Yao et al. 1986; Janes and Mann 1987; Wilson et al. 1988; Govender et al. 1989) is encountered often enough for sporotrichosis to be considered in the differential diagnosis of patients presenting with chronic joint disease. Reviews have

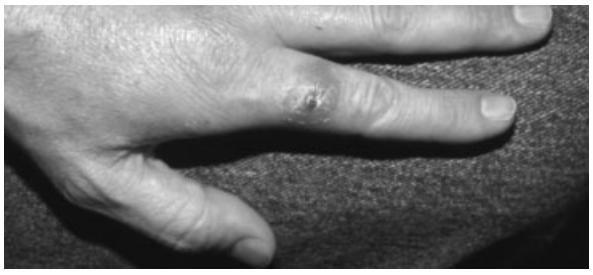

Figure 4 *Solitary cutaneous sporotrichosis lesion on the finger of a professional rose gardener*

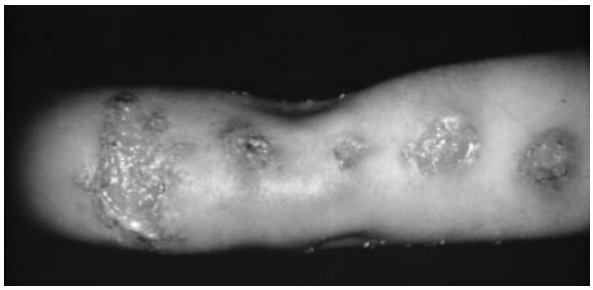

Figure 5 *Lymphangitic sporotrichosis ulcers develop in an ascending fashion along channels of the lymph system. (Courtesy T.G. Mitchell)*

noted that 1–14 percent of cases of sporotrichosis involve one or more joints (Rowe et al. 1989; Ortiz and Lefkovitz 1991). Joint infection also has been noted in the absence of accompanying skin lesions (Ortiz and Lefkovitz 1991).

Sporothrix arthritis is a rare disease and should be considered to be a monoarthritis, with only 51 cases reported in the English literature. Most of the patients have been middle-aged men who have a significant alcohol intake (77 percent). Seventeen joints have been affected and in 20 percent the knees were involved (Howell and Toohey 1998)

Dissemination, which can involve the CNS, has historically been relatively rare (Smith et al. 1981; Selman and Hampel 1982; Friedman and Doyle 1983; Morgan et al. 1984; Aronson 1992). However, cases of sporotrichosis now being seen in AIDS patients are usually disseminated, fatal infections (Lipstein-Kresch et al. 1985; Kurosawa et al. 1988; Shaw et al. 1989; Heller and Fuhrer 1991; Bolao et al. 1994; Donabedian et al. 1994). Extracutaneous sporotrichosis can also occur in organ transplant recipients who are receiving immunosuppressive therapy for the prevention of organ rejection (Gullberg et al. 1987; Agarwal et al. 1994).

Sixty-three widely distributed cutaneous lesions were observed in a female host with both humoral and cellular immunity within normal limits, but who was under treatment with long-term prednisone (Severo et al. 1999).

Although the typical clinical presentation of sporotrichosis is that of a chronic cutaneous and subcutaneous infection, *S. schenckii* has also caused eye infections (Schell 1986; Rippon 1988), sinusitis, infection of the vocal cords (Smith et al. 1981; Agger and Seager 1985), pyelonephritis (Agarwal et al. 1994) and pulmonary infections. Pulmonary sporotrichosis has been increasingly documented (Rohatgi 1980; Friedman and Doyle 1983; Nusbaum et al. 1983; Pluss and Opal 1986; Pueringer et al. 1986; Heller and Fuhrer 1991) and is suspected of being more common than is generally realized (Lavalle and Mariat 1983; England and Hochholzer 1985, 1987; Gerding 1986; Pluss and Opal 1986; Pueringer et al. 1986; Watts and Chandler 1987; Rippon 1988; Haponik et al. 1989; Farley et al. 1991).

A recent 30-year review at one institution found that 4 percent of sporotrichosis cases were pulmonary infections (Rowe et al. 1989). This form of the disease follows inhalation of conidia that have become airborne from the mold as it grows upon organic substrata in nature. The infection is insidious and sometimes asymptomatic. Symptoms include low-grade fever, weight loss, night sweats, productive cough with or without hemoptysis, and pleuritic pain. Radiological examination of the lungs can reveal adenopathy, nodules or cavitation, usually involving the upper lobes, or diffuse involvement similar to that seen with tuberculosis (Comstock and Woolson 1975; Rohatgi 1980; Pluss and Opal 1986). Ethanol abuse is found in conjunction with some cases of sporotrichosis, especially pulmonary and joint ínfections, but the reasons for this apparent association are not known.

HISTOPATHOLOGY

Histological examination of lesions reveals a central necrotic region with associated infiltration of neutrophils, macrophages, and giant cells. Yeast cells are usually not seen using routine histological methodology, but can be detected through examination of serially sectioned tissue that has been stained with special fungal stains. This approach reveals areas of host tissue reaction to the fungus and indicates where the yeast cells are likely to be found. The yeast cells of *S. schenckii* are often solitary and quite round in shape, 3–5 μm in diameter, mostly without apparent budding (Figure 6). Asteroid bodies (Figure 7) and cylindrical 'cigar-shaped' buds are often cited as characteristic microscopic features in cases of sporotrichosis, but both these can be difficult to demonstrate in many cases. Asteroid bodies are stellate-shaped conglomerations which develop from host–parasite interactions and can be seen in other diseases in addition to sporotrichosis.

Electron micrography of asteroid bodies obtained from cases of sporotrichosis suggests that materials from disintegrated host cells, particularly polymorphonuclear neutrophils, are deposited upon the surface of the fungal

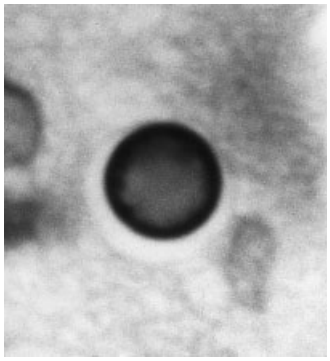

Figure 6 S. schenckii *cells in infected tissue are rare in number, round to ellipsoidal, 3–5 μm, yeast-like, with or without obvious budding.*

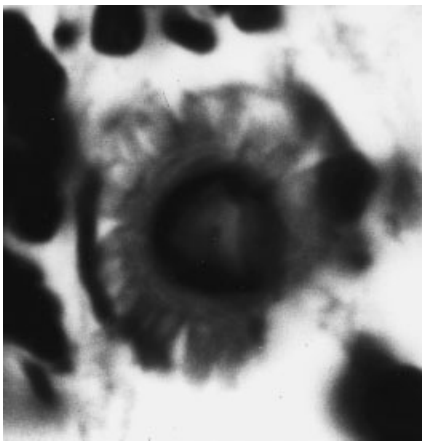

Figure 7 *Asteroid bodies consist of degenerate materials from the host immune response that accumulate in a ray-like manner upon the yeast cells of* S. schenckii, *giving them a stellate appearance.*

cell and are subsequently crystallized through an unknown process. The result is a multilayered accretion which features an outer layer of low electron density that appears to contain fragments of cell membrane-Iike material (Hiruma et al. 1991, 1992a). In lesions where the fungus is abundant, cells of *S. schenckii* can be within the macrophages, and sometimes exhibit a staining artifact of a halo or capsule. In these instances, sporotrichosis can be mistaken for *Histoplasma capsulatum* var. *capsulatum* (Nusbaum et al. 1983; Farley et al. 1991; Aronson 1992).

The characteristic infiltrates in three zones (central suppurative, tuberculoid, and peripheral round cell) were found in 27.27 percent, the asteroid bodies in 18 percent, and the yeast cells in 32 percent. The histopathological findings usually offer clues to the diagnosis and in just a few cases when the yeast cells are found, it can be diagnostic (Ruiz-Esmenjaud et al. 1996).

Asteroid bodies and yeast cells with budding, highly suggestive of the disease, were seen in the cytological and histological preparations obtained with fine-needle aspiration (FNA) cytology (Zaharopoulos 1999).

IMMUNOLOGY AND SEROLOGY

Data suggest that exposure to *S. schenckii* is widespread in many populations, and that the host immune response is able to resolve most inoculum challenges. Antigens prepared from *S. schenckii* have permitted small-scale studies of skin-test reactivity to be conducted in areas of both high and low endemicity (Ajello 1970). Results from studies of a prison population and a hospital population in the USA showed delayed hypersensitivity reactions occurring in 11 percent and 10 percent of individuals, respectively. In comparison, people involved in high-risk occupations were found to have a much greater prevalence of skin-test positivity. Of workers tested in a plant nursery, 33 percent were reactive; for those workers employed for at least 10 years, the rate of positive skin tests was 58 percent. In Brazil, skin testing in a small group from the general population yielded a reactivity rate of 24 percent. These findings suggest that exposure to *S. schenckii* is more common than the number of clinical infections indicates, and that most infections are asymptomatic and self-limiting. Occasional clinical observations of apparent spontaneous remission support this notion for both cutaneous and pulmonary infections (Powell et al. 1978; Bargman 1981; Iwatsu et al. 1985; Pluss and Opal 1986; Pueringer et al. 1986; Ramani et al. 1991).

In 50 patients, a metabolic antigen was used to elicit delayed hypersensitivity skin reaction and delayed hypersensitivity skin reactions were positive in 78 percent and culture in 94 percent (Espinosa-Texis et al. 2001). Immunodiffusion has been positive in 80 percent, complement fixation in 40 percent and also Western blot has been performed (Arenas 2003)

Animal models of sporotrichosis have also provided data which suggest that acquired resistance to infections may occur. Experimentation has indicated that exposure of mice to viable cells of *S. schenckii* can confer increased resistance to a subsequent intravenous challenge with the fungus (Shiraishi et al. 1992). In addition, sensitized spleen cells which were transferred into fresh mice also conferred increased resistance to intravenous challenge. Further testing showed that T-cell-mediated macrophage activation led to increased killing of *S. schenckii* cells from a subsequent challenge, and conversely that pretreatment with a macrophage inhibitor reduced resistance to intravenous challenge. Other studies using the mouse model have also revealed that *S. schenckii* can secrete extracellular proteases, and that mouse antibodies formed against these enzymes correlate with the course of disease progression (Yoshiike et al. 1993). Furthermore, it has been shown, in the mouse model, that the cellular immune response becomes depressed between weeks 4 and 6 of infection, and is a harbinger of worsening disease (Carlos et al. 1992).

The infectivity of this fungus between isolates from human cutaneous and pulmonary sporotrichosis after subcutaneous injection into the footpad showed restricted fungal infection and, after intravenous or intraperitoneal injection of yeast forms, three of four isolates from cutaneous sporotrichosis were unable to establish infection, but the development of systemic infection was observed only with *S. schenckii* isolates obtained from the human lung lesion (Tachibana et al. 1999a)

The cell-mediated immune response against infection with *S. schenckii* has been investigated in vivo and in vitro. There have been few studies reporting the role of cytokines in this infection. In this study it was shown that immune lymph node cells expressed message for interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-10 (IL-10) after stimulation

with heat-killed *S. schenckii*. These results suggest that acquired immunity against *S. schenckii* is expressed mainly by macrophages activated by CD4+ T cells, exerting protective immunity against *Sporothrix* via enhanced fungicidal activity (Tachibana et al. 1999b)

Differences in human serological response to sporotrichosis have been demonstrated for patients with cutaneous disease compared with patients with extracutaneous (joint, pulmonary, and CNS) disease (Scott and Muchmore 1989). In tests using a single strain of *S. schenckii* for antigen preparation, twice as many antibodies were detected in the sera of patients with extracutaneous infection. It is not clear how this may relate to pathogenicity, but the ability to detect antibodies that are specific for extracutaneous sporotrichosis could potentially provide a useful means for serological diagnosis of this complication.

Antibodies to *S. schenckii* antigens can routinely be detected in most cases of sporotrichosis through the use of complement fixation and latex agglutination tests, but such tests require the use of specialized, labile, and relatively expensive reagents. Therefore, as a practical matter, it is easier and cheaper to diagnose most infections by culture and microscopic examination of infected tissue. In the diagnosis of CNS sporotrichosis, however, detection of antibodies has succeeded in several patients when a culture-based diagnosis had failed. In such cases serological testing offers an invaluable tool (Scott et al. 1987).

A concanavalin A (conA)-binding fraction has been isolated from the *S. schenckii* cell wall (SsCBF) and the immunodominance of the *O*-glycosidically linked oligosaccharides in the reactivity of SsCBF with IgG from patients with sporotrichosis has been observed. These findings suggest that the SsCBF is a species-specific antigenic fraction recognized by human sera and with potential application in the serological diagnosis of sporotrichosis as well as in the epidemiologic survey of the systemic form of this mycosis (Loureiro et al. 2000).

A predominant antigen gp84 has been demonstrated by Western blot with a conA-horseradish peroxidase (HRP) conjugate (Lima and Lopes-Bezerra 1997)

LABORATORY DIAGNOSIS

The quickest means of diagnosing most mycoses is by immediate microscopic examination of infected host material. This approach is usually futile with sporotrichosis because the cells of *S. schenckii* normally occur very sparsely within infected material. However, painstaking histological examination of multiple tissue sections can often establish the diagnosis. Another approach to diagnosis by microscopy is the staining of histological tissue sections with a species-specific fluorescent antibody conjugate which can quickly provide a specific identification of *S. schenckii* (Kaplan and Gonzalez-Ochoa 1963); these fluorescent antibody reagents are, however, available only at certain reference laboratories and are not commercially offered. The usual form of *S. schenckii* in tissue is that of a yeast, but, in rare cases, superficial crusts from lesions or specimens from pulmonary infection have shown hyphal filaments (Maberry et al. 1966; Lopes et al. 1992).

By indirect immunofluorescence, parasite elements were demonstrated in 50 patients corresponding with a sensitivity and specificity of 100 percent. In this work indirect immunofluorescence was the most effective diagnostic method for sporotrichosis followed by culture, hypersensitivity skin reaction, and histopathology (Espinosa-Texis et al. 2001). It has been found that anti-*Sporothrix* antibodies are specific in sporotrichosis. Immunohistochemistry was performed in 25 paraffin-embedded biopsy specimens of sporotrichosis in which asteroid bodies (AB) were demostrated, ten of them with a positive culture. The AB were extracellular eosinophilic structures, 15–35 μm in diameter, and located within abscesses, with a central yeast. The yeast stains with the anti-*Sporothrix* antibody, while the spicules do not. Visualization of the spicules alone can lead to the demonstration of the AB in adjacent sections, and thus could be a useful clue in the diagnosis of sporotrichosis (Rodríguez and Sarmiento 1998). Immunohistochemistry has been a useful tool as is immunofluorescence when a yeast or AB is present (M. Heurre, personal communication) (Figure 8).

The most sensitive means of diagnosing sporotrichosis is recovery of the fungus in culture. Infected material is inoculated onto fungal culture media that are formulated to allow the growth of *S. schenckii* while selectively inhibiting the growth of fungal contaminants and the normal microbiota that could be present. Cutaneous specimens consist of pus or biopsied tissue from the lesion, whereas pulmonary specimens consist of expectorated sputum or material collected by means of a bronchoscope. If meningitis is suspected, cerebrospinal fluid is obtained for culture via a lumbar puncture. As cultures are often negative in cases of CNS infection, serological testing together with culture is recommended

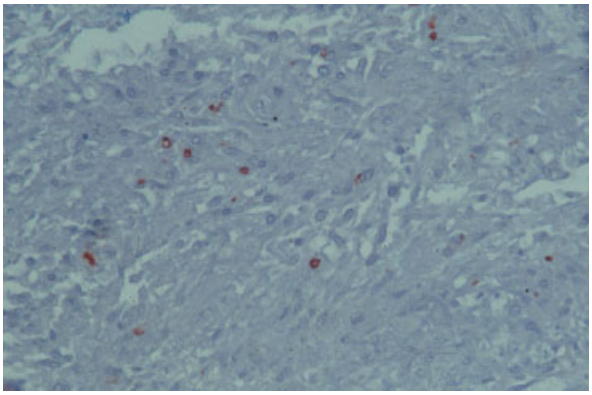

Figure 8 *Sensitivity by immunohistochemistry is as high as immunoflorescence for* Sporothrix *yeasts.*

(Scott et al. 1987). Serology should also be considered in any case where sporotrichosis is strongly suspected but cultures are negative.

MOLECULAR BIOLOGY

RFLP analysis of mitochondrial DNA (mtDNA) has been reported to be a useful method of identification, taxonomy, typing, and epidemiology of *S. schenckii*. Originally based on the phylogeny of 14 mtDNA types of *S. schenckii*, types 1–14 were constructed by estimating sequence divergences of mtDNA using restriction patterns with *Hae*III. Based on genetic distance, they have been clustered into two main groups: group A and group B. Isolates of group A exist mainly in North, South, and Central America, including Costa Rica (Ishizaki et al. 1996), and most of the Japanese isolates belong to group B (Takeda et al. 1991; Ishizaki et al. 1998)

S. schenckii strains have been classified into 23 types and clustered into two groups, group A and group B, on the basis of RFLP of mtDNA. The geographical distribution of mtDNA types was very characteristic. All isolates from Spain belonged to group B in contrast to North and South America where group A was predominant (Kawasaki et al. 2000) (Figure 9).

mtDNA types based on RFLP patterns with *Hae*III have been investigated also in China. In addition to types 1–23, a new mtDNA type (type 24) was found. Type 24 was divided into two subtypes (24A and 24B) based on RFLP with *Eco*RV. Seventy-seven isolates in China consisted of 58 isolates of type 4 (91 percent), five of type 6, one of type 5, one of type 20, and two of type 24, and classified into group A (types 1–3, 11, 14–19, 22 and 23) and group B (types 12, 13, 20, 21, and 24). Most isolates in China belong to group B and Korean isolates presumably belong to group B (Lin et al. 1999).

As we can see, group B contains most of the Japanese isolates and group A most of the American types. Group A is more branched compared with group B, where most of the types seem to be more closely genetically related and more mtDNA variability is found in group A, probably because the isolates have different geographic origins, although most of them come from the American continent. The molecular evolutionary explanations for the high variability are not clear. It will start to emerge with the molecular study of other genetic characters such as DNA sequences of mitochondrial and nuclear genes. mtDNA diversity was analyzed in 42 clinical isolates from Mexico, Guatemala, and Colombia, and six new types were found. Most of the strains belong to types 14 and 30, the former restricted

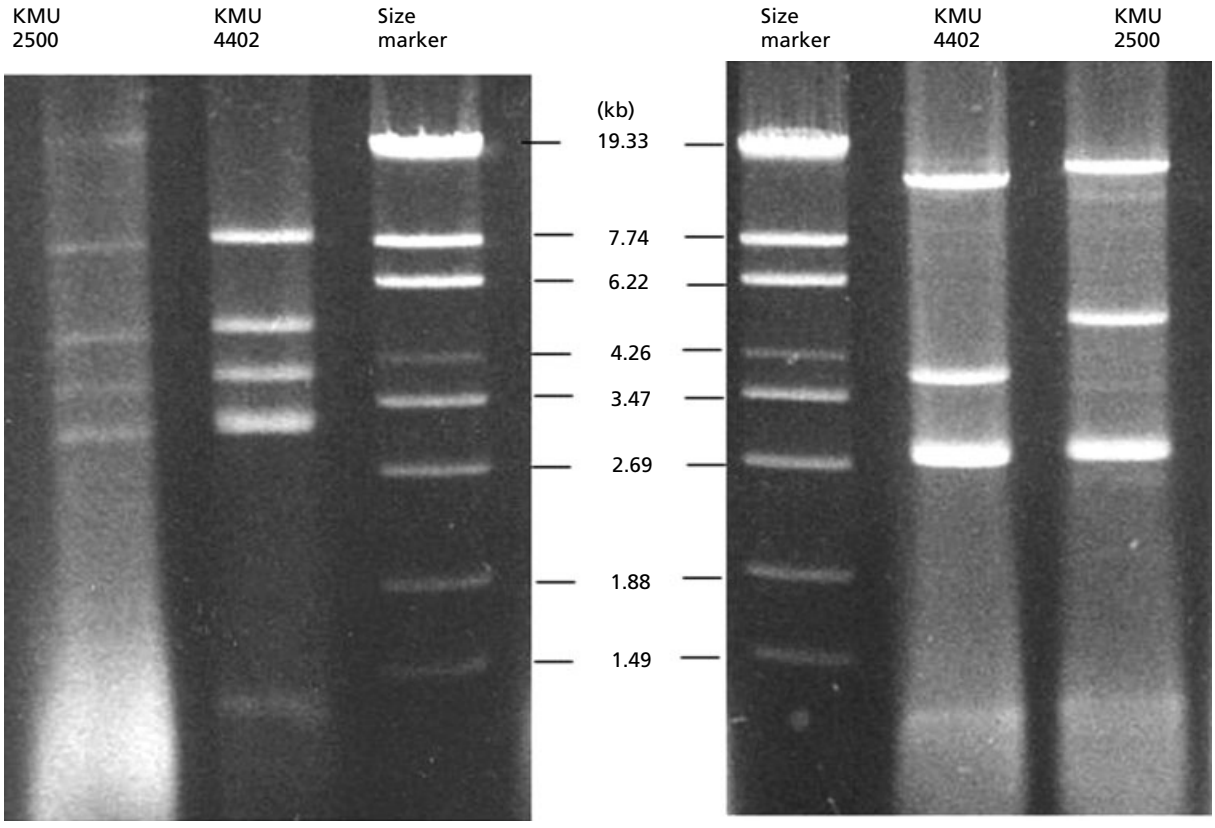

Figure 9 *RFLP patterns of mtDNA of* S. schenckii.

to Mexico, whereas the latter was distributed in Mexico, Guatemala, and Colombia. The new types (25–30) were identified in Mexico, Guatemala, and Colombia. These types were clustered in group A (types 26, 28–30) and group B (type 27). It seems that the variability in *S. schenckii* mtDNA is due in part to restriction site changes; however, the variation in size could also indicate length mutations (Mora-Cabrera et al. 2001).

Other clinical isolates were investigated for mtDNA type RFLP patterns with *Nae*III. The 62 isolates in South Africa comprised types 3 (9.7 percent), 4 (1.6 percent), 11 (9.7 percent), 17 (77.4 percent), and 23 (1.6 percent), whereas the 23 Australian isolates comprised types 3 (26 percent), 4 (56.5 percent), 7 (8.6 percent) and 21 (8.6 percent). The isolates in South Africa mainly belong to group A, with the predominance of type 17 (77 percent), and isolates in Australia mainly belong to group B.

The phylogenetic tree of mtDNA types and the worldwide distribution of types 3 and 4, suggest that these two types are ancestral types (or closely related), and that the others diverged from them over the course of continental drift. It may be that the divergence of mtDNA types occurred in South Africa earlier than in Australia (Ishizaki et al. 2000).

Partial cDNA cloning of a putative membrane transporter protein gene expressed in *S. schenckii* is reported and also DNA polymorphism of the isolated gene is demonstrated using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis. Also based on mtDNA diversity, *S. schenckii* has been classified into three groups located on the branches of the phylogenetic tree constructed by digestion profiles of mtDNA with restriction enzymes. The correlation of the results of PCR-SSCP analysis with the mtDNA diversity might indicate linkage of the mutation of the membrane transporter protein gene with the evolution of *S. schenckii*, and although the precise mechanism must be elucidated by further investigation, database searches also showed homology with the sugar transporter protein family (Sugita 2000)

Clinical isolates of fixed and lymphocutaneous forms from Mexico, Guatemala, and Colombia, as well as environmental isolates from Mexico, were studied by analyzing their phenotypic characteristics (conidial length, thermotolerance by percent growth inhibition at 35 and 37°C, median lethal dose (LD50), and genotypic characteristics [by random amplified polymorphic DNA (RAPD) analysis polymerase chain reaction (PCR)]. In general, the highest virulence, as determined by measurement of the LD50 for mice, was observed for the Mexican environmental isolates. *Sporothrix* fell into four major groups by hierarchical cluster analysis. A principal component analysis yielded three distinct groups, depending on the geographical origins of the isolates. This study revealed that isolates from Colombia had low thermotolerance at 35 and 37°C and could be associated with superficial skin lesions in patients with fixed clinical forms of sporotrichosis. Distinct patterns dependent on geographical origin were also revealed by RAPD analysis PCR, but these had no relation to the clinical form of the disease (Mesa-Arango et al. 2002).

TREATMENT

Uncomplicated lymphocutaneous sporotrichosis historically has been treated by oral administration of saturated solutions of potassium iodide, an inexpensive treatment that has proved effective in most patients. In some cases, the application of topical heat has been shown to cure lymphocutaneous disease (Hiruma et al. 1992b). A partial explanation for the efficacy of topical heat therapy has been provided by laboratory investigation. When yeast cells of *S. schenckii* were incubated in serum with polymorphonuclear neutrophils at 40°C as compared with 37°C, there was no difference in the rates at which the fungal cells were engulfed by the neutrophils. Once engulfed, however, the rate of killing by the neutrophils was significantly greater at 40°C than at 37°C (Hiruma and Kagawa 1986).

In one study of 58 patients, potassium iodide was equally effective whether administered once or three times daily, although side effects were slightly increased (Cabezas et al. 1996).

Potassium iodide cures approximately 90 percent of immunocompetent patients with sporotrichosis and relapses are rare. A pregnant woman taking excess quantities of iodine may result in possible goiter, hypothyroidism, and fetal death. Potassium iodide is currently a category D drug, meaning that it should not be used in pregnant or breastfeeding women unless the benefits of taking it outweigh the risks. Care must be taken in patients with renal failure and other potential causes of hyperkalemia, or in children with cystic fibrosis as they are susceptible to goitrogenic effects of iodide, or in patients with thyroid disease (Sanchez 2000; Sterling and Heymann 2000). Potassium iodide may cause the Wolff–Chaikoff effect (WCE), the cessation of thyroid hormone synthesis subsequent to the administration of iodide, but also iodine excess may lead to thyrotoxicosis (Jod–Basedow effect) and, rarely, iodine excess may cause an acute thyroiditis manifested by a painful, enlarged thyroid gland (Heymann 2000).

Modern antifungal compounds have been shown to be highly effective in curing lymphocutaneous sporotrichosis. Itraconazole and, to a lesser degree, fluconazole are orally administered drugs that are both effective; however, they are quite expensive and unavailable in many parts of the world (Sharkey-Mathis et al. 1993; Ghodi et al. 2000)

Forty-three Japanese cases of sporotrichosis were treated with itraconazole for 3 months, adult patients received 100 mg/day, whereas young patients under 5

years of age were treated with 50 mg/day; 88 percent have been assessed as cured or improved (Noguchi et al. 1999).

Terbinafine, another of the new antifungals, has been used topically for dermatophytosis, and preliminary results from oral use in cases of sporotrichosis have been promising (Hull and Vismer 1992).

A review article reported that 18 patients with sporotrichosis enrolled in five separate studies were cured by terbinafine at doses of 125–500 mg/day for a median of 12 weeks (range 4–37). They received terbinafine for up to 1 month after clinical resolution of lesions or for a maximum period of up to 6 months (Hay 1999; Pérez 1999). A total of 63 patients with cutaneous or lymphocutaneous sporotrichosis were randomized in a comparative study to evaluate the efficacy and safety of two doses of terbinafine, 28 were treated with 500 mg/day and 35 with 1 000 mg daily during a maximum of 24 weeks. Tolerance was similar in both groups, but 6 cases relapsed in the first group and none in the second. There were two drug adverse events related to the 1000 mg dose group, one case of gastrointestinal hemorrhage and one of progressive abdominal pain (Chapman et al. 2004).

Systemic forms including osteoarticular, pulmonary, and meningeal involvement can be life-threatening and very difficult to treat (Wescott et al. 1999).

Complicated cases of sporotrichosis are those that involve one or more joints, or that have disseminated to other organs, such as the CNS. These cases are very difficult to treat, and require the intravenous administration of amphotericin B. These patients may also need a prolonged subsequent regimen of an oral antifungal azole to prevent reactivation of the infection. In the case of AIDS patients, sporotrichosis (as with other fungal infections) can potentially be suppressed but cannot be cured, so lifelong suppressive antifungal therapy is required.

In patients infected with HIV, sporotrichosis may require systemic administration of amphotericin. The disease is potentially life-threatening. In one case amphotericin therapy was reinstituted but discontinued because of renal complications and amphotericin B lipid complex was then initiated at a dose of 5 mg/kg/day and was tolerated well, resulting in progressive resolution of all lesions. Lesions have recurred, despite the oral administration of itraconazole, fluconazole, or terbinafine. Blood levels of itraconazole are diminished by a number of agents that stimulate its hepatic metabolism, such as carbamazepine and rifampin (Ware et al. 1999) (see Table 1).

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Eumycetomas

RODERICK J. HAY

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INTRODUCTION

The ability to form granules in vivo is a property possessed by a variety of microorganisms. Usually these granules are small, visible, microbial aggregates composed of cellular masses which are frequently filamentous. They probably represent the formation in vivo of colonies. However, their small size and the discrete nature of the granules are characteristic. The best examples are the 'grains' of a fungal mycetoma or eumycetoma and the sulphur granules of actinomycosis. A number of fungi produce clusters of hyphae in vivo, and these range in size from large fungus balls, which are easily visible in gross pathological material such as the aspergillomas, to dermatophytes, which can be visualized occasionally by microscopy within foreign body giant cells. Strictly speaking, these are not true granules and the nomenclature will be discussed briefly at the end of this section.

Mycetomas are chronic subcutaneous infections caused by fungi or actinomycetes (Table 1), known as eumycetomas and actinomycetomas respectively (Mahgoub and Murray 1973). The organisms are traumatically implanted into the deep dermis or subcutaneous tissue from the natural environment and cause a subcutaneous infection characterized by the formation of large aggregates of fungal or actinomycete filaments known as grains. A dense infiltrate of polymorphonuclear leukocytes and other inflammatory cells accumulates around these structures (Chandler and Watts 1987). This abscess either discharges onto the skin surface through draining sinuses or may involve contiguous structures such as the bone, causing chronic osteomyelitis. Mycetoma is a chronic disease process which causes considerable deformity and disability. It does not often spread beyond the locality of the initial site of infection and is seldom fatal. As there are pronounced differences in the treatment and prognosis of mycetomas resulting from different organisms, in particular between the eumycetomas and actinomycetomas, identification of the causative organisms plays a key part in their management.

The name mycetoma has been applied erroneously to a number of other conditions including those in which a large fungus ball is formed and dermatophyte pseudomycetomas (Ajello et al. 1980). The formation of a fungus ball, exemplified by the disease aspergilloma, usually occurs within a cavity formed as a result of disease. Lung cavities are most often affected although fungus balls may occur in the paranasal sinuses. The fungi find their way to this site either through inhalation or bloodstream spread, but not through implantation. Sinuses affecting the skin are not formed and the pathogenesis of this form of fungal infection is different from that of a true mycetoma. Dermatophytes may produce granules composed of mycelial elements which often develop within giant cells (Ajello et al. 1980). Once again, the pathogenesis is completely different from mycetoma. Infections do not follow implantation of environmental organisms but follow invasion from a site

Table 1 *Causes of eumycetoma*

White grains	Black grains
Acremonium falciforme	*Curvularia lunata*
Acremonium kiliense	*Curvularia geniculata*
Acremonium recifei	*Exophiala jeanselmei*
Aspergillus nidulans	*Leptosphaeria senegalensis*
Aspergillus flavus	*Madurella mycetomatis*
Chaetospaeronema larense[a]	*Madurella grisea*
Cylindrocarpon cyanescens[a]	*Phialophora parasitica*
Fusarium moniliforme	*Pyrenochaeta romeroi*
Fusarium solani	
Neotestudina rosatii	
Scedosporium apiospermum	

a) Very rare causes of mycetoma.

on the skin, particularly the hair follicle. For this reason granules formed by dermatophytes are sometimes referred to as pseudomycetomas.

HISTORY

Mycetoma, Madura foot, is a disease of great antiquity and was probably known by the ancient Indian writers as padavalmita (foot anthill) (Sran et al. 1972). Changes suggestive of mycetoma have also been found in ancient skeletal remains in Israel (Spigelman and Donoghue 2001). The earliest medical description of the condition in Western literature appears to be that of the German traveller and physician, Kaempfer (1651–1716) and formed part of his doctoral thesis at the University of Leyden in Holland in 1694 (Kaempfer 1694). The thesis, 'Disputatio physica medica inauguralis exhibens decadem observationum', contains a section concerned with a disease called 'Perical' or 'ulcerous hypersarcosis of the feet' which was indigenous to Malabar in southern India. He described a tumorous disease generally affecting the feet which, as a result, would enlarge to three or four times the normal size. A feature of the condition was said to be the presence of oozing and granules. However, it was not fatal and caused considerable deformity often as a result of treatment – generally caustics or the application of heated metal. In 1842, Gill, an English surgeon working in the Madurai dispensary, once again in southern India, was said to have mentioned a condition that may have been mycetoma in a dispensary report (Gill 1842, cited in Carter 1874). The disease was first described in the medical literature by Godfrey (1846), who was the garrison surgeon in Bellary, Madras, India. He described four patients and his paper was published in *The Lancet* entitled 'Diseases of the foot not hitherto described.' Godfrey reported this new condition as 'morbus tuberculosis pedis' and the gross deformity, but non-fatal nature, of the condition was emphasized (Godfrey 1846). In 1859, Eyre drew attention once again to the small characteristic pigmented specks seen within mycetomas and mentioned in Kaempfer's original article (Eyre 1859). Originally these were thought to be caused by blood, but their presence was noted to be a characteristic of this particular infection. Although many cases involved the feet, other sites were also affected; for instance, Minas (1860) first described lesions of the hand and pointed out that the grains in lesions could be either black or pale but that the former were more common. The bone changes were first described by Ballingali (1855).

The causative organisms were first described in 1860 by Carter who suggested that the small bodies were 'fungus particles.' He also proposed that these were fungi of plant origin (Carter 1861). He believed that the white particles or grains were merely degenerate elements of the black form. In order to clarify the etiology of mycetoma, Carter enlisted the help of the Reverend Berkley who isolated a red mold from a lesion. Berkeley named this fungus *Chionyphe carteri*, a new species (Berkley 1862). The true identification of this organism remains unknown as it does not correspond to any fungus recognized as pathogenic today. In 1874, Carter produced a monograph on mycetoma, 'On mycetoma or the fungus disease of India,' using this disease name for the first time (Carter 1874). He also appears to have described the pathological features of an infection caused by the organism now known as *Madurella mycetomatis*. The first isolation of a fungal species recognized today was by Brumpt (1906), who cultured the organism *Madurella mycetomi (-atis)*, from a black grain infection.

The pathogenesis of mycetoma still eluded investigators. For instance, Bidie also carried out detailed studies of the disease in Bombay and studied the grains which he also believed to be caused by fungi that had entered the body as spores (Bidie 1862). However, the most likely explanation for the true portal of entry of organisms came in 1893, when Bocarro found a thorn embedded in the inflammatory mass and proposed that the infection was usually introduced by a minor injury (Bocarro 1893). Although until this time most of the cases originated from India, it soon became clear that cases were being reported from Algeria, the USA, and Sudan.

In 1894, two observations showed that actinomycetes were also causes of this infection. Boyce and Surveyor (1894) observed that the size of filaments within certain grains of mycetomas were fine and more likely to be bacterial in origin. In the same year, Vincent (1894) isolated an organism that he named *Streptothrix madurae* (probably *Actinomadura madurae*) from an Algerian case.

PATHOGENESIS

A unifying theory for the pathogenesis of mycetoma is hampered by the lack of an effective animal model for

eumycetomas, although there are experimental models for actinomycete infections such as nocardiosis (mice) and *A. madurae* (goats). However, there is evidence to connect mycetoma with implantation of microorganisms from the environment coupled with an extraordinary series of adaptive mechanisms which are of potential benefit to the survival of the organisms in nature.

Some of the causative fungi have been isolated from the natural environment. For instance, *Leptosphaeria senegalensis* has been identified in the natural environment in acacia thorns (Segretain and Mariat 1969). In addition, it is occasionally possible to see plant material, probably originating from a thorn in some lesions of mycetoma (Cameron et al. 1973). These appear as pieces of lignified tissue containing fungal hyphae. Basset and co-workers removed thorn material from two cases and cultured *L. senegalensis* from one and *Pyrenochaeta romeroi* from the other (Basset et al. 1965).

There is no evidence to suggest that the infection is specifically associated with an underlying host predisposition and, even though some patients appear to have defective T-lymphocyte-mediated responses to the fungi, these have been described in those with extensive disease, suggesting that they may have followed rather than caused the infection (Mahgoub et al. 1978). Other investigators have failed to find any changes in T-lymphocyte function in patients with disease (Bender et al. 1987). There is also nothing to suggest that other less debilitating changes in immune function occur in mycetoma patients, although in one study a high frequency of diabetes mellitus was reported among patients (Hay and Mackenzie 1983). The rare cases reported in immunosuppressed patients may even be atypical with the formation of clusters of hyphae rather than frank grains (Meis et al. 2000) This has also not been mentioned by others (Mahgoub and Murray 1973). By contrast, there is evidence that fungi in mycetomas may develop a number of different adaptive changes in vivo, such as cell wall reduplication or unfolding of the cytoskeleton, or deposition of extra- or intracellular melanin, all of which may affect the ability of the host inflammatory response to destroy the organisms. Cell wall reduplication gives rise to an 'onion skin' appearance around cells in eumycetoma grains caused by different species under electron microscopic examination (Hay and Collins 1983; Wethered et al. 1987). This appears to follow intrahyphal growth of mycelial elements. Adjacent cell walls also fuse, particularly at the rim of the grain where, in addition, there is evidence of deposition of human immunoglobulin. The contribution of the latter to the microstructure of grains is unknown. In the case of *M. mycetomatis*, cells within the grain may be surrounded by electron-dense homogeneous material, fungal melanin. This is very resilient to physical extremes and is difficult to section using a microtome (Findlay and Vismer 1987). Once again it is thought to provide a protection for living cells within the fungal grain.

As yet there is little evidence among the fungal causes of mycetoma of immunomodulation as has been demonstrated with the actinomycete, *Streptomyces somaliensis*, which produces an extracellular protease that affects the ability of macrophages in vitro to kill bacteria (Nasher et al. 1987).

EPIDEMIOLOGY

The epidemiology of the geographical distribution of cases of mycetoma is well documented. As a result of a worldwide postal survey, Mariat (1963) produced evidence that mycetomas were found throughout a range of countries, mainly confined to tropical regions, and that infections outside these areas were rare and usually occurred as imported infections. The countries reporting the highest prevalence of infection were Mexico where *Nocardia brasiliensis* was the dominant organism and Senegal where most infections were caused by fungi. In addition to these countries, India and Sudan have a higher than average prevalence of infection (Mahgoub and Murray 1973; Hay et al. 1992). In these two areas, *M. mycetomatis* is the most common reported cause of mycetoma. *Nocardia* spp. and *M. mycetomatis* were also the most common organisms documented in the Mariat survey, accounting for 32 and 19 percent of the total, respectively. Recently, *M. mycetomatis* DNA has been identified in soil (23 percent) and thorn (5 percent) samples from Sudan but not from the Netherlands (Ahmed et al. 2002). In addition, mycetomas occur throughout central and northern South America, around the Saharan region and Central and South Africa, the Middle East, India, and Pakistan. Infections are less frequently seen further east. The main endemic areas for mycetomas are relatively arid and have a short rainy season, with a constant temperature with little fluctuation with different seasons or at night (Gonzalez-Ochoa 1962). In temperate areas, mycetoma infections are rare and usually caused by fungi rather than actinomycetes. There are reasons for suspecting the accuracy of some of these data. In the UK, for instance, over half the fungal causes of imported mycetomas resulted from pigmented fungi that are sterile and produce nonpigmented grains in vivo (Hay and Mackenzie 1983). They cannot therefore be assigned to a single species or genus. There is evidence from the analysis of the protein profiles of some of these agents, which appear to be very similar in gross morphology, using polyacrylamide gels, that this is a heterogeneous group of fungi (Zaini et al. 1991).

There are variations within the endemic areas although these principally affect the actinomycetes where, for instance, *S. somaliensis* is mainly found in hot and dry climates with less than 50–250 mm of rain per

year. Among the fungi, *Pyrenochaeta romeroi* is also found in areas with higher rainfall levels (Mahgoub and Murray 1973). As has been described above, the fungi that cause mycetomas are associated with vegetation such as acacia trees (Segretain and Mariat 1969). This is compatible with the description of the endemic areas because they are mainly seen in regions with low rainfall.

Mycetoma is most often seen in men, the male:female ratio of infections being approximately 4:1. It is mainly a disease of rural workers aged between 16 and 45 years. Although they may not give a history of an initiating trauma, their occupation predisposes to such an event. Women with mycetomas are also usually from rural areas and work in the fields. However, occasionally mycetomas are found in nonmanual workers.

CLINICAL APPEARANCES

The clinical appearances of mycetoma are characteristic (Abbott 1956; Mahgoub and Murray 1973). The incubation period is unknown as most patients do not give a history of a trauma that initiated the lesions. However, in some cases, it is clear that infection started over 8 years after a traumatic injury (Maiti et al. 2002).

The earliest sign of a mycetoma is the appearance of a hard subcutaneous nodule (Figure 1). This is painless and usually found on exposed sites such as the arms and legs. More than 95 percent of mycetomas start at these areas. As the lesion enlarges, sinuses appear on the skin surface (Figure 2). These present as papules 0.2–0.5 cm in diameter or pustules that discharge their contents and then dry up, leaving a small scar. Sometimes individual lesions are painful before the release of purulent material. At an early stage, pain and tenderness are intermittent and unpredictable. As the lesion increases in size, the whole area becomes hard, swollen, and, in time, limbs become grossly deformed. In advanced mycetomas there is often localized sweating over lesions (R.J. Hay, personal observation). In some cases pain becomes prominent in the later stages, but conversely, patients may have very extensive lesions without symptoms.

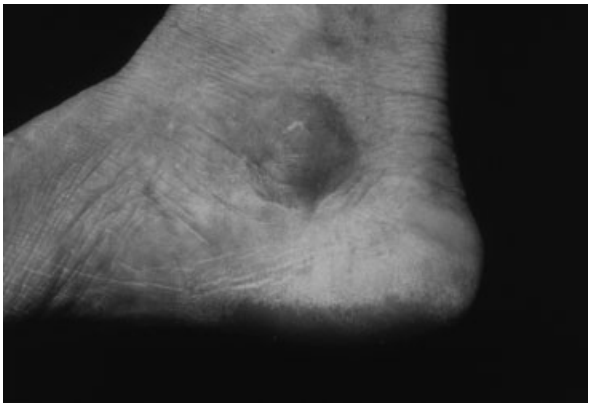

Figure 1 *Early mycetoma before the development of sinuses*

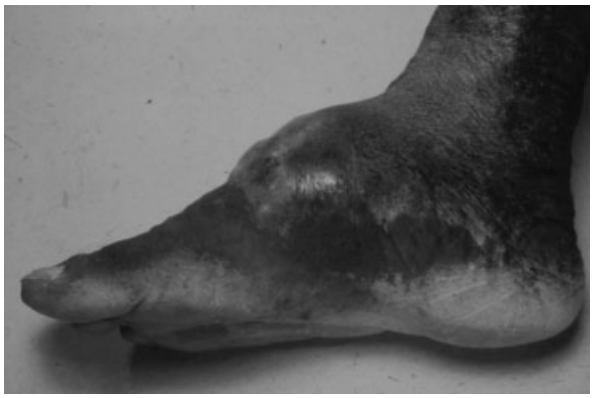

Figure 2 *Mycetoma of the foot caused by* Madurella grisea

Occasionally mycetomas may be found on the scalp and thorax, although these are most often caused by actinomycetes, namely *S. somaliensis* and *Nocardia* spp., respectively. It is not possible to distinguish clinically with any degree of certainty between the different causes of mycetoma. The lesions usually remain localized and are locally aggressive. Actinomycetomas usually evolve more rapidly than eumycetomas, but this cannot be relied on as a means of distinguishing between the two types. Very rarely, lesions may spread to lymph nodes, although this is most likely to occur with *S. somaliensis* (El Hassan and Mahgoub 1972). Metastatic mycetomas are exceptionally rare and, on the few occasions where two lesions are seen, these may have been caused by separate infections. Occasionally cystic mycetomas confined to the bone and without draining sinuses have been described (Fahal et al. 1998). The main complications of eumycetomas are deformity which can lead to considerable disability, fungal osteomyelitis, and secondary bacterial infection. The last is not common but may cause an acute exacerbation of the swelling and increased purulent discharge from sinuses combined with pyrexia.

DIFFERENTIAL DIAGNOSIS

Mycetoma must be distinguished from other conditions in which draining sinuses may develop. These include actinomycosis which is usually restricted to sites where the causative organisms are carried, such as the mouth and jaw. The grains in actinomycosis are soft and yellowish. Botryomycosis, a bacterial disease, may also be confused with mycetoma, although there is usually wider internal spread and patients have a pronounced fever. Carcinomas, such as squamous carcinomas, chondrosarcoma, and Kaposi's sarcoma, may have to be distinguished where necessary by biopsy.

Diagnostic imaging

The radiological appearances of mycetomas are well described (Davies 1958; Cockshott 1968). They include soft tissue swelling, endosteal cavitation with small cystic lesions, and periosteal bone erosion (Figure 3). New bone growth is often seen in longstanding lesions along the periosteum. Hyperostosis may occur in the skull and other areas and, in advanced lesions, pathological fractures and bone resorption can be seen. Once again it is difficult to distinguish between eumycetomas and actinomycetomas. Cystic lesions are often fewer and larger in eumycetomas. Other methods of detection include bone scanning and echo scanning. Bone scans may be more useful at detecting early bone involvement than x-rays, although not widely available in the endemic areas. Ultrasound scans show the marked tissue and bone swelling with hyperreflective areas (Fahal et al. 1997). However, magnetic resonance imaging provides a clearer picture of both bone and soft tissue distribution (Czechowski et al. 2001)

Laboratory diagnosis

The diagnosis of eumycetoma is confirmed by the demonstration of grains in lesions and their identification. This is important in order to provide information on prognosis and treatment. For instance, it is important to differentiate eumycetomas, which often respond poorly to chemotherapy, from actinomycetomas, which respond well. In addition, there are differences in responses within the eumycetomas. Infections caused by *M. mycetomatis* respond to ketoconazole in about 50 percent of cases. Likewise, it is worth using intravenous miconazole in infections caused by *Scedosporium apiospermum*.

The methods of laboratory diagnosis of granule-forming fungi are no different from those used for other fungal infections: direct microscopy, culture, serology, and histopathology. The key to the diagnostic process is the extraction of grains from lesions. This can be accomplished in one of two ways.

First, an unruptured pustule is identified, cleaned with an alcohol swab and gently pierced with a sterile needle. The edges of the sinus beneath are squeezed, and a small drop of pus and blood is extruded. This is then spread on a glass slide where it is possible to identify small (200 μm to 2 mm) granules that are white/yellow, black, or red. These are then processed for microscopy, culture, and histopathology (see section on Histopathology below). Often it is necessary to open more than one pustule and it is important to scrape beneath the edge of the scab because grains may be trapped there.

Deep biopsy is the second approach. A superficial biopsy removing the mouth of a sinus track is seldom effective because the amount of material is usually insufficient to identify grains, and the chances of the specimen containing a grain are low because the mouth of the sinus merely represents a pathological thoroughfare. A large deep biopsy, which is usually taken under a general anaesthetic, on the other hand, provides a large amount of material from which it may be possible to identify grains. These should be picked out of the tissue for culture and for histopathology – segments containing visible grains should be sent for processing.

Fine needle aspiration can also sometimes provide suitable diagnostic material (El Hag et al. 1996)

Direct microscopy

Grains are mounted in 5–10 percent potassium hydroxide and allowed to stand for 1–2 hours. The material is then gently flattened by pressure on the cover slip. Grains must be distinguished from clusters of neutrophils, but it is possible to make out their structure using this method, as well as the type of pigmentation. Extracellular melanin produced by *M. mycetomatis*, for instance, forms into irregular clusters of pigmentation. The presence of hyphae in grains can be seen under high power objective (Figure 4). If broad hyphae are not seen, it is likely that the grain is caused by an actinomycete.

Culture

Provided the appropriate material is selected, culture is usually successful. It is best to use conventional media such as Sabouraud's agar for primary isolation, using plates or tubes both with and without antibiotics such as penicillin and streptomycin (Mahgoub and Murray 1973; Mariat et al. 1977). The rate of growth is highly variable and therefore cultures should be kept for at least 3 weeks before they are discarded as negative.

The criteria for recognition are no different from those used with other fungi. Colonial morphology, patterns of sporulation, pigmentation, and growth rates

Figure 3 *X-ray of eumycetoma of foot caused by a* Fusarium *species*

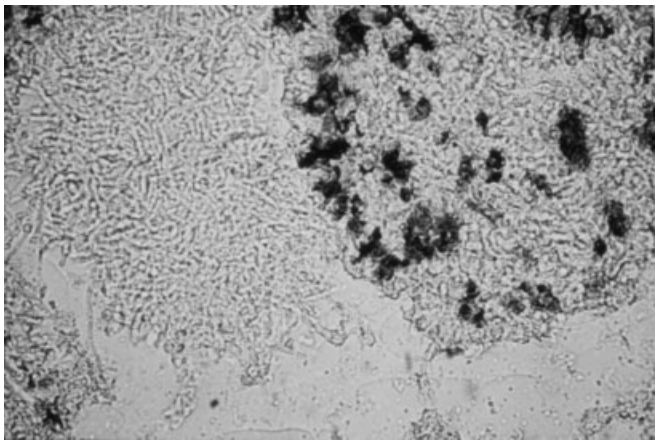

Figure 4 *Grains of* Madurella grisea. *Magnification × 300; stain: KOH mount.*

are all important. Media used range from Sabouraud's to Czapek–Dox or Lactrimel (Borelli and Feo 1966). Failure to sporulate is common and a variety of media should be used. The main means of diagnosis is through colonial characteristics. However, some physiological tests have been carried out on the more common mycetoma agents (Table 2).

At present there are few studies and no established molecular diagnostic tests although these have been applied to certain organisms, notably *M. mycetomatis* (Lopes et al. 2000). The cultural characteristics of the main organisms are discussed below

ACREMONIUM SPECIES

Most of these fungi implicated in mycetoma were previously described as members of the genus *Cephalosporium*. The main species implicated in mycetoma are *Acremonium kiliense*, *Acremonium falciforme*, and *Acremonium recifei*. Colonies of all three are slow growing and downy. The color is white to pink. On the undersurface there is usually a clear pigmentation that is pink to rose in color. The hyphae are not pigmented. Conidia are borne on aerial hyphae and appear in clusters bound by a gelatinous material. These conidia are single or multicellular and fusiform with a slight curve; some conidia appear as a shallow crescent. They are about 10 × 4 μm in length and width. The different species vary in conidial structure.

A. falciforme is mainly crescentic and nonseptate, but has some two- or three-celled conidia; *A. kiliense* has straight short conidia; *A. recifei* is mainly crescentic and nonseptate.

EXOPHIALA JEANSELMEI

Previously known as *Torula*, *Pullularia*, or *Phialophora jeanselemi*, this fungus forms a distinct colony that changes with time from a predominance of yeast-like cells, which form a glabrous mucoid colony with dark pigmentation, to a downy colony containing a lot of mycelium with aerial hyphae. Conidia are produced by annellation. These are formed and are often seen in clusters at the tip of an annelide. Conidia are oval and about 5 × 1 μm in size (Nielsen et al. 1968).

LEPTOSPHAERIA SPECIES

These fungi can be recognized by their more rapid growth to produce a downy colony with a gray to black reverse pigmentation. With *L. senegalensis*, asci are produced on older colonies and these contain eight oval ascospores that are long, measuring up to 30 μm in length. The ascospores of *Leptosphaeria tompkinsii* are shorter.

MADURELLA SPECIES

M. mycetomatis has other generic synonyms such as *Streptothrix* and *Glenospora*, as well as other *Madurella*

Table 2 *Physiological characteristics of common fungal causes of mycetoma*

Species	Starch	Gelatin	Glucose	Galactose	Lactose	Maltose	Sucrose
Scedosporium apiospermum	+	+	+	var	–	–	var
Madurella mycetomatis	+	±	+	+	+	+	–
Madurella grisea	+	–	+	+	–	+	+
Pyrenochaeta romeroi	+	±	+	+	–	+	+
Leptosphaeria senegalensis	+	?	+	+	var	+	+
Exophiala jeanselmei	–	–	+	+	–	+	+

species. It was originally named as *M. mycetomi* but this was subsequently changed to the form used today: *M. mycetomatis*.

The rate of growth of *M. mycetomatis* colonies is very variable, but may start very slowly. Colonies are often glabrous initially and of a white to tan color, with deep folds that develop a down, resulting from hyphal proliferation over the surface. This is cream to gray in color. Diffusible brown pigment can be seen on the undersurface. Conidial formation is best seen on cornmeal agar. *M. mycetomatis* produces either small flask-like phialides which produce small round conidia (2–4 μm in diameter) or elongated pyriform conidia (3–5 μm). In old colonies there are numerous black sclerotia 1 mm in diameter.

Madurella grisea

The colonies are similar to *M. mycetomatis* initially, although there is no diffusible pigment. Once again a small downy cover forms after 2–3 weeks. Colonies are sterile, although on poor media pycnidia form. Recognition is difficult as the fungus is sterile and some concern has been expressed that isolates identified as *M. grisea* may not form a single species. Protein patterns analyzed from extracted fungi by polyacrylamide gels suggest that their protein patterns are not homogeneous. At present there have been no published studies that employed genetic techniques to compare different isolates.

NEOTESTUDINA ROSATII

This was formerly named *Zopfia rosatii*. The organism produces a slow-growing yellow to brown-colored colony which is covered by folds. On certain media such as cornmeal agar older colonies (after 3 weeks) produce asci. The ascostromas are easily distinguished because of their black color. Asci are long, up to 30 μm, and have thick walls; they contain eight two-celled ascospores which are 10 μm in length (Segretain and Destombes 1961).

PYRENOCHAETA SPECIES

P. romeroi produces a dark downy colony with a black to tan undersurface. The colony expands rapidly and produces pycnidia. These have thick outer walls with setae. Pycnidia are 40–100 μm by 50–120 μm in size and contain elliptical and yellowish pycnidiospores which are about 1 μm in diameter. These are produced by phialides in chains. A new species, *Pyrenochaeta mackinnonii*, differs in the size of pycnidia and pycnidiospores (Borelli 1976).

SCEDOSPORIUM APIOSPERMUM

This fungus is the imperfect state of *Pseudallescheria boydii*. It is synonymous with *Monosporium apiospermum*. The colonies are rapidly growing and a lot of fluffy aerial mycelium is produced which is white at first, but with time becomes gray or brown. The underside of the culture is usually gray. Sectoring or contour formation may occur on the colonial surface. Conidia are produced singly or in clusters on anellophores or laterally on conidiogenous hyphae. These conidia are distinctive, large, lemon-shaped and with a yellowish color. They vary from 4–9 to 6–10 μm in size. Some colonies produce asci. The ascospores are lightly pigmented and round to oblong (6 × 6 μm).

The isolation of unusual organisms

The other fungal causes of mycetoma are very rare and have been described on a single or, at the most, two occasions. It is difficult to decide whether an isolated organism is a novel cause of mycetoma or a contaminant. Theoretically, as most of these agents are of low virulence, it is possible for other environmental organisms to be implicated in mycetomas. If the organism is isolated at least twice from grains, and these are compatible with a fungal infection, it is likely that they are the cause of the infection. Such rare organisms include *Pseudochaetosphaeronema larense* (*Chaetosphaeronema larense*) (Borelli and Zamora 1973), *Cylindrocarpon (Phialophora) cyanescens* (Zoutman and Sigler 1991), *Phialophora parasitica* (Pracharktam et al. 2000), and *Pyrenochaeta mackinnonii* (Borelli 1976). Other fungi described as causes of eumycetoma are *Corynespora cassiicola* and *Plenodomus avramii*.

Serodiagnosis

The value of serodiagnosis in the diagnosis of mycetomas is debatable. Certainly it cannot be used as a screening process for suspicious-looking lesions, given that weak positive reactions may occur in otherwise healthy individuals. There are also no commercial systems for the detection of antibody or antigen in mycetoma.

The techniques used have generally depended on either immunodiffusion or counterimmunoelectrophoresis (Murray and Mahgoub 1968; Mariat et al. 1977); there is no enzyme-linked diagnostic system such as an enzyme-linked immunosorbent assay (ELISA), which has been developed specifically for measuring antibodies to mycetoma as a diagnostic measure, even though investigators have used ELISA to study the pathogenesis of infection (Wethered et al. 1988). An immunodiffusion assay has been established in a few laboratories and provides useful help for following the course of treatment where initial reactions are strongly positive. This is more often seen with actinomycetes such as *Nocardia* spp. or *S. somaliensis* (Murray and Mahgoub 1968). Both these groups give strongly positive responses which can be quantitated and followed as a guide to treatment outcome or relapse. Generally, for most eumycetomas, the serological responses are less

reliable and their monitoring only occasionally forms a useful part of the management of these infections.

HISTOPATHOLOGY

Provided that the limitations of histopathology in the diagnosis of mycetoma are recognized, this is a useful procedure. It can be performed either on grains extracted from lesions and fixed immediately in formalin or on biopsy material. If grains are used, it is important to ensure that the laboratory staff can also identify the relevant material in the specimen pot, otherwise it will be discarded. For most purposes, recognition is based on the appearances of grains using the hematoxylin and eosin (H&E) stain (Winslow and Steen 1964; Mahgoub and Murray 1973; Mariat et al. 1977; Destombes 1978; Chandler and Watts 1987). Specific fungal stains such as the methenamine silver and periodic acid–Schiff (PAS) stains are useful to ensure that fungi are present, but are seldom helpful for specific diagnosis. Actinomycete filaments also take up silver-based stains.

A scheme for the identification of mycetomas is shown in Table 3. The key elements for recognition are the presence of pigmentation and whether it is intramural or extracellular, the size and shape of grains, and their affinity for H&E. Although, with practice, it is possible to separate some of the black grain eumycetomas by their appearances in H&E, this is not the case with pale grain fungi. There is some evidence that infections caused by *S. apiospermum* show clusters of vesicular expansion of hyphae at the grain periphery and that this is less common with other nonpigmented agents of mycetoma (Figure 5). However, this is not completely reliable as a means of distinguishing among different organisms. The definitive diagnosis is made by culture.

Dermatophyte hyphal clusters are often referred to as pseudomycetomas. They seldom produce sinuses, but clusters of fungal hyphae are seen in giant cells in the dermis rather than subcutaneous fat or fascia (Chandler and Watts 1987). This mode of pathogenesis is somewhat similar to that seen with *S. somaliensis* which also starts as an intracellular infection (Nasher et al. 1987). However, with dermatophytes, the fungal hyphae are discrete and unaltered, in contrast to those seen with other fungi, where there are gross cell wall changes. They also originate from an endogenous infection and are not implanted from the environment. In some cases, pseudomycetoma caused by dermatophytes appears to follow persistent hair shaft infection whereas, in others, it is a manifestation of disseminated subcutaneous disease. Both types are rare.

Electron microscopy does not play a role in the diagnosis of mycetomas although it is useful for studying the pathogenesis of infection. As described above, a characteristic of these infections is the change in cell wall structure with gross thickening and fusion of adjacent cells. The cell walls often show an 'onion skin' appearance, which is probably the result of intrahyphal growth of fungi (Hay and Collins 1983).

Immunofluorescence has not been developed as a means of identification of mycetomas. This is partly the result of autofluorescence of grains which makes recognition difficult. Theoretically there is no reason why nonfluorescent techniques, such as immunoperoxidase staining, should not be used for mycetomas as there is no evidence that there is antigenic variation in vitro versus in vivo that could lead to false-negative reactions. However, reagents for such tests are not available at present. Likewise there is no established place for molecular genetic techniques such as Southern hybridization or restrictive fragment length polymorphism (RFLP) analysis in the diagnosis of these infections.

Table 3 *Characteristics of the grains of the common eumycetoma agents*

Color	Species	Size (μm)	Shape	Appearances in H&E[a]
White (pale)	*Scedosporium apiospermum*	500–1500	Irregular	Prominent vesicles. Round–oval Eosinophilic fringe
	Neotestudina rosatii	500–1000	Regular oval or round, lobed	Compact with cement-like matrix, basophilic hyphae
	Acremonium or *Fusarium* spp.	500–2000	Regular oval or round	Dense packing of hyphae which stain poorly
	Aspergillus nidulans	200–1000	Lobed	Pale, poor staining of fungal hyphae, hyaline
Black	*Madurella mycetomatis*	500–5000	Irregular lobed	Hard, cement-like matrix Hyphae swollen and indistinct
	Madurella grisea	500–2000	Irregular	No cement, swollen hyphae Individual elements distinct
	Leptosphaeria senegalensis	500–2000	Arc-like. Round	Distinct hyphal rim, diffuse central hyphae
	Exophiala jeanselmei	200–1000	Arc-like	Dense hyphal rim, neutrophil invasion common

a) These appearances are features described in H&E stained sections. They are variable.

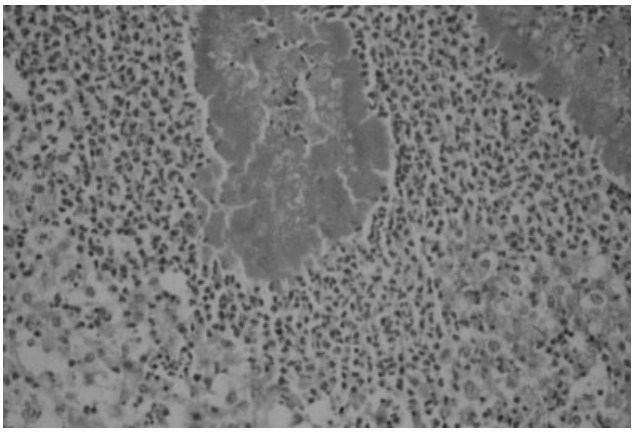

Figure 5 *Grains of* Scedosporium apiospermum. *Magnification × 350; stain: H&E*

TREATMENT

Treatment of eumycetomas with chemotherapy is difficult and their management often involves deciding from conservative approaches involving observation, a trial of chemotherapy, and surgery (Hay et al. 1992).

Conservative management

Eumycetomas are seldom life-threatening and are often asymptomatic. Where chemotherapy is not available, it may be preferable to review patients regularly without active intervention. Relief of pain and dressings to sinuses may be required from time to time.

Chemotherapy

The range of antifungals that can used with success in mycetomas is not great. In some cases, responses have been seen with amphotericin B, either in conventional formulation or in combination with lipids or liposomes; ketoconazole may also be effective. Reports of individual successes with itraconazole and terbinafine have been recorded, although in no case has there been sufficient follow-up to establish cure (Paugam et al. 1997; Hay 1999). Of all these approaches, more is known about ketoconazole, which in daily doses of 200 mg has been recorded as a successful therapy for mycetomas caused by *M. mycetomatis*. The response rate is variable though and probably depends on the extent of the infection, but in about half the cases successful results can be obtained. Therapy is continued for at least 3 months and 12–18 months may be required. In other patients a trial of therapy is a wise choice as this can be guided by the in vitro sensitivity pattern [minimal inhibitory concentration (MIC)] of the organism isolated. Although this approach has some logic, in vitro sensitivity is not a good guide to cure or remission and it is probably more realistic to regard this approach as palliative in slowing enlargement of lesions. As such, it has clinical value even though it is seldom curative. Griseofulvin is seldom active against mycetoma agents in vitro and yet it appears to slow the progress of mycetomas in some patients. It is possible that this is because it is an active inhibitor of leukocyte chemotaxis and suppresses the accumulation of neutrophils in lesions (Yousef and Hay 1987).

There is no published experience on the use of immunological therapy such as cytokines – granulocyte–macrophage colony-stimulating factors – in mycetoma.

Surgery

The ultimate method of eradicating a fungal mycetoma is surgical excision (Mahgoub and Murray 1973). However, it is important to recognize that this has to be sufficiently radical to ensure an adequate margin of excision, otherwise relapse is inevitable. Local excision of mycetomas on the foot may cause practical problems if there is insufficient tissue remaining to allow adequate weight-bearing support, and pain on standing or walking may result. In other words, the extent and method used have to be considered carefully. The definitive curative process is adequate amputation, again ensuring that the margins are well clear of infected bone or subcutaneous tissue. Amputation has to be carefully considered together with the patient, taking into consideration the site, the resulting disability and its effect on employment or social life, the availability of good prostheses, and the discomfort or deformity caused by the lesion. In many cases this may be the best approach, but it should be arrived at after proper consideration of all the alternatives and after informed discussion with the patient. It may be preferable to accept a measure of disability with an untreated mycetoma if the alternative is an operation that would deprive the patient of his or her livelihood.

APPARENT GRANULE FORMATION BY OTHER FUNGI

In addition to mycetomas, granule-like structures may be formed by a number of different organisms in vivo. Usually this is a pathological phenomenon and not seen normally with infections caused by these organisms. The exception is where mycelial fungi grow within spaces or cavities such as pathological cavities in the lung. Under these conditions a number of different fungal species produce a fungus ball. *Aspergillus* spp. are the most common causes, but other organisms from *Coccidioides immitis* to *Pseudallescheria boydii* have been implicated and it is possible that all mycelial fungi have the propensity to form into balls given the appropriate conditions in vivo. However, two specific and different examples of in vivo granule formation are seen with the dermatophytes and *Scedosporium apiospermum*.

Pseudomycetomas caused by dermatophytes

These have been defined in the Introduction. As their pathogenesis is completely different to that seen with mycetomas, they are termed 'pseudomycetomas' (Ajello et al. 1980). The aggregates are formed of loose collections of dermatophyte mycelium, usually packed into giant cells. This phenomenon has been seen after hair shaft infections caused by both endothrix and ectothrix pathogens; it may also occur in the rare disseminated forms of dermatophytosis.

Strictly speaking, these collections are randomly aggregated hyphae and are not granules at all.

Systemic infections caused by *Pseudallescheria boydii* (*Scedosporium apiospermum*)

This organism is a known cause of mycetoma. Occasionally the rare systemic infections caused by this organism produce a compact aggregate of hyphae like a microcolony. This cannot be distinguished as a 'granule' in gross pathological specimens and is once again best designated as a pseudogranule.

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Protothecosis

R. SCOTT PORE

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ETIOLOGIC AGENTS

The etiologic agents of protothecosis are algae:

- *Prototheca wickerhamii* Tubaki and Soneda 1959
- *Prototheca zopfii* Krüger 1894
- *Chlorella* spp. Beyerinck 1890 may cause infections.

HISTORY

Prototheca spp. were discovered by Zopf (Krüger 1894a, b) and are related to *Chlorella* spp. Species of both genera reproduce only asexually by similar internal formation and subsequent release of spheroidal progeny cells from a parental sporangium. Species of both genera can subsist saprophytically in wastewater (Pore 1983). Comparison of nutritional and cell wall composition data has led to a proposed phylogenetic relationship between the chlorophyll-free *P. wickerhamii* and the photosynthetic *Chlorella protothecoides* Krüger (Pore 1972; Conte and Pore 1973). Confirmation of this relationship came from DNA base composition data (Huss et al. 1988), and sequence similarities between the 16S-like ribosomal RNA (rRNA) and the genetic DNA that separated these two species from the other *Chlorella* spp. tested (Huss and Sogin 1990). On the one hand, *Chlorella* spp. are polyphyletic, but DNA-binding homologies showed heterogeneity among *Prototheca* spp. as well (Huss et al. 1988). Although it has been postulated that *Prototheca* spp. could be nonphotosynthetic mutants of extant *Chlorella* spp. (see Cooke 1968 for references to this mutation theory), there is no evidence that such is the case. Considerable evolutionary diversity would appear to separate the species of these two genera (Nedelcu 2001); with the lone exception that *C. protothecoides* is the postulated phylogenetic ancestor of *P. wickerhamii* (Ueno et al. 2003). From this summary it may be concluded that *Prototheca* spp. are achlorophyllous descendents of *Chlorella* spp., but evolutionary distinctions and further taxonomic hierarchies are speculative.

The discovery of *P. zopfii* during the late nineteenth century notwithstanding, proof of its infectious potential was not obtained until 1952, when it was shown to cause bovine mastitis (Lerche 1952). *P. zopfii* is now known to be the agent of several different animal infections. *Prototheca* spp. stand as the best, if not the only, examples of algae with infectious capabilities in animals and humans. The index human case of dermal protothecosis of the foot (Davies et al. 1964) may also have been caused by *P. zopfii*; however, nearly all subsequent human infections have been caused by *P. wickerhamii*, a microbe belatedly discovered in studies conducted separately by Phaff, Soneda and Wickerham (Tubaki and Soneda 1959). Finally, a few reports incriminate *Chlorella* spp. themselves as infectious agents (see the section on *Chlorella* spp. below).

EPIDEMIOLOGY

Ecology

P. wickerhamii and *P. zopfii* are ubiquitous inhabitants of wastewater (household and municipal sewage), in

which they grow and thrive, ranging in densities up to 10^6 cells/ml (Pore and Boehm 1986). Likewise, they are common in animal waste and agricultural sewage. They also achieve a high population density in some types of tree slime flux (Krüger 1894a, b; Pore 1986). Both species have been isolated in Asia, Europe, North and South America. From these sources they contaminate aquatic systems and food (Pore 1983), and may subsequently be eaten by humans and animals. They pass undigested through the gastrointestinal tract of normal human and monogastric animals tested, with little if any multiplication, and are excreted to resume their saprophytic lifecycle (Pore and Shahan 1988). In a few cases, however, sustained colonization of the gut by *P. wickerhamii* was suspected (see section on Intestinal infection below). Canine protothecal enteritis is a recognized syndrome, and a prelude to systemic involvement (section on Canine infection below). Because of the importance of endemic protothecal mastitis in dairy cattle, a question remaining to be answered is, to what degree are ingested *Prototheca* spp. killed by digestion in ruminants?

Epidemiologic studies by Schuster and Blaschke-Hellmessen (1983), Blaschke-Hellmessen et al. (1987), Pore et al. (1987), and Anderson and Walker (1988) have indicated that *P. zopfii* may be isolated routinely from secreted milk, bulk milk collections, and dairy farm environments. Recent evidence that *P. zopfii* could survive milk pasteurization (Melville et al. 1999) is also of epidemiologic concern.

Relative to the high concentration of *P. wickerhamii* in municipal wastewater and the opportunity for occupational exposure, Clark et al. (1985) did not detect elevated protothecal antibody titers in sewage treatment workers tested. Organically enriched water, in general, not just wastewater, has commonly been incriminated as the source of skin and wound infections, the largest category of human protothecosis cases, but the case reports were anecdotal in nature.

Infections

More than 100 human infections have been documented in the medical literature during the previous 50 years (Krcmery 2000) and, even though many cases may have escaped diagnosis or case reporting, human protothecosis is still considered to be a rare disease. Protothecosis is worldwide in occurrence, but its incidence is low and the disease is not thought to be communicable. Animal infections, on the other hand, may be common, as in bovine protothecal mastitis, but in the past they were mainly undiagnosed by veterinary scientists.

HOST RESPONSES

Clinical presentations of protothecosis

CUTANEOUS

Protothecosis, following naturally occurring skin inoculation, may be the best-documented type of infection by *Prototheca* spp. In several cases, aquariums or wastewater were implicated as the source of the infections, but more frequently no such aqueous source was discovered. The primary cutaneous lesion has not been described, but after several weeks or more, a circumscribed or progressive, indurated, papular rash develops as an early manifestation. Inflammation is usually moderate and the primary symptoms may be pruritus and skin blemishes, although eczematoid reactions may occur. Such primary infections may resolve in some patients, although this has not been documented, and all current reports indicate that the disease is progressive over a period of many years; characteristically exemplified by spreading cutaneous nodules (Figure 1). Well-illustrated recent reports of cutaneous protothecosis include those of Cho et al. 2002, Piyophirapong et al. 2002 and Chao et al. 2002. The infections are not always benign, but may cause burning irritation (Tyring et al. 1989), pseudoepitheliomas or lymphocutaneous spread of granulomatous lesions for up to 18 years (Yip et al. 1976). Some patients are immunocompromised and may develop acanthosis or ulcerative skin lesions (Wolfe et al. 1976; Dagher et al. 1978; Thianprasit et al. 1983; Tejada and Parker 1994; Polk and Sanders 1997; Chao et al. 2002; Piyophirapong et al. 2002).

The index case of protothecosis was a progressive infection of the epidermis and dermis of the foot of an African rice farmer in Sierra Leone (Davies et al. 1964). It started as a small itchy, weeping, raised lesion, which,

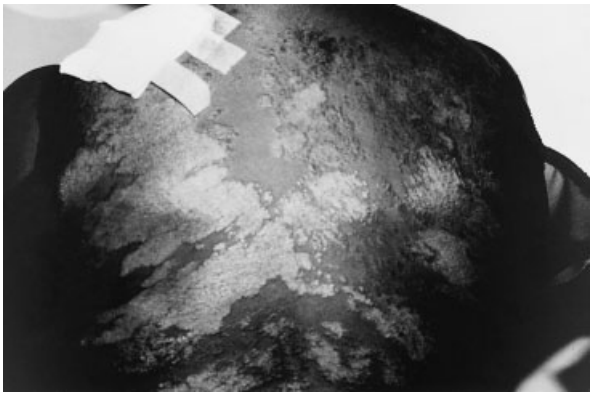

Figure 1 *Progressive cutaneous protothecosis of several years' duration. The spreading, raised plaques lacked pigmentation and were culturally and histopathologically positive for* P. wickerhamii. *The case was reported by Venezio et al. (1982). (Photograph courtesy of John Williams and John Phair)*

5 years later, became a well-demarcated, dry, atrophic, depigmented, papular dermatitis. After 2–3 years, complicated by secondary bacterial infections, the foot had the mossy appearance of elephantiasis. Subsequently, the algal pathogen caused femoral lymphadenitis (Davies and Wilkinson 1967). Although this patient was infected by *P. zopfii* (synonym used was *Prototheca segbwema*), almost all subsequent human infections have been attributed to *P. wickerhamii*. Another well-documented case (Venezio et al. 1982) of several years' duration developed from papular lesions into depigmented scaling plaques covering 50 percent of the skin surface (Figure 1). This case and two others were considered to be the sole reported cases of 'disseminated cutaneous protothecosis' (Thianprasit et al. 1983; Wirth et al. 1999), and all three patients had severe, defective neutrophil functions.

Cutaneous protothecosis may be a superficial form of wound protothecosis, local trauma has been recalled by patients (Chao et al. 2002). From such wounds papules followed by an extensive spreading granulomatous eruption of the skin can develop (Mars et al. 1971; Mayhall et al. 1976; Tang et al. 1995; Kim et al. 1996). *P. wickerhamii* was also the cause of an ulcerating cutaneous papulopustular lesion on the leg of an immunocompromised patient with metastatic adenocarcinoma of the bones. Like the index case, the algae were localized in small foci within the lesions, but the dermatitis was progressive (Klintworth et al. 1968). Raised, plaque-like, verrucose lesions with ulceration have been reported. Forty-five human case reports of all types of protothecosis were reviewed by Nelson et al. (1987) and 34 additional cases of cutaneous infections have been reviewed by Boyd et al. (1995). Resolution of cutaneous infections after antibiotic therapy left considerable residual scarring and atrophy of the skin (Tyring et al. 1989). In three instances patients had simultaneous cutaneous and systemic infections (Cox et al. 1974; Mohabeer et al. 1997; Marr et al. 1998). An infection of the nasopharyngeal mucosa complicated prolonged endotracheal intubation of an insulin-dependent diabetic individual receiving high-dose systemic dexamethasone (Iacoviello et al. 1992). Ulcerations of the hard palate, esophagus, and tongue were inflamed and necrotic, and contained clusters of algal cells that stained lightly with hematoxylin and eosin (H&E), but intensely with periodic acid–Schiff (PAS) and methylene blue.

WOUND INFECTION

Wounds may also be the cause of deeper infections. Accidental dermal and subdermal traumatic inoculation of *P. wickerhamii* during surgery to relieve carpal tunnel syndrome was thought to be the cause of suppurative tenosynovitis (Pegram et al. 1983; Moyer et al. 1990). Again, almost all such human infections were caused by *P. wickerhamii* as annotated by Nelson et al. (1987), along with the source of the infections. Cellulitis, with an aspirate of necrotizing fibrinopurulent exudate containing *P. wickerhamii* occurred in a renal transplant recipient who also received immunosuppressants (Mezger et al. 1981). Subcutaneous wound infections are known to involve tendons and bones in humans (Sirikulchayanonta et al. 1989), dogs (unpublished data) and cats (Coloe 1982). Opportunistic wound infections are probably the second most frequent form of human protothecosis. Three cases of protothecal peritonitis secondary to peritoneal dialysis demonstrate the susceptibility of the compromised abdominal cavity opportunistic infection (O'Connor et al. 1986; Gibb et al. 1991; Sands et al. 1991).

OLECRANON BURSITIS

The relatively high incidence of protothecal olecranon bursitis has led to its recognition as a separate clinical entity. Although the circumstances for traumatic implantation were confirmed in many instances, a number of patients inexplicably reported no prior trauma. The bursa evidently provides a favorable environment for *P. wickerhamii*, and the preexistence of inflammatory conditions and the needle delivery of steroid therapeutics may provide the necessary conditions for infection in some of the cases (Cochran et al. 1986). An underlying immunodeficiency is not a necessary condition for an infection, however (Ahbel et al. 1980). Otherwise normal patients were found to have tender, swollen, olecranon bursas. Aspirated bursal contents were serosanguineous and culturally positive for *P. wickerhamii* (Nosanchuk and Greenberg 1973) or, in a single case, for *P. zopfii* (Naryshkin et al. 1987). Protothecal olecranon bursitis was usually less acute than corresponding bacterial infections. Nelson et al. (1987) have compiled many such protothecal olecranon bursitis cases. Finally, in one instance, olecranon bursitis caused by *P. wickerhamii* was antecedent or coincident with systemic infection (Marr et al. 1998).

INTESTINAL INFECTION

Prototheca portoricensis (another synonym for *P. zopfii*) was proposed as the suspected cause of tropical sprue after its isolation from the feces of two Puerto Rican patients (Ashford et al. 1930). This original observation was never again repeated on sprue patients, despite the efforts of several investigators over the intervening years (Klipstein and Schenk 1975), but the account by Ashford and colleagues does stand as an early attempt to incriminate algae as etiologic agents of infections. In fact, *P. wickerhamii* and *P. zopfii*, originating in the diet, may subsequently be isolated from human and animal feces (Pore and Shahan 1988). *P. wickerhamii* (notably not *P. zopfii*) was isolated on prototheca isolation medium (PIM) from the stools of nine of 500 patients tested (unpublished data). Follow-up was not done for

all nine patients, but for one of the patients, a nutritionally normal, breast-fed, 1-year-old with nonbloody diarrhea of 2 months' duration, the organism was repeatedly recovered on PIM for the final 2 weeks of the diarrhea. At one point in this case fecal dilution plate counting revealed 25 colony-forming units (c.f.u.) per gram of wet stool. A dietary source of the alga was discounted and the microorganism disappeared from the feces near the end of the diarrheal episodes. This patient did not develop any related adverse health problems during 18 subsequent years. All of the other patients also had episodic chronic diarrhea and two of the patients had colony counts of more than 100 c.f.u. per fecal smear on PIM (100 × 15 mm Petri dish). In no case, however, was a causal relationship of the *P. wickerhamii* with the diarrhea established. One infant had pica, but environmental studies on this patient plus four of the others revealed no ingestion source of *P. wickerhamii. P. wickerhamii* was also isolated on PIM from the stools of two Philippine patients, one having acute diarrhea. One culture collection strain of *P. wickerhamii* (CDC B-1280) is labeled 'human intestine.'

An additional case of infant diarrhea, possibly caused by *P. wickerhamii*, has been reported (Casal et al. 1983). In another instance *P. wickerhamii* was repeatedly isolated from the stools of a patient with a well-documented cutaneous protothecosis, but enteritis was not evident or, if evident, not reported (Venezio et al. 1982). Only one case report exists with strong evidence of intestinal protothecosis. This patient with underlying chronic mucocutaneous candidiasis developed a 4-year symptomatology including abdominal pain, diarrhea, and a non-necrotizing granulomatous mass partially obstructing the colon. Histological demonstration of protothecosis was made, but the species was not determined (Raz et al. 1998). Three years later the symptomatology continued and the protothecosis was related to polyps in the colon and ileum. Two patients with systemic protothecosis had a history of antecedent diarrhea (Cox et al. 1974; Heney et al. 1991), and intestinal protothecosis in dogs is not uncommon (see the section on Canine infection below).

SYSTEMIC INFECTION

There are several accounts of human patients with multiorgan protothecosis. A patient having Hodgkin's disease and multiple predisposing factors for secondary infection, and chronic diarrhea of a year's duration, had a mixed *P. wickerhamii*, *Torulopsis glabrata* sepsis, the source of which was probably the long-term Hickman catheter (Heney et al. 1991). Several case reports are available in which the patients had both cutaneous and systemic protothecoses (Cox et al. 1974; Mohabeer et al. 1997; Marr et al. 1998). The first was a rare, chronic, disseminated infection involving the gallbladder, surface of the liver, and the duodenum, in an otherwise normal patient operated upon for symptomatic sclerosing cholangitis (Chan et al. 1990). Two other patients had involvement of the liver, viscera and central nervous system (Matsuda and Matsumota 1992; Takaki et al. 1996). As there was antecedent diarrhea in two cases (Cox et al. 1974; Heney et al. 1991) and a postulated intestinal route of infection or involvement in two others (Chan et al. 1990; Matsuda and Matsumota 1992), these human cases may be compared with the frequently encountered visceral canine protothecosis, which is associated with bloody diarrhea prior to multiorgan dissemination (see the section on Canine infection below). Finally, one case of protothecal endocarditis in a premature infant was reported (Buendia et al. 1998).

IMMUNODEFICIENCY, OPPORTUNISM

In at least half of the published protothecosis cases the patients had a preexisting immune dysfunction or had received immunosuppressive therapy. This was particularly true of the cutaneous and disseminated infections in humans, but has not been as apparent in olecranon bursitis (Woolrich et al. 1994). Corticosteroid treatment appeared to be a significant predisposing factor in several recent cutaneous cases (Chao et al. 2002), and in one otherwise immunocompetent patient, protothecosis developed at the site of cutaneous corticosteroid injection (Kim et al. 1996); another patient who was corticosteroid dependent developed olecranon bursitis with possible subsequent dissemination (Marr et al. 1998). A myasthenia gravis patient receiving corticosteroids developed algemia (prototheca sepsis), but recovered with amphotericin B and gradual elimination of immunosuppressive therapy (Mohabeer et al. 1997). Thirteen cases of protothecosis in cancer patients, several with lymphoid malignancies, have been summarized (Torres et al. 2003). *P. wickerhamii* caused meningitis in one human immunodeficiency virus (HIV)-positive patient (Kaminski et al. 1992) and cutaneous protothecosis in six other AIDS patients (Laeng et al. 1994; Woolrich et al. 1994; Carey et al. 1997; Polk and Sanders 1997; Cunliffe and DiPersio 2000; Piyophirapong et al. 2002). Although other predisposing factors were also present, AIDS was thought to be relevant to the protothecosis. Regarding dogs, immunodeficiency was found to be a significant precondition in several systemic infections (Thomas and Preston 1990). Experimental mouse infections were facilitated by pretreatment with corticosteroids (Pore 1971) as was a naturally occurring disseminated mastitis in a cow (Taniyama et al. 1994).

Animal infections

BOVINE MASTITIS

After its original description and experimental reproduction in Germany (Lerche 1952), protothecal bovine

mastitis has gained worldwide recognition. Caused by *P. zopfii,* protothecal mastitis is mildly inflammatory in comparison to bacterial mastitis; however, it is invasive, reaching the supramammary lymph nodes, chronic, and results in reduced milk production. Twenty-three of 93 dairy cows in one herd were culled (Frank et al. 1969), and 400 cases were reported in New York State (Mayberry 1984). Ten of 192 infected cows in a Danish herd had decreased milk production (Bodenhoff and Madsen 1978), and five of 130 cows were culled for protothecosis, after calving, in the UK (Spalton 1985). A report from Hungary lists 223 cases in 32 herds over 2 years (Janosi et al. 2001b). The only pathogen present in three cases (Dion 1979), and in 10 other cases of mastitis (Chengappa et al. 1984), was *P. zopfii*.

The ubiquity of *P. zopfii* in the dairy farm environment (Schuster and Blaschke-Hellmessen 1983; Blaschke-Hellmessen et al. 1987), and its frequency in milk collections (Pore et al. 1987), provided the foundation for epidemiologic studies (also refer to the section on Ecology below) of bovine mastitis in a herd of 263 cows (Anderson and Walker 1988) and a 52-herd survey (Costa et al. 1998). In the later study, *P. zopfii* was the most common mastitis pathogen recovered (41.2 percent), and this included bacteria. In another large herd, 248 cows were culled for protothecal mastitis. The advanced age of the cows and prior antibiotic treatment for bacterial mastitis were found to be significant risk factors (Tenhagen et al. 1999). Two additional risk factors were found to be high capacity machine milking and poor herd management (Corbellini et al. 2001b; Janosi et al. 2001a). A review of protothecal bovine mastitis included a more comprehensive discussion of pathogenesis and possible control measures (Pier et al. 2000).

Despite the postulated decrease in milk production, protothecal mastitis is an underreported dairy cow disease. Factors responsible include:

- the reluctance of dairy farmers to admit to mastitis in their herds
- insufficient information about protothecal mastitis etiology
- the relatively low white blood cell counts in the milk
- the lack of a treatment regimen
- the ease of culling and its economic advantages.

Both mastitic and non-mastitic dairy cows have been found to have elevated levels of anti-*P. zopfii* antibodies (Jensen et al. 1998), and a recently developed enzyme-linked immunosorbent assay (ELISA) test based on elevated serum IgA and IgG, and whey IgA against *P. zopfii* (Roesler and Hensel 2003) presents the opportunity for screening infected herds and advancing epidemiologic research.

CANINE INFECTION

After chronic hemorrhagic diarrhea in a domestic dog that was attributed to *Prototheca* spp., necropsy revealed small, white, granulomatous nodules containing numerous cells of *Prototheca* spp. in the intestinal mucosa and wall, peritoneal cavity, myocardium, kidneys, liver, and spleen (Buyukmihci et al. 1975). Notable in this case was necrotizing granulomatous chorioretinitis leading to blindness. Similarly, Cook et al. (1984) described a dog presenting with blindness and deafness, that had had a previous bloody diarrhea and ultimately developed multiorgan system involvement. In this case, *P. zopfii* was identified by fluorescent antibody stains as it was in a similar third case (Gaunt et al. 1984). In a fourth example, blindness caused by microabscesses containing *P. wickerhamii* under the detached retina was preceded by an enterocolitis (Font and Hook 1984). It appears, based on a review of 12 such cases, that hemorrhagic diarrhea and disseminated protothecosis is a distinctive syndrome that can be caused by either *P. wickerhamii* or *P. zopfii* after protothecal colitis in dogs (Migaki et al. 1982). Similar cases were reported and the syndrome reviewed (Rakich and Latimer 1984; Thomas and Preston 1990; Rallis et al. 2002). Attention was drawn to the fact that the syndrome is most common in the Collie breed and the similar features of immunodeficiencies in both canine and human protothecoses. However, not all dogs with disseminated protothecosis had documented antecedent intestinal infections (Tyler et al. 1980), so the syndrome and its epidemiology need further elucidation. Myocardial infection may also be a significant manifestation of systemic canine protothecosis (Moore et al. 1985). To summarize, early recognition of canine protothecosis syndrome should include manifestations of chronic diarrhea and/or acute blindness, this indicating the most opportune time for therapeutic intervention (Hollingsworth 2000).

OTHER ANIMAL INFECTIONS

Kaplan (1978) compiled information on 42 case reports of protothecosis in animals and 18 in humans. He included several cases attributable to *Chlorella* spp. as well (Kaplan et al. 1993). Disseminated protothecosis in cattle has also been described (Migaki et al. 1969), and it may be acute, causing massive necrotic mastitis, kidney infarction, pulmonary artery thrombosis and colitis (Taniyama et al. 1994). In this last instance a cow was simultaneously infected with *P. zopfii, Aspergillus* sp. and a zygomycete (Taniyama et al. 1994). Cutaneous protothecosis caused by *P. wickerhamii* in two cats has some of the features of human infections (Dillberger et al. 1988), and a spreading mucocutaneous infection of the nares of a dog has been well documented (Sudman et al. 1973); otherwise, descriptions of cutaneous infections in animals have been rare. One cutaneous dog infection was thought to be the primary manifestation of a case of systemic protothecosis (Ginel et al. 1997).

Experimental infection

P. zopfii was pathogenic for mice after intraperitoneal inoculation (Schiefer and Gedek 1968). Weakly inflammatory, but chronic, infections of the kidney capsule and kidney tubules were the prominent manifestations (Pore 1971), and hydrocortisone treatment enhanced the experimental infection of mice by *P. wickerhamii* (Pore 1971). de Camargo et al. (1980) were less successful and only obtained intratesticular infections in laboratory animals, but Horiuchi and Masuzawa (1995) successfully demonstrated a mouse model for cutaneous protothecosis. In this experimental model *P. wickerhamii* caused epithelioid cell granulomas in BALB/c mice while ICR mice were less vulnerable. Experimental mastitis in cows, after introduction of *P. zopfii* through the teat canal, led to well-documented progressive pyogranulomatous infections (Lerche 1952; Frank et al. 1969; McDonald et al. 1984). The experimental infections mimicked natural infections and *P. zopfii* was subsequently isolated from secreted milk. Schiefer and Gedek (1968) produced mastitis with *Prototheca moriformis* – another potential cause of bovine mastitis (Pore et al. 1987). *P. zopfii* was lethal for immunosuppressed mice in one study (Jensen and Aalbaek 1994). Finally, cells of *Chlorella* spp. persisted in the peritoneal cavity of rats with minimal pathogenesis (Rogers et al. 1980), but even *Prototheca* spp. were not very aggressive pathogens in normal laboratory rodent models of infection.

Immunology

About half the patients with protothecosis have immunodeficiencies prior to or coincident with their protothecosis. These immunodeficiencies differ, but commonly fall within the same category as those for patients with increased susceptiblity to opportunistic fungal infections. One opportunistically predisposed patient was simultaneously infected with *P. wickerhamii* and the fungus *Exophialia jeanselmei* (*Phialophora gougerotti*) (McAnally and Parry 1985), while another patient had *P. wickerhamii* and *T. glabrata* (Heney et al. 1991). A typical example of predisposition was a patient receiving long-term prednisolone for systemic lupus erythematosus who developed cutaneous protothecosis after an injury. The combination of oral prednisolone and cyclophosphamide in a patient with systemic lupus erythematosus was a contributing immunosuppressive factor for another patient with cutaneous protothecosis (Thianprasit et al. 1983). A significant number of protothecosis patients received local corticosteroid injections in soft tissues or the olecranon bursa (Nelson et al. 1987) or in skin lesions (example of two cases; Walsh et al. 1998). Patients with malignancies and other immunosuppressive factors above have been reviewed (Torres et al. 2003). Corticosteroids may predispose patients to opportunistic protothecosis and mycoses by suppressing T-cell-mediated immunity, as do cytotoxic agents and other immunosuppressants. Cutaneous protothecosis has been described more than once in patients receiving combination immunosuppressive therapy to combat graft rejection (Wolfe et al. 1976), as has chemotherapy-induced neutropenia in a patient with acute myelocytic leukemia (Wirth et al. 1999). Corticosteroids, antilymphocyte globulin, and cytotoxic drugs predispose patients to opportunistic bacterial and fungal infections as well as cutaneous abscesses caused by *P. wickerhamii*. There is evidence that *P. wickerhamii* itself may be immunoinhibitory (Perez and Ginel 1997).

Intracellular killing of *P. wickerhamii* by human polymorphonuclear neutrophils (PMNs) in vitro was enhanced in the presence of humoral factors that stimulated oxidative metabolism and degranulation (Phair et al. 1981). Subsequent research linked this finding to a case of protothecosis in a patient who had a specific defect in PMN killing of *P. wickerhamii* (Venezio et al. 1982), but nonspecific acquired immunity in this case was normal. Likewise, a severe oxidative burst defect in peripheral blood neutrophils was found to be contributory to protothecosis in an AIDS patient (Carey et al. 1997). Similarly, a Collie dog with disseminated protothecosis had a serum-mediated defect in PMN chemotaxis (Rakich and Latimer 1984). Natural killer cell activity was suppressed in one patient with cutaneous protothecosis (Tyring et al. 1989). Finally, one patient with disseminated protothecosis, who had developed a high specific IgG titer to *P. wickerhamii*, possessed a normal cell-mediated immune system (Chan et al. 1990), but another with a high serum titre had a blast transformation deficiency specific for the microorganism (Cox et al. 1974). These patient studies indicate that protothecosis, like several of the mycoses, may not be ameliorated by the humoral immune system and indicate that nonspecific leukocyte defects are paramount susceptibility factors.

Treatment

SURGERY

Surgical excision has been a successful means of treating localized cutaneous protothecosis (Thianprasit et al. 1983; Cunliffe and DiPersio 2000), wound protothecosis (Sirikulchayanonta et al. 1989; Tejada and Parker 1994) and protothecal and olecranon bursitis (Ahbel et al. 1980; Vernon and Goldman 1983; Naryshkin et al. 1987), often combined with an antibiotic. Wound infections have responded to débridement plus amphotericin B (Moyer et al. 1990; Walsh et al. 1998), and intrabursal amphotericin B alone was successful (Cochran et al. 1986). Removal of a right atrial mass followed by amphotericin B resolved a case of protothecal endocarditis in a premature infant (Buendia et al. 1998).

AMPHOTERICIN B

Amphotericin B alone has been used successfully to treat cutaneous infections (Mayhall et al. 1976; Thianprasit et al. 1983; Carey et al. 1997; Chao et al. 2002), a mucosal infection of the pharynx (Iacoviello et al. 1992) and a case of algemia, together with the removal of a Hickman catheter (Heney et al. 1991). Amphotericin B, followed by ketoconazole therapy, was successful in a case of systemic human protothecosis (Chan et al. 1990), but failed in a similar case in a dog (Moore et al. 1985). Amphotericin B alone was not efficacious in a case of cutaneous protothecosis (Yip et al. 1976); but amphotericin B has also led to cures (Chao et al. 2002). Combined with transfer factor, amphotericin B was effective in a case of disseminated human protothecosis (Cox et al. 1974). Intestinal protothecosis did not respond to amphotericin B alone (Raz et al. 1998). Tetracycline has had postulated synergistic activity with amphotericin B (Venezio et al. 1982; Tyring et al. 1989; Sands et al. 1991). Aminoglycosides and tetracycline were good inhibitors in susceptibility tests (Shahan and Pore 1991), but they lacked efficacy in a mucocutaneous dog infection (Sudman et al. 1973). Although tetracycline after surgery (Sirikulchayanonta et al. 1989) and oral tetracycline plus topical amphotericin B (Tyring et al. 1989) are reported to be successful therapeutic agents, tetracycline alone has also failed as a treatment for protothecosis (Wolfe et al. 1976). So, in light of increasing success with azoles (see the next section), tetracycline therapy is not promising. In patients with specific neutrophil killing defects, it may be advantageous to use a cidal agent such as amphotericin B (Carey et al. 1997). The same may be true for patients developing chemotherapy-induced neutropenia (Torres et al. 2003), along with amelioration of the neutropenia (Wirth et al. 1999).

AZOLES

Recent reports of successful therapy of cutaneous protothecosis with oral azoles are promising. A case of cutaneous eczematous dermatitis of the eyelid was successfully treated with oral ketoconazole over a 4-month period (Kuo et al. 1987), as was a post-surgical wrist infection (Pegram et al. 1983), but failures of ketoconazole treatment have also been reported (McAnally and Parry 1985; Walsh et al. 1998). Oral fluconazole has proven successful (Follador et al. 2001) as has 200 mg/day oral itraconazole (Boyd et al. 1995; Tang et al. 1995; Okuyama et al. 2001; Cho et al. 2002). Instances of failure of itraconazole (Carey et al. 1997) and fluconazole (Walsh et al. 1998) have also been reported. In one instance, a cutaneous infection that did not respond to itraconazole was successfully resolved with fluconazole (Kim et al. 1996), and another with amphotericin B (Carey et al. 1997). Itraconazole alone was not satisfactory for intestinal protothecosis, but in combination with interferon-gamma (IFN-γ), the patient improved (Raz et al. 1998). Favorable treatment results were achieved with fluconazole for peritonitis (Gibb et al. 1991).

In a report of five cutaneous cases compromised by steroid treatment, successful resolution of each separate case occurred by using ketoconazole, fluconazole, or itraconazole (Chao et al. 2002). In the other two cases, intravenous amphotericin B or intralesional amphotericin B resulted in cures. In the case of AIDS-related cutaneous infections, oral ketoconazole was not successful, but 2 g intravenous amphotericin B for 10 weeks resulted in resolution that was maintained for 3 years (Piyophirapong et al. 2002).

RESISTANCE

Currently, the management of disseminated protothecosis with conventional antibiotics remains an open question, as exemplified by the patient who received three different azoles and amphotericin B, but remained infected (Takaki et al. 1996). Even though azoles and polyenes may be efficacious in humans, azoles give unreliable susceptibility data, and the development of resistance has been noted (Takaki et al. 1996). *P. wickerhamii* is uniformly sensitive to amphotericin B in in vitro susceptibility tests (Segal et al. 1976), but an exception has been noted (Takaki et al. 1996). Notably, both *P. wickerhamii* and *P. zopfii* are resistant to 5-fluorocytosine (Pore 1973). Likewise, both species may be resistant to fluconazole and itraconazole, at least according to the E-test (Blaschke-Hellmessen 1996; Linares et al. 1998), but this finding is in need of confirmation in light of the successful therapies reported above. Similarly to the situation with yeasts, successful therapy of protothecosis with azoles does not necessarily correspond to conventional minimum inhibitory concentration (MIC) susceptibility testing results, which can be equivocal, yet azoles are often efficacious therapies. Finally, it should be kept in mind that all infections responding to antibiotics were caused by *P. wickerhamii*.

LABORATORY DIAGNOSIS

Isolation and culture

FROM BLOOD, CSF, AND TISSUE

P. wicherhamii and *P. zopfii* grow aerobically on conventional Sabouraud's and blood agar media after 24–48 hours at room temperature up to 35°C. The small colonies are grossly indistinguishable from yeast colonies. As these two pathogenic *Prototheca* spp. may be sparse in clinical specimens, a large, fresh, preferably unrefrigerated specimen should be processed for culture. Notably, *Prototheca* spp. similar to *Cryptococcus* spp., are uniformly inhibited in vitro by common culture medium additives such as cycloheximide, tetracycline

(Mezger et al. 1981), and aminoglycosides (Shahan and Pore 1991).

FROM STOOLS

As *Prototheca* spp. may be overgrown by faster growing microorganisms and hence usually unrecoverable from contaminated specimens, PIM (Table 1), which contains phthalate to suppress bacteria and 5-fluorocytosine to suppress yeasts, was found to be a good selective isolation medium for *Prototheca* spp. from contaminated sources (Pore 1973). A subsequent modification included the addition of rose Bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein sodium salt) for further suppression of fungal and bacterial growth (Pore 1983). Specimens may be smeared or streaked with a swab and, after 48–72 hours, yeast-like colonies will become visible with the aid of a low-power stereomicroscope. Not all yeast species are suppressed by PIM, so suspicious colonies should eventually be examined at magnifications of 400 × with a compound microscope.

Microbiology

MORPHOLOGY

Colonies have a yeast-like appearance, but microscopically, budding is absent. Reproduction of *Prototheca* spp. is via the formation of a variable number of sporangiospores (commonly two to eight) within a sporangium (Figure 2). The sporangiospores are released through a characteristic split in the sporangial wall and usually disperse (Figure 3). The reproductive morphology is identical to that of most *Chlorella* spp. and is the basis for a postulated phylogenetic relationship to the algae; however, *Prototheca* spp. lack plastids

Table 1 *Prototheca isolation medium (PIM)*

Ingredients	Amount
Distilled water	1 l
Potassium hydrogen phthalate[a]	10 g
Sodium hydroxide	0.9 g
Magnesium sulfate	0.1 g
Potassium phosphate, monobasic	0.2 g
Ammonium chloride	0.3 g
Thiamine	0.001 g
Rose Bengal	0.0025 g
Glucose	10 g
Purified agar	15 g

a) Adjust to pH 5.1 ± 0.1 with NaOH before sterilization; autoclave for 15 min; after sterilization add 5-fluorocytosine (0.25 g) (Pore 1973). The potassium hydrogen phthalate is the unmetabolizable buffer; it also serves to enhance growth in an unknown way and suppresses growth of most bacteria and fungi in contaminated specimens. A modification of this medium (Pore et al. 1987) is an enrichment medium for the recovery of *P. zopfii* from milk and the environment. *C. protothecoides,* but not other species of *Chlorella*, also grows on PIM. Another modification (Pore 1985, 1998) is the filter-sterilized, PIM, minimum essential medium for assimilation and identification to species.

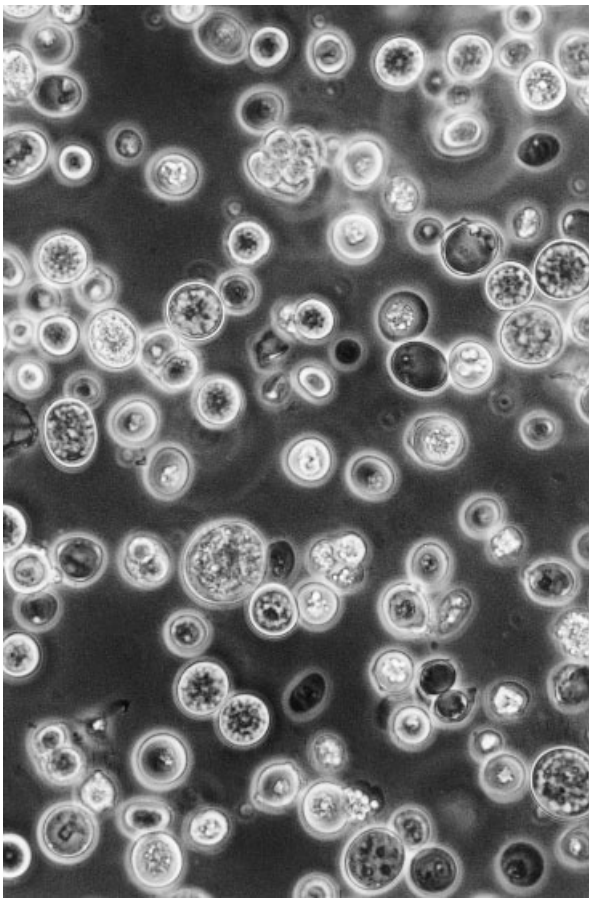

Figure 2 P. wickerhamii*; typical stages of the lifecycle in culture. Phase contrast; magnification × 700*

or vestigial plastids as is often erroneously reported (Figure 4). Examination of published electron micrographs (Klintworth et al. 1968; Patni and Aaronson 1974; Joshi et al. 1975; Kaplan 1978; Cheville et al. 1984; Font and Hook 1984; Jensen et al. 1998) supports the claim that no leukoplasts or thallocoid structures are present. The sporangium is spheroidal or an enlarged version of the sporangiospore. The sporangiospores are usually spheroidal or ellipsoidal, but reniform and falcate strains occur. The fact that a Dauer cell stage resists staining with methylene blue and rose Bengal is of significance in identification (Pore 1998). Sexual stages are not found (Pore 1985). Three other species – *P. moriformis*, *Prototheca stagnora*, and *Prototheca ulmea* – which have been isolated from nature, but not from animal infections, all have a large capsule that may give a resemblance to *Cryptococcus* spp. (de Camargo and Fischman 1979; Pore 1985, 1986).

PHYSIOLOGY

Chlorophyll is not present in the *Prototheca* spp. Glucose is assimilated oxidatively and the assimilation of a few other carbon substrates has taxonomic significance (Pore 1985). D-Lactic acid is produced from glucose by

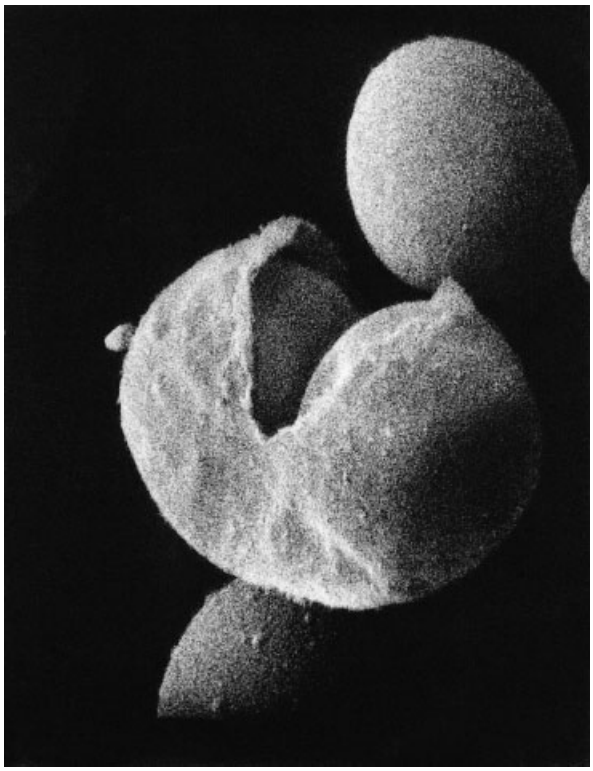

Figure 3 P. wickerhamii *releasing sporangiospores from the sporangium. Scanning electron micrograph; magnification* × *9 350. (Courtesy of Phillip Allender)*

P. zopfii (Barker 1935; Ueno et al. 2002) and other species also produce acid, but not gas, from glucose (unpublished results). Ueno et al. (2002) do report CO_2 production during fermentation as a function of temperature. Ammonium chloride can be assimilated as a sole nitrogen source, but nitrate can not (Pore 1972). Frequently, authors erroneously report starch to be present, but glycogen is the storage carbohydrate (Manners et al. 1973) and starch is absent (Pringsheim 1963). Thiamine (Anderson 1945) and oxygen (Barker 1935) are required for growth. *Prototheca* spp. may store lipids up to 10 percent by weight (Atkinson et al. 1972; Sud and Feingold 1979). The fatty acids 16:0, 18:1, and 18:2 (palmitic, oleic, and linoleic acids) were the predominant extractable lipids of *P. zopfii* grown on glucose or hexadecane (Boehm 1984). Growth on fatty acids (Barker 1935) may well be a reason for the ubiquity of *Prototheca* spp. in fatty acid-enriched wastewater (Pore and Boehm 1986). Assimilation of hexadecane and other hydrocarbons is a notable feature of some strains of *P. zopfii* (Walker and Pore 1978; Pore 1983; Boehm 1984). The cycloartnol-ergosterol biosynthetic pathway of *P. wickerhamii* differs from the lanosterol-ergosterol pathway of yeasts, yet azoles remain effective inhibitors of growth (Mangla and Nes 2000). Ergosterol was not detected in *Prototheca* spp. (unpublished results).

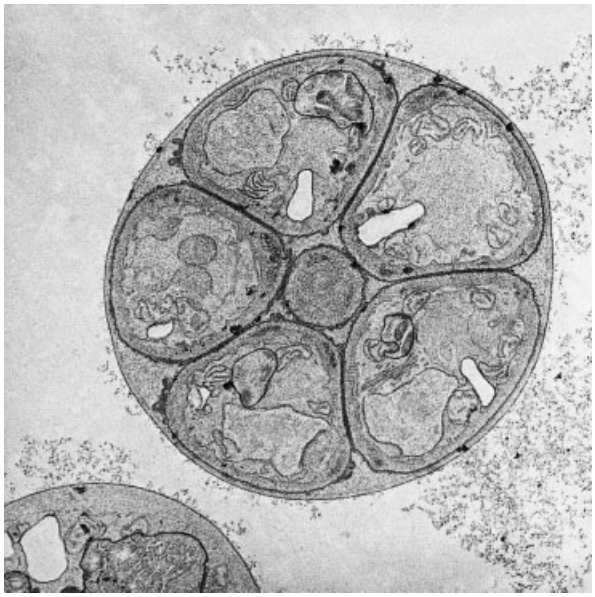

Figure 4 P. wickerhamii *sporangium thin section demonstrating five sporangiospores. Nuclei, mitochondria, Golgi and glycogen storage organelles are present. Transmission electron micrograph; magnification* × *16 250*

The outermost trilaminar aspects of the cell walls of *Prototheca* spp. resist acid and alkaline hydrolysis and contain neither cellulose (as sometimes erroneously reported) nor chitin (Conte and Pore 1973). Atkinson et al. (1972) proposed that sporopollenin could be the refractory compound in the cell walls responsible for resistance to hydrolysis, but the current status of this proposal is that sporopollenin is not the compound in the outermost nonhydrolysable trilaminar cell wall layers (Puel et al. 1987). Regardless of the resolution of this question, the unique cell wall of *Prototheca* spp. and its role in pathogenesis and ecology is a relevant topic for speculation. The presence or absence of carotenoids, accessory photosynthesis pigments, and the purported monomers of sporopollenin would all help in the resolution of the question. Another potentially significant component of the cell envelope of *P. zopfii* is an immunoreactive linear β(1,6)-galactopyranosyl (Manners et al. 1973; Roy et al. 1981). Significant quantities of similar galactans can be easily extracted and purified from the capsules of *P. zopfii* and *P. moriformis* grown in bioreactors. These viscous, polyanionic galactans contain variable amounts of arabinose and rhamnose (unpublished results) and could have medical or industrial value. Likewise, *P. zopfii* and *P. moriformis* have been shown to produce commercial quantities of L-ascorbic acid (Running et al. 2002).

Identification

Five species of *Prototheca* are currently valid, but only two are documented pathogens. Table 2 provides the minimum information needed to differentiate *P. wickerhamii* from *P. zopfii*. For a complete means to

Table 2 *Abbreviated key to differentiate the pathogenic species of* Prototheca[a]

Species	Sporangiospore diameter on PIM[b] (μm) [usual results]	Trehalose assimilation on PIM[c] [no exceptions]
P. wickerhamii	<4.3	+
P. zopfii	>5.7	–

a) Based on complete key for the separation of all five species (Pore 1985, 1998).
b) *Prototheca* isolation medium.
c) Modified for assimilation testing (Pore 1985, 1998).

differentiate all five species the reader is referred to Pore (1985, 1986, 1998).

PROTOTHECA WICKERHAMII

P. wickerhamii grows on PIM after 3–5 days as a white yeast-like colony. In many respects the colony resembles *Candida* spp. (Figure 5a). The small, usually smooth colonies (Figure 5b) characteristically develop a tan pigmentation with advanced age (2–10 weeks) on Sabouraud's medium. All cell stages are spheroidal in culture. Sporangiospores are 2.5–4.5 μm (mean 3.2), sporangia are 7–13 μm (mean 9.4), and Dauer cells are 5.5–8.5 μm (mean 6.5) in diameter. Temperature optima are usually above 25°C and pathogenic strains grow at 37°C. It is the only species that assimilates trehalose (Arnold and Ahearn 1972). The API 20C carbohydrate assimilation (Padhye et al. 1979) and the API ZYM tests (Casal et al. 1985) are of value for clinical identification. A monograph containing a complete taxonomic description was prepared by Pore (1985).

PROTOTHECA ZOPFII

After growing for 3–5 days on PIM, the colonies are whitish with a wrinkled, yeast-like appearance (Figure 5b). The colonies are faster growing and larger than those of *P. wickerhamii* and the sporangia, ranging up to 25 μm in diameter, give a 'ground-glass' appearance when colonies are observed with a stereomicroscope (magnification × 25). Cells are commonly spheroidal or ellipsoidal, waxy, and hydrophobic. Newly released sporangiospores are spheroidal (4.5–7.5, mean 6.5 μm) or ellipsoidal (3–7 × 5–8, mean 5.5 × 6.5 μm). Dauer cells are spheroidal (8.5–14, mean 11 μm) or ellipsoidal (6–11 × 8.5–13, mean 9.5 × 11.5 μm), and sporangia are spheroidal (14–25, mean 17.3 μm) or ellipsoidal (11–20 × 14–23, mean 14.5 × 16.5 μm), but morphology may vary depending on substrate and environmental conditions. Some strains grow at 37°C. *P. zopfii* is the only unencapsulated species capable of growing at pH 5.1 in the presence of acetate (Pore 1985), or assimilating 1-propanol (Arnold and Ahearn 1972). Notably, only 80 percent of the strains tested grew in the presence of clotrimazole 50 μg/ml (Pore 1985), invalidating the use of clotrimazole for identification. No clinically derived strains of *Prototheca* spp. have been described as having a capsule, but spontaneous variants of *P. zopfii* in laboratory culture may produce a polysaccharide capsule; these variants are indistinguishable from *P. moriformis* by conventional morphological and physiological tests (Pore 1985). Several synonyms and varieties have been proposed for the pathogenic *Prototheca* spp., but *P. portoricensis, P. segbwema*, and *Portotheca salmonis* have been reduced to synonymy with *P. zopfii* (Pore 1998). Finally, an evaluation of morphological variability in *P. zopfii* was undertaken (Blaschke-Hellmessen et al. 1985).

CHLORELLA SPP.

In one report algae were isolated from cattle with lymphadenitis (Rogers et al. 1980) and in another from sheep liver (Zakia et al. 1989), but identification of genus and species was inconclusive. All other green algal infections were based on isolation or histopathological observations, and identification of species was not carried out. In previous taxonomic studies, identification of species followed the key points of Shihira and Krauss

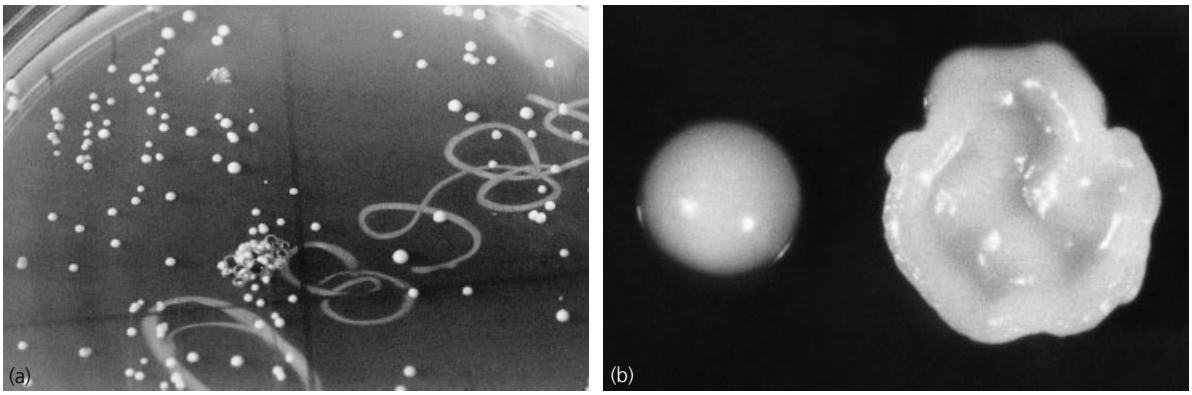

Figure 5 (a) *Primary isolation of* P. wickerhamii *from fecal (human) smear after 72 hours on PIM.* **(b)** P. wickerhamii *usually has a smooth, round colony after 72 hours on PIM:* P. zopfii *produces a larger wrinkled colony.*

(1965), who included *C. protothecoides*, the postulated extant ancestor of *P. wickerhamii,* as subsequent studies have shown (Pore 1998; Ueno et al. 2003). Except for the presence or absence of photosynthetic pigments, the physiological similarities, like the morphological similarities, between *C. protothecoides* and *P. wickerhamii* are remarkable. *C. protothecoides* even inhabits wastewater (Pore and Boehm 1986).

Histopathology

PROTOTHECA WICKERHAMII

P. wickerhamii may be rare to abundant in biopsied specimens of infected tissue, and histopathological detection is the usual means of diagnosis of protothecosis. The algal cells may be seen with the aid of H&E, Papanicolaou's, methylene blue, safranin, lactophenol cotton blue, or Gram's stains, but special stains, such as PAS and Gomori's methenamine silver (GMS), facilitate and confirm detection and identification (Figure 6). A comprehensive selection of color illustrations of the histopathology has been published

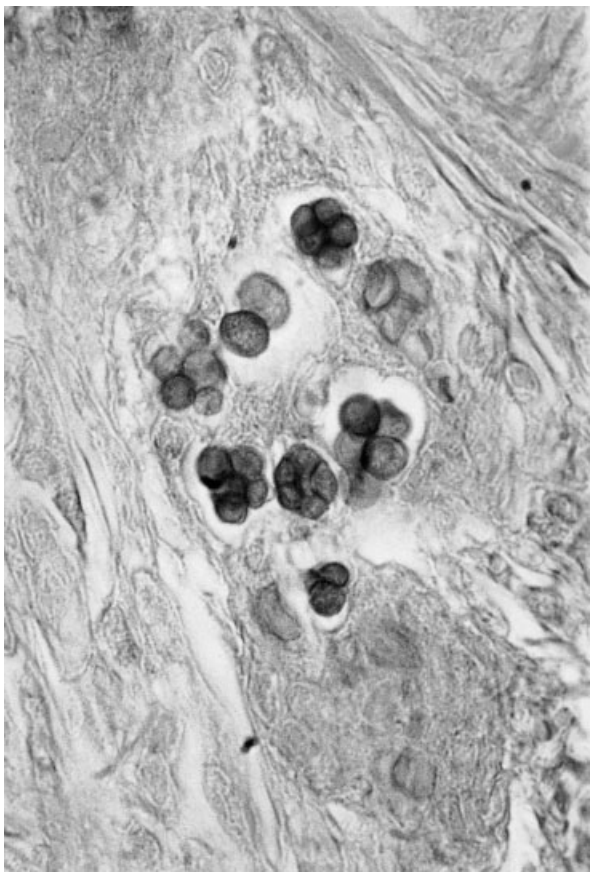

Figure 6 P. wickerhamii *in dermis stained by Gridley's silver stain method. In this instance the older sporangial walls are unstained and the sporangiospores alone are strongly stained. Magnification × 700*

(Chandler et al. 1980). Unlike in vitro size data, speciation on the basis of cell size in tissue is not definitive because of a size overlap of the sporangia of the two species, imprecise dating of the age of the algae, and the compounding influence of host tissue effects. Single cells range from 2 to 10 μm in diameter and sporangia from 10 to 30 μm, larger than in vitro sporangia and clearly overlapping the size range for *P. zopfii*. Empty, collapsed sporangia may be common, and could be difficult to differentiate from fungal debris. *P. wickerhamii* is unencapsulated; however, it was reported to stain weakly with mucicarmine (Kuo et al. 1987) – similar to the purported acid fastness; these may be staining artifacts, not observed by all investigators.

Infected epidermal and dermal responses include hyperkeratosis, parakeratosis, acanthosis, and papillomatosis (Davies et al. 1964). Granulomatous inflammatory reactions in the dermis and soft tissues were associated with multinuclear giant cells containing intracellular *P. wickerhamii*, a notable feature, and surrounded by lymphocytes, plasma cells, eosinophils, and a few polymorphonuclear leukocytes (Mars et al. 1971). Intraepidermal to mid-dermal depositions of the algae may be pathognomonic in cutaneous manifestations (Yip et al. 1976). *P. wickerhamii* may cluster in microabscesses, in which case the lesions show central necrosis and caseation. By contrast, other reporters have not always detected notable multinucleated giant cell response nor microabscess formation, but characterize the histopathological response as a mixed inflammatory response (Walsh et al. 1998).

The distinctive internal cleavage of the spheroidal sporangia, described as morula or being mulberry like, is considered pathognomonic for protothecosis; alternatively, sporangia that break open to release two to eight or more sporangiospores into the tissue are also indicative of *P. wickerhamii* (Figure 7). The sporangia are usually smaller, and the sporangiospores fewer and larger than those of *Coccidioides immitis* and *Rhinosporidium seeberi*. Juxtaposed algal cells of different sizes may falsely resemble budding, but a distinctive isthmus between two cells is never seen. Reports of internal starch granules and leucoplast lamellae are erroneous; *Prototheca* spp. have neither, although they do have refractive glycogen and lipid granules (unpublished observations).

Immunofluorescent reagents have led to a means for the histopathological identification of protothecosis and the differentiation of *P. wickerhamii* from *P. zopfii* (Sudman and Kaplan 1973). Many case reports would not have been published without the taxonomic corroboration obtainable with these serological reagents, but these reagents have limited availability and staining should be interpreted with caution. The best current method of speciation is still from the morphological and physiological traits of cultured isolates (see Table 2).

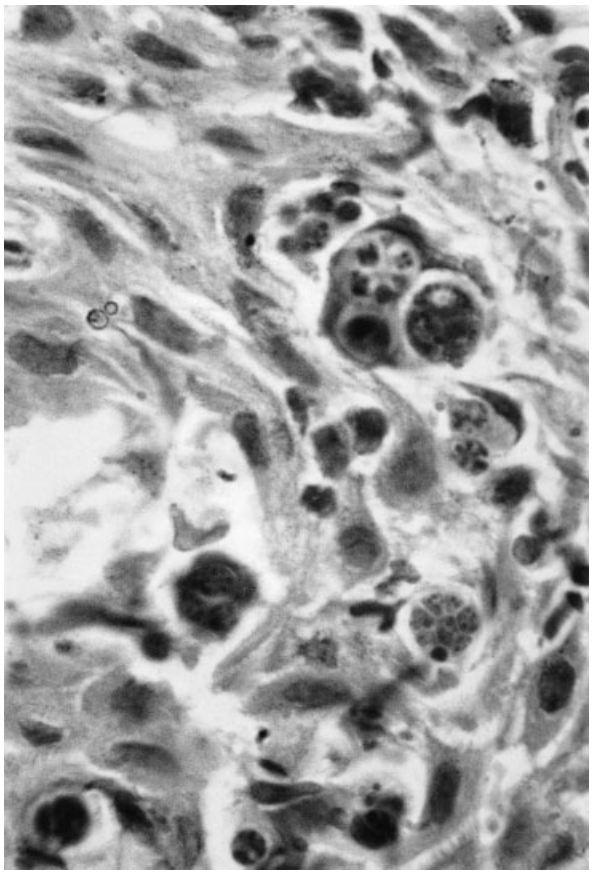

Figure 7 P. wickerhamii *in dermis with little tissue inflammation demonstrated by H&E staining. Several sporangia contain sporangiospores, the best histopathological identifying characteristic. Magnification × 700*

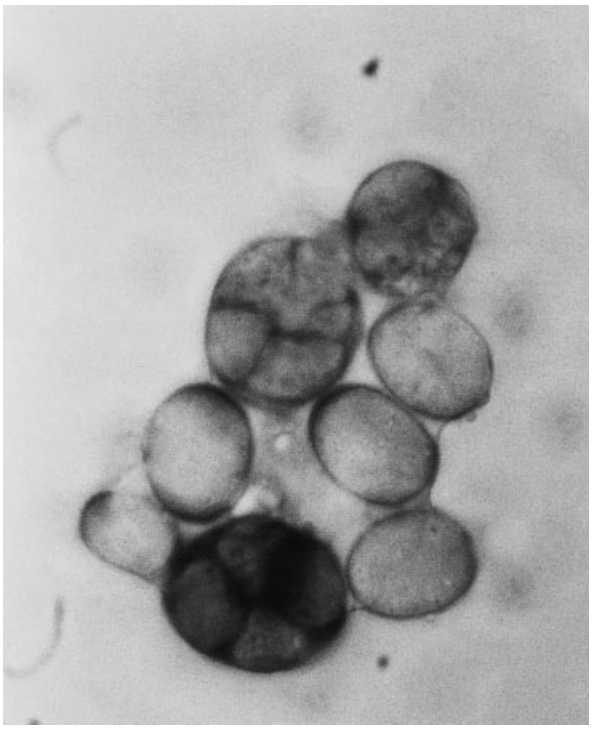

Figure 8 P. zopfii *is rare in secreted bovine milk without enrichment culture (see Table 1), but it is large and distinct when stained with methylene blue. Magnification × 700*

PROTOTHECA ZOPFII

P. zopfii causes chronic progressive pyogranulomatous lesions in the bovine mammary gland and associated lymph nodes. The microorganisms cluster in alveolar lumina and epithelia (Pore et al. 1987). They may also cause acute destruction of blood vessels, acini, and connective tissues in the udder, as well as mildly inflammatory, but highly destructive and necrotic, lesions of other organs (Taniyama et al. 1994; Jensen et al. 1998). The algal cells can be cultured from secreted milk (Figure 8). After experimental inoculation via the teat canal, living and degenerated *P. zopfii* cells were found in enlarged macrophages in granulomas, but not in associated neutrophils or epithelial cells (Cheville et al. 1984). Mastitis is almost solely caused by *P. zopfii*, whereas *P. zopfii* or *P. wickerhamii* causes dog infections equally frequently. Only three human infections with *P. zopfii* are known (Davies et al. 1964; Naryshkin et al. 1987; Kwok and Schwartz 1996); another was associated with a case of onycholysis (Magerman et al. 1991).

P. zopfii is unpigmented and the shrunken basophilic cytoplasm (seen by H&E staining) is within a large, unstained, spheroidal cell wall. However, the specific fungal stains, based on GMS or PAS, do stain the cell wall, even though the wall is chitin free. Specific immunohistochemical staining has helped to elucidate the intracellular sites of *P. zopfii* in macrophages and neutrophils from mastitic infections (Jensen et al. 1998; Corbellini et al. 2001a). Although cells resembling fungi may be seen, budding is absent and sporangial reproduction is present instead. All stages in the formation of two to six endospores are frequently seen within a sporangium after thin sectioning. In tissue, the sporangiospores of *P. zopfii*, but not the sporangia, are fewer and larger than those of *C. immitis* and *R. seeberi*. Although *P. zopfii* sporangia and sporangiospores are also larger than those of *P. wickerhamii,* in vitro, no comparable size data are available for the two species in histological material, so differentiation on the basis of size should be done with caution, if at all.

CHLORELLA SPP.

Histopathological identification to species or even genus is not possible among the small unicellular green algae. Green lymph nodes in sheep and cattle that histopathologically contain microorganisms resembling *Prototheca* or *Chlorella* spp. have been interpreted as chlorella infections based on the green appearance of these nodes (Chandler et al. 1980; Rogers et al. 1980; Jones et al. 1983; Connole 1990; Kaplan et al. 1993; Seniw et al. 1997). Reports of green tissues from animals are,

however, anecdotal and hard to interpret, and green lymph nodes have been found in a case of bovine protothecosis (Migaki et al. 1969). Chlorophyll is soluble in alcohol and absent from most histopathological preperations. In at least one instance, however, the infectious microorganisms contained electron microscopic evidence of chloroplasts (Kaplan et al. 1993) – definitive evidence of a green alga, although not necessarily *Chlorella* spp., but conclusive evidence that the microorganisms were not *Prototheca* spp. Finally, not all *Chlorella* spp. contain starch granules as is sometimes erroneously reported; *C. protothecoides* is such an example. *C. protothecoides* is an alga that stores glycogen, as does *Prototheca* spp. (unpublished observations). Many other *Chlorella* spp. species do store starch, and such PAS-stainable granules in the cytoplasm may after all prove to be an identification characteristic (Chandler et al. 1980).

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Pythiosis

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Members of the oomycetous genus *Pythium* are ecologically and physiologically unique. They occur in soil and aquatic habitats worldwide. *Pythium* species are important plant pathogens causing seed decay, preemergent and post-emergent damping off, root rot of seedlings, and rot of stored foodstuffs. In addition, some species of *Pythium* have been reported to cause disease in fish. *Pythium insidiosum*, however, is the only member of the genus that has been recognized as a mammalian pathogen. Chandler et al. (1980) coined the term pythiosis to include all clinical and pathological manifestations caused by *P. insidiosum* in mammals. In horses, the disease has been long known as bursatee, leeches, granular dermatitis, hyphomycosis destruens equi, phycomycosis, espundia, summer sores, and swamp cancer. Pythiosis in mammals is characterized by the development of cutaneous, subcutaneous, blood vessel, and intestinal lesions and, less frequently, by the involvement of bones and lungs. If not treated, the disease progresses rapidly, becoming life-threatening.

HISTORY

The first reported cases of equine pythiosis were described in the middle of the last century when veterinarians studied cutaneous granulomas among equines in India. The etiology of the disease was not established. The first well-documented cases, in which the 'fungal-like' nature of the infection was suggested, were those published by Smith (1884), Fish (1895–96), and Drouin (1896). In Indonesia, de Haan and Hoogkamer (1901), working with several equines with cutaneous granulomas, isolated the etiologic agent of pythiosis for the first time. Their isolate, however, could not be identified because it did not sporulate on their media. These investigators named the disease hyphomycosis destruens. This name was modified later by de Haan (1902) to hyphomycosis destruens equi. In 1924 another Dutch scientist, working with horses in Indonesia, completed the most comprehensive papers on equine pythiosis ever published (Witkamp 1924, 1925). He also isolated a nonsporulating organism from equine cutaneous granulomas and covered several aspects of the disease, including its clinical signs, pathology, microbiology, animal inoculation, diagnosis, treatment, and immunology. Unfortunately, this classic work remained largely unnoticed, in part because of its Dutch language, but also because of the contemporaneous finding that cutaneous granulomas in horses may also be caused by nematode species of the genus *Habronema* (Ransom 1911). Thus, the terms used to describe pythiosis were also indiscriminately used for equine cutaneous habronemiasis.

The early findings on the 'fungal' nature of cutaneous granulomas in equines were obscured by the new

hypothesis of equine habronemiasis, and the presumed mycotic etiology was forgotten. Thirty-seven years later, Bridges and Emmons (1961) isolated a sterile, filamentous microorganism from equine cases in Texas and Florida, similar to those that had been studied by de Haan and Hoogkamer and Witkamp earlier in the century. They believed that they had isolated 'a species of *Mortierella*' – a zygomycete. The filamentous isolate was named *Hyphomyces destruens*, based on the disease name previously coined by de Haan and Hoogkamer (1901, 1903). It was not clear, however, if the authors intended to introduce a new binomial for the etiologic agent of cutaneous granulomas in horses. In 1974, Austwick and Copland reported that an organism, isolated from horses in Papua New Guinea afflicted with swamp cancer, formed biflagellate zoospores after transfer to a petri dish of sterile water to which a sterilized decayed piece of rotten maize silage had been added. They concluded that the organism should be classified in the oomycete genus *Pythium* but they failed to provide a scientific name for their isolate.

Later, Ichitani and Amemiya (1980) isolated a filamentous microorganism from a Japanese horse with granular dermatitis. Based on the production of smooth oogonia and aplerotic oospores, they identified their isolate as *Pythium gracile*. In 1987, de Cock et al., working in Costa Rica with numerous isolates of a *Pythium* sp., recovered from horses that had espundia (a regional name used for pythiosis), concluded that these isolates belonged to an undescribed species of *Pythium* and the binomial *P. insidiosum* was validly introduced. They also reported that other isolates from cows, dogs, horses, and humans, including the one described as *P. gracile*, also belonged to the single species *P. insidiosum*. Almost concurrently an isolate, from horses in Australia, was also described as a new species and named *Pythium destruens* by Shipton in 1987. Later this isolate was studied by Mendoza and Marin (1989) and it was found to be indistinguishable from the strains isolated from other pythiosis cases; it thus became a synonym for *P. insidiosum*. Recently, Schurko et al. (2003a, b), using molecular tools, confirmed that *P. insidiosum* is the only etiologic agent in the genus causing pythiosis in mammals.

Since 1961 several new cases of pythiosis have been reported in equines (Habbinga 1967; Hutchins and Johnston 1972; Connole 1973; McMullan et al. 1977; Murray et al. 1978; Miller and Campbell 1982b; Mendoza and Alfaro 1986), cats (Bissonnette et al. 1991; Thomas and Lewis 1998), cattle (Miller et al. 1985; Santurio et al. 1998), dogs (Pavletic et al. 1983; Foil et al. 1984; Thomas and Lewis 1998; Graham et al. 2000), humans (Thianprasit 1986, 1990; Rinaldi et al. 1989; Sathapatayavongs et al. 1989; Chetchotisakd et al. 1992; Triscott et al. 1993; Virgile et al. 1993; Imwidthaya 1994b), and in captive bears and a camel in South Carolina and Florida zoos respectively (personal communications, L. Kaufman and A.A. Padhye, and J.F.X. Wellehan).

TAXONOMY AND MORPHOLOGICAL FEATURES OF *PYTHIUM INSIDIOSUM*

Pythium insidiosum is an organism classified in the kingdom Straminipila, class Oomycetes, order Pythiales, and family *Pythiaceae* (Dick 2001). Although Dick (2001) suggests the term Peronosporomycetes to rename the class Oomycetes in the kingdom Striminipila, for convenience, we will continue using the term Oomycetes throughout this chapter. This was motivated also by his position to treat the straminipilans as members of the kingdom Fungi. In recent years, however, strong phylogenetic evidence has been generated indicating that the straminipilans are not fungi, but protistal microbes very close related to algae and plants (Herr et al. 1999; Baldauf et al. 2000). Patterson in 1989 proposed the term Stramenopila. This name has been widely used. However, Dick (2001) recently modified it to Straminipila. He stated that the term Stramenopila is a bad derivation from the latin 'stramini'= a straw and 'pilus'= a hair. So, the straminipilans are organisms bearing tubular tripartite hairs. Therefore, the right spelling of this term should be Straminipila. Dick (2001) also indicated that the spelling 'straminopile,' appearing in the *Dictionary of fungi* (Hawksworth et al. 1995), was an unintentional misprint corrected in the 2001 edition of the same dictionary (Kirk et al. 2001).

In culture, *P. insidiosum* develops fungal-like sparsely septate hyphae; thus it erroneously has been referred to as a microorganism belonging to the kingdom Fungi. All of its phylogenetic features, however, correlate with the kingdom Straminipila (Baldauf et al. 2000; Martin 2000; Dick 2001). On this basis, *P. insidiosum* should only be referred to as a protist or a parafungal organism. As with other oomycetes, *P. insidiosum* grows relatively well on a variety of media. On cornmeal agar, colonies are colorless to white and submerged, with short aerial filaments, and a finely radiate pattern. The hyphae range between 4 and 10 μm in diameter with perpendicular lateral branches. Cross-septa are only occasionally observed in young hyphae, but they are abundant in old viable hyphae. Appressoria and hyphal swellings, measuring 12–28 μm in diameter, are common in laboratory cultures (Figure 1a, b).

Production of zoosporangia is only possible in water cultures incubated at 28–37°C. Zoosporangia increase in number when *P. insidiosum* is placed in contact with pieces of grass leaves in water containing a minimal quantity of different ions (including Ca^{2+}) (Mendoza and Prendas 1988). Early-stage sporangia cannot be differentiated from vegetative hyphae. At maturity, sporangial protoplasm flows into a discharge tube and

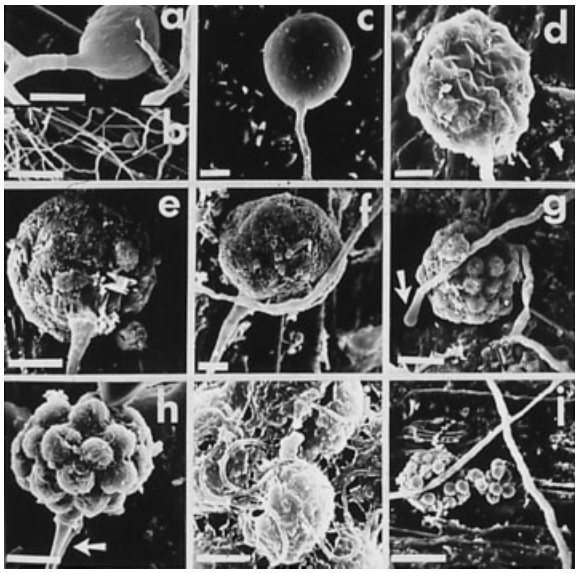

Figure 1 *Transmission electron microscopy of* Pythium insidiosum*'s different stages of development. The formation of globose vesicles and hyphae on a leaf are the first steps in the colonization of a plant host (a, b). The electron photographs show sporangial formation leading to the release of secondary-type zoospores (c–j). Bar:* **(a, c)** *28 μm,* **(b)** *120 μm,* **(d)** *20 μm,* **(e)** *30 μm,* **(f)** *15 μm,* **(g)** *32 μm,* **(h)** *18 μm,* **(i)** *15 μm,* **(j)** *50 μm. (Reproduced, with permission, from Mendoza et al. 1993)*

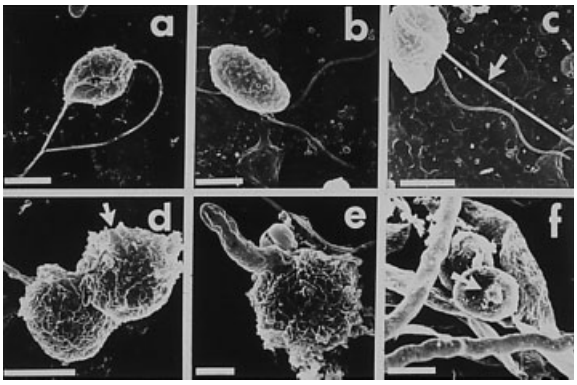

Figure 2 *Transmission electron microscopy of* Pythium insidiosum*'s motile zoospores (a–c). Encysted zoospores are characterized by their globose morphology (d–f). Arrows indicate the place once occupied by the flagella. An amorphous substance used to bind the zoospores to the host's tissue is evident over the surface of encysted zoospores. Zoospores that encyst away from leaf or animal tissues do not secrete this amorphous material* **(f)**. *Bars:* **(a, b)** *9 μm,* **(c, d, f)** *10 μm,* **(e)** *5 μm. (Reproduced, with permission, from Mendoza et al. 1993)*

forms a vesicle. It is globose and hyaline, and measures 20–60 μm in diameter. Through progressive cleavage, biflagellate zoospores are formed inside the vesicle (Figure 1c). The internal developmental process within the undifferentiated vesicle and the release of zoospores takes about 35 minutes. The zoospores mechanically break the vesicle's wall and upon emergence swim for about 20 minutes and then encyst (Figure 1j). The zoospores are reniform and of the secondary biflagellate type. The two flagella arise from the deeper part of a lateral groove and seem to emerge from a common point. They are unequal in length. The anterior shorter flagellum is of the tinsel type and is covered with mastigonemas (small hair-like structures). The posterior flagellum is of the whiplash type and lacks mastigonemas (Figure 2a–c). After encystment, the zoospore's flagella are detached and it becomes spherical. When chemotactically stimulated by a suitable substrate (plant or animal tissue), the zoospores secrete anamorphous sticky material which covers their surface, and they become attached to the plant's or animal's surface. This substance, which may be a glycoprotein, has been implicated as a potential virulence factor in establishing infection (Mendoza et al. 1993) (Figure 2d–f).

In contrast to other species of the genus, only a few strains of *P. insidiosum* have been reported to produce oogonia. Thus, the mechanism implicated in the process of oogonial formation in *P. insidiosum* remains obscure. When oogonia develop in vitro, they are intercalary, smooth, and subglobose. They have a rigid fertilization tube measuring 23–30 μm in diameter and may have one to three declinous antheridia per oogonium. The antheridia are attached over their entire length to the oogonium (de Cock et al. 1987; Shipton 1987). The oospores are aplerotic, or almost plerotic, and are pressed to one side of the oogonium by the rigid fertilization tube. They measure 20–25 μm in diameter (Figure 3k) (de Cock et al. 1987; Shipton 1987). The morphology of the mature oogonia is the basis for speciating members of the genus *Pythium*.

Schurcko et al. (2003a, b) using phylogenetic tools, reported that in the analyses done on 23 isolates of *P. insidiosum* from the Americas, Asia, and Australia, all clustered together according to their geographical areas (Figure 4). This study showed that *P. insidiosum* is more closely related to each other than to any other *Pythium* species, and quite different from the genera *Phytophthora* and *Lagenidium*. Interestingly, the third cluster of *P. insidiosum* strains was made of isolates from Thailand and the USA. Schurcko et al. (2003b) suggested that these unique isolates could belong to new species very close related to *P. insidiosum*. However, they did not treat these cryptic variants as such.

Recently, Grooters (2003) found that clinical cases initially thought to be pythiosis in dogs were not caused by *P. insidiosum*, but by a new species in the genus *Lagenidium*. She based her findings on morphological and molecular studies. In a recent phylogenetic study, however, Schurko et al (2003a, b) described at least three cryptic strains of *P. insidiosum*. These strains formed a sister clade with the other 20 *P. insidiosum* isolates studied by them. They stated that all *P. insidiosum*, and the other *Pythium* spp, were closer to the

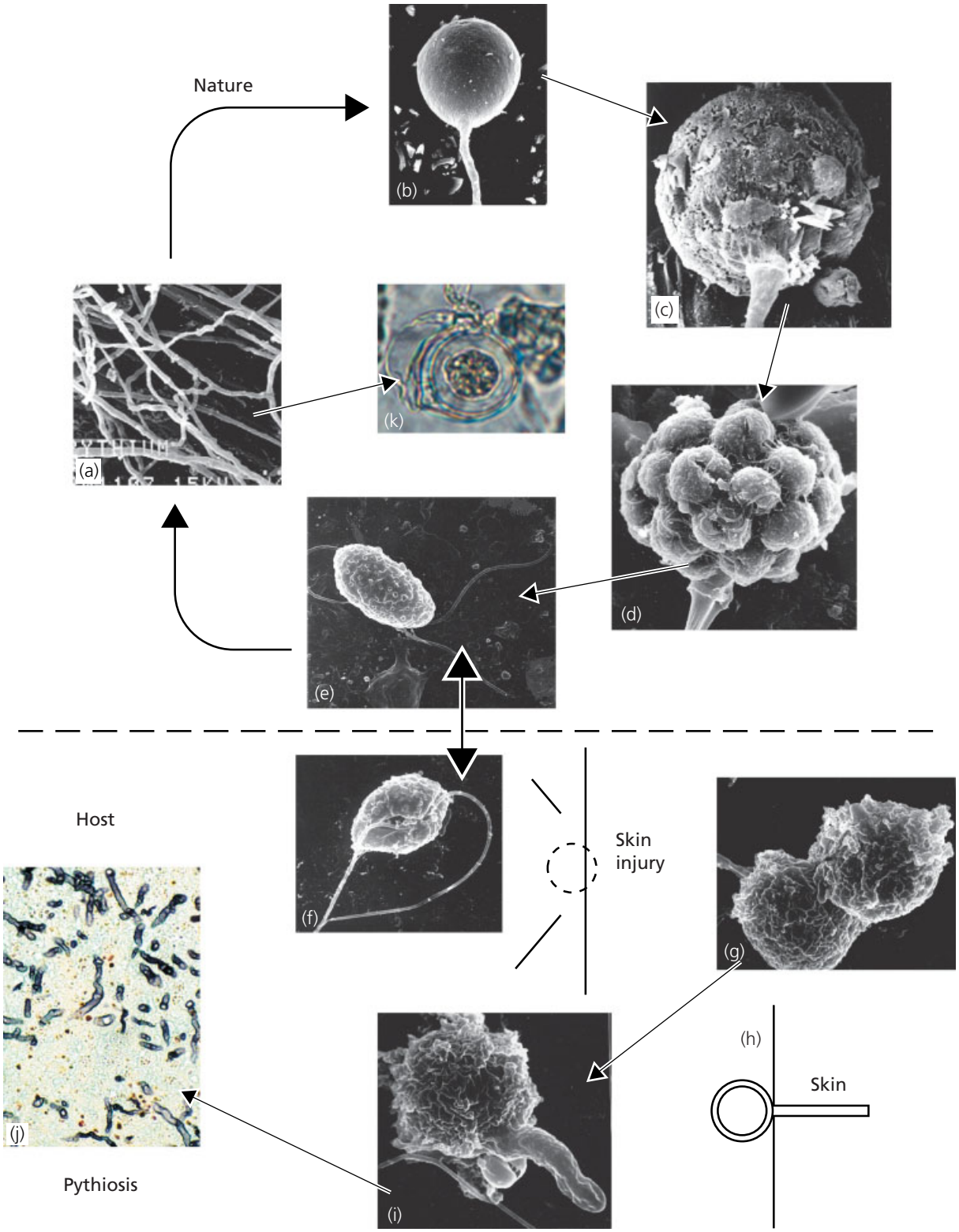

Figure 3 *(a–e, k, in upper panel) The life cycle of* Pythium insidiosum *in nature. Plant tissue is first colonized by* **(a)** *hyphae of* P. insidiosum*, and then* **(b–d)** *the differentiation of sporangium into mature stages leads to* **(e)** *zoospore release.* **(f)** *The zoospores swim to locate another plant or may be attracted by injured animal tissue. The encysted zoospores were (g, dots) attached to tissue by a sticky substance, germinate* **(h)**, *invade the host* **(i)**, *and cause pythiosis.* **(j)** *The formation of masses, called kunkers, occurs only in horses. The production of oospores occurs in nature and they may serve as* **(k)** *resistant spores.*

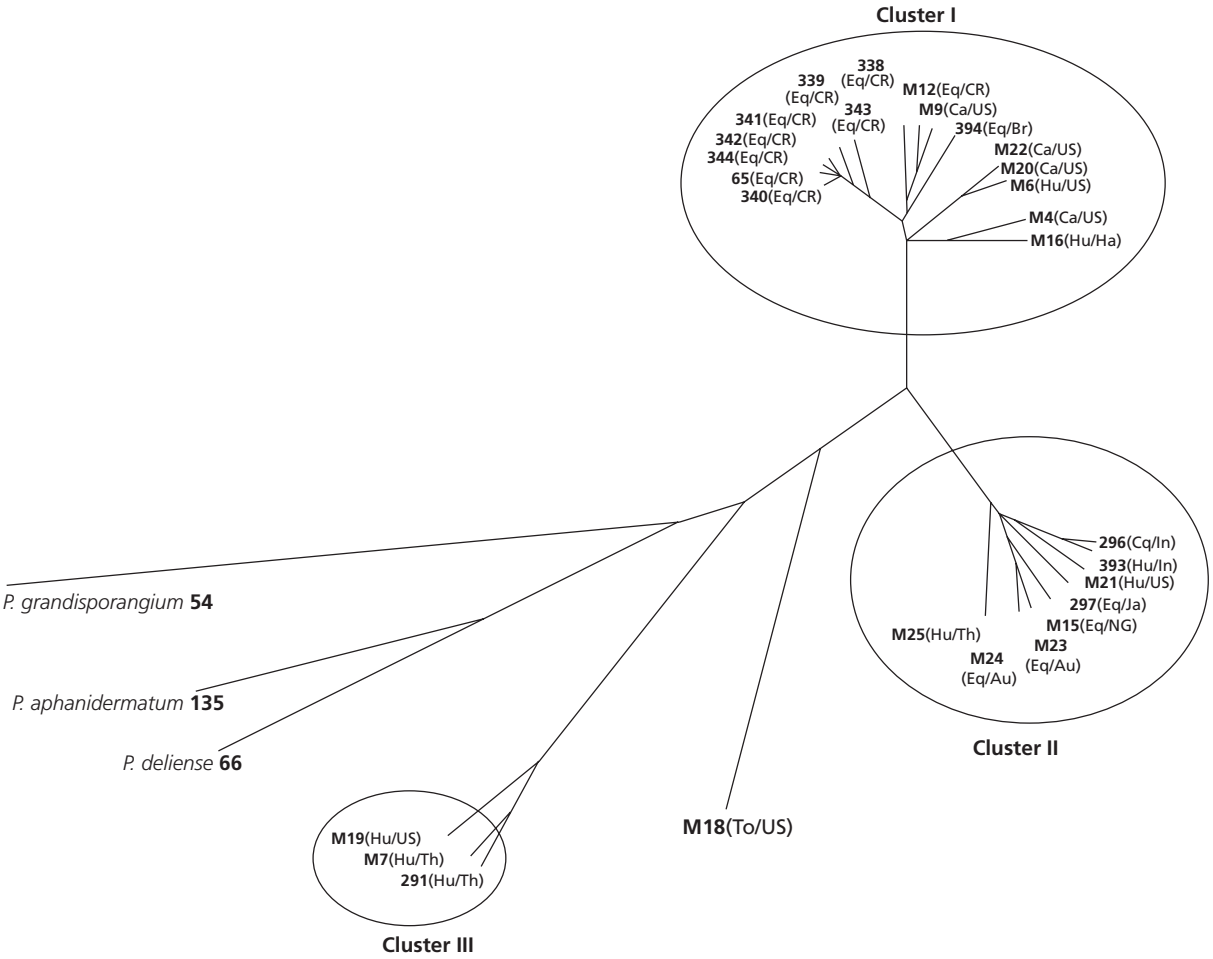

Figure 4 *The phylogenetic tree shows an unrooted unweighted pair-group method average (UPGMA) phenogram depicting the clusters formed by the 23* Pythium insidiosum *isolates investigated by Schurko el at. (2003a). The numbers and letters in bold identified the strains used to build the tree and in parenthesis the origin and geographical location of the strains. Cluster I contains all the strains from the Americas, cluster II, strains from Asia and cluster III (including M18) has a mixture of Asian and American isolates. (Reproduced, with permission, from Schurko et al. 2003a)*

genus *Lagenidium* and far away from the genus *Phytophthora*. Thus, it could be that the strains studied by Grooters (2003) may be related to the third group described by Schurko et al (2003a, b) rather than being new members of the genus *Lagenidium*. However, this conjecture needs verification.

EPIDEMIOLOGY

Pythium spp. are ecologically versatile microorganisms. They occur in practically all soils and wet environments. They are among the most destructive phytopathogens, inflicting economic losses on a wide variety of crops. However, of all the described species (about 120) only one, *P. insidiosum*, has been implicated as an etiologic agent of mammalian disease. Since the last century, water has been related to cases of cutaneous granulomas in equines. It was noted that, after horses had grazed for a lengthy period in stagnant water, they frequently developed pythiosis. The term 'bursattee,' derived from the Indian words 'bururs' or 'bursat,' meaning rain or rain sore, was used in India to describe this condition. Moreover, equine pythiosis has been referred to as swamp cancer in some areas of the world. Thus, a relationship between wet habitats and this disease had been suspected from early in its history. In Australia, equine pythiosis is usually observed in late summer and autumn after the formation of large bodies of stagnant water from the remnants of the previous winter's rains and high seasonal temperatures – conditions that favor the rapid growth of *P. insidiosum* (Miller and Campbell 1982b; Shipton 1985). Miller (1983) noted that, under laboratory conditions, the zoospores of *P. insidiosum* are attracted to animal hair and tissue. He used horse hair as bait to isolate *P. insidiosum* from swampy areas in Australia. He speculated that this oomycete lives in wet habitats and that perhaps it may require a plant to complete its life cycle. Although an unidentified water lily was suggested as a possible plant host (Miller 1983), other findings (Mendoza and Prendas 1988; Chaiprasert

et al. 1990; Mendoza et al. 1993) indicate that, during its life cycle, *P. insidiosum* may preferentially use grass tissue, but in environments free of gramineae, it may complete its life cycle on other plants.

DISTRIBUTION

Pythiosis is a disease of the temperate, subtropical, and tropical areas of the world. It has been reported in Argentina, Australia, Brazil, Colombia, Costa Rica, Haiti, India, Indonesia, Japan, New Guinea, New Zealand, South Korea, Thailand, the USA, and Venezuela. Verified cases of this disease have not been reported in Europe. A case of cutaneous granulomas in a horse with all the features of pythiosis was, however, published by Drouin (1896) in France. The geographical location and tropical climate of Africa seemingly would make it an ideal region for pythiosis. Nevertheless, cases from that continent have yet to be reported. In the Americas, the disease has been diagnosed in North, Central, and South America, as well as the Caribbean islands.

In the USA, the disease is more common in states near the Gulf of Mexico: Alabama, Florida, Louisiana, Mississippi, and Texas. In addition, sporadic cases have been diagnosed in nearby states such as Georgia, Missouri, North Carolina, South Carolina, Tennessee, and as far off as Illinois, Indiana, New York, and Wisconsin – all temperate regions of the USA near the Canadian border. Cases of pythiosis have not as yet been described from Mexico. In Central America, Costa Rica is the country with the largest number of documented cases of pythiosis in horses. This disease is most prevalent in the Atlantic region. Some cases have been diagnosed along its Pacific coast, however. The disease has been observed in other Central American countries – Guatemala, Nicaragua, and Panama (personal communications), but there are no recorded cases in the literature. In the Caribbean region, a case of human pythiosis was recently recorded in Haiti, so the disease may also be present in the other islands of that tropical region (Virgile et al. 1993). Northern Argentina, Brazil, Colombia, and Venezuela have reported cases of equine pythiosis since 1965. The Pantanal region of Brazil, the world's largest freshwater wetland, may have the highest incidence and prevalence of equine pythiosis, but systematic studies are not available to support this impression (dos Santos and Londero 1974; Santurio et al. 1998; Leal et al. 2001).

Australia, the Pacific islands, and Asia have reported cases of pythiosis since the last century. In Australia, it has been found in Maitland, New South Wales, the coast of Queensland, the Northern Territory, and Western Australia. In Indonesia, the disease occurs in Borneo, Java, and Sumatra. In Japan, pythiosis is known to occur along the southern coast of Kyushu and the Ryukyu Islands. A case of intestinal pythiosis was recently reported in a dog from South Korea, suggesting that the disease could also be present in North Korea and China (Sohn et al. 1996). In Thailand, cases of human pythiosis have been found in the northern and southeastern parts of the country. Figure 5 shows the currently known distribution of pythiosis around the world.

LIFE CYCLE OF *PYTHIUM INSIDIOSUM*

P. insidiosum, as with other *Pythium* spp., is an organism of aquatic environments, although it can also be found in soil as a result of its ability to produce resistant spores (de Cock et al. 1987; Mendoza et al. 1993). To maintain its life cycle in nature, *P. insidiosum* requires a low concentration of ions, a pH near neutrality, and a plant host (Shipton et al. 1982; Shipton

Figure 5 *Map showing the distribution of pythiosis in the tropical, subtropical, and temperate regions of the world.*

1983, 1985). As for other zoosporic fungal-like organisms (Endo and Colt 1974), *P. insidiosum* probably uses plants to produce sporangia and release zoospores to colonize other plants and expand its ecological niche, but it has not been demonstrated to cause pathology in plants. Early studies on the life cycle of this oomycete have shown that *P. insidiosum* is present in stagnant waters and may possibly require the Australian water lily or other plants to complete its life cycle (Miller 1983). It was also found that its zoospores may play an important role in the propagation of infections among plants and animals. More recently, other investigators (Mendoza et al. 1993) confirmed that *P. insidiosum* has a special tropism for animal and plant tissues, and that, upon release of zoospores, several mechanisms are activated that permit plant and animal tissue invasion. Encysted zoospores are surrounded by an amorphous material that has adhesive properties. This substance attaches the zoospores to the host's surface. They further hypothesized that chemotactic factors may signal zoospores to produce this material. Figure 3 illustrates various stages of *P. insidiosum*'s life cycle in detail.

CLINICAL AND PATHOLOGICAL SIGNS OF INFECTIONS CAUSED BY *PYTHIUM INSIDIOSUM*

The pathogenesis of pythiosisis not clear, in part because the disease is not reproducible in natural hosts. Laboratory data obtained from strains of *P. insidiosum* indicated that zoospores have a tropism for animal and plant tissues (Miller 1983; Mendoza et al. 1993). Thus, the zoospores, as motile propagules, in all likelihood, play a role as infecting agents in natural infections. However, reports of pythiosis in animals and humans with no history of contact with stagnant water open the possibility of inoculation by other propagules of *P. insidiosum* (hyphae, resting spores, oogonia) (Rinaldi et al. 1989; Fischer et al. 1994).

When an animal or human enters swampy areas inhabited by *P. insidiosum*, its zoospores would be attracted by any open wound on their skin and encyst on the exposed tissue. On the basis of electron microscopic observations (Mendoza et al. 1993), the zoospores are known to secrete a sticky substance that allows them to make and maintain tight contact with the host during the initial stages of invasion. Stimulated by the temperature of the host's skin, the encysted zoospores develop a germ tube, often directed towards injured tissue (Mendoza et al. 1993), and mechanically penetrate the tissue (see Figure 2e). Ravishankar and Davis (2001) experimentally established that *P. insidiosum* cannot penetrate normal skin, but has the potential to successfully penetrate injured skin. Their study strongly supports previous speculations. *P. insidiosum* initiates a cell-mediated immune response in its host, mostly in the form of eosinophils and a few neutrophils (Miller and Campbell 1983; Mendoza and Alfaro 1986), but the immune response cannot prevent the propagation of *P. insidiosum*. The eosinophils then, in an effort to phagocytose the microorganism, degranulate over the hyphae. New eosinophils fuse around the degranulated eosinophils and are also degranulated in turn. A mass known as a 'kunker' is thus formed. The eosinophilic material (Splendore–Hoeppli-like phenomenon) around *P. insidiosum* hyphae is a main feature of the infection in equines but not in the other species. Kunkers are composed of degranulated eosinophils interlaced with the viable hyphae of *P. insidiosum*. The lower panel of Figure 3 summarizes the probable mechanism of infection. The tissue damage observed in acute and chronic cases has been attributed to the release of chemicals from the degranulated eosinophils and mast cells (Miller 1981; Miller and Campbell 1983; Mendoza et al. 2003).

Pythiosis in non-primates

HORSES

Since the beginning of this century, the clinical features of pythiosis in horses have been well described by several authors (de Haan and Hoogkamer 1901, 1903; Witkamp 1924; Bridges and Emmons 1961; McMullan et al. 1977; Miller and Campbell 1982b; Chaffin et al. 1995). There are no reports of predisposition with regard to sex or age of the afflicted animals. Lesions caused by *P. insidiosum* are found on any part of a horse's body, although they are more common on the lower limbs because these are the first areas to come in contact with swampy water. In addition, the lesions in horses can also be found on the thorax, abdomen, neck, shoulders, genitalia, and head. Pythiosis often occurs as a single lesion but unusual cases with multicentric granulomas have been encountered. When lesions develop on the extremities (particularly near the joints), lameness is a frequent finding. In chronic pythiosis, emaciation and secondary infections are also common. There are no reports of animal-to-animal, or animal-to-human transmission of infection.

Lesions are circular in shape, from 5 to 500 mm in diameter (Miller and Campbell 1982b; Mendoza and Alfaro 1986), with a characteristic serosanguineous discharge (Figure 6a, b). Intense pruritus is one of the characteristic clinical features of the disease in horses. Ulceration of the tissue is common in large lesions. Smaller ones often show one or more small ulcers. As the disease progresses, small coral-like masses called kunkers, which contain viable hyphae of *P. insidiosum*, are found in the sinuses and surface of the infected tissue. They vary in size and form (10–90 mm in diameter) (Figure 7). Metastasis of *P. insidiosum* through lymphatic vessels to regional lymph nodes (Connole 1973; Murray et al. 1978), lungs (Witkamp

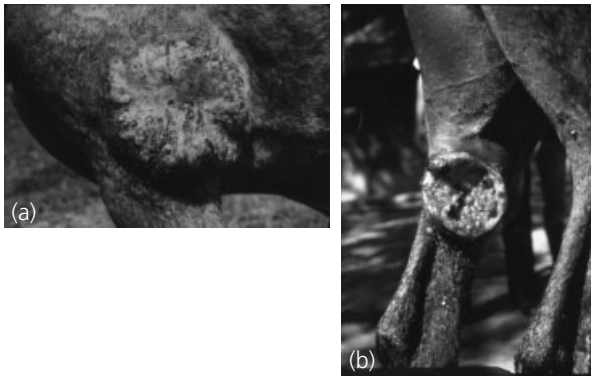

Figure 6 *Clinical features of cutaneous granulomas caused by* Pythium insidiosum *in two horses (a, shoulder; b, leg). Note the circular shape typical of pythiosis granulomas in horses.*

1924; McMullan et al. 1977; Goad 1984), or bones (Mendoza et al. 1988; Alfaro and Mendoza 1990; Eaton 1993; Neuwirth 1993) is common (Figure 8). Equine intestinal pythiosis, resulting from direct inoculation of propagules of *P. insidiosum* in mucous membranes, has been reported (Brown and Roberts 1988; Morton et al. 1991; Purcell et al. 1994).

Histopathologically, in the early stages of pythiosis, abundant microabscesses with an inflammatory reaction, composed mainly of eosinophils, a few neutrophils, lymphocytes, and macrophages, are present in the subcutaneous lesions. In chronic cases, eosinophilic granulomatous tissue with giant cells is often recorded. In central areas of inflammation, sequestered eosinophilic masses (kunkers) are detectable. Sections of the kunkers stained with Gomori's methenamine silver reveal the presence of aseptate hyphae 6–10 μm in diameter along with cellular debris (Figure 9).

DOGS

Canine infections were first reported in dogs with cutaneous and gastrointestinal lesions from the Gulf of Mexico in the USA (Heller et al. 1971; Miller et al. 1983; Pavletic et al. 1983; Foil et al. 1984). Subcutaneous pythiosis lesions have been recorded also on the legs, face, and tail in dogs (Figure 10) (Foil et al. 1984; Thomas and Lewis 1998). Systemic pythiosis with involvement of internal organs has also been reported in dogs (Foil et al. 1984; Thomas and Lewis 1998). The pruritic

Figure 8 *Posterior view of a bone in the front leg of a horse affected by* Pythium insidiosum*, with lysis and exostosis of the metacarpal, carpal, and distal radius, and the first phalanx bones.*

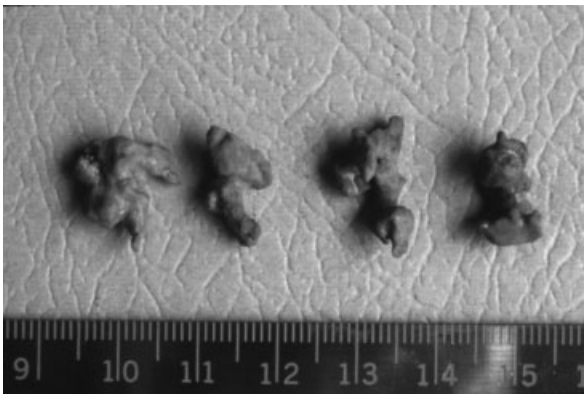

Figure 7 *This shows four irregularly shaped, firm, coral-like masses called kunkers which were removed from a cutaneous granulomatous lesion in a horse with pythiosis. (Reproduced, with permission, from Mendoza and Alfaro 1986)*

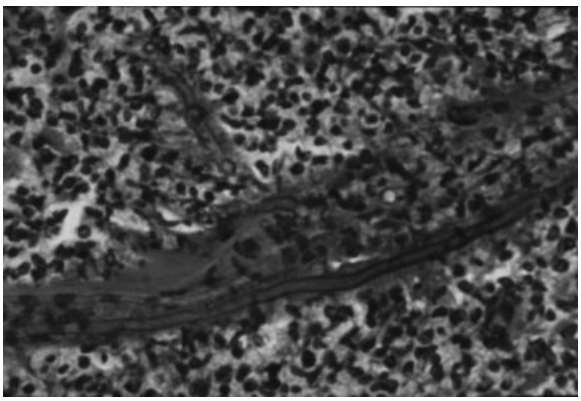

Figure 9 *Photomicrograph of a kunker's tissue section, showing the aseptate hyphae of* Pythium insidiosum *and cellular debris. Periodic acid–Schiff stain; magnification × 9 200.*

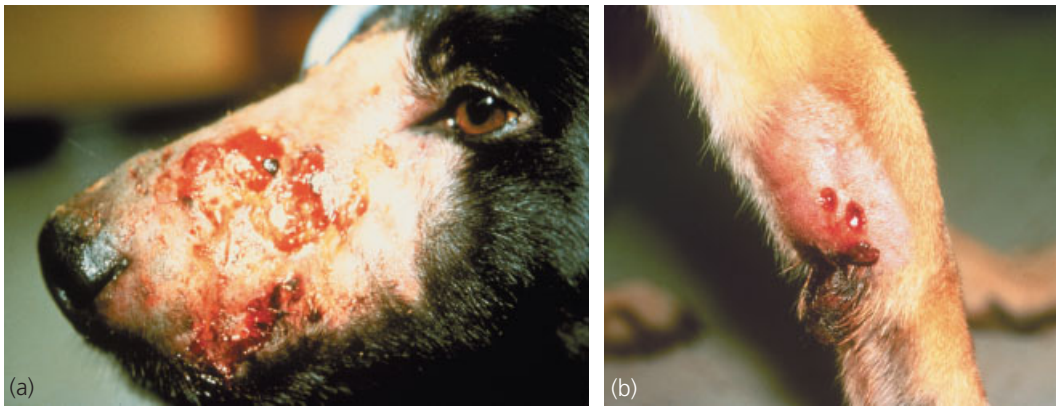

Figure 10 (a, b) *Skin lesions caused by* Pythium insidiosum *on two dogs with subcutaneous infection. (Courtesy of Dr Randall C. Thomas)*

skin lesions were denuded of hair and perforated by fistulous sinus tracts discharging a serosanguineous exudate. Histopathological findings included multifocal areas of necrosis with moderate neutrophils and macrophages. Discrete granulomatous infiltrates with eosinophilic material are surrounded by eosinophils, neutrophils, and epithelioid cells. Giant cells have also been reported in chronic cases. Hyphae which measured 4.4–8.6 μm in diameter were detected in these tissues.

Gastrointestinal pythiosis in dogs is characterized by vomiting, weight loss, and sporadic diarrhea (Miller et al. 1983, 1985; Pavletic et al. 1983; Thomas and Lewis 1998). Formation of hard gastrointestinal granulomatous masses, areas of mural thickness, and mucosal ulceration are common in most cases. Lesions can spread to adjacent tissue such as that of the pancreas, uterus, and mesenteric lymph nodes. Histopathologically, the mucosa shows ulceration, atrophy, and epithelial cell hyperplasia. The submucosa is thickened and the muscular mucosa contains focal granulomas. Neutrophils, eosinophils, plasma cells, macrophages, epithelioid cells, and giant cells are observed in the affected tissues. The hyphae of *P. insidiosum* are difficult to detect in hematoxylin–eosin-stained sections, but with Gomori's methenamine silver stain, hyphae between 2.5 and 8.9 μm in diameter are readily detected. Recently, cases of canine pythiosis have been recorded in Australia (English and Frost 1984) and in the USA (Miller et al. 1985).

Grooters (2003) stated that there were clinical cases similar to those observed in canine pythiosis, caused by an undescribed species in the genus *Lagenidium*. She based her findings on histopathological, cultural, and molecular analysis. However, her data have yet to be confirmed by others (see earlier under Taxonomy and morphological features of *Pythium insidiosum*).

Cats and cattle

Few cases of pythiosis in cats (Bissonnette et al. 1991; Thomas and Lewis 1998) and calves (Miller et al. 1985; Santurio et al. 1998) have been described in the literature. Bissonnette et al. (1991) was the first to diagnose a case of pythiosis in a cat with facial swelling, but none of its internal organs was involved (Figure 11a). *P. insidiosum* was isolated from the affected tissue. Histopathological features, similar to those recorded in horses and dogs, were observed in this case.

A total of five calves with pythiosis have so far been reported with the disease. Showing multiple focal ulcers and fistulous tracts, draining a watery purulent exudate and with swelling of fetlock joints, they were diagnosed with pythiosis (Miller et al. 1985; Santurio et al. 1998). Miller and colleagues (1985) reported that *P. insidiosum* was isolated from two of the studied calves, whereas *P. insidiosum*-like hyphae were found in the tissue of a third case. Histological sections from that case showed granular encrustations around the hyphae. The perihyphal deposit was composed of granular material similar to that of the Splendore–Hoeppli phenomenon. Similar observations were described by Santurio et al. (1998) in calves from the Pantanal region of Brazil.

More recently, several cases of pythiosis in calves were also described in the State of Apure, Venezuela (Figure 11b) (personal communication, Rosa Cristina Perez). Serological testing done in these animals was consistent with *P. insidiosum*. In addition, histopathological and DNA sequencing analysis from the infected tissues confirmed the presence of this pathogen in the infected calves.

Human pythiosis

Most human cases have occurred in Thailand with only a few reports of such cases in Australia, Haiti, New Zealand, and the USA. Two human cases of subcutaneous pythiosis were mentioned by de Cock et al. (1987), but details about those early cases were not available. The first five cases of this disease in humans involved two women and three men in the rural areas of northern Thailand (Sathapatayavongs et al. 1989). Ten

Figure 11 (a) *Lesions on a cat from Florida, USA with subcutaneous pythiosis (arrow).* **(b)** *A subcutaneous lesion on the limb of a Venezuelan calf caused by* Pythium insidiosum. *(Courtesy of Dr Randall C. Thomas and Rosa Cristina Perez)*

more cases were later reported from the same areas of Thailand (Chetchotisakd et al. 1992; Wanachiwanawin et al. 1993). Most were associated with a hemoglobinopathic syndrome. Clinically, progressive gangrene and pain in the extremities were the main findings. Strikingly, all cases showed large vessel arteritis (Figure 12). Angiographic analysis indicated occlusion of the iliac, popliteal, and femoral arteries. Treatment by amputation was successful in seven of the 15 cases. Fatal invasion of the abdominal arteries with occlusion of the infrarenal part of the aorta was also recorded. So far, the majority of cases of human pythiosis have occurred in Thailand, with some 90 new patients reported since the first case in 1987 (personal communication, Theerapong Krajaejun).

Three patients with subcutaneous orbital pythiosis were diagnosed in the USA (Rinaldi et al. 1989). One was a healthy boy in Texas who had accidentally been struck in his right eye. He developed progressive peri-

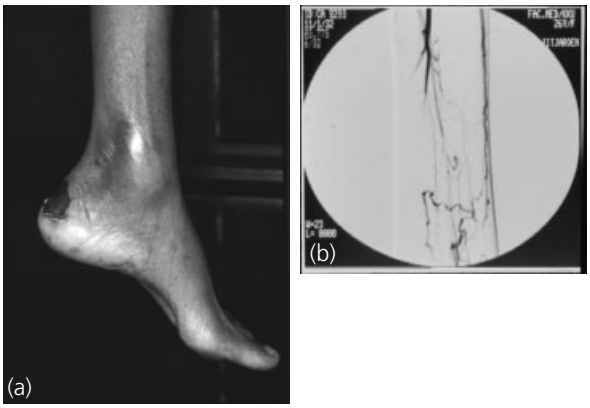

Figure 12 (a) *Dry gangrenous lesion on the left leg caused by* Pythium insidiosum *in a Thai thalassemic patient.* **(b)** *Digital subtraction angiography of the same patient showing occlusion of the left superficial femoral artery. (Courtesy of Dr Ploenchan Chetchotisalkd, Thailand)*

orbital oedema with chemosis, erythema, and periorbital cellulitis (Figure 13). *P. insidiosum* was isolated from biopsied tissue. Details of the second case were not discussed. The third case was recorded by Shenep et al. (1998) on a 2-year-old boy from Tennessee. Two additional cases of pythiosis with a similar clinical history were reported in Australia (Triscott et al. 1993). These cases involved an 11-year-old and a 14-year-old boy. Both developed periorbital swellings which rapidly developed into an orbital tumour. The diagnosis was made histopathologically using an immunoperoxidase staining assay (see section on Immunohistochemical assays below). In addition to vessel arteritis and subcutaneous pythiosis in humans, cases of keratitis have also been recorded in Haiti (Virgile et al. 1993), New Zealand (Fraco and Parr 1997), and Thailand (Kunavisarut et al. 1988; Imwidthaya and Methitrairut 1992; Imwidthaya 1995).

Triscott et al. (1993) described in detail the histopathology of subcutaneous pythiosis in humans. They found that the histological features were similar to those of equine pythiosis. Eosinophilic granular masses of about 7 mm in diameter, containing degenerating eosinophils, eosinophilic granules, hyphae, and intact eosinophils at the edge of the granulomatous areas were the main findings. They also reported a mixed inflammatory cell infiltrate comprising eosinophils, lymphocytes, neutrophils, macrophages, and mast cells. As in equine pythiosis, the hyphae of *P. insidiosum* were restricted to the areas of the eosinophilic granular masses. Kunkers, as recorded in equine pythiosis, have not, however, been detected in tissue from human cases.

IMMUNOLOGY

The development of an immune response to *P. insidiosum* antigens during equine infections has been known since early in the century (Witkamp 1924, 1925).

Figure 13 *Severe orbital swelling resulting from* Pythium insidiosum *infection in a healthy boy. (Courtesy of Drs Michael G. Rinaldi and Steven Seidemseld, USA)*

In those studies, Witkamp described precipitin and complement fixation antibodies, and a cellular immunity response to a skin test in horses with active pythiosis. Miller and Campbell (1982a), using a similar approach, confirmed this early work. At least three antigens were prepared. A trypsin–hyphal antigen detected one precipitin band by double immunodiffusion (ID) in all horses with the disease. By contrast, only 82 percent of the horses were positive to a complement fixation test. The skin test, using a precipitate protein antigen, was positive in 64 percent of the clinically infected horses and in 31 percent of normal horses inhabiting the enzootic areas. The finding that normal animals reacted positively in a delayed hypersensitivity skin test indicates that subclinical pythiosis may occur in some horses. They also reported detectable levels of *P. insidiosum* antibodies at the day of birth in foals born to mares with active pythiosis, suggesting that their foals received some degree of passive immunity against *P. insidiosum*. Horses with a history of pythiosis but negative to skin tests were considered anergic. Analogous immunological studies in other areas also confirmed these findings (Mendoza and Alfaro 1986; Grooters et al. 2002; Krajaejun et al. 2002).

Immunization (immunotherapy) with antigens derived from *P. insidiosum* was shown to have curative properties in horses afflicted with the disease (Miller 1981; Mendoza and Alfaro 1986; Hensel et al. 2003; Mendoza et al. 2003). Fifty percent of infected horses responded to immunization. Changes from an eosinophilic infiltrate, before immunization, to a mononuclear response (macrophages, T lymphocytes), after immunization, suggested that cellular immunity was playing a major role in the clearance of *P. insidiosum* from immunized horses. Mendoza et al. (2003) suggested that a switching from a T helper 2 (Th2) to a T helper 1 (Th1) response was behind the curative properties of the immunotherapeutic antigens used by these investigators.

Animals in the early stages of pythiosis react positively to a delayed hypersensitivity skin test using a culture filtrate antigen (CFA) and are cured by immunization with precipitated protein antigens. These observations, together with the fact that sera from humans and animals reacted positively in ID and Western blot tests, indicated that cellular and humoral immunity were active in the early stages of the disease. Animals became anergic when the disease reached chronicity (>2 months).

Systemic human pythiosis in Thailand, on the other hand, has been diagnosed mainly in thalassemic patients (Imwidthaya 1994a). This may indicate that the disease usually occurs in debilitated patients. However, there is no such parallel in animals with this disease. Moreover, in the USA and Australia, four subcutaneous human cases of pythiosis were recorded in healthy individuals (Rinaldi et al. 1989; Triscott et al. 1993). Thus, the predisposition to *P. insidiosum* infection in patients with thalassemia hemoglobinopathy syndrome is intriguing and merits further investigation.

LABORATORY DIAGNOSIS

Clinical material from suspected cases of pythiosis should be sent directly to the laboratory for processing. Storage of tissue samples at 4°C during transportation may result in the death of *P. insidiosum*. If the samples cannot be delivered immediately, it is recommended that they should be vigorously washed several times in sterile distilled water, and then transported in sterile distilled water plus antibiotics (ampicillin + streptomycin). The most suitable tissue from which to isolate *P. insidiosum* from horses is the coral-like structures known as kunkers. These masses are removed and repeatedly washed in distilled water. The tissue or kunkers are then cut into small pieces, placed on Sabouraud's dextrose agar (SDA) and incubated at 37°C for 24–48 hours. Parts of the pieces of the kunkers should be placed in 10 percent KOH for direct microscopy examination. Wet mounts are of value for the early detection of *P. insidiosum*, which appear as hyaline sparsely septated hyhae 4.0–9.0 μm in diameter.

Several media have been used for the isolation of *P. insidiosum*. The most common is SDA. *P. insidiosum* grows rapidly at 37°C on that medium. In 24 hours it develops flat colonies that are 20 mm in diameter. Microscopically, however, the hyphae are found to be sterile at this stage. The hyphae are branched at approximately 90° angles and are usually coenocytic, although occasional septa are observed in tissue sections and in old cultures. To induce zoospore production, it is recommended that boiled grass leaves be placed on *P. insidiosum* cultures. After 24 hours of incubation at 37°C, the leaves are immersed in dilute salt solution containing calcium and incubated 2–3 hours at 37°C (Mendoza and Prendas 1988). Zoosporangia containing motile zoospores will be readily observed at the edges of the leaves. Oogonium production is very rare in *P. insidiosum*, so specific identification is based on serological tests and DNA testing (Mendoza et al. 1987; Badenoch et al. 2001; Grooters and Gee 2002; Schurko et al. 2003a).

Histopathology

In tissue sections, the hyphae of *P. insidiosum* in tissue sections are often difficult to differentiate from those found in cases of zygomycosis caused by fungal species of the orders Mucorales and Entomophthorales. In such cases, the diameter of the hyphae may be of help. The hyphae of *P. insidiosum* are between 3.0 and 10.0 μm in diameter, whereas those of *Basidiobolus ranarum* and *Conidiobolus coronatus* are broader, being about 5–15 μm in diameter. However, the isolation of *P. insidiosum* and the use of serological tests are the ultimate basis for the diagnosis of pythiosis. In some cases, clinical data can also be useful. For example, clinically pythiosis and zygomycosis caused by *B. ranarum* occur in the same anatomic areas in horses, so it is often difficult to differentiate between them, although infections caused by *C. coronatus* occur primarily in a horse's nostrils. This clinical difference may be of help, if serological tests and isolation of the etiologic agent are not possible.

DNA-based diagnostic assays

The identification of *P. insidiosum* in the laboratory and histopathological preparations is entirely based on its sexual oogonia and hyphae-like morphological features. However, several fungi have also morphological similarities with the filamentous structures of this oomycete, and the development of oogonia in agar plates is difficult. To overcome these facts, DNA methodologies have recently been used for its diagnosis and identification (Badenoch et al. 2001; Grooters and Gee 2002; Reis et al. 2003). Badenoch et al. (2001) were the first to use DNA sequencing to identify *P. insidiosum* hyphae from a patient with keratitis. These authors suggested that molecular tools could be important in differentiating *P. insidiosum* from the filamentous fungi. Later, Grooters and Gee (2002) developed nested primers that specifically amplified 105 base pairs from *P. insidiosum*'s genomic DNA. More recently, Reis et al. (2003) obtained several amplicons of *P. insidiosum*'s 18S small-subunit rDNA sequences from three cases of horses with systemic pythiosis. These studies indicated that DNA-based technology is ideal to identify *P. insidiosum* from fixed tissues and cultures, and could be successfully used more frequently in the future.

Serology

Serological methods for diagnosing *P. insidiosum* infections were developed in the beginning of the century (Witkamp 1925). Miller and Campbell (1982a) and Mendoza and Alfaro (1986) based their immunological studies of equine pythiosis on Witkamp's early work. They found that immunodiffusion and complement fixation tests were useful in the diagnosis of pythiosis. Moreover, a skin test was shown to be effective in determining subclinical infections in animals with active disease.

IMMUNODIFFUSION TEST

Two antigens have been used to diagnose *P. insidiosum* infections by immunodiffusion. One was prepared from the hyphae of *P. insidiosum* digested with trypsin (Miller and Campbell 1982a). This trypsin antigen detected one precipitinogen in all animals with active pythiosis. However, its shelf life was short. The other antigen was obtained from concentrated CFAs (Mendoza et al. 1986). It detected three to six precipitins in sera from horses and humans with pythiosis. One of them was the precipitinogen reported earlier in an ID test with trypsin antigens. Healthy individuals were always negative with the ID test. Chronic equine cases of pythiosis showed no bands in the ID test. These animals also did not react to skin tests and were considered anergic. The ID test detects pythiosis in its early stages (3 days), indicating that antibodies against the antigens of *P. insidiosum* are developed early in the course of infection.

No crossreactivity was recorded using the CFA of *P. insidiosum* and sera from patients with zygomycosis or other mycotic and bacterial diseases. The ID test was specific for pythiosis. Thus, the presence of a precipitin band in ID is suggestive of this disease. The number of precipitins in ID does not correlate with the severity of the disease. Horses treated and recovered from the disease showed no precipitin bands after 2 months of successful treatment. Some bands of non-identity were recorded when sera from horses with pythiosis reacted to the antigens of *B. ranarum (haptosporus)* or *C. coronatus* (Kaufman et al. 1990). The CFA proved to be

more sensitive, stable and useful, not only in diagnosing the disease in cats, cattle, dogs, horses, and humans, but also in monitoring response to treatment (Mendoza et al. 1986; Imwidthaya and Srimuang 1989; Pracharktam et al. 1991). In addition, complement fixation has been previously used (Miller and Campbell 1982a). However, this test is no longer available.

WESTERN BLOT

Culture-filtrated antigens in the ID test detected at least six precipitinogens. However, ID is too insensitive and also unable to identify major antigenic immunogens. Western blot analysis was introduced to determine which of the antigens of *P. insidiosum* were of importance during infection (Mendoza et al. 1992a). It was found that equine IgG recognized almost all of the cytoplasmic proteins of *P. insidiosum*. Several protein antigens of 28, 30, and 32 kDa) and other immunogens were found to be immunodominant (Figure 14). Negative results in Western blot were recorded using sera from healthy horses or sera from horses with various other diseases. Equine immunoglobulin G against the 32, 30, and 28 kDa and other immunodominant antigens was found to persist at least for a year in horses cured by immunotherapy. This suggests that these prominent antigens may also be important as protective immunogens in horses. It was later found that the addition of cytoplasmic antigens, containing the 28, 30, and 32 kDa immunodominant proteins, to the available *Pythium* vaccine enhanced its curative properties (Mendoza et al. 2003). This finding suggested that these proteins may play a major role in the immunotherapy of pythiosis. In equine chronic pythiosis, Western blot fails to detect the cytoplasmic antigens of *P. insidiosum*, indicating that anti-*P. insidiosum* IgG is absent from their sera. This supports the concept that horses with the chronic disease are anergic. IgG from horses with active pythiosis recognized only the 44 kDa cytoplasmic antigen of *C. coronatus*, confirming that the Western blot test is very specific in horses. This test is useful for analyzing immunoglobulin classes during infection. Recently, a single-step immunoblot (SIB) assay to diagnose pythiosis in horses was shown to be sensitive, specific, and easy to perform (Rosa 1993). Studies using human sera in Western blot are not yet available.

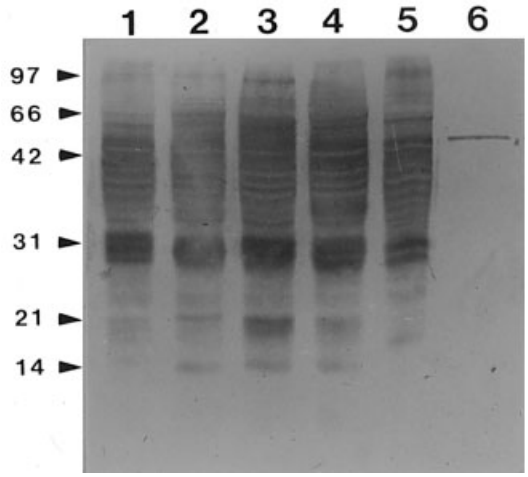

Figure 14 *Immunoblot analysis of the CFAs from different strains of* Pythium insidiosum *(lanes 1–5) and* Conidiobolus coronatus *CFAs (lane 6) after reacting with sera from horses with pythiosis. The 28, 30, and 32 kDa proteins are observed as prominent bands. A band of crossreaction was observed against* C. coronatus*'s CFA (lane 6). (Reproduced, with permission, from Mendoza et al. 1992a)*

ENZYME-LINKED IMMUNOSORBENT ASSAY

The enzyme-linked immunosorbent assay (ELISA) test was originally designed to detect anti-*P. insidiosum* IgG in proved cases of pythiosis with a negative ID (Rosa 1993; Mendoza et al. 1997). More recently, the test was shown to be helpful in cases of a dog and cat with the disease (Mendoza et al. 1997; Grooters et al. 2002). In Thailand, at least another in-house assay was developed using antigens from Thai isolates (Krajaejun et al. 2002). These ELISAs showed high sensitivity and specificity to diagnose pythiosis and were also suitable to monitor the response to treatment, confirming previous observations (Mendoza et al. 1997). In addition, some healthy horses from enzootic regions reacted positively in low titers, suggesting that subclinical infection may occur.

The ELISA developed to detect pythiosis in humans (Krajaejun et al. 2002) and animals (Mendoza et al. 1997; Grooters et al. 2002) both showed 100 percent sensitivity and specificity. These ELISAs were capable of discriminating between sera from apparently healthy individuals or heterologous infections and patients with active pythiosis.

IMMUNOHISTOCHEMICAL ASSAYS

Two tests have been developed for the immunodetection of *P. insidiosum* hyphae in tissue: an immunofluorescence test (Mendoza et al. 1987) and an immunoperoxidase assay (Brown and Roberts 1988; Triscott et al. 1993). The immunofluorescence assay was shown to be entirely specific for *P. insidiosum* in the detection of its hyphae in cat, dog, and human tissues. However, in equine pythiosis, tissue sections of kunkers containing hyphae showed a uniform fluorescence throughout the sections. This was attributed to the exoantigens released by the hyphae of *P. insidiosum* into the matrix of the kunkers. Immunofluorescence has also been used to differentiate *P. insidiosum* from other species of *Pythium*.

The immunoperoxidase assay was performed on the tissue of dogs, horses, and humans with the disease (Brown and Roberts 1988; Triscott et al. 1993; Fischer et al. 1994). *P. insidiosum* in those tissues stained positively after applying anti-IgG peroxidase. Tissue sections

from cases of zygomycosis caused by *B. ranarum* and *C. coronatus* were negative in these assays. Both assays were found to be sensitive and specific.

ANIMAL INOCULATION

All attempts experimentally to reproduce pythiosis in dogs, horses, and mice have failed (Patino-Meza 1988). Early workers, however, found that rabbits were susceptible to infection by the propagules of *P. insidiosum* (Witkamp 1924). Amemiya (1969, 1982), working with strains isolated from horses with granular dermatitis in Japan, confirmed the susceptibility of rabbits to hyphal inoculation. In his work, he found that subcutaneous inoculation of *P. insidiosum* hyphae gave rise to nodules that contained hyphae and inflammatory cells at the injection sites (Figure 15). Intravenous injection caused systemic infections in the inoculated rabbits with granulomatous necrotizing masses in the aortas, intestines, livers, and lungs.

Miller and Campbell (1983) evaluated motile zoospores as inocula in cortisone-treated and non-cortisone-treated rabbits. They found that both groups were extremely susceptible to infection by the zoospores of *P. insidiosum*. All inoculated animals developed necrotizing hepatitis or embolic nephritis, thus demonstrating that *P. insidiosum* does not require a debilitated or immunocompromised host to induce infections. In striking contrast, numerous authors have failed to reproduce the disease in horses and dogs, even after exposing them to motile zoospores by submerging the animals in tanks of seeded water which simulated the conditions encountered in swamps. The predisposing factors required to develop pythiosis in dogs, horses, and humans remain unknown. Mendoza et al. (2003), based on clinical data, however, argued that humans and animals are resistant to pythiosis. They also suggested that perhaps a defect in the immune response is what makes a host susceptible, which in part explains the low occurrence of the infection in the endemic areas.

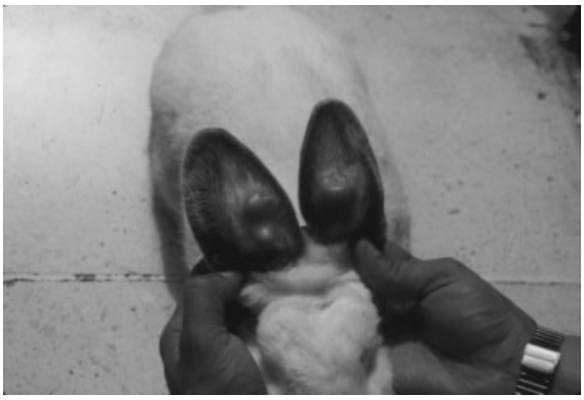

Figure 15 *Experimental pythiosis in a rabbit. Subcutaneous nodules and systemic dissemination of* Pythium insidiosum *are the main features of such experimental infections.*

TREATMENT

Treatment of infections caused by *P. insidiosum* in animals and humans is difficult. Three therapeutic methods are, however, often used for this disease: surgery, drugs, and immunotherapy.

Surgery

Radical surgery has been successfully used in cases of equine pythiosis since the last century. It consists of removal of the lesions and their kunkers, followed, in some cases, by cauterization (Habbinga 1967; McMullan et al. 1977). This method is very popular and frequently used by veterinary practitioners, although it is not always successful. In general, the response to surgery is limited. Moreover, lesions on the limbs of equines are not easily treated by this method because of their delicate anatomic structure. Surgical removal of tumor-like lesions in dogs with intestinal pythiosis has been reported, but survival beyond 3 months was extremely rare in the treated dogs (Fischer et al. 1994; Thomas and Lewis 1998). A common shortcoming of surgical treatment is recurrence as a result of incomplete removal of infected tissue.

Radical surgery has also been used in human cases with arteritis caused by *P. insidiosum*. Amputation of the extremities is a drastic procedure of the last resort, which is used to treat patients with severe *Pythium* arteritis. However, this method has proved to be only partially successful because most patients finally died of disseminated abdominal arteritis (Sathapatayavongs et al. 1989; Wanachiwanawin et al. 1993). Early detection of affected vessels using angiography is important in determining the most appropriate amputation site.

Chemotherapy

Two main groups of drugs are commonly used to treat pythiosis: iodide and amphotericin B. Iodides have been used since early in the century in equine pythiosis with contradictory results. Although some authors reported cures with intravenous injections of potassium iodide (0.75 g/45 kg in weight) (Gonzalez et al. 1979) or sodium iodide (1.0 g/15 kg in weight) (Hutchins and Johnston 1972), other investigators reported failures (de Haan and Hoogkamer 1903; Hartsfield 1971; Murray et al. 1978). Similar observations were recorded in humans. Patients with arteritis caused by *P. insidiosum* did not respond to potassium iodide, but patients with subcutaneous pythiosis seem to have responded well to this drug (Thianprasit 1990). The main drawback of iodide and amphotericin B is their toxicity (Murray et al. 1978).

Amphotericin B is the drug of choice in many mycotic infections. As a result of the lack of ergosterols in the cytoplasmic membrane of members of the genus

Pythium, however, one could predict that amphotericin B would be ineffective in infections caused by *P. insidiosum*. Nevertheless, in equine pythiosis, amphotericin B has been used with some success. Eight cases of equine pythiosis were cured out of a total of 10 horses (McMullan et al. 1977) after intravenous injection of amphotericin B (0.38–1.47 mg/kg). Topical application of this drug had little or no effect on equine cases (Miller et al. 1983). The use of amphotericin B has been limited partly as a result of the cost of therapy, the poor rate of success, and its toxic side effects. Amphotericin B in humans with pythiosis gave contradictory results as well. For example, intravenous injections of amphotericin B (0.1 mg/kg) were ineffective in cases with arteritis (Wanachiwanawin et al. 1993) and in one case of subcutaneous infection (620 mg/kg, over a 6-week period) (Rinaldi et al. 1989). It was effective in two subcutaneous pythiosis cases when it was used in combination with 5-fluorocytosine (amphotericin B 0.5 mg/kg/day, 5-fluorocytosine 150 mg/kg/day) (Triscott et al. 1993). A combination of itraconazole and terbinafine saved the life of a boy in Tennessee with subcutaneous pythiosis (Shenep et al. 1998). However, this combination has been also used in dogs and other animals with contradictory results.

Immunotherapy

Immunization of equines with products derived from *P. insidiosum* cultures was reported to have curative properties in Australia (Miller 1981) and Costa Rica (Mendoza and Alfaro 1986). Two antigens, referred to in the literature as Miller's and Mendoza's vaccines (Newton and Ross 1993), have been used for immunization. The Australian vaccine was prepared from sonicated hyphal antigens, whereas the Costa Rican vaccine used precipitated proteins from CFAs. Immunotherapy of horses with pythiosis using Miller's vaccine alone gave a success rate of 53 percent. An increase in the percentage of cured cases was obtained when immunization was followed by surgical removal of cutaneous lesions. Similar results were obtained using Mendoza's vaccine (Figure 16). Nevertheless, significant reduction in the swelling, caused by the vaccine at the site of injection, an undesirable side effect of Miller's vaccine, was the main feature of Mendoza's vaccine. Moreover, the vaccine described by Miller was unstable, losing its curative properties after storage at 4°C. By contrast, Mendoza's vaccine was found to be effective even 18 months after its preparation.

Recently Mendoza et al. (2003) introduced new immunotherapeutic immunogens to treat pythiosis in both humans and animals. This new formulation contains exo- and endoproteins extracted from cultures of *P. insidiosum* and was able to cure chronic and acute cases of the disease (Thitithanyanont et al. 1998; Hensel et al. 2003; Mendoza et al. 2003). These studies suggest

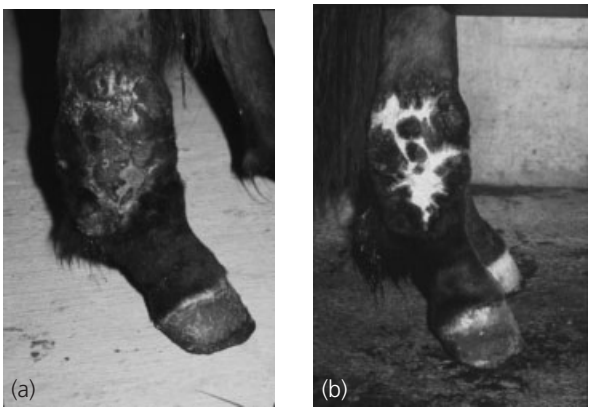

Figure 16 (a) *A horse with pythiosis before immunotherapy;* **(b)** *the same horse after successful immunotherapy. (Reproduced, with permission, from Mendoza and Alfaro 1986)*

that hyphal antigens may contain products that are involved in the enhancement of the immunological response to immunization. Horses with the disease that failed to respond to immunization did not develop swellings at the injection sites. All in all, this finding suggests that the response to immunization is directly related not only to the immunostatus of the infected hosts, but also to the type of immunogens used.

Based on histopathological data, the hyphae of this oomycete are always sequestered within kunkers. Thus, it has been postulated that perhaps some antigens are downregulated during infection (Mendoza et al. 2003). Reports on the histological changes that take place after immunotherapy had shown that the initial eosinophilic inflammatory reaction, typical of equine pythiosis, changed to a mononuclear response composed mainly of macrophages and T lymphocytes (perhaps cytotoxic lymphocytes) (Miller 1983; Mendoza and Alfaro 1986). The antigens, used during immunotherapy, apparently trigger a Th1 immune response with macrophages and cytotoxic lymphocytes that eventually clear the organism from tissues. This contention is supported by the fact that cultures of biopsied tissue in immunized horses are always negative for hyphae. This confirms, in part, the observation that the digestion of the kunkers by the mononuclear inflammatory cells seems to destroy the hyphae of *P. insidiosum* (Miller 1981; Mendoza et al. 1992b). Complications associated with immunotherapy are mainly an inflammatory reaction at the injection site, secondary bacterial contamination, and lameness in lesions located on limbs. It was reported that some horses cured by immunization relapsed one year later. This suggests that, if the vaccine has prophylactic properties, it is of short duration. The prophylactic and curative properties of *P. insidiosum* immunogens have been recently evaluated by Santurio and Leal (2003) in an animal model. These investigators confirmed that the antigens derived from this oomycete have, indeed, curative and prophylactic properties.

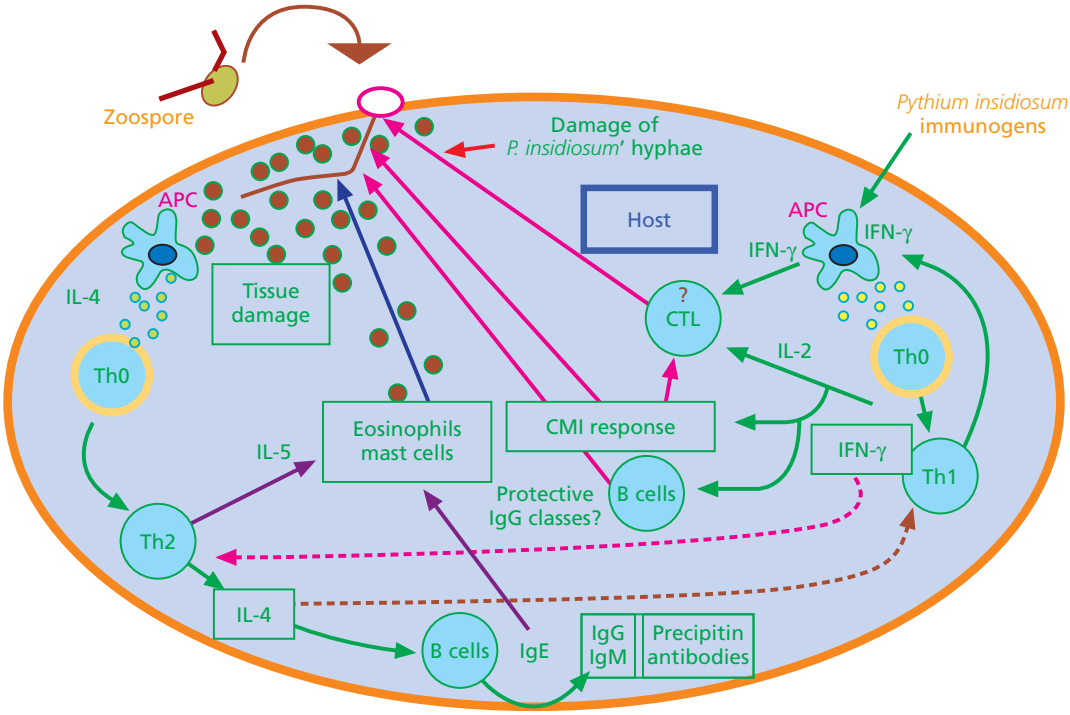

Figure 17 *Proposed working hypothesis of the pathogenic mechanisms involved during natural infection and those triggered by immunotherapy. In this model, pythiosis begins when the host contacted a zoospore (or other propagules of the pathogen) through a wound.* Pythium insidosum *will develop hyphae-like filaments that penetrate the host. Once in the tissues, it will release antigens that will lock the immune system into a Th2 response and which contain typical cellular and other mediators including IL-4 that could downregulate the Th1 response (brown dotted arrow) and cause pythiosi (blue arrow) (left side of the figure). By contrast, after immunotherapy, IFN-γ is immediately triggered, which in turn activate a Th1 immunity and downregulates the Th2 response (pink dotted arrow). The mononuclear response (putative natural killers and cytotoxic lymphocytes, cell-mediated immunity) is believed to be responsible for the killing of the pathogen in the infected tissues (red arrows). APC, antigen presenting cell. (Reproduced, with permission, from Mendoza et al. 2003)*

The mechanism involved in the response of horses to natural infection and to immunotherapy using *P. insidiosum* products has been recently addressed by Mendoza et al. (2003). In their model, *P. insidiosum*, after contact with the host, will release exoantigens that will lock the immune system into a Th2 response (eosinophils, mast cells, IgE, IL-4, IL-5, precipitin, IgG, and IgM). They propose that the degranulation of the eosinophils and mast cells is responsible for the tissue damage observed during natural infection (Figure 17). By contrast, after injection with the immunotherapeutic antigens of *P. insidiosum*, the host will trigger a Th1 response with interferon-gamma (IFN-γ) that eventually downregulates the Th2 response, and a prominent mononuclear response. These investigators speculated that the events triggered by the immunotherapeutic antigens ultimately kill the pathogen in the infected tissues, and therefore are more likely responsible for the cure of the infected hosts (Figure 17).

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Lobomycosis

SINÉSIO TALHARI AND ROGER PRADINAUD

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This disease was first described in 1931 by Lobo in Recife, Brazil as blastomycosis queloidiforme, following observations in a 52-year-old Amazonian man who had multinodular keloid-like lesions in the lumbar region. Further cases have been identified in Brazil and other countries in the Amazon region, and Central America. Later sporadic and isolated cases have been described in Europe and North America.

The only case described in Europe (France) was of a 24-year-old aquarium employee who presented with Lobo's disease on the dorsum of his hand 3 months after contact with an infected dolphin (Symmers 1983). A single report from the USA was of a patient who probably contracted the infection in Venezuela (Burns et al. 2000). The total number of registered patients reached 464 by December 2002 (Baruzzi et al. 1979; Silva and Brito 1994; Pradinaud and Talhari 1996; Opromolla et al. 1999; Saint-Blancard et al. 2000; Fischer et al. 2002).

The etiologic agent responsible for Lobo's initial case was classified as *Glenosporella loboi* by Fonseca Filho and Area Leão in 1940. Other names that have been proposed include *Glenosporopsis amazonica*, *Paracoccidioides loboi*, *Blastomyces loboi*, *Loboa loboi*, and *Lacazia loboi*.

Lobomycosis has been described in dolphins: *Tursiops truncatus* (bottle-nosed dolphin) in Florida (Migaki et al. 1971; Symmers 1983) and *Sotalia guianensis* in the Surinam river. The disease identification in dolphins has widened the geographical distribution since seven cases have now been reported in Florida and single cases in the Gulf of Mexico, Surinam, and the Gulf of Gascony, off the coast of France.

The human disease affects the tropical zone of the New World and Table 23.1 illustrates the distribution.

There is a clear gender predilection as 90 percent of cases have been reported in men. This is most likely to be related to occupational exposure. However, in the Amazonian Caiabi Indian population, the prevalence in women reaches 32 percent, probably as a consequence of their role in agricultural and forest activities (Baruzzi et al. 1979).

The age range of patients at the time of diagnosis is between 40 and 70 years. Their case histories, however, suggest that clinical manifestations began many years before. Some cases have also been reported in children: a 5-year-old whose lesions evolved from the age of one; there is also a report of disease in a 2-year-old Caiabi Indian.

ETHNIC BACKGROUND

There is no ethnic predominance as all races appear to be equally susceptible. However, lobomycosis is very well documented in the Mato Grosso (Brazil) Caiabi Indians where it was first recognized in 1915. By 1979, 60 cases had been reported in this particular tribe, representing 31 percent of all Brazilian cases (Baruzzi et al. 1979). It has therefore been suggested that this tribe might be genetically predisposed. However, the fact that no new cases have been identified after movement of these tribes to a new habitat in Xingu National

Table 23.1 *Geographical distribution of Lobo's disease*

Country	No. of cases
Brazil	296
Colombia	50
Surinam	34
Costa Rica	21
Venezuela	20
French Guiana	17
Panama	13
Peru	3
Bolivia	3
Ecuador	2
Guyana	2
Mexico	1
Europe: France/Gulf of Gascony	1
USA	1
Total	464

Park points to an environmental or exposure-related etiology.

OCCUPATIONAL EXPOSURE AND INOCULATION

Farmers exposed to harsh conditions and aquatic environments represent most of the reported cases. Other high-risk activities include gold mining, fishing, and hunting.

Patients often report a bite or sting from an arthropod, snake or sting-ray, or otherwise trauma from a cutting instrument. Probably, the fungus is an aquatic saprophyte, which would explain disease in aquatic mammals such as the dolphin. There are no reports of person-to-person spread, even with intimate contact. However, there are reports of voluntary inoculation (Borelli 1968) and accidental surgical inoculation of a patient (Azulay et al. 1975).

CLINICAL FEATURES

Lobomycosis is characterized by variably sized dermal nodules, either lenticular or in plaques, which can attain the size of a small keloid-like cauliflower. They can be either hyper- or hypopigmented, and occasionally achromic. A number of different types of lesions may be seen at various stages of disease progression (Figures 1 and 2). Infiltrative lesions are often a feature of early disease and can simulate tuberculoid leprosy or burn scars, especially in the Caiabi Indian population (Figure 3). Verruciform lesions represent advancing pathology and occur most commonly on the lower extremities and, like other exotic mycoses, can give the 'mousy-foot' appearance. Ulcerative lesions may also represent advanced pathology and on healing result in sclerotic or atrophic scars. Most of the ulcerated lesions are caused by trauma. A few cases of dermohypodermic lesions could be referred to as having a gumma-like appearance, but the characteristic softening is absent and therefore these lesions are more typical of nodules. True gummatous lesions are rarely observed. Patients, however, usually present with lesions of various types.

Figure 1 *Isolated and confluent keloid-like lesions*

The disease generally does not affect the epidermis and therefore skin lesions usually have a smooth and shiny appearance, especially in the facial region. Fine telangiectasia and sometimes 'black dots,' a feature of chromoblastomycosis and other vegetating dermatoses, can be observed, the 'black dots' corresponding to transepidemic elimination of necrotic fungi. Some lesions, especially on the lower extremities, can be keratotic and vegetating. An extreme case was that of a patient whose skin resembled a rock covered with seashells. On the sole of his foot, lesions appeared crater-like.

Any area of the body is potentially susceptible as infection commonly follows trauma. However, the most commonly affected areas include the lower extremities (31 percent), the ears (26 percent), and the upper extremities (20 percent), and the disease is usually unilateral (Figures 4 and 5).

Figure 2 *Isolated and confluent papules and nodules*

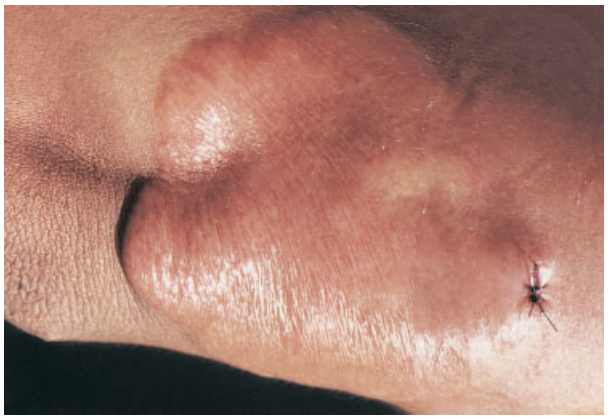

Figure 3 *Infiltrative plaque*

Disease varies in extent and local or distant spread of lesions can occur. Local spread probably occurs in part by autoinoculation, producing small satellite nodules at the periphery of the mother lesion. The disease can invade lymph nodes proximal to the affected area but reports, however, vary as to how commonly this occurs, ranging from less than 10 percent (Azulay et al. 1975) to more than 25 percent (Wiersema 1971). Distant skin metastases occur via transdermal and lymphohematological routes. Cutaneous dissemination can result in confluent lesions and plaques (Figures 6 and 7), with some patients developing hundreds of lesions. There has been a single report of visceral pathology in a native Costa Rican whose lesions evolved over a period of 47 years. He presented with left lower extremity lymphangitis secondary to left testicular tumoral spread, which necessitated orchidectomy. Histology revealed a multicellular granuloma actively phagocytosing the cells of the fungal pathogen and sparing the scrotum.

Figure 4 *Classic infiltrative nodules of the auricle, on one side only, which are indolent and have evolved over many years.*

Figure 5 *Infiltrative lesions of a woman's face. This is a very unusual location of the disease. (Courtesy of Arival C. Brito, Belém*

The clinical presentation in dolphins is similar to that in humans. They also develop keloidal nodules, either confluent or in plaques, as well as verrucous and vegetating lesions.

EVOLUTION AND PROGNOSIS

Disease onset is usually insidious and therefore difficult to document. The increase in either size or number of lesions is a slow and progressive process, which can continue for up to 40–60 years. The presenting complaint is usually esthetic or functional disability. The disease progresses in a benign fashion and patients usually die of intercurrent illnesses. At present, however, it appears incurable as new lesions usually continue to appear despite treatment either in proximity to, or distant from, the initial lesion. Interestingly, spontaneous regression in three Caiabi Indians has been reported.

Figure 6 *Numerous papular and tumor lesions on the arm and forearm.*

Figure 7 *Numerous papules, plaques and tumoral lesions on the foot, leg and thigh.*

DIAGNOSIS

The clinical diagnosis is apparent to physicians who have seen previous cases of lobomycosis and the patient is known to reside in an endemic area. The differential diagnosis includes mainly leprosy, anergic cutaneous leishmaniasis, chromoblastomycosis, paracoccidioidomycosis, and Kaposi's sarcoma.

Diagnosis may also be established by a skin smear or biopsy.

Skin smear

A skin smear permits direct visualization of the parasite, which is abundant in lesions. It is quick and easy and entails scraping a tumoral lesion with a scalpel blade in order to obtain dermal tissue for microscopy. Lobomyces are yeast-like rounded thick-walled cells which range from 5 to 15 μm in diameter and occur in chains of two to ten cells (Figure 8). Granules (five to eight) are present in the center and there is usually one that is larger than the others. They appear mobile due to Brownian motion, especially in a fresh specimen.

In old lesions, one can observe the presence of a single large inert granule. The cells are united by little cell bridges. In most cases, each cell's cytoplasm is clearly demarcated, although, at the end of the chain, one can occasionally see a budding cell, with continuous cytoplasm between the mother and daughter cell.

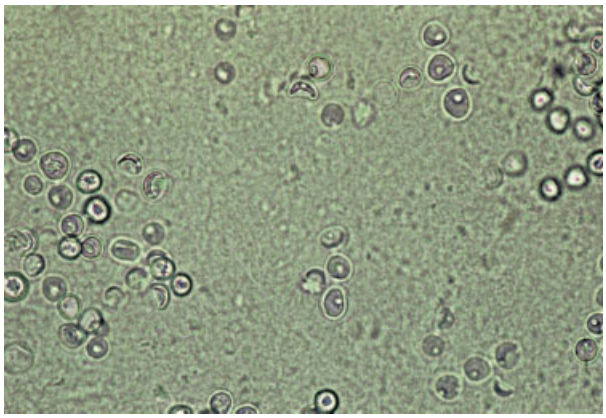

Figure 8 *Numerous cells of* P. loboi *in a smear of dermal scrapings: the diagnosis can be confirmed in the office within a few seconds. Multiple buds and chains are connected by tubes; five to eight mobile protoplasmic granules are seen in active fungus.*

Histopathology

Histopathology is pathognomonic. The epidermis is usually atrophic, although it can also be hyperplastic, vegetating, and keratotic. Lesions in dolphins are identical to those in human skin (Destombes and Ravisse 1964; Symmers 1978; Arrese et al. 1988; Sesso and Baruzzi 1988; Sesso et al. 1988).

In the dermis a thin band of subepithelial collagen, analogous to lepromatous leprosy's 'Unna band' is usually intact. The remainder of the dermis is occupied by a progressively fibrous, diffuse, inflammatory granuloma. The granuloma is composed of histiocytes and numerous giant cells, which are often clustered in small groups separated by interstitial tissue. The histiocytes, and more often the multinucleated giant cells, can be seen to have numerous phagocytosed thick-walled cells clearly distinguishable by periodic acid-Schiff (PAS), or Gomori's/Grocott's, or Gridley's silver stains (Figures 9 and 10). Although the cells can sometimes appear empty, they often contain vacuolized inclusions that are birefringent in polarized light. Budding can be observed but hyphae are always absent. These yeast-like cells, which abound in the cytoplasm of giant cells, occasionally appear free floating in the stroma of the granuloma. It is important to note that necrosis and suppuration are never present in the granuloma (unless there is nearby epidermal damage). Histochemical stains reveal polysaccharides in the inflammatory granuloma,

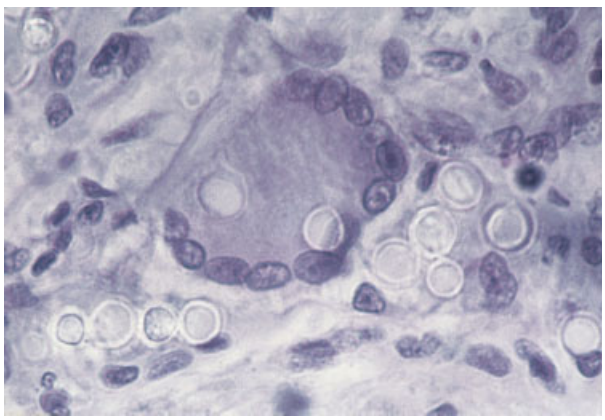

Figure 9 *Pathology: foreign body giant cell and polymorphous infiltrate with histiocytes. Parasites are abundant throughout the whole granuloma. Hematoxylin and eosin stained section.*

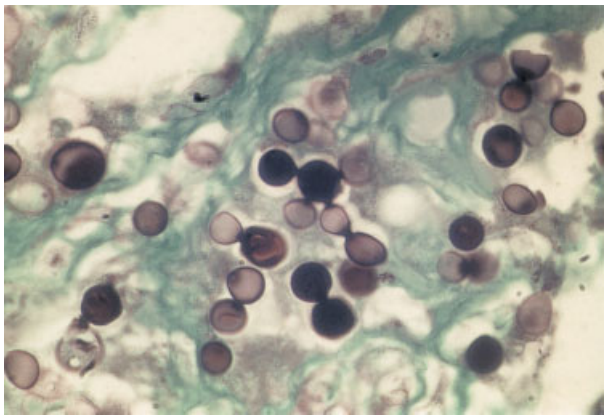

Figure 10 *Gomori's–Grocott's stain on the same histological section as seen in Figure 9. (Courtesy of A.R. Gradelha)*

which are absent in the deeper areas, especially in the hypodermis. The latter is usually intact.

Disease spread to the lymph nodes produces a similar inflammatory infiltrate with histiocytes and multinucleated giant cells. The cytoplasm of these cells is often xathomatized and contains the same abundance of yeast cells as in the dermis.

Electron microscopic studies demonstrate small spines 1 μm in size on the external glycoprotein membrane which itself measures approximately 0.8 μm. The fungus is multinucleated and central vacuolized inclusion bodies have been identified as triglyceride and polysaccharide glycogenic particles (Furtado et al. 1967; Grimaud et al. 1974).

Culture

There have been many attempts to culture *L. loboi*, but all have been unsuccessful. Sampaio (1974) obtained encouraging results by spreading the cells of *L. loboi* in a 199 TC, containing 5 percent bovine fetal serum penicillin 100 units/ml, amphotericin B μg/2.5 ml, and 2.5 percent phytohemagglutinin at 37°C. The inoculum, which had been taken from the lesion of a patient, was preserved for 5 months in 4°C distilled water.

Animal inoculation has been used with varying success. Armadillo (*Euphractus sexcintus*) inoculation has been more successful and resulted in nodular lesions histologically identical to human pathology (Sampaio and Dias 1977). L. Ajello (unpublished data) has also successfully inoculated the footpads of mice at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA.

IMMUNOLOGY

Patients with Lobo's disease have normal humoral immunity but a partial cellular immune deficiency reflected in absent delayed hypersensitivity responses to dinitrochlorobenzene, streptococcus, staphylococcus, trichophyton and candida antigens (Pecher and Fuchs 1988). In addition, some form of secondary immunodeficiency has been suggested in a dolphin with lobomycosis but to date, lobomycosis in conjunction with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS) has not been reported in humans.

Antigens modified for intradermal use can be used to evaluate the potential of asymptomatic infection due to *L. loboi*. However, these have also given positive results with paracoccidioidomycosis, mycetomas caused by *Nocardia brasiliensis* and in a single case of eczema. This may be due to antigenic relationships between *L. loboi* and other mycological agents which have been demonstrated for *Histoplasma capsulatum* var. *capsultatum* and var. *duboisii*, *Blastomyces dermatitidis*, *Candida albicans*, and the mycelial form of *Coccidioides immitis* (Silva 1978).

When Jorge Lobo described the first case of lobomycosis in 1931, he suggested that the etiologic agent is a fungus similar to *Paracoccidioides brasiliensis*. Taborda et al. (1999) have shown that it is indeed close to *P. brasiliensis* and have designated it *Lacazia loboi*. They suggest that *L. Loboi* is a sister taxon of *P. brasiliensis* as demonstrated on the 18S rDNA phylogenetic tree. However, paracoccidioidin skin tests are negative in cases of lobomycosis.

TREATMENT

The optimal treatment for localized lesions is wide surgical excision ensuring that margins are free of infection to avoid recurrence. It is worth noting that instruments contaminated during an operation can lead to reinfection. Furthermore, in black patients surgical excision carries the risk of inducing true keloids.

The only treatment of choice for disseminated infections is chemotherapy. Clofazamine at doses of 100 and 200 mg daily has been tried in several studies (Silva 1978; Azulay 1995, personal communication; Talhari et al. 1988) as it has shown efficacy with a variety of fungi and actinomycetes (especially the *Nocardia* spp.) and has anti-inflammatory properties in processes involving foreign body granulomas. Of the 16 treated cases in the various studies, four demonstrated 30–80 percent improvement following 2–8 months of therapy. However, compliance to drug therapy, especially when required for prolonged periods, is low as the disease appears to be well-tolerated by patients. However, Niemel (1990, personal communication) reported lack of clinical efficacy of clofazamine in a patient on long-term therapy for *Mycobacterium leprae* who had coincidental *L. loboi* infection. Ketoconazole therapy for lobomycosis has proved to be disappointing.

Of interest is that a dolphin with lobomycosis has been successfully treated with miconazole (Dudok Van Heel 1977). There has been a recent report of successful

treatment of a localized plaque lesion on the face with a combination of both clofazamine 100 mg and itraconazole 100 mg daily for a one year period (Fischer et al. 2002). The patient remained disease-free both clinically and histopathogically after a follow-up period of 4 years. Therefore, dual therapy with clofazamine and itraconazole may be a promising treatment for plaque lesions only.

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Rhinosporidiosis

SARATH N. ARSECULERATNE AND LEONEL MENDOZA

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THE ETIOLOGIC AGENT

'There is a certain air of romance attached to the name ''Rhinosporidium''' (Thomas et al. 1956), arising from many synonyms given to its picturesque developmental stages and from its enigmatic nature.

Rhinosporidiosis, the disease that *Rhinosporidium seeberi* causes in humans and animals, presents, as the name implies, with chronic, granulomatous polyps, predominantly in nasal and other respiratory sites. It was first observed by Malbran in Buenos Aires in 1892. Seeber's thesis (1900) first reported the disease, in Argentina. Comprehensive descriptions of the organism (Minchin and Fantham 1905; Beattie 1906; Tirumurti 1914; Ashworth 1923; Karunaratne 1964) and clinical aspects of rhinosporidiosis have been made (Allen and Dave 1936; Karunaratne 1964). While important advances have been made since this chapter was first written in 1998, unresolved enigmas yet remain. Since the organism has yet to be cultured in vitro, the classical Koch's postulates are as yet unfulfilled. Recently, however, using molecular tools, Herr et al. (1999b) found that *R. seeberi* was not a fungus but an unusual protist that shared phylogenetic features with microbes that cause infections in fish.

NOMENCLATURE

The diverse names and descriptions of *R. seeberi* on which tentative classifications were based, were chronologically reviewed by Ashworth (1923) and de Mello (1954) (Table 1). The nomenclature of the developmental stages and their contents is given in the section on Light microscopical and ultrastructural features below.

TAXONOMY

The taxonomy of *R. seeberi* (Ashworth 1923; de Mello 1954; Karunaratne 1964) had been, until recently, controversial. Confusion had arisen undoubtedly from the absence of pure isolates of the organism due to its intractability to culture. *R. seeberi* was initially classified as a sporozoon, then as a phycomycete, and when the doubts deepened, it was placed in an orphanage of anomalous fungal and fungal-like organisms, and even as a cyanobacterium. With the advent of phylogenetic analyses, its dignity was restored, by grouping it with *Dermocystidium*, the rosette agent, *Ichthyophonus*, and *Psorospermium* in a new Class – the Mesomycetozoea, formerly Ichthyosporea (Mendoza et al. 2002), a name which signifies the location of this group at the point when the animals and fungi diverged some 2.5 billion years ago.

Table 1 *Nomenclatural history of* R. seeberi *(Wernicke 1903) Seeber 1912*

Year	Event
1892	Malbran observed the organism in a nasal polyp, and regarded it as a sporozoon, but did not publish his findings
1900	Seeber's (1900) thesis described the organism (cysts, spores containing 'sporozoites') in a nasal polyp; he regarded it as a sporozoon allied to the Polysporea of the Coccidia, but did not name the organism
1900	Wernicke (see de Mello 1954) named the organism *Rhinosporidium seeberi*
1903	O'Kinealy (1903) described a nasal polyp and Vaughan described its histology (cyst with a pore, filled with sporules)
1903	Belou (see Ashworth 1923; de Mello 1954) described the organism under the name *Coccidium seeberia* Wernicke 1900
1904	Minchin and Fantham (1905) studied O'Kinealy's (1903) rhinosporidial tissue and named the organism *Rhinosporidium kinealyi*
1912	Seeber (see Ashworth 1923; de Mello 1954) endorsd the generic name *Rhinosporidium* of Minchin and Fantham, and the specific name *seeberi* of Wernicke, and established the identity of *R. kinealyi* with *R. seeberi*
1913	Zschokke (1913) recorded a similar organism in a nasal growth in a horse; he named it *Rhinosporidium equi*
1923	Ashworth (1923) renamed the species *R. seeberi* instead of Belou's *R. seeberia* and considered *R. kinealyi* as identical with *Coccidium seeberia*. He finally validated the designation '*Rhinosporidium seeberi* (Wernicke 1903), emend. Ashworth,' which is now the valid name of the organism. Ashworth also concluded that 'all examples of *Rhinosporidium* recorded from man appear to be referable to one species'
1936	Ciferri et al. (1936) established the identity of *R. seeberi* and *R. equi*, a view endorsed by Ashworth (1923)
1940	Carini (1940) described cysts with spores in skin nodules in frogs (*Hyla rubra*) and created a new genus *Dermosporidium* for the organism which he regarded as having close affinities with, but different from, *R. seeberi*. The relationship of *R. seeberi* to Carini's *Dermosporidium* remained obscure until 1999 (see Herr et al. 1999a)
1958	Vanbreuseghem (1958) included two synonyms: *Rhinosporidium ayyari* (see Allen and Dave 1936) and *Rhinosporidium amazonicum* Aben-Athar 1944
1999	The new classification of *Rhinosporidium seeberi* and its affinities were proposed by Herr et al. (1999b)

Previous attempts to classify *R. seeberi* (see Ashworth 1923) are of interest in the larger context of taxonomy, especially in the use of morphology as a criterion for its taxonomic classification. For instance:

- Minchin and Fantham 1905 – affinities with Neosporidia and on the structure of its simpler 'spore', with the Haplosporidia
- Beattie 1906 – Neosporidia, allied to the Sarcosporidia based on its striated capsule, a framework separating the 'spores', and polar development of the 'spores'
- Ridewood and Fantham 1907 – Order Haplosporidia, Sub-order Polysporulea, based on the numerous 'spores'
- Laveran and Pettit 1910 noted the resemblance of the trout pathogen *Ichthyosporidium*, regarded as a haplosporidian, to *Rhinosporidium*. The organism described by Laveran and Pettit was regarded as a phycomycete, allied to the Chytridineae, by Plehn and Mulsow (1911). The affinity of *R. seeberi* to the Chytridineae was in doubt, because the Chytridineae have flagellated stages
- Ashworth (1923) related *R. seeberi* to the phycomycetes 'as the thallus is formed of a single cell,' and to the Chytridineae on account of the absence of a mycelium and because 'sexual organs are not generally formed so that the spores are usually asexual;' the absence of such flagellation in *R. seeberi* was noted, while from the spores of most Chytridineae, a flagellated stage emerges. He further placed it provisionally in the sub-order Olpidiaceae in which the trophic stage is spherical or elliptical and because 'each such cell becomes transformed into one sporangium only' as in *R. seeberi*
- Vanbreuseghem (1973) failed to identify *R. seeberi* as an alga
- Herr et al. (1999b), through phylogenetic analysis of the organism's 18S SSU rDNA, classified *R. seeberi* in a new clade which they named the Mesomycetozoea. This new group includes fish and amphibian pathogens in the former DRIP clade (*Dermocystidium*, the rosette agent, *Ichthyophonus*, and *Psorospermium*). Cavalier-Smith (1998 quoted by Mendoza et al. 2001a) included the mesomycetozoeans in a class Ichthyosporae but based on the

fact that 'not all members of this group are fish pathogens and that preliminary analysis showed that *R. seeberi* may have chitin synthase genes, render this epithet inappropriate,' Mendoza et al. (2002) amended the term to Mesomycetozoea. The finding of Herr et al. (1999b) on the phylogenetic placement of *R. seeberi* was recently confirmed by Fredricks et al. (2000).

The current classification of *R. seeberi* according to Mendoza et al. (2001a), modifying the proposal of Cavalier-Smith (1998), is as follows (Figure 1):

Kingdom – Protozoa
Sub-kingdom – Neozoa
Infra-kingdom – Neomonada
Phylum – Neomonada
Sub-phylum – Mesomycetozoa (formerly Ichthyosporea)
Class – Mesomycetozoea
Order – Dermocystida
Family – Rhinosporideaceae (Mendoza et al. 2002)
Dermocystidium spp.
Rhinosporidium seeberi
Rosette agent
Order – Ichthyophonida
Family – Ichthyophonae (Mendoza et al. 2002)
Ichthyophonus
Psorospermium

The taxonomy of *R. seeberi* has finally been resolved (Herr et al. 1999b; Fredricks et al. 2000). These phylogenetic analyses showed that *R. seeberi* is not a fungus but a unique protist that shares morphological and phylogenetic features with several previously orphaned aquatic microbes, located at the divergence between animals and fungi. More information about the mesomycetozoeans could be found in a recent review of this class by Mendoza et al. (2002). In that review, two Orders were recognized – Dermocystida (to which

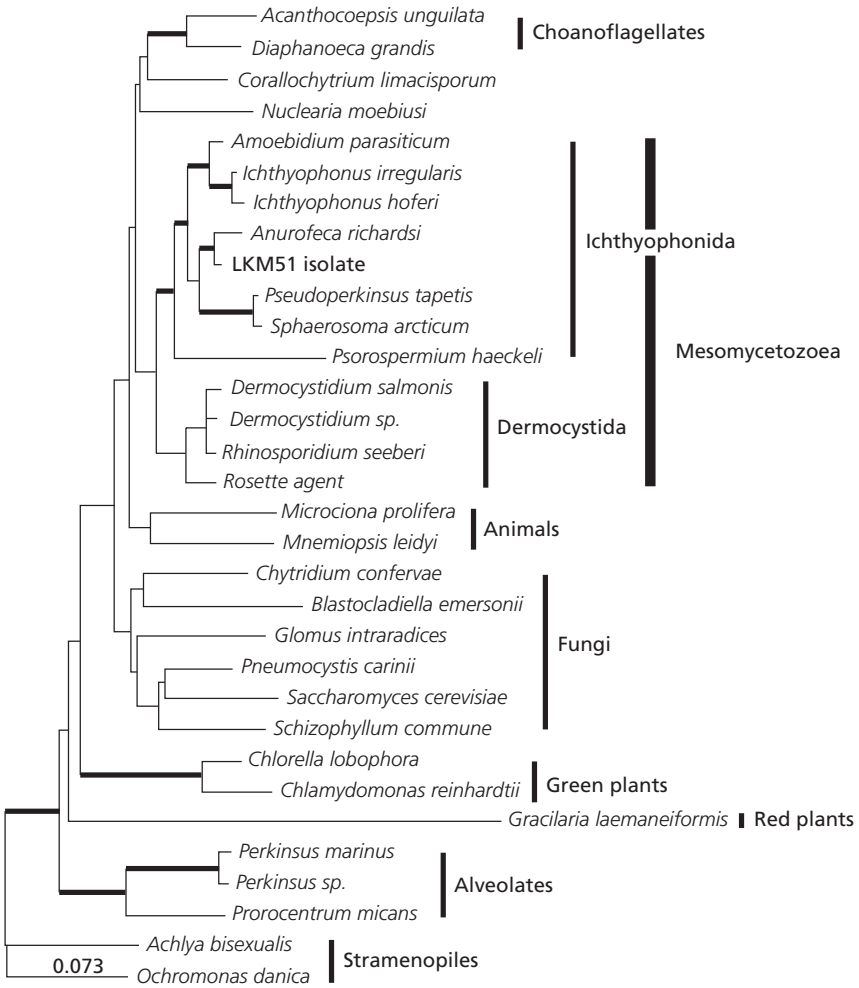

Figure 1 *Phylogenetic analysis of* R. seeberi *and other microbes using the 18S SSU rDNA molecule and distances estimated by maximum likelihood. The thickened branches are supported 90 percent of 1000 bootstrapped. The scale for percentage nucleotide substitution is given on the branch to* Ochromonas danica. *In the phylogenetic tree,* R. seeberi *is the sister clade to* Dermocystidium *spp. and closely related to the Rosette Agent in the Order* Dermocystida *(Mendoza et al. 2002).*

R. seeberi belongs) and the Ichthyophonida, with the commonality of several features that *R. seeberi* shares with the other mesomycetozoeans.

BIOLOGY

The absence of a method for the in vitro propagation of *R. seeberi* undoubtedly accounts for the paucity of data on the biology of *R. seeberi*. Vanbreuseghem (1973) commented that African rhinosporidial endospores are smaller than Indian ones, although the organism 'looks similar in sections of very different origins.' We have no evidence (Arseculeratne et al. 2001) that endospores from rhinosporidial tissues from different patients in Sri Lanka show differences in size.

Karunaratne (1964) postulated, from epidemiologic data, that a saprobic form might exist in soil or water; Gori and Scasso (1994) referred to a yeast form in tissues. There is no evidence for an alternate life cycle in any intermediate host.

Random amplified polymorphic DNA (RAPD) analysis of DNA from six strains of *R. seeberi* from patients with differing clinical presentations showed a heterogeneity in strains of *R. seeberi*, with the six strains falling into three distinct groups on banding patterns (Appuhamy et al. 2002); there was, however, no correlation of the patterns with the clinical picture of the disease. Variations, especially in virulence properties, if they exist, might be relevant to the wide spectrum of clinical features, as well as to the variations seen in histopathology of rhinosporidial granulomata (see Arseculeratne et al. 2001).

Ecology

It is generally held, from cogent epidemiological evidence, that the main natural habitat of *R. seeberi* is ground water in ponds and lakes, or in soil that is contaminated with such water. This evidence includes:

- a history given by the majority of Asian patients of exposure to these sources
- the predominant occurrence of lesions in nasal and ocular sites
- the reports of Noronha (1933) and of Mandlik (1937) that rhinosporidiosis of the nose was closely associated with the occupation of sand-gathering from river beds
- the outbreaks of rhinosporidiosis in humans who had bathed in the same lake (Vukovic et al. 1995), and in swans inhabiting the same lake (Kennedy et al. 1995)
- the higher prevalence in human males, through occupational exposure to soils and soil dust
- a history of trauma from grass or wood splinters (Karunaratne 1964) sometimes related to agricultural occupations
- the occurrence of rhinosporidiosis in animals with an aquatic habitat, such as river dolphin, geese, swans and ducks
- the frequency of anterior urethral rhinosporidiosis in Muslim males (Ingram 1910; Dhayagude 1941; Gahukamble et al. 1982), which was attributed to infection conveyed by pieces of stone or brick, probably contaminated by soil-borne *R. seeberi*, applied to absorb the last drops of urine.

Many reports on microscopic examination of deposits of stagnant water, silt, manure, and water plants in endemic areas have not yielded confirmation of the hypothesis of the aquatic habitat of *R. seeberi*. Histological studies on samples from aquatic animals such as fish, snails, crabs, and frogs have also yielded negative results. Amphibia exposed to natural waters did not develop rhinosporidiosis (Reddy and Lakshminarayana 1962); deposits from ground waters in which rhinosporidial patients had bathed failed to induce rhinosporidiosis after subcutaneous injection in the following amphibia – *Kaloula taprobanica*, *Tamanella obscura*, *Bufo melanostictus*, *Polypidatus cruciger*, *Rana temporaria* (Arseculeratne 1999, unpublished data).

Deposits of water from a reservoir in Sri Lanka in which many rhinosporidial paients had bathed, however, showed bodies on periodic acid–Schiff (PAS)-stained smears, that were compatible in size and shape with rhinosporidial endospores, with the presence within these bodies of structures that were also compatible with the electron-dense bodies (EDBs), Figure 2). Indirect immunofluorescence tests, however, with anti-rhinosporidial antibody, gave extensive nonspecific labeling of amorphous, possibly mineral deposits in the water, which made identification of specifically-labeled rhinosporidial bodies difficult. Polymerase chain reaction (PCR) studies on these deposits are in progress in our laboratory.

It should be added that microscopic methods which rely on the morphology of *R. seeberi* as observed in histopathological sections have assumed that the morphology of the tissue stages is similar to that of the saprobic pathogen. There is no evidence, on the other hand, that *R. seeberi* is a dimorphic organism, as some classical fungi are.

Cultural methods applied to material from aquatic habitats have also uniformly proved to have been unsuccessful, undoubtedly due to the uncultivability of the pathogen in vitro.

A reservoir of *R. seeberi* in animals such as horses and cattle, from which transmission to humans is thought to occur, has been postulated (Weller and Riker 1930; Rao 1938), although it is probable that soil or ground water is the common source of infection for these animals, and for humans who work with them.

Reports that the 'causative organism of rhinosporidiosis, viz. *Microcystis aeruginosa*' was isolated from

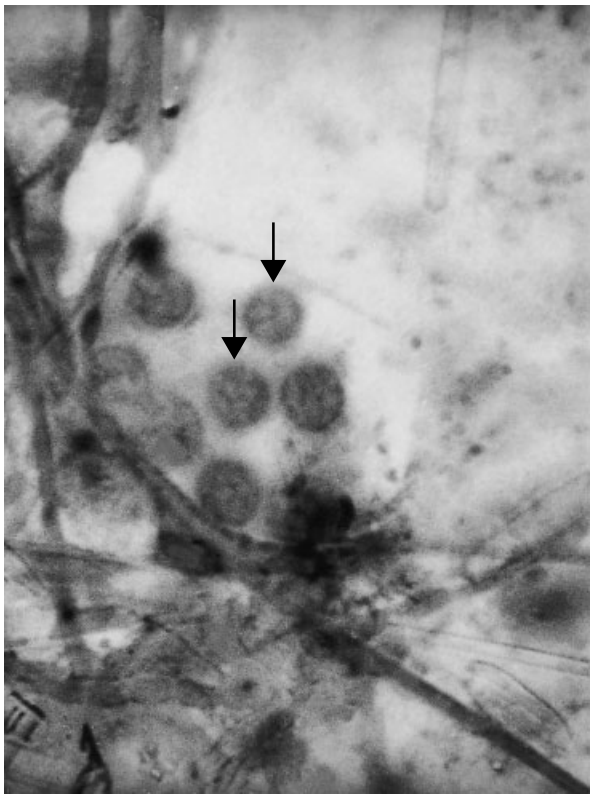

Figure 2 *Smear of deposit from reservoir water in which rhinosporidial patients had bathed, showing spherical PAS-positive bodies (arrows) with intracellular structures, compatible in size and shape with rhinosporidial endospores. PAS; initial magnification ×1 000.*

aquatic environments were recently published (Ahluwalia et al. 1997; Ahluwalia 1999). These investigators erroneously concluded that the causative agent of rhinosporidiosis was the aquatic cyanobacterium *Microcystis aeruginosa*, ubiquitously found in stagnant ground waters. *M. aeruginosa* proved to be an effective red-herring on account of:

- its presence in the same type of waters which are the putative habitat of *R. seeberi*
- its presence as 'round bodies', a feature shared with *R. seeberi*
- its isolation from rhinosporidial polyps. It needs to be pointed out that the surface of rhinosporidial polyps, especially in nasal sites, is rough, a feature which allows the retention of contaminant aquatic micro-organisms. Moreover, the sporangia of *R. seeberi* are often invaded by host-cell infiltrates which include phagocytes; these phagocytes could contain *Microcystis* which could be visualized microscopically and be isolated in culture from rhinosporidial polyps
- no genetic or immunological homologies between *Microcystis* and *R. seeberi* were reported by Ahluwalia. Refutations of Ahluwalia's views were made by Arseculeratne (2000b) and by Mendoza et al. (2001b) on the basis of
  - non-contamination of the organisms's DNA with human DNA
  - absence of control samples from normal people inhabiting the same areas from where the author's water samples were collected
  - absence of cultures of endospores and sporangia free of bacteria after multiple washes and filtration
  - improper sampling of rhinosporidial tissue
  - ultrastructural studies including the morphology of rhinosporidial mitochondria
  - inability of *M. aeruginosa* to cause rhinosporidiosis in experimental animals
  - the absence of reactivity of this cyanobacterium with human and experimental anti-*R. seeberi* antibody in immunofluorescence tests
  - absence of reactions with anti-rhinosporidial sera in immunoblots against *M. aeruginosa* extracts
  - the absence of amplification in PCR tests with primers based on the sequence data published by Herr et al. (1999b). A previous report on the culture of a mycelial fungus as the cause of rhinosporidiosis (Krishnamoorthy et al. 1989) had similar flaws.

Light microscopical and ultrastructural features

In parallel with its disputed taxonomy for many decades, a variety of inconsistent and overlapping terms has been applied to the morphological structures of the organism in its developmental stages (Table 2). Kennedy et al. (1995) proposed a new terminology for the standardization of the ontogenic nomenclature of the *R. seeberi*; these terms are used in this chapter. It is to be noted that these terms represent stages in a continuum of development of the organism; some of these terms, such as electron-dense body, should be regarded as tentative until more definitive studies, as suggested in the following discussion, are available.

Consensual descriptions of the light-microscopical and ultrastructural features of the different ontogenic stages of *R. seeberi*, as they appear in rhinosporidial tissues of humans and animals, have been made by many authors; rhinosporidial endospores and sporangia in various stages of development are uniformly found in histopathological material from diseased humans (Figure 3) and animals.

The 'life cycle' of *R. seeberi*, as deduced from histopathological appearances (Tirumurti 1914; Ashworth 1923; Kutty and Teh 1974; Savino and Margo 1983; Thianprasit and Thagernpol 1989; Kennedy et al. 1995), is represented in Figure 4. All developmental stages of *R. seeberi* are readily identified in most hematoxylin and eosin (H&E)-stained histopathological sections, irrespective of the location of the rhinosporidiosis and the species of the host.

Table 2 *New terminology of the ontogenic stages of* R. seeberi[a]

Earlier terminology	New terminology
Developmental stages	
Spore, immature spore, endospore, sporoblast, early trophic stage, spherule, conidium, sporule	Immature endospore
Mature spore, sporont, pansporoblast, merozoite, yeast phase in tissue, spore morula	Mature endospore
Trophocyte (early, intermediate, late), trophic stage, trophozoite, precleavage phase, immature sporangium, granular stage, sporocyst, cyst, spherule, sporangium	Juvenile sporangium
Endosporulating stage, sporulation phase, trophic sporangium	Intermediate sporangium
Mature trophocyte, mature cyst, endosporulating stage, post-cleavage phase, post-cleavage sporangium, adult stage	Mature sporangium
Intracytoplasmic structures in juvenile sporangium	
Lipid droplets	Lipid bodies
Multilamellar bodies	Laminated bodies
Intracytoplasmic structures in mature endospore	
Lipid bodies, cytoplasmic vacuoles	Lipid globules
Spherules, electron-dense circular structures, protrusions of cell wall, electron-dense inclusions, germinative bodies, sporozoites, spores, sporules, spherical bodies, spore morulae	Electron-dense bodies

a) Adapted from Kennedy et al. 1995.

THE LAMINATED (MULTILAMELLAR) BODY

Several authors have described these bodies as consisting of concentric rings of electron-dense material around a core of chromatin, and seen in juvenile and intermediate sporangia in human and animal rhinosporidial tissues (Kutty and Teh 1974; Savino and Margo 1983; Thianprasit and Thagernpol 1989; Kennedy et al. 1995). Thianprasit and Thagernpol (1989) regarded the laminated body (LB) as the precursors of the endospores. Apple (1983) found these bodies in mature endospores and suggested that, after discharge from the sporangia, the endospores released the LB and from these arose the juvenile sporangia in continuation of the life cycle of *R. seeberi* (see also Kennedy et al. 1995). Kutty and Gomez (1971) described the LB as being 1–1.5 μm in size, derived from the EDBs, and liberated from the endospores; they concluded that the LB is the earliest infective stage, giving rise to the juvenile sporangium. Further work is necessary to elucidate the ontogenic significance of the LBs.

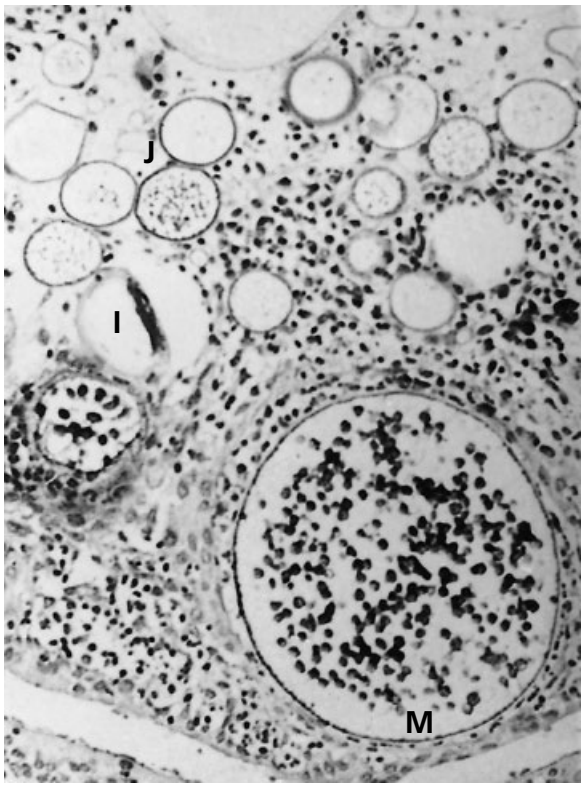

Figure 3 *Developmental stages of* R. seeberi *in rhinosporidial tissue. M, mature sporangium, with surrounding cell infiltrate; I, intermediate sporangium; J, juvenile sporangium. H&E; initial magnification ×400*

ENDOSPORES

The endospores are produced by endosporulation in the sporangia, and are thought to be the asexual propagules of *R. seeberi*; there is no evidence of a sexual stage in the life cycle of *R. seeberi*. After nuclear divisions in the juvenile sporangia, endospores are formed by the condensation of the cytoplasm around the nuclei with the formation of cell walls; 'surfaces of contact remain distinguishable as a sort of network between the spores' (Ashworth 1923). The development of endospores from a germinal part of the inner sporangial wall was postulated by Minchin and Fantham (1905) (see also Beattie 1906; Savino and Margo 1983). Ashworth (1923) and Moses et al. (1991) noted the attachment of endospores to the inner aspect of the sporangial wall; Figure 5 illustrates the immature endospores and their probable origin from this site. Mature endospores were reported to be in the center of the sporangium (Ashworth 1923;

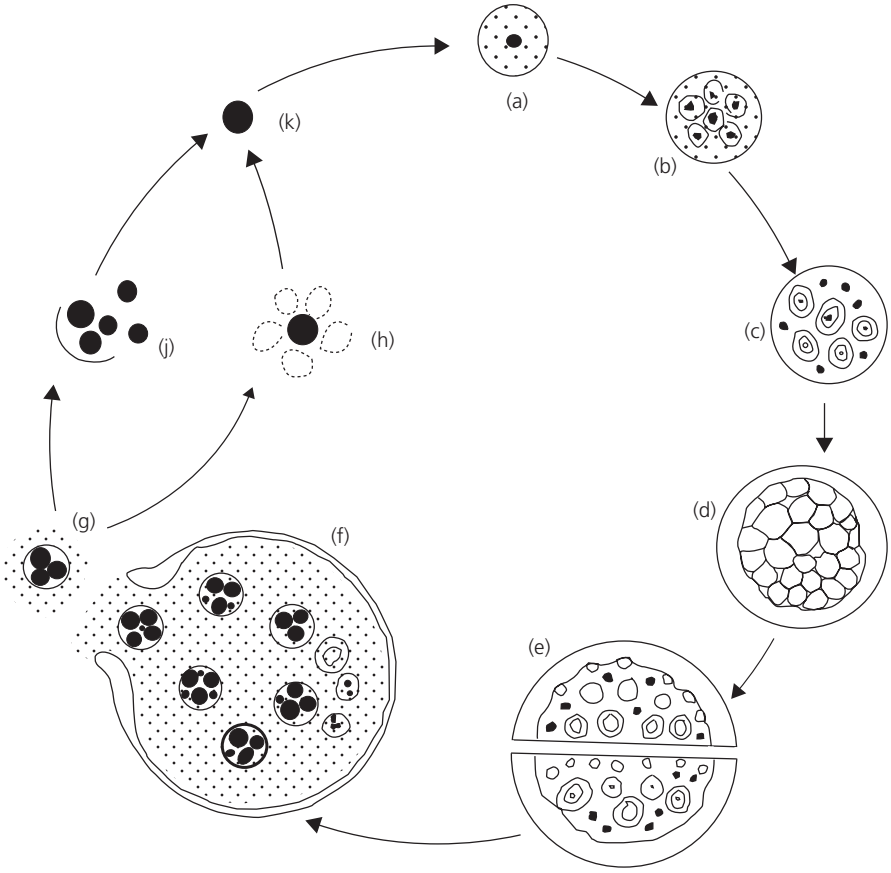

Figure 4 *Diagram (not to scale) of the life cycle of* Rhinosporidium seeberi. **(a)** *Juvenile sporangium;* **(b)** *juvenile sporangium with vesicles containing chromatin;* **(c)** *juvenile sporangium with laminated bodies;* **(d)** *endosporulating immature sporangium with the formation of endospore walls;* **(e)** *immature endospores formed on inner sporangial wall, maturing centripetally (upper half of figure) or formed at the center of the sporangium, maturing centrifugally;* **(f)** *mature sporangium and matrix containing mature endospores with EDBs, departing through annulus and pore;* **(g)** *mature endospore with EDBs;* **(h)** *endospore with disappearing 'nutritive spherules' (Ashworth 1923);* **(j)** *endospore liberated after dissolution of wall of 'spore morula' (Tirumurti 1914);* **(k)** *free endospore*

Shrewsbury 1933; Savino and Margo 1983; Noor-Sunba and al-Ali 1989; Mendoza et al. 1999), indicating a centripetal direction of maturation. Mature endospores may also be seen laterally (Figure 6); their location at the pore end of the sporangium (Beattie 1906; Ingram 1910; Tirumurti 1914; Ashworth 1923) is probably in preparation for their discharge through the sporangium's exit pore.

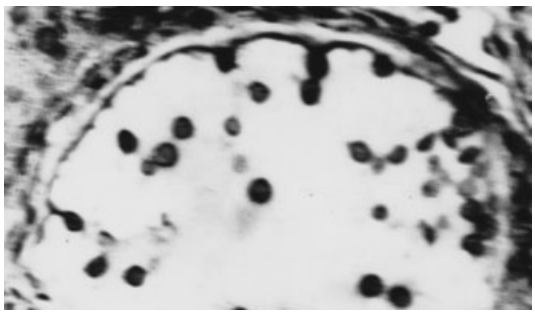

Figure 5 *Intermediate thick-walled sporangium with formation of new endospores on inner sporangial wall. PAS stain; initial magnification ×400*

The mature endospores are spherical, measuring approximately 10–12 μm in diameter. Vanbreuseghem (1973) described endospores in Indian rhinosporidial tissue to be larger than those in African tissue. Endospores have a thick wall that is PAS positive (Figure 7) and contains chitin (Karunaratne 1964), as do fungi. Ultrastructurally, they were found to have a fibrillar coat (Figure 8, Arseculeratne et al. 1999, unpublished data) which, on an analogy with the fimbriae of gram-negative bacteria, could mediate the hemagglutination of red blood cells and adherence to mammalian cells (HEp-2) in culture, described below. The finely granular cytoplasm contains a vesicular nucleus. The most striking microscopical feature is the presence of several spherical bodies of approximately 1–1.5 μmin diameter; these include the EDBs (see Contents of endospores, below). Immature and mature endospores (see Figure 6), some of which appear to be degenerate, are present in mature sporangia (see Table 3).

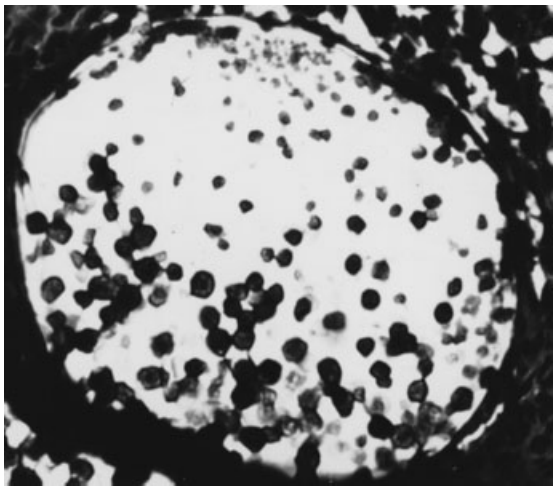

Figure 6 *Mature sporangium with atypical walls, containing mature endospores adjacent to the sporangial wall, with immature or degenerate endospores on the opposite side. H&E; initial magnification ×400*

Kutty and Teh (1975) described the endospore as having a prominent Golgi apparatus, endoplasmic reticulum, mitochondria, vacuolated spaces in the cytoplasm with an irregular plasma membrane, and the EDBs.

We have not detected motility of endospores in wet mounts of material from nasal or palpebral rhinosporidiosis; flagella or organs of motility were not seen on scanning electron microphotographs of endospores from different patients (Arseculeratne et al. 2002, unpublished data). The scanning electron microphotographs of Noor-Sunba and al-Ali (1989) also do not reveal flagella or organs of motility on the endospores; nor did Kutty and Teh (1974) note flagella on the sporangia.

Beattie (1906) described free endospores as having residual matricial material from the sporangium, which

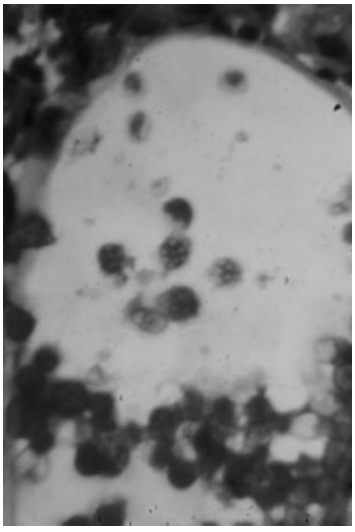

Figure 7 *Mature endospores with PAS-positive walls and internal EDBs. PAS stain; initial magnification ×400*

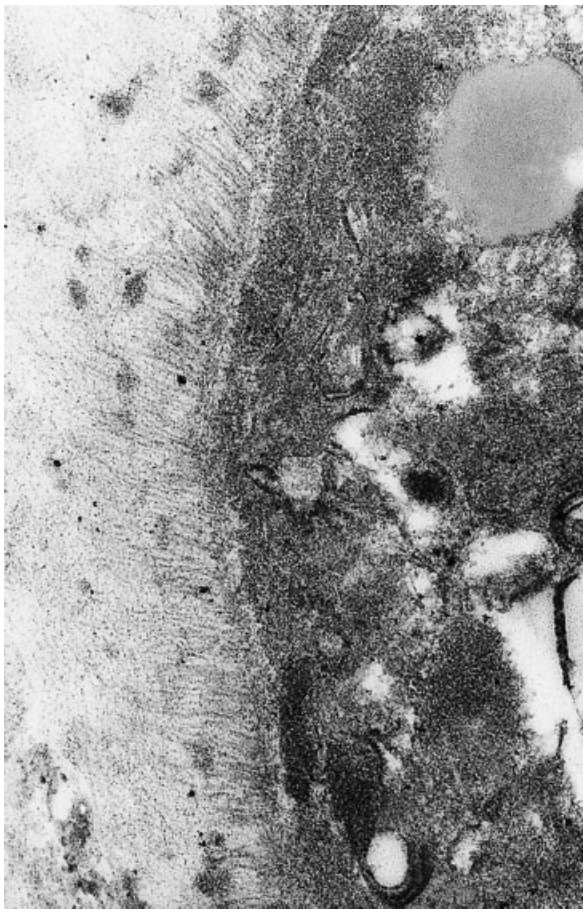

Figure 8 *Electron photomicrograph of endospore showing fibrillar coat.*

appears as an envelope around the endospore and trailing off, giving the appearance of a '. . . tail to a comet, the head being the spore-morulae' (endospore). It was pointed out (Arseculeratne 2000a) that in cytodiagnosis of rhinosporidiosis from smears, especially from nasopharyngeal sites, the residual cytoplasm of the epithelial cells (Figure 9a) could mimic the tail-like mucoid, matricial material around the endospore (Figure 9b). The rhinosporidial endospores with the mucoid covering could be distinguished from epithelial cells by the PAS stain as the nuclei of the latter do not stain magenta with this procedure, whilst the endospore stains strongly positive.

Contents of endospores: the electron-dense body

The most controversial morphological elements of *R. seeberi* are the electron-dense bodies (EDB). They are the most prominent inclusions of the endospores, and are 1–1.5 μm, spherical bodies appearing in clusters (Shrewsbury 1933) of 15–20 EDBs. Kennedy et al. (1995) described their ultrastructural features as 'irregular membrane-bound, vacuolated structures of various

Table 3 *Morphological characteristics of rhinosporidial sporangia*

Sporangium	Characteristics
Juvenile	6–10 μm in diameter Unilamellar, PAS-positive, mucicarmine and GMS-negative wall Fibrillar or granular, PAS-positive, mucicarmine and GMS-negative cytoplasm Chromatin present as a single nucleus or as multiple nuclei, each within a vesicle Laminated bodies present Lipid globules present
Intermediate	100–150 μm in diameter Bilamellar wall, inner cellulose, PAS- and mucicarmine-positive Outer wall PAS-, mucicarmine- and GMS-negative No organized nucleus, lipid globules present Mature endospores absent
Mature	100–450 μm in diameter Thinner bilamellar outer wall Inner wall PAS- and mucicarmine-positive, GMS-variable Outer wall PAS-, mucicarmine- and GMS-negative Immature and mature endospores present, embedded in matrix Mature endospores with lipid globules and electron-dense bodies

GMS, Gomori's methanamine silver; PAS, periodic acid–Schiff.

sizes containing electron-dense granular material.' Their periphery stains deep magenta with the PAS stain (Figure 7) and they can be clearly seen in smears or wet mounts of the mucoid discharge on rhinosporidial growths (Figure 10). The EDBs have also been seen in the endospores in rhinosporidial tissue of dogs (Easley et al. 1986).

The EDBs have been shown to be yellow-fluorescent with acridine orange, Feulgen-positive and DNAase-sensitive (Vanbreuseghem et al. 1955; Lakshmanan et al. 1978; Savino and Margo 1983; Moses et al. 1991). We noted that these bodies when released by ultrasonic treatment of Percoll-purified endospores or in their native location within endospores, fluoresced yellow

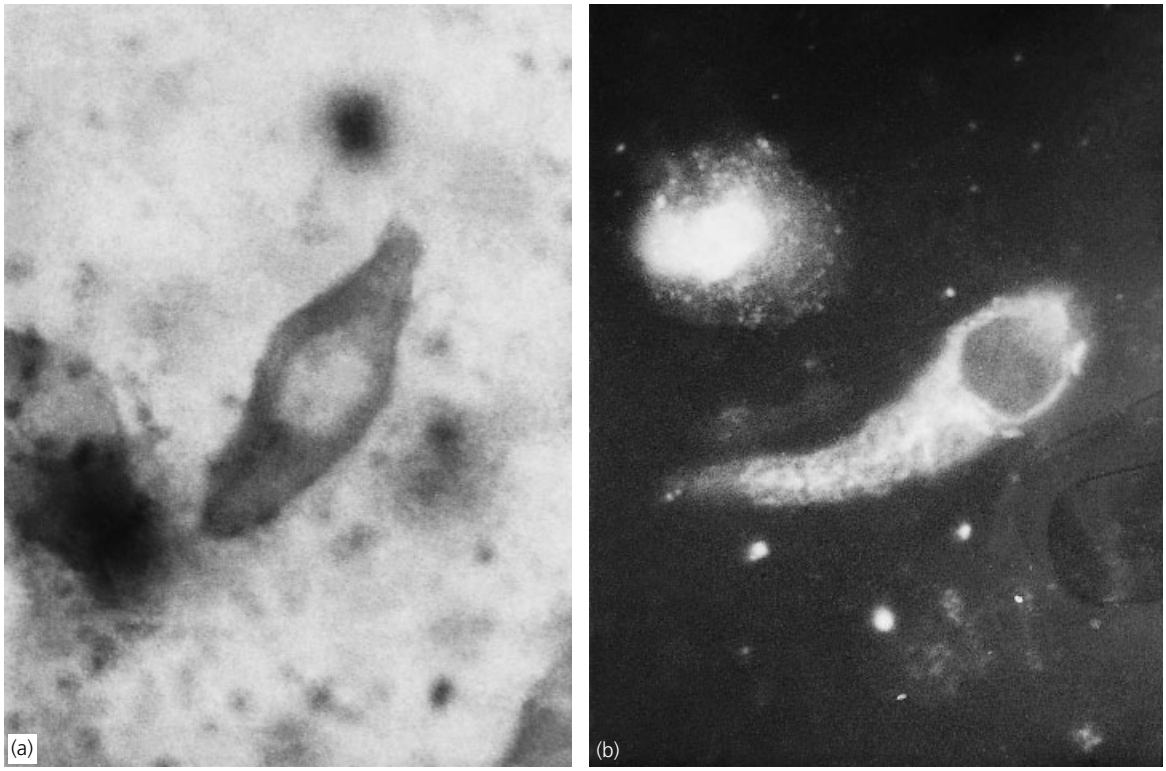

Figure 9 (a). *Normal bovine, nasal epithelial cell showing nucleus with residual cytoplasm in elongated fashion. PAS; initial mgnification ×1 000. Compare with* **(b)**. **(b)**. *Smear from human nasal rhinosporidiosis, showing endospore with residual matricial material from sporangium, mimicking an epithelial cell; indirect immunofluorescence with antirhinosporidial antibody. Compare with* **(a)**. *PAS; initial magnification ×1 000*

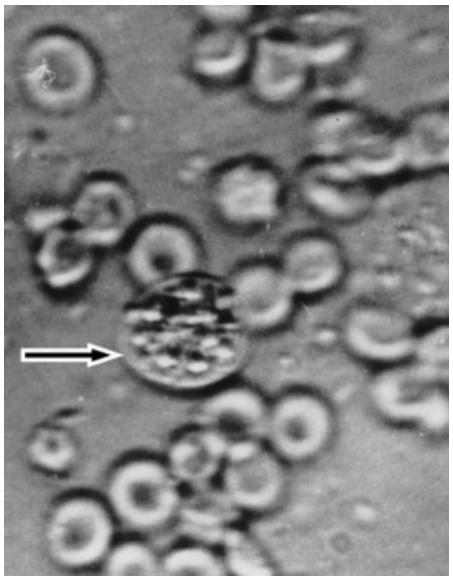

Figure 10 *Mature 10–12 μm endospore (arrow) containing EDBs, amidst red blood cells, in unstained wet mount of mucoid discharge from nasal rhinosporidiosis; initial magnification ×1 000*

with acridine orange, suggesting the presence of nucleic acid; acridine orange, however, is also claimed (Chayen and Bitensky 1991) to stain mast cell granules, cartilage matrix (red fluorescence), keratin (red fluorescence), vascular elastic elements (yellow fluorescence), but these elements have not been described in EDBs.

Lakshmanan et al. (1978) concluded from histochemical tests that the EDBs stained deeply with bromophenol blue, indicating that their contents are proteinaceous, as are the walls which also stain positively with Bismark Brown; deep staining of the wall of the EDB with Sudan Black B was interpreted as indicating that the wall of the EDB had lipoprotein. The EDB also stained deeply with methyl green and was Feulgen-positive. These workers concluded that: '. . . the spherule is made up of a lipoprotein coat with a protein matrix and a strongly Feulgen-positive centre' and that the EDB does contain deoxyribonucleic acid; they cite Vanbreuseghem et al. (1955) as also having demonstrated DNA in the EDB. Beattie (1906) had also suggested that the EDB are indeed the 'spores' and describes them as containing a 'well-defined nucleus which reacts to the chromatin stains.' Minchin and Fantham (1905) noted 'a central spot' in the 'refractile granule' (EDB), which stains with hematoxylin and concluded that it is 'a chromatic nucleus.' Teh and Kutty (1975) concurred with this view. Karunaratne (1964) noted that Vanbreuseghem et al. (1955) considered the EDB to be active bodies, the actual spores; this interpretation would then mean that the 'spore' of Ashworth (1923), now named the endospore, is really a collection of the ultimate true spores and hence meriting the term 'spore-morula' used by Beattie (1906) and Tirumurti (1914).

Kutty and Gomez (1971) recognized that '. . . one of the interior ''spherical bodies''' shows 'a smaller and darker sphere enclosed within it . . .'; this 'would appear to represent a nucleus with a nucleolus.' This view is compatible with our observation that when the next stage of development, the juvenile sporangium, occurs, only one larger spherical body or very rarely two, appears within the juvenile sporangium (Figure 11). If this interpretation is correct, it would imply that this EDB is the ultimate generative, infective unit of *R. seeberi* as these and other authors have concluded. Lakshmanan et al. (1978) came to a similar conclusion: '. . . the spherules could be the initial inciting agent of the disease.'

We have found that endospores from fresh, unfrozen homogenates of rhinosporidial tissues stained deeply purple-blue in the cytoplasm and the EDBs with 3-[4,5-dimethyl thiazol-2-yl]-2-5-diphenyl tetrazolium bromide (MTT) (Figure 12; Arseculeratne and Atapattu 2003a). This dye is used in tests of the viability of diverse cells in culture (as in lymphoproliferative assays on lymphocytes – Mosmann 1983), yeasts, and mycelial fungi. The formation of the purple-blue colored formazan from MTT is due to the reduction of MTT by mitochondrial dehydrogenases and this phenomenon in the EDB, while being also shown in the endospore's

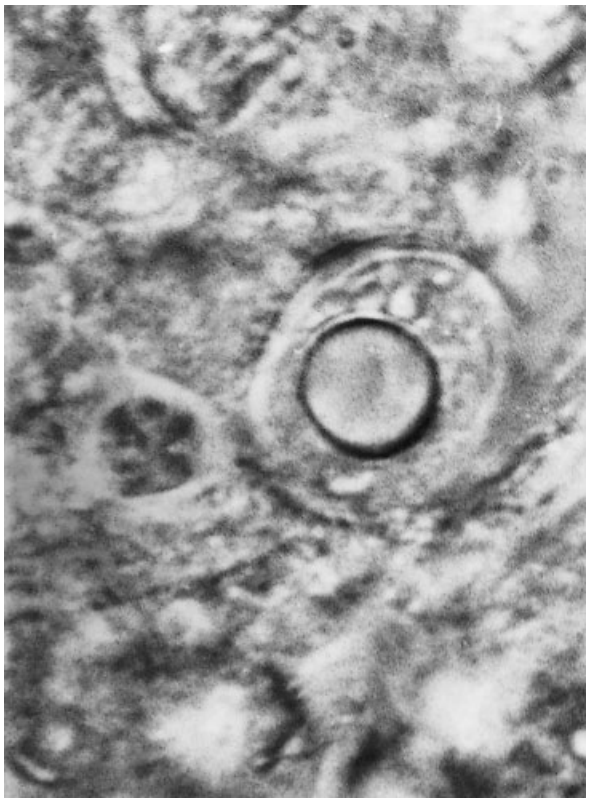

Figure 11 *Developing endospore in prejuvenile sporangium stage, showing single body (probably an EDB) which will develop into a juvenile sporangium. Wet mount of endospores in suspension; initial magnification ×1 000.*

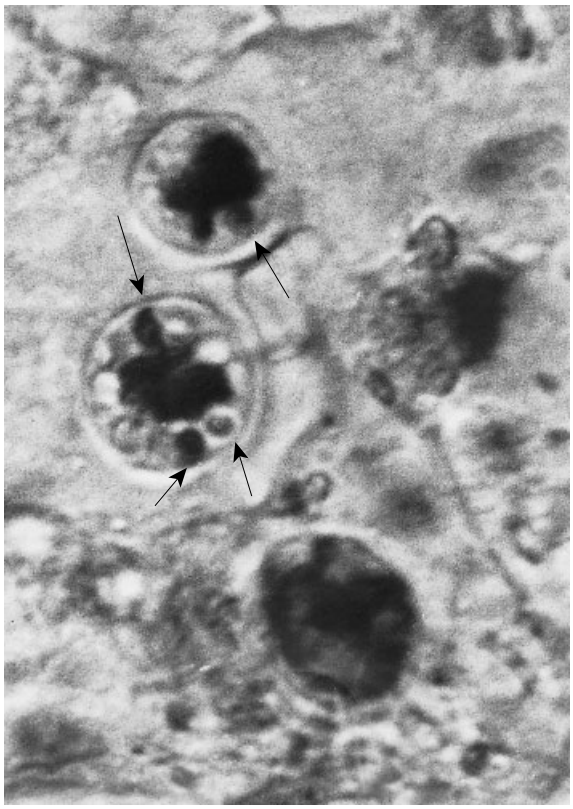

Figure 12 *Fresh endospores after incubation with MTT showing deep (purple-blue) staining of cytoplasm, and spherical internal bodies (arrows), probably EDBs. Other bodies of similar shape and size are MTT-negative and could be the protein nutritive spherules of Ashworth (1923) or the lipid bodies of Kennedy et al. (1995); initial magnification ×1 000*

cytoplasm (which contains mitochondria as described by Kutty and Teh 1975), would therefore indicate vital activity in these sites. If this conclusion is valid, then it is further evidence that the EDBs are indeed viable units.

These conclusions on the generative function of the EDB differ from those of Ashworth (1923) that the EDB are proteinaceous nutritive reserves; Shrewsbury (1933), too, disputed Ashworth's view. Easley et al. (1986), on the other hand, agreed with Ashworth (1923) that the EDBs are reserve bodies rather than mere 'protrusions of a morula-like cell wall of the spore' (Bader and Grueber 1970) or germinative bodies (Teh and Kutty 1975).

A basic question therefore, which needs resolution through an experimental model of pathogenicity, is what is the ultimate generative unit of *R. seeberi*? Is it the endospore, as most authors have assumed, or is it the EDB?

Other contents of the endospore

Eosinophilic, lipid globules were described (Kennedy et al. 1995) in the cytoplasm of the mature endospore and were regarded as nutritive reserves; they were absent in immature endospores. Some spherical bodies that do not stain with MTT (Figure 12) could be the protein nutritive bodies postulated by Ashworth (1923) or the lipid bodies described by Kennedy et al. (1995). In correlation with MTT-stained endospores, transmission electron microphotographs (Figure 13) also revealed the EDBs that possessed an electron-dense core bounded by a thick electron-dense wall, in contrast to other bodies, perhaps the nutritive (protein or lipid) bodies that were electron-lucent and without a wall.

SPORANGIA

Table 3 summarizes the principal morphological features of *R. seeberi*'s sporangia. These, of varying size and shape and in various stages of development, are abundant in most rhinosporidial tissues. Sporangia were classified according to their maturity by Kennedy et al. (1995).

The juvenile sporangium (formerly, the trophocyte)

This developmental stage is the least controversial, and has been identified free in the stroma of infected tissue or even within mature sporangia (Karunaratne 1964). It is a thin-walled sphere of 10–15 μm diameter in its early stages (Figure 14), enlarging to about 100 μm (Bader

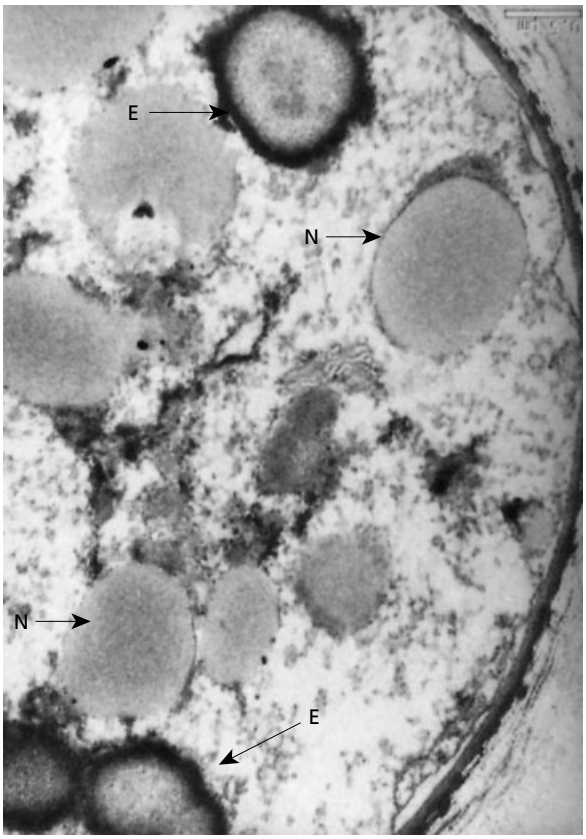

Figure 13 *Transmission electron microphotograph of Percoll-purified mature endospore showing EDBs with a relatively thick wall (E) and electron-lucent bodies, perhaps nutritive bodies without such walls (N); initial magnification × 16 000*

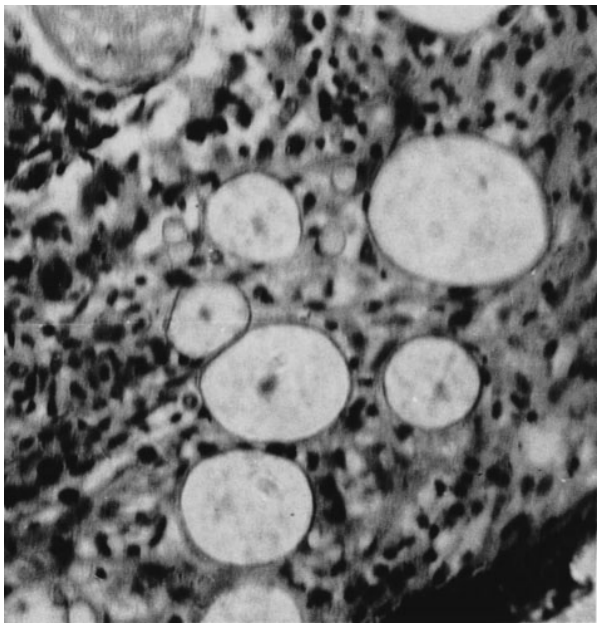

Figure 14 *Thin-walled, early juvenile sporangia, each with a single nucleus and granular cytoplasm. H&E; initial magnification ×400*

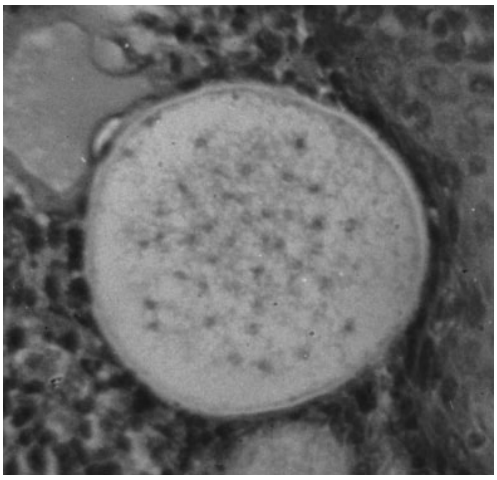

Figure 15 *Juvenile sporangium in the stage of nuclear division. H&E; initial magnification ×400*

and Grueber 1970; Savino and Margo 1983; Kennedy et al. 1995). Bader and Grueber (1970) found its wall to be laminated and that, histochemically, it consisted of a 'chitinous membrane incorporating PAS-positive neutral polysaccharides of a hemicellulose character.' It has variable intracelluar appearances according to its stage of development. Kutty and Teh (1975) recognized early, intermediate, and late stages of the juvenile sporangium.

The chromatin is either organized into a prominent single nucleus (Figure 14) with a nucleolus (Kutty and Teh 1975), or may be fragmented and diffuse (Tirumurti 1914). Kennedy et al. (1995) observed 'an aggregate of clumped and granular electron-dense material . . . and an interrupted double layered nuclear membrane . . . as the only nuclear remnants identified.' When the juvenile sporangium is about 50–60 μm in diameter, nuclear division (Figure 15) results in the appearance of chromatin within the vesicles (Figure 16) (Kutty and Teh 1974; Kennedy et al. 1995).

Kennedy et al. (1995) described ocular rhinosporidiosis in swans in which juvenile sporangia had unilamellar walls ranging from 2.4 to 9.9 μm in thickness and the wall was of electron-dense material which merged with the outer capsule of 0.9–1.5 μm thickness, which consisted of fibrils and hazy electron-dense material. This outer layer was considered in other descriptions as the outer of a bilamellar wall. In later stages, the filamentous structures are replaced by a 'faint encapsulation.' Kutty and Teh (1975) noted the similarity of the radiating structures on the surface to structures on the surface of *Cryptococcus neoformans* and suggested that this appearance was 'additional evidence in support of its (*R. seeberi*'s) fungal nature . . . .' The pitfalls of using morphological similarities in taxonomy have been pointed out earlier in this discussion in relation to the phenomenon of 'morphological convergence' in fungi.

The granular or fibrillar cytoplasm (Kennedy et al. 1995) contains endoplasmic reticulum, mitochondria, lipid globules, membrane-bound vacuolated structures containing electron-dense material that stained positively with the Feulgen stain, and microbodies that are probably nutritive in nature and are used up as the juvenile sporangium matures. Laminated bodies, with two or more concentric rings surrounding a core, ranging from 1.4 to 2.6 μm were also noted throughout the cytoplasm.

The intermediate sporangium

Intermediate (immature) sporangia (Figure 17) are spherical bodies that measure approximately 100–150 μm in diameter. Kennedy et al. (1995) identified a bilamellar wall with an overlying, third capsular layer which thinned as the sporangium matured. Thianprasit and Thagernpol (1989) described this stage to possess a trilaminated wall that included an outer capsule of a 'myriad of curvilinear structures;' the capsule was considered to be the third layer – hence trilamellar. Some descriptions of intermediate sporangia exclude this layer in recognizing the sporangium's wall to be bilamellar. The subcapsular, outer chitinous layer is thicker than the capsular layer and is similar, structurally, to the unilamellar wall of the juvenile sporangium. The new inner layer, probably of cellulose, is electron-lucent and thinner, and is formed when the sporangium is about 100 μm in diameter. The intermediate sporangium contains immature endospores in the cytoplasm that is granular or fibrillar. An intermediate sporangium that is about 140 μm in diameter is said to contain several thousand endospores (Ashworth 1923). Laminated bodies have also been noted in the intermediate sporangia.

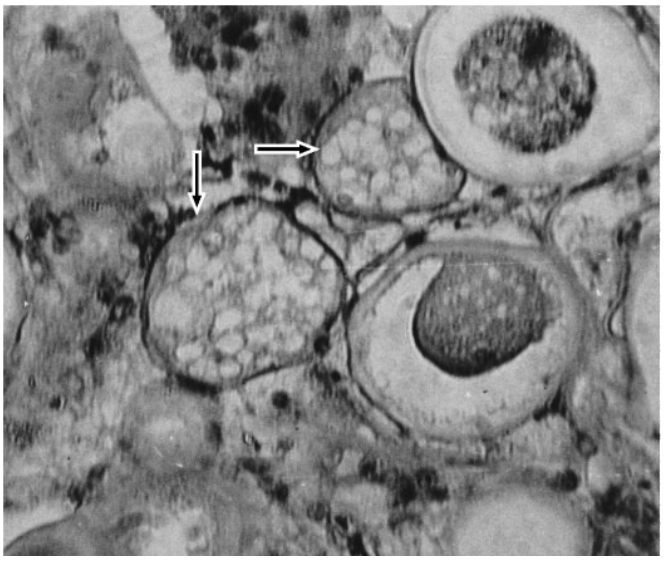

Figure 16 *Juvenile sporangia (arrows) with vesicles, after nuclear division. H&E; initial magnification ×400*

The mature sporangium

This spherical body measures approximately 150–400 μm in diameter, and is located in the stroma of the rhinosporidial polyps, as well as intraepithelially (Figure 18) and in downgrowths of the squamous epithelium into the subepidermal tissue (Figure 19).

The mature sporangium has a trilamellar (Savino and Margo 1983) wall that is thinner (1–3 μm) but is similar, tinctorially, to that of the intermediate sporangium. The outer wall contains chitin, whilst the inner wall has cellulose (Ashworth 1923; Shrewsbury 1933; Karunaratne 1964; Jain 1967), and is more translucent. The possession of chitin is a property that is shared with the fungi; the presence of a chitin-synthase gene in *R. seeberi* was reported by Herr et al. (1999a). Striations of the inner sporangial wall were noted by Beattie (1906) and by Ashworth (1923), although their existence was disputed by Minchin and Fantham (1905), and by Kutty and Teh (1974). A specialized, thickened (15 μm) area of the sporangial wall is the annulus (Figure 20) which surrounds the exit pore (Beattie 1906; Ingram 1910; Tirumurti 1914; Ashworth 1923; Karunaratne 1964; Grover 1970; Mendoza et al. 1999), which is the aperture through which the endospores are extruded.

When the sporangium matures, the wall becomes thinner while the annulus surrounding the pore remains thick (Figure 20). With maturation, the bilamellar (Savino and Margo 1983; Thianprasit and Thagernpol

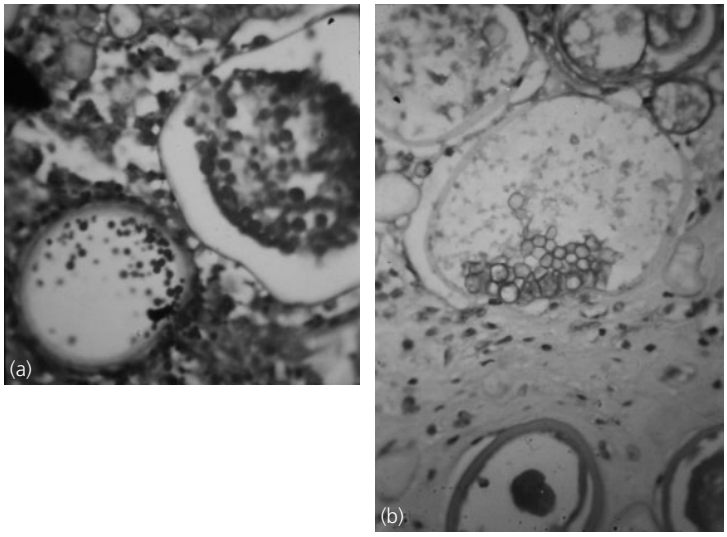

Figure 17 (a) *Intermediate (thick-walled) and mature (thin-walled) sporangia in rhinosporidial tissue. PAS stain; initial magnification ×400.* **(b)** *Intermediate, thick-walled sporangium with new formation of endospores on inner wall. PAS; initial magnification ×400*

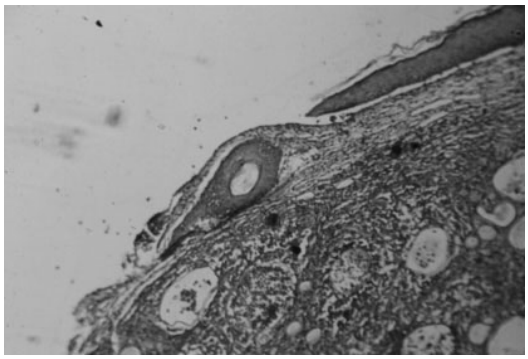

Figure 18 *Intraepithelial effete, thick-walled sporangium in subcutaneous rhinosporidiosis. PAS; initial magnification ×200*

1989) wall retains filamentous radiations or 'curvilinear tubular structures' on the outer surface; the 'dense network of threads' in the cell wall was thought to consist of cellulose (Bader and Grueber 1970).

The sporangial walls are PAS-positive as are the walls of the endospores, while also staining with Alcian Blue, indicating the presence of acid mucopolysaccharides; other tinctorial properties were described by Rao (1966) and Jain (1967). PAS-positivity decreases with maturation of the sporangial wall (Jain 1967). Rao (1966) and Jain (1967) considered the presence of acid mucopolysaccharides to confer selective permeability to nutrients, on the sporangial wall. It is relevant that Mendoza et al. (1999) postulated that sporangia apparently absorb nutrients through the walls during the active process of extrusion of the endospores. Extensive histochemical studies on the different developmental stages were also made by Bader and Grueber (1970).

The walls of degenerate sporangia could have very thick, homogeneous walls (Beattie 1906; Karunaratne 1964), or the wall could be absent, and when the sporangium is empty of endospores, it could pose a diagnostic problem in histopathology (Arseculeratne et al.

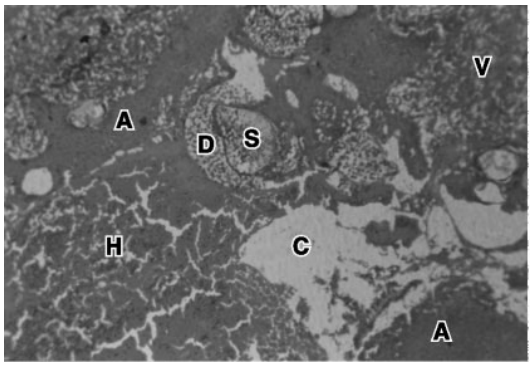

Figure 19 *Degenerate sporangium (S) in nasal rhinosporidiosis; it is encircled by ancanthotic squamous epithelium (A) surrounded by dense cell infiltrate (D), in vascular stroma (V) with hemorrhage (H) and cystic spaces (C). H&E; initial magnification ×400*

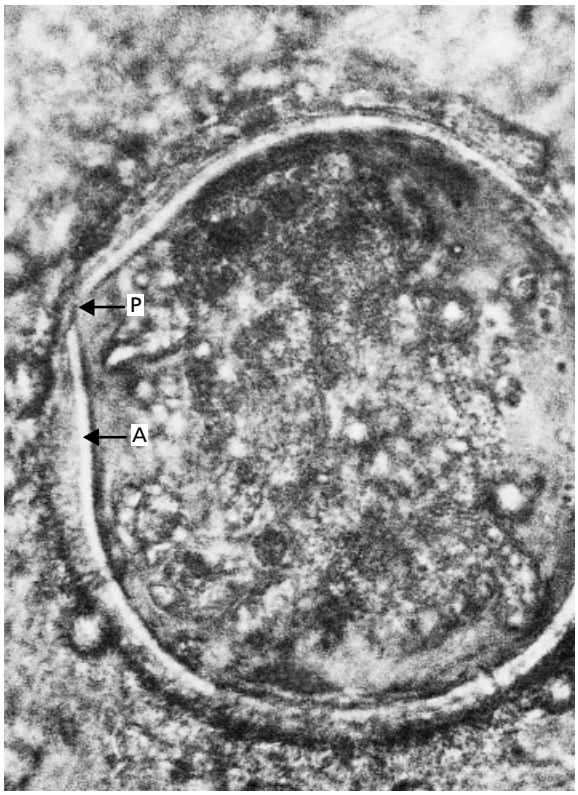

Figure 20 *Intermediate sporangium with annulus (A) and pore (P). Wet mount; initial magnification ×400*

2001). Empty, degenerate sporangia may collapse on themselves, giving characteristic linear markings that represent infoldings of the wall. A homogeneous, eosinophilic, vacuolated material sometimes fills the empty sporangia (Figure 21; Jimenez et al. 1984). Degenerate sporangia could be seen to have within them infiltrates of host-inflammatory cells (Figure 22) and multinucleated giant cells (Figure 23).

Herr et al. (1999b) reported that *R. seeberi* possesses mitochondria with flat cristae, a finding that contrasted with that of Fredricks et al. (2000). However, recently Mendoza et al. 2001a) presented new evidence that supports Herr et al.'s (1999b) observations. The presence of mitochondria with flat cristae in *R. seeberi* is also in agreement with the presence of mitochondria with flat cristae in the other members of the Order Dermocystida (the Rosette Agent and *Dermocystidium*).

Ashworth (1923) estimated that a mature sporangium contains about 16 000 endospores, many of which fail to mature and then appear morphologically and tinctorially abnormal.

The mature endospores within a mature sporangium are embedded in an intrasporangial matrix and appear spaced (Figure 24). The matricial mucoid material stains with Alcian Blue, suggesting the presence of mucopolysaccharides (Savino and Margo 1983; Thianprasit and Thagernpol 1989) that were considered as

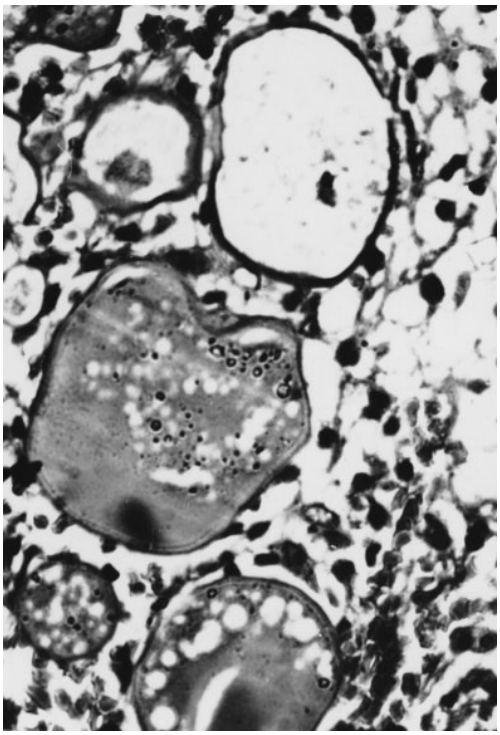

Figure 21 *Degenerate, thin-walled sporangia that are empty or contain vacuolated, homogeneous eosinophilic material; nasal rhinosporidiosis. H&E; initial magnification ×400*

inhibitors of phagocytosis of *R. seeberi* in the host tissue (Thianprasit and Thagernpol 1989); it was also thought to inhibit the penetration of drugs (Woodard and Hudson 1984). Residual portions of this mucoid material might be represented by an envelope on the endospores that leave the sporangium; this matricial residue might also appear as the 'tail of a comet' (Beattie 1906; Figure 9b), and show intense staining in immunofluorescence tests (Atapattu et al. 1999), suggesting the high antigenicity of this material. Smears of crushed polyps reveal the endospores in masses embedded in the

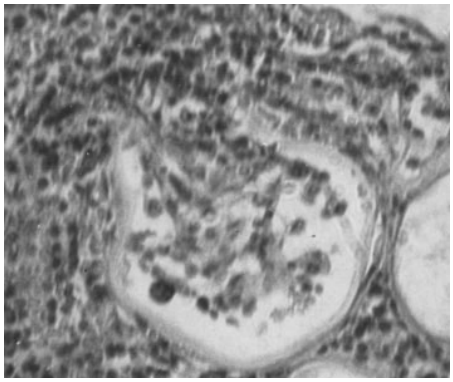

Figure 22 *Invasion of an effete sporangium by fibrocellular stromal tissue in a submental rhinosporidial mass in disseminated rhinosporidiosis. H&E; initial magnification ×400*

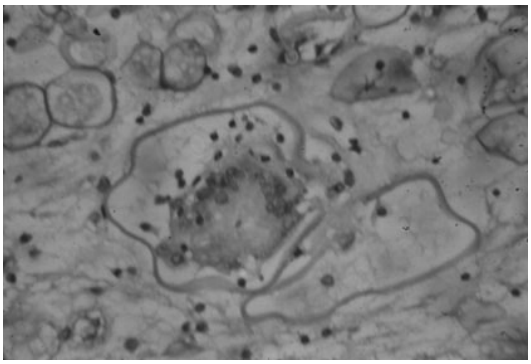

Figure 23 *Degenerate sporangium invaded by a multinucleated giant cell. PAS; initial magnification ×400*

mucoid matrix or individually as unenveloped bodies (Figure 25). The role of the matrix in the release of endospores is referred to below.

The large, 400–450 μm, subepithelial mature sporangia give the typical strawberry-like appearance to the rhinosporidial polyps, the surface of which is studded with yellow, pinhead-sized spots representing the sporangia.

The sporangia of *R. seeberi* could resemble the spherules of *Coccidiodes immitis* but can be differentiated from the latter by being larger, having thicker walls, and with more numerous endospores and an exit pore. The sporangium of *R. seeberi* also differs in staining with

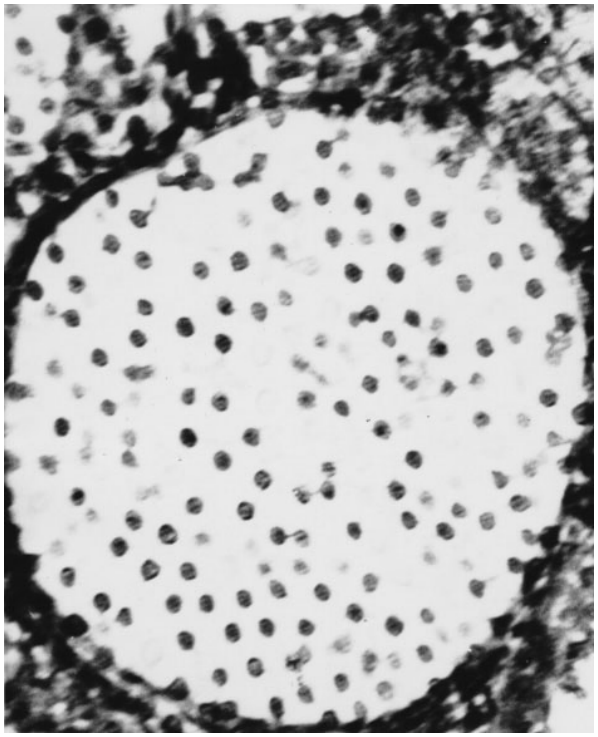

Figure 24 *Mature endospores, separated from each other by matrix in mature sporangium; nasal rhinosporidiosis. H&E; initial magnification ×400*

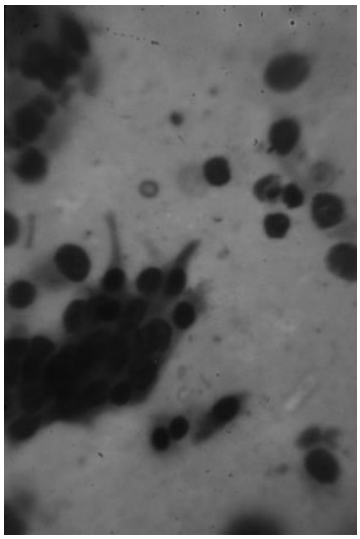

Figure 25 *Smear of homogenate of nasopharyngeal rhinosporidial growth, showing mature endospores, unenveloped or embedded* en masse *in homogeneous material which also appears as envelopes, bipolar wisps or 'tails' on individual endospores. Leishman's stain; initial magnification ×1 000.*

mucicarmine, unlike those of *C. immitis* (Kwon-Chung and Bennett 1992).

Release of endospores from sporangia

Before extrusion, mature endospores are relocated close to the pore. The mature sporangium's pore is surrounded by a thickened annulus of the sporangial wall (Figure 20), through which the endospores are extruded into the surrounding tissues to perpetuate the 'life cycle' of *R. seeberi.* de Mello's (1954) review included a figure (Figure 23) that illustrated a lid or operculum that closed the pore; a 'film' or 'thin disc' covering the pore was also referred to by Ashworth (1923) and by Karunaratne (1964). A pore might not be visualized in some sections of tissue (see Bader and Grueber 1970); this is attributable to the plane of sectioning of the sporangium falling outside the level of the pore.

It had been been assumed that the escape of endospores resulted from rupture of the sporangium from external tissue pressure (Bader and Grueber 1970) or from within the sporangia (Ashworth 1923; Karunaratne 1964) with the endospores being 'shot-out' from the sporangium after its rupture (Beattie 1906) or after the 'thin film over the pore gives way' (Ashworth 1923). Evidence, also documented on a video recording, was produced for the operation of an active, vital mechanism, probably osmotic, for the extrusion of the endospores (Mendoza et al. 1999). de Mello (1954), too, postulated an osmotic mechanism. The endospores are extruded in an orderly procession (Figure 26) and not explosively, *en masse*, through the exit pore.

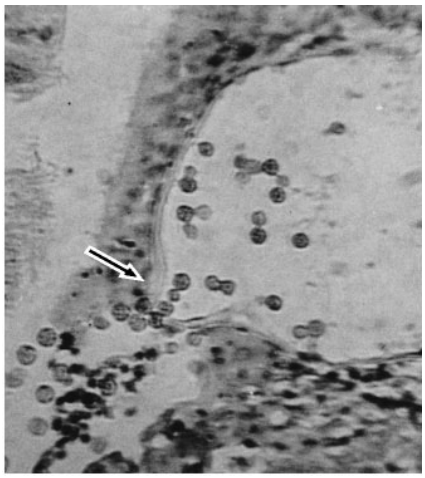

Figure 26 *Mature sporangium in nasal rhinosporidiosis with mature endospores exiting through the annulus and pore (arrow). H&E; initial magnification ×400*

Isolation and purification of developmental stages

The inability to obtain pure preparations of the developmental stages of *R. seeberi*, free of contamination with tissues of the host or with environmental microorganisms, has been the cause of the lack of unequivocal observations and firm conclusions on the biology and immunology of the pathogen, as well as on immunity mechanisms in diseased hosts. Crude preparations of rhinosporidial bodies, derived from homogenized polyps, have been used after sedimentation, coarse filtration, or pipetting-out from the homogenates. These preparations have probably had contamination from host material and the use of such crude 'antigens' could have led, for instance, to the identification of 'rhinosporidial antigenemia' on immunoprecipitation in gel-diffusion tests (Chitravel et al. 1982). It is more likely that the precipitin reactions that were observed were between contaminating human antigens and the corresponding antibodies from the animal sera which were obtained by immunization against these crude rhinosporidial preparations derived from human tissue.

A method was described (Atapattu et al. 1999) for the isolation, in the pure state, of the different developmental stages of *R. seeberi*, from homogenates of rhinosporidial tissue. This method is based on centrifugation of deposits of tissue homogenates, in density gradients of Percoll (Figure 27). The EDBs may be obtained by controlled disintegration of the endospores by ultrasonic or enzymic (chitinase) means. PAS-stained smears of chitinase-treated suspensions of endospores (calcium phosphate buffer at pH 6) showed that the intensity of PAS positivity of the endospore's wall decreased, with better visualization of their EDBs (Arseculeratne 2002, unpublished data).

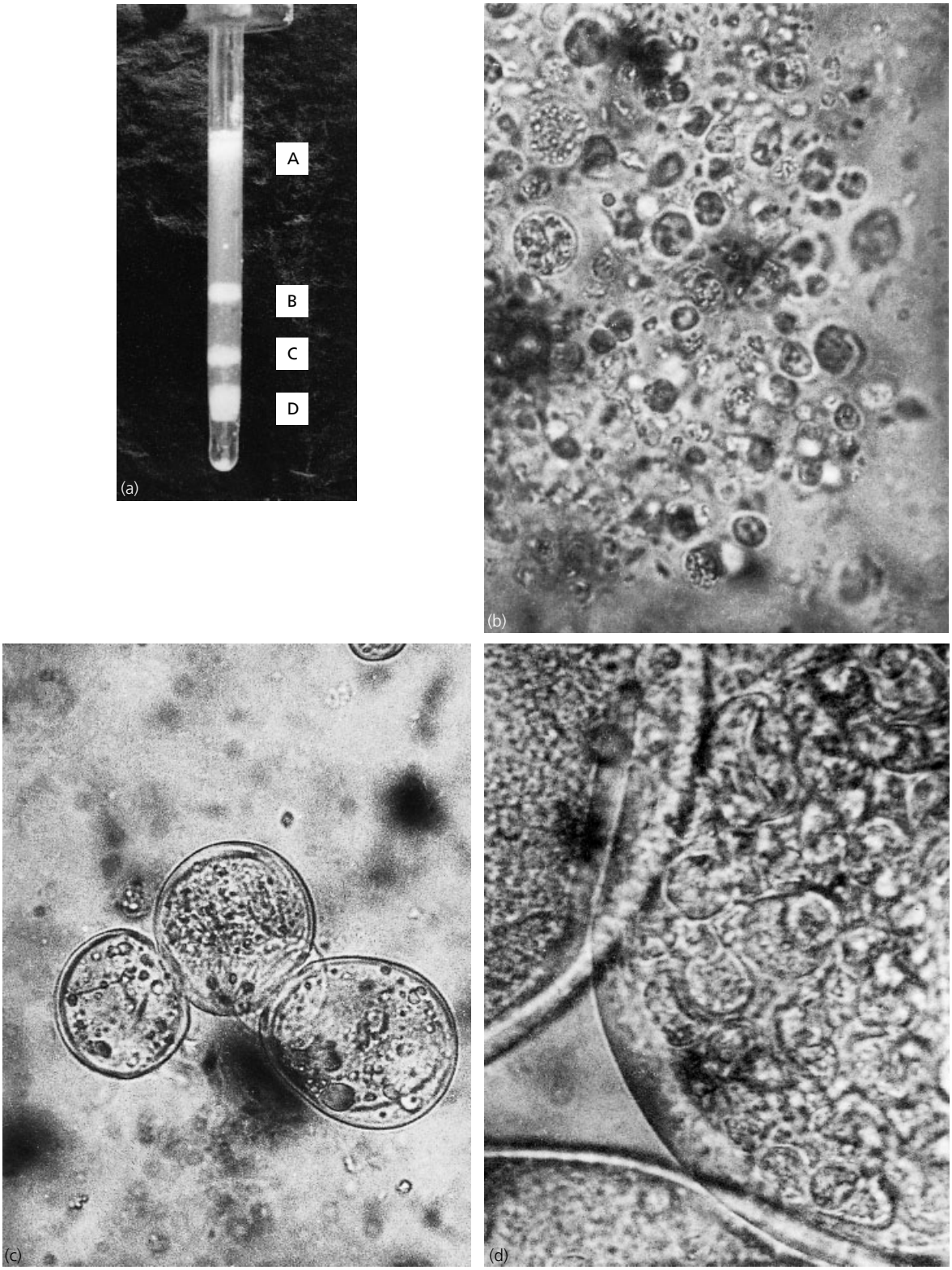

Figure 27 (a) *Fractionation of homogenate of rhinosporidial tissue in Percoll of graded density. A, small endospores; B, larger endospores; C, juvenile sporangia; D, intermediate sporangia. Mature sporangia were not visualized, probably after rupture.* **(b)** *Small and large endospores after fractionation in Percoll. Wet mount; initial magnification ×400.* **(c)** *Juvenile sporangia after fractionation in Percoll. Wet mount; initial magnification ×400.* **(d)** *Intermediate sporangia after fractionation in Percoll. Wet mount; initial magnification ×400.*

When aseptic conditions are used in the Percoll purification, the isolated rhinosporidial bodies are free even of bacterial contamination. These preparations would permit investigations, particularly on the immunology and experimental pathogenicity of *R. seeberi*.

VIABILITY OF ENDOSPORES

The question of the viability of the endospores, in homogenates that have been stored at low temperatures or after purification in Percoll, and used for inoculation of the test animals, is a problem that has a bearing on experiments on the pathogenicity of *R. seeberi*. Homogenates of human or animal rhinosporidial tissues have been used by many workers to attempt infection of experimental animals. With congenitally immunodeficient mice (Arseculeratne et al. 2000), the homogenate was mixed with sonicates of Percoll-purified endospores with their ultrasonically liberated EDBs. With the inability to culture *R. seeberi*, in vitro, and the absence of a method for the direct assessment of the viability of these bodies at the time of experimentation, the viability of the endospores and sporangia was assumed in all these experiments. Nigrosin-eosin or nigrosin-neutral red was found to be useful in determination of viability through non-uptake of the dye (Jain 1967). Arseculeratne and Atapattu (2003a) described the use of Evan's blue in a simple test for the assessment of the morphological integrity of *R. seeberi*'s endospores; it is based on their staining by the vital dye Evan's blue (Figure 28); unfrozen, fresh endospores were also found to stain deeply (purple-blue) with MTT (Figure 12; Arseculeratne and Atapattu 2003a), which is used to assess the viability and hence proliferation of lymphocytes in lymphoproliferative assays for cell-mediated immune competence, and for assessing the viability of fungi. Endospores, after autoclaving, formalinization, and trypsinization, failed to take up the Evan's blue or MTT, whilst endospores from freshly homogenized human rhinosporidial tissue stained deeply, in its cytoplasm and EDBs. *Toxoplasma* behaves in a similar manner in the Sabin–Feldman test (Sabin and Feldman 1948) when dead cells fail to stain with methylene blue. Purified endospores after prolonged storage at –20°C retained their staining with Evan's blue but failed to stain with MTT (Arseculeratne and Atapattu 2003a). Apparently, staining with Evan's blue reveals the morphological integrity of the endospores, especially the EDBs, whilst MTT staining indicates their viability on account of the need for enzymic mechanisms (mitochondrial in eukaryotic cells) to reduce the MTT to the purple-blue colored formazan.

Biological activities of endospores

HEMAGGLUTINATION

Percoll-purified suspensions of endospores from human rhinosporidial tissues were found to cause hemagglutination of red blood cells from humans, a species that is susceptible to infection with *R. seeberi*, and from rats (Figure 29), that have not been reported as susceptible; the hemagglutination occurred as well at 37°C as at 4°C, and was reactivated after agitation and disruption of the clumps of hemagglutinated red cells after first incubation (Arseculeratne and Atapattu 2003b).

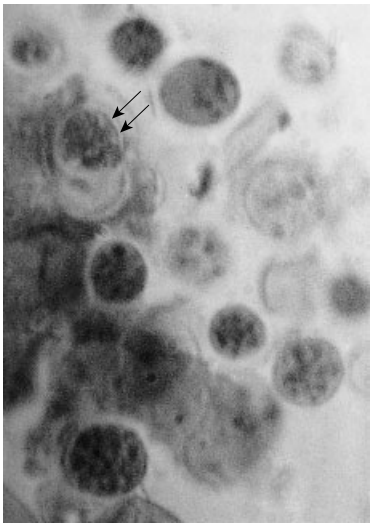

Figure 28 *Fresh endospores stained with 0.2 percent Evan's blue in PBS, showing deeply stained bodies probably EDBs, with moderately stained cytoplasm, and unstained spherical bodies (arrows); initial magnification ×1 000.*

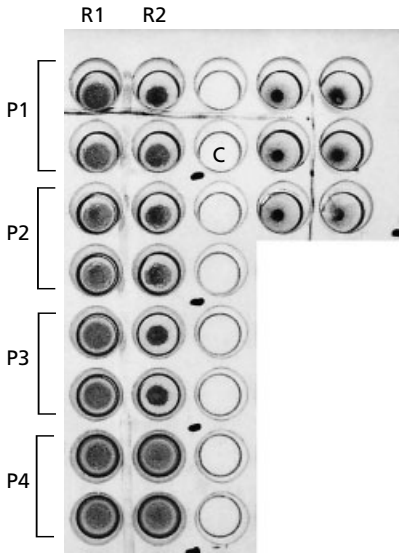

Figure 29 *Hemagglutination of rat red blood cells by purified rhinosporidial endospores. C, control red cells in PBS; R1, R2, rats from which red cells were obtained; P1, P2, P3, P4, purified endospores from four rhinosporidial patients.*

ADHERENCE

Endospores were reported to show strong adherence to epithelioid cells from a human rectal tumor, in vitro (Levy et al. 1986). Endospores also showed adherence to HEp-2 cells (derived from human laryngeal carcinoma) in culture in Minimum Essential Medium (MEM) with 10 percent fetal calf serum (Figure 30); an intriguing feature of the adherent or free endospores in mixtures with these mammalian cells was the appearance of bodies that were similar in size and shape to the EDBs on the surface of the endospore (Figure 31; Arseculeratne and Atapattu 2003b), suggesting that the EDBs were in the process of extrusion from the endospores on contact with the HEp-2 cell. Similar 'protrusions' of the endospore's cell wall were described by Bader and Grueber (1970) and by Teh and Kutty (1975), whilst the latter authors considered the protrusions to contain EDBs. The significance of this phenomenon remains to be elucidated. The HEp-2 cells that showed adherent endospores numbered only about half of the total cells present and a similar phenomenon, attributed to variations in the density of receptors for adherence on the buccal cell-surface, was reported for *Candida albicans* (Sandin et al. 1987).

It was previously pointed out (Atapattu et al. 2000) that the endospore had a fibrillar surface as revealed on transmission electron microscopy (Figure 8), which might, on a parallel with the adhesive fimbriae of gram-negative bacilli, be the agent responsible for the adherence to mammalian cells and for hemagglutination.

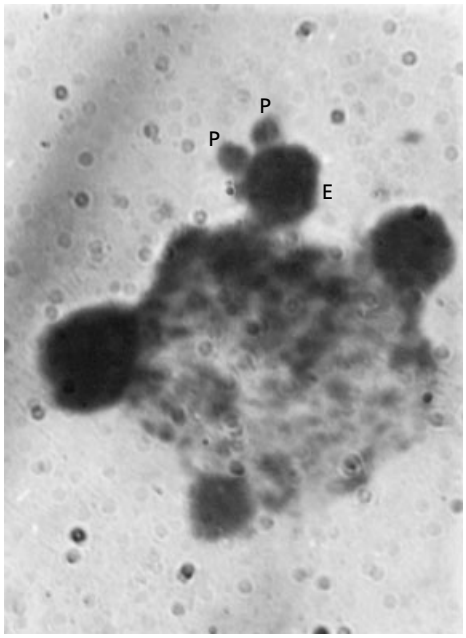

Figure 30 *Adherence of purified endospores to HEp-2 cells in MEM with 10 percent fetal calf serum. One endospore (E) has two protrusions (P) suggestive of extruding EDBs. PAS stain; initial magnification ×1 000.*

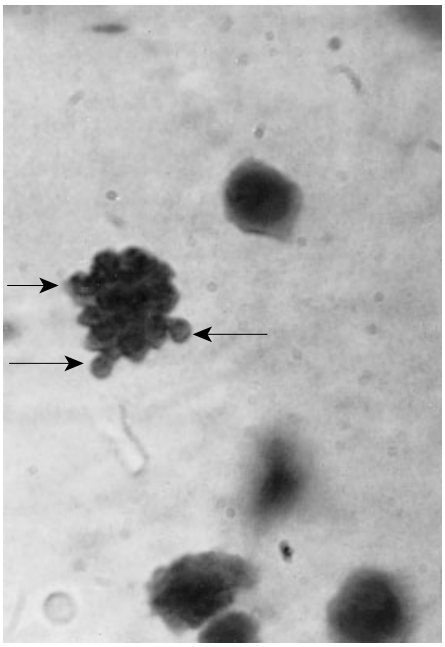

Figure 31 *Protruding bodies (arrows), compatible in size and shape with EDBs, attached to endospore in suspension with HEp-2 cells. PAS; initial magnification ×1 000.*

Sensitivity of *R. seeberi* to antimicrobial drugs and biocides

On account of the inability to culture *R. seeberi* in vitro, conventional tests of sensitivity to antimicrobial drugs and to biocides have not been possible with *R. seeberi*. There have been many agents that were tested clinically. Quinine hydrochloride (Wright 1922), salts of antimony and bismuth, iodine, and pentamidine (Rajam et al. 1955) griseofulvin, amphotericin B, and topical steroids (Jimenez et al. 1984; Ho and Tay 1986), local and systemic antibacterial antibiotics and radiotherapy (Satyanarayana 1966) were clinically ineffective, as were other compounds (see de Mello 1954). Allen and Dave (1936), discussing the treatment of rhinosporidiosis, recorded negative results in therapy with a variety of other compounds. The only agent that is now used in therapy is dapsone (4,4'-diaminodiphenyl sulphone, DDS), and the evidence for its efficiacy was obtained from clinical observations and microscopic examination of the pathogen in rhinosporidial tissue from treated patients (discussed under Treatment below). Since leprosy, which also occurs in the same regions of rhinosporidial endemicity (in Sri Lanka), is also treated with this drug, an alternative drug for rhinosporidiosis will be advantageous, in the event of the development of resistance of *Mycobacterium leprae* to dapsone. An additional justification for an alternative drug is that dapsone precipitates hemolytic crises in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, which is common in the regions of rhinosporidial endemicity in Sri Lanka.

The development of sporangia from endospores in aqueous suspension under storage (described under in vitro culture, below) was used in an attempt to test the sensitivity of *R. seeberi* to various drugs in vitro (Arseculeratne 1999, unpublished data). Viability of *R. seeberi* was deduced from the formation, over a period of 1–2 weeks, of juvenile sporangia of at least 50 µm in diameter as observed microscopically in flat-bottomed wells of cell culture trays. It was difficult to standardize the optimum conditions for sporangial development from the endospores, and the method was abandoned. A further disincentive to pursue this technique was the long period of observation required; the development of sporangia from endospores, or 'maturation' as Easley et al. (1986) termed the phenomenon, was noted to be upto 35 days while Grover (1970) noted the development within 10 days.

The Evan's blue test of the morphological integrity of endospores has been used to investigate their sensitivity to various biocides and antimicrobial agents (Arseculeratne 2002). The EDBs of untreated endospores stained intensely while the cytoplasm had a lesser intensity of staining (Figure 28). The end point used was the loss of staining of the EDBs and the cytoplasm, as in endospores that were treated with autoclaving and formalinizing. Chloroxylenol, chlorhexidine, Betadine, cetrimide, Tincture Iodine, and 10 percent formalin produced marked loss of staining with Evan's blue. Endospores treated with these agents and stained with MTT also showed a similar pattern of loss of staining (Arseculeratne 2002, unpublished observations).

CULTURE

The terms 'growth' and 'development' need definition in discussing the literature on culture of *R. seeberi*. By 'growth,' in this discussion, is meant the increase in size of a given stage, e.g. the enlargement of a sporangium. By 'development' is meant the sequential formation of the ontogenic stages, e.g. the development of an endospore into a juvenile sporangium. 'Development' may also be equated with 'maturation,' a term used by Easley et al. (1986). 'Serial propagation' refers to the repetition of the cycle of development of each stage to the mature sporangium, the release of its endospores and the repetition of the cycle. These differentiations might serve to avoid confusion in deducing from observations on the formation of sporangia from endospores in vitro, that true, cyclical development of the ontogenic stages occurred.

Culture on inanimate media

Numerous attempts are recorded in the literature as not having produced definite evidence of serial propagation of *R. seeberi*, on inanimate culture media (Shrewsbury 1933; Dhayagude 1941; Satyanarayana 1960; Reddy and Lakshminarayana 1962; Kennedy et al. 1995). A wide variety of conventional bacteriological culture media has been used unsuccessfully, sometimes with exotic additives such as horse dung, prune juice, and hydrocele fluid, and incubated under various conditions of temperature and aerobiosis. Claims have, however, been made of successful culture in inanimate media (see Karunaratne 1964), but none of these reports provided unequivocal evidence for serial propagation of *R. seeberi*, in vitro. What appears to have been interpreted as propagation was in fact the maintenance of morphological integrity and continuation of the development of the endospores for a limited period under suitable conditions such as low temperature, and an environment such as that in monolayer cultures of cells. The limited ontogenic development is not indicative of serial propagation of *R. seeberi*.

Other reports of allegedly successful culture of *R. seeberi* in vitro (e.g. the mycelial fungus of Krishnamoorthy et al. 1989) dealt with contamination by environmental microorganisms or by organisms that were confused with *R. seeberi* (e.g. *Microcystis aeruginosa* of Ahluwalia et al. 1997; Ahluwalia 1999).

Grover (1970) suspended material from human rhinosporidial tissues in the synthetic medium TC 199 at 4°C and observed a rapid increase in the number of endospores and the formation of sporangia; the organism was maintained for 4 months. No mycelium was formed. We have made similar observations with Percoll-purified endospores (Arseculeratne et al. 1996, unpublished data) in phosphate-buffered saline or in tissue culture media, although the development did not proceed beyond the stage of immature sporangia; at no stage did we observe endosporulation in, and endospore-release from, these sporangia. Various conditions, such as temperature (4 °C, room temperature ambient at 28–30 °C), bubbling of air through the suspension, alternative suspending media (distilled water, phosphate-buffered saline pH 7.2, natural ground water) were tested, but the conditions for reproducible ontogenic development are as yet obscure. This limited in vitro development has therefore been unable to provide the basis for a standardizable, reproducible method for the assay of sensitivity of *R. seeberi* to antimicrobial agents.

Growth in cell cultures

Cultures of mammalian cells have also been tested for their ability to support the serial propagation of *R. seeberi*.

Levy et al. (1986) described attempts to grow the organism from canine nasal rhinosporidiosis in monolayers of a cell line from an epithelioid human rectal tumor (HRT); they reported strong adherence of the organism 'within minutes' to the cells. They also claimed

to have observed, after 2 days, 'polyp-like structures' consisting of HRT cells and mature sporangia releasing endospores into the medium. The analogy between their polyps in cell cultures, which appeared within 2 days, and the polyps in clinical cases appears doubtful, not only in respect of the time interval of several months required for the development of the natural lesions, but also because the clinical polyps are consequent to the host's tissue response (epithelial proliferation, edema, with cellular infiltration into the fibrovascular stroma) surrounding the multiple sporangia and endospores. These authors regarded the need for contact with mammalian cells for the complete development of the pathogen, and the absence of such cells as a reason for the failure of culture of the pathogen on inanimate culture media. Easley et al. (1986) documented similar results. There was focal proliferation of the tumor cells around the organism, the numbers of which increased; the authors claimed that this 'reproduction' continued for over 3 months in subculture. No independent confirmation of these results has been reported.

Whether these observations were similar to those reported by Grover (1970) is not clear. The development of endospores into sporangia, which was earlier reported by Grover (1970), was also noted by Levy et al. (1986), who added that this development occurred also in the absence of the HRT cells.

We have observed the development of immature sporangia from endospores that were added to cultures of HEp-2 or HeLa cells in MEM with 10 percent fetal calf serum, or even in phosphate buffered saline (PBS) pH 7.2 thawed after storage at −20°C, although the formation of mature sporangia with mature endospores was not observed.

Kennedy et al. (1995), however, did not note any evidence of growth of *R. seeberi* in fresh rhinosporidial tissue, in culture media with 10 percent fetal bovine serum.

IMMUNOLOGY

Data on the immunology of the developmental stages of *R. seeberi* were unavailable until recently, attributable to the absence of a method for obtaining pure cultures of the organism in vitro. The method described above for the purification of the developmental stages of *R. seeberi* from homogenates of rhinosporidial tissue, using graded dilutions of Percoll, now permits the isolation of these stages, free of contamination from host tissue and contaminant microorganisms. EDBs may also be obtained from the endospores by controlled sonic disintegration.

Herr et al. (1999c) used immunoelectron microscopy to demonstrate an antigen that first appears immediately under the mature sporangial wall; this antigen was apparently absent in sporangia of earlier stages of maturation and in endospores. The authors stated that this was 'the first report in which an antigenic material with a potential role in the immunology of rhinosporidiosis has been detected.' This finding also suggests the occurrence of antigenic variation in *R. seeberi* that might be a means of immune evasion of this pathogen.

A further finding that is relevant to the pathogenesis of rhinosporidiosis, in particular to its chronicity, recurrence, and dissemination, is that sonic extracts of purified rhinosporidial endospores and sporangia showed evidence on immunoblots of the presence of human immunoglobulins (IgG, IgM, and to a lesser degree IgA) in the rhinosporidial antigens; this suggests that either immunoglobulins were bound to the rhinosporidial antigens or that these antigens had epitopes that cross-reacted with those on human immunoglobulins (Atapattu et al. 2000). Azadeh et al. (1994) reported the presence of IgG and IgA on walls of early and mature sporangia and in endospores; IgM was absent. This phenomenon might also contribute to immune evasion by *R. seeberi*.

The phenomenon of binding by pathogens of host proteins has also been described in bacteria (Boyle 2000) and in schistosomes (Roitt et al. 1993).

PATHOGENICITY IN EXPERIMENTAL ANIMALS

An experimental animal model of rhinosporidiosis has yet to be established. This lack has contributed to the paucity of information on immunity mechanisms in rhinosporidiosis, some aspects of the developmental 'life cycle' and the in vivo response of *R. seeberi* to antimicrobial drugs.

Rhinosporidiosis occurs in many species of farm, domestic, and wild animals. Some of these species (cats, goats, horses, bovines), conventional experimental animals (guinea pigs, mice, rabbits, rats), and other animals such as monkeys and snails, and even a human volunteer (Habibi et al. 1944, quoted by Karunaratne 1964) have been inoculated with homogenates or powdered dry material from rhinosporidial tissue, through diverse routes – subconjunctival, subcutaneous, intradermal, inreaperitoneal, intravaginal, intratesticular, as well as by scarification of the skin, buccal and nasal mucosa, and through the gills of fish, the mantle and mouth parts of snails. No sustained or progressive disease occurred (see Karunaratne 1964). The chorioallantoic membrane, yolk sac, and the amniotic cavity of developing chick embryos also failed to support growth of *R. seeberi*. On a superficial resemblance of the lesions and tissue forms produced by *R. seeberi*, to those of a *Dermosporidium* spp. in frogs, Karunaratne and Dissanaike (A.S. Dissanaike, 1995, personal communication) injected homogenized material from human rhinosporidiosis into frogs (*Rana temporaria*) but no progressive disease ensued. We have also found five genera/species of amphibia to be insusceptible to rhinosporidial

infection (Arseculeratne 1999, unpublished data). Since the frog pathogen *Dermocystidium* is now included with *Rhinosporidium* in the proposed, new Order Dermocystida of the Class Mesomycetozoea (Herr et al. 1999b; Mendoza et al. 2002), it is relevant that Carini (1940) failed to produce the disease in the frog *Hyla rubra*, with the frog pathogen.

Negative results were also produced by intraperitoneal injection of suspensions of endospores and sporangia in 5 percent hog gastric mucin or in 100 percent Percoll in mice (Arseculeratne, 2000, unpublished data).

Iatrogenically immunosuppressed animals (steroids, Jain 1967; cheek pouch of hamsters under steroids, Grover 1970; cyclophosphamide in C3H mice, Arseculeratne 1998, unpublished data; x-irradiated, Satyanarayana 1960) also proved to be insusceptible. Congenitally immunodeficient mice also failed to support the growth of *R. seeberi*; Levy et al. (1986) stated that 'aged spores' failed to produce infection in nude mice when the 'spores' were inoculated, on the scarified nasal mucosa, intramuscularly or intraperitoneally. Congenitally immunodeficient SCID and nude mice failed to support the growth of endospores, sporangia and EDBs released from endospores, purified from human rhinosporidial tissue (Arseculeratne et al. 2000).

A uniform finding in all these experiments was that the injected material showed only degenerate sporangia that were subsequently invaded by fibrous tissue, and that the endospores had disappeared in approximately 3 weeks.

The failure to establish infection might have resulted from nonspecific immunity, especially through macrophages, which might have prevented the initial establishment of infection during the first few days after inoculation, rather than from specific adaptive immune responses that would have been operative later.

A second factor that might be involved in the failure of tests of experimental pathogenicity is the viability of the rhinosporidial endospores and sporangia that were obtained from homogenized tissues. Using the MTT dye reduction test described above, we observed that Percoll-purified endospores which had been stored at −20°C for several months, failed to stain with the dye, indicating a reduction of viability (Arseculeratne and Atapattu 2003a). The age of the endospores used as inocula in the experiments reported in the literature has not been stated and the possibility remains that low viability of the endospores contributed to the negative results.

A further factor that might be absent in inocula prepared from rhinosporidial tissue is concomitant microbiota – hydrophilic microorganisms in the putative natural aquatic habitat of *R. seeberi*; it is conceivable that some of these organisms might be synergistic with *R. seeberi* as has been demonstrated of *Wolbachia* spp. with filarial nematodes, lactobacilli with *Trichomonas vaginalis*, and *Staphyococcus aureus* with *Candida albicans*.

RHINOSPORIDIOSIS

Rhinosporidiosis is an infective disease in the sense that the pathogen is detectable in the lesions in rhinosporidiosis. No evidence has, however, been adduced that it is transmissible as there has been no unequivocally documented instance of cross-infection between members of the same family or between animals and humans.

Cases have been overwhelmingly sporadic but two outbreaks have been described. In Serbia an outbreak of ocular and nasal rhinosporidiosis in 17 humans was reported by Vukovic et al. (1995), whilst Kennedy et al. (1995) described an outbreak of ocular and nasal rhinosporidiosis in captive swans in Florida, USA.

EPIDEMIOLOGY

Geographical distribution

Sporadic cases of the disease have been reported from more than 60 countries of different geographical characteristics (Table 4), although it occurs predominantly in tropical and subtropical regions. Southern India and Sri Lanka have had the highest incidence; other countries where the disease is endemic include Argentina, Brazil, USA (Texas), and Uganda. Incidence rates, even in hyperendemic countries, vary with region; in India, South India has a high incidence whereas the disease is rare in North India (Paul et al. 1978). In Sri Lanka, the predominant focus is the dry zone. The south central region in the USA is an endemic-enzootic region (Wallin et al. 2001). Karunaratne (1964) noted that ocular rhinosporidiosis (oculosporidiosis) was commoner in South Africa.

In Western temperate and Middle Eastern countries, most cases of human rhinosporidiosis occurred in expatriate Indians who probably acquired the disease in their native lands (Al-Hili 1985; Matusik et al. 1986; Mears and Amerasinghe 1992; Ohgaki et al. 1994). On account of this feature, increasing international movement of persons from countries with endemic rhinosporidiosis might result in a more frequent occurrence of rhinosporidiosis in nonendemic countries; the disease might then merit the term 'an emerging infection.' Yet a few cases of rhinosporidiosis have been reported in persons, living in the West, who have never traveled to endemic areas (Wright 1907; Weller and Riker 1930; Lasser and Smith 1976; Woodard and Hudson 1984; Gaines et al. 1996). The disease in all these cases, irrespective of the country of occurrence, has shown remarkably uniform clinical and pathological features.

Reasons for endemicity

It has also to be explained why the disease is of high endemicity in certain regions of southern India and in

Table 4 *The geographical distribution of rhinospiridiosis in humans and animals*

Region	Countries
Africa	Burundi, Cameroons, Chad, Congo, Egypt, Ethiopia, Gabon, Ghana, Guinea, Ivory Coast, Kenya, Liberia, Madagascar, Malwai, Martinique, Mozambique, Nigeria, Rwanda, South Africa, Sudan, Tanzania, Uganda, Zaire, Zambia, Zimbabwe
North America	Canada, Mexico, USA
Central America	Belize, El Salvador
Caribbean Islands	Cuba, Guadeloupe, Tobago, Trinidad
South America	Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, Paraguay, Uruguay, Venezuela
Asia	Bangladesh, India, Indonesia, Japan, Laos, Malaysia, Pakistan, Papua New Guinea, The Philippines, Sri Lanka, Thailand, Vietnam
Europe	Germany, Great Britain, Italy, Latvia, The Netherlands, Poland, Russia, Serbia, Spain, Switzerland
Middle East	Bahrain, Iran, Israel, Saudi Arabia, Turkey

the dry zone of Sri Lanka. If indeed ground waters are the natural habitat, then the chemical and physical characteristics of these waters need definition. In addition, as pointed out above, other aquatic microorganisms might also be relevant to a possible synergistic action in the establishment of natural rhinosporidiosis.

Seasonal incidence

Differences of opinion exist on the seasonality of the disease. Paul et al. (1978) stated that in Jammu, India, the disease appears to be met with towards the later months of the year (see also Jain 1967). Ratnakar et al. (1992) reported a seasonal variation in ocular and nasal disease. There was no seasonal feature in other reports (Balachandran et al. 1990); indeed, in view of the slow growth of the lesions, a seasonal variation might be obscured. The time of arrival of the patients, especially farmers, at hospital might rather depend on weather and monsoons and on exigencies of occupation.

Incubation period

Rhinosporidial growths are of variable duration, from 8 days to 35 years (Allen and Dave 1936; de Mello 1954), with a shorter duration in ocular than in nasal disease (Karunaratne 1964). When a preceding event such as trauma has been noted, the incubation period has been as short as 6–16 days (Jimenez et al. 1984); more commonly it has been months or a few years. In most cases, it is difficult to estimate the incubation period on account of the slow growth of the polyps and hence clinical manifestation

Differences in race and religion of patients

From Sri Lankan data corrected for population ratios, Karunaratne (1964) recorded differences in the occurrence of nasal and ocular rhinosporidiosis between racial and religious groups; it was noted that nasal and ocular rhinosporidiosis was commoner in the Muslims with the comment that their religion 'enjoins the thorough washing out of the nose before entering the mosque for prayer, and I am given to understand that this may include the mechanical cleansing of the nostril with the finger,' an ideal scenario for the inoculation of finger-borne *R. seeberi* from the water. Hindus predominated among patients with nasal rhinosporidiosis in India (Agarwal 1966). Penile or urethral rhinosporidiosis was, however, predominant among Muslims (Tirumurti 1914). Kurup (1931) concluded that 'customs, ways, manners and sanitary conditions' were important in its etiology; in his series, the incidence was greatest among the Muslims on the west coast of India.

Occupation and socioeconomic status of patients

Occupationally, most patients, especially in the large Indian series, have been agricultural workers and persons of the lower socioeconomic strata. Of particular interest is the occurrence of rhinosporidiosis in river-sand workers (Noronha 1933; Mandlik 1937) which supports the claim that the pathogen has an aquatic habitat, with sand particles providing mucosal abrasions for its implantation. From Sri Lankan data, Karunaratne (1964) postulated an etiologic link with work that involved contact with mud in paddy fields, although the occurrence of the disease in patients without such an occupation makes this a doubtful link. Caniatti et al. (1998), reporting four dogs with nasal rhinosporidiosis, also suggested mud in rice fields as the source of the pathogen. Indian data do not support that view.

Patients have often been poor, with a low standard of living, and among them farmers and laborers predominated (Allen and Dave 1936; Elles 1941). In India, the disease was found in 'all socio-economic strata and among all communities and religious groups' (Moses et al. 1990); this is of interest on account of the putative aquatic habitat of *R. seeberi* and the suggestion that water in communal pools, which are used for ablutions before religious rites in some communities, is a vehicle of transmission.

Age and sex differences of patients

Most patients in the African, Sri Lankan (Karunaratne 1964) and Indian series (Moses et al. 1988) have been under 25 years of age, with a range from 4 months to 90 years (Sharma et al. 1962), whilst most patients were young adults (15–40 years); this feature is probably linked to occupation.

In patients under 15 years of age, nasal and ocular rhinosporidiosis occurred more frequently in females (Karunaratne 1964). In adults, a male predominance has been noted in most series, African, Sri Lankan, and Indian, although in other series (Moses et al. 1988, 1990) no sex difference was noted in the occurrence of either nasal or ocular disease. In other series, for example of 1 241 patients, 84 percent were males (Kwon-Chung and Bennett 1992). The male preponderance in older age groups was, however, not seen in younger patients. The preponderant male incidence might have an occupational basis, although the male preponderance in animal rhinosporidiosis would argue against an occupational basis for the preponderance of the disease in human males. Karunaratne (1964) noted that nasal rhinosporidiosis was commoner in males whereas ocular rhinosporidiosis was more common in females. Occupational exposure to the postulated water-borne pathogen is insufficient to explain the predominant occurrence in males, in view of the exposure of females, in one series of cases, to the same environment (Wright 1922). A male predominance has been noted in swans (Kennedy et al. 1995), as in humans, although Moses and Balachandran (1987) found no sex difference in 103 farm animals.

Sources of the pathogen

The evidence for ground waters as the natural habitat and source of *R. seeberi* is impressive:

- rhinosporidiosis in patients with a history of having bathed frequently in such waters
- the occurrence of an outbreak in persons who bathed in the same lake (Vukovic et al. 1995)
- the clustering of the disease in river-sand workers (Noronha 1933; Mandlik 1937)
- the occurrence of the disease especially in upper respiratory and ocular sites
- the occurrence of nasal and ocular disease in animals that habitually come in contact with such waters, especially the epizootic disease in swans (Kennedy et al. 1995); see also Ecology, above.

MODE OF INFECTION

Rhinosporidiosis in upper respiratory sites is thought to be initiated by the implantation of endospores from the aquatic habitat into the respiratory mucosa, aided by abrasions caused, for instance, by sand particles in river-sand workers who dive into the water (Mandlik 1937) or by vigorous cleansing of the anterior nares with the fingers (Forsyth 1924; Karunaratne 1964); it is relevant to the initiating role of abrasions caused by sand tha workers who did not enter the water, but merely worked on the river banks, did not show the disease (Jain 1967). Trauma to the nose even in the absence of exposure to stagnant water, has been recorded as an antecedent event (Weller and Riker 1930; Jimenez et al. 1984). 'Autoinoculation' of the mucosa by endospores released from sporangia or after surgery was claimed (Karunaratne 1964) to be responsible for satellite polyps in the adjacent regions. On the same basis, it has been claimed that polyps on the skin result from implantation of endospores by scratching with contaminated fingers, although the rarity of skin polyps in children who might be expected to scratch themselves as often as adults makes this explanation improbable. Polyps on the skin on the other hand could occur through hematogenous dissemination from respiratory sites; lesions on the skin, however, without precedent respiratory rhinosporidiosis have been recorded (Madhavan et al. 1978).

No clear evidence of transmission between humans, even between those living in close contact, has been recorded (Wright 1922); the disease has occurred in members of the same family, although they were not living together (Allen and Dave 1936). Karunaratne (1963) documented three cases among family members who lived together, but such an occurrence could have also been rather the result of exposure to a common source of the pathogen.

Karunaratne (1964) used the term 'transepithelial infection' to refer to the implantation of endospores through minor abrasions in the skin or mucosae, through occupationally acquired injuries, from sand particles, and vegetable matter contaminated with soil; dust laden with the endospores is thought to be the vehicle of infection in ocular rhinosporidiosis. Bader and Grueber (1970) agreed with the concept of transepithelial infection though Ashworth (1923) disputed it.

The mode of infection in ocular rhinosporidiosis is even more obscure; Kaye's (1938) patients gave no history of bathing in ponds, exposure to animals, or trauma. Ocular rhinosporidiosis, as it occurs in dry regions in the Middle Eastern countries, is believed to have been due to the dustborne pathogen. Dust storms were suggested as the possible vehicle although ocular disease has been recorded in wet regions as well (Moses et al. 1990).

From a case of vulval rhinosporidiosis in a woman and penile rhinosporidiosis in her male sexual partner, Symmers (1966) postulated a sexual mode of transmission; Palaniswamy and Bhandari (1983) and Sasidharan et al. (1987) found no evidence for such transmission.

There is no evidence that humans can acquire the disease from animals (Lasser and Smith 1976). Humans and animals probably acquire the pathogen from the common source of ground waters. From the similarity noted between *Ichthyosporidium* and *Rhinosporidium* spp. (Laveran and Pettit 1910), Allen and Dave (1936) surmised that *R. seeberi* might also be a parasite of fish and that animals and humans could acquire the pathogen from inland waters which harbor infected fish. Reddy and Lakshminarayana (1962) found no evidence for the existence of *R. seeberi* in inland fish.

There is evidence for trauma as a predisposing factor; Moses and Balachandran (1987) reported that in 19 cattle with rhinosporidiosis, all had nasal disease at the site of the passage through the nasal septum, of the guiding nose-rope. Trauma followed soon after by exposure to stagnant water, featured in a case of nasal rhinosporidiosis (Atav et al. 1955), and Symmers (1966) documented a case with trauma and simultaneous contact with stagnant water shortly preceding the appearance of the lesion at the traumatized site. The occurrence of rhinosporidiosis at a site of a closed injury that precluded exposure to soil, dust, or water, as with the rhinosporidial mass over the tibia (Figure 43, p. 31) could have resulted from bloodborne endospores which extravasated at the traumatized site. Trauma to the nose without exposure to soil or ground water, followed by nasal rhinosporidiosis, has also been reported (Weller and Riker 1930; Jimenez et al. 1984). Finger-borne inoculation in anterior nasal passages has been suggested (Forsyth 1924; Karunaratne 1964), although the development of lesions in the nasopharynx cannot be thus explained.

Penile and anterior urethral rhinosporidiosis in the male is thought to result from the application, by some communities in whom rhinosporidiosis in these sites is commoner, of a stone or piece of brick to absorb the last drops of urine (Kurup 1931; Kutty and Unni 1969).

Predisposition to infection

A history of bathing in ground waters is common in Sri Lankan patients. On the other hand, whilst numerous persons bathe in such waters, only a small proportion of these bathers acquire the disease, suggesting the existence of predisposing host factors. The only host factor that has been investigated is blood group; Kameswaran and Lakshmanan (1999) reported that in India, group O had the highest incidence (70 percent) in rhinosporidial patients followed by group AB, although groups A, B, and O are distributed 'fairly equally,' whilst group AB is rare. Jain (1967) found no association of incidence with blood groups. In our series of 21 Sri Lankan patients, the pattern differed: group O, 43 percent; group A, 29 percent; group B, 19 percent; and group AB, 10 percent; with a population distribution of 43 percent, 21 percent, 26 percent, and 5 percent, respectively. HLA or genetic typing of rhinosporidial patients would be of interest.

The possible existence of host factors that predispose to infection is suggested by the development of rhinosporidiosis in boys but not in girls who lived under the same conditions, using the same swimming baths (Karunaratne 1936).

CLINICAL FEATURES

Sites

The most common (70 percent) sites of solitary growths are the vestibule of the nose (Figure 32), the septum, and the inferior and middle turbinates and the floor of the nasal acvity. Bilateral (Figure 33) or multiple polyps are less common. Other respiratory sites are the adjacent sinuses, nasopharynx, fauces, soft palate, inner end of the Eustachian tube, tonsil and larynx, and occasionally the trachea and bronchi. Nonrespiratory sites with single or multiple polyps (10 percent) are the skin of the face (Figure 34), scalp, body (superficially, subfascially, or intramuscularly), external ear, vulva, vagina, rectum, glans and fossa navicularis of the penis, and the external urethral meatus of males and females. It is claimed (Kutty and Unni 1969) that in anterior urethral rhinosporidiosis in the male, infection is initiated by the rubbing of stones to absorb the last drops of urine by certain communities; the stone could cause abrasions in the epithelium while containing the soil or water-based pathogen,

Pulmonary lesions have been rare and rhinosporidiosis in the anterior buccal cavity has not been reported; this rarity is conceivably associated with continual flushing of the cavity with food and fluids, leaving insufficient contact time for implantation of the organism. Rhinoporidiosis has, however, occurred in the parotid gland

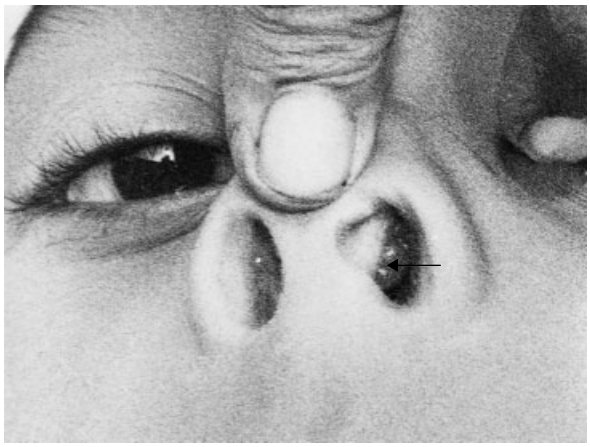

Figure 32 *Early, strawberry-like, pedunculated rhinosporidial polyp (arrow) in nasal vestibule. (Courtesy Dr P. Balasooriya, Sri Lanka)*

Figure 33 *Bilateral rhinosporidial masses obstructing anterior nares, in advanced disease. (Courtesy Dr P. Balasooriya, Sri Lanka)*

(Mahadevan 1952), a rare site, infection in which was thought to have arisen through the Stenson's duct from the oral cavitiy. Approximately 15 percent of all cases of rhinosporidiosis are in ocular sites (oculosporidiosis) with palpebral conjunctival lesions being the commonest (Figure 35). Ocular polyps are painless, granular, often sessile masses; they could occur in the limbus, canthus, and lacrimal duct and sac with spread to the nose (Kirkpatrick 1916). With involvement of the bulbar conjunctiva, scleral 'melting' and staphyloma formation are complications (Castellino et al. 2000). The lacrimal apparatus – lacrimal sac, nasolacrimal duct – could also be involved (Kameswaran 1966). Obstruction of the lacrimal duct may lead to suppurative dacryocystitis with secondary infection. The idea that the initial focus of infection is the lacrimal duct is probably invalid as nasal lesions have been on the contralateral side. Ocular cases have occurred predominantly in dusty regions rather than in wet agricultural areas, while also being preceded by injuries to the eye.

Sites in disseminated cases include the brain (Alessandrini 1926 quoted by Karunaratne 1964), bones of limbs

Figure 34 *Verrucous rhinosporidial polyps on the face of a man aged 41 years. (Courtesy Dr P. Balasooriya, Sri Lanka)*

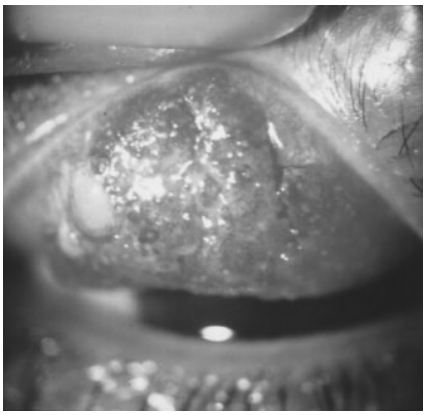

Figure 35 *Sessile rhinosporidial mass on upper palpebral conjunctiva, with a fluid-filled vesicle on outer surface of the eyelid. The vesicle disappeared after surgical excision of the rhinosporidial mass. (Courtesy Dr C.R. Seimon, Sri Lanka)*

(Nguyen-Van-Ut et al. 1959; Chatterjee et al. 1977; Aravindan et al. 1989; Gokhale et al. 1997; Figure 36), the skin, and the abdominal viscera.

Rhinosporidial polyps on nonrespiratory sites of the body, notably the skin and subcutaneous tissues, could occur without primary lesions in respiratory or ocular sites (Madhavan et al. 1978).

Symptoms

The presenting symptoms depend on the site of the disease. In respiratory sites, the commonest symptoms are obstruction to breathing, and bleeding even with minimal trauma of sneezing or nose-blowing. Changes in voice occur in laryngeal disease. Pain is uncommon.

PATHOLOGY

Macroscopic appearances

The macroscopic appearances vary with the site of rhinosporidial polyps. Nasal polyps are typically strawberry-like masses with pinhead-sized, cream-colored spots due to mature subepithelial sporangia. Polyps on the skin, and in respiratory sites, could be sessile or pedunculate and non-infiltrating into deeper tissues; this latter feature makes the radical excision of pedunculate polyps possible with minimal spillage of endospores and uncommon recurrence, while the excision of sessile polyps might be incomplete, with contamination of the adjacent mucosa by endospores leading to recurrence. Most polyps are lobulated and, in the nasopharynx, the lobes appear variegated with smooth, nongranular surfaces or granular and vascular in other areas; histopathological diagnosis is easier with the latter portions of the growth. Polyps with a verrucous surface might

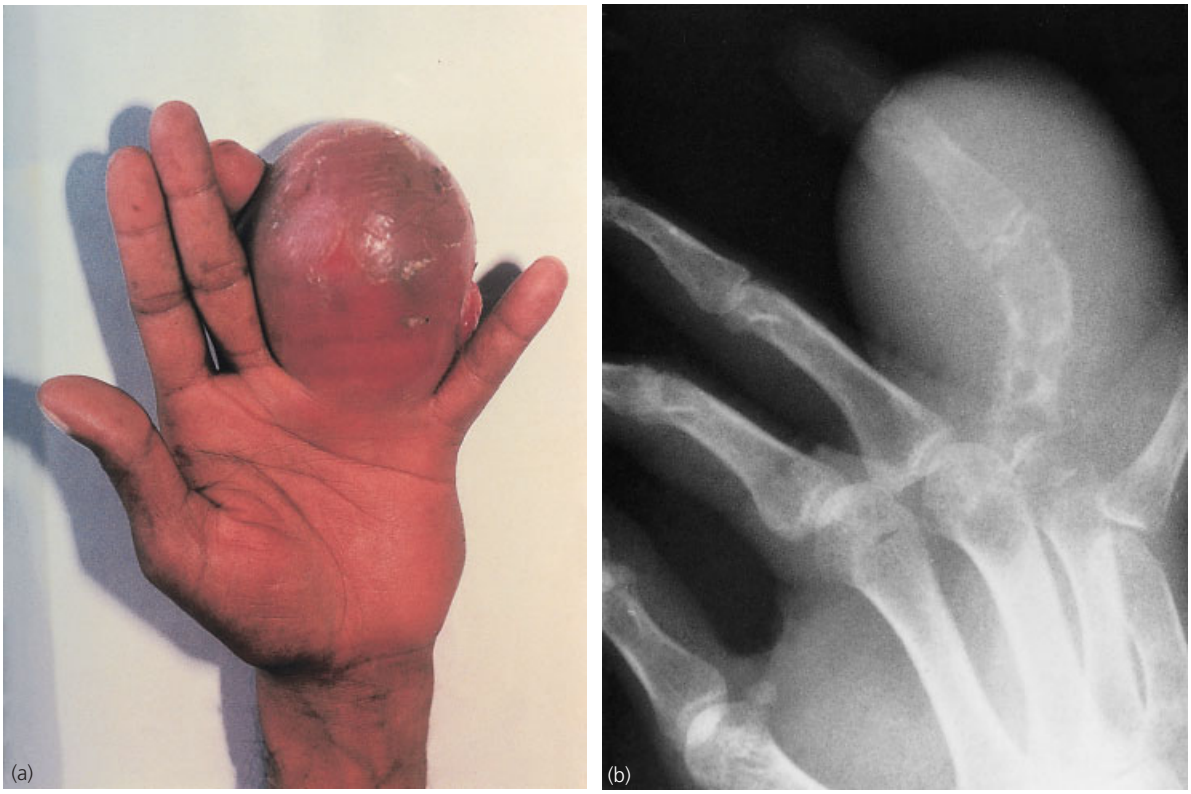

Figure 36 (a) *Globular rhinosporidial mass on finger, in a patient with disseminated rhinosporidiosis. (Courtesy Dr S. Gokhale, India).* **(b)** *Radiograph of fingers of patient in* **(a)**, *showing destruction and fracture of bones of the affected finger. (Courtesy Dr S. Gokhale, India)*

entrap other microorganisms from the aquatic habitat; this is of importance in sampling for purposes of culture in that contaminating microorganisms which appear in culture might be erroneously incriminated as the cause of rhinosporidiosis (see Krishnamoorthy et al. 1989; Ahluwalia et al. 1997; Ahluwalia 1999).

Polyps in respiratory sites are usually covered with mucus in which numerous endospores may be detected on stained smears. Growths on the skin of the body might undergo ulceration and de-epithelialization, and secondary infection, and then mimic malignant tumors. Pus formation is uncommon. Bones, as in the foot, hand or limbs, which underlie rhinosporidial masses, could show erosion or fractures on radiographs (Chatterjee et al. 1977; Gokhale et al. 1997; Figure 36b).

Disseminated rhinosporidiosis is relatively rare, and presents with masses of varying size on the face, trunk, limbs, and in the viscera including the liver, spleen, brain, and kidneys (Rajam et al. 1955; Agrawal et al. 1959). Rhinosporidial endospores and sporangia in these cases have been detected microscopically in the blood vessels. Lymph node enlargement, even in disseminated rhinosporidiosis, is curiously uncommon (Chatterjee et al. 1977), although it is possible that lymph nodes have not been examined in patients with disseminated disease.

Microscopic appearances

The histopathological pattern in human rhinosporidiosis shows wide variation, especially in the stroma and infiltrating cells (Arseculeratne et al. 2001). The various developmental stages of the organism can be seen in a single histological section (Figure 3) and their presence, rather than the host's tissue response, affords a definitive diagnosis.

The epithelium overlying mature sporangia is attenuated or hyperplastic with keratinization, and acanthotic with downgrowths that surround the sporangia (see Figure 19). This phenomenon is further discussed under Transepidermal elimination below. Proliferation of respiratory (ciliated, columnar) epithelium results in a papillary appearance, with many goblet cells and hypersecretion of mucus (Karunaratne 1964). Growths on the skin may be denuded of epithelium and ulcerated.

Endospores and sporangia (Figure 18) have been seen intraepithelially (Noronha 1933; Karunaratne 1964); it is uncertain whether the presence of sporangia, intraepithelially, results from the development of intraepithelial endospores after 'transepithelial infection' (Karunaratne 1964) or from transepidermal elimination (see below), with the organism being 'thrust into the epidermis from the subjacent connective tissue' (Ashworth 1923; Figures 37 and 38). Endospores

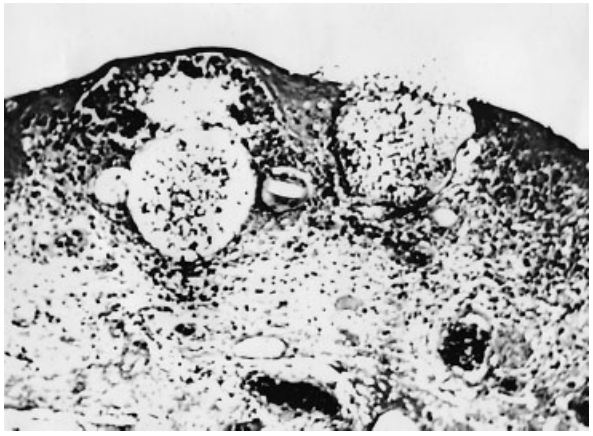

Figure 37 *Late stage of 'transepidermal elimination' of sporangia and endospores in ocular rhinosporidiosis. Note thinned epithelium. H&E; initial magnification ×400*

occur in both the mature sporangia as well as free, extrasporangially, in the tissues.

Fibromyxomatous or fibroedematous reactions occur in the subepithelial stroma with few or absent sporangia (Figure 39). Tirumurti (1914) noted myxomatous degeneration, especially in nasal polyps. Mild-to-moderate inflammatory cell infiltrates accumulate around a multitude of sporangia (Figure 40) and, more prominantly, around fee endospores. The cells include lymphocytes, plasma cells, histiocytes, neutrophils (especially around free endospores and with secondary infection), and rarely eosinophils; these are in varying proportions depending on the chronicity of the polyp. The paucity of eosinophils contrasts rhinosporidiosis with opportunistic, mycelial deep mycoses; there is also no eosinophilic precipitate (probably of antigen/immunoglobulin complexes) (Grover 1970), the Splendoré–Hoeppli phenomenon, seen around hyphae of *Entomorphthora coronata* (a cause of nasal polyps in horses) and *Basidiobolus ranarum*, and in other zygomycotic tissues (Williams 1969).

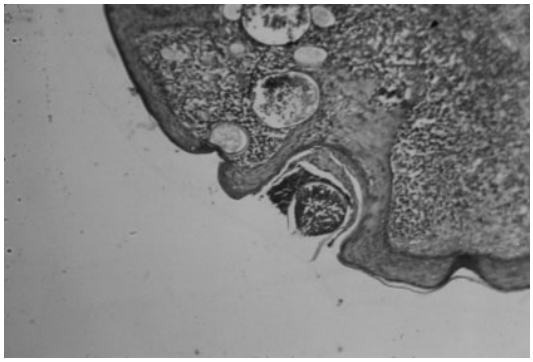

Figure 38 *Subcutaneous rhinosporidiosis with down-growth of squamous epithelium encircling a degenerate sporangium, illustrating 'transepidermal elimination' of degenerate sporangia. PAS stain; initial magnification ×200*

Figure 39 *Acanthotic squamous epithelium (a), fibromyxomatous stroma (f), with sparse cell infiltration in an area with scarce sporangia, in nasopharyngeal rhinosporidiosis. H&E; initial magnification ×400*

With secondary infection, neutrophils are numerous. Stromal microabscesses may occur (Jimenez et al. 1984). The predominant mononuclear cell in the stroma is the plasma cell (Kaye 1938). In more chronic lesions, lymphocytes and macrophages predominate. Invading connective tissue (Figure 22) or multinucleate giant cells (Figure 23) may be seen within degenerate or empty sporangia, which may also be calcified. Stromal giant cells occasionally contain juvenile sporangia (Karunaratne 1964). In a rare case of fatal, disseminated visceral rhinosporidiosis, Rajam et al. (1955) found relatively insignificant histopathology or evidence of inflammatory reactions around the sparse sporangia and endospores in the viscera.

Marked vascularity, resulting in focal hemorrhage in the polyp and severe external hemorrhage following surgery or trauma, occurs with the very vascular polyps, especially in nasal rhinosporidiosis (see Figure 19). Empty spaces (Figure 19) occur in the stroma and are derived from the destruction of tissue elements or from dilated glands or ducts in glandular tissues (Karunaratne 1936). They are commoner in nasal rhinosporidiosis (Beattie 1906). There is minimal fibrosis which is more marked in lesions that are regressing under dapsone therapy (Job et al. 1993).

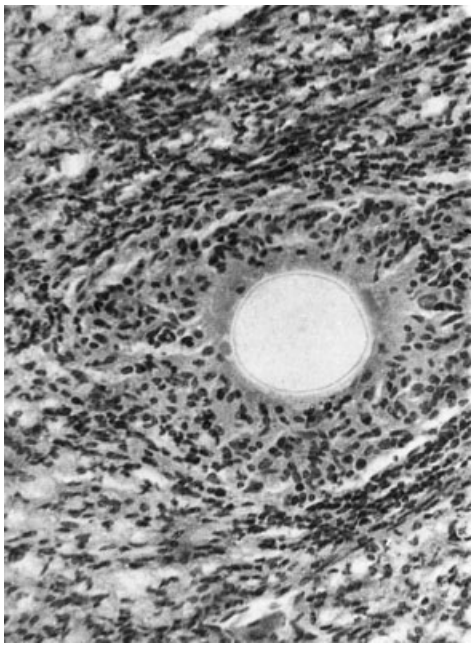

Figure 40 *Mononuclear cell infiltration around degenerate sporangium. H&E; initial magnification ×400*

Erosion of bone (Figure 36b) or cartilage with perforation of the nasal septum has been recorded (Atav et al. 1955; Chitravel et al. 1990; Gokhale et al. 1997).

TRANSEPIDERMAL (TRANSEPITHELIAL) ELIMINATION OF RHINOSPORIDIAL ENDOSPORES

This term was applied to the presence of rhinosporidial sporangia in subepithelial sites, with their endospores exiting through the breached epithelium (Figures 37 and 24.38). In only three reports (Thianprasit and Thagernpol 1989; Azadeh et al. 1994; Kennedy et al. 1995) has this term been used.

In what may be construed as the sequence of this process, the early stages of this phenomenon are characterized by the formation of downgrowths of the deeper layers of the epidermis to surround the mature dermal and subdermal sporangia (Figures 19 and 24.38). These downgrowths often appear histologically as 'whorls' (Arseculeratne et al. 2001; see Lever and Schaumberg-Lever 1990) and have also been described as a response to chemical irritants (Lever and Schaumberg-Lever 1990); their presence in rhinosporidial tissues is therefore not a specific reaction in this disease, and it might alternatively be regarded as a nonspecific defense reaction against irritants. This phenomenon was thought to represent a nonspecific defense mechanism of the host for the expulsion of rhinosporidial sporangia and endospores. Thinning of epithelium (Figure 37) which overlies the sporangia was thought to result from pressure from the underlying sporangia.

On a parallel with the amphibian skin pathogen *Batrachochytrium* (Daszak et al. 1999), it was postulated (Arseculeratne et al. 2001) that the phenomenon in rhinosporidiosis could, on the other hand, be the pathogen's mechanism for endospore dispersal. The apparent migration of the mature sporangia towards the surface of the epithelium could be due to the dissolution of the tissues by histolytic enzymes of *R. seeberi* rather than through a mere effect of pressure which forces the sporangium towards the epithelium. Broz and Privora (1952) described a similar appearance in the granulomatous lesions in frogs caused by *Dermocystidium*: 'When the cyst reaches the surface of the skin, it becomes exposed, owing to ulceration of the epithelium, and its membrane ruptures, setting the spores free.' *Dermocystidium* is now included with *Rhinosporidium* in the proposed Class Mesomycetozoea (Herr et al. 1999b). In Figure 2 of Wallin et al.'s (2001) paper on rhinosporidiosis in a cat, 'the ''nipple-like'' projection', of the mature sporangium projected into the nasal cavity; this appearance recalls that described by de Mello (1954) of the sporangial pore that is directed towards the surface of the epithelium.

The conceivable defensive role of the epithelium on the part of the host, together with the apparent migration of the sporangia towards the surface of the epithelium in the postulated pursuance of the release of the endospores on the part of the pathogen, constitute the phenomenon of 'transepidermal (epithelial) elimination' of sporangia and endospores.

MODE OF SPREAD

Rhinosporidial infection spreads locally, regionally, and to distant, anatomically unrelated sites.

From upper respiratory sites, especially the anterior nares where the disease most often begins, local and regional spread occurs in the nasal passages, extending to the nasopharynx, lacrimal apparatus, and paranasal sinuses; in these instances, the spread is probably through autoinoculation (Weller and Riker 1930; Karunaratne 1936) of adjacent sites by endospores released spontaneously from adjacent polyps or after surgery. In Ingram's case (1910), the initial growth in the external urethral meatus spread to involve the glans within a period of 2 years, thus spreading more slowly than in other sites. Dissemination refers to the occurrence of lesions in sites such as the limbs and viscera that are anatomically unrelated or distant from the initial foci in the upper respiratory passages.

Lymphatic spread has not been documented, although postulated (Ashworth 1923) in cutaneous and subcutaneous rhinosporidiosis (Agrawal et al. 1959; Agarwal 1966), although such spread will not explain dissemination to anatomically unrelated sites. None of the numerous reports on rhinosporidiosis has described the occurrence of lymphadenitis. de Mello (1954) stated that 'lymphatic nodes are not affected,' nor did Pumhirun et al. (1983) find enlarged lymph nodes in their case of nasopharyngeal and facial rhinosporidiosis. The presence

of enlarged inguinal and epitrochlear lymph nodes in a case of disseminated rhinosporidiosis was noted by Agrawal et al. (1959) though the histology of the nodes was not commented on. Neither Chatterjee et al. (1977) nor Agarwal (1966) who studied 27 cases, found enlargement of lymph nodes in their cases of disseminated disease.

Inguinal lymphadenitis was recently described (Arseculeratne et al. 2001) in a Sri Lankan patient with disseminated disease involving the lower limbs. The criteria on which lymph node involvement was made included the location of the mass in the site of inguinal lymph nodes, the histological presence of a capsule in the mass, trabeculae from the capsule towards the center of the mass, a subcapsular sinus, and masses of lymphocytes which probably represented the cortical follicles (Figure 41).

Lymphadenitis in rhinosporidiosis is apparently rare, contrasting with its frequency in systemic mycotic disease; this rarity might be based on mechanisms of immune evasion in rhinosporidiosis which are discussed below.

There is also histological evidence for hematogenous spread of rhinosporidiosis to anatomically distant sites; Rajam et al. (1955) identified endospores and sporangia in the lumen of renal blood vessels, urine, ascitic fluid, and viscera; these authors' case is of interest in that growths were not found in the nose, nasopharynx, or urethra, which are common initial sites of rhinosporidiosis. Sporangia with endospores were also found within blood vessels in the case of Ho and Tay (1986). Figure 34 illustrates facial polyps in a Sri Lankan patient that developed a few years after excision of a nasal polyp; amongst 20 other rhinosporidial masses on the rest of this patient's body was a pedunculated mass on the chest wall (Figure 42) and a large subcutaneous mass that appeared at the site of a previous closed injury on the leg (Figure 43). Hematogenous spread to these sites could have followed the nasal infection.

Concurrent diseases

Infections or malignancies concurrent with rhinosporidiosis, in the same or different sites or adjacent tissues, have been recorded; in conjunctival rhinosporidiosis with papillomavirus (Noor-Sunba and al-Ali 1989); papovavirus infection (Noor-Sunba et al. 1988); squamous carcinoma of the tongue (Karunaratne 1936), Ahluwalia and Bahadur 1990); leprosy with nasal (Allen and Dave 1936; Rosenbaum and Gan 1948) and cutaneous rhinosporidiosis (Porichha et al. 1986; Jayakumar et al. 1995); syphilis (Satyanarayana 1960); rhinoscleroma (Al-Serhani et al. 1998) in humans; and schistosomiasis in bovines (Rao 1938). Rhinosporidiosis coexisted in 50 percent of bullocks with schistosomiasis of the nose (Datta 1965). Jain (1967) quoted Patnaik that rhinosporidiosis was not coexistent with schistosomiasis; in Sri Lanka, bovine nasal scrapings, from abattoir samples, showed schistosomes in approximately 75 percent of buffaloes and in 40 percent of cattle, while none had concomitant rhinosporidial endospores (Arseculeratne and Rajapakse 2001, unpublished data).

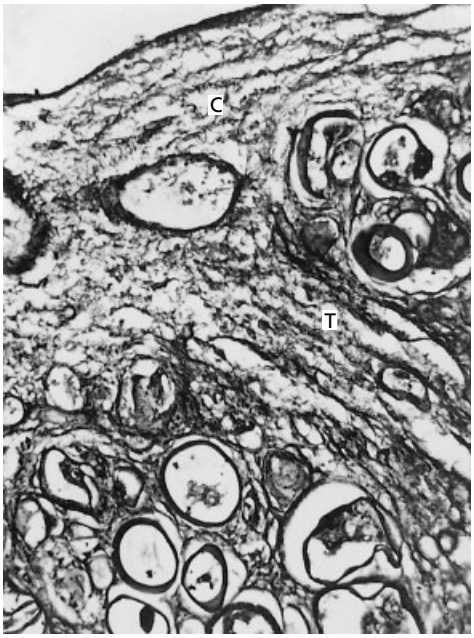

Figure 41 *Inguinal lymphadenitis in disseminated rhinosporidiosis affecting lower limbs. C, capsule of lymph node; T, trabeculae from capsule to parenchyma, with degenerate sporangia in node's cortex. Reticulin stain; initial magnification ×400*

Figure 42 *Globular, compact, pedunculated rhinosporidial mass (2 cm × 3 cm) on posterior chest wall in a case of disseminated rhinosporidiosis.*

Figure 43 *Rhinosporidial mass on the tibia mimicking a malignant tumor, a nonulcerated rhinosporidial lump on the knee, and an ulcerated rhinosporidial mass on the hand, in a patient with disseminated rhinosporidiosis.*

It is not clear whether the concurrence of these diseases with rhinosporidiosis has a causal, sequential, or coincidental basis. In view of the occurrence of suppressor immune reactions in rhinosporidiosis, discussed under Immunity below, local or generalized cell-mediated immunity (CMI) anergy might occur with some of these associated diseases. In cats, rhinosporidiosis was not associated with the feline leukemia and feline immunodeficiency viruses (Wallin et al. 2001). There is no evidence in the voluminous literature that rhinosporidiosis is an opportunistic infection, although Stricker et al. (1999) reported a case of a human immunodeficiency virus (HIV)-positive man who had an unusual infection which these authors claimed to be rhinosporidiosis; their histopathological section photograph, however, does not reveal the different stages of *R. seeberi*'s development which are invariably seen in rhinosporidial tissue (Figure 3). In the extensive literature on clinical rhinosporidiosis, from diverse environments, such an association between an identified immunodeficient state and rhinosporidiosis has not been recorded.

IMMUNITY

Information on mechanisms of nonspecific and specific adaptive antirhinosporidial immunity would be necessary to explain some predominant clinical characteristics of the disease – chronicity, spontaneous regression, recurrence after surgery, and occasional dissemination. Such data on mechanisms of antirhinosporidial immunity were scarce until recently, undoubtedly due to the absence of a method for obtaining pure preparations of *R. seeberi*, uncontaminated by host tissue and other microorganisms, and consequently the lack of reagents for suitable immunological tests in diseased hosts as well as in vitro. A further lack was an experimental model of rhinosporidiosis.

Only one report is available on mechanisms of antirhinosporidial humoral immunity (Chitravel et al. 1982) that described the use of crude preparations of endospores and sporangia obtained from human polyps. Homogenates after filtration and centrifugation were used with Freund's complete adjuvant as antigens for the preparation of antirhinosporidial antibodies. These authors were not able to demonstrate antirhinosporidial antibody in patients by double diffusion tests in gels or counterimmunoelectrophoresis (CIE), but reported that rhinosporidial antigen(s) were detected in rhinosporidial patients' sera by gel diffusion tests. Kwon-Chung and Bennett (1992) pointed out that Chitravel's gel precipitin lines could have been due to human antigens that contaminated the crude rhinosporidial antigen preparations and which reacted with antihuman antibody from the experimental animals that were immunized with the crude antigen preparations. If these preparations did not contain mature sporangia, then new rhinosporidial antigens which appear only in maturing sporangia (Herr et al. 1999c), might also have been excluded. Crude preparations of endospores and sporangia were also used by Reddy and Lakshminarayana (1962) in agglutination tests, which, however, gave inconclusive results, as agglutination was caused by sera from normal humans as well as from patients with rhinosporidiosis.

New information on cell-mediated and humoral antirhinosporidial immunity in human rhinosporidial patients and in experimental mice is now available and is reviewed below.

Cell-mediated immune responses

Cell-mediated immune responses (CMI) in rhinosporidiosis was investigated by Reddy and Lakshminarayana (1962) by the 'cutaneous' injection of crude preparations of rhinosporidial endospores. Transient erythematous reactions were produced in normal persons as well as in rhinosporidial patients, and these authors concluded that these reactions were nonspecific.

The only definitive report on cell-mediated immunity in rhinosporidial patients was that of Chitravel et al.

(1981); they used the Leukocyte Migration Inhibition (LMI) test and found that, in patients with disease of less than 9 years' duration, LMI was detectable but that the inhibition decreased when the disease was of longer duration. These authors suggested that immunological 'unresponsiveness or tolerance' might explain this finding. Our data on antirhinosporidial CMIR in mice, discussed below, elucidates Chitravel's results.

CMI responses in human rhinosporidiosis were recently described (de Silva et al. 2001). Two approaches were used:

1. immunohistochemistry with monoclonal antibodies against specific markers on cells in human rhinosporidial tissues
2. in vitro lymphoproliferative responses (LPR) of peripheral blood lymphocytes from rhinosporidial patients to the T-cell mitogen concanavalin A (conA) and to sonic extracts from Percoll-purified rhinosporidial endospores and sporangia.

The cell infiltrate in human rhinosporidial polyps showed similar patterns despite their origin from different clinical presentations and of differing durations. In composition, the infiltrates were mixed; neutrophils were abundant, CD20+ B cells were present in considerable numbers with plasma cells, CD68+ macrophages (Figure 44), some of which were foamy, and CD3+ T lymphocytes, were numerous. CD4+ helper T lymphocytes were scarce, while the presence of CD8+ T-cytotoxic/suppressor lymphocytes was marked; CD8+ cytotoxic/suppressor T lymphocytes were found specially around and within mature sporangia (Figure 45).

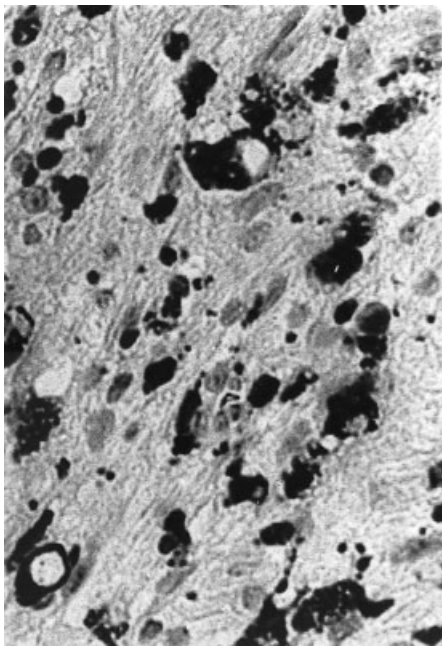

Figure 44 *CD68+ macrophages in rhinosporidial stroma, visualized with monoclonal antibodies-Dako Fast-red. (Reproduced with permission from* Mycopathologia*, 2001;***52***: 59–68)*

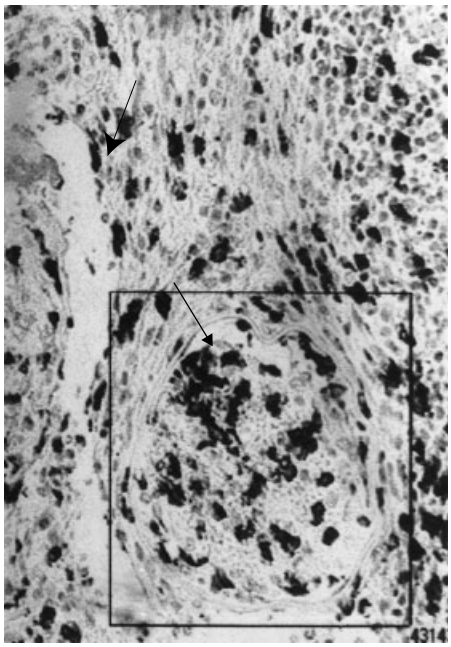

Figure 45 *CD8+ T lymphocytes in rhinosporidial stroma and within a mature, degenerate sporangium (arrow), visualized with monoclonal antibodies-Dako Fast-red. (Reproduced with permission from* Mycopathologia*, 2001;***52***:59–68)*

There were many TIA1-positive lymphocytes of the cytotoxic subtype located especially around the sporangia. CD56/57+ NK lymphocytes were less numerous than CD8+ T lymphocytes and were also located around mature sporangia.

In LPR assays in vitro (de Silva et al. 2001), rhinosporidial lymphocytes showed stimulatory responses (stimulation indices (SI) >1, Figure 46) with conA, but the majority of these samples showed significantly diminished responses to rhinosporidial antigens, indicating that suppressor responses could occur in human rhinosporidiosis. The SI of lymphocytes from control (nonrhinosporidial) persons and the SI of lymphocytes from rhinosporidial patients, to conA showed no significant differences, indicating that the rhinosporidial patients had general adequate cell-mediated immune competence. There was, however, no correlation between the intensity of the depression of the LPR-SI to rhinosporidial antigen and the site, duration of the disease, the number of lesions, or to the presence of dissemination. These results demonstrate that while on the one hand a CMIR does develop in human rhinosporidiosis, suppressor responses, on the other hand, also occur.

In some patients, the peripheral blood lymphocytes showed a significantly lower proliferative response to rhinosporidial antigen than to conA, suggesting that the suppression was antigen-specific; the rhinosporidial antigen did not suppress the response to conA when the two agents were mixed, nor was the antigen toxic to the lymphocytes.

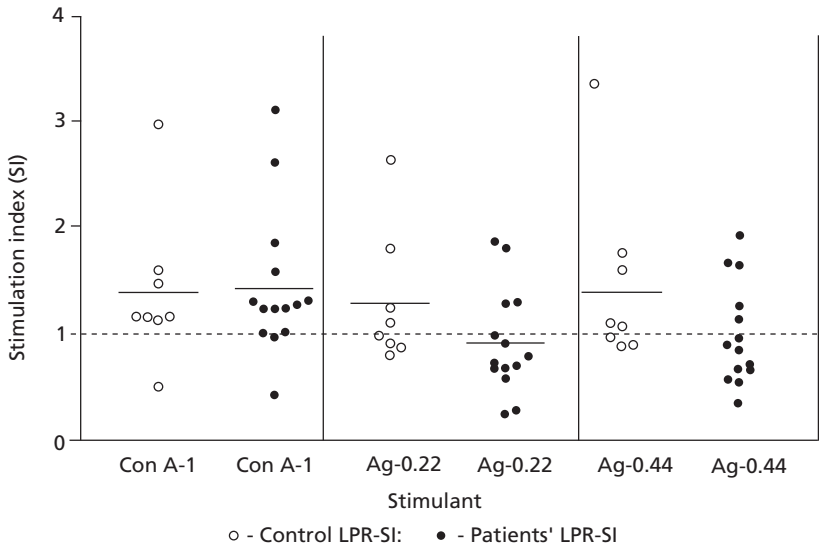

Figure 46 *Scatter of LPR-SI of control subjects and rhinosporidial patients to conA and rhinosporidial extract (RhA) as stimulants. Horizontal bars represent mean values. Open circles, control LPR-SI; filled circles, patients' LPR-SI. (Reproduced with permission from* Mycopathologia, *2001;***52***:59–68)*

The delayed-type hypersensitivity (DTH) Mantoux reactivity to PPD-S from *Mycobacterium tuberculosis* was markedly depressed (to approximately 25 percent) in most Sri Lankan patients with rhinosporidiosis (with or without prior bacille Calmette–Guérin (BCG) vaccination), although the Mantoux reactivity was far more pronounced in normal and BCG-vaccinated populations (75 percent); no correlation was found between Mantoux-negativity and the clinical state of rhinosporidiosis – whether respiratory or in other sites, localized or disseminated, or with single or multiple polyps – as with the lymphoproliferative responses in vitro (de Silva et al. 2001). Rhinosporidial antigen prepared from purified, sonicated endospores and sporangia from the polyps of one patient with disseminated disease, injected intradermally in the same patient, gave a negative skin reaction, correlating with the significantly depressed lymphoproliferative response (SI < 1) in this patient.

The CMI anergy demonstrated in rhinosporidial patients and in in vitro tests needs further study for the determination of whether the suppression is a result of general, nonspecific suppression of CMI or is antigen-specific.

CMI responses in experimental animals have hitherto not been examined. CMIR to *R. seeberi* in experimental mice were recently studied by the quantitative footpad response of the DTH, with histopathology of the treated footpads for confirmation of the DTH-type response. Jayasekera et al. (2001) demonstrated that sonicated suspensions of rhinosporidial endospores and sporangia, used for sensitization and challenge, evoked well-marked DTH-type footpad responses of a magnitude similar to that evoked by T-dependent antigens such as sheep red blood cells. The footpads challenged with rhinosporidial extracts showed histopathology (Figure 47) which was typical of DTH reactions in the mouse.

An interesting finding in the mouse experiments was that, in comparison with the results after a single sensitizing dose of rhinosporidial antigen(s), repeated sensitization resulted in the decrease in the intensity of the DTH footpad response; conversely, the humoral immune response was significantly elevated. This phenomenon of immune deviation has been ascribed (Mosmann and Sad

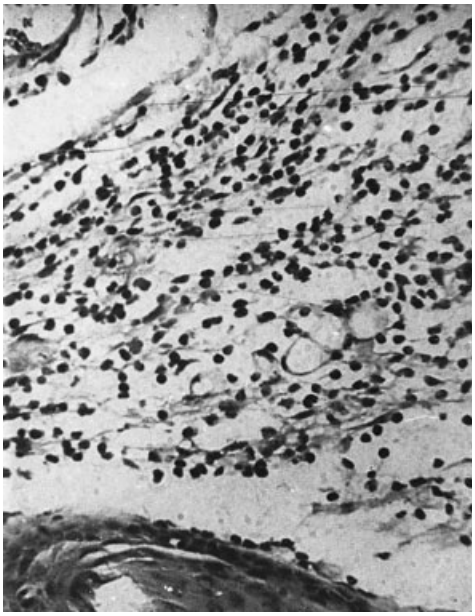

Figure 47 *Footpad of mouse showing DTH reaction to challenge following sensitization with extract of rhinosporidial endospores/sporangia, with infiltrate of neutrophils, macrophages and lymphocytes. H&E; initial magnification ×400*

1996) to the switch, on repeated administration of antigens, from activation of CD4+ Th0 cells to the production of CD4+ Th2 cells which encourage antibody production, after an initial production of CD4+ Th1 cells which induce DTH. The ensuing decrease of DTH reactivity with the switch from Th1 to Th2 might contribute to decreased antirhinosporidial cell-mediated immunity and might explain the chronicity, recurrence, and dissemination in clinical rhinosporidiosis. This finding might also explain Chitravel et al.'s observation (1981), that the intensity of LMI waned when the duration of the disease was greater than 9 years.

Humoral immune responses

Jain (1967) used a suspension of 'spores' as antigen, injected subcutaneously in humans, and concluded that '... this disease does not produce any sensitizing antibodies' as no reactions were produced in either one hour or after 3 days.

Reddy and Lakshminarayana (1962) used suspensions of endospores and sporangia from homogenized rhinosporidial tissues, in agglutination tests for experimental antirhinosporidial antibody and observed nonspecific 'clumping.' The same antigen was used in 'cutaneous' tests on patients and normal persons, but no specific reactions were detected.

Chitravel et al. (1982) reported that antirhinosporidial antibody was not detected in patients by immunodiffusion or CIE; they used suspensions of endospores and sporangia, that were ground with glass powder. Precipitin lines were, however, detected in our (Arseculeratne et al. 1999) CIE tests with antigens released by ultrasonic disintegration of Percoll-purified endospores/sporangia, and human rhinosporidial sera, and the possibility remains that Chitravel's antigen preparations had insufficient amounts of soluble antigen. Antirhinosporidial humoral immune responses (HIR) was also detected in experimental mice (Jayasekera et al. 2001) and rabbits (Atapattu et al. 1999) by indirect immunofluorescence tests which used Percoll-purified, sonically disrupted endospores and sporangia as antigen. Rhinosporidial patients' sera, tested by indirect immunofluorescence, had appreciable titers (over 1/320) whereas only two of 30 clinically nonrhinosporidial persons showed low (1/30) titers; these two persons were from an area endemic for rhinosporidiosis and might have been subclinically exposed to the organism with consequent immunization. Antibody titers of over 1/320, especially of IgG and IgM, were also detected in patients' sera in immunodot blot assays on nitrocellulose paper, with specific antihuman immunoglobulin phosphatase-conjugated tracers (Arseculeratne et al. 1999 and unpublished data 2002).

It can be concluded that *R. seeberi* shows a variety of mechanisms of evasion of immune responses, which might explain some hitherto enigmatic aspects of rhinosporidiosis, viz. chronicity, recurrence, and dissemination:

- antigenic variation, based on the findings of Herr et al. (1999c)
- immune suppression as demonstrated by de Silva et al. (2001)
- immune distraction: the cell-infiltration patterns in rhinosporidial tissues in some cases indicate that infiltrates occur in some areas of the tissue in which rhinosporidial bodies are absent, suggesting that the infiltrates were in response to free rhinosporidial antigen. If this were the case, the possibility of immune distraction by free antigen in 'mopping up' of antirhinosporidial antibody might conceivably contribute to immune evasion by *R. seeberi* (Arseculeratne et al. 2001)
- immune deviation: experimental studies on CMIR in mice to *R. seeberi* (Jayasekera et al. 2001) suggest the occurrence of immune deviation which might further contribute to immune evasion through decreasing antirhinosporidial CMI reactivity, if, indeed, antirhinosporidial CMIR is protective
- binding of host immunoglobulins (Atapattu et al. 2000)
- antigen sequestration: the thick wall of the endospores which contains chitin and cellulose is conceivably impermeable to release of antigens or to immune destruction of the endospores, as perhaps with encysted parasites (Abbas et al. 2000). Selective permeability, however, to smaller molecules probably exists in rhinosporidial endospores, as shown by the entry of Evan's blue and MTT into endospores as described above. Moreover, the endospore of *R. seeberi* is not comparable with the encysted resting stage of amebae but is a vegetative stage in its life cycle.

It is interesting that several of these mechanisms of evasion of immune responses have also been described in parasitic infections, notably malaria (Goonewardene et al. 1990; see also Kierszenbaum 1994) and in fungal infections (chromomycosis, Fuchs and Pecher 1992; see also Cuff et al. 1986 on *Candida albicans*).

DIAGNOSIS

Urgency in diagnosis of rhinosporidiosis arises from its mimicry of malignant tumors (Figure 43).

Clinical clues to diagnosis

The macroscopic features of polyps and the clinical presentation, especially of nasal polyps, facilitate diagnosis. Rhinosporidiosis presents as single, or less frequently multiple, polyps in respiratory or ocular sites. The polyps are strawberry-like, red due to vascularity, with a granular surface that has yellowish, pinhead-sized spots. The polyps are friable and fragile, very vascular,

and bleed easily on handling. Lobulation, especially in nasopharyngeal sites, is common. Growths on the skin are often multiple and wart-like.

Differential diagnosis varies with the site. Urethral rhinosporidiosis needs differentiation from caruncles; nasal rhinosporidiosis from hypertrophic rhinitis, allergic polyps, vascular polyps, angiomas and fibromas, carcinoma (Ravi et al. 1992; Lourenco and Costa 1996), granulomatous leishmaniasis, leprosy, rhinoscleroma, and mycotic granulomas. Rhinosporidial lesions on the skin of the body may simulate viral warts, sarcomas, and leprous, tuberculous, or cryptococcal nodules.

Disseminated rhinosporidiosis is not uncommon. A history of nasal polyps and their surgical excision is often given, and the disseminated lesions appear several months or even years after the primary lesions. Growths of diverse appearances – pedunculated or sessile polyps or masses – appear on the body, face, scalp, limbs, and in the viscera (lungs, kidneys, liver, spleen, and brain). Rhinosporidial masses are accompanied by osteolysis and fractures of underlying bones (Figure 36b; Gokhale et al. 1997). Lymphadenitis, though hitherto unreported in rhinosporidiosis, was detected in a case of disseminated rhinosporidiosis in Sri Lanka as described above.

The contents of closed, subcutaneous lumps need microscopic examination of needle aspirates. The surface of mucosal polyps is often coated with mucus that contains numerous endospores which afford a convenient method for cytodiagnosis with PAS or silver-stained smears.

DIFFERENTIAL DIAGNOSIS

Rhinosporidiosis mimics, macroscopically, several infective and noninfective diseases. Noninfective diseases include:

- in the rectum: hemorrhoids and polyps
- in the nasal passages: papillary adenocarcinoma, hypertrophic rhinitis, beningn polyps
- in the eye: pterygia, mucocele of the lacrimal sac
- penile and urethral sites: squamous carcinoma, condylomata
- on the body: sarcoma, verruca vulgaris, fibroma, angiofibroma.

Infective diseases include rhinoscleroma, cryptococcosis, chromo(blasto)mycosis, histoplasmosis, infections caused by *Chrysosporium (Emmonsia) parvum* vars. *parvum* and *crescens*, blastomycosis, and paracoccidiodomycosis. The agents of these diseases have distinguishing morphological and tinctorial features and may also be cultured in vitro and in experimental animals, especially mice.

HISTOPATHOLOGY

The definitive diagnosis of rhinosporidiosis is by histopathology on biopsied or resected tissues. A histopathological diagnosis is based on the classical descriptions of rhinosporidial pathology, of Beattie (1906), Tirumurti (1914), Ashworth (1923), and Karunaratne (1964). Most histological sections contain the different developmental stages of the pathogen (Figure 4), and these appearances are more specific than the tissue responses – stromal or cell infiltrates – of the host; useful stains are H&E, PAS, mucicarmine, and Gomori's methenamine silver.

Autofluorescence of the sporangia and endospores, in sections of paraffin-embedded tissues, has been noted (Senba and Yamashita 1985; Moses et al. 1991) and merits consideration in tests that involve the use of fluorescent tracers.

Notable features of rhinosporidial histopathology include variations in the host responses:

- within the same specimen
- in different sites of the same and in different hosts, in respect of the density of the developmental stages
- in the stromal and cellular response (Arseculeratne et al. 2001).

Well-marked cell infiltrates, especially of macrophages and lymphocytes, occur around both intact, apparently viable sporangia that contain intact endospores as well as around degenerate, empty sporangia. Cell infiltrates could also be sparse around degenerate or intact sporangia in other regions of the same tissue and in samples from different patients. Kennedy et al. (1995) described similar histopathological variations in rhinosporidial tissues from swans; three patterns were identified:

- a severe, focal or diffuse acute and chronic inflammatory cell infiltrate
- granulomatous inflammation with epithelioid histiocytes and foreign body-type giant cells
- a fibroblastic reaction with paucity of sporangia and an inflammatory cell infiltrate, that was infrequent.

Rhinosporidial endospores and sporangia in disseminated cases involving the kidneys and liver might not be associated with a surrounding inflammatory cell infiltrate, although these bodies in the lung and subcutaneous tissues were found to have been surrounded by well-marked cell infiltrates (Agrawal et al. 1959).

Problems in histopathological diagnosis

Despite the ease of histopathological diagnosis through the ubiquitous developmental stages of *R. seeberi* in most rhinosporidial tissue, there are, however, problematic instances from which arise confusion, misdiagnosis, or a false-negative diagnosis. These instances which were described elsewhere (Arseculeratne et al. 2001) consisted of:

- rhinosporidial tissue in which rhinosporidial bodies were absent despite the presence of marked cell

infiltrates while other, more vascular, portions of the polyp, however, had rhinosporidial bodies; this problem occurs especially in the variegated polyps of nasopharyngeal rhinosporidiosis. Selection of typical portions of such polyps for histology might minimize the occurrence of a false-negative diagnosis
- atypical sporangia in which the well-marked, thick wall was absent
- the presence of only fragments of the sporangial wall, while endospores might or might not be present
- the absence of typical rhinosporidial bodies although PAS-positive bodies which are similar to the rhinosporidial endospores might be present indicating incipient or slow-growing rhinosporidiosis.

A noteworthy feature in rhinosporidial histopathology is the absence of the Splendoré–Hoeppli (antibody-mediated) reaction around rhinosporidial bodies. This reaction is often present in (mycelial) mycotic and some bacterial infections. This absence is remarkable in that rhinosporidial patients show high titers of anti-rhinosporidial antibody.

Differential histopathological diagnosis

A problem in differential histopathological diagnosis is that of subcutaneous spherulocystic disease (myospherulosis). Hutt et al. (1971) described it as 'reminiscent of an endosporulating fungus such as rhinosporidiosis;' McClatchie and Bremner (1969) noted it as a 'distant cousin of rhinosporidiosis.' The cysts of myospherulosis remain unstained with Gomori methenamine silver or with mucicarmine and no spores are present inside the cysts. The bodies in myospherulosis were regarded as erythrocytes, altered by exogenous or endogenous fat or lipids, or as derived from malignant cells or macrophages (Chau et al. 2000, see also Ali et al. 2001).

Histopathological differential diagnosis might also consider coccidiodomycosis in which *C. immitis* has mature stages which consist of large, thick-walled bodies which are smaller than the sporangia of *R. seeberi*, but which also contain endospores which are also smaller than those of *R. seeberi* and do not contain EDB.

CYTODIAGNOSIS

Cytodiagnosis is a useful method in diagnosis on aspirates from nonulcerated lumps in extrarespiratory sites (Kamal et al. 1995; Kavishkar et al. 1998), smears of the endospore-laden mucoid covering on the surfaces of polyps in the respiratory passages and from ulcerated polyps elsewhere in the body, washings of sinuses and bronchi, bronchial brushings, or fine-needle aspirates from bones (Pai et al. 1996). With suitable stains such as Gomori methenamine-silver, Papanicolaou's, PAS, Mayer's mucicarmine, and other stains, diagnostic features, especially of the endospores with their unique EDBs, are revealed (Ashworth 1923; Allen and Dave 1936; Kaye 1938; de Mello 1954; Rajam et al. 1955; Thomas et al. 1956; Vanbreuseghem 1958; Khan et al. 1969; Fortin and Meisels 1974; Chaudary et al. 1986; Jaiswal et al. 1992; Van der Coer et al. 1992; Gori and Scasso 1994; Maru et al. 1999). Wet mounts of the mucoid material on rhinosporidial polyps would also show the endospores with their EDBs (Figure 10).

In posterior respiratory sites, respiratory (columnar, ciliated) epithelial cells with their large nuclei could mimic endospores with the 'tail of the comet' appearance that is caused by the residue of the intrasporangia, mucoid material; caution was advised (Arseculeratne 2000a) in the identification of endospores in these sites (Figure 9). The PAS stain is particularly useful in this differentiation; the epithelial cell's nucleus is PAS-negative whereas the endospore stains markedly magenta.

Allen and Dave (1936) used nasal smears for monitoring antirhinosporidial chemotherapy, although it is noteworthy that the presence of endospores in nasal smears did not always correlate with the presence of nasal growths; smears from nasal passages after resection of the polyps have shown endospores with subsequent recurrence of the growth.

TREATMENT

Spontaneous regression of rhinosporidial polyps has been noted in humans (Forsyth 1924; Allen and Dave 1936; Karunaratne 1964; Anand et al. 1975) and in swans (Kennedy et al. 1995); it is rare, and surgical excision of the polyp, in accessible sites, remains the mainstay of treatment. Cauterization of the base of the excised polyp, especially in sessile growths, is recommended (Desmond 1953; Samaddar and Sen 1990; Ravi et al. 1992) to prevent, or at least minimize, recurrence. Excision with a snare was considered to result in dissemination and recurrence (Khan et al. 1969). Spillage of endospores by mere handling of the polyp or from its cut surface is probably responsible for the recurrence or development of new lesions on adjacent sites; this process was termed 'autoinoculation' (Karunaratne 1964). Satyanarayana (1960) esimated the recurrence rate after surgery at 11 percent, whilst Sasidharan et al. (1987) reported a recurrence rate of 25 percent in penile rhinosporidiosis.

Cryosurgery has also been used. Extensive growths have necessitated amputation, of the penis in disease of the glans, or of the foot or hand. Since rhinosporidial polyps in respiratory sites are very vascular, extensive hemorrhage needs caution (Khan et al. 1969).

Numerous antimicrobial agents – antimony compounds, bismuth, iodine, and pentamidine (Rajam et al. 1955), antifungal agents griseofulvin, amphotericin B, and topical steroids (Jimenez et al. 1984; Ho and Tay 1986), 5-fluorocytosine, potassium iodide, ketoconazole), quinine hydrochloride (Wright 1922), paludrine, pentamidine, antibacterial antibiotics, and deep x-ray therapy

(Satyanarayana 1960) – have not been demonstrated to have any effect. Trivalent or pentavalent compounds of antimony have been reported to have been of some effect and Wright (1922) claimed that ocular rhinosporidiosis responded to 2 percent tartar emetic (antimony potassium tartrate). Allen and Dave (1936) found 'neostibosan' to have been effective, with the disappearance of endospores from nasal secretions. Dapsone is the only drug that has been found, on clinical and microscopic grounds, to have a proven beneficial effect; it minimized recurrence and produced firmer, more fibrous, and less vascular nasal and nasopharyngeal polyps (Nair 1979); recurrence occurred in 28.6 percent of dapsone-treated patients whereas 93 percent of untreated patients required surgery for recurrent disease after a 3-year period. Histologically and ultrastructurally, it caused the arrest of the maturation of the sporangia and promotion of a granulomatous response with fibrosis (Job et al. 1993, see also Krishnan 1979; Venkateswaran et al. 1997). Its therapeutic role is, essentially, as an adjunct to surgery.

Failure of drug therapy was attributed to the impenetrability of the sporangial wall (Woodard and Hudson 1984).

RHINOSPORIDIOSIS IN ANIMALS

Several domesticated species [dogs (Easley et al. 1986), cats (Moisan and Baker 2001; Wallin et al. 2001), parrots], farm species [bovines (buffaloes and cattle), goats (Jain 1967), horses, mules, ducks, geese, swans (Kennedy et al. 1995)], and wild animals [the river dolphin *Inia geoffrensis* (Lipscomb 1998)], and water fowl have shown natural rhinosporidiosis. The only epizootic, of ocular and nasal rhinosporidiosis, occurred in swans (Kennedy et al. 1995). Nasal disease is more common in mammals. Rhinosporidiosis in animals is comparable with the disease in humans in many respects (Karunaratne 1964):

- predominance in males
- occurrence in India, South America, and South Africa
- general characteristics of the growths
- histopathological patterns and their variations.

In swans, Kennedy et al. (1995) described the epithelium as attenuated or acanthotic. The degree of acute or chronic inflammation was mild to moderate and showed three patterns:

1. a severe, focal to diffuse, acute and chronic cell infiltrate
2. a granulomatous reaction with epithelioid histiocytes and foreign body-type multinucleate giant cells commonly found centered around ruptured or collapsed, crescent-shaped sporangia
3. the least common pattern with fibroblasts usually associated with a paucity of sporangia and inflammatory cells.

These patterns are similar to those in human, equine (Rippon 1988), and canine (Easley et al. 1986) rhinosporidiosis.

There are no data on immunological responses in naturally occurring rhinosporidiosis in animals.

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Blastomycosis

ARTHUR F. DI SALVO AND BRUCE S. KLEIN

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ETIOLOGIC AGENT

Blastomyces dermatitidis is the etiologic agent of blastomycosis. This thermally dimorphic, presumably terricolous, fungus causes a primary acute or chronic infection of the respiratory system. In many instances the disease manifests itself as cutaneous lesions, but the fungus may infect almost any organ. *B. dermatitidis* exists in a yeast-like form in tissues or when incubated at 37°C. In nature or when incubated at 25°C on Sabouraud's glucose agar (SGA), it appears as a white, cottony mold. A definitive identification is made by the isolation of the etiologic agent. Prompt diagnosis requires a knowledgable physician and an astute microbiologist.

HISTORY

In 1894, Gilchrist, Assistant Dermatologist at Johns Hopkins Hospital in Baltimore, MD, presented a preliminary report on the first patient with blastomycosis (Gilchrist 1894). Formalinized tissue from the patient was referred to him as a case of scrofuloderma (cutaneous tuberculosis). The clinical appearance, according to the referring physician, was classic for this disease. The cutaneous manifestations of tuberculosis were common at that time and justified the diagnosis. Gilchrist examined the tissue histologically but was unable to find tubercle bacilli. However, he described the 'numerous curious bodies' that he saw in the excised tissue. Gilchrist called this disease process a protozoan dermatitis but expressed the opinion that the organisms were more probably of plant origin.

By the time he published an extended report on this patient he was convinced that the causative agent was a 'blastomycete,' a general term used at that time to refer to the yeasts (Gilchrist 1896). The histopathological description was thorough and complete. The illustrations in that paper demonstrate all the classic characteristics of this organism; the thick-walled, budding yeast-like cells with a broad base are clearly shown.

In his second report, also published in 1896, Gilchrist, with his colleague Stokes, described the remaining salient features of blastomycosis in another patient (Gilchrist and Stokes 1896). As these physicians had the opportunity to see this second patient, tissue specimens were taken for culture as well as histopathology. The typical unicellular tissue-form cells of *B. dermatitidis* were again described together with a complete histopathological account. The cultures from the diseased tissue grew in both their yeast and mycelial forms. Their observation of hyphae with distal and lateral cells or conidia on the mycelium led them to conclude that this agent was a filamentous fungus and not a true yeast.

The authors also observed that the mycelial form was not seen in tissues. They were inclined to separate *B. dermatitidis* from the other pathogenic 'blastomycetes' which later came to be known as species of *Candida* and *Cryptococcus*. As part of the investigation, they inoculated a dog intravenously with a pure culture of the patient's isolate. From the postmortem examination of this dog, they observed and reported on the

gross and microscopic features of bilateral pulmonary blastomycosis.

Extended observations of the isolate from the second patient were published in 1898 (Gilchrist and Stokes 1898). They detailed the budding mechanism, the various tissue stains which aided in the visualization of the organism, and the inflammatory reaction in the tissue. Extensive experiments with the isolate were also performed. They described the septations in the hyphae, observing the morphological variations of the yeast-like cells and mycelium, but did not recognize the thermal cause for the two forms. The isolates were found to be relatively inert biochemically.

In the early 1900s, both pulmonary and cutaneous blastomycosis were frequently misdiagnosed as manifestations of tuberculosis. At that time mycobacterial infections were more prevalent in the general population and there was considerably more medical knowledge of tuberculosis and its etiologic agent. Those clinicians who became familiar with blastomycosis were able to make the correct diagnosis.

During the next 10 years, many case reports of blastomycosis appeared in the literature. Ricketts, later of Rocky Mountain spotted fever fame, published an extensive review of blastomycosis in 1901 (Ricketts 1901). He reviewed the subtle differences between the blastomycetic diseases which were then known: cryptococcosis (*Cryptococcus neoformans*), coccidioidomycosis (*Coccidioides immitis*), and blastomycosis. Ricketts concluded that these were similar diseases with different clinical manifestations and there was enough similarity to state that, if not caused by the same organism, the agents were sufficiently related to be in the same genus. He conceded that thrush (*Candida albicans*) and sporotrichosis (*Sporothrix schenckii*) were different disease entities.

Ricketts described 12 new cases of blastomycosis with a review of their clinical courses, therapy, attempts at serology, and exquisite histopathological details on each patient. He recognized the mycelial and yeast forms of the organism and noted that the mycelial form never appeared in tissue. On one patient, he provided the first histological report of both 'small forms' (4–6 μm) and 'large forms' (20–30 μm) of *B. dermatitidis* in potassium hydroxide (KOH) preparations from fresh tissue. Unfortunately, the cultures from this patient did not grow. Cultures from tissue of another patient, however, were incubated in both a 'brood oven' incubator and the laboratory at room temperature. He described the differences in gross colonial morphology between the two cultures but did not present a microscopic comparison. The brood oven culture was described as having the appearance of 'a heap of earth worms.' He also failed to recognize that temperature was the cause for the two forms.

The thermal basis for dimorphism was first described by Hamburger in 1907 (Hamburger 1907). He specifically reported that the incubator temperature was 37°C and that room temperature varied between 16°C and 24°C. The gross colonial morphology and the microscopic characteristics of both forms were described. He converted the mycelial form of *B. dermatitidis* to the yeast form and vice versa simply by changing the temperature of incubation. Hamburger stated that temperature is the most important factor for this transformation in morphology, and recommended that duplicate cultures of clinical material should be made to accommodate growth at either temperature (see below).

In 1914, Stober published the first attempts to define the ecology and epidemiology of blastomycosis (Stober 1914). From his investigation of the social aspects of several of the patients and his personal inspection of their residence, he made some observations on the probable source of infections. Many patients, Stober reported, lived in close proximity to damp earth, rotted wood, and wet, cardboard-lined walls. He also associated blastomycosis with plumbers who allegedly came in contact with the mold on timbers in cellars where they worked. He grew a white mold from one of the homes but did not provide sufficient mycological features of the isolate to show that this was *B. dermatitidis*. Thus, Stober probably performed the first ecological studies of *B. dermatitidis* and was the first investigator to recognize the ecological association of this mold with the environment. His environmental observations are similar to the associations we make today with the probable natural reservoir of *B. dermatitidis*. This paper also provided a thorough study of the pathological, mycological, and clinical features of systemic blastomycosis. He described the course of the illness in patients as well as the therapy and prognosis of this mycosis.

ECOLOGY

Knowledge of the ecology of the etiologic agent is essential for the investigator to understand the epidemiology of blastomycosis. Although blastomycosis and its specific etiologic agent, *B. dermatitidis*, were first described in 1894, the mystery of the precise ecological niche occupied by this terricolous fungus remains unresolved. The natural reservoir of the three other major systemic fungal pathogens was firmly established by the middle of the twentieth century. *C. immitis* was associated with the deserts of the southwestern USA and northwestern Mexico in 1932 (Stewart and Meyer 1932), *Histoplasma capsulatum* var. *capsulatum* was isolated from infested soil in 1949 (Emmons 1949), and the association of *C. neoformans* with pigeon droppings has been known since 1951 (Emmons 1951). The ecological niche of *B. dermatitidis* cannot be described with certainty but there is sufficient evidence to describe the probable habitat. The first isolation of *B. dermatitidis* from nature was not reported until 1961 (Denton et al. 1961). The fungus was found in a collection of soil, tobacco, and organic debris

obtained in a tobacco-drying barn in Lexington, KY, USA. However, it was later found that the outbuilding from which the fungus was isolated had formerly housed a dog with blastomycosis. Although this isolation did not establish the ecological niche of this organism, it did prove the value of the investigational method used to recover the agent and demonstrated that *B. dermatitidis* could survive in certain soil for at least 2 years.

Isolation from nature

There have been only a few credible reports of the isolation of this fungus from a natural habitat (Denton et al. 1961; Denton and Di Salvo 1964, 1979; Bakerspigel et al. 1986; Klein et al. 1986, 1987a; Baumgardner and Paretsky 1999). Each of these isolations was made from material that was rich in organic matter including animal feces, dust, plant fragments, minerals, or insect remains. The environmental samples usually contained soil, or were near the soil. The epigeal substrate from which the fungus has been isolated was moist, shaded from direct sunlight, mixed with organic material, and with a pH of less than 6.0. A more specific ecological association for *B. dermatitidis*, as with *H. capsulatum* var. *capsulatum* and *C. neoformans*, is not yet apparent.

Environmental isolations

In 1964, Denton and Di Salvo reported the first isolation of *B. dermatitidis* from naturally contaminated environmental sites (Denton and Di Salvo 1964). Of 356 samples collected along the flood plain of the Savannah River in Augusta, GA, USA, 10 yielded the fungus. The samples were collected in abandoned sheds which had previously provided shelter to mules, chickens, dogs, or rabbits. Persistent attempts to re-isolate the geophilic fungus from the positive sites were not successful. Only one site yielded the fungus from a sample collected 10 months later (Denton and Di Salvo 1979).

In 1986, Klein et al. reported the isolation of *B. dermatitidis* from a beaver lodge located in Eagle River, WI, USA, in association with an outbreak of blastomycosis among a large number of school children (Klein et al. 1986). This was the first account of an isolate in direct association with human infection and confirmed what was long suspected: the source of infection for blastomycosis was a saprophytic mold in nature. In the same year, Bakerspigel and associates reported the isolation of *B. dermatitidis* from a patient with systemic disease who worked in a shed in Ontario, Canada (Bakerspigel et al. 1986). The shed contained large amounts of diatomaceous earth and the air was often dusty. To control this dust, the area was sprinkled with water on a daily basis. After soil sampling at this worksite, these investigators were successful in isolating *B. dermatitidis* from the environment. In 1987, Klein et al. reported the isolation of *B. dermatitidis* from soil collected at two new sites in Wisconsin where additional outbreaks of blastomycosis had occurred (Klein et al. 1987a). The patients in both these latter outbreaks were involved with the disruption of soil. Thus, it has been shown without a doubt that *B. dermatitidis* is an edaphic organism. In 1999, Baumgardner and Paretsky reported the isolation of *B. dermatitidis* from environmental material. The samples were collected from a woodpile located near a dog kennel, which housed dogs with a confirmed diagnosis of blastomycosis. The significant feature of this isolation is that, for the first time, the organism was isolated from the environment using an in vitro method (Baumgardner and Paretsky 1999).

Water association

Some investigators claim that the ecological niche of *B. dermatitidis* has an association with water. There has been some support for this hypothesis from both epidemiologic and experimental studies. The first isolates of *B. dermatitidis* from nature came from the alluvial flood plain of the Savannah River (Denton and Di Salvo 1964). In 1969, Borelli and his colleagues described the distribution of the fresh water salamanders of the genus *Necturus*, which coincided with the distribution of patients with blastomycosis in the USA. They suggested that these salamanders may play a role in the ecology of *B. dermatitidis* (Borelli et al. 1969). Furcolow et al. published an extensive investigation of the prevalence and incidence of *B. dermatitidis* in dogs and human beings (Furcolow et al. 1970). They reviewed the literature from 1885 until 1968 and found a high prevalence of human and canine cases south of the Ohio River and east of the Mississippi River. From an analysis of these findings they hypothesized an ecological relationship between *B. dermatitidis* and the proximity to water.

The conidia of *B. dermatitidis* are difficult to separate from their conidiophores. McDonough et al. demonstrated that the conidia are readily released from the mycelium in the presence of moisture (McDonough et al. 1976). Subsequently, McDonough and Kuzma, in an epidemiologic study of blastomycosis in Wisconsin, found that many of the human and canine subjects lived near bodies of water. This led them to suggest that foggy weather may contribute to the release of conidia in nature and the infection of susceptible hosts (McDonough and Kuzma 1980).

Archer also studied canine blastomycosis in Wisconsin (Archer 1985). He found that 68 percent of the 200 dogs with this mycosis lived within 500 meters of water. In a similar account from Canada, Harasen and Randall reported the location of eight dogs with blastomycosis in a small town in Saskatchewan (Harasen and Randall 1986). The dogs all resided within two blocks of a creek, which was a resting place for migrating Canada geese

(*Branta canadensis*). Thus, three purported requirements for the ecological niche of *B. dermatitidis* in nature were satisfied (soil, moisture, and organic material). Bakerspigel et al. isolated *B. dermatitidis* from the soil in a workshed in which there was a constant spray of water for dust control. The atmosphere had essentially 100 percent humidity (Bakerspigel et al. 1986). Finally, six of the 13 outbreaks of human blastomycosis occurred along rivers or lakes in the USA.

Geographical distribution

The geographical distribution of histoplasmosis and coccidioidomycosis has been defined by the use of skin test surveys, soil isolations, and serological studies. But these tools have not been available for the investigation of blastomycosis. Neither the skin test nor the serological antigens have sufficient specificity to be reliable, and the methods available to isolate *B. dermatitidis* from natural substrates, utilizing animal inoculation, are cumbersome and have a low sensitivity. The present understanding of the geographical distribution of *B. dermatitidis* comes from published case reports and a few outbreaks which attracted investigation.

These reports and studies usually reflect the interest of clinicians, epidemiologists, and mycologists who publish their experiences, rather than a true reflection of the distribution of *B. dermatitidis*. From the investigations of the outbreaks listed above, it can now be said with some certainty that the desiccation of the substrate, followed by a disturbance of the site by anthropurgic activity or natural phenomena (wind or storms), results in the creation of an aerosol of infectious particles. From a detailed review of the literature and careful interpretation of the evidence, it appears that autochthonous cases of blastomycosis occur in North America and Africa with a possible focus in central India (Figure 1). First thought to be restricted to the North American continent, sporadic case reports of blastomycosis from various parts of the world began to appear in the 1940s. Many of these reports were the result of erroneous identification of the etiologic agent resulting from incomplete studies of the isolate. Other reports did not present sufficient data to validate the claim that these were autochthonous cases. Di Salvo reviewed the original publications of all reports of blastomycosis from outside the USA (Di Salvo 1992a). Each case was re-examined as to the details of diagnosis. Those with insufficient diagnostic information (clinical, cultural, or histological) were discarded. The remaining reports in that publication present the current knowledge of the geographical distribution of *B. dermatitidis*.

With culture and tissue confirmation it became clear that Africa was an endemic area for this arcane mycosis. There have been scattered reports of autochthonous cases of blastomycosis from the Middle East and Europe, but these involved a small number of patients and, in some cases, insufficient information was presented to make a definitive statement of endemicity. It is curious that in North America, the disease occurs almost exclusively east of the 100th meridian which is approximately through the center of the USA (Figure 2). There has been a recent report of two patients with blastomycosis from Colorado who were both involved with the relocation of prairie dogs. Although the two patients previously resided in the

Figure 1 *Worldwide distribution of patients with documented evidence of blastomycosis. Detailed maps of each of these areas appeared in a previous publication (Di Salvo 1992a). (From Di Salvo 1992a, with permission. Copyright by South Carolina Department of Health and Environmental Control)*

Figure 2 *The 100th meridian passes through the center of the USA and Canada. This figure also shows the location of the 13 documented outbreaks of blastomycosis (Di Salvo 1992b). (From Di Salvo 1992b, with permission. Copyright by South Carolina Department of Health and Environmental Control)*

endemic area, circumstantial evidence (temporal onset, substantial disruption of soil, and excess rainfall immediately preceding the anthropurgic activity), despite lack of antibody evidence and negative soil sampling, is convincing that these were autochthonous cases (Hannah et al. 2001). This finding suggests that a previous patient with blastomycosis from Colorado may have also been an autochthonous case of this mycosis instead of reactivation from residency in the endemic area more than 50 years previously (Ehni 1989). The number of cases is extensive in the USA, and moderate in Canada, with only one well-documented patient from Mexico.

There is a need for a simpler, more efficient, in vitro method to isolate or identify the etiologic agent of blastomycosis in the environment. Until this is available much of the ecology of *B. dermatitidis* will remain speculative.

EPIDEMIOLOGY

The infectious particles of *B. dermatitidis* are its mycelial fragments and conidia; the respiratory system is the portal of entry. Particles which are between 0.5 and 5.0 μm can readily pass through the respiratory tract and enter the alveoli of the lungs. The conidia of *B. dermatitidis* vary in size from 2.0 to 10 μm and the mycelial fragments may be even smaller; thus these particles can readily reach the alveoli of humans and other animals. When the host's resistance fails, an infectious process is established. Garrison and Boyd presented in vitro evidence to demonstrate the conversion of the conidia to yeast-like cells (Garrison and Boyd 1978), whereas Denton and Di Salvo demonstrated the infectivity of conidia in vivo (Denton and Di Salvo 1968). The incubation period for blastomycosis is 4–8 weeks after an inhalation exposure and a clinical dermal lesion will be obvious 1–5 weeks after a trauma (Di Salvo 1992b).

Seasonality

A substantial number of publications, which reported the time of year when cases of *B. dermatitidis* occurred, have been reviewed (Di Salvo 1992b). Each of these

papers surveyed a large number of human beings or dogs with blastomycosis. From this analysis, there does not appear to be any seasonal occurrence of this mycosis. The evidence is difficult to evaluate because the time of exposure or onset of illness usually cannot be determined with any accuracy. Cutaneous lesions may appear at various intervals after the initial exposure, which is frequently unknown; pulmonary infection may be asymptomatic or unrecognized and there may be prolonged time lapses before a definitive diagnosis is made.

Occupational risk

No association can be made between *B. dermatitidis* and a specific occupation. Ten selected papers published between 1955 and 1988 were reviewed (Di Salvo 1992b). Each paper reported on a large series of patients in which occupation was specified. There were a total of 771 patients with blastomycosis in the analysis. It was obvious that most of the victims of this disease had an occupation or avocation involving outdoor activity. There are many additional single case reports of individuals with an environmental exposure to wood or soil who became infected with *B. dermatitidis*. Thus, it is not the specific occupation that renders a person susceptible to infection with *B. dermatitidis*, but the exposure of an individual to the probable ecological niche of the fungus.

Age, race, and gender

There is no recognized age, race, or gender susceptibility. People of all races and all age groups, male or female, are susceptible to this mycosis. The predominance of patients in the fourth, fifth, and sixth decades of life is consistent with an occupation or avocation that places these individuals at greater exposure to the elusive environmental niche of this fungus. A similar reasoning can be applied to the gender of the patients. Males may be infected more than females, but this bias reflects the workforce more probably exposed to the natural nidus of *B. dermatitidis* or the greater male orientation in the past to outdoor activities. Unlike coccidioidomycosis, race susceptibility for *B. dermatitidis* is not evident. Each report of such predilections merely reflects the population at risk or being studied (Di Salvo 1992b).

Transmission

It is generally accepted that the respiratory system is the portal of entry for all forms of blastomycosis except direct transcutaneous inoculation (Schwarz and Baum 1951). Blastomycosis is not ordinarily transmitted from person to person or animal to human. With the exception of one report in which two young men working side by side acquired pulmonary disease, there has been no evidence of aerosol transfer (Procknow 1966). The two patients had no other association except at work, thus leading the investigator to believe that there may have been human-to-human transmission. Exposure to the same environmental source of *B. dermatitidis* at the worksite was not absolutely ruled out. There have been two reports with strong evidence to support transplacental passage of *B. dermatitidis* (Watts et al. 1983; Maxson et al. 1992) and two well-documented cases of conjugal transmission of blastomycosis (Farber et al. 1968; Craig et al. 1970; Dyer et al. 1983).

Transcutaneous blastomycosis caused by accidental inoculation through dog bites during examination or treatment is an occupational hazard of veterinarians. There are several well-documented reports of humans becoming infected from animals in this way (Di Salvo 1992b). Two other reports of transmission from dogs to humans were, most probably, a common exposure to a contaminated site of *B. dermatitidis* (Schwartzman et al. 1959; Lieberman 1963). Similarly, pathologists have acquired infections with *B. dermatitidis* through the percutaneous route by self-inoculation during a postmortem examination of a patient with the infection (Di Salvo 1992b).

Skin test

There is currently no reliable skin test available for *B. dermatitidis*. The dermal hypersensitivity to fungal antigens has been a significant tool for delineating the geographical distribution of *H. capsulatum* var. *capsulatum* and *C. immitis*. Information gleaned from these studies has also established the prevalence of the asymptomatic disease caused by these agents. Unfortunately, the delayed-type hypersensitivity skin test antigen for blastomycosis has not been useful. The crude extracts, made from either the mycelial form (blastomycin) or the yeast form of the organism, have had the same deficiencies as the serological tests: a lack of sensitivity and specificity.

The blastomycosis skin test has presently been abandoned by the medical community because it is not suitable for either diagnostic or epidemiologic studies. For these reasons the US Food and Drug Administration (FDA) removed the product from the US market in 1972.

Many scientists have endeavored to develop an improved skin test antigen through the development of purified chemical extracts of specific antigenic fractions, but a successful product has yet to be achieved. Recent studies by Abuodeh and Scalarone have produced encouraging results with regard to the development of a useful skin test antigen (Abuodeh and

Scalarone 1994, 1995). Preparations from a yeast-form isolate were efficacious with respect to their sensitivity to delayed-type hypersensitivity responses in *B. dermatitidis*-sensitized guinea pigs; but there was cross-reaction in animals sensitized with the *capsulatum* variety of *H. capsulatum*. Additional studies with a more purified derivative elicited a more specific reaction (Bono et al. 1995). These investigations may lead to the development of a useful epidemiologic tool in the future.

Animal disease

Next to humans, dogs are the most frequent animal infected with *B. dermatitidis*. Canines, as a surrogate for humans, have served as sentinels for human disease in epidemiologic studies for many years. Menges and his colleagues reported on an extensive study of canine and human blastomycosis in 1965 (Menges et al. 1965). The results of that study indicated that both humans and dogs were exposed to the same natural nidus of *B. dermatitidis* where they inhaled the infectious particles. These investigators also concluded that there was no evidence of animal-to-human transmission. Similar studies have supported these conclusions and have shown that in the endemic areas for blastomycosis, both dogs and humans have a similar geographical distribution of the disease (McDonough and Kuzma 1980; Archer et al. 1987; Baumgardner et al. 1995). Canine blastomycosis has been called the harbinger of human blastomycosis (Sarosi et al. 1979). Legendre has reviewed animal blastomycosis in detail (Legendre 1992).

MYCOLOGY

Nomenclature

The etiologic agent of blastomycosis is the thermal dimorphic species *B. dermatitidis* (Gilchrist and Stokes 1898). It has long been recognized that this binomial is taxonomically incorrect. This specific epithet, first used by Gilchrist and Stokes in 1898, has had several synonyms proposed over the years. All have been rejected (Conant 1939). Although medical mycologists agree that *B. dermatitidis* does not conform to the taxonomic code, the traditional, long-time use of the binomial has justified its continued use.

Identification

An isolate of *B. dermatitidis* is identified by several morphological characteristics. The classic means of identification is the in vitro conversion of this dimorphic fungus from the yeast form to the mycelial form and/or vice versa. Temperature variation is the method used to demonstrate the thermal morphological change. Incubation of yeast colonies on SGA at 25–30°C will yield growth of the mycelial form of the mold in 10–14 days. Conversely, the cultivation of mycelium on an enriched medium, such as brain–heart infusion agar with 5 percent blood (see Laboratory diagnosis), and incubation at 37°C will yield yeast colonies in 3–5 days.

The culture of *B. dermatitidis* from clinical material requires 2–4 weeks for growth at 25°C on SGA. The typical colony of most isolates of *B. dermatitidis* is a white, aerial, cottony, filamentous mold. The colony may vary from a flat, dull colony of mostly vegetative hyphae to a heaped fungal mass with hyphal tufts. After 2–3 weeks the culture usually becomes more typical in appearance. The mycelial-form colony is white in the early stages of growth but may become tan to light brown as the colony ages. Concentric rings of growth are frequently seen in older colonies, i.e. 4–8 weeks of age (Figure 3).

Microscopically, the mycelium and the asexual propagules (conidia) are evident. The hyphae are narrow (6–7 μm wide), hyaline, branching, septate mycelia. New growth occurs at the hyphal tip. The unicellular conidia are formed on thin, lateral conidiophores or directly on the hyphae (sessile). They are slightly oval to round with a smooth wall and vary from 3 to 6 μm in diameter. These structures are readily seen in a slide culture or a wet mount preparation. *B. dermatitidis* cannot, however, be identified solely by its microconidia, which are

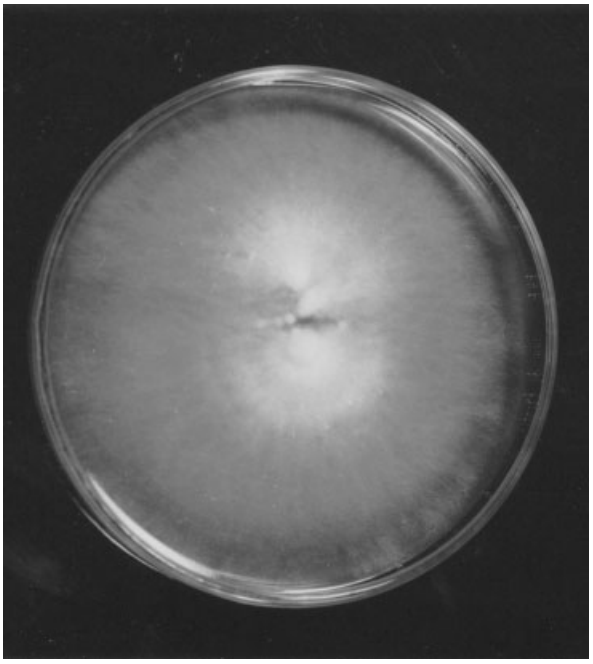

Figure 3 Blastomyces dermatitidis *mycelial form colony on Sabouraud's glucose agar. Note the concentric rings of growth. This colony, incubated at 25°C, was about 4 weeks old.*

indistinguishable from those of many other fungal saprophytes and pathogens (Figure 4).

At 37°C the yeast-like, or tissue, form grows in about 7–10 days when cultured on enriched media (usually brain–heart infusion agar containing 5 percent blood). The colony appears as a buttery-like, cerebriform, soft colony with a tan color. A wet mount in lactophenol cotton blue can be prepared from the culture. Microscopic examination shows typical yeast cells, 8–12 μm in diameter, with a thick wall (frequently referred to as a 'double wall') and a single bud with a wide base of attachment to the mother cell, characteristic of this species (Figure 5). Electron microscopy studies have shown that the cell wall consists of two layers separated by a clear zone (Daniel et al. 1979). The yeast forms contain two to four nuclei per cell (Clemons et al. 1991) while the conidia are uninucleate.

The yeast cells, when found without buds, may be confused by less experienced laboratorians with the immature spherules of *C. immitis*, the yeast forms of *H. capsulatum* var. *duboisii*, *C. neoformans*, or an unbudded yeast-form cell of *Paracoccidioides brasiliensis*.

A defining characteristic of *B. dermatitidis* and several other dimorphic fungi is the fact that their yeast forms will convert into mycelial forms when incubated at 25°C and, conversely, mycelial forms can be changed to the yeast forms when incubated at 37°C. Many strains of *B. dermatitidis* do not readily convert to the yeast form. Several transfers, 3–4 days apart, aid in the transformation, although sometimes the transformation is not complete and transitional forms are seen (Figure 6).

There are no diagnostic biochemical tests which will aid in the identification of the yeast or mycelial forms of *B. dermatitidis*. Morphology is the preliminary or presumptive method of identification and the specific nucleic acid probe is confirmatory.

Teleomorph

Ajellomyces dermatitidis, the ascomycetous or sexual state of this pathogen, was first described by McDonough and Lewis (1968) . It is a heterothallic member of the family *Gymnoascaceae*. The perfect state is characterized by its globose gymnothecia with spiralled hyphae radiating from their centers. A network of secondary peridial hyphae develops from the spirals. When mature, the tan gymnothecia measure 200–350 μm in diameter. The hyaline, evanescent asci produced in the gymnothecia contain eight smooth, spherical, hyaline, or light-tan ascospores that are uninucleate and measure 1.5–2.0 μm in diameter. It is interesting to note that the teleomorph of *H. capsulatum* is in the same genus as that of *B. dermatitidis*.

African forms

The autochthonous isolates of *B. dermatitidis* from Africa are similar to each other and appear to be distinct from North American isolates (Di Salvo 1992b). It has been suggested that isolates from these two different parts of the world are probably the same species, but different serotypes. McDonough crossed African isolates with North American isolates with the production of cleisothecia that lacked ascospores, thus indicating that the two isolates were closely related but not unequivocally the same species (McDonough 1970). However, four African strains studied by Kwon-Chung

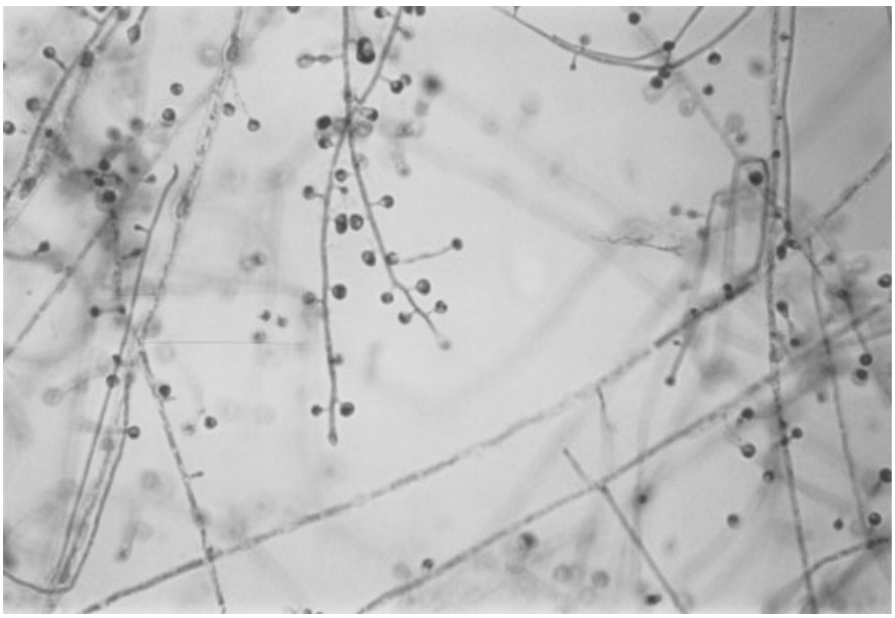

Figure 4 *Microscopic view of* Blastomyces dermatitidis *mycelium and conidia. Agar at 25 C,°13 days old. Magnification* × *112*

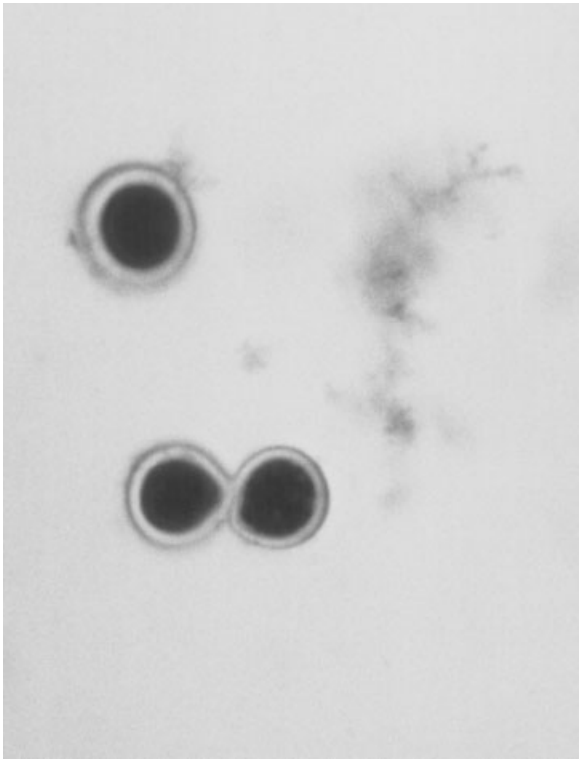

Figure 5 *Microscopic view of the yeast form of* Blastomyces dermatitidis *in a methylene blue wet mount. Note the figure-of-eight appearance of two cells of equal size. The broad base of attachment, single bud and 'double contour' wall are obvious. Magnification × 160*

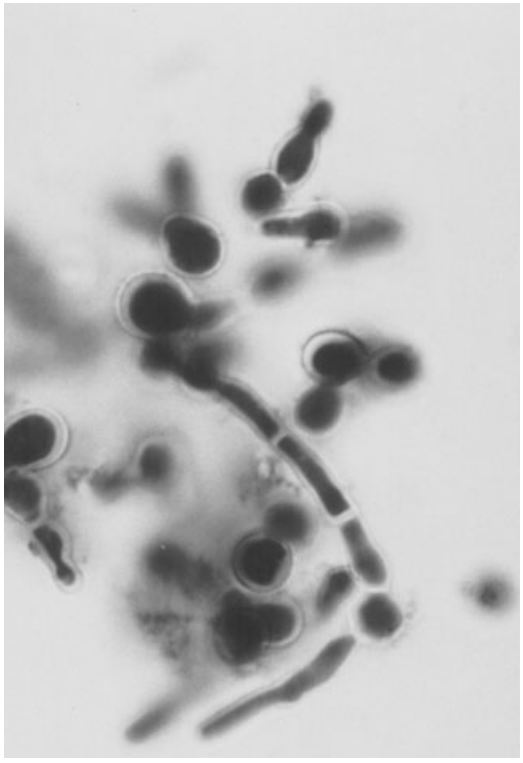

Figure 6 *Conversion of the mycelial form of* Blastomyces dermatitidis *to its yeast-form by incubation at 35°C. Note that the conversion was not complete on the first transfer. Some fragments of mycelium are seen intermingled with the yeast forms. At 48 hours; magnification × 160*

did not cross with her North American tester strains (Kwon-Chung 1971).

Sudman and Kaplan investigated the antigenic relationship between North American and African strains of this fungus using fluorescent antibody reagents (Sudman and Kaplan 1974). The conclusion of this study was that, although the strains share certain antigens, the isolates from these two geographical areas were antigenically different. Kaufman et al. (1983) examined the exoantigens of a large number of strains of *B. dermatitidis* from various parts of the world. They reported that the African isolates did not produce the A exoantigen which was evident in all other isolates. They also described gross and microscopic differences in the colonial morphology between African and North American isolates. Some African strains have echinulate conidia as opposed to the smooth-walled conidia of North American strains. These investigators concluded that there are two serotypes of *B. dermatitidis*.

Vermeil et al. (1982), in a comparative study of these geographically distinct strains, reported that the conidia from the African strains were gathered in clusters. They also observed that the mycelial form of the African strains did not convert to the yeast form as readily as the North American strains. Using the enzymatic activity of the yeast-form as a biotyping tool, Summerbell et al. (1990) failed to show any differences between two African strains and 18 North American strains based on these criteria. The taxonomy and biology of *B. dermatitidis* were recently reviewed by Sugar (1992).

Biochemistry

Comparative studies on the chemical composition of yeast and mycelia have enhanced understanding of the biochemistry of *B. dermatitidis*. Lipids make up about 9 percent of the dry weight of yeast cells (Beck and Hauser 1938). About 75–80 percent of extractable lipids from yeast and mycelia are oleic and linoleic acids. Oleic acid predominates in yeasts, while a higher percentage of linoleic acid is recovered from mycelia (Domer and Hamilton 1971). Studies to localize lipids found that about 2 percent of the yeast cell wall was lipid, while negligible lipid was found in the mycelial cell wall. The lipid content of yeasts is interesting, as several studies have noted differences in lipid content between virulent and avirulent isolates of *B. dermatitidis*. Lipid content directly correlated with virulence of the isolate in mice in one study (Di Salvo and Denton 1963). However, in another study, a spontaneous avirulent mutant had a three- to fourfold increase in total lipid content,

primarily composed of an increase in fatty acids, when compared to the virulent strain (Brass et al. 1982).

Other differences in cell wall composition between yeast and mycelia have been studied in an effort to understand dimorphism and virulence. The cell walls of both yeast and mycelia contain chitin and glucan. The glucan in yeast cells is 95 percent α-glucan, while that of mycelia is 60 percent α- and 40 percent β-glucan (Kanetsuna and Carbonell 1971). This shift in predominant type of glucan is similar to *P. brasiliensis* and is thought to be secondary to activation of α-glucan synthase with increased temperature (Kanetsuna et al. 1972). The chitin isolated from *B. dermatitidis* mycelia is similar to chitin obtained from *H. capsulatum* and *P. brasiliensis* (Gow and Gouday 1983). Chitin is a stable cell wall component during dimorphic change. Its role in dimorphism is suggested by electron microscopic studies showing different chitin fibril arrangements in yeast and mycelial cell walls. In yeast, the chitin fibrils are noted to be tightly interwoven in a random orientation, while in mycelia, they are arranged in a more longitudinal manner in many areas of the cell wall (Kanetsuna 1981). Isolation of the enzyme chitin synthetase from the yeast and mycelial forms indicates similar biochemical characteristics, but the yeast form enzyme is in latent form requiring activation by protease, whereas the mycelial form enzyme is in active form (Shearer and Larsh 1985).

The dimorphism of *B. dermatitidis* is primarily temperature-dependent. However, studies into the mechanism of dimorphic transition indicate a more complex regulation other than by temperature alone. Yeasts grown at 37° C can revert to mycelia in the presence of cAMP phosphodiesterase inhibitors, theophylline, or 3-isobutyl-1-methylxanthine. Using electron microscopy to study yeasts at 37°C, cAMP phosphodiesterase is localized to nuclear and mitochondrial membranes and to endoplasmic reticulum and vacuolar areas. By contrast, similar studies of the yeast 24 hours after lowering the incubation temperature to 25°C show decreased enzyme activity of cAMP phosphodiesterase in vacuolar areas. This suggests that increased levels of intracellular cAMP may be required for dimorphic transition to the yeast form (Paris and Garrison 1984). Other physiological changes that occur with mycelial to yeast transition upon change of temperature are related to mitochondrial respiration. Three stages in the transition are described:

1. immediately after an increase in temperature, partial or complete uncoupling of oxidative phosphorylation, leading to decreases in ATP levels, respiration rate, and electron transport components
2. a dormant period of 4–6 days with absent to low rates of respiration
3. a recovery phase marked by increase in respiration, increased ATP, and electron transport components and the appearance of yeast cell morphology.

During stage 2, the presence of cysteine is required for the operation of the sulfhydryl-induced shunt pathway. These stages of form transition are shared by *H. capsulatum* and *P. brasiliensis* (Medoff et al. 1987). Nutritionally dependent dimorphism is also indicated by reported conversion of mycelial to yeast form at 26°C on partially defined media.

HOST RESPONSES

Clinical forms

There are two clinical forms of blastomycosis: cutaneous and systemic or disseminated disease. It was thought that these were two clinically distinct syndromes until 1951 when Schwarz and Baum (1951) clearly demonstrated that the common cutaneous form is largely a manifestation of active or inactive systemic disease.

CUTANEOUS DISEASE

Cutaneous blastomycosis may be a primary transcutaneous disease or secondary to systemic infection. Primary transcutaneous blastomycosis is the result of the direct inoculation of *B. dermatitidis* into the skin, and therefore it is commonly seen as a solitary lesion. This type of disease is unusual and frequently occurs as the result of an accidental inoculation. It is considered an occupational hazard for mycologists, pathologists, and veterinarians (Schwarz 1983). There have been several reports of laboratory accidents which occurred during the course of experimental studies. Accidental self-inoculation during postmortem examinations of patients who have died from blastomycosis has also been reported. Similarly, veterinarians are at risk from transcutaneous inoculation as a result of accidents which occur during postmortem examinations, surgery, or when bitten by animals, usually dogs, infected with *B. dermatitidis*. As the accidents occur when working with the organism or infected tissue, most inoculations occur on the hands (Schwarz 1983). When first noted, the wound site is tender and inflamed. The lesion starts as a papule and promptly develops into a pustule; lymphangitis or regional lymphadenopathy may or may not occur, but the progression of the infection stops at the axilla. There are usually no constitutional symptoms and the course of disease is self-limited. The lesions heal spontaneously in several weeks to several months, and systemic antifungal therapy is not required. The pus from the lesions contains many yeast form cells which are readily visible in KOH wet mounts. This observation, together with knowledge of the previous trauma, is diagnostic. The incubation time for transcutaneous inoculation blastomycosis is from 1 to 5 weeks (Di Salvo 1992b).

Secondary cutaneous blastomycosis occurs as a result of the hematogenous spread of infection from another

focus, usually the lungs; therefore the lesions are frequently multiple. Although cutaneous lesions result from disseminated blastomycosis, the initial pulmonary disease may not be obvious and the cutaneous lesions may be the only presenting symptoms. This cutaneous manifestation may occur spontaneously or develop at the site of a subsequent injury. The eliciting trauma may be as slight as a bump to an extremity, where transient circulating blastomycetes localize in the damaged tissue. The skin lesions appear as indolent, ulcerated, or verrucous lesions and may resemble a malignancy. Typically a lesion ulcerates and heals with scarring. Generally there is a sharp edge at the periphery of the lesion where invasive activity is occurring. The boundary frequently consists of minute abscesses which often serve as a good source of clinical material for direct examination or culture. Healing is obvious in the central area. As opposed to primary inoculation blastomycosis, the regional lymph nodes are not involved in secondary cutaneous blastomycosis (Figure 7).

SYSTEMIC DISEASE

The systemic disease takes many clinical forms. Acute primary pulmonary blastomycosis probably occurs more frequently than is reported. The symptoms are those of an influenza-like illness with fever, chills, and a productive cough, and chest x-rays will show nonspecific pulmonary disease. The etiology is usually not suspected and, if considered, the patient is closely observed without the administration of therapy. Untreated, acute pulmonary blastomycosis may be self-limited and resolve within a month or progress to chronic pulmonary disease or systemic disease. The diagnosis of acute primary pulmonary blastomycosis is frequently made retrospectively when the disease progresses to the chronic form of pulmonary infection, secondary cutaneous lesions develop, or the patient is recognized as a member of a cohort exposed at a common point source. The incubation period of blastomycosis acquired by the respiratory route is 4–8 weeks (Di Salvo 1992b).

Figure 7 *This patient has a cutaneous lesion of secondary blastomycosis. Six months before the appearance of the lesion, he experienced an acute, undiagnosed, pulmonary infection.*

The most common form of pulmonary blastomycosis is a slowly progressing chronic pneumonia which persists for many months. The symptoms consist of fever, chills, cough, chest pain, hemoptysis, night sweats, and weight loss. The differential diagnosis includes tuberculosis, histoplasmosis, coccidioidomycosis, other non-infectious forms of chronic lung disease, and malignancy. Pleural effusions do occur, but they are an unusual manifestation of blastomycosis (Jay et al. 1977; Kinasewitz et al. 1984; Failla et al. 1995).

Although pulmonary and cutaneous manifestations are the most common form of the disease, there have been many reports of infection of practically every other organ. Systemic blastomycosis may be limited to the lungs or, if disseminated, involve almost any organ in the body. Dissemination may occur by the hematogenous, lymphatic, or direct extension routes (Schwarz and Baum 1951) and the symptoms vary according to the organ systems affected by the disease.

Miliary blastomycosis is an unusual systemic manifestation of this fungous infection. The symptoms consist of a rapid onset of severe dyspnea and respiratory failure due to adult respiratory distress syndrome (ARDS). The sporadic occurrence of this rare form of blastomycosis is considered a medical emergency and the outcome is frequently fatal. An aggressive therapeutic regimen is imperative if the patient is to survive (Shaw et al. 1976; Rippon et al. 1977; Griffith and Campbell 1979; Stelling et al. 1984; Renston et al. 1992; Meyer et al. 1993). It is thought that this clinical entity may represent endogenous reactivation (Berger and Kraman 1981; Stelling et al. 1984).

The male urogenital system seems to be highly susceptible to blastomycosis. This includes the kidney, prostate, testes, epididymis, and seminal vesicles. The prostate is frequently involved whereas the urinary bladder itself is rarely infected. Prostatitis caused by *B. dermatitidis* was extensively reviewed by Schwarz (1982).

Osteomyelitis is a frequent complication of blastomycosis (Denton et al. 1967) and arthritis may be the presenting symptom in disseminated blastomycosis (George et al. 1985; Robert and Kauffman 1988). Blastomycosis of the central nervous system, although not common, does occur. Pleocytosis is usually lymphocytic (Carmody and Tappen 1959; Buechner and Clawson 1967), although a largely polymorphonuclear response has been reported in one patient (Harley et al. 1994). Blastomycosis of the larynx, adrenals, heart, eye, paranasal sinuses, tongue, uterus, ovary, etc., has also been

reported. The liver, spleen, and gastrointestinal tract are only rarely involved.

REACTIVATION BLASTOMYCOSIS

It has been suggested that reactivation of latent blastomycosis can occur. Several observations support this concept which is well documented in tuberculosis. Subsequent to the course of benign self-limited disease, in some patients a period of clinical and radiological resolution of the mild pulmonary symptoms has been reported. At some later time, ranging from months to years, the patients exhibit signs of systemic blastomycosis (Laskey and Sarosi 1978). It was reported that one patient, from Colorado, developed fatal progressive pulmonary blastomycosis 40 years after leaving the endemic area (Ehni 1989). However, as a result of a subsequent report of two patients with proven blastomycosis from Colorado, which has been considered outside the endemic area, reactivation disease in that patient should be reconsidered (Hannah et al. 2001). The source of the sequestered organisms, unlike tuberculosis, is not known. Such occurrences reinforce the admonition that a careful patient history is essential when an endemic fungal disease is suspected. There is insufficient evidence to determine if exogenous reinfection occurs.

SUBCLINICAL DISEASE

It is becoming more apparent that there is a form of blastomycosis that is subclinical or an acute, mild, self-limited disease (Denton et al. 1967; Recht et al. 1979; Sarosi et al. 1986; Vaaler et al. 1990). This concept has been reinforced during studies of common source outbreaks of blastomycosis (Sarosi et al. 1974; Centers for Disease Control 1979; Klein et al. 1986). The diagnosis of asymptomatic patients was determined while investigating a cohort of exposed individuals. Many of these individuals recovered spontaneously from their illness. The only evidence of *B. dermatitidis* infection was detected by antigen-specific immune studies, serological testing, and an abnormal chest x-ray. In some instances, the revelation of a previous history of pulmonary disease, compatible with blastomycosis, was revealed after the diagnosis of systemic blastomycosis was confirmed.

OPPORTUNISTIC INFECTIONS

B. dermatitidis may act as an opportunistic fungus in immunocompromised patients. Sporadic cases of blastomycosis in such patients were reported before 1990. In most patients, the underlying conditions were hematological malignancies and corticosteroid therapy (Schwarz and Salfelder 1977; Recht et al. 1982). Additional patients with blastomycosis, who were compromised by sarcoid, renal transplants, and Hodgkin's disease, have also been reported (Önal et al. 1976; Chow et al. 1981; Hii et al. 1990; Winquist et al. 1993).

The number of acquired immunodeficiency syndrome (AIDS) patients with blastomycosis is limited when compared with those patients superinfected with the other systemic fungi. *Candida* spp. and cryptococcal infections are the most prevalent mycoses in AIDS patients. Histoplasmosis is common among AIDS patients in the endemic area for that disease and coccidioidomycosis is frequent among the population where *C. immitis* is found. There have been only a few reports of AIDS (or other immunocompromised) patients with blastomycosis. The first account of this combination was made by Chiu et al. (1988). The only collective, comparative review was published by Pappas et al. (1992). Their series of 15 AIDS patients with blastomycosis reported that the disease was usually disseminated, survival was short and the central nervous system was frequently involved (Pappas et al. 1992). While only a limited number of such patients have been reported to date (Kitchen et al. 1989; Herd et al. 1990; Fraser et al. 1991; Harding 1991), Pappas et al. have reported that an increasing proportion of blastomycosis patients seen at their medical center (24 percent seen between 1978 and 1991) have underlying impairment of immunity (Pappas et al. 1993). Mortality rates in such patients are high and exceed 30 percent. Maintenance antimycotic therapy may be required for AIDS patients with blastomycosis as it is for those with cryptococcosis.

As with the other endemic mycoses, some AIDS patients with blastomycosis had not lived in an endemic area for several years. These findings support the case for subclinical infection with sequestration of a quiescent nidus of infection. Endogenous reactivation occurs when host defenses are compromised as in the case of these AIDS patients.

It is interesting to note that, in 1914, Stober commented that, in humans, host resistance must be substantially diminished for an infection with *B. dermatitidis* to occur (Stober 1914). The detailed clinical aspects of blastomycosis were recently reviewed (Causey and Campbell 1992; Pappas and Dismukes 2002).

Radiology

There are no diagnostic radiological features of blastomycosis. Pulmonary infiltrates, consolidation, cavities, miliary and intermediate nodules, and mass-like lesions have been reported, but the distribution of this pathology differs among studies (Pfister et al. 1966; Halvorsen et al. 1984; Sheflin et al. 1990; Brown et al. 1991). Although pathologists claim that pleural effusions are an uncommon finding postmortem (Schwarz and Salfelder 1977), some radiologists claim that 20 percent of their blastomycosis patients had signs (blunting of the

costophrenic angle) that were consistent with fluid (Sheflin et al. 1990).

The location of the pulmonary abnormalities also varied with the reports. One or both lungs may be affected. Bilateral disease is more common, although the abnormalities may occur in either the right or left lung. Upper or lower lobes may be involved but more often the disease is limited to the upper lobes.

Pathology

GROSS PATHOLOGY

The gross pathology of the dermal lesions in primary and secondary blastomycosis has been adequately characterized in the clinical description of the disease. The two types of lesions are distinct and an exposure history will support the diagnosis of primary cutaneous blastomycosis.

There is no characteristic gross appearance of organs with blastomycetic lesions. When the patient has had disseminated disease the lesions will vary with the extent of the pathology and the organs involved. The lungs are the most frequently affected organ in blastomycosis. Clinically, radiologically and grossly, the lung lesions frequently resemble carcinoma. Schwarz and Baum (1951) found that the lungs were usually heavy, i.e. 2000 g for both. The cut surface is gray with small pneumonic foci, but the typical cavities, usually seen in other fungal disease or tuberculosis, are not found (Schwarz and Baum 1951).

The bronchial tree may be ulcerated and is often filled with exudate which contains many organisms. Cavitary lesions are not common. Pleuritis is frequent but pleurisy is unusual. Calcified nodules, common in histoplasmosis, are not found and caseation of the lymph nodes is not common. The gross lesions of other organs most closely resemble a tumor upon inspection. Histological examination and culture are required to confirm the diagnosis. Yeast forms of *B. dermatitidis* have been found in blood vessels and their presence is evidence that fungemia does occur (Schwarz and Salfelder 1977).

HISTOPATHOLOGY

Histological stains

The universal histological stain in general use in the pathology laboratory is the hematoxylin and eosin (H&E) stain. This reagent is used on all diagnostic tissue sections. It will stain many, but not all, fungi. The advantage of H&E is its capability to reveal tissue structures and the inflammatory reaction as well as the invading fungus. *B. dermatitidis*, however, is sometimes difficult to visualize in tissue sections stained by this method.

There are three special stains commonly used for *B. dermatitidis* and other fungi. The special stains provide a sharp delineation of the fungus and demonstrate its typical characteristics. Gomori's methenamine–silver nitrate (GMS) stain outlines the fungal cell walls which are stained a deep black whereas the intracellular portions of the yeast cells are rose colored. The tissue background is light green, providing an effective contrast with the organism. This stain is especially useful for the inexperienced pathologist and when there is a paucity of organisms in the infected tissue. A combination of GMS and H&E has the advantages of both stains: readily visualized organisms and the opportunity to evaluate the host tissue reaction. With the periodic acid–Schiff (PAS) stain the fungus is red with a pink or light green background depending on the counterstain. It outlines *B. dermatitidis* distinctly and is preferred by some pathologists for the study of fungal histopathology. The Papanicolaou stain, commonly used in exfoliative cytology, also stains *B. dermatitidis* very well. The translucent cell wall is obvious whereas the cytoplasm is granular and stains a pale pink (Trumbull and Chesney 1981). The yeast forms can be readily visualized with ordinary cervical cytology screening procedures (Dyer et al. 1983).

The direct fluorescent antibody (DFA) test is a serological tool that is useful for the rapid and specific identification of certain fungi. It is specific for *B. dermatitidis*, when evaluated with the morphological characteristics of the suspect organism. DFA is not a stain, but a visible antigen–antibody reaction which fluoresces and outlines the fungus cell (Kaplan and Kaufman 1963). These reagents will stain only the yeast form and therefore this tool is most useful as an adjunct to histopathology. A fluorescence microscope and specific filters are required. Unfortunately, these reagents are not commercially available, so specimens must be sent to a reference laboratory that offers the desired analysis.

Nested polymerase chain reaction (PCR) has been investigated on tissue from dogs infected with *B. dermatitidis* (Bialek et al. 2003). Formalin-fixed, paraffin-embedded tissues were examined for evidence of this organism. PCR amplification of the gene encoding the unique *BAD1* adhesin was found to be as sensitive as, and even more specific than, PCR amplification of 18S rDNA for detection of *B. dermatitidis* in canine tissue. Neither was PCR crossreactivity seen in human tissue from biopsy proven cases of histoplasmosis

Morphology

Morphology is the key to the histological identification of *B. dermatitidis*. Individual cells or budding cells may be seen. The single bud with a wide base and a thick wall ('double contoured') is pathognomonic of blastomycosis. The fungus cells are 8–12 μm in diameter and round or globose in shape. Careful examination may reveal that the yeast cells contain several nuclei, with a retracted cytoplasm within the cell wall. The yeast cells may be free in the tissue or within giant cells. The inflammatory reaction is a mix of poly-

morphonuclear cells and a granulomatous reaction. Early lesions are usually suppurative with a polymorphonuclear infiltrate. As the inflammatory process proceeds, a granulomatous or mixed response with multinucleate giant cells appears in the lesion. The giant cells contain nuclei both in the centre and the periphery of the cell (Figure 8).

Size variation

The microscopist should be aware that both small and large forms of *B. dermatitidis* do occur. Small forms, 2–10 μm in diameter, have been described (Manwaring 1949; Tompkins and Schleifstein 1953; Tuttle et al. 1953; Schwarz and Salfelder 1977). These diminutive cells resemble those of the *capsulatum* variety of *H. capsulatum* in size, but they posses the characteristic broad base of *B. dermatitidis*. *H. capsulatum* yeast forms are usually elliptical and are more apt to be seen within mononuclear or polymorphonuclear leukocytes rather than giant cells. Large forms of *B. dermatitidis* have also been reported. Watts et al. (1990) described a case of pulmonary blastomycosis in which cells of *B. dermatitidis* as large as 40 μm in diameter were found in lung tissue. Walker et al. (2002) described culturally confirmed cutaneous lesions with yeast cells 20–40 μm in diameter. One of 10 wild strains of *B. dermatitidis* isolated from soil also produced large yeast forms. When recovered from experimentally infected mice, most cells from this isolate were 25–30 μm in diameter (Denton and Di Salvo 1964). Histologically, these aberrant forms have frequently been found mixed with yeast cells of normal size and morphology. In those instances, where cultures have been made, the yeast forms grew in the normal size range (W. Kaplan 1988, personal communication). It is interesting to note that Stober described small forms (diameter 3–5 μm) and large forms (diameter 25–30 μm) in 1914 (Stober 1914).

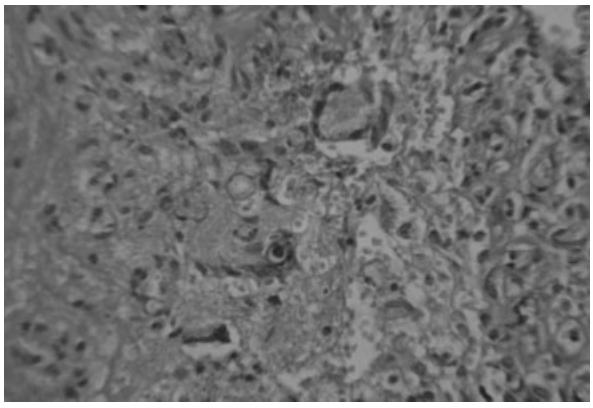

Figure 8 *A microscopic view of a tissue section with a bone lesion caused by* Blastomyces dermatitidis. *Note the giant cell containing nuclei and cells of the yeast form. As a result of the large size of the tissue-form of this organism, the yeast invoked a foreign body tissue reaction.*

Mycelial forms

Mycelial forms of *B. dermatitidis* have occasionally been seen in tissue (Hardin and Scott 1974; Kaufmann et al. 1979; Atkinson and McCurley 1983) and bronchial washings (Herd et al. 1990). The latter case was an AIDS patient, and the mixture of tissue and mycelial forms found in this patient was similar to that found in an AIDS patient with coccidioidomycosis. These hyphal forms of *B. dermatitidis* are usually short, septate, and hyaline, with occasional branches which resemble the pseudomycelium of *C. albicans*.

Skin

The common histological finding in cutaneous blastomycosis is pseudoepitheliomatous hyperplasia with squamous cell proliferation and acanthosis. Intraepithelial and dermal microabscesses with budding yeasts are seen. Giant cells may or may not be present and the organisms can be found within or outside the giant cells. In chronic cutaneous blastomycosis eosinophils may be seen.

Other organs

Abscesses with few or numerous organisms may be seen or the yeast-like cells may be dispersed in the lesion. The host inflammatory reaction may be both suppurative and granulomatous. As in the cutaneous lesions, the organisms are frequently found in giant cells. In bones, a granulomatous osteomyelitis is usually seen.

Caveat

For the inexperienced pathologist, it may be difficult to make a definitive diagnosis of *B. dermatitidis* if the typical morphological forms are not present in the tissue sections being examined. This occurs when there are only a few fungal cells present or when budding yeasts are not found. *B. dermatitidis* may then be difficult to distinguish from the *duboisii* variety of *H. capsulatum*, *C. immitis* (immature spherules), *P. brasiliensis* (look for multiple buds, thin walls, narrow attachment of daughter cells), or *C. neoformans* if a large capsule is not present (look for a capsule or use the mucicarmine stain which tints the smallest amount of the polysaccharide capsule). *Candida* spp., and sometimes *S. schenckii*, will appear as round cells. The DFA can resolve many of these problems if no typical forms are present. A comprehensive review of the pathology of blastomycosis can be found in Chandler and Watts (1992).

Host–pathogen interactions

DIMORPHISM AND VIRULENCE

Transition to a yeast form is a requirement for pathogenesis. Medoff et al. (1986) treated *H. capsulatum*

mycelia with the sulfhydryl inhibitor *p*-chloromercuriphenylsulfonic acid (PCMS), to prevent conversion to the yeast form, and showed that inoculation with this material no longer caused disease in a murine model of infection. His findings imply that yeast-form-specific factors are essential for pathogenicity of dimorphic fungi. To date, few form-specific genes have been identified in *B. dermatitidis* and other dimorphic fungi. One of the best studied in *B. dermatitidis* is *BAD1* (*Blastomyces* adhesin; formerly WI-1). A number of molecular and genetic tools applied to *B. dermatitidis* have aided the study of this yeast-form-specific virulence factor, as described below. Another yeast-form specific gene of *B. dermatitidis* is *bys 1* (*Blastomyces* yeast-specific), but its function is unknown (Burg and Smith 1994). α-Glucan is an additional differentially expressed product of *B. dermatitidis*. The cell wall glucan content of *B. dermatitidis* yeast is 95 percent α-glucan (Kanetsuna and Carbonell 1971), which is situated in the outer wall. By contrast, the proportion of α- and β-glucan is more evenly distributed in the cell walls of the mold. Hence, these polymers are differentially expressed in the yeast and mold forms of the fungus. In *B. dermatitidis*, as well as in *P. brasiliensis* and chemotype II strains of *H. capsulatum*, the loss of yeast cell wall α-(1,3)-glucan in genetically related variants correlates with the loss of virulence in animal and cell culture models of infection (Hogan et al. 1996).

TOOLS FOR GENETIC MANIPULATION OF *B. DERMATITIDIS*

Study of *B. dermatitidis* pathogenicity has benefited from the application of molecular biological tools (Brandhorst et al. 2002). High-voltage electric pulse was used first for introducing foreign DNA into *B. dermatitidis* (Hogan and Klein 1997). Transformation efficiency by electroporation is low, however (range, 1–42 c.f.u./μg transforming DNA). Where numerous transformants must be screened, a more efficient gene-transfer method is desirable. *Agrobacterium tumefaciens*-mediated gene transfer, developed originally for transformation of plant species, has been adapted for use with *B. dermatitidis* (Sullivan et al. 2002), and routinely generates transformants with a frequency of up to 10^3 per 10^7 per target yeast. Transforming DNA chiefly integrates into a single chromosomal locus. This method of DNA transfer thus offers the advantages of single-site integration, desirable for insertional mutagenesis, and a high yield of transformants.

The *Escherichia coli* hygromycin phosphotransferase gene (*hph*), coding for hygromycin B resistance, serves as the staple dominant marker for transformation. Chlorimuron ethyl resistance, encoded by the *sur* (sulfonylurea resistance) gene from *Magnaporthe grisea*, is another effective dominant selectable marker and has been useful in retransformation of previously manipulated, hygromycin-resistant strains. G418 resistance also has been employed, yielding three options for antibiotic selection-based transformation (Brandhorst et al. 2002). Auxotrophic strains of *B. dermatitidis* have become available recently, when strains with spontaneous and UV-induced mutations in the *ura5* gene for uracil production were isolated using 5-fluoro-orotic acid selection (Sullivan et al. 2002). Complementation of this auxotrophy offers more efficient selection than antibiotic resistance.

Homologous recombination takes place in *B. dermatitidis*, but its rate relative to the usual mode of illegitimate recombination is uncertain. To knock out the *BAD1* gene by homologous recombination, the *hph* selection marker was placed downstream of a truncated span of *BAD1* promoter. This strategy may have functioned as a promoter trap. Homologous replacements of *BAD1* represented about 1–2 percent of hygromycin B-resistant transformants (Brandhorst et al. 1999). Targeted knockout of genes, while attainable by more conventional techniques, may be enhanced by such a strategy. Reporters for studies of gene regulation have been developed for *B. dermatitidis*. β-Galactosidase activity can be measured in extracts of *B. dermatitidis* transformed with this reporter, and *BAD1*–β-galactosidase reporter fusions have been used to confirm that *BAD1* is transcriptionally regulated (Rooney et al. 2001). A β-glucuronidase reporter is also functional in *B. dermatitidis* (Brandhorst et al. 2002).

IMPORTANCE OF BAD1 IN PATHOGENESIS

Manipulation of the *BAD1* locus is an example of the application of new molecular genetic tools. *BAD1* was successfully deleted from *B. dermatitidis* strain ATCC 26199 by allelic replacement. The knockout strain had diminished capacity to bind macrophages and murine lung tissue, compared to the parent strain, and most importantly, was found to be attenuated in a murine model of blastomycosis (Brandhorst et al. 1999). Binding and virulence defects were rescued by re-expression of *BAD1* in trans in the knockout strain. The indispensable role of *BAD1* in pathogenesis was thus uncovered using the molecular genetic tools now available in *B. dermatitidis*.

Sequence analysis of the *BAD1* gene predicts a product consisting of a short amino-terminal region, a 24-amino acid repeat, present in 30 tandem copies within the core of the protein, and a carboxyl-terminal region that possesses homology to epidermal growth factor (Hogan et al. 1995). The C-terminal EGF domain is important for BAD1 localization on the yeast cell surface (Brandhorst et al. 2003). The tandem repeat of BAD1, which constitutes over 75 percent of the protein's primary structure, mediates binding of macrophage CR3 and CD14 receptors (Newman et al. 1995). BAD1 binding to phagocytes has also been found to modulate host immune responses. For example, BAD1

downregulates the production of tumor necrosis factor-alpha (TNF-α), which is normally stimulated in response to the infection, and critical for host defense (Finkel-Jimenez et al. 2001). A *BAD1* knockout strain evokes the production of TNF-α in vivo in an experimental model of infection and in vitro during co-culture with macrophages and neutrophils, whereas the isogenic, wild-type strain (and purified *BAD1*) curtails TNF-α production, promoting progressive infection. BAD1 modulates TNF-α production partly through inducing transforming growth factor-beta (TGF-β), which antagonizes phagocyte TNF-α responses (Finkel-Jimenez et al. 2002). Hence, in addition to promoting binding of the fungus to cells and tissue, BAD1 enhances pathogenicity by allowing *B. dermatitidis* to evade host immunity by modulating it in a manner that favors fungal survival over host survival.

BAD1 AND FORM TRANSITION

BAD1 is only found on the surface of the yeast form of *B. dermatitidis*, and not on the surfaces of hyphal filaments or conidia that make up the mold form (Rooney et al. 2001). BAD1 is not a prerequisite for the form transition to yeast. Strains from which BAD1 has been deleted undergo form transition in both directions with no hindrance. Given the established role of BAD1 in pathogenesis, these observations help explain how the thermally triggered form transition leads to establishment of a virulent state. Once conversion to yeast has begun, BAD1 rapidly accumulates on the surface of the organism. An analysis of yeast and mycelial morphotypes employing both Northern hybridization and reporter genes fused to the *BAD1* promoter has shown that the form-specific regulation of *BAD1* is tightly controlled at the level of transcription (Rooney et al. 2001). Hence, a virulence gene – *BAD1* – has proven to be under transcriptional control that is tied, either directly or indirectly, to the temperature-induced transition from mold to yeast.

Many African strains of *B. dermatitidis* are naturally devoid of BAD1, as they lack the gene for this protein (Klein et al. 1997). When the BAD1 coding region, under the control of its native promoter, was transferred into an African strain of *B. dermatitidis*, the heterologously expressed BAD1 retained form-specific expression and control at the level of transcription. The same phenomenon was observed when the *BAD1* gene was transferred into *H. capsulatum*, which also lacks a native *BAD1* locus, indicating that the form-specific mechanism of transcriptional control is conserved (Rooney et al. 2001). Sequence analysis of the upstream region of *BAD1* identified regions of homology with the promoter of the *H. capsulatum* yeast-form-specific gene *yps-3*. It seems likely that other, as yet undefined, yeast-form-specific genes may be controlled by the same transcriptional regulatory mechanism.

HOST DEFENSE MECHANISMS

Polymorphonuclear leukocytes (PMN) efficiently phagocytose and kill the conidia of *B. dermatitidis*. By contrast, Drutz and Frey (1985) demonstrated that PMNs do not ingest *B. dermatitidis* yeast cells. Instead they surround and attach to extracellular yeasts. The lack of phagocytosis is attributed to the size of the yeast cells. PMN killing of conidia and yeast is mediated by oxidative mechanisms. Killing of the yeast is thought to involve an extracellular process, whereby PMNs attach to the yeast and empty granule contents in the extracellular space (Diamond and Krzesicki 1978). Brummer and Stevens (1982) have reported that PMNs augment growth of yeast in vivo and in vitro, rather than kill the fungus, fueling controversy about the role of PMNs in host defense.

Alveolar macrophages readily ingest conidia, but have difficulty both ingesting and killing yeast. Peripheral monocytes and especially monocyte-derived macrophages ingest and kill conidia and yeast more efficiently. Macrophage functions are enhanced with cells from immunized patients (Bradsher et al. 1985). Killing of yeast cells by interferon-gamma (IFN-γ)-activated macrophages does not depend on products of the oxidative burst (Brummer and Stevens 1987).

Bradsher described an in vitro test to quantify cell-mediated immunity in patients with blastomycosis (Bradsher and Alford 1981). The *Blastomyces* alkali-soluble water-soluble (ASWS) antigen developed by Cox (Cox and Larsh 1974) was used to measure lymphocyte migration inhibition and lymphocyte transformation. An outbreak of blastomycosis in which 48 patients were identified with acute pulmonary blastomycosis provided an opportunity to evaluate this assay in humans (Klein et al. 1990). Most patients developed specific cellular immunity between 3 months and 21 months after exposure to *B. dermatitidis*. The assay may offer an epidemiologic tool to elucidate the geographical distribution of blastomycosis. A detailed discussion of the immunology of *B. dermatitidis* has been published by Klein (1992).

As early as 1914, results of autogenous vaccines were reported. Stober described three patients (Stober 1914). One patient did well and two improved for a short period of time, then relapsed and died. There has been no recent investigation into the immunization of humans. Mice were shown to be protected against a lethal challenge with *B. dermatitidis* yeast cells by immunization (recovery from nonlethal subcutaneous infection) (Morozumi et al. 1982). Protection was mediated by cells and not serum, supporting the previous studies by Cozad and Chang (1980), which established that cellular immunity mediates protection against *B. dermatitidis*. More recently, Wuthrich et al. (2000) described a genetically engineered attenuated vaccine strain of *B. dermatitidis* that harbors a targeted deletion

of *BAD1*. The vaccine confers sterilizing immunity against infection with isogenic and nonisogenic strains in a murine model, and has allowed investigation of the cellular molecular mechanisms of acquired immunity in the murine system. As in prior work, T cells rather than B cells are largely responsible for vaccine immunity, particularly $CD4^+$ T cells. Type 1 cytokines including IFN-γ, TNF-α, and granulocyte-macrophate colony-stimulating factor (GM-CSF) are instrumental in immunity mediated by these cells in normal hosts (Wuthrich et al. 2002). There is sufficient redundancy in immune-deficient hosts so that an absence of one or more of these products can be offset by a compensatory response in one of the others. For example, IFN-γ deficiency can be compensated by TNF-α, and conversely, TNF-α deficiency can be compensated by GM-CSF (Wuthrich et al. 2002). This theme has been extended to studies in $CD4^+$ T-cell-deficient hosts (Wuthrich et al. 2003), where $CD8^+$ T cells subsume the role of T-cell help, producing the type 1 cytokines needed for host defense and sterilizing immunity against *B. dermatitidis* (or *H. capsulatum*). These studies raise the prospect that engineered vaccines can protect against infection with *B. dermatitidis* or related fungi, possibly even in immune-deficient hosts (Casadevall and Pirofski 2003).

LABORATORY DIAGNOSIS

The diagnosis of blastomycosis in a patient frequently requires a combination of several techniques. The clinical signs and symptoms are nonspecific. Some commercial serological tests lack specificity and most lack sensitivity. The histological picture may not be clear as a result of the morphological similarity with other mycotic agents and nonmycotic artifacts. A presumptive diagnosis can frequently be made by integrating patient history, the clinical symptoms, a wet mount examination, and serology. The only absolute laboratory diagnostic criterion for blastomycosis is the isolation and identification of *B. dermatitidis*.

As the fungal etiologic agent is not always apparent on clinical presentation of the patient, universal methods for fungus culture are performed if a mycotic disease is suspected. The techniques for the isolation and identification of mycotic agents are described in detail in Laboratory diagnosis. Table 1 describes the appropriate clinical material to be selected for the isolation of *B. dermatitidis*. The application to blastomycosis will be described briefly.

Direct examination

The observation of yeast cells in an adequate clinical sample is a quick way to establish a preliminary diagnosis. However, isolation and culture are essential to confirm the diagnosis. The specimens to be submitted to the laboratory depend on the manifestations of the disease: skin scrapings or pus from skin lesions; sputum in cases of pulmonary disease; and biopsy tissue from any lesion. Urine, even in the absence of urinary tract symptoms, is frequently a good source of the organism in males. The yeasts are most probably liberated from an infected prostate gland. Although hematogenous dissemination is common in blastomycosis, blood cultures have only rarely yielded this fungus (Musial et al. 1987).

Direct microscopic examination of the clinical material can readily be performed. A typical cutaneous lesion shows central healing with microabscesses at the periphery. A pus specimen may be obtained by nicking the top of a microabscess with a scalpel. The exuded purulent material is mixed in a drop of 20 percent KOH on a microscope slide. A coverslip is applied and the slide is heated gently to clear the tissue and purulent material without damage to the fungus. The wet mount is then examined under the microscope. *B. dermatitidis* appears as a round yeast cell with double wall and often with a single bud. Usually the cells are uniform in size, 8–12 μm, readily seen in pus or infected tissue with KOH. The apparent double wall and broad-based bud are characteristic. Bud scars are sometimes seen at the side of the juncture of the mother and daughter cells. Both cells are frequently the same size and appear as a figure-of-eight (see Figure 5).

Culture

The preferred medium for the isolation of *B. dermatitidis* is Emmons' modification of SGA. The modification includes the addition of antibiotics to inhibit bacterial contamination, a decrease in glucose from 4.0 percent to 2.0 percent and a final pH of 6.9. The reduced sugar content is more favorable for the recovery of *B. dermatitidis*. Samples submitted for culture are incubated at both 35–37°C and 25–30°C. All cultures should be held 4–6 weeks before discarding as negative.

PRESUMPTIVE DIAGNOSIS

A presumptive identification of *B. dermatitidis* is based on the colonial and microscopic morphology as described above and the conversion of the isolated form to the converse. The dimorphic attribute applies to only a few human pathogens and the structures of one form or the other are sufficiently distinct to the experienced mycologist to allow the identification of the isolate. In recent years several means of verifying the identity of *B. dermatitidis* have been developed: exoantigen, DNA probe, and DFA.

EXOANTIGEN

A simple, specific, and sensitive test to identify the mycelial form of dimorphic fungi is the exoantigen test

(Kaufman et al. 1983). This is a double immunodiffusion reaction in agar performed by using an aqueous extract of a 7–10-day-old mycelial culture as the unknown antigen. This unknown antigen solution is precipitated against a known, standardized antiserum. A sharp, clear precipitin band of identity is formed where the antigen and the homologous antiserum meet. This test, with the proper antiserum, is 100 percent specific for *B. dermatitidis* and can also be applied to the identification of other dimorphic fungi. It is interesting that nonviable mycelial cultures of *B. dermatitidis* can be identified by this method (Di Salvo and Wooten 1982) and *B. dermatitidis* can be specifically identified even when the extracted culture is contaminated (Di Salvo et al. 1981).

DIRECT FLUORESCENT ANTIBODY

The direct fluorescent antibody (DFA) test for *B. dermatitidis* was developed by Kaplan and Kaufman (1963). There are several advantages of this rapid procedure to identify yeast forms of the fungus. The technique may be applied to tissue sections or to a yeast-form culture. Tissue, suspected of being malignant, is often removed at surgery and placed in formalin. Later, when the possibility of a fungous infection is being considered, clinical material for culture is not available. If the organisms in the tissue are not typical because of unusual shapes or size, the morphology may not be sufficient for a definitive diagnosis. Where morphology among yeasts can be very confusing, DFA is specific. When applied to unknown yeast organisms, DFA will fluoresce positive as soon as growth is visible. The stain will identify *B. dermatitidis* whether or not the organisms are viable.

NUCLEIC ACID PROBES

A simplified test using nucleic acid probes has recently been developed for the identification of *B. dermatitidis* cultures. The probes may be applied to either the mycelial or yeast forms of the organism. The single-stranded DNA probe is combined with a chemiluminescence label for detection. If the unknown organism is *B. dermatitidis*, the DNA probe will hybridize with the RNA of the target organism. The labelled DNA/RNA hybrid is then detected for identification of the fungus. In three evaluations of the nucleic acid probe, the sensitivity ranged between 87.8 percent and 100 percent. Two studies reported 100 percent specificity (Scalarone et al. 1992; Stockman et al. 1993). The third evaluation tested 17 strains of *P. brasiliensis*, a morphologically similar dimorphic fungus (Padhye et al. 1994). These investigators found that 10 of the 17 *P. brasiliensis* strains hybridized with the *B. dermatitidis* probe. This crossreaction is not an obstacle if the morphology is distinctive for the suspected organism. The commercially available reagents are expensive, but the test requires less than 2 hours to confirm the identity of the isolate. Any rapid means of identification is welcome by mycologists because the traditional methods required to isolate and identify dimorphic fungi require 4–6 weeks. In the future, the nested PCR assay for detecting *B. dermatitidis BAD1* sequences, developed by Bialek et al. (2003), may fulfill this criterion.

A comprehensive discussion of specific culture methods for each type of clinical material has been published (Goodman 1992). Detailed diagnostic laboratory procedures for the microbiologist are described in that publication.

Serodiagnosis

As fungal pulmonary infections cannot be etiologically classified clinically, the serological tests for fungal infections are requested, and performed, as a group. Most diagnostic laboratories in the USA perform fungal serology as a battery for the endemic mycoses blastomycosis, histoplasmosis, and coccidioidomycosis.

There are two serological tests in general use which detect antibodies to *B. dermatitidis* and are used for the diagnosis of blastomycosis: the complement fixation (CF) test and the immunodiffusion (ID) precipitin test. The CF test usually requires 2–3 months for antibody to be detected. Both the sensitivity and the specificity of the CF test are poor. However, the results are quantitative and may aid in evaluating the progression of disease or efficacy of therapy. The ID or precipitin test may be positive in up to 80 percent of the patients with blastomycosis, 2–3 weeks after the onset of symptoms, and its specificity is close to 100 percent. A positive test indicates recent or active disease. The enzyme immunoassay (EIA) test is a more recent development in the diagnosis of blastomycosis. In one small trial of a commercial diagnostic kit, the procedure was shown to have a sensitivity of 100 percent and a specificity of 85.6 percent (Sekhon et al. 1995).

An outbreak of blastomycosis among a group of 95 individuals exposed to a point source of *B. dermatitidis* provided an opportunity to study the serology of this disease. A comparison of the CF, ID, and EIA tests on 47 patients meeting the case definition was reported (Klein et al. 1987b). They found these three tests to have a sensitivity of 9, 28, and 77 percent, respectively, and a specificity of 100, 100, and 92 percent. The low sensitivity in this study may be the result of the number of asymptomatic or mild cases of blastomycosis identified in this cohort. The EIA testing was performed at the Centers for Disease Control and Prevention (CDC) using their reagents, not the commercially available kit. As the day of exposure was known with certainty, which is not usually the case in blastomycosis, the time required for the development of antibody could be determined reliably. The conclusion of this study was that, in this instance, the EIA antibody was detected as

early as 13 days and peaked at 50–70 days after the onset of symptoms. The ID antibody peaked at the same time but did not appear earlier. Therapy shortened the antibody window, but the three tests did not appear to have any prognostic value. A report by Kaufman (1992) specifically described the present immunodiagnosis of blastomycosis in greater detail.

A noncommercial radioimmunoassay has been reported that incorporated BAD1, which is a target of both antibody and T-cell responses (Klein and Jones 1990). Sensitivity and specificity were 85 and 97 percent, respectively, in 68 patients with blastomycosis, and 73 patients with other systemic mycoses. Follow-up studies demonstrated that BAD1 tandem repeats are the target of the antibody response in patients and that these sequences are present in the A antigen of *B. dermatitidis*. BAD1 and A antigen of *B. dermatitidis* are thus similar antigens, if not the same, although detailed biochemical studies of the antigens demonstrated that carbohydrate on A antigen (lacking on BAD1) accounts for crossreactivity with sera from histoplasmosis patients or rabbit histoplasmosis immune sera (Klein and Jones 1994).

TREATMENT

Not all patients with blastomycosis require antifungal therapy. It is customary to observe the patient with suspected mild pulmonary blastomycosis while the cultures are pending. In many patients, the pneumonia will clear spontaneously. If progression of the disease is obvious, an antimycotic agent should be considered. There are two major classes of antimycotic agents used for blastomycosis: the polyenes and azoles.

The polyene amphotericin B, introduced in the 1960s, is still considered the gold standard of therapy for blastomycosis and other systemic mycoses. This drug must be administered intravenously for several weeks. The initiation of therapy requires hospitalization, close observation of the patient, and monitoring of renal function. Amphotericin B has significant side effects in many patients, but relapses are few after an adequate course of therapy (about 2 g). The mechanism of action of this agent is believed to be an alteration of the permeability of the cell membrane of the yeast cells which allows essential constituents of the fungus to leak from the cell with subsequent death of the fungus. It is still considered the drug of choice by many infectious disease physicians, particularly in patients with severe disease or underlying impairments of immunity.

The azoles cause fungistasis by blocking the enzyme which converts lanosterol to 14-demethyllanosterol. This biochemical sequence is the forerunner of ergosterol synthesis. The triazole, itraconazole, is presently the drug of choice for mild cases of blastomycosis. This oral medication can be administered on an outpatient basis and is less toxic than amphotericin B. Some physicians still debate whether antimycotic therapy is necessary after surgical excision of pulmonary lesions (Edson and Keys 1981). More conservative clinicians tend to provide the additional antibiotic therapy. For immunocompromised patients, particularly those with AIDS, or in life-threatening infection, amphotericin B is still the drug of choice. A detailed account of the therapy of blastomycosis with various antimycotic agents has recently been published by Bradsher et al. (2003). Additional information on antifungal therapy is presented in Principles of antifungal therapy.

The recognition of patients infected with blastomycosis requires a detailed patient history and knowledge of the ecology and epidemiology of *B. dermatitidis*.

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Coccidioidomycosis

DEMOSTHENES PAPPAGIANIS

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Coccidioides immitis (a second species, *Coccidioides posadasii* sp.nov., has been proposed by Fisher et al. 2002), is a soil-inhabiting fungus which, usually following inhalation, causes coccidioidomycosis. It usually occurs naturally only in certain areas of the western hemisphere. The disease, in its various manifestations, has also been called coccidioidal granuloma, San Joaquin fever, valley fever, and desert rheumatism. As a result of its persistent geographical distribution, coccidioidomycosis is an 'endemic systemic mycosis.' *C. immitis* undergoes a morphological change from its hyphal/arthroconidial form in nature, or in the usual laboratory culture, to a spherule-endospore form in vivo. It is thus grouped with the dimorphic systemic pathogenic fungi.

Recent reviews of coccidioidomycosis and coccidioides are provided in *Seminars in Respiratory Infections* (Ampel 2001) and by Cole et al. (2004).

Coccidioides species have been included as a 'select agent' of bioterrorism.

ETIOLOGIC AGENT

History

During the nineteenth century, physicians in the central valleys of California described febrile maladies related to disturbance of the soil, which probably included coccidioidomycosis (Thompson 1969). The first definitive written word on the disease emerged in 1892 when Posadas, in Buenos Aires, reported on the disease in an Argentinian soldier. The disease had begun some 4 years earlier, with its first manifestations being cutaneous involvement; a physician, Dr Bengolea, described it as 'mycosis fungoides,' a neoplastic disease which involves the skin, but it became evident that this was a progressive, systemic, and altogether different disease. Posadas had noted microscopically an organism that resembled a protozoan, belonging to the group referred to as coccidia that are characterized by a cyst containing endocystic cells. Two years after the initial report by Posadas, a second case was reported, originating in California (Rixford 1894). In fact, in the next several decades this was the site of occurrence and recognition of most of the cases.

As a result of its resemblance to the protozoan coccidia, the organism was subsequently named *Coccidioides immitis* (coccidia-like, *im*, not; *mitis*, mild). [Interestingly, no additional case was recognized in Argentina until 35 years after Posadas' report (Negroni 1967)]. The fungal nature of the organism was unrecognized until 1900 at which time a filamentous mold, believed for several years to have been a contaminant of cultures obtained from patients' tissues and exudates, was shown by inoculation of laboratory animals to change its morphological form into one resembling a protozoan (Ophüls and Moffitt 1900). This provided proof of the diphasic fungal nature of *C. immitis*.

Significant migration of individuals into the endemic areas of the southwestern USA occurred in the 1930s, as a result of catastrophic ecological and economic changes elsewhere in the USA. With the start of World War II in the early 1940s, military troops were also brought into endemic areas. These two groups of largely susceptible individuals permitted refinement of the knowledge about coccidioidomycosis (Smith et al. 1946a). In 1956, specific antifungal therapy in the form of amphotericin B was introduced for the treatment of coccidioidomycosis

(Fiese 1957). The azoles, introduced in the 1980s, significantly altered the approach to treatment.

Mycology

C. immitis Rixford and Gilchrist, 1896, is diphasic and polymorphic (Figure 1). The hyphal arthroconidial form is the saprophytic or saprobic form, found in nature and the usual laboratory cultures, and infrequently in tissues of the infected host (arthroconidia were formerly referred to as arthrospores). Airborne arthroconidia are inhaled and convert to the spherule-endospore ('parasitic') phase in the infected mammalian host. Special cultural conditions permit the production of the spherule-endospore phase in vitro (Converse 1955). The taxonomic placement of *C. immitis* has customarily been in the Fungi Imperfecti (Deuteromycotina) owing to an absence of a demonstrable conventional sexual conjugational stage. However, Burt et al. (1996) have described 'cryptic sex' (which may resemble parasexuality) as providing a means of exchanging genetic information. There are variations in the phenotype of the mycelial phase but not the spherule-endospore phase. Several features have, however, indicated that coccidioides probably fits in the division Ascomycotina (Ascomycetes), family *Onygenaceae*, and order Onygenales (Currah 1985): the hyphae are septate and have septal pores close to structures that resemble Woronin bodies; thallic arthroconidia are produced from specialized hyphae (i.e. whole hyphal cells become thick walled spore-like structures) which alternate with empty, degenerate 'disjunctor' cells (Sigler and Carmichael 1976) (Figures 1 and 2). Four pairs of chromosomes are reportedly present.

The percentage of guanine–cytosine (49.1–49.61 percent) was similar in the spherule and hyphal forms of three strains of *C. immitis* [compared with 47.3 percent and 48 percent in *Histoplasma capsulatum* and *Blastomyces dermatitidis*, respectively, both of which are ascomycetes (Pappagianis et al. 1985)]; based on homology/relatedness studies of the 18S ribosomal gene, *C. immitis* was shown to be closely related to *H. capsulatum* and *B. dermatitidis* (as well as *Trichophyton rubrum*), providing affirmation of taxonomic affiliation with ascomycetes (Bowman et al. 1992). The DNA studied by restriction enzyme analysis allowed separation of *C. immitis* into two groups, I and II (Zimmermann et al. 1994). Fisher et al. (2002) concluded that group I corresponded to a new species, *Coccidioides posadasii* ('non-California') and group II, *C. immitis*

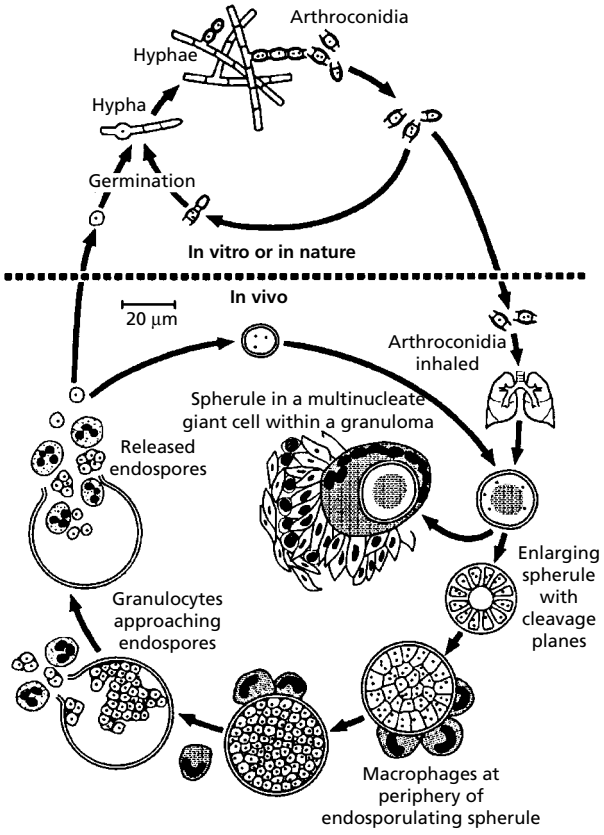

Figure 1 *Diagram illustrating the morphological forms of* Coccidioides immitis *in its saprobic form and its 'parasitic' form in vivo. The spherule/endospore form is depicted as evolving after inhalation of the arthroconidia, and the usual cellular reaction to the in vivo forms. (Adapted from Forbus and Bestebreurtje (1946) and others)*

Figure 2 *Chains of arthroconidia, some of which are separated by 'disjunctor' cells which undergo degeneration, first of the cytoplasm and later of the nuclei and cell wall.*

('California') species. There is no evidence that disease or its laboratory diagnosis associated with *C. immitis* or *C. posadasii* differs, or that response to therapy differs; and epidemiologic and experimental data indicate that immunity to these putative species is cross-protective (Pappagianis 1999).

The vegetative hyphal form can grow on a variety of media, ranging from those with sparse organic substrates (ammonium acetate suffices as a source of carbon and nitrogen) to the usual rich clinical laboratory culture media: blood agar, Sabouraud's, etc. On suitable media (e.g. 1 percent glucose/0.5 percent yeast extract/2 percent agar) arthroconidia are formed in 5–7 days at 34–37°C. These arthroconidia, usually 2–4×5–6 μm can be barrel-shaped, cylindrical, or oval and, when terminal on a hypha, they can be bullet-shaped. They form in the chain of cells of specialized hyphae developed from short hyphae 1–2 μm in diameter that are borne laterally on vegetative hyphae (2–4 μm in diameter). The arthroconidia, usually multinucleate and having a very refractile inner and outer wall, alternate with degenerating ('disjunctor') cells which share only the outer wall with adjacent arthroconidia (Huppert et al. 1982) (Figures 1 and 2). This thinner wall readily fractures, leading to disarticulation and, in appropriate currents of air, dispersion of individual or short chains of arthroconidia. When deposited on a suitable (nonviable) substrate with adequate moisture, the arthroconidia will germinate unilaterally or bilaterally to give rise to hyphae which, in turn, can produce arthroconidia. These airborne arthroconidia are highly infectious and it is necessary to be constantly aware of the potential infectious hazard to laboratory workers handling cultures, particularly from cultures more than 3 days old.

When the arthroconidia reach the airways of a human or other species (or are injected parenterally), and in the presence of host inflammatory cells (Baker and Braude 1956) and increased concentrations of CO_2 (Lones and Peacock 1960), the arthroconidia shed an outer wall layer and all but one nucleus, then become round and enlarge to produce a spherule. Nuclear division is followed by formation of cleavage planes of cell wall material which originates in the peripheral cell wall. It is likely that formation of the larger spherule from the smaller endospore involves several coordinated enzyme activities involved in the synthesis and breakdown of cell wall substances: synthases of chitin, glucan, mannan; lysis by chitinase, chitobiase, glucanase, mannosidase and proteases. In about 48 hours in vivo, the progressive growth and segmentation yield a mature spherule 20–150 μm in diameter with variable numbers, from a few to hundreds, of endospores 2–4 μm in diameter (Figure 3). These endospores can be released from the mature spherule, although the mechanism of release is uncertain but may involve proteases (Resnick et al. 1987; Yuan and Cole 1987), glucosidase (glucanase) (Kruse and Cole 1992) and chitinase (Johnson and Pappagianis 1992); these act upon the spherule wall and its internal projection around the endospores (Figure 4). The chitinase, and possibly also the glucanase and a protease, are important antigens in the induction of antibodies that have a significant role to play in the serodiagnosis of coccidioidomycosis (see section on Immunoserology, below). The released endospores enlarge in vivo and produce endosporulating spherules, which, in turn, can release another generation of endospores; in this way the pathogenic events continue. It is possible to demonstrate a similar formation of endosporulating spherules from an inoculum of arthroconidia or endospores in a synthetic medium (Converse 1955; Levine et al. 1960).

The metabolic activities have been summarized (Pappagianis 1988b). Expression of certain genes and their ultimate protein products (e.g. enzymes such as aldehyde reductase) can be specifically associated with the morphological (i.e. parasitic) spherule-endospore phase (Delgado et al. 2004). Some coccidioidal enzymes have been cloned and expressed by recombinant technology, e.g. chitinase (Zimmermann et al. 1996) and aspartyl protease (Johnson et al. 2000). *C. immitis* is catalase positive in both hyphal and spherule/endospore forms; it grows aerobically and readily uses glucose and other carbohydrates as carbon sources. Under anaerobic conditions, however, some metabolism can be carried out, and spherules produce more CO_2 than is produced by hyphae (Lones and Peacock 1960). The arthroconidia and hyphae can carry out endogenous respiration for many hours in the absence of an added carbon source. Both hyphae and spherules contain enzymes of the tricarboxylate cycle with the exception of α-ketoglutaric dehydrogenase. Isocitric lyase and malate synthase were, however, found in the hyphal form, suggesting that the glyoxylate bypass (conservative metabolism of fatty acids) can be utilized in the absence of 2-oxoglutaric dehydrogenase (Lones 1967). Endospores, but not spherules, use malate as a carbon source.

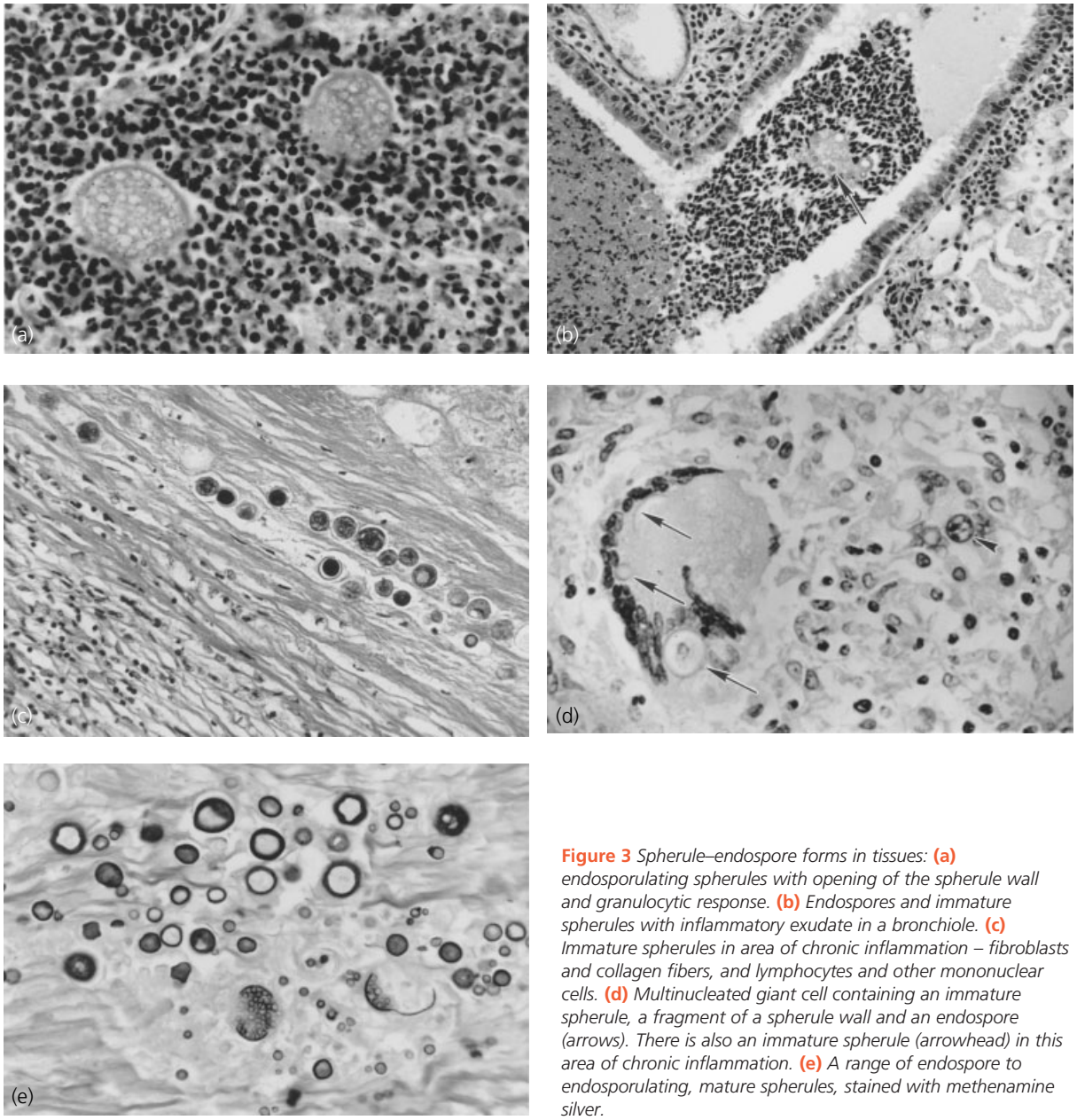

Figure 3 *Spherule–endospore forms in tissues:* **(a)** *endosporulating spherules with opening of the spherule wall and granulocytic response.* **(b)** *Endospores and immature spherules with inflammatory exudate in a bronchiole.* **(c)** *Immature spherules in area of chronic inflammation – fibroblasts and collagen fibers, and lymphocytes and other mononuclear cells.* **(d)** *Multinucleated giant cell containing an immature spherule, a fragment of a spherule wall and an endospore (arrows). There is also an immature spherule (arrowhead) in this area of chronic inflammation.* **(e)** *A range of endospore to endosporulating, mature spherules, stained with methenamine silver.*

The hyphae can utilize starch or xylose but the spherule/endospore form does not. Ammonium, nitrate, urea, acetamide, and several amino acids can be utilized as sources of nitrogen.

Ecology/epidemiology/geographic distribution

These aspects have been reviewed (Pappagianis 1988b). Coccidioidomycosis occurs primarily in arid and semi-arid areas of the western hemisphere where *C. immitis* lives in the soil, on the surface, and to a depth of 30 cm (Egeberg and Ely 1956). In the hottest periods of the year the soil temperature at the surface and to a depth of 1 cm can reach 60°C, which is lethal to *C. immitis* within minutes. Disturbance of the soil, with the creation of dust by winds or excavation or earthquakes (Schneider et al. 1997) throws arthroconidia up into the air. These conidia are hydrophobic and have a greater buoyancy with slower settling in air than would be expected for their size, ~2×5 μm. This allows them to be airborne for up to hundreds of kilometers. Such conidia-laden dust, which leads to infection, is most likely to occur in the hot dry seasons when the soil has become low in moisture.

The relative confinement of *C. immitis* to specific geographic areas, which has no precise explanation, makes coccidioidomycosis an 'endemic mycosis.' The

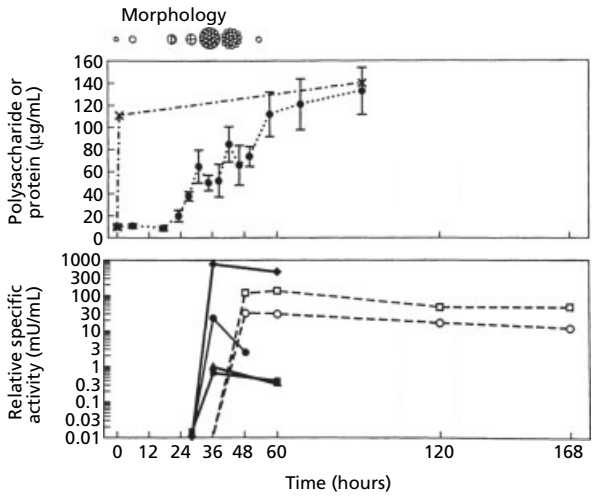

Figure 4 *Sequence of appearance of polysaccharide, protein and carbohydrases and proteases during the evolution in vitro of endospore to spherule to endospore. Top: filled circles, protein; crosses, polysaccharide. Bottom: filled circles, chitinase; filled squares, β-1,3-glucanase; filled triangles, β-1,6-glucanase; filled diamonds, β-1,3-glucanase; open circles, protease azocollytic; protease elastase. (From Pappagianis (1989) with permission)*

Figure 5 *Geographical distribution of* Coccidioides immitis *sites where coccidioidomycosis is usually acquired. (From Pappagianis (1980) with permission)*

regions fall between 40° North 120° West (Northern California) and 40° South 65° West (Argentina), and include the southwestern USA, California, Arizona, New Mexico, Texas, and small areas of Nevada and Utah, southern and northeastern (Petersen et al. 2004); Mexico; Honduras; Guatemala; Colombia; Venezuela; Paraguay; Argentina and, recently recognized, Brazil (state of Piaui). The name 'San Joaquin fever' or 'valley fever' is derived from a central region of California in which the disease is highly endemic (Figure 5).

Many, but not all, of the regions where the fungus is found conform to a bioclimatic area referred to as the Lower Sonoran Life Zone (Maddy 1957). *C. immitis* exists in areas that have relatively low annual rainfall (12.5–50 cm), hot summers (e.g. as in Phoenix, Arizona, a mean temperature 26–32°C) and relatively mild winters, though there is occasional snow at some elevations (900–1000 meters) where *C. immitis* is found. The soil is often sandy and alkaline (Swatek 1970). During the hot, dry season the soil has a high concentration of mineral salts which is better tolerated by *C. immitis* than by other microorganisms in the soil. Despite its survival in hot, dry, desert-like areas, where the relative humidity is usually no lower than 20 percent, it does not survive well at lower, e.g. 10 percent, relative humidity.

Rainfall has two influences: first, rain wets the soil, diminishing the airborne dust and fungal conidia, thereby reducing infections (Smith et al. 1946a); second, rainfall moistening the soil and *C. immitis* contained therein leads to germination of the fungal arthroconida; this supports a new round of vegetative growth, producing another crop of the infectious arthroconidia. Roughly, the higher the rainfall, the greater the number of cases of the infection that present in the succeeding hot, dry season (Pappagianis 1994). Although there is a greater risk of infection associated with soil-disturbing occupations, overall more cases are observed in persons not occupationally exposed, conforming to the easy movement of airborne arthroconidia.

Increased travel or immigration has led to increased numbers of cases among those entering the endemic areas (Ampel et al. 1998; Pappagianis 1994). Such cases have been observed in Europeans, Canadians, and Asians who have briefly visited the endemic western hemisphere, and in those who live elsewhere in countries with endemic *C. immitis* but who visit the endemic areas. A few infections have occurred in people who have never visited the endemic areas, but who have received or been exposed to exported products from these areas (Symmers 1967).

In addition to humans, a variety of other species have been infected with *C. immitis* (Table 1). There is a wide range of responses among animals in which infections have been recognized: cattle, sheep, and swine appear to be resistant to progressive forms of the disease; they are often infected and have mediastinal lymph node involvement while remaining clinically healthy. On the other hand, domestic horses

and llamas often have severe disease (Ziemer et al. 1992), though recently subclinical infections have been recognized in some domestic horses (Higgins et al. 2004). Przewalski (non-domesticated) horses have been recognized as having severe infections (Terio et al. 2003). Primates and monkeys have generally had severe disease, although arrest of the disease with antifungal therapy has been achieved. Domestic dogs appear to have disease patterns – mild to severe dissemination, especially involving the osteoarticular tissues – as seen in humans. Marine mammals (sea otters, sea lions, and recently a dolphin) which reside in the Pacific Ocean or its bays, are unique among these hosts. How they are infected is uncertain though the arthroconidia can travel long distances in the air, and their buoyancy and hydrophobicity could lead to their flotation on sea water and aerosolization by wind and/or wave action.

HOST RESPONSE

Clinical forms

Infection with *C. immitis* usually follows inhalation of the arthroconidia. Rarely, traumatic introduction of the organism into the skin leads to infection and disease (Wilson et al. 1953). In both instances, the regional lymph nodes are often involved: hilar in pulmonary infection, and inguinal in infection of the lower extremities. (Human-to-human transmission of *C. immitis* ordinarily does not occur, but has followed transplantation of organs from individuals with coccidioidomycosis

Table 1 *Animal species with naturally acquired coccidioidomycosis*

Animal	Animal
Aardvark[a] (*Orycteropus aferi*)	Lemur, ringtail[a] (*Lemur catta*)
Armadillo, nine banded[b] (*Dasypus novemcinctus*)	Lion, mountain[a] (*Felis concolor*)
Baboon[a] (*Papio* sp.), mandrill (*Mandrillus* sp.)	transvaal[a] (*Panthera leo krugeri*)
Badger[a] (*Taxidea taxus*)	Llama[a] (*Lama glama*)
Bear, Malayan sun[a] (*Ursus malayanus*)	Monkey[a] – tropical American (*Cebus hypoleucus*)
Binturong[a] – a civet (*Arctitis binturong*)	colobus (*Colobus guereza*)
Burro (*Equus assinus*)	guenon DeBrazza (*Cercopithecus neglectus*)
Cat, domestic (*Felis catus*)	sooty mangabey (*Cerocebus atys*)
Cattle, domestic (*Bos taurus*)	spider[c] (*Ateles* sp.)
Cheetah[a] (*Acinonyx jubatus*)	wooley[c] (*Lagothrix* sp.)
Chimpanzee[a] (*Pan troglodytes*)	bonnet macaque (*Macaca radiata*)
Chinchilla[a] (*Chinchilla lanigera*)	Celebes macaque (*Macaca maurus*)
Coyote[a,b] (*Canis latrans*)	lion tail macaque (*Macaca silenus*)
Deer, white-lipped[a] (*Cervus albirostris*)	rhesus (*Macaca mulatta*)
Dog (*Canis familiaris*)	Okapi[a] (*Okapia johnstoni*)
Dog, Cape or African hunting[a] (*Lycaon pictus*)	Onager[a] (*Equus hemionus onager*)
Dolphin, Pacific bottle-nosed[b] (*Tursiops gilli*)	Otter, river[a] (*Lutra canadensis*)
Ferret[a,c](*Mustela* sp.)	sea[b] (*Enhydra lutris*)
Gazelle (*Gazella thomsonii*)	Rhinoceros, Northern White[a] (*Ceratotherium simum cottoni*)
Genet[a,c](*Genetta felina*)	black (*Diceros bicornis*)
Gorilla, mountain[a] (*Gorilla gorilla beringeri*)	Rodents[b] – pocket mouse (*Perognathus baileyii*,
lowland[a] (*Gorilla gorilla gorilla*)	*P. penicillatus*, *P. intermedius*)
	grasshopper mouse (*Onchyomys torridus*)
Horse, domestic (*Equus caballus*)	ground squirrel (*Citellus harrisi*)
Horse[a], Przewalski (*Equus przewalski*)	kangaroo rat (*Dipodomys merriami*)
Human (*Homo sapiens*)	Sea lion[a,b] (*Zalophus californianus*)
Impala[a] (*Aepyceros melampus*)	Sheep (*Ovis aries*)
Jackrabbit[b] (*Lepus californicus*)	Skunk, hog-nose[a,c] (*Mephitis* sp.)
Kangaroo[a] (*Macropus rufus*)	Snake, Sonoran Gopher[b] (*Piticophis melanoleucus affinis*)
wallaroo[a] (*Macropus robustus or erubescens*)	Swine (*Sus scrofa*)
Kiang[a] (*Equus hemionus kiang*)	Tapir[a] (*Tapirus terrestris*)
Kit fox[b] (*Vulpes velox*)	Tiger[a], Bengal and Sumatran (*Panthera tigris*)
Lemur, red ruffed[a] (*Varecia variegata* v. *suber*)	Zebra[a], Grevy's (*Equus grevyi*)

This compilation includes cases detected by Raymond Reed, DVM and Kathryn Orr, DVM.
a) Zoo or captive animals.
b) In the wild.
c) Species name uncertain.

who served as donors (Tripathy et al. 2002; Wright et al. 2003.)

It is estimated that some 60 percent of individuals infected by the respiratory route are asymptomatic or have such a mild illness that medical attention is not sought (Figure 6). Their exposure to *C. immitis* is diagnosed from the presence of a positive skin test, or from detection of a chronic pulmonary residual lesion, 'coccidioidoma' or cavity, detected incidentally in a chest radiograph. Occasionally a previously healthy individual may present with hemoptysis or other complaint that can be referred to a cavity.

Of the 40 percent who develop symptoms, most will have an acute febrile illness beginning 7–28 days (average 10–16 days) after exposure to the organism. The clinical presentation has been reviewed in the literature (Galgiani 1993). Chest pain, often pleuritic and sometimes severe, occurs in 75 percent. The following symptoms occur in descending order: cough, fever, malaise, rash, sore throat, headache, arthralgia and/or myalgia. Anorexia, weight loss, and night sweats are encountered in some patients. These signs and symptoms resemble those of many other diseases and have led to designation of coccidioidomycosis as 'flu-like.'

The acute-stage pulmonary lesions, detected by chest radiograph, are also similar to those found in other diseases: small or large single or multiple infiltrates (the latter can resemble metastatic carcinoma); hilar and/or mediastinal adenopathy; transient cavity, pleural, or pleuropericardial effusions; miliary or reticulonodular lesions (each of which is prognostically unfavorable).

Various rashes occur, but erythema nodosum and erythema multiforme are most commonly seen (Quimby et al. 1992).

Of the 40 percent with symptomatic infections, most will recover completely. These patients are immune to exogenous reinfection as are those individuals who have incurred asymptomatic infections. About 10 percent of these patients will, however, be left with a pulmonary residual nodule or cavity, usually detected several months or years later. Infrequently, a chronic progressive fibrocavitary disease resembling apical tuberculosis or histoplasmosis capsulati evolves. Metapulmonary spread of the disease (dissemination) is usually clinically apparent, but can occur silently, although infrequently, to the eyes (Rodenbiker et al. 1981) or kidneys (Petersen et al. 1976). Such dissemination occurs and becomes manifest in the first few weeks to months after onset of primary infection; for example, meningitis became evident about 5 weeks after the initial onset of symptoms (Pappagianis 1988a). Dissemination may not, however, be noted for some years and can occur in the absence of any previously recognized pulmonary focus. In the southwestern USA where coccidioidomycosis is endemic, the overall rate of dissemination in symptomatic cases is 5–7 percent but the rate varies with ethnicity or race. Thus, the primary infection disseminates in one to two per 100 symptomatic adult white males, at a slightly higher rate in Mexicans and Native Americans (Indians) (approximately 5 per 100), but at a markedly higher rate in African-Americans (>20 per 100) and Filipinos (>25 per 100) and other Asians (30 per 100) (Gifford et al. 1936/1937; Smith et al. 1946a, b; Pappagianis 1988b).

Additional risk factors that heighten the risk of dissemination are the following:

- group B blood (Deresinski et al. 1979)
- HLA class II (Louie et al. 1999)

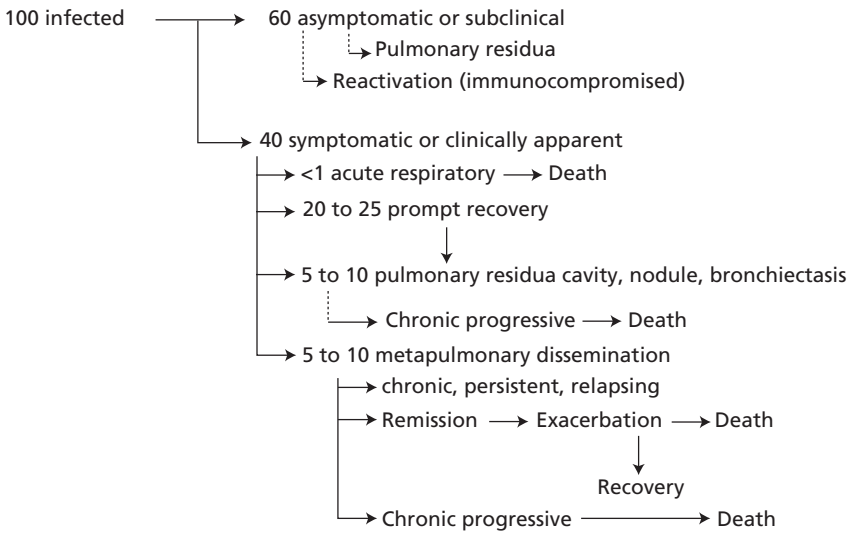

Figure 6 *Forms of coccidioidomycosis in humans. Broken lines indicate infrequently occurring phenomena.*

- pregnancy, particularly when acute primary coccidioidomycosis is acquired during the third trimester (Peterson et al. 1993)
- hematopoietic neoplasms, e.g. lymphoma, AIDS (Deresinski and Stevens 1974; Fish et al. 1990)
- immunosuppression for organ transplant (Cohen et al. 1982; Riley et al. 1994)
- trauma creating a site of lessened resistance (Pappagianis 1985).

Dissemination can affect virtually any tissue and organ, although the gastrointestinal mucosal surface (Weisman et al. 1976) and endocardium have rarely been affected. The skin, bones, and meninges are affected (Huntington 1971). The involvement of various tissues and organs in disseminated disease assures that coccidioidomycosis can be confused with a great variety of infections, neoplasia, connective tissue disorders, etc.; in fact, it has become a 'great imitator' similar to syphilis. A history of travel or residence in 'endemic areas' or (rarely) exposure to products exported from such areas, should prompt consideration of coccidioidomycosis in the differential diagnosis. The coexistence of other diseases (infectious, neoplastic, 'connective tissue,' etc.) must not, however, be overlooked despite confirmation of coccidioidomycosis (Huth 1994).

Immunology

C. immitis can be a primary pathogen producing disease in normal individuals or opportunistic disease in immunocompromised individuals. It is unique among the systemic pathogenic fungi, because its replication in the infected host results in spherules containing tens to hundreds of endospores each of which can, in turn, produce mature endosporulating spherules. Contrast this with the single or few progeny produced by the budding of other systemic pathogens. The mechanism(s) by which *C. immitis* causes disease has not been defined. It may involve toxic action (Gale et al. 1967), enzymatic activity, e.g. proteases (Lupan and Nziramasanga 1986; Resnick et al. 1987; Yuan and Cole 1987; Johnson et al. 2000), and immunological mechanisms: complement-mediated/hypersensitivity/cytokines may contribute to pathogenesis (Magee and Cox 1995).

Mannose is a prominent component of coccidioidal cells (Pappagianis et al. 1961) and may play a role in immune responses via macrophage mannose receptors (Ampel et al. 2004, unpublished).

The immune interactions of host and *C. immitis* have been well reviewed (Cox and Magee 1998). The immunology can be divided roughly into the following categories:

- innate responses of the host
- specific humoral or T-cellular immunity
- induction of protective immunity.

The precise initial events in the interaction between the previously uninfected host and infective arthroconidia have not been well defined. The conidia are of such dimensions that at least some of them may elude the upper respiratory filtration and mucociliary escalator to reach the small bronchi, bronchioles, and alveoli. [Bronchoscopic examination had shown that primary endobronchial disease existed in the apparent absence of pneumonitis (alveolitis) (Birsner 1954).] Although the portal of entry is usually respiratory, infrequently infections have resulted from traumatic introduction of arthroconidia through the skin (Wilson et al. 1953). Experimentally, subcutaneous or intraperitoneal injection of conidia is less likely to result in the severe, progressive, and lethal disease than that induced by respiratory challenge.

In the mouse lung, the arthroconidia elicit a response which includes both macrophages and polymorphonuclear leukocytes (PML). The presence of PML appears to contribute to conversion of arthroconidia to spherules (Baker and Braude 1956). The outer wall of the arthroconidia, before being shed in the evolution to spherules, can, however, have a toxic effect inhibiting phagocytosis and destruction of the arthroconidia by human neutrophil granulocytes (Drutz and Huppert 1983). Mature spherules, by virtue of their size, and packets of newly released endospores embedded in a matrix appear to resist phagocytosis until individual endospores are released from this matrix (Frey and Drutz 1986).

Nevertheless, PMLs may provide an impediment to some morphological forms of *C. immitis* after the activation of complement and production of chemotactic substances in the absence of antibody (Galgiani et al. 1980). This may result after antigen–antibody precipitation as in a fibrin clot (Pappagianis and Zimmer 1990), induction of an 'oxidative burst' with production of antimicrobial H_2O_2 and hypochlorous acid (induced by arthroconidia and by immature but not mature spherules) (Galgiani 1995), production of cationic peptides (Segal et al. 1985), or production of lysozyme which is lethal to *C. immitis* spherules in vitro (Collins and Pappagianis 1973). Some studies suggest that enhanced phagocytosis by PMLs may result after immunization (Wegner et al. 1972).

The induction of a *C. immitis*-specific T-lymphocyte response underlies the major protective response to *C. immitis* (reviewed by Cox and Magee 1998). This has been derived from studies in humans and in experimental animals. A high rate (~100 percent) of humans with primary acute coccidioidomycosis who recovered had positive skin test (delayed-type hypersensitivity) to coccidioidin compared with only about 70 percent of those with unifocal disseminated (extrapulmonary) disease and about 30 percent with multiple sites of dissemination (Smith et al. 1948). Anergy was associated with a poor prognosis in those patients with disseminated disease, and may result from the following:

- excessive fungal antigen or of immune complexes
- induction of suppressor cells
- relative increase of Th2 lymphocytes over Th1 (the latter of greater importance in cell-mediated immunity) (Corry et al. 1996)
- depression of the immune response by fungal cell products.

[However, 15–20 percent of patients with chronic coccidioidal pulmonary cavities are nonreactive to skin tests with coccidioidin, but do not have a poor prognosis or a risk of dissemination from their pulmonary lesion (Smith et al. 1948; Winn 1968).]

It has been proposed that part of the protective immune response to *C. immitis* is related to an opsonizing effect of IgM reacting with surface antigen of *C. immitis* (Pappagianis and Zimmer 1990); but the higher IgG titers are associated with a poorer prognosis (see section on Immunoserology, below) It appears that induction of a *C. immitis*-specific T-lymphocyte reponse underlies the major protective induced immune response to *C. immitis*. In experimental animals, several lines of evidence indicate that immune T lymphocytes and lymphokines are crucial in the protection against *C. immitis* (Beaman et al. 1983). The immune T lymphocytes act not by a direct killing action on *C. immitis* but rather through cytokine activation of monocyte/macrophages (Beaman et al. 1983; Slagle et al. 1989; Ampel et al. 1992; Ampel 2003). Experimentally, interferon-gamma was associated with resistance, interleukins 4 and 10 with susceptibility to *C. immitis* (Magee and Cox 1995; Fierer et al. 1998). Various levels of deficiency of T cell or competency of macrophages may exist in patients who are at higher risk of disseminated coccidioidomycosis. For example, it needs to be determined whether anticoccidioidal enzymatic activities are significantly different in macrophages and this can provide a possible explanation of differences in susceptible versus resistant or immunized hosts.

Resistance (immunity) to second exogenous infection follows subclinical infections (detected by positive skin test) and complete recovery from clinically apparent infection only. Only four cases of second exogenous (re)infection have been documented in >30 000 cases of coccidioidomycosis. Only one was a naturally acquired infection but in an immunocompromised individual. Three others were laboratory acquired (Pappagianis 1999). Endogenous reactivation of a previously arrested, non-disseminated, coccidioidal infection can occur as a result of immunosuppression: iatrogenic as with organ transplantation, from infection with HIV, or through development of a hematopoietic–lymphoreticular malignancy. Reactivation of a previously disseminated coccidioidal infection, even after apparently successful antifungal therapy, is not uncommon (Stevens 1995).

It had been demonstrated by Rixford (Rixford and Gilchrist 1896) that prior infection with *C. immitis* rendered a dog resistant to (parenteral) reinfection. The resistance conferred upon humans by prior infection led to experimental immunization in laboratory animals. In 1951 it was demonstrated by C. E. Smith that injection of the hyphal/arthroconidial form of living avirulent *C. immitis* in mice induced resistance to intraperitoneal challenge. Injection of virulent *C. immitis* in mice or monkeys provided protection against lethal respiratory challenge (Pappagianis et al. 1960). Killed *C. immitis* also afforded protection (Friedman and Smith 1956; Levine et al. 1960). Of the various morphological forms used in nonviable vaccines, the mature spherule (particularly its cell wall) proved to be the most efficacious (Levine et al. 1961). These immunizations did not prevent infection following respiratory challenge but did prevent progressive, lethal disease. After tests for safety of the killed spherule vaccine, tests for protective efficacy in humans showed that the killed spherule vaccine did not prevent clinically apparent coccidioidomycosis (Pappagianis and the Valley Fever Vaccine Study Group 1993). The inadequate response of humans may have resulted from the relatively small dose of antigen that had to be used because of the inflammatory effect of whole spherules injected intramuscularly. It is possible that isolation and/or synthesis of the immunogenic component away from irritating cell wall components, e.g. chitin, may provide a tolerable and effective vaccine for humans.

Some candidate subcellular vaccines that have exhibited protection against respiratory challenge in mice include derivatives of mechanically disrupted spherules (Zimmermann et al. 1998), a soluble proline-rich antigen (PRA) developed by Galgiani and colleagues (Shubitz et al. 2002), which proved to be the same as antigen 2 (Ag2) developed by Cox and colleagues (Zhu et al. 1996); and antigens including coccidioides-specific antigen (CSA) of Cole et al. (2004). Recombinant peptide forms of Ag2/PRA and CSA, when mixed or chemically combined, have the appropriate properties and immunogenicity that may permit them to be tested in nonhuman primates and humans.

Screening immunization of a cDNA library has led to another antigen with protective activity in mice (Ivey et al. 2003).

Histopathological response

Coccidioidal lesions can generally be termed pyogranulomatous, but in fact a variety of histopathological responses can be detected: tuberculosis-like granulomata, caseation necrosis with or without (more often) calcification, hyalinization-fibrosis, fibrinous pleuritis. Endospores attract PML whereas the mature spherules evoke a mononuclear/macrophage response (Figure 1). Thus, abscesses and tubercles may be side by side (Huntington 1971). *Coccidioides* in the skin evokes a proliferative ('pseudoepitheliomatous') response in the epidermis. The synovial membranes produce a villous

response in coccidioidal arthritis. Bones have lytic lesions (occasionally proliferative) in the human, lytic or proliferative in dogs. In old pulmonary lesions (coccidioidomas), immature (non-endosporulating) spherules are often embedded in fibrocaseous material. In the absence of identifiable *C. immitis*, the granulomatous reaction may resemble that of other diseases such as sarcoidosis. Peripheral (bloodstream) eosenophilia can occur and on occasion the acute coccidioidal pulmonary disease can resemble 'eosenophilic pneumonia' of other etiologies (and disastrous therapy with adrenocorticosteroids may be applied).

In coccidioidal meningitis, the cerebrospinal fluid (CSF) exhibits a pleocytosis which, early on, may have a preponderance of PML and eosenophilia, but usually the lymphocytes predominate. Cerebral arteritis can accompany coccidioidal meningitis.

Treatment

Most patients with coccidioidomycosis could be managed successfully without specific antifungal therapy but, if necessary, the disease can be treated medically or surgically. The decision to treat or not to treat, and the mode of therapy depend on the clinical stage, on risk factors in the hosts, and the type and location of lesions. The variability in the clinical course of the disease, whether treated or untreated, has made evaluation of therapy difficult. More than one therapeutic modality may have to be applied.

MEDICAL THERAPY

Currently available therapy includes preparations of the polyene amphotericin B (AMB), including lipid or liposomal forms, and azoles. The azoles are fungistatic. The AMB under some circumstances can be fungicidal but with clinical concentrations is usually fungistatic. There is a lack of well controlled therapeutic studies which has led to uneven and sometimes uncertain application of therapy. Between the introduction of AMB in the treatment of coccidioidomycosis in humans in 1956 (Fiese 1957), and the introduction of the azoles 25 years later, the usually benign course of primary coccidioidomycosis prompted a conservative approach – to avoid the undesirable side effects of parenteral AMB, particularly nephrotoxicity and adverse effects on the hematopoietic system. Miconazole, also given parenterally, had a short-lived application in disseminated coccidioidomycosis. After the introduction of the azoles that could be taken by mouth [ketoconazole (KTZ), fluconazole (FLU), and itraconazole (ITR)] and were relatively free of undesirable side effects, these oral medications were used initially to treat disseminated disease (Tucker et al. 1990a, b; Diaz et al. 1991; Galgiani 1993; Galgiani et al. 1988, 2000). In some locations, the routine early use of the relatively benign triazoles has become customary in the treatment of suspected or confirmed cases of coccidioidomycosis without controlled evaluation of the efficacy at this stage of the disease, i.e. it is not known whether such treatment hastens recovery or prevents complications such as extrapulmonary spread of the disease or development of pulmonary residual lesions. Despite uncertainty regarding the usefulness of treatment in acute primary coccidioidomycosis, the presence of certain threatening conditions (severe intrapulmonary disease, risk factors for dissemination) and unfavorable laboratory values (such as peripheral blood eosinophilia and climbing complement fixation (CF) titer) gives the justification for treatment.

KTZ is now infrequently used in human coccidioidomycosis in the USA. Variconazole, a triazole as are FLU and ITR, has had limited use in coccidioidomycosis but has appeared effective even against meningitis following failure of FLU therapy (Cortez et al. 2003).

Treatment is almost always indicated for patients with demonstrated or surmised disseminated disease, or with rapidly progressive primary pulmonary disease. AMB is indicated for the latter, for widespread extrapulmonary lesions, infected pregnant women (the AMB appears to have little adverse effect on the fetus), and osseous lesions. [The place of the azoles in the treatment of humans with osseous lesions is not well defined (ITR may be preferred), but in the dog KTZ appears to lead to an improvement in osteoarticular disease (Wolf and Pappagianis 1981).] For more indolent disseminated disease, the azoles can be used, and which to use may be guided by the side effects (greater with KTX) or expense (greater with FLU than with KTZ or ITR). However, other properties may also influence the selection: excellent penetration of FLU into the CSF (appropriate for treatment of meningitis); short half-life of KTZ compared with those for FLU and ITR (Tucker et al. 1990a). Significant interaction with other drugs requires close attention when the azoles are used (e.g. with rifampin, cyclosporin, warfarin, and terfenadine). Acquisition of resistance to FLU by *C. immitis* has been suggested but not proven.

Two major problems accompany medical therapy: the long-term therapy of coccidioidomycosis (usually months or years) is very expensive, and there is uncertainty as to when to stop treatment. Relapses have followed seemingly successful treatment, especially with the azoles (Dewsnup et al. 1996).

FUNGICIDAL AGENTS

These are needed, because currently used chemotherapeutic agents have not killed cells in the host. Experimentally, nikkomycin Z, an inhibitor of synthesis of cell wall polysaccharide by chitin synthase, has proved to be curative (Hector et al. 1990) (Figure 7).

SURGICAL THERAPY

Surgical approaches are indicated as diagnostic and therapeutic procedures for numerous coccidioidal lesions.

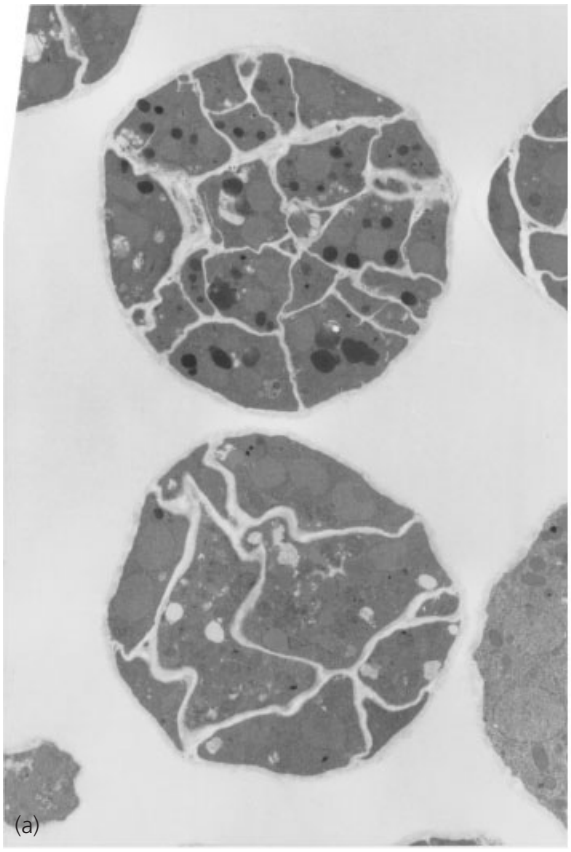

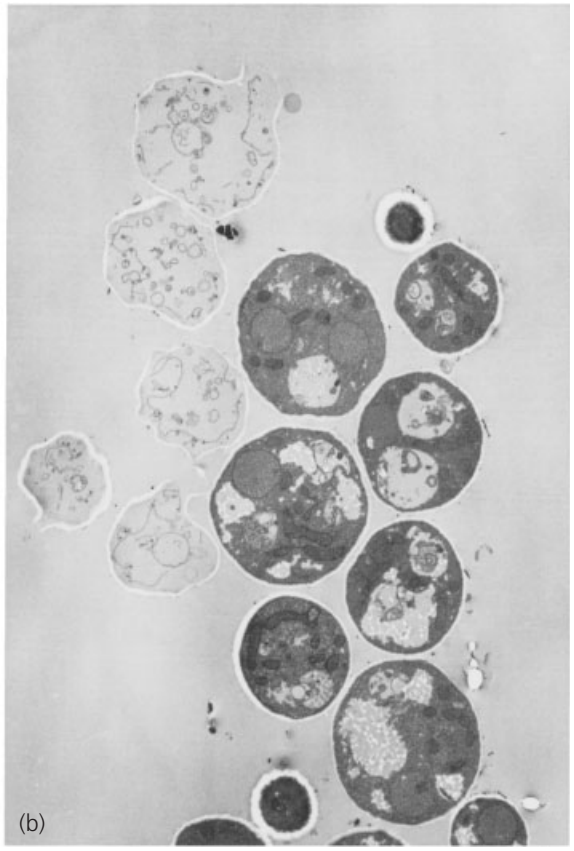

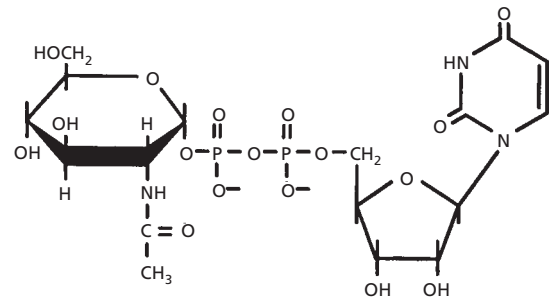

UDP-*N*-acetylglucosamine

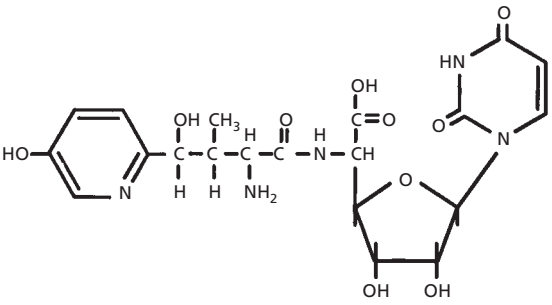

(c) Nikkomycin Z

Figure 7 (a) *Transmission electron micrograph of 33-hour-old spherules produced in vitro, untreated* **(a)** *and those exposed to nikkomycin Z 2 μg/mL for 24 hours* **(b)**. *Untreated spherules exhibit an intact cell wall and segmentation towards formation of endospores. In the presence of nikkomycin there is a decrease in the size of the spherules, little or no cell wall was formed and the ghosts of some spherules are collapsing. (From Pappagianis (1989) with permission.)* **(c)** *Comparative structure of antifungal nikkomycin Z and UDP-N-acetylglucosamine.*

Biopsy (this may be excisional) of a solitary pulmonary nodule is in the diagnostic category; this type of nodule must be distinguished from neoplastic or other infectious nodules which have not yielded a diagnosis with non-invasive procedures (Forseth et al. 1986). Coccidioidal pulmonary cavities, and their associated complications such as hemorrhage, rupture into the pleural space, or formation of a bronchopleural fistula, may require surgical correction (Cunningham and Einstein 1982).

Drainage of abscesses and empyema, removal of infected bone or joint tissue, and immobilization of a joint by arthrodesis are additional therapeutic surgical procedures (Bried and Galgiani 1986). Stabilization of vertebrae infected with *C. immitis* has been accomplished (Herron et al. 1997).

Surgical placement of prostheses, such as insertion of an Ommaya reservoir and catheter into the lateral ventricle or cisterna magna, may help in the treatment of coccidioidal meningitis (Zealear and Winn 1967).

Surgical procedures are usually carried out with concomitant antifungal medical therapy.

LABORATORY DIAGNOSIS

Direct examination

Microscopic demonstration of *C. immitis*, particularly the endosporulating spherules, provides the most direct and secure method of diagnosis. Detection may be attempted with bronchial washings, exudates, and sputum (the last is often unreliable because of confusing artifacts). Synovia or other body fluids, e.g. pleural, pericardial, or peritoneal, may reveal presence of the organism though the corresponding tissue is more likely to yield *C. immitis* microscopically (or by culture). The endosporulating spherules or other forms can be identified in preparations treated with potassium hydroxide or stained with hematoxylin and eosin (H&E) or Papanicolaou stain, but they may be more readily distinguished when stained by methenamine silver or periodic acid–Schiff (PAS), particularly when the latter is used without a counterstain (Huntington 1971). *C. immitis* can be visualized in gram-stained sputum or body fluids, but this has not been a widely advocated method. Various sized forms of *C. immitis* may be seen, and in some lesions, particularly chronic ones, small immature spherules without endospores may be observed and can resemble *Blastomyces dermatitidis* (Figure 3). The presence of chitin in the cell wall permits fluorescent staining of *C. immitis* in sputum or other body fluids using Calcofluor white M2R (Polysciences Inc.) or its equivalent Fluorescent Brightener 28 (Sigma Chemical Co.). Fluorescent antibody staining (of limited availability) has shown a desirable specificity and can be used when the usual histopathological examination has not yielded a definitive answer.

In some tissues, e.g. pulmonary cavities, the hyphal form may be observed but is not definitive for *C. immitis* and further confirmatory studies are required, e.g. conversion of hyphal to endosporulating spherule by inoculation of laboratory animals or special culture media. A wide range of tissue reactions is evoked by *C. immitis* – suppurative, granuloma, pyogranuloma, caseation necrosis with or without calcification, hyaline or fibrotic changes. Thus, even if the characteristic endosporulating spherules are not seen, the diagnosis of coccidioidomycosis should not be rejected based on the nature of the host response.

Culture

C. immitis is hardy and non-exacting in its nutritional requirements, being able to grow in simple media containing salts and ammonium acetate or ammonium lactate as its sources of energy, carbon, and nitrogen. It can grow in media with an acidic pH <3.0 and alkaline pH >9.0). (The tolerance of a high pH may underlie its ability to survive in the alkaline soils of the endemic areas, and in the selective action of NH_4OH added to yeast extract/phosphate medium.) It can grow at 22°C and at 40°C, but its optimum for hyphal growth is about 30°C. In vitro, the conversion from hyphal to spherule/endospore form is enhanced by incubation at 40°C but it can be maintained in the spherule/endospore phase at 37°C. Exposure to 46°C for 2 hours is tolerated but it is killed in 4 minutes at 60°C. Inoculation of sputum, exudates, tissues, onto many of the commonly used culture media will yield satisfactory growth, including: sheep blood agar, brain heart infusion, beef infusion glucose, Sabouraud's glucose agar, glucose/yeast extract agar. The glucose (2 percent)/yeast extract (1 percent) agar (2 percent) provides excellent yields of arthroconidia whereas the peptone-containing Sabouraud's glucose agar can diminish the yield of arthroconidia.

Culture of the blood may yield the organism, but patients with coccidioidal fungemia are frequently very ill and in the preterminal stage of their coccidioidomycosis. Care must be taken to permit recovery of *C. immitis* in specimens that may contain other organisms. Treatment of sputum with alkali or hypochlorite for recovery of *Mycobacterium tuberculosis* can inactivate *C. immitis*. Recovery of *C. immitis* may, however, be enhanced by mucolytic agents such as *N*-acetyl-1-cysteine and dithiothreitol. Competing organisms can be minimized by the addition of the antifungal cycloheximide and antibacterial chloramphenicol. Before diagnostic animal inoculation, specimens can be treated with penicillin G and streptomycin (or comparable aminoglycoside) to reduce possible infection with bacteria. Isolation from the soil can entail similar plating or inoculation of laboratory animals – mice being the most convenient.

Faint grayish-white growth becomes visible in 48 hours on several media; it becomes clearly evident as a

cottony to weblike growth in 48–72 hours. Although rarely necessary, it is advisable to retain cultures for 2–3 weeks before discarding them as negative.

Typical gross morphology is exhibited by most isolates of *C. immitis* but atypical colonial growth does occur. For this reason and because the hyphal/arthroconidial form of *C. immitis* resembles other nonpathogenic fungi, it is essential to carry out definitive identification. This can be achieved by demonstrating the spherule/endospore phase after inoculation into laboratory animals or into special culture media.

More recently, a DNA hybrid protection assay (Gen-Probe, Inc.) has proved to be a sensitive and rapid way to identify isolates as *Coccidioides* (Beard et al. 1993). Ribosomal RNA (rRNA) is extracted from the suspect organism and allowed to react with specific DNA from *C. immitis* to which is attached a potentially luminescent acridinium ester. If the rRNA is of coccidioidal origin, it will react with its complementary DNA, and this protects the ester linkage against hydrolysis, thus yielding luminescence. Heterologous rRNA will not interact with the coccidioidal DNA, and the acridinium ester will be hydrolyzed and thus not yield luminescence. The method appears to be quite specific and requires very little of the mycelial growth form.

Another in vitro test which has served for several years as a specific test for *C. immitis* is the exoantigen test (Standard and Kaufman 1977). The suspect culture is grown for several days on a slant culture. An aqueous solution of thimerosal is added to the slant to sterilize the culture and to extract antigen. The antigen-containing supernatant is then tested by immunodiffusion using specific anticoccidioidal antibody to demonstrate the presence of antigen specific for authentic *C. immitis*. A 19 kDa polypeptide with serine protease activity may represent the '*Coccidioides immitis*-specific' antigen (Pan and Cole 1995).

The indispensable condition for morphological identification is demonstration of endosporulating spherules. This can be achieved by intraperitoneal or intranasal inoculation of mice or intratesticular inoculation of guinea pigs. If the animals become overtly ill, tissue with visible lesions can be examined microscopically in a squash preparation or permanent histological sections. If no illness develops, mice should be sacrificed at 7–10 days. If no lesions are evident, subcultures should be made from the tissues, and additional mice sacrificed at 14 and 21 days. Intraperitoneal inoculation usually suffices but some isolates of lesser virulence may only be recoverable after intranasal inoculation.

Immunoserology

Immunological tests have been very useful in epidemiologic studies and in the diagnosis and management of cases of coccidioidomycosis [reviewed by Pappagianis 2001 (in Ampel 2001) and Pappagianis and Zimmer 1990]. They represent sensitive and specific tests which can assist in the diagnosis when there is not ready access to sputum or tissue, and to obviate invasive procedures. In the early application and evaluation of these procedures, conversion to skin test positivity appeared to precede the detection of antibody (Smith et al. 1956). However, more recent studies have indicated that antibody can be detected before the skin test becomes positive. Therefore, a negative skin test should not preclude a diagnosis of coccidioidomycosis nor the use of serological tests (Table 2).

Skin testing can be carried out with lysate-culture filtrate of the hyphal phase (coccidioidin) or lysate of the spherule/endospore phase (spherulin) (Levine et al. 1973). Although some individuals yield immediate-type reactions, the delayed cutaneous hypersensitivity reaction (readings at 24–48 hours, therefore earlier than tuberculin reactivity) represents the evidence of past or current infection. Only recent conversion to positivity is of assistance in the diagnosis of an acute primary coccidioidal infection. A reaction in a healthy individual denotes resistance to exogenous reinfection. Cross-reactivity in individuals previously infected with *H. capsulatum* can be observed, but does not represent a serious problem.

Failure to react to the skin test (anergy) may develop in about half the patients with disseminated coccidioidomycosis. However, 15–20 percent of patients with non-disseminated disease, e.g. a single chronic pulmonary cavity or nodule, fail to react (Winn 1968).

In vitro lymphocyte stimulation by coccidioidal antigen is a correlate of skin test responsiveness but has not supplanted the simple intracutaneous test.

In primary non-disseminating coccidioidomycosis, a regular sequential response is noted, indicated by production of two kinds of antibody: a precipitin and a complement-fixing antibody (Figure 8). The former was originally detected by a tube precipitin (TP) test (soluble, heat-stable antigen present in coccidioidin yielded a precipitate when mixed with serum of a patient with early, acute coccidioidomycosis) (Smith et al. 1956). It was later shown that the reaction could

Table 2 *Application of serologic tests in coccidioidomycosis*

	Detection of	
	IgM	IgG
Tube precipitin (TP)	Yes	No
Latex particle agglutination (LPA)	Yes	No[b]
Immunodiffusion = TP (IDTP)	Yes	No
Complement fixation (CF)	No (rare[a])	Yes
Immunodiffusion = CF (IDCF)	No	Yes
Counterimmunoelectrophoresis (CIE)	Yes	Yes
Enzyme-linked immunoassay (EIA)	Yes	Yes

a) Rarely positive CF in presence of positive IDTP (IgM, and absence of detectable IgG by IDCF).
b) Occasional positive LPA in presence of detectable IgG but not IgM by immunodiffusion.

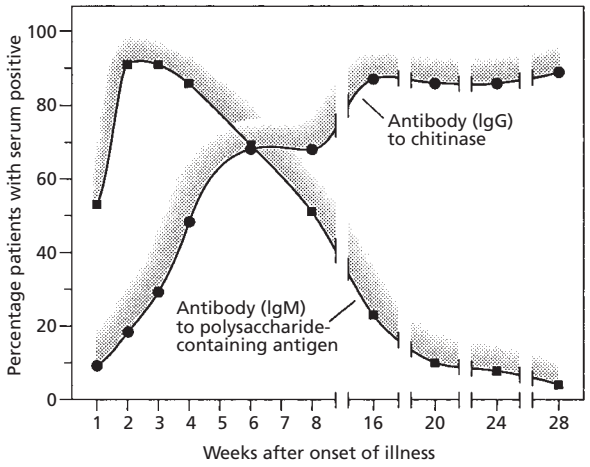

Figure 8 *Sequential antibody response in humans infected with* Coccidioides immitis. *The shaded zones represent the greater sensitivity, though not precisely defined, of more recent immunodiffusion and enzyme immunoassay tests for the detection of the IgM or IgG than the results (solid squares and circles) obtained using a TP and CF test by Smith et al. (1956).*

also be detected by agar gel double immunodiffusion (ID). The reaction is referred to as immunodiffusion corresponding to the tube precipitin (IDTP) test; and the responsible antibody was found to be IgM. This antibody could also be detected by antigen-coated latex particles and by enzyme-linked immunoassay (EIA). The antigen that reacts with the IgM appears to be mainly polysaccharide but has also been described in connection with a 120 kDa glucosidase (Kruse and Cole 1992), or a 21 kDa proteinase (Resnick et al. 1987) that can be released into the culture medium (Zimmer and Pappagianis 1989). This IgM antibody is detectable in more than 50 percent of patients with acute coccidioidomycosis by a week after the onset of symptoms. It is subsequently detectable (between 2 and 3 weeks of illness) in more than 90 percent of patients, and gradually fades although, with currently available methods, more than 10 percent of patients still have detectable antibody 6 months after onset of illness. There is no clear correlation between its titer and prognosis. It has been detected in the blood of newborns whose mothers had coccidioidomycosis and the anti-coccidioidal IgM, but this did not denote presence of the disease in the neonate. Presence of the IgM in the CSF has been infrequent but only associated with coccidioidal meningitis.

The antibody appearing later was originally detected by CF (Smith et al. 1956) and its titer clearly correlated with the extent and severity of the disease. It did not yield a precipitate with its corresponding antigen in a liquid milieu, but in a gel (agar, agarose, Gellan, Kelco of Merck & Co., Inc.) the antibody with its corresponding antigen produced a precipitate referred to as immunodiffusion corresponding to complement fixation (IDCF) (Figure 9). The antibody can also be detected by EIA (Pappagianis and Zimmer 1990; Gade et al. 1992). The responsible antigen, a protein of approximately 110 kDa, or 48 kDa under reducing conditions (Zimmer and Pappagianis 1988), is a chitinase (Johnson and Pappagianis 1992), which is released by the maturing then rupturing spherule. [The coccidioidal DNA which encodes for this chitinase has been cloned in *Escherichia coli* which has produced enzymatically and serologically active chitinase (Zimmermann et al. 1996).] The corresponding antibody appears to be primarily IgG. Its titer generally rises with worsening of the disease, and decreases with improvement of the patient. A titer above 1:16 is often associated with metapulmonary dissemination, e.g. to the skin, a bone or even the meninges (Smith et al. 1956). With this last, there can be lethal disease even though the CF titer in the serum is less than 1:16. In most patients with meningitis, antibody can also be detected in the CSF but the serum must always be tested along with the CSF

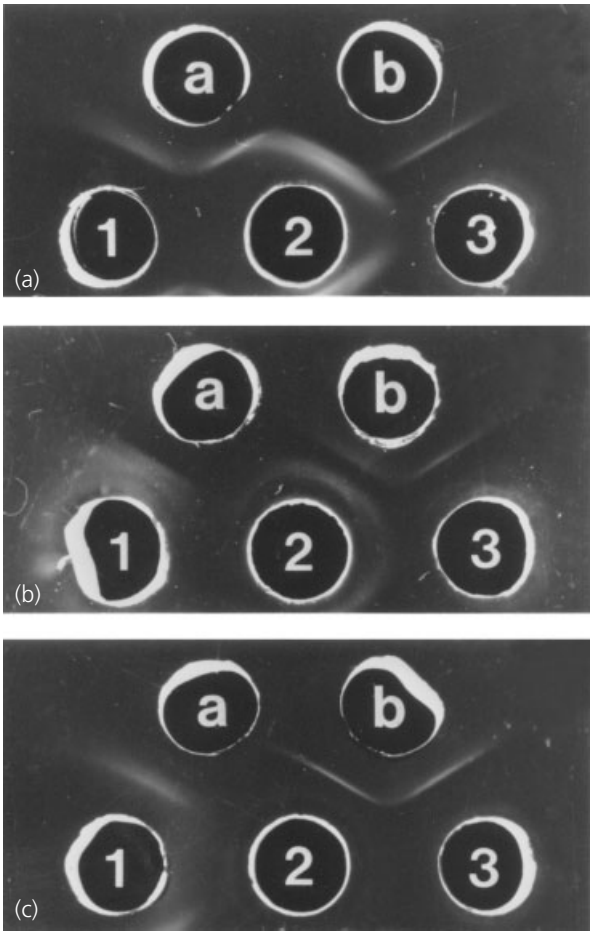

Figure 9 *Agar gel immunodiffusion indicating the detection of the usual antibodies (IgM and IgG) in human serum at different stages after infection. Well 2, patient's serum; wells 1 and 3, control IgM- and IgG-positive sera, respectively. Wells a and b contained coccidioidal antigen.* **(a)** *Early infection, IgM present;* **(b)** *later stage, both IgM and IgG present;* **(c)** *even later, only IgG detectable.*

because the CSF of some patients may be seronegative early in the course of meningitis. Testing synovial and other cavity fluids may be helpful in following the disease in corresponding anatomic sites.

Although the serological reactivity may obviate the need for invasive procedures, mediastinoscopy, or bronchoscopy, when possible, and recovery of the organism must be attempted. It is important to remember that in suspected early coccidioidomycosis, the tests for IgM are needed because the coccidioidal IgG may not be detected at that stage.

In the usual patient, serological reactivity is of considerable assistance for diagnosis and prognosis. However, some patients with immunosuppressive conditions (iatrogenic as for organ transplantation, or AIDS) may fail to produce a detectable antibody response (Fish et al. 1990). However, appropriate testing (concentrating serum prior to testing) has reduced seronegativity in AIDS patients with coccidioidomycosis to less than 10 percent.

It has been shown that detection of antigen may be accomplished before the detection of the antibodies described above, and this may provide an additional early serodiagnostic test and one useful in immunocompromised patients (Galgiani 1992).

Histopathology

Gross lesions may provide a clue to the histopathological changes which may involve a pyogranulomatous response with granulocytes, including both neutrophils and eosinophils, macrophages, multinucleate giant cells with palisading epithelioid cells, caseation necrosis, and hyalinization or fibrosis with lymphocytes at the periphery. The granulomatous changes, fibrosis and, occasionally, calcification, may represent chronic lesions, but only the presence of *C. immitis* distinguishes these from lesions of many other diseases. Vasculitis with perivascular inflammation and endarteritis obliterans has been described in association with coccidioidal meningitis/encephalitis.

The suppurative reaction may be extensive, but microabscesses only a few hundred micrometers in diameter may be found adjacent to granulomas. The former probably represent a response to released endospores, the latter a response to the larger spherules. Occasionally, the surface of mature spherules stained with H&E demonstrates a rough eosinophilic deposit similar to the Splendore–Hoeppli phenomenon associated with other fungi.

In addition to H&E and Papanicolaou's stains, the polysaccharide stains PAS and methenamine silver are useful for detection of *C. immitis*. Methenamine silver may stain the *C. immitis* diffusely dark, so that the internal structure, e.g. endospores in a spherule may not be clearly visible. PAS can provide better definition of the spherule/endospore phase. The so-called 'Schiff-positive dust' (Huntington 1957) stained by PAS can occasionally be seen in the cytoplasm of multinucleate giant cells and may represent remains of cell wall polysaccharide, digested autolytically by *C. immitis*, or by host cell enzymes, e.g. lysozyme (Collins and Pappagianis 1973), or chitinase. Calcofluor, which produces fluorescence with chitin in the cell wall, can be used to detect *C. immitis* in bronchoalveolar lavage specimens.

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Histoplasmosis

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Histoplasma capsulatum, like Caesar's Gaul, is tripartite. Taxonomically, it has been subdivided into three varieties, each with its own distinctive and defining characteristics. The varieties are validly referred to as *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. capsulatum* var. *farciminosum*. As might be expected, each of them causes a distinct type of histoplasmosis, designated respectively as histoplasmosis capsulati, histoplasmosis duboisii, and histoplasmosis farciminosi. They also have a varied geographical distribution. Rather than divide the genus *Histoplasma* into varieties and classes, Kasuga and colleagues propose seven phylogenetic species based on genetic isolation (Kasuga et al. 2003). The *capsulatum* variety is cosmopolitan, being endemic in all the world's continents. The *duboisii* variety has the most limited areas of endemicity, being restricted essentially to the central part of the African continent between the tropics of Cancer and Capricorn, and to the offshore island of Madagascar in the Indian Ocean. *H. capsulatum* var. *farciminosum* is not known to occur or to have been introduced anywhere in the New World. Its endemic areas are currently located in Africa, eastern Europe, the Middle East, Asia (including Japan and the Philippines), and the Far East. All three varieties occur as saprophytic, mitosporic molds (Fungi imperfecti) in their ecological niches in nature or in the laboratory cultures at room temperature (about 25°C). In the tissue of their mammalian hosts, however, or when incubated at 37°C on rich media, they transform into unicellular yeast-like budding organisms.

ETIOLOGIC AGENTS: HISTORICAL BACKGROUND AND NATURAL HISTORY

Histoplasma capsulatum var. *capsulatum* – Darling 1906

The genus *Histoplasma* was established in 1906 when Darling (1906) described the first case of histoplasmosis capsulati in Panama and named its etiologic agent *H. capsulatum*. He was under the impression that this organism was a protozoan closely related to the species of *Leishmania*, but 'the differences are so marked and the lesions so unusual that I feel the case is a unique one.' The mycotic nature of this 'protozoan' was inferred by da Rocha-Lima of Brazil, who, while in Germany, compared histological sections from Darling's Panamanian human case with sections from east African cases of equine epizootic lymphangitis (histoplasmosis farciminosi) as well as visceral leishmaniasis (kala-azar). The equine cases (*Equus asinus* × *caballus* and *E. caballus*) were known to be caused by the fungus *Cryptoccocus farciminosus*, now designated *H. capsulatum* var. *farciminosum*. As a result of these comparative histological studies, da Rocha-Lima concluded that Darling's *H. capsulatum* was a fungus and not a protozoan, based on the intracellular occurrence of the unicellular tissue forms of the organisms and similarities in their morphology and tinctorial reactions (da Rocha-Lima 1912, 1912–13).

It remained for De Monbreun (1934) of Vanderbilt University in Nashville, TN to isolate the *capsulatum*

variety of *H. capsulatum* for the first time. He clearly described and depicted the in vitro mycelial and unicellular forms of this mold and proved that it was dimorphic. He also reproduced the critical clinical and histopathological features of Darling's histoplasmosis capsulati in two monkeys (*Macacus rhesus*) after intravenous injection of the yeast form of his historic isolate.

Development of the histoplasmin skin test also was an important milestone in our understanding of histoplasmosis (Palmer 1945). These studies provided the initial evidence that histoplasmosis often caused a benign and usually asymptomatic infection characterized by pulmonary calcification. Subsequently this approach established our understanding of the geographical distribution of histoplasmosis in the USA (Edwards et al. 1969).

The astuteness of De Monbreun as an investigator was revealed when he stated that 'the saprophytic form of *Histoplasma capsulatum* probably exists free in nature.' This probability turned into reality in 1949 when Emmons of the National Institutes of Health in Bethesda, MA reported the isolation from two soil samples collected under the edge of an outbuilding on a Virginia farm where histoplasmosis capsulati had been proven in 7 of 43 trapped brown rats (*Rattus norvegicus*) (Emmons et al. 1947).

The geophilism of *H. capsulatum* var. *capsulatum* and its growth as a saprophyte in soil were irrefutably established by Emmons when he detected and photographed the diagnostically important tuberculate macroconidia of that fungus in the supernatant of suspensions of soil specimens that had been proven to be positive for that mold by passage through laboratory mice (Emmons 1949).

The saprophytic existence of *H. capsulatum* in avian and chiropteran habitats was established respectively by field studies (Zeidberg et al. 1952; Emmons 1959). Although birds are not known to be infected by the *capsulatum* variety (Mengis and Habermann 1955), transitory infections in chickens (*Gallus gallus*) and pigeons (*Columba livia*) were, however, reported by Schwarz et al. (1957) when their birds were inoculated intravenously with 500 000 yeast-form cells/ml of the *capsulatum* variety – a highly improbable type of challenge to occur in nature. The innate resistance of birds, perhaps, may be attributed to their normally high body temperatures of 42–43°C.

Bats of a wide variety of genera (24) and species (33) have been found to be infected by *H. capsulatum* var. *capsulatum* (Schacklette et al. 1962; Ajello et al. 1995, 1996) and one species (*Nyceteris hispida*) by the *duboisii* variety in Nigeria (Gugnani et al. 1994).

Among humans, isolated cases and outbreaks of histoplasmosis capsulati almost invariably can be traced to sites associated with accumulations of bird and bat guanos in many parts of the world (Ajello 1964; Sacks et al. 1986). The *capsulatum* variety of *H. capsulatum* thrives in such soils, especially in caves, chicken coops with dirt floors, attics, and the hollow walls of homes, industrial plants, schools, church steeples, etc., and the roosts of gregarious birds, such as blackbirds, oil birds, pigeons, seagulls, and starlings (Ajello 1964; Smith and Furcolow 1964; Dean et al. 1978).

Disturbances of such sites create aerosols laden with infectious propagules of *H. capsulatum* var. *capsulatum*. When inhaled, these aerosols result in infections varying in degrees of severity depending on the site of the inoculum and the immunological status of the individuals involved.

A possible explanation for the predilection of the *capsulatum* variety for avian bat habitats was suggested by Vining and Weeks (1974). These investigators found significant chemical differences between soils positive and negative for this fungus. The positive soils were richer in nitrogen and phosphorus as well as organic matter and water-holding capacity. Previously, Smith and Furcolow (1964) had found that growth-stimulating substances were present in infusions of starling feces.

Knowing the relationship between avian and chiropteran habitats and the *capsulatum* variety of *H. capsulatum*, decontamination procedures for clearing sites found to be infested by that fungus have been developed (Smith et al. 1964; Tosh et al. 1967). Formalin at a concentration of 3 percent was found to be the most effective agent. Application procedures and costs for decontaminating a 3.23 hectare (7 acre) bird roost site were presented by Bartlett et al. (1982). The total cost of this project was $US75 000.

For a long time *H. capsulatum* was considered to be an asexual mold classified in the Fungi imperfecti or Deuteromycetes grouping where all fungi without known sexual states were classified under a system based on spore groups developed by Saccardo (1899). However, in 1972, Kwon-Chung discovered that the variety *capsulatum* of *H. capsulatum* was heterothallic and described its perfect state. It proved to be an ascomycete and was named *Emmonsiella capsulata* (Kwon-Chung 1972). Isolates of the *capsulatum* variety were found to be one or the other of two mating types designated as (a) and (b) or (+) or (−). When the opposite mating types are paired on appropriate media, cleistothecia are formed that are loosely composed of a core group of ascogenous hyphae surrounded by tightly coiled hyphae. When mature, the cleistothecia contain club-shaped asci (3–5×10–16 μm), each of which contains eight globose ascospores measuring 1.5 μm in diameter. Four of the eight ascospores will turn out to be of the (+) mating type and four of the (−) mating type (Kwon-Chung and Bennett 1992, pp. 497–8).

Kwon-Chung (1975) investigated the genetic relationship of the *capsulatum* variety to the *duboisii* variety. It was thus determined that isolates of the *duboisii* variety successfully mated with the appropriate opposite mating types of the *capsulatum* variety giving rise to a teleomorph identical to *E. capsulata*.

The ascospores from the cleistothecia of the *duboisii* matings did not germinate. When injected into mice, however, the ascospores proved to be pathogenic, converting into yeast-form budding cells. The tissues from the experimentally infected mice, when cultured, gave rise to colonies typical of *Histoplasma* (Kwon-Chung and Hill 1981). In 1986, Vincent et al. demonstrated that the restriction enzyme digest pattern of mitochondrial DNA from the *capsulatum* and *duboisii* varieties supported their varietal status.

In 1979, McGinnis and Katz compared herbarium specimens of *Ajellomyces dermatitidis* and *E. capsulata* and concluded that the differences between these two genera were too insignificant to warrant maintenance of the genera as separate entities. As a result, *E. capsulata* was reduced to the status of a synonym of *A. capsulatus*. The species of *Ajellomyces* are classified in the order Onygenales, family *Onygenaceae* of the phylum Ascomycota (Sigler 1993).

Histoplasma capsulatum var. *duboisii* – Vanbreuseghem (1952) – Ciferri 1960

Although much is known about the natural history of the *capsulatum* variety, relatively little is known about the *duboisii* variety in respect to its niche in nature. This variety was first isolated in culture and identified as the cause of a new form of histoplasmosis by Duncan in 1943. He did not, however, publish his findings until 1947 and 1958 (Duncan 1947, 1958). In the meantime, Vanbreuseghem, in a case report by Dubois et al. (1952), described the agent as a new species of *Histoplasma* and named it *Histoplasma duboisii*. Duncan, in his 1958 paper, fully described the clinical, histological, and mycological features of the disease that had been contracted by an English mining engineer who had worked for 6 years in the Gold Coast Colony now known as Ghana. Duncan found that the mycelial form of his African isolate was indistinguishable from that of *H. capsulatum*. It was recognizable as being different by the large size of the tissue forms of the pathogen, which measured 8–12 μm and up to 15 μm in the longer axis. These dimensions were in striking contrast to the in vivo measurements of the tissue-form cells of the *capsulatum* variety, which predominantly range in size from 3 to 4 μm in diameter. It is as well to recall, however, that some isolates of the *capsulatum* variety also produce large yeast-like cells of 6–17 μm in diameter in vitro and in vivo, as emphasized by Drouhet and Schwarz (1956). As a matter of fact, these two investigators stated that 'the great variability found in the strains studies does not seem to indicate justification of assembling the African strains with large cells in a distinct species.' Such an opinion was shared by Ciferri in 1960, who named it *H. capsulatum* var. *duboisii*.

Four years later, the disease term 'histoplasmosis duboisii' was coined by Cockshott and Lucas (1964). They succinctly stated that the 'American isolates and African isolates have negligible differences between their mycelial phases but distinct morphological differences in their parasitic stages whether naturally acquired or experimentally induced infections.' In 1975, Kwon-Chung solidly established the validity of the status of *H. duboisii* as a variety of *H. capsulatum* by successfully mating the tester strains of *A. capsulatus* (as *E. capsulata*), the teleomorphic state of the *capsulatum* variety, with varieties of the *duboisii* variety.

In the disseminated cases of histoplasmosis duboisii, a characteristic clinical picture is presented, revealing bone and subcutaneous abscesses and pleomorphic skin lesions. A definitive diagnosis rests on the demonstration of large, ovoid, budding yeast cells in pus exudate from granulomas and other clinical materials.

The *duboisii* variety of *H. capsulatum* can be readily isolated from clinical material on a variety of media. In its mycelial form grown at 25°C, the isolates cannot be distinguished from the *capsulatum* variety as stated previously. Only by converting the mycelial isolates to their yeast form at 37°C on blood or brain–heart infusion agars can the *duboisii* variety be identified. The diagnostic large-form unicellular cells (12–15 μm in diameter) are formed in significant numbers only in cultures held for several weeks at 37°C.

It has long been surmised that the *duboisii* variety was geophilic, but its isolation from soil was not achieved until 1994. In that year, Gugnani et al. reported the first isolation of that variety from eight of 45 soil specimens collected in an eastern Nigerian bat cave in the state of Anambra. It is noteworthy that one of the eight isolates was recovered by direct plating of a 10^{-5} dilution of a soil suspension on an unspecified agar plate (either Sabouraud's dextrose agar or yeast extract agar supplemented with antibiotics).

In addition, the duboisii variety was recovered from the intestinal contents of one of 35 bats comprising the species *Nycteris hispida* – the hairy slit-faced bat – and *Tadarida* (*Chaerephora*) *pumila* – the lesser mastiff bat. The positive bat was one of the hairy slit-faced bats that are found in most of Africa south of the Sahara (Nowak 1991). Thus, another nonhuman mammalian host of this variety has been added to those previously known: *Papio cynocephalus cynocephalus* – the yellow baboon (Curtois et al. 1955); *Papio cynocephalus anubis* – the olive baboon (Butler and Hubbard 1991); and *Papio cynocephalus papio* – the red baboon (Walker and Spooner 1960).

In a personal communication from P.K.C. Austwick (August 1980), one of us (L.A.) was informed that two aardvarks (*Oryceteropus capensis*), one in the London Zoo and another in the Copenhagen Zoo, were histologically confirmed to have lungs infected by *H. duboisii*. The aforementioned baboons and bat, as well as the soil burrowing aardvarks, may act and serve as sentinels for the presence of the *duboisii* variety in a

given territory. This would parallel the role played by badgers (*Meles meles*) in Denmark (Jansen et al. 1992), Germany (Rapp et al. 1992; Grosse et al. 1997), and Switzerland (Burgisser et al. 1961) in revealing endemic foci of the *capsulatum* variety of *H. capsulatum* in these respective countries.

An epizootic of histoplasmosis duboisii in a Texan colony of 3 400 baboons casts a new light on the epidemiology of this disease (Butler et al. 1988; Butler and Hubbard 1991). Twenty-one cases of simian histoplasmosis duboisii developed over a period of 4 years in the primate colony in San Antonio, TX. Eight of the baboons had been born in the USA whereas of 13 wild-caught simians, six (*P. cynocephalus papio*) had been imported from Senegal, a west African country known to be endemic for *H. capsulatum* var. *duboisii*, and the remaining seven (four *P. cynocephalus cynocephalus* and three *P. cynocephalus anubis*) had been captured in Kenya, an east African country with no known endemic cases of histoplasmosis duboisii. The first infection was diagnosed in one of the Senegalese baboons, 18 months after entry into the USA. The Kenyan baboons developed their infections 10–12 years after being colonized in Texas.

In their 1991 report, Butler and Hubbard stated that the incubation period in their baboons was at least 9 months with a possible upper-end incubation period of 18 months. The most likely route of transmission from the Senegalese import was a direct contact brought about by the grooming habits of the baboons. This social habit involves licking of lesions or picking and ingesting material from lesions on other animals.

In humans, however, in analogy with the *capsulatum* variety, histoplasmosis duboisii is acquired via the respiratory route (Gatti and DeVroey 1991) and, as revealed by Gugnani et al. (1994), soil is the natural habitat occupied by this etiologic agent.

Histoplasma capsulatum var. *farciminosum* (Rivolta 1873) Weeks, Padhye et Ajello 1985

The *farciminosum* variety of *H. capsulatum* has the distinction of having been the first of the three varieties to have come to medical attention and to have been identified as the etiologic agent of a widespread endemic disease known variously as the farcy of Africa, the farcy of Naples and, most commonly, epizootic lymphangitis.

The disease had long been confused with glanders, a highly contagious disease of asses, horses, and mules caused by *Pseudomonas mallei*. In 1873, Rivolta of Pisa, Italy, noted the presence of yeast-like organisms in clinical material taken from horses suffering from epizootic lymphangitis, which he considered to be a species of *Cryptococcus*. The yeast cells noted in the pus taken from the lymph nodes of a horse were unicellular, oval, and budding. In size, these cells measured 2.5–3.5×2.3 μm. Ten years later in 1883, he and Micellone named the organism *C. farciminosum* (Rivolta and Micellone 1883). However, it was not until 1895 that this organism was successfully isolated and cultured by Marcone of Naples. The isolate developed as a sterile mold in a medium composed of horse serum, glucose, glycerin, and sucrose (Marcone 1895).

It remained for Redaelli and Ciferri to recognize that the agent of epizootic lymphangitis was a dimorphic mold and that it was appropriate to transfer the organism to Darling's genus *Histoplasma* on the basis that in tissues the in vivo form cells of *C. farciminosum* were indistinguishable from those of the *capsulatum* variety of *H. capsulatum*. They accordingly transferred Rivolta's *C. farciminosum* to the genus *Histoplasma* as *H. farciminosum* (Ciferri and Redaelli 1934).

In 1985, in studying Egyptian isolates from horses, Weeks et al. induced the production of numerous microconidia and moderate numbers of tuberculate macroconidia. This was accomplished by incubating the cultures in the dark at 30°C for a period of 8–12 weeks on soil-extract agar. The smooth to rough-walled microconidia were pyriform to subglobose and measured 3–5×2.5–3.5 μm. The macroconidia were globose to subglobose (8–12×1–1.5 μm) with thick walls. For the first time, these investigators found that the mycelial cultures of *H. farciminosum* produced micro- and macroconidia that are characteristics of the members of the genus *Histoplasma*. In addition, the close resemblance of the yeast forms of the *capsulatum* and *farciminosum* varieties and the antigenic relationship shown to exist between them by Standard and Kaufman (1976) led to the conclusion that *H. farciminosum* was best treated as another variety of *H. capsulatum*.

Unlike the other two varieties of *Histoplasma*, authenticated cases of histoplasmosis farciminosi have only been known to occur in equines: asses (*Equus asinus*), horses (*E. caballus*), and mules (*E. asinus* × *caballus*). It is pertinent to note that only in the African continent do the three varieties of *H. capsulatum* coexist.

HOST RESPONSE

Human infection with *H. capsulatum* var. *capsulatum* is usually clinically unrecognized, identified only by skin test positivity or pulmonary or splenic calcifications. Symptomatic cases present with a variety of clinical manifestations ranging from an acute pulmonary disease, to a chronic pulmonary infection, or a progressive disseminated form. Among symptomatic cases with no underlying lung disease or immunosuppressive disorders, self-limiting illnesses develop in more than 90 percent of individuals. Chronic pulmonary histoplasmosis develops in those with underlying emphysema, and progressive disseminated disease in those at the extremes of age or

who are immunosuppressed. In this section the main clinical manifestations of histoplasmosis capsulati are examined.

Clinical forms

ACUTE SELF-LIMITED HISTOPLASMOSIS CAPSULATI

After low-inoculum exposure, the infection is asymptomatic in at least 90 percent of cases (Wheat and Kauffman 2003). Among symptomatic patients, the most common clinical presentation is acute pulmonary histoplasmosis characterized by fever, cough, and chest pain. Chest x-rays show mediastinal lymphadenopathy with infiltrates (Figure 1). Most patients recover in a few weeks, but some experience prolonged fatigue. Heavy exposure causes diffuse pulmonary involvement, which is often accompanied by respiratory failure (Kataria et al. 1981). Patients may also experience symptoms as a result of compression of the mediastinal structures by enlarged lymph nodes (Loyd et al. 1988). Patients with acute histoplasmosis may experience rheumatological syndromes characterized by arthritis or arthralgia, and erythema nodosum or erythema multiforme (Rosenthal et al. 1983). Pericarditis is another inflammatory complication of acute histoplasmosis, and both pericarditis and rheumatological syndromes occur in less than 10 percent of cases (Wheat et al. 1983). Rarely, constrictive pericarditis may develop.

Figure 1 *Chest x-ray showing right hilar lymphadenopathy with adjacent infiltrate in acute pulmonary histoplasmosis capsulati*

CHRONIC PULMONARY HISTOPLASMOSIS CAPSULATI

Chronic pulmonary histoplasmosis capsulati occurs in individuals with underlying bullous lung disease and is characterized by recurrent pulmonary symptoms, progressive lung infiltrates, fibrosis, and cavitation (Figure 2) (Goodwin et al. 1976; Wheat et al. 1984). Most patients experience recurrent illnesses highlighted by progressive radiological worsening and respiratory deterioration. Chest x-rays show cavity enlargement, formation of new cavities, and spread to new areas of the lungs. Rarely, bronchopleural fistula or hematogenous dissemination may develop.

PROGRESSIVE DISSEMINATED HISTOPLASMOSIS CAPSULATI

Hematogenous spread outside the lungs occurs during the acute infection but is rarely recognized clinically. These patients recover with the development of varying degrees of immunity to the *capsulatum* variety of *H. capsulatum*. Progressive disseminated disease occurs in about one in 2 000 acute infections (Sathapatayavongs et al. 1983), mostly in patients who are immunosuppressed or at the extremes of age (younger children and older adults). Common clinical manifestations include fever, weight loss, and respiratory symptoms. Physical examination frequently reveals hepatomegaly and/or splenomegaly. Laboratory tests usually show anemia, leukopenia, thrombocytopenia, and elevated hepatic enzymes and bilirubin. Shock with hepatic, renal, and

Figure 2 *Chest x-ray showing bilateral upper lobe fibrotic infiltrates in a patient with underlying emphysema complicated by chronic pulmonary histoplasmosis capsulati*

respiratory failure and coagulopathy may complicate severe cases (Wheat et al. 1990b). Meningitis, cerebritis, and focal brain or spinal cord lesions occur in 10–20 percent of cases, as either manifestations of widely disseminated infection or isolated findings (Wheat et al. 1990a). Other frequent sites of dissemination include the oral mucosa, gastrointestinal tract, skin, kidneys, and adrenal glands (Figure 3). Endocarditis is a rare manifestation of disseminated histoplasmosis. Chest x-rays usually show diffuse infiltrates but are normal in 25–33 percent of cases (Figure 4).

MEDIASTINAL FIBROSIS

Mediastinal fibrosis represents an abnormal fibrotic response to past infection and is a rare finding (Goodwin et al. 1972; Loyd et al. 1988; Davis et al. 2001). Recurrent hemoptysis is a common symptom and respiratory failure often ensues. Serological tests may be positive and staining of tissues may show the small tissue-form cells of the *capsulatum* variety of *H. capsulatum* in half to two-thirds of cases, although cultures are usually negative, supporting the belief that fibrosing mediastinitis represents an aberrant inflammatory response rather than a progressive infection. The superior vena cava, airways, pulmonary arteries and veins, and the esophagus are most commonly involved, but any mediastinal structure can be trapped in these fibrotic masses (Goodwin et al. 1972; Loyd et al. 1988). Chest x-rays may be normal or show only mediastinal widening, but computed tomography (CT) reveals restriction of mediastinal structures. Pulmonary angiograms may show obstruction of the pulmonary arteries (Figure 5). Calcification is often present. The diagnosis requires demonstration of obstruction of mediastinal structures by CT scan, and cannot be made based on the presence of fibrosis histopathologically in biopsy specimens, as this finding is not specific for fibrosing mediastinitis.

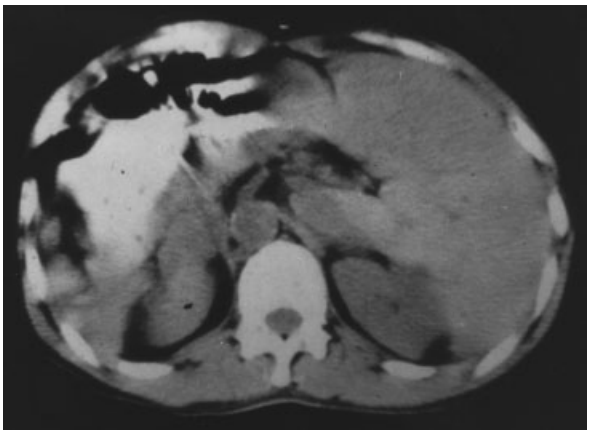

Figure 3 *Bilateral adrenal enlargement caused by disseminated histoplasmosis established by fine needle aspiration biopsy showing organisms consistent with* H. capsulatum *var.* capsulatum

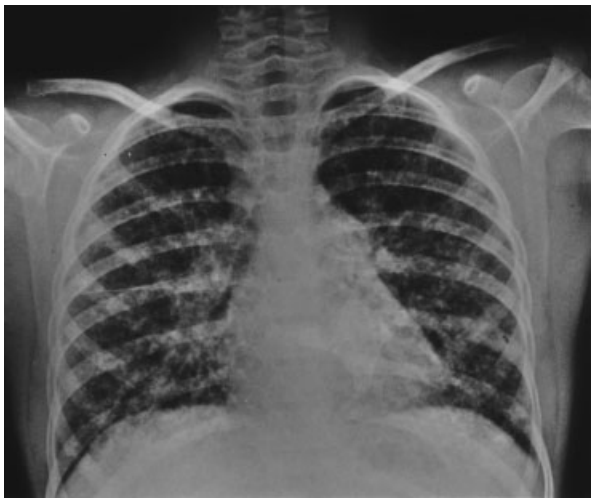

Figure 4 *Chest x-ray showing diffuse miliary infiltrate in a patient with disseminated histoplasmosis capsulati*

HISTOPLASMOMA AND BRONCHOLITHIASIS

Rarely, patients may develop a slowly enlarging pulmonary nodule, which has been called an 'enlarging histoplasmoma' (Goodwin and Snell 1969), causing concern about neoplasia. Lesions range in diameter from 8 to 35 mm and enlarge slowly (2 mm/year). Histologically, they are characterized by a necrotic center surrounded by a fibrous-like capsule. Organisms may be seen in the necrotic center but usually cannot be isolated

Figure 5 *Pulmonary angiogram showing obstruction of right pulmonary artery in a patient with fibrosing mediastinitis*

in culture. As these lesions or mediastinal nodes calcify, they may erode into adjacent bronchi, causing broncholithiasis (Arrigoni et al. 1971). Patients may experience recurrent hemoptysis and expectorate small gravel-like particles of tissue. Bronchial obstruction or tracheoesophageal fistula may also complicate broncholithiasis.

PRESUMED OCULAR HISTOPLASMOSIS CAPSULATI

This distinct clinical entity is usually seen in histoplasmin skin test-positive individuals residing in endemic areas for histoplasmosis capsulati, but scientific evidence that *H. capsulatum* var. *capsulatum* causes this syndrome is not very convincing (Spaeth 1971; Ciulla et al. 2001). However, the eye may also be involved in patients with disseminated histoplasmosis (Specht et al. 1991). Ocular histoplasmosis and presumed ocular histoplasmosis are discussed in Oculomycosis.

Immunology

Histoplasma capsulatum var. *capsulatum* can be a primary pathogen producing systemic disease in normal individuals or opportunistic infection in immunocompromised hosts. The infection is acquired by inhalation of the infectious propagules of *H. capsulatum* into the respiratory tract. The conversion from mycelial to yeast form is critical because it is the yeast form in which *H. capsulatum* var. *capsulatum* exists as a facultative intracellular parasite of the mononuclear cells in the susceptible host (Schwarz 1981). The virulence factor(s) and the mechanisms(s) by which *H. capsulatum* causes disease are not clearly defined. The disease process may involve an endotoxin-like substance (Salvin 1952), collagenases and elastases (Okeke and Miller 1991), siderophores (Burt 1982), and immunological mechanisms: complement-mediated/hypersensitivity and cytokines may contribute to pathogenesis (review by Howard 1981a; Lane et al. 1994; Zhou et al. 1995). In addition, the ability of live *H. capsulatum* var. *capsulatum* yeast cells to neutralize the acidic environment (pH 5.0) of phagolysosomes, enabling them to survive within macrophages, contributes to the virulence of the organism (Eissenberg et al. 1993). The host's ability successfully to mount a series of complex but interrelated cellular responses in the lung is responsible for the recovery and possible development of resistance to reinfection. The cells of the immune system 'talk' to each other by direct cell–cell interactions, cytokine production, and associated signal transduction. The salient features of the natural (innate) and acquired immunity are discussed in the next section.

NATURAL (INNATE) IMMUNITY

Although the precise initial events in the interaction between the previously uninfected host and infective conidia are not well defined, the innate cellular resistance to histoplasmosis capsulati is mediated by polymorphonuclear leukocytes (PML), macrophages, and natural killer (NK) cells. The inhaled infectious units (conidia and mycelial fragments) of *H. capsulatum* var. *capsulatum* lodge within the terminal bronchioles and alveoli. The organism changes from mycelial to yeast-form cells, the form in which the *capsulatum* variety of *H. capsulatum* exists as a facultative intracellular parasite in the susceptible host (Schwarz 1981). The initial cellular infiltrate is composed predominantly of PMLs, which changes to a predominance of macrophages and lymphocytes with progression of the inflammatory response. The microconidia and yeast cells of the invading pathogen bind to the PMLs via the CD11/CD18 family of integrins (adhesion-promoting glycoproteins) without prior opsonization (Schnur and Newman 1990). After binding, the ingestion of *H. capsulatum* var. *capsulatum* is rapid and maximum phagocytosis occurs within 1 hour. The PMLs from guinea pigs and mice exhibit significant fungicidal activity to both the conidia and yeast cells of *H. capsulatum* in vitro (review by Howard 1981a; Kondoh et al. 1989). The killing of conidia and yeast cells is mediated by the combined effect of hydrogen peroxide, halides, and myeloperoxidase (Howard 1981b).

Although there is some controversy regarding their fungicidal activity, human PMLs exert strong fungistasis against the *capsulatum* variety of *H. capsulatum* (Schnur and Newman 1990; Brummer et al. 1991). Phagocytosis of opsonized yeast cells by PMLs stimulates the respiratory burst with intracellular production of H_2O_2 and O_2^-. This phenomenon appears to be unique to PML–*H. capsulatum* interaction, because O_2^- is not released by monocytes upon phagocytosis of the yeast cells. These findings suggest that PMLs may be more important in early host defenses against *H. capsulatum* than previously recognized.

Normal macrophages recognize, bind, and phagocytose *H. capsulatum* conidia and yeast cells, thereby providing a permissive environment for multiplication of the organism in the yeast form (Figure 6). The yeast cells attach to human macrophages via the CD11/CD18 family of integrins – the same receptors by which they bind to PMLs (Schnur and Newman 1990). Once ingested, the *capsulatum* variety of *H.capsulatum* yeast cells confront a hostile environment, which occurs by stimulation of the respiratory burst and phagolysosomal fusion, although the yeast cells survive and grow within normal human and murine macrophages (Figure 6). How *H. capsulatum* survives within macrophages is not known. However, the ability of live yeast cells to neutralize the acidic environment (pH 5.0) required for the fungicidal activity of many lysosomal enzymes has been suggested as a possible mechanism (Eissenberg et al. 1993).

There is considerable evidence to indicate that NK cells participate in early host defenses against *H. capsulatum*. var. *capsulatum*. NK cells from lungs and spleens

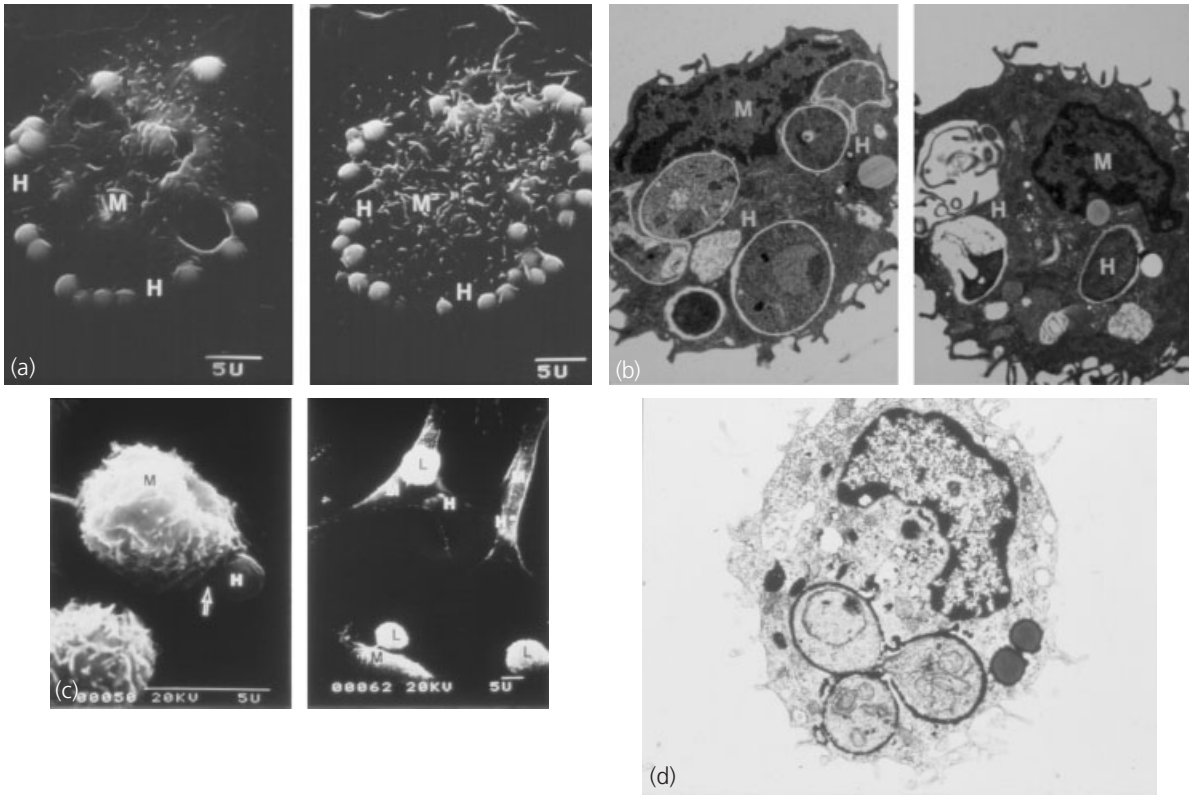

Figure 6 *Ultrastructural morphology of interaction of* H. capsulatum *var.* capsulatum *with murine macrophages 1 hour after incubation at 37°C.* **(a)** *Scanning electron micrograph (SEM) of normal (left; magnification ×19 500) immune (right; magnification ×14 300) macrophages showing phagocytosed yeast cells. Note the activated macrophage with ruffled membranes which has ingested more yeast cells.* **(b)** *Transmission electron micrograph (TEM) of normal (left) and immune (right) macrophages with phagocytosed yeast cells. Note the activated macrophage has degraded yeasts (magnification ×62 000).* **(c)** *SEM of the attachment of lymphocytes (L) to peritoneal macrophages (M) from immune mice (left). Two macrophages also contain phagocytosed histoplasma yeast cells (H) (magnification ×10 250) SEM of an activated murine macrophage (M) engulfing yeast cells of* H. capsulatum *(H) (right; magnification ×25 000).* **(d)** *TEM of a macrophage with ingested yeast cells, showing positive acid phosphatase staining (dark areas) around ingested cells indicating phagosome–lysosome fusion (magnification ×62 000)*

of normal mice are cytotoxic to *H. capsulatum* yeast cells in vitro (Yamada et al. 1982; Raman et al. 1989b). The cytotoxicity is enhanced by addition of anti-histoplasma antibody to NK cell–yeast cell cultures, demonstrating antibody-dependent cellular cytotoxicity (ADCC) by NK cells to *H. capsulatum*. Likewise, NK cells isolated from human peripheral blood of both histoplasmin-positive and histoplasmin-negative subjects exhibit natural as well as ADCC to *H. capsulatum* (Tewari et al. 1981; Choudhury et al. 1989). Although the mechanism of NK cell cytotoxicity is not known, the NK cells bind to *H. capsulatum* by microvilli (Figure 7), in a similar way to that previously described for binding of NK cells to tumor cell targets (Frey et al. 1982). The binding is followed by exocytosis of NK cell granules and the cytotoxicity is presumably expressed by associated signal transduction.

Additional evidence for involvement of NK cells in innate resistance to histoplasmosis capsulati is provided by NK cell depletion and adoptive transfer experiments. Depletion of NK cells by administration of anti-NK cell antibody (anti-asialo GM1) enhanced susceptibility of mice to histoplasmosis as assessed by challenge with *H. capsulatum* yeast cells. (Raman et al. 1989a). Furthermore, adoptive transfer of NK cells to cyclophosphamide-treated mice (NK cell deficient) restored the in vitro NK cell activity of their lung and spleen cells and protected them from a subsequent challenge with *H. capsulatum* (Raman et al. 1990).

The yeast-form cells of *H. capsulatum* var. *capsulatum* have a predilection for the reticuloendothelial system and, in fact, the term 'reticuloendotheliosis' was formerly used as a synonym for histoplasmosis capsulati. It is noteworthy that yeast cells were seen in the endothelial cells lining blood and lymph vessels and in alveolar lining of the lungs by Darling (1906), in the first reported human case of histoplasmosis in Panama (review by Schwarz 1981). In an attempt to elucidate the mechanism involved in extrapulmonary dissemination of the organism, an in vitro experimental model has been developed to study the interaction of *H. capsulatum* var. *capsulatum* with human umbilical vein endothelial cells,

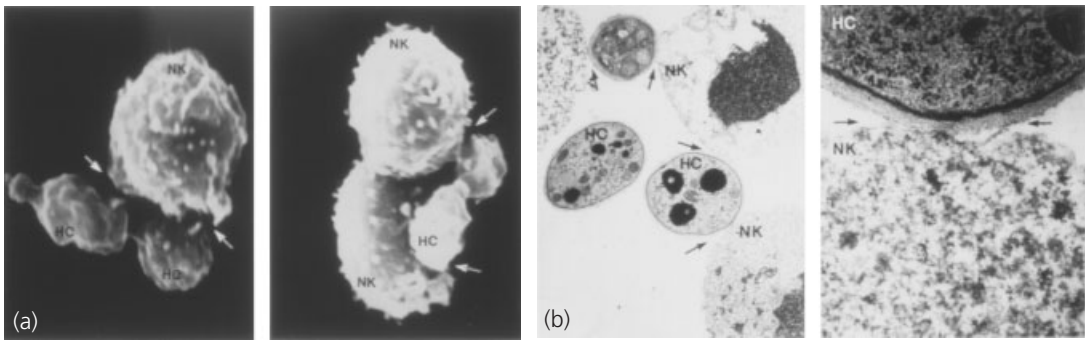

Figure 7 *Interaction of* H. capsulatum *var.* capsulatum *with splenic NK cells from mice in culture.* **(a)** *SEM showing binding of two NK cells to a budding yeast cell (left) and one NK cell to two budding yeasts (right) (magnification ×25 000).* **(b)** *Binding of an NK cell to the cell wall of a yeast cell (left; magnification ×77 000) and conjugate formation between NK cells and yeast cells. Note degranulation of NK cells (right; magnification ×51 300)*

mouse pulmonary cells, and mouse heart endothelial cells, in monolayer cell cultures (Bedrosian et al. 1986; Tewari et al. 1986; Hughes et al. 1994). The endothelial cells maintain a cobblestone arrangement and other characteristics described for endothelial cells, but are much larger than macrophages. Both human and mouse endothelial cells adhere to, phagocytose, and kill yeast cells of *H. capsulatum* (Figure 8). Unlike macrophages, where maximum phagocytosis is observed within 1 hour, optimum endothelial cell phagocytosis requires 4–6 hours. However, only 25–30 percent of cells in monolayer cultures react with *H. capsulatum*, suggesting a heterogeneous nature for endothelial cells. Ultrastructural studies provide supporting evidence for morphological degradation and killing of yeast cells (Figure 8). These findings indicate that endothelial cells may have a critical role in host defenses to histoplasmosis capsulati.

The role of humoral factors in natural resistance to histoplasmosis is not clearly defined. Nevertheless, heat-labile and/or heat-stable serum opsonins enhance phagocytosis of *H. capsulatum* var. *capsulatum* yeast cells by PMLs, but not by macrophages (review by Deepe and Bullock 1992). Furthermore, normal serum inhibits growth of *H. capsulatum* yeast cells and the inhibition is attributed to transferrin, which binds iron and makes it unavailable for nutritional requirement of the organism (Sutcliffe et al. 1980).

ACQUIRED IMMUNITY

H. capsulatum var. *capsulatum* is a highly immunogenic mold and acquired immunity is expressed by induction of a spectrum of cellular as well as humoral immune responses. However, it is generally accepted that the primary host defense mechanism activated in response to *H. capsulatum* is the cell-mediated arm of the immune response. Several lines of evidence support this concept:

- Congenitally athymic (nude) mice lacking T cells are more susceptible to *H. capsulatum* var. *capsulatum* infection (Williams et al. 1978; Miyaji et al. 1981).
- Lymphokines from immune mice or IFN-γ can activate macrophages for enhanced antifungal activity to *H. capsulatum* (Wu-Hsieh and Howard 1984, 1987).
- Adoptive transfer of T cells but not B cells or serum from immunized mice protects naive animals against a lethal challenge with *H. capsulatum* (Tewari et al. 1977, 1978; Williams et al. 1981).
- Transfer of *H. capsulatum*-reactive CD4 cells and to a lesser extent that of CD8 cells confers immunity to naive mice against histoplasma yeast cells (Allendoerfer et al. 1993; Deepe 1994).
- Immunized mice exhibit a delayed-type hypersensitivity (DTH) response to histoplasmin, and lymphocytes proliferate in response to histoplasmin in culture (Tewari et al. 1982; Khardori et al. 1986; Sharma et al. 1986).

How T cells contribute to protective immunity is not well understood. The most logical mechanism by which T cells augment the protective immune response is by release of macrophage-activating lymphokines which, in turn, arm the macrophages for more efficient antifungal activity. Although T cells can produce a number of regulatory cytokines, IFN-γ is the only cytokine that has been shown to arm murine macrophages for antifungal activity against *H. capsulatum* (Wu-Hsieh and Howard 1987). Abrogation of protective immunity by administration of anti-IFN-γ antibody to mice (Zhou et al. 1995) and failure of IFN-γ to knock out mice to control *H. capsulatum* infection (Wu-Hsieh and Howard 1984) further support the role of IFN-γ in histoplasmosis capsulati. In addition to CD4 cells, CD8 and NK cells produce IFN-γ and thus could potentially produce IFN-γ in *H. capsulatum* infection. However, co-culturing of human monocyte-derived macrophages with IFN-γ does not enhance their anti-*H. capsulatum* activity (Fleishmann et al. 1990), but they are activated by interleukin 3 (IL-3), granulocyte–macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (Newman and

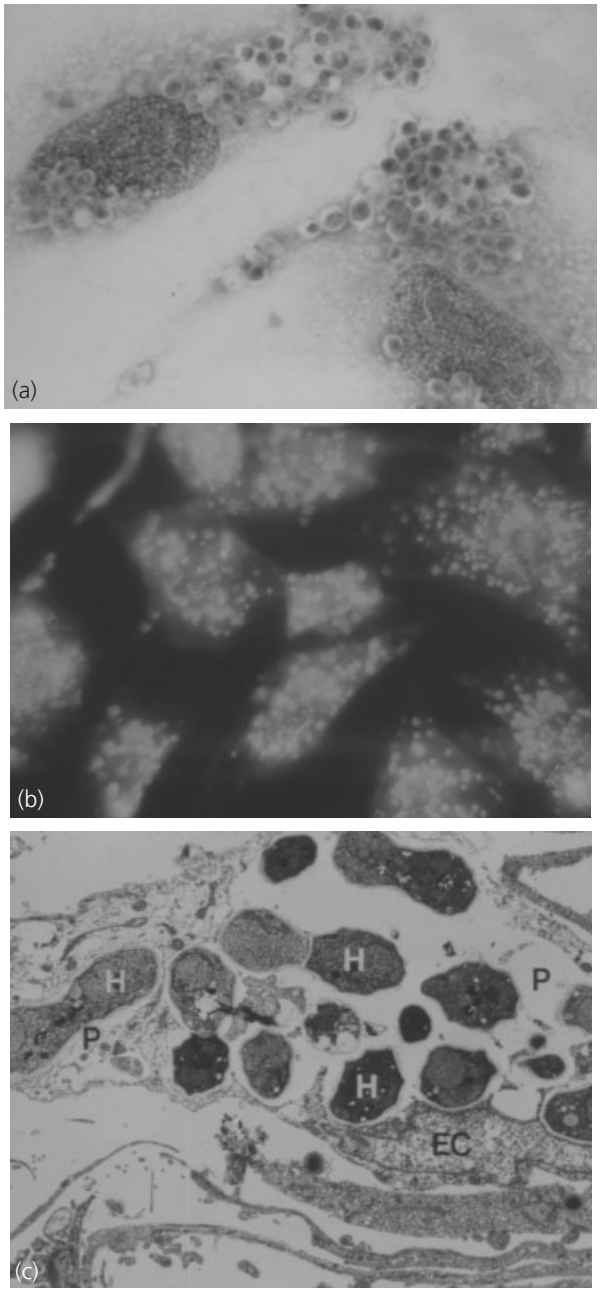

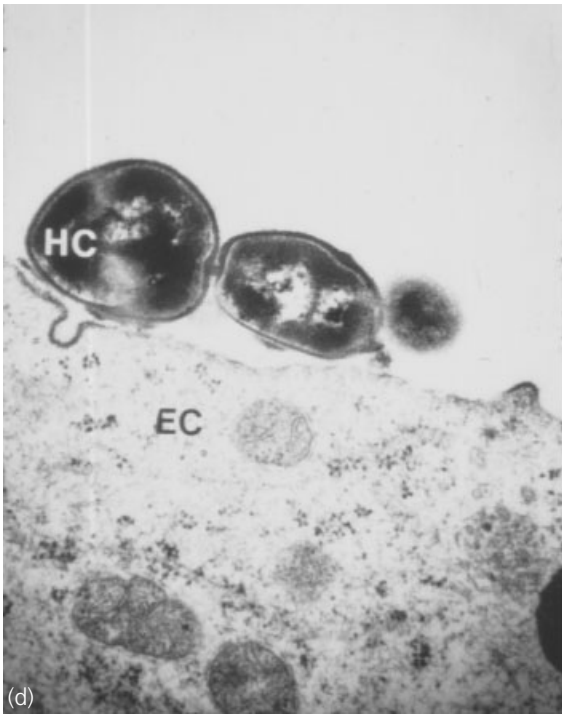

Figure 8 *Interaction of human umbilical cord endothelial cells (EC) with* H. capsulatum *var.* capsulatum *(HCH) in culture.* **(a)** *An endothelial cell monolayer, 4 hours after incubation with histoplasma yeast cells. Note phagocytosis of yeast cells by EC (Giemsa stain; magnification ×3 900).* **(b)** *Acridine orange-stained EC monolayer showing numerous orange-colored lysosomes (magnification ×3 400).* **(c)** *TEM of a phagocytic EC with numerous H yeast cells in phagosomes (P). Note intact as well as degraded HC within phagosomes (magnification ×12 000).* **(d)** *TEM of an adherent HC yeast cell to an EC. Note morphological alteration in HC even before ingestion (magnification ×34 000)*

Gootee 1992), indicating differences in signaling mechanisms for macrophage activation between the two species.

Administration of interleukin 12 (IL-12) or neutralizing antibody to interleukin 4 (IL-4) has a protective effect on *H. capsulatum* infection in mice, demonstrating the antagonistic role of these cytokines (Zhou et al. 1995). Treatment of *H. capsulatum*-infected mice with neutralizing antibodies to IL-12, IFN-γ or tumor necrosis factor α (TNF-α), enhances the severity of the disease. Recently treatment with tumor necrosis factor (TNF) inhibitors has been shown to predispose to severe histoplasmosis in man (Wood et al. 2003). Moreover, IL-12 treatment increases the endogenous production of IFN-γ in infected mice, suggesting that the protective effect of IL-12 is primarily mediated through production of IFN-γ. Recently, Zhou et al. (1997) also demonstrated the protective effect of IL-12 against histoplasmosis capsulati in immunodeficient severe combined immunodeficiency disease (SCID) mice. The effect was further increased by combining IL-12 with amphotericin B. The protective effect is associated with increased production of IFN-γ, TNF-α, and nitric oxide from spleen cells.

Macrophages from immunized mice or macrophages activated in vitro have ruffled membranes with high affinity surface receptors and are far more phagocytic than normal macrophages (see Figure 6). The activated macrophages also have more lysosomal enzymes and the ability to produce more reactive nitrogen and oxygen intermediates (RNI-NO, NO_2^-, and ROI-H_2O_2, O_2^-). However, it is generally considered that activated macrophages exert fungistatic but not fungicidal activity against the *capsulatum* variety of *H. capsulatum*,

although ultrastructural studies indicate morphological degradation of the ingested organisms. Neutralization of the acidic environment of phagolysosomes (pH 5.0) has been proposed as a possible mechanism for survival of *H. capsulatum* within macrophages (Eissenberg et al. 1993).

The opsonization of microconidia and yeast cells with anti-*H. capsulatum* antibodies increases the phagocytic activity of PMLs, but the enhancing effect of antibodies is not observed in phagocytosis of the organism by macrophages (review by Deepe and Bullock 1992). The antibody-dependent cellular cytotoxicity of both murine and human NK cells to *H. capsulatum* yeast cells, and thus antibodies, may have a contributory role in host defenses (Tewari et al. 1982; Choudhury et al. 1989).

The inhalation of a small number of *H. capsulatum* conidia, which frequently occurs in individuals living in an endemic area for histoplasmosis capsulati, results in asymptomatic or mild self-limiting infections with concomitant development of resistance to reinfection. This had also been observed in experimental animals in the early 1950s and provided the major rationale for development of a vaccine for histoplasmosis (review by Salvin 1965). The immunity induced by sublethal infection of experimental animals persisted longer than that induced by killed organisms or fractions thereof. However, only a low level of protection was observed in mice immunized with killed cells and the immunogenicity was attributed to cell wall constituents. Feit and Tewari (1974) demonstrated that immunization of mice with *H. capsulatum* yeast ribosomes or ribosomal proteins elicited a high degree of immunity, protecting up to 90 percent of animals from a lethal challenge. The immunity was comparable to that obtained by a sublethal infection with live yeast cells (Tewari 1975). Furthermore, a cytoplasmic membrane fraction or membrane proteins were as immunogenic as ribosomes (Tewari and LaFemina 1983; Tewari et al. 1989). Recently, Deepe and his associates isolated a glycoprotein (HIS-62) from the cell walls and cell membranes of *H. capsulatum* var. *capsulatum* yeast cells, cloned the gene encoding this antigen, and prepared a recombinant protein that was shown to be a member of the heat-shock protein Hsp60 family. Immunization of mice with the native as well as with the recombinant protein conferred significant protection against a lethal challenge with *H. capsulatum* yeast cells (Gomez et al. 1991, 1995). However, another protein (80 kDa) from *H. capsulatum* var. *capsulatum* with homology to Hsp70 did not provide significant protection. In view of the concern of using heat-shock proteins as a vaccine in humans, four recombinant polypeptides were prepared from Hsp60, but these recombinants did not confer significant protection against histoplasmosis capsulati (Deepe et al. 1996).

There is a strong correlation between protective immunity and delayed hypersensitivity in histoplasmosis. Lymphocytes from immunized mice proliferate in response to histoplasmin and the proliferative response is used as an in vitro correlate of DTH responses (Tewari et al. 1982; Deepe et al. 1996). Furthermore, there is a strong correlation between footpad reactivity (DTH reaction) to histoplasmin and production of macrophage migration inhibition factor (MIF) when peritoneal exudate cells from immunized mice are cultured with histoplasmin (Tewari et al. 1989). It is noteworthy that both the DTH reaction and MIF production decayed faster than the protective immunity in immunized mice. The immunized mice showed footpad reactivity (DTH) responses to histoplasmin which peaked between 21 and 28 days after immunization, and thereafter showed a gradual decline, reaching the control level at 105 days (Khardori et al. 1986; Sharma et al. 1986). There was, however, no decline in protective immunity for up to 105 days, the maximum observation period used in these studies (Figure 9). It is noteworthy that protective immunity persisted longer than the DTH reaction and MIF production, demonstrating a dichotomy between the two manifestations of the immune response in histoplasmosis capsulati.

Treatment

Histoplasmosis capsulati is a systemic fungal infection with a wide spectrum of clinical manifestations in different individuals. The therapy of the disease has to be clinically determined after taking into consideration the immunocompetence of the host, site of infection, and the extent of the disease. Initially, amphotericin B was the only effective drug available for treatment. However, toxicity of the drug limited its use to patients with severe disease. The development of azoles such as ketoconazole and, more recently, itraconazole, which have lower toxicity and can be given orally, has allowed the clinician options for treatment of patients with less severe disease. The current recommendations for treatment are presented in this section.

ACUTE PULMONARY

Patients with extensive acute pulmonary histoplasmosis capsulati benefit from antifungal therapy and those who are hypoxic may also require adjunctive corticosteroid therapy (Table 1) (Wheat and Kauffman 2003). Patients with less extensive disease but who remain symptomatic for a month or more may also benefit from therapy. While the benefit of therapy for patients with more severe, diffuse disease seems to be clear, based upon the author's experience and the anecdotal reports of others, the effect in patients with localized disease and persistent symptoms is uncertain. Amphotericin B should be used in severely ill patients requiring hospitalization (Table 2). Itraconazole is highly effective in individuals with milder illnesses or as a follow-up

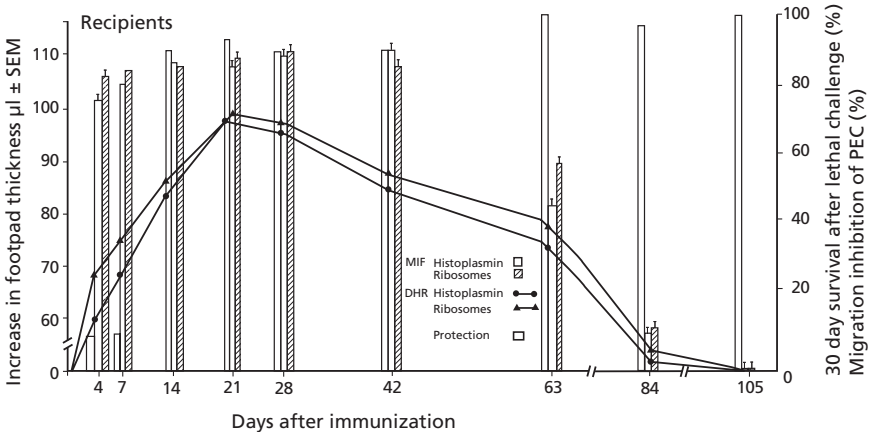

Figure 9 *Relationship of protective immunity, footpad reactivity, and production of macrophage MIF by peritoneal exudate cells from mice immunized by sublethal infection with yeast cells of* H. capsulatum *var.* capsulatum *(105 cells, s.c.). Mean ± SEM of five mice per time period for delayed hypersensitivity and MIF and 20 mice per group for protective immunity (10 mice/group per experiment)*

therapy after an initial response to amphotericin B (Wheat et al. 1995).

MEDIASTINAL GRANULOMA

Mediastinal granuloma may produce obstructive symptoms or fistula. Occasional patients with these complications improve after antifungal therapy (Savides et al. 1995), but some may require surgical resection (Gilliland et al. 1984). The benefit of therapy in such cases, however, is difficult to prove. Prophylactic antifungal therapy or surgery to prevent fibrosing mediastinitis is not indicated because progression of granulomatous to fibrosing mediastinitis is rare (Loyd et al. 1988).

RHEUMATOLOGICAL AND PERICARDITIS

Patients with these inflammatory manifestations may respond to aspirin or nonsteroidal anti-inflammatory agents, but also may require treatment with corticosteroids (Rosenthal et al. 1983; Wheat et al. 1983). Antifungal therapy is not required, but should be considered in those receiving corticosteroids at higher than physiological doses for more than 3 weeks, in order to reduce the risk for dissemination during corticosteroid-induced immunosuppression. Rarely, joints and the pericardium are sites of disseminated infection, in which case treatment would be necessary.

Table 1 *Summary of indications for treatment of histoplasmosis capsulati*

Treatment indicated	Treatment not indicated
Acute pulmonary, diffuse	Pericarditis
Acute pulmonary, localized with prolonged symptoms	Rheumatological
Chronic pulmonary	Fibrosing mediastinitis
Disseminated	Histoplasmoma
Symptomatic mediastinal granuloma	Presumed ocular histoplasmosis

CHRONIC PULMONARY

Treatment of chronic pulmonary histoplasmosis capsulati improves survival, reduces symptoms, promotes radiological healing, and eradicates *H. capsulatum* from the sputum (Sutliff et al. 1964). Most patients (about 80 percent) respond well to itraconazole 200–400 mg daily (Dismukes et al. 1992). Itraconazole should be continued for at least a year and until maximal clinical and radiological benefits have been achieved. Amphotericin B may be needed initially in patients with severe respiratory insufficiency. Relapse is common (about 25 percent) after discontinuation of treatment, emphasizing the need for prolonged follow-up.

DISSEMINATED

Disseminated histoplasmosis is usually fatal if untreated (Furcolow 1963; Sathapatayavongs et al. 1983). Liposomal amphotericin B induced a more rapid response than the standard deoxycholate formulation, and improved survival in patients with the acquired immunodeficiency syndrome (AIDS) (Johnson et al. 2002). The high cost of the liposomal formulation may preclude its use in all situations, however. Amphotericin B and itraconazole induce a favorable response in 85–90 percent of patients (Wheat et al. 1990b, 1995; Dismukes et al. 1992). Itraconazole should be administered for at least a year in most patients. Relapse is common in patients who are immunosuppressed, including those with AIDS. The level of histoplasma antigen declines during therapy and increases with relapse, providing a useful tool for monitoring the response to therapy (Wheat et al. 1991). Treatment should be continued until antigenemia and antigenuria have resolved or at least reached a stable low level (<2 units). Lifelong maintenance treatment is needed in patients with AIDS who do not achieve a good CD4 cell response to potent antiretroviral therapy. If the CD4 cell count increases to

Table 2 *Recommendations for antifungal therapy of histoplasmosis capsulati*

Disease severity	Drug	Dose	Duration
Mild	Itraconazole	200 mg t.i.d. × 3 days or 200 mg q.d or b.i.d.[a]	3–24 months[b]
Moderate or severe	Amphotericin B or liposomal amphotericin B	0.7–1 mg/kg/day or 3 mg/kg/day for liposomal preparation	3 days to 2 weeks based on severity[c]

a) The itraconazole dose should begin as a loading dose followed by a treatment dose of 200 mg once or twice daily. Blood levels should be measured after 1 week of therapy and the dosage should be modified to maintain blood levels between 1 and 10 μg/ml.
b) Treatment duration ranges from 3 months in patients with acute pulmonary histoplasmosis to 12–24 months in patients with disseminated or chronic pulmonary histoplasmosis. If treatment is initiated with an amphotericin B formulation, itraconazole may be used to complete the recommended duration of therapy. Patients with AIDS may require lifelong treatment if they fail to achieve CD4 cell improvement to >150 cells/ml in response to potent combination antiretroviral therapy.
c) Amphotericin B dosage is 0.7–1 mg/kg/day whereas the dosage of the liposomal preparation is 3 mg/kg/day.

150 cells/mm^3 or higher, and the antigen levels in urine and serum decline to <2 units, itraconazole may be stopped after one year. Maintenance therapy also may be required in other immunocompromised individuals with persistent immunosuppression, or those who have relapsed after extended courses of amphotericin B or itraconazole.

The response to therapy in patients with meningitis is inferior to that in other types of histoplasmosis: 20–40 percent of patients with meningitis die and up to half of responders relapse after therapy is stopped. The optimal treatment for *Histoplasma* meningitis is unknown, but an aggressive approach is recommended because of the poor outcome. Although the best therapy is still not known, it seems reasonable to begin therapy with liposomal amphotericin B, 3–5 mg/kg/day, for 2–3 months and until CSF cultures are negative and CSF antigen levels are negative, followed by itraconazole, 200 mg twice or three times daily for at least another year. Lifelong maintenance therapy may be needed in patients who relapse or in those whose CSF findings do not return to normal. Patients who fail chronic itraconazole therapy have these options: fluconazole 800 mg daily, voriconazole 200 mg twice daily, and posaconazole.

MEDIASTINAL FIBROSIS

Antifungal treatment usually does not influence the course of mediastinal fibrosis (Goodwin et al. 1972; Loyd et al. 1988; Davis et al. 2001). However, a few patients have shown improvement after treatment with ketoconazole (Urschel et al. 1990). In such cases, differentiation between fibrosing and granulomatous mediastinitis may have been difficult. Thus, a 3-month trial of itraconazole is reasonable, especially in patients with positive serological tests and elevated erythrocyte sedimentation rates, or findings that may be more consistent with granulomatous mediastinitis. It is not likely to be helpful, however, since the pathogenesis is fibrosis rather than infection or inflammation. Resection of the fibrotic tissue may be helpful but has a high operative mortality (about 25 percent) and probably should be reserved for patients with severe and progressive manifestations (Mathisen and Grillo 1992). A nonprogressive course is common, precluding the need for surgical intervention (Goodwin et al. 1972; Loyd et al. 1988).

PRESUMED OCULAR HISTOPLASMOSIS CAPSULATI

Presumed ocular histoplasmosis does not represent an active infection and would not be expected to respond to antifungal therapy, although no prospective trials have been reported (Ciulla et al. 2001).

LABORATORY DIAGNOSIS

Direct examination

Rounded to oval yeast cells measuring 2–4 mm in diameter, characteristic of *H. capsulatum* var. *capsulatum*, may be seen by fungal staining of biopsy specimens from pulmonary, mediastinal, or extrapulmonary tissues. Less commonly, organisms may be observed by fungal staining of sputum, sterile body fluids, or peripheral blood smears. In disseminated histoplasmosis capsulati, the highest yield is from bone marrow (>50 percent) (Figure 10). Organisms may be overlooked in patients with histoplasmosis leading to false-negative results, and staining artefacts or other organisms may be mistaken for *H. capsulatum* in patients with other diseases, emphasizing the importance of the skill and experience of the examiner for accurate interpretation of fungal stains. *Candida (Torulopsis) glabrata*, *Blastomyces dermatitidis*, capsule-deficient strains of *Cryptococcus neoformans*, *Penicillium marneffii*, *Toxoplasma gondii*, and *Pneumocystis carinii* may be misidentified in routine clinical laboratories as the tissue-form cells of the *capsulatum* variety of *H. capsulatum*. Mucicarmine staining for capsular material may be used to distinguish *C. neoformans* from *H. capsulatum*, but capsule-deficient strains may be misidentified by this technique. In such cases, melanin staining is positive with *C. neoformans* but negative with *H. capsulatum*.

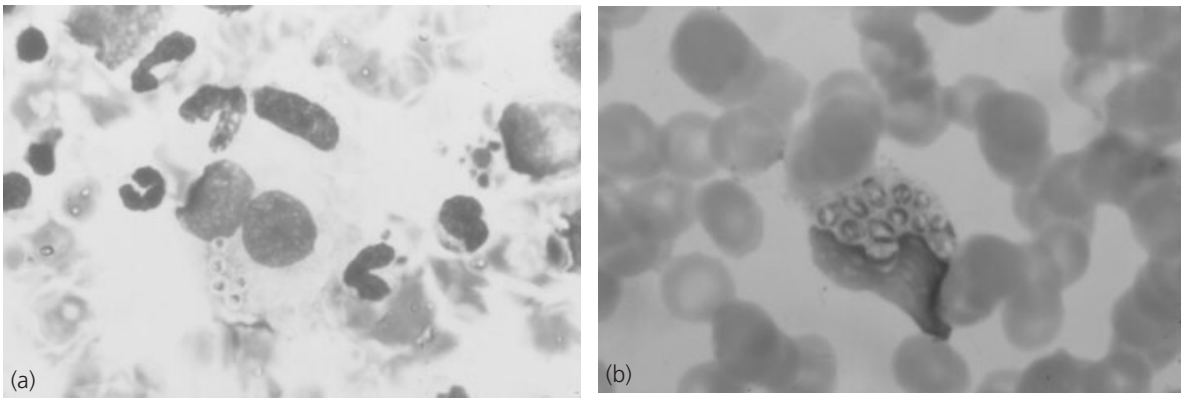

Figure 10 (a) *Bone marrow smear from a young girl with disseminated histoplasmosis capsulati showing budding yeast cells (2–4 μm) in a phagocytic monocyte Giemsa stain; magnification ×3 400).* **(b)** *Peripheral blood smear of an AIDS patient with disseminated histoplasmosis capsulati showing numerous yeast cells (2–4 μm) in a monocyte (Giemsa stain; ×3 400)*

Culture

Cultures are most useful in patients with disseminated or chronic pulmonary histoplasmosis, being positive in 50–85 percent of cases. In disseminated disease, the highest culture yield is from bone marrow or blood, being positive in over 75 percent of cases (Sathapatayavongs et al. 1983; Wheat et al. 1990b). Organisms can be found in sputum or bronchoscopy specimens from 60–85 percent of patients with cavitary histoplasmosis if multiple specimens are obtained (Wheat et al. 1984). The sensitivity of culture is only 10–15 percent in patients with other forms of histoplasmosis capsulati (Williams et al. 1994). Slow growth (2–4 weeks) may delay the diagnosis in patients with positive cultures (Williams et al. 1994). The development of specific nucleic acid probes has facilitated the identification of *H. capsulatum* var. *capsulatum* in cultures with atypical morphology (Stockman et al. 1993).

Immunoserology

This section will concentrate on three tests that are commonly used: the immunodiffusion and complement fixation tests for detection of antibodies and an immunoassay for demonstration of histoplasma polysaccharide antigen in body fluids. The precipitin bands (H and M) are demonstrated by the immunodiffusion test using histoplasmin as the antigen. H precipitin bands can be demonstrated in less than 25 percent of patients and clear during the first 6 months after infection, whereas M bands appear in about 75 percent of individuals and may persist for several years after the resolution of infection (see Figure 5) (Wheat et al. 1982; Williams et al. 1994). The complement fixation test is performed with both histoplasmin and whole yeast cell antigens. Complement fixation titers of 1:8 or higher are found in most patients with active histoplasmosis capsulati, whereas titers of 1:32 or higher are more suggestive of active infection. Both the immunodiffusion and complement fixation tests should be performed to obtain the highest sensitivity for diagnosis (Figure 11).

Enzyme immunoassay or radioimmunoassay methods for detection of antibodies are more difficult to interpret, because of higher background positivity rates. They have not been validated adequately to recommend their use in place of immunodiffusion and complement fixation methods (Wheat 1993). Antibodies require 4–8 weeks to develop after acute infection and may be negative when the patient is first seen. Furthermore, serological tests may be falsely negative in up to one-third of immunocompromised patients (Williams et al. 1994). False-positive results are caused by antigenic cross reactions with *B. dermatitidis*, *Coccidioides immitis*, and *Paracoccidioides brasiliensis* (Wheat et al. 1986). M precipitin bands and low titers (1:8 or 1:16) of complement-fixing antibodies may require years to clear, causing confusion in patients with other diseases.

Histoplasma polysaccharide antigen is found in the blood, urine, and bronchoalveolar lavage fluid of more than 90 percent of individuals with disseminated histoplasmosis capsulati, and in the urine of 75 percent of those with extensive pneumonitis after heavy acute exposure (Wheat et al. 1986; Wheat and Kauffman 2003). Antigen may be found in the cerebrospinal fluid of 25–50 percent of patients with meningitis caused by histoplasmosis (Figure 11). It is important to recognize that lower levels of crossreactions may be seen in patients with histoplasmosis duboisii, blastomycosis, paracoccidioidomycosis, and penicilliosis marneffei. Antigen levels decline during treatment and increase with relapse, providing a tool for monitoring therapy (Wheat et al. 1991). Antigen levels should be monitored every 3–6 months, and at the time of suspected relapse.

Histoplasmin skin test antigen is a culture filtrate of the mycelial form of *H. capsulatum* var. *capsulatum* but

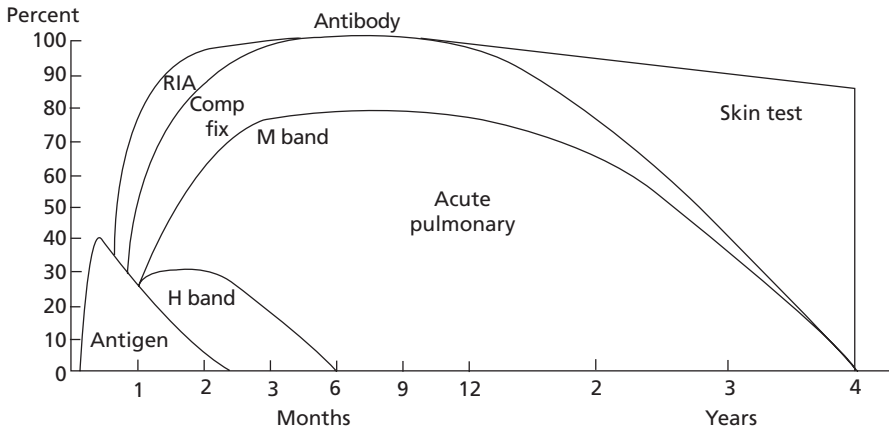

Figure 11 *Time course of diagnostic tests for histoplasmosis capsulati in patients with acute pulmonary disease. Antigen refers to histoplasma antigen in urine. H band and M band refer to precipitants measured by immunodiffusion. Comp fix refers to titers of at least 1:8 to either the yeast or mycelial antigen; Skin test refers to histoplasmin skin test reactivity; RIA refers to measurement of antibodies by radioimmunoassay showing that antibodies may become detectable somewhat earlier than by complement fixation of immunodiffusion.*

is no longer available. Histoplasmin skin testing is not recommended for diagnosis, because of high rates of positivity (50–80 percent) in endemic areas and false-positive results in patients with other fungal diseases (Edwards et al. 1969). Furthermore, skin tests may also be falsely negative in more than half of patients with disseminated disease and skin tests boost the levels of anti-histoplasma antibody, causing confusion in the interpretation of serological tests (Campbell and Hill 1964). The skin test is, however, very useful in epidemiologic studies for determining the prevalence of histoplasmosis capsulati.

Molecular diagnostics

Molecular methods have been developed for diagnosis of histoplasmosis. DNA probes are commercially available and widely used in the clinical mycology laboratory, reducing the time for definitive identification of positive cultures. Research is in progress to develop molecular diagnostic methods to detect *H. capsulatum* var. *capsulatum* DNA in tissue sections and body fluids. To date, while studies suggest that these methods will prove useful, none has been validated by comparison to other established procedures. These are promising methods, particularly for diagnosis of specimens containing granulomas but with negative fungal stains, but require more evaluation before they can be recommended for use in the clinical laboratory. Furthermore, how they will compare with antigen detection in body fluids for rapid diagnosis of histoplasmosis requires further investigation. Nevertheless, polymerase chain reaction (PCR) methods are offered at several commercial laboratories, but should not be accepted as accurate, or used for making the diagnosis of histoplasmosis in patients, until they are validated.

Histopathology

The tissue response in histoplasmosis capsulati follows the general course of histopathological reactions described as granulomas. The early lesions in the lung are areas of pneumonia containing a large number of macrophages and lymphocytes with occasional epithelioid cells and multinucleated giant cells. At this stage, tissue-form yeast cells are easily seen within macrophages. Central areas begin to show necrosis early in the evolution of the lesion, and are occasionally accompanied by transudation of fluid into the necrotic areas (Figure 12). With the progression of the lesion, tissue architecture is lost and its outer margins exhibit epithelioid cells together with multinucleated giant cells, lymphocytes, and plasma cells (Goodwin et al. 1981). Proliferation of fibroblasts, deposition of collagen around margins of the lesion, and calcification are usually observed in old, localized granulomas. In 'histoplasmoma,' the outer portion of fibrosis may consist of multiple layers of fibrous tissue (Goodwin and Snell 1969).

In cavitary pulmonary histoplasmosis capsulati, the cavity lining is necrotic without a well-localized outer margin of fibrosis. Histoplasma yeasts seen in the cavity lining are pleomorphic, with occasional short hyphal forms. The inflammatory response varies considerably from one area of the lungs to another; some areas have well organized granulomas with whorls of epithelioid cells and necrosis, whereas other areas have caseous necrosis. Demonstration of *H. capsulatum* is very difficult with hematoxylin and eosin (H&E) staining and is almost impossible in areas without necrosis. Gridley's stain is useful for demonstration of yeast cells. The periodic acid–Schiff (PAS) stain can be negative despite the presence of silver-staining yeast cells by Gomori's

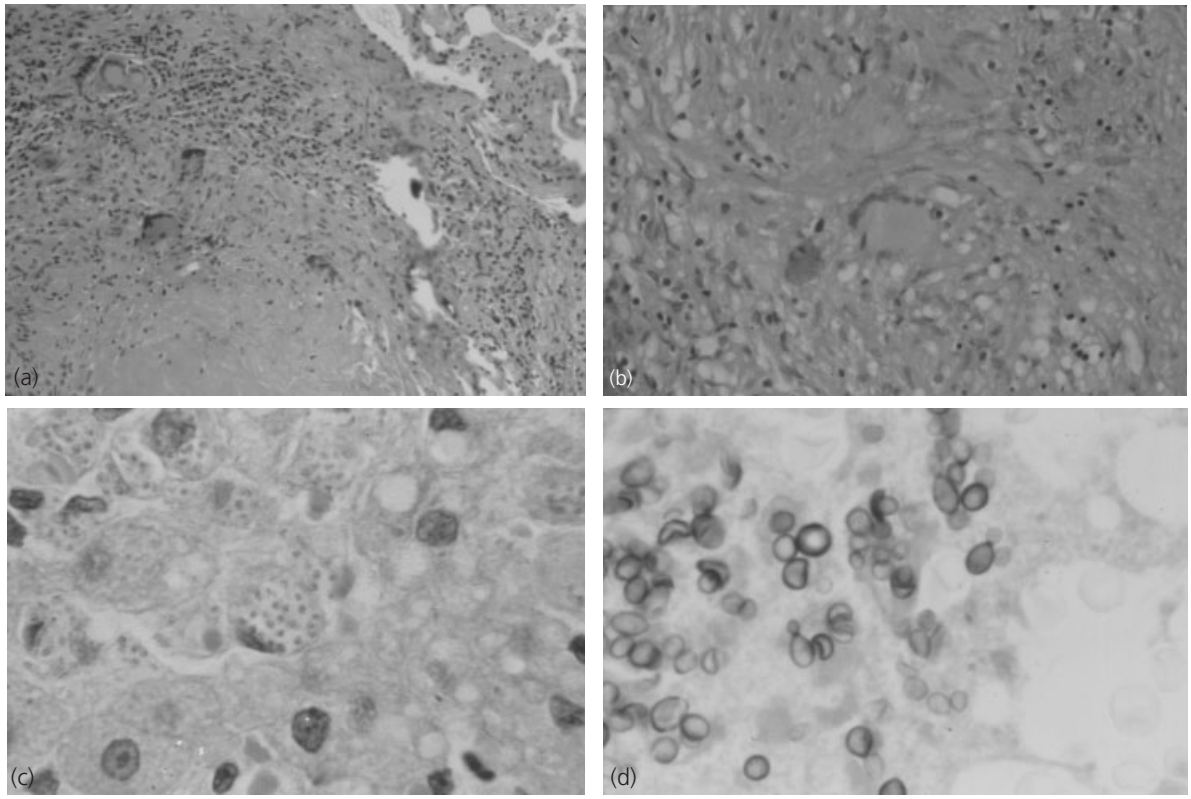

Figure 12 *Histopathology of histoplasmosis capsulati.* **(a)** *Cross-section of the lung from a patient with histoplasmosis showing granulomatous inflammatory response consisting of mononuclear cells and giant cells with a central area of necrosis (H&E stain; magnification ×1 200).* **(b)** *Cross-section of a mediastinal lymph node showing caseous granulomatous lymphadenitis resulting from* H. capsulatum *var.* capsulatum. *Note the epithelioid cells and giant cells around the necrotic centre (H&E stain; magnification ×1 200).* **(c)** *Cross-section of the liver showing numerous intracellular yeast cells in histiocytes with associated mononuclear cell infiltration (H&E stain; magnification ×1 200).* **(d)** *Cross-section of a lung granuloma showing intact and some degraded yeast cells (GMS stain; magnification ×1 200). (Courtesy of Dr Joan Barenfanger, MMC, Springfield, IL)*

methenamine silver (GMS) stain – the preferred technique for recognition of the yeast cells of the three varieties of *H. capsulatum*. The organisms from old lesions may not grow on culture (Figures 13 and 14).

A critical issue in pulmonary pathology is the distinction of infection from non-infectious granulomas in the lung. In Wegener's granulomatosis, necrotizing vasculitis of blood vessels is prominent, even in areas without necrosis of surrounding tissues. Vessel walls are destroyed and infiltrated with acute and chronic inflammatory cells, mixed with multinucleated giant cells and histiocytes. If one considers only the lungs and not the lesions of Wegener's granulomatosis in the upper airway or kidney, the tissue response can be quite similar to that of chronic pulmonary histoplasmosis capsulati. Lymphomatoid granulomatosis differs in that the vessel necrosis is less prominent, cellular infiltrates are present in all layers of the vessels, and the lesions are predominantly angionecrotic. Lesions of sarcoidosis can also be mistaken for those of histoplasmosis capsulati. Within infectious diseases, tuberculosis most closely resembles histoplasmosis, histopathologically. In cryptococcosis, necrotic areas contain large collections of yeasts that can be easily recognized by special stains. Furthermore, cryptococcal granulomas have yeast cells throughout the lesion. *C. immitis* is the fungus with an inflammatory response that mimics that of histoplasmosis, but spherules of *C. immitis* are easy to find and are distinctive. Likewise, *B. dermatitidis* yeast cells have thick, double refractile cell walls with a broad-based septum between the mother and daughter cells, and are usually associated with a mixed suppurative and granulomatous tissue reaction.

Involvement of hilar lymph nodes with necrotizing granulomas and calcification is common in pulmonary histoplasmosis (Figure 12). Patients who progress to fibrosing mediastinitis have increasing effacement of lymph node architecture with caseous necrosis and dense bands of collagen about the node (Goodwin et al. 1972). With progression of the lesion, fibrotic compression of pulmonary veins, superior vena cava, bronchus or pulmonary arteries can occur. Caseous nodes can adhere and rupture into the esophagus or, rarely, the bronchus. In patients with pericarditis, thickened pericardium shows nonspecific inflammation more often than a granulomatous response or *H. capsulatum*

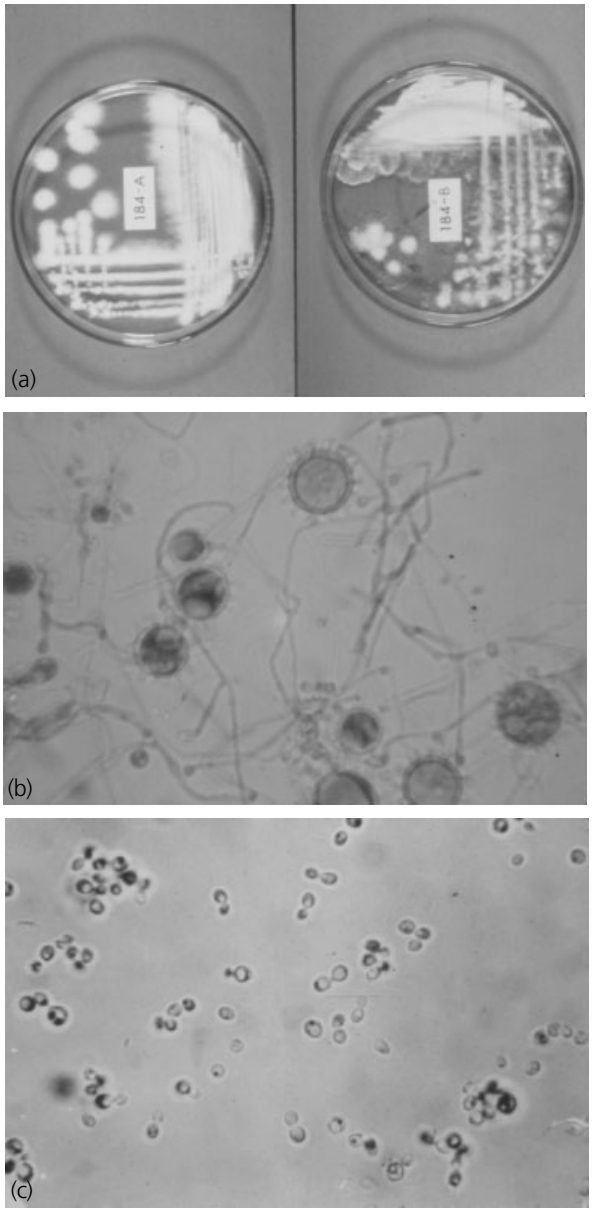

Figure 13 *Morphology of* H. capsulatum *var.* capsulatum. **(a)** *Mycelial colonies of brown (left) and albino (right) types at 25°C after 4 weeks (Cotton blue stain).* **(b)** *Microscopic appearance of* H. capsulatum *in mycelial form showing branching septate hyphae with microconidia (2–4 μm) and tuberculate macroconidia (9–15 μm) (Cotton blue stain; magnification ×3 400.)* **(c)** *Yeast form growth of* H. capsulatum *var.* capsulatum *on brain–heart infusion agar slants at 37°C after 48 hours. Morphology of* H. capsulatum *var.* capsulatum. **(d)** *Microscopic appearance of* H. capsulatum *var.* capsulatum *yeast cells (2–5 μm). (Cotton blue stain; magnification ×3 400)*

yeast cells. Occasionally, caseous subcarinal nodes may be attached to the pericardium.

The most prominent lesions of progressive disseminated disease are extrapulmonary. There is a diffuse scattering of infected macrophages throughout the mononuclear phagocytic system (MPS), often referred to as the reticuloendothelial system; granulomas are generally formed poorly, if at all. The organs rich in MPS are most frequently involved. Necrosis is less prominent in lesions outside the lungs because necrosis perhaps reflects an adequate host defense that is compromised in disseminated disease. A notable exception is the adrenal glands where necrosis is a prominent feature. Fungal stains demonstrate a large number of histoplasma yeast cells in lesions with diffuse infiltration by macrophages. In patients with AIDS and other severely immunocompromised hosts, the phagocytic cells are usually engorged with yeast cells, presumably as a result of lack of adequate cytokine production and signaling required for killing of the ingested organisms.

Some extrapulmonary sites have their peculiar lesions. The infected cardiac valves often have large yeast forms or short hyphal cells. The histological characteristics of an embolized vegetation of a peripheral artery can readily be confused with candidal infection. In the CNS, histoplasmosis capsulati may present only as a meningitis or one or more cerebral granulomas. In meningitis, the organisms can be so sparse that histological sections may miss the diagnosis unless cultures of basilar meninges are done.

HISTOPLASMA CAPSULATUM VAR. *DUBOISII* INFECTION

H. capsulatum var. *duboisii* is endemic to the tropical regions of Africa and the island of Madagascar. It was

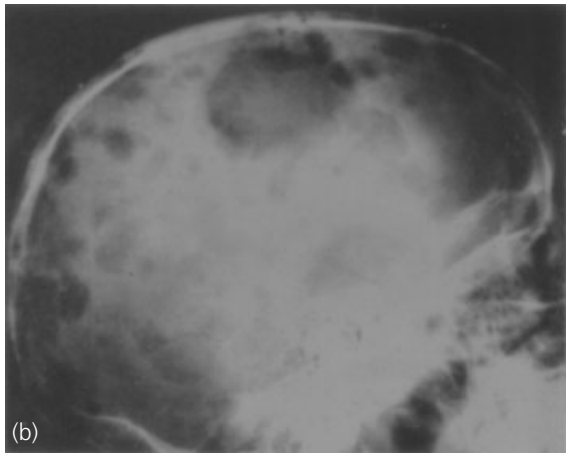

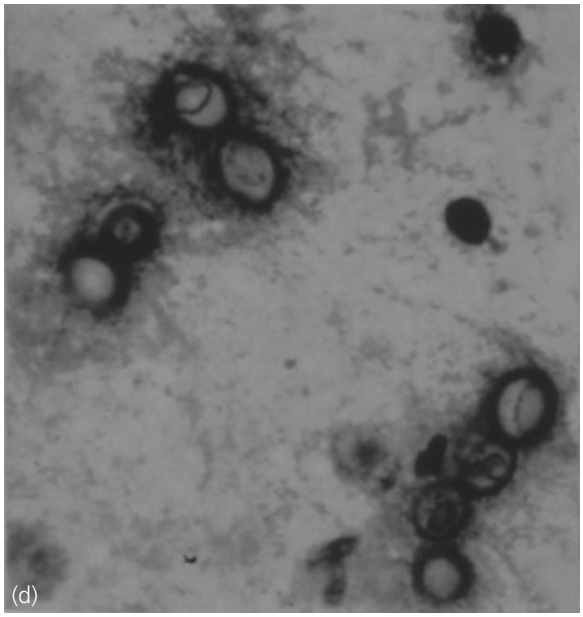

Figure 14 (a) *Head of a male child with disseminated form of histoplasmosis duboisii showing multiple subcutaneous abscesses over the bone defects shown in* **(b)**. **(b)** *x-Ray of the skull with multiple osteolytic defects resulting from granulomas in the diploe which have extended to involve both the inner and outer tables of the vault.* **(c)** *A female patient with two large abscesses on the back caused by* H. capsulatum *var.* duboisii. **(d)** *Yeast cells of* H. capsulatum *var.* duboisii *in pus (PAS stain; magnification ×800). (Courtesy of Dr Adatukumbo Lucas, Ibadan Nigeria)*

contrasted to *H. capsulatum* var. *capsulatum* by Houston (1994). Cases have also been diagnosed in travelers who have visited those areas and immigrants from central Africa. Several cases have been reported in people with AIDS. Exposure to bat or bird droppings appears to be a risk factor for acquisition of histoplasmosis duboisii. Isolation of the *duboisii* variety from the environment was conclusively established in 1994 by Gugnani et al.

The clinical manifestations of *H. capsulatum* var. *duboisii* infections differ from those seen in patients infected with the *capsulatum* variety of *H. capsulatum*. Although the infection is acquired by inhalation, the most common clinical manifestations in histoplasmosis duboisii involve skin, bone, and subcutaneous tissues (Cockshott and Lucas 1964). Multiple osteolytic lesions are commonly found in rib, vertebra, femur, humerus, tibia, skull, and wrist (Williams et al. 1971). The granulation tissue from a vertebral lesion may cause compression of the spinal cord, resulting in paraplegia. Skin lesions may be papular, nodular, ulcerative, circinate, and eczematoid. Formation of subcutaneous abscesses from underlying bone lesions are common in histoplasmosis duboisii. With the enlargement of nodular or papular lesions, the centre ulcerates. Patients with only skin or bone lesions may have an indolent disease course, and may sometimes recover without any treatment.

If present, the pulmonary lesions of histoplasmosis duboisii, in chest radiographs, may have a diffuse

reticular pattern extending throughout both lung fields or localized with or without involvement of mediastinal lymph nodes. These radiographic findings are similar to that observed in *H. capsulatum* var. *capsulatum* infections. The patients with a mild form of the disease presumably recover without any treatment.

In patients with the disseminated form of histoplasmosis duboisii, multiple lesions are usually present in the liver, spleen, lymph nodes, bone marrow, and other visceral organs. Fever, anemia, and loss of weight are common accompaniments. Such patients follow a progressive, wasting course and their disease may prove fatal if untreated. At autopsy, numerous nodular lesions are found throughout the reticuloendothelial system. This feature is remarkably similar to that observed in patients with histoplasmosis capsulati (Wheat et al. 1981).

Microscopic examinations of tissue sections show numerous organisms in areas of granulomatous inflammation (Williams et al. 1971). The giant cells contain large, thick-walled yeast cells that are larger than those of the *capsulatum* variety. An acute inflammatory response may be seen in ulcerated skin lesions and in soft tissue abscesses. The well-organized granulomata with their caseous centres and surrounding epitheloid cells, which are commonly associated with pulmonary histoplasmosis capsulati, are rarely observed in histoplasmosis duboisii.

Diagnosis is made by fungal stains that reveal ovoid, thick-walled yeasts measuring 12–15 μm in diameter that are distinct from those of *H. capsulatum* var. *capsulatum* (2–5 μm in diameter). Morphologically, *H. capsulatum* var. *duboisii* and var. *capsulatum* are identical in culture; however, they can be distinguished by differences in the size of their yeast-form cells in experimentally infected animals. Treatment of histoplasmosis duboisii is similar to that of *H. capsulatum* var. *capsulatum* infections. Both amphotericin B and itraconazole are effective (Eichmann and Schar 1996) in HIV-positive as well as in HIV-negative individuals (Figure 14).

HISTOPLASMA CAPSULATUM VAR. *FARCIMINOSUM*

As has been emphasized, histoplasmosis farciminosi is an equine disease. Clinically, it is a virulent disease notable for the suppuration of the superficial and subcutaneous lymphatic vessels, especially in the neck and limbs of the infected animals. Other parts of the body are more rarely involved – flanks, back. In general, the initial lesions are most frequently associated with those parts of the body which are most apt to be subject to wounds by kicks, contusions, and harness galls. The ulcerations are painful and usually pruritic. The infected lymphatic vessels become dilated and form a linear series of abscesses which in time rupture and discharge serosanguineous pus for several weeks. Noncutaneous lesions may occur in the mucous membranes and proliferate along the nasal septum to the pharynx, larynx, and trachea. Ocular involvement may also occur, especially on males. Such infections are characterized by watery discharge from one or both eyes with blepharitis that may lead to the closing of the affected eyes (Singh 1966). Dissemination of histoplasmosis farciminosi is essentially through the lymphatic ducts. Involvement of the internal organs has not been documented.

Diagnosis is based on the detection of the tissue-form cells of the dimorphic *H. capsulatum* var. *farciminosi*. These unicellular hyaline cells are 2.5–3.5×2.0–3.0 μm. They are best detected in pus smears on tissue sections through staining with the combined GMS–H&E stain or the PAS stain. The yeast-like cells are found to be intracellular within phagocytes. As noted previously, they are indistinguishable from the tissue-form cells of the *capsulatum* variety of *Histoplasma* in respect of size, form, and staining reactions. Confirmation of isolating the etiologic agent can be achieved by the use of a soil extract medium as reported by Weeks et al. (1985) and by converting the mycelial form to its yeast-like form by growing the isolates at 37°C in brain–heart infusion agar with or without 5 percent sheep blood.

Treatment is empirical as no consistent effective treatment has been developed. Surgery for extirpation of early lesions has been effective in some instances. Recently administration of amphotericin B has been recommended (Chermette and Bussieras 1993). For further information on histoplasmosis farciminosi, the readers are referred to the 1980 publication of Chandler, Kaplan, and Ajello.

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Paracoccidioidomycosis

GIL BENARD AND MARCELLO FRANCO

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ETIOLOGIC AGENT

History

Paracoccidioidomycosis, a term officially recognized in 1971 in Medellín, Colombia, during a meeting of mycologists, is a deep mycosis which may be manifested as an overt disease or occur as an asymptomatic form. The mycosis is of great interest for Latin American countries and is caused by a dimorphic fungus *Paracoccidioides brasiliensis*, which still has not revealed its perfect, sexual phase (Lacaz 1994a).

In 1908, in the city of São Paulo, Brazil, Adolpho Lutz (1855–1940), one of the greatest scientists in Brazilian medicine, described this morbid entity for the first time in two patients, who presented with extensive oropharyngeal lesions associated with cervical lymphadenitis. Lutz was able to isolate the microorganism in culture; the parasite's colony showed gross resemblance to the fur of a white rat, grew as a filamentous microorganism in various media at room temperature, and had a dimorphic nature. As Lutz never saw endogenous sporulation, he considered the fungus to be different from *Coccidioides immitis*, a fungal agent previously described in Argentina (Lutz 1908).

Between 1909 and 1912, Alfonso Splendore (1871–1953), a distinguished Italian bacteriologist, observed new cases of the disease, gave a more complete description of its causative agent and of the clinico-histopathological features of the disease, and assigned the causative agent to the *Zymonema* genus under the name *Zymonema brasiliense* (Splendore 1912).

Later on, the disease was baptized 'Brazilian blastomycosis' and soon after, 'South American blastomycosis' due to the observation of cases in other South American countries.

In 1923, Floriano Paulo de Almeida (1898–1977), a researcher working at the University of São Paulo Medical School, confirmed that the agent differed from that of coccidioidomycosis, and, in 1930, after a series of systematic studies, established a new genus within the kingdon of fungi, i.e. *Paracoccidioides*. He revalidated the name of the species created by Splendore and officially assigned the name of the new fungus, *Paracoccidioides brasiliensis*, according to the rule established by Linneus (Campos and Almeida 1927).

Synonymy of the disease then proliferated, with the coining of terms such as paracoccidioidic granuloma, paracoccidioidic granulomatosis, malignant ganglionic granuloma of blastomycetic origin, neotropical blastomycoid granulomatosis, adenomycosis, Lutz disease, and Lutz–Splendore–Almeida disease.

As a result of its geographical distribution, the mycosis was also designated as South American blastomycosis. After the First Pan-American Symposium on Paracoccidioidomycosis, held in Medellin, Colombia, in 1971, the term paracoccidioidomycosis became well established, and is nowadays the denomination universally accepted (PAHO, 1972).

Mycology

The etiologic agent of paracoccidioidomycosis, *P. brasiliensis*, is a dimorphic fungus only known in its asexual state (anamorph). The dimorphism depends primarily on the temperature.

In its parasitic phase and in culture at 35–37°C, the growth is yeast-like. The fungal elements are rounded

cells up to 5–40 μm in diameter, with a well-defined refringent apparently double wall and reproduction by single or multiple buds, which are connected to the parent cells by a narrow bridge. Sometimes, in the lesions, the parasite presents as small diminute yeast cells, measuring about 3–5 μm (Lacaz 1994b).

The type of reproduction of *P. brasiliensis* is characterized by the formation of chromatin masses in the cytoplasm, which may cross already existing small openings in the cell walls, or newly formed ones, dragging the cytoplasmatic mass and the enveloping membrane (Lacaz 1994b).

The variability in size and number of the daughter cells and their connection to the parent cells are characteristic. The large cells, with multiple exosporulation, which in cross-section resembles a pilot's wheel, are pathognomic.

In culture at room temperature, the growth is mycelial. The colonies do not grow rapidly and start to develop after 20–30 days of incubation: they become velvety and cracked, and may show glabrous, cerebriform, and brownish features. Under the microscope, the colonies show fine, septated, interlaced, mycelial hyaline hyphae, 0.8–2.5 μm in diameter. Round to subspherical, 20–50 μm in diameter, terminal, and intercalary chlamydoconidia may be observed (Lacaz 1994b) (Figure 1).

In the parasitic state of *P. brasiliensis*, the blastoconidium has a blastic development which is characterized by the blowing-out of a conidiogenous cell or of a fertile hypha to form a conidium. The electron microscopy studies suggested that both walls of the conidiogenous cell are used in the formation of the conidial wall, which means that the conidia can be considered as holoblastic. Regarding the order of arrangement and the position of the conidium, the terms used for *P. brasiliensis* will be botryose, asynchronous (Lacaz 1994b).

It should be mentioned that *P. brasiliensis* is a eukaryotic cell or eukaryote, showing a cell wall made up by chitin as visualized by periodic acid–Schiff (PAS). This wall can be well detected by electron microscopy.

The molecular aspects of *P. brasiliensis* dimorphism, diagnosis, epidemiology, taxonomy, and genetics have recently been extensively reviewed (San-Blas et al. 2002).

Molecular tools have been used to discover genes with differential expression according to the fungal phase. It is important to stress that glucose polymers are arranged mainly as α-1,3-glucan in the cell wall of the pathogenic yeast phase (95 percent) of the fungus whereas β-1,3-glucan is the only glucose polymer found in the mycelial cell wall (San-Blas 1979). These features have a remarkable importance in pathogenicity and dimorphism of *P. brasiliensis*.

The synthesis in vitro of β-glucan by β-1,3-glucan synthase requires UDP-glucose as the preferred nucleotide precursor. So far, only one related *P. brasiliensis* gene, *FKSP61*, has been cloned and sequenced (Pereira et al. 2000).

In addition, two other DNA fragments have been shown to correspond to distinct glucan synthase genes, which indicates that genes encoding glucan synthases appear to comprise multigene families.

On the other hand, no α-1,3-glucan synthase gene has been reported in *P. brasiliensis*. This polysaccharide is organized as the outer capsule in the yeast phase of the fungus and replaces almost entirely the β-glucan that comprises the neutral polysaccharide of the vegetative mycelial phase, behaving like a virulence factor.

Chitin represents a major structural component of the cell wall with functions in morphogenesis and virulence. Five chitins synthase genes, representing different classes of enzyme, are active in *P. brasiliensis* (Niño-Vega et al. 2000b).

The best studied proteins and genes related to fungal morphogenesis include the heat shock proteins (Hsp). The Hsp 70 gene isolated in *P. brasiliensis* is differentially expressed during transition from the mycelial to yeast form (Silva et al. 1999).

The synthesis of polyamines is a metabolic process that appears to be involved in the dimorphic process; this process is catalyzed by ornithine decarboxylase (ODC). The *PbrODC* gene has been cloned and sequenced in *P. brasiliensis*.

Several *P. brasiliensis* DNA sequences of potential diagnostic use have been reported, such as a species-specific 110 bp DNA fragment, a species-specific 14-base DNA probe originated from the 28S ribosomal gene of the fungus, a specific 418 bp fragment from the 5.8S rDNA flanking internal transcribed spacer regions, and primers derived from the sequence of the gene coding for the immunodominant gp43 antigen (Goldani et al. 1995; Sandhu et al. 1997; Imai et al. 2000).

The molecular methods used for identifying *P. brasiliensis* are also used for epidemiologic screening and in molecular taxonomic studies. Random amplified polymorphic DNA (RAPD) analysis of *P. brasiliensis* strains have indicated that DNA variation correlates with geographical areas but not with pathological findings (Calcagno et al. 1998). RAPD analysis also achieved discrimination that reflected degree of virulence. Altogether these data suggest that *P. brasiliensis* may consist of several genetically distinct groups under a single morphological species (Molinari-Madlum et al. 1999).

The integration of data between morphological features, virulence, and DNA variation might eventually lead to a more appropriate species differentiation concept in the fungus.

Molecular methods have also been useful to redefine the taxonomy of *P. brasiliensis*. Phylogenetic comparison based on large subunit (28S) ribosomal rDNA sequences has placed the fungus as belonging in the order Onygenales, family *Onygenaceae* (phylum Ascomycota) (Leclerc et al. 1994).

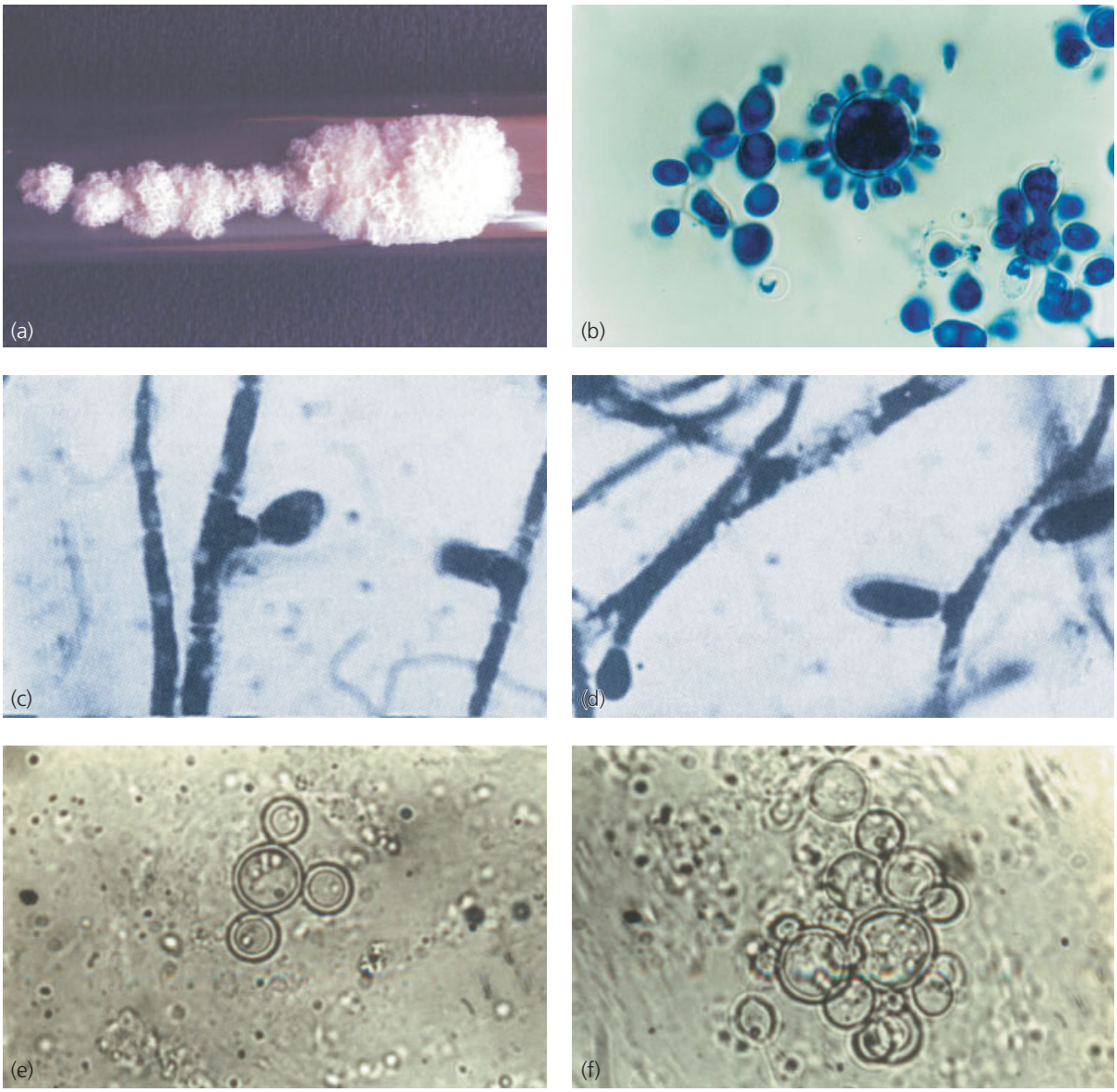

Figure 1 (a) *Macroscopic aspect of a* Paracoccidiodes brasiliensis *culture at 37 C ön Fava Netto's medium.* **(b)** *Yeast-like forms on lactophenol culture grown at 35°C.* **(c, d)** *Mycelial form grown at 25°C on Sabouraud agar, showing fertile hyphae producing terminal conidia.* **(e, f)** *Direct examination: KOH mount of sputum showing yeast-form-like cells.*

Four or five chromosomes of variable molecular weights have been reported in *P. brasiliensis* depending on the samples and the techniques used for karyotyping. The data so far indicate that the fungus has chromosomal polymorphism, due to genetic translocations or large-scale deletions, as is the case for other pathogenic fungi. The phenomenon may play a role in promoting the genetic variability and accelerated evolution of different isolates (Montoya et al. 1999). The genome size of *P. brasiliensis* is around 45.7–60.9 Mbp and the nuclei of the yeast cells may be diploid (Cano et al. 1998).

The *P. brasiliensis* genome project is under way. There has been constructed a *P. brasiliensis* DNA library from the yeast pathogenic forms and sequenced 13 490 Expressed Sequence Tags (EST) and clusters. Sequence analyses of these clusters have already identified several virulence factors (Goldman 2002). In addition, a similar approach is aiming at identifying expressed genes in mycelium and yeast forms. Seven genes that code for members of the Hsp family have been identified which could potentially be involved in the dimorphic and thermoregulated transition of the fungus. Genes coding to multidrug resistance proteins have also been identified. These issues will only be further addressed as gene-disruption and/or RNA interference approaches become available for the pathogen (Felipe et al. 2002).

Ecology and epidemiology

TRANSMISSION AND ECOLOGICAL NICHE

Paracoccidioidomycosis is acquired most commonly by inhalation of propagules from the filamentous phase of the infecting agent present in the nature (Borelli 1963–64). The true prevalence and incidence of paracoccidioidomycosis are not precisely known. Many factors contribute to this situation. The mycosis is not a notifiable disease in the countries where it is endemic. Cases are sporadic; neither epidemic outbreaks nor interhuman transmission have been reported to date. Familiar incidence is rare. Moreover, the ecological niche of the fungus is not yet solved (Restrepo et al. 2001). The long time of incubation, sometimes decades, in the most common form of the disease, the chronic form, makes the delimitation of the areas where the disease was probably acquired difficult. Acute/subacute form cases, more rarely seen, make this task somewhat easier. The presence of a significant percentage of children under 14 years of age with the acute/subacute form within a circumscribed region would imply that it is an endemic region, because such children have less frequently moved away from their living place as compared to adult patients The regions considered endemic in Latin America are despicted in Figure 1.

In the face of these difficulties, Borelli (1963–64) created the concept of 'reservárea' for the place in endemic areas where man acquires the infection, and consequently where the fungus has its habitat, in opposition to 'endemic area,' which refers to the place where the disease is diagnosed.

Previous estimates of incidence were based on the number of cases per year diagnosed in traditional mycoses services and encompassed mostly the period from 1940 to 1980. Based on these data, Londero and Ramos (1990) had estimated an annual incidence of one to three cases per 100 000 habitants in highly endemic areas, while Franco et al. (1989) estimated an annual incidence of four new cases per million habitants. However, this may not hold valid because of the increasing urbanization of the population in the Latin American countries in the last decades. A recent study analyzed 3 181 deaths from paracoccicioidomycosis in Brazil, based on 16 years of sequential data. The mean annual mortality rate was 1.45 per million inhabitants, representing the eighth most common cause of death from predominantly chronic or recurrent infectious and parasitic diseases; the data justified classifying the systemic mycosis as a major health problem in Brazil (Coutinho et al. 2002).

The *P. brasiliensis* habitat still is a puzzle and has been reviewed recently (Restrepo et al. 2001). It is presumed that the fungus lives in its saprophytic filamentous phase in humid, protein-rich soils, in areas with small temperature variations, where it would produce the arthroconidia and aleurionoconidia. Once man disturbs this habitat, these small infectious propagules, which are less than 5 μm in diameter, would disperse in the air, be inhaled, and reach the terminal bronchi and alveoli. Thus the strong link that exists between agricultural activity and paracoccidioidomycosis infection and disease. Recently, the role played by deforestation in increasing both exposure and incidence rates has been documented in Brazil in Amerindians and in children (Coimbra et al. 1994; Rios-Gonçalves et al. 1998; Fonseca et al. 1999). Unexpectedly, however, there is only a small number of successful recoveries of *P. brasiliensis* from the soil (Franco et al. 2000). One possible reason comes from a recent study on the action of agricultural pesticides on *P. brasiliensis*, showing that its in vitro growth was inhibited by concentrations of pesticides that can usually be found in agricultural soils (Ono et al. 2001).

Borelli (1963–64) has also previously suggested that the fungus survives in cavities in the underground, associated with rodents' holes and armadillo's burrows. Although there is still no information regarding rodents, the hypothesis is consistent with the recent findings of fungal isolation from the nine bands armadillos (*Dasypus novemcinctus*) (Naiff et al. 1986; Cadavid and Restrepo 1993; Bagagli et al. 1998; Silva-Vergara and Martinez 1999). Moreover, skin surveys of domesticated animals such as cows, horses, and sheep revealed a high proportion of positive responses to paracoccidioidin (Costa and Fava Netto 1978). Serological studies in dogs showed high percentages of positive tests, mainly in dogs from rural areas as compared to urban animals (Mós and Fava Netto 1974; Ono et al. 2001). Up to very recently, the enigma of why *P. brasiliensis* could not be found in autopsies of dogs highly exposed to the fungus as demonstrated by high titers of specific antibodies was explained in principle by the assumption that dogs are extremely resistant to paracoccidioidomycosis, as opposed to coccidioidomycosis. However, a recently proven case of paracoccidioidomycosis in a dog will reshape this discussion (Ricci et al. 2002).

A careful study in Colombia by Restrepo's group attempted to characterize the ecological factors that prevail in the 'reservárea.' Altitude from 1 000 to 1 499 meters above sea level, rainfall from 2 000 to 2 999 mm, presence of humid forests and of coffee and tobacco crops were significantly associated with the disease (Calle et al. 2001). In Brazil more than 800 cases have been notified in the last 6 years in an area at the border of the amazonic region that had experienced an intense migration process and had become a new agricultural frontier (Lima and Durlacher 2003).

AGE AND GENDER

The disease predominates in men aged 30–50 years. It decreases gradually over 60 and abruptly in the young.

This is because the chronic form of the disease is the most frequent presentation of the mycosis, the acute/subacute form representing usually less than 10 percent of the cases. Males are afflicted with much greater frequency than females, with a ratio 14:1 or more, depending on the patient series. The reason for the lower incidence in women was elegantly explained by observations showing that *P. brasiliensis* mycelia and conidia possess an estrogen receptor and that estrogens inhibit the mycelia/conidia to yeast transformation (Restrepo et al. 1997; Aristizábal et al. 1998). These data fit well with the finding that the acute/subacute form of the disease affects equally both genders before puberty, but preferentially men after puberty (Silvestre et al. 1997), and with the knowledge that infection is acquired at an early age with no gender differences (Londero et al. 1987). In the review published by Londero et al. (1996), which included children up to 14 years of age, there was a slight predominance of males in the older group. In endemic areas, skin test surveys showed that positive reactions start before completion of the first decade of life, reaching their peak by 15–19 years of age, and only decrease after the age of 50, probably due to immunological senescence. However, paracoccidioidomycosis disease has occasionally been seen in fertile adult women; in these cases a search for immunosuppressive-associated conditions must be undertaken, with some reports of a disseminated disease in pregnant women (Martinez et al. 1993).

OCCUPATION AND RACE

Almost half of the reported cases in adults occur in individuals whose occupations require extensive exposure to the soil. Among the agricultural activities, work in coffee plantations has been traditionally linked to the mycosis (Naiff et al. 1988; Coimbra et al. 1994). In a recently published epidemiologic survey of 584 cases from an endemic region in São Paulo State, Brazil, bricklaying and masonry were, after agricultural activities, the second most important risk factors, which suggests that digging and excavating may also favor the encounter with fungus from under the ground, as first pointed out by Borelli in 1972 (Blotta et al. 1999). No ethnicity susceptibility has been documented. Although a few studies had previously suggested some link between HLA and disease susceptibility, the influence of genetic traits on the mycosis has thus far not been elucidated (Dias et al. 2000).

GEOGRAPHICAL DISTRIBUTION

The mycosis was limited to continental Latin America until 1987, when cases were reported in the Caribbean islands of Trinidad, Grenada, and Guadeloupe (Restrepo-Moreno 1994). Paracoccidioidomycosis is distributed heterogeneously in the endemic region; areas of high endemicity may be localized next to areas of very low incidence. The highest numbers of cases have been reported from Brazil, Colombia, and Venezuela (Restrepo 1985); 7 000 of the approximately 10 000 patients reported from that period were natives of Brazil (Figure 2). Cases diagnosed outside Latin America referred to individuals who, with no exception, once visited or lived in endemic areas from Latin America up to 10–40 years before (Ajello and Polonelli 1985). Within endemic areas, 10–50 percent of healthy adult individuals react to paracoccidioidin, suggesting previous exposure to *P. brasiliensis*. Caution in the interpretation of these skin test surveys comes from studies showing that concomitant histoplasmin reactivity significantly increases the rate of paracoccidioidin reactivity when crude paracoccidioidal antigens are used. The use of the specific gp43 antigen has significantly reduced the percentage of skin-reactive persons in areas where *Histoplasma capsulatum* is endemic (Kalmar et al. 2004). Recent molecular biology studies have indicated that there are at least five different groups of *P. brasiliensis* strains and that they correspond closely with the borders of the various endemic areas and countries (Niño-Vega et al. 2000a).

OTHER FACTORS

Alcoholism was shown to be a predisposing factor to paracoccidioidomycosis. It also probably accounts for a worse prognosis for this infection, at least because of the poorer compliance to treatment of such patients (Martinez and Moya 1992). Undernourishment is another factor commonly associated with paracoccidioidomycosis (Restrepo and Benard 2004).

HOST RESPONSES

Most authors consider that the infection takes place through the inhalation of fungal propagules and that the lungs are the primary focus of the lesions. In most cases, the initial pulmonary infection does not cause noticeable symptoms. Once the conidia reach the terminal bronchi or alveolar spaces, the conidia to yeast transformation takes place (McEwen et al. 1987). In some cases, the fungus disseminates by the lymphohematogenous route, producing distant subclinical and quiescent foci. Apparently, the local defenses are capable of controlling fungal spread, but some viable yeast cells may remain silent in such foci in the pulmonary/mediastinal lymph nodes or even elsewhere. Rarely, the initial pulmonary infection overcomes the host's immune defenses, causing an acute/subacute disease, the juvenile type, with predominant involvement of the reticuloendothelial system. Most often, the quiescent foci remain so throughout life, as demonstrated by the high number of subclinical infections versus the low disease incidence of this mycosis in endemic regions (Wanke and Londero 1994). Nevertheless, the most common clinical presentation of the disease is the chronic form, or adult-type disease,

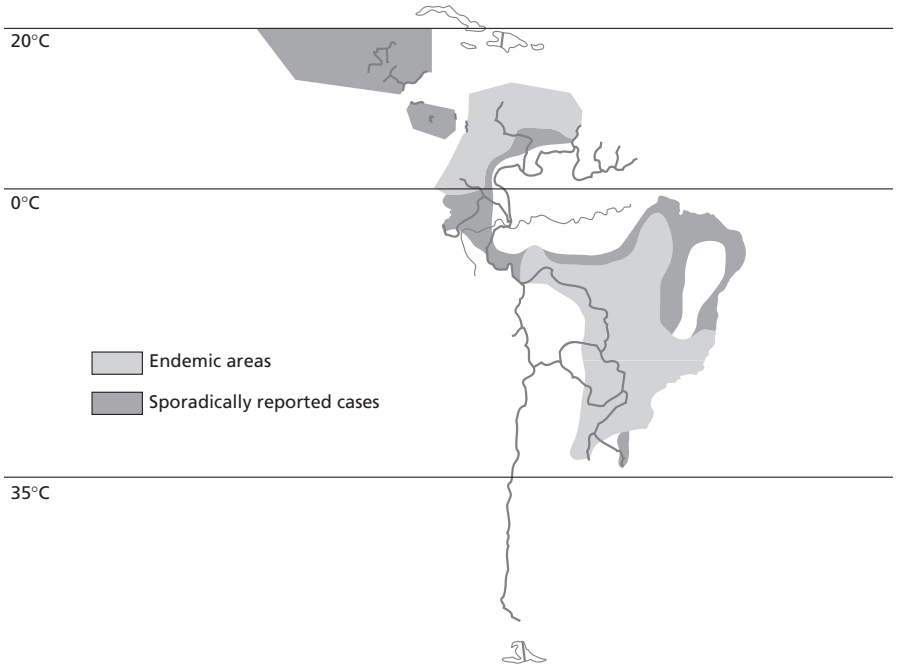

Figure 2 *Geographical distribution of paracoccidioidomycosis.*

believed to result from fungal reactivation in these foci, which not infrequently occurs when the patient has already left the endemic area, as demonstrated by the patients who developed the disease many years after they have migrated to countries outside Latin America (Figure 3).

Actually, paracoccidioidomycosis, either in the acute/subacute or chronic form, is more frequently a disseminated disease, even though clinical manifestations appear to be restricted to a sole organ (Yamaga et al. 2003). However, determination of the degree of dissemination is certainly influenced by the availability of diagnostic procedures. Thus, as a polymorphic disorder that frequently involves more than one organ system, any topographic classification is ineffective. The currently accepted classification of the mycosis takes into consideration not only the organs involved but also the host's immune condition and the disease's

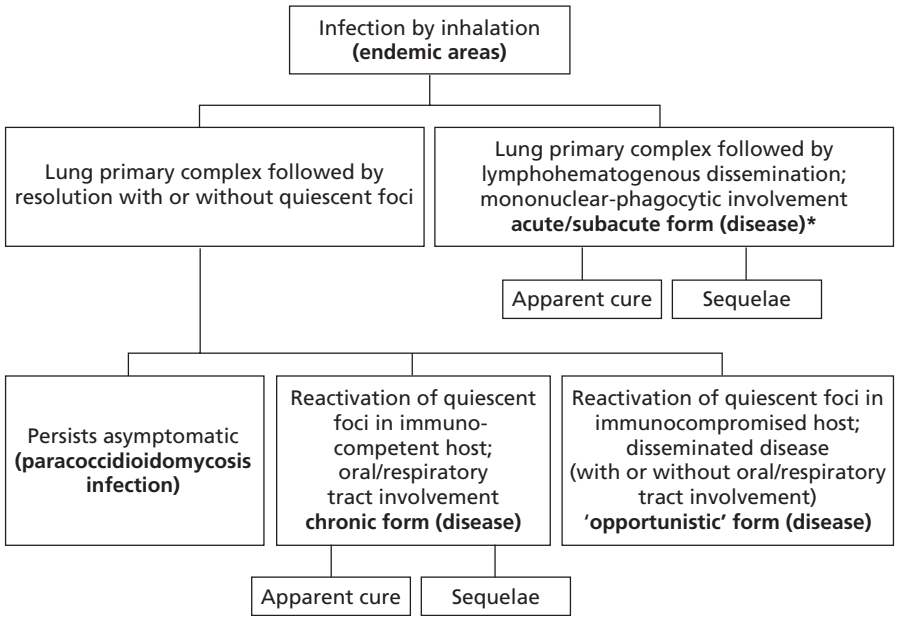

Figure 3 *Classification of paracoccidioidomycosis.*

natural history (Franco et al. 1987). The different outcomes that may result from the *P. brasiliensis*–host interaction are shown in Figure 1. Those individuals who remain infected are categorized as having only the subclinical form. The disease that develops soon after the initial infection is classified as the acute/subacute form. The acute/subacute pattern predominates in children and young adults. According to the severity of the process, acute/subacute patients are assigned to two subgroups, severe and moderate. The chronic progressive form of paracoccidioidomycosis, more common in adult men over 30 years of age, may remain localized in the lungs, the unifocal disease, or may disseminate from its primary foci, the multifocal disease. The chronic form can be mild, moderate, or severe (Franco et al. 1987).

Clinical forms

PARACOCCIDIOIDOMYCOSIS INFECTION

P. brasiliensis-infected persons would correspond to up to 10 percent of the population in countries where paracoccidioidomycosis is endemic, according to previous skin-test surveys (Wanke and Londero 1994). However, there are only sparse reports on the encounter of the fungus in quiescent foci, usually calcified lymph nodes, accidentally or in autopsies of individuals from these countries who died of unrelated causes (reviewed in Restrepo 2000). There are also anedoctal case reports of a pulmonary regressive form (Restrepo et al. 1976; Londero 1986). The study of the morphology and viability of the dormant yeast cells from a patient's residual lesion demonstrated a large proportion of aberrant cells, but there were still 5 percent viable cells (Restrepo 2000). Analysis of some immunological parameters from a small number of infected individuals revealed a Th1-type immune profile that distinguishes them from patients with this disease or cured individuals (Mamoni et al. 2002). Another example is a recent serological survey of blood donors from the countryside of a Brazilian endemic region, employing a more sensitive test, which disclosed that 12 percent of the putatively healthy donors produce specific antibodies (Botteon et al. 2002). The reasons why in some of these infected persons the fungus reactivates at some moment and provokes the chronic form of the disease are not known. A thorough clinical and immunopathological study of persons living in endemic areas is still lacking and is needed before any definitive statement on the concept of paracoccidioidomycosis infection can stand.

ACUTE/SUBACUTE FORM OF THE DISEASE

The time elapsed from infection to the onset of symptoms in the acute/subacute form is not precisely known, but has been estimated to be short, a few months. The acute form involves preferentially the reticuloendothelial system. Fever, malaise, weight loss, and emaciation may be seen in the acute/subacute form. Actually, only a minor proportion of the cases reported have had lung symptomatology. However, this involvement may be underestimated because the severe extrapulmonary lesions tend to minimize the more discrete respiratory manifestations (Londero 1986). Frequently, the mycosis is misdiagnosed as ganglionar tuberculosis or certain lymphomatous disorders. However, induced sputum or other maneuvers may reveal the characteristic *P. brasiliensis* yeast cells (Restrepo et al. 1989). New imaging methods, such as gallium and computerized tomography, have allowed detection of incipient or discrete interstitial pulmonary lesions not revealed by plain radiographs (Funari et al. 1999; Yamaga et al. 2003).

Mucosal lesions are rather exceptional, but skin involvement is more common and tends to be multiple, in contrast with the chronic form (Restrepo and Benard 2004). The lesions represent hematogenous spread of the fungus. In this case, they may appear as ulcerated or ulcerovegetative lesions, papules, crust-covered ulcers, usually at the same stage of development. Sporadic reports of septic shock due to septicemia by *P. brasiliensis* show that the fungus can be bloodborne.

Almost every patient with acute/subacute paracoccidioidomycosis exhibits involvement of the superficial and/or deep lymph node chains. Lymph nodes vary in size, number, consistency, and location; with time, they liquefy, forming abscesses or fistulae (Figure 4).
The spleen and liver are frequently involved. Splenic lesions are nodular or miliary. Gross hepatic lesions may not be apparent, but histopathological examination regularly reveals fungal invasion of this organ (Teixeira et al. 1978). Abdominal lymph node involvement is also common. Hypertrophied lymph nodes, usually generalized but particularly periaortic, around the hepatic hilum and in the retroperitoneum, can be detected by radiological examination. Patients may complain of abdominal masses, lymph node enlargement, diarrhea, vomiting, abdominal distension and/or pain, and ascites. Coalescent masses may become palpable and may result in extrinsic compression of adjacent structures, leading to jaundice (by compression of the biliary duct), pancreatitis, or intestinal obstruction (Martinez et al. 1979a).

The intestinal mucosa may be affected and the involvement may be secondary to blockade of the regional lymphatic flow, with retrograde progression of *P. brasiliensis* to the mucosa, a process resulting in mycotic enteritis (Martinez et al. 1979b). In this case, the submucosal inflammatory process is granulomatous, fungal cells are visualized, and the intestinal changes may vary from dilated loops, edema, congestion, nodule formation to multiple mucosal ulcers (Martinez et al. 1979a).

Figure 4 *Subacute form of paracoccidioidomycosis in:* **(a)** *a young adult man: enlarged cervical suppurated lymph nodes;* **(b)** *a girl: suppurated axilar lymph node.*

Recent studies have also shown bone marrow infiltration mainly, but not exclusively, in the acute/subacute form of the disease (Resende 2000). The histological pattern is variable but appropriate staining always displays fungal cells. Bone marrow invasion is frequently associated with marked eosinophilia (Shikanai-Yasuda et al. 1992a). Bone and joint lesions are frequent, and appear closely related to bone marrow infiltration. The lytic lesions mainly locate at the diaphyseal or metaphyseal–epiphyseal regions of the long bones, but ribs, skull, phalanges, and vertebral lytic lesions have also been documented. A pathological fracture may occasionally occur.

CHRONIC FORM

Most often, chronic-form patients present a history of a few to several months of cough, either productive or not, weight loss, anorexia. Fever may be absent or present in low grade and nocturnal, as in tuberculosis, which is the main differential diagnosis. Occasionally, these respiratory symptoms are mild and the patient seeks medical care only when lymphohematogenous dissemination results in lymph node involvement or distant cutaneous lesions. Plain films taken during admission reveal the primary, pulmonary focus. The pulmonary involvement may be present as micronodular or miliary, nodular, infiltrative or interstitial, cavitary, fibrotic, or mixed patterns (Valle et al. 1992) (Figure 5). Emphysematous areas, pleural thickening, and enlarged hilar and mediastinal lymph nodes can also be observed. Right ventricular hypertrophy may be found in cases of long duration. Lung involvement is probably secondary to a chronic lymphangitic process due to the fungus itself and to the host's response represented by formation of granulomas and fibrosis, the latter predominating at the perihilar region (Tuder et al. 1985). This aspect correlates with the butterfly-like (peri-hilar) micronodular and interstitial infiltration observed on plain films.

Alternatively, in some patients the main complaints may be dysphagia and hoarseness. Generally they are associated with lung infiltrates on the x-ray, and in these cases the decrease in the nutritional status is more prominent due to the patient's impairment in swallowing. The lesions in the oropharyngeal and laryngeal mucosa may be infiltrative, ulcerated, nodular, or vegetative, and usually have a granulomatous aspect (Sant'Anna et al. 1999) (Figure 6). The base of the ulcerated lesions is usually covered by small abscesses

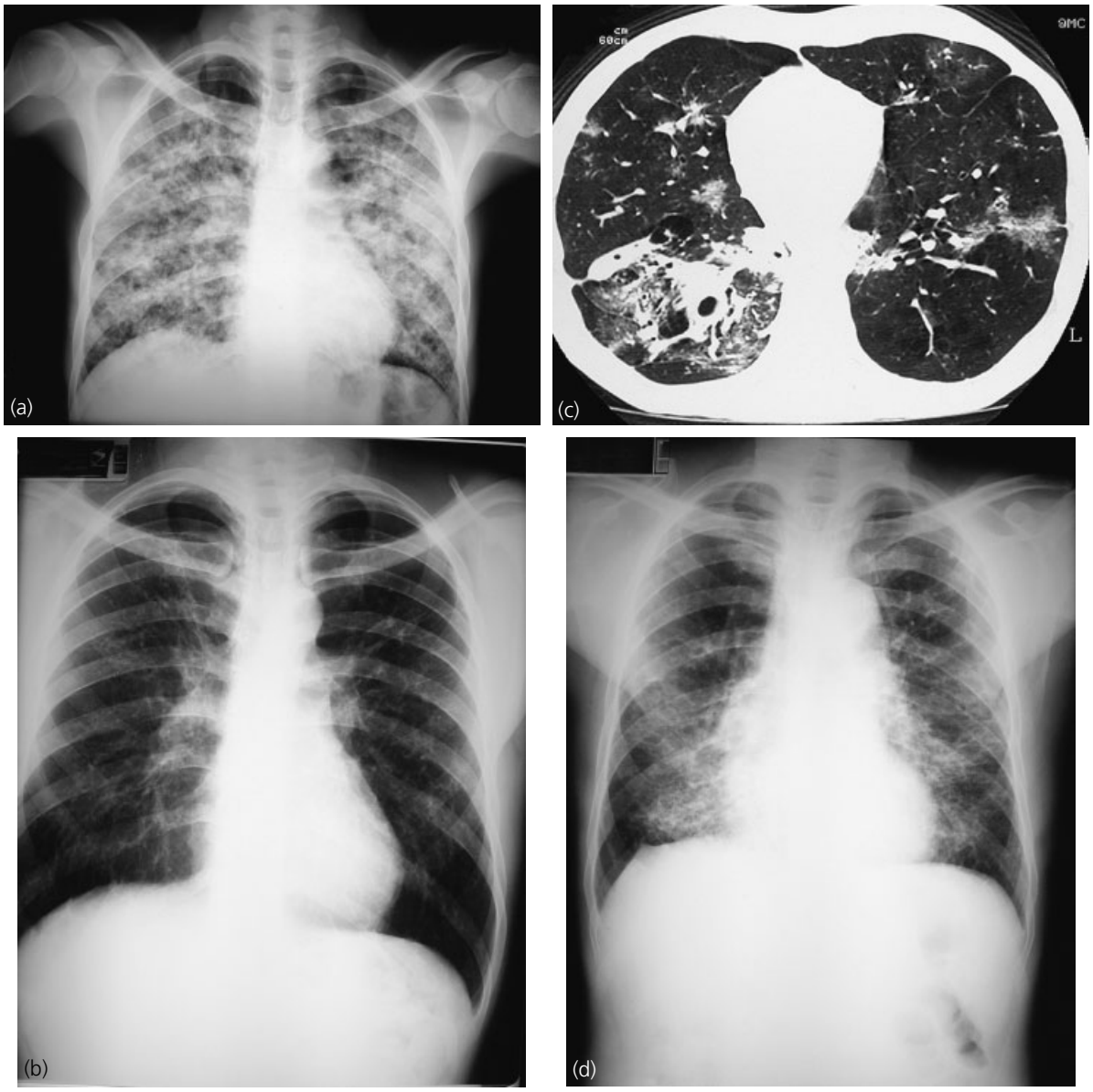

Figure 5 *Pulmonary involvement in the chronic form paracoccidioidomycosis:* **(a)** *extensive pneumonic involvement of the lungs and* **(b)** *the same patient, after 2 years of treatment, with discrete fibrotic, mainly perihilar, lines;* **(c)** *computed tomography showing diffuse pleuropathy with bronchocentric nodules, ground-grass opacities, cavities of several sizes, airspace consolidations, and centrilobular opacities (<1 mm) associated with bronchiectasis and parenchymal distortion.* **(d)** *Micronodular and reticular bilateral infiltrates predominating in the middle and lower fields.*

(the mulberry-like lesions) that probably represent fungal dissemination through the lymphatic system, as they are usually accompanied by regional lymph node involvement (Castro et al. 1999a). Cutaneous lesions are mostly represented by contiguous involvement of the perioriﬁcial mucosal lesions or draining lymph nodes or may represent hematogenous spread in patients with long-lasting disease. The latter, thus, have no preferential localization. The adrenals are often involved in patients with the chronic form of the mycosis, but only a small proportion of them will suffer from adrenal hypofunction or insufficiency (Addison's disease) (Del Negro et al. 1980). The glands containing multiple, granulomatous foci, and diffuse necrosis may be seen in the most severe cases. Recently, silent CNS granulomatous-like lesions attributable to paracoccidioidomycosis have been reported in a small survey of patients using magnetic resonance imaging; the clinical importance of this finding awaits confirmation (Castro et al. 1999b). On the other hand, granulomatous involvement of the spinal cord has been reported in a sizable number of patients and is a severe condition because it is frequently associated with significant, often irreversible, neurological impairment.

Figure 6 *Chronic form paracoccidioidomycosis in an adult man: a lips lesion with the moriform stomatitis aspect and a facial skin ulcer.*

'OPPORTUNISTIC' FORM

Paracoccidioidomycosis, as an apparently opportunistic infection in a patient with cancer, was first documented by Lacaz et al. in 1948. The association with hematological malignancies, predominantly, or other types of cancer, generally resulted in a disseminated form of the mycosis with clinical features of both the acute/subacute and chronic forms (Marques and Shikanai-Yasuda 1994). Unless the clinician is armed with a high degree of suspicion, the diagnosis of the mycosis is delayed. The same applies to AIDS patients. This subject has been recently reviewed (Benard and Duarte 2000). It is believed that in most instances it represents reactivation from quiescent foci as a result of the failure of the immune surveillance in blocking the parasite multiplication. The two reasons for the lower than expected number of cases of this mycosis in patients with the acquired immunodeficiency syndrome (AIDS) are supposedly that the two infections have non-overlapping epidemiologic features (e.g. urban versus rural) and that many patients infected with the human immunodeficiency virus (HIV) are on continuous antifungal regimens which, although directed at other fungal infections, also have a suppressive effect on *P. brasiliensis*. Paracoccidioidomycosis mainly afflicts those patients with low (<200 cells/μl) CD4 counts. Nonetheless, it has seldom been the cause of death. As with other fungal opportunistic infections, the mycosis responds adequately to the usual antifungal treatments.

SEQUELAE

Mycologically, successfully treated patients may persist with high morbidity. In the acute/subacute form, disabsorptive syndromes may ensue due to the digestive involvement that resolves by intense fibrosis and obstruction of the abdominal lymphatics, aggravating the nutritional status of the patients, resulting in immunodeficiency and opportunistic infections with high mortality rates (Shikanai-Yasuda et al. 1992b; Benard et al. 1996). Patients may also present with relapsing ascites or chylothorax. In the adult form, fibrosis, either pulmonary, tracheal, labial, or adrenal, leading to, respectively, emphysema, permanent tracheostomy, microstomy, and Addison's disease, are the cause of serious problems in patients who respond to therapy (Mendes et al. 1994). Thus, despite the recent advances in the treatment, these sequelae still preclude the complete recovery in several patients.

Immunology

The initial events of the host–parasite interaction are unknown due to our inability to detect the precise moment when the initial infection occurs. The data collected up to now indicate that the host–parasite interaction in paracoccidioidomycosis is complex, and both arms of the immune system participate in the host's response. On the one hand, the patients have a polyclonal activation of the humoral system with high serum concentrations of specific IgA, IgG isotypes, and IgE (Yarzábal et al. 1980; Biagioni et al. 1984; Baida et al. 1999; Biselli et al. 2001; Mamoni et al. 2002). However a protective role for antibodies has not yet been demonstrated. On the other hand, there is hyporesponsiveness of the patients' T-cell-mediated immunity characterized by decreased lymphocyte proliferation and absence of hypersensitivity skin-test responses, the intensity of which parallels the severity of the disease and correlates inversely with the amount of circulating anti-*P. brasiliensis* antibodies (Musatti et al. 1976; Motta et al. 1985). This imbalance was particularly noted when antigen-specific responses were analyzed (Benard et al. 1996, 1997; Oliveira et al. 2002). Several studies showed that a marked imbalance in the cytokine secretion pattern underlies this picture: lack or strongly decreased secretion of Th1-type cytokines (IL-2, IL-12, and IFN-γ) associated with normal or increased secretion of Th2 cytokines (IL-10, IL-5, and IL-4) (Karhawi et al. 2000; Benard et al. 2001; Oliveira et al. 2002). Previous studies on animal models of paracoccidioidomycosis had already shown that Th2-type responses dominated over Th1-type responses (Kashino et al. 2000). Recently, the role of IL-10 and IL-12 in the regulation of the host's immune responses has been emphasized (Romano et al. 2002). In situ studies of patients' biopsy lesions have shown overexpression of TGF-β, a cytokine with strong immunosuppressive activity (Neworal et al. 2001; Parisi-Fortes et al. 2001). Furthermore, high levels of specific antibodies of the IgE and IgG4 (switch dependent upon IL-4) isotypes were found in patients with the acute/subacute form of the disease as well as in chronic cases with severely disseminated disease, denoting the Th2 control of the humoral immune response in these patients (Biselli et al. 2001; Mamoni et al. 2002). Another aspect in favor of Th2 control was the observation of peripheral and bone marrow eosinophilia in severe cases, an abnormality linked to increased production of IL-5 (Oliveira et al. 2002). Thus, apparently in

the acute/subacute disease, the immune response fails to control the proliferation of the fungus from the beginning of the infectious process due to a profoundly downregulated Th1 response concomitant to upregulated Th2 immune response. In chronic less severe cases, the initial infection is apparently controlled, which allows a better balance of the immune response, with lower antigenic challenge and a less marked Th2 immune response. Apoptosis of the antigen-specific T cells has also been linked to the depressed cell-mediated immune response (Cacere et al. 2002). Patients with apparent cure demonstrate strong lymphocyte proliferative responses as well as positive skin tests, probably in consequence of the recovery of the fungal antigen-induced Th1 type reactivity (Benard et al. 1996; Karhawi et al. 2000).

The cytokine imbalance also affects the macrophages, which represent a crucial cell in the host defense mechanism against *P. brasiliensis*. It has been shown that the intracellular killing of *P. brasiliensis* yeast cells and conidia by this cell occurs only when previous activation has taken place (Gonzalez et al. 2000), which in humans has been shown to be mediated by the synergistic action of TNF-α and IFN-γ (Anjos et al. 2002). The main fungal antigen, gp43, appears to directly affect macrophage functions (Benard et al. 2001; Popi et al. 2002). Polymorphonuclear neutrophils (PMN) also participate in the immune response to the fungus, but their role is less well understood. PMNs are found in some paracoccidioidal lesions, specially in those representing acute inflammation or microabscesses (Sandoval et al. 1996); in experimental models they appear early in the inflammatory process and may play a role in the control of the initial infection (Burger et al. 1996). PMNs can also be activated by proinflammatory cytokines to have enhanced anti-*P. brasiliensis* activity (Kurita et al. 2000; Rodrigues et al. 2001) .

Treatment

The disease caused by *P. brasiliensis* can be managed successfully with oral antifungal drugs. Many drugs are available, and the list is still increasing. Historically, sulfa drugs were introduced in 1940 (Ribeiro 1940), followed by intravenous amphotericin B, in 1958 (Lacaz and Sampaio 1958). The use of azole derivatives (miconazole, soon discarded, ketoconazole, itraconazole, fluconazole, and more recently, voriconazole) was introduced in the 1970s. The treatment is divided into an attack phase and maintenance treatment. Two different drugs can be used for each phase, or the same drug can be used for the two phases but at different dosages (Mendes et al. 1994). Among the reasons for this two-phase prolonged treatment is the chronic progressive nature of the disease and the knowledge that all currently available drugs, albeit with apparently different efficacy, are only fungistatic. In parallel, measures in support of the general condition are required, because cellular immune mechanisms may be affected by malnutrition, concomitant infections, or by the disease process itself, and they are crucial to controlling the fungus dissemination.

Sulfadiazine is used at 60–100 mg/kg daily doses divided in four equal parts to a maximum daily dose of 6 g. Water intake and urine alkalinization (usually by bicarbonated water intake) are recommended to prevent crystalluria and tubular deposits of sulfadiazine. Duration of the attack treatment is dictated by the patient's response, once clinical and serological improvement has been attained; in the chronic form, 2–6 months are required; in acute/subacute cases, the duration of treatment should be longer. Slow-acting sulfa drugs (sulfamethoxypyridazine or sulfadimethoxine), 1 g/day, can be provided as maintenance treatment (Mendes et al. 1994). Sulfadiazine is usually recommended for mild to moderate cases, but our own experience is that it can be used for severely ill acute/subacute cases with good results. It can be used for patients who have been treated initially with amphotericin B. Sulfadiazine has been replaced in many services by trimethoprim-sulfamethoxazole (80 mg of trimethoprim and 400 mg of sulfamethoxazole per tablet, two tablets at 8- or 12-hour intervals) due to its easier posology and availability. This combination has the advantage of permitting alternative parenteral administration whenever necessary. Maintenance treatment in these cases can be achieved by using half the dose of the attack treatment or by using slow-acting sulfas.

Amphotericin B is quite effective, but it should be reserved for severely disseminated cases or in whom gastrointestinal involvement may impair drug absorption. A total dose of 1–2 g is usually required to achieve substantial clinical improvement. There are no sufficient data regarding effectiveness of the new amphotericin B lipid formulations in paracoccidioidomycosis.

As to the imidazole derivatives, patients can be treated with 200–400 mg ketoconazole once daily for periods of 6–12 months and low relapse rates have been documented; however, other studies indicated higher relapses rates and the need for 18 months or more of treatment to achieve good results (Del Negro 1982; Restrepo et al. 1983; Marcondes et al. 1984; Dillon et al. 1985; Marques et al. 1985). These discrepant results may be related to differences in severity of disease among the studies and to problems in drug absorption. Indeed, caution should be urged regarding its absorption, which is dependent on acid gastric pH. Poor absorption has been described with concomitant use of antacids, or when the drug is taken with meals (Mendes et al. 1994). Liver toxicity as well as testosterone synthesis blockade, clinically manifested or not by gynecomastia or sexual impotence, should also be evaluated, especially during prolonged therapy.

With the advent of the new triazole, itraconazole, which was shown to be less toxic and more potent in vitro, ketoconazole tends to be used less. Itraconazole is administered in 100 mg capsules that should be given with a meal; a 100–200 mg regimen for adults, and half dosage for children, daily for 6 months, have been shown to be effective in reducing all active lesions in the large majority (98 percent) of patients, including acute/subacute and chronic forms (Mendes et al. 1994). It is important to remember that both ketoconazole and itraconazole penetrate the brain barrier poorly, thus CNS lesions should be carefully discarded. Instead of amphotericin B, we have used itraconazole, at higher doses (300–400 mg adults, 100–200 mg children) for some severe cases with good results, provided there is no involvement of the digestive tract that could hinder its absorption. Some other authors (Marques 2002; Shikanai-Yasuda et al. 2002a) suggest that patients should be kept on maintenance therapy with slow-acting sulfas after clinical and mycological cure to prevent relapses, while other investigators argue that this is not required (Telles 2002; Tobón et al. 2002). Side effects have been few and include transient elevation of hepatic enzymes (Tobón et al. 1995). A randomized trial with sulfadiazine, ketoconazole, and itraconazole for the treatment of patients with moderate severity disease failed to show higher efficacy of one regimen over the others. However, long-term follow-up was not evaluated (Shikanai-Yasuda et al. 2002b). Experience with fluconazole is far less extensive than with itraconazole, and due to its higher cost is still used less. It may be useful in severely ill patients who must be treated intravenously. There is only one report on the successful use of terbinafine in an adult patient (Ollague et al. 2000).

New perspectives in paracoccidioidomycosis treatment include the new itraconazole formulations (oral solution and intravenous formulations) that will probably further extend its indications. A multicentric prospective trial on voriconazole, 400 mg daily for 6 months, showed encouraging results (F.Q. Telles Filho, M.A. Shikanai-Yasuda and L.Z. Goldani, 2003, unpublished observations). This drug will probably be an excellent option due to its efficacy and low incidence of side effects. Nevertheless, some challenges in the treatment of patients with this mycosis still remain. Neither the clinical and laboratory criteria that allow the transition from the attack to the maintenance treatment phases nor the parameters that could be used to end treatment thus assuring that the patient is definitively cured have been fully standardized. The gradual decrease in the titers of the serological tests currently available is one of the laboratory parameters most frequently used; restoration of some cellular immunity responses, such as reactivity to the paracoccidioidin skin test, is also used. Newer methods, e.g. antigenemia detection and molecular biology approaches, will probably be useful.

LABORATORY DIAGNOSIS

Direct examination

The direct microscopic observation of the characteristic *P. brasiliensis* multiple-budding yeast forms in 10 percent potassium hydroxide preparations of clinical specimens is the most recommended way for diagnosing the mycosis (Lacaz et al. 2002). Usually the sources are sputum, secretion, or scraps/debris from ulcerated skin and mucosal lesions, draining material from suppurating lymph nodes or abscesses, bronchioalveolar fluid, etc. Bronchioalveolar, articular, cerebrospinal fluid or other fluids must be centrifuged before examination. For bone marrow aspirates, Giemsa staining is recommended. When the first examination of the sputum is negative, it can be homogenized with an equal volume of 4 percent sodium hydroxide or *N*-acetyl-L-cysteine and then centrifuged again. Visualization of the multiple-budding yeast cells (pilot's wheel) with the well-defined refringent double wall is mandatory, because the *P. brasiliensis* yeast cells may range from a few microns to 30–40 μm, and when small forms predominate they may be mistaken for *Blastomyces dermatitidis*, capsule-deficient *Cryptococcus neoformans*, *H. capsulatum* yeast cells, endospores, and small *C. immitis* empty spherules or even *Pneumocystis carinii* (Silletti et al. 1996).

More recently, several molecular biology diagnostic tests based on polymerase chain reaction (PCR) are being implemented which will probably open a new field in the rapid and specific diagnosis of paracoccidioidomycosis (Motoyama et al. 2000; Lindsley et al. 2001; Sano et al. 2001). For example, the gene for gp43 from 10 *P. brasiliensis* yeast cells/ml of sputum samples could be amplified, providing sufficient accuracy for the diagnosis of the mycosis (Gomes et al. 2000).

Cultures

Cultures from every patient's specimen should be obtained whenever possible. However, they are far less useful in diagnosis compared to direct examination since other more rapidly growing microorganisms such as bacteria, yeasts (especially *Candida*), and contaminant molds eventually present in the samples inhibit their growth. Isolation should be attempted by the use of modified Sabouraud agar, yeast extract agar plus antibiotics and cycloheximide, brain–heart infusion (BHI) agar plus blood and antibiotics (without cycloheximide), or tryptic soy agar incubated at 37°C and room temperature. Cultures should be observed for up to 4 weeks. As the mycelial form is not characteristic, subcultures in BHI, Fava Netto's agar, or peptone–yeast extract–glucose agar, for up to 14 days at 35–37°C, allow yeast conversion and the definitive diagnosis (Lacaz et al.

2002). In cases of severe disseminated disease, mainly those with the acute/subacute form, blood cultures may become positive (Negroni 1993).

Serology

Patients with paracoccidioidomycosis not only have a polyclonal B-cell activation but also produce high amounts of anti-*P. brasiliensis* antibodies, which are long-lasting and generally correlate with the severity of the disease. Once having started the treatment, serological titers tend to fall, accompanying the clinical improvement. Many serological tests have been developed and extensively studied. They are highly useful for both diagnosis (especially in patients with only deep-seated lesions) and treatment follow-up of patients. Agar gel immunodiffusion (ID), complement fixation (CF), and counterimmunoelectrophoresis (CIE) tests have been employed extensively (Mendes-Giannini et al. 1994). The CF tends to be used less often due to its difficult and time-consuming technique. Also available are indirect immunofluorescence, indirect hemagglutination, enzyme-linked immunosorbent assay (ELISA), and the dot blot immunobinding and Western blotting (Mendes-Giannini et al. 1984, 1992; Taborda and Camargo 1993, 1994; Martins et al. 1997). Some of the newer tests have employed purified antigens, which are well characterized and more specific. The use of these purified antigens has resulted in improvement in the efficiency of the serological tests presently available. However, they are still not easily available for most diagnostic centers. The best characterized and most used is the 43 kDa glycoprotein (Puccia and Travassos 1991). ID, CF, and CIE, which employ crude antigens derived from the yeast phase, can detect $\geqslant$95 percent of active cases. Most authorities recommend the use of two different tests, since none of these low-cost, widely used tests *per se* guarantees more than 90 percent sensitivity when employed alone. ID, for example, is very simple and specific. A conjoint effort of several diagnostic centers assessing the value of this test using a reference crude antigen (Ag7) demonstrated it to be 84.3 percent sensitive and 98.9 percent specific (Camargo 1994). Patients with very localized, benign disease may present negative results with these tests, but when tested with more sensitive techniques, e.g. Western blot or gp43-ELISA, the diagnosis is usually reached. Crossreactivity is a less common problem, but may occur between the patients' sera and *Histoplasma* and *Lacazia loboi* antigens (Puccia and Travassos 1991).

The value of serological tests in the follow-up of patients under treatment has also been addressed by several centers. Serial serological evaluation, usually 3 months apart, together with the clinical, mycological, and radiological surveys, can guide the treatment schedule, and two negative results in two of the serological tests routinely employed is an additional criterion that helps in the difficult decision of therapy discontinuation (Restrepo and Benard 2004). The ID, CF, CIE, ELISA, and Western blot tests were shown to be able to document the decline in antibody levels that parallels the clinical improvement: serum antibodies became undetectable approximately 2 years after cessation of therapy, depending on the clinical form (Fava Netto 1961; Ferreira-da-Cruz et al. 1990; Mendes-Giannini et al. 1994; Bueno et al. 1997; Martins et al. 1997; Del Negro et al. 2000). Thus, in this circumstance, rising antibody levels mean a relapse. Nonetheless, a considerable number of patients maintain, sometimes all life long, the so-called 'healing titers,' i.e. the persistence of low serological titers in the tests. This is more frequent in patients with the acute or chronic multifocal form disease (Del Negro et al. 2000). The debate on the clinical relevance of this phenomenon, whether or not it represents the persistence of residual active lesions, thus eventually predicting future relapses, is inconclusive. Part of this problem has been lately addressed by the search of tests for antigenemia detection. Although not yet completely standardized, preliminary results of antigenemia detection in blood and urine reveal that this is a promising tool (Gómez et al. 1998; Salina et al. 1998). It will also be very helpful in the diagnosis of immunocompromised patients, specially with AIDS, in whom the search of antibody response is not a completely reliable diagnostic tool. Antigenemia follow-up studies would also permit a more precise determination of improvement as antigen load decreases with treatment response.

Histopathology

The histopathological features of paracoccidioidomycosis are similar to those of other systemic mycoses and it is essentially characterized by a granulomatous inflammation (Montenegro and Franco 1994).

It was first asserted by Motta that granulomatous inflammation response is assumed to be the most evolved form of the host tissue reactivity against the invading fungal cells. *P. brasiliensis* granuloma generally centers around one or more fungal cells and is made up of giant cells and epithelioid cells. Polymorphonuclear leukocytes may be observed close to the fungi in the central area; surrounding the epithelioid cells, there is a halo of mononuclear cells and frequently of eosinophils. Fungal antigens tend to be trapped inside epithelioid granulomata. In addition to central suppuration, *P. brasiliensis* granulomata may show central necrosis of the coagulation type.

This type of inflammation represents an immune-specific tissue response of the host in an attempt to destroy, block, and circumscribe the parasite and to prevent its multiplication. Accordingly the two clinicohistopathological forms of the disease are classified as

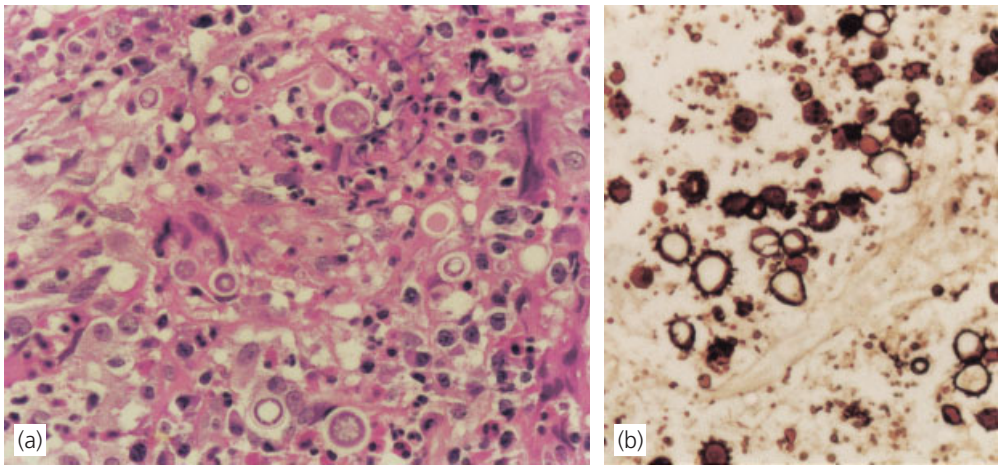

Figure 7 **(a)** *Epithelioid granulomatous inflammation in lymph node with numerous fungal cells in hematoxylin and eosin.* **(b)** *The pathognomic multiple-budding yeast cells are Gomori methenamine silver stain strongly positive.*

the hyperergic and anergic poles. The anergic pole, as seen in cases associated with AIDS, is characterized by disseminated infection, with a mixed suppurative and looser granulomatous inflammation, showing extensive areas of necrosis and large numbers of fungal cells. On the other hand, the hyperergic pole is defined by a more benign, localized infection with persistent cellular immune responses and histopathology showing compact epithelioid granuloma with few fungi (Figures 7 and 8).

Skin and mucous membrane lesions usually exhibit pseudoepitheliomatous hyperplasia and intraepithelial microabscesses. Particularly in ulcerated skin lesions or ruptured lymph nodes, the granulomatous inflammation is associated with a mixed pyogenic component with the formation of intraepidermic microabscesses.

Due to the chronic nature of the infection and the dynamics of the host–parasite relationship, the histopathology of the disease may show areas of extremely active disease characterized by pyogenic reaction and loose granulomata, rich in budding fungal cells, intermingled with areas with compact granulomas, rare fungal cells, and variable degrees of fibrosis. This mixed aspect can be observed either in lymph nodes, skin, or pulmonary lesions, suggesting that the disease evolves through localized new bouts of fungal multiplication and tissue invasion, whereas the adjacent older lesions are on their way to fibrotic resolution. Computer tomographic imaging of the lung confirmed this aspect by depicting areas with alveolar condensation alongside fibrotic and emphysematous zones.

As the morphology of the parasitic forms of *P. brasiliensis* is pathognomonic, the diagnosis of paracoccidioidomycosis is frequently first established by the histopathology examination of a biopsy and further confirmed either by culture or serology. If the parasite is

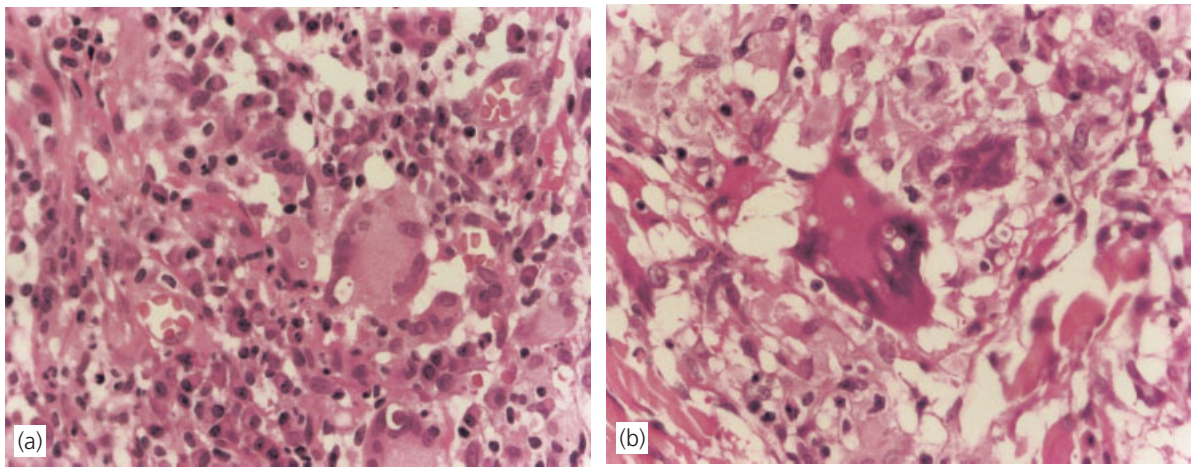

Figure 8 *The two polar histopathological forms of paracoccidioidomycosis:* **(a)** *compact epithelioid granuloma with few fungal cells and* **(b)** *loose, exudative granulomatous inflammation.*

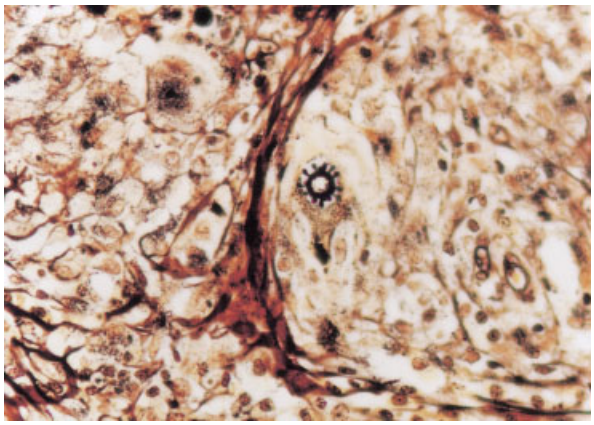

Figure 9 *The pathognomic pilot-wheel yeast form of* P. brasiliensis *in Grocott methenamine silver stain.*

abundant, it may be identified by hematoxylin and eosin stain and confirmed by a special fungal stain such as Grocott silver methenamine (Figure 9). When the disease is chronic, most of the fungal cells are found inside the macrophages, but free yeast cells predominate in disseminated cases. Pauciparasitic forms of the *P. brasiliensis* granuloma may occur, which shows a sarcoidic pattern with rare yeast forms. In these cases, the diagnosis may rely on epidemiologic data plus molecular diagnostic tools such as PCR on paraffin-embedded tissue. On the other hand, some cases may show predominance of diminute parasitic yeast forms which may be taken by those of *H. capsulatum*. Diagnosis then should rely on culture or on the use of immunohistochemistry with specific polyclonal or monoclonal anti-*P. brasiliensis* antibody.

Residual lesions of the pulmonary primary complex of the disease have rarely been described (Londero 1986; Angulo-Ortega and Pollak 1971) and they may present as fibrogranulomatous, fibrocaseous, and calcified lesions in the lungs and/or in the regional lymph nodes.

In old inactive fibrotic lesions, the remaining fungi have the morphology of nonviable cells and require a long time to multiply in microaerophilic conditions, which could explain the long latency period needed for the mycosis to become manifested in the chronic form patients (Restrepo 2000).

Immunohistochemical techniques and monoclonal antibodies to T-lymphocyte subsets have been used to characterize the tegumentary and lymphatic granulomatous lesions from patients with paracoccidioidomycosis. In the granuloma, T cells formed a peripheral mantle around the centrally aggregated macrophages. The majority of the lymphocytes were T-helper cells with few suppressor cells, indicating that those cells were actively involved in the pathogenesis of the lesions and in disease control. The phagocytic histiocyte is lys+ S-100−, whereas the antigen-presenting histiocyte is lys− S-100+. In the granulomata, the histiocyte population was predominantly lys+ with few S-100+ cells, a pattern similar to that of other immunogenic granulomata. It is possible that the interplay between antigen-presenting cells and lymphocytes in *P. brasiliensis* granuloma would result in the release of T-cell stimulating factors, such as IL-2. Lymphokines released by activated lymphocytes would attract, fix, and activate macrophages in the inflammatory foci. The activated macrophages would then show enhanced killing of *P. brasiliensis*, secrete cytokines, and further differentiate into epithelioid cells. Altogether, the effector cells in the *P. brasiliensis* granuloma are: natural killer cells, lymphocytes, T-cytotoxic cells, activated neutrophils and eosinophils, activated macrophages, epithelioid cells, giant cells, and B-lymphocytes (Moscardi-Bacchi et al. 1989).

In accordance with these data, studies on protective immunization in paracocciciodomycosis have indicated a positive correlation between elevated cellular immune responses and restriction of infection and in the numbers of fungal cells in granulomata (Meira et al. 1996).

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Penicilliosis

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ETIOLOGIC AGENT

Penicillium marneffei is the only species known to be dimorphic among the over 200 described species of the genus *Penicillium*. This fungus is currently only known to be endemic in southeast Asia. It can cause infections of the reticuloendothelial system in humans and lower animals.

Infections have been reported in apparently immunocompetent subjects but, more often, in immunocompromised patients undergoing corticosteroid treatment or affected by such underlying diseases as tuberculosis, Hodgkin's lymphoma and the acquired immunodeficiency syndrome (AIDS). The interest of medical mycologists in this mycosis has increased since it appeared in association with human immunodeficiency virus (HIV) infections in patients traveling or living in southeast Asia. As a result of the recent enormous increase of HIV infection in that region, *P. marneffei* has become one of the principal new emerging fungal pathogens. The disease that it causes is also known as penicilliosis marneffei.

HISTORY

P. marneffei was first described by Capponi et al. in 1956 as an etiological agent infecting the reticuloendothelial system of Chinese bamboo rats (*Rhizomys sinensis*). It was isolated by Capponi from the hepatic lesions of one of the Chinese bamboo rats maintained in captivity for experimental infections at the Pasteur Institute of Indochina at Dalat (now Vietnam). The organism was identified as a new species by Segretain (1959a), who named the fungus *Penicillium marneffei* in honor of Hubert Marneffe, then Director of the Pasteur Institute of Indochina. In the same year, Segretain (1959b) also described the first human infection that was caused by the accidental puncture of his finger by a needle used to inoculate hamsters. Nine days later, a small nodule appeared at the site, followed by lymphangitis and axillary adenopathy.

The first spontaneous infection in a human was reported in 1973 in an American minister with Hodgkin's disease, who had traveled in southeast Asia one year before (Di Salvo et al. 1973). *P. marneffei* was incidentally found in an isolated splenic abscess when the patient underwent splenectomy for management of a lymphoma. The fungus was isolated from the spleen and observed in histological sections.

A second imported case in the USA was described in a 59-year-old man who had traveled extensively in the Far East. The disease, a focal pulmonary infection, was recognized following pneumonectomy one month before death (Pautler et al. 1984).

In the same period penicilliosis marneffei was reported in southeast Asia in subjects living in Thailand (Jayanetra et al. 1984), Hong Kong (So et al. 1985; Yuen et al. 1986), and the Guangxi region of southern China (Deng and Connor 1985; Wei et al. 1985). Most of the cases in the Guangxi region were observed between 1964 and 1983 and had been originally misdiagnosed as histoplasmosis capsulati (Deng and Connor 1985).

During the period 1988–89, disseminated *P. marneffei* infections began to be observed in AIDS patients who had traveled to or were living in southeast Asia (Ancelle et al. 1988; Peto et al. 1988; Piehl et al. 1988; Coen et al. 1989; Sathapatayavongs et al. 1989; Stern et al. 1989). The recent rapid rise in the number of reported cases of penicilliosis marneffei coincides with the explosive epidemic of AIDS in northern Thailand where this mycosis is now estimated to be the third most common HIV-related opportunistic infection after tuberculosis and cryptococcosis.

With the aim of identifying the natural habitat of *P. marneffei* and its mode of transmission, several studies on wild bamboo rats have been carried out in different regions of southeast Asia where most patients with autochthonous infection have been observed (Deng et al. 1986, 1988; Wei et al. 1987; Li et al. 1989; Ajello et al. 1995; Chariyalertsak et al. 1996b).

Figure 1 *The hoary bamboo rat* Rhizomys pruinosus **(a)** *(reproduced, with permission, from Nowak 1991) and the large bamboo rat (*Rhizomys sumatrensis*)* **(b)** *(Chariyalertsak et al. 1996b).*

EPIDEMIOLOGY

Ecology

The natural habitat of *P. marneffei* is probably soil. Since the first discovery of *P. marneffei* as an agent of disease of captive wild Chinese bamboo rats (*Rhizomys sinensis*) in Vietnam (Capponi et al. 1956), these rodents have been erroneously regarded by some investigators as a potential source of human infection.

Epidemiologic investigations were therefore focused on wild rodents in the Asian areas where the disease was suspected to be endemic (Deng et al. 1986, 1988; Wei et al. 1987; Li et al. 1989; Ajello et al. 1995; Chariyalertsak et al. 1996b). The animals were trapped and portions of their internal organs cultured. Table 1 lists the bamboo rat species trapped in different Asian countries and found positive for *P. marneffei*. The most studied animals are hoary bamboo rats (*Rhizomys pruinosus*), which have been found to be apparently healthy carriers of the fungus (Figure 1). Among the internal organs, the lungs had the highest positivity (89–83 percent), followed by the liver (83–33 percent), spleen (78 percent), mesenteric lymph nodes (39 percent) and pancreas (33 percent) (Deng et al. 1986; Ajello et al. 1995).

The isolation of the fungus from four species of bamboo rats in China, Thailand, and Vietnam confirmed the value of these wild rodents as epidemiologic markers. However, there is no evidence that the disease is transmitted to humans by the bamboo rat because

Table 1 *Bamboo rat species trapped in different southeast Asian areas and found positive for* Penicillium marneffei

Bamboo rat species	Positive/examined No. (%)	Country	References
Rhizomys sinensis (Chinese bamboo rat)	1/1	Vietnam	Capponi et al. 1956
	2/2	Southeast China	Deng et al. 1988
Rhizomys pruinosus (hoary bamboo rat)	114/179 (64)	Southeast China	Wei et al. 1987
	37/41 (90)	Southeast China	Deng et al. 1988
	15/16 (94)	Southeast China	Li et al. 1989
	6/8 (75)	Thailand	Ajello et al. 1995
Cannomys badius (bay bamboo rat)	6/31 (19)	Thailand	Ajello et al. 1995
	3/61 (5)[a]	Thailand	Chariyalertsak et al. 1996b
Rhizomys sumatrensis (large bamboo rat)	13/14 (93)	Thailand	Chariyalertsak et al. 1996b

a) *P. marneffei* were isolated from three of 10 reddish-brown *C. badius*. All 51 grayish-black *C. badius* were negative for *P. marneffei*.

these animals live far away from humans in remote mountain areas where few people reside (Deng et al. 1986). In addition, few people are in contact with, or consume, them. Both humans and bamboo rats are probably infected from a common environmental source. *P. marneffei* was isolated from soil collected from the burrows of hoary bamboo rats in China by Deng et al. in 1988 and in Thailand by Chariyalertsak et al. (1996b) from the burrow of *R. sumatrensis*. The modified flotation method (Vanittanakom et al. 1995) combined with mouse inoculation was applied in the isolation in Thailand.

By the analysis of 550 patients with disseminated *P. marneffei* infection diagnosed at Chiang Mai University Hospital in northern Thailand between 1991 and 1994, a consistent significant seasonal variation in the incidence of penicilliosis marneffei was observed. The infection was significantly more frequent in the rainy season (May to October) than in the dry season (Chariyalertsak et al. 1996a).

A case-control study performed in the same region did not suggest bamboo rats as a reservoir for infection in humans, nor could a significant association be demonstrated between *P. marneffei* infection and exposure to bamboo, a plant commonly growing in rural areas in northern Thailand and often eaten by Thais (Chariyalertsak et al. 1997). This study identified an age range of 16–30 years and occupational or other exposure to soil, especially during the rainy season, as factors independently associated with an increased risk for *P. marneffei* infection.

Infection in humans

The route of acquisition of *P. marneffei* has not yet been definitively established, but a respiratory portal of entry would be consistent with infections caused by other dimorphic fungal pathogens that produce conidia in their saprophytic habitats in nature. The attachment of *P. marneffei* conidia to the bronchoalveolar epithelium is facilitated by the recognition of extracellular matrix proteins, laminin and fibronectin, by a protein present on the surface of the conidia (Hamilton et al. 1999).

Studies of bamboo rats and human infections with *P. marneffei* have indicated that this organism is endemic in Thailand, the Guangxi region of China, Hong Kong, Vietnam, and Indonesia (Ajello et al. 1995). It is also suspected to be endemic in neighboring countries such as Cambodia, Laos, Malaysia, Myanmar (Burma), and, as recently reported, the Manipur State of India (Singh et al. 1999) and Taiwan (Hsueh et al. 2000). A specific skin test would be extremely useful to establish the precise geographical distribution of *P. marneffei*.

Penicilliosis marneffei has been associated with living in rural areas and with predisposing factors in the human hosts, such as cell-mediated immunodeficiency caused by lymphoproliferative disorders, corticosteroid treatment, and, most importantly, HIV infection (Drouhet 1993).

This fungal disease was a rare event in southeast Asia until the end of the 1980s (Table 2) when it began to be reported in some patients affected with AIDS who were living or had been traveling in the Asian endemic regions (Ancelle et al. 1988; Peto et al. 1988; Piehl et al. 1988; Coen et al. 1989; Sathapatayavongs et al. 1989; Stern et al. 1989). But it was from 1991, with the spread of the AIDS pandemic across the endemic areas, that *P. marneffei* emerged as a significant pathogen among HIV-infected Asian natives, especially in Thailand where the mycosis is accounting for 15–20 percent of all AIDS-related illness (Supparatpinyo et al. 1998). The disease was designated an indicator of AIDS by the Thai Ministry of Public Health (Patamasucon 1992). The possibility of spread of the fungus to the nearby nonendemic countries, as suggested in a report from Taiwan (Hsueh et al. 2000), is worrying. Penicilliosis marneffei will remain a significant public health problem in those endemic Asian countries where the incidence of AIDS is foreseen to continue its dramatic rise. At times, this fungal infection has been reported in Europe and in other nonendemic countries (Table 3). Some of the patients were native of, or had been living in, southeast Asia, but most of them had just been visiting the endemic areas for a short period of time, suggesting that exposure to the fungal propagules is probably high and the infection may be easily acquired. A non-Asian case in a Congolese HIV-positive physician suspected to have acquired penicilliosis during a training course at the Pasteur Institute in Paris (France), although not working directly with the fungus, was considered suggestive of the highly infective nature of *P. marneffei* airborne conidia (Hilmarsdottir et al. 1994). A second case in a patient native of Ghana and living in Germany, who had never been to Asia and for whom the possible source of contamination has not been found, arouses the suspicion that other natural habitats outside Asia might exist (Lo et al. 2000). Attempts to identify different genotypes of strains isolated from human and natural sources have been undertaken (Vanittanakom et al. 1996; Hsueh et al. 2000; Imwidthaya et al. 2000; Trewatcharegon et al. 2001). Further studies, however, are needed to analyze the existence of multiple genotypes of *P. marneffei* and their geographical variations.

From the history of heterochthonous cases, it appears that the period between infection in the endemic areas and manifestation of clinically active disease is extremely variable, ranging from a few weeks to 11 years, which suggests reactivation in the presence of immunosuppression (Peto et al. 1988; Jones and See 1992).

Penicilliosis marneffei occurs mainly in adults; however, cases have also been reported in children with or without AIDS, living in endemic areas (Jayanetra et al. 1984; Deng and Connor 1985; Yuen et al. 1986;

Table 2 Penicillium marneffei *infections from southeast Asia as of Nov 2002*

Country where diagnosed	No. of cases Non-AIDS	References	No. of cases AIDS	References
Cambodia			2	Bailloud et al. 2002
China	8	Deng and Connor 1985	1	Liao et al. 2002
	1	Wei et al. 1985		
	1	Li et al. 1985		
	4	Deng et al. 1988		
	3	Li et al. 1991		
	5	Deng and Ma, 1995		
Hong Kong	1	So et al. 1985	2	Tsang et al. 1991
	1	Yuen et al. 1986	2	Tsui et al. 1992
	1	Tsang et al. 1988	1	Ko 1994
	1	Chan et al. 1989	1	Chang et al. 1998
	2	Chan and Woo 1990	19	Wong and Lee 1998
	1	Lo et al. 1995		
	1	Wong et al. 2001c		
India			4	Singh et al. 1999
			46	Ranjana et al. 2002
Malaysia	1	Saadiah et al. 1999	1	Rokiah et al. 1995
Taiwan	1	Wang et al. 1989	1	Liu et al. 1994
	8	Hsueh et al. 2000	1	Chang et al. 1995
			1	Chiang et al. 1998
			1	Hung et al. 1998
			16	Hsueh et al. 2000
Thailand	5	Jayanetra et al. 1984	5443	HIV/AIDS report, Thailand, 2002; Sathapatayavongs et al. 1989; Chiewchanvit et al. 1991; Thongcharoen et al. 1992; Supparatpinyo et al. 1992,1994; Sirisanthana and Sirisanthana 1993,1995; Louthrenoo et al. 1994; Srison et al. 1995; Vanittanakom and Sirisanthana 1997; Kantipong et al. 1998; Ukarapol et al. 1998; Chokephaibulkit et al. 2001
	5	Supparatpinyo et al. 1992		
	1	Supparatpinyo et al. 1994		

Table 3 Penicillium marneffei *infections diagnosed in nonendemic areas, reported as of Nov 2002*

Country where diagnosed	No. of cases	References	Country where diagnosed	No. of cases	References
Australia	1	Jones and See 1992	Germany	1	Sobottka et al. 1996
	1	Heath et al. 1995		1	Rimek et al. 1999
Canada	1[a]	Sekhon et al. 1994		1	Lo et al. 2000
	1[a]	Manion et al. 1991	Italy	1	Coen et al. 1989
Japan	1	Mohri et al. 2000	Sweden	1	Julander and Petrini 1997
			Switzerland	1	Kronauer et al. 1993
Belgium	2	Depraetere et al. 1998		2	Borradori et al. 1994
France	1	Ancelle et al. 1988		2	Garbino et al. 2001
	1	Stern et al. 1989	The	1	Hulshof et al. 1990
	2	Hilmarsdottir et al. 1993	Netherlands	2	Kok et al. 1994
	1	de Truchis et al. 1991	UK	1	Peto et al. 1988
	1	Hilmarsdottir et al. 1994		1	McShane et al. 1998
	2	Grise et al. 1997		1	Vilar et al. 2000
	1	Lachaud et al. 1998		1	Bateman et al. 2002
	1	Miegeville et al. 1998	USA	1[a]	Di Salvo et al. 1973
	1	Valeyrie et al. 1999		1[a]	Pautler et al. 1984
	1	Rosenthal et al. 2000		1[a]	Piehl et al. 1988
				1	Nord et al. 1998

a) Non-AIDS patients.

Deng et al. 1988; Sirisanthana and Sirisanthana 1993; Kwan et al. 1997). *P. marneffei* infection appears to be an important problem in children who acquired HIV infection perinatally (Sirisanthana and Sirisanthana 1995). The clinical presentation of this mycosis in children is reported to be similar to the infection in adults.

MYCOLOGY

Morphology and physiology

P. marneffei grows in culture at 25°C on Sabouraud's dextrose agar as a mycelial fungus typical of the genus, producing rapidly growing greenish–yellow sporulating colonies, with a pink or red center and dark green edges. A characteristic brick-red pigment is released into the medium. On potato dextrose agar or Sabouraud's dextrose agar the pigment appears after 24–72 hours of incubation at 25–30°C (Figure 2). Little or no pigment is produced at 35–37°C. At this higher temperature colonies are glabrous, off-white, and yeast-like.

Microscopically, the mycelial form of growth is characterized by sinuous smooth-walled conidiophores (diameter 1.5–2 μm, length 70–100 up to 175 μm), borne on aerial hyphae, which have terminal, typically divaricate, verticils, either symmetrical or asymmetrical and seldom ramified (Figure 3). Metulae (7–11 × 2.5–3.5 μm) originate from the apex of the conidiophore in groups of four to five. Sometimes one of the metulae is much longer than the others and is usually septate. The phialides, arranged in verticils of four to six, are the same size as the metulae, and have a flask-like shape gradually tapering to a slender collar. Conidia are ellipsoidal to globose (2–3 μm), smooth walled, often with prominent disjunctors, and arranged in short disordered chains. The terminal conidia of the conidial chains are sometimes larger than the ones beneath them, which is a characteristic of *P. marneffei* (Corda's phenomenon) (Segretain 1959b).

P. marneffei requires an organic source of nitrogen for mycelial growth in a well-defined, synthetic, chemical medium. Casein hydrolysate, peptone, and asparagine are utilized, whereas $NaNO_3$ and $(NH_4)_2PO_4$ are not. Glucose (optimal growth), lactose, xylose, maltose, levulose, and mantel are used as carbon sources. Sucrose is weakly used (Segretain 1959a). The fungus is sensitive to cycloheximide. *P. marneffei* has proved difficult to maintain in culture, and pigment production may be reduced by prolonged storage. More extensive study of the biochemical properties of *P. marneffei* and their possible use in strain biotyping has been reported (Wong et al.

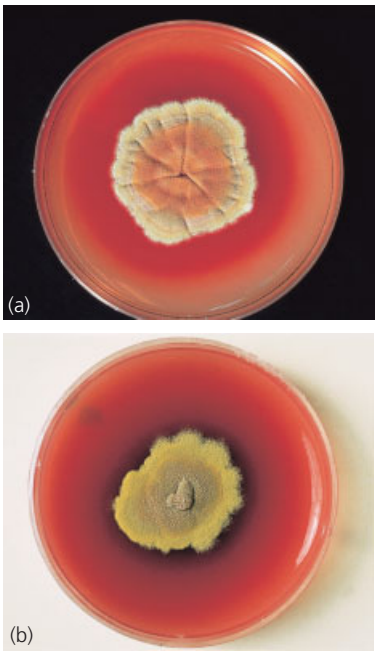

Figure 2 *Colony of* Penicillium marneffei *on* **(a)** *Sabouraud's dextrose agar and* **(b)** *potato dextrose agar after 10 days of incubation at 28°C*

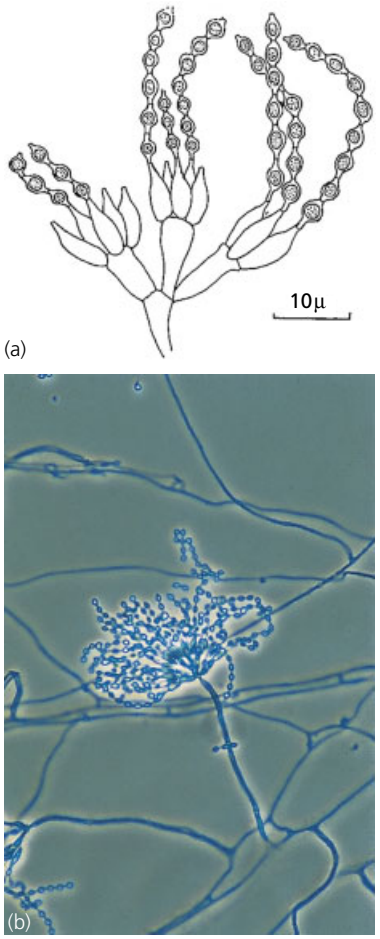

Figure 3 *Conidiophore of* Penicillium marneffei *from Segretain's drawing of the type strain* **(a)** *and microscopy of the mold form of this fungus (lactophenol cotton blue mount)* **(b)**. *Magnification, × 600*

2001a). In addition, some enzymatic activities were examined in *P. marneffei* human isolates (Youngchim et al. 1999). Both mycelial and yeast form expressed several interesting enzymes, for examples, acid phosphatase, esterases, and lipases, which might be implicated with virulence in this fungus.

The fungus is characterized by its thermal dimorphism. When grown at 37°C on 5 percent sheep blood agar, wort agar, or in liquid synthetic media containing maltose or glucose and amino acids, *P. marneffei* undergoes a transformation to a yeast-like single-cell form, also named arthroconidium.

Hyphae first become shorter, develop more septa and branches, and cease to produce conidia. After 2 weeks there is a gradual shift to spherical or ellipsoidal yeast-like cells which are 2–6 μm in diameter and divide by transverse septation (fission) in a somewhat similar fashion to *Schizosaccharomyces* spp.

The in vitro yeast-like form resembles that found in rodents and humans. Globose, ovoid, and elongated yeast-like cells measuring 2–3 × 2–6.5 μm are seen free and within macrophages. Extracellular, elongated, and sausage-shaped cells up to 8–13 μm long, and more rarely short hyphae, no longer than 20 μm, are also observed. The multiplying cells usually have a single central septum and occasionally two.

The cells of *P. marneffei* are poorly stained by hematoxylin and eosin (H&E). Their cell wall, however, is well stained by periodic acid–Schiff (PAS) and Gomori's methenamine silver (GMS) stains. Initially, *P. marneffei* yeast forms were mistaken for *Histoplasma capsulatum* var. *capsulatum* yeast cells (Deng and Connor 1985). The principal differences between the tissue forms of these two dimorphic fungi are presented in Figures 4 and 29.5.

The ultrastructural characteristics of *P. marneffei* in golden hamsters were described by Drouhet and colleagues who showed the evolution of the fungal cell in liver macrophages (Drouhet et al. 1988). The various stages of the multiplication process (formation of the septum and division by fission) were recognized (Figure 6). The yeast-like cells were often included in phagolysosomes, and some were surrounded by concentric calcified layers as in an onion bulb. This aspect of the host defense reaction has also been observed in animal models and human infections caused by *Histoplasma capsulatum* var. *capsulatum*.

Identification

In the first mycological description of *P. marneffei* (Segretain 1959a), the new species was classified in the genus *Penicillium*, section Asymmetrica, subsection Divaricata, according to Raper and Thom's classification (Raper and Thom 1949). Subsequently, however, *P. marneffei* was assigned to the subgenus Biverticillium of

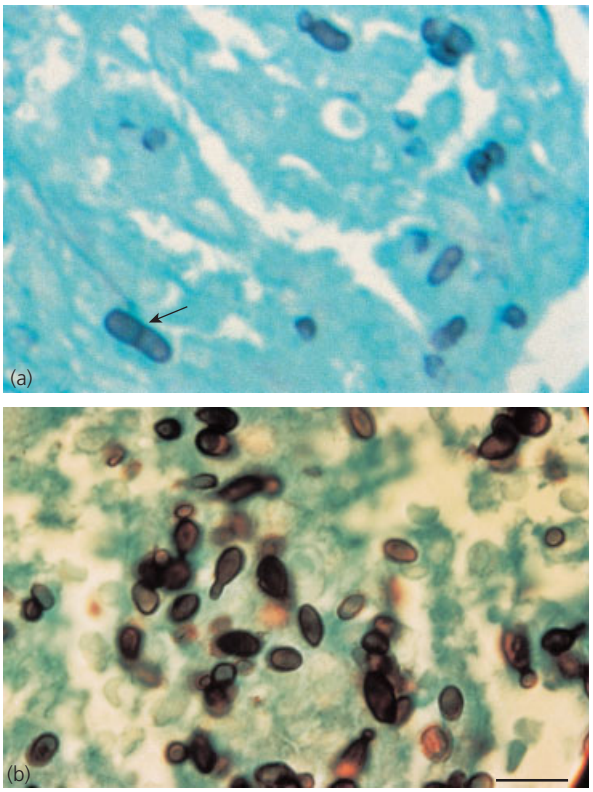

Figure 4 (a) *Oval, round, and sausage-shaped cells of* Penicillium marneffei, *some with septum (arrows), in a tissue section.* **(b)** *Uniform round and ovoid budding cells of* Histoplasma capsulatum *var.* capsulatum *in a tissue section. GMS stain; bar, 10 μm.*

Pitt's taxonomy because of its frequently biverticillate conidiophores and on the basis of its poor growth on 25 percent glycerol nitrate agar (Pitt 1979). Phylogenetic analysis also confirmed that *P. marneffei* is closely related to the *Penicillium* species in the subgenus *Biverticillium*, and the sexual *Talaromyces* species with an asexual biverticillate penicillium state (LoBuglio and Taylor 1995).

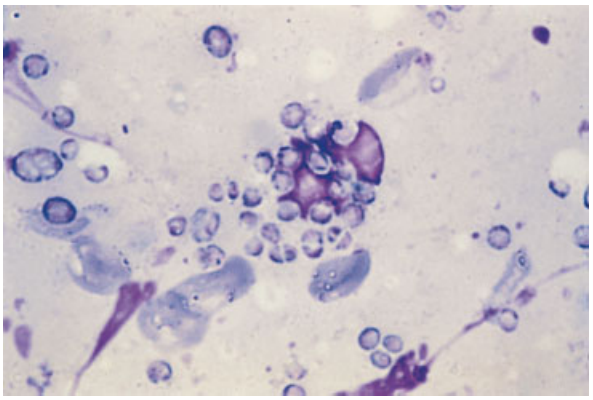

Figure 5 *Wright stain of blood smear showing intracellular* Penicillium marneffei *yeasts with septa, magnification × 1 500*

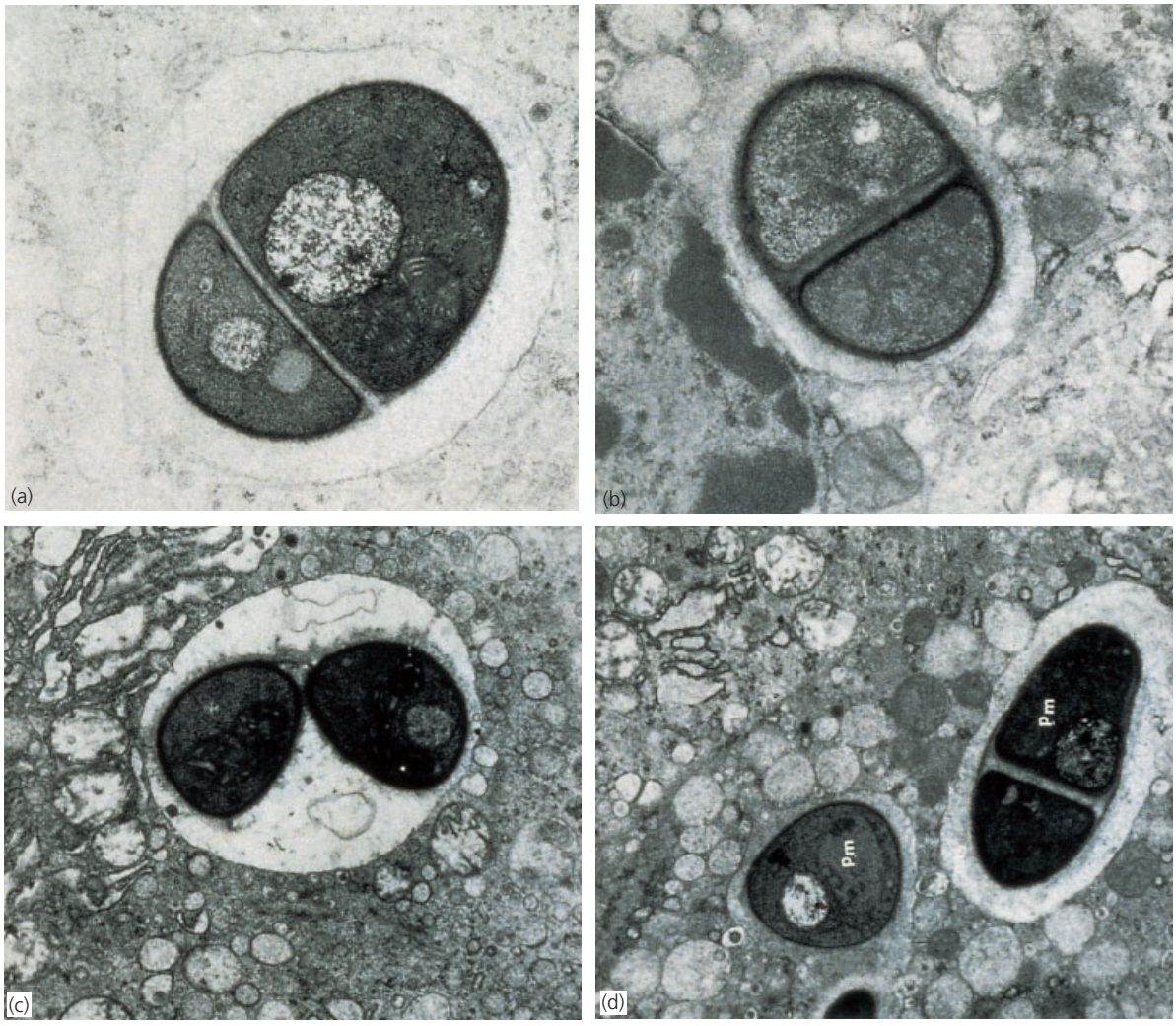

Figure 6 *Ultrastructure of yeast-like cells of* Penicillium marneffei *at various stages of division by fission within vacuoles of macrophages:* **(a, b)** *magnification ×126 500;* **(c, d)** *magnification ×73 600. (Reproduced, with permission, from Drouhet 1993)*

The above-described macro- and micromorphology of *P. marneffei* is relatively characteristic, and identification of the fungus is not considered difficult. A reddish pigment is, however, produced by other *Penicillium* species from which *P. marneffei* must be differentiated (Table 4).

Another approach to the differentiation of *P. marneffei* from other penicillia consists of the use of an exoantigen test (Sekhon et al. 1989). The exoantigen extract from a slant culture of *P. marneffei* is tested by a reverse microimmunodiffusion procedure against rabbit anti-*P. marneffei* reference serum in the presence of appropriate reference antigens. The production of two precipitin lines of the same identity as the *P. marneffei* reference system confirms identification.

A significant criterion in the identification of this fungus is based on the observation that *P. marneffei* is the only species of the *Penicillium* genus which, on enriched media at 37°C, converts into a schizogenous yeast form.

HOST RESPONSES

Clinical presentation

The clinical presentation of penicilliosis marneffei is similar in patients with and without HIV infection. Predominant signs and symptoms are chills, persistent fever, lymphadenopathy, hepatosplenomegaly, leukocytosis, anemia, persistent cough, weakness, and weight loss. Skin lesions and subcutaneous abscesses have often been reported. This clinical presentation is relatively nonspecific. It may be suggestive of tuberculosis or other systemic mycoses. Patients with AIDS present similar manifestations caused by HIV as well as other opportunistic infections or lymphoma.

P. marneffei usually causes an invasive disseminated disease, characterized in AIDS patients by a more rapid onset and more severe symptoms. Focal infections have been described rarely. The spleen, lung, or nasopharynx

Table 4 *Main characteristics of* Penicillium *spp. producing reddish diffusible pigments*

***Penicillium* spp.**	***Penicillium* subgenus**	**Penicilli**	**Conidia**	**Pigment**
P. citrinum	*Furcatum* sect. *Furcatum*	Bi-(mono-ter) verticillate divaricate	Smooth or very finely roughened	Yellow–brown to reddish brown
P. janthinellum	*Furcatum* sect. *Divaricatum*	Bi-(mono-ter) verticillate strongly divaricate	Smooth to finely roughened	Orange–reddish to vinaceous-purple
P. marneffei	*Biverticillatum*	Bi-(ter) verticillate divaricate	Smooth	Brick-red
P. purpurogenum	*Biverticillatum*	Biverticillate symmetrical appressed	Smooth, finely roughened or verrucose	Red to purple–red
P. rubrum	*Biverticillatum*	Biverticillate symmetrical appressed	Smooth	Red to purple–red

was reported as the only involved organ in HIV-negative patients (Di Salvo et al. 1973; Pautler et al. 1984; Deng et al. 1988; Chan et al. 1989). Among the AIDS population, one patient was reported to have a localized *P. marneffei* infection of the lung, which was diagnosed in the very early stage by a normal chest x-ray (Romaña et al. 1989).

Data on the extent of organ involvement are incomplete because several cases have been poorly described. An analytical overview of the clinical aspects of the first 88 cases, half of them in AIDS patients, has been published (Drouhet 1993). Pulmonary manifestations, mostly persistent cough and dyspnea, and occasionally chest pain and hemoptysis, associated with bronchopneumonia, pulmonary abscess or pulmonary infiltrates, have been reported in 70 percent of HIV-negative and 86 percent of HIV-positive patients. In some cases, however, respiratory involvement has been found in the presence of a normal chest x-ray. In addition to lymphadenopathy in 45–56 percent of patients and hepatosplenomegaly in 43–50 percent, lesions have frequently been observed in other body sites such as the meninges, kidney, pericardium, and digestive tract – expressions of hematogenous spread of the infection.

Cutaneous and subcutaneous involvement has been reported in 68–71 percent of patients with or without AIDS (Supparatpinyo et al. 1994; Drouhet and Dupont 1995). Papules, rashes, acne-like pustules, and nodules are usually multiple, and located on the face, trunk, and extremities. Some of the papules have central necrotic umbilications resembling molluscum contagiosum (Figure 7). A more rapid appearance of multiple cutaneous lesions is observed in AIDS patients.

Bone and joint infections have also occurred in patients with disseminated disease, affected or not with AIDS (Jayanetra et al. 1984; Deng and Connor 1985; Chan and Woo 1990; Li et al. 1991; Louthrenoo et al. 1994; Deng and Ma 1995; Pun and Fang 2000). Arthritis affected both the large peripheral joints and the small finger joints, and osteomyelitis affected the flat bones of the skull, ribs, and the long bones. Most of the patients had multiple osteolytic lesions associated with swelling of the soft tissue and pain, particularly of the joints.

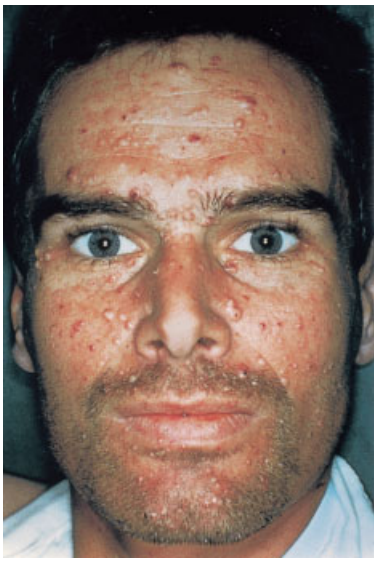

Figure 7 *Skin lesions in HIV-positive patient infected with* Penicillium marneffei. *Some of the papules have a central umbilication resembling lesions of molluscum contagiosum. (Reproduced, with permission, from Kronauer et al. 1993)*

Histopathology

The host response to *P. marneffei* invasion mainly involves the mononuclear phagocytic cells of the lung and the reticuloendothelial system, and resembles the response in acute histoplasmosis capsulati. In the early lesions, *P. marneffei* yeast-like cells are engulfed by histiocytes where they proliferate within the phagocyte's vacuoles. As the lesions progress, necrosis develops in the central areas, and fungal cells are released into the cytoplasm. Infiltration of neutrophils is followed by abscess formation. A granulomatous reaction takes place, especially in the organs of the reticuloendothelial system, with epithelioid cells, lymphocytes, plasma cells, and giant cells surrounding the necrotic area. Slowly evolving granulomas in the lung may lead to fibrosis and cavitation. Calcifications, such as those seen in experimental and human pulmonary histoplasmosis capsulati, have not been observed in human patients with

penicilliosis marneffei, but have been reported in liver macrophages of experimentally infected golden hamsters (Drouhet et al. 1988). The fungal cells were surrounded by concentric calcified layers like onion bulbs, which represented a host defense response.

A totally different histopathological pattern – an anergic and necrotizing reaction involving mainly the lung, liver, and skin – is associated with compromised immunity of the host. The reaction is characterized by necrosis surrounded by histiocytes, which are distended by the engulfed proliferating fungi and are unable to form phagocytic vacuoles. This histopathological feature indicates a progressive disseminated infection.

In anergic as well as in pyogranulomatous lesions, special stains such as PAS or GMS are essential to distinguish penicilliosis marneffei from tuberculosis and other systemic mycoses as the fungi stain poorly in H&E preparations.

Immunology

The inhaled *P. marneffei* conidia are known to recognize fibronectin and to bind laminin of the bronchoalveolar epithelium via a sialic acid-containing receptor, avoiding entrapment by the mucus and removal from the respiratory tract (Hamilton et al. 1999). This adherence represents the first step of the host–parasite interaction. After attachment to the bronchoalveolar epithelium, *P. marneffei* conidia are easily phagocytized by macrophages.

The response of phagocytic cells to *P. marneffei* was studied with a macrophage culture system, using murine J774 macrophages and culture conditions suitable to reproduce the transformation of the fungus from the conidial to the characteristic in vivo yeast-like form. The results showed that following phagocytosis of *P. marneffei* conidia, nonstimulated murine macrophages are damaged by the fungus, which, growing intracellularly, undergoes transformation into a yeast-like form. By contrast, macrophages stimulated with interferon-gamma (IFN-γ) (50 U/ml) and lipopolysaccharide at 1 μg/ml significantly inhibit the intracellular growth of the conidia and kill the fungus. This correlates with the amount of nitric oxide produced by stimulated macrophages, suggesting that the L-arginine-dependent nitric oxide pathway has an important role in the murine host defense against *P. marneffei* (Cogliati et al. 1997). Killing of intracellular *P. marneffei* conidia was also shown to occur in IFN-γ-lipopolysaccharide-activated human THP1 cells (Taramelli et al. 2000). An increase in pH within the phagocytic vacuole, by threatening the *P. marneffei*-infected human THP1 and mouse J774 macrophages with chloroquine, induced macrophage killing of this fungus (Taramelli et al. 2001). The increase in intravacuolar pH may directly reduce fungus growth or may inhibit pH-dependent yeast virulence factors. It has been shown that both mycelial and yeast form of *P. marneffei* expressed acid phosphatase activity (Youngchim et al. 1999). This enzyme has been considered one of the virulence factors for intracellular pathogen (Baca et al. 1993).

Other polymorphonuclear (PMN)-activating cytokines have been proven to enhance PMN inhibitory effects on germination and morphological changes of *P. marneffei*, but only granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and IFN-γ enhance PMN activity from being fungistatic to fungicidal (Kudeken et al. 1999). The killing mechanism of GM-CSF-stimulated neutrophils on *P. marneffei* was not mediated by a superoxide-dependent mechanism, but through exocytosis of granular enzymes which were largely heat-labile (Kudeken et al. 2000).

The ability of unopsonized *P. marneffei* to parasitize mononuclear phagocytes without stimulating the production of tumor necrosis factor (TNF)-alpha, observed in vitro using human leukocytes, may be critical for host defenses as it enhances the virulence of this intracellular parasite (Rongrungruang and Levitz 1999).

Iron availability was reported to affect the immunity to, and the pathogenicity of, *P. marneffei*, as iron overloading significantly reduces the antifungal activity of macrophages, whereas exogenous iron enhances, and iron chelators inhibit, the extracellular growth of *P. marneffei* (Taramelli et al. 2000).

It was shown that the immunocompetent host generates a cell-mediated response against *P. marneffei* (Deng et al. 1988) and that the failure of a CD4+ T-cell-dependent immunity in AIDS patients contributes to the development of disseminated systemic infection (Viviani and Tortorano 1990; Supparatpinyo et al. 1992).

The role of T lymphocytes in host defenses against *P. marneffei* was studied in mice experimentally depleted of CD4+ or CD8+ T cells (Viviani et al. 1993a). The results suggest that CD4+ T cells and, to a lesser extent CD8+ T cells, play a role in the protective host response against the fungus. *P. marneffei* strains differed in their virulence for mice. Depending on this virulence, CD4+ T-cell-mediated protective immunity acts to slow or halt multiplication of the fungus in the viscera. It is therefore likely that AIDS patients are susceptible to systemic *P. marneffei* infection because they are unable to generate a CD4+ T-cell response. Furthermore, CD8+ T cells, which are spared by the virus, may be insufficient to eliminate the organisms in developing granulomas. Further evidence that cell-mediated immunity plays a central role in a host defense mechanism against *P. marneffei* infection was provided in work involving experimentally infected mice (Kudeken et al. 1996).

In the mouse model, CD4+ T cells play an important role in eradicating *P. marneffei* from infected sites (Kudeken et al. 1997). Interleukin-12 (IL-12) protects mice against this infection. The induction of IL-12

synthesis by human peripheral blood mononuclear cells is promoted by a phosphoprotein, osteopontin (OPN), after stimulation with *P. marneffei* (Koguchi et al. 2002). Furthermore, the OPN production by monocytes is regulated by GM-CSF. It is suggested that the possible mechanism is mediated by mannoprotein in the OPN production upon stimulation by *P. marneffei*. The mannoprotein (Mp1p) is known as an abundant cell wall antigen of the yeast cells of this fungus (Cao et al. 1998a). Thus, the OPN may promote a Th1 response through the mannoprotein-induced IL-12 secretion by human macrophages stimulated with *P. marneffei* and contribute to host defense against this pathogen. In addition, IFN-γ renders murine macrophages highly active in killing *P. marneffei* yeast cells by promoting the release of nitric oxide (Kudeken et al. 1998).

LABORATORY DIAGNOSIS

The high mortality rate in the first reported cases of *P. marneffei* infection is attributable to a delay in diagnosis because the mycosis was often misdiagnosed as tuberculosis and treated accordingly. The recently improved survival rate can be ascribed to clinicians' and microbiologists' increased awareness of the disease.

Diagnosis rarely requires invasive procedures even though biopsy of lymph nodes, lung, liver, and bone marrow provides specimens rich in fungal organisms trapped by the reticuloendothelial cells. Biopsy is recommended in the presence of skin nodules or subcutaneous abscesses. Skin lesions should be sought carefully because they may facilitate diagnosis. Biopsy samples must always be processed for both microscopy and culture. Other laboratory tools including polymerase chain reaction (PCR) and methods for detecting specific antibody against and antigen of the fungus in clinical specimens have been developed.

Microscopic examination

A variety of clinical specimens is appropriate to diagnose this condition. The clinical specimens include bone marrow aspirates, blood, lymph node biopsies, skin biopsies, skin scrapings, sputum, bronchoalveolar lavage pellets, pleural fluid, liver biopsies, cerebrospinal fluid, pharyngeal ulcer scrapings, palatal papule scrapings, urine, kidney, pericardium, stomach or intestine, and stools (Vanittanakom and Sirisanthana 1997). Rapid bedside diagnosis can be made by microscopic examination of Wright's stained bone marrow aspirates and/or touch smear of skin biopsy or lymph node biopsy specimens (Supparatpinyo et al. 1994). The yeast cells of *P. marneffei* could be seen in the peripheral blood smear of a patient with fulminant infection (Supparatpinyo and Sirisanthana 1994).

P. marneffei can be seen in histopathological sections stained with H&E, GMS, or PAS. They must be discriminated from *Leishmania donovani*, *Pneumocystis carinii*, and *Histoplasma capsulatum* var. *capsulatum*, which also frequently cause disease in patients with AIDS. *P. marneffei* superficially resembles the *H. capsulatum* var. *capsulatum* in tissue when yeast-like cells are clustered within macrophages or histiocytes, but the two organisms multiply differently. *P. marneffei* replicates by fission, instead of budding, and the considerable variation in size and shape of the extracellular yeasts (enlarged, occasionally septate, tubular or sausage shaped) makes *P. marneffei* easily distinguishable from the budding tissue-form cells of *H. capsulatum* (see Figures 4 and 5).

A specific indirect fluorescent antibody reagent was developed to facilitate the rapid detection and identification of *P. marneffei* in histological sections (Kaufman et al. 1995). This antiglobulin preparation has proved to be specific because it failed to react with the yeast and hyphal forms of *H. capsulatum* and with a variety of other fungi, including *Aspergillus* spp. Specific IgM monoclonal antibodies were also produced from mice immunized with *P. marneffei* mycelial culture filtrate and proven strongly reactive in immunofluorescence staining of the fungus in tissue samples (Trewatcharegon et al. 2000).

Cultures

P. marneffei can be cultured from various clinical specimens. Bone marrow culture is the most sensitive (100 percent), followed by skin biopsy culture (90 percent), and blood culture (76 percent) (Supparatpinyo et al. 1994). A blood culture method, the lysis centrifugation system (Isolator System, Wampole Laboratories, Cranbury, NJ), has proved highly effective for the isolation of *P. marneffei*, especially in AIDS patients who frequently have concomitant bacteremia.

P. marneffei grows easily in routine culture media containing antibacterial agents. The fungus is inhibited by cycloheximide, which has therefore to be excluded from Sabouraud's dextrose agar. Inoculated Petri dishes should be incubated at 25°C and 37°C to demonstrate thermal dimorphism. The fungus grows in a mycelial phase at 25°C after 3–5 days of incubation. The mycelial phase produces red pigment which diffuses into the agar (Figure 2). Aged colonies are deep red. Microscopic examination shows typical structure of the genus *Penicillium* (see Figure 3). At 35–37 C° on Sabouraud's agar or on brain–heart infusion agar, colonies are glabrous, off-white and yeast like, and the diffusible red pigment may be poorly produced (Figure 8, Figure 9). Pigment may also be masked by the color of media such as blood or chocolate agar. Microscopically, yeast cells of *P. marneffei* are

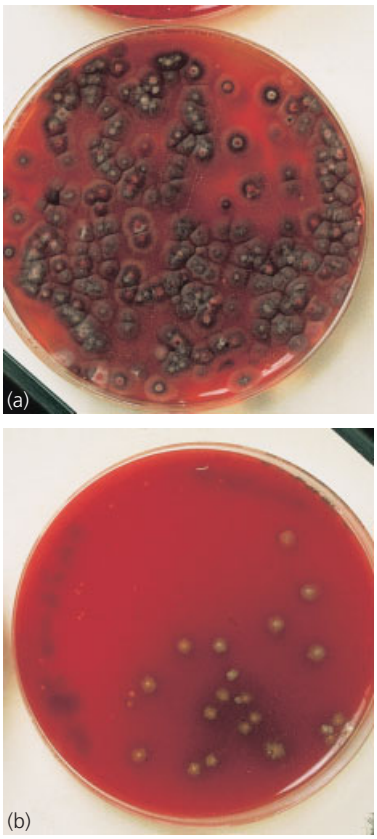

Figure 8 *Colonies of* Penicillium marneffei *grown from blood (Isolator system blood culture) on* **(a)** *Sabouraud's dextrose agar and* **(b)** *blood agar, after 7 days at 37°C.*

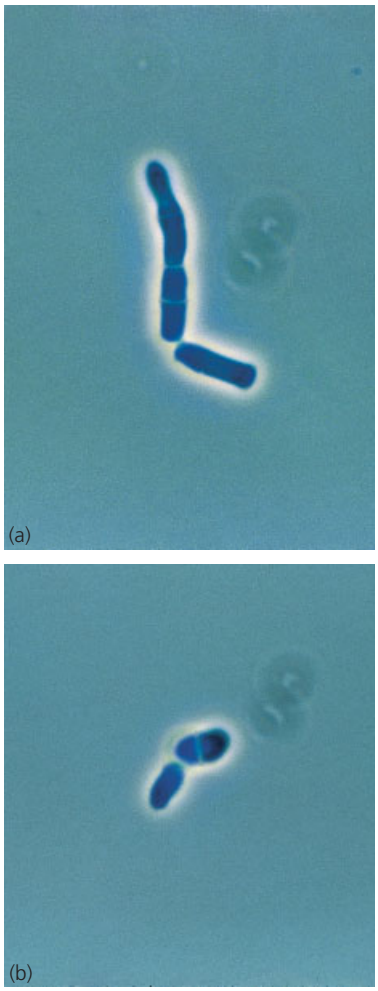

Figure 9 *Yeast cells of* Penicillium marneffei *cultured at 37 C ° showing arthroconidia-like cells; magnification ×1 500 (Chariyalertsak et al. 1996b).*

globose to oval with single septum, or arthroconidia-like cells.

Serodiagnosis

ANTIBODY DETECTION

Several studies were carried out to detect reliable reagents for antibody and antigen detection in body fluids. Although antigen is more likely to be detected than antibodies in patients with impaired immunity, antibodies to *P. marneffei* were shown to be produced by AIDS patients and may be useful for monitoring the efficacy of treatment. A microimmunodiffusion test, using a mycelial culture filtrate antigen of *P. marneffei* (Sekhon et al. 1982), has been used to monitor the serological response of an AIDS patient with penicilliosis marneffei (Viviani et al. 1993b). Sera drawn early in the course of the disease gave positive antibody reactions whereas sera taken 3–5 months after the start of therapy were negative. In another study of the immunodiffusion test, using yeast culture filtrate antigen, only two of 17 patients infected with *P. marneffei* were positive antibody reactions in their serum specimens (Kaufman et al. 1996). An indirect fluorescent antibody test for detecting IgG antibody, using germinating conidia and yeast cells as antigens (Yuen et al. 1994), could detect the IgG titer of 160 or more in patients infected with *P. marneffei*.

Thereafter, several tests to detect antibodies to *P. marneffei* have been developed using crude antigen preparation from yeast phase and mold phase, purified proteins from yeast phase, and recombinant antigen. An immunoblot analysis of sera from 33 *P. marneffei*-infected AIDS patients, 29 non-infected AIDS patients, 25 AIDS patients from nonendemic areas, and 84 healthy individuals revealed the specificity of secreted yeast phase proteins of 54 and 50 kDa in the diagnosis of penicilliosis marneffei (Vanittanakom et al. 1997). Other diagnostic antigens of *P. marneffei* included antigens with relative molecular masses of 61 kDa (Jeavons et al. 1998) and 38 kDa (Chongtrakool et al. 1997), which were prepared from purified cytoplasmic yeast antigen and from acetone-precipitated culture filtrate of mold form, respectively. In addition, a gene (*MP1*) encoding a highly antigenic cell wall mannoprotein (Mp1p) has been cloned and the purified recombinant antigenic mannoprotein produced and tested in an

enzyme-linked immunosorbent assay (ELISA) for antibody detection (Cao et al. 1998a, b). The sensitivity of these antigenic preparations is difficult to compare as different methods have been used and different patient populations studied. Recently, recombinant Mp1p protein vaccine was used for generation of protective immune responses against *P. marneffei* infection using a mouse model, and compared with *MP1* DNA vaccine delivered by live-attenuated *Salmonella typhimurium* (Wong et al. 2002). The intramuscular *MP1* DNA vaccine offered the best protection after challenge with *P. marneffei* yeast cells intravenously.

The scanty antibody response of immunosuppressed patients or the presence of antibodies in patients previously exposed to the fungus may limit the usefulness of the antibody detection. Tests for antigen detection would be of major clinical value, mainly to diagnose promptly the infection in patients without skin lesions, to identify AIDS patients with an initial infection and to predict relapse.

ANTIGEN DETECTION

In order to assess the presence of antigen in body fluids of patients affected with *P. marneffei*, rabbit polyclonal antibodies against arthroconidial filtrate (or yeast phase) were used in an immunodiffusion and a latex agglutination (LA) test and, despite the crude antigen preparation used to raise the antibody reagent, the tests showed a surprisingly high specificity, 100 percent (Kaufman et al. 1996). Encouraging results were also obtained with the antibody reagent against the recombinant mannoprotein Mp1p, that was used to test sera from AIDS and non-AIDS patients with penicilliosis in an ELISA assay with a specificity and sensitivity of 100 and 65 percent, respectively (Cao et al. 1999). The combined use of antigen and antibody tests was proven to increase the diagnostic sensitivity from 76 to 82 percent (Kaufman et al. 1996) and from 65 to 88 percent (Cao et al. 1999). The ELISA system using the recombinant Mp1p was further tested with sera from eight *P. marneffei*-infected HIV-positive patients and seven *P. marneffei*-infected HIV-negative patients (Wong et al. 2001b). Serum antigen titers were found to be higher in HIV-positive patients, whereas serum antibody titers were found to be higher in HIV-negative patients. A urinary antigen detection assay was also studied using a rabbit hyperimmune IgG preparation which, at a cutoff titer of 1:40, had a diagnostic sensitivity of 97 percent and specificity of 98 percent (Desakorn et al. 1999). A dot blot ELISA and a LA test were developed further for detecting *P. marneffei* antigenuria by using the same polyclonal antibody and compared with the ELISA (Desakorn et al. 2002). All tests appeared to be highly specific. The sensitivities of the tests were as follows: dot blot ELISA, 94.6 percent; ELISA, 97.3 percent; and LA test, 100 percent. The LA test will be evaluated for the detection of antigenemia and for the treatment follow-up.

PCR

Oligonucleotide primers for selective amplification of *P. marneffei* DNA were designed from the nuclear ribosomal DNA (rDNA) internal transcribed spacer region (LoBuglio and Taylor 1995). Using the nested PCR, the test was 100 percent successful in amplifying *P. marneffei* DNA. Thereafter, nucleotide sequence from the 18S rDNA of this fungus was determined (Vanittanakom et al. 1998). An oligonucleotide probe was designed and proved to be specific for *P. marneffei* in the PCR-hybridization reaction, regardless of whether the fungus was isolated from humans or natural habitats. This PCR-hybridization technique could be used to detect *P. marneffei* DNA in EDTA-blood samples collected from AIDS patients with penicilliosis. Recently, new PCR primers were designed based on the 18S rDNA sequence, and were used in a single and nested PCR methods for the rapid identification of *P. marneffei* (Vanittanakom et al. 2002). Very young culture of *P. marneffei* (2-day-old filamentous colony, 2 mm in diameter) could be performed by this assay. The application of this method for early diagnosis of the disease needs to be studied further. Another method which was developed to provide rapid identification of dimorphic fungi including *P. marneffei* was a PCR-enzyme immunoassay (PCR-EIA) method (Lindsley et al. 2001). The DNA was amplified by using universal fungal primers which was directed to the conserved regions of rDNA. The PCR amplicons were then detected colorimetrically in an enzyme immunoassay format for specific fungal species. The probes developed in this test were found to be highly specific and should prove to be useful in differentiating these organisms in the clinical setting.

THERAPY

P. marneffei infection is fatal in the absence of treatment, as documented by a fatality rate of 91.3 percent in non-AIDS patients and 100 percent in AIDS patients (Drouhet 1993; Supparatpinyo et al. 1993). In addition, a high rate (50 percent) of relapse is reported after successful primary therapy (Supparatpinyo et al. 1998).

In vitro studies have shown that most *P. marneffei* strains are very sensitive to the antifungals used in systemic treatment, mostly to amphotericin B, ketoconazole, itraconazole, voriconazole, and terbinafine (Sekhon et al. 1992; Drouhet 1993; Supparatpinyo et al. 1993; Boon-Long et al. 1996; McGinnis et al. 1997, 2000).

The antifungal drugs mostly used in the treatment of *P. marneffei* infection are amphotericin B, itraconazole, and ketoconazole. At present, the treatment regimen

recommended in Thailand consists of a 2-week course of intravenous amphotericin B, followed by itraconazole (400 mg/day) for 10 weeks, given on an outpatient basis (Sirisanthana et al. 1998). The short course of amphotericin B was designed to lower the incidence of adverse drug effects and to reduce the length of hospital stay. As *P. marneffei* is highly susceptible to itraconazole, patients with mild to moderately severe infection can be given itraconazole 400 mg/day as primary therapy (Supparatpinyo et al. 1998). Secondary prophylaxis with itraconazole 200 mg/day is well tolerated and should be the standard care for patients with AIDS successfully treated for *P. marneffei* infection (Supparatpinyo et al. 1998). Itraconazole (200 mg/day) has been proved to be safe and effective as primary prophylaxis to prevent *P. marneffei* infection in patients with advanced HIV infection, especially those with CD4+ lymphocyte counts of <100 cells/μl (Chariyalertsak et al. 2002).

FUNGAL GENETICS

P. marneffei is a dimorphic fungus which displays a temperature-dependent dimorphism. In host cells or at 37°C, the fungal hyphae undergo phase transition via the arthroconidiation to form fission yeast cells. The genetic factors that express during phase transition may be involved in the molecular mechanisms of fungal cell morphogenesis and pathogenesis. A homolog of the *Aspergillus nidulans abaA* gene has been cloned from *P. marneffei* and shown that it is involved in both asexual conidiation at 25°C and dimorphic hyphal-yeast switching (Borneman et al. 2000).

Another gene, *cflA*, which is a homolog of the *Saccharomyces cerevisiae cdc42*, has been cloned from *P. marneffei* (Boyce et al. 2001). *cflA* is required for polarized growth of the vegetative mycelium at 25°C and for correct cellular morphology and separation of yeast cells at 37°C. However, it does not appear to be involved in development or in dimorphic switching. Furthermore, a *gasA* gene encoding a Gα subunit has been cloned from *P. marneffei*, and investigated as a key regulator of asexual development (Zuber et al. 2002). By contrast, *gasA* mutants have no apparent defect in dimorphic switching or yeast-like growth. Borneman et al. (2002) has cloned and characterized an APSES protein-encoding gene from *P. marneffei* that has a high degree of similarity to *Aspergillus nidulans stuA*. This gene is required for metula and phialide formation of *P. marneffei* but is not required for dimorphic growth.

A search for phase-specific genes in *P. marneffei* has been investigated by using differential display technique (Cooper and Haycocks 2000). Many of the genes whose expression displays during mold-to-arthroconidium transition are related to energy metabolism. Expression of the gene which encodes malate synthase is upregulated in the arthroconidial or yeast phase as compared to the mold phase (Cooper and Haycocks 2000). The authors plan to assess the role of those genes in the virulence of *P. marneffei* in murine model by creating specific gene-disruption mutants.

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Candidiasis

ESTHER SEGAL AND DANIEL ELAD

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The genus *Candida* comprises about 200 species, of which close to 20 have been associated with pathology in humans or animals (Meyer et al. 1998; de Hoog et al. 2000), including species, such as *Candida famata* or *Candida inconspicua*, which have been added more recently to the list of potential pathogens. This chapter focuses only on the major pathogenic species:

- *Candida albicans*
- *Candida dubliniensis*
- *Candida glabrata*
- *Candida guilliermondii* and its teleomorph *Pichia guilliermondii*
- *Candida kefyr* and its teleomorph *Kluyveromyces marxianus*
- *Candida krusei* and its teleomorph *Issatchenkia orientalis*
- *Candida lusitaniae* and its teleomorph *Clavispora lusitaniae*
- *Candida parapsilosis*
- *Candida tropicalis*

HISTORIC NOTES

Based on detailed historic reviews of the genus *Candida* (Odds 1988; Rippon 1988; Kwon-Chung and Bennett 1992), the following significant data regarding the genus and the diseases caused by it may be outlined.

The first known description of *Candida* infection, oral candidiasis (thrush) in two patients with other underlying disease, may be found in Hippocrates' 'Epidemics' from the fourth century BC. The first descriptions of thrush in modern medicine were made by Rosen von Rosenstein in 1771 and by Underwood in 1784, who identified the infection as a pediatric problem and accordingly described it in books dealing with such entities. Veron in 1935 suggested that the gastrointestinal tract of newborns might become infected during birth. Although Lagenbeck, in 1839, described the fungus in a case of oral thrush observed in a patient suffering from typhus, he misidentified it as the causative agent of the underlying disease. The correct association between oral thrush and the fungus was made only 3 years later, in 1842, by Gruby who classified the microorganism as *Sporotrichum*.

During the following decades various pathological conditions were shown to be associated with yeasts. The fungus was isolated by Bennett in 1844 in the sputum of a tuberculotic patient, by Wilkinson in 1849 from vaginal candidiasis, by Robin in 1853 from a systemic infection and by Zenker in 1861 from a brain infection in a debilitated patient, in whom the fungus spread hematogenously from an oral infection. In 1875, Hausemann established the possibility of infant infection during birth by demonstrating the analogy between the causative agent of oral and vaginal thrush. Additional pathological entities caused by *Candida* were described at the beginning of the twentieth century: onychomycosis by Dubendorfer in 1904, dermatitis by Jacobi in 1907, chronic mucocutaneous candidiasis by Forbes in 1923, and cystitis by Rafin in 1910. Later, in 1928, Conner described osteomyelitis, in 1940 Joachim and Polayes

described endocarditis, and in 1943 Suthin noted the association between pathology of the endocrine system and *Candida* infections. Castellani, in 1912, while describing 'tea tasters' cough,' was probably the first to suggest the possibility that *Candida* species other than *C. albicans* may be involved in pathological processes.

The nomenclature of the yeast isolated from the patients, changed often. Robin in 1853 named it *Oidium albicans*, Quinquad in 1868 *Syringospora robinii*, and Reess in 1875 *Saccharomyces albicans*. Mycological studies by Grawitz, published in 1877, described the various morphological forms of *Candida*. The first binomial to gain wide acceptance over a long period, and which is sometimes still used, albeit wrongly, was *Monilia albicans*, which was suggested by Zopf in 1890. The criteria defined by Saccardo for the genus *Monilia* permitted the inclusion of certain fungi isolated from rotting vegetation into the genus. Berkhout, in 1923, after recognizing the differences between *Monilia* spp. isolated from rotting plants and fruit and those isolated from medical cases, established the genus *Candida* to accommodate the latter. This was accepted as the official name of the genus by the Eighth Botanical Congress in Paris in 1954.

Two major medical events have revived the interest in fungal diseases in general and *Candida* infections in particular. The first was the introduction of antibacterial drugs in the second half of the twentieth century. These drugs, especially those having a broad spectrum of activity, may act as predisposing factors for mycotic infections by causing an imbalance of the host's natural microflora in favor of fungi, upon which they have no inhibitory activity. The second event was the increase in the prevalence of immunosuppressed patients during the last few decades, as a result of chemotherapy or disease [acquired immunodeficiency syndrome (AIDS)], which led to a parallel increase in the incidence of *Candida* infections in general, and the less pathogenic non-*C. albicans* species in particular.

These events, in conjunction with the development of more sophisticated biological research techniques, led to a highly intensive study of the pathogenic mechanisms of *Candida* infection, such as adhesion, production of proteinases, and interaction with the defenses of the host and, consequently, to new concepts for diagnosis and management. Another result of the intense interest in *Candida* was the increased demand for more efficient drugs with fewer side effects. Recent reports indicating the possibility of innate or acquired resistance of a number of *Candida* spp. to several antimycotic drugs (see section on Treatment below) has emphasized the importance of in vitro antimycotic susceptibility testing and has prompted efforts toward the standardization of these procedures and their interpretation.

The extensive use of antimycotic drugs, particularly azoles, for prolonged therapeutic courses has led to changes in the relative prevalence of various *Candida* species, with a decrease in the proportion of *C. albicans* as the etiologic agent of candididiasis and an increase in the proportion of non-*albicans* species such as *C. glabrata* or *C. krusei* (Krcmery and Barnes 2002).

An additional recent aspect is the development of molecular technologies in the use of taxonomy, resulting in changes of the status of certain *Candida* spp. and the recognition of new species such as *C. dubliniensis* by Sullivan and colleagues in 1995 (Sullivan et al. 1995; Gutierrez et al. 2002). This species was discovered in the course of an epidemiologic survey of *Candida* in human immunodeficiency virus (HIV)-positive patients. It was identified initially as an 'atypical *Candida albicans*' strain that did not hybridize with a *C. albicans* specific probe (Sullivan et al. 1993). Subsequently, it was recognized as a separate taxon (Sullivan et al. 1995).

TAXONOMIC COMMENTS

Most yeast species dealt with in this chapter often went through name changes, usually following the discovery of synonymy among species, discovery of teleomorphic stages or nonvalidity of binomials. For a comprehensive treatise on the taxonomic aspects of yeasts the reader is referred to Kurtzman and Fell (1998) and de Hoog et al. (2000).

The more recent changes in nomenclature are described below, some of them based on molecular biological methods which have been applied in search of a better understanding of taxonomic relationships between yeast species. The analysis of small ribosomal subunit sequences has shown (Barns et al. 1991; Hendriks et al. 1991) that *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *Candida viswanatii* form one subgroup with a more distant connection to *C. guilliermondii*. *C. kefyr* and *C. glabrata* are located on two different but interconnected branches, while *C. lusitaniae* and *C. krusei* are on a different branch each. These findings are in agreement with those of the coenzyme Q analysis (Yamada and Kondo 1972) which assesses the number of isoprene units per ubiquinone molecule, according to which *C. albicans* and *C. tropicalis* and *C. guilliermondii* share the same system (Q9), *C. kefyr* and *C. glabrata* share the Q6 system, whereas *C. lusitaniae* and *C. krusei* have Q8 and Q7 systems, respectively.

Candida species, exhibiting a teleomorphic stage, are considered ascomycetous fungi (Kurtzman 1993). Meyer et al. (1998) divided the *Candida* species on the basis of physiological characteristics into 12 groups. The pathogenic *Candida* species dealt with in this chapter are included in group VI. They do not assimilate inositol, erythritol, and nitrate and grow at 40°C.

Candida albicans

The most significant change in the taxonomy of this species was the acceptance of the present binomium in

place of *Monilia albicans* (see section on Historic notes below). The synonymy between *C. albicans*, *C. langeronii*, and *C. clausenii* has been confirmed by molecular biological methods (Mahrous et al. 1992; Wickes et al. 1992) as has been that of *Candida stellatoidea* (Kwon-Chung and Bennett 1992).

Two serotypes, A and B, based on differences between the mannan component of the cell wall have been described (Hasenclever and Mitchell 1961). Serotype A was found to be antigenically related to *C. tropicalis*, whereas serotype B was related to *C. stellatoidea*, currently integrated into the *C. albicans* species. Additional attempts of subspeciation of *C. albicans* have been carried out, including various biotyping and chemotyping methods as well as susceptibility to 'killer factors' (Polonelli et al. 1985). Recent efforts are focused on typing of *C. albicans* isolates by restriction enzyme profiles, karyotyping, and other molecular biological techniques (see section on Genetics below).

The use of molecular technologies yielded new insights on the possible sexual mating of *C. albicans*, identifying a mating type-like locus (Hull and Johnson 1999; Hull et al. 2000; Magee and Magee 2000).

Candida dubliniensis

Phylogenetic trees have shown that *C. dubliniensis* is a discrete taxon within the *Candida* genus (Gutierrez et al. 2002). *C. dubliniensis* differs in its DNA fingerprint pattern from *C. albicans* (Sullivan et al. 1993, 1995). In addition, pulse field gel electrophoresis showed that *C. dubliniensis* has 10 or more chromosomes whereas *C. albicans* has seven to eight. *C. dubliniensis* differs by 2.3 percent from *C. albicans* in the V3 variable region of the large ribosomal RNA genes.

Candida glabrata

The taxonomic affiliation of this species is the most controversial among those described in this chapter as this yeast was classified in the genus *Torulopsis* (Kwon-Chung and Bennett 1992). The principal point of argument was whether the ability or inability to produce pseudohyphae/hyphae is a cogent reason to differentiate between two genera (*Candida* and *Torulopsis*) or not. This controversy was complicated by additional arguments on whether the name *Torulopsis* was validly published before *Candida* and the importance of keeping the two genera divided to avoid confusion among the mycologists (Odds 1988). It is currently generally accepted that this yeast should be assigned to the genus *Candida*.

Candida guilliermondii

Two varieties of *C. guilliermondii* have been described: *C. guilliermondii* var. *guilliermondii* and *C. guilliermondii* var. *membranaefaciens*. Both variants have teleomorphs: *Pichia guilliermondii* and *Pichia ohmeri*, respectively (Kurtzman 1998a).

Candida kefyr

C. kefyr is the anamorph of *Kluyveromyces marxianus*. *C. kefyr* was formerly designated as *Candida pseudotropicalis*, considerd to be the anamorph of *Kluyveromyces fragilis*. The two teleomorphs and their anamorphs were found to be conspecific (Kwon-Chung and Bennett 1992) and since *K. marxianus* and *C. kefyr* have precedence on *K. fragilis* and *C. pseudotropicalis*, respectively, they have been accepted.

Candida krusei

C. krusei is the anamorph of *Issatchenkia orientalis*. *C. krusei* differs from other pathogenic yeast species in a number of characteristics, such as its ability to grow on vitamin-free media (Odds 1988), the formation of flat, spreading colonies on Sabouraud's agar, cell wall mannan composition (Kogan et al. 1988), coenzyme Q numbers (Yamada and Kondo 1972) and other properties recently reviewed (Samaranayake and Samaranayake 1994).

Candida lusitaniae

C. lusitaniae is the anamorph of the ascomycetous yeast *Clavispora lusitaniae*. Genetic variability among clinical isolates was described (Merz et al. 1992). The involvement of *C. lusitaniae* as an opportunistic pathogen in humans was initially reported by Holzschu et al. (1979).

Candida parapsilosis

Nakase et al. (1979) showed that *C. parapsilosis* isolates could be divided into form I (arabinose positive) and form II (arabinose negative). The latter was shown subsequently to consist of anascosporogenous strains of *Loderomyces elongisporus* (Hamajima et al. 1987).

Candida tropicalis

The synonymy between *C. tropicalis* and *C. paratropicalis* has been confirmed by molecular biological methods (Wickes et al. 1992).

MYCOLOGICAL CHARACTERISTICS

Morphology

With a few exceptions (described below), macroscopic and microscopic cultural characteristics of the yeasts dealt with in this chapter are not specific enough to be a

basis for differentiation. Colonies cultured on glucose peptone agar (GPA), incubated at 25°C for 3 days, have a diameter of 2–3 mm, are white to cream colored, smooth or umbonate, and may become wrinkled after further incubation, as well as dull to glistening. Intraspecific variability of these characteristics is significant enough to preclude their use as criteria for species identification. In spite of the basic morphological similarity between various *Candida* spp., a number of characteristics, which are species specific, are evident on certain media, such as corn meal agar (CMA).

CANDIDA ALBICANS

On CMA, after 3 days of incubation, *C. albicans* will produce true- and pseudomycelium, grape-like groups of blastospores clustered at the septa and, typically for this species, at the end of the hyphae or short lateral branches, chlamydospores.

When incubated for 2 hours in 10 percent serum at 37°C, typical cell elongations – germ tubes – are formed. They differ from other, similar outgrowths produced by several other species by the absence of a constriction at the tube's base.

An interesting phenomenon is the 'phenotypic switching' described initially by Slutsky et al. (1985). It was demonstrated that strains of *C. albicans* possess the ability to undergo phenotypic switching expressed in colony morphology variations, such as white–opaque (Slutsky et al. 1987), traits associated with virulence (see section on Virulence factors below). This phenomenon was extensively investigated, including various genetic aspects (Soll 2002).

CANDIDA DUBLINIENSIS

C. dubliniensis is morphologically very similar to *C. albicans*. The main similarities are germ tube and chlamydospore production, while the main differences are the color produced when grown on ChromAgar medium and it does not grow well, or not at all, at 45°C (Gutierrez et al. 2002). Additional, subtler differences may be noted (Al-Mosaid et al. 2001).

CANDIDA GLABRATA

A characteristic of this species is the absence of hyphae or pseudohyphae on CMA.

CANDIDA GUILLIERMONDII (ANAMORPH) AND *PICHIA GUILLIERMONDII* (TELEOMORPH)

On CMA, formation of pseudohyphae may vary according to the strain from abundant to sparse. Blastospores may develop in small chains or in clusters. True hyphae are not produced.

P. guilliermondii is heterothallic. On 5 percent malt extract agar, asci containing one to four hat-shaped ascospores, which are liberated shortly after their generation, are formed. The majority of *C. guilliermondii* natural isolates, however, do not produce the teleomorph (Kurtzman 1998a).

CANDIDA KEFYR (ANAMORPH) AND *KLUYVEROMYCES MARXIANUS* (TELEOMORPH)

Colonies of this species, cultured on GPA and incubated for 3 days at 25°C, are relatively small: 1–2 mm. On CMA, in addition to pseudohyphae, blastospores may be observed. Occasionally the blastospores may be elongated and reach a length of up to 16 μm, a characteristic they share with *C. krusei*.

Kluyveromyces ascii contain one to four reniform ascospores, which tend to agglutinate after their release (Lachance 1998).

CANDIDA KRUSEI (ANAMORPH) AND *ISSATCHENKIA ORIENTALIS* (TELEOMORPH)

Colonies of *C. krusei* cultured on GPA become flat-topped after 5–7 days of incubation. Most blastospores are elongated and may reach 25 μm in length after a week. In Sabouraud's broth, *C. krusei* grows on top of the medium as a thick pellicle that may be observed after 2–3 days' incubation at 25°C (Figure 1).

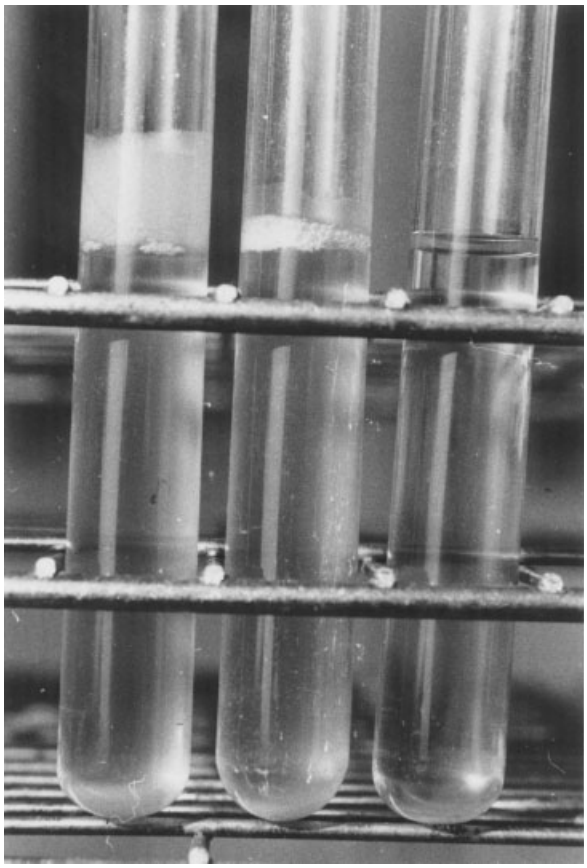

Figure 1 C. krusei, C. tropicalis, *and* C. albicans, *in Sabouraud's dextrose broth. Note the large pellicle of* C. krusei *(left) and small pellicle of* C. tropicalis *(center).*

Issatchenkia orientalis The taxonomic connection between *I. orientalis* and *C. krusei* has been confirmed only by comparison of genetic material (Wickes et al. 1992). Asci of *I. orientalis* contain one, occasionally two, smooth, spherical ascospores (Kurtzman 1998b).

CANDIDA LUSITANIAE (ANAMORPH) AND *CLAVISPORA LUSITANIAE* (TELEOMORPH)

On CMA, blastospores in verticillate chains are born on branching, often curved, pseudohyphae.

Clavispora lusitaniae is heterothallic. One to four ascospores, sometimes containing an oil droplet, are contained in each ascus. The ascospores are clavate (hence the name *Clavispora*), a characteristic unique to the genus. The spores might be slightly echinulate, a characteristic sometimes seen only by electron microscope (EM) examination. The type strain of *C. obtusa* (CBS 1944), which unlike *C. lusitaniae* does not assimilate lactate or ferment galactose, produced *Cl. lusitaniae* teleomorphs when mated with α mating types of *C. lusitaniae* (Rodriguez de Miranda 1979).

CANDIDA PARAPSILOSIS

On CMA, after 3 days incubation, long, regularly branching pseudohyphae with a 'pine forest' aspect are formed. Production of pseudomycelium may be, however, slight in some strains.

CANDIDA TROPICALIS

C. tropicalis grows on the surface of Sabouraud's dextrose broth as a thin pellicle which becomes evident after 2 days of incubation at 25°C (Figure 1).

Physiology

Candida spp. metabolize glucose via the hexose monophosphate pathway under aerobic conditions (assimilation), or via the Embden–Meyerhof pathway in anaerobiosis (fermentation). Additional metabolic mechanisms such as mitochondrial oxidative phosphorylation and the Krebs cycle and protein synthesis by 80S ribosomes (composed by a 60S and a 38S subunit) do not differ from those described in other eukaryotic cells (Braude 1986).

The enzymatic apparatus of *Candida* spp. is complex and has been extensively researched and reviewed (Odds 1988). The practical importance of *Candida* enzymes is significant since a number of them, such as proteases and those involved in sterol synthesis, may be directly involved in pathogenesis, or serve as target for antimycotic drugs, respectively.

Among catabolites secreted by some *Candida* spp. are acids, fatty acids, and alcohols, including ethanol.

Growth temperature seems to have an important influence upon morphogenesis of several *Candida* spp. While temperatures around 25°C promote primarily the formation of chlamydospores in *C. albicans*, higher temperatures, such as those present in potential hosts (around 37°C but sometimes reaching 43°C), will promote the formation of pseudohyphae (Braude 1986). Not all *Candida* spp. are able to grow at 37°C and higher temperatures, and consequently such capabilities are considered important pathogenic factors that separate potentially pathogenic strains from environmental saprophytes.

Genetics

A significant amount of data regarding the genomes, including genome size, number of chromosomes, and allocation of genes to specific chromosomes, of medically important *Candida* spp. has been accumulated during the last decade as recently summarized by Magee and Chibana (2002). Since *C. albicans* was studied more intensively than other species, this section focuses primarily on this microorganism. A significant recent development in *Candida* genetics is the *Candida albicans* Genome Project, targeted at the recognition of the fungus' genes (www-sequence.stanford.edu/group/candida).

The medically important *Candida* species have been traditionally assigned to the Deuteromycetes as only an anamorph state was known. Over the years, however, an ascomycetous teleomorph state was discovered for a number of *Candida* species (see section on Taxonomic comments below). *C. albicans* was considered as a species with no recognized sexual state. However, recent studies indicated that mating might occur in this species as well (Hull and Johnson 1999; Hull et al. 2000; Magee and Magee 2000). The *Candida* spp. dealt with in this chapter are considered diploid, except for *C. glabrata* (Doi et al. 1992; Kitada et al. 1995). Classical genetic studies, particularly those of *C. albicans*, were hampered due to its diploid nature and the lack of sexual reproduction. Nevertheless, molecular-genetic techniques enabled investigators to carry out studies revealing significant information regarding the genetics of *C. albicans*, and, to some extent, of other *Candida* spp. as well.

The principal findings related to *C. albicans* regarding the number of chromosomes, isolation of various mutations including site-directed mutagenesis, identification and sequencing of various genes, as well as allocation of part of them to specific chromosomes, DNA amplification, genetic cloning and gene expression are as follows.

The DNA content of *C. albicans* (nuclear and mitochondrial) was estimated to be in the range of 40 pg/cell (Riggsby et al. 1982). As cytological methods for demonstration of chromosomes are difficult, most karyotyping studies are based on electrophoretic methods (Magee and Magee 1987).

An important characteristic of *C. albicans* and other *Candida* spp. is the electrophoretic karyotype variability, which led to inconsistent results regarding the number of chromosomes (Magee and Magee 1987; Magee et al.

1988; Lasker et al. 1989; Wickes et al. 1991; Altboum 1994). The current notion is that *C. albicans* has eight pairs of chromosomes (Chibana et al. 2000). A physical mapping of chromosome 7 has been reported by Chibana et al. (1998).

Various types of mutants of *C. albicans* have been isolated spontaneously or following treatment with chemical or physical mutagens (Kirsch et al. 1990; Altboum 1994). These include auxotrophic strains, strains resistant to various substances, or temperature-sensitive and respiratory-deficient mutants expressed as 'petite' phenotype (Figure 2). The most frequently induced mutations were in genes involved in the biosynthesis of amino acids and nucleic acids expressed phenotypically by auxotrophic strains. Various techniques, including the widely applied method of complementation of homologous mutations in *Saccharomyces cerevisiae* (Malathi et al. 1994; Rosenbluh et al. 1985) have been used to isolate *C. albicans* genes from genomic libraries. This led to the isolation of a significant number of genes, some of which have been sequenced and/or located on chromosomes (Magee et al. 1988; Lasker et al. 1989; Rustchenko-Bulgac et al. 1990). Isolation of defined mutants and the corresponding genes led to development of transformation methods that resulted in gene expression. An important development in the area of mutagenesis was the introduction by Fonzi and Irwin (1993) of the targeted mutagenesis method, allowing the isolation and identification of specific mutants and the corresponding genes.

Genetic-molecular studies of other species besides *C. albicans* focused primarily on karyotyping (Asakura et al. 1991; Lott et al. 1993; Doi et al. 1994) or use of molecular techniques for epidemiological typing (Carruba et al. 1991; Doebbeling et al. 1991, 1993; Branchini et al. 1994) or phylogeny (Hendriks et al. 1991; Kurtzman 1993). Isolation of mutants (Gleeson et al. 1990) and transformation in *C. tropicalis* (Sanglard and Fiechter 1992), as well as isolation and sequencing of the *SAP* gene (Togni et al. 1991; Ganesan et al. 1991) in this species have been reported. In addition, cloning of genes encoding for acid proteases in *C. parapsilosis* (de Viragh et al. 1993) and genetic manipulation in *C. glabrata* (Zhou et al. 1994) have also been reported. Furthermore, efforts have been focused on use of molecular methods, primarily polymerase chain reaction (PCR), for diagnostic purposes attempting identification and detection of various species (Lehmann et al. 1992; Burgener Kairuz et al. 1994; Maiwald et al. 1994).

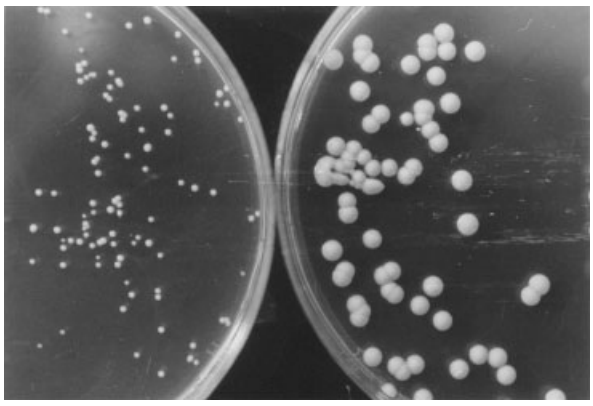

Figure 2 *'Petite' mutants of* C. albicans*: left, mutant; right, parent strain*

ECOLOGY AND EPIDEMIOLOGY

Candida spp. are currently considered the fourth most common cause of hospital acquired systemic infections in North America, accounting for 10 percent of all bloodstream infections (Richardson and Warnock 1997; Sullivan and Coleman 2002), and which may lead to mortality rates of up to 35 percent (Wenzel 1995). Although most *Candida* spp. have been isolated from animal and other environmental sources (Mok et al. 1984; Mok and de Carvalho 1985; de Hoog et al. 2000), human infections are usually endogenous. *Candida* spp. can be isolated from various sites on or in the human body of 30–50 percent of asymptomatic individuals (Richardson and Warnock 1997), primarily the gastrointestinal (GI) tract, the oropharynx, the vagina, and the skin (Mok and da Silva 1984; de Hoog et al. 2000). Variations regarding the presence of *Candida* spp. in healthy individuals may be a function of various factors such as climate, age, or diet of the surveyed population. As summarized by Odds (1988), the range of *Candida* isolations from the oral cavity is 1.9–41.4 percent, the GI tract 0–55 percent and the vagina 2.2–68 percent. The same source (Odds 1988) indicates that mean values of data from a number of epidemiologic surveys reveal that *C. tropicalis* and *C. glabrata* are the second most frequent species isolated, from the oropharynx in the case of the former, and from the vagina and the GI tract the latter.

C. albicans is generally considered the major pathogen among the *Candida* species. Although an increase in the prevalence of non-*albicans* species has been noted during the last decade (Hazen 1995; Krcmery and Barnes 2002; Nguyen et al. 1996) it is still usually the most frequently isolated species (Edmond et al. 1999; Pfaller and Diekema 2002), albeit the differences have become less substantial. Recent surveys indicate (Krcmery and Barnes 2002; Pfaller 2003) that besides *C. albicans*, the four most common *Candida* spp. isolated from candidemia patients include *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. About a dozen more rarely isolated additional non-*albicans* *Candida* spp., such as *C. guillermondii*, *C. kefyr* or *C. lusitaniae*, have been reported as causes of candidiasis (Krcmery and Barnes 2002; Pfaller and Diekema 2002). Particu-

larly noteworthy is the identification of a novel *Candida* species – *C. dubliniensis* – as a pathogen (see section on *Candida dubliniensis* below).

Analogy between two isolates based primarily on phenotypic morphological and physiological characteristics is often insufficient. Therefore, efforts have been made during recent years to apply molecular biological techniques (Pfaller 1992b; Sullivan and Coleman 2002), such as pulsed-field gel electrophoresis (Pfaller et al. 1998), randomly amplified polymorphic DNA analysis (Lehmann et al. 1992; Lin and Lehmann 1995; Holmberg and Feroze 1996), and restriction fragment length polymorphism (Joly et al. 1996, 1999; Lockhart et al. 1997), to epidemiological studies of various *Candida* spp. This approach is exemplified in a recent study of Singh et al. (2002), who by using contour-clamped homogenous electrical field analysis in a survey on vaginitis caused by *C. krusei*, identified two genotypes (Singh et al. 2002).

It is generally accepted that the incidence of yeast infections has increased during the last decades (Pfaller and Wenzel 1992). Several reasons have been mentioned as predisposing to this increase, such as immunosuppression (pathological and iatrogenic) and increase in the use of broad-spectrum antibacterials, to mention but two. However, other factors, such as higher awareness and, more importantly, improved and easily accessible diagnostic tools, may have contributed significantly to the observed epidemiologic changes.

In addition to the overall increase of *Candida* infections, epidemiologic shifts in the relative prevalence of the different *Candida* spp. and emergence of new pathogens has occurred (Hazen 1995; Pfaller and Diekema 2002). Among the possible factors contributing to these shifts, an important role has been attributed to antifungal drugs. It is believed that the introduction of various antimycotic agents into extensive use has resulted in the selection of specific species that are inherently less susceptible to the specific drug (most known examples are *C. glabrata* and *C. krusei* exhibiting resistance to fluconazole). In addition, more recently, other factors, such as demographic parameters or even specific genetic susceptibility, have been suggested as contributing to the change in the relative proportions of the different *Candida* spp. involved in human pathology (Pfaller 2003).

In view of the scope of this chapter, the following sections deal only briefly with epidemiologic aspects of the major pathogenic *Candida* spp., and the reader interested in more detailed information is referred to specific literature.

Candida albicans

C. albicans is isolated more rarely than other *Candida* spp. from environmental sources. This might be a result of this species' relatively high adaptation to a parasitic way of life and consequent loss of some of the capacities possessed by other species. Two serotypes have been recognized: serotype A is more prevalent than serotype B among clinical isolates (Auger et al. 1979). Studies with *C. albicans* isolated from immunosuppressed patients (Brawner et al. 1992) indicated a higher incidence of serotype B. These finding may, however, differ according to the geographical area. Interestingly, a correlation between susceptibility to flucytosine and serotype has been noted: while serotype A isolates are susceptible, serotype B isolates often exhibit resistance (Auger et al. 1979).

Outbreaks of *Candida* infections traced back to hospital personnel or contaminated materials, in risk populations such as neonates (Shian et al. 1993; el-Mohandes et al. 1994) or coronary bypass patients (Isenberg and Tucci 1989) have been reported.

Candida dubliniensis

The initial isolation of *C. dubliniensis* was from the oral cavity (Sullivan et al. 1995) and subsequently from other sites including vagina (Coleman et al. 1997), urine (Polacheck et al. 2000), and blood (Meis et al. 1999). Although the original isolates were from HIV-positive patients, later this yeast was cultured from non-HIV carriers receiving chemotherapy, following bone marrow transplantation (Meis et al. 1999), or broad-spectrum antibacterial treatment (Polacheck et al. 2000). It was also reported recently by Jabra-Rizk et al. (2001) that *C. dubliniensis* was isolated from healthy children.

It is interesting that *C. dubliniensis* may develop resistance to fluconazole and this may possibly have contributed to the emergence of this species in HIV carriers/AIDS patients (Sullivan et al. 1999).

Candida guilliermondii

C. guilliermondii has been isolated from various environmental sources such as swimming pools (Aho and Hirn 1981), soil, sand, and seawater (Boiron et al. 1983), as well as amphibians (Mok and de Carvalho 1985), wild and domestic birds (Mangiarotti et al. 1993), and mammals, including the human cutaneous surface. In addition, *C. guilliermondii* has been isolated from the hands of hospital personnel (Strausbaugh et al. 1994). In another report of nosocomial infections caused by this yeast in a neonatal intensive care unit, the source of infection was the heparin solution used to flush 'butterfly needles' used for blood sampling (Yagupsky et al. 1991).

Candida glabrata

This species is one of the most prevalent among those isolated from the oral cavity (Stenderup 1990) or vagina of healthy individuals (Odds 1988). The prevalence of

this species among those causing human disease has increased significantly (Borg von-Zepelin et al. 1999; Pfaller and Diekema 2002), becoming an important cause of candidemia. In addition, it is a major pathogen involved in candidal vaginitis (Sobel 1998) or denture stomatitis (Cumming et al. 1990). It is generally believed that increased prevalence of this species as a pathogen is due to selective pressure resulting from its resistance to fluconazole (Richardson and Warnock 1997). In addition, it has been recently suggested that other factors, such as age or geographical area, may also play a role (Pfaller 2003).

Candida kefyr

This species has been involved only rarely in human pathology. Several cases of disseminated yeast infections caused by *C. kefyr* have been described and this species was isolated sporadically from vaginal, urinary, ear, and GI infections (Davis 1986).

Candida krusei

C. krusei has been isolated from various environmental sources (Do Carmo Sousa 1969) but only rarely from the mucosal surfaces of healthy carriers (Odds 1988). The involvement of *C. krusei* in pathological processes has increased in the last few years and may continue to do so, considering its resistance to fluconazole, widely used as an antimycotic prophylactic drug. In fact, a comparison between the incidence of disseminated *C. krusei* in 1989 and 1990, the year in which fluconazole prophylaxis was introduced, showed that the latter practice led to an increase of such cases while no such change was observed for *C. albicans* and *C. tropicalis* cases (Wingard et al. 1991). Indeed, *C. krusei* became an important cause of difficult to treat cases of candidemia (Pfaller 2003). Among mucocutaneous *Candida* infections it is an important cause of vaginitis (Singh et al. 2002).

Candida parapsilosis

C. parapsilosis has a wide distribution in nature and has been found to be part of the normal human skin flora especially subungual spaces (Weems 1992).

C. parapsilosis has been reported as a contaminant of high concentration glucose solutions and prosthetic material (Branchini et al. 1994). A number of nosocomial infections with this yeast species have been reported. It was isolated from cases of sepsis in an intensive care nursery (Shian et al. 1993; el-Mohandes et al. 1994), it was reported to be prevalent in patients with indwelling devices or those fed parenterally (Herrero et al. 1992; Martino et al. 1993; Krcmery and Barnes 2002), possibly as a result of its tendency to form biofilms on catheter tips (Kuhn et al. 2002). Nosocomial infections were connected with the presence of *C. parapsilosis* on hospital equipment, personnel hands, and pigeon guano on window sills (Greaves et al. 1992; Oie and Kamiya 1992; Sanchez et al. 1993), and infection of high-risk patients connected with such contamination (Greaves et al. 1992; Sanchez et al. 1993). Cases of endophtalmitis caused by *C. parapsilosis* following lens implants has been reported (Kauffman et al. 1993).

Candida tropicalis

Odds (1988) reports that according to some surveys, *C. tropicalis* is a relatively prevalent pathogenic yeast species in the oral cavity of asymptomatic carriers. *C. tropicalis* has been shown to be involved in various human pathologies such as fungemia (Pfaller 2003) post-surgery infections, osteomyelitis (Ferra et al. 1994), endocarditis (Shmuely et al. 1997; Gerritsen et al. 1998). In addition to endogenous infections, which are the most common, cases of fungemia in a neonatal intensive care unit have been connected to the presence of the fungus on the hands of personnel (Finkelstein et al. 1993).

Candida lusitaniae

C. lusitaniae was first described as part of the normal microbial flora of warm-blooded animals by van Uden and do Carmo-Sousa (Sanchez et al. 1992). *C. lusitaniae* has been isolated from various animal sources (pigs, mastitis milk, bird droppings), alimentary products (citrus peel juice), and clinical specimens (sputum, feces, urine) (Merz 1984).

The prevalence of *C. lusitaniae* among yeast species isolated from clinical cases is relatively low. During a 15-month study, only 0.64 percent of the isolated yeast species were identified as *C. lusitaniae* (Merz 1984). Nosocomial colonization of the digestive or urinary tracts has been reported and the possibility of transmission by hands of hospital personnel suggested (Sanchez et al. 1992; Strausbaugh et al. 1994). Restriction enzyme analysis of *C. lusitaniae* isolates cultured in the same study showed that although most patients carry the same strain throughout the period of colonization, occasionally, two different strains may be present in different body sites (Sanchez et al. 1992).

The distribution of the two mating types of *Cl. lusitaniae* is unequal: a ratio of 1:6 between the 'a' and the 'α' mating types has been reported.

PATHOGENICITY AND PATHOGENESIS

Virulence factors

In addition to previously available information regarding virulence properties of *Candida* spp. (Cutler 1991; Odds 1994; Segal 1994b), newer studies with this subject have

been summarized in several review articles in recent literature (Staib et al. 2000; Calderone and Fonzi 2001; Haynes 2001; Navarro-Garcia et al. 2001; Soll 2002). While many of the reviews describe investigations concentrating on specific virulence attributes, such as adherence to host tissues (Cotter and Kavanagh 2000; Sundstrom 2002), proteinases (De Bernardis et al, 2001; Monod and Borg-von Zepelin 2002), phospholipases (Ghannoum 2000), or biofilm formation (Waldvogel and Bisno 2000; Ramage et al. 2001), the most intriguing facet of the recent studies is the focus on isolation and/or expression of specific virulence genes (Staib et al. 2000; Hoyer 2001; Navarro-Garcia et al. 2001). Although the majority of these reviews report on research involving *C. albicans*, studies on additional *Candida* spp., such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, or *C. dubliniensis* have also been reviewed (Haynes 2001; Gutierrez et al. 2002).

The virulence traits of *Candida* spp., particularly of the major pathogen, *C. albicans*, to which possible roles in pathogenesis of candidiasis have been attributed, are believed to be associated primarily, with:

- the ability of the fungus to bind (attach, adhere) to host tissue as an initial step in the recognition and interaction with the host. Special attention has been given more recently to an associated phenomenon – biofilm formation, as a significant factor in the pathogenesis of candidiasis, affecting adversely the host's response to infection and causing difficulties in therapy
- the production of specific enzymes that could facilitate tissue penetration and invasion, such as secretory aspartyl proteinases (SAPs) and phospholipases
- yeast–hyphal morphogenetic transformation, which could also facilitate penetration and, in addition, assist the microbe to evade the host's defense system
- phenotypic switching
- various immunomodulatory effects of fungal determinants, which could contribute to reduced activity of the host's defense system.

Although the above-mentioned mechanisms of virulence are supported by evidence provided by various experimental studies and epidemiologic–clinical observations, it should be emphasized that assessment of microbial virulence under actual in vivo conditions is a complex issue, since virulence involves a combination of traits, while in experimental systems a single characteristic is assessed under given conditions.

The ability of *Candida* to attach to host tissues has been extensively studied both in vitro and in vivo, the latter mostly in experimental animal models, and reviewed in several articles (Segal 1987a, 1994b; Calderone and Braun 1991; Kennedy et al. 1992; Segal and Sandovsky-Losica 1995, 1997). As stated above, the more recent studies and reviews summarizing those studies (Cotter and Kavanagh 2000; Sundstrom 2002) concentrate on genetic aspects related to adhesion or association with the host (Hoyer 2001). As it is outside the scope of the present text to exhaust in detail the subject, only the principles and the major conclusions drawn from the various studies are detailed.

In vitro systems showed that *Candida* can adhere to a variety of substrates, such as exfoliated human epithelial cells (buccal, vaginal, dermal) (Segal and Sandovsky-Losica 1995), human tissue lines (endothelial, Hela) (Hostetter 1994a, b) animal tissue segments (oral, gastrointestinal) (Segal and Sandovsky-Losica 1995) or inert surfaces (various polymers used for indwelling medical devices) (Waldvogel and Bisno 2000). These experiments revealed that a gradient in the level of adherence could be noted, which correlates with the pathogenicity of the *Candida* spp. in man and animals. Thus, *C. albicans* was, generally, the most adherent *Candida* species. However, intraspecies variation expressed by different adhesion ability of various isolates of the species was also noted (Samaranayake et al. 1994). In addition, it was shown that mutants with reduced adherence exhibited also decreased pathogenicity in vivo (Roth-Ben Arie et al. 2001). Adhesion to tissues was found to be dependent on various environmental conditions affecting the fungus, and influenced by various host factors, such as fungal surface hydrophobicity, growth medium, and growth conditions, or hormonal and immune status of the host, respectively (Kennedy 1988).

Animal experimental models and ex vivo systems have confirmed many of the data derived from the in vitro systems. They showed that the attachment of the fungus to the host's tissues initiates colonization and the infectious process. This concept was the basis for attempts to prevent development of infection by intercepting the binding of the fungus to host's tissues (Segal and Sandovsky-Losica 1997). Such an approach could be considered as a novel prophylaxis modality for prevention of disease.

Studies aimed at identifying the fungal surface molecules involved in the binding process led to the recognition of several putative adhesins: mannan (Calderone and Braun 1991), the mannoproteins – primarily their proteinous moiety (Douglas 1987) – and chitin (Segal and Sandovsky-Losica 1995, 1997). Miyakawa et al. (1992) suggested that antigen 6 of the mannan might be associated with adhesin activity. Different adhesins may, possibly, exist on different *Candida* species (Bendel and Hostetter 1993). The counterpart receptor molecule on the host cell is, depending on the type of cell, apparently, fucosyl, glucosamine, fibronectin or arginine-glycine-asparagine (RGD) (Hostetter 1994a, b).

More recent studies reported several additional adhesins in *C. albicans*, such as Alsp and Hwp1p. Hoyer's group described a family of surface glycoproteins, the Alsp (Hoyer 2001), that is involved in fungal binding and may act as adhesins, thus being considered virulence attributes, playing a role in pathogenesis. It was also

found that at least nine genes encode these molecules. Another probable adhesin, a cell wall protein as well, the Hwp1p, was described (Staab and Sundstrom 1998; Tsuchimori et al. 2002). It was shown in these studies that mutants deficient in these adhesins were less virulent. Gale et al. (1998) have demonstrated the linkage of adhesion, hyphal growth, and virulence of *C. albicans* to the *INT1* gene. Int1p, the integrin-like protein, is considered a *C. albicans* virulence factor (Hostetter 2000).

Regarding other *Candida* spp., Epa1p, a cell wall protein acting as an adhesin in *C. glabrata*, was reported (Cormack et al. 1999). *ALS* genes have been reported for *C. tropicalis* and *C. dubliniensis* (Hoyer et al. 2001).

C. albicans also possesses surface components (e.g. iC3b), which are associated with binding to fibrinogen and complement (Calderone and Braun 1991). This interaction and speculated molecular mimicry mechanisms of mammalian integrins by *C. albicans* surface proteins (Hostetter 1994a) may affect the binding to neutrophils and thereby phagocytosis, which in turn will affect the host's defense system, and should thus be considered as additional factors playing a role in the pathogenesis of candidiasis.

As sequelae of attachment, microorganisms can grow in colonial communities and produce the so-called 'biofilms,' instead of single entities – the 'planktonic forms.' Biofilm production was shown for *Candida* spp. (Kumamoto 2002), particularly for *C. albicans* (Chandra et al. 2001). The biofilms contain extracellular materials (ECM), composed of proteins, carbohydrates, and other substances (Baillie and Douglas 2000). Other significant characteristics of biofilms are the poorer response of the pathogen in the biofilm to antimicrobials (Ramage et al. 2002) and the difficulties of the hosts' defense systems to cope with the microbe, resulting in difficulties of eradication of infection. *Candida* biofilm formation has important clinical ramifications, as biofilms are formed on inert surfaces of medical devices (Sanchez-Sousa et al. 2001), such as catheters, artificial dentures, prosthetic valves, the use of which is known to be associated with increased risk for development of systemic infections.

The factors involved in fungal tissue penetration and invasion, which may follow the initial step of interaction between the fungus and host cell, are related to the morphogenetic change from yeast to hyphae, and to production of hydrolytic enzymes (Odds 1994). Among the latter, the SAPs (Ruchel et al. 1992) and phospholipases (Ogawa et al. 1992) seem to be the major enzymes playing a significant role in pathogenesis of candidiasis (Ghannoum 2000; Hube 2000).

SAPs can be found following adhesion of *C. albicans* to epithelial cells. As in the case of adhesion, SAP production was exhibited, primarily, by pathogenic *Candida* spp. Specifically, while enzyme activity was revealed by *C. albicans*, *C. tropicalis*, and *C. parapsilosis* (Sono et al. 1992; Fusek et al. 1994) other *Candida* spp. exhibit it only partially. Enzymes were detected in vivo during infection (Ruchel et al. 1991) and in association with phagocytosis (Borg and Ruchel 1990). SAP-deficient mutants were less pathogenic in inducing experimental infection and unable to invade tissue (Kwon-Chung et al. 1985; Ross et al. 1990). It is believed that SAP production may facilitate invasion through degradation of keratin and collagen.

Currently, the SAP family consists of nine enzymes, for which genes have been isolated (Hube and Naglik 2001), and for which correlations with specific clinical forms have been described. Thus, *SAP2* has been associated with vaginal candidiasis, while other SAPs (e.g. *SAP1–SAP6*) have been associated with systemic infection (Hube et al. 1997; Sanglard et al. 1997). A most recent review by Monod and Borg-von Zepelin (2002) states that 10 genes of *C. albicans* SAPs have been cloned thus far. Of interest are the studies in AIDS patients undergoing antiretroviral treatment with inhibitors of aspartic proteinases, that revealed a decrease in incidence of oropharyngeal candidiasis in these patients (Egger et al. 1997), possibly due to inhibition of production of Sap protein, involved in the fungal adherence to the mucosal surface (Borg-von Zepelin et al. 1999).

Phospholipases have been shown to be fungal virulence factors (Ghannoum 2000; Hube 2000; Monod and Borg-von Zepelin 2002; Sundstrom 2002). Phospholipases have been shown for *C. albicans* and other *Candida* spp., such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, or *C. krusei* (Ghannoum 2000). As in the case of the SAP enzymes, a role in the adherence of *Candida* to host cells and tissue penetration has been attributed to phospholipase production (Ghannoum 2000). Two genes encoding phospholipases in *C. albicans* have been cloned (Leidich et al. 1998; Sugiyama et al. 1999) and disruption of the genes affected the fungal virulence (Leidich et al. 1998). It is interesting in this context to mention the study of Hube and colleagues (Hube 2000) reporting on ten genes encoding lipases that, the authors suggest, may have a role in the microorganism's virulence.

The importance of the transition to the hyphal form as a virulence factor, recognized for many years, has been corroborated more recently by molecular studies (Madhani and Fink 1998). The transformation into the hyphal form is noted during active infection (Odds 1988; Edwards 2000). It is believed that phospholipases concentrated at the hyphal tip may be related to the greater invasiveness of this form as compared to the yeast. In addition, the hyphae, being larger than the yeast form, are more resistant to phagocytosis (Diamond and Krzesicki 1978), and thus the morphological change contributes to increased pathogenic potential of the fungus. An interesting recent observation reported by Sundstrom (2002) indicates the presence of the adhesin Hwp1 only on the surfaces of germ tubes and mature hyphae, and not on that of the other morphological

forms of *C. albicans*. Furthermore, it was also shown by the same group of investigators (Sundstrom et al. 2002) that this cell wall component contributed to the yeast's virulence in murine oropharyngeal candidiasis. Recent molecular studies on *C. albicans* dimorphism (Liu 2001) revealed the association between specific genes involved in the morphological transition to hyphae and the fungus' virulence. A *Candida CLA4* mutant, with reduced yeast to hyphae transition ability, was found to have a lower virulence in mice (Madhani and Fink 1998). Cyclic AMP has been shown to be involved in blastospore-hypha transition; thus the cAMP signaling pathway plays a role in pathogenesis (Bahn and Sundstrom 2001).

Phenotypic switching associated with morphological colony changes from smooth to rough or fuzzy type, white to opaque, and other morphological changes is a phenomenon described first in the 1980s (Slutsky et al. 1985, 1987). Later studies associated phenotypic switching with differences in antigenicity (Anderson et al. 1990), pathogenic potential (Jones et al. 1994), and indicated genetic regulation (Perez-Martin et al. 1999). Phenotypic switching is considered as a potential virulence characteristic, as it may express the fungal plasticity, contributing to its adaptability to various anatomic sites of the human body and thereby possibly enabling the variety of clinical entities it causes, as well as play a role in the transition from commensalisms to pathogenicity (Soll 2002). Furthermore, phenotypic switching may assist the microbe to evade the host's defense systems, in analogy to the bacterial 'phase transition' phenomenon.

Several investigators (Cassone 1989; Domer and Garner 1989; Garner et al. 1990; Domer et al. 1992) described immunomodulatory activity of fungal surface components. An immunosuppressive effect of *C. albicans* in experimental models was noted, which was attributed particularly to manann (Domer 1989; Domer and Carrow 1989).

Should such a mechanism or a similar one occur in clinical situations, this could be added to the arsenal of candidal pathogenic traits contributing to the pathogenesis of the infection.

This section can be concluded by the belief that the current technology of transcript profiling using DNA arrays, which are already commercially available, may lead in the future to identification of additional virulence traits and, most importantly, possibly those expressed in vivo.

Pathogenesis of candidiasis

In discussing pathogenesis of candidiasis, it has to be kept in mind that *Candida* is a human commensal, so that the infectious source is mostly endogenous (Kennedy 1989). The GI tract is considered a major reservoir for candidiasis, e.g. in vaginitis or diaper rash. Moreover, from the GI tract the fungus can invade the bloodstream following damage to the GI mucosa, such as induced by anticancer treatment (irradiation or chemotherapy) (Cunningham et al. 1985) or major surgery, and spread hematogenously into various organs, causing deep-seated/disseminated infection. It is believed that *Candida* may also be able to cross the intact GI mucosa by a process termed 'persorption' (Krause et al. 1969) following fungal overgrowth, which can result from changes in the normal balance of the microbial flora due to extensive antibiotic treatment. Although previous experimental studies, such as those reported by Sandovsky-Losica et al. (1992), have already shown that systemic candidiasis originating from the GI tract follows adhesion to its mucosa (Sandovsky-Losica and Segal 1990; Sandovsky-Losica et al. 1992), more recent investigations have revealed additional aspects of this interaction. Wiesner et al. (2002), using enterocyte cultures, reported that the *INT1* gene was involved in adherence of *C. albicans* to enterocytes. Moreover, this involvement was more marked for blastospores than the hyphal form (Wiesner et al. 2002). However, *Candida* can be introduced from exogenous sources as well (Edwards 2000). These may include introduction through various catheters and lines, or other indwelling/prosthetic medical devices (Khatib and Clark 1995). This route is of particular importance in the development of deep-seated and systemic candidiasis as most of those therapeutic modalities are used primarily in compromised hosts whose defense systems are unable to combat the introduced pathogen. An interesting observation was made by Rangel Frausto et al. (1994) regarding nosocomial transmission of yeasts. The survival and transmission of *Candida* spp. on and by human volunteers has been assessed and it has been shown that, although the half-life of the microorganisms was relatively low, more than 10^3 colony-forming units of the yeast remained on the hands of the volunteers after 45 minutes (Rangel Frausto et al. 1994).

Once *Candida* entered the bloodstream, whether from exogenous or endogenous source, the microorganisms have to adhere to the endothelial surface of the blood vessels, before dissemination into tissues (Klotz 1992). This process involves endocytosis, endothelial invasion, and response to the invading pathogen expressed in production of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) or interleukin (IL)-6 (Orozco et al. 2000). It was also noted that germ tubes are better endocytosed than blastospores (Phan et al. 2000).

It is noteworthy that proinflammatory cytokines have been shown recently to be involved in the pathogenesis of candidiasis (Brieland et al. 2001). In a model using immunocompetent mice, Brieland and colleagues compared the pathogenesis of intravenous (IV)-induced infection by *C. albicans* versus that of *C. glabrata*,

analyzing organ colonization and cytokine production. They found that while kidneys of *C. glabrata* infected animals were colonized, the animals nevertheless, survived, contrary to those infected by *C. albicans*, that were colonized as well and succumbed to the infection. The authors suggest that the difference is associated primarily with a more rapid induction of the proinflammatory cytokines in response to *C. glabrata* than to *C. albicans*.

Person-to-person transmission is not a predominant mechanism of pathogenesis in candidiasis, and is expressed primarily in oral thrush of newborns acquired during birth from their mothers affected by vaginal infection, or noted more rarely in sexual transmission from vaginitis patients to their male partners.

The normal innate mammalian defense system against *Candida* infection includes intact dermal surfaces, intact mucosa, unspecific humoral factors, and the immune system – humoral and cellular (Greenfield 1992; Levitz 1992).

The first line of defense, which protects, primarily, before the mucocutaneous forms of candidiasis, is the unbreached skin and mucosa (Edwards 2000). As detailed under the section on Clinical entities below, risk factors, which increase the susceptibility to these types of *Candida* infections, include maceration of skin, changes of microbial flora, trauma to skin/mucosa, hormonal changes affecting skin integrity or local defense and/or excess of carbohydrates on surfaces, as seen in diabetics. It is believed that the skin, in addition to its function as a barrier, possesses an inflammatory–immunological activity as well (Wagner and Sohnle 1995). The latter may involve the specific skin cells – epidermal Langerhans cells and keratinocytes – which may function as antigen-presenting cells, engage in phagocytosis or/and produce various cytokines. As indicated above, breaching the intactness of the skin (e.g. through intravenous catheters) or of the GI mucosa can lead also to introduction of the organism into the bloodstream, either from exogenous or endogenous (GI tract) sources, respectively. Thus, damage to the integuments may be considered as a risk factor for the development of systemic candidiasis. Additional nonspecific, nonimmune factors to which a possible role in defense has been attributed include iron-binding proteins, such as transferrin or lactoferrin (Nikawa et al. 1993).

The second line of defense, which comes into action following fungal penetration, includes phagocytic and candidacidal activity of polymorphonuclear (PMN) (primarily, neutrophils) and mononuclear (primarily, monocytes) cells (Marodi et al. 1991a, b; Greenfield 1992; Levitz 1992). These processes involve myeloperoxidase and superoxide production, or cationic proteins. PMN activity is of crucial significance in protection against the deep-seated forms of *Candida* infections, as can be judged by epidemiologic data that indicate that an increased rate of these infections is associated with neutropenia or defective PMNs (see sections on Clinical entities and Immunity below).

As to specific activity of the immune system, despite numerous experimental studies and clinical observations there is no clearcut concept of the activity of the various elements of the system and the mechanisms underlying that activity. T lymphocytes involved in cell-mediated immune response seem to be of significance, particularly in defense against mucocutaneous forms of candidiasis (Domer and Carrow 1989). Patients with T-cell deficiencies, such as AIDS patients, are especially prone to these infections. The role of these cells in resistance to systemic candidiasis is more controversial (see discussion in the section on Immunity below). Opsonizing antibodies (IgG) seem to contribute to phagocytosis and thereby to defense. An additional humoral element of the immune system involved in defense is, apparently, the complement cascade (Gelfand et al. 1978).

ANTIGENICITY, IMMUNE RESPONSE, AND IMMUNITY

The immune response of the mammalian host to *Candida* infection, the antigens eliciting this response, and the eventual resulting immunity, as well as the defense mechanisms involved in the process, constitute complex issues which have attracted extensive research over the years, research which has not always led to unequivocal conclusions. Nevertheless, certain pertinent generalizations can still be drawn.

Antigenicity

In discussing the topic of antigenicity, one should bear in mind that the definition of the term should be in the frame of a specific context; whether:

- in relation to antigens which elicit immune responses in general
- as antigens which elicit a response during infection, a response which can be measured and used as a diagnostic tool
- as antigens which confer resistance to the infection and may be considered 'protective antigens.' As in many microbial systems, the antigens eliciting a given response or having a protective role may be different.

In defining the antigenicity of *Candida*, primarily that of *C. albicans*, most studies, particularly earlier investigations, concentrated on the cell wall. Those studies indicated that the mannoprotein complex, particularly the polysaccharidic moiety of the *Candida* cell wall, is the major antigenic component. It was postulated that the backbone structure of the mannan confers the antigenic properties to the whole group, and the specific antigenicity of a given species or variety is apparently

due to the different side chains of the mannan molecule (Reiss 1986; Reiss et al. 1992). This hypothesis was the background of extensive experimental studies of Tsuchiya et al. (1965), and over the years of other investigators (Miyakawa et al. 1986) as well. These studies, using agglutination assays with anti-*Candida* sera, culminated in the development of an antigenic profile of *Candida* spp. of medical interest. The presence of heat-labile and heat-stable antigens, which could be common or specific to a particular species or several species, was shown. A commercial kit based on this principle enables us to differentiate among the most commonly encountered pathogenic *Candida* spp. Limited serotyping of *C. albicans* into two serotypes, serotype A and serotype B, as described by the studies of Hasenclever and Mitchell (1961), was also based on a similar principle. Summaries of studies conducted in the last decade on *Candida* spp. cell wall, such as that of Chauhan et al. (2002), contribute to a more detailed knowledge of its composition and molecular structure. It became evident that, besides the traditionally recognized polysaccharides, other components such as chitinase and glycolipids can be considered important cell wall constituents. Glycosylphosphatidylinositol (GPI) was found to play a role in anchoring proteins to the cell wall polymers (Fonzi 1999). In addition to cell wall composition and structure, studies were also aimed at characterizing cell wall antigens. Ponton described several types of antigens expressed either on the surface of germ tubes, blastoconidia, or both (Ponton et al. 1993, 2002). La Valle et al. (2000) studied the 65 kDa mannoprotein of *C. albicans* and found it to be a major immunodominant antigen. The group of Poulain concentrated on phospholipomannans (Poulain et al. 2002; Trinel et al. 2002). They showed that *C. albicans* can synthesize specific β-1,2-oligomannosides which have antigenic properties.

If antigens are to be used as reagents for diagnostic purposes, it is of importance to define the aim. If the aim is to differentiate between colonization of superficial mucocutaneous surfaces and deep-seated organ involvement, it would seem reasonable not to employ *Candida* cell wall antigens. Since *Candida* is an ubiquitous microorganism and human commensal, it elicits immune responses in the host to the fungal cell wall to which there is constant exposure. On the other hand, a reasonable approach would be to search for antigens of the *Candida* cytosol, which are released during infection and elicit a measurable immune response. This rationale was the basis for development of immunodiagnostic tests in which the reagent was a cytoplasmic *Candida* extract, standardized by its protein content (Reiss et al. 2002). More defined antigens, mostly of proteinaceous nature, to which the above-mentioned characteristics have been attributed, have also been suggested. These include the enolase, a protein with catabolic enzymatic activity (Franklin et al. 1990); a 47–48 kDa protein described by Matthews et al. (1988), who believe that it has some protective role in AIDS patients and which could be identical with the enolase, and more recently La Valle et al. (1995) described a 70–80 kDa heat shock protein (HSP) with immunogenic activity, and which the authors suggest as a possible reagent in diagnosis. Undefined nonprotein antigens, probably a glycoprotein, and the polysaccharide mannan, have been believed to be released during systemic *Candida* infections and consequently tests aimed at the detection of these antigens for diagnostic purposes were devised (the Ramco and Pastorex kits, respectively).

It should be noted that some of the antigens mentioned above, such as the 65 kDa protein or the oligomannosides, may be considered as potential reagents with diagnostic value as well (La Valle et al. 2000; Sendid et al. 2002).

The identification of antigens, the response to which would induce protection to the infection, is a subject even more complicated and controversial than the identification of antigens of diagnostic relevance. This is so despite experimental attempts, as mentioned later, to induce protection against experimental infection by immunization with various *Candida* preparations. These have included whole live organisms, live-attenuated *Candida* cells and those killed by different methods or noncellular preparations (Segal 1987b, 1991). Among the latter can be included antigens prepared by the author with *Candida* ribosomal fractions, which induced, in animal models, significant protection against systemic candidiasis (Segal 1989). The protection is, apparently, due primarily to the ribosomal-protein(s) moiety. Interestingly, specific ribosomal proteins, the L7/L12 of *Brucella* spp., have been shown to be immunogenic and to elicit a delayed-type hypersensitivity (DTH) response (Bachrach et al. 1994).

It is noteworthy that a recent study of Mizutani et al. (2000) suggested that a membrane fraction of *C. albicans* may act as an antigen which confers $CD4^+$ T-cell-mediated resistance to systemic candidiasis in mice. The 65 kDa mannoprotein described by La Valle et al. (2000) was also able to induce a cell-mediated immune (CMI) response (La Valle et al. 2000). A more recent study of Dromer et al. (2002) reported on the ability of synthetic analogs of β-1,2-oligomannosides of *C. albicans* to prevent intestinal colonization by the fungus.

Immune response

Exposure to *Candida* species stimulates both humoral and CMI responses. Anti-*Candida* antibodies can be detected in experimental animal infections, in humans with naturally acquired infections, as well as in healthy individuals harboring the fungus in a carrier state. The antibodies represent the different immunoglobulin types – IgG, IgM, IgA or IgE – albeit not all immunoglobulin types can be detected in all the clinical entities, under all conditions, and in different body fluids.

IgG and IgM are generally found in sera of patients with deep-seated forms of candidiasis, except if the patients are highly immunosuppressed and unable to mount an immune response. IgG and IgM antibodies can also be found in the mucocutaneous forms of candidiasis, as well as in asymptomatic carriers.

Anti-*Candida* IgE antibodies have been found in serum and in other body fluids in situations associated with allergy. In addition to measuring IgE antibodies in serum and other body fluids by serological tests, these antibodies can be revealed by skin testing with a *Candida* antigen. Skin inoculation with a *Candida* antigen may reveal an immediate type hypersensitivity (ITH) reaction resulting in the appearance of a flare-like erythema at the inoculation site 20–30 minutes after the test inoculation. The ITH response will be, generally, found in allergic states. An interesting observation was made by Witkin (Witkin et al. 1989) who noted the presence of IgE antibodies in women with vaginitis, indicating a possible role or association of ITH in the pathogenesis of vaginal infection.

IgA antibodies have been found in sera and vaginal secretions of patients with candidal vulvovaginitis (Schonheyder et al. 1983; Sobel 1985) and in vaginal fluids from experimentally infected rats (Polonelli et al. 1994; De Bernardis et al. 2002). However, the differences in the titers of IgA antibodies in patients in comparison to those of healthy individuals, or in other conditions with candidal colonization, are not discriminatory enough to permit the use of detection of this type of antibodies as a reliable diagnostic tool for candidal vaginitis.

As to CMI, a *Candida* infection or an asymptomatic colonization stimulates the response of the cellular immune system (Domer and Carrow 1989; Baum 1994). The CMI response can be detected by in vitro assays as well as by in vivo measurements. The current concept of the chain of events culminating in the expression of CMI postulates that PMNs produce IL-12, which stimulates Th1 cells to release interferon-gamma (IFN-γ) and IL-2, which activate phagocytes. In vivo measurements include skin testing with a candidal antigen (candidin). The CMI response thus measured will be expressed in a DTH reaction, revealing an indurated erythema at the site of the test-antigen inoculation, apparent about 48 hours postinoculation. As *Candida* do colonize healthy individuals, the skin test for detection of a DTH response will be positive in most of the tested individuals and cannot, therefore, serve as a tool for diagnosis of candidiasis. It can, however, indicate that the individual tested possesses an adequate cell-mediated immune system in order to mount the response to the antigen with which he/she was tested. Indeed, the *Candida* skin test is used to evaluate the CMI competence of patients. A negative candidin test will generally point to the inadequacy of the patient's immune system to mount a DTH response, and will suggest that the patient is immunodeficient or immunosuppressed. However, as there are different responses to different *Candida* antigens, this test must be interpreted with caution. CMI responses can be, as mentioned earlier, detected by several in vitro assays. The most commonly used assay is the lymphocyte transformation/proliferation test, which measures the in vitro stimulation of T lymphocytes with *Candida* antigens, indicating the sensitization of the CMI system by the fungus. This assay or other in vitro assays for detection of CMI response to *Candida*, similar to the skin test for in vivo evaluation of DTH, do not have marked value for diagnosis of candidiasis. However, as in the case of the skin test, the in vitro assays are useful for evaluation of the cell-mediated immune status of the individual. Moreover, the use of more than a single evaluation method, as for example a skin test and the in vitro lymphocyte transformation assay or the macrophage migration inhibition assay, may enable one to define more specifically the putative immune defect of the tested patient. This was demonstrated by Kirkpatrick (1989) in a series of studies of patients with chronic mucocutaneous candidiasis (CMC) in whom a differentiation of the individual immune defect(s) has been shown, revealing, for example, incompetence in antigen recognition versus inability to mount an immune response. The differentiation of different T-lymphocyte subsets (e.g. CD4, CD8) may also be of clinical relevance in evaluating the patients' immune status and possible susceptibility to *Candida* infection.

Immunity

Immunity to *Candida* infection, including the innate and adaptive defense system, is also a topic that has attracted considerable research interest. As can be judged by recent reviews (Romani 2002a, b) detailed, in-depth information on defense systems against candidiasis is emerging from studies undertaken during the last decade. Nevertheless, it is still difficult to draw clear and unequivocal conclusions. This might be due to the complexity of the topic and, partially, to the fact that most experimental evidence has been derived from animal models, among which divergences exist, and which do not necessarily correspond to human situations.

The contemporary view as to the innate versus adaptive immunity is that both should be looked upon as an integrated system. Immunity to *Candida* is currently believed to depend on various cells, including polymorphonuclear and mononuclear leukocytes, keratinocytes, and dendritic, epithelial, and endothelial cells. In addition, humoral elements, including antibodies, cytokines, and complement, are involved (Romani and Kaufmann 1998).

Among the T cells, the Th1 subset is the predominant cell population responsible for protection (Romani 1999). However, an important role in the immune response should be attributed to the Th2 subset as well.

It is believed that immunity is affected by the subtle interactions between the two subsets and their cytokines/chemokines. The current paradigm regarding events occurring following exposure to *C. albicans* yeasts includes the production, by PMNs, dendritic or other cells, of cytokines, which in turn induce Th1 cells to secrete cytokines activating effector cells. Among the cytokines, IL-2, IL-12, TNF-α, IFN-γ were reported to be of significance in protection against *Candida*. However, in superficial infections, local immunity at different anatomic sites apparently plays a significant role in immune response and defense to *Candida* (De Bernardis and Boccanera 2002).

MUCOSAL AND CUTANEOUS CANDIDIASIS

CMI plays a major role in the defense against mucosal and cutaneous *Candida* involvement. Indeed, CMI deficiencies as noted in AIDS or CMC patients result in increased susceptibility for development of cutaneous, nail, or mucosal candidiasis (Edwards 2000). As stated above, investigations of underlying mechanisms of CMI (Fukazawa et al. 1994) revealed the involvement of $CD4^+$ T lymphocytes, various cytokines, such as IFN-γ, IL-2 and 12, granulocyte-macrophage colony-stimulating factor (GM-CSF) (Smith et al. 1990; Watanabe et al. 1991; Romani and Kaufmann 1998; Romani 1999). Macrophages may act as effectors exerting their candidacidal effect following activation by IFN-γ produced by the Th1 CD4+ subset. It is also believed that oxygen radicals or cationic proteins or peptides released from the activated cells may be associated with the antifungal effect. In addition to CMI, antibodies have been shown to have a role in immunity as deduced from studies on experimental vaginitis in laboratory animals (De Bernardis and Boccanera 2002). A study by Fidel et al. (1995) pointed out, interestingly, that systemic CMI seems not to have a protective role in vaginitis (De Bernardis et al. 2002). Thus, apparently, defense against specific forms of superficial candidiasis may depend on different immune mechanisms, emphasizing the possibility of immunological compartmentalization.

DEEP-SEATED CANDIDIASIS

Immunity to deep-seated/systemic candidiasis does involve T-cell CMI as well; however, additional cellular and humoral elements seem to contribute significantly. This conclusion can be derived from the clinical–epidemiologic observations as to the individuals particularly susceptible to these forms of *Candida* infection. While, as indicated above, individuals with defects in the CMI system are especially prone to mucosal and cutaneous candidiasis, the other candidiasis forms are present mostly in neutropenic patients (Edwards 2000). It is, thus, evident that the PMNs are of importance in defense against these *Candida* infections. The role of granulocytopenia as a risk factor for systemic candidiasis was also demonstrated in experimental animal models, in which a neutropenic state was induced, revealing increased susceptibility to *Candida*. It is interesting that some studies (Fukazawa et al. 1994) have shown the involvement of both the Th1 and Th2 subsets of the CD4+ T cells in systemic *Candida* infection. A complex and not fully elucidated interaction between the two subsets takes place.

Extensive research has been devoted to the elucidation of the intricate role of cytokines in immunity to systemic *Candida* infections. The studies indicated that a major role might be attributed to IL-12, TNF-α (Mencacci et al. 1998), transforming growth factor beta (TGF-β) (Spaccapelo and Romani 1995) and IFN-γ (Cenci and Mencacci 1998) that are required for Th1 response induction (Mencacci et al. 1998). It should also be noted that different cytokines are induced by different *Candida* antigens (Cassone and De Bernardis 1998). Of interest are recent findings of Brieland et al. (2001). These researchers investigated the role of proinflammatory and anti-inflammatory cytokines in murine systemic candidiasis and showed that TNF-α played a significant role in protection against infection.

Phagocytosis and killing of *Candida* involve complement, antibodies, and various cytokines, such as IFN-γ or tumor necrosis factor (TNF) (Diamond et al. 1991) and granulocyte colony-stimulating factors (Kullberg and Anaissie 1998; Kullberg et al. 1998). Differences in the activity of phagocytes against the hyphal versus the yeast form have been described indicating increased efficacy of PMNs towards the blastospores (Romani and Bistoni 2002). It was also shown that phagocytosis of *C. albicans* yeasts by PMNs involve a mannose receptor. PMNs are known for their ability to kill *Candida* via oxidative burst, enzymatic activities such as myeloperoxidase, and defensins (Domer and Carrow 1989; Greenfield 1992). Intracellular killing of the phagocytized fungus seems to be more efficient in the presence of opsonins. Yeast cells surviving in murine macrophage may develop a germ tube, perforate the phagosome, and cause the cell's destruction (Kaposzta et al. 1999). Studies have shown the presence of specific receptor sites on *C. albicans* for CR2 and CR3 of the complement (Calderone et al. 1994; Hostetter 1994b), which could be involved in phagocytosis.

It has been postulated by Matthews (Matthews et al. 1988) that anti-*Candida* antibodies found in sera of AIDS patients directed against a 47 kDa candidal antigen might have a protective role (see the following section on Immunization below).

Immunization

Experimental animal models have indicated that sublethal *Candida* infections may induce partial immunity to reinfection (Giger et al. 1978; Oblack and Holder 1979), an observation that indicated that obtainment of

adaptive immunity could be feasible. This was the rationale for various attempts to induce protection against *Candida* infections by immunization with a fungal antigen. Various fungal preparations, including live, attenuated or killed organisms, subcellular components, and defined antigens have been explored as possible immunogens (Segal 1994a). Various routes and procedures of immunization have been investigated as well. It is not practical, nor is it purposeful, within the scope of this chapter to detail all these studies, and the specifically interested reader should refer to relevant bibliography.

It should be noted that although, despite the efforts, no clinically available anti-*Candida* vaccine of proven efficacy has yet been obtained, some recent developments seem promising. These are related, particularly, to the possibility of using anti-*Candida* antibodies in immunotherapy and/or immunoprophylaxis of systemic candidiasis, based on the observations of Matthews and colleagues (Matthews et al. 1988) indicating a possible role for anti-*Candida* antibodies in resistance to systemic candidiasis. A specific *Candida* antigen, the heat shock protein 90 (HSP90), was identified as an immunodominant antigen that elicits protective antibodies (Matthews et al. 1991, 1995). Use of molecular engineering technology enabled production of a human antibody, which is being evaluated in human clinical trials (Matthews et al. 2000; Matthews and Burnie 2001; Rigg et al. 2001) (under the designation 'Mycograb') as an adjunct to antifungal therapy and possible immunoprophylaxis in compromised patients (Matthews and Burnie 2001). Other investigators have also suggested the usefulness of anti-*Candida* antibodies (Cutler et al. 2002). Cutler and colleagues demonstrated that antibodies to a mannan–protein complex, encapsulated in liposomes, protected mice against challenge with the fungus.

Various attempts to immunize against candidiasis have been undertaken for several decades and have been summarized previously (Segal 1987b, 1991). Among these were studies on the ribosomal fraction of *C. albicans* as immunogen (Segal 1987b, 1991), revealing their protectivity. Based on this background, Levy et al. (1989) found that supplementing *C. albicans* ribosomes with a cell wall fraction from *Klebsiella pneumoniae*, an adjuvant permissible for human use, augmented the efficacy of the vaccine.

The group of Segal (Eckstein et al. 1997) investigated the use of liposomal particles as carriers of the *C. albicans* ribosomes. The efficacy of ribosomes incorporated into liposomes, with and without addition of lipid A, was compared to that of ribosomes, and incomplete Freund's adjuvant (IFA), revealing that the liposomal preparations were as effective as ribosomes and IFA. Thus, *C. albicans* ribosomal particles have the potential for human use.

Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was found to be a surface antigen (Gil-Navarro et al. 1997) that binds to fibronectin and laminin (Gozalbo et al. 1998) and is expressed in vitro and in infected tissues (Gil et al. 1999). The possibility to generate 'humanized' antibodies, using *Candida* GAPDH as an immunogen, has been recently suggested (Priel et al. 2002).

PATHOLOGY AND HISTOPATHOLOGY

Many *Candida* spp., notably *C. albicans*, are part of the normal microflora of various mucosal surfaces. Consequently, pathology caused by these yeasts is a result of one or more underlying predisposing factors which:

- impair, to various degrees, the immune response to these microorganisms (metabolic diseases, AIDS, immunosuppressive chemotherapy)
- produce an imbalance, in favor of the fungal microflora (antibacterial drugs), or
- damage the integrity of the integument (surgery, intravenous catheters).

The macroscopic and microscopic appearance of lesions caused by *Candida* spp. is primarily influenced by the interaction of three factors:

1. the site of infection
2. the pathogenicity of the infecting microorganism and to a lesser extent its species
3. the competence of the host's immune system.

Consequently, pathological and histopathological features of *Candida* infections are highly variable: the same microorganism may cause dissimilar pathological lesions in patients whose immune system is intact and in those who are in a state of immunosuppression. Nevertheless, certain common pathological characteristics of *Candida* infection may be described (Salfelder 1990; Luna and Tortoledo 1993)

Superficial infections

Superficial infections result from invasion of the superficial layers of skin and/or mucosae by the microorganism. Macroscopically these infections are characterized by the formation of a grayish plaque, surrounded by edema, which, at histopathological examination, consists of the infecting microorganism, neutrophils, and cell debris.

In deeper ulcerative lesions of the mucosae, such as those of the digestive system (especially the esophagus) often observed in immunosuppressed patients, the infecting microorganism may be present in the submucosa and may reach blood vessels and be disseminated hematogenously. The cellular reaction to the infection is limited or absent, and necrosis, typically accompanied by hemorrhage, are the salient microscopic features of *Candida* infection in these patients.

A special form of superficial infection, chronic mucocutaneous candidiasis, is characterized by the formation of warty proliferation of the skin, which results from hyperkeratosis and epithelial hyperplasia. Granulomatous tissue may be formed in the subcutis.

Deep infections

Several parenchymatous organs may be involved. These infections are characterized by microabscesses, macroscopically similar to miliary tuberculosis in which the microorganism may be individuated as blastospores and/or pseudohyphae (the latter absent in *C. glabrata* infections), neutrophils, and mononuclear cells, and a necrotic center. Granulomata with giant cells and lymphocytes may be formed in chronic infections.

Although various organs (such as liver, spleen, and brain) (Figure 3) may be affected in disseminated *Candida* infections, kidneys seem to be target organs of *C. albicans*, presenting nephritis, pyelonephritis, and, more rarely, papillary necrosis. Infection may be ascending or hematogenous. The former is a frequent sequel to prolonged catheterization and antibacterial therapy. It is characterized by small white lesions, especially in the medullar part of the kidney. Histopathological examination of these lesions reveals the presence of the infecting agent in the tubules, invading the surrounding parenchyma through the tubule's wall. When the spread of the infecting agent is hematogenous, the lesions are evenly distributed between cortex and medulla, lesions in the lower urinary tract are typically absent, and, microscopically, fungal elements may be noticed in the glomeruli.

Candida endocarditis, often a sequel to intravascular catheterization or prosthetic valve implantation, is characterized by vegetations on the surface of the endocardium, especially the mitral valves. The composition of these vegetations may vary and they consist of blood clots, cell debris, and fungal elements. Microabscesses may develop in the myocardium and evolve, in chronic cases, to granulomata.

Liver and spleen lesions are typically larger than those observed in other organs and may be detected by computerized tomography or other related techniques. Microbscesses characterize the acute stage of the infection, whereas granulomatous lesions without many organisms may develop in the chronic forms.

CLINICAL ENTITIES

Candida species can cause a range of clinical forms, from superficial manifestations involving skin, nails, and mucosal surfaces to deep-seated infections involving various internal organs and to disseminated disease (Odds 1988; Rippon 1988; Kwon-Chung and Bennett 1992; Bodey 1993; Edwards 2000). For the sake of simplicity, clinical entities can be subdivided into two large groups:

1. mucocutaneous candidiasis
2. deep-seated candidiasis.

Mucocutaneous candidiasis

Mucocutaneous candidiasis can be further subdivided into:

- cutaneous infections
- nail infections
- mucosal infections,

each group including a number of specific clinical forms.

It is evident that such a division is, to a certain degree, artificial, since in certain clinical forms, more than a single system can be involved. For example, patients suffering from CMC can present clinical manifestations on the glabrous skin, on mucosal surfaces, and in the nails. This may also apply, to some degree, to nail infection, which can include also the surrounding skin area (paronychia).

CUTANEOUS INFECTIONS

Based on the dermatological classifications (Goslen and Kobayashi 1993), the major syndromes considered here are:

- candidal intertrigo
- interdigital candidiasis
- perianal (diaper) rash
- candidids
- CMC.

As tabulated by Odds (1988), *C. albicans* is the most commonly isolated species from most forms of cutaneous involvement. However, other pathogenic *Candida* spp., such as *C. tropicalis*, *C. guilliermondii*, *C. kefyr*, *C. krusei* and *C. parapsilosis* are also known to be involved. If not otherwise specifically stated, the descriptions given below apply to *C. albicans*, as well as to other *Candida* spp.

Candidal intertrigo

Candida intertrigo is believed to be the most common clinical form of the cutaneous infection, as *Candida* spp. readily colonize skin folds, particularly in moist and macerated sites (Hymes and Duvic 1993; Edwards 2000). These may include ear folds or the submammary, crural, and axillary areas. The lesions are erythematous, with vesicles and pustules, in combination with pruritus.

These infections are frequently associated with specific predisposing conditions (Odds 1988; Wagner and Sohnle 1995), such as obesity, diabetes mellitus (Montes 1992), various endocrine disturbances, HIV infection

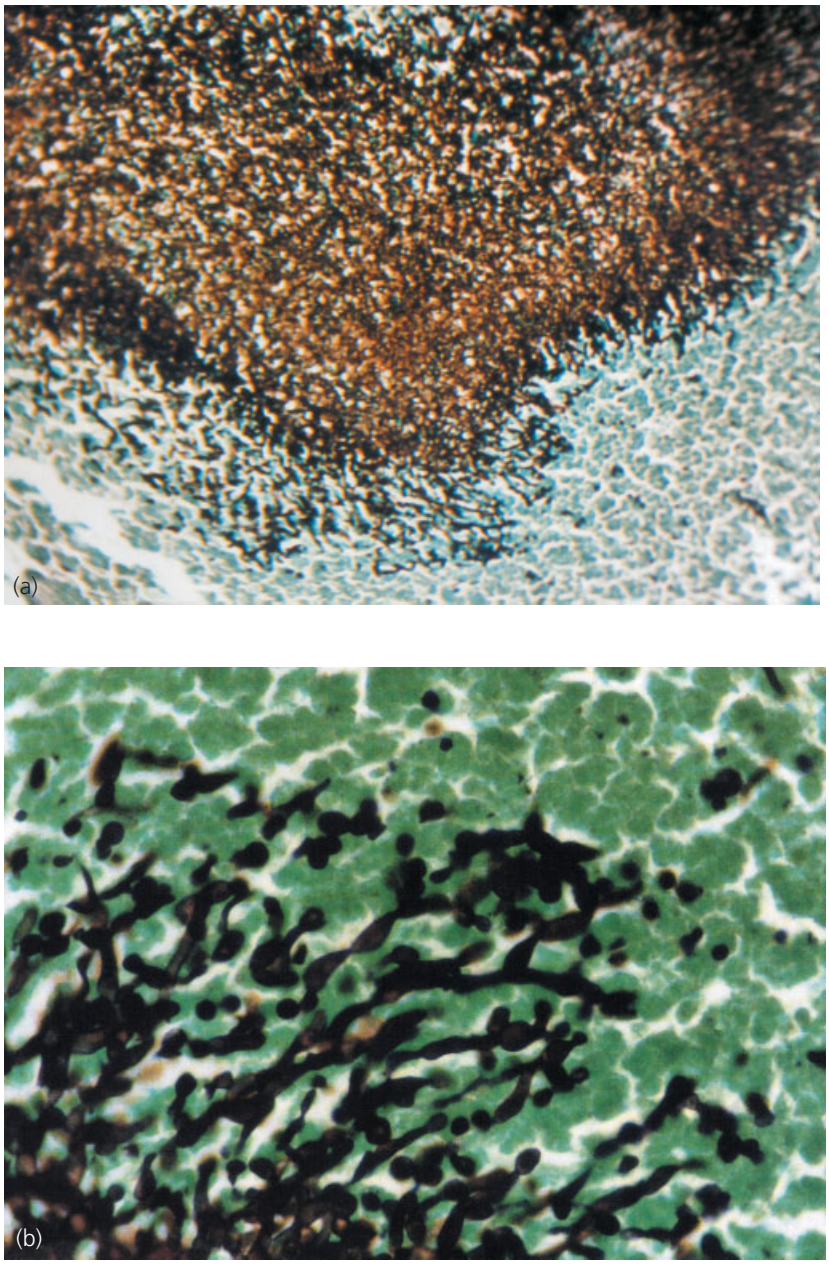

Figure 3 *Hepatic candidiasis:* **(a)** *candidal lesion;* **(b)** *candida mycelial and yeast elements. GMS. (Courtesy Professor Salfelder, Venuzuela)*

(Odds et al. 1990) or various iatrogenic factors including antibacterial and steroid therapy.

Interdigital candidasis

Among the intertriginous areas affected by *Candida*, the skin folds between the fingers of the hands should be particularly mentioned. In this form the skin folds between the fingers are macerated and itching. This condition is associated with excessive exposure to moisture, and thus seen particularly among dishwashers, bartenders, and fruit cannery workers. A similar erosive rash may occur between the toes in hot climates.

Perianal (diaper) rash

Another clinical form associated with *Candida* is that involving infants wearing diapers, in whom rashes are seen in the perianal area and on the buttocks. It should, however, be pointed out that diaper rash is not associated exclusively with *Candida*, and it is possible that the organisms are secondary to a preexisting inflammatory condition.

Candidids

A less commonly seen clinical form involves lesions mediated by the host's immune response to the infection – the candidids (Rippon 1988). These may be considered allergic responses, similar, albeit less frequent, to that noted in dermatophyte infections (the dermatophytids). Generally, with the treatment of the *Candida* infection, the candidid reaction clears.

Chronic mucocutaneous candidiasis

This paragraph will refer only to the cutaneous manifestations of chronic mucocutaneous candidiasis (CMC), while the other manifestations (mucosal and nail), which can also be associated with this form, as stated above, are covered later under the appropriate sections.

CMC is a relatively rare condition, however extensively studied, particularly by Kirkpatrick (1989, 1993), since it is considered the most severe clinical entity of the superficial candidiasis forms, and associated with a set of specific predisposing factors. Most of the cases are known to be caused by *C. albicans*.

The predisposing factors include, generally, congenital immunological or endocrinological defects. The immunological defects are mostly in CMI, which can be either at the stage of general or specific antigen recognition, and/or response to the antigen. This can be expressed in defects of T-lymphocyte activation and/or cytokine production leading to impairment of macrophage activation.

Clinically, the entity is characterized by the presence of persistent lesions, with high rate of recurrence, starting in early childhood and possibly persisting throughout the individual's lifetime. Lesions can be seen on various skin sites, and are not limited particularly to skin folds as in the other cutaneous forms of candidiasis. CMC can appear in a generalized form or can be localized and assume a form of hyperkeratotic lesions – '*Candida* granuloma.'

An interesting feature of CMC, from which conclusions as to the immune-defense mechanisms in candidiasis have been drawn, relates to the phenomenon that CMC patients, who may suffer from extensive cutaneous, nail, and/or mucosal infections, do not develop deep-seated or systemic candidiasis. It should be added in this context that AIDS patients, who frequently also suffer from severe and repeated mucocutaneous candidiasis, and may thus be considered in a similar category as CMC patients (Filler and Edwards 1993), do not generally develop systemic candidiasis.

This observation led to the hypothesis that, apparently, the immune-defense mechanism in candidiasis differs for the mucocutaneous versus the deep-seated forms (Domer and Lehrer 1993). It is thus believed that, while the resistance to the mucocutaneous forms is based, primarily, on intact and adequately functioning CMI, particularly, that associated with T-cell activity, resistance to deep-seated and systemic candidiasis involves additional factors. The CMC condition, in addition to serving as a relevant 'in vivo model' for providing valuable insights on the immune-defense mechanisms in candidiasis, was also one of the few entities in which various immunomodulating substances, such as transfer factor (Kirkpatrick et al. 1976; Mackie 1979) or thymosin (Wara and Amman 1978), have been evaluated as possible therapeutic approaches.

NAIL INFECTIONS

Under this section two syndromes are discussed: *Candida* onychomycosis and paronychia (Haneke 1986; Hymes and Duvic 1993).

Paronychia

Paronychia is an infection of the nail folds. It is a condition characterized by an inflammation with painful, erythematous, and swollen nail folds. It generally appears around the nails of the hands and less frequently on those of the feet. Nail plate involvement can follow, but not necessarily. *C. albicans* is the major etiologic agent; however, *C. parapsilosis* and *C. guilliermondii* are also recognized agents of this syndrome. Secondary bacterial infections are a known complication.

Paronychia is often seen in patients who also suffer from erosio interdigitalis. Similar to erosio interdigitalis, it is associated with excessive water immersion and is therefore seen in a similar population segment: dishwashers, bartenders, and fruit cannery workers. It can thus be considered, to some degree, an occupational disease. CMC patients often suffer from paronychia. Paronychia is more frequent among women than among men.

Onychomycosis

As in paronychia, involvement of the nail itself by *Candida* is more often seen in nails of the hands. The affected nails may become discolored, eroded, brittle, detached from the nail bed, and painful. Generally there is less accumulation of debris under the nail plate than in dermatophyte onychomycosis; however, the condition is more painful. In most instances the infection begins with the nail fold involvement, although as indicated above, not all the paronychia cases do lead to onychia. Candida onychomycosis is caused by the same *Candida* species as paronychia, and is more frequent among women. It is seen in CMC patients and in immunosuppressed patients or those with symptomatic peripheral vascular disease.

MUCOSAL INFECTIONS

Involvement of mucosal surfaces by *Candida* is considered the most frequent clinical manifestation of

candidiasis. Although the term mucosal surfaces encompasses in the strict sense of the word mucosal membranes of various organs, what is generally understood by the term mucosal candidiasis is involvement, primarily, of the oral and vaginal mucosa. Thus, the following section covers *Candida* infections of these two mucosal sites, while additional mucosal manifestations will be dealt elsewhere, under the appropriate sections.

Oral candidiasis

Candidiasis of the oral mucosa (Samaranayake and MacFarlane 1990; Lynch 1994; Cannon et al. 1995) is a disease recognized since antiquity, which has gained renewed significance more recently as an infection frequently seen in AIDS patients and in other conditions. As cited by Samaranayake and Macfarlane (1990) in their monograph on oral candidiasis, the renewed interest in this infection is expressed in the large number of scientific papers published in the last two decades.

Although *C. albicans* is, as in other types of *Candida* infection, the most frequently isolated etiological agent, additional *Candida* spp., such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. guilliermondii*, are also implicated in oral candidiasis (Bodey 1993). It should be emphasized, however, that these species can also be isolated from the oral cavity of normal individuals without obvious pathology. This observation, which demonstrates the complexity of the diagnosis, is relevant also to other anatomic sites, such as the vagina.

Oral candidiasis can be clinically classified into several different forms:

- acute pseudomembranous candidiasis
- acute atrophic candidiasis
- chronic atrophic candidiasis
- chronic hyperplastic candidiasis
- angular cheilitis.

Oral candidiasis of several clinical forms, mostly of chronic nature, is seen in patients with CMC. It can assume the pseudomembranous or hyperplastic form, with or without angular cheilitis. It can also, similar to the situation in AIDS patients, spread to the esophageal mucosa.

Acute pseudomembranous and acute atrophic candidiasis

Acute pseudomembranous candidiasis, known also under the term oral thrush, is manifested by white-gray lesions on the gums, tongue, or oral mucosa, which can appear as single lesions or as confluent large plaques. The lesions, particularly when covering larger areas, may be painful, and disturb intake of food. They may spread to the mucosa of the esophagus, as seen in a significant number of AIDS patients, and cause dysphagia. In addition to AIDS or cancer patients and otherwise debilitated individuals, the infection is generally present in infants, particularly those born to mothers suffering from vaginal candidiasis, and in the elderly.

Acute atrophic candidiasis is characterized by painful, erythematous mucosa, particularly on the tongue, which can be associated with loss of the tongue papillae, affecting food intake. Acute atrophic candidiasis may follow the acute pseudomembranous form with the disappearance of the pseudomembranous lesions. It can appear as a sequelae of antibiotic treatment.

Chronic atrophic and hyperplastic candidiasis

Chronic atrophic oral candidiasis, known also as denture stomatitis, is frequently seen in elderly individuals (Cumming et al. 1990), particularly those wearing dentures, and is reported in a very high proportion of this group. It is believed to be the most common clinical form of oral mucosa involvement. Clinically it is characterized by erythema and/or edema of the mucosa under the dentures, and generally, contrary to acute atrophic candidiasis, is not painful.

The chronic hyperplastic form, termed also *Candida* leukoplakia, is a much rarer condition. It presents with white plaques, which can appear on various sites of the buccal mucosa, and contrary to the acute pseudomembranous form, can not be removed. The importance of this clinical entity lies in the association with possible transformation into a malignant state.

Angular cheilitis

Clinically this form is characterized by erythema and fissures at the folds of the corners of the mouth. It may accompany other clinical forms of oral candidiasis, such as denture stomatitis or oral thrush. When accompanying denture stomatitis its appearance is attributed to unfitting dentures, which create the appropriate physical conditions for development of this type of infection. It is also believed that angular cheilitis may be associated with avitaminosis.

VAGINAL CANDIDIASIS

Candidal vulvovaginitis (Odds 1988; Sobel 1992a, b, 1993; Edwards 2000) is a common female infection, primarily during the fecund period. It is believed to be the most frequent or second most frequent vaginal infection, depending on geographical area, and its incidence is estimated in the range of 5–20 percent. The incidence increases in particular groups, such as pregnant or diabetic women, those using oral contraceptives or who are post antibiotic treatment. The predisposing factors believed to be associated with this specific condition can be associated primarily with the host and also with some virulence attributes of the pathogen. The former may include hormonal effects as seen in pregnancy, immunological or nutritional factors (Witkin et al. 1983, 1986, 1989). The latter may be correlated with certain fungal

factors, such as increased adherence ability (Segal et al. 1984).

Besides *C. albicans*, *C. glabrata* and *C. tropicalis* are the most frequently isolated *Candida* spp. (Horowitz et al. 1992) both from vulvovaginitis patients and from healthy carriers. Again, as already stated, here too the problem of microbiological diagnosis is demonstrated.

Clinically the syndrome includes complaints of vulvovaginal pruritus and discharge, which can be thick and 'curd-like' or thin. The infection may be associated with erythema of the vulvovaginal mucosa and possibly also of the perianal area. The lesions on the mucosal surface are basically adherent plaques. The condition may cause discomfort and pain during intercourse.

An important feature of candidal vulvovaginitis is the recurrence of the infection, although the majority of vulvovaginitis cases will respond to treatment. Chronic, recurrent vaginal candidiasis poses a medical problem and was quite extensively studied, as to possible risk or predisposing factors, and fungal reservoir (Sobel 1992b).

Sexual transmission to a male partner is known (Sobel 1993). In the male the infection presents, generally, as a balanitis with lesions and erythema on the penis.

Deep-seated candidiasis

The term deep-seated candidiasis (Odds 1988; Bodey 1993; Edwards 2000) refers to infection of visceral organs and possibly to multiple-organ or disseminated disease. Basically, any organ or system can be affected, thus the clinical entities may include: candidiasis of the gastrointestinal tract, respiratory system, central nervous system, renal and urinary tract, cardiovascular system; hepatosplenic candidiasis; hematogenous disseminated disease; ocular infections; and a variety of other specific manifestations. As it is impossible to cover all the clinical manifestations, the description here is limited to the major clinical entities.

CANDIDIASIS OF THE GASTROINTESTINAL TRACT

Esophagitis

This syndrome (Musial et al. 1988) includes painful dysphagia and possibly chest pain. Additional symptoms may include nausea and/or vomiting. White patches, which resemble those of oral candidiasis, can be noted by endoscopy on the esophageal mucosa. Esophagitis may be associated with presence of oral candidiasis, but may also present as a separate clinical entity without oral involvement.

The frequency of this syndrome increased since the appearance of AIDS. It is estimated that 10–30 percent of AIDS patients with oral candidiasis may suffer also from candidal esophagitis. The infection is also seen in cancer patients following anticancer therapy.

Gastrointestinal candidiasis

As stated earlier, *Candida* spp., primarily *C. albicans*, *C. glabrata*, or *C. tropicalis*, colonize the gastrointestinal (GI) tract in a significant proportion of normal individuals (Stone et al. 1973; Bolivar and Bodey 1985; Odds 1988) as judged by fungal isolation from feces. An increase in the percentage of colonized individuals is noted among hospitalized patients (Kusne et al. 1994).

Clinical involvement of mucosal surfaces of the stomach and/or small and large intestine, with mucosal white plaques and ulcerations are found primarily in cancer patients. A large survey conducted by Eras et al. (1972) on cancer patients, including postmortem studies, revealed involvement of stomach and intestines.

As already mentioned, *Candida* GI colonization and infection are believed to play a role in the pathogenesis of development of disseminated candidiasis. The GI tract can serve as a reservoir for the fungus, from where it can spread, particularly if there is a breach in the intactness of the mucosal lining. In patients with malignancies, there are several factors contributing to an increased risk of dissemination. These include increased *Candida* GI colonization, as these patients are generally treated with antibiotics, which change the GI flora in favor of *Candida*, and impairment of the GI mucosa, mostly due to anticancer therapy. Furthermore, such individuals may also have deficiencies in the activity of the immune system, both due to the neoplastic process itself and as possible sequelae of the anticancer treatment, therefore being unable to overcome the infectious agent once it was introduced. Thus, GI colonization and infection in cancer patients or in other categories of debilitated individuals, such as organ transplant patients (Kusne et al. 1994), is of particular significance and should be monitored and, if possible, controlled.

The possibility that *Candida* colonization may cause diarrhea is speculative, although reports in this regard are found in the literature (Talwar et al. 1990; Enweani et al. 1994; Levine et al. 1995).

Candidiasis of the liver, spleen, and other organs

Hepatosplenic candidiasis, which can be considered also as part of a disseminated infection, became a recognized clinical entity recently (Haron et al. 1987; Thaler et al. 1988; Edwards 2000). It is seen primarily in leukemics, with the hepatosplenic involvement becoming apparent generally during recovery from the neutropenic state, while the infection started during the patient's neutropenic period. This clinical entity is difficult to diagnose and manage. Diagnosis can be assisted by computed tomography (CT) scans demonstrating lesions in the liver and the spleen, and by biopsied tissues revealing the presence of fungal elements. Most cases are caused

by *C. albicans*; however, infections by other species have also been described (Pines et al. 1994).

Other organs of the GI system affected by *Candida* include the gallbladder and pancreas. In this connection peritonitis due to *Candida* should also be mentioned, which occurs particularly in patients on peritoneal dialysis (Yuen et al. 1992; Amici et al. 1994).

CANDIDIASIS OF THE RESPIRATORY SYSTEM

Respiratory *Candida* infections involving the lungs or bronchial system appear predominantly in patients with underlying primary diseases (Masur et al. 1977; Gueteau et al. 1991). Bronchopneumonia can originate from hematogenous spread of the fungus as part of a disseminated infection or from introduction of the pathogen into the lung. Diagnosis is difficult as *Candida* can be found in the sputum of individuals without candidiasis, so that it is pertinent to demonstrate the presence of the fungus in bronchopulmonary tissues.

CANDIDIASIS OF THE CARDIOVASCULAR SYSTEM

Candida spp. can cause clinical manifestations in various organs of the cardiovascular system (Musial et al. 1988; Kwon-Chung and Bennett 1992; Edwards 2000): the pericardium, myocardium or endocardium, with endocarditis being the best-known clinical entity.

Endocarditis (Hallum and Williams 1993; Wilson H.A. et al. 1993) is seen primarily in intravenous drug addicts and in individuals with impaired or prosthetic heart valves. It was also described in patients following various cardiac surgery procedures or as sequelae of anti-cancer therapy. Due to the increase in drug addiction and frequency of cardial surgery, an increase in the incidence of *Candida* endocarditis was noted.

Endocarditis can be caused by *C. albicans* and other species, such as *C. parapsilosis* (Cancelas et al. 1994) and *C. tropicalis*. Interestingly, non-*albicans Candida* infection is seen mostly in heroin addicts. It is possible that the infection originates from contaminated heroin. In the other susceptible groups, such as those with impaired or prosthetic valve patients, the infection may originate from introduction of the fungus during the surgical procedure, with the prosthetic or impaired valve serving as a suitable substrate to which *Candida* can readily adhere and form the typical vegetations.

Clinically, candidal endocarditis is difficult to differentiate from that caused by bacteria. It may, however, have a more prolonged onset; e.g. in post-surgery patients it may become apparent months later. The patients present with fever, heart murmurs, possible blood vessel obstruction, splenomegaly, and typical endophthalmitis. Pathologically, *Candida* endocarditis is characterized by large vegetations, from which emboli may be released.

RENAL AND URINARY TRACT CANDIDIASIS

Lower urinary tract infection

Candida lower urinary tract infection (UTI) (Musial et al. 1988; Gentry and Price 1993) is quite frequently seen in association with indwelling catheters, and may originate from the GI tract or genital flora. It is, thus, more frequently seen in females. The infection is also found in diabetics.

The symptoms are not unique and the course of the infection can be mild or more severe. An interesting clinical feature is the possible formation of fungal masses, 'fungus balls' (Scerpella and Alhalel 1994), which may cause obstruction and impair normal urine flow.

A basic problem associated with the diagnosis of this entity is the significance of candiduria in respect of discriminating infection from colonization due to contamination with flora of adjacent anatomic sites. Quantitative assessment of *Candida* colony-forming units (CFU.) in urine may be helpful, but does not always provide a definitive unequivocal answer.

Renal infection

Renal candidiasis (Edwards 2000), can, theoretically, originate from hematogenous *Candida* dissemination or as an ascending UTI. In reality, however, the latter is quite a rare event, and most of the cases of renal involvement come from hematogenous spread.

Pathologically, as mentioned in the relevant section, renal candidiasis is characterized by microabscess formation, mostly evident in the cortex of the kidneys. Similar pathology is noted in experimental *Candida* infections in animal models. The kidneys are a target organ for the fungus in infections induced by different ways of inoculation (intraperitoneal or intravenous).

CENTRAL NERVOUS SYSTEM CANDIDIASIS

Central nervous system (CNS) involvement by *Candida* (Walsh et al. 1985) is uncommon and is limited predominantly to *C. albicans*. The most susceptible individuals are AIDS patients and premature infants (Fakes 1984; Baley et al. 1986; Hughes et al. 1993; Edwards 2000). The CNS is generally seen as part of disseminated candidiasis involving in most cases primarily the meninges, although abscess formation in brain tissue was also reported.

Candida meningitis patients may present with symptoms similar to those seen in meningitis of other etiology, including various neurological abnormalities. *Candida* organisms may be detected microscopically in direct smears from the cerebrospinal fluid (CSF). In addition, abnormal protein and/or sugar values may be found in the CSF.

DISSEMINATED CANDIDIASIS AND CANDIDEMIA

Disseminated candidiasis can be defined as a multiorgan infection including possible candidemia, although blood cultures do not always yield the fungus. As already stated in the previous sections, disseminated candidiasis may include involvement of the CNS, kidneys, heart, eyes, or other organs and systems. As previously indicated, hepatosplenic candidiasis, although dealt with in an other section, is a specific clinical manifestation of disseminated infection.

Disseminated candidiasis caused by *C. albicans* and other species is associated with debilitation. It is seen in cancer patients (Musial et al. 1988), particularly those with acute leukemia (D'Antonio et al. 1992), in patients post-surgery, particularly GI and cardiac surgery, in transplant patients, particularly bone marrow transplants, in premature infants (Walter et al. 1990), burn patients (Green et al. 1994), and drug addicts.

Candidemia may present with the unspecific symptoms of a septic state, including fever. Presence of cutaneous and ocular involvement may be helpful diagnostic criteria. Candidemia patients may present (Edwards 2000) with nodular lesions on the skin and typical white lesions in the retina. These criteria are of particular importance, as cultural evidence of fungal etiology may be lacking.

Ocular *Candida* infections

Candida species can affect both the outer and inner eye (Langenhaeck and Zeyen 1993; Romano and Madjarov 1994). Infection may originate from hematogenous dissemination or from direct fungal introduction, the first, generally resulting in inner-eye infection, and the latter in clinical manifestations of the outer parts of the eye. Both categories of eye involvement are caused by *C. albicans* and some other *Candida* species, such as *C. parapsilosis*, *C. krusei*, and *C. glabrata*.

Outer-eye infections could also be considered in the section on superficial mucocutaneous candidiasis. These include infections of the conjunctiva (conjunctivitis), cornea (keratititis), eyelids (blepharitis), or lacrimal duct (lacrimal canaliculitis). Such infections may be associated with some ocular trauma, such as surgery, injury, or even the use of contact lenses. *Candida* infections may also follow other ocular infections, such as herpes simplex, or appear as sequelae of corticosteroid or antibacterial treatment.

Endophthalmitis, as indicated above, is generally a result of hematogenous fungal spread (McQuillen et al. 1992; Cohen and Montgomerie 1993), although it may also result from an exogenous source. The infection can cause loss of vision and is characterized by presence of typical white cotton-like lesions, which, as indicated, are an important diagnostic criterion for disseminated candidiasis.

ANIMAL DISEASE

Candida spp. may constitute part of the normal microflora in various anatomic sites, such as the digestive and genital tracts, skin, and ears (Yeruham et al. 1999; Duarte et al. 2001), of animals. Consequently, their involvement in pathological processes is secondary, often as a sequel to prolonged antibacterial chemotherapy, stress, and substandard management practices such as overcrowding and bad nutrition. Immune insufficiencies, of primary importance as predisposing factors to human mycotic infections, seem to have a more limited significance in animals. Nevertheless, the possibility of correlation between hereditary immunodeficiency in foals (McClure et al. 1985) and *Parvovirus*-induced immunosuppression in dogs (Anderson and Pidgeon 1987) has been suggested.

Most *Candida* infections, which have been described in animals, can be grouped into three principal categories as follows.

Candidiasis of the digestive tract

Esophagogastric candidiasis has been reported in various animal species. *C. albicans* and other non-specified *Candida* spp. have been isolated from pigs suffering from ulcers related to altered keratinization of the esophagogastric tract more frequently than from healthy animals (Kadel et al. 1969). The ability of *C. albicans* to utilize keratin as sole nitrogen source has been suggested as an explanation for this observation (Kapica and Blank 1957). Other possible predisposing factors to esophagogastric candidiasis in swine may be bad management, overcrowding, and antibacterial chemotherapy.

Other animals from which *Candida* spp. were isolated from esophagogastric lesions are foals (Gross and Mayhew 1983) and calves (Cross et al. 1970; Elad 1993). *C. glabrata* was found to colonize the abomasums of preweaned calves, possibly being involved in neonatal calf diarrhea (Elad et al. 1998). Colonization was more frequent and shedding more intensive in calves fed with milk replacers than in those fed dam's milk (Elad et al. 2002).

Candida spp., especially *C. albicans*, may be part of the normal flora of the avian digestive tract. However, they may be also involved as primary pathogens or as secondary invaders of damaged mucosa (such as is often the case in force-fed geese), in infections of various parts of this system, especially the crop. These infections may unsettle the function of the digestive tract and cause vomiting, increase in crop emptying time, and impaction. Antibacterial chemotherapy is assumed to play an important role as a prediposing factor to avian candidiasis by changing the microbial balance. The outcome depends primarily on the immune competence of the

host (Ritchie et al. 1994), influenced by environmental factors such as stress and captivity. *Candida* infections of birds, in sites other than the digestive tract, have been reported sporadically (Ritchie et al. 1994). In addition to *C. albicans*, other *Candida* spp. have been reported to be involved in avian candidiasis (Moretti et al. 2000), albeit more rarely.

Candidiasis of the urogenital tract

Candida spp. have been reported to be part of the normal flora of the genital tract of various animals (Chengappa et al. 1984), but have been associated with cases of bovine abortion (Foley and Schlafer 1987) and reduced fertility in cattle (Panangala et al. 1978; Sinha et al. 1980; Sutka 1983; Foley and Schlafer 1987; Tainturier et al. 1993) and horses (Abou-Gabal et al. 1977). The isolation of *Candida* spp. from aborted bovine fetuses is rare relative to other pathogens, including hyphomycetes, but has a worldwide distribution (Foley and Schlafer 1987). *C. tropicalis*, *C. albicans*, *C. krusei*, and *C. parapsilosis* are among the species isolated from such cases.

C. albicans as the causetive agent of cloaca and vent infections of geese and turkeys has been reported (Beemer et al. 1973; Marius-Gestin et al. 1987).

Candida mastitis

Candidal mastitis is usually self-limiting with spontaneous recovery and the infection has usually no systemic consequences. *Candida* spp. have been isolated from milk sampled from cattle with and without signs of mastitis. Most cases are sporadic but outbreaks have been reported (Elad et al. 1995). These were usually associated with intramammary instillation of contaminated antibacterial preparations, usually home made under nonhygienic conditions (Kirk et al. 1986). However, heavy environmental contamination with the yeast, in conjunction with insufficient milking hygiene, have been suspected of precipitating such outbreaks (Elad et al. 1995). All the yeast species dealt with in this chapter (and many others) have been isolated from cases of mycotic mastitis. *C. krusei* seems, however, to be more prevalent than other species in many reports. This might be the consequence of this species' relatively high environmental prevalence, especially in silage, where it may thrive during certain stages of anaerobiosis development, by using lactic acid as carbon source (Burmeister and Hartman 1966).

In addition, unique yeast infections, such as pyothorax (Fales et al. 1984) and urocystitis (Fulton and Walker 1992) in two cats, enteritis in a dog (Anderson and Pidgeon 1987), and keratitis in a horse (Desbrosse 1994), all caused by *C. albicans*, have been reported. In wild animals, *C. albicans* has been isolated from marine mammals (Sweeney et al. 1976), wild birds, marsupials, and reptiles.

LABORATORY DIAGNOSIS

Laboratory diagnosis of candidiasis depends upon the nature of the different clinical entities, specifically, whether they are mucocutaneous or deep-seated forms. Thus, the discussion of laboratory diagnosis will be in reference to the two groups of clinical manifestations.

Mucocutaneous candidiasis

Laboratory diagnosis of the different clinical forms of mucocutaneous candidiasis, which follows the clinician's diagnosis of suspected *Candida* involvement of the skin, nails, or mucosal surfaces, includes, generally, two steps:

1. direct examination of pathological specimen to demonstrate fungal presence
2. isolation of the fungus in culture and its identification.

DIRECT EXAMINATION

Skin and nail infections

Clinical specimens from diseased skin or nails can be collected by scraping the affected area, or, in some instances, by the use of swabs; material from nails can also be obtained by cutting the infected nail (Merz and Roberts 2003).

The preferred method for direct examination of clinical specimens from cutaneous and nail candidiasis is the wet mount technique. Specimens have, in most cases, to be treated with a keratinolytic substance, generally 10–30 percent KOH, which facilitates microscopic examination of the specimen. It is also helpful to add Parker's ink or the lactophenol cotton blue stain, which enables easier demonstration of the fungal elements. A valuable development in this regard involves the use of fluorochromes, such as calcofluor white or analogous products, with affinity for chitin and glucan, which makes the demonstration of fungal elements with a fluorescent microscope relatively simple, not requiring specific expertise (occasionally, nonspecific fluorescence, e.g. cotton fibers, may occur). Stained mounts, such as Gram staining of dermal and nail specimens, or histopathological sections are, generally, not recommended for routine use.

Direct microscopic examination of infected material is expected to reveal the presence of budding yeasts, pseudohyphae, and/or hyphae. The finding is indicative of candidiasis, but does not result in most cases in a definite speciation. The latter can be obtained only by isolation of the fungus in culture and its identification.

Mucosal infections

Specimens from patients with vaginal and oral infections can be collected by swabs (Merz and Roberts 2003) and kept, preferably, in transport medium before being processed in the laboratory. Specimens from oral lesions can also be obtained by scraping.

Processing of the specimen does not generally require treatment with a keratinolytic substance. Both wet and fixed mounts can be used. Wet mounts can be unstained, prepared in saline or water, or stained with lactophenol cotton blue or calcofluor white. Fixed mounts can be stained by Gram, methylene blue or Giemsa. As in the case of skin and nail infections, histopathological sections are not necessary for routine diagnosis of candidal vulvovaginitis or oral candidiasis.

Microscopic examination of specimens from vulvovaginitis or oral candidiasis will demonstrate the presence of budding yeasts, pseudohyphae, and/or hyphae. Demonstration of hyphal elements in direct microscopic mounts is important for the diagnosis, as both the vagina and the mouth are colonized also normally by *Candida* spp., and it is believed that the presence of the hyphal elements noted directly in the clinical specimen, in addition to the yeasts, is an indicator of infection (Figure 4). However, it should be taken into account that *C. glabrata*, a significant non-*albicans* species involved in vaginal infections, does not produce hyphae or pseudohyphae in clinical specimens.

In mucosal infections as well, microscopic examinations serve only as indications of candidiasis, without defining the exact etiology, which can be established only by culture. However, in this context, the significance of the direct examination for diagnosis should be emphasized. Since *Candida* spp. can be found in the mouth and vagina of healthy individuals, positive cultures without direct demonstration of the fungus in the clinical specimen are not always sufficient for diagnosis.

CULTURE

Isolation of *Candida*

The routine medium used for isolation of fungi in culture (Hazen and Howell 2003) from mucocutaneous infections is Sabouraud dextrose agar (SDA) supplemented with antibiotics (chlomphenicol, gentamicin, and/or tetracycline) to prevent bacterial overgrowth. It is also recommended to use an additional isolation medium – SDA to which cycloheximide (Actidione) has been added as well, a medium on which contamination by airborne molds can be limited. It is, however, not recommended to use the latter medium (commercially known as Mycosel or as Mycobiotic agar) as a single isolation medium, since some *Candida* are sensitive to cycloheximide and will not grow.

Candida from mucocutaneous sources are, generally, relatively easily isolated in culture. Cultures can be incubated at 28°C or/and at 37°C, and *Candida* colonies will be apparent within 2–3 days; in some cases growth will be noted already after 24 hours, but may take in others more than 3 days.

Identification and speciation

The description in this section is also relevant to the parallel one in diagnosis of deep-seated *Candida* infections.

It is advisable to obtain pure cultures prior to the identification process and to check by microscopy that the colonies reveal yeasts. Identification of *Candida* and

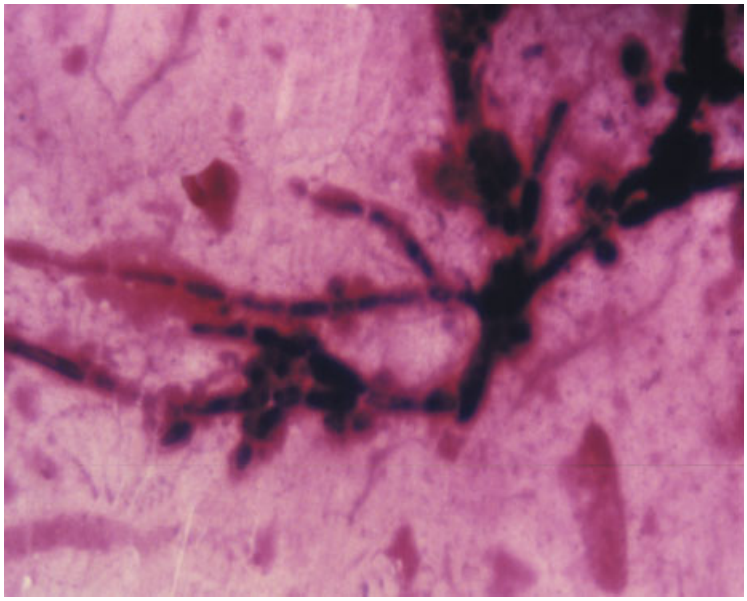

Figure 4 Candida *vaginitis: Gram's stain of clinical specimen*

its speciation is, generally, based on the following categories of criteria: macroscopic/microscopic morphology and physiological/biochemical characteristics; serology for antigenic identification of *Candida* spp. or serotypes can also be applied, although this is not done routinely.

Use of morphology for identification

Macroscopic morphology of *Candida* spp. (Kwon-Chung and Bennett 1992; de Hoog et al. 2000) on routine isolation medium (SDA), is rather similar. *Candida* spp. appear on SDA as smooth, in some species (e.g. *C. krusei*) more dry, cream colonies. Although fine differences between the species can be noted, they are, generally, very difficult to interpret and influenced by subjective judgment, and thus are not believed to be a valid criterion for routine *Candida* speciation.

A number of investigators (Quindos et al. 1992) have undertaken to develop specific media, which would enable, already at the isolation stage, investigators to differentiate, on the basis of macroscopic morphology, the most commonly encountered pathogenic *Candida* spp. The systems were, however, not specific enough or too difficult to standardize for a reliable method. An important development was introduced by Odds and Bernaerts 1994, consisting of the CHROMagar system, which uses chromogenic substances. It is based on the reaction between specific enzymes of the different species and chromogenic substrates, which results in formation of differently colored colonies. This system recommended by the producers for isolation and identification, permits, based on the color of the colonies, fast presumptive identification of *C. albicans*, *C. krusei*, *C. tropicalis* (Figure 5), and others. Of particular notice is *C. dubliniensis*, which grows on this medium in a darker shade of green than *C. albicans*, a characteristic that is helpful in differentiating between the two species.

Microscopic morphology of the culture on SDA isolation medium is also not reliable for species identification. The *Candida* spp. will appear as budding yeasts, and although some differences in the size and shape (oval or more elongated) do exist between the species (see section on Mycological characteristics below), they are too subtle to serve as a reliable, repeatable tool for routine diagnosis.

The commonly used differential medium both for genus identification as well as speciation, is the CMA plate (Dalmau plate) supplemented with Tween 80 or rice agar (de Hoog et al. 2000). Subcultures on CMA plates, made from the SDA isolation medium by furrowing the CMA plates, and incubated for 2–5 days at 28°C, will reveal, upon direct (inverted microscope) or mount microscopic examination, the presence of pseudo- and true hyphae, a characteristic of the genus *Candida* (except for *C. glabrata*, see section on Taxonomic comments, below). In addition, microscopic examination of CMA plates will also reveal species-specific characteristics, such as chlamydospores in *C. albicans* and *C. dubliniensis*, and the typical morphology of the different species (see description under section on Mycological characteristics, below). Both unstained and stained wet or fixed mounts, respectively, can be used to demonstrate the microscopic morphology formed on the CMA plates.

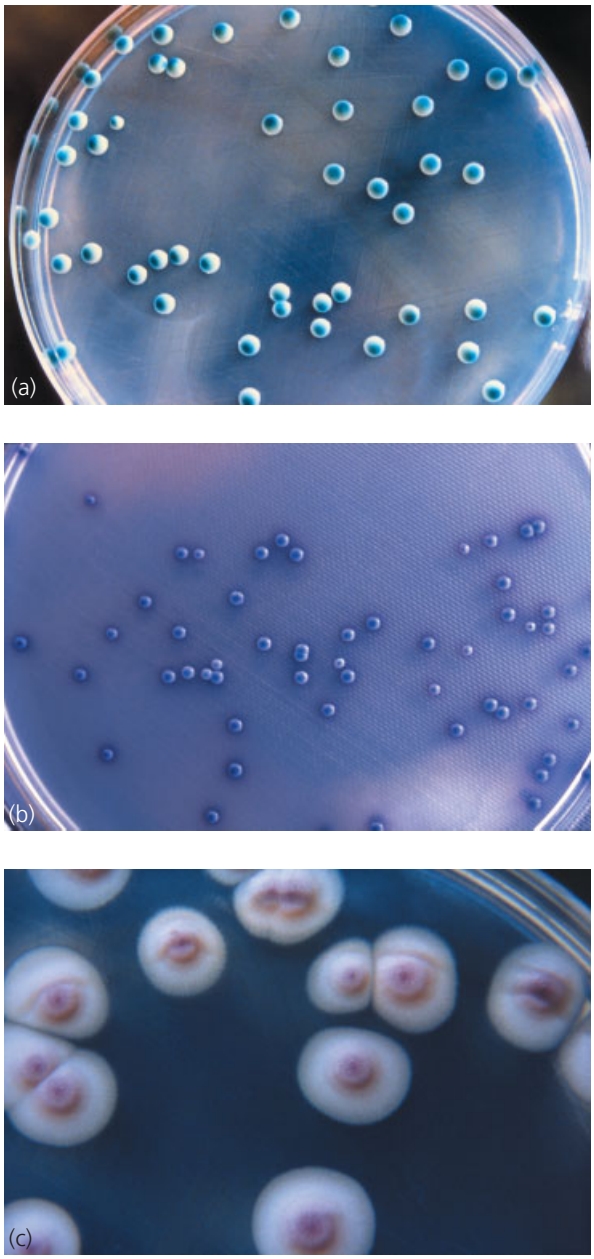

Figure 5 Candida *species on CHROMagar* Candida*:* **(a)** C. albicans*;* **(b)** C. tropicalis*;* **(c)** C. krusei

In the context of morphology, as a basis for species identification, the so-called 'germ tube test' (Merz and Roberts 2003) should be mentioned. The principle of this test is the ability of *C. albicans* or *C. dubliniensis* blastospores to produce, under defined conditions (generally, in serum incubated at 37°C for 2 hours),

germ tubes. The formation of germ tubes and chlamydospores is indicative of these two species and therefore offers reliable and easily performed tests for routine diagnosis.

Physiological/biochemical characterization

The genus *Candida* and the different *Candida* spp., similar to other yeasts, can be characterized by the pattern of utilization of specific carbohydrate and nitrogen substances (see section on Mycological characteristics below). As mentioned earlier, *Candida* spp. can utilize carbohydrates both oxidatively (assimilation) and anaerobically (fermentation). Yeasts possessing the ability to ferment a given carbohydrate do also assimilate that substance, but not necessarily vice versa. Thus, biochemical identification of *Candida* spp. is based primarily on assimilation and fermentation tests.

Various techniques have been developed for assessing the assimilation patterns. The classical Wickerham and Burton (1948) method assessed assimilation by determining the ability of a given yeast isolate to grow in a set of defined minimal liquid media supplemented with different carbohydrates. This method, which, although precise, was laborious and time consuming and therefore not suitable for routine use, was replaced in the clinical laboratory by the auxanographic technique (Hazen and Howell 2003). This technique employs minimal media agar plates on which paper disks impregnated with different carbohydrates are placed, and the growth ability of the yeast around a specific disk is an indication of the yeast's ability to assimilate that carbohydrate. This method is simpler and quicker, and therefore more adapted for routine use. Modifications of this principle are used by various commercial kits, such as the API 20C, API ID32C (Figure 6), Vitek, Minitek, Uni-Yeast-Tek, and others. These commercial systems are easy to use and some are automated. A number of studies focused on comparison of the various methods (Fenn et al. 1994; Riddle et al. 1994). Other methods, such as MicroScan, have also been described (Land et al. 1991; St Germain and Beauchesne 1991).

Fermentation tests are more difficult to perform. The classical tests involved liquid media supplemented with different carbohydrates, a color indicator to assess pH changes to measure acid formation, and a tool to assess gas production (generally an inverted small tube, the Durham tube), a criterion of fermentation. There are several modifications for assessment of gas production, such as use of semisolid media or a wax layer on top of the liquid medium. Most of the commercial kit systems do not use fermentation assays but rely on assimilation tests.

Other identification methods

Unlike diagnosis of mucocutaneous candidiasis, in the deep-seated forms isolation of the fungus may be problematic, its identification, once isolated, is relatively straightforward by the described methods. Nevertheless, additional methodologies for identification have been described.

Candida spp. can also be identified serologically, using specific antisera. A commercially available set of sera (Iatron System), prepared against *Candida* cell wall antigens (see section on Antigenicity below) enables quick species identification of the commonly encountered pathogenic *Candida* spp. by simple slide agglutination. However, serological identification is not used as a standard routine method in clinical laboratories for diagnosis of candidiasis.

Other non-culture identification methods may include molecular techniques (Buchheidt et al. 2000; Alexander 2002; Sullivan and Coleman 2002) such as the use of

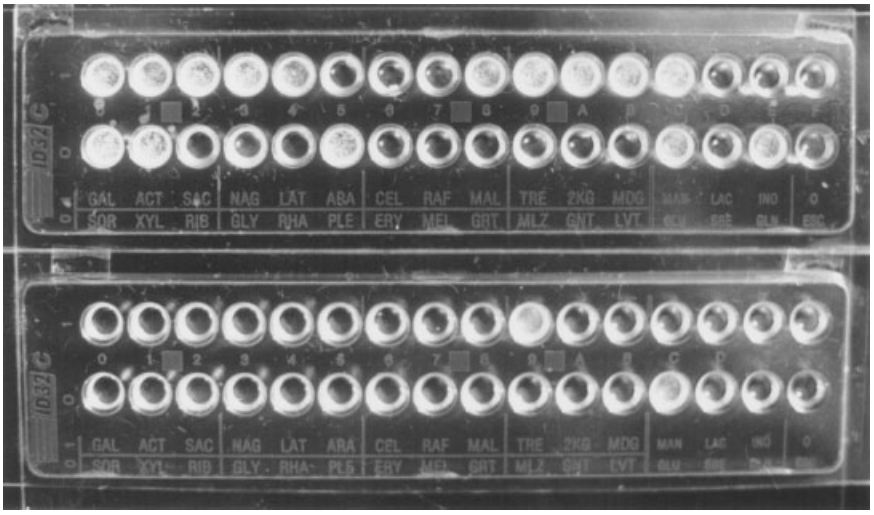

Figure 6 Candida *spp. in API 32C: upper panel,* C. albicans*; lower panel,* C. glabrata

electrophoretic patterns of DNA, RNA profiling, restriction enzyme analysis (Velegraki et al. 1999), and PCR (Niesters et al. 1993; Maiwald et al. 1994; Santos et al. 1994). Common molecular probes used by a number of investigator groups for identification of *Candida* at genus or species level are ribosomal DNA primers, including the large or small rRNA subunits (Haynes and Westerneng 1996). Additional probes described include SAP DNA (Flahaut et al. 1998), the *ERG11* gene involved in ergosterol biosynthesis (Posteraro et al. 2000), and others.

However, once again, these too do not yet, generally, constitute part of routine diagnosis.

Deep-seated candidiasis

Although the basic principles involved in the diagnosis of deep-seated and disseminated candidiasis are similar to those of the mucocutaneous candidiasis, the diagnosis of the deep-seated entities is more difficult and complicated, including various problems at the different steps of the process. While laboratory diagnosis of mucocutaneous candidiasis is based on direct examination of clinical specimen, followed by isolation and identification of *Candida* spp., that of the deep-seated forms involves in addition also use of immunodiagnostic and other noncultural methods.

DIRECT EXAMINATION

Due to the nature of the different clinical forms, collection of clinical specimens may be problematic. In some clinical forms it is difficult to obtain a sample from the infected organ or the specimen can be collected only by invasive methods. In others, such as in the case of samples from the respiratory tract, the demonstration of *Candida* in sputum, a sample which is easily obtainable, poses a problem in interpretation, as *Candida* is part of the oral flora; thus to have meaningful results, it is necessary to obtain samples by the invasive procedures of bronchial lavage or biopsy. Some clinical specimens, such as CSF, may require procedures of concentration due to the possibility of paucity of the pathogen in the clinical sample (Konneman et al. 1997). However, if samples from normally sterile sites are obtained, demonstration in such samples of the presence of yeasts and/or hyphal elements, indicative of *Candida*, is important for the establishment of diagnosis.

Direct demonstration of *Candida* in specimens from deep-seated candidiasis can be done both by wet and fixed mounts, aided by calcofluor white. Tissue biopsies can be prepared and stained by histopathological techniques (Salfelder 1990), using the periodic acid–Schiff (PAS) or the Gomori methamine silver (GMS) stain. An additional method using an immunohistochemical technique was described by Marcilla et al. (1999). These investigators used monoclonal antibodies (3H8), in identification of *C. albicans* (Marcilla et al. 1999).

CULTURE

Isolation of *Candida* species

A basic problem is the difficulty in isolating *Candida* in culture from specimens of deep-seated candidiasis, particularly if the infection is localized in an internal organ. The specimen that is most easily obtainable, which leads to the highest isolation rates, is blood cultures. However, even these are characterized by a relatively high proportion of failure to isolate the fungus (Jones 1990; Pfaller 1992a; Reiss and Morrison 1993). The most recommended methods for blood cultures include the lysis-centrifugation isolator system (Wilson M.L. et al. 1993; Kirkley et al. 1994), which apparently yields the highest rates of positive cultures (Telenti and Roberts 1989), the Bactec radiometric or nonradiometric systems (Yagupsky et al. 1990), and use of biphasic media (Richardson and Carlson 2002). Other specimens from normally sterile sites with lesser rates of positive cultures may include CSF, tissue biopsies, or bronchial washings. Such specimens are inoculated on Sabouraud's agar with antibiotic but without cycloheximide. Growth conditions are similar to those for specimens from mucocutaneous candidiasis. However, it is advisable to wait much longer periods (up to 3 weeks) before a decision is made that a culture is negative.

Identification of *Candida* species

Identification of the *Candida* isolated is done as in the case of mucocutaneous candidiasis. It is based, primarily, on macroscopic and microscopic morphology, and on physiological/biochemical characteristics of the isolates.

Immunodiagnosis

The basis for immunodiagnosis is the patient's immune response to *Candida*, as expressed by presence of antibodies or CMI, and/or presence of microbial antigens in the patient's body fluids. Contrary to the mucocutaneous forms of candidiasis, in which the use of direct examination of the clinical material and culture is quite satisfactory for diagnosis, in the deep-seated entities, these methods, as indicated above, are in many instances not sufficient. Thus, the search for additional approaches, such as the use of immunodiagnostic techniques or other non-culture methods, is warranted, and indeed this led to the development of some diagnostic procedures, primarily in the area of immunodiagnosis (de Repentigny 1992; Reiss et al. 2002).

Immunodiagnosis of candidiasis is based, primarily, on detection of the humoral immune response as expressed in antibody production, and detection of fungal antigens. As a consequence two types of assays are involved in immunodiagnosis:

1 tests for detection of antibodies, primarily in serum
2 tests for detection of the fungal antigen(s) in body fluids, again, primarily in serum.

Tests for detection of antibodies

The classical serology included assays for detection of serum antibodies by various modifications of the agglutination test. However, in view of the ubiquitous and commensal nature of *Candida* spp., antibodies to *Candida* detected by the agglutination technique, which generally represent antimannan antibodies, can be found in healthy individuals or in those with superficial infections (Lew 1989). Thus, this test is not discriminatory enough to be useful in the diagnosis of deep-seated candidiasis. By contrast, tests determining the presence of antibodies raised to *Candida* internal antigens, which, it is assumed, are released into the patient's body fluids, primarily during invasive infection, could be more discriminatory and therefore more useful for diagnosis. In addition, it should be considered that immunocompromised patients, who are the principal candidates at risk to develop systemic infections, have difficulties in mounting an antibody response at detectable levels.

Tests aimed at detecting antibodies to *Candida* antigens released and/or expressed during deep-seated infections, include the gel immunodiffusion (ID) or counter-immunoelectrophoresis (CIE) techniques to detect antibodies to *Candida* cytoplasmic antigens, which are standardized by their protein content (Reiss et al. 2002). Other systems used include the enzyme-linked immunosorbent assay (ELISA) or latex-agglutination (LA) test. Detection of antibodies to defined antigens believed to circulate in body fluids during infection, such as enolase (Mitsutake et al. 1994), heat shock proteins (47 kDa – breakdown of HSP90) Walsh and Chanock (1997), and more recently, specific mannosides (Poulain et al. 2002), although not widely used, has also been evaluated. Low sensitivity is the major problem of using antibody-detection methods in diagnosis, which may be either the result of the use of more discriminatory, less sensitive tests, such as the ID or the poor immune status of a significant proportion of the candidiasis patients.

Although attempts to use techniques that demonstrate the different immunoglobulin types for diagnostic purposes have been described (Fegeller et al. 1990), the presence of IgM anti-*Candida* antibodies as an indicator of a recent infection has, apparently, contrary to other microbial infections, no proven validity. Thus, the differentiation between types of immunoglobulins has limited diagnostic value in candidiasis and, consequently, is generally not carried out for this purpose. Moreover, determination of the specific immunoglobulin type cannot be used effectively for differentiating between the mucocutaneous *Candida* infections and the deep-seated candidiasis forms. The possibility of using antibody detection as a valid diagnostic means is dependent on the specific serological test, which measures the response to a specific candidal antigen rather than a specific immunoglobulin type. It should be emphasized, however, that precipitins detected by the immunodiffusion or counterelectrophoresis tests represent generally IgG antibodies, while other tests, such as hemagglutination, used in some laboratories, can detect the different immunoglobulin types (Fegeller et al. 1990).

Tests for detection of antigens

Detection of *Candida* antigens in body fluids is an important diagnostic tool, particularly in the immunocompromised patients who have difficulties in mounting antibodies at a detectable level (Walsh and Chanock 1997).

Although various techniques have been described (de Bernardis et al. 1993; Reboli 1993), currently the most widely used tests include LA and ELISA (Fujita and Hashimoto 1992). Some of these are commercially available, such as the Cand-Tec or Pastorex LA systems or the lately introduced Platelia system, a modified ELISA method. Antigen is generally detected in serum; however, it can be found also in other body fluids, such as urine (Ferreira et al. 1990). The antigens detected by the different systems include mannan (Pastorex LA), an undefined glycoprotein (Cand-Tec LA), a 47 kDa protein (HSP), enolase (ELISA), and specific mannosides (Platelia) (Walsh and Chanock 1997; Sendid et al. 2002). Generally, the antigen-detection tests are more significant for diagnosis as they detect active infection. Although as a rule they are sensitive tests, they still may not be sensitive enough to detect all the cases of antigenemia, particularly since some of the antigens are cleared quickly from the bloodstream. Another source for false-negative results may come from cases where the infective agent is one of the rarer *Candida* spp., due to the specificity of the antigenic determinants, which are absent or not recognized by the detecting reagent, which is generally an anti-*C. albicans* serum. It has been suggested that concurrent determination of both antigens and antibodies in clinical samples may increase the sensitivity of the diagnosis of systemic candidiasis (Sendid et al. 2002).

CMI as evaluated in vivo by a skin test to measure DTH to candidal antigen, or by in vitro assays such as lymphocyte transformation, are valid tools in assessing the immune competence of the patient, but do not contribute much to diagnosis of candidiasis.

In summary, although the immunodiagnostic techniques are associated with problems of standardization and interpretation, and cannot be used as the sole methods for laboratory diagnosis, they are, nevertheless, a helpful adjunct in the complex diagnosis of deep-seated candidiasis. The use of a combination of antigen-detection and antibody-detection tests is helpful in diagnosing the infection in both the immunocompetent and the non-immunocompetent patient.

Other nonculture methods

Since, as indicated above, in spite of the existence of culture and immunodiagnostic techniques, the diagnosis of deep-seated candidiasis is still problematic, the search for more sensitive and/or more specific methods continued. These included the possibility of detecting non-antigenic fungal metabolites released during infection into the patients' body fluids and, more recently, detection of fungal nucleic acids by sensitive molecular techniques.

Detection of fungal metabolites

Most studies in this area concentrated on detection of arabinitol as a candidal metabolite which is released during infection into the patient's body fluids, and which could possess a diagnostic value (Reiss and Morrison 1993). As arabinitol was first described by Kiehn and Bernard in 1979 as a fungal metabolite detected in the serum of patients with disseminated candidiasis, the techniques to detect this metabolite are in general applicable for serum, although arabinitol can be found also in urine. Arabinitol is released by most of the pathogenic *Candida* species, except *C. krusei* and *C. glabrata* (and not by *Cryptococcus neoformans*).

The techniques to detect arabinitol include the original method by which the metabolite was first detected, gas-liquid chromatography (GLC), and the more recent techniques involving enzymatic-fluorometric measurements (Reiss and Morrison 1993; Switchenko et al. 1994). Moreover, a kit for the latter technique has been described (Tokunaga et al. 1992). Serum arabinitol levels can also be elevated in patients with renal insufficiency and some other clinical conditions. In order to overcome this problem, determination of the serum arabinitol/creatinine ratio is advised.

Another *Candida* metabolite, suggested by Miyazaki et al. (1995) to be useful for diagnosis, is the cell wall component (1→3)-beta-D-glucan for which commercial kits already exist (Hossain et al. 1997). It should, however, be remembered that glucan is found in other fungi as well, therefore its value as a diagnostic tool is debatable (Verweij et al. 1998; Chryssanthou et al. 1999).

Molecular biology techniques

These techniques are based on detection of candidal DNA in patients' body fluids involving the use of specific DNA probes. This approach became more promising as a possible diagnostic tool with the development of DNA amplification techniques, particularly PCR. Recent review articles suggest the usefulness of such methodologies in diagnosis (Reiss et al. 2000; Turin et al. 2000).

Various possible probes have been described, such as the actin gene, the gene encoding cytochrome P450 14-lanosterol demethylase (Burgener Kairuz et al. 1994), the chitin synthetase gene (Jordan 1994), SAP genes (Flahaut et al. 1998), mitochondrial DNA or the candidal DNA repetitive elements (CARE). A widely used probe consists of the rRNA gene complex (Maiwald et al. 1994; Kappe et al. 1998). Some of these probes are species specific while others have wider specificity (Niesters et al. 1993). Although most of the studies aim for fungal detection in serum, some involve other clinical specimens such as urine or tracheal aspirate.

As already indicated in the section on Other identification methods above, such DNA probes can also be used for species identification after these have been isolated in culture. An example of such an approach is a study by Weissman et al. (1995) who used the benomyl resistance gene for *C. albicans* molecular identification.

Successful use of molecular techniques for detection of the fungal DNA in clinical specimens could possibly provide a sensitive means for early diagnosis. However, presently, these techniques are still mostly at the investigatory stage, and do not provide easily applicable methods for routine clinical use.

TREATMENT

Susceptibility testing

Susceptibility testing of fungi to antimycotic drugs, including that of yeasts, was until recently performed only rarely. This was a result of several factors:

- the cases necessitating systemic antifungal treatment were relatively rare
- the number of antifungal drugs was limited
- the incidence of resistance to the drugs was rare
- most importantly, correlation between in vitro and in vivo results was questionable.

These factors have changed: a marked increase in the incidence of mycotic diseases, especially disseminated infections, has been noted, the number of available drugs has increased, and continues to increase, and an innate or acquired resistance of certain yeast species to several drugs has been reported. Thus, collaborative efforts have been initiated, which have culminated in a proposal of a standard for broth dilution susceptibility testing of yeasts (but so far not yeast forms of dimorphic fungi) published as National Committee for Clinical Laboratory Standards (NCCLS) document M27-A2 (National Committee for Clinical Laboratory Standards 2002). The acceptance and use of these standards may, in analogy with similar standards for antibacterial drugs, lead to a better predictive value of in vitro susceptibility testing of antimycotic drugs.

The performance of in vitro fungal susceptibility testing poses considerable difficulties, including the test's procedure (i.e. necessity of adjusting the medium to the

drug, estimation of inoculum size) and particularly their interpretation (Cook et al. 1990; Odds 1993). Several additional problems make reliable antifungal susceptibility testing complicated. Among these are:

- the fact that different morphological cell forms of the same fungus may show different susceptibility to the same drug (azoles seem to be more efficient in inhibiting hyphal forms than blastospores of *C. albicans* (Fromtling 1988; Walsh and Pizzo 1988)
- low water solubility and diffusibility of the drugs as well as their instability (especially polyenes)
- the determination of endpoint minimum inhibitory concentration (MIC) in liquid media, is complicated for some drugs, especially 5-fluorocytosine and the azoles, by the 'trailing' phenomenon: a certain turbidity is noted in the broth above the MIC, indicating a partial growth inhibition (Espinel-Ingroff et al. 1992; Fromtling et al. 1993). To overcome this problem, it has been suggested that it is best to ignore the slight turbidity above the MIC or to use a 1:5 dilution of the drug-free control culture as a reference of 80 percent growth inhibition (Espinel-Ingroff et al. 1992).

Several methods may be implemented in the testing of fungal susceptibility, but only one, the macrodilution technique, has been standardized so far, and is detailed in the NCCLS publication.

Other methods include various microdilution techniques, the results of which, according to preliminary investigations (Espinel-Ingroff et al. 1992, 1995; Tiballi et al. 1995; To et al. 1995), do not diverge significantly from those of the macrodilution method. Tests on solid media include the agar dilution and agar diffusion methods. The diffusion method may be implemented by using drug-impregnated disks or strips, the latter giving an indication of the MIC. This is the principle of the commercially available Etest (Figure 7). In a comparison between results obtained by the Etest and the NCCLS standard method, differences for certain species or specific drugs were shown and the comparison varied, according to the drug tested, between 71 percent and 84 percent (Colombo et al. 1995).

In spite of these advances in standardization of antifungal susceptibility test methods, the most troublesome problem remains the predictive value of the results for therapy. In fact, discrepancies between in vitro results and therapy success still afflict antibacterial susceptibility tests, more than a decade after their standardization, making them useful primarily for the indication of drugs to which the tested microorganism is resistant and thus probably useless for therapeutic purposes. The increase in amphotericin B (Dick et al. 1980; Hadfield et al. 1987) and/or azole-resistant (see section on Prophylaxis below) clinical isolates of yeasts, suggests that indication of possible resistance will presumably also be the primary function of antimycotic susceptibility tests.

Therapy

The therapy of candidiasis, similar to laboratory diagnosis, depends on the type of clinical manifestation and is different for the mucocutaneous forms as opposed to the deep-seated entities. Thus, the topic of therapy will be discussed in the light of the two groups of clinical forms (Richardson and Warnock 1997; Edwards 2000).

THERAPY OF MUCOCUTANEOUS CANDIDIASIS

Patients suffering from cutaneous or mucosal *Candida* infections can in principle be successfully treated topically. The specific drugs include, primarily, the polyene nystatin, and the azoles miconazole, clotrimazole, and ketoconazole (Georgopapadakou and Walsh 1994). The polyene amphotericin B in a formulation for topical administration, and additional azole compounds, such as econazole, sulconazole, and terconazole, have also been used for treatment of mucocutaneous candidiasis (Richardson and Warnock 1997; Edwards 2000; Stevens and Bennett 2000).

Nystatin (Brajtburg et al. 1990) is poorly soluble in water, and the specific formulation may vary according to the clinical entity. Thus, nystatin can be administered as a suspension or gel for treatment of oral candidiasis, and it can also be applied in the form of a cream or suppository, for dermal or vaginal infections, respectively. The duration of treatment may also vary according to the clinical entity. So for nystatin, suppositories of 100 000 units/ml, a 14-day course, is recommended in the treatment of vaginitis (Bennett 1995), while the treatment of oral infection by nystatin suspension of similar concentration may require 2–3 weeks (Richardson and Warnock 1997). For the treatment of cutaneous candidiasis, such as candidal intertrigo or diaper rash, nystatin may be used as topical preparation in combination with anti-inflammatory agents. *Candida* nail infections do not, generally, respond to topical nystatin treatment, although such treatment may be helpful for the accompanying paronychia. Nystatin, as other polyenes, acts on the fungal cytoplasmic membrane through complexing with the ergosterol in the membrane. In the form of topical preparations it is nontoxic and usually devoid of side effects, except for the bitter taste of the suspension used in treatment of oral infection.

The azoles (Fromtling 1988) miconazole, clotrimazole, and ketoconazole can also be used in different formulations, such as lotion, cream, suppository, powder, or spray. They can be applied for the treatment of both cutaneous and mucosal involvement, but are not useful in treating nail infections. The advantage of the azole compounds over nystatin is that the necessary treatment duration is shorter. So a treatment regimen for candidal vulvovaginitis with miconazole cream would require a 7-day use, and for some azoles (e.g. tioconazole) even a

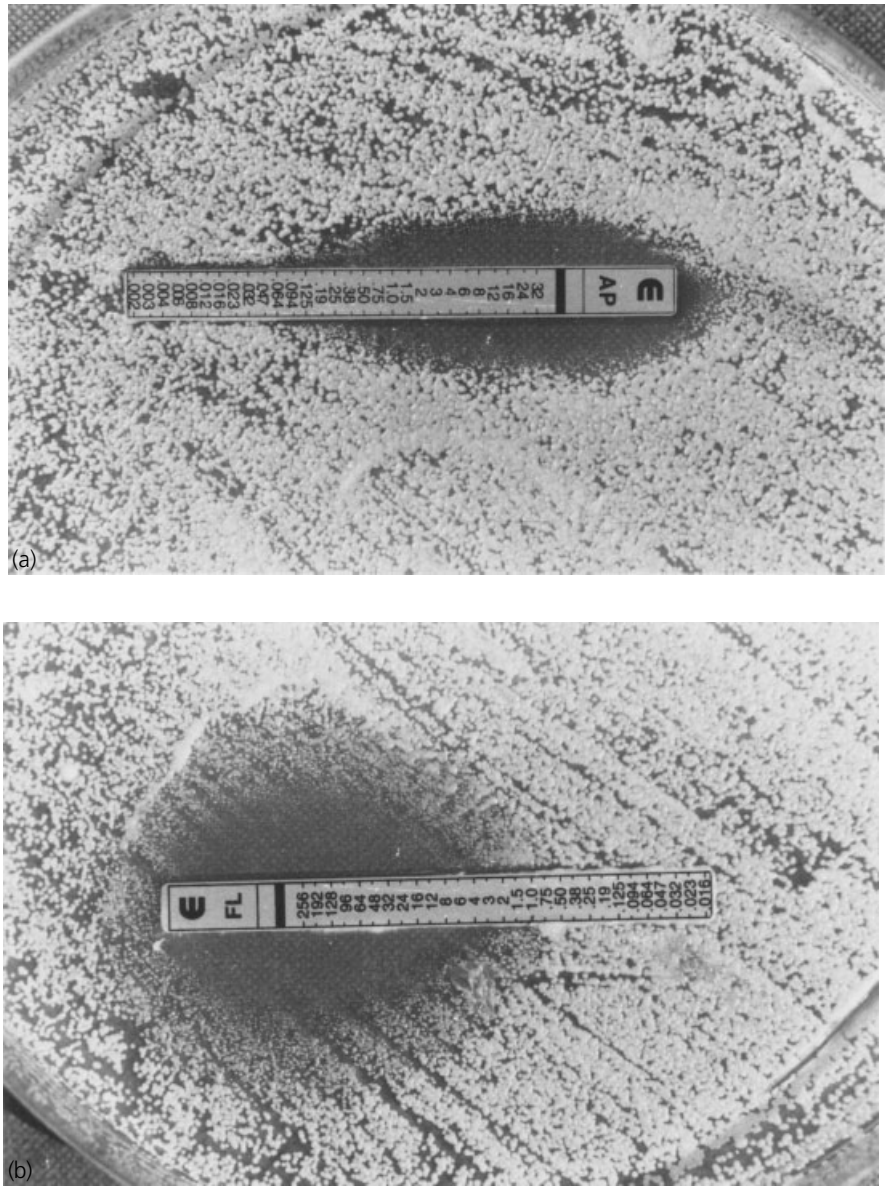

Figure 7 C. albicans *susceptibility testing by Etest:* **(a)** *amphotericin B (AP);* **(b)** *fluconazole (FL). Note the partial inhibition zone around the fluconazole strip.*

single-day treatment could be sufficient in comparison to a 14-day course for nystatin. An additional advantage of the azoles is their broad antifungal spectrum, which permits the use of these drugs even without laboratory confirmation of the candidal etiology of the infection. The azoles, as the polyenes, act on the fungal cytoplasmic membrane, however, through another mechanism – interference in the biosynthesis of ergosterol. Similar to the polyenes, they too, basically lack side effects when used as topical formulations.

Topical treatment will not suffice in nail infections, in CMC patients, in AIDS patients with *Candida* infections, or in cases of recurrent vulvovaginitis. These may require systemic oral therapy. The oral administration of the drugs can replace or be an adjunct to the topical treatment. The major antifungals administered orally for treatment of mucocutaneous *Candida* infections are primarily the triazoles: fluconazole and itraconazole (Bennett 1995). The use of these drugs is currently preferred over that of the oral preparation (tablets) of ketoconazole, as the use of the latter (see below) is associated with more adverse side effects and is not recommended for the treatment of vulvovaginitis (Stevens and Bennett 2000).

Tablets of fluconazole (Zervos and Meunier 1993) have been extensively used for therapy of oral and esophageal candidiasis in AIDS patients (Dupont and Drouhet 1988). Administration of 50–100 or 100–200 mg/day for 2 weeks is effective in the treatment of oral or esophageal candidiasis, respectively. However,

the problem in these patients is the high rate of relapses of the infection. Thus, fluconazole has been used for prolonged periods, as a prophylactic means of preventing relapses of the infection (Just-Nubling et al. 1991). Long-term administration and prophylactic use of fluconazole are also applied for the management of CMC and prevention of recurrences in cases of recurrent vaginal infections, respectively.

Oral use of fluconazole is basically not associated with major side effects (nausea or abdominal pain have been noted in a small percentage of individuals; Bennett 1995). However, prolonged administration of the drug can lead to induction of resistance and was expressed in the appearance of fluconazole-resistant *C. albicans* isolates (Sanguineti et al. 1993; Newman et al. 1994). In addition, fluconazole was also found to be inherently ineffective against certain *Candida* spp., such as *C. krusei* and *C. glabrata* (Hitchcock et al. 1993; Richardson and Warnock 1997). As a consequence, *Candida* speciation seems to be important in regard to therapy and it would be advisable to perform sensitivity testing prior to commencement of treatment.

Itraconazole (Sporanox) has a similar profile of use: AIDS patients, CMC, or vaginitis (Rees and Philips 1992). It has also been applied in treatment of *Candida* nail infections (Hay et al. 1988). As with fluconazole, itraconazole use is apparently devoid of significant adverse reactions. Cross-resistance with fluconazole has been reported (Richardson and Warnock 1997).

As indicated above, the application of the two triazoles is currently preferred over the oral use of ketoconazole (Nizoral), the first azole for systemic administration, due to possible side effects associated with the use of this drug. Administration of ketoconazole can be, albeit rarely, associated with severe hepatotoxicity (Lewis et al. 1984). Ketoconazole use can also interfere with steroid hormone synthesis and be expressed in reduced levels of testosterone (Hanger et al. 1988). Milder, but much more frequent, side effects include nausea and vomiting, at higher doses of the drug. Another problem associated with ketoconazole use is the relatively low absorption of the drug in AIDS patients due to lower gastric acidity in these patients (Richardson and Warnock 1997).

Nystatin can also be used in the form of tablets. Since, as indicated above, nystatin is poorly soluble in water, it is not well absorbed by the GI mucosa. Nystatin tablets have been used as an adjunct to topical treatment in the therapy of recurrent vaginal candidiasis. The rationale of this therapeutic approach is that oral use of nystatin may lead to clearance or reduction of the *Candida* load in the GI tract, and thereby eliminate a possible reservoir for relapse of the vaginal infection.

In CMC patients, who tend to develop relapses, which are explained by their underlying immune defect that renders them susceptible to the infection, an additional therapeutic approach has been investigated. Attempts have been made to potentiate the immunoreactivity of such patients by use of immunomodulatory agents (Kirkpatrick 1993), such as 'transfer factor,' levamisole, or thymosine. The most extensively studied immunomodulator was the transfer factor, a leukocyte extract, which was able to transfer to CMC patients the ability to mount a DTH response, an ability that they lacked.

Additional antimycotic drugs, such as the allylamine terbinafine (Perea et al. 2002), have been shown to have in vitro activity against some *Candida* spp., but have not been widely adopted for clinical use to treat infections with the fungus.

DEEP-SEATED CANDIDIASIS

It is evident that therapy of the deep-seated forms of *Candida* infections requires systemic administration of the antimycotics. The gold standard of antifungals suitable for treatment of systemic mycoses was, and still is, amphotericin B (AMB) (Sarosi 1990). AMB (Fungizone) has a wide antifungal spectrum, it is effective against molds, yeasts, and dimorphic fungi (Bennett 1995). Although, as a rule, resistance to AMB is not a phenomenon restricting the use of this agent, reports on resistant strains of *C. tropicalis* and *C. lusitaniae* are found in the literature (Richardson and Warnock 1997). Resistance of *C. lusitaniae* to AMB may emerge during treatment (Pappagianis et al. 1979; Merz 1984).

AMB is a polyene that acts like other polyenes, through binding to the ergosterol in the fungal cytoplasmic membrane (Georgopapadakou and Walsh 1994), which leads to increased membrane permeability, and subsequently to fungal cell death. As other polyenes, it is water insoluble and consequently not well absorbed from the GI tract and not applicable for oral use, but has to be administered parenterally. The clinically applicable parenteral AMB formulation consists of a lyophilized powder of 50 mg AMB, 41 mg sodium deoxycholate and 25 mg of sodium phosphate, made up in water supplemented with 5 percent of glucose. This formulation is given in the form of a slow (several hours) IV infusion (Edwards 2000).

The dose and duration of AMB treatment of deep-seated candidiasis is dependent upon the specific clinical entity, and the category of patients involved: non-immunosuppressed, granulocytopenic, or otherwise immunosuppressed. Nevertheless, a protocol has been adopted, which in general terms can be outlined as involving treatment with 0.5–1 mg/kg/day of AMB for a duration of 6–10 weeks. In view of the possible side effects associated with AMB therapy, it is recommended to initiate the treatment at a low dose, and monitor the patient's response before dose increase.

Treatment with AMB is associated with considerable side effects. These may include acute reactions and cumulative effects. The acute reactions may include, fever, chills, hypotension, nausea, and vomiting. These

side effects can be ameliorated or prevented by some supportive measures, such as use of steroids and/or Ibuprofen. The major risk of AMB treatment is its cumulative effect – the nephrotoxicity (Sabra and Branch 1990). AMB therapy causes renal impairment in most patients. Thus, AMB treatment requires careful monitoring of renal functions. Although the renal impairment can be reversible several months after cessation of treatment, irreversible damage does also occur. Other, less common, not immediate, effects may include anemia.

The problem of AMB therapy associated with its significant toxicity seems to be overcome to some extent by newer formulations involving AMB–lipid complexes (Janoff 1990; Meunier 1989; Fielding 1991). Three major formulations of this type are currently available: AMB with dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol (ABLC, Abelcet), AMB with cholesterol sulfate (ABCD, Amphocil), and a liposomal AMB preparation (AmBisome) (Chopra et al. 1991). The AMB–lipid complexes are characterized by lower nephrotoxicity. They can, therefore, be administered at significantly higher doses (5 mg/kg/day), doses which are not tolerated with the traditional AMB formulation. The major drawback of these formulations is their current extremely high cost, which hampers their use on a wider scale. It should be added, in this context, that trials with mixtures of AMB and Intralipid (Chavanet et al. 1992; Caillot et al. 1993; Nath et al. 1999), which significantly lower the cost of the treatment, have been carried out.

Disseminated candidiasis can also be treated by a combination of AMB and 5-fluorocytosine (5-FC) (Polak 1987). 5-FC (flucytosine, Ancobon) is a fluorinated pyrimidine, which acts as an antimetabolite (Diasio et al. 1978). Following deamination it converts into 5-fluorouracil (5-FU), which is incorporated into RNA resulting, subsequently, in impaired protein synthesis. Additional conversion of 5-FU into 5-fluorodeoxyuridilic acid, which can act as an inhibitor of thymidilate synthetase, may result in inhibition of DNA synthesis.

5-FC is water soluble and absorbed quite well by the GI mucosa following oral administration (tablets, capsules); it is usually given at a dose of 150 mg/kg/day (Bennett 1995). It is also well distributed in the body fluids, reaching significant levels in CSF. In patients with no underlying major renal dysfunction, 5-FC treatment leads rarely to side effects, which may include nausea, vomiting, and diarrhea. However, more serious side effects, such as leukopenia, thrombocytopenia, and hepatotoxicity, are also known, particularly among patients with underlying renal or hematological impairments. In such patients careful monitoring is required. The major disadvantage of 5-FC treatment is the frequent occurrence of resistant strains during the course of treatment, and even the presence of primary resistant strains (Stiller et al. 1982) being a recognized phenomenon. Thus, laboratory assessment of the fungal isolate's sensitivity to 5-FC prior to commencement and during the course of treatment, as well as careful monitoring of serum levels of the drug, is recommended.

5-FC is generally not administered as a single therapeutic agent, but rather given in combination with AMB. It is believed that the combined treatment may enable the use of lower AMB doses, thereby reducing possible side effects, without compromising its therapeutic efficacy. The combined treatment is particularly recommended in *Candida* meningitis, due to the high passage of 5-FC through the blood–brain barrier.

The triazoles fluconazole and itraconazole have been considered as possible alternatives to AMB, or additional agents in the treatment of deep-seated candidiasis. The triazoles act as the other azoles (imidazoles) by interfering in the biosynthesis of ergosterol, through inhibition of lanosterol demethylation, involving binding to cytochrome P450 enzymes. The advantage of the triazoles over the imidazoles is that they affect less the synthesis of human sterols (Hanger et al. 1988) and, therefore, are less associated with side effects. As a consequence, use of triazoles would seem preferable over the imidazole ketoconazole, which is also suitable for systemic (oral) use and could be considered for treatment of deep-seated candidiasis. (Kauffman et al. 1991, 1993; Meunier et al. 1992).

Fluconazole is active against most *Candida* spp. However, resistant *C. albicans* strains have been isolated, primarily from AIDS patients under prolonged fluconazole treatment (Richardson and Warnock 1997). Of clinical significance is also the phenomenon of certain species, such as *C. krusei* and *C. glabrata*, which reveal initial insensitivity to the drug. Epidemiologic data point to an increase in incidence of systemic candidiasis due to these species (Hazen 1995; Nguyen et al. 1996; Krcmery and Barnes 2002), which may possibly be the result of selective pressure of fluconazole use.

Fluconazole is available in two forms, tablets (50, 100, 200 mg) for oral use and a formulation for IV use. Fluconazole is quite soluble and well absorbed from the GI tract after oral administration (Bennett 1995). It is also well distributed in the human body sites, including the CNS; its level in the CSF can reach 70 percent of that in the serum. Its use is rarely followed by serious side effects, but nausea, vomiting, and diarrhea have been recorded in a small percentage of treated individuals.

Fluconazole dose and treatment duration depend on the specific clinical entity and take into consideration the patient involved. Generally, doses of 200–400 mg/day (higher doses of up to 800 mg/day have also been reported) for 6–8 weeks are used. Of the deep-seated *Candida* infections, fluconazole has been used extensively and successfully in treatment of esophagitis; it has also been applied in treatment of endocarditis, hepatosplenic candidiasis or in candidemia of non-immunosupressed patients (Bennett 1995).

Itraconazole can be used orally in the form of capsules and, more recently, as solution and parenterally

by IV administration. It is less well absorbed from the GI tract than fluconazole, and crosses the blood–brain barrier less well. Cross-resistance with fluconazole does, apparently, exist, and reports on resistance of *C. glabrata* have been published (Dermoumi 1994). Itraconazole has been used in treatment of *Candida* esophagitis. In view of the recent introduction of the IV formulation, its role in other deep-seated *Candida* infections is not yet clear.

It is noteworthy that voriconazole, the most novel licensed triazole, has activity against non-*albicans* species resistant to fluconazole, such as *C. glabrata* and *C. krusei*.

Another more recent antifungal drug approved for use in treatment of candidemia is the lipopeptide (echinocandin) caspofungin. Caspofungin is a semisynthetic compound acting as a glucan synthesis inhibitor. It is administered intravenously and is the first antifungal drug active on the fungal cell wall. Combinations of caspofungin and azole are currently being evaluated by in vitro studies (Roling et al. 2002).

At present, most clinicians feel that AMB therapy, particularly that using AMB–lipid complexes, is still preferable for treatment of systemic candidiasis in severely ill immunosuppressed patients.

Immunotherapy as an adjunct to antimicrobial treatment has also been undertaken. This included the use of cytokines, such as for example, granulocyte colony-stimulating factor (G-CSF). However, as these attempts were sporadic and limited, it is difficult to draw conclusions as to the efficacy of this approach (Stevens 1998; Edwards 2000).

Prophylaxis

Vaccination is considered a most effective measure for prevention of microbial diseases. The last decade has seen several attempts to prevent *Candida* infections in humans by employing anti-*Candida* antibodies (Rigg et al. 2001). However, this approach is still experimental and has not yet reached the stage of a commercial vaccine. Thus, other alternatives, primarily the use of antifungals on a prophylactic basis in patients at risk, seem to be a reasonable approach. Since one of the principal aims in the earlier studies was to reduce the fungal load, particularly in the GI tract, which is a major source for *Candida*, and thereby reduce the risk of infection, nystatin was used in these attempts.

Later studies, with few exceptions (Damjanovic et al. 1993), focused on the triazoles (Thunnissen et al. 1991; Wingard et al. 1991, 1993; Goodman et al. 1992; Cesaro et al. 1993; Winston et al. 1993; Chandrasekar and Gatny 1994), mostly fluconazole (Thunnissen et al. 1991). The population groups in whom prophylactic attempts have been undertaken include, primarily, leukemia patients (Winston et al. 1993), neutropenic individuals (Cesaro et al. 1993), bone marrow transplants (Wingard et al. 1991), and neonates in intensive care units (Damjanovic et al. 1993), all of which are considered at high risk for the development of candidiasis.

These studies indicated that prophylactic use of antifungals could reduce *Candida* colonization (Chandraseker and Gatny) or GI carriage (Damjanovic et al. 1993), and possibly also infection (Goodman et al. 1992). However, they also pointed to the important observation that the possible preventive effect was mostly noted in regard to *C. albicans* and not to the non-*albicans* species: *C. krusei* (Wingard et al. 1991; Goodman et al. 1992; Winston et al. 1993), *C. guilliermondii*, *C. parapsilosis* (Cesaro et al. 1993), and *C. glabrata* (Wingard et al. 1993; Chandrasekar and Gatny 1994). Thus, an increase in candidiasis due to non-*albicans* species has been noted in at-risk patients (Hazen 1995; Nguyen et al. 1996; Krcmery and Barnes 2002).

It is noteworthy that, while the use of nystatin would have an effect solely in preventing *Candida* infections, the use of triazoles is expected to have a broader spectrum of activity. While this approach still continues, studies comparing the efficacy and toxicity of different compounds have been published (Walsh 2002). Walsh and colleagues compared voriconazole and liposomal AMB in neutropenic patients and reached the conclusion that 'voriconazole is a suitable alternative to amphotericin B preparations for empirical therapy in patients with neutropenia.' Another study by Wolff et al. (2000), which compared fluconazole with low-dose AMB in bone marrow transplants, found both antifungals have been effective in prophylaxis, with better tolerance of the triazole.

A more recent study of Bow et al. (2002) undertook a meta-analysis including several azoles and AMB, reaching the conclusion that antifungal prophylaxis reduced morbidity and mortality caused by fungi.

It has recently been indicated (Sobel 2003) that the echinocandins, being characterized by their action on the fungal cell wall, show promise for prophylactic use in high-risk patients.

In addition, other possible prophylactic measures that have been suggested include a more cautious use of venous catheters, a risk factor for candidemia (Nucci 2003), use of immunomodulatory means to boost the immune system such as granulocyte transfusions (Van Burik 2003) or cytokines (e.g. G-CSF) (Rodriguez-Adrian et al. 1998; Stevens 1998).

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Serious infections caused by uncommon yeasts

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There are more than 500 species in almost 50 genera of yeast, or yeast-like organisms, that have been described in the world's literature. This vegetative morphological form must convey advantages to the organism, since yeast have arisen in several different branches in the evolution of fungi. This is exemplified by many ascomycetous and basidiomycetous yeast genera.

Members of the asexual genus *Candida*, and one of the sexual basidiomycetous *Cryptococcus* spp., *Cryptococcus neoformans*, cause most serious human yeast infections. In addition, however, there are less common yeast genera that may also cause human infection. These include members of the genera *Blastoschizomyces*, *Hansenula*, *Malassezia*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, *Trichosporon*, and *Ustilago*.

Most members of these genera have caused fungemia with or without organ invasion in immunocompromised patient populations. Although uncommon, these infections are an important clinical entity as they are often difficult to treat, due to both the compromised nature of the patient and the refractoriness of the organisms to standard antifungal regimens.

SPECTRUM OF CLINICAL DISEASE

Most of these organisms are associated with environmental sources and are less frequently encountered as colonizers of humans. The major exception is the genus *Malassezia*, which is a very common component of the normal skin flora of humans and many animal species. The other genera are not usual components of our normal flora, but may be found as transient colonizers. Persistent colonization, when it occurs, is seen more often in hospitalized or immunocompromised patients. Colonization may then serve as a precursor to infection.

Given the relative rarity of these infections most reported information is in the form of case reports and case series. Derived from these reports, Table 1 summarizes the types of infections caused by individual genera. The most common infection is fungemia, but there are also reports of solid organ involvement with most of these yeast. The host characteristics of patient populations who are more at risk for infection are described in Table 2. As apparent from this table, most of these infections occur in immunocompromised patient populations.

Blastoschizomyces

Blastoschizomyces capitatus, formerly included in the genus *Trichosporon*, is known to be an environmental organism. Nevertheless, an observational report that represents the largest collected series of infection with this organism failed to reveal a common environmental exposure (Martino et al. 1990b). In this study, 20 patients with evidence of *B. capitatus* colonization or infection were identified, yet no environmental source was found, nor was the isolation of *B. capitatus* from environmental cultures related to cases of colonization or infection (Martino et al. 1990b). There was also no evidence for a common source of transmission through food, equipment, or personnel. Given the rarity of clinical disease with *B. capitatus*, risk factors for the development of infection are not clearly defined, but notably the single case series (Martino et al. 1990b) and multiple case reports (Martino et al. 1990b; Girmenia et al. 1991;

Table 1 *Types/sites of infection*[a]

Type or site of infection	*Blastoschizomyces*	*Hansenula*	*Malassezia*	*Rhodotorula*	*Saccharomyces*	*Sporobolomyces*	*Trichosporon*	*Ustilago*
Fungemia	++	++	++	++	++	–	++	+
Pneumonia	+	++	+	–	+	–	++	–
Peritonitis	–	–	+	+	–	–	+	–
Meningitis	+	–	–	+	–	–	–	–
Brain abscess	+	–	–	–	–	–	+	–
Endophthalmitis	–	–	–	+	–	–	+	–
Endocarditis	+	+	–	+	–	–	+	–
Hepatosplenic infection	+	–	–	–	+	–	+	–
Lymphadenitis	–	+	–	–	–	+	–	–
Bone marrow infiltration	–	–	–	–	–	+	–	–
Osteomyelitis	+	–	–	+	–	–	+	–
Renal/urinary infection	+	+	–	–	–	–	+	–

+ Reported only in isolated case reports.
++ Reported in larger case series or observational studies.
a) *Hanseniospora* is not included because it has not been shown to cause infection (see section on Microbiology below).

D'Antonio et al. 1994; Pagano et al. 1996) describe the development of disease in patients with hematological malignancies, the vast majority of whom were neutropenic at the time of development of infection.

Twelve of the 20 patients in Martino's case series had *B. capitatus* infection, rather than simply colonization. Each of these 12 presented with fever in the setting of fungemia or solid organ invasion. Sites of solid organ involvement included lung, liver, spleen, kidney, central nervous system, and heart (Martino et al. 1990b). Lung involvement was manifest by productive cough and radiographic studies showing infiltrates (which appeared to be mycetomas in some) (Martino et al. 1990a, b). Hepatic, renal, and/or splenic involvement appeared as focal, nodular lesions seen by imaging studies, and laboratory evaluation demonstrating abnormal liver function tests (Martino et al. 1990a). Central nervous system involvement was manifest clinically by neurological deficits and radiographically by the appearance of focal intracerebral lesions. Myocarditis and endocarditis were both diagnosed at autopsy in one patient with symptomatic heart failure (Martino et al. 1990a). In contrast to the patients with infection, those with transient colonization in this series had *B. capitatus* in stool or urine cultures with no evidence for invasive disease.

In additional reports, other types of infection have included osteomyelitis and diskitis, which developed after *B. capitatus* fungemia (D'Antonio et al. 1994), and

Table 2 *Defined characteristics of susceptible patient populations*

Population	*Blastoschizomyces*	*Hansenula*	*Malassezia*	*Rhodotorula*	*Saccharomyces*	*Trichosporon*	*Ustilago*
Neonate	–	–	+	–	–	+	–
Malignancy	+	+	–	+	+	+	–
Central venous catheter	–	+	+	+	+	+	+
Immunosuppressive medications	–	+	+	+	–	+	–
Burns	–	–	–	+	–	+	–
Dialysis	–	–	–	+	–	–	–
Surgery	–	+	–	+	+	–	–
HIV infection	–	–	–	–	+	–	–

meningitis (Girmenia et al. 1991). Both these infections were in children who had received bone marrow transplants for acute leukemia. An adult leukemia patient with hepatosplenic *B. capitatus* infection (DeMaio and Colman 2000) and a pediatric leukemia patient with *B. capitatus* osteomyelitis (Cheung et al. 1999) have also been reported.

Diagnosis of *B. capitatus* infection is usually made by culture of blood, or other affected body site. Biopsy may be needed to show invasive disease; e.g. in patients with pulmonary symptoms and positive sputum cultures, lung biopsy may be used to show histological evidence of invasion.

Treatment of disseminated *B. capitatus* infection requires systemic antifungal therapy. Fluconazole and flucytosine have good in vitro activity (Vendetti et al. 1991). In clinical practice, amphotericin B with or without flucytosine has frequently been used (Martino et al. 1990a, b; D'Antonio et al. 1994). Fluconazole was used as treatment in the case of meningitis: symptoms resolved and the organism was eradicated from the cerebrospinal fluid. Three months after fluconazole was discontinued, however, the patient was found on autopsy to have persistent evidence of meningeal invasion with *B. capitatus* (Girmenia et al. 1991). After a course of amphotericin B, itraconazole was used as continuation treatment in the child with osteomyelitis (D'Antonio et al. 1994). A crucial aspect of treatment is also likely to be resolution of neutropenia; one case report describes a lack of response to antifungal therapy until the patient was treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Pagano et al. 1996), another describes the effective use of interferon-gamma (IFN-γ) for hepatosplenic infection after the failure of amphotericin B alone (DeMaio and Colman 2000).

Hansenula

The more commonly recognized *Hansenula* species, *Hansenula anomala*, is an environmental colonizer that may be isolated from organic substances including vegetables, fruit, and soil. Less common is *Hansenula polymorpha* which may also be found colonizing environmental sources (Lodder 1970; McGinnis et al. 1980).

The first reported case of human infection with *H. anomala* was of a child with interstitial pneumonia (Csillag et al. 1953). Subsequent cases include additional patients with pulmonary involvement (Csillag and Brandstein 1954; Kane et al. 2002), neonatal ventriculitis (Murphy et al. 1986), endocarditis (Nohinek et al. 1987), and urinary tract infection (Qadri et al. 1988). Most reported cases have described fungemia without end-organ involvement (Milstoc and Siddiqui 1986; Murphy et al. 1986; Klein et al. 1988; Yamada et al. 1995; Ma et al. 2000; Wong et al. 2000). *H. anomala* has also been reported as the actual etiologic agent in cases of oral 'candidiasis' (Cameron et al. 1993).

Risk factors for *H. anomala* infection appear to be similar to those described for other opportunistic fungal infections. These include central venous catheters, use of broad-spectrum antibacterials and hyperalimentation, immunosuppression/neutropenia, and surgery (Klein et al. 1988; Thuler et al. 1997).

Diagnosis of *Hansenula* infection is usually by isolation of the organism in culture of the infected site. Treatment of systemic *H. anomala* infection should include removal of the central venous catheter, if one is present, and use of a systemic antifungal agent. The optimal agent and dose have not been established. Parenteral amphotericin B is commonly used, and in vitro studies demonstrate susceptibility to this agent (Klein et al. 1988; Goss et al. 1994). There is, however, one report of amphotericin B failure, with subsequent successful sterilization of blood cultures by use of ketoconazole (Wong et al. 2000). Lipid complex amphotericin was successfully used to treat *H. anomala* in one patient with pneumonia, fungemia, and acute renal failure (Kane et al. 2002). The utility of fluconazole is unclear since there are reports of its successful use (Hirasaki et al. 1992; Goss et al. 1994; Yamada et al. 1995), but also reported instances of in vitro fluconazole resistance (Yamada et al. 1995), clinical failure of fluconazole treatment (Yamada et al. 1995), of the development of *H. anomala* infection in a patient already taking fluconazole for treatment of a *Candida* urinary tract infection (Alter and Farley 1994), and of breakthrough fungemia with *H. anomala* despite fluconazole prophylaxis (Krcmery et al. 1998).

There is only one clearly documented case of tissue infection with *H. polymorpha*: a child with chronic granulomatous disease developed hilar lymphadenopathy (McGinnis et al. 1980). *H. polymorpha* was recovered from cultures of hilar, posterior mediastinal, and paratracheal lymph nodes. The patient was successfully treated with amphotericin B.

Malassezia

Among the *Malassezia* species, *Malassezia furfur* is the most commonly identified cause of human infection. Infections with *Malassezia pachydermatis* are less common. There are a few case reports of *Malassezia sympodialis* infection. The most common types of *Malassezia* infection are superficial cutaneous and folliculitis (see Superficial diseases caused by *Malassezia* species).

The major type of serious infection caused by *M. furfur* is central venous catheter-related fungemia. Neonates, especially those receiving lipid infusions (Sizun et al. 1994) and immunosuppressed patients, both children and adults (Barber et al. 1993; Morrison and Weisdorf 2000) are the patient populations most at risk for infection. Early reports suggested that *M. furfur* fungemia was almost always associated with the use of

parenteral lipid infusions, but later reports describe the infection in patients without this risk factor (Myers et al. 1992; Barber et al. 1993). The clinical findings of *M. furfur* fungemia are nonspecific and the same as those seen with other types of bloodstream infection: fever, leukocytosis, thrombocytopenia (Barber et al. 1993).

In many reports of *M. furfur* infection, the organism is isolated only from blood cultures drawn through the central venous catheter (i.e. peripheral blood cultures are negative), and solid organ involvement is absent. There are, however, cases reported of pneumonia (Redline and Dahms 1981; Richet et al. 1989), peritonitis (Wallace et al. 1979; Gidding et al. 1989), and an intracardiac mass (Schleman et al. 2000) caused by *M. furfur*.

Although venous catheter colonization clearly occurs, all *M. furfur* fungemia should be treated, since prediction of catheter colonization versus true systemic infection is not possible. As for the other uncommon yeast infections, treatment of serious *M. furfur* infections is not standardized, but usually involves removal of the central venous catheter and use of systemic antifungal therapy (Marcon and Powell 1992; Morrison and Weisdorf 2000). Intravenous amphotericin B is the most commonly utilized antifungal agent.

M. sympodialis is a human colonizer and is felt to be a pathogen despite a paucity of reports of human infection. The strongest evidence for a pathogenic role comes from the isolation of the organism from the scalp of a patient with the acquired immunodeficiency syndrome (AIDS) with a focal skin infection (Simmons and Gueho 1990), as a central venous catheter-related infection in a surgical-oncology patient (Kikuchi et al. 2001), and as the cause of malignant otitis externa in a man with diabetes mellitus (Chai et al. 2000). It is also possible that *M. sympodialis* is under-recognized as a cause of infection since most laboratories do not distinguish it from *M. furfur*.

M. pachydermatis is also recognized as a cause of human infection. Essentially all reported cases are of fungemia in neonates (Marcon and Powell 1992; Lautenbach et al. 1998; Chryssanthou et al. 2001). There is also at least one reported occurrence of fungemia in an immunosupressed adult with a central venous catheter. As with *M. furfur* and other yeasts, central venous catheters and the administration of intravenous lipids may be risk factors for infection (Welbel et al. 1994). Removal of the venous catheter and administration of amphotericin B are both components of treatment of this infection as well. There are also reports of susceptibility to fluconazole and itraconazole, and resistance to flucytosine (Chryssanthou et al. 2001).

Rhodotorula

Rhodotorula species have been isolated from many environmental sources including soil, water, fruit juice, milk products, shower curtains, toothbrushes, and fiberoptic bronchoscopy equipment (Kwon-Chung and Bennet 1992; Whitlock et al. 1992; Warren and Hazen 1995). Infections due to the genus are rare, but are most frequently due to *Rhodotorula rubra*. Infections due to *Rhodotorula minuta* (Gregory and Haller 1992; Goldani et al. 1995; Pinna et al. 2001; Cutrona et al. 2002) and *Rhodotorula glutinis* (Pien et al. 1980; Casolari et al. 1992; Gaytan-Martinez et al. 2000; Dorey et al. 2002; Hsueh et al. 2003) have also been reported.

As with the other genera, the most commonly described infection due to *Rhodotorula* is fungemia. There are also reports of endocarditis (Naveh et al. 1975), meningitis (Pore and Chen 1976; Ahmed et al. 1998; Huttova et al. 1998), ventriculitis (Donald and Sharp 1988), peritonitis (Eisenberg et al. 1983; Wong et al. 1988; Pennington et al. 1995), and eye infections (Casolari et al. 1992; Gregory and Haller 1992; Guerra et al. 1992; Muralidhar and Sulthana 1995).

Rhodotorula fungemia most commonly occurs in patients with central venous catheters (Kiehn et al. 1992; Alliot et al. 2000; Kiraz 2000). Risk factors for the development of this infection are the same as those described for other opportunistic fungal bloodstream infections (immunosuppression, neutropenia, broad-spectrum antibacterials, hyperalimentation, burns, and surgery) (Marinová et al. 1994), but may also include endocarditis (Pien et al. 1980). The clinical presentation of *Rhodotorula* fungemia is nonspecific, with findings that may be seen in any bloodstream infection: fever, chills, tachycardia, and hypotension (Pien et al. 1980). Of note, many reports of *Rhodotorula* fungemia describe bloodstream infections that are polymicrobial.

Since *Rhodotorula* infections other than fungemia are rare, few general conclusions can be drawn about their clinical presentation. *Rhodotorula* peritonitis has only been reported in patients undergoing peritoneal dialysis and is notable for a propensity to lead to peritoneal fibrosis (Donald and Sharp 1988). Eye infections due to *Rhodotorula* species have included chronic postoperative endophthalmitis (caused by *R. minuta*) (Gregory and Haller 1992), keratitis (*R. glutinis*) (Casolari et al. 1992; Guerra et al. 1992), and dacryocystitis (Muralidhar and Sulthana 1995).

When deciding on treatment, it must be recognized that *Rhodotorula* may be an environmental contaminant. In one series where *Rhodotorula* was isolated from blood cultures (lysis centrifugation method) in 36 patients, only 23 were felt to have clinically significant fungemia. In the remainder there was only one colony of yeast isolated on one culture plate, and no clinical evidence of infection (Kiehn et al. 1992). In vitro susceptibility testing has shown that *Rhodotorula* is sensitive to flucytosine, moderately sensitive to amphotericin B, miconazole, ketoconazole, and itraconazole, and often resistant to fluconazole (Kiehn et al. 1992; Marinová et al. 1994; Galan-Sanchez et al. 1999). Treat-

ment of fungemia has historically included systemic antifungal treatment and/or removal of a central venous catheter. Current recommendations for treatment are not standardized, but usually involve removal of the central catheter when feasible (Pien et al. 1980) and the use of amphotericin B at doses of 0.7 mg/kg/day (Kiehn et al. 1992). It may also be reasonable to add flucytosine to this regimen. *Rhodotorula* peritonitis has often been treated with intraperitoneal amphotericin B (Eisenberg et al. 1983; Wong et al. 1988), but this may exacerbate the risk of peritoneal fibrosis, and therefore intravenous amphotericin B may be preferable.

Saccharomyces

Saccharomyces cerevisiae is commonly known as baker's yeast or brewer's yeast, and is used in baking bread or making beer. Human colonization may occur at any mucosal surface, but happens more commonly in patients with underlying illnesses (Greer and Gemoets 1943; Kiehn et al. 1980; Aucott et al. 1990). A recent case series showed that increasing length of hospitalization was also a risk factor for greater likelihood of colonization with *S. cerevisae* (Salonen et al. 2000).

Clinical infections most commonly occur at the sites of mucosal colonization (thrush, esophagitis, vulvovaginitis), but invasive infections have also been reported. Overall, these infections occur in patients with significantly compromised immune systems. *S. cerevisiae* is thus a weak pathogen at best, but one that can clearly cause invasive opportunistic disease in an immunocompromised host. The specific underlying diseases associated with invasive *S. cerevisiae* infection include malignancy (Aucott et al. 1990), AIDS (Sethi and Mandell 1988; Doyle et al. 1990), myelodysplastic syndromes (Aucott et al. 1990; Oriol et al. 1993), burns (Eschette and West 1980), and rheumatoid arthritis (Feld et al. 1982). Clinical characteristics that may also be associated with disease are those described for other opportunistic fungal infections [invasive procedures/surgery (Dougherty and Simmons 1982; Chertow et al. 1991), hyperalimentation (Aucott et al. 1990), broad-spectrum antibacterial therapy (Aucott et al. 1990), and the presence of central venous catheters (Cimolai et al. 1987)]. Isolated fungemia is most common, but solid organ involvement may occur. Documented sites of deep infection include the lungs (Aucott et al. 1990), liver (Aucott et al. 1990), and joints (Feld et al. 1982). Empyema (Chertow et al. 1991) and peritonitis (Dougherty and Simmons 1982) have also been reported. There are also several case reports of *S. cerevisiae* fungemia after therapeutic use of *Saccharomyces boulardii* (Cesaro et al. 2000; Perapoch et al. 2000)

Clinical features of *S. cerevisiae* infection are not specific to the organism, but instead are determined by the site of infection. Blood cultures are diagnostic of fungemia, but since the organism is a common colonizer of mucosal surfaces, histological confirmation of tissue invasion is often necessary in order to diagnose solid organ involvement (Aucott et al. 1990).

Successful treatment of invasive *S. cerevisiae* infection with oral azoles has been noted in case reports (Dougherty and Simmons 1982; Cairoli et al. 1995), but there are also reports of azole-resistant *S. cerevisiae* (Salonen et al. 2000), and, in general, fungemia and/or solid-organ infection should be treated with parenteral amphotericin B.

OTHER *SACCHAROMYCES* SPECIES

S. boulardii is a *Saccharomyces* species, not usually considered a pathogen, that has been used in the treatment of bacterial diarrhea. In this setting, there have been reported cases of fungemia due to *S. boulardii* (Zunic et al. 1991; Pletincx et al. 1995; Hennequin et al. 2000; Rijnders et al. 2000; Lherm et al. 2002; Riquelme et al. 2003). Treatment should involve discontinuation of *S. boulardii* and institution of antifungal therapy.

Sporobolomyces

Sporobolomyces is usually recovered only from soil, occasionally from other environmental sources (leaves, bark, grasses, fruit) and rarely as a cause of human infection. Case reports describe one instance of mycetoma in which *Sporobolomyces roseus* was isolated (Janke 1954), one patient with dermatitis due to *Sporobolomyces holsaticus* (Bergman and Kauffman 1984), and several infections that appear due to *Sporobolomyces salmonicolor*. The reports of *S. salmonicolor* in which the organism is acting as pathogen have included one report of a positive culture from a removed nasal polyp (Dunnette et al. 1986), lymphadenitis (Plazas et al. 1994) and bone marrow involvement in AIDS patients (Morris et al. 1991), and a prosthetic cranioplasty infection (Morrow 1994). In addition, there is one reported case of extrinsic allergic alveolitis being caused by *Sporobolomyces* spp. in a veterinary student who developed fever, cough, and dyspnea when exposed to straw from which the organism was isolated: the patient had serum precipitans against *Sporobolomyces* and a positive intradermal skin test (Cockcroft et al. 1983). Furthermore, there is a description of an outbreak of asthma after a thunderstorm during which time there had been an increase in the number of *Sporobolomyces* spores in the air, suggesting the organism may have contributed to this event (Packe and Ayres 1985).

Given the rarity of clinical infection due to *Sporobolomyces* spp., there is clearly no standard therapy. Treatment which has been used successfully includes amphotericin B (Morrow 1994), amphotericin B followed by ketoconazole (Morris et al. 1991), and amphotericin B followed by fluconazole (Plazas et al. 1994).

Trichosporon

Trichosporon beigelii is a part of normal soil flora, and is occasionally found as colonizer of the oropharynx, skin, and nails. The organism was initially noted to cause superficial infections of the hair shaft (see Superficial diseases caused by *Malassezia* species), then recognized as a pathogen capable of causing invasive disease (Watson and Kallichurum 1970). Most reported cases of disseminated infection have been in patients who are immunosuppressed in the setting of hematologic or solid organ malignancy, or solid organ transplantation (Sarfati et al. 1983; Murray-Leisure et al. 1986; Ness et al. 1989; Mirimoto et al. 1994; Tashiro et al. 1994). A series from Japan described 43 patients identified with disseminated *T. beigelii* infection: 37 (86 percent) of these patients had an underlying hematological malignancy. The majority of these patients (26 of 43) had been profoundly neutropenic (ANC $<100/mm^3$) prior to the development of infection (Tashiro et al. 1994). Notably, nearly all the patients in this series without a hematological malignancy were receiving systemic corticosteroids. Other populations in which *T. beigelii* fungemia has been described include premature infants (Giacoia 1992; Fisher et al. 1993; Singh et al. 1999), burn patients (Hajjeh and Blumberg 1995), and patients with the human immunodeficiency virus (HIV) infection (Anuradha et al. 2000; Girija et al. 2001).

Both a colonized gastrointestinal tract and central venous catheters are considered potential portals of entry for this infection. Pulmonary involvement is, however, the most common site of end-organ disease (Tashiro et al. 1995). Chest x-rays may show diffuse interstitial infiltrates or patchy reticulonodular involvement (Hoy et al. 1986; Tashiro et al. 1995). Signs and symptoms are similar to those of other fungal pneumonias in immunosuppressed patients: persistent fever in the face of antibacterial therapy associated with dyspnea, cough, and bloody sputum production (Tashiro et al. 1995). Other organs that are less commonly involved in disseminated *T. beigelii* infection include the brain (Watson and Kallichurum 1970), eyes (Sheikh et al. 1974), heart (Sidarous et al. 1994), liver (Haupt et al. 1983), spleen (Bhansali et al. 1986), bone (Goodman et al. 2002), and peritoneum (Anuradha et al. 2000). It is also important to note, however, that *T. beigelii* funguria may be a benign finding, not associated with invasive infection, in renal transplant patients (Lussier et al. 2000). There is also one report of a breast implant infection with *T. beigelii* in an immunocompetent patient (Reddy et al. 2002).

Cutaneous findings in association with deep *T. beigelii* infection have included macules, papules, vesicopustules, and nodules which may be localized to the extremities or found dispersed over the entire body (Piérard et al. 1992; Hsiao et al. 1994). Clinically, these lesions may appear similar to those seen in disseminated candidiasis. Cellulitis may also occur (Libertin et al. 1983). Skin biopsy with culture and/or histological studies may be necessary to clarify the diagnosis.

Disseminated trichosporon infection is often diagnosed by blood cultures. In the face of end-organ disease, diagnosis may also be made by biopsy of the affected site with culture or histopathological studies. Histology most frequently shows both yeast forms and hyphal elements that are larger than those seen in *Candida* infections (up to 10 μm). An example is illustrated by renal involvement in a neutropenic patient with leukemia (Figure 1). Arthrospores may be seen in some infections. Another distinguishing histological feature is that *T. beigelii* is commonly found arranged in a radial pattern (Tashiro et al. 1994). Immunoperoxidase staining has also been used as a diagnostic modality since discrimination between *Candida* and *T. beigelii* in tissue sections may occasionally be difficult (Tashiro et al. 1994). Of note, serum from patients with disseminated *T. beigelii* infection may show cross-reactivity with the latex agglutination test commonly used to diagnose infection with *Cryptococcus neoformans*.

Although intravenous amphotericin B is used as standard treatment of disseminated trichosporonosis, there are many reports of therapeutic failures (Walsh et al. 1990; Fisher et al. 1993; Tashiro et al. 1994). In one series, nine of 11 patients with trichosporon fungemia died of systemic fungal infection despite therapy with amphotericin B (Krcmery et al. 1999). In vitro susceptibility testing has demonstrated that although *T. beigelii* is inhibited by standard concentrations of amphotericin B, the levels required for the lethal effects on the organism are much higher. For example, using seven clinical isolates of *T. beigelii*, Walsh demonstrated that the organism was inhibited by standard concentrations of amphotericin B (< 2.0 μg/ml). With both a macrodilution technique and timed kill studies, however, they found that the majority of strains were not killed unless

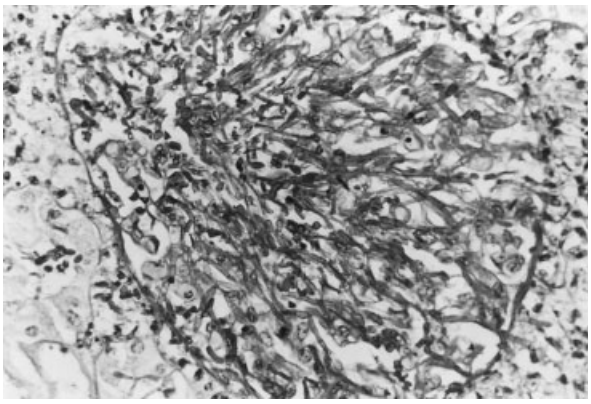

Figure 1 *Kidney involvement with* T. beigelii *in a neutropenic patient with leukemia (periodic acid–Schiff (PAS) 300×)*

extremely high concentrations of amphotericin B (> 20 μg/ml) were used (Walsh et al. 1990). Thus, amphotericin B is a static agent at attainable concentrations, making treatment difficult in patients who lack neutrophils to combat the infection. Other therapeutic options which have been considered include: the use of high-dose amphotericin B, combination treatment with amphotericin B and flucytosine or rifampin or fluconazole, liposomal amphotericin B, and systemic azoles (Walsh et al. 1990; Anaissie et al. 1992; Hajjeh and Blumberg 1995; Canales et al. 1998; Cawley et al. 2000). Although there are in vitro data showing synergism between terbinafine and azoles against *T. beigelii* (Ryder 1999), to our knowledge there are no clinical reports of its use.

OTHER *TRICHOSPORON* SPP.

In the past, species other than *T. beigelii* were only rarely reported as the cause of clinical infections. The taxonomy of this genus has now been redefined (see section on Microbiology below). *Trichosporon capitatum*, *Trichosporon fermentans*, and *Trichosporon penicillatum* are now classified as *Blastoschizomyces* and *Geotrichum* species. In addition, newly defined species, including *Trichosporon asahii*, *Trichosporon inkin*, *Trichosporon louberi*, *Trichosporon mucoides*, and *Trichosporon pullulans*, are being reported with increased frequency. There may be an impact on human infection since virulence may vary among these species. There are several case reports of invasive *T. pullulans* infection: one of pneumonia and the remainder of intravascular catheter-related fungemia in neutropenic patients (Shigehara et al. 1991; Kunová et al. 1995; Krcmery et al. 1999). Patients with *T. pullulans* fungemia have developed the infection while receiving ketoconazole and itraconazole antifungal prophylaxis (Kunová et al. 1995, 1996). There are also reports of bone marrow transplant patients receiving fluconazole prophylaxis who have nevertheless acquired serious *Trichosporon* infections (Moretti-Branchini et al. 2001). In one patient *T. asahii* caused fatal fungemia, despite the use of amphotericin. In the other, *T. inkin* caused a vascular catheter infection. One case report also describes a *T. inkin* lung abscess (Piwoz et al. 2000). Table 3 summarizes these sites of infection, and other case reports of infection with trichosporon species other than *T. beigelii* (Abliz et al. 2002; Fournier et al. 2002; Gokahmetoglu et al. 2002; Meyer et al. 2002; Crowther et al. 2003; Nettles et al. 2003; Padhye et al. 2003).

Ustilago

Ustilago species are known as pathogens of corn and other grains. In corn, they are also known to induce tumor formation (Banuett 1995). *Ustilago maydis* has been described as an allergen causing asthma and *Ustilago esculenta* has been reported as a cause of hypersensitivity pneumonitis (Yoshida et al. 1996). There is one report of a central venous catheter infection with *Ustilago*. This infection occurred in a man with a chronic indwelling venous catheter who visited a farm frequently. The organism was isolated from the catheter, and subsequently from blood cultures 3 days after the catheter was removed. The infection resolved without use of an antifungal agent, but susceptibility testing showed that amphotericin B, fluconazole, and itraconazole should each have been effective (Patel et al. 1995).

MICROBIOLOGY

In order to understand the phylogenetic position of a yeast species, the sexual, teleomorph, stage needs to be demonstrated. This may be difficult since some species are heterothallic, requiring two genetically different, but compatible, strains for sexual reproduction to occur. In addition, there are data that suggest that some fungi, including yeast species, have lost their ability to reproduce sexually.

For this discussion, the less common yeast will be divided by their known or implied teleomorph classification. The ascomycetous yeast include members of the genera *Hansenula*, *Haseniospora*, and *Saccharomyces*. The basidiomycetous yeast include species of *Blastoschizomyces*, *Malassezia*, *Rhodotorula*, *Sporobolomyces*, *Trichosporon*, and *Ustilago*. In the absence of asci/ascospore or basidia/basidiospore production, other characteristics can be used to define their phylogenetic positions. This separation may be very important, as there are data that some cell wall active antifungals may not inhibit cell wall synthesis of all classes of fungi. Simple tests that can be performed in a clinical laboratory include testing for urease activity and determining staining with DBB. Methods such as DNA reassociation

Table 3 *Sites of* Trichosporon *infection*

Site	*T. asahii*	*T. inkin*	*T. louberi*	*T. mucoides*	*T. pullulans*
Pulmonary	–	+	–	–	+
Bloodstream/disseminated	+	+	–	+	+
Renal/urinary	–	–	+	–	–
Peritoneum	–	+	–	–	–
Liver/spleen	+	–	–	–	–

Table 4 *Characteristics of ascomycetous (non-*Candida*) yeast recovered from clinical specimens*

Characteristic	*Hansenula* spp.	*Hanseniospora* spp.	*Saccharomyces* spp.
Budding	Multilateral with narrow attachments	Bipolar, elongated daughter cells	Multilateral
Hyphae	± Pseudohyphae, rarely true	Absent to extensive	± Pseudohyphae
Ascospores	Hat-shaped	Spherical to hat-shaped	Spherical
Temperature	± 37°C	± 37°C	± 37°C
Fermentation	± (species dependent)	+	+
Nitrate utilization	+	–	–

studies, G/C content and rRNA/DNA sequencing are also available in research laboratories to elucidate phylogenetic relationships.

Ascomycetous yeast

Ascomycetous yeasts that have been recovered from clinical specimens, and may lead to human infection, include species of *Hansenula*, *Haseniospora*, and *Saccharomyces*. Characteristics of these yeasts are shown in Table 4. All three genera are urease negative and DBB negative.

HANSENULA

Although there are at least 30 species of *Hansenula* that have been described, the two most frequently encountered are *H. anomala* (*Candida pelliculosa*) and *H. polymorpha*. Key characteristics are the lack of urease, presence of hat-shaped ascospores, ability to utilize nitrate, compatible carbohydrate assimilation patterns and both species are fermenters. (Kurtzman 1984; Warren and Hazen 1995). Clinically relevant *Hansenula* spp. are shown in Table 5.

HANSENIOSPORA

There are at least eight recognized species of *Hanseniospora*, but only three may be encountered in clinical specimens (*Hanseniospora uvarum*, *Hanseniospora valbyensis*, and *Hanseniospora guilliermondii*) (Table 6). To date, none is known to have caused actual infection. Identification is based on ascospore production, lack of urease and nitrate utilization, fermentation, and compatible carbohydrate assimilation patterns (Smith 1984).

SACCHAROMYCES

Pseudohyphae or chains of budding yeast cells may be present. Asci and ascospores (usually four) are seen in many strains. There are seven species within this genus of which *S. cerevisiae* is the one associated with humans. Identification of this species can be made by carbohydrate assimilation patterns and microscopic morphology (ascospores can be seen in approximately 60 percent of strains). The genus cannot utilize nitrates, and all species are capable of fermenting sugars (Yarrow 1984; Warren and Hazen 1995).

Basidiomycetous yeast

Members of six genera of basidiomycetous yeast have been reported to cause human infection. Characteristics of these yeasts are shown in Table 7. Most are positive for urease activity and DBB staining.

BLASTOSCHIZOMYCES

Blastoschizomyces is a basidiomycetous genus with a single species, *B. capitatus*. This species was originally classified in the genus *Trichosporon*, which shares many similarities. Identification of *B. capitatus* is established by morphology with the production of true hyphae and annelloconidia, lack of urease activity, growth up to 42–45°C, growth in the presence of cycloheximide, and compatible carbohydrate assimilation patterns (Salkin et al. 1985; Warren and Hazen 1995).

MALASSEZIA

All *Malassezia* species but one (*M. pachydermatis*) require exogenous fatty acids for growth. Identification to the level of genus may be made based on the char-

Table 5 *Clinically relevant* Hansenula *species*

Characteristic	*H. anomala*	*H. polymorpha*
Ascospores	1–4, hat-shaped	1–4, less hat-shaped, more hemispherical
Hyphae	Pseudohyphae may be produced, but no true hyphae	Neither pseudo- or true hyphae
Growth at 37°C	±	+
Specific carbohydrate assimilation patterns	+	+
Fermentation	+	+

Table 6 *Clinically relevant* Hanseniospora *species*

Characteristic	*H. uvarum*	*H. valbyensis*	*H. guilliermondii*
Ascospores	1–2, spherical	2, rarely 4, hat- to helmet-shaped	1–4, mostly 4, hat- to helmet-shaped
Hyphae	Pseudohyphae, ± extensive	Pseudohyphae, ± extensive	Pseudohyphae, ± extensive
Growth at 37°C	–	–	+
Specific carbohydrate assimilation patterns	+	+	+
Fermentation	+	+	+

acteristics in Table 7; more detailed information for speciation is provided in Superficial diseases caused by *Malassezia* species).

RHODOTORULA

Rhodotorula is a common yeast in environments associated with water. A key characteristic is the production of carotenoid pigments that convey an orange/pink color to the colonies. No *Rhodotorula* species produce true hyphae. Identification is based on the pigment, urease activity, nonfermenter, lack of utilization of inositol, and nitrate utilization. Some strains may produce capsular-type material. There at least eight species; some are the anamorph stage of the genus *Rhodosporidium*. Of the eight, *R. rubra*, *R. glutinis*, and *R. minuta* are encountered. They can be speciated by the characteristics noted in Table 8 (Fell et al. 1984; Warren and Hazen 1995).

SPOROBOLOMYCES

Sporobolomyces is another pigmented, basidiomycetous yeast genus. Key characteristics of the genus include the production of carotenoid pigments, presence of budding, true hyphae, forcibly discharged ballistospores, presence of urease activity, and the inability to ferment carbohydrates. There are at least seven species and perhaps the anamorph stage of *Sporidiobolus*. Of the seven species, *S. holsaticus*, *S. roseus*, and *S. salmonicolor* have been recovered from clinical specimens. Separation of these two species is based on morphology, temperature optima, and carbohydrate assimilation patterns. See Table 9 (Fell and Statzell Tallman 1984).

TRICHOSPORON

Trichosporon is a genus characterized by the production of septate hyphae, abundant arthrospores, budding cells (that may be difficult to discern), and an inability to ferment carbohydrates. Most also express urease activity. *T. beigelii* (or *T. cutaneum*, which is the correct taxonomic name) is the most common species in this genus associated with humans, as both a colonizer and as a pathogen. Three species associated with humans have been re-evaluated and transferred to other genera; *T. capitatum* has been transferred to the genus *Blastoschizomyces*, and *T. penicillatum* and *T. fermentans* have been transferred to the genus *Geotrichum*. Most other species are environmental and rarely associated with humans.

In the past, the asexual genus *Trichosporon* encompassed a very heterogenous group of species that were the anamorph stages of both ascomyceterous and basidiomycetous fungi. Over the last 20 years, this genus has received significant taxonomic evaluation and revision. This genus now is considered a basidiomycetous yeast genus based on cell wall structure, positive DBB staining, septal pore morphology, urease production, similar rRNA sequences, and similarity of a complex polysaccharide antigen cross-reactive with the capsular antigen of *Cryptococcus neoformans* (Kreger-van Rij 1984).

Table 7 *Characteristics of basidiomycetous (non-cryptococccal) yeast recovered from clinical specimens*

Characteristic	*Blastoschizomyces*	*Malassezia*	*Rhodotorula*	*Sporobolomyces*	*Trichospsoron*	*Ustilago*
Colony pigmentation	–	–	+	+	–	–
Budding	May be present (anellospores)	Unipolar	Multilateral	Multilateral	May be difficult to observe	Present
Hyphae	True	±	Usually absent	True and pseudo	True	–
Other spores	Arthrospores	–	–	± ballistospores	Arthrospores	Absent
Temperature	up to 45°C	37°C	± 37°C	± 37°C	± 37°C	–
Lipid-dependent	No	All, except *M. pachydermatis*[a]	No	No	No	No
Urease	–	+	+	+	Most positive	No
Fermenter	No	No	No	No	No	

a) *M. pachydermatis* is lipophilic, but not lipid-dependent.

Table 8 *Clinically relevant* Rhodotorula *species*

Characteristic	*R. rubra*	*R. glutinis*	*R. minuta*
Hyphae	Rudimentary, pseudohyphae may be produced	Usually absent, pseudohyphae if present	Absent
Growth at 37°C	±	±	±
Nitrate utilization	–	+	–
Specific carbohydrate assimilation patterns	+	+	+

Table 9 *Clinically relevant* Sporobolomyces *species*

Characteristic	*S. holsaticus*	*S. roseus*	*S. salmonicolor*
Hyphae	Extensive true hyphae, rare pseudohyphae	Absent	Absent–present, pseudo or true
Ballistospores	Usually produced	usually abundant	Usually produced
Growth at 37°C	–	–	±
Nitrate utilization	+	+	+
Specific carbohydrate assimilation patterns	+	+	+

Identification of *Trichosporon* spp., especially *T. cutaneum* (*beigelii*), currently requires morphological and biochemical studies (for details, see Superficial diseases caused by *Malassezia* species). Morphologically, abundant true hyphae are produced within 24–72 hours that give rise to arthrospores which are initially rectangular in shape. Blastospore production is usually more difficult to observe. They may be produced along the true hyphae, or in chains from pseudohyphae, or they may even bud off from the arthrospores. Most species including *T. cutaneum* (*beigelii*) are urease positive, do not ferment carbohydrates, but assimilate many carbohydrates. Assimilation patterns are commonly used for identification of members of this genus.

Gueho et al. 1992 have performed an extensive evaluation of 101 strains representing the full range of species recovered from humans, animals, and environmental sources. Characteristics used for assessment included morphology, ultrastructure morphology, physiological parameters, ubiquinone systems, G/C content of DNA, DNA/DNA reassociation percentages, and partial sequences of 26S rRNA. A total of 19 taxa were delineated within this genus using data from all of the characteristics. Members of six of the 19 taxa were associated with humans: *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. mucoides*, *T. ovoides*, and *T. inken*. Fortunately, speciation within this genus can be accomplished using temperature studies, assimilation reactions, and cylcoheximide susceptibility or resistance. DNA-related studies by Sugita et al. 1995 confirmed the studies of Gueho et al. 1992. Human infections caused by *T. cutaneum* (*beigelii*) were caused by at least four different DNA-based species. When DNA association percentages of 10 clinical strains of *Trichosporon* were determined, seven of the eight isolates recovered from blood or urine specimens were *T. asahii* and one was *T. ovoides*. Of the two strains recovered from superficial infections, one was *T. cutaneum* and one was *T. montevideense*. Clinical studies with identification to the levels of these taxa will be needed to determine whether there are differences in virulence or treatment associated with any of these new taxa.

USTILAGO

Ustilago is a yeast-like phase of an important plant disease called the smuts. They are recognized by their elongated budding cells.

CONCLUSIONS

With growing populations of immunocompromised patients, it is likely that the uncommon yeast described in this chapter and other species will become ever more important. Further changes in epidemiology and spectrum of disease may be noted in upcoming years with the increasing use of extended-spectrum azoles and cell wall acting echinocandins. Accurate recognition of the causative organisms of specific infections, continued reporting of efficacy of different therapeutic options, and the use of standardized in vitro susceptibility testing should allow ongoing refinements in the treatment of the patients affected by such infections.

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Cryptococcosis

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Cryptococcus neoformans is an encapsulated heterobasidiomycetous fungus that has become a major human pathogen and a common infection in certain immunocompromised hosts (Perfect and Casadevall 2002). Cryptococcosis, the disease resulting from infection with *C. neoformans*, varies from a localized skin lesion or asymptomatic colonization of the respiratory tree to a widely disseminated life-threatening infection, which may infect all organs of the body. However, *C. neoformans* has a special propensity for invading the central nervous system and cryptococcal meningoencephalitis is the primary clinical presentation for the life-threatening stage of this infection.

HISTORY

Historically, human cryptococcosis was first described in 1894 when Busse and Buschke independently reported on the same case of a 31-year-old woman with a history of enlarged lymphatic glands who had developed a large ulcer over her tibia (Buschke 1895). Busse observed yeast-like forms in histological sections of the lesion and he was able to culture the yeast, which he initially referred to as *Saccharomyces*. The patient died and was found at autopsy to have multiple abscesses in the lungs, spleen, kidneys, bones, and skin. No mention of symptoms attributable to the central nervous system (CNS) or examination of the CNS was made in this patient. The first isolation of *Cryptococcus* from the environment was reported in 1894 when Sanfelice isolated the yeast from peaches and named it *Saccharomyces neoformans* (Sanfelice 1894). A few more reports of isolation of this fungus from humans and animals occurred, and then in 1901 Vuillemin renamed the yeast *Cryptococcus hominis* to distinguish it from *Saccharomyces* spp. because it did not form ascospores (Vuillemin 1901).

The first published case of cryptococcal meningitis was diagnosed in a 29-year-old woman with leptomeningeal involvement described by Verse in 1914 (Verse 1914). Two cases of meningitis were then described in 1916 by Stoddard and Cutler and, on complete pathological inspection of the CNS tissue obtained postmortem, these investigators observed yeast forms with surrounding areas of clearing in the tissue (Stoddard and Cutler 1916). This finding was initially misinterpreted as evidence of tissue histolysis and they renamed the yeast *Torula histolytica*, and the syndrome was referred to clinically as torulosis. There was confusion over the fact that one yeast was now considered under three different genera (*Saccharomyces*, *Cryptococcus*, and *Torula*) until 1935 when Benham published a comprehensive study of yeasts that contained 22 cryptococcal strains including the original Busse–Buschke strain (Benham 1935). Based on morphology, fermentation, and serological studies, she concluded that all the cryptococcal isolates should be considered as one species and she designated the name as *Cryptococcus hominis*. Unfortunately, the term 'torulosis' continued to be used for another quarter of a century despite Benham's proposals to call the

disease cryptococcosis. *C. neoformans* is now considered the most valid name based on priority since Sanfelice first proposed the species name in 1894, and for the last 40 years *C. neoformans* has been the name used to designate the yeast causing cryptococcosis. In 1976, Kwon-Chung discovered and characterized the sexual stage of this basidiomycete and the teleomorph was named *Filobasidiella neoformans* (Kwon-Chung 1976). Finally, the entire genome of *C. neoformans* was sequenced in 2003.

Cryptococcosis was considered a clinical rarity in the early 1900s. In Littman and Zimmerman's textbook on cryptococcosis, published in 1956, they had found just over 300 cases of cryptococcosis in the world's medical literature before 1955 (Littman and Zimmerman 1956). The number of cases of cryptococcosis has dramatically increased since the 1950s. This increase results partly from greater awareness of *C. neoformans* as a pathogen, but mostly the increase parallels the ever-enlarging population of immunocompromised individuals as a result of aggressive chemotherapies, organ transplantation, and the acquired immunodeficiency syndrome (AIDS). In many medical centers throughout the world, *C. neoformans* is the most commonly identified pathogen obtained from cerebrospinal fluid (CSF) cultures in adults.

MYCOLOGY

There are 19 species within the genus *Cryptococcus*, and they have been found on every continent and in or on a variety of terrains and animals. All members of the genus are nonfermentative, assimilate inositol, and generally produce urease. Although occasional cases of other cryptococci such as *Cryptococcus laurentii* and *Cryptococcus albidus* are alleged to cause human infections (Lynch et al. 1981; Horowitz et al. 1993), *C. neoformans* should be considered the only cryptococcal species that is routinely pathogenic for humans. Isolation of other cryptococcal species from clinical specimens should generally require both culture and histological proof of tissue invasion before attributing disease to them.

Serotypes

C. neoformans can be subclassified into four serotypes and three varieties. The serotypes are based on variation in capsular epitopes and thus different capsular agglutination reactions (Ikeda et al. 1982). These serotypes are designated as A, B, C, and D (Evans 1950; Evans and Kessel 1951; Vogel 1966) and there are diploid or hybrid strains designated AD. There are specific polyclonal or monoclonal antibodies that can recognize each of the serotypes. Prior to the molecular age of *C. neoformans*, serotype A and D strains were included under var. *neoformans* but today are separated into two varieties, *neoformans* and *grubii*, and the serotype B and C strains were considered under var. *gattii*. The varieties differ in their biochemical properties (Table 1) such as the ability of var. *gattii* strains to use glycine or proline as sole sources of nitrogen whereas var. *neoformans*/*grubii* strains cannot (Dufait et al. 1987). The var. *gattii* strains are also resistant to the chemical canavanine, whereas var. *neoformans*/*grubii* strains are usually sensitive (Polacheck and Kwon-Chung 1986). The ability to utilize glycine and the resistance to canavanine were exploited to distinguish var. *gattii* from var. *neoformans*/*grubii* strains. A medium called CGB contains canavanine (C) and has glycine (G) as the sole nitrogen and carbon source. When variety *gattii* strains are grown on CGB agar, glycine is metabolized and results in the formation of ammonia which alkalinizes the medium and converts the bromothyol blue (B). The variety *neoformans*/*grubii* strains usually cannot metabolize glycine and those rare isolates that can are almost always inhibited by the canavanine that is also present in the medium. Urease activity is another difference between the two varieties in that var. *gattii* urease is sensitive to dicationic chelating agents such as EDTA, but var. *neoformans*/*grubii* urease is resistant. This difference can be important if isolates are grown on media containing EDTA and then subjected to a rapid urease test. The test can be falsely negative for var. *gattii* strains. There have only been rare reported cases of *C. neoformans* infections with urease-negative strains (Ruane et al. 1988; Bava et al. 1993). With recent DNA typing methods and other genetic features, some mycologists have even proposed that var. *gattii* be actually reclassified as a separate species and named *Cryptococcus bacillospora*.

The only reliable differences in morphology between these varieties are in the teleomorph stage (see section on Life cycle and genetics below). There is no credible way to distinguish the yeast forms of the varieties based on morphology. However, var. *gattii* basidiospores tend to appear more bacilliform or rod-shaped oval when compared with var. *neoformans*/*grubii* basidiospores.

Table 1 *Comparison of* C. neoformans *var.* neoformans *with var.* gattii

Variety	Serotypes	Canavanine resistance	Glycine as sole nitrogen source?	Shape of basidiospores
neoformans	A and D	No	No	Round
gattii	B and C	Yes	Yes	Elliptical

There are significant differences in the ecology and epidemiology between the two varieties and these will be further discussed in those sections.

Through a variety of genetic analyses, it has been demonstrated that the serotype A and D strains have significantly diverged from each other. It is estimated from population genetic studies that these serotypes have separated from each other over 18 million years ago (Xu et al. 2002). To account for significant genetic diversity, there is the proposal to separate these serotypes into two varieties (Franzot et al. 1999) which we use in this chapter. Serotype A was proposed to be named var. *grubii* and serotype D would be var. *neoformans*. The varietal status for the serotype AD strains is uncertain, but there is evidence that most of these strains represent diploids and/or hybrids. These findings show that these varieties still have the ability to mate with each other. As previously mentioned, Kwon-Chung identified the sexual stages of *C. neoformans* and these findings allowed for the completion of the fungal life cycle. Furthermore, *C. neoformans* can also be divided into eight distinct molecular genotypes: VN 1–4 for serotype A and VG 1–4 for serotypes B and C (Meyer et al. 2003). Serotype D isolates have proven more difficult to cluster into distinct genotypes.

Life cycle and genetics

The life cycle of *C. neoformans* involves two distinct forms: asexual and sexual. First, in the asexual stage *C. neoformans* exists as yeast cells and reproduces by simple budding (Figure 1). These haploid, unicellular yeasts are the only forms of *C. neoformans* that have been recovered from the environment and human infections. These yeast-like forms exist in one of two mating types, 'a' or 'alpha', and when yeasts of opposite mating type are combined under certain conditions they can undergo conjugation to produce the perfect (sexual) state (Kwon-Chung 1975). Generally, these matings occur in the laboratory on nonenriched media such as V8 juice or hay-infusion agar, and temperatures must be held under 37°C. In fact, room temperature, minimal water exposure, and environmental P_{CO_2} concentrations are the best conditions for mating to occur. These observations suggest that there are regulatory controls for mating that respond to certain environmental signals such as nitrogen starvation and temperature. Recent work has begun to dissect the genes and pathways in *C. neoformans* that control this bipolar mating system (Hull and Heitman 2002). The mating type loci for several serotypes have been sequenced and annotated. Genes for pheromones and their receptors have been identified and the signaling networks that control mating have been determined and studied.

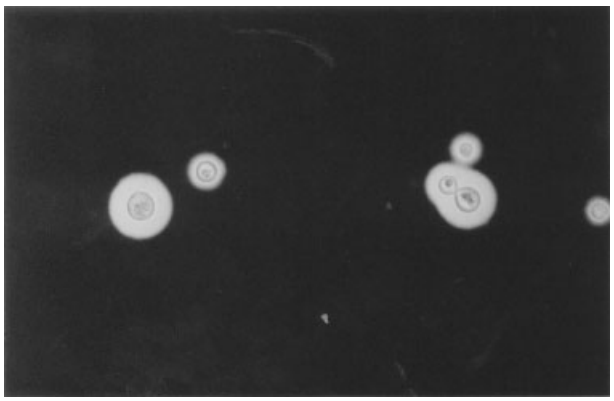

Figure 1 *India ink preparation of the yeast forms of* C. neoformans. *The India ink particles serve to outline the large polysaccharide capsules of the yeasts, making them appear as clear areas surrounding the yeasts. Note the one mother yeast cell with the attached daughter cell that is budding.*

Conjugation between the 'a' and 'alpha' mating strains results in the formation of the teleomorph (sexual) stage which consists of dikaryotic hyphae that bear true clamp connections (Figure 2). Some of these hyphae develop specialized terminal structures called basidia, and the formation of these structures is the basis of classifying the teleomorphs in the phylum Basidiomycota. Furthermore, careful study of the hyphae, basidia, and spore chains showed them to be similar to members of the genus *Filobasidium*. Meiosis occurs at the terminal portion of the basidium and uninucleate basidiospores are formed. These basidiospores bud off at the ends of the basidia in a basipetal fashion to form four chains of spores. These spores are initially unencapsulated but can quickly develop capsules when released from the basidia onto agar and begin budding as yeasts and thus complete the sexual life cycle. The perfect state of *C. neoformans* has been observed only in the laboratory and never conclusively shown to occur in nature. In fact, it is unknown to what extent, if any, sexual reproduction presently occurs in nature. Some investigators have hypothesized that the basidiospores produced by the perfect state may be the infectious propagule for humans because they are ideally suited in size (1–2 μm) for inhalation into the alveoli (Ajanee et al. 1996). Strains of the appropriate mating types of serotype A or D can mate with each other (A × A, A × D or D × D), and the resulting teleomorph is called *Filobasidiella neoformans* var. *neoformans* (Kwon-Chung 1975). Likewise, serotype B and C strains can mate with each other (B × B, B × C or C × C), and the teleomorphs in this case are called *Filobasidiella neoformans* var. *bacillispora* (Kwon-Chung 1975). The teleomorphs of the two varieties can be distinguished on morphological grounds on the basis of their basidiospores. The var. *neoformans* produces chains of spherical basidiospores, whereas the var. *bacillispora* produces oval, bacilliform basidiospores (Figure 2).

When the mating types of *C. neoformans* strains recovered from either the environment or patient samples have been determined, there has always been a large excess of the 'alpha' mating type over 'a' mating type strains

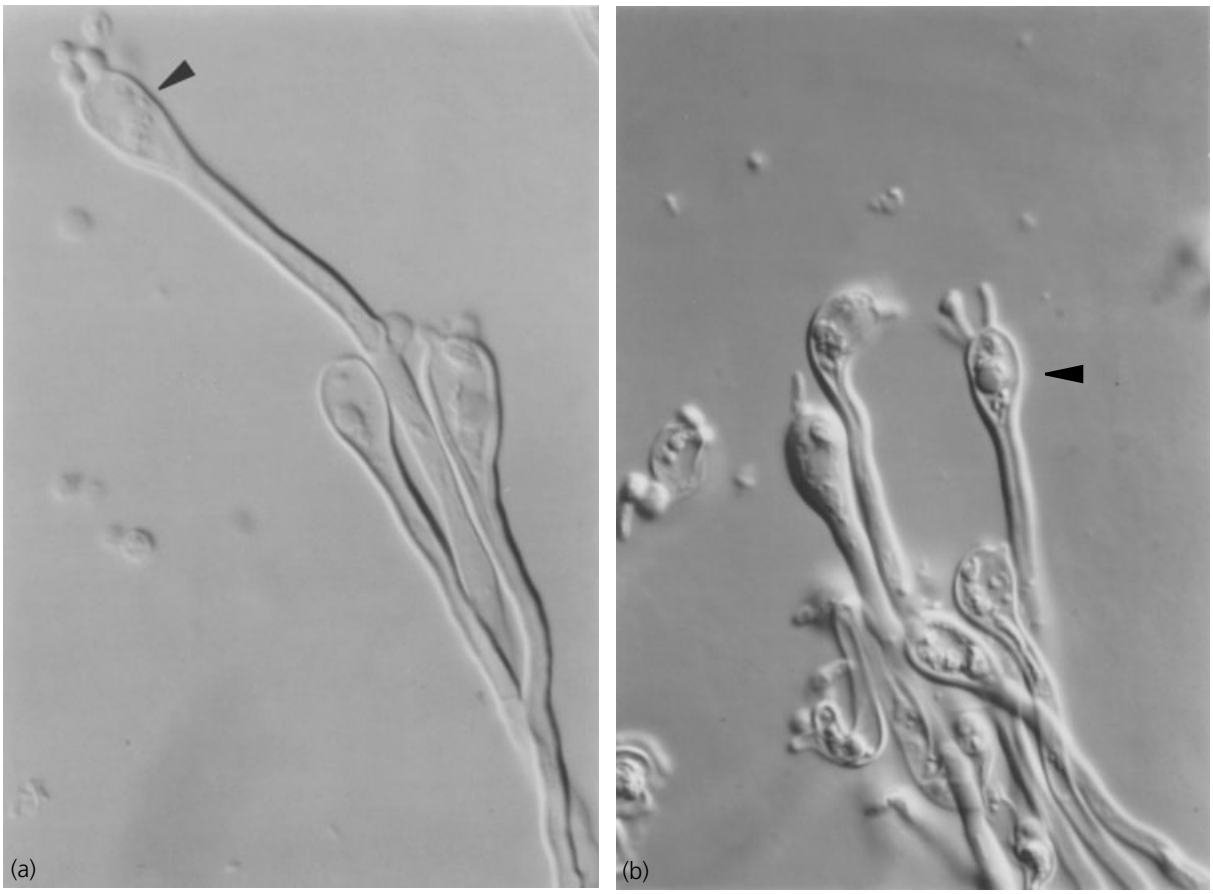

Figure 2 *The teleomorph stages of* **(a)** C. neoformans *var.* neoformans *and* **(b)** C. neoformans *var* gattii. *This stage results from mating between two isolates of the opposite mating type, and is characterized by hyphae containing nuclei from each parent (dikaryotic hyphae). At the end of some hyphae, specialized structures called basidia (arrow) can form. Meiosis occurs in the basidia, and the resulting haploid basidiospores bud off at the apex. Note the shape of the basidiospores of each variety. The basidiospores of var.* neoformans *are round, whereas those of var.* gattii *are elliptical.* **(a)** *Courtesy of Wiley Schell;* **(b)** *courtesy of Michael McGinnis*

(i.e. >95 percent) (Kwon-Chung and Bennett 1978). There has been some evidence for recombination in nature among strains, but the reason for this biased difference in mating type remains unclear. One possibility for predominance of the 'alpha' mating type comes from Wickes et al. (1996), in which they found that several haploid 'alpha' mating type strains were able to form hyphal structures on starvation media. The haploid fruiting formed by these strains produced basidia and basidiospores that were indistinguishable from those produced by hyphae resulting from matings. This ability to form basidiospores by haploid fruiting was independent of serotype, and was only seen in alpha mating type strains. However, in a recent investigation MAT 'a' strains have been observed to produce haploid fruiting (Tscharke et al. 2003), but MAT 'alpha' strains seem to more commonly perform this morphological change.

Growth and Identification

C. neoformans can be grown on a variety of agars, and develops into white, mucoid colonies that usually become visible to the naked eye within 48 hours of incubation. The mucoid appearance of the colonies is caused by the yeast cell's production of a well-defined polysaccharide capsule. The optimal growth temperature of most cryptococcol strains ranges between 30 and 35°C, and the maximally tolerated temperature is approximately 40°C. *C. neoformans* var. *grubii* generally is more thermotolerant than the other two varieties (Martinez et al. 2001). On rich media at 30°C the doubling time for most cryptococcal strains is between 2.5 and 6 hours. The yeast forms are round to oval with diameters between 2.5 and 10 μm and hyphae are never seen with the yeast forms in nutrient media, although pseudohyphal and hyphal forms can sometimes be found when the yeast cells are stressed by either harsh environmental conditions such as elevated temperatures or under nutrient-poor conditions.

In the clinical microbiology laboratory, *C. neoformans* can be readily differentiated from other yeasts on the basis of morphology and biochemical tests. The finding of a mucoid colony on an agar plate is usually the first clue to the presence of *C. neoformans* from a clinical

specimen. This suspicion is further heightened by observing encapsulated, budding yeasts on an India ink preparation of the colony (see Figure 1). The rapid urease test is another presumptive means for identifying the yeasts as *C. neoformans*. Virtually all cryptococci (both *neoformans* and non-*neoformans* species) have the enzyme urease and thus can hydrolyze urea to ammonia, and increase the ambient pH. This property can be detected by using various broths containing urea and a pH marker. As cryptococci have large amounts of urease, positive results using such tests can be seen within 15 minutes (Zimmer and Roberts 1979). The identification of *C. neoformans* specifically can be confirmed by using various biochemical tests, and most clinical laboratories use a battery of biochemical tests contained in commercially available kits. Among the cryptococci, *C. neoformans* is the only one that possesses prominent laccase activity, and testing for its presence is another means of accurate identification. This feature can be detected by culturing the isolated yeasts on special agars such as niger seed agar, birdseed agar, caffeic acid, or dopamine agar, and examining for the growth of brown or black colonies (melanin-positive). These assays are particularly helpful when attempting to identify cryptococci from environmental sources with contamination by other fungi and bacteria. However, most clinical microbiology laboratories do not use these special agars because of the expense of keeping them, and instead depend on the biochemical testing. The yeast forms of cryptococci can be identified in histopathological specimens by using mucicarmine or alcian blue staining of histopathological specimens to demonstrate that the yeast forms possess a polysaccharide capsule (Figure 3). There may be strains with a variety of capsule sizes in tissue but only rarely is a true hypocapsular strain detected in tissue. Finally, a Fontana–Masson stain, although not specific for melanin, can demonstrate production of melanin by these yeasts in tissue.

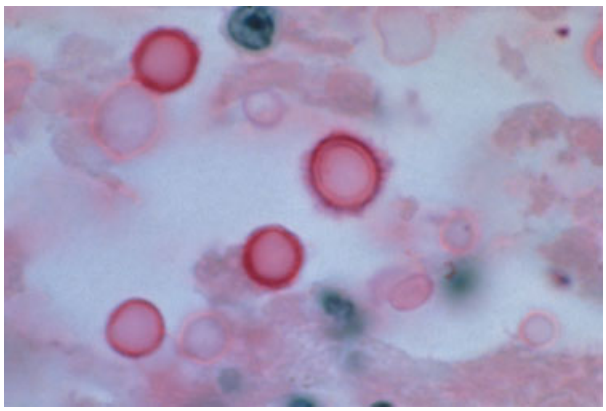

Figure 3 *Histopathology slide of the lung of a patient with cryptococcal pneumonia. The yeasts are easily identified in tissue and the bright red staining around the yeast body demonstrates how this mucicarmine stain identifies the polysaccharide capsule of* C. neoformans.

Capsule

The polysaccharide capsule that surrounds *C. neoformans* is easily observed when yeast cells from the site of infection are examined under the microscope in a suspension of India ink (the ink particles serve to outline the capsule, and make it appear as a clear area or halo surrounding the yeasts) (see Figure 1). Capsule thickness can vary, and in some isolates the capsule can account for more than 50 percent of the diameter of the yeast cells. The capsule is an important virulence determinant for *C. neoformans*, and it is considered to be one of the 3 'classic' virulence factors of cryptococci (the other two are melanin production and growth at 37°C which are discussed in the sections on Melanin production and Growth at 37°C below). Mutant cryptococci that are either hypocapsular or acapsular are consistently less virulent in animal models than encapsulated strains (Chang and Kwon-Chung 1994), and are more easily taken up by white blood cells in in vitro assays (Bulmer et al. 1967; Kozel and Cazin 1971; Fromtling et al. 1982; Kozel and Gotschlich 1982). Infections caused by capsule-free or poorly encapsulated strains are associated with less severe disease (Farmer and Komorowski 1973; Levinson et al. 1974; Milchgrub et al. 1990). The major polysaccharide present in the capsule is glucuronoxylomannan. It is the variable structure of this polysaccharide that accounts for the different serotypes. The glucuronoxylomannans of the serotypes differ in the degree of mannosyl substitution and in the molar ratios of mannose, xylose, and glucuronic acid (Turner and Cherniak 1991). This capsule material can be released from the yeast and extrudes into various tissues such as blood and CSF. It is this antigen that is detected by the cryptococcal antigen test which is so effective for diagnosis in the clinics (see section on Meningoencephalitis below).

Several physiological conditions have been linked to the growth and regulation of the capsule in *C. neoformans*. The P_{CO_2} concentration of the surrounding environment, ferric iron availability, and presence of serum can dramatically influence capsular size (Granger et al. 1985; Vartivarian et al. 1993; Zargoza et al. 2003). Cryptococcal polysaccharide synthesis and capsule size are increased additively by physiological concentrations of CO_2, limitation of ferric iron, and addition of serum. Most *C. neoformans* strains appear to produce large capsules at physiological P_{CO_2} concentrations and small capsules when exposed to environmental P_{CO_2} concentrations. This environmental regulation of the capsule appears to be congruent with the yeast's needs as a pathogen. For example, a small capsule is required for deposition in the host's airways from their environmental source, but a large capsule is needed for protection against the host's defenses. As *C. neoformans* does not produce hydroxamate siderophores for iron acquisi-

tion (Jacobson and Petro 1987), but responds to iron in the environment, it is likely that there are specific iron receptors on the surface of *C. neoformans*; iron is extremely important to the growth of *C. neoformans* (Jacobson and Vartivarian 1992). Several genes that are essential for capsule production and regulation have recently been identified (Chang and Kwon-Chung 1994, 1999; Moyrand et al. 2002). It is also clear that there are signaling pathways such as the cAMP pathway that control the expression of capsule production (Alspaugh et al. 1997) and the capsule appears to attach to the cell wall through glucan linkages (Reese and Doering 2003). Understanding the formation of this unique structure is underway, but further insights will require continued focus on molecular and biochemical aspects of the formation and maintenance of this imposing physical structure.

The impact of the capsular polysaccharide on host immunity can be profound. For instance, it has been shown to have the following effects on immunity:

- antiphagocytosis
- depletes complement
- decreases antibody responsiveness
- inhibits leukocyte migration
- dysregulates cytokine secretion
- produces brain edema
- enhances human immunodificiency virus (HIV) infection
- interferes with antigen presentation
- produces selectin and tumor necrosis factor receptor loss
- creates highly negative charge around cells
- extrudes itself into the intracellular environment with potential local toxicity for cellular organelles (Casadevall and Perfect 1998).

Since a major component of the yeasts' growth in the host is intracellular (Feldmesser et al. 2001), the negative impact of the polysaccharide extruded into the interior of a phagocytic host cell is likely to be profound. The capsule is a major virulence factor through its multipotential ability to abrogate a successful host immune challenge.

Melanin production

The presence of a functional laccase(s) enzyme in *C. neoformans* is unique among other members of this genus. This enzyme participates in the conversion of diphenolic compounds into melanin. The production of this pigment by a simple biochemical pathway is both a means for identifying *C. neoformans* in the laboratory and also a major virulence factor. It is thought that this enzyme is bound on the inner aspect of the cytoplasmic membrane, and the primary gene encoding for this enzyme has been identified and characterized (Polacheck et al. 1982). A wide variety of diphenolic substrates can be utilized by the cryptococcal laccase, including catecholamines such as dopamine and norepinephrine. The ability to produce melanin by a variety of pathogenic fungi has been touted as a potential protectant from host cell damage. Cryptococcal mutants lacking laccase activity have been found to be attenuated in animal models of infection (Salas et al. 1996) and are also killed by the epinephrine oxidative system in the presence of a transition metal ion and hydrogen peroxide compared to the more resistant laccase-positive cells (Kwon-Chung et al. 1982; Rhodes et al. 1982; Polacheck et al. 1990). The melanin produced by degradation of these catecholamines accumulates in the cell wall and may provide protection against a variety of host oxidative stresses (Jacobson and Emery 1991). Importantly, the ability to form melanin in the host has been demonstrated (Nosanchuk et al. 2000a; Rosas et al. 2000). The ability to degrade naturally occurring catecholamines such as norepinephrine to an antioxidant to protect the yeast when it is in the catecholamine-rich CNS may partially explain its propensity to infect the central nervous system. Besides melanin's antioxidant properties, it may also provide other protections for the yeast including:

- cell wall support or integrity
- alterations in cell wall charge
- interference with T-cell response
- abrogation of antibody-mediated phagocytosis
- protection from temperature changes and antifungal agents (Casadevall and Perfect 1998).

Growth at 37°C

A very basic trait for all pathogenic fungi is their ability to grow well at 37°C. In fact, mutants of *C. neoformans* that cannot grow well at 37°C are avirulent even when they possess capsules and laccase activity (Kwon-Chung et al. 1982). Cryptococcal strains differ in their rates of growth at 37°C and their virulence is directly related to their rate of growth at physiological temperatures. Very high mammalian body temperatures of 39–40°C will significantly slow the growth rate of most *C. neoformans* strains and at this temperature the yeasts will actually begin to produce intracellular vacuolization with aberrant budding patterns and pseudohyphal structures. The varieties *gattii* and *neoformans* appear to be more sensitive to high temperatures than var. *grubii*, and at 40°C most strains of var. *gattii* and var. *neoformans* will lose viability within 24 hours (Martinez et al. 2001). Furthermore, it is clear that high-temperature growth is under genetic control with the isolation of a series of temperature-sensitive mutants. Recent work, beginning with the findings of Odom et al., showing that calcineurin in *C. neoformans* was important to high-temperature growth (Odom et al. 1997), has begun to unravel the molecular networks and specific genes necessary for *C. neoformans*

to grow well at mammalian body temperatures (Alspaugh et al. 2000; Erickson et al. 2001). This includes both the signaling pathways of calcineurin and *RAS* and includes biochemical pathways such as the stress protectant sugar trehalose and certain vacuolar functions (Erickson et al. 2001).

ECOLOGY

C. neoformans was first identified in nature by Sanfelice in 1894 from samples of peach juice (Sanfelice 1894). Although fruits are currently not thought to be a significant environmental source for this fungus, certain species of trees are a potential reservoir or habitat for it. *C. neoformans* var. *grubii* can also be found in soil contaminated by bird guano. Its ability to survive within scavenging predators of the soil has led to studies of *C. neoformans* growth in ameba (Steenbergen et al. 2001), dictosteylium (Steenbergen et al. 2003), and nematodes such as *Caenorhabditis elegans* (Mylonakis et al. 2002).

C. neoformans var. *neoformans* and var. *grubii*

Emmons isolated *C. neoformans* from soil, pigeon droppings, and pigeon nests in the 1950s (Emmons 1951, 1955). Since those initial reports, this fungus has been found in soil samples from around the world. The soils that most commonly yield *C. neoformans* var. *neoformans* and var. *grubii* have been frequented by birds, especially pigeons and chickens. For instance, this fungus has commonly been isolated from roosting sites of pigeons in which their droppings had accumulated over many years. Guano from other birds such as canaries, parrots, and turkeys has also yielded *C. neoformans* var. *neoformans* and var. *grubii*. It is not known why there is a frequent association of pigeons with *C. neoformans* because pigeons rarely develop disease with *C. neoformans* in nature. This probably results from the fact that the normal body temperature for a pigeon is above 40°C and most cryptococci are inhibited by these elevated temperatures (Littman and Borok 1968). Pigeons can, however, harbor the yeast as saprophytes in their gastrointestinal tracts. In fact, when force-fed *C. neoformans*, pigeons had viable yeasts cultured from their crops for more than 2 months (Swinne-Desgain 1976). Thus birds could act as a vector for spread of the fungus in the environment.

This fungus can metabolize low-molecular-weight nitrogenous compounds, such as creatinine, which are present in bird guano, and it has been postulated that there is a natural selection for *C. neoformans* to inhabit and grow in this niche. The exact role of pigeon guano in the pathogenesis of human infections is obscure. Only rarely can one elicit a history of intense pigeon exposure from patients with cryptococcosis (Fessel 1993), and there have never been outbreaks of the disease traced back to pigeon-roosting areas. However, there is a case report of infection in an immunosuppressed patient linked to a strain found in a pet bird's cage (Nosanchuk et al. 2000b).

C. neoformans var. *neoformans* and var. *grubii* have occasionally been isolated from rotting vegetation and wood of trees (Pal and Mehrotra 1985). A report from Brazil demonstrated that var. *grubii* isolates could be cultured from areas of wood decay existing on living trees (Lazera et al. 1996). It is probable that decaying wood of certain trees is one natural ecological niche for *C. neoformans* var. *neoformans* and var. *grubii*.

C. neoformans var. *gattii*

As opposed to var. *neoformans* and var. *grubii*, var. *gattii* has never been cultured from bird guano. There have, however, been several environmental niches identified for var. *gattii* strains. Ellis and Pfeiffer were able to culture *C. neoformans* var. *gattii* from vegetation associated with river red gum trees (*Eucalyptus camaldulensis*) and forest red gum trees (*Eucalyptus tereticornis*) in Australia (Ellis and Pfeiffer 1990; Pfeiffer and Ellis 1992). These trees were exported from Australia to various parts of the world, and it was reasoned that var. *gattii* may have been exported along with the trees. These investigators were able to culture a strain of var. *gattii* from a river red gum tree in San Francisco, CA (Pfeiffer and Ellis 1991). There may also be an association with var. *gattii* release of spores with the flowering of the trees since cultures of air samples taken from under flowering trees can yield cryptococci, whereas samples taken when the trees are not in flower do not. Areas of the world that have river red gum trees in the environment also have a fair number of var. *gattii* cryptococcal strains involved in their cases of cryptococcosis. However, a recent significant outbreak of cryptococcosis on Vancouver Island in British Columbia demonstrated that other trees such as fir and oak will also contain var. *gattii* strains (Stephen et al. 2002) and possibly become a vector for human disease.

EPIDEMIOLOGY

Cryptococcosis is a worldwide infection which only rarely causes disseminated disease in healthy individuals. Most patients with cryptococcosis have evidence of some underlying immunocompromising condition. The most common of these underlying conditions worldwide is AIDS, followed by prolonged treatment with corticosteroids, organ transplantation, malignancies, and sarcoidosis (Pappas et al. 2001; Perfect and Casadevall 2002). However, recent estimates are that if patients with HIV infection are excluded, approximately 20 percent of patients with cryptococcosis will present with no apparent underlying disease or risk factor (Pappas et al. 2001).

Cryptococcosis is not a routinely reportable disease in the USA, so accurate nationwide estimates of prevalence are difficult to determine. However, in 1992, before the availability of highly active antiretroviral therapy (HAART), an incidence of 4.9 cases of cryptococcosis per 100 000 population was reported in both San Francisco, CA and Atlanta, GA (Pinner et al. 1993). Between 1992 and 1994 in four large metropolitan areas within the USA, the incidence of infection ranged between 0.2 to 0.9 cases/100 000 population. With widespread use of fluconazole and the introduction of HAART, this lower rate of infection has continued in the US cities. Cryptococcosis in AIDS within developed countries has generally focused on a group of patients with less access to medical care (Mirza et al. 2002). Prior to HAART it was estimated that 6–10 percent of all patients with AIDS in the USA, western Europe, and Australia were diagnosed with cryptococcosis, making it the fourth most common opportunistic infection in this population (Kovacs et al. 1985; Powderly 1993). The rate of cryptococcal meningitis in patients with AIDS is even higher in sub-Saharan Africa where rates of 15–30 percent have been encountered (Clumeck et al. 1984; Van de Perre et al. 1984; Powderly 1993), and HAART has yet to make an impact on the incidence of cryptococcal disease in many of these countries. It can be anticipated that the numbers of cases of cryptococcal meningitis in the world will continue to mirror the worldwide control of the HIV epidemic. In addition, in developed countries more individuals are undergoing organ transplantation and aggressive cancer chemotherapeutic regimens and thus the pool of at-risk patients continues to enlarge.

Interestingly, there are only rare reports of outbreaks of cryptococcosis traced to a common source such as the recent Vancouver Island outbreak (Stephen et al. 2002), and this is unlike the occasional known outbreaks of infection from an environmental source with the classic dimorphic fungi. There have been no documented cases of spread of infection from person to person through routine contact, but there are several cases of cryptococcosis produced by transplantation of infected tissue (Beyt and Waltman 1978; Kanj et al. 1996). *C. neoformans* occasionally has been introduced into a host through direct trauma with a fomite (Glaser and Garden 1985; Casadevall et al. 1994), although inhalation into the lung is the most common route of initial infection. Cryptococcosis appears to be primarily a disease of adults because it is uncommon to produce disease in children, even in those with AIDS. The reason for the reduced incidence of disease in children is unclear and it occurs despite the fact that studies have shown that children are exposed to this fungus at a young age (Chen et al. 1999; Goldman et al. 2001).

The frequency of serotypes or varieties of *C. neoformans* strains that have been found in cases of cryptococcosis varies according to geographical location and whether the patient has HIV infection as a predisposing condition. Kwon-Chung and Bennett did an extensive review of 725 clinical isolates of *C. neoformans* obtained from around the world before the AIDS epidemic (Kwon-Chung and Bennett 1984). These investigators found that, overall, var. *neoformans* and *grubii* were far more common than var. *gattii*, and accounted for more than 80 percent of all isolates, and of these isolates 80 percent were var. *grubii*. The var. *gattii* isolates were almost exclusively found in tropical and subtropical areas such as southern California, Hawaii, Brazil, Australia, Southeast Asia, and Central Africa. This geographical restriction is most probably the result of the ecological relationship of var. *gattii* with certain trees such as eucalyptus and firs. Serotype D strains (var. *neoformans*) predominately come from Europe, especially Denmark, Germany, Italy, and Switzerland. Isolates from the UK consisted of 87 percent serotype A and 13 percent serotypes B and C, whereas isolates from continental USA, excluding southern California, were 81 percent serotype A, 7 percent serotype D, and 6 percent serotypes B and C (some had no identifiable serotype)(-Bennett et al. 1977; Friedman 1983; Kwon-Chung and Bennett 1984). Before the AIDS epidemic, the serotypes of cryptococcal strains isolated from patients living in southern California had a higher proportion of serotypes B and C when compared with the rest of the USA. It is important to keep in mind that these isolates were all obtained before the AIDS epidemic. The vast majority of cryptococcal isolates from patients with AIDS have been var. *neoformans* or var. *grubii*, regardless of geographical location. This is most strikingly demonstrated by data from Central Africa. Over 90 percent of isolates obtained before 1970 from Central Africa were var. *gattii*. However, among the many cryptococcal strains obtained from patients with AIDS in Central Africa, there has been only one var. *gattii* strain identified (Kapend'a et al. 1987). The reason(s) why patients with AIDS are more likely to be infected by var. *neoformans* or var. *grubii* strains is not known. It is hypothesized that differences in infection patterns are the result of certain epidemiologic factors of exposure. For instance, HIV remains a predominantly urban infection; patients with AIDS may not be exposed to the rural environmental niches of var. *gattii* strains. However, it may also be possible that var. *neoformans* or var. *grubii* strains more commonly produce dormant infections that are reactivated when immunity is impaired during HIV infection compared with var. *gattii* in which disease may occur primarily during initial infection.

PATHOPHYSIOLOGY AND IMMUNOLOGY

The exact pathophysiology of cryptococcosis is not known for certain, but a reasonable scheme starts with the host coming into contact with cryptococci from the environment through inhalation of small yeasts or basi-

diospores, with their subsequent deposition in the pulmonary alveoli. It is advantageous for the infecting propagules to be as small as possible because this would favor deposition into the lung, and it is for this reason that some investigators have postulated that the infectious propagules of *C. neoformans* are the basidiospores produced during the sexual cycle or with haploid fruiting. Basidiospores are smaller than the yeast cells obtained in clinical specimens and appear to be the ideal size for inhalation. With identification of the haploid fruiting process, spores can be made in the environment without the need for mating. However, there have been small poorly encapsulated yeasts of sizes consistent with basidiospores isolated from the environment (Neilson et al. 1977; Ruiz and Bulmer 1980), and it must be stressed that the basidiospore structures have yet to be directly observed in nature.

Once the yeasts or spores arrive at the alveoli, the earliest innate immune response elicited is composed of alveolar macrophages, which is then followed by the arrival of polymorphonuclear cells, activated macrophages, and lymphocytes. Data from both experimental animal and human experiments show that the protective components of the immune system for cryptococcosis are primarily cell-mediated, and are composed of activated alveolar macrophages, natural killer (NK) cells and both CD4+ and CD8+ lymphocytes. Several studies have indicated that CD4+ and CD8+ cells are especially important in containing the spread of cryptococci from the site of initial infection in the lung to the central nervous system (Hill and Harmsen 1991; Mody et al. 1993, 1994).

Patients with intact immune systems can mount an effective, coordinated response to cryptococcal infections, and the result is a granulomatous-type inflammatory lesion which is primarily driven by the Th1 cytokine responses. A series of studies have identified the positive features of IL-2, IL-12, and interferon-gamma for an effective immune response against *C. neoformans*. A Th2 response is associated with an ineffective inflammatory reaction. Most primary infections of the lungs are asymptomatic in immunocompetent individuals because they are readily contained by the immune system. As cryptococci are widespread in nature and yet cryptococcosis is a rare event, it is assumed that subclinical primary infections are common in the general population. Unfortunately, there is not a standardized skin test to prove this hypothesis. Besides the host's immune status, there may be some contribution of inoculum size or particular virulence of infecting strain as to whether or not immunocompetent individuals become symptomatic during primary infection.

Elegant postmortem studies have shown the existence of pulmonary foci and hilar lymph nodes containing yeasts during autopsies in individuals who had no antecedent respiratory complaints (Baker and Haugen 1955; Baker 1976). These focal areas of quiescent infection have the potential to disseminate should the host's immune system become impaired. The widespread and acute presentation of cryptococcosis in patients with AIDS shows that the lack of an effective cell-mediated immune response with deterioration in numbers and functions of CD4+ cells results in a rapid dissemination of yeasts throughout the body with a higher fungal burden in tissues compared to apparently immunocompetent individuals. Disseminated cryptococcosis in the immunocompromised population may represent either reactivation of latent infection or in some cases a primary infection with immediate disease due to an initial ineffective immune response.

Although the cell-mediated arm of the immune system is the primary host reaction in responding to invading cryptococci, antibodies directed against *C. neoformans* can be protective. For instance, passive immunization of mice with monoclonal antibodies directed against the capsule can protect and modulate infection (Mukherjee et al. 1992, 1993, 1994). The basis for the humoral protection may be the result of opsonization or decreased levels of glucuronoxylomannan in tissue. As a result of the protection afforded by antibodies in animal models of infection, there remains hope of developing a vaccine for use in people at high risk for cryptococcosis or the use of serotherapy in the treatment of active disease. Both polyclonal and monoclonal anticryptococcal antibodies have been infused and evaluated in patients, but further studies are needed to define their clinical effectiveness.

C. neoformans appears to have some type of neurotropism in humans because of its common propensity to cause meningoencephalitis. The reasons for this unique presentation are not known. There is some speculation that because of its ability to use catecholamines as substrates for melaninogenesis, *C. neoformans* has a survival advantage in the CNS where these products occur in large amounts. It is also possible that this yeast has special surface properties or has the ability to reside within host cells and traverse across the blood–brain barrier within them. Once the yeast is in the CNS compartment, the relatively immunologically sequestered environment of the CNS allows it to grow easily.

CLINICAL PRESENTATION

Meningoencephalitis

Cryptococcal meningoencephalitis is the most frequently encountered manifestation of cryptococcosis. The term 'meningoencephalitis' is more appropriate than meningitis because pathological inspection of human cases shows that the underlying brain parenchyma is generally involved, although radiographically it may not be

apparent. The clinical presentation of this infection in AIDS versus non-AIDS patients is slightly different (Table 2). This clinical difference results from the more severe immunosuppression generally observed in patients with AIDS. It is a manifestation of a higher burden of yeasts and a reduced host inflammatory response. When cryptococcal meningoencephalitis is diagnosed in the setting of HIV infection, it is almost always in patients with CD4 lymphocyte counts lower than 100 cells/mm^3. The features of CNS cryptococcosis in patients with AIDS collected from several different series are shown in Table 3 (Kovacs et al. 1985; Eng et al. 1986; Zuger et al. 1986; Chuck and Sande 1989; Clark et al. 1990a; Saag et al. 1992). This infection was the AIDS-defining illness for 60 percent of the patients in these series. The onset of symptoms was usually over a period of 1–2 weeks, and the three most common symptoms were headache, fever, and malaise, seen in 83, 75, and 68 percent of patients, respectively. Symptoms such as stiff neck, photophobia, and vomiting were seen only in a minority of patients. The initial physical examination revealed altered mentation in 24 percent of the patients and focal neurological deficits in 6 percent (Table 3). Rarely, patients with cryptococcal meningitis and severe immunosuppression can present in a fulminant fashion with rapidly developing coma and death.

By contrast, patients without AIDS usually have symptoms for a longer period of time. Those less common patients with no obvious underlying risk factors can have symptoms lasting for months before a diagnosis is made. However, most patients present with signs and symptoms of subacute meningoencephalitis. Headaches, fever, lethargy, coma, personality changes, and memory loss can develop over weeks. However, there can be tremendous variability in the clinical presentation. Some patients may present with severe headaches for only a few days, intermittent headaches for months, or no headaches at all. The presence of a headache may, in fact, be an important positive prognostic factor by allowing an earlier diagnosis (Dismukes et al. 1987).

It is generally thought that there is no difference in the clinical presentation of patients with cryptococcal meningoencephalitis based on the serotype of the infecting strain. However, the possibility of strain influence should not be entirely neglected. Reviews of cryptococcosis in Australia compared the clinical features of patients with infections caused by the varieties (Speed and Dunt 1995). In contrast to the patients with infection resulting from var. *neoformans* or var. *grubii* strains, the patients with var. *gattii* were more likely to have neurological complications at presentation such as hydrocephalus and cranial nerve deficits. These differences might be explained by the possibility that var. *gattii* strains are generally less virulent than var. *neoformans* or var. *grubii*. Thus, less virulent cryptococcal strains might allow a more robust immune response by the host. The active inflammatory response may account for the insidious presentation and focal neurological signs. It is again interesting to note that in several large reviews of *C. neoformans* there were few infections caused by var. *gattii* strains in highly immunosuppressed patients.

Lung

Despite the probability that most pulmonary infections with cryptococci are asymptomatic, hundreds of cases of symptomatic pulmonary cryptococcosis in apparently immunocompetent hosts have been described in the medical literature (Kent and Layton 1962; Newberry et al. 1967; Tynes et al. 1968; Warr et al. 1968; Hammerman et al. 1973; Rosenheim and Schwarz 1975; Paillas et al. 1982; Henson and Hill 1984; Cohen 1985; McDonnell and Hutchins 1985; Diamond and Levitz 1988). Campbell's comprehensive review of the English medical literature up to 1965 remains the most complete description of primary pulmonary cryptococcosis (Campbell 1966). This review suggests that primary pulmonary cryptococcosis in the immunocompetent host may present asymptomatically in 32 percent, i.e. abnormal findings on a chest x-ray taken for other reasons. However, most patients described in this review presented with symptoms including cough (54 percent), chest pain (46 percent), sputum production (32 percent), weight loss and fever (26 percent each), and hemoptysis (18 percent). Other presentations including dyspnea, night sweats, and superior vena cava obstruction are rarer (Menon and Rajamani 1976; Lehmann et al. 1984). Patients may simply present with airway colonization (Duperval et al. 1977), but this colonized state is frequently observed in patients with underlying chronic lung disease.

Table 2 *Comparison of cryptococcal meningoencephalitis in AIDS versus non-AIDS patients*

Findings	Patients with AIDS	Patients without AIDS
Duration of symptoms	Usually <2 weeks	Usually <2 weeks
Positive India ink of CSF	Approx. 75%	Approx 50%
CSF antigen titer (1:1,024	Common	Rare
Serum antigen positive	Common	Common
CSF white blood cells (20/μl	Very common	Rare
Extraneural involvement	Common	Rare
Opening pressure (200 mm	Common	Less common

Table 3 *Clinical features of cryptococcal meningoencephalitis in patients with AIDS*

Clinical feature	Frequency among patients with AIDS (%)
Headache	83
Fever	75
Malaise	68
Vomiting	42
Meningismus	32
Altered mentation	24
Photophobia	19
Focal neurological deficit	6

Immunocompromised patients with cryptococcal pneumonia may have a more rapid symptomatic clinical course than that seen in immunocompetent hosts. Although *C. neoformans* typically enters through the lungs, in the immunocompromised host it tends to disseminate rapidly or reactivate from a primary focus, eventually to establish infection within the CNS; hence these patients may present with a meningitis syndrome rather than a pulmonary syndrome. However, overwhelming cryptococcal pneumonia with adult respiratory distress syndrome can also occur on initial presentation (Kent and Layton 1962; Henson and Hill 1984; Murray et al. 1988). These immunocompromised patients include those with HIV infection, cirrhosis, diabetes, Cushing's syndrome, sarcoidosis, chronic leukemia, lymphoma, those undergoing treatment with glucocorticoids, and solid organ transplant recipients (Kerkering et al., 1981; Blanc et al. 1982; Kramer et al. 1983; Christoph 1990; Cameron et al. 1991). Kerkering et al., in a classic retrospective review of pulmonary cryptococcosis, described 41 patients with pulmonary infection, 34 of whom had an underlying immunocompromising condition other than HIV (Kerkering et al., 1981). Twenty-nine of these patients developed disseminated disease, but only one of the patients with disseminated disease was identified as immunocompetent. Unlike immunocompetent hosts who may have an inapparent pneumonitis, 83 percent of the immunosuppressed patients had constitutional symptoms. The most common presenting symptoms were:

- fever 63 percent
- malaise 61 percent
- chest pain 44 percent
- weight loss 37 percent
- dyspnea 27 percent
- night sweats 24 percent
- cough 17 percent
- hemoptysis and headache 7 percent each.

Subsequent to the diagnosis of pulmonary cryptococcosis, dissemination to the meninges occurred in 25 patients within 2–20 weeks of the pulmonary presentation. All the patients had abnormal chest x-rays. The most common findings were alveolar or interstitial infiltrates, followed by single or multiple coin lesions, masses, cavitary lesions, and pleural effusions. In this review the outcome of infection clearly supported the recommendation that immunodeficient hosts with pulmonary cryptococcosis require antifungal therapy (Kerkering et al. 1981).

Pulmonary cryptococcosis is less common than meningitis as a presenting complaint in patients with AIDS, but is well described. Pulmonary cryptococcosis in patients with HIV infection has slightly different manifestations than in other types of immunocompromised hosts (Wasser and Talavera 1987; Khardori et al. 1988; Chechani and Kamholz 1990; Clark et al. 1990b; Cameron et al. 1991). In one series of pulmonary manifestations of AIDS, *C. neoformans* was the implicated pathogen in approximately 10 percent of the cases (Suster et al. 1986). Almost all patients with AIDS present with symptoms, including:

- fever 81 percent
- cough 63 percent
- dyspnea 50 percent
- weight loss 47 percent
- headache 41 percent
- occasionally pleuritic chest pain and hemoptysis.

Dissemination of cryptococci, particularly to the meninges or blood, occurred in 94 percent of the patients with pulmonary disease, based on the results from two studies (Wasser and Talavera 1987; Cameron et al. 1991). Physical examination findings were not stated in most reports, but may include lymphadenopathy, rales, tachypnea, and splenomegaly (Cameron et al. 1991). Concomitant second infections with other opportunistic pathogens, including *Pneumocystis jiroveci*, *Mycobacteriurn avium-intracellulare*, cytomegalovirus, and *Histoplasma capsulatum* var. *capsulatum*, can occur in conjunction with pulmonary cryptococcosis (Chechani and Kamholz 1990; Clark et al. 1990b; Cameron et al. 1991). In addition, *C. neoformans* pneumonia may occur as a consequence of adjunctive steroid therapy for *P. jiroveci* (Lambertus et al. 1990) and its clinical presentation can be confused in AIDS patients with pneumocystis pneumonia.

Skin

It has been known for over 50 years that *C. neoformans* infection can produce a variety of skin lesions. Littman and Zimmerman (1956), in their classic monograph, described the observations of Cawley, Grekin, and Curtis (Cawley et al. 1950) on cutaneous cryptococcosis. It was noted that skin lesions could appear as acneiform lesions, purpura, papules, vesicles, nodules, tumors, abscesses, ulcers, superficial granulomas, plaques resembling ecchymoses, and sinus tracts. Recent case reports in AIDS patients have shown an

even more expanded variety of skin presentations to include herpetiformis- (Borton and Wintroub 1984) or molluscum contagiosum-like lesions (Concus et al. 1988). Furthermore, cellulitis (Gauder 1977; Mayers et al. 1981) around an intravenous catheter, which may appear similar to a bacterial infection, can be caused by *C. neoformans* in patients at high risk for infection (Sarosi et al. 1971; Schupbach et al. 1976). The impressive array of cutaneous presentations of infection emphasizes the particular need for skin biopsy and proper histopathology of all new skin lesions for diagnosis in immunocompromised patients. These sentinel lesions may be the first presentation of disseminated cryptococcosis, and the identification of cutaneous cryptococcosis represents disseminated disease in most cases. However, direct inoculation of *C. neoformans* into the skin with production of primary infection has been described (Neuville et al. 2003) and it has been linked to both direct trauma with contaminated fomites and/or laboratory or clinical accidents (Glaser and Garden 1985; Casadevall et al. 1994). There are some cryptococcal strains which appear to have a tropism for skin in animal models and *C. neoformans* var. *neoformans* appears to infect the skin more commonly than the other varieties. Finally, in solid organ transplant recipients with the use of immunosuppressives like tacrolimus, some institutions have begun to observe more infections with *Cryptococcus* limited to the skin (Singh et al. 1997).

Prostate

The prostate gland is known to be a site for cryptococcal infection (Braman 1981). Infection at this site typically does not cause symptoms of prostatitis, but these yeasts have been isolated from prostate tissue and blood after urological procedures (Plunkett et al. 1981). *C. neoformans* has been described to cause a penile ulcer (Perfect and Seaworth 1985) and a vulvar lesion (Blocker et al. 1987), but there have been no reports of conjugal spread of this infection. The significance of genitourinary infection for clinical disease may reside in the prostate's potential as a protected sanctuary for this yeast during drug treatment. In careful follow-up of patients with AIDS-associated cryptococcosis, it has frequently been found that routine urine or seminal fluid cultures for cryptococci after prostatic massage were positive at the end of an extended course of therapy (Larsen et al. 1989; Staib et al. 1989). However, it is not certain that the prostatic foci of infection are the reasons why some patients with cryptococcosis relapse after therapy is stopped.

Eye

In patients with disseminated cryptococcosis, ocular involvement with *C. neoformans* is not a rare event (Blachie et al. 1985). Before HIV infection, one series found ocular signs and symptoms in approximately 45 percent of patients (Okun and Butler 1964). From ocular palsies to involvement of the retina (Crump et al. 1992), which can also be simultaneously infected with other pathogens such as HIV or cytomegalovirus (Doft and Curtin 1982), ocular cryptococcosis is a sight-threatening infection. In fact, most cases of cryptococcal endophthalmitis lead to severe visual loss with only an occasional case successfully managed (Denning et al. 1991a). In about a quarter of cases, eye involvement may present before the diagnosis of cryptococcal meningoencephalitis (Crump et al. 1992).

There have been recent descriptions of catastrophic visual loss in patients without evidence of cryptococcal endophthalmitis (Johnston et al. 1992; Rex et al. 1993). Funduscopic examination is either normal or demonstrates papilledema. Investigators have suggested that two pathogenic mechanisms exist for this complication. One group has rapid visual loss in a period as short as 12 hours and a clinical syndrome suggestive of optic neuritis with the optic nerve possibly infiltrated with yeasts. Few therapeutic maneuvers have been successful for this form of visual loss. The second group presents with slow visual loss which typically begins later in therapy and slowly progresses over weeks to months. In this group, symptoms may be related to increased intracranial pressure and treatment of these pressure conditions with shunts, medications, or optic nerve fenestrations may halt progression of the visual loss.

The eye may also be the entry site for *C. neoformans* infection. For example, one case of a corneal transplant transmitting this yeast from the donor to recipient was reported with eventual development of meningitis in the patient (Beyt and Waltman 1978); a second case of cryptococcal keratitis after a keratoplasty has been described (Perry and Donnenfeld 1990); and, finally, the observations that meningitis was only identified after eye involvement have been documented in some cases (Granger et al. 1986; Birkmann and Bennett 1988). All these findings suggest the possibility that trauma to the eye with *C. neoformans* contaminated debris could be a portal of entry for *C. neoformans* in some clinical cases of infection.

DIAGNOSIS

Meningoencephalitis

The clinical diagnosis of cryptococcal meningoencephalitis can be very difficult given the subacute onset of symptoms and the nonspecific presentation that many patients can have. The most important point for the diagnosis is to appreciate the signs and symptoms referable to the CNS in high-risk patients, and to consider *C. neoformans* as a possible pathogen in cases of apparently immunocompetent individuals presenting with subacute to chronic meningitis.

A lumbar puncture is necessary for the definitive diagnosis of cryptococcal meningoencephalitis. The opening pressure may be quite elevated, especially in patients with AIDS-associated infections. About two-thirds of patients with AIDS-associated cryptococcal meningoencephalitis described in the literature had initial spinal taps with opening pressures greater than 200 mmH_2O. Examination of the CSF with an India ink preparation will show the typical encapsulated yeast forms in more than 75 percent of patients with AIDS and approximately 50 percent of non-AIDS cases. White blood cell counts of the CSF are characteristically low in AIDS-associated cases (<20 cells/mm^3) and somewhat higher in non-AIDS cases (50–350 cells/mm^3) with a mononuclear cell predominance. Protein and glucose levels in the CSF are usually only slightly abnormal in AIDS-associated cases but cases of cryptococcal meningitis can present with very elevated protein levels and prominent hypoglycorrhachia. On the other hand, the CSF glucose and protein levels, as well as the cell counts, can all be completely normal in some severely immunosuppressed patients with cryptococcal meningoencephalitis.

The diagnosis of meningoencephalitis is firmly established by culture of the yeasts from the CSF. In patients with AIDS-associated infections, CSF cultures are almost always positive because of the high burden of organisms associated with severe immune suppression. In non-AIDS patients, 90 percent will have positive cultures and this rate can be further increased by the culturing of large volumes of CSF (15–20 ml). For those cases where the India ink examination is negative, the cryptococcal polysaccharide antigen assay in the CSF is an important adjunct to diagnosis. Results of the polysaccharide antigen test may be obtained soon after the lumbar puncture is performed and can suggest the presence of the infection before the cultures become positive in the laboratory. The antigen test is both very sensitive and specific (>90 percent). In all patients, the antigen test is positive in more than 90 percent of cases, but false-negative results are slightly more common in non-immunosuppressed patients. On the other hand, false-positive tests can result from infections caused by the fungus *Trichosporon beigelii* or possibly other bacteria (McManus and Jones 1985; Westernik et al. 1987; Chanock et al. 1995). Testing for cryptococcal polysaccharide antigen in serum can also be performed and, in patients with AIDS, the sensitivity of using serum for the antigen test is useful as a screening test in patients who do not initially receive a lumbar puncture in geographical areas with a high incidence of cryptococcal disease. The antigen test can be quantified by testing serial dilutions of CSF or serum. In general, the higher the antigen titer, the higher the burden of yeasts and this finding may help in assessing prognosis. Patients with high polysaccharide antigen titers at the beginning or end of therapy are at higher risk for treatment failure. However, the polysaccharide antigen titers cannot be precisely used to follow therapy and to predict relapse in patients (Powderly et al. 1994) and the use of polysaccharide antigen titers to make treatment decisions can be faulty.

On some occasions, the diagnosis of cryptococcal meningoencephalitis is made only after *C. neoformans* is recovered from another body site. This is especially true in patients with AIDS who have a much higher likelihood of having extraneural disease involving the respiratory tract, urinary tract, or bloodstream at the same time as a CNS infection. There are occasional cases in which a blood culture that was sent during the workup of fever of uncertain etiology in a patient with advanced HIV infection returned positive for *C. neoformans*. Then a subsequent lumbar puncture confirmed the diagnosis of a CNS infection. Blood cultures are positive for cryptococci in about two-thirds of AIDS-associated cases of meningoencephalitis, but occasionally a blood culture is positive without evidence of CNS involvement. However, all patients with a positive serum cryptococcal antigen or blood culture should have a lumbar puncture performed to examine for CNS infection and, in patients that are severely immunosuppressed, these findings should support the diagnosis of disseminated infection and treatment administered for invasive disease.

Radiological imaging of the brain by computed tomography (CT) or magnetic resonance imaging (MRI) is helpful when there is a concern about performing a lumbar puncture in a patient with focal neurological signs or symptoms. It is also useful when it shows significant hydrocephalus because this is an indication for placement of a ventricular shunt. Mass lesions can be seen in about 10 percent of patients with cryptococcal meningoencephalitis. Caution should be exercised in patients with AIDS and mass lesions because lymphomas and cerebral toxoplasmosis can coexist with cryptococcal meningitis. The mass lesions caused by cryptococci may not always be true abscesses or granuloma, but may be gelatinous, cyst-like areas resulting from collection of yeasts and antigen with sparse granulomatous inflammation. It is also important to note that, in following patients with cryptococcomas, these brain lesions may worsen radiographically despite effective therapy and this likely reflects an improved inflammatory reaction during elimination of infection (Hospenthal and Bennett 2000).

Pneumonitis

Diagnosis of infection in immunocompetent hosts has been made antemortem by lung biopsy, for histopathology and/or culture, cytopathology, sputum cultures, and serum antigen testing, in a patient with an abnormal chest x-ray (Smith et al. 1976; Kauffman et al. 1981; Fouret et al. 1985; Davies and Sarosi 1987; Murata et al. 1989). Several publications on cryptococcal pneumonia have reported patients with asymptomatic colonization of the respiratory tree by *C. neoformans* (Tynes et al. 1968; Warr et al. 1968; Hammerman et al. 1973; Subramanian et al. 1982). This condition generally

occurs in patients with an underlying lung disease such as chronic obstructive pulmonary disease. Hence, a sputum culture positive for *C. neoformans* in a patient, with a lack of clinical symptoms or chest x-ray findings of an infiltrative process, needs to be interpreted carefully and may not represent clinical disease. In the normal host, pulmonary *C. neoformans* generally does not disseminate outside the lung, and thus blood, urine, or CSF cultures, and serum or CSF cryptococcal polysaccharide antigens, will be negative. Nevertheless, all patients with cryptococcal pneumonia and/or pulmonary colonization should be evaluated for disseminated disease (Kerkering et al. 1981; Lehmann et al. 1984). Clinicians might consider following patients without symptoms, with no apparent immunocompromising conditions and a negative serum polysaccharide antigen titer, without checking for CNS involvement since their chance of meningitis is very low. All others should be considered for lumbar puncture to rule out meningitis.

Radiographically, *C. neoformans* pneumonia in the normal host may present with well-defined, noncalcified, single or multiple lung nodules, indistinct to mass-like infiltrates, hilar and mediastinal lymphadenopathy, occasionally pleural effusions, and, more rarely, cavitation (Hunt et al. 1976; Young et al. 1980; Zlupko et al. 1980; Paillas et al. 1982; Feigin 1983; McAllister et al. 1984; Murata et al. 1989). Single or multiple peripheral nodules are the most common radiological findings (Figure 4), and many of these infections are discovered when the nodules are aspirated or removed to rule out malignancy (Khoury et al. 1984). These infiltrates may resolve without any specific therapy.

Diagnostic studies in HIV-infected patients with cryptococcal pneumonitis should include arterial P_{O_2}, chest x-ray, cultures, and serum cryptococcal polysaccharide antigen detection. Mild-to-moderate hypoxemia can be found, although both normal P_{O_2} and profound hypoxemia have been documented with cryptococcal pneumonia (Clark et al. 1990b; Cameron et al. 1991). Adult respiratory distress syndrome has also occurred during this infection (Perla et al. 1985; Murray et al. 1988; Similowski et al. 1989). Chest radiographs most often reveal interstitial infiltrates, either focal or diffuse, and lymphadenopathy; unlike immunocompetent and other types of immunocompromised hosts, nodular and alveolar infiltrates are more unusual (Miller and Edelman 1990; Clark et al. 1990b; Cameron et al. 1991). Large masses and pleural effusions are also unusual in AIDS patients (Cameron et al. 1991). As the most common radiological picture is one of interstitial infiltrates, *C. neoformans* pneumonia can easily be confused with *P. jiroveci* pneumonia, a common cause of interstitial infiltrates in patients with AIDS (Loerinc et al. 1988). Cryptococcal serum antigen detection can be extremely helpful while awaiting culture results. As patients with AIDS frequently have disseminated cryptococcosis, positive serum or CSF cryptococcal antigen tests are also frequently detected (Clark et al. 1990b; Cameron et al. 1991).

MANAGEMENT

Meningoencephalitis

For an invasive mycosis, the clinical management of cryptococcosis has been relatively well studied and there is substantial evidence-based data to make therapeutic recommendations. In fact, a group of experts in medical mycology have developed guidelines for the therapy of cryptococcosis (Saag et al. 2000). Despite the studies, there remains a wide range of options and consequences

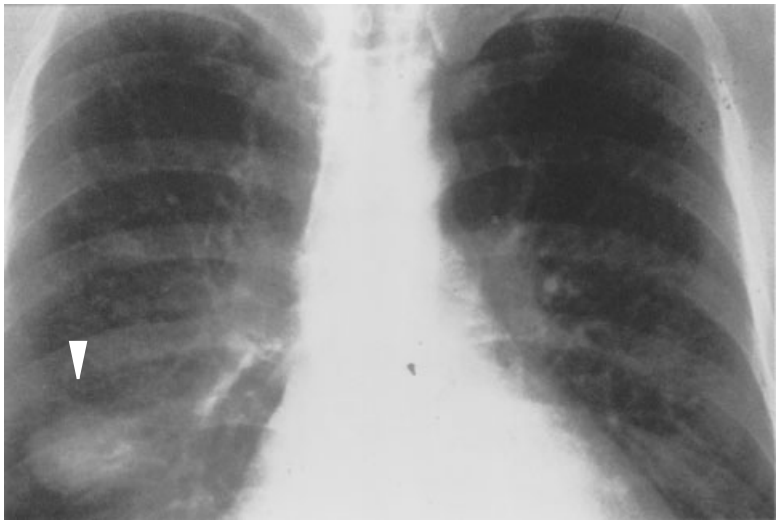

Figure 4 *A chest x-ray from a patient with an asymptomatic nodule in the right lower lung field (arrow). The nodule was removed because of suspicion for cancer, and was found to be caused by infection with* C. neoformans. *Most cases of pulmonary cryptococcosis are asymptomatic, and it is not unusual to find nodules secondary to pulmonary cryptococcosis on chest x-rays taken for other reasons.*

in the management of cryptococcosis in many cases algorithms cannot be followed.

Amphotericin B therapy converted cryptococcal meningitis from a uniform fatal infection to one that is curable. Amphotericin B therapies prior to the AIDS epidemic reported therapeutic successes in 60–70 percent of patients (Sarosi et al. 1969). Recent studies have suggested that higher daily doses of amphotericin B may be more effective (deLalla et al. 1995) and with the use of lipid formulations of amphotericin B, such as AmBisome at 4 mg/kg/day, the use of a polyene as a single agent results in a reasonable treatment outcome (Leenders et al. 1997).

In early studies, flucytosine was used alone in the treatment of cryptococcal meningitis (Utz et al. 1972). However, enthusiasm for flucytosine as a single agent was quickly discouraged because of the rapid development of drug resistance. Fluconazole has outstanding CNS pharmacokinetics and has been used extensively in the treatment of cryptococcal meningitis (Stern et al. 1988; Berry et al. 1992; Saag et al. 1992; Yamaguchi et al. 1996). Because of concern about its slow CSF sterilization, its use has tended to occur after the burden of yeast has been reduced with other drug regimens. Despite its poor penetration into CSF, itraconazole has been used in the successful management of cryptococcal meningitis (Denning et al. 1989; Viviani et al. 1989). New extended-spectrum azoles such as voriconazole can treat cases of cryptococcosis (Perfect et al. 2003), but it is not certain how these azoles will improve on fluconazole treatment. The echinocandins, a new class of antifungal agents, do not appear to be effective in treatment of cryptococcal infections.

The most recent strategy for the management of cryptococcal meningitis uses a combination of antifungal drugs in sequence. Years of study have shown that conventional amphotericin B and flucytosine together are excellent for the initial management of infection (Utz et al. 1975; Bennett et al. 1979; Dismukes et al. 1987; Schmitt et al. 1988; Larsen et al. 1990; DeGans et al. 1992). The addition of flucytosine to amphotericin B helps sterilize the CSF quicker than amphotericin B alone or combinations of amphotericin B and fluconazole. It has also been associated with fewer cases of relapse compared to a single agent (Saag et al. 1999). A large comparative study in AIDS patients for the management of cryptococcal meningitis has also been adopted in the treatment of non-AIDS patients with cryptococcal meningitis (van der Horst et al. 1997; Pappas et al. 2001). The regimen used in this study was amphotericin B at 0.7 mg/kg/day and flucytosine 100 mg/kg/day for at least 2 weeks of induction therapy and then switching to oral fluconazole at 400–800 mg/day alone for 8 weeks. Ideally, a lumbar puncture would be performed after 2 weeks of therapy with extension of the amphotericin B therapy if the CSF cultures are still postive. After these 2.5–3 months of therapy, the fluconazole at 200–400 mg/day is continued for 6 months to a year in non-AIDS patients and in AIDS patients suppression therapy had been recommended for a lifetime prior to HAART. In the suppressive stage of treatment, there are data that suggest that the use of fluconazole may be better than itraconazole (Saag et al. 1999). With the advent of HAART and thus improvement in immunity with antiretroviral therapies, the need for lifelong suppression in patients who have reduced their viral load, increased their CD4 cells ($\geqslant$200 cells/ml), and been free of clinical cryptococcal disease for 1–2 years is being questioned. Recent studies have supported stopping fluconazole in this patient population where the underlying disease is being controlled with HAART (Martinez et al. 2000; Vibhagool et al. 2003).

The issue of discontinuing chronic suppressive therapy was evaluated by a retrospective observational study of 100 patients with a definitive diagnosis of cryptococcal meningitis who subsequently had CD4 counts above 100 cells/μL while receiving HAART (Mussini et al. 2004). Prior to discontinuation of chronic suppressive antifungal therapy, the median duration of HAART in this patient cohort was 26 months, the median duration of maintenance therapy was 33 months, and the median time the CD4 count had been above 100 cells/μL was one month. Forty-one patients had a positive serum cryptococcal antigen at the time maintenance therapy was discontinued. Over a median follow-up of 28 months, there were two relapses of cryptococcal meningitis and two cases of extrameningeal cryptococcosis. The relapse in one patient occurred at a CD4 count of 46 cells/μL, but the other three patients all had negative serum cryptococcal antigen test results at the time therapy was discontinued, and at the time of relapse or extrameningeal disease had undetectable HIV viral loads and CD4 counts above 100 cells/μL (two above 400 cells/μL). These data demonstrate that it may be possible to withdraw chronic suppressive therapy in select patients, but that there remains a small, but significant, risk for recurrence of cryptococcosis.

Site of infection

Unlike cryptococcal meningitis, pulmonary cryptococcosis has not been prospectively studied and most data come from retrospective examination of patient outcomes. Azoles such as fluconazole at 200–400 mg/day for 3–6 months are generally safe and effective (Dromer et al. 1996; Yamaguchi et al. 1996; Pappas et al. 2001). In fact, fluconazole can be used in the treatment of both symptomatic and asymptomatic patients with *C. neoformans* isolated from the lung or sputum. In seriously ill patients, the use of amphotericin B or the combination of amphotericin B plus flucytosine may be more attractive. Surgery has been recommended for a large CNS lesion (>3 cm) and lung abscesses with *C. neoformans*

var. *gattii*, but this strategy should only be followed on an individual case basis. Treatment of other sites of cryptococcal infection should be determined by their location and host factors. Amphotericin B-containing regimens should be considered as induction therapy when there is a high burden of yeasts such as during severe immune depression, but otherwise fluconazole is a reasonable choice.

Immune status of host

Every attempt to improve immunity of the host during treatment needs to be made. On a practical basis this may mean an attempt to reduce immunosuppressive agents, for instance, reduction of prednisone doses to 20 mg/day or less per day. Treatment with HAART can help reconstitute the immune cells of patients with HIV infection and should be encouraged in the management of cryptococcal meningitis, but the timing of a HAART regimen in relationship to stage of treatment remains uncertain. For instance, it is clear that a reconstitution syndrome can occur in the central nervous system or other sites when patients with cryptococcosis are started on HAART (Jenny-Avital and Abadi 2002). This syndrome is marked by evidence of sterile inflammation at this site of infection and this could add to new symptoms in the patient. In patients with increased intracranial pressure, the immune reconstitution may actually worsen symptoms and immune reconstitution syndrome may actually improve with the use of corticosteroid therapy to reduce symptomatic inflammation. Better guidance into what is the best time for HAART therapy in patients with cryptococcosis is needed, but it may be reasonable to start HAART after several months of antifungal therapy to control the initial yeast burden of infection.

Suppressive therapy

Relapse rates in AIDS patients with cryptococcal meningitis before HAART approached 50–60 percent when therapy was stopped within the first 6 months. Fluconazole suppressive therapy with 200 mg/day in AIDS patients has dramatically reduced these numbers to less than 5 percent (Bozette et al. 1991) and it is probably better than intermittent intravenous amphotericin B and/or daily suppression with itraconazole (Powderly et al. 1992). Since non-AIDS patients also relapse (10–15 percent) in the first 6 months after therapy is stopped, this suppressive strategy is similar to the AIDS patients' strategy with 6–12 months of fluconazole after induction therapy being used. In AIDS patients, suppressive therapy has been used for a lifetime, but recent data have studied the discontinuation of drugs after 1–2 years in those with a satisfactory response to HAART (Martinez et al. 2000; Vibhagool et al. 2003).

Role of intracranial pressure

Patients with a high burden of yeasts and polysaccharide antigen can develop increased intracranial pressure before, during, or after treatment (Denning et al. 1991b; Graybill et al. 2000). In this syndrome of early increased intracranial pressure, the patient can suddenly develop systemic hypertension, decreased sensorium, cranial neuropathies, visual loss, worsening headaches, and mental confusion. It is suggested that this elevated pressure is related to reduced CSF resorption, brain edema, and CSF outflow resistance. This increased pressure may respond to CSF withdrawal techniques (lumbar–peritoneal shunts, repeated lumbar taps) (Van Gemert and Vermeulen 1991), but the preciseness of management for this life-threatening complication remains unclear. On the other hand, development of classic noncommunicating hydrocephalus weeks or months after treatment is probably related to inflammation and scar tissue from the original infection and should respond to a ventriculoperitoneal (VP) shunt. A VP shunt can be placed successfully while cryptococcal meningitis is being effectively treated. However, shunts should not be placed until adequate antifungal treatment has begun (Yadav et al. 1988; Ingram et al. 1993).

Antigen titers

CSF polysaccharide antigen titers generally report the burden of yeasts at initial diagnosis, and high antigen titers do predict poor prognosis. However, in an individual patient the reliability of following antigen titers to guide therapy is unclear (Powderly et al. 1994), and thus it is difficult to use titer changes during therapy to make clinical decisions.

Cytokines and specific antibodies

Disseminated cryptococcosis generally occurs during an episode of immunosuppression. Granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-2, IL-12, and interferon-gamma can positively interact with host cells to inhibit or kill cryptococci. Both GM-CSF and interferon-gamma have been examined in open human trials, but it remains uncertain whether these biological modifiers will have a positive impact on the outcome of this infection. Interferon-gamma treatment has been studied prospectively; there was a trend toward a faster reduction in yeast counts in the CSF but no impact on outcome. Specific monoclonal antibodies and serum therapy have a positive effect on the prevention and treatment of experimental cryptococcosis. Early safety studies with a monoclonal antibody have been completed in humans, but efficacy data with the use of antibodies are lacking. It remains important to continue examination on the impact of biolo-

gical enhancers and strategies for management of infection since there is still a 10–25 percent acute mortality rate for cryptococcal meningitis during early therapies even in medically advanced countries.

PROGNOSTIC FACTORS

The most important prognostic factor for a patient with cryptococcosis is the nature of their underlying illnesses. Certainly, those patients with underlying malignancies or AIDS have a much poorer prognosis than those patients with no apparent risk factors for cryptococcosis. In one comparison of patients with cryptococcal meningoencephalitis, patients with malignancies as an underlying risk factor actually had a much shorter median survival rate than patients with AIDS (2 versus 9 months) (White et al. 1992), and this observation was made before the use of HAART, which even further improves the survival of patients with AIDS and cryptococcosis.

In patients without AIDS, several clinical features have been identified which correlate with treatment failure (Diamond and Bennett 1974; Bennett et al. 1979; Dismukes et al. 1987). Patients who have died during therapy have been more likely to have presented with:

- positive India ink examinations of the CSF
- CSF white blood cell counts of less than 20 cells/mm^3
- initial CSF or serum cryptococcal antigen titers of more than 1:32
- extraneural sites of infection
- high opening pressures (>350 mmH_2O) on lumbar puncture (Diamond and Bennett 1974).

Patients who relapsed after treatment have been characterized by:

- persistently low CSF glucose concentrations after 4 weeks of therapy
- low initial CSF white blood count, ⩾ post-treatment CSF, or serum antigen titers of 1:8 or more
- treatment with corticosteroids with at least 20 mg prednisone or its equivalent daily after completion of therapy (Diamond and Bennett 1974).

In patients without AIDS as an underlying risk factor, the following factors predicted a favorable response:

- normal mental status, headache, CSF white blood count over 20 cells/mm^3
- absence of another underlying disease
- negative CSF India ink preparations (Dismukes et al. 1987).

Although these observations were made more than 15–25 years ago, they still likely identify patients with a high burden of yeasts, poor mental status, difficult-to-treat underlying disease on presentation, and risk for failure of treatment.

In patients with AIDS as a risk factor, significant pretreatment predictors of death during therapy include:

- abnormal mental status
- a CSF antigen titer of more than 1:1 024
- a CSF white blood count of less than 20 cells/mm^3 (Saag et al. 1992).

Diastolic hypertension has also been associated with earlier death in these patients (Fan-Havard et al. 1992), and this finding is probably reflective of increased intracranial pressure. The AIDS patient with cryptococcal meningitis at highest risk for complications has:

- high burden of yeasts
- poor inflammatory response
- increased intracranial pressure ⩾350 mmH_2O.

PREVENTION

Trying to prevent cryptococcosis in the general population is an unrealistic task given its rarity. However, in certain high-risk populations, antifungal prophylaxis is a consideration. Some data have been collected from patients with AIDS who were given chronic fluconazole for prophylaxis (Nightingale et al. 1992). Patients receiving fluconazole 200 mg/day had cryptococcosis rates of 1 percent over 35 months, compared with patients given topical clotrimazole who had rates of 7 percent (Powderly et al. 1995). However, general recommendations for fluconazole prophylaxis in patients with AIDS can be made only after very careful consideration of costs, impact on survival, prevalence of the infection, and the potential development of azole resistance in other fungal species. It is rarely considered today in the era of HAART.

Immunization is an attractive form of preventing cryptococcosis. A cryptococcal glucuronoxylomannan tetanus toxoid conjugate vaccine was developed, and it appeared to be highly immunogenic in murine models (Devi et al. 1991). There are now several *C. neoformans* immunogenic proteins in which genes have been cloned and recombinant proteins made. Thus progress toward vaccine development is being made. Trials in humans have not yet been done with any product, but it seems that, for patients at highest risk for cryptococcosis, any vaccine will have to be administered at a time when their immune systems are competent enough to make a protective response. Without a standardized skin test, it is more difficult to determine who is at risk and the magnitude of potential need.

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Systemic zygomycosis

DAVID H. ELLIS

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INTRODUCTION

The Zygomycetes are a class of relatively primitive, fast growing, widely distributed, terrestrial fungi which are largely saprobic on plant debris and in soil. Many species are common environmental contaminants, often causing food spoilage; some are sources of food in the Orient; and a few are pathogens of plants, insects, and, more rarely, of humans. Several species are thermophilic, capable of growing at well over 40°C. Hyphae are coenocytic and mostly aseptate, making them clearly distinguishable from those of other filamentous fungi. Although the absence of hyphal cross-walls or septa facilitates rapid growth and sporulation by allowing the uninhibited absorption and translocation of nutrients, together with cellular organelles between growth sites, it is also a significant disadvantage in that hyphal elements are prone to physical damage and subsequent death. By comparison, when septate hyphae, which are typically found in higher fungi belonging to the Basidiomycetes and Ascomycetes, are damaged, the pores between adjacent compartments are plugged, thus preventing death of the whole hyphal strand.

Thus, in human cases of zygomycosis, reliable culture identification of the causative fungus is made in less than 50 percent of the cases reported in the literature (Scholer et al. 1983). Asexual reproduction is by nonmotile sporangiospores, by modified sporangial units functioning as conidia, or by true conidia (Hesseltine and Ellis 1973). Sexual reproduction is isogamous through the formation of zygospores. However, most isolates appear to be heterothallic, requiring mating by two distinct strains for the formation of zygospores. Thus, in pure culture the sexual or zygosporic state is of limited taxonomic value, although recent mating experiments, leading to the formation of zygospores, have been used to confirm the identity of three zygomycetous fungi isolated from clinical specimens (Weitzman et al. 1995). Occasionally, resting spores known as azygospores, which develop from a single gametangium without any evidence of sexual fusion, may also be observed in some species (Benjamin 1979). At present, the classification and identification of zygomycetous fungi are based primarily on the asexual or anamorphic state, which is characterized by the production of sporangia and sporangiospores.

Sporangiospores are distinguished from other asexual fungal spores, in that they are cleaved out of the cytoplasmic contents of the sporangium without the wall of the sporangium being directly involved in the formation of the spore wall (Benjamin 1979). Other asexual reproductive structures occasionally observed in some species are chlamydoconidia and yeast-like blastoconidia. Chlamydoconidia are thick-walled resting structures formed endogenously within the hyphae. Yeast-like growth usually occurs only under specific culture conditions, especially anaerobiosis or high glucose concentrations, and has been reported in species from a number of genera, including *Cokeromyces*, *Mucor*, and *Rhizomucor* (Benjamin 1979). As a result of the above characteristics, the Zygomycetes, as a class, have long been considered a rather natural group. This grouping has been supported by the composition of the cell wall which is chitosan–chitin in nature (Hesseltine and Ellis 1973) and by molecular evidence (Kwon-Chung 1994). More recent molecular phylogeny studies have provided

strong support for the monophyly of the Mucorales, with the exception of *Mortierella* spp., which appear to be misclassified within the Mucorales (Voigt et al. 1999). Accordingly a new order, the Mortierellales, has been proposed to accommodate *Mortierella* (Cavalier-Smith 1998; Voigt and Wostemeyer 2001). Based on the 18S gene tree topology, *Absidia corymbifera* and *Rhizomucor variabilis* also appear to be misplaced taxonomically. *A. corymbifera* is strongly supported as a sister group of the *Rhizomucor miehei–Rhizomucor pusillus* clade, while *R. variabilis* is nested within *Mucor* (Voigt et al. 1999). To date, some 870 species of the Zygomycetes have been described (Kirk et al. 2001). Until 1979, the Zygomycetes were divided into two orders: the Mucorales and the Entomophthorales (Onions et al. 1981). Hesseltine and Ellis (1973) gave a comprehensive key to the groups and genera and accepted 14 families in the Mucorales. Based on phylogenetic relationships, Benjamin (1979), O'Donnell (1979), and Jefferies (1985) produced a new classification of the Zygomycetes at ordinal level, creating seven orders and 30 families. Currently 10 orders and 32 families are recognized (Kirk et al. 2001); with three orders containing genera and species of medical importance: the Mucorales, Mortierellales, and Entomophthorales. The term 'zygomycosis' is used in this chapter to describe any infection caused by a member of the Mucorales or Mortierellales. However, it should be noted that some authors still prefer to use the term 'mucormycosis' to describe mycoses caused by members of the Mucorales and Mortierellales and entomophthoromycosis (or entomophthoramycosis – the spelling used seems to depend upon the author) to describe mycoses caused by species belonging to the Entomophthorales. In general, fungi in the order Mucorales cause the more severe forms of disease, whereas those in the order Entomophthorales cause disease of the nasal mucosa and subcutaneous tissue (Goodman and Rinaldi 1991). Recent reviews of the Zygomycetes of medical interest have been made by Ribes et al. (2000), Schipper and Stalpers (2003), and Dromer and McGinnis (2003).

Mucorales

The Mucorales (commonly called bread or pin molds) consist of fungi that reproduce asexually by means of nonmotile sporangiospores borne in sporangia (multispored), merosporangia (spores aligned in rows), sporangiola (containing few spores), or as one-spored sporangia or conidia. Sporangia generally have a central columella, which may extend below the sporangium to form a funnel-shaped apophysis. Several species also develop stolons and rhizoids.

Most of the species known to cause human or animal infections belong to a few genera within the family *Mucoraceae*, and are readily recognizable by their rapid growth rate, filling a Petri dish within 3–5 days, and their characteristic appearance, colonies usually being loosely floccose and of a gray or brownish-gray color (Table 1). These include members of the genera *Absidia*, *Apophysomyces*, *Mucor*, *Rhizomucor*, and *Rhizopus*. *Rhizopus oryzae* (*arrhizus*) is the most frequent infectious agent reported, followed by *Rhizopus microsporus* var. *rhizopodiformis*, *Absidia corymbifera*, and *Rhizomucor pusillus* (Scholer et al. 1983). These four species account for more than 80 percent of culture-proven cases of zygomycosis. Other mucoraceous species reported less frequently include *R. microsporus* var. *microsporus*, *Mucor ramosisimus*, *Mucor circinelloides*, *Mucor indicus*, and *Apophysomyces elegans*. Members of several other genera belonging to other families, such as *Cunninghamella bertholletiae* (*Cunninghamellaceae*), and *Saksenaea vasiformis* (*Saksenaeceae*), are also occasionally reported.

Mortierellales

This is a newly created order to accommodate a single family, the *Mortierellaceae* (Cavalier-Smith 1998). All structures are generally more delicate than in the Mucorales and the sporangia have no columellae and are split into numerous, few, or single spores. *Mortierella*

Table 1 *Families and genera of the Mucorales of medical importance*

Family	Genera	Key identification features
Mucoraceae	*Absidia*, *Apophysomyces*, *Mucor*, *Rhizomucor*, *Rhizopus*	Multispored sporangia with columella which may or may not show an apophysis. Sporangia may be globose, pyriform, or ellipsoidal in shape
Cunninghamellaceae	*Cunninghamella*	Single-spored, globose to ovoid, echinulate sporangiola (conidia) borne on globose to clavate fertile vesicles
Saksenaeaceae	*Saksenaea*	Flask-shaped sporangia with columella and simple, darkly pigmented rhizoids
Syncephalastraceae	*Syncephalastrum*	Cylindrical merosporangia with spores in one row borne on globose vesicles
Thamnidiaceae	*Cokeromyces*	Dimorphic with yeast growth at 37°C, sporangiola with few spores borne on long recurved pedicles arising from a fertile vesicle

wolfii is probably the only species pathogenic to animals (Zycha et al. 1969; Domsch et al. 1980; de Hoog and Guarro 1995), although Scholz and Meyer (1965) have reported a case of pulmonary infection in cattle caused by *Mortierella polycephala*.

ETIOLOGIC AGENTS OF MEDICAL IMPORTANCE

By definition, all pathogenic zygomycotic species will grow at 37°C, with the possible exception of the *M. circinelloides* group. Thus occasional reports of infections caused by species unable to grow at temperatures above 35°C must be regarded as questionable. Usually laboratory incubation temperatures range between 26 and 35°C; however, growth temperature studies at 40 or 45°C can also be helpful. Most isolates are heterothallic, i.e. zygospores are absent, so identification of those species is based primarily on sporangial morphology. This includes the arrangement and number of sporangiospores, shape, color, presence or absence of columellae and apophyses, as well as the arrangement of the sporangiophores and the presence or absence of rhizoids. Tease mounts are best, using a drop of 95 percent alcohol as a wetting agent to reduce air bubbles. There are very few molecular techniques currently in use for the identification of zygomycetes, most studies have been directed at phylogeny. (For detailed descriptions of species, keys to taxa, and additional information, see Hesseltine and Ellis 1964, 1966; Ellis and Hesseltine 1965, 1966; Nottebrock et al. 1974; O'Donnell 1979; Domsch et al. 1980; McGinnis 1980; Onions et al. 1981; Scholer et al. 1983; Samson et al. 1995; Goodman and Rinaldi 1991; Ribes et al. 2000; Schipper and Stalpers 2003.)

Absidia corymbifera (Cohn) Saccardo & Trotter

The genus *Absidia* is characterized by a differentiation of the hyphae into arched stolons bearing more or less verticillate sporangiophores in the raised part (internode), and rhizoids formed at the point of contact with the substrate (at the node). This feature separates the species of *Absidia* from those of the genus *Rhizopus*, where the sporangia arise from the nodes and are, therefore, found opposite the rhizoids. The sporangia are relatively small, globose, pyriform, or pear shaped, and are supported by a characteristic funnel-shaped apophysis. This distinguishes the genus *Absidia* from the genera *Mucor* and *Rhizomucor*, which have large, globose sporangia without an apophysis. *Absidia* currently contains 21 mostly soil-borne species (Zycha et al. 1969; Domsch et al. 1980; Schipper 1990; Schipper and Stalpers 2003). *A. corymbifera* is the only species of *Absidia* known to cause disease in humans and animals. *Absidia ramosa*, previously reported as a pathogenic species, has now been reduced to synonymy with *A. corymbifera* (Nottebrock et al. 1974; Scholer et al. 1983).

A. corymbifera (synonym: *A. ramosa*) is a relatively rare agent of human zygomycosis (Kwon-Chung and Bennett 1992); it is more often reported as an animal pathogen (Smith 1989; Jensen et al. 1989, 1990; Jensen 1992; Knudtson and Kirkbride 1992; Guillot et al. 2000). However, it was probably responsible for the first reported case of human pulmonary zygomycosis (Furbringer 1876) and it is occasionally reported from this site (the lungs) (Murphy and Bornstein 1950; Darja and Davy 1963; Lake et al. 1988; Leleu et al. 1999). However, *A. corymbifera* is most often associated with cutaneous infections in both immunocompetent patients, often following localized trauma (Roger et al. 1989; Marshall et al. 1997; Scalise et al. 1999; Seguin et al. 1999; Narain et al. 2001; Thami et al. 2003), and immunosupressed patients, including bone marrow transplant (Hagensee et al. 1994; Jantunen et al. 1996; Leong et al. 1997; Leleu et al. 1999; Paterson et al. 2000), idiopathic aplastic anemia (Cloughley et al. 2002), neonates (Amin et al. 1998; Buchta et al. 2003), and AIDS patients (Chavanet et al. 1990; Hopwood et al. 1992). Lopes et al. (1995) described an unusual case of cutaneous infection from a leukemic patient, which probably resulted from latent osteomyelitis. It has also been reported as the causative agent of meningitis that followed a penetrating head injury (Mackenzie et al. 1988), renal infection in intravenous drug-using AIDS patients (Smith et al. 1989; Torres-Rodriguez et al. 1993), and rhinocerebral infection in neutropenic patients (Manso et al. 1994; Ryan et al. 2001). *A. corymbifera* has a worldwide distribution mostly in association with soil and decaying plant debris (Domsch et al. 1980).

Colonies are fast growing, floccose, white at first, becoming pale gray with age, and up to 1.5 cm high. Sporangiophores are hyaline to faintly pigmented, simple or sometimes branched, arising solitary from the stolons, in groups of three, or in whorls of up to seven. Rhizoids are very sparingly produced and may be difficult to find without the aid of a dissecting microscope to examine the colony on the agar surface. Sporangia are small (10–40 μm in diameter) and are typically pyriform in shape with a characteristic conical columella and pronounced apophysis, often with a short projection at the top (Figure 1). Sporangiospores vary from subglobose to oblong–ellipsoidal (3.0–7.0×2.5–4.5 μm) and are hyaline to light-gray and smooth walled. Zygospores formed by compatible mating strains are reddish-brown, thick walled (60–100×45–80 μm) and have flat projections and one to three equatorial ridges. Isolates are thiamine dependent and have an optimum growth temperature of 35°C, with a maximum of 48–52°C. Key laboratory diagnostic features are growth at 40°C and the formation of small pyriform sporangia with characteristic conical columellae and pronounced apophyses.

Figure 1 Absidia corymbifera *showing a typical pyriform-shaped sporangium with a conical columella and pronounced apophysis. Magnification ×330*

(For additional taxonomic descriptions, see Ellis and Hesseltine 1965; Hesseltine and Ellis 1966; Nottebrock et al. 1974; O'Donnell 1979; Domsch et al. 1980; McGinnis 1980; Scholer et al. 1983; Schipper 1990; Samson et al. 1995; de Hoog et al. 2000; Schipper and Stalpers 2003.)

Apophysomyces elegans Misra, Srivastava & Latas

The genus *Apophysomyces* was erected by Misra et al. (1979) to accommodate a new mucoraceous fungus with prominent funnel-shaped apophyses, which they named *A. elegans*. It is readily distinguishable from other zygomycetes of medical importance, especially the morphologically similar, strongly apophysate pathogen *Absidia corymbifera*. *A. elegans* has sporangiophores with distinctive funnel- or bell-shaped apophyses and hemispherical columellae, a conspicuous pigmented subapical thickening which constricts the lumen of the sporangiophore below the apophysis, and distinctive foot cells. However, *A. elegans* may resemble *S. vasiformis* in gross colony morphology and in its failure to sporulate on routinely used media (Ellis and Ajello 1982; Padhye and Ajello 1988).

A. elegans is an emerging human pathogen of apparently immunocompetent patients and is usually associated with invasive soft tissue infections complicated by burns or wounds contaminated by soil (Winn et al. 1982; Wieden et al. 1985; Newton et al. 1987; Cooter et al. 1990; Huffnagle et al. 1992; McGinnis et al. 1993; Weinberg et al. 1993; Holland 1997; Burrell et al. 1998; Kimura et al. 1999; Page et al. 2001; Blair et al. 2002). Other reports include infections of the kidney and bladder (Lawrence et al. 1986; Okhuysen et al. 1994; Naguib et al. 1995), abdominal wall (Lakshmi et al. 1993), rhinocerebral infection (Brown et al. 1998; Garcia-Covarrubias et al. 2001), and osteomyelitis (Eaton et al. 1994; Meis et al. 1994). Interestingly, most cases occur in apparently immunocompetent patients, indicating that traumatic implantation of the fungus through the skin is the most important predisposing factor. *A. elegans* is a soil fungus with a tropical to subtropical distribution. It was initially isolated from soil samples collected from a mango orchard in northern India (Misra et al. 1979) and subsequently from soil in northern Australia (Cooter et al. 1990).

Colonies are fast growing, white becoming creamy white to buff with age, downy with no reverse pigment, and are composed of broad, sparsely septate (coenocytic) hyphae typical of a zygomycetous fungus. Sporangiophores are unbranched, straight or curved, slightly tapering towards the apex, up to 200 μm long, 3–5 μm in width near the apophysis, and hyaline when young but developing, as the culture ages, a sepia to brown pigmentation and a conspicuous subapical thickening 10–16 μm below the apophysis (Figure 2). Sporangiophores arise at right angles from the aerial hyphae and often have a septate basal segment resembling the 'foot cell' commonly seen in *Aspergillus* spp. Rhizoids are thin walled, subhyaline, and predominantly unbranched. Sporangia are multispored, small (20–50 μm in diameter), typically pyriform in shape, hyaline at first, sepia colored when mature, columellate and strongly apophysate (Figure 2). Columellae are hemispherical in shape and the apophyses are distinctively funnel or

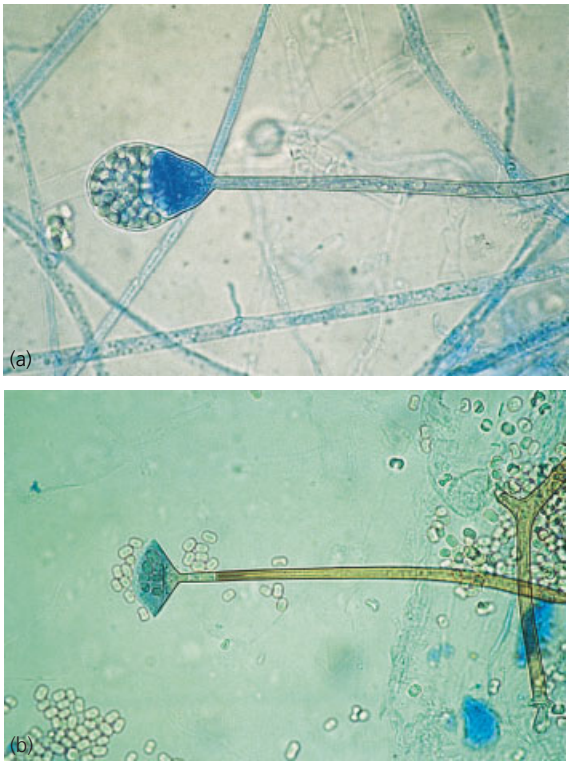

Figure 2 Apophysomyces elegans*:* **(a)** *young sporangium;* **(b)** *mature sporangium showing distinctive funnel-shaped apophyses and a conspicuous pigmented subapical thickening below the apophysis. Magnification ×495*

bell shaped. Sporangiospores are smooth walled, mostly oblong, occasionally subglobose (3–4×5–6 μm), and subhyaline to sepia *en masse*. Good growth occurs at 26, 37 and 42°C. Key laboratory diagnostic features include rapid growth at 42°C (thermotolerant), and the formation on nutrient-deficient media of characteristic 'cocktail glass' sporangia. (For additional taxonomic descriptions, see Misra et al. 1979; Ellis and Ajello 1982; Wieden et al. 1985; Lawrence et al. 1986; Padhye and Ajello 1988; Cooter et al. 1990; McGinnis et al. 1993; de Hoog et al. 2000.)

Cokeromyces recurvatus Poitras

C. recurvatus is the type species of the genus *Cokeromyces* and is usually found in soil or isolated from rodent or lizard dung (Shanor et al. 1950; Benny and Benjamin 1976). It has been reported on several occasions from humans as a colonizer of the vagina (Rippon and Dolan 1979; McGough et al. 1990; Kwon-Chung and Bennett 1992; Kemna et al. 1994) and once from the urinary bladder (Axelrod et al. 1987). *C. recurvatus* has also been reported as causing severe diarrhea in bone marrow transplant recipients (Alvarez et al. 1995; Tsai et al. 1997) and pleuritis and peritonitis in a patient with a ruptured peptic ulcer (Munipalli et al. 1996). Its validity as a human pathogen is, however, doubtful because there has not been any evidence of tissue invasion in any cases. *C. recurvatus* is one of the few zygomycetous fungi that exhibits thermal dimorphism by growing at 37°C in a yeast-like form characterized by the development of large, spherical cells, bearing one to many buds, similar in appearance to those of *Paracoccidioides brasiliensis*, but growing as a mold when incubated at lower temperatures.

Colonies grow moderately at 25°C and are flat, radially wrinkled, tan to brown, becoming grayish with the production of sporangiospores. Sporangiophores are mostly unbranched, 9 μm wide and produce terminal vesicles up to 30 μm in diameter; rhizoids are absent. Sporangiola have few spores, and are globose to obovoid, with a columella, but no apophysis; they are borne terminally on long, recurved, and contorted pedicles which arise from the fertile vesicle formed at the apex of the sporangiophore. Sporangiospores are smooth walled and ovoid to ellipsoid in shape (2.5×4.5 μm). Zygospores are produced in abundance (homothallic) and are dark brown, globose (33.5–54.5 μm in diameter) with rough walls resulting from sharply pointed projections. Isolates readily convert from mold-like to yeast-like colonies when incubated at 35–37°C on enriched media such as brain heart infusion agar with 5 percent sheep blood. *C. recurvatus* has a maximum growth temperature of 42°C, is thiamine dependent, has a positive assimilation of nitrate, negative assimilation of sucrose, negative fermentation of glucose and is sensitive to cycloheximide (Kemna et al. 1994). Key features include dimorphic growth as a yeast at 37°C, sporangiola borne terminally on long recurved stalks, and the development of abundant zygospores at 25°C. (For further information see Zycha et al. 1969; Goodman and Rinaldi 1991; Kwon-Chung and Bennett 1992; Kemna et al. 1994; de Hoog et al. 2000.)

Cunninghamella bertholletiae Stadel

The genus *Cunninghamella* is characterized by white to gray, rapidly growing colonies, producing erect, straight, branching sporangiophores. These sporangiophores end in globose or pyriform vesicles from which several one-celled, globose to ovoid, echinulate or smooth-walled sporangiola develop on swollen denticles. Chlamydoconidia and zygospores may also be present. *Cunninghamella* species are mainly soil fungi of the Mediterranean and subtropical zones, and they are only rarely isolated in temperate regions. The genus now contains seven species, with *C. bertholletiae*, the only one known to cause disease in humans and animals (Domsch et al. 1980).

Once again, there has been some confusion as to the correct name of this zygomycete. Many medical mycologists (McGinnis 1980; Weitzman 1984; Rippon 1988) preferred the name *C. bertholletiae* because of the thermophilic nature of the human isolates that grow at temperatures as high as 45°C. However, Samson (1969) and Domsch et al. (1980) preferred the name *Cunninghamella elegans* and Lunn and Shipton (1983) went further and reduced *C. elegans* (*C. bertholletiae*) to a variety of *Cunninghamella echinulata*, i.e. *C. echinulata* var. *elegans*. However, *C. bertholletiae* is currently the most acceptable name; *C. elegans* differs by having purely gray colonies and by not growing at temperatures above 40°C (Weitzman and Crist 1979; de Hoog et al. 2000).

C. bertholletiae is a common soil fungus found throughout the temperate regions of the world. It is a rare cause of zygomycosis in humans, often associated with traumatic implantation (Boyce et al. 1981) and immunosuppression (Robinson et al. 1990). Pulmonary infections are being reported with increasing frequency in cancer patients (Kwon-Chung et al. 1975; Kiehn et al. 1979; Ventura et al. 1986; Reed et al. 1988; Maloisel et al. 1991; Cohen-Abbo et al. 1993; Dermoumi 1993; Kontoyianis et al. 1994; Mazade et al. 1998; Rickerts et al. 2000). Reports of pulmonary infection in immunocompetent patients are rare (Zeilender et al. 1990). Disseminated disease has been reported by Sands et al. (1985) and Iwatsu et al. (1990), and rare cases of rhinocerebral infection (Brennan et al. 1983) and sinusitis (Ng et al. 1994) have also been reported. Infections have also been reported in association with desferrioxamine therapy (Rex et al. 1988; Sane et al. 1989; Maloisel et al. 1991), transplant recipients (Kolbeck et al. 1985; Nimmo et al. 1988; Darrisaw et al. 2000; Garey et al. 2001; Zhang et al. 2002), and in AIDS patients (Mostaza et al. 1989).

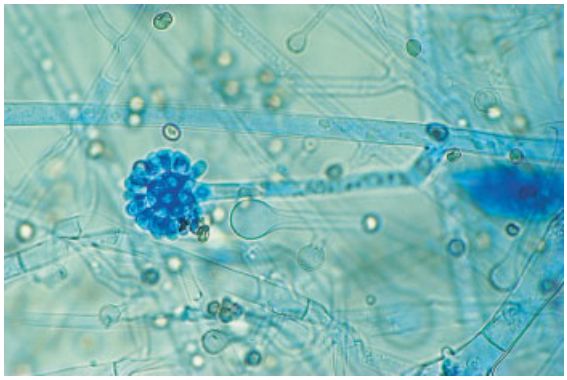

Figure 3 Cunninghamella bertholletiae*: microscopic morphology showing the formation of single-celled sporangiolas from a swollen vesicle. Magnification ×495*

Colonies are very fast growing, white at first, but becoming rather dark gray and powdery with development. Sporangiophores are up to 20 μm wide, straight, with verticillate or solitary branches. Vesicles are subglobose to pyriform, with the terminal ones being up to 40 μm and the lateral ones 10–30 μm in diameter. Sporangiola are globose (7–11 μm diameter), ellipsoidal (9–13×6–10 μm), verrucose or short echinulate, hyaline singly but brownish *en masse* (Figure 3). Temperature is at optimum 25–30°C and has a maximum up to 50°C. Key features include growth at 40°C and the presence of one-celled, globose to ovoid, echinulate 'conidia' borne on terminal or lateral globose to clavate fertile vesicles. (For additional information see Samson 1969; Weitzman and Crist 1979, 1980; Domsch et al. 1980; McGinnis 1980; Lunn and Shipton 1983; Weitzman 1984; Sands et al. 1985; Iwatsu et al. 1990; de Hoog et al. 2000.)

Mortierella wolfii Mehrotra & Baijal

The genus *Mortierella* has now been placed in a separate order, the Mortierellales (Cavalier-Smith 1998), and is characterized by gray to yellowish-grey, rapidly growing colonies, often spreading in overlapping 'waves' or lobes, which produce small delicate sporangia lacking a columella, on simple or branched sporangiophores (Figure 4). Sporangia may be one- or multispored and a collarette is typically left after the sporangial wall dissolves. Sporangiospores are one celled, globose to ellipsoidal, and chlamydoconidia may also be present. *Mortierella* spp. are common soil fungi and the genus contains about 90 recognized species; however, *M. wolfii* is probably the only species pathogenic to animals (Zycha et al. 1969; Domsch et al. 1980; de Hoog et al. 2000), although Scholz and Meyer (1965) have reported a case of pulmonary infection in cattle caused by *Mortierella polycephala*.

M. wolfii is an important causal agent of bovine mycotic abortion, pneumonia, and systemic mycosis in Australia, Europe, New Zealand, and the USA (di Menna et al. 1972; Carter et al. 1973; Wohlgemuth and Knudtson 1977; MacDonald and Corbel 1981; Neilan et al. 1982; Komoda et al. 1988; Smith 1989; Knudtson and Kirkbride 1992; Done et al. 1994; Johnson et al. 1994; Uzal et al. 1999). Confirmed human infections have not been documented. *M. wolfii* has also been isolated from soil, rotten silage, hay, and coal spoil tips (Mehrotra and Baijal 1963; Austwick 1965). Seviour et al. (1987) have described a specialized medium, silage extract agar, to stimulate sporulation of isolates of *M. wolfii*.

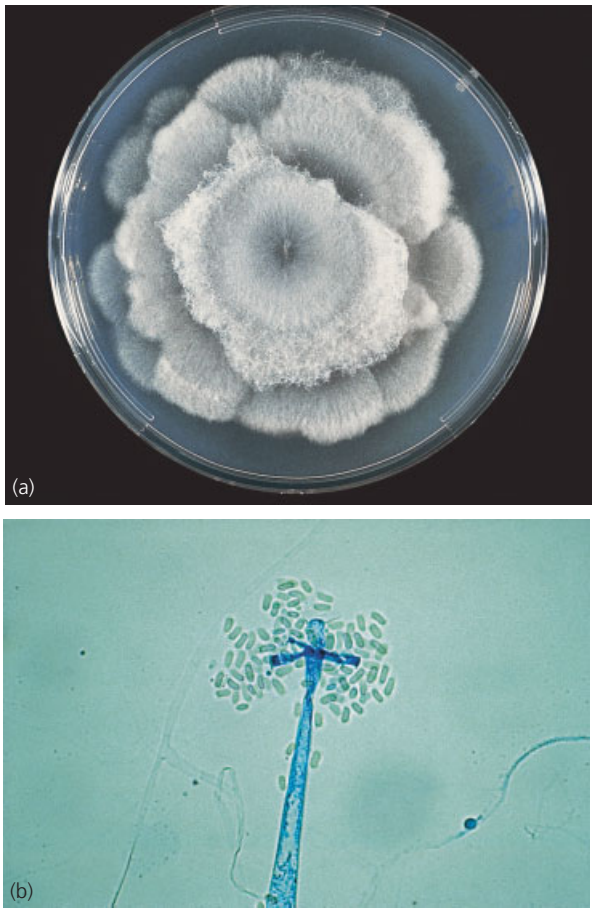

Figure 4 Mortierella wolfii*: **(a)** culture showing typical lobed growth pattern; **(b)** sporangium with acrotonous (terminal) branches. Magnification ×330*

Cultures are fast growing, white to grayish-white, downy, often with a broadly zonate or lobed (rosette-like) surface appearance, and no reverse pigment. Sporangiophores are typically erect, delicate, 80–250 μm in height, 6–20 μm wide at the base, arising from rhizoids or bulbous swellings on the substrate hyphae, and terminating with a compact cluster of short acrotonous (terminal) branches. Sporangia are usually 15–48 μm in diameter, with transparent walls, and a conspicuous collarette is usually present after dehiscence of the sporangiospores (Figure 4). Columellae are generally lacking and sporangiospores are single-celled,

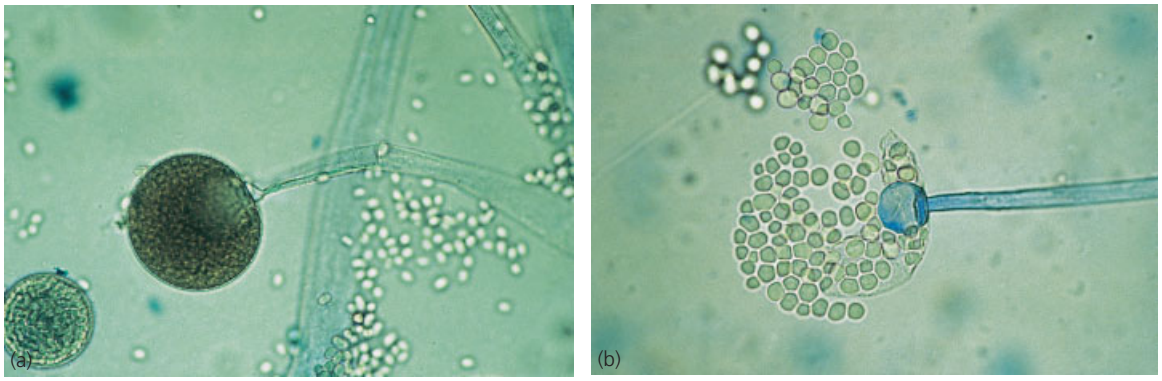

Figure 5 Mucor *species:* **(a, b)** *sporangia, columella and sporangiospores. Magnification ×495*

short–cylindrical, 6–10×3–5 μm, with a double membrane. Chlamydoconidia with or without blunt appendages (ameba-like) may be present. Zygospores have not been observed. Isolates grow well at 40–42°C, with a maximum of 48°C. Key features include rapid growth at 40°C (thermotolerant) and characteristic delicate acrotonous branching sporangia without columellae. (For additional information, see Mehrotra and Baijal 1963; Zycha et al. 1969; Domsch et al. 1980; Seviour et al. 1987; de Hoog et al. 2000.)

Mucor species

The genus *Mucor* can be differentiated from *Absidia*, *Rhizomucor*, and *Rhizopus* by the absence of stolons and rhizoids. Colonies are very fast growing, cottony to fluffy, white to yellow, becoming dark gray, with the development of sporangia. Sporangiophores are erect, simple or branched, forming large (60–300 μm in diameter), terminal, globose to spherical, multispored sporangia, without apophyses and with well-developed subtending columellae (Figure 5). A conspicuous collarette (remnants of the sporangial wall) is usually visible at the base of the columella after sporangiospore dispersal. Sporangiospores are hyaline, gray or brownish, globose to ellipsoidal, and smooth walled or finely ornamented. Chlamydoconidia and zygospores may also be present. The genus contains about 50 recognized taxa, many of which have widespread occurrence and are of considerable economic importance (Zycha et al. 1969; Schipper 1978a; Domsch et al. 1980). However, only a few thermotolerant species are of medical importance and human infections are only rarely reported (Table 2). Most infections reported list *M. circinelloides* and similar species such as *M. indicus* (*M. rouxii*), *Mucor ramosissimus*, and *Mucor amphibiorum* as the causative agents. However, *Mucor hiemalis* and *Mucor racemosus* have also been reported as infectious agents, although their inability to grow at temperatures above 32°C raises doubt as to their validity as human pathogens and their pathogenic role may be limited to cutaneous infections (Scholer et al. 1983; Goodman and Rinaldi 1991; Kwon-Chung and Bennett 1992; de Hoog et al. 2000). Key laboratory diagnostic features are the presence of large, spherical, non-apophysate sporangia with pronounced columellae and conspicuous collarettes after sporangiospore dispersal. (For additional information, see Schipper 1978a; Domsch et al. 1980; McGinnis 1980; Onions et al. 1981; Scholer et al. 1983; Rippon 1988; Goodman and Rinaldi 1991; Samson et al. 1995; de Hoog et al. 2000; Schipper and Stalpers 2003.)

MUCOR AMPHIBIORUM SCHIPPER

This species has been reported as the causative agent of zygomycosis in several amphibia (Frank et al. 1974; Schipper 1978a; Speare et al. 1994, 1997; Berger et al. 1997) and in free-living platypuses (*Ornithorhynchus anitinus*) (Obendorf et al. 1993; Connolly et al. 2000). Human infections have not been reported. Colonies are

Table 2 *Maximum temperature of growth for the reported pathogenic species of* Mucor

Species	Maximum temperature (°C)	Pathogenicity
M. amphibiorum	36	Animals, principally amphibians
M. circinelloides	36–40	Animals, occasionally humans
M. hiemalis	30	Questionable cutaneous infections only
M. indicus	42	Humans and animals
M. racemosus	32	Questionable
M. ramosissimus	36	Humans and animals

grayish-brown, slightly aromatic, and do not grow at 37°C (maximum temperature for growth is 36°C). Sporangiophores are hyaline, erect, and mostly unbranched, rarely sympodially branched. Sporangia are dark brown, up to 75 μm in diameter, and are slightly flattened with a diffluent membrane. Columellae are subglobose to ellipsoidal or pyriform, up to 60×50 μm, with small collars. Sporangiospores are smooth walled, spherical, and 3.5–5.5 μm in diameter. Zygospores, when formed by compatible mating types, are spherical to slightly compressed, up to 70×60 μm in diameter, with stellate projections. *M. amphibiorum* is distinguished by poor ramification of the sporangiophores and by globose sporangiospores. Ethanol and nitrates are not assimilated (Schipper 1978a; Scholer et al. 1983; de Hoog et al. 2000).

MUCOR CIRCINELLOIDES VAR. TIEGH

M. circinelloides is a common and variable species that includes four forms: *circinelloides*, *lusitanicus*, *griseocyanus*, and *janssenii* (Schipper 1978a; Scholer et al. 1983). It has been reported as a rare cause of cutaneous infections in humans (Fetchick et al. 1986; Berenguer et al. 1988; Wang et al. 1990; Fingeroth et al. 1994; Chandra and Woodgyer 2002) and from mycoses in cattle and swine (Morquer et al. 1965), birds (Porges et al. 1935; Marjankova et al. 1978), and a platypus (Stewart et al. 1999). Colonies are floccose, pale grayish-brown, and grow poorly at 37°C (maximum growth temperature 36–40°C). Sporangiophores are hyaline and mostly sympodially branched with long branches erect and shorter branches becoming circinate (recurved). Sporangia are spherical, varying from 20 to 80 μm in diameter, with small sporangia often having a persistent sporangial wall. Columellae are spherical to ellipsoidal and are up to 50 μm in diameter. Sporangiospores are hyaline, smooth walled, ellipsoidal, and 4.5–7×3.5–5 μm in size. Chlamydoconidia are generally absent. Zygospores are only produced in crosses of compatible mating types and are reddish-brown to dark brown, spherical with stellate spines, up to 100 μm in diameter and have equal to slightly unequal suspensor cells. *M. circinelloides* differs from other species of *Mucor* in its formation of short circinated (coiled), branched sporangiophores bearing brown sporangia and its ability to assimilate ethanol and nitrates (Schipper 1976; Scholer et al. 1983; Samson et al. 1995; de Hoog et al. 2000; Schipper and Stalpers 2003).

MUCOR HIEMALIS WEHMER

This species has been reported from a few cases of human cutaneous infection (Neame and Rayner 1960; Costa et al. 1990; Prevoo et al. 1991). However, with a maximum growth temperature of 30°C its pathogenicity is questionable. It is most commonly recovered from soil (Schipper 1973). Colonies are grayish-ochraceous in color. Sporangiophores are hyaline, erect, mostly unbranched, with occasional sympodial branching. Sporangia are yellow to dark brown, up to 80 μm in diameter and have a diffluent membrane. Columellae are ellipsoidal, 30×38 μm in diameter. Sporangiospores are hyaline, smooth walled, ellipsoidal, sometimes flattened on one side, and 5.5–9×2.5–5.5 μm in size. Oidia may be present in the substrate hyphae, chlamydoconidia are uncommon, and zygospores are not formed in pure culture. *M. hiemalis* is similar to *M. circinelloides* but produces larger sporangiospores, usually has unbranched sporangiophores, and is unable to grow at 37°C (Schipper 1976; Domsch et al. 1980; Samson et al. 1995; de Hoog et al. 2000; Schipper and Stalpers 2003).

MUCOR INDICUS LENDNER

M. indicus, usually referred to in the medical literature by its synonym *M. rouxii* (Calmette)Wehmer *sensu* Webmer, has been reported from human gastric (Douvin et al. 1975; Borg et al. 1990) and pulmonary (Krasinski et al. 1985) infections and from a case of necrotizing fasciitis (Mata-Essayag et al. 2001). Oliver et al. (1996) reported an interesting case of an hepatic infection in a bone marrow transplant recipient who had ingested contaminated naturopathic medicine. Colonies are characteristically deep yellow, aromatic, and have a maximum growth temperature of 42°C. Sporangiophores are hyaline to yellowish, erect or, rarely, circinate, and repeatedly sympodially branched, with long branches. Sporangia are yellow to brown, up to 75 μm in diameter, with diffluent membranes. Columellae are subglobose to pyriform, often with truncate bases, up to 40 μm high. Sporangiospores are smooth walled, subglobose to ellipsoidal, and 4–5 μm in diameter. Chlamydoconidia are produced in abundance, especially in the light. Zygospores, when formed by crosses of compatible mating strains, are black, spherical, up to 100 μm in diameter, with stellate spines and unequal suspensor cells. *M. indicus* differs from other species of *Mucor* by its characteristic deep-yellow colony color, growth at over 40°C, assimilating ethanol, but not nitrate, and being thiamine dependent (Schipper 1978a; de Hoog et al. 2000; Schipper and Stalpers 2003).

MUCOR RACEMOSUS FR.

This species has been infrequently reported as a causative agent of animal and human zygomycosis (Scholer et al. 1983). With a maximum growth temperature of only 32°C, however, its pathogenicity is questionable. Colonies are light grayish-brown in color. Sporangiophores are hyaline, erect, and show both sympodial and monopodial branching. Sporangia are brownish, up to 80–90 μm in diameter, and the membrane is mostly diffluent but persistent in smaller sporangia. Columellae are subglobose to pyriform, often with truncated bases,

light brown with collars. Sporangiospores are hyaline to pale brown, smooth walled, subglobose to broadly ellipsoidal, 8–10 μm in diameter. Chlamydoconidia are formed abundantly, but zygospores are not produced in pure culture. *M. racemosus* differs from other species of *Mucor* by the formation of chlamydoconidia in sporangiophores, its inability to grow at 37°C, and its positive assimilation of sucrose (Schipper 1976; Samson et al. 1995; de Hoog et al. 2000).

MUCOR RAMOSISSIMUS SAMUTSEVICH

M. ramosissimus has been isolated from mucocutaneous (Vignale et al. 1964), cutaneous (Weitzman et al. 1993), and rhinocerebral (Bullock et al. 1974) infections and from a case of septic arthritis in a neonate (Sharma et al. 1994). Colonial growth is restricted; it is grayish and does not grow at 37°C (maximum temperature for growth is 36°C). Sporangiophores are hyaline, slightly roughened, tapering towards the apex, and are erect with repeated sympodial branching. Sporangia are gray to black, globose or somewhat flattened, up to 80 μm in diameter and have very persistent sporangial walls. Columellae are applanate (flattened), up to 40–50 μm in size, and are often absent in smaller sporangia. Sporangiospores are faintly brown, smooth walled, subglobose to broadly ellipsoidal, 5–8×4.5–6 μm in size. Oidia may be present in the substrate hyphae, chlamydoconidia and zygospores are absent. Assimilation of ethanol is negative and that of nitrate is positive. *M. ramosissimus* differs from other species of *Mucor* by its low, restricted growth on any medium, extremely persistent sporangial walls, columellae that are applanate or absent in smaller sporangia (often resembling *Mortierella* species), short sporangiophores that repeatedly branch sympodially as many as 12 times, and the occurrence of racket-shaped enlargements in the sporangiophores (Hesseltine and Ellis 1964; Schipper 1976; Scholer et al. 1983; de Hoog et al. 2000; Schipper and Stalpers 2003).

Rhizomucor species

The genus *Rhizomucor* is distinguished from *Mucor* by the presence of stolons and poorly developed rhizoids at the base of the sporangiophores and by the thermophilic behavior of its three species: *R. miehei*, *R. pusillus*, and *R. tauricus*. All three of these species are potential human and animal pathogens and were originally classified in the genus. *R. variabilis* as described by de Hoog et al. (2000) is not thermophilic and is probably a degenerate culture of *M. hiemalis* (Voigt et al. 1999). *R. pusillus* is cosmopolitan and both *R. miehei* and *R. pusillus* have been reported as pathogens to humans and animals, the latter to the greater extent (Cooney and Emerson 1964; Hesseltine and Ellis 1973; Schipper 1978b; Scholer et al. 1983; de Hoog et al. 2000; Schipper and Stalpers 2003).

RHIZOMUCOR MIEHEI (COONEY AND EMERSON) SCHIPPER

R. miehei (synonym: *Mucor miehei* Lindt) has been reported as a rare cause of bovine mastitis (Scholer et al. 1983) and is similar in most respects to *R. pusillus* (described in the next section). However, all strains are homothallic, forming numerous zygospores, which are reddish-brown to blackish-brown, globose to slightly compressed, up to 50 μm in diameter, with stellate warts and equal suspensor cells. Colony color is a dirty gray rather than brown, and sporangia have spiny walls, are up to 50–60 μm in diameter, with columellae rarely larger than 30 μm in diameter. Growth is stimulated by thiamine, with no assimilation of sucrose and maximum temperature of growth is 54–58°C. Key features include growth at 45°C, the formation of numerous zygospores, a dirty gray culture color and a partial growth requirement for thiamine. (For further information, see Cooney and Emerson 1964; Schipper 1978b; Scholer et al. 1983; de Hoog et al. 2000.)

RHIZOMUCOR PUSILLUS (LINDT) SCHIPPER

R. pusillus (synonym: *Mucor pusillus* Lindt) is a rare human pathogen. It has been reported from cases of pulmonary (Nicod et al. 1952; Zagoria et al. 1985; Latif et al. 1997; Bjorkholm et al. 2001; Ma et al. 2001), disseminated (Meyer et al. 1973; Kramer et al. 1977; Severo et al. 1991; Gonzalez et al. 1997), and cutaneous types of infection (Wickline et al. 1989). Erdos et al. (1972) have documented a case of endocarditis and St-Germain et al. (1993) reported three cases of pulmonary and one of disseminated infection in patients with leukemia. It is more often associated with animal disease, especially bovine abortion (Smith 1989). *R. pusillus* has a worldwide distribution and is commonly associated with compost heaps. This thermophilic zygomycete is readily recognizable by its characteristic compact, low growing (2–3 mm high), gray to grayish-brown colored mycelium, and by the development of typical, sympodially branched, hyaline to yellow–brown sporangiophores (8–15 μm in diameter), always with a septum below the sporangium. Sporangia are brown or gray, globose (40–60 μm in diameter), each possessing an oval or pear-shaped columella (20–30 μm), often with a collarette (Figure 6). Sporangiospores are hyaline, smooth walled, globose to subglobose, occasionally oval (3–5 μm), and are often mixed with crystalline remnants of the sporangial wall. Chlamydoconidia are absent. Zygospores are rough walled, reddish-brown to black, 45–65 μm in diameter, and may be produced throughout the aerial hyphae in matings between compatible isolates. Temperature growth range is: minimum 20–27°C; optimum 35–55°C; maximum 55–60°C. There is positive assimilation of sucrose and no thiamine

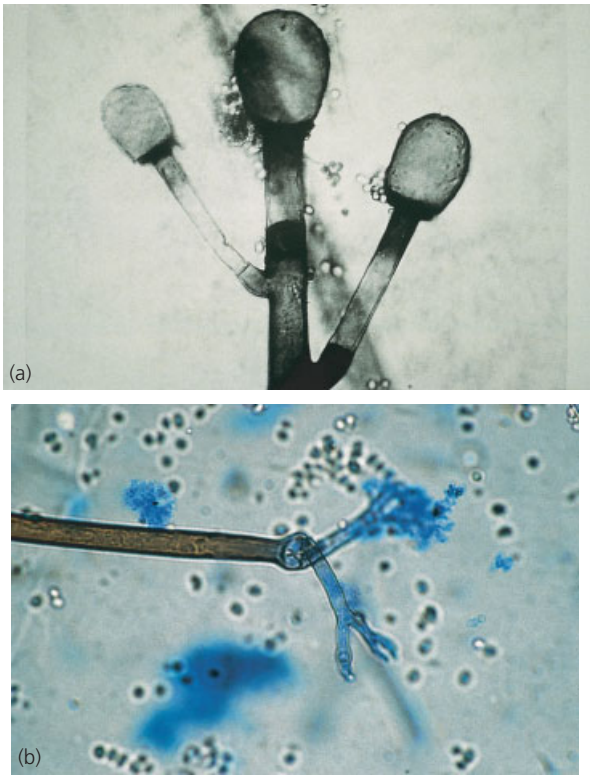

Figure 6 Rhizomucor pusillus: **(a)** *branched sporangiophores and columellae;* **(b)** *primitive rhizoids. Magnification:* **(a)** *×330 and* **(b)** *×495*

dependence. Key features include growth at 45°C, poorly developed stolons and rhizoids, branching sporangiophores with a septum below the sporangium, dark-colored sporangia without apophyses, and smooth-walled globose to subglobose sporangiospores. (For additional information, see Cooney and Emerson 1964; Schipper 1978b; Domsch et al. 1980; Ellis and Keane 1981; McGinnis 1980; Scholer et al. 1983; de Hoog et al. 2000; Schipper and Stalplers 2003.)

Rhizopus species

The genus *Rhizopus* is characterized by the presence of stolons and darkly pigmented (pheoid) rhizoids, the formation of sporangiophores singly or in groups from nodes directly above the rhizoids, and apophysate, columellate, multispored, generally globose sporangia. After spore release, the apophyses and columella often collapse to form an umbrella-like structure. Sporangiospores are globose to ovoid, one celled, hyaline to brown, and striate in many species. Colonies are fast growing and cover an agar surface with a dense cottony growth which is at first white, becoming gray or yellowish-brown with sporulation. Species of *Rhizopus* are often associated with soil, plant material, fruit, and similar substrates. Many species grow well at high temperatures, some are used in food fermentations, whereas others are pathogenic to humans and animals. (For descriptions of species, keys to taxa, and additional information, see Domsch et al. 1980; McGinnis 1980; Onions et al. 1981; Scholer et al. 1983; Schipper 1984; Schipper and Stalpers 1984, 2003; Ellis 1985, 1986; Rippon 1988; Kwon-Chung and Bennett 1992; Samson et al. 1995; de Hoog et al. 2000.)

In the past, numerous attempts have been made to clarify the species concepts of the genus *Rhizopus* (Inui et al. 1965; Zycha et al. 1969; Hesseltine and Ellis 1973). Three excellent revisions with easy-to-use keys have been produced by Schipper (1984), Ellis (1985, 1986), and Schipper and Stalpers (2003). Basically, three groups have been recognized: the 'stolonifer' group, the 'oryzae' group, and the 'microsporus' group. The GC values of the three groups have been defined by Frye and Reinhardt (1993), and temperature growth studies at 30, 36, and 45°C are characteristic for each of the groups.

The 'stolonifer' group has sporangia up to 275 μm in diameter and grows at 30°C, but has a maximum growth temperature of 36°C. Species in this group include *Rhizopus sexualis* and *Rhizopus stolonifer*. The latter has been unconvincingly implicated in human infection (Ferry and Abedi 1983), although with a maximum growth temperature of only 32°C its pathogenicity is thus questionable.

The 'oryzae' group has been reduced to a single species that is able to grow at 40°C but not at 45°C, and has sporangia not exceeding 240 μm in diameter. There is no doubt that *R. oryzae* and *Rhizopus arrhizus* are synonymous, the contentious issue being which species name to use (Schipper 1984; Ellis 1985; Frye and Reinhardt 1993). Briefly, *R. arrhizus* was described before *R. oryzae* and some authors would therefore give that name priority (Ellis 1985, 1986; Rippon 1988; Goodman and Rinaldi 1991). However, other authors consider the original identity of *R. arrhizus* as somewhat doubtful and at best an extreme or atypical form of *R. oryzae*, because it shows applanate columellae that are very unusual in *R. oryzae* (Domsch et al. 1980; Scholer et al. 1983; Schipper 1984; Kwon-Chung and Bennett 1992). The taxonomic treatment of Schipper (Schipper 1984; Schipper and Stalpers 2003) is used in this chapter; however, the synonym *R. arrhizus* is commonly used in the medical literature. *R. oryzae* is an important human pathogen.

The 'microsporus' group has simple rhizoids, and smaller sporangia up to 100 μm in diameter and grows at both 40 and 45°C. This group contains four species: *Rhizopus homothallicus*, *Rhizopus azygosporus*, *Rhizopus schipperae*, and *Rhizopus microsporus*, with the latter subdivided into three varieties, namely *R. microsporus* var. *microsporus*, *R. microsporus* var. *oligosporus*, and *R. microsporus* var. *rhizopodiformis* (Table 3). All are thermophilic and *R. microsporus* is a well-recognized pathogen of humans and animals.

Table 3 *Differentiation of pathogenic* Rhizopus microsporus *isolates*

Species	Growth at 45°C	Growth at 50°C	Main species characteristics
R. azygosporus	Good	No	Abundant azygospores
R. microsporus var. *microsporus*	Good	No	Sporangiospores angular to ellipsoidal and distinctly striate, up to 5–6 μm diameter
R. microsporus var. *oligosporus*	Restricted	No	Sporangiospores globose, up to 9 μm diameter or more, heterogeneous
R. microsporus var. *rhizopodiformis*	Good	Good	Sporangiospores globose rarely over 5 μm in diameter minutely spinulose
R. schipperae	Good	No	Abundant chlamydoconidia and restricted sporulation

RHIZOPUS AZYGOSPORUS YUAN & JONG

R. azygosporus is closely related to *R. microsporus* (Yuan and Jong 1984) and has been reported as the causative agent of three fatal cases of gastrointestinal infection in premature babies (Woodward et al. 1992; Schipper et al. 1996). Previously, this fungus was only known from its type culture, which has been isolated from tempeh, a solid fermented soybean food from Indonesia (Yuan and Jong 1984). Colonies are whitish to gray–black, producing pale brown simple rhizoids. Sporangiophores are brownish, up to 350 μm high, and 6–14 μm wide. Sporangia are grayish-black, spherical, and 50–100 μm in diameter. Columellae are subglobose to globose. Sporangiospores are ovoid to ellipsoidal, 4–5 to 6–7 μm in diameter with faint striations. Azygospores are pale to dark brown, spherical to subglobose, 30–70 μm in diameter, with coarse conical projections. All strains produce abundant azygospores in unmated isolates as a species characteristic. There is good growth at 45°C with a maximum of 46–48°C. (For further information, see Yuan and Jong 1984; Schipper et al. 1996; de Hoog et al. 2000.)

RHIZOPUS MICROSPORUS VAR. *MICROSPORUS*

R. microsporus var. *microsporus* is a rare cause of human infection (Kerr et al. 1988; Kwon-Chung and Bennett 1992). An unusual case of cellulitis in the leg of a diabetic patient was reported by West et al. (1995) and a cutaneous case in a patient with idiopathic thrombopenic purpura was reported by Kobayashi et al. (2001). Colonies are pale brownish-gray producing simple rhizoids. Sporangiophores are brownish, up to 400 μm high and 10 μm wide, but most are smaller and are produced in pairs. Sporangia are grayish-black, spherical, up to 80 μm in diameter. Columellae are subglobose to globose to conical. Sporangiospores are angular to broadly ellipsoidal to lemon-shaped, quite equal in size, up to 5–6 μm in diameter and are distinctly striate. Zygospores, formed by crosses of compatible mating strains, are dark red–brown, spherical, up to 100 μm in diameter, with stellate projections and unequal suspensor cells. There is good growth at 45°C, with a maximum of 46–48°C. (For further information, see Scholer et al. 1983; Schipper and Stalpers 1984, 2003.)

RHIZOPUS MICROSPORUS V. TIEGH. VAR. *OLIGOSPORUS* (SAITO) SCHIPPER AND STALPERS

R. microsporus var. *oligosporus* is a rare cause of human zygomycosis (Tintelnot and Nitsche 1989). Colonies are pale yellowish-brown to gray and sporulation is often poor. Rhizoids are subhyaline and simple. Sporangiophores are brownish, up to 300 μm high, and 15 μm wide, with one to three produced together. Sporangia are black, spherical, up to 100 μm in diameter. Columellae are subglobose to somewhat conical. Sporangiospores are subglobose to globose, up to 9 μm in diameter, almost smooth, with larger spores often irregular in shape (Figure 7). Chlamydoconidia are abundant, hyaline, single or in chains, spherical, ellipsoidal, or cylindrical, 7–35 μm in diameter. Zygospores are not known. There is growth at 45°C with a maximum of 46–48°C. (For further information, see Scholer et al. 1983; Schipper and Stalpers 1984; de Hoog and Guarro 1995; Samson et al. 1995.)

RHIZOPUS MICROSPORUS V. TIEGH VAR. *RHIZOPODIFORMIS* (COHN) SCHIPPER AND STALPERS

R. microsporus var. *rhizopodiformis* is the second most frequently isolated zygomycete, accounting for between 10 and 15 percent of reported human cases (Scholer et al. 1983; Kwon-Chung and Bennett 1992). It is most commonly isolated from cutaneous infections with several reports citing the use of contaminated Elastoplast bandages or wooden tongue depressors as the environmental source of the fungus (Baker et al. 1962; Ellis 1978; Gartenberg et al. 1978; Bottone et al. 1979; Everett et al. 1979; Mead et al. 1979; Sheldon and Johnson 1979; Myskowski et al. 1983; West et al. 1983; Parfrey 1986; Norden et al. 1991; Paparello et al. 1992; Mitchell et al. 1996; Verweij et al. 1997; Holzel et al. 1998). Gastrointestinal infections are also commonly reported (Neame and Rayner 1960; Waller et al. 1993; Kimura et al. 1995), but disseminated and rhinocerebral forms of infection are rare (Rosenberger et al. 1983;

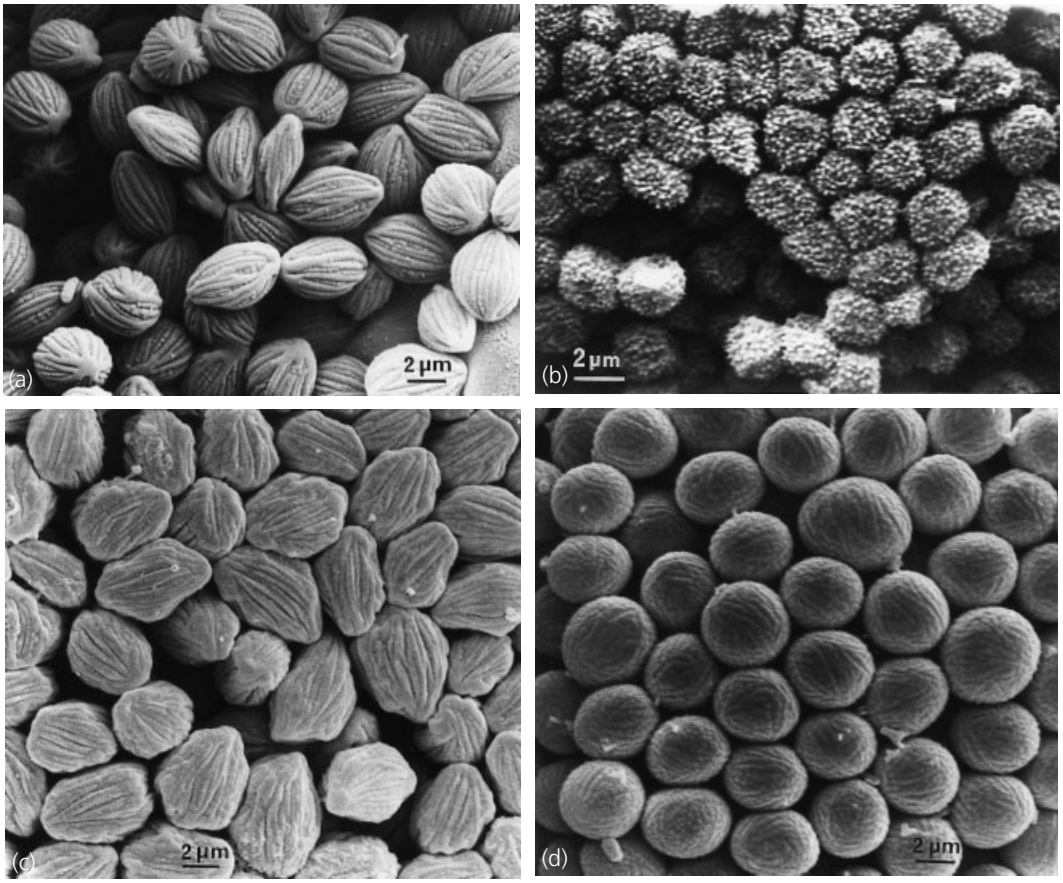

Figure 7 Rhizopus *spp.: sporangiospore ornamentation by scanning electron microscopy.* **(a)** R. microsporus *var.* microsporus*;* **(b)** R. microsporus *var.* rhizopodiformis*;* **(c)** R. oryzae *(*R. arrhizus*).* **(d)** R. microsporus *var.* oligosporus

Rangel-Guerra et al. 1985; Iqbal and Scheer 1986; Levy et al. 1986). *R. microsporus* var. *rhizopodiformis* is also an important pathogen in animals, especially in swine and bovine abortion (Dion and Sandford 1985; Smith 1989). Colonies are dark grayish-brown, up to 10 mm high with simple rhizoids. Sporangiophores are brownish, up to 500 µm high and 8 µm wide, with one to four produced together. Sporangia are bluish to grayish-black, spherical and up to 100 µm in diameter. Columellae are pyriform, comprising 80 percent of the sporangium. Sporangiospores are subglobose to globose, quite equal in size, up to 6 µm in diameter, and minutely spinulose (Figure 7). Zygospores, when formed by crosses of compatible mating strains, are reddish-brown, spherical, up to 100 µm in diameter, with stellate projections and unequal suspensor cells. There is good growth at 45°C with a maximum of 50–52°C (Bottone et al. 1979; Scholer et al. 1983; Schipper and Stalpers 1984, 2003; Ellis 1986; Polonelli et al. 1988; Waller et al. 1993; de Hoog et al. 2000).

RHIZOPUS ORYZAE WENT & PRINSEN GEERLIGS

R. oryzae (synonym: *R. arrhizus*) is the most common causative agent of zygomycosis, accounting for some 60 percent of the reported culture positive cases, and nearly 90 percent of the rhinocerebral form of infection (Scholer et al. 1983; Hyatt et al. 1992; Kwon-Chung and Bennett 1992; Hofman et al. 1993; Attapattu 1995; Adler et al. 1998; Romano et al. 2002). Pulmonary infections (Muhm et al. 1996; Yokoi et al. 1999; Vincent et al. 2000) and primary cutaneous infections (Johnson et al. 1993; Linder et al. 1998; Song et al. 1999) have also been reported. Cerebral infections have occasionally been diagnosed in leukemia patients (McGinnis 1980) and drug addicts (Oliveri et al. 1988; Fong et al. 1990). Sanchez et al. (1994) reviewed infections in AIDS patients and Winkler et al. (1996) reported a case of gastric mucormycosis in a renal transplant patient. This species has rarely been reported from animals (Smith 1989; Perelman and Kuttin 1992). *R. oryzae* (*arrhizus*) has a worldwide distribution with a high prevalence in tropical and subtropical regions. It has been isolated from many substrates, including a wide variety of soils, decaying vegetation, foodstuffs, and animal and bird dung. *R. oryzae* is often used in the production of fermented foods and alcoholic beverages in Indonesia, China, and Japan. However, it also produces the ergot alkaloid agroclavine which is toxic to humans and animals (Domsch et al. 1980; Samson et al. 1995).

Colonies are very fast growing at 25°C, reaching 5–8 mm in height, with some tendency to collapse, white and cottony at first, becoming brownish-gray to blackish-gray depending on the amount of sporulation. Sporangiophores are up to 1 500 μm in length and 18 μm in width, smooth walled, nonseptate, simple or branched, arising from stolons opposite rhizoids usually in groups of three or more (Figure 8). Sporangia are globose, often with a flattened base, grayish-black, powdery in appearance, up to 175 μm in diameter, and with many spores. Columella and apophysis together are globose, subglobose or oval, up to 130 μm in height, and soon collapse to an umbrella-like form after spore release (Figure 8). Sporangiospores are angular, subglobose to ellipsoidal, with ridges on the surface, and up to 8 μm in length. Chlamydoconidia may be formed singly or in chains and are hyaline, smooth walled, spherical to ovoid, and 10–35 μm in diameter. Zygospores, when formed by crosses of compatible mating strains, are red to brown, spherical or laterally flattened, 60–140 μm in diameter, with flat projections and unequal suspensor cells. There is no growth at 45°C, but good growth at 40°C. Key features include growth at 40°C, sporangiophores often more than 1 mm in height, rhizoids with secondary branching, and sporangia between 100 and 240 μm in diameter (Domsch et al. 1980; Scholer et al. 1983; Schipper 1984; Ellis 1985; Oliveri et al. 1988; Samson et al. 1995; de Hoog et al. 2000).

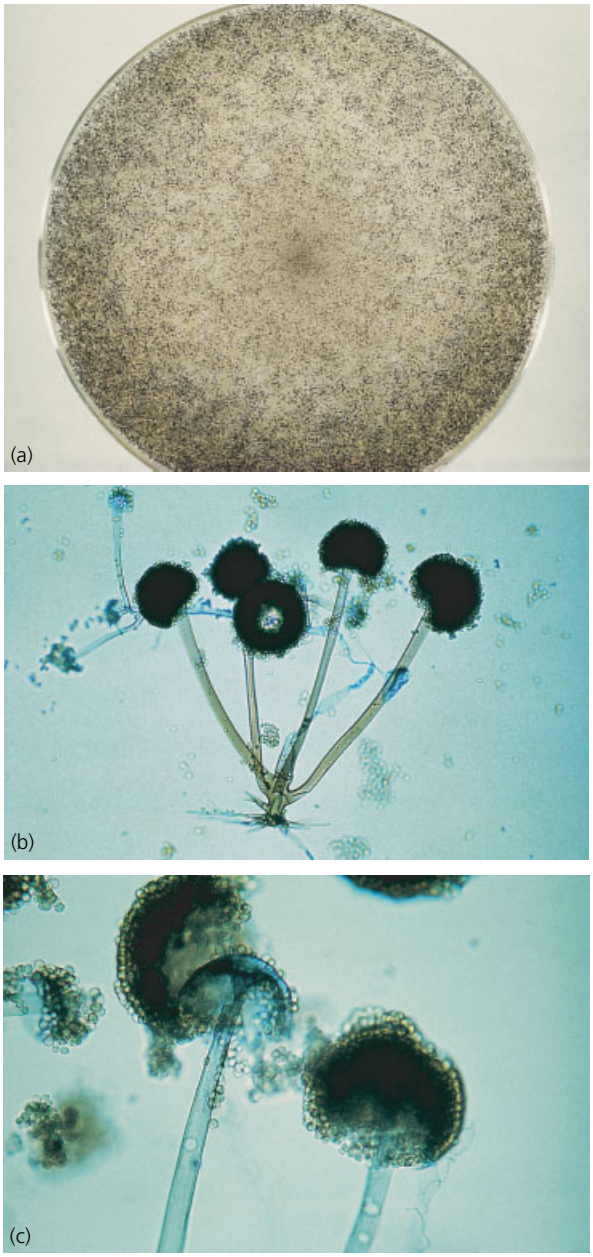

Figure 8 Rhizopus oryzae (arrhizus)**: (a)** *culture showing abundant pin-head sporangia;* **(b)** *sporangiophores, rhizoids, and sporangia. Magnification ×80.* **(c)** *Sporangium with collapsed columell and sporangiospores. Magnification ×330*

RHIZOPUS SCHIPPERAE WEITZMAN, MCGOUGH, RINALDI & DELLA-LATTA

R. schipperae belongs to the *R. microsporus* complex and is closest to *R. microsporus* var. *microsporus* (Weitzman et al. 1996). It was recently isolated from the bronchial wash and lung specimens from a patient with myeloma (Weitzman et al. 1996) and from paranasal and gastrointestinal specimens from a patient suffering heatstroke (Anstead et al. 1999). Distinguishing characteristics include the production of sporangia on a limited range of media, best on Czapek Dox agar [Oxoid (CM97), Basingstoke, Hants, UK], with clusters of up to 10 sporangiophores arising from each bundle of rhizoids and the formation of abundant, large, mostly rounded chlamydoconidia on all media. Colonies on Czapek Dox agar are grayish-white producing hyaline to pale brown simple rhizoids. Sporangiophores are brownish, 100–460 μm in length, 5–15 μm wide and may be produced singly, in pairs, or in aggregates of up to 10. Sporangia are grayish-black, spherical, and are up to 80 μm in diameter. Columellae are subglobose to subglobose–conical. Sporangiospores are subglobose to ovoid, 5–6 to 6–7 μm in diameter with faint striations. Chlamydoconidia are abundant, terminal or intercalary, globose, oval to ellipsoidal, up to 20 μm in diameter. Zygospores are unknown. Growth is on potato dextrose agar at 45°C, but not at 48–50°C, with optimal development observed at 30–35°C (Weitzman et al. 1996).

RHIZOPUS STOLONIFER (EHRENB:FR) VUILL.

R. stolonifer has been implicated only once in human infection (Ferry and Abedi 1983), and with a maximum growth temperature of only 32°C its pathogenic role is questionable. Colonies are whitish with black spots of sporangia and dark sporangiophores; they produce well-developed complex rhizoids. Sporangiophores are brownish, up to 2 000 μm high and 20 μm wide, and are usually produced in groups of one to three. Sporangia are blackish, spherical, up to 275 μm in diameter.

Columellae are conical and up to 140 μm in height. Sporangiospores are angular to globose to ellipsoidal, up to 13 μm in length, and distinctly striate. Zygospores, when formed by crosses of compatible mating strains, are black, spherical, up to 200 μm in diameter, with stellate projections and unequal suspensor cells. There is no growth at 33°C, but good growth and sporulation at 15–30°C. (For additional information, see Domsch et al. 1980; Onions et al. 1981; Schipper 1984; Samson et al. 1995; de Hoog et al. 2000.)

Saksenaea vasiformis Saksena

The genus *Saksenaea* is characterized by the formation of flask-shaped sporangia with columellae and simple, darkly pigmented rhizoids. *S. vasiformis* is the only known species and appears to have a worldwide distribution in association with soil (Saksena 1953; Hodges 1962; Ajello et al. 1976). *S. vasiformis* is an emerging human pathogen (Holland 1997) that is most often associated with cutaneous or subcutaneous lesions after trauma (Oberle and Penn 1983; Ellis and Kaminski 1985; Parker et al. 1986; Pritchard et al. 1986; Padhye et al. 1988; Goldschmied-Reouven et al. 1989; Tauphaichitr et al. 1990; Mathews et al. 1993; Bearer et al. 1994; Lye et al. 1996; Chakrabarti et al. 1997; Wilson et al. 1998). It has also been associated with rhinocerebral infection (Kaufman et al. 1988), cranial infection (Ajello et al. 1976; Dean et al. 1977), osteomyelitis (Pierce et al. 1987), necrotizing cellulitis (Patino et al. 1984), and disseminated types of infection (Torrell et al. 1981; Hay et al. 1983; Solano et al. 2000). *S. vasiformis* has also been reported from a case of bovine cranial zygomycosis (Hill et al. 1992) and from cases of invasive infection in a captive killer whale and a number of dolphins (Robeck and Dalton 2002).

Laboratory identification of this fungus may be difficult or delayed because of the mold's failure to sporulate on primary isolation media or on subsequent subculture onto potato dextrose agar. Sporulation may be stimulated by the use of nutrient-deficient media, such as cornmeal–glucose–sucrose–yeast extract agar or Czapek Dox agar, or by using the agar block method described by Ellis and Ajello (1982), Ellis and Kaminski (1985), and Padhye and Ajello (1988).

Colonies are fast growing, downy, white with no reverse pigment, and are made up of broad, nonseptate hyphae typical of a zygomycetous fungus. Sporangia are typically flask shaped with a distinct spherical venter and long neck, arising singly or in pairs from dichotomously branched, darkly pigmented rhizoids (Figure 9). Columellae are prominent and dome shaped. Sporangiospores are small, oblong, 1–2×3–4 μm in size, and are discharged through the neck after the dissolution of an apical mucilaginous plug. Key features include the formation of unique flask-shaped sporangia and failure to sporulate on primary isolation media. (For additional information, see Saksena 1953; Ellis and Hesseltine 1966; Chien et al. 1992; Kwon-Chung and Bennett 1992; de Hoog et al. 2000.)

Figure 9 Saksenaea vasiformis *showing a distinct flask-shaped sporangium. Magnification ×495*

Syncephalastrum racemosum

The genus *Syncephalastrum* is characterized by the formation of cylindrical merosporangia on a terminal swelling of the sporangiophore. Sporangiospores are arranged in a single row within the merosporangia. *S. racemosum* is the type species of the genus and a potential human pathogen; however, well-documented cases are lacking. Kamalam and Thambiah (1980) reported a case of cutaneous infection and Turner (1964) reported one of bovine mycotic abortion. It is often isolated from soil and dung in tropical and subtropical regions, and it can be a persistent laboratory contaminant. The sporangiophore and merosporangia of the *Syncephalastrum* spp. can also be mistaken for an *Aspergillus* sp. if the isolate is not looked at carefully.

Colonies are very fast growing, cottony to fluffy, white to light gray, becoming dark gray with the development of sporangia. Sporangiophores are erect, stolon-like, often producing adventitious rhizoids, and show sympodial branching (racemose branching) producing curved lateral branches. The main stalk and branches form terminal, globose to ovoid vesicles which bear finger-like merosporangia directly over their entire surface (Figure 10). At maturity, merosporangia are thin walled, evanescent, and contain globose to ovoid, 5–10(18) μm, smooth-walled sporangiospores (merospores). Optimum growth temperature is 20–40°C. Key features include the production of sympodially branching sporangiophores with terminal vesicles bearing merosporangia. (For additional information, see Domsch et al. 1980; McGinnis 1980; Onions et al. 1981; Samson et al. 1995; de Hoog et al. 2000.)

HOST RESPONSES

In general, members of the Mucorales cause the more severe forms of zygomycosis, *Mortierella* (Mortierellales)

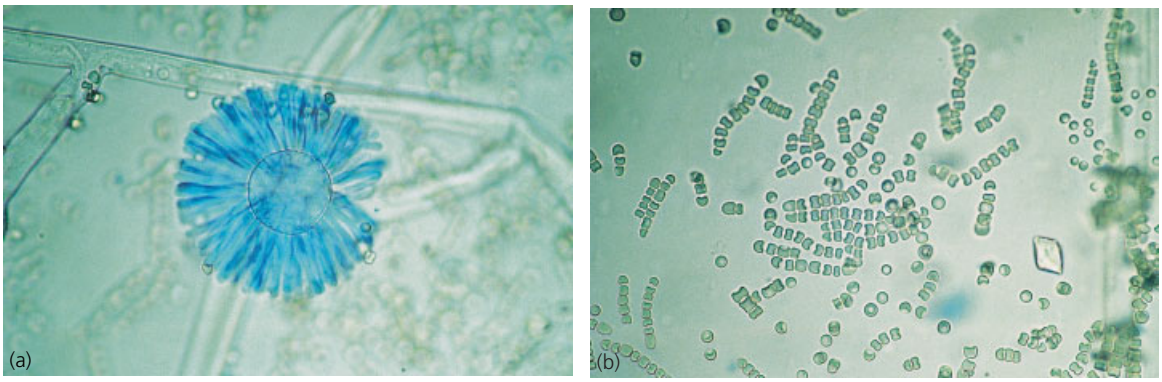

Figure 10 Syncephalastrum racemosum: **(a)** *terminal vesicle and merosporangia;* **(b)** *merospores. Magnification ×495*

is not a human pathogen, and the Entomophthorales cause more chronic disease of the nasal mucosa and subcutaneous tissue (see Subcutaneous Zygomycosis). Infections are rare in the immunocompetent patient and usually present as cutaneous lesions after the traumatic implantation of fungal elements through the skin. Lesions usually remain localized around the initial site of the inoculation and respond well to local débridement and antifungal therapy. However, zygomycosis in the debilitated patient is the most acute and fulminate fungal infection known. The disease typically involves the rhinofaciocranial area, lungs, gastrointestinal tract, skin, or, less commonly, other organ systems (Ribes et al. 2000). It is often associated with acidotic diabetes, starvation, severe burns, intravenous drug abuse, and other diseases such as leukemia and lymphoma, immunosuppressive therapy, or the use of cytotoxins and corticosteroids, therapy with desferrioxamine (an iron-chelating agent for the treatment of iron overload), and other major trauma. Infections are increasingly being reported from HIV-positive patients (Teira et al. 1993; Nagy-Agren et al. 1995; van den Saffele and Boelarert 1996). Symptoms usually develop rapidly depending on the site of infection, the immune status of the host, and degree of pathology. The infecting fungi, primarily *R. oryzae*, have a predilection for invading vessels of the arterial system, causing embolization and subsequent necrosis of surrounding tissue. A rapid diagnosis is extremely important if management and therapy are to be successful (see reviews by Rippon 1988; Goodman and Rinaldi 1991; Kwon-Chung and Bennett 1992; Ribes et al. 2000; Dromer and McGinnis 2003).

Rhinocerebral zygomycosis

Rhinocerebral infection is the most common form of the disease and is usually associated with acute, uncontrolled diabetes mellitus or acidosis (Abramson et al. 1967; Baker 1971; Rippon and Dolan 1979; Kline 1985; Reich et al. 1985; Oakley et al. 1986; Huddle et al. 1987; Ryan-Poirier et al. 1988; Bessler et al. 1994; Hopkins and Treloar 1997; Adler et al. 1998; Dokmetas et al. 2002; Romano et al. 2002). Other predisposing factors include steroid-induced hyperglycemia, especially in patients with leukemia and lymphoma, renal transplant recipients, and those receiving concomitant treatment with corticosteroids and azathioprine, and in patients with chronic alcoholism (Meyer et al. 1972; Meyer and Armstrong 1973; Hammer et al. 1975; Lyon et al. 1979; Blitzer et al. 1980; Fisher et al. 1980; Kilpatrick et al. 1983; Carbone et al. 1985; Rippon 1988; Sica et al. 1993; Nussbaum and Hall 1994; Alloway et al. 1995; Schuster and Stern 1995; Shpitzer et al. 1995; Ryan et al. 2001). Rare cases of rhinocerebral infection have also been reported in two elderly, nonketotic diabetic patients with periorbital cellulitis and blindness (O'Brien and McKelvie 1994), a patient with burns (Stern and Kagan 1999), and in an otherwise healthy patient (Radner et al. 1995). Rhinocerebral zygomycosis is usually an acute, rapidly progressive, fulminant disease, typified by local tissue necrosis progressing to sinusitis with invasion of the arterial walls, and leads to invasion of the periorbital tissue and brain. Several species have been reported as causative agents, but most human cases are caused by *R. oryzae* (Carbone et al. 1985; Rangel-Guerra et al. 1985; Parfrey 1986; Rinaldi 1989; Ribes et al. 2000). Infections usually begin in the upper turbinates or paranasal sinuses after the inhalation of sporangiospores, and may involve the orbit, palate, face, nose, or brain (Abidi et al. 1984; Morduchowicz et al. 1986; Kwon-Chung and Bennett 1992). Initial symptoms include fever, unilateral headache, nasal or sinus congestion or pain, and a serosanguineous nasal discharge. However, patients are rarely seen during early stages of infection and most present in a comatose state or with symptoms of advanced disease, such as periorbital or perinasal swelling, and a thick, bloody nasal discharge. Other symptoms include ophthalmoplegia with ptosis and proptosis, decreased visual acuity, and facial paralysis. Abnormal mental status often indicates brain involvement. If the fungus invades the palate, a black, necrotic, plaque-like lesion or fistula is often formed (Lehrer et al. 1980;

Table 4 *Agents of human zygomycosis and clinical manifestations*

Clinical manifestation	Pathogenic species reported
Rhinocerebral	*R. oryzae* (most common), *A. corymbifera*, *C. bertholletiae*, *S. vasiformis*, *A. elegans*, *M. ramosissimus*
Pulmonary	*R. oryzae* (most common), *A. corymbifera*, *C. bertholletiae*, *R. pusillus*, *R. schipperae*, *M. indicus*
Gastrointestinal	*A. corymbifera* (most common), *R. oryzae*, *R. azygosporus*, *R. microsporus* var. *rhizopodiformis*, *M. indicus*, *A. corymbifera*
Cutaneous	*R. microsporus* var. *rhizopodiformis*, *R. oryzae*, *R. pusillus* (most common), *A. corymbifera*, *C. bertholletiae*, *A. elegans*, *S. vasiformis*, *R. microsporus* var. *microsporus*, *R. microsporus* var. *oligosporus*, *M. circinelloides*, *M. hiemalis*, *M. ramosissimus*, *S. racemosum*
Disseminated	*R. oryzae*, *R. pusillus*, *A. corymbifera*, *C. bertholletiae*, *S. vasiformis*, *A. elegans*

Bigby et al. 1986; Kotzamanoglou et al. 1988; Rippon 1988; Goodman and Rinaldi 1991; Kwon-Chung and Bennett 1992; Ribes et al. 2000; Dromer and McGinnis 2003) (Table 4).

Pulmonary zygomycosis

This form of disease is progressive and usually fatal within 2–3 weeks. Infections result by inhalation of sporangiospores into the bronchioles and alveoli, leading to pulmonary infraction and necrosis with cavitation. Hematogenous dissemination to other organs, particularly the brain, often occurs (Meyer et al. 1972; Carbone et al. 1985; Rangel-Guerra et al. 1985; Bigby et al. 1986; Parfrey 1986; Rothstein and Simon 1986; Lake et al. 1988; Rippon 1988; Rinaldi 1989; Goodman and Rinaldi 1991; Kwon-Chung and Bennett 1992; Ribes et al. 2000). Predisposing conditions include: hematological malignancies, lymphoma and leukemia, or severe neutropenia, treatment with cytotoxins and corticosteroids, desferrioxamine therapy, uncontrolled diabetes, organ transplantation, and AIDS (Winston 1965; Schwartz et al. 1982; Hoffman 1987; Chavanet et al. 1990; St-Germain et al. 1993; Piliero and Deresiewicz 1995; Rickerts et al. 2000; Vincent et al. 2000). Clinical symptoms include unremitting fever and a rapidly progressive pneumonia. Hemoptysis and pleuritic chest pain may also be present. The progressive development of lung infiltrates despite broad-spectrum antibacterial treatment is a common radiological finding (Bartram et al. 1973; Skahan et al. 1991; Richardson and Warnock 1993). *R. oryzae* is the most common causative agent, followed by *A. corymbifera*, *C. bertholletiae*, and *R. pusillus* (Ventura et al. 1986; Reed et al. 1988; Rippon 1988; Ribes et al. 2000).

Gastrointestinal zygomycosis

This is a rare entity, usually associated with severe malnutrition, particularly in children, and gastrointestinal diseases which disrupt the integrity of the mucosa. Primary infections probably result after the ingestion of fungal elements and usually present as necrotic ulcers (Watson 1957; Neame and Rayner 1960; Isaacson and Levin 1961; Michalik et al. 1980; Agha et al. 1985; Carbone et al. 1985; Parfrey 1986; Gordon et al. 1988; Ismail et al. 1990; Skahan et al. 1991; Thomson et al. 1991; Mooney and Wagner 1993; Reimund and Ramos 1994; Vadeboncoeur et al. 1994; Hughes et al. 1995; Kimura et al. 1995; Singh et al. 1995; Winkler et al. 1996). Invasive gastrointestinal zygomycosis has been reported in liver transplant patients (Mazza et al. 1999; Vera et al. 2002), a renal transplant patient (Tinmouth et al. 2001), heart–lung and heart transplantation (Knoop et al. 1998), and in neonates (Amin et al. 1998; Nissen et al. 1999). An unusual case was reported from a patient with emphysematous gastritis (Cherney et al. 1999) and another in an immunocompetent host following surgery (Carr et al. 1999). Lesions are most common in the stomach, colon, and ileum (Richardson and Warnock 1993). Symptoms vary depending on the site and extent of disease.

Nonspecific abdominal pain and hematemesis are typical. Necrotic ulcers develop and peritonitis follows if intestinal perforation occurs. Intestinal infections are usually fatal within 2–3 weeks as a result of bowel infarction, sepsis, or hemorrhagic shock (Lyon et al. 1979; Rippon 1988; Rinaldi 1989; Richardson and Warnock 1993). *A. corymbifera* is probably the most frequently isolated mucoraceous causative agent, but *R. azygosporus* has been reported as the causative agent in three cases of gastrointestinal infection in premature babies (Woodward et al. 1992; Schipper et al. 1996). A rare case of colonization of gastric cancerous ulcer by *R. microsporus* var. *rhizopodiformis* has also been reported by Kimura et al. (1995).

Cutaneous zygomycosis

Clinical manifestations of cutaneous zygomycosis vary considerably depending on the status of the host and the portal of entry. Cutaneous lesions are typically single, nonspecific, and include plaques, pustules, ulcerations, deep abscesses, and ragged necrotic patches. There is often a mixed suppurative and necrotizing inflammatory reaction in the dermis and subcutaneous tissue (Goodman and Rinaldi 1991). Primary cutaneous zygomycosis is usually caused by the traumatic implantation of fungal elements through the skin, especially in

patients with extensive burns, diabetes, or steroid-induced hyperglycemia, and in cases of major trauma resulting from automobile accidents or machinery (Wilson et al. 1976; Tomford et al. 1980; Sands et al. 1985; Venezio et al. 1985; Parfrey 1986; Johnson et al. 1987; Rippon 1988; Vainrub et al. 1988; Rinaldi 1989; Tintelnot and Nitsche 1989; Skahan et al. 1991; Adam et al. 1994; Hicks et al. 1995; Liao et al. 1995; Lopes et al. 1995; Seguin et al. 1999; Song et al. 1999; Chandra and Woodgyer 2002). Cases have arisen at insulin injection sites, spider bites, entry sites of intravenous or peritoneal catheters, or operative wounds (Kwon-Chung and Bennett 1992; Baraia et al. 1995; Leong et al. 1997). In patients with burns, the initial clinical signs include fever, swelling, and changes in the appearance of the burn wound leading to severe underlying necrosis and infarction (Bruck et al. 1971; Cooter et al. 1990). Necrotizing cutaneous lesions have occurred in patients who have had contaminated surgical dressings or Elastoplast bandages applied to their skin (Gartenberg et al. 1978; Dennis et al. 1980; Patterson et al. 1986; White et al. 1986; Kerr et al. 1988; Lakshmi et al. 1993). An outbreak in neonates has been reported, caused by the use of wooden tongue depressors as splints to immobilize limbs (Mitchell et al. 1996; Tonks 1996). Cutaneous infections have also been reported from adhesive tape used to secure endotracheal tubes (Dickinson et al. 1998; Alsuwaida 2002) and from a snapdragon skin patch test (Blair et al. 2002). Reports of primary cutaneous infections in neonates appear to be increasing over time (du Plessis et al. 1997; Amin et al. 1998; Linder et al. 1998; Oh and Notrica 2002; Buchta et al. 2003). Many species have been reported as causative agents with *R. microsporus* var. *rhizopodiformis*, *R. oryzae*, and *R. pusillus* being the most common (Rippon 1988). *C. bertholletiae* has also been reported as causing a cutaneoarticular infection in an AIDS patient (Mostaza et al. 1989).

Most of the reported infections with *S. vasiformis* and *A. elegans* have been localized to the site of trauma in a previously normal host (Kwon-Chung and Bennett 1992). In the immunocompetent patient, infections usually remain localized around the site of the initial trauma and respond well to local débridement and antifungal therapy (Song et al. 1999; Kimura et al. 1999); however, in the immunosuppressed patient, infections may rapidly disseminate and require much more aggressive management. Cutaneous and subcutaneous zygomycosis may also be the result of hematogenous spread or direct invasion from other organs, and usually indicates a very poor prognosis (Goodman and Rinaldi 1991; Ribes et al. 2000).

Disseminated zygomycosis

Systemic infections may originate from any of the above, but are usually seen in neutropenic patients with pulmonary infection and less commonly from the gastrointestinal tract, burns, or other cutaneous lesions (Ingram et al. 1989; Richardson and Warnock 1993). Recent reports have also indicated an association between desferrioxamine use and disseminated zygomycosis, particularly in dialysis patients (Windus et al. 1987; Boelaert et al. 1988; Rex et al. 1988; Ryan-Poirier et al. 1988; Sane et al. 1989; Goodman and Rinaldi 1991; Prokopowicz et al. 1994). The most common site of spread is the brain, but metastatic necrotic lesions have also been found in the spleen, heart, and other organs (Tuder 1985; Iqbal and Scheer 1986; Bosken et al. 1987; Kalayjian et al. 1988; Rippon 1988; Rozich et al. 1988; Rinaldi 1989; Severo et al. 1991; Teira et al. 1993; Fortun et al. 1995; Paterson et al. 2000; Eucker et al. 2001). Infections are usually diagnosed postmortem unless there are metastatic cutaneous lesions. Cerebral infection after hematogenous dissemination results in abscess formation and infarction, and is thus distinct from the rhinocerebral form of infection (Richardson and Warnock 1993). Patients usually present with sudden onset of focal neurological deficits or coma. Traumatic implantation leading to brain abscess has been reported in intravenous drug abusers and AIDS patients (Pierce et al. 1982; Lawrence et al. 1986; Parfrey 1986; Woods and Hanna 1986; Kasantikul et al. 1987; Cuadrado et al. 1988; Oliveri et al. 1988; Smith et al. 1989; Stave et al. 1989; Fong et al. 1990; Hopkins et al. 1994). Sporadic reports of other miscellaneous types of zygomycosis include endocarditis (Virmani et al. 1982; Zhang et al. 2002), osteomyelitis (Echols et al. 1979; Pierce et al. 1987; Eaton et al. 1994; Meis et al. 1994), renal infections (Langston et al. 1973; Low et al. 1974; Levy and Bia 1995; Melnick et al. 1995; Utas et al. 1995; Gonzalez et al. 1997), and a case of fatal penile necrosis in a patient with undiagnosed diabetes (Williams et al. 1995).

LABORATORY DIAGNOSIS

As zygomycosis is such an aggressive and rapidly fatal infection, it is essential to make an early diagnosis, although at times this may be very difficult. The diagnosis is usually made by demonstrating the characteristic ribbon-like, aseptate hyphae in tissue and culture of the causative agent. Despite being recognized as common laboratory contaminants, zygomycetes are infrequently isolated in the clinical laboratory. Therefore, in patients with any of the above predisposing conditions, especially diabetes or immunosuppression and/or clinical symptoms, the isolation of any zygomycete fungus should be regarded as potentially significant. Obviously, in patients without predisposing conditions, the isolation of a zygomycete from a nonsterile site, such as skin or sputum, must be interpreted with caution, especially in the absence of direct microscopic detection in clinical specimens.

Clinical material

Clinical material may include:

- skin biopsies from cutaneous lesions
- sputum and needle biopsies from pulmonary lesions
- nasal discharges, scrapings, and aspirates from sinuses in patients with rhinocerebral lesions
- biopsy tissue from patients with gastrointestinal and/or disseminated disease.

However, as most specimens are collected from abscesses and necrotic tissue, a biopsy usually provides the best specimen; swabs are not recommended. Ideally, tissue specimens should include both normal tissue, and the center and edge of the lesion. Tissue samples should be kept moist with saline or brain–heart infusion broth and be transported to the laboratory as soon as possible. It is essential that both direct microscopy and culture be performed on all specimens. It should also be noted that zygomycetous fungi have coenocytic hyphae, which will often be damaged and become nonviable during the biopsy procedure (especially scrapings and aspirates), or by the chopping up or tissue grinding processes in the laboratory. This is why zygomycetous fungi which are clearly visible in direct microscopic or histopathological mounts are often difficult to grow in culture from clinical specimens. If on clinical and/or radiological evidence zygomycosis is suspected, excessive tissue damage when collecting the specimen should be avoided if possible and in the laboratory the tissue should be gently teased apart and inoculated directly onto the isolation media.

Direct microscopy and histopathology

Microscopy is the most rapid diagnostic method and the presence in clinical specimens of broad, 10–15 μm wide, infrequently septate, thin-walled hyphae, which often show focal bulbous dilatations and irregular branching, is diagnostic for zygomycosis (Figure 11). Scrapings, sputum, and exudates may be examined using 10–20 percent KOH and Parker ink or Calcofluor mounts. The demonstration of zygomycetous hyphae on direct microscopy from such nonsterile sites is essential in order to determine the significance of a positive culture. Tissue sections should be stained with hematoxylin and eosin (H&E), Gomori's methenamine silver (GMS) (Grocott 1955), and periodic acid–Schiff (PAS) stains. Staining reactions may be variable; however, unlike *Aspergillus* spp. mycelium, zygomycete hyphae are usually clearly visible in H&E-stained sections. Nevertheless, a GMS stain should always be performed (Chandler et al. 1980; Rippon 1988). Apart from the presence of the characteristic ribbon-like hyphae, the main histopathological features of zygomycosis are necrosis with acute and chronic infiltrates and involvement of blood vessels. Blood vessel invasion is quite frequent, resulting in thrombi, infarction, and necrosis of blood vessels. Occasionally, sporangia with well-delineated columellae and sporangiospores may be seen in tissue sections of nasal and pulmonary tissue that are well aerated (Chandler et al. 1980; Salfelder 1990; Ribes et al. 2000).

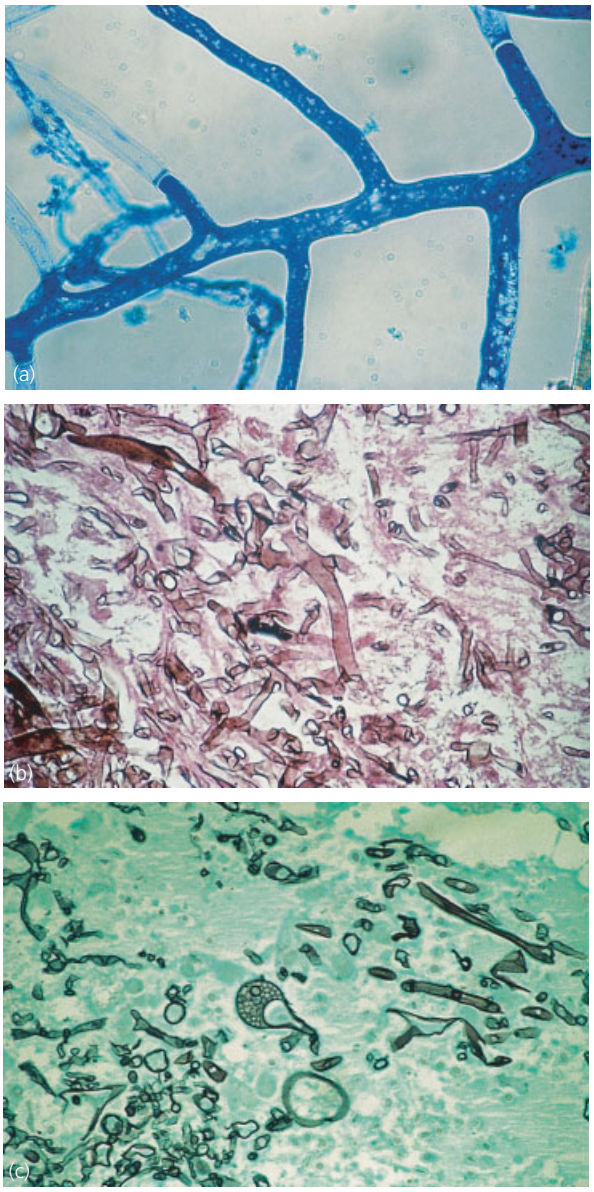

Figure 11 *Tissue morphology in zygomycosis:* **(a)** *lactophenol cotton blue mount showing distinctive wide infrequently septate hyphae;* **(b)** *H&E-stained section of infected tissue showing broad, infrequently septate, thin-walled hyphae with focal bulbous dilatations and irregular branching, typical for those species belonging to the Mucorales;* **(c)** *GMS-stained tissue section showing typical zygomycotic hyphae and by chance a sporangium of* Absidia corymbifera. *Magnification ×330*

Culture

Nutritionally, members of the Mucorales are not particularly fastidious and will grow on most routine fungal

culture media provided they do not contain cycloheximide (actidione). However, the use of antibacterial antibiotics such as chloramphenicol and gentamicin in primary isolation media is recommended to prevent bacterial contamination. Media containing dextrose as a carbon source and ammonium or peptone as nitrogen sources are preferred (Scholer et al. 1983). Sabouraud's dextrose agar containing antibiotics is usually used as the primary isolation medium, with potato dextrose agar frequently used for culture maintenance and identification purposes. Thiamine is the only growth factor required by some species, such as *A. corymbifera*; however, sufficient amounts are usually present in agar containing peptone (Scholer et al. 1983). Most mucoraceous species sporulate profusely and are fast growing, often pushing the lid off the petri dish in a few days. However, laboratory identification of some species, especially *A. elegans* and *S. vasiformis*, may be difficult or delayed because of their failure to sporulate on primary isolation media or on subsequent subculture onto potato dextrose agar. Sporulation may be stimulated by the use of nutrient deficient media, such as cornmeal–glucose–sucrose–yeast extract agar, Czapek Dox agar, or by using the agar block method described by Ellis and Ajello (1982) and Ellis and Kaminski (1985).

Immunology and serology

Several studies have investigated the ability of members of the Mucorales to cause infection in normal, cortisone-treated and alloxan-induced diabetic animals (Waldorf and Diamond 1989). Diabetic animals develop a rapidly fatal infection when challenged with *R. oryzae*, *R. pusillus*, *R. microsporus* var. *oligosporus*, and *A. corymbifera* (Reinhardt et al. 1981). Intranasal or itrasinus inoculation of *R. oryzae* into streptozotocin-induced diabetic mice results in typical pulmonary or rhinocerebral infections (Waldorf et al. 1984b). Unlike other models of zygomycosis (Waldorf et al. 1982), these diabetic models lead to induction of specific enhanced susceptibility to infection by *Rhizopus* spp., resembling the predisposition to zygomycosis which occurs in uncontrolled human diabetic patients (Waldorf and Diamond 1989). *Rhizopus* spp. spore germination occurs in tissues of diabetic or cortisone-treated mice after intranasal inoculation, but not in normal animals (Lundborg and Holma 1972; Smith 1976; Waldorf et al. 1982, 1984a; Waldorf and Diamond 1984). Thus, prevention of zygomycosis appears to require inhibition of sporangiospore germination by the bronchoalveolar macrophage, thereby preventing conversion of the fungus to its hyphal form, although resident bronchoalveolar macrophages are unable to kill *R. oryzae* spores (Lundborg and Holma 1972). The invasive hyphal elements seen in tissue are too large to be ingested by phagocytic cells, and it would appear that neutrophils have an important role to play in killing hyphae of *R. oryzae* (Diamond and Clark 1982; Schaffner et al. 1986). Isolates of *R. oryzae* also produce an alkaline protease with proteolytic activity within the normal physiological pH range. However, further studies are needed to examine the role of enzymes and toxins in the virulence of these fungi (Waldorf and Diamond 1989). In general, however, most data suggest that pathogenicity of the zygomycetes depends primarily on the susceptibility of the host rather than on the pathogenic potential of the fungus.

The development of serological assays for the early diagnosis of zygomycosis has proved to be very difficult. Sera from proven cases are difficult to obtain because infections are infrequently diagnosed. Only weak antibody responses are detected in humans, probably related to the depressed immunological status of the patients and the rapid progressive course of infection, which often leads to death before significant production of specific antibodies. However, some laboratories have developed 'in house' immunodiffusion and ELISA tests for the detection of antibodies to zygomycetes (Jones and Kaufman 1978; Waldorf et al. 1982; Lawrence et al. 1986; Wysong and Waldorf 1987; Kaufman et al. 1989, 1990). Exoantigen tests for the rapid identification of nonsporulatng strains of *A. elegans* and *S. vasiformis* have been developed (Lombardi et al. 1989). The development of more sensitive DNA-based diagnostic methods, antigen detection, or specific serological procedures may prove to be more valuable in the future (Dromer and McGinnis 2003).

TREATMENT (MANAGEMENT/THERAPY)

The successful management of infections caused by mucoraceous zygomycetes requires an early diagnosis, control or reversal of any predisposing factors or underlying disease, antifungal therapy, and aggressive surgical débridement which may have to be repeated until all infected necrotic tissue is removed.

Amphotericin B is the drug of choice and full-dose therapy of 1.0 or 1.5 mg/kg/day is necessary. Lower dosages of 0.8–1.0 mg/kg/day and/or alternate day therapy may be considered after the patient has been stabilized with no new areas of necrosis developing (Andriole and Bodey 1994). Some patients may require a total dose of up to 4 g (Sugar 1992). Liposomal amphotericin B is increasingly being used, as it is much better tolerated than conventional amphotericin B and doses as high as 3–5 mg/kg/day may be given (Grauer et al. 1993; Munckhof et al. 1993; Berenguer et al. 1994; Lim et al. 1994; Ng and Denning 1995; Strasser et al. 1996; Saltoglu et al. 1998; Bjorkholm et al. 2001; Cagatay et al. 2001; Cloughley et al. 2002; Ma et al. 2001; Handzel et al. 2003; Kofteridis et al. 2003). Amphotericin B is generally continued for 8–10 weeks. To date other antifungal

agents such as 5-fluorocytosine, the azoles, or the echinocandins have had no role in the management of zygomycosis. However, recent in vitro data (Sun et al. 2002a, b; Dannaoui et al. 2003) and in vivo data for the new triazole agent posaconazole have demonstrated promising activity against the zygomycetes (Greenberg et al. 2003; Tobon et al. 2003). Antifungal susceptibility testing of these fungi is also not reliable and has an uncertain place in guiding therapeutic decisions.

In the diabetic patient prompt correction of acidosis is essential and with early diagnosis and treatment, 50–85 percent of patients with rhinocerebral zygomycosis can be cured (Pillsbury and Fischer 1977; Meyers et al. 1979; Lehrer et al. 1980). The best prognosis is among those without extension into the brain or the internal carotid artery. The most critical decisions all concern balancing the extent of surgery between that necessary to control progressive disease and that which causes unnecessary loss of the eye or gaping operative wounds (Kwon-Chung and Bennett 1992). By contrast, few leukemia patients with the infection recover (Richardson and Warnock 1993).

Treatment of pulmonary, gastrointestinal, or disseminated zygomycosis has been successful too rarely to judge appropriate therapy, but intravenous amphotericin B and decreased immunosuppression appear important in survival (Kwon-Chung and Bennett 1992). In normal patients with localized cutaneous lesions surgical débridement is often sufficient alone, although intravenous treatment with amphotericin B is also indicated. Adjunctive therapies using G-CSF (Sahin et al. 1996; Gonzalez et al. 1997; Leong et al. 1997; Ma et al. 2001; Slavin et al. 2002), GM-CSF (Leleu et al. 1999; Garcia-Diaz et al. 2001; Ma et al. 2001; Mileshkin et al. 2001; MacKenzie et al. 2002), interferon-gamma (Okhuysen et al. 1994), and hyperbaric oxygen have been used with increasing frequency (Ferguson et al. 1988; Okhuysen et al. 1994; Kajs-Wyllie 1995; Bentur et al. 1998; Gonzalez et al. 2002).

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Aspergillosis

MALCOLM D. RICHARDSON

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The accumulation of data on many aspects of *Aspergillus* spp. and aspergillosis justifies a text of major proportions. Many monographs, reviews, and journal supplements have more than adequately covered the organisms and the complete disease area (Raper and Thom 1965; Rinaldi 1983; Al-Doory and Wagner 1985; Walsh and Pizzo 1988; Cohen 1991; Barnes and Denning 1993; Brakhage et al. 1999; Latgé 1999). More recent overviews of aspergillosis can be found in Marr et al. (2002), Richardson and Warnock (2003), Wiederhold et al. (2003), and a supplement of *Clinical Infectious Diseases* (Advances against aspergillosis 2003). Arguably the most comprehensive resource is the *Aspergillus* website (www.aspergillus.man.ac.uk). The content of this website is updated regularly. The aim of this chapter is to present the reader with a summary of the organisms and an overview of the major clinical manifestations in order to present a contemporary account of the pathogenesis of the disease, new diagnostic procedures, and new perspectives on treatment.

BRIEF DESCRIPTION OF THE GENUS

Aspergillus is a very large genus containing over 185 species to which humans are constantly exposed. Only a small number of these species have, however, been associated with disease. Of these, over 95 percent of all infections are caused by three species: *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*. Several more species have been reported in association with aspergillosis cases, including *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus oryzae*, *Aspergillus ustus*, and *Aspergillus versicolor*.

Of the documented species of *Aspergillus*, *A. fumigatus* causes the large majority of cases of both invasive and non-invasive aspergillosis. Indeed, the allergic forms of the disease appear to be almost exclusively caused by this organism. Both aspergilloma and invasive aspergillosis are also caused by *A. flavus* and *A. niger*. More recently, cases of invasive aspergillosis caused by *A. terreus* and *A. nidulans* have been reported.

The fungi classified in the genus *Aspergillus* are anamorphic (asexual) filamentous organisms which reproduce by means of asexual spores termed conidia. Teleomorphic (sexual) forms of many aspergilli have been described. The aspergilli produce conidia in a basipetal fashion, which results in a chain of asexual conidia (the youngest conidium at the base and the oldest at the tip of the chain). The conidiogenous cell is termed a phialide. The base of the conidiophore, where it originates from the parent vegetative hypha, is termed a foot cell.

The conidiophore is hyphal-like and enlarges at its apex to form a swollen vesicle. Phialides may arise directly from the vesicle (uniseriate) or from sterile cells called metulae (biseriate); in some species, such as *A. flavus*, both conditions may exist in the same head. In the past, phialides were incorrectly referred to as sterigmata, a term that should be restricted to structures formed by Basidiomycetes.

A number of reference sources on the mycology of the aspergilli culminated in the publication in 1965 of a

manual by Raper and Thom (1965) in which the total number of known species and varieties were classified into 18 groups, 132 species, and 18 varieties. Since that time numerous new species and varieties have been described.

The identification of species of *Aspergillus* is not easy. Several excellent guides are available to aid in the identification of the common species of medically important aspergilli (Raper and Thom 1965; de Hoog et al. 2000). The genus *Aspergillus* is characterized by septate hyphae from which arise nonseptate conidiophores; although these conidiophores may be rough or smooth, hyaline or pigmented, they all terminate in a swollen vesicle. One or two rows of phialides are formed on the surface of the vesicle. Chains of round to oval, variously pigmented conidia are produced from the tips of the phialides. The shape of the vesicle and the arrangement of the phialides thereon determine the shape of the conidial head, from columnar to radiate, a key feature in speciating members of the genus. Finally, pigmentation of the conidia is the main factor determining the color of the colony. Following growth on appropriate media, observation of these macroscopic and microscopic features is usually sufficient to identify the organism.

The restricted list of pathogenic species of the genus suggests that these organisms may share some properties not found in other species which confer on them an intrinsic pathogenic advantage. One obvious feature that these human pathogens must share is the ability to grow efficiently at 37°C. The production of proteolytic enzymes is another property or virulence mechanism that has been investigated in the pathogenesis of invasive and allergic disease. The ability to produce elastase has been correlated with mouse virulence in strains of *A. fumigatus*. Likewise, isolates from cases of invasive aspergillosis in humans all showed the ability to digest elastin, regardless of the species. Finally, antigens with protease activity have been isolated from *Aspergillus* spp. and have been shown to react with sera from patients with allergic aspergillosis, aspergilloma, and invasive disease.

Most of the species of *Aspergillus* reproduce asexually and are classified with the Deuteromycetes (Fungi Imperfecti). However, some species, in addition to their asexual reproduction, have the characteristic of reproducing sexually by the formation of ascospores, which typifies Ascomycetes. However, for the purpose of classifying all fungi which reproduce asexually within one genus, similar to that for *Aspergillus* spp., and to avoid confusion by initiating new genera, most mycologists agree that the generic name *Aspergillus* should be applied to all species of these fungi, regardless of whether an ascosporic stage has been observed. It has been suggested that finding the sexual stage in a fungus merely completes its characterization but does not justify its inclusion in another genus; therefore, the names of the ascosporic species (the sexual stage) should be used as synonyms for certain *Aspergillus* spp., whenever this is applicable.

Accurate identification of any species of *Aspergillus* requires the isolation of a pure culture and its examination on a culture medium of known composition. Differences in the nature and composition of the culture media produce marked changes in growth characteristics and colony color, as well as in the dimensions and morphology of the microscopic components, which may lead to an inaccurate identification. Even though aspergilli can grow on almost every microbiological culture medium (liquid, solid, or semisolid), various authors have proposed standardized and reproducible formulae for media which in their experience have provided uniform cultures over long periods and which are of value for comparative studies. These media include Czapek Dox agar which is widely used as a routine medium for comparative studies and was used extensively by Raper and Thom (1965) and their coworkers. A medium used extensively for primary isolation from clinical specimens is malt extract agar on which most aspergilli sporulate freely.

In identifying aspergilli, it is important to keep in mind that there is variation among strains within species, as well as among species within a genus. Therefore, characteristics of the various groups of aspergilli may overlap. Within each strain, species, or group there are certain characteristics which can be identified.

There are several key features which are frequently consistent enough to be used to speciate individual isolates. As a result of the overlapping of criteria for identification of certain species, however, they may be classified within more than one group. The criteria in Table 1 are useful when identifying unknown isolates to species level.

The 18 groups of aspergilli classified by Raper and Thom (1965) are: *Aspergillus clavatus*, *Aspergillus glaucus*, *Aspergillus ornatus*, *Aspergillus cervinus*, *Aspergillus restrictus*, *A. fumigatus*, *Aspergillus ochraceus*, *A. niger*, *Aspergillus candidus*, *A. flavus*, *Aspergillus wentii*, *Aspergillus cremeus*, *Aspergillus sparsus*, *A. versicolor*, *A. nidulans*, *Aspergillus ustus*, *Aspergillus flavipes*, and *A. terreus*.

Although more than 180 species are found within the genus, three species, *A. fumigatus*, *A. flavus*, and *A. terreus*, account for most cases of invasive aspergillosis. The ability to distinguish between the various clinically relevant species has diagnostic value, as certain species are associated with higher mortality and increased virulence and vary in their resistance to antifungal therapy. A molecular method has been developed to identify *Aspergillus* at the species level using the 18S and 28S rRNA genes for primer binding sites (Henry et al. 2000). The contiguous internal transcribed spacer (ITS) regions, ITS 1-5.8S-ITS 2, from reference strains and clinical isolates of aspergilli were amplified, sequenced, and compared with non-reference strains in GenBank.

Table 1 *Morphological criteria for the identification of* Aspergillus *spp.*

Criteria
Growth condition
Medium, temperature, light, age and source
Colony characteristics
Rate of growth
Pattern of growth
Color and texture
Basal mycelium
Surface mycelium
Abundance and arrangement of heads, sclerotia, cleistothecia
Reverse color
Conidial stage
Heads
Manner in which borne
Color
Form
Overall dimension
Vesicle
Shape
Dimension
Color
Fertile area
Phialides
Development
Arrangement – uniseriate or biseriate
Primaries – dimension and color
Secondaries – dimension and color
Conidiophore
Length
Diameter
Wall characteristics
Conidia
Dimension
Wall characteristics
Color
Hülle cells and other elements
Shape
Dimension
Sclerotia or sclerotium-like structures
Form and structure
Dimension
Color
Ascosporic stage
Cleistothecia
Origin
Form and structure
Quantity present
Dimension
Color
Asci
Shape
Dimension
Ascospores
Pattern
Dimension
Color

(*Continued over*)

Table 1 *Morphological criteria for the identification of* Aspergillus spp. (*Continued*)

Adapted from Raper and Thom (1965).

ITS amplicons from *Aspergillus* spp. ranged in size from 565 to 613 bp. Comparison of reference strains and GenBank sequences demonstrated that both ITS 1 and ITS 2 regions were needed for accurate identification of *Aspergillus* at the species level. Intraspecies variation among clinical isolates and reference strains was minimal. Other pathogenic molds demonstrated less than 89 percent similarity with *Aspergillus* ITS 1 and 2 sequences. A blind study of 11 clinical isolates was performed and each was correctly identified. The clinical usefulness of this rapid approach to identification has still to be determined. Further reviews of the major pathogenic species of aspergilli can be found in de Hoog et al. 2000; Brakhage and Langfelder 2002; Denning 2000; Varga and Töth 2003.

MORPHOLOGY

Description of colony appearance

Colonies of aspergilli may be black, brown, yellow, red, white, green, or other colors depending on the species and the growth conditions (Figures 1 and 2). The colony color always depends on the color of the microscopic components of the fungus, e.g. the color of the vegetative hyphae, the conidial heads, and the sexual structures if they are present. In addition to the color of the aerial parts of the fungal colonies, there may be pigmentation of the underlying medium, which may be different from the

Figure 1 A. fumigatus *(right) and* A. flavus *(left) cultures*

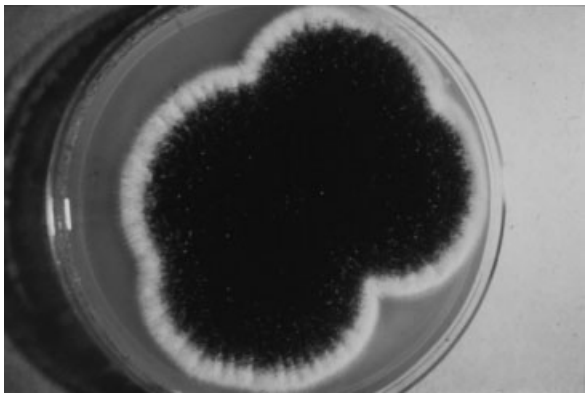

Figure 2 A. niger *culture*

color of the aerial parts of the colonies. Therefore, at any one time colonies may have one or more shades.

Even though most species of aspergilli are fast-growing fungi, there is a great deal of variation in their rate of growth. Growth rate is an important characteristic for identification of the species, so colony diameter at a certain age, under standard conditions, is a useful feature to note in the identification.

The marginal appearance of the colonies is another important characteristic. Margins may appear as heavy and sharply delineated, thin and diffuse, smooth and entire, irregularly lobed, submerged, or aerial. Also, the texture of the colonial surface may be velvety, floccose, or granular, with or without zonation. Zonation is usually expressed by alternating production of either conidial heads and sclerotia or conidial heads and cleistothecia (depending on the species).

The mycelium

The mycelium of the *Aspergillus* spp. is similar to that of most other fungi. It is well developed, with branching hyaline and septate hyphae. The hyphae may be thin or dense, and light or heavily sporulating. The cells of the hyphae are usually multinucleated. The mycelium can produce copious levels of enzymes and some produce mycotoxins. The mycelial form of *Aspergillus* spp. is characterized by vigorous growth and an abundant production of conidia carried on long, erect conidiophores. These arise from a specialized cell within the vegetative hyphae known as a 'foot cell'.

The conidial head

The conidial head of aspergilli is conventionally considered to comprise the conidia, the phialides, the vesicle, and the conidiophore, which arise from the foot cell (Figures 3, 4, 5, 6 and 7). In most species,
the shape, the size, and the color of the conidial heads within the same colony show no variation.

In the early stages of fungal growth, certain cells in the submerged or aerial parts of the vegetative mycelium enlarge and form a heavy wall. These foot cells will form a branch, which is always produced at a right angle to the hyphal cells. Not all aspergilli have distinct foot cells. The branch formed, which develops into a conidiophore, terminates in a swollen head known as a vesicle. The length of the conidiophore (between the foot cell and the vesicle) and the nature of its wall (smooth,

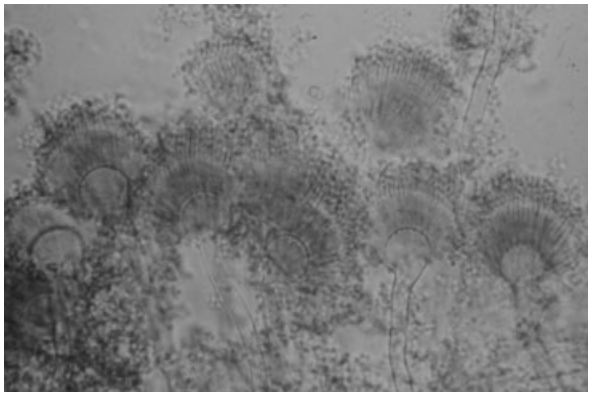

Figure 3 A. fumigatus *sporing head*

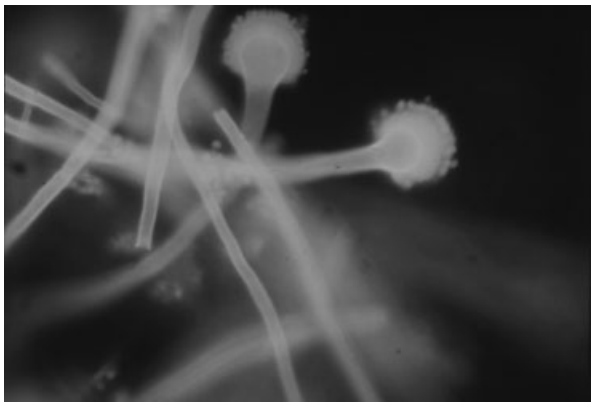

Figure 4 A. fumigatus *sporing heads stained with Calcofluor white and viewed under the fluorescence microscope*

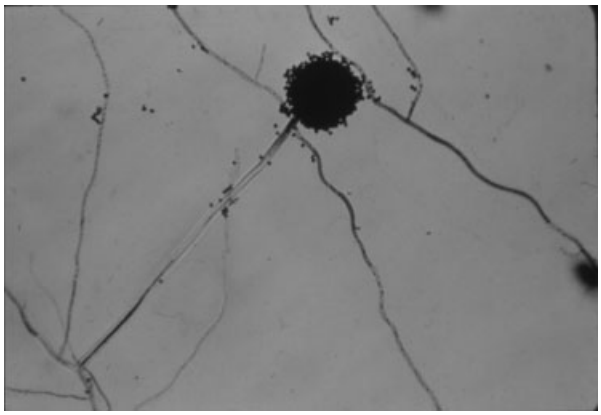

Figure 5 A. niger *sporing head*

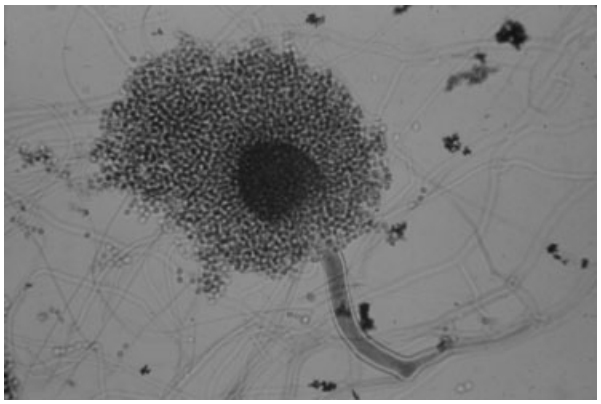

Figure 6 A. clavatus *sporing head*

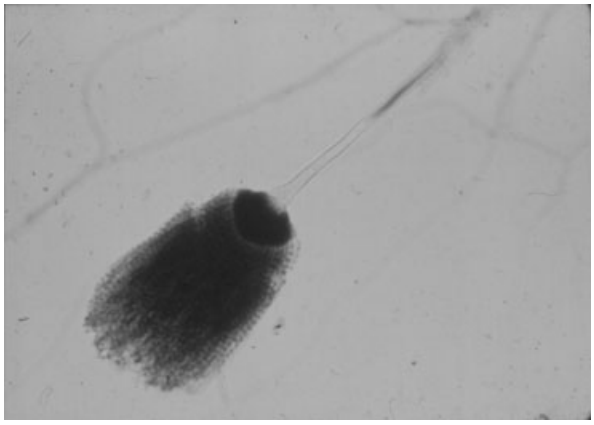

Figure 7 A. terreus *sporing head*

rough, echinulate, or pitted) vary from one species to another and are considered important characteristics of the species. Also, the conidiophore may be septate or nonseptate, in addition to being uniform throughout in length or greater in diameter at the base of the foot cell. Certain species, such as *A. glaucus*, may show branched conidiophores.

The vesicles vary in shape and size. They may be globose, hemispherical, elliptical, or elongated and clavate. They may have a very thin or a relatively thick wall. They are mostly hyaline, but in a few species they may appear pigmented. The lumen of the vesicle is usually a part of the conidiophore without any septum separating the vesicle from the conidiophore. These vesicles are usually borne upright on the conidiophore, with a few exceptions where they are formed at an angle to its main axis.

Depending on the species, certain areas of the vesicle surface become fertile and give rise to a layer of conidium-producing cells, which are called 'phialides'. These phialides, in some species, will cover the entire surface of the vesicle, whereas in others they may cover the upper half or three-quarters. They may vary in color in different species, from hyaline to lightly or darkly pigmented. The phialides are usually cylindrical, and are produced in different sizes and shapes, although they are usually uniform within the same culture of each species. They are produced perpendicular to the point of origin on the vesicle surface.

The cylindrical body of the phialides narrows at the apex to form a conidium-producing tube. However, depending on the species, the phialides may be formed in one layer (primary phialides), or each of these primary ones will bear one or more phialides (secondary phialides) in one linear surface. The size, shape, and length of the secondary phialides are usually different from those of primary origin. When there are secondary phialides, however, they will be the ones that narrow to form a conidium-producing tube. In some species, some of the primary phialides have been found to be septate.

Nuclear division starts within the conidium-producing structures of the phialides as a preliminary stage of conidia formation. One of the new nuclei moves upwards to the conidium-producing tube; this is followed by the formation of a septum separating the new nucleus from the rest of the cylinder. Another division follows within the cylinder and one of the newly formed nuclei moves upwards towards the tube, pushing the first nucleus (which has become a conidium by this time) out of the tube, but remaining attached to its tip. As this process continues, newly formed conidia push previously formed conidia out of the tube, thereby forming a chain of conidia at the tip of each phialide. The conidia remain attached to each other within each chain by a connective bridge, which is usually not visible with a light microscope. There are variations among the various species in the flexibility of these bridges, which affects the ease with which conidia are freed from each other.

Conidia are usually globose with a rough surface and are found in various sizes; they are mostly uninucleate. However, some have been found to contain up to 12 nuclei. The length of conidial chains, their density of packing and their orientation around the conidial heads vary from species to species and are considered part of the characteristics of the species and group. The individual color of the conidia (which may be hyaline), the collective color of the conidial mass, as well as the color of the aerial hyphae, are species characteristics that give the shade observed in the colonies.

Sexual reproduction

Many species of *Aspergillus* can reproduce sexually. Sexual reproduction in the ascosporic aspergilli is mainly by the formation of ascospores within asci. The mature ascospores are usually either in the shape of a double convex lens or two symmetrical valves, with variations among the different species. In members of the *A. nidulans* group, the color of the ascospores varies from red–brown to purple–red or violet; in the *A. ornatus* group, individual species display shades of red–brown at maturity; otherwise the ascospores are usually hyaline.

The usual number of ascospores per ascus is eight in most species.

The cleistothecium (ascocarp), where the asci are formed, varies in size, color, shape, and appearance in different species. They may appear naked or surrounded by a layer of specialized chlamydospores known as Hülle cells. These cells (which have no known function) are usually borne in hyphae as terminal or intercalary cells similar to chlamydospores. They may be globose (as in *A. nidulans*), subglobose, or elliptical in shape with very thick walls, which may occupy the whole cell body.

THE CAUSAL ORGANISMS AND THEIR HABITAT

Most human infections are caused by *A. fumigatus*, but *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus* have also been implicated. These molds are widespread in the environment. They are common soil inhabitants and are also found in large numbers in dust and decomposing organic matter. Their spores are often found in the outside air (Goodley et al. 1994; Anderson et al. 1996).

A. fumigatus is thermotolerant, able to grow over a temperature range from below 20°C up to 50°C, and grows well at over 40°C. It abounds in vegetable matter decomposing in warm environments, such as self-heating hay and composts. Strains of *A. fumigatus* can be distinguished from one another by analysis of polymorphic DNA markers (Aufauvre-Brown et al. 1992; van Belkum et al. 1993; de Hoog et al., 2000, and reviewed in Warnock et al. 2001). This approach has allowed environmental strains to be associated with particular disease entities (Symoens et al. 1993; Buffington et al. 1994; Girardin et al. 1994a, b; Tang et al. 1994; Mondon et al. 1995).

Inhalation of conidia leads to a variety of disease patterns. Atopic subjects may develop asthma. In nonatopic subjects, the presence of damaged lung tissue may result in the growth of a fungus ball (aspergilloma). Inhalation of massive doses of the conidia may lead to alveolitis. Aspergillosis also occurs in many species of birds and mammals, domesticated and wild, infected primarily by inhalation and starting as a pulmonary disease, although sometimes involving other organs. *A. fumigatus* is also one of several molds implicated in bovine mycotic abortion, probably initiated by inhalation, but with alimentary infection from spores ingested in moldy fodder as another possible route.

Nosocomial outbreaks of aspergillosis have become a well-recognized complication of construction work in or near hospital wards in which neutropenic patients are housed. In several reported outbreaks, building works adjacent to the unit in which patients were accommodated led to contamination of the air. In other outbreaks, the ventilation system for the unit drew contaminated air from neighboring building sites, or became contaminated in some other way (Goodley et al. 1994; Anderson et al. 1996; Cornet et al. 1999; Thio et al. 2000, and reviewed in Warnock et al. 2001).

The factors influencing the recovery of *Aspergillus* from the air and a comparison of the instruments available for sampling air are reviewed by Morris et al. 2000.

CLINICAL MANIFESTATIONS

Conidia of *A. fumigatus* can, because of their small size (<5 μm) and aerodynamic properties, bypass the upper respiratory tract defenses and may reach distal regions of the lung (Amitani et al. 1992, 1995). In this region the host defenses rely on phagocytic cells to remove the conidia efficiently.

An understanding of the spectrum of clinical manifestations seen in aspergillosis is also dependent on an insight into the ways in which *Aspergillus* spp. can transform from a saprophyte to a parasite. The ability of *A. fumigatus* to invade living tissue is dependent upon a number of virulence attributes (reviewed in Bouchara et al. 1995; Latgé 2001). Several reports suggest that the virulence of *A. fumigatus* is associated with the production of secreted proteases and elastases (Monod et al. 1993; Frosco et al. 1994; Moser et al. 1994; Reichard et al. 1994). Extensive colonization of lung tissue by *A. fumigatus* has suggested a key role for fungal proteases during infection. However, work by Jaton-Ogay et al. (1994) demonstrated that a serine alkaline protease (ALP) was not essential for invasion of lung tissue when ALP-negative mutants were shown to cause comparable mortality and to invade lung tissue in immunocompromised mice, as well as the parent strain (Monod et al. 1993; Tang et al. 1993). These data contrast with the work of Kolattukudy et al. (1993) who observed that an ALP-deficient mutant obtained by nitrosoguanidine treatment was less virulent than a wild-type strain of *A. fumigatus*. As proteases do not appear to play a role in the establishment of infection, other hypotheses for virulence of *A. fumigatus* have been put forward. One proposal (Jaton-Ogay et al. 1994) is that *A. fumigatus* has an ecological niche in the bronchi characterized by nutrient and temperature conditions favorable to its saprophytic growth; this results because, although this fungus accounts for only a small proportion of the airborne mold spores, it is the one isolated from lung and sputum most frequently. Consequently, invasive aspergillosis may occur when the host phagocytic defenses have been weakened by immunosuppression.

Inhalation of the conidia of *Aspergillus* spp. can give rise to a number of different clinical forms of aspergillosis, depending on the immunological status of the host (Cohen 1991; Soubani and Chandrasekar 2002; Richardson and Warnock 2003). However, there are no currently accepted classification schemes so the terminology used here may not always correspond to that adopted by other texts. Moreover, some clinical entities defy precise classification and the pathological features

at the time of diagnosis may not be known. In non-compromised individuals, *Aspergillus* spp. can act as potent allergens or cause localized infection of the lungs or sinuses.

In neutropenic patients, there is widespread growth of the fungus in the lungs and dissemination to other organs often follows. This condition is usually fatal, even if diagnosed during life and treated. It must, however, be emphasized that, with early diagnosis and treatment, a small but significant number of patients is cured.

The following sections deal, in turn, with the clinical manifestations of aspergillosis in the various organ and tissue systems, pointing out, where possible, those features that may assist in the early and definitive recognition of the disease.

Allergic aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is characterized by recurrent pyrexia, cough, wheezing, sputum plugs containing aspergilli, and recurrent pulmonary infiltrates (Patterson et al. 1982; Ikemoto 1992; Greenberger 2002a, b, 2003; Moss 2002). This is an uncommon condition, most often seen in atopic individuals who develop bronchial allergic reactions (asthma) following inhalation of *Aspergillus* spores (Singh et al. 2003). Mucus plugs then form in the bronchi, leading to atelectasis. The illness may be mild, but it is an episodic condition and can often progress to bronchiectasis and fibrosis. ABPA is a specific disease seen in about 5 percent of asthmatic individuals; the pathology is thought to be a type III and IV reaction to inhaled conidia of *A. fumigatus*. Dual skin reactions (immediate and late) and IgG and IgE antibodies are also recognized. ABPA is thought to result from type I and III, and perhaps type IV hypersensitivity reactions to antigens released from the fungus colonizing the bronchial tree.

Against this pathological and immunological background a number of major criteria for the diagnosis of ABPA are recognized:

- asthma (reversible airway obstruction)
- eosinophilia of sputum and blood
- recurrent pulmonary infiltrates
- allergy to antigens of *Aspergillus* spp. by skin test (immediate weal–flare reaction, type I) and late reaction (Arthus, or type III).

Other criteria are:

- *Aspergillus* spp. in sputum
- raised total IgE and specific IgE and IgG in serum
- history of recurrent fever or pneumonia
- history of coughing up plugs.

The clinical course of ABPA is characterized by frequent exacerbations of cough, wheezing, and fever, with eosinophilia of sputum and blood. Allergy or infection or both may trigger the exacerbations. A significant number of patients with ABPA have clinically and immunologically demonstrable atopy. The incidence of atopy is particularly high in those patients with early-onset asthma (first decade of life).

The expectoration of sputum containing brownish mucus plugs is another feature of ABPA. Microscopy shows that the plugs are made up of eosinophils and septate hyphae embedded in mucus and fibrinous debris. Culture of the sputum may yield *A. fumigatus*. However, this is not pathognomonic, because aspergilli may occasionally be isolated from the sputum of normal people.

The most frequent symptoms seen in ABPA include fever, intractable asthma, productive cough, malaise, and weight loss. Expectoration of brown eosinophilic mucus plugs containing *Aspergillus* mycelia is common.

The radiological findings range from small, fleeting, unilateral, or bilateral infiltrates with ill-defined margins (often in the upper lobes) and hilar or paratracheal lymph node enlargement, to chronic consolidation and lobar contractions. Bronchiectasis represents the later stage of the disease. Bronchial obstruction secondary to mucus plugs often produces radiological signs and is believed to be the first step in the development of inflammation and infection that eventually lead to fibrosis. Correlation is poor between radiological and clinical findings.

The clinical features of ABPA appear to result from reaginic (IgE) and precipitating (IgG) antibodies (Brummund et al. 1987; Marchant et al. 1994). Specific IgE is believed to cause the asthma, eosinophilia, and immediate skin reaction, whereas precipitating antibodies are believed to cause the pulmonary infiltrations, damage to the bronchial wall, and late skin reaction. Serum IgE is significantly elevated in ABPA but not in other hypersensitivity lung diseases caused by, for example, inhaled organic dusts.

Reviews by Cockrill and Hales (1999), Wark et al. (2001), Chetty (2003), and Kauffman (2003) summarize the role of cellular immunity in ABPA and give very clear guidelines regarding diagnosis and treatment.

ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS AND CYSTIC FIBROSIS

An association between ABPA and cystic fibrosis was first reported by Mearns et al. (1965). ABPA occurs with an incidence of 10–11 percent in patients with cystic fibrosis. Nearly one-half of cystic fibrosis patients have a positive immediate weal and flare skin test to *A fumigatus* and *A. fumigatus*-specific IgE (Marchant et al. 1994) and IgG without evidence of ABPA (Knutsen et al. 1994). Aspergillus allergy appears to be coincident with *Pseudomonas aeruginosa* colonization; hence it is difficult to distinguish the effects of either organism. The diagnosis of ABPA is, however, clearly important from a clinical viewpoint because of its asso-

ciation with severe proximal bronchiectasis and a far more rapid decline in lung function in cystic fibrosis.

A greater understanding of the association between ABPA and cystic fibrosis is provided by the identification of mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) gene in patients with ABPA but without clinical criteria for cystic fibrosis. Miller and colleagues analysed the entire coding region in 11 individuals who met criteria for the diagnosis of ABPA but had normal sweat electrolyte tests (Miller et al. 1996). Of the 11 patients, six had minor mutations of the CFTR gene, suggesting that CFTR plays an etiologic role in a subset of patients with ABPA. Recurrence of ABPA after lung transplant has also been described in a patient with cystic fibrosis (Fitzsimmons et al. 1997). An exhaustive review of ABPA and cystic fibrosis has been published recently (Stevens et al. 2003).

ASPERGILLUS SPP. AS FUNGAL ALLERGENS

Aspergillus spp. antigens have been studied thoroughly as allergens because *Aspergillus* conidia were among the first to be recognized as important aeroallergens (Kauffman et al. 1984).

Two antigens (18 and 20 kDa) with potential for the diagnosis of allergy have been purified from *A. fumigatus* by chromatography (reviewed in Ikemoto 1992). The 18 kDa antigen was isolated from the mycelium of ten different strains of *A. fumigatus*. Monospecific polyclonal rabbit antiserum directed against this antigen reacted with antigens of 26, 28, 44, and 46 kDa as well (Latgé et al. 1991). The characteristics of the 18 kDa antigen suggest that it is similar to Ag-2 or Ag-10 described previously from counterimmunoelectrophoresis patterns. The 20 kDa allergen is a glycoprotein that appears to be different from antigens Ag-3, Ag-5, Ag-7, and Ag-13. Another glycoprotein allergen, designated gp55, was recently isolated; its allergenic activity was sensitive to protease but not to deglycosylation. On the basis of the amino-terminal protein sequence, this appears to be a novel protein.

The amino acid sequence of the 18 kDa protein (Asp fI) has been determined partially (Burnie and Matthews 1991). Asp fI mRNA was detected in *A. fumigatus* but not in seven other *Aspergillus* spp. Asp fI shows extensive sequence homology (95 percent) to mitogillin (a cytotoxin) produced by *A. restrictus*. A cross-inhibition radioimmunoassay with a murine monoclonal antibody and human IgG and IgE antibodies revealed that Asp fI and mitogillin are indistinguishable antigenically. The simultaneous toxicity and allergenicity of Asp fI have significant implications for understanding the etiology of ABPA. Asp fI cDNA from *A. fumigatus* has now been cloned, and the recombinant allergen has been expressed and used in skin test trials. A recombinant *A. fumigatus* protein (65 kDa) expressed from a cDNA clone has also been shown to bind IgE from ABPA patients. On the basis of cDNA and deduced amino acid sequence homology to Hsp90 from other organisms, this allergen is apparently a heat-shock protein of the Hsp90 family (Burnie and Matthews 1991). The presence of non-allergenic homologs provides a significant opportunity to study the basis of allergen epitopes by focusing on the differences between these proteins, i.e. Hsp90 from *A. fumigatus* (allergenic) and Hsp90 from humans (non-allergenic). These important achievements provide a significant aid to standardizing *Aspergillus* spp. extracts and diagnosing *Aspergillus*-related diseases. However, optimal methods for the preparation of relevant antigens have still to be defined (Little et al. 1993). For detailed descriptions of those *Aspergillus* antigens approved by the allergen nomenclature committee see Banerjee and Kurup (2003) and Kurup et al. (2000).

Infection of the paranasal sinuses

Aspergillosis of the sinuses includes a number of diseases ranging from a benign non-invasive form to an aggressively invasive type. To rationalize a number of classification schemes, Talbot et al. (1991) have defined four basic types: saprophytic aspergillus colonization of a previously abnormal sinus, allergic aspergillus sinusitis, subacute or chronic invasive aspergillus sinusitis, and fulminant invasive aspergillus sinusitis. These clinical entities have in many ways pulmonary counterparts: aspergilloma, ABPA, chronic necrotizing pulmonary aspergillosis, and invasive pulmonary aspergillosis, respectively (Chang et al. 1992; De Carpentier et al. 1994; Schubert 2001; Dhiwakar et al. 2003).

Aspergillosis is currently the most common fungal infection of the paranasal sinuses (see reviews by Blitzer and Lawson 1993, Drakos 1993, and Richardson and Warnock 2003). Most patients who have developed aspergillus sinusitis have no underlying disease, although invasive rhinosinusitis has been seen in patients with acute leukemia (Talbot et al. 1991). In 20 cases of orbital aspergillosis, only one patient had diabetes. Thirty-seven patients with sino-orbital aspergillosis presented with no systemic disease. There have been only occasional reports of sinus aspergillosis arising in diabetes and leukemia patients (Peterson and Schimpff 1989). The disease runs a more fulminant course in immunocompromised patients; the mortality rate was reported as 100 percent in bone marrow transplant recipients. There is a more recent association of aspergillus sinusitis in patients with nasal allergies.

The nose and paranasal sinuses have local factors that may promote fungal infection, including nasal polyps, recurrent bacterial infections and chronic rhinitis with stagnation of nasal secretions. Some authors have suggested that occlusion of the nasal ostia of the sinuses creates an anaerobic environment that may promote fungal pathogenicity. Other reports have challenged this

concept, citing aspergillus infections in such well-aerated regions as the nose, bronchi, and external ear. Other underlying factors include prolonged antibiotic therapy for sinusitis and the greater use of antibiotics and immunosuppressive agents.

Two different forms of sinusitis due to *Aspergillus* spp. have been recognized. Acute sinusitis is a life-threatening condition encountered in immunocompromised patients. The clinical presentation is similar to that of rhinocerebral mucormycosis. The presenting symptoms include fever, nasal discharge, headache, and facial pain. Necrotic lesions develop on the hard palate or nasal turbinates and disfiguring destruction of facial tissue may occur. The infection can spread into the orbit and brain, causing thrombosis and infarction. Paranasal aspergillus granuloma formation is a slowly progressive condition. It is most common in the tropics, where *A. flavus* is the most common cause, although cases have been reported from temperate climates. Affected individuals usually complain of longstanding symptoms of nasal obstruction and headache, suggesting chronic sinusitis, but are otherwise normal. Patients present with unilateral facial pain and headache, or with facial swelling and proptosis. The swelling is firm but not usually tender. In the later stages of this condition, upward spread of the fibrosing paranasal granuloma results in focal cerebral or orbital infection. The typical radiological finding is a dense filling defect within the maxillary or ethmoid sinuses with erosion of the surrounding bone. This can be confirmed by computed tomography (CT) or magnetic resonance imaging (MRI). A third form of aspergillus sinusitis, termed allergic fungal sinusitis (AFS), has recently been described. Up to 7 percent of patients requiring sinus surgery may have AFS.

Aspergillus sinusitis is a worldwide disease; the largest number of cases occur in hot dry climates. It may be that a hot, dry, dusty climate produces chronic nasal inflammation, allowing an ingrowth and tissue damage by the fungus and its metabolites, followed by the immunological reaction of the host to the fungal antigens. In hot, dry environments aspergillus infection has a more virulent course.

Generally, only one sinus is involved with *Aspergillus* spp., most commonly the maxillary sinus. The others, in decreasing order of frequency, are the ethmoid, sphenoid, and frontal sinuses. In a review of 103 cases (cited by Blitzer and Lawson 1993), 67 patients were found to have solitary sinus involvement (46 maxillary, eight sphenoidal, eight nasal, three ethmoidal, two frontal). Aspergillosis of the sphenoid sinus can be confusing, with reported stone formation and cavernous sinus syndrome; it can also mimic a pituitary tumor. When the disease becomes invasive, it usually spreads from the maxillary sinus to the ethmoid and then extends to the orbit and nasal cavity. Most cases of orbital aspergillosis originate in the maxillary and ethmoidal sinuses. Nasal, postnasal, sinus, and orbital symptoms are found in upper respiratory tract infections with *Aspergillus* spp. The nasal symptoms are associated with a mucoid or mucopurulent discharge, swollen nasal mucosa, enlarged turbinates, and occasional polyps or facial pain. In some cases, the secretions are gelatinous, containing necrotic tissue. Irrigation of the maxillary antrum may be difficult, and viscous or greasy material may appear in the lavage fluid. Repeated antral irrigations and antibiotics do not provide symptomatic or radiological improvement. Surgical access to the antrum reveals a thickened or polyploid antral lining, granulation tissue, and necrotic or gelatinous material, often in a cheese-like mass of brownish or greenish material. The administration of systemic corticosteroids seems to be a major factor in predisposing humans to aspergillus infection. Aspergillosis of the nose and paranasal sinuses can be classified into two types: invasive and non-invasive. In the non-invasive form, the patient complains of a nasal obstruction, rhinorrhea, and a sensation of fullness of the face. The sinus involved is opaque radiologically, without invasion. These cases appear to respond well to Caldwell–Luc procedures with an excellent prognosis. In patients with the invasive form, the disease involves the nose, orbit, palate, turbinates, and skin. Patients have pain, proptosis, and blurred vision. Radiologically, there is bone destruction, usually involving the floor of the medial orbital wall. The erosion may involve the basisphenoid and pituitary fossa, the temporal bone and pterygopalatine fossa, the alveolus with loosening of teeth, and the frontal bone. CT scanning has been very useful in defining the full extent of the disease (Ashdown et al. 1994; Krennmair et al. 1994).

Aspergillus sinusitis often produces a mixture of high- and low-density areas within the sinuses. Bone windows allow a very accurate assessment of possible invasion. MRI can be used for better definition of the high and low signals produced by the mycetoma. Despite surgical therapy, recurrences occur and multiple surgery is necessary to eradicate the disease. Direct extension or hematogenous spread from an infected sinus may cause vascular invasion. The invasive form also involves bone or meninges. In one series there were 17 deaths among 103 patients, all of which were caused by intracranial extension. Interestingly, in almost all these cases, there was no known underlying disease. Invasive sinus aspergillosis has also been reported to be associated with facial nerve paralysis, optic neuritis, arteritis, and rupture of the carotid artery. Involvement of the skull base can simulate a malignancy. Histopathologically, epithelium is ulcerated, with a dense inflammatory infiltrate of lymphocytes, plasma cells, and neutrophils. There may be bone necrosis. The granulomas have been classified as proliferative with pseudotubercles in a fibrous stroma, necrotizing with large areas of edematous necrosis, and mixed.

The diagnosis of paranasal sinusitis is nonspecific and often confusing. The differential diagnosis includes

bacterial sinusitis, malignant tumors, tuberculosis, syphilis, osteomyelitis, Wegener's granulomatosis, and rhinoscleroma. Treatment for the non-invasive form consists of sinusotomy and curettage of all diseased and necrotic tissue. This is usually curative on its own. The invasive form, however, requires radical surgical débridement and intravenous amphotericin B. Azole antifungals may have a role in treatment. Early work with itraconazole suggests that it may also have a role. Despite these measures, however, multiple recurrences requiring several procedures are the rule.

Initially, AFS was attributed to *Aspergillus* spp.; it is now clear, however, that many environmental fungi are associated with the disease (Buzina et al. 2003). *Bipolaris* spp. are the most common of this group, but *Exserohilum*, *Curvularia*, and *Alternaria* spp. have also been identified by culturing tissue. The typical patient is an immunocompetent, atopic, young adult with a longstanding history of allergic rhinitis, nasal congestion, headache, nasal polyposis, asthma, and/or recurrent sinusitis. There is a male preponderance of 2 or 3:1. The patient may have had several sinus operations over a period of years. Visual changes may have occurred, and even facial deformity has been reported as a consequence of the expansile nature of the alterations of the sinus. This form of sinusitis is initiated by hypersensitivity to fungal antigens. Immunologically, the serum IgE is increased, and the patient demonstrates a positive cutaneous skin test to the fungus. Elevated serum fungal allergen-specific IgE and IgG precipitins can be demonstrated. A non-contrast CT scan will frequently demonstrate multiple sinus involvement, with areas of high signal within the sinus. This is almost pathognomonic. Bone expansion is commonplace. Bone destruction is reported 30–50 percent of the time. At surgery, dense, inspissated, rubbery, green–brown, allergic mucoid material, and hyperplastic mucosa and polyps are noted. This mucus contains an increased number of eosinophils and Charcot–Leyden crystals, epithelial cells, and cellular debris. Areas of bone may include posterior sphenoid, ethmoid septa, laminar papyracea, and the medial antral wall. The dura, however, remains intact.

Treatment is conservative surgical drainage. Endonasal approaches to the ethmoid, sphenoid, and frontal sinuses, and the Caldwell–Luc approach to the maxillary sinuses, are reasonable choices. Systemic antifungals should be avoided unless there is definite evidence of tissue invasion or there is orbital or intracranial invasion.

The diagnosis and incidence of AFS has been revised recently by Ponikau and colleagues (1999) and Buzina et al. (2003). The objective of these studies was to re-evaluate the current criteria for diagnosing AFS and to determine its incidence in patients with chronic rhinosinusitis (CRS). This prospective study evaluated the incidence of AFS in 210 consecutive patients with CRS with or without polyposis, of whom 101 were treated surgically. A novel method of patient sampling is described where each nostril was lavaged with saline. The histopathological examination of mucin for fungi and eosinophils is emphasized. Fungal cultures of nasal secretions were positive in 202 (96 percent) of 210 consecutive CRS patients. Allergic mucin was found in 97 (96 percent) of 101 consecutive surgical cases of CRS. Allergic fungal sinusitis was diagnosed in 94 (93 percent) of 101 consecutive surgical cases with CRS, based on histopathological findings and culture results. IgE-mediated hypersensitivity to fungal allergens was not evident in the majority of AFS patients. The data from this report indicate that the diagnostic criteria for AFS are present in the majority of patients with CRS with or without polyposis. The authors propose that because the presence of eosinophils in the allergic mucin, and not a type 1 hypersensitivity, is the common feature in the pathophysiology of AFS, the clinical entity AFS should be renamed eosinophilic fungal rhinosinusitis. Molecular techniques have also been applied (Willinger et al. 2003).

Aspergilloma

This is the most familiar of the localized infections produced by *Aspergillus* spp. Typically, aspergillomas develop in old tuberculous cavities, but any cause of lung necrosis acts as a suitable environment. Fungus ball (aspergilloma) formation usually occurs in patients with residual lung cavities following tuberculosis, sarcoidosis, bronchiectasis, pneumoconiosis, and ankylosing spondylitis, or where there is a neoplasm of the lungs. Hemoptysis is the only serious complication. Fungus balls are usually located in the upper lobes. Less frequently, they occur in the apical segments of the lower lobes. Spontaneous lysis has been reported to occur in up to 10 percent of cases. Patients are often asymptomatic, but may present with chronic cough, malaise, and weight loss. Hemoptysis is the most common symptom, occurring in 50–80 percent of cases, and can, on occasion, be massive and life threatening (Jewkes et al. 1983). Chest x-rays will reveal a characteristic oval or round mass with a radiolucent halo or crescent of air over the superior aspect (Figure 8). The mass can often be shown to move as the patient changes position (Figure 9). CT will help to delineate the lesion.

Chronic pulmonary aspergillosis

A number of clinical entities have been described, including chronic necrotizing pulmonary aspergillosis (CNPA), semi-invasive aspergillosis, chronic invasive pulmonary aspergillosis, symptomatic pulmonary aspergilloma, and aspergillus pseudotuberculosis. This distinction between subacute invasive pulmonary aspergillosis, CNPA, and aspergilloma has not been rigorously defined, and an overlap in clinical and radiological features bwtween these different entities probably exists (Denning et al. 2003). In the course of their clinical practice, Denning and colleagues have observed patients

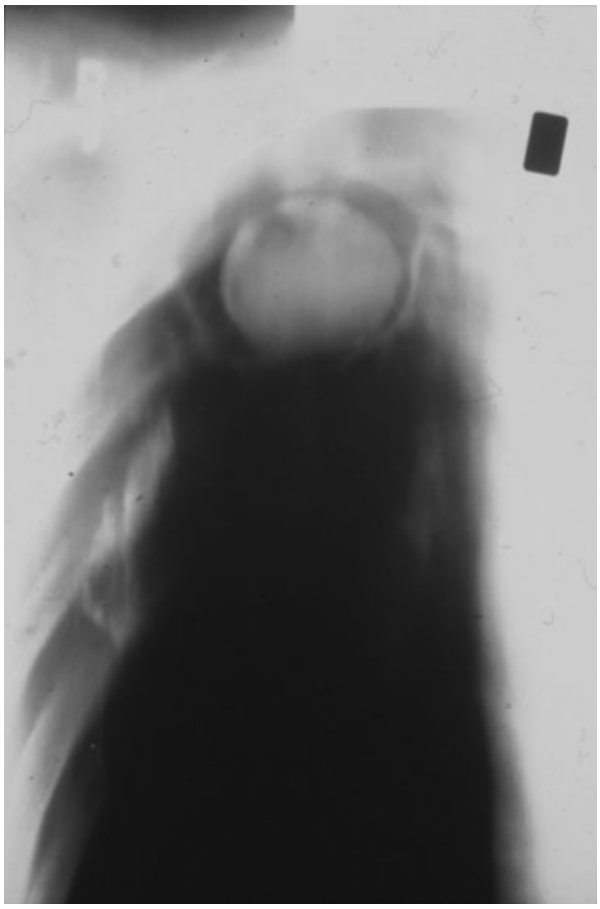

Figure 8 *Radiological appearance of an aspergilloma*

who have chronic pulmonary disease undoubtedly caused by *A. fumigatus*, with clinical features often different from that described in the older literature. Three distinct radiological patterns have been proposed. The first is characterized by the formation and expansion of multiple cavities, some containing fungus balls, which has been termed chronic cavitary pulmonary aspergillosis (CCPA). In some cases, this progresses to marked and extensive pulmonary fibrosis, which has been termed chronic fibrosing pulmonary aspergillosis (CFPA). In these patients, pleural involvement has been seen, either as direct invasion of the pleural cavity or as fibrosis. The third proposed category comprises those patients where there is progressive enlargement of a single cavity, usually with a thin wall, in some cases to substantial dimensions, occurring slowly over months or rapidly in weeks. This last group of patients appear to have slowly progressive invasive aspergillosis, a disease entity similar to or identical to the previously described CNPA. These patients appear to differ slightly from those with CCPA and CFPA because they usually have minor or moderate degrees of immune dysfunction, such as diabetes or corticosteroid use. The authors propose that this condition be called subacute invasive pulmonary aspergillosis, interchangeable with CNPA (Denning et al. 2003).

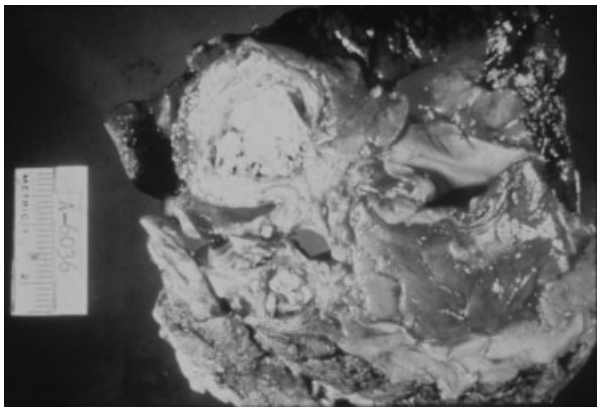

Figure 9 *Gross pathological appearance of an aspergilloma*

This condition usually occurs in middle-aged or older men with chronic or previously treated lung disease such as tuberculosis (reviewed by Binder et al. 1982). Pleural spread has been reported, but dissemination beyond the lung does not occur. The most frequent symptoms include fever, productive cough, malaise, and weight loss, often lasting for months before diagnosis. The radiological findings include a chronic progressive infiltrate representing parenchymal necrosis involving the upper lobes or the superior segment of the lower lobes. Cavitation is common and about 50 percent of patients develop single or multiple fungus balls.

Infection of the central nervous system

It is much more common for cerebral aspergillosis to occur after hematogenous dissemination of infection from the lungs than as a result of direct spread from the nasal sinuses. The brain is involved in about 10 percent of cases of disseminated aspergillosis, but cerebral infection is seldom diagnosed during life (Haran and Chandy 1993; Ashdown et al. 1994).

The symptoms of cerebral aspergillosis are gradual in onset. Confusion, behavioral alterations, and reduced consciousness in a neutropenic patient should suggest the diagnosis. Multiple brain lesions with infarction caused by cerebral arterial thrombosis often result in focal neurological signs, fits, and raised cerebrospinal fluid (CSF) pressure.

The CSF findings are normal in 50 percent of cases. In the remainder, the protein concentration may be raised, but the glucose concentration is usually normal. On occasion, a marked pleocytosis is seen. It is most unusual to recover the fungus from the CSF.

CT is often helpful in locating the lesions, but the findings are nonspecific. Meningitis is a most unusual manifestation of CNS aspergillosis.

Invasive aspergillosis

Various forms of invasive infection have increased markedly in number over the past few years (Anaissie et al.

1989; Walsh 1990; Cohen et al. 1993; Denning 1994b; Williamson et al. 1999; Dimopoulos et al. 2003; Richardson and Warnock 2003). Although the lung is the most common site of infection, aspergillosis can disseminate to virtually any body site, and indeed the true extent of this spread is often only apparent postmortem. Invasive aspergillosis is almost always seen in the setting of the immunocompromised host and is often fatal, even if diagnosed during life and treated. Those at risk include neutropenic patients with hematological malignancies, transplant recipients, and children with chronic granulomatous disease. There is widespread growth of the fungus in the lung tissue, resulting in hemorrhagic infarction. Hematogenous dissemination to other organs often follows.

The increase in the incidence of invasive aspergillosis is exemplified in a report of infections in 60 consecutive adults undergoing unrelated donor bone marrow transplantation over a 7-year period (Williamson et al. 1999). T-cell depletion was employed in 93 percent. There was a high incidence of invasive aspergillosis (17 cases), despite the use of itraconazole or amphotericin B prophylaxis. Ten of these cases occurred beyond 100 days. Two patients (11 percent) with invasive aspergillosis survived. Invasive aspergillosis was significantly associated with bacteremia and multiple viral infections.

The most common presentation in the neutropenic patient is an unremitting fever (higher than 38°C) which fails to respond to broad-spectrum antibacterial treatment. Pleuritic chest pain is not unusual. Cough may be present, but sputum production is usually minimal. Hemoptysis is uncommon.

The radiological findings are nonspecific, but the earliest lesions are single or multiple opacities. These usually progress to diffuse bilateral consolidation, or cavitation, or large wedge-shaped peripheral lesions, representing hemorrhagic infarction. These last suggest aspergillosis and their detection is sufficient justification for treatment. In 10 percent of patients with proven aspergillosis, the chest x-ray has been normal within a week of death. CT often reveals nodular lesions in patients with normal chest x-rays.

Expectoration of necrotic tissue from an infarcted lesion can leave behind what looks like a fungus ball in the lung. However, this should not be confused with the classic benign condition seen in the non-immunocompromised patient.

Hematogenous dissemination of infection from the lungs to the brain, gastrointestinal tract, and other organs occurs in up to 30 percent of patients.

The risk factors associated with invasive aspergillosis are in general similar to those for disseminated candidosis, with an even higher emphasis on prolonged neutropenia and corticosteroid treatment, particularly in combination with other immunosuppressive drugs as used in organ and bone marrow transplant recipients.

The degree and duration of granulocytopenia together are the most important predisposing host factor. The risk of developing an aspergillus infection seems to be low during the first 2 weeks of neutropenia, but after the second and third week the risk increases dramatically. Cell-mediated immunodeficiency, as in patients undergoing bone marrow transplantation or with corticosteroid therapy, is an additional risk factor.

Pulmonary CT may detect nodular lesions, sometimes with a surrounding zone of intermittent attenuation, in patients with a normal chest x-ray, and has therefore been considered extremely valuable (McWhinney et al. 1993; Gotway et al. 2002).

Specific diagnostic aspects of invasive aspergillus infections in allogenic bone marrow transplant recipients have been described also by Jantunen and colleagues (Jantunen et al. 2000). The charts of 22 consecutive patients with invasive aspergillosis transplanted in 1989–95 were reviewed. Invasive aspergillosis was diagnosed 69–466 days (median 131 days) after bone marrow transplantation. In 16 patients (73 percent), a definite or probable diagnosis of invasive aspergillosis was made during life. Respiratory symptoms were the presenting feature in half of the patients, followed by neurological symptoms (27 percent). Chest x-ray revealed single or multiple nodular lesions in ten patients; cavitation was observed in five patients. Tissue biopsy was the most common method of diagnosis (nine patients: lungs, six; liver, one; subcutaneous tissue, one; brain, one). Five cases of invasive aspergillosis were detected by nine guided fine-needle lung biopsies in eight patients and without complications. Bronchoalveolar lavage (BAL) was performed in 14 patients, with findings suggestive of invasive pulmonary aspergillosis in eight cases. Lungs were the most common organ affected (90 percent), followed by the central nervous system (CNS) (41 percent). More recent reviews and clinical surveys include those by Marr et al. (2002), Guermazi et al. (2003), and Wiederhold et al. (2003).

Acute invasive pulmonary aspergillosis

This is the most common form of invasive aspergillus infection and carries a very high mortality rate, in some studies about 95 percent (Gerson et al. 1984; Weinberger et al. 1992; Gentile et al. 1993; Ribrag et al. 1993; Richard et al. 1993; Saugier-Veber et al. 1993; Walmsley et al. 1993, and reviewed by Marr et al. 2002, Richardson and Warnock 2003, and Wiederhold et al. 2003). This type of infection may present in several forms. A bronchopneumonia with solitary or multiple infiltrates, fever, and lack of response to treatment with antibiotics is one common manifestation of acute pulmonary aspergillosis. Invasive aspergillosis of the upper airways has been described (Logan et al. 1994). Also a lobar pneumonia which resembles a bacterial infection may occur.

In the early stages of acute aspergillus bronchopneumonia, the small, patchy infiltrates may be undetectable

with ordinary chest x-rays, and in these cases CT has proved valuable for the detection of these lesions (Palmer et al. 1991; Aquino et al. 1994; Blum et al. 1994). Even in cases where the aspergillus infection presents as nonspecific infiltrates on conventional chest x-rays, a CT scan may give very valuable clues to the diagnosis by revealing a wedge-shaped, peripheral halo zone of intermediate CT attenuation surrounding a nodular infiltrate (Taccone et al. 1993). This halo is the CT correlate of hemorrhage and edema surrounding an infarct caused by thrombosis (Figure 10).

Bronchopneumonic aspergillus infiltrates later give rise to abscess formation, and not infrequently cavitary lesions may develop, often responding with neutrophil recovery (Pai et al. 1994). CT is also valuable for the demonstration of such cavitary lesions. These latter lesions may sometimes give rise to a secondary aspergilloma. Sudden life-threatening hemoptysis may supervene.

Another major manifestation of acute pulmonary aspergillosis is hemorrhagic pulmonary infarction caused by invasion and thrombosis of a large pulmonary artery. The classic clinical manifestation is sudden onset of fever, and a pleural friction rub, suggesting pulmonary thromboembolism. If uncontrolled, the aspergillus infection may extend by invasive growth into neighboring organs and structures, e.g. the mediastinum, ribs, vertebrae, esophagus, and pericardium (Ribrag et al. 1993).

The clinical manifestations described above in acute pulmonary aspergillosis are not diagnostic for this infection (Saugier-Veber et al. 1993). The diagnosis can only be made with certainty by histopathological demonstration of the fungus in a fine-needle lung biopsy, but this is not always possible in acutely ill patients, not infrequently with severe hemorrhagic diathesis. Also the tissue obtained may show only necrotic debris from infarcted areas without the characteristic aspergillus hyphae. Often, therefore, the diagnosis may only be strongly suspected by judicious interpretation of the clinical and radiological manifestations, in the presence of the most important risk factors for invasive aspergillus infection. In this setting the presence of *Aspergillus* spp. in tracheobronchial secretions should not be ignored and ascribed to contamination or colonization. It increases the likelihood of pulmonary aspergillosis.

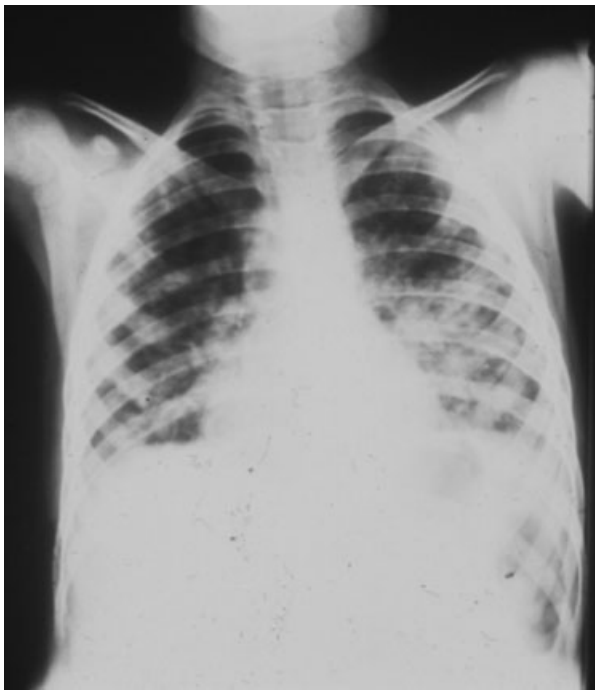

Figure 10 *Radological appearance of acute invasive aspergillosis*

Aspergillus can more readily be found in sinus washings and biopsies from necrotic lesions in the sinuses. The use of surveillance cultures is not common practice in most hospitals but may be useful. The value of antigen and antibody detection in the serological diagnosis and prognosis of invasive aspergillosis is discussed below. The value of imaging in the diagnosis of invasive aspergillosis is highlighted in reviews by Worthy et al. (1997) and Berger (1998).

Systematic CT scanning appears to allow earlier diagnosis of invasive aspergillosis. A number of studies have shown that the halo sign was highly indicative of invasive pulmonary aspergillosis in neutropenic patients. The image occurs early in the disease and predicts aspergillosis before typical cavitation. The experience of one center highlights the value of CT scanning in the early diagnosis of invasive aspergillosis. Caillot and colleagues have systemically performed CT scanning over the past 7 years in febrile neutropenic patients with pulmonary x-ray infiltrates (Caillot et al. 1997). This approach allowed the investigators to detect suggestive CT halo signs in 92 percent of 23 histologically proven and 14 highly probable invasive aspergillosis patients, compared with 13 percent before the period of analysis. The mean time to invasive pulmonary aspergillosis diagnosis was reduced dramatically from 7 ± 5.5 to 1.9 ± 1.5 days. Other centers have reported similar findings (Franquet et al. 2001).

Aspergillus fungemia

Aspergillus fungemia is encountered infrequently. In most published studies of fungemia, it is seldom mentioned. The interpretation of these data is difficult because media contamination, yielding false-positive results of cultures, often occurs. Therefore, determining the importance of aspergillus fungemia in the immunocompromised patient is particularly difficult. A review of the literature of aspergillus fungemia by Duthie and Denning (1995) suggests that both clinical and laboratory criteria have to be critically appraised before assigning the isolation of *Aspergillus* spp. from blood into one of the following: a genuine case of fungemia or, where the organism has been isolated from the blood but in an atypical clinical setting, a case not compatible

with disseminated infection (pseudofungemia). In their review of 30 cases of genuine fungemia and 34 cases of pseudofungemia, and the inclusion of two further cases of genuine infection from their own practice, a number of features emerge. A wide range of media and blood culture systems was used to isolate *Aspergillus* spp. The median time to positive blood culture was 8.5 days (range 1–27 days) in the genuine cases. Genuine aspergillus fungemia was observed more often after cardiac surgery or during neutropenia than in other settings. Other patients at risk for aspergillus fungemia were similar to those at risk for invasive aspergillosis, including patients with the acquired immunodeficiency syndrome (AIDS). Of 19 patients who were treated, seven (44 percent) survived. In the group of patients with aspergillus pseudofungemia, there were no deaths, and cultures of additional specimens from the same patient were not positive. The conclusion to be drawn from the numbers of disseminated aspergillosis is that aspergillus fungemia occurs at a far greater rate than is normally detected. Development of an optimal blood culture system may be justified for the increasing numbers of high-risk patients. Many modifications of classic blood culture systems for optimizing the isolation of fungi have been described, but very few of these have resulted in an increased isolation rate of *Aspergillus* spp. It is possible that agitation of some of the static systems may enhance the recovery of hyphal elements of this organism (Duthie and Denning 1995).

Cerebral aspergillosis

The most important organ affected in disseminated aspergillosis, apart from the lungs, is the brain (invasive aspergillosis infection), because of both its frequency and its staggering mortality. The clue to the interpretation of this clinical manifestation is the striking tendency for aspergillus hyphae to invade cerebral vessels, causing thrombosis and infarction. Accordingly, the typical manifestations of cerebral aspergillosis are focal neurological deficits including hemiparesis, focal seizures, and cranial nerve palsy. In addition, progressive signs of CNS depression may be seen. Solitary or multiple abscesses may form in an infarcted area, and a CT or MRI scan will show abnormalities consistent with infarctions or abscesses. Although the clinical and radiological manifestations of cerebral aspergillosis are not diagnostic, the presence of CNS manifestations as described in a febrile, immunocompromised patient with pulmonary infiltrates must be considered highly suggestive of disseminated aspergillosis.

Ocular infections

Three forms of ocular infection with *Aspergillus* spp. have been recognized. The traumatic implantation of the fungus into the eye may result in a corneal ulcer which can progress to perforation. Endophthalmitis is an uncommon condition, but it has been described in drug abusers, patients with endocarditis, and organ transplant recipients. It can arise following ocular trauma or hematogenous spread of the fungus. Hematogenous spread is more usual in immunocompromised patients. The symptoms include ocular pain and impaired vision.

Orbital aspergillosis can develop as an extension from infection of the paranasal sinuses. The presenting symptoms include orbital pain, proptosis, and loss of vision. In 25 percent of cases the infection spreads into the brain and causes death (Sugata et al. 1994).

Endocarditis and myocarditis

Aspergillus endocarditis tends to occur in patients undergoing open heart surgery, although it has also been described as a complication of parenteral nutrition and drug addiction. The aortic and mitral valves are the most frequent sites of infection (Motte et al. 1993). It often gives rise to large friable vegetations and large emboli are common.

The symptoms and clinical signs are similar to those of bacterial endocarditis, with prolonged fever and abnormal heart murmurs. More specific features include large friable vegetations. Emboli that obstruct major arteries, particularly those of the brain, occur in about 80 percent of cases.

Myocardial infection with abscess formation or mural vegetations may occur as a result of hematogenous dissemination. Myocarditis has been reported in about 15 percent of patients dying with disseminated aspergillosis. It can result in nonspecific ECG abnormalities or congestive heart failure.

Osteomyelitis

Aspergillus osteomyelitis is an uncommon condition, but children with chronic granulomatous disease seem to be at particular risk. In these children, spread from an adjacent lung lesion is usual, and the ribs and spine are the most common sites of aspergillus infection. In adults, the spine is also a very common site of infection, but hematogenous spread of the fungus may be more common. Paraplegia can occur.

Otomycosis

Otomycosis is the name given to growth of *Aspergillus* spp., usually *A. niger* or *A. fumigatus*, within the external auditory canal (Paulose et al. 1989). Patients present with decreased hearing, itching, pain, or discharge from the canal. Otoscopy reveals greenish or black fuzzy growth on the cerumen or debris in the auditory canal. Treatment usually consists of careful cleaning of the canal and the application of topical nystatin suspension or ointment morning and evening for 2–3 weeks. Imidazole creams such as econazole nitrate also

give excellent results. The course is chronic with acute episodes, especially in summer, and intermittent remissions. With antifungal treatment the prognosis is good.

Aspergillus spp. may invade the external auditory canal of immunocompromised patients, extending into contiguous bone or even brain.

Skin infections

Two different forms of cutaneous aspergillosis have been reported in immunocompromised patients (Carlisle 1978; McCarthy et al. 1986; Allo et al. 1987). Cutaneous lesions may arise at catheter insertion sites and as the source of a subsequent disseminated infection. The lesions begin as erythematous to violaceous, edematous, indurated plaques which evolve into necrotic ulcers covered with a black eschar.

In about 5 percent of patients with aspergillosis, hematogenous spread of infection gives rise to cutaneous lesions. These may be single or multiple, well-circumscribed, maculopapular lesions which become pustular. They evolve into ulcers with distinct borders covered by a black eschar. The lesions enlarge and may become confluent.

Primary cutaneous aspergillosis requires direct inoculation and is usually seen in immunocompromised hosts (reviewed in Groll et al. 1998b; Walsh 1998). Primary cutaneous infection has been reported at intravenous catheter sites, under occluded skin, in surgical wounds and burns, and in damaged skin, for example, venisection sites and pyoderma gangrenosum. Other cases have been reported where patients were nursed on a spinal rotorest bed designed to obviate the need for turning patients with unstable spinal injuries. The bed therefore rotates to alter sites of pressure, but the skin remains occluded with the bed sheets. Another case of primary cutaneous aspergillosis has been reported in a tetraplegic patient where there were erythematous and violaceous papules and plaques studded with pustules (Galimberti et al. 1998). It is anticipated that as the numbers of immunocompromised hosts continue to increase, the numbers of primary cutaneous aspergillosis will continue to increase in parallel with a rise in cases of invasive pulmonary aspergillosis.

Infections of the gastrointestinal tract

Gastrointestinal tract infection has been detected in 40–50 percent of patients dying with disseminated infection. The esophagus is the most frequent site of involvement, but intestinal ulcers also occur and these often result in bleeding or perforation.

Hepatic and splenic infection

This has been seen in up to 30 percent of patients with disseminated aspergillosis. The symptoms include liver tenderness, abdominal pain, and jaundice, but many patients are asymptomatic. CT scans will reveal numerous, small, radiolucent lesions scattered throughout the liver. Modest elevations in alkaline phosphatase or bilirubin concentrations can often be detected.

Aspergillosis in solid organ transplants

The incidence of invasive aspergillosis varies according to the organ transplanted, with renal transplant recipients being least at risk. Lung transplantation for cystic fibrosis carries a high risk of invasive aspergillosis (Bag 2003). In some units preoperative colonization remains a contraindication to transplantation. In early studies in lung transplant recipients, 27.5 percent developed a fungal infection postoperatively, and invasive aspergillosis accounted for 9.8 percent. Other reviews show the following pattern of incidence: liver recipients, 1–4.5 percent; kidney recipients, 0.5–2.2 percent; lung or lung–heart recipients, 18 percent (Panackal et al. 2003).

Other case series indicate that the incidence of invasive aspergillosis in liver transplant recipients appears to be 1.5–14.7 percent and the time period of highest risk post-transplant has been reported to be between 14 and 100 days. The predictors for invasive fungal infections complicating orthotopic liver transplantation (OLT) include severe renal insufficiency, prolonged operative time (>11 h), retransplantation, and colonization within 3 days of OLT. If a number of these predictors are present then the incidence of invasive fungal infection can be as high as 67 percent.

The risk factors for invasive aspergillosis in solid organ transplant recipients include: pulse steroid administration, the use of OKT3, antibiotic usage, organ failure, retransplantation, thrombocytopenia, and hospital construction. Recent data support the association between cytomegalovirus (CMV) and fungal infections in liver transplant recipients. CMV infection has been found to be present in 28.5 percent of cases of invasive aspergillosis. The use of antilymphocyte globulin, antithymocyte globulin, OKT3 monoclonal antibody, and the number of episodes of acute rejection treated are all associated with an increased risk of fungal infections.

Aspergillosis in solid organ transplant recipients present nonspecifically and their signs and symptoms often overlap with those of other infectious and non-infectious processes. In any infection diagnosed in a solid organ transplant recipient, a careful search should be made for metastatic infection, especially of the skin, the skeletal system, and the CNS. Pulmonary symptoms predominate – including nonproductive cough, pleuritic chest pain, dyspnea and low-grade fever. Chest radiology may suggest a patchy pneumonia, cavitary lung disease, or a pulmonary embolus; the chest x-ray may also be normal. From the lungs, *Aspergillus* spp. may disseminate to almost any organ, including the brain,

liver, spleen, kidney, thyroid, heart, blood vessels, bone and joints, among others. *Aspergillus* spp. may also invade the paranasal sinuses, gastrointestinal tract, or skin; rarely, it may gain entry through an intravenous catheter. *Aspergillus* spp. may cause peritonitis in renal transplant recipients on continuous ambulatory peritoneal dialysis (CAPD), or in liver transplant recipients with intra-abdominal abscesses. Endophthalmitis may occur, usually in conjunction with endocarditis. Other unusual presentations of aspergillus infections in solid organ transplant recipients include tracheobronchitis, with infection limited to the anastomotic site and large airways in heart–lung and lung transplant recipients, and wound infections.

LUNG TRANSPLANTATION

Aspergillus infections, documented in 6–8 percent of lung transplant recipients, remain among the most significant opportunistic infections after lung transplantation (Kubak 2002). Lung transplant recipients have a unique predisposition for and clinical manifestations of aspergillus infection. Direct communication of the transplanted organ with the environment, impairment in local host defenses (e.g. mucociliary clearance and cough reflex), disruption of lymphatic drainage, ischemic airway injury, altered alveolar phagocytic function, and an overall greater requirement of immunosuppression render lung transplant recipients uniquely susceptible to aspergillus airway colonization and invasive disease. Isolated tracheobronchitis and bronchial anastomotic infections are entities distinct from aspergillus pneumonia. In one study, although the early mortality of patients with bronchial anastomotic aspergillus infections did not differ significantly from patients without these infections, their long-term survival was reduced.

Aspergillus infections in lung transplant recipients have been reported largely as institutional experiences with few patients or case reports. Consequently, the risk factors, role of aspergillus airway colonization, and variables influencing outcome have not been well defined.

A recent review has identified a total of 159 cases of aspergillus infections in 40 published reports (Singh and Husain 2003). Of these, 87 were individually detailed cases, and 72 were summarized in reports containing 2–14 cases. The type of lung transplantation was reported in 66 patients. Overall, 51.5 percent (34 of 66) of the patients with aspergillus infections were single lung transplant recipients, 34.9 percent (23 of 66) were bilateral lung transplant recipients, and 13.6 percent (9 of 66) were heart–lung transplant recipients. Patients ranged in age from 19 to 64 years (median 48 years), and 64 percent were male.

The median incidence of aspergillus infection was 6.2 percent and ranged from 2.2 to 20 percent. Aspergillus infections occurred a median of 3.2 months and up to 5 years after lung transplantation. Twenty-six percent of all infections occurred within 1 month, 51 percent within 3 months, and 72 percent within 6 months of lung transplantation. Sixteen percent of the infections occurred after 6 months, and only 12 percent were documented after 12 months of transplantation.

Time of onset of infections varied significantly for different types of lung transplant recipients. The median time to onset after transplantation was 0.7 months for heart–lung, 3.9 months for bilateral lung, and 5 months for single lung transplant recipients (P=0.046). Single lung transplant recipients developed aspergillus infections significantly later after transplantation than all other patients (median 4.9 months versus 2.1 months, P=0.019). Sixty-eight percent (15 of 21) of the single lung as compared with 35 percent (9 of 26) of the bilateral lung transplant recipients developed aspergillus infection at >3 months after transplantation (P=0.021). Time of onset also differed for various types of aspergillus infections. Invasive pulmonary or disseminated aspergillosis occurred significantly later than tracheobronchitis (P=0.03). Of the aspergillus infections occurring within 3 months of transplantation, 75 percent (21 of 28) were tracheobronchitis or bronchial anastomotic infections, 18 percent (5 of 28) were invasive pulmonary infections, and 7 percent (2 of 28) were disseminated invasive infections (P=0.06). Other variables (e.g. age, chronic obstructive pulmonary disease as underlying lung disease, antilymphocyte globulin use for induction, use of antifungal prophylaxis, prior rejection or CMV infection) did not differ for patients with early- versus late-onset aspergillus infections.

Of 78 lung transplant recipients, 29 (37 percent) had tracheobronchitis, 25 (32 percent) had invasive pulmonary aspergillosis, and 16 (20 percent) had bronchial anastomotic infection. Only 10 percent (8 of 78) of the patients had disseminated invasive aspergillosis. Of these, 4 percent (3 of 78) had multiple sites of dissemination and 5 percent (5 of 78) had a single extrapulmonary site of infection documented; these included osteomyelitis in two patients (involving pelvis in one and vertebrae in the other), and thoracic wound infection, endophthalmitis, and retroperitoneal abscess in one patient each. Eight percent (3 of 36) of the patients with invasive aspergillosis were diagnosed only at autopsy.

Fifty-eight percent (19 of 33) of the single lung as compared with 45 percent (14 of 31) of all other patients had invasive aspergillosis (P=0.3). Single lung transplant recipients were older (P=0.006) and more likely to have chronic obstructive pulmonary disease as an underlying illness (P=0.05). Of the patients with invasive pulmonary aspergillosis, 67 percent (16 of 24) were single lung, 21 percent (5 of 24) bilateral lung, and 13 percent (3 of 24) heart–lung transplant recipients.

Whether invasive aspergillosis first occurred in the native or transplanted lung could be ascertained in ten patients. In nine of these, invasive aspergillosis was first documented in the native lung. The underlying lung disease in these patients was chronic obstructive

pulmonary disease in five, 1-antitrypsin deficiency in three, idiopathic pulmonary veno-occlusive disease in one, and unknown in one patient. Invasive aspergillosis occurred a median of 7 months post-transplant in these patients (range 18 days to 5 years). Seven of these 10 patients died.

In total, 91 percent of the aspergillus infections were due to *A. fumigatus*, 2 percent (1 of 40) to *A. flavus*, 2 percent (1 of 40) to *A. niger*, and 5 percent (2 of 40) were mixed infections due to two *Aspergillus* spp. Non-*A. fumigatus* spp. were associated with 10 percent (3 of 29) of tracheobronchial infection episodes, but were never associated with invasive aspergillus infections. Only 15 percent (4 of 27) of the aspergillus infections were accompanied by fever. Fever was significantly more likely to be documented in patients with invasive as compared with localized tracheobronchial aspergillus infections; 50 percent (2 of 4) of the patients with disseminated aspergillosis, 20 percent (2 of 10) of those with invasive pulmonary aspergillosis, and 0 percent (0 of 13) of those with tracheobronchial or bronchial anastomotic infections were febrile ($P = 0.046$). Prior rejection episodes were documented in 50 percent, and use of antilymphocyte preparation or OKT3 in 38 percent of the lung transplant recipients with invasive pulmonary aspergillosis. The radiographic appearance of pulmonary aspergillus infections was a focal consolidation in 40 percent (4 of 10), cavitary lesions in 30 percent (3 of 10), and nodules or mass-like lesions in 30 percent (3 of 10).

Data on aspergillus colonization could be assessed in 395 patients in 25 studies that had numerator and denominator data available. *A. fumigatus* was the most common species cultured (63 percent, 174 of 276), followed by *A. niger* (12 percent, 34 of 276), *A. versicolor* (9 percent, 26 of 276), *A. flavus* (8 percent, 21 of 276), *A. nidulans* (4 percent, 10 of 276), *A. glaucus* (3 percent, 7 of 276) and *A. terreus* (1 percent, 4 of 276). In 60.9 percent (14 of 23) of the patients, the first positive culture for *Aspergillus* was detected within 3 months of transplantation, in 4.3 percent (1 of 23) between 3 and 6 months, in 26.1 percent (6 of 23) between 6 and 12 months, and in 8.7 percent (2 of 23) at >12 months after transplantation. Of the patients with an airway culture positive for *Aspergillus*, 7.4 percent (15 of 207) had tracheobronchitis or bronchial anastomotic infection, and 20.1 percent (41 of 202) had invasive aspergillosis upon first detection of *Aspergillus*. An additional 5.7 percent (11 of 193) of the patients with a positive airway culture progressed to aspergillus infection at a later time point. These included 3.1 percent (6 of 193) with progression to tracheobronchitis, and 2.6 percent (5 of 193) with progression to invasive aspergillosis. The mortality rate was 62.5 percent (5 of 8) in those with progression due to infection and 1 percent (1 of 105) in those who remained colonized.

Overall mortality in lung transplant recipients with aspergillus infections was 52 percent (59 of 113) and varied significantly with site of infection, type of lung transplantation, time of onset of infection, and antifungal therapy employed. Mortality rate was 23.7 percent (9 of 38) for patients with tracheobronchial or bronchial anastomotic infections, 81.9 percent (18 of 22) for patients with invasive pulmonary aspergillosis, 67 percent (2 of 3) for those with disseminated invasive infection, and 33 percent (1 of 3) for patients with only an extrapulmonary site of infection (P=0.0001). Mortality for single lung transplant recipients (60 percent, 18 of 30) was significantly higher than for bilateral lung (40 percent, 6 of 15) or heart–lung transplant recipients (56 percent, 3 of 9; P=0.03). Patients with late-onset aspergillus infections had significantly higher mortality than those with early-onset infections (57 percent, 12 of 23 versus 28 percent, 7 of 25; P=0.045). However, when only the patients with invasive aspergillosis were analyzed, the mortality rate did not differ for those with late (73 percent, 8 of 11) versus early-onset aspergillus infections (71 percent, 5 of 7). Age (44.7 versus 45.5 years), rejection (76 percent, 16 of 21 versus 47.1 percent, 8 of 17), or antilymphocyte globulin use (34.6 percent, 9 of 26 versus 34.5 percent, 10 of 29) did not differ significantly in patients with aspergillus infection who lived versus those who died, respectively.

Of lung transplant recipients with invasive aspergillosis, 18 received amphotericin B, five received a liposomal formulation of amphotericin B, and one patient received amphotericin B plus itraconazole. Mortality was significantly higher in patients with invasive aspergillosis who received amphotericin B (83 percent, 15 of 18) as compared with those who received a liposomal formulation of amphotericin B or amphotericin B plus itraconazole (33 percent, 2 of 6; P=0.038). Mortality rate, however, did not differ for patients with tracheobronchitis or bronchial anastomotic infections who received amphotericin B (33 percent, 2 of 17) as compared with the other regimens (19.3 percent, 5 of 26, P >0.20). Only four patients underwent pneumonectomy for the treatment of invasive aspergillosis; all were single lung transplant recipients and three of four of these patients died. Of the four patients who underwent pneumonectomy, one each had received amphotericin B, a liposomal formulation of amphotericin B, and amphotericin B plus itraconazole as antifungal therapy; the antifungal therapy was unavailable in one patient.

Although previous reports have alluded to a higher incidence of aspergillus infections in single lung transplant recipients, the report by Singh and Husain (2003) has highlighted the unique characteristics and overall greater impact of aspergillus infections after single lung transplantation. Single lung transplant recipients were significantly older, more likely to have chronic obstructive pulmonary disease as an indication for lung transplantation, or have developed aspergillus infections later after transplantation, tended to have a higher incidence of inva-

sive aspergillosis, and had higher mortality than all other lung transplant recipients with aspergillus infections.

In the vast majority of the single lung transplant recipients, invasive aspergillosis was documented in the native lung, suggesting that the native lung may harbor a nidus for *Aspergillus* and serve as a source of infection in these patients. It is known that single lung transplant recipients are more likely to have chronic obstructive pulmonary disease, a condition which predisposes to airway colonization with *Aspergillus*. A molecular epidemiologic study in lung transplant recipients that used DNA primers for strain typing documented that, although the clinical strain of a single lung transplant recipient was identical to the one collected at home, the isolates in other lung transplant recipients were deemed to be more likely of nosocomial origin. Thus, aspergillus infections in single lung transplant recipients likely represent reactivation of a preexisting focus. The higher mortality (despite pneumonectomy) in these patients may be related to the higher incidence of invasive pulmonary as compared with tracheobronchial infections.

Characterized by endobronchial lesions ranging from erythema to ulceration and pseudomembrane formation, tracheobronchitis is an entity observed specifically in lung transplant recipients. Lesions in the vicinity of or involving the anastomotic site can result in bronchopleural fistula and fatal hemorrhage. Bronchopleural fistulas were documented in 4.4 percent (2 of 45) of the lung transplant recipients with tracheobronchial aspergillus infections. A majority of the patients in whom tracheobronchitis or bronchial anastomotic infections occurred were bilateral lung transplant recipients. Bilateral lung and right lung transplant recipients in a previous study were documented to have a higher incidence of bronchial anastomotic infection. It was proposed that bilateral lung transplant recipients may be more likely to have greater impairment of cough reflex and mucociliary clearance, leading to a higher risk of colonization and subsequent infection of the anastomotic sites. Also, bilateral lung transplant recipients undergo longer operations and may have a higher risk of ischemia in the area of anastomosis.

Two observations relevant to clinical and radiographic manifestations of aspergillus infections in lung transplant recipients deserve mention. Fever was documented in only 15 percent of the patients with aspergillus infections. Patients with invasive pulmonary or extrapulmonary aspergillosis were more likely to be febrile than those with tracheobronchitis or bronchial anastomotic infections. Second, aspergillus lung infections frequently lacked a characteristic radiographic appearance and presented most often with focal areas of patchy consolidation or infiltrate. Nodular lesions were documented in 30 percent of the patients with pulmonary aspergillosis in our study and in 27 percent in a previous report. Characteristic CT findings (e.g. halo sign) were distinctly unusual.

An optimal antifungal prophylactic strategy in lung transplant recipients has not been defined. The antifungal agent employed and the duration of prophylaxis remain controversial in these patients. Aerosolized amphotericin B and itraconazole are currently the most frequently employed antifungal prophylactic agents in lung transplant recipients. Given the variability in the route of administration, dosages, and duration of prophylaxis, the efficacy of either agent cannot be discerned incontrovertibly. However, the analysis described here allows the following conclusions that may be relevant in the approach toward antifungal prophylaxis or designing antifungal prophylactic trials. Tracheobronchial or anastomotic infections were the most frequently occurring infections within 3 months; the median time to onset was 2.7 months. Antifungal prophylaxis, if employed, may be limited to 3 months in most patients. Such a strategy utilizing an 'effective' antifungal agent could potentially prevent 62 percent of the trancheobronchial, 36 percent of the invasive pulmonary, and 50 percent of the disseminated infections. Invasive pulmonary aspergillosis, however, occurred a median of 5.5 months after transplantation. Single lung transplant recipients were significantly more likely to develop aspergillus infections after 3 months, and the native lung may have been the source in these patients. Thus, a longer duration of prophylaxis, employing a systemic antifungal agent able to achieve adequate pulmonary levels, may be preferable in these patients.

In summary, the review has highlighted an overall greater morbidity and poorer outcome in single lung transplant recipients with aspergillus infections. The single lung transplant recipients with aspergillus infections were older, more likely to have chronic obstructive pulmonary disease as an underlying illness and to have developed aspergillus infections later after transplantation, and tended to have a higher incidence of invasive aspergillosis than other lung transplant recipients. Overall mortality in lung transplant recipients with aspergillus infections was 52 percent; single lung transplant recipients and patients with late-onset aspergillus infections had significantly higher mortality. The unique epidemiologic characteristics and variables influencing outcome delineated in the review have implications relevant for the management and for designing antifungal prophylactic studies in lung transplant recipients in the future.

HEART TRANSPLANTATION

Most systemic fungal infections in heart transplant recipients are caused by *Aspergillus*, second in incidence to CMV. The infection manifestation is pneumonia occurring around 35 days post-transplantation. In one study in 844 heart transplant recipients between the years 1980 and 1998, there were 21 definite cases of invasive pulmonary aspergillosis and four probable. The median time to onset was 46 days. The presentation and diag-

nostic features included fever/cough, single or multiple pulmonary nodules, abnormal radiological findings (nodules), and a positive culture. Neutropenia was not present (Montoya et al. 2003).

Further information on aspergillosis in solid organ transplant recipients can be found in Kubak (2002) and Singh (2003).

Invasive aspergillus infection in AIDS

Invasive aspergillus infections appear to be rather uncommon in individuals with AIDS, although the incidence appears to be increasing (Minamoto et al. 1992; Lortholary et al. 1993; Khoo and Denning 1994). The rarity of invasive aspergillosis during the first few years of the AIDS epidemic led to its exclusion as an AIDS-defining illness. The low incidence of this infection in patients with AIDS is highlighted by the report from the Memorial Sloan Kettering Cancer Center, New York (Pursell et al. 1992). Of the 972 patients with AIDS who were observed over a 10-year period, *Aspergillus* spp. were isolated from various respiratory sites from 45 patients before death. Invasive aspergillosis was documented postmortem in four of these patients and was strongly suspected in an additional patient in whom a postmortem examination was not performed. Traditional risk factors for the development of invasive disease (neutropenia, hematological malignancy, and/or corticosteroid use) were present in all the patients with invasive aspergillosis. A review of the literature up to 1992 reveals reports of 13 cases of invasive aspergillosis in patients with AIDS.

In a report from France (Daleine et al. 1993) where respiratory secretions from 614 patients positive for the human immunodeficiency virus (HIV) over a 2-year period were analyzed retrospectively, it was found that the prevalence of *Aspergillus* spp. isolated was 2.7 percent in BAL specimens (21 of 757), 15.1 percent in sputum specimens (3 of 53), 12.6 percent in bronchial aspirates (11 of 87), 7 percent in protected brush specimens (2 of 28) and 16.6 percent in lung biopsy specimens (4 of 24). A total of 20 patients (rate = 3.14 percent) had lung specimens positive for *Aspergillus* spp.

Patients with advanced HIV infection certainly have profound underlying immunosuppression and are exposed to aspergillus conidia, so it would appear that these infections should be more common. One explanation for the low prevalence may be the fact that neutrophils and macrophages are the main immunological defenses against *Aspergillus* spp. and that these components of the host defense system are more or less spared in HIV infection compared with the defects in T lymphocytes. However, in vitro, neutrophils from children with HIV appear to show impaired ability to kill hyphae of *A. fumigatus* (Roildes et al. 1993a).

One explanation may be that these infections are now being diagnosed more frequently than in the past because of less reluctance to perform bronchoscopies and lung biopsies. Other explanations may relate to an increased prevalence of risk factors for invasive aspergillosis in the HIV-infected population. One consideration is whether advanced HIV infection itself is a risk factor, and whether certain pulmonary infections, such as tuberculosis or *Pneumocystis carinii* pneumonia, may lead to pulmonary damage that predisposes to invasive aspergillosis. Aerosolized pentamidine may also be a risk factor, because most patients with invasive aspergillosis reported in the literature had received this form of therapy. Finally, it should be considered whether prolonged treatment with certain antifungal agents having little activity against *Aspergillus* spp., and thus possibly leading to colonization, is an additional risk factor. It must be emphasized, however, that, as yet, there is little to support the establishment of anything but neutropenia and steroid treatment as risk factors for invasive aspergillosis infection in people infected with HIV.

The clinical presentation of invasive aspergillosis in the HIV-infected population ranges from isolated cutaneous involvement to widely disseminated disease. Most patients reported thus far have advanced HIV infection, with CD4 counts below 50/mm^3 and histories of other opportunistic infections. Pulmonary infections are the most common manifestation of invasive aspergillosis in patients with AIDS (Miller et al. 1994). The lungs are involved as the sole site in about 60–80 percent of all cases, and lung involvement with extrapulmonary disease occurs in about 25 percent. Extrapulmonary infection in the absence of lung involvement has been seen in about 10–15 percent of cases.

Extrapulmonary invasive aspergillosis has been seen in virtually all organs of the body in patients with AIDS (Kempner et al. 1993). The most common site has been the CNS (Woods and Goldsmith 1990); focal abscesses, meningitis, and spinal cord lesions have all been described. Only a few patients with CNS lesions have been intravenous drug users; most probably result from hematogenous spread, presumably from the lungs, but there is an occasional case in which it seems likely that spread from the sphenoid sinus occurred, with resulting basilar meningitis. The heart has been the next most commonly involved extrapulmonary organ; many pathological descriptions have shown multiple myocardial abscesses. Gastrointestinal aspergillosis has been described in one patient, with scattered ulcers extending from the stomach to the colon. Abscesses of the kidney, liver, spleen, and peripancreatic spaces have all been reported in the literature; most were diagnosed postmortem.

Invasive pulmonary aspergillosis in neonates

Only a few cases of invasive aspergillosis have been reported in neonates and infants <3 months of age. A

case report from Frankfurt, Germany reviews 43 cases of invasive aspergillosis during the period 1955–96 (Groll et al. 1998a). Eleven of the 44 patients had primary cutaneous aspergillosis, 10 had invasive pulmonary aspergillosis, and 14 had disseminated disease. Most infections were nosocomial in origin. Prematurity (43 percent), proven chronic granulomatous disease (14 percent), and a complex of diarrhea, dehydration, malnutrition, and invasive bacterial infection (23 percent) accounted for the majority of underlying conditions. At least 41 percent of the patients had received corticosteroid therapy before diagnosis, but only one patient had been neutropenic. Among patients who received medical and/or surgical treatment, outcome was relatively favorable, with an overall survival rate of 73 percent. From this review it is evident that invasive aspergillosis may occur in neonates and young infants. The current recommendation regarding treatment is high-dose amphotericin B and appropriate surgical interventions.

A North American experience of aspergillosis in children is illustrative (Abbasi et al. 1999). Sixty-six patients with culture-documented aspergillus infection were identified from a 34-year period. The most common underlying diagnosis was leukemia. Risk factors included neutropenia, immunosuppression, and prior antibiotic therapy. On the basis of clinical presentation, 23 patients were believed to have disseminated disease and 43 to have localized disease. The lung was the most frequently affected organ. Overall mortality was 85 percent within the first year of diagnosis. Patients who presented with disease in sites other than the lungs fared better than patients with initial pulmonary involvement. A further North American experience is reviewed by Wright et al. (2003).

EPIDEMIOLOGY OF ASPERGILLOSIS

Aspergillus spp. grow well on virtually any organic debris. They are, therefore, found naturally in leaves, grains, soil, and composts. Compost is a particularly rich source for the thermotolerant species. *A. fumigatus* is often the predominant fungal member of the compost microbiota, with more than 10^8 conidia per gram of compost material. Likewise, numbers of airborne conidia in such places as hay barns have been estimated to be as high as $10^9/m^3$. These numbers are exceptional, however, and the number of conidia in outdoor air probably rarely exceeds $200/m^3$. Interestingly, indoor air usually contains larger numbers of aspergillus conidia than outdoor air sampled over the same period. This has led hospital epidemiologists to advocate the use of high-efficiency filtration units to reduce to undetectable levels the number of airborne particles in the rooms of patients at risk. Various hospital routines may increase the level of contamination. Bursts of airborne conidia have, for example, been detected during cleaning procedures (Rhame et al. 1984). In general, however, with the exception of those individuals with occupational exposure, everyday exposure to conidia of *Aspergillus* spp. may be of little concern to most of the population. Nosocomial outbreaks of invasive aspergillosis are often attributed, correctly or incorrectly, to an environmental point source, such as a contaminated air vent or a demolition project (Walsh and Pizzo 1988; Walsh and Dixon 1989). The interrelationship between isolates of *A. fumigatus* from outside air with strains implicated in clusters of invasive disease has been analyzed by various molecular techniques (Loudon et al. 1993, 1994; Symoens et al. 1993; Girardin et al. 1994b; Goodley et al. 1994; Tang et al. 1994; Kennedy et al. 1995; Anderson et al. 1996).

HOST DEFENSE

Host defense to invasive aspergillosis is primarily an innate mechanism. By contrast, in the allergic forms of aspergillosis, the acquired responses appear to contribute more to the pathology than to the control of disease. In immunocompromised patients, various defects in neutrophil function have been recognized in, for example, patients with chronic granulomatous disease or patients with prolonged neutropenia induced by cytotoxic drugs. Elucidation of the host defense mechanisms in aspergillosis has been the focus of many studies (reviewed by Rhodes 1993, Kauffman and Tomee 2002).

Innate mechanisms

COLONIZATION OF THE RESPIRATORY MUCOSA

Initial interaction with host cells and colonization of mucosal surfaces are now considered crucial steps in the establishment of infection. Ligand-mediated adherence may be a component of this process. For example, fibrinogen, which plays a key role in inflammatory reactions and in the coagulation pathway, appears to be a good candidate for mediating adherence. Various studies have shown that, among filamentous fungi belonging to different groups (opportunistic fungi, strictly saprophytic or phytopathogenic fungi, and dermatophyte or related species), only the pathogenic aspergilli, particularly *A. fumigatus*, bind crude fibrinogen (reviewed by Bouchara et al. 1988, 1995, and Annaix et al. 1992). The binding was detected mainly at the surface of the conidia. Further studies demonstrated that the site of maximum fibrinogen binding was on the outer conidial surface but that binding sites also lay in the inner part of the wall and in cytoplasmic granules (Annaix et al. 1992). Other virulence determinants of *A. fumigatus*, relevant in vivo, may include proteases and elastase enzymes (Kolattukudy et al. 1993; Jaton-Ogay et al. 1994; Bouchara et al. 1995).

Aspergillus spp., unlike many other fungal species, can colonize the respiratory mucosa. This can occur in the normal airway, and the individual may become sensitized, causing ABPA (see section on Allergic aspergillosis below). More commonly, the fungus colonizes damaged airways, such as in patients with healed tuberculosis, bronchiectasis, and cystic fibrosis (see section on Allergic aspergillosis below). Although the type III hypersensitivity reaction to aspergillus antigens in the bronchial wall in ABPA and the importance of the neutrophil in host defense against *Aspergillus* spp. are well appreciated (see section on Immune response below), the mechanisms of colonization of the respiratory mucosa are poorly understood. Amitani and colleagues (1992) have shown that culture filtrates of most clinical isolates of *A. fumigatus* studied slow ciliary beat frequency and damage human respiratory epithelium in vitro. These changes appeared to occur concurrently. The characteristics of a factor purified from a culture filtrate of one clinical isolate of *A. fumigatus* has been described (Amitani et al. 1995). The ciliary-inhibitory activity was heat labile and associated with both high- and low-molecular-weight factors. As a result of purification of the low-molecular-weight activity, one such component was identified as gliotoxin, a known metabolite of *A. fumigatus*. It is suggested that *A. fumigatus* produces a number of biologically active substances which slow ciliary beating and damage epithelium and which may influence colonization of the airways.

INTERACTION WITH ALVEOLAR MACROPHAGES AND POLYMORPHONUCLEAR LEUKOCYTES

Numerous reviews of the acquisition and pathogenesis of invasive aspergillosis have identified common features which appear to be responsible for placing patients at risk for these infections (Holden et al. 1994). Neutropenia and broad-spectrum antibiotics and corticosteroids are consistently identified as key predisposing factors. Likewise, the recognition that patients with chronic granulomatous disease, in which phagocytic cells fail to produce the respiratory burst, are susceptible to invasive aspergillosis adds further weight to the concept that the presence of normally functioning phagocytes is the key factor in defense against invasive disease. It has been shown, in a murine model, that both neutrophil and monocyte function have to be ablated for invasive infection to develop. Bronchoalveolar macrophages are believed to serve as the first line of defense and to be responsible for the ingestion and intracellular killing of inhaled conidia (Henwick et al. 1993). Those conidia escaping the macrophages and going on to germinate and form hyphae are the target of attack by the granulocytes, which adhere to the hyphae, degranulate, and kill the fungus.

Our knowledge of the role that phagocytic cells play in the eradication of *A. fumigatus* has been advanced only recently. The published studies on the ability of phagocytic cells to kill conidia of *A. fumigatus* have produced divergent and conflicting results, largely as a result of the many different in vitro assays of phagocytosis and intracellular killing being used (Ibrahim-Granet et al. 2003).

One important aspect of host defense to *Aspergillus* in relation to phagocytosis in the alveoli may be the role of surfactant proteins. Surfactant protein A (SP-A) and surfactant protein D (SP-D) are thought to play important roles in pulmonary defense. These proteins are members of the C-type lectin superfamily, which also includes serum mannose-binding protein, conglutinin, and collectin 43. SP-A and SP-D are synthesized by type II cells and Clara cells in the lung and share many structural features. The host defense role played by SP-A and SP-D very much depends on the target organs and specific surfactant protein involved. Recent reports have shown that these proteins bind to and enhance killing and clearance of the organism in vitro (Madan et al. 1997; Allen et al. 1999). It is suggested that SP-D specifically plays a major role in the recognition of *Aspergillus* conidia in alveolar fluid (Allen et al. 1999).

ANTIPHAGOCYTIC ACTIVITY OF *ASPERGILLUS*-DERIVED PRODUCTS

It has been shown over the past 10 years that spores and spore diffusates of *A. fumigatus* suppress the production of superoxide anion and hydrogen peroxide by rodent phagocytic cells and that diffusates of *A. fumigatus* spores inhibit phagocytosis of antibody-coated radiolabeled sheep red blood cells by mouse peritoneal exudate cells. Similar experiments with human cells have shown that spore diffusates of *A. fumigatus* reduce the migration of human polymorphonuclear leukocytes (PMNL) and decrease the capacity of mouse peritoneal exudate cells to spread on glass. In addition, it has been shown that human pulmonary macrophages can bind and kill *A. fumigatus* conidia. More recently, Murayama and colleagues (1998) showed that *Aspergillus*-derived products had a suppressive effect on PMNL-mediated damage of *A. fumigatus* hyphae. The conclusion drawn from these studies is that *A. fumigatus* produces a variety of substances, some of which may suppress antifungal (anti-*A. fumigatus*) activity of human alveolar macrophages and PMNLs.

RECENT PROGRESS IN AN UNDERSTANDING OF HOST DEFENSE MECHANISMS AGAINST INVASIVE ASPERGILLOSIS

The fact that invasive aspergillosis disproportionately afflicts immunocompromised patients indicates the critical importance of the immune status of the host in this infection, but the defense mechanisms against this pathogen remain incompletely understood. Recent work suggests that the chemokine ligand monocyte chemotactic protein-1, also designated CC chemokine ligand-2 (MCP-1/CCL2), may be necessary for effective host defense against invasive aspergillosis (Morrison et al.

2003). It was found that there was a rapid and marked induction of MCP-1/CCL2 in the lungs of neutropenic mice with invasive aspergillosis. Neutralizing MCP-1/CCL2 resulted in twofold greater mortality and greater than threefold increase in pathogen burden in the lungs. Neutralization of MCP-1/CCL2 also resulted in reduced recruitment of natural killer (NK) cells to the lungs at early time points, but did not affect the number of other leukocyte effector cells in the lungs. Ab-mediated depletion of NK cells similarly resulted in impaired defenses against the infection, resulting in a greater than twofold increase in mortality and impaired clearance of the pathogen from the lungs. This study suggests that MCP-1/CCL2-mediated recruitment of NK cells to the lungs may be a critical early host defense mechanism in invasive aspergillosis and indicates that NK cells may be an important and previously unrecognized effector cell in this infection.

Another area of innate host defenses against *Aspergillus* are defensins. Defensins and other antimicrobial peptides act in the innate defense of epithelial surfaces. Human β-defensin 1 (hBD-1) has recently been shown to be expressed in airway epithelial cells and so has been implicated as a primary component of antibacterial activity in human lung. β-Defensins have been implicated in host defense of human airway epithelia. Antibacterial activity associated with low-molecular-weight, salt-sensitive, and heat-stable factors has been detected in the surface fluid of primary cultures from normal airway epithelia, and expression of hBD-1 messenger RNA has been detected in human lung. Hence, hBD-1 has been implicated as a major component of host defense in human airways. Furthermore, inactivation of hBD-1 in the lungs of cystic fibrosis patients may be an important cause for the onset of chronic lung infection. It is tempting to speculate that β-defensins have a role to play in the pathogenesis of pulmonary aspergillosis. Research in this area is continuing.

Modulation of innate mechanisms

There has been some concern expressed regarding the immunosuppressive effects of cyclosporin A on the function of peripheral blood phagocytes. Cyclosporin A has been reported to induce immunosuppressive effects on the fungicidal activity of murine polymorphonuclear monocytes against blastoconidia of *Candida albicans* in vivo and has rendered the animals susceptible to subsequent challenge with this organism (Vecchiarelli et al. 1989; Roildes et al. 1993b, c). Roildes et al. (1994a, b) addressed this issue by looking at the effects of cyclosporin A on phagocytic defenses against *A. fumigatus* in vitro and ex vivo. Cyclosporin A at therapeutically relevant concentrations did not suppress antifungal activity of phagocytes except that of circulating monocytes. However, cyclosporin A did appear to induce significant immunosuppression of phagocytes' antifungal function at relatively high concentrations in vitro, especially when combined with corticosteroids. This observation has potential implications for patients receiving these immunosuppressive agents.

Treatment with recombinant human interferon-γ is known to augment the ability of monocytes and neutrophils from patients with chronic granulomatous disease (CGD) to generate superoxide. This observation prompted Rex et al. (1990) to look at the effect of interferon-γ on neutrophils, from patients on interferon-γ therapy for CGD, in their ability to damage aspergillus hyphae. Neutrophils from patients receiving interferon-γ produced significantly more damage to hyphal elements than those from the placebo group. The authors conclude that interferon-γ therapy improves the ability of CGD neutrophils to damage *A. fumigatus* hyphae in an in vitro assay.

Immune response

In colonizing and in invasive forms of aspergillosis, the study of the immune response has centered on the humoral response as an aid to determining the diagnosis or prognosis of patients. In ABPA, studies have attempted to explain the pathology of the disease, which appears to be caused primarily by that very response. In all studies on the humoral response to aspergillus antigens, progress has been hampered by the lack of common, reference antigens which are capable of detecting responses to all common species and by which results obtained in multiple centers may be compared.

HUMORAL RESPONSE IN ALLERGIC ASPERGILLOSIS

Patients with extrinsic allergic alveolitis or hypersensitivity pneumonitis develop IgG precipitin antibodies. The pulmonary pathology appears to be mediated by immune complex deposition, perhaps in conjunction with cell-mediated mechanisms. By contrast, IgE appears to play a major role in the pathophysiology of ABPA. The patients have elevated total IgE, which rises and falls with exacerbations and remission of clinical symptoms. Specific anti-aspergillus IgE is also demonstrable in ABPA patients and IgG anti-aspergillus precipitin antibodies are also produced by ABPA patients, with almost 100 percent having positive serological tests for these antibodies (Brummund et al. 1987). Pulmonary immune complex deposition, secondary to elevated IgG antibodies, may account for much of the inflammatory component seen during active phases of ABPA.

Significantly elevated levels of specific *A. fumigatus* IgG have been also been detected in cystic fibrosis patients with ABPA (Murali et al. 1994).

HUMORAL RESPONSE IN ASPERGILLOMA

Patients with aspergilloma, almost without exception, mount a strong IgG response to aspergillus antigens,

judging by the multiple precipitin bands formed on agar gel double diffusion or counterimmunoelectrophoresis to extracts of the infecting fungus. The response does not appear to provide any protection to the patient, however. In cases of aspergilloma in which the fungus ball has formed secondary to cavitation of an invasive lesion, the ability of patients to produce antibodies is governed more by their degree of immunosuppression than by the type of disease.

HUMORAL RESPONSE IN INVASIVE ASPERGILLOSIS

As a result of their profound degrees of immunosuppression, patients with invasive aspergillosis mount little to no humoral response to the fungus when the standard serological tests are used. However, the use of purified antigens in immunoblotting procedures appears to detect antibody in immunocompromised individuals (Hearn et al. 1995). Antibody responses in these patients do not appear to confer any degree of protection in invasive aspergillosis.

CELLULAR RESPONSE IN ALLERGIC ASPERGILLOSIS

The presence of bronchocentric granulomatosis as a pathological hallmark of ABPA indicates that there must be a cell-mediated component in the response of these patients to *Aspergillus* spp. Lymphocyte proliferation in response to antigens of *A. fumigatus* has been demonstrated in some ABPA patients. Likewise, fractionation of antigenic extracts of *A. fumigatus* yielded components that elicited positive-type (type IV) skin reactivity in, and proliferation of lymphocytes from, sensitized guinea pigs.

CELLULAR RESPONSE IN ASPERGILLOMA

There is little evidence to support the presence of a cell-mediated response in aspergilloma patients. In general, the pathology of aspergillomas is one in which an inflammatory response, either acute or chronic, is minimal. Host defense against this form of infection has not been well studied.

CELLULAR RESPONSE IN INVASIVE ASPERGILLOSIS

The fungicidal activity of neutrophils against hyphae involves the release of granular contents on the surface of the organism. Both macrophages and neutrophils use oxidative and non-oxidative fungicidal mechanisms including myeloperoxidase and myeloperoxidase-independent oxidants. The granular constituents of neutrophils have all been implicated as antifungal agents and include: lactoferrin, lysozyme, cathepsin G, azarocidin, and defensins. Interferon-γ and granulocyte-macrophage colony-stimulating factors (GM-CSF) both augment oxidant release and fungicidal activity. Specific antibodies or T lymphocytes do not appear to be of major importance in host protection against *Aspergillus* spp. Complement facilitates neutrophil damage to hyphae and monocyte killing of conidia.

The role of T cells in invasive disease has been investigated in experimental systems, with little consensus as to its importance. For example, nu/nu mice fare no worse than their nu/+ littermates when infected with conidia of *A. fumigatus*. Nu/+ can be immunized protectively against the fungus, however, and the protective immunity can be transferred to nu/nu mice recipients with adherent peritoneal cells from the immunized nu/+ mice. This suggests a role for T cells and macrophages in the acquisition of immunity to aspergillosis, but the issue is still open to investigation. In humans, reviews of cases of invasive aspergillosis have not suggested that T-cell defects were primary predisposing factors for disease but rather that granulocytopenia is the risk factor most commonly associated with infection. Additional recent evidence on the incidence of invasive aspergillosis in patients with AIDS also suggests that, even in the face of profound loss of CD4+ T cells, this fungal infection rarely occurs (see above), except when patients become neutropenic, secondary to azidothymidine (AZT) therapy.

Immune regulation in aspergillosis

Regulation of the immune response to aspergillosis has been little studied. Of interest are the descriptions of metabolic products of the fungus that are immunomodulatory in vitro. One of the best characterized of these is gliotoxin, produced in vitro by *A. fumigatus*. Gliotoxin is a potent inhibitor of macrophage phagocytosis and also appears to inhibit the ability of spleen cells to induce major histocompatibility complex (MHC)-restricted cytotoxic cells. *A. fumigatus* also produces phospholipid compounds that are active in inhibiting the activation of complement via the alternative pathway. These factors have the potential to influence both the innate and immune responses that provide adequate host defense to *Aspergillus* spp. in the normal host. It is not known whether gliotoxin or the complement inhibitor is, however, produced in biologically significant quantities in vivo.

DIAGNOSIS

Establishing the diagnosis of aspergillosis in a compromised patient is difficult because the clinical presentation is nonspecific and the fungus is seldom isolated from blood or other body fluids, or from sputum. Interpretation of serological test results is also difficult because failure to detect precipitins in a compromised individual does not mean that aspergillosis is not present (Saugier-Veber et al. 1993). Nor is the detection of circulating antigen a consistent finding in such patients (Jones and McLintock 2003; McLintock and Jones 2004).

The problems associated with the diagnosis of invasive aspergillosis are highlighted by the diagnostic criteria that have to be fulfilled before patients are eligible for inclusion in clinical trials of new antifungal agents. Very often these criteria are either presumptive or definitive depending on whether tissue diagnosis has been achieved. The following patient selection criteria are required by the European Organization for Research in the Treatment of Cancer (EORTC):

- persistence of fever: ⩾38°C for 3–5 days despite broad-spectrum antibiotics, unless biopsy-proven aspergillosis is documented before day 5 of fever
- current negative blood cultures for bacterial pathogens
- no presumptive diagnosis of infections caused by viruses, acid-fast bacilli, legionellae, Q fever, psittacosis, mycoplasmas, chlamydia pneumonia, and *Pneumocystis* sp.
- appropriate physical symptoms and signs suggestive of invasive aspergillosis such as:
  pulmonary: cough, dyspnea, hemoptysis, crepitation, hypoxia, chest pain
  sinus: headache, nasal discharge, facial swelling, tenderness, cellulitis
  liver: may be asymptomatic, discomfort in the right upper quadrant, jaundice, hepatomegaly, abnormal liver function tests
  CNS: headache, focal fits, focal neurological deficit, confusion, altered conscious level, abnormal CSF findings
  other sites: these include bone, kidney, skin, pleura, eyes, spine, external ear, endocarditis, pericardium, joints, adrenal, peritoneal, gastrointestinal tract, lymph node, and thyroid, as appropriate
- appropriate radiological investigations:
  pulmonary: pulmonary infiltrates either nonspecific or suggestive (nodules, cavities)
  sinus: mucosal thickening, opacification/clouded sinus spaces, fluid levels, bone destruction
  liver: nodules/abscesses demonstrated by computed tomography, ultrasonography, or MRI
  spleen: nodules/abscesses demonstrated by computed tomography, ultrasonography, or MRI
  CNS: nodules/abscesses demonstrated by computed tomography, ultrasonography, or MRI
  other sites: as appropriate
- positive identification of *Aspergillus* spp. either by histology or by culture from an appropriate site (i.e. involved organ):
  pulmonary: bronchial washing/bronchoalveolar lavage/tracheal aspirate/anterior nares/transbronchial biopsy/radiologically guided fine-needle aspiration (FNA)/open lung biopsy
  sinus: nasal eschars/sinus aspirate/sinus biopsy
  liver: FNA/laparotomy/laparoscopic guided biopsy
  spleen: FNA/laparotomy/laparoscopic guided biopsy
  CNS: FNA/stereotactic biopsy, CSF
  other sites: appropriate sampling.

A positive blood culture (highly unlikely in aspergillosis) in the presence of radiological and clinical features can be used as an alternative mycological confirmation.

Entry into clinical trials often depends on direct tissue evidence but obviously some patients will be too ill or considered at high risk (thrombocytopenia) for invasive procedures, e.g. open lung biopsy.

Definitively diagnosed invasive aspergillosis requires all of the above criteria to be satisfied and *Aspergillus* spp. should be isolated from the organ/site involved.

Microscopy

Microscopic examination of sputum preparations is often helpful in the diagnosis of allergic aspergillosis, because abundant septate mycelia with characteristic dichotomous branching are usually seen.

Microscopic examination of sputum is seldom helpful in patients with suspected invasive aspergillosis, but examination of BAL specimens is often rewarding (Delvenne et al. 1993) (Figure 11). Typical mycelium may also be detected in wet preparations of necrotic material from cutaneous lesions or sinus washings, but isolation of the etiologic agent in culture is essential to confirm the diagnosis.

The most reliable method for the diagnosis of acute invasive aspergillosis is the examination of stained tissue sections. The detection of nonpigmented, septate filaments which show repeated dichotomous branching is characteristic of aspergillus infection (Figures 12, 34.13 and 34.14). However, other less common organisms, such as *Fusarium* spp. and *Scedosporium apiospermum*, appear similar. More precise identification can sometimes be achieved with immunochemical staining methods. One of the main limitations in using this approach has been crossreactivity between species, and even monoclonal antibodies may suffer the same problems. In one report it has been shown that murine monoclonal antibodies specifically recognize cytoplasmic antigens of *A. fumigatus*, *A. flavus*, and *A. niger*

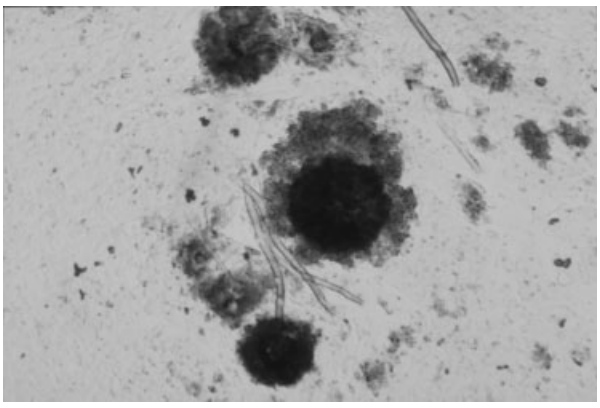

Figure 11 *Direct microscopy of sputum from a patient with allergic bronchopulmonary aspergillosis caused by* A. niger

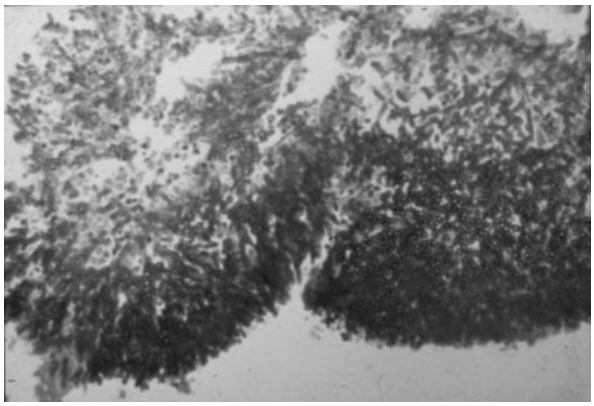

Figure 12 *Histopathological appearance of an aspergilloma*

(Fenelon et al. 1999). These antibodies were used to identify *Aspergillus* spp. in frozen sections of tissue from cases of invasive aspergillosis by immunofluorescence, and in paraffin-embedded clinical specimens by immunofluorescence and immunoperoxidase staining.

Optical brighteners, such as Calcofluor white, which label glucan and chitin in the fungal cell wall, have been used to highlight fungal elements in a variety of clinical specimens for the past 20 years. Further refinements to this technique for the recognition of aspergillus hyphae in respiratory secretions have been reported by Ruchel and Schaffrinski (1999).

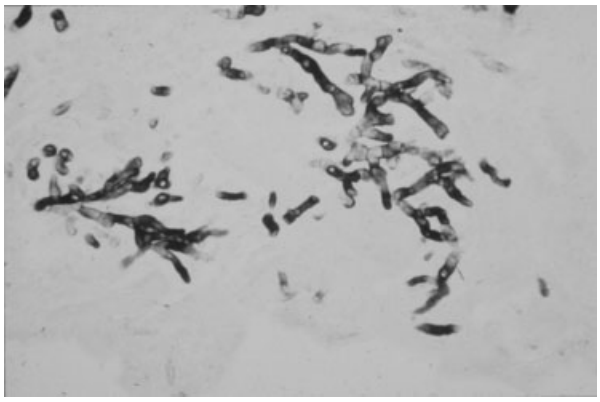

Figure 13 *Histopathological appearance of an* A. fumigatus *infection of the paranasal sinuses*

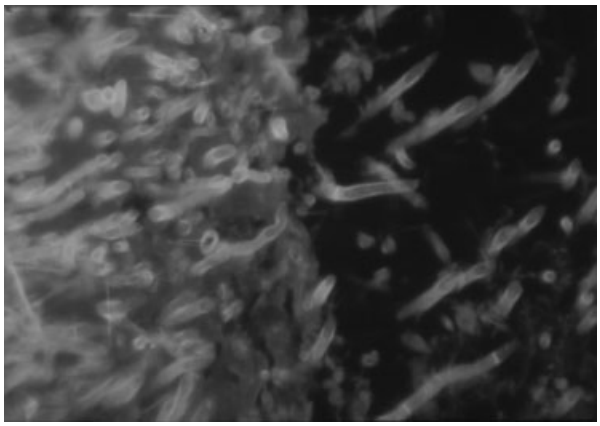

Figure 14 *Postmortem tissue from a case of upper airway aspergillosis stained with Calcofluor white. This technique provides a very rapid staining method for biopsy specimens.*

Culture

The definitive diagnosis of aspergillosis depends upon the isolation of the etiologic agent in culture. The fungus may be recovered from sputum specimens from patients with allergic aspergillosis, but cultures from patients with other forms of aspergillosis are less successful. Moreover, because *Aspergillus* spp. are commonly found in the air, their isolation must be interpreted with caution (Nalesnik et al. 1980). Their isolation from sputum is more convincing if multiple colonies are obtained on a plate, or the same fungus is recovered on more than one occasion. Often the number of colonies isolated is small which may result from the filamentous character of the organism. Positive culture may also be a sign of transient exposure to inhaled spores. If sputum cannot be obtained from an immunocompromised patient with a lung infiltrate, alveolar lavage specimens should be obtained. Isolation of an *Aspergillus* sp. from such specimens is often indicative of infection, but is positive in less than 60 percent of cases. *Aspergillus* spp. may be recovered from sputum or bronchial lavage specimens, especially in patients with diffuse pulmonary infiltrates, whereas recovery of fungus from patients with focal lesions is more difficult. A positive culture is indicative of infection but may also merely represent colonization (Delvenne et al. 1993). However, the isolation of *A. fumigatus* from respiratory tract specimens in heart transplant recipients is highly predictive of invasive aspergillosis (Munoz et al. 2003). It has been estimated that around 40 percent of neutropenic patients from whom *Aspergillus* spp. are isolated do not have invasive disease (McWhinney et al. 1993).

Aspergillus spp. are seldom recovered from blood, urine, or CSF specimens, although cultures of blood have been positive in occasional patients with endocarditis. More often, however, their isolation is the result of contamination. It has not been established whether lysis-centrifugation is any more useful than traditional blood culture methods in the diagnosis of aspergillosis.

The diagnosis of aspergillus sinusitis is less difficult to establish than infection of other sites. The fungus can usually be isolated from sinus washings or biopsies of the necrotic lesions in the nose or palate.

Skin tests

Skin tests with *A. fumigatus* antigen are useful in the diagnosis of allergic aspergillosis. Patients with uncomplicated/extrinsic asthma caused by *Aspergillus* spp. give

an immediate type I reaction. Those with allergic bronchopulmonary aspergillosis give an immediate type I reaction and many also give a delayed type IV reaction.

Serological tests

Many potential systems for the immunodiagnosis of aspergillosis have been described (Kurup and Kumar 1991; Barnes 1993; Kappe and Seeliger 1993). Those based on detection of antibody to the organism have been very successful in allergic aspergillosis and aspergilloma, and those used for the detection of fungal antigen have great potential for the diagnosis of invasive aspergillosis (Manso et al. 1994). To understand the potentials and limitations of serology in aspergillosis, it is necessary to review the extensive literature on the antigens of *Aspergillus* spp. and how this knowledge has been applied to the design of immunoassays for improved diagnosis.

ANTIGENS OF *ASPERGILLUS* SPP.

A major problem that has prevented the development of standardized serological tests is the vast heterogeneity of antigen types and the lack of understanding of their relevance in vivo. Most of the antigens identified in crude extracts from *A. fumigatus* grown in vitro lack specificity (Hearn 1992; Latgé et al. 1993). For example, Western blot experiments have demonstrated that circulating antibodies from sera of either aspergilloma patients or non-infected individuals bind to most antigens secreted in glucose–peptone culture medium (Latgé et al. 1993). In this study, the levels of anti-*A. fumigatus* antibodies were 10–100 times higher in aspergilloma patients than in control individuals. Furthermore, antigens of *A. fumigatus* crossreact with other fungi (Kumar and Kurup 1993).

The presence of detectable levels of anti-*Aspergillus* spp. antibodies in every individual is thought to result from the continuous inhalation of conidia from the atmosphere. However, normal individuals inhale only conidia whereas the development of aspergillosis results from mycelial growth in lung tissue. Although conidial and mycelial antigens are very similar, growth-phase-specific antigens have been demonstrated in *Aspergillus* spp. (Kauffman et al. 1984). In addition, expression of different mycelial antigens in vitro is highly dependent on the nutrient composition of the culture medium (Moutaouakil et al. 1993). These findings suggest the existence of antigens, released during mycelial growth in vivo, which can only be recognized by antibodies in patients infected with *Aspergillus* spp. To identify such antigens, Sarfati et al. (1995) probed immunoblots of extracts from kidneys of mice infected intravenously with *A. fumigatus* with sera from patients with aspergilloma. A limited number of antigens were detected with molecular masses of 31, 36, 56, 84, 88, and 200 kDa. A 88 kDa antigen secreted by *A. fumigatus* has been described by Kobayashi et al. (1993). Antigens of this type have been characterized as belonging to two classes:

1. galactomannan- and/or galactofuran-containing glycoproteins
2. antigenic proteins without the galactofuran epitope.

These studies have confirmed that antigens bearing a galactofuranosyl epitope are present both in vivo and in vitro. An exhaustive review of *Aspergillus* antigens can be found in Latgé (1999).

TESTS FOR ANTIBODIES TO *ASPERGILLUS* SPP.

Detection of specific precipitating antibodies to *Aspergillus* spp. by double diffusion, counterimmunoelectrophoresis or enzyme-linked immunosorbent assay (ELISA) has provided the basis for the most frequently used serological tests for the diagnosis of aspergillosis (Hopwood and Evans 1991; Barnes 1993). Some of the tests are available commercially from a number of sources and are technically simple to perform and to interpret. The presence of one or more weak precipitin bands is one of the diagnostic criteria accepted for the diagnosis of ABPA. Published studies would suggest that 70–100 percent of patients with ABPA are positive for IgG-precipitating antibodies directed against *Aspergillus* spp., depending on the antigen used and whether or not serum was concentrated before testing. Furthermore, specific IgE is a reliable indicator of ABPA in cystic fibrosis (Marchant et al. 1994). In addition, rates of positivity in patients with extrinsic allergic alveolitis are also very high. Precipitins are present in 98–99 percent of aspergilloma patients, and multiple bands, often three or more, are routinely present. In patients with invasive aspergillosis, however, the experience with detecting precipitating antibodies has been disappointing. Immunoblotting is a sensitive technique that has been used to detect antibodies in sera from patients with invasive aspergillosis and patients with ABPA. Although the correlation between the sizes of the recognized antigens has not been exact, it has great research potential for determining which antigens may be the most appropriate for future testing. This information, in conjunction with ELISA data, may allow wider availability of a more sensitive and specific test for aspergillosis.

More recently, a number of highly sensitive methods for the detection of very low levels of antibodies in serum samples have been described. It has been reported that all serum specimens from patients with invasive aspergillosis contain antibodies to a 58 kDa concanavalin A-binding antigen identified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. However, the specificity of this antigen has not been well documented (Fratamico and Buckley 1991). Both antigen and antibody levels have been monitored successfully in a number of cases

of invasive aspergillosis (Manso et al. 1994; Hearn et al. 1995). With these highly sensitive methods for antibody detection, a problem of specificity arises because IgG antibodies to aspergillus antigens can be detected in a proportion of healthy individuals.

Immunoblotting in conjunction with SDS-PAGE has been used to detect serological responses to aspergillus antigens. Piechura and coworkers (1985) have used two-dimensional electrophoresis and isoelectrofocusing to analyze complex mixtures of aspergillus antigens. Hearn et al. (1995) have extended these studies by using immunoblotting to detect specific IgG antibodies to aspergillus antigens in serum specimens from patients with different types of aspergillosis. Different *A. fumigatus* extracts, including culture filtrates, surface components, and mycelial fractions, were tested. All these preparations were shown to be highly reactive antigenically when tested previously by precipitin and ELISA procedures. In particular, patients with invasive aspergillosis were capable of mounting a response to culture filtrates, surface washes, and mycelial extracts of *A. fumigatus* (a total of 12 antigenic fractions); 11 were reactive with serum specimens from patients with aspergilloma. Eight of these antigens showed good responses with serum specimens from patients with ABPA, which were used to assess the sensitivity of IgG detection. No measurable activity was detected in 18 negative control serum specimens, whereas 11 of 13 patients with proven, highly probable, or probable cases of invasive aspergillosis had anti-*Aspergillus* spp. IgG to multiple antigenic preparations. Patients with invasive aspergillosis who were capable of mounting a substantial humoral response to aspergillus antigens gave an antibody profile with five antigenic preparations, which seemed to be characteristic of the disease. The authors concluded that this approach was highly sensitive and may allow the selection of fractions that are both highly antigenic and specific for the detection of antibody to aspergillus antigens. This study also indicates that the use of a spectrum of antigenic molecules is advisable, given the variability observed in the immune responses of individual patients.

DETECTION OF ASPERGILLUS ANTIGEN

Antibody production in the immunocompromised host with invasive aspergillosis is invariably difficult to detect. Therefore, methods have been sought for diagnosing this infection which would rely on the measurement of a cell component of fungal origin and thereby be independent of the host's ability to respond (Richardson 1987; Barnes 1993). Aspergillus galactomannan circulates during infection and appears in the urine, presumably after clearance by a receptor-mediated process by Kupffer's cells in the liver. In addition to galactomannan, at least seven other *Aspergillus*-related antigens have been detected, by immunoblotting, in the urine of patients with invasive disease (Ansborg et al. 1994).

Various immunoassay formats have been designed to detect antigen, either free or in immune complexes in serum, bronchoalveolar fluid, or urine. In two of the largest studies on patient samples, a radioimmunoassay for antigen detection showed 74 percent sensitivity and 90 percent specificity, and either of two ELISA procedures used to measure antigenemia or antigenuria, with a rat monoclonal antibody (EB-42) specific for galactomannan, showed greater than 95 percent sensitivity and specificity. The value of granulocyte-macrophage (GM) detection by the Pastorex *Aspergillus* latex agglutination (LA) test (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) has been evaluated by a number of groups (Haynes and Rogers 1994; Hopwood et al. 1995; Verweij et al. 1995a, b, c) and the test showed sensitivities of up to 95 percent with serum samples from patients with a high index of suspicion for invasive aspergillosis. The latex test was also found to yield positive results earlier than conventional microbiological procedures for 68 percent of patients with proven invasive aspergillosis. However, these observations have not been confirmed by others (Warnock et al. 1991), and a sensitivity as low as 38 percent has been reported (Warnock et al. 1991; Hopwood et al. 1995). In the Verweij et al. (1995b) study, the LA test yielded positive results only during advanced stages of infection in most patients with suspected invasive aspergillosis and did not contribute to an early diagnosis. This study showed that an ELISA using a monoclonal antibody to GM detected GM in serum up to 5 days earlier than the LA test did. Although both LA and ELISA failed to detect one proven infection, the ELISA detected GM in two additional patients for whom the LA test continued to yield negative results. Moreover, GM was detected in more serum samples by ELISA than by LA. This suggests that monitoring sequential serum samples from high-risk patients during neutropenia may allow the diagnosis of invasive aspergillosis to be made at an earlier stage of infection. Furthermore, GM has been detected by ELISA in BAL fluid, but the antigen may appear sooner in serum (Verweij et al. 1995a). Galactofuran detection also has been described and may be an additional marker of invasive aspergillosis (Stynen et al. 1995).

One problem long associated with the increased sensitivity of immunoassays such as ELISA or LA has been the occurrence of false-positive results (Kurup and Kumar 1991; Warnock et al. 1991; Kappe and Schulze-Berg 1993). Therefore, to identify a genuine elevation of antigen level in serum, positive ELISA results should be found for at least two consecutive serum samples.

The studies by Verweij and colleagues (1995a, b, c) are encouraging and form the basis for a number of recommendations for the immunodiagnosis of invasive aspergillosis:

- antigen detection in serum at regular intervals by the double sandwich ELISA may allow the early diag-

nosis of invasive aspergillosis in immunocompromised patients
- twice-weekly collection and testing of serum samples from a patient during periods of neutropenia should be sufficient to detect an increase in the GM in serum early in the course of infection
- where there is a positive ELISA result, serum samples should be tested in order to exclude the possibility of a false-positive result
- confirmation of suspected invasive aspergillosis should be obtained by chest x-ray, computed tomography, or BAL.

Although antigen detection for the early diagnosis of invasive aspergillosis holds great promise for the future, determination of its full diagnostic potential awaits widespread availability and evaluation. In addition, greater effort has to be made in the search for immunodominant antigens.

Another approach to the diagnosis of aspergillosis that is independent of a host immunological response is the detection of fungal metabolites in the body fluids of patients. The presence of high levels of D-mannitol in the serum and tissues of experimentally infected animals has been shown to correlate with the presence and extent of invasive aspergillosis. Likewise, the presence of oxalic acid in BAL fluid has been proposed as a presumptive marker for aspergillosis.

Further information regarding the Platelia *Aspergillus* sandwich ELISA has been reported. Galactomannan has been detected by this test in BAL fluid specimens from patients with invasive aspergillosis and an excellent correlation has been found between serum and BAL fluid results (Verweij et al. 1995a). The detection of galactomannan in both BAL fluid and serum from a patient suspected of having invasive aspergillosis seems to provide strong evidence for the presence of this disease. The concentration of galactomannan in serum has been shown to correspond to the tissue burden of *Aspergillus* (Stynen et al. 1995), and there is some evidence that the course of the antigen titer is closely related to clinical outcome (Rohrlich et al. 1996; Verweij et al. 1997). Indeed, the amount of circulating galactomannan decreased in six of ten pediatric patients during antifungal treatment (Rohrlich et al. 1996) and in patients with invasive aspergillosis following bone marrow transplantation who responded to antifungal therapy (Patterson et al. 1995; Verweij et al. 1997).

Until now the sandwich-ELISA has been evaluated predominantly with serum samples from patients receiving treatment for hematological malignancies. The presence of a well-defined period of increased risk for invasive aspergillosis means that the sandwich ELISA can be used to screen patients at the time of risk of acquiring this infection. This approach may allow patients with invasive aspergillosis to be identified promptly and to benefit from early antifungal treatment.

The combination of antigen testing by ELISA and early CT scanning has been shown to improve diagnosis and outcome in immunocompromised patients (Caillot et al. 1997). Twenty-three histologically proven and 14 highly probable cases of invasive aspergillosis in 37 hematology patients were analyzed retrospectively. BAL fluid was positive in 22 (69 percent) of 32 cases. When BAL fluid was tested for antigen, the *Aspergillus* ELISA test was positive in 83 percent of cases. Starting from October 1991, early thoracic CT scans were routinely performed in febrile neutropenic patients with pulmonary infiltrates on chest radiograph. This approach allowed the investigators to recognize suggestive CT halo signs in 93 percent of patients, compared with 13 percent before this date. The mean time to a diagnosis of invasive pulmonary aspergillosis was reduced from 7 to 1.9 days.

The ELISA for detection of galactomannan becomes positive at an early stage of infection. Early detection is probably the most important feature of this assay, because the detection of antigenemia dictates the initiation of therapy. Recent studies, reviews, and recommendations highlight the usefulness of the galactomannan assay (Maertens et al. 1999, 2002; Denning 2000; Dupont et al. 2000). However, a number of important issues have to be addressed, for example false-negative reactivity, influence of host factors, and the degree of angioinvasion. Furthermore, some reports disagree with the consensus view (reviewed by Jones and McLintock 2003).

Other ELISA formats for the detection of *Aspergillus* antigen have been designed. Chumpitazi and colleagues developed an inhibition enzyme immunoassay (inhibition-EIA) to monitor for the occurrence of invasive aspergillosis in sera from 45 immunocompromised patients (Chumpitazi et al. 2000). The test used rabbit polyclonal antibodies and a mixture of components from *A. fumigatus*, containing three predominant antigens with molecular masses of 18 000, 33 000, and 56 000 Da. Circulating antigens were found in five of seven proven cases of invasive aspergillosis due to *A. fumigatus*. In two of the five positive cases, antigenemia was detected with inhibition-EIA earlier than with x-ray or other biological methods. No antigens were detected in the sera from two patients with proven invasive aspergillosis due to *A. flavus* and *A. terreus*, nor in the sera from four patients with probable invasive aspergillosis. Circulating antigens were not detected in the control group, composed of 30 healthy adult blood donors. Four of the 32 at-risk patients examined, although they displayed no definite evidence of invasive aspergillosis, gave a positive result in this test. The sensitivity, specificity, and positive predictive value of inhibition-EIA were 71.4, 94.4, and 71.2 percent, respectively. The data were compared with those obtained by an LA assay of galac-

tomannan that was positive in only one patient with probable invasive aspergillosis. The higher sensitivity obtained by inhibition-EIA may well be as a result of its ability to detect circulating antigens other than galactomannan in the sera of immunocompromised patients with invasive aspergillosis. It would be useful for the inhibition ELISA to be compared with the Platelia ELISA for galactomannan.

DETECTION OF *ASPERGILLUS* DNA BY POLYMERASE CHAIN REACTION (PCR)

The limitations of antibody detection and the problems of sensitivity associated with antigen detection have prompted the evaluation of the PCR for the diagnosis of invasive aspergillosis (Alexander 2002; Kawazu et al. 2003). The main advantages of PCR appear to be that it detects low burdens of fungal genetic material and warns of the presence of possible invasive aspergillosis. A number of PCR techniques have been developed to detect either individual species or general primer-mediated methods to detect filamentous fungi in general (Melchers et al. 1994; Montone and Litzky 1995). Various clinical specimens have been analyzed by these methods, including sputum, whole blood, and BAL fluid. Early reports described methods to amplify the gene for *A. fumigatus* 18 kDa ribonucleotoxin (Reddy et al. 1993). Subsequently, a PCR, based on universally conserved sequences within fungal large rDNA, including that of *A. fumigatus*, was described (Haynes et al. 1995). Primers to sequences of large subunit rDNA genes, which are universally conserved within the fungal kingdom, were capable of amplifying DNA from 43 strains representing 20 species (12 strains) of medically important fungi (Haynes et al. 1995). Sequence analysis of the products from *A. fumigatus* allowed the design of specific primers which only amplified homologous DNA. This approach allowed the detection and identification of *A. fumigatus* within 8 hours from simulated specimens.

The sensitivity of PCR is an important issue. The lower limit of detection reported for *A. fumigatus* corresponds to 10–100 c.f.u. per sample (Reddy et al. 1993; Spreadbury et al. 1993; Tang et al. 1993). Of equal concern is the question of false positives. In a competitive PCR assay applied to BAL samples, this problem was highlighted (Bretagne et al. 1995). Here a competitive, internal control was incorporated into a PCR designed for the detection of *Aspergillus* sp. DNA in BAL samples. For this purpose, a 1 kb mitochondrial DNA fragment of *A. fumigatus* was sequenced. The primers used allowed amplification of *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger* DNAs, but not DNA of other fungi and yeasts. BAL samples from 55 consecutively enrolled patients were tested. Of 28 immunocompromised patients, six were PCR positive, three died of invasive pulmonary aspergillosis and their BAL cultures yielded *A. fumigatus*, and three were culture negative and did not develop invasive pulmonary aspergillosis. Of 15 HIV-positive patients and nine immunocompetent patients, five and four, respectively, were both PCR positive and culture negative, and none developed aspergillosis. Therefore, in this evaluation, PCR confirmed invasive pulmonary aspergillosis in three patients but gave positive results for 25 percent (12 of 49) of the patients who did not have evidence of aspergillosis. The authors conclude that the predictive value of PCR-positive results seems low for patients at risk for aspergillosis and that the risk of contamination of reaction buffers or biological samples with aspergillus conidia was high and was a major consideration if the potential diagnostic benefit of PCR was going to be realized.

There have been a number of reports on the use of PCR for the detection and identification of *Aspergillus* spp. Einsele and colleagues reported an oligonucleotide primer pair consisting of a consensus sequence for a variety of fungal pathogens, whilst the species-specific probes used for species identification were derived from a comparison of the sequences of the 18S rRNA genes of *Aspergillus* and *Candida* spp. (Einsele et al. 1997).

Verweij and coworkers sought to compare the sensitivity and utility of the double-sandwich ELISA for *Aspergillus* galactomannan with that of a PCR-based system in BAL fluid samples from 19 patients who were treated for hematological malignancies and suspected of having invasive pulmonary aspergillosis (Verweij et al. 1995a). ELISA was also performed with serum samples. All patients had fever and pulmonary infiltrates on the chest radiograph on the day that the BAL fluid was obtained. *Aspergillus* spp. were detected by PCR or ELISA in five of seven patients who had radiological evidence of invasive aspergillosis.

While most PCR systems for detection of aspergillosis use whole blood or BAL as a source, the possibility of using serum was investigated by Yamakami et al. (1996). Employing a nested PCR method, these investigators worked with two sets of oligonucleotide primers derived from the sequence of the variable regions V7–V9 of the 18S rRNA genes of *A. fumigatus*. The DNA fragment of interest was detected in the serum of mice with disseminated aspergillosis and in patients with invasive aspergillosis.

Walsh and colleagues reported the use of single-strand conformational polymorphism (SSCP) as a technique for detecting and delineating differences between fungal species and genera (Walsh et al. 1995). These investigators used a 197 bp fragment amplified from the 18S rRNA gene, which is common to all medically important fungi. After amplification, the fragments were denatured and run on an acrylamide-glycerol gel at room temperature or 4°C for 4.5 or 4 hours. The SSCP patterns of major and minor bands at room temperature permitted a distinction to be made between strains of *A. fumigatus* and *A. flavus*. The SSCP banding pattern among different strains of the same *Aspergillus* sp. was also consistent.

A variety of PCR protocols for human samples have been published, including PCR assays, methods detecting one species, members of a fungal family, or several species. As the incidence of fungal species in various infections is relatively low and identification of the infecting agent to the species level is required to guide appropriate treatment, the only efficient and economic approach may be a single protocol that is able to detect and identify many species. However, the protocols detecting several species use labor-intensive blotting protocols and sequential hybridizations with various radiolabeled probes for differentiation of species, which make these approaches impractical for routine laboratory use. In a recent study, a PCR method is described that is based on the sequence variation of the fungal ITS region (Hendolin et al. 2000). The method includes multiplex liquid hydridization with species-specific probes, and it employs nonradioactive and automated PCR product detection on a fluorescent automated DNA sequencer. Nonhybridized products are easily recognized on the sequencer and they can be used for identification by sequencing. The method has been applied to the analysis of tissue samples from a range of clinical specimens from deep sites. Total DNA was extracted from deep-tissue samples from 13 patients with suspected or proven fungal infection, and from nasal polyp tissue from seven patients with CRS. Broad-range primers were used to amplify the fungal ITS region (ITS 1-5.8S rRNA-ITS 2). Eight common fungal pathogens were identified by multiplex liquid hybridization with species-specific probes. The nonhybridized products were identified by sequencing either directly, or after segregation by cloning to allow assessment of mixed fungal populations. Twenty (95 percent) of 21 tissue specimens yielded PCR products, whereas culture resulted in only eight (38 percent) positive specimens. All culture or direct-microscopy positive specimens were PCR positive. Sequencing the nonhybridized PCR products identified an infecting agent in seven specimens, and sequences of unknown fungal origin were detected in three specimens. *Phoma glomerata* was detected by PCR in two CRS patients but culture was negative.

Another development has been the report of a two-step PCR assay that specifically amplifies a region of the 18S rRNA gene that is highly conserved in *Aspergillus* spp. (Skladny et al. 1999). A number of primers with the least homology to equivalent human or *Candida* gene sequences were screened for the pairs that gave the highest sensitivity and specificity. No crossreaction with numerous fungal and bacterial pathogens was observed. The assay allowed direct and rapid detection of down to 10 fg of *Aspergillus* DNA corresponding to 1–5 c.f.u./ml blood. A total of 315 blood and BAL samples from 140 subjects, including 93 patients at risk for invasive disease, were screened. The authors found 100 percent correlation between positive histology, culture, or high-resolution CT findings and PCR results. The test specificity was 89 percent. The method appears to be simple, specific, rapid, and inexpensive.

Other PCR formats have been adapted for the detection of *Aspergillus* DNA. A nested PCR test targeting the large ribosomal subunit genes of *Aspergillus* spp. was evaluated retrospectively on 175 serum samples from 37 bone marrow transplant recipients, 70 percent of whom received grafts from unrelated donors (Williamson et al. 2000). Six patients had proven infection, seven had probable infection, and three had possible infection, using the revised EORTC case definitions. These 16 patients were all PCR positive (57 out of 93 samples tested). Two additional patients who did not fulfill current diagnostic criteria, but in whom invasive aspergillosis was thought clinically probable, were also PCR positive (five of nine samples). Invasive aspergillosis was unlikely in the remaining 19 patients, four of whom were PCR positive on a single occasion (four of 70 samples). Three samples were inhibitory to PCR. The sensitivity of PCR in diagnosing patients with invasive aspergillosis was 100 percent, specificity was 79 percent, and positive predictive value was 80 percent, using the criterion of a single positive result. If two positive results were required, these values became 81 percent, 100 percent, and 100 percent, respectively. The median duration of infection documented by PCR was 36 days (range 3–248 days) in 17 of 18 patients (94 percent) who did not survive. Positive PCR results predated the institution of antifungal therapy in two-thirds of patients. Four patients became PCR positive during pretransplant conditioning therapy.

Many of these PCR formats are labor intensive and require a minimum of one working day to perform. The Light Cycler technique combines rapid in vitro amplification of DNA in glass capillaries with real-time species determination and quantification of DNA load. Loeffler and colleagues have established a quantitative PCR protocol for *A. fumigatus*, as well as for *C. albicans* (Loeffler et al. 2000). The sensitivity of the assay was comparable to those of previously described PCR protocols (5 c.f.u./ml). Specific detection of *A. fumigatus* could be achieved. The assay showed a high reproducibility of 96–99 percent. The assay was linear in a range between 10^1 and 10^4 aspergillus conidia. The Light Cycler allowed quantification of the fungal loads in clinical specimens from patients with hematological malignancies and histologically proven invasive fungal infections. In blood specimens spiked with aspergillus conidia, a sensitivity of 5 c.f.u./ml was demonstrated. All samples were also found to be PCR positive by PCR-ELISA analysis.

DETECTION OF CELL WALL β-1,3-D-GLUCAN

The cell wall of aspergillus hyphae, and the cell walls of other pathogenic fungi consist of mannans and glucans. The detection of circulating β-1,3-D-glucan is another

investigative strategy for diagnosis of invasive aspergillosis.

The plasma concentration of β-1,3-glucan has been measured at the time of routine cultures in febrile episodes (Obayashi et al. 1995). With a plasma cutoff value of 20 pg/ml, 37 of 41 episodes of proven fungal infections (confirmed at postmortem or by microbiological methods), including aspergillosis, were detected. All of the 59 episodes of nonfungal infections, tumor fever, or collagen-vascular diseases had concentrations below the cutoff value (specificity 100 percent). Of 102 episodes of fever of unknown origin, 26 had plasma glucan concentrations of more than 20 pg/ml. Of these, 102 cases are taken as nonfungal infections, the positive predictive value of the test was estimated as 59 (37 of 63), the negative predictive value as 97 percent (135 of 139), and the efficacy as 85 percent (172 of 202). Although a positive result does not indicate the specific cause of the detected fungal infection, this approach is very encouraging and warrants more extensive investigation in selected patient populations. More recent studies have confirmed the usefulness of this approach (Hossain et al. 1997). The test has now been reformatted as an ELISA (Glucatell – Associates of Cape Cod Inc.). Ostrosky-Zeichner and coworkers reported in an abstract from a multicenter trial evaluating the usefulness of the Glucatell assay as an aid to diagnosis of invasive fungal infections in humans (ICAAC 2003: www.iccac.org). The study evaluated the assay in 170 healthy controls and 163 patients with either proven or probable invasive fungal infections; the assay was able to provide a very high specificity and a high predictive value. This assay may be a useful diagnostic adjunct to cultures in the earlier diagnosis of invasive fungal infections.

CONCLUSIONS

Considering the differences in patient populations, conditions of specimen collection, and controls, no comparisons can be legitimately drawn between different antigen and PCR detection systems. The fact that these approaches have been found consistently to enhance the diagnosis of invasive aspergillosis is very encouraging. The recent approval of the Platelia *Aspergillus* galactomannan ELISA by the FDA in the United States should facilitate further understanding. A more comprehensive review of recent developments in the serological and immunological diagnosis of invasive aspergillosis is given by Yeo and Wong (2002), Jones and McLintock (2003), and McLintock and Jones (2004).

HISTOLOGICAL FEATURES

An extremely important characteristic of the histopathology of invasive aspergillosis is the striking tendency of the fungal hyphae to invade large and small arteries and veins, causing inflammation, thrombosis, and infarction. A variety of clinical manifestations and signs of organ dysfunction reflects this angiotropic behavior of *Aspergillus* spp. Aspergillus hyphae can be seen in thrombosed vessels and necrotic tissues, with regular septation and dichotomous branching at about 45°, advancing in the same direction. Aspergillus hyphae stain poorly with hematoxylin and eosin, and are best highlighted with Gomori's methenamine silver. A varying admixture of inflammatory cells is seen depending on the immune status of the patient.

Prognostic markers in aspergillosis

In allergic forms of aspergillosis, antibody titers may be of some prognostic value. It has been suggested that precipitins may decrease with successful corticosteroid therapy. IgE levels are, however, the most useful parameter to follow in the treatment of ABPA patients. The total IgE often rises before a clinical relapse, and the duration of therapy may be judged based on the fall in IgE level.

Tests for aspergillus precipitins are often helpful in the diagnosis of the different forms of aspergillosis that can occur in the noncompromised patient. Precipitins can be detected in up to 70 percent of patients with allergic aspergillosis and over 90 percent of patients with aspergillomas.

The precipitin test is also useful for the diagnosis of chronic necrotizing aspergillosis of the lung and other invasive forms of aspergillus infection, such as endocarditis, provided that the patient is not immunosuppressed.

The detection of precipitins in a neutropenic patient with unresponsive fever or a lung infiltrate is often sufficient to prompt the initiation of therapy, but it must be stressed that a positive test result is not proof of infection. Nor does a negative precipitin test result preclude the diagnosis of aspergillosis in an immunocompromised patient, because such individuals are often incapable of mounting a detectable serological response.

Tests for the detection of circulating aspergillus antigen in blood and urine offer an alternative means of diagnosing aspergillosis in the immunocompromised patient. Changing titers of galactomannan may predict treatment outcomes or indicate the progression of disease. However, aspergillus galactomannan is rapidly cleared from the circulation and frequent sampling is required for optimal detection of antigen.

Levels of antigenemia and antigenuria may correlate with the clinical course in invasive aspergillosis. Data in animal studies and some from patient series would suggest that antigen levels rise as the clinical condition worsens. Also, some studies have shown that efficacious antifungal therapy causes antigen levels to fall; however, this has not been confirmed in all studies. At this time, it is probably premature to derive any universal correlates for antigen levels in this disease.

In conclusion, rapid serological and molecular diagnostic methods facilitate the early diagnosis of invasive fungal infection and would appear to be most useful when used prospectively to screen high-risk patients. However, in order to determine the optimal approach to treatment it is essential that these tests are incorporated into management strategies and their impact on incidence of invasive fungal infection and clinical outcome evaluated in further clinical trials (Jones and McLintock 2003).

Future directions of diagnostic techniques

In the field of medical mycology, DNA-Chip technology appears to be the most promising molecular technique and has several potential applications, including:

- pathogen identification either of single species or part of a panel (as for example fungi causing fungemia)
- detection of antifungal resistance alleles
- virulence factors in bacteria; fungal typing in cases of nosocomial infection or for molecular epidemiologic purposes
- host–pathogen interaction with gene expression arrays.

It is likely that the panel strategy will be increasingly applied to the diagnosis of systemic fungal infections. Currently, most of the applications of DNA-Chip technology have been in the field of cancer, human genetics, or the monitoring of cellular gene expression. A DNA-Chip or DNA-Microarray is a small surface, usually glass or silica, which is covered by hundreds to hundreds of thousands of different oligonucleotides. Each oligonucleotide is represented on the Chip as millions of copies. These Chips are used in hybridization reactions to detect nucleic acids generated from a sample by an amplification technique. We are only at the beginning of the story of the use of DNA-Chips in the field of microbial diagnostics. Small microarrays have been used to identify bacteria in blood cultures using the 23S ribosomal gene. Very few fungal applications have been described. A DNA-Chip test requires approximately 6 hours to be performed. First DNA or RNA must be extracted from the sample using standard existing procedures. Amplification is then made with PCR, reverse transcriptase polymerase chain reaction (RT-PCR), or nucleic acid sequence based amplification (NASBA). The amplification products are cleaved to fragments of approximately bases and labelled with a fluorescent marker. The labelled products are then hybridized on the chip and the chip is thoroughly washed. Finally, using a confocal laser reader, the fluorescence, which is proportional to the amount of hybridized material on an individual oligonucleotide, is measured on each printed spot. One particular technique that is currently being evaluated for fungal diagnostics is arrayed primer extension (APEX). This is an integrated system with multiplex primer extension on a DNA array, fluorescence imaging, and data analysis. Fungal DNA is amplified by PCR, digested enzymatically, and annealed to the immobilized primers, which promote sites for template-dependent DNA polymerase extension reactions using four fluorescently labeled dideoxynucleotides. This and other methodologies will hopefully be developed into commercially available tests for the mycology laboratory.

MANAGEMENT

A number of new reviews and monographs have been published which should be consulted for detailed aspects and recommendations regarding the treatment of diseases caused by *Aspergillus* (Chiller and Stevens 2000; Stevens et al. 2000a; De Marie 2000; Judson and Stevens 2001; Reichenberger et al. 2002; Advances against aspergillosis 2003; Richardson and Jones 2003; Richardson and Warnock 2003; Anonymous 2004). Further information can be found on the *Aspergillus* website: www.aspergillus.man.ac.uk. A detailed account of the clinical pharmacology of all drugs currently used and those being developed for treating aspergillosis has been published by Groll et al. 1998b.

Allergic aspergillosis

Mild disease may not require treatment. Prednisone is the drug of choice because it is effective in reducing symptoms, improving chest x-rays, and clearing positive sputum cultures. The usual dosage regimen is 1.0 mg/kg/day until x-rays are clear, then 0.5 mg/kg/day for 2 weeks. The same dose is given at intervals of 48 hours for another 3–6 months, and then the dose is tapered off over another 3 months. The initial regimen should be resumed if the condition recurs. Bronchodilators and postural drainage may help to prevent mucus plugging. Treatment with antifungal drugs is not known to be helpful (Ikemoto 1992). However, more recent reports suggest that antifungal treatment may be helpful (Leon and Craig 1999; Salez et al. 1999; Chiu 2003; Stevens et al. 2000b, 2003).

Aspergilloma (fungus ball) of the lung

Surgical resection is indicated if massive or recurrent hemoptysis should occur. On occasion, segmental or wedge resection will suffice, but lobectomy is usually required to ensure complete eradication of the disease (Jewkes et al. 1983).

If surgical intervention is contraindicated, endobronchial instillation or percutaneous injection of amphotericin B may be helpful (Jewkes et al. 1983; Yamada et al. 1993). The optimum dosage has not been determined, but 10–20 mg amphotericin B in 10–20 ml distilled water

instilled two or three times per week for about 6 weeks has proved successful. Larger doses (40–50 mg) have been instilled into lung cavities using percutaneous catheters. Itraconazole does not appear to be effective (Campbell et al. 1991).

The treatment of mild-to-moderate bleeding and asymptomatic patients remains controversial, but observation without intervention may be the best approach to management. Recent reports suggest that antifungal treament may be useful in the management of aspergilloma (Kaestel et al. 1999; Ikemoto 2000; Kawamura et al. 2000; Regnard et al. 2000).

Chronic necrotizing aspergillosis of the lung

Treatment with an antifungal drug, such as amphotericin B, is often the first step in management, but surgical resection of necrotic lung and surrounding infiltrated tissue may also be required. The long-term prognosis is poor.

Infection of the paranasal sinuses

In some cases of paranasal aspergillus granuloma, surgical removal of infected material, with drainage and aeration, is curative. Often, however, the condition will recur, necessitating further surgical intervention. The long-term results are generally poor. Postoperative treatment with itraconazole appears promising as a means of preventing relapse. The drug should be given at a dosage of 200 mg/day for at least 6 weeks.

Neutropenic patients with acute aspergillus sinusitis require surgical débridement and treatment with amphotericin B (1.0 mg/kg/day).

Endophthalmitis

Patients with aspergillus endophthalmitis should be treated with intravenous amphotericin B (1.0 mg/kg/day). Surgical débridement and intravitreal instillation of amphotericin B (5 μg doses two or three times) may also be required.

Endocarditis

Aspergillus endocarditis requires aggressive medical and surgical treatment. Treatment with high-dose amphotericin B (1.0 mg/kg/day) alone is ineffective. Infected tissue and prostheses must be removed.

Osteomyelitis

Surgical débridement of necrotic tissue is important in the management of aspergillus osteomyelitis. Most patients with vertebral osteomyelitis undergo simple débridement as part of their initial diagnostic procedure. Later procedures include radical débridement with bone grafting. Both medical and surgical treatment are required if ribs are infected.

Treatment with itraconazole (400 mg/day) has proved successful in several patients with aspergillus osteomyelitis.

Cutaneous aspergillosis

High-dose amphotericin B (1 mg/kg/day) is the treatment of choice. Débridement of cutaneous lesions that arise at catheter insertion sites should be delayed until the neutrophil count has recovered.

Acute invasive aspergillosis

The successful management of acute invasive aspergillosis in the neutropenic patient depends on the prompt initiation of antifungal treatment (within 96 hours of the onset of infection) (Denning and Stevens 1990; Denning 1994a; Keating et al. 1994). The prognosis is poor if the neutrophil count does not recover.

The drug of choice for the treatment of disseminated aspergillosis is amphotericin B. There are numerous regimens for the administration of this drug, but there is widespread agreement that in neutropenic patients it is important to give the full dose of amphotericin B from the outset. High doses must be used (at least 1.0 mg/kg/day).

The optimum duration of treatment has not been established, but amphotericin B should be continued at least until the neutrophil count is more than 0.5×10^9/l. Thereafter treatment should be continued until symptoms resolve and relevant radiological abnormalities (on x-rays and CT scans) disappear. The shortcomings of current methods of diagnosis often require clinicians to proceed to amphotericin B treatment without waiting for formal proof that a neutropenic patient who has persistent fever (>72–96 hours duration), and is unresponsive to antibacterial drugs, has aspergillosis. Empirical treatment should be initiated with the usual test dose (1 mg) of amphotericin B. If possible, the full therapeutic dosage level should be reached within the first 24 hours of treatment (Fraser and Denning 1993).

Neutropenic patients who recover from aspergillosis may suffer from reactivation of the infection during subsequent periods of immunosuppression. One solution to this problem is to begin empirical treatment with amphotericin B (1 mg/kg/day) not less than 48 hours before antileukemic treatment is commenced. The drug should then be discontinued until the neutrophil count has recovered.

During the 1980s, several investigators incorporated amphotericin B into lipid vehicles and showed a reduction in the toxic adverse effects without loss in the effec-

tiveness of amphotericin B. Initially, such formulations were produced locally and used at single hospitals. In more recent years, lipid complexes of amphotericin B have become commercially available. These formulations include:

- the liposome AmBisome (Gilead Sciences, Foster City, CA, USA)
- a lipid vesicle – amphotericin B colloidal dispersion: Amphotec, Amphocil (InterMune, Brisbane, CA, USA)
- an amphotericin B–lipid complex: Abelcet (Enzon, Bridgewater, NJ, USA).

All of these preparations differ in size, structure, and pharmacokinetics, and, to a certain extent, in the clinical efficacy in the treatment of invasive aspergillosis (reviewed by Tollemar and Ringden 1995 and Barrett et al. 2003).

The treatment of invasive aspergillosis in bone marrow transplant patients has been reviewed recently by Marr et al. 2002; Richardson and Warnock 2003; Wiederhold et al. 2003, and treatment guidelines are published as *Therapeutic guidelines in systemic fungal infections* (Richardson and Jones 2003) and on Clinical Mycology Online (www.clinical-mycology.com).

AMBISOME (LIPOSOMAL AMPHOTERICIN B)

Liposomal amphotericin B (AmBisome) is well tolerated and doses as high as 3–5 mg/kg/day have been administered without significant side effects. Some 10 000 patients have been treated worldwide over the past 6 years. Administration of the drug in this form has sometimes eradicated aspergillus infection in neutropenic patients, and it should be considered in patients who have failed to respond to the conventional parenteral formulation, or who have developed side effects that would otherwise necessitate discontinuation of the drug.

A representative study highlighting the use of AmBisome in invasive aspergillosis is that by Mills et al. (1994). In a single-center study, the diagnosis of aspergillosis was proved in 21 of 116 patients with signs indicative of invasive aspergillosis (with four patients diagnosed postmortem). Of these 21 patients, 13 (62 percent) obtained complete or excellent partial resolution. Of the 17 patients with aspergillosis confirmed in life, the response was 77 percent (13 of 17). In eight patients, liposomal amphotericin B was discontinued when the neutrophil count was below 1×10^9/l, without recrudescence of the infection. Complete hematological remission was confirmed in nine responders on recovery from chemotherapy; three were found to have persistent disease and two had recurrent aspergillosis following subsequent chemotherapy. Of the eight nonresponders, four had refractory malignancy and four died before assessment was possible. In a further report, Ng and Denning (1995) detail the results from 34 centers in the UK of 17 cases of definite aspergillosis treated with AmBisome. The overall response rate was 59 percent (10 of 17). Mean (±SD) cumulative doses of AmBisome received by the responders and those who failed to respond were 2 081±1 189 mg and 1 207±839, respectively. Mean durations of treatment were 17±8 days and 7±4 days, respectively. All six patients who received therapy for more than 14 days responded, whereas 75 percent of those (three of four) who were treated for 5 days or less died. The average maximum daily dose received/tolerated by the group as a whole was 3±1 mg/kg/day (range 1–5 mg/kg/day).

AmBisome was used as 'salvage' therapy in seven patients who received conventional amphotericin B as first-line treatment but either failed to respond or developed renal insufficiency as a result of treatment. The response rate among this group of patients was 43 percent (three of seven). Of ten of those who did not receive prior therapy with conventional amphotericin B, eight (80 percent) responded to AmBisome therapy.

The data concerning the efficacy of AmBisome in the treatment of mycologically proven cases of invasive aspergillosis, in the analysis by Ng and Denning (1995) of compassionate use, are similar to those reviewed by Tollemar and Ringden (1995) (17 of 29 or 60 percent cure/improvement). This survey emphasizes the point that those patients who responded to AmBisome tended to have received a larger (cumulative) dose for a longer period of time than those in whom treatment failed. Among the patients who had proven invasive aspergillosis, those in whom AmBisome was used as salvage therapy had a less favorable prognosis than those who had never been treated with conventional amphotericin B. AmBisome (up to 5 mg/kg/day) was well tolerated in this study, even in one patient who had previously had a severe drug reaction after conventional amphotericin B therapy.

A notable feature of AmBisome is the very low incidence of acute infusion-related adverse reactions; in fact, where such reactions do occur patients appear to tolerate subsequent doses much more readily. There is no requirement for test dosing, slow escalation, or premedication.

A number of recent clinical evaluations and reviews have reinforced the earlier perception of AmBisome being a safe and effective treatment for invasive aspergillosis (Ribaud and Gluckman 1996; Coukell and Brogden 1998; Ringden et al. 1998). An illustrative study is that of Leenders and colleagues who reported a comparison between AmBisome 5 mg/kg/day with conventional amphotericin B 1 mg/kg/day (Leenders et al. 1998). Among patients with suspected or documented pulmonary aspergillosis, 11 (42 percent) patients on AmBisome and six (21 percent) patients on conventional amphotericin B had a complete response. Other trials have been reported by Andström et al. (1996) and Clark et al. (1998).

More recent information can be found in Marr et al. (2002) and Wiederhold et al. (2003). High-dose AmBisome has become an attractive therapeutic option for the management of invasive aspergillosis, although further studies are needed to assess this approach (Walsh et al. 2001; Martin et al. 2003).

AMPHOCIL/AMPHOTEC

Amphocil [amphotericin B colloidal dispersion (ABCD)] is formed from equimolar amounts of amphotericin B and cholesterol sulfate; it has a disk-like form with a mean size of 122 nm and is rapidly taken up by the liver. Despite the lack of published studies, ABCD has been used in a number of centers and the data presented in abstract form (Oppenheim et al. 1995). The effectiveness of ABCD has been studied in a number of animal models of infection. In a rabbit model of invasive aspergillosis, conventional amphotericin B, at a dosage of 1.5–4.5 mg/kg/day, was more effective in eradicating hepatic and renal infection than ABCD at dosages from 1.5 to 4.5 mg/kg/day (reviewed by Warnock 1995). Infection persisted in the lungs even when animals were treated with a lethal 4.5 mg/kg dose of conventional amphotericin B. However, ABCD could be administered at a much higher dosage (15 mg/kg/day) and this eradicated the infection. In a second report involving a rabbit model of invasive aspergillosis (reviewed by Warnock 1995), animals treated with ABCD had better survival rates (33 percent and 70 percent at dosages of 1 and 5 mg/kg/day) than those treated with conventional amphotericin B (14 percent at 1 mg/kg/day).

The clinical data are still too limited to allow firm conclusions to be drawn, but published information on the compassionate use of ABCD in patients with invasive aspergillosis compares favorably with that for conventional amphotericin B, but not with AmBisome. The collective global experience has been reviewed by Barrett et al. (2003). Of patients at 45 centers, 168 were entered into an open-label, non-comparative evaluation of the efficacy and safety of ABCD in confirmed systemic mycoses. To be included in the study, patients had either to have a systemic fungal infection that had failed to respond to conventional amphotericin B or to be unsuitable for conventional amphotericin B because of nephrotoxicity from conventional amphotericin B or other drugs or because of preexisting renal impairment. A total of 97 patients were evaluable for clinical efficacy of whom 33 percent had invasive aspergillosis. Thirty-four per cent of cases of aspergillosis responded. In a separate analysis of 21 patients with proven fungal infections treated with Amphocil in two UK hospitals, a clinical response was seen in eight of 13 patients with aspergillosis.

Further US experience is reported by White et al. (1997). Eighty-two patients with proven or probable aspergillosis who were treated with Amphocil were compared retrospectively with 261 patients with aspergillosis who were treated with conventional amphotericin B. Response rates (48.8 percent) and survival rates (50 percent) among Amphocil-treated patients were higher than those (23.4 percent and 28.4 percent, respectively) among amphotericin B-treated patients. This study suggested also that in the treatment of aspergillosis, Amphocil caused fewer nephrotoxic effects than conventional amphotericin B and that the efficacy of Amphocil was at least comparable with that of amphotericin B.

More recent information about the use of Amphocil in aspergillosis can be found in Noskin et al. 1999, Roland 1999, and Barrett et al., 2003.

ABELCET

Abelcet [amphotericin B lipid complex (ABLC)] consists of amphotericin B complexed with two lipids, dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, in a 1:1 drug:lipid molar ratio. In animal models, ABLC has been shown to be at least as effective as amphotericin B and substantially less toxic.

Two key studies represent the current clinical experience with ABLC. As part of a 225 patient study where ABLC was used at 5.0 mg/kg/day, complete response or improvement was achieved in 60 percent (43 of 72) of aspergillosis cases (reviewed by Barrett et al. 2003). Among the different patterns of invasive aspergillosis, the response rate was greatest for sinus aspergillosis (83 percent), followed by pulmonary aspergillosis (58 percent) and extrapulmonary aspergillosis (52 percent). Extrapulmonary aspergillosis included single-organ infection (n=14) and disseminated infection (n=13). The response to ABLC in single-organ extrapulmonary aspergillosis showed a trend towards a more favorable response in nine of 14 cases (64 percent) versus five of 13 (38 percent) in disseminated aspergillosis. There was also a trend towards a more favorable response in all nondisseminated aspergillosis (38 of 59 or 64 percent) versus disseminated aspergillosis cases (five of 13 or 38 percent). The authors note that responses were obtained in patients with disseminated aspergillosis unresponsive to or intolerant of conventional amphotericin B, and that this response was encouraging considering that the mortality rate in this disease approaches 100 percent.

The second major study of the use of ABLC in aspergillosis is where the efficacy, nephrotoxicity, and mortality of patients with invasive aspergillosis treated with ABLC (5 mg/kg/day) in an emergency program were compared with those of a historical control group of patients treated with conventional amphotericin B (reviewed by Barrett et al. 2003). The patients selected (n=151) were those who had failed to respond to antifungal therapy, and had nephrotoxicity or preexisting renal insufficiency. The historical control patients (n=122) had received 500 mg or more of conventional

amphotericin B or had nephrotoxicity. All patients met the definitions for definite or probable aspergillosis. A distinction between the different disease patterns of aspergillosis is not made. ABLC-treated patients showed greater probability of survival (P=0.0233) (median of 52 versus 31 days) and higher response rates (40 percent versus 23 percent, P=0.002) than patients treated with conventional amphotericin B. Among patients with serum creatinine of 221 μmol/l or more when ABLC treatment began, a significant decrease from baseline levels was observed between week 2 and week 5 (P=0.004). The authors conclude from this study that ABLC appears to be associated with at least equal, and possibly better, efficacy and clearly less toxicity compared with conventional amphotericin B in the treatment of disseminated aspergillosis.

Data on ABLC are limited, although the formulation seems to have an acute toxicity profile (pyrexia, chills) very similar to that of amphotericin B. As clinical experience with this drug is restricted to a few centers, it will be some time before more definitive statements can be made regarding overall toxicity.

A number of studies have confirmed the usefulness of ABLC in the treatment of severe invasive candidosis and as second-line treatment of invasive aspergillosis, cryptococcosis in HIV patients, and miscellaneous fungal infections (Oravcova et al. 1995; Franklin et al. 1997; Mehta et al. 1997). The drug appears to be cleared rapidly from the serum. High tissue levels, especially in the lung, have been recorded in animals, followed by rapid clearance of the drug from the lung area over 24 hours. Human data regarding accumulation of ABLC in lung tissue appears to be based on postmortem tissue from one patient. The initial clinical trials were conducted in the USA (reviewed by Lister 1996). The size and quality of these studies varied considerably and the conclusions that can be drawn are limited in the absence of confirmatory studies.

The recommended dosage of ABLC is 5 mg/kg and this should be infused over a period of 2 hours for at least 2 weeks. Response to treatment times is highly variable. This formulation of amphotericin B has been administered to individual patients for up to 11 months, to a cumulative dosage of 50 g without significant toxic side effects.

Very few studies evaluating the effectiveness of ABLC in invasive aspergillosis have been described. Mehta et al. (1997) report that five of seven evaluable patients with aspergillus pneumonia responded to ABLC (daily dose of 5 mg/kg).

A further study regarding the safety and efficacy of ABLC has recently been reported by Walsh et al. (1998). ABLC was evaluated in 556 cases of invasive fungal infection treated through an open-label, single-patient, emergency-use study of patients who were refractory to or intolerant of conventional antifungal therapy. This large clinical trial demonstrated a significant improvement in renal function following the initiation of therapy with ABLC, particularly in patients with amphotericin B-induced nephrotoxicity or primary renal dysfunction. Among the 291 mycologically confirmed cases evaluable for therapeutic response, there was a complete or partial response to ABLC in 55 of 130 cases of aspergillosis. Among the different patterns of invasive aspergillosis, the response rate was greatest for single-organ extrapulmonary aspergillosis and for sinus aspergillosis, followed by pulmonary and disseminated aspergillosis.

As clinical experience with this drug is restricted to a few centers, it will be some time before more definitive statements can be made regarding overall efficacy and toxicity.

There is some concern regarding the incidence and severity of toxicity of ABLC. Ringden et al. (1998) reported their experience with ABLC for proven or suspected invasive fungal infection in 19 patients with a variety of hematological conditions. ABLC was discontinued in 14 of the 19 patients due to side effects. These included renal insufficiency, increase in bilirubin, erythema, increased alanine aminotransferase (ALAT), fever and chills, severe vomiting, and a variety of other drug-related effects. The authors express the opinion that ABLC is far more toxic than AmBisome. In their experience, AmBisome was discontinued in only six of one series of 187 patients. However, it is pointed out that the dose of AmBisome used was much lower in most patients.

Further experience with Abelcet is reported by Boyle and Swenson (1999), Linden et al. (1999), Martino et al. (1999a, b), Walsh et al. (1999b), and reviewed by Barrett et al. (2003).

ITRACONAZOLE

Itraconazole is comparable in vitro to amphotericin B in efficacy. Itraconazole capsules are tolerated with an acceptable level of toxicity. Treatment with itraconazole (400 mg/day) has sometimes proved successful in neutropenic individuals with invasive aspergillus infection (Jennings and Hardin 1993). However, absorption of the drug from the gastrointestinal tract can be a problem and blood concentrations must be measured at regular intervals. Recently, there have been several reports on the successful treatment of invasive pulmonary aspergillosis in neutropenic patients (reviewed by Beyer et al. 1994a; Denning et al. 1994). The accumulated data on itraconazole are highlighted by a small randomized trial where itraconazole (capsules) 400 mg/day was compared with amphotericin B at a dose of 0.6 mg/kg/day in 32 patients with suspected or proven fungal infection (reviewed by Beyer et al. 1994b). Invasive pulmonary aspergillosis was suspected or documented in 13 of 32 (41 percent) of the patients. The overall response rate was similar, with 63 percent in the itraconazole arm and

56 percent in the amphotericin B arm, but all three fatalities with documented invasive pulmonary aspergillosis were treated with amphotericin B. Patients with candida infections responded better to amphotericin B, whereas patients with aspergillus infections had a better response to itraconazole, although the response was not significant. The median treatment duration of 20 days with itraconazole and 13 days with amphotericin B was short, and the responses to conventional amphotericin B and itraconazole were associated with neutrophil recovery in most patients. Itraconazole has also been successfully used in the treatment of invasive pulmonary aspergillosis in patients who did not respond to amphotericin B (see Beyer et al. 1994b). The use of oral itraconazole plus intranasal amphotericin B for prophylaxis of invasive aspergillosis has been described (Todeschini et al. 1993).

More recent studies reaffirm the value of itraconazole in the treatment of invasive aspergillosis, especially where an early diagnosis is achieved. An illustrative study is that of Caillot et al. (1997) where systematic use of CT added to the early diagnosis of invasive aspergillosis. Twenty-six of 37 patients with proven or highly probable invasive aspergillosis were improved or cured by antifungal treatment. Twenty-two patients received itraconazole (median dose 400 mg/day) for a median length of 302 days (range 103–1 185). No severe adverse reactions were seen. The plasma levels of itraconazole were determined in 16 patients, and in 14 of them the residual levels of itraconazole were greater than 0.6 μg/ml during the first 20 days of treatment. In 13 cases, amphotericin B (median dose 1.3 mg/kg/day) was combined with itraconazole for a median duration of 9 days (range 5–20).

There is limited experience with the new oral solution formulation of itraconazole as first-line treatment of invasive aspergillosis – 400–600 mg/day for 4 days then 200 mg twice daily. An alternative approach appears to be the use of high-dose itraconazole solution (400–600 mg) following 7–14 days of AmBisome.

An intravenous formulation of itraconazole is currently being evaluated and has been licensed in the UK, the USA, and The Netherlands. Data are available only in abstract form at the moment. Further studies are being conducted to determine the efficacy of this formulation in the treatment of aspergillosis and as empirical therapy for fever of unknown origin. The current dosing schedule of the intravenous formulation is as follows:

- Day 1 and 2: 1-hour infusion 200 mg twice daily
- From day 3 onwards: a single 1-hour infusion 200 mg each day. Safety of use for periods longer than 14 days has not been established.

Recent reports on all formulations of itraconazole include: Popp et al. (1999); Prentice et al. (1999); Persat et al. (2000); Caillot (2003). Additional information and guidelines can be found on the *Aspergillus* website (www.aspergillus.man.ac.uk), and in *Therapeutic guidelines in systemic fungal infection* (Richardson and Jones 2003). Recent findings on itraconazole are found in a *Journal of Antimicrobial Chemotherapy* supplement (in press).

VORICONAZOLE

A new azole antifungal, voriconazole (Pfizer), formulated in both oral and intravenous forms, is currently being evaluated in clinical studies. It exhibits potent antifungal activity against clinical isolates of *Aspergillus* spp. in vitro. It appears to be more active in vitro compared to amphotericin B (Clancy and Nguyen 1998). For *A. fumigatus* the minimum inhibitory concentration (MIC) has been shown to be in the range 0.03–0.5 μg/ml and the MIC_{90} to be 0.25 μg/ml. In experimental aspergillosis in mice, oral voriconazole 30 mg/kg body weight per day appears to significantly delay or prevent mortality (Murphy et al. 1997). A number of clinical evaluations have demonstrated the effectiveness of voriconazole in invasive aspergillosis (initial clinical experience reviewed by De Pauw and Meis 1998). Caillot et al. (1997) described three patients who were cured and one who improved with voriconazole treatment (median dose 6 mg/kg/day; median duration 63 days). One patient failed to respond to a 26-day treatment course with voriconazole.

Further animal data have been reported by Chandrasekar et al. (2000). Their work compared the efficacies of amphotericin B and voriconazole against pulmonary aspergillosis in a guinea pig model. Voriconazole-treated animals had significantly better survival rates and a decreased fungal burden in the lungs compared with controls. Although no statistical difference was seen between the efficacies of voriconazole and amphotericin B, a trend favoring voriconazole was noted.

Currently, voriconazole is licensed for the treatment of patients aged 2 years or over with invasive aspergillosis. It may be administered either by mouth or intravenously, depending on the condition of the patient. It also appears to be active against other filamentous fungi besides *Aspergillus* (Perfect et al. 2003).

A review of the clinical efficacy of voriconazole in invasive aspergillosis can be found in Muijsers et al. (2002), and the *Drugs and Therapeutic Bulletin* (Anonymous 2004). The conclusion is that voriconazole appears to be a reasonable choice as a second-line therapy, not least because it offers the potential for intravenous or oral treatment of a broad spectrum of fungal infections, in both adults and children. However, there is no convincing published evidence to justify the claim that voriconazole is superior to amphotericin B at increasing survival rates in patients with invasive aspergillosis (Anonymous 2004).

CASPOFUNGIN

Caspofungin is a synthetic lipopeptide. As one of the echinocandins it inhibits the synthesis of β-1,3-D-glucan, an essential component of fungal but not mammalian cells. Its range of activity is restricted to *Aspergillus* and *Candida* spp. Caspofungin is licensed for the treatment of invasive aspergillosis in adults who have failed to tolerate or respond to conventional or lipid formulations of amphotericin B and/or itraconazole, and for the treatment of candidosis in non-neutropenic adults.

Published evidence on the efficacy of caspofungin in invasive aspergillosis is limited to an uncontrolled retrospective study, published only in abstract form (Maertens et al. 2002). This involved 83 patients with presumed invasive aspergillosis who had either failed to tolerate or not responded to unspecified antifungal therapy, and were given intravenous caspofungin for up to 162 days. A favorable response (i.e. resolution of, or improvement in, attributable clinical and radiological or bronchoscopic features) was reported in 45 percent of patients.

In conclusion, caspofungin appears to be better tolerated than conventional amphotericin B but there is little published evidence to justify its use in invasive aspergillosis (Anonymous 2004).

IN VIVO EFFECTS OF COLONY-STIMULATING FACTORS ON NEUTROPHIL FUNCTION AND INVASIVE ASPERGILLOSIS

The assessment of the effects of colony-stimulating factors on neutrophil function is contradictory, largely because of the diversity of methods used and the growth phase of the organism used. In vitro, granulocyte colony-stimulating factor (G-CSF) appears to be a weaker stimulus to the neutrophil metabolic burst than GM-CSF, tumor necrosis factor (TNF), or various interleukins (IL). Phagocytosis and fungicidal activity appear to be enhanced by exposure of normal effector cells to G-CSF, GM-CSF, or IL-8 (Roildes et al. 1993b, c; Richardson and Patel 1995). Pre-exposure to concentrations of IL-8 achievable in vivo significantly enhances phagocytic ingestion of *A. fumigatus* conidia (Richardson and Patel 1995). Furthermore, IL-8-primed neutrophils showed enhanced phagocytic activity to the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine.

CLINICAL TRIALS OF COLONY-STIMULATING FACTORS IN INVASIVE ASPERGILLOSIS

Closely paralleling preclinical trials in experimental infections, a number of studies have shown that macrophage colony-stimulating factor (M-CSF), G-CSF, and GM-CSF can reduce the severity and duration of chemotherapy-induced neutropenia; these growth factors are a useful adjunct to antifungal therapy (Spielberger et al. 1993). Undoubtedly, there will soon be other investigations of the benefits and risks of using colony-stimulating factors to enhance neutrophil production and upregulate their function. Furthermore, it is anticipated that this will produce a rapid growth in our understanding of the role of neutrophils in response to invasive aspergillosis. A limited number of studies have demonstrated the safe concomitant use of amphotericin B, granulocyte transfusions, and GM-CSF. Concern has been raised about the use of GM-CSF during serious fungal infection because this cytokine can inhibit neutrophil migration into the inflammatory zone measured in a standardized skin chamber assay.

The use of cytokines, together with standard and new formulations of amphotericin B, should be considered for granulocytopenic patients with such invasive fungal infections as invasive aspergillosis, particularly when there has been no response to antifungal therapy alone, and when bone marrow recovery is not expected for at least 10–14 days (Iwen et al. 1993). In a series of cases reported by Bodey et al. (1993), there were two patients with aspergillosis who were treated with GM-CSF and amphotericin B. A patient with underlying breast cancer, who had received a bone marrow transplant, contracted an aspergillus pneumonia. This patient only partially responded. A second patient with acute myeloid leukemia and *A. flavus* sinopulmonary infection failed to respond to combination treatment. The dose range of GM-CSF used in this series was 100–759 $\mu g/m^2$/day, based on initial phase 1 trials. This was considered to be too high because a number of patients developed capillary leak syndrome. It is advocated that doses in the range 15–30 $\mu g/m^2$ be used in future trials. However, cytokines should be used cautiously in the setting of persistent acute myeloid leukemia because the issues surrounding such treatment remain unresolved at the present time.

EMPIRICAL TREATMENT OF PRESUMED INFECTION

Persistent fever suggests an occult fungal infection. The likelihood of this increases with the number of preceding febrile episodes. It has been shown that 44 percent of patients suffering their fourth bout of fever had a fungal infection as the cause.

Patients with prolonged neutropenia (more than 7–10 days) or receiving high doses of glucocorticoids during neutropenia, and a high probability of invasive aspergillosis should be given empirical systemic antifungal therapy. This recommendation is based on numerous reviews and clinical studies. Such patients, with fever persisting despite antibiotic therapy or presenting at the onset of a febrile episode with findings suggesting the possibility of an invasive infection, should be started on antifungals. In this situation amphotericin B must be used. If possible, the full therapeutic dosage level (1.0–1.5 mg/kg/day of the conventional formulation) should be reached within the first 24 hours of treatment. There

is no need for gradual escalation of dosage, nor is there evidence to support the clinical prejudice that a lower dose can be used in suspected invasive disease.

Should the conventional formulation be contraindicated, one of the lipid-complexed formulations should be used instead. For example, in a Europe-wide randomized, double-blind comparative trial of the liposomal formulation of amphotericin B (AmBisome) versus conventional amphotericin B in the empirical treatment of both pediatric and adult febrile neutropenic patients, AmBisome (3 mg/kg/day) was as effective as conventional amphotericin B in the prevention of proven treatment-emergent fungal infections (Prentice et al. 1997). Of significance was the reduction in overall drug-related toxicity by two- to sixfold in patients treated with AmBisome compared with conventional amphotericin B. Severe drug-related adverse reactions were almost absent in patients treated with AmBisome. Nephrotoxicity, in patients not receiving concomitant nephrotoxic agents, was seen only in 3 percent of patients treated with AmBisome, compared with 23 percent of patients on conventional amphotericin B. Time to develop nephrotoxicity was longer in patients on AmBisome compared with conventional amphotericin B.

A significantly larger study, which formed the basis of a successful new drug application to the Federal Drug Administration (FDA) for the use of AmBisome in empirical therapy of presumed fungal infections in febrile, neutropenic patients has been reported (Walsh et al. 1999a). This study was a multicenter, double-blind trial that compared AmBisome (3 mg/kg/day) with conventional amphotericin B (0.6 mg/kg/day). Nearly 700 adult and pediatric patients with neutropenia and pyrexia of unknown origin (PUO) were randomized to receive either conventional amphotericin B or AmBisome. Therapeutic success, measured by a combination of factors including resolution of fever, absence of emergent fungal infection, and patient survival for at least 7 days after therapy, was equivalent between the two groups. There were significantly fewer proven treatment-emergent fungal infections in patients treated with AmBisome than in patients treated with conventional amphotericin B. Similar to the Prentice et al. (1997) report, there was a significant reduction in the frequency of infusion-related fever and in the development of nephrotoxicity. The conclusions that can be drawn from the Walsh et al. (1999a) study are that AmBisome was equivalent to conventional amphotericin B for empirical antifungal therapy in neutropenic patients, but superior in reducing proven treatment-emergent fungal infections, nephrotoxicity, and infusion-related toxicity.

The duration of treatment will differ from patient to patient. If the patient responds and a diagnosis of fungal infection is established, a full course of treatment should be given. More often, however, the patient responds and/or the neutrophil count recovers, but a firm diagnosis is not obtained. In this situation it is reasonable to discontinue amphotericin B when the neutrophil count goes above 1×10^9/l, the fever and other symptoms and signs resolve, and relevant radiological abnormalities return to normal.

Neutropenic patients who recover from invasive aspergillosis may suffer from reactivation of the infection during subsequent periods of immunosuppression. One solution to this problem is to begin empirical treatment with amphotericin B (1 mg/kg/day) not less than 48 hours before antileukemic treatment is commenced. This drug should be continued until the neutrophil count has recovered.

It is important to remember that empirical antifungal therapy does not preclude further investigation for other occult causes of fever (drug-induced fever, viral infection, possibly *Pneumocystis*, rarely parasites or mycobacteria). In addition, a change in fever pattern, including abatement of previously continuous fevers, which frequently follows initiation of amphotericin B therapy, cannot be taken as a guarantee of a favorable response to empirical antifungal therapy. Furthermore, efforts to document fungal infection during empirical therapy in order to adapt antifungal therapy to specific agents and their clinical symptoms should not be abandoned.

Recent reports, reviews, and guidelines regarding empirical treatment of fever in neutropenia include: Hamacher et al. (1999); Roland (1999); Wingard et al. (1999); Prentice et al. (2000); Marr (2002); Richardson and Jones (2003).

A recent review of voriconazole and caspofungin in the *Drugs and Therapeutics Bulletin* (Anonymous 2004) recommends that neither caspofungin nor voriconazole be used for empirical treatment of fever in patients with neutropenia.

TREATMENT OF INVASIVE ASPERGILLOSIS IN NEONATES

High dose (1.0–1.5 mg/kg/day) amphotericin B remains the cornerstone of treatment for both suspected and proven invasive aspergillosis. In neonates, the pharmacokinetics of amphotericin B are characterized by extreme variability between individuals and a comparatively lower clearance rate. However, no correlations between plasma concentrations and pharmacological effects have ever been established, and there is no evidence that these pharmacokinetic characteristics result in any clinical consequences. Indeed, several case series indicate that amphotericin B is usually tolerated without nephrotoxicity at daily doses of up to 1 mg/kg/day, even in very low-birthweight infants, and daily dosages of up to 1.5 mg/kg have been safely administered. It is recommended that a starting dosage of at least 1 mg/kg daily be used, in view of the high mortality associated with invasive aspergillosis. Because it has the potential to cause cardiac arrhythmias, amphotericin B should be

infused under careful monitoring, in particular in the presence of hyperkalemia and/or renal impairment.

Liposomal amphotericin B (AmBisome) has been tolerated at dosages of up to 5 mg/kg/day in more than 50 term and preterm infants (Groll et al. 1998b; Scarcella et al. 1998; Weitkamp et al. 1998). Published data regarding the efficacy of either Abelcet or Amphocil are lacking. The collective opinion is that AmBisome should be the salvage agent of choice in neonates refractory to or intolerant of conventional amphotericin B. A starting dose of 5 mg/kg/day is recommended for proven infections, with dosage adjustment only to limit toxicity (Groll et al. 1998b). Where there is fever of unknown origin without conclusive evidence of invasive aspergillosis, then empirical/pre-emptive AmBisome should be given at a rate of 3 mg/kg/day.

Itraconazole as the oral solution may be indicated in a stable patient with residual lesions. The drug is absorbed by preterm neonates. The recent licensing of intravenous itraconazole offers the opportunity to treat the condition parenterally.

COMBINATION THERAPY

Published data on the use of the new antifungals caspofungin or voriconazole in combination with other antifungal drugs is limited to case reports or case series. Further investigation of the efficacy and safety of caspofungin and voriconazole in combination therapy is needed (for recent reviews see: Lewis and Kontoyiannis 2001; Antoniadou and Kontoyiannis 2003; Steinbach et al. 2003).

PROPHYLAXIS

Old and new approaches to prophylaxis include aerosolized amphotericin B, low-dose intravenous amphotericin B, liposomal amphotericin B, and oral itraconazole (for general reviews see Prentice et al. 2000; Singh 2000; Cornely et al. 2003; Richardson and Warnock 2003). Guidelines can be found in Richardson and Jones (2003).

Amphotericin B inhalation

Aerosolized amphotericin B appeared to be promising in some of the early clinical trials, which have included patients undergoing bone marrow transplantation, and the incidence of aspergillus infections in some series dramatically decreased (compared with historical controls) (Jorgensen et al. 1989; Connealy et al. 1990; Jeffery et al. 1991; Myers et al. 1992; Beyer et al. 1993, 1994a, b). Unfortunately, these results have not been confirmed. A more recent study shows that, in contrast, aerosolized amphotericin B was poorly tolerated (Erjavec et al. 1997). Here, the value of inhaled amphotericin B against invasive pulmonary aspergillosis was evaluated in 61 neutropenic episodes in 42 patients treated for a hematological malignancy. Each patient was assigned to receive amphotericin B in doses escalating to 10 mg thrice daily but only 20 (48 percent) patients managed to complete the schedule regimen. One patient tolerated the full dose initially, but had to discontinue treatment when dyspnea developed as a result of pneumonia and acute respiratory distress. Another 22 patients (52 percent) experienced side effects, including eight (19 percent) who reported mild coughing and dyspnea but who tolerated the full dose, and three (7 percent) patients whose dose was reduced to 5 mg thrice daily. Another six (14 percent) patients could tolerate only 5 mg thrice daily, and five (12 percent) others stopped treatment because of intolerance. The study indicated that elderly patients and those with a history of chronic pulmonary obstructive disease were more likely to develop side effects during inhalation. Twelve (28 percent) patients developed proven or possible invasive fungal infection but no correlation was established between infection and the total amount of amphotericin B inhaled. The investigators concluded that prophylaxis with inhaled amphotericin B does not appear useful in preventing invasive pulmonary aspergillosis in neutropenic patients and is tolerated poorly. The type of nebulizer would appear to be a key issue in the delivery of amphotericin B aerosols throughout the respiratory tract. Many types of nebulizers exist. The nebulizer used by Erjavec and colleagues was capable of producing particles of 0.5–5.5 μm, sufficiently small to reach most components of the respiratory tree including the terminal bronchioles and alveolar sacs. However, nothing is known regarding the level of amphotericin B that is achieved in the pneumocytes of the alveolar epithelium that lines the alveolar ducts and sacs.

The question of safety and tolerance of inhaled amphotericin B has been investigated as part of a study to evaluate the benefit of aerosolized amphotericin B as prophylaxis against invasive aspergillosis (Dubois et al. 1995). The investigators looked specifically at oxygen saturation levels, peak flow values, and symptoms of patients given amphotericin B. Data are presented on a series of 18 patients and 132 amphotericin B administrations. Four (22 percent) of the patients stopped treatments because of nausea and vomiting which were believed to be due to the inhaled amphotericin B. For the remaining patients, no treatment was stopped because of symptoms or physiological changes caused by amphotericin B, although there were nine instances of clinically significant bronchospasm as defined by a drop in peak flow of 20 percent or more, nine clinically relevant increases in cough, and three clinically relevant increases in dyspnea. The findings from this study led the authors to draw the following conclusions. First, most prophylactic amphotericin B was well tolerated and did not cause any clinically or physiologically significant changes. Second, inhaled amphotericin B might in

some cases cause nausea and vomiting, which would lead to discontinuation of therapy. Third, asthmatic subjects are more likely to experience significant drops in peak flow values. The authors advocate that peak flow should be monitored and bronchodilators should be used prior to amphotericin B inhalation for known asthmatic subjects. Fourth, an occasional patient with no known history of asthma will consistently experience adverse symptoms with amphotericin B inhalation. It is clear from this study that whilst side effects do occur, neither the frequency nor severity of reactions appears to preclude the use of inhaled amphotericin B in the majority of patients. However, the specific benefit as prophylaxis against pulmonary aspergillosis has still to be resolved. It is possible that nebulized liposomal amphotericin B (AmBisome) may provide greater protection. In terms of drug levels in alveolar sacs and lung tissue of mice, aerosolization and delivery of liposomal amphotericin B was shown to be far superior to that of conventional amphotericin B and that liposomal amphotericin B was more effective than conventional amphotericin B when given as a prophylactic aerosol in clearing mouse lungs of *Aspergillus*, although this was very much inoculum concentration dependent (Allen et al. 1994).

Oral itraconazole prophylaxis (see below) combined with intranasal amphotericin B is another approach (Todeschini et al. 1993). Here, itraconazole capsules (200 mg/day) combined with intranasal amphotericin B (10 mg/day) was prescribed as prophylaxis in 164 patients. The incidence of proven aspergillosis was lower, as was the mortality rate. However, more experience is needed before this drug combination can be recommended for daily clinical practice, especially as the oral solution formulation of itraconazole will undoubtedly supersede the capsule form for routine prophylaxis.

Low-dose amphotericin B

The value of low-dose amphotericin B (0.15–0.25 mg/kg/day) as prophylaxis against *Aspergillus* spp. in patients undergoing allogeneic bone marrow transplantation has been explored in a number of studies (Rousey et al. 1991; Perfect et al. 1992). The data do not suggest a benefit compared with historical controls. For example, in a placebo-controlled prospective study involving 188 patients who underwent autologous bone marrow transplantation, amphotericin B (0.1 mg/kg daily) did not prevent aspergillus infection compared to the placebo group. One aspergillus infection was documented in each group. Prophylaxis was discontinued once an individual developed a persistent fever for 5 days despite treatment with broad-spectrum antibiotics. Suspected fungal infections were treated empirically with amphotericin B 0.6 mg/kg/day. In this study amphotericin B at 0.1 mg/kg/day did not prevent the emergence of aspergillosis compared with placebo.

The conclusions drawn from these earlier studies of the benefit of low-dose amphotericin B as prophylaxis against invasive aspergillosis are not clear. Overall, low-dose amphotericin B does not appear to provide a protective effect compared with placebo, but there does appear to be a possible effect in decreasing mortality.

Liposomal amphotericin B

The proven efficacy and improved toxicity profile of liposomal amphotericin B (AmBisome) prompted Tollemar and colleagues to evaluate the role of this lipid formulation of amphotericin B in preventing fungal infections after bone marrow transplantation (Tollemar et al. 1993). Thirty-six patients received the drug, at a dosage of 1 mg/kg for between 1 and 11 weeks, and 40 patients received no treatment. Fungal colonization decreased in the AmBisome group whereas it increased in the placebo group. By the end of prophylaxis, eight of 24 (33 percent) patients who received AmBisome were colonized compared with 18 of 29 (62 percent) patients in the placebo group. Presumed fungal infection occurred in five of 36 patients given AmBisome. None of these infections was fatal. In the control group, seven patients developed presumed fungal infection and two died. Proven fungal infection occurred in one patient receiving AmBisome (*Candida guilliermondii*) compared with three patients receiving placebo (*C. guilliermondii*, two; *C. albicans*, one). The study showed possible benefits regarding the reduction of proven fungal infection in the patients given AmBisome prophylactically. A beneficial effect of prophylaxis was found as none of the patients with a suspected fungal infection in the AmBisome group died with proven fungal infection compared with two placebo-treated patients.

The value of AmBisome as a prophylactic agent has been examined further by Kelsey et al. (1999) where AmBisome 2 mg/kg was compared with placebo in patients undergoing chemotherapy or bone marrow transplantation. Prophylaxis began on day 1 of chemotherapy and continued until neutrophils regenerated or infection was suspected. Of 161 evaluable patients, 74 received AmBisome and 87 received placebo. Proven fungal infections developed in no patients on AmBisome and in three patients on placebo. Suspected fungal infections requiring intervention with systemic antifungal therapy (usually amphotericin B) occurred in 31 patients (42 percent) on AmBisome and in 40 on placebo (46 percent)

Itraconazole

Itraconazole is an orally active triazole with a wide spectrum of activity which has been successfully used in the prevention of aspergillus infection.

Itraconazole has been compared with ketoconazole and nystatin in neutropenic patients in retrospective

analyses, and was found to be superior (reviewed by Beyer et al. 1994b). In a placebo-controlled, randomized trial of itraconazole prophylaxis, 400 mg/day reduced the overall incidence of proven fungal infection to nine of 83 neutropenic episodes (11 percent), compared with 15 of 84 episodes (18 percent) in the placebo arm (Beyer et al. 1994b). However, it should be noted that this effect was mainly the result of a reduction of systemic candida infections and was not statistically significant. The incidence of suspected or proven cases of invasive pulmonary aspergillosis was similar in both the prophylactic itraconazole (five of 83 or 6 percent) and the placebo (four of 84 or 5 percent) arms.

The absorption of itraconazole from the gastrointestinal tract varies within a wide range and is largely unpredictable, so itraconazole doses higher than 400 mg or therapeutic drug monitoring might be necessary for effective antifungal prophylaxis with this drug. There are a number of drugs that interfere with the absorption of itraconazole, among which are agents such as antacids that are commonly used in neutropenic patients. Further trials including larger numbers of patients are needed to make a definitive statement on the effectiveness of itraconazole for the prophylaxis of invasive pulmonary aspergillosis. To facilitate the prophylactic use of this agent, the availability of an itraconazole formulation with improved and more predictable bioavailability would clearly be beneficial.

ITRACONAZOLE ORAL SOLUTION

The known problems associated with the bioavailability of the capsule form in neutropenic patients have prompted the development of an oral solution of itraconazole. This bioavailablilty problem is thought to arise because gastrointestinal mucositis (following intensive cancer chemotherapy or radiotherapy) can make drug dissolution and absorption erratic, leading to abnormally low blood levels and lack of drug efficacy in some cases. It appears that this formulation has improved pharmacokinetics and more predictable bioavailability in neutropenic patients (Prentice et al. 1994; Michallet et al. 1998). In this study trough serum concentrations of itraconazole were measured weekly during prophylaxis with itraconazole liquid, 2.5 mg/kg b.i.d. in neutropenic patients undergoing treatment for hematological malignancies. From week 2 of prophylaxis, at least 84 percent of patients had itraconazole plasma concentrations higher than 250 ng/ml; this concentration is considered the minimum level required for antifungal efficacy. These pharmacokinetic findings have been confirmed by a much larger study where the value of prophylactic itraconazole solution was compared with fluconazole suspension (see below).

There has been a considerable expansion in the literature on the use of the itraconazole oral solution as prophylaxis against aspergillosis (see, for example, Foot et al. 1999; Glasmacher et al. 1999a, b; Kibbler 1999; Menichetti et al. 1999; Harrousseau et al. 2000; and reviewed in a *Journal of Antimicrobial Chemotherapy* supplement (in press).

ITRACONAZOLE LIQUID COMPARED WITH ORAL AMPHOTERICIN B PROPHYLAXIS

A randomized, double-blind, double-dummy study compared the efficacy of itraconazole liquid with oral amphotericin B capsules in patients with hematological malignancy and profound neutropenia (Harrousseau et al. 2000). The incidence of fungal infections was compared between groups and comparisons were also made of the frequency and time and initiation of intravenous amphotericin B rescue therapy. A total of 557 patients were randomized to receive either itraconazole liquid, 2.5 mg/kg body weight b.i.d. (n=281) or oral amphotericin B, 2×250 mg capsules q.i.d (n=276). Prophylaxis was initiated with chemotherapy and continued until the neutrophil count recovered or another study point was reached, up to a maximum of 8 weeks. Although not reaching statistical significance, there was a trend in favor of itraconazole liquid for proven invasive aspergillosis (five versus nine cases) as well as overall documented deep fungal infections including aspergillosis (eight versus 13 cases). One of eight patients died in the itraconazole group in contrast to five of 13 in the amphotericin B group. In this study plasma levels of itraconazole were measured and adequate levels (>250 ng/ml) were attained in at least 80 percent of patients 10–14 days after the start of treatment. After 4 weeks of prophylaxis, these levels were reached in 96 percent of patients.

ITRACONAZOLE LIQUID COMPARED WITH FLUCONAZOLE PROPHYLAXIS

Itraconazole in its liquid suspension form (5 mg/kg) has been compared with fluconazole suspension (100 mg) in a randomized trial where either itraconazole or fluconazole was given from the start of cytoreduction to neutrophil recovery in 581 neutropenic episodes in 445 adults receiving a bone marrow transplant or chemotherapy for hematological malignancy (Morgenstern et al. 1999). The dose of fluconazole suspension was chosen based on accepted practice at the time the study commenced. There were more proven cases of invasive aspergillosis in patients given fluconazole prophylaxis compared with itraconazole (four in the fluconazole arm, none in the itraconazole arm), demonstrating the superiority of itraconazole in suppressing invasive aspergillosis in immunocompromised patients. However, there were more gastrointestinal disturbances associated with itraconazole. These are most likely due to the cyclodextrin vehicle used in the solution formulation.

SAFETY OF ITRACONAZOLE LIQUID FOR ANTIFUNGAL PROPHYLAXIS

Safety information from data-on-file studies and summaries in abstract form is available for about 1 000 patients.

Adverse experiences observed frequently in these patients included nausea, vomiting, mucositis, diarrhea, and fever. Biochemical abnormalities frequently occurring for all treatment groups included decreases in calcium, potassium, and uric acid and increases in urea and bilirubin. The incidence and nature of adverse events reported in these studies to be definitely related to prophylactic drug treatment were similar for itraconazole liquid, oral amphotericin B, and placebo and were mainly gastrointestinal in nature.

ITRACONAZOLE ORAL SOLUTION AND CHILDREN

Itraconazole oral solution has been evaluated in neutropenic children undergoing an allogeneic bone marrow transplant (Foot et al. 1999). In an open study of antifungal prophylaxis in 103 children, patients received 5.0 mg/kg itraconazole oral solution per day. Prophylaxis was started at least 7 days before the onset of neutropenia and continued until neutrophil recovery. No proven systemic fungal infections occurred during the study but 26 patients received intravenous amphotericin B for antibiotic-unresponsive PUO. One patient received amphotericin B for mycologically confirmed esophageal candidosis. Three patients developed suspected oral candidosis but none was mycologically proven and no treatment was given. Serious adverse events occurred in 27 patients. The most common adverse events attributable to itraconazole were vomiting, abnormal liver function, and abdominal pain. This study indicates that itraconazole oral solution may be used as antifungal prophylaxis in neutropenic children.

PREVENTION

Prevention of aspergillosis is relatively difficult. Simple precautions – such as eliminating potted plants from patients' rooms and using barriers during hospital construction – are recommended. The use of high-efficacy particulate air (HEPA) filters appears to be the only currently effective means of decreasing the incidence of aspergillus infection.

The principles of environmental control of nosocomial aspergillosis are complex given that even HEPA units are not completely effective in preventing disease. Fungal exposure would be more precisely studied using a personal air sampler for the patient, but there is no fungal sampler currently available which can be used in this way, and there are also severe technical limitations on the duration of the sampling time of available fungal samplers (Morris et al. 2000; Thio et al. 2000). Alternatively, a systematic program of longitudinal patient and environmental surveillance may predict cases of invasive aspergillosis. Indeed, there appears to be a correlation between the recovery of *Aspergillus* spp. from the nose and mouth of patients in an open hematology ward and an elevated number of conidia in the air (Richardson et al. 2000).

The relationship between aspergillosis in predisposed patients and building work is also complex. Hospitals are buildings of continuous change and adaptation, so construction is an inevitable prospect which may extend throughout the year. Whether or not this activity is complicated by an outbreak of infection in the susceptible patients nearby, or is a risk related directly to the amount of disruption or some other factor, is unknown. In one study, an increase in the number of patients with invasive aspergillosis could not be explained by an increase in the number of aspergillus conidia in the outside air (Leenders et al. 1998). Genotyping of the isolates from the unit where the survey was carried out showed that clonally related isolates were persistently present for more than 1 year. Clinical isolates of *A. fumigatus* obtained during the outbreak period were different from the clones normally persistent in that environment.

Currently, the environmental mycology of most outbreaks of nosocomial aspergillosis is poorly defined. However, the development of molecular biology techniques more directly applicable to *Aspergillus* spp. may help resolve some of these difficulties.

Aspergillus spp. have a major reservoir in organic debris, bird droppings, dust, and building material. The principal approach in the prevention of aspergillosis is to minimize patients' exposure to aspergillus conidia by filtering air or initiating some form of patient isolation (Sherertz et al. 1987; Rhame 1991; Hay 1993). Further steps consist of elimination of obvious sources of aspergilli, such as removing plants from the surrounding environment of a patient. In some instances surface disinfection with copper-8-quinolinolate has been reported to be effective. Susceptible patients should not be treated in areas where there is construction or demolition activity, and if such activities are under way, measures should be instigated to seal these sites to prevent air exchange with the patients' environment.

Certain foodstuffs, such as cereals, nuts, and spices, e.g. ground black pepper, have been found to be contaminated with aspergilli and should not be offered to patients at risk of developing invasive pulmonary aspergillosis (reviewed by Beyer et al. 1994b). Although outbreaks of invasive aspergillosis have been associated with construction within or around a hospital, the precise source of the fungus is occasionally difficult to trace with certainty (Opal 1986; Iwen et al. 1994; Anderson et al. 1996). There have been few studies that have prospectively examined the aeromycology in and around a hospital during major building alterations and then compared these findings with samples from patients and the incidence of invasive aspergillosis (reviewed by Beyer et al. 1994b).

In a seminal study (Goodley et al. 1994), the authors took advantage of the opportunities that arose during

widespread building operations around their hospital where several groups of patients seemed at risk of fungal infection – in wards for renal transplantation, bone marrow transplantation, oncology, and intensive care. Air samples were taken in these wards (by SAS Sampler, pbi International, Milan, Italy) and various outdoor sites around the hospital, at specific sites throughout the hospital, sequentially throughout the year as well as in particular areas during periods of construction activity. Nasal swabs were also taken from patients for comparison with the air sampling results. The most commonly isolated fungal species was *A. fumigatus*. Nasal swabs were positive in 12 of 188 samples – 11 *A. fumigatus* and one *Aspergillus sydowi*. Most of the air samples cultured less than 10 c.f.u./m^3 throughout the year. A peak of higher counts occurred in March (190 c.f.u./m^3, confirmed at various sites) which could not be explained either by building work or by meteorology. Eight of the positive nasal swabs were obtained during March; three cases of invasive aspergillosis developed through the year and did not seem to be related to the spell of higher spore counts. One of the buildings was demolished, but there was no significant rise in spore counts and no change in the background pattern of fungal isolation in the wards or the corridors. Air sampling was repeated over the following year when a peak was recorded in June at 90 c.f.u./m^3, and very similar low levels throughout the rest of the year. The authors' interpretation of the results was that, because cases of invasive aspergillosis seemed to develop at low spore levels, then all highly susceptible patients should have protective isolation (HEPA ventilation and sterile management procedures). Routine nasal swab sampling was not proposed as an alternative to air sampling. Avoidance measures appear to be appropriate if minimal exposure is the only component necessary to induce invasive aspergillosis in transplant recipients.

The debate concerning the value of laminar air flow (LAF) rooms in protecting high-risk patients from *Aspergillus* has continued. Wald and coworkers conducted a retrospective analysis of aspergillosis in bone marrow transplant recipients (Wald et al. 1997). One of the conclusions of this study was that the use of LAF rooms is protective against early, but not late, aspergillosis, during the transplant course. The conclusions in this study were questioned by Viscoli (1998), who was of the opinion, based on personal experience and the existing literature, that even if it was shown that LAF rooms were associated with a reduced risk of early aspergillosis, it is unlikely that this benefit resulted from the use of the LAF room system as a whole, but rather by the air filtration through the HEPA filters. The opinion was offered that the use of LAF rooms in bone marrow transplant recipients should not be encouraged without convincing supporting data.

A preventative strategy has been proposed by a consortium of The Mycologic Study Group of the French Society of Hematology and The Research Group on Fungal Infections, and The European Group for Research on Biotypes and Genotypes of *Aspergillus* Network (Gangneux et al. 2002). The salient points of this strategy are as follows:

- Air-control measures remain crucial for the reduction of environmental dissemination of fungal conidia. The consortium's premise is that the nosocomial origin of aspergillosis has been convincingly demonstrated in epidemic situations. A number of investigations have shown that the concentration of *Aspergillus* spp. and other fungi in the air in a hematology unit correlated with the incidence of invasive aspergillosis in nonepidemic situations. Thus, it is strongly recommended that patients who are at high risk of infection benefit from the measures put forth by the Centers for Disease Control and Prevention, that is, the use of HEPA filtration, use of LAF systems, high rates of room-air change, use of positive pressure, and use of well-sealed rooms.
- Air-control efficiency must be monitored. Several years ago, a regular monitoring of environmental fungal contamination (for *Aspergillus* and other airborne fungal species) was started, with two major goals: to detect increases in conidia density and to assess air-control efficiency. Environmental monitoring requires the following:
  - air sampling with an efficient biocollector
    surface sampling with contact petri dishes or swabs, which is a simple and efficient monitoring method that can detect minor contamination, even when concomitant air samples test negative for fungi
  - sampling of patient rooms that are equipped with HEPA filters, with or without LAF, and sampling of all parts of the ward that are provided with air filtration and positive pressure (particularly corridors)
  - use of guidelines for patient management and cleaning procedures in protective environments, which should be adapted according to the results of monitoring.
- Determination of the baseline values of air and surface concentrations of *Aspergillus* spp. and other fungal conidia is essential for valuable assessment of any further increase in fungal contamination. Given the genetic diversity of *A. fumigatus* isolates, and given the current limitations of molecular typing methods to localize the fungal source or to date the infection, environmental sampling is not, in the authors' opinion, simply a means of comparing environmental isolates with clinical isolates.
- The sources and routes of conidia transmission are unclear. It has been suggested that aspergillosis is waterborne, and that nosocomial aspergillosis can be airborne from water sources in the hospital setting.

However, this is not a unversal finding. It is apparent that the environmental risk of invasive aspergillosis linked to water should be interpreted according to the local situation, namely, the source of water (underground versus surface water). In addition, because humidity favors fungal growth, high fungal densities near water sources may simply reflect the presence of conidia in the air or on surfaces and indicate the need for new cleaning procedures. Furthermore, the biotope of *A. fumigatus* differs from that of *Fusarium* and *Acremonium* spp.

Alternative sources of conidia inhalation should not be neglected. These sources include the clothing of visitors and medical staff as well as personal and medical materials. Therefore, specific preventive measures may be appropriate. The gastrointestinal route of infection is rarely considered; however, a high rate of food contamination by filamentous fungi, such as *Aspergillus* spp. and zygomycetes, has been reported. The existence of isolated gastrointestinal filamentous fungal infection without pulmonary or disseminated infection supports the hypothesis that, in addition to the risk of conidia inhalation, contact with food or water can lead to conidia absorption.

The authors' conclusions and recommendations in this consensus document are many-fold. Aspergillosis mainly occurs in neutropenic patients. Air-control measures are presently the most effective way of significantly reducing the incidence of nosocomial aspergillosis. Although they are expensive and only partially effective, these measures should not be called into question, as shown by their protective effect against early infections after bone marrow transplantation. However, the efficiency of air-control measures should be continuously monitored by regular measurement of environmental levels of fungal conidia. This approach is strongly recommended in units that use air-control measures, as are specific investigations of cases of aspergillus infection. However, there should be more investigation and control of alternative sources of contamination. Prevention of delayed acquired aspergillosis is even more difficult: control of environmental sources of contamination and follow-up of aspergillus colonization are difficult outside the hospital setting, and, until now, there has been no demonstration that any antifungal drug or cytokine can significantly prevent or reduce the risk of aspergillosis in patients who are at risk of infection.

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Deep phaeohyphomycosis

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The incidence of fungal infections in humans has been rising for the past several decades due to a number of diverse reasons, some of which include social and geopolitical factors (Dixon et al. 1996; Cooper 2002). The primary dynamic leading to this marked increase in fungal infections has been the expanding population of immunocompromised individuals. Moreover, the etiologic agents in many of these cases are misplaced saprotrophs, i.e. opportunistic pathogens. Such organisms tend to be benign towards humans and animals, rarely causing disease. These fungi exist in nature by gathering nutrition via consuming organic debris or through parasitic relationships with living plants. Yet, under the proper conditions, these opportunists can readily exploit a dysfunctional mammalian immune system to cause severe cutaneous, subcutaneous, and deep-seated disease.

One group of opportunistic fungal pathogens is characterized by the single shared characteristic of possessing a darkly pigmented appearance. The pigment has been demonstrated to be a melanin produced via a pentaketide pathway (Wheeler and Bell 1988). Traditionally, the dark color of these fungi caused them to be designated as 'dematiaceous'. However, the use of the word 'dematiaceous' to describe melanized fungi has been argued to be epistemologically incorrect (Pappagianis and Ajello 1994). Subsequently, the term 'phaeoid' has been used to replace the prior designation. Nonetheless, the descriptive word 'dematiaceous' has been so entrenched within the mycological literature that it is still used today in synonymy with the term 'phaeoid'. In this chapter, both expressions will be used interchangeably. Despite the semantic arguments, an important point needs to be emphasized with regard to the use of either designation – both have no real taxonomic significance. Rather, these terms refer to a heterogeneous collection of generally unrelated fungi that share the common phenotypic trait of being darkly colored under normal laboratory conditions.

The phaeoid (dematiaceous) fungi are a curious group of organisms capable of causing a wide array of clinical pathologies ranging from the superficial to the deep-seated infections (Perfect et al. 2003; Sanche et al. 2003). The focus of this chapter is placed upon those molds that cause the latter type in humans and animals. Such infections have been designated as visceral, systemic, or disseminated phaeohyphomycosis. In this chapter, these terms are reserved to describe a particular type of phaeohyphomycosis. The more general phrase, deep-seated phaeohyphomycosis (DSP), is proffered in their place. As discussed below, DSP can be divided into disseminated phaeohyphomycosis as well as a second clinical entity, cerebral phaeohyphomycosis. While sharing many features, these two categories of disease caused by dematiaceous fungi are disparate in several epidemiologic, etiologic, and clinical aspects. These differences are presented, as are the general diagnostic and intervention methods for both types of DSP. Also, the potential relationship of certain biological features to virulence is discussed. However, a comprehensive description regarding the mycology and taxonomy of the etiologic agents of DSP is not presented. For this, as well as a fuller discourse pertaining to superficial, cutaneous,

and subcutaneous diseases caused by pigmented fungi, the reader is referred to White piedra, black piedra and tinea nigra; and 18, Chromoblastomycosis in this volume as well as several recent reviews (Kwon-Chung and Bennett 1992; Matsumoto and Ajello 1998; Brandt and Warnock 2003; Perfect et al. 2003; Sanche et al. 2003; Schell et al. 2003).

PHAEOHYPHOMYCOSIS – HISTOPATHOLOGICAL AND CLINICAL CONCEPTS

Dematiaceous fungi are etiologic agents responsible for a number of diverse diseases in humans and animals (Table 1). Though darkly pigmented in culture, not all these fungi appear so in vivo. In addition, studies have indicated that some fungal pathogens traditionally considered not to be phaeoid (e.g. *Cryptococcus neoformans*) do apparently either make minute amounts of melanin or have the ability to do so in vivo (Kimura and McGinnis 1998). This has led to a range of difficulties and confusion in the diagnosis of these diseases and their corresponding causative agents (Kwon-Chung and Bennett 1992).

To help resolve these problems, Ajello and colleagues proposed the terms 'phaeohyphomycosis' and 'hyalohyphomycosis' to distinguish between those fungal infections caused by phaeoid and hyaline molds, respectively, and which exhibit a predominant filamentous morphology in vivo (Ajello et al. 1974). In particular, these medical mycologists envisioned phaeohyphomycosis to encompass cutaneous, subcutaneous, and systemic infections in which the etiologic agent 'develops in the host tissues in the form of dark-walled dematiaceous septate mycelial elements.' Thus, this designation defined the infection based upon its histopathology rather than its clinical presentation. However, the original definition was limited to hyphomycetous fungi, i.e. a class of molds taxonomically placed within the form-division Fungi Imperfecti [Deuteromycota; asexual (mitosporic) fungi]. As new cases of phaeohyphomycosis were reported, it became clear that not all were hyphomycetes. Subsequently, Ajello amended the definition of phaeohyphomycosis to include infections caused by fungi belonging not only to the form-class Hyphomycetes, but also those grouped within the form-class Coelomycetes (form-division Fungi Imperfecti) and division Ascomycota [fungi that form sexually derived spores (meiospores) within an ascus] (Ajello 1981).

As conceived, phaeohyphomycosis was intended to exclude the well-established disease states that also featured the formation of phaeoid hyphae in tissue, e.g. tinea nigra, chromoblastomycosis, sporotrichosis, and eumycotic mycetoma. These latter diseases are distinct, separate entities having their own unique clinical and histopathological presentation in addition to etiologic differences. The reader is referred to the appropriate chapters within this book (White piedra, black piedra and tinea nigra; Chromoblastomycosis; Sporotrichosis; and Eumycetomas, respectively) for a more in-depth discussion of these disease states.

As a cosmopolitan disease, phaeohyphomycosis mainly afflicts adults among whom many are immunocompromised. Underlying conditions that contribute to infection by dematiaceous fungi include tuberculosis, diabetes, cancer, tissue transplantation, and use of corticosteroids or other immunosuppressive drugs, surgery, and a number of other conditions including acquired immunodeficiency syndrome (AIDS) due to the human immunodeficiency virus (HIV) (Table 2).

Many cases of phaeohyphomycosis appear in the perfectly healthy and may have no evident exposure history. These non-life-threatening infections include focal-subcutaenous, mycotic keratitis, and paranasal sinus infections. Subcutaneous phaeohyphomycosis is a common site of infection and is generally thought to occur as a result of traumatic implantation of fungal material from contaminated plants or soil. The most common outcome of this type of inoculation is a localized cyst or abscess that appears primarily as a single, discrete and asymptomatic or mildly painful nodule. From this entity, granulomatous reactions may occur and the disease may on occasion progress to produce verrucose plaques reminiscent of chromoblastomycosis.

Table 1 *Fungal infections with phaeoid etiologies*

Infection
Black piedra
Chromoblastomycosis
Eumycotic mycetoma
Phaeohyphomycosis
Pseudallescheriasis
Sporotrichosis
Tinea nigra
Zygomycosis

Table 2 *Risk factors associated with phaeohyphomycosis*

Risk factor
Bone marrow transplantation
Cancer
Cardiac surgery
Chronic ambulatory peritoneal dialysis
Corticosteroid therapy
Diabetes
Fungal sinusitis
HIV infection
Intravenous drug use
Neutropenia
Solid organ transplantation
Trauma to skin or soft tissues
Tuberculosis

Mycotic keratitis also is a common site for this fungal infection where asexual spores of these fungi that are common in our environment can contaminate a corneal abrasion following trauma. Paranasal sinus infections are also frequently diagnosed. They present with sinuses impacted with a phaeohyphomycotic ''fungoma'' seen in biopsy. Frequently, there is an allergic host response to the fungus as evidenced by the presence of eosinophils. Rarely is this type of sinus infection highly invasive. These non-life-threatening infections are caused by the same genera/species that cause deep-seated infections implying the importance of the host in the dynamics of the phaeoid fungus/host interactions.

By comparison, DSP is rare. Recently, Revankar and colleagues (Revankar et al. 2002, 2004), as well as Filizzola et al. (Filizzola et al. 2003), published reviews focused on DSP. Case reports from 1996 through 2002 were obtained from citations listed in the Medline database (National Library of Medicine, Bethesda, MD). Additional cases of DSP were reviewed from other references cited in these reports. Though not fully comprehensive, since non-English language literature was generally excluded, these reviews clearly separate DSP into two broad groups of infections. In one category of DSP, infection is disseminated throughout the body and may involve the central nervous system (CNS). Most individuals affected with disseminated disease tend to have decreased host immunity or possess other risk factors. A second distinct type of DSP is generally restricted to the CNS. Curiously, about half of the victims of cerebral phaeohyphomycosis do not appear to possess underlying conditions or risk factors predisposing them to infection. Both types of DSP are discussed in greater detail below.

It is significant to note, however, that DSP appears to involve only a subset of fungal species listed as etiologic agents of phaeohyphomycosis (Matsumoto et al. 1994; Matsumoto and Ajello 1998). Of the more than 100 species among the 60 genera associated with infection by dematiaceous fungi, about a third are known to cause DSP (Table 3). [The specific epithets presented in Table 3 and elsewhere in the text represent the currently accepted nomenclature for a particular fungal species. Previously employed binomials for fungal species causing phaeohyphomycosis, as well as a detailed mycological description of these etiologic agents, can be readily found in several publications (Kwon-Chung and Bennett 1992; Matsumoto and Ajello 1998; Perfect et al. 2003; Sanche et al. 2003; Schell et al. 2003).] This list should not be considered comprehensive since new cases of DSP are likely to be reported that involve species not listed. Moreover, not all medical mycologists may agree with the inclusion of certain fungi as agents of phaeohyphomycosis (see discussion below). Nonetheless, the fungal species presented here typify those that have been adequately documented as the major etiologic agents of DSP.

Table 3 *Fungal species associated with DSP* [a]

Fungal species
Acropophialophora
Alternaria infectoria
Amium leproinum
Aureobasidium pullulans
Bipolaris
B. australiensis
B. hawaiiensis
B. specifera
Bipolaris spp.
Chaetomium
C. atrobrunneum
C. globosum
C. perlucidum
C. strumarium
Cladophialophora
C. bantiana
C. devriesii
C. modesta
Cladophialophora spp.
Cladosporium cladosporoides
Curvularia
C. geniculata
C. lunata
C. pallescens
Exophiala
E. castellanii
E. jeanselmei
E. mansonii
Exserohilum rostratrum
Fonsecaea pedrosoi
Hormonema dematioides
Lecythophora mutabilis
Microascus cirrosus
Myceliophthora thermophila
Nodulisporium spp.
Ochroconis gallopavum
Phaeoacremonium parasiticum
Phialemonium curvatum
Phialophora richardsiae
Rhamichloridium obovoideum
Rhinocladiella atrovirens
Scopulariopsis
S. brumptii
Scopulariopsis spp.
Scytalidium dimidiatum
Wangiella dermatitidis

a) Data collected from Revankar et al. (2002, 2004); Barron et al. (2003); Filizzola et al. (2003); Teixeira et al. (2003).

The list of phaeohyphomycotic agents given in Table 3 is notable in its exclusion of *Scedosporium* spp. [*Scedosporium apiospermum* (anamorph of *Pseudallescheria boydii*) and *Scedosporium prolificans* (formerly *Scedosporium inflatum*)] as agents of DSP.

The inclusion of these fungi as a cause of phaeohyphomycosis has been a source of controversy and needless consternation (Matsumoto et al. 1994). Some investigators persist in embracing *Scedosporium* spp. in the etiology of phaeohyphomycosis (Revankar et al. 2002, 2004; Schell et al. 2003). Others seemingly do not agree with this position, yet continue to include this organism in the description of possible causative agents (Matsumoto and Ajello 1998; Perfect et al. 2003). Moreover, some recent publications clearly state that *Scedosporium* is an agent of hyalohyphomycosis (McGinnis and Ajello 1998; Tadros et al. 1998; Walsh 1998; Torres and Kontoyiannis 2003) or the form-genus is conspicuously omitted as a phaeohyphomycotic agent (Sanche et al. 2003). The confusion is further compounded by the fact that several authors of the above-cited publications have taken both positions.

Perhaps the source of this chaos lies in the misapplication of the true, intended definition of phaeohyphomycosis. As noted above, this clinical entity is based upon the histopathological observation of 'dark-walled dematiaceous (i.e. phaeoid) septate mycelial elements' in diseased tissue (Ajello et al. 1974; Ajello 1981). As dematiaceous fungi, *Scedosporium* spp. do form a melanin-like color in culture (Schell et al. 2003). These species also stain positive for melanin in vivo, using the Fontana–Masson silver stain, which is specific for such pigments (Wood and Russel-Bell 1983). The histopathology of tissues infected with *Scedosporium* spp. is also similar to phaeohyphomycosis, but more strongly resembles the dichotomous hyphal branching seen in aspergillosis (Chandler and Watts 1987; Cooper and Salkin 1993). However, the hyphae observed in these tissues are hyaline except when aggregates are formed such as in a pulmonary 'fungoma' (fungus ball). Brown conidia are sometimes observed in the latter. Yet, a fungoma due to *Scedosporium* clearly represents a separate clinical presentation that tends not to be invasive (Cooper and Salkin 1993). Therefore, despite the occasional presence of pigmentation, inclusion of *Scedosporium* spp. as etiologic agents of phaeohyphomycosis is inconsistent with the present definition of this disease state. Unless the description of this term is amended formally, clinicians should be aware that not all phaeoid fungi are agents of phaeohyphomycosis. Indeed, some melanized fungi cause infections better attributed to markedly different, classical presentations (e.g. sporotrichosis, chromoblastomycosis, etc.). At the risk of eliminating more fungi listed in Table 3, if a particular mold species does not consistently form dark-colored hyphae in vivo, it should be considered an agent of hyalohyphomycosis. Such is the case with *Scedosporium* spp. and is a position more in line with the accepted definitions first proposed by Ajello and colleagues (Ajello et al. 1974; Ajello 1981).

This same argument, however, might be used against other dematiaceous fungi generally acknowledged by medical mycologists to be phaeohyphomycotic agents. For example, species of *Bipolaris*, as well as other fungi, do not always produce pigmented fungal elements in tissue. In one such case involving *Bipolaris spicifera*, the clinical isolate did form dark-colored hyphae in experimentally infected mice (Yoshimori et al. 1982). Given the ever-increasing spectrum of pathogenic dematiaceous fungi and the inconsistent nature of pigment production in vivo, perhaps the definitions of phaeohyphomycosis and hyalohyphomycosis need to be re-evaluated. However, except in the clinical presentation of fungoma, *Scedosporium* spp. do not produce visible melanin in vivo despite the large number of reports of disease involving these species. Collectively, these cases certainly outnumber those of phaeohyphomycosis caused by the most frequently encountered dematiaceous fungi in which melanin is consistently visible in tissues.

CLINICAL MANIFESTATIONS OF DSP

DSP can be divided into the separate clinical presentations of systemic disease and cerebral involvement. These different manifestations share the same histopathological concept – the presence of darkly pigmented hyphae in vivo. In broad terms, many of the symptoms, treatment outcomes, and types of patients are common to both. Yet, each does have unique aspects that distinguish one from the other. The following succinct descriptions compare both the mutual and exclusive aspects of disseminated and cerebral phaeohyphomycosis.

Disseminated phaeohyphomycosis

Compared to cutaneous and subcutaneous forms of phaeohyphomycosis, there are relatively few reports of disseminated disease by phaeoid fungi in the medical literature. Although infrequent in morbidity, disseminated phaeohyphomycosis deserves special consideration as a separate disease type given its level of mortality (Revankar et al. 2002). Disseminated disease results by the spread of the dematiaceous pathogen from a previously colonized or infected body site (Kwon-Chung and Bennett 1992; Brandt and Warnock 2003; Perfect et al. 2003; Sanche et al. 2003). Trauma or surgery is typically a cause of cutaneous or subcutaneous infection sites. In addition, dissemination can originate from the lungs or infected sinus tissue following inhalation of fungal cells or conidia. Under the appropriate circumstances, direct extension from the sinus or hematogenous spread of the fungus to one or more distant sites of the body can result in visceral infections of the heart and heart valves, brain, joints, bone, kidney, liver, lymphatics, pancreas, and other organs. Cases of dialysis-associated peritonitis have also been shown to be caused by dematiaceous fungi.

Recently, Revankar and co-workers (Revankar et al. 2002) reviewed 72 case studies of disseminated phaeohyphomycosis published in the English language literature between 1996 and 2001. The authors considered each of these cases valid based upon the following criteria:

- the presence of a clinical syndrome consistent with disseminated disease
- histopathological evidence of a true infection
- the recovery and identification of a dematiaceous fungus.

Included in this review were 30 infections caused by *S. prolificans*. As previously noted, infection by this fungus is better termed hyalohyphomycosis. Therefore, the 30 cases cited by these investigators are excluded from the following discussion. The remaining 42 infections, though drawn from a selected pool of reports, do represent a general overview of disseminated disease caused by dematiaceous fungi.

Most of the 27 species cited by Revankar et al. (2002) as etiologic agents are described in single case reports. This reflects the uncommon nature of disseminated phaeohyphomycosis. Of the 42 patients studied, 35 (83 percent) possessed some risk factor predisposing them to infection, including two cases of AIDS. In addition, some of the apparently normal individuals exhibited eosinophila. This suggests that certain patients have developed a poor TH2 response, thereby hindering cell-mediated immunity and inadvertently promoting dissemination of the infection. Clinically, the most common symptoms among the 42 affected individuals included fever, skin manifestations, respiratory complaints, and neurological problems. Sepsis and gastrointestinal symptoms were also recorded. With regard to the sites of infection, half the cases involved the lungs. Other significantly affected organs included the brain, heart/heart valve, kidney, liver, and lymph nodes. In some instances, the spleen and pancreas were involved. Death resulted in 29 (70 percent) of the 42 cases, including five patients who did not receive antifungal therapy. The remaining 13 patients received surgical intervention, antifungal therapy, or both. Amphotericin B alone or in combination with other drugs and/or surgery was used in 10 cases. However, specific therapeutic recommendations based upon the outcome of these treatment regimens are not possible given the general resistance of dematiaceous fungi to amphotericin B as well as the number of diverse species implicated in these infections. Yet, the results do make it clear that no single drug or combination therapy improved mortality rates.

Cerebral phaeohyphomycosis

Cerebral infections by phaeoid fungi represent a specialized type of DSP. For instance, most of the commonly encountered dematiaceous fungi causing cerebral phaeohyphomycosis produce mainly brain abscesses as opposed to disseminated disease (Kwon-Chung and Bennett 1992; Filizzola et al. 2003; Sanche et al. 2003; Revankar et al. 2004). These fungi include *Cladophialophora bantiana*, *Ochronconis gallopavum*, *Ramichloridium obovoideum*, *Wangiella dermatitidis*, *Curvularia* spp., and *Bipolaris* spp. Studies of human infections and in animal models have shown with certainty that *C. bantiana* is neurotropic, i.e. it has a propensity to cause infection of the CNS (Dixon et al. 1987a, b). The remaining major etiologic agents also possess neurotropic tendencies (Walsh et al. 1987). Moreover, whereas disseminated phaeohyphomycosis mainly afflicts individuals having immunodeficiencies or other risk factors, nearly half of patients with cerebral phaeohyphomycosis are immunocompetent. There does appear to be some symptomatic overlap in that disseminated phaeohyphomycosis can also involve the CNS. However, the latter type of phaeohyphomycosis is usually accompanied by disease of multiple organs and typically originates by hematogenous spread from a primary focal infection. Cerebral phaeohyphomycosis, particularly in apparently normal hosts, usually does not involve other organs. Hence, these observations and the limited etiologic spectrum lend special consideration to cerebral phaeohyphomycosis as a separate type of DSP.

CNS infections by phaeoid fungi are distributed worldwide. Presumably, most infections that lead solely to brain abscess or meningitis occur initially from the inhalation of fungal propagules. Following colonization in the lung, the fungus is transferred via a hematogenous route to the CNS. Alternatively, chronic fungal sinusitis may serve as the cause of CNS invasion (Sanche et al. 2003; Schell et al. 2003). Phaeohyphomycotic agents commonly associated with this condition include species of *Bipolaris*, *Exserohilum*, *Curvularia*, *Alternaria*, and *Cladosporium*.

Clinical symptoms of cerebral phaeohyphomycosis include headache, fever, seizures, and neurological deficits. In contrast to bacterial infections of the CNS, these symptoms do not always occur together. Sudden onset of blindness may also occur mainly in those infections by *Bipolaris* or *Curvularia* due to compression of the optic nerve (Walsh 1998). Again, immunocompetent patients tend not to have signs of organ infection other than the CNS.

Abscesses in cerebral phaeohyphomycosis may occur singly or as multiple masses (Perfect et al. 2003; Sanche et al. 2003). The frontal lobe is the most common site for abscess formation, but other areas of the brain can be affected. Another type of presentation, either alone or in conjunction with abscess development, is meningitis. Reports of dematiaceous fungi in CSF, though very rare, have been documented.

Two independent surveys of the English-language literature from 1996 through 2002 have been published that describe authentic cases of cerebral phaeohypho-

mycosis. One survey focused solely on infection in the immunocompetent host (Filizzola et al. 2003), whereas the other was broader in scope (Revankar et al. 2004). Though not fully comprehensive, these two reviews provide a well-documented synopsis of the current status of cerebral phaeohyphomycosis. Collectively, both study groups cited 109 cases of cerebral phaeohyphomycosis. Each report was considered bona fide if it met specified clinical, histopathological, and mycological criteria that define phaeohyphomycosis. However, a single case due to the hyalohyphomycotic agent *S. prolificans* was incorrectly included (Revankar et al. 2004). Therefore, among the 108 valid case reports, a total of 25 phaeoid fungal species were identified. The number of infected males was three times that of females. Of all the patients afflicted with cerebral phaeohyphomycosis, 55 percent were immunocompetent. Only a single individual was HIV positive among those predisposed to infection. Overall mortality was 73 percent amid the 97 patients for whom an outcome was reported. Nearly the same mortality was reported for both apparently normal and at-risk individuals (74 and 71 percent, respectively). When cases caused by selected etiologic agents are considered, some marked differences in mortality exist (Table 4). With regard to *C. bantiana*, which was responsible for 44 percent of the cases reported, overall survival of the 42 patients followed was 29 percent. Interestingly, compromised individuals survived at a higher rate (45 percent) than normal hosts (23 percent) (Filizzola et al. 2003; Revankar et al. 2004). Though not quite significantly different (P=0.06), and the underlying reasons unclear, the limited data set does seem to suggest that immunocompetent persons with cerebral phaeohyphomycosis due to *C. bantiana* face a greater mortality risk. Comparable overall levels of mortality resulted from infections with *Bipolaris* spp. and *O. gallopavum*, though much fewer cases have been observed. By contrast, virtually all cases of cerebral phaeohyphomycosis involving *R. obovoideum*, *W. dermatitidis*, *Fonsecaea pedrosoi*, and *Chaetomium* spp. were fatal regardless of host immune status.

HISTOPATHOLOGY AND DIAGNOSIS OF DSP

Phaeohyphomycotic lesions exhibit distinct histopathologies depending upon the clinical form of the disease (McGinnis 1983; Chandler and Watts 1987). Moreover, the pathological features are similar regardless of the etiologic agent. In DSP, lesions of a given organ may occur as single or multiple abscesses that broadly resemble the subcutaneous cystic form of phaeohyphomycosis. Alternatively, infection may consist of an inflammatory infiltrate with the presence of necrosis. When formed, abscesses are usually circumscribed and encapsulated granulomas, the walls of which comprise epithelial histiocytes and giant cells surrounded by connective tissue. A pyogenic inflammatory response is common as well. The centers of the granulomas contain necrotic debris, fibrin, and degenerated polymorphonuclear cells.

Within the necrotic debris and inflammatory infiltrates, as well as in the giant cells, fungal elements can be observed. The morphology of these fungi may consist of septated hyphae (2–6 μm in diameter), pseudohyphae (moniliform hyphae), budding yeasts, enlarged subglobose cells (up to 25 μm in diameter), chlamydoconidia, or spherical cells divided internally in a single plane. The latter resemble the muriform (sclerotic) cells characteristically found in chromoblastomycosis (McGinnis 1983; Chandler and Watts 1987), but differ in having thinner cell walls and a single planate septum. Lesions may contain one or more of the above structures. The brown pigment in the walls of these fungal cells may be visible in hemaotoxylin and eosin-stained tissue sections. This is not always the case, however. For example, the fungal elements of the species of *Bipolaris*, *Curvularia*, and *Alternaria* often appear hyaline in vivo although these fungi do form melanin in culture. In such circumstances, the Fontana–Mason stain will help reveal if a potential dematiaceous fungus is present (Wood and Russel-Bell 1983). Still, fungi other than those causing phaeohyphomycosis (and the similar clinical entity, chromoblastomycosis) also stain positive using this method (Kimura and McGinnis 1998).

Table 4 *Survival rates of cerebral phaeohyphomycosis patients infected with selected etiologic agents*[a]

	Number of deaths per total number of patients				
Fungal species	Number of cases	Normal host	Compromised host	Outcome unknown	Overall death rate (%)
C. bantiana	48 (44%)	24/31	6/11	6	71
R. obovoideum	13 (12%)	4/4	5/5	4	100
Bipolaris spp.	9 (8%)	2/4	3/4	1	63
F. pedrosoi	5 (5%)	1/1	2/3	1	75
Chaetomium spp.	5 (5%)	0/0	5/5	0	100
O. gallopavum	5 (5%)	0/0	3/5	0	60
W. dermatitidis	4 (3%)	4/4	0/0	0	100

a) Data taken from Filizzola et al. (2003) and Revankar et al. (2004).

A presumptive diagnosis of DSP can be made if brown fungal elements are observed in vivo and are accompanied by a clinical presentation consistent with the disease. However, such observations do not indicate which pathogen is involved. It is critical to the therapeutic intervention in cases of systemic and cerebral phaeohyphomycosis that the etiologic agent be identified. Because phaeoid fungi can occur as contaminants, documentation that an infection is present requires that cultures be obtained from otherwise sterile body sites or from tissue in which the histopathology is consistent with DSP. For a fuller treatment regarding culture and identification methods for other major species of melanized fungi that cause phaeohyphomycosis, several resources can be consulted (McGinnis 1980; Dixon and Polak-Wyss 1991; Matsumoto and Ajello 1998; Sanche et al. 2003; Schell et al. 2003). In addition to identifying isolates by morphological characteristics, other phenotypic measurements that can be used include nitrate and carbohydrate assimilation tests, exoantigen tests, proteolytic activity, growth temperature response, etc. Generally, the biochemical and exoantigen methods have limited applications in the identification of dematiaceous fungi.

It is important to note that there is a paucity of molecular methods to identify dematiaceous fungi. Virtually all are based upon the identification or amplification of specific DNA from ribosomal or internal transcribed spacer regions (Abliz et al. 2003, 2004a, b; Hall et al. 2004). One commercially available method, the MicroSeq D2 large-subunit rDNA sequencing kit, shows some promise. Of the 80 phaeoid fungi tested, representing 36 separate species, 52 (65 percent) yielded identifications consistent with those derived by classical phenotypic methods (Hall et al. 2004). Also, this molecular-based identification method appears equivalent to the phenotypic procedures in terms of costs. However, more isolates of the same and different species need to be tested to determine the true efficacy of this kit. Despite the generally good results with this kit, though, it remains essential for the near future in medical mycology that requisite laboratory skills in the identification of fungi by more traditional phenotypic means continue to be developed and maintained.

TREATMENT OF DSP

The mortality rate of individuals having DSP, even if treated, is grimly high compared to that of persons receiving therapy for other forms of phaeohyphomycosis. Moreover, because publications on DSP have reported individual cases of disease and treatment, if any, there is relatively little information to recommend global treatment strategies. The situation is further exacerbated by the fact that these case reports collectively describe a diverse spectrum of species rather than a series of frequently encountered pathogens. It seems that each case of DSP should be approached therapeutically based upon its own particular circumstances. Nonetheless, the collective evidence discussed below seems to suggest some general guidelines for treatment of DSP as well as hope that newer antifungal agents will aid in the cure of infections caused by the phaeoid fungi.

Treatment of disseminated phaeohyphomycosis by surgical means is typically impractical. Prior to the development of effective azole and echinocandin drugs, chemotherapy instituted using amphotericin B, flucytosine, or a combination of both yielded mixed results (Revankar et al. 2002). The advent of azole and echinocandin classes of drugs, however, has added to the available antifungal armamentarium. The so-called older azole drugs, such as itraconazole, are very effective against dematiaceous fungi in vitro (McGinnis and Pasarell 1998b; McGinnis et al. 1998). In one landmark study, nine of 17 patients with phaeohyphomycosis showed a marked positive response to itraconazole therapy (Sharkey et al. 1990). In addition, many of the newer azoles show good promise both in vitro (Espinel-Ingroff 1998a, b; McGinnis and Pasarell 1998a) and in vivo (Graybill et al. 2004; Negroni et al. 2004). In a mouse model of disseminated phaeohyphomycosis due to *W. dermatitidis*, posaconazole reduced tissue burden and prolonged survival. Additionally, posaconazole provided a complete cure to a woman with a 12-year case of relapsing phaeohyphomycosis due to *Exophiala spinifera* that disseminated upon pregnancy. Though small in number, these experiences suggest that therapeutic regimens employing azoles alone may be effective in the treatment of systemic phaeohyphomycosis.

Cerebral phaeohyphomycosis should be treated aggressively given the rather poor clinical outcome in both normal patients and those at risk (Filizzola et al. 2003; Perfect et al. 2003; Revankar et al. 2004). For single brain abscesses, surgical debridement may be essential for survival. Even partial removal may facilitate recovery, particularly when combined with antifungal therapy. When surgery is contraindicated, a concerted therapeutic approach must be taken. Yet, treatment failures occur even with high doses of amphotericin B and flucytosine. This may be due in part to the innate or acquired resistance of dematiaceous fungi to these drugs. As with disseminated phaeohyphomycosis, the newer azoles and echinocandins show great potential in treating cerebral infections. Murine models of cerebral phaeohyphomycosis due to either *R. obovoideum* (Al-Abdely et al. 2000) or *C. bantiana* (cited in Sanche et al. 2003) indicated that posaconazole was superior to amphotericin B and itraconazole in prolonging survival and reducing fungal counts in the brain. In addition, Trinh and colleagues (Trinh et al. 2003) recently reported that voriconazole and caspofungin markedly delayed the progression in a premature infant of a fatal cerebral infection caused by *C. bantiana*.

In summary, the treatment options for DSP are generally limited due to the efficacy of available antifungal agents and the limitations of surgical interventions. The newer antifungals, especially posaconazole, appear to hold promise for future advances in curing these infections. Perhaps a more concerted effort to clinically assess the azoles in the treatment of DSP needs to be undertaken, and in particular comparing the in vitro susceptibility of the different causative agents with patient outcome. It seems critical that such 'real world' data be collected and analyzed in order to determine if the promise of the newer antifungals will be fulfilled. In addition, another avenue ripe for exploration is immune modulation. A complete understanding of the immune response to DSP is lacking. More information in this area is needed before potential protocols can be established to enhance or target a host's immune system towards prohibiting and eliminating infections by dematiaceous fungi.

BIOLOGICAL ASPECTS RELATED TO PATHOGENESIS

Given the wide array of phylogenetically diverse fungi causing DSP, it is unlikely that there will be a great number of universal themes pertaining to the underlying molecular mechanisms responsible for virulence. In fact, as in most pathogenic fungi, virulence is not dependent upon one or two factors acting alone, but rather two or more modalities acting in conjunction with each other. Recent reviews have emphasized these same points (van Burik and Magee 2001; Cole 2003).

The dematiaceous fungi do, however, share a few common themes associated with virulence. These factors are best exemplified by *W. dermatitidis*, which can serve as a paradigm for most phaeoid fungi (Szaniszlo 2002). This fungus is polymorphic and is a cause of subcutaneous and deep-seated phaeohyphomycosis. Moreover, under the appropriate culture conditions, or through the use of particular morphological mutants, *W. dermatitidis* will produce muriform-like cells that resemble the 'sclerotic body' characteristic of chromoblastomycosis Hence, this fungus may serve as a model to study this disease as well. Two particular attributes of *W. dermatitidis* that contribute to virulence are briefly summarized below.

Perhaps one of the best-studied virulence factors in *W. dermatitidis* is melanin. The genetics and biochemistry of the pentaketide biosynthetic pathway leading to melanin production has been extensively characterized in this pathogen (Cooper and Szaniszlo 1997; Feng et al. 2001; Szaniszlo 2002). Prior to the cloning of a key gene in this pathway, melanin-deficient mutants of *W. dermatitidis* were shown to be significantly less virulent in a murine infection model than the pigment wild-type strain (Dixon et al. 1987b, 1989a, 1992). Subsequently, a genetically derived null mutant, defective in the gene encoding polyketide synthase (*WdPKS1*), was shown to be similarly less virulent (Feng et al. 2001). The proposed mechanism by which melanin functions in pathogenesis relates to experimental findings that this pigment acts as an antioxidant, thereby promoting survival of the oxidative bursts produced by phagocytes (Jacobson and Tinnell 1993; Schnitzler et al. 1999; Jacobson 2000). Melanin may also bind host hydrolytic enzymes that target the fungal plasma membrane. These experimental results have dramatic implications for other dematiaceous fungi given that melanin production in most of these species involves the same biochemical pathway (Wheeler and Bell 1988). Indeed, melanins have been shown to be important virulence factors in a phylogenetically diverse number of fungi pathogenic for humans, animals, and plants (Langfelder et al. 2003).

Chitin synthase represents another virulence factor in *W. dermatitidis* and, by extension, the phaeoid fungi in general. Chitin is a crucial cell-wall polymer that plays a significant role in the morphogenesis of nearly all fungi. This polysaccharide and the enzymes involved in its biosynthesis have been extensively studied in *W. dermatitidis* (Szaniszlo 2002). Null mutants were derived in five different chitin synthase genes. In addition, mutants bearing various combinations of these mutations were generated. Collectively, these mutations produced a wide range of morphological and growth phenotypes. When selected mutants were incubated at temperatures equivalent to that found in infection, cellular development was significantly altered or stopped. Moreover, certain mutants able to grow well at these temperatures and not exhibiting observable cellular abnormalities were less virulent than the wild-type strain in both immunocompetent and immunocompromised mouse models of infection (Wang et al. 1999, 2001; Liu et al. 2004). In short, chitin represents not only a target for the treatment of disease caused by *W. dermatitidis* and like fungi, but it also serves as yet another factor involved in pathogenesis.

SUMMARY AND FUTURE DIRECTIONS

DSP is characterized by the clinical presentation of disseminated and cerebral infections. Each has its own unique characteristics, although they share some similar histopathological features. They are both caused by a wide variety of fungal species having high levels of mortality even though most patients receive surgical and/or chemotherapeutic treatment. In the future, it is essential for medical mycologists, research scientists, and clinicians to make a concerted effort to develop additional intervention strategies employing newer antifungal agents that seem to show promise in early trials. Such strategies may focus upon the discovery of potential virulence factors and exploiting any unique fungus-specific features they present. Incorporated into these efforts should be the development of modern and more

efficient molecular diagnostics for DSP, although current skills in the more traditional areas of fungal culture and identification must be maintained as well as instilled in future generations of medical mycologists.

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Deep hyalohyphomycosis

CAROLINE B. MOORE AND DAVID W. DENNING

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THE CONCEPT OF HYALOHYPHOMYCOSIS

Definition

The clinical nomenclature 'hyalohyphomycosis,' first used by Ajello (1982), serves as an umbrella term grouping together infections caused by molds that lack melanin in their cell walls (known as hyaline, non-dematiaceous or non-pheoid), which do not have other specific, well-established names, such as aspergillosis. This terminology allows for the inclusion of future non-melanized filamentous etiologic agents or disease states, thus avoiding the need to create a multitude of unnecessary names. Furthermore, it is resistant to the many taxonomic changes that inevitably happen (Matsumoto et al. 1994).

Causative organisms

Currently known agents of hyalohyphomycosis are many and varied, presently totaling more than 20 genera and 70 species (Table 1). Some of these species are well documented in causing disease (Table 2); others have been encountered less often.

The hyalohyphomycetes comprise a heterogeneous group characterized by production of colorless septate hyphae in host tissue. Hyphae are parallel-walled, typically showing irregular branching at both 45° and 90°. Hyphal diameter can vary substantially (2–8 μm), but is more slender than that of the Zygomycetes. Colony color can also differ widely, but is never darkly pigmented.

They are opportunistic pathogens, normally found as saprophytes in soil and vegetative matter, and frequently identified as plant pathogens. The most common agents of disease are species of *Fusarium*, *Scedosporium*, *Acremonium*, and *Paecilomyces*.

Identification of hyaline filamentous fungi is not always straightforward, and this chapter does not intend to provide an exhaustive text on the subject. Key characteristics of common genera are provided. However, the reader is referred to excellent guides on the identification of medically important species (Sutton et al. 1998; de Hoog et al. 2000; Larone 2002). Additional resources can be found at www.doctorfungus.org and www.clinicalmycology.com. Expert help may also prove advantageous.

Categories of disease

Hyaline hyphomycetes are capable of causing a wide spectrum of disease that can be further divided into superficial, deep tissue, and disseminated infections. This chapter aims to concentrate on deep-seated infection, and as such, does not give detail on other forms of disease, including those of the eye (see Oculomycosis).

Deep infections may involve the lungs, sinuses, heart, liver, spleen, kidney, bones, or central nervous system (Table 2), and are commonly acquired through the respiratory tract, gastrointestinal tract (e.g. after major surgery), or blood vessels (e.g. catheter-related). Skin breakdown or toenail onychomycosis can be the origin of infection in invasive fusarial disease (Boutati and Anaissie 1997). Major trauma may also result in

Table 1 *Currently known agents of hyalohyphomycosis*[a]

Genera and species (synonym)	Genera and species (synonym)	Genera and species (synonym)
Acremonium	***Fusarium***	***Scedosporium***
A. alabamense	*F. anthophilum*	*S. apiospermum*
A. atrogriseum	*F. aquaeductuum*	*S. prolificans*[b]
A. blochii	*F. chlamydosporum*	***Schizophyllum***
A. curvulum	*F. dimerum*	*S. commune*
A. falciforme	*F. equiseti*	***Scopulariopsis***
A. kiliense	*F. napiforme*	*S. acremonium*
A. potronii	*F. nygamai*	*S. asperula*
A. recifei	*F. oxysporum*	*S. brevicaulis*
A. roseogriseum	*F. polyphialidicum*	*S. brumptii*[b]
A. strictum	*F. proliferatum*	*S. candida*
Aphanoascus	*F. sacchari*	*S. fusca*
A. fulvescens	*F. semitectum* (*F. incarnatum*)	*S. koningii*
Beauveria	*F. solani*	***Scytalidium***
B. bassiana	*F. subglutinans*	*S. hyalinum*
B. brongniartii	*F. tabacinum*	***Trichoderma***
Cephaliophora	*F. verticillioides* (*F. moniliforme*)	*T. citrinoviride*
C. irregularis	***Myriodontium***	*T. harzianum*
Chrysonilia	*M. keratinophilum*	*T. koningii*
C. sitophila	***Neocosmospora***	*T. longibrachiatum*
Chrysosporium	*N. vasinfecta*	*T. pseudokoningii*
C. zonatum	***Ovadendron***	*T. viride*
Coprinus	*O. sulphureo-ochraceum*	***Tritirachium***
C. cinereus	***Paecilomyces***	*T. oryzae*
Cylindrocarpon	*P. crustaceus*	*T. roseum*
C. cyanescens	*P. javanicus*	***Tubercularia***
C. destructans	*P. lilacinus*	*T. vulgaris*
C. lichenicola	*P. marquandii*	***Verticillium***
C. vaginae	*P. taitungiacus*	*V. serrae*
Engyodontium	*P. variotii*	***Volutella***
E. album	*P. viridis*	*V. cinerescens*

a) Species are often unidentified in published reports.
b) Generally regarded as dematiaceous.

implantation. Infection can remain localized in deep tissue and organs, or can disseminate hematogenously or via the lymphatic system. Secondary dissemination to the skin is more frequently seen with some of the fungi described here than with disseminated aspergillosis.

Patients at risk

Recent advances in medical procedures, therapy, and education have been extremely successful in increasing survival in patients, whom, historically, would have had

Table 2 *Common manifestations of deep infection caused by principal agents of hyalohyphomycosis*

	Dissem.	Skin	Blood	Lung	Brain	Sinus	Heart	Liver/spleen	Kidney	Bones/joints
Fusarium spp.	✓✓	✓✓	✓✓	✓	✓	✓	✓	✗	✗	✓
Scedosporium apiospermum	✓	✓	✓	✓✓	✓✓	✓	✓	✗	✓	✓✓
Scedosporium prolificans[a]	✓✓	✓	✓✓	✓✓	✓✓	✓	✓	✓	✓	✓✓
Acremonium spp.	✓	✓✓	✓✓	✓	✓	✗	✓	✗	✗	✓✓
Paecilomyces spp.	✓	✓✓	✓	✓	✓	✓	✓	✗	✓	✓
Scopulariopsis spp.	✓✓	✓✓	✗	✓	✓	✓	✓✓	✗	✗	✗

✓✓ common site of infection of this species; ✓ less commonly reported; ✗ rarely or not reported to date; Dissem., propensity for disseminated infection.
a) Generally regarded as dematiaceous; included to enable comparison between species.

limited treatment options and a poor prognosis. As a direct result, the incidence of infection with opportunistic fungal pathogens, such as hyaline molds, has increased substantially worldwide. Whilst overall a relatively small number of infections are encountered, associated mortality is disproportionately high. Persistent neutropenia is probably the key risk factor for infection with hyaline molds. Highly susceptible patients include those with underlying hematological malignancies, bone marrow transplant recipients, and solid organ transplantation. Interestingly, hyalohyphomycosis in individuals infected with the human immunodeficiency virus (HIV) appears to be relatively uncommon. HIV specifically targets the T-lymphocyte population whereas hyaline mold pathogens are primarily granulocyte-controlled infections (Matsumoto et al. 1994).

Immunocompetent individuals may also be infected by these pathogens. Common predisposing factors include near-drowning or penetrating trauma (Walsh et al. 2004).

DIAGNOSIS

Early diagnosis is important for a successful outcome in invasive hyalohyphomycosis; this, however, can be problematic. Such agents of disease are commonly encountered as laboratory contaminants. Furthermore, isolation of the fungus, particularly from respiratory tract sites, may simply represent colonization, which may or may not progress to infection depending primarily on the immunological status of the host and the virulence of the organism. Consequently, a positive culture from a nonsterile site, such as sputum, must be interpreted with caution. Nevertheless, since these infections can be rapidly devastating, isolation of the fungus must not be automatically dismissed without investigation, particularly in the immunosuppressed host. Definitive diagnosis requires the histological detection of hyphae in normally sterile body fluids and/or tissue sections, together with a positive culture whose morphology matches that seen by microscopy. However, biopsies may be contraindicated in some patients due to severe thrombocytopenia (Hennequin et al. 2002).

Microscopy

Direct microscopy may be helpful in preliminary diagnosis. It will rarely indicate which fungus is responsible, but should distinguish the zygomycetes and hyalohyphomycetes. The test is rapid and the diagnostic yield may be higher than culture alone (Denning et al. 2003). If both microscopy and culture are positive, the likelihood of invasive disease is much higher. Sputum, bronchial washings, and aspirates should be examined using 10 percent potassium hydroxide and Parker ink (1:1), Gram stain, or, preferably, a fluorescent chitin dye such as Calcofluor (to increase sensitivity). Body fluids and exudates should be concentrated by centrifugation and the sediment similarly examined microscopically.

Histology

Hyphae are difficult to detect in hematoxylin and eosin (H&E)-stained sections due to poor staining, and any delay waiting for the results of subsequent specialized fungal stains can have fatal consequences. Therefore, tissue biopsies should be stained with a fungal stain in parallel with standard stains. Periodic acid–Schiff (where cell walls will be red) or a silver stain such as Gomori methenamine silver or Grocott stain (which will stain cell walls black) is recommended. Absence of melanin in the cell wall can usually be confirmed with a melanin stain such as the Fontana–Masson stain, although unstained or H&E-stained specimens should reveal melanin pigment, if present. In histological preparations, it can be almost impossible to distinguish between the various genera of hyaline molds, and agents of hyalohyphomycosis may be misidentified as *Aspergillus* spp. Moreover, histological appearance of fungi may be altered after antifungal therapy (Walts 2001; Denning et al. 2003). Immunohistological staining and in situ hybridization have both been used on tissue sections to differentiate *Fusarium* and *Scedosporium* spp. from other hyalohyphomycetes, including *Aspergillus* spp. However, antibodies to hyaline hyphomycetes, other than *Aspergillus*, are not commercially available (Kaufman et al. 1997; Hayden et al. 2003).

Culture

Since direct microscopy or histopathology may not be reliable means of distinguishing between the various genera of hyaline molds, all specimens should be cultured using a primary isolation medium, such as Sabouraud glucose agar. Cycloheximide should be avoided since many fungal species are sensitive to this compound. Furthermore, prior antifungal therapy could reduce the yield or sensitivity of culture. On occasion, isolates may appear colonially atypical or fail to sporulate on primary isolation media. Subculturing on to less nutritionally rich agars, such as half-strength cornmeal or potato dextrose agar, may help induce sporulation. Some organisms grow rather slowly; therefore, it would be prudent to incubate cultures for 4–6 weeks, generally at 30°C.

Definitive identification of the etiologic agent can then be accomplished using macroscopic and microscopic morphology. Identification is crucial for guiding antifungal therapy since intrinsic resistance to one or more drugs has been noted in many genera (Table 3). Acquired resistance is less well characterized. The National Committee for Clinical Laboratory Standards (NCCLS)(National Committee for Clinical Laboratory

Table 3 *Summary of in vitro susceptibility patterns of common hyalohyphomycetes to licensed and investigational systemic antifungal agents*

	FCZ	ITZ	KCZ	MCZ	VOR	POS	AMB	5FC	CASP	MICA	ANID
Fusarium spp.	–	–	–	–	+	+/–	+	–	–	–	–
Scedosporium apiospermum	–	+	+/–	+	+	+	–	–	+/–	+/–	+/–
Scedosporium prolificans[a]	–	–	–	–	+/–	–	–	–	+/–	+/–	+/–
Acremonium spp.	–	–	–	–	+	+	+	–	+	?	+/–
Paecilomyces lilacinus	–	–	–	–	+	+	–	–	–	+	?
Paecilomyces variotii	–	+	+	+	+	+	+	+	+	+	?
Scopulariopsis spp.	–	–	+/–	+/–	+/–	?	–	–	+	?	?

AMB, amphotericin B; ANID, anidulafungin; CASP, caspofungin; 5FC, flucytosine; FCZ, fluconazole; ITZ, itraconazole; KCZ, ketoconazole; MCZ, miconazole; MICA, micafungin; POS, posaconazole; VOR, voriconazole.
+, susceptible; –, not susceptible; +/–, some isolates susceptible, limited susceptibility; ?, unknown.
a) Generally regarded as dematiaceous; included to enable comparison between species.

Standards 2002) has developed a standardized method, M38-A, for susceptibility testing of these organisms. The E-test method (AB Biodisk, Sweden) has also been investigated, achieving 70–100 percent agreement depending on drug and organism, with the M38 method (Pfaller et al. 2000; Espinel-Ingroff 2001). However, due to the relative infrequency of such organisms, these tests are unlikely to be clinically validated for some time. Such testing should be performed by a specialist laboratory.

Other tests

In contrast to other mold infections, including aspergillosis, *Fusarium*, *Scedosporium*, *Acremonium*, and *Paecilomyces* can be isolated relatively easily from routine blood cultures. This is most likely due to the expression of unicellular structures in infected tissues (adventitious forms), which are thought to facilitate hematological dissemination more easily than hyphal elements (Boutati and Anaissie 1997; Pontón et al. 2000). Furthermore, use of a specific fungal medium may provide a reduction in detection time compared to standard aerobic medium (Hennequin et al. 2002).

To date, no commercially available serologically based methods are available to aid in the diagnosis of any specific hyalohyphomycosis. Assays exist for the detection of 1,3-β-D-glucan, a component of most fungal cell walls, including the hyaline molds. Whilst these may be useful in diagnosis of fungal infection, they are unable to distinguish between different fungal genera (Obayashi et al. 1995). A number of molecular tests, generally polymerase chain reaction (PCR)-based, have been developed both for diagnosis of systemic mycoses from clinical samples, and for identification of fungal cultures, including *Fusarium* and *Scedosporium* spp. (Hue et al. 1999; Chen et al. 2002; Pryce et al. 2003).

PREVENTION

Nosocomial hyalohyphomycete infections have been reported. Potential reservoirs include hospital water supplies, soil of potted plants, and nearby landscaping or construction work. Contaminated drinking water or foodstuffs such as nuts and spices may also be hazardous (Summerbell et al. 1989; Berenguer et al. 1997; Anaissie et al. 2001; Shaw 2002; Dignani and Anaissie 2004). Of particular note, *Paecilomyces* spp. are resistant to standard sterilization methods and outbreaks have been associated with contaminated skin lotion (Orth et al. 1996). Every attempt should be made to ensure immunocompromised patients avoid exposure to potential environmental sources.

Fluconazole prophylaxis is routinely used in immunocompromised patients, especially bone marrow transplant recipients, and has been successful in reducing the frequency of *Candida* infections. However, a higher prevalence of mold infections at autopsy has been recorded in marrow transplant patients, possibly due to increased early post-transplantation survival (van Burik et al. 1998). Moreover, empirical treatment in febrile neutropenic patients is now generally regarded as accepted practice since therapeutic delay can result in increased morbidity and mortality. The drug of choice is usually amphotericin B. Many agents of hyalohyphomycosis are clinically resistant to this agent (Table 3), and invasive infection may develop regardless of therapy (Merz et al. 1988). Consequently, a high index of suspicion should be maintained amongst neutropenic patients receiving antifungal prophylaxis or empiric therapy.

PRINCIPAL HYALINE MOLDS CAUSING DEEP INFECTIONS

Fusarium species

BRIEF DESCRIPTION OF THE GENUS

Members of the genus *Fusarium* are anamorphic (asexual) filamentous organisms that propagate by producing conidia (asexual spores). Sexual states (teleomorphs) have been described in some of the fusaria.

Conidiogenous cells (termed phialides) are formed on aerial septate hyphae or short, branched conidiophores that are often in dense clusters (sporodochia). Microconidia and macroconidia are typically produced, although microconidia may be absent in some species. Some species also contain blastoconidia. In addition, hyaline or pale chlamydospores, produced intercalary or terminally, singly or in chains, may be seen in some species. In comparison to plant-dwelling strains, clinical isolates often exhibit a reduced morphology.

MORPHOLOGY

For the majority of species, colonies grow rapidly (40–80 mm within 7–10 days) although some species such as *Fusarium dimerum* are rather slower growing (less than 20 mm in 7–10 days). Initially colonies may be white but darken with age to shades of cream, purple, pink, red, or yellow (Figure 1). Similarly, the reverse may be pale but can darken to purple, red, green, blue, or brown as the culture matures. Sporodochia may not always be seen in culture. If present, they are often cream, orange, or tan. Colony texture can vary, but is usually floccose, woolly, or cottony.

Large smooth sickle- or canoe-shaped macroconidia (5–73×1.5–6 μm) with one to five transverse septa (Figure 1) are produced in basipetal fashion (growing from the base) from monophialides or sporodochia, accumulating in slimy masses. These conidia are thick-walled with a basal foot cell and an often pointed apical end. Macroconidia, typically used in identifying the genus and species, may only be apparent in the later stages of growth; thus care must be taken not to confuse young cultures, where only microconidia are evident, with *Acremonium* spp.

Microconidia are produced also in basipetal series on mono- or polyphialides, accumulating in small slimy heads or, sometimes, chains (Figure 1). They are generally unicellular, although two- or three-celled varieties are occasionally observed, and variable in size and shape (4–26×1.5–11 μm, smooth, oval, subspherical or pyriform, straight or curved with a rounded or flat base) (Nelson et al. 1994; Sutton et al. 1998; de Hoog et al. 2000).

Molecular methods, such as 28S rRNA gene sequencing, have been used to identify *Fusarium* spp. (Hennequin et al. 1999).

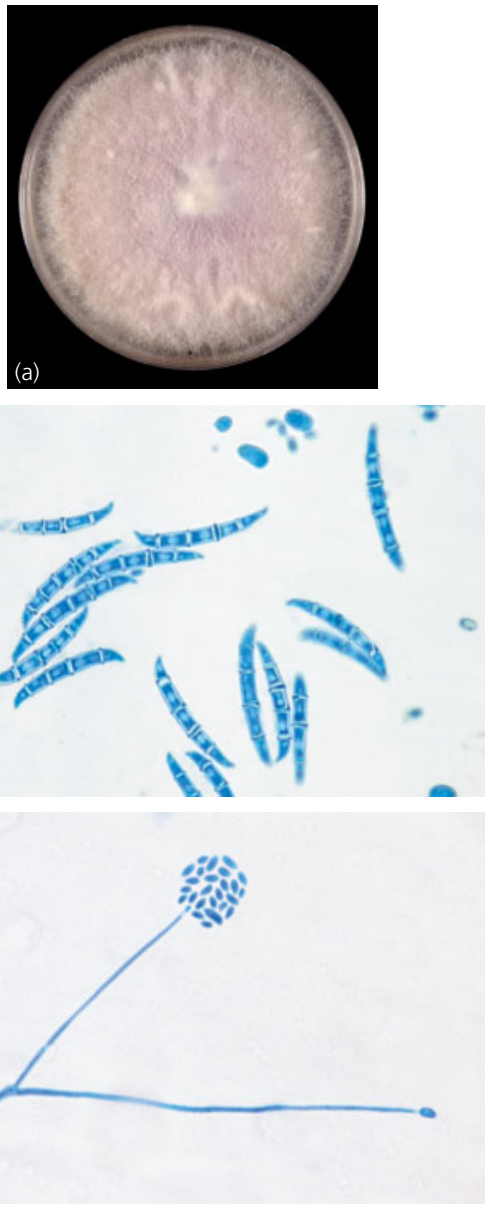

Figure 1 (a) *Culture of* Fusarium *sp. (Sabouraud glucose agar, 30°C, 7 days) and* **(b)** *microscopic appearance showing macroconidia and* **(c)** *microconidia. © Salford Royal Hospitals NHS Trust*

EPIDEMIOLOGY

Fusarium spp. are frequently found as saprophytes on organic debris and in soil. They are commonly implicated as plant pathogens, and indeed may cause economically devastating disease. Conversely, *Fusarium venenatum* (previously misidentified as *Fusarium graminearum*) is a commercially useful organism, being used for mycoprotein production for human consumption (Quorn).

Some species are more commonly found in tropical or temperate climates, whilst others are more geographically widespread, including desert and arctic areas. Despite worldwide distribution, most cases of invasive fusariosis have been reported from the United States and Southern Europe, particularly France. Moreover, *Fusarium* spp. are more abundant in the air during the rainy summer–autumn months, coinciding with increased infection rates (Nelson et al. 1994; Boutati and Anaissie 1997; Girmenia et al. 2000).

More than 50 species of *Fusarium* have been described, the taxonomy of which is confusing and under revision. Only a small number of species, however, have been docu-

mented as causing human infection (Table 1). The most virulent appears to be *Fusarium solani*, which, not surprisingly, is also most commonly isolated (in approximately 50 percent of cases). *Fusarium oxysporum* and *Fusarium verticillioides* (*moniliforme*) are also commonly encountered. Other rarer species include *Fusarium proliferatum* and *F. dimerum* (Mayayo et al. 1999; Pontón et al. 2000).

Fusarium spp. are the most common cause of fungal keratitis and were previously considered to cause only corneal, skin, and nail infections (not discussed in this chapter). However, they are now recognized as having the ability to cause disseminated disease and, indeed, in some centers, have emerged as the most common non-*Aspergillus* mold pathogen (Anaissie et al. 1988; Marr et al. 2002). Disseminated fusarial infection was first described in 1973 in a child with acute leukemia (Cho et al. 1973). One US institution recorded a significant increase from 0.5 to 3.8 cases per year from 1975 to 1995 (Boutati and Anaissie 1997). Incidence rates in other centers worldwide have remained more stable (5.1 and 6.3 mean cases per year for the periods 1981–90 and 1991–96, respectively) (Girmenia et al. 2000).

Fusarium spp. are able to produce several mycotoxins. Alimentary toxic aleukia is one of the most important mycotoxicoses. During the 1930s to 1950s, this disease was widespread in Siberia due to overwintering cereal grains on the field. Upon ingestion of contaminated food, mycotoxins cause tissue breakdown and suppress cellular and humoral immunity, resulting in bone marrow failure and potentially fatal disease (Nelson et al. 1994). It is currently unknown whether such toxins are expressed in human tissue during disseminated infection. Adherence factors may also be important in catheter-related infections (Pontón et al. 2000; Dignani and Anaissie 2004).

RISK FACTORS AND CLINICAL MANIFESTATIONS

Fusarium spp. have been documented as causing infections in almost any organ, including mycetoma, keratitis, pulmonary infections, endocarditis, catheter infections, brain abscess, and fungemia (Table 2).
Skin and soft tissue infections may be indolent, locally invasive, or manifestations of systemic disease. Toenail onychomycosis and skin lesions can both be a source of dissemination in immunosuppressed individuals; thus a thorough skin and nail examination should be performed in patients prior to commencement of immunosuppressive therapy. Endogenous endophthalmitis may also be an indication of disseminated infection (Castagnola et al. 1993; Velasco et al. 1995; Boutati and Anaissie 1997; Camin et al. 1999; Nucci et al. 2003).

Deep infections within the immunocompetent patient setting usually remain localized, and include continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis, or septic arthritis and osteomyelitis, generally as a result of trauma or surgery. Disseminated infection has occurred rarely in immunocompetent patients; risk factors include those with extensive burns (Guarro and Gené 1995; Dignani and Anaissie 2004).

Risk factors for invasive disease include persistent neutropenia and graft-versus-host disease (GVHD) (Walsh et al. 2004). Patients with underlying hematological malignancies are most susceptible, with more than 90 percent of reported cases of fusariosis occurring in this population, half of which were acute leukemic patients (Pontón et al. 2000). Hematopoietic stem cell transplant recipients may be at particular risk, especially those who have received grafts with human leukocyte antigen-mismatched or unrelated donors or have undergone multiple transplants (Marr et al. 2002; Nucci 2003). Fusarial infection in solid organ transplant recipients appears to be less prevalent, with only eight cases reported until 2003, five in renal transplant recipients (Nucci 2003).

Invasive disease initially presents as persistent fever (>90 percent) refractory to broad-spectrum antibiotics (and perhaps also to antifungal drugs) in a profoundly neutropenic patient, possibly with pulmonary infiltrates and/or sinusitis (70–80 percent), similar to invasive aspergillosis. However, unlike aspergillosis (<10 percent of cases), metastatic cutaneous lesions may be seen in 70–90 percent of cases, sometimes preceding fungemia (1–10 days). Moreover, skin lesions are characteristic, evolving from subcutaneous nodules to erythematous lesions, later becoming necrotic, and may provide the sole diagnostic sign in >50 percent of patients. In further contrast to aspergillosis, routine blood cultures are positive in 40–60 percent of cases. Antemortem diagnosis is achieved in >90 percent of cases (Merz et al. 1988; Martino et al. 1994; Boutati and Anaissie 1997; Nucci and Anaissie 2002; Dignani and Anaissie 2004).

TREATMENT AND PROGNOSIS

In patients with catheter-associated infection, such as those with CAPD peritonitis, catheter removal is required, in addition to systemic antifungal treatment. Localized infection in the solid organ transplant recipient may respond to surgical resection, together with long-term antifungal therapy. Favorable response rates may be achieved with such localized infections. Nonetheless, timely treatment is crucial if disseminated infection is to be avoided (Velasco et al. 1995).

The overall mortality rate associated with invasive fusarial infection remains very high (52–70 percent). Persistent neutropenia and corticosteroid administration are significant prognostic factors. In those who do not recover from neutropenia, the death rate will be almost 100 percent, despite antifungal therapy. Attributable mortality in solid organ transplant recipients with fusariosis appears to be lower, possibly because

these infections tend to be more localized (Groll and Walsh 2001; Nucci et al. 2003; Dignani and Anaissie 2004).

Optimal therapy has yet to be established. Since outcome is poor unless the neutrophil count increases, efforts to modulate the host response by administration of growth factor [granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF)] and/or colony-stimulating factor (CSF)-stimulated granulocyte transfusion and, if possible, concomitant reduction of immune suppressive therapy may be beneficial (Spielberger et al. 1993; Boutati and Anaissie 1997; Hennequin et al. 1997).

Fusarium spp., particularly *F. solani*, are resistant to most of the established antifungal drugs (Table 3). Amphotericin B shows some activity in vitro, although in vivo data may be contradictory (Pujol et al. 1997; Arikan et al. 1999; Guarro et al. 1999; Ortoneda et al. 2002a). Nevertheless, satisfactory response, or at least control of the infection allowing recovery of immune function, may sometimes be achieved with high doses of conventional or, preferably, liposomal amphotericin B (Boutati and Anaissie 1997; Letscher-Bru et al. 2002).

Voriconazole has also been used to effectively treat invasive *Fusarium* infection, and recently was approved for such indications (Consigny et al. 2003; Vincent et al. 2003). This efficacy is supported by in vitro and in vivo data, although *F. solani* may be less susceptible (Clancy and Nguyen 1998; Arikan et al. 1999; Paphitou et al. 2002; Graybill et al. 2003).

Posaconazole shows modest in vitro and in vivo activity (Lozano-Chiu et al. 1999; Paphitou et al. 2002), whilst *Fusarium* spp. are intrinsically resistant to echinocandin agents (Pfaller et al. 1998; Tawara et al. 2000; Arikan et al. 2001).

Combination therapy, often amphotericin B with an azole agent, has shown promising activity in vitro and has been used clinically with some degree of success (Durand-Joly et al. 2003; Ortoneda et al. 2004).

Scedosporium species

BRIEF DESCRIPTION OF THE GENUS

The genus *Scedosporium* contains two medically important species, *Scedosporium apiospermum* and *Scedosporium prolificans*. *S. prolificans* (formerly *Scedosporium inflatum*) is regarded as dematiaceous (darkly pigmented), but has been included within this chapter to ease comparison between the two species.

S. apiospermum is the asexual state of teleomorph *Pseudallescheria boydii*, which is homothallic (sexual reproduction requiring only a single organism). The synanamorph *Graphium eumorphum* may also exist in some strains.

The sexual form of *S. prolificans* has been described as belonging to the genus *Petriella*.

MORPHOLOGY

Colonies of *S. apiospermum* spread rapidly (40 mm within 7–10 days), initially white but becoming dark gray or brown, with a hairy, woolly, or cottony texture (Figure 2). A pale reverse is initially seen, developing brown or black areas.

The teleomorph *P. boydii* produces spherical brown fruiting bodies, 140–200 μm in diameter, known as cleistothecia, which contain asci and ascospores. However, it is rare for clinical strains to produce these sexual structures; prolonged incubation may be required to induce their formation. In the asexual form, cylindrical conidiogenous cells (termed annellides) arise from branching septate hyphae (2–4 μm in diameter) and produce slimy masses of conidia. Conidia (6–12×3.5–6 μm) are unicellular, smooth, and oval with a flat base, initially colorless or pale becoming brown and thick walled. Conidia are sometimes produced directly on the hyphae (sessile). In the *Graphium* state, conidiophores are sometimes bundled together, forming synnemata, producing clusters of conidia at their apical tip (Figure 2). Both asexual forms may exist in a single isolate.

Colonies of *S. prolificans* also grow rapidly (20 mm within 7–10 days), initially with a moist, almost yeast-like texture, later developing tufts of aerial hyphae. The

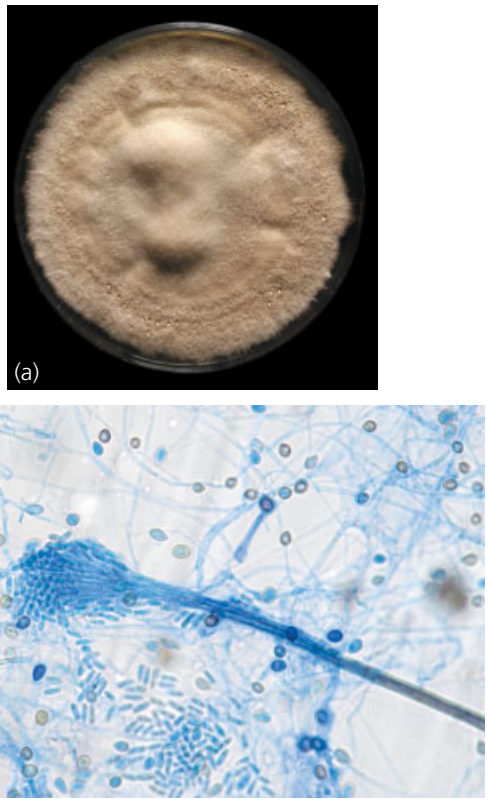

Figure 2 (a) *Culture of* Scedosporium apiospermum *(Sabouraud glucose agar, 30°C, 14 days) and* **(b)** *microscopic appearance showing synnemata (*Graphium *state). © Salford Royal Hospitals NHS Trust*

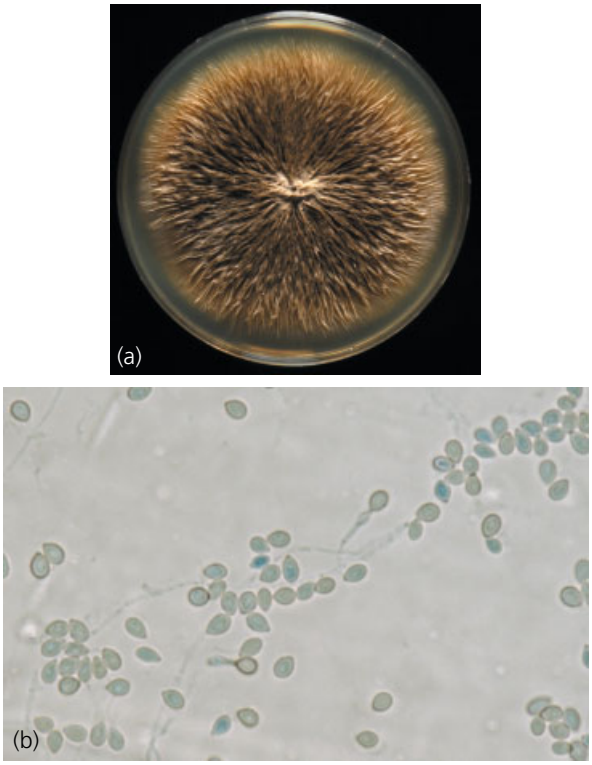

Figure 3 (a) *Culture of* Scedosporium prolificans *(Sabouraud glucose agar, 30°C, 14 days) and* **(b)** *microscopic appearance.* *© Salford Royal Hospitals NHS Trust*

colony color may be olive-gray or black with a gray to black reverse, more darkly pigmented than that of *S. apiospermum* (Figure 3).

Conidiogenous cells of *S. prolificans* are characteristically flask-shaped with an inflated base, often with an elongated tip (Figure 3). Single-celled, smooth, oval, flat-based, olive-brown conidia (3–7×2.5 μm) accumulate in slimy heads. Sessile conidia, which are darker and rounder, may also be seen (de Hoog et al. 2000).

S. apiospermum and *S. prolificans* also differ in their physiology: unlike *S. prolificans*, *S. apiospermum* is able to assimilate ribitol, xylitol, and L-arabinitol. Furthermore, *S. apiospermum* can grow on cycloheximide-containing media (e.g. Mycosel), whereas *S. prolificans* is inhibited by the compound. Molecular methods have also been used to discriminate between the two species (Wedde et al. 1998).

EPIDEMIOLOGY

Scedosporium spp. occur worldwide. *S. apiospermum* is frequently found as a saprophyte in soil, sewage, manure, and contaminated water. The natural habitat of *S. prolificans* appears to be soil, while it has been isolated from the hospital environment during building works. Both species have historically been important causes of subcutaneous infection in immunocompetent individuals (not discussed here), normally following minor trauma such as splinters or thorns. They are, however, becoming increasingly implicated in invasive disease. In vivo work suggests that *S. prolificans* is more virulent than *S. apiospermum*. Interestingly, most cases of disseminated *S. prolificans* infection have been recorded in Spain and Australia; the reasons for this distribution remain unclear (Cano et al. 1992; Berenguer et al. 1997; Bouza and Muñoz 2004).

RISK FACTORS AND CLINICAL MANIFESTATIONS

Scedosporium infection may involve almost any organ (Table 2). Reports of *S. apiospermum* infection include invasive pulmonary disease, sinusitis, brain abscess, endocarditis, fungemia, mycetoma, and peritonitis (Jabado et al. 1998; Nesky et al. 2000; Castiglioni et al. 2002; Raj and Frost 2002; Bouza and Muñoz 2004). *S. prolificans* has also caused renal and splenic abscess, in addition to pulmonary infections, endocarditis, fungemia, and many other manifestations (Marin et al. 1991; Carreter de Granda et al. 2001; Simarro et al. 2001; Bouza and Muñoz 2004).

For both species, manifestations in immunocompetent individuals are usually localized, and include subcutaneous infections, septic arthritis, osteomyelitis, and sinusitis. Pulmonary infection and endocarditis in the normal host have also been reported (Sobottka et al. 1999; Greig et al. 2001; Tirado-Miranda et al. 2001; Levine et al. 2002). The main risk factors are surgery or trauma, although intravenous drug use has also been reported as such (Berenguer et al. 1997; Bouza and Muñoz 2004). *S. apiospermum* has caused fatal pulmonary, cerebral, and disseminated infection in near-drowning victims in polluted water and sewage (Dworzack et al. 1989). In addition, either species may colonize the airway of cystic fibrosis patients, triggering allergic disease in some (Cimon et al. 2000).

Patients at high risk of disseminated disease include those receiving corticosteroids or with prolonged neutropenia. Indeed, at least 90 percent of cases are associated with persistent neutropenia. Those with hematological malignancy, particularly acute leukemia, or hematopoietic stem cell transplant recipients are especially at risk (Berenguer et al. 1997; Revankar et al. 2002). The incidence of *S. apiospermum* infection in solid organ transplant recipients is approximately 0.1 percent, with lung transplant recipients most at risk (Castiglioni et al. 2002).

Clinical signs of invasive infection are typically similar to those produced by *Aspergillus* or *Fusarium*: unremitting fever (>90 percent) unresponsive to broad-spectrum antibiotics (and possibly also to antifungal agents) in a persistently neutropenic patient, followed by pulmonary involvement (approximately 75 percent). Indeed, with both *Scedosporium* spp. the lung is the most common site of infection in immunocompromised patients. Frequent dissemination to other organs is seen, with a particular propensity for the central nervous system.

Neurological symptoms may be noted (approximately 30–40 percent for *S. prolificans*, possibly even higher with *S. apiospermum*), in addition to widespread metastatic cutaneous lesions (25 percent), which typically appear as nodules, possibly becoming necrotic. Metastatic eye involvement may also occur (approximately 10 percent). Dissemination is often seen in solid organ transplant patients, in contrast to fusarial infection in such patients (Berenguer et al. 1997; Bouza and Muñoz 2004). Positive blood cultures are obtained in approximately 80 percent of *S. prolificans* infections (Revankar et al. 2002), whereas the isolation rate is much lower for *S. apiospermum*. Occasional case reports suggest that indwelling central venous catheters may also serve as a portal of entry, particularly with *S. prolificans* (Berenguer et al. 1997).

TREATMENT AND PROGNOSIS

Successful outcomes are often achieved in immunocompetent patients since disease tends to be more localized. A maximal dose of antifungal treatment in combination with surgical debridement and/or drainage, if appropriate, is usually the therapy of choice (Wood et al. 1992; Berenguer et al. 1997).

On the contrary, in the immunocompromised host, *Scedosporium* usually results in a rapidly disseminated infection that is almost universally lethal (85–100 percent) (Wood et al. 1992; Berenguer et al. 1997; Castiglioni et al. 2002; Marr et al. 2002; Revankar et al. 2002). These data indicate that resolution of neutropenia is absolutely crucial for survival, particularly since these organisms are resistant to many antifungal drugs (Table 3).

Therapeutic choices are very limited. As with *Fusarium* infections, administration of hematopoietic growth factors may help with neutropenic resolution. Immunosuppressants should also be reduced if clinically justifiable (Idigoras et al. 2001).

S. prolificans is intrinsically resistant to amphotericin B. Most *S. apiospermum* isolates are resistant in vitro and treatment failures have been widely reported (Ellis 2002; Meletiadis et al. 2002). Indeed, *Scedosporium* infection can often develop during amphotericin B treatment (Berenguer et al. 1997; Safdar et al. 2002). Interestingly, in vitro studies have shown that antifungal drugs, including amphotericin B, cooperate with polymorphonuclear leukocytes to cause increased damage to *Scedosporium* hyphae (Gil-Lamaignere et al. 2002). Moreover, combination therapy of liposomal amphotericin B and G-CSF prolonged survival in an in vivo disseminated *S. prolificans* model (Ortoneda et al. 2002b).

Itraconazole and miconazole both show some activity in vitro and have been used clinically with some degree of success against *S. apiospermum*, but rarely so with *S. prolificans* infections. Intravenous miconazole, however, has toxic side effects (Nomdedeu et al. 1993; Cuenca-Estrella et al. 1999; Meletiadis et al. 2002; Bouza and Muñoz 2004).

Voriconazole was recently indicated for *Scedosporium* infections, and numerous case studies have demonstrated efficacy against *S. apiospermum* (Girmenia et al. 1998; Muñoz et al. 2000; Nesky et al. 2000; Poza et al. 2000). In vitro data confirm activity against *S. apiospermum*, and to a lesser extent, *S. prolificans* (Cuenca-Estrella et al. 1999; Carrillo and Guarro 2001).

Posaconazole activity against *S. prolificans* is poor. However, in vitro and in vivo responses against *S. apiospermum* have been noted (Espinel-Ingroff 1998; Carrillo and Guarro 2001; Meletiadis et al. 2002; González et al. 2003). In addition, Mellinghoff et al. (2002) report successful posaconazole treatment of *S. apiospermum* brain abscesses in a leukemic patient, after failure of itraconazole, amphotericin B, and ketoconazole.

S. apiospermum and *S. prolificans* appear moderately susceptible in vitro to the echinocandin agents, but no clinical data exist (Espinel-Ingroff 1998; Uchida et al. 2000).

Combination antifungal therapy may provide additional therapeutic options. Voriconazole in combination with terbinafine has been used successfully to treat disseminated *S. prolificans* infection (Howden et al. 2003). In vitro studies confirm synergy with azole/terbinafine combinations (Meletiadis et al. 2003). In addition, liposomal amphotericin B combined with itraconazole produced a favorable outcome in *S. apiospermum* infection, but not *S. prolificans* (Barbaric and Shaw 2001).

Other species

Many other species, particularly *Acremonium*, *Paecilomyces*, and *Scopulariopsis*, have been documented rarely as causing deep tissue or disseminated infection (Table 1).

MORPHOLOGY

The growth rate of *Acremonium* spp. is moderate (10–30 mm in 7 days), generally slower than *Fusarium* spp. Colonies are flat, occasionally raised in the center, glabrous to velvety and may be pink, yellow, or white with a colorless, pinkish, or pale brown reverse. Microconidia (3–10×1.5–3 μm) accumulate at the tips of narrow tapering phialides (de Hoog et al. 2000).

Paecilomyces spp. grow rapidly (30–50 mm in 7 days), initially white, becoming brownish or violet, with a powdery to floccose texture. Conidiophores with characteristic tapering phialides and chains of conidia are observed (de Hoog et al. 2000).

Colonies of *Scopulariopsis* spp. are typically white, becoming light or dark brown, with a cream to brown reverse. They grow moderately quickly (30 mm in 7 days) with a powdery to velvety texture. Conidiophores,

annellides, and often rough-walled conidia arranged in chains are seen (de Hoog et al. 2000).

EPIDEMIOLOGY

Acremonium, *Paecilomyces*, and *Scopulariopsis* spp. inhabit soil and plant material; some species of the latter two can also be isolated from insects. All three genera occur worldwide. They may be encountered as contaminants, but are also capable of causing infection, especially in the immunocompromised host.

Each genus contains a number of species, although only a few have been reported as human pathogens (Table 1). Most *Acremonium* infections have been caused by *Acremonium kiliense* or *A. strictum*. Other species include *Acremonium alabamense*, *Acremonium falciforme*, and *Acremonium recifei*. There are two medically prevalent species of *Paecilomyces*, *Paecilomyces lilacinus* and *Paecilomyces variotii*. Other species include *Paecilomyces javanicus* and *Paecilomyces marquandii*. *Scopulariopsis* infections are primarily caused by *Scopulariopsis brevicaulis*, although other species have been noted, including *Scopulariopsis acremonium*, and *Scopulariopsis candida* (Herbrecht et al. 2002; Steinbach et al. 2004; Walsh et al. 2004).

RISK FACTORS AND CLINICAL MANIFESTATIONS

Acremonium, *Paecilomyces*, and *Scopulariopsis* spp. have been documented as causing a wide spectrum of disease (Table 2).

In the immunocompetent patient, ocular infection (not discussed in this chapter) is the main clinical manifestation of *Acremonium* and *Paecilomyces* spp., typically following traumatic or surgical injury. *Acremonium* is one of the main causative agents of mycetoma, whereas the most common presentation of *Scopulariopsis* infection is onychomycosis, generally of the toenail (neither discussed) (Guarro et al. 1997; Chan-Tack et al. 1999; Steinbach et al. 2004). Nonetheless, *Acremonium* and *Paecilomyces* have been reported as causing peritonitis, endocarditis, and sinusitis (Guarro et al. 1997; Heitmann et al. 1997; Aguilar et al. 1998; Kovac et al. 1998; Nayak et al. 2000; Manzano-Gayosso et al. 2003). Endocarditis caused by *Scopulariopsis* has also been documented (Chen-Scarabelli and Scarabelli 2003).

In the immunocompromised individual, the spectrum of disease includes fungemia and pulmonary and cerebral infections (Boltansky et al. 1984; Guarro et al. 1997; Sillevis Smitt et al. 1997; Sellier et al. 2000; Nedret Koç et al. 2002; Kantarcioğlu et al. 2003; Steinbach et al. 2004).

Similar to *Fusarium* and *Scedosporium* spp., predisposing factors for such invasive disease includes persistent neutropenia and corticosteroid use. Hematopoietic stem cell and organ transplant recipients are most at risk (Guarro et al. 1997; Steinbach et al. 2004; Walsh et al. 2004). Furthermore, clinical presentation is also comparable to other invasive hyalohyphomycoses. Cutaneous lesions may be the first indication of infection, although blood cultures are often positive during *Acremonium* and *Paecilomyces* infections (Guarro et al. 1997; Pontón et al. 2000; Steinbach et al. 2004).

TREATMENT AND PROGNOSIS

Since these infections occur rarely, few clinical data exist to confirm optimal treatment. Localized infection may be resolved by surgery and antifungal therapy. On the contrary, complete recovery from disseminated infection depends almost entirely on recovery of the neutrophil count. Choice of antifungal drug effective against these agents is limited (Table 3); hence combination therapy has generally been utilized.

Amphotericin B, often in combination with an azole, has typically been used against *Acremonium* infection. In vitro activity is seen with some isolates, although clinical response is variable. Higher doses may improve the success rate (Guarro et al. 1997; Groll and Walsh 2001). Voriconazole and posaconazole are active in vitro against some species. More importantly, clinical successes have been reported in patients with hematological malignancies who had failed amphotericin B therapy (Herbrecht et al. 2002; Mattei et al. 2003). Of the limited in vitro data available, caspofungin appears active against *Acremonium* spp., though no clinical data exist (Espinel-Ingroff 1998; Pfaller et al. 1998).

Species identification of *Paecilomyces* is important due to variations in antifungal susceptibility (Table 3). *P. lilacinus* is resistant in vitro to amphotericin B and itraconazole, whilst *P. variotii* is susceptible (Radford et al. 1997; Aguilar et al. 1998; Espinel-Ingroff et al. 2001). Amphotericin B, either alone or in combination with other agents, has been the most widely used regimen against *Paecilomyces* infections. However, a 40 percent failure rate is seen (Aguilar et al. 1998). Voriconazole and posaconazole are active in vitro against some *Paecilomyces* spp. (Radford et al. 1997; Wildfeuer et al. 1998; Espinel-Ingroff et al. 2001; Uchida et al. 2001). Furthermore, voriconazole has been used with partial success against *P. lilacinus* skin and soft tissue infections in immunocompromised individuals who had failed amphotericin B and/or itraconazole therapy (Hilmarsdóttir et al. 2000; Martin et al. 2002). Caspofungin appears active in vitro against *P. variotii*, although *P. lilacinus* is resistant (Del Poeta et al. 1997). Interestingly, caspofungin in combination with itraconazole was used successfully against progressive cutaneous *P. lilacinus* infection (Safdar 2002). Micafungin shows good activity in vitro against both *P. variotii* and *P. lilacinus*, although this has not been substantiated clinically (Uchida et al. 2000). Terbinafine has been used successfully in a soft tissue infection caused by *P. lilacinus* in a heart transplant recipient (Clark 1999).

Historically, amphotericin B has been the therapy of choice in invasive *Scopulariopsis* infections, but is often

ineffective (approximately 60 percent), correlating with in vitro resistance (Aguilar et al. 1999; Steinbach et al. 2004). Itraconazole also shows poor in vitro activity, although a few case studies report success (Radford et al. 1997; Espinel-Ingroff et al. 2001; Steinbach et al. 2004). Variable in vitro responses to voriconazole and clinical failure of voriconazole combined with caspofungin have been reported (Wildfeuer et al. 1998; Espinel-Ingroff et al. 2001; Cuenca-Estrella et al. 2003; Steinbach et al. 2004). In vitro and clinical response to caspofungin was documented against *Scopulariopsis* endocarditis (Chen-Scarabelli and Scarabelli 2003). Recurrent subcutaneous infection caused by *S. brevicaulis* in a liver transplant recipient was controlled with surgery and long-term terbinafine therapy (Sellier et al. 2000).

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Pneumocystis pneumonia

MELANIE T. CUSHION

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A NOTE ABOUT NOMENCLATURE

Since the publication of the previous (9th) edition of this book in 1998, distinct species within the genus *Pneumocystis* have been described. The species which infects human beings was described as *Pneumocystis jirovecii*, while *Pneumocystis carinii* was retained as the name of the species first identified in rats (Frenkel 1999). The species infecting mice was named *Pneumocystis murina* (Keely et al. 2004) and another species found in rats was given the binomial, *Pneumocystis wakefieldiae* (Cushion et al. 2004a). *P. jirovecii* as the name for the human species is controversial (Stringer et al. 2002, 2003; Hughes 2003) and its validity will be decided by the International Botanical Congress in 2005. In this chapter, *Pneumocystis carinii* is used in the section on History below to refer to organisms from any host. Throughout the remaining sections, care is taken to use the correct species assignations when known. The term '*Pneumocystis*' is used when referring to the organisms in general or when the host of origin is not known.

HISTORY

Pneumocystis carinii was first described in Brazil by Carlos Chagas in 1909, while studying infections with *Trypanosoma cruzi* in humans and animal models (Chagas 1909). He interpreted sickle and rounded forms within cyst forms of the organism as male and female stages of trypanosoma. An Italian investigator who agreed with this description, Antonio Carini, sent slides from the lungs of rats infected by *Trypanosoma lewisi* to associates in Paris. Dr and Mrs Delanoë at the Pasteur Institute reviewed the slides and proved conclusively in 1912 that the organism was not a trypanosome, but an entirely different species of protozoan parasite (Delanoë and Delanoë 1912). Since the original diagnostic slides were contributed by Dr Carini, the organism species name bore his surname while the genus recognized both the tropism for the lung (pneumo-) and the characteristic developmental form (-cyst) of the microbe.

Throughout the next three decades, little notice was paid to these organisms, although their presence was observed in the lungs of several mammalian species. Epidemics of the pneumonia were first reported in the 1940s and were especially virulent in European orphanages that housed poorly nourished and sickly children in crowded conditions following World War II (Gajdusek 1957). The pneumonia was characterized by a prominent plasma cell infiltrate in the lung interstitium and the presence of an alveolar exudate described as 'honeycombed' upon histological examination. In 1942, van der Meer and Brug unambiguously reported the presence of the organisms in the lungs of humans (van der Meer and Brug 1942). In 1951, Vanek established the etiologic agent of interstitial plasma cell pneumonia as *Pneumocystis* by demonstration of the organisms in alveolar exudate of children dying of the pneumonia (Vanek 1951). By 1955, the first cases of infection were reported in the USA, although the American

pediatricians were reluctant to accept the etiologic agent as *Pneumocystis* (Gajdusek 1957).

Improved conditions associated with economic recovery in Europe led to a decline in cases of the pneumonia by the 1960s. In 1958, Ivady and Paldy reported the successful treatment of infants with interstitial plasma cell pneumonia (Ivady and Paldy 1958). Previously, this family of cationic diamidine compounds was routinely used for therapy of African trypanosomiasis, or 'sleeping sickness.' This drug became generally accepted as the treatment of choice for *Pneumocystis* pneumonia until the 1970s when it was supplanted by the less toxic and highly efficacious dual regimen of trimethoprim-sulfamethoxazole (TMP-SMX). Extending the previous work of Dutz (1970) and Frenkel (Frenkel et al. 1966), Hughes et al. showed that this combination was highly effective for both prophylaxis and therapy of the infection (Hughes et al. 1974a).

During the 1960s and 1970s, the major target populations for *Pneumocystis* pneumonia were children with primary immunodeficiency diseases and patients of all ages receiving corticosteroids and other immunosuppressive therapy to prevent rejection of organ transplants and for the treatment of cancer. Most patients were responsive to TMP-SMX and its widespread use was associated with a dramatic decline in the number of cases of *Pneumocystis* pneumonia at most major medical centers in the USA.

Pneumocystis pneumonia (PCP) once again became a focus of medical attention during the 1980s as the most common opportunistic infection of individuals with the acquired immunodeficiency syndrome (AIDS) (Mills 1986). Unlike the previous subpopulations infected with *Pneumocystis*, AIDS patients experienced a more subtle clinical presentation, slow response to treatment, recurrent infections, and a high rate of adverse reactions to TMP-SMX.

During the early years of the AIDS epidemic, only a rudimentary knowledge of the organism's basic biology, structural and antigenic characteristics, and molecular genetics existed. Despite a lack of continuous in vitro propagation system and undefined life cycle, almost three decades of concerted basic and applied research has resulted in a better understanding of the family of organisms once known singularly as *Pneumocystis carinii*. Sequence analysis of the 16S-like ribosomal RNA gene as well as protein-encoding genes revealed members of the *Pneumocystis* genus to be fungi rather than protozoons as once thought. In response, pharmaceutical companies began development of novel anti-*Pneumocystis* compounds targeted to unique fungal processes, such as cell wall synthesis (Schmatz et al. 1990, 1995). Molecular and antigenic studies support species distinctions for organisms residing in different mammalian hosts, and in some cases, for different species of *Pneumocystis* within the same mammalian host. The process of naming and describing individual species has begun.

Although the incidence has declined significantly from 1992 through 1997 for each of 15 of the 26 specific AIDS-defining opportunistic infections (OI) including PCP ($P<0.05$), PCP remains as the most common AIDS-defining OI to occur first, in 36 percent of individuals infected with the human immunodeficiency virus (HIV) (Jones et al. 1999). PCP was the most common AIDS-defining OI (274 cases per 1 000 person-years), and the most common AIDS-defining OI to have occurred during the course of AIDS (53 percent of persons who died with AIDS had PCP diagnosed at some time during their course of AIDS).

The administration of highly active anti-retroviral therapy (HAART) targeting the HIV infection reduced dramatically the incidence and mortality of *P. jirovecii*. However, a growing body of evidence indicates that *P. jirovecii* populations are evolving resistance to current therapies, with specific mutations in the target genes of sulfamethoxazole (dihydropteroate synthase) (Beard et al. 2000; Huang et al. 2000; Navin et al. 2001) and atovaquone (cytochrome bc_1) (Walker et al. 1998; Armstrong et al. 2000; Kazanjian et al. 2000, 2001, 2004). Eradication of *Pneumocystis* with the currently available therapies is unlikely. Concomitant with the lack of knowledge of the infection's transmission and life cycle, history predicts a re-emergence of PCP. In this chapter, the current state of knowledge in many areas of *Pneumocystis* investigation will be reviewed. Care was taken to include key historical studies as well as the most recent reports on each subject.

THE ORGANISM

Pneumocystis are eukaryotic fungal organisms found in low number in the lungs of mammalian hosts with intact immune systems and in more abundance in homeotherms with compromised immune status. Most mammalian species have been reported to harbor organisms with morphology ascribed to *Pneumocystis*, although marine mammals and some exotic species have not been adequately evaluated. *Pneumocystis* has not been detected in surveys of poikilotherms or birds, although antibodies were detected in one study measuring the serological responses of chickens to rat-derived *Pneumocystis* (Settnes et al. 1994).

Morphology and life cycle

The nomenclature associated with descriptions of the life cycle stages and morphological features of *Pneumocystis* bears many remnants of its previous classification as a protozoan parasite. In this chapter, terms more suitable for a fungus are introduced (Cushion et al. 1997b; Ruffolo 1994). Because *Pneumocystis* has been refractory to all attempts to propagate it in vitro, histochemical and ultrastructural analyses of organisms found in human and

rat lungs have significantly contributed to the current understanding of its life cycle and morphology.

MORPHOLOGY

The morphology of *Pneumocystis* found in the lungs of various mammalian hosts is quite similar, with only subtle ultrastructural differences reported between rabbit-, mouse-, and rat-derived organisms (Dei-Cas et al. 1994; Nielsen et al. 1998). Most investigators have relied on the immunosuppressed rat model of PCP for morphological analyses because of the ease of obtaining tissue for study.

Three developmental forms of *Pneumocystis* are generally recognized: the trophic stage, precyst, and cyst. A filamentous phase has not been identified and attempts to detect an environmental cycle in which amplification takes place have been unsuccessful. Proposed life cycles have relied exclusively on microscopic observations of developmental forms found in infected mammalian lungs.

The trophic form

The trophic form of *Pneumocystis* is the smallest of its life cycle stages. These forms range in size from 1.5 to 5.0 μm and are ellipsoid in shape when viewed by light microscopy of unfixed preparations, but can appear ameboid in appearance after fixation and analysis by electron microscopy (Ruffolo 1994) (Figure 1a). Trophic forms are thought to reproduce by binary fission, with some evidence of this process provided by electron microscopy (Campbell 1972; Richardson et al. 1989; Yoshida 1989). This stage of the organism has been subdivided by some investigators into 'small' and 'large' categories, the primary characteristics of each category being:

- overall size
- the presence or absence of tubular extensions on the cell surface
- size of nucleus
- number of glycogen granules and cellular inclusions.

Since the smaller trophic form is presumed to be the direct product of excystation (spore release), it is reduced in size (1.5–2.0 μm) and contains fewer inclusions. The larger trophic form is thought to represent the vegetative stage and thus has more cellular inclusions and a larger nucleus as it proceeds towards mitotic division. Cytoplasmic organelles include a nucleus (~0.5–1.0 μm) bounded by a typical nuclear envelope with pores, which at times is associated with a nucleolus; usually a single mitochondrion with lamellar cristae which can be branched or irregular in shape (Palluault et al. 1991; Ruffolo et al. 1989); rough endoplasmic reticulum usually contiguous with the nucleus; ribosomes; occasional vacuoles; and glycogen granules. Smooth endoplasmic reticulum and Golgi-like vesicles were described in *Pneumocystis* from neonatal rabbits (Palluault et al. 1990).

Freeze fracture and transmission electron microscopy have revealed that the trophic forms are circumscribed by two distinct unit membranes separated by a thin electron-lucent space (Campbell 1972; De Stefano et al. 1990b). A dense matrix associated with the outermost membrane contains polysaccharides, the predominant surface antigen of the organism [the major surface glycoproteins (MSG)] and several classes of host-derived molecules, e.g. cardiolipin, fibronectin. The entire complex spans 20–50 nm. The lack of staining with methenamine silver (used for diagnosis of fungal infections and PCP) by this form of the organism is probably due to the almost nonexistent electron-lucent layer thought to be composed in part of β-glucan (De Stefano et al. 1990a). Usually present only on the surface of large trophic forms is a feature called 'tubular expansions or extensions' (Yoshida 1989; Dei-Cas et al. 1991). Ultrastructural studies show them as long, thin, coiled, membranous structures filled with electron-dense granular material, the function of which is not known but suggested to be a means of adherence to host cells or for uptake of nutrients (Figure 1c, d).

The observation that some trophic forms are ameboid in appearance has led some investigators to assume that at least this stage is motile (Campbell 1972; Haselton et al. 1981). By contrast, studies with live organisms have not supported this contention (Shiota 1984; Cushion et al. 1988), and the presence of cytoskeletal elements indicative of motility (e.g. microtubules, microfilaments supporting the membrane) has not been detected in trophic forms.

The precyst

The precyst (sporocyte) is recognized as an intermediate stage of the sexual phase of reproduction leading to cyst development. It is presumed that a mating event first occurs to provide a zygote which initiates sporogenesis. The zygotic nucleus undergoes meiosis and a subsequent mitosis within the sporocyte. Sporocyte development has been characterized as early, intermediate, and late. An early sporocyte has an oval shape, a cell wall of 40–50 nm containing the two unit membranes with a widening electron-lucent space, and contains several mitochondria which may be clumped around the diploid nucleus (Figure 2a, b). Ultrastructural studies of this early stage have revealed the only morphological evidence for sexual reproduction of *Pneumocystis*, the presence of synaptonemal complexes, indicating a meiotic prophase (Matsumoto and Yoshida 1984). During the intermediate sporocyte stage, the zygotic nucleus meiotically divides to yield four nuclei, while the cell becomes more spherical and the electron-lucent space between the two membranes continues to enlarge. Cytoplasmic inclusions characteristic of this stage include spindle microtubules, which appear to arise from a dense body called the nucleus-associated organelle (NAO) (Matsumoto and Yoshida 1984). The

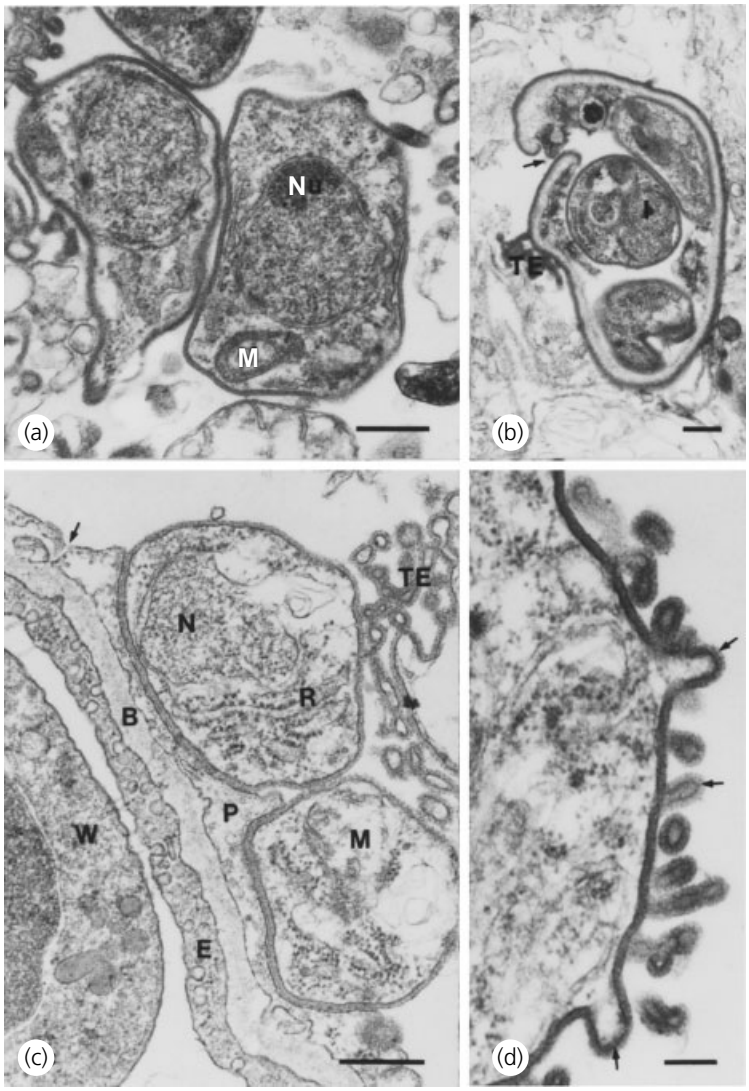

Figure 1 *Trophic forms of* P. carinii. **(a)** *The small trophic forms lack tubular extensions. A prominent nucleolus (Nu) is adjacent to the nuclear envelope of the trophic form on the right. A mitochondrion (M) and endoplasmic reticulum (R) can be observed in the same trophic form.* **(b)** *Mature intracystic bodies (ICB) (spores) are thought to be released through ruptured cyst (spore case) walls (arrow). A mature ICB (I) has the same structure as the small trophic form. Tubular extensions (TE) are also present.* **(c)** *Small trophic forms adhere tightly to the type I pneumocyte (P). The alveolar endothelium is discontinuous (arrow). Tubular extensions (TE) are evident on the alveolar lumenal side of the trophic forms. The type I pneumocyte lies adjacent to the basement membrane (B) which separates the endothelial cells (E) that line the capillary. A white blood cell (W) appears in the capillary lumen. A nucleus (N) is also present.* **(d)** *The surface of the trophic form consists of two unit membranes and a thick external glycocalyx. Tubular extensions show continuity with the cell surface (arrows). The bar lines represent 0.5 μm in Figures 1, 2 and 3, except in Figure 1* **(d)** *and Figure 2* **(b)**, *where it represents 0.2 μm. Figures 1, 2 and 3 are modifications of those appearing in Schrager et al. (1993).*

late sporocyte is defined by its spherical shape and prominent three-layer cell wall (80–120 nm). This form contains a total of eight nuclei resulting from mitotic division of the products of meiosis, aggregated mitochondria, and the presence of unit membranes within the cytoplasm, apparently for compartmentalization of the organelles into spores and completion of sporogenesis.

The cyst stage

The cyst stage (spore case, ascus) is the end product of sporogenesis. This form is spherical, 5–8.0 μm in diameter, and has a thick cell wall composed of the three layers (Figure 2c). Eight spores are commonly found within the case. *Pneumocystis* asci are of the protunicate type, characterized by a thin delicate wall and passive discharge of their ascospores. The other two types of asci found in the ascomycetes, unitunicate and bitunicate, actively discharge their spores by forcible ejection (Liu and Hall 2004). Biochemical analyses and staining reactions provide strong evidence for the presence of substantial amounts of glucan in the spore case cell wall and there is some evidence that chitin or at least, *N*-acetylglucosamine may also be

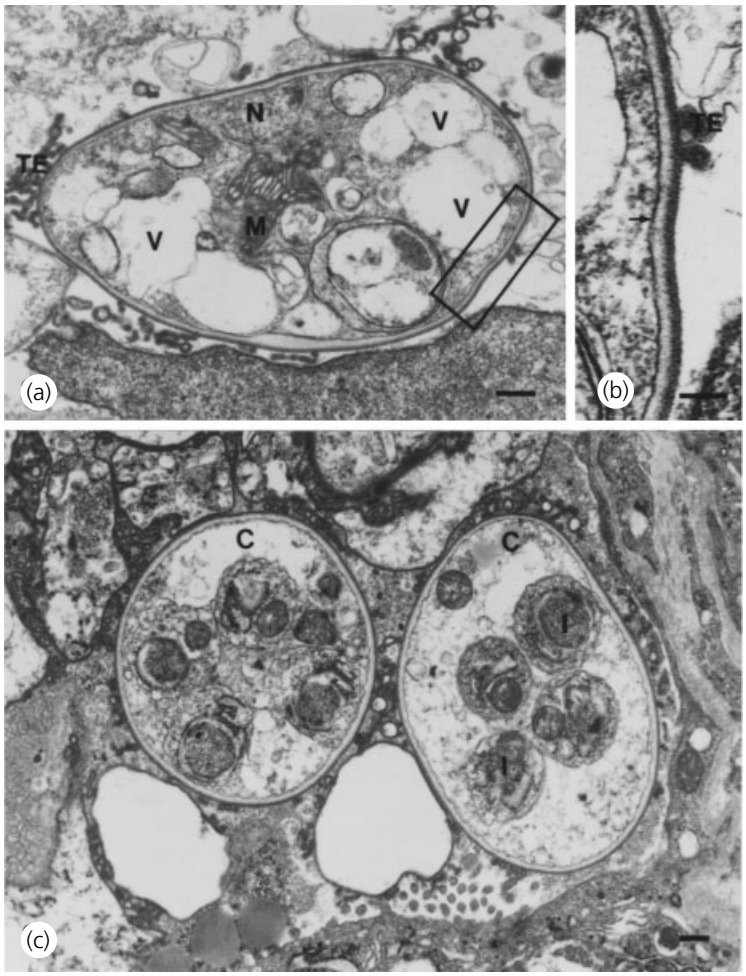

Figure 2 *Precyst and cysts (sporocytes and spore cases).* **(a)** *Aggregation of mitochondria (M) are a characteristic of precysts (sporocytes). Lamellar cristae can be seen in these organelles. Vacuoles (V), tubular extensions (TE), and a nucleus (N) are also present.* **(b)** *Higher magnification of the sporocyte surface within the rectangle in* **(a)** *shows the plasma membrane (arrow) and a thickened cell wall consisting of an inner electron-lucent region and an outer electron-dense layer in which the second unit membrane is enmeshed. Tubular extensions are reduced in number in precysts (sporocytes) and cysts (spore cases–I.C).* **(c)** *It is presumed that as the spore cases mature, nuclei and cytoplasm are segmented into ICB/spores (I) within the spore cases.*

present (Garner et al. 1991; De Stefano et al. 1992). The spores are spherical, 1–2 μm in diameter, contain the same organelles of the small trophic forms, and are bounded by two unit membranes. One irregularity of the spore case cell wall can be observed at one of the poles, where a thickening of the electron-lucent space is apparent by electron microscopy and by light microscopy of methenamine silver stained organisms or interference contrast microscopy of unstained organisms (Ruffolo et al. 1986). Membranous particles, mitochondria, and unincorporated cytoplasm are often present at this location.

The process of spore release is not well documented, except by Shiota (1984) who described the excystation of spherical and elongated intracystic bodies using light microscopic techniques. Prior to release, the spores have been reported to exhibit a variety of shapes, such as:

- spherical, which imparts a rosette array within the spore case
- large and irregular shapes that seem to be tightly packed into the case
- elongated banana-shaped spores that often exhibit dense cytoplasm
- ellipsoidal forms that sometimes appear as if attached to stalks.

Contractile-like movement was observed in some light microscopic studies of the ellipsoidal and elongated shaped spores (Shiota 1984; Cushion et al. 1988) and subsequently documented by videomicroscopy (Newsome et al. 1991). Neither the function of the various shapes nor the significance of the movement is understood. Empty spore cases are frequently observed by light and electron microscopy and assume a crescent shape. In some, a rent in the wall is evident (Figure 3). Within

Figure 3 *Collapsed cyst (spore case). A cresent-shaped collapsed spore case (C). A trophic form (T) is also shown. The spores have been released, presumably through a rent in the cell wall (arrow). The residual cytoplasm has begun to degenerate.*

the collapsed thick wall, a remaining spore may be observed as well as residual cytoplasm.

In addition to the archetypal thick-walled form, a thin-walled cyst has been described by some investigators (Vossen et al. 1978; Matsumoto and Yoshida 1986). This stage contains large irregularly shaped 'daughter forms' that fill the entire cell which is delimited by a cell wall lacking the inner electron-lucent layer.

LIFE CYCLE

The intrapulmonary life cycle of *Pneumocystis* likely involves an asexual phase of cell division by the apparent haploid trophic form and sexual phase (sporogenesis) leading to the production of a thick-walled reproductive cyst (spore case, ascus) containing eight intracystic bodies (spores) (Figure 4). An additional asexual cycle leading to formation of a thin-walled cyst (endogeny) has been proposed (Vossen et al. 1978; Matsumoto and Yoshida 1986).

Several life cycles of *Pneumocystis* have been described. All relied upon organism morphologies observed in infected lungs by techniques of light and electron microscopy and not by direct observations made from replicating organisms (Walzer et al. 1989). Thus, the complete life cycle of *Pneumocystis* is not known, but has been deduced from static images and based on the life cycles of other protists, especially parasitic protozoa, but more recently, fungi (Cushion 2004b). It is hoped that a clearer understanding of the fungal nature of *Pneumocystis*, coincident with improvement in ex vivo culture conditions, will lead to definition of its life cycle.

The asexual replication of the trophic forms appears to resemble binary fission, rather than budding, a process used by many but not all yeasts. *Schizosaccharomyces pombe* is one yeast that divides by binary fission and also has a sexual phase initiated by morphologically indistinguishable mating types. *S. pombe* is phylogenetically related to *Pneumocystis* (see below) and it is conceivable that they share some similarities between their life cycles.

In 1984, Matsumoto and Yoshida provided ultrastructural evidence for sexual reproduction (Matsumoto and Yoshida 1984). Synaptonemal complexes, zipper-like conformations which occur during pairing of homologous chromosomes, were observed in a *Pneumocystis* life cycle stage the authors called an 'early precyst.' If sexual reproduction does take place, cyst development would necessarily follow a mating event. Ultrastructural observation of binucleate trophic forms has been interpreted as resulting from conjugation (Yoshida 1989), but could just as easily represent binary fission. The trophic forms/trophozoites involved in this process have been postulated to be 'isogametes' because of the lack of morphological characteristics to distinguish male or female forms. However, isogametes are usually associated with a protozoan life cycle. Rather, the trophic forms may be more akin to the mating types of yeast which are, in general, morphologically indistinct, but are defined by pheromones and receptors encoded by mating type-specific genes (Herskowitz 1989). For example, in the ascomycete family, *Saccharomyces cerevisiae* has two mating types, 'α' and '**a**,' and *S. pombe* has 'minus (−)' and 'plus (+).' Since the mating process in yeast is usually initiated by an environmental stress, e.g. nitrogen starvation, perhaps sporogenesis of *Pneumocystis* is induced by unfavorable conditions in the mammalian lung brought about by an increasing organism burden or depletion of nutrients.

Molecular genetic techniques have led to significant advances in our understanding of the *Pneumocystis* life cycle. Like that in yeast, the sexual cycle likely involves coordination of mating types. The process is initiated by attachment of the pheromone from one mating type to the other's receptor. In yeast, the receptor *STE2* in **a** cells is recognized by **α**-factor and the receptor *STE3* of α cells is recognized by **a**-factor. The complementary binding results in signaling to the interior of the cells via a heterotrimeric G protein complex, then to a mitogen-activated kinase cascade. Activation of Ste12p stimulates the cells into expression of mating type genes, cell cycle arrest, fusion of mating types, and nuclear fusion (Herskowitz 1989). Within the genome of *P. carinii* lies homologs to *STE3*, *STE2*, *STE11*, *STE12*, *STE20*, and Gpa1(Smulian et al. 1996, 2001), suggesting that the organisms do indeed mate and can transduce this signal via the appropriate signal cascade. The *P. carinii* Ste12 homolog (which also has homology to the Ste11 of *S. pombe*) was shown to be phosphorylated by a map kinase of *P. carinii* (PCM) (Vohra et al. 2003b). A homolog of the **a** mating

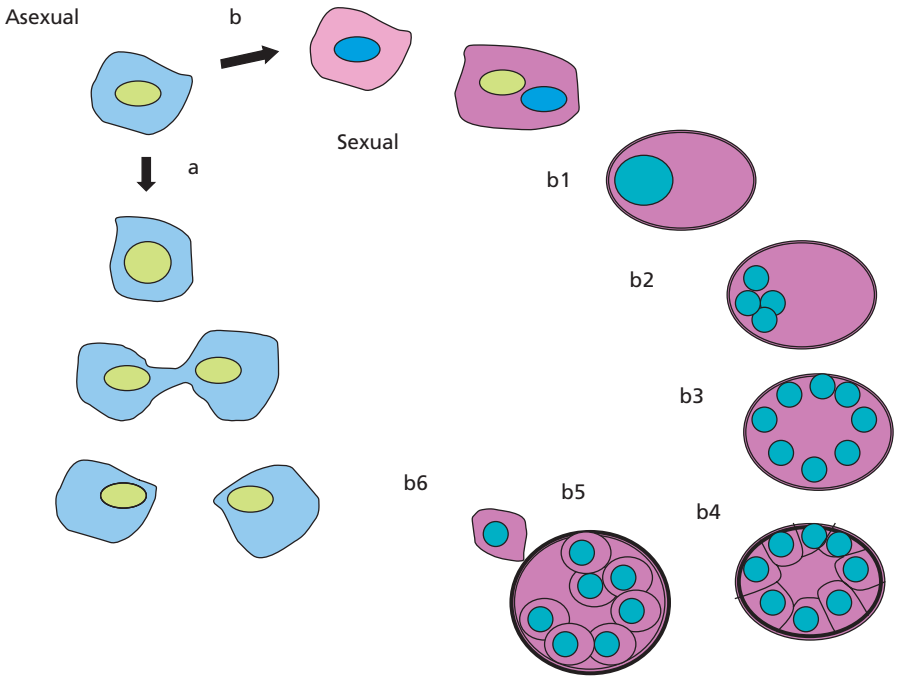

Figure 4 *Proposed life cycle of* P. carinii. *Trophic forms of opposite mating type (shown as blue or pink) can undergo asexual binary fission* **(a)** *or enter into sexual reproduction via mating* **(b)**. *Asexual phase* **(a)**. *Trophic forms replicate by mitosis where the haploid nucleus replicates, cytoplasmic volume increases, and the cell splits by simple cell division. No budding has been observed in the life cycle of* Pneumocystis. *Sexual phase. Haploid trophic forms (mating types, blue and pink) conjugate to form a diploid zygote (early phase sporocyte) (b1) which undergoes meiosis (b2) and subsequent mitosis (b3) to form eight haploid nuclei (late phase sporocyte). The spores are formed by compartmentalization of nuclei and cytoplasmic organelles, e.g. mitochondria (b4). Release is thought to occur through a rent in the cell wall (b5). The ascospores then emerge and become the vegetative stage, or trophic forms, which presumably can enter either phase of division (b6).*

factor was found flanking the *STE3* gene of *P. carinii* (Smulian et al. 2001). The linkage of an **a**-factor pheromone with an **a**-factor receptor has not been reported for any other member of the fungi. Despite the lack of in vitro system and thus, any molecular tools to evaluate function, investigators in the field have relied upon expression in heterologous systems like yeast and *S. pombe*, with generally good rates of success (Thomas et al. 1998; Fox and Smulian 1999; Vohra et al. 2003a). This approach will be invaluable for definition of the complete replication cycle and other pathways of *P. carinii*.

Putative gene homologs that function in mitotic and meiotic replication in other fungal systems have been identified through the *Pneumocystis* Genome Project (Porollo et al. 2004) and by investigators in the field (Thomas et al. 1996, 2001; Gustafson et al. 1999; Vohra et al. 2003b). A partial summary of the homologous genes involved in cell division and replication from the Expressed Sequence Tag (EST) portion of the Genome Project has been reported (Cushion 2004b).

HOST RANGE

Early surveys of wild and domestic animal populations used microscopic methods for detection of *Pneumocystis*. Presence of organisms consistent with the morphology of the cyst form of *Pneumocystis* in the lungs of mammals was used as the diagnostic criteria. Later studies have relied upon the polymerase chain reaction (PCR) with primers targeting the mitochondrial large subunit (mtLSU) of the ribosomal RNA in most cases. Both standard and nested PCR techniques have been used. Table 1 summarizes the majority of these reports. The organisms were detected in phylogenetically disparate classes of mammals spanning rodents, canines, felines, ungulates, and primates. A recent study using nested PCR surveyed a large number of diverse species of nonhuman primates and found evidence supporting the coevolution of the *Pneumocystis* species with their distinct host (Hugot et al. 2003). A striking finding of this study was the genetic diversity of *Pneumocystis* populations as well as their prevalence within the primates (20–100 percent). *Pneumocystis* was also detected with high frequency in wild shrews, ranging from 30–70 percent of the animals sampled (Laakkonen and Henttonen 1995; Laakkonen and Soveri 1995; Bishop et al. 1997). The presence of *Pneumocystis* has not been reported in any of the poikilotherms, although a detailed survey of such animals is absent from the literature. Studies of members of the Aves kingdom have not found evidence of *Pneumocystis* using both microscopic and molecular genetic methods, although some antibodies to rat-derived *Pneumocystis*

Table 1 Pneumocystis *detected in mammals*

Animal	Reference	Country	Prevalence (if available)
Wild rats	Yoshida and Ikai 1979	Japan	
	Shimizu et al. 1985	Japan	20.5% (8/39)
	Settnes and Lodal 1980	Denmark	22% (33/150)
Misc. rodents	Lainson and Shaw 1975	Brazil	
	Kucera et al. 1971	Czech Rep	
	Laakkonen et al. 2001a	USA	
Ferret	Stokes et al. 1987	USA	
Shrew	Laakkonen and Henttonen 1995	Finland	
	Laakkonen and Soveri 1995		
	Laakkonen et al. 2001a, b	USA	
Sloth	Lainson and Shaw 1975	Brazil	
Hare	Blazek and Pokorny 1963	Czech Rep	
	Poelma 1972	Netherlands	17% (75/437)
Rabbit	Sheldon 1959	USA	
	Mata 1959	Mexico	
	Soulez et al. 1988	France	
Dog	Carini and Maciel 1916	Italy	
	Farrow et al. 1972	Australia	
	Copland 1974	New Guinea/Australia	
	McCully et al. 1979	South Africa	
	Davalos 1963	Mexico	2.7% (3/108)
	Zavala and Rosado 1972	Mexico	6.3% (4/63)
	Botha and van Rensburg 1979	South Africa	
	Yoshida and Ikai 1979	Japan	15% (2/13)
	Settnes and Hasselager 1984	Denmark	
	Sedlmeier and Dahme 1955	Germany	
Cat	Settnes and Hasselager 1984	Denmark	4% (3/75)
	Zavala and Rosado 1972	Mexico	12.6% (10/79)
Goat	McConnell et al. 1971	South Africa	
Swine	Kucera et al. 1968	Czech Rep	
	Seibold and Munnell 1977	USA	
	Jecny 1973	Russia	
	Bille-Hansen et al. 1990	Denmark	
Cattle, sheep	Jecny 1973	Russia	
Horse (foals)	Shively et al. 1973, 1974	USA	
(CID)	Perryman et al. 1978	USA	
	Ainsworth et al. 1993	USA	100% (5/5)
	Peters et al. 1994	UK	
	Ewing et al. 1994	USA	100% (3/3)
	Perron LePage et al. 1999	France	
	Jensen et al. 2001	Denmark	100% (6/6)
Non-human primates			
Marmosets	Richter et al. 1978	USA	11.3% (50/441)
	Hugot et al. 2003	France	28.6% (10/35)
Owl monkeys	Long et al. 1975	USA	
	Chandler et al. 1976	USA	
Owl-faced monkey	Hugot et al. 2003	France	100% (1/1)
White-nosed monkey	Hugot et al. 2003	France	100% (1/1)
Squirrel monkey	Hugot et al. 2003	France	20% (1/5)
Swamp monkey	Hugot et al. 2003	France	100% (1/1)
Chimpanzee	Chandler et al. 1976	USA	
Macaque	Matsumoto et al. 1987	Japan	
	Norris et al. 2003 (SIV+)	USA	100% (11/11)
	Hugot et al. 2003	France	40% (4/10)
	Guillot et al. 2004	France	
Tamarins	Hugot et al. 2003	France	
		French Guyana	75% (9/12)
Lemurs	Hugot et al. 2003	France	100% (2/2)

(*Continued over*)

Table 1 Pneumocystis detected in mammals (*Continued*)

Animal	Reference	Country	Prevalence (if available)
Sakis	Hugot et al. 2003	French Guyana	100% (2/2)
Zoo animals	Poelma 1975	Netherlands	
Red kangaroo			
Tree shrew			
Senegal galago			
Demidoff's galago			
Brown howler monkey			
Woolly monkey			
Long-haired spider monkey			
White-eared marmoset			
Chimpanzee			
Three-toed sloth			
Palm squirrel			
Red panda			
Fennec fox			
Tree hyrax			
Large-toothed hyrax			

were detected in the sera from some birds (Settnes et al. 1994). Temperature may play a role in the organism's proclivity for mammals, as birds maintain a higher basal temperature than mammals, at approximately 41°C, and poikilotherms have variable temperatures only slightly higher than the ambient temperature. As discussed later in this chapter, the organism populations harbored by the different species of mammals are genetically and phenotypically distinct, warranting the formal process of naming of these *Pneumocystis* species.

TAXONOMY AND PHYLOGENY

Since the publication of the previous, 9th edition of this text in 1998, there has been a general acceptance within the scientific community that members of the genus *Pneumocystis* are bonafide fungi, albeit 'atypical' fungi. Individual gene sequence comparisons and the fungal homologies of a large number of putative genes produced by the *Pneumocystis* Genome Project have contributed in large part to this consensus. With the question of 'What are they, exactly?' (Stringer 1996) apparently settled, the issue of describing distinct species within the genus has become the focus of attention. In this section, a summary of the controversy regarding the fungal versus protozoan nature of *Pneumocystis* will be presented. The newly named species within the genus will be highlighted as well as the studies and observations that lead to these distinctions.

Beginning with its first identification as a life cycle stage of a trypanosome by Carlos Chagas in 1909, the taxonomy and phylogenetic classification of *Pneumocystis* has been fraught with controversy. The Delanöes believed it to be a protozoan and that assertion was generally accepted by the rather uninterested scientific community. After *P. carinii* was found to be the etiologic agent of epidemic interstitial plasmacellular pneumonia in the 1950s, a renewed effort to classify it was initiated. Application of the tools available at that time, including vitro culture, microscopic analysis, histological staining, and animal inoculation experiments, resulted in two divided opinions; that *P. carinii* was a protozoan or that it was a fungus (Gajdusek 1957).

Ultrastructural studies of the 1960s and 1970s reached 'conclusions' similar to those of the earlier microscopists, that *Pneumocystis* was a protozoon or a yeast, based on the 'characteristic' cellular structures of the cyst and trophic forms. Exploiting the immunosuppressed rat model of pneumocystosis established by Frenkel in 1966, investigators were able to compare organisms obtained from rats with those from humans. In 1967, Barton and Campbell concluded that the organisms identified as *Pneumocystis* in rat lungs most likely were protozoons based on the presence of a protozoan-like pellicle and predicted motility as indicated by morphological features identified as 'pseudopodia' in the trophozoites (Barton and Campbell 1967, 1969). In 1972, Campbell confirmed the structural similarities of rat and human-derived *Pneumocystis* and again underscored the protozoan nature of the organism, adding the presence of filopodia as an additional feature supporting this contention (Campbell 1972). In contrast, Vavra and Kucera in 1970 reached the conclusion that *Pneumocystis* was a yeast after extensive ultrastructural analyses of rat and human organisms (Vavra and Kucera 1970). They found the morphology of the intracystic bodies within the cyst similar to ascospores within the ascus of yeasts like *Saccharomyces*. In addition, the organization of the cell membrane and wall were likened to those observed in *Rhodotorula* or *Coccidioides immitis*, and the lamellar cristae of the mitochondria reminiscent of the yeast organelle. Placement in the Ascomycetes was

tentatively proposed with verification once a culture system was found.

More recent ultrastructural studies echo the same conundrum. In 1984, Matsumoto and Yoshida not only provided evidence for meiosis as a means of reproduction for *Pneumocystis* (the presence of synaptonemal complexes) but proposed a life cycle with striking similarity to that of a protozoan parasite with isogamous gametes (Matsumoto and Yoshida 1984). Studies by Ruffolo and colleagues (Ruffolo et al. 1989) reported the morphology of the cristae in *Pneumocystis* mitochondria to be lamellar, rather than vesicular, and the process of daughter cell formation more like that of a fungus. There are no morphological features of *Pneumocystis* entirely consistent with either a yeast or protozoan classification. Ultrastructural analysis of the cyst wall by De Stefano et al. (1990b) confirmed an earlier finding of Bommer (1962) which described two unit membranes, a plasma membrane delimiting the daughter forms as well as an outer membrane enmeshed within the dense carbohydrate matrix of the cyst wall. A two-membrane outer layer is more reminiscent of protozoons than any common fungus. Most investigators recognized the limitations of ultrastructural analyses for taxonomic purposes, especially in the case of *Pneumocystis* which cannot be cultured in vitro to provide further verification of their observations.

Compositional analyses of the lipids and carbohydrates of *Pneumocystis* have not yet yielded any signature molecules that would define its taxonomic placement. Rather, these studies confirmed the atypical nature of this family of organisms. Instead of finding ergosterol as the main structural sterol, as is the case with most yeast, cholesterol was the bulk sterol in *P. carinii* (Kaneshiro et al. 1989). Kaneshiro et al. have suggested that cholesterol (C27), is probably totally scavenged from the host. By contrast, another study provided evidence that cholesterol could be synthesized, at least in part, by the organism (Zhou et al. 2002). Gas chromatography-mass spectrometry (GC-MS) analyses of the monosaccharide fragments of the carbohydrates of the cyst wall and cytoplasmic contents of *P. carinii* identified glucose as the major constituent; mannose and galactose were present in equal ratios; *N*-acetyl-D-glucosamine was found in lesser amounts, while trace amounts of ribose and sialic acid were present. Sugars were representative of about 8 percent of the cyst wall components, a somewhat lower amount than most fungi (De Stefano et al. 1990a). In contrast to the rat-derived carbohydrate analyses, high-pressure liquid chromatography (HPLC) analyses conducted by Lundgren et al. found mannose, glucose, galactose, and glucosamine to be present in *P. jirovecii* at equimolar concentrations (Lundgren et al. 1991). The presence of chitin was inferred by lectin binding (Garner et al. 1991), but enzymes involved in chitin biosynthesis have not yet been detected in its genome.

Molecular biological analyses

There is overwhelming genetic evidence supporting the fungal identity of *Pneumocystis*. Whereas consideration of morphological characteristics, biochemical composition, or drug susceptibilities produced no definitive phylogenetic association, the use of molecular genetic tools initiated in 1988 provides a distinct answer to the question of the phylogenetic placement of this complex of organisms. *Pneumocystis* have been placed in the Phylum Ascomycota; the Subphylum Taphrinomycotina (syn. Archiascomycotina); Class Pneumocystidomycetes; Order Pneumocystidales; and Family *Pneumocystidaceae* (Eriksson and Winka 1998). The Taphrinomycotina are a paraphyletic assemblage of basal taxa and the identity of the closest extant relative of *Pneumocystis* remains unsettled, with phylogenetic results varying by gene sequence used and method for construction. The morphologies and biochemical characteristics of the fungi within this group are highly diverse and include such members as the fission yeast *S. pombe*, the plant pathogen *Taphrina deformans*, and *Neolecta vitellina*, the only member with a fruiting body structure (Liu and Hall 2004).

SMALL SUBUNIT RRNA SEQUENCES

Sequence comparison of rRNA genes is the most widely used method for studying phylogenetic relationships among prokaryotic and eukaryotic microbes (Vandamme et al. 1996). Both the large and small nuclear ribosomal RNA genes contain regions that are conserved and evolve at a slower pace as well as other regions that are more rapidly evolving and have increased sequence variation. Comparisons of sequences at the more conserved regions are used to predict more inclusive relationships such as kingdom, while the variable regions are used to define the closer associations, e.g. genus, species. Two laboratories reported the fungal nature of *P. carinii* based on comparisons of the DNA gene sequence of the small subunit ribosomal RNA (16S) gene (Edman et al. 1988) and of cDNA from the same gene of *P. carinii* (Stringer et al. 1989). Construction of several phylogenetic trees based on 16S-like sequence infer a relationship of *P. carinii* with the ascomycetes *S. cerevisiae*, *S. pombe* or *T. deformans* (Gargas et al. 1995) (Figure 5).

5S RNA SEQUENCE

Sequencing of the 5S RNA gene showed a relationship with the 'Rhizopoda/Myxomycota/Zygomycota group' (Watanabe et al. 1989). The 16S-like genes have replaced the 5S genes for phylogenetic analyses because of associated evolutionary clock anomalies and ambiguities due to the short 120 bp sequence length.

MITOCHONDRIAL GENE SEQUENCES

The use of the mitochondrial genome as an evolutionary clock has been questioned because of their supposed

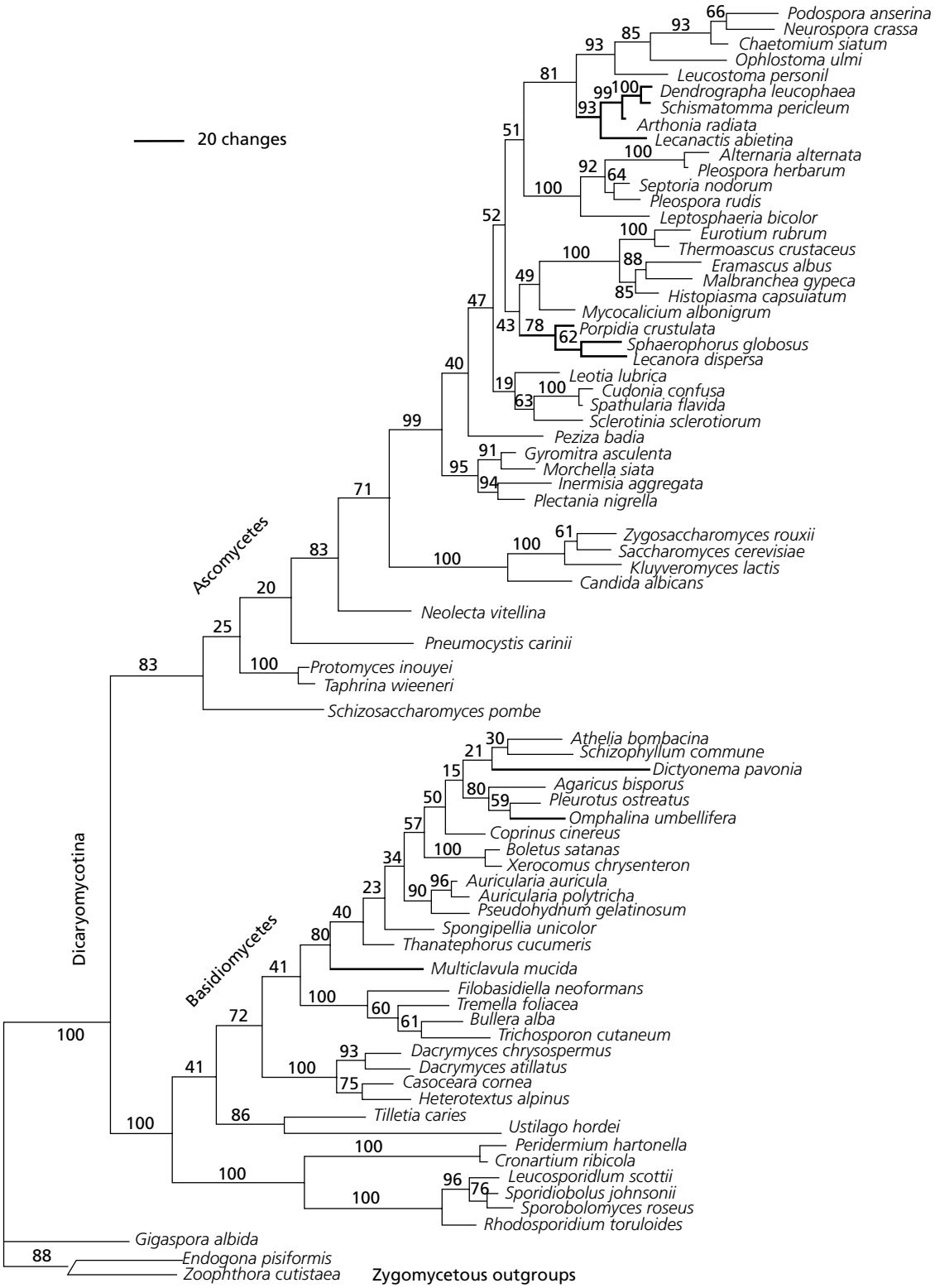

Figure 5 *Phylogeny of* Pneumocystis carinii. *Reprinted from Gargas et al. 1995*

bacterial origins and endosymbiotic lifestyles that probably arose more than once throughout microbial evolution. Nonetheless, comparison of several *Pneumocystis* mitochondrial genes supports the fungal identity of the organism. Comparison of the putative amino acid sequences of the entire genes or portions of the mitochondrial cytochrome oxidase II, apocytochrome *b*, *NADH 1*, *NADH 2*, *NADH 3*, *NADH 6* genes from

P. carinii with like genes from three fungi and two protozoans showed greater homology to the fungi than to the protozoans (Pixley et al. 1991). Comparison of the mitochondrial small subunit rRNA secondary structures of representative members of the fungi and protozoa with *Pneumocystis* sequence supported its fungal identity. Wakefield et al. (1992) employed a strategy whereby oligonucleotide primers were targeted to a sequence specific for a portion of the large subunit of the mitochondrial RNA of *P. carinii*. A series of fungi representing genera of Ascomycota and Basidiomycota were subjected to the PCR under conditions of reduced stringency. Members of the ustomycetous red yeast fungi were found to produce amplicons with these primers. Comparisons of gene sequences from these basidiomycetes with those of *P. carinii* inferred a relationship among these organisms. However, placement of *Pneumocystis* among the basidiomycetes has not been upheld by recent data from the *Pneumocystis* Genome Project (pgp.cchmc.org) or by comparisons of other genes (Liu and Hall 2004). Rather, BLAST analyses (Altschul et al. 1990) of cDNA and genomic sequences of *P. carinii* show that the greatest majority of putative genes have homology to the ascomycetes, *S. pombe*, *S. cerevisiae* and *Neurospora crassa* (Cushion et al. 2004b).

ELONGATION FACTOR 3

Identification of the *Pneumocystis* gene for translation elongation factor 3 (EF3) provided strong evidence for a fungal ancestry (Ypma-Wong et al. 1992). This factor is essential for protein synthesis in fungi but not in other eukaryotes. Alignment of the deduced amino acid sequence showed 57 percent identity with *S. cerevisiae* and *Candida albicans* EF3 proteins, while comparison of the *S. cerevisiae* and *C. albicans* amino acid sequences showed a 78 percent identity. These data suggest that *Pneumocystis*, although a member of the fungi, is significantly divergent from other fungi.

RNA POLYMERASE II

Slowly evolving genes that encode proteins are being routinely used to infer phylogeny. These genes are usually those in replication, transcription, and translation of genetic material and did not arise from horizontal transfer (Sicheritz-Ponten and Andersson 2001). One such gene is that encoding the second subunit of the nuclear DNA-dependent RNA polymerase II (*RPB2*). This and other protein-encoding genes have been found to resolve internal branches that were unresolved in rDNA trees (Liu and Hall 2004). Analyses of the *RPB2* gene from *P. carinii*, and those from 60 other fungi including seven basiodiomycetes as outgroups, supported the small subunit rRNA trees in its placement in the Taphrinomycotina, but differed in its closest fungal affinities. Maximum parsimony with bootstrap values greater than 40 percent showed a closer affinity with *S. pombe*, while Bayesian inference with a posterior probability of 97 percent placed *P. carinii* with the plant pathogen *T. deformans* (Liu and Hall 2004).

COMPARISONS OF OTHER GENE SEQUENCES

Several other protein-encoding genes from *Pneumocystis* have been sequenced and compared with like genes from protozoans and fungi. Edlind et al. found the β-tubulin gene to be more homologous with the genes of filamentous fungi (89–91 percent) such as *N. crassa* than with those of yeasts such as *S. pombe* (Edlind et al. 1992). The thymidylate synthase (*TS*) and dihydrofolate reductase (*DHFR*) genes in protozoa are fused and encode a bifunctional protein (Garret and Coderre 1984). In the yeasts and mammals evaluated thus far, these genes are separate. In 1989, it was shown that *Pneumocystis* encodes these proteins with separate genes found on separate chromosomes (Edman et al. 1989a, b). Construction of phylogenetic trees with limited numbers of fungal and protozoal members using *TS* and *DHFR* sequences supported the association of *Pneumocystis* genes with ascomycetes and basidiomycetes. Similar studies with transcription factor IID and a P-type cation-translocating ATPase gene (Stringer 1993) also supported the fungal identity of *Pneumocystis*.

GENETIC DIVERSITY OF *PNEUMOCYSTIS* FOUND IN DIFFERENT MAMMALIAN HOSTS

It has long been appreciated that there are differences among the antigenic reactivities of *Pneumocystis* obtained from the lungs of different mammalian hosts (Graves et al. 1994). Molecular genetic evidence supports these initial findings. Comparison of the srRNA and mitochondrial large ribosomal subunit RNA, and *TS* gene sequences between rat, human, mouse, ferret, and pig *Pneumocystis* shows differences similar to those observed between different yeast species (Keely et al. 2004). Likewise, the sequences of genes from the multigene family encoding the MSG of *Pneumocystis* are quite distinct, supporting the observed antigenic reactivities of the expressed proteins (Gigliotti et al. 1993a; Kovacs et al. 1993; Stringer et al. 1993; Wada et al. 1993; Keely et al. 1994; Sunkin et al. 1994). Comparison of the sequence of a portion of the 5-enolpyruvyl shikimate phosphate synthase, a gene in the *arom* locus, among rat-, human-, mouse-, rabbit-, and ferret-derived *Pneumocystis* showed divergence at the nucleotide level ranging from 7 to 22 percent and at the deduced amino acid sequence level of 7–26 percent (Banerji et al. 1995). The human and rat *Pneumocystis* α-tubulin gene were 92 percent identical in their predicted amino acid sequence and 26 percent divergent in the nucleotide sequence (Stringer 1993). Table 2 illustrates some of these differences.

Table 2 *Pairwise distances (percent) of six* Pneumocystis *genes*

	SODA[a]		*DHPS*		*DHFR*		*mtrRNA*		*AROM*		*TS*	
Species compared	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**	**LSU**	**SSU**	**nt**	**aa**	**nt**	**aa**
P. murina/*P. carinii*	16	4	6	6	17	19	8	10	7	7	6	5
P. murina/*P. jirovecii*	27	23	15	19	31	34	20	18	17	19	21	17
P. carinii/*P. jirovecii*	33	25	16	18	36	39	24	23	18	20	22	14
P. jirovecii/primate[a]	10	2	10	11	19	21	15	12	NA	NA	NA	NA

Modified from Keely et al. 2004.
AROM (5-enolpyruvylshikimate-3-phosphate synthase); *DHFR* (dihydrofolate reductase); *DHPS* (dihydropteroate synthase); *mtrRNA* (mitochondrial large subunit rRNA); NA, No sequence available; *SODA* (manganese co-factored superoxide dismutase); *TS* (thymidylate synthase)
a) *P. jirovecii* was compared to non-human primate-derived *Pneumocystis* from owl-monkey and macaque.

Electrophoretic karyotypes from rat, mouse, human, and ferret *Pneumocystis* have been shown to be distinct (Stringer and Cushion 1998; Weinberg and Bartlett 1991) (see Figure 6). The rat *Pneumocystis* profiles from *P. carinii* and *P. wakefieldiae* ranged between 700 and 300 kb with 13–15 bands, with estimated total genome sizes of 8.2 and 7.7 Mb, respectively. *P. murina* from mouse contained 15–16 bands within a shorter range of 600–350 kb but with a similar estimated genome size of 8.2 Mb. The human *P. jirovecii* karyotype of 12 bands ranged in size from 800 to 350 kb with a unique band at approximately 800 kb and an estimated genome size of 7.0 Mb. *Pneumocystis* from ferret produced the most distinct karyotype, with about 13 bands separating between 525 and 700 kb and one large band at ~850 kb with the largest estimated genome size for *Pneumocystis* species to date, 11.0 Mb.

Supporting the concept of species specificity were the negative results from several animal studies in which *Pneumocystis* obtained from one host was inoculated or injected into different immunosuppressed recipient hosts. For example, Gigliotti et al. attempted to inoculate severe combined immunodeficiency disease (SCID) mice with *Pneumocystis* derived from immunosuppressed ferrets and tracked the infection with *Pneumocystis*-specific molecular markers (Gigliotti et al. 1993b). No cross-infection could be detected.

GENETIC DIVERSITY WITHIN A SINGLE HOST

Rat

The presence of two genetically distinct *Pneumocystis* residing in the same infected rat lung was described in 1992 by detection of divergent 5.8S and 26S rRNA sequences (Liu et al. 1992). That these two 'sequovars' were from two discrete genomes was reported in 1993 using a combination of pulsed-field gel electrophoresis (PFGE), Southern hybridizations, and gene sequence comparisons (Cushion et al. 1993). The karyotpe profiles of the two populations, termed 'prototype' and 'variant' at that time, produced by PFGE were distinct (Figure 6) and hybridization with eight single-copy gene probes to the Southern blotted profiles resulted in localization to chromosomes of different sizes (Cushion et al. 2004a). Of note, 13 patterns of karyotypic profiles have been identified from rats infected with the 'prototype' population (Rebholz and Cushion 2001) while only a single profile has emerged for the 'variant' organism. The most common profile of 'prototype,' form 1, is illustrated in Figure 6. The form 1 genome was chosen as the first *Pneumocystis* to be sequenced (Cushion and Arnold 1997). Both populations were often found within the same rat colony (identified by PFGE profiles), and many of the infections (within the same rat lung) were composed of both organisms (Cushion et al. 1993).

The small subunit rRNA sequences of these two *Pneumocystis* populations were 6.6 percent divergent in a highly variable region of the gene. The sequence differences in each population have been reported at several other loci and ranged from 4 to 31 percent (Table 3). Such levels of divergence are associated with species differences among other fungal genera. Calculations based on the rates of change of the small subunit rRNA genes and the internal transcribed spacer (ITS) regions of the nuclear ribosomal locus estimate that these two species diverged 22–15 million years ago (Cushion et al. 2004a).

The MSG that are on the surface of all *Pneumocystis* organisms are encoded by a multigene family. MSG genes from each of the *Pneumocystis* populations in rats were found to be genetically distinct, as evidenced by the lack of hybridization of a probe from prototype to variant *Pneumocystis* populations separated by contour clamped homogeneous electrical field (CHEF) (Cushion et al. 1993) and by sequence comparisons that showed 35 percent divergence (Cushion et al. 2004a). Moreover, the genetic elements associated with regulation of the transcription of MSG genes from each of these populations were found to be quite different (Schaffzin and Stringer 2000). These genetic differences were verified at a protein level with immunoblotting techniques (Vasquez et al. 1996).

Other animals

Two *Pneumocystis* populations coinfecting ferrets have been found to vary in sequence at the *arom* locus by a similar level of divergence (15 percent) observed between *P. carinii* and *P. wakefieldiae* (Banerji et al. 1994). These

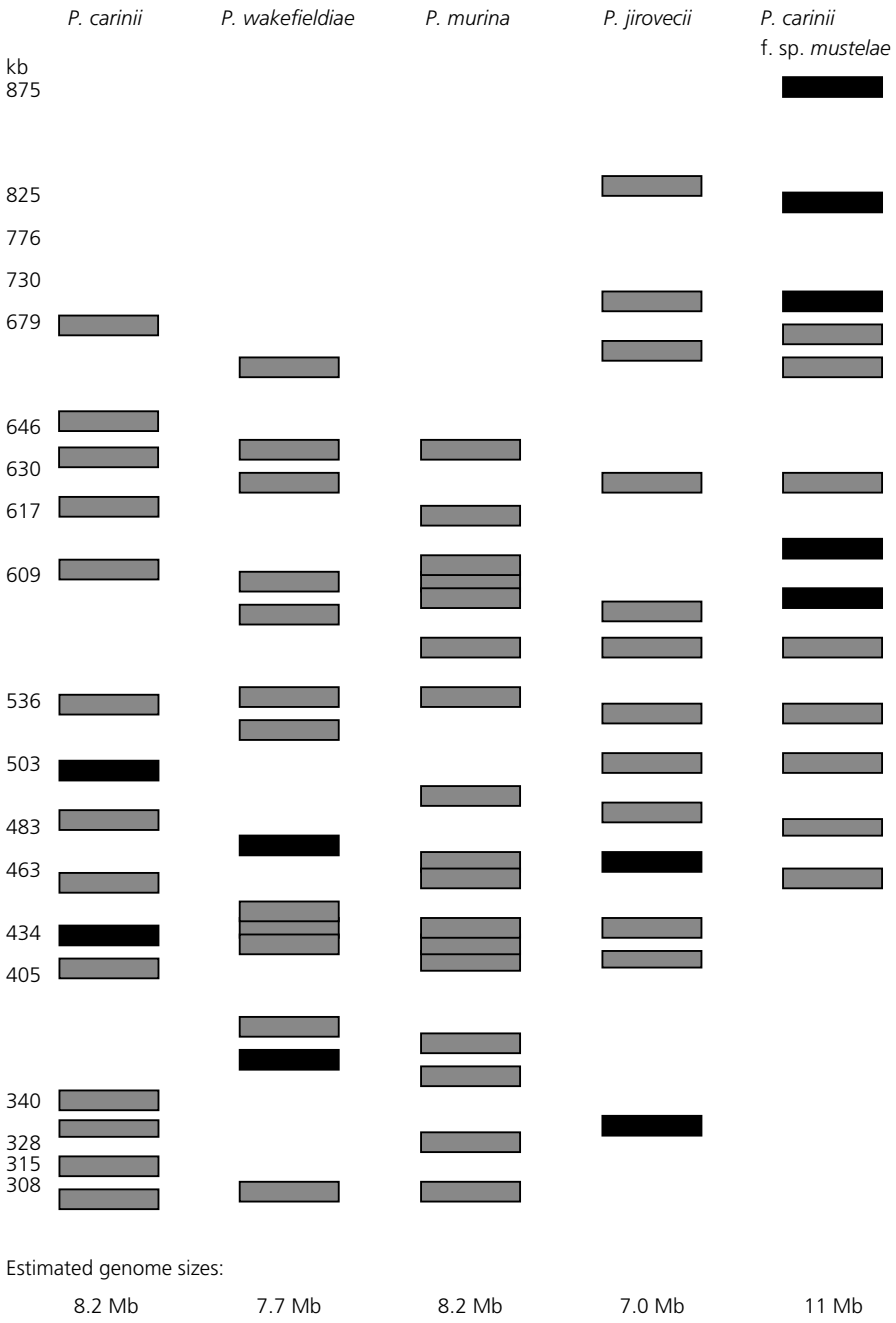

Figure 6 *Schematic of electrophoretic karyotypes from different species and putative species of* Pneumocystis. *Profiles were based on the migration of chromosome-sized bands using the pulsed field technique of CHEF. Sizes of chromosomes were calculated by linear regression using concatenated lambda phage DNA. Reprinted with permission from Cushion et al. 2004b, p. 165*

two sequences were 17 and 18 percent divergent from the *P. carinii* and *P. wakefieldiae arom* gene sequences.

PCR amplification directed to the mtLSU of *Pneumocystis* was used to amplify DNA purified from the lung tissue of a female Cavalier King Charles Spaniel diagnosed with PCP. Two sequences of about 350 bp each had 86.9 percent identity to each other and identity with other *Pneumocystis* species that ranged from 87 to 73 percent (English et al. 2001). The 13 percent divergence between the two sequences is similar to differences observed between the two rat *Pneumocystis* species (Cushion et al. 2004a), and indicates that these may be from two distinct genomes infecting the dog. Further studies of the organisms from the ferret and dog will require additional sequence data to validate these potential species.

Human *Pneumocystis*

The level of genetic divergence among *Pneumocystis* organisms infecting humans appears to be less than

Table 3 *Percentage divergence between DNA sequences of* P. carinii *and* P. wakefieldiae

DNA sequence	Percent divergence	Reference
Thymidylate synthase	6	Keely et al. 1994
TATA binding protein (TBP)	9	Sunkin and Stringer 1996
α-Subunit of guanine nucleotide binding protein	17.5	Smulian et al. 1996
BiP precursor	17	Stedman and Buck 1996
Heat-shock protein 70	18	Stedman et al. 1998
Mitochondrial large subunit rRNA	14	Sinclair et al. 1991
Nuclear small subunit rRNA	6.6	Edman et al. 1988; Stringer et al. 1989; Liu and Leibowitz 1993
26S rRNA	5	Liu et al. 1992
Internal transcribed spacer regions I and II	30, 31	Ortiz-Rivera et al. 1994, 1995
5.8S	4	Liu et al. 1992

that between the two species found in rats (Nimri et al. 2002). However, significantly more genotypes of *P. jirovecii* have been identified based on differences of a few nucleotides, but only one karyotypic profile has been reported for human *Pneumocystis* (Stringer et al. 1993).

A number of different genetic loci have been used to type human infections, but most studies employ the ITS of the nuclear rRNA genes, the mtLSU, and more recently, the *DHPS* gene, the target of sulfamethoxazole (Beard 2004). Use of these markers has been used to:

- show that humans can be infected by a single *P. jirovecii* genotype or mixed genotypes
- explore the distribution of genotypes among geographic locations and association of genotypes with underlying disease states
- detect the presence of potential mutations associated with drug resistance.

The ITS regions of the nuclear rRNA operon have produced the most diversity thus far. This locus in *Pneumocystis* consists of genes encoding the 18S, 5.8S, and 26S subunits. The ITS1 and ITS2 regions are located between the 18S and 5.8S, and the 5.8S and 26S genes, respectively (Liu and Leibowitz 1993). These regions evolve more rapidly than the genes that they flank and are useful for detection of differences among closely related populations of organisms (Chen et al. 2000). To date, 15 types of ITS1 sequences (types A through O) and 14 types of ITS2 sequences (a to n) have been reported, for a total (in combination) of more than 60 genotypes (Lee et al. 1998; Nimri et al. 2002). Despite dramatic geographic differences, ranging across nine countries and spanning four continents and numbering greater than 300 specimens, the most common genotype of *P. jirovecii* was 'Eg' followed by type 'Ne' (Lee et al. 1998; Nimri et al. 2002).

Four genotypes occur in the *P. jirovecii* mtLSU loci with two polymorphisms each at nucleotides 85 and 248 (Beard et al. 2000). Genotypes 1 (85:C/248:C) and 2 (85:A/248:C) were those most frequently detected in a study evaluating geographic variation among *P. jirovecii* populations, while genotypes 3 (85:T/248:C) and 4 (85:C/248:T) were found in less than 10 percent of the 324 samples analyzed (Beard et al. 2000).

Four distinct genotypes are also found in the *P. jirovecii DHPS* gene, at locations in which the same mutations conferred resistance to sulfa-based drugs in other pathogens (Skold 2000), namely (165:A/171:C), genotype 1; (165:G/171:C), genotype 2; (165:A/171:T), genotype 3; and (165:G/171:T). All these mutations are nonsynonymous, resulting in amino acid substitutions. Genotype 1 encodes a threonine at amino acid position 55 and a proline at position 57; genotype 2 has an alanine at position 55 and a proline at 57; genotype 3, a threonine at 55 and a serine at 57; and genotype 4 contains an alanine at 55 and a serine at 57. Combination of the the four genotypes of the mtLSU and the *DHPS* predicts 16 possible combinations, of which 14 have been observed (Beard et al. 2000).

New *Pneumocystis* nomenclature

In 1976, Frenkel proposed that a distinction be made between the organisms obtained from humans and those from rats on the basis of inability to cross-infect and due to serological reactivities (Frenkel 1976). The suggested names, *Pneumocystis jiroveci* for the human organism and *Pneumocystis carinii* for the one found in rats, did not gain wide acceptance.

The cumulative evidence provided by genetic data and cross-infection studies led a large group of *Pneumocystis* investigators to reconsider the issue of nomenclature at the 3rd International Workshop on *Pneumocystis*, Cleveland, Ohio, June 1994. It was the consensus of the group that sufficient evidence for speciating the organisms had not been obtained and a provisional system of nomenclature was proposed until other requirements as outlined by the Code were met to initiate species distinctions. In sexually reproducing organisms, individuals within a species are interfertile and reproductively isolated from individuals of other species. As the reproductive capacity of *Pneumocystis* is not fully understood

and the organism cannot be cultured or cloned, taxonomic hierarchy relying on historical methods may not be forthcoming. It was proposed that the organisms found in each mammalian species be given a tripartite name based on the host of origin and in accordance with the International Botanical Code of Nomenclature for 'physiological variants' (The Pneumocystis Workshop 1994; Greuter 2000). For example, the name provisionally given to *Pneumocystis* from mouse was *Pneumocystis carinii* f. sp. *muris*. Occasionally these names may still be found in the literature.

In 1999, Frenkel began the formal process required for describing new species of *Pneumcoystis* (Frenkel 1999). He retained his originally proposed name for the species found in humans, *P. jirovecii*, and the name for the most common organism found in rats as *P. carinii*. Subsequently, the other species found in rats was described and named *P. wakefieldiae* (Cushion et al. 2004a), followed by description of the species in mouse as *P. murina* (Keely et al. 2004). There are now a total of four formally described *Pneumocystis* species in the fungal phylum Ascomycota, subphylum Taphrinomycotina (O.E. Eriksson and Winka 1997), Order Pneumocystidales (O.E. Erikss. 1994), Class Pneumocystidomycetes (sensu O.E. Erikss. and Winka 1997), Family Pneumocystidaceae (O.E. Erikss. 1994), Genus *Pneumocystis* (Delanoë and Delanoë 1912) (Table 4).

These species were described using a combination of molecular and phenotypic characteristics following a phylogenetic species concept (Taylor et al. 2000) rather than standard methods of species using mating crosses. Phylogenetic analyses with neighbor-joining trees applied to five *Pneumocystis* taxa, seven *Taphrina* taxa, *S. pombe*, and *S. cerevisiae* separated the archiascomycetes (*Taphrina*) cluster from the hemiascomycete cluster (*S. pombe*, *S. cerevisiae*), with the *Pneumocystis* cluster between the two, strongly supporting monophyly for the three groups (Keely et al. 2004). Within the *Pneumocystis* group, *P. murina* forms a monophyletic clade with the other two species found in rodents, *P. carinii* and *P. wakefieldiae*, with a higher affinity for *P. carinii* (Figure 7). Based on estimates using the rDNA and *DHFR* genes as well as taking into consideration the divergence of the hosts, it appears that *P. murina* has been living in the mouse for about 30–40 million years (Keely et al. 2004), while the *Pneumocystis* group probably diversified about 100 million years ago.

Table 4 *The taxonomic hierarchy of* Pneumocystis

Level	Taxa
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Taphrinomycotina
Order	Pneumocystidales
Class	Pneumocystidomycetes
Family	*Pneumocystidaceae*
Genus	*Pneumocystis*
Species	*jirovecii, carinii, wakefieldiae, murina*

IN VITRO PROPAGATION

The single most important need in *Pneumocystis* research is the establishment of a continuous in vitro propagation system. Since the 1940s a multitude of investigators have attempted to culture primary isolates from mammalian hosts onto various artificial substrates, monolayer and cell tissue cultures, and organ cultures, without success. Even in this new century, the ex vivo culture of any *Pneumocystis* species defies the efforts of scientists. At best, the organism is able to replicate five- to tenfold after inoculation from the host, with only a few passages of diminishing numbers (Cushion 1989). The lack of in vitro propagation has hampered basic biological studies and also more sophisticated molecular genetic techniques. Many approaches for in vitro growth have been tried, some of which are summarized here. Other reviews are recommended to the interested reader for further details (Cushion 1989; Sloand et al. 1993; Armstrong and Cushion 1994b).

Monolayer-based systems

A relatively large number of cell lines and conditions have been explored with little improvement over the tenfold increase in number of *Pneumocystis* most frequently reported (Sloand et al. 1993). Laboratories currently using tissue culture systems routinely employ the human lung epithelioid A549 cell line, the human lung fibroblast line, HEL, or the mink lung cell line, Mv 1 Lu. Growth rates are variable within a laboratory and among laboratories. The use of monolayer-based systems for in vitro drug screening remains problematic. A viability stain must be included in the organism counts over the short-term culture period; dead *Pneumocystis* can stain with Giemsa/Diff-Quik and lead to misinterpretation of a drug's effects. Certain drugs detrimentally influence the viability and metabolic processes of the monolayer cells, leading to an incorrect conclusion of efficacy for *Pneumocystis*. Evaluation of the uptake of radiolabeled precursors is also confounded by the presence of monolayer cells. Monolayer cell systems have been useful for studying the mechanisms of *Pneumocystis* adhesion (Limper et al. 1991, 1993) and should provide additional information in this area of research and other host cell–pathogen interactions.

Cell-free systems

In the late 1980s and early 1990s, a number of cell-free systems were evaluated for growth and drug-screening capabilities. An increased incorporation of [^{35}S]methionine

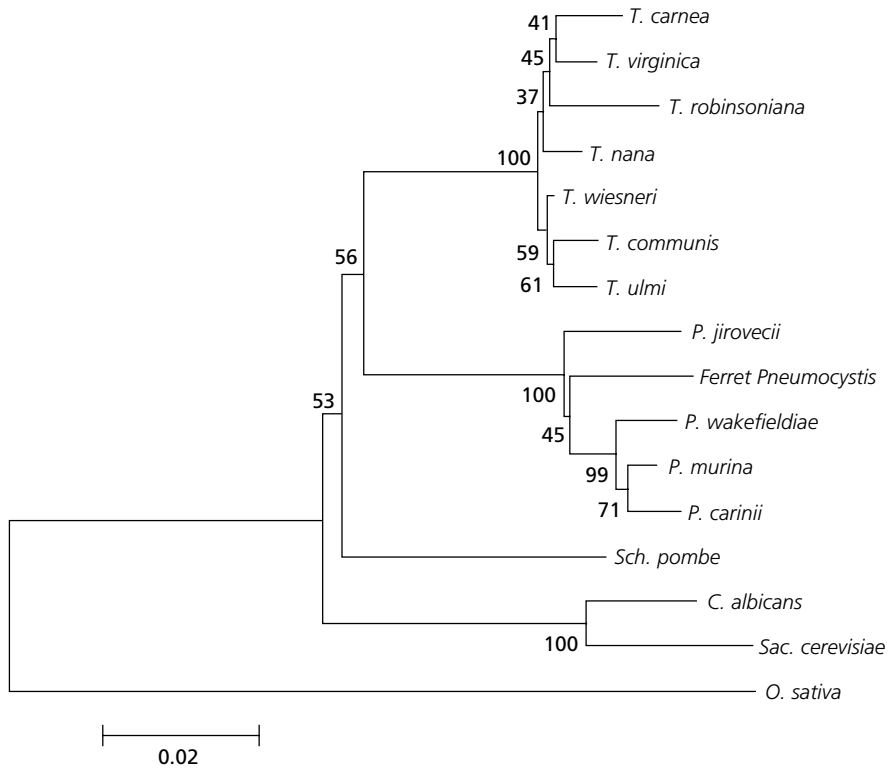

Figure 7 *Phylogenetic tree depicting the relationships between* Pneumocystis *species and among higher fungi. The tree was constructed using the neighbor-joining method, pairwise deletion, and Kimura two-parameter (K2P) in MEGA version 2.1 software (Kumar et al. 2001). One thousand bootstrap replications were performed and the values (expressed as percentages) are shown at the nodes. Bar indicates K2P distance. Reprinted with permission from Keely et al. (2004)*

into *Pneumocystis*-specific proteins over time and a five- to sevenfold increase in organism nuclei were observed in organisms maintained in a neopeptone-based medium (Cushion and Ebbets 1990). *Pneumocystis* from nude mice increased fourfold in a primary culture containing Dulbecco's MEM medium supplemented with fetal bovine serum and reducing agents (Tegoshi and Yoshida 1989). Four serial passages of declining growth were observed. Comley and Sterling (1994) used rat *Pneumocystis* to demonstrate incorporation of *para*-aminobenzoic acid (pABA) and de novo synthesis of folates in an RPMI-1640 based medium. In this system, *Pneumocystis* were most viable in the first 24 hours of ex vivo maintenance as shown by pABA uptake, but organisms that stained with Giemsa persisted throughout the 3-day culture period. Exposure to pentamidine at high levels (100 μM) resulted in a dramatic decrease in radiolabel incorporation, but not in organism number.

A cell-free system using a collagen-coated porous membrane support matrix that could maintain the continuous cultivation of *Pneumocystis* from rats was described in 1999 (Merali et al. 1999). The key ingredients included *S*-adenosylmethionine, an iron component in the form of ferric pyrophosphate, *N*-acetylglucosamine, pABA, putrescine, and horse serum. Temperature of incubation was lower than previously reported, 31°C, and the pH was higher at 8.8. The doubling times ranged from 19 to 65 hours, depending on the inocula density. The addition of *S*-adenosylmethionine was thought to be an essential component and it was later reported that *P. carinii* was auxotrophic for this compound (Merali et al. 2000). Although enthusiasm for the method was initially very high, other laboratories were unable to repeat the success of the originating laboratory (Larsen et al. 2002; Cushion and Walzer 2004).

In 1994, a cell-free system for the maintenance of *Pneumocystis* from rats using an ATP bioluminescent assay was reported (Chen and Cushion 1994a). Organisms cultured in an RPMI-based system with fetal bovine serum and nutritional additives were responsive to the standard anti-*Pneumocystis* compounds, TMP-SMX and pentamidine, and could increase modestly (three- to fivefold) over a 7-day period. Refinement of the system using a multisample luminometer permitted large numbers of compounds to be screened per year, resulting in a profile of responses to more than 60 compounds representing several classes of drugs (Cushion et al. 1997a). More recent improvements to the system include standardization of inocula by ATP content, karyotypic profile, and use of cryopreserved organisms (Collins and Cushion 2001). The latter modification reduced reliance upon availability of organisms

from the immunosuppressed animal model and permitted a series of assays to be performed with a single characterized 'lot' of organisms.

BIOCHEMISTRY AND METABOLISM

The lack of a continuous culture system has compromised metabolic studies in two significant ways. First, the veracity of the incorporation of radiolabeled precursor molecules or the identification of biochemical constituents can be difficult to interpret due to the presence of contaminating host cells that may take up the same compounds or contribute to the biomolecular pools. Second, the interpretation of results in a suboptimal in vitro environment must acknowledge the tenuous state of organisms after isolation from the mammalian lung and subsequent placement in less than adequate culture conditions. Nonetheless, investigators have persevered and the results of these biochemical analyses are summarized here.

The presence of gene homologs representing many metabolic processes has been identified by sequencing of the *P. carinii* genome and should help discern the actual metabolic capacities of these organisms(Cushion 2004a, b; Porollo et al. 2004). In this section, the potential gene homologs identified by the *Pneumocystis* Genome Project or by other laboratories belonging to metabolic cycles that have been explored by standard biochemical approaches are noted. For a more comprehensive summary of potential metabolic cycles in *Pneumocystis*, the reader is directed to the following resources: Cushion 2004a, b; Porollo et al. 2004.

General and intermediate metabolism

Early studies using standard biochemical approaches provided evidence for an operational glycolytic pathway in *Pneumocystis* (Pesanti and Cox 1981; Mazer et al. 1987; Pesanti 1989). Recent genomic data lend strong support for a typical glycolytic pathway in *P. carinii*. Homologs to fungal enzymes in the pathway found in its genome include hexokinase, phosphofructokinase, fructose-biphosphate aldolase, triose phosphate isomerase, phosphoglycerate kinase, pyruvate kinase (Porollo et al. 2004), and enolase (Fox and Smulian 2001).

Several lines of investigations indicate that *Pneumocystis* respires aerobically. The presence of intact mitochondria observed by light microscopy and at the ultrastructural level, cytochemical and electrophoretic techniques, as well as incorporation studies predict a functional tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and an active electron transport chain (Pesanti and Cox 1981; Mazer et al. 1987; Pesanti 1989). Cytochrome activities were predicted by inhibition studies with cyanide (Pesanti 1984; Chen and Cushion 1994b), hydroxynaphthoquinones (Dohn and Frame 1994; Hughes et al. 1990), and antimycin A (Chen and Cushion 1994a). The synthesis of ATP was verified by direct analysis using bioluminescence detection (Chen and Cushion 1994a). Gene sequences isolated from mitochondrial DNA of *Pneumocystis* indicate that *Pneumocystis* contains the genes necessary for mitochondrial processes (Pixley et al. 1991; Cushion and Smulian 2001). *Pneumocystis* has been shown to use oxygen at a low rate, perhaps due to unfavorable assay conditions (Pesanti 1984).

All the genes necessary for a functional citric acid cycle in *P. carinii* have been identified in genomic and cDNA sequences by homology to fungal genes (Porollo et al. 2004) and include pyruvate dehydrogenase, citrate synthetase, aconitase, isocitrate dehydrogenase, alpha ketoglutarate, succinyl-coA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase. Likewise, the various subunits of the complexes necessary for oxidative phosphorylation are present in its mitochondrial genome, including seven of the NADH dehydrogenase (complex I); the succinate dehydrogenase of complex II; cytochrome *c*–coenzyme Q oxidoreductase of complex III; cytochrome oxidase of complex IV; and the ATP synthase subunits (Porollo et al. 2004).

Sterol metabolism

Sterols are thought to be used for two purposes in fungal cells, in the maintenance of cell architecture and function of membranes and as a requirement in cellular processes: 'metabolic sterols' (Benveniste 2002). Yeast sterols are distinct from those in animal cells, with ergosterol and 24(28)-dehydroergosterol predominating in yeast membranes.

Kaneshiro and colleagues performed direct biochemical analyses that confirmed cholesterol, rather than ergosterol, was found as the most abundant bulk sterol in *Pneumocystis* preparations (Kaneshiro et al. 1989). The activity of a key enzyme in the biosynthesis of mevolonate, 3-hydroxy-3-methylglutaryl coenzyme A reductase, was detected by biochemical methods and could be inhibited by lovastatin (Kaneshiro et al. 1994a, b). Incorporation of radiolabeled squalene and mevolonate into specific *Pneumocystis* sterols provided evidence that the organism was capable of its own sterol biosynthesis (Kaneshiro et al. 1994a). Incorporation studies with radiolabeled serine, ethanolamine, and choline provided evidence that the organism could metabolize phospholipid head groups and de novo synthesize sphingolipids (Florin-Christensen et al. 1995). Furlong et al. (1994) provided chromatographic and mass spectrometry data indicating the presence of phytosterols in *Pneumocystis*-infected rat lungs and verified the earlier findings of the lack of ergosterol and cholesterol as the predominate bulk sterol.

Inhibitor studies provided evidence that at least a portion of the sterol pathway of *P. carinii* may be operational. Terbinafine, an inhibitor of squalene

epoxidase, was shown to be effective against *Pneumocystis* infections in vivo and to inhibit organism growth in vitro (Contini et al. 1994, 1996; Cirioni et al. 1995). Likewise, Urbina et al. (1997) could inhibit sterol C-24 alkylation in vitro using targeted inhibitors and subsequently decrease proliferation of the organisms in short-term culture as well as inhibit the biosynthesis of its sterols. An in vitro study utilizing the bioluminescent ATP assay was conducted to probe several enzymatic steps of the sterol biosynthetic pathway in *P. carinii* (Kaneshiro et al. 1999b). Coincident with these inhibition studies, several orthologs of the genes in this pathway were identified in gene databases generated by the *Pneumocystis* Genome Project (Porollo et al. 2004). Significantly, the inhibitory functions probed by the ATP assay could be associated with the presence of the gene in the *Pneumocystis* genome. Thus, inhibitors targeted to the enzymatic steps driven by *erg1* (squalene epoxidase), *erg7* (oxidosqualene-lanosterol cyclase), and *erg6* [*S*-adenosyl-Lmethionine:sterol C-24 methyltransferase (SAM:SMT)], resulted in decreased organism viability. The lanosterol demethylase gene of *P. carinii* was recently sequenced (Morales et al. 2003) and its presence could explain the inhibition shown by treatment with proprietary inhibitors GR 40317A, GR 42539X, and GR 40665X (Kaneshiro et al. 2000) but not with standard azoles in clinical use. The precise structure of the *P. carinii erg11* gene homolog awaits further study, but such structural information should reveal the specific properties that render it refractory to standard azoles.

Orthologs to HMG-CoA reductase 1 and 2 (Hmg1, 2, *S. cerevisiae*) genes have recently been detected in the *P. carinii* genome (Porollo et al. 2004). Although the compounds targeting this step were ineffective at reducing the ATP pools of the *P. carinii* in this study, more recent studies performed in our laboratory showed strong inhibition of ATP with the HMG-CoA reductase inhibitor, simvastatin (Collins et al. 2003). Curiously, a homolog to *erg4*, which encodes the enzyme that catalyzes the last step in ergosterol biosynthesis [the sterol C-24(28) reductase], was identified in the *P. carinii* genome (Porollo et al. 2004). Whether this genomic sequence is functional, and what role it plays, if any, in ergosterol biosynthesis, is not known at this time. Members of the genus *Pneumocystis* may not be typical fungal organisms due to the lack of ergosterol as their major sterol, but these organisms still have a functional sterol biosynthetic pathway that can be exploited for drug development.

A rare C_{32} sterol, the C-24-alkylated lanosterol derivative 24(*Z*)-ethylidinelanost-8,24(28)-3β-ol, was detected in large amounts in *P. jirovecii* (Kaneshiro et al. 1999a). Given the trivial name, pneumocysterol, this compound was reported to comprise almost 50 percent of the organism-specific sterols in some samples derived from human lungs. Although present in other *Pneumocystis* species, pneumocysterol appears to be most abundant in human organisms.

As a direct result of the *Pneumocystis* Genome Project, genes associated with sterol biosynthesis encoding *erg7* (AF285825) and *erg6* (AY032981) (Kaneshiro et al. 2001a; Zhou et al. 2002), were sequenced. The *erg6* gene of *P. carinii* was cloned, expressed in *Escherichia coli*, and was able to complement the null mutant in *S. cerevisiae* (Kaneshiro et al. 2001a). The sterol-binding motif of the *P. carinii* SAM:SMT product was novel and reported to have high-affinity binding properties of lanosterol. Furthermore, the enzyme was able to transfer methyl groups from *S*-adenosyl-L-methionine (SAM) to the C-24 position of both lanosterol and 24-methylenelanosterol, thus defining it as an enzyme capable of two methyl group transfers (Kaneshiro et al. 2001a). The oxidosqualene cyclase (Erg7p) encoded by the *ERG7* gene converts oxidosqualene to lanosterol, the first cyclic component of sterol biosynthesis.

The isoprenoid biosynthetic pathway leading to the production of squalene and other compounds includes a branch giving rise to the polyprenyl chain of coenzyme Q (ubiquinone). Coenzyme Q homologs function in the mitochondrial electron transport chain and offer a potential drug target in some parasite–host situations. Kaneshiro et al. (1994b) identified the homolog CoQ_{10} as the predominant species in *Pneumocystis*-infected lung homogenates and further showed that the ubiquinone could be de novo synthesized by incorporation of radiolabeled mevalonic acid into the molecule. Most higher animals contain CoQ_{10} as the sole endogenous ubiquinone, except in the rat, where CoQ_9 is the major homolog (Ellis 1994). Coenzyme Q analogs that function as antiparasitic drugs include the 8-aminoquinolones and hydroxynapthoquinones. Atovaquone, a hydroxynapthoquinone which is used for the treatment of moderate PCP, is an analog of coenzyme Q and blocks mitochondrial electron transport by binding to cytochrome *b* of the bc_1 complex (Gutteridge 1991). In addition, atovaquone was recently reported to inhibit *P. carinii* coenzyme Q biosynthesis (Cushion et al. 2000; Kaneshiro et al. 2001b).

FATTY ACIDS

Pneumocystis lives in an environment in which it is bathed in lipids comprising mainly saturated fatty acids. Purified preparations of *Pneumocystis* have been shown to take up the radiolabeled and fluorescently labeled fatty acids oleic, linoleic, palmitic, stearic acids, and phospholipids (Paulsrud and Queener 1994; Sleight et al. 1994). An epoxy fatty acid, *cis*-9,10-epoxyoctadecanoic acid (epoxy stearate), was identified in purified *Pneumocystis* preparations (Ellis et al. 1994). This unusual fatty acid has been detected in rust fungi where it is a major component. It is interesting to note that like *Pneumocystis*, the rust fungi also lack ergosterol.

Transport

Little is known of the transport or efflux systems of *Pneumocystis*, but these functions are likely important in determining the efficacy of therapeutic compounds. Studies to identify mechanisms of amino acid and glucose uptake were recently conducted (Basselin-Eiweida et al. 2001). Simple diffusion was found as the mechanism for translocation of aspartic acid. By contrast, Michaelis–Menten first-order kinetics, indicating the presence of carrier-mediated mechanisms, was exhibited by the neutral amino acids glutamine, leucine, and serine; the basic amino acid arginine; and the aromatic amino acid tyrosine. Leucine, glutamine, and serine used the same carrier system, as illustrated by competition assays. Exogenous glucose was necessary for efficient amino acid uptake, while other sugars, e.g. galactose, fructose, were less effective. Neutral, basic, and aromatic acids alike were taken up by facilitated diffusion. This conclusion was drawn from observations regarding the effects on uptake with reduced sodium or potassium ions; the lack of inhibition of transport by oubain and valinomycin; and the lack of effect when inhibitors to ATP synthesis and maintenance of the electrochemical ion gradients and membrane potentials were added to the assay. Transport of arginine appeared to be facilitated by both a high-affinity carrier and a low-affinity carrier. In addition, *P. carinii* may possess a separate carrier for aromatic amino acids that may also transport neutral amino acids.

Transport of glucose, and the inhibitor, 2-deoxyglucose, utilized two facilitated transport systems (Basselin-Eiweida et al. 2001). The high-affinity transporter was inhibited by protein synthesis inhibitors, suggesting active transport. The properties of the low-affinity system indicated it was likely to operate by facilitated diffusion, probably in environments of high exogenous glucose levels. Importantly, these investigators noted the dramatically slow uptake rate of both amino acids and glucose by *P. carinii*. They conjectured that *P. carinii* may be capable of synthesizing most of these compounds, negating the requirements for high-affinity transporters or such mechanisms.

Polyamines

Polyamines are low molecular weight polycations thought to play an important role in the regulation of cell growth and differentiation (McCann et al. 1986). *Pneumocystis* appear to have functional polyamine biosynthesis (Merali and Clarkson 1996). However, they are unable to regulate polyamine catabolism as a result of uncontrolled activity of the key enzyme spermidine/spermine-N^1-acetyltransferase (SSAT) (Merali 1999). This results in a net loss of polyamines from the cell and back conversion to smaller polyamines which could make it vulnerable to compounds that interfere with polyamine biosynthesis. α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of the first and rate-limiting step in polyamine biosynthesis, the conversion of ornithine to putrescine by the enzyme ornithine decarboxylase. This compound was effective in reducing *Pneumocystis* infection in immunosuppressed rats in one laboratory (Clarkson et al. 1990) but not in another (Walzer 1994). Ornithine decarboxylase activity was not detected in an early study by Pesanti et al. (1988), but later, Clarkson et al. (1990) reported measurable activity. To date, a fungal homolog for the gene encoding ornithine decarboxylase has not been identified in the EST library or the genome of *P. carinii*, but the gene encoding an inhibitor of the enzyme ornithine decarboxylase antizyme has been detected in the EST and genomic libraries (Ivanov et al. 2000; Porollo et al. 2004).

S-Adenosylmethionine (AdoMet) is a key molecule in both methylation reactions and polyamine biosynthesis. Most AdoMet is used for transmethylation reactions which transfer the *N*-methyl group of the methionine group to other molecules such as proteins, lipids, or DNA which forms new molecules in some cases (e.g. lecithin in the case of complex lipids), or regenerates the methionine. The remaining AdoMet (~2–5 percent) enters into polyamine biosynthesis after decarboxylation (Merali et al. 2000). AdoMet synthetase transfers the adenosyl group of ATP to methionine to form AdoMet and release of P_i and PP_i. Recently, it was reported that the long-term culture of *P. carinii* was dependent upon exogenous supplementation of AdoMet (Merali et al. 1999). Further studies by these investigators described the lack of AdoMet synthetase activity by *P. carinii* in an in vitro system and characterized two transporters, one high affinity and one low affinity, that were capable of transporting exogenous AdoMet into the cells (Merali et al. 2000). They concluded that *P. carinii* was auxotrophic for AdoMet, a finding unprecedented in the fungal kingdom. Supporting their discovery was the apparent reduction of plasma AdoMet concentration in *P. carinii*-infected rats that was correlated with infection burden (Merali et al. 2000). Translation of the effect to the clinical setting confirmed the reduction of plasma AdoMet in patients suffering from PCP, but not in those with bacterial pneumonia, tuberculosis, or cryptococcal meningitis (Skelly et al. 2003). These findings are very intriguing and demand further investigation. On the one hand, replication of the success reported by the authors of these studies using a culture supplemented with AdoMet has not been reproduced in other laboratories (Cushion and Beck 2001; Larsen et al. 2002) and the presence of a fungal gene homolog encoding AdoMet synthetase in the genome of *P. carinii* also seems to contradict the assertion that the organism does not have the capacity to synthesize AdoMet (Porollo et al. 2004).

However, expression of the gene has not been verified and the clinical and experimental findings of plasma AdoMet depletion in infected hosts lends support for the salvage of this compound by these organisms.

Replication

Thermal denaturation studies and gene sequence data show the genome of rat-derived *Pneumocystis* to be adenine and thymine rich (Stringer and Cushion 1998). These initial findings have been supported by the sequencing of over 3 000 genes by the *Pneumocystis* Genome Project. The current estimate of genome-wide A+T content is ~70 percent.

The ploidy of the *Pneumocystis* genome has not been definitively defined. Cytofluorometric evaluations of cyst and trophic forms using the DNA-intercalating stain 4′,6-diamidino-2-phenylindole (DAPI) indicated that 90 percent of the trophic forms contained the same DNA content as those within the cyst (Yamada et al. 1986). Later studies using a number of nuclear intercalating dyes and image analysis confirmed the apparent haploid state of the trophic forms and ascospores within the cysts (Wyder et al. 1994); however, there was some variation observed in the nuclear content, suggesting that there may be other states of ploidy. By contrast, two-dimensional separation of apparently homologous chromosomes by pulsed-field gel-separated chromosomes predicted a diploid nucleus (Cornillot et al. 2002).

There are conflicting data on the incorporation of precursors into *Pneumocystis* nucleic acids. One early study found organisms sustained in a balanced salt solution could incorporate [^{3}H]uridine into TCA-precipitable material, although the uptake was not inhibited by the anti-*Pneumocystis* drug pentamidine (Pesanti and Cox 1981). These investigators reported no uptake of radiolabeled thymidine or hypoxanthine. By contrast, organisms incubated in a low pH minimal medium were able to incorporate [^{3}H]uridine, uracil, thymidine, and hypoxanthine as well as [^{35}S]methionine (Cushion and Ebbets 1990). More recently, the ability of *Pneumocystis* to incorporate 5-bromodeoxyuridine in the lungs of rats and mice was evaluated to determine whether a salvage pathway for thymidine was operational (Vestereng and Kovacs 2004). No incorporation was observed by either *P. murina* or *P. carinii*, although host cells readily took up the analog, suggesting that if *Pneumocystis* possesses a thymidine salvage pathway it was not active during infection in the mammalian host.

Analysis of a partial EST database from *P. carinii* revealed about 11 percent of the genes identified as fungal homologs were associated with the transcription process, including initiation factors, regulators, repressors, terminators, chromatin remodeling proteins, and histone proteins and regulators (Cushion et al. 2004b; Porollo et al. 2004). In addition, sequencing of its genome revealed a multitude of fungal homologs to DNA repair genes such as *rad2*, *rad8*, *rad15*, *rad16*, *rad18*, *rad26*, *rad32*, and *rad50*; repair helicases *rhp26*, *rhp54*, and *rhp57*; and *MSH2* (Porollo et al. 2004).

Carbohydrates

Besides the chromoatographic analyses of monomeric sugars in the cell wall of *Pneumocystis*, described above, other studies suggest the presence of more complex carbohydrate polymers. The lytic action of the enzyme preparation, Zymolyase, which contains as its major component β-1,3-glucan laminaripentaohydrolase, and the staining of the cyst wall with aniline blue suggest the organism maintains a glucan layer, at least in the cyst form. The gene encoding the *P. carinii* β-1,3-glucan synthetase gene *Gsc1* was cloned and shown to mediate the polymerization of uridine 5′-diphosphoglucose into β-1,3-glucan (Kottom and Limper 1999, 2000). The glucan in the cyst wall of *Pneumocystis* was implicated as a factor in the detrimental inflammatory response in the lungs of its mammalian hosts (Hahn et al. 2003). Compounds targeting glucan synthesis were found to arrest the replication of cysts in the lungs of treated animals and offer an attractive alternative target for chemotherapy of infection (Schmatz et al. 1990, 1995). In addition to β-glucan, direct synthesis of α-1,4-glucan in short-term culture was shown by biochemical methods, GC-MS, and uptake experiments (Williams et al. 1991). α-Glucan in the cell wall plays a key role in fission yeast morphogenesis, maintenance of the characteristic cylindrical shape, and separation of the mother and daughter cell after mitotic replication (Konomi et al. 2003).

PNEUMOCYSTIS INFECTION

Incidence of PCP

In the pre-AIDS era, defined as those decades prior to 1980, the number of reported cases of PCP in humans was fewer than 100 (Walzer et al. 1974; Kovacs et al. 2001). In the decades that followed, a dramatic increase in cases was observed due to the onset of the AIDS epidemic in 1982. In 1991, the incidence of PCP peaked at 20 000 cases, with a subsequent decline due to the recommendation for prophylaxis of HIV-positive patients with a CD4 cell count of less than 200/μL from a Public Health Service task (1989). This decline was even more dramatic after 1995 when widespread HAART was instituted, targeting the HIV virus and permitting immune reconstitution of patients (Kaplan et al. 2000). After 1995, PCP declined at an average of 21.5 percent per year (Kovacs et al. 2001). In spite of such dramatic decreases in PCP cases, this pneumonia still remains the most common life-threatening infection associated with HIV-infected persons. Of significant

concern is the increasing incidence of mutations in genes targeted by the mainstays of therapy, TMP-SMX and other secondary therapies such as atovaquone.

Description of clinical features

RISK FACTORS

A decrease in cell-mediated immunity is the single most important risk factor for the development of PCP. Reduction in the cellular immune response can be potentiated by:

- administration of immunosuppressive agents, such as corticosteroids or cancer chemotherapeutic agents
- congenital conditions like the severe combined immunodeficiency disease syndrome (SCIDS)
- starvation or malnutrition
- most recently, infection with the human immunodeficiency virus (HIV).

Quantification of the number of circulating CD4+ lymphocytes has long been recognized as an indicator of the level of immunosuppression and of the risk of developing pneumocystosis (Masur et al. 1989; Phair et al. 1990). Coordination of large center studies (Multicenter AIDS Cohort Study and Pulmonary Complications of HIV Infection Study) determined that the risk for *P. carinii* pneumonia sharply increases when the CD4+ lymphocyte counts reach 200/μL in individuals not on a prophylactic regimen, while in those with less than 200/μL, the risk becomes five times greater as compared with patients maintaining counts above 200/μL.

Although depression of cell-mediated immunity is clearly a risk factor in the development of PCP, there are clinical and experimental data implicating a role for humoral immunity. Patients with only B-lymphocyte abnormalities and mice with B-cell defects have been reported to develop PCP (Saulsbury et al. 1979; Rao and Gelfand 1983; Marcotte et al. 1996) while passive antibody administration aids in the clearance of the infection in experimental animal models (Gigliotti and Hughes 1988; Roths and Sidman 1993; Gigliotti et al. 2002).

CLINICAL PRESENTATION

Adults

PCP in adults commonly presents with increasing dyspnea, a nonproductive cough, inability to breathe deeply, substernal tightness, and often with night sweats. A fever of 38.5°C and tachypnea are often encountered. Rarely, there is sputum production or hemoptysis. Physical examination of the lungs may find little deviation from normal, but various degrees of respiratory distress, small respiratory volumes and fine basilar rales may be present. AIDS patients have a more insidious progression to clinical disease than patients with immune depression unrelated to HIV, approximately 25 days versus 5–10 days (Dohn and Frame 1994).

The chest radiograph may appear normal or indicative of severe disease. Subsequent follow-up of normal radiographs by high-resolution computed tomography (CT) scan of the chest will show a ground glass attenuation if PCP is present (Gruden et al. 1997). Diffuse, symmetric interstitial infiltrates are most commonly encountered, while focal infiltrates, lobar consolidations, cavities, and nodules are less common. In early infection, the infiltrates may be widely distributed but consolidation and air bronchograms develop with progression of the disease. Increased incidence of apical infiltrates and pneumothoraces have been associated with aerosolized prophylactic pentamidine administration (Stansell and Hopewell 1995). Pneumatoceles have been observed with increasing frequency in cases of prolonged PCP (Stansell and Hopewell 1995). The degree and severity of abnormalities on the chest radiograph are prognostic and can be correlated with higher mortality.

Impaired oxygenation is the primary abnormality detected by laboratory methods (Walzer 2004b). Many patients exhibit a widening of the alveolar–arterial oxygen gradient (A–a/D_{O_2}) proportional to the severity of disease and respiratory alkalosis (Forrest et al. 1999). However, a significant number of patients can present with a normal (A–a)D_{O_2} gradient at rest. Other physiological changes include hypoxemia, impaired diffusing capacity, alterations in lung compliance, total lung capacity, and vital capacity (Walzer 2004b). Analyses of bronchoalveolar lavage fluids (BALF) have revealed a generalized fall in surfactant phospholipids and phosphatidylcholine, but an increase in surfactant proteins (SP) A and D and sphingomyelin has been observed (Phelps and Rose 1991; Hoffman et al. 1992; Limper et al. 1994; Mason et al. 1998; Martin and Wright 1999). SP-A appears to inhibit phagocytosis of *Pneumocystis* (Koziel et al. 1998b) in some studies, while in others, it was shown to mediate binding (McCormack et al. 1997). SP-D has also been shown to bind to the organism's surface glycoproteins and facilitate adherence to macrophages (Limper et al. 1995; O'Riordan et al. 1995).

Children

The presentation in children usually involves cyanosis, flaring of the nasal alae, mild cough usually without fever, and intercostal retractions in severe disease (Walzer 2004b). In contrast to increased risk of the pneumonia in adults with CD4+ counts of 200/μL or below, 27 percent of children 1 year of age or less who developed *Pneumocystis* had CD4+ counts greater than 1000/μL (Anonymous 1995), due to the higher overall CD4 count in children.

EXTRAPULMONARY PNEUMOCYSTOSIS

Prior to the AIDS era, reports of extrapulmonary spread of *Pneumocystis* were rare (Telzak et al. 1990; Ng et al.

1997). Although an incidence of 1–3 percent of extrapulmonary cases has been reported in the post-AIDS era, it is likely there are a significant number of cases that go unreported.

Most extrapulmonary *Pneumocystis* is detected as an incidental finding during postmortem examination. It is rarely considered as a cause of the clinical manifestations associated with the HIV syndrome. Extrapulmonary spread of *Pneumocystis* is found most commonly in patients in the late stages of AIDS who have not received prophylactic treatment or only aerosolized pentamidine (Walzer 2004b).

The frequency of involvement in organs other than the lungs or body sites in a series of 52 patients, 40 of whom were HIV positive and 12 who were not infected, was lymph nodes (44 percent); spleen (33 percent); liver (31 percent); bone marrow (33 percent); adrenal glands (19 percent); gastrointestinal tract (19 percent); genitourinary tract (17 percent); thyroid (17 percent); heart (15 percent); pancreas (13 percent); eyes (12 percent); ears (12 percent); skin (4 percent); and other sites (2–3 percent) (Telzak and Armstrong 1994). The incidence of *Pneumocystis* in extrapulmonary sites in HIV-positive patients was evenly distributed at about 33 percent among the lymph nodes, spleen, liver, and bone marrow. By contrast, the preferential site for extrapulmonary *Pneumocystis* in non-AIDS patients was the lymph nodes with 75 percent of the 12 patients showing involvement. Infection of multiple extrapulmonary sites has been associated with a rapidly fatal outcome. Areas of focal involvement present as retinal cotton wool spots; polyploid lesions in the external auditory canal; thyroid mass; pancytopenia from bone marrow necrosis; pleural effusion; and numerous hypodense low attenuation lesions in the spleen or other organs on CT scans. Involvement of pleural and peritoneal spaces often resulted in calcification of these surfaces. Histopathological findings of fine needle aspirates or biopsies of these lesions showed areas of necrosis with exudative foamy material. Staining with methenamine silver or fluorescently labeled monoclonal antibodies revealed characteristic *Pneumocystis* morphologies (Walzer 2004b).

PCP IN PATIENTS WITHOUT UNDERLYING IMMUNOSUPPRESSION

PCP has been rarely reported in individuals with intact immune systems. However, two reports describe the infection in the elderly. In the first, a 67-year-old man presented with tachypnea, chills, and fever (Ludmerer and Kissane 1991). The chest radiograph showed bilateral pulmonary infiltrates and a biopsy confirmed the diagnosis of PCP. No evidence of an underlying immunosuppression was detected in laboratory tests. In the second report, a cluster of five elderly patients (ages 66–78 years) presented during a 3-month period at the New York Hospital–Cornell Medical Center with somewhat atypical symptoms of *P. carinii* pneumonia (Jacobs et al. 1991). Only two had fever and the chest radiographs revealed focal, lobar infiltrates or pleural effusions rather than bilateral involvement. No obvious immune impairment was detected, although it was suggested that subtle decreases in immune function associated with age may predispose the elderly population to this infection.

Autopsy studies conducted on individuals without predisposing immune suppression showed little evidence for infection with *P. jirovecii*, either by standard histological methods (Cushion 2004c) or by use of PCR (Peters et al. 1992). However, limited sample population numbers and the small amounts of lung tissue analyzed could explain these negative results.

More recent studies employing nested PCR for detection of *P. jirovecii* in bronchoalveolar fluids or oropharyngeal washes have reported positive amplicons from patients without underlying disease as well as from patients with a wide variety of disease states not including infection with HIV (reviewed by Cushion 2004c). The accumulating evidence from these studies suggests that colonization of humans by *P. jirovecii* is not a rare event and some subpopulations may serve as reservoirs of the infection (discussed in the section on Epidemiology below).

Pathology and pathogenesis

In the advanced diffuse form of pneumocystosis, all lobes of the lung are involved. They manifest a rubbery consistency, are bulky and densely consolidated. The cut surfaces appear yellowish to reddish gray, are granular and dry with destruction of air spaces. In less severe disease, there are foci of consolidation and areas of atelectasis admixed with compensatory hyperinflation. The nodular form of pneumocystosis is rare and typified by solitary and multiple nodules with some cavitation (Walzer et al. 1989).

Histopathological examination reveals two characteristic traits:

- alveolar interstitial thickening
- a frothy eosinophilic honeycombed exudate in the lumens (Figure 8a).

The interstitial thickening is due to hyperplasia and hypertrophy of the type II pneumocytes, interstitial edema, mononuclear cell infiltration, and in some cases, mild fibrosis (Walzer et al. 1989). The cellular infiltrates are limited in the alveolar septa but more apparent in the perivascular and peribronchial regions. The honeycomb exudate usually contains organisms when stained with methenamine silver to visualize the cyst form. Organisms cannot be visualized with hematoxylin and eosin. As the disease progresses, there may be hyaline membrane formation accompanied by interstitial fibrosis and edema. Histopathological assessment of the disease in AIDS and non-AIDS patients revealed a higher organism burden but less tissue damage in the former

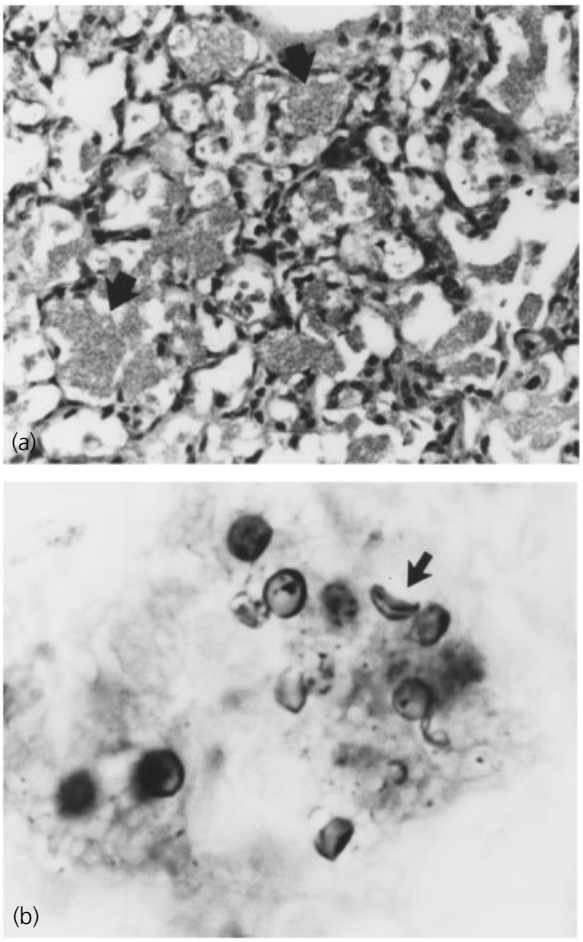

Figure 8 *Diagnostic histopathology.* **(a)** *Hematoxylin and eosin-stained lung sections. Note the characteristic honeycombed exudate, a hallmark of fulminant* P. carinii *pneumonia. (Original magnification 100×)* **(b)** *Cup-shape morphology of cysts (spore cases) visualized by Grocott's methenamine silver staining. (Original magnification 250×) Photographs courtesy of Paul Steele, MD, University of Cincinnati College of Medicine, Department of Pathology*

patient group (Dohn and Frame 1994). In some patients, the alveolar exudate may not be present or microcalcifications, granulomas, or cavitary lesions instead may appear. Ultrastructural studies have detected alveolar-capillary leakage and type I cell damage associated with attachment and presence of the organism (Yoneda and Walzer 1980).

Diagnosis

PCP should be considered in any immunocompromised patient who presents with fever, respiratory symptoms, or infiltrates on chest radiogram. Because these symptoms can be associated with a multitude of infectious and non-infectious conditions, definitive diagnosis must be made by morphological or histopathological demonstration of the organism. An algorithm for evaluation of patients with suspected PCP has been published by the National Institutes of Health in 2001 (Kovacs et al. 2001) and remains a helpful guide in establishing a diagnosis and for initiation of treatment (Figure 9). This algorithm includes screening with a non-invasive sampling method, induced sputum. It is likely an updated algorithm will be issued in which alternative non-invasive techniques such as oral washes or oral swabs will be usd for DNA extraction and subsequent amplification by PCR.

Patients with a clinical presentation consistent with PCP and with a chest radiograph demonstrating reticular or granular infiltrate can be placed on therapy immediately or the diagnosis confirmed with a procedure to collect respiratory secretions for microscopic evaluation. Those with a negative chest radiograph with a high degree of suspicion should undergo additional tests.

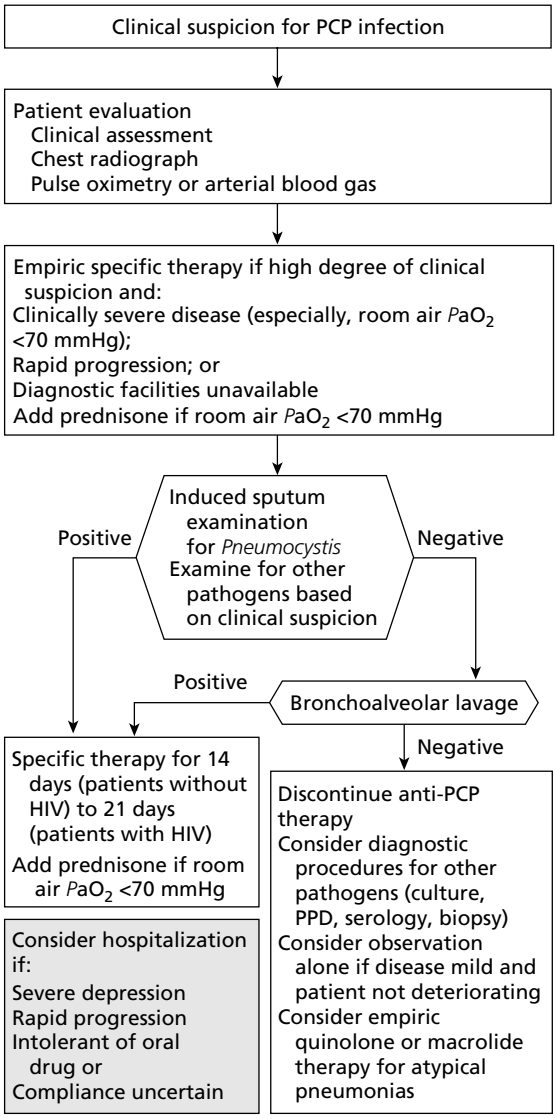

Figure 9 *Algorithm for evaluation of patients with suspected* Pneumocystis *pneumonia. Reprinted with permission from Kovacs et al. (2001)*

High-resolution CT scan of the chest or pulmonary function can be used to identify patients without the infection (Huang 2004; Walzer 2004b).

METHOD OF SAMPLE COLLECTION

The diagnosis of pneumocystosis relies on efficient sampling of the infected area. The organism can be detected in a variety of respiratory and other samples including induced sputum, BALF, tracheal aspirate fluid, tissue obtained by transbronchial biopsy, bronchial brushes, pleural fluid, oropharyngeal washes, and swabs. Generally, samples collected from HIV-infected patients provide a higher diagnostic yield than non-HIV-infected patients because of their increased organism burden.

Open thorax lung biopsy and transbronchial biopsy

Open thorax lung biopsy is the most invasive of the sampling procedures and requires the use of an operating room and general anesthesia. Fiberoptic bronchoscopy is the most common invasive procedure for collection of diagnostic tissue, but like open thorax lung biopsy, is rarely performed today. These procedures may be indicated after nondiagnostic bronchoscopy, suspicion of an infection concomitant with pneumocystosis, or for diagnosis of Kaposi's sarcoma.

Bronchoalveolar lavage (BAL)

BAL alone is more efficacious than brushings or washings, carries little morbidity, and has become the method of collection in most institutions. Sensitivity can be increased if multiple lobes are sampled and combined (Baughman 1994; Baughman et al. 1994; Walzer 2004b). Twenty to thirty milliliters of the lavage fluid is collected and concentrated by centrifugation. The pellet is smeared onto glass slides for fixation followed by histochemical staining.

Induced sputum

Deep inhalation of a 3 percent sodium chloride solution by the patient results in osmotic accumulation of fluid in and irritation of the respiratory passages, which can improve the organism yield, especially in AIDS patients. Depending on the institution, the patient base, and technical expertise, the diagnostic yield can range from 50 to 90 percent (Kroe et al. 1997; Caliendo et al. 1998). There are many fine technical points that must be considered during the procedure to provide the best diagnostic yield, including the use of toothpaste, which should be avoided, and the use of mucolytic agents to liquefy the sample (Cushion 2003). Although the procedure is non-invasive and relatively inexpensive, drawbacks to its use are the lack of information concerning other infections or disease processes that may be present in the lung and low diagnostic yield in non-AIDS patients.

Nasopharyngeal aspirates

Recent studies with neonates have increased the popularity of this sampling method (Vargas et al. 2001, 2003). Detection of *P. jirovecii* from these samples relies mostly on amplification of its DNA by PCR, but in some cases organisms have been detected (Vargas et al. 2001). An advantage of the procedure is that these samples are commonly used for diagnosis of repiratory viruses and can concomitantly be evaluated for the presence of *P. jirovecii*. The aspirates are collected with a suction catheter after instillation of 1–1.5 mL of nonbacteriostatic saline (pH 7.0) in each nostril. The sample can then be treated with a mucolytic agent and subsequently stained or prepared for the PCR by DNA extraction

MICROSCOPIC IDENTIFICATION

A variety of stains can be employed for the detection of *Pneumocystis*. Variations of the methenamine silver stain (e.g. Grocott's, Gomori's) are the most widely used for diagnosis with tissue sections, but can also be used with body fluids such as BALF. A positive reaction results in a distinctive black cup-shaped morphology of the cysts against green-colored host cell architecture (Figure 8b) (Cushion 2003). In some reactions, the cysts will appear with a darkened structure resembling a 'double comma,' or 'parentheses' within the cyst. There is no budding associated with *Pneumocystis*, and this feature can be used to differentiate *Pneumocystis* infection from other respiratory fungal diseases such as histoplasmosis. Only the cyst form of the organism can be visualized with these stains, which is a drawback to their use. If the trophic form predominates in an infection, the organism burden can be greatly underestimated. The silver stain and other stains that selectively stain the wall of *Pneumocystis* cysts (e.g. toluidine blue O, cresyl echt violet) are favored among pathologists because they provide a sharp contrast between background host cells and organism. The Papanicolaou stain, frequently used for staining cytopathological specimens, can demonstrate the foamy exudate surrounding the organisms, although the organisms stain poorly. Likewise, the gram stain produces a negative reaction with poorly defined organism morphology.

Most laboratories now use rapid variants of the Giemsa stain for accelerated diagnoses. The most rapid modification of the methenamine silver stain takes approximately 30 minutes to perform, while the rapid Giemsa-like stains (e.g. Diff-Quik^{R}, LeukoStat^{R}) take 30 seconds (Cushion et al. 1985). With the Giemsa stains, all forms of the organism are stained, which permits rapid assessment of the total organism burden. The nuclei appear reddish-purple in contrast to the light blue cytoplasm. The cyst wall excludes the dyes and appears

as a circumscribed clear zone around the cyst contents. This stain not only permits visualization of all forms of *Pneumocystis*, but also permits assessment of the sample quality by demonstration of host cells such as alveolar macrophages and neutrophils.

Commercial kits employing fluoresceinated monoclonal antibodies against surface antigens of both cyst and trophic forms are widely used for diagnosis. Although laboratories must be equipped with epifluorescent microscopes, the reaction is specific for the organism and usually can be readily visualized against the background host cells. Depending on the monoclonal antibody supplied with the kit, staining may target only the cyst form or may target all forms of the organism. Since trophic forms are more numerous than cyst forms, it is recommended that laboratories use kits with antibodies directed to all forms of the organism, such as 2G2 or 3F6, which are available from DAKO Corp., Carpinteria, CA and Chemicon International, Inc. (Kovacs et al. 2001). Fluorescein isothionate is the typical fluorophore conjugated to the antibody in a direct assay, or to the detection antibody in an indirect assay. Organisms appear a brilliant apple green in color. Immunohistochemical techniques employing antibodies specific for *Pneumocystis* have been used with success with tissue sections, although silver stains can often provide the same information. Immunohistochemistry, however, provides confirmation of diagnosis in unusual settings such as extrapulmonary pneumocystosis.

CULTURE

Detection of *Pneumocystis* by amplification using artificial media or tissue culture is not an option since there is no continuous in vitro culture system for *Pneumocystis*.

SERUM TESTS

Serum antibody detection has had little success in the diagnosis of pneumocystosis in immunocompromised patient populations (Walzer and Cushion 1989). The high prevalence of circulating anti-*P. carinii* antibodies in the general population has confounded attempts to establish a serodiagnostic test. Prior exposure to the organism cannot be distinguished from active disease.

Other serological tests sometimes used for diagnosis are similarly nonspecific. Serum lactic dehydrogenase (LDH) levels are often elevated in PCP and decline with successful treatment. However, similar increases can be associated with other diseases (Benson et al. 1991) and thus, LDH levels must be used in concert with other diagnostic modalities.

OTHER LABORATORY TESTS

Several other diagnostic tools have been used for diagnosis of PCP. Arterial blood gas (ABG) is an important laboratory test for PCP patients, due to the frequency of impaired gas exchange. Typically, patients with PCP will have a decreased arterial oxygen tension with an increased arterial–alveolar gradient, and respiratory alkalosis. The ABG can be used to direct decisions regarding patient admissions, administration of adjunctive corticosteroids, and to assess PCP therapy (Huang 2004). Gallium scans and diethylenetriamine pentaacetic scans are imaging techniques reported to be abnormal in patients with PCP, but again, are nonspecific for PCP (Huang 2004).

Pulmonary function tests are useful in those patients where there is a clinical suspicion for PCP, but in which there is a normal chest radiograph (Huang 2004). Patients with PCP will often demonstrate a restrictive ventilatory defect with decreased lung volumes and increased airflows. Measurement of the diffusing capacity for carbon monoxide, DL_{CO}, is a sensitive indicator of alveolar-capillary block (Sankary et al. 1988). In patients with PCP, this is decreased on average from 89 to 100 percent (Coleman et al. 1984; Hopewell and Luce 1985; Shaw et al. 1988; Camus et al. 1993). Although such decreases are not definitive, a normal DL_{CO} would strongly suggest the absence of PCP.

MOLECULAR METHODS

Amplification of *Pneumocystis* DNA by PCR offers a dramatically increased level of detection and the ability to genotype the infecting organism populations. Although it is not yet routinely used in the clinical laboratory, it will likely eclipse standard microscopic methods because of its specificity, sensitivity, and relative low cost. In addition to diagnosis, PCR is routinely used for investigations of colonization in normal populations and those with underlying disease states (Helweg-Larsen et al. 2002; Huang et al. 2003; Morris et al. 2004).

P. jirovecii is routinely detected in DNA extracted from human samples by PCR using primers targeting the mtLSU (Wakefield et al. 1990b). These primers are: pAZ102-E (5′-GATGGCTGTTTCCAAGCCCA-3′) and pAZ102-H (5′-GTGTACGTTGCAAAGTACTC-3′) and result in a product of 346 bp. The mtLSU gene is present in multiple copies per organism and provides the most sensitive target for detection. The reaction can be a direct one, or nested, using a second set of primers directed to a region within the product of pAZ102-E and pAZ102-H (Wakefield et al. 1990a). The internal primers are: pAZ102-X (5′-GTGAAATA-CAAATCGGACTAGG-3′) and pAZ102-Y (5′-TCAC-TTAATATTAATTGGGGAGC-3′), which produce a 267 bp product(Vargas et al. 2000). The nested approach is used for samples with anticipated low template levels.

TREATMENT

Trimethoprim-sulfamethoxazole (TMP-SMX)

The combination therapy of TMP-SMX is the therapy of choice for all forms of pneumocystosis (Walzer 2004b). TMP-SMX, which inhibits the folic acid pathway at two distinct steps, is administered orally or intravenously in a dose of 15–20 mg/kg/day trimethoprim (TMP) and 75–100 mg/kg/day sulfamethoxazole (SMX) in three or four divided doses for 21 days in HIV-infected patients and for 14 days in non-infected patients (Walzer 2004b). The parenteral preparation is warranted for gravely ill patients or those with gastrointestinal disturbances. Serum levels of 5–8 μg/mL in adults and 3–5 μg/mL TMP in children and 100–150 μg/mL SMX in both are recommended for maximum efficacy, although there is some question as to the value and practicality of this practice (Walzer 2004b). A significant number of AIDS patients (50–80 percent) experience adverse reactions to this regimen, with gastrointestinal complaints and skin rashes being the most common problems (Stein and Stevens 1995). Other adverse reactions include fever, neutropenia, thrombocytopenia, nausea and vomiting, pancreatitis, nephritis, hyperkalemia, metabolic acidosis, anaphylactoid reaction, and hepatic disturbances (Walzer 2004b). It is thought that the adverse reactions are due to the sulfonamide component, but TMP has been implicated as the cause of skin rashes and hyperkalemia (Velazquez et al. 1993).

Pentamidine

Two parenteral drugs, the cationic diamidine pentamidine isethionate and the dihydrofolate synthase inhibitor trimetrexate, are considered the major alternatives to oral TMP-SMX for moderate to severe pneumocystosis in hospitalized patients (Barry and Johnson 2001).

Pentamidine is administered parenterally as a single daily dose of 4 mg/kg/day or, in some patients, a lower dose of 3 mg/kg/day has been shown to be effective with fewer side effects (Conte et al. 1990). About 50 percent of patients experience adverse effects including local reaction at the site of administration; systemic effects including dizziness, flushing, and cardiac arrhythmias. Hypotension occurs in a significant number of patients and requires vigorous supportive therapy. Nephrotoxicity is a frequent side effect but is usually reversible. The risk of hyperglycemia is related to pentamidine dose, length of administration, and serum levels exceeding 100 ng/mL (Waskin et al. 1988). Other side effects reported were pancreatitis, dysglycemia, hypocalcemia, hypomagnesemia, neutropenia, and hepatic disturbances (Walzer 2004b). Administration of pentamidine via the aerosolized route at 300 mg every month via Respiragard II nebulizer system efficiently delivers high doses of the drug to infected areas, but has been shown to be less effective than other drugs in the treatment of pneumocystosis, probably because it is not well distributed to all lobes of the lungs (Kovacs et al. 2001). It is used primarily as a prophylactic measure.

Trimetrexate

Trimetrexate, a lipid-soluble derivative of methotrexate, was shown to be less effective than TMP-SMX in a controlled trial of hospitalized patients, but better tolerated (Sattler et al. 1994). Trimetrexate is delivered intravenously as a single daily dose of 45 mg/m^2. Bone marrow suppression is the primary side effect associated with this therapy, but administration of folinic acid (20 mg/m^2 every 6 hours) has been found to ameliorate or prevent toxicity. Other side effects reported have been skin rash, peripheral neuropathy, and liver function abnormalities (Walzer 2004b).

Alternatives

Several alternative regimens have been recommended for patients who cannot tolerate or who fail TMP-SMX. These include dapsone at 100 mg daily in combination with TMP taken orally at 320 mg every 8 hours. Atovaquone, a hydroxynaphthoquinone, is another alternative which is administered at 1 500 mg orally per day. Although not as effective as TMP-SMX, it is better tolerated. Dapsone and its combinations appear to be the most efficacious alternative therapies, although some patients still manifest reactions to dapsone which is structurally similar to sulfonamides.

The combination of primaquine and clindamycin has been shown to be most effective against mild to moderate pneumocystosis (Black et al. 1994) but is contraindicated in glucose-6-phosphate dehydrogenase (G6PD) deficiency in which hemolysis has been reported as a major adverse reaction (Korraa and Saadeh 1996). For this purpose, atovaquone has been recommended for patients with G6PD deficiency and who are intolerant to TMP-SMX or pentamidine. The recommended doses are clindamycin at 600 mg (Walzer 2004b) or 300–450 mg (Kovacs et al. 2001) every 6 hours intravenously, with 15–30 mg of primaquine per day orally. Treatment may be initiated with intravenous clindamycin, then switched to oral administration.

Corticosteroids

Adjunctive corticosteroid treatment within the first 72 hours of anti-*Pneumocystis* of moderate to severe

pneumocystosis has resulted in improved survival for many patients (Masur 1992). This important finding led to the recommendation by a panel of experts that steroids be added to the treatment of all patients with moderate to severe pneumocystosis (i.e. arterial oxygen pressure of <70 mmHg, alveolar arterial gradient of >35 mmHg) in the following dose regimen: prednisone 40 mg orally twice daily, days 1–5; 40 mg once daily, days 6–10; and 20 mg once daily, days 11–20 (Anonymous 1990). Steroids are thought to reduce the host inflammatory response, which may then reduce the decline in oxygenation often associated with the first few days of therapy. The principal side effects associated with steroid administration were oral candidiasis, exacerbation of herpes simplex infections, and metabolic effects, such as hypoglycemia.

Mutations in *DHPS* and cytochrome *b*. Is resistance occurring?

TMP-SMX is a combination of two antifolates: trimethoprim (TMP), which inhibits the dihydrofolate reductase enzyme (*DHFR*), and sulfamethoxazole (SMX), which inhibits the activity of dihydropteroate synthase (*DHPS*), in folic acid synthesis. *DHFR* reduces dihydrofolic acid to tetrahydrofolic acid, which can be converted to the kinds of tetrahydrofolate cofactors used in 1-carbon transfer chemistry. *DHPS* catalyzes the condensation of pABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate. Since many pathogens, inlcuding *Pneumocystis*, must make their own folates and their mammalian hosts do not, this combination has a high therapeutic index.

Mutations in the dihydropteroate synthase gene (*DHPS*), the target of the sulfa component of TMP-SMX, have been identified in over 50 percent of the *P. jirovecii* samples in surveyed geographic areas (Beard et al. 2000). These point mutations have been associated with sulfa and sulfone resistance in bacterial pathogens (Lopez et al. 1987; Fermer and Swedberg 1997; Kai et al. 1999) and malaria (Brooks et al. 1995). In 1997, the first evidence for such mutations in *P. jirovecii* was identified (Lane et al. 1997). The nucleotide polymorphisms all resulted in changes in the amino acid sequence of the enzyme protein, suggesting evolutionary selection was occurring, perhaps in response to drug pressure. These changes were at amino acids 55 and 57. The pterin substrate binds to threonine at site 55 and arginine at 56. The mutations at position 55 usually substitute alanine for the threonine, which lacks the hydroxyl group needed for binding. The wild-type proline at site 57 is replaced with serine, which may influence the three-dimensional position of Arg56, critical for binding to pABA and sulfa, and the pterin moiety.

Subsequent to these first observations, several studies have been published showing the dramatic spread of the mutant *DHPS* genotypes among *P. jirovecii* infecting human populations around the globe (Helweg-Larsen et al. 1999; Santos et al. 1999; Beard et al. 2000; Huang et al. 2000; Nahimana et al. 2003; Calderon et al. 2004b; Kazanjian et al. 2004; Montes-Cano et al. 2004) and the association of the mutant genotypes with sulfa prophylaxis and potential resistance (Ma et al. 1999; Armstrong et al. 2000; Huang et al. 2000; Kazanjian et al. 2000; Calderon et al. 2004b). In each study, significantly more *DHPS* mutations occurred in sulfa-treated patients than in non-treated study groups. The appearance of the *DHPS* mutations is a relatively recent event, with no evidence for the polymorphisms prior to 1993 (Kazanjian et al. 1998, 2000).

Although the presence of the mutant *DHPS* genotypes and their association with prior sulfa exposure is clear, less clear is how these mutations respond to TMP-SMX therapy. Studies with contrasting results showed a significant decline in survival of patients with the mutations or treatment failure with a sulfa-containing drug (Helweg-Larsen et al. 1999; Nahimana et al. 2003) while other studies have shown no effect in patient outcome (Huang et al. 2000; Kazanjian et al. 2000). Such study outcomes can be confounded by such variables as infections with mixed genotypes, for example. Direct evidence showing a correlation of the *DHPS* gene of *P. carinii* containing the mutations and increased resistance to sulfa was recently demonstrated in a heterologous yeast system, providing evidence that it is likely these mutations will contribute to some decrease in sensitivity to these compounds (Iliades et al. 2004; Meneau et al. 2004).

Last, the encroachment of *P. jirovecii DHPS* mutations into human populations is not relegated to those infected with HIV, rather these mutations are now being reported in association with other types of underlying diseases such as chronic bronchitis (Calderon et al. 2004a). It is apparent that *P. jirovecii DHPS* mutants now enjoy widespread prevalence among the general human population. Whether these mutations will confer a greater survival rate with a higher level of resistance to the sulfa compounds or increased virulence remains to be seen. It should be noted though, that *P. jirovecii* is now being reported in association with a variety of underlying disease states and the issue has been raised as to whether its presence represents a comorbidity factor (De La Horra et al. 2004; Morris et al. 2004).

Atovaquone is a second-line therapeutic for mild to moderate PCP and is used as a prophylactic agent as well. As a ubiquinone mimic, atovaquone binds to cytochrome *b* in the mitochondria, affecting mitochondrial electron transport and parallel processes such as ATP and pyrimidine biosynthesis. Some evidence indicates it may also inhibit ubiquinone synthesis in *Pneumocystis* (Kaneshiro 2001). Mutations in the cytochrome *b* gene of *P. jirovecii* in many patient isolates have been reported (Walker et al. 1998; Kazanjian et al. 2001) and

their association with atovaquone exposure documented (Kazanjian et al. 2001). This finding is not altogether surprising since resistance develops in almost all patients receiving atovaquone as monotherapy for the treatment of malaria (Looareesuwan et al. 1996). Unlike the prevalence of two major mutations in *DHPS*, several polymorphisms have been identified in the cytochrome *b* gene of *P. jirovecii*. Like similar studies conducted with the *DHPS* gene, mutations in the cytochrome *b* gene of *Pneumocystis* expressed in a yeast system was shown to confer a resistant phenotype in vitro (Hill et al. 2003; Kessl et al. 2004).

Despite strong evidence supporting the emergence of new *P. jirovecii* genotypes induced by selective drug pressure, there is no reason as yet to change treatment or prophylactic regimens, and dosing schedules as outlined above remain the recommended procedures(Kovacs et al. 2001).

EPIDEMIOLOGY

As the populations at risk for HIV infection have changed since the 1980s, so too have the demographics of PCP. The medically underserved African American and female populations in the USA are now considered at risk (Justice et al. 2001; Kilbourne et al. 2001; Rabeneck et al. 2001). In these populations, PCP remains a leading opportunistic infection (Centers for Disease Control and Prevention 1999; Fisk et al. 2003). In countries considered to be 'developing' (i.e. Africa, Asia, the Phillipines, and Central and South America), the number of those infected with HIV has increased dramatically, sometimes infecting a third of the population in some sub-Saharan countries. Although PCP rarely occurred in African adults in the first decade of the AIDS pandemic, the incidence has risen dramatically as it has in other developing countries, reaching a 25–80 percent coinfection rate with tuberculosis and a mortality rate ranging from 20 to 80 percent (Fisk et al. 2003).

Moreover, PCP is occurring more frequently in other patient groups that in some cases were rarely affected before, including transplantation recipients, especially in lung transplantation patients (Kubak 2002); patients receiving high dose corticosteroids for brain neoplasms, inflammatory or collagen-vascular disorders, and especially patients with Wegener's granulomatosis (Russian and Levine 2001); in association with asthma (Kuitert and Harrison 1991; Abernathy-Carver et al. 1994; Sy et al. 1995; Dziedziczko and Banach-Wawrzenczyk 1997; Contini et al. 1998); chronic obstructive pulmonary disease (Palange et al. 1994); cystic fibrosis (Royce and Blumberg 2000; Sing et al. 2001); Epstein–Barr virus infection (Stiller et al. 1992); lupus erythematosus (Wainstein et al. 1993; Nguyen et al. 1995; Ward and Donald 1999); rheumatoid arthritis (Oien et al. 1995; Ohosone et al. 1997; Rolland et al. 1998); thyroiditis (Guttler et al. 1993); and ulcerative colitis (Khatchatourian and Seaton 1997; Quan et al. 1997).

The complete life cycle of any *Pneumocystis* species is poorly understood and this has confounded epidemiological studies. The current concepts of the life cycle are discussed in the section on The organism below, but it should be emphasized that neither the route of exit from the lung nor the process it uses to travel to the alveoli is known. There is good evidence that the infection is acquired by an airborne route (Walzer et al. 1977; Hughes 1982), but the agent of transmission has not been identified. In fact, the transmission and acquisition phases are almost completely unknown. Although recent studies have detected *Pneumocystis* DNA in neonatal human and rat populations, an assessment of potential reservoirs in the general population is only in its infancy. Significantly, there is no information about replication within the non-immunocompromised mammalian lung nor its transmission or acquisition among intact hosts.

Seroepidemiologic surveys have shown that the majority of adults in the USA and Europe have anti-*P. carinii* antibodies and healthy children seroconvert at 4 years of age or less (Pifer et al. 1978; Peglow et al. 1990). The primary infection was once thought to be asymptomatic or with generalized symptoms that may be mistaken for a mild viral infection. However, recent studies have shown the high prevalence of *P. jirovecii* in neonates with respiratory symptoms (Vargas et al. 2001), calling into question this widespread assumption. Moreover, reports of the association of *P. jirovecii* in cases of sudden infant death syndrome (SIDS) (Vargas et al. 1999; Morgan et al. 2001; Chabe et al. 2004) also suggest that the organism may be more virulent than once thought. Serological studies have shown that exposure to *P. carinii* is worldwide, although the seroreactivities to specific organism antigens varied among geographic regions (Smulian et al. 1993a).

The evidence on hand supports transmission by immunocompetent individuals or those with subclinical infections. First, more than 75 percent of the human populations surveyed in the USA and Europe are seropositive to *Pneumocystis* antigens by 4 years of age, implying that the organisms are ubiquitous, or at least plentiful, and exposure is a common occurrence. The sheer number of *Pneumocystis*-seropositive immunocompetent individuals argues against the fulminant PCP-infected host as the primary source of infection, as these patients are rare (in relation to the general public) and often hospitalized or have limited access to the general population. More recent data strongly support the concept of host–host transmission. A geographic study of *P. jirovecii* genotypes showed that the genotype with the double mutation in *DHPS* was the most prevalent in the populations surveyed, replacing the wild-type (Beard et al. 2000). The fact that these mutations were not observed before 1993 (Kazanjian et al. 1998, 2000) but had spread over the globe throughout the proceeding

10 years (Miller et al. 2003; Kazanjian et al. 2004; Montes-Cano et al. 2004) argues for a selective pressure and host-dependent spread. Direct evidence is provided by the rat animal model of PCP. Infection with *Pneumocystis* is widespread in the healthy non-immunosuppressed rat populations available from commercial vendors (Icenhour et al. 2001), arguing for highly efficient transmission and maintenance of the infection within these healthy populations. The transmission of the organism from mother to offspring occurs within 1–2 hours after birth in rats (Icenhour et al. 2002) and animals within an infected colony remain positive for *P. carinii* amplicons throughout their residence.

Studies now being conducted in human populations, both immunosuppressed and non-immunsuppressed, point to certain reservoirs of infection in the general population, including patients with underlying disease states besides HIV or overt immunosuppression; neonates; and possibly pregnant women. This is an active and concerted area of research and basic epidemiologic principles for *Pneumocystis* infection should emerge within the next few years.

Asymptomatic colonization

Probable asymptomatic colonization has been shown for both healthy and HIV-infected individuals. Nested or single round PCR amplification targeting the small subunit and large subunit mtLSU of *P. jirovecii* showed 35 of the 47 BAL samples from HIV-infected patients contained *P. jirovecii*- specific DNA; 18 of these came from patients who did not have clinical evidence of *P. jirovecii* pneumonia, suggesting asymptomatic colonization occurred in these patients. In a larger study, 17 of 93 (18 percent) consecutive BALF samples from HIV-negative patients undergoing diagnostic bronchoscopy were found to be positive for the presence of *P. jirovecii* by PCR (Maskell et al. 2003). Analysis of potential risk factors showed only glucocorticoid use [and not lung cancer or chronic obstructive pulmonary disease (COPD)] was associated with the presence of *P. jirovecii*.

The state of pregnancy may facilitate asymptomatic colonization. A study of 33 pregnant healthy women in their third trimester versus 28 healthy women within 15 days of their menstrual cycle showed the presence of *P. jirovecii* DNA in five (15.5 percent) of deep nasal swabs from the pregnant women as compared to none in the non-pregnant women (Vargas et al. 2003). Evidence for the early acquisition of *Pneumocystis* in neonates with the mother as a potential source of organisms is accumulating in both human studies (Miller et al. 2002) and animal studies (Icenhour et al. 2002).

Other factors appear to favor colonization with *P. jirovecii*. In a large study of 367 patients with suspected bacterial pneumonia, the presence of *P. jirovecii* (detected by the same PCR methods discussed above) was statistically associated with old age, concurrent disease, and steroid treatment (Helweg-Larsen et al. 2002).

HOST RESPONSES

Impaired cellular immune function has long been held to be the primary predisposing factor for development of PCP with the CD4 cell playing a central role. The risk of developing PCP in HIV-infected patients increases with a decline in the number of circulating CD4 cells. It is formally recommended that chemoprophylaxis be instituted when the CD4 count in adults falls below 200/mm^3 (Centers for Disease Control and Prevention 2002). However, the decrease in CD4 cells rarely occurs without perturbation of other immune responses and there is now considerable evidence that defects in humoral immunity also contribute to the development of PCP.

Antigens

Immunoblotting studies have identified the major antigens of *Pneumocystis* from humans and animal models that elicit serum IgG antibody responses (Walzer 2004a). There are two distinct antigenic regions that are similar across species lines, the MSGs that migrate between 95 and 120 kDa and a broad-based band at 35–55 kDa that likely contains many discrete antigens.

Each *Pneumocystis* organism encodes about 100 different MSG genes distributed among each of its chromosomes as detected by hybridization of Southern blotted electrophoretic karyotypes (Stringer and Keely 2001). These antigens were localized to the surface of the organism (Kovacs et al. 1989) and have been shown to evoke strong humoral and cell-mediated responses (Smulian and Theus 1994) and to interact with host proteins on lung cells such as fibronectin, vitronectin, and SP-A (Pottratz and Martin 1990a; Limper et al. 1991, 1993; Pottratz et al. 1991; Zimmerman et al. 1992) and the mannose receptor on macrophages (Ezekowitz et al. 1991). It is thought that the MSGs function in a variety of ways:

- by promoting adherence to host lung cells and to other *Pneumocystis* organisms
- by complexing with host molecules to avoid the host immune responses
- by modulating the species of surface MSGs to circumvent the immune response
- perhaps by functioning as adhesions during the mating response (Cushion 2004b).

A sequence common to nearly all MSG mRNAs, the upstream conserved sequence (UCS), has been identified and is present in a single copy per organism genome and resides on a single chromosome (Wada et al. 1995; Sunkin and Stringer 1996). It is thought that the MSG

genes are transcribed only when adjacent to the UCS and the genes are translocated from subtelomeric regions on each chromosome via reciprocal exchange, gene conversion, or a recombination event. The UCS encodes a signal peptide thought to aid in chaperoning the molecule to the endoplasmic reticulum and golgi for processing and ultimately the cell surface where they are the most abundant surface protein.

The MSGs are heavily glycosylated, highly immunogenic, and have been shown to contain protective B- and T-cell epitopes (Gigliotti and Hughes 1988; Roths and Sidman 1992, 1993; Gigliotti et al. 2002). Immunizations with native or recombinant MSG have resulted in conflicting results; some report a protective effect, while others found no such protection (Theus and Walzer 1997; Gigliotti et al. 1998; Theus et al. 1998; Lasbury et al. 1999). Besides the multigene family of MSGs, other multiply repeated genes are present at the telomeric ends of the chromosomes (Schaffzin et al. 1999; Keely et al. 2001; Ambrose et al. 2004). These include the *PRT* family of genes that encode kexin-like subtilisin proteases that are likely involved in processing the signal peptide on the nascent MSG and the MSG-related proteins (MSR) that encode proteins similar but distinct to the MSG. These genes are grouped in arrays at the telomeric ends that vary in number of each gene family member present (Keely et al. 2001). The *PRT* genes exist as a multiple gene family in *Pneumocystis* infecting rats, but only as a single copy in *P. murina* (Lee et al. 2000) and *P. jirovecii* (Kutty and Kovacs 2003), illustrating that there are significant differences among the species of *Pneumocystis*.

The other major antigen group includes proteins that migrate as a broad band between 35 and 45 kDa in *P. jirovecii* (Walzer and Linke 1987) and 45–55 kDa in *P. carinii* and *P. wakefieldiae* (Vasquez et al. 1996). A gene encoding an antigen within the 45–55 kDa region of rat-derived *Pneumocystis* (p55) was cloned and shown to be a single copy gene which hybridized to a chromosome band of about 670 kbp on southern blotted electrophoretic karyotypes (Smulian et al. 1992). The function of this antigen is unclear but has been shown to elicit a cell-mediated response in rats previously exposed to *Pneumocystis* (Smulian et al. 1993b; Theus et al. 1994). An immunodominant epitope was mapped to the 3′ portion of the molecule which contains a 7 amino acid repeat motif. The cells responding to p55 were shown to be CD4+ cells which secreted a Th1 cytokine pattern. The corresponding *p55* gene from *P. murina* was recently cloned and characterized (Ma et al. 2003) and shown to be in single copy as well.

Humoral Immunity

The importance of the humoral arm of the immune response in the control of *Pneumocystis* infection has been shown by several experimental animal studies and indicated by specific immunodeficiencies. *Pneumocystis* infection has been reported in B-cell-deficient mice and other animals (Marcotte et al. 1996; Walzer et al. 1997; Lobetti 2000; Macy et al. 2000) and in patients with hypogammaglobulinemia (Walzer et al. 1989; Esolen et al. 1992). Passive immunotherapy with monoclonal antibodies or hyperimmune sera raised against *Pneumocystis* decreased the organism burden in immunosuppressed animal models (Gigliotti and Hughes 1988; Roths and Sidman 1993). Immunization by intratracheal administration of organisms in CD4-depleted mice was shown to be protective against subsequent *Pneumocystis* challenge possibly by stimulating serum antibodies (Harmsen et al. 1995; Garvy et al. 1997b; Gigliotti et al. 1998; Pascale et al. 1999). Development of spontaneous PCP in colonies of mice with only B-cell defects suggests a role for the humoral branch in the control of the infection (Marcotte et al. 1996). It is clear that macrophages avidly bind and degrade the organism and addition of antisera and complement enhances the rapid internalization and degradation (Smulian and Theus 1994).

Several studies have shown that the organism elicits a strong humoral response. Early studies used the indirect fluorescent antibody assay (IFA), the enzyme-linked immunosorbent assay (ELISA), and complement fixation assay with whole or fractionated *Pneumocystis* organisms or crude antigen preparations to show that most of the human population had antibodies to *Pneumocystis* (Walzer 2004a). Later studies using immunoblotting techniques expanded these studies to evaluate antibodies to specific antigens (Buhl et al. 1993; Lundgren et al. 1993a, b; Smulian et al. 1993a; Chen et al. 1994; Elvin et al. 1994; Smulian and Walzer 1994). Such studies showed that exposure to *P. jirovecii* in humans occurs early in life. By age 3 years, most children have developed antibodies to the organism (Meuwissen et al. 1977; Pifer et al. 1978; Peglow et al. 1990; Vargas et al. 2001; Daly et al. 2002). A study which examined 680 serum samples from healthy adults in several different areas of the world showed 76 percent reacted with the *P. jirovecii* antigens by immunoblotting techniques (Smulian et al. 1993a). Most (60 percent) of the reactivity was observed in the area of 35–45 kDa while only about 30 percent of the sera reacted at ~95 kDa, the area at which the MSG family migrates. Of note, the frequency of antibodies to the MSG region exhibited significant geographic variation, suggesting that different strains of *P. jirovecii* may be predominant in a given area. The high prevalence of serum antibodies and the ubiquitous nature of *Pneumocystis* have confounded efforts to develop serodiagnostic assays. Most serological surveys have shown the frequence and/or level of antibodies to *Pneumocystis* among HIV-infected patients and other immunocompromised hosts were similar to those in healthy controls (Smulian and Walzer 1994). The frequent presence of antibodies in

most human populations has contributed to the belief that humoral immunity contributes little to the host defense against *Pneumocystis* infection.

The MSG family of genes expressed in part or as the whole recombinant protein have been used to probe the immune response in humans. One laboratory expressed a highly conserved region of the carboxy terminus of an MSG gene that was recognized by all serum samples, and thus could not discriminate among patients with active disease, previous episodes, or healthy controls (Mei et al. 1998). More recently, three overlapping fragments that spanned the entire human MSG gene were generated: *MSGA*, *MSGB*, and *MSGC* (Daly et al. 2002). Recognition of *MSGA* and *MSGB* was significantly lower in HIV-positive patients than normal healthy controls. *MSGC* was recognized by patients with previous episodes of PCP. Responses did not vary by geographic location. These data are important because they show for the first time that different regions of the MSG molecule can identify previously unrecognized differences among populations. Serological response may yet play a role in defining disease state.

Cellular immune response

Evidence for the critical role of T cells in resistance to *Pneumocystis* infection has come from observations of the susceptibility of hosts with T-cell deficiencies to pneumocystosis and directly through reconstitution experiments. Infection with HIV, congenital syndromes with T-cell defects, and administration of drugs to suppress the immune system are all factors that place a mammalian host at risk for pneumocystosis. Except for syndromes with specific T-cell defects, the other immunosuppressive states include a more global immune depression. However, adoptive transfer studies, cell depletion, and reconstitution experiments in rodents and the use of knockout mice models have provided good evidence that the primary defect for development of PCP is CD4 cell dysfunction. CD4 cells also interact with B cells and other cells via the CD40–CD40 ligand pathway. Disruption of these interactions resulted in development of PCP in young children with the hyper-IgM syndrome (Levy et al. 1997) and in impaired clearance of *P. murina* from the lungs of mice (Wiley and Harmsen 1995).

Compelling evidence for the critical role of CD4 cells in resolution of pneumocystosis comes from adoptive transfer experiments in which splenic T cells from normal healthy animals or those exposed to *Pneumocystis* were transferred to animals depleted of CD4 cells. The recipient animals went on to clear or substantively reduce their *Pneumocystis* burden (Graves et al. 1994). Chronic administration of anti-CD4 antibodies to BALB/c mice permitted the development of pneumocystosis following inoculation of *Pneumocystis* organisms (Shellito et al. 1990). SCID mice were able to resolve the infection after transfer of splenic cells from genetically matched healthy donors (Harmsen and Stankiewicz 1991; Roths and Sidman 1993). However, when the splenic populations were divided into CD8+ and CD4+ populations, transfer of the former cell population was not effective in decreasing the organism burden (Roths and Sidman 1992). When the SCID mice that were reconstituted with splenic populations were then treated with anti-CD4 antibody, the mice developed *Pneumocystis* infection, while those receiving anti-CD8 antibodies were able to resist infection. *Pneumocystis*-infected SCID mice infused with splenic cells depleted of B cells could not resolve the infections (Harmsen and Stankiewicz 1991), suggesting the CD4 cell was not the only immune effector cell involved in mechanisms of resistance. These studies provide strong evidence for the critical role of CD4 cells in resistance and recovery from PCP but suggest a concerted effort by several immune compartments.

CD8 cells accrue in the lungs of experimental animals with pneumocystosis and can be found in the BALF from human patients infected with *P. jirovecii*. CD8 cells have been shown to contribute to host defenses in experimental mouse models (Beck et al. 1996; Beck and Harmsen 1998). BALB/c mice depleted of CD4 lymphocytes, CD8 lymphocytes, or both CD4 and CD8 lymphocytes, and infected with *P. murina*, showed moderate infection in mice depleted of CD4 lymphocytes while mice depleted of CD8+ lymphocytes cleared the inoculum. This result would seem to show that CD8 lymphocytes are unnecessary for defense when CD4 lymphocytes are available. However, mice depleted of both CD4 and CD8 lymphocytes developed PCP with a significantly higher organism burden than mice depleted of CD4 lymphocytes alone. Thus, CD8 lymphocytes function in the defense against *Pneumocystis* in vivo in the context of depleted populations of CD4 lymphocytes. CD8 taken from the lungs of the CD4-depleted mice proliferated in response to *P. murina* antigen and produced interferon-gamma (IFN-γ) in vitro. Thus the mechanism of host defense by CD8 cells likely involves IFN-γ.

Alveolar macrophages serve as the first line of defense against *Pneumocystis* and clear the organism from the alveoli (Koziel et al. 1994; Limper et al. 1997). The nature of the binding occurs via the mannose macrophage receptor (Ezekowitz et al. 1991) and this binding may be enhanced by host-derived molecules (Pottratz and Martin 1990a, b; Limper et al. 1993) or via the gp120 molecule on the organism's surface (Pottratz et al. 1991). More recent studies showed that additional components were necessary for the macrophage: *Pneumocystis* interaction including β-glucan (Vassallo et al. 1999), which resulted in stimulation of tumor necrosis factor alpha (TNF-α) release; extracellular matrix (Fishman et al. 1991); surfactant proteins (Limper et al.

1995; O'Riordan et al. 1995; McCormack et al. 1997); and Fc receptors (Ezekowitz et al. 1991). Hidalgo et al. (1992) were able to demonstrate induction of the oxidative burst in alveolar macrophages isolated from rats and in a cell line (NR8383) and that the evolution of hydrogen peroxide could be enhanced by complement. The release of nitric oxide by mouse alveolar macrophages was elevated in CD4-depleted *Pneumocystis*-infected mice, but the levels did not correlate with either clearance or severity of infection (Shellito et al. 1996). Urokinase-type plasminogen activator, a protease that facilitates recruitment of inflammatory cells, was shown to participate in the clearance of *Pneumocystis* from the lung (Beck et al. 1999). It is interesting to note that *P. carinii* contains a gene that encodes enolase, a function of which is to activate plasminogen (Fox and Smulian 2001), which may serve to regulate host plasminogen activator. Infection with HIV impairs the mannose receptor-mediated binding and phagocytosis of *Pneumocystis* and also changes the cytokine response (Kandil et al. 1994; Koziel et al. 1998a).

The modulation of cytokine profiles by *Pneumocystis* has primarily been studied in animal models. The proinflammatory cytokines TNF-α, and interleukin (IL)-1 play important roles in host defense against this infection, especially in the early stages (Chen et al. 1992a; Garvy et al. 1997a; Kolls et al. 1997; Limper 1997). The role of IL-1 in the clearance of infection was evaluated in the SCID mouse model (Chen et al. 1992b). Reconstituted SCID mice were able to clear the infection and IL-1 activity in the lung homogenate supernates correlated with an increase during infection and a reduction to baseline after clearance. Antibody to IL-1 could inhibit organism clearance, implicating this cytokine as an important host defense against *Pneumocystis* infection. It is interesting to note that secretion of IL-1 by human macrophages from patients with HIV is significantly reduced, perhaps mediating in part the susceptibility to the infection (Smulian and Theus 1994). Activated CD4 cells produce IFN-γ which has been shown to mediate activation of macrophages. Administration of aerosolized IFN-γ to CD4-depleted mice infected with *Pneumocystis* results in a dramatic reduction in organism burden (Beck et al. 1991), suggesting a role in organism clearance. IFN-γ may mediate this response through induction of nitric oxide synthesis in macrophages (Ishimine et al. 1995) or by other means such as TNF-α release. Investigations into the molecular mechanisms of the roles of some cytokines have produced some puzzling results. In one report, deletion of IFN-γ or TNF-α receptor genes did not adversely affect clearance of *Pneumocystis* infection, while elimination of both receptor genes resulted in severe disease (Rudmann et al. 1998). Some cytokines appear to be produced in response to *Pneumocystis* infection (IL-6), but their role, if any, in host defense remains unclear (Chen et al. 1993), while others seem to modulate the host inflammatory response, such as IL-10 (Ruan et al. 2002; Qureshi et al. 2003) or have no apparent role (IL-4, G-CSF) (Garvy et al. 1997b).

The roles of the costimulatory molecules CD2 and CD28 were investigated after mice with the double knockouts spontaneously developed *P. murina* pneumonia (Beck et al. 2003). Mice inoculated with *P. murina* could develop the pneumonia with the loss of CD28 alone, but the organisms eventually cleared. However, mice deficient in both CD2 and CD28 accumulated CD8+ T cells in their lungs in response to infection and demonstrated markedly reduced specific antibody titers. Cytokine profiles suggested IL-10 and IL-15 may be important for the response to *P. murina*.

Although it may seem apparent that a host response to *Pneumocystis* would be a positive effect, increasing evidence implicates a detrimental role in some cases. Immune reconstitution has been associated with marked cellular inflammatory response, rise in proinflammatory cytokines and chemokines, decreased lung compliance, and impaired oxygenation (Chen et al. 1992c; Wright et al. 1997, 1999b). Adoptive transfer of CD4 cells leads to an early hyperinflammatory response, even in the context of profound immunosuppression by corticosteroids (Roths and Sidman 1992; Theus et al. 1995, 1998). CD4 cells expressing the CD25 phenotype appear to be the cell type mediating this response (Hori et al. 2002). A role for the CD8 cell in the pathological inflammatory response has also been implicated. Immunocompetent mice depleted of CD4 cells exposed to *P. murina* develop the infection with a concomitant influx of CD8 cells and increase in neutrophil numbers, experience altered alveolar-capillary permeability, surfactant dysfunction, and impaired respiration (Wright et al. 1997, 1999a, b, 2001). Depletion of the CD8 cells in these CD4-depleted mice dramatically reduced the associated pathology. In rats, the early hyperinflammatory response after adoptive transfer of sensitized CD4 cells could be ameliorated by addition of CD8 cells (Theus et al. 1995; Theus and Walzer 1997). It appears that both cell types contribute to the pathology of *Pneumocystis* infection, and modulation of this response will require further knowledge of the immune circuitry and host immune status.

ANIMAL MODELS OF INFECTION

Animal models of PCP have played an instrumental role in the scientific investigation of this organism. Without a continuous supply of organisms furnished by an in vitro culture system, researchers have relied on animal models for a source of organisms and for assessment of drug responses. The observations, made in the 1950s and 1960s, that the pneumonia could be provoked in rats and rabbits by corticosteroid administration (Weller 1956; Sheldon 1959; Frenkel et al. 1966) led to the current models used today. This subject is reviewed thoroughly

by Armstrong and Cushion (1994a). The animal models of pneumocystosis have been shown to be strikingly similar to human pneumocystosis in pathology, organism morphology, and most of the basic biology known to date. Drug responses are similar with only few exceptions (Walzer 1994).

There are basically two types of animal models of pneumocystosis. The first type is a provocation of organisms resident within the lung or acquired from ambient air by chronic administration (e.g. 6–8 weeks) of corticosteroids or another immunosuppressant. The immunosuppressants can be administered in the drinking water (e.g. dexamethasone 1 mg/L) or by injection (e.g. Depo-Medrol, 4 mg/kg/IM or SC). This immunosuppressive regimen is associated with wasting, increased debility, and marked involution of all lymphoid tissues (Walzer et al. 1984a, b). Lymphopenia in the rat model was found to result in inverted CD4/CD8 cells, similar to that in AIDS patients. Oftentimes, an antibiotic (e.g. tetracycline) is concurrently administered to prevent secondary bacterial infections resulting from the immunosuppressed state of the animals. Administration of a low-protein diet (8 percent) has been reported to exacerbate the infection in the rat model (Hughes et al. 1974b). It is presumed that organisms within the lung begin to replicate or replicate without clearance once the immune system is sufficiently suppressed. This has been shown by experiments in which rats are received into barrier facilities that exclude all contact with ambient air, are immunosuppressed, and develop the infection (Cushion et al. 1993). The only source of organisms are those within the lungs of the animals. Rats receive the infectious propagules through an airborne mode, as shown by development of the infection in immunosuppressed *Pneumocystis*-naive animals placed in the same room (but not in contact) with *Pneumocystis*-infected rats (Walzer et al. 1977; Hughes 1982).

The second approach to establishing *Pneumocystis* in animal models is via inoculation. A surgical procedure in which the organisms are instilled into the trachea of lightly anesthetized, immunosuppressed rats (Bartlett et al. 1987), or mice (Bartlett et al. 1991), or instillation via a feeding cannula (Boylan and Current 1992) is used for inoculation. An immunosuppressant regimen as described above is required for replication of the inoculated organisms.

Both methods are useful for studying different aspects of the infection. The 'naturally' acquired infection permits an evaluation of the pathological processes that more closely mimic the actual disease progression. The inoculated model affords standardization in drug evaluations, a consistent supply of organisms, and the ability to use a genetically defined population.

Several species of animals have been used for the study of pneumocystosis by employing either one or both of these techniques. Rats and mice are the models used by most researchers. The ferret model had been used because of its larger size, permitting BAL, but now is rarely employed for studies (Armstrong and Cushion 1994a).

Other approaches to reducing the immune response are gaining favor, especially those designed to preferentially deplete the CD4 cell populations. CD4 cell depletion has been accomplished by administration of anti-CD4 monoclonal antibodies administered on a weekly basis or by implantation of a hybridoma which secretes monoclonal antibodies to this population (Shellito et al. 1990; McFadden et al. 1994; Thullen et al. 2003).

Animals rendered immunodeficient by genetic defects have been used on a more limited basis, but afford a model with fewer manipulations than the previously described ones. Many colonies of immunodeficient mice or rats are often infected with *Pneumocystis*. Homozygous scid/scid mice are especially prone to the infection as well as nude mice and rats (Roths et al. 1990; Sidman and Roths 1994). Neonate rabbits can be naturally infected with *Pneumocystis* without administration of immunosuppressants during their first few weeks of life (Soulez et al. 1988). This model has primarily been used for ultrastructural studies.

CONCLUSIONS

The placement of *Pneumocystis* in the Kingdom Fungi is now generally accepted. The genus has been placed within the Ascomycetes within its own family and order. Four species have been described: *P. jirovecii* which infects humans (Frenkel 1999); *P. carinii* and *P. wakefieldiae* from rats (Frenkel 1999; Cushion et al. 2004a); and from mice, *P. murina* (Keely et al. 2004).

Experimental models and human epidemiologic studies have implicated neonates as potential reservoirs of infection, as well as members of the general population that may be only slightly immunocompromised. Association of *P. jirovecii* with respiratory symptoms and with SIDS calls into question the concept of primary infection as a benign event. The association of *P. jirovecii* with underlying disease states such as COPD suggests its presence may be a comorbidity factor.

P. jirovecii has evolved mutations in the *DHPS* gene which have been linked to sulfa prophylaxis. The *DHPS* mutations in *P. jirovecii* have been reported in many different countries and continents, evidencing the rapid spread of these strains. Mutations in the cytochrome *b* gene, the target of atovaquone, have been reported, raising the specter of drug-resistant organisms.

The *Pneumocystis* Genome Project and efforts of individual investigators have contributed to a better understanding of the metabolic capacity of these organisms and the molecular processes of replication. Genes associated with mating, meiosis, cell cycle, sterol metabolism, heme biosynthesis, and energy production have

been identified. Identification of unique cycles or genes should provide new strategies and desperately needed drug targets.

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Adiaspiromycosis and other infections caused by *Emmonsia* species

LYNNE SIGLER

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The mycosis caused by *Emmonsia* species is called adiaspiromycosis. The disease name is based on the cells found in tissue which have been termed adiaspores. Adiaspores are formed in vivo when inhaled conidia, which initially measure about 4 μm diameter, enlarge to form thick-walled spherule-like cells reaching 15–300 μm diameter. The size of the adiaspores is dependent upon the species involved, with those of *Emmonsia crescens* being larger than those of *Emmonsia parva* (Table 1). Although adiaspores of *Emmonsia* spp. may resemble the spherules containing endospores produced by *Coccidioides immitis* (Table 1), adiaspores do not form internal spores, nor do they further replicate. Infections usually occur in animals, and many species of burrowing animals, including rodents, small carnivores, insectivores, marsupials, and edentates, throughout the world, have been found to be infected. Adiaspiromycosis in humans usually involves the lung, is often self-limited, benign and asymptomatic, with rare cases progressing to a fatal outcome. Diagnosis is based most commonly on radiological and histopathological findings. Available evidence suggests that *Emmonsia* spp. are weak pathogens and that pulmonary impairment and elicitation of symptoms is dependent upon the number of conidia inhaled. *Emmonsia* spp. are only rarely reported as the cause of extrapulmonary infection; one such report involved *Emmonsia pasteuriana*, described as a new pathogen in 1998.

ETIOLOGIC AGENTS

The genus *Emmonsia* currently includes three species: *E. parva* (Emmons & Ashburn), Ciferri and Montemartini (1959), *E. crescens* Emmons and Jellison (1960), and *E. pasteuriana* Drouhet, Gueho & Gori (Drouhet et al. 1998). The taxonomic history of *Emmonsia* spp. has been complicated. Mycologists have disagreed about the appropriate genus for the species, and about their relationship to the dimorphic pathogens. Recently, classical mating experiments and DNA sequence comparisons have provided evidence to confirm that the closest relative to *Emmonsia* spp. is *Blastomyces dermatitidis* Gilchrist & Stokes. Molecular techniques also helped to classify an *Emmonsia*-like isolate that differed in producing yeast-like cells, rather than adiaspores, in vivo. *E. pasteuriana* is known thus far only from a single case of disseminated cutaneous infection in a patient with the acquired immunodeficiency syndrome (AIDS). Other species formerly assigned to *Emmonsia*, i.e. *Emmonsia brasiliensis* and *Emmonsia ciferrina*, have been reclassified (Padhye and Carmichael 1968).

ETYMOLOGY

The term adiaspore comes from the Greek verb *speirein* (*a* – negative, *dia* – through, *speirein* – to scatter or

Table 1 *Comparison of* Emmonsia *spp.*[a] *with* Blastomyces dermatitidis, Paracoccidioides brasiliensis, Histoplasma capsulatum, *and* Coccidioides immitis

	Ecology and distribution	Mycelial form	Teleomorph	Parasitic form in vivo	Confirmatory tests	Figure showing morphology in vitro and in vivo
Emmonsia crescens	Many animal hosts (about 120 species of rodents, lagomorphs, carnivores, insectivores, herbivores, marsupials), rarely soil. Worldwide except Africa, Australia	Narrow hyphae forming solitary sessile conidia and 1–3 conidia at the ends of narrow, often slightly swollen stalks. Conidia subglobose, often slightly broader than long, 2.5–4 μm long and 3–5 μm wide, smooth to finely roughened	*Ajellomyces crescens*	Oval or globose adiaspores, 50–500 μm, commonly 300 μm in diameter, walls 10–70 μm thick	Maximum growth temperature 37°C (lower for some strains), forming adiaspores measuring 20–140 μm. Exoantigen tests may show nonspecific precipitin lines against *B. dermatitidis* and *H. capsulatum* antisera	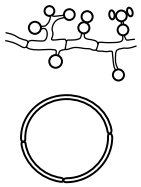
Emmonsia parva	Few animal hosts (about 21 species of rodents, lagomorphs, carnivores, and marsupials), rarely soil. Geographically restricted (parts of USA, Kenya, Europe, Australia)	Narrow hyphae forming solitary sessile conidia and 1–3 conidia at the ends of narrow, often slightly swollen stalks. Conidia subglobose, often slightly broader than long, 2.5–4 μm long and 3–5 μm wide, smooth to finely roughened	Not known; relationship to *Ajellomyces* inferred	Oval or globose adiaspores, 10–40 μm in diameter, walls thin	Maximum growth temperature 40°C, forming adiaspores measuring 8–20 μm diameter. Exoantigen tests may show nonspecific precipitin lines against *B. dermatitidis* and *H. capsulatum* antisera	Similar to *E. crescens*, but smaller adiaspores
Emmonsia pasteuriana	Known only from human host in Italy	Narrow hyphae forming solitary sessile conidia and 1–3 (sometimes up to 8) conidia at the ends of narrow stalks, often swollen distally to form a vesicle-like structure. Conidia subglobose to oval, often slightly broader than long, 2–5.5 μm long by 2–3.5 μm. Conidia subglobose, smooth to finely roughened or tuberculate	Not known; relationship to *Ajellomyces* inferred	Small and large yeast cells; small cells oval to pyriform (2–4 μm), often intracellular; occasionally larger, thick-walled (8–15 μm); all with narrow-based buds; budding mostly unipolar, sometimes bipolar	Converts to yeast form at 37°C on enriched media; no adiaspores formed	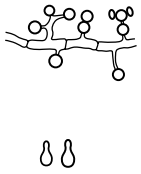
Blastomyces dermatitidis	River and forest soil, beaver dams, habitat poorly known. North America in areas along Mississippi and Ohio River valleys, eastern and central Canada, rarely Africa, Asia and Middle East	Narrow hyphae forming solitary sessile conidia and 1, uncommonly 2, conidia at the ends of narrow, usually unswollen stalks. Conidia mostly pyriform, 4–5 μm long and 3–4 μm wide	*Ajellomyces dermatitidis*	Large yeast cells, 10–12 μm in diameter, double contoured wall and single broad-based bud	Converts to yeast form at 37°C on enriched media; yeast cells large, thick-walled with broad-based buds. DNA probe and exoantigen test	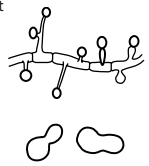
Paracoccidioides brasiliensis	Rarely isolated from soils (coffee plantation, rain forest), armadillos, habitat considered to be forest areas of moderate climate, medium to high annual rainfall, in Central and South America	Narrow hyphae often remaining sterile; occasionally forming lateral or terminal pyriform conidia or intercalary arthroconidia that are slightly swollen (2–5.5 μm long and 2–3.5 μm wide)	Not known; relationship to *Ajellomyces* inferred	Thick-walled central cell up to 30 μm in diameter, bearing 1 to many daughter cells of varying size and joined to mother cell by narrow pedicel, occasionally occurring in short chains	Converts to yeast stage at 37°C on enriched media. DNA probe and exoantigen test	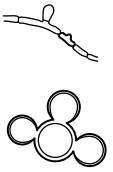
Histoplasma capsulatum var. *capsulatum*	Soil associated with dung of birds and bats. North America in areas along the Mississippi, Missouri, and Ohio River valleys of the USA, eastern Canada and Mexico, rarely in Central and South America, Asia and Europe	Narrow hyphae forming solitary sessile smooth or roughened, microconidia and tuberculate macroconidia at the ends of unswollen stalks. Microconidia pyriform, 4–5 μm long and 3–4 μm wide. Macroconidia broadly pyriform, 8–17 μm long and 8–13 μm wide or globose, 8–13 μm diameter	*Ajellomyces capsulatus*	Intracellular, rarely extracellular, small budding cells, 2–5 μm in diameter	Converts to yeast form at 37°C on enriched media; yeast cells small, oval, with narrow-based buds. DNA probe and exoantigen test	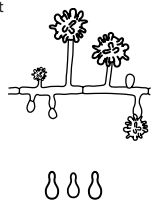
Coccidioides immitis	Soil of arid regions, animal burrows, has been found in mixed infections with *E. parva* in lungs of rodents and other burrowing animals. Geographically restricted to arid regions of southwest USA, Mexico, and parts of Central and South America	Hyphae forming alternate arthroconidia. Arthroconidia cylindrical 3–8 μm long and 3–5 μm wide	Not known; placement within the *Onygenaceae* inferred	Spherules 30–60 (–80) μm with walls 2 μm thick and containing endospores 2–5 μm in diameter	DNA probe and exoantigen test	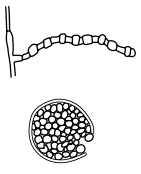

a) Modified from Sigler 1998, 2003 and based on data from Sigler 1996; figures copyright L. Sigler.

disperse), for the spherule which enlarges from the inhaled conidium (Emmons and Jellison 1960).

HISTORY

The first observation of infected animals and the suggestion of a fungal etiology are generally attributed to Kirschenblatt (Kirschenblatt 1939; Jellison 1969; Schwarz 1978; Rippon 1988) who, in 1939, applied the name *Rhinosporidium pulmonale* to the cyst-like bodies observed in lungs of several rodents. However, the name has not been used because it was not accompanied by the required Latin diagnosis or confirmed as applicable to a fungus by culture. The first valid name for the fungus dates from 1942. The earliest record of infection dates from a rodent specimen deposited in 1845 in a Swedish museum (Jellison 1969).

Infection in North America was first recorded in animals in Arizona. During the 1930s and 1940s, mycologists conducted surveys to define the natural habitats and endemic areas of the dimorphic systemic pathogens. These involved isolation of the fungi from soil, dung, or tissues of trapped birds and animals, and analysis of human exposure by measuring reactivity to antigens through skin tests. In 1942, Emmons reported that soil and desert rodents were natural reservoirs for *Coccidioides immitis* in regions of Arizona. Later that year, Emmons and Ashburn (1942) reported that some animals exhibited the presence of a second fungus forming spherule-like structures measuring up to 20 μm in diameter but lacking internal spores. Lungs of some animals contained both these cells and spherules of *C. immitis*. Thinking that their fungus had features typical of a zygomycete, Emmons and Ashburn (1942) named it *Haplosporangium parvum* and distinguished it from other species of the genus by the smaller size of the solitary sporangia. Some mycologists then considered *C. immitis* also to be a zygomycete bacause of the internal cleavage of the spherules into endospores (Emmons 1942; Baker et al. 1943). Congruences between *C. immitis* and *H. parvum*, including similarities in tissue morphologies, animals with dual infections, and skin test crossreactivity, led Emmons and Ashburn to speculate on a possible genetic relationship between them, even though *H. parvum* more closely resembled *B. dermatitidis* and *Histoplasma capsulatum* in cultural features.

Five years later, Dowding (1947) observed similar but much larger cells within lungs of rodents captured over a wide range in Alberta, Canada. The cells reached diameters of 300 μm and were often aggregated into pearly white nodules visible by gross examination. Cultures of the Alberta isolates grew poorly at 37°C, but conidia enlarged to form swollen cells as much as ten times greater in diameter and with walls up to 10 μm thick; on return to room temperature, these enlarged cells germinated to form hyphae. Dowding disagreed that *H. parvum* was most closely related to *C. immitis* because of the different type of conidia formed (i.e. arthroconidia in *C. immitis* versus solitary ones in *H. parvum*) and the lack of internal spores within spherules. Dowding also suggested that *H. parvum* was more closely related to *B. dermatitidis*, *H. capsulatum*, and *Paracoccidioides brasiliensis* and that these fungi, including *C. immitis*, formed a 'natural group.' She was the first to suggest a close biological relationship among the dimorphic pathogens. The placement of *H. parvum* in the zygomycete genus *Haplosporangium* was also challenged by Carmichael (1951). He suggested that the species was aligned most closely with *B. dermatitidis*; it formed conidia of the type that he later called 'aleuries' or aleurioconidia, and had affinities to members of the ascomycete family *Gymnoascaceae* (Ascomycota) (Carmichael 1951, 1962).

Ciferri and Montemartini (1959) made the necessary transfer of *H. parvum* to a new hyphomycete genus *Emmonsia* with *E. parva* as the type species. Emmons and Jellison (1960) described *E. crescens* for morphologically similar isolates that formed larger cells in vivo, for which they coined the term 'adiaspore.' They distinguished *E. crescens* from *E. parva* by the larger multinucleate (rather than uninucleate) adiaspores and by the lower maximum growth temperature. However, mycologists have disagreed on whether these differences were sufficient to recognize distinct species or varieties, and on the most appropriate generic name for the agents of adiaspiromycosis. For example, Carmichael (1962) re-evaluated the genus *Chrysosporium* and included in it many fungi producing aleurioconidia, including *B. dermatitidis* and the species of *Emmonsia*. While some mycologists accepted his proposed name *Chrysosporium parvum* with two varieties, *C. parvum* var. *parvum* and *C. parvum* var. *crescens* (McGinnis 1980; Boisseau-Lebreuil 1981; Rippon 1988; McGinnis and Rinaldi 1995), others argued for use of the genus name *Emmonsia* with the varieties *E. parva* var. *parva* and *E. parva* var. *crescens* (von Arx 1973; van Oorschot 1980; Kwon-Chung and Bennett 1992).

Recent evidence confirms that *Emmonsia* is the appropriate name for the agents of adiaspiromycosis, that the taxa should be considered species rather than varieties, and that there is a close relationship between *Emmonsia* spp. and *B. dermatitidis*. *Emmonsia* spp. and *B. dermatitidis* could be accommodated in the same genus but have been retained in separate genera mainly due to the differences in their parasitic forms (i.e. adiaspores versus yeast cells) and the clinical syndromes that they elicit. That distinction becomes less clear with the finding that the recently described *E. pasteuriana* demonstrates budding cells. Teleomorphs (meiotic or sexual stages) of *E. crescens* and *B. dermatitidis* are in the ascomycete genus *Ajellomyces*, as is that of *H. capsulatum*. The genus *Ajellomyces* is currently classified in the order Onygenales of the Ascomycota. Anamorphs (mitosporic or asexual stages) of onygenalean fungi are

commonly aleurioconidia or arthroconidia that are released by fracture or dissolution of all or part of the supporting hyphal cell (Carmichael 1962; Sigler and Carmichael 1976; Sigler 1997, 2003). For further discussion on relationships between *Emmonsia* spp. and the dimorphic fungi, see section on Teleomorphs and relationships below.

EPIDEMIOLOGY

Ecology, distribution, and animal infection

Even though *E. parva* was discovered first, *E. crescens* appears to be the primary agent of adiaspiromycosis in both animals and humans. However, it is difficult to verify the species involved in some literature reports due to uncertainty caused by nomenclatural controversy. The species can be verified only when size of the adiaspores observed in tissues is given. *E. crescens* has been reported worldwide from rodents, insectivores, carnivores, lagomorphs, and marsupials. Approximately 118 animal hosts were identified in detailed records compiled up to the 1970s and sporadic cases of animal infection have been reported subsequently (Dvorák 1968; Jellison 1969; Doby et al. 1970; Dvorák et al. 1973). *E. parva* is geographically more restricted and associated with fewer animal hosts. It has been recorded from about 21 species of rodents, lagomorphs, carnivores, and marsupials in the USA, Kenya, (former) Czechoslovakia, the former Soviet Union, and Australia (Jellison 1969; Doby et al. 1970; Krivanec and Mason 1980; Mason and Gauhwin 1982). Factors affecting geographical distribution are largely unknown. In North America, *E. crescens*-infected animals have been recorded from 13 states and four Canadian provinces, whereas *E. parva*-infected animals have been found in Arizona, Texas, Montana, and Utah. The original Arizona survey revealed a high prevalence of *E. parva* infection (33 percent of 303 animals) (Emmons and Ashburn 1942), but a survey of almost 1 800 animals collected over several years in (former) Czechoslovakia yielded 260 infected animals, of which only four showed infection with *E. parva* (Krivanec 1977). *E. crescens* has been found in New Zealand in a marsupial introduced from Australia (Johnstone et al. 1993), but it appears to be absent in neighboring Australia (Connole 1990). There, *E. parva*-like adiaspores have been observed in wombats, but culture yielded atypical nonsporulating isolates (Krivanec and Mason 1980; Mason and Gauhwin 1982). There is some basis to suggest that species could be introduced through transport of infected animals (Peterson and Sigler 1998).

Similarly to *B. dermatitidis*, the habitat for *Emmonsia* spp. is not well known, but animals are presumed to acquire infection by inhalation exposure to conidia in soil of wooded areas. Long-term studies in the Czech Republic have revealed that the highest prevalence of infection (33 percent) occurs in rodents from wooded windbreaks separating cultivated fields, compared with rodents from the fields themselves or from small plots of deciduous woods (Hubálek et al. 1995). Infection is absent in rodents that do not build their nests in soil (Hubálek 1999). Carnivores may play a role in distributing the organism by liberating adiaspores trapped in the tissues of their prey (Krivanec et al. 1975b; Krivanec and Otcenášek 1977). Viable adiaspores of *E. crescens* survive passage through the digestive tracts of mice, birds, and carnivores (Krivanec et al. 1975a; Krivanec and Otcenášek 1977; Hejtmanek 1985), as evidenced by the isolation of the fungus from the dung of coyotes for which rodents are a major dietary constituent (Sigler and Flis 1998). There are only a few records of direct isolation from soil and inanimate materials including moldy straw and bird's nest, all from Alberta, Canada (Sigler and Flis 1998; Peterson and Sigler 1998). Indirect isolation from soil occurred on at least one occasion when an *E. parva*-like isolate was recovered following experimental inoculation of soil into mice during a search for *H. capsulatum* in Missouri (Menges and Habermann 1954; Jellison 1969). However, molecular analysis revealed that the Missouri isolate is genetically distinct (Peterson and Sigler 1998).

In addition to habitat, factors affecting prevalence of infection are species and age of animal, season, geographical area, and altitude (Doby et al. 1971; Hubálek et al. 1995, 1998; Hubálek 1999). *E. crescens* infection is higher in mustelid carnivores, ranging from 30 to 76 percent of animals, than in small rodents and insectivores (1–50 percent) and large carnivores (<1 percent) (Doby et al. 1971; Krivanec et al. 1975a, b; Krivanec and Otcenášek 1977); however, the numbers of carnivores surveyed have been small. An 11-year survey of 10 081 small mammals from the Czech Republic found adiaspiromycosis in 13 percent of animals and a higher prevalence in rodents (13 percent of juveniles; 20 percent of adults) than in insectivores (2 percent of juveniles; 4 percent of adults) (Hubálek 1999). Infection is higher in adult rodents than in juveniles, but there is no difference in infection rates between adults according to sex (Hubálek et al. 1995). Dvorák et al. (1967, 1969) found seasonal variation with the highest incidence in spring. Hubálek et al. (1993) found the highest incidence in spring for voles and in winter for mice, reflecting differences in the animals' digging and burrowing activities. They also suggested that the peak of fungal proliferation may occur in spring when mean soil temperature, 5 cm below the surface, ranges from 3.4 to 12.2°C.

There have been few reports of illness or death of animals naturally infected with *Emmonsia* spp., but severely emaciated animals have been found postmortem to be heavily infected (Jellison 1969; Simpson and Gavie-Widen 2000). Hubálek et al. (1995)

determined the mean intensity of infection of *E. crescens* as 177.2 adiaspores per juvenile and 212.5 per adult rodent, with the number of adiaspores ranging from one to 4 618, and adiaspore size varying between 46 and 451 μm (mean 239.4 μm in adults). Lung tissues of some of these animals showed adiaspores of two distinct sizes, suggesting that they had been reinfected. Rare reports concern domestic or farm animals including two dogs (Al-Doory et al. 1971; Koller et al. 1976), a goat (Koller and Helfer 1978), and a horse (Pusterla et al. 2002). Findings were incidental in the goat and one dog, but the horse and a second dog demonstrated advanced pulmonary infection. In the case of the dog, which also had degenerative arthritis, organisms were found only in the lung and lymph nodes at necropsy (Al-Doory et al. 1971). The isolated fungus was inoculated into mice intraperitoneally, resulting in pulmonary lesions and recovery of the same fungus in culture. These findings are more suggestive of blastomycosis, known to be common in dogs, but the cells in tissue were described as irregular in size and nonbudding.

Experimental infection has been induced in dogs, guinea pigs, mice, rabbits, rats, and sheep, but not birds, by inoculating conidial suspensions, mainly of *E. crescens*, via intranasal, intraperitoneal, subcutaneous, or intravenous routes (Jellison 1969). Intravenous inoculation induces adiaspore development in all soft tissues, especially in the liver, lungs, and spleen. Golden hamsters were found to be less susceptible than other animals to developing adiaspiromycosis (Boisseau-Lebreuil 1975), and infectivity as well as persistence of adiaspores was greater after subcutaneous inoculation with *E. crescens* isolates than with *E. parva* isolates (Carmichael 1951). Time since injection, the strain used, the resistance of the animal, and the inoculation route are factors generally affecting the development of the adiaspores (Carmichael 1951; Dvorák et al. 1973; Slais 1976; Boisseau-Lebreuil 1977). Enlargement of conidia to adiaspores reaching 50–100 μm occurs within 2 weeks of intraperitoneal inoculation with *E. crescens* (Dvorák et al. 1973; Krivanec et al. 1974; Hejtmanek 1985). Experimental infection was obtained for *E. pasteuriana* only in golden hamsters following intratesticular inoculation; other routes of inoculation in the hamster, mice, and guinea pigs were unable to induce infection (Drouhet et al. 1998; Drouhet and Huerre 1999).

Human adiaspiromycosis

The number of recorded human infections is low, with approximately 50 cases of pulmonary or disseminated adiaspiromycosis reported from Central and South America, Europe, USA, and North Africa (Kwon-Chung and Bennett 1992; England and Hochholzer 1993; Sigler 1998). As with animals, infection occurs following inhalation exposure to conidia. Eleven patients whose case histories were reviewed by England and Hochholzer (1993) were adult white males from Argentina, Brazil, Germany, Guatemala, Spain, and the USA. Four of these individuals may have acquired the infection following inhalation of dust through farming, woodworking, or bird-handling activities. Eighteen cases of infection have occurred in Brazil in adult males between the ages of 26 and 62 (Barbas Filho et al. 1990; de Almeida Barbosa et al. 1997; Martins et al. 1997; dos Santos et al. 1997; Moraes et al. 2001). Three of these patients developed acute pulmonary infection following cleaning or renovation of buildings or the handling of rodent and bat carcasses or nesting material (Barbas Filho et al. 1990; de Almeida Barbosa et al. 1997). Soil dust from a garden shop was thought to have been the source of exposure for a 2-year-old Finnish girl (Nuorva et al. 1997). Of 14 case histories reviewed by Schwarz (1978) from the Czech Republic, France, Guatemala, Honduras, Russia, and Venezuela, four involved women and four involved boys aged 10 and 11 years. Most cases of adiaspiromycosis occur in non-AIDS patients. Underlying conditions have included tuberculosis, yellow fever, cancer of the bladder, diffuse lymphoma, chronic bronchitis, steroid-dependent emphysema, atherosclerosis, aortic atheroslcerotic aneurysm, and mycocardial infarction (Kodousek 1974; Schwarz 1978; Bambirra and Nogueira 1983; England and Hochholzer 1993; Moraes et al. 2001).

Pulmonary and disseminated infection have been reported in a few patients with AIDS (Echavarria et al. 1993; Gori et al. 1998; Turner et al. 1999). The histopathological presentations in these patients are dissimilar to classical adiaspiromycosis, suggesting that different *Emmonsia* spp. may be involved (Drouhet et al. 1998; Gori et al. 1998). The unusual features of these cases are discussed in the section on Infections in AIDS patients below.

HOST RESPONSES

Clinical manifestations of adiaspiromycosis

Adiaspiromycosis in humans generally involves the lungs and is often self-limited, benign, and asymptomatic. Functional disability and onset of symptoms is dependent upon the number of conidia inhaled (Barbas Filho et al. 1990). A heavy or repeated exposure may result in acute pulmonary disease with bilateral granulomatous lesions (de Almeida Barbosa et al. 1997). Patients are either asymptomatic or present with nonproductive cough, dyspnea on exertion, and low-grade fever; less common findings are hemoptysis, pain, chills, malaise, weight loss, night sweats, and crackles in the lungs (Barbas Filho et al. 1990; England and Hochholzer 1993;

de Almeida Barbosa et al. 1997). Almost all cases of human pulmonary adiaspiromycosis involve *E. crescens* (sometimes reported as *E. parva* var. *crescens*) (Schwarz 1978; Kwon-Chung and Bennett 1992; England and Hochholzer 1993; de Almeida Barbosa et al. 1997).

Diagnosis of infection is based most commonly on radiological (Figure 1) and histopathological findings without confirmation by culture. Diagnosis may be complicated by symptoms that mimic those of other fungal diseases, tuberculosis, sarcoidosis, Hodgkin's disease, or farmer's lung, or the co-occurrence with mycobacterial or other infections (England and Hochholzer 1993; de Almeida Barbosa et al. 1997). This may lead to failure to perform fungal culture, but even if adiaspiromycosis is suspected, fungal culture of sputum, bronchial washings, or bronchoalveolar lavage is often unsuccessful. Adiaspores may be discovered incidentally. In the first case of human infection, a single adiaspore was found in a nodule from a patient who had aspergillosis and cystic disease of the lung (Doby-Dubois et al. 1964). If numerous, adiaspores may be present in multiple granulomas involving both lungs (Schwarz 1978; England and Hochholzer 1993). Three forms of infection have been recognized (Kodousek et al. 1971; Vojtek et al. 1972; Schwarz 1978; England and Hochholzer 1993; de Almeida Barbosa 1997). These include:

1. a solitary or localized granuloma
2. a sporadically disseminated form
3. an acute, densely disseminated type with clinical symptoms.

The most prevalent findings in chest x-rays or computed tomography scans are diffuse bilateral reticulonodular infiltrates with areas of honeycombing (Barbas Filho et al. 1990; Peres et al. 1992; England and Hochholzer 1993). Most patients recover spontaneously or with antifungal therapy and/or prednisone, but in a few cases, the infection progresses to a fatal outcome (Peres et al. 1992; England and Hochholzer 1993; de Almeida Barbosa et al. 1997). Extrapulmonary infection is uncommon. Adiaspores of *E. crescens* were observed in two sections taken from an appendix of an 11-year-old boy with bronchitis (Kodousek 1974).

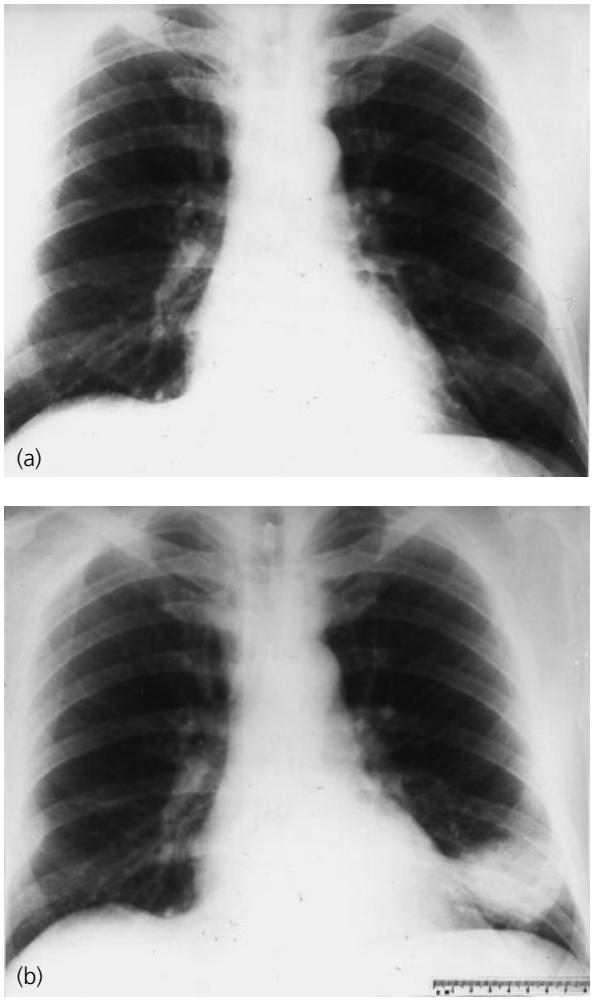

Figure 1 *Progressive radiological findings in a 43-year-old man with localized adiaspiromycosis.* **(a)** *Minimal infiltrate in the lower lobe of the left lung;* **(b)** *mass-like lesion 4 months later. (From the Armed Forces Institute of Pathology Photograph, Neg. Nos 76-924-1 and 76-924-3, respectively. Reproduced with the permission of Dr D.M. England from England and Hochholzer 1993. Work of the US Government. Not subject to copyright in the USA)*

Infections in AIDS patients

Three patients with AIDS have been described recently as having infection caused by *Emmonsia* spp. In all cases, the fungus was grown in culture. This is in contrast to the failure to culture the fungus from non-AIDS patients with pulmonary adiaspiromycosis. Unusual findings in the case described by Echavarria et al. (1993) were dissemination involving bone, bone marrow, and lungs and extensive osteomyelitis. The fungus was identified as *E. parva* based on the smaller adiaspores, measuring between 25 and 35 μm in diameter. Although grown from pus, sputum, and bone marrow, the fungus was later lost to further investigation as to its relationship with other *Emmonsia* spp. Turner et al. (1999) described pulmonary adiaspiromycosis in a male with advanced HIV infection who was negative for both *Pneumocystis carinii* and mycobacterial infection. The fungus was grown from a bronchoalveolar lavage and a protected brush specimen and identified as *E. parva* by formation of adiaspores at 37°C. Fungal elements were reported to be present in the specimens, but their nature was not described nor were they illustrated in the report. The third case involved a female who developed disseminated cutaneous mycosis; histopathology revealed small yeast-like cells rather than adiaspores (Drouhet et al. 1998; Gori et al. 1998)

(Table 1). The fungus in culture demonstrated morphological features compatible with *Emmonsia*, but the isolate was found to be genetically distinct and described as a new species, *E. pasteuriana* (Drouhet et al. 1998).

Unconfirmed reports concerning *Emmonsia* species

Some reports which have been attributed to *Emmonsia* spp. are doubtful. In cases of skin (Thammayya and Sanyal 1973; Kamalam and Thambiah 1979) and corneal infection (Wagoner et al. 1999), the fungal elements in tissue are incompatible with those of *Emmonsia* spp. In two reports of deep infections (Stillwell et al. 1984; Warwick et al. 1991), one of endocarditis (Toshniwal et al. 1986), and one of sinusitis (Levy et al. 1991), the isolated organism was identified in each report only as *Chrysosporium* species; however, the discussions in each report implicate *E. parva* (as *Chrysosporium parvum*) and the reports have been cited by subsequent authors under that etiology. None of these cases can be confirmed as adiaspiromycosis. The fungus causing sinusitis described by Levy et al. (1991) is suspected to be the basidiomycete *Schizophyllum commune* (Sigler and Verweij 2003).

Histopathology

In histopathological examination of biopsy specimens, the adiaspores of *E. crescens* are generally found within granulomas, with each granuloma containing one or occasionally two adiaspores (Figure 2). In a severe infection, the pleural surface may appear grossly as grayish-white and thickened and marked by numerous discrete white nodules measuring up to 1 mm in size, with the intervening pulmonary tissue unchanged (Watts et al. 1975). Adiaspores appear as round or oval cells, ranging from 50 to 500 μm in diameter (commonly <300 μm), either lacking internal contents or appearing granular, and having walls between 10 and 70 μm thick (Kodousek 1974; Chandler et al. 1980; Hejtmanek 1985; England and Hochholzer 1993). The wall appears either trilaminar, staining positive with Gomori's methenamine silver, Gridley, and periodic acid–Schiff stains, or bilaminar when stained with hematoxylin and eosin (Watts et al. 1975). Adiaspores may be surrounded by a few macrophages and lymphocytes or by a granulomatous inflammatory reaction composed of epithelioid cells, macrophages, lymphocytes, plasma cells, scattered neutrophils, and fibrous tissue; eosinophils are uncommon (Albassam et al. 1986; England and Hochholzer 1993). Multinucleated giant cells may be seen in some granulomas. England and Hochholzer (1993) noted that patients with more acute infections of short duration (<2 months) presented with necrotic and suppurative granulomas, having adiaspores that averaged 100 μm and showed a paucity of T cells, B cells, and KP-1 macrophages on immunohistochemistry, whereas patients with infections of longer duration had non-necrotizing granulomas containing larger adiaspores (average diameter 155 μm) and demonstrated good immunoreactivity for T cells and KP-1-positive macrophages. Adiaspores in stages of collapse or being broken down by inflammatory cells can sometimes be observed (Albassam et al. 1986; England and Hochholzer 1993). The in vivo structures of other fungi, including the spherules of *C. immitis* or nonbudding yeast cells of *B. dermatitidis* (Table 1) and even *Curvularia* species in allergic bronchopulmonary disease, as well as parasites and plant cells, have been suggested as possible confounding factors in the differential diagnosis of adiaspiromycosis (England and Hochholzer 1993). Spherules of *C. immitis* are smaller on average (30–60 μm), have walls about 2 μm thick, and contain endospores 2–5 μm in diameter.

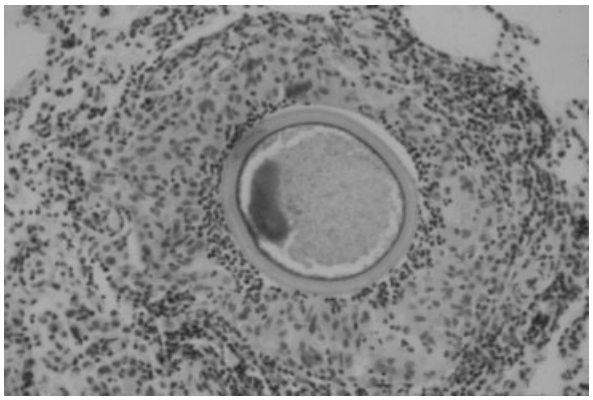

Figure 2 *Histological section of lung of striped skunk showing inflammatory reaction around adiaspore of* E. crescens. *Hematoxylin and eosin; note thick fungal cell wall. (Reproduced, with permission, from Albassam et al. 1986)*

In naturally infected animals, adiaspores occur mostly in lungs within solitary or multiple non-necrotic, sometimes coalescing, granulomas; rarely, adiaspores are extrapulmonary and involve the lymph nodes (Albassam et al. 1986). In severely infected animals, pearl-white or grayish-white nodules measuring up to 1 mm in diameter can be seen on gross examination (Figure 3) (Dowding 1947; Albassam et al. 1986). As with human infections, adiaspores may be found incidentally when only a few are present and the animal is asymptomatic. Large numbers of adiaspores have been found in lungs of trapped rodents with higher numbers in adults compared with juveniles (mean 212.5 versus 177.2) (Hubálek et al. 1995). In the first case of disseminated pulmonary disease in a horse, biopsy examination revealed multiple granulomas composed of an outer layer of epitheloid macrophages and occasional giant cells, and an inner neutrophilic focus surrounding spherical adiaspores of *E. crescens*, 30–80 μm in diameter; at

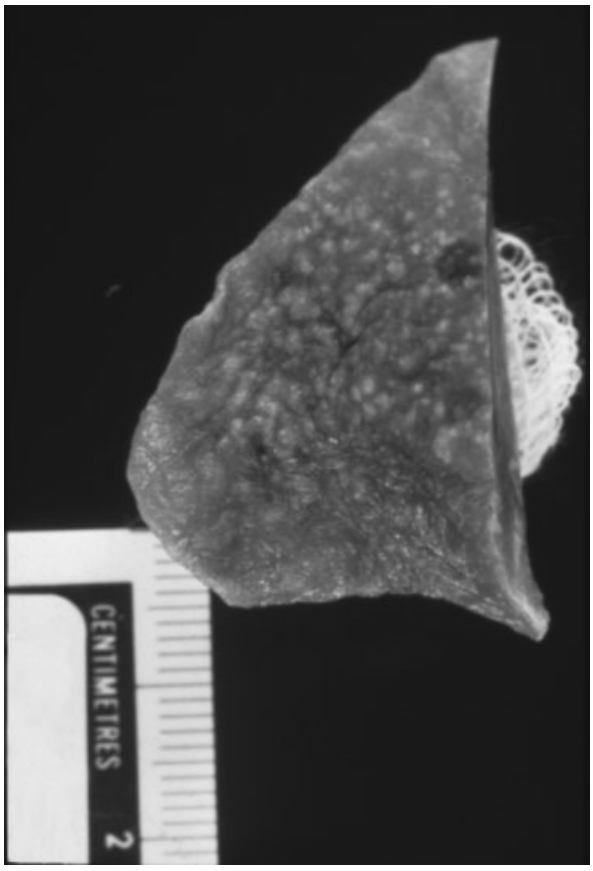

Figure 3 *Formalin-fixed lung of mature striped skunk (*Mephitis mephitis*) showing multiple nodules caused by* Emmonsia crescens. *(Reproduced, with permission, from Albassam et al. 1986)*

postmortem, the lung parenchyma showed diffuse infiltration with 1–2 mm diameter miliary, white nodules (Pusterla et al. 2002). Different inflammatory responses and the absence of reaction around some adiaspores have been explained as arising from repeat exposure, the time involved in maturation of adiaspores or varying antigenicity of the adiaspore wall (Albassam et al. 1986). In animals experimentally infected by intraperitoneal inoculation (Figure 4), between one and several adiaspores develop within granulomas in the liver, spleen, kidney, peritoneum, or other organs (Hejtmanek 1985).

Cytochemistry and structure of adiaspores and hyphae

The adiaspores of *E. crescens* have a complex wall structure, especially when immature (England and Hochholzer 1993) (Figure 5). Cytochemistry has revealed three layers, of which the outer is thinnest and contains the proteins, arginine, tyrosine, and tryptophan, and traces of hydrophilic lipids. The inner wall is thickest and consists of neutral mucosubstances (Hejtmanek 1985). Using transmission electron microscopy, the three layers are almost amorphous or appear as fibrils of different electron densities (Watts et al. 1975; Hejtmanek 1985; Albassam et al. 1986). The innermost layer consists of finer fibrils than the middle layer, while the outer layer appears as irregular heterogeneous fibrils of higher electron density (Watts et al. 1975; Albassam et al. 1986). The middle zone has been described as a fenestrated layer composed of discontinuous dense fibrillar lamellae (Watts et al. 1975). Neutral and acid mucins have been located in the inner and middle layers by colloidal iron and mucicarmine stains (England and Hochholzer 1993). Adiaspores developing on agar demonstrate the hydrolytic enzymes, alkaline and acid phosphatases, nonspecific esterase, aminopeptidase, and respiratory enzymatic activity, including glucose-6-phosphate dehydrogenase, diaphorase, and peroxidase (Kodousek and Hejtmanek 1971; Hejtmanek 1985). Similar enzymatic activity has been reported for hyphae that have cell walls containing chitin, septa with simple pores and Woronin bodies, mitochondria, glycogen granules, vacuoles, lipid bodies, granular endoplasmic reticulum, and numerous nuclei in the hyphal tips (Hejtmanek 1985).

There has been disagreement about the internal structure of adiaspores and about their propensity to divide internally or to replicate by budding. Some authors have indicated that the adiaspores of *E. parva* are uninucleate whereas those of *E. crescens* are multinucleate, and that this factor is associated with unipolar or multipolar budding (Emmons and Jellison 1960; Dvorák et al. 1973; Hejtmanek 1985). Others have found adiaspores to be commonly empty (England and Hochholzer 1993) or to contain lipid vacuoles, mitochondria, and ribosomes with (Hejtmanek et al. 1974) or without (Albassam et al. 1986) nuclei. A form of 'budding' could be induced in the adiaspores of *E. crescens* by changing in vitro growth conditions of immature adiaspores (Emmons 1964), but most investigators now agree that, in the host, adiaspores demonstrate neither budding nor internal replication.

IMMUNODIAGNOSIS

There is no reliable serological test for the diagnosis of adiaspiromycosis. Environmental exposure to *Emmonsia* spp. was first detected by positive skin-test reactions to antigens prepared from culture extracts in a group of school children, some of whom also demonstrated reactivity to coccidioidin (Emmons and Ashburn 1942). Delayed-type hypersensitivity reactions to antigens of *E. crescens* and precipitating antibodies in sera can be demonstrated in humans with infections (Tomšíková et al. 1970, 1974; Simecek et al. 1982). Antigenic activity has been found to vary among strains and to be greater in the parasitic stage (Tomšíková et al. 1972, 1976, 1978). Both strain- and species-specific antigens were

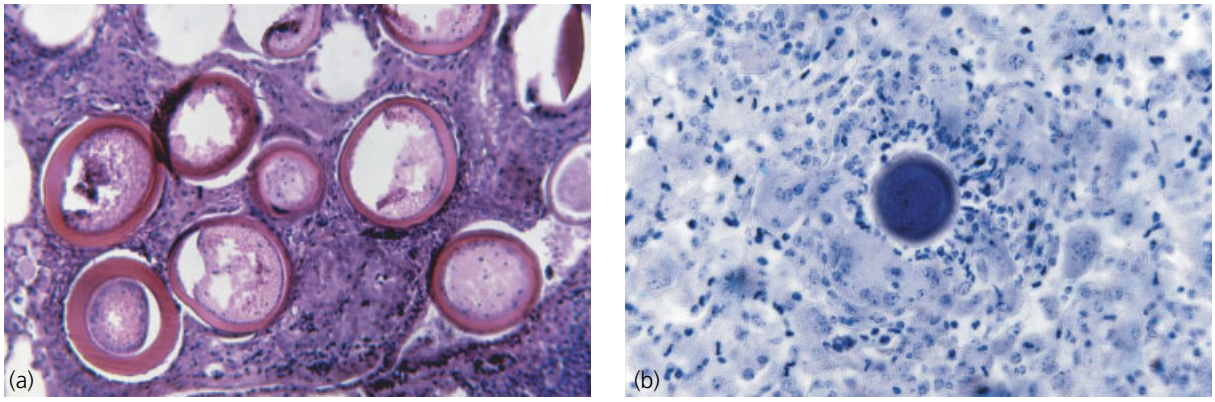

Figure 4 *Histological sections of mouse lung after experimental inoculation showing* **(a)** *numerous thick-walled adiaspores of* E. crescens *and* **(b)** *solitary thin-walled adiaspore of* E. parva.

detected by immunodiffusion tests, using antisera raised against partially purified cytoplasmic antigen (Cano and Taylor 1974). A passive cutaneous anaphylaxis test in young guinea pigs detected two possible cases of human infection among donors of 83 sera collected from trappers and mammologists who handled a variety of animals in their work (Jellison et al. 1962). Immunization with specific antiserum achieved some inhibition of adiaspore development in experimentally infected animals (Tomšíková et al. 1982).

Immunological crossreactivity has been demonstrated between *Emmonsia* spp. and *B. dermatitidis* and *H. capsulatum* (Tomšíková et al. 1970; Al-Doory et al. 1971; Sekhon et al. 1986), but not with *C. immitis* (Pusterla et al. 2002). Nonspecific precipitin lines are produced in exoantigen tests between *E. parva* antisera and the A antigen of *B. dermatitidis* or the H and M antigens of *H. capsulatum*, but the exoantigen is considered reliable in differentiating among the species (Sekhon et al. 1986; Kwon-Chung and Bennett 1992; Padhye et al. 1994). A commercially available DNA probe for *B. dermatitidis* was found to show crossreactivity with *P. brasiliensis* but not with *E. parva* (Padhye et al. 1994). In exoantigen tests, no crossreactivity was found between *E. pasteuriana* and *B. dermatitidis*, *H. capsulatum*, or *C. immitis* (Gori et al. 1998).

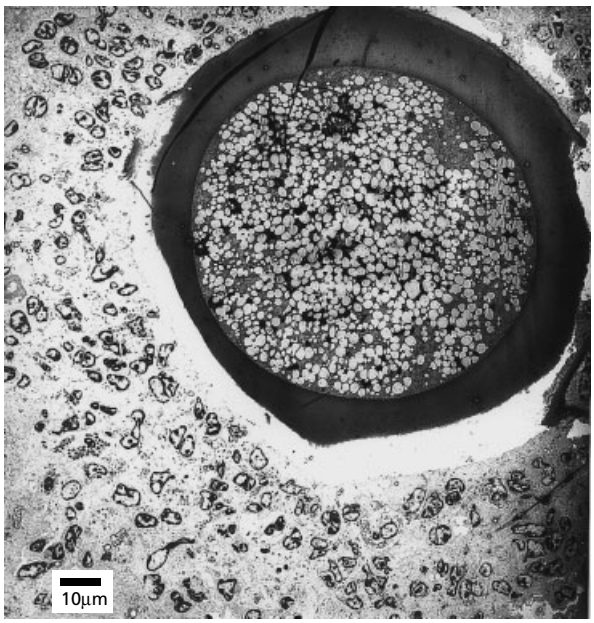

Figure 5 *Electron micrograph of an adiaspore in lung of striped skunk showing thick outer wall, large central lipid vacuoles, glycogen clumps, and outer mitochondria. Uranyl acetate and lead citrate; bar, 10 µm. (Reproduced, with permission, from Albassam et al. 1986)*

LABORATORY DIAGNOSIS

Direct examination and isolation

Diagnosis of adiaspiromycosis is generally made by observation of characteristic adiaspores by histopathology. Based on the larger size of the adiaspores, most reports concern *E. crescens*. The fungus is rarely cultured from specimens in cases of human infection. The differential clinical diagnosis is complicated by mimicking of other syndromes (including other fungal infections, tuberculosis, sarcoidosis, idiopathic granulomatous disease, etc.) and this may lead to failure to perform fungal culture. However, even if adiaspiromycosis is suspected, fungal culture of sputum, bronchial washings, or bronchoalveolar lavage is often negative (Barbas Filho et al. 1990; England and Hochholzer 1993).

Most *Emmonsia* isolates have been recovered from dissected animal lung tissue. In the case of a horse with disseminated pulmonary adiaspiromycosis, culture was negative from lung tissue and from transtracheal

aspirate, but etiology of the fungus as *E. crescens* was confirmed by the adiaspores measuring 30–80 μm and by polymerase chain reaction (PCR) assay of the tissue (Pusterla et al. 2002). The sequence of the PCR-amplified DNA fragment from the lung was found to be identical to an LSU-rDNA sequence on deposit in GenBank for an *E. crescens* isolate (accession number AF038335, Peterson and Sigler 1998). *Emmonsia*-like isolates are occasionally cultured from human respiratory or cutaneous specimens, but the significance of these isolates is difficult to evaluate when information on results of direct examination is lacking (Peterson and Sigler 1998). The fungus is occasionally isolated from other substrates including soil, dung, or straw.

By contrast, culture is usually successful from specimens of patients with AIDS. In the osteomyelitic infection attributed to *E. parva*, direct examination of pus showed oval to elongated thick-walled cells lacking internal contents; the fungus was isolated from pus, sputum, and bone marrow (Echavarria et al. 1993). The fungus was grown from a respiratory specimen of a male patient with pulmonary infection and advanced AIDS (Turner et al. 1999). *E. pasteuriana* was isolated with some difficulty from a cutaneous lesion in the Italian patient with AIDS (Gori et al. 1998). Although disseminated cutaneous lesions were present, these were all culture negative except for the last biopsy taken just before death.

Mycology

The colonial and microscopic features of *Emmonsia* spp. resemble those of their closest relative, *B. dermatitidis*. *Emmonsia* spp. differ in their propensity to form one to three conidia at the ends of narrow, often slightly swollen stalks, in the conidia being flattened and as broad as they are long, and in their growth responses at higher temperature (Table 1). Conidia are aleurioconidia, i.e. solitary conidia released by lytic disintegration of part of the supporting hyphal cell (Carmichael 1962; Sigler, 1996, 1997, 2003). The colonial features of *Emmonsia* spp. are shown in Figures 6 and 38.7 and compared with a typical colony of *B. dermatitidis* shown in Figure 8. Microscopically, *Emmonsia* spp. appear similar to each other (Figure 9) (Table 1). Conidia are single celled and formed on the sides of the hyphae (sessile) or at the ends of a short narrow stalks which are either nonswollen or slightly swollen at the end closest to the conidium. Commonly, two or three conidia are arranged at the tips of spine-like pegs arising by secondary proliferation from the swollen cell. Conidia appear slightly flattened and slightly broader than long (subglobose or oval) or are pyriform and have narrow basal scars. Conidia of *E. crescens* and *E. parva* have smooth walls

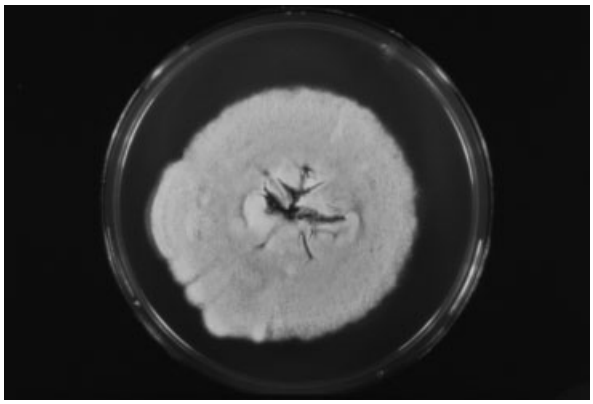

Figure 6 *Velvety, buff colored colony of* E. crescens *on phytone yeast extract agar after 28 days at 25°C (UAMH 7365). Note that colonies demonstrate variation in color and texture.*

and measure 2.5–4 μm long and 3–5 μm wide, but they may swell in age and become finely roughened. Up to eight conidia are formed in *E. pasteuriana* and they measure 2–5.5 μm long by 2–3.5 μm wide; conidia are smooth to finely roughened or appear tuberculate by scanning electron microscopy.

Emmonsia spp. grow well on routine mycological media including Sabouraud's glucose agar without cycloheximide, but conidiation is enhanced on media such as potato dextrose agar (PDA). *Emmonsia* spp. are slightly to moderately inhibited on media containing cycloheximide at a concentration of 400 μg/ml; *E. crescens* is less tolerant than *E. parva* (Sigler 1996). Isolates of both species vary in growth rates, morphology, and color (Otcenášek and Zlatanov 1975; Sigler 1996). On PDA, colonies reach diameters of 4.5–8 cm after 4 weeks at 25°C and may be glabrous (smooth or bald), woolly, velvety, or coarsely powdery. Colonies are flat, raised in the center, or radiately folded and the color varies from white to yellowish-white, buff, or tan (Sigler 1996). Two main colony

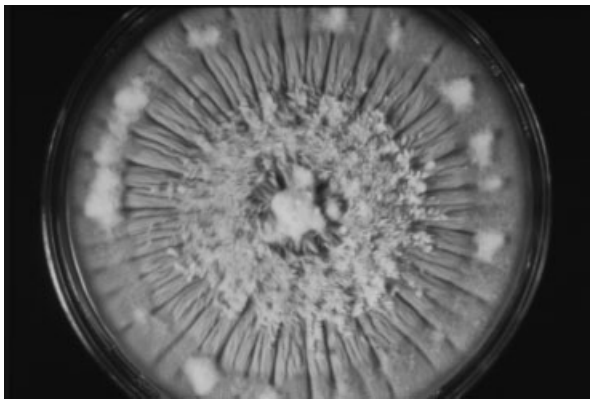

Figure 7 *Glabrous to hairy yellowish-white colony of* Emmonsia parva *on phytone yeast extract agar after 28 days at 25°C (UAMH 134). Note that colonies demonstrate variation in color and texture.*

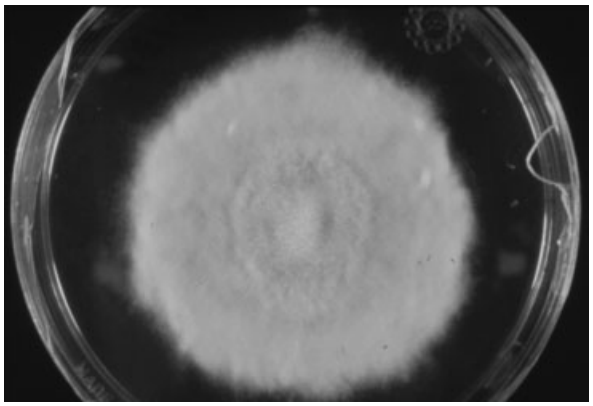

Figure 8 *Buff colored colony of* B. dermatitidis *on phytone yeast extract agar after 28 days at 25°C (UAMH 5438). (Compare with Figure 6)*

types may be observed, but intermediate forms are common (colony colors are described according to the color charts of Kornerup and Wanscher 1978). One type is yellowish-white to orange-white (4A2/5B3), with a densely woolly center dotted with exudate droplets, and having a broad glabrous margin. The reverse of the colony is grayish-brown (6D3). A second type is pale orange to grayish-orange (5A3/B5), with a coarsely powdery center and an irregular margin. Exudate droplets are present or absent and the reverse is reddish-brown (8E4). Colonies of *E. pasteuriana* are similar in being yellowish-white (4A2), densely woolly, furrowed, or zonate with powdery or glabrous sectors.

Emmonsia spp. are distinguished by their growth responses at higher temperature. In vitro, isolates of *E. crescens* are almost completely inhibited at 37°C and some strains show restricted growth even at 30°C (Boisseau-Lebreuil 1981; Sigler 1996). At 35–37°C, conidia in the residual inoculum develop into globose swollen cells or adiaspores which measure 20–140 μm in diameter (average 59 μm) (Figure 10). These cells are thinner walled than those formed in vivo and have sometimes been given other names (e.g. chlamydospores or trophocytes) (Carmichael 1962; Dvorák et al. 1973). Maturing adiaspores germinate from many sites if transferred to culture media at lower temperatures (25°C) (Emmons 1964; Boisseau-Lebreuil 1970; Hejtmanek 1976). At 37°C, isolates of *E. parva* form colonies ranging from 9 to 77 mm in diameter after 21 days but are almost completely inhibited at 40°C (Sigler 1996). Adiaspores formed at this temperature are smaller than those of *E. crescens* and range from 8 to 20 μm in diameter (mean 12 μm). Complete conversion of hyphae to adiaspores is rarely achieved. When grown on enriched media such as brain heart infusion agar at 37°C, *E. pasteuriana* converts to a yeast phase consisting of oval to lemon-shaped, small (2–4 μm) yeast-like cells having narrow-based buds (Table 1) but conversion is incomplete and hyphal forms are present.

Teleomorphs and relationships

Recently, classical mating experiments and DNA sequence comparisons have provided evidence that *Emmonsia* spp. are anamorphs (mitotic, asexual stages) of *Ajellomyces* spp. and that their closest relative is *B. dermatitidis*. Species of *Ajellomyces* are heterothallic and teleomorphs (meiotic, sexual stages) form when compatible isolates are mated. Ascocarps are composed of branched, anastomosing, pale-brown hyphae, in which individual cells are somewhat diamond shaped, and terminate in coiled appendages. Ascospores are small (<1–1.5 μm diameter) and covered with minute spiny projections. The teleomorph of *B. dermatitidis* is *Ajellomyces dermatitidis* (McDonough and Lewis 1967, 1968); that of *H. capsulatum* was described first as *Emmonsiella capsulata* but later renamed as *Ajellomyces capsu-*

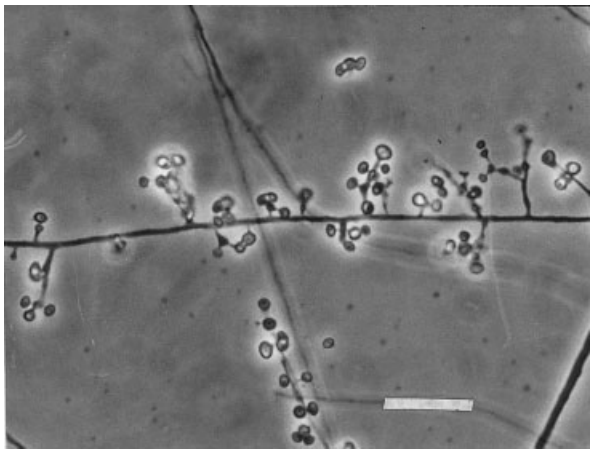

Figure 9 *Microscopic morphology of* E. crescens *(UAMH 7365). In* Emmonsia species*, one to three conidia are borne at the ends of stalks which are commonly slightly swollen. Bar, 20 μm. (Reproduced, with permission, from Sigler 1996)*

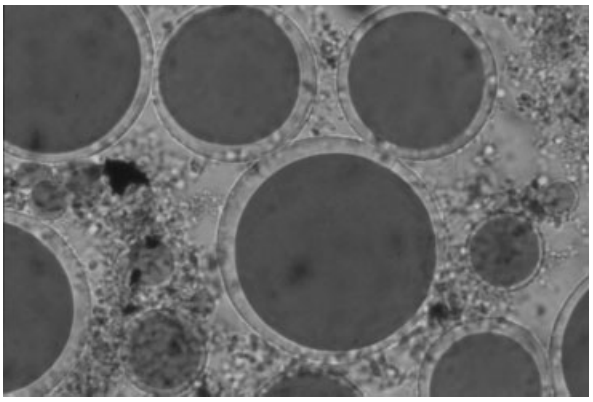

Figure 10 *Adiaspores of* Emmonsia crescens *formed in vitro on phytone yeast extract agar at 37°C (UAMH 7365) and stained with acid fuchsin in lactic acid.*

latus (Kwon-Chung 1972; McGinnis and Katz 1979). Matings among 12 strains of *E. crescens* resulted in a teleomorph described as *Ajellomyces crescens* (Sigler 1996) (Figures 11–14). However, no fertility was seen among mated isolates of *E. parva* or when isolates were paired with those of *E. crescens*.

Phylogenetic trees derived from comparison of DNA sequences obtained from the ribosomal rRNA gene confirm the relationship among these species. *Emmonsia* spp. and *B. dermatitidis*, together with *H. capsulatum*, *Paracoccidioides brasiliensis*, and *Lacazia loboi* (not known in culture), are placed as members of the monophyletic *Ajellomyces* clade (Bowman and Taylor 1993; Leclerc et al. 1994; Drouhet et al. 1998; Peterson and Sigler 1998; Herr et al. 2001; Untereiner et al. 2002; Sigler 2003). Although *Ajellomyces* was classified in the family *Onygenaceae,* order Onygenales (Currah 1985), phylogenetic analyses of DNA sequence data have shown the *Ajellomyces* clade to be a lineage distinct from the *Onygenaceae* (Sugiyama and Mikawa 2001; Untereiner et al., 2002). The new family *Ajellomycetaceae* has been proposed for this lineage (Untereiner et al. 2004). Although the *Ajellomyces* clade is well supported, the relationships within it are not clearly resolved. *P. brasiliensis* is in the clade, but its position is not well defined. *C. immitis* is placed within the *Onygenaceae* and apart from *Ajellomyces* species; its closest relative is a nonpathogenic species *Uncinocarpus reesii* (*Malbranchea* anamorph) (Sigler and Carmichael 1976; Bowman and Taylor 1993; Pan et al. 1994; Sugiyama and Mikawa 2001).

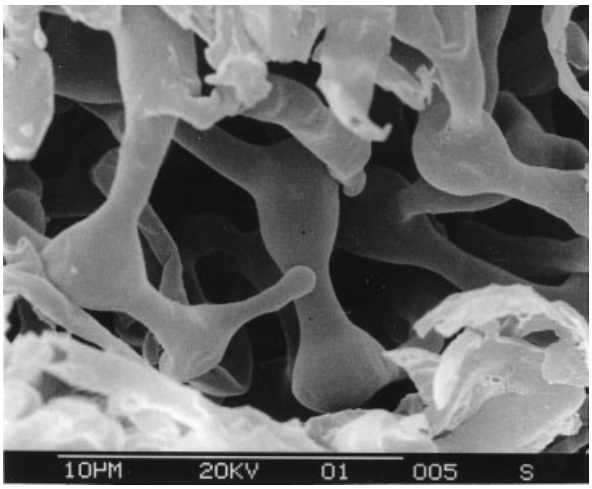

Figure 12 *Scanning electron micrograph showing the swollen, diamond-shaped cells of the hyphae composing the ascocarp (UAMH 128 × 129). (Reproduced, with permission, from Sigler 1996)*

Phylogenetic analysis of *Emmonsia* isolates based on LSU-ITS rDNA sequences placed *E. crescens* isolates into two groups according to their geographical origin and found greater than expected variation among isolates identified as *E. parva*, leading to the suggestion that additional species exist, and that some human associated isolates may represent undescribed species (Peterson and Sigler 1998). *E. parva* isolates originally obtained by Emmons from rodents in Arizona were found to be genetically closer to *B. dermatitidis* than to *E. crescens*. Guého et al. (1997), comparing LSU rDNA sequences, found almost the same level of

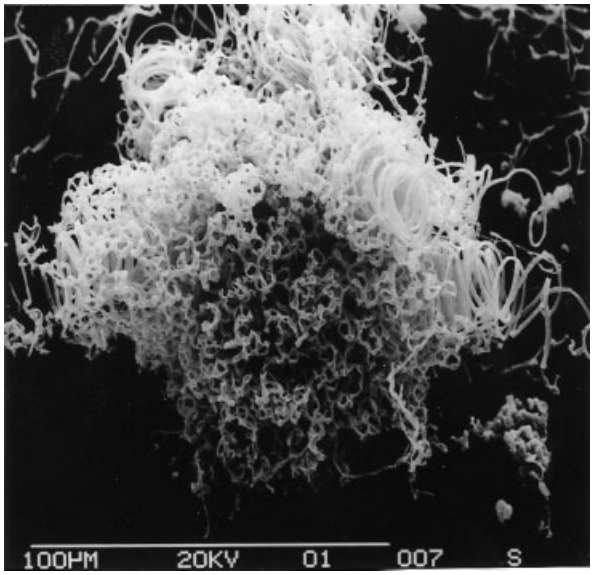

Figure 11 *Scanning electron micrograph of an ascocarp of* Ajellomyces crescens, *the sexual stage of* Emmonsia crescens, *obtained by mating sexually compatible strains. Note the helically coiled appendages arising from the ascocarp (UAMH 127 × 349). (Reproduced, with permission, from Sigler 1996)*

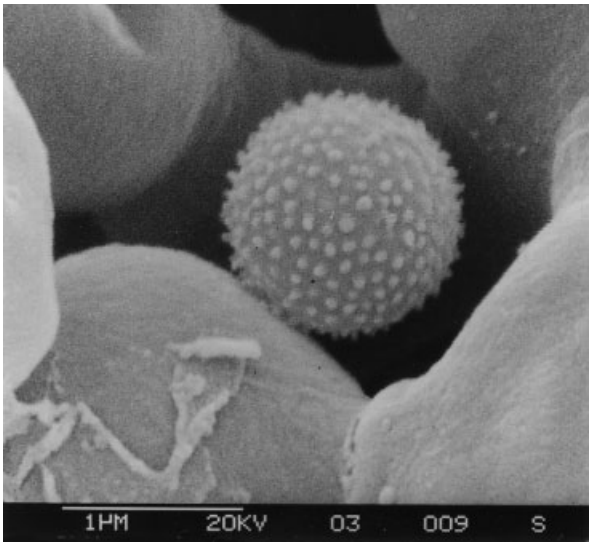

Figure 13 *Scanning electron micrograph of ascospore of* Ajellomyces crescens. *Note that ascospores are tiny measuring 1–1.5 μm in diameter and have short spiny projections (UAMH 127 × 349). (Reproduced, with permission, from Sigler 1996)*

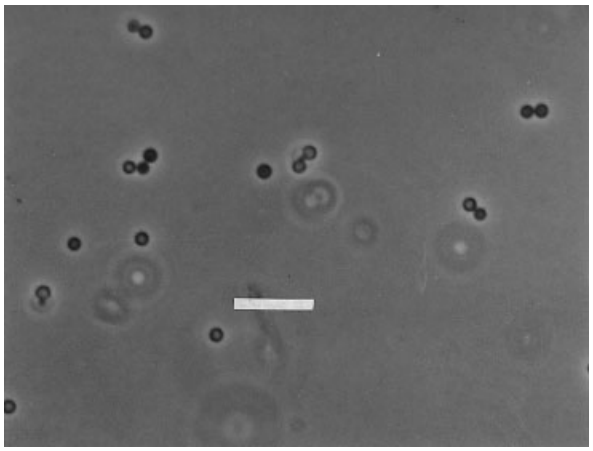

Figure 14 *Ascospores of* Ajellomyces crescens *examined by light microscopy (UAMH 128 × 349). Bar, 10 μm. (Reproduced, with permission, from Sigler 1996)*

genetic diversity between African and North American isolates of *B. dermatitidis* as between the latter and *E. parva*. A later study showed that *E. pasteuriana*, which demonstrates a yeast form in vivo, is closer to *E. crescens* than to *B. dermatitidis* (Drouhet et al. 1998). The close relationship between *Blastomyces* and *Emmonsia*, and the finding that some human-associated *Emmonsia* spp. produce budding cells in vivo, suggests that these anamorph taxa should be combined. Similar arguments have been made previously and species of both genera were at one time placed in *Chrysosporium*. An impediment to combining the species in *Blastomyces* comes from the fact that the *Blastomyces* Gilchrist & Stokes is illegitimate according to the Botanical Code. To have this medically important genus retain priority over *Emmonsia*, it must first be conserved (Peterson and Sigler 1998).

Differential diagnosis

Diagnosis of adiaspiromycosis is generally based on radiological presentations and the finding of characteristic adiaspores in tissue. In culture, isolates of *Emmonsia* spp. resemble those of *B. dermatitidis*, but cultures are rarely established from patients with pulmonary adiaspiromycosis. *E. crescens* and *E. parva* do not convert to a yeast form, and their strongly inhibited growth and formation of adiaspores in culture at 37°C or 40°C, respectively, may aid in identification (Sigler 1996). *B. dermatitidis* isolates, by comparison, convert to the yeast form typical for the species (large thick-walled cells with broad-based buds) and are more tolerant of cycloheximide (Table 1). *B. dermatitidis* isolates can be confirmed by the DNA probe or exoantigen test (Table 1). Microscopically, conidia of *B. dermatitidis* are usually solitary at the ends of stalks with rare proliferation to form another conidium, in contrast to those of *Emmonsia* spp. which often demonstrate secondary proliferation (Figure 9).

TREATMENT

The optimum therapeutic protocol for patients with pulmonary adiaspiromycosis is not clear, but association of a steroid with an antifungal drug appears to aid recovery (de Almeida Barbosa et al. 1997). These authors reviewed the outcome of therapy for three Brazilian patients and case histories for 16 other patients. Two of three Brazilian patients improved without therapy. A third did not respond to itraconazole alone but recovered when prednisone was added to the regimen. A few patients have shown improvement after therapy with steroids alone (Kodousek et al. 1971; Vojtek et al. 1971), amphotericin B alone (de Almeida Barbosa et al. 1997) or in combination with flucytosine (Quilici et al. 1977) or prednisone (de Almeida Barbosa et al. 1997), ketoconazole (Severo et al. 1989; Moraes et al. 2001), and antituberculosis agents (Simecek et al. 1982). Improvement in one case followed therapy with thiobendazole, although in vitro susceptibility tests demonstrated that *E. crescens* was resistant at concentrations up to 128 μg/ml, whereas *E. parva* was sensitive at 1 μg/ml (Barbas Filho et al. 1990). Mycelial stages of both *E. crescens* and *E. parva* are sensitive to imidazoles by in vitro susceptibility tests (Otcenášek and Ditrich 1983). Although *E. crescens* appears less sensitive to amphotericin B in vitro (minimal inhibitory concentration 4 μg/ml) (Otcenášek and Hubálek 1968), enlargement of conidia into adiaspores in experimentally infected mice was prevented by treatment with amphotericin B (Otcenášek and Dvorák 1967).

Infection in two AIDS patients responded to therapy with amphotericin (Echavarria et al. 1993) or amphotericin followed by fluconazole (Turner et al. 1999). However, a third patient whose CD4 counts were very low, died after a short course of therapy with amphotericin (Gori et al. 1998).

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History of human parasitology

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The history of parasitology embraces three interconnected fields: zoology, with its interests in parasites for their own sakes, veterinary medicine, and human medicine, particularly tropical medicine. During our relatively short history on Earth we humans have acquired about 300 species of helminth worms and over 70 species of protozoa (Ashford and Crewe 2003) of which a small proportion cause some of the most important diseases in the world, particularly in the tropics. In this chapter it is only possible to deal with those parasites that are the most common and which cause the most important diseases.

HUMAN EVOLUTION AND PARASITIC INFECTIONS

Human evolution and the acquisition of parasitic infections cannot be separated and, in order to understand the history of parasitology, we have to delve deeply into the past. It is now widely accepted that our species, *Homo sapiens*, emerged somewhere in Africa, but we do not know exactly where or when; opinions on this subject are extreme. Molecular evidence dates our origins to somewhere between 150 000 years ago in eastern Africa (Tishkoff et al. 2001) and 60 000 years ago in the northern part of southern Africa (Wells 2002). The details are not really relevant to us here, but what is important is that most authorities agree that our early ancestors spread from Africa throughout the world in several waves (Templeton 2002) until, at the end of the last Ice Age, about 11 000 years ago, humans had migrated to, and inhabited, virtually the whole of the Earth. During these migrations humans were not alone and brought with them various parasites as 'heirlooms,' inherited from primate ancestors in Africa, and 'souvenirs,' acquired from animals encountered during these migrations. Agriculture, the practice of domesticating animals, and the development of settlements and cities increased the opportunities for the transmission of infections between animals and humans and between humans. Later, the opening up of trade routes brought parasitic infections to new geographical regions, for example, colonization and the slave trade brought new parasites to the New World from the Old World (Desowitz 1997).

For many years the distribution of parasites in the past has been largely a matter of speculation, but we have now begun to learn a lot about the past history of parasitic infections from studies of archeological artifacts such as the presence of helminth eggs or protozoan cysts in coprolites (fossilized or desiccated feces) and naturally or artificially preserved bodies. From such studies a new science, palaeoparasitology, has emerged and some examples of these discoveries will be discussed later.

Written records

Direct evidence concerning our knowledge of parasites in the past is fragmentary, so we have had to rely heavily on the written record. The first written records date from a period of Egyptian medicine between 3000 and 400 BC, particularly the papyri of 1500 BC discovered by Georg Ebers at Thebes (Bryan 1930). Many diseases, specifically fevers, are described in detail in the writings of Greek physicians between 800 and 300 BC, the best known being the collected works known as the *Corpus*

Hippocratorum (see Jones and Whithington 1948–1953). Physicians from other civilizations also made significant contributions to our understanding of parasitic diseases and there are extensive writings from China (3000–300 BC), India (2500 – 200 BC), Rome 700 BC – 400 AD, and the Arab Empire (latter part of the first millennium). Two important Arabic medical works that include a great deal of information about diseases caused by parasites are *Al-hawi* by Rhazes (850–923) and *Al canon fi al tib* by Avicenna (980–1037).

Religious and superstitious beliefs in the Dark and Middle Ages held back medical progress in Europe, and it was not until the Renaissance that scientific and medical revolution freed scientists from the chains of superstition and religion and unleashed a period of spectacular investigation that gradually led to the great discoveries of the 19th century. These include the renunciation of the theory of spontaneous generation and the development of the 'germ theory', by Louis Pasteur; the demonstration that diseases are caused by bacteria, also by Pasteur; the discovery of viruses, by Pierre Emile Roux; the introduction of methods of preventing diseases caused by microorganisms, by Robert Koch; and the incrimination of vectors in the transmission of parasites, by Patrick Manson.

Tropical diseases

Most commentators date the origin of tropical diseases as a distinct branch of medicine to 1898 when Sir Patrick Manson, now universally regarded as the 'father of tropical medicine,' published the first edition of his *Tropical Diseases* (Manson 1898). The important tropical diseases described by Manson included a number caused by parasites – malaria, sleeping sickness, amoebic dysentery, kala azar, and bilharzia – but Manson did not consider parasitology to be a distinct branch of tropical medicine.

Michael Warboys has suggested that 'the dominant tradition of the history of tropical diseases has been to celebrate the discoveries of the etiologies of the classic group of vector-borne parasitic diseases in the period 1870–1920' (Warboys 1993). There is, however, much more to the history of tropical diseases than this and it is now clear that the work of those who investigated parasitic diseases in the centuries before Manson should not be dismissed as irrelevant to our understanding of these diseases. Nor should the discoveries of the 20th-century workers be regarded as mere finishing touches to previous knowledge. Scientific progress during any period of history depends on the availability of techniques and of individuals capable of using and interpreting the information that becomes available. The remarkable thing about the last decades of the 19th century was the coming together of so many new ideas and personalities.

Parasitic diseases have always held a fascination for travelers, clinicians, and scientists. Some, such as sleeping sickness, are unique to the tropics; others, such as malaria, are of particular importance in the tropics but also extend into more temperate regions; still others, such as hookworm, have worldwide distribution (either now or in the past). The present distribution of parasitic diseases tends to reflect the success of hygiene and control measures in the more developed parts of the world rather than any clear geographical or climatic restriction. Thus, many parasites that were once widespread are now found in only the warmer and poorer parts of the world and are considered as 'exotic' diseases when imported into the cooler northern and southern latitudes where they were once prevalent. The term 'tropical diseases' has now become one of convenience rather than a definitive description

The history of parasitology has been well served in the scientific literature, particularly in works devoted to tropical medicine. As well as the publications already mentioned there is a considerable amount of information about the history of parasitology in the more general works by Scott (1939), Ackernecht (1965), Brothwell and Sandison (1967), Campbell et al. (1992), Chernin (1977), Cox (1996), Mack (1991), Norman (1991), Ranger and Slack (1992), and Ransford (1983). More specifically, there are a number of publications dedicated to the history of parasitology, including those of Cook (2001), Cox (2002, 2004a), Foster (1965), Garnham (1970), Hoeppli (1956, 1959), Kean et al. (1978), Moore (1976), and Warboys (1983). The most comprehensive work on the history of any aspect of parasitology, one that will be impossible to emulate, is David Grove's *A history of human helminthology* (Grove 1990), which contains over 800 pages of detailed accounts of all the discoveries in human helminthology and allied fields. *The Wellcome Trust illustrated history of tropical diseases* (Cox 1996) contains chapters on most of the important human parasitic infections. Foster's *A history of parasitology* (Foster 1965) covers some, but not all, important parasitic infections. In addition, there are a number of important monographs mainly relating to the associations between parasitic infections and human endeavor and welfare, and these will be mentioned in the appropriate places.

Helminth worms, being large and easily recognizable, and common parasites of domesticated and wild animals, very soon attracted the attention of zoologists who, schooled in the concepts of comparative zoology, treated parasites in the same way as any other group of animals and tended to ignore the fact that they also caused disease. Veterinarians and clinicians, on the other hand, were quick to note the implications of these infections and to draw parallels between parasitic infections in domesticated animals and humans and discoveries in the field of zoology. To this day, helminthology is usually regarded as a branch of zoology. Protozoa, being

microscopic, had to await the development of the microscope before they were first recognized; the history of the protozoa, therefore, parallels that of other microorganisms and has largely been the province of medicine rather than zoology. There is thus a dichotomy that is not always apparent in that the interest of most helminthologists has centered on the worms themselves and, to a lesser extent, the diseases they cause whereas, in contrast, scientists interested in parasitic protozoa have tended to focus predominantly on diseases. There are other obvious reasons for this dichotomy; for example, the fact that helminths are multicellular organisms and can be examined outside their hosts has facilitated physiological and biochemical studies, whereas isolating protozoa from their host cells has proved to be very difficult. These differences in approach have had a marked effect on our understanding and interpretation of the history of parasitology. Although discoveries about the helminths and protozoa originally tended to run on parallel paths, these paths converged at the end of the 19th century when Patrick Manson introduced the medical and veterinary world to the concept of vector-borne parasitic diseases and brought entomologists into the field of parasitology. Thereafter, research and teaching institutions have traditionally considered protozoology, helminthology, and entomology as three interrelated fields.

The history of parasitology embraces some of the most exciting discoveries ever made in medicine, and these findings have had an impact well outside their immediate field. For example, the discovery of the vector-borne nature of the filarial worms influenced ideas about the transmission of malaria which, in turn, led to the discovery of the mosquito-borne transmission of yellow fever, the whole field of arboviruses, and the implication of other vectors in trypanosomiasis, relapsing fever, and plague. Similarly, the discovery that trypanosomes caused diseases in both animals and humans opened up a new field of zoonoses (infections common to animals and humans) and the study of reservoir hosts for a number of infections.

Many of the great discoveries in parasitology were made over a relatively short period, in the 30 years between 1885 and 1915, when major advances were also being made in our understanding of other infectious diseases. The great personalities of this period made discoveries in a number of fields, and their findings and ideas fed off one another. The names of Pasteur, Koch, Bruce, and Manson occur time and time again in the history of parasitology and microbiology.

The history of parasitic diseases can be considered as a number of phases: the recognition of the disease; the discovery of the organism involved; the connection between the organism and the disease; the consolidation of all these strands; and the application of this knowledge to control schemes.

So vast is the field of human parasitology, with its many and far-reaching discoveries, that it is not possible to do justice to the whole subject and in the rest of this chapter only the most significant aspects will be discussed. No attempt will be made to discuss the parasites or the diseases they cause other than briefly, and the reader is referred to other chapters in this volume to provide the necessary background information and details.

HELMINTHS AND HELMINTH INFECTIONS

Discovery of the organisms

Given the ubiquitous nature and large size of the helminth worms, it would be surprising if our earliest ancestors had not been aware of the more common types; evidence for this assumption comes from the fact that in primitive tribes in Sarawak and North Borneo most people are aware of their intestinal roundworms and tapeworms (Hoeppli 1959). Much has been made of quotations from the Bible on the subject of helminth worms, but these are often open to several interpretations; the only conclusion that can be safely drawn is that those who contributed to the Bible were aware of the existence of parasitic worms (i.e. we cannot conclude that they possessed any detailed knowledge of the subject). With the first written records by physicians we are on firmer ground. The Egyptian medical papyrus usually referred to as the *Papyrus Ebers*, dating from around 1500 BC, refers to intestinal worms and these records are backed by the discovery of calcified helminth eggs in mummies dating from 1200 BC. The Greeks, Aristotle (384 – 322 BC) in particular, were familiar with a range of parasites from humans and also domesticated and wild animals. Greek and Roman physicians, including Hippocrates (460 – 375 BC), Celsus (25 BC – AD 50), and Galen (129 – 200 AD), were familiar with the human roundworms, *Ascaris lumbricoides* and *Enterobius vermicularis*, and tapeworms. In fact, the terms 'roundworm' (helmins strongyle) and 'ribbon worm' (helmins taenia) can be traced back to the earliest Greek records (Grove 1990). Paulus Aegineta (625–690) clearly described *Ascaris*, *Enterobius*, and tapeworms, and gave good clinical descriptions of the infections they caused. With the decline and fall of the Roman Empire, the study of medicine switched to Arabic physicians. Avicenna recognized not only *Ascaris*, *Enterobius*, and tapeworms but also the Guinea worm, *Dracunculus medinensis*, a worm that had been recorded in parts of the Arab world, particularly around the Red Sea, for over 1 000 years.

The medical literature of the Middle Ages is very limited, but the existence of parasitic worms was widely

recorded and, in many cases, these were regarded as the possible causes of a number of diseases. Unfortunately, many of these 'worms' were fictitious, as were the symptoms ascribed to their presence, for example toothache and heart attacks (Hoeppli 1959). On the other hand, Chinese physicians believed that humans should harbor at least three worms to be in good health, and this belief that worms were beneficial also extended into Europe (Foster 1965).

The history of helminthology really took off in the 17th and 18th centuries following the re-emergence of science and scholarship during the Renaissance. In his *Systema Naturae*, Linnaeus described six helminth worms: *Ascaris lumbricoides*, *Ascaris vermicularis* (= *Enterobius vermicularis*), *Gordius medinensis* (= *Dracunculus medinensis*), *Fasciola hepatica*, *Taenia solium*, and *Taenia lata* (= *Diphyllobothrium latum*) (Linnaeus 1758). Thereafter there was a gradual accretion of new species; nine species were identified by 1782, with the addition of *Trichuris trichiura*, *Taenia saginata*, and *Echinococcus granulosus*. This number remained virtually unchanged in various texts published between 1800 and 1819 but was subsequently extended by the inclusion of *Trichinella spiralis* and *Loa loa* by 1845; *Ancylostoma duodenale*, *Schistosoma mansoni*, and *Hymenolepis nana* by 1855; and *Strongyloides stercoralis*, *Wuchereria bancrofti*, *Clonorchis sinensis*, *Fasciolopsis buski*, *Paragonimus westermani*, *Heterophyes heterophyes*, *Schistosoma haematobium*, and *Dipylidium caninum* between 1885 and 1890 (see Grove 1990).

By the beginning of the 20th century, 28 species had been recorded in humans, a number that has grown to >300 species including accidental and very rare records (Coombs and Crompton 1991; Ashford and Crewe 2003). Of these, about 80 can be regarded as fairly common and about 20 very common. The description of a new species of helminth worm in humans is now very unusual, but advances in biochemistry and molecular biology permit the identification of minute but significant differences between parasites and this is likely to lead to the creation of additional species and subspecies from within the current list.

Our understanding of parasitology could not really progress until the theory of spontaneous generation had been disproved. Doubts had been raised about the validity of this concept in the 17th century, but it still persisted until the definitive experiments of Pasteur at the end of the 19th century. A number of helminthologists were among those who had begun to doubt the concept, including Francesco Redi and Edward Tyson who, in the 17th century, went so far as to describe sexual reproduction in worms. Studies on the biology and life cycles of helminth worms effectively destroyed the theory of spontaneous generation (see Farley 1977) well before Pasteur delivered its deathblow and promulgated the 'germ theory' of infection.

Table 1 lists some of the most important events in our knowledge and understanding of parasitic worms. Note the massive activity that occurred at the end of the 19th and beginning of the 20th centuries.

ASCARIS AND ASCARIASIS

Ascaris lumbricoides, the large roundworm, is one of the six worms listed by Linnaeus and its name has remained unchanged ever since. Human infections are worldwide, with >250 million people infected. The presence of this worm, which is often voided in the feces, is very obvious and its existence has been recorded from Egyptian medical papyri of 1500 BC, the works of Hippocrates in the 5th century BC (see Jones and Whithington 1948–1953), Chinese writings of the 2nd and 3rd centuries BC, and the texts of Roman and Arabic physicians from the 1st to 10th centuries AD (see Grove 1990; Goodwin 1996b). Direct evidence of the antiquity of human infections comes from the discovery of eggs of *Ascaris* in human coprolites dated 227 BC from Peru (Horne 1985), in Egyptian mummies from 1938 –1600 BC (Cockburn et al. 1998) and in Chinese mummies from 1368 – 1644 BC (Dexian et al. 1981). This worm is so common that it is impossible to say who discovered it, but the first scientific accounts of its anatomy were by Edward Tyson (1683) and Francesco Redi (1684). The mode of infection by the ingestion of eggs was established by Casimir Davaine (1862) and Giovanni Grassi (1881) who infected himself and later found eggs in his feces. The whole life cycle, including the migration of the larval stages around the body, was worked out in 1922 by Shimesu Koino who also infected himself and deduced the life cycle when he found eggs in his sputum (Koino 1922).

TRICHINELLA AND TRICHINOSIS

Trichinosis, also known as trichinellosis and trichina infection, is caused by the nematode worm *Trichinella spiralis* and is usually acquired by eating infected pork. Foster (1965) considers this to be one of the most interesting parasitic infections and more likely to be responsible for some religious traditions of avoiding pork than the possibility of tapeworm infection (see below). Although the association with pigs had been recognized since the earliest times, the encysted larvae in the muscle were not seen until 1821 and even then their presence was not associated with disease. The story of the association between the worm and human infection is described in full by Foster (1965) and Grove (1990) and can only be summarized here. It revolves around the discovery of the encysted larvae of the worm in humans in 1835 by James Paget, a medical student at St Bartholomew's Hospital in London; this discovery led to intensive investigations by some of the world's most important parasitologists including Rudolf Leuckart, Rudolph Virchow, Thomas

Table 1 *Important events in the history of our knowledge and understanding of parasitic worms.*

Date	Event
c. 5000 BC	Hookworm ova in a human (?) coprolite from Brazil
c. 2500 BC	*Paragonimus* ova in human coprolites from Chile
c. 2330 BC	*Ascaris* ova coprolites from Peru
c. 2000 BC	*Taenia* ova in an Egyptian mummy
c. 1500 BC	*Papyrus Ebers* (Egypt): reference to roundworms (*Ascaris lumbricoides*), threadworms (*Enterobius vermicularis*), and tapeworms (*Taenia* spp.)
1250–1000 BC	*Schistosoma haematobium* eggs in Egyptian mummies
1300–1234 BC	Biblical references to *Dracunculus medinensis* in Red Sea region
c. 800 BC	*Ancylostoma* worms in a Peruvian mummy
c. 600–700 BC	Records of *Dracunculus medinensis* worms from Mesopotamia
c. 400 BC	*Corpus Hippocraticorum* (Aphorisms and Epidemics): references to roundworms (*Ascaris lumbricoides*), threadworms (*Enterobius vermicularis*, *Echinococcus granulosus*, and hydatid disease
500–400 BC	Hookworm eggs in coprolites from Brazil
c. 384–322 BC	Aristotle refers to cysticerci in tongues of pigs and distinguishes between large and flat worms (*Taenia*), cylindrical worms (*Ascaris*), and thin worms (*Enterobius*)
c. 300 BC	Chinese descriptions of threadworms, tapeworms, hookworms, and hookworm disease
200–300 BC	*Chinese Book of Plain Questions* mentions *Ascaris*
167 BC	*Clonorchis* ova from China
AD 20	Celsus recognizes tapeworms taenia, tinea, and taeniola (*Taenia* spp.), vermes cucurbitini (tapeworm proglottids), 'hailstones' (cysticerci) and roundworms, lumbrici teretes (*Ascaris lumbricoides*)
AD 27–39	Pliny describes tapeworms in Egyptians (presumably *T. saginata* because pork was not eaten) and *Tinea rotunda* (Ascaris)
130–200	Galen recognizes three types of worms: roundworms (*Ascaris lumbricoides*), threadworms (*Enterobius vermicularis*), and tapeworms (*Taenia* spp.) and also cysticerci in livers of slaughtered animals
c. 900	Hookworms in a pre-Columbian Andean mummy
c. 1000	Avicenna (Ibn Sina) refer to 'four kinds of worms'
c. 1340	Johannes Actuarius (a Byzantine physician) describes *Trichocephalus trichiuris* (=*T. trichiura*).
1379	Jehan de Brie refers to *Fasciola hepatica* from sheep
1592	Dunus discovers *Diphyllobothrium latum*
1602	Plater distinguishes betweem *Diphyllobothrium* and *Taenia*
1634	Herbert describes *Dracunculus medinensis* worms in the leg
1674	Welsch makes a detailed study of *Dracunculus medinensis* and dracunculiasis
1683	Tyson describes the anatomy of *Ascaris lumbricoides*
1683	Tyson discovers the scolex of *Taenia solium*
1684	Redi describes helminths from domestic and wild animals and arranges them according to their hosts
1684	Redi recognizes parasitic nature of *Echinococcus* cysts
1700	Andry publishes *De la génération des vers dans le corps de l'homme*, the first textbook on medical parasitology, in which he suggests that worms are derived from 'seeds'. He also draws *Taenia saginata*, but calls it *T. solium*, and describes *Diphyllobothrium latum*
1766	Palas realizes that *Echinococcus* cysts are tapeworm larvae
1770	Mongin describes *Loa loa* and the clinical features of loiasis
1793	Chisholm observes transmission of *Dracunculus medinensis*
1808–10	Rudolphi publishes his text *Entozoorum, sive verminum intestinalium, historia naturalis*, regarded as the first textbook on helminthology.
1819	Bremser describes *Enterobius vermicularius*
1819	Rudolphi describes the larvae of *Dracunculus medinensis*
1833	Hilton finds but does not describe *Trichinella spiralis* in human muscle
1835	Paget discovers and Owen reports on the larvae of *Trichinella spiralis* in human muscle
1843	Dubini recognizes the hookworm *Ancylostoma duodenale*
1843	Gruby and Delafond find microfilariae in blood of dogs
1843	Busk discovers *Fasciolopsis buski*
1846	Leidy describes *Trichinella spiralis* in pig muscle
1847	Fujinami describes Katayama disease caused by *Schistosoma japonicum*
1850	Bilharz discovers *Schistosoma haematobium*
1853	von Siebold describes *Echinococcus granulosus* tapeworms in dogs fed with hydatid cysts from sheep

(*Continued over*)

Table 1 *Important events in the history of our knowledge and understanding of parasitic worms.* *(Continued)*

Date	Event
1855	Küchenmeister demonstrates that *Taenia solium* infections are acquired by eating pork
1859	Virchow finds adult worms of *Trichinella spiralis* in a dog fed on infected muscle
1863	Bastian gives a detailed description of *Dracunculus medinensis*
1863	Demarquay finds microfilariae of *Wuchereria bancrofti* in fluid from a scrotal tumor
1863	Naunyn infects dogs with *Echinococcus* hydatid cysts from a human
1863	Cobbold suggests that snails might be the intermediate hosts of schistosomes
1864–9	Cobbold's book *Entozoa* is published
1864	Harley suggests that snails might be the intermediate hosts of schistosomes
1867	Leuckart describes life cycle of *Echinococcus granulosus*
1868	Wucherer finds microfilariae of *Wuchereria bancrofti* in the urine of a patient with hematuria
1869	Fedchenko finds *Dracunculus medinensis* in *Cyclops*
1872	Lewis sees microfilariae of *Wuchereria bancrofti* in human blood
1874	McConnell describes *Clonorchis sinensis*
1875	O'Neill discovers microfilariae of *Onchocerca volvulus* in skin nodules
1876	Normand discovers larvae and adults of *Strongyloides stercoralis*
1876	Joseph Bancroft finds and recognizes adult *Wuchereria bancrofti* worms
1877	Manson demonstrates that *Wuchereria bancrofti* is transmitted by mosquitoes
1877	Bavay describes *Strongyloides stercoralis* larvae and adult worms
1877	Perroncito establishes the bovine source of *Taenia saginata* infections
1879	Perroncito realizes that conditions in mines favor the development of hookworm larvae
1879	Ringer discovers *Paragonimus westermani*
1881	Manson suggests that *Paragonimus westermani* develops in a snail
1881	Leuckart and Thomas independently describe the life cycle of *Fasciola hepatica*
1881	Braun finds larvae of *Diphyllobothrium latum* in fish and induces infections in dogs
1893	Manson reports on the finding of adult *Onchocerca volvulus* worms
1890	Mackenzie finds larvae of *Loa loa*
1895	Argyll-Robertson suggests that *Loa loa* is transmitted by blood-sucking insects
1900	Low, acting on a suggestion by Bancroft, demonstrates microfilariae in mosquito mouthparts and confirms transmission through bite of mosquito
1904	Katsurada describes *Schistosoma japonicum* from a cat
1904	Fujinami finds *Schistosoma japonicum* in a human
1905	Looss elucidates the life cycle of *Ancylostoma duodenale*
1912	Leiper demonstrates transmission of *Loa loa* by flies of the genus *Chrysops*
1913	Turkhud completes the life cycle of *Dracunculus medinensis*
1914	Miyairi and Suzuki describe the life cycle of *Schistosoma japonicum* in snails
1914	Leiper distinguishes between *Schistosoma mansoni* and *S. haematobium* and identifies their snail intermediate hosts
1915	Kobayashi realizes that *Clonorchis sinensis* uses a fish host
1917	Janicki and Rosen independently implicate copepods and fish in the life cycle of *Diphyllobothrium latum*
1922	Koino describes the life cycle of *Ascaris lumbricoides*
1926	Blacklock elucidates the life cycle of *Onchocerca volvulus* in blackflies
1927	Lichtenstein and Brug recognize *Brugia malayi*

References are given in the text and in Grove (1990). Note that the dates of particular discoveries do not always coincide with the dates of publications. This is because in the 19th century and earlier, scientists often made their findings known at meetings or in letters before the publication of the definitive paper. In some cases the reference refers to a later and fuller report. Some of the earlier dates are very approximate.

Cobbold, Richard Owen, and Gottlob Küchenmeister. Although the discovery was made by Paget, much of the credit has been attributed to Owen who not only wrote the definitive report (Owen 1835) but also played down Paget's role in the discovery. The adult worm itself was discovered by Rudolph Virchow and Friedrich Zenker and it was the latter who finally recognized the clinical significance of the infection and realized that humans became infected by eating uncooked pork (see Grove 1990; Bundy and Michael 1996).

HOOKWORMS AND HOOKWORM INFECTION

Human hookworm infections caused by *Ancylostoma duodenale* and *Necator americanus*, the former originating in Asia and the latter in Africa, have been asso-

ciated with humans for over 5 000 years (Hoeppli 1959). Hookworms reached America before the 5th century BC and larvae have been found in coprolites dating from this period (Ferreira et al. 1980). Hookworm disease has been recognized since the earliest records began and the Egyptian papyrus of 1500 BC, the works of Hippocrates in the 5th century BC, and Avicenna in the 10th century all give accurate descriptions of the disease; its history has been reviewed by Foster (1965), Grove (1990), and Ball (1996). Adult *A. duodenale* worms were first recognized by Angelo Dubini in 1843 (Dubini 1843) and credit for the discovery that humans become infected through the skin goes to Arthur Looss who accidentally infected himself and realized that the infection begins when larvae in the soil penetrate the skin, usually of the foot (Looss 1898).

The connection between the presence of worms and hookworm disease was established in 1854 by Wilhelm Griesinger (Griesinger 1854). The clinical signs of hookworm infection – anemia, greenish-yellow pallor, and lassitude – had been observed by Hippocrates and his followers and many early physicians (see Grove 1990) and associated with miners, but it was not until 1879 that Edoardo Perroncito, working with miners in the St Gothard tunnel, realized that the conditions in mines favor the development of the larval stages (Perroncito 1880). The importance of hookworm infections in the USA in the early years of the 20th century led to the establishment of a number of Schools of Public Health and eventually to the creation of the World Health Organization (Ettling 1990).

STRONGYLOIDES AND STRONGYLOIDIASIS

Strongyloidiasis is caused by two species of intestinal nematodes, of which *Strongyloides stercoralis* is the more common and important species. The life cycle involves both parasitic and free-living generations. Parthenogenic female worms lay eggs that hatch within the host and the larvae that are passed out in the feces adopt a free-living existence in the soil from which they penetrate the skin of a new host. Alternatively, the larvae can mature to the infective stage in feces on the skin and reinfect the host through the skin, or mature to the infective stage without leaving the gut and penetrate the gut wall. It is not surprising, given the absence of eggs and confusion with other free-living species of nematodes, that *S. stercoralis* was not recognized until 1876 when both the larvae and the disease, strongyloidiasis, were discovered by Louis Normand who later also found the adult worms and sent them for identification to Arthur Bavay who realized what they were and described them (Bavay 1877). In 1883 Rudolf Leuckart described the alternation of generations involving both parasitic and free-living phases (Leuckart 1883). The discovery that infection with *Strongyloides* occurred through the skin was made by Paul Van Durme based on the work of Looss mentioned above, who had shown that *A. duodenale* infects its host in this way (Van Durme 1901–1902). However, Van Durme was probably working with the other species, *S. fuelleborni* (see Grove 1990), and the mode of infection by *S. stercoralis* was established by Looss who infected himself by putting larvae on his skin and later detecting larvae in his feces (Looss 1905). The phenomenon of autoinfection was described by Friedrich Fülleborn, working with dogs in Hamburg in 1914 (Fülleborn 1914). For nearly a century *S. stercoralis* received little attention, until it was realized that it could cause disseminated infections in immunosuppressed patients, including those infected with the human T-lymphocyte virus type 1 (HTLV-1); although at one time thought to be implicated, *S. stercoralis* is no longer regarded as a major concomitant of acquired immunodeficiency syndrome (AIDS) (see Gutierrez 2000).

LYMPHATIC FILARIASIS

Lymphatic filariasis is caused by infection with the nematode worms *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*, which are transmitted by mosquitoes. The discovery of the life cycle by Patrick Manson in 1877 is widely regarded as the most significant discovery in tropical medicine, with implications that reached far beyond helminthology into such diverse areas as malaria and the arboviruses. The massive swellings of the limbs associated with lymphatic filariasis cannot have gone unnoticed by our early ancestors, and descriptions of what was almost certainly this disease date back to 2000 BC in Egypt and to Nok sculptures of the Sudan and West Africa from 500 AD (see Grove 1990; Nelson 1996). The larval stages of the filarial worms, microfilariae, were first seen in the blood of dogs in 1843 (Gruby and Delafond 1843) and in humans by Demarquay (1863). This latter discovery attracted the attention of a number of eminent parasitologists, including Wilhelm Greisinger, Theodor Bilharz, and Otto Wucherer but it was a relatively unknown Scottish clinician, Timothy Lewis, who realized that the worms were associated with filariasis (Lewis 1872). The mode of transmission remained a mystery until Patrick Manson discovered that the mosquito was involved (Manson 1878).

The story of Manson's discoveries has been told many times (see Manson-Bahr 1962; Foster 1965; Service and Wilmott 1978; Chernin 1983; Grove 1990; Eldridge 1992; Nelson 1996). Manson, then working in Amoy in China, found microfilariae in the blood of dogs and humans; he hypothesized that these parasites in the blood might be transmitted by bloodsucking insects. Accordingly, he fed mosquitoes on the blood of his gardener, who was harboring the parasites, and subsequently was able to detect larval stages in the mosquitoes (Manson 1878). Manson, probably prompted by Fedchenko's discovery of the life cycle of *Dracunculus medinensis* (see below),

mistakenly believed that the parasites escaped from the mosquito into water and that human infections were acquired through contact with contaminated water. The actual mode of transmission, through the bite of a mosquito, was established only when suggestions by the Australian parasitologist, Thomas Bancroft, were followed up by Manson's assistant George Carmichael Low (Low 1900). Manson was instrumental in the foundation of the London School of Hygiene and Tropical Medicine in 1899 and the Royal Society of Tropical Medicine and Hygiene in London in 1907.

ONCHOCERCA AND ONCHOCERCIASIS

The history of onchocerciasis, or river blindness, caused by the nematode worm *Onchocerca volvulus*, is a relatively short one and there are few reliable early records. This is partly because there are so many causes of blindness in the tropics with which this condition can be confused. Accounts of the history of onchocerciasis are given by Grove (1990) and Muller (1996b). The skin lesions associated with this infection were well known in West Africa and called 'craw craw' by the time John O'Neill first identified nematode larvae in the lesions of those suffering from this condition in 1875 (O'Neill 1875). Adult worms were not seen until some years later when Leuckart received specimens from a German doctor in Ghana and sent them to Manson who, with due acknowledgements, described them in *Davidson's hygiene and diseases of warm climates* (Manson 1893).

For a number of years, this parasite was considered to be a rare curiosity until the connection between the worm and the skin lesions was made by Rodolfo Robles in Central America (Robles 1917). Robles was also the first person to suggest that blackflies might be involved in the transmission of the infection but it was not until 1923–1926 that Donald Breadalbane Blacklock demonstrated conclusively that blackflies were indeed the vectors (Blacklock 1926). In 1928, there came the first report of microfilariae in histological sections of the eyes of blind individuals (Ochoterena 1928) and between 1928 and 1935 a flurry of activity clearly established the role of *Onchocerca* infection as a cause of blindness (see Grove 1990). The 20th century has been one of triumph over this disease, first with the use of insecticides to control the blackfly larvae and later with the development and free distribution of a very effective drug, ivermectin, making the eradication of onchocerciasis a real possibility.

LOIASIS

Loa loa, the nematode that causes loiasis, is a large worm that occasionally passes through the eye and must have been recognized from the earliest times. There are few early records but there is no doubt that Huighen van Linschoten became aware of this worm during the course of his researches into Guinea worm at the end of the 16th century (van Linschoten 1610). The adult worm was first seen by a French surgeon, Mongin, in 1770 (Mongin 1770) and the microfilariae were recognized in 1890 by Stephen Mackenzie who referred his material to Patrick Manson (see Manson 1891). The mode of transmission remained a mystery until 1895 when, basing his ideas on Manson's earlier work on lymphatic filariasis, Douglas Argyll-Robertson predicted that the vector would be a bloodsucking insect but failed to find any parasites in his suspected insect hosts (Argyll-Robertson 1895). In 1912 Robert Leiper threw the list of possible vectors wide open and conclusively demonstrated that the vectors were actually tabanid flies belonging to the genus *Chrysops* (Leiper 1913). One of the signs of loiasis is the presence of swellings, known as Calabar swellings, particularly on the arms and legs. Argyll-Robertson suspected that they were caused by the worm and this was eventually accepted by Patrick Manson who was unsure as to whether the swellings were caused by the blockage of lymphatics or as a result of a pathological reaction to some product of the worm (Manson 1910).

DRACUNCULUS AND DRACUNCULIASIS (GUINEA WORM DISEASE)

Dracunculiasis, caused by the nematode *Dracunculus medinensis*, is the only parasitic infection of which a good description is given in the Bible; most observers agree that the 'fiery serpents' that afflicted the children of Israel in the region of the Red Sea between 1300 and 1234 BC were dracunculus worms (The Bible, Numbers 21, Verse 6; Foster 1965). There are also numerous records of this worm in second millennium papyri from Egypt; writings from Mesopotamia in the 7th century BC; and later records by Greek, Roman, and Arabic physicians (see Grove 1990; Tayeh 1996). One of the best descriptions is that by Avicenna in his book *Al canon fe al tib*. Avicenna recognized that this was a worm and not a rotten vein, as had previously been maintained (hence the old name 'Medina vein'). The presence of the worm in the leg was formally recognized by Thomas Herbert in 1634 (Herbert 1634). Throughout the 18th and early 19th centuries the conviction strengthened that dracunculiasis was caused by the worm *D. medinensis* (listed as *Gordius medinensis* by Linnaeus in 1758) and that infection was acquired through drinking water as suggested by the Dutch navigator Huighen van Linschoten in 1610 (van Linschoten 1610). On the other hand, George Busk (Busk 1846) and H. J. Carter (Carter 1855) argued that the infection could be acquired through the skin. The actual life cycle was unravelled by a Russian scientist, Aleksej Fedchenko, who suspected that water fleas, *Cyclops*, were involved and that infection was acquired by accidentally consuming these invertebrates, but was unable to induce infections in experimental animals by feeding them infected water fleas (Fedchenko 1870). It was not

until 1905 that Robert Leiper demonstrated that *Dracunculus* larvae could survive in digestive juices (Leiper 1906) and the whole life cycle was finally elaborated by Dyneshvar Turkhud in 1913 (Turkhud 1914). The 20th century was one of considerable success in controlling this infection, which is now well on the way to being eradicated.

SCHISTOSOMES AND SCHISTOSOMIASIS

Human schistosomiasis, or bilharzia, affects between 200 and 300 million people and is caused by several species of *Schistosoma* each giving rise to a characteristic form of the disease. Calcified eggs of *Schistosoma haematobium*, the cause of urinary schistosomiasis, have been found in Egyptian mummies dating from 1250 to 1000 BC (Contis and David 1996) providing direct evidence that these schistosomes were present in ancient Egypt. The most obvious sign of urinary schistosomiasis is blood in the urine, and this cannot have gone unobserved by the ancient Egyptians. Inevitably there have been numerous attempts to find descriptions of this condition in the medical papyri and many historians have described the disease as 'aaa' recorded in over 50 early papyri, including the *Papyrus Ebers*. In some papyri, 'aaa' occurs together with the initial hieroglyph interpreted as a penis discharging blood, suggesting schistomiasis haematobia (Ebbell 1937). This interpretation is widely quoted in historical and parasitological textbooks but no passages from the papyri link 'aaa' with the bladder or urine and the discharge from the penis might equally represent semen and not blood. This subject is discussed by Nunn and Tapp (2000), who abandon 'aaa' as a possible ancient Egyptian word for schistosomiasis. However, as schistosomiasis was almost certainly common and widespread in ancient Egypt, it is curious that the Egyptians did not have a word for it unless it was so common that it was ignored. It should be mentioned here that there have been a number of other suggestions as to what 'aaa' might be, including hookworm infection.

The worm itself, *S. haematobium*, was first described by Theodor Bilharz in 1851 and the popular name for all forms of schistosomiasis, 'bilharzia,' now honours his discovery (Bilharz and von Siebold 1852–53). The connection with the urinary disease was made a year later by Bilharz working with Wilhelm Griesinger (Bilharz 1853). The search for the intermediate stages in the life cycle of *S. haematobium* took a long time. A number of experienced parasitologists, including Arthur Looss, Prospero Sonsino, and Thomas Cobbold, working at the end of the 19th century, all failed to infect snails and it was not until 1915 that Leiper demonstrated the complete life cycle in the snail host (Leiper 1916).

Our knowledge of the history of intestinal schistosomiasis, caused by *S. mansoni*, dates back to conclusions reached by Manson in 1902 that there were two species of *Schistosoma* in humans (Manson 1902). At the time this was not universally accepted and it was Leiper who firmly established the existence of *S. mansoni* as a separate species in 1915 (Leiper 1916).

The third important form of schistosomiasis, Katayama disease, caused by *S. japonicum*, was first recognized by Akira Fujinami in 1847 in a report that did not become available until 1909 (Fujinami and Nakamura 1909). The worm itself was discovered by Fujiro Katsurada in 1904 (Katsurada 1904). Development in the snail host was described by Miyairi and Suzuki (1913) 2 years before Leiper independently described the life cycle of *S. haematobium*.

The history of such an important disease as schistosomiasis involves a great number of observations, events, and individuals, and a detailed account of the history is given by Grove (1990). There are shorter accounts by Hoeppli (1959), Foster (1965), and Goodwin (1996a). A bibliography covering the early history of schistosomiasis is given by Warren (1973) and there is an excellent account of schistosomiasis in the context of British and American imperialism by Farley (1991).

LIVER AND LUNG FLUKES

Several flatworms or flukes, probably over 100 as adults or larvae, infect humans, of which the most important are *Paragonimus westermani*, causing paragonimiasis; *Clonorchis sinensis*, causing clonorchiasis; and *Opisthorchis* spp., causing opisthorchiasis. Virtually all the important discoveries about the parasites themselves were made over a period of less than 45 years, between 1874 and 1918, and all were based on observations that had been made on other parasitic flukes such as *Fasciola hepatica* in sheep and others of zoological rather than medical interest. The various discoveries were made by a large number of people and often reported in obscure publications, and no attempt will be made here to list the individual achievements; for this the reader is referred to Grove (1990) and Muller (1996a).

The histories of these infections as diseases begin with the discovery of the worms and continue with the elaboration of the various life cycles. The adult worm of *P. westermani* was discovered in the lungs of a human in Taiwan in 1879 by B.S. Ringer who wrote to Manson who later described his find (see Manson 1881). The presence of eggs in the sputum was recognized independently by Erwin von Baelz in 1880 (von Baelz 1880) and by Manson in 1881 who, having read Ringer's report, realized the connection with the worms he had seen and postulated that a snail might act as an intermediate host (see Manson 1881). A number of Japanese workers, including Koan Nakagawa, Sadamu Yokogawa, Harujiro Kobayashi, and Keinosuke Miyairi, reported on the whole life cycle in the snail *Semisulcospira* between 1916 and 1922 (see Grove 1990).

The human liver fluke, *C. sinensis*, was first recognized by James McConnell in 1875 (McConnell 1875). The

whole life cycle remained a puzzle until Kobayashi discovered that there was a second intermediate host, a fish (Kobayashi 1915), and the snail host was discovered by Masatomo Muto in 1918 (Muto 1918). Kobayashi's discovery that the second intermediate host could be an important food fish had an immediate impact on the control of this infection.

The first records of *Opisthorchis* adult worms in humans were made by Konstantin Wingradoff (1892) and the snail and fish hosts were described by Hans Vogel (1934).

CESTODES AND CESTODIASIS

Humans harbor about 55 species of tapeworms, about 40 adult and 15 larval stages. The most important are the adults of *Diphyllobothrium latum* (the broad tapeworm), *Taenia solium* (the pork tapeworm), *T. saginata* (the beef tapeworm), and the larval stages of the dog tapeworm, *Echinococcus granulosus*. The large size of the adult worms and their common occurrence attracted the attention of all the major medical writers including the authors of the *Papyrus Ebers* and the *Corpus Hippocratorum* and physicians such as Celsus and Avicenna.

It is generally held that the broad tapeworm *D. latum* was first recognized by Felix Plater (sometimes spelled Platter) at the beginning of the 17th century (see Foster 1965; Grove 1990). By the middle of the 18th century it was apparent that individuals harboring *D. latum* were those whose diet was mainly fish, but it was not until the life cycles of other tapeworms of zoological interest had been elaborated that further progress became possible. The existence of three hosts in the life cycle of *D. latum* (human, fish, and copepod) confused the issue until 1917 when Janicki and Rosen independently worked out the whole of the life cycle (Janicki and Rosen 1917). Plater had already provided an excellent description of the disease, and the only significant subsequent discovery was made in 1948 when it was realized that this worm has an affinity for vitamin B_{12}, thus accounting for the pernicious anemia associated with this infection (see von Bonsdorff 1977).

The scientific study of the taeniid tapeworms can be traced to the work of Edward Tyson who, in the late 17th century, studied not only the tapeworms of humans but also those of dogs and other animals. This gave him the opportunity to recognize the 'head' (scolex) of a tapeworm for the first time, to describe the anatomy and physiology of the adult worms, and to lay the foundations for our knowledge of the biology of the taeniid tapeworms of humans (Tyson 1683). Although the distinction between broad and taeniid tapeworms had been recognized by the 17th century, the existence of two species of *Taenia* in humans presented problems because, even to the trained eye, the distinctions between *T. solium* and *T. saginata* are not obvious. This led to confusion between the two worms until a century after the work of Tyson when, in 1782, Johann Goeze noted the similarities between the heads of tapeworms in humans and pigs (Goeze 1782). It was not until 1855 that Gottlob Küchenmeister demonstrated experimentally that eating infected pork gave rise to tapeworms in humans (Küchenmeister 1855) although the presence of cysts in pork had been noted throughout recorded history and is mentioned by Aristotle in the 4th century BC, Hippocrates, and Galen (see Foster 1965; Grove 1990). It has been suggested that, even before these discoveries, people had long been aware of the dangers of infected pork and that this accounted for the Jewish and Islamic prohibitions on the consumption of pork (Küchenmeister 1857) but there is no real evidence for this and the same arguments have been put forward for *Trichinella spiralis*.

The history of our knowledge of *T. saginata* parallels that of *T. solium*, and Goeze and Küchenmeister are credited with recognizing the differences between the two species of *Taenia* in humans. Several years after the implication of pigs as the intermediate hosts of *T. solium*, J.H. Oliver noted tapeworm infections in individuals who had eaten 'measly' beef (Oliver 1871) and the role of cattle as intermediate hosts of *T. saginata* was confirmed in a more controlled experiment in 1877 (Perroncito 1877). Together with observations on the pork tapeworm, these discoveries had a massive impact on the control of tapeworm infections in humans, as they led to a restriction of the amount of infected meat consumed by humans.

Humans are also host to two important kinds of larval tapeworm, cysticerci of the pork tapeworm *T. solium* and hydatid cysts of the dog tapeworm *E. granulosus*. The demonstration of the life cycle of *T. solium* threw new light on the nature of cysticercosis, and it was soon apparent that humans could become infected with the larval stages of *T. solium* when they ingested eggs. This made humans, in effect, dead-end intermediate hosts for this parasite, which also used humans as its definitive hosts. Although, for ethical reasons, the conclusive experiments could not be carried out, by the middle of the 19th century various experiments with animals and observations on humans had established without doubt that cysticercosis was caused by the ingestion of the eggs of *T. solium* (Küchenmeister 1860).

Infections with the larval stages of the dog tapeworm *E. granulosus* cause hydatid disease in humans. These cysts have been recognized from the earliest times and there are accurate descriptions by Hippocrates and Galen among others (see Grove 1990) but it was not until 1684 that Redi recognized the parasitic nature of these cysts (Redi 1684). Pierre Pallas was the first to suggest that these cysts were the larval stages of tapeworms (Pallas 1766) and in 1853 Carl von Siebold demonstrated that echinococcus cysts from sheep gave rise to adult tapeworms when fed to dogs (von Siebold 1853). The whole story was completed in

1863 when Bernhard Naunyn found adult tapeworms in dogs fed with hydatid cysts from a human (Naunyn 1863).

PROTOZOA AND PROTOZOAL INFECTIONS

Discovery of the organisms

Because of their small size, it was not possible to see the unicellular eukaryotic organisms we know as protozoa until the invention of the microscope and Antony van Leeuwenhoek's observations towards the end of the 17th century (Dobell 1960). All commentators agree that the first person to see a parasitic protozoan was Leeuwenhoek, who observed *Giardia* in his own stools. His written descriptions and his clinical observations leave little doubt that this flagellate is indeed the organism that he saw (Dobell 1960). Leeuwenhoek also described, very accurately, a number of other protozoa including flagellates and ciliates in frogs. The protozoa were recognized as a phylum of the animal kingdom a century later (Müller 1786) but it was not until over 140 years after Leeuwenhoek's observations that other parasitic protozoa were described and these were the large and conspicuous gregarines of insects (Dufour 1828), the beloved introduction to parasitic protozoology of generations of parasitologists. Among the conspicuous parasites in the blood are the trypanosomes, and the first to be recognized was from the blood of a fish (Valentin 1841); it is interesting to note that it was more than 50 years before similar parasites were seen in humans. The first associations between blood parasites and disease were those recorded by Griffiths Evans, who found trypanosomes in the blood of horses suffering from surra (Evans 1881). David Bruce found similar organisms in cattle, suffering from nagana, and dogs in 1894–7 (see Bruce 1915). Amebae are also conspicuous protozoa and those in humans were first seen in 1849 by Gros who recognized the nonpathogenic *Entamoeba gingivalis* from the mouth (see Dobell 1919); the pathogenic *E. histolytica* was recognized for the first time by Alexandrovitch Lösch in 1875 (Lösch 1875).

The detection of protozoa living in red blood cells was not possible until the development of adequate staining techniques; the first to be seen were malaria parasites by Charles Laveran in 1880 (Laveran 1880); *Babesia* in cattle by Theobald Smith and Frederick Kilbourne in 1893 (Smith and Kilbourne 1893); and *Theileria* in cattle by Charles Lounsbury in 1904 (Lounsbury 1904).

Leishmania parasites were discovered independently by P.F. Borovsky in 1898, William Leishman in 1903, Charles Donovan in 1903, and James Homer Wright in 1903. The first leishmania parasites associated with New World leishmaniasis were discovered by Gaspar Vianna in 1911.

The remaining important protozoan parasites of humans were not detected until the beginning of the 20th century when, in a flurry of activity, *Trypanosoma cruzi* was identified as the cause of the condition that is now called Chagas disease (Chagas 1909). In a remarkable discovery that was to have much wider implications, *Toxoplasma gondii*, a parasite with the widest known host range including humans, was found in an obscure mammal, the gundi, by Charles Nicolle and Louis Manceaux in 1909.

Table 2 lists some of the most important events in our knowledge and understanding of parasitic protozoa. Note that the considerable activity that occurred during the second half of the 19th century mirrors what was also being done in the field of helminthology.

AMOEBAE AND AMOEBIASIS

Humans harbor nine species of intestinal amoebae of which only one, *Entamoeba histolytica*, is a pathogen. *E. histolytica* causes two forms of the same disease: amoebic dysentery, resulting from the invasion of the gut wall, and hepatic amoebiasis caused by extra-intestinal amoebae in the liver. There is circumstantial evidence that both forms of the disease were recognized from the earliest times, but there are so many causes of both dysentery and liver disease that these records are open to other interpretations (see Bray 1996). The history of amoebiasis has been described by Dobell (1919), Foster (1965) and Bray (1996). James Annersley (1828) is credited with the first accurate descriptions of both forms of the disease and was the first to suggest the connection between them. From the middle of the 19th century, several workers observed amoebae in the stools of patients with amoebic dysentery but credit for the discovery of *E. histolytica* is now given to Alexandrovitch Lösch (Lösch 1875). The association between the amoeba and liver abscesses was finally determined shortly afterwards when Stephan Kartulis demonstrated amoebae in both intestine and liver (Kartulis 1886). Many workers did not believe that *E. histolytica* could cause disease, as all intestinal amoebae were generally thought to be harmless. It was therefore suggested that humans might harbor two morphologically similar amoebae, one pathogenic and one not (see Dobell 1919). This situation has only been resolved comparatively recently using biochemical techniques that clearly show that there are indeed two species: *E. histolytica*, which can cause disease, and *E. dispar*, which cannot (see Classification and introduction to the parasitic protozoa).

GIARDIA AND GIARDIASIS

Giardia holds a special place in the affection of all protozoologists because the parasite that causes giardiasis, *Giardia duodenalis* (also known as *G. intestinalis* or *G. lamblia*) was the first parasitic protozoan ever to

Table 2 *Important events in our knowledge of parasitic protozoa.*

Date	Event
1681	Leeuwenhoek discovers *Giardia duodenalis* in his own feces
1773	Muller describes *Cercaria tenax* (*Trichomonas tenax*) from human mouth
1786	Müller recognizes Protozoa as a phylum of the Kingdom Animalia
1841	Valentin sees trypanosomes in the blood of fish
1848	Gros describes *Entamoeba gingivalis* from a human mouth
1853–4	Devaine describes *Cercomonas hominis* (*Trichomonas* and *Chilomastix*) from human feces
1857	Nägli describes a microsporidian from silkworms
1859	Lambl describes *Giardia duodenalis*
1870	Lewis describes *Entamoeba coli* from a human
1875	Lösch describes *Entamoeba histolytica*
1878	Lewis describes *Trypanosoma lewisi* from a rat
1879	Leuckart creates the phylum Sporozoa
1880	Laveran sees the malarial parasite *Plasmodium* in human blood
1881	Evans finds trypanosomes in the blood of a horse suffering from surra
1885	Cunningham sees *Leishmania* parasites in cutaneous lesions
1887	Metchnikov recognizes the coccidial nature of the malaria parasites
1891	Nepveu sees African trypanosomes in human blood
1893	Smith and Kilborne describe *Babesia* from cattle and incriminate ticks in its transmission
1894	Bruce sees trypanosomes in the blood of cattle and suggests a connection with tsetse flies
1897	Ross demonstrates the mosquito transmission of malaria parasites
1898	Bignami, Grassi, and Bastianelli describe mosquito stages of human malaria parasites
1898	Borovsky sees *Leishmania* parasites in a cutaneous lesion
1902	Dutton finds *Trypanosoma brucei gambiense* in human blood
1903	Wright describes *Leishmania* from cutaneous lesions
1903	Leishman and Donovan independently describe *Leishmania* from kala azar patients
1904	Bruce suggests that tsetse flies transmit African trypanosomes
1908	Nicolle and Manceaux and Splendore independently discover*Toxoplasma gondii*
1909–12	Chagas discovers *Trypanosoma cruzi*, its role in South American trypanosomiasis and transmission by bugs
1909	Kleine describes the cyclical transmission of trypanosomes
1910	Stevens and Fantham recognize *Trypanosoma brucei gambiense*
1911	Vianna describes *Leishmania braziliensis* in the New World
1912	Brumpt describes the transmission of *Trypanosoma cruzi* via the feces of bugs
1912	Tyzzer describes *Cryptosporidium* from a mouse
1921	Sergent and Sergent show that *Leishmania* parasites are transmitted by sandflies
1937	Wolf and Cowan implicate *Toxoplasma gondii* in human disease
1941	Adler and Ber demonstrate that leishmaniasis is transmitted through the bite of a sandfly
1948	Shortt and Garnham describe liver stages of malaria parasites
1959	Matsubayashi and colleagues describe a human infection with the microsporidian, *Encephalitozoon*
1976	Nime and colleagues and Meisel and colleagues independently describe human infections with *Cryptosporidium*
1969	Hutchison and colleagues describe life cycle of *Toxoplasma gondii*
1979	Ashford finds *Cyclospora* in a human
1982	Krotoski and colleagues discover *Plasmodium* hypnozoites

References are given in the text. Note that the dates of particular discoveries do not always coincide with the dates of publications. This is because in the 19th century and earlier scientists often made their findings known at meetings or in letters before the publication of the definitive paper. In some cases the reference refers to a later and fuller report.

be seen. An account of the history of giardiasis is given by Farthing (1996).

The first good accounts and illustrations of *Giardia* were by Vilém Lambl (1859), but the parasite received little attention until the war of 1914–1918 when troops returning to the UK with diarrhea were found to have, in their feces, *Giardia* cysts which caused similar infections in laboratory animals (Fantham and Porter 1916). In 1921 the distinguished British protozoologist Clifford Dobell suggested that *Giardia* could be a serious pathogen (Dobell 1921) and in 1926 Reginald Miller observed diarrhea and malabsorption in children infected with *Giardia* (Miller 1926). It still took some time before the association between infection with

Giardia and disease became widely recognized, following the detailed studies of Robert Rendtorff who produced unambiguous evidence linking the parasite with disease (Rendtorff 1954).

In the 300 years since *Giardia* was first discovered it has become recognized as a common and serious pathogen worldwide. It is still not known how many species infect humans and what role, if any, is played by reservoir hosts in the epidemiology of the infection.

AFRICAN TRYPANOSOMES AND SLEEPING SICKNESS

The story of African sleeping sickness is told briefly by Hoare (1972) and in more detail by Foster (1965), Nash (1969), Lyons (1992), Williams (1996), and Cox (2004b). The early records of African trypanosomiasis or sleeping sickness are vague. The first definitive accounts are those given by an English naval surgeon, John Atkins, in 1721 (see Atkins 1734) and Thomas Winterbottom, who coined the term 'negro lethargy,' in 1803 (Winterbottom 1803). Many explanations were put forward as causes of sleeping sickness, but the real basis of the disease was not forthcoming until Pasteur had established the germ theory towards the end of the 19th century. The first clues came from observations on surra, a horse disease, and nagana, a wasting disease of cattle. Although trypanosomes had been seen in the blood of fishes, frogs, and mammals several years before, it was not until 1881 that Griffith Evans found trypanosomes in the blood of horses and camels suffering from surra and suggested that the parasites might be the cause of the condition (Evans 1881).

In 1894, David Bruce, a British army surgeon, was sent to Zululand (now part of South Africa) to investigate an outbreak of nagana in cattle. Given the scientific climate of the period, Bruce suspected a bacterial cause but instead found trypanosomes in the blood of diseased cattle; he later demonstrated that these caused nagana in cattle, horses, and dogs. Bruce also noticed that infected cattle had spent some time in the fly-infested 'tsetse belt' and that the disease was similar to that in humans suffering from the 'fly disease' of hunters (Bruce 1895, 1915). Bruce also showed that the infection was acquired from tsetse flies, but mistakenly thought that transmission was purely mechanical.

It was later discovered that the trypanosomes that cause nagana and surra are not the same as those that cause human sleeping sickness. Although organisms that were certainly trypanosomes had been seen in human blood by Gustave Nepveu in 1891 (see Nepveu 1898) it was not until 1902 that Everett Dutton found the trypanosome that causes Gambian or chronic sleeping sickness (*Trypanosoma brucei gambiense*) in humans (Dutton 1902). In 1910 *T. b. rhodesiense*, the cause of Rhodesian or acute sleeping sickness, was described by J.W.W Stephens and Harold Fantham (Stephens and Fantham 1910). The role of the tsetse fly in the transmission of sleeping sickness remained controversial until Friedrich Kleine, a colleague of Robert Koch, demonstrated cyclical transmission, but he mistakenly thought that there was a sexual stage in the life cycle (Kleine 1909).

The persistence of trypanosomes in the blood and the existence of successive waves of parasitemia have attracted the attention of all those who have worked with trypanosomiasis. This phenomenon was described in detail by Ronald Ross and David Thomson in 1911 (Ross and Thompson 1911). The underlying mechanism and the way in which the parasite evades the immune response, now called 'antigenic variation,' were not elaborated until the work of Keith Vickerman in 1969 (Vickerman and Luckins 1969); this discovery precipitated a vast amount of interest in trypanosomes as cells and not only as causes of disease. Other 20th-century investigations have been concerned with refining the early discoveries and establishing the epidemiology of African sleeping sickness, leading to the development of methods for the control of both the human and animal forms of trypanosomiasis and the development of new and effective drugs.

SOUTH AMERICAN TRYPANOSOMIASIS: CHAGAS DISEASE

The earliest records of Chagas disease in South America are from 2 500-year-old mummies that show clear signs of the destructive nature of the disease (Rothammer et al. 1985). There are also a number of possible early written records, but the signs and symptoms of Chagas disease are so vague that it is difficult to know whether or not many of the conditions attributed to this infection are really valid. The early history of Chagas disease is described by Guerra (1970) and Miles (1996) and is passed over here because, from a scientific and medical viewpoint, the history really begins with a remarkable series of discoveries that were made between 1907 and 1912 by Carlos Chagas. Chagas not only discovered the trypanosome that causes the disease but also demonstrated its life cycle in the bugs that transmit it and described the disease which, although it affected millions of people, had until then remained enigmatic. Chagas's discoveries have been extensively described elsewhere (Wenyon 1926; Scott 1939; Guerra 1970; Lewinsohn 1979; Kean et al. 1978; Leonard 1990; Miles 1996). Briefly, Chagas, who was then in charge of an antimalaria campaign in Brazil, noticed that the bugs that infested the poorly constructed houses harbored flagellated protozoa; these could experimentally infect monkeys and guineapigs, in the blood of which a new trypanosome, *Trypanosoma cruzi*, was subsequently detected (Chagas 1909). Chagas suspected that these bugs might also transmit the parasite to humans and confirmed this when he found the trypanosomes in the blood of children

suffering from an acute febrile condition (Chagas 1911). In 1912, Chagas demonstrated that *T. cruzi* was maintained in a number of reservoir hosts (Chagas 1912) thus completing in less than 5 years a cycle of discovery that had taken many years for earlier workers in other fields. The disease that affects some 18 million people now commemorates his name.

One thing that Chagas did not get right was the actual mode of transmission by the bug, as he thought that the trypanosomes were transmitted via the bite of the insect; it was left to Emile Brumpt to demonstrate that transmission was via the fecal route (Brumpt 1912). The links between infection with *T. cruzi* and the various signs of Chagas disease, such as megacolon, megaesophagus, and cardiac failure, were not determined until the work of Fritz Koberle in the 1960s (Koberle 1968). Exactly how the damage to heart and nerves is caused and whether or not there is an autoimmune component are still controversial issues.

LEISHMANIA AND LEISHMANIASIS

Leishmaniasis, caused by several species of *Leishmania*, is transmitted by sandflies and occurs in various forms in both the Old World and New World. Human leishmaniasis was, and still is in some cases, acquired accidentally from naturally infected wild animals. The conspicuous cutaneous lesions have been the subject of numerous records dating back to 2500–1000 BC (see Manson-Bahr 1996). Detailed descriptions of the Old World forms are given by the Arab physicians including Avicenna in the 10th century, and missionaries were well aware of this disease in the New World in the 16th century (see Lainson 1996).

The history of Old World leishmaniasis is described by Hoare (1938), Garnham (1987), and Manson-Bahr (1996). The discovery of the parasite responsible for the Old World cutaneous disease has been a matter of some controversy. There is no doubt that the organism was actually seen in 1885 by David Cunningham (Cunningham 1885) and in 1898 by a Russian military surgeon, P.F. Borovsky (see Hoare 1938), but credit for its discovery is usually given to James Homer Wright (Wright 1903). Credit for the discovery of *Leishmania donovani*, the parasite that causes visceral leishmaniasis (kala azar), goes to William Leishman and Charles Donovan who independently discovered the parasite in the tissues of patients suffering from this condition (Donovan 1903; Leishman 1903). Borovsky's discoveries were unknown to Wright and to Leishman and Donovan, but the names of the two latter workers are commemorated in the popular designation of the intracellular parasites as Leishman–Donovan bodies.

The search for a vector was a long one, partly because of the small size of the sandfly vectors. Although enough epidemiological evidence to incriminate sandflies belonging to the genus *Phlebotomus* had accumulated by the mid 1910s, it was not until 1921 that the experimental proof of transmission to humans from a sandfly was demonstrated by the Sergent brothers, Edouard and Etienne (Sergent et al. 1921). Infection through the bite of a sandfly was not finally demonstrated until 1941 (Adler and Ber 1941).

Leishmaniasis also occurs in the New World; the disfiguring conditions caused have been recognized in sculptures since the 5th century and in the writings of the Spanish missionaries in the 16th century (see Lainson 1996). Until 1911, it was thought that the New World forms of leishmaniasis were the same as those of the Old World, but in that year Gaspar Vianna found that the parasites in South America differed from those in Africa and India and created a new species, *Leishmania braziliensis* (Vianna 1911). Since then a number of other species, causing cutaneous and mucocutaneous leishmaniasis, unique to the New World have been described suggesting that this is where they emerged independently (see New World leishmaniasis). There is, however, some controversy about the status of New World visceral leishmaniasis which was once thought to be caused by *L. donovani*, a species imported from the Old World possibly with the Spanish military and missionaries. This parasite has long been regarded as a separate species, *L. chagasi*, named in honor of the great Brazilian parasitologist Carlos Chagas, but the validity of *L. chagasi* as a New World species has been challenged by biochemical and molecular studies and it is now thought to be *L. infantum*, a species related to *L. donovani*, and thus of Old World origin (Maurício et al. 2000).

Following the discovery of the sandfly transmission of Old World leishmaniasis, the vectors in the Old World were also thought to belong to the same genus, *Phlebotomus*, but in 1922 it was discovered that the genus involved was actually *Lutzomyia*. Over the last two decades the complex pattern of species of parasite, vector, reservoir host, and disease has been painstakingly elaborated by Ralph Lainson and his colleagues (see Lainson 1996 and New World leishmaniasis).

MALARIA

Malaria ranks among the most important infectious diseases in the world. Its history extends into antiquity and has been reviewed many times by, for example, Garnham (1966), Harrison (1978), Bruce-Chwatt (1985, 1988), McGregor (1996), Nye and Gibson (1997), Dobson (1999), and Poser and Bruyn (1999). Malaria almost certainly evolved with humans during our evolution from our primate ancestors; similar parasites are common in monkeys and apes. The disease probably originated in Africa and spread with human migrations, first throughout the tropics, subtropics, and temperate regions of the Old World and then to the New World with explorers, missionaries, and slaves. The

periodic fevers of malaria are characteristic and are mentioned in the records of every civilized society from China in 2700 BC, through the writings of Greek, Roman, Assyrian, Indian, Arabic, and European physicians up to the 19th century. The earliest detailed accounts are those of Hippocrates in the 5th century BC, and for the next 700 years much of what we know about malaria relates to the disease in Greece, Italy, and throughout the Roman Empire. Thereafter, references to malaria become commonplace in Europe and elsewhere.

The science of malariology could not take off until the end of the 19th century, with the establishment of the germ theory and the birth of microbiology; a start was then made in earnest to discover the cause of the disease that was threatening many parts of the European empires. The story of the discovery of the malaria parasite and its mode of transmission is one of the most exciting in the history of infectious diseases and, like all good stories, is full of triumphs, disappointments, and rivalries (see McGregor 1996).

In 1880, a French army surgeon, Charles Laveran, looking for a bacterial cause of malaria, found and described malarial parasites in human blood and the parasite was immediately causally linked with the disease (Laveran 1880). The mode of transmission was not at all clear and numerous suggestions were put forward, none of which stood up to any detailed analysis. The actual incrimination of the mosquito as a vector was largely due to the intuition of Patrick Manson. In 1877 Manson had demonstrated the mosquito transmission of lymphatic filariasis (Manson 1878), in 1893 Smith and Kilbourne had shown that ticks transmit piroplasms (Smith and Kilbourne 1893), and in 1894 Bruce had implicated tsetse flies in the transmission of African trypanosomiasis (Bruce 1895). Thus, it was evident to Manson that a vector might be involved in the transmission of malaria and he postulated that it could be a mosquito (Manson 1894). Manson was unable to undertake this investigation himself, and secured the services of Ronald Ross, an army surgeon working in India. After several false starts using the wrong kinds of mosquito, *Culex* and *Aedes* instead of *Anopheles*, Ross, who had already demonstrated that bird malaria parasites developed in mosquitoes, found developing parasites in a mosquito fed on the blood of a patient suffering from malaria (Ross 1897). The rest of the life cycle in the mosquito was worked out by the Italian scientists Amico Bignami, Battista Grassi, and Giovanni Bastianelli (see Bastianelli and Bignami 1900; Fantini 1999). There is considerable controversy about the respective roles of Ross and the Italian workers in the discovery of the life cycle of the malaria parasite in the mosquito, and the arguments have been well rehearsed elsewhere. Nevertheless, most commentators agree that it was Ross who incriminated the mosquito as the vector of malaria parasites in birds and that he and the Italian workers established that this was also the case for the human malaria parasites independently at about the same time (see Dobson 1999; Fantini 1999).

The life cycle in humans, however, remained incompletely understood and nobody knew where the parasites developed during the first 10 days after infection, as they could not be found in the blood. In part this was due to too much reliance on a mistake by the influential German scientist Fritz Schaudinn who, in 1903, described the direct penetration of red blood cells by the infective sporozoites injected by the mosquito (Schaudinn 1903). The question was not resolved until 1947 when Henry Shortt and Cyril Garnham, working in London, showed that a phase of division in the liver preceded the development of parasites in the blood (Shortt and Garnham 1948); thus the whole life cycle had taken over 50 years to elucidate. There remained one further problem; what caused the long prepatent period between infection and the appearance and reappearance of parasites in the blood seen in some temperate strains of *P. vivax*? This led to the discovery in 1982 of dormant exoerythrocytic stages, hypnozoites, by Wojciech Krotoski, working with Garnham's team (Krotoski et al. 1982).

The last 50 years have been dominated by the control of the malaria parasite and its vectors by drugs and insecticides and the search for a vaccine. For a fascinating and amusing insight into the problems, solutions, intrigues, and personalities involved, the reader is referred to the popular book *The malaria capers* by Robert Desowitz (Desowitz 1991).

TOXOPLASMA, TOXOPLASMOSIS, AND INFECTIONS CAUSED BY RELATED ORGANISMS

The history of toxoplasmosis is a relatively recent one despite the fact that the organism that causes the disease, *Toxoplasma gondii*, is the most common human parasite world wide and one that infects the widest range of hosts (see Toxoplasmosis). The history of toxoplasmosis has largely escaped the attention of the major reference books, but the basic events are well covered by Dubey and Beattie (1988) and Moulin (1993). *T. gondii* was discovered, largely by accident, by Charles Nicolle while searching for a reservoir host of *Leishmania* in a North African rodent, *Ctenodactylus gondi* (Nicolle and Manceaux 1909). At about the same time, Alfonso Splendore, working in São Paulo, discovered the same parasite in rabbits (Splendore 1909) and subsequently there have been numerous records from mammals and birds both in the wild and in captivity. The association with human disease was not made until 1937 when Arne Wolf and David Cowen reported on a congenital infection (Wolf and Cowen 1937). This report stimulated a vast amount of research, quickly leading to the knowledge that *T. gondii* is actually a very common parasite of humans but rarely causes disease, and that the parasite can cross the placenta and damage the fetus. The life cycle of *T. gondii* remained

elusive until 1970 when William Hutchison and his colleagues demonstrated that this parasite is a stage in the life cycle of a common intestinal coccidian of cats (Hutchison et al. 1970), an observation quickly confirmed by other workers who had been working on this problem at the same time (see Cox 2002).

The parasitological significance of this discovery was that until then it had been assumed that the intestinal coccidians of vertebrates had only one host. The discovery of the *T. gondii* life cycle initiated a massive search for similar phases in the life cycles of other coccidian parasites. As a result, a number of protozoa that had not been properly identified could be classified as stages in the life cycle of poorly understood coccidians that had occasionally been encountered in the intestine and tissues of humans and other animals and in which transmission depends on predator–prey relationships (see *Sarcocystis*). The species that infect humans are *Sarcocystis hominis*, acquired from cattle, and *S. suihominis*, acquired from pigs, and a complex of species collectively known as *S. lindemanni*. There is a detailed account of the nature and discovery of these species by Tadros and Laarman (1982).

CRYPTOSPORIDIUM, *CYCLOSPORA*, AND *ISOSPORA*

Humans also harbor three other species of coccidia, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and *Isospora belli*, once regarded as rare and accidental curiosities but now identified as possible pathogens in AIDS patients and immunocompromised individuals. All have simple single-host life cycles. *Cryptosporidium parvum* was discovered in 1912 by the American parasitologist Edward Ernest Tyzzer in the gastric glands of laboratory mice in which he had previously found another species, *C. muris* (Tyzzer 1912). The first sporadic human cases were recorded independently in 1976 by Nime and colleagues (Nime et al. 1976) and Meisel and colleagues (Meisel et al. 1976) but from 1981 onwards numerous new cases began to be recognized in AIDS patients. *C. parvum* is not very host-specific, and the source of these infections is probably drinking water contaminated with cattle feces. *Cryptosporidium* infections are now known to be very common and have caused a number of epidemics. There are histories of human cryptosporidiosis by McDonald (1996) and in Dubey et al. (1990).

Cyclospora cayetanensis, another coccidian that is mainly associated with AIDS, was discoved in 1979 by the English parasitologist Richard Ashford who found an unidentified coccidian in patients in Papua New Guinea (Ashford 1979), but it received little attention until 1986 when it was found again in the stools of patients with HIV (Soave et al. 1986). In 1993, this parasite was formally named *Cyclospora cayetanensis* (Ortega et al. 1993) and since then it has been identified as the cause of a number of outbreaks of diarrhea and fatigue in both immunocompetent and immunosuppressed individuals but the reservoir host, if any, is not known.

Isospora belli, the last of this group of parasites, was discovered in 1915 (Woodcock 1915) and is another coccidian frequently found in asymptomatic immunocompetent individuals but is associated with diarrhea in AIDS patients.

MICROSPORIDIANS

Microsporidians are common spore-forming parasites of vertebrates closely related to the Fungi (Cavalier-Smith 1998) but traditionally regarded as parasites. During the 19th century microsporidians attracted considerable attention, mainly as parasites of invertebrates, following the discovery of spores of *Nosma bombycis* by Nägeli in 1857 while investigating an outbreak of a disease called pébrine in the silkworm *Bombyx mori* (Nägeli 1857) and subsequent detailed investigations by Louis Pasteur in 1870 (see Vallery-Radot 1924). In the last decade of the 19th century, and throughout the 20th century, increasing numbers of species of microsporidians were discovered in fish, amphibians, reptiles, birds, and mammals (see Canning and Lom 1986). There have also been several sporadic reports of what might have been human microsporidial infections, but it is difficult to interpret the various structures that have been described as spores. The first human case was probably that of '*Encephalitozoon chagasi*' in a newborn baby in 1927 (Torres 1927) but this cannot be confirmed because the original material has been lost. The parasite named *Encephalitozoon hominis* by Wolf and Cowen (1937) is now known to have been *Toxoplasma gondii*. The first authenticated record of human microsporidiosis was in 1959 when Hisakichi Matsubayashi and his colleagues in Japan found an *Encephalitozoon* sp. in a boy with convulsions (Matsubayashi et al. 1959). Thereafter, there have been increasing numbers of reports of cases of microsporidian infections in humans. In 1985 a new microsporidian, *Enterocytozoon bieneusi*, was found in an AIDS patient (Desportes et al. 1985) since when about 14 species, belonging to seven genera, have been associated with fulminating infections in immunodepressed patients, some with less serious infections in immunocompetent individuals (Canning 2001), and the number of cases, particularly in AIDS patients, continues to rise. Very little is known about the transmission and epidemiology of the microsporidians. There is a detailed account of the microsporidians in

Microsporidiosis.

SUMMARY AND CONCLUSIONS

The history of parasitology is a fascinating one, and parasites have been the subject of some of the most exciting discoveries in the field of medicine. In this chapter, it has not been possible to do more than merely

touch on the major events, each one of which has represented the culmination of years of observation, conjecture, and experimentation. The personalities involved have been among the most eminent of their generations, but the cooperation and competition that existed between them would require a book on its own. The history of parasitology to a large extent reflects the availability of new concepts and techniques; thus each discovery has been the product of its own time and has been possible only because particular individuals have been willing and able to exploit new knowledge as it became accessible. There is more to the history of parasitology than this, because most of the great discoveries were made by individuals who were not looking in the same direction as everybody else. The conviction that diseases arose from decay and the air we breathe held back the discovery of the microbial causes of diseases despite the fact that, with hindsight, all the clues had been there. Had the helminthologists been able to persuade others not only that worms do not arise de novo, but also that they cause disease, the germ theory would have emerged two centuries earlier. The germ theory, with its emphasis on the search for bacterial causes of disease, held back the discovery of the protozoal causes of disease; in another context, it also inhibited a logical approach to nutritional disorders. Those who broke through the barriers of preconceived ideas, no matter how fashionable, were the ones who made the greatest discoveries. The history of 20th-century medicine will pinpoint the mistakes made in the study of AIDS and bovine spongiform encephalopathy (BSE) and will, almost certainly, tell us that our approaches to such subjects as vaccines and drugs were misplaced. For the present, it is widely assumed that all the important discoveries have been made; moreover, the climate of research at the beginning of the 21st century favors large teams working on relatively small projects. It is difficult to imagine that any future discoveries in the field of parasitology will engender the excitement of many of those outlined in this chapter, but there will certainly be new inventions and developments. There will, for example, be new drugs and new vaccines; new species will be described, and molecular techniques will be used to unravel details about both the parasites and the diseases they cause. These developments will bring fame and fortune, membership of learned societies, and possibly Nobel Prizes, but the most important achievements will probably go unsung. The campaign for the eradication of Guinea worm is reaching its final stages, the eradication of river blindness is well on its way, there is now an ambitious program to eradicate lymphatic filariasis and, in many parts of the world, malaria is being controlled by the use of insecticide-impregnated bed nets. These events, which will probably not be mentioned in any publications other than specialized reports, will be much more important to those suffering from these diseases than any other more spectacular discoveries or advances. The future history of parasitology will be, and should be, written in terms of the development of the underprivileged parts of the world that have borne the burden of these diseases for far too long.

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World-wide importance of parasites

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PARASITIC DISEASES AND GLOBAL HEALTH

Introduction

This chapter describes the prevalence and overall distribution of parasitic infections in the world, the burden they constitute to the human population, and the costs they represent to individuals and society.

In 1993, life expectancy in the least developed countries of the world was 43 years, compared with 73 years in developed countries. Although life expectancy was projected to increase to 79 years by the year 2000 in developed countries, it was expected to remain less than 60 years in 45 developing countries (WHO 1995a). About 51 million people died in the world in 1993, 39 million of them in the developing countries. Worldwide, communicable diseases were responsible for c. 40 percent of the total number of deaths (20 million). In contrast, 80 percent (16 million) of deaths in developing countries were due to infectious and parasitic diseases (WHO 1995a). Five years later, communicable diseases, including bacterial, viral, parasitic, and zoonotic diseases, still caused 13.3 million (25 percent) of the 53.9 million deaths that occurred in 1998. In that year, communicable diseases were the main cause of death among children aged 0–4 years (63 percent of all deaths), and of premature deaths (48 percent of all deaths) in ages 0–44 years. They were also the main cause of death (45 percent of all deaths) in low-income countries such as those from South East Asia and Africa (WHO 1999). Six diseases were the leading infectious disease killers: acute respiratory infections (including pneumonia and influenza), acquired immune deficiency syndrome (AIDS), diarrheal diseases, tuberculosis, malaria, and measles (WHO 1999). Even in 2001, 19 percent of the 56.554 million deaths that occurred in the world were caused by infectious or parasitic diseases. Lower respiratory tract infection was the main killer (3.871 million deaths), followed by HIV/AIDS (2.866 million), diarrheal diseases (2.001 million), tuberculosis (1.644 million), malaria (1.124 million), and measles (745 thousand deaths) (WHO 2002).

Communicable diseases occur worldwide. Poverty and its accompanying features (lack of sanitation, malnutrition, illiteracy, and overcrowding) all contribute to most of the parasitic diseases of public health importance. Although infectious and parasitic diseases are more significant causes of morbidity and mortality among the poor, no-one is exempt from infection. Internal and external parasites are among the most common pathogens worldwide and anyone can be exposed to them at home or when they travel.

The burden of parasitic diseases

A comprehensive study by the World Bank (1993a) attempted to measure the burden that diseases, as a group or individually, represent in different regions of the world. The measurement took into account both losses from premature death (defined as the difference between the actual age of death and the age at which death would have occurred in a population with a low mortality) and the effects of losses caused by weakened health. The effects of the loss of healthy life were quantified for the diseases listed in the 109 categories

included in the International Classification of Diseases (WHO 1977) and for approximately 95 percent of possible causes of disability (World Bank 1993a). To evaluate losses from illness, the incidence of cases by age, sex and demographic region was estimated and the number of years of healthy life lost was obtained by multiplying the expected duration of the disease by a severity weight that estimated the severity of the disability in comparison with loss of life caused by the illness (World Bank 1993a). The global burden of disease estimated in this manner was quantified using a unit called the disability adjusted life year (DALY), one DALY being defined as the loss of 1 year of healthy life to disease. The relative global burden of a specific disease was estimated by comparing the DALYs lost from different diseases or groups of diseases. In many areas of the world, communicable diseases continue to impose a significant burden that is greater in developing countries than in countries with established market economies. Injuries also cause greater losses in developing countries than in developed countries (Figure 1).

Parasitic diseases contribute heavily to the burden produced by communicable diseases, their contribution being comparable with such common diseases as tuberculosis, sexually transmitted diseases, diseases preventable by vaccination, and acute respiratory infections. Malaria is the parasitic disease responsible for the greatest burden worldwide; in Africa its burden is almost as great as that produced by acute respiratory infections. Worm infections are also a significant burden in Africa and some other regions (Figure 2) (World Bank 1993b).

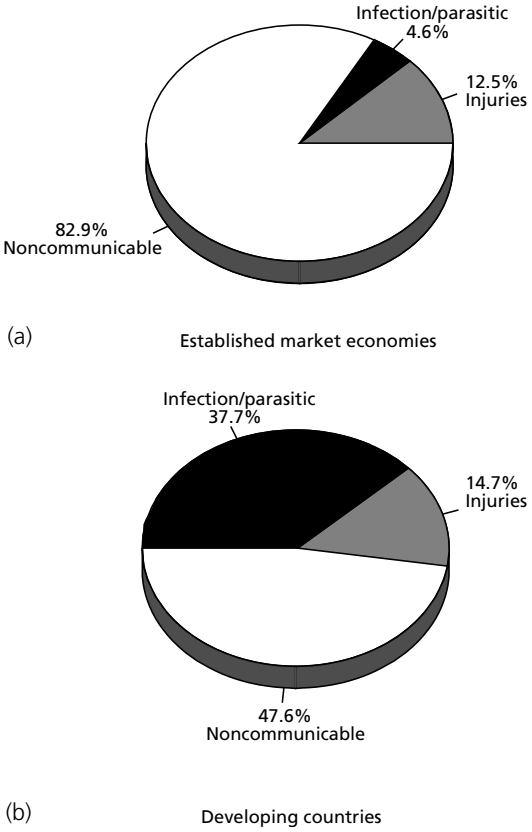

Figure 1 *Percentage distribution of DALYs lost by overall cause in* **(a)** *countries with established market economies and* **(b)** *developing countries (data from World Bank 1993b).*

The burden of infectious diseases, including parasitic infections, is borne mainly by the poorest people in the poorest countries of the world. Parasitic diseases are thus contributing to inequalities that exist both within and between societies (Evans and Jamison 1994). In Figure 3, the DALYs lost as a result of infectious diseases of microbial and viral origin are compared with the losses caused by parasitic diseases in developing countries and in countries with established market economies. Although the burdens produced by diseases of viral or microbial origin are the highest, the burden produced by parasitic diseases is also great (World Bank 1993b). The effects and burdens produced by parasitic diseases vary greatly from region to region. Different parasitic diseases are responsible for the bulk of the burden in different geographical areas; for example, malaria and schistosomiasis are the diseases that cause the highest burden globally, but Chagas disease produces the highest burden in Latin America and the Caribbean (Figure 4).

The burden of different communicable diseases also varies with age (Table 2.1). Among parasitic diseases in 1990, malaria produced the highest burden in children under 5 years of age, and intestinal worms produced the highest burden in children aged 5–14 years (Table 2.1). The situation remained the same in 2001: independent of age, malaria was still the parasitic disease that produced the highest burden among all parasitic diseases (Table 2.2) (WHO 2002).

Disease burdens vary not only with age and geographical region but also among demographic groups. Not surprisingly, a significant proportion of the deaths caused by communicable diseases occurs in developing countries (Tables 2.3 and 2.4) and the individuals at greatest risk are those living in rural areas and in urban squatter settlements. Such people are usually poor and lack adequate housing, safe water supplies, and good waste-disposal systems. Workers involved in mining and timber exploitation are also often exposed to parasitic diseases as an occupational hazard (Rosenfield et al. 1984).

Population movement and parasitic diseases

Population movements have helped to raise awareness of parasites as a worldwide public health problem. In the early 1990s, 500 million people crossed international borders by air, 18–20 million refugees were generated from conflicts and 20–30 million people were displaced

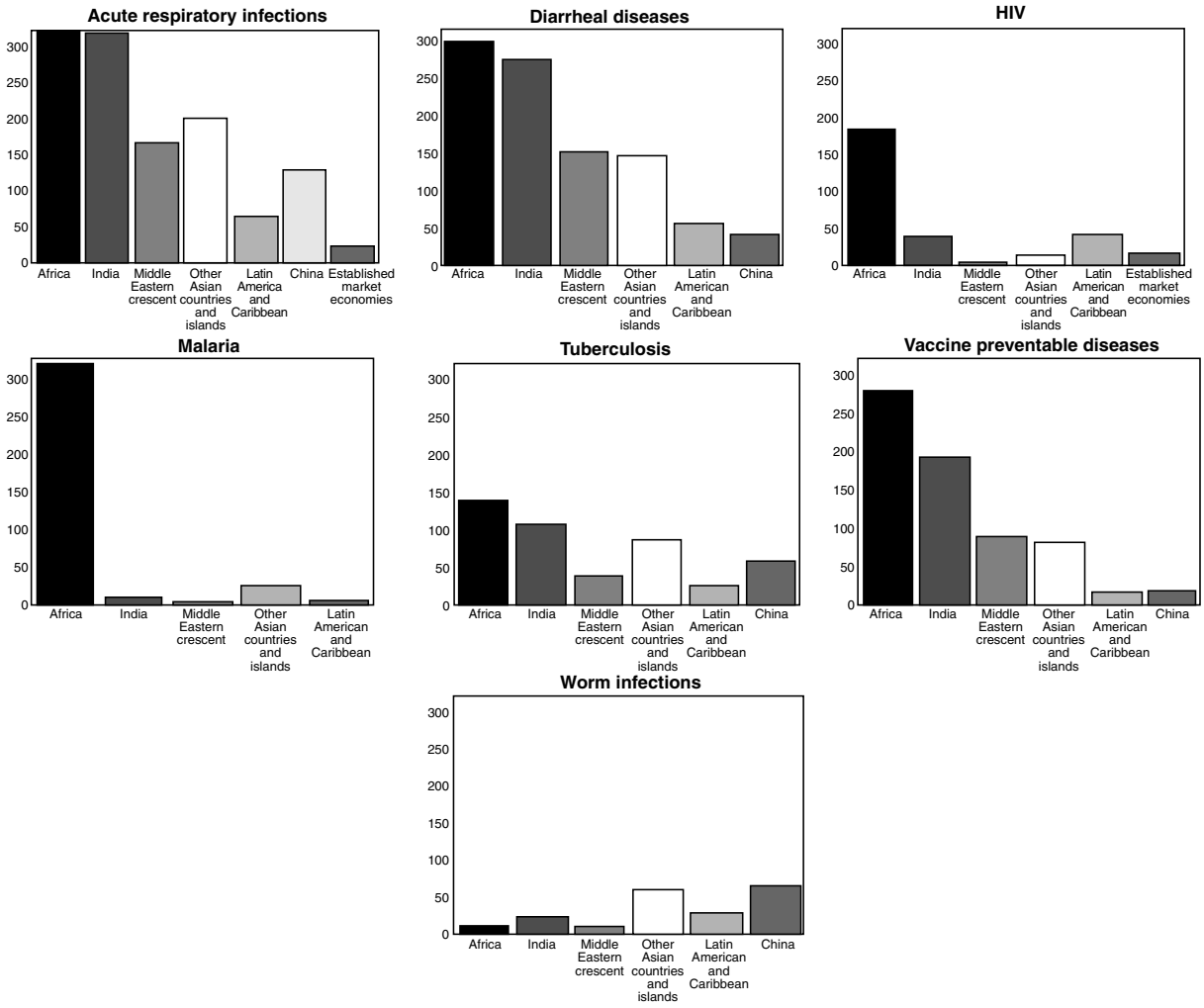

Figure 2 *Distribution of DALYs lost by selected communicable diseases by region, 1990 (hundreds of thousands of DALYs lost) (data from World Bank 1993b).*

for other reasons (Wilson 1995; WHO 1995b). Of the 5.2 billion people in the world, 1 in every 134 was forced to leave their home in 1992 (WHO 1995b) and an estimated 70 million people, mostly from developing countries, work in countries to which they have migrated (Wilson 1995). Many of these people have carried parasites to their new homes, sometimes requiring treatment themselves and sometimes serving as sources of infection to others. As a result of these population shifts, medical personnel in many advanced countries have gained personal experience of parasitic diseases for the first time.

The USA exemplifies the problems faced by the countries with immigrants with intestinal parasites. It is estimated that more than 600 000 immigrants enter the USA each year from countries where intestinal parasites are endemic: Asia, the Middle East, sub-Saharan Africa, Eastern Europe, Latin America, and the Caribbean. At entry, people with parasitic infections may be asymptomatic, and stool examination is not sensitive enough as a screening method for detecting all of those infected. There is, however, a broad-spectrum antiparasitic drug, albendazole, which is safe and effective in eradicating many intestinal worms; it would be possible to screen all immigrants for parasites and to treat those infected or to treat all immigrants presumptively (400 mg of albendazole per day for 5 days). Another possibility would be to do nothing unless the immigrants have symptoms (watchful waiting). Estimates of the costs of these three types of intervention were made, and effectiveness was expressed both in terms of the cost of treatment per DALY averted and in terms of the cost per hospitalization averted. Presumptive treatment of all immigrants at risk for parasitosis, when compared with watchful waiting, would avert at least 870 DALYs, prevent at least 33 deaths and 374 hospitalizations, and save at least US$4.2 million per year. Screening compared with watchful waiting would cost US$159 236 per DALY averted. It was therefore concluded that presumptive treatment of all immigrants at risk of parasitosis would save lives and money, while universal screening and treatment only of those with positive stools,

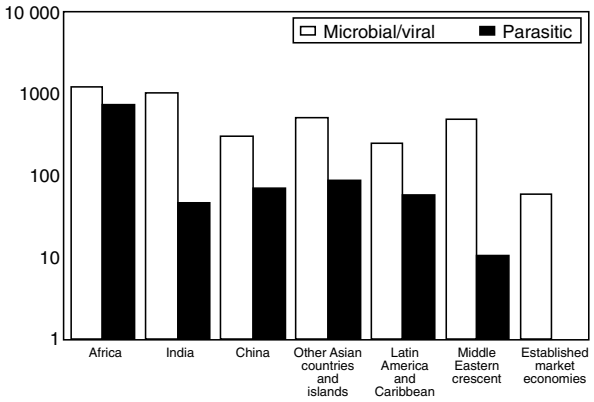

Figure 3 *Burden of disease caused by parasites (including malaria, African and American trypanosomiasis, schistosomiasis, leishmaniasis, filariasis, onchocerciasis, ascariasis, trichuriasis, and hookworm) and by microbial and viral infections (including tuberculosis, sexually transmitted diseases, HIV, diarrheal diseases, pertussis, poliomyelitis, diphtheria, measles, tetanus, meningitis, hepatitis, and acute respiratory infections) by region, 1990 (hundreds of thousands of DALYs lost) (data from World Bank 1993b).*

would save lives but would be less cost-effective (Muennig et al. 1999).

In addition to international migration, many people also migrate between regions of a country; in the last six decades, economic hardship in the rural areas of developing countries has stimulated migration to urban areas within the same country. As a result, c. 45 percent of the world's population now live in cities, and diseases that were traditionally considered to be 'rural' have spread into the cities, usually affecting the most deprived populations. This phenomenon has brought many people within the orbit of public health services for the first time and diseases previously not noticed have now made themselves apparent. It has also increased demands on already overburdened public health services. From their bridgeheads in the slums, the parasites may spread to other urban areas, reaching new hosts through vectors, through fecal contamination of soil, food, and water, and through transfused blood.

Transfusion-acquired malaria (especially *Plasmodium vivax* malaria) and Chagas disease pose particular problems as they are both easily transmitted by blood transfusion. Other parasites, such as the African trypanosomes (particularly *Trypanosoma gambiense*), *Babesia microti*, and the filarial worms such as *Brugia malayi*, *Loa loa*, *Wuchereria bancrofti*, *Mansonella ozzardi*, and *M. perstans*, may also be transmitted by blood transfusion but fortunately this mode of transmission is rare even in the endemic countries. It is not surprising that malaria has resulted from blood transfusion as plasmodia can remain viable in refrigerated blood for up to 10 days. In developing countries, transfusion malaria is most common in people who have received blood sold by low-income donors whereas in developed countries, transfusion malaria results from the migration of carriers

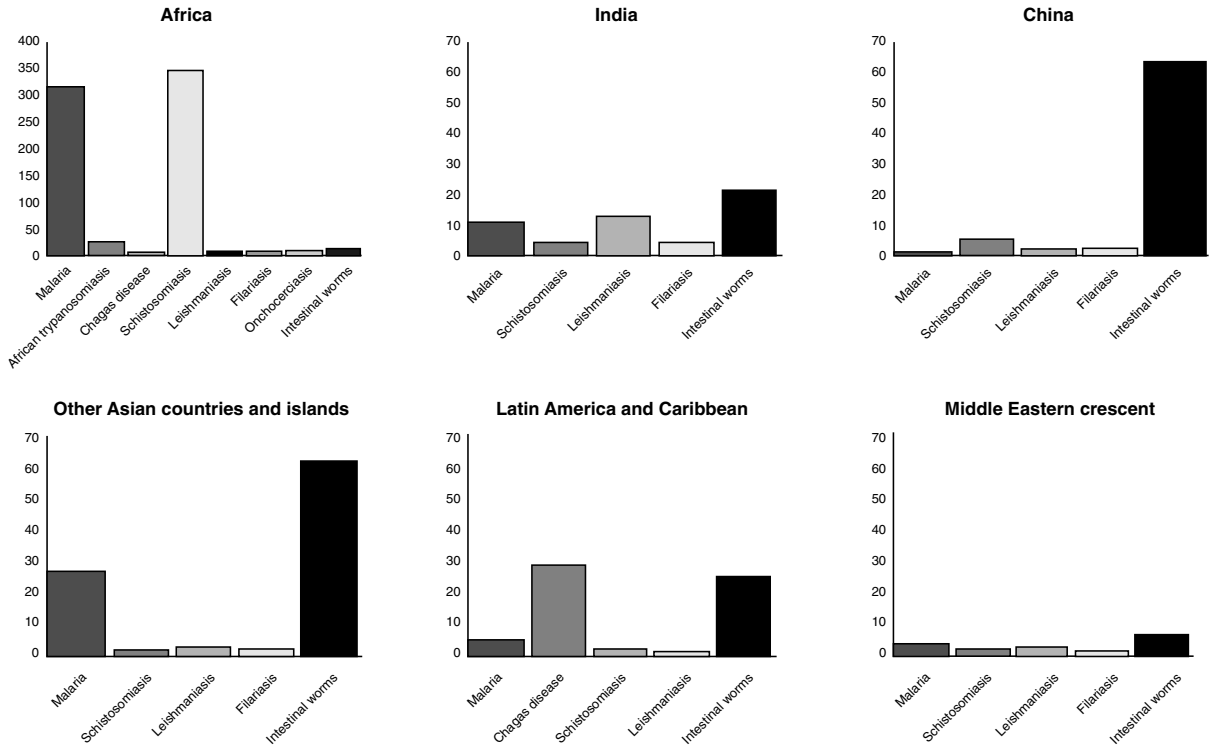

Figure 4 *Burden of disease caused by various parasitic diseases by regions, 1990 (hundreds of thousands of DALYs lost) (data from World Bank 1993b).*

Table 2.1 *Main causes of disease burden in children from developing countries, 1990*

Disease	DALYs lost (millions) ⩽4 years old	%	Disease	DALYs lost (millions) 5–14 years old	%
Acute respiratory infections	93.2	18	Intestinal helminths[b]	16.8	11.8
Diarrheal diseases	82.5	16	Vaccine preventable diseases	11.8	8.3
Vaccine preventable diseases[a]	55.1	10.6	Acute respiratory infections	10.4	7.36
Malaria	24.3	4.7	Diarrheal diseases	9.3	6.5
Tuberculosis	2.6	0.5	Tuberculosis	6.8	4.85
			Malaria	6.5	4.57
Total DALYs lost[c] (millions)	518.0			142.0	

Adapted from World Bank 1993b
a) Pertussis, polio, measles and tetanus; b) *Ascaris* and *Trichuris*; c) Communicable, noncommunicable and injury DALYs.

from endemic areas. To avoid such infections, blood donation is not permitted in some countries from those who have had malaria or who have visited or lived in malarious areas. Individuals with positive serology for *T. cruzi*, the causative agent of Chagas disease, have been found among the 7 million or more legal immigrants from South and Central America to the USA. Their presence poses a risk of transmission of Chagas disease by blood transfusion. A similar risk exists in Europe where more than 250 000 immigrants from Central and South America now live (Schmunis 1994). Transfusion-transmitted *T. cruzi* infection has been reported in

Table 2.2 *DALYs lost (millions) due to nine 'highest worldwide burden of disease' communicable diseases (all ages) 2001*

Acute respiratory infections	HIV/AIDS	Diarrheal diseases	Malaria	TB	Measles	Tropical diseases[a]	Pertussis	Sexually transmitted diseases
94	88	62	42	36	26	13	12	12

WHO 2002
a) African trypanosomiasis, Chagas disease, schistosomiasis, leishmaniasis, lymphatic filariasis, and onchocerciasis

Table 2.3 *Deaths by cause and demographic group according to types of country and age group, 1990*

	Developing countries		Formerly socialist economies of Europe and established market economies		
Disease	Deaths (thousands) ⩽4 years	Deaths (thousands) ⩾5 years	Deaths (thousands) ⩽4 years	Deaths (thousands) ⩾5 years	World
Acute respiratory infections	2 710	1 274	21	309	4 314
Diarrheal diseases	2 474	392	4	3	2 873
Tuberculosis	71	1 907	0	38	2 016
Malaria	633	294	0	0	926
African trypanosomiasis	5	51	0	0	55
Chagas disease	0	23	0	0	23
Schistosomiasis	1	37	0	0	38
Leishmaniasis	7	46	0	0	54
Onchocerciasis	0	29	0	0	30
Ascariasis	0	13	0	0	0
Trichuriasis	0	10	0	0	9
Hookworm	0	6	0	0	0
Lymphatic filariasis	0	0	0	0	0

Data from World Bank 1993b

Table 2.4 *Annual deaths by selected infectious and parasitic disease*

Selected infectious or parasitic disease	Deaths (thousands)				
	1990[a]	1993[b]	1995[c]	1999[d]	2001[e]
Acute respiratory infections (upper and lower)	4 314	4110[f]	4 416	4 010	3 947
HIV/AIDS		700	1 063	2 673	2 866
Diarrheal diseases	2 873	3 010[f]	3 115	2 213	2 001
Tuberculosis	2 016	2 709	3 072	1 669	1 644
Malaria	926	000	1 423	1 086	1 124
Diseases preventable by vaccination		1 679	2 100	1 554	1 318
Sexually transmitted diseases				178	189
Meningitis		245	35[g]	124	173
Hepatitis		933[g]	1 156[h]	171[h]	127[i]
African trypanosomiasis	55	55	20	66	50
Chagas disease	23	45	45	21	13
Leishmaniasis	54	197	20	57	59
Schistosomiasis	38	200	80	14	15
Onchocerciasis	30	35	47	0	0
Ascariasis	13	60	60	3	4
Hookworm	6	90	65	7	4
Trichuriasis	9		10	2	2
Total world deaths		51 000	51 882	55 965	56 554

a) World Bank 1993b; b) WHO 1995a; c) WHO 1996; d) WHO 2000a; e) WHO 2002; f) Children under 5 years; g) Meningococcal; h) HBV; i) HBV and HCV.

Canada, the USA, and Spain (Villalba et al. 1992; Schmunis 1991). Infection may occur in nonendemic areas by means other than transfusion; for example, infection in a migrant was the cause of a case of congenital *T. cruzi* infection in Sweden.

As air travel has become more common, imported malaria has been seen with increasing frequency in Canada, the Caribbean, Europe, Oceania, and the USA and it has been stated that patients with malaria are now more often seen in temperate London (England) than in tropical Jamaica (West Indies) (Alleyne 1983). In the UK, 8 353 cases of imported malaria were diagnosed between 1987 and 1992 (WHO 1999) and more than 1 000 cases of imported malaria are detected yearly in the USA. For travelers in most areas of Asia, the species of *Plasmodium* responsible for malaria is usually *P. vivax*, whereas for travelers in Africa and some parts of Asia it is *P. falciparum*, and either or both species may occur in travelers in the Americas. There is a constant risk that travelers infected with plasmodia who return to areas where there are *Anopheles* mosquitoes susceptible to infection may start local transmission (Maldonado et al. 1986; Phillips-Howard 1991; López-Antuñano and Schmunis 1993). Malaria has also been reported in individuals living in nonmalarious areas who have never left that area and have never had a blood transfusion. Most cases have been acquired by people living near or passing through airports and have probably originated in airplane-transported infected mosquitoes.

Food-borne trematodes, such as *Opisthorchis* spp., are responsible for diseases in migrants from South East Asia and the eastern Pacific, and a high prevalence has been reported in refugees in camps in Cambodia and Laos and in Thai immigrant workers in Kuwait and China. Pulmonary paragonimiasis has been diagnosed in refugees from South East Asia residing in France and in the USA. Liver flukes have been found in immigrants from Asia to Europe and the USA (WHO 1995b). Ethiopian refugees in Israel also harbor liver flukes. The round worm *Strongyloides stercoralis* and the protozoa *Giardia duodenalis* and *Entamoeba histolytica* are among the parasites often acquired by migrants and by travelers to developing countries.

NON-HELMINTH PARASITES

African trypanosomiasis (sleeping sickness)

Trypanosomiasis, caused by *T. brucei gambiense* and *T. brucei rhodesiense*, is endemic in rural areas of 36 countries of sub-Saharan Africa, particularly in West, Central, and East Africa in areas where the tsetse fly is present. Sleeping sickness caused by the trypanosomes has eliminated entire communities in Africa. Outbreaks of sleeping sickness produced by *T. b. gambiense* continue to occur in Angola, Congo, Sudan, Uganda, and Zaire and *T. b. rhodesiense* is a serious threat in Tanzania and Mozambique. Sporadic cases of sleeping sickness have also been reported from some southern African countries (WHO 1986, 1994a). Even tourists to

certain areas of Africa can be affected, as shown by recent cases in UK travelers returning from East Africa (Moore et al. 2002). It is estimated that over 55 million people in 36 countries are at risk of acquiring sleeping sickness and that 250 000–300 000 people are infected. The yearly incidence is difficult to ascertain because surveillance is poor in the c. 200 confirmed foci of endemic sleeping sickness. The number of cases reported annually varies from 20 000 to 25 000 (WHO 1986, 1994a), and the estimated annual mortality 20 000 to 70 000 from 1990 to 2001. (World Bank, 1993b; WHO 1995a, 1996, 2000a, 2002).

The impact of the animal trypanosome disease, nagana, is also great, depriving much of Africa of milk, meat, and draught oxen for plowing and transport. The cost of these problems is more than US$4.5 million per year (Kabayo 2002).

Projects for economic development, if planned without proper consideration for their effects on the environment and on health, can cause unexpected health problems. Development projects for producing electricity and improving agriculture may change the local ecology and facilitate the spread of trypanosomiasis and other parasitic infections. For instance, they may encourage people to migrate to areas where their contact with parasites and their vectors is increased. Dams may displace villages, forcing people into tsetse-infested grasslands. Extensive lake margins may encourage growth of vegetation in which tsetse flies can breed, increasing the risk of bites among people who come to collect water. Such exposures may cause recrudescence of infection and even bring about full-blown epidemics (WHO 1986). Political upheavals and droughts may also stimulate life-style changes, many of which facilitate the spread of diseases. One of the most common consequences of civil disorder and of drought is the mass movement of people. While in transit, they are vulnerable to disease and many may become infected if they migrate into or across trypanosomiasis endemic areas.

If untreated, both forms of African trypanosomiasis are fatal, but there are important differences between the two forms of the disease. *T. b. gambiense* infections may run a protracted course of several years and infected individuals may serve as sources of infection if they migrate to areas where susceptible hosts and vectors are present. The disease caused by *T .b. rhodesiense* in East Africa causes death in weeks or (at most) a few months and, therefore, carriers pose less of a threat to individuals in uninfected areas. Melarsoprol has been used for the treatment of African trypanosomiasis for decades and eflornithine, which is much more expensive but has fewer side-effects and improves the overall cure rate, has been introduced for treatment more recently. An economic study in Uganda indicated that melarsoprol treatment is associated with an incremental cost per life and DALY saved of US$209 and US$8, respectively. Each additional life saved by switching from melarsoprol alone to a combination of melarsoprol and eflornithine would cost an extra US$1 033 per life saved, and an extra US$40.9 per DALY gained. Shifting from this second alternative to treatment of all patients with eflornithine would lead to an incremental cost per life saved of US$4 444 and an incremental cost of US$166.8 per DALY gained (Politi et al. 1995).

Programs for control of sleeping sickness are based on a combination of active and passive medical surveillance and on vector control. Costs include those incurred as a result of death and loss of productivity due to illness and those incurred as a result of the cost of treatment and disease control. The estimated cost from loss of income due to premature death from sleeping sickness is US$615–761 per person, varying in different areas and among different segments of society; the cost per individual is higher for those with a higher income. In 1984–85, the cost of surveillance was estimated at US$0.79–1.39 per person depending on the strategy used. The cost of treatment, including hospitalization, was US$35, US$88 and US$133 per person for treatment with pentamidine, suramin, and melarsoprol, respectively. Costs for prevention using screens and traps varied from US$2.30 to 11.50 per person. Ground-spraying costs were estimated at US$200–1000 per km. The cost per person protected by screens and traps depended on the number of people served per screen or trap and on the density of the population in the area under surveillance (WHO 1986).

American trypanosomiasis (Chagas disease)

Chagas disease occurs frequently in South and Central America, from Mexico in the north to Argentina and Chile in the south. Most of the people infected with *T. cruzi* in the Americas live in poor rural or semi-urban areas. Infected individuals have been detected in all Central and South American countries, including a few in French Guiana, Guyana, and Surinam. The only Spanish-speaking countries in the western Hemisphere where human *T. cruzi* infections have not been found are Cuba and the Dominican Republic. American trypanosomiasis also occurs as a zoonotic disease in the USA, where its range extends to Northern California in the west and to Maryland in the east.

Chagas disease is a major public health problem in Latin America. About 16–18 million people were considered to be infected and 90 million others exposed to the risk of infection in the 1980s. More recent data suggest that there are 11 million infected individuals (Table 2.5) and that 2–3 million have the cardiac and/or hollow visceral lesions that characterize the chronic stage of the disease. Many infected individuals show few or no clinical symptoms of disease and thus may not seek treatment. Infected individuals who are not treated

Table 2.5 *Infection by* Trypanosoma cruzi *in the Americas based on data for 1980–85 or later estimates*

Country	Endemic area (thousands km^2)	% total country area	Estimated population at risk		Estimated infected population		Estimated yearly incidence
			Number (thousands)[a]	% total population	Number (thousands)	% total population	
Argentina	1 946	70	6 900	23	2 333	7.2	[b]
Bolivia	1 300	100	2 834	55	1 134	22.2	86 676
Brazil	3 615	42	41 054	32	1 961	1.21	[b]
Chile	350		1 800	15	142	1.06	[b]
Colombia	200	18	3 000	10	900–1 300	3.3–4.02	39 162–31 330
Costa Rica	...	...	1 112	45	130	5.3	4 030–3 320
Ecuador	100	35	3 823	41	30–450	0.34 to 4.09	7 488–13 365
El Salvador	...	...	21	43	322	6.9	10 048
Guatemala	...	...	4 022	52	730	9.8	30 076–28 387
Honduras	...	...	1 824	42	300	7.4	9 891–11 490
Mexico	...	...	...	...	152–540	0.18–0.63	14 2880
Nicaragua	...	...	...	...	67	1.82	5 016–2 660
Panama	...	...	898	42	220	10.6	7 130–5 346
Paraguay	...	...	1 475	45	397	11.59	14 680
Peru	120	9	6 766	34	643	3.47	19 072–24 320
Uruguay	125	71	975	33	37–51	1.25–1.59	[b]
Venezuela	697	76	11 392	68	800–1 200	7.42–4.14	17 9703–22 960

Data from Schmunis 1994, 2000

a) Total population the year the study was made or average population for the years the study was made (Schmunis 1994). Incidence data from 1990 and 1997 (Hayes and Schofield 1990; Schofield and Dujardin 1997);

b) Incidence data from these countries are not included because the beneficial effect of control programs is not considered in the calculation (Hayes and Schofield 1990).

... No data.

early after infection or in the early chronic stage of the infection will remain infected throughout their lives. The majority of cases of human infection occur in rural areas, particularly where houses are colonized by the triatome bugs that transmit the infection. The extent of vector transmission in the human population is related to socioeconomic status; poor people living in inadequate housing being most commonly infected. The infection persists wherever living conditions permit intimate contact between the triatome bugs and the human host. Prevalence in the general population is 0.34 percent in Ecuador, 1.25 percent in Uruguay, 11.5 percent in Paraguay, and 22.2 percent in Bolivia. Incidence estimates range from 3 320–4 030 for Costa Rica to 22 960–179 703 for Venezuela (Table 2.5) (Hayes and Schofield 1990; Schmunis 1994; Schofield and Dujardin 1997).

Transfusion is the second most common mode of acquiring the infection. Infection rates in blood donors vary from 6 to 12 percent in some areas of Argentina, Brazil and Chile and from 2 percent in Honduras to 48 percent in Bolivia. In 1997, six countries had more than 99 percent screening coverage for *T. cruzi*, but seven countries still showed risk of transfusion-transmitted infection that year (Schmunis et al. 2001). The risk of receiving an infected blood unit and acquiring a transfusion-transmitted infection has been reduced with time due to improvements in screening coverage (Schmunis 1999)

Congenital transmission, the third most common route of acquiring the infection, occurs in at least 3–5 percent of babies born to infected mothers (Schmunis 1994).

Chagas disease is a debilitating and incapacitating chronic condition that develops in 20 percent of those infected with *T. cruzi*, killing from 23 000 (World Bank 1993b) to 45 000 (Moncayo 1993) people every year in the early 1990s. Recent estimates suggest that mortality has decreased to 13 000 per year (WHO 2002). Chagasic cardiopathy was ranked third as a cause of disability in a rural area of Brazil where *T. cruzi* infection is endemic (Dias et al. 1985) and was also the main cause of early retirement in the region (Lopes and Chapadeiro 1986). In another endemic area of Brazil, 4–9 percent of all incapacity benefits received by individuals 30–50 years old were attributed to the effects of Chagas disease (Zicker and Zicker 1985). The number of productive years lost because of the disease among individuals 15–64 years of age in endemic areas of Brazil was estimated to be 2.275 and 1.369 per 100 000 population for males and females, respectively (Pereira 1984). Using data from the World Bank (1993b) study and from other sources, it is possible to compare the burden and general costs of Chagas disease with those of other diseases (World Bank 1993b). The disease burdens produced in 1990 by malaria (35 700 000 DALYs) and schistosomiasis (4 500 000 DALYs) world-wide are higher than that produced by Chagas disease (2 740 000 DALYs), but the Chagas disease burden is higher than those produced by leishmaniasis, African trypanosomiasis, leprosy, filariasis, and onchocerciasis (Figure 4). In Latin America, the burden of Chagas disease exceeds that of the so-called tropical diseases (i.e. malaria, schistosomiasis, and leishmaniasis) and intestinal worms. In Latin America, tropical diseases together produced a disease burden that was only about one-quarter of that caused by Chagas disease. The disease burden caused by Chagas disease is, moreover, the fourth highest among all infectious diseases that occur in the region. Only acute respiratory infections, diarrhea, and AIDS produced higher disease burdens. The situation has improved since 1990, and the burden of Chagas disease is now considered to be 676 000 DALYs (WHO 2002).

As the affected population is mostly at a low socioeconomic level, the cost of treatment is usually provided by the State. In Brazil in 1987, the estimated cost for pacemakers and surgery to correct enlarged viscera was US$250 million and the cost of lost labor by the 75 000 chagasic workers represented a loss of another US$625 million (Dias 1987). These costs did not include consultation, care, and supportive treatment, costs for which amounted to another US$1 000 per patient per year (Schofield and Dias 1991), or disability payments which, in one Brazilian State in 1987, were US$400 000 (Dias 1987). In Argentina, treatment of an acute case cost US$591.80; treatment of an asymptomatic case cost US$ 174.49; and treatment of one with cardiac lesions cost US$603.62 (del Rey et al. 1995). In the same country, the cost for 30 months of treatment of 128 patients who had cardiac lesions was US$350 000 (Evequoz 1993). If all patients from Bolivia who needed treatment were treated, that country would spend US$215 000 each year for treatment of patients with acute Chagas disease; US$21 million for treatment of chronic disease and US$186 000 for treatment of congenital disease. In 1994, direct costs to Bolivia as a result of deaths from the disease were US$343 000 per year and annual indirect costs of morbidity and mortality were estimated at US$43.8 and 57.5 million, respectively (Human Development Ministry 1994). In Chile, the yearly cost of the disease was estimated at US$37 million in addition to the cost of pacemakers and surgery for implantation of pacemakers (Apt 1991).

In addition to the costs of treatment, there are also costs associated with the control of vectors responsible for Chagas disease. The annual cost for vector control through insecticide spraying, housing improvement, and health education in countries infested with the vector *T. infestans* is large: US$100 000 for Uruguay; US$300 000 for Chile; US$18 million for Argentina, and US$25 million for Brazil.

Brazil spent US$516 682 000 in prevention and control activities, mostly for vector control, from 1975 to 1995. In 1978, it was estimated that there were 3 573 000 individuals infected with *T. cruzi*, 3.1 percent of the

population; by 1995 the seropositivity rate had decreased to 1.3 percent, 1 961 000 infected individuals. Between 1975 and 1995, vector control prevented 277 000 new infections and 88 000 deaths while the expenditures prevented (expected benefits) were US$847 million. The loss of 1 620 000 DALYs, 41 percent from averted deaths and 59 percent from averted disability (Akhavan 2000), was also prevented. These data indicate the benefit of vector control. Sustained vector-control activities were responsible for the interruption of *T. cruzi* vectoral transmission in Uruguay in 1997, Chile in 1999, and in nine of the 11 endemic states of Brazil in 2000–02.

Leishmaniasis

Leishmaniasis is considered to be endemic in Africa, in most countries of the Americas and Asia, and in many countries of Europe. Most cases of cutaneous leishmaniasis (more than 90 percent) occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria. More than 90 percent of visceral leishmaniasis cases occur in Bangladesh, India, Nepal, and Sudan (WHO 1994b). In all its forms, there are 12–13 million cases of leishmaniasis worldwide; the annual incidence is 600 000 new clinical cases, and 350 million people are at risk of infection. The development of new territories, explosive migration, rapid urbanization and the associated environmental changes, are expanding the areas where human cutaneous leishmaniasis commonly occurs. Leishmaniasis is transmitted by sandflies and the clinical manifestations of infection vary depending on the species of *Leishmania* involved (WHO 1990a).

Cutaneous leishmaniasis is characterized by skin ulcers that heal slowly and leave scars. The species causing cutaneous leishmaniasis in the Old World are *L. tropica*, *L. major*, and *L. aethiopica*; the latter is also the etiological agent of diffuse leishmaniasis. In the New World, cutaneous leishmaniasis is mainly caused by *L. braziliensis*, *L. mexicana*, *L. amazonensis*, *L. venezuelensis*, *L. panamensis*, and *L. guyanensis*. *L. braziliensis* and *L. panamensis* are mainly responsible for mucocutaneous leishmaniasis, a condition in which cutaneous lesions spread to the mucosa of the mouth and nose and *L. mexicana* and related forms are responsible for diffuse cutaneous leishmaniasis in the New World (WHO 1990a; Rey 1991).

Cutaneous leishmaniasis is not usually life threatening, but clinical visceral leishmaniasis (kala azar) is often fatal if untreated. Visceral leishmaniasis is caused by *L. donovani* and *L. infantum* in the Old World and *L. infantum chagasi* in the New World. Although most infections are asymptomatic or subclinical, those associated with malnourishment often result in severe disease. In the Indian subcontinent, 400 000 new cases are estimated to have occurred each year with a case fatality rate of 5–7 percent. Large epidemics of kala azar occurred in India in 1978, 1982, and 1987 (WHO 1990a). An epidemic in Southern Sudan was first recognized in mid 1988 and, by 1993, 300 000–400 000 people were at risk of infection and 40 000 were thought to have died. In some villages, the population decreased by 30–40 percent as a consequence of the epidemic (WHO 1993a). Overall it is estimated that visceral leishmaniasis kills at least 50 000 people per year.

Prevention of cutaneous leishmaniasis is based on:

- case detection and treatment
- the destruction of sandflies and their breeding places
- the prevention of contact between humans and sandflies.

In addition to these measures, the canine reservoir must also be eliminated to prevent visceral leishmaniasis in the Mediterranean and American regions. Treatment costs (drug only) for cutaneous and mucocutaneous leishmaniasis vary from US$60 to 70 per person, in different regions. Control costs for visceral leishmaniasis are US$100 per person. When other related costs are included, the cost of each case of visceral leishmaniasis amounts to US$250. When treatment with amphotericin is needed (because of failure of other drugs to bring about cure) the cost of each case increases by US$45 (WHO 1990a, 1993a).

An epidemic of visceral leishmaniasis in Sudan provided an opportunity to obtain accurate data on excess mortality, the costs of intervention measures and their effects, thus making it possible to express the cost-effectiveness of intervention as the cost per DALY averted. The cost-effectiveness ratio of US$18.40 per DALY places the treatment of visceral leishmaniasis in Sudan among health interventions considered ‘very good value for money’ (i.e. interventions of less than US$25 per DALY) (Griekspoor et al. 1999).

Malaria

Malaria is the most important protozoal disease affecting humans. People suffering from malaria mostly live in poverty in rural areas where *Anopheles* mosquitoes are present. Over 40 percent of the world’s population remains exposed to varying degrees of risk from malaria and one or more types of malaria occur in 90 regions of the world (WHO 1994c, d, e). The global incidence of malaria is c. 120 million clinical cases each year, the parasite being carried by 300–400 million people. Countries in tropical Africa account for more than 80 percent of all clinical cases and more than 90 percent of all carriers (WHO 1992a, 1995a). The reporting of incidence figures for large regions tends to mask trends within countries and among countries in a region. The malaria situation has improved in some countries but has deteriorated in others. In India, the reduction in reported malaria cases from 9 million to 5 million between 1976 and 1984 was largely due to a

return to levels that had occurred before a peak just prior to 1976.

Some areas are particularly susceptible to epidemics of malaria. These include the 'frontier areas' in South East Asia and South America that have experienced substantial population increases and are now being rapidly developed. In the highly endemic areas of Africa, there has been little change in levels of infection, but epidemics associated with weather and population movement have occurred in areas of low endemicity (WHO 1992a). Worldwide distribution of malaria and major problem areas are shown in Figure 5 (WHO 1993b). Of the 5.3 million cases reported to the World Health Organization in 1992 (excluding the African Region), 75 percent were concentrated in six countries: Afghanistan, Brazil, Colombia, India, Sri Lanka, and Vietnam (WHO 1994c). Within these countries, malaria is concentrated in certain areas (WHO 1992a). In 1994, 27 million cases were reported from Africa, 1.1 million from the Americas, 3.5 million from South East Asia, 2.1 million from the western Pacific, 321 000 from the eastern Mediterranean Region, and 91 000 from Europe (WHO 1997a).

Severe and fatal malaria infections are usually caused by *P. falciparum*, the predominant species of malaria in tropical Africa *P. falciparum* is less common in the rest of the world. For certain areas, including 'frontier areas' and areas with civil war or other conflicts, illegal trade and mass movements of refugees, malaria mortality is probably greatly underestimated (WHO 1994c). For example, 1 428 deaths from malaria were reported from the Americas in 1986 but the annual malaria mortality for the Brazilian Amazon region alone (a 'frontier area') was between 6 000 and 10 000. Implementation of early diagnosis and prompt treatment through strengthening the local health services, has drastically decreased mortality by falciparum malaria in the Amazon Region of South America.

The vast majority of deaths from malaria occur in Africa, where the death toll so caused is now estimated to be at least 1 million per year in children (WHO 1993b). Between 1970 and 1975, malaria was responsible for 20–30 percent of infant mortality in Kenya and Nigeria and, in the Gambia, mortality from malaria was 6.3 per 1 000 per year in infants and 10.7 per 1 000 per year in children under 4 years of age (WHO 1992a).

Resistance of *P. falciparum* to the antimalarial drug chloroquine was detected almost simultaneously in Brazil, Colombia, Venezuela, and Thailand in the late 1950s and the 1960s. Several countries have now reported the widespread presence of *P. falciparum* strains resistant not only to chloroquine but also to amodiaquine, pyrimethamine in combination with long-acting sulfonamides, and to other drugs. The degree of resistance varies from country to country (López-Antuñano and Schmunis 1993). Resistance to *P. vivax* to chloroquine has been shown in Indonesia, Papua New Guinea, Myanmar, and Vanuatu (WHO 1997a).

P. falciparum has a high prevalence in areas that border jungles where the population is very mobile owing to expansion of agricultural and mining activities. In South America, these areas also have a high frequency of infection with *P. falciparum* strains resistant to antimalarial drugs. In such areas, migration favors contact between carriers and others who are susceptible to the infection. Exposure of large numbers of susceptible people to infected vectors gives rise to epidemics with high rates of morbidity and mortality. In these areas, the breakdown of the social structure, the absence of health service facilities, and the resultant failure of diagnosis and treatment of infected people adds to the risk of infection and death from malaria.

In many of the nations that are promoting national development and experiencing expanding populations, new roads are opened into jungle areas that are undergoing agricultural development and from which natural resources are being extracted. One of the detrimental consequences of such development is an increase in the incidence of malaria, mainly because of internal migrations. The destinations of the migrants are often determined by roads planned by development officers in remote cities without any thought for public health considerations. The migrants use the new roads to penetrate into jungle areas where disease vectors abound; here they often construct temporary dwellings that lack even minimum sanitary facilities. These areas often lack basic health and education programs as well as political and administrative organization, leaving the impoverished migrants vulnerable to disease. Some types of development program are particularly likely to increase the risk of malaria; for example, flood-based irrigation systems used for growing rice and banana crops. Poorly built roads, dams, and irrigation canals can also produce anopheline breeding sites, particularly during long periods of rain.

For most countries, the cost of malaria has been enormous. In the USA, it was estimated to be US$500 million in 1938 (Williams 1938). In Greece prior to the 1940s, the annual costs of malaria were huge: US$12 million for the loss of working days and earnings (using the minimum wage for the calculations), US$3.5 million for treatment and US$7.5 million for deaths (Livadas and Athanassatos 1963). In India, annual costs were US$50 million, US$60 million, and US$340 million, respectively (Sinton 1938). In the 1950s, lost wages amounted to US$60 million per year in Indonesia and US$14 million per year in the Philippines, where treatment costs were US$25 million per year and costs due to deaths were US$15 million per year (Wernsdorfer and Wernsdorfer 1988). The cost of deaths associated with malaria was estimated at US$157 million in Mexico for the period 1949–1963 (Wernsdorfer and Wernsdorfer 1988). In Paraguay, the economic potential

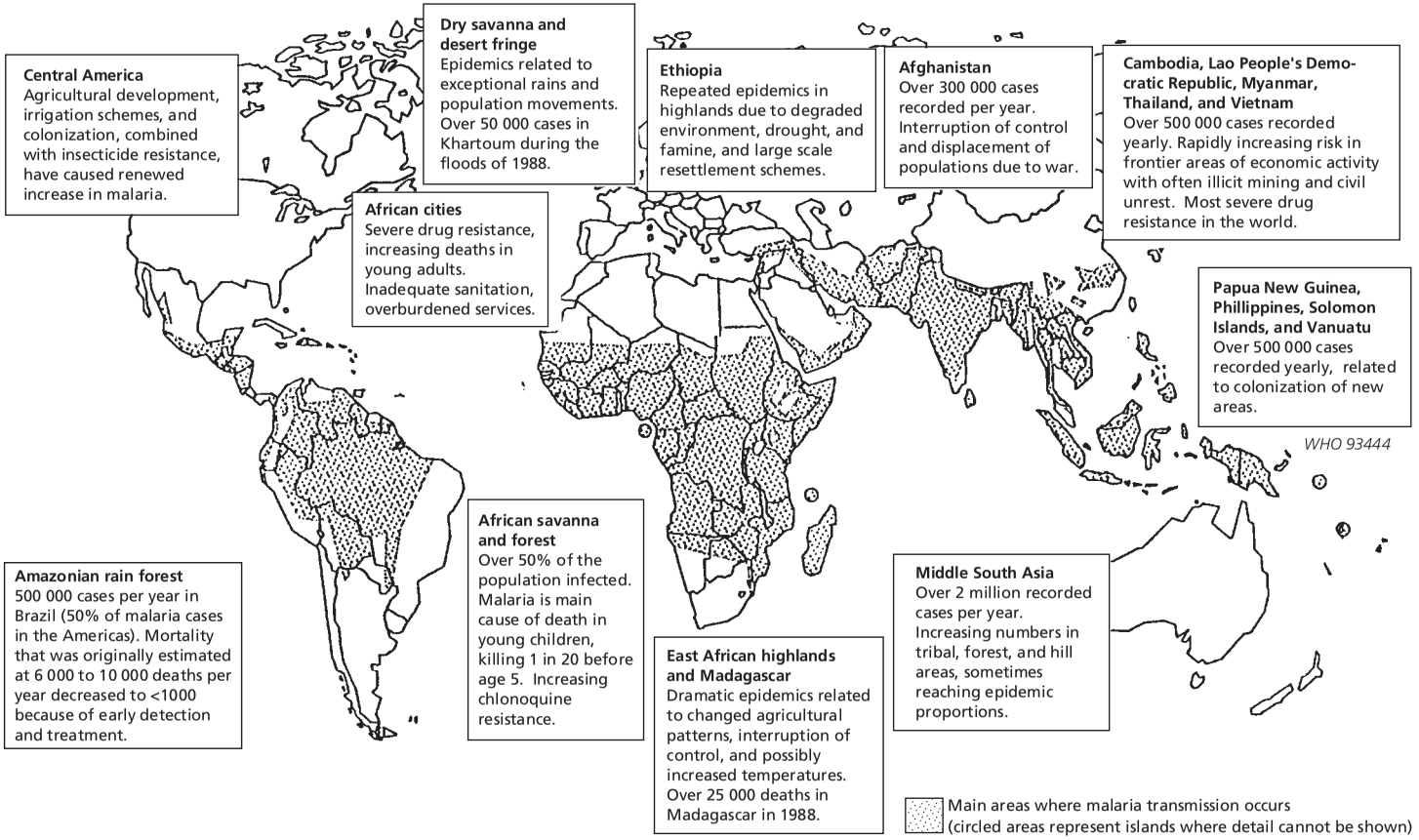

Figure 5 *Malaria distribution and problem areas (reproduced with permission from WHO 1993b).*

of a family with malaria was 74–86 percent of the potential of families without the disease (Conly 1975). In Peru in 1998, there were 77 000 cases of malaria with 43 deaths and the cost to the Peruvian economy was US$37.9 million that year, 1.5 percent of the total health expenditure. The government cost was US$ 9.3 million, 1 percent of the public health expenditure, The costs to families, who are mostly poor, amounted to US$26.6 million, averaging US$475 per family. The cost to families per malaria case was US$95, each family having an average of five cases (Ministry of Health 2000).

High malaria prevalence with its concomitant high social and economic costs interferes with the productivity and stability of agricultural settlements (Sawyer 1993). In Africa, the estimated average time lost by adults due to one episode of malaria were 6.2 days, 4.3 days, and 4 days in Sudan, Sri Lanka, and Burkina Faso respectively; in Kenya, Malawi, Nigeria, and Rwanda, on the other hand, it was 2–3 days (WHO 1999). In 1985 in Africa, a malaria episode in a person cost US$1.80 directly and US$9.80 indirectly. Another study showed that the cost of providing treatment was US$0.21 per child and US$0.63 per adult. The direct expenditures incurred as a result of malaria by low-income and high-income households during a year in Malawi were US$19.13 and 19.84, respectively and the indirect costs were US$2.13 and 20.61, respectively (Ettling et al. 1994). In the sub-Saharan countries only, the direct cost of malaria is estimated to be US$500 million annually (WHO 1999). Thus, it is not surprising that direct and indirect costs of malaria in Africa as a whole were estimated as US$800 million in 1987 (WHO 1993b) and were predicted to reach US$1.8 billion by 1995 (WHO 1995a).

The high costs caused by malaria are not limited to Africa. In Thailand in 1952, at the beginning of the intensification of the antimalarial program, the loss in agricultural production caused by malaria was US$1.52–1.90 million. Fourteen years later, this figure had decreased to US$0.30–0.37 million per year (Kühner 1971). The cost of the malaria control program was US$17 million in 1980 (Kaewsonthi and Harding 1984). Resistance to DDT and other insecticides and to chloroquine and other drugs has significantly increased the costs associated with malaria prevention and control.

In areas where the population is relatively stable, malaria can often be controlled by vector source reduction and the protection of households from vectors by various methods including indoor spraying with residual insecticides and insecticide-impregnated bednets. For migrant populations, the best approach for prevention and control is promotion of self-protection; for example, using screened tents, insecticide-impregnated bednets, and insect repellents. The efficacy and relative cost-effectiveness of insecticide-treated bednets for the control of malaria in children under 5 years of age have recently been demonstrated by several large-scale trials (WHO 2000b). However, it has been suggested that long-term use of insecticide-treated bednets in areas of high transmission could lead to mortality rebound in later childhood. This would reduce the cost-effectiveness of the intervention and, in the extreme, could lead to negative overall effects (Coleman et al. 1999).

In all situations, early detection and treatment will decrease morbidity and prevent mortality. Selection of the appropriate control measures requires assessment of the risk factors present in the area. In most cases, it is necessary to use several control measures in an integrated fashion (López-Antuñano and Schmunis 1993).

Among infants, severe anemia and malaria are often associated. The cost and effectiveness of control measures to prevent both anemia and malaria have been evaluated based on data from the expanded program on immunization (EPI) and not on the malaria control program. For severe anemia, and from the perspective of the health provider, the cost-effectiveness ratios were, respectively, US$8, 9, and 21 per DALY for malaria chemoprophylaxis with Deltaprim (a combination of 3.125 mg pyrimethamine and 25 mg dapsone) + iron, Deltaprim alone, or iron supplementation alone. For malaria prevention, Deltaprim + iron cost US$9.7 per DALY and Deltaprim alone cost US$10.2 per DALY. The cost-effectiveness ratios ranged from US$9 to 26 for severe anemia prevention and from US$11 to 12 for the prevention of clinical malaria. These measures were highly cost-effective, as defined by the World Bank's proposed threshold of less than US$25 per DALY for comparative assessments. Furthermore, all the preventive interventions were less costly than current malaria and anemia control strategies that rely on clinical case management. This economic analysis supports the inclusion of both malaria chemoprophylaxis and iron supplementation delivered through the EPI as part of the control strategies for these major killers of infants in parts of sub-Saharan Africa (Alonzo Gonzalez et al. 2000).

The success of malaria control programs has been uneven in some countries of the Americas. Such is the case in Brazil: malaria transmission was controlled elsewhere in the country by 1980 but not in the Amazon Basin where cases increased steadily until 1989 to almost half a million a year, and the coefficient of mortality quadrupled during the period 1977–1988. A reorganization of program activities from 1989 to 1996 facilitated a change towards earlier and more aggressive case treatment and more concentrated vector-control measures. The epidemic stopped expanding during 1990–1991 and reversed during 1992–1996. The total cost of the program from 1989 through mid-1996 was US$616 million: US$526 million for prevention and US$90 million for treatment. Compared to what would have happened in the absence of the program, nearly 2 million cases of

malaria and 231 000 deaths were prevented; the lives saved were almost equally due to preventing infection and to case treatment. Converting the savings in lives and morbidity into DALYs yields almost 9 million DALYs, 5.1 million from treatment and 3.9 million from prevention. Nearly all the gain came from controlling deaths and therefore from controlling *P. falciparum*. The overall cost-effectiveness was US$2 672 per life saved or US$69 per DALY, which is low compared to most previous estimates and compares favorably with many other disease-control interventions. Contrary to much previous experience, case treatment appears to be more cost-effective than vector control, particularly where *P. falciparum* is prevalent and unfocussed insecticide spraying is relatively ineffective. Halting the epidemic, by better targeted vector control and emphasizing treatment, paid off in much reduced mortality from malaria and in significantly lower costs per life saved (Akhavan et al. 1999).

In the Americas, good records of the cost of malaria control programs have been kept for several years and these indicate a continuous increase in the funds spent on antimalarial activities. Unfortunately, funding from international organizations and bilateral agencies has steadily decreased (Figure 6) (López-Antuñano and Schmunis 1993; PAHO 1994, 1995). Table 2.6 shows the overall budget for the health sector, the budget devoted to public health and the budget for malaria control programs in countries of the Americas in 1993 (PAHO 1994). From 1996 to 1999, expenditures by the 21 American countries with active control programs were US$263 million from the national budget and US$23 million from external contributions or loans. In 2000, countries spent US$105 million on malaria; US$97 million from the national budget and US$8.2 million from external contributions or loans (Organizacion Panamericana de la Salud 2000). Despite high expenditures for malaria control, the problem of malaria in the Americas remains unsolved.

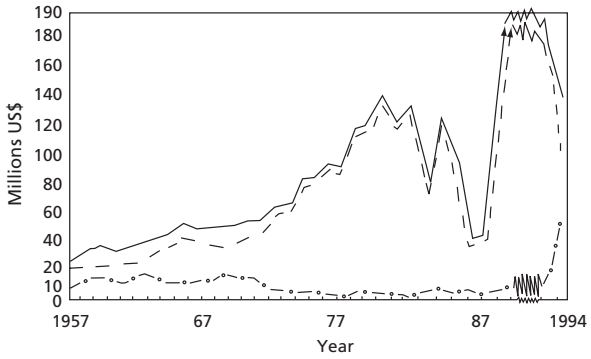

Figure 6 *Funds for malaria programs in the Americas, 1957–93. Total funds ——; funds provided by local governments - - -; funds provided by international or bilateral aid agencies -.-. Data from López-Antuñano and Schmunis 1993; PAHO 1994, 1995.*

Intestinal protozoa

Intestinal protozoa are the etiological agents of several widespread parasitic diseases, the most common of which are caused by *Entamoeba histolytica* and *Giardia duodenalis* (also known as *G. lamblia*).

Amebiasis, caused by *E. histolytica*, is a cosmopolitan infection transmitted by the fecal–oral route. Its greatest impact is in Africa and Asia. In Africa, Egypt, Morocco and countries located between 10°N and 10°S are severely affected. Prevalence is high in Asia, particularly in Bangladesh, Myanmar, China, India, Iraq, the Republic of Korea, and Vietnam. The amebae in these countries are highly pathogenic. Amebiasis is also a problem in Mexico and other Latin-American countries. On the other hand, in Europe and the USA, amebic infection is often asymptomatic or benign in spite of prevalence rates as high as 2–5 percent (WHO 1981). In 1984, it was estimated that 500 million people worldwide were infected with *E. histolytica* and that 40–50 million individuals had clinical symptoms of amebiasis, including diarrhea, dysentery, and liver disease. Worldwide amebiasis causes 40 000–100 000 deaths per year (WHO 1981, 1987, 1993c) and in 1996 was responsible for 48 000 cases (incidence) and 70 000 deaths (WHO 1997b).

The highest prevalence of *G. duodenalis* occurs in the tropics and subtropics where sanitation is poor. Transmission may be direct by ingestion of feces or indirect through ingestion of contaminated water or food. Travelers to tropical Africa, Mexico, Russia, South East Asia, southern Asia, and western South America are at a high risk of acquiring giardiasis (Wolfe 1992). Giardiasis may infect 200 million people worldwide and may produce symptoms in 500 000 individuals every year (Walsh and Warren 1979; WHO 1987, 1993c, 1997b). In developing countries, *G. duodenalis* is one of the first pathogens to infect infants and peak prevalence rates of 15–20 percent occur in children under 10 years old (Hill 1993). In developed countries, outbreaks frequently occur in child-care settings (Thompson 1994) and in adults who drink contaminated water. From 1977 to 1988, giardiasis was the leading cause of outbreaks of water-borne disease in the USA, causing an estimated 4 600 hospital admissions annually (Lengerich et al. 1994). Homosexual males constitute a special risk group. *Giardia duodenalis* may cause acute or chronic diarrhea, steatorrhea, loose stools, malabsorption of fat (Benenson 1990), and growth retardation and malnutrition in children (Farthing et al. 1986). Its major impact is on children under 3 years old, the undernourished, and the immunocompromised. It is also a cause of morbidity in adults (Farthing 1993).

Prevention of amebiasis and giardiasis is based on the implementation of good personal hygiene, appropriate disposal of human waste, and adequate handling and treatment of water supplies. The combination of filtra-

Table 2.6 *Funds assigned to the health sector, public health, and malaria prevention and control programs in the Americas 1993*

Country (geographical sub-region)	Budget for health sector (US$)[a]	Budget for public health (US$)[a]	% assigned for public health	Budget for malaria programs (US$)[a]	% of health sector	Loans or grants for malaria programs (US$)[a]
Mexico	13 376 794 032	1 494 709 452	11.17	28 441 613	0.21	...
Belize	9 717 172	1 655 934	17.04	477 919	4.92	100 000
Costa Rica	1 166 108 757	50 479 687	4.33	1 714 017	0.15	344 310
El Salvador	887 956 536	84 883 721	9.56	1 220 930	0.14	1 023 255
Guatemala	134 204 319	93 324 434	69.54	2 434 719	1.81	166 985
Honduras	805 829 807	83 904 586	10.41	2 016 013	0.25	283 072
Nicaragua	...	...	...	...	...	301 647
Panama	344 163 190	172 081 595	50.00	3 719 976	1.08	71 000
Haiti	973 600	...	...	20 000	2.05	250 000
Dominican Republic	...	...	...	599 334	...	517 815
French Guiana	...	...	...	538 535	...	...
Guyana	...	...	...	91 973	...	...
Brazil	...	1 130 947 000	...	97 124 000	...	5 500 000
Bolivia	96 404 722	35 846 246	37.18	187 066	0.19	...
Colombia	...	738 829 332	...	13 524 381	...	...
Ecuador	204 632 124	100 307 839	4.90	4 963 244	0.24	...
Peru	5 380 095 982	...	...	...	...	...
Venezuela	10 735 841 686	851 385 818	7.93	6 976 914	0.06	4 600 000
Argentina	...	...	...	1 826 000	...	...
Paraguay	225 233 859 410	83 547 789 813	37.09	6 405 522	0.003	...
Total	260 218 581 338	88 386 145 456	33.97	172 282 156	0.066	13 158 084

Reproduced from PAHO 1994 with permission.
a) conversion based on United National exchange rates at 31 December 1993.
... No data.

tion and chlorination usually eliminates these organism from water, but higher concentrations of chlorine and longer exposure times are needed than those required for the elimination of bacteria. Boiling is an effective method of purifying drinking water for personal use.

There are few estimates of the cost of amebiasis, but one calculation suggested that in Mexico in 1984, expenditure for the treatment of invasive amebiasis was 1.6 percent of the budget of the Health Ministry (WHO 1987). In the developing countries, the costs associated with giardiasis are difficult to estimate because there are no hard data on the number of cases of diarrhea induced by giardiasis. An estimate made on the basis of the number of admissions to hospitals for giardiasis in the USA suggests direct costs of US$3–5 million annually.

THE HELMINTH WORMS

Dracunculiasis (Guinea worm disease)

Dracunculiasis occurs in 16 sub-Saharan countries in Africa, as well as in India and Pakistan. It afflicts the ten African countries considered to be among the least developed countries of the world, where the gross domestic product ranges from US$450 to 550 per inhabitant per year. The number of cases reported in the 1980s worldwide was 5–10 million and this decreased to 3 million in 1992, to 229 773 in 1993, and to 164 973 in 1994. Niger, Nigeria, and Sudan accounted for two-thirds of all cases from Africa (WHO 1993c, 1995c). During 1998, there were almost 72 000 cases reported from Africa, the most affected countries being Ghana, Nigeria, and Sudan (WHO 1999).

Dracunculiasis causes disability as a result of the migration of the parasite through the host. This migration results in localized pain, mainly in the areas around the joints. When the worm emerges through the skin, the pain at the site is severe and is accompanied by general symptoms of fever, joint pain, nausea, and vomiting. There is a painful edema followed by a blister and then an ulcer at the site of penetration. Extraction of the worm may cause disability for 2–4 weeks.

The success of the eradication campaign now underway in many countries is based on the provision of potable drinking water through the construction of safe wells. In infected areas, prevention is implemented through health education programs that convey the message that drinking contaminated water is the origin of the disease, that individuals with open blisters and ulcers should not be permitted to come into contact with any source of drinking water, and that water must be treated (by boiling, chlorinating or filtering) to eliminate the larvae of the crustaceans that act as the intermediate hosts. In Nigeria, control of the intermediate host costs US$3.00 per household for water filters and US$1 per capita for cement ring wells. This amounts to c. 30 percent of the estimated potential losses suffered in a season due to incapacity caused by the disease (Guyatt and Evans 1992).

Filariasis

About 750 million people in 76 countries live in areas of endemic filariasis, an estimated 90 million of them are infected with filariae. Of these, 72.8 million are infected with *Wuchereria bancrofti* and 5.8 million are infected with a variety of species of *Brugia* (WHO 1993c). There are 300 000 individuals infected with filariae in seven countries of the Americas; 48.6 million in eight countries of South East Asia (4.8 million infected with *Brugia*); 3.9 million in 14 countries or territories of the western Pacific (1 million infected with *Brugia*); and 25.6 million in 40 countries in Africa (WHO 1992b). Almost 40 million people worldwide suffer from lymphedema, elephantiasis or hydrocoele caused by bancroftian filariasis, 14 million of them in Africa and 21 million in South East Asia. Worldwide, *Brugia* is believed to cause lymphedema and elephantiasis in 2.8 million people of whom 1 900 000 are in South East Asia and 900 000 are in the western Pacific (WHO 1994f).

Clinical manifestations of bancroftian and brugian filariasis vary widely in different geographical areas from asymptomatic microfilaremia and malaise to acute symptoms characterized by adenolymphagitis with fever, hydrocoele, lymphedema, elephantiasis, and chyluria, the last four being characteristic of chronic bancroftian filariasis (WHO 1992b). In India, it is estimated that an infected person suffers 4.47 acute attacks per year if they have bancroftian filariasis and 2.20 acute attacks per year if they have brugian filariasis. Each attack has an average duration of 4 days and the numbers of working days lost per year per person are 17.43 and 8.91, for bancroftian and brugian filariasis, respectively (WHO 1992b).

The vectors of filariasis are *Culex*, *Anopheles*, *Aedes*, and *Mansonia* mosquitoes. Slum dwellers with poor housing and sanitation are at the highest risk of bancroftian filariasis transmitted by *C. quinquefasciatus* whereas the rural poor suffer more from filariasis transmitted by other mosquitoes. Control measures are directed towards reduction of vector density and interruption of contact between human and vector by the application of a combination of chemical or biological control measures, environmental management, and personal protection measures (WHO 1992b). Treatment is another factor in control and involves annual administration of a single dose of diethylcarbamazine (DEC) or a combination of DEC and ivermectin (WHO 1994g). Ivermectin is the drug of choice for both individual treatment and mass-treatment programs for the control of onchocerciasis (Ottesen 1994) and a single dose can also be used for treatment or as a control measure for lymphatic filariasis (Brown et al. 2000). The combinations of DEC plus ivermectin or ivermectin plus

albendazole caused a decrease in microfilaremia greater than that achieved with either drug alone (Ismail et al. 1998; Beach et al. 1999). Thus, combinations of DEC or ivermectin with albendazole could be effective for mass treatment of lymphatic filariasis and at the same time would decrease the burden produced by soil-transmitted helminth infections. Control costs vary with the scope of coverage and the strategy or combination of strategies used. Control costs are US$1.8 million in China; US$1 million in India and Thailand; US$500 000 in Egypt and Malaysia; and from US$27 000 to 300 000 in French Polynesia, Papua New Guinea, and Sri Lanka (WHO 1994g).

Between 75 and 122.9 million people are at risk of onchocerciasis (river blindness), which is endemic in 28 countries in Africa and six countries in the Americas. In Africa, 17.7 million people are infected with *Onchocerca volvulus*, of whom 500 000 have damaged vision and 267 000 are blind. In the Americas, 140 000 people are infected with *Onchocerca* of whom 750 are blind (WHO 1995d). Many individuals infected with *O. volvulus* are asymptomatic. Symptoms of acute dermal or ocular infection are rare clinical manifestations of chronic established infection, the most severe consequences of which are reduction of the visual field and blindness. The impact of the disease is seen mainly in sub-Saharan countries where, in some areas, up to 40 percent of adults may have severe visual impairment. This results in an inability of the family to support itself and a decrease in agricultural output that leads to unstable communities (WHO 1995d).

Control activities were originally based on control of the vector of onchocerciasis, the blackfly *Simulium*, but are now based on yearly administration of single doses of ivermectin to those at risk. Evaluation of a community-directed, ivermectin-treatment program in Uganda indicated that desired treatment coverage of the population eligible to take the drug, was achieved in 42.6–51.0 percent of the communities. The annual cost per person treated with ivermectin was higher in the districts with small populations (fewer than 15 000) than in those with large populations (more than 40 000), US$0.40 as opposed to US$0.10 or less (Katabarwa et al. 1999). The costs of ivermectin distribution vary between US$0.10–5 per person per year (WHO 1995d). Implementation of control measures has been successful in many areas and has resulted in interruption of transmission in 11 countries of West Africa. Levels of infection in children have been significantly reduced and the numbers of infected people decreased from 1 million in 1975 to 10 000 in 1992. Since the inception of the prevention program, 100 000 people have avoided blindness.

Food-borne trematode infections

Food-borne trematode infections have their highest prevalence in South East Asia and in the western Pacific; they are also present in areas of Africa, the Americas, and Europe. The geographical areas involved are expanding as are the populations at risk as a result of improvements in transportation that favor population movements and trade. It is estimated that food-borne trematode infections affect 40 million people throughout the world (WHO 1995b). The food-borne parasites of greatest public health importance are *Fasciola*, *Clonorchis*, *Paragonimus*, and *Opisthorchis*.

Human fascioliasis, caused by *F. hepatica* and *F. gigantica*, has been reported from Bolivia, China, Ecuador, Egypt, Iran, Peru, Portugal, and Spain; and 2.4 million people are infected. Clonorchiasis has been observed in China, Hong Kong, Macao, Republic of Korea, Russia, and Vietnam and estimates suggest that 7 million people are infected. Opisthorchiasis, caused by *O. viverrini* and *O. felinus*, occurs in Kazakhstan, Laos, Russia, Thailand, and Ukraine; 10 million people are infected. Of less importance from the public health viewpoint are the intestinal flukes that cause echinostomiasis, fasciolopsiasis, heterophyiasis, metagonimiasis, and nanophyetiasis. These infections are found in China, Egypt, Japan, Republic of Korea, Russia, and Thailand and c. 1.2 million people are infected (WHO 1995b).

The epidemiology of these diseases is determined by ecological and environmental factors. Aquatic or semi-terrestrial snails are the intermediate hosts and the reservoir hosts are fish, crustaceans or mammals. Human infection occurs from ingestion of raw or uncooked fish, shellfish or aquatic plants that harbor the infective metacercariae (Figure 7). In some areas, industrial production of cultured fish and shellfish may be related to the spread of food-borne trematodes (Benenson 1990; WHO 1995b).

To prevent human *Fasciola* infection, infection of livestock must also be controlled, as humans are only accidental hosts. The consumption of watercress or other aquatic plants from areas where sheep or cattle graze should be avoided, as should be the use of animal feces for fertilizing water plants. Mollusk hosts should be eliminated when possible. The best method for prevention of infection with *Clonorchis* and *Opisthorchis* is to avoid eating raw fish and to use sanitary methods for disposal of feces. Infection with these parasites is serious as they not only produce liver disease but also increase the risk of cholangiocarcinoma. *P. westermani* and several other related species cause lung fluke disease. These parasites have freshwater crabs as their intermediate hosts and prevention is effected by thoroughly cooking crustacea, by disposal of sputum and feces in a sanitary manner and by snail control when feasible (Benenson 1990). In migrants to developed nations, lung fluke infection is often misdiagnosed as tuberculosis. Programs for control of food-borne trematodes should be multisectoral, integrating the activities related to human health, food safety, aquaculture, agriculture, and education (WHO 1995b).

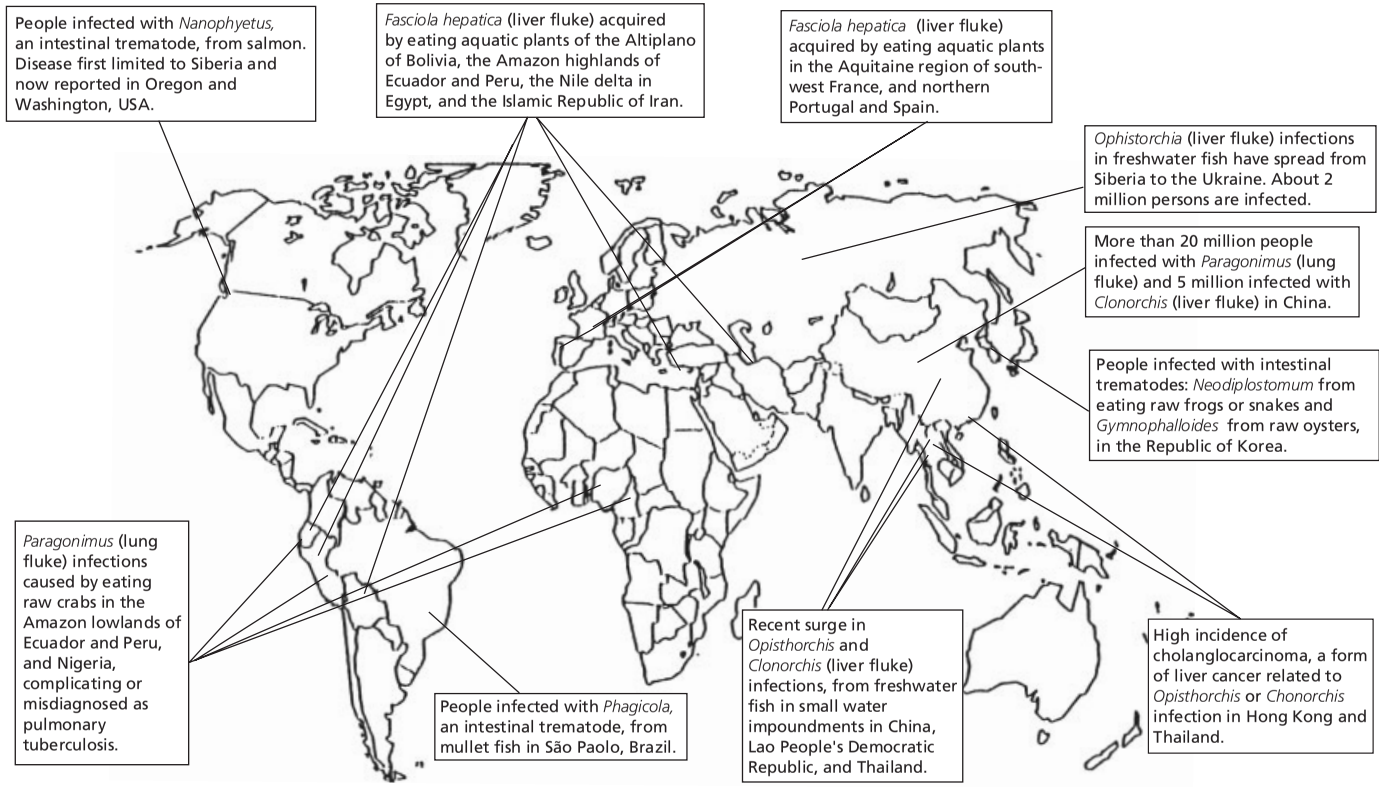

Figure 7 *Food-borne trematode infections: the global distribution is changing with the environment and with changes in human behavior (reproduced with permission from WHO 1995b).*

The economic burden of these diseases stems from a number of direct and indirect costs associated with the infections, including morbidity, loss of productivity, absenteeism, and cost of healthcare in infected people as well as losses in agricultural and aquacultural enterprises. The wages lost annually in Thailand as a result of opisthorchiasis in those aged 15–60 years are estimated at US$60 million and the cost of medical care is US$19.4 million. In addition, the cost of control for a 6-year period (1988–1993), with a coverage of 3 million people, was US$8.3 million, including costs for fecal examinations, drugs, and the promotion and training of personnel for community participation in control programs. In the Republic of Korea, control programs provide medical examinations and treatment for 15 million people and cost an estimated US$2.5 million per year (WHO 1995b).

Intestinal worms: *Ascaris*, hookworm, and *Trichuris*

Ascaris lumbricoides infections have been reported during the last few years from more than 150 of the 208 states and countries of the world. Hookworms and the whipworm, *Trichuris trichura*, are as widely distributed as *Ascaris* (Bundy and Cooper 1989; WHO 1987). These parasites are soil-transmitted helminths of global importance. Strongyloidosis has a patchy global distribution and is less prevalent than ascariasis. *Ascaris* has high prevalence in the subtropical and tropical areas of Africa, Asia, and the Latin-American countries. Although common in humid parts of the world, *Trichuris* is also found in temperate climates. Hookworm infection has almost been eradicated from Europe and the USA. The hookworm *Necator americanus* occurs in the Americas, equatorial Africa, southern Asia, South East Asia, Polynesia, and Australia. *Ancylostoma duodenale*, another hookworm, is more common in Africa and in northern and south-western Asia. The ranges of both species of hookworm (and of other parasites of global importance) often overlap.

During 1977–1978, 800–1 000 million people were infected with *Ascaris* and, of these, 1 million had symptoms; hookworm infected 700–900 million people, of whom 1.5 million had symptoms; and *Trichuris* affected 500 million people of whom 100 000 had symptoms. The number of deaths due annually to *Ascaris* and hookworms in this period were approximately 20 000 and 50 000, respectively (WHO 1993c). Subsequently, it was reported that *Ascaris* and *Trichuris* cause clinical symptoms in 214 and 133 million people, respectively, and that hookworm causes clinical symptoms in 96 million (WHO 1995a). More recent estimates for *Ascaris* are 1 400–1 500 million infected, 120–220 million cases (Albonico et al. 1999) and 4 000 deaths (WHO 2002). An estimated 1 049 million people harbor *Trichuris* (Crompton 1999); 114 million of them are preschool children and 233 million are children aged 5–14 years (Chan 1997). It is estimated that 27 million school-age children are infected in sub-Saharan Africa, 7 million in India, 42 million in China, 70 million in the rest of Asia, 39 million in Latin America and the Caribbean, and 18 million in the Middle-Eastern Crescent (Stephenson et al. 2000). Deaths attributed to *Trichuris* and hookworm were 2 000 and 4 000, respectively (WHO 2002).

The impact of these parasites on public health is related to the severity of the infection; heavy infections cause malabsorption and large blood and protein losses that are compounded in the context of poor nutritional status. Heavily infected people are also at risk of developing complications such as intestinal obstruction or anemia. The impact of these parasitic diseases is difficult to evaluate, as they may cause effects such as retarded physical growth, retarded development of cognitive skills, low participation in education, and poor performance (Evans and Jamison 1994). None of these effects is easy to isolate and measure routinely. Although there is a relationship between parasitic infections and human malnutrition, it is sometimes difficult to determine which is the primary factor because there are similarities in the socioeconomic conditions in which these conditions both occur (Crompton and Nesheim 1982).

Ascaris is mainly prevalent in young people (i.e. children aged 6–12 years). *Trichuris* infections peak in early life and then generally remain steady, whereas hookworm prevalence peaks in adolescence. Heavy worm burdens of *Ascaris* and *Trichuris* are found in children of primary school age (WHO 1981, 1987).

In many communities, 15 percent of the infected population harbor more than 60 percent of the worms. As morbidity is usually related to intensity of infection, heavily infected individuals have a greater risk of developing disease symptoms, and they also cause the most contamination of the environment (Anderson 1986). In addition, individuals with a heavy worm burden are more likely to re-acquire heavy infection after treatment and to suffer from polyparasitism (Anderson and Medley 1985; Bundy and Cooper 1989). The growth and nutrition of children are adversely affected by *Ascaris* and *Trichuris* infection and there are remarkable improvements in appetite, food intake, digestion, absorption, and growth in infected children who receive treatment (Cooper et al. 1990; Stephenson et al. 1989). Hookworm infection often causes iron-deficiency anemia, which may reduce productivity in affected individuals. The infection and the resulting anemia may impair mental development in children, negatively influencing their cognitive performance. Infection may also complicate pregnancy (Stephenson 1984; Prescott and Jancloes 1984; WHO 1987).

The cost of infection by intestinal worms is huge. In the late 1970s, US$4.4 million was annually lost in Kenya in the form of nutrients wasted as food unab-

sorbed by worm-infested people. In the same country in 1976, the cost incurred by the government for treatment of parasitic intestinal infections was US$339 000 (costs for examining patients and for drugs) and the cost incurred by affected families US$394 000 (cost for drugs, lost wages, and for transportation). These figures seem to be low as 88 804 patients were admitted to hospitals in Kenya that year as a result of ascariasis (Stephenson 1984; WHO 1987).

Programs for prevention of infection with intestinal worms must be based on the use of several strategies, focusing on improvements in basic sanitation, waste disposal, health education, and health services. In the short term, immediate action can be taken by administering the drug albendazole, which decreases morbidity and mortality. One strategy is to select the target population and then treat only those who are infected. This approach requires screening the entire population. It has been estimated that in Rwanda, application of this strategy would cost US$600 000 in salaries, excluding costs for the collection and handling of samples. Treatment of infected individuals (about two-thirds of the population for round worms and one-third for flat worms) would cost US$64 000 per year. An alternative program using chemotherapy (piperazine and niclosamide) on all individuals has been estimated to cost US$440 000 per year (De Schaepdryver 1984). Improving basic environmental sanitation and treatment facilities would be considerably more expensive than either of these policies. Control of these parasites requires a massive effort to raise the standard of living of the affected population if the benefits of any treatment programs are to have a lasting effect.

Schistosomiasis

Schistosomiasis is the most important and prevalent of the water-borne parasitic diseases. It is a major health risk in the rural areas of 74 developing countries. Five species of *Schistosoma* affect humans:

1. *S. mansoni* occurs in 36 countries in Africa, nine in the Americas, and seven in the eastern Mediterranean
2. *S. japonicum* is found in China, Indonesia, the Philippines, and Thailand
3. *S. mekongi* occurs in Cambodia and Laos
4. *S. intercalatum* is present in five countries in Central Africa
5. *S. haematobium* is prevalent in 51 eastern Mediterranean and African countries and in India and Turkey.

The first four of these species cause intestinal schistosomiasis and *S. haematobium* causes urinary schistosomiasis. It is estimated that more than 200 million people residing in rural and agricultural areas are infected with schistosomes and that 500–600 million people are exposed to infection. The estimated mortality was about 200 000 per year (WHO 1985, 1993d, 1994h) but has now decreased to c. 17 000 (WHO 2002).

The prevalence of schistosomiasis within countries varies widely: in some countries, the entire population is exposed; in others, there are only scattered foci of disease. Morbidity also varies widely, the foci of clinical disease usually being highly localized. The incidence of clinical schistosomiasis may be significantly higher in countries where the prevalence of the infection is greater than 40 percent (WHO 1985). In the past 10 years, the incidence and prevalence of schistosome infection have decreased in some areas but the infection has become more widespread in others. Large hydroelectric and agricultural irrigation projects (some involving the creation of artificial water reservoirs) and associated population movements are considered to be responsible for many of the increases.

Rates of infection with schistosomes are higher for men than for women because cultural and social biases in behavior and occupation cause men to be more exposed to contaminated water. Of the individuals infected with *S. haemotobium,* 60–70 percent are aged 5–14 years and this group is also the most heavily infected group in areas where *S. haemotobium* is endemic. Hematuria is present in 80 percent of infected children. The prevalence and intensity of urinary disease is lower in older age groups. In contrast, *Schistosoma japonicum* infection has no typical age prevalence and intensity distribution (WHO 1985, 1993d). In areas endemic for *S. mansoni*, the highest prevalence of infection is in those aged 10–24 years, but the prevalence in older groups may also be high. Usually, the most heavily infected individuals are those aged 10–14 years. A significant proportion of these individuals (those with an egg count of fewer than 800 eggs per gram of feces) have hepatomegaly and splenomegaly (WHO 1985, 1993d). Poverty, ignorance, substandard housing, and poor sanitation are the main factors that maintain these endemic foci.

Several studies have been made on the public-health impact of schistosomiasis. In these studies, the following parameters have been assessed: school performance, physical fitness, growth, productivity, and earnings. Although results have not been conclusive (Tanner 1989) there is consensus that in north-eastern Brazil, Egypt and Sudan there is a severe reduction of the work capacity of the rural population as a consequence of schistosomiasis (WHO 1994h).

The economic impact of schistosomiasis is partly a result of the large number of working days lost per infected person per year. This loss is 4.4 days and 40 days per year per person for those infected with *S. haematobium* and *S. japonicum*, respectively. About 10 percent of people infected with *S. mansoni* develop severe symptoms. The number of days of life lost because of schistosomiasis is c. 600–1 000 per case

(Walsh and Warren 1979). The global burden caused by schistosomiasis was 4 500 000 DALYs in 1990 (World Bank 1993b), but decreased to 1 760 000 DALYs in 2001 (WHO 2002).

The annual per capita cost for control of schistosomiasis depends on the nature of the program used. In Central Liberia, focal application of a molluscicide (niclosamide) was combined with mass chemotherapy. A three-dose metrifonate mass treatment was applied in one area with *S. haematobium* infections only, concurrent metrifonate and niridazole mass treatment in one dose was applied in another area with *S. haematobium* infections only, while in another area with both *S. haematobium* and *S. mansoni*, praziquantel (1 dose) was used. The costs per capita protected were US$3.33 for metrifonate, US$1.53 for metrifonate and niridazole combined, and US$1.67 for praziquantel. These figures do not include costs of parasitological examinations (Saladin et al. 1983). In the case of Saint Lucia, the costs were US$1.10 per person per year for chemotherapy, US$3.70 for snail control, and US$4.50 for a clean water supply (US Congress 1985). Costs per protected person were estimated at US$0.70–3.10 per year in three African countries. This amount may seem little, but it should be taken into account that total health expenditures per person in these countries varied from US$1 to US$3 per person per year (Gryseels 1989). Case detection of infected individuals in Sudan cost US$0.03 per capita per year, just for operating the health services. In Brazil, the costs, including laboratory diagnosis, consultation, and drugs, were US$3.73 (Guyatt and Evans 1992). The introduction of specialized programs, instead of using the general health services for control of schistosomiasis, increased the annual cost per person from US$1.50–6.53 (WHO 1993d). A schistosomiasis control program for control of *S. haematobium* or *S. mansoni* in a rural area with a population of 100 000 will have a 5-year cost of US$3.70–25.91 per capita. The variation is determined by the prevalence, intensity and rate of infection in the population (Rohde 1989).

Miscellaneous worms of regional importance

In addition to the parasites of global importance, there are others of regional or national relevance. These are the roundworms *Capillaria philippinensis*, *Enterobius vermicularis*, and *S. stercoralis*, the cestodes *Hymenolepis nana*, *Taenia saginata*, *T. solium*, and *Diphyllobothrium latum* and the trematode *Fasciolopsis buski* (WHO 1987). It has been estimated that there are 80–100 million cases of *Strongyloides*, 400 million of *Enterobius* and 78 million of *Taenia* worldwide (WHO 1990b; Warren et al. 1993). The most widespread of these worms is *S. stercoralis* which is prevalent in tropical and subtropical areas and is a severe problem in South America and the Caribbean. *Enterobius* is cosmopolitan, but human infections are most common in temperate regions and it is often found in developed countries in the northern hemisphere (WHO 1981, 1987). Morbidity is usually low. Taeniasis, caused by *T. saginata*, is also cosmopolitan but is most prevalent in African countries south of the Sahara, eastern Mediterranean countries, and in parts of the former Soviet Union. Lower prevalences are found in Europe, India and southern Asia, Japan, the Philippines, and much of Latin America. The lowest prevalences are found in Australia, Canada, and the USA (WHO 1979). *T. solium* is mainly restricted to poor socioeconomic areas of central and southern Africa, Mexico, Central and South America, and southern Asia. Sporadic cases are found in southern Europe (WHO 1979). Human cysticercosis, caused by the larval stages of *T. solium* in tissues, is far more important as a public health problem than is human taeniasis (i.e. the adult form in the intestine). The highest prevalences of human cysticercosis are found in some areas of Africa, Asia, and South America (WHO 1981, 1987). In Mexico, hospitalization and treatment costs for humans with neurocysticercosis was US$17 million annually (Murrell 1991).

EXPANDING THREATS

New diseases may emerge as a result of changes in ecology, human demographics, and human behavior. Factors such as international travel and commerce, introduction of new technology and industry, microbial adaptation and change, breakdown of public health measures, and social disorder all affect human health (Institute of Medicine 1992; Morse 1995). As a result, several parasites, such as *Anisakis*, *Babesia*, *Cryptosporidium*, *Cyclospora*, *Giardia*, microsporidians, *Plasmodium*, *S. stercolaris*, and *Toxoplasma gondii* may be poised for an increase in importance (Institute of Medicine 1992; Wurtz 1994). This list could also be extended to include a number of other parasites. Some of these parasites (i.e. *Giardia*, *Plasmodium*, *Strongyloides*, and *Schistosoma*) and the factors that favor their emergence or reemergence, have been mentioned before. For example, schistosomiasis has increased in incidence in many areas after the construction of dams. A brief review of the others follows.

Anisakis is a common parasite of marine mammals and fish and the etiological agent of herring worm disease (anisakiasis) in humans, who are incidental hosts. Humans are infected by larvae present in undercooked fish, squid or octopus. The larvae localize in the gastrointestinal tract and then migrate upwards, attaching to the oropharynx. Many cases have been observed in Japan, where it is customary to consume raw fish. Infected fish can be transported around the world and the incidence of infection is increasing because of the growing trend in the consumption of raw fish. Cases have been reported in the Americas, Asia,

Europe, and the South Pacific (WHO 1987; Benenson 1990; Institute of Medicine 1992). Prevention is effected by not eating raw fish.

Babesia microti, a protozoan transmitted by nymphs of the *Ixodes* tick, infects red blood cells, and is carried mainly by deer mice. Human babesiosis is usually a result of infection by *B. microti* but other species of *Babesia* have also been implicated. Babesiosis in humans has been observed in Mexico, Europe, and the USA. The emergence of cases in the USA is linked to reforestation and a resultant increase in the deer population and the number of deer mice. Preventive measures include the elimination of deer mice in the vicinity of human habitations and the use of tick repellents (Benenson 1990).

The coccidian *Cryptosporidium parvum* was recognized as a human pathogen less than 30 years ago. Cryptosporidia live intracellularly in the gut cells of humans and domestic animals such as cattle. Transmission is by the fecal–oral route with direct person-to-person and animal-to-person transmission and indirect water-borne transmission. The parasite is found worldwide and its importance as a human pathogen was recognized as a result of several small outbreaks that were either water-borne or food-borne and one massive outbreak associated with drinking water (the latter affected more than 400 000 people in Wisconsin, USA). In immunocompetent individuals, the infection may be asymptomatic or it may produce an acute or persistent diarrhea. Individuals immunosuppressed because of infection with human immunodeficiency virus (HIV) or from other causes have been the most severely affected, experiencing cryptosporidiosis as an aggressively opportunistic infection. Prevention should be achieved by improving surveillance of water purification plants to detect malfunction and by applying high standards of personal hygiene. Appropriate measures include the sanitary disposal of human feces and animal excreta. Chemical disinfection of water is ineffective against cryptosporidia because the oocysts (the infective stage) are very resistant to chlorine (Benenson 1990; Fraser 1994; Colley 1995).

Described in 1979, *Cyclospora cayetanensis*, another coccidia, is now recognized as producing long-lasting diarrhea in humans in North, Central and South America, and the Caribbean, as well as in South East Asia and Eastern Europe. It is transmitted through contaminated water or food, but is highly unlikely to be transmitted from person to person because oocysts require days or weeks to sporulate and become infectious (Ortega et al. 1993; Wurtz 1994). An outbreak of c. 850 cases occurred in Canada and USA (Centers for Disease Control 1996). *C. cayetanensis* may be the origin of persistent diarrhea in immunocompromised patients with AIDS. Prevention is similar to that of the other food- or water-borne diseases.

Microsporidia are normally parasites of animals other than humans. Four genera of microsporidia, *Encephalitozoon*, *Enterocytozoon*, *Nosema*, and *Trachipleistophora*, have been recognized as human pathogens worldwide, usually affecting the immunosuppressed, including those with HIV. *Encephalitozoon* has been reported to cause systemic infection with central nervous system and kidney involvement; *Nosema* is considered to be the cause of keratitis; and *Trachipleistophora* has been recognized as an agent of myositis. *Enterocytozoon*, which causes the majority of microsporidial infections in humans, produces chronic diarrhea in AIDS patients. The mode of transmission of microsporidia to humans is unknown (Desportes et al. 1985; Canning and wHollister 1987; Shadduck and Greeley 1989; Centers for Disease Control 1990; Orenstein et al. 1990; Cali et al. 1991; Madi et al. 1991; Molina et al. 1993; Pol et al. 1993).

Toxoplasma gondii is a coccidian parasite of cats that produces disease opportunistically in immunosuppressed humans on all continents. Infection is also common in immunocompetent individuals, but most of them are asymptomatic. Infection occurs as a result of eating infected raw or undercooked meat from the intermediate hosts (sheep, swine, chicken, goats, cattle or birds) or by ingestion of food or water contaminated with oocysts from cat feces. Transplacental infection occurs when pregnant women acquire the infection during pregnancy. Prevention is based on personal hygiene, avoiding eating raw or undercooked meat, and avoiding contact with cat feces. Patients with AIDS must receive prophylactic treatment to prevent disease caused by *Toxoplasma* (Benenson 1990; Wong and Remington 1994). Human toxoplasmosis in the USA is estimated to be an annual economic/public health burden of more than US$400 million. (Murrell 1991).

CONCLUSION

Infectious disease must be viewed as a global problem, a problem exacerbated by dynamic ecological changes brought about by technological, socioeconomic, environmental, and demographic changes, as well as by microbial change and adaptation (Institute of Medicine 1992). The mobility of modern humans and the evolution of parasites (including drug-resistant strains) mean that parasitic diseases are a threat in both developing and developed regions.

Parasitic infections cause human suffering and economic losses worldwide but the burden is greatest in underdeveloped countries. Millions of people are at risk of infection, disease, and death from protozoan and metazoan parasites. These parasites cause social problems and their effects are increased by the conditions in disordered societies. Their presence hampers educational, political, and economic development, imposing a tremendous burden on the already precarious health services in the poor areas of the world. This situation constitutes a serious public health concern and

Table 2.7 *Global estimates of parasitic infections and disease burden*

Infection	Infected population[a]	Number of cases[a]	Population at risk[a]	Burden of disease (thousands of DALYs)[b]
African trypanosomes[c]	250–300*	250–300*	50–55†	1 598
American trypanosomes[d, e]	16–18†	3.2–3.6†	60†	649
Amebiasis[f]	500†	40–50†		
Ascariasis[g]	785–1 500†	120-220†		1 181
Clonorchiasis[h]	7†		289.3†	
Dracunculiasis[i]	164*		100†	
Enterobiasis[j]	400†			
Fascioliasis[h]	2.4†		180†	
Lymphatic filariasis[k]	78.6–90†	5†, l	751†	5 644
Giardiasis[m]	200†	500*		
Hookworms[n]	750–1 000†	1.5–96†		1 825
Intestinal flukes[h]	1.3[a]			
Leishmaniasis[o]	12–13†	600*	350†	2 357
Malaria[p]	500†	250–450†	2 400†	42 280
Onchocerciasis[q]	17.5†	270*, r	75–80†	987
Opisthorchiasis[h]	10.3†		63.5†	
Paragonimiasis[h]	20.5†		194.8†	
Schistosomiasis[s]	200†		400–500†	1 760
Strongyloidosis[j]	80–100†			
Taeniasis[t]	70†			
Trichuriasis[u]	750–1 049†	133†		1 649

a) Numbers in thousands (*) and millions (†); b) WHO 2002; c) WHO 1994d, 1995a; d) Schmunis 1994; e) 400 000 cases of heart and hollow viscera disease annually (WHO 1995a); f) WHO 1987, 1993c, 1997b; g) WHO 1990b, 1993c, 1995a; Albonico et al. 1999; h) WHO 1995b; i) WHO 1995c; j) WHO 1990b; k) WHO 1992b, 1993c, 1994f; l) disabled; m) WHO 1987, 1993c; n) WHO 1993c, 1995a; o) WHO 1990a, 1993c, 1995a; p) WHO 1993b, 1995a; q) WHO 1995c; r) Blind; s) WHO 1985, 1993d, 1994h; t) Warren et al. 1993; u) WHO 1990b, 1993c, 1995a; Crompton 1999; Stephenson et al. 2000.

inhibits the healthy development of individuals and social groups (Tables 2.4 and 2.7).

In developed countries, worms and protozoa present increased threats because of population movement, trade, and changing life-styles. Implementation of prevention and control strategies requires a systematic multidisciplinary and multisectoral approach.

For many diseases, there is sufficient knowledge to develop successful control programs. The following factors are vital:

- knowledge of the means of spread of the parasite and of any reservoirs
- a means of detecting both clinically apparent and asymptomatic infection
- a system of treatment or a method of interrupting spread.

In addition, the political will to implement control programs is also necessary but is often lacking in societies that lack cohesion and where social and economic chaos prevail. Meanwhile, failure to use classic public health measures while waiting for a 'miraculous' system of cure will retard progress. Because of the complexity and cost of controlling infectious diseases, the worldwide burden caused by parasitic infections is only likely to decrease in the context of both social and economic development.

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Epidemiology of parasitic infections

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INTRODUCTION

Epidemiology is the study of patterns of infection and associated disease within populations or defined communities. It is interdisciplinary in character, drawing on concepts and methods from a wide variety of biomedical fields including molecular biology, immunology, genetics, population biology, statistics, and mathematics, plus the specific fields associated with a particular infection, such as virology, bacteriology, parasitology, and vector biology. It is a quantitative field of medicine relying on accurate surveillance and measurement of infection plus disease, and dependent on statistical methods for hypothesis testing, parameter measurement, and description, plus mathematical techniques for the provision of a theoretical template to facilitate the interpretation of observed pattern or experimental outcome. A sound and detailed knowledge of the biology of the host and infectious agent, however, is an essential prerequisite for the successful application of such quantitative tools.

The past decade has witnessed significant changes in the manner in which epidemiological research is conducted, largely arising from the availability of new methods of measurement or detection of infection or disease linked to rapid advances in the disciplines of molecular biology and immunology. One example is the use of polymerase chain reaction (PCR) methods to detect the presence of very low concentrations of a particular pathogen within the human host. Such techniques enable quantitative measures to be made of parasite load or burdens within infected patients, in addition to the more precise determination of the prevalence (proportion of hosts infected) of infection. New immunological tools greatly facilitate the detection of current or past infection with non-invasive sample collection procedures (such as saliva-based assays for antibodies specific to viral, bacterial, and parasite infections) being of particular value in field-based studies.

Advances in other areas have also made very important contributions to epidemiology, particularly in the field of genetics. Molecular methods and the increased availability of genome sequence data for both pathogen and host open new avenues for the study of the determinants of pathogenesis and the coevolution of the parasite and its human or vector host. Increasingly, linkage or segregation analyses are beginning to find associations between the occurrence of disease, or persistent infection, and particular genotypes within human populations. Molecular or genetic epidemiology will undoubtedly be a major growth area in the coming decade. Associated population genetic studies can define the frequency of predisposition to infection or disease within defined populations.

There has also been a growing realization amongst researchers in this area that conventional statistical approaches to epidemiology, which have been so successful in improving understanding of non-infectious disease problems, are less appropriate for the study of the population level consequences of infection and immunity. Increasingly, concepts and methods that have evolved in evolutionary biology, ecology, and population genetics are being used to study the population biology, transmission dynamics, and evolution of infectious diseases within human communities. Such approaches recognize that the interaction between parasite and host

is a very dynamic one, in terms of transmission and persistence, and of the evolution of both organisms (with that of the pathogen having the potential to take place much more rapidly than that of the human host).

Observed patterns of infection and disease in human communities are determined by the interplay between the variables that control susceptibility to infection, its typical course, and the likelihood of serious disease within an individual, and the variables that control the rate of transmission between people. The former include innate susceptibility, the distribution of latent and infectious periods plus the duration of acquired immunity, while the latter include behavioral, social, environmental, and demographic factors. Researchers are typically concerned with the recondite biological and clinical details that make each infection unique. This chapter focuses on the principles that determine observed epidemiological patterns, independent of the type of infectious agent under study. Historically, work on infectious disease is often compartmentalized into distinct areas based on the taxonomy of the infectious agent, namely, virology, bacteriology, and parasitology. However, the principles governing the spread, persistence, and evolution of all infectious agents are the same. A simple example is provided by the notion of reproductive or transmission success. If we consider the host as the basic unit of study, then the reproductive number of an infection (R_0) is the average number of secondary cases of infection generated by one primary case in a susceptible host population (Anderson and May 1991). The magnitude of this parameter reflects the potential for transmission and evolution, and the degree to which control measures must inhibit transmission if an infection is to be eradicated in a given population.

The real world is of course replete with complications – economic and social as well as biological – and it is easy to lose sight of generalities when grappling with the details that ultimately make each infectious agent unique. However, it is often the case in epidemiology that the influence of a few factors dominates the generation of observed pattern. Understanding the key principles that underpin spread and persistence helps in the identification of these factors.

This chapter highlights the key processes that influence the transmission, persistence, and control of protozoan and helminth parasites with a focus on malaria, intestinal nematodes, schistosome flukes, and the filarial worms. Bear in mind, however, that the principles are equally relevant to the study of viral and bacterial infections (Anderson and May 1991).

BASIC CONCEPTS

Microparasites and macroparasites

A distinction is made between microparasites and macroparasites to cut across conventional taxonomic lines in order to focus on the population biology of the infectious agent.

MICROPARASITES

Microparasites may be thought of as those agents which have direct reproduction – usually at very high rates – within the host (Anderson and May 1991). They tend to be characterized by small size and a short generation time. Recovery from infection is usually associated with acquired immunity against reinfection with the same strain of parasite for some time, and often for life. For many viruses, bacteria, and protozoa, however, immunity may be highly strain specific although a degree of cross-protection may arise via shared antigens. Although there are important exceptions, the duration of infection is typically short relative to the expected life span of the human host. This feature, combined with acquired immunity, means that microparasite infections are typically of a transient nature in individual hosts. Most viral, bacterial, and (in a less certain manner) many protozoan parasites fall broadly into the microparasitic category.

For such infectious agents, it makes sense to stratify the host population into a few classes namely: susceptibles, infecteds but latent (i.e. non-infectious), infecteds and infectious, and immune individuals. A reasonable operational definition of a microparasite is an organism whose population biology can, to a sensible first approximation, be described by such a compartmental model. An essential feature of the framework is that no account is taken of the degree or severity of the infection (i.e. the abundance of the infectious agent within the host). The reality of heterogeneity between individuals with respect to genetic background, behavior, and environment is replaced by the abstraction of some average 'infected' or 'immune' individual.

It is often necessary to distinguish between infection and disease. The period between the point of infection and the appearance of symptoms of disease is termed the incubation period. The duration of symptoms of disease is not necessarily synchronous with the period during which an infected host is infectious to susceptible individuals. Furthermore, the host may be infected but not infectious. The period from the point of infection to the beginning of the state of infectiousness is termed the latent period. For some infections such as malaria, intermittent bouts of infectiousness may occur. For others, such as *Human immunodeficiency virus* (HIV), the degree of infectiousness may vary widely throughout the incubation period. The sum of the latent and infectious periods is referred to as the average generation time of the infection. The term average refers to the fact that latent, infectious, and incubation periods are often very variable between individuals due to a variety of factors such as host and parasite genetic background and the size of the infecting inoculum of the infectious agent.

Most epidemiological and demographic parameters – human birth and death rates, disease-induced death rate, recovery rates, rate of loss of immunity – can be measured directly by appropriate observational studies. The transmission rate, however, combines many biological, social, and environmental factors, and is thus rarely amenable to direct measurement. The best way is to infer this rate indirectly via longitudinal or cross-sectional cohort based studies to directly measure the incidence of infection.

MACROPARASITES

Macroparasites may be thought of as those having no direct reproduction within the definitive host. This category embraces helminths and arthropods, where transmission stages produced within or on the host pass to the exterior to complete development to the next stage of often complex life cycles which may involve more than one host (e.g. the schistosome flukes and the filarial worms). Macroparasites are typically larger than microparasites and have much longer generation times, often being an appreciable fraction of the human host's life span. When an immune response is elicited, it usually depends on the past and present number of parasites harbored, and tends to be of a relatively short duration once parasites are removed (e.g. by chemotherapy) from the host. Thus, macroparasitic infections are typically persistent in character, with hosts continually being reinfected. The evocation of an immune response and the pathology induced by infection both depend on the burden of parasites harbored by the host. This means that to quantify levels of infection and morbidity in the population it is necessary to measure the distribution between individuals of parasite numbers per host. As shown in Figure 1, parasite numbers are rarely uniformly or randomly distributed. Typically, the distributions are highly heterogeneous with most people harboring few parasites and a few harboring many. Compartmental models based on divisions into susceptible and infected persons are therefore inappropriate descriptions of the epidemiology of macroparasitic infections. The appropriate framework for study is one that records the prevalence (fraction infected) of infection, the average intensity of infection and the distribution of parasite numbers within the human population.

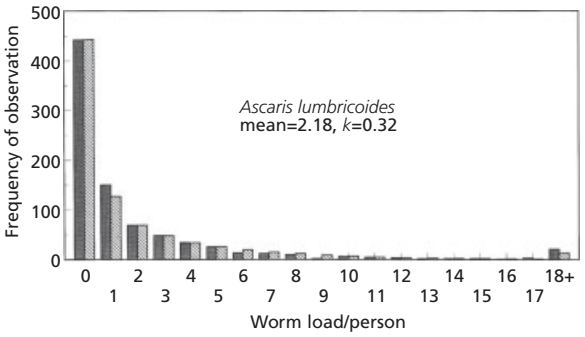

Figure 1 *The frequency distribution of* Ascaris lumbricoides *in a fishing community in Pulicat, Tamil Nadu, India (after Elkins et al. 1986). The negative binomial probability distribution provides a good empirical description of the observed pattern with mean = 2.8 worms per person and* k *= 0.32. Dark bars, observed value; hatched bars, expected value*

The division into microparasites and macroparasites is rather crude. Many infectious agents are not easily forced into this dichotomous scheme. Many protozoan parasites such as the *Plasmodium* species that induce the disease malaria may, to a good approximation, have their epidemiology described by a microparasitic compartmental framework (i.e. prevalence of infecteds, susceptibles, and immunes), although their patterns of persistence within the human population, with individuals being repeatedly infected, are characteristic of macroparasites. However, the dichotomy serves as a useful starting point for highlighting the population biology and epidemiology, as opposed to emphasizing conventional taxonomic categorizations.

Basic reproductive number of infection, R_0

For a microparasite, the basic or case reproductive rate, R_0, is defined as the average number of secondary cases of infection produced by one primary case in a susceptible population. When a microparasitic infection first invades a human community, the fraction susceptible decreases over time. Eventually, some sort of equilibrium state is reached (endemic infection) which may involve oscillatory fluctuations in prevalence or incidence of a seasonal or longer term nature. At this equilibrium (or oscillating equilibrium) the rate at which susceptible infants or children are infected is exactly balanced by the rate at which new susceptibles are born into the community and each infection will on average produce exactly one secondary case. That is, at equilibrium, the effective reproductive rate $R = 1$. If we assume that the human community mixes homogeneously (independent of age, sex, residence location, etc.), the effective reproductive rate R is equal to the basic reproductive rate, R_0, discounted by x, the fraction of the human community that is susceptible, R, is equal to R_0x. Given that at equilibrium $R = 1$, then

$$R_0 = 1/x \qquad (1)$$

where x is the fraction susceptible at equilibrium. This simple relationship provides a method of estimating the value of R_0 from serological or other data on age-specific susceptibility.

For a macroparasite, R_0 is the average number of female offspring (in the case of a dioecious parasite), produced throughout the lifetime of a mature female parasite, which themselves achieve reproductive maturity in the absence of density-dependent constraints. In the absence of these constraints, which include competition

for space or other resources within the human host and acquired immunity dependent on parasite load, parasite numbers within the population would grow exponentially provided $R_0 > 1$. In practice, macroparasite populations tend to be rather stable through time and a variety of density-dependent factors act to generate such stability.

For most common viral and bacterial infections and some protozoa (e.g. *Toxoplasma*), serological tools are available to establish whether or not a person has acquired and recovered from infection at some time in their past. Age-stratified cross-sectional serological surveys or longitudinal cohort studies provide the best information for estimating the magnitude of R_0 in a given community. A diagrammatic serological profile is shown in Figure 2. The magnitude of R_0 can either be estimated from this profile by calculating the fraction of the total population susceptible to infection or equally simply from the following expression:

$$R_0 = (L - M)/(A - M) \qquad (2)$$

where L is life expectancy, M is the average duration of maternal antibody-derived protection (typically 6 months for many common viral infections such as measles), and A is the average age at infection. The key quantity is A, which can be estimated directly from the serological profile (Grenfell and Anderson 1985). If the value of A is small, R_0 is large, and the infection has high transmission efficiency, while, conversely, if A is large, R_0 is small, and the infection has low reproductive success. Transmission success of a given parasite can vary widely in value between different locations and at different times of the year.

Serological surveys provide a wealth of information about the transmission dynamics of infectious agents. They not only provide information on the values of M (duration of maternally derived protection) and A (average age at infection), but they can also guide decisions on the optimum age at which to vaccinate and on the fraction who should be immunized to block transmission. Carrying out large-scale surveys to include infants, children, and adults can be difficult if blood samples are required to extract serum for the evaluation of the presence or absence of antibodies specific to particular antigens. Techniques based on the testing of saliva for secreted antibodies (Perry et al. 1993) have been developed for a variety of infectious agents and these provide epidemiologists with a powerful tool for the study of transmission. Serum or saliva samples must be collected at random within the community, taking due note of important stratifications within the population such as age, sex, ethnic background, residence location, etc. However, problems can arise if the duration of measurable antibody production following infection is not lifelong. This is thought to be the case for many bacterial and protozoan infections which makes serological surveys of less value in determining past and current patterns of transmission. We require more sophisticated techniques to detect immunological markers of past infection with antigenically variable parasitic organisms. In general, however, the collection of serological data, finely stratified according to age, is of vital importance in any quantitative assessment of transmission and the potential impact of control measures. Ideally, age-stratified cross-sectional surveys should be conducted each year, such that a profile of herd immunity can be constructed prior to and post the introduction of any intervention program.

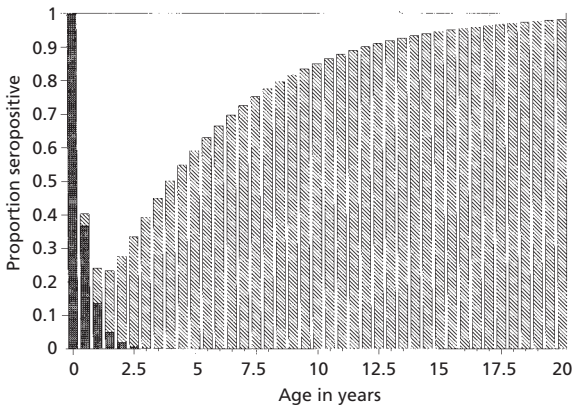

Figure 2 *Diagrammatic representation of a cross-sectional seroepidemiological survey, stratified by age, for the presence of antibodies specific to parasite antigens. Dark bars, maternal antibodies; hatched bars, antibodies from infection*

The measurement and interpretation of the magnitude of R_0 can be made complicated by genetic variability within the parasite population when such variability is reflected by different phenotypic properties such as transmissibility or antigenic characteristics (Gupta and Day 1994). Consider a protozoan that induces lifelong immunity in those who recover from infection. In such cases R_0 is inversely related to the average age at infection, A, by a simple relationship

$$R_0 = L/A \qquad (3)$$

where L is human life expectancy. However, lifelong immunity is rarely observed in parasitic protozoa due largely to the existence of many distinct antigenic strains of a particular parasite species. In such cases, recovery from infection by one strain may or may not confer a degree of resistance to another strain depending on their degree of 'antigenic' relatedness. Immunity may then only develop as a consequence of exposure to many different antigenic types or strains circulating in a given locality. This appears to be the case for the malaria parasite *Plasmodium falciparum*. Young children in hyperendemic malaria zones may experience many clinical attacks of malaria per year (Greenwood et al. 1991). Although older children develop a functional but non-sterilizing immunity manifest as a reduction in clinical episodes, a substantial reduction in parasite load is only

observed in adults after a long period of exposure to the genetical diverse population of parasites. If the delay in the development of immunity is a direct consequence of the antigenic diversity within the parasite population (as opposed to parasite evasion or modulation of the host's immunological attack) then we would expect to see a slower rise by age of immunological (seroepidemiology) evidence of exposure to a particular strain, but a much faster rise in the exposure to 'malaria', where the latter is defined as the experience of any one of several strains. When n strains are independently circulating in a defined population, the average age on first exposure to malaria, A_n, is

$$A_n = 1/\sum_{i=1}^{n} R_{0i} \qquad (4)$$

Here it assumed that strain-specific immunity is life-long and R_{0i} defines the case reproductive number of strain i. This equation makes clear that a low average age at first infection (often the case for *P. falciparum*) can arise when the transmissibilities of each of many strains circulating is low. These conclusions are well supported by field observation on the rise with age in the fraction of a population in Madang, Papua New Guinea who had experienced infection with five antigenically distinct strains of *P. falciparum* (Gupta et al. 1994b) (Figure 3).

The relationship between prevalence and intensity of infection

We have briefly noted that many sources of heterogeneity influence the distribution of parasites within a human community (Figure 1). The precise degree of heterogeneity, measured either by a statistic such as the variance/mean ratio (V/M) of parasite load per host, or the aggregation parameter, k (which varies inversely with the degree of aggregation) of the negative binomial probability distribution (which provides a good empirical model of many observed distributions), determines the relationship between the prevalence and mean intensity of infection (Figure 4a). As the degree of contagion or aggregation increases, the prevalence reaches a plateau, well below 100 percent, as the mean parasite load rises. For a given species of parasite, observed patterns of heterogeneity are often remarkably constant independent of the study community or geographical region (Anderson and May 1985, see Figure 4b). For helminth parasites in particular, worm load is directly correlated with the likelihood of symptoms of disease. As such parasite aggregation determines the prevailing burden of disease in a given population for a fixed mean worm load, the greater the degree of parasite aggregation, the smaller the number of individuals in the population showing symptoms of disease. However, for extreme aggregation the severity of disease will be greater in those showing symptoms due to their very high worm loads.

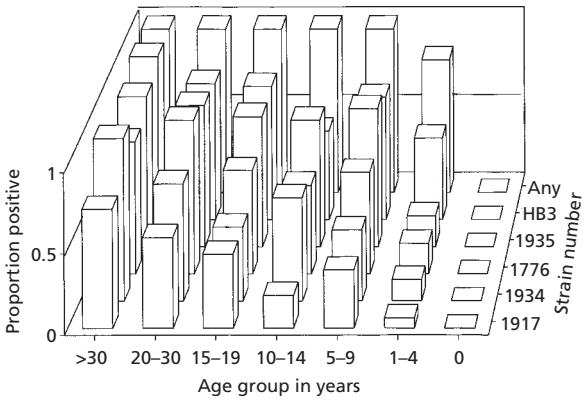

Figure 3 *Age-stratified serological survey for presence of past infection by a series of distinct isolates or strains (serotypes) of* Plasmodium falciparum *from a community in Papua New Guinea (data from Gupta et al. 1994b). The isolates differ in parasite induced erythrocyte surface antigens (PIESA). The proportion exposed to any type is recorded at the back of the graph (= Any)*

Heterogeneity and predisposition to infection

The epidemiological measures of prevalence and average intensity of infection are summary statistics of the frequency distribution of parasite numbers per host (Figure 1). The form of these distributions is of great significance to the burden of disease in a population and the regulating constraints acting on the net rate of parasite transmission. For helminths and protozoa, the distributions are highly aggregated or contagious in form such that most individuals harbor few parasites and a few individuals harbor many. In other words, the variance in parasite load is much greater in value than the mean.

Parasite aggregation may arise as a consequence of a wide variety of factors, either acting alone or concomitantly (Anderson and Gordon 1982). These include heterogeneity in exposure to infection (due to social, environmental, or behavioral factors, or to aggregation in the spatial distribution of infective stages or infected intermediate hosts), differences in susceptibility to infection (due to genetic or nutritional factors, or to varying past experience of infection), or to variability in parasite survival within different individuals (due to genetic, immunological, or nutritional factors). Which factors are of major importance as determinants of observed patterns is often difficult to ascertain in particular settings. Heterogeneity in the human behavioral patterns associated with exposure to infection is often very important as is well illustrated by water contact patterns in relation to *Schistosoma mansoni* transmission (Fulford et al. 1996) (see Figure 6, see p. 7). However, host genetic background is undoubtedly of greater impor-

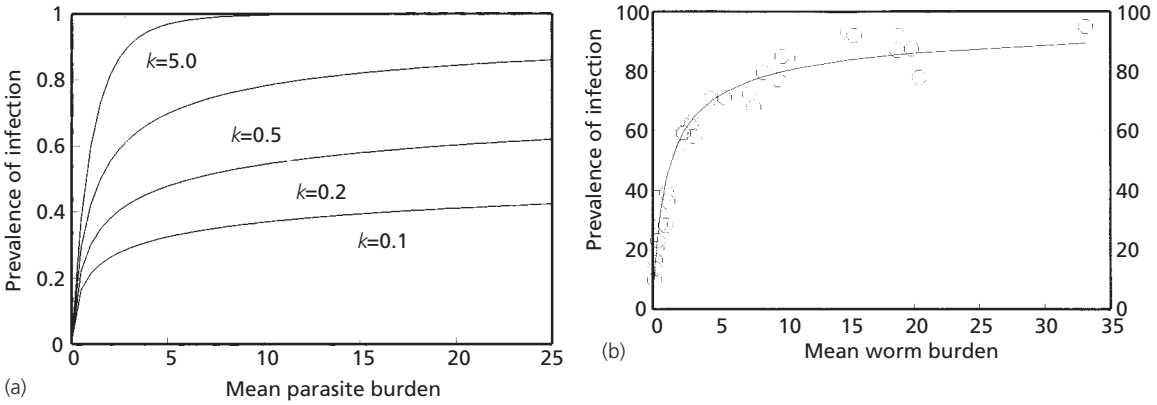

Figure 4 (a) *The relationship between the prevalence and mean intensity of infection predicted by the negative binomial distribution for different values of the aggregation parameter* k *(where* k → *0 reflects extreme aggregation and* k → *5 reflects a random distribution of parasite numbers per host).* **(b)** *A comparison of the observed and expected (on the basis of the negative binomial model with* k = *0.586) relationship between the prevalence and intensity of infection with* Ascaris lumbricoides *in different human communities. The circles represent observed values; the continuous line is the expected value (after Guyatt et al. 1990).*

tance as well illustrated by the practice of using inbred strains of rodent hosts in the experimental study of many parasitic infections in order to reduce variability in the typical course of infection.

A growing number of studies are pointing to genetic factors in human communities as key determinants of susceptibility to infection and disease. For example, severe clinical disease in schistosomiasis is typically the consequence of heavy infection, the occurrence of which is determined largely by the susceptibility or resistence of individuals (Wilkins et al. 1987). The intensity of infection in a particular study site in Brazil has been shown to be influenced by a major gene leading to the hypothesis that worm burden is largely controlled by the genetic background of the human host concomitant with the degree of exposure to infection (Abel et al. 1991). The gene, referred to as *sm1*, is located on chromosome 5, and the region in which segregation analysis indicates it lies contains several candidate genes that encode immunological molecules that are known to play important roles in the acquisition of resistance to *S. mansoni* (Marquet et al. 1996).

The discovery of a clear genetic marker for predisposition to heavy infection in the study of Marquet et al. (1996) in the case of schistosomes may point the way to a better understanding in general of predisposition to helminth infection and associated disease. Epidemiological studies based on the quantification of worm load, chemotherapeutic treatment, and the monitoring of patterns of re-infection post treatment, have provided firm evidence of predisposition to heavy infection for a variety of parasites including *Ascaris lumbroides* (Elkins et al. 1986), *Necator americanus* (Schad and Anderson 1985), *Trichuris trichiura* (Bundy et al. 1987), and *Schistosoma mansoni* (Bensted-Smith et al. 1987). Undoubtedly, however, genetic factors act concomitantly with the host behaviors to determine parasite load.

Aggregated distributions of parasite numbers per host are also of importance for protozoans such as the malarial parasites. Observed heterogeneities include the distribution of oocysts in mosquito populations, the number of distinct antigenic types or strains per human host or per mosquito, and the frequency distribution of mosquito bites per person (Paul et al. 1995; Dye and Hasibeder 1986; Anderson 1994). If there is significant heterogeneity in exposure/susceptibility to infection then in epidemic situations, the fraction of the population infected will depend critically on the degree of heterogeneity in exposure. This point is illustrated in Figure 5 which records the relationship between the fraction infected in an epidemic and the magnitude of transmission success of the parasite (R_0), as a function of the degree of heterogeneity in the rate of exposure to infection defined by the coefficient of variation (where cv = 0

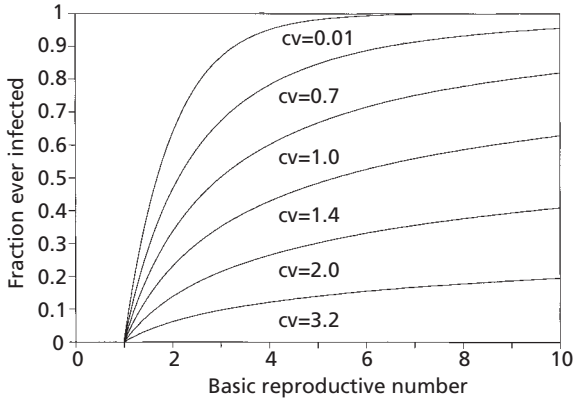

Figure 5 *The predicted relationship between the fraction of a population infected by a microparasite over the course of an epidemic in a virgin population and the degree of heterogeneity in the human behaviors that dictate exposure to infection as measured by the coefficient of variation (cv) where cv = 0 reflects a random distribution (see Anderson and May 1991).*

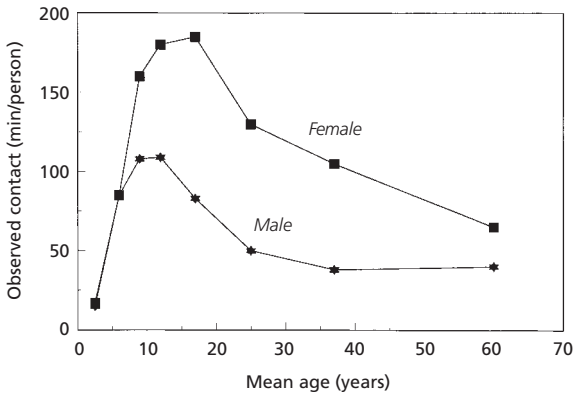

Figure 6 *Recorded water contact rates, stratified by age and sex in a Kenyan community where* Schistosoma mansoni *is endemic. The vertical axis records the arithmetic mean duration of water contact per individual (Kintengei community (1990–1992), after Fulford et al. 1996).*

reflects a random distribution of exposure) of the frequency distribution. An example of variation in exposure to infection is shown in Figure 6.

Irrespective of the generative mechanisms of such patterns, aggregation of parasites within human communities has important implications for the epidemiology and control of these infections. Firstly, it enhances the likelihood of an individual parasite finding a mate of the opposite sex (Figure 7). It therefore enhances the net reproductive rate of the total parasite population. Second, it increases the net regulatory impact of density-dependent constraints on parasite establishment, survival, and reproduction (often immunologically mediated). Third, it results in severe symptoms of disease typically occurring in a relatively small fraction of the total population. Finally, it has important implications for the design of control programs (i.e. chemotherapy can be focused on those predisposed to heavy infection).

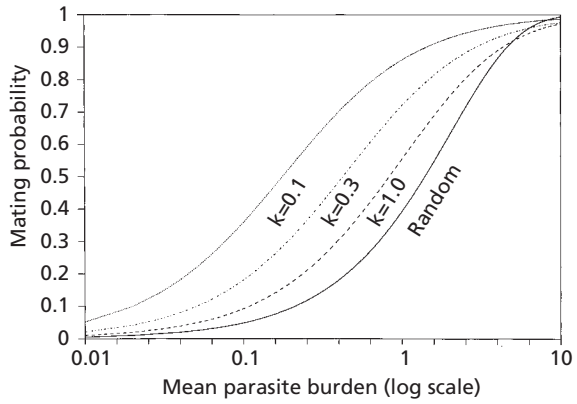

Figure 7 *The dependence of the probability that a female worm is mated, on the mean worm burden per person, for various assumptions concerning the distribution of parasite numbers per host for a monogamous parasite. The distributions are the negative binomial (= aggregated) with various* k *values and the Poisson (= random) distribution (after May 1977).*

If host genetic background has a major influence on immunocompetence, however, as suggested by work on *S. mansoni* (Marquet et al. 1996), then work towards vaccine development must take account of the fact that the aim of immunization must be to protect those least able to acquire immunity.

TRANSMISSION DYNAMICS

Observed epidemiological pattern is generated by the population or transmission dynamics of the parasite within a defined community. Before turning to the key processes influencing transmission, a brief comment is made on observed patterns of prevalence and intensity of infection.

Cross-sectional surveys of prevalence and intensity of infection

A variety of methods may be employed to assess the manner in which the prevalence or intensity of infection changes over time or between different strata (such as age groups) of the population, depending on the type of infectious agent under study. These range from molecular and immunological methods to the direct counting of either parasite eggs in fecal material (intestinal nematodes) or infected red blood cells in blood samples (i.e. for *Plasmodium*). The majority of published studies are cross-sectional and stratified by age owing to the long period of study required to monitor longitudinal changes in a cohort of people over their average life span.

As illustrated in Figures 8–10, changes in both prevalence and intensity with age (= time) are typically convex in form with the latter showing a greater degree of convexity than the former. Changes in prevalence as individuals age are a poor reflection of average parasite load due to the high degree of heterogeneity in the frequency distribution of parasite numbers per person. Convex patterns of change with age may arise either as a result of age-related changes in exposure to infection (due to behavioral factors) (see Figure 6) and/or increased resistance to infection (where acquired immunity may influence establishment or parasite survival once in the host) in older individuals with considerable past experience of infection. With respect to convexity in prevalence an additional factor may be of importance. A decay in prevalence in older age groups could arise, independent of any change in the average worm load, if the degree of parasite aggregation increases with age. Much evidence supports this notion and it may arise due to heterogeneity in genetic make-up within the host population where some individuals develop a good degree of immunity after limited exposure while others remain predisposed to heavy infection. In general, convexity is a common feature for all of the major

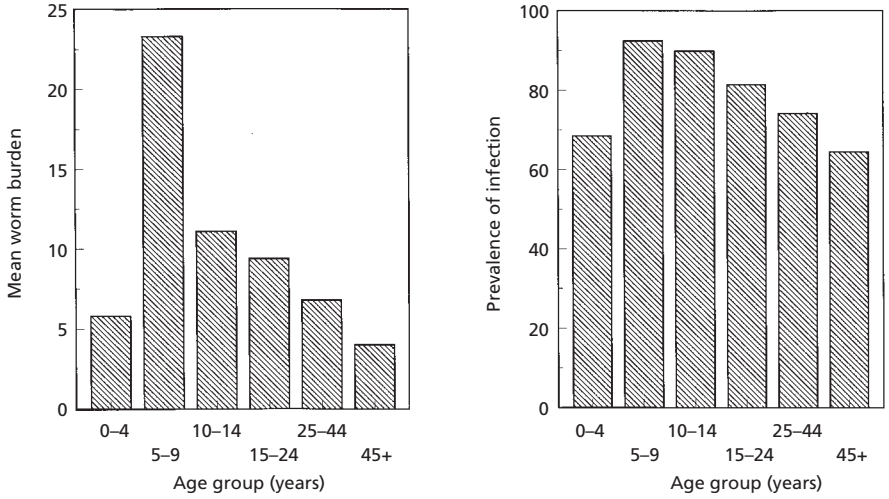

Figure 8 *Cross-sectional survey of the prevalence of infection and mean worm burden in a community in southern India infected with the intestinal nematode* Ascaris lumbricoides *(after Elkins et al. 1986)*

parasitic infections and may arise from ecological (= behavioral) or immunological processes, or a combination of both.

Studies in which age-related changes in exposure have been quantified (i.e. water contact patterns in the case of schistosomes, vector biting rates for malaria and the filarial worms) suggest that a combination of both factors acts to determine the observed convex profiles. A good indication of the significance of the slow build up of immunity with repeated exposure as individuals age is provided by comparative studies of the degree of convexity as a function of the overall net intensity of transmission in a defined community. For intestinal nematodes (Anderson and May 1991), schistosomes (Anderson 1987), and malaria (Boyd 1949), the degree of convexity is greater in areas of high transmission intensity and less in areas of low average exposure (Figure 11). The issue of why immunity to parasite infection builds up slowly as individuals age is discussed later in this chapter.

In summary, observed patterns of infection with parasitic organisms tend to reveal three key points, namely, heterogeneity in parasite burdens per person, predisposition to heavy or light infection, and convexity in the manner prevalence or intensity change as individuals age.

Life cycles and reproductive success

Microparasite and macroparasite life or developmental cycles may be direct or indirect. In the latter case one or more intermediate hosts may be involved, such as the molluskan intermediate host of the schistosomes and the mosquito vector of the malaria parasites. The transmission potential of a parasite with a complex life cycle is determined by many distinct population-determining

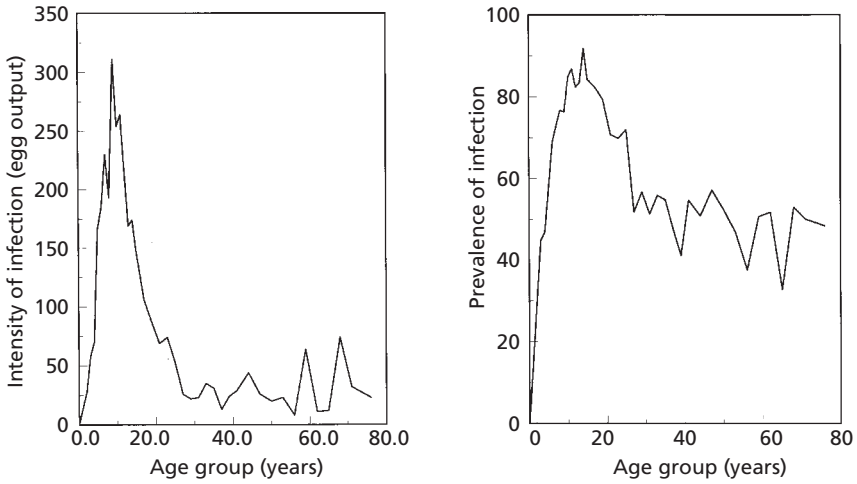

Figure 9 *Cross-sectional survey of the prevalence of infection and the mean intensity of infection (based on fecal egg counts) in a community in Tanzania infected with* Schistosoma haematobium *(after Bradley and McCullough 1973)*

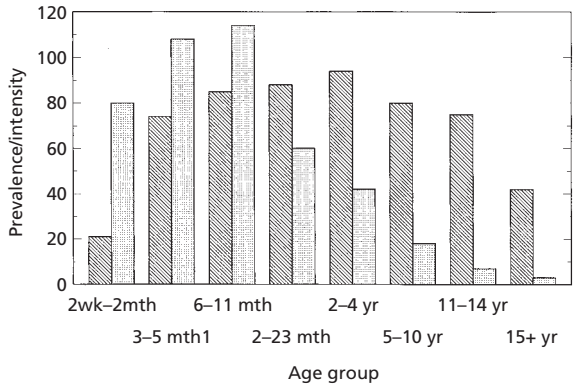

Figure 10 *Cross-sectional survey of the prevalence (dark bars) and intensity (lighter bars) of* Plasmodium falciparum *(after Davidson and Draper 1953)*

rate processes that influence the many distinct developmental stages. Examples of the population and rate processes involved in some direct (intestinal nematodes) and indirect cycles are portrayed in Figures 12–14.

The overall transmission or reproductive success is best defined by the basic reproductive number, R_0, of the parasite. For a direct life cycle intestinal nematode with a free-living larval or egg stage as the transmission stage (Figure 12), the magnitude of R_0 in a human community of size N is defined as follows (see Anderson and May 1991):

$$R_0 = [s\lambda\beta N d_1 d_2]/[(\mu + \mu_1)(\mu_2 + \beta N)] \qquad (5)$$

The individual parameters are defined in Table 1. Provided $R_0 > 1$, the parasite population will persist in the host population. However, unlike many directly transmitted viral or bacterial infections, the critical host density for persistence of most protozoa and helminths is typically very low. Infections such as intestinal nematodes or malaria parasites were therefore able to persist endemically in small hunter-gatherer societies in areas such as the Amazon basin or Papua New Guinea (Tyrrell 1977) prior to the encroachment of civilization into remote rain forest habitats.

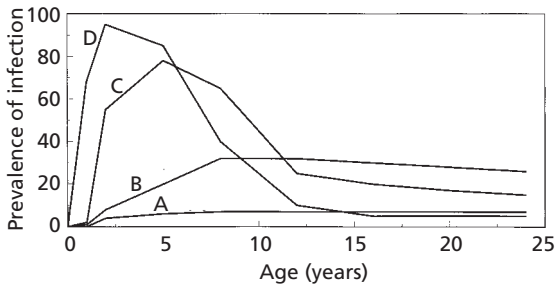

Figure 11 *Prevalence of acute malaria infection versus age in years in populations with differing levels of endemicity. A, low endemicity; B, moderate endemicity; C, high endemicity; D, hyperendemicity (after Boyd 1949)*

In the absence of regulating constraints on population growth, the parasite population growth rate Λ is given approximately by:

$$\Lambda = (R_0 - 1)/(\mu^{-1} + \mu_1^{-1}). \qquad (6)$$

This expression makes clear the role of net reproductive success (R_0) and adult parasite life expectancy μ_1^{-1} as important determinants of re-infection rates, or parasite population recovery, after the cessation of control measures. Populations of short-lived parasites (i.e. malaria) will bounce back rapidly to pre-controlled levels once control measures cease, while long-lived species may take many years to recover (Table 2).

For microparasites with indirect life cycles (Figure 14) such as the *Plasmodium* species, the magnitude of R_0 is determined by rate processes acting on the human and vector populations. For malaria R_0 is defined as follows (see Aron and May 1982):

$$R_0 = [ma^2bc/\mu_2\gamma]exp(-\mu_1 T_1 - \mu T_2) \qquad (7)$$

where m is the ratio of female mosquitoes per human host, a is the rate of biting on humans by a single mosquito (the 'human biting rate' defined as the number of bites per unit of time), b is the proportion of infectious bites that produce a patent infection, γ^{-1} is the average duration of infectiousness over the course of infection in the human host, c is the proportion of bites by a susceptible mosquito on infected people that produce a patent infection in the vector, μ_1^{-1} is mosquito life expectancy, μ_2^{-1} is human life expectancy, T_2 is the latent period in the mosquito (before it becomes infectious to humans), and T_1 is the latent period in humans (before gametocytes appear) (Table 3). In practice, the effective time the insect vector has to transmit is much shorter than its life expectancy due to the fact that the latent period of infection in the vector is often long by comparison with life expectancy (Table 3). This is the reason why the prevalence of infection in vectors is often very low (a few percent or less), since few survive long enough for the infection to mature. If $R_0 > 1$, the infection is endemic in the human population.

Vector densities often fluctuate widely over the different seasons of a year due to the influence of environmental factors, such as rainfall on breeding success and life expectancy. The magnitude of m in eq. (5) may therefore fluctuate widely such that at certain times of the year $R_0 > 1$, whilst at others (the dry season) it falls below unity in value. In such circumstances the endemic persistence of malaria depends on the survival of the infection in the human host. Areas of low transmission are much more sensitive to seasonal changes in mosquito or vector density.

More generally, the longest lived stage in the parasite's life cycle plays a key role in ensuring the long persistence of the organism. Control measures aimed at

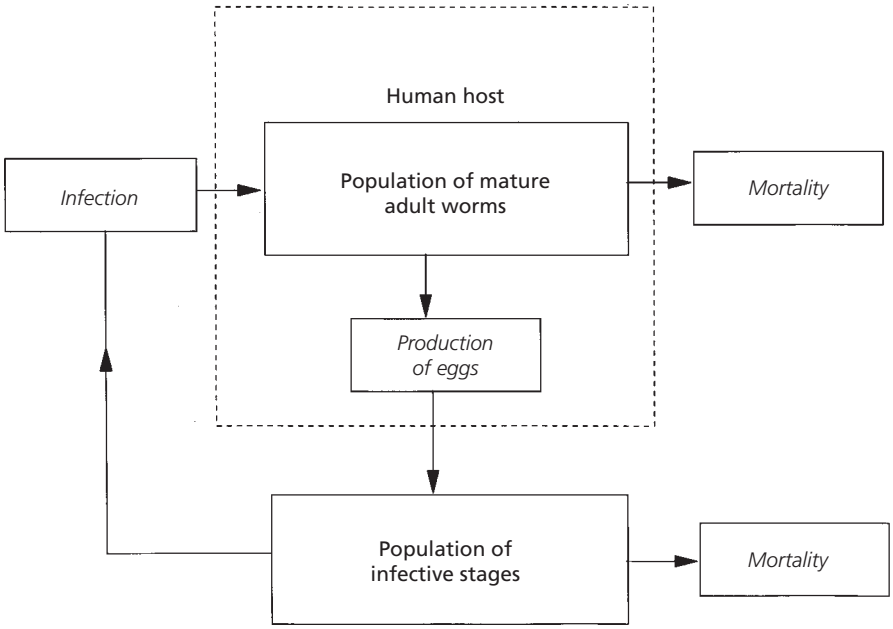

Figure 12 *Flow chart of the principal population and rate processes involved in the life cycle of a directly transmitted intestinal nematode (macroparasite)*

different stages in the cycle (e.g. insecticides acting on the vector or chemotherapeutic agents acting on the infection in the human host) may have similar effects on transmission success, but very different effects on the ability of the parasite population to recover rapidly post the cessation or interruption of control measures.

Regulating constraints on parasite population growth

A characteristic of many parasitic infections is their stability or robustness to perturbation, whether induced by environmental changes or control interventions.

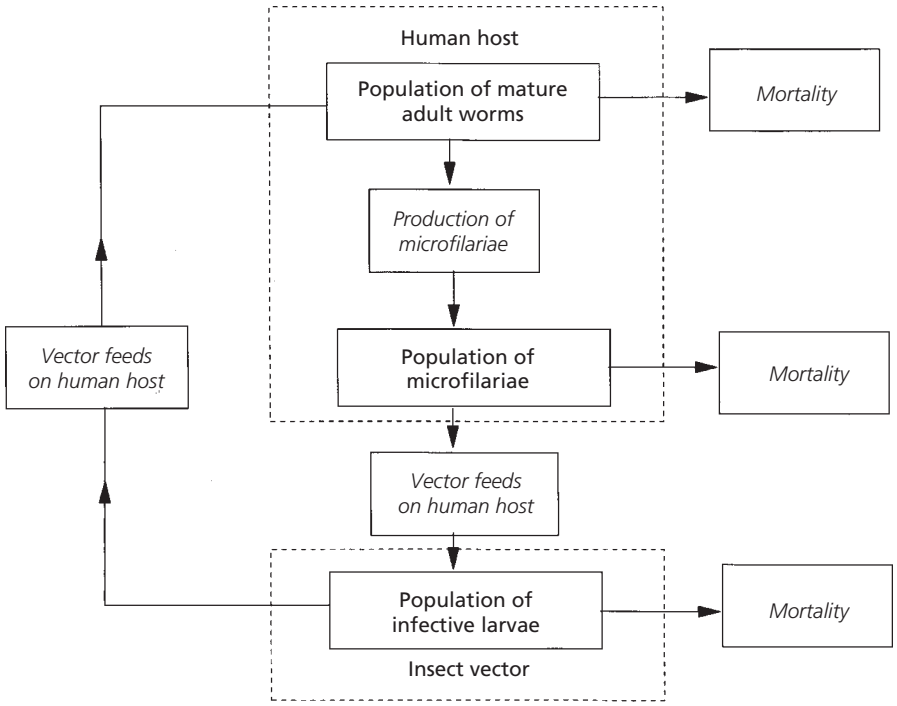

Figure 13 *Flow chart of the principal populations and rate processes involved in the life cycle of an indirectly transmitted filarial nematode (macroparasite)*

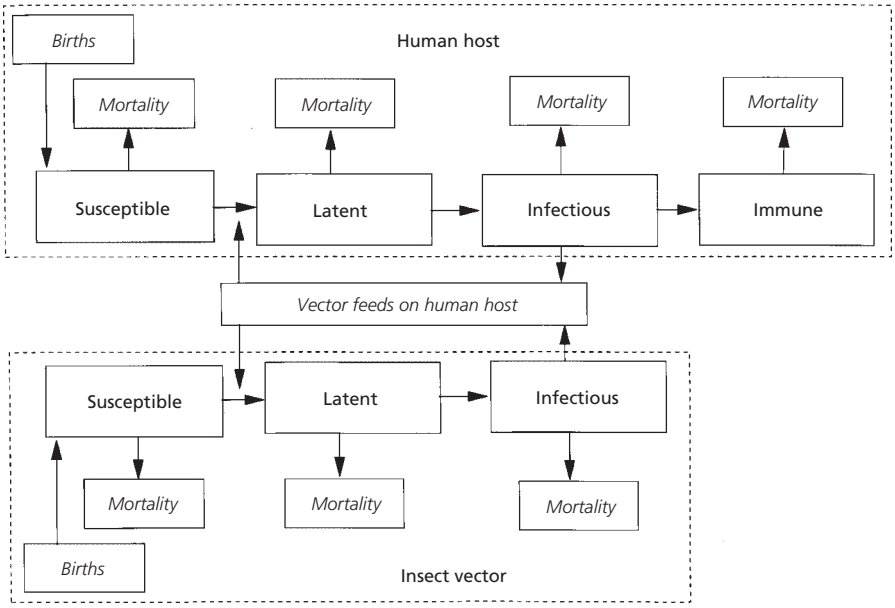

Figure 14 *Flow chart of the principal population and rate processes involved in the life cycle of a malarial parasite (vector transmitted microparasite)*

Helminth infections are particularly remarkable in this sense, where after the cessation of mass chemotherapy, parasite abundance typically returns, over a time span of a year or so, to pre-control levels. In part this is a consequence of their transmission potential (e.g. very high fecundity) and in part due to the long life expectancy of the mature worms in the human host (Table 2). In the absence of density-dependent constraints on population growth, however, provided $R_0 > 1$, their abundance would grow exponentially. As originally noted by Malthus in the context of human population, resource limitation must eventually regulate the rate of growth until some sort of equilibrium is attained. This may be oscillating in character but for most parasites, if we ignore short-term seasonal fluctuations, endemic states appear to be stable and non-oscillatory. For parasites occupying an environment created by another living organism, the constraints on growth are many and varied and act on establishment, fecundity, and survival. An example is presented in Figure 15, where the fecundity of *S. mansoni* is plotted as a function of the intensity of infection. They may arise via competition for limiting resources such as food or space or, more commonly, as a result of the non-linear action of the host's immunological responses. The non-linear nature of such responses is reflected by modest or little response at very low parasite densities, and strong responses at high densities. At very high parasite (or antigen) densities, in some cases the effectiveness of the immune response decays as depicted in Figure 16, where T cell proliferation in response to different concentrations of *P. falciparum* antigens is plotted. This may be due to modulation by the parasite, the accumulation of parasite

Table 1 *The rate processes that determine the magnitude of the basic reproductive number, R_0, of a direct life cycle intestinal nematode (see Anderson and May 1991)*

Parameter	Definition
s	Sex rate of adult worms
λ	Per capita fecundity of female worms
β	Per capita transmission rate of infective stages to the human host
d_1	Proportion of adult worms that attain sexual maturity
d_2	Proportion of transmission stages that survive to the infective stage
μ^{-1}	Life expectancy of the human host
μ_1^{-1}	Life expectancy of adult worms
μ_2^{-1}	Life expectancy of infective larvae
N	Human population size

Table 2 *Helminth parasite life expectancy in the human host (see Anderson and May 1985 for source references)*

Parasite	Life expectancy (years)[a]
Enterobius vermicularis	<1
Trichuris trichiura	1–2
Ascaris lumbricoides	1–2
Necator americanus	2–3
Ancylostoma duodenale	2–3
Schistosoma mansoni	3–6
Schistosoma haematobium	3–6
Wuchereria bancrofti	3–5
Onchocerca volvulus	8–10

a) Rough approximations, owing to the practical difficulties inherent in estimation.

Table 3 *Latent periods and vector life expectancies (see Anderson and May 1991 for source references)*

Parasite	Latent period (days)	Vector	Life expectancy (days)
Plasmodium falciparum	11 days (24°C)	*Anopheles gambiae*	8–15
Trypanosoma brucei	15–35	*Glossina morsitans*	28–32
Yellow fever virus	10–12	*Aedes aegypti*	8–14
Onchocerca volvulus	14	*Simulium damnosum*	10–20
Wuchereria bancrofti	13–15	*Anopheles funestus*	10–15
Schistosoma mansoni	20–42 (22°C)	*Biomphalaria glabrata*	7–42

secreted toxins or some sort of malfunctioning of the host induced regulatory constraints on the immune response.

Immunity to parasitic infections

One of the major features of the epidemiology of parasite infections is evidence for repeated infection as individuals age in areas of endemic exposure despite abundant evidence of immune recognition and attack. This is a puzzle of both practical and fundamental significance. Protozoa and helminths have complex genomes and elaborate developmental cycles, and have evolved a wide array of mechanisms to evade or modulate immunological attack (Maizels et al. 1993 and Immunology and immunopathology of human parasitic infections).

As mentioned earlier, the observed aggregated distributions of parasite number per host may reflect differences in innate susceptibility to infection or in the ability to mount effective immunological responses. Work on the population dynamics of the interaction between subsets of T helper cells (Th1 and Th2 subsets) suggests that early exposure to infection in infancy (or even in the womb via maternal experience) may in part determine predisposition to heavy or light infection where high exposure generates tolerance with elevated Th2 responses concomitant with high helminth loads (Schweitzer and Anderson 1992). The quantitative details of the host's ability to recognize and respond to particular parasite antigens (influenced by the genetic make-up of the host) is of importance in determining whether the host is persistently susceptible or develops protective immunity.

To tease out the factors that control observed patterns of infection and immunity in communities, epidemiological study must turn more and more to detailed longitudinal studies of changes in parasite numbers and immunological responses specific to parasite antigens. For the blood-dwelling schistosome flukes in humans (*S. mansoni* and *S. haematobium*), circulating specific and non-specific serum IgE correlates both positively with accumulated past experience of infection and negatively with re-infection rates after drug treatment (Hagan et al. 1987). For the gut-dwelling nematode *Trichuris trichiuria*, comparison of age-dependent isotope responses and the age-profile of worm burden in two endemic communities with very different levels of transmission suggests that parasite-specific secretory IgA responses best reflect accumulated past experience of infection, whereas serum IgG isotopes simply correlate positively with current worm burden (Needham et al. 1994) (Figure 17).

How specific immunological responses to protozoa and helminths operate to generate resistance (effector mechanisms) is poorly understood. Some information can be drawn from epidemiological studies via associa-

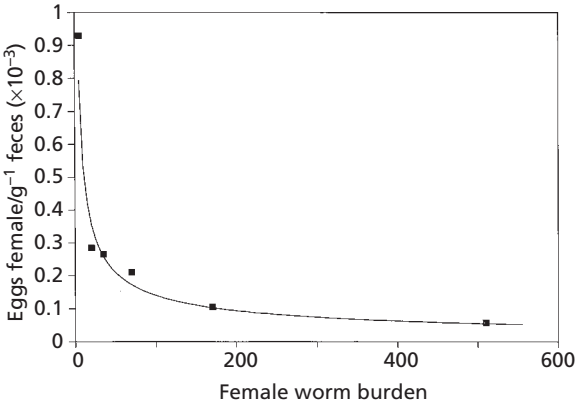

Figure 15 *Density-dependent fecundity in* Schistosoma mansoni *based on autopsy data collected by Cheever (1968) (see Medley and Anderson 1985)*

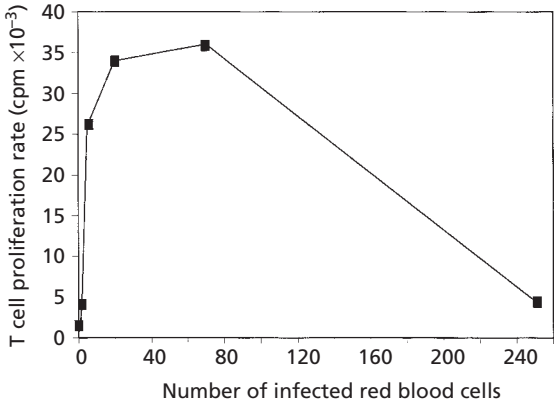

Figure 16 *Dose–response curve of the influence of antigen concentration (number of* P. falciparum *infected red blood cells) on T-cell proliferation rates (after Jones et al. 1990)*

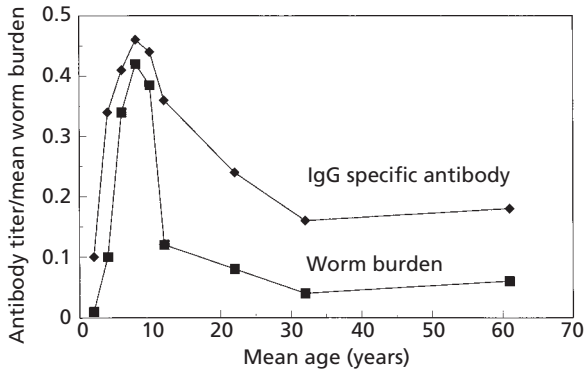

Figure 17 *Age-related changes in the burden of* Trichuris trichiura *and IgG antibodies specific to parasite antigens in a community in St Lucia, West Indies (after Needham et al. 1994)*

tion of parasite load with different types of response, or by following immune responses following drug treatment and during re-infection. A general pattern seems to be that individual responses mounted to protozoan and helminths rarely operate in isolation and resistance is typically a multi-component response (see Immunology and immunopathology of human parasitic infections).

The many and exciting opportunities presented by advances in molecular biology for the study of immunity to parasites are parallelled by the challenge of an ever-expanding descriptive literature. At present our understanding of how immunity (as reflected by resistance to infection and increased rates of parasite clearance from the host) works for any single protozoan or helminth parasite of humans is limited despite abundant descriptive study (see Immunology and immunopathology of human parasitic infections). The population ecology of the immune system is a very non-linear world and future research will need to emphasize the measurement and quantification of the many rate parameters that control the humoral and cellular responses to parasite antigens, and the functional dependencies between parasite and immune system variables that determine the dynamics of infection both within the host and in the community of hosts. To achieve this for the major infections of human communities will require more detailed longitudinal studies that combine conventional measures of parasite burden with immunological and molecular studies of parasite genetic diversity and the dynamics of immune responses under repeated exposure to infection.

Antigen diversity and strain structure in parasite populations

The maintenance of antigenic diversity is a key strategy adopted by many infectious agents to evade immunological attack in the host and to facilitate the invasion of hosts who have prior experience of infection. Advances in molecular biology have provided many new tools for the study of genetic diversity within parasite populations and these have stimulated a shift in emphasis in epidemiological research towards a more evolutionary approach to the study of infection and immunity. Evolution is at the core of the relationship between host and pathogen. In seeking explanations of observed epidemiological pattern, in the search for new drugs and vaccines, or indeed in the study of immunological responses to infection, the enormous potential of viruses, bacteria, protozoa, and helminths to evolve rapidly (either via mutation or recombination events) on timescales much shorter than the generation time of the human host, is often inadequately appreciated. In many cases, particularly for microparasites, such evolution may take place on a timescale faster than that required to mount an effective immunological response. Genetic variation in microparasites, via antigenic change, is often a central pillar of the pathogen's strategy to persist in the host in the face of immunological attack. Persistence enhances the likelihood of transmission to new hosts in the population. This strategy is particularly common amongst persistent viral or protozoan infections, such as HIV or *Plasmodium* or *Trypanosoma* species, where the immune system acts as the main selective force driving the emergence and reproductive success of 'escape' antigenic variants. One spin-off of the rapidly expanding databases on the antigenic diversity of important human pathogens (sequence databases), has been the development of statistical techniques to examine relatedness between different parasite isolates via the construction of phylogenetic trees (Harvey 1996). These techniques often enable inferences to be drawn (about who acquired infection from whom) via sequence data from parasite isolates from different patients. In the coming decade, sequence information on genetic variation within pathogen populations will increasingly be linked to phenotype, as mirrored by reproductive success of the parasite or the pathogenesis induced by different genetic variants.

At the between-host level, antigenic variation for both the microparasites and macroparasites may permit repeated infection of individual hosts as is evident in *P. falciparum* infection in humans. The slow build up of cross-reactive (across parasite strains) immunological responses after repeated exposure to different antigenic strains will influence the duration of infection, the infectiousness of a person and in some cases the likelihood of the occurrence of disease. In many cases the delay in the development of immunity to parasitic infection may be a consequence of the antigenic diversity of the parasite in which protective immunity only develops after exposure to many different antigenic types or strains circulating in a given locality. The antigenic genetic constitution of a parasite population may vary from one locality to another (Gupta and Day 1994).

Where many strains persist in a given community the question arises of how this diversity is maintained. A

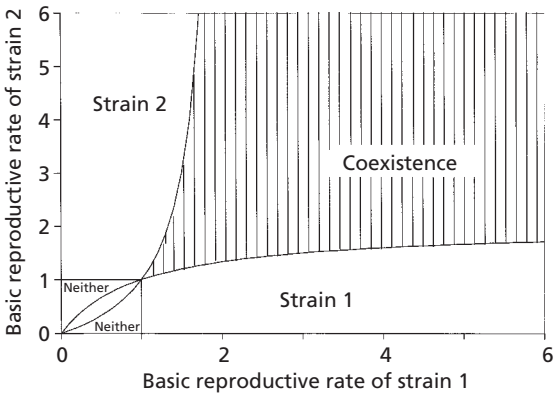

Figure 18 *The influence of the degree of cross-immunity (c = 0, total cross-immunity, c = 1, no cross-immunity, with c set at 0.5) on the pattern of coexistence of two strains of a microparasite as a function of their respective transmission success as measured by the basic reproductive number,* R_0 *(after Gupta et al. 1994a)*

simple view might be that the strain with the greatest reproductive success would, over time, competitively displace other strains. However, recent work shows that this is not necessarily the case, provided a degree of cross-immunity builds up to antigens shared by all strains. In the case of complete cross-immunity, competitive exclusion occurs, while in the case of no cross-immunity (unlikely in reality), all strains can persist since they circulate independently in the host population (Gupta et al. 1994a). For intermediate cases (probably the norm), coexistence is possible provided the reproductive successes (the R_0) of the various strains do not differ too widely in magnitude (Figure 18). Long-term studies of temporal and spatial trends in the prevalence of different parasite strains (e.g. *P. falciparum*, African trypanosomes, and some helminth species) are in progress, but as yet little is understood about longitudinal trends in parasite diversity within defined human communities (Anderson 1994; Snow et al. 1993). What has become clear, however, is that superinfection of an infected host by a new strain appears common for many of the microparasitic organisms, including HIV and malaria (Paul et al. 1995; Robertson et al. 1995).

The observation that many distinct strains persist in human communities over long periods of time raises a further question concerning the role of genetic exchange in the maintenance of parasite strain structure. Many parasites have the opportunity for the exchange of genetic material because of, for example, sexual processes in the case of *P. falciparum*, coinfection of the same host cell for HIV, or transformation in bacteria. The persistence of strain structure plus the frequent opportunity for genetic exchange appears paradoxical, because it would seem that the former would be lost rapidly as a result of genetic recombination. This issue has recently been resolved by theoretical and observational studies which reveal that dominant polymorphic antigenic determinants (that is, those that elicit the most effective immune responses) will be organized into non-overlapping combinations of antigenic determinants as a result of selection by the host's immune system, thereby defining a set of distinctive independently transmitted strains (Gupta et al. 1996). Dominant polymorphic antigen determinants will be in linkage disequilibrium, despite frequent genetic exchange, even though they may be encoded by several unlinked genes. By contrast, weaker polymorphic determinants within the same parasite population will be in linkage equilibrium. This suggests that the detection of non-random association between epitope regions of parasite antigens can be employed as a strategy for identifying the dominant polymorphic antigens. To date, analyses of linkage disequilibrium patterns within infectious agent populations have, for the most part, only addressed the question of clonality (Walliker 1989; Tibayrenc et al. 1990).

The population dynamics and genetics of a parasite population that is structured into strains by a dominant immune response may be influenced at other levels by weaker responses to both conserved and other polymorphic determinants. For example, the typical age distribution of *P. falciparum* prevalence (see Figure 10) suggests that although the variant surface antigens (VSA) (which undergo antigenic variation within the host) categorize the population into strains (each with a given repertoire of expressed antigens), some degree of infection blocking immunity to all strains is eventually established after repeated exposure to a conserved antigen (Gupta and Day 1994).

More generally, these and other studies reveal the importance of a better understanding of the population genetics of parasite and host populations for the interpretation of epidemiological patterns. Molecular epidemiological studies that address the question of the linkage between genetic diversity in the parasite and the incidence of infection and associated disease are likely to be a major focus in the epidemiology of infectious diseases in the coming decade. The occurrence of disease in infected patients may often be linked to the subtle interplay between the genetic background of the host and that of the invading pathogen.

CONTROL OF INFECTION AND DISEASE

At present, the options for the community-based control of the major tropical parasitic infections are either chemotherapy (e.g. for malaria and the helminth infections) or interventions to prevent exposure to infection (e.g. impregnated bed nets to prevent contact with biting vectors, safe water supplies, and good sanitation to reduce contact with the transmission stages of intestinal nematodes). Vaccines are not available for any of the key parasitic infections and early trial results with candidate products have been disappointing. To date, no effective vaccine has been developed for an antigenically

variable infectious agent that provides long protection duration, irrespective of whether the target agent is a virus, bacterium or protozoan. In research on candidate vaccines, most attention is directed at achieving high efficacy. However, duration of protection is of equal significance in terms of potential effectiveness within a community-based immunization program.

For both chemotherapy and interventions to reduce the likelihood of transmission, the difficulty of the task of achieving long-term control is related to the magnitude of the transmission intensity in a defined community as measured by R_0. To block or prevent transmission, the magnitude of R_0 must be reduced to less than unity in value. As control measures are introduced, and the value of R_0 progressively declines over time, significant changes in the prevalence of infection are only likely to occur as the critical point of $R_0 = 1$ is approached (Figure 19).

For microparasite infections, community-based chemotherapy or chemoprophylaxis (in the case of malaria) is expensive and beyond the reach of most communities in poor tropical regions. In addition, treatment tends to select for resistant strains of the parasite, as well illustrated by malaria. In many regions of the world, but particularly in South-East Asia, multi-drug resistant strains of *P. falciparum* are a major cause of morbidity and mortality. Some take a pessimistic view that with the decline in the rate of development of new antimalarials (because of drug development costs and the endemism of the infection in poor developing, as opposed to wealthy developed countries) the battle to control malaria by community-based drug use is likely to be futile in the longer term. As such, many see the future in terms of vaccines although current progress does not promote optimism for rapid advances in this area. The main hope at present lies in an integrated approach involving vector control by insecticides, the promotion of community-wide impregnated bed net use, and drug treatment to prevent mortality (see Control of parasites, parasitic infections and parasitic diseases).

Epidemiological study of the emergence and persistence of drug resistant parasites has been limited in scope and scale to date. Detailed long-term longitudinal studies (ideally cohort based) are required in defined areas to measure the frequency of the genes that determine resistance to defined drugs. In parallel with such molecular epidemiological studies, quantitative information must be acquired on patterns of drug use to define the intensity of selection that induces the observed change in resistance frequency. Much more thought must be given to defining optimum methods for the use of a number of different drugs, both for the individual patient and for the community, to maximize the effective life expectancy of a drug and minimize the rate of evolution of resistance.

A more encouraging picture emerges for the helminth infections where community-wide use of anthelmintics (often targeted at the child age groups in which the intensity of infection and associated morbidity is greatest) has been very effective in parasite control. To reduce R_0 to less than unity in value, the proportion of the population that must be treated per unit of time (i.e. monthly or yearly) must exceed a critical value, g, where

$$g = 1 - exp[(1 - R_0)/A]/h \qquad (8)$$

Here, A is the life expectancy of the parasite in the human host and h is the efficacy of the drug (Anderson and May 1991). To take a specific example, namely *Ascaris lumbricoides* for R_0 values around 3 and with $A = 1$ year, a drug of 95 percent efficacy would have to be administered to more than 91 percent of the population each year to eradicate the infection (Figure 20). However, if morbidity control is the aim, a much more targeted program of treatment of heavy infection in schoolchildren would suffice. Recent studies show that additional benefits accrue from the treatment of heavy infection, such as improved rates of growth in children,

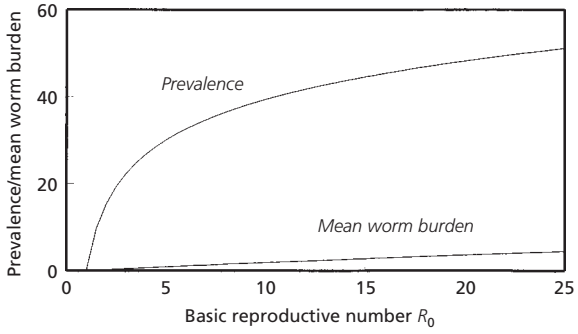

Figure 19 *The relationship between the prevalence and intensity of a macroparasitic infection as a function of the magnitude of transmission success as measured by* R_0. *Below the point* $R_0 = 1$, *the parasite is unable to persist in the host population.*

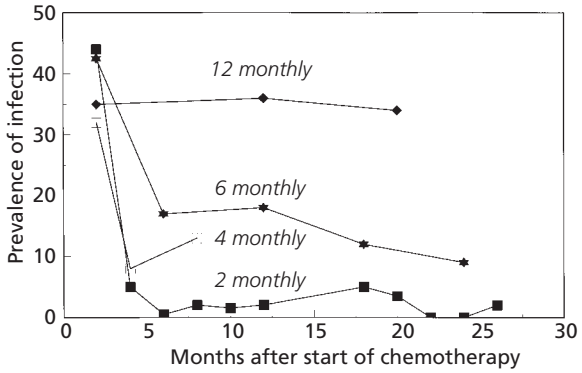

Figure 20 *The influence of different monthly intervals between rounds of mass chemotherapy on the prevalence of* Ascaris lumbricoides *in a study population in Korea (after Seo and Chai 1980)*

improved cognitive function, and a net reduction in transmission in the community as a whole (Nokes and Bundy 1994). A major practical advantage of targeting treatment to children is their accessibility during school attendance. The frequency of treatment following the introduction of control will depend on rates of re-infection as defined by the magnitude of R_0. For long-lived species with limited transmission success, such as the schistosome and filarial worms, intervals of a few to many years may suffice (Butterworth et al. 1991). Conversely, for the short-lived worms such as the intestinal parasites, yearly or more frequent treatment is required (Thein-Hliang et al. 1984).

Long-term control of all parasites in areas of endemic infection will require continual intervention or chemotherapeutic treatment unless the intensity of control is sufficient to eradicate the parasite. For the important infections in tropical regions, eradication is difficult to envisage given limited resources for public health measures. Vaccines are ideally required and their development must remain a research priority in the coming years (see Control of parasites, parasitic infections and parasitic diseases).

The scientific assessment of the effectiveness of a given intervention program in both epidemiological and economic terms, is often poor despite large expenditure in the implementation of a specific program. More rigorous approaches to intervention study design and evaluation are required that meld quantitative epidemiology study pre- and post-intervention, with assessments of cost and benefit. Cost–benefit analyses of different control options must be based on a framework that captures the interaction between the transmission dynamics of the parasite and the intensity of the control measure. This relationship is invariably non-linear and hence intuition alone will not suffice to accurately deduce the potential impact of a given policy. The key problem, however, is often in the design of the intervention program. Detailed statistical analyses are required to determine the correct sample size (whether in units of people or communities) required to detect a defined level of change in the incidence of infection or disease following intervention.

The practical aspects of the control of parasitic diseases are discussed in more detail in Control of parasites, parasitic infections, and parasitic diseases.

CONCLUSIONS

The interdisciplinary character of epidemiological research implies that advances in a wide variety of disciplines will influence our understanding of the processes that control the spread and persistence of infectious agents in human communities in the coming years. These include those in molecular biology, immunology, mathematics, genetics, information handling, storage, and retrieval. Modern desktop computers plus associated software provide a striking illustration of how advances in one field of science can influence progress in epidemiological study. Database construction and handling for a large cohort intervention study is now a simple task, as are comparative studies of sequence data from different patients or pathogen isolates, or detailed statistical analyses seeking associations between host and parasite variables.

However, in parallel with such technical advances, whether in data management or the measurement plus detection of infection or immunity, there is an associated need to broaden the conceptual template on which epidemiological study is based. Until recently, much epidemiological research on infectious agents ignored the very dynamic nature of the interaction between host and parasite populations, both with respect to the dynamics of transmission and evolution. A failure to take account of population biology and evolution may even influence the more practical end of epidemiology, namely, cost–benefit analyses of different control interventions. Such analyses must be based on measures of the impact of control on both transmission success and parasite evolution.

Direct and indirect benefits arise from vaccinating or drug treating individual patients. The direct benefits to the patient are obvious, but the indirect benefits relate to the removal of a host that can potentially pass infection to others. As such, net benefit to the community includes an element related to the reduction in transmission success of the parasite. Drug use may promote the rate of spread of drug resistant parasite strains and hence reduce the period over which a given drug is an effective treatment.

A key problem in all epidemiological studies is the interpretation of heterogeneity in patterns of infection and disease within human communities. It is in this area that recent advances in biological research, particularly at the molecular genetic level, are likely to promote the greatest change in epidemiological research. Genetic diversity in host and pathogen populations is now measurable on the scale required for population-based comparisons and linkage analyses. There seems little doubt that such work will begin to provide answers to such fundamental questions as to why some are predisposed to specific infections and associated disease, while others are not.

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Immunology and immunopathology of human parasitic infections

F.E.G. COX AND DEREK WAKELIN

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INTRODUCTION

The human immune system is concerned with defense against invading organisms and the removal of malignant cells. The role of the immune system is to deliver an appropriate immune response at the right time and place and it is not surprising, therefore, that different infectious agents should elicit different immune responses. In order to understand the nature of infectious agents, particularly parasites, it is necessary to distinguish between microparasites and macroparasites (see Epidemiology of parasitic infections). Microparasites are small and multiply within their vertebrate host, often inside cells, thus posing an immediate threat unless contained by an appropriate immune response. Viruses, bacteria, and protozoa are microparasites, typically inducing an infection in which the host becomes infected, then experiences a latent period during which the parasites multiply. This is followed by a period of disease and discomfort during which the immune response brings the infection under control and, finally, a gradual recovery and long-term resistance to reinfection. Macroparasites (i.e. helminths) are large and most do not multiply within their vertebrate host and, therefore, do not present an immediate threat after initial infection. However, the host must protect itself from overwhelming infections and reinvasion by infective stages and can only do so by eliciting an appropriate immune response. Immune responses to protozoa (microparasites) and helminths (macroparasites) are therefore very different from one another.

Although protozoa and helminths are very different in terms of the immune responses they elicit, the infections they cause have a great deal in common, usually being long-lasting and, over a period of years, inducing immunopathological changes that may be more dangerous than the infection itself. In short, the parasite invades the host, is recognized as foreign and elicits an apparently appropriate immune response. However, parasites have all evolved mechanisms for evading the immune response and the net result is a situation in which the infection is only partially controlled. As the parasite may not be totally eliminated, the host continues to mount increasingly complex immune responses until the parasite kills the host, the host overcomes the infection or there is an uneasy compromise. During the course of all infections, parasites die or are killed and parasite molecules deposited on host cells may elicit autoimmune responses which, in turn, contribute to the pathology of the infection. Some parasites can avoid eliciting an immune response by mimicking host molecules which, if effective, can be very successful but, if unsuccessful, can initiate an autoimmune reaction. The majority of parasitic infections also elicit powerful inflammatory responses that may alter, render nonfunctional or even destroy host tissues.

Virtually all parasitic infections are long-term and chronic, and it was once thought that there were

no effective, protective immune responses against parasitic infections in humans. It is now clear, however, that some form of immunity is the rule. The evidence for this comes from a number of sources:

- in endemic areas, the majority of people experience clinical immunity to parasitic infections and the prevalence of infection falls with age while immunological parameters increase
- the uneasy compromise that exists after recovery can be broken, for example by immunosuppressive drugs or concomitant infections
- in every experimental model investigated, immunity can be demonstrated and there is no reason why humans should be different.

IMMUNE RESPONSES TO PARASITIC INFECTIONS

The overall immune response

The immune response to any parasite, as with any other infectious agent, begins when the host becomes infected and the parasite is recognized by the cells of the immune system. In order to initiate an immune response, molecules of the parasite must come into contact with the cells of the immune system and, if this does not happen, no immune response will be initiated. Many parasites invade host cells and tissues and thus come into direct contact with the immune system; most intestinal parasites pass through host tissues before reaching their final location. Even for intestinal parasites that do not breach the integrity of gut wall, such as the protozoan flagellate *Giardia duodenalis*, there is the potential for direct antigen uptake across the mucosa and the possibility of evoking an immune response. Once a parasite is recognized as foreign, the next series of events is initiated. The events involved in recognition are complex and much of what we know about them is concerned with peptide antigens. This chapter contains no detailed discussion of carbohydrate antigens although these play an important role in many antiparasite responses. In the simplest situation, the first event that occurs is when parasite peptide antigens are processed by antigen-presenting cells (APC) and transported to the surface of the cell in combination with specific major histocompatibility complex (MHC) molecules. The whole complex, consisting of the MHC molecule and the peptide, is then recognized by antigen receptors on helper T lymphocytes and the immune response proper is initiated. These early steps are known as antigen processing and presentation and these events determine the eventual outcome of the immune response. A number of cell types, particularly macrophages and dendritic cells, can act as APCs. In many parasitic infections, macrophages are involved and, as the antigen on the surface of the APC binds to the receptor on the helper T lymphocyte, the macrophage releases T-cell-activating molecules known as cytokines. In response, the helper T cells produce other cytokines that, in their turn, activate the effector cells of the immune system. The specific target of the immune attack is the antigen that initiated the immune response in the first place but parasites, particularly helminths, are very large targets and the immune system has to recognize a large number of different surface antigens. Not all of these are involved in protection, and some are even able to deflect the immune response away from more vulnerable targets. Much research has been devoted to the identification and characterization of protective antigens in order to try to understand how the immune response to parasites works and how to target vaccines to relevant molecules. Considerable effort has also been put into identifying antigens that are irrelevant to protection, or even counter-protective, or are involved in the evasion of the immune response and the pathology of the disease.

Although the immune responses to parasites are complex, the individual components are relatively simple and there is nothing unique or special about immunity to parasites except for the degree of complexity. In this context, it is convenient to consider the protective immune response against infectious organisms as having three arms:

1. cytotoxic T (Tc) cells
2. activated macrophages
3. antibody.

The Tc cells plus activated macrophages constitute what is conventionally called cell-mediated or antibody-independent immunity, while antibody constitutes humoral or antibody-dependent immunity (Figure 1). The Tc cells engage target cells expressing antigens in combination with class I MHC molecules and bring about their cytotoxic activity by creating pores in the membrane of the target cell, which then ruptures. Tc cells are frequently involved in viral infections but are seldom implicated in parasitic infections. Natural killer (NK) cells constitute a heterogeneous collection of lymphocytes that are neither B nor T cells, are not specific to a particular antigen and do not involve MHC class I molecules. NK cells seem to be able to kill target cells directly by forming pores in the target cell membrane, which ruptures as in Tc cell killing. NK cells are being increasingly implicated in protection against parasitic infections, not necessarily as effector cells but as the sources of important regulatory molecules.

Activated macrophages, macrophages that have been sensitized and triggered by signals such as lipopolysaccharide and interferon-gamma (IFN-γ) are among the most important cells involved in protection against parasites and can kill target cells either intracellularly or extracellularly. If it is small, the target cell can be

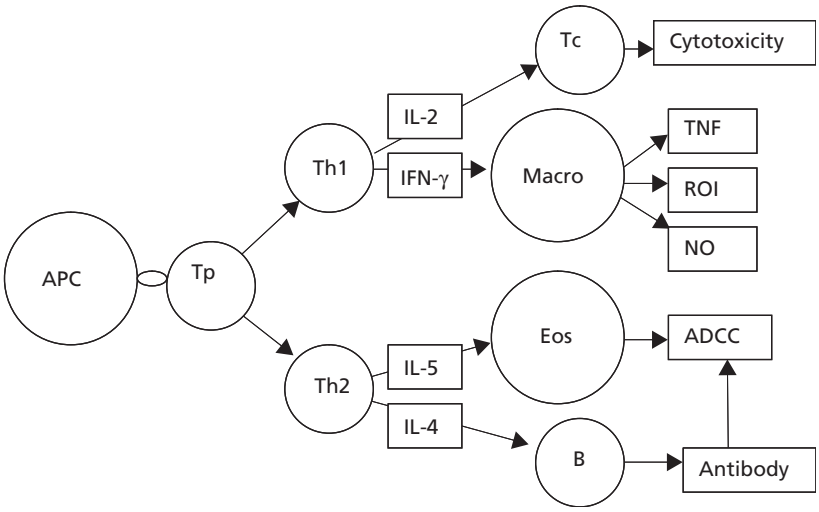

Figure 1 *Diagrammatic representation of the main features of the overall immune response. Antigen is recognized and processed by APCs and presented to precursor T (Tp) cells that carry receptors for the antigen. Depending on the nature of the antigen, and how it is presented, the Tp cells differentiate into Th1 or Th2 cells. Th1 cells produce a number of cytokines including IL-2 and INF-γ. IL-2 activates Tc cells that kill target cells carrying the original antigen in an MHC restricted manner. INF-γ activates resting macrophages (Macro) making them more phagocytic and causing them to release a variety of molecules, including TNF-α, ROI, and NO. ROI and NO are involved in the intracellular killing of ingested parasites or the destruction of parasites in close proximity to the activated macrophage. Th2 cells release a different set of cytokines, including IL-4 and IL-5. IL-4 is involved in B cell differentiation and activation and the release of antibodies specific to the antigen. IL-5 activates eosinophils (Eos). Antibodies may act in cooperation with cells such as eosinophils and macrophages by acting as a bridge bringing the activated cell and the parasite together and facilitating the release of toxic molecules directly onto the surface of the parasite, a process known as ADCC.*

phagocytosed and destroyed in the phagolysosome via a number of pathways including:

- proteolytic enzyme activity
- reactive oxygen intermediates (ROI) (superoxide, hydrogen peroxide and hydroxyl radicals) that destroy the target cell though a process of lipid peroxidation
- nitric oxide (NO) that reacts with the superoxide anion to produce highly toxic peroxynitrite and hydroxyl radicals.

Macrophages also produce tumor necrosis factor-alpha (TNF-α), which has a synergistic effect with NO. These molecules, among other things, interfere with membrane integrity, DNA structure and mitochondrial function and bring about the destruction of, or damage to, target cells. One of the key features of the activation of macrophages is that the various toxic molecules produced are released not only into the phagolysosome but also extracellularly and are thus able to destroy cells in the close vicinity of the activated macrophage. Macrophages are, therefore, able to kill large cells or multicellular organisms, especially if the macrophage is closely bound to the target via, for example, an antibody bridge as described below. These toxic molecules can also damage host cells, and their release in an inappropriate place or in excess is a major cause of immunopathology. TNF-α, for example, is highly toxic and can cause cachexia, hemodynamic dysfunction, neurotoxicity, acidosis, and renal failure; IFN-γ can cause tachycardia, leukopenia, and malaise, and can enhance the effects of TNF-α. The roles of TNF-α, oxygen-derived free radicals, NO, and other molecules in pathogenesis are reviewed by Clark and Cowden (2003).

The most important cells with direct effector functions in immune responses are Tc cells, activated macrophages and B lymphocytes (Figure 1). Each of these cells has its specific role in the immune response and can act concurrently or sequentially, with or without other cells, to bring about the immobilization or destruction of the invading organism. Other cells commonly seen in response to parasitic infections, particularly helminths, include eosinophils (Figure 1). Eosinophilia is a characteristic of a number of helminth infections but the role of these cells in human infections is enigmatic. Much of what we know about the part played by eosinophils in parasitic infections has been derived from histological studies but the elucidation of the role of interleukin (IL)-5, the key eosinophil growth and development factor, has made it possible to examine the role of these cells in more detail. It is now clear that eosinophils do play an important role in resistance to a range of helminth infections in mice, rats, dogs, primates, and humans, and that they are involved in the killing of the larval, but not the adult, stages of these parasites (Meeusen and Balic 2000).

The humoral immune response is concerned with B cell products, i.e. immunoglobulin (Ig) antibodies. The

four major classes, IgA, IgG, IgM, and IgE, occur in different situations and are effective against parasites in different ways although there is some sharing of functions. Antibodies are directly effective against invading organisms in four main ways: neutralization, agglutination, complement activation, and facilitated opsonization. IgA occurs as two subclasses, IgA1 and IgA2, each of which can occur in a monomeric, dimeric or, occasionally, polymeric form. IgA is essentially an antibody found in secretions where it protects the mucous surfaces of the gut, respiratory tract and urinogenital tract; it also occurs in breast milk. Its main functions are the neutralization of bacterial toxins and the inhibition of the invasion of the mucosa, either by agglutinating the infectious agents or blocking attachment to host cells. IgG and IgM are found in the serum and extravascular spaces. IgM, a pentameric immunoglobulin that occurs as a single subclass, is the first antibody produced in response to antigenic stimulation; it is very good at agglutinating microorganisms with repeated epitopes and at activating complement via the classic pathway. IgG occurs as four subclasses (IgG1, IgG2, IgG3, and IgG4), and usually replaces IgM; it is the most common antibody in secondary immune responses. All subclasses can agglutinate microorganisms. IgG1, IgG2, and IgG3 can activate complement, IgG3 being the most effective. IgG1 and IgG4 can pass across the placenta, and IgG1 and IgG3 can bind to macrophages and neutrophils via their Fc portions. IgE has an affinity for mast cells, basophils and eosinophils and, when bound to an antigen, causes cross-linking, resulting in the release of pharmacologically active substances from the cells to which it is bound.

Antibodies seldom work in isolation from other components of the immune system. IgG and IgM fix complement and initiate complement-mediated lysis of the target cell. IgG and IgE bind to macrophages, eosinophils, and mast cells, via the Fc portion, and to the target cell by the Fab portion, and thus facilitate the destruction of the target cell by toxic molecules released by the effector cell, a process known as antibody dependent cell-mediated cytotoxicity (ADCC).

The immune responses evoked during infections with parasites are not unusual and are essentially the same as those involved in immunity to other infectious organisms. What is different is the complexity of the immune response in reaction to diverse antigens expressed by different stages of complicated life cycles often in different locations in the host. This leads to a plethora of antibody-independent and antibody-dependent responses which, over a period of time, can give rise to misdirected immune responses leading to pathological changes instead of protection. For example, the production of TNF-α in malaria is beneficial but in excess is detrimental; in schistosomiasis potentially protective responses lead to the formation of harmful granulomas around nonthreatening eggs deposited in tissues. For these reasons, current interest in immunity to parasites is being concentrated on an understanding of the overall control of the immune response which is brought about by the interactions of the various cytokines involved in a cytokine network.

The cytokine network in parasitic infections

Cytokines are low molecular weight protein mediators that are involved in the growth, differentiation and repair of cells and occupy a central position in the regulation of the immune response. Since their discovery in the 1960s, the study of cytokines has revolutionized understanding of both the control of the immune response and the nature of the pathology associated with diseases caused by infectious organisms. This has become a massive field and is covered in more detail in Cytokines in the Immunology volume of this series and, in the context of infectious diseases, in Kotb and Calandra (2003). The cytokine network acts as a series of signals between the cells of the immune system. Many cytokines are growth or activation factors and without them the immune system could not function. An understanding of the cytokine network has permitted immunologists to unravel the interactions that come into play during an immune response and to consider individual components separately before considering them in the context of the response as a whole. Four main groups of cytokines are involved in the immune response: interleukins, interferons, transforming growth factors, and tumor necrosis factors (Tables 1 and 2). All of these have been intensively studied, particularly the interleukins; about 30 interleukins have been described but there is a considerable degree of overlap in structure and function, particularly concerning the 'over-16s', giving rise to some confusion and concern (Eberl 2002). Among the most important cytokines in the context of immunity to infectious diseases are IL-1–6, 8–10, 12, 13, and 15–18, interferon-alpha (IFN-α), interferon-beta (INF-β) and IFN-γ, TNF-α and tumor necrosis factor-beta (TNF-β). Other cytokines involved, directly or indirectly, in infectious diseases include a group known as chemokines (including IL-8) that are responsible for the induction and migration of subsets of leukocytes. Many cytokines occur in more than one form, e.g. IL-1a and IL-1b, with subtly different functions. Cytokines are produced by many cell types and act on many cell types, including the ones that produce them, in some cases promoting cell activity and in other cases inhibiting it. Thus, there is a network of cytokine activity that can be very difficult to unravel. Nevertheless, it is possible to distinguish two distinct pathways of cytokine activity:

1 the pro-inflammatory pathway (mainly involving IL-1, IL-6, IL-12, IL-15, IL-18, IFN-γ and TNF-α) that leads to cell-mediated immune responses

Table 1 *Interleukins involved in immunity to parasitic infections*

Cytokine	Important source cell(s)	Important target cell(s)	Important action(s)	Role in immunity to parasitic infections
IL-1	Macrophages and virtually all nucleated cells	Macrophages, T cells, B. cells, eosinophils, neutrophils	Lymphocyte activating factor. Stimulation of immune response, increase in production of other cytokines	Involved in all parasitic infections
IL-2	Th1 cells	Th and Tc cells	T cell growth factor. Growth and proliferation of T cells, induction of cytotoxic T cells	Cytotoxic killing of liver stages of *Plasmodium*
IL-3	T cells	Hemopoietic and other cells	Multicolony stimulating factor. Hemopoiesis	Involved in all parasitic infections
IL-4	Th2 cells	B cells, macrophages, mast cells, and other cell types	Proliferation of B cells, direction of immune response to Th2 pole, immunoglobulin switching, inhibition of inflammation	Involved in antibody production in all parasitic infections. Production of IgE. Expulsion of gastro-intestinal nematodes, Inhibition of cell mediated immunity in leishmaniasis
IL-5	T cells, mast cells, eosinophils	Eosinophils, B cells	Eosinophil differentiation factor. Production of eosinophils	Eosinophilia in helminth infections, ADCC killing of helminth larval stages
IL-6	T cells, monocytes, macrophages, and other cell types	B cells, myeloid cells, and other cell types	B cell stimulatory factor. B cell growth and differentiation	
IL-7	Bone marrow cells	Pre-B and pre-T cells	Lymphopoietin. Growth of pre-T and pre-B cells	
IL-8	Monocytes and macrophages	Neutrophils	Neutrophil chemoattraction	
IL-9	Th2 cells	T cells, mast cells	T cell growth factor. Synergizes with IL-4 in antibody production	
IL-10	Th2 cells, monocytes, macrophages	Th1 cells, NK cells, macrophages	Immunoregulation. Enhancement of Th2 responses, inhibition of Th1 cell and macrophage function, inhibition of IL-12, IL-13 and IFN-γ	Inmunity in leishmaniasis. Downregulation of immunity in filariasis
IL-11	Bone marrow cells	Pre-B cells	Promotion of pro-inflammatory cytokines IL-1, IL-6 and NO	
IL-12	Macrophages	T cells, NK cells	NK cell stimulatory factor. Promotion of Th1 responses, inhibition of IL-4 and IL-10	Enhancement of immunity to intracellular protozoa, e.g. *Leishmania*, *Trypanosoma cruzi*, *Toxoplasma*
IL-13	Th2 cells, other T cells, eosinophils, mast cells	B cells, monocytes	Proliferation of B cells, promotion of Th2 differentiation, inhibition of Th1 activities	Expulsion of gastro-intestinal nematodes
IL-14	T cells	B cells	Proliferation of B cells, inhibition of antibody secretion	
IL-15	Monocytes	T cells, NK cells	T cell growth factor. Promotion of Th1 cell activities, enhances cytotoxic activities of T cells and NK cells	
IL-16	T cells	T cells	Chemoattraction	
IL-17	T cells	Macrophages	Initiation of inflammatory responses	
IL-18	Activated macrophages		Induction and enhancement of IFN-γ and NK cell cytotoxicity	Regulation of mast cell and Th2 cytokines in trichinellosis

There is a considerable degree of cross-function among the post-16 cytokines (IL-17 to IL-27) (see Eberl 2002). Most of these cytokines have not been thoroughly investigated in parasitic infections.

Table 2 *Properties of important cytokines in immunity to parasitic infections*

Th1 cytokines	Th2 cytokines	Pro-inflammatory cytokines	Counter-inflammatory cytokines	Cytokines that can lead to pathology[b]
IFN-γ[a]	IL-4[a]	IL-12	IL-4	IL-1
IL-2	IL-5[a]	IL-15	IL-10	IL-6
IL-3	IL-3	IL-18	TGF-β	IL-8
TNF-α	IL-13	IFN-γ		IL-12
TNF-β	IL-6			TNF-α
GM-CSF	IL-10			MIF
	TGF-β			

a) These are the most important cytokines.
b) For example, increased vascular permeability, tissue damage, circulatory collapse, multi-organ failure etc.

2 a pathway (involving IL-3, IL-4, IL-5 and IL-13) that leads to the activation of the humoral immune response, the production of antibodies and the activation of inflammatory cells.

These distinctions are not absolute and some cytokines can have contrasting functions, for example IL-6 is a pro-inflammatory cytokine that can also indirectly damp down such responses through its ability to control the production of acute phase proteins. In all infections there are well-regulated pathways of cytokine activity that lead to protection but these are nearly always accompanied by counter-protective pathways that damp down potentially damaging side-effects (Figure 2). Many cell-mediated immune responses, for example, are characterized by the production of dangerous molecules such as TNF-α and NO that can cause pathology but are modulated by other cytokines such as IL-4, IL-10, and IL-13.

From the moment an immunologically intact host is infected with a parasite or any other infectious agent, the host begins to mount an appropriate protective immune response. The key cells are the so-called T helper (Th) lymphocytes characterized by the possession of a CD4 surface marker. At first, these are uncommitted but gradually differentiate into Th1 and Th2 cells, each characterized by the cytokines they produce, until eventually they become fully differentiated; when this happens, they are mutually exclusive. The Th1 cells produce T1 cytokines that drive the immune response towards antibody-independent immune responses, in particular IL-2 that drives the immune response towards the production of CD8 Tc cells and IFN-γ that drives the response towards the activation of macrophages. Th2 cells, on the other hand, produce IL-4, IL-5, IL-10, and IL-13 that lead to the activation of B cells, and the subsequent production of antibody, and to the proliferation and differentiation of eosinophils. A third subset of Th cells, Th3 or T-regulatory (Tr) cells produce cytokines such as IL-10 and transforming growth factor-beta (TGF-β) that down-regulate the activities of other Th cells and, therefore depress or even suppress the immune response. In short, the Th1 responses represent the cell-mediated arm of the immune response and the Th2 responses represent the humoral arm. Tc cells are ideally suited for the destruction of virus-infected cells, IFN-γ-activated macrophages are involved in the killing of intracellular pathogens and the antibody produced by B cells is most effective against extracellular pathogens such as helminth worms. The role of Th1 and Th2 cells in a number of infectious diseases is well discussed in the various contributions in Romagnani (1996) and Kotb and Calandra (2003). Parasites are no different from other pathogens in that they inevitably induce some kind of immune response except that Tc cells are less involved in parasitic infections than in viral infections. In general, it is widely accepted that protective Th1 responses predominate in infections caused by intracellular protozoa whereas Th2 responses are more important in immunity to extracellular helminth infections. With reference to specific examples, toxoplasmosis (like tuberculosis) is highly polarized towards the Th1 pole, leishmaniasis (and leprosy) tend towards the Th1 pole, malaria tends towards the Th2 pole and helminth

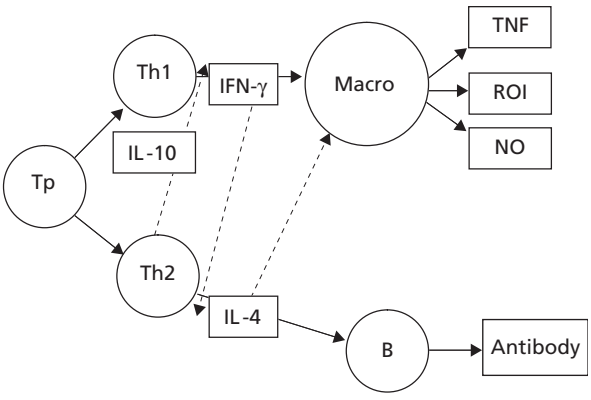

Figure 2 *Diagrammatic representation of the cytokine cross-regulation of the immune response. IFN-γ, as well as having a positive role in the activation of macrophages (Th1 response) has a negative effect on Th2 responses. Similarly, the Th2 cell products IL-4 and IL-10 inhibit Th1 responses. Positive pathways are here indicated by solid lines and negative pathways by broken lines.*

infections are polarized to the Th2 pole (Garraud et al. 2003). In addition, the mutual exclusivity mentioned above frequently results in extreme polarization in which one arm of the immune response is protective and the other counter-protective. However, these are generalizations and the details of each individual immune response can differ from time to time or from stage to stage of an infection. It is also important to bear in mind that the Th1/Th2 paradigm is not a rigid set of rules and that there may be switches in the patterns of Th1 and Th2 cytokines during the course of an infection (Allen and Maizels 1997).

Th cell subsets and the cytokine network were originally identified and defined in mice (Mossman and Coffman 1989) but virtually every component has since been shown to apply to humans as well (Romagnani 1991); parasitic infections provided the first evidence for human Th cell dichotomy. Currently, extrapolations are made from mouse to human and are tested before any definite assumptions are made.

The polarization of T cells towards cell-mediated or antibody-mediated responses does not depend entirely on the infectious agent involved but can be modulated by pre-existing factors including the cytokines present locally. For example, the presence of IL-12 or IFN-γ drives the immune response towards the Th1 pole whereas IL-4 drives it towards the Th2 pole (Ma et al. 1997). Initial or subsequent polarization involves the interaction of a number of regulatory cytokines some of which act as growth and differentiation factors. What is important here is that these cytokines, and also effector molecules, act nonspecifically. It is, therefore, possible for one infectious agent to be caught up in the cytokine network generated in response to another acquired previously, concurrently or subsequently (see Cox 2001).

Another important factor in determining the outcome of an infection is whether or not the established infection is inducing a Th1 or a Th2 response. The Th1 immunological milieu involves a number of molecules and the cells that produce them, in particular, NK cells, IL-12, and IFN-γ. The initiation of a new immune response in such a situation is gradually forced towards the Th1 pole and, if the superimposed agent is controlled by Th2 responses, it is at an advantage in such a situation. The converse applies if IL-4 predominates in the immunological milieu at the time of the second infection.

By the beginning of the 1990s, a general pattern emerged in which it appeared that, in the majority of protozoal infections in experimental models, Th1 cells were protective and Th2 cells were counter-protective, whereas in helminth infections the opposite was the case, Th2 cells were protective and Th1 cells counter-protective (Cox and Liew 1992). It is now clear that the interplay between cytokines in the immune response to parasites is not as simple as it once seemed to be and can vary over the duration of the infection, at different sites in the host, between different species or strains of hosts and between different strains of parasite (Allen and Maizels 1997). Nevertheless, the Th1/Th2 dichotomy is a useful concept when attempting to unravel the complex immune responses that occur in parasitic infections; this can be illustrated by reference to NO, a reactive gas with many functions that has assumed a central role in our understanding of the immunology and immunopathology of parasitic infections. Its role in cell signaling has been known for a long time but it is only in the past decade that its role in immunity to infection has been understood (Clark and Rockett 1996). There are two important isoforms of NO: constitutive and inducible. Constitutive NO is found in a variety of cells. Inducible NO is produced by macrophages under the control of the enzyme inducible nitric oxide synthase (iNOS). NO is a powerful antimicrobial compound and is capable of killing a range of organisms including bacteria, protozoa, and helminth worms. It acts on iron–sulfur clusters in macromolecules and represses DNA synthesis and iron-containing enzymes, including those involved in respiration. Nitric oxide synthase is up-regulated by IFN-γ and TNF-α and is down-regulated by IL-4, IL-5, and IL-10. NO is, therefore, a key molecule in the immune response to parasitic infections and its production and activity are favored by Th1 responses. However, NO is also potentially dangerous and is inhibited by Th2 responses (James 1995).

It is important to understand the cytokine system in parasitic infections because of the complexity of the immune responses involved when coping with highly evolved and antigenically diverse pathogens, and in order to interpret events leading to protection on one hand and pathology on the other. Interpretations from only a few years ago of the immune response to parasites now seem very simplistic and new discoveries have called for a reinterpretation of many of the facts observed. On the other hand, new discoveries have enabled us to understand much more clearly many of the phenomena previously observed. In the remainder of this chapter, these points are illustrated by reference to the most important parasitic infections in terms of human parasitic diseases.

IMMUNITY TO PROTOZOA

Characteristics of protozoa in relation to the immune response

Unlike most viral and bacterial infections, protozoal infections tend to be chronic and to be associated with inappropriate immune responses, immunodepression to superimposed infections and immunopathological damage. This pattern has parallels with the patterns found in mycobacterial infections, leprosy, and

tuberculosis, in which most individuals usually develop some immunity to the clinical disease while never actually eliminating the infection altogether. The immune response patterns to these infections are also seen in bacterial infections, such as listeriosis, and in sepsis. Leprosy, tuberculosis, and many protozoal infections present similar problems and the study of protozoa is now providing important clues as to what is happening in other infections (see Kotb and Calandra 2003). Our increasing knowledge of immune responses to protozoa is also beginning to explain the immunopathology associated with such infections because long-term chronic infections continually stimulate the immune system, which responds inappropriately and can eventually begin to attack the body itself. It is now clear that an understanding of the roles of cytokines is central to understanding the protective immune response, immunopathology and the possibility of developing vaccines. In this context, the use of inbred strains of laboratory mice has been invaluable and the ease with which genes can be deleted or inserted makes it possible to dissect these immune responses in great detail, to identify the roles of particular cytokines, and to extrapolate from mice to humans.

Infections with *Leishmania* species in inbred strains of mice have become the paradigms for studies on T cell subsets and the cytokine network as a whole. Early investigations using *L. major* in mice revealed that there is antagonism between the products of Th1 cells (IFN-γ) and Th2 cells (IL-4 and IL-10), the former leading to protection and the latter to counter-protection. It was later shown that the effector molecule involved in parasite killing was NO. These early observations subsequently proved to be very simplistic but provided a basis for more detailed investigations of leishmaniasis and of other parasitic protozoa. *Toxoplasma gondii* is also a parasite of macrophages and, like *Leishmania*, is controlled by IFN-γ-activated macrophages and the production of NO, TNF-α synergizes with IFN-γ in this killing. Malaria is a complex infection and immunity involves both Th1 and Th2 responses; parasite killing involves both antibodies and NO, which is also responsible for much of the pathology associated with severe malaria. Studies on cytokines have now extended to all the protozoa that infect humans, and research in this area is probably one of the most intense and productive areas of parasite immunology. It is probably true to say that in most, if not all, infections either the Th1 or the Th2 response is protective whereas the other is counter-protective and can lead to pathological changes or switching off the protective immune response.

Protozoa are microparasites that multiply within their vertebrate hosts by dividing at a genetically determined rate; thus, no matter how small the initial infective dose, the infection will eventually overwhelm the host unless it is controlled. The only ways in which this rate of multiplication can be curtailed are exhaustion of the cells available for infection or intervention of an immune response. In protozoal infections, some kind of acquired immunity is the rule and the main reason why this does not eliminate the infection is because all protozoan parasites have evolved ways of evading the consequences of the immune responses that they have evoked (Zambrano-Villa et al. 2002). Protozoal infections, therefore, tend to be long and chronic. The general pattern is, typically, a latent period during which few or no parasites can be detected, a phase of logarithmic increase, a crisis during which the infection is brought under control, and a rapid or gradual decline leading to a chronic infection with the possibility of subsequent recrudescences and long-term immunopathological damage.

All protozoal infections are accompanied by a number of immunological changes including the production of specific antibodies, usually IgM followed by IgG, and cell-mediated responses that can be measured in various ways. It is often not appreciated that many of these immunological responses have little or nothing to do with protection. All protozoa are antigenically complex and are, therefore, recognized as foreign so a variety of immune responses is inevitably initiated. These responses are often either due to irrelevant antigens or to relevant antigens that the parasite is able to use as part of its repertoire of immune evasion techniques. For example, antibodies to the dominant antigen of the malaria sporozoite deflect the immune response away from more vulnerable targets; in leishmaniasis, antibodies merely represent the stimulation of a counter-protective Th2 response.

In summary, protozoal infections are accompanied by changes in immunological parameters that, in some cases, mirror recovery but do not necessarily represent a protective mechanism. Unfortunately, a vast amount of the literature on the immunology of protozoal infections is devoted to these 'will-o'-the-wisps' and this has tended to obscure and confuse valid and relevant observations.

The importance of protective immune responses to protozoa has been highlighted by the consequences of co-infections with human immunodeficieny virus (HIV). Protozoal infections such as cryptosporidiosis, cyclosporiasis, and also microsporidiosis, once thought to be rare and harmless, are now recognized as concomitants of acquired immune deficiency syndrome (AIDS) with major pathological potential (see Intestinal coccidia: cryptosporidiosis, isosporiasis, cyclosporiasis, and 25, Microsporidiosis). HIV is known to adversely affect the immune response to a number of other protozoal infections including leishmaniasis, toxoplasmosis, and Chagas disease (Ambroise-Thomas 2001), but does not seem to affect the outcome of malaria or African trypanosomiasis. The occurrence of such protozoal infections in immunosuppressed and immunocompromised patients has become an important aspect in

the management of AIDS patients. Thus, in many parts of the world, an understanding of the immune response to parasitic protozoa has taken on a new significance.

Immunity to intestinal protozoa

The gastrointestinal tract serves as a habitat for a number of organisms including viruses, bacteria, protozoa and helminth worms. In normal, healthy individuals the mucus layer of the gastrointestinal tract passively protects the underlying cells from invasion. In addition, there is a specialized mucosal immune system consisting of mucosa-associated lymphoid tissue (MALT), intra-epithelial lymphocytes, macrophages and T and B lymphocytes in the lamina propria together with secreted noncomplement fixing immunoglobulin, IgA. Singly or together, these elements provide the first line of defense against invading organisms that gain entry to the body via the alimentary tract. Thus, the immunology of the gut can be considered as a discrete component of the overall immune system. Once an organism has broached the intestinal defenses, it effectively no longer occupies the gastrointestinal compartment of the body and becomes subject to attack by other components of the immune system. It is necessary to consider immunity to intestinal protozoa against this background. In this context, it is important to note that many experimental studies have involved the administration of parasite antigens by routes other than the intestinal one; thus, the immune responses generated have not necessarily been appropriate. The literature on immune responses to intestinal protozoa has been confused by results of such experiments and many of the conclusions drawn probably have little validity. This section, therefore, stresses what is known about immunity to intestinal protozoa in humans and underemphasizes the contributions made by animal and in vitro studies. The comprehensive reviews available will be cited.

INTESTINAL AMEBAE

Entamoeba histolytica is the only ameba of importance about which anything concerning the immunology is known. There is some controversy as to whether or not all strains of this parasite are pathogenic (see Amebiasis: *Entamoeba histolytica* infections) but, in thecontext of this chapter it is assumed that they are not. Most strains of *E. histolytica* are nonpathogenic and liveas harmless commensals in the large intestine where they do not appear to elicit any immune response. Pathogenic forms can invade the mucosa and submucosa, causing deep ulcers, and may be carried in the bloodstream to the liver, where they cause large abscesses. Immune responses, indicated by the production of specific antibodies, are elicited at various stages after invasion but there is no real evidence that recovery from infection induces any protection against subsequent infections nor, at the population level, is there any age-dependent diminution in prevalence that can be ascribed to the acquisition of immunity. In patients with invasive amebiasis, there is a transient secretory immune response followed by the production of serum antibodies (Pérez-Montfort and Kretschmer 1990). The secretory immunoglobulin, IgA, is probably the most important antibody in the gut. Mucosal IgA antibodies directed against a galactose lectin correlate with the development of resistance in children (Haque et al. 2002) but this resistance is very short-lived (Stanley 2001). There is no correlation between serum antibodies, predominantly IgG, and the severity of the infection or recovery. It is not clear whether the apparent absence of acquired immunity is due to some defect in the immune system or to the fact that the amebae have evolved ways of evading the humoral immune response. Evasive mechanisms include the capacity to degrade IgG and secretory IgA, to resist complement lysis and to redistribute and shed surface antigens (see Zambrano-Villa et al. 2002). In some but not all individuals with invasive amebiasis, there is a transient cell-mediated immune response as detected by skin tests, but it is not at all clear what role cellular immune responses play. The epithelial cells that the amebae invade can produce a variety of chemokines and cytokines as well as mediators such as NO so it is possible that cell-mediated immune mechanisms could play some role in protection. Cell-mediated responses are depressed during *E. histolytica* infections; in vitro studies show that amebae can lyse target cells, including those of the immune system, by inserting ion channels called amebapores into their membranes. *E. histolytica* can also promote the production of the Th1 cytokines, IL-4 and IL-10, and block the production of IL-18 (Que et al. 2003), two mechanisms known to inhibit cell-mediated immune responses. In conclusion, there is some evidence that humans mount an effective immune response against *E. histolytica*, that this involves secretory mucosal IgA, and that any immunity is transient. However, the fact that recurrences of amebic ulcers are rare could suggest that there is some kind of longer-lasting immune response; this warrants some attention. Various aspects of amebiasis, including immunology, are reviewed by Stanley (2001), Petri (2002), and Haque et al. (2002) (see also Amebiasis: *Entamoeba histolytica* infections).

There have been a number of attempts to develop vaccines against *E. histolytica* including DNA vaccines incorporating genes for a galactose lectin; this vaccine elicits a cell-mediated immune response (Gaucher and Chadee 2002). The possibilities and problems associated with developing vaccines against amebiasis are discussed by Miller-Sims and Petri (2002).

INTESTINAL FLAGELLATES

Giardia duodenalis has been long considered to be a harmless commensal of the small intestine but increasing evidence that it can cause malabsorption and severe diarrhea has stimulated interest in immune responses to this parasite (see Giardiasis). A number of experimental studies with *G. muris* in mice clearly demonstrate that there is acquired immunity and protection against subsequent challenge and immunity is genetically controlled. In mice, the key molecule is secretory mucosal IgA. Mice can also be infected with the human parasite *G. duodenalis* and, in this model, there is an IL-4-dependent, Th2-type protective response involving the production of IgA (Bienz et al. 2003; Zhou et al. 2003). In humans, the evidence for acquired immunity is more circumstantial but comes from a number of sources. There is usually spontaneous clearance and resistance to reinfection; those living in endemic areas are less affected than visitors, young children are more affected than older ones or adults, and immunodepressed individuals tend to experience long chronic infections (reviewed by Janoff and Smith 1990; Nash 1994; Kulda and Nohynkova 1995; Faubert 1996; see also Giardiasis). All the available evidence suggests that the effector mechanism or mechanisms involve secretory IgA but the case is by no means proven. IgA is the most characteristic *Giardia*-specific antibody in patients with acute giardiasis and individuals with hypogammaglobulemia tend to experience chronic infections, but there is little direct evidence of any IgA antiparasitic effects. On the other hand, specific antibodies are found in human milk and saliva; breast-fed infants in endemic areas seem to acquire some degree of protection (Walterspiel et al. 1994). Other antibodies may also be involved, for example, current infections are accompanied by high levels of specific IgM but this is quickly replaced by IgA in acute infections and after re-exposure. Overall, it seems that immunity to giardiasis is similar to immunity to a number of bacterial infections in which secretory IgA in breast milk protects the new born infant until it can produce its own antibodies. IgA, which cannot fix complement, presumably adversely affects the parasites indirectly by preventing attachment to the villi. *G. duodenalis* can undergo antigenic variation although the actual mechanism is not understood (Nash 1997). The variant specific surface antigens constitute a family of unusual proteins and it is more likely that they have a biological role, possibly protecting them against intestinal proteases, than any immune evasive mechanism (Nash 2002). There have been reports of giardiasis in AIDS patients, but *Giardia* is not considered to be a major concomitant of HIV infections (Janoff et al. 1988). There is no vaccine against giardiasis and most observers do not believe that one is necessary. The immunoprophylaxis and immunotherapy of giardiasis is reviewed by Olson et al. (2002).

Although the human intestine is parasitized by a number of other flagellates including *Chilomastix mesnili*, *Dientamoeba fragilis*, and *Trichomonas hominis*, these are not normally regarded as important pathogens and virtually nothing is known about any immunological responses to them.

INTESTINAL COCCIDIANS

Little is known about the immune responses of humans to any of the three intestinal coccidians, *Cryptosporidium parvum*, *Isospora belli*, or *Cyclospora cayetanensis*. Cryptosporidiosis is the only important disease caused by any of these parasites and has only been recognized as such during the last 20 years (reviewed by Martins and Guerrant, 1995; see also Intestinal coccidia: cryptosporidiosis, isosporiasis, cyclosporiasis). In healthy individuals, the infection is an unpleasant but self-limiting one, lasting less than a month and characterized by mild to severe watery diarrhea. In AIDS patients, the infections are more persistent, the symptoms much more severe and death may result (Adal et al. 1995). This suggests that normally there is immune involvement and specific antibodies have been detected in the serum of infected individuals; characteristically, IgM is produced first and then replaced by IgG, levels of which begin to decline after about a year. Nothing is known about the possible mechanisms or relevance of these immunological responses.

INTESTINAL CILIATES

Balantidium coli, the only intestinal ciliate of humans, is comparatively rare. Nothing is known about the immune responses to this ciliate and, by analogy with *Entamoeba histolytica*, it is unlikely that there is any reaction unless the mucosa and submucosa are invaded.

Immunity to protozoa inhabiting the urinogenital tract

The only protozoan that inhabits the human urinogenital tract is *Trichomonas vaginalis*, which parasitizes both men and women. The nature of the infection and the disease caused in men is poorly documented, but vaginitis caused by this parasite is an important sexually transmitted disease in women, in whom it persists for long periods unless treated (reviewed by Honigberg and Burgess 1994; see also *Trichomonas* infections). There are no really appropriate animal models of human trichomoniasis and, although experimental infections in guinea pigs and mice have provided some information (e.g. complement-dependent and

complement-independent killing in animals given intraperitoneal, intradermal or subcutaneous injections), little that is really relevant can be derived from these observations (Ackers 1989). Similarly, observations on *T. foetus* in cattle have been largely irrelevant to human trichomoniasis. In humans, secretory IgA antibodies to *T. vaginalis* have been detected in vaginal secretions. Antibodies produced in response to infection diminish with time and do not appear to confer any protection against reinfection. Epidemiological evidence, derived from many studies in sexually transmitted disease clinics, suggests that infections with *T. vaginalis* tend to be persistent, that reinfection frequently occurs after cure and that the prevalence of the infection does not plateau or fall off in the older age groups. All this suggests that there is little or no acquired immunity and that specific immune responses play an insignificant role in limiting this infection. The reasons why there appears to be no immunity to *T. vaginalis* are not clear, although it has been suggested that this parasite has considerable antigenic diversity; whether or not it can also undergo antigenic variation is an open question (Alderete 1983).

Immunity to macrophage-inhabiting protozoa

INTRODUCTION

Three groups of parasitic protozoa inhabit macrophages at some stage during their life cycles: *Leishmania* spp., *Toxoplasma gondii*, and *Trypanosoma cruzi*. Each has evolved its own way of evading the destructive mechanisms of the macrophage: leishmanias survive in the fused phagolysosome; *T. gondii* prevents phagosome–lysolysosome fusion and *T. cruzi* escapes from the phagosome into the macrophage cytoplasm. In addition, all these parasites also possess enzymes that counteract the various toxic molecules present in the phagolysosome. Because of their ability to avoid destruction by macrophages, they all escape attack by one of the main arms of the immune system and this enables them to give rise to long chronic infections. On the other hand, none of these parasites is able to survive in IFN-γ activated macrophages, thus they live on a knife edge by inhabiting cells that can sustain them but which also have the capacity to destroy them. *Leishmania* spp. are totally dependent on macrophages whereas *T. gondii* and *T. cruzi* are also able to parasitize other cell types. This section deals with immunity to *Leishmania* spp. and *Toxoplasma gondii*. *T. cruzi* is discussed later under Immunity to tissue-inhabiting protozoa.

Leishmania spp.

About 20 species of *Leishmania* are known to infect humans in the New and Old Worlds and the number is gradually increasing. Leishmaniasis can be conveniently classified into three forms, cutaneous, mucocutaneous, and visceral, but these categories are not absolute (see Old World leishmaniasis and 17, New World leishmaniasis). As these parasites are very common and as the majority of those infected (particularly with the cutaneous forms) do recover, albeit slowly, the immunology has been intensively studied, mainly with the ultimate aim of producing a vaccine. *L. major*, the causative agent of Old World cutaneous leishmaniasis, and *L. donovani*, the causative agent of visceral leishmaniasis, have been the most studied species, mainly because of the ease with which they can be maintained in genetically characterized laboratory mice. In particular, certain strains of mice (e.g. C57Bl) are relatively resistant to infection and develop small self-healing cutaneous lesions whereas others (e.g. BALB/c) are more susceptible and develop overwhelming visceralizing infections (reviewed by Liew 1990; Alexander and Russell 1992; Liew and O'Donnell 1993; Bogdan and Röllinghoff 1998, 1999; Tacchini-Cottier et al. 2003). Developments in molecular biology, particularly the use of knock-out mice, have made it possible to dissect the immune response to *L. major* in considerable detail and the *L. major*–mouse model has become a paradigm for all leishmanial infections. *L. major* infections in resistant mice tend to resemble most *L. major* infections in humans in the New World and *L. major* infections in susceptible mice resemble the condition known as diffuse cutaneous leishmaniasis (DCL). Models of visceral leishmaniasis, caused by *L. donovani*, and infections with the New World species have not been so extensively investigated but, in general, extrapolations from mice and hamsters to the human situation have tended to confirm the universal nature of the immune response involved. Immunity to leishmaniasis is cell-mediated and involves Th1 cytokines. Leishmanial parasites survive and multiply within normal resting macrophages but are killed by activated macrophages through a mechanism involving NO. NK cells and the cytokines IL-12, IL-18, TNF-α, and TNF inducers play major roles in this process by up-regulating the IFN-γ-induced NO killing. In essence, the protective immune response in leishmaniasis is a classical Th1-type cell-mediated response. However, this protective response is counteracted by an equally effective Th2 response involving IL-4, IL-10, IL-13, and TGF-β. IL-4 and IL-13 counteract the macrophage-activating effects of IFN-γ, IL-10 down-regulates macrophage activity, and TGF-β blocks IFN-γ-activated macrophage activity. The net result is that NO production is inhibited and parasite killing reduced. Resistant strains of mice tend to have high levels of IFN-γ and low levels of IL-4 and IL-10, whereas the opposite is the case in susceptible strains. The overall control of the immune response, therefore, depends on the activation of one of two subsets of T lymphocytes: activation of Th1 cells leads to the production of IFN-γ and protection whereas activation of Th2 cells produces IL-4 and IL-10 and is

counter-protective (see Reiner and Locksley 1995; Scott 1996). It is now clear, however, that this is not the whole story; the actual control of immunity to leishmaniasis is much more complicated and the role of IL-12 and the interplay between IL-12 and IL-4 are crucial to the outcome of the infection (Scott and Farrell 1998). It is also important to note that in mice infected with *L. donovani*, the dichotomy between Th1 and Th2 responses is not nearly so clear cut (Kaye 1995) possibly indicating important differences in antigen processing and presentation in the skin and in the viscera (Moll 1993; Solbach and Laskay 1995, 2000; Ritter and Korner 2002). It is also significant that although IL-12 enhances the production of chemokines important in protection against *L. major* infections (Zalph and Scott 2003), in *L. mexicana* infections in mice there is an alternative pathway leading to the production of IFN-γ that is independent of IL-12 (Buxbaum et al. 2002). Overall, it is now clear that although the general patterns of immunity are similar, there are significant differences between the actual mechanisms involved with different species of *Leishmania* and to some extent this may account for differences in the pathologies associated with different species.

Although the distinctions between the Th1 and Th2 subsets are not so clear cut as in mice, this pattern is also true for human leishmaniasis including visceral leishmaniasis (Kemp et al. 1996). In patients with fulminating *L. donovani* infections, IL-4 and IL-10 levels tend to be high and IFN-γ levels low; in cutaneous infections (in which recovery is normally the rule) IFN-γ levels are high, and in chronic mucocutaneous infections IL-4 levels are high. All these facts are in accordance with well-established clinical observations such as the development of strong delayed type hypersensitivity (DTH) indicating macrophage activation, which correlates with recovery from cutaneous leishmaniasis; diffuse cutaneous leishmaniasis is characterized by the absence of DTH; and high levels of nonprotective IgM and IgG antibodies (resulting from Th2 cell activity) are seen in visceral leishmaniasis (see Blackwell 1993). In localized mucocutaneous leishmaniasis, Th1 cytokines predominate whereas in the more destructive forms there is a mixture of Th1 and Th2 responses, although the Th2 cytokine, IL-4, tends to predominate in accordance with the well-established paradigm (Pirmez et al. 1993). In humans, the nature of the immune response is also determined by numerous extraneous factors such as the species or strain of parasite involved, the genetic makeup of the host or host population, the number of bites by infected sandflies, and the number of parasites injected.

There have been a number of experimental vaccines developed against leishmaniasis and there have been some encouraging results in humans (Greenblatt 1988; Convit et al. 1989; Modabber 1995; Handman 1997). Vaccines that have been used or considered for use include attenuated strains, killed parasites, parasite extracts or metabolic products and, more recently, DNA vaccines. The use of relatively crude vaccines is still going on. Vaccines based on autoclaved *L. major* and *L. infantum* plus bacille Calmette–Guérin (BCG) have been successful in protecting dogs against these infections (Mohebali et al. 1999) and multiple injections of autoclaved *L. major*, with or without BCG, elicit immune responses that suggest that BCG could boost the immune response in humans (Alimohammadian et al. 2002). The possibility of using such vaccines against *L. donovani* in humans is discussed by Dube and Srivastava (2002). Two kinds of vaccine, one using genetically attenuated *L. major* and the other using autoclaved parasites, produced encouraging results in monkeys; both protocols are safe in humans but require further improvement before vaccine development can be contemplated (Amaral et al. 2002). A better characterized vaccine using five *L. infantum* antigenic determinants plus BCG gave 90 percent protection in dogs (Molano et al. 2003). These promising results have encouraged others to develop more advanced vaccines such as recombinant (de Carvalho et al., 2003) or DNA vaccines in mice infected with *L. major* (López-Fuertes et al. 2002; Méndez et al. 2002) or *L. donovani* (Sukumaran et al. 2003). Currently there are many leads but no effective and safe commercially available vaccines against leishmaniasis.

Toxoplasma gondii

Toxoplasma gondii is a common parasite of cats, which can be transmitted to virtually all warm-blooded animals (Dubey and Beattie 1988; see also Toxoplasmosis). The parasites infect macrophages in which they survive by preventing phagosome–lysosome fusion; they undergo rapid division to form merozoites known as tachyzoites. The tachyzoites escape from the host cell and invade new cells. After a short time, this rapid division slows down and the parasites transform into slowly dividing bradyzoites, which remain dormant in cysts in various tissues, including the brain, where they may die and become calcified. If the infected individual is subsequently immunocompromised in some way, the dormant parasites in the cysts may become activated and give rise to a fulminating infection. In humans, most infections are benign or inapparent but if the parasite crosses the placenta it can seriously damage the fetus. *Toxoplasma gondii* infections are also now becoming serious concomitants of AIDS (Ambroise-Thomas and Pelloux 1993). The most obvious features of human *T. gondii* infections are that individuals acquire the infection relatively early in life and after recovering from it are immune to reinfection. Acute infections are characterized by specific IgM and chronic or recovered infections are characterized by high levels of specific IgG. *T. gondii* infections in all mammals except cats seem to be very similar so there

is no major problem in extrapolating from studies in laboratory animals to humans. Much of what is known about immunity to toxoplasmosis has been derived from studies in mice mainly because of the ease with which various components of the immune response can be manipulated in these animals (Gazzinelli et al. 1993; Alexander and Hunter 1998; Wille and Hunter 2003). There have also been numerous parallel studies using sheep, as toxoplasmosis is a serious veterinary problem associated with abortions. The immune response varies throughout the infections. First, there is the innate immune response during which the infected resting macrophages produce small amounts of IL-1, IL-12, IL-18 and TNF-α. These cytokines stimulate NK cells to produce IFN-γ which activates the infected macrophage and brings about NO-mediated parasite killing. This innate immunity is, however, of limited efficacy and duration and the infection then moves into its acute phase during which the infected macrophages produce IL-12 that stimulates both CD4 and CD8 lymphocytes to produce IFN-γ which, in turn, stimulates the production of NO in the infected cell and subsequent parasite killing. During the chronic phase of the infection, CD8 cells seem to be the main source of IFN-γ and the infection is tolerated at a low level until the parasites die and become calcified. If, however, an individual is immunocompromised in some way during the chronic phase of the infection, there is a rapid and acute recrudescence of the parasitemia, which can be fatal. In addition to the Th1-type immune response, there is evidence of some Th2-type activity during which tachyzoites are coated with antibody and are either prevented from invading host macrophages or are unable to prevent phagosome–lysosome fusion if invasion is successful. As pointed out above, antibody levels are indicative of acute or chronic infections.

The key molecule in immunity to toxoplasmosis is NO, which kills the target cell by interfering with DNA and metabolic pathways. NO is a very toxic molecule and over-production can cause pathological damage to host cells and tissues and can also cause immunosuppression. In toxoplasmosis, the pathology is manifested in toxoplasmic encephalitis and retinopathies. The host, however, is able to moderate these adverse effects by producing IL-6, IL-10, and TGF-β, which inhibit the activity of IFN-γ and hence the production of NO. However, TGF-β also promotes the replication of the parasite (Nagineni et al. 2002). The roles of IL-6 and IL-10 are equivocal in that IL-6 actually promotes parasite proliferation which, with IL-10, can contribute to toxoplasmic encephalitis. IFN-γ plays a central role in toxoplasmosis as it not only induces microbicidal activity but also promotes cyst formation and prevents cyst rupture. There are numerous possible sources of IFN-γ, both T cell and non-T cell, and at least five mechanisms whereby IFN-γ-activated macrophages can affect the parasite – including the production of NO and ROI and limiting the supply of iron to the parasite (Suzuki 2002). The immune responses to *T. gondii* are very complex; immunity to infection and the deleterious effects of inflammation are two sides of the same coin and it is essential for the host to maintain a balance between the various cytokines. In order to understand the mechanisms involved in the control of the cytokine network, recent research has moved away from the cytokines themselves to the signaling pathways and the ways in which *T. gondii* can disrupt macrophage function. *T. gondii* induces the activation of two transcription factors, the signal transduction activator of transcription (STAT)-1 and nuclear factor kappa B (NFκB), but blocks their translocation so that the macrophage cannot then produce IL-12 or TNF-α (Denkers et al. 2003).

It is not easy to make direct extrapolations from mice to humans but the available evidence suggests that the mechanisms of immunity in humans are similar to those in mice; for example, IFN-γ inhibits replication of the tachyzoites in macrophages from infected AIDS patients (Delemarre et al. 1994). In vitro studies using human cells have also confirmed murine studies. Using *T. gondii* - infected human myelomonocytic cells, Belloni et al. (2003) have confirmed that TNF-α and IFN-γ act synergistically and have also shown that TNF-α production is inhibited by the parasite and that this is caused by the shedding of TNF-α receptors. In retinochorditis, one of the most common manifestations of toxoplasmosis, the parasite actually inhibits the production of TGF-β suggesting that the interplay between *T. gondii* and retinal cells may play an important role in this condition (Nagineni et al. 2002). The role of NO in neurodegeneration is also controversial. NO is known to damage neuronal cells but this rarely happens during *T. gondii* infections, possibly because the parasite is able to inhibit the production of NO by microglia through a pathway involving prostaglandin-E and IL-10 (Rozenfeld et al. 2003). Whatever the mechanism, immunity is clearly very important in humans because the majority of the world's population will be infected at some time and that those that are immunoincompetent (e.g. the fetus) or immunosuppressed are at particular risk from a fulminating infection. Toxoplasmosis is now regarded as the most common cause of focal central nervous system infection in AIDS patients (Mariuz et al. 1994).

There is no vaccine against toxoplasmosis in humans. Commercial vaccines based on attenuated strains of *T. gondii* have been developed for use in sheep (Buxton 1993), but such a vaccine would be unacceptable for human use. Our understanding of the cytokine network and the development of DNA vaccines have encouraged the possibility of vaccines that might be acceptable for human use and there have been some successes in mice immunized with a plasmid containing a *T. gondii* surface antigen and IL-2 (Chen et al. 2002). Also being developed are DNA vaccines designed to activate CD8 cells (Scorza et al. 2003) and DNA vaccines designed to

stimulate Th1-type responses and the production of IFN-γ (Fachado et al. 2003). However, a vaccine suitable for use in humans is a long way away.

Immunity to blood-inhabiting protozoa

INTRODUCTION

Four important groups of protozoan parasites inhabit the human bloodstream:

1. the African trypanosomes *Trypanosoma brucei gambiense* and *T. b. rhodesiense*
2. the American trypanosome *Trypanosoma cruzi* (but only transiently, it is best regarded as a tissue parasite)
3. the malaria parasites *Plasmodium falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*
4. in rare and accidental infections, *Babesia* spp.

The bloodstream is a dangerous place for a parasite to live as there is continual and intimate contact with the cells and molecules of the immune system, particularly IgM and IgG antibodies and phagocytic cells. The ability to survive in what should be a very hostile environment has attracted the attention of numerous scientists. African trypanosomes have been particularly intensively studied in laboratory animals and this activity has generated a vast literature much of it, unfortunately, largely irrelevant to the human disease. In this section, only those aspects of the immune response directly concerned with human infections are discussed.

AFRICAN TRYPANOSOMES

The African trypanosomes that infect humans are *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, both related to *T. b. brucei*, a trypanosome of wildlife and domesticated animals that does not infect humans (see African trypanosomiasis). Little is known about the acquisition of immunity to trypanosomes in humans, but there is epidemiological evidence that immunity to *T. b. gambiense* does develop, albeit slowly, in human populations (Khonde et al. 1995). *T. b. brucei* has been widely used as a model for both the human and animal disease, largely because it is the only subspecies that is easy to maintain in laboratory mice and because of its similarities to the human forms. Infections with all three subspecies are similar; the parasites live and multiply in the blood and infections are characterized by successive waves of parasitemia representing different antigenic variants. Each trypanosome is covered with a thick glycoprotein coat, the variant surface glycoprotein (VSG). There is an almost limitless repertoire of VSGs, each known as a variable antigen type (VAT), that arise in 1 in 10^6 cells in laboratory syringe-passaged populations and 1 in 100 cells in field isolates. The normal pattern of events is that the parasites multiply in the blood, are destroyed by an antibody-mediated immune response specific to the major VAT, and are replaced by another population of parasites carrying a new VAT (Barry 1997). Each wave of parasitemia is accompanied by an increase in antibody specific to the VAT that preceded it and is responsible for the removal of most of the trypanosome population leaving a minor population that gives rise to the next wave of parasitemia; subsequent waves appear every 5–10 days. The antibody class involved in the destruction of the trypanosomes is IgM, which facilitates agglutination, complement-mediated lysis, and phagocytosis by macrophages and neutrophils. Later in the infection, VSG-specific IgG antibodies are involved in clearing trypanosomes from the tissues (see Sternberg 1998). The destruction of the trypanosomes, however, results in the release of a number of toxic molecules and internal and external antigens that adhere to or accumulate in various host tissues resulting in complex-mediated damage and resultant immunopathological changes which have been well documented in animal studies but have been little investigated in humans.

There is no evidence of protective cell-mediated immunity in African trypanosomiasis but there is some limited evidence that NO, which has an alternative role (see below), can kill trypanosomes possibly only in combination with TNF-α (Mansfield et al. 2001).

Trypanosomes are also enmeshed in the cytokine network and molecules involved in the immune response such as (TGF-β) and IFN-γ can act directly or indirectly on parasites; IFN-γ acts as a growth factor for *Trypanosoma brucei* (see Mansfield et al. 2001). Trypanosomes can also secrete or excrete products that affect cells of the immune system, for example trypanosome-derived lymphocyte triggering factor (TLTF) and trypanosomal macrophage activating factor (TMAF). TLTF induces lymphocytes to produce IFN-γ, which activates macrophages and promotes trypanosome growth, TMAF also stimulates macrophage activity (see Sternberg 1998; Hamadien et al. 2000). The production of TLTF has been demonstrated in the human trypanosomes *T. b. gambiense*, *T. b. rhodesiense* (Bakhiet et al. 1996). All the African trypanosomes are, therefore, potentially caught up in a series of interactions in which trypanosome-derived factors activate both T lymphocytes and macrophages. The net effect is that lymphocyte produced IFN-γ enhances trypanosome growth while other trypanosome-derived molecules induce macrophages to produce molecules that inhibit lymphocyte activation (Figure 3).

Trypanosome infections are accompanied by severe immunodepression to superimposed antigens and concurrent infections even though their hosts are capable of mounting effective specific immune response against trypanosome antigens. Although this phenomenon has been most extensively studied in animals, there is evidence that it also occurs in patients with trypanosomiasis (Greenwood et al. 1973). The underlying basis of

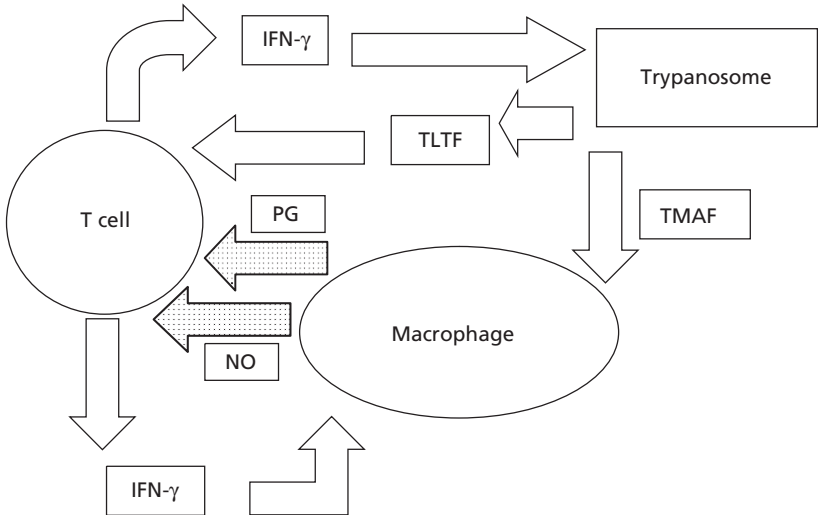

Figure 3 *Diagrammatic representation showing some of the interactions that exist between African trypanosomes and the cytokine network. Trypanosomes produce a number of factors including TMAF and TLTF that stimulate macrophages and lymphocytes respectively. IFN-γ, a product of stimulated lymphocytes, can act as a trypanosome growth factor but prostaglandins (PG) and NO produced by macrophages inhibit lymphocyte activity and hence the production of IFN-γ. Trypanosomes are therefore enmeshed in a network of cytokines and effector molecules that both stimulate and inhibit their growth and development. Positive signals are here indicated by open arrows and inhibitory ones by stippled arrows. (From Cox (2001) with permission from Cambridge University Press)*

this immunodepression is not at all clear but it is thought that impaired B-cell responses may result from overactivation and clonal depletion of B cells from lymph nodes during the course of infection. T-cell activity is also suppressed and this is thought to result from the development of immunosuppressive macrophages and the production of NO and prostaglandins.

The whole field of the immunology of trypanosome infections is a very controversial one and the traditional view that anti-VSG antibody is entirely responsible for the remission of the infection, albeit temporarily, has been challenged by several workers including Mansfield (1994), Mansfield et al. (2001), and de Baetselier (1996) based on in vitro observations and studies in mice. Mansfield suggests that macrophage-derived NO affects the survival of the trypanosomes and also inhibits T-cell function, leading to immunodepression. de Baetselier maintains that the trypanosomes induce CD8 T lymphocytes to produce IFN-γ and macrophages to produce TNF-α and that these synergize to produce toxic molecules that both adversely affect the trypanosomes and cause immunodepression. It is now clear that the traditional picture of antibody-mediated immune responses eliminating variant specific antigens and clearing successive parasitemias is not the whole picture (Mansfield et al. 2001). It is also not at all clear what the situation is in humans, whether or not there is any immunodepression and, if so, if it has any affect with respect to intercurrent infections such as HIV.

MALARIA PARASITES

Malaria is generally recognized as the most important parasitic disease, both in terms of mortality and morbidity (see Malaria). Humans harbor four species of malaria parasite, *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, in order of clinical importance. *P. falciparum* is the most intensively studied parasite and, unless otherwise specified, is the only one considered here. The control of malaria has largely depended on the combined use of insecticides and drugs but resistance has made these ineffective and attention has turned to the possibility of developing a vaccine. An important spin-off of this work has been the realization that much of the pathology of malaria is closely associated with immunity, in particular the involvement of cytokines. Research into immunity and immunopathology has resulted in a massive literature reaching tens of thousands of papers, mostly based on rodent models and in vitro systems, much of it, particularly many of the extrapolations made, irrelevant or peripheral to the human situation (Cox 1988). Until recently, our understanding of the ways in which humans react to infection with human malaria parasite has remained a mystery, but success in cloning the *Plasmodium* genome and the application of molecular techniques directly to the human malaria parasites is beginning to pay dividends. However, there are a number of things that we do not understand and our ignorance of these areas hinders our ability to develop vaccines or ways of ameliorating the pathology associated with the disease.

The starting point in our understanding of immunity to malaria is that from epidemiological studies it is known that:

- malaria infections are long-lived, 1–2 years in the case *P. falciparum* and 3–50 years for *P. malariae*

- individuals can be reinfected after natural recovery or cure
- there is a gradual build up of immunity over a period of many years
- any immunity fades quickly
- immunity is largely strain-specific
- children born to immune mothers are themselves resistant to that particular strain of malaria.

In summary, immunity to malaria can be considered to be the rule, although it is often incomplete and may take many years and numerous exposures to the bite of infected mosquitoes to develop. Thus, there is evidence of some immunity and the real challenge is to try to understand the mechanisms involved in order to develop effective vaccines.

In order to understand the immunology of malaria, it is necessary to describe the life cycle (see Malaria for further details). The life cycle of *P. falciparum* begins when the infective stages, sporozoites, are injected by a mosquito directly into a capillary in the skin. The sporozoites circulate in the blood for 30–45 min before they enter liver cells where they undergo a phase of pre-erythrocytic schizogony involving repeated nuclear divisions resulting in the production of about 30 000 uninucleate merozoites after 6–7 days. The merozoites flood into the blood and actively attach to and invade red blood cells within about 20–30 seconds and initiate the erythrocytic stage of the life cycle (see Bannister and Mitchell 2003). Inside the circulating red blood cell, the merozoite feeds on hemoglobin and transforms into a uninucleate feeding stage called a trophozoite; the nucleus then begins to divide and, in *P. falciparum* infections but not in other species, the infected cells disappear from the circulation and adhere to endothelial cells of various internal organs. This process is known as sequestration and is very important in the pathology of the disease. Nuclear division occurs until a schizont containing about 16 merozoites is formed; the merozoites break out of the cell and invade new cells, a process that is repeated almost indefinitely. The liberation of parasites from the red cell is accompanied by the release of a number of toxic molecules, some of host origin and some of parasite origin, that are responsible for some of the pathology associated with malaria. Some young merozoites do not develop into schizonts but into male and female gametocytes that circulate in the peripheral blood until they are taken up by a mosquito when it feeds. Within the mosquito, the gametocytes mature into male and female gametes, fertilization occurs and a zygote called an ookinete is formed. Finally, there is another phase of multiplication resulting in the formation of sporozoites that migrate to the salivary glands and are injected when the mosquito feeds on a new host. The life cycles of the other malaria parasites of humans differ in the length of the pre-erythrocytic and erythrocytic phases, the numbers of merozoites produced and the absence of sequestration. Some strains of *P. vivax* have a persistent dormant pre-erythrocytic phase.

Each stage in this complex life cycle is antigenically distinct and a potential target for immune attack. The malaria parasite is most susceptible when the sporozoite is circulating in the blood, when merozoites are liberated from the liver into the bloodstream before they invade the red blood cells, and when merozoites have been released from red blood cells. The sporozoite is the obvious first target for immune attack. It is the first stage in the infection and the parasites are free in the blood for up to 30–45 min. The sporozoite possesses an immunodominant 40–60 kDa protein surface coat, the circumsporozoite protein (CSP), characterized by a repeat region consisting of four amino acids repeated 37 times, and three or four copies of a smaller repeat dispersed throughout the molecule. In *P. falciparum*, the amino acids of the dominant repeat are asparagine–alanine–asparagine–proline (NANP), and the minor repeat is asparagine–valine–aspartate–proline (NVDP). Other malaria parasites have similar repeat regions but with different amino acid sequences. The CSP is very antigenic and elicits a strong antibody response and has, therefore, been a favorite for vaccination studies. When exposed to antibody, however, the surface molecules are cross-linked and the sporozoite sheds the coat and escapes; thus, it is now thought that the repeat sequence may be a mechanism whereby the parasite evades the immune response by putting up a powerful 'smoke screen' that deflects the response away from more important targets. The repeat region of the CSP is not the only possible target; there are a number of other sporozoite antigens from nonrepeat regions and also those involved in the penetration of the hepatocytes. One of the most important is the thrombospondin related adhesion protein (TRAP) that is involved in sporozoite motility (Sultan et al. 1997).

It was previously thought that, once in the liver, the parasite was safe from immune attack but, in murine malaria models, there is now evidence that there is a Tc cell response to the early stages in hepatocytes and that what is recognized are parasite antigens in the presence of a hepatocyte MHC class I molecule (Tsuji and Zavala 2003). Although we do not know what happens in humans, there is evidence that in West Africa the outcome of infection is controlled by the MHC class I molecules, thus there has been increased interest in antigens associated with the liver stages. The best studied pre-erythrocytic-stage antigens are the liver-stage antigens (LS-A), LS-A1, and LS-A3 but at least 15 more have been identified some of which have some homologies with antigens of other stages in the life cycle (Grüner et al. 2003).

The erythrocytic stages, particularly the merozoites, have received the most attention. They are the easiest to study, they are responsible for much of the disease and

they are obvious targets for attack. A vast amount of information about these stages has been accumulated and many of the antigens involved have been characterized and cloned. The best studied antigen is the merozoite surface protein (MSP)-1, found in all the malaria parasites studied to date; it is a glycoprotein with a molecular weight of c. 190–195 kDa. Experimentally, antibodies against MSP-1 block red blood cell invasion, thus this could be a major target for immune attack and for exploitation as a vaccine. Unfortunately, there is considerable diversity in MSP-1 between isolates, and immunity to one isolate does not confer immunity to another. Other *P. falciparum* antigens of importance are apical membrane antigen (AMA)-1, erythrocyte binding antigen (EBA)-175, serine rich antigen (SERA), and ring-infected erythrocyte surface antigen (RESA), all of which are involved in binding to or invading red blood cells. The erythrocyte membrane protein (EMP), PfEMP-1, found on the surface of late-infected red cells is involved in sequestration and undergoes antigenic variation and is, therefore, thought to be in part responsible for the recrudescences that occur in falciparum malaria (Gatton et al. 2003). The actual role of PfEMP-1 is unclear but it is thought that sequestration prevents infected erythrocytes from circulating through the spleen thus protecting them from the killing mechanisms that appear to operate there. Antibodies against PfEMP-1 prevent sequestration and facilitate parasite destruction in the spleen.

The antigens associated with the sexual stages would seem to be unlikely targets for immune attack but, in fact, they are very important. There are a number of antigens associated with the gametocytes, the male and female gametes and the zygote and, unlike most of the other antigens of malaria parasites, they are very susceptible to antibody attack. This only happens when the gametocytes are taken up by a mosquito together with antibodies in the serum. When the gametocytes escape from the infected red cell they are recognized by the antibodies and are either killed or inactivated. In either case, the infection in the mosquito cannot continue. The antigens of most interest are: a 250 kDa antigen on the gametocytes, gametes and zygote; a 25 kDa antigen on the gametes and zygote; and a 48 kDa antigen on the zygote and oocyst.

The targets for immune attack are, therefore, numerous and complex and the immune responses involved must also be correspondingly numerous and complex. Virtually all Th1 and Th2 components of the immune system are involved at some stage during an infection. Although many details are missing, we now have a very good idea of the overall immune response during the blood stages of a malaria infection. The first time an individual becomes infected with malaria, parasite antigens stimulate NK and T cells to produce low levels of IFN-γ that activate macrophages to produce TNF-α, ROI, and NO. All three molecules have been implicated in the killing of intra-erythrocytic parasites but the strongest evidence supports the role of NO as the key molecule in this process (see Clark and Cowden 2003). Whatever the actual mechanism, these non-antibody molecules, possibly in combination, bring the infection under control with few clinical symptoms. Where the destruction of parasites occurs is not known but it does not seem to be in the circulating blood: the most likely site is the spleen and evidence for this comes the observation that individuals who have had their spleens removed often have difficulty in controlling the parasitemia. It is possible that the flow of blood is slowed down in the spleen and the infected erythrocytes come into close contact with macrophages lining the blood vessels thus causing the release of pro-inflammatory cytokines and NO that damage the intra-erythrocytic parasites while infected cells and free merozoites are phagocytosed.

On exposure to a subsequent infection, the primed T cells produce larger amounts of IFN-γ that induces macrophages to produce excessive amounts of the pro-inflammatory cytokines, IL-12, IL-18, and TNF-α, leading to the symptoms associated with severe malaria. In individuals who recover, the immune system switches to the production of antibodies, mainly IgG2 and IgG3, which impede red blood cell invasion, inhibit parasite multiplication, enhance phagocytosis of merozoites and infected cells, and neutralize toxins (Garraud and Perraut 2003). At the same time, Tr cells produce anti-inflammatory cytokines, IL-10 and TGF-β, which down-regulate the production of TNF-α by macrophages and bring about amelioration of the symptoms of severe malaria. It is important to note that the different roles of Th1 and Th2 cytokines do overlap, for example IL-4, IFN-γ, and TGF-β are involved in the production of IgG1, IgG2a, and IgGb respectively (Stavnezer 1996).

The erythrocytic stages are not the only targets for immune attack; the early stages in the liver are other possible targets. The completion of the *Plasmodium* genome project has resulted in the description of a plethora of liver-stage antigens (see Grüner et al. 2003; Taylor-Robinson 2003). Immunity to liver stages is very difficult to investigate in humans but the only antigen expressed specifically by infected hepatocytes is LSA-1, thought to be the target for Tc cells or NK cells (Schmieg et al. 2003). Interest in the sporozoite antigens has also not waned, although attention has shifted towards the possibility of incorporating such antigens into multicomponent vaccines. Similarly, antigens from the sexual stages are now being considered as possible components of multistage vaccines (see below).

The complexity of the immune response means that inevitably some of the reactions will be adverse ones and the main manifestations of severe malaria, metabolic acidosis, cerebral malaria and anemia correlate with circulating levels of the pro-inflammatory mediator TNF-α. There is now incontrovertible evidence that

severe malaria is caused, in part at least, by NO elicited during the protective immune response (Clark and Rockett 1996). The arguments that have gradually led to this conclusion are complex; further information is to be found in the comprehensive review by Clark and Cowden (2003). It is now also clear that in cases of severe malaria, NO as evidenced by the presence of iNOS, is present in many tissues of the body suggesting that the disease is a multi-organ one (Clark et al. 2003). The realization that protective immune responses may have deleterious side-effects has serious implications for the development of a vaccine against malaria.

The development of a vaccine must take into consideration what stage of the parasite life cycle to attack and what kind of antigens to use. The life cycle of the malaria parasite is very complex and includes a number of stages that are biochemically and immunologically distinct. Immune responses against all these stages have been detected but immunity against one does not protect against the next. In effect, malaria infections consist of a sequence of different infections, not just one.

The antigens are complex: each stage possesses a large number of different antigens some of which are protective and some of which are not. In addition, malaria parasites have evolved a number of ways to evade the immune response so some of the antigens identified could be counter-protective. Research on malaria vaccines has paralleled or, in some cases, led research on viral and bacterial vaccines and the emphasis has changed with our increasing understanding of the immune response as a whole. The traditional approaches have been to identify and characterize potentially protective surface antigens and to use these as:

- subunit antigens on their own
- as a basis for synthetic vaccines
- as recombinant molecules expressed in a suitable vector.

All three methods have been tried with varying success over the past two decades and this subject has been extensively reviewed (Hoffman 1996; Facer and Tanner 1997; Good et al. 1998; Miller and Hoffman 1998; Ballou et al. 1999; Anders and Saul 2000; Plebanski and Hill 2000). More recently, there has been a switch to the possibility of DNA vaccines and prime-boost methods of immunization whereby the host is first immunized with a DNA vaccine expressing the requisite antigens and subsequently with a recombinant vector expressing the same antigens.

Sporozoites are obvious targets, being the first stages to enter the body and circulating in the blood for a short time before entering the liver; a vaccine against the sporozoite should prevent infection. The first vaccines against malaria consisted of irradiated sporozoites (Clyde et al. 1973) and it soon became clear that the immune response was directed against the NANP repeat region of the major surface protein of the sporozoite, CSP. The NANP repeat was then used as a basis for recombinant vaccines expressed in *E. coli* (Ballou et al. 1987) and for synthetic vaccines (Herrington et al. 1987) in a number of trials with potentially promising results – about 20–30 percent of volunteers were protected. However, these trials also revealed that there was little correlation between antibody levels and protection. It is now widely accepted that the sporozoite repeat antigen is not a good vaccine candidate because if even one sporozoite escaped into the liver an infection would be initiated. In addition, the epidemiological evidence does not support the concept that a sporozoite vaccine would be effective. Returning to vaccines based on irradiated sporozoites, it was found that high levels of irradiation prevented sporozoites from invading liver cells and that exposure to such sporozoites was not protective, so attention moved on to sporozoite antigens involved in hepatocyte invasion. A promising recombinant vaccine called RTS,S and consisting of part of the CSP fused to the hepatitis B surface antigen was developed in the Gambia with excellent results (over 70 percent efficacy); unfortunately, this protection faded after about 15 weeks (Bojang et al. 2001). Modifications of this vaccine are now being developed for possible clinical trials. All these vaccine trials were based on eliciting antibody responses but attention has now turned towards T-cell responses (Taylor-Robinson 2003). It was once thought that there was no immune response against the liver stages but it is now apparent that there are immune responses in the liver that block the invasion of the hepatocytes and probably kill the early stages of the parasite within the hepatocytes (Aidoo and Udhayakumar 2000). Several liver-stage antigens are now recognized, particularly LSA-1 and LSA-3, as possible targets for immune attack and there have been several attempts to incorporate them into multicomponent vaccines. The most promising developments are concerned with DNA vaccines and the current front runner is a vaccine based on a string of 20 T- and B-cell epitopes derived from *P. falciparum* pre-erythrocytic-stage antigens fused with the TRAP molecule plus the modified vaccinia virus Ankara (MVA) (Moorthy and Hill 2002). This DNA ME-TRAP vaccine is now undergoing clinical trials with different prime-boost regimens (McConkey et al. 2003). Similar vaccines with the fowlpox virus FP9 substituted for MVA and the possibility of boosting the response to RTS,S are also at various phases of development and clinical trial (Moorthy and Hill 2002).

Blood stages are obvious vaccine targets because there are so many of them and because they cause the disease. A number of potential antigens have been recognized, either associated with the infected red cell, intra-erythrocytic parasites, merozoites or secreted soluble antigens. The prime targets are the antigens involved in adhesion and red blood cell invasion. A vaccine against the adhesion molecule, PfEMP-1, would

prevent or decrease the pathology associated with sequestration but, because of the ability of PfEMP-1 to undergo antigenic variation, it is not a suitable vaccine candidate. Attention has, therefore, focused on antigens involved in red cell invasion. A recombinant form of MSP-1 has been assessed in Phase I and Phase II (safety and efficacy) trials but was found to produce adverse hypersensitivity effects (Keitel et al. 1999). However, MSP-1 together with other antigens, is being considered as a component of multicomponent vaccines incorporating the genes for antigens from various stages of the life cycle, including the sporozoite, liver, erythrocytic and sexual stages (Holder 1999; Shi et al. 1999). A number of multicomponent vaccines have been developed of which the most extensively studied is one based on three blood-stage antigens (MSP-1, MSP-2, and RESA), which has produced some encouraging results in field trials in Papua New Guinea (Genton et al. 2003).

Currently, the only vaccine that has been subject to extensive field trials is SPf66, a synthetic vaccine consisting of a polymer of three merozoite antigens: Pf83 (an 83 kDa peptide representing part of MSP-1), Pf55, and Pf35. These antigens, none of which has been identified with any of the known major malaria antigens, are linked together by the sporozoite antigen NANP (Valero et al. 1993). In a number of extensive trials in Colombia, Ecuador, and Venezuela, the results obtained indicated that the vaccine was safe and immunogenic and, although it did not prevent malaria, it reduced the number of malaria episodes. It has also undergone extensive trials in natural populations of children in Africa and adults in Thailand but, unfortunately, it did not prevent malaria in children in trials in Tanzania (Beck et al. 1997) or The Gambia (Bojang et al. 1998). SPf66 is the only malaria vaccine that has so far been approved by the WHO but has now been discontinued in its original form.

One of the possible vaccine components is the sexual stage in the mosquito (Targett 1995). At first, this might seem to be a very strange target for attack but it is both logical and feasible. The principle is to immunize individuals with sexual-stage antigens to raise antibodies that inactivate the sexual stages when taken up in the serum by a mosquito. This has been called the transmission blocking or 'altruistic' vaccine because it does not protect the person immunized although it does prevent transmission. Pfs25 and Pfs28, antigens on the surface of the malaria zygote, are the most favored candidates for such a vaccine and a recombinant fusion protein, TBV25-28, containing these two antigens has been developed and considered for clinical trials (Gozar et al. 2001). Up to now, the idea of such a vaccine would have been unthinkable but there is no reason why such a transmission blocking antigen should not be added to another vaccine such as SPf66.

Numerous other possibilities are currently being pursued and it will probably be necessary to have a multipronged approach to the development of a malaria vaccine. It may not be necessary to produce sterile immunity but it might be possible to reduce the severity of the infection. It may, therefore, be possible to consider novel approaches such as vaccinating against the disease rather than the infection and there is some evidence that it is possible to immunize laboratory animals against the toxins produced by malaria parasites (Schofield et al. 2002).

The development of a malaria vaccine has been a long and frustrating search with many false starts and unachievable promises. The future, however, is brighter and depends on scientific co-operation rather than competition which is now forthcoming (Medaglini and Hoeveler 2003; www.niaid.nih.gov/dmid/malaria/malariavac.htm).

BABESIAL INFECTIONS

Babesiosis, although common in wild and domesticated animals, is a rare accidental infection in humans (see Babesiosis of humans). Little is known about immune responses in humans except that immunologically intact individuals are susceptible to infection with the rodent babesia *Babesia microti*. Immunity seems to be similar to that seen in malaria in that it involves the activation of macrophages and NK cells and the production of NO (Aguilar-Deflin et al. 2003). Such infections are accompanied by raised specific antibody levels, even in the presence of persisting parasitemias, a phenomenon common in many protozoal infections. Babesiosis caused by *B. microti* is more serious in immunologically compromised patients and the European form, caused by *B. divergens*, has been reported only from splenectomized individuals, suggesting that an intact immune system is required to keep this infection under control (see Telford et al. 1993). Although nothing is known about the ways in which *Babesia* spp. survive in humans, it is known that there are a number of ways in which these parasites survive in other hosts; these include antigenic variation, cytoadhesion to avoid immune attack (as in malaria infections), binding of host proteins to infected red blood cells and immunodepression (Allred 2003). The persistence of babesial infections and the ability to sequester suggest that the pathology of babesiosis might resemble that seen in malarial infections (see Clark and Cowden 2003).

Immunity to tissue-inhabiting protozoa

Several parasitic protozoa occur in human tissues, including the following:

- the ubiquitous coccidian *Toxoplasma gondii*, in a variety of nucleated cells, including macrophages, and the brain
- *Trypanosoma cruzi* in muscle and nerve cells
- *Sarcocystis* spp. in muscle
- microsporidians in various tissues including the brain

- opportunistic amebae in the brain.

Of these, *T. gondii*, already discussed, and *T. cruzi* are the most important and, partly because of the ease with which they can be maintained in laboratory animals, have been the most intensively studied. The remaining tissue-inhabiting protozoa cause long, chronic and usually harmless infections but these may become fulminating in immunologically compromised individuals such as those co-infected with HIV.

CHAGAS DISEASE (NEW WORLD TRYPANOSOMIASIS)

Chagas disease, caused by the trypanosome *Trypanosoma cruzi*, is confined to South and Central America (see WHO 2002; New World trypanosomiasis). *T. cruzi* infects more than 150 species of mammals and most of the research done with this parasite has been carried out in rodents, particularly mice, in which the pattern of immunity that emerges is complex and is influenced by the genetic make-up of both the parasite and host. Chagas disease is one of the most insidious of all parasitic diseases. The infection is transmitted by blood-sucking bugs and is typically acquired in childhood; the victim may experience little more than a localized swelling and a transient fever but, in some individuals, possibly as many as 20–40 percent, the infection may be acute and life threatening. Adults who become infected tend to develop a life-long chronic infection which can result in death many years later. The trypanosomes divide at the site of the bite and circulate briefly in the blood before entering macrophages, in which they develop into nonflagellated amastigotes and survive by escaping from the phagolysosome and multiplying in the cytoplasm of the cell. From there, they escape and invade other macrophages or a variety of other nucleated cell types, including muscle cells (particularly cardiac muscle) and nerve cells. There is a strong immune response in individuals who survive an acute infection but this is only partially effective and does not eradicate all the parasites; the infection, therefore, persists throughout the life of the host. During the course of the infection, the host mounts specific antibody responses involving all subclasses of immunoglobulins. As in most other infections, there is an early rise in IgM followed by increasing levels of IgG, which persist throughout the infection and are, therefore, useful for diagnosis.

The use of genetically modified mice has made it possible to examine the immune response to *T. cruzi* in some detail (Tarleton et al. 2000). In mice, high levels of IgG2a, IgM, and IgE persist for about 13 weeks after which IgG2a and IgM levels begin to decline but, together with IgG1 and IgG2b, remain elevated throughout infection (Rowland et al. 1992). Studies in mice have also provided clues about cell-mediated immune responses to *T. cruzi* (reviewed by Takle and Snary 1993; Kierszenbaum and Sztein 1994; Kirchoff 1994). Protective immunity to *T. cruzi* is T-cell dependent and cell-mediated (Wrightsman et al. 2002). The main defense mechanism is provided by macrophages activated by TNF-α, IFN-γ, and IL-12 resulting in the production of NO, which is responsible for the killing of intracellular parasites (see Machado et al. 2000; Talvani et al. 2003). However, this activity can be inhibited by IL-4, IL-10, and TGF-β (Munoz-Fernandez et al. 1992; Gazzinelli et al. 1992; DosReis 1997; Reed 1998). There is no clear-cut Th1 and Th2 polarity in *T. cruzi* infections and it appears that Th1 cells are required for the control of infection and that Th2 cells may be involved in the persistent parasitemia and severity of disease (Kumar and Tarleton 2001).

The long-term persistence of parasitemia is a characteristic feature of Chagas disease and it is clear that *T. cruzi* survives for the whole lifetime of patients with chronic Chagas disease as evidenced by the fact that, at autopsy, parasites have been detected in heart muscle (Brener 1994), smooth muscle and adrenal glands (Teixeira et al. 1997). The parasite can only survive by evading the immune response and this is partially achieved inactively by forsaking macrophages and invading nonphagocytic cells. *T. cruzi* also evades the immune response actively in a number of ways. Antibody attack is prevented in two ways: the inhibition of antibody binding, and the inhibition of complement activation. Antibody binding is inhibited by the overproduction of nonprotective IgM that interferes with the binding of potentially protective IgG; complement activation is inhibited by means of a surface glycoprotein that binds C3 (the third component of complement) and thus inhibits both the classical and alternative complement pathways (Beucher et al. 2003). *T. cruzi* also employs a variety of immune evasion mechanisms to enable it to avoid cell-mediated immune responses (Zambrano-Villa et al. 2002). The production of IL-12 and TNF-α (essential for parasite killing) is inhibited by a parasite surface antigen, AgC10, that binds to the surfaces of macrophages (de Diago et al., 1997); another parasite molecule promotes the production of IL-10 and TGF-β (Sztein et al. 1990) while also inhibiting the production of IL-12 (Silva et al. 1998). Immunosuppression is also characteristic of *T. cruzi* infections and this is brought about, in part, by the induction of a population of suppressor cells (Tarleton 1998). In the context of immune evasion, it might be to the advantage of the parasite to mimic host antigens but one possible outcome of the possession of shared antigens is autoimmunity, which some authorities consider to be characteristic of this infection.

Chagas disease is life-long and usually chronic and is accompanied by the gradual destruction of the infected cells, giving rise to cardiac failure and loss of control of the digestive system later in life, possibly 20–30 years later (Tarleton 2001). Whether or not Chagas disease is

an autoimmune disease is a matter of controversy (Leon and Engman 2001), but the histopathological picture does suggest an autoimmune phenomenon. Sequestered antigens released when infected cells are destroyed and uninfected cells onto which *T. cruzi* antigens have been adsorbed are potential causes of autoimmunity. In experimental animals, autoantibodies appear after nerve cells have been destroyed, antibodies to rat ganglia cross-react with *T. cruzi* and anti-*T. cruzi* antibodies cross-react with mammalian tissues. Comparable observations have been made in patients with Chagas disease, for example the C-terminus of the *T. cruzi* ribosomal P protein shares epitopes with host cells (Mahler et al. 2001) and myosin shares epitopes with the *T. cruzi* B-13 antigen with evidence of some association with chronic chagasic cardiopathy (Cunha-Neto and Kalil 2001). There is further information on the pathology of Chagas disease in New World trypanosomiasis.

In summary, immunity to *T. cruzi* involves some of the most complex immunological responses encountered in any infection involving antigens, host cells, chemokines and cytokines (see Teixeira et al. 2002); the interplay between immunity, immune evasion, and autoimmunity is likely to take many years to elucidate. In the meantime, although some experimental vaccines have been developed, the possibility of a protective vaccine that does not cause any autoimmunity against this important human pathogen seems remote.

MICROSPORIDIANS

Microsporidiosis, caused by at least eight species belonging to five genera (see Microsporidiosis) is a rare but increasingly recognized condition in immunologically compromised patients, particularly those suffering from AIDS (Bryan 1995; Deraedt and Molina 1995). As the condition is virtually unknown in immunologically competent individuals, there must be some immune response that keeps these infections under control, but definite evidence for this, and its mechanisms, is lacking. Apart from the epidemiological evidence, most of our knowledge comes from animal and in vitro studies, which might not be relevant to what happens in humans. It is clear that perturbation of T-cell function is a major cause of immunological failure leading to microsporidiosis, but as T cells are central to so many immunological responses, it is difficult to draw firm conclusions about the nature of natural or acquired immunity to microsporidiosis in humans.

SARCOCYSTIS AND RELATED ORGANISMS

Little is known of these rare infections in humans (described in *Sarcocystis*) in terms of the immune responses that they evoke in their natural hosts, laboratory animals or humans. All form thick-walled cysts with few signs of any immunological reaction or immunopathology.

OPPORTUNISTIC AMEBAE

Although these free-living amebae (belonging to the genera *Acanthamoeba*, *Balamuthia*, and *Naegleria*) are sometimes grouped with the intestinal amebae, it is convenient to consider them briefly here. As there have been relatively few human infections, there is very little information available and it is not known whether or not individuals mount a protective immune response. If they do, it is not clear whether this protects against subsequent infection (which, in any case, would be very unlikely). Virtually all that is known is that specific antibodies have been detected in the sera of infected individuals (see Opportunistic amebae). Information that has accrued from observations in animal models has been reviewed by John (1993).

IMMUNITY TO HELMINTHS

Characteristics of helminths in relation to the immune response

Helminths (worm parasites) confront the immune system with problems that are quite different from those posed by protozoa. These problems are largely the consequences of greater size and structural complexity, but are compounded by three additional factors:

1. worms have the ability to move actively through the body of the host
2. as part of their life cycles, many undergo sequential developmental changes in the host, during which their structure and antigenic make-up may change dramatically
3. their bodies are covered by layers that are more complex and less vulnerable to immune attack than conventional plasma membranes.

Thus, although helminths occupy many of the sites in the body that are exploited by protozoa (other than the truly intracellular), the immune mechanisms required to destroy them in or remove them from these sites must often be qualitatively different. Unlike protozoa, the majority of helminths do not replicate within the host. In these circumstances, an increase in the worm burden carried (and the associated danger of pathology) occurs only when the host is repeatedly infected. Under conditions of natural infection, immune responses are, therefore, most important in preventing or reducing reinfection. There are, however, some helminths in which the numbers of parasite stages present in the body do increase after infection. These include:

- tapeworms, such as *Taenia solium* and *Echinococcus*, in which an initial infection may produce large numbers of larval stages
- the blood flukes (*Schistosoma*), in which eggs become trapped in tissues

- the nematodes, *Strongyloides* and *Trichinella*, in which larvae accumulate in the body.

In such cases, the development of the immune response can be considered analogous to that which occurs in protozoan infections.

Adult stages of helminths range in length from a few millimeters to several meters. Larval stages may be less than 1 mm in length, but even the smallest larva is too large to be phagocytosed or lysed by a single cell; cytotoxic activity, therefore, requires multiple interactions between the parasite surface and inflammatory cells. Such interactions can be mediated by both complement and antibody, ADCC being a common response to invasion by worms.

In addition to being large, helminths are complex multicellular organisms with well developed organs and tissues. When alive, they present the immune system with a diverse range of antigens, both on their surfaces and in excretory and secretory products. When they die, many more antigens may be released. Although immune responses are made to the majority of these antigens, they may have little or no effect on the parasite for some or all of the following reasons:

- the molecules concerned may not be essential for worm survival
- in the living worm, these molecules may be inaccessible to effector mechanisms
- the response to released antigens may take place at a considerable distance from the parasite
- the parasite may actively move away from the immune response generated at a particular site
- the antigens may elicit inappropriate immune responses
- the parasite may evade, inactivate or down-regulate immune effectors.

In general then, although there are important exceptions involving larval stages, worms are likely to be much less easily controlled by immediate interactions with immune effector mechanisms than are protozoa. Binding of antibodies or surface activation of complement rarely kills worms directly; effective immunity is more often the indirect result of immunologically specific recognition events. Among the more important of these are ADCC, immune-mediated inflammation, and antibody-mediated inhibition of enzyme activity.

The result of ADCC reactions is the adherence of large numbers of cells and the release onto the surface of a variety of enzymes and short-range mediators. These may harm the parasite directly, either by destroying the integrity of the surface layer or by causing damage to deeper tissues. The effectiveness of such attack is influenced by the nature of the surface available. For example, whereas flukes such as schistosomes have relatively delicate cytoplasmic surfaces, nematodes have tougher collagenous cuticles that provide them with considerable protection against external damage. Larval nematodes may be killed by ADCC, but adults are often unaffected. Even the theoretically more vulnerable schistosomes have multiple evasion strategies that result in their surfaces being difficult to damage. In addition, effective ADCC requires not only that cells bind to the target, but also that the cells maintain contact, which is sometimes difficult when the target is an active worm.

With the important exceptions of tapeworms and acanthocephalans, most helminths feed actively and digest their food source enzymatically, either within their own intestines or externally before uptake. The enzymes used in these processes can be important targets for the host immune response, as are those used by worms to penetrate the host and to move through its tissues. The interaction of antibodies with target parasite enzymes can inhibit their activity, which can prevent successful invasion, migration, growth, and development and may result in worm death. Indeed, the complex nutritional, environmental, and behavioral requirements of worms make any host-mediated interference with normal function a potential means of increasing resistance.

By mechanisms that are not fully understood, the range of immune responses elicited by infections with helminths typically show a number of important differences from those generated by protozoan infections. Both in humans (Yazdanbakhsh et al. 2001, 2002) and experimental rodents (Gause et al. 2003), helminth antigens elicit responses that are predominantly mediated through cytokines released from lymphocytes of the Th2 subset. Prominent among these responses are the increased production of reaginic antibody (IgE), mast cells and eosinophils. Immediate hypersensitivity reactions, in which these components are involved, are therefore characteristic accompaniments of worm infections, but rare in protozoa. The inflammatory consequences of such reactions can be very effective forms of host defense and provide substantial resistance to infection. They result in structural, functional, and biochemical changes in the tissues surrounding the worms and lead to the release of powerful biologically active mediators. If antigenic stimulation and inflammation persist, worms may become the focus of cellular accumulations that trap them and that may ultimately kill them. As well as endangering the parasite, such chronic reactions may also cause severe host pathology. In a number of infections (e.g. schistosomes, filarial nematodes, larval cestodes), the parasites release factors that lead to down-regulation of the immune response, IL-10 playing an important modulatory role. In this way, parasites protect themselves from the host response and prolong their survival.

One of the most interesting experimental models of helminth infection is that of the intestinal nematode *Trichuris muris* in mice. As with the protozoan

Leishmania major, there is a reciprocal relationship between the Th subset response and immunity but in the case of *T. muris*, Th2 activity leads to resistance and Th1 to continuing susceptibility – i.e. the opposite of what happens in *L. major* infection. The dependence of the host response to *T. muris* on specific cytokines is elegantly demonstrated by the fact that the phenotypes of genetically susceptible and resistant mice can be reversed by injection of the appropriate cytokines or anticytokine reagents (Else et al. 1994). Although these 'helminth-type' and 'protozoan-type' patterns are clear cut in certain experimental models, in the majority of species of both groups the interrelationship between Th1 and Th2 responses appears to be more complex.

It should, therefore, be clear that although protozoa and helminths are grouped together as 'parasites' and both are powerfully immunogenic to the hosts, if they are to be successfully controlled by immune responses rather different strategies are required and quite distinct mechanisms may be involved.

Immunity to intestinal helminths

The characteristics of the intestine as an environment for parasites have already been described (see Immunity to intestinal protozoa). Despite the many defenses that can act there, the intestine is one of the commonest environments for parasitic worms and it is colonized by representatives of all the major groups: digeneans (flukes), cestodes (tapeworms), and nematodes (roundworms). Relatively little is known of immune responses to intestinal flukes and tapeworms in humans. More is known about nematodes, but most of this information concerns responses that can be measured peripherally, i.e. antibodies, proliferative and cytokine responses of circulating lymphocytes, delayed hypersensitivity and inflammatory responses. For an insight into responses operative at the intestinal level, we are heavily dependent on data obtained from work with experimental models.

INTESTINAL NEMATODES

A very large number of nematode species live in or infect via the intestine. Collectively, they are among the commonest of all parasites, infecting an estimated one-quarter of the world's population (see

Gastrointestinal nematodes – *Ascaris* hookworm, *Trichuris*, and *Enterobius*). The major intestinal nematodes of humans (*Ascaris*, hookworms, *Strongyloides*, and *Trichuris*) all mature in the intestine, but reach that site in a variety of ways. *Trichuris* infections are established by ingestion of eggs and the worms develop directly in the large intestine. *Ascaris* also infects orally, but after hatching, the larval stages penetrate the intestinal mucosa and migrate via the liver and lungs before returning to the small intestine to mature. The larvae of hookworms and *Strongyloides* characteristically infect through the skin and then migrate to the small intestine. In addition to the intestine itself, immunity to worms with migratory stages may, therefore, operate at several sites in the body. The mechanisms likely to be effective are determined by the characteristics of each organ system involved. Immune and inflammatory responses in parenteral sites such as the skin, liver, and lungs share a number of common features. Worms moving through these sites are likely to be in intimate contact with potential effectors, such as antibodies (IgM, IgG), complement, biologically active mediators and cytotoxic cells, and can therefore be damaged directly. In contrast, worms living in the intestine itself may have a quite different relationship with the immune system. Not only does the intestine have distinctive immune mechanisms, but unless worms penetrate into the mucosa they are unlikely to be accessible to some of the effector mechanisms that operate in the tissues. Those effectors that may affect intestinal worms include the antibody isotypes that are secreted into the lumen (IgA and IgM) or leak from the mucosa (IgG), and mediators such as amines, prostaglandins, leukotrienes and reactive oxygen and nitrogen metabolites that are produced by inflammatory cells. Intestinal worms, like all parasites, are wholly dependent on the physical and physicochemical characteristics of their environment, and these conditions may change dramatically when the intestine is inflamed as a result of infection. Such changes can themselves contribute to immunity by making the local habitats of the parasites unsuitable for their continued survival. As already mentioned (see The cytokine network in parasitic infections, and Characteristics of helminths in relation to the immune response), worm infections selectively elicit Th2 subset responses and the cytokines produced by these cells generate powerful responses, components of which (e.g. IgE, mast cells, and eosinophils) are known to be potent mediators of intestinal inflammation.

Experimental systems in laboratory animals show that intestinal worms can be very effectively controlled by host immune responses (Gause et al. 2003). It is much less clear whether or not intestinal worms in humans are similarly controlled. Infections are not only very common, they are often persistent. Individuals may acquire worms early in life, retain worms for long periods, and be easily reinfected. Epidemiological studies indicate that although the prevalence of infections often remains high as people age, the intensity of infection (numbers of worms) can decline significantly (Bundy 1995). This may indicate the operation of protective immune responses, although it is obvious that behavioral changes will also be involved, as transmission of all the major intestinal worms is dependent on some degree of contact with fecal or fecally contaminated material. Cross-sectional analysis of infected populations shows that the parasite burden is aggregated into

relatively few individuals, most having small numbers of worms. Again, this may indicate an effective immunity or may reflect behavioral influences. It is well established that individuals are predisposed to a particular level of infection, i.e. they reacquire worm burdens after chemotherapy that are similar to those existing before treatment. In some, this predisposition results in heavy infections (i.e. the individuals are susceptible); in others, only light infections are acquired (i.e. they appear resistant). Predisposition suggests the operation of genetically determined levels of immunity to infection, and evidence supporting this interpretation is now beginning to appear (Williams-Blangero et al. 2002).

Knowledge of immune responses to intestinal nematodes of humans is much less detailed than that of responses to filarial worms and schistosomes (see Immunity to filarial nematodes, and Schistosomes). In general, correlative immunoepidemiological studies show that serological responses correlate positively with intensity and duration of infection, but certain isotypes appear to be associated with protective immunity. These include IgA responses in *Trichuris* infections (Bundy and Medley 1992) and IgE responses in hookworm (*Necator*) and *Trichuris* infections (Pritchard et al. 1995; Faulkner et al. 2002). The functional roles of antiworm antibodies are uncertain. ADCC responses may be generated against stages migrating through tissues, but the adults in the intestine are large and well protected organisms. Antibodies may interfere with feeding activities, particularly where, as in hookworms and possibly *Trichuris*, these depend on the worm releasing enzymes into the host's tissues. Antibodies may also reduce the effectiveness of worm secretions that interfere with the operation of host defense mechanisms, such as anti-enzymes, antioxidants and immunomodulators (Pritchard 1995). Recent studies have begun to analyze cellular and cytokine responses (Cooper et al. 2000; Geiger et al. 2002; Turner et al. 2002). So far, these have not shown the same clear-cut correlation of protective immunity with T2 cytokines that has emerged from work in animal models (see below). Geiger et al. (2002), for example, found that parasite-free individuals showed higher T1 cytokine production (TNF-α, IL-12, IFN-γ) than infected individuals, whereas T2 cytokines were present equally in parasite-free and infected patients. Both this group and Turner et al. (2002) recorded production of IL-10 in infected individuals, suggesting that chronic infections may be associated with activity of Th3 or Tr cells.

Experimental studies in rodents have given detailed pictures of the nature and operation of immune responses against intestinal nematodes. These may operate during the course of primary infections to expel worms from the intestine. On reinfection, protective responses operate more quickly and may prevent the establishment of infection almost completely. In all cases, immunity is Th cell-dependent, and Th2 cells play the major role, polarization of the response to this subset being critical for protection (Gause et al. 2003). In manipulative experiments, it has been shown that IFN-γ can prevent, and IL-4 together with IL-13 can promote, the normal expression of immunity; IL-10 production is also important to ensure a Th2 bias. The roles of the co-stimulatory and signaling membrane molecules that regulate T-cell activity and define whether host responses are protective or otherwise (e.g. B7.1-B7.2, CD40L, STAT 4/6 and chemokine ligands) have now been fully defined (Gause et al. 2003; deSchoolmeester et al. 2003). Analysis has also extended to the role of intracellular signaling pathways (Artis et al. 2002).

Responses to infection are accompanied by acute inflammatory changes that result in structural and functional modifications of the intestinal mucosa, and in some species (e.g. *Nippostrongylus*, *Strongyloides*, *Trichinella*) these act as important effector mechanisms, (Artis and Grencis 2001). Although IgE antibodies and eosinophilia accompany the intestinal inflammation induced by infection in rodents they do not seem to have a significant role, and deletion of these responses by using antibodies against the relevant cytokines does not affect the expression of immunity. In contrast, mast cells do play an important role against certain species (Helmby and Grencis 2002). In other parasites (e.g. *Trichuris*) antiworm IgA and IgG1 antibodies seem to be important, probably by interfering with important metabolic or behavioral activities. In worms that migrate through the tissues to reach the intestine (*Nippostrongylus*, *Strongyloides*), immunity acts against the intestinal stages during a primary infection, but against tissue stages in subsequent infections, larvae being trapped in inflammatory foci in the lungs or the skin. Similar mechanisms appear to act against migrating larval hookworms in immune mice (Girod et al. 2003).

Experimental models have not only allowed the study of protective responses, they have also shed light on situations in which immunity does not operate or operates at best ineffectively. Some nematodes in mice (like nematodes in humans) establish chronic, long-term infections. With one species (*Heligmosomoides polygyrus*), this is due to immunomodulatory influences exerted by adult worms, which effectively suppress the potentially protective responses elicited by the larval stages. Immunomodulation is associated with the release of particular molecules in the secretions of adult worms. Full expression of this immune suppression is under strong host genetic influences, which determine the speed and nature of antiparasite responses. *Trichuris muris* can also establish chronic infections in certain strains of inbred mice. In most strains, infection elicits Th2 responses that result in effective immunity and worms are lost before they can become mature. In certain strains, the initial Th2 response is switched to a Th1 response and the host is then not only unable to eliminate the existing infection but remains susceptible

to subsequent infections as well, a situation that has many parallels with human *Trichuris* infections (Artis and Grencis 2001). Chronic worm infections are often associated with immunopathological changes to the intestine, which almost certainly reflect the effects of persisting, inappropriate immune responses.

Immunity to filarial nematodes

Filarial nematodes (members of the Filarioidea) are important human parasites in many subtropical and tropical countries (see Lymphatic filariasis, and Onchocerciasis). All are transmitted by insect vectors, which act as intermediate hosts allowing development of larvae to the infective stage. These larvae are transmitted when the insect bites, the initial stages of infection, therefore, involve the skin and dermal tissues. In some species, the adults remain in the superficial layers of the body but in others, the adult worms live within much deeper tissues; both are, therefore, theoretically susceptible to direct interaction with immune effectors such as antibody, complement and cytotoxic cells. There are several species of filariae that infect humans, many of which are of low pathogenicity or of only local significance. The most important species are those in which the adult worm lives in the lymphatic tissues (the genera *Wuchereria* and *Brugia*) and those in which the adults live in the skin (*Onchocerca*). In the former, adult females liberate embryos (microfilaria larvae) that circulate in the blood; female *Onchocerca* release microfilaria into the skin. Biting insects pick up these larvae while feeding, the larvae undergo molts to reach the infective third stage, and these then re-enter the human at the next blood meal. In the lymphatic species, pathology is caused by the adult stages, whereas in *Onchocerca* microfilaria are the primary cause of pathology.

It is characteristic of all filarial infections that they are long-lived; worms survive for many years and release millions of microfilaria. Many individuals in areas where these infections are endemic continue to accumulate worms over long periods. These two features suggest that there is little or no immunity, but more detailed epidemiological studies suggest otherwise. Even in areas where the overall prevalence is high, some individuals are parasitologically negative, show no pathology but do have antiparasite immune responses, indicating exposure to infection. The majority show signs of infection, of pathology, or both. Some are heavily infected, with many circulating microfilaria, but may have relatively little overt pathology; some have severe pathology but may have few or no microfilaria in the blood; others may have low microfilaremia but show abnormal pathology indicative of hypersensitivity reactions. Collectively, these data show that filarial infections do stimulate immune responses; in some individuals these responses appear to be fully protective, but in others, immune responses contribute to the development of pathologic changes. This spectrum of responsiveness reflects a variety of patterns of Th cell and cytokine activity as well as parasite-induced immune suppression.

Analysis of protective responses to filarial nematodes has been carried out in vitro, using human cells and sera, and in vivo, using a variety of animal models. It has been shown in several cases that immunity can effectively operate through ADCC reactions directed against antigens present on the cuticular surface. The filarial cuticle is relatively delicate, compared with that of the intestinal nematodes for example, and may play a significant role in the uptake of nutrients. Antigens on the cuticle are known to be targets for antibodies, particularly IgG and IgE isotypes, and these facilitate adherence of cells such as eosinophils, macrophages, and neutrophils. Under experimental conditions, ADCC reactions can kill all stages, from microfilaria to adult; under conditions of natural infection it is most likely that a primary target is the incoming infective larva. Although the cuticle appears to be a major target for immune responses, other protective antigens have also been identified. Antigens derived from internal tissues, including muscle proteins, can elicit protective responses when used to immunize experimental animals.

Immunity to filaria is T-cell dependent and involves the activity of Th cells. In humans, putative immunity correlates with production of T-cell dependent antibody isotypes (IgE and IgG3), and these isotypes are also required for effective ADCC. Production of eosinophils, a contributor to cytotoxicity, is also T dependent. Studies in experimental animals have confirmed the need for Th cell activity, and shown that in some cases the Th2 subset is functionally important, IL-4 being a key cytokine (Lawrence 1996; Volkmann et al. 2001). There are some interesting exceptions to these conclusions (e.g. Le Goff et al. 2002) and these raise the perennial problem as to how representative of human responses are those measured in experimental animals. However, reduced IL-4 production is often found in heavily infected individuals but present in putative immunes. A factor complicating interpretation of immune responses to filarial worms is the demonstration that they harbor symbiotic *Wohlbachia* bacteria (Taylor 2002). These play an important role in worm development and fertility; if the bacteria are removed by antibiotic treatment worms may die. *Wohlbachia*, however, releases a lipopolysaccharide-like endotoxin that can affect both the nature of the immune response and the consequential immunopathological consequences.

Some of the most interesting data concerning immune responses to filarial infections in humans are those that attempt to correlate patient status with particular components of the immune system (Nutman 2001). It is now widely accepted that, both for lymphatic filariasis and onchocerciasis, there are individuals in populations in endemic areas who neither develop patent infections

nor show any signs of diseases, yet are immunologically positive in the sense of having antiparasite antibodies and antigen-specific T-cell activity. Such individuals are often referred to as 'endemic normals', although the use of this term is debatable. The fact that these individuals remain parasitologically negative despite exposure to infected vectors implies effective control of incoming infections and it is thought that this control operates against the infective larval stages. Experimental data from *Brugia* infections in cats, as well as data from rodent models, support this interpretation; indeed there is good evidence that irradiated infective larvae can induce an effective immunity when used as a vaccine and that this immunity is Th2-dependent (Bancroft et al. 1994).

The suggestion that some individuals do develop immunity to filarial infections raises the question of why the majority apparently do not, despite evidence for immune responses to the parasites. Those who show parasitological or pathological evidence of infection can be divided into a number of categories (Freedman 1998; Nutman 2001). In lymphatic filariasis, the extremes are patients who are microfilaremic and asymptomatic, and those who are microfilaria negative but show obstructive pathology. Another category shows atypical allergic hyper-responsiveness (tropical pulmonary eosinophilia). The lymphocytes of those with high microfilaremia often appear to be unresponsive to parasite antigens when tested in vitro (as is also the case with cells from patients with heavy skin burdens of the microfilaria of *Onchocerca*). There is evidence that altered T-cell-subset activity (including Tr cells) and cytokine production are contributory factors (Maizels et al. 1995; Satoguina et al. 2002; Steel and Nutman 2003) but released parasite products themselves can be directly immunosuppressive (Allen and MacDonald 1998; Pfaff et al. 2002). The existence of phosphorylcholine on filarial molecules is associated with altered immune responsiveness, mediated through IL-10 (Houston et al. 2000) and IL-10 is increasingly recognized as an important modulator of the antiparasite response leading to an altered balance between Th1 and Th2 activity. One consequence of this is the high levels of circulating antiworm IgG4 and IgE. These isotypes are found in both microfilaremic and elephantiasis patients but in the former, the ratio between the isotypes is biased to IgG4 and in the latter, the bias is towards IgE. The high levels of IgG4 may inhibit allergic responsiveness in the microfilaremic patients, whereas high IgE may promote pathological responses leading to obstructive pathology. IgE may, therefore, be important in protection against larval stages, through mediation of ADCC, but it may also contribute to immunopathology via hypersensitivity reactions to adult worms. Other IgG isotypes are low in microfilaremic individuals but more prominent in the elephantiasis group, in whom they may again contribute to pathology.

The T-cell anergy seen in patients heavily infected with lymphatic filarias or with *Onchocerca* appears similarly to be due to down-regulation of T-cell responsiveness by the cytokine IL-10 (Mahanty et al. 1997; Doetze et al. 2000). This is released from both Th2 cells, monocytes and macrophages, but predominantly the latter. Microfilaremic patients show high IL-10 production and low antigen-specific T-cell proliferation; symptomatic patients produce little IL-10 and their T cells proliferate well when exposed to parasite antigen. These results suggest that Th1 responses are somehow involved in antiparasite activity, which may be both beneficial, giving immunity against larvae, and harmful, via inflammatory responses to adult worms. IL-4 secretion appears to be linked with that of IL-5, lymphatic filariasis being characterized by high peripheral eosinophilia. In filarial infections there is, therefore, a complex, parasite-induced interplay between Th subsets that results in modulation of antibody and cellular responsiveness and determines both the parasitological and pathological outcomes of infection (Figure 4).

Immunity to flukes

Several species of digenean Platyhelminthes are parasitic in humans. They can be divided into four categories, based on their locations within the body:

1. the lung flukes (*Paragonimus*)
2. liver flukes (e.g. *Clonorchis*/*Opisthorchis*)
3. intestinal flukes (many genera)
4. blood flukes (*Schistosoma*).

Both lung and liver flukes can be highly pathogenic, with infections resulting in severe tissue damage (see Lung and liver flukes). Serological and cellular responses to these parasites are well described, but are not easily correlated with protective immunity. Little is known of immune responses to the human intestinal flukes, although infections of mice with *Echinostoma* have shown that an effective immunity can operate. The most intensively studied flukes are the schistosomes.

SCHISTOSOMES

Adult schistosomes live in the blood vessels of the host. Infections are initiated by penetration through the skin of larval stages that have developed in aquatic snails. To reach their final location, the larvae (schistosomula) migrate from the skin via the lungs to the liver, where they develop into mature male and female worms. These form permanent pairs, and then move to the mesenteric veins around the intestine or the vesical veins around the bladder. Once there, the worms can survive for prolonged periods (possibly years) during which time the female releases large numbers of eggs. These must leave the body and hatch in water so that larvae can find and

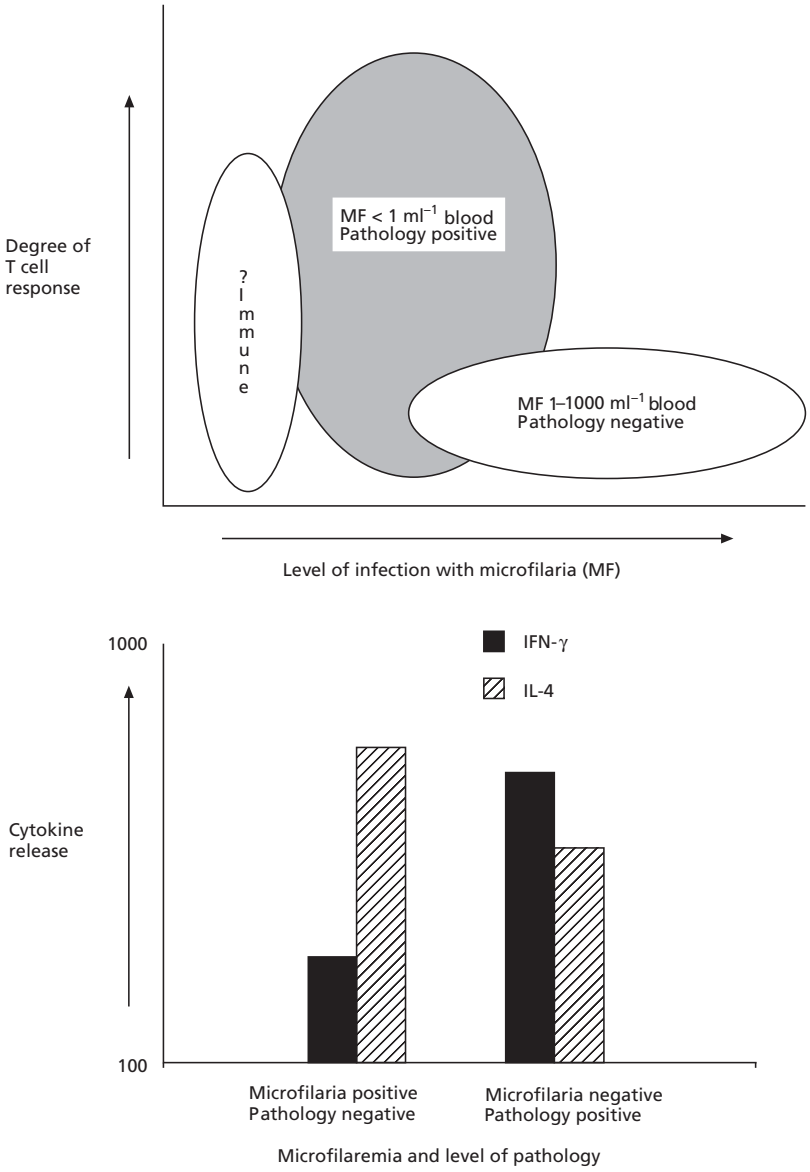

Figure 4 *Diagrammatic representation of the variations in T cell responsiveness and Th cell subset activity in patients infected with the lymphatic filaria* Brugia malayi. *T cell responses, reflected in level of proliferation when stimulated in vitro with antigen, are much higher in individuals without obvious infection (putatively immune) and in patients showing obstructive pathology than in those showing heavy microfilaremia but little pathology. Cytokine profiles, measured by in vitro cytokine production, indicate that heavy microfilaremia is associated with a predominant Th2 response (i.e. IL-4 production), whereas pathology in the absence of microfilaremia is associated with a predominant Th1 response (i.e. IFN-γ is the major cytokine). Data from Maizels et al. 1995*

penetrate a suitable snail. Eggs develop in the host's body and the contained larvae release enzymes that facilitate their passage out of blood vessels, through tissues into the lumen of the gut or bladder. Release from the body therefore occurs with feces or urine. There are three major species infecting humans: *Schistosoma japonicum*, *S. mansoni* (both in mesenteric veins), and *S. haematobium* (vesical veins).

Schistosomiasis is a multifaceted, immunopathological disease, but the most important components are the hypersensitivity responses initiated by eggs that are trapped in tissues (see Schistosomes: general). Granulomata form around the eggs, particularly those in the liver, and cause considerable local tissue damage. By blocking blood flow, granulomata can cause portal hypertension and serious vascular abnormalities, some of which (e.g. esophageal varices) may be life threatening.

Schistosome infections are common wherever climatic and environmental conditions favor snail development and where socioeconomic conditions allow fecal and urinary contamination of water that is used for drinking, bathing, washing or working. Infections are acquired early in life and are long-lasting; the disease is most

serious in older children and young adults. Initial infections appear to generate little or no protective immunity, but the intensity of infection declines with age and it is now accepted that this decline has an immunological as well as a behavioral component.

Experimental models have primarily used *S. mansoni*, which infects a variety of rodents and primates – work with other species is covered in Schistosomes: Asian. Primate studies defined the phenomenon of 'concomitant immunity' – the host is immune to reinfection while continuing to harbor the adult worms from an initial infection. Concomitant immunity was interpreted as showing that resistance mechanisms operated against the early schistosomula stage, but that adult worms were unaffected. In essence, this view still holds although there is now evidence that adults are also affected by immunity (Pearce and MacDonald 2002). Early studies also focused on effector mechanisms that resulted in damage to the tegumental surface which, in the larval stages at least, appeared vulnerable to direct damage by complement-mediated mechanisms and damage brought about by ADCC. The ability of adult worms (and indeed later larval stages) to survive in the face of responses capable of destroying early schistosomula depends on intrinsic changes in the tegument that render it less easily damaged, and the acquisition of host molecules (including glycolipids, immunoglobulin, and Fc receptors) that provide an immunological 'disguise'. In addition, schistosomes have a number of active evasion strategies that interfere with host immune mechanisms.

Experimental models, both in vivo and in vitro, indicate that there are multiple mechanisms through which resistance can be expressed. There is still no clear consensus about the mechanisms underlying protective immunity, although it seems clear that ADCC can be an important component of protective immunity. Several antibody isotypes and cell populations can participate in this response; eosinophils are major killer cells and IgG, IgE, and IgA are important isotypes. Interactions between parasites and macrophages may also be important, both in terms of ADCC and through the release of mediators capable of damaging the worms. A major difficulty, which has still to be resolved, is the relevance of data from animal experiments and from in vitro studies to the immune responses likely to occur in humans living in endemic areas (Capron 1998).

It is clear that schistosome infections elicit powerful CD4 T cell-dependent responses, the balance between Th1 and Th2 components being critical to the outcome (Hoffman et al. 2002). Data from experimental models show that the initial response is Th1 biased, switching to a Th2-biased response when egg laying begins. This switch is mediated by changes in local dendritic cells and co-stimulatory molecules as well as an altered cytokine environment in which IL-4 plays an important role (Gause et al. 2003) and is triggered by egg-associated carbohydrate antigens. These have specific glycan determinants that appear selectively to boost Th2 responses both to themselves and to bystander antigens (Okano et al. 2001). The antigens that can initiate protective immunity include molecules present at the surface of the tegument, the targets for ADCC as well as internal antigens. The latter include structural proteins, e.g. paramyosin, as well as a number with enzyme activity, e.g. glutathione S-transferase (GST) and triosephosphate isomerase. Several antigens have been defined and produced as recombinant or synthetic molecules and have been exploited as vaccine candidates (Bergquist et al. 2002). Trials have been carried out in rodents, bovines, and primates, with some degree of success achieved, largely in terms of reducing worm egg production and thus reducing egg-induced pathology. Clinical trials have been carried out using GST as the major antigen (Capron et al. 2001). However, not everyone is convinced about the need for and feasibility of a vaccine (Gryseels 2000) and this topic has been fiercely debated (Hagan et al. 2000).

The demonstration that immunity does play a part in determining the age-related decline in infection seen in endemic areas has come from detailed population surveys; these have monitored levels of reinfection after chemotherapy, as well as degrees of water contact. They have shown that individuals may effectively resist reinfection despite frequent contact with infected waters. Immunological studies on these populations have identified significant correlations between protective immunity and levels of antiworm IgE, but have also shown that other isotypes (particularly IgM, IgG2, and IgG4) can act as blocking antibodies and interfere with the expression of immunity (Hoffman et al. 2002). In young children (under 10 years) blocking antibodies predominate; in older children, protective IgE predominates. It is assumed that IgE may act in ADCC and that blocking antibodies interfere with the optimal operation of this protective mechanism.

Invasion and development of schistosomes are associated with distinct immunopathological phases. Penetration of the skin by cercariae causes a dermatitis, which may be severe in individuals sensitized by prior infection. The developmental stages may also be associated with allergic symptoms. This is particularly true in the case of *S. japonicum* infection in which fever, eosinophilia, lymphadenopathy, splenomegaly, and intestinal disturbances form the syndrome known as 'Katayama fever'. The hypersensitivity responses that lead to granuloma formation around trapped eggs have been studied in great detail, particularly in *S. mansoni* infections. Granuloma formation is the result of a complex series of cellular interactions involving T and B cells, antigen-presenting cells, eosinophils, and macrophages These interactions are coordinated through adhesion molecules, co-stimulatory molecules, chemokines, and cytokines (Sandor et al. 2003). Both Th1 and Th2 cells

contribute to granuloma formation, but down-regulation of Th2 activity occurs as infection progresses and this results in modulation of granuloma formation – IL-10 can play an important role in this process (Sadler et al. 2003). Granulomas eventually become surrounded by fibrous material, which helps to localize the lesion, but if excessive can lead to severe pathology. A balance between Th1 and Th2 responses appears to be critical: if either dominates, pathological levels of fibrosis may result. In the mouse, severe fibrosis appears to be Th2-mediated whereas in humans, a Th1 bias has been implicated (Fallon et al. 2000a; Hoffman et al. 2002; Pearce and MacDonald 2002), although IFN-γ has been associated with protection and TNF-α with aggravation of this disease (Henri et al. 2002). The severity of fibrosis in infected hosts is strongly influenced by genetic factors (Dessein et al. 1999).

S. mansoni and *S. japonicum* eggs leave the host's body via the intestine, which may become extensively damaged as tissues become sensitized to the eggs. Work in mice has shown that the enteropathy associated with this phase of infection is aggravated by Th2-dominated responses (Fallon et al. 2000b). In *S. haematobium*, the eggs of which leave via the bladder, inflammatory responses may produce severe pathological changes. There is a definite, but unexplained, association between such changes and the development of bladder cancers.

Immunity to tapeworms

Humans may act as hosts for adult stages of a number of tapeworm species. Some of these (e.g. *Taenia saginata* and *T. solium*) occur only in humans, others (e.g. *Diphyllobothrium latum*) occur in other hosts as well. Infections with adult tapeworms are acquired, in almost all cases, by ingestion of larval stages present in the bodies of intermediate hosts; for example, in the case of *T. saginata* by ingesting cysticerci present in infected beef and in the case of *D. latum* by ingesting plerocercoids present in infected fish. One species, *Hymenolepis nana*, has a unique life cycle in which development from the egg to the adult can take place within a single host. This species can, therefore, be locally quite common particularly in children. Very little is known of immune responses to adult tapeworms in humans; experimental studies with *H. nana* and other hymenolepids in rodents have shown that protective responses can act in the intestine against the adult stage.

LARVAL TAPEWORMS

In general, adult cestodes cause relatively little pathology, whereas larval tapeworms (metacestodes) can be highly pathogenic. In most cases, larvae are acquired by the accidental ingestion of eggs released from adult tapeworms in the intestines of other host species or from larval stages carried in transport hosts used for food. Larvae of the pork tapeworm *T. solium*, however, can develop from eggs released by adults in the intestine of the same or other humans.

The larval infections of greatest clinical importance are those caused by *T. solium* (cysticercosis), *Echinococcus granulosus* (hydatid disease), and *E. multilocularis* (alveolar echinococcosis) (see Larval cestodes). In all of these, the metacestodes formcyst-like structures in host tissues. Cysticercosis occurs wherever *T. solium* infections are common in the natural intermediate host, the pig. If infected pig meat is eaten raw or undercooked, the larval stages (cysticerci) become activated in the intestine, attach to the mucosa, and transform into the adult worm. When mature, the adult begins to shed segments (proglottids) that pass out with feces. As these decay, they release infective eggs that may contaminate water or vegetables, which then become a source of human infection. It is also thought that eggs can hatch directly in the intestine of a host harboring an adult worm (autoinfection). By whatever route infection is achieved, eggs hatch, then larvae penetrate the intestinal wall and migrate via the bloodstream. Cysticerci can form in the muscles, where they are relatively harmless, and in the central nervous system (CNS), where they can cause severe symptoms.

Larvae of *Echinococcus* spp. are acquired by accidental ingestion of eggs deposited in the feces of dogs or wild carnivores such as foxes. The eggs hatch in the small intestine, the larvae penetrate the mucosa and migrate via the blood, and develop in internal organs such as the lungs, liver, peritoneal cavity, and CNS. Larvae of *E. granulosus* grow into large fluid-filled cysts (hydatid cysts), whereas larvae of *E. multilocularis* form a pseudo-malignant mass of proliferating vesicular structures.

The immunological relationships between larval tapeworms and their hosts are complex and not well understood. Metacestodes often elicit little inflammation, they can persist for long periods and some can metastasize. All of these characteristics imply minimal or ineffective immune responses, yet it is quite clear that infections are immunogenic. There are many descriptions of cellular and serological responses to metacestodes in humans, from which some correlations with protective immunity can be drawn. There has been considerable work on infections in animal models, from which have come more precise insights into determinants of resistance and susceptibility. Immune responses can be considered as operative early or late in infection. The term 'early' describes responses against the hexacanth larva or onchosphere that is released from the egg and which undertakes the initial migration into the tissues; 'late' describes responses against the subsequent developing cystic metacestode stages.

In the early stages of larval development, the tapeworms have an unprotected external layer called the

tegument. They are, therefore, susceptible to damage by complement-fixing antibodies (e.g. IgG1 and IgG2 isotypes in mice; IgG2a in rats) and by complement- and antibody-mediated cellular cytotoxicity. Experimental studies show that host immunity can destroy larvae as they cross the mucosa or after they penetrate into other organs, but killing does not occur during an initial infection and the larvae become successfully established; early immunity is, therefore, most effective against reinfection. As the larvae develop, they acquire an external protective layer formed of both host and parasite components. They also produce sulfated polysaccharides and proteoglycans that interfere with host complement activation. In consequence, the larvae become insusceptible to the responses that can damage earlier life-cycle stages. Late immunity appears to operate exclusively through cytotoxic mechanisms, though these have little significant effect on larvae when they are fully developed. Larval tapeworm infections, like schistosome infections, therefore, show concomitant immunity, the host harboring fully developed primary infection larvae while being resistant to reinfection.

There is considerable evidence that larval tapeworms exert a variety of immunosuppressive effects on the host. For example, larval secretions (specifically glycoconjugates) inhibit T-cell proliferative responses and IL-2 production, prevent the accumulation of inflammatory cells, and interfere with normal macrophage functions (Dematteis et al. 2001; Rodriguez-Sosa et al. 2002). Infections, particularly with *E. granulosus*, generate powerful hypersensitivity responses, which can be life threatening if there is release of cyst fluid during surgical removal procedures.

Studies with *Echinococcus* spp. in experimental rodents and in humans are beginning to identify some of the key immunological events that may determine the outcome of infection (Gottstein and Felleisen 1995). As with many infections, there are striking genetically determined differences in the susceptibility and resistance of different strains of mice to this parasite. Mice that have greater resistance show responses that seem to indicate a dominant influence of Th1 cells, and the granulomata that develop around the mass of larval tissue contain a high proportion of CD8 T cells. In contrast, more susceptible mice, in which parasite growth is unchecked, have Th2-dominated responses and a high proportion of CD4 T cells in the granulomata. It is suggestive that similar data have come from studies of humans in endemic areas, some of whom, though susceptible to infection, seem to be resistant to disease. In these individuals, CD8 T cell numbers in the granulomata are high and peripheral lymphocytes secrete large amounts of the Th2 cytokine IL-5. Thus, Th1- and Th2-mediated mechanisms play a crucial role in the complex relationship between parasite and host (Vuitton 2003) and manipulation of the balance between the two may have clinical benefits (Godot et al. 2003).

There is no immediate prospect of immunoprophylaxis against larval tapeworm infections in humans, but the development of a recombinant vaccine against the larval stages of *Taenia ovis* in sheep has stimulated similar studies on vaccines against *Echinococcus* and *T. solium* cysticercosis (Lightowlers et al. 1996; Lightowlers 2003). Interestingly, one candidate antigen is paramyosin, also used in vaccine studies with schistosomes and filarial nematodes (Vazquez-Talavera et al. 2001). The most important practical application of immunological studies at present is the development of improved immunodiagnosis; larval tapeworm infections being difficult to diagnose definitively, even with modern scanning techniques.

In contrast to our current knowledge about immunity to intestinal nematodes, relatively little is known about responses to intestinal tapeworms. The human species (*Taenia*, *Diphyllobothrium*) appear to generate little or no protective immunity, although hosts do make immune responses (see Intestinal tapeworms). There is a considerable body of work using hymenolepid tapeworms in experimental rodents and this has given a significant body of data (Andreassen et al. 1999). Many aspects of the intestinal response in these models (e.g. mastocytosis, goblet cell hyperplasia, eosinophil infiltration, IgE) resemble those seen in nematodes (Ishih and Uchikawa 2000; Starke and Oaks 2001), but in the rat (the natural host) these are not linked to worm loss. In the mouse, this parasite is rejected and recent work shows that the mechanisms involved, as with nematodes in mice, are Th2-dependent (McKay and Khan 2003).

RESPONSES TO PARASITES MAY INTERACT WITH OTHER DISEASES

The powerful immune responses induced by parasites may be expected to influence the susceptibility of infected individuals to other diseases. It has been known for many years that the presence of one parasite may alter responses to a second, but in recent years there has been increased interest in broader aspects of parasite–disease interactions, in part triggered by the hygiene hypothesis. This states that early exposure to the antigens of infectious agents and environmental organisms can affect the balance of Th cell responses made subsequently, and thus influence susceptibility to allergic and autoimmune diseases (Rook 2000). Particular attention has been paid to the possible consequences of infections with intestinal helminths because of the ability of such infections to polarize towards Th2-dominated responses. It has been proposed that, because these worms are widespread in children in the developing world, they may be an important factor underlying the reduced prevalence of autoimmune and (paradoxically) allergic diseases in these regions (Cooper 2002) and there is some experimental evidence to

support this (e.g. Bashir et al. 2002). However, the position regarding the influence of parasites on autoimmune disease seem less clear cut (Agersberg et al. 2001). Evidence also suggests that infections with other worms such as schistosomes and *Onchocerca* can modulate responses to mycobacteria, responses to which are a powerful component of the hygiene hypothesis (Sewell et al. 2002; Stewart et al. 1999). Experimental data suggest that the strong Th2-biased responses associated with intestinal worms may alter susceptibility to autoimmune inflammatory diseases such as colitis and Crohn's disease (Elliot et al. 2000; Khan et al. 2002).

Worm infections have been associated with reduced responses to vaccines, although this may be more a reflection of an immune suppression (Cooper et al. 2001). They have also been linked to increased susceptibility to HIV and human T-cell lymphotropic virus (HTLV) infection, although the evidence here is sometimes contradictory (Ayash-Rashkovsky et al. 2002; Satoh et al. 2002; Wolday et al. 2002). A reciprocal effect of HIV infection and increased susceptibility to reinfection with *Schistosoma mansoni* has been described (Karanja et al. 2002). Recently, there has been interest in the interactions between intestinal worm infections and malaria. In endemic areas, where humans are commonly co-infected, epidemiological evidence has proposed both an increased incidence of *P. falciparum* and protection against cerebral malaria (Nacher et al. 2001, 2002).

The immune responses to infection with protozoa also influence infections with other infectious agents including viruses, bacteria, helminth worms, and other protozoa. There are numerous examples from experimental infections (see Cox 2001) but there is very little direct evidence that these interactions are important in humans, largely because of the difficulties inherent in carrying out the necessary definitive studies. The best-known example is Burkitt's lymphoma; the causative agent of this childhood lymphoma, the Epstein–Barr virus, is common all over the world but only causes tumors in areas where malaria is endemic (De The 1985) presumably as a consequence of malaria-induced immunodepression. There have been sporadic reports of enhanced bacterial infections in children infected with malaria (Walsh et al. 2000) and it has been known for some time that it is difficult to vaccinate children with malaria against tetanus, typhoid, or bacterial meningitis (Williamson and Greenwood, 1978). Acquired and innate immunodeficiencies also have an adverse outcome on infections with parasitic protozoa and the interactions between HIV and protozoa have been extensively studied. HIV infections are associated with enhanced *Toxoplasma gondii*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Isospora belli*, *Leishmania* spp., and *Trypanosoma cruzi* infections (see Ambroise-Thomas 2001). In all these cases, the parasitic infection is enhanced, and may even be fatal, in individuals infected with HIV. The most intensively studied interaction is the HIV/*Leishmania* combination, in which both organisms parasitize macrophages: HIV enhances the protozoan infection and vice versa, and the actual outcome of the dual infection is determined by a complex and changing balance between a number of different cytokines (Wolday et al. 1999). Interestingly there is very little evidence that HIV infections are enhanced in individuals infected with malaria although the reverse does seem to occur (Chandramohan and Greenwood 1998).

Our understanding of the Th1/Th2 dichotomy that has run as a thread throughout this chapter has opened up new prospects for the control of parasitic infections but it is clear that much more needs to be known about what happens in humans, particularly in those co-infected with another infectious agent, before any immunologically based preventative or therapeutic measures can be implemented.

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Control of parasites, parasitic infections, and parasitic diseases

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INTRODUCTION AND CONTEXT

Since the publication of the 9th edition of Topley and Wilson in 1998, the landscape of global health has changed considerably. These changes have been driven by a series of initiatives that address broader development issues. These initiatives are as listed below.

1 Changed political awareness of international health issues with an increasing political commitment to poverty alleviation as a core component of agreed International Development Targets and Millennium Development Goals.
2 Changes in international organization policy and focus generated by changed leadership. In particular, the initiatives of the World Health Organization (WHO) in recognizing the need for public–private partnerships for health development; in defining priorities; and in launching initiatives such as Roll Back Malaria and the Tobacco-Free Initiative.
3 The establishment of the Global Fund for human immunodeficiency virus (HIV)/acquired immune deficieny syndrome (AIDS), tuberculosis (TB) and malaria following the initiative of the UN Secretary General. There is awareness that with the relentless problems of these diseases, even a fund of approximately US$2 billion will have limited impact since the estimated needs of HIV/AIDS, TB, and malaria come to an annual figure of c. US$10 - billion.
4 The new approach to donor-funding of health in poor countries where sector-wide approaches to financing (SWAP) (Cassels 1997) have been developed to enable a more coordinated approach to financing. This prevents donors from influencing, via special projects, the overall national health policy and plans. This so-called 'basket' funding recognizes that ownership rests with the country; that donors all contribute to the 'basket'; that, once committed, control of resources is lost; that priorities are established through policy dialogue; that partnership relations are strengthened. The SWAPs approach is also layered onto the increasing decentralization of national budgets to district-level management in many countries. However, the approaches of SWAPs to discourage project-specific or disease-specific funding appears at odds with the approaches of the Global Fund where disease-specific projects and programs are applied for.
5 Over recent years, large international nongovernmental development organizations (NGDO) have become increasingly active in disease control implementation and policy. Médecins Sans Frontières (MSF), Save the Children Fund (SCF), and Oxfam have been vociferous on issues of equity and access to drugs and in criticizing 'vertical' programs and drug donation programs. Notwithstanding policy papers introduced by NGDOs, MSF has advocated the establishment of a drugs for neglected disease initiative which, while necessary and laudable, must recog-

nize that in many circumstances such drugs require delivery through disease-specific methodologies and disease control is of necessity through approaches that are antipathetic to perceived policy wisdom of SWAPs and decentralization (e.g. trypanosomiasis or leishmaniasis).

6 There has been increasing recognition that infectious diseases are more prevalent and inflict a greater burden of disease on the poorest quintile of the population. The poorest 20 percent would benefit proportionately more if there was a pro-poor focus in tackling infectious diseases compared with other health interventions (Gwatkin et al. 1999).

7 There has been, in parallel with the recognition of the need for increased partnership, an expansion of public–private partnerships in health. These are summarized by Widdus (2001) who identifies some 70+ such initiatives. The diversity of objectives, financing, governance, and management of these alliances prevents any significant generalizations about best practice and how lessons can be learned as most of the alliances/partnerships are disease- or intervention-specific. The nature of some of these partnerships has been examined in more detail by Reich (2002), where some of the different models and experiences are discussed. Many of these have been developed over the past decade and have been funded by extensive long-term commitments from new donors, drug donations, long-term commitment from the private sector, and a recognition that relatively cheap interventions sustained by health systems can bring long-term health benefits. The establishment of the Bill and Melinda Gates Foundation as a key player in global health has greatly enhanced the opportunities for both research and intervention in parasitic infections. This Foundation, recognizing the importance of alliances that were embedded in its approach to program support, commissioned the study 'Building *Successful* Global Health Alliances', which outlines the parameters, criteria, and experiences of such alliances and creates a template through which to measure success (McKinsey 2002).

8 The introduction in 1993, following the World Development Report (World Bank 1993), of the concept of global burden of disease has been further expanded in a series of publications. Such data has enabled an assessment of likely change in global disease burden between 1990 and 2020. These projections suggest that as a proportion of global disease burden, only malaria remains a significant burden as a parasitic infection. Malaria falls in global burden importance from a ranking of 11th to a predicted 26th over this period. The majority of the projected changes in global burden are in the increased burden of cerebrovascular events, depressive illness, conflict-related conditions, road traffic accidents, and cancers. The disability-adjusted life years (DALY) burden of parasitic disease is projected to remain largely stable while the surge of noncommunicable disease burden due to the epidemiological transition, diet, and lifestyle change associated with urbanization, substance abuse, environmental degradation, population growth, and increased conflict are projected to be proportionally greater contributions. The DALY burden and overall public health importances of major parasitic disease are shown in Table 1.

9 The impact of nonhealth-sector roles, their financing and policies (e.g. education, agriculture, transport, natural resources) within government will have significant impact on health outcomes. Of particular importance will be levels of investment and achievement of education targets in an attempt to provide universal primary education by 2015; the importance of increasing the proportion of females in primary education will be of particular significance in achieving targets of improving maternal, child, and infant mortality figures. The complexities of the interactions and interrelationships are summarized in Figure 1

10 The interest in and, indeed, fear of emerging diseases (defined as either new infections of humans or re-emerging infections (where a rapid increase in incidence is seen of an existing infection or in a new geographical area) has been an important element in debate on health policy as epidemics of West Nile fever, Lyme disease, and Hantavirus have been recognized in the USA. Considerable additional resources have become available for research on such emerging agents. Recent quantitative analysis of the risk of emergence allied to the nature of the organism's mode of transmission and source have been provided by Taylor et al. (2001). They note that viruses, bacteria, and protozoa are more likely to emerge than macroparasites (e.g. helminths); that c. 75 percent of emergent organisms are from zoonotic sources and that emergences are independent of the mode of transmission. It should be noted, however, that despite the emphasis in some circles of the importance of such agents, they are not predicted to play a significant role in the global burden of disease estimates as a proportion of global DALYs. Such conclusions are based on the current definition of species. However, the capacity now exists to identify species complexes/groups and a level of intra-species variation is becoming apparent as a result of molecular analyses. The new discipline molecular epidemiology, which has been applied to vectors and causative parasites, clearly suggests that the absolute numbers of genetically distinct parasites and vectors, irrespective of subspecific variation, is much greater than hitherto recognized, emphasizing both the degree of biodiversity in microorganisms and its importance in the strategies of parasite and vector control.

Table 1 *Overview of public health importance of parasitic infection (see also Remme et al. 2002)*

Disease / Parasite	Population at risk (millions)	No. of endemic countries	No. of infected (millions)	Estimated deaths (humans × 1000)	DALYs female	DALYs male	Total DALYs
Malaria	2 000	90	300–500	1 080	182.3	17.5	357.3
Leishmaniasis	350	82	12	41	12	8.6	20.6
Lymphatic filariasis	750	65	119	No direct mortality	5.6	2.9	7.5
Guinea worm disease	140	18	c. 0.12	No direct mortality			
Onchocerciasis	122	34	17.6	No direct mortality	3.7	2.7	6.4
African trypanosomiasis	50	36	0.02–0.30	50	9	8.8	17.8
Chagas disease	90	19	16	21	14.8	12.6	27.4
Schistosomiasis	500–600	74	200	11	29.9	15.4	45.3
Ascaris infection			1 000		53.8	51.4	105.2
Trichuris infection			900		32.2	30.9	63.1
Hookworm infection			500		5.8	5.6	11.4
Entamoeba infection			500	40–100			
Giardiasis			200				
Taeniasis	40		15				
Neurocystocercosis			50	50			
Food-borne trematodes			500				
Fascioliasis	180.25	8	2.39				
Clonorchiasis	289.26	6	7				
Opisthorchiasis	63.60	5	10.30				
Paragonimiasis	194.80	5	20.60				
Other intestinal flukes		6	1.28				

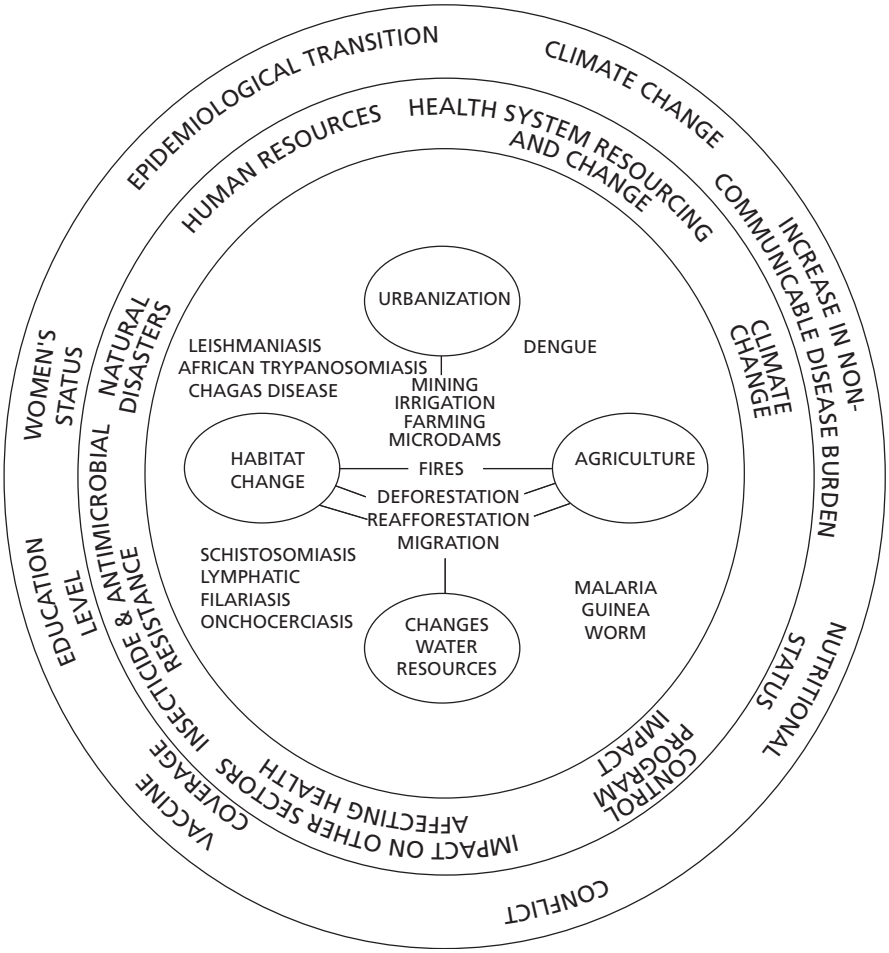

Figure 1 *Relationships and interactions between sectors intervention, environment, and vector-borne infection (Molyneux 2001)*

11 The suggestions that global climate change will have a widespread impact on health as mean temperatures rise over the next decades have provoked studies on the projected change in distribution of vector-borne infections. It is generally agreed (from different climate models) that the mean rise of temperature over the next 100 years will be of the order of 2–4°C. The impact of these changes, particularly on the distribution of *Plasmodium falciparum* malaria has been projected by various groups (IPCC 2001; Rogers and Randolph 2000; Hay et al. 2002; Patz et al. 2002) although little consensus is available (Patz et al., 2002). In addition, the role of El-Niño events has been studied and identified with changes in epidemic patterns in different regions of several vector-borne infections (dengue in Indonesia, malaria in most of Africa, Colombia, and India).

12 In many countries, the resources allocated to health remain disproportionately small compared with other sectors. The recent Macroeconomics and Health Report (WHO 2001) has made a significant contribution to the recognition that investment in health is critical to furtherance of human development and that significant returns on investment in health are obtained in the development process. Ill health is both a cause and consequence of poverty.

13 Civil unrest and disturbances, conflict and consequent migration, social disruption, and disruption of infrastructure including health services have had profound consequences for many populations over the last decade, particularly when associated with the end of the cold war. The impact of these events is summarized by Murray et al. (2002) and Supplement 1 to *Lancet* **360** (December 2002). The study of global burden of disease (Murray and Lopez 1996) suggests that the trend in disease burden associated with conflict will continue to rise to 2020. Molyneux (1997) summarized the impact of conflict on parasite infections; Table 2 updates available information on parasite infections in relation to conflict.

Epidemics may also be provoked by ecological, climatic, and environmental change; urbanization; human population movement resulting from civil unrest and conflict; reduced surveillance; and drug or insecticide resistance. Policymakers recommend that health systems are restructured to include a generalized 'horizontal' pattern of healthcare, insurance systems, and

Table 2 *Conflict related change in parasitic diseases*

Disease	Organism/location	Change
African trypanosomiasis (sleeping sickness)	*Trypanosoma brucei gambiense*	Epidemics in Democratic Republic of Congo (DRC) and Angloa over recent decades, associated with destruction/disruption of health services (Ekwanzala et al. 1996). Epidemics spread in north-western Uganda following conflict-related migration from Sudan.
	T. b. rhodesiense	Disruption of cotton and coffee production in Busoga during the Amin regime resulted in spread of *Lantana*, providing breeding sites for *Glossina fuscipes* and initiating transmission of acute sleeping sickness in peri-domestic environments, with cattle acting as reservoir hosts; restocking following cattle raiding induced epidemics following importations of infected cattle.
Visceral leishmaniasis	*Leishmania donovani*	Epidemics in southern Sudan: a changed ecological situation associated with an increase in *Phlebotomus orientalis* populations in maturing *Acacia / Balanites* woodland initially provoked the epidemics, which were left largely uncontrolled because of civil war, migration of infected populations, scarcity of treatment centers, and availability of drugs (Ashford and Thomson 1991; Seaman et al. 1992)
Cutaneous leishmaniasis	*Leishmania tropic*	Resurgence of *L. tropica* in Afghanistan (Kabul) following an increase in urban population density of non-immunes because of conflict (Ashford et al. 1992).
	Leishmania major	Movement of populations to Khartoum because of conflict and drought, and establishment of transmission among peri-urban reservoir of non-immunes living in shanties.
Malaria	Refugee camps in Africa	Refugee populations settled at lower altitude, in sites with relatively high rainfall; refugees with inadequate immunity or exposed to different strains of *Plasmodium falciparum*; absence of drugs and no control of *Anopheles gambiae*.
	Refugee camps in Afghanistan and Pakistan	Malaria epidemics in camps (controlled by spraying tents to control *A. stephensi* and *A. culicifacies*), exacerbated by increase in prevalence of *Plasmodium falciparum*.
	Cambodia	Mass deportation of urban non-immunes to forced labor in rice fields and forests; conscription for construction of defenses in border areas where multi-drug-resistant malaria commonly occurs.
Onchocerciasis	Sierra Leone	Weekly aerial larviciding against *Simulium* suspended because of security problems and local conflict. Levels of transmission consequently increased.
	Guinea Bissau, Sudan, Sierra Leone	Civil unrest prevented distribution of ivermectin with consequent resurgence in incidence.
	DRC, Central African Republic	Suspension or reduction of ivermectin distribution because of conflict.
	Liberia and Angola	Planning of program for community-directed distribution of ivermectin was retarded by collapse of national structures; remaining (passive) distribution by nongovernmental donors.
Dracunculiasis	Ghana	Increase in reported cases 1 year after local conflict failed to contain cases from previous year.
	Sudan	Civil war prevents adequate case-finding, case-containment, water-supply control, and filter distribution.

user charges, and decentralization of management to district level (or equivalent). Such restructuring reduces the ability of the system to respond to factors that lead to epidemics. It must be borne in mind that so called 'vertical' parasitic disease control activities (e.g. onchocerciasis control and the lymphatic filariasis, Chagas' disease, and Guinea worm eradication programs) have been remarkably successful, onchocerciasis control being judged particularly cost effective (Benton and Skinner 1990). Because of the complexity of the biological systems inherent in parasitic infections, particularly those that are vector-borne, such diseases are not easily amenable to control by a strictly 'horizontal' health system approach.

This chapter draws together information on the control of parasites, parasitic infections, and the diseases caused by parasites. The title distinguishes between the control of an organism (parasite, vector or ectoparasite) at the level of the individual and at the level of the community. An example of the paradox (individual vs. community) is the conflict between the treatment of individual patients compared with the need to control or even eradicate disease. It is important to emphasize these distinctions as they are frequently ignored by

policy makers, health workers, and scientists seeking to develop new approaches and tools for control.

The changing health environment has already been emphasized, but it is important to recognize that approaches to control are dependent on an accurate knowledge of the problem. This requires biological, medical, and epidemiological inputs to define the etiology (causative organisms), the vectors (if vector-borne), the parameters and mode of transmission (vector-borne, water-borne, aerosol, oro–fecal, venereal). Systems of surveillance, monitoring and evaluation are required to define prevalence and trends of infection and disease. Without such fundamental information, a control strategy cannot be appropriately designed and implemented. Biological information needs to be supplemented by consideration of issues such as:

- logistics
- cost-effectiveness
- potential for integration within existing programs
- past successes or failures
- input from governmental sectors other than health (e.g. agriculture, forestry, education, water, other natural resources, wildlife)
- acceptability of an intervention to the target communities
- potential for ecological damage
- priority rating afforded by the Ministry of Health (MOH)
- availability of human resources for implementation
- potential of research to provide improved products within a particular timescale.

The overall framework of approaches towards parasitic disease control is summarized in Figure 2.

CONCEPTS OF CONTROL AND ERADICATION

A distinction must be maintained between the terms 'control' and 'eradication'; the latter is often used inappropriately and it should be employed with caution. In 1988, the International Task Force for Disease Eradication (ITFDE) was formed to evaluate systematically the potential for eradication of candidate diseases and to identify specific barriers to eradication. The criteria used to asses the feasibility of eradication are provided in

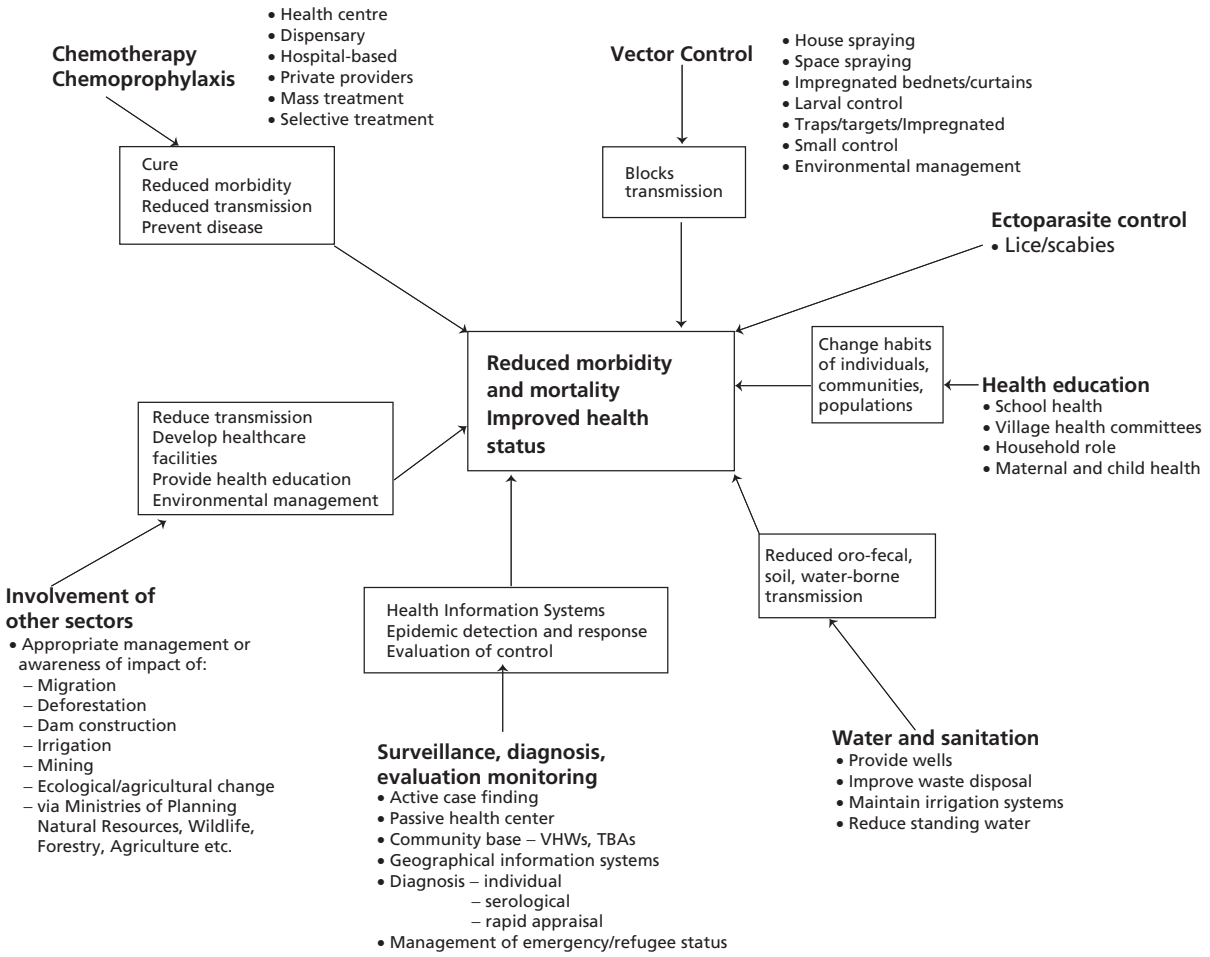

Figure 2 *Framework for the control of parasitic diseases*

Table 3 *Criteria for assessing eradicability of diseases or conditions (Dowdle and Hopkins 1998)*

General category	Specific criteria	Examples
Scientific feasibility	Epidemiologic vulnerability	Existence of nonhuman reservoir; ease of spread; natural cyclical decline in prevalence; naturally induced immunity; ease of diagnosis; and duration of any relapse potential.
	Effective, practical intervention available	Vaccine or other primary preventive, curative treatment, and means of eliminating vector. Ideally, intervention should be effective, safe, inexpensive, long lasting, and easily deployed.
	Demonstrated feasibility of elimination	Documented elimination from island or other geographic unit.
Political will / Popular support	Perceived burden of the disease	Extent, deaths, other effects; true burden may not be perceived; the reverse of benefits expected to accrue from eradication; relevance to rich and poor countries.
	Expected cost of elimination or eradication	Especially in relation to perceived burden from the disease.
	Synergy of eradication efforts with other interventions	Potential for added benefits or savings or spin off effects.
	Necessity for elimination rather than control.	

Table 3. The ITFDE has recently been reconstituted to evaluate the present situation as new developments have taken place over the last decade. The ITFDE defined eradication as 'reduction of the worldwide incidence of a disease to zero as a result of deliberate efforts obviating the necessity for further control measures.' The original ITFDE reviewed more than 90 diseases, 30 of them in depth, and concluded that dracunculiasis, rubella, poliomyelitis, mumps, lymphatic filariasis, and cysticercosis could probably be eradicated using existing technology. The term 'elimination' is increasingly being used to replace the term 'eradication,' which should be only used in a global context. The Dahlem conference held in Berlin in 1997 (Dowdle and Hopkins 1998; WHO 1998) also considered these issues in some detail and introduced the term 'extinction' to classify an organism that no longer exists (in contrast to the causative organism of smallpox, which has been eradicated as a cause of disease but still exists in stocks retained in secure laboratories). 'Elimination' is now regarded as meaning removal of an organism from a defined geographical region (local eradication) which creates problems for quantification of achievement towards the goal. The accepted position is that the disease is not eradicated but no longer requires ongoing investment in control and is maintained at a level where the problem is no longer a significant health burden. A new concept, 'elimination of a disease as a public health problem' has been introduced through World Assembly Resolutions. The definitions used in this chapter are from Dowdle and Hopkins (1998) and are as follows:

- Control: Reduction of disease incidence, prevalence, morbidity or mortality to a locally acceptable level as a result of deliberate efforts; continued intervention measures are required to maintain the reduction.
- Elimination of disease: Reduction to zero of the incidence of a specified disease in a defined geographical area as a result of deliberate efforts; continued intervention measures are required.
- Elimination of infection: Reduction to zero of the incidence of infection caused by a specified agent in a defined geographical area as a result of deliberate efforts; continued measures to prevent re-establishment of transmission are required.
- Eradication: Permanent reduction to zero of the worldwide incidence of infection caused by a specific agent as a result of deliberate efforts; intervention measures are no longer needed.
- Extinction: The specific infectious agent no longer exists in nature or the laboratory

The classic eradication program was that of smallpox, which achieved its target in 1977. To date, no parasitic disease has been eradicated, although attempts to eradicate Guinea worm are underway, through the program Global 2000 (Hopkins et al. 2002). Nevertheless, successful local eradication (correctly, elimination) has been achieved in some restricted geographical or epidemiological situations:

- onchocerciasis has been eliminated from several parts of Kenya and from the Nile at Jinja in Uganda by using the insecticide dichlorodiphenyltrichloroethane (DDT) to remove the local vectors (*Simulium neavei* and *S. damnosum*, respectively)
- the Onchocerciasis Control Programme (OCP) in West Africa has achieved the same goal by targeting particular cytoforms of the *S. damnosum* complex using aerial application of insecticides.

Local elimination has also been achieved as detailed below:

- the malaria vector *Anopheles gambiae* from Brazil in the late 1930s by Soper using larviciding measures and house spraying with pyrethrum, a success repeated in the early 1940s after the same species had been introduced into Egypt
- *Glossina palpalis*, the tsetse fly, the vector of human trypanosomiasis was eliminated from the island of Principe in 1905 by trapping flies using sticky back packs on plantation workers
- animal trypanosomiasis has been eliminated from parts of North East Nigeria by ground spraying of tsetse resting sites with persistent doses of DDT
- *Aedes aegypti*, the vector of yellow fever, has been eliminated from parts of Central and South America
- local antimosquito spraying has eliminated lymphatic filariasis from the Solomon Islands with no evidence of any resurgence over a 20-year period
- filariasis due to *Brugia malayi* has been eliminated from Sri Lanka through selective treatment with the drug diethylcarbamazine (DEC), antilarval measures (removal of host plants by herbiciding) and environmental improvements
- chemotherapeutic approaches have eliminated filariasis due to *Wuchereria bancrofti* from Japan, South Korea, and Taiwan in Asia, and Suriname and Trinidad and Tobago in the Americas (WHO 1994)
- filariasis has also been eliminated as a public health problem in large areas of China
- a long-term 'elimination' program has been successful against hydatid disease in Iceland
- malaria was eliminated from Sardinia by DDT spraying as well as in other marginal areas of distribution.

One noticeable feature of these successes is that many examples refer to islands or isolated populations or areas where the parasite is at the edge of its geographical range. Clearly, the advantages of isolation and a greater ability to control animal or human population movements are important. Elimination or global eradication of any disease is difficult to achieve and costs increase per case detected, controlled or averted as the end-point is reached.

However, the high cost of eradication or local elimination programs may be justified as these costs are time limited, whereas disease control implies a long-term commitment. Any control program must be cost-effective and should reduce the target disease to a level at which costs are sustainable by the local community (or by public or private healthcare systems). Control seeks to bring the problems to a level at which the disease is no longer of public health importance with morbidity at an acceptable level within the community, an absence of mortality and, if appropriate, greatly reduced levels of disability. To translate the level of control achieved to eradication or elimination status requires *either* a vastly increased cost per case treated or prevented which, for financial and ecological reasons, may never be feasible, *or* the development of a more effective intervention.

PUBLIC–PRIVATE PARTNERSHIPS IN DISEASE CONTROL

There have been significant developments of public–pri-private partnerships (PPP) for disease control over recent years. These have, in part, been generated by the new policies of the Director-General of WHO, Dr. Gro-Harlem Brundtland, following her election in 1998 but also by the recognition that such partnerships represent a response to the changing environment in international health and that no single organization can undertake or be responsible for all aspects of health and its delivery. Recent PPPs in parasite control include:

- African Programme for Onchocerciasis Control (APOC)
- OCP (1974–2002)
- Onchocerciasis Elimination Programme in the Americas (OEPA)
- Roll Back Malaria
- Multilateral Initiative on Malaria
- Medicines for Malaria Venture
- Global Allliance to Eliminate Lymphatic Filariasis
- Global School Health Initiative
- Partnership for Parasite Control
- Global Alliance for African Human Trypanosomiasis
- Guinea Worm Eradication Programme (also known as 'Global 2000').

Each of these PPPs has different characteristics (e.g. governance, financing, time frame, links with the private sector) and different disease-specific objectives. Widdus (2001), Buse and Walt (2000a, b), Reich (2002) and Buse and Waxman (2001) provide a detailed analysis of the underlying principles of these alliances as well as case studies of functionality.

COMPONENTS OF CONTROL

Components of control are listed under the following headings:

- Situation analysis
- Definition of objectives and strategy
- Options and responsibilities at different levels of health system
- Planning and resourcing
- Evaluation and monitoring
- Implementation and integration of selected methods of control.

Situation analysis

- Perform desk study of published and unpublished reports to assess problems in the context of country, region, and district.

- Acquire information on prevalence and incidence.
- Appraise the validity of information.
- Evaluate current epidemiological situation by passive surveillance at health centers or by questionnaires (e.g. using the postal system).
- Observe changes over time and predict future change.
- Define the structure of health services and their existing capacity, human resources available, and needs for training and capacity building.
- Establish the priority afforded to the disease by the government, the MOH, the district management teams, and the communities.
- Establish links to other sectors or organizations in planning for control (e.g. other ministries, development organizations, nongovernmental organizations (NGO).
- Assess the influence of other activities such as development projects on planned programs.
- Carry out spot surveillance of local prevalence, vectors and, if applicable, animal reservoirs.
- Use rapid assessment methodologies (e.g. for schistosomiasis, onchocerciasis, filariasis, or loiasis).
- Assess the available methods for prediction of epidemics using remote sensing or climate prediction available to other sectors (e.g. agriculture, natural resources, environment).
- Establish a national task force composed of various stakeholder groups to address the problem.

Definition of objectives and strategy/options and responsibilities at different levels of health system

- Analyse cost-effectiveness of different control approaches and options.
- Select appropriate methodology and define control requirements.
- Establish an inventory of personnel and facilities (including estimation of training needs and requirements for equipment and drugs).
- Establish feasibility in the context of other health needs.
- Contrast epidemic ('firefighting') problems for which vertical, rapid intervention is necessary with endemic situations for which a long-term approach and integration are required (Table 4).
- Establish emergency response capacity to address predicted epidemic risk.

Planning and resourcing

- Define the expected contribution from the government.
- Develop national plan.
- Evaluate targeted approaches to donors in the context of donor priorities and prevailing national policy.
- Define appropriate timeframes for implementation of plans.

Table 4 *Role of different levels of the health system in parasitic disease control*

Level	Role
Community	Identification of suspects/patients
	Follow-up of patients
	Coordination of any appropriate vector-control activities (e.g. bednet distribution to vulnerable groups/reimpregnation)
	Facilitation of cooperation, local logistics
	Communication by village health committees
District	Passive detection and treatment
	Parasitological/serological diagnosis
	Treatment and minimum clinical care
	Follow-up of microscopy
Regional	Active surveillance
	Confirmatory diagnosis
	Data collection
	Technical supervision of vector control
	Distribution of reagents and materials for vector control
Ministry/country level	Situation analysis/policy position
	National strategy and plan
	Establish stakeholder group/national task force
	Financing
	Training needs and responsibility
	Health education
	Distribution of technical information, equipment, drugs, materials
	Purchase of equipment and supplies
	Human resource management

- Define the relationship of the action to overall health plans and budgets.
- Establish links with appropriate international reference centers for technical support; control of an epidemic may merit application for emergency status to provide rapid funding (e.g. requests for therapeutic drugs and insecticides from international aid agencies and NGDOs).
- Establish drug supply line following identification of sources; initiate quality assurance mechanisms; define tax status of drugs (e.g. donated products).
- Define role of the nongovernment sector (e.g. private providers, NGOs) in control policy.
- Ensure adequate information exchange about control policy between different bodies and individuals involved.
- Undertake knowledge, attitudes, and practice (KAP) studies as a basis to inform approaches to social mobilization strategies.
- Train personnel (including management) through courses, instruction of trainers, educational materials, and health education programs.
- Assess community acceptability and the perceived priority of any involvement that will require resource input from the communities (e.g. role and views of village health workers (VHW), volunteers, traditional birth attendants (TBA), community leaders, school teachers).
- Define the management structure of the program and its relationship with existing management structures.
- Assess capacity available (managerial, financial, technical) and ensure capacity building is embedded in planning.

Evaluation and monitoring

- Assess progress towards objectives (prevalence distribution, vector status).
- Establish sentinel site / baseline data in defined units.
- Define appropriate methods for epidemiological evaluation, e.g. parasitological, serological, and vector sampling methods.
- Carry out longitudinal surveys or spot surveys at indicator villages.
- Adjust the program in the light of results.
- Establish process indicators at national and subnational level.

Examples of the value of assessing longitudinal trends in disease control are illustrated in Figures 3a–e, 4a, b, p. 14, and 5, p. 15. These show trends in parameters that measure the impact of implementation of selected methods of control on onchocerciasis, Chagas' disease, and Guinea worm either by measures that indicate change of incidence or by directly measuring vector populations and hence transmission since the program began.

Implementation and integration of selected methods of control

CHEMOTHERAPY AND CHEMOPROPHYLAXIS

- Assess the availability and quality of drugs and the distribution system.
- Establish relationship between national bodies, donation programs, and NGDO community to define operational relationships (e.g. onchocerciasis, lymphatic filariasis, African trypanosomiasis, schistosomiasis, trachoma programs).
- Assess or monitor drug resistance (e.g. East African network for antimalarial drug resistance).
- Assess the role of private providers and control of quality and price (e.g. malaria drug policy).
- Utilize other systems for distribution (e.g. schools, agricultural extension workers, other health or government workers, NGOs, committees).

VECTOR AND RESERVOIR CONTROL

- Assess availability, cost, and appropriateness of insecticides.
- Assess availability of skills to monitor insecticide resistance.
- Assess availability and effectiveness of alternative chemicals.
- Assess capacity for management of the control program.
- Establish relationships with other sectors to provide support for environmental control measures.
- Assess acceptability and feasibility of reservoir control.
- Assess environmental acceptability of interventions.
- Assess personal protection (e.g. bednets, sustainability of a bednet program/retreatment modalities).
- Establish policy in relation to bednet distribution (e.g. vulnerable groups, social marketing).
- Investigate opportunities for integration if appropriate (e.g. malaria and lymphatic filariasis in Africa; dengue and filariasis in the Pacific; leishmaniasis, Chagas' disease, and malaria via bednets in Latin America).

ENVIRONMENTAL MANAGEMENT

- Ensure effective linkages between health and other sectors.
- Assess potential impact on other diseases.

IMMUNOPROPHYLAXIS

- Assess availability of vaccines.
- Assess capacity to manage the program and efficacy of cold chain.

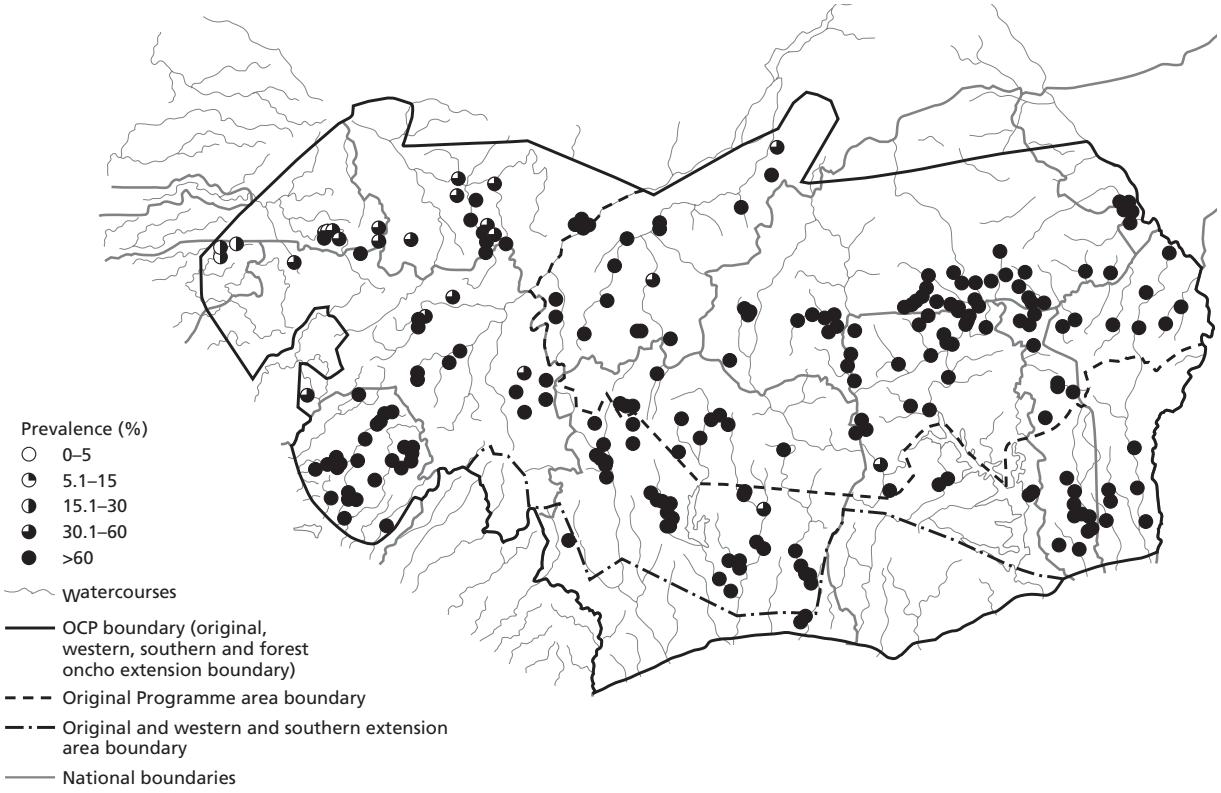

(a)

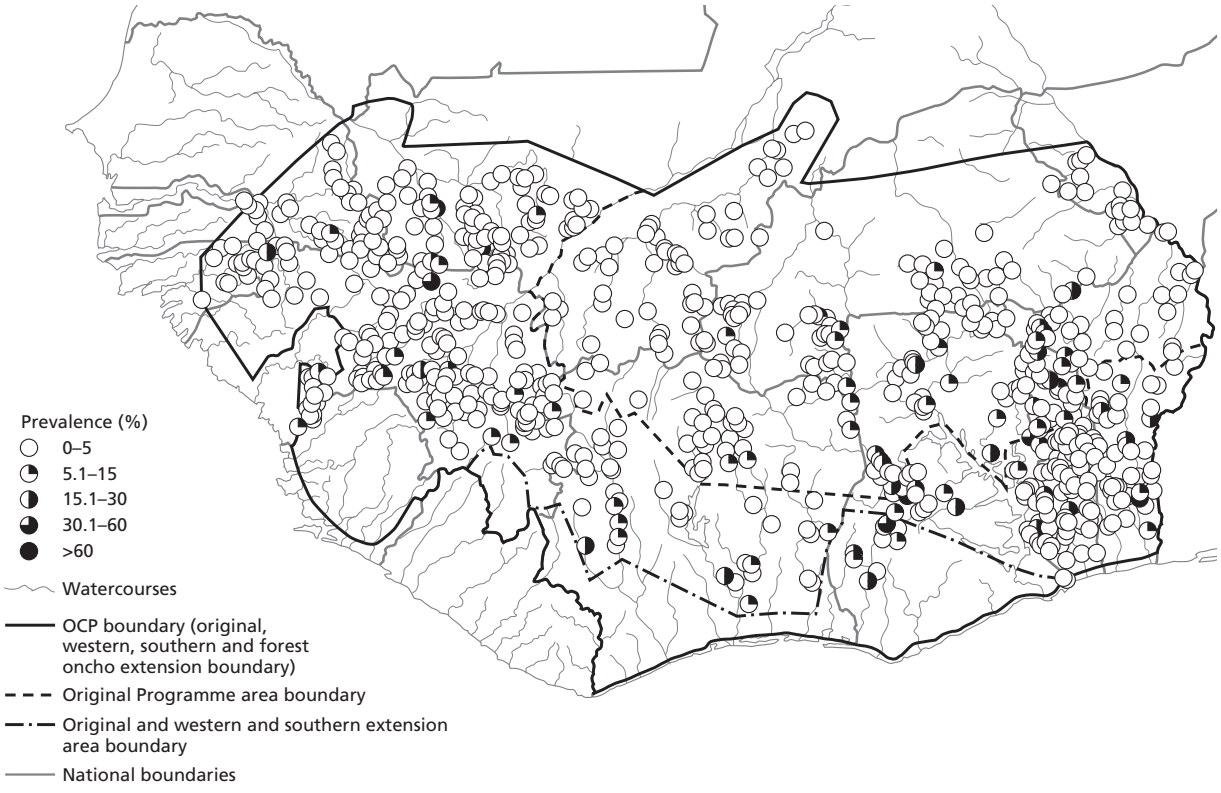

(b)

Figure 3 *Maps showing epidemiological changes in the prevalence and transmission of* Onchocerca volvulus *in countries of the OCP 1994–2001.* **(a)** *Epidemiological situation of villages evaluated before the control.* **(b)** *Prevalence of microfilariae in villages evaluated 2000–01 in OCP (Continued over)*

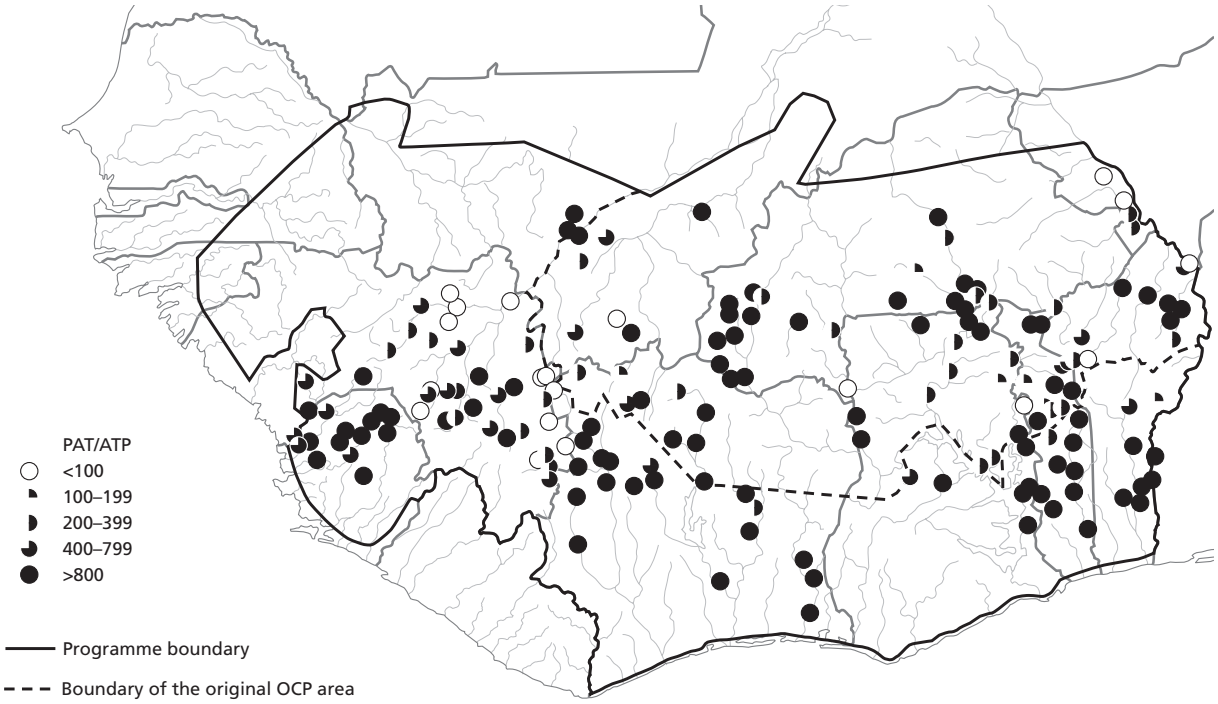

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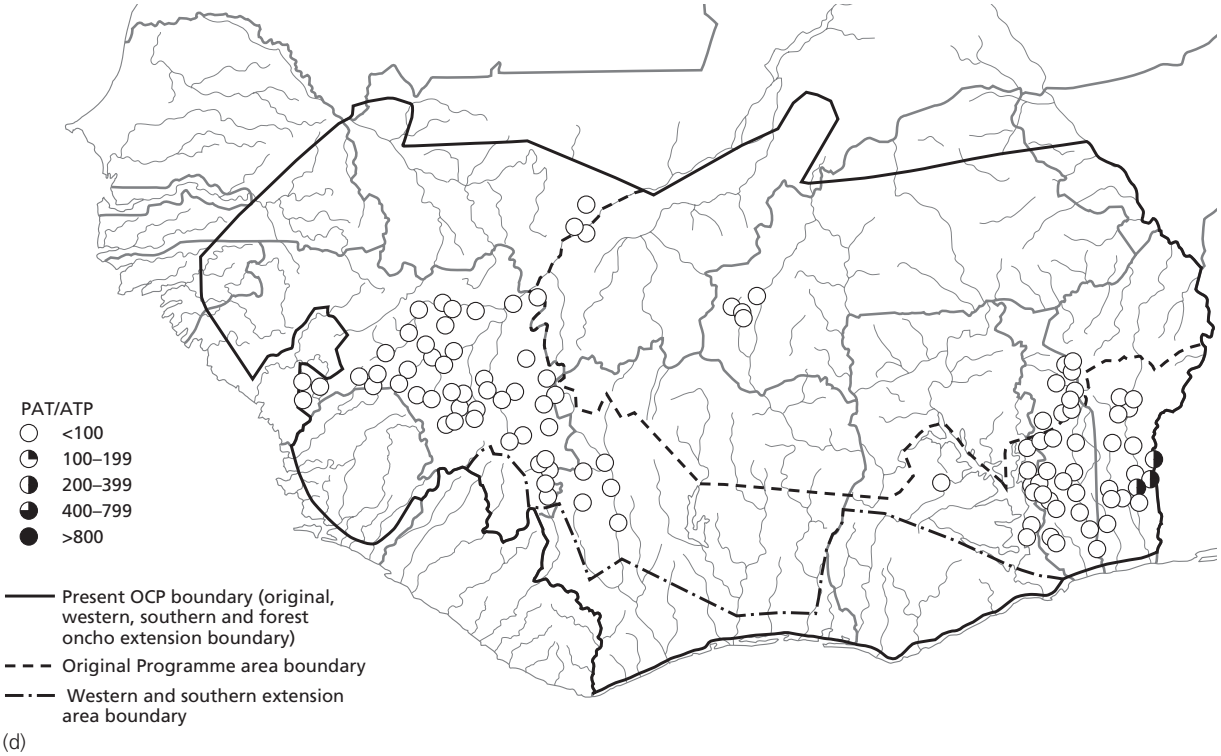

(d)

Figure 3 *Maps showing epidemiological changes in the prevalence and transmission of* Onchocerca volvulus *in countries of the OCP 1994–2001 (Continued).* **(c)** *Annual transmission potential of* Onchocerca volvulus *in West Africa, pre-control 1974.* **(d)** *Annual transmission potential of* Onchocerca volvulus *in West Africa, post-control 2001 (Continued over)*

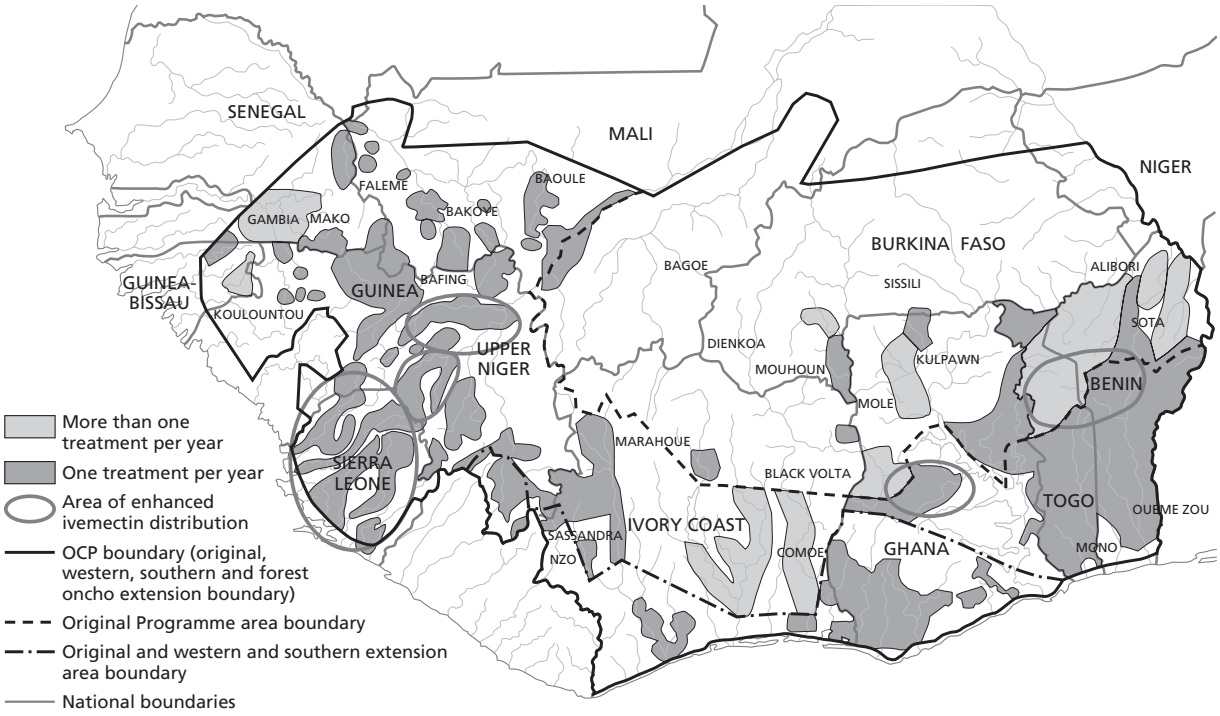

(e)

Figure 3 *Maps showing epidemiological changes in the prevalence and transmission of* Onchocerca volvulus *in countries of the OCP 1994–2001 (Continued).* **(e)** *Distribution of (ivermectin) in OCP area. Note the extensive distribution in areas formerly in extension areas with 'core area' free of onchocerciasis except in restricted area.*

- Establish links to existing programs (e.g. expanded program of immunization (EPI) to utilize the cold chain).

HEALTH EDUCATION / SOCIAL MOBILIZATION

- Use media resources, including radio, television, and video.
- Use posters and drama sessions oriented around the local environment and traditions.
- Ensure participation of teachers, local leaders, health workers, local medical practitioners, religious leaders.
- Ensure link to KAP and feedback to inform social mobilization strategy.

STRATIFICATION OF PARASITIC DISEASES IN RELATION TO ELIMINATION AND CONTROL

Control programs often involve specific approaches to arrest the transmission of infection (e.g. via vector control) or to prevent or cure a disease. Although such programs have been successful in the past, integrated approaches are now recognized as being more appropriate for reducing prevalence and incidence. This is important if the strategy is aimed at alleviation of a disease problem in a community or population rather than in an individual. Integrated control is based on coordinated planning and detailed knowledge from many different areas: scientific, technical, intersectoral, financing, and managerial. An approach termed 'stratification' has been used in malaria control; it means that the strategy is modified according to different epidemiological situations (WHO 1993a). Malaria stratification has been taken a step further by those with particular interests in different environments and geographical regions, a process known as 'micro-stratification' (Rubio-Palis and Zimmerman 1997). While stratification has been most widely discussed with reference to malaria (Table 5, p. 29), the process is equally applicable to other parasitic diseases: leishmaniasis (WHO 1990), onchocerciasis, filariasis (WHO 1992a), schistosomiasis, and African trypanosomiasis. Tables 5–10, p. 29–39 illustrate the concept of stratification in the planning of control in selected parasitic diseases.

APPROACHES TO CONTROL

Control of a parasitic infection can be focused on the individual, with a view to alleviating pain, reducing disability or avoiding death, while at the same time reducing the parasite load and transmission within a community. Such an approach will be less cost-effective than larger-scale control programs that employ methods such as vector control, reservoir-host control or mass drug distribution. Large-scale measures have a public health objective that, while reducing individual suffering, also reduces community morbidity and mortality. This

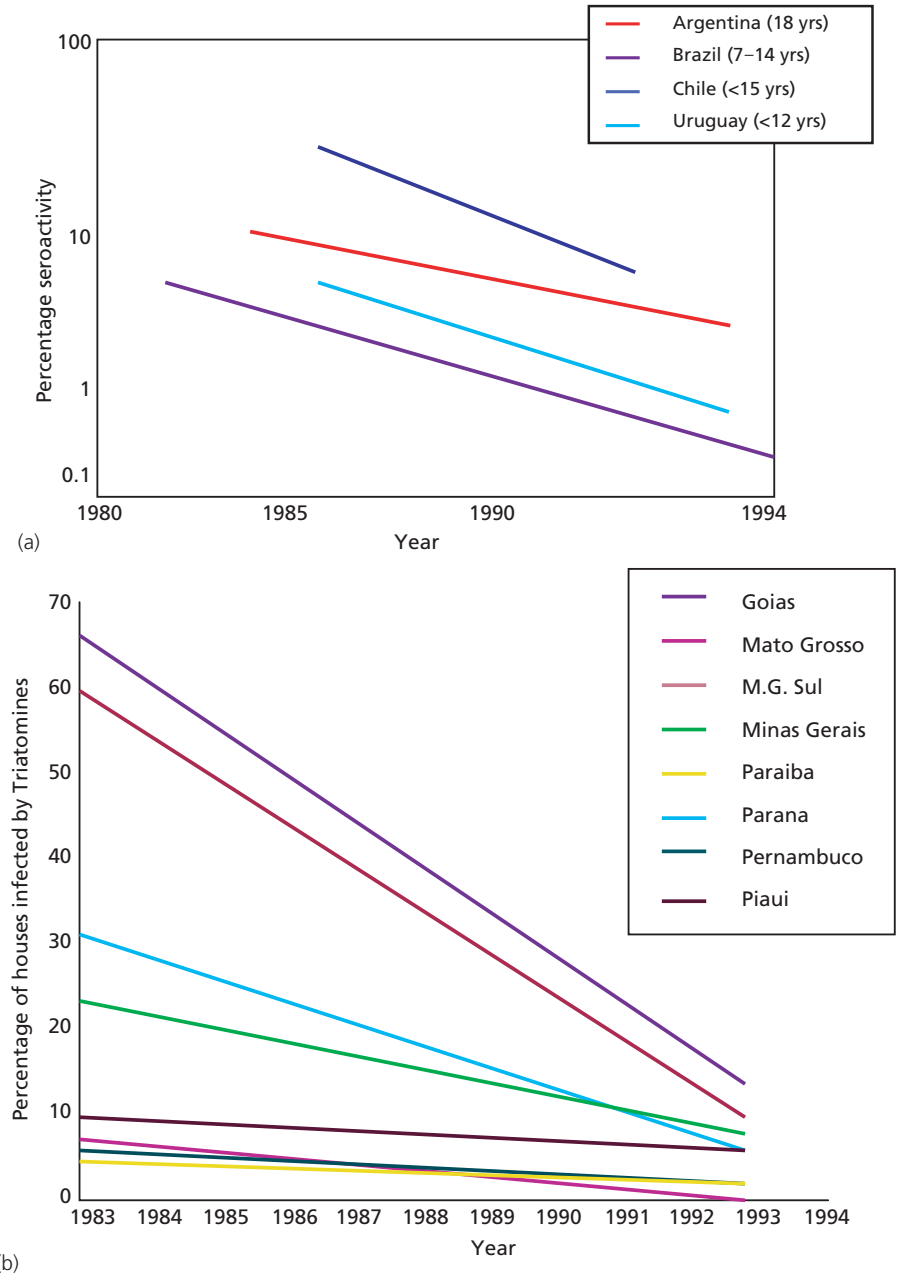

Figure 4 *Southern Cone Initiative.* **(a)** *Elimination of transmission: incidence of Chagas disease 1980–94 (Moncayo 1999).* **(b)** *Interruption of transmission: Brazil, endemic states 1983–93 (Gadelha 1993).*

provides socioeconomic benefits through improved agricultural productivity, improved cognitive function, better nutrition as a result of increased agricultural output of a more varied diet, and enhanced population mobility allowing for additional earning opportunities. Control of animal parasitic diseases also has benefits for human populations through increased protein availability and higher income from the sale of higher-quality livestock enhancing both local and national economies. Parasitic disease control programs vary in scale but they have generally been targeted at two different types of disease situation:

1. endemic disease in which long-term chronic infections have persisted in communities (e.g. onchocerciasis, hydatid disease (*Echinococcus* infection), schistosomiasis, Guinea worm (dracunculiasis), Chagas disease, and filariasis
2. epidemic disease where rapid intervention is required to prevent widespread morbidity and mortality.

Epidemics are frequently predictable but if health facilities are ill-equipped or nonexistent, high mortality may occur before control can be instigated. Recent examples of parasitic disease epidemics are described in Table 11, p. 40.

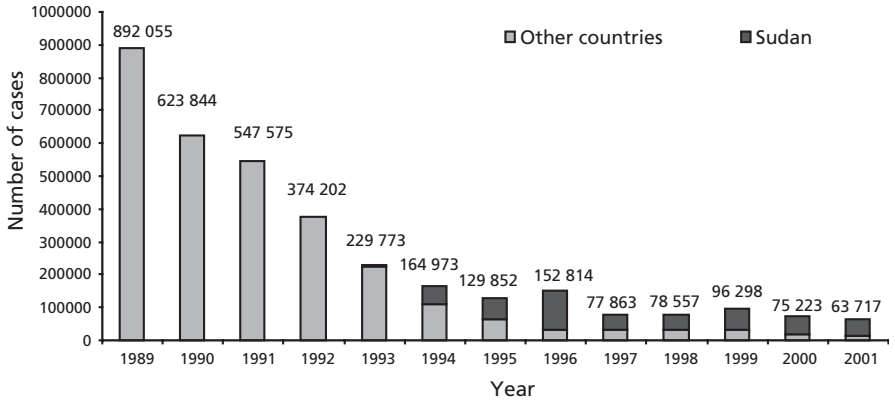

Figure 5 *Declining trend in dracunculiasis prevalence 1989–2001 (WHO 2002b)*

Anthropogenic and environmental changes frequently result in epidemics of parasitic disease. Several reviews have identified the primary drivers of these changes (Molyneux 1997, 2003a, b) as listed below:

- Movements of non-immune populations in areas where transmission occurs. Such movements may be of an organized nature (e.g. mobilization of the workforce in Brazil to exploit forest resources has resulted in malaria epidemics). Alternatively, they may occur without formal organization (e.g. movements of workers involved in mining for gold or gems in the Amazon and South East Asia).
- Climatic changes (e.g. temperature change is considered to be a cause of highland malaria in Kenya and Ethiopia). Unusual levels of rainfall following periods of drought result in epidemics of malaria in East and South Africa.
- Urbanization results in populations being exposed to new organisms as vectors establish in new habitats and peridomestic reservoirs act as sources of infection. Health services are grossly inadequate or nonexistent and service providers are often only NGDOs or faith groups. The increasing impact of urbanization on various different forms of leishmaniasis has been highlighted by WHO (2002a): for zoonotic cutaneous leishmaniasis (*L. guyanensis* and *L. brasiliensis*) in Brazil, Colombia, and Venezuela; for anthroponotic cutaneous leishmaniasis (*L. tropica*) in cities of south west Asia (Afghanistan, Iran, Syria, and Iraq); for zoonotic visceral disease (*L. infantumi*) in north-east Brazil where drought-induced migration to cities in which peri-urban shanty settlements provide conditions for transmission between humans and dog reservoirs via *Lu. longipalpis*.
- Changes in vegetation such as the development in Uganda of thickets of *Lantana*, which provided a habitat for *Glossina fuscipes* thus provoking epidemics of Rhodesian sleeping sickness. Another example is deforestation, which has resulted in exposure to leishmaniasis in the Amazon (Walsh et al. 1993).
- Development projects, particularly those involving water resource development, frequently exacerbate the health problems of the local or incoming population (Birley 1995; Hunter et al. 1993).
- Conflict, civil unrest, and associated population disruption have profound impacts on parasitic diseases and epidemics are frequently associated with such events. Disease-causing organisms with a capacity for rapid adaptation and reproduction that are associated with vectors having characteristics as generalists are more prone to create health problemsin conflict environments (Molyneux 1997). Tables 2 and 15, p. 43, summarize recent examples of changes in parasitic-disease epidemiology associated with conflict and characteristics of generalist vectors.
- Agricultural development projects, particularly those linked to irrigated agriculture and development of monocultures are associated with changed patterns ofinsect-borne infection, especially malaria (Ijumba and Lindsay 2001), Japanese encephalitis, leishmaniasis.

Common themes which operate in the above settings and are associated with changing vector-borne diseases include:

- epidemics associated with generalist vectors (characteristics of generalist vectors are listed below)
- animal reservoirs or mixing vessels associated as food sources with generalist vectors
- animal reservoirs that may be domestic, wild or intensively reared
- reduced biodiversity (often associated with the impact of hydrological change and deforestation/reforestation), which encourages expansion of adaptable generalist vectors and reservoirs
- ratio of *P. vivax*: *P. falciparum* changing with increasing *P. falciparum* (see also Table 15, p. 43)
- extractive activities (uncontrolled) generating the development of antimalaria resistance

- hydrological impacts over variable time-frames
  malaria and Japanese encephalitis – acute
  schistosomiasis / dracunculiasis – medium
  filariasis – chronic
- deforestation/reforestation impacts via
  behavior of humans, animal reservoirs, and vectors
  edge/interface effects/fragmentation patterns
  degree and type of reforestation
  loss of biodiversity
  loss of forest causing elimination of vector species
  common pattern of change occurring within different vector complexes.

Characteristics of generalist vectors include:

- wide geographical distribution
- species complexes or species groups
- capacity to feed on a range of available hosts
- ability to exploit peridomestic and peri-urban settings
- abiltiy to exploit new pre-imaginal habitats
- efficient vectors with high vectoral capacity
- no transovarial transmission.

Control of animal reservoir hosts

Many parasitic diseases are zoonoses, defined as 'those diseases and infections (the agents of) which are naturally transmitted between (other) vertebrate animals and man' (WHO, 1979). A list of recognized parasitic zoonoses is provided by the WHO (1979). Ostfeld and Keesing (2000) provide an updated list of vector-borne infections of potential public health importance, while a recent analysis of all emergent and re-emergent infections (Taylor et al. 2001) has identified that 75 percent of emerging pathogens are zoonotic and that such organisms are more than twice as likely to emerge as nonzoonotic ones. However, viruses and protozoa are more likely to emerge than macroparasites such as the helminths. The important zoonoses for which reservoir control can have a cost-effective impact are discussed below. The presence of an animal reservoir host may be a major impediment to control of a disease, particularly if the habits and habitats of the animal hosts prevent intervention either on the grounds of practicality or for reasons such as protected status of the host (e.g. primates or endangered species). The International Commission for Disease Eradication recognizes that the existence of a reservoir host precludes the likelihood of eradication or elimination of the infection (see Table 3).

LEISHMANIASIS

Among the most important examples of control programs targeted against animal reservoir hosts was that aimed at the great gerbil *Rhombomys opimus*, the reservoir host of *Leishmania major* in the former Soviet Union; a variety of techniques were employed to eliminate this rodent in the central Asian republics of Uzbekistan, Kazakhstan, and Turkmenia. Burrow systems identified by aerial or ground surveys were destroyed by deep plowing. Alternatively, poisonous baits of zinc phosphide mixed with wheat and vegetable oil were introduced into every third or fourth hole. The effects of the zinc phosphide were enhanced by prior application of the anticoagulant, dicoumuarol, 5–7 days earlier. Because of its high toxicity, zinc phosphide had to be inserted directly into the rodent burrows. Elimination of *R. opimus* can also be achieved by flooding burrows in irrigation schemes. Reinvasion can be prevented by canal construction and agricultural development.

The fat sand rat *Psammomys obesus* (reservoir of *L. major* in the Near East and North Africa) has not been effectively controlled as it is not granivorous and because anticoagulants are too expensive for large-scale use. The other reservoir hosts of *L. major* (*Meriones* spp. and *Tatera* spp.) are not easily controlled. Large-scale control of hyraxes (reservoirs of *L. aethiopica*) needs to be organized at a local level using shooting and trapping.

The animal reservoirs of visceral leishmaniasis (*L. infantum* and *L. i. chagasi*) are domestic and wild canids. The instigation of dog control for rabies control in China has reportedly reduced the incidence of visceral leishmaniasis to almost zero. Elimination of stray dogs is justified for many reasons and although shooting and poisoning are effective they are not acceptable to some communities. In some countries (e.g. France) screening of domestic dogs for *Leishmania* spp. by serological examination in parallel with clinical examination, permits infected animals to be identified and either destroyed or treated. Treatment is not, however, entirely effective and an alternative approach is to administer a single prophylactic treatment shortly before the peak transmission season. Control of canine leishmaniasis could be integrated with rabies control by:

- registration of dogs
- regular evaluation and surveillance
- serological diagnosis
- mobilization and motivation of the dog-owning community.

However, a new approach has been the use of insecticide (deltamethrin)-impregnated collars on domestic dogs; this reduced the proportion of sandflies that took blood meals and survived by over 90 percent for at least 8 months after collars were applied (Killick-Kendrick et al. 1997). The importance of controlling canine leishmaniasis is underlined by the finding that human *L. infantum* infection is more widespread in southern Europe than was previously thought and by the fact that visceral leishmaniasis is an HIV-associated disease. In South America, and to a lesser extent in Europe, the presence of wild canid reservoirs necessitates a continuous commitment to control.

There is limited opportunity to control the wide range of reservoir hosts of New World cutaneous leishmaniasis. The WHO (1990) suggests that control of the opossum (*Didelphis marsupialis*) could be achieved by using baited pitfall traps in urban and peri-urban environments or in disturbed primary forest; this would result in control of *L. guyanensis*. *T. cruzi* might also be tackled by the same measures in peri-urban situations, but the cost-effectiveness of such measures remains to be validated. The WHO suggests an environmental management approach combining primary forest clearance with insecticide application, in order to create a vector-free and reservoir-free zone around villages. The transmission of *L. guyanensis* has been greatly reduced by these measures in French Guyana.

HYDATID DISEASE

Dogs and other canids are the definitive hosts of *Echinococcus granulosus*. Several countries have instigated dog control and surveillance and chemotherapeutic treatment (with praziquantel) to eliminate adult worms or to reduce access of dogs to larval stages in offal. Major hydatid control campaigns have been introduced in a number of countries including Australia, Iceland, New Zealand, Cyprus, Argentina, and Uruguay. The major measures applied are:

- preventing dogs from gaining access to raw offal at abattoirs and farms or to dead animals in the field
- reducing parasite loads by culling dog populations in combination with mass treatment.

The application of such measures resulted in a rapid reduction of cystic infections in livestock in Cyprus, where over 30 000 dogs were eliminated over a 2-year period. A parallel health education program was also introduced to inform the public about appropriate practices for feeding dogs and for slaughtering animals. A particular problem in hydatid control is that dog feces are used for specific purposes in some areas of the world; for instance, the Turkana of northern Kenya use dog feces to lubricate necklaces, and such feces are used for leather curing in Lebanon. Further problems arise from the inappropriate disposal of animal or even human corpses, and from close relationships between humans and dogs (e.g. 'nurse dogs' in Kenya, or family pets in England). In such cultures, the elimination of dogs cannot be envisaged.

Alveolar hydatid disease (caused by *E. multilocularis*) is a problem in mountainous areas of Europe, northern USA, Siberia, and China, where the fox is the main definitive host and larval (cystic) stages are found in microtine rodents (usually the genus *Microtus*). Health-education campaigns in Europe have successfully discouraged the consumption of wild fruits, particularly blueberries, which can be contaminated with fox feces. In China, reduction of the canine population has resulted in reduced transmission to humans; however, changes in land use associated with loss of forest has increased incidence of alveolar hydatid disease in other areas.

TAENIASIS, CYSTICERCOSIS, AND NEUROCYSTICERCOSIS

Cysticercosis of the central nervous system (neurocysticercosis) is caused by the larval stage (cysticerci) of the pork tapeworm *Taenia solium*. Human beings act as definitive hosts and swine as intermediate hosts. Pigs become infected when they ingest human feces containing *T. solium* eggs, which develop in the muscle and brain into cysticerci. When people eat the undercooked pork of an infected animal, they develop tapeworm infection. Human beings can, however, become infected by directly ingesting *T. solium* eggs from human feces. These eggs then develop into cysticerci, which migrate mostly into muscle (causing cysticercosis) and into the central nervous system where they can cause seizures and other neurological symptoms of neurocysticeriosis. Human cysticercosis infections are acquired by the feco–oral route in areas with poor hygiene and sanitation.

Neurocysticercosis has serious morbidity and is also a leading cause of epilepsy, which has profound social, physical, and psychological consequences. The diagnosis of neurocysticercosis involves the interpretation of nonspecific clinical manifestations, such as seizures, often with characteristic findings on computer-assisted tomography (CAT) scans or nuclear magnetic resonance (NMR) imaging of the brain, and the use of specific serological tests. Diagnostic criteria based on objective data (clinical, imaging, immunological, and epidemiological) have been proposed for different levels of the healthcare system but are not generally used in areas endemic for the disease. Lack of awareness and differences in quality and availability of medical services mean a lack of reporting. Human carriers of *T. solium* are diagnosed by the detection of proglottids or eggs in feces, or by more sensitive methods such as the detection of *Taenia* antigens in stools or specific antibodies in serum.

Human cysticercosis is a disease associated with poverty in areas where people eat pork and traditional pig husbandry is practiced. It is endemic in the Andean area of South America, Brazil, Central America and Mexico, China, the Indian subcontinent, South-East Asia, and subSaharan Africa. The spread of the disease is facilitated by poor hygiene, inadequate sanitation, and the use of untreated or partially treated wastewater in agriculture.

To control cysticercosis, the following intervention strategies are available (Lightowlers 1999):

- Case management, reporting, and surveillance. People with neurocysticercosis usually present with nonspecific neurological symptoms such as epilepsy,

for which adequate case management should be available in health services. Such management requires a consensus on standardized criteria and guidelines for early differential diagnosis in peripheral healthcare structures (with emphasis on resource-poor areas) as well as guidelines for possible treatment or referral to the next level of the healthcare system. Better surveillance and reporting would lead to a more accurate understanding of the extent of the problem and to the identification of foci of transmission.

- Identification and treatment of individuals who are direct sources of contagion (human carriers of adult tapeworm) and their close contacts, combined with hygiene education and better sanitation, will interrupt or reduce the cycle of direct person-to-person transmission, an approach that has been successfully applied to other contagious diseases.
- Universal or selected treatment with praziquantel (10 mg/kg body weight) has significantly reduced the prevalence of human taeniasis in areas where *T. solium* infection is endemic (Mexico). To limit reinfection of humans by the intermediate hosts, treatment should be accompanied by veterinary sanitary measures, meat inspection, improvement of pig husbandry, and treatment of infected animals. Single-dose therapeutic agents, for example oxfendazole, have recently become available, with no deleterious effects on pigs or on the meat product. Animal vaccines are under development.
- Long-term success is more likely when anthelminthic chemotherapy programs are integrated into a wider intersectoral approach to increase public awareness and hygiene practices. Additional measures to sustain the impact of specific interventions include the provision of clean water and sanitation, and health education. Improvement in living conditions, legislation, modernization of swine husbandry, and improvement of meat inspection, have reduced transmission in many industrialized countries.

The International Task Force for Disease Eradication declared *Taenia solium* a potentially eradicable parasite, for the following reasons (CDC 1993):

- the life cycle requires humans as definitive hosts
- tapeworm infections in humans are the only source of infection for pigs, the natural intermediate host
- the transmission of infection from pigs to human beings can be controlled
- no reservoir for infection exists in wildlife.

The strategic use of anthelminthics against adult worms in people and the larval parasite in swine, combined with health education and regulation of pig slaughter, will interrupt transmission, but this approach has yet to be proven in any well-monitored intervention trials.

Taeniasis and cysticercosis do not lead to sudden, large-scale international epidemics and do not seem to constitute an appropriate subject for international notification although Roman et al. (2000) argue that neurocysticercosis should be made an international reportable disease. As with other potentially eradicable diseases, significant progress has been made over the past decade in developing tools, refining strategies, and defining the public health problems. Sotelo (2003) argues that eradication remains an attainable goal, the achievement of which requires more advocacy, resources, national commitment, and global structures. Health ministries should be encouraged to set up surveillance and reporting systems, and adopt an active approach towards prevention and control of these diseases.

Community participation in parasitic disease control

The drive towards primary healthcare following the Alma-Ata declaration of 1978 provoked a greater degree of involvement of communities in healthcare through:

- the use of community leaders to support various programs
- the identification of personnel to undertake health activities on a voluntary basis
- emphasis on the importance of such activities in community well being.

The topic of community participation has been reviewed by Curtis (1991) who provides a series of examples in vector-borne disease control. MacCormack (1991) provides an insight into the underlying principles of sustainable vector control in a community context and reviews the factors that influence success and failure. She emphasizes that much of the success achieved in small pilot programs has depended on particular characteristics such as:

- leadership
- a responsive, well motivated, well educated community
- incentives from agencies and insecticide manufacturers
- ease of communication.

Following initial success, there is a danger that a 'hot' project will fall into a steady state as enthusiasm and donor support wane while the project life-cycle faces inevitable problems. The scaling-up of pilot projects to national ones within a primary healthcare context presents additional challenges. For instance, the community may be affected by the replacement of local leaders with national bureaucracy. In establishing a functional link between the communities and the health systems, each group must be trained to understand the social role on the one hand and technical skills on the other. Communities' local knowledge about insects should be

exploited to aid in vector control. Appropriate control methods, and the importance of maintaining them, must then be clearly explained to all those involved at the local level.

It must also be established whether unpaid community labor can be sustained over time; although it has been achieved in pilot programs, doubts exist about longer term sustainability (Walt 1988). Much is likely to depend on the community structure and its relationship with those in authority, who are perceived as those most likely to benefit. If, for example, a cost-recovery system operates, the volunteers are less able to collect fees from their social superiors. Professional interaction between technicians and volunteers can also fuel conflicts based on perceptions about status.

The outcome of community participation in any project depends on the numerous complex social interactions existing within the community environment. The interaction between weak and strong groups, and the impact of participation on such group relationships, are of critical importance (Antia 1988). It is valuable to define the boundaries of the community involved because individuals tend to identify with a particular locale; this is despite the inherent social instability of most villages, which results from factors such as migration, schooling, and marriage. For practical reasons, the community is usually defined by a geographical boundary such as an urban neighborhood or an agricultural village while nomadic groups represent a mobile community.

Communities differ in how they function and are stratified; for example, they may be democratic, autocratic or under military control. In a democratic environment, obtaining consensus may be a slow process, but the likelihood of sustainability is high. MacCormack concludes that community participation in vector control will be sustainable only if the assessment of the costs-to-benefits ratio takes account of 'opportunity costs' (the value of activities people would undertake if they had not committed themselves to a particular control activity). Sustainability will be enhanced if the following conditions apply: activities are linked to community priorities; skills training enhances community well being; and preventative work links to curative or care outcomes in the primary healthcare setting, or can produce income (Rajagopalan et al. 1987).

Dedicated control programs often achieve good results, but they are often part of a research project. Therefore, coverage of the population in need is relatively small, but delivery costs are high, as are recurrent costs as a proportion of overall costs. This leaves little financial input for operational expenses. In situations in which the frequency of the intervention is limited (e.g. once a year) the commitment of dedicated resources is difficult to justify.

Community-based treatments are usually better targeted and tend to involve volunteers, TBAs, and primary healthcare workers. Increasingly, other types of groups are also becoming involved; examples include women's groups, faith groups, civil society organizations (CSO), and NGDOs. The NGDO community has become increasingly involved in onchocerciasis control. Over the past decade, as the programs in Africa and the Americas have expanded using the donated drug Mectizan® (ivermectin), the momentum for NDGO involvement came from the organizations committed to blindness control that recognized the value of ivermectin as a tool for reducing morbidity associated with onchocercal eye disease (Drameh et al. 2002). At present, NGDOs provide some 25 percent of the resources required for national onchocerciasis programs, and 12 international NGDOs are active in some 20 countries in Africa through the APOC and the countries from the former OCP. The key element of the approach to control is community-directed treatment with ivermectin (CDTI), which is regarded as the key driver in ensuring sustainability of this program. The progress in the APOC program is documented in *Annals of Tropical Medicine and Parasitology*, volume 96, 2002, Supplement 1.

CDTI CHALLENGES

Amazigo et al. (2002) review the challenges presented by CDTI strategies. This approach is based on the principle of community participation but also ensures empowerment by allowing communities to decide who should be the community directed distributors (CDD) and how to plan ivermectin distribution (e.g. dates, location model of distribution). The replacement of the community-based treatment system with the community-directed approach has been encouraged as the latter is likely to be more sustainable, provides community ownership and empowerment and reduces costs to the health system. CDTI enables communities to organize distribution in line with cultural norms and organizational structures – such as kinship and clan structures in Uganda (Katabarwa et al. 2000), while stimulating basic healthcare infrastructure in remote areas (Hopkins 1998). Amazigo et al. (2002) articulate challenges for CDTI into managerial, technical, and sociopolitical headings. These can be summarized as shown below.

Managerial

- Develop drug collection mechanisms/including issues of delay and wastage.
- Integrate with health services including use of CDTI in other health problems and sharing infrastructure and personnel.
- Strengthen infrastructure at all levels to manage and sustain CDTI.

Technical

- Achieve and maintain 65 percent coverage of the total population within all villages (e.g. achieve

100 percent geographic coverall of meso- and hyperendemic villages).
- Address refusals caused by perceptions of adverse reactions.
- Conduct an accurate census using a standard formula for calculating such a census.
- Establish commitment to self-monitoring especially by communities and use the results to improve projects.
- Undertake operational research that identifies problems and apply results.

Sociopolitical

- Increase recognition of role of CDDs and thereby prevent attrition.
- Determine and provide appropriate incentives and motivation for CDDs.
- Involve women as CDDs and in community decisive making.
- Increase involvement and build capacity of local NGDOs.
- Plan and achieve post APOC financial support.
- Achieve balance between cost-recovery strategies compared with guaranteed affordability at all levels; reduce costs.
- Develop advocacy to gain support from political leaders.

The experience that the CDTI concept has provided to date regarding community involvement in onchocerciasis control in Africa gives valuable insights into the link between communities and health systems, which are applicable and generalizable to other programs and have wider policy implications (e.g. cost recovery). However, CDTI may not be applicable in other settings where a high degree of coverage is needed and where health services themselves are better resourced than in rural Africa or in urban settings or conflict situations. The APOC has experienced the value of and problems presented by CDTI in conflict environments where the necessity of convincing communities to engage in onchocerciasis control is a priority: identifying partners prepared to work in insecure areas, and population movement. The needs of CDTI (i.e. high coverage, need for census, selection of CDDs, drug logistics, and monitoring) are difficult to achieve during conflict and civil unrest. Treatment can be given on specific days organized by governments such as independence days, Head of State's birthday, local festivals (e.g. to celebrate harvest or onset of rain). The use of such occasions may reduce the need for intense monitoring to ensure high coverage and compliance. There are no dedicated staff costs, administrative costs are absorbed by the system, transport costs are minimal, compliance is high, and costs of monitoring and evaluation are low. These arguments have been developed to justify such approaches to lymphatic filariasis control in Papua New Guinea. Treatment with DEC was simplified by creating tablets based on a calculation of the average weight of adults and children; the appropriate amount of drug was formulated into one blue tablet for adults and one red tablet for children. This eliminated the need for scales for weighing, allowed the treatment to be administered by untrained personnel and improved compliance (by using a single tablet). The estimated cost per treatment was US$0.8 per child and US$1.2 per adult (not including costs for transportation, health-promotion materials, monitoring, and coordination). In seeking to develop community treatment of any disease, several issues (e.g. devising reporting forms for illiterate communities, drug collection points) need to be studied through locally organized operational research.

Because of the pressures on health systems, it is necessary to use personnel resources at the level of the community to participate in various phases of control activities. This is particularly relevant in the maintenance phase of programs when the community has seen benefits from intervention phases; this promotes confidence between the program participants, the communities themselves, and in health systems. Parasitic disease control programs are increasingly becoming dependent on the involvement of human resources without specific technical knowledge or with only limited training.

In the Chagas' disease control program, Garcia-Zapata and Marsden (1986) have described the value of community involvement in establishing vigilance over the presence of vector bugs following the attack phase. Bug information posts were linked to schools because it was recognized that children were sensitive to the renewed presence of triatomine bugs. Reappearance of bugs is the basis for initiating selective spraying at the local level. If given adequate information, local isolated farmers could also act as notifiers of bug presence. Simple devices for bug monitoring have been developed in Argentina. Similarly, successes in programs to control onchocerciasis, lymphatic filariasis, and Guinea worm have been due to the commitment of communities.

CONTROL OF BRUGIAN FILARIASIS

A focus of filariasis caused by *Brugia malayi* existed in the Indian coastal state of Kerala, in the Cherthala region of Alleppey. There was a high prevalence of the disease with serious clinical manifestations of elephantiasis. The vectors in the area were *Mansonia* mosquitoes that breed on certain water plants (*Pistia*, *Eichhornia*, and *Salvinia*), to which their larvae attach for respiration. The local people believed that vegetation in ponds improved the quality of the water, which was used for domestic purposes. Also, the water plants are used as green manure on coconut plantations (Rajagopalan et al.

1987) and, therefore, the community were reluctant to use chemical larvicides or herbicides.

An extensive education and awareness program was initiated via the media, targeting various sectors of the community. A group of sociologists was used to pass on the necessary technology and to create interest. The Filariasis Patients' Association, headed by a former school teacher, developed a school health-education program. This campaign was targeted towards the female population of Cherthala, who were often not chosen as brides because of filariasis; the effectiveness of the campaign was enhanced by a high level of literacy among the local women. The main control measure was the removal of water plants. The community first had to be persuaded that alternative green manure fertilizers were adequate and that ponds could be cleared without financial loss. As no government organization was available to supervise the weed removal, this was done by the people themselves. To provide motivation, the community was encouraged to culture fish that could be sustained only in weed-free ponds. Species of fast-growing edible fish were purchased from the State Fisheries Department and distributed free to those who agreed to remove weed from their ponds. The economic benefits derived from the fish motivated the community to keep the ponds free of weed, thereby removing the vector habitat.

The program is organized by the peoples' Filariasis Control Movement (FILCO), which supports the following variety of approaches to ensure that the whole community is involved:

- in six secondary schools, Student Filariasis Control Clubs run programs to detect and treat filariasis
- all educational institutions contribute voluntary labor to improve the environment
- the National Cadet Corps and National Service Scheme provide services for filariasis control
- the interns of medical colleges are involved in clinical and diagnostic night camps
- the Departments of Fisheries and Agriculture popularize fish culture and alternative fertilizers.

To publicize the message, hoardings depicting filariasis patients were erected in areas where they would have most impact (markets, hospitals, bus stations, railway stations). The slogan employed was 'Remove weeds and protect yourselves and your future generations from filariasis.' These messages were reinforced by the distribution of films depicting the suffering of a filariasis patients.

In parallel with environmental measures to reduce vector levels, screening and treatment of cases, previously under government control, are now undertaken by voluntary organizations. For instance, 75 filariasis detection and treatment centers are now screening the endemic area and positive individuals are being treated with DEC.

Brugian filariasis appears to have been eliminated from Sri Lanka over the last two decades as a result ofselective chemotherapy using DEC and vector control of *Mansonia* host plants using herbicides. Extensive epidemiological investigations have indicated that *Brugia malayi* is no longer present in previously endemic areas.

GUINEA WORM ERADICATION

Guinea worm infection (dracunculiasis) is the subject of a global eradication campaign, initiated in 1980. The International Commission for the Certification of the Eradication of Dracunculiasis was established by the WHO in the mid 1990s to certify the absence of transmission in member states who applied for such status. Prior to such certification, a process of field visits by members of the Commission must be undertaken to validate the information submitted by the country. A pre-certification period of three years is necessary to be sure that transmission has ceased in previously endemic areas. A reward system is also introduced to ensure suspect cases are thoroughly investigated, and posters plus health education messages via media are used to promote the campaign. To date, elimination of transmission has been achieved in India and Pakistan, and both countries have been certified free of transmission. Much of the success in India can be attributed to the development of safe water supplies during the International Water Decade with the drive to ensure that every village had access to safe water. However, the disease remains endemic in 13 African countries while four countries in Africa (Cameroon, Chad, Kenya, and Senegal) and Yemen are in pre-certification status. While there has been a significant reduction in incidence in many countries, Sudan remains the country with the biggest problem due to civil unrest and population movement over the last two decades; Sudan accounts for around 75 percent of all global cases (Hopkins and Withers 2002). The impact of Guinea worm disease is concentrated at harvest and planting times when infected individuals are incapacitated and unable to farm or to attend school for up to 3 months at a time. The infection is painful, and secondary infections often result when the worms emerge through the skin of the extremities. As there are no effective drugs or vaccines, Guinea worm must be eradicated through a series of measures that require community involvement – filtering drinking water; treating bodies of water with the insecticide temephos to kill copepods, providing safe water, and case isolation (containment) to prevent contamination of water sources. The proportion of cases contained is an important measure of the success of the surveillance system, of the involvement of communities, and as a predictor of the likelihood of cases being detected the following season. In the pre-certification stage, it may be necessary to introduce rumor registers and provide

rewards when detected cases are confirmed. Progress in the eradication program is shown in Figure 5.

The eradication strategy is based on the establishment of a national program in each affected country to survey every affected village, to estimate numbers of cases, and to initiate plans. It is then necessary to establish village-based surveillance, using VHWs to report cases on a monthly basis. Health education encourages the use of cloth filters and the prevention of contamination of water sources. Some villages can also be targeted for provision of safe water while the establishment of a community-based surveillance system (with monthly supervision) might be broadened to cover other major endemic conditions. *Dracunculus*-free certification can be issued to clear areas. To date, there has been considerable success with this approach and incidence has decreased dramatically. VHWs are directly involved in measures that benefit health and increase agricultural productivity and school attendance. Such success increases confidence within communities and motivates them to become involved in other self-sustaining health interventions. A new intervention using portable plastic drinking straws with a filter inserted has been introduced in Sudan to prevent ingestions of copepods.

Community surveillance methods for eradication require the establishment of village development committees whose members are responsible for case notification, health education, distribution and maintenance of filters, and patient care. In Mauritania, this role has been assigned to females who undertake the roles of water gathering and distribution. A potential weakness is that village eradication activities are dependent on monthly supervision from the health personnel at a sub-district level. Each village requires monthly visits, usually integrated with the extended vaccination program or other health programs. Dracunculiasis can be eradicated but needs considerable community commitment from VHWs; in Burkina Faso and Mali, these workers are rewarded by distribution of cloth in recognition of their contribution to the program.

COMMUNITY MALARIA CONTROL IN PONDICHERRY, INDIA

In the coastal areas of Pondicherry, South India, malaria has been controlled by a combination of approaches organized by the Vector Control Research Centre (VCRC). These approaches have achieved good control of the vector *A. subpictus* and also produced income for the local population. Vector control was achieved by removing the algae *Enteromorpha* from coastal lagoons where they sheltered mosquito larvae from fish and other predators. The algae were collected in the pre-monsoon season and provided the raw material for producing paper to be sold by villagers. The lagoon became a focus of mosquito-breeding during the dry season and the villagers were persuaded to deepen one of the ponds in order to allow the other to drain into it, the mud from the deepened pond being used to fill low-lying areas. The deeper pond was then used for prawn culture, which provided extra income for the village.

Control of vectors was also necessary in other breeding sites, particularly the earthenware pots in which coconut husks are retted to make coir. Control was achieved by introducing larvae of the mosquito *Toxorhynchites* which predate larvae of vector mosquitoes. The water filled pits where *Casuarina* trees are grown also act as breeding sites and so these were stocked with the larvivorous fish *Gambusia*. This action was encouraged by using science clubs in schools to stock the pits.

Involving the community initially faced resistance from villagers who were divided among themselves and inherently suspicious of officialdom. These communities placed a higher priority on services such as water, electricity, and roads rather than vector control, except in situations in which malaria epidemics occurred. Only after the VCRC had intervened and asked government departments to provide electricity and a water supply did the villagers have enough confidence to allow vector control projects to commence. The success of this program, as that of filariasis control, was largely due to the initiative of active locally based indigenous scientists (VCRC 1992).

CONTROL OF PARASITIC INFECTIONS THROUGH SCHOOL-AGE CHILDREN

Population increases and investments in education have increased the global population of school-age children. This group is a particularly important entry point for improving health status by using education to create norms of healthy behavior and lifestyle. The school also provides access to a population with a high prevalence of helminth infection in whom mass drug distribution can rapidly reduce morbidity, enhance nutritional status, and improve cognition and hence school performance. Urbanization has increased enrolment into schools, but also promotes conditions such as lack of adequate waste disposal, overcrowding, and the absence of clean water, which may encourage increased transmission of helminth diseases.

A WHO pamphlet (WHO 1995a) estimated that of a global total of 1.2 billion school-age children, 700 million were registered in school with 400 million attending each day, and enrolment still increasing. The target parasitic diseases susceptible to control are schistosomiasis, *Ascaris lumbricoides* infection, *Trichuris trichiura* infection, hookworm infection (*Necator americanus* and *Ancylostoma duodenale*), and infection by the food-borne trematodes (*Clonorchis*, *Opisthorchis*, and *Paragonimus*). Many school-age children have mixed infections, providing justification for mass interventions based on effective single doses of drugs such as the benzimidazoles (mebendazole and albendazole) and praziquantel; these eliminate intestinal helminths and schistosomes.

The epidemiological characteristics of each particular situation require investigation; prevalence and intensity vary even in adjacent areas, as do the pattern and rate of reinfection. It is assumed that reinfection will occur and require retreatment, although emphasizing the importance of personal hygiene should reduce the rate of reinfection. There is thus a need for field studies to ascertain whether intervention for both schistosomiasis and intestinal helminths is warranted. Rapid assessment of hematuria is used to evaluate levels of morbidity due to *S. haematobium* while other approaches are being tested for *S. mansoni* and intestinal helminths.

Drugs for the treatment of intestinal helminths and schistosomes, and their treatment schedules and efficacy, are described by WHO (1995b). Albendazole and praziquantel have now been used in combination; combined drug delivery offers advantages in terms of lower cost and simplification of therapy. This results in improved compliance and improved drug efficacy by potentiation of the therapeutic effect. Trials have provided no evidence of adverse effects of combined treatment with praziquantel and albendazole. Phase I clinical trials showed a four-fold increase in the bioavailability of albendazole in the presence of praziquantel, while the bioavailability of praziquantel was unaffected by the administration of albendazole. Studies on the safety and efficacy of combined treatment have shown no increase in adverse effects and no reduction in efficacy.

The WHO emphasizes the importance of targeting chemotherapy for intestinal helminths at school-age children, as they harbor the most intense infections of *Ascaris*, *Trichiuris*, and *Schistosoma* although intensities of the different worm infections vary considerably between individuals. Treatment of this group achieves the maximum return in terms of reducing morbidity, and schools provide the most accessible entry point for maximum coverage. The WHO also stresses the value of targeting schoolchildren for oral antischistosomal drugs; programs should seek to provide complete coverage of the school-age population. Such approaches could be combined with other helminth control and also with other health-oriented activities such as immunization, nutritional programs, maternal and child health activities, and general health education as part of the school curriculum. Retreatment should not be undertaken for schistosomiasis more than once a year. Epidemiological studies need to be established to evaluate the criteria for retreatment frequency in each area.

The integration of helminth control into the education sector not only fulfils the immediate objective of infection control, but also brings broader health issues to the attention of a group who can strongly influence the behavior of future generations. WHO policy promotes school health programs, with de-worming as an entry point and with an emphasis on personal hygiene. The integration of school-based and community-based chemotherapy into the primary healthcare system will improve coverage and promote optimal retreatment schedules. Another potential benefit may be gained through training personnel at the periphery, who will develop activities based on disease-specific interventions to reduce prevalence and intensity of infection; this will reduce morbidity through coverage of schools and other entry points. While this approach to infection control will undoubtedly improve the health of schoolchildren and, to some extent, contribute to lower transmission rates over time, greater impact will be achieved if health education, improved water supplies, sanitation, and environmental management are integrated into the activities involved in community development.

Role of vector control in disease control

INSECT CONTROL

The role of insects in the transmission of parasitic infections has been recognized since Manson discovered that filaria parasites were transmitted by mosquitoes. From the latter half of the last century onwards, recognition followed that numerous other infectious diseases – sleeping sickness, malaria, Chagas disease, and onchocerciasis as well as various viral infections – were also insect-borne or tick-borne. Knowledge of biology of the vector (particularly of breeding sites, habitat of pre-adult stages, adult resting sites, biting behavior and host preferences, migration capacity and longevity) is a key factor in assessing whether or not appropriate control can be directed at the vector. Early attempts to target malaria by controlling mosquitoes depended on knowledge of larval stages and breeding sites. The removal of breeding sites (source reduction) was the only approach available to reduce vector populations; it was achieved by applying oil and copper compounds and by the rigorous removal or destruction of larval habitats by mechanical methods (WHO 1995c). Tsetse flies were controlled by trapping or destroying savanna and riverine habitats of *Glossina morsitans* and *G. palpalis* group flies, respectively. In some countries, game animals (the favored hosts of *Glossina*) were eliminated by shooting prior to the introduction of cattle. Removal of game animals rapidly reduced the tsetse population in large areas of South Africa, Uganda, and Zimbabwe, providing additional land for ranching.

The advent of chemical insecticides in the 1940s provided new tools for vector control that became incorporated into programs designed to control, if not eradicate, numerous diseases. DDT, initially the most widely used of these insecticides, was (together with chloroquine) a major tool in the campaign for malaria eradication launched by the WHO in 1955. In parallel, DDT was also being utilized in:

- tsetse-control programs for both human and animal trypanosomiasis
- control of *Simulium* (blackfly) larvae to control river blindness
- the successful elimination of *S. damnosum* in Uganda.

Meanwhile, another chlorinated hydrocarbon, benzene hexachloride (BHC), also known as lindane, was successfully used against triatomine bugs in Chagas disease control.

Recognition of the environmental side-effects of chlorinated hydrocarbons, through direct toxicity on nontarget insects and bio-accumulation, led to a suspension of their widespread use and a drive to utilize other chemical classes of insecticide. This was accelerated by the realization that resistance of mosquitoes to DDT was posing problems for malaria control, particularly in indoor house-spraying programs. Attempts were made by the environmental lobby to include DDT in the international treaty to ban persistent organic pollutants (POP); however, the public health utility of DDT has been recognized through the efforts of the WHO which, in the light of impending resistance to synthetic pyrethroids, advocated for its continued acceptability for indoor house-spraying against *Anopheles* vectors of malaria. Hence, DDT can still used in some public health control programs for indoor house-spraying; however, caution is needed and its use should be monitored and the potential for side-effects studied (Curtis 1994). While pyrethroids such as lambacyhalothrin are widely advocated for indoor house-spraying, the major emphasis in malaria vector control now is the drive to increase use, coverage and retreatment of permethrin-treated bednets as part of the Roll Back Malaria strategy. Ambitious targets have been set for the coverage of vulnerable groups (pregnant women, children under 5) but significant issues remain to be resolved in respect of payment for these insecticide-treated nets, subsidy for vulnerable groups, methods for ensuring re-treatment regularly takes place, the role of permanently impregnated nets, and how sustained coverage through the commercial sector is to be achieved given the policy that nets should be available free.

There are, however, many examples where vector control has had a profound public health impact. Outside the domain of parasitic disease, the elimination of yellow fever by source reduction of *Aedes aegypti* in Central America illustrates what can be achieved through intensive and well-organized campaigns of vector control. The elimination of *A. gambiae* from Brazil, following its establishment from West Africa in the 1930s, represented not only an important public health achievement but also a major contribution to the development of Brazil, given the efficiency of *A. gambiae* as a vector of malaria in Africa.

Currently, intensive programs are underway to eliminate *Triatoma infestans* (the vector of *Trypanosoma cruzi*) by domiciliary spraying using the synthetic pyrethroids (deltamethrin and lambacyhalothrin) in the Southern Cone countries of South America. This has had a major impact on transmission, as determined by standardized serological procedures and community-based bug-monitoring systems in Brazil, Argentina, Paraguay, Chile, and Uruguay. Figure 4(a–d) demonstrates the success of the program in terms of impact on transmission by incidence and by levels of house infestation. Applied research has been geared towards providing additional methodologies to support government-organized control schemes such as fumigant canisters, insecticidal paints, and simple bug-monitoring devices for surveillance of reinvasion. The Chagas disease control programs do not benefit from having an effective chemotherapeutic agent. This has extended the anticipated time for reducing Chagas disease as a public health problem because no drug or vaccine is likely to become available in the foreseeable future. Hence, the current approach of intensive vector control supported by national governments is the only feasible one (WHO 1991) with screening for infective bloodmeals.

To achieve the objectives of the OCP, vector control has been the core strategy in 11 countries in West Africa. The program, initiated in 1974, was based on larviciding the breeding sites of *S. damnosum* complex blackflies, initially in six West African countries. The strategy was based on the need to break the life cycle for the lifetime of the adult *Onchocerca volvulus* which, in 1974, was considered to be 18–20 years. At that time, only one insecticide (the organophosphate, temephos) had the appropriate chemical characteristics to satisfy the environmental prerequisite of avoiding long-term damage to the riverine ecology. Two major problems faced the program early in its existence: migration of savanna blackflies from outside the program area, and resistance of some blackfly populations to temephos. These problems were solved by extending the area of the program westwards and southwards and by engaging in a research program that rapidly developed alternative insecticides. The extensive activities of the OCP and its management and structures are described by Samba (1994), Molyneux (1995), and Boatin et al. (1997). At present, seven larvicides from different chemical classes are in operational use They are used on a rotational basis in accordance with parameters such as cost-effectiveness, environmental side-effects, river discharge rates, carry distance of insecticide, and risk of resistance development. The seven insecticides in current use (Hougard et al. 1993; Yameogo et al. 2004) are:

- *Bacillus thuringiensis israeliensis* H–14 serotype toxin (Bti)
- temephos, phoxim, and pyraclofos (all organophosphates)
- carbosulfan (a carbamate)
- permethrin (a pyrethroid)

- etofenprox also known as vectron (a pseudopyrethroid).

While vector control has been successful in breaking the onchocerciasis transmission cycle, the necessary duration of vector control has been reduced by the widespread use of ivermectin; this became available for use as a microfilaricide suitable for use in communities in 1986 (Remme 1995). Epidemiological studies indicate that the duration of adult worm life is 14 years and that distribution of ivermectin in hyperendemic areas on an annual basis rapidly reduces microfilarial loads and also has a rapid impact on ocular morbidity. Hence, combined vector control and chemotherapy reduce the duration of control to a 12-year period although ivermectin alone, at least in the African endemic areas, appears not to prevent transmission (Winnen et al. 2002).

Increasing numbers of displaced persons and refugees pose special problems for organizations responsible for management of disease. Intervention through vector control is an important component, as there is great potential for rapid disease outbreaks in crowded, unsanitary environments that support traumatized, malnourished, displaced individuals who are exposed to vector-borne infection or ectoparasites. Epidemics are sometimes most effectively controlled through vector control. Thomson (1995) provides details of how such epidemics should be controlled, with specific reference to relief organizations. The conflict-associated parasitic disease problems are shown in Table 2.

SNAIL CONTROL

The elimination of snails for the control of schistosomiasis remains an important component of control activities and three approaches have been used: chemical, environmental, and biological. Projects in several countries have shown that chemical control using molluscicides (e.g. niclosamide) in combination with other methods can reduce or eliminate transmission. Chemical molluscicides must be environmentally acceptable and must not produce adverse side-effects if they enter the food chain. In addition, they must be cost-effective, snail-specific, and easily applied. During the 1970s, snail control, either by chemical methods or through environmental management (weeding, irrigation-canal construction, and maintenance), was a key control component. The advent of safe and effective chemotherapy for schistosomiasis (oxamniquine, praziquantel) capable of reducing morbidity through population-based campaigns has shifted emphasis away from molluscicide. Snail control is now considered as one of several approaches in integrated morbidity control, contributing to reduction in transmission at times of peak transmission. Target levels of reduction of snail populations should exceed 95 percent and must be maintained throughout the main season of transmission. Molluscicides can be used to destroy snails in breeding sites in transmission foci, reducing transmission in recreational areas such as lakes. They are also useful in the following ways (McCullough 1992):

- as a means of reinforcing community involvement
- to reduce transmission in areas where there is a special risk (e.g. fishing villages in the Volta Lake, Ghana)
- to eliminate newly introduced snail populations
- for total elimination of snails in isolated focal transmission sites (e.g. oases in North Africa)
- to prevent the establishment of dense populations in irrigation schemes.

Biological control of the snail hosts of schistosomes has been attempted by introducing other snail genera or species to out-compete the natural hosts but the impact of this approach on schistosomiasis morbidity has been limited.

Population-based chemotherapy, combined with local and seasonal use of molluscicides, will be the central features of future schistosomiasis control in high priority foci. Molluscicide must be planned effectively and better strategies and delivery systems are required to improve cost-effectiveness. New, inexpensive but effective synthetic molluscicides are needed in addition to bayluscide, the only compound currently commercially available (WHO 1993b).

Role of chemotherapy and chemoprophylaxis

Drugs are the mainstay for controlling many diseases, but antiparasitic drugs present a considerable challenge for researchers interested in rational drug development. Investment by multinational drug companies in new agents for use in the tropics and the developing world is limited because the markets themselves determine the research and investment strategies of the pharmaceutical industry.

Parasite infections pose a variety of other problems for chemotherapeutic control:

- the organisms rapidly develop drug resistance
- effective compounds have a narrow therapeutic index and hence side-effects are common
- parasites can invade specific sites of the body making drug delivery difficult
- helminth parasites can be relatively large and require proportionally more drug than microscopic organisms
- compounds may be difficult to obtain when required and manufactured only infrequently, particularly those compounds used for treatment of diseases that affect relatively small numbers of individuals
- some currently used compounds contain toxic elements such as arsenic and antimony

- many compounds must be administered under medical supervision, require hospitalization, and are neither accessible nor affordable by individuals.

Despite these problems, some compounds are highly effective for controlling parasitic infections and for the treatment of individuals. New drugs for human diseases have also originated from alternative sources over the last decade. For instance, veterinary research produced ivermectin, used for treating onchocerciasis, lymphatic filariasis and scabies; ivermectin was originally marketed for the treatment of helminth infections of animals. Eflornithine, a drug that can cure late stage chronic sleeping sickness, was originally developed as an anti-tumor drug. The artemisine derivative qinghaosu, produced from the Chinese herb *Artemisia* and used as a cure for fever in China for 2 000 years, is now widely available for malaria treatment. Its availability is often uncontrolled, which will encourage rapid development of resistance. In addition, as with many well-used drugs, fake products are becoming widely available. This is of concern because the drug has valuable curative properties and, therefore, should only be introduced in appropriate circumstances in relation to the current drug-resistance spectrum of malaria parasites in each region. New formulations of existing drugs have been developed, such as encapsulated liposomes that provide slow release of a drug; a slow-release formulation of amphotericin B (AmBisome) has been developed to reduce toxicity and enhance efficacy in leishmaniasis treatment.

A distinction must be made between drug treatment initiated to alleviate individual suffering, and mass treatment strategies that seek not only to improve the well being of individuals, but also that of communities. This latter objective is achieved by ensuring that no parasites can be transmitted by vectors, or that no parasites remain to reinfect the population. Drugs must, therefore, be regarded as having several functions in control. Gutteridge (1993) provides a summary of the drugs available for parasitic diseases and the rationale for their use. Tables 12, 13 and 14 summarize the drugs available for treatment of the diseases discussed in this chapter.

A major problem in many parasitic diseases is that drug resistance frequently arises. Management of drug resistance in parasitic disease is difficult as there are few alternative compounds available. Strategies for reducing the likelihood of the development of drug resistance in malaria and helminth diseases are well documented and include the following:

- appropriate dosage regimes
- control of drug quality and distribution (in malaria)
- incorporation of vector control to reduce the development or spread of resistance.

Mechanisms responsible for development of resistance (Gutteridge 1993) include:

- the ability of the organism to metabolize the drug to an inactive form
- changes in the permeability of the organism so that the drug is no longer taken up
- development of alternative biochemical pathways
- increased levels of target enzyme production
- a change in the biochemical target so that the drug cannot bind as well as it did to the original compound.

The ability of microorganisms to change and adapt rapidly will clearly represent a continuing problem and drug resistance is unlikely to be avoidable. Hence, any disease control strategy based on chemotherapy should anticipate such a problem and should seek to reduce the rate at which resistance develops, while also promoting strategies for management of resistance if it occurs.

In many developing countries, drugs are available through many sources. From the perspective of the consumer, the availability of drugs at a health facility is a major indicator of quality of care. In the poorest households, up to 50 percent of expenditure on health is on drugs, many of variable quality (counterfeit, expired). In the developing world, pharmaceutical expenditure accounts for 20–30 percent of recurrent costs; it is second only to personnel costs (World Bank 1994). Supply of drugs for the control of parasites is usually organized in the public sector through government-formulated essential drug lists. The WHO essential drug list (WHO 1992b) and program includes drugs for the treatment of infection with parasites. The concept of an essential drug list is that such drugs should be available at all times, in adequate amounts, and in the appropriate dosage forms (WHO 1992b). Several important drugs used for parasite disease control are often unavailable, despite being specified on essential drug lists.

While governments have a responsibility to provide essential drugs to different levels of the health service, private providers play a major role in many countries where private expenditure on drugs considerably exceeds public expenditure. In Africa, c. 70 percent of the population has no regular access to essential drugs and drug stocks often run out as a result of problems with management, logistics or finances. This is particularly true in peri-urban and urban centers, even to the extent that hospitals may run out of drugs. Drug shortages are less frequent in commercial facilities and such supplies are more readily available in urban centers, but the perception of higher quality product availability in the private sector allows for prices to be inflated. Private suppliers are also frequently not subject to quality control. In the case of antimalarials, which are among the most frequently required drugs, there is overpricing and supply is irregular while fake and adulterated products are promoted by importers. Antimalarials are often supplied without diagnostic confirmation.

The problems of drug supply, cost (generic versus brand name), quality control, distribution, irrational prescribing, noncompliance, and availability have been highlighted by the World Bank (1993, 1994). These problems potentially apply to all drugs, and awareness of them and the ways to circumvent them must be addressed if chemotherapy or chemoprophylaxis is to be a more effective part of disease control. Other problems may arise if vertical programs involving donated drug distribution are developed; for example, the distribution of ivermectin for onchocerciasis and albendazole for filariasis. Such an approach may conflict with the development of revolving funds to sustain drug supplies at primary healthcare centers, as might distribution for control of schistosomiasis or intestinal helminths targeted through school health programs. In the increasing number of emergency situations, the agencies involved need to be aware of the potential needs of the affected populations as part of their response planning. In this context, a predictive approach to supplying drugs (and insecticides) is necessary and must be based on a knowledge of the ecology of the area and the likely disease problems.

The use of reliable, systematic reviews of evidence of effectiveness to inform policy is becoming recognized as a vital contribution to enabling resources to be used appropriately. The Cochrane Collaboration has developed a series of systematic reviews of randomized control trials that provide a reliable assessment of the effectiveness of various healthcare interventions. This approach has been promoted because traditional reviews are unsystematic and neither respect scientific principles nor control for bias and random error. The approach involves worldwide partners and is designed to build on enthusiasm for the process, to minimize duplication, to avoid bias, to maintain an electronic database, and to ensure wide access in order to make the information available to decision makers. A list of the Cochrane reviews and protocols can be found at www.cochrane.org/cochrane/revabstr/g070index.htm.

The Parasitic Diseases Group is currently compiling a register of published and unpublished trials in parasitic diseases, identified by hand-searching relevant journals, using editors in China, Chile, the USA, and the UK. The review topics include malaria chemoprophylaxis in pregnancy, the use of artemisine derivatives in malaria, chemotherapy of filariasis, and the treatment of neurocysticercosis and giardiasis.

Role of diagnosis in control

Diagnosis of an infection or disease is a fundamental concept of curative medicine. In some parasitic infections, identification of more than simply the presence of the organism is essential if the appropriate treatment is to be given. For example, in sleeping sickness it is important to confirm the stage of the disease, as well as the presence of the parasite; this is because the drug-based treatment for late stage disease (melarsoprol) can be fatal. Hence, treatment is not initiated without confirmation of central nervous system involvement. In other diseases with high prevalence of infection in which mass treatment campaigns are in operation, diagnosis of the individual is not important. Diagnosis of malaria can only be confirmed by microscopical diagnosis; this is rarely undertaken in many settings in Africa. Clinic-based presumption diagnosis is often not malaria and this fact, together with first-line treatment-seeking behavior outside the formal public health sector, means few patients are correctly diagnosed. This results in over-consumption of antimalarials with unwanted consequences for drug resistance as well as increased cost to poor people.

In some diseases, such as schistosomiasis, onchocerciasis, and lymphatic filariasis, for which the overall prevalence is high, it is important that available resources are concentrated on those populations most in need. Therefore, the concept of rapid appraisal of disease burden in various communities has developed so that interventions can be better targeted. Ngoumou et al. (1994) described such an approach to the rapid mapping of hyperendemic onchocerciasis using nodule frequency, which correlates closely with community microfilarial load, as a means of targeting ivermectin distribution. The frequency of hematuria can be used as an indicator for the prevalence of schistosomiasis in school children. Recently, the rapid assessment of prevalence of eye worm (RAPLOA) technique, which uses the local name for the tropical eye-worm symptoms with a photograph of the worm passing through the eye or the presence of Calabar swelling, has been developed in communities in southern Nigeria and Cameroon. Recognition of the condition is valuable in prediction of endemicity.

There is, therefore, a trend away from 'gold standard' parasitological diagnostic techniques when disease-control programs are underway. This does not diminish the importance of diagnosis of the individual infection in need of specific treatment. The need to maintain diagnostic parasitological skills remains, and is particularly relevant, in the provision of diagnostic staff for microscopic diagnosis of malaria. Identification of parasites and differential diagnosis of *Plasmodium* species are important not only in determining treatment but also in saving unnecessary expenditure on drugs by defining whether or not a fever is of malarial origin. Despite the continuing need for skilled microscopists for malaria diagnosis, the amount of training provided is often inadequate, as are the quality of microscopes and staining materials available.

Serological diagnosis for screening populations has been used for surveillance of sleeping sickness. An antibody detection test, the card agglutination test for trypanosomiasis (CATT), and antigen detection tests are used

to screen populations. In those suspected of infection, parasites are detected by examination of blood or lymph by direct or concentration techniques. Similarly, a direct agglutination test (DAT) has been developed to identify suspected cases of visceral leishmaniasis; this should be followed by a parasitological diagnosis, usually by the examination of bone marrow aspirate or splenic biopsy. These techniques are painful and risky, respectively, and not applicable to large numbers of cases.

Diagnostic developments include tests to detect antigens in urine – dip-stick techniques based on enzyme-linked immunosorbent assay (ELISA) systems and using color change as an indicator of the presence of antigens. In the case of urinary schistosomiasis, a color test is used to indicate the presence of blood.

Lymphatic filariasis due to *Wuchereria bancrofti* (and *Brugia malayi*) poses particular problems for diagnosis as the parasites appear in the blood only at night, when it is often difficult to take blood smears. Methods for detection of parasite antigen (Weil et al. 1997) or DNA represent more effective ways of obtaining information about the endemic status of filariasis and of monitoring the impact of control. Nevertheless, while such testing is being evaluated, parallel confirmatory night-time blood samples also need to be taken for monitoring and evaluation purposes at sentinel sites in control programs. Evaluation of lymphatic filariasis programs would also benefit from the availability of a test to detect antibodies resulting from exposure to infective larvae (L3) as a measure of effectiveness of transmission control. The importance of the continuing of monitoring and evaluation throughout a program allows programs to be determined by different methodologies (Figure 3(a–c) and Figure 4(a, b)). It is important to define the role of different age groups in the population in order to focus on incidence following interventions as a true measure of the impact and efficacy of the intervention while recognizing the sensitivity and specificity of any test as well as the sampling frame involved and the sensitivity of the expected population size to census error in defining real incidence prevalence or coverage if an arbitrary figure of success is selected.

There is also a need to develop an alternative diagnostic test to detect onchocerciasis in areas where the disease has been controlled, as skin snipping is no longer acceptable and both prevalence and microfilarial load are low. Here, it is important to determine if transmission is occurring and, although this is undertaken by examining blackflies, this may not be feasible. The ideal requirement for onchocerciasis serological diagnosis would be a test in humans to detect the pre-patent developing adult worm stage of the infection when few if any microfilaria have reached the skin.

The role of diagnosis in the control of parasites varies depending on how the question is approached; for instance, the following factors may need to be compared:

- treatment at the level of the individual for cure versus treatment at the level of the community for control
- safe drug versus toxic drug as a life-saving option
- expensive intervention versus cost-effective intervention
- cheap drug versus free drug
- monitoring control versus obtaining baseline data on prevalence pre-intervention.

The appropriate diagnostic approach depends on the particular situation.

Control of intestinal parasites

The impact on morbidity of intestinal helminths and, to a lesser extent, protozoal infections due to *Entamoeba* and *Giardia*, has been highlighted by the recognition that their public health impact was previously grossly underestimated (Table 1). There is now a body of evidence suggesting that intestinal helminths (*Ascaris*, *Trichuris*, and hookworms) have significant effects on growth, nutritional status, and school performance in children. The opportunity to provide alleviation of worm burdens through single dose, effective, inexpensive drugs (albendazole, mebendazole) is currently being exploited. This approach provides direct benefits as the worm burden is removed, reducing morbidity, and also possibly reducing the rate of transmission. Repeat treatment ensures that burdens are maintained below levels associated with morbidity, preventing protein–energy malnutrition and iron-deficiency anemia.

It is recommended that treatment for intestinal helminth infections is administered without prior screening as prevalence is high. The school-based approach is one of a range of strategies that can be used for helminth control at the community level; it can be integrated into existing systems such as maternal and child healthcare, family planning, water supply, and sanitation, and should be reinforced through health education. In areas where schistosomiasis is endemic, control approaches can be combined, reducing costs, improving compliance, and facilitating the logistics of control. In the longer term, health education, improved environmental sanitation, and safe water supplies are the components essential for the reduction of morbidity caused by intestinal parasites transmitted by the oro–fecal route or through the skin. In school-based programs, it is vital to train teachers about prevention and control, and these topics should be included in the curriculum.

There are continuing plans in some countries for major parasitic disease control programs. China has commenced a national de-worming program for a population of 200 million children; in Mauritius, the World Food Programme is including de-worming in a school meals initiative.

Table 5 *Stratification of malaria epidemiology in relation to approaches to control (after WHO 1993a)*

Malaria type (geographical distribution)	Epidemiological characteristics	Operational	Action required	
			Disease management	Prevention
Savanna malaria. Sub-saharan Africa, Papua New Guinea.	*Plasmodium falciparum* dominant parasite. Perennial transmission but varying seasonality depending on distance from equator. Mortality and morbidity in young children and pregnant women. Drug resistance increasing problem.	Limited coverage by health services. Malaria control programs inadequate. Deficiency of appropriate capacity. Recognize interactions between HIV/AIDS and malaria treatment, control and policy. Increase access of poor to effective service	Increased interest of government, NGO, and private sector in malaria disease. Strengthen capacity for management of severe and complicated malaria. Improve diagnostic services, particularly in view of increased drug costs. Introduce appropriate national drug policies.	Implement impregnated bednets and curtains. Chemoprophylaxis for pregnant women unless precluded by drug resistance.
Malaria of highland or desert fringe (Africa, South East Asia highland; Sahel, South Africa, and South Pacific).	Risks of epidemics due to climate, conflict, changing agriculture practice or migration patterns. Drug resistance an increasing problem	Health services not available or limited. Terrain and distance present obstacles to control. Poor management of malaria where not historically a problem.	Establish epidemic preparedness, strategies, and policies. Active case detection and treatment of fever may be justified. Health services must be made aware of risk potential.	Vector control might reduce or eliminate transmission, particularly indoor residual spraying
Malaria of plains and valleys outside Africa (Central America, China, Indian sub-continent)	Variable, mainly moderate transmission. *P. vivax* may dominate. Strong seasonal variation. Drug resistance established.	Vector control not effective. Inadequate disease management. Health services inadequate; private services available.	Ensure responsibility of malaria control with general health services. Strengthen epidemiological systems.	Improve vector control if cost-effective. Environmental management might have impact hence intersectoral links necessary. Bednets can be useful (China).
Agricultural development projects. All malarious areas.	Increased transmission sometimes associated with irrigation. Seasonal malaria outbreaks in non-immune immigrant workers. Predictability of impact of change difficult to assess.	Insecticide resistance in cotton-growing areas. Finance for malaria control more likely to be available.	Establish services for treatment or strengthen existing ones.	Environmental management should be considered. Site and construct habitation appropriately. Impregnated bednets for labour force. Larvivorous fish in rice growing areas. House spraying and chemoprophylaxis appropriate for work force. Personal protection.

(*Continued over*)

Table 5 *Stratification of malaria epidemiology in relation to approaches to control (after WHO 1993a) (Continued)*

Malaria type (geographical distribution)	Epidemiological characteristics	Operational	Action required	
			Disease management	Prevention
Urban and peri-urban malaria (Africa, South America, South Asia).	Transmission and immunity variable. Increasing drug resistance.	Relatively good access to health services. Antimalarials available from different sources. High human population density. Breeding sites identifiable.	Standardize and harmonize treatment practices. Introduce drug quality and control systems. Facilities specific for malaria treatment required.	Personal protection and prevention. Impregnated bednets.
Malaria of forest and forest fringes South East Asia, South America.	Focally intense transmission. Occupational risk groups (e.g. mining). Severe multi-drug resistance. Non-immune migrant labour at high risk.	Health services inadequate or absent. No or limited social organization in communities. Variety of drugs sold via different outlets. Vector-control efficacy doubtful.	Treatment needs to be continually adjusted depending on experience of response to drug regimes. Establish communication between medical staff involved in treatment. Information, education, and communication of the private sector providers.	
War zone malaria. Conflict/ refugee emergency malaria.	Displacement of parasite-carrying population or non-immunes. Environmental degradation increases mosquito breeding.	Disruption of vector control. Limited curative services available. Drug distribution only via humanitarian assistance. Drugs provided may be inappropriate.	Awareness by responsible authorities of malaria risk. Drugs for treatment available via emergency programs must be appropriate to drug resistance pattern prevailing. Establish treatment facilities.	Refugees and soldiers may be protected by personal protection/chemoprophylaxis for vulnerable groups. Environmental measures and chemical control in refugee camps.

Table 6 *Stratification of epidemiology of African and South American trypanosomiasis in relation to control*

Disease and geographical locality	Epidemiological characteristics	Operational approach	Disease management	Required prevention
Rhodesiense sleeping sickness. East and Central Africa. *T. b. rhodesiense* Vector: *Glossina morsitans*, *S. pallidipes*. *G. fuscipes* in Uganda and Kenya epidemics	Usually zoonosis with range of game and domestic animal reservoirs. Transmission associated with entry into areas where savanna flies feed on game animals. Vector control inappropriate, occupation-associated transmission (e.g. honey gatherers, fishermen, poachers). Epidemics associated with human-to-human transmission by *G. fuscipes* with domestic cattle as reservoir hosts.	Acute disease. Passive case detection in local health facilities by routine diagnostic techniques. Need for active case detection and treatment centers in epidemics.	Accurate disease stage diagnosis; availability of drugs; recognition of toxicity of arsenicals and provision of treatment and care for serious adverse events	Avoidance of high risk areas. No vectors or reservoir control directly targeted but animal trypanosome control by vector control may have impact. Treatment of domestic livestock with trypanocides may reduce human cases. Epidemics stemmed by active case detection and vector control to reduce transmission (traps and targets)
Gambiense sleeping sickness. West and Central Africa. *T. b. gambiense*. Vector: riverine flies, *G. palpalis* group	Person-to-person transmission via riverine flies. Animal reservoirs of limited importance. Site associated transmission around high humidity areas.	Active case detection followed by treatment. Diagnosis by low parasitemias insensitive hence serological tests of value for screening. Vector control to arrest epidemic and halt transmission.	Provide diagnostic facilities at primary health care (PHC) and district level. Ensure drug availability. Maintain surveillance to detect early cases facilitating easier treatment. Provide adequate medical support for treatment and resuscitation in view of risk of side-effects to melarsoprol.	Vector control by community (traps); residual spraying of resting sites. Maintain surveillance to reduce epidemic risk. Prophylaxis with pentamidine no longer employed.
Chagas Disease. South and Central America. *T. cruzi*. Vector: triatomine bugs *Triatoma infestans* in Southern Cone	Zoonosis transmitted by intradomiciliary bugs. High vector infection rate. Many reservoir hosts. Acute disease in children but chronic in adults.	Diagnosis of chronic disease difficult and no effective remedy if diagnosed. Acute disease treatable but drugs expensive and toxic.	Limited efficacy of chemotherapy provides poor prognosis with death through cardiomyopathy. Transfusion disease averted by control of blood via trypanocidal additive (gentian violet).	Vector control by indoor house spraying or other insecticidal approaches – paints, fumigant cans. Improved housing provides beneficial long-term effect.

Table 7 *Stratification of epidemiology and control of leishmaniasis*

Disease and geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Old World visceral leishmaniasis. Indian subcontinent. *L. donovani*. Vector: *P. argentipes*.	Anthroponotic subclinical cases frequent. No animal reservoir. Endemic with periodic epidemics.	Capacity to diagnose is limited. Antileishmanial drugs expensive, toxic, and not easily available. Inadequate reporting and case follow-up. Parasitological diagnosis to be available at district level with serological examination at PHC level if serodiagnostic capacity exists.	Improve diagnostics. Active case detection to reduce human reservoir.	Possible use of impregnated materials or residual insecticide application in epidemics to reduce transmission by sandflies.
East Africa. *L. donovani*. Vectors: *P. orientalis*; *P. martini*.	Reservoir hosts (rodents, carnivores) likely but importance unknown. Suggested association with termite hills in Kenya. Epidemic in S. Sudan associated with movement of population and development of *Acacial Balanites* woodland.	Passive case detection and treatment. Annual incidence requires to be checked by active surveillance. S. Sudan epidemic places major demands on availability of Pentostam worldwide.	Improved diagnostics and drug availability.	Reservoir or vector control not feasible.
Visceral leishmaniasis with canine reservoir or assumed canine reservoir. North Africa, Middle East, Central and South America. *L. infantum*. Vectors: *Larroussius* subgenus; *P. perfiliewi*, *P. perniciosus*, *P. ariasi*. New World *Lutzomyia longipalpis*.	Zoonotic disease with foxes and dogs as reservoir hosts. Endemic over wide area; epidemics are infrequent. Sporadic cases over wide area with subclinical cases likely to be common. Found more frequently in children .	HIV-associated increase in cases in southern Europe through intravenous drug users. Vector control using insecticide impregnated collars on dogs. Diagnosis and treatment of dogs in endemic areas of Mediterranean and S. America has little impact. Reservoir control useful but has limited impact where feral reservoir hosts exist.	Ensure a minimum capacity for passive case detection and treatment.	Serological monitoring and treatment of dogs in Mediterranean. Use of insecticide impregnated collars on dogs.

(*Continued over*)

Table 7 *Stratification of epidemiology and control of leishmaniasis (Continued)*

Disease and geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Anthroponotic cutaneous leishmaniasis. Old World urban centers. *L. tropica*. Vector: *P. sergenti*.	Predominantly found in densely populated settlements. Person-to-person transmission by *P. sergenti*. Incidental hosts not important. Transmission seasonal after sandfly peak.	Passive case detection as a minimum to reduce human reservoir. Integrate any vector control activities (house spraying, impregnated nets, and targets) with malaria control (but rarely sympatric).	Ensure awareness of disease; transmission, diagnosis, and treatment. Ensure drug availability.	Human reservoir control by active case detection. Vector control by residual insecticides in houses where cases found. Check susceptibility of vector to insecticide. Malaria control by house-spraying has impact as do bednets; use of impregnated materials feasible.
Zoonotic cutaneous leishmaniasis. Arid rural areas of Old World. *L. major*. Vectors: *P. papatasi*, *P. dubosqi*.	Reservoir (rodent)/sandfly transmission systems: *Rhombomys*/*P. papatasi* (Asia). *Psammomys*/*P. papatasi* (Middle East/N. Africa). *Arvicanthis*/*P. dubosqi* (sub-Saharan Africa). Settlements around rodent colonies at risk; also workers (e.g. construction) who enter arid environment with rodent colonies.	Passive case detection and treatment with drugs. Live vaccines used in military situations. Rodent control/environmental control possibly effective. Self-healing lesions with total immunity, but high level of disfigurement.	Awareness at health facilities. Availability of drugs.	Leishmanization has been used but 2.5 percent have large nonhealing lesions. Vector control not a cost-effective option. Environmental measures to prevent rodent establishment (e.g. plowing, poisons, flooding).
Zoonotic cutaneous leishmaniasis. East African highlands. *L. aethiopica*. Vectors: *P. longipes*, *P. pedifer*.	Association with hyraxes. Human cases associated with the proximity to hyrax colonies.	Case detection. Epidemiological knowledge of high-risk areas by health services.	Awareness at PHC level of clinical presentation; availability of antileishmanials.	Hyrax eradication possible locally. Vector control ineffective.
'Uta' cutaneous leishmaniasis. Andean highlands/western Cordilleras. *L. peruviana*. Vector: *Lu. peruensis*.	Putative reservoir hosts are rodents (*Akodon* and *Phyllotis*) and dogs. Seasonal transmission. Lesions predominantly in children below school age.	Passive surveillance. Case monitoring.	PHC awareness and case reporting.	Value of vector control is unknown except where proven peridomestic transmission.
Cutaneous leishmaniasis in Northern South America. *L. guyanensis* Vector: *Lu. umbratilis* (possibly *Lu. whitmani* and *Lu. anduzei*).	Forest edentates (Sloths *Choleopus* and anteater *Tamandua*). Reservoir hosts have high infection rate. *Didelphis* (oppossum) secondary peridomestic reservoir hosts where ecology has altered.	Humans infected by encroaching into forest or by establishment of habitation at forest edge.	Case detection and treatment. Availability of antileishmanials.	Vector control of limited impact. Clearing forest up to 300m from habitation reduces incidence.

(*Continued over*)

Table 7 *Stratification of epidemiology and control of leishmaniasis (Continued)*

Disease and geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Cutaneous leishmaniasis in Central America/Northern South America. *L. panamensis*. Vectors: *Lu. trapidoi*, *Lu. gomezi*, *Lu. ylephiletor*, *Lu. panamensis*.	Also associated with forest penetration or association with secondary forest. Variety of reservoir hosts: edentates, primates, dogs, and rodents.	Passive case detection; awareness of risk for those entering or living in forest.	Case detection and treatment. Availability of antileishmanials.	Clearing of forest around villages reduces transmission. Vector and/or reservoir control not practicable.
Cutaneous and mucocutaneous leishmaniasis in Central and South America. *L. braziliensis*. Vectors: *Lu. wellcomei*, *Lu. intermedia*, *Lu. whitmani*.	Infection associated with forest activities and clearing land. Perennial transmission with high incidence in at-risk groups. Reservoir host not proven and many suspected vectors. In urban/peri-urban environment dogs, horses and pigs have been incriminated as reservoir hosts.	Espundia (mucocutaneous lesions) develop after varying periods following self-healing primary lesions.	Passive case detection; availability of drugs. Essential care of espundia cases to reduce sequelae of lesions. Commercially available vaccine probably not efficacious.	No vector or reservoir control applicable in forest but vector control possible in peri-urban situations, personal protection might help if affordable.
Cutaneous leishmaniasis in South and Central America. *L. mexicana* complex. Vectors: *Lu. olmeca*, *Lu. flaviscutellata*.	Variety of ecological situations – wet forest in Igapo, Brazil; dry forest in Yucatan; fringe forest in Dominican Republic. Rodent and marsupial reservoir hosts; highly dispersed and diverse.	Associated with penetration into forest environments.	Passive case detection, PHC, awareness, and availability of drugs.	Reservoir and vector control not possible. Personal protection in forest; reduce risk by not sleeping in forest. Health education.

Table 8 *Stratification of epidemiology and control of onchocerciasis*

Geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Africa				
South of Sahara. Former OCP countries.				
1 Original areas: Benin, Burkina Faso, Cote d'Ivoire, Ghana, Mali, Niger, Togo.	1 Savannah woodland of northern tropics. Hyper-endemic villages close to rivers. Historically associated with high blindness rate resulting in depopulation. Major migratory vectors in savanna are *S. damnosum* and *S. sirbanum*. Annual transmission potential of more than 100 was associated with high blindness risk. Disease controlled by vector control since 1974 except in limited areas of Togo (Oti basin)	1 Vector control by weekly aerial larviciding for 14 years eliminates adult worms in human reservoir. Ivermectin twice per year for foci with inadequate vector control.	1 Vector control applied due to lack of availability of community-based drug until 1986. Thereafter, ivermectin for morbidity control to reduce community microfilarial load (CMFL) and arrest development of ocular lesions.	1–6 Maintain surveillance system for recrudescence using tests for early detection of transmission. Institute ivermectin if appropriate. Establish appropriate surveillance via integration into public health systems. Possible detection of early transmission by detection of infective larvae in blackflies. Maintenance of ivermectin distribution. Ensure high and sustainable delivery via community-directed treatment assisted by NGOs.
2 Extension areas: southern areas of Ghana, Togo, Benin; western extension to Guinea, Guinea Bissau, Sierra Leone, Senegal.	2 In forest areas, less blinding form of *O. volvulus* transmitted by forest vectors *S. soubrense*, *S. yahense*, *S. sanctipauli*, *S. squamosam*, *S. leonense* (Sierra Leone), which are less efficient and nonmigratory or less migratory.	2 Combined vector control and Ivermectin for 12 years projected to reduce disease to nonsignificant level. Ivermectin alone in areas of low CMFL (<10) where no invasion of *Simulium* threatens original OCP area.	2 Ivermectin throughout for extended periods up to 20 years as ivermectin does not interrupt transmission.	
3 Non-OCP countries: East of Benin to Sudan.		3 Ivermectin through community-based distribution systems.	3 Impact of ivermectin on skin disease and other manifestations of *O. volvulus* infection to be assessed.	
4 West and Equatorial Africa: Guinea-Bissau to Gabon; Rain forest.		4 Ivermectin through community-based distribution systems.		
5 Zaire Basin.		5 Ivermectin through community-based distribution systems.		
6 East Africa highlands: Ethiopia to Southern Malawi.		6 Focal vector control could achieve eradication of *S. damnosum* or *S. neavei*. Ivermectin through community-based distribution systems.		

(*Continued over*)

Tabl 8 *Stratification of epidemiology and control of onchocerciasis (Continued)*

Geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Central America				
Guatemala; Mexico in well defined foci. Cross-border movement of migrant workers may spread the disease.	Anthrophilic *S. ochraceum* in highland foci; *S. metallicum* more zoophilic also involved. Inefficient as vector due to buccal armature: but compensated by vector abundance.	Control by ivermectin distribution via national programs through twice-yearly distribution.	Ivermectin-based.	Ivermectin distribution over years will reduce morbidity of skin and anterior segment eye disease
South America				
1 Brazil, S. Venezuela	1 Primary vectors: *S. oyapockense* and *S. guianense*. Secondary vector: *S. exiguum*.	Vector control not feasible. Integration of ivermectin with other programs.	Ivermectin-based.	Ivermectin distribution over years will reduce morbidity of skin and anterior segment eye disease.
2 N. Venezuela	2 Primary vector: *S. metallicum*. Secondary vector: *S. exiguum*.			
3 Ecuador, Columbia.	3 Primary vector: *S. exiguum*.			
Yemen				
Distributed along Wadis.	Vector: member of the *S. damnosum* complex (*S. rasyani*)	Ivermectin-based control initiated.	Ivermectin.	Ivermectin control embarked on.

Table 9 *Stratification of epidemiology and control of lymphatic filariasis (LF) (see Molyneux and Zagaria 2002)*

Geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Tropical America: N E Brazil, Guyana, Surinam, French Guyana, Haiti, Dominican Republic, Costa Rica, Trinidad and Tobago.	Focal transmission by *Culex quinquefasciatus*. Varying levels of endemicity: Guyana endemic and about to launch program based on DEC salt. Haiti and Dominican Republic using DEC + albendazole. Surinam, Trinidad and Tobago, and Costa Rica have eliminated public health problem by DEC selective treatment	Limited control programs organized through Ministry of Health (in Brazil SUCAM). Urban mosquito control difficult. Strategy based on annual DEC + albendazole treatment of all eligible population. Introduction of home care for lymphedema	Opportunities for introduction of new approaches. Treatment using topical antibiotics, daily washing with soap and water of affected limbs thorough interdigital drying, limb elevation, and breathing. Single annual dose DEC + albendazole donated by GlaxoSmithKline	Very limited control implemented except in Recife. Potential for all appropriate measures to reduce transmission by single annual treatment of DEC + albendazole or DEC salt.
Tropical Africa: broad transmission zone in sub-Saharan Africa.	Urban filariasis *Culex quinquefasciatus* transmitted in E. Africa and Madagascar. Filariasis transmitted by *A. gambiae* complex and *A. funestus* in E. Africa and W. Africa. Mapping ongoing elsewhere to define implementation units for national control programs	Up-scaling control programs; nine countries active. Strategy based on ivermectin + albendazole where onchocerciasis and filariasis are co-endemic. DEC + albendazole elsewhere (Kenya, Zanzibar and Comoros).	Disease problems have been underestimated; hydrocoele surgery significant burden on district hospitals (30 percent of surgical interventions). Increase the development of home-based lymphedema management.	Pilot projects in Zanzibar using polystyrene beads for larval *Culex* control; impregnated bednets and, in urban areas, *Bacillus sphaericus* larval toxins. Development lingages and evaluate bednet programs on LF as part of malaria control.
Middle-East: Egypt	Urban and peri-urban transmission in Cairo and Nile delta. Resurgence since control relaxed in 1965 resulting in prevalence of c. 20 percent. *Culex molestus* and *C. quinquefasciatus* vectors.	DEC + albendazole campaigns active in all endemic villages in the Nile Delta (at-risk population 2.4 million) three rounds of drug distribution completed by end 2002. Vector control limited.	As above	Vector control likely to have limited impact; chemotherapeutic preventive approach will reduce transmission
Indian sub-continent: South Asia, India, Sri Lanka, Bangladesh, Nepal, Vietnam, Thailand, Myanmar.	*C. quinquefasciatus*, *Anopheles* species. Urban and semi-urban with high clinical morbidity particularly in India.	Variable depending on country and resources available. Chemotherapy via DEC + albendazole or DEC fortified salt. Mosquito control of limited impact.	As above. Surgery for serious deformities widely practiced in India, especially for hydrocoele, to reduce morbidity. Heavy cost burden on surgical services.	Mosquito control measures of limited impact. Define impact of DEC + albendazole on morbidity

(*Continued over*)

Table 9 *Stratification of epidemiology and control of lymphatic filariasisLF (see Molyneux and Zagaria 2002) (Continued)*

Geographical locality	Epidemiological characteristics	Operational approach	Disease management	Prevention
Pacific rim and islands	*Aedes* vector on Pacific islands. *Anopheles punctulatus* in Papua New Guinea, Vanuatu. *C. quinquefasciatus* in China. In the Solomon Islands *W. bancrofti* eliminated by malaria vector control in 1970s.	DEC + albendazole once a year for at least 5 years	As above.	Vector control difficult although some success in trials with *B. sphaericus* biocides. DEC salt and DEC + albendazole. Bednets contribute to reducing transmission in *Anopheles* settings (e.g. Vanuatu). Solomon Islands remain LF-free after elimination by indoor spraying of DDT in antimalaria campaign in 1980s
South Asia, India, Malaysia, Indonesia, China, Philippines, Vietnam. *Brugia malayi*, *Brugia timori*	*Mansonia*, *Anopheles*. In some areas animal reservoirs exist (cats and *Presbytis*, leaf monkeys).	Community participation in environmental vector control (weeding and fish culture) and chemotherapeutic control in India; use of DEC + ivermectin and DEC salt in same manner as for *Wuchereria bancrofti* filariasis.	As above. Treatment using topical antibiotics, hygiene of infected limbs in Kerala, India to ameliorate acute episodes.	Chemical control against *Mansonia* difficult due to variable adult behavior and larval association with weeds (dependence on environmental control).

Table 10 *Stratification of epidemiology and control of schistosomiasis*

Geographical locality	Epidemiological features	Operational approach	Disease and management	Prevention
Africa, South America, and Caribbean				
Schistosoma mansoni, *S. haematobium* widespread in Africa. *S. intercalatum* is limited in West and Central Africa. *S. mansoni* in South America and Caribbean	*S. mansoni/Biomphalaria* transmitted. *S. haematobium/ Bulinus* transmitted. *S. mansoni* has peak prevalence in 10–24-year-olds; heaviest parasite loads 10–14 age group. *S. haematobium* peak prevalence and intensity 10–14-year-olds.	Linkage to other sectors in planning prevention and control via appropriate water-resource management. Population movement to be monitored in context of potential disease impact after exposure to new water-related development. Development of link to schools for chemotherapeutic control and for health education. Assessment of potential for molluscicide.	Can result in significant morbidity and mortality, placing considerable burden on curative services. Severity of symptoms generally depends on intensity of infection. *S. mansoni* symptoms associated with hepatic, splenic, and intestinal systems. *S. haematobium* pathology concentrated in genital and urinary systems and lower intestine. Use of hematuria as rapid assessment tool for intervention priority.	Primary prevention is reduce access to contaminated water via improved management of water resources; involvement of other sectors in planning development projects. Chemotherapy assists in reducing infection reservoir; snail control may supplement control operations and be planned into irrigation schemes or impoundments. Health education via schools and community health providers. Maintenance of drug availability.
Middle East/North Africa				
S. mansoni and *S. haematobium*.			Praziquantel can reverse symptoms of severe disease. Carcinomas associated with schistosomal disease but precise etiology not established.	
South East Asia, China, Philippines, Thailand				
S. japonicum, *S. mekongi* in Kampuchea Laos.	*S. japonicum* transmitted by amphibious *Oncomelania* with many animal hosts (cattle, dogs, rats, pigs). *S. japonicum* has no typical age prevalence or intensity; variable depending on epidemiology. *S. mekongi* transmitted by *Neotricula* snails (dog reservoir).	National programs in China developed via communist structures in integrating chemotherapy and snail control. In Philippines, approaches to control through snail control, case detection and treatment, environmental sanitation, and health education. Redirection with increased emphasis with availability of praziquantel.	*S. japonicum* disease similar to *S. mansoni* with most severe disease between ages 2 and 40. Praziquantel effective in treatment.	

Operational phases of control operations: Phase 1 – planning data gathering; planning; resource allocation. Phase 2 – attack/intervention. Phase 3 – maintenance.

Table 11 *Recent epidemics of parasitic diseases, causes, impact and control*

Disease and geographical location	Putative cause	Epidemiological impact	Control problems and approaches
Malaria			
Madagascar, Highland malaria in Ethiopia, Kenya.	Change in agricultural patterns, interruption of control, environmental degradation (changing larval habitats), resettlement schemes , temperature change, and increased rainfall following period of drought. Non-immune population.	Over 25 000 deaths in Madagascar in 1988.	Institute epidemic preparedness policy. Ensure availability of appropriate drugs.
Forest-fringe malaria in South East Asia, South America.	Penetration into forest for gem and gold mining. Non-immune population exposed.	Epidemics in N.E. Kenya in 1999 following El Niño rains	Establish vector-control capacity and acquire insecticides.
Refugee camps.	New breeding sites; non-immune populations move to lower altitudes (e.g. Rwanda).		Inform and support local health structures and NGDOs.
Leishmaniasis			
Visceral leishmaniasis in South Sudan.	Ecological changes provide enhanced breeding sites for *P. orientalis* in *Acacia/ Balanites* woodland.	5 000 cases/year; 100 000 at risk – 75 percent mortality in children reported.	NGO-initiated treatment centers. Provision of emergency drugs. Limited diagnostic facilities. High incidence of post-kala azar dermal leishmanoid PKDL. Treatment established. Availability of drugs problematic.
Cutaneous leishmaniasis in Khartoum.	Population movement and breakdown of health services in conflict zone.	Outbreaks in Khartoum with 100 000 cases due to *L. major*.	Chemotherapy; vector control; rodent control.
Cutaneous leishmaniasis in Kabul, Afghanistan.	Lack of diagnostic capacity, drugs, and means of delivery.	New urban foci of transmission in *P. sergenti*. 4 000 cases in urban areas and 6 000 from provinces.	Active case detection and treatment; insecticide treatment of houses in cities; health education units.
Trypanosomiasis			
T. b. rhodesiense epidemic in Busoga, Uganda.	Change in ecology due to encroachment of *Lantana* following civil disturbance and change agricultural practices.	Up to 8 000 cases/year in 1980.	Surveillance and treatment. Establishment of treatment centers. Vector control via impregnated traps.
T. b. gambiense in Zaire–South Sudan, Angola	Breakdown of surveillance through disruption of health services.	Increased incidence.	Increase surveillance and treatment but coverage limited, drugs not available.
Schistosomiasis			
S. haematobium and *S. mansoni* epidemics increase associated with water-impoundment and rice irrigation throughout Africa.	Non-immune migrant population exposed for first time. *Bulinus senegalensis* breeds in irrigation systems; increase in snail populations. *Biomphalaria* increases. Major dam projects (Aswan, Egypt; Diama, Senegal and Akosombo, Ghana) are a well-recognized cause but micro-dams in West Africa provide additional and extensive new problems. War and refugee migration.	Increased incidence, high morbidity in non-immune populations.	Increase availability of chemotherapy. Target if possible through schools. Snail control or environmental management if feasible. Health education.

Table 12 *Widely used tools for parasitic disease control – diagnostics*

	Diagnostic tools		
Disease	**Personal diagnostic test (direct)**	**Personal diagnostic test (indirect)**	**Community assessment**
American trypanosomiasis		Serodiagnostic test using synthetic peptides.	Agglutination test for blood bank screening.
Dracunculiasis	Direct observation of worm emergence.		Use of village volunteers to identify cases and contain them.
Food-borne trematode infections	Kato technique (i.e. cellophane-fecal thick smear).	Intradermal screening for paragonimiasis.	
Leishmaniasis	Cutaneous: parasitological diagnosis by smear, culture.	ELISA immunofluorescence.	
	Visceral: parasitological diagnosis by bone-marrow puncture, spleen aspiration.	DAT.	
Lymphatic filariasis	Night blood films for nocturnally periodic forms; day blood films for diurnally periodic forms.	Assays to detect circulating filarial antigen by immunochromatographic test. 'Brugia Rapid' has similar characteristics for *Brugia*.	Hydrocoele survey; questionnaires to district hospitals for records; community assessment of lymphoedema rates.
Taeniasis, cysticercosis, neurocysticercosis	Fecal smear.	Coproantigen detection; CAT scan; NMR scan.	High prevalence of late-onset epilepsy; local pig husbandry, meat inspection, contaminated carcass detection.
Loa loa	Day blood films, presence of tropical eye worm or Calabar swelling.	No immunological methods available.	RAPLOA; use of remote sensing to determine areas of risk of high prevalence.
Malaria	Light-microscopic examination of Giemsa-stained blood films.	See Malaria.	
Onchocerciasis	Skin snips.	DEC patch test as methodology for rapid diagnosis (mini Mazzotti).	Rapid assessment of nodule prevalence.
Schistosomiasis	Kato technique for: *S. intercalatum*, *S. japonicum*, and *S. mansoni*.	Quantitative urine filtration technique for *S. haematobium*.	Direct observation for gross hematuria, indicative of heavy *S. haematobium* infection in children in endemic areas.
			Detection of microhematuria by reagent strips (*S. haematobium*).
Sleeping sickness	Blood film.	Lymph gland palpation.	
	CSF examination.	CATT.	
	Bone-marrow aspiration.	Capillary tube centrifugation.	

Table 13 *Widely used tools for parasitic disease control – chemotherapy*

Disease	Treatment tools		
American trypanosomiasis	Acute phase: nifurtimox.	Blood bank transmission control: crystal violet/sodium ascorbate to kill parasites in infected blood.	
Dracunculiasis	Treatment aimed only at superinfections.		
Food-borne trematode infections	Praziquantel.		
Leishmaniasis	Pentavalent antimonials.	Amphotericin B.	Pentamidine.
Lymphatic filariasis	DEC + albendazole outside areas of onchocerciasis endemicity.	Ivermectin + albendazole in onchocerciasis-endemic areas.	
Malaria	Sporontocidal: proguanil, pyrimethamine, atovaquone. Tissue schizontocidal: primaquine, proguanil, tetracycline. Gametocytocidal: primaquine, quinine, mefloquine, chloroquine, amodiaquine. Blood schizontocidal: quinine, mefloquine, halofantin, chloroquinine, amodiaquine, atovaquone, artemisinins, pyronaridine, tetracycline.	Sulfadoxine/pyrimethamine or sulfalene/pyrimethamine, mefloquine.	Severe disease: quinine (+ tetracycline where needed). Artemether where quinine resistance.
Onchocerciasis	Ivermectin (single dose mass administration once a year). In the Americas, twice yearly treatment given.		
Schistosomiasis	Praziquantel.	Metrifonate.	Oxamniquine.
Sleeping sickness	Early stage: pentamidine (*T. b. gambiense*). Suramin (*T. b. rhodesiense*).	Advanced stage: melarsoprol for both forms. Eflornithine if available for melarsoprol-resistant cases of *T. b. gambiense*.	For arsenical-resistant cases of *T. b. gambiense* (mainly): eflornithine; nifurtimox has been produced for drug-resistant cases on humanitarian grounds.
Taeniasis, cysticercosis, neurocysticercosis	Praziquental for *T. solium*.	Praziquental given as mass chemotherapy can reduce prevalence of cysticercosis.	

Table 14 *Widely used tools for parasitic disease control – vector control*

Disease	Vector control tools		
American trypanosomiasis	Indoor residual spraying with pyrethroids.	Pyrethroid paint.	Fumigant canisters with pyrethroids.
Dracunculiasis	Temephos.		
Food-borne trematode infections	Environmental management, including land reclamation and drainage.	Niclosamide (molluscicide).	Biological control (natural predators).
Leishmaniasis	Residual insecticide spraying (DDT, pyrethroids).	Insecticide-treated nets have impact where transmission is in houses (Middle East *L. tropica* urban foci and in South America).	Insecticide-impregnated collars for dogs, which are reservoir hosts of *L. infantum* (Gavgani et al. 2002).
Lymphatic filariasis	Impregnated bednets in areas where disease is transmitted by *Anopheles*.	Polystyrene beads (if feasible) in settings where disease is transmitted by *Culex*; integrated control in Pacific where dengue control for *Aedes* is implemented.	Pyrethroid-impregnated bednets and curtains.
Malaria	Pyrethroid-impregnated bednets and curtains.	Indoor residual spraying (DDT, organophosphates, carbamates, pyrethroids).	Environmental management.
Onchocerciasis	Larviciding though aerial spraying (in rotational use: *B.t* H-14, temephos, pyraclofos, pyrethroids).	Ground larviciding employed in some settings where access is easy; *Simulium neavei* eliminated from several areas of East Africa by larvicides; deforestation reduces *S. neavei* populations.	
Schistosomiasis	Niclosamide (molluscicide).	Environmental management of irrigation systems.	
Sleeping sickness	Pyrethroid-impregnated screens.	Traps; pyrethroid-impregnated and non-impregnated.	

Table 15 *Examples of changes in* Plasmodium falciparum *:* P. vivax *ratios associated with anthropogenic change (conflict, irrigation, mining)*

Location	Projected changes	Reference
Tajikistan	Health systems disruption, conflict migration, chloroquine resistance in *P. falciparum*.	Pitt et al. (1998)
Afghanistan/Pakistan	Chloroquine resistance in *P. falciparum*.	Rowland and Nosten (2001)
Sri Lanka, Mahaweli	Irrigation on large scale.	Amerasinghe and Indrajith, 1994
Thar Desert, Rajasthan, India	Irrigation on large scale. Establishment of *Anopheles culcifacies* as efficient *P. falciparum* vector and dominance over *An. stephensi* (a poor vector).	Tyagi and Chaudhary (1997)
Amazonia, Brazil	New breeding sites for efficient vectors *An. darlingi* through mining, deforestation, road building.	Marques (1987)

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Diagnosis of parasitic infections

STEPHEN H. GILLESPIE

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INTRODUCTION

The ubiquity of human parasites is such that their diagnosis should be of importance to all involved in the management of patients with infection. Yet this branch of diagnostic microbiology is often strangely neglected. Parasite diagnosis frequently poses unique problems requiring novel solutions. The diversity of agents requires, on the part of the diagnostician, an encyclopedic knowledge covering pathogens that are imported and genuinely rare. Also, it requires the application of the full range of microbiological diagnostic methods. The majority of parasite diagnoses are made in a laboratory whose major interest is bacteriology but there are a number of important differences in the approach required for success. This chapter outlines the factors necessary for successful diagnosis of parasitic infections.

Life-cycle and natural history

Humans often form only one part of a complex parasitic life cycle. It is important to understand the way in which the organisms interact with the host if optimal diagnosis is to be obtained. For example, *Toxocara canis* is the ascarid parasite of canids and is unable to complete its life cycle in humans and other paratenic hosts (Gillespie et al. 1993). The diagnosis can be made in canids by examining stools for the presence of the characteristic eggs (Habluetzel et al. 2003). In contrast, in paratenic hosts such as the human, this approach will fail because parasite development is blocked at the L2 larval stage and adults cannot be found in the stool; it is thus essential to diagnose this infection by serological means.

Giardia has a simple life cycle which may only involve humans (and potentially an animal reservoir). The diagnosis is made simply by examining stools for the presence of *Giardia* cysts. However, a proportion of cases may not be diagnosed by this means and symptoms consistent with giardiasis may persist. In this instance, the fact that the protozoa preferentially infect the duodenal–jejunal junction means that this site can be sampled directly, either by duodenoscopy and biopsy or by the string test (see String test below).

Knowledge of the life cycle and natural history can indicate when to sample. For example, patients who have been exposed to schistosomiasis by swimming in contaminated water may present soon after their return from an endemic country. They may already have the symptoms and signs of Katayama fever. However, it would be futile to attempt to make a diagnosis by microscopic examination of stool or urine as it is likely that adults will not have matured and begun to deposit eggs. Later in the infection, the excretion of eggs may not be regular: in *Schistosoma haematobium* infection, eggs are preferentially excreted around mid-day. To obtain maximum yield, urine should be collected at this time. Similar considerations apply to the diagnosis of filarial infections where these parasites have become adapted to the circadian rhythms of the host. In areas where mosquitoes are night-biting, the maximum diagnostic yield can be obtained by sampling between midnight and 2 a.m. (Simonsen et al. 1997).

The pathogenic mechanisms of the parasite may affect the choice of specimen. *Plasmodium falciparum* produces knobs on the surface of the red cell that make it adhere to capillary endothelium, a point of considerable importance in the pathogenesis of cerebral

malaria (Waller et al. 1999). It also has importance for laboratory diagnosis as capillary blood has a higher diagnostic yield than the venous blood traditionally sampled by doctors in developed countries. The microfilariae of *Onchocerca volvulus* can be found in the skin, but their distribution is not uniform. Knowledge of the normal distribution of microfilariae and the variations in different geographical locations will increase the diagnostic yield and reduce the number of (painful) pinch biopsies required to make a diagnosis (Scheiber et al., 1976).

Resources

The world is ill-divided, especially when it comes to infectious diseases. The poorest members of the world community have a disproportionate number of the world's parasites. Thus, those with the greatest need of parasite diagnosis have the least economic resource to acquire it (World Bank 1993). This financial fact of life is one that those charged with parasite diagnosis must bear in mind in delivering diagnostic services. It means that resources should be targeted on the most important clinical problems. For example, concentration techniques increase the yield of positive results for protozoal cysts and helminth eggs, but the cost of reagents and the expense of purchase and maintenance of a centrifuge may mean that simple wet preparations should be employed (Ramsay et al. 1991). Such compromises may not be as damaging as a superficial assessment might suggest. For individuals living in an endemic area and constantly at risk of acquiring infection, sensitive diagnosis may result in many treatment courses where the balance of benefit between parasite clearance and drug adverse events is uncertain.

The same considerations must be made in relation to technology. A range of diagnostic approaches can be applied to protozoa and helminths, but many of these methods are too expensive to be applied in the areas where the infections are most common.

Objectives of parasitic diagnosis

PATIENTS FROM NON-ENDEMIC AREAS

Many individuals now travel to countries where tropical parasites are endemic. Returning travelers will have some clear diagnostic demands. They may be very concerned about the risk of tropical parasites, no doubt enhanced by lurid stories from hardened expatriates. Their objective in presenting for diagnosis is to exclude the risk of an infection which is not yet causing symptoms (MacLean and Libman 1998). It may be that such a disease is evolving but the traveler is presenting before the infection has had time to become patent. Moreover, as infections in travelers are often light, the disease may present in a more subtle way than that described in the textbooks of parasitology and tropical medicine.

Travelers often lack the immunity that inhabitants of endemic countries possess. This may have a significant impact on the natural history of infection and a change in the speed at which diagnosis is required. *P. falciparum* is rapidly progressive in immunologically naïve subjects. Thus, when malaria is suspected in such a patient, the diagnosis of malaria should be made rapidly.

Patients from an endemic country with *Entamoeba* cysts in their stool rarely need further investigation. However, for a patient living in a country where this disease is rare, it would be useful if a species-specific diagnosis could be made. It is normal practice to attempt to eradicate cyst passage in returning travelers. To prevent unnecessary treatment in patients with *Entamoeba dispar* infection, a species-specific diagnosis is required (Sanchez-Guillen et al. 2002).

PATIENTS IN ENDEMIC AREAS

Most health facilities in endemic areas experience a severe shortage of resources for medical services. These scarce resources are often disproportionately directed towards clinical services. This frequently means that laboratories charged with the task of making a diagnosis lack the necessary resources to do so. Hospital managers often do not recognize that resources spent in obtaining an accurate diagnosis may produce savings in medical and pharmacy costs. Faced with this, it is essential that laboratory managers choose wisely which diseases to target and which methods to employ. Many patients have had to travel long distances to access healthcare. This means that, where possible, methods should be employed that provide a rapid diagnosis. The patients are now often required to make a financial contribution towards the cost of their laboratory costs and this may also serve to limit the scope of testing. These considerations do not necessarily mean that a poor quality service is the inevitable result. Although recent diagnostic developments have achieved similar sensitivity and specificity as a thin blood film examined by a trained microscopist, the latter remains the optimum diagnostic method for most parts of the world (Humar et al. 1997). Also, although limited resources probably limit stool examination to a direct saline wet preparation, this method will reliably diagnose the individuals that are most heavily infected with helminths and are thus most likely to benefit from treatment (Ramsay et al. 1991).

DIAGNOSIS FOR PUBLIC HEALTH

The approach to diagnosis required for public health purposes is often very different from that for routine curative services. In many instances, the questions posed are quite dissimilar and this has an impact on the methods employed. For example, for a patient who is thought to have schistosomiasis, the identification of a single egg in stool or urine is all that is required to initiate therapy. However, in a schistosomiasis control

program, although this sort of result will be useful in quantifying the number of patients with infection, it is more likely that it will be necessary to quantify the worm burden of people living in infected communities. Thus, for *S. mansoni* infections, it would be necessary to perform a Kato-Katz investigation that would give a useful estimate of the number of eggs per gram, and thus the overall worm burden (Katz et al. 1972). To characterize the nature of hookworm infection, culture is necessary but this would have no impact on the management of the patient's illness (Harada and Mori 1955). In addition, parasite field studies often need to process specimens from a very large number of patients in a short time and methods must be adapted to this requirement.

Serology has limited value in the diagnosis of parasitic infections in endemic areas, but serological techniques are often employed in public health surveys as a way of rapidly determining the number of patients who have current or past infection with a particular pathogen.

In determining the epidemiology or epizoology of parasitic infections, it may be necessary to employ complex typing techniques to characterize pathogens identified in different communities. For example, the identification of a species of *Leishmania* might be sought so that its method of transmission could be better understood for control purposes. To achieve this, microsatellite DNA analysis and multilocus enzyme electrophoresis can be employed (Banuls et al. 2002; Barker 2002). Such techniques are not required for diagnosis in routine practice.

DIAGNOSIS FOR RESEARCH

Good-quality field research of parasitic infection should ask a very specific question for which diagnostic methods may be required. For example, if the nature of drug-resistant malaria is under investigation, it might be necessary to deploy DNA amplification techniques to determine the presence of various drug-resistance genes (Dorsey et al. 2001).

SPECIMENS

Any tissue or body fluid can be used for the diagnosis of parasitic infections. However, it is essential that the right specimen is obtained for diagnosis and in the right condition. It is pointless to spend time searching a stool for the presence of amebic trophozoites unless the stool is both fluid and fresh: amebic trophozoites rapidly lose motility as the temperature falls. Although females of *Trichinella spiralis* are found in the intestine and produce symptoms of diarrhea, the diagnosis of trichinosis is made by serology or by the examination of a muscle biopsy (see *Trichinella*).

Stool samples

A minimum of three samples of stool is necessary to make a diagnosis of intestinal infection. As excretion of parasite eggs and cysts can be intermittent, it is preferable that specimens be sent on different days. To identify the trophozoite stage of *Entamoeba* and *Giardia*, freshly passed stools should be examined. If this is not possible, it may be necessary to fix the specimen for later examination. It may not be possible to make a diagnosis of amebiasis from a stool sample but motile trophozoites may be observed in scrapings from intestinal ulcers visualized on sigmoidoscopy.

Duodenal/jejunal biopsy or Enterotest may be needed to sample the jejunal fluid to diagnose giardiasis (Goldsmid and Davies 1978). A summary of the parasites that can be diagnosed using intestinal samples is listed in Table 1. Each of the modalities of laboratory diagnosis may need to be employed.

MACROSCOPIC EXAMINATION

It is essential to note the consistency of the stool to be examined. This can guide the technologist as to whether or not there is purpose in trying to identify trophozoites

Table 1 *Methods and pathogens that can be identified in intestinal specimens using these methods*

Sample	Method	Examples of pathogens identified
Stool	Microscopic examination of unstained and stained preparations	Protozoa: *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Isospora belli*, Microsporidia
		Helminths: *Trichuris trichiura*, hookworms, *Ascaris lumbricoides*, *Schistosoma* spp.
	EIA, nucleic acid amplification	*Cryptosporidium parvum*, *E. histolytica*, *G. lamblia*
	Culture	*Strongyloides stercoralis*, speciation of hookworm
Adhesive tape sample, anal swab	Direct microscopy	*Enterobius vermicularis*
String test, duodenal fluid examination	Microscopy, EIA PCR	*G. lamblia*, *Cryptosporidium parvum*, *S. stercoralis*
Sigmoidoscopy	Direct examination and ulcer scrapings	*E. histolytica*
Proctoscopy	Rectal biopsy	*Schistosoma mansoni*

or cysts. Also, a negative result is often more easily explained to a clinician suspecting diarrheal disease if the fact that the stool is formed is noted. The presence of proglottids can be confirmed by macroscopic examination of the stool and sometimes adult ascarid worms can be found in stools.

MICROSCOPIC EXAMINATION

Direct saline wet preparations have the virtue of speed and cheapness. Unfortunately, this method of examination is less sensitive than the concentration techniques. Direct saline preparations are required if the trophozoite stages are to be seen. Quantification of the number of eggs present in stools may be achieved by variants of the Kato-Katz method (Katz et al. 1972).

Staining of stools can improve the diagnostic yield by making the target pathogen more easy to identify. Modified acid fast preparations or the use of UV illumination for the examination of auramine-stained preparations make the identification of *Cryptosporidium* and *Isospora* much easier (see Intestinal coccidia: Cryptosporidiosis, isosporiasis, cyclosporiasis). In the same way, modified trichrome, Starey-Warthin, and calcofluor white may assist the diagnosis of microsporidiosis (see Microsporidiasis). Rapid specific diagnosis can be obtained by the use of direct immunofluorescence (Kuczynska et al. 2003). For the diagnosis of microsporidiosis, direct immunofluorescence is probably essential. These techniques have proved of particular value for the diagnosis and quantification of intestinal protozoa in water supplies.

To improve the sensitivity of diagnosis, stools should be concentrated. A wide variety of methods is reported in the literature. Many of these methhods require expensive reagents and equipment such as a centrifuge, but others have been adapted for resource-poor communities (Ramsay et al. 1991). Different techniques are required for different pathogens and the optimal method should be employed for any specific target pathogen in, for example, a research study. To overcome the need to employ more than one concentration method, many laboratories choose to use an all-purpose method that gives good all-round performance. The formol–ether method and its variants are widely used as they provide good concentration of protozoal cysts and helminth eggs while also removing fecal debris (Allen and Ridley 1970). Commercially prepared centrifugation tubes can make this technique easier to apply.

IMMUNOLOGICAL AND MOLECULAR DIAGNOSTICS

Enzyme immunoassays can also be used to process stool samples. These methods have the advantage that they can be automated. Commercially produced diagnostic tests are now available for most intestinal protozoan pathogens (Garcia et al. 2000). In the same way, primer pairs for the main intestinal protozoa are now available and provide enhanced sensitivity. With the appearance of real-time polymerase chain reaction (PCR) equipment, it is now possible to make a rapid specific diagnosis of intestinal protozoal infections using these techniques (Bialek et al. 2002).

CULTURE METHODS

One of the major differences between parasitology and most other branches of microbiology is the fact that culture methods are rarely used. However, there are a limited number of situations where cultures of parasites are essential for a complete diagnosis. *Strongyloides* larvae are difficult to identify in stools, and an improved yield can be obtained by culturing stools. Cultures can reveal the presence of larvae when their numbers are low (Arakaki et al. 1990). Culture is also essential to permit species identification of hookworms (Harada and Mori 1955).

The viability of schistosome eggs can be determined by microscopic examination to see the cilia of the 'flame' cell. Alternatively, it is possible to hatch the miracidium from the eggs – the ultimate proof of viability. Fresh stool or urine specimens are placed in ten volumes of unchlorinated water (e.g. spring water) in an Erlenmeyer flask. The flask is covered and the sediment examined after 24 hours for the presence of miracidia.

SIGMOIDOSCOPY

The value of ulcer scrapings for diagnosis of amebiasis has already been discussed. In addition to this, rectal biopsy samples obtained at sigmoidoscopy or proctoscopy can be valuable in the diagnosis of schistosomiasis, especially in travelers when the number of eggs present in stools may be small (Harries et al. 1986).

STRING TEST

This technique depends on a length of nylon 'string' with a weighted end. This is swallowed and travels to the upper part of the jejunum. After a period of time, the string is pulled up and the fluid expressed can be examined. Bile-staining of the string can be read as proof that the string has traveled as far as the jejunum. Commercial versions of this device are available and the method can be used to make a diagnosis of giardiasis, strongyloidiasis, and cryptosporidiosis (Beal et al. 1970).

PINWORM

Pinworms, *Enterobius vermicularis*, lay their eggs on the skin around the anus. The optimum diagnostic yield is obtained from the perianal skin. Eggs can be harvested by using adhesive cellulose tape and then examined microscopically. Several commercial kits are available that make the diagnosis of pinworm more easy (Wagner and Eby 1983).

Urogenital samples

MACROSCOPIC EXAMINATION

Urine should be examined macroscopically where overt hematuria associated with urinary schistosomiasis can be observed. In cases of lymphatic filariasis, abnormal connections between the thoracic duct and ureters can permit leakage of lymph into the urine. This phenomenon, chyluria, is demonstrated by the excretion of milky urine after the patient has taken a fatty meal.

MICROSCOPIC EXAMINATION

Eggs of *Schistosoma haematobium* are mainly excreted into the urine were they can be demonstrated by microscopic examination. It is possible to improve the sensitivity of the method by expressing a larger volume of urine through a micropore membrane. Eggs are trapped in the membrane and can be seen under the microscope. An estimation of the worm burden can be obtained for research purposes by filtering a standard volume, usually 10 ml (Peters et al. 1976).

The diagnosis of vaginitis or urethritis caused by *Trichomonas vaginalis* is usually made by examination of vaginal discharge fluid or urethral pus. A simple saline wet preparation may permit the observation of the characteristic motile protozoa (see Figure 13.1, p. 256). Diagnosis can be made more sensitive by the use of direct immunofluoresence and culture (Draper et al. 1993).

CULTURE

Urine from patients infected with *S. haematobium* can be incubated overnight and the sediment observed for the presence of viable miracidia. The diagnosis of trichomoniasis can be enhanced by culture of vaginal and urethral secretions (see *Trichomonas* infections).

Blood

Blood is a vital specimen for the diagnosis of parasitic infections because many organism have a blood stage as part of their life cycle. These are summarized in Table 2. It is essential that the specimens are taken at the right time. Malaria parasites are characteristically detectable in the blood during a fever and for some hours afterward. When a patient presents with fever and a history of residence or travel in an endemic area, it is essential to take a blood film for malaria immediately; however, a minimum of three samples should be obtained when the patient is febrile. Failure to follow this rule may result in a false negative diagnosis. In cases of lymphatic filariasis, there is diurnal variation in the presence microfilariae in blood. For this reason, it is essential to obtain blood samples at the correct time and to take the samples into bottles containing ethylenediaminetetraacetic acid (EDTA) (Simonsen et al. 1997).

CONCENTRATION TECHNIQUES

Many parasites may be present in blood in low numbers, which makes concentration techniques essential. The simplest of these is the thick film: red cells stacked on top each other are lysed by the hypotonic Field's stain – this allows a large number of red cells to be scanned quickly for the presence of the characteristic ring-shaped merozoites of malaria parasites. Centrifugation and fluorescence microscopy can be used to enhance the sensitivity of diagnosis. Using fluorescent antibodies makes this technique more sensitive for the diagnosis of *P. falciparum* and *P. vivax* (Forney et al. 2003). Buffy-coat concentration may permit the diagnosis of *Leishmania donovani* and African trypanosomes (Woo 1970). An improved concentration technique for African trypanosomes is to centrifuge blood through a DEAE column (Lumsden et al. 1979). To concentrate microfilariae, a measured volume of blood can be filtered through a micropore membrane (Bell 1975; Desowitz et al. 1973). The membrane is then stained and examined microscopically to enable species identification. This approach has the dual advantage of increasing the sensitivity of the technique while permitting the numbers of organisms present to be counted.

CULTURE

There are few culture techniques for blood samples because most of the organisms require the intervention of intermediate vectors. However, specimens of blood and buffy coat can be cultured for *Leishmania* on media such as Schneider's insect medium. After two weeks, promastigotes can be visualized. The sensitivity of blood culture for *Trypanosoma cruzi* can be improved by

Table 2 *Protozoan and helminth parasites that can be identified in blood*

Specimen	Technique	Pathogens
Whole or EDTA blood	Thick and thin films	*Plasmodium* spp., *Leishmania donovani* and *L. infantum*, *Trypanosoma brucei rhodesiense* and *T. b . gambiense*. *Babesia* spp., various microfilaria
Buffy coat	Stained film	*Leishmania* spp., *Trypanosoma* spp., microfilariae
EDTA blood	DEAE concentration	Trypanosomes
	Membrane filtration	Microfilariae

xenodiagnosis. Uninfected triatomid bugs are permitted to feed on a suspected patient for approximately 30 minutes. The bugs are then kept and sacrificed monthly for 3 months. The guts are dissected and examined microscopically for the presence of trypanosomes.

Biopsy specimens

In many instances, the life-cycle of the parasite means that the only effective means of diagnosis is biopsy. For example, *Leishmania donovani* can be obtained from concentrated buffy coat cells but this is less sensitive than biopsy of bone marrow or splenic fluid where the organisms are concentrated. For many parasitic infections, biopsy is the only practical means of making the diagnosis (e.g. cutaneous leishmaniasis or onchocerciasis). A list of the most common parasitic infections diagnosed by biopsy is listed in Table 3.

SKIN

Punch biopsies or 'skin snips' are the investigation of choice for onchocerciasis. Specimens should be obtained from areas of skin that exhibit the characteristic dry 'chicken skin' appearance that has been associated with infection. In addition to any suspect areas, samples should be taken from the shoulder area, from the buttocks and from the calves even when the skin appears normal. The biopsies are then placed in saline for transport to the laboratory. The microfilariae start to emerge after 30 minutes to 1 hour and can be identified by their characteristic movement. In addition, stained preparations permit the identification of species. The diagnostic yield can be increased by giving the patient a small dose of diethylcarbamazine, which stimulates the migration of the microfilariae.

The diagnosis of cutaneous leishmaniasis depends on a biopsy of the suspected ulcer. Material can be obtained for microscopy or culture using a slit skin smear. A touch preparation from a biopsy of the ulcer edge can be made. Alternatively, the biopsy material can be cultured in Schneider's insect medium for 2 weeks.

MUSCLE

Trichenella spiralis larvae are located in the striated muscle. Diagnosis can be made by muscle biopsy and then pressing the material between two slides for examination under the microscope.

BRAIN

Biopsy of the brain may form part of the diagnostic process for a number of parasitic infections where various stages may be found in the brain. Biopsy may be performed to diagnose the etiology of ring-enhancing lesions in the brain demonstrated by computed tomography (CT) scans or magnetic resonance imaging (MRI). This situation often arises in patients with late stage human immunodeficieny virus (HIV) infection with low CD4 cell counts. The differential diagnosis includes lymphoma and cerebral toxoplasmosis. Other parasites that may be observed in brain biopsy include tissue stages of *Taenia solium*, *Echinococcus multilocularis*, *Entamoeba histolytica*, and eggs of various schistosomes.

IMMUNOLOGICAL DIAGNOSIS

Antibody detection

Immunological diagnosis is an attractive approach to parasitic diagnosis but has only established itself in a limited number of diagnostic areas. This is not surprising since the immune response to protozoan and metazoan pathogens is often complex and many of these organisms have evolved methods to subvert the immune response.

Table 3 *Summary of techniques and pathogens identified with biopsy specimens*

Specimen	Technique	Pathogen
Skin	Slit skin smear	*Leishmania* spp.
	Pinch biopsy	*Onchocerca volvulus*
Brain	Microscopic examination of stained smears, culture, EIA PCR	*Toxoplasma gondii*, *Naegleria fowleri*, *Acantamoeba*, microsporidia, cysticercosis, *Echinococcus* spp.,
Muscle	Direct microscopy	*Trichinella spiralis*
Eye	Microscopy of stained samples	Microsporidia, *Acanthamoeba*
Vitreous or aqueous	EIA	*Toxocara canis*
Lymph node	Microscopic examination of stained material	*Trypanosoma* spp., *Toxoplasma gondii*, *Leishmania donovani*
Liver	Microscopic examination of stained preparation	*Toxoplasma*, *Naegleria*, *Entamoeba histolytica*, Microsporidia, *Opisthorchis sinensis*
	Examination of cyst fluid by microscopy and EIA	*Echinococcus granulosus*
Bone marrow	Microscopy of stained material and culture	*Leishmania donovani* and other species

Patients in many tropical countries have raised concentrations of polyclonal, low-affinity antibody that tends to produce false positive results in many immunological tests. Also, patients in endemic countries have life-long exposure to pathogens and many have serological evidence of previous infection making it impossible to identify a new infection.

TOXOPLASMOSIS

Toxoplasmosis is one disease for which immunological diagnosis is the method of choice. Although the organism is relatively easy to culture for sufficiently sensitive diagnosis, animal inoculation is required and, therefore, this approach to investigation is limited to the processing of precious specimens. Evidence of recent toxoplasmosis can be demonstrated using the Sabin-Feldman dye test or one of many commercial assays to detect immunoglobulin (Ig)M (Sabin and Feldman 1948; Evans and Ho-Yen 2000). The age of the infection can also be determined by measuring the affinity of serum IgA.

AMEBIASIS

Antibodies to *E. histolytica* can be detected by a variety of techniques including indirect immunofluorescence assay (IFA) and indirect hemagglutination assay (IHA). These titers are often negative in patients with intestinal disease but are elevated in patients with invasive infection. Acute diagnosis of amebic abscess can be assisted by the use of the cellulose acetate precipitin test, which becomes positive much earlier than the IFA and IHA.

MIGRATING WORMS

Most of the organisms responsible for larval migration are unable to complete their life cycle in the human host. As a consequence, it may not be possible to identify these organisms by conventional phenotypic methods. An example of such a parasite is *Toxocara canis*; the diagnosis is made by detection of antibodies specific to the excretory secretory antigens (de Savigny et al., 1979). Other examples include *Trichinella*, dog hookworm infection, and gnathosomiasis.

RETURNING TRAVELERS

Although of little value in endemic countries, many serological tests are of diagnostic value in returning travelers because the time frame of their exposure to the infective agents is known. In addition, the degree of the infection is frequently light, which makes diagnosis by conventional morphological methods more difficult. As travelers are unlikely to have been exposed to organisms such as schistosomiasis or filariasis previously, the detection of antibodies to such parasites could be diagnostic. Alternatively, because many travelers seek medical help to exclude tropical infections, a negative result may be seen as even more valuable in that it allows the patient to be reassured.

EVALUATION OF SPACE-OCCUPYING LESIONS

Detection of antibodies to *T. solium*, *Echinococcus granulosis* and *Echinococcus multilocularis* may assist the difficult diagnosis of these infections. Several enzyme immunoassays (EIA) have been described (Feldman et al. 1990).

Antigen detection

Direct detection of parasite antigens, as opposed to the detection of antibody responses, has found a regular place in diagnosis.

MALARIA

A simple dipstick presentation kit is available for the rapid diagnosis of malaria. Although the cost of this kit may restrict its application in resource-poor countries, it does provide a rapid and reliable diagnosis of *P. falciparum* and *P. vivax* malaria without the need for a microscope or EIA reader. It is probably a little less sensitive than the results obtained by an expert microscopist. It is indicated for the rapid diagnosis of malaria in laboratories that do not process many specimens for malaria. It would allow a rapid reliable diagnosis that could be confirmed by a reference facility.

INTESTINAL PROTOZOA

Commercially produced kits with a IFA or EIA format are available for the diagnosis of *Giardia lamblia, Cryptosporidium parvum,* Microsporidia, and *Entamoeba histolytica* infection. The latter method is especially valuable as *E. histolytica* and *E. dispar* cannot be distinguished under the light microscope. These methods appear to be at least as sensitive as light microscopy and, if the EIA format is employed, also permit large numbers of specimens to be processed (Ong et al. 1996).

MOLECULAR DIAGNOSIS

Although many molecular diagnostic methods have been described, molecular diagnostics have little part to play in diagnosis for the majority of parasitic infections. However, they do have considerable application in public health microbiology and in research when it is necessary not only to identify the presence of a pathogen but also to determine its subtype. Molecular methods can also be used to investigate the mechanisms of resistance to antiparasitic drugs. This has proved especially valuable for malaria and for intestinal nematode infections.

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Cellular organization of parasitic protozoa

HEINZ MEHLHORN

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DEFINITION OF THE CELL

The organic entities on Earth exist in the following forms: various types of cells; short sequences of stable proteins (prions); extracellular genomes (viroids, or naked RNA molecules); and protein capsules, or capsids, containing relatively short molecules of DNA or RNA often surrounded by an additional membrane (bacteriophages and viruses). Prions, viroids, phages, and viruses lack their own metabolism and the ability to reproduce independently, whereas all types of cells are true living systems with metabolic ability and the capacity for independent reproduction. All cells share the following common attributes:

- They are enclosed by a cell membrane.
- Their systems of reproduction use DNA for information storage and RNA for directing cellular organization.
- Their genomes may undergo accidental change (i.e. mutate).
- They can use chemical bond energy or light energy to run their metabolic systems.
- They can detect and respond to environmental signals and can receive, recognize, and transmit signals and impulses.

Cells may also be motile and, in the case of eukaryotes, may have flowing cytoplasm (Mehlhorn and Ruthmann 1992; Alberts et al. 2002; Hausmann et al. 2003).

Two basic types of true cells are distinguishable: prokaryotes and eukaryotes. There are no transitional forms in existence today, and thus these two forms are quite distinct. Prokaryotes always occur as functionally single cells with no specialization, whereas eukaryotes may consist of a single cell (e.g. Protozoa) or may be multicellular organisms made up of differentiated (specialized) cells (e.g. Metazoa). If prokaryotic organisms (such as mycoplasms and bacteria) do aggregate, they occur as chains or clusters of unspecialized cells. In contrast, eukaryotes can consist of many cells functioning in a highly integrated fashion. Some significant differences between the cellular components of eukaryotes and prokaryotes are listed in Table 1.

Prokaryotic and eukaryotic cells represent the units of life. It was recognized by Virchow in 1855 that they are the smallest units capable of maintaining the continuity of life or, as he expressed it in Latin, '*Omnis cellula e cellula*' (every cell derives from a cell).

UNICELLULAR EUKARYOTES

The higher protists (those that resemble plants, fungi, and animals) are all composed of eukaryotic cells. Eukaryotes may be unicellular in all their developmental stages, as are the protists, or unicellularity may be limited to certain developmental stages, such as the generative forms (the oocytes and spermatids) of plants and animals. Protists have followed many evolutionary

Table 1 *Differences between prokaryotes and eukaryotes*

Attributes	Prokaryotes	Eukaryotes
Cell nucleus		+
DNA		
Amount	Low (up to 1.4 $\times$ 10^{-2} pg/cell)	High (up to 1.6 $\times 10^{-2}$ – 96 pg/cell)
Organization	Circular	Linear (chromosomes) plus circular elements
Recombination	Conjugation	Meiosis and syngamy
Introns		+
Cell division		
Speed	Quick (20 min)	Slow (hours)
Mode	By formation of septa	By mitosis and cytokinesis
Organelles		
Ribosome type (subunits)	70S (30S + 50S)	80S (40S + 60S)
Membrane-bound organelles (mitochondria, plastids, Golgi, etc.)		+
Microtubules		+
Membrane-bound flagella (9 $\times$ 2 $\times$ 2 pattern)		+
Use of actomyosin for movement		+
Endo- and exocytotic activity (i.e. movement)		+

+ = present

paths in the development towards multicellularity, and the following protists represent intermediate steps in this process:

- protist forms with many nuclei per cell
- stable, double nucleated forms, such as *Giardia*
- forms with cell aggregation including some green algae, like *Volvox*
- forms with chains of dividing organisms, e.g. microsporidians
- forms that have multicellular stages, e.g. Myxozoa.

Note that although the Myxozoa, or myxosporidians, have long been classified with the protists, and are mentioned here, it is now accepted that they should be classified with the Metazoa (see Cavalier-Smith 1998). Even highly differentiated Metazoa retain vestiges of their unicellular origin, as shown by their development from unicellular eggs, some of which may develop even if they are not fertilized. They also have the ability to reconstruct their whole bodies from a single cell, as do the sponges (phylum Porifera).

The group termed protists is comprised exclusively of unicellular eukaryotic organisms that may be phototrophic, autotrophic, or heterotrophic. The Protozoa are named after the Greek term for 'first' (*proto*) and 'animal' (*zoon*). They are heterotrophic, lacking the ability to use light and inorganic materials to obtain energy and to synthesize structural components. Therefore, they must obtain preformed organic compounds, and on this basis may be considered to be animals. Apart from a few sedentary species, most Protozoa are motile. Because they have difficulty in retaining water, partly because of their small size, most live in aquatic (or at least moist) environments. Although the majority of protozoans are free living, many species are mutualists, commensals, or true parasites. Some are highly pathogenic to their plant or vertebrate hosts and hence are relevant to veterinary and human medicine and agriculture (Mehlhorn 2002).

GENERAL MORPHOLOGY OF PARASITIC PROTOZOA

The classification of the Protozoa remains in flux, as new data continue to be obtained (see Classification and introduction to the parasitic protozoa and Mehlhorn 2002). Some of the attributes of the protozoans are, however, beyond dispute. For example, they are all organized according to the basic pattern of the eukaryotic cell (Figure 1), the same type being found in all metazoan cells.

Eukaryotic cells consist of a membrane-bound cytoplasm containing one or more nuclei and various organelles that are also often membrane bound, their compartments and membranes acting as sites where reaction processes can occur (Alberts et al. 2002).

Cell membrane

The parasitic protozoans are surrounded by a membrane 4–10 nm thick (the unit membrane or plasmalemma) (Figures 2 and 3). In living cells this membrane always forms a closed sac or vesicle.

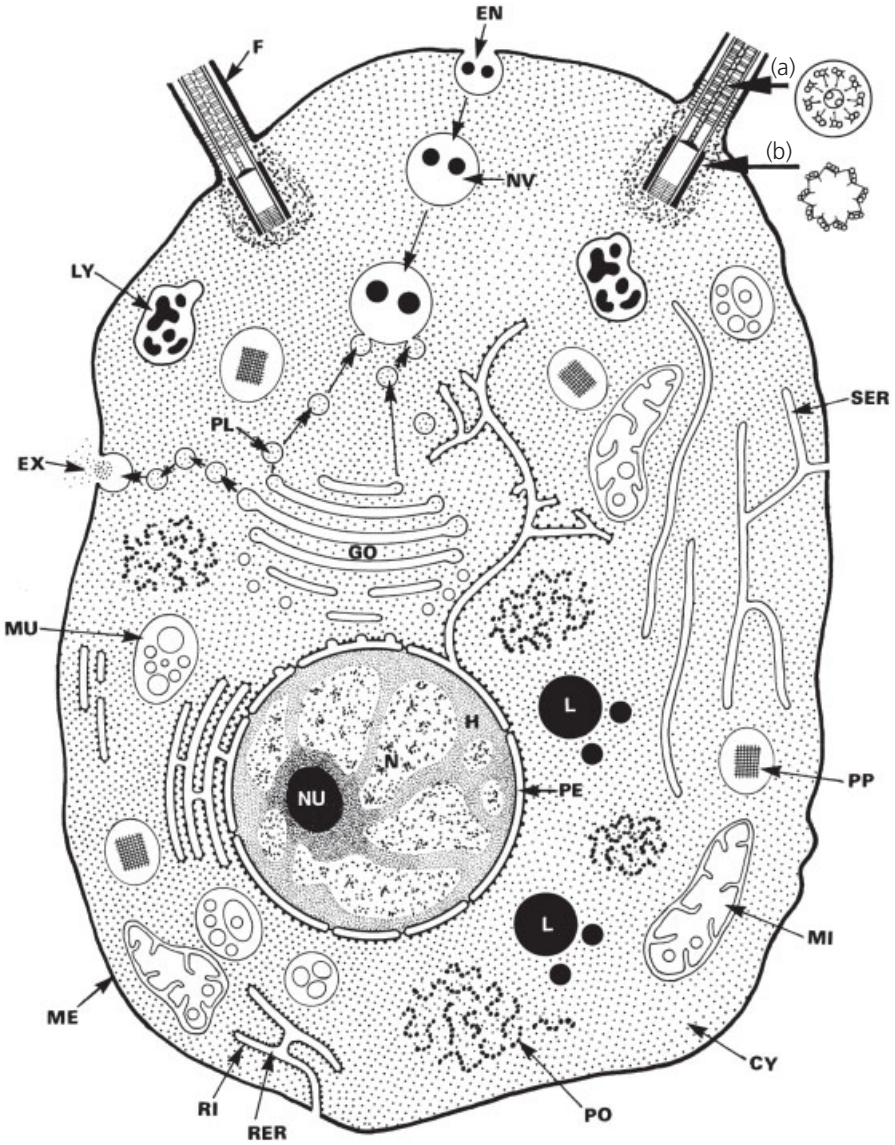

Figure 1 *Fine structure of a typical eukaryotic cell,* **(a)** *cross-section of the free flagellum:* **(b)** *cross-section of the basal body. B, basal body; CT, cytoplasm; EN, endocytosis; EX, exocytosis; F, flagellum; GO, Golgi apparatus; H, heterochromatin; L, lipid; LY, secondary lysosome (phagolysosome); ME, cell membrane (plus surface coat); MI, mitochondrion; MU, multivesicular body; N, nucleus; NU, nucleolus; NV, food vacuole (endocytotic vacuole); PE, perinuclear space; PL, primary lysosomes; P0, polyribosomes (chains); PP, peroxisome with protein crystal; RER, rough endoplasmic reticulum; RI, ribosome; SER, smooth endoplasmatic reticulum.*

The membrane is composed of species-specific amounts of various proteins and a double layer of lipids (Figure 2). It is physiologically and morphologically asymmetric, presenting a 'p face' (directed towards the cytoplasm) and an external 'e face.' High-magnification electron microscopy reveals that it is trilaminar (composed of three layers) (Figure 3). It is semipermeable, i.e. only certain types of molecules may cross it (Gennis 1989).

Several models have been created to explain how this biomembrane functions. The most widely accepted is the fluid mosaic model of Singer and Nicolson (1972) in which it is proposed that the membrane consists of a relatively stable double layer of lipid molecules within which proteins float like icebergs. At least some of the proteins may be structured to form pores or channels (Figure 2). The membranes expand by additive inclusion of vesicles formed inside the cytoplasm and shrink by formation of endocytotic vesicles (Figure 1) (Neupert and Lill 1992).

There are several systems for transporting substances through the cell membrane into the cytoplasm. Passage may occur by permeation (nonmediated transport), a process that is dependent on concentration gradients. Active transport (mediated transport) using motile carriers may also occur. In this system a protein binds

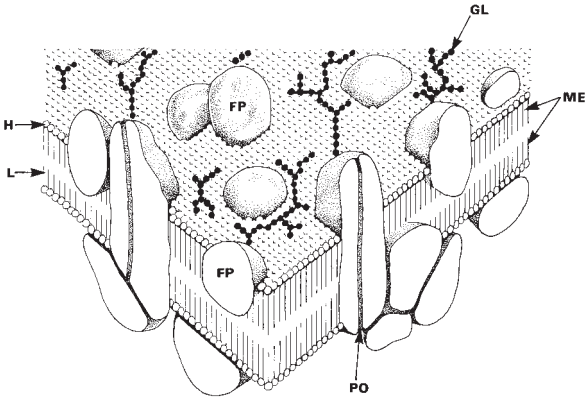

Figure 2 *Cell membrane of a typical eukaryotic cell following the fluid mosaic model of Singer and Nicolson (1972). Proteins (FP) float in a membrane that consists of a double layer of lipid molecules each with a hydrophilic component (H) and two lipophilic layers (L). The proteins, which may form pores (PO), often anchor glycoproteins, and together form the glycocalyx (GL).*

the molecule to be transported and then the complex moves actively from one side of the membrane to the other. Movement depends on changes in the electric charge that are linked with the binding and release of the transported molecule. The fixed pore (Figure 2) is a protein structure that stretches through the membrane. The molecules to be transported pass through the space, or channel, formed between the subunits of the pore. All forms of active transport require energy, which is derived from various metabolic reactions.

In addition to the direct transport of molecules through the cell membrane, other mechanisms exist for internalization of materials by cells. For example, relatively large organic materials may be internalized by the formation of endocytotic vesicles (Figure 2), a process termed pinocytosis for the uptake of liquids and phagocytosis for the uptake of solid particles. Within the vacuoles the material is degraded and then transported into the cytoplasm by mechanisms similar to those used by the cell membrane. Alternatively, it may be released by dissolution of the membrane. Some protozoans have developed special places for the uptake of food, called cytostomes. Many cells contain clathrin-coated pits that are involved in the receptor-mediated endocytosis of macromolecules, but our knowledge of coated pits in Protozoa remains limited (Morgan et al. 2002), although clathrin-dependent endocytosis has been seen in trypanosomes.

The cell membranes of both Protozoa and Metazoa are involved in many other tasks apart from transport functions, for example cell recognition. When cells come into contact with each other, recognition occurs via special receptors in the membranes that perceive chemical signals given off by other cells. Cell membranes also participate in excretion and secretion, these processes occurring by mechanisms similar to those for the intake of materials (e.g. bloodstream trypanosomes excrete trypanopains and oligopeptidases in order to cross the blood–brain barrier; Londsdale-Eccles and Grab 2002). Membrane-bound structures exist that mediate the joining of cells. These are needed for fusion of gametes and similar structures and are also found at places where flagella are attached to the surfaces of protozoa. The undulating membranes and recurrent flagella of trypanosomes and trichomonads are joined to the cell by means of such cell junctions (Figure 4; see also Figure 29a). Cell junctions also play a role in attachment of many parasites to the surfaces of host cells. For example, trypanosomes use junctions for attachment to the vector's intestine.

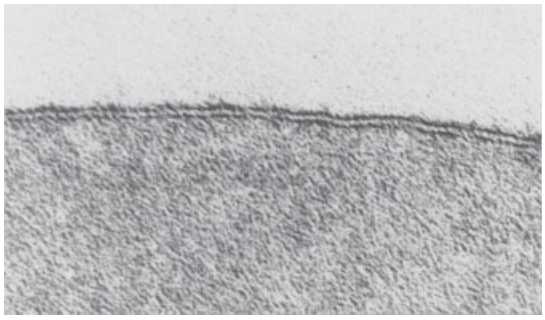

Figure 3 *Transmission electron micrograph of a section through the membrane of an erythrocyte showing the three layers of the membrane (× 100 000).*

Pellicle and cytostomes

Many protozoans have more than one limiting membrane for at least some of their developmental stages (Table 2). In some groups the main cell membrane is underlined by one or more 'inner' membranes that are often derived from the endoplasmic reticulum (Table 2, Figure 5).

Like the outer membranes, these inner membranes are species specific and possess distinct inner and outer surfaces. The characteristics of the inner membranes have been revealed by the methods of freeze fracture and negative staining (Figure 6). The single outer membrane and the membranous complexes often have subpellicular microtubules (c. 25 nm in diameter) beneath them.

These shape-stabilizing cell boundary complexes are called pellicles (Figures 4 and 5). The motile stages of trypanosomes and *Leishmania* species form a pellicle that consists of one membrane plus underlying microtubules (Figure 7), whereas the motile stages of coccidians (sporozoites, merozoites, ookinetes, kinetes) have a pellicle consisting of three membranes plus underlying microtubules (Figure 6).

In ciliates the pellicle is composed of an outer membrane, a system of alveolar sacs, longitudinal micro-

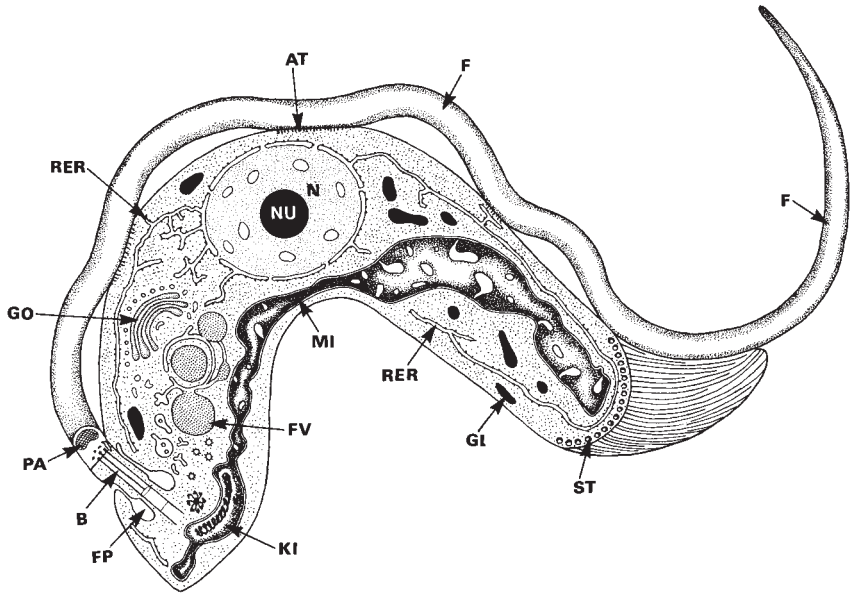

Figure 4 *Longitudinal section through a trypomastigote of* Trypanosoma *sp. AT, attaching zone of the flagellum; B, basal body; F, flagellum; FP, flagellar pocket (with cytostomal activity); FV, food vacuole; GL, glycosomes; GO, Golgi apparatus; KI, kinetoplast (containing DNA filaments); MI, mitochondrion; N, nucleus; NU, nucleolus; PA, paraxial rod; RER, rough endoplasmic reticulum; ST, subpellicular microtubules.*

Table 2 *Types of limiting membranes of some parasitic protozoans*

Species	Stage	Single membrane	Two or more membranes	Pellicle with subpellicular microtubules	Cyst wall
Trichomonas vaginalis	Trophozoite	+			
Giardia lamblia	Trophozoite	+		Ventral side	
	Cyst	+			+
Trypanosoma brucei group	Epi- and trypomastigotes			+	
Leishmania spp.	A-, promastigotes			+	
Entamoeba histolytica	Magna-form	+			
	Minuta-form	+			
	Cyst	+			+
Pneumocystis carinii	Trophozoite	+			
	Cyst	+			+
Eimeria spp.	Sporozoites			+	
	Merozoites			+	
	Oocysts				+
	Sporocysts	+			+
	Meronts	+			
	Male gametes	+			
	Female gametes of some species	+	+		
Toxoplasma gondii	Sporozoites			+	
	Merozoites			+	
	Oocysts	+			+
	Sporocysts	+			+
	Meronts	+			
	Male gametes	+			
	Gamonts	+			
	Female gametes		+		

+ = present

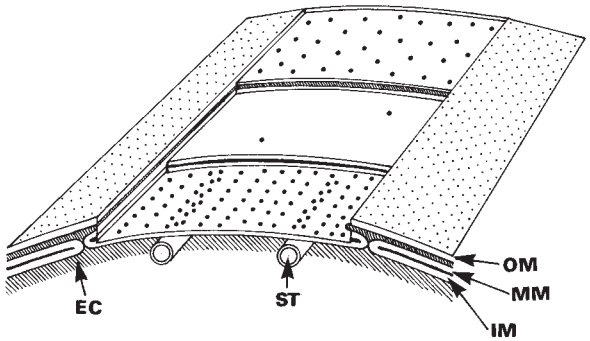

Figure 5 *Three-layered pellicle of the invasive stage of a sporozoan as revealed by freeze etching. Note that each of the membranes has unique intramembranous particles. EC, ectoplasm; IM, inner membrane; MM, middle membrane; OM, outer membrane; ST, subpellicular microtubules.*

tubules, and kinetodesmal fibres. This complex pellicle produces a stable base to hold rows of cilia (see Figure 18).

In the free-living euglenids the plasmalemma is reinforced by longitudinal microtubules and by a dense underlying epiplasm. The number of subpellicular microtubules is often stable within a species and may thus be used for species definition. For example, the merozoites of the tissue cyst-forming Coccidia (e.g. *Sarcocystis*, *Toxoplasma*, *Besnoitia*, *Frenkelia*) always have 22 microtubules and various *Eimeria* species have 24, 26, 28, 30, or 32 microtubules, respectively. In some parasites, such as the trypanosomatids, the number of subpellicular microtubules varies with the size of the individual parasite form, i.e. a long slender form with a small diameter has fewer microtubules than a form of the same species with a larger diameter (Figure 7).

The subpellicular microtubules may run from one pole to the other, as they do in gregarines and trypanosomes, or they may be restricted to limited portions of the pellicle. In ciliates, for example, they are restricted to the front half of the cell and in the coccidian motile stages they are localized to the front two thirds of the cell (Figure 8).

In the motile stages of the Coccidia the microtubules are anchored to an anterior polar ring. At the attachment point there is an interruption of the two inner membranes (Figure 9). If two or more polar rings are present (species specific), it is the outer one that is connected to the microtubules. The subpellicular microtubules are usually kept in contact with the inner surface of the pellicular membrane by means of side arms. The exact role of the microtubules in movement has not yet been identified.

The membrane of the eukaryotic cell can take up material by various methods including permeation, active transport and endocytosis. Endocytosis (see Figure 1) may occur at any point on the cell membrane or may occur only at predisposed places. For example, in *Giardia* trophozoites, endocytosis occurs only in the dorsal region, the region opposite the ventral sucker; in trypanosomes, endocytosis occurs in the flagellar pocket (Morgan et al. 2002) and in sporozoans it is found at small cytostomes called micropores (Figure 10) (Mehlhorn 2002).

Large endocytic elements (cytostomes or 'cell mouths') are characteristic of many ciliates (see Figure 31e) (Hausmann et al. 2003).

Cytostomes are reinforced by various structures (Figure 10), for instance by bundles of microtubules in trypanosomes and ciliates. After the phagosomes enter the cytoplasm the contents are digested and resorption of the necessary molecules occurs. The residue may be voided to the outside or stored as an inclusion. The process of excretion, called exocytosis, may occur anywhere on the surface or, as in ciliates, at a specialized place called the 'cell anus' or cytopyge. Exocytosis is a process similar to endocytosis but in reverse.

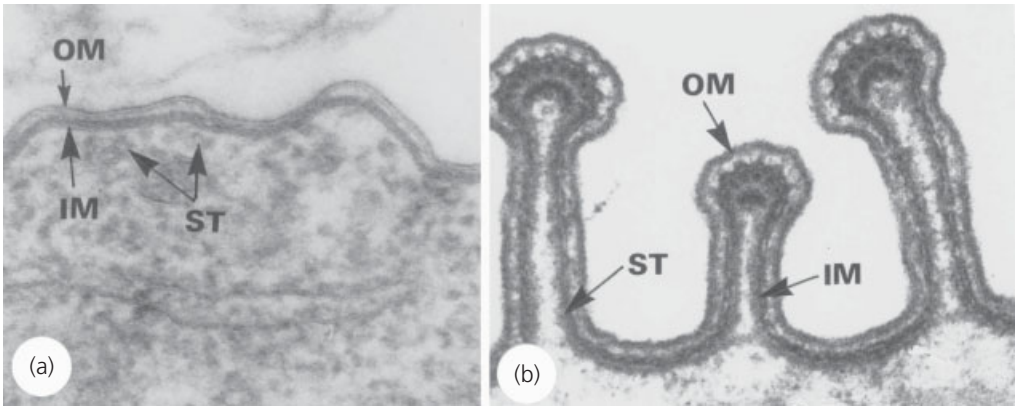

Figure 6 *Transmission electron micrographs of sections through the three-layered pellicle of a coccidian* **(a)** *and a gregarine* **(b)**. *Note that the two inner membranes appear mainly as a thick layer. IM, two inner membranes; OM, outer membrane; ST, subpellicular microtubule (×70 000).*

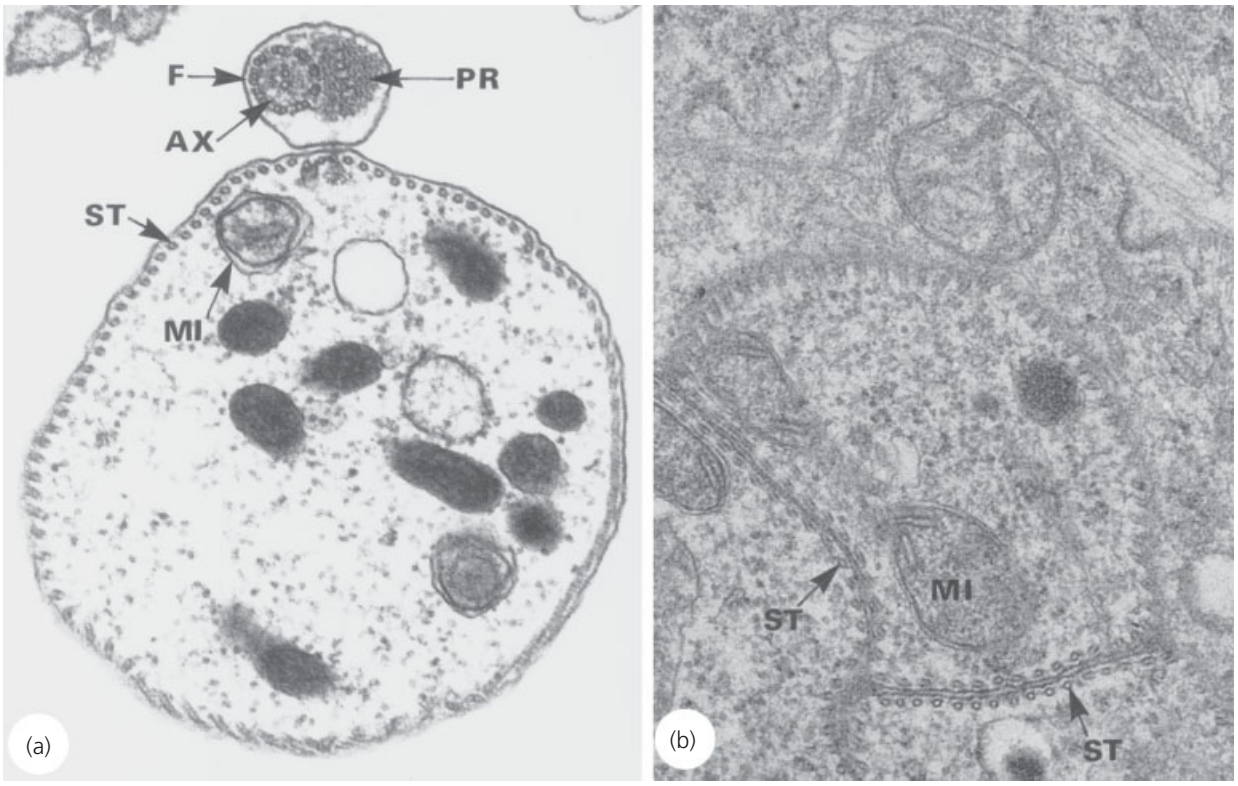

Figure 7 *Transmission electron micrographs of cross-sections through a trypomastigote of* Trypanosoma vivax **(a)** *and an amastigote of* Leishmania donovani **(b)**. *Note that in both cases the pellicle consists of a single cell membrane plus subpellicular microtubules (ST). AX, axoneme; F, flagellum; MI, mitochondrion; PR, paraxial rod (×35 000).*

Cyst wall

Many parasitic protozoans (Table 2) are capable of undergoing encystation, which involves formation of a cyst wall either outside or inside the cell membrane. This cyst wall may be single layered or multilayered.

Walls formed outside the plasmalemma are produced by exocytosis of materials. Cyst walls have two main

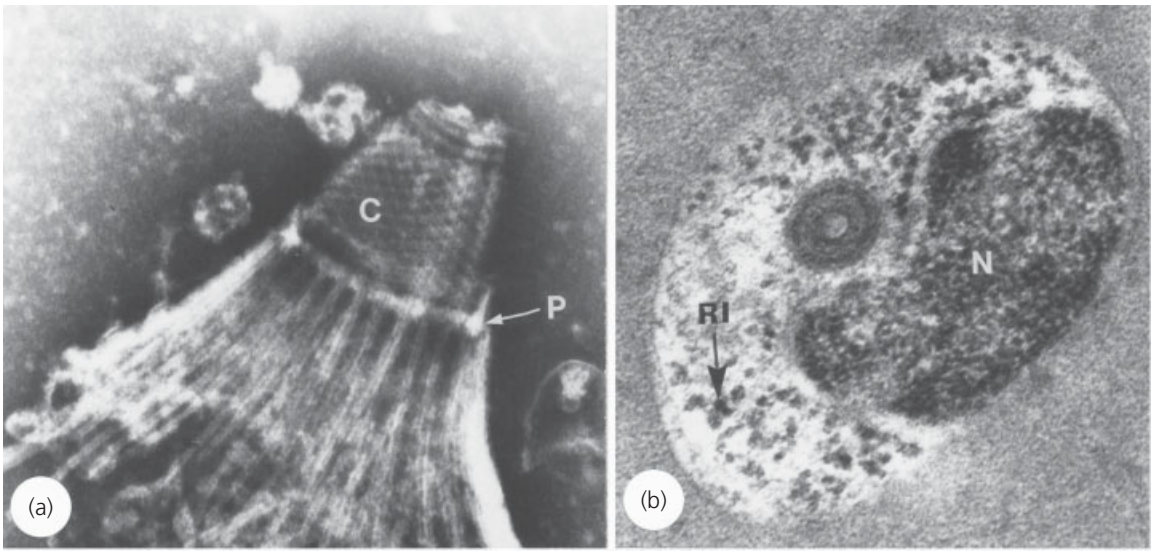

Figure 8 *Transmission electron micrographs:* **(a)** *Negatively stained apical pole of a merozoite of* Sarcocystis. *Note the protruded conoid (C), and the polar ring (P), to which the subpellicular microtubules are attached (×50 000).* **(b)** *Cross-section of a micropore (in an erythrocytic stage of* Theileria annulata*, the agent of Mediterranean coast fever of cattle). N, nucleus; RI, ribosome (×35 000).*

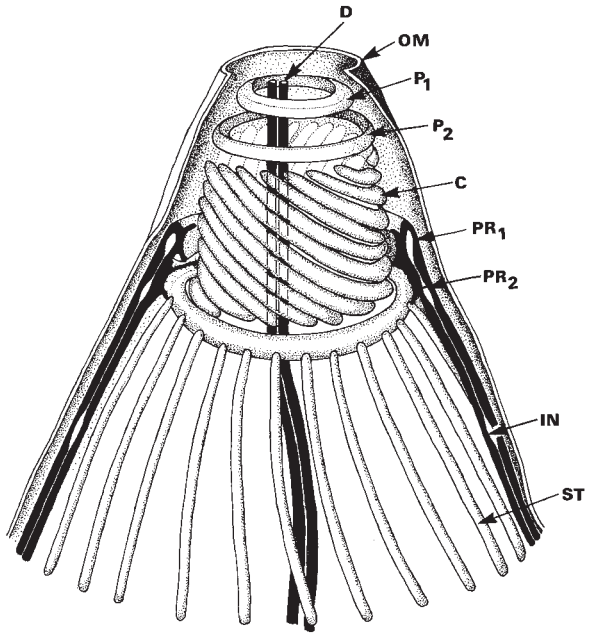

Figure 9 *Coccidian merozoite showing the apical pole and its protruded conoid. This structure is not present in hemosporidians and piroplasms. C, conoid consisting of microtubules; D, ductules of the rhoptries; IN, inclusion between the two inner membranes of the pellicle; OM, outer membrane of the three-layered pellicle; P1, P2, preconoidal rings; PR1, PR2, polar rings (the posterior one is connected with the subpellicular microtubules); ST, subpellicular microtubule.*

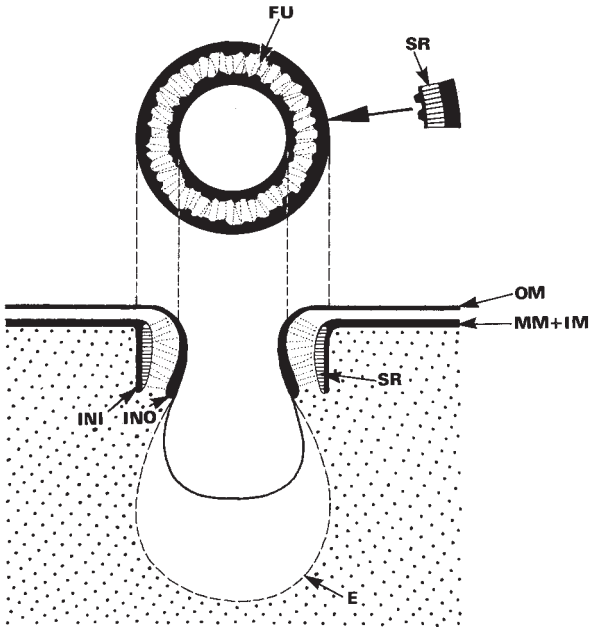

Figure 10 *A sporozoan micropore in longitudinal and tangential sections. E, enlargement during food uptake; FU, filamentous elements; IM, inner membrane of the pellicle; INI, interruption of the inner membrane (forms of the outer ring in tangential section); INO, dense material along the inner ring (invaginated outer membrane); MM, middle membrane; OM, outer membrane of the pellicle; SR, dense material between the inner and middle membranes.*

tasks: to protect the organism against unfavorable environmental conditions when passing from one host to another and to create spaces for reorganization and nuclear division. Cyst walls may also aid the parasite in transmission from one host to another by facilitating attachment to host-cell surfaces.

The cyst wall has one layer in cysts of *Entamoeba histolytica*, *Giardia* species, and some ciliates, such as the fish parasite *Ichthyophthirius multifiliis* and the human ciliate parasite *Balantidium coli*. There are usually two types of cysts in the sages of Coccidia in feces: the oocysts and the sporocysts. The oocyst wall is usually double-layered, but in a few species it may have four layers. It is formed by two types of cyst wall-forming bodies. The sporocyst wall usually has only a single layer (Figure 11).

The chemical composition of cyst walls varies according to the species, although proteins are usually the basic component. The cyst walls of *E. histolytica* and *G. lamblia* contain proteins which are keratin-like or elastin-like albuminoids composed of lysine, histidine, arginine, tyrosine, glutamic acid, and glycine. The double-layered oocyst wall of the sporozoans is periodic acid–Schiff (PAS) positive. The outermost layer of these oocysts consists mainly of fatty alcohols (e.g. hexacosanol), some phospholipids, and fatty acids. It has no carbohydrates or proteins. The inner layer is composed of glycoproteins and contains most of the carbohydrates found in the oocyst wall. These carbohydrates are composed of mannose, galactose, glucose, and hexosamine. The oocyst wall is highly resistant to the passage of potassium dichromate, sodium hypochloride, sulfuric acid, and sodium hydroxide and these chemicals are therefore used in storage and cleaning of oocysts. The oocyst wall is permeable to oxygen, carbon dioxide, ammonia, methylbromide, carbon disulfide, and various organic solvents. It is highly susceptible to mechanical pressure and therefore may easily be ruptured by shearing forces. Thus mechanical rupture of oocysts in the gizzard of the avian host is likely to be the normal method of excystation of avian coccidians. The micropyle (a preformed opening in some species) is probably used as rupture point to facilitate the escape of sporocysts. The sporocysts in the oocyst are bound by a double-layered wall, the outer layer of which is relatively smooth. The sporocysts of eimerians have an opening for exit of the sporozoites. This is closed by the Stieda body, which can be dissolved by trypsin. The sporocysts of *Isospora* and the tissue cyst-forming Coccidia (*Sarcocystis*, *Toxoplasma*) have sutures on which the excystation fluids act causing collapse of the sporocyst wall (Figure 11g).

The cyst walls of Myxozoa and Microspora are at least double-walled in most species (Figure 12) and the layers can be resolved by electron microscopy. In Myxozoa the wall consists of valves that open at preformed sites to release the infectious sporoplasm.

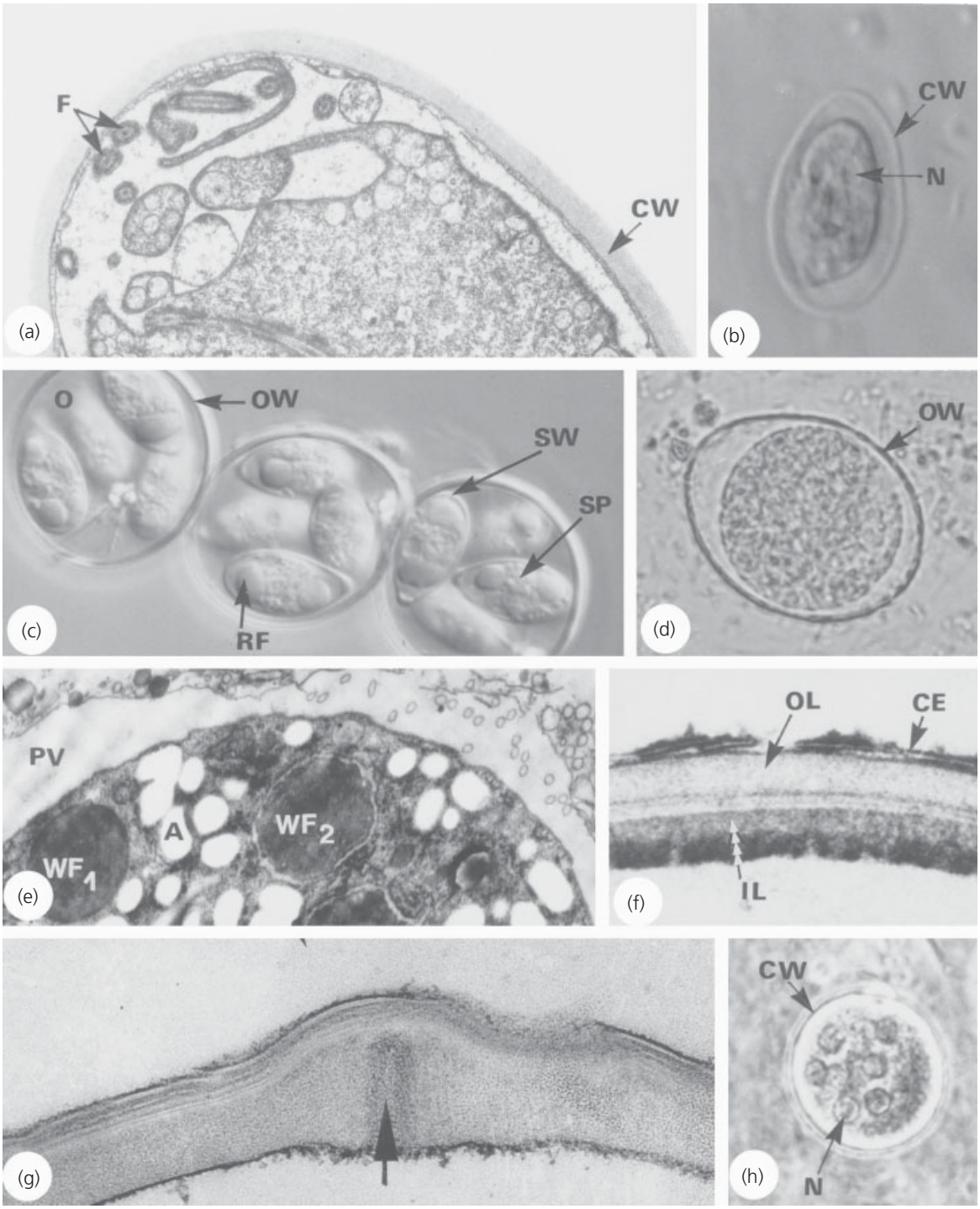

Figure 11 *Light (b, c, d, h) and electron micrographs (a, e, f, g) of cysts and cyst walls. (a, b)* Giardia lamblia. *CW, cyst wall; F, flagellum; N, nucleus.* **(c)** Eimeria *oocysts (0) containing sporocysts (SP). OW. oocyst wall; RF, refractile body of sporozoites; SW, sporocyst wall.* **(d)** Isospora *sp., unsporulated oocysts. OW, oocyst wall.* **(e)** Eimeria maxima*; macrogamete with wall forming bodies I and 2 (WFI, WF2). A, amylopectin; PV, parasitophorous vacuole. (1) Oocyst wall of* Eimeria *and* Isospora *spp. CE, cell membrane; IL, OL, inner and outer layer of oocyst wall.* **(g)** *Sporocyst wall in* Toxoplasma *and* Sarcocystis *spp., with the pre-formed suture (arrow) that ruptures during the excystation process.* **(h)** Entamoeba coli*; eight-nucleated cyst. CW, cyst wall; N, nucleus.*

The Microspora have developed a hollow tube that is protruded from the surface of the wall. It penetrates into a host cell and the infectious sporoplasm is passed through it. The exospore layer in Microspora is proteinaceous and is 15–100 nm thick, depending on the species. The endospore layer is chitinous and

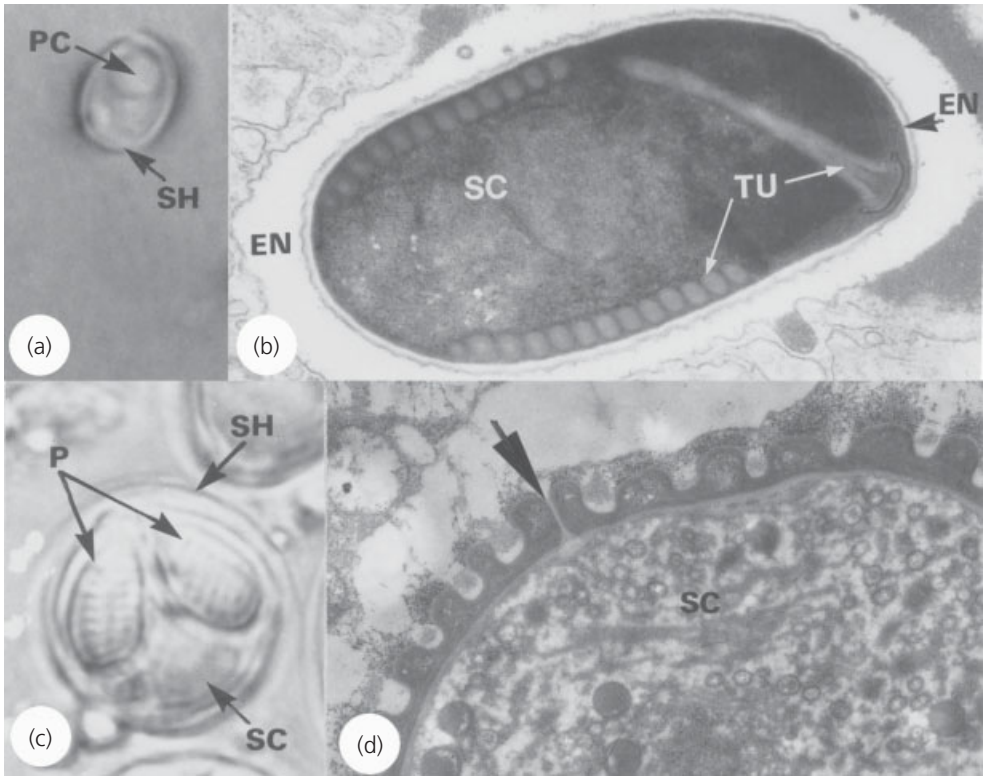

Figure 12 *Light* **(a, c)** *and electron micrographs* **(b, d)** *of* Microspora **(a, b)** *and* Myxozoa **(c, d)**. *Note that the microsporidian wall (*Nosema *sp.) is entirely closed, while that of* Myxozoa **(d)** *(*Myxosoma *sp.) has two valves that leave a small opening (arrow). EN, endospore; EX, exospore; P, polar capsule with solid filaments; PC, polar capsule with a hollow tubule; SH, shell; SC, sporoplasm; TU, tubule.*

c. 150–200 nm thick. The spores are gram-positive (i.e. stain reddish purple with the Gram stain), a fact that is of diagnostic value. They are also stained light blue by Giemsa.

Surface coat

The plasma membrane of eukaryotic cells is strikingly asymmetric (Figure 2). The outer and inner layers are clearly delineated and the polypeptides on each surface (Figures 13 and 14) are distinct. Glycolipids and glycoproteins are present only on the external surface. The peripheral layer is rich in carbohydrate and is called the glycocalyx or surface coat. Molecules of glycosylphosphatidylinositol (GPI) serve as parasite-specific anchors for numerous surface proteins. The thickness of this layer varies with the species and with the developmental stage of the organism. Not only is the surface coat composed of glycoproteins and glycolipids, but various glycoproteins and proteoglycans (acid mucopolysaccharides) may also be adsorbed to it (Figure 13).

The surface coat may be a rather delicate coating, a mass of delicate filaments or a thick mat. Whatever its structure, it has several functions in the life of the organism: (1) it acts as a mechanical or chemical barrier; (2) it plays a role in recognition and adhesion to other cells; (3) it contains enzymes that act on substances in the environment; and (4) it contains molecules that can act as antigens and thus plays an important role in immunological processes.

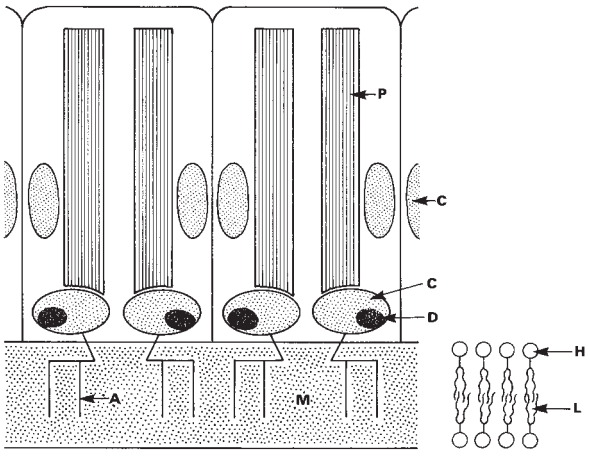

Figure 13 *Arrangement of the components of the surface coat along the surface of a trypanosome. A, anchor of a variant surface glycoprotein molecule; C, carbohydrate; D, cross-reacting determinant; H, hydrophilic component of the VSG molecule; L, lipophilic component; M, membrane consisting of two layers of lipid molecules; P, protein.*

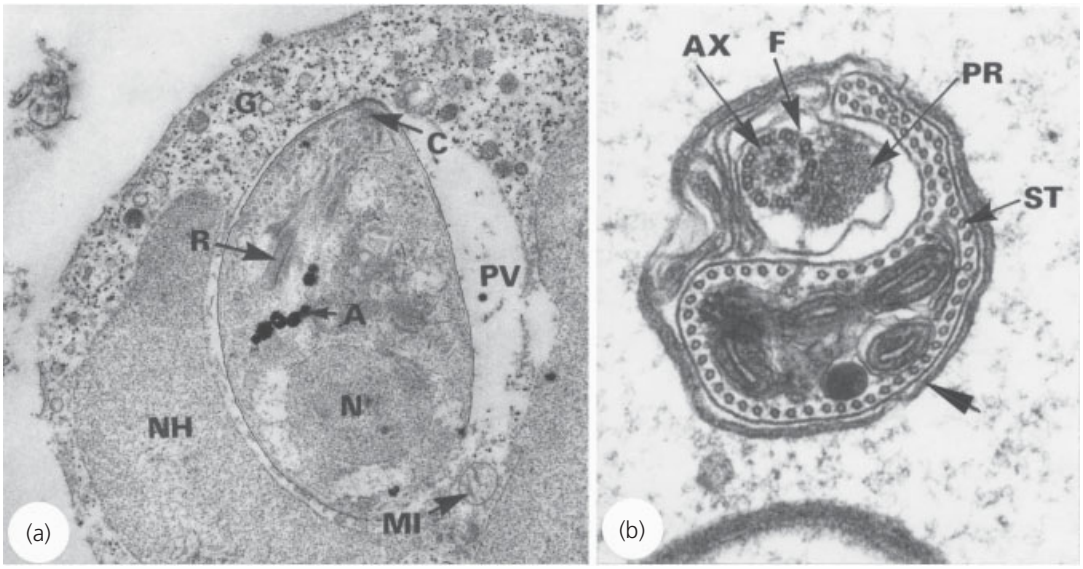

Figure 14 *Transmission electron micrographs of the surface coat:* **(a)** Toxoplasma gondii*; the tachyzoite within a host cell vacuole (PV) shows a slight positive Thièry reaction along its surface (arrow). Note the presence of amylopectin (A) granules in the parasite and of glycogen (G) in the host cell. C, conoid; MI, mitochondrion; N, nucleus; NH, nucleus of the host cell; R, rhoptry (×7 000).* **(b)** Trypanosoma vivax*. Cross-section through a trypomastigote stage showing the discharge of the surface coat (arrow). AX, axoneme; F, flagellum; PR, paraxial rod; ST, subpellicular microtubule (×25 000).*

The surface coat may change its composition as the parasite develops from stage to stage. In addition, many parasitic Protozoa, in particular the trypanosomes, have developed sophisticated methods of antigenic variation. This may be achieved by selective activation of different genes at different times. Organisms of the *Trypanosoma brucei* group have up to 1 000 genes that may become activated during the production of variant surface glycoproteins (VSGs). This selective activation results in changes in the variable antigen types (VATs) displayed and hinders the host defense against these blood-inhabiting flagellates. *Plasmodium* may also display variant antigen types, but there are fewer variants than those displayed by trypanosomes. Such different surface antigens (SAGs) are found in many of the coccidians, too (Lyons et al. 2002). The action of these genes results in antigenic variation and the production of immunologically different strains of protozoans; this explains why most antiprotozoal vaccines provide only limited protection, restricted to certain localities. The development of potent vaccines against protozoan parasites depends on the discovery of species-specific antigens with invariant epitopes that are accessible to the immune system during the parasite's life. In addition, they must be essential to the parasite's survival, possibly playing an important role in cell recognition, adhesion, immune evasion, metabolism, or cell invasion.

Cytoplasm

The cytoplasm in protozoa is generally divided into two zones: the peripheral, electron-lucent ectoplasm (hyaloplasm) and the denser central endoplasm. The endoplasm contains the cell organelles and the nucleus. This differentiation is particularly prominent in the amoebae and in the gregarines (Figures 15 and 16) but is not apparent in all species.

In some species, endoplasm and ectoplasm cannot even be distinguished by electron microscopy. Microgametes of most sporozoans, apart from those of the piroplasms, have a very reduced cytoplasm. They are comprised mainly of flagella, a mitochondrion, and a nucleus (Figure 17) (Mehlhorn 2002; Ferguson 2002).

The cytoplasm of most cells has a high viscosity and stability and a prominent cytoskeleton. Contractile elements of this cytoskeleton are responsible for the cytoplasmic flow and these include actin filaments with a diameter of c. 6 nm. These filaments are composed of a double chain of globular bodies. Cytoskeletons may also contain 10 nm filaments or intermediate filaments which,

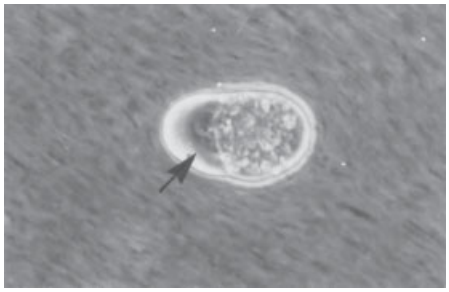

Figure 15 *Light micrograph of a trophozoite of* Entamoeba histolytica*. Note the presence of hyaline ectoplasm in the pseudopodium (arrow) and along the outer surface (×1 000).*

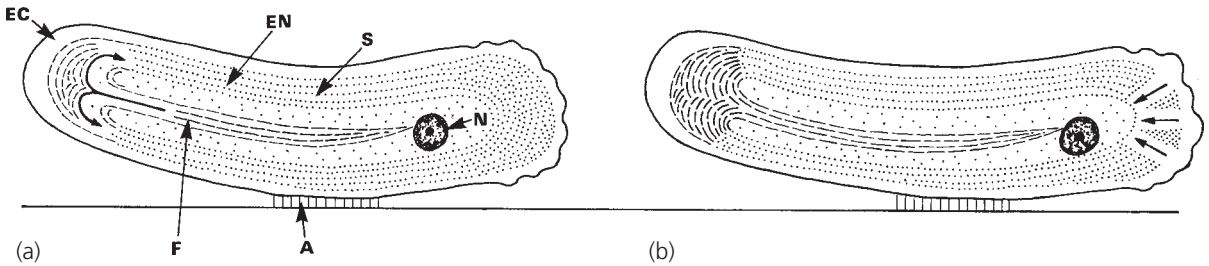

Figure 16 *Amebic movement brought about by a steady forward flux of the central liquid endoplasm and its transformation into a more stable form as it moves posteriorly and laterally. The origin of the forces needed for such movement is explained by either apical* **(a)** *or posterior* **(b)** *contractions (arrows). A, zone of adhesion to the surface; EC, hyalinic ectoplasm; EN, dense, granulomatous endoplasm; F, fluid portion of endoplasm; N, nucleus; S, stable, nonfluid portion of endoplasm.*

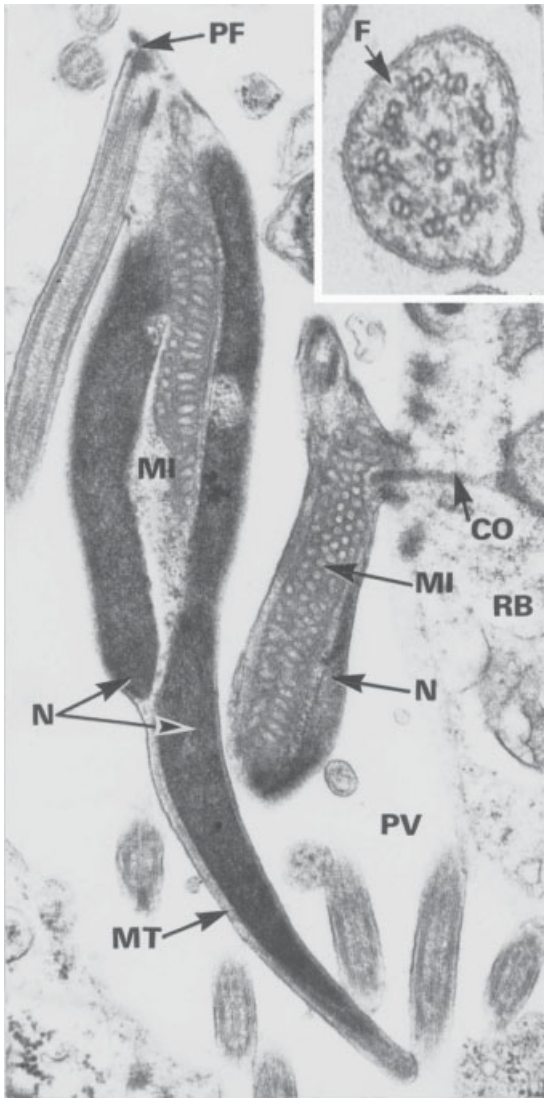

Figure 17 *Transmission electron micrographs of a longitudinal section through a coccidian microgamete (*Sarcocystis *sp.) and a cross-section through a flagellum (inset). CO, connection of the nucleus to the microgamont; F, flagellum; MI, mitochondrion; MT, microtubule; N, nucleus; PF, perforatorium (apical pole); PV, parasitophorous vacuole; RB, residual body (×25 000; inset ×100 000).*

to date, have been found only in the cells of higher vertebrates. The various types of filaments are organized into the microtubules of the cytoskeleton. The tubules have an outer diameter of 25 nm and an inner diameter of 15 nm. They are composed of protofilaments that are visible in cross-section. The protofilaments consist of α and β tubulin elements in a helical arrangement. The microtubules are polymerized at particular points called microtubule organizing centers (MTOCs). These centers exist at centromeres, centrioles, and at certain places in membranes. They also occur as constituents of flagella and cilia (see Figures 28 and 29).

The processes that bring about motility of the cytoplasm are well documented for metazoan cells, but for protozoans they are still poorly understood (Figure 17). In all cases, it is probably the aggregation of actin with myosin and tropomyosin to form an actomyosin complex that leads to movement, as proposed in the early sliding filament model of Huxley and Hanson (1954). This ATP-dependent system may produce relatively rapid movements, as occur in amoeba (20 μm s^{-1}) and in the sporozoites and merozoites of sporozoans. In addition to the cytoskeletal system, most parasitic protozoans have developed unique skeletal elements composed of combinations of the usual cytoskeleton elements. These structures include the following:

- subpellicular microtubules of trypanosomes and the motile stages of the Coccidia (see Figures 4 and 5)
- kinetodesmal fibrils of ciliates (Figure 18a)
- bundles of cytoplasmic microtubules in gamonts of piroplasms (Figure 18b)
- combined microtubules and filaments observed in the ventral disc of the diplomonadids (Figure 18c)
- crystalloid protein densifications that occur below the membrane of giardial trophozoites, at the apex of eimerian microgametes (Figure 18d) and also in the gamonts of piroplasms (Figure 18b)
- axostyles and pelta, occurring prominently in the trichomonads and consisting of one or more parallel rows of microtubules (Figure 19)
- costa and parabasal filaments, the filamentous, sometimes striated elements (Figure 19) that line the

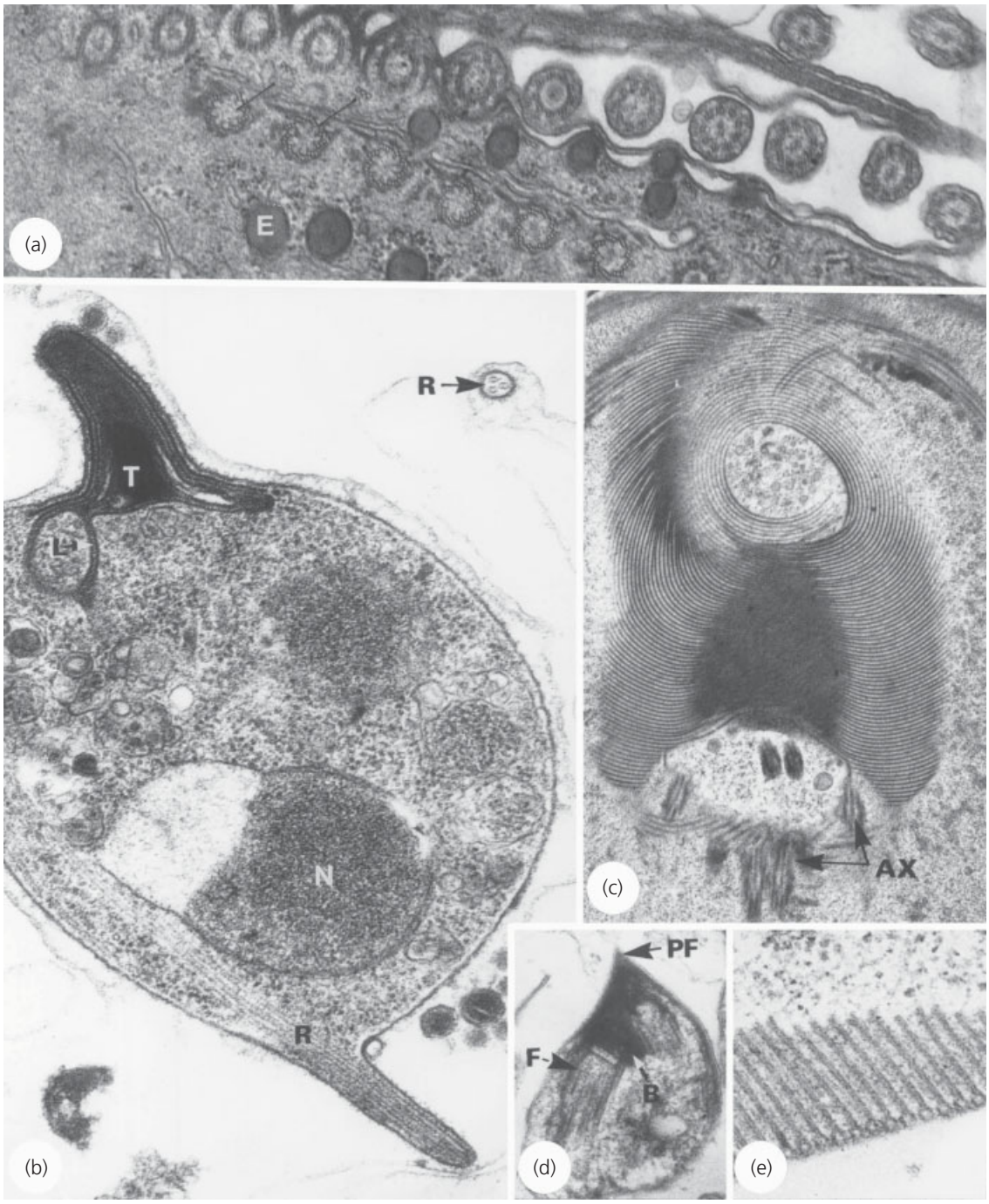

Figure 18 *Transmission electron micrographs:* **(a)** *Cross-section through the base of a cilium of Ichthyophthirius multifiliis showing interconnecting microtubules (arrows). E, Extrusomes (×30 000).* **(b)** Babesia canis*, longitudinal section through a microgamont. Note the thorn-like dense apex (A), the microtubule-containing ray (R), and the labyrinthine structure (L) at the base of the thorn (T). N, nucleus (×25 000).* **(c)** *Tangential section through the ventral disc of* Giardia lamblia*. Note the helically arranged rows of material. AX, axoneme (×20 000).* **(d)** *Perforatorium of the microgamete of eimerians, a structure needed for entering the macrogamete. B, basal body; F, flagellum; PF, perforatorium.* **(e)** *Cross section through the disc of* Giardia lamblia*, showing microtubules attached to filaments (×95 000).*

recurrent flagellum or the Golgi apparatus in trichomonads

- paraxial rods, which consist of a network of microfilaments that run along the axonemal microtubules of the flagella of Kinetoplastida (Figures 4, 7, and 29a)
- conoids, which are found in the motile stages of some Coccidia, such as in the genera *Eimeria*, *Sarcocystis*,

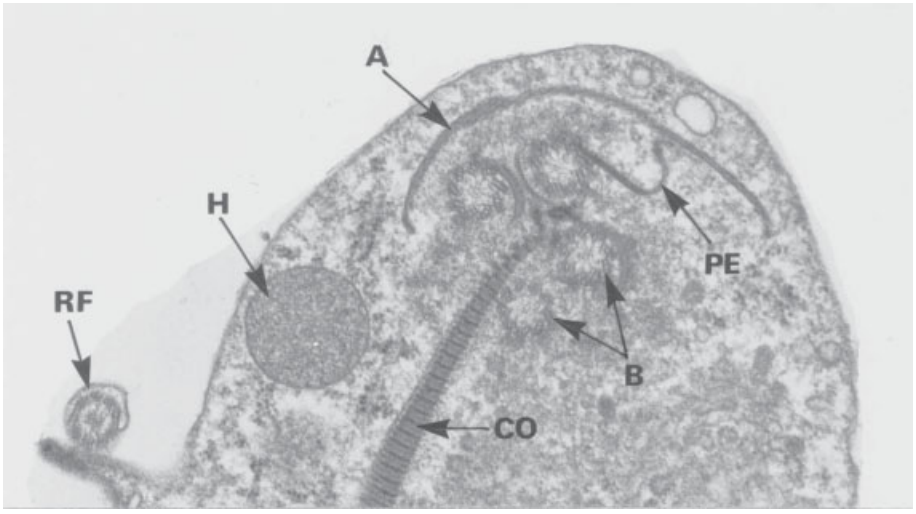

Figure 19 *Transmission electron micrographs of the apical pole of* Trichomonas vaginalis *showing the line of microtubules making up the axostyle (a), the basal bodies of the four anterior free flagella (b) and the costa (c) (×15 000). H, hydrogenosome; PE, pelta; RF, recurrent flagellum running along a surface fold.*

and *Toxoplasma* (see Figures 8 and 9a), but which are always absent from the hemosporidians (e.g. genera *Plasmodium*, *Theileria*, *Babesia*).

The conoid (Figure 9a) is a hollow truncated cone composed of spiralling microtubules 25 nm in diameter. Two accessory structures, the conoidal or preconoidal rings, form an integral part of the conoid and they are connected with each other by a canopy-like membrane. During cell penetration the conoid protrudes through the anterior polar ring system. The conoid is apparently involved in penetration of host cells.

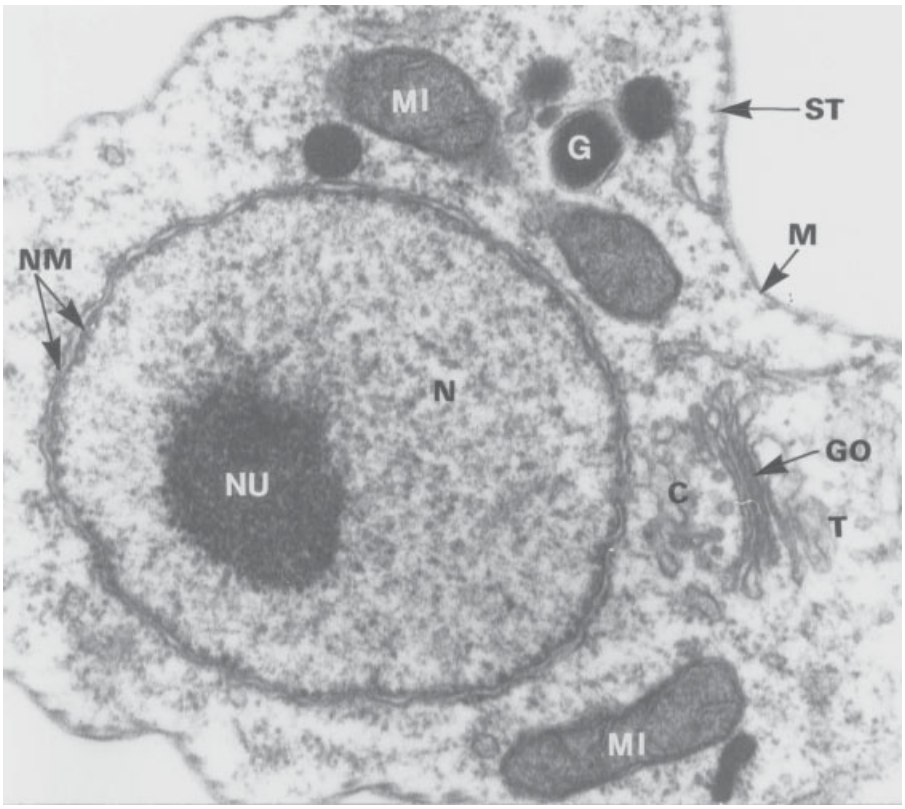

Figure 20 *Transmission electron micrograph of a developing stage of* Crithidia *sp. The section is in the region of the nucleus (×25 000). GO, Golgi apparatus; M, cell membrane MI, mitochondrion; N, nucleus; NM, nuclear pore; NU, nucleolus; ST, subpellicular microtubules; T, trans side of the Golgi apparatus.*

CYTOPLASMIC INCLUSIONS

The endoplasm contains a variety of organelles and other structures within which metabolic processes occur.

Nucleus

Protozoa possess at least one well-developed nucleus that is usually spherical to ovoid (Figure 20) and enclosed by a double-layered membrane containing pores (Figures 1 and 21). The pores are c. 50–70 nm in diameter with a central opening made up of eight subunits.

When viewed by light microscopy, the nuclei may appear vesicular or compact. The karyoplasm is composed of structural and enigmatic proteins: the chromatin (DNA) which may be organized into chromosomes; and the nucleolus. No membranes exist within the nucleus. There is usually only one nucleolus but in some species, and in certain developmental stages of other species, there may be several. The nucleolus is the site of synthesis of the large RNAs (28S, 18S, and 5.8S) and of the precursors of the ribosomes. It has two zones; one is composed of filaments 5–8 nm wide and the other is composed of granules 15 nm wide. The granular zone is situated at the periphery of the nucleolus. During nuclear division the nucleoli arc often dissolved and the chromosomes may condense and become visible (e.g. in ciliates) or they may remain stretched out and invisible (e.g. in the eimerians and the hemosporidians). The nuclear membranes are retained in the protozoans during division. Separation of the chromosomes is carried out by a spindle apparatus, the appearance, arrangement, and placement of which is species specific. The apparatus always consists of microtubules and filaments.

In ciliates, two morphologically distinct nuclei occur: a generative micronucleus and a somatic macronucleus (see Figure 31e). In other parasitic species, and in some specific stages such as the meronts, sporonts, and gamonts of the coccidia and hemosporidia, there may be many nuclei, all of which may have a similar appearance. Morphologically similar nuclei may have similar functions or they may have different functions, as in the Myxosporea. During binary division the karyoplasm is usually completely distributed between the two daughter nuclei. In some cases, e.g. during the formation of microgametes in some coccidians, only one functional nucleus is produced and a part of the nucleus is left behind (see Figure 32c, e).

Mitochondria and related structures

Mitochondria contain the enzymes for oxidative phosphorylation and the tricarboxylic acid cycle, and are bounded by two membranes. Most parasitic Protozoa have one of three basic types of mitochondria, distinguished by characteristic infoldings of the inner

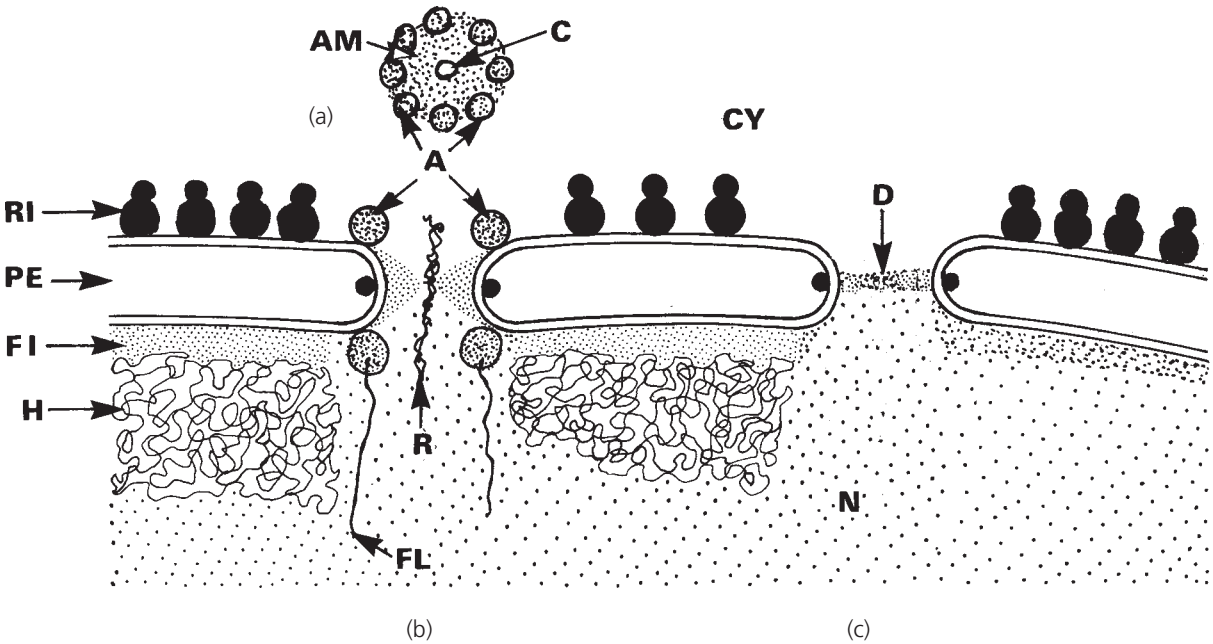

Figure 21 *Nuclear pores (diameter c. 80 nm):* **(a)** *Surface view showing the eight outer peripheral annuli.* **(b)** *Longitudinal section of an active pore.* **(c)** *Longitudinal section of an inactive pore. A, annular element; AM, amorphous material; C, central channel (diameter 15–20 nm) also called central granulum when RNA passes through; CY, cytoplasm; D, diaphragm; Fl, filamentous layer; FL, filaments connected at inner annular elements; H, heterochromatin; N, nucleoplasm (karyoplasm); PE, perinuclear spore; R, RNA; RI, ribosome (with subunits).*

membrane, which may be tubular, sack-like, or cristae-like (Figure 22).

Mitochondria reproduce by division and are therefore said to be semiautonomous organelles. This form of reproduction is possible because mitochondria have their own DNA. In most mitochondria this DNA is composed of two circular strands arranged in a supercoil. Some species have a single, large mitochondrion that contains more DNA, e.g. species of *Trypanosoma* and *Leishmania*. These organisms have 5 percent of their DNA in a single structure called the kinetoplast (Figures 4 and 7.22a) which is located close to the basal body of the flagellum. Kinetoplastid flagellates have no infoldings in this region of the mitochondria, providing space for the thousands of minicircles (0.3–0.8 μm in length) and the few maxicircles (9–11 μm in length) that make up their mitochondrial DNA; this region stains with Giemsa solution and is visible as a deep purple dot. Only the DNA of the maxicircles seems to be transcribed. During cell division the kinetoplast is always reproduced before nuclear division occurs. Trichomonads, some amoeba (e.g. *Entamoeba histolytica*), and Microspora have no mitochondria, but some amoeba contain symbiotic bacteria that may function as mitochondria. The trichomonads, which are anaerobic, have microbodies called hydrogenosomes. These spherical organelles have two closely attached membranes surrounding a granular matrix (Figure 19). The enzyme system of these bodies differs from that of mitochondria, as they metabolize pyruvate from glycolysis into acetate, carbon dioxide, and hydrogen. In ciliates, hydrogenosomes with more easily visible double membranes are present, as well as mitochondria.

The motile stages of sporozoans (i.e. merozoites and sporozoites, Figures 23 and 7.24a) have a Golgi-adjunct body (also called a double-walled organelle) close to the nucleus (Scholtyseck and Mehlhorn 1972).

In several species and genera (e.g. *T. gondii*, *Sarcocystis*, and *Plasmodium*) this organelle contains genes of the photosystem II and resembles a plastid, or a remnant of one, in terms of shape and activity (Hackstein et al. 1995). This body is now called the apicoplast and has been found to contain a third genome (in addition to that of the nucleus and mitochondria).

Endoplasmic reticulum, ribosomes, and golgi apparatus

The endoplasmic reticulum (ER) is a large system of tubes and sacs that runs throughout the cell. It connects the nuclear space with the cell interior and with the cell surface (Figures 1 and 25). There are two types of ER: rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (sER).

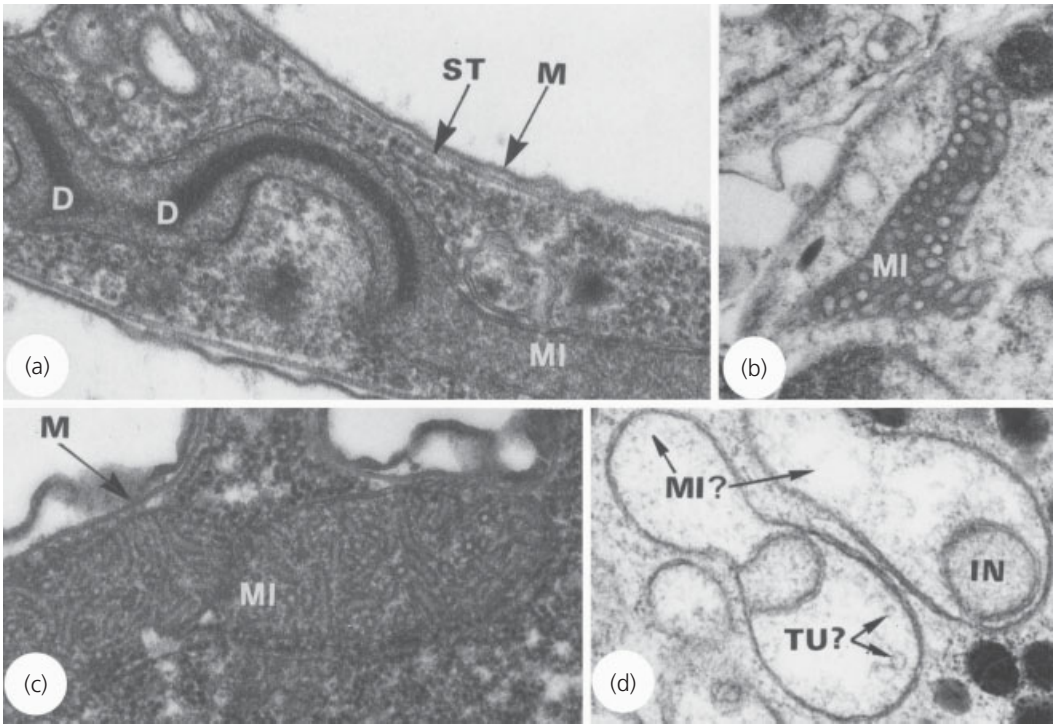

Figure 22 *Transmission electron micrographs of mitochondria or similar structures:* **(a)** Trypanosoma vivax*, kinetoplast in division (mitochondrion with cristae) (×25 000).* **(b)** Sarcocystis *microgamont with a mitochondrion of the sacculus type (×25 000).* **(c)** Ichthyophthirius multifiliis*; a trophozoite with a mitochondrion of the tubular type (×40 000).* **(d)** Theileria *sp.; mitochondria-like structures within kinetes. D, DNA filaments; IN, invagination; M, cell membrane; MI, mitochonidrion; ST, subpellicular microtubule; TU, tubule (×30 000).*

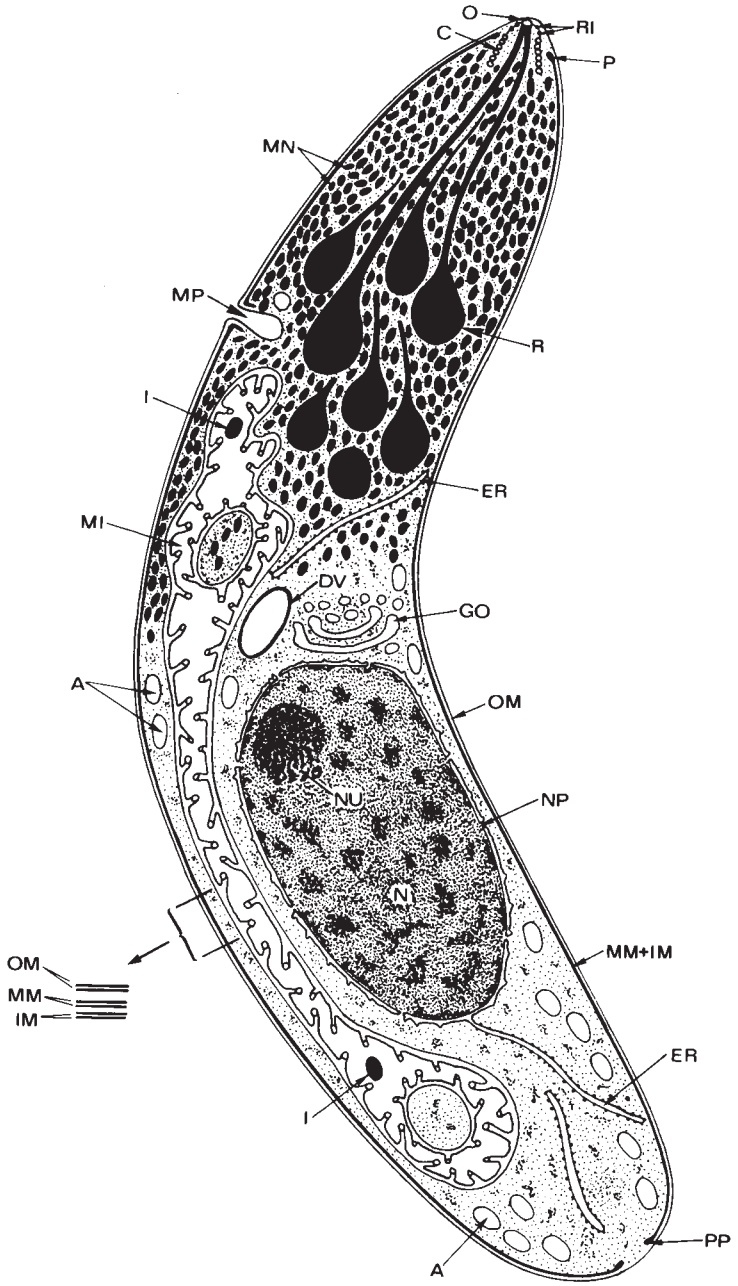

Figure 23 *Longitudinal section through a typical coccidian merozoite. A, amylopectin; C, conoid; D, dense, spherical bodies; DW, double-walled vesicle; ER, endoplasmic reticulum; GO, Golgi apparatus; I, dense inclusion; IM, inner membrane; MI, mitochondrion; MM, middle membrane; MN, micronemes; MP, micropore; N, nucleus; NP, nuclear pore; NU, nucleolus (karyosome); O, opening of the conoid; OM, outer membrane of pellicle; P, anterior polar ring; PP, posterior polar ring; R, rhoptries; RI ring-like elements of the conoidal canopy*

The rER is characterized by the presence of ribosomes (Figures 1 and 25) along its outer surface, whereas these are lacking in the sER. The rER and the sER may be interconnected.

The ribosomes consist of RNA and protein. The ribosomal RNA represents about four-fifths of the total cellular RNA. There are several types of ribosomes, depending on the nature of their subunits (Table 1; Figure 20); these subunits can be released by appropriate treatment of the ribosomal proteins. Several ribosomes often align in a chain to form polyribosomes, or polysomes (Figures 1 and 25), where they are collectively active in protein synthesis. Ribosomes occur along the surface of the rER, either singly or as polysomes. They also occur in the cytoplasm and inside the mitochondria. In Protozoa, ribosomes have a diameter of c. 30 nm and are composed of two subunits with sedimentation characteristics of 60S and 40S. The intact

ribosome is of the 80S type. Mitochondrial ribosomes are of the 70S type or, in ciliates, 80S. The mitochondrial ribosomes are similar to those of prokaryotes (Bielka 1982) (see Table 1).

The Golgi apparatus is a flat sac with swollen regions (Figures 1 and 26), from which vesicles are formed (Farquhar and Palade 1981). Several Golgi apparatuses together form dictyosomes. These organelles are always situated close to the rER and the two organelles act together as functional units in the production and transport of membrane and in the formation and transport of all types of macromolecular proteins and lipids (Gal and Raikhel 1993). The Golgi apparatus is never covered by ribosomes. Although many parasitic protozoans have prominent dictyosomes, certain developmental stages of protozoans have only a single Golgi apparatus which is difficult to detect in electron micrographs. The principal function of the Golgi apparatus is the transport of secretions and excretions formed on the rER. These reach the Golgi apparatus at its cis side and are transported within vacuoles that are cut off at its periphery (the trans region) (Figure 26). Endocytotic vesicles such as food vacuoles also come into contact with the Golgi apparatus and the rER system at points where they fuse

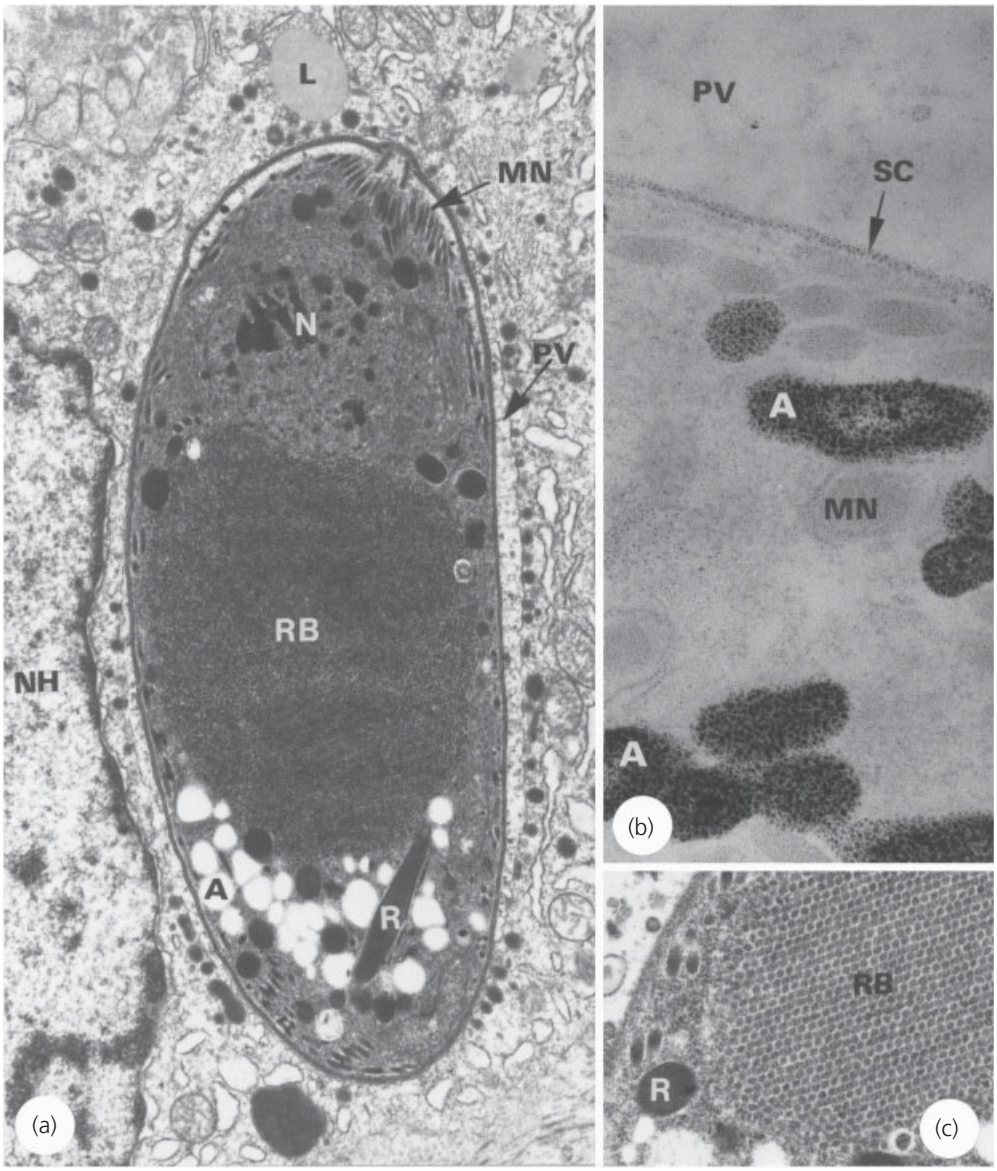

Figure 24 *Transmission electron micrograph of storage materials in coccidians:* **(a)** *Longitudinal section of a sporozoite of* Cystoisospora felis. *A, amylopectin; L, lipid; MN, micronemes; N, nucleus; NH, nucleus of the host cell; PV, parasitophorous vacuole; RB, refractile body (consisting of protein granules); RH, rhoptry; SC, surface coat (×12 000).* **(b)** *The Thiéry reaction shows polysaccharides (e.g. amylopectin) within granules (A) and along the surface (SC) of a meiotic (×65 000).* **(c)** *Cross-section of* C. felis *showing structure of refractile body. RB, refractile body; R, rhoptry (×30 000).*

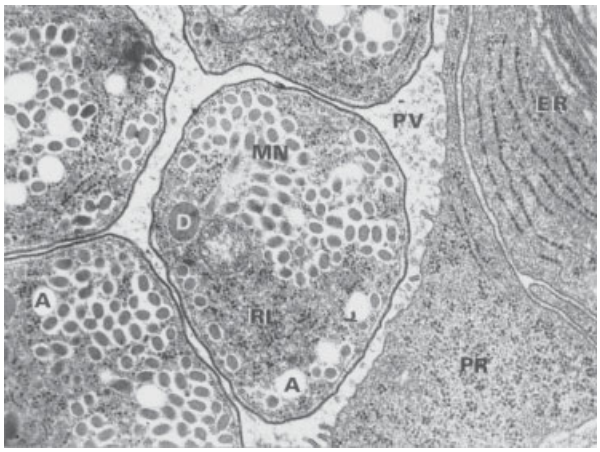

Figure 25 *Transmission electron micrograph of a cross-section through a mass of merozoites of* Eimeria. *The cytoplasm of the host cell adjacent to the parasitophorous vacuole (PV) shows the rough endoplasmic reticulum (ER) and many polyribosomes (PR). A, amylopectin; D, dense body; MN, micronemes; RI, ribosomes (×30 000).*

with enzyme-containing vacuoles produced by the Golgi apparatus; this is how digestive enzymes and other products enter the endocytotic vacuoles.

Lysosomes

Lysosomes are vesicles measuring 0.2–0.5 μm, bound by a single membrane. They are derived from the sER and are formed and released from the secretion side of the Golgi apparatus (the trans side). They contain enzymes such as phosphatases, proteinases, lipases, and nucleases, and have an internal pH of 4–5. When first released they are called primary lysosomes. After fusion with the endocytotic vesicles their enzymes become active and the vesicle is then called a secondary lysosome. In these secondary lysosomes, or phagolysosomes, the ingested food is dispersed (Figures 1 and 26). Another type of secondary lysosome is the autolysosome, which is involved in the disintegration of cellular waste material, thus providing the function of disposal of debris. In *Leishmania* species so-called megasomes (reaching the size of the nucleus) may occur as special type of lysomal vacuole.

Microbodies

Microbodies are ubiquitous in eukaryotic cells. Several types of microbodies have been described (de Souza 2002):

- Hydrogenosomes (Benchimol 1999) are mostly spherical and occur in trichomonads. They reach a diameter of about 0.5–1 μm, are bound by two closely attached membranes, and reproduce by binary fission. However, they do not contain nucleic acid. Thus their proteins are synthesized in free ribosomes and post-translationally inserted into the organelle. As shown by biochemical methods, hydrogenosomes produce ATP and the molecular hydrogen that gives them their name.
- Peroxisomes (called glycosomes in trypanosomatids). These spherical, dense-appearing organelles are membrane-bound, randomly distributed throughout the cell, and reach a size of 0.7 μm diameter. Their name comes from the fact that they usually contain enzymes which use molecular oxygen to remove hydrogen atoms from organic substrate via an oxidative reaction, thus producing hydrogen peroxide

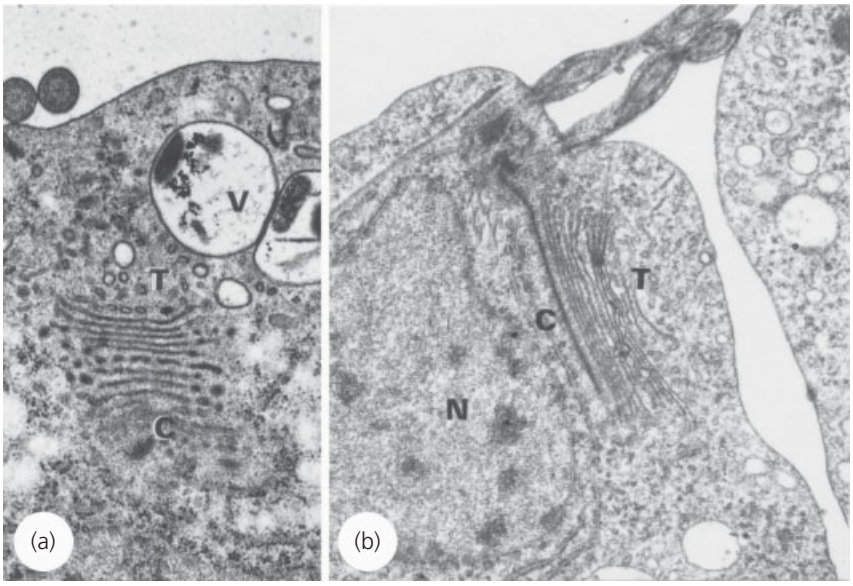

Figure 26 *Transmission electron micrograph of the Golgi apparatus in* Trichomonas vaginalis. *Note the active forms* **(a)** *and the inactive ones* **(b)** *(×20 000). C,* cis *side of Golgi; N, nucleus; T,* trans *side of Golgi; V, food vacuole.*

(H_2O_2). Furthermore, the peroxisomes contain catalase, which utilizes the H_2O_2 to oxidize a variety of other substrates by the so-called peroxidative reaction. In the case of the peroxisomes of trypanosomatids it has been clearly shown that they contain glycolytic enzymes being involved in the conversion of glucose to 3-phosphoglycerate (Opperdoes 1987). These bodies (originally named glycosomes) do not possess a genome and are thus encoded by nuclear genes as are the other peroxisomes. Reproduction occurs via binary fission.

- Acidocalcisomes (formerly designated in light microscopy as 'volutin granules') occur in large numbers in trypanosomes as spherical organelles of about 200 nm in diameter in all regions of the cell. They are limited by a 6-nm thick membrane and are involved in the storage and exchange of calcium, magnesium, and zinc ions (Docampo and Moreno 2001).
- Glyoxisomes of many protozoans process lipids for energy production. The reservosomes of *Trypanosoma cruzi* are spherical, reaching diameters of 0.7 μm and include all macromolecules ingested during the endocytic processes in the flagellar pocket. Also cruzipain, the major cysteine proteinase in the cell, is accumulated with reservosomes.

Rhoptries, micronemes, and dense bodies

The osmiophilic rhoptries, micronemes, and dense bodies are characteristic of the motile stages of sporozoans. They are usually located in the area between the nucleus and the apical pole. Rhoptries vary considerably in number (2–20) and in shape, appearing club-shaped (*Eimeria*), teardrop shaped (*Plasmodium*), or elongated (*Sarcocystis*, *Toxoplasma*). The anterior neck portions of rhoptries are narrow and duct-like and are found at the extreme tip of the cell running through the conoid if one is present (Figures 8 and 24). Rhoptries release enzymes (Rhop 1–20) during the penetration process, after which they appear partly empty with a sponge-like interior. Micronemes are small rod-like structures 50–90 × 300–600 nm with rounded ends. These structures usually occupy the anterior regions of the motile stages of the sporozoans and are often arranged in bundles (Figures 24 and 27). The function of micronemes is not completely understood, although it is known that they contain at least seven proteins, which are used during adhesion of the parasite to the host cell as is the case in the so-called circumsporozoite protein (CSP) of *Plasmodium* species. Dense bodies (0.2 μm) are found at the anterior part of penetrating coccidian stages. At least six different proteins (GRA 1–6) are released during penetration into the parasitophorous vacuole (as defense against the digestive enzymes of the host cell?). They disappear during the reproductive process in meronts, macrogamonts, and microgamonts.

Wall-forming bodies

Parasites that produce walled cysts, such as amoebae, *Giardia*, coccidians, *Balantidium*, Myxozoa, and Microspora, develop wall-forming bodies of various types (see Figures 11e and 12). In amoebae, diplomonadids, Microspora, and Myxozoa, the contents of the wall-forming bodies fuse outside the cell after being excreted by exocytosis. This fused material forms an external cyst wall (see Figure 12). In macrogametes of coccidia the wall-forming bodies fuse in the region immediately below the cell membrane, thus producing an internal cyst wall (see Figure 11).

One or two different types of wall-forming body may occur in coccidia of the various genera. For example, in *Eimeria* and *Isospora* the macrogametes have two types of wall-forming bodies: (1) electron-dense bodies that give rise to the outer layer of the oocyst wall; and (2) sponge-like bodies that fuse to produce the inner layer of the oocyst wall. The entire oocyst wall is produced

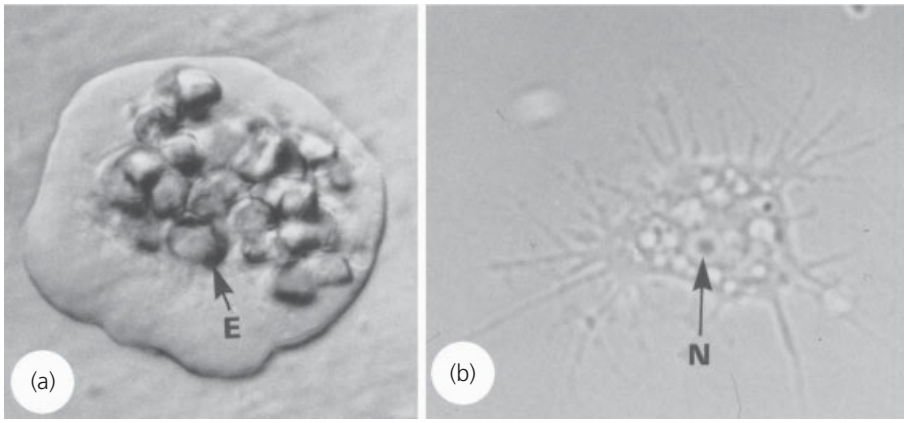

Figure 27 *Light micrographs showing amoebae with lobopodia* **(a)** *and filopodia (h):* **(a)** Entamoeba histolytica*, magna form. E, erythrocyte (×2 000).* **(b)** Acanthamoeba castellanii. *N, nucleus (×1 200).*

inside the cell membrane (see Figure 11e). The oocysts of *Sarcocystis* and the sporocysts of all coccidians are bound by a smooth wall that is formed by fusion of a single type of electron-dense wall-forming body (see Figure 11g). The cyst walls of all stages of Coccidia provide good protection against adverse environmental conditions. Whether they are single or double walled, the oocysts and sporocysts of all *Eimeria* have openings, whereas the sporocysts of *Isospora* and of the tissue cyst-forming coccidia have sutures in their walls (Figure 12d).

Vacuoles and storage elements

All parasitic protozoa have various types of vacuoles, the largest of which are the food vacuoles (see Figure 1); these disintegrate during digestion. Vacuoles that serve as storage elements contain crystalloid proteins (Figure 24a), lipids, or carbohydrates (Figure 24c). Lipid-containing vacuoles appear slightly grey in electron micrographs and are present mainly in resting stages such as cysts. Large amounts of protein are found in the refractile bodies of sporozoan sporozoites (Figure 24a) and in *Plasmodium* ookinetes. Carbohydrates are generally stored in the form of granules of glycogen or amylopectin (Figures 24a and 25). Amylopectin-containing granules appear as brilliant white areas in electron micrographs of gregarines, coccidians, and some endoparasitic ciliates. These granules are scattered throughout the cytoplasm of sporozoans of all developmental stages except microgametes, and are particularly numerous in the cytoplasm of macrogametes and oocysts (see Figure 11e). Glycogen (see Figure 14a) is present as small randomly distributed granules in the cytoplasm of *Tritrichomonas foetus* and as a large mass in the cytoplasm near the nucleus in *Entamoeba* or *Iodamoeba* cysts.

Locomotor systems

All protozoa are motile in at least one stage of their life cycle. The different species have developed distinct locomotor systems during their evolution.

Some protozoans use pseudopodia as locomotory organs. These structures, which may occur as thick lobopodia or as fine filopodia (Figure 27), are produced by the cytoplasmic movement mediated by the activity of the calcium-regulated actomyosin complexes. The contraction of filaments of actomyosin causes pressure on the cytoplasm at the posterior pole and this initiates a forward flow of cytoplasm at the apical pole. At the apical pole the amoeboid movement is enhanced by a transformation of the local cytoplasm from a stable gel form to a more liquid sol form (see Figure 16). Pseudopodia are most prominent in the various types of amoeba but may also be found in motile metazoan cells such as leucocytes.

Many protozoans use flagella or cilia as locomotor organs (Hausmann et al. 2003). These structures are constructed according to a common plan. Flagella are longer and generally less numerous than cilia, but their basic structures are similar (Figures 1, 28, and 29).

Both types of organelles are c. 0.2–0.4 μm in diameter and both possess an axoneme (an arrangement of nine pairs of outer microtubules and a single pair of central microtubules) which is anchored to a basal body (kinetosome) that resides inside the cortical cytoplasm of the cell (Figures 1 and 29d). The basal bodies are similar to centrioles in having nine sets of three microtubules arranged in a ring-like pattern. The basal bodies may be connected to filamentous elements such as the kinetodesmal filaments of cilia. Several species of Protozoa have a rod-like structure consisting of a network of protein filaments inside the flagellum (Figures 4, 7, and 29a). These rods lie beside the axoneme, probably adding to the thickness and stability of the flagellum. This enhancement may be particularly important for protozoans living in viscous media such as blood or intestinal fluids. Although flagella and cilia are constructed according to a general blueprint, in different protozoans the paired outer microtubules in the flagella and cilia may differ in shape (Figure 28). The A tubule, which is furnished with two dynein arms, typically possesses 13 protofilaments, whereas the B tubule has only nine protofilaments and shares three to four of the protofilaments of the A tubule. The dynein arms act as enzymes for breaking down ATP and are proposed to represent the motor system in the 'gliding filament' theory. This theory postulates that the movement of flagella and cilia is initiated by the gliding of microtubules along each other using the dynein arms as linking elements. The central regions of flagella are stabilized by spike-like elements

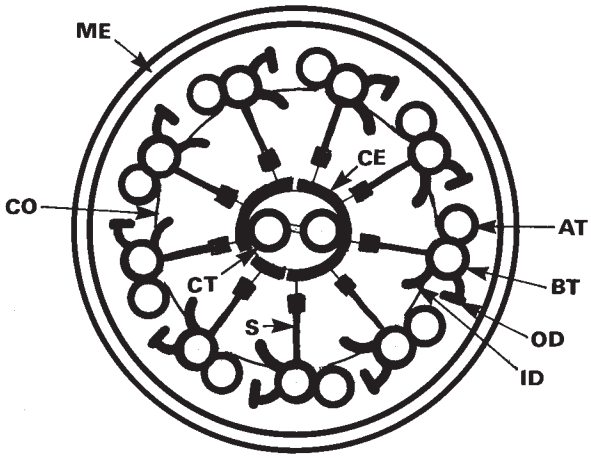

Figure 28 *Diagrammatic representation of a cross-section through a cilium or flagellum. AT, A tubules (consisting of 13 subunits); BT, B tubules; CF, central sheath; CO, connection between the outer pairs of microtubules; CT, central tubule; ID, inner dynein arm; ME, cell membrane; OD, outer dynein arm; S, spike.*

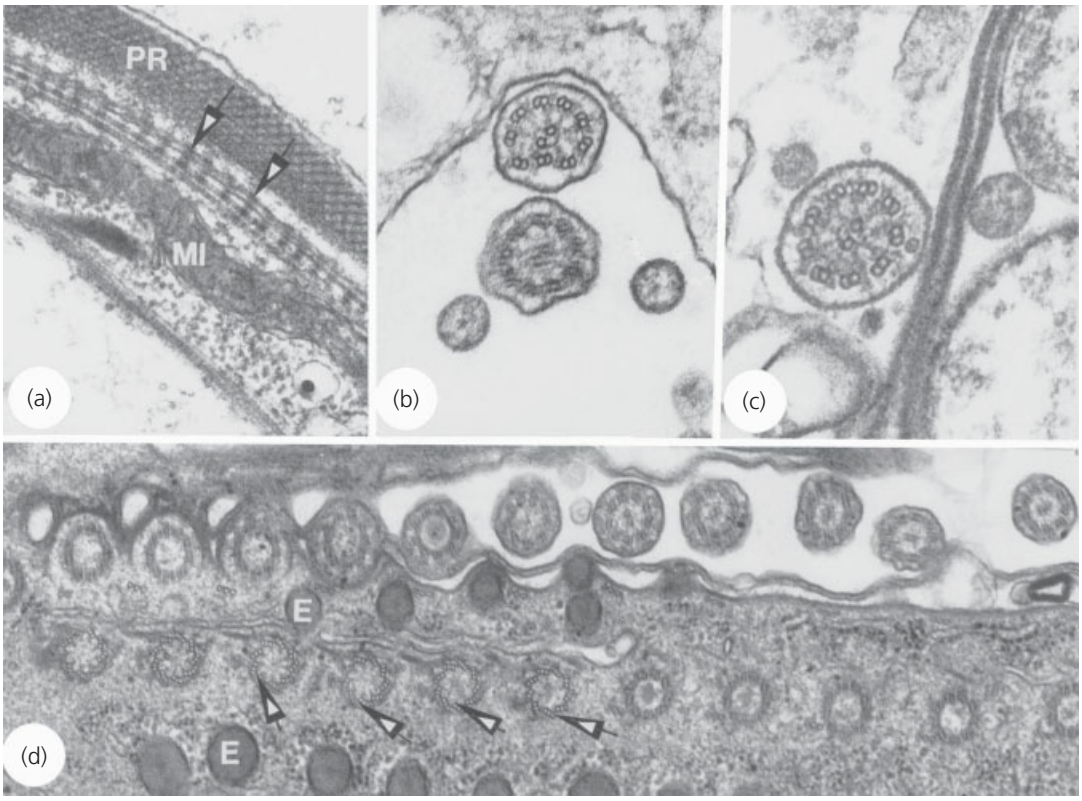

Figure 29 *Transmission electron micrographs of flagella* **(a–c)** *and cilia* **(d)**. **(a)** Trypanosoma vivax*, longitudinal section of a flagellum, attached to the surface by semidesmosomes (arrows) (×23 000).* **(b, c)** *Cross-section through the flagella of the gametes of* Eimeria *and* Sarcocystis *species showing the typical 9 × 2 + 2 pattern (b, ×25 000; c, ×40 000).* **(d)** *Cross-section through the basal bodies of cilia showing the typical 9 × 3 tubule arrangement (arrows). E, extrusomes; MI, mitochondrion; PR, paraxial rod of the flagellum.*

(Figure 28) in addition to the rod mentioned above. In the trichomonads and in the trypomastigote stages of trypanosomes, a flagellum may be connected to the cell surface by desmosomes (Figure 29). The recurrent flagella are attached in this manner and when the attached flagellum pulls the plasmalemma away from the body, the undulating membrane is created. The recurrent flagella never run inside the cytoplasm, but the axonemes of *Giardia* trophozoites run inside the cytoplasm for several micrometers (Figure 18c).

The invasive stages of sporozoans, i.e. the merozoites and sporozoites, have three types of movement: gliding, twisting, and bending. Only the first of these leads to active displacement of the organisms; the other two only change the direction of movement. The gliding form of movement is extremely rare in eukaryotic cells. It is temperature sensitive and cytochalasin B sensitive, the latter property suggesting the participation of actin in the process. The gliding movement may be related to the capping phenomenon in sporozoans. In capping, the organisms aggregate materials on their surfaces and move them towards the posterior pole, from where they release them into the surroundings. A parasite floating in a liquid could move forward using this type of action. The most studied of the capping phenomena is the circumsporozoite reaction of *P. falciparum* sporozoites.

Surface interactions and penetration

Parasites living inside host cells use a variety of means, either active or passive, to enter the host cell. In active processes the parasite's motility mechanisms play a role, whereas in passive processes the host cell internalizes the parasite by a phagocytic mechanism in the same way that food particles are ingested. In all cases, however, entry occurs after an initial recognition step in which some structure on the parasite surface reacts with and binds to some structure on the host-cell surface.

Different types of invasion processes are used by different intracellular pathogens. Some, such as the Microspora, penetrate the host-cell plasmalemma to enter the host-cell cytoplasm, whereas members of *Mycoplasma* and viruses invade by membrane fusion. Many parasites enter their host cells by phagocytosis. After entry by phagocytosis, different parasites follow different paths: the *Leishmania* develop in phagolysosomes; the mycobacteria inhibit fusion of the lysosomes with the phagosomes; and *T. cruzi*, some species of *Leishmania*, and all schizonts of *Theileria* (Piroplasmea) escape from the phagosome into the host-cell cytoplasm.

Some phagocytic processes are quite complex. Some organisms, such as malaria parasites, members of the genera *Eimeria*, *Sarcocystis*, and *Toxoplasma* actively

invade by forming a moving junction between the host membrane and the parasite. After completion of the process, the parasite rests in a parasitophorous vacuole bound by the invaginated host-cell membrane. This parasitophorous vacuole membrane may disintegrate after the parasite has penetrated; for instance, stages of *Babesia* and *Theileria* are located directly in the cytoplasm of their host cells (red blood cells) shortly after invasion is completed (Figure 30a, b).

MODES OF REPRODUCTION

In parasitic protozoans, cell division occurs during both vegetative and sexual development (in which it occurs during the formation of gametes). Cell division may occur in production of gametes of both sexes, as in the gregarines, or only during production of the male gamonts, as in the Coccidia.

Binary fission

The most basic type of multiplication is binary fission (Figure 31). This process produces two daughter cells after duplication of the organelles of the mother cell. The axis of cell division is a characteristic feature of the various groups within the Protozoa, and different types of division can be distinguished: irregular, amoeba-like, longitudinal, oblique, and transverse. In microsporidians (e.g. *Nosema*) binary fissions occur after nuclear divisions in an irregular fashion, the cytoplasm being divided irregularly and different sizes of daughter cells being formed. After freeing, however, the daughter cells all develop a similar shape. In rhizopods such as *Entamoeba* and *Acanthamoeba*, cell division is irregular with respect to the cytoplasm but the constriction always occurs perpendicular to the spindle axis; this is called ameba-like division (Figure 31a). In flagellates such as the diplomonads, trichomonads, and trypanosomatids, and in sporozoans such as the Coccidia, the axis of cell division runs longitudinally and the process is therefore called longitudinal division. In flagellates the polarity of the division axis is determined by the initial axis of duplication of the basal bodies of the flagella (Figure 31b). Unusual binary fissions occur in a few species of sporozoans. For instance, the process by which the merozoites of piroplasms bud from the parent cell, one at a time, could be considered to be an irregular form of binary fission. The processes of endodyogeny in those Coccidia that form tissue cysts is a very peculiar form of longitudinal division (Figure 31d). In this process, two daughter cells are formed within a mother cell, arching over and parallel to the axis of the dividing nucleus. The daughter cells produce the inner two pellicular membranes de novo from the membranes of the endoplasmic reticulum and take over the outer membrane of the mother cell's pellicle. The two inner membranes of the mother cell disintegrate. In trichomonads, the axis (Figure 31c) of cell division is longitudinal at the beginning of the process when the flagellar basal bodies and the nucleus are reduplicated. Later, the divided nuclei move into positions opposite each other and as a result an oblique, or even a transverse, cytoplasmic division occurs.

In opalinid flagellates, which are characterized by oblique rows of 'cilia,' binary fission occurs during the sexual and asexual developmental phases (Figure 31f). The axis of the cytoplasmic division initially runs longitudinally, but soon becomes parallel to the oblique rows of cilia. This is called oblique division. It is intermediate between the longitudinal fission of flagellates and the transverse division of ciliates.

In ciliates such as *Balantidium* and *Ichthyophthirius*, binary fission starts with the duplication of the cytostomal kinetosomes (basal bodies) and is followed by the division of the macronuclei and micronuclei. The axis of cytoplasmic division runs perpendicular to the axis of the nuclear spindles. Cell constriction may occur simultaneously with nuclear division or there may be a time lag. This process is also called oblique division, or, as the division proceeds across the rows of the cilia, it is also described as homothetogenic fission (Figure 31e).

Rosette-like multiplication

If division of the cytoplasm is incomplete or delayed after nuclear division, the pair of newly formed nuclei may divide again before cytoplasmic division occurs. This may result in the simultaneous development of more than two offspring. In some cases the offspring remain attached at their posterior poles for some time, forming a mass that may appear as a rosette in stained preparations. Studies of this process by light microscopy were originally misinterpreted and the process was considered to be a multiple division. Rosettes of this type are frequently found in cultures of trichomonads and trypanosomes. They are also relatively common in preparations of *T. gondii* collected during the acute phase of infection, when endodyogenies are often repeated inside parasitophorous vacuoles of macrophages and reticuloendothelial cells.

Multiple divisions

Multiple divisions are characteristic of amoebae, sporozoans, and Microspora, but are rarely observed in the other groups of parasitic protozoans. The formation of daughter cells by multiple divisions may be initiated by three main types of mother cells (Figure 32a–f).

A multinuclear type of mother cell may initiate multiple divisions. In this case, daughter cell formation starts at the end of a phase of repeated nuclear division in the mother cell. In this way, multinucleate plasmodia

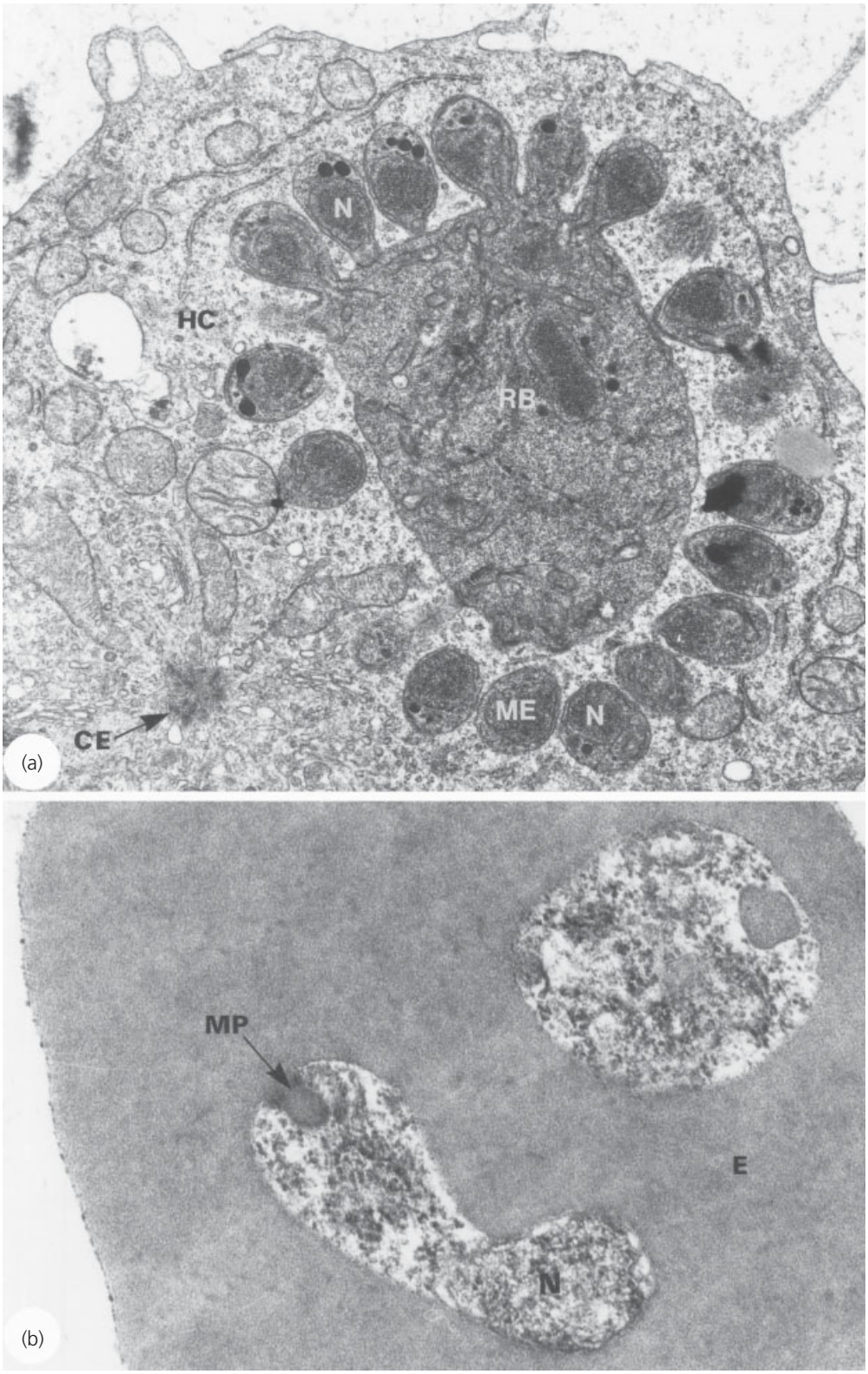

Figure 30 *Transmission electron micrographs of piroplasms (*Theileria annulata*) situated in the cytoplasm of their host cells.* **(a)** *A meront within a lymphocyte. The host cell was in the process of dividing; note the centriole (CE) and the spindle (×12 000).* **(b)** *A slender trophozoite and a spherical gamont in a bovine erythrocyte. CE, centriole; E, erythrocyte; HC, host cell cytoplasm; ME, merozoite, MP, micropore; N, nucleus; RB, residual body (×40 000).*

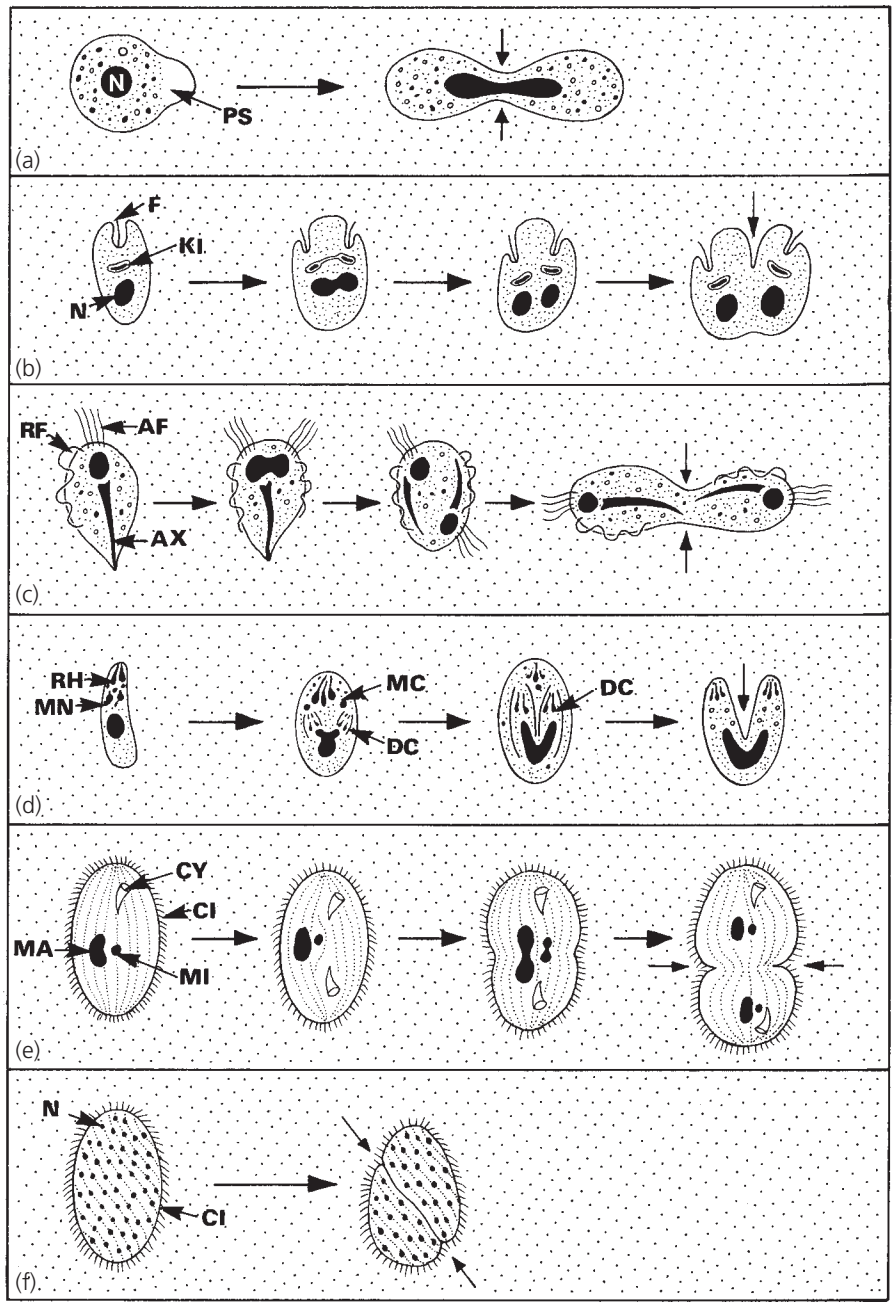

Figure 31 *Different types of binary fission (invaginations are shown by small arrows):* **(a)** Amoeba *type; division without fixed axis.* **(b)** *Trypanosomatid type (here* Leishmania*): longitudinal division.* **(c)** *Trichomonad type (here* T. vaginalis*): note that there is a longitudinal division only at the beginning.* **(d)** *Endodyogeny of tissue cyst forming* Coccidia *(e.g.* Toxoplasma*,* Sarcocystis*): inner development of daughter cells.* **(e)** *Ciliata type (e.g.* Balantidium*): cross-division.* **(f)** *Opalinata type: oblique division. AF, anterior free flagellum; AX, axostyle; B, basal body of flagellum; Cl, cilium; CY, cytopharynx; DC, daughter cell; F, short flagellum in a pocket; Kl, kinetoplast; MA, macronucleus; MC, mother cell; Ml, micronucleus; MN, micronemes; N, nucleus; PS, pale pseudopodium; RF, recurrent flagellum; RH, rhoptries.*

are produced before cytoplasmic division starts. The occurrence of such multinucleate forms led to the naming of the genus *Plasmodium*. Multinucleate stages are also found in the life cycles of *E. histolytica*, in meronts and sporonts of gregarines, and in some stages of some coccidians. They occur in all gamonts of gregarines, but only in the microgamonts of some Coccidia. The cytoplasm of the plasmodia may or may not be divided, but when it is divided the pattern is always species specific. In eimerian and theilerian meronts and in the sporonts of hemosporidians and of some piroplasms, daughter cell formation closely follows the last nuclear division. In eimerian microgamonts, a microgamete is formed around each nucleus and an electro-

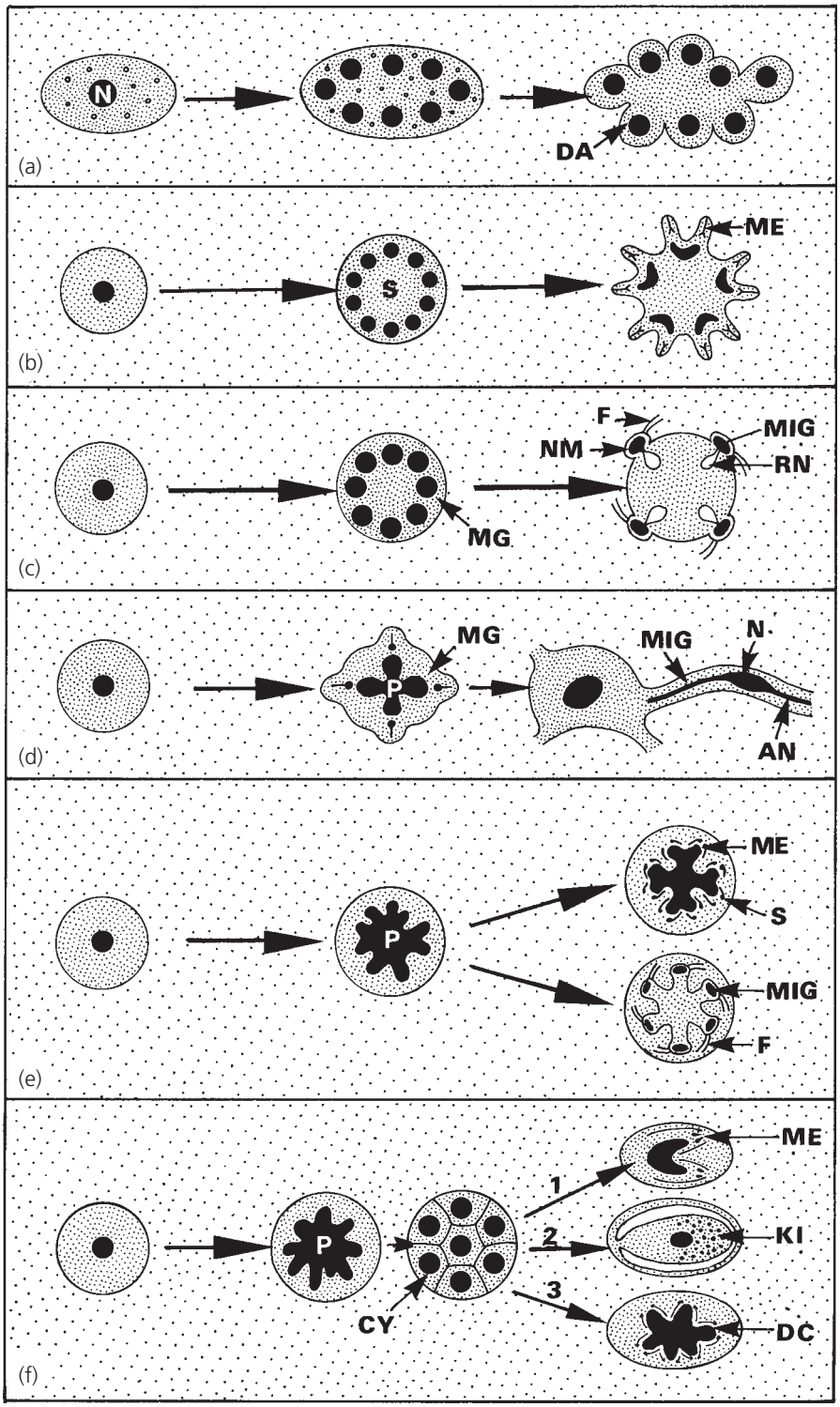

Figure 32 *Diagrammatic representation of multiple division:* **(a)** Ameba *sp.: formation of vegetative stages after excystation.* **(b)** *Formation of merozoites by meronts of species of* Eimeria, Plasmodium, Theileria, *etc.* **(c)** *Formation of microgametes, e.g.* Eimeria *spp.* **(d)** *Formation of microgametes of* Plasmodium *spp. and other hemosporidia (e.g. exflagellation).* **(e)** Sarcocystis *sp.: formation of merozoites by meronts (1) and of microgametes by microgamonts (2).* **(f)** *Formation of cytomeres. These may each develop: (1) two merozoites (some* Eimeria *spp.); (2) a single kinete (*Babesia *spp.); (3) many parasitic stages in the life cycle of various coccidians, i.e. merozoites of* Globidium *spp. or sporozoites of species of* Plasmodium, Babesia, *and* Theileria. *AN, axoneme (flagellum of microgamete); CT, cytomere; DA, daughter amoeba; DC, daughter cell; F, flagellum; KI, kinete; ME, merozoite anlage; MG, microgamont; MIG, microgamete; N, nucleus; NM, nucleus of microgamete (dense part); PN, polymorphous, multilobulated nucleus; RN, residual nucleus (light part); S, schizont.*

nlucent remnant of the mother cell's karyoplasm is left behind. Only occasionally do the nuclei of the mother cell divide after cytoplasmic division starts and therefore it is rare for two microgametes to originate from a single nucleus of the microgamont.

A uninuclear type of mother cell may initiate multiple division. In this case, daughter cell formation starts without a preceding phase of nuclear division, but the nucleus of the parasite has generally grown considerably and developed a lobulate surface before daughter cell formation occurs. When division of this giant nucleus begins, daughter cell formation occurs simultaneously. The daughter cells incorporate portions of the giant nucleus, the chromosomal components of which have been increased by a preceding endomitosis. This process of nuclear splitting occurs during the merogony of some cyst-forming coccidians and has been described as endopolygeny. The microgamonts of some species of *Sarcocystis* and of *Plasmodium* produce their microgametes in this way. A similar process of simultaneous nuclear splitting and daughter-cell formation is found in sporonts of some piroplasms during the production of sporozoites.

There is another type of multiple division that is intermediate between these two types. In these cases, a large mother cell with a lobated giant nucleus is divided into numerous uninuclear cytomeres. The single limiting membrane of these cytomeres originates from the surface membrane of the original cell or from the endoplasmic reticulum of the mother cell. Such cytomeres are regularly formed in meronts of some species of *Eimeria* such as *E. tenella*, in some species of *Globidium* and in sporonts of all species of *Babesia*, *Theileria*, *Plasmodium*, and *Hepatozoon*. The uninuclear cytomeres ultimately produce the daughter cells. They give rise to two merozoites in *Eimeria*, to a single kinete in *Babesia* and *Theileria*, to many merozoites in *Globidium*, and to sporozoites in *Plasmodium*, *Babesia*, and *Theileria*. Cytomeres merge in Myxozoa.

Additional information concerning the composition, metabolism, genetics, and molecular biology of the cell can be found in de Puytorac et al. (1987), Alberts et al. (2002), Darnell et al. (1994), Mehlhorn (2002), and Hausmann et al. (2003).

CONCLUSION

This chapter illustrates that the Protozoa are a heterogeneous group, with wide variations in morphology and life cycle. The parasitic protozoans are probably polyphyletic in origin and this is also probably true of the Eukaryota (see Classification and introduction to the parasitic protozoa). There is no good reason to assume that the Eukaryota are of a single evolutionary line, but rather they may have arisen by mutation and probably symbiosis from various prokaryotic predecessors at more than one time.

There are only a few features that are shared by all parasitic protozoans; they are all basically unicellular organisms and they are all eukaryotic. Based on their common unicellular eukaryotic plan, a vast array of forms has developed producing a wide variety of organisms. These organisms have successfully inhabited a great range of ecological niches, some of which, to our detriment, are located in the human or animal body (Mehlhorn 2002).

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Classification and introduction to the parasitic protozoa

F.E.G. COX

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There are >200 000 named species of protozoa, of which nearly 10 000 are parasitic in invertebrates and in almost every species of vertebrate. It is therefore not surprising that humans should act as hosts to protozoa, but what is surprising is that we should harbor so many. During our short evolutionary history we have acquired >70 species belonging to >30 genera as heirlooms from our primate ancestors or souvenirs from the animals with which we have come in contact. Many of these protozoa are relatively harmless, but a few cause some of the most important diseases of tropical countries such as malaria, sleeping sickness, Chagas disease, and leishmaniasis, which together threaten over one quarter of the population of the world. Many also occur in temperate regions and some are increasingly being recognized and implicated as major pathogens in immunocompromised patients, particularly those suffering from HIV infections or undergoing immunosuppressive therapy.

From a practical viewpoint it is convenient to regard the protozoa as organisms that lie somewhere between the prokaryotic and higher eukaryotic organisms, sharing some of the characteristics of each. They are small, mostly 1–150 μm, the parasitic forms tending towards the lower end of this range, have short generation times, high rates of reproduction and a tendency to induce immunity to reinfection in those hosts that survive; features of infections with microparasites such as bacteria. On the other hand, protozoa are undoubtedly eukaryotic cells with organelles and metabolic pathways similar to those of their hosts.

Structurally, each protozoan is the equivalent of a single metazoan cell with its plasma membrane, nucleus, nuclear membrane, chromosomes, endoplasmic reticulum and, in most cases, mitochondria, Golgi body, ribosomes and various specialized structures adapted to meet particular needs (see Cellular organization of parasitic protozoa). Parasitic protozoa are not simple or degenerate forms and their particular adaptations frequently include complex life cycles and specialized ways of entering their hosts and maintaining themselves once they have gained access. Their nutrition, physiology, and biochemistry are largely geared to the parasitic habit and are specialized rather than degenerate. Sexual reproduction occurs in some protozoa and, in the parasitic forms, is particularly important in the sporozoans in which it provides for apparently limitless variation and adaptability.

NATURE OF THE PARASITIC PROTOZOA

The definition of Protozoa as single-celled eukaryotic organisms is very simplistic and the group has always been an enigmatic one, largely because of the small size of the cells, the relative lack of morphological features,

and the absence of any meaningful evolutionary history. In order to understand the background to the currently used classifications it is necessary to consider briefly the history of the subject, the changes that have occurred, and the reasons for these changes. It is generally agreed that the single-celled organisms that we now call protozoa were first recognized in 1676 by Antony van Leeuwenhoek, who described them as 'little animals' or 'animalcula' (Dobell 1960). The term Protozoa, meaning 'first animals', was introduced by Goldfuss in 1818 (Goldfuss 1818) and has been in use in a modified form ever since, although Goldfuss included a number of metazoans such as sponges and bryozoans within the group. The term 'single-celled animals' came into common usage after the botanist Matthias Jacob Schleiden and the zoologist Theodor Schwann had elucidated their cell theory in 1838 (see Cole 1926). By the middle of the 19th century, most biologists had realized that single-celled organisms included some that had greater affinities with plants than with animals and, in order to avoid the preconceptions inherent in the use of the term Protozoa, Hogg introduced the term 'Protoctista' to embrace those forms that had animal, plant, or no clear affinities (Hogg 1860). Haeckel also considered that all unicellular forms represented a primitive group for which he coined the term 'Protista' (Haeckel 1876). In 1938, Copeland grouped together all single-celled organisms that did not have obvious animal or plant affinities and used Haeckel's term Protista, which he later changed to Hogg's Protoctista on grounds of priority (Copeland 1938). All three terms, Protozoa, Protista, and Protoctista, are currently in use but this is not an appropriate place to discuss or to comment on the validity of these terms, the background of which is discussed in more detail by Lipscomb (1991) and Scamardella (1999). The term Protozoa is now almost universally used by parasitologists.

PARASITIC PROTOZOA AND PARASITIC INFECTIONS

Before attempting to classify the parasitic protozoa, it is necessary to introduce them and to comment briefly on the infections they cause. Traditionally, the parasitic protozoa of humans are listed in what used to be regarded as their taxonomic order but, as will be explained later, this is no longer appropriate. Nevertheless, listing them according to their normally accepted groupings is a convenient and familiar starting place.

Parasitic amoebae

Several species of amoebae are common in humans in most parts of the world but only one, *Entamoeba histolytica*, is an important pathogen. *E. histolytica* invades the small intestine and colon and may be carried to the liver and other parts of the body causing amoebic dysentery or liver abscesses. Four other intestinal amoebae are commonly found all over the world: *E. dispar*, once regarded as a nonpathogenic race of *E. histolytica*, resembles the pathogenic form; *E. coli*, the most common amoeba of humans, is a harmless commensal, as are *Endolimax nana* and *Iodamoeba butschlii*. Humans are occasionally infected with other intestinal amoebae, *E. hartmanni*, *E. moshkovskii*, and *E. chattoni*. *E. gingivalis* occurs in the mouth and is sometimes associated with infected gums.

BLASTOCYSTIS

Cysts of *Blastocystis hominis* are increasingly being recognized in human feces. At one time this protozoan was thought to be a simple contaminant of fecal samples but it is now known to infect the intestine where it occurs in several forms including amoeboid and cystic but not flagellated forms. *B. hominis*, which occupies an unique position in the classification of the protozoa, was not considered in the 9th edition of Topley and Wilson.

Facultative amoebae and flagellates of humans

Several free-living amoebae occasionally infect humans, sometimes with fatal results. Species of *Acanthamoeba* can cause upper respiratory tract infections in immunocompromised individuals. *Balamuthia mandrillaris* has been found in the brain. *Naegleria fowleri* and other *Naegleria* species, which are flagellates with amoeboid phases in their life cycles, have been implicated in primary meningoencephalitis in otherwise healthy individuals.

INTESTINAL AND RELATED FLAGELLATES

A number of flagellates occur in the alimentary canals of humans. Large infestations can build up, but the infections are seldom harmful although some may cause gastrointestinal disorders. Flagellates similar to those in the intestine can occur in other parts of the body, such as the urogenital system, and these may cause more serious infections. Eight species of flagellate are ubiquitous and common parasites of the human gastrointestinal tract or urogenital system. Few do any real harm, but some occasionally give rise to unpleasant symptoms which can usually be easily treated. The most important by far is *Giardia duodenalis*, also known as *G. lamblia*, *G. intestinalis*, or *Lamblia intestinalis*. Three species of trichomonads are common in all parts of the world: *Trichomonas vaginalis* in the vagina and urethra of women and in the urethra, seminal vesicles, and prostate of men; *T. tenax* in the mouth; and *Pentatrichomonas hominis* in the cecum and large intestine. *Enteromonas hominis* is cosmopolitan and harmless.

Chilomastix mesnili is rare and harmless. *Dientamoeba fragilis* is transmitted through the eggs of the pinworm *Enterobius vermicularis* and may cause diarrhea.

TRYPANOSOMES OF HUMANS IN SOUTH AMERICA

Trypanosoma cruzi occurs in South and Central America and is infective to c. 100–150 species of wild and domesticated mammals. The vectors are bugs belonging to the family *Reduviidae*. In the human host, the flagellated trypanosomes enter various cells, particularly macrophages, muscle cells, and nerve cells, where they round up and multiply as nonflagellated amastigote forms. The disease, which can last for the lifetime of the host, is called Chagas disease and takes various forms depending on where the amastigotes develop, the most serious consequences being cardiac failure due to parasites in the heart muscles or the loss of the nervous control of the alimentary canal due to parasites in the nervous system. *T. rangeli*, which is usually harmless, occurs in humans, primates, cats, and dogs in Central and South America where it is transmitted by bugs and, although the parasite is quite different from *T. cruzi*, the two are occasionally confused.

TRYPANOSOMES OF HUMANS IN AFRICA

The African trypanosomes are found in the blood and are transmitted from host to host by tsetse flies belonging to the genus *Glossina*. The only form found is the flagellated form and, unlike the New World species, there are no amastigote forms. Two forms (subspecies) infect humans; *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, the former in West and Central Africa where it causes chronic sleeping sickness and the latter in the savannah of East Africa where it causes acute sleeping sickness. If the trypanosomes cross the blood–brain barrier they can cause coma and death. Closely related species occur in horses, cattle, and game animals.

LEISHMANIA SPECIES

Leishmania species, which are closely related to the trypanosomes, are transmitted by sandflies and cause serious diseases in humans. The parasites, which infect macrophages, occur only in the nonflagellated amastigote form. The typical infection is a long-lasting or nonhealing cutaneous lesion but in many species, and in particular individuals, the parasites may invade subcutaneous or deeper tissues, causing hideous and permanent disfiguration. The most serious disease, kala azar, involves the macrophages of organs such as the liver and can result in multiorgan system failure. Leishmaniasis is now known to be caused by a complex of about 20 species. As the morphology of all these parasites is similar, identification tends to be based on isoenzyme and DNA techniques. In the Old World, the main species causing cutaneous leishmaniasis are *L. tropica* and *L. major* and the species causing visceral leishmaniasis are *L. donovani* and *L. infantum*. In the New World cutaneous and mucocutaneous leishmaniasis are caused by at least 13 distinct species of *Leishmania* and visceral leishmaniasis is caused by *L. infantum* (at one time thought to be a separate species, *L. chagasi*, and now probably best referred to as *L. infantum chagasi*).

COCCIDIA: *TOXOPLASMA* AND RELATED COCCIDIA

Coccidia are common parasites of the epithelial cells of the gut of vertebrates. The most common species in humans is *Toxoplasma gondii*, a parasite of felids with a very wide range of intermediate hosts, including humans. In cats and other felids the life cycle is confined to the gut, but if the infective cysts passed in the feces are ingested by other warm blooded animals, multiplication occurs in various cells throughout the body. Infections are normally symptomless, but in the fetus or in immunosuppressed patients they may be very serious and they may occasionally cause ocular damage in healthy individuals. Humans also act as definitive hosts to two intestinal species related to *Toxoplasma*, *Sarcocystis hominis*, and *S. suihominis*, the respective intermediate hosts of which are pigs and cattle. Humans can also act as accidental, dead-end, intermediate hosts to several other species of *Sarcocystis*, cysts of which occur in the muscles. *Isospora belli* is another coccidian with a life cycle similar to that of *Toxoplasma* in cats but which occurs in the intestine of humans in which it is very rare.

CRYPTOSPORIDIUM

Coccidians belonging to the genus *Cryptosporidium* are relatively common parasites in the intestinal and respiratory tracts of mammals, birds, and reptiles. *C. parvum* causes gastrointestinal disorders in cattle, sheep, and humans. The infection is unpleasant but not normally dangerous in humans except in immunocompromised individuals, such as acquired immunodeficiency syndrome (AIDS) sufferers, in whom it can be fatal.

CYCLOSPORA

Cyclospora cayetanensis is an intestinal coccidian that causes a self-limiting infection with diarrhea and malabsorption. First recorded in 1986, it has since been found in both immunologically competent and immunocompromised patients in many parts of the world. The actual route of infection is unknown, although water and meat have been implicated. It is also not clear whether or not there is any other host apart from humans.

MALARIA PARASITES

The malaria parasites belong to the same phylum as the coccidians but to a different group, the hemosporidians, members of which, as the name implies, are parasitic in the erythrocytes of vertebrates. The malaria parasites of mammals all belong to the genus *Plasmodium* and are transmitted by female mosquitoes of the genus

Anopheles. Human malaria, one of the most important diseases in the world with >500 million people at risk in tropical and subtropical regions, especially Africa, is caused by four species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The disease is characterized by periodic fevers. *P. falciparum* causes malignant tertian malaria and is the most common and serious of all the forms of malaria, often causing cerebral damage resulting in death.

PIROPLASMS

The piroplasms are parasites of the erythrocytes of vertebrates and the vectors are ticks. *Babesia* species live in the blood of vertebrates and can cause serious disease, babesiosis. *B. microti* is common in wild rodents and occasionally infects humans in North America and sporadically elsewhere but the infections are not usually fatal. In Europe, splenectomized humans are sometimes infected with *B. divergens*, a cattle species, and the infection may be fatal.

CILIATES

Parasitic ciliates occur in most groups of vertebrates and invertebrates but only one species, *Balantidium coli*, infects humans. *Balantidium coli* is a common parasite of pigs in all parts of the world and has also been recorded in a number of other mammals including rats, dogs, camels, monkeys, apes, and c. 1 000 humans. The ciliate lives in the lumen of the large intestine and may invade the gut wall, where it produces ulcers resembling those caused by *Entamoeba histolytica*, although most cases are asymptomatic.

MICROSPORIDIANS

There are some 700 species of microsporidians in nearly all groups of vertebrates and invertebrates, particularly fish and arthropods. All microsporidians possess an inherent ability to multiply in their vertebrate hosts but this seems to be curtailed in humans, except in those individuals whose immune system is compromised. The first human case of microsporidiosis was recorded in 1959 and since then several new genera and species have been recorded, mainly in AIDS sufferers and others undergoing immunosuppressive therapy. Microsporidiosis is extremely rare in humans. *Encephalitozoon cuniculi*, a parasite of peritoneal macrophages that also spreads to other parts of the body including the brain, occurs in rodents, rabbits, dogs, and other carnivores and primates and there are increasing numbers of records from humans. Of the other species, few have been recorded from any animals although these must almost certainly be the sources of human infection. *Encephalitozoon hellem* parasitizes the cornea and conjunctiva and has been recorded in the viscera. *Encephalitozoon intestinalis* is an intestinal form that also occurs in a variety of cells in different parts of the body. *Enterocytozoon bieneusi* is also an intestinal form associated with chronic diarrhea in AIDS patients. *Vittaforma corneae* infects the cornea. *Trachipleistophora hominis* has been recorded from the cornea and skeletal muscle of AIDS patients. The microsporidians are now thought to be more closely related to the fungi than the protozoa, but are retained in this volume because this is where they have been traditionally placed.

PNEUMOCYSTIS

The taxonomic position of *Pneumocystis* has long been uncertain but it is now clearly acknowledged to be a fungus (see Cavalier-Smith 2001). *Pneumocystis carinii*, which is normally a harmless parasite in the lungs but may be fatal in immunocompromised people, has previously been regarded as a protozoan and was included in this volume in the 9th edition of Topley and Wilson. In the present edition it is considered with the other fungi in the Medical Mycology volume,

Pneumocystis pnuemonia.

KINGDOMS IN DISPUTE

In his 1758 classification of all living things, Carl Linnaeus recognized two kingdoms, Animalia and Plantae. By the middle of the 19th century, Hogg (1860) and Haeckel (1876) had argued for the creation of an additional kingdom to accommodate single-celled organisms. Haeckel is often credited with the concept of three kingdoms of living things: Animalia (animals), Plantae (plants), and Protista (protozoa), which was expanded to four kingdoms, Animalia, Plantae, Protoctista, and Mychota (prokaryote organisms) by Copeland (1938). With the removal of the fungi from the plant kingdom the erection of a fifth kingdom, Fungi, was necessary and the viruses were added by Jahn and Jahn (1949) who recognized six kingdoms: Archetista (viruses), Monera (bacteria and blue green algae), Metazoa (multicellular animals), Metaphyta (multicellular plants), Fungi (fungi), and Protista (unicellular organisms). This classification did not recognize the fact that the single-celled eukaryotic organisms were not a homogeneous assemblage and that there were unicellular members in the animal and plant kingdoms that could not logically be separated from the Metazoa and Metaphyta. In other words, there was an argument for the creation of groupings such as Protozoa and Protophyta within the animal and plant kingdoms. Nevertheless, Jahn and Jahn's classification became widely adopted. With the removal of the viruses, now considered to be nonliving, there emerged what is now known as Whittaker's five-kingdom classification: Monera (prokaryotes), Animalia, Plantae, Fungi, and Protista (Whittaker 1969). Whittaker's five-kingdom scheme has stood the test of time fairly well but, as studies at the biochemical and molecular levels identified an increasing number of problems with the conventional ideas about single-celled

organisms, it became necessary to adopt a rather more radical approach such as that set out by Cavalier-Smith (1993) and largely adopted by Corliss (1994). Conventionally, since the time of Linnaeus, the term 'kingdom' has been used as the highest category in the taxonomic hierarchy but Corliss, echoing the views of many protozoologists, accepted that the 'lower' eukaryotes could no longer be accommodated within a single kingdom and advocated the use of a superior category, the 'Empire,' containing six eukaryote kingdoms: Archezoa, Protozoa, Chromista (unicellular plant-like organisms), Plantae, Fungi, and Animalia. The first three contain only single-celled organisms, but a number of single-celled organisms also occur in the kingdom Plantae, a few in the kingdom Fungi, but none in the kingdom Animalia. One problem was that in this classification the kingdom Protozoa was not equivalent to any previous grouping with that name. In 1998, Cavalier-Smith came down in favor of six kingdoms – Bacteria, Protozoa, Chromista, Plantae, Fungi, and Animalia – effectively separating the protozoa from both the plant and animal kingdoms (Cavalier-Smith 1998). This broad classification will be adopted in this chapter.

INSTABILITY IN THE PHYLA AND CLASSES

When the Animalia was recognized as a kingdom, the Protozoa could be classified as a phylum and the subordinate groups as classes. However, when the status of the Protozoa was raised to that of a kingdom, the subordinate groups automatically became phyla and certain groups that had previously enjoyed ordinal status became elevated to classes or even phyla. This caused a great deal of confusion and requires some explanation. Goldfuss (1818) recognized three great groups of protozoa (amoebae, flagellates, and ciliates) on the basis of their mode of locomotion and this number was increased to four by the addition of the sporozoans by Bütschli (1883–5). These four groups persisted in various guises until comparatively recently, as the Rhizopoda or Sarcodina (amoebae, protozoa that move by means of pseudopodia), Mastigophora (flagellates, protozoa that move by means of flagella), Ciliophora or Ciliata (ciliates, protozoa that move by means of cilia), and Sporozoa (sporozoans, spore-forming protozoa that do not have any obvious means of locomotion). The Sporozoa sometimes included microsporidians and myxosporidians, often grouped together as cnidosporidians. This is essentially the traditional classification used in classical works by Calkins (1901), Craig (1926), Wenyon (1926), Kudo (1954), and Manwell (1961). By the beginning of the 1960s, with the availability of increasingly sophisticated ways of studying protozoa, it was becoming clear that this classification was unworkable and in 1964 the Society of Protozoologists published a revised classification (Honigberg et al. 1964). This grouped the Sarcodina and Mastigophora together as the Sarcomastigophora and removed the myxosporidians and microsporidians from the Sporozoa, with which they had been traditionally classified. One particularly controversial action was to remove the piroplasms from the Sporozoa and place them among the Sarcomastigophora, something that was ignored by virtually all parasitologists. The Society of Protozoologists' 1964 classification, with slight modifications (e.g. the replacement of the piroplasms among the Sporozoa) was widely accepted and appeared in standard textbooks such as those by Baker (1969) and Levine (1973). Subsequent changes involving *Toxoplasma* and related organisms were gradually incorporated into the 1964 scheme and a consensus classification, such as that of Baker (1977), gradually emerged.

This consensus did not last long, and rapid and major developments in our understanding of the protozoa necessitated a new classification. In 1980, the Society of Protozoologists published its second classification (Levine et al. 1980). The working party responsible for this classification recognized that the protozoa did not represent an assemblage of primitive organisms but embraced a number of diverse and distinct groups, and recognized seven phyla: Sarcomastigophora, Apicomplexa (essentially equivalent to the Sporozoa), Ciliophora, Microspora, Myxozoa, Ascetospora, and Labyrinthomorpha. Nevertheless, the affinities with the traditional classification remained clear and a modification which took into account only the parasitic forms was set out by Cox (1981) and used in information retrieval systems and parasitology textbooks such as Cox (1982), Kreier and Baker (1987), and Mehlhorn (1988). Like its predecessors, this scheme failed to keep up with developments at the ultrastructural, molecular, and biochemical levels and by the beginning of the 1990s Sleigh (1991) had decided to ignore the concept of phyla and simply recognize four 'groups': the flagellated protozoa, the amoeboid protozoa, the ciliated protozoa, and the sporozoans. This approach, also adopted by Cox (1991, 1993), has the merit of simplicity and clear links with the traditional classification, but is merely a classification of convenience and does not stand up to rigorous analysis at either the evolutionary or molecular level.

GENERA AND SPECIES: OASES OF STABILITY

The names of the genera and species of protozoa that infect humans have remained remarkably consistent throughout the upheavals that have characterized the classification of the protozoa over the past few years. The main changes have been the addition of the coccidians *Cryptosporidium* and *Cyclospora*, largely as concomitants of HIV infections, *Balamuthia* as an accidental infection, and *Blastocystis*. In addition, previously unidentified tissue parasites have now been identified as *Sarcocystis* spp. Another important change has been the

growth in the number of species in the genus *Leishmania*. More recently we have seen the removal of the enigmatic species *Pneumocystis carinii*, now classified among the fungi, and the microsporidians *Brachiola*, *Encephalitozoon*, *Enterocytozoon*, *Microsporidium*, *Nosema*, *Septata*, and *Trachipleistophora*, which now rightly belong with the fungi but are retained in this volume for the sake of continuity.

The increase in the number of parasitic protozoa recorded from humans and the accumulation of knowledge about their biology has resulted in the creation of taxonomic and other groupings at the subgenus and subspecies levels, largely as a result of an unwillingness to tamper with well-established genera and species. As the parasitic protozoa have traditionally been classified with the animal kingdom, the International Code of Zoological Nomenclature applies to the naming of the subordinate groups, subgenera, and subspecies. However, the rules have not been applied rigorously and several apparent anomalies have arisen and the use of subgenera, species, and subspecies across the whole of the parasitic protozoa has not been uniform.

Among the amoebae, the only controversy relates to the most important parasite, *Entamoeba histolytica*. For many years it has been known that this parasite exists in two forms, one pathogenic and one nonpathogenic. This has resulted in some workers suggesting that the pathogenic form sensu strictu should be called *E. histolytica* and the nonpathogenic form something different, for example *E. dispar* as suggested by Brumpt (1925). Until relatively recently, most scientists were content to use one name, *E. histolytica*, but isoenzyme and other biochemical and molecular studies have established that there are real differences between the pathogenic and nonpathogenic forms and this has resulted in the reintroduction of *E. dispar* for the nonpathogenic form. Unfortunately, things are not that simple, and there have been reports that some of the biochemical criteria used are not stable (Spice and Ackers 1992). Nevertheless, it seems sensible to use the two specific names for which there is some precedent. Another apparently harmless species resembling *E. histolytica* is *E. hartmanni*, and there have been occasional reports of infections with another similar parasite, *E. moshkovskii*. There is currently some stability in the classification of amoebae belonging to the genus *Entamoeba*, but there may be other revisions on the way.

Giardia duodenalis is another intestinal parasite that causes a range of symptoms and what actually constitutes this species remains to be resolved, mainly because of the genetic variability that exists even within cloned lines (Thompson et al. 1993). There is little agreement about what the species name should actually be: *G. intestinalis* and *G. duodenalis* tend to be used interchangeably in western Europe and Australia, *G. lamblia* is used in the USA, and *Lamblia intestinalis* is sometimes used in eastern Europe. Electrophoretic analysis suggests that the species in humans is morphologically *G. duodenalis*, which parasitizes a number of mammals, but that it could be afforded specific status as *G. intestinalis* on grounds of host specificity (Mayrhofer et al. 1995).

The genus *Leishmania* has received the most attention. Originally there were two Old World species, the cutaneous form *L. tropica* and the visceral form *L. donovani*. *L. tropica* was subsequently differentiated as two varieties, *L. tropica* var. *minor* and *L. tropica* var. *major*, and later subspecies, *L. t. minor* and *L. t. major*, which have now been raised to species level as *L. tropica* and *L. major*. Two subspecies of *L. donovani*, *L. d. donovani* and *L. d. infantum*, have now been raised to species level, as has *L. donovani* var. *archibaldi*. It was originally thought that the leishmanias in the New World were varieties of the Old World forms and the cutaneous form was called *L. tropica* var. *mexicana*, later changed to *L. mexicana*, and the mucocutaneous form was called *L. tropica* var. *braziliensis*, subsequently *L. braziliensis*. The New World visceral form of *L. donovani* subsequently became *L. d. chagasi* and then *L. chagasi*, but is now thought to be the same as the Old World species *L. infantum* (Maurício et al. 2000) which, in this volume, is referred to as a subspecies, *L. infantum chagasi*. The cutaneous forms have undergone massive revisions as more and more subspecies or species have been discovered. Initially, each new discovery was assigned either to *L. mexicana* or to *L. braziliensis* as a subspecies, for example *L. b. braziliensis*, but current opinion favors the use of species. Recognition of the fact that these species could and should be grouped as the 'mexicana complex' and the 'braziliensis complex' led to the creation of two subgenera; *Leishmania*, which embraces all the Old World species and members of the 'mexicana complex' and *Viannia*, which includes the 'braziliensis complex.' A detailed account of this classification and its background is given by Lainson and Shaw (1987). It should be pointed out here that the classification of the New World leishmanias is now based on morphological, behavioral, geographical, clinical, biochemical, and molecular criteria (see also New World leishmaniasis).

Subgenera have also been used for the classification of the trypanosomes, *Trypanosoma* spp. Large numbers of trypanosomes parasitize all groups of vertebrates and, in an attempt to put some kind of order into what was becoming an increasingly heterogeneous genus, Hoare, in 1966, proposed the creation of a number of subgenera as a framework within which to classify the trypanosomes of mammals (see Hoare 1972). These subgenera were placed in two major groups; Stercoraria, containing *Megatrypanum*, *Herpetosoma*, and *Schizotrypanum*, and Salivaria, containing *Duttonella*, *Nannomonas*, *Pycnomonas*, and *Trypanozoon*. The New World trypanosome of humans, *T. cruzi*, is classified in the subgenus *Schizotrypanum* and *T. rangeli* is placed in the subgenus

Herpetosoma, whereas the Old World forms, *T. brucei gambiense* and *T. b. rhodesiense*, are placed in the subgenus *Trypanozoon* (see for example Lumsden and Evans 1976). However, the use of *Trypanosoma (Schizotrypanum) cruzi* and *Trypanosoma (Trypanozoon) brucei* is clumsy and the practice has now been all but abandoned.

The classification and nomenclature of the human trypanosomes is still not entirely satisfactory at the level of genus and species. There are real differences between *T. cruzi* and *T. brucei* and, although it would seem logical to classify them as separate genera, this cannot be done in isolation. Any changes would result in serious consequences for the classification of all the other trypanosomes of vertebrates and great disruption to information retrieval systems if one of the two generic names were to change.

The malaria parasites belonging to the genus *Plasmodium* have also been classified into subgenera largely on the same grounds as the trypanosomes; the numbers recorded in all groups of higher vertebrates, and a perceived need to bring some order into an increasingly complex situation. Nine subgenera have been proposed: *Plasmodium*, *Vinckeia*, *Laverania*, *Haemamoeba*, *Giovannolaia*, *Noyella*, *Huffia*, *Sauramoeba*, and *Carinia*, of which three occur in mammals, four in birds, and two in lizards (Garnham 1966). The human species, *Plasmodium falciparum*, was placed in the subgenus *Laverania* whereas the others, *P. malariae*, *P. ovale*, and *P. vivax*, were placed in the subgenus *Plasmodium*. As far as human parasites are concerned, the use of subgenera served very little purpose and has fallen into disuse.

CLASSIFICATION BELOW THE SPECIES LEVEL

There is a perceived need for some sort of category below that of species to differentiate between genetically distinct forms of parasites that produce markedly different diseases in humans, and the use of subspecies has been common among protozoologists. In the genus *Leishmania*, in which it was most widely used, this practice has fallen into disuse and there has been an incremental drift upwards towards separate species status. This tendency has been resisted by those working with the African trypanosomes and *Trypanosoma cruzi*, the causative agent of Chagas disease in South America. There is no doubt, however, that there are many different forms of *T. cruzi* and that their epidemiology and the diseases they produce are significantly different but, although there seems to be a de facto case for the consideration of subspecies, there has been little enthusiasm for such a move.

The only consistent use of subspecies is in the case of the African trypanosomes. Human sleeping sickness in Africa is caused by two different trypanosomes both closely related to a natural parasite of equines and game, *Trypanosoma brucei*. The two human forms have sometimes been recognized as species, *T .gambiense* and *T. rhodesiense* (Levine 1973) but, using morphological, clinical, epidemiological, biochemical, and molecular criteria, there can be no doubt about the close relationship between the human and the animal forms and the logical conclusion is that the animal form should be classified as *Trypanosoma brucei brucei* and the human forms as *T. brucei gambiense* and *T. brucei rhodesiense*. This approach has been adopted by most workers in the field and by information retrieval services. The widespread use of these subspecies was partly responsible for the demise of the use of subgenera because most authors and editors did not want to be bothered with names like *Trypanosoma (Trypanozoon) brucei gambiense*.

Toxoplasma gondii is another parasite that seems to exist in two forms, a virulent one and an avirulent one, and it is also very interesting to note that a single strain of *T. gondii* probably accounts for all the virulent forms worldwide (Sibley and Boothroyd 1992). There are, however, no serious moves to create any subspecies.

MOLECULAR AND BIOCHEMICAL APPROACHES TO THE CLASSIFICATION OF THE PARASITIC PROTOZOA

Fortunately there are a number of alternative approaches other than morphology and life cycles to the classification of protozoa, including the application of the techniques of biochemistry and molecular biology. Such techniques are increasingly being used to resolve phylogenetic, and consequently taxonomic, questions. The most widely used techniques can be roughly divided into those that are enzymatic or genetic. This topic is too extensive to be discussed in detail here and there is a useful review by Barta (2001), a comprehensive phylogenetic tree of parasites by de Meeŭs and Renaud (2002), and accounts of particular applications in a number of papers in a symposium volume mainly devoted to leishmaniasis (Alvar and Baker 2002).

The first widely used technique was the use of isoenzyme profiles, which proved to be extremely useful tools for distinguishing between apparently identical parasites. Isoenzymes are enzymes that perform the same functions but have different mobilities in electric fields and can thus be distinguished very easily; populations of parasites with identical isoenzyme patterns are called zymodemes. This technique has been successfully used to distinguish the various species of *Leishmania* and the results obtained have correlated well with epidemiological, clinical, and molecular findings. Isoenzymes have also been used to distinguish between the subspecies of African trypanosomes and pathogenic and nonpathogenic forms of *Entamoeba histolytica* and *Toxoplasma gondii*, and have also been used extensively in studies on *Giardia duodenalis* and *Cryptosporidium parvum*.

Useful as they may be, isoenzyme techniques have the major disadvantage of being reliable only for distinguishing between organisms that are closely related (Richardson et al. 1986) and DNA and RNA technology is increasingly being used both for the diagnosis of parasitic infections and for resolving taxonomic and phylogenetic problems. Briefly, both DNA and RNA can be used to determine evolutionary distances, as nucleotide sequences tend to diverge over time and to evolve at a more predictable rate than do morphological characters. Differences in nucleotide sequences can, therefore, be compared and the more similar the sequences in two organisms the more likely it is that they are related; large numbers of such studies can be used to create realistic phylogenetic trees. Different kinds of RNA, particularly small nuclear RNA (16S and 18S SnRNA) and small subunit ribosomal RNA (SSUrRNA) have been extensively used for taxonomic and phylogenetic investigations and, although data are currently based on very few taxa, these techniques have been very useful. Johnson and Baverstock (1989) were the first to attempt to produce a comprehensive phylogenetic tree of the protozoa with special reference to the parasitic forms, using data derived from SSUrRNA. RNA sequences have confirmed the differences between pathogenic and nonpathogenic strains of *E. histolytica* determined by isoenzyme studies (Petri et al. 1993). Similar studies have also identified similarities between the Old World trypanosome *T. cruzi* and New World *T. brucei* and have distanced both from *Leishmania* species, thus confirming the current classification of the kinetoplastid flagellates (Maslov and Simpson 1995). SSUrRNA are the most commonly used sequences employed in systematic studies and, as far as the protozoa are concerned, the validity of such studies has been confirmed by comparing results obtained with SSUrRNA and those obtained using amino acid sequences of protein-encoding genes (Baldauf et al. 2000).

Ribosomal RNA studies using 18S rRNA have also shown that *Toxoplasma* and *Sarcocystis* are monophyletic and thus derived from a common ancestor (Ellis et al. 1995).

DNA methodology has also proved to be very useful. DNA probes have been extensively used for studies on *Leishmania* species both for diagnosis and for determining relationships. Initial studies that distinguished between the New World forms *L. mexicana* and *L. braziliensis* have now been extended to all species and, coupled with isoenzyme studies, have been largely responsible for the present classification of the genus. The development of the polymerase chain reaction (PCR) has revolutionized the use of DNA techniques in parasitology and has been used to confirm the existence of virulent and avirulent strains of *T. gondii* (Guo and Johnson 1995). Other widely used techniques include restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD). One example of the application of such techniques has been the establishment of the fact that *Leishmania chagasi* in the New World is the same as *L. infantum* in the New World (Maurício et al. 2000).

Increasingly, biochemical and molecular criteria are being used for the identification of new species and it is now unlikely that any future descriptions of protozoa lacking good morphological characteristics will be published and accepted unless the descriptions are soundly based at the molecular and genetic levels.

No matter how carefully any one particular species is characterized, this is of little use to any overall classification, which needs to take into account the characteristics of a diversity of organisms. One of the most powerful tools available for the study of evolution is parsimony analysis (Sober 1988; Stewart 1993). Essentially what this means is that the simplest explanation consistent with the available data should be used to reach conclusions about phylogenetic relationships. The concept is not a new one and many biologists are familiar with it in another guise, that of Occam's razor, which holds that entities should not be multiplied beyond necessity, i.e. where there is a simple explanation there is no need to look for a complex one. Parsimony analysis relies on the availability of raw data and, in the phylogenetic studies with which we are concerned here, these include morphology, life cycles, and molecular data. The ideal is to create taxa that can be ranked in a hierarchical order that reflects the underlying phylogeny. This approach has obvious advantages but also less obvious disadvantages: two taxa may possess characters that are only superficially similar; too much emphasis may be placed on too few examples; and the rates of morphological or molecular change may not be the same in the different groups under consideration. The most satisfactory examples of parsimony analysis are where traditional and molecular approaches produce the same overall pattern, but these may present the greatest difficulties if the various criteria used (e.g. morphological and molecular) are both based on the same invalid assumptions. On the other hand, where different approaches produce conflicting results it is possible that in seeking an explanation for the discrepancies a better understanding of the relationships will emerge.

CLASSIFICATION OF THE PARASITIC PROTOZOA OF HUMANS

In recent years, the availability of a number of molecular markers has made it possible to analyze relationships between protozoans and to draw conclusions that would not have been possible using morphological characters alone. So far, there has been remarkable agreement between the different approaches and, in most cases, molecular techniques have confirmed the more traditional findings; in other cases they have clarified

long-standing controversies. In the long run, it is likely that molecular classifications of the protozoa will be the norm. What most parasitologists require, however, is a convenient, familiar, and stable framework within which to work and communicate, as most are concerned with only relatively few species of protozoa and those interested in the parasitic protozoa of humans need to be familiar with only around 20 genera and 50 species. It is therefore important that any classification should reflect modern thinking about the classification of the protozoa as a whole, while retaining sufficient traditional material to permit easy reference to past papers, textbooks, and information retrieval systems. There have been a number of attempts to do this and it is convenient to consider three classifications, those of the 1980s, 1994, and 2000s. The 1980s classifications are largely based on the Society of Protozoologists' second classification (Levine et al. 1980), the 1994 classifications are represented by the interim classification of Corliss (1994), and the 2000s classification is based on the comprehensive system drawn up by Cavalier-Smith (1993) and subsequent modifications. The 1980 and 1994 systems are set out below in order to set the scene for the more recent classification and to provide some continuity between the different systems several of which are still in use.

Traditional 1980s classification

Below is an outline version of a traditional 1980s classification. This classification is provided simply as a background for further discussion and to focus on the central problem of protozoan classification; the need to satisfy protozoologists on one hand and the need to be useful to parasitologists, especially medical parasitologists, on the other. The detailed classification by Lee et al. (1985) is typical of those used in the 1980s.

Kingdom Animalia
Subkingdom Protozoa
Phylum Sarcomastigophora (the amoebae and flagellates)
Subphylum Mastigophora. Intestinal, tissue, and blood-dwelling flagellates, e.g. *Giardia*, *Chilomastix*, *Trichomonas*, *Dientamoeba*, *Leishmania*, *Trypanosoma*
Subphylum Sarcodina. Obligate and facultative amoebae, e.g. *Entamoeba*, *Iodamoeba*, *Endolimax*, *Acanthamoeba*, *Naegleria*
Phylum Apicomplexa (the sporozoans)
Class Sporozoea
Subclass Coccidia
Order Eucoccidiida
Suborder Eimeriina, e.g. *Isospora*, *Sarcocystis*, *Toxoplasma*, *Cryptosporidium*
Suborder Haemosporina, e.g. *Plasmodium*
Subclass Piroplasmea, e.g. *Babesia*
Phylum Microspora, e.g. *Encephalitozoon*, *Nosema*
Phylum Ciliophora, e.g. *Balantidium*

1994 'Interim user-friendly classification'

In 1994, in order to meet various and sometimes conflicting requirements, Corliss proposed a 'utilitarian and user-friendly classification' which took into account the numerous advances that had been made since the 1980 classifications while maintaining much that was traditional (Corliss 1994). In this classification, Corliss recognizes six kingdoms with the majority of single-celled organisms generally accepted as protozoa in two of them, the Archezoa and Protozoa. In the Archezoa there are three phyla, two of which contain parasites, and in the Protozoa there are 14 phyla, six of which contain parasites. This classification, which is outlined below, was accepted by a majority of protozoologists and was used in the 9th edition of Topley and Wilson.

Empire Eukaryota
Kingdom Archezoa
Phylum Metamonada
Class Trepomonadea
Order Diplomonadida, e.g. *Giardia*
Order Enteromonadida, e.g. *Enteromonas*
Class Retortamonadea
Order Retortamonadida, e.g. *Chilomastix*, *Retortamonas*
Phylum Microspora
Class Microsporea
Order Microsporida, e.g. *Encephalitozoon*, *Enterocytozoon*, *Nosema*
Kingdom Protozoa
Phylum Percolozoa
Class Heterolobosea
Order Schizopyrenida, e.g. *Naegleria*
Phylum Parabasala
Class Trichomonadea
Order Trichomonadida, e.g. *Dientamoeba*, *Trichomonas*
Phylum Euglenozoa
Class Kinetoplastidea
Order Trypanosomatida, e.g. *Leishmania*, *Trypanosoma*
Phylum Ciliophora
Class Litostomatea
Order Trichomonatida, e.g. *Balantidium*
Phylum Apicomplexa (Sporozoa)
Class Coccidea
Order Eimeriida e.g. *Cryptosporidium*, *Cyclospora*, *Isospora*, *Sarcocystis*, *Toxoplasma*
Class Haematozoea
Order Haemosporida, e.g. *Plasmodium*
Order Piroplasmida, e.g. *Babesia*
Phylum Rhizopoda
Class Lobosea
Order Acanthopodida, e.g. *Acanthamoeba*, *Balamuthia*
Class Entamoebidea

Order Euamoebida, e.g. *Endolimax*, *Entamoeba*, *Iodamoeba*

Kingdom Chromista

Kingdom Plantae (the plant kingdom sensu strictu)

Kingdom Fungi (the fungi sensu strictu)

Kingdom Animalia (the animal kingdom sensu strictu)

This classification retained many of the familiar features of more traditional classifications and served as a transitional phase between the older classifications and the newer ones outlined below.

2000s Classification

Although the Corliss classification served the protozoological community well, subsequent developments in molecular biology have provided us with a much clearer understanding of phylogenetic relationships involving the single-celled organisms, to such an extent that the status of the various groups of protozoa has had to be reappraised. Comparison of SSUrRNA and protein sequences (Sogin and Silberman 1998; Doolittle 1999; Baldauf et al. 2000) have made it possible to arrange organisms within groups based on evolutionary distances. Such studies by themselves, however, do not necessarily reflect hierarchical arrangements. An alternative approach has been to consider the megaevolution of eukaryotic organisms, particularly the acquisition of prokaryotic organelles, for example mitochondria and chloroplasts, by a process of symbiogenesis,. Such studies, coupled with the realization that organelles can have been not only acquired but also modified, multiplied, or lost, have made it possible to arrange all living organisms within a realistic and evolutionarily and taxonomically sound overall system (Cavalier-Smith 1998). It is also clear that RNA and protein sequences broadly reflect the hierarchical systems based on evolutionary principles, thus providing, for the first time, a system of classification of the protozoa that is comparable with systems devised for higher eukaryotes. This scheme will therefore be adopted here and the major elements are easily recognizable as those in earlier classifications. In this classification there are six kingdoms – Bacteria, Protozoa, Animalia, Fungi, Plantae, and Chromista – of which the Protozoa constitute the basal eukaryotic kingdom. This system re-establishes the validity of the kingdom Protozoa with 13 phyla of which seven contain parasites that infect humans, but has necessitated the redistribution of some taxa, including some parasites of humans.

KINGDOM PROTOZOA. Unicellular eukaryotic phagotrophic, nonphotosynthetic organisms without cell walls

Subkingdom 1 Archezoa. Single-celled eukaryotic organisms exhibiting various prokaryotic features in their ribosomes and tRNA; lacking plastids, mitochondria, Golgi bodies, and cytoplasmic inclusions, e.g. hydrogenosomes and peroxisomes.

Phylum Metamonada. Unicellular intestinal flagellates with two, four or eight (occasionally more) flagella.

Class Trepomonadea. Intestinal flagellates; one or two karyomastigonts each with one to four flagella; no contractile axostyle; few cell-surface cortical microtubules.

Order Diplomonadida: *Giardia duodenalis*

Order Enteromonadida: *Enteromonas hominis*

Class Retortamonadea. Intestinal flagellates; cortical microtubules over entire body surface

Order Retortamonadida *Chilomastix mesnili*, *Retortamonas intestinalis*

Phylum Parabasalia. Unicellular flagellates with one or more nuclei and numerous flagella; characteristic complex parabasal body equivalent to Golgi body; no mitochondria.

Class Trichomonadea. Intestinal and related flagellates; typically four to six flagella, noncontractile axostyle.

Order Trichomonadida: *Dientamoeba fragilis*, *Trichomonas vaginalis*, *T. tenax*, *Pentatrichomonas hominis*

Subkingdom 2 Neozoa. Single-celled eukaryotic organisms typically possessing plastids, mitochondria, Golgi bodies, and cytoplasmic inclusions including hydrogenosomes and peroxisomes.

Phylum Percolozoa. Unicellular, nonpigmented organisms typically possessing one to four flagella, mitochondria and peroxisomes but lacking Golgi bodies.

Class Heterolobosea (flagellated amoebae). Amoeboflagellates; trophic form amoeboid; temporary flagellated phase in life cycle.

Order Schizopyrenida: *Naegleria fowleri*

Phylum Euglenozoa. Unicellular flagellates with one to four flagella, possess Golgi body and mitochondria.

Class Kinetoplastea (kinetoplastid flagellates). Unicellular flagellates with one to two flagella; prominent kinetoplast (DNA-containing body) within a single mitochondrion.

Order Trypanosomatida: *Leishmania donovani*, *L. infantum*, *L. major*, *L. tropica*, *L. braziliensis*, *L. mexicana*, *L. aethiopica*, *L. amazonensis*, *L. colombiensis*, *L. garnhami*, *L. guyanensis*, *L. lainsoni*, *L. lindenbergi*, *L. naiffi*, *L. panamensis*, *L. peruviana*, *L. pifanoi*, *L. shawi*, *Trypanosoma cruzi*, *T. brucei gambiense*, *T. brucei rhodesiense*, *T. rangeli*

Phylum Amoebozoa. Unicellular nonflagellated organisms with pseudopodia used for both feeding and locomotion.

Class Amoebaea. Amoebae with lobose pseudopodia and mitochondria; typically free-living but facultative parasites.

Order Acanthopodida: *Acanthamoeba castellani*, *Acanthamoeba* spp., *Balamuthia mandrillaris*

Class Entamoebidea. Amoebae with lobose pseudopodia; secondary loss of mitochondria, peroxisomes or hydrogenosomes; obligate parasites.

Order Euamoebida: *Entamoeba histolytica*, *E. coli*, *E. dispar*, *E. hartmanni*, *E. gingivalis*, *E. moshkovskii*, *E. chattoni* (=*E. polecki*), *Endolimax nana*, *Iodamoeba buetschlii*

Phylum Sporozoa (sporozoans). Unicellular organisms possessing at some stage an apical complex composed of polar rings, rhoptries, micronemes, and typically a conoid; elaborate life cycles involving a sexual process; all parasites.

Class Coccidea. Sexual stages small and intracellular usually in epithelial cells of vertebrate host; resistant oocyst.

Order Eimeriida: *Cryptosporidium parvum*, *Toxoplasma gondii*, *Cyclospora cayetanensis*, *Isospora belli*, *Sarcocystis hominis*, *S. suihominis*

Order Piroplasmida: *Babesia microti*, *B. divergens*, *B. gibsoni*

Order Haemosporida: *Plasmodium falciparum*, *P. malariae*, *P. ovale*, *P. vivax*

Phylum Ciliophora (ciliates). Unicellular organisms characterized by possession of many cilia used for locomotion and with complex oral ciliature used for feeding; two kinds of nuclei, one or more polyploid macronuclei and one or more diploid micronuclei.

Class Litostomatea. Mainly free-living ciliates with inconspicuous oral ciliature.

Order Vestibuliferida: *Balantidium coli*

KINGDOM CHROMISTA. Unicellular photosynthetic filamentous or colonial, organisms (in part 'algae'); some with secondary loss of plastids.

Subkingdom Chromobiota

Phylum Bigyra

Class Blastocystea: *Blastocystis hominis*

KINGDOM FUNGI. Eukaryotic heterotrophic organisms lacking plastids but possessing cell walls containing chitin and β-glycans.

Phylum Microspora (Microsporidians)

Class Microsporea: *Encephalitozoon cuniculi*, *E. hellem*, *E. intestinalis*, *Enterocytozoon bieneusi*, *Nosema ocularum*, *N. corneum*, *Brachiola connori*, *B. vesicularum*, *B. algerae*, *Microsporidium ceylonensis*, *M. africanum*, *Vittaforma corneae*, *Trachipleistophora hominis*, *T. anthropophthera*, *Pleistophora ronneafiei*.

KINGDOMS AND SUBKINGDOMS

The most important change from the 1980 and 1994 classification is that the Protozoa becomes a kingdom in its own right and is no longer regarded as a subkingdom of the kingdom Animalia as in the 1980s classification. The kingdom Protozoa now embraces the 1994 kingdoms Archezoa and Protozoa and it is now accepted that the kingdom Protozoa is the basal eukaryotic kingdom and has equal status with the other eukaryotic kingdoms, Chromista, Fungi, Plantae, and Animalia. As far as parasitologists are concerned this move is to be welcomed as it brings together most of the single-celled organisms traditionally regarded as protozoa within a single higher taxon.

ARCHEZOA

In the Corliss 1994 classification it was recognized that the flagellated protozoa, far from being classified in the same phylum as the amoebae as in the 1980s classification, did not constitute a coherent group and needed to be separated in some logical way. A number of amitochondrial flagellates and amoebae, together with the microsporidians, were placed in a distinct primitive kingdom, the Archezoa, the affinities of which were at that time obscure. In the present classification, the subkingdom Archezoa retains the phylum Metamonada and has added to it the phylum Parabasalia previously classified in the 1994 kingdom Protozoa. However, it is now apparent that the phylum Microspora has close affinities with the Fungi and should be removed from the subkingdom Archezoa and kingdom Protozoa (see below).

FLAGELLATED ARCHEZOANS: *GIARDIA*, *ENTEROMONAS*, *CHILOMASTIX* AND *RETORTAMONAS*, *DIENTAMOEBA*, *TRICHOMONAS*, AND *PENTATRICHOMONAS*

The flagellated species *Giardia duodenalis*, *Enteromonas hominis*, *Chilomastix mesnili*, and *Retortamonas intestinalis* (parasites of the human intestine) have been traditionally classified with a number of other flagellates within the subphylum or phylum Mastigophora. In this classification, as in the 1994 classification, they have been placed in the phylum Metamodada in what is now the subkingdom Archezoa. Most protozoologists, largely basing their evidence on ribosomal RNA, now regard *Giardia* as very primitive and consider that it represents a very early stage of eukaryote evolution and that in any overall evolutionarily sound scheme it should be placed in some basal, pivotal position. Parallels between *Giardia* and the other genera listed suggest that they should all be grouped together in the same phylum and that *Giardia* and *Enteromonas* should be in one class and *Chilomastix* and *Retortamonas* in another. The 1994 classification separated these flagellates from members of the Trichomonadida, containing *Dientamoeba fragilis* (parasitic in the intestine), two species of *Trichomonas*, *T. vaginalis* and *T. tenax*, and *Pentatrichomonas hominis* (parasitic respectively in the urogenital system, mouth, and intestine). *Dientamoeba* has no flagellum and is permanently amoeboid and was thus once classified with the amoebae, but there is now no doubt that it is a

flagellate and should be classified with *Trichomonas*. Corliss (1994) pointed out that some protozoologists believed that the whole of the phylum Parabasala (now Parabasalia), which contains the Trichomonadida, should be classified with the Archezoa, bringing all these flagellated protozoa of humans closer together; in the present classification this has been done and they are assigned to the phylum Parasbasalia in the subkingdom Archezoa.

AMOEBOFLAGELLATES: *NAEGLERIA*

These freshwater forms are occasional accidental parasites of humans. In previous classifications they have been classified with other amoebae such as *Entamoeba* and *Acanthamoeba*, or with the flagellated protozoa. There is now no doubt that these organisms should be classified with a number of other free-living flagellated protozoa in a separate phylum, Percolozoa, as in the 1994 and present classifications.

AMOEBAE: *ACANTHAMOEBA*, *ENDOLIMAX*, *ENTAMOEBA*, AND *IODAMOEBA*

The arguments for keeping all these amoebae together in one phylum, Rhizopoda in the 1994 classification and Amoebozoa in the present one, are sound. There are also sound arguments for placing *Acanthamoeba* spp. (accidental parasites of humans) in one class, Amoebaea, and the obligate species, *Entamoeba histolytica*, in another, Entamoebidea. The class Amoebaea is equivalent to Lobosea in the 1994 classification. This classification is essentially a traditional one and should be acceptable to parasitologists.

BLOOD AND TISSUE FLAGELLATES: *LEISHMANIA* AND *TRYPANOSOMA*

These important genera include *Trypanosoma brucei gambiense*, *T. b. rhodesiense*, *T. cruzi*, and several species of *Leishmania*. They have long been recognized as comprising a distinct assemblage which has been given several names, ranging from the order Kinetoplastida to the phylum Kinetoplasta. In this classification, they are placed close to their free-living relatives, in the phylum Euglenozoa, which should be satisfactory for both parasitologists and protozoologists.

CILIATED PROTOZOA: *BALANTIDIUM*

The phylum Ciliophora contains ciliated protozoa that all possess distinctive patterns of ciliary distribution and can thus be classified satisfactorily on morphological grounds. *Balantidium coli* is the only ciliate that infects humans and, although in the past its taxonomic position has been uncertain, it is now accepted by most protozoologists that it is correctly classified in the phylum Ciliophora and class Litostomatea. *B. coli* is not an important parasite and is readily recognized, so its classification is of little interest to most parasitologists. However, what is of interest is the fact that the ciliates are now thought to be more closely related to the sporozoans than was previously believed when the protozoa were divided between the four major groups, amoebae, flagellates, sporozoans, and ciliates.

SPOROZOANS

Sporozoa is a traditional term applied to a large group of sexually reproducing, spore-forming protozoa, most of which have recognizably similar morphology at the electron microscope level and similar life cycles. This group originally contained a heterogeneous collection of spore-forming single-celled organisms including, at one time, the microsporidians, here classified in the kingdom Fungi. In the 1970s, a new parasite genus, *Perkinsus*, containing parasites of marine molluscs that were quite different from any of the other sporozoans, was allocated to this group and the phylum Sporozoa was downgraded to a class and the name of the phylum changed to Apicomplexa. The phylum Apicomplexa containing two classes Sporozoea, identical with the taxon Sporozoa established by Leuckart (1879), and Perkinsea containing the genus *Perkinsus* (see Levine et al. 1980). It is now clear that *Perkinsus* spp. do not belong with the sporozoans and should be classified close to, or with, the dinoflagellates (Siddall et al. 1997; Herrán et al. 2000) therefore the taxon Apicomplexa is redundant and should not be used. The traditional sporozoans (that is, all except the members of the class Perkinsea) possess sufficient common morphological features and life cycles, supported by studies at the molecular level, to justify inclusion in a single phylum. In the classification advocated here, Sporozoa replaces the phylum Apicomplexa and many parasitologists will be pleased to see the return of the phylum Sporozoa, which has both priority and common usage. References to Apicomplexa or apicomplexans should now revert to Sporozoa or sporozoans, the original names.

COCCIDIANS: *ISOSPORA*, *SARCOCYSTIS*, AND *TOXOPLASMA*

Members of this group are either parasites that undergo the whole of their life cycle in a single host, typically in the epithelial cells of the gut, or divide a similar cycle between two hosts. Until the early 1970s these parasites were known only from miscellaneous stages in their hosts but since then they have been classified as a tight assemblage on the grounds of morphology, life cycles, and molecular similarities. The species recognized in humans are *Isospora belli*, *Sarcocystis hominis*, *S. suihominis*, and *Toxoplasma gondii*. Although there may be some controversy about their exact identity and relationships, there is no doubt that in this classification they are satisfactorily accommodated in the phylum Sporozoa, Class Coccidea, and Order Eimeriida.

HEMOSPORIDANS: *PLASMODIUM* AND *BABESIA*

These parasites occur in the blood of their vertebrate hosts and have been extensively studied, largely because members of the genus *Plasmodium* cause malaria and members of the genus *Babesia* cause important diseases in cattle. In the 1994 classification, the class Haematozoea contains two orders; Haemosporida, containing members of the genus *Plasmodium*, and Piroplasmida, containing members of the genus *Babesia* which are rare and accidental parasites of humans. There can be little dispute about the classification of the Haemosporida, which is conventional and well accepted. The piroplasms have had a more checkered history, originally being described as sporozoans as long ago as 1889 (Smith and Kilborne 1893), regarded as a suborder by Wenyon (1926), transferred to the amoebae (Honigberg et al. 1964), elevated to the level of a subclass of the class Sporozoa (Levine et al. 1980) and to a class (Cox 1991). In the present classification the redundant class Haematozoa disappears and brings the malaria parasites and piroplasms together as equivalent orders. However, superficial similarities might mask fundamental differences and these may in time be sufficient to justify further separation of the two groups, either as subclasses or classes.

BLASTOCYSTIS

Blastocystis hominis has had a checkered taxonomic history since its discovery nearly 100 years ago and has been classified with the fungi, plants, flagellates, amoebae, and sporozoans. Molecular techniques have now established that it belongs close to the sporozoans and ciliates but in a separate kingdom, Chromista (Silberman et al. 1996; Cavalier-Smith 1998; Arisue et al. 2002). *Blastocystis* is the only parasitic member of this kingdom and is included in this volume for convenience and because of its affinities with other protozoa.

MICROSPORIDIANS: *BRACHIOLA*, *ENCEPHALITOZOON*, *ENTEROCYTOZOON*, *MICROSPORIDIUM*, *NOSEMA*, *PLEISTOPHORA*, *TRACHIPLEISTOPHORA*, AND *VITTAFORMA*

These tissue-inhabiting parasites have also had a checkered history, at one time being classified with the Sporozoa, later in a group with the myxosporidians and in the 1980s and 1994 classifications in a phylum of their own, the Microspora. As these parasites have become increasingly recognized as significant concomitants of HIV infections it is important that they should not be neglected on some classificatory sideline, and they are now thought to be more closely related to fungi than to protozoa (Hirt et al. 1999; Cavalier-Smith 2001). Accordingly they are now classified in the eukaryotic kingdom Fungi but are retained in this volume because they are traditionally considered together with the protozoa. It should be noted that *Pneumocystis carinii* was formerly considered to be a protozoan but has now been formally assigned to the Fungi, and this will probably also be the case with the microsporidians in due course.

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Amebiasis: *Entamoeba histolytica* infections

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INTRODUCTION

There are six species of amebae commonly found in the human gastrointestinal tract. Of these, four belong to the genus *Entamoeba*: *E. histolytica*, *E. hartmanni*, *E. coli*, and the recently redescribed *E. dispar*. The rest represent separate genera: *Endolimax nana* and *Iodamoeba buetschlii*. *Dientamoeba fragilis*, long considered another intestinal amebae has now been shown to be an aberrant trichomonad. Only *E. histolytica* is of medical importance, since it is the causative agent of amebiasis, a potentially fatal disease. Other amebae are of interest mainly because their trophozoites may be difficult to distinguish by light microscopy from trophozoites of *E. histolytica*. *E. dispar*, however, deserves special mention because it is morphologically indistinguishable from *E. histolytica* and accounts for most asymptomatic infections previously assigned to *E. histolytica*.

Because of its clinical importance, most of this chapter is devoted to *E. histolytica*, although reference to the non-invasive *E. dispar* is necessarily included in those sections where differentiation between the two species is relevant. At the end of the chapter, brief consideration is given to the other intestinal amebae of humans, as well as to *E. moshkovskii* (the 'Laredo-type' that can be found in humans), *E. polecki* from pigs, *E. invadens* from reptiles, and *E. chattoni* from monkeys. Additional information may be obtained from Martínez-Palomo (1982, 1993), Espinosa-Cantellano and Martínez-Palomo (2000), Haque et al. (2003), Stanley (2003), and Stauffer and Ravdin (2003).

For the last 70 years, two of the most puzzling aspects of the biology of *E. histolytica* were the unexplained variability of its pathogenic potential and the restriction of human invasive amebiasis to certain geographical areas despite the worldwide distribution of the parasite. The main debate centered on the question of whether there were one or two species of *E. histolytica*. In 1925, the French parasitologist Brumpt proposed that invasive amebiasis is produced by a species of ameba with a worldwide distribution that is biologically distinct but morphologically similar to nonpathogenic amebae (Brumpt 1925). Apart from some isolated studies carried out by Simic, there was nothing to refute or confirm this hypothesis for almost 50 years (Simic 1931). However, in the 1970s, differences in surface properties were found between strains of *E. histolytica* isolated from carriers and those obtained from patients with invasive amebiasis (Martínez-Palomo et al. 1973; Trissl et al. 1977, 1978). Starting in 1978, Sargeaunt and colleagues applied an isoenzyme technique to thousands of isolates of

amebae obtained from several continents (Sargeaunt et al. 1982). This technique is based on the analysis of band patterns obtained after gel electrophoresis of the enzymes hexokinase and phosphoglucomutase. It revealed that invasive amebiasis is produced by strains that have characteristic isoenzyme patterns (zymodemes) distinct from those obtained from amebae harbored by most carriers. Moreover, monoclonal antibodies specifically recognized amebae belonging to pathogenic zymodemes (Strachan et al. 1988; Petri et al. 1990; Tachibana et al. 1990), and molecular probes were able to distinguish between pathogenic and nonpathogenic isoenzyme patterns (Tannich et al. 1991a).

The application of molecular biology to the field finally resolved the problem, and it is now accepted that there are two species: *E. histolytica*, previously known as pathogenic or invasive *E. histolytica*, and *E. dispar* formerly denominated as nonpathogenic or noninvasive *E. histolytica*. For a comprehensive review of the history of the debate, as well as the scientific evidence that supports the conclusion of the distinct natures of *E. histolytica* and *E. dispar*, refer to Spice and Ackers (1992), Diamond and Clark (1993), and Clark and Diamond (1993).

CLASSIFICATION

According to the Committee on Systematics and Evolution of the Society of Protozoologists, the *Entamoeba* are grouped in the family Endamoebidae, order Amoebida, subclass Gymnamoebia, class Lobosea, superclass Rhizopoda, subphylum Sarcodina, phylum Sarcomastigophora (Levine et al. 1980). However, in the 15 years since the last taxonomic scheme for the Protozoa was adopted, new data on the ultrastructure and on the molecular biology of numerous species have rendered it obsolete, and have indicated the necessity for revision of the classification of Protozoa (Cox, 1992; Corliss 1994; see also Classification and introduction to the parasitic protozoa). In Corliss's proposed classification, the amebae are all placed in the phylum Rhizopoda, class Entamoebidea. Within this class, they are classified in the order Endamoebida and the family Endamoebidae. *Dientamoeba* is now considered a flagellate.

The factors currently considered to be useful for differentiation of *Entamoeba* from other amebae have been summarized by Neal (1988) and include intrinsic characteristics such as morphology, type of nuclear division, type of movement, type of physiology, antigenic nature, nature of DNA, variability of isoenzymes, susceptibility to drugs, and extrinsic characteristics such as host specificity, virulence factors, behavior in laboratory hosts, and clinical effects. Riboprinting has also been applied to distinguish different *Entamoeba* species from other intestinal protozoa.

STRUCTURE AND LIFE CYCLE

The trophozoite

The motile form of *E. histolytica*, the trophozoite (Figure 1), is a highly dynamic and pleomorphic cell, the form and motility of which are strongly affected by changes in temperature, pH, osmolarity, and redox potential. Actively motile amebae are elongated in form, with protruding lobopodia and a trailing uroid, whereas resting trophozoites tend to be spherical. The diameter of the cell varies between 10 and 60 μm, not only due to the pleomorphism of the parasite, but also to the feeding conditions: amebae obtained directly from intestinal or liver lesions are generally larger (20–40 μm) than those found in nondysenteric stools or in cultures (10–30 μm). The cell surface has numerous circular openings of diameter 0.2–0.4 μm that correspond to the mouths of micropinocytic vesicles. These openings are absent from the large protruding stomas of macropinocytic channels (2–6 μm in diameter) and from lobopodia. The uroid, when present, appears as a tail formed of irregular folds of the membrane and filiform processes called filopodia. Filopodia can also be found in other regions of the cell

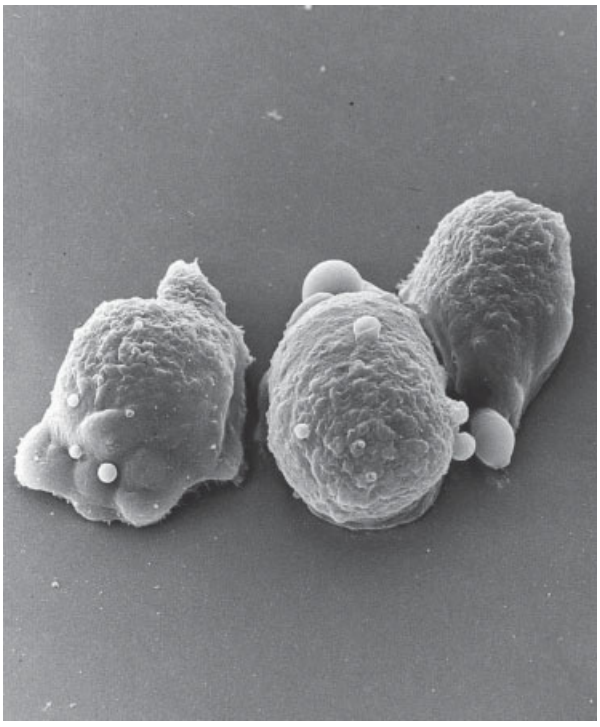

Figure 1 *Scanning electron micrograph of* E. histolytica *cultured trophozoites. Note the rough appearance of the cell surface, except in the regions of pseudopod extension. The parasite on the left shows a trailing uroid opposite to the pseudopod formations and abundant filopodia at the site of membrane attachment. A phagocytic opening of the membrane is clearly evident in the trophozoite located at the center of the micrograph.*

surface and are more frequently observed in amebae in monoxenic cultures or in contact with epithelial tissues (Martínez-Palomo 1982).

THE PLASMA MEMBRANE

The plasma membrane of *E. histolytica* is c. 10 nm thick and is covered by a uniform surface coat composed mainly of glycoproteins (Figure 2). The binding of the lectin concanavalin A to the surface of the trophozoite suggests a high content of sugar residues (i.e. mannose, glucose). In actively motile trophozoites, a thin layer of material is deposited on the substrate, leaving a trail of microexudate with cytochemical properties similar to those of the surface coat (Martínez-Palomo 1982).

Interaction of the trophozoite plasma membrane with specific ligands induces a dramatic redistribution of surface components that accumulate at the uroid and are later released into the medium. This capping of surface molecules, suggested as a mechanism of evasion of the humoral immune response, occurs through a sliding mechanism that involves both actin and myosin and is regulated by calmodulin and a myosin light-chain kinase (Espinosa-Cantellano and Martínez-Palomo 1994).

THE CYTOPLASM

The cytoplasm of *E. histolytica* trophozoites is characterized by the absence of most of the differentiated organelles found in other eukaryotic cells (i.e. mitochondria, Golgi apparatus, rough endoplasmic reticulum, centrioles, and microtubules). Instead, the cytoplasm contains abundant vacuoles of extremely variable size, with diameters 0.5–9.0 μm (Figure 3). Some of these vacuoles have been identified using biochemical and ultrastructural techniques. They include phagocytic vacuoles, macropinocytic and micropinocytic vacuoles, lysosomes, residual bodies, and autophagic vacuoles. Food vacuoles can be observed filled with starch or bacteria in amebae in xenic and monoxenic cultures. Amebae recovered from dysenteric stools usually contain ingested red blood cells; ingestion of red blood cells has traditionally been considered the best evidence of the invasive nature of the parasite. The lysosomes of amebae differ from those of other eukaryotes in that the contained enzymes are bound to the lysosomal membrane rather than being free in the vacuolar compartment (Martínez-Palomo 1986). Our knowledge of this complex vacuolar system is mainly of the vacuoles involved in endocytic processes (Batista et al. 2000). The nature and functions of other vacuoles remain to be determined.

Although no structures resembling classical mitochondria are present in the amebic cytoplasm, the *E. histolytica* genome encodes molecular chaperone cpn60 and pyridine nucleotide transhydrogenase (Yu and Samuelson 1994; Clark and Roger 1995) that are associated with mitochondria in other eukaryotes. Cpn60 has been localized in one or two small (1–2 μm) vacuoles in the cytoplasm of *E. histolytica*. Targeting of cpn60 to this compartment is dependent on short signal peptides that can be replaced with mitochondrial targeting sequences from other protozoa. The crypton or mitosome seems to be a mitochondrial remnant whose function remains unknown.

A lattice of tubules and vesicles superficially resembling smooth endoplasmic reticulum can be found, although very seldom, in the cytoplasm of *E. histolytica* trophozoites. The lattice is made up of extremely thin tubules approximately 20 nm in diameter, forming irregular whorls or parallel arrays. In contrast to the 10 nm thick plasma and vacuolar membranes, the membrane enclosing these tubules is only 6 nm thick (Martínez-Palomo 1982).

Ribosomes appear to be mostly ordered in helical arrays approximately 300 nm long and 40 nm in diameter. In cysts and resting cultured trophozoites, the helices aggregate in large crystalline inclusions that can be several micrometers in length, forming the classic chromatoid body with a hexagonal packing pattern. Whether ribosomes in helices and in chromatoid bodies are functionally mature ribosomes or ribosomal precursors remains to be established.

Despite the striking motility and plasticity of the trophozoite and the important role that the cytoskeleton

Figure 2 *Plasma membrane of a trophozoite of* E. histolytica *seen in a transmission electron micrograph of a cryofixed, cryosubstituted sample. A thick surface coat made of fibrilar material is clearly revealed with these techniques. The surface components here shown are usually lost during ordinary techniques used for fixation and embedding. The section was counterstained with only uranyl acetate and lead citrate.*

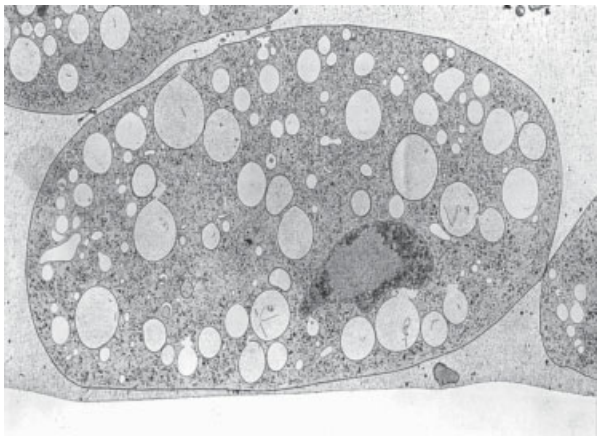

Figure 3 *Transmission electron micrograph of an* E. histolytica *trophozoite in culture. The ultrastructural appearance of the trophozoite is mainly characterized by the lack of mitochondria, a Golgi system, endoplasmic reticulum and cytoplasmic microtubules. Cryofixation and cryosubstitution of the sample provide a better preservation of cytoplasmic components than do standard procedures.*

plays in the invasion process, little was known about the structural organization of the cytoskeleton until recently. Prior to improvements in microscopy, only scarce microfilaments resembling actin (7 nm in diameter) were visible by transmission electron microscopy. The development of cryofixation and cryosubstitution allows much better preservation of biological materials and produces fewer artifacts than chemical fixation. Filaments with the appearance and size of actin have been visualized massed below the plasma membrane and in pseudopodial extensions and phagocytic stoma. Moreover, filaments with the size and appearance of myosin, which had only been observed in immunofluoresence preparations, have been observed with transmission electron microscopy. These studies have confirmed the absence of microtubules in both the cytoplasm and nuclei of nondividing amebae. Microtubules are known to be present in the nuclei of dividing trophozoites (González-Robles and Martínez-Palomo 1992).

Finding cylindrical particles in the cytoplasm of *E. histolytica* generated considerable interest. These particles are found in trophozoites obtained from such diverse sources as colonic exudates from patients with acute amebic colitis, human liver lesions, and axenic and polyxenic cultures. These particles are commonly arranged in rosettes; in thin sections of amebae one or two rosette conglomerates are usually found surrounding a finely granular specialized area of the cytoplasm. Each conglomerate is c. 1 μm in diameter and is composed of 9–30 cylindrical bodies. The bodies vary in size and may be up to 250 nm long and 90 nm wide. They are surrounded by a membrane that is 7 nm thick. They tend to be bullet-shaped, although occasionally they appear rounded at both ends. The morphological similarity of the cylindrical particles to rhabdoviruses, and the cytochemical detection of a dense RNA-containing core in the rosette arrangements of the former have led to speculation of a possible viral nature. There is, however, no conclusive evidence to date about the nature and biological significance of these structures.

THE NUCLEUS

The nucleus of *E. histolytica* is 4–7 μm in diameter. The nuclear membrane is double and is interrupted by numerous nuclear pores c. 65 nm in diameter. The membrane is lined by a thin, uniform layer of granules that gives the nucleus the appearance of a ring in optical section. Chromatin clumps are usually uniform in size and evenly distributed inside the nuclear membrane although in some cells the chromatin appears concentrated on one side as a crescentic mass. The karyosome or endosome is a small, spherical mass c. 0.5 μm in diameter and located in the central part of the nucleus. Intranuclear bodies 0.2–1.0 μm in diameter are frequently observed but their nature and function are unknown. Nuclear division proceeds without dissolution of the nuclear membrane and involves the participation of thick microtubule spindles, but the precise mechanism is unknown. Fluorescence microscopy with the spreading technique has estimated that the number of chromosomes is about 30–50, which agrees with the results obtained from pulse-field gel electrophoresis (Willhoeft and Tannich 2000).

The cyst

Cysts have been studied far less extensively than trophozoites, mainly due to our inability to induce encystation in axenic cultures. Thus, most studies on *Entamoeba* cysts have been carried out on *E. invadens*, a parasite of reptiles that can be made to encyst in culture (reviewed in Eichinger 2001). *E. histolytica* cysts are round or slightly oval hyaline bodies 8–20 μm in diameter. They are surrounded by a refractile wall which is 125–150 nm thick and apparently composed of fibrillar material. This forms a tight mesh and may give rise to several lamellae (Figure 4). In *E. invadens*, the cyst wall has been shown to contain chitin. The plasma membrane frequently has deep invaginations; polyribosomes and vacuoles containing dense fibrogranular material can be observed close to its cytoplasmic face. Food vacuoles tend to disappear as the cyst matures. Staining with iron-hematoxylin makes the cytoplasm appear vacuolated with numerous glycogen deposits that decrease in size and number as the cyst matures. Chromatoid bodies, which are aggregated ribosomes, can be identified inside the cytoplasm as rod-shaped structures with blunt or rounded ends. Iodine stains allow the clear visualization of 1–4 small nuclei.

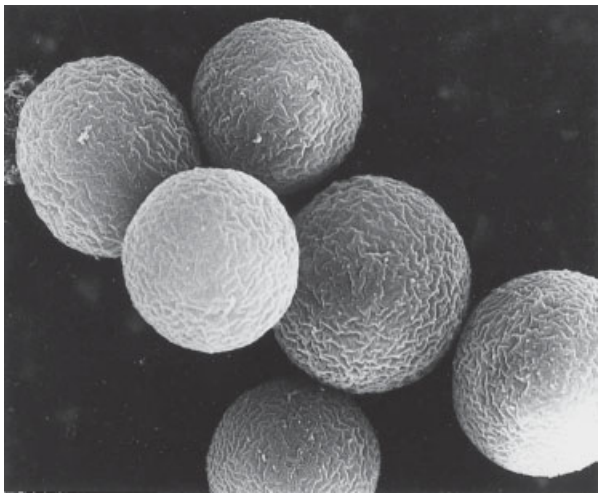

Figure 4 *Scanning electron micrograph of* E. histolytica *cysts recovered from feces of a human carrier. The cyst wall allows the parasite to survive in a relatively hostile environment outside the host.*

The life cycle

Trophozoites dwell in the colon, where they multiply and encyst typically producing four-nucleated cysts. These appear in the formed stools of carriers as round or slightly oval hyaline bodies with a refractive wall. When a cyst is ingested, the cyst wall is dissolved in the upper gastrointestinal tract and the parasite excysts in the terminal ileum, eventually giving rise to eight uninucleated trophozoites. Cysts do not develop within tissues, but the invasive form of the parasite, the trophozoite, can penetrate the intestinal mucosa and disseminate to other organs. Trophozoites are short-lived outside the body and do not survive passage through the upper gastrointestinal tract. In contrast, cysts may remain viable in a humid environment and stay infective for several days.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Biochemistry

METABOLISM

The metabolism of the parasite is puzzling since *E. histolytica* is a facultative aerobe with peculiar glycolytic enzymes that are also found in other amitochondriate eukaryotes like *Naegleria*, and in certain bacteria. Glucose and, to a lesser extent, galactose are the main sources of energy. The uptake of glucose occurs via a specific transport system that provides c. 100 times the amount incorporated by endocytosis. This transport system is the rate-limiting step in glucose consumption. Glycogen is the main form of storage.

Glucose-6-phosphate is degraded to pyruvate via the Embden-Meyerhof pathway. A unique aspect of glycolysis in *Entamoeba* is the utilization of inorganic pyrophosphate (PPi), generally considered an end-product of metabolism, as an energy source in several glycolytic reactions. The genes coding for several of these enzymes have been cloned, including pyrophosphate-dependent phosphofructokinase (PPi-PFK) (Huang et al. 1995) and pyruvate phosphate dikinase (PPDK) (Bruchhaus and Tannich 1993; Saavedra-Lira and Pérez-Montfort 1996). In addition, the primary structure of enolase, which catalyses the conversion of 2-phosphoglycerate to phosphoenol pyruvate, has also been reported (Beanan and Bailey 1995).

The end-products of pyruvate degradation depend on the degree of anaerobiosis: under aerobic conditions (5 percent oxygen concentration) acetate, ethanol, and carbon dioxide are formed, whereas in an anaerobic environment, only ethanol and carbon dioxide are produced. The enzymatic activities involved in this process include a pyruvate synthase, aldehyde dehydrogenase (ALDH) and both NAD+-linked and NADP+-linked alcohol dehydrogenase (ADH). The genes encoding an NADP+-dependentADH and ALDH have been cloned (Kumar et al. 1992; Zhang et al. 1994). The presence of a multifunctional NAD+-dependent acetaldehyde/alcohol dehydrogenase, previously found only in anaerobic and facultative anaerobic bacteria, has been reported (Bruchhaus and Tannich 1994; Yang et al. 1994). It has been suggested that during aerobiosis, electrons are transferred from reduced substrates via a succession of carriers (including flavins, ferredoxin, other FeS proteins and ubiquinone) to molecular oxygen, which is reduced to water (Weinbach 1981; Huber et al. 1987; Ellis et al. 1994). The final electron acceptor under anaerobic conditions is not known.

Little is known about nucleic acid, lipid, and protein metabolism (Reeves 1984; McLaughlin and Aley 1985). *E. histolytica* is a purine auxotroph but can apparently synthesize pyrimidines. The parasite seems to be able to synthesize some of its lipids, including cholesterol and isoprenoids. At least three phospholipase activities (A1, A2, and L1) have been detected. Enzymes involved in inositol metabolism have been identified (Lohia et al. 1999), as have some of the early enzymes participating in the biosynthesis of glycosylated proteins (Vargas-Rodríguez et al. 1998; Villagómez-Castro et al. 1998).

E. histolytica is thought to be unique among eukaryotes in that it does not contain glutathione or glutathione-dependent enzymes (Fahey et al. 1984). In other organisms, the primary function of glutathione is to protect against oxygen toxicity. High concentrations of cysteine and other thiols have been identified in *Entamoeba*, which could carry out the functions of glutathione and its dependent enzymes (Fairlamb 1989). In addition, the presence of a disulphide oxidoreductase that binds flavin adenine dinucleotide (FAD) as a

cofactor and can reduce alkyl hydroperoxides and hydrogen peroxide through NADH or NADPH has been suggested as a means of protection against damage by active oxidizing agents (Bruchhaus and Tannich 1995).

PLASMA MEMBRANE AND SURFACE MOLECULES

The biochemical characterization of cellular membranes, and particularly of the plasma membrane, is important for the understanding of host–parasite interactions. Progress in this field has been hampered by the presence of potent proteases, the unavailablity of suitable enzyme markers in the parasite, and the continuous turnover of surface membranes by endocytosis.

Membrane lipids have been studied both in whole extracts of trophozoites and in isolated plasma and internal membrane fractions (Aley et al. 1980). Cholesterol is enriched in the plasma membrane fraction to a molar ratio of 0.87 with phospholipid, a finding consistent with previous reports in other types of cells. Phosphatidylethanolamine predominates over phosphatidylcholine in the plasma membrane, although the latter is the most abundant lipid in internal vesicles. An unusual phospholipid, ceramide aminoethyl phosphonate (CAEP) has been demonstrated in internal vesicles, but is mostly concentrated in the plasma membrane, where it accounts for 35 percent of the total lipid content (Aley et al. 1980). This may have biological importance, as this compound is resistant to hydrolysis and may protect the parasite against the action of its own phospholipase.

Isolated plasma membranes with surface glycoproteins stabilized by concanavalin A treatment yielded a total of 12 glycoproteins ranging from 12 to 200 kDa in molecular weight. These were identified by radiolabeling and autoradiography (Aley et al. 1980). When Percoll density gradients were used to separate these glycoproteins after they had been labeled by lactoperoxidase iodination, 16 bands were identified, nine of which comigrated with ^{125}I-concanavalin A-labeled peptides, a result suggesting they had a glycoprotein nature. The discrepancy in the number of glycoproteins found by these two procedures may be due to the action of amebic proteases activated differently by various lysis conditions. Such proteases have been shown to affect the electrophoretic profile of whole lysates (Espinosa-Cantellano and Martínez-Palomo 1991).

Several surface molecules that mediate adhesion to cells or intestinal mucus are recognized by sera from patients cured of invasive amebiasis. They include:

- a 260 kDa N-acetyl-D-galactosamine inhibitable lectin formed of two subunits, one of 170 kDa and one of 35 kDa (Petri et al. 1989, 1990)
- an N-acetylglucosamine inhibitable lectin of 220 kDa (Rosales-Encina et al. 1987)
- a 112 kDa surface adhesin (Arroyo and Orozco 1987).

Antibodies raised against these proteins partially inhibit adhesion and phagocytosis of target cells in vitro, suggesting that the proteins participate in amebic adherence. The 112 kDa adhesin seems to be composed of two subunits, an adhesin and a protease encoded by adjacent genes (García-Rivera et al. 1999). In addition, *E. histolytica* expresses two lipophosphoglycan (LPG)-like molecules that may also participate in adhesion (Isibasi et al. 1982; Moody-Haupt et al. 2000). Conflicting reports on whether *E. dispar* synthesizes LPG have been published, one group having identified only one of the two molecules (Moody et al. 1998), while the other did not find any LPG even after using different methods of extraction in several isolates (Bhattacharya et al. 2000). Other surface antigens identified by sera from patients include peptides of 30, 96, and 125 kDa, a serine rich *E. histolytica* protein and a lipopeptidophosphoglycan (reviewed in Espinosa-Cantellano and Martínez-Palomo 1991).

Among the enzymes identified in the plasma membrane of *E. histolytica* are Ca2+-adenosine triphosphatase (ATPase), phospholipase A, neuraminidase and a metallocollagenase thought to play an important role during parasite invasion (reviewed in Espinosa-Cantellano and Martínez-Palomo 1991). Cysteine proteases are the most abundant proteases and have also been implicated in the pathogenicity of *E. histolytica* trophozoites (Keene et al. 1990). Four major cysteine proteases have been reported:

1. a 56 kDa cysteine protease (Keene et al. 1986)
2. a 26–29 kDa histolysin (Luaces and Barret 1988)
3. a 22–27 kDa amebapain (Scholze et al. 1992)
4. a 16 kDa cathepsin B-like protease (Lushbaugh et al. 1985).

Three different cysteine protease genes have been described. Protein sequences of purified enzymes suggest that one gene encodes amebapain and a second one histolysin. The amebic cysteine proteases are secreted enzymes and are, therefore, mainly located in the internal vesicles but some have been identified on the surface of the trophozoite. The identity of the higher molecular mass proteases was uncertain, but recent reports have started to answer this question. Cysteine proteases of 35 and 48 kDa were identified as products of EhCP2 and EhCP1, respectively, after purification and partial N-terminal sequencing (Hellberg et al. 2000). In addition, a novel 60 kDa cysteine protease, probably involved in erythrophagocytosis (Spinella et al. 1999) and a 50 kDa protease forming a tight complex with a 75 kDa adhesin (García-Rivera et al. 1999) have been reported in the parasite.

LYTIC FACTORS

E. histolytica derives its name from its ability to lyse virtually every tissue in the human body and in the

Figure 5 *Scanning electron micrograph of guinea pig cecal mucosa. A microulceration has been experimentally produced by trophozoites of* E. histolytica. *This lesion exemplifies the earliest stage in the development of intestinal invasive amebiasis.*

bodies of experimental animals (Figure 5). Lysis of target cells involves contact-dependent as well as contact-independent mechanisms. Initial attachment of the trophozoite occurs via the amebic adhesins described above. It has been suggested that, once attached, the parasite releases an active peptide, the amebapore, which is capable of inserting ion channels into liposomes and which possesses cytolytic and bactericidal activities (Leippe et al. 1994; Bruhn and Leippe 1999). *E. dispar* also possess channel-forming proteins that have been called disparpores, but seem to have different activities (Nickel et al. 1999). During the process of tissue invasion, degradation of extracellular matrix components (ECM) possibly involves specific receptors. A receptor common for fibronectin and laminin has been identified in trophozoites of *E. histolytica* (Talamás-Rohana et al. 1994). The actual degradation of ECM occurs as a result of action by the collagenase and secreted cysteine proteases that have been shown to be active against a wide variety of ECM proteins, including collagen, fibronectin, laminin, and some proteoglycans. Interestingly, the expression of cysteine protease genes has been found to be 10–100-fold higher in pathogenic *E. histolytica* than in nonpathogenic *E. dispar* strains (Tannich et al. 1991b).

Molecular biology and genetics

The total DNA content is 0.5 pg per cell, all of which is concentrated in the nucleus; there are no DNA-containing organelles (Byers 1986). Molecular analysis indicates that there is a complex karyotype with 31–35 chromosomal bands, ranging in size from 0.3 to 2.2 megabases and 14 linkage groups each with a ploidy of at least 4 (Willhoeft and Tannich 2000).

In most other unicellular eukaryotes, rDNA is present intrachromosomally. In contrast, rDNA in *Entamoeba* seems to exist exclusively as extrachromosomal circular molecules (Bhattacharya et al. 1989; Huber et al. 1989). This peculiar characteristic is also shared by *Naegleria*.

Various circular DNA molecules that are not circular rDNA have been detected by Southern blot analysis (Dhar et al. 1995). The fact that such circular DNA exists in the nucleus is supported by the identification (through hybridization with various circular DNA clones) of protein-coding regions in exonuclease-resistant DNA (Lioutas et al. 1995). The origin and function of these circular DNA elements remains to be established but their presence could explain, at least partially, the large variations in number and size of the karyotype of the parasite. The first *E. histolytica* gene was cloned in 1987 (Edman et al. 1987; Huber et al. 1987) and since then, numerous genes have been identified, their sequences analyzed, and their products characterized. An *E. histolytica* Genome Project has rapidly advanced. The genome is highly repetitive and introns are found in 15 percent of genes encoding proteins (Mann 2002).

Despite impressive advances in our understanding of the molecular biology of *Entamoeba*, many questions in the field are far from being resolved. *E. histolytica* has been successfully transfected and this allows studies on gene regulation, on the biological significance of various molecules for the survival of the parasite and on the mechanisms by which it produces disease (Nickel and Tannich 1994; Purdy et al. 1994).

CLINICAL ASPECTS

The term ‘amebiasis’ includes all cases of human infection with *E. histolytica*, but only a proportion of cyst-releasing individuals have symptoms due to the penetration of the parasite into the tissues, a condition known as ‘invasive amebiasis.’ The large group of infected, asymptomatic individuals, previously described as having ‘luminal amebiasis,’ is now thought to be composed mainly of *E. dispar* carriers.

Invasive amebiasis is a potentially fatal condition and there is a relatively high mortality among patients with severe forms of the disease. On a global scale, it ranks second after malaria as a cause of death among people with parasitic infections produced by protozoa (Walsh 1984).

CLINICAL MANIFESTATIONS

An epidemic of amebic dysentery that occurred in Chicago in 1933 provided an opportunity for studying the incubation period of the disease. Contamination of the water supply of two large hotels in the area was responsible for this outbreak, which resulted in around 1 400 cases of amebic dysentery. Although the complete history could not be obtained from all patients, reliable data were available from 391 cases. In these, the incubation periods ranged from 1 to 19 weeks, divided as follows: less than 1 week (6.7 percent), 1–4 weeks (59 percent), 4–8 weeks (24.7 percent), 9–13 weeks (7.4 percent), and more than 13 weeks (2.2 percent). Severe infections tended to have a shorter incubation period, most of them being reported within 1–6 weeks after initial exposure (US Treasury Department 1936).

Depending on the affected organ, the clinical manifestations of amebiasis are intestinal or extraintestinal. Both localizations can occur at the same time, but they are usually manifested separately.

Intestinal amebiasis

There are four clinical forms of invasive intestinal amebiasis, all of which are generally acute:

1. dysentery or bloody diarrhea
2. fulminating colitis
3. amebic appendicitis
4. ameboma of the colon.

The first of these is considered to be relatively benign, but the other three are severe forms of the disease and require prompt medical attention.

AMEBIC DYSENTERY

Dysenteric and diarrheic syndromes account for 90 percent of the cases of invasive intestinal amebiasis. Their various clinical manifestations depend on where the lesions are located within the rectosigmoid or higher regions of the colon. In people with this form of the disease, rectosigmoidoscopy may reveal superficial ulcerations extending over limited areas of the terminal portion of the large intestine. Patients with dysentery have an average of 3–5 mucosanguineous evacuations per day with moderate colic pain preceding discharge, they also have rectal tenesmus. In patients with bloody diarrhea, evacuations are also few, but the stools are composed of liquid fecal material stained with blood and, although there is moderate colic pain, there is no rectal tenesmus. Fever and systemic manifestations are generally absent. These syndromes constitute classic ambulatory dysentery and can easily be distinguished from diseases of bacterial origin. The clinical course is moderate and symptoms disappear rapidly with treatment; spontaneous remissions are occasionally observed after several days (Sepúlveda and Treviño-García Manzo 1986; Martínez-Palomo and Ruíz-Palacios 1990).

FULMINATING AMEBIC COLITIS

In contrast to the dysenteric syndrome, fulminating amebic colitis is an extremely severe, rapidly evolving clinical condition with necrotic ulcerous lesions extending over large areas, even the entire colon. These can affect all layers of the intestinal wall. Evacuations, preceded by intense colic pain, are frequent (20 or more in 24 h) and consist of fecal material mixed with blood or occasionally blood alone. Rectal tenesmus tends to be constant and acute. Systemic manifestations include abdominal discomfort, anorexia, and nausea. High fever (39–40°C) is usually present, accompanied by a weak, rapid pulse and low blood pressure. The patient suffers from dehydration and prostration, and may even develop shock. Peritonitis is a common complication due to the perforation of the intestinal wall (Sepúlveda and Treviño-García Manzo 1986; Martínez-Palomo and Ruíz-Palacios 1990).

AMEBIC APPENDICITIS

The symptoms of this condition are similar to those of bacterial appendicitis: acute pain and rigidity in the lower right quadrant of the abdomen, fever, tachycardia, and nausea. In more than two-thirds of cases of amebic appendicitis, patients have ulcerous lesions of the cecum. In these cases, diarrhea, often bloody, is also present (Sepúlveda and Treviño-García Manzo 1986; Martínez-Palomo and Ruíz-Palacios 1990).

AMEBOMA

Amebomas are pseudotumoral lesions, whose formation is associated with necrosis, inflammation and edema of the mucosa, and submucosa of the colon. Amebomas always co-exist with amebic ulcerations. These are generally single, but occasionally multiple, masses, usually found in the vertical segments of the large intestine: the cecum, the rectosigmoid region, and the hepatic and splenic angles of the colon. The condition is usually acute with dysentery or bloody diarrhea, abdominal pain, and a palpable mass in the corresponding area of the abdomen. If the lesion is located in the rectosigmoid region, it can be identified by endoscopy (Sepúlveda and Treviño-García Manzo 1986; Martínez-Palomo and Ruíz-Palacios 1990).

Extraintestinal amebiasis

E. histolytica can infect almost every organ of the body, including the liver, brain, lung, and skin. By far the most frequent form of extra-intestinal amebiasis is the amebic liver abscess which, due to its great clinical and epidemiological importance, is the most extensively discussed here.

Thoracic complications of amebiasis, including pleuropulmonary, pericardial, and mediastinal manifestations, are secondary to liver abscess. Involvement of the central nervous system is rare and lesions are single or multiple small areas of softening in the left cerebral hemisphere. Cutaneous amebiasis is usually a complication of intestinal amebiasis with dysentery. It may occur in the perianal region, or it may appear in the skin surrounding a fistula in cases of liver abscesses that involve the abdominal wall.

AMEBIC LIVER ABSCESS

This condition results from the migration of *E. histolytica* trophozoites from the colon to the liver via the portal circulation. The time lapse between penetration of the mucosa of the large intestine and damage to the hepatic parenchyma is unknown. It has been observed clinically and confirmed in a large series of autopsies that amebic colitis is found in only one-third of cases of hepatic abscess. Amebic liver abscesses have been reported in patients of all ages, but predominate in adults between 20–60 years. It has a marked preference for the right lobe of the liver and it is at least three times more frequent in males than in females. Interestingly, it is 10 times more common in adults than in children (Sepúlveda and Treviño-García Manzo, 1986).

The signs and symptoms of amebic hepatic abscess vary but, in general, the onset is abrupt with pain in the right hypochondrium radiating towards the right shoulder and scapular area. The pain usually increases with deep breathing, coughing, and while stepping on the right foot during walking. When the abscess is localized to the right lobe, symptoms include irritative cough, sometimes productive, and a pleuritic type of pain. Abscesses in the upper left lobe can cause epigastric, sometimes dyspneic pain, at times spreading to the base of the neck and to one or both shoulders. A sharp increase in pain centered in the precordial region strongly suggests that the abscess has penetrated into the pericardial space. This complication is nearly always fatal due to cardiac tamponade. Localization of the abscess in the vicinity of the diaphragm can lead to perforation of the pleura, causing pleurisy or empyema. If the bronchi become involved, pus may be vomited leading to clinical improvement. If the lung is involved, there may be pulmonary consolidation and abscess formation. When located in the inferior part of the liver, the abscess can penetrate into the peritoneal cavity and into neighboring organs (Sepúlveda 1970).

Fever between 38 and 40°C is found in all patients with amebic liver abscess. The patient commonly has chills and profuse sweating in the afternoon and at night. Other symptoms include anorexia, nausea, vomiting, and, of course, diarrhea (with or without blood), and dysentery. On physical examination, the cardinal sign of amebic liver abscess is painful hepatomegaly. Digital pressure and fist percussion often produce intense pain in the liver region. On palpation, the liver is soft and smooth, in contrast to the rough hard irregular character of the liver in patients with cirrhosis and hepatocarcinoma (Sepúlveda 1970). Jaundice is present in 8 percent of the patients that respond well to treatment. When jaundice is severe, multiple abscesses should be suspected (Figure 6). Ascites and hepatic coma occur rarely, but prognosis is poor in these patients.

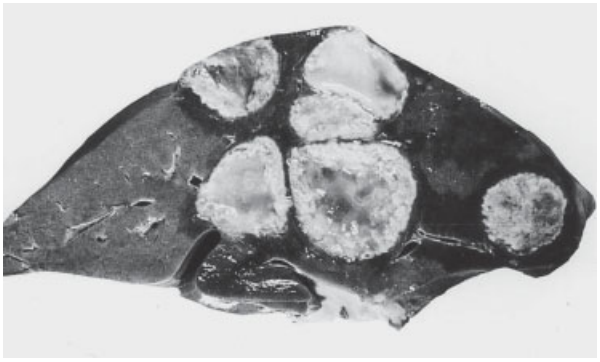

Figure 6 *Macroscopic appearance of amebic liver abscesses. When viewed microscopically, these lesions represent areas of liquefactive necrosis, rather than true abscesses.*

IMMUNOLOGY

Humoral immune response

The time between infection with *E. histolytica* and the appearance of local antibody responses remains unknown. Coproantibodies have been found by indirect hemagglutination assay (IHA) in c. 80 percent of patients with amebic dysentery, compared to 2 percent of healthy controls and 4 percent of patients with nonamebic intestinal parasitic infections; this figure was obtained at the time when patients came in for treatment, but 3 weeks later it fell to 55 percent. This coincided with an increase in serum antibodies suggesting that the humoral immune reaction in people with invasive intestinal amebiasis is initiated by a short and transient local secretory response, followed by an increase in systemic antibodies (reviewed in Pérez-Montfort and Kretschmer 1990). Immunoglobulin (Ig)A-type anti-*E. histolytica* antibodies have also been found in human milk, colostrum, and saliva, and in the bile of intracecally immunized rats. The protective role of secretory IgA (and IgE) in amebiasis has not been established. In fact, *E. histolytica* trophozoites have been shown to degrade secretory IgA (Kelsall and Ravdin 1993).

Circulating antibodies to *E. histolytica* can be detected as early as 1 week after the onset of symptoms in humans and experimental animals. All immunoglobulin classes are involved, but there seems to be a predominance of IgG. In contrast to findings in animals, the

levels of circulating antibodies in humans do not necessarily correlate with the severity of the disease. High titers of antibodies tend to appear early in the disease and to persist after invasive amebiasis is cured and in patients whose subclinical amebic infection is controlled (Knobloch and Mannweiler 1983). Antibody levels fall in different people at different rates following treatment. The detection of antibody in a patient depends on the sensitivity of the test used. Thus, if antibodies are measured by the sensitive techniques of IHA or by enzyme-linked immunosorbent assay (ELISA), antibodies can be detected more than 3 years after an invasive amebic episode in the absence of any recurrent infection (reviewed in Kretschmer 1986).

Complement may also be involved in limiting amebiasis, a role supported by the observation that experimental animals decomplemented with cobra venom factor are significantly more susceptible to development of amebic liver abscess than are normal animals. Also, the addition of cobra venom factor to normal human serum decreases the lysis of complement sensitive strains (Reed and Gigli 1990). Moreover, amebae are capable of activating complement through the classical and alternative pathways, even in the absence of antibody (Calderón and Schreiber 1985). This activation is lethal in vitro to the non-invasive *E. dispar*, and to *E. histolytica* strains that have been axenized and have partially lost their virulence. However, fresh isolates from patients with colitis or amebic liver abscesses are resistant to complement-mediated lysis (Reed et al. 1983) and repetitive treatment with human serum may induce complement resistance of axenic *E. histolytica* in culture (Calderón and Tovar 1986) suggesting that some amebae have developed a mechanism not yet understood for evasion of complement lysis. These observations may explain the conflicting reports of complement levels in humans and in experimental animals with invasive amebiasis: some suggest decreased levels of complement whereas others suggest elevated levels.

The deleterious effects of antiamebic antibodies on in vitro cultured trophozoites have been studied extensively. Immune sera can inhibit both erythrophagocytosis by *E. histolytica* and the cytotoxic effect of amebae on cells of cultured lines. At high concentrations, immune serum produces rapid lysis of trophozoites (reviewed in Kretschmer 1986). In apparent contrast to these observations, amebae may survive exposure to antibody in some circumstances. Incubation of amebae with immune serum induces the rapid redistribution of surface-bound antigens to the uroid of the cell and the caps thus formed can be either released or endocytosed. This process is both temperature and pH dependent, occurs through a sliding mechanism that involves both actin and myosin, and is regulated by calmodulin and a myosin light-chain kinase (Espinosa-Cantellano and Martínez-Palomo 1994). The parasite, which has thus shed the antibody–antigen complexes, apparently emerges undamaged and free of potentially harmful antibodies.

Taken together, these observations suggest that although a rapid humoral immune response is mounted by the host upon invasion by *E. histolytica*, the parasite has developed efficient evasion mechanisms that include resistance to complement and a mechanism for capping and shedding surface antigens. It is, therefore, considered that humoral antibodies are not protective against *E. histolytica*, a conclusion further supported by the observation of high rates of reinfection in persons with elevated antibody titers and by the detection of high levels of antibodies in patients with symptomatic amebiasis.

Cellular immune response

Even though the basic components of a local cellular immune response, such as mononuclear phagocytes and lymphocytes, are regularly present in early intestinal amebic lesions, their role in the establishment or prevention of invasive infection is not well understood. Evidence of systemic cell-mediated immunity has been confirmed in vivo through delayed hypersensitivity skin reactions, and in vitro through lymphokine (macrophage inhibition factor (MIF)), blastogenic response, leukocyte adherence inhibition, and lymphocytotoxic assays. Many patients, however, fail to react to delayed skin tests with amebic antigens during the early stages of the disease, apparently due to a state of specific unresponsiveness. Such unresponsiveness has been detected by in vitro assays, which show a transient failure to produce MIF, and by a reduction of lymphocyte cytotoxicity in patients in early stages of untreated hepatic amebiasis (reviewed in Kretschmer 1986). Thus tissue invasion by *E. histolytica* must be preceded by and be associated with some degree of T-cell suppression as a result of either selection or induction of suppressor T cells. Supporting the selective mechanism is the observation of increased susceptibility to invasive amebiasis of patients and experimental animals immunosuppressed by mechanisms mediated by suppressor T cells. The coincidence of malnutrition and amebic liver abscess in over 90 percent of clinical and autopsy cases and the significantly increased HLA-DR3 antigen levels found in Mexican patients with amebic liver abscess also suggest that T-cell mediated suppression is a factor in invasive amebiasis. The inductive proposal, on the other hand, is based on the observation that cell-free extracts of the parasite can exhaust, and thus suppress, the cellular immune response of the host (Kretschmer 1986).

The existence of an early transient anergy state does not necessarily preclude a protective role for cell-mediated immunity against invasive amebiasis. In fact, a state of cell-mediated immunity may be responsible for the rarity of recurrences of amebic liver abscesses in humans: 0.04 percent recurrences versus 0.2 percent

amebic liver abscess per initial infection per year calculated in Mexico City (Kretschmer 1986), although a study of 2 013 cases of amebic liver abscess in Vietnam did not support the concept of a long-lasting immunity against recurrent liver abscess (Blesmann et al. 2002).

The existence of a partial cellular, rather than humoral, immunity against extraintestinal amebiasis is based on the following findings:

- immunization studies and studies of the effect of immunosuppression of experimental animals on infection
- the results of a few studies of passive transfer of immunity with cells
- the cytolytic effect of activated lymphocytes and macrophages (irrespective of the presence of antibodies or complement) against *E. histolytica*.

This conclusion is further supported by studies on severe combined immunodeficient (SCID) mice, in which 100 percent developed liver abscesses when challenged intrahepatically with *E. histolytica* trophozoites (Cieslak et al. 1992). The actual defense strategy seems to depend heavily on macrophages; depressing macrophage function with silica or antimacrophage serum increases the development of experimental amebiasis, whereas enhancing macrophage function with bacille Calmette-Guérin (BCG) decreases such development. Moreover, congenitally athymic nu/nu mice (which are devoid of T lymphocytes) develop amebic liver abscesses or intestinal amebic disease only after macrophage blockade with silica. *E. histolytica* may possess mechanisms to evade or modify the action of macrophages. The parasite has been found to release a small molecular weight factor that can inhibit the locomotion of monocytes, but not of polymorphonuclear cells (reviewed in Kretschmer 1986).

PATHOLOGY

A detailed description of the pathological changes in amebiasis is beyond the objectives of this chapter, but a brief summary of the main characteristics of intestinal and hepatic lesions is given. For a comprehensive review of the pathology of the disease, refer to Pérez-Tamayo (1986).

Invasion of the colonic and cecal mucosa begins in the interglandular epithelium. Cell infiltration around invading amebae leads to rapid lysis of inflammatory cells and tissue necrosis (Figure 7); thus, acute inflammatory cells are seldom found in biopsy samples or in scrapings of rectal mucosal lesions. Ulcerations may deepen and progress under the mucosa to form typical flask ulcers. These extend into the submucosa producing abundant microhemorrhages, the presence of which explains the finding of hematophagous amebae in stool specimens and in rectal scrapings. Such ameba are the best indication of the amebic nature of a case of dysentery or bloody diarrhea. The ulcers are initially superficial, with hyperemic borders, a necrotic base, and normal mucosa between the sites of invasion. Further progression causes the loss of mucosa and submucosa; the lesion can extend into the muscle layers and eventually cause rupture of the serosa.

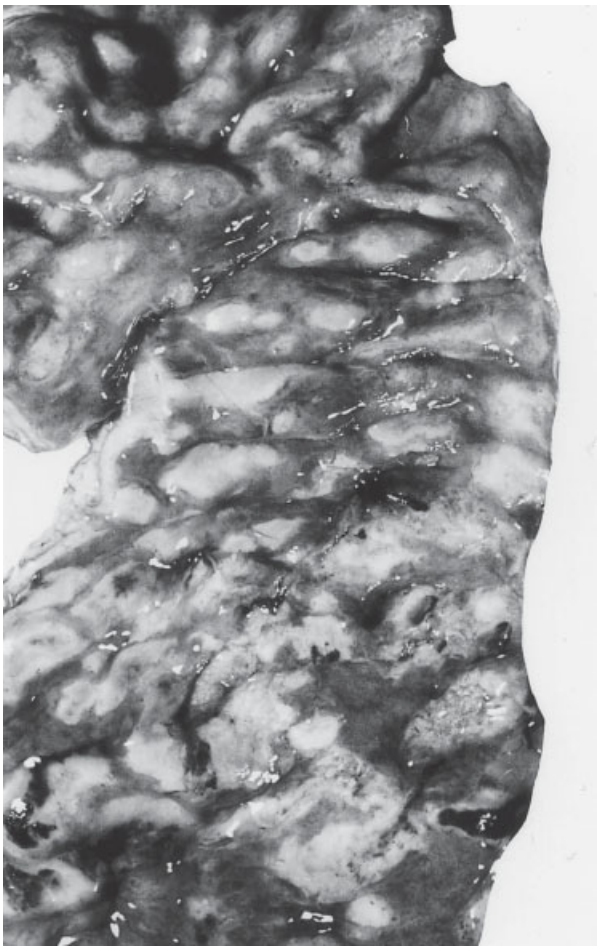

Figure 7 *Surface view of the mucosa of the colon in a fatal case of amebic colitis. Large and confluent ulcerations have destroyed most of the mucosal layers of the large intestine.*

Complications of intestinal amebiasis include perforation, direct extension to the skin and hematogenous dissemination, mainly to the liver. The presence and extent of liver involvement bears no relationship to the degree of intestinal amebiasis, and these conditions do not necessarily coincide. The early stages of hepatic amebic invasion have not been studied in humans. In experimental animals, inoculation of *E. histolytica* trophozoites into the portal vein produces multiple foci of infection in the liver with neutrophil accumulation around parasites, followed by focal necrosis and granulomatous infiltration. As the lesions grow in size, the granulomas gradually become necrotic and coalesce, necrotic tissue occupying progressively larger portions of the liver. Hepatocytes close to the early lesions degenerate and become necrotic, but direct contact of hepatocytes

with amebae is rarely observed. The lesion can eventually consist of large areas of liquefied necrotic material surrounded by a thin capsule of fibrous appearance (Tsutsumi et al. 1984).

DIAGNOSIS

Traditionally, the diagnosis of invasive intestinal amebiasis has been based on the microscopic identification of *E. histolytica* trophozoites in rectal smears or recently evacuated stools and on the results of rectosigmoidoscopy. However, the microscopic detection of amebae has several drawbacks. The procedure is tedious and time consuming; it requires a skilled technician; the sensitivity is relatively low; several samples must be taken to detect cyst passers, and fresh samples are needed for the detection of trophozoites. Trophozoites are most likely to be found in material obtained during rectosigmoidoscopy such as the bloody mucus and in the yellowish exudate covering the mucosal ulcerations. The finding of motile trophozoites containing ingested red blood cells confirms the diagnosis of amebic infection in patients with the clinical gastrointestinal symptoms. Problems arise when cysts alone are identified in stools of healthy or diarrheic individuals, as the cysts may be those of the nonpathogenic *E. dispar*, which are morphologically indistinguishable from cysts of the pathogenic *E. histolytica* (Martínez-Palomo 1993).

The standard for the differentiation of *E. histolytica* from *E. dispar* was, until recently, the determination of their isoenzyme pattern by gel electrophoresis. This procedure is expensive, slow, and requires the cultivation of amebae from feces, which is not routinely applicable. New diagnostic methods are now based on the detection in stool of *E. histolytica*-specific antigen or DNA and by the presence of antiamebic antibodies in serum (reviewed in Tanyuksel and Petri 2003).

Monoclonal antibodies found by ELISA and immunofluorescence assays of fecal samples have been tested. Although the ELISA was reported to have high sensitivity and specificity in a study of 701 samples, it could not discriminate between pathogenic *E. histolytica* and nonpathogenic *E. dispar* (del Muro et al. 1987). The immunofluoresence test, on the other hand, proved to be much faster than the test based on isoenzyme determination, but still required cultivation of the organism, and detected only the motile form of the parasite (Strachan et al. 1988). An ELISA based on monoclonal antibodies directed against cross-reactive and specific *E. histolytica* epitopes of the amebic galactose adhesin is now available commercially. The authors reported that the test gives 93 percent specificity and 96 percent sensitivity in the detection of infection with pathogenic *E. histolytica* (Haque et al. 1994).

Molecular probes provide another approach for the detection of the parasite in stools. A study with a specific probe of 123 stool samples from patients in Mexico City revealed a 93 percent specificity and 100 percent sensitivity for detection of amebae in stools, although it could not distinguish between amebae with pathogenic and nonpathogenic isoenzyme patterns (Samuelson et al. 1989). A study carried out in the south-eastern state of Chiapas in Mexico, using polymerase chain reaction (PCR) to amplify specific pathogenic and nonpathogenic samples resulted in 98 percent specificity and 96 percent sensitivity for the identification of *E. histolytica* in stools of 201 randomly selected individuals (Acuña-Soto et al. 1993).

A comparison of the ELISA and PCR methods to differentiate *E. histolytica* from *E. dispar* in stool samples revealed that the ELISA test is less sensitive (Gonin and Trudel 2003). The specificity of PCR was confirmed in a study of a large number of isolates from both species obtained in three different continents (Zaki et al. 2002). In addition, a closed-tube, real-time PCR assay has given improved results over traditional PCR methods (Blesmann et al. 2002).

The diagnosis of invasive intestinal amebiasis may be confirmed by endoscopy. In patients with benign dysentery, examination by rectosigmoidoscopy reveals small superficial ulcerations of a linear or oval shape, covered by a yellowish exudate and surrounded by a normal or hyperemic mucosa. In patients with fulminating colitis, the ulcers are large and tend to be confluent, often with a necrotic appearance. The intervening mucosa shows intense inflammation and signs of hemorrhage. Endoscopy should be carried out with great care in these cases, as there is a risk of intestinal perforation. If an ameboma is situated in the terminal portion of the colon, it can be visualized by rectosigmoidoscopy. If it is located elsewhere, it is necessary to use colonoscopy or radiography and a barium enema to identify the pseudotumoral mass, which is often surrounded by ulcerations (Sepúlveda 1970).

The diagnosis of amebic liver abscess is sometimes difficult. In endemic areas or when there is a history of travel to such places, amebic abscess should always be suspected in patients with spiking fever, weight loss, and abdominal pain in the upper right quadrant or epigastrium, as well as in patients with tenderness in the liver area. The presence of leukocytosis, a high level of alkaline phosphatase and an elevated right diaphragm (visible in a chest X-ray) suggests the presence of an hepatic abscess. The diagnosis is confirmed by ultrasonography or by computerized axial tomography (CAT) scans. The latter is the most precise method for identifying hepatic abscesses, particularly when they are small, and it is of great value in the differential diagnosis of other focal lesions of the liver (Sepúlveda and Treviño-García Manzo 1986).

Serological tests for antiamebic antibodies are positive in c. 75 percent of patients with invasive colonic amebiasis and in more than 90 percent of patients with amebic liver abscesses. Circulating antibodies have been

detected by virtually all known serologic tests, including tests based on immunofluorescent antibodies (IFA), IHA, radioimmunoassay (RIA), counterimmunoelectrophoresis (CIE), and ELISA. The latter is the most sensitive (it gives no false negatives in cases of amebic liver abscess), it is reasonably specific (3.6 percent false positives in controls living in endemic areas), and remains positive longest (more than 3 years in the absence of recurrent infection) (reviewed in Kretschmer 1986). The IFA test also deserves special mention because it is much simpler than all other tests, and when carried out in conjunction with IHA gives a 100 percent positivity in cases of amebic liver abscess. The Center for Disease Control in Atlanta, Georgia, has chosen the IHA as its standard serologic reference test for amebiasis. Titers below 1:256 are considered nonspecific. Because of its relative simplicity and efficiency, the CIE is particularly well suited for epidemiologic surveys.

Although serological techniques are useful in the diagnosis of invasive intestinal amebiasis and amebic liver abscess and as a tool for epidemiological studies of the disease, they do not aid in the diagnosis of simple intestinal infection. In endemic areas, the high prevalence of antiamebic antibodies in the general population reduces the usefulness of serologic tests for diagnosis and other tests must be performed before establishing the diagnosis of invasive amebiasis. Attempts have been made to discriminate serologically between present and past invasive amebiasis, using antibodies to specific antigens in sera of:

- patients with active or cured amebic liver abscesses
- individuals who are asymptomatic cyst carriers (Ximénez et al. 1993).

An antigen-detection ELISA in serum has proved to be useful (Haque et al. 2003).

EPIDEMIOLOGY AND CONTROL

Infection by *E. histolytica* is ubiquitous, but the highest incidence is usually found in communities with poor socioeconomic conditions and inadequate sanitation. In such communities, the infection is typically endemic. According to the World Health Organization, approximately 500 million people (not including those in the People's Republic of China) are infected with a 10 percent annual morbidity index (Walsh and Martínez-Palomo 1986), and up to 110 000 deaths annually can be attributed to complications of the disease (WHO, 1985). However, the actual frequency of amebiasis due to *E. histolytica* has been difficult to establish because there is a tendency to overestimate it in endemic areas, where cases of dysentery or bloody diarrhea are often misdiagnosed as amebiasis. In nonendemic areas with a low incidence of the disease, there is a tendency to overlook the presence of *E. histolytica* in stools.

In areas with high levels of poverty, illiteracy, and overcrowding, plus inadequate and contaminated water supplies and poor sanitation, direct fecal–oral transmission occurs frequently and the endemic character of the disease is maintained. Although difficult to implement, programs for the control of the disease require the improvement of environmental sanitation, health education, and early detection and treatment of infected people.

EPIDEMIOLOGY

Serological surveys

Antiamebic antibodies are relatively long-lasting in the serum of recovered patients and although antibodies cannot be used to discriminate between persons with recent and past infection, or between those infected with *E. histolytica* and *E. dispar*, detection of antibodies in the population has proved to be a valuable tool in epidemiological prevalence studies. A national seroepidemiological survey was undertaken in all 32 states of Mexico, with a probability-based sampling of almost 68 000 individuals who were representative of the population in age, socioeconomic status, and urban or rural origin. Antibodies to *E. histolytica* were detected by IHA assays in 8.41 percent of the population, ranging from 9.80 percent in the South Pacific region to 6.26 percent in the north-eastern parts, with a higher prevalence in rural (9.89 percent) than in urban (7.93 percent) areas. Frequency of presence of antibodies increased during the first decade of life, reaching the highest prevalence in children aged between 5–9 years. Antibody was detected more frequently in women (9.34 percent) than in men (7.09 percent). There was a clear correlation between high seroprevalence, low socioeconomic and educational levels, and inadequate housing conditions, although positive samples were found in all groups (Caballero-Salcedo et al. 1994).

A previous national serological survey of nearly 20 000 serum samples from 46 Mexican urban regions detected an average frequency of amebic seropositivity of 5.95 percent (Gutiérrez et al. 1976). The differences between these studies do not necessarily reflect an increase in seroprevalence in the Mexican population, but may be due to differences in the populations studied (the latter including only urban areas) and the methodology applied (IHA versus CIE).

Human susceptibility

The main reservoir of *E. histolytica* is human, although morphologically similar amebae may be found in primates, dogs, and cats. The parasite has been transmitted to various mammalian species, but it is doubtful that species other than humans serve as significant reservoirs of the parasite. Human susceptibility to infection

appears to be widespread, but most individuals harboring the parasite do not develop disease. It is now known that most of these asymptomatic carriers are infected with the noninvasive *E. dispar* although *E. histolytica* has been positively identified in some asymptomatic persons. In a semi-rural area south of Durban, South Africa, 1 percent of apparently healthy individuals were carriers of pathogenic *E. histolytica* and a 1-year follow-up revealed that 90 percent of these carriers remained asymptomatic and underwent spontaneous cure, the remaining 10 percent developed amebic colitis (Ghadirian and Jackson 1987). The factors involved in the development of disease are unknown, but probably include host as well as parasite characteristics.

Frequent luminal amebic infections have been reported in male homosexual populations in North America, northern Europe, and Japan. The spread in this group is associated with specific sexual practices; prevalence rates are as high as 32 percent and most reported cases are asymptomatic or present diffuse symptoms. *E. dispar* has been most frequently identified, except in Japan, where pathogenic *E. histolytica* is not uncommon (Takeuchi et al. 1990).

Incidence of invasive amebiasis

There are a few epidemiological reports of invasive intestinal amebiasis that are accompanied by microscopic identification of *E. histolytica* trophozoites and rectosigmoidoscopy studies showing intestinal ulcerative lesions; these reports provide divergent data. The frequency of invasive intestinal amebiasis has been reported to range from 2.2 percent to 16.2 percent in persons with acute diarrhea or dysentery, and the case-fatality rate to range from 0.5 percent in uncomplicated cases to 40.2 percent in persons with amebic dysentery complicated by peritonitis – these figures are drawn from Mexico, Nigeria, Venezuela, and South Africa (reviewed in Muñoz 1986). Although a large number of cases were included in these studies, the populations sampled were biased by including only those admitted to hospitals and healthcare centers.

The frequency of amebic liver abscess is a reliable measure of rates of liver infection, as liver lesions can be identified clinically, in the laboratory, or through post-mortem studies. Liver abscess occurs in c. 2 percent of adult amebiasis patients in endemic areas (Sepúlveda 1982). Elsdon-Dew tabulated the geographical distribution of persons with amebic liver abscesses and concluded that the condition is prevalent in West and South East Africa, South East Asia, Mexico, and the western portion of South America (Elsdon-Dew 1968). Amebic hepatic abscess is at least ten times more common in adults than in children. It predominates in adults aged between 20 and 60 years and within this group, three times as many men suffer from this condition as women. The reasons for the notable differences in the frequency and severity of invasive amebiasis according to age and sex are unknown.

The differentiation between *E. histolytica* and *E. dispar* in areas of high endemicity has already provided relevant epidemiologic information in Mexico (Sánchez-Guillén et al. 2002), Côte d'Ivoire (Heckendorn et al. 2002), Ecuador (Gatti et al. 2002), and Vietnam (Blesmann et al. 2002)

CHEMOTHERAPY

The use of amebicides in the treatment of patients with amebiasis has contributed greatly to reducing the morbidity and mortality of the disease. Antiamebic drugs may be classified into three groups:

1 luminal amebicides
2 tissue amebicides
3 mixed amebicides.

The most frequently used amebicides with luminal action are di-iodohydroxyquin (650 mg orally three times daily for 20 days), diloxanide furoate (500 mg orally three times daily for 10 days) and paromomycin (500 mg orally three times daily for 5–10 days).

Amebicides effective in both tissues and the intestinal lumen include metronidazole and the nitroimidazole derivatives tinidazole and ornidazole. In addition to being active in both tissues and the intestinal lumen, these drugs have the advantage that they are given orally and are most effective therapeutically. They are reasonably well tolerated and despite reported carcinogenic effect in rodents and their mutagenic potential in bacteria, no such effect has been detected in humans. They are, therefore, the drugs of choice in the treatment of invasive amebiasis in spite of some unpleasant, but not serious side-effects. Metrodinazole is given orally 500–800 mg three times a day for 5 days to people with invasive intestinal amebiasis and for 5–10 days to persons with liver abscesses. Tinidazole or ornidazole are administered orally 2 g once a day for 1–3 days. For further details on the chemotherapy of amebiasis, refer to Martínez-Palomo and Ruíz-Palacios (1990).

There is no drug resistance in amebiasis. Those few reports of failed treatment refer to advanced cases in which most of the liver parenchyma was replaced by the amebic abscess and treatment was administered too late. In vitro studies performed with fresh isolates from symptomatic patients and asymptomatic carriers, as well as axenically cultured pathogenic amebae, confirmed drug sensitivity in all cases (Burchard and Mirelman 1988).

Two severe forms of invasive intestinal amebiasis require surgery in addition to chemotherapy: toxic megacolon and amebic appendicitis. Amebic liver abscesses should be treated by chemotherapy; percutaneous or surgical drainage is indicated in cases of imminent rupture.

One of the most striking features of invasive amebiasis, whether intestinal, hepatic or cutaneous, is

that after adequate treatment amebic lesions heal without scarring. There is no apparent explanation of this peculiar aspect of the pathology of the disease.

VACCINATION

Evidence for the existence of acquired immunity against amebiasis is essentially based on two sets of observations. The first is the widely held knowledge that recurrence of amebic liver abscess is exceedingly rare. Although no recent data are available, estimates in the early 1970s in Mexico City indicated that 0.04 percent of patients recovered from an amebic liver abscess had recurrences during a 7-year follow-up period (versus a prevalence of 0.1–0.2 percent of amebic liver abscesses per year in the general population) (reviewed in Kretschmer 1986). The second set of observations supporting acquired immunity is drawn from studies on experimental animals that are refractory to amebic hepatic reinvasion after spontaneous or induced recovery. SCID mice can be partially protected by passive immunization procedures (Cieslak et al. 1992) and partial protection of intact mice has also been obtained through immunization with crude and fractionated preparations of *E. histolytica* and with live trophozoites. Local and systemic antibody responses have been reported to occur in experimental animals after immunization with fixed trophozoites and with an immunogenic peptide fused to the cholera toxin B subunit (Moreno-Fierros et al. 1995; Zhang et al. 1995).

There is also evidence against the development of immunity to invasive amebiasis. Recurrence of amebic hepatic abscesses was documented in a study in Vietnam (Blesmann et al. 2002). The rise in morbidity and mortality due to *E. histolytica* with increasing age, for example, casts some doubt on the concept of effective acquired protective immunity. In addition, it has been observed that not all antibodies are protective, as passive immunization with purified antiamebic immunoglobulins fails to protect hamsters against intrahepatic challenge with *E. histolytica* (Campos-Rodríguez et al. 1995). It has, therefore, been suggested that induction of protective immunity depends on the recognition by antibody of specific molecules. Recombinant peptides of three *E. histolytica* antigens show promise for prevention of experimental amebic liver abscess (reviewed in Stanley 1996): the serine-rich *E. histolytica* protein (SREHP), the 170 kDa subunit of N-acetyl-D-galactosamine inhibitable lectin, the 29 kDa cysteine-rich antigen. Much work still needs to be undertaken before an antiamebic vaccine is produced.

INTEGRATED CONTROL

Transmission of amebiasis may be accomplished through a variety of mechanisms. Asymptomatic carriers passing large numbers of cysts in their stools are important sources of infection, particularly if they are engaged in the preparation and handling of food. The cysts may remain viable and infective for a few days in feces, but are killed by desiccation and temperatures greater than 68°C, so boiled water is safe. The amount of chlorine normally used to purify water is insufficient to kill cysts; higher levels of chlorine are effective, but the water thus treated must be dechlorinated before use. Houseflies and cockroaches ingest cysts present in feces and can pass them from their guts following periods as long as 24 hours.

Fecal contamination of springs, unprotected shallow wells, and streams may occur as a result of surface runoff. Contamination may also result from discharge of sewage into rivers. Occasionally, siphonage of sewage into the water supply system has been responsible for outbreaks of infection. Freshening of vegetables and fruit with contaminated water and using human excreta as fertilizer may produce heavy contamination, a particularly serious problem with vegetables and fruit that are usually eaten raw (Walsh and Martínez-Palomo 1986).

The basic means of preventing amebic infection is the improvement of living conditions and education in countries where invasive amebiasis is prevalent. The main targets should be improvements in sanitation of the environment, detection and treatment of infected persons, and health education.

The most effective preventive measure is the adequate disposal of human feces through proper drainage systems or the use of septic tanks. Purified water should be distributed through pipelines to avoid contamination. In areas where amebic infection is common, drinking water should be boiled for several minutes, or filtered or chlorinated with higher levels of chlorine that those used to eliminate bacterial contamination. People should be instructed to clean and freshen vegetables carefully with uncontaminated running water, because treatment with iodine, chlorine, or silver solutions gives unreliable results. Food handlers should be periodically checked for intestinal infection and treated if found positive. Houseflies and cockroaches should be controlled and food adequately protected from them.

Cases of invasive amebiasis require prompt chemotherapy and it was previously recommended that all asymptomatic carriers of *Entamoeba* be treated. With the development of methods to differentiate between *E. histolytica* and *E. dispar*, *E. histolytica* carriers may be identified and should be treated, whereas *E. dispar* carriers should be monitored closely for the possibility of superinfection with *E. histolytica*. Whether such a course of action will be practicable for general control of amebiasis remains to be seen.

As part of health education of the population, hygienic practices such as hand washing after defecation and before eating, boiling of drinking water, and

avoiding the consumption of raw vegetables and exposed food should be constantly reiterated in schools and healthcare units, and through periodic campaigns in the mass media.

Health personnel should be given training to improve the accuracy of their examination of stools. Doctors should be constantly reminded of the problem of amebiasis and informed of advances in diagnostic and therapeutic procedures; their active participation in prevention programs should be encouraged.

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Other intestinal amebae

JOHN P. ACKERS

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INTRODUCTION AND SCOPE

The indubitably pathogenic protozoa that inhabit the human intestine have, quite rightly, received the bulk of our attention, both in research terms and in this volume. Nevertheless, interms of numbers of persons infected, these pathogenic species are dwarfed by the unobtrusive, largely nonpathogenic and little studied organisms that are the subject of this chapter: *Entamoeba coli*, *E. gingivalis*, *E. moshkovskii*, *E. chattoni*, *E. polecki*, *Endolimax nana*, and *Iodamoeba bütschlii*.

In other chapters, the classification, immunology, epidemiology, and any progress towards the development of a vaccine are dealt with separately for each organism. So little is known about these aspects of most of the species considered here, however, that it would be tedious to repeat our ignorance for each one. These aspects are, therefore, considered briefly together before moving on to consider the clinical features of infection by each of these organisms.

CLASSIFICATION

Classically, all the *Entamoeba* are placed in one of the two great branches of the phylum Sarcomastigophora – the subphylum Sarcodina ('ameba'); and within this subphylum successively in the superclass Rhizopoda, class Lobosea, subclass Gymnamoebia, order Amoebida, and sub-order Tubulina (Levine et al. 1980) as are *Endolimax* and *Iodamoeba*. The overall classification of the protozoa is unsatisfactory and that of ameboid organisms perhaps particularly so. However, in the case of the organisms included in this chapter, molecular taxonomy seems to confirm that they are more similar to each other (albeit only distantly related) than to any other major group of eukaryotes and may legitimately be placed in the family Entamoebidae (Silberman et al. 1999). Figure 1 shows that the number of nuclei in the mature cyst, long one of the bases of microscopical diagnosis, is a taxonomically valid character (note, however, that *I. bütschlii* was not included in this analysis).

None of these organisms possess mitochondria and it had been speculated that they and some other anaerobic protozoa were examples of surviving pre-mitochondrial eukaryotes ('Archezoa'). Enough genes of mitochondrial origin have now been observed in all the well-studied members of this group to make it virtually certain that they are descended from organisms that did possess this organelle and although no investigation has been made of the organisms described here, it seems very unlikely that the same is not true of them as well.

IMMUNOLOGY

Essentially, nothing is known of human immune responses to these parasites, although their ubiquity strongly suggests that protective immunity develops only after multiple infections, if at all. This is supported by some data from the Gambia (Bray and Harris 1977), which shows that the percentage of cyst-positive feces increases with age for all the gut protozoa studied (which included *E. coli*, *Endolimax nana*, and *I. bütschlii*)

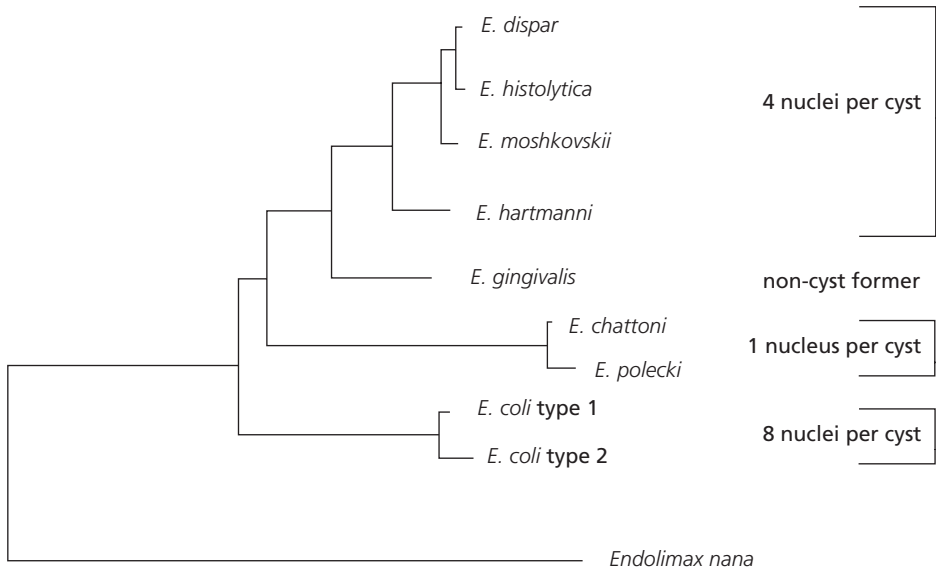

Figure 1 *Phylogenetic tree based on small sub-unit ribosomal RNA sequences, showing the relationship between the* Entamoeba *spp. discussed in this chapter and the number of nuclei in the mature cyst. (Courtesy of Dr Graham Clark, London School of Hygiene & Tropical Medicine; adapted from Silberman et al. 1999)*

except for *Giardia intestinalis*. Importantly, there is no evidence that any of these organisms can become clinically significant even in the presence of severe immunosuppression or clinical acquired immune deficiency syndrome (AIDS). It need hardly be added that there is no current work directed towards developing a vaccine against any of these organisms.

BIOCHEMISTRY AND MOLECULAR BIOLOGY

Again, there is virtually nothing known about the biochemistry of any of these organisms. All may be cultivated (with difficulty in some cases) in complex media in the presence of bacteria (Sargeaunt and Williams 1982; Diamond 1983) but only *E. moshkovskii* can currently be grown axenically (Diamond et al. 1995) and this has greatly hindered studies of metabolic pathways. The very limited amount of published molecular biological data suggests that these organisms may well share the peculiarities (such as ribosomal RNA genes carried on circular episomes) of *E. histolytica*. A comparative *Entamoeba* genome sequencing project, which includes *E. moshkovskii*, is underway at the Sanger Institute (www.sanger.ac.uk/Projects/Comp_Entamoeba/) and this will greatly increase our knowledge of at least this species.

EPIDEMIOLOGY AND LIFE-CYCLES

With the exception of *E. gingivalis* (see below), infection with all of these organisms is assumed to be acquired by oral ingestion of fecally contaminated food or drink. Although no human volunteer studies have been carried out to prove this, the epidemiology of the infections and their close similarities to *E. histolytica*/*dispar*, where this has been demonstrated, support this. The life cycle is simple: excystment occurs, possibly as with *E. histolytica* in the small bowel, and the organisms then live as trophozoites in the lumen of the large bowel. There is no evidence that any of these organisms can invade the mucosa while the host is alive although they may do so rapidly after death (Vogel et al. 1996). Responding to unknown stimuli, trophozoites round up and secrete a resistant wall, becoming the cysts which, when shed in the feces, are responsible for transmitting the infection. Nuclear division frequently occurs following encystment to produce the characteristic numbers of nuclei so important in microscopical diagnosis. Although morphologically similar cysts are found in the feces of many wild and domestic animals, with the further exception of *E. chattoni* and *E. polecki* (see below), there is no evidence that animal reservoirs are important in the epidemiology of human infections (but also no evidence that they are not).

In a fascinating study, Garrido-Gonzalez et al. (2002) measured the cyst output from a number of infected persons. The results are complex but, in summary are thus:

- cyst excretion varies greatly between patients but a small group of persons produce a majority of the cysts
- cyst excretion varies widely over time and tends to occur in bursts

- in mixed infections cyst excretion by both species is affected
- different parasites produce different average numbers of cysts with *Endolimax nana* > *E. coli* > *E. histolytica*/*dispar* .

These results, which are supported by earlier and largely forgotten studies, clearly have implications for both diagnosis and prevention strategies.

DIAGNOSIS

In anything other than research settings, diagnosis of all these organisms except *E. gingivalis* is by microscopical examination of feces and is based on cyst morphology. Detailed protocols may be obtained from standard texts (Garcia and Bruckner 1997) or from the Centers for Disease Control and Prevention (CDC) (www.dpd.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm) but, in outline, specimens should be stained with iodine and, if possible, a cyst concentrate should also be examined. Measurement of cysts is essential for accurate diagnosis, along with nuclear number and morphology.

Since it produces no cysts, diagnosis of *E. gingivalis* relies on trophozoite morphology; however, this is not distinctive (see below). As in all such cases, the trophozoites need either to be examined very shortly after the specimen is obtained or fixed immediately before being stained and examined at leisure.

Cultivation of these organisms, although not usually feasible routinely, has two advantages – it provides a (probably modest) increase in sensitivity and, more importantly, it allows much more accurate species diagnosis to be made based on methods such as isoenzymes, riboprinting and polymerase chain reaction (PCR) although this is exclusively carried out in a research setting. Protocols that allow the extraction of DNA directly from fecal samples and its use for PCR-based diagnosis of *E. histolytica* and *E. dispar* are now in routine use and could certainly be adapted for the nonpathogenic ameba if there were any demand for this.

CLINICAL ASPECTS

All of these organisms are regarded as nonpathogenic (occasional reports to the contrary are given below) and the advice from CDC is that persons infected with *E. coli*, *E. polecki*, *Endolimax nana* or *I. bütschlii* should not be treated (www.cdc.gov/ncidod/dpd/parasites/amebae/factsht_amebae.htm). Their presence is, however, evidence that close contact with fecally contaminated material has occurred and might prompt a more thorough search for pathogenic species. There seems no reason to doubt that the luminal amebicides such as paromomycin and diloxanide furoate would be effective if used. As would be anticipated, metronidazole appears to be effective in eliminating oral infection with *E. gingivalis* in the small number of reported cases where this was attempted (Sefer et al. 1989; el Azzouni and el Badry, 1994).

Clinical manifestations

ENTAMOEBA COLI

Entamoeba coli competes with *Endolimax nana* for the title of the world's commonest intestinal protozoan, with average incidence figures of 27 percent in a large number of older surveys (Levine 1973) and up to 40 percent reported in some populations; even in 1987, 4 percent of routine fecal samples taken in the US contained this parasite (Kappus et al. 1994). Apparently identical cysts have been found in the feces of many primates and, rarely, of other mammals. Typical cysts (Figure 2) are easy to identify being large (15–25 μm in diameter) and having eight smallish nuclei of one-sixth to one-eighth the cyst diameter. Peripheral chromatin is less regularly spread around the nuclear membrane than in *E. histolytica* cysts; chromidial bars, if present, have splintered rather than rounded ends. Occasionally, cysts may contain fewer than eight, or up to 16 nuclei; cyst size may be quite variable (Garrido-Gonzalez et al. 2002) and those 15 μm or less in size may be harder to distinguish from *E. histolytica*/*dispar*.

Trophozoites are usually 20–25 μm long with granular cytoplasm containing ingested bacteria, yeasts and other debris, but very rarely erythrocytes; in infected humans, trrophozoites are found in the cecum and colon. The single nucleus contains a large karyosome; blunt pseudopodia are extended in a sluggish, randomly directed manner. Although easily cultivated in bacteria-

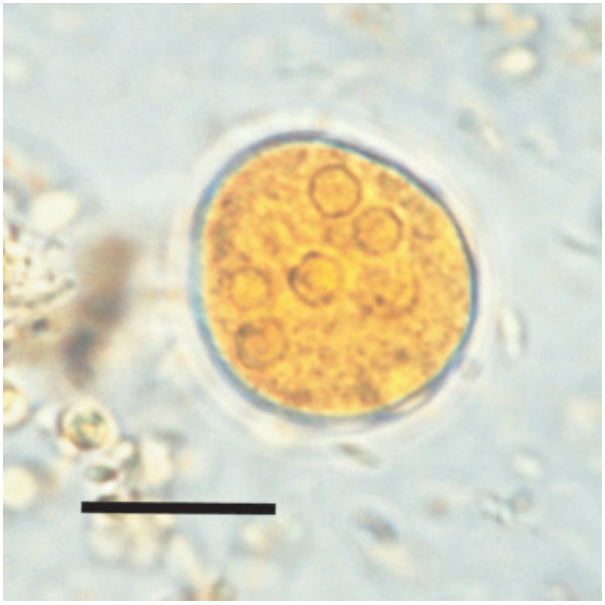

Figure 2 *Cyst of* E. coli*, wet preparation stained with iodine. Bar = 10 μm.*

containing media, very little work has been devoted to this common parasite and almost nothing is known of its cell biology. After being cultured, it may be distinguished from *E. histolytica* by isoenzyme analysis (Sargeaunt and Williams 1978), several monoclonal antibodies and riboprinting (Clark and Diamond 1992).

E. coli is normally regarded as wholly harmless, although two isolated reports (Wahlgren 1991; Corcoran et al. 1991) have suggested that it might be an occasional cause of diarrhea. In this, as in so many other cases, the evidence consists of symptomatic patients in whom no other pathogen could be identified and in whom treatment resulted in clinical cure accompanied by loss of the infection. There are a number of weaknesses with this kind of argument – however thoroughly cases of diarrhea are studied, in about 25 percent of them no pathogen can be demonstrated, and drugs such as metronidazole have a very broad spectrum of activity against a wide variety of gastrointestinal organisms. However, while *E. coli* clearly does not produce illness in the majority of those who carry it, that is not say that it cannot be pathogenic in some.

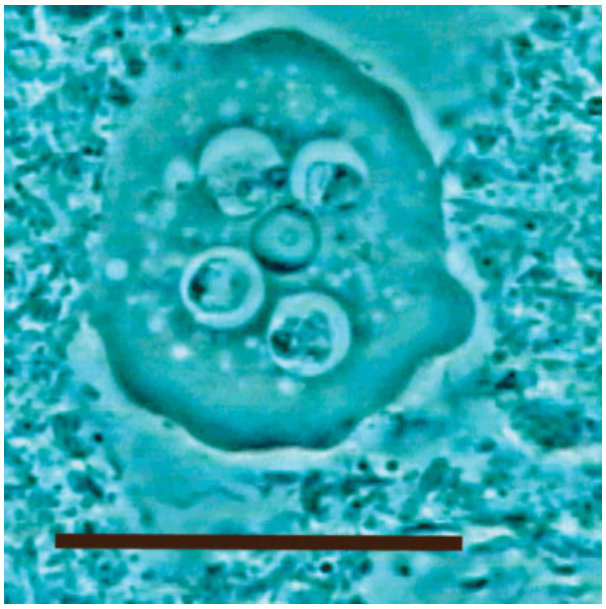

Figure 3 *Trophozoite of* E. gingivalis *in scrapings from a case of severe periodontal disease. Bar = 10 μm. (Courtesy of Dr Mark Bonner, Institut International de parodontie, Quebec)*

ENTAMOEBA GINGIVALIS

E. gingivalis is unusual among the *Entamoeba* in two respects – first, it inhabits the mouth rather than the large bowel; secondly, no cyst of *E. gingivalis* has ever been found. Transmission is presumably by direct mouth-to-mouth contact and, since the trophozoite is never exposed to desiccation or other unfavorable conditions, a resistant cyst is not needed. No selection pressure to maintain the ability to encyst therefore exists and so, like most redundant functions, it has probably been lost (Clark and Diamond 1997).

Trophozoites of this species may be recovered from the mouths of a significant percentage of patients with periodontal disease (Levine 1973; Favoreto and Machado 1995) and, although it is normally regarded as a harmless commensal that finds an ideal home among diseased gums (Pomes et al. 2000), some believe that it has a directly pathogenic role. Frequency of isolation appears to increase with age, possibly reflecting deteriorating condition of the gums and *E. gingivalis* has been implicated in the severe periodontal disease suffered by some human immunodeficiency virus (HIV)-positive patients (Lucht et al. 1998) and may even spread outside the oral cavity following radiotherapy (Perez Jaffe et al. 1998). *E. gingivalis* has also been recovered from the vaginas of a small number of women using intra-uterine devices and who have concomitant bacterial infections (deMoraes Ruehsen et al. 1980; Clark and Diamond 1992). In these cases, *E. gingivalisis* is presumed to have been acquired by orogenital contact.

The trophozoite of *E. gingivalis* (Figure 3) is about 10–20 μm long with a typical *Entamoeba* nucleus. A number of xenic media for its cultivation exist and it may be distinguished from other species by isoenzyme patterns, riboprinting, and PCR (Kikuta et al. 1996) By light microscopy, the trophozoites are not distinguishable from *E. histolytica* except in that they are frequently seen to have ingested both erythrocytes and leukocytes whereas *E. histolytica* when isolated from patients (but not in vitro) appears to ingest only the former (Dao 1985).

ENTAMOEBA MOSHKOVSKII

This organism was first isolated from the sewers of Moscow in 1941, but has subsequently been recovered from the same source in many parts of the world (Levine 1973). Its presence in sewers was a great mystery because no human excretors had ever been found. However, it is now known to be ubiquitous in both fresh and brackish anoxic environments. It may be grown in axenic culture and its optimum growth temperature is about 24°C.

Up to 1998, seven isolates of organisms (originally assumed to be *E. histolytica*) with the unusual property of being able to grow at room temperature and in hypotonic media had been recovered from asymptomatic human carriers. These organisms, referred to as atypical, *E. histolytica*-like or 'Laredo-type' (after the original isolate) are of low pathogenicity in laboratory animals, resistant to a number of drugs and differ antigenically from classical *E. histolytica* or *E. dispar* (Goldman 1969). In 1980, Sargeaunt et al. showed that the isoenzyme patterns of five such isolates were all very similar to that of two *E. moshkovskii* strains; Sargeaunt et al. (1982) and Clark and Diamond (1991) proved that the 'Laredo-type' and *E. moshkovskii* are the same species.

Both trophozoites and cysts of *E. moshkovskii* resemble those of *E. dispar*. The active trophozoites are usually 11–13 μm long; the cysts spherical, 7–17 μm in diameter and containing a large glycogen vacuole when fresh (Figure 4). The mature cysts contain four nuclei; chromidial bodies, if present, have rounded ends (Levine 1973). Unless they are cultured and their isoenzymes or other defining properties are tested, the organisms are likely to be identified as *E. dispar*. Epidemiologically, *E. moshkovskii* is presumably either a free-living species that is an occasional human parasite (apparently, it may be adapted to grow well at 37°C (Lachance 1959)) or a true human parasite which, because of its tolerance of low temperature and hypotonicity, can persist indefinitely outside its host(s). The latter possibility has been rendered much more likely by the recent and wholly unexpected finding that over 20 percent of a group of pre-school children in Bangladesh were infected with *E. moshkovskii* (Ali et al. 2003). The nested PCR diagnostic procedure developed by these authors should make it much easier to see if this situation exists in other populations.

One other Laredo-type human isolate – *E. ecuadoriensis* – is known; it possesses the same culture tolerance as *E. moshkovskii* but by both isoenzyme analysis (Sargeaunt et al. 1980) and riboprinting (Clark and Diamond 1991) is clearly distinct from all other known species of *Entamoeba*.

ENTAMOEBA POLECKI AND *ENTAMOEBA CHATTONI*

Entamoeba polecki (for which some authors prefer the name *Entamoeba suis* (Levine 1973) is a common parasite of the cecum and colon of pigs, where it apparently causes little or no pathology. The trophozoites may be 10–25 μm long (although they usually measure 12–18 μm) with vacuolated cytoplasm; the cyst (Figure 5) is usually 9–15 μm in diameter with a single nucleus even when mature. The nuclear endosome is usually quite large; glycogen granules and chromidial bodies may or may not be present (Noble and Noble 1952). The nucleus is 25–33 percent of the diameter of the whole cyst, compared with the relatively larger (33–50 percent) nucleus of the immature, uninucleate cyst of *E. histolytica*. However, the differences are subtle and, indeed, the parasites grown from one such specimen were isoenzymically indistinguishable from *E. histolytica* (Sargeaunt et al. 1980).

Entamoeba chattoni is an equally common parasite of wild primates (Levine 1973; Jackson et al. 1990), which is also found in captive animals. The cyst (Figure 6) is 6–18 μm in diameter, usually containing only one nucleus with a very variable appearance.

Both species have occasionally been reported from human feces; *E. polecki* most often in SE Asia (Desowitz and Barnish 1986; Muller et al. 1987; Barnish and Ashford 1989) or in refugees from that region (Chaker et al. 1982; DeGirolami and Kimber 1983), although it has also been reported from France (Masure et al. 1980) and Venezuela (Chacin Bonilla et al. 1992). No definite pathology has been linked to this organism. A small number of human infections with *E. chattoni* have also been described, mostly in those with occupational exposure to primates (Burrows and Klink 1955; Sargeaunt et al. 1992); some patients had diarrhea but whether or not this was due to the ameba is not clear.

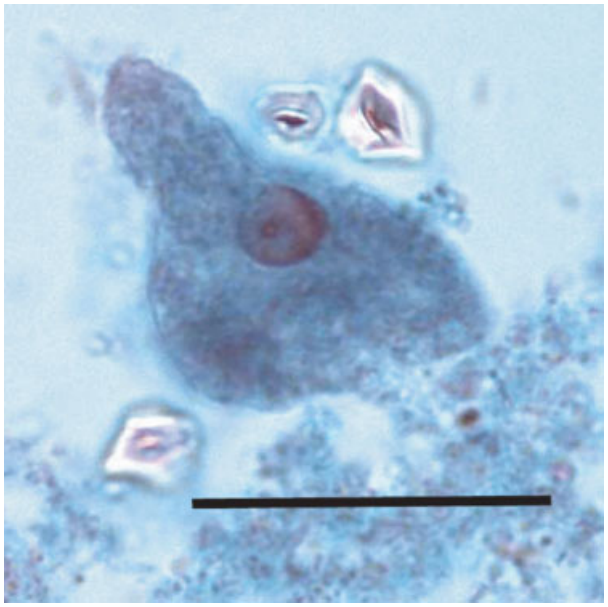

Figure 4 *Trophozoite of* E. moshkovskii *in culture. Bar = 10 μm. (Courtesy of John Williams, London School of Hygiene & Tropical Medicine)*

Figure 5 *Cyst of* E. polecki, *wet preparation stained with iodine. Bar = 10 μm. Note that these cysts and those of* E. chattoni *(Figure 6) are not morphologically distinguishable. (Courtesy of John Williams, London School of Hygiene & Tropical Medicine)*

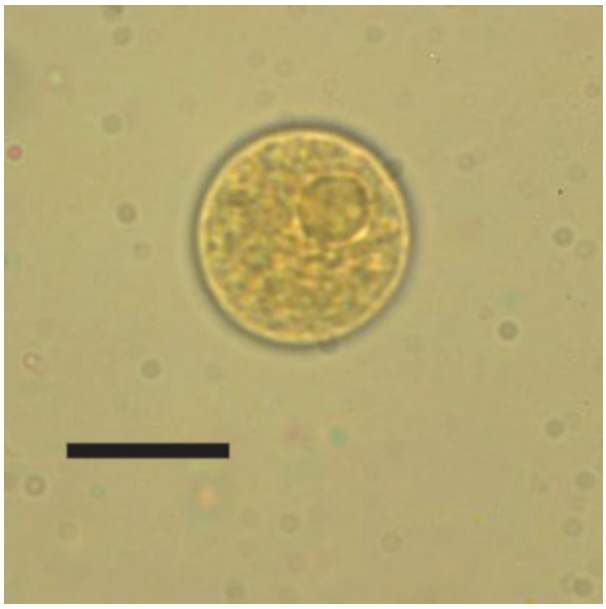

Figure 6 *Cyst of* E. chattoni*, wet preparation stained with iodine. Bar = 10 μm. (Courtesy of Dr Jaco Verweij, Centrum voor Infectieziekten, Afdeling Parasitologie, Universiteit Leiden)*

Although human infections with *E. polecki* and *E. chattoni* seem to be linked to exposure to different reservoir hosts, the cysts of both species are microscopically indistinguishable (Burrows 1959). Recently, therefore, species-specific PCR primers were developed to distinguish reliably between the two (Verweij et al. 2001). A most surprising result to emerge from this work was that, of 12 human fecal samples obtained from the Netherlands that were microscopically positive for uninucleate *Entamoeba* cysts, only one was infected with *E. polecki* and two with *E. chattoni*. The remainder were infected with two distinct *Entamoeba* species which, while clearly clustering with *E. polecki* and *E. chattoni*, were identical with neither. These results suggest that other related *Entamoeba* species can also infect humans; the authors suggest that, for the present, all such uninucleate cysts should be reported as '*E. polecki*-like.'

ENDOLIMAX NANA

Endolimax nana is a small, frequently reported ameba that is diagnosed in humans about as often as *E. coli* (Levine 1973; Kappus et al. 1994); similar if not identical parasites are also found in primates and pigs (Levine 1973). Both cysts and trophozoites possess a characteristic limax nuclear morphology that must be recognized to make an accurate diagnosis of fecal specimens. Limax nuclei have a large, irregularly-shaped mass of chromatin (the karyosome or endosome), which may be either centrally or eccentrically placed but there is no peripheral chromatin, so the nuclear membrane is all but invisible. The ring-shaped nucleus typical of *Entamoeba* and *Giardia* species is not seen; instead, wet preparations stained with iodine reveal the nuclei as dark, soft-edged specks which 'blink' into and out of view as the microscope is focused up and down.

Endolimax nana cysts may be ellipsoidal, spherical or irregular with a longest dimension of 6–10 μm, although it is usually less than 10 μm (Figure 7). They are thin-walled and almost always contain four limax-type nuclei, never more – smaller, thick-walled bodies with more than four dark nuclei are of fungal origin. Trophozoites are usually 8–10 μm long with a single limax nucleus and granular cytoplasm; they move sluggishly by extending blunt pseudopodia. The organism may be cultivated in bacteria-containing media and an isoenzyme pattern has been published (Sargeaunt and Williams 1979); otherwise nothing is known of the metabolism.

This organism is normally regarded as nonpathogenic although there are isolated reports of gastrointestinal symptoms in AIDS patients for which no other cause could be found (Rolston et al. 1986; Peters et al. 1987). Reports of *Endolimax nana* being responsible for urticaria (Veraldi et al. 1991) and rheumatism (Burnstein and Liakos 1983; Alarcon Segovia and Abud Mendoza 1985) have yet to be confirmed.

IODAMOEBA BÜTSCHLII

Trophozoites and, above all, cysts of *I. bütschlii* contain glycogen that is colored deep brown in the usual iodine-stained wet preparations and gives the genus its name. Trophozoites are usually 12–15 μm long with a single nucleus containing a large karyosome and granules visible in permanent stained preparations; the cytoplasm may be coarse with ingested bacteria and glycogen-

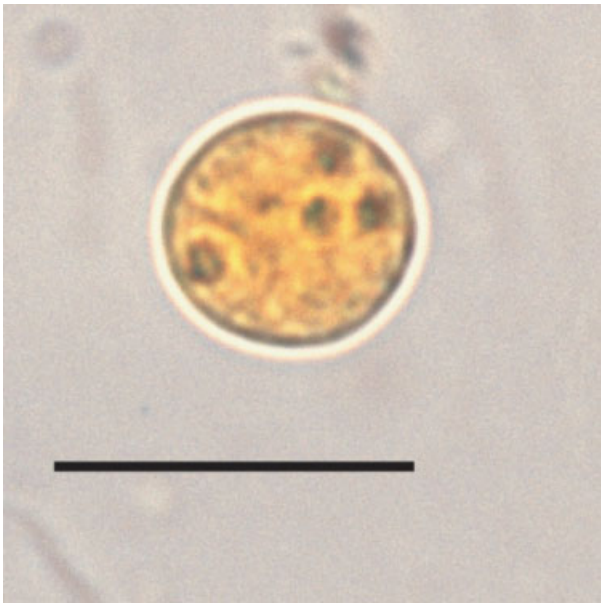

Figure 7 *Cyst of* Endolimax nana*, wet preparation stained with iodine. Bar = 10 μm. (Courtesy of John Williams, London School of Hygiene & Tropical Medicine)*

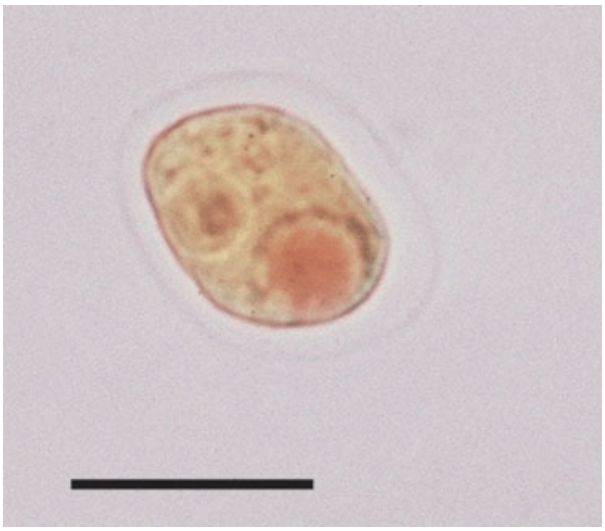

Figure 8 *Cyst of* I. bütschlii*, wet preparation stained with iodine. Bar = 10 μm. (Courtesy of John Williams, London School of Hygiene & Tropical Medicine)*

containing vacuoles. Cysts (Figure 8) are usually 9–12 μm across, often round or oval but not unusually very irregular with a single, limax-type nucleus containing a large central or eccentric karyosome. The glycogen vacuole is large and compact with a clearly defined edge; it stains deeply with iodine when the cysts are fresh but may disappear if they are stored for more than a few days. Distinction from other species is normally easy but may be difficult if immature, uninucleate *E. histolytica*/*E. dispar* cysts are present; these often contain glycogen stores, which will be used up as they mature, but this glycogen is usually present as a more diffuse, less deeply staining mass.

I. bütschlii is not rare in human feces but is usually reported in only a few percent of fecal samples from developing countries or from male homosexuals; identical cysts are found in the feces of several primates and the organism is (or was) very common in pigs all over the world. All such parasites are assigned to the same species but no evidence of human infections from animal reservoirs has been presented. The isoenzyme profile of an isolate has been published (Sargeaunt and Williams 1979).

I. bütschlii is not normally regarded as a pathogen (a disseminated infection and cerebral granuloma attributed to it were probably caused by an *Acanthamoeba* species, see Opportunistic amebae) but has occasionally been proposed as a cause of diarrhea (Andrew 1947; Rolston et al. 1986).

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Opportunistic amebae

DAVID T. JOHN

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INTRODUCTION

Free-living amebae of the genera *Naegleria*, *Acanthamoeba*, and *Balamuthia* can cause disease in humans and other animals. Normally, they live as phagotrophs in aquatic habitats where they feed on bacteria but as opportunists, they may produce serious infection of the central nervous system (CNS) and the eye. The term amphizoic (from Greek 'amphi' meaning 'on both sides') has been proposed to describe the ability of these amebae to live in two worlds, as free-living organisms and as endoparasites (Page 1974).

Naegleria fowleri is responsible for a rapidly fatal infection involving the CNS and called primary amebic meningoencephalitis (PAM). Infection occurs most often in healthy young people who have a recent history of swimming in freshwater. Several species of *Acanthamoeba* cause disease. *Acanthamoeba* may produce a chronic CNS infection known as granulomatous amebic encephalitis (GAE) or an eye infection referred to as *Acanthamoeba* keratitis. Human infections originally attributed to *Hartmannella*, another free-living ameba, were actually caused by *Acanthamoeba*. *Balamuthia mandrillaris*, a leptomyxid ameba (Visvesvara et al. 1993), causes a chronic CNS infection similar to that produced by *Acanthamoeba*, also termed GAE. Additional reports have described eye infections caused by *Hartmannella* and *Vahlkampfia* (Aitken et al. 1996; Inoue et al. 1998), a fatal CNS infection caused by *Hartmannella vermiformis* (Centeno et al. 1996) and a nonfatal encephalitis caused by *Sappinia diploidea* (Gelman et al. 2001).

CLASSIFICATION

The classification of the Protozoa is in a state of flux and, following the precedents set out elsewhere in this volume (see Classification and introduction to the parasitic protozoa), the scheme proposed by Corliss (1994) and widely adopted by scientists working with free-living protozoa is used in this chapter. On the basis of the traditional classification published by the Society of Protozoologists (Lee et al. 1985), all the opportunistic amebae were classified in the phylum Sarcomastigophora and subphylum Sarcodina, which contained two classes, class Lobosea and class Acarpomyxea. Class Lobosea contained two orders: order Amoebida (family Acanthamoebidae, e.g. *Acanthamoeba*) and order Schizopyrenida (family Valkampfidae, e.g. *Naegleria*). Class Acarpomyxea contained the order Leptomyxida (family Leptomyxidae, e.g. *Balamuthia*). In Corliss' classification both *Acanthamoeba* and *Balamuthia* are classified in the phylum Rhizopoda, class Lobosea, and *Naegleria* has been moved to the phylum Percolozoa and placed in the class Heterolobosea and order Schizopyrenida.

STRUCTURE AND LIFE-CYCLES

Morphology

The nuclei of the opportunistic amebae are characterized by a large central nucleolus or karyosome, and a nuclear membrane without chromatin granules. These features are especially useful when examining histological sections as they readily distinguish the opportunistic amebae from *Entamoeba histolytica*, the most important parasitic ameba of humans.

NAEGLERIA

The trophozoites of *N. fowleri* are known as limax amebae (from the Latin for 'slug'). These amebae are elongate and move in a directional manner by eruptive, blunt pseudopodia, called lobopodia. Actively moving amebae average about 22 μm in length (range 15–30 μm); inactive, rounded forms range from 9 to 15 μm in diameter (Carter 1970).

Trophozoites of pathogenic *Naegleria* have distinctive phagocytic structures known as amebostomes (Figure 1) (John et al. 1985). Amebostomes are used for engulfment and vary in number, depending on the strain. By transmission electron microscopy, amebostomes appear to be densely granular in contrast to the highly vacuolated body of the ameba. Amebostomes are visible by light microscopy but appear as thick-walled vacuoles. Similar food cups or phagocytic stomata occur on *E. histolytica* (Gonzalez-Robles and Martinez-Palomo 1983).

Reproduction in *Naegleria* is by simple binary fission of the trophozoite. Nuclear division is promitotic, which means that the nucleolus and the nuclear membrane persist during nuclear division (karyokinesis). The nucleolus elongates, forming a dumbbell-shaped structure, and divides into two polar masses, or nucleoli. During this process, the nuclear membrane remains intact (Page 1988).

When *Naegleria* are suspended in distilled water or non-nutrient buffer, they transform into temporary flagellated forms. The typical *N. fowleri* flagellate is a bluntly elongate cigar- or pear-shaped cell with two flagella emerging from beneath the anterior rostrum (Figure 2). Of the different species of *Naegleria*, *N. fowleri* flagellates are the most uniform, generally having two flagella (John et al. 1991).

The cysts of *N. fowleri* (Figure 3) are spherical, often clumped closely together, and are 7–15 μm in diameter (Carter 1970; Page 1988). Examination of cysts by electron microscopy reveals an average of fewer than two mucoid-plugged pores or ostioles per cyst and a relatively thin cyst wall (Schuster 1975), a feature that makes *N. fowleri* cysts susceptible to desiccation.

ACANTHAMOEBA

A feature that readily distinguishes the amebae of *Acanthamoeba* from those of *Naegleria* is the presence of acanthopodia (from Greek 'acanth-' meaing 'spine or thorn'), tapering spike-like pseudopodia (Figure 4). In contrast to *Naegleria*, which has rapid, directional locomotion, *Acanthamoeba* moves slowly on a broad front without direction. Trophozoites of *Acanthamoeba* are larger than those of *Naegleria* and average about 24–56 μm in length (Lewis and Sawyer 1979). Nuclear division in *Acanthamoeba* is metamitotic (i.e. the nucleolus and the nuclear membrane disintegrate during early karyokinesis), a pattern similar to that of dividing metazoan cells (Page 1988).

Considerable variation in cyst morphology occurs among the different species of *Acanthamoeba*, resulting in the naming of new species. Page (1967) recognized four species of *Acanthamoeba*; by 1976 he had recorded seven. On the basis of morphology and isoenzyme analysis, De Jonckheere (1987) identified 17 species of *Acanthamoeba*, of which seven have been associated with human infection. The cysts of *Acanthamoeba* are double-walled and, therefore, quite resistant in the environment. The cyst wall is made up of an outer, wrinkled or rippled ectocyst and an inner endocyst. The encysted ameba conforms to the shape of the endocyst.

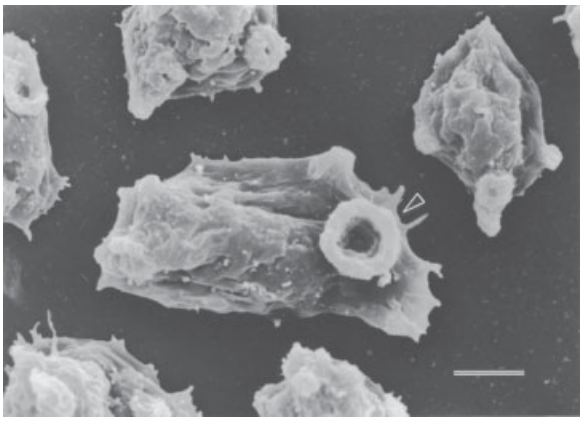

Figure 1 *Scanning electron micrograph (SEM) of a* Naegleria fowleri *ameba with a single amebostome (arrowhead). Bar = 5 μm*

Figure 2 *SEM of a* Naegleria fowleri *flagellate with two flagella emerging from beneath the anterior rostrum. Bar = 3 μm*

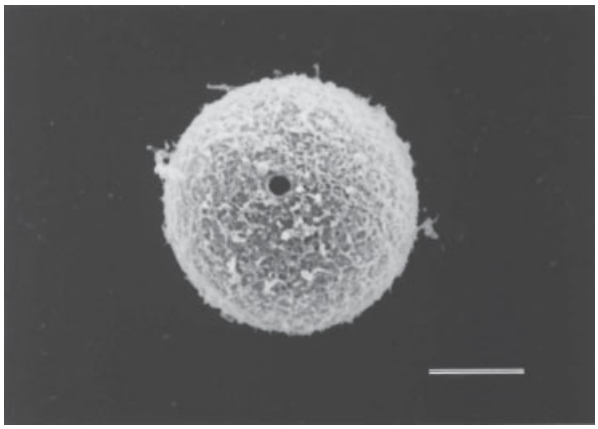

Figure 3 *SEM of a* Naegleria fowleri *cyst with a single pore or ostiole. Bar = 3 μm*

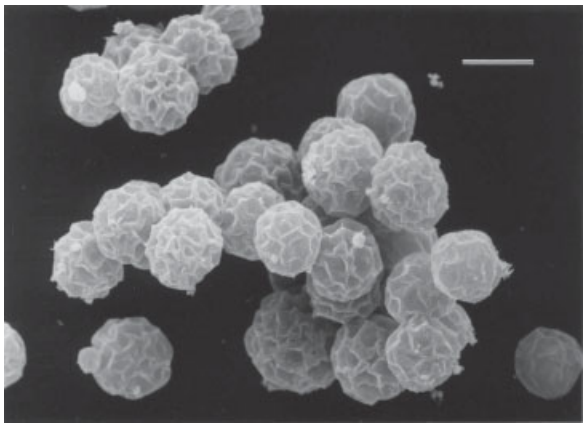

Figure 5 *SEM of pathogenic* Acanthamoeba polyphaga *cysts. Bar = 10 μm*

The characteristic wrinkled appearance of *Acanthamoeba* cysts is readily seen in culture (Figure 5) and in histological sections (see Figure 11).

BALAMUTHIA

The trophozoites of *B. mandrillaris* are mostly irregular or branching in shape (Figure 6), with some limax forms. Their length ranges from 12 to 60 μm (mean c. 30 μm) (Visvesvara et al. 1993). These amebae exhibit little directional motility, although a spider-like walking movement may be seen with amebae grown in Vero cell culture. Nuclear division is metamitotic, as in *Acanthamoeba*. Cysts are irregularly round and are 6–30 μm in diameter (mean 15 μm) with two walls. The inner cyst wall is thin and spherical and the outer cyst wall is thick and wavy or wrinkled (Figure 6) much like the cysts of *Acanthamoeba* (Visvesvara et al. 1993).

Life-cycles

Naegleria, *Acanthamoeba*, and leptomyxid amebae are distributed worldwide in freshwater and soil. *Acanthamoeba* and some leptomyxids are found in marine environments as well. Life-cycles are simple; a feeding trophozoite, or ameba, a resting cyst and, in *Naegleria*, a transient flagellate.

NAEGLERIA

The life-cycle of *N. fowleri* is illustrated in Figure 7. The term 'ameboflagellate' is used to describe amebae that can transform into flagellates. When *Naegleria* amebae are placed in a non-nutrient medium, such as distilled water or buffer, they differentiate into transient, nonfeeding, nondividing flagellates that, after a time, revert back to amebae. Amebae also encyst when conditions are appropriate and, later, excyst in a favorable environment.

The invasive stage of *N. fowleri* is the ameba and infection is acquired by exposure of the nasal passages to the amebae in fresh water. The amebae invade the nasal mucosa, the cribriform plate and the olfactory bulbs of the brain. It is likely that flagellates or cysts of

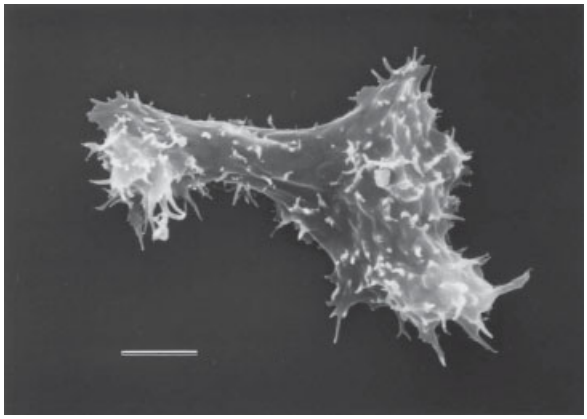

Figure 4 *SEM of a pathogenic* Acanthamoeba castellanii *ameba displaying numerous acanthopodia. Bar = 5 μm*

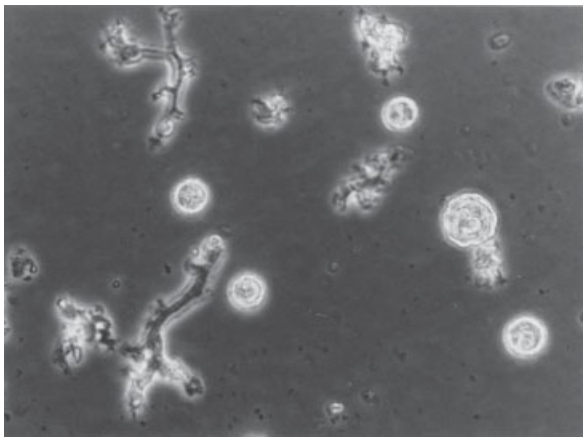

Figure 6 *Phase-contrast micrograph of* Balamuthia mandrillaris *amebae and cysts. (Courtesy Dr Govinda S. Visvesvara, Centers for Disease Control and Prevention, Atlanta, Georgia)*

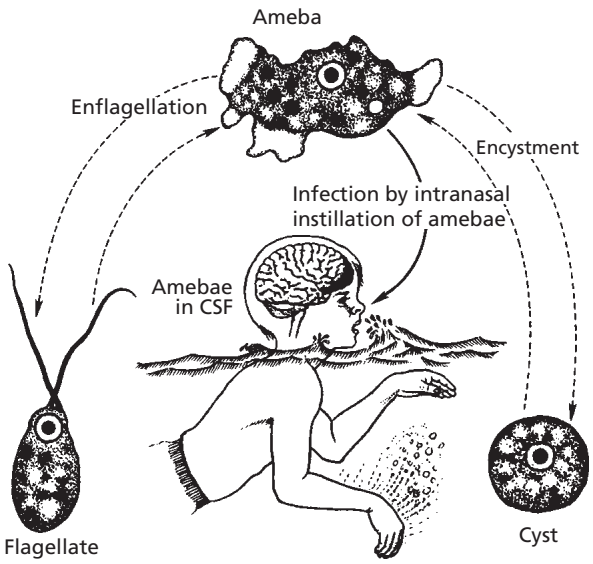

Figure 7 *Life cycle of* Naegleria fowleri *and human infection*

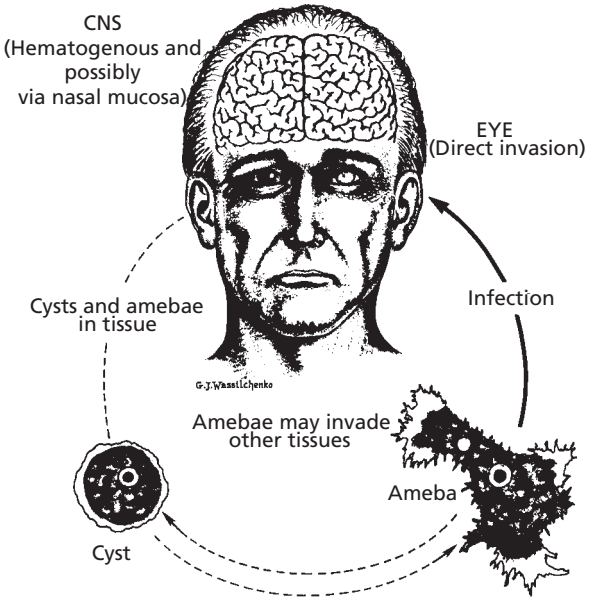

Figure 8 *Life cycle of* Acanthamoeba *spp. and human infection*

N. fowleri could enter the nose of a swimmer as readily as amebae. However, flagellates would revert quickly to amebae or the amebae could escape from cysts, the point being that amebae are the invading organisms. Flagellates or cysts of *N. fowleri* have never been found in tissue or cerebrospinal fluid.

ACANTHAMOEBA AND *BALAMUTHIA*

Figure 8 illustrates the life-cycle of *Acanthamoeba* and shows human involvement. The free-living cycle of the ameba and cyst is also reflected in human infection, in which both amebae and cysts are seen in tissue, in contrast to naeglerial infection in which only amebae occur. *Acanthamoeba* seems to be truly amphizoic in all respects. Amebae and cysts of *Balamuthia* are also found in tissue.

Human infection by *Acanthamoeba* involves the CNS, the eye and other organs. Although amebae of pathogenic *Acanthamoeba* are able to invade the nasal mucosa and cause fatal CNS disease in experimental animals, this is not thought to be the usual route of invasion in human infection. Invasion of the CNS seems to be by way of the circulation, the amebae originating from a primary focus elsewhere in the body, possibly the lower respiratory tract, ulcers of the skin or mucosa or other wounds. The modes of invasion by leptomyxid amebae are thought to be the same. GAE tends to occur in persons who are debilitated, chronically ill or immunocompromised. *Balamuthia* infections occur in both immunosuppressed and immunocompetent persons. In contrast, *Acanthamoeba* keratitis usually occurs in healthy individuals and infection is by direct invasion of the cornea through trauma to the eye or the wearing of contaminated contact lenses.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Most research on the cell and molecular biology of free-living amebae has been with nonpathogenic rather than pathogenic species. Nonetheless, some observations can be made and comparisons drawn between the two groups.

Cell differentiation

Cell differentiation is the process by which amebae become cysts or flagellates and, of course, then become amebae again. *Acanthamoeba*, *Balamuthia*, and *Naegleria* all produce cysts, but only *Naegleria* produces flagellates. Thus, free-living amebae provide useful models for studying the developmental biology and molecular mechanisms controlling morphogenesis and differentiation in eukaryotic cells.

ENCYSTMENT

Encystment is an adaptive mechanism that enables the organism to survive conditions that would kill the ameba. Factors thought to induce cyst formation include starvation, drying and various chemicals. Excystment is the process by which amebae exit from their protective cysts. It is generally held that unfavorable conditions induce encystment and that favorable conditions stimulate excystment.

There are no definitive studies that describe the conditions for encystment and excystment in *N. fowleri*. Chemical factors that induce encystment in *A. castellanii* include inhibitors of DNA synthesis – fluorodeoxy-

uridine, mitomycin C, trenimon (Neff and Neff 1972) and hydroxyurea (Rudick 1971); the mitochondrial inhibitors – diminazine (berenil), ethidium bromide, erythromycin, and chloramphenicol (Akins and Byers 1980); and acetate and glucose starvation (Byers et al. 1980). Medium from encysting cultures of *A. castellanii* contains an extracellular encystment-enhancing activity that stimulates cyst formation in early log phase cultures (Akins and Byers 1980). Encystment-enhancing activity is required for encystment in cultures induced by diminazine and by glucose starvation, but not when it is induced by total deprivation of nutrients (Akins et al. 1985).

ENFLAGELLATION

Although spontaneous enflagellation occurs in cultures of *Naegleria*, especially exponential phase cultures of *N. fowleri*, synchronized enflagellation may be achieved by suspending amebae in distilled water or non-nutrient buffer. Enflagellation in *Naegleria* apparently occurs not as an obligatory phase in the life cycle but as a response to changes in the environment. Factors affecting enflagellation in *N. fowleri* include: nutrient depletion, temperature, phase of growth, and culture agitation. Maximum enflagellation in axenically grown *N. fowleri* occurs 4–5 hours after suspension in buffer (Cable and John 1986). For axenically grown nonpathogenic *N. gruberi*, maximum enflagellation occurs somewhat sooner, c. 1.5 hours after suspension in buffer (Fulton 1977).

Cyclohexamide and actinomycin D completely prevent enflagellation in *N. fowleri* when added before enflagellation commences but, when added after initiation of enflagellation, both prevent further differentiation and cause existing flagellates to revert to amebae (Woodworth et al. 1982a). The ultrastructure of *N. fowleri* flagellates is that of a typical eukaryotic protist. There is a distinct nuclear membrane and a prominent nucleolus, numerous vacuoles and cytoplasmic inclusions, pleomorphic mitochondria and some rough endoplasmic reticulum. Basal bodies, rootlets, and flagella are formed quickly after an initial lag of 90 minutes (Patterson et al. 1981).

Macromolecular composition

Changes in cell composition of *N. fowleri* are related to culture age. For shaken axenic cultures, average cell mass remains constant during logarithmic growth at 150 pg per ameba but decreases by 30 percent during the stationary phase at 96 hours. During logarithmic growth, 80–85 percent of the cell dry mass is protein (120 pg per ameba) (Weik and John 1978). Cell dry mass and protein of *N. fowleri* are c. 70 percent of values reported for *N. gruberi* (Weik and John 1977). The majority of *N. fowleri* polypeptides have molecular masses within the range of 20–60 kDa (Woodworth et al. 1982b). During logarithmic and stationary phases of growth of *N. fowleri*, carbohydrate content averages 15 pg per ameba and RNA is c. 18 pg per ameba (Weik and John 1978). By comparison, *N. gruberi* carbohydrate averages c. 35 pg per ameba and the RNA content is 8 pg per ameba (Weik and John 1977). The more than two-fold higher RNA value for the smaller pathogenic *N. fowleri* reflects different biosynthetic capabilities and maintenance of a larger ribosome complement. Total DNA in *N. fowleri* is 0.2 pg per ameba during logarithmic growth; it doubles during transition from exponential phase to stationary phase and then gradually decreases almost to initial levels. The peak in DNA content corresponds to an increase in the average number of nuclei per ameba; nuclear number then decreases as cells enter the stationary phase (Weik and John 1978). In *N. gruberi*, the total DNA content of 0.2 pg per cell increases by 50 percent during exponential growth (Weik and John 1977).

RESPIRATORY METABOLISM

As an opportunistic pathogen, *N. fowleri* lives in the brain, an oxygen-rich environment, and would thus be expected to have an aerobic metabolism. The use of the synonym '*N. aerobia*' (Singh and Das 1970) recognized the aerobic nature of the organism, in contrast to the anaerobic nature of strictly parasitic amebae. Unlike *E. histolytica*, an anaerobic parasite that lacks mitochondria, *N. fowleri* lives in aerobic aqueous environments and has many mitochondria.

Whole-cell respiration rates were measured polarographically throughout the growth cycle of *N. fowleri* (Weik and John 1979a). In agitated cultures, amebae consume 30 ng atoms O per minute per mg of cell protein during logarithmic growth. Under similar conditions, *N. gruberi* amebae consume 80 ng atoms O per minute per mg of cell protein (Weik and John 1979b). The lower oxygen consumption, and presumably oxygen requirement, of *N. fowleri* probably explains the presence of the pathogen in heated waters in which dissolved oxygen concentrations are substantially reduced. The respiratory rate gradually declines during the stationary phase. The reduction in respiratory rate may involve respiratory control as increases in respiratory rate did not occur despite the addition of oxygen (Weik and John 1979a). The respiratory process of isolated *N. fowleri* mitochondria is similar to that of classic mammalian cell mitochondria. Oxidation is coupled to phosphorylation (adenosine triphosphate (ATP) formation) as shown by the two- to three-fold increase in respiration on addition of a phosphate acceptor or an uncoupling agent. The spectra of oxidized and dithionite-reduced mitochondria show distinct absorption bands of flavins and c-type, b-type and a-type cytochromes (Weik and John 1979a).

Genetics

It has generally been assumed that reproduction in *Acanthamoeba* and *Naegleria* is asexual and that sexuality, or genetic exchange, does not occur. In the past, genetic studies have been hampered because of difficulty in visualizing the very small, numerous chromosomes of these amebae, which may number 16 for *Naegleria* (De Jonckheere 1989) and 80 for *Acanthamoeba* (Volkonsky 1931). Fulton (1970) reported that one strain of *N. gruberi* had twice the ploidy of another strain, based on cell and nuclear volume and DNA content. Cariou and Pernin (1987) and Pernin et al. (1992) provided evidence for diploidy and genetic recombination in *N. lovaniensis*. De Jonckheere (1989) compared the electrophoretic karyotypes of all the species of *Naegleria* and concluded that, although karyotype analysis cannot be used to identify *Naegleria* species, it is useful for studying gene localization and genetic exchange. No genetic studies on the opportunistic amebae have been published. Clark and Cross (1987) described a self-replicating ribosomal DNA plasmid in *N. gruberi*, making it the third eukaryotic genus to have a nuclear plasmid DNA. Additionally, they sequenced the small-subunit rRNA gene of *N. gruberi* and showed that circular rRNA genes are a general feature of schizopyrenid (ameboflagellate) amebae, including, presumably, *N. fowleri* (Clark and Cross 1988).

CLINICAL ASPECTS

Clinical manifestations

PAM

PAM typically occurs in healthy children or young adults with a recent history of swimming in freshwater. The disease is rapidly fatal, usually resulting in death within 72 hours of the onset of symptoms. Infection follows entry of water containing amebae or flagellates into the nose. It has been suggested that inhalation of cysts (e.g. during dust storms) could lead to infection (Lawande et al. 1979). From the nasal mucosa, the amebae penetrate the cribriform plate and travel along the olfactory nerves to the brain. They invade the olfactory bulbs and then spread to the more posterior regions of the brain. Within the brain, the amebae provoke inflammation and cause extensive destruction of tissue (Carter 1970; Martinez 1985).

The clinical course is dramatic. Symptoms begin with severe frontal headache, fever (39–40°C) and anorexia followed by nausea, vomiting, and signs of meningeal irritation, frequently evidenced by a positive Kernig's sign. Involvement of the olfactory lobes may cause disturbances in smell or taste and may be noted early in the course of the disease. Visual disturbance may occur. The patient may experience confusion, irritability, and restlessness and may become irrational before lapsing into a coma. Generalized seizures may also occur. In order of frequency of occurrence, the more important symptoms are (Carter 1970; Martinez 1985):

- headache
- anorexia
- nausea
- vomiting
- fever
- neck stiffness.

GAE

GAE usually occurs in debilitated or chronically ill persons, some of whom may be undergoing immunosuppressive therapy. Infection by *Balamuthia* may occur in the immunocompetent. The underlying conditions reported in GAE are Hodgkin's disease, systemic lupus erythematosus, diabetes mellitus, glucose-6-phosphate dehydrogenase deficiency, alcoholism, and AIDS. Some of the victims of GAE are not debilitated or immunocompromised, but are otherwise healthy individuals. GAE is a disease not as well defined as that caused by *N. fowleri*. The course of infection is subacute or chronic, lasting weeks, months or even years, and is characterized by focal granulomatous lesions of the brain. The onset of GAE, unlike that of PAM, is insidious with a prolonged clinical course (Carter et al. 1981; Martinez 1987).

Acanthamoeba infection probably occurs through the lower respiratory tract or through ulcers of the skin or mucosa. Invasion into the CNS is by hematogenous spread from the primary focus of infection. As there are no lymphatic channels in the brain, invasion of the brain must be via the bloodstream (Martinez 1987). Even though some *Acanthamoeba* isolates are able to produce a CNS infection after intranasal instillation in mice, there is no proof that similar invasion occurs in the human disease. The portal of entry and spread in *Balamuthia* infection is believed to be the same as that for *Acanthamoeba*. The incubation period is not known, but probably lasts weeks or months and, during the prolonged clinical course, single or multiple space-occupying lesions develop. An altered mental state is a prominent feature in GAE. Headache, seizures, and neck stiffness occur in about half of the cases. Nausea and vomiting may also be noted (Martinez 1987).

ACANTHAMOEBA KERATITIS

Acanthamoeba keratitis is a chronic infection of the cornea caused by several species of *Acanthamoeba* including *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. polyphaga*, and *A. rhysodes* and infections are being diagnosed with increasing frequency (Stehr-Green et al. 1989). Infection is by direct contact of the cornea with amebae, which may be introduced through minor

corneal trauma or by exposure to contaminated water or contact lenses. The wearing of contact lenses and the use of home-made saline solutions are important risk factors. Saline solutions contaminated with protein residues from contact lenses promote the growth of bacteria and yeast which, in turn, are a source of food for the amebae. Amebae attach to the contact lenses stored in contaminated solutions and are transferred to the eye when lenses are placed over the cornea. Amebae become established as part of the conjunctival flora and may invade the corneal stroma through a break in the epithelium or through the intact epithelium. They produce an infection that progresses to *Acanthamoeba* keratitis, which usually develops over a period of weeks to months, and is characterized by:

- severe ocular pain (often out of proportion to the degree of inflammation)
- affected vision
- a stromal infiltrate that is frequently ring-shaped and composed predominantly of neutrophils.

Acanthamoeba keratitis is a serious ocular infection which, if not properly managed, can lead to loss of vision and even loss of the eye.

IMMUNOLOGY

The factors responsible for susceptibility and innate resistance to infection by pathogenic free-living amebae are undefined. Relatively few human infections have occurred, even though large numbers of individuals must have been exposed to amebae. Most cases of PAM occur in otherwise healthy children or young adults. *Acanthamoeba* keratitis also generally occurs in healthy individuals. In contrast, GAE usually occurs in persons who are chronically ill, debilitated or immunosuppressed, although *Balamuthia* infections may occur in the immunocompetent.

Antibodies to opportunistic amebae have been detected in human sera and include agglutinating antibodies against pathogenic and nonpathogenic *Naegleria* (Marciano-Cabral et al. 1987; Reilly et al. 1983). The agglutinating activity was specific for each species, indicating exposure to each of the *Naegleria* species. Antibodies to both pathogenic and nonpathogenic *Acanthamoeba* and *Naegleria* were detected in normal human sera by indirect fluorescent antibody testing of 93 serum samples. All were positive, with titers ranging from 1:5 to 1:80 (Cursons et al. 1980). An avidin-biotin horseradish peroxidase assay has been used to detect antibodies to pathogenic and nonpathogenic *Naegleria* in samples of human serum (Dubray et al. 1987). Antibodies were detected in 88 percent of sera from 115 hospital patients. Antibodies were identified as immunoglobulin (Ig)G and IgM, with IgG antibody titers ranging from 1:20 to 1:640. Radioimmunoassay detected antibody to *N. fowleri* in a pool of normal human sera (Tew et al. 1977). Antibody titers were nearly nine times greater against intracellular antigens than against cell surface antigens. The apparent widespread occurrence of antibodies to pathogenic free-living amebae in human sera may reflect the global distribution of these organisms. Alternatively, it may represent cross-reacting antibodies to antigens that have yet to be identified.

Serum from a victim of PAM in New Zealand was reported to have a low level of serum IgA, although IgG and IgM levels were normal (Cursons et al. 1979). In contrast, normal serum levels of IgA were found in a child who died of naeglerial infection in England (Cain et al. 1979). Because serum IgA levels may not be an accurate reflection of secretory IgA concentrations, both patients may have had deficient levels of secretory IgA. As *N. fowleri* invades the nasal mucosa, it seems reasonable to suggest that secretory IgA may play a role in protection. Except for the serological surveys described above, all other information on immune responses to pathogenic free-living amebae has come from experimental laboratory studies, mostly with animals (reviewed by John 1993).

PATHOLOGY

Pathogenicity

Pathogenicity is the ability of a microorganism to produce disease, whereas cytopathogenicity is the ability to produce pathologic change in cells or a cytopathic effect in vitro in cultured cells. The proposed mechanisms of cytopathogenicity for *N. fowleri* include phagocytosis, release of cytolytic substances and the presence of a biologically active component, *Naegleria* ameba cytopathogenic material (NACM).

Phagocytosis is a basic function of amebae. It causes the destruction of cells, whether in cell culture or in human tissue and is, therefore, intimately involved in pathogenesis. Brown (1979) called the piecemeal engulfment of mouse embryo cells by *N. fowleri* trogocytosis (from the Greek for 'to nibble'). It is now known that trogocytosis is accomplished by amebostomes (John et al. 1985) that the amebae use to engulf particles of various sizes, including cultivated mammalian cells. In addition to phagocytosis and trogocytosis, *N. fowleri* seems to injure cultivated mammalian cells by another contact-mediated means. Marciano-Cabral et al. (1990) reported cytolytic factors on the membranes of amebae that lyse B103 rat nerve cells on contact. Lysis is followed by engulfment of cellular debris. Cytopathic activity was enhanced by the divalent cations calcium or magnesium.

Various phospholipases (phospholipase A, lysophospholipase, sphingomyelinase) have been identified in culture media in which *N. fowleri* is grown (Cursons et al. 1978; Hysmith and Franson 1982). The phospholipase activities from cultures of pathogenic *Naegleria* are

much greater than from cultures of nonpathogenic *Naegleria*. Phospholipases have also been reported to be present in media in which *A. culbertsoni* is grown. The amounts are greater in pathogenic *A. culbertsoni* cultures than in those of nonpathogenic *A. castellanii* (Cursons et al. 1978). Other enzymes, some with a possible role in pathogenesis, occur in *N. fowleri* extracts. These are various hydrolases, including acid phosphatase, several glycosidases, and elastase.

A cytopathic effect has been attributed to NACM, obtained from lysed amebae (Dunnebacke and Dixon 1989). NACM is isolated from lysates of *Naegleria* by centrifugation, filtration, and lyophilization and has been obtained from *N. fowleri*, *N. gruberi*, and *N. jadini*, but not from *Acanthamoeba*. NACM kills cells of a variety of avian and mammalian cell lines. After cultures are inoculated, there is a long latent period (4–10 days) followed by a short period (under 24 hours) during which the monolayer is destroyed. The cytopathic effect has been maintained in cell cultures through nine serial passages. NACM is a protein with a molecular mass of 36 000 kDa and an isoelectric point of pH 4.2. Monoclonal antibodies to NACM prevent its cytopathic activity. Fluorescent staining shows NACM to be located at the tips of the ameba's pseudopodia and in the peripheral cytoplasm. It forms ring-shaped structures resembling amebostomes (Dunnebacke and Dixon 1989).

PAM

The gross pathologic findings in PAM are remarkably constant. The cerebral hemispheres are usually edematous and swollen, meninges are diffusely hyperemic with a slight purulent exudate and the cortex contains many focal superficial hemorrhages. There is severe involvement of the olfactory bulbs, with hemorrhage, necrosis, and purulent exudate (Carter 1972; Martinez 1985).

Microscopic examination reveals many amebae in the subarachnoid and perivascular spaces. Presumably, the perivascular spaces provide a path of migration for the amebae, and the blood vessels supply the oxygen needed by these aerobic organisms. Small numbers of amebae are found clustered within the brain tissue and in the purulent exudate of the meninges and brain substance. Within the exudate, some amebae may be seen engulfed by macrophages. Many amebae contain phagocytosed cellular debris and erythrocytes. The purulent exudate contains numerous polymorphonuclear and mononuclear leukocytes (Carter 1972; Martinez 1985). Figure 9 shows *N. fowleri* amebae with engulfed erythrocytes in brain tissue; cysts do not occur in tissue.

The cortical gray matter is a preferred site for the ameba's development. Consequently, severe involvement occurs in the cerebral hemispheres, cerebellum, brain stem, and upper portions of the spinal cord. Encephalitis may be a result of light amebic invasion and inflammation or massive invasion with purulent, hemorrhagic necrosis. Typically, the olfactory bulbs are extensively invaded, with hemorrhage and an inflammatory exudate; the involvement here is greater than in other areas of the brain. Infection of the central nervous system with *N. fowleri* may be described best as an acute, hemorrhagic, necrotizing meningoencephalitis (Carter 1972; Martinez 1985). Focal demyelination in the white matter of the brain and spinal cord may occur (Chang 1979; Duma et al. 1971). Curiously, demyelination may occur in the absence of amebae or cellular infiltrate. Chang (1979) suggests that demyelination may be caused by a phospholipolytic enzyme or enzyme-like substance produced by actively growing amebae present in the adjacent gray matter.

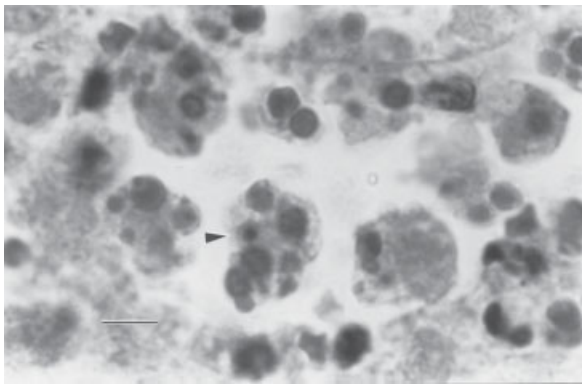

Figure 9 *Histological section of brain with* Naegleria fowleri *amebae containing engulfed erythrocytes (H&E stain). Prominent nucleolus visible within nucleus of one ameba (arrowhead). Bar = 10 μm*

GAE

In contrast to naeglerial infection, which is characterized by a diffuse meningoencephalitis, *Acanthamoeba* and *Balamuthia* CNS disease is a focal granulomatous encephalitis. Martinez (1980) gave a summary of the neuropathological features for 15 patients with GAE. In affected areas, the leptomeninges contain a moderate amount of purulent exudate. The cerebral hemispheres show moderate or severe edema with foci of softened tissue and associated hemorrhagic necrosis. Lesions are usually multifocal and more posterior, including the upper portion of the spinal cord. The olfactory bulbs are not usually involved. Lesions of the CNS in GAE are characterized by necrosis with hemorrhagic foci and localized leptomeningitis. The chronic inflammatory exudate over the cortex comprises mostly mononuclear cells with a few polymorphonuclear leukocytes. The brain substance may have a prominent granulomatous reaction with foreign-body giant cells, which are never seen in naeglerial infection. The multinucleated giant cells may not be present in immunosuppressed patients (Carter et al. 1981; Martinez 1987).

Amebae reach the brain via the bloodstream, invasion of the CNS is thus centrifugal, from the deeper tissues

toward the brain surface. Trophozoites and cysts (Figures 10 and 11) occur in most infected tissues and around blood vessels. *Acanthamoeba* and *Balamuthia* reach the CNS by hematogenous spread from a primary focus of infection elsewhere in the body, most probably the skin, mucosa or lungs. Within the infected primary tissues, there occurs a chronic granulomatous reaction like that seen in the brain, with multinucleated giant cells, trophozoites, and cysts. Similar lesions have been described from other tissues including prostate, thyroid, uterus, and pancreas, probably resulting from hematogenous dissemination of amebae from the primary focus in the skin or lungs, or possibly even a secondary CNS lesion (Martinez 1987).

ACANTHAMOEBA KERATITIS

Ocular infections with *Acanthamoeba* are characterized by chronic progressive ulcerative keratitis (Cohen et al. 1985). During early corneal infection, there may be pseudodendritic figures in the epithelium or just beneath the epithelium in the anterior stroma (Johns et al. 1987). In advanced cases of *Acanthamoeba* keratitis, there may be a marked stromal infiltrate and necrosis. The whitish inflammatory infiltrate, often appearing ring-shaped around the corneal ulcer, consists mainly of polymorphonuclear leukocytes and macrophages, with a few lymphocytes (Mathers et al. 1987). Although granulomatous inflammation has been described in *Acanthamoeba* keratitis, in most of the reports, neutrophils (not lymphocytes) are the predominant infiltrating cells. Corneal ulceration may progress to perforation (Lindquist et al. 1988).

DIAGNOSIS

Laboratory diagnosis

The laboratory diagnosis of infection by the opportunistic amebae depends on the recovery and identification of amebae in cerebrospinal fluid (CSF), brain tissue or corneal scrapings. Amebae in a clinical specimen may also be cultivated on non-nutrient agar spread with gram-negative bacteria and later transferred to liquid medium with antibiotics for axenic growth.

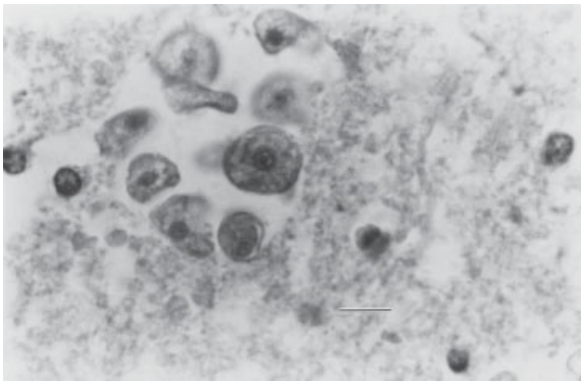

Figure 10 *Histological section of brain with* Acanthamoeba castellanii *amebae (H&E stain). Bar = 10 μm*

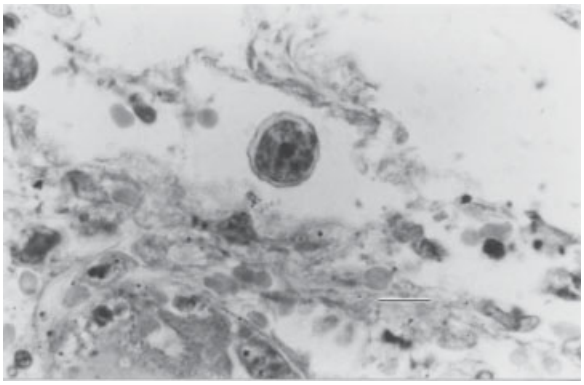

Figure 11 *Histological section of brain with* Acanthamoeba castellanii *cyst exhibiting typical wrinkled ectocyst (H&E stain). Bar = 10 μm*

PAM

The diagnosis of PAM is made by microscopic identification of living or stained amebae in CSF; motile amebae are readily seen in simple wet-mount preparations. Amebae can be distinguished from other cells by their limax shape and progressive movement. It is not necessary to warm the slide as amebae remain fully active at room temperature. Refrigeration of the spinal fluid is not recommended because this may kill the amebae.

Spinal fluid smears may be stained with Wright or Giemsa stains. The bacterial Gram stain is of little value because heat fixing destroys the amebae, which causes them to stain poorly and appear as degenerating cells. Giemsa- or Wright-stained amebae have considerable amounts of sky-blue cytoplasm and relatively small, delicate, pink nuclei. Mononuclear leukocytes, on the other hand, have large purplish nuclei with only a small amount of sky-blue cytoplasm. In cytospin preparations of CSF, the amebae tend to be rounded and flattened, without pseudopodia. Occasionally, enlarged teardrop-shaped food vacuoles appear to radiate from the nucleus but this is an artefact induced by the cytospin procedure (Benson et al. 1985). Amebae may be cultivated by placing some of the CSF on non-nutrient agar (1.5 percent) spread with a lawn of washed *Escherichia coli* or *Enterobacter aerogenes* and incubated at 37°C. The amebae grow on the moist agar surface and use the bacteria as food, producing plaques as they clear the bacteria.

Clinically, PAM closely resembles fulminating bacterial meningitis, and the laboratory findings are also similar. The CSF is purulent or sanguinopurulent, with leukocyte counts (predominantly neutrophils) ranging from a few hundred to more than 20 000 cells mm^{-3}.

Spinal fluid glucose levels are low and protein content is generally increased. Typically, Gram-stained smears and cultures of spinal fluid are negative for bacteria (Carter 1972; Martinez 1985).

GAE

The laboratory diagnosis of GAE is made by identifying amebic forms of *Acanthamoeba* or *Balamuthia* in the CSF, or amebae and cysts in brain tissue. Whereas *N. fowleri* is readily cultured from CSF, *Acanthamoeba* and *Balamuthia* are not. *Acanthamoeba* has only rarely been isolated from patients with GAE. *A. culbertsoni* and *A. rhysodes* have been cultured from CSF, and *Acanthamoeba* sp. and *A. palestinensis* have been cultured from aspirated and biopsied brain material, respectively. Leptomyxid amebae have been cultivated twice from biopsy material (Visvesvara et al. 1990; Gordon et al. 1992).

As with *N. fowleri*, *Acanthamoeba* may be cultured on non-nutrient agar spread with washed *E. coli* or *E. aerogenes*. *Balamuthia* must be grown in tissue culture, preferably Vero cell (Visvesvara et al. 1993). *Acanthamoeba* and *Balamuthia* do not have a flagellate stage but amebae are identified by their small spiky acanthopodia or branching shape, respectively, and cysts are readily identified by their distinctive double-walled wrinkled appearance. Species identification may be made by using the indirect fluorescent antibody technique (IFAT) and specific antisera against *Acanthamoeba* spp. or *B. mandrillaris*. The species of *Acanthamoeba* identified most frequently from cases of GAE have been *A. castellanii* and *A. culbertsoni*.

ACANTHAMOEBA KERATITIS

Acanthamoeba keratitis is diagnosed by identifying amebae cultured from corneal scrapings or by histological examination of infected corneal tissue. As in GAE, *Acanthamoeba* may be cultured from corneal scrapings on non-nutrient agar spread with gram-negative bacteria. Cultures of corneal material should be incubated at 30°C rather than 37°C. Of the five species of *Acanthamoeba* identified as capable of causing eye infections, only *A. hatchetti* has not been cultured from clinical material. Species identification is based on indirect immunofluorescent antibody staining. The two species most frequently identified in *Acanthamoeba* keratitis have been *A. castellanii* and *A. polyphaga*. *A. castellanii* is the species that has most often been identified in cases of *Acanthamoeba* GAE and ocular infection.

Rapid diagnosis of *Acanthamoeba* keratitis may be made by identifying amebae or cysts in corneal scrapings using procedures for Giemsa staining, calcofluor white staining (Wilhelmus et al. 1986), and IFAT (Epstein et al. 1986). The calcofluor white procedure and IFAT both require the use of fluorescence microscopy and IFAT also requires an antiserum to *Acanthamoeba*.

Histopathological preparations of corneal tissue may be stained using the conventional hematoxylin and eosin (H&E) procedure or by the more specialized staining procedures of Heidenhain's hematoxylin, Gomori's chromium hematoxylin, periodic acid–Schiff, Bauer chromic acid-Schiff, and silver methenamine (McClellan et al. 1988). The special staining techniques are useful for demonstrating the presence of cysts in corneal tissue. IFAT and calcofluor white staining also may be used (Silvany et al. 1987).

Herpes simplex keratitis is the disease most commonly mistaken for *Acanthamoeba* keratitis (Johns et al. 1987; Mannis et al. 1986; Moore and McCulley 1989). The single most consistent clinical symptom of *Acanthamoeba* keratitis is severe ocular pain, which is not characteristic of an infection limited to the cornea and generally not present in persons with herpes simplex keratitis. Additional distinguishing features of *Acanthamoeba* keratitis include a history of direct exposure to soil or water, wearing contact lenses, scleritis and failure of cultures from the inflamed eye to reveal bacteria, fungi or viruses (Mannis et al. 1986).

EPIDEMIOLOGY

Literature review

Human infection by *Naegleria* and *Acanthamoeba* has been reported worldwide, as has the environmental isolation of these opportunistic amebae (reviewed by John 1993). Although leptomyxid amebae have been identified as a cause of human disease (Visvesvara et al. 1990; Lowichik et al. 1995) and have been cultivated twice from tissue (Visvesvara et al. 1990; Gordon et al. 1992), the environmental isolation of pathogenic leptomyxid amebae has been reported only once (John and Howard 1995).

PAM

Most of the reports of PAM have been from the developed rather than the developing nations, probably because of greater awareness rather than greater incidence. Australia, Czechoslovakia, and the USA have reported 75 percent of all cases of PAM. In the USA, most reported cases have been from the coastal states of Virginia, Florida, and Texas, accounting for 67 percent of the cases.

The majority of patients with naeglerial infection have had a history of recent swimming in freshwater during hot summer weather. In Richmond, Virginia, infection in 14 of 16 cases was probably acquired in two man-made lakes located within a few miles of each other (Callicott Jnr 1968; Duma et al. 1971; dos Santos 1970). Over a 3-year period, 16 young people died in Czechoslovakia after swimming in the same heated and chlorinated

indoor swimming pool (Červa et al. 1968). Similar fatal cases have been reported following swimming in:

- swimming pools in Belgium, England, and New Zealand
- hot springs in California and New Zealand
- lakes in Arkansas, Florida, Missouri, Nevada, South Carolina, and Texas
- streams in Belgium, Mississippi, and New Zealand
- an irrigation ditch in Mexico.

Individual references for the above cases may be found in John (1993).

Infection has not always been acquired by swimming. The South Australian and northern Nigerian cases occurred in arid regions where swimming is unusual; the proposed means of infection were bathing and face washing (Anderson and Jamieson 1972; Lawande et al. 1980) or inhalation of dust-borne cysts (Lawande et al. 1979).

N. fowleri has been isolated from a variety of environmental sources in Africa, Asia, Australia, New Zealand, Europe, and North and South America, with 33 references describing 211 isolates (for specific references, see John 1993; John and Howard 1995). *N. fowleri* is more readily isolated from warm, clean water than from warm, organically enriched water. The average water temperature has been c. 30°C (range 15–45°C). Although laboratory animals may succumb to naeglerial infection, there is no evidence for an animal reservoir of *N. fowleri*.

GAE

There have been even fewer cases of *Acanthamoeba* CNS infection than of *Naegleria* infection. More than half of these have been reported from the USA. Unlike naeglerial infection, GAE is not associated with swimming. Infections often occur in persons who are debilitated or immunosuppressed including patients with acquired immunodeficiency syndrome (AIDS). In one of the AIDS patients, the clinical features were more like those of PAM than of GAE, presumably because of immunosuppression (Wiley et al. 1987). With the current increase in the number of immunosuppressed persons, it is likely that there will be a corresponding increase in disseminated *Acanthamoeba* infections.

GAE caused by the leptomyxid, *B. mandrillaris*, was first described in a pregnant mandrill baboon that died of meningoencephalitis at the San Diego Zoo (Visvesvara et al. 1990). Amebae were cultivated from brain tissue and used to prepare immune serum for indirect immunofluorescence assays; these revealed 16 human cases of leptomyxid infection originally identified as *Acanthamoeba* infections. Leptomyxid infections were also identified in a sheep, a horse, and a gorilla. Since the original description, leptomyxid infections continue to be reported in immunocompetent and immunosuppressed humans, including patients with AIDS.

At the time of writing, the world literature contains 26 references that describe the environmental isolation of 144 pathogenic *Acanthamoeba* isolates (for specific references see John 1993; John and Howard 1995). These have been recovered from a variety of sources at somewhat lower average temperatures than for the isolation of *N. fowleri* (below 25°C). Only one report describes the environmental recovery of pathogenic leptomyxid isolates and these do not appear to be *Balamuthia* (John and Howard 1995). As with *N. fowleri*, there is no evidence for an animal reservoir of pathogenic *Acanthamoeba* or *Balamuthia*.

ACANTHAMOEBA KERATITIS

The first cases of *Acanthamoeba* keratitis were reported from the UK in 1974 (Nagington and Watson 1974) and the USA in 1975 (Jones et al. 1975) and were associated with trauma to the eye or exposure to contaminated water. Since 1985, there has been a dramatic increase in the number of cases associated with the wearing of contact lenses; the greatest increase occurred in wearers of soft contact lenses (Stehr-Green et al. 1989). The continued increase in the number of contact lens wearers, and of those not properly caring for their lenses, will undoubtedly result in an increase in the incidence of *Acanthamoeba* keratitis, mainly in the developed nations where the wearing of contact lenses is widespread.

CHEMOTHERAPY

Overview

Although *Acanthamoeba* keratitis may be treated with antimicrobial agents, virtually all cases of PAM and GAE have been fatal because there is no effective treatment.

PAM

At present, there is no satisfactory treatment for PAM. The antibiotics used to treat bacterial meningitis are ineffective in naeglerial infection, as are the antiamebic drugs. Amphotericin B, a drug of considerable toxicity, is the antinaeglerial agent for which there is evidence of clinical effectiveness. The four known survivors of PAM, children from Australia (Anderson and Jamieson 1972), the UK (Apley et al. 1970), India (Pan and Ghosh 1971), and the USA (Seidel et al. 1982), were treated with amphotericin B, given intravenously and intrathecally. The patient in the USA was also given parenteral miconazole and oral rifampicin (Seidel et al. 1982). In experimental infections, tetracycline acts synergistically with amphotericin B to protect mice (Thong et al. 1979). Goswick and Brenner (2003) describe the effective treatment of experimental PAM in mice with azithromycin. Treatment was initiated 72 hours after infection and was

continued for 5 days, with protection being 100 percent and greater than for amphotericin B-treated mice. Interestingly, the patient who recovered from *Sappinia diploidea* encephalitis also was treated with azithromycin (Gelman et al. 2001).

Amphotericin B is administered intravenously at high doses; 1–15 mg/kg of body weight daily for 3 days and then 1 mg/kg per day for 6 days. Additionally, amphotericin B may be given intrathecally and miconazole is administered intravenously (Carter 1972; Seidel et al. 1982). Amphotericin B is a polyene compound that acts on the plasma membrane, disrupting its selective permeability and causing leakage of cellular components. On exposure to amphotericin B, amebae round up and fail to form pseudopodia. Membrane-related changes, evident by electron microscopy, include enhanced nuclear plasticity, increased amounts of smooth and rough endoplasmic reticulum, decreased food vacuole formation and production of blebs on the plasma membrane (Schuster and Rechthand 1975).

GAE

As with naeglerial infection, there is no satisfactory treatment for GAE, partly because most cases have been diagnosed after death and there has not been adequate opportunity to evaluate therapeutic regimens. There are three reports of persons having recovered from *Acanthamoeba* CNS infection. A 7-year-old girl with a single *Acanthamoeba*-induced granulomatous brain tumor recovered following total excision of the mass and treatment with ketoconazole. *A. palestinensis* was cultured from the brain biopsy material (Ofori-Kwakye et al. 1986). The second report involved a 40-year-old man with *Acanthamoeba* meningitis who recovered following treatment with penicillin and chloramphenicol. *A. culbertsoni* was repeatedly cultured from the patient's CSF (Lalitha et al. 1985). The third case, for whom complete recovery cannot be claimed because the patient returned home and was not followed-up, was a 30-year-old man with chronic meningoencephalitis who was treated with sulfamethazine and from whose CSF *A. rhysodes* was cultured (Cleland et al. 1982).

Because the cysts of *Acanthamoeba* and *Balamuthia* form in tissues, a potentially effective drug for GAE must be able to destroy cysts as well as amebae. Otherwise, a possible relapse could occur after the course of treatment has ended.

ACANTHAMOEBA KERATITIS

Most of the earlier cases of *Acanthamoeba* keratitis required corneal transplants in order to manage the disease. Even so, there were reported instances of surgical enucleation. With present therapies, *Acanthamoeba* keratitis can be managed by medical treatment alone if infection is identified soon enough (Moore and McCulley 1989). The first successful medical cure of *Acanthamoeba* keratitis was reported by Wright et al. (1985) and involved the use of a combination of dibromopropamidine and propamidine isethionate ointment and drops and neomycin drops. The success of this treatment has been confirmed by others. Signs of toxicity of propamidine and dibromopropamidine have been reported in one patient (Yeoh et al. 1987), but when treatment was discontinued, there seemed to be a recurrence of the *Acanthamoeba* keratitis.

In addition to topical propamidine, other successful treatment regimens have used:

- topical miconazole and systemic ketoconazole (Wilhelmus et al. 1986)
- topical miconazole and neosporin with epithelial debridement (Lindquist et al. 1988)
- topical clotrimazole (Driebe Jnr et al. 1988)
- oral itraconazole with topical miconazole and surgical debridement (Ishibashi et al. 1990)
- topical polyhexamethylene biguanide (Larkin et al. 1992).

PREVENTION AND CONTROL

Vaccination

There is no vaccine to protect against infection by the opportunistic amebae. Numerous attempts have been made to immunize mice against infection by *N. fowleri* or *Acanthamoeba*, without producing solid immunity (for details of the studies, see John (1993)).

Integrated control

Because of the relationship of swimming to naeglerial infection, many swimming areas have been subjected to intense investigation. Although *N. fowleri* has been isolated from many such areas, not all sampling efforts have yielded *N. fowleri*. Obviously, there are factors that favor the development of *N. fowleri* in swimming areas, including a warm temperature, the presence of an adequate food supply, insufficient residual free chlorine, minimal competition from other protozoans and probably optimal pH and oxygen levels. With the present limited understanding of the ecology of *N. fowleri*, practical measures for the prevention and control of the infection include education of the public, awareness within the medical community and adequate chlorination of public water supplies, including swimming facilities. Adequate chlorination requires a continuous free residual chlorine level of 0.5 mg per liter of water (Derreumaux et al. 1974) and remains the single most effective disinfectant system for controlling opportunistic amebae in public waters. This level of chlorination has effectively controlled the *N. fowleri* problem in the public water supplies of South Australia (Dorsch et al. 1983). The risk of acquiring naeglerial infection through

swimming in Florida's freshwater lakes is estimated at c. 1 in 2.6 million exposures (Wellings 1977). Considering the millions of persons who swim outdoors each summer, it is truly remarkable that there are not more cases of PAM.

Factors that have been associated with *Acanthamoeba* keratitis in contact lens wearers are:

- using nonsterile, home-made saline
- disinfecting lenses less frequently than recommended
- wearing lenses while swimming (Stehr-Green et al. 1987).

Contact lens wearers should closely follow the manufacturer's recommendations for wear, care, and disinfection of lenses. Home-made saline solutions remain an important risk factor associated with *Acanthamoeba* keratitis. Contamination of the contact lens care system with bacteria or fungi encourages the survival and growth of *Acanthamoeba*. Amebae, including *A. polyphaga* and *A. hatchetti*, have been isolated from laboratory eyewash stations, especially stations containing reservoirs (Bier and Sawyer 1990; Tyndall et al. 1987). Eyewash stations with reservoirs should be flushed weekly, otherwise they potentially present a health hazard to users, particularly those wearing contact lenses.

With the expected increase in the number of contact lens wearers, there will undoubtedly be a corresponding increase in the number of cases of *Acanthamoeba* keratitis. As immunosuppression becomes more widespread (not just because of AIDS but also because of organ transplantation, cancer chemotherapy, congenitally acquired immunodeficiency, and suppression resulting from the indiscriminate release of toxic chemicals and carcinogens into the environment), the possibility of more CNS and disseminated *Acanthamoeba* infections also increases.

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Giardiasis

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INTRODUCTION

Giardia was first seen by Leeuwenhoek in his own stool specimens in 1681, but was not described until 1859 by Lambl. In the early 1930s, Clifford Dobell translated a number of Leeuwenhoek's works from Dutch and Latin. In the account of his own giardiasis infection, Leeuwenhoek writes (Dobell 1920):

> All the particles aforesaid (description of stool debris) lay in a clear transparent medium, wherein I have sometimes also seen animalcules a-moving very prettily (trophozoites); some of 'em a bit bigger, others a bit less, than a blood-globule, but all of one and the same make. Their bodies were somewhat longer than broad, and their belly which was flatlike, furnisht with sundry little paws, wherewith they made such a stir in the clear medium and among the globules, that you might e'en fancy you saw a pissabed (woodlouse or sow-bug) running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but slow progress.

However, there is no evidence that either Leeuwenhoek or Lambl saw or recognized the cyst forms of the organism; they were first noted by Grassi in 1879 and were thought to be coccidia. Only later did Grassi associate the cyst form with the flagellated trophozoite form (Meyer 1990). The history of giardiasis is given in History of human parasitology.

Giardia lamblia is worldwide in distribution, apparently more prevalent in children than in adults, and more common in warm climates than in cool ones. It is the most commonly diagnosed flagellate in the intestinal tract, and it may be the most commonly diagnosed intestinal protozoan in some areas of the world with an estimated 2.8×10^8 cases per year (Lane and Lloyd 2002).

CLASSIFICATION

Although various criteria have been used to differentiate species of *Giardia*, including host specificity, various body dimensions, and variations in structure, there is still considerable debate over the appropriate classification and nomenclature of this group of organisms. Based on work by Filice (1952) on structural variations, three groups have been proposed: amphibian *Giardia* (represented by *G. agilis*), the muris group from rodents and birds (represented by *G. muris*), and the intestinalis group from a variety of mammals (including humans), as well as birds and reptiles (represented by *G. duodenalis*). Despite disagreement concerning the names *intestinalis* and *lamblia*, both continue to be used to describe this organism. Meyer prefers to use *Giardia duodenalis*, which is often followed by the name of the animal from which the organism was obtained (Meyer 1990).

Ten strains from human- and animal-source *G. duodenalis* were evaluated using an isoelectric focusing technique. Banding patterns obtained from trophozoite total cell proteins demonstrated both similarities and differences. These findings confirm the heterogeneity of this group of *Giardia* spp., from animal and human hosts and from hosts within the same geographical area (Isaac-Renton et al. 1988; Ey et al. 1996; McIntyre et al. 2000; Monis et al. 1999; Sulaiman et al. 2003, 2004).

In a recent study from Australia, ribosomal RNA sequencing revealed differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. Results from the study suggest that zoonotic transmission of *Giardia* infections between humans and dogs does not occur frequently in the particular communities studied. The dog-associated sequences have not been reported before, suggesting that there may be a new *G. duodenalis* subgroup (Hopkins et al. 1997). However, in a recent study in India, *G. duodenalis* isolates recovered from humans and dogs living in the same locality were characterized at three different loci; the SSU-rDNA, elongation factor 1-alpha (ef1-α), and triose phosphate isomerase (*tpi*) gene. Analysis of the *tpi* gene placed canine *Giardia* isolates within the genetic groupings of human isolates (Assemblages A and B). Epidemiological data showing a highly significant association between the prevalence of *Giardia* in humans and the presence of a *Giardia*-positive dog in the same household provided further evidence for zoonotic transmission (Traub et al. 2004).

Molecular classification tools have been very valuable in delineating the pathogenesis and host range of *Giardia* isolates obtained from humans and a variety of other mammals. Molecular typing indicates that most animal parasites are not infective to humans but those that are can be genotypically classified into assemblage A or B (Lane and Lloyd 2002). In one study, assemblage A isolates were solely detected in patients with intermittent diarrheal complaints, while assemblage B isolates were present in patients with persistent diarrheal complaints (Homan and Mank 2001). Restriction fragment length polymorphism (RFLP) analysis has distinguished two groups (designated groups I and II) within assemblage A (Amar et al. 2002). Currently, assemblages A (groups 1 and 2), and B (groups 3 and 4) contain human pathogens, while C through G are found in other mammals (Adam 2001). The phylogeny of the organism has not been finalized, but there is a growing opinion that *Giardia* may not be an ancient eukaryote, but that it may be derived from a more complex mitochondria-containing protozoan (Lane and Lloyd 2002).

Within the USA and other areas throughout the world, the term *Giardia lamblia* has been commonly used for many years and refers to those organisms found in humans, as well as other mammals. In some geographic areas, this designation tends to eliminate any confusion, since the majority of health workers are used to this name and continue to report the presence of the organism using it. Others feel the designation *G. duodenalis* is taxonomically correct and refers to the many 'species' containing the 'duodenalis' double medial bodies. As long as both laboratory staff and physicians use the same species designation, the choice is optional.

STRUCTURE AND LIFE CYCLE

The life cycle is illustrated in Figure 1 and is similar to the simple, direct life cycle of most intestinal protozoa. Both the trophozoite and cyst are included in the life cycle of *G. lamblia*. Trophozoites divide by means of longitudinal binary fission producing two daughter trophozoites. The trophozoites are the intestinal dwelling stages and attach to the epithelium of the host villi by means of the ventral disc. The attachment is substantial and results in disc impression prints when the organism detaches from the surface of the epithelium. Trophozoites may remain attached or may detach from the mucosal surface. Since the epithelial surface sloughs off the tip of the villus every 72 h, the trophozoites apparently detach at that time.

There are several theories on possible mechanisms for attachment by the ventral disk; they include microtubule mediation, hydrodynamic action, contractile protein activity, and the interaction of lectins with surface bound sugars. Based on conflicting information, the attachment process probably depends on multiple mechanisms. A study by Katelaris and colleagues indicates that attachment to enterocyte-like differentiated Caco-2 cells is primarily by cytoskeletal mechanisms that can be inhibited by interfering with contractile filaments and microtubules; attachment by mannose binding lectin also seems to mediate binding (Katelaris et al. 1995).

The most common location of the organisms is in the crypts within the duodenum. For reasons that are not known, cyst formation takes place as the organisms move down through the colon.

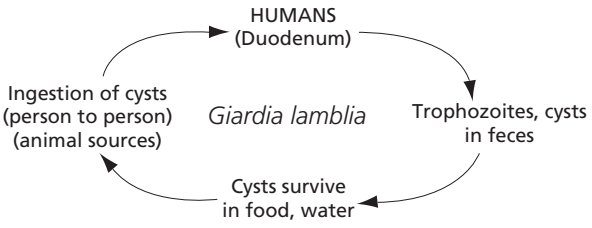

Figure 1 *Life cycle of* Giardia lamblia. *(Adapted from Garcia 2001)*

The *Giardia* encystation process can be divided into three parts:

1 stimulus for encystation
2 synthesis and intracellular transport of cyst wall components
3 assembly of the extracellular cyst wall.

It has been suggested that a cholesterol-regulated process similar to that in mammalian cells occurs during the induction of encystation in *Giardia*. The formation of the cyst wall during encystation is characterized by the appearance of dense encystation-specific vesicles (ESV). These ESVs transport cyst wall components to the plasma membrane of the encysting cell and release their contents to the cell exterior during cyst wall formation. Information also suggests that the cyst wall contains only two cyst proteins; these proteins appear to be common components of the cyst walls of a variety of intestinal parasitic protozoa.

The *Giardia* cyst wall is composed of an outer filamentous portion and an inner membranous portion. The process begins with the appearance of cyst wall antigens that appear on small protrusions of the trophozoite surface; these eventually form cap-like structures and may cover the entire surface of the trophozoite. Based on the filamentous nature of the cyst wall and its constant thickness, it may form from successive layers of cyst wall materials. The cyst wall has also been found to be somewhat flexible, but rigid enough to maintain its shape after the excysted trophozoite has left the cyst (Lujan et al. 1997).

Based on results by Halliday et al. (1995). *Giardia* appears to take up conjugated bile salts by active and passive transport mechanisms like the mammalian ileum. As conjugated bile salts are known to promote encystation, these uptake mechanisms may play an important role in the survival of the cyst stage and ultimate completion of the life cycle of the parasite (Das et al. 1997; Healy 1990). Cholesterol starvation may also play a role in stimulating encystation (Lujan et al. 1996). The trophozoites retract the flagella into the axonemes, the cytoplasm becomes condensed, and the cyst wall is secreted (Erlandsen et al. 1996). As the cyst matures, the internal structures are doubled so that when excystation occurs, the cytoplasm divides to produce two trophozoites. Excystation would normally occur in the duodenum or appropriate culture medium (Bingham and Meyer 1979). If kept cool and moist, cysts can remain viable for several months, but they cannot survive if moisture is lacking.

At the light microscopic level and seen on permanent stained fecal smears stained with trichrome or iron-hematoxylin stains, the trophozoite is usually described as teardrop-shaped from the front with the posterior end being pointed (Figures 2 and 3). Examined from the side, the trophozoite resembles the curved portion of a spoon. The concave portion is the area of the sucking disc (Figure 2). There are four pairs of flagella, two nuclei, two axonemes, and two slightly curved bodies called the median bodies. The trophozoites usually measure 10–20 μm in length and 5–15 μm in width (Figure 3 and Table 1)

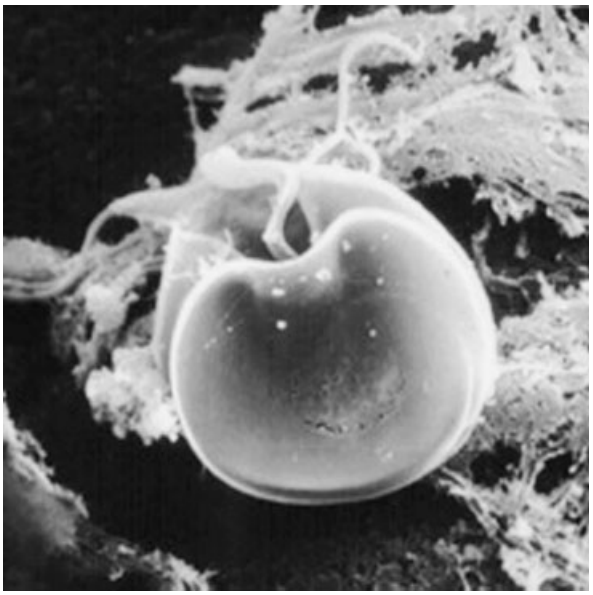

Figure 2 *Scanning electron micrograph of* Giardia lamblia *trophozoite on the mucosal surface. (Courtesy of Marietta Voge) (Garcia 2001)*

The cysts may be either round or oval and contain four nuclei, axonemes, and median bodies (Figure 3). Often, some cysts appear to be shrunk or distorted and one may see two halos, one around the cyst wall itself and one inside the cyst wall around the shrunken organism. The halo effect around the outside of the cyst is particularly visible on permanent stained smears. Cysts normally measure 11–14 μm in length and 7–10 μm in width (Figure 3 and Table 2).

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Organisms in the genus *Giardia* are described as aerotolerant anaerobes that use substrate-level phosphorylation, iron-sulfur protein- and flavoprotein-mediated electron transport for the production of energy (Jarroll and Lindmark 1990). *Giardia* lacks mitochondria and microbodies, but contains lysosome-like organelles that accumulate ferritin and stain positively for acid phosphatase and aryl sulfatase (Upcroft and Upcroft 1998).

In the presence of exogenous glucose, intact *Giardia* trophozoites produce ethanol and acetate as organic end-products: aerobically, six times more acetate than ethanol is produced; anaerobically, twice as much ethanol as acetate is produced. In both situations, carbon dioxide is the only gaseous end-product that has been detected. Based on the pattern of carbon dioxide

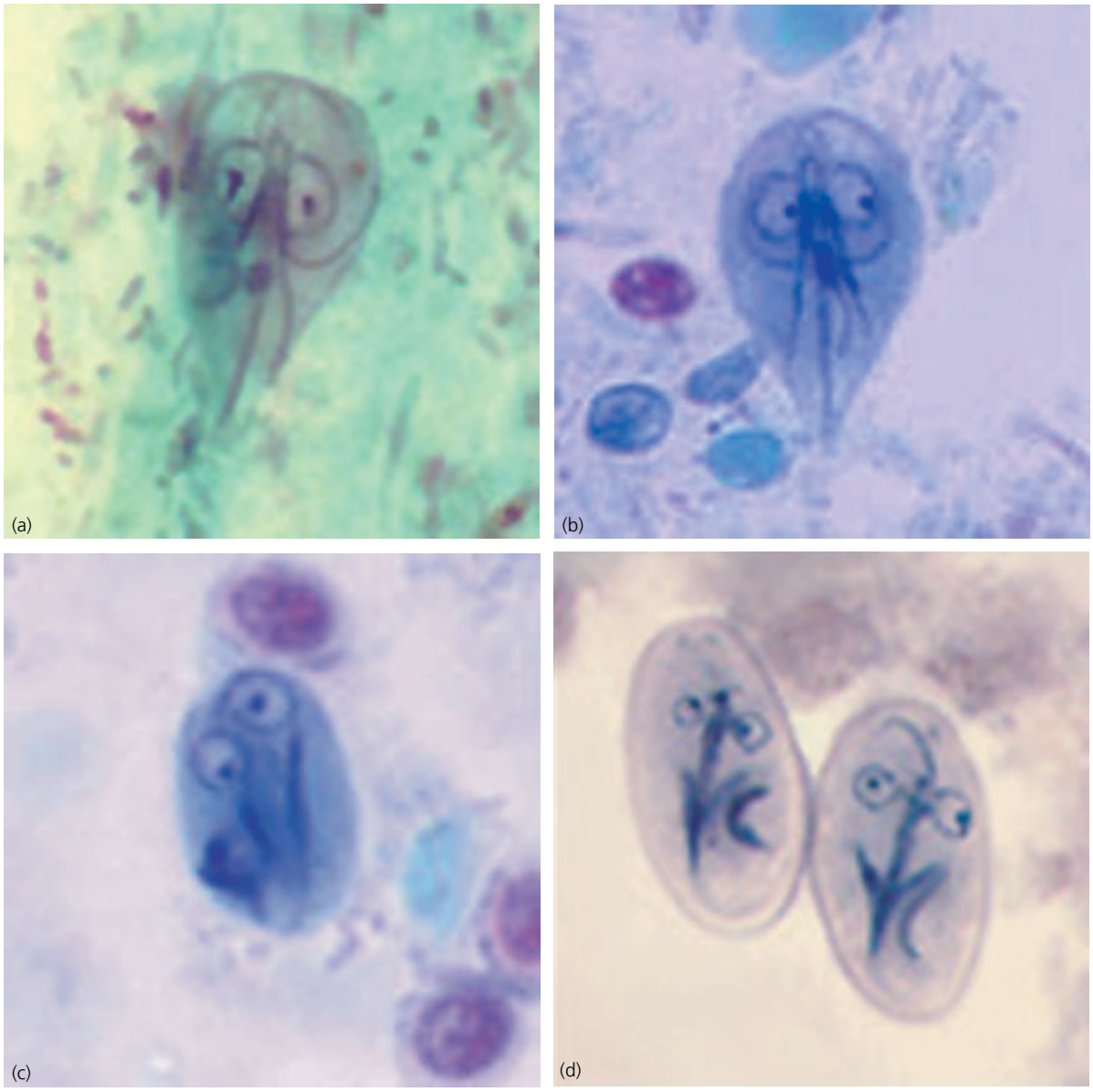

Figure 3 Giardia lamblia *(a, b) trophozoites stained with Wheatley's trichrome;* **(c)** *cyst stained with Wheatley's trichrome;* **(d)** *cysts stained with iron hematoxylin.*

production, glucose is metabolized using the Embden-Meyerhof-Parnas (EMP) pathway and the hexose monophosphate (HM) pathway. A number of energy metabolism enzymes have been identified and are reviewed by Jarroll and Lindmark (1990).

Carbohydrate and energy metabolism studies have also been conducted using the cyst stage; enzyme data confirm that specific activities occur regardless of the life-cycle stage involved. Studies also show that the cyst stage respires at c. 10–20 percent of the rate observed in trophozoites; respiration is not stimulated by exogenous glucose, but by exogenous ethanol. It is also interesting to note that cyst respiration decreases as water temperature decreases. These findings may explain increased cyst viability with decreased storage temperature.

In summary, a proposed energy metabolic pathway for *Giardia* indicates oxidation of endogenous and exogenous glucose incompletely to ethanol, acetate, and carbon dioxide. Energy is produced through substrate-level phosphorylation; iron-sulfur proteins and flavins are involved in electron transport. There is no evidence for oxidative phosphorylation or a cytochrome-mediated electron transport system (Jarroll and Lindmark 1990).

The fact that *Giardia* shares many metabolic attributes of the anaerobic bacteria provides a wide range of drug targets for future consideration. An example is the utilization of the amino acid arginine as an energy source, using the arginine dihydrolase pathway, which occurs in a number of anaerobic bacteria (Brown et al. 1998).

Table 1 Giardia lamblia *trophozoite morphology*

Organism stage	Shape and size	Motility	Nuclei	Flagella	Other features
Giardia lamblia trophozoite	Pear-shaped, 10–20 μm; width 5–15 μm	'Falling leaf' motility may be difficult to see if organism in mucus	Two nuclei Not visible in unstained mounts	Four lateral, two ventral, two caudal	Sucking disc occupying ½–¾ of ventral surface pear-shaped front view; spoon-shaped side view

Culture studies indicate that *Giardia* trophozoites have the capacity to synthesize isoprenoid lipids de novo, as well as to incorporate preformed lipids; also, steryl esters are abundant and increase during encystation, while triglycerides are low (Ellis et al. 1996; Gibson et al. 1999). The phospholipids present include phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and sphingomyelin. Neutral lipids present include sterols, mono-, di-, and triacylglycerides. Fatty acids can be incorporated into phospho- and neutral lipids (Jarroll and Lindmark 1990). Increased fatty acid unsaturation and the accumulation of storage lipids are consistent with the parasite differentiation into a cyst stage that can survive outside the host.

In the trophozoite, nucleic acid requirements are met by the salvage of pyrimidines, purines, and their nucleosides. Various carrier-mediated mechanisms have been proposed.

In contrast to trophozoites of *Entamoeba* and the trichomonads, *Giardia* has very few carbohydrate-splitting hydrolases. It has also been found that *Giardia* trophozoites exhibit increased levels of chitin synthetase activity during encystment (Jarroll and Lindmark 1990).

Although antigenic analysis shows promise in helping to identify and classify *Giardia*, both common and different antigens have been recovered in axenic cultures. The use of monoclonal antibodies in the study of antigens has proved useful; technical methods also involve quantitation, an important requirement in this type of analysis. Another potential problem involves antigenic variation; a single *Giardia* trophozoite can give rise in vivo, as well as in vitro, to trophozoites with varying surface antigens. Immune pressure may play a role in antigenic variation, but the speed with which antigenic changes take place after infection suggests that other mechanisms may also be involved. The antigenic profile of *Giardia* in culture may also change if the trophozoites harbor endosymbionts; the presence of these organisms may influence both antigenic and chemotaxonomic determinations (Meyer 1990).

Giardia lamblia is known to undergo surface antigenic variation. The antigens involved belong to a group of variant-specific surface proteins (VSP) that are unique cysteine-rich zinc finger proteins. Selection by immune-mediated processes is a possibility, especially because switching occurs at the same time that humoral responses are first detected. The purpose of antigenic variation may be the presentation of a wide variety of VSPs to the various hosts, increasing the chances of successful initial infection or reinfection (Nash 1997). Since VSPs are resistant to the effects of intestinal proteases, they may allow the organism to survive in the protease-rich small intestine (Nash 2002). Thus, the two major hypotheses concerning antigenic variation are evasion of the host immune system and allowing the parasites to survive in different intestinal environments; certainly these hypotheses are not mutually exclusive (Adam 2001).

CLINICAL ASPECTS

From available data, it appears the incubation time for giardiasis is c. 12–20 days. Because the acute stage usually lasts only a few days, giardiasis may not be recognized as the cause of the symptoms observed, which may mimic acute viral enteritis, bacillary dysentery, bacterial or other food poisonings, acute intestinal amebiasis or 'traveler's diarrhea' (toxigenic *E. coli*). However, the type of diarrhea plus the lack of blood, mucus, and cellular exudate is consistent with giardiasis.

Clinical manifestations

Nausea, anorexia, malaise, low-grade fever, and chills may accompany onset. There may be a sudden onset of

Table 2 Giardia lamblia *cyst morphology*

Organism stage	Shape and size	Motility	Nuclei	Flagella	Other features
Giardia lamblia cyst	Oval, ellipsoidal or may appear round 8–19 μm; usual range 11–14 μm; width, 7–10 μm	Nonmotile	Four nuclei – not distinct in unstained preparations; usually located at one end	Flagella not visible	Longitudinal fibers in cysts may be visible in unstained preparations; deep staining median bodies usually lie across the longitudinal fibers; there is often shrinkage and the cytoplasm pulls away from the cyst wall; there may also be a 'halo' effect around the outside of the cyst wall due to shrinkage caused by dehydrating reagents

explosive, watery, foul-smelling diarrhea with flatulence and abdominal distention. Other symptoms include epigastric pain and cramping. There is increased fat and mucus in the stool, but no blood. Weight loss often accompanies these symptoms. The acute infection usually resolves spontaneously, although in some patients, particularly children, the acute symptoms may last for months. Acute giardiasis must be differentiated from other acute viral, bacterial, and protozoal agents.

The acute phase is often followed by a subacute or chronic phase. Symptoms in these patients include recurrent, brief episodes of loose, foul stools; there may be increased distention and foul flatus. Between mushy stools, the patient may have normal stools or may be constipated. Abdominal discomfort continues to include marked distention and belching with a rotten egg taste. Chronic disease must be differentiated from amebiasis and other intestinal parasites such as *Dientamoeba fragilis*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Isospora belli*, microsporidia, *Strongyloides stercoralis*, and from inflammatory bowel disease and irritable colon (see also Intestinal coccidia: cryptosporidiosis, isosporiasis, cyclosporiasis, Microsporidiosis, and *Strongyloides* and *capillaria*). Based on symptoms such as upper intestinal discomfort, heartburn and belching, giardiasis must also be differentiated from duodenal ulcer, hiatus hernia, gallbladder, and pancreatic disease.

Various types of malabsorption have been described including steatorrhea, disaccharidase (lactase, xylase) deficiency, Vitamin B_{12} malabsorption, hypocarotenemia, low serum folate, and protein-losing enteropathy. Lactose intolerance may persist after effective therapy, particularly in patients from ethnic groups with a predisposition for lactase deficiency. This should be considered prior to retreatment, especially if the patient continues to have diarrhea, but post-treatment negative stool specimens.

Although there is speculation that the organisms coating the mucosal lining may act to prevent fat absorption, this does not completely explain why the uptake of other substances normally absorbed at other intestinal levels is prevented (Tandon et al. 1977). Occasionally the gall bladder may also be involved, causing gall-bladder colic and jaundice. *G. lamblia* has also been identified from bronchoalveolar lavage fluid (Stevens and Vermeire 1981) and the urinary tract (Meyers et al. 1977). Reasons for such a dramatic variation in host susceptibility are not understood. Host factors such as nutritional status and both systemic and mucosal immunity may also play a role.

IMMUNOLOGY

Recent studies document antigenic variation with surface antigen changes during human infections with *Giardia*, and although the biological importance of this work is not clear, it suggests that this may provide a mechanism enabling the organism to escape the host's immune response (Nash et al. 1990). Also one would suspect that the more rapid the rate of change, the more likely that a chronic infection would be seen. Certain surface antigens may allow the organisms to survive better in the intestinal tract and might not be immunologically selected (Nash 1997).

Although patients with symptomatic giardiasis usually have no underlying abnormality of serum immunoglobulins, a high incidence of giardiasis has been shown to occur in patients with immunodeficiency syndromes, particularly in common variable hypogammaglobulinemia (Ament et al. 1973). Giardiasis was found to be the most common cause of diarrhea in these patients and was associated with mild to severe villus atrophy. Successful treatment of giardiasis led to symptomatic cure and improvement in mucosal abnormalities, with the exception of nodular lymphoid hyperplasia.

Acute (polymorphonuclear leukocyte and eosinophils) and chronic inflammatory cells have been found in the lamina propria together with increased epithelial mitoses – findings that revert to normal after therapy. The presence of these inflammatory cells has been linked to epithelial cell damage. The range of patient response is from normal to almost complete villous atrophy. Crypt mitosis is also increased based on the degree of villous damage, however, it has also been reported that mucosal changes return to normal within several days of treatment and that patients with giardiasis have reduced surface area compared with uninfected individuals.

Although no definitive pattern has been identified, it appears that lamina propria plasma cells producing immunoglobulin (Ig)M and IgE increase, whereas local production of IgA seems to be suppressed. More severe villous damage has also been reported in patients with diminished gamma globulins.

During the acute phase of giardiasis, jejunal active antigen uptake is increased, leading to delayed recruitment of mucosal and connective mast cells. These changes may influence the increased incidence of hypersensitivity reactions and allergic disease associated with *Giardia* infection (Hardin et al. 1997). Severe malabsorption has also been linked with isolated levothyroxine malabsorption, leading to severe hypothyroidism (Seppel et al. 1996) and secondary impairment of pancreatic function (Carroccio et al. 1997). In both cases, therapy with metronidazole led to complete remission of symptoms.

PATHOLOGY

Although the organisms in the crypts of the duodenal mucosa may reach very high densities, they might not cause any pathology. The organisms feed on the mucous secretions and do not penetrate the mucosa as *G. muris* does in mice where trophozoites have been recovered in

the deeper tissues (Owen et al. 1981). Although some organisms have been seen in biopsy material inside the intestinal mucosa, others have been seen attached only to the epithelium. In one in vitro study using cultured epithelial cells, the organisms showed no toxic or invasive effect (Chavez et al. 1986). Another study by Chen and colleagues has demonstrated a *G. duodenalis* cysteine-rich surface protein (CRP136) that has 57 percent homology with the gene encoding the precursor of the sarafotoxins, a group of snake toxins from the burrowing adder known to cause symptoms similar to those of humans acutely infected with *Giardia* (Chen et al. 1995). Thus, CRP136 represents the first evidence for a potential *Giardia* toxin. In symptomatic cases, there may also be irritation of the mucosal lining, increased mucus secretion, and dehydration (Erlandsen 1974; Peterson 1957). In a recent study, it appears that *G. lamblia* may cause intestinal pathophysiology by disrupting tight junctional ZO-1 and increasing epithelial permeability. Apical administration of epidermal growth factor (EGF) prevents these abnormalities and reduces epithelial colonization by the live parasites (Buret et al. 2002).

With the advent of acquired immune deficiency syndrome (AIDS), there was speculation that *G. lamblia* might be an important pathogen in this group, but clinical findings to date have not confirmed this fear (Meyer 1990; Smith et al. 1988). Although giardiasis has certainly been reported in AIDS patients, clinical experience does not suggest a more pathogenic role for *G. lamblia* in these patients than in other non-AIDS individuals.

Isoenzyme studies, primarily designed to assist in organism identification and classification, have also provided additional information regarding pathogenicity, implication in waterborne outbreaks, and human disease. In one study where isoenzyme patterns of 32 isolates of *Giardia* obtained from both humans and animals were examined, there was no obvious correlation between clinical symptoms and isoenzyme patterns. Isolates from asymptomatic individuals were found in the same zymodemes (isoenzyme groups) as isolates from symptomatic hosts. This study also confirmed previous observations regarding genetic heterogeneity and demonstrated significant differences between isolates from within a single region and other widely separated geographic locations (Proctor et al. 1989). A study from Australia provided data on two *Giardia* demes derived from children with similar chronic symptoms both of which appear to be pathogenic (Upcroft et al. 1995).

DIAGNOSIS

Generally, routine laboratory test results are normal, including hematology; eosinophilia is rare. Malabsorption of fat, glucose, lactose, xylose, carotene, folic acid, and vitamin B_{12} is occasionally present in some patients. Diagnosis of giardiasis is usually based on identification of the organisms from stool or duodenal aspirate specimens (Table 3).

Routine stool examinations are normally recommended for the recovery and identification of intestinal protozoa. In the case of *G. lamblia*, because they are attached so securely to the mucosa by means of the sucking disk, even a series of five to six stools may be examined without recovering the organisms. These parasites also tend to be passed in the stool on a cyclical basis. The EnteroTest capsule may be helpful in recovering the organisms, as may duodenal aspirates. Although cysts can often be identified on the wet stool preparation, many infections may be missed without the examination of a permanent stained smear (Collins et al. 1978; NCCLS 1997; Garcia 2001). If material from the string test (EnteroTest) or mucus from a duodenal aspirate are submitted, they should be examined as a wet preparation for motility; motility may be represented by nothing more than a slight flutter of the flagella because the organism will be caught up in the mucus. After diagnosis, the rest of the positive material can be preserved and processed for permanent staining. Experience in the clinical laboratory setting has shown

Table 3 *Laboratory diagnosis of giardiasis – key points*

Laboratory diagnosis
Even if a series of three stool specimens (ova and parasite examinations) are submitted and examined correctly, the organisms may not be recovered and identified.
Motility on wet preparations may be difficult to see because the organisms may be caught up in mucus.
Any examination for parasites in stool specimens must include the use of a permanent stained smear (even on formed stool).
Duodenal drainage either alone or in combination with the EnteroTest capsule may be very helpful in organism recovery. This technique does not take the place of the ova and parasite examination.
Immunodiagnostic procedures have been found to be more sensitive than the routine stool methods (ova and parasite examination) and are now being used routinely by some laboratories. These procedures are normally performed on order; if negative and the patient remains symptomatic, additional testing (O&P examination, special stains for the coccidia and microsporidia) can be ordered. Other considerations such as cost, training, equipment availability, batching of tests, and number of requests also play a role in test selection.

that the examination of duodenal fluid after a series of negative stool examinations rarely confirms a positive infection. This may be due to parasite location or small numbers. Because of these considerations, the examination of intestinal fluid should not replace routine stool examinations, all of which should include the use of permanent stained smears (Garcia 2001).

Procedures have also been developed using the enzyme-linked immunosorbent assay (ELISA) to detect *Giardia* antigen in feces. The ELISA is at least as sensitive as microscopic wet examinations (Addis et al. 1991; Nash et al. 1987). A fluorescent method using monoclonal antibodies has also proved extremely sensitive and specific in detecting *Giardia* in fecal specimens (Garcia et al. 1992). Many of these newer methods are being used to screen patients suspected of having giardiasis or those who may be involved in an outbreak situation (Garcia and Shimizu 1997; Aldeen et al. 1998).

A number of studies have compared the newer immunoassay products, with excellent and comparable results. Several new products are available in a cartridge format (Figure 4). Some of these use an immunochromatographic strip-based detection system for the detection of *Giardia lamblia* and *Cryptosporidium parvum*, which can be used with fresh, frozen and/or formalinized fecal specimens (Garcia and Shimizu 2000; Katanik et al. 2001; Garcia et al. 2003). A third cartridge system uses enzyme immunoassay for the detection of the *Entamoeba histolytica*/*E. dispar* group, *Giardia lamblia*, and *Cryptosporidium parvum*, which requires fresh or frozen fecal specimens (Garcia et al. 2000). Results have demonstrated that ELISA for detection of copro-antigens in a single stool sample may be almost as sensitive for the detection of giardiasis as repeated microscopy on two sequential stool samples (Mank et al. 1997).

In a study of nine different immunoassay kits for the detection of *Giardia lamblia*, the sensitivity of ELISA ranged from 94 to 99 percent; the specificity was 100 percent. All direct fluorescence assay results were in agreement with both 100 percent specificity and 100 percent sensitivity; a total of 60 specimens positive for *Giardia*, 40 specimens positive for both *Giardia* and *Cryptosporidium*, and 50 negative fecal specimens were tested. In addition to specificity and sensitivity, factors such as cost, simplicity, ease of interpretation of results, equipment, available personnel, and number of tests ordered are also important considerations prior to method selection (Garcia and Shimizu 1997).

In another study of nine commercially available ELISAs for detection of *Giardia lamblia* in fecal specimens, all except one were found to be rapid, sensitive, and specific. Sensitivities and specificities ranged from 88.6 to 100 and 99.3 to 100 percent, respectively (Aldeen et al. 1998).

Occasionally, after multiple stool examinations and examination of intestinal fluid have been negative, a small bowel biopsy may confirm the suspect diagnosis of giardiasis. It is recommended that the biopsy be taken from the area of the duodenojejunal junction; but biopsies from multiple duodenal and jejunal sites are preferred. Fluid smears and touch preparations can be air dried, fixed in methanol, and stained with Giemsa stain. The organisms appear somewhat purple and the epithelial cells appear pink. Routine histological procedures should also be performed with very careful screening of the material; trophozoites are very difficult to see and may be present in very few of the sections. Normally, the trophozoites are more likely to be seen attached to the microvillous border within the crypts.

Radiological findings from the small intestine may be normal in giardiasis, but abnormalities can occur and are usually seen in the duodenum or jejunum. Findings may include thickened mucosal folds, increased secretions, and a pattern of edema and segmentation, none of which is diagnostic for giardiasis. Approximately 20 percent of patients with giardiasis may exhibit these findings after routine barium examination. It is always recommended that the stools be collected for routine examinations prior to the patient receiving barium as organisms may be difficult to identify in the stool for several days to more than a week afterwards. Because giardiasis may not produce any symptoms at all, demonstration of the organism in symptomatic patients may not rule out alternative diagnoses such as peptic ulcer, celiac disease, strongyloidiasis, and possibly carcinoma.

Unfortunately, serodiagnostic procedures for giardiasis do not yet fulfill the criteria necessary for wide clinical use, particularly since they may indicate either past or present infection. In contrast, the detection of antigen in a stool or visual identification of the organisms using immunodiagnostic reagents indicates current infection. With the increase in numbers of *Giardia* infections and awareness of particular situations such as nursery school settings, additional detection assays have been developed as rapid and reliable immunodiagnostic procedures (Keystone et al. 1978; Craft 1982; Sealy and Schuman 1983; Addis et al. 1992; Garcia and Shimizu 1997; Aldeen et al. 1998; Garcia and Shimizu 2000; Garcia et al. 2000; Katanik et al. 2001; Garcia et al. 2003).

Recent studies using indirect fluorescent assay (IFA) to measure anti-*Giardia* humoral responses demonstrated that more than 34 percent of asymptomatic patients have a titer equal to or less than 1:500, while more than 29 percent of the symptomatic patients have a titer of 1:8000 or higher. The circulating antiparasite IgM and IgA, measured by ELISA, were significantly higher in symptomatic patients and correlated with higher cyst output (Soliman et al. 1998).

The secretory immune response to membrane antigens during *Giardia lamblia* infection in humans may be important in future studies in the diagnosis of giardiasis; these antigens were not recognized by saliva samples from healthy individuals (Rosalesborjas et al. 1998).

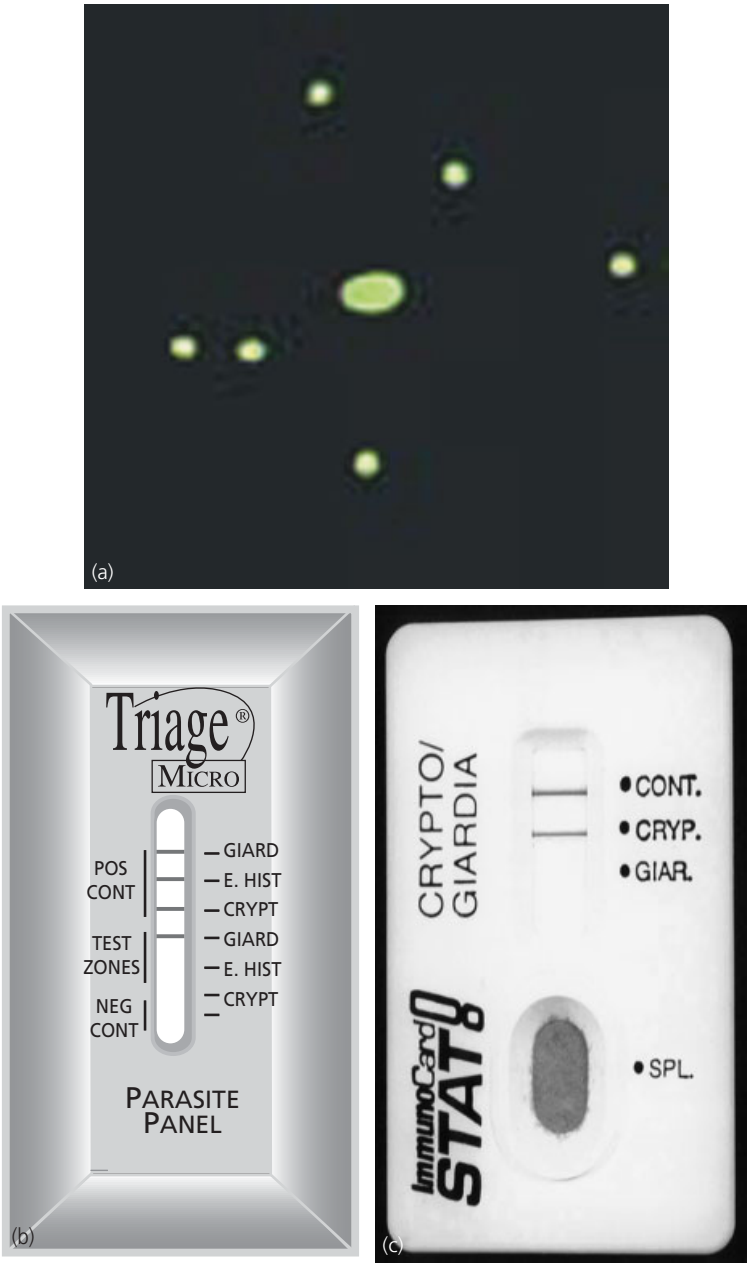

Figure 4 *Fecal immunoassays for the detection of* Giardia lamblia*:* **(a)** *Merifluor* Cryptosporidium/Giardia *combination FA reagent (Meridian Bioscience, Cinncinnati, OH) for the detection of* Giardia lamblia *cycsts (large oval structure) and* Cryptosporidium parvum *(small round objects) –can be used with preserved stools;* **(b)** *Triage® Paasite Panel (BIOSITE Diagnostics, San Diego, CA) cartridge format for the detection of* Giardia lamblia*,* Cryptosporidium parvum*, and the* Entamoeba histolytica/E. dispar *group – requires fresh stools;* **(c)** *immunochromatographic cartidge format (Meridian Bioscience, Cinncinnati, OH) for the detection of* Giardia lamblia *and* Cryptosporidium parvum.

EPIDEMIOLOGY

Giardiasis is one of the most common intestinal parasitic infections in humans and is distributed worldwide in developed and developing countries. There are, however, differences in the numbers of infections, not only between countries but also within geographic regions. Infections seem to be more common in children than adults, and other social, environmental, climatic, and economic factors also play a role in disease prevalence. Susceptibility to infection with *Giardia* is influenced by sex, age, environmental conditions, socioeconomic conditions, occupation, nutritional status, gastric acidity, and overall host immune status.

Transmission occurs through ingestion of as few as ten viable cysts. Infection can be acquired from food or water and directly person-to-person by the oro-fecal route. Often there are outbreaks due to poor sanitation

facilities or breakdowns as evidenced by travelers and campers (Kettis and Magnius 1973; Knaus 1974; Lopez et al. 1978; Moore et al. 1969). There has also been an increase in the prevalence of giardiasis in the male homosexual population, probably due to anal and oral sexual practices (Phillips et al. 1981; Schmerin et al. 1978).

Geographical distribution varies considerably worldwide with reported prevalence figures ranging from a low of 5 percent in Indonesia to 43 percent in the Seychelles; in the US, the figure is c. 7.4 percent (Islam 1990). When reviewing reported prevalence figures, it is always important to remember that data varies considerably, depending on the sensitivity and specificity of the diagnostic methods used. In areas of the world in which *Giardia* is endemic, the majority of infections in children occur in those under 10 years of age. A decrease in infections seen in children older than 10 years may reflect acquired immunity and behavioral changes that decrease potential environmental exposure to the organism. High population density and overcrowding probably contribute to higher prevalences of giardiasis. In low-income populations residing on the United States–Mexico border with no sewerage and water services, households with children under 5 years of age were 1.3 times at risk for *Giardia* infection when compared with *Cryptosporidium*. These findings highlight the differences in the transmission of these two pathogens and emphasize the need for interventions related to hygiene, water supply, and sanitation (Redlinger et al. 2002).

Although seasonal patterns have been identified for some infectious diseases, limited information is available for giardiasis. Some data suggest an association with the cooler, wetter months of the year; this is not surprising if one considers the issue of environmental conditions advantageous to cyst survival.

It has been documented that certain occupations may place an individual at risk of infection and these include sewage and irrigation workers who may become exposed to infective cysts. In situations where young children are grouped together (e.g. nursery schools), there may be an increased incidence of exposure and subsequent infection of both children and staff.

The possible association between gastric acidity and giardiasis has been debated for some time; decreased gastric acid production may predispose to infection with *Giardia*. It is thought that normal gastric acidity acts as a barrier to the establishment of an infection; patients who have had a gastrectomy are prone to infection with *Giardia*. Although achlorhydria is associated with blood group A, and some evidence suggests that this group is more susceptible to giardiasis, subsequent evidence has not confirmed this association. Since reduction in gastric acid also occurs as a result of malnutrition, these factors may be linked and, as a group, increase the susceptibility to infection with this organism. This link between malnutrition and giardiasis may also be explained by the impairment of the host's immune system.

The issue of breast milk in modifying infection with *Giardia* has also been discussed (Islam 1990). Lower incidence of giardiasis in children up to and including 6 months of age may be related to an association with breast-feeding and some protection against infection through secretory IgA. However, lower incidence may also be related to decreased exposure to *Giardia* in breast-fed infants.

Giardiasis is one of the more common causes of traveler's diarrhea and has been recorded from all parts of the world. It has also been speculated that visitors to areas endemic for *Giardia* are more likely to present with symptoms than individuals who live in the area; this difference is probably due to the development of immunity from prior, and possible continued, exposure to the organism (Soliman et al. 1998; Isaac-Renton et al. 1999).

In the USA, there have been a number of outbreaks attributed to either resort or municipal water supplies in Oregon, Colorado, Utah, Washington, New Hampshire, and New York (Craun 1990; Kirner 1978; Shaw et al. 1977; Wright et al. 1977). Most waterborne outbreaks have occurred in water systems using surface water sources (Dykes et al. 1980). High rates of infection have also been reported from hikers and campers who drank stream water: because some of these areas are remote from human habitation, infected wild animals, especially beavers, are suspected as being a possible source of infection (Dykes et al. 1980). In addition to beavers and muskrats, *Giardia* infects a number of different animals, including dogs, cats, cattle, sheep, pigs, goats, gerbils, rats, and gorillas; however, the role of some of these species in zoonotic giardiasis is uncertain (Halliday et al. 1995; Karanis et al. 1996; Graczyk et al. 2002; van Keulen et al. 2002).

Use of immunomagnetic beads to separate *Giardia* cysts from environmental surface waters, followed by DNA release and polymerase chain reaction (PCR) amplification of the target giardin gene has been used to improve the reliability of the detection of this parasite with improved sensitivity (Mahbubani et al. 1998). Another approach involves continuous separation channel centrifugation, which can simultaneously concentrate multiple pathogens as small as one micron with high and reproducible efficiency in a variety of water matrices (Borchardt and Spencer 2002). Unfortunately, cryptosporidial oocysts and *Giardia* cysts have also been found in bottled water in Brazil (Franco and Cantusio Neto 2002). It is now obvious that a large portion of the total organism loads in watercourses and in drinking water reservoirs result from rainfall and extreme runoff events. Thus, regular water samples are inadequate for accurately estimating the microbial contamination of watercourse systems. The procedures for raw-water surveillance within the context of multiple-barrier protection and risk assessment must

include sampling during extreme runoff situations (Kistemann et al. 2002). A new dye, Beljian red, has been shown to discriminate *Giardia* cysts seeded into water samples from those naturally present in the sample; this approach should be useful in determining the specific recovery of protozoan parasites from environmental samples (Ferrari et al. 2003).

The phagocytic capacity of hemocytes of the Asian freshwater clam, *Corbicula fluminea*, for *Giardia* cysts may be another useful mechanism for indicating contamination of waste waters and agricultural drainage with these parasites (Graczyk et al. 1997).

Although control efforts have emphasized water treatment issues, a recent study suggests that a number of species of rotifers ingested varying numbers of *Giardia* cysts, which were retained within the rotifers' bodies throughout the observation period. This type of ingestion may represent another means of reducing water contamination (Trout et al. 2002).

With the recently recognized efficacy of ultra-violet (UV) irradiation against *Cryptosporidium* oocysts, the inactivation of *Giardia* cysts was also investigated, as was cyst ability to repair UV-induced damage following typical drinking water and wastewater doses of 160 and 400 JM(−2). The infectivity reduction of *Giardia* cysts at these UV doses remained unchanged after exposure to repair conditions. Thus, UV disinfection at practical doses achieves appreciable (greater than 4 log) inactivation of cysts in water with no evidence of DNA repair leading to infectivity recovery (Linden et al. 2002).

During the past few years, giardiasis has received much publicity. With increased travel, there has been a definite increase in symptomatic giardiasis within the USA (Addis et al. 1992). Various surveys show infection rates of 2–15 percent in various parts of the world.

CHEMOTHERAPY

If giardiasis is diagnosed, the patient should be treated; however, the therapy is controversial, depending on geographical location in the world. In the majority of cases, giardiasis can be eliminated with the use of quinacrine, but its side-effects include: nausea, vomiting, headache, and dizziness. Vertigo, excessive sweating, fever, pruritis, corneal edema, myalgias, and insomnia have also been reported.

Metronidazole is also very effective and is approved by the World Health Organization (WHO, 1995) but is listed as the second drug of choice by the Centers for Disease Control in the United States because of potential carcinogenicity in rats and mutagenic changes in bacteria. Although these changes have never been demonstrated in humans and the issue is still debated, metronidazole is not recommended for pregnant women. The most frequent side-effects are gastrointestinal and include: nausea, vomiting, diarrhea, and crampy abdominal pain. Additional complaints may include metallic taste, headache, dizziness, drowsiness, lassitude, paresthesias, urticaria, and pruritis. Alcohol should be avoided when taking this medication. Although standard therapy usually cures the infection, some immunocompromised patients, including patients with AIDS, as well as some immunocompetent patients, have giardiasis that is refractory to therapy. Therapy with a combination of quinacrine and metronidazole has been reported to be effective (Nash et al. 2001).

Tinidazole has also been used and has proved more effective than metronidazole as a single dose (Jokipii and Jokipii 1979). Furazolidone is often used for treating children and paromomycin has been suggested for use in pregnancy (Davidson 1990).

In the absence of a parasitological diagnosis, the treatment of suspected giardiasis is a common question with no clear-cut answer. The approach depends on the alternatives and the degree of suspicion of giardiasis, both of which will vary among patients and physicians. It is not recommended that treatment be given without good parasitological evidence particularly since relief of symptoms does not allow a retrospective diagnosis of giardiasis; the most commonly used drug, metronidazole, also targets other organisms besides *Giardia*.

The third question involves treatment of asymptomatic patients. Generally, it is recommended that all cases of proven giardiasis be treated because the infection may cause subclinical malabsorption, symptoms are often periodic and may appear later, and a carrier is a potential source of infection for others. Certainly, in areas of the world where infection rates are extremely high, as well as the prospect of reinfection, the benefit per cost ratio also has to be examined.

INTEGRATED CONTROL

Because of the potential for wild animal, and possibly other domestic animal, reservoir hosts, other measures as well as personal hygiene and improved sanitary measures have to be considered (Brightman and Slonka 1979; Hewlett et al. 1982). Iodine has been recommended as an effective disinfectant for drinking water, but it must be used according to the appropriate directions (Jarroll et al. 1980; Jarroll et al. 1981; Zemlyn et al. 1981). Filtration systems have also been recommended, although they have certain drawbacks, such as clogging. Water-treatment systems tend to be unavailable in developing countries, many of which do not have piped water available to the general population. Boiling the water is effective, but carries with it the cost of fuel, another consideration in many parts of the world. Improved personal hygiene, routine hand-washing, better preparation and storage of food and water, control of insects that may come in contact with infected stools and then contaminate food or water, and treatment of symptomatic and asymptomatic individuals

would all lead to a decrease in the overall numbers of infections with this parasite.

Although the potential for a vaccine has been discussed, prospects are poor for the development of actual vaccine reagents in the near future. Control measures will continue to be centered on environmental and personal hygiene issues.

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Trichomonas infections

DONALD E. BURGESS

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INTRODUCTION

Of the three to five trichomonad species found in humans, only *Trichomonas vaginalis* regularly displays the capacity for producing disease. Human trichomoniasis persists and is essentially cosmopolitan in its occurrence despite the availability of effective chemotherapeutic agents and little evidence of the development of significant drug resistance. A sexually transmitted disease, trichomoniasis caused by *T. vaginalis* ranges from a mild or inapparent infection to one causing a chronic and substantial level of inflammation in the reproductive tract of women and significant urethritis in men (Krieger et al. 1993a). Rare cases of perinatal infection of females born to infected mothers (Sobel 1992; Smith et al. 2002; Szarka et al. 2002) and respiratory trichomonad infections (Walzer et al. 1978) have been reported, the latter possibly attributable to trichomonads other than *T. vaginalis*.

CLASSIFICATION

T. vaginalis is a parasitic protozoan and is classified as follows:

Class Trichomonadea, Kirby, 1947

Order Trichomonadida, Kirby, 1947 emend. Honigberg, 1974. There are typically 4–6 flagella, which may be recurved, free or attached to an undulating membrane (when present). There are usually no true cysts.

Family Trichomonadidae, Wenyon, 1926. Characterized by possession of: a cytostome; 3–5 free flagella (one flagellum on the margin of an undulating membrane); and an axostyle protruding through the posterior of the cell.

Genus Trichomonas, Donné, 1837. Trophozoites that typically possess: 4 free flagella (one along the outer margin of the undulating membrane); a costa at the base of the undulating membrane; and an axostyle. No cysts are present.

STRUCTURE AND LIFE CYCLE

The shape of *T. vaginalis* is typically pyriform in culture, although ameboid shapes are evident in parasites adhering to mammalian cells (Nielsen and Nielsen 1975). Light- and electron-microscopic studies (Honigberg and King 1964; Nielsen 1975), summarized by Honigberg and Burgess (1994), indicate that *T. vaginalis* is c. 9.7 × 7 μm and that nondividing organisms have four anterior flagella. In addition to the flagella (four anterior and one recurrent) the undulating membrane and the costa also originate in the kinetosomal complex at the anterior of the parasite. Internal organelles include a prominent nucleus and a rigid structure, the axostyle, that runs through the cell from the anterior to posterior end (Figure 1).

Figure 1 Trichomonas vaginalis *from culture, stained with Giemsa's stain, showing axostyle (left) and undulating membrane (arrow). Four distinct anterior flagella are visible (right). Bar = 10 μm. (Courtesy of M. Tighe, VMBL)*

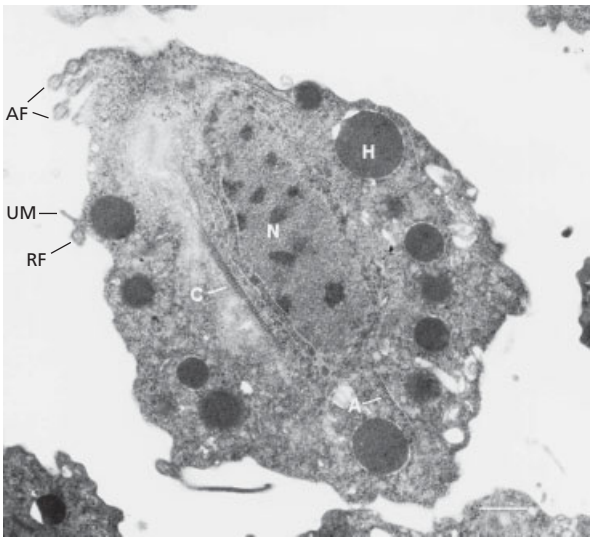

Figure 2 *Transmission electron micrograph of* Trichomonas vaginalis *from culture showing: axostyle (A), costa (C), hydrogenosome (H), nucleus (N), anterior flagella (AF), recurrent flagellum (R), undulating membrane (UM). Bar = 1 μm. (Courtesy of A. Blixt, VMBL)*

A respiratory organelle, the hydrogenosome (Lindmark and Müller 1973), is present and appears by light microscopy as a paraxostylar and paracostal chromatic granule. The hydrogenosome is seen as an osmiophilic, dense granule by electron microscopy (Nielsen 1975, 1976) (Figure 2).

The life-cycle of *T. vaginalis* is simple in that the trophozoite is transmitted through coitus and no cyst form is known. The trophozoite divides by binary fission giving rise to a population in the urogenital tract of humans.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Anaerobic metabolism produces most of the parasite's energy. Earlier work on the biochemistry of *T. vaginalis* (reviewed in Shorb 1964; von Brand 1973; Honigberg 1978) indicated that several sugars (e.g. maltose, glucose) are fermented by the parasite through a glycolytic pathway. Subsequent studies revealed that transport of glucose across the cell membrane occurred by facilitated diffusion (ter Kuile and Muller 1992). The metabolism of *T. vaginalis* differs from that of eukaryotic cells with mitochondria in three major areas:

1 the importance of inorganic pyrophosphates in carbohydrate catabolism
2 the importance of sulfur proteins in metabolism
3 the disposal of electrons by the formation of molecular hydrogen (Searle and Müller 1991; Müller 1992).

Trichomonads lack both cytochrome-mediated electron transport and the associated electron transport-linked phosphorylation, as well as the familiar mitochondrion that houses this system in other eukaryotic cells. Instead, the site of fermentative carbohydrate metabolism is the hydrogenosome (Lindmark and Müller 1973; Müller 1992), an organelle thought to have arisen by endosymbiosis with an anaerobic bacterium, or conversion of mitochondria (Lahti et al. 1992). Hydrogenosomes isolated from *T. vaginalis* have an NADH/ferredoxin oxidoreductase activity and these organelles ferment pyruvate to acetate, malate, molecular hydrogen and cxarbon dioxide. Pyruvate is also converted to acetyl-CoA via ferridoxin–oxidoreductase activity (Johnson et al. 1990; Land et al. 2001). This fermentation is dependent on adenosine diphosphate (ADP), inorganic phosphate, magnesium and succinate, and carbon dioxide-dependent carboxylation of pyruvate to malate by malate dehydrogenase. Detergent disruption (Triton X100) stops pyruvate-dependent formation of molecular hydrogen (this effect is overcome by adding exogenous ferredoxin), indicating that the structural integrity of the hydrogenosome is crucial to its effective functioning (Steinbuchel and Müller 1986).

The mechanism of development of anaerobic resistance to metronidazole also is controlled by hydrogenosomes in that metronidazole competes for hydrogen ions as an electron acceptor. In metronidazole-resistant *T. vaginalis* the expression levels of the hydrogenosomal enzymes (pyruvate:ferredoxin oxidoreductase, ferridoxin,

malic enzyme, and hydrogenase) are reduced dramatically which is likely to eliminate the ability of the parasite to activate metronidazole (Land et al. 2001).

Hydrolytic enzymes

Proteinases of *T. vaginalis* have been known since the 1970s and detailed investigations of these enzymes have been made during the last 10 years. Cysteine proteinases seem to be particularly prevalent, as dithiothreital stimulates the activity of several proteinases from *T. vaginalis*. These range from 20 to 110 kDa (Lockwood et al. 1987), the lower molecular weight proteinases being released from the cell (Lockwood et al. 1984, 1987, 1988; North et al. 1990). The release of proteinases from *T. vaginalis* is of interest in the context of its ability to elicit varying degrees of inflammation and the possible role of these enzymes in pathogenesis. There are reports of cell-detaching factors released by the parasite into the growth medium (Garber et al. 1989; Lushbaugh et al. 1989) some of which are reported to have trypsin-like activity. These factors are active on human and hamster cells, causing them to detach and round up. Release of cell-detaching factors and proteinases from *T. vaginalis* clearly implies that these parasite products could degrade proteins such as laminin, vitronectin and other components of the extracellular matrix, thus effecting the release of host cells from tissue.

The substrate specificity and structure of some of the proteinases of *T. vaginalis* are now being determined. Parasite proteinases were not inhibited by pepstatin, phenyl methyl sulphonyl fluoride or EDTA, but they were inhibited by iodoacetic acid, antipain, leupeptin and N-a-p-tosyl-L-lysine chloromethyl ketone. Lower molecular sizes of proteinases were released from the parasite; proteinases of 25, 27, and 34 kDa specifically hydrolyzed synthetic substrates with arginine–arginine residues whereas other proteinases had activity over a wide substrate range (North et al. 1990). Recent cloning studies of cysteine proteinase genes of *T. vaginalis* indicate that at least four distinct genes are present and that they have considerable homology (up to 45 percent identity) with cysteine proteinase genes of *Dictyostelium discoideum*. Three of the proteinase genes were present as single copies, whereas the other was present as a multiple copy gene. The amino acid sequence data indicated that these proteinases were of the L/cathepsin H/papain type (Mallinson et al. 1994).

Nucleic acids

Nucleic acid synthesis in trichomonads differs markedly from that in mammalian cells, as trichomonads do not synthesize either purines or pyrimidines de novo. *T. vaginalis* does not incorporate purine-ring precursors into nucleic acids but rather directly salvages purine nucleosides through adenosine and guanosine kinases. Neither inosine nor hypoxanthine are incorporated and no interconversion between adenylate and guanylate occurs (Heyworth et al. 1982). Trichomonads are also unable to synthesize pyrimidines from aspartate, orotate and bicarbonate, the typical de novo pathway. Neither thymidylate synthetase nor dihydrofolate reductase activity has been found. Conversion of cytidine by cytidine phosphotransferase and nucleotide kinases to cytidine monophosphate (CMP), cytidine diphosphate (CDP) and cytidine triphosphate (CTP) allows *T. vaginalis* to salvage exogenous cytidine, uridine, uracil and, to some extent, thymidine into its nucleotide pool (Wang and Cheng 1984). Cytidine is converted to uridine by cytidine deaminase, whereas uracil is converted to uridine by uridine phosphorylase before conversion into nucleotides. Nucleosides (but not nucleotides) are transported across the cell membrane of *T. vaginalis*. The nucleosides adenosine, guanosine and uridine are transported across the cell membrane by a two-carrier, facilitated transport mechanism at a rate apparently sufficient to sustain growth (Harris et al. 1988). Intracellularly, nucleosides are rapidly converted to nucleotides so that transport is not affected by any appreciable build up of nucleosides. Neither bases nor D-ribose affect uptake of nucleosides, suggesting that the carriers operating in *T. vaginalis* are similar to those in other parasitic protozoa such as *Plasmodium berghei* (Hansen et al. 1980) and *Leishmania donovani* (Aronow et al. 1987).

CLINICAL ASPECTS

T. vaginalis is site-specific, usually surviving in only the urogenital tract of humans although rare cases of pneumonia in newborns have been reported (Smith et al. 2002; Szarka et al. 2002). Approximately 10 percent of vulvovaginitis is due to infection with *T. vaginalis* (Sobel 1992). In men, clinical features of the infection may include urethral discharge and inflammation of the tissues lining the reproductive/urinary tract (Krieger et al. 1993a), but it is more usual for men to display no symptoms.

A female patient infected with *T. vaginalis* may have no obvious clinical signs or may suffer vaginitis of varying severity. Asymptomatic patients can also carry the infection and remain at risk of developing symptomatic disease at a later date (Krieger et al. 1988). Clinical features that may develop in 50–90 percent of infected women include: purulent vaginal discharge, pruritus, and dyspareunia. Leukorrhea and dysuria are occasionally seen. Vaginal discharge is observed in 50–75 percent of diagnosed women and is considered malodorous in only c. 10 percent of these patients. Although the discharge has often been described as yellow–green and frothy, only c. 8 percent of patients produce a discharge characterized as frothy and c. 59 percent produce a discharge characterized as

purulent. Diffuse vulvar erythema and copious vaginal discharge are often present. Punctate hemorrhages of the cervix may result in a strawberry-like appearance, apparent by inspection in only 1–2 percent of patients but apparent in 45 percent of patients examined by colposcopy (Wølner-Hanssen et al. 1989). Pruritus, particularly vulvar itching, is a frequent symptom (25–50 percent of patients) and may be severe (Sobel 1992).

IMMUNOLOGY

Some of the earliest investigations into immunity centered on reports that sera obtained from a variety of mammals can lyse *T. vaginalis* and agglutinate the parasite. Although earlier investigators considered lysis to be dependent on antibody action, *T. vaginalis* is now known to activate complement by the alternative pathway (Gillin and Sher 1981). An extensive study of normal human, bovine, sheep, horse, swine, and dog sera against *T. vaginalis* (Reisenhofer 1963, summarized by Honigberg 1970) showed that these sera had low (equal to or less than 1:64) but consistently detected agglutinating activities. Current understanding of immunity to *T. vaginalis* is unsatisfactory and it is not clear whether induced immunity is protective and if so, how. Although there is evidence that protection may be achieved by immunization of laboratory animals, strong protective immunity does not seem to follow natural infection in humans. Immunosuppressed persons, such as those with acquired immune deficiency syndrome (AIDS), do not develop fulminating *T. vaginalis* infections despite the high prevalence of the parasite in the general population. A recent study of patients infected with *T. vaginalis* and human immunodeficiency virus (HIV) indicated no evidence of increased levels or longevity of parasite infection in these patients compared to patients with *T. vaginalis* but without HIV (Cu-Uvin et al. 2002). These observations may indicate that innate immunity involving chemotaxis and subsequent influx of neutrophils are much more important than acquired immunity in controlling infections with *T. vaginalis* since neutrophils are often the most numerous leukocytes present in sites of infection.

Innate immunity

Innate immune responses to *T. vaginalis* are well documented and may be important in controlling infection as well as in inflammation during infection. Both macrophages and polymorphonuclear neutrophils (PMN) have been shown to be capable of killing *T. vaginalis* in vitro (Rein et al. 1980) and PMNs have been reported to be attracted to a factor(s) secreted by live *T. vaginalis* (Mason and Forman 1980). Leukocytic vaginal discharge was previously considered to be an important clinical finding expected in women infected with *T. vaginalis*, but more recent studies indicate no significant difference between the numbers of infected and uninfected women with vaginal discharge (Fouts and Kraus 1980). Even so, recruitment of macrophages and PMNs to the site of infection (by the sensitized T lymphocytes and the lymphokines they produce, or by parasite-derived factors that are directly chemotactic for PMNs) could be important elements in protective immunity and the pathological changes present in persons with trichomoniasis.

Acquired immunity

Early experimental studies on acquired immune responses to *T. vaginalis* examined various immunizations of laboratory animals and evaluated antibody from patients with *T. vaginalis* infection. Guinea pigs given intraperitoneal inoculations of vaginal material containing trichomonads produced antibody responses, as did rabbits given formalin-killed parasites (Riedmuller 1932; Tokura 1935). In mice, intramuscular inoculation induced protection against challenge infection that lasted at least 15 weeks (Kelly and Schnitzer 1952; Schnitzer and Kelly 1953). Intramuscular injection of *T. vaginalis* protected up to 100 percent of mice against an intraperitoneal challenge, but much less protection was afforded against a subcutaneous challenge (Kelly et al. 1954). The protective effect of sera from infected humans was shown in experiments in which human sera were transferred to mice inoculated intraperitoneally with *T. vaginalis*. Similar mouse inoculation experiments with human sera or rabbit hyperimmune sera indicated that these antibodies were both protective. Levels of protective antibodies in the sera of patients quickly diminish following elimination of infection (Teras and Nigesen 1969; reviewed in Honigberg 1970). The conclusion from these and subsequent observations (Sobel 1992) is that although an immune response to *T. vaginalis* occurs in infected patients and produces circulating antibody against the parasite, the response is short-lived and does not provide protection against re-infection.

Antibody responses during infection with *T. vaginalis* have been evaluated in numerous surveys by several immunoassays (summarized in Honigberg and Burgess 1994). Although some infected patients do not have detectable anti-*T. vaginalis* antibodies, most display secreted and circulating (serum) antibodies. Secretory antibody levels in the reproductive tract seem to increase in women infected with *T. vaginalis* and parasite-specific antibodies occur in the urogenital tract of persons harboring *T. vaginalis*. Immunoglobulin IgA antibody, specific for *T. vaginalis*, was present in vaginal secretions of 76 percent of 29 infected women and 42 percent of 19 apparently uninfected women with higher levels in the infected patients (Ackers et al. 1975). Subsequent reports have verified the presence of

parasite-specific antibody in vaginal secretions. Street et al. (1982) reported that 73.2 percent of infected women had anti-*T. vaginalis* IgG or IgA antibodies, compared to 41 percent of uninfected women. Additional studies on a women with proven *T. vaginalis* trichomoniasis have confirmed these findings (Romia and Othman 1991; Alderete et al. 1991a).

Early evidence that a cellular immune response is elicited in patients infected with *T. vaginalis* is based on the observation that such patients develop positive delayed-type hypersensitivity (DTH) reactions to whole parasite antigen preparations (Adler and Sadowsky 1947; Aburel et al. 1963). Later it was shown that blood lymphocytes from individuals with active infections proliferated in vitro when stimulated with whole cell antigen preparations; lymphocytes from uninfected persons gave minimal responses (Yano et al., 1983a, 1983b; Mason and Patterson 1985). Similar results have been obtained in early studies of DTH reactions (summarized in Honigberg and Burgess 1994) and lymphocyte proliferation responses in mice (Mason and Gwanzura 1988). These results, together with the findings in patients, indicate that systemic sensitization of T lymphocytes can occur during infection.

Antigens

Early immunological studies of *T. vaginalis* indicated that there were antigenic differences between geographically distinct strains of this parasite. In a series of studies in the 1960s, four serotypes (TLR, TN, TRT, and TR) were described by the use of cross-agglutination and complement fixation methods. These serotypes had common and unique antigens and were present in a large geographical area of central and eastern Europe (reviewed by Honigberg 1970). In another study, agglutination, complement fixation and fluorescent antibody methods were used to demonstrate three serotypes of *T. vaginalis* in one geographical area of the former USSR (Andreeva and Mihov 1976). This report was among the first to describe molecular differences between strains of *T. vaginalis* and to attempt to correlate these with serological type; on the basis of the electrophoretic patterns of ten strains of *T. vaginalis*, five different antigenic groups were detectable. Since this initial work, numerous antigens of *T. vaginalis* have been described, some being shared between strains and others being strain-specific.

Subsequently, antigens of *T. vaginalis* have been partially characterized including limited information about function. In one study, 20 immunogenic polypeptides, ranging from 20 to 200 kDa, were detected with rabbit antisera (Alderete 1983) many of which were determined to be on the parasite surface (Alderete et al. 1985). Additional studies using monoclonal antibodies prepared against one clone of *T. vaginalis*, showed that these monoclonal antibodies reacted with surface epitopes on four of nine distinct parasite clones (Torian et al. 1984; Connely et al. 1985). Western blot analysis of reactions indicated the presence of both conserved and heterogeneous epitopes and antigens (Torian et al. 1988).

Some isolates of *T. vaginalis* possess a 270 kDa surface antigen (P270) that is strongly expressed, whereas other isolates express little P270 on their surface (Alderete et al. 1986a, b). Expression of higher amounts of P270 correlates with lower *T. vaginalis* cytotoxicity toward HeLa cells, suggesting that the degree of expression of P270 may be a useful virulence marker. A 6-amino acid epitope of P270 (DREGRD) binds experimental antibodies specific for P270, as well as a high percentage of the antibody in serum from infected patients, suggesting that P270 could be an immunodominant antigen (Alderete and Neale 1989; Dailey and Alderete 1991). Additional *T. vaginalis* antigens have been detected, including a 230 kDa surface antigen that binds to vaginal antibodies present in infected patients (Alderete et al. 1991a) and proteinases that elicit serum and secreted antibodies (Alderete et al. 1991b; Bozner et al. 1992). Four antigenic surface molecules have also been implicated in the adhesion of *T. vaginalis* to vaginal epithelial cells, their expression being upregulated during attachment to host cells (Arroyo et al. 1993). Antibodies to these molecules protect target cells from parasite-mediated cytotoxicity (Arroyo et al. 1992) suggesting that anti-adhesion immune responses could be important in protecting against the pathogenic effects of *T. vaginalis*.

PATHOLOGY

Although *T. vaginalis* was first described by Donné (1836), its role as a cause of vaginitis was not recognized for another 80 years (Höhne 1916). The early report of Hogue (1943) on the pathogenic effects of *T. vaginalis* on cells in cultures suggested that the parasite could directly damage host cells. The clinical features, including pathogenicity, of human urogenital trichomonads have been reviewed (Jírovec and Petru 1968; Brown 1972; Catterall 1972; Honigberg 1990; Honigberg and Burgess 1994). Trichomoniasis is a frequently encountered, sexually transmitted disease (Lossick and Kent 1991) and a common cause of vaginitis and exocervicitis (Heine and McGregor 1993; Sobel 1992). Children born to women with trichomoniasis may suffer from low birth weight (discussed by Heine and McGregor 1993; Gibbs et al. 1992; Cotch et al. 1991), probably as a result of tissue damage from the cytopathic effect of this parasite on mammalian cells (Alderete and Perlman 1983; Krieger et al., 1985a).

Histological findings

Histological findings in women with trichomonad cervicitis have been described (Koss and Wolinska 1959); up

to one-third of biopsies of female patients infected with *T. vaginalis* show no histological changes. Patients displaying changes had increased vascularity of the squamous epithelium and the presence of the trichomonads was often accompanied by distension of blood vessels within papillae. The 'strawberry cervix' (Lang and Ludmir 1961; Fouts and Kraus 1980) appearance of the exocervix is due to the local extravasation of blood resulting in petechiae (rather than to ulceration) and is not usually due to inflammation. There is more pronounced dilation of epithelial vessels in patients with trichomonad cervicitis compared with those with nontrichomonad-induced cervicitis. Edema of the squamous epithelium is accompanied by separation of epithelial cells from each other, inflammation of the squamous epithelium, and desquamation. Perinuclear haloes may develop that can be confused with the koilocytotic atypia caused by human papilloma virus infection (Quinn and Holmes 1984); these are often confined to the basal layers of the epithelium. A proportion of epithelial cells display a variety of other abnormalities including enlarged nuclei, hyperchromasia and binucleation. Pyknotic nuclei are evident in the damaged epithelium indicating necrosis and a purulent exudate often coats the epithelial surface; such findings are rare in nontrichomonad cervicitis. Biopsy material from 11 women infected with *T. vaginalis* was examined by Nielsen and Nielsen (1975) in an electron microscope study. Generally, there was evidence of chronic nonspecific inflammation with subepithelial infiltration by neutrophils and lymphocytes. Three of the patients had cervical erosion; the lumens of the glands were packed with neutrophils. In cases of more severe inflammation, neutrophils were present in deeper layers of the epithelium, the neutrophils near the surface being arranged in lacunae. *T. vaginalis* was occasionally found on the surface of the stratified squamous epithelium in clusters situated in areas of shallow depression in the epithelial surface. The trichomonads were often seen on the vaginal surface in a dense mantle, but were not attached to the epithelium in observations in seven patients. Ameboid trichomonads attached only to necrotic epithelial cells with the undulating membrane on the surface away from the substrate. In the areas of contact between parasites and epithelial cells, there was interdigitation of cytoplasmic projections from the parasite and host cell, which is evidence of intimate contact between the parasite and host during human infection. In women with florid vaginitis, the secretion typically contains numerous motile trichomonads, thus rendering diagnosis reasonably easy. In women with latent trichomoniasis, in which the parasite has retreated into the cervical region and in which bizarre and atypically hyperplastic cells may be present in smears, the trichomonads are more difficult to find. In the diffuse or patchy inflammatory secretion, the trichomonads may not be motile. *T. vaginalis* is often difficult to recognize in routinely fixed and stained smears (such as those stained with hematoxylin and eosin). Antibody-based immunohistochemical procedures have been developed that compare favorably with wet mount methods (Kreiger et al. 1988).

Mechanism of pathogenicity

Studies of the cytopathic effects of *T. vaginalis* on a variety of cell lines in vitro provide support for a cytopathic mechanism in which both parasite–host cell contact and soluble factors play a role. It was previously known that efficient killing of several target cell lines by *T. vaginalis* required close approximation or contact between parasite and target (Krieger et al. 1985a; Alderete and Garza 1985, 1988). Evidence that parasite adhesion to target cells is important for induction of damage of targets has come from experiments in which various treatments, (Arroyo and Alderete 1989), low temperature (4°C) (Alderete and Garza 1985), cytochalasin D (Krieger et al. 1985a), trypsin (Alderete and Garza 1985), or cysteine proteinase inhibitors lowered adhesion of parasites to targets. These studies suggested that proteinaceous surface structures synthesized by the parasite played a role in adhesion. Four antigenic surface molecules have specifically been implicated in the adhesion of *T. vaginalis* to host cells (Enbring and Alderete 1992) and parasites in contact with HeLa cells or vaginal epithelial cells increase their expression of these adhesion molecules (Arroyo et al. 1993). Although these reports indicate that intimate association of *T. vaginalis* to targets leads to efficient killing in these experimental systems, they do not prove the absence of one (or more) soluble mediators responsible for host cell damage. There are several reports of soluble factors produced by *T. vaginalis* that are active against nucleated host cells (Garber et al. 1989; Lushbaugh et al. 1989; Pindak et al. 1986). Although most of the cell-detachment factors remain uncharacterized there is a recent report of a soluble lytic factor with phospholipase activity (Lubick and Burgess 2004). When homogeneous preparations of such molecules become available, the precise structure and function of such factors will be established and a better understanding of the mechanisms of pathogenesis of trichomoniasis at the molecular level will be possible.

DIAGNOSIS

Microscopy and culture procedures are the most widely used methods for the detection of *T. vaginalis* in men and women. The most commonly employed procedures are:

- direct microscopic examination of fresh (stained or unstained) material in wet mounts, accomplished with the aid of bright-field, dark-field, or phase-contrast microscopy

- microscopic examination of fixed and stained preparations, usually smears, stained with Giemsa's stain
- cultivation employing a variety of media.

A combination of the wet mount and culture procedures is both efficient and cost-effective in detection of *T. vaginalis*-positive cases among both women and men. Reports of an improved culture device, InPouch™ (BioMed Diagnostics Inc.), indicate that it compares favorably with standard culture procedures in a clinical setting (Draper et al. 1993; Levi et al. 1997; Barenfanger et al., 2002).

Immunological methods have not been used widely for diagnosis of *T. vaginalis* infection although the relative specificity and sensitivity of individual techniques has been improved recently. In a comparison of several methods, direct detection of *T. vaginalis* using fluorescein-labeled monoclonal antibody compared favorably with wet mounts, staining of fixed material and culture (Krieger et al. 1988). Immunohistochemical methods detected the related trichomonad, *Tritrichomonas foetus*, in sections of formalin-fixed tissue from the reproductive tract of infected cattle more easily than standard microscopic methods (Burgess and Knoblock 1988; Rhyan et al. 1995).

Antibody and antigen detection

Several types of enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlman 1971) have been developed for *T. vaginalis*, either to measure patient antibodies or to detect antigens of *T. vaginalis* in clinical samples. In one example, whole-cell antigen preparations of *T. vaginalis* (Street et al. 1982) together with aqueous extracts (Alderete 1984) were used as antigens to detect antibodies in serum and vaginal secretions of patients. Affinity-purified, rabbit antibodies to *T. vaginalis* have been used as both capture and detection antibodies in a sandwich ELISA to detect antigens of *T. vaginalis* (Watt et al. 1986). This assay had a sensitivity of 77 percent, a specificity of 100 percent, and was more sensitive than microscopic examination of wet mounts, but less sensitive than culture methods for detection of infection. Detection of *T. vaginalis* antigens in fluids on vaginal swabs by ELISA had a sensitivity of 93.2 percent and a specificity of 97.5 percent in a study of 44 culture-positive patients in a study group of 482 (Yule et al. 1987). Employing a monoclonal anti-*T. vaginalis* antibody as a capture and detection antibody, a sensitivity of 89 percent and a specificity of 97 percent have been achieved (Lisi et al. 1988).

Molecular methods

Nucleic acid hybridization methods for detection of *T. vaginalis* (Rubino et al. 1991) have sensitivity and specificity as good as culture methods. Riley et al. (1992) developed a polymerase chain reaction (PCR) method, using primers that produced a 102. bp product, termed A6p. They used the method to test 24 isolates of *T. vaginalis*, all of which reacted positively and similar results have been obtained with another PCR method using a different target (Kengne et al. 1994). In both cases, PCR procedures produced no false positive reactions with template DNA from sources such as human cells, other parasitic protozoa and viruses. A PCR assay using primers based on beta-tubulin sequences of *T. vaginalis* detected 22 of 23 culture-positive samples (Madico et al. 1998). Also, PCR methods were able to detect a higher prevalence in males than was detected by culture methods (Schwebke and Lawing 2002) and positive PCR results have recently been suggested as diagnostically useful where trichomoniasis may be undertreated (Wendel et al. 2002).

EPIDEMIOLOGY AND CONTROL

Trichomoniasis persists throughout the world, despite the availability of effective chemotherapeutic agents and seemingly adequate awareness of the disease on the part of physicians, public health workers and the general population (Schwebke 2002). In recent years, there has been a focus on *T. vaginalis*, due partly to heightened awareness of sexually transmitted diseases generally, as well as the fact that people with *T. vaginalis* infections seem to be at higher risk of HIV infection than are uninfected persons.

Infections with *T. vaginalis* are quite common; the prevalence of *T. vaginalis* in nonselected female populations is probably 5–20 percent. Most estimates of prevalence are based on data from selected patient populations (e.g. those attending sexually transmitted disease clinics); such estimates are unlikely to be representative of the population at large. Even so, the data indicate that there has been a slow decline in the prevalence of *T. vaginalis* infections over the past 20 years. In 1972, more than 180 million women and men were estimated to be infected with *T. vaginalis* worldwide (Brown 1972), including c. 2.5 million women in the USA and 1 million women in the UK (Catterall 1972). *T. vaginalis* infection prevalence in American women is estimated at 3 million cases annually (Rein 1990) and some investigators consider trichomoniasis to be the most common sexually transmitted protozoan disease (Levine 1991) and perhaps even the most common sexually transmitted disease (Hammill 1989). Although estimates in nonselected male populations are not as certain as for women, the prevalence in men is probably c. 50–60 percent of that in women (Rein and Müller 1989). One study noted a prevalence of 11 percent in men attending an STD clinic (Krieger et al. 1993b) which is comparable to the 17 percent recently detected in a similar study (Schwebke and Lawing 2002).

CHEMOTHERAPY

Treatment of vaginal trichomoniasis relies on the 5-nitroimidazole compounds: metronidazole, tinidazole, and ornidazole. Metronidazole is the only one of these currently recommended by the WHO (Schwebke 1995). Tinidazole with metronidazole have comparable efficacies when used in a single-dose regimen (Gabriel et al. 1982). Metronidazole acts by interfering with DNA synthesis, apparently as the result of the production of a transient cytotoxic intermediate form (Lossick 1990). Daily treatment for 7 days or treatment by a single 2 g dose gave equivalently high cure rates (more than 90 percent) (Lossick 1982). The daily treatment regimen consists of oral administration of 250 mg of metronidazole three times daily for 7 days, producing a cure rate of c. 95 percent (Lossick 1990; Sobel 1992). Although the multiple-dose regimen is efficacious for the patient even without treatment of the sexual partner, it suffers from a degree of noncompliance. The single-dose regimen is particularly effective if used with simultaneous treatment of sexual partners, but it does not protect against the relatively frequent event of prompt reinfection by untreated sexual partners. Because of the multifocal nature of infection with *T. vaginalis*, systemic (rather than local) treatment is preferred. In fact, endogenous reinfection by parasites harbored in the urethra and periurethral glands is known to occur when local treatment is attempted (Rein and Muller 1989).

VACCINATION

There is no vaccine currently available for use against *T. vaginalis*. The search for vaccines against protozoa is complicated by their ability to evade and modulate host immune responses. *T. vaginalis* may be able to evade the immune response as it exists largely outside the host in the lumen of the reproductive tract. Effector mechanisms such as secretory antibody (sIgA) may prove to be crucial in protection against the cytotoxic effects of this parasite on host cells. Many basic questions concerning the immune response to *T. vaginalis* remain unanswered and such lack of knowledge impedes vaccine development. For example, we do not know the mechanisms by which natural infections are eliminated, nor which target antigens elicit protective immunity. We do not even know if an effective protective immunity develops during natural infection. The absence of an appropriate laboratory animal model for trichomoniasis has delayed progress in answering these questions. The natural history of infection by *T. vaginalis* in humans suggests that adaptive immune responses during infection do not play a significant role in controlling infections. Indeed, the most prevalent leukocyte response in the reproductive tract during infection is by neutrophils and chemotaxis of neutrophils by components of *T. vaginalis* has been reported (Mason and Forman 1980). These findings, together with the pattern of frequent reinfection, suggest that effective immunological memory may not be established in most infected persons. Other explanations for the absence of protective immunity after infection are the presence of a considerable number of distinct antigenic types of *T. vaginalis* (Krieger et al. 1985b), or the occurrence of some form of antigenic drift over time, as has been observed with certain surface antigens (Alderete et al. 1986b). A combination of these phenomena could produce a re-infection pattern similar to that seen with the rhinoviruses that cause the common cold. If such drifts occur, new antigenic profiles in the parasite population would occur so rapidly that the host could not effectively respond to the ever-changing epitopes being displayed.

INTEGRATED CONTROL

Control of trichomoniasis due to *T. vaginalis* should include:

- thorough examination to determine infection status of the patient
- appropriate treatment of the patient and sexual partner(s)
- continued surveillance by public health agencies to provide updated prevalence estimates.

Current knowledge of the immune response to *T. vaginalis* is insufficient to predict whether the development of a vaccine represents a realistic goal. A rational vaccine development strategy should include research toward understanding the roles of certain surface antigens and secreted antigenic molecules (e.g. proteinases) and identification of new target antigens.

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Dientamoeba fragilis and other intestinal flagellates

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INTRODUCTION

The digestive tract of humans can serve as habitat for several species of flagellates belonging to protozoan orders Trichomonadida, Diplomonadida, and Retortamonadida. Besides *Giardia* and the trichomonads discussed in Giardiasis and *Trichomonas* infections, these are *Dientamoeba fragilis*, *Enteromonas hominis*, *Chilomastix mesnili*, and *Retortamonas intestinalis*. All are confined to the large intestine, the habitat with low digestive activity and an abundance of the intestinal flora. Although diverse in morphology and subcellular organization, these flagellates are united by their ability to live and multiply under anaerobic or microaerophilic conditions. All seem to depend on associated bacterial biocenosis for both nutrition and adjustment of proper physicochemical conditions. However, their metabolism remains unknown since they are rarely obtained in a condition suitable for biochemical experiments. Also, genomic studies are scarce and limited to sequence analyses of rRNA genes for phylogenetic investigations. The life cycles of most of these organisms are simple and direct and do not involve sexual processes.

Typically, these organisms alternate the proliferative trophozoite, multiplying by binary fission in the host intestine, and the resting resistant cyst that is discharged with feces and serves to transmit the infection. *Dientamoeba* does not produce cysts and is an exception from this generalization. Infection with the cyst-producing flagellates is acquired through contaminated food and water or by direct fecal–oral transmission. Just one of the species discussed (*D. fragilis*) is an enteropathogen of clinical importance; the remaining species are harmless commensals. Some pathogenic potential was ascribed to *Chilomastix* but has not been proved. An increase in the flagellate population is often secondary to a pathological condition of another etiology, indicating merely that the intestinal biocenosis is out of balance. Still, even the nonpathogenic intestinal flagellates deserve consideration both from the diagnostic standpoint and as indicators of a poor standard of personal hygiene and sanitation.

DIENTAMOEBA FRAGILIS

Classification

Dientamoeba fragilis Jepps and Dobell, 1918 is an unusual flagellate with no flagella and was originally classified as an ameba. However, further morphological studies by Dobell (1940) revealed its relationship to *Histomonas meleagridis*, a turkey pathogen related to the trichomonads. Dobell noticed the presence of centrodesmus (centrodesmose), a fiber connecting the nuclei of the binucleate forms of *Dientamoeba* and later identified as the extranuclear spindle characteristic for the mitosis of Trichomonadida. Trichomonad affinities were unequivocally proven by the electron microscopic studies of Camp et al. (1974) and further confirmed by molecular phylogenies based on a complete sequence of the small subunit rRNA gene (Silberman et al. 1996). Biological, clinical, and diagnostic aspects of *Dientamoeba* have been recently reviewed (Johnson et al. 2004).

Structure and life cycle

Dientamoeba occurs in a trophozoite stage only and does not encyst. Low resistance of trophozoites to unfavorable environmental conditions, failure to infect humans and primates with cultured trophozoites (Dobell 1940), and the close relationship with *Histomonas* led to the assumption that *Dientamoeba*, like *Histomonas*, can develop in a helminth vector. Burrows and Swerdlow (1956) proposed the pinworm *Enterobius vermicularis* as a probable vector and pinworms eggs as a vehicle for *Dientamoeba* transmission. Uninucleate ameboid cells have been observed in pinworm eggs (Burrows and Swerdlow 1956; Ockert 1990) but convincing direct evidence of their identity with *Dientamoeba* is lacking.

Trophozoites of *Dientamoeba* usually measure 7–12 μm in diameter although they can vary within a wider range (3.5–22 μm). They are uninucleate or binucleate with predominance of binucleate forms (60–80 percent) resulting from protracted telophase of the parasite mitosis. Nuclei are rounded with a central group of chromatin granules and delicate nuclear membrane without adjacent peripheral chromatin (Figure 1). The extranuclear mitotic spindle can be seen in properly stained binucleate forms as a fibril extending between the nuclei (Figures 1 and 2c).
Dientamoeba produces blunt pseudopodia and feeds by phagocytosis of bacteria or other particles such as rice starch in culture (Figure 2a, b).

Electron microscopy shows a profoundly reduced but recognizable pattern of the trichomonad cell (Camp et al. 1974), with typical association of striated parabasal fibrils and Golgi dyctiosomes into the parabasal apparatus, presence of the extranuclear mitotic spindle, and double-membrane-bound organelles corresponding to hydrogenosomes. *Dientamoeba* lacks kinetosomes, microtubular structures such as axostyle and pelta, and

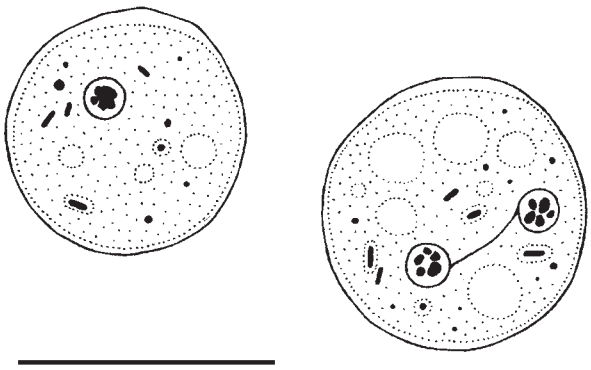

Figure 1 Dientamoeba fragilis*: uninucleate and binucleate trophozoites. Note the typical arrangements of chromatin granules within the nuclei and a fiber representing the extranuclear mitotic spindle (paradesmose) connecting the nuclei in the binucleate form. Based on iron-hematoxylin stained specimens. Bar = 10 μm.*

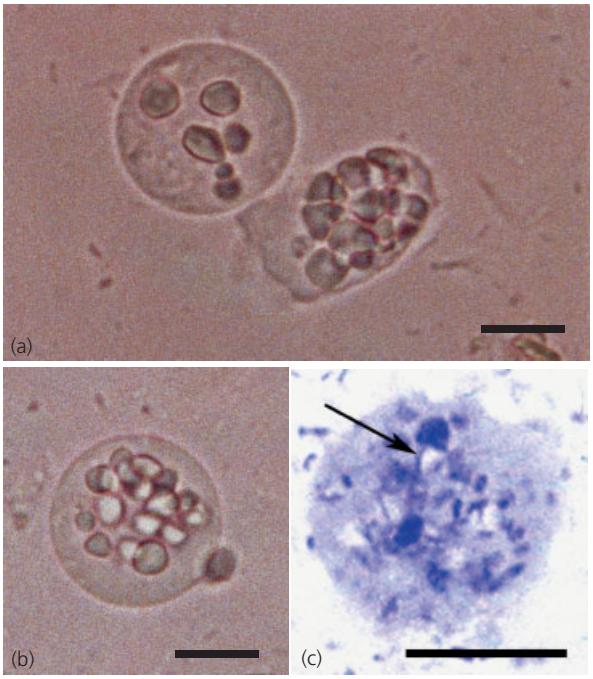

Figure 2 Dientamoeba fragilis*:* **(a, b)** *fresh mount from culture, trophozoites with ingested rice starch grains;* **(c)** *binucleate trophozoite with extranuclear spindle (arrow). Giemsa–Romanowski stain. Bar = 10 μm.*

any type of centriole. Microtubule organizing centers of the mitotic spindle are attached at the inner side of the base of parabasal fibrils that assume polar position in the mitotic cells. The cytoplasm contains glycogen granules and numerous food vacuoles (secondary lysosomes) with ingested particles in different phases of digestion. Mitochondria are absent.

Biochemistry and molecular biology

No experimental data are available on the biochemistry of *Dientamoeba*. The presence of hydrogenosomes and susceptibility to metronidazole suggest that the pyruvate-metabolizing pathway linked to ferredoxin-mediated electron transport known to operate in the hydrogenosome of trichomonads, is also functional in *Dientamoeba*.

The gene for small subunit ribosomal RNA (SSU rRNA) was cloned and sequenced for phylogenetic studies (Silberman et al. 1996) and fragments of this gene obtained by polymerase chain reaction amplification with trichomonad-specific primers, were used for riboprinting of *Dientamoeba* isolates (Johnson and Clark 2000). The genotyping of *Dientamoeba* based on direct amplification from human stool samples was reported by (Peek et al. 2004).

Clinical aspects

Despite controversies about the primary role of *D. fragilis* in the etiology of gastrointestinal disorders, suffi-

cient evidence is available to demonstrate that the organism is pathogenic (reviewed by Yang and Scholten 1977; Ockert 1990; Windsor and Johnson 1999). Symptomatic infections with *Dientamoeba* occur in both children and adults. The most frequent symptoms are abdominal pain, intermittent diarrhea, flatulence, and fatigue, sometimes accompanied by nausea, vomiting, and anorexia. The disease usually has a chronic, recurrent character and often is associated with a peripheral eosinophilia that is more common in children than in adults (Spencer et al. 1979, 1982). Acute diarrhea or cases presenting as eosinophilic or ulcerative colitis have also been observed (Cuffari et al. 1998; Shein and Gelb 1983). Disappearance of symptoms after elimination of *Dientamoeba* by chemotherapy from patients where *D. fragilis* was the only pathogen detected, have been reported repeatedly (Kean and Malloch 1966; Spencer et al. 1979; Ockert 1990). *Dientamoeba* can cause diarrhea in those with acquired immune deficiency syndrome (AIDS). Although the prevalence of *Dientamoeba* infection is higher in groups that are positive for human immunodeficieny virus (HIV) than in groups not infected with HIV (Mendez et al. 1994), there is no conclusive evidence that the pathogenic effect is more severe in HIV-positive patients than it is in HIV-negative individuals (Lainson and da Silva 1999).

Immunology

No systematic study on the immune response to the infection with *D. fragilis* has been published so far. Peripheral eosinophilia has been reported repeatedly, but its significance is not known. Chan et al. (1996) employed indirect immunofluoresence assay in a seroprevalence study of *Dientamoeba* infection in children and confirmed by immunoblotting with representative sera that the dominant antibody response is directed against a 39 kDa protein of *D. fragilis*.

Pathology

Histopathologic studies on appendices removed from young patients infected with *Dientamoeba* showed changes in the intestinal wall that varied from suppurative appendicitis to lymphoid hyperplasia and marked fibrosis in connective tissue of the submucosa (Burrows et al. 1954). The authors concluded that *D. fragilis* is noninvasive and assumed that irritation caused by the protracted presence of the parasite can induce low-grade inflammatory changes in the appendix that ultimately lead to fibrosis. In contrast, Shein and Gelb (1983) observed by sigmoidoscopy an invasive ulcerative process with numerous punctiform ulcers in the intestine of a woman infected with *Dientamoeba*. Flat ulcers and both acute and chronic inflammatory changes were demonstrated on biopsy but *Dientamoeba* was absent in aspirates from the areas of ulceration. According to Ockert (1990) inflammatory changes of the mucosa of rectum and sigmoid region of colon were relatively frequent in patients infected with *D. fragilis*, usually displaying a picture of low-grade chronic proctitis.

Diagnosis

Infection by *D. fragilis* should be considered in differential diagnosis in patients with abdominal pain, diarrhea, flatulence, vomiting, and unexplained eosinophilia. Unfortunately, awareness of the infection is low and methods used in many laboratories are unsuitable for the detection of this parasite. Examination of permanently stained smears prepared from fresh stools is mandatory for successful laboratory diagnosis of *Dientamoeba*; cultivation significantly increases parasite detection. It is essential that the fecal specimens are preserved and fixed or inoculated in suitable media soon after defecation, as survival time of *Dientamoeba* trophozoites is short. The collected specimens must not be refrigerated. Routine coprological concentration techniques are useless because *D. fragilis* does not form cysts, and trophozoites are destroyed during the procedure. Chan et al. (1993) reported employment of indirect immunofluoresence to facilitate detection of *D. fragilis* in stools.

Schaudin/polyvinyl alcohol fixative/preservative followed by iron hematoxylin or trichrome stain or a non-mercury-based alternative EcoFix followed by EcoStain (Garcia and Shimizu 1998), as recommended for differentiation of intestinal amebae, are suitable methods for microscopic diagnosis of *Dientamoeba*. The main diagnostic characters are nuclear morphology and presence of binucleate cells. The extranuclear spindle can be recognized occasionally and, if visible, can support the diagnosis unequivocally.

Diagnostic cultivation can be performed in the simple biphasic medium of Dobell and Laidlaw or in the more complex Robinson's medium (Clark and Diamond 2002). The Dobell–Laidlaw biphasic medium is essentially a slant of coagulated horse serum overlaid with Ringer's solution containing native egg white and supplemented with rice starch powder. By using this medium, Ockert (1990) reported an increase in detection efficiency in a large (over 1000) random sample of people in Halle, Germany from c. 2 percent diagnosed from stained fecal smears alone to c. 39 percent diagnosed from a combination of microscopy and cultivation. *Dientamoeba* usually multiplies in a primary culture and often survives for several subcultures, but successful establishment of a long-term culture is rare. Robinson's medium may facilitate establishment of cultures as it is designed to exert partial control over the concomitant bacterial flora. It is preconditioned by inoculation of a standard strain of *Escherichia coli* and unwanted associates are suppressed by erythromycin added to the medium at each subculture.

Epidemiology

DISTRIBUTION

Infections with *D. fragilis* occur worldwide (Windsor and Johnson 1999) with higher prevalence in children than in adults, and in women than in men. Although some published data are difficult to compare due to different sensitivities of the detection methods used, it is apparent that the infection is relatively frequent. In studies based on large samples of stools examined for parasites over 2–5 years, the incidence of the infection varied from 4 to 30 percent (Yang and Scholten 1977; Ockert 1990; Windsor et al. 1998). Prevalence studies in Germany and the USA (Ockert 1990; Grendon et al. 1995) showed the highest frequency of infection in children aged 6–9 years (47 percent), with variations between 10 and 82 percent observed in individual communities of children in Germany (Ockert 1990). Higher infection rates are usually linked to crowded conditions and poor standards of personal hygiene. Reports on the highest prevalence in adults (up to 50 percent) concern inmates of certain mental institutions and other closed or semicommunal groups (Wenrich 1944; Windsor and Johnson 1999). Interestingly, infections by the cyst-forming intestinal protozoa are most prevalent in a different age group (3–5-year-olds) and co-infections with *Dientamoeba* occur less frequently than those among other intestinal flagellates and amebae. Reported high prevalence and differences in age and sex distribution of subjects infected with *Dientamoeba* and other intestinal protozoa suggest that factors involved in *D. fragilis* transmission are unique to this species.

TRANSMISSION

The transmission of *Dientamoeba* is not completely resolved. The relatively high prevalence of the parasite is in sharp contrast to the absence of a resistant cyst stage and the fragility of the trophozoite, which does not survive in water (Wenrich 1944), and its viability in feces decreases rapidly. Moreover, the trophozoites were found to die in a solution simulating gastric juice (Yang and Scholten 1977), and attempts to infect a human volunteer and primates with trophozoites from culture given per os were unsuccessful (Dobell 1940). Thus, transmission by fecally contaminated food or water as well as direct fecal–oral transmission is unlikely.

In analogy with the closely related poultry pathogen *Histomonas meleagridis*, dissemination of *D. fragilis* infection by eggs of a helminth vector was proposed (Dobell 1940) and indirect evidence has been accumulated that the pinworm *Enterobius vermicularis* might be the hypothetical vector (Burrows and Swerdlow 1956; Yang and Scholten 1977; Ockert 1990). Association of infections with *D. fragilis* and pinworm has been observed at histological examination of human appendices (Burrows and Swerdlow 1956; Ockert 1990; Červa et al. 1991) as well as by routine examination for parasites, if proper detection methods were used (Yang and Scholten 1977; Ockert 1990). Statistical treatment confirmed that the frequency of these co-infections is significantly higher than would be expected at random, independent distribution of the parasite. Also, the increased prevalence of *Dientamoeba* in young people and women reflects a similar distribution of pinworm infections. Moreover, Ockert (1990) performed successful experimental self-infections with *D. fragilis* by ingesting washed pinworm eggs obtained from children infected with both *Enterobius* and *Dientamoeba*. There is also a report on *Dientamoeba* infection that followed accidental acquisition of enterobiosis by a person collecting perianal samples for pinworm diagnosis (Burrows and Swerdlow 1956).

Burrows and Swerdlow (1956) observed ameboid cells resembling mononuclear *Dientamoeba* trophozoites in eggs of pinworm females from subjects infected with *D. fragilis,* but not in worms from patients free from *Dientamoeba* infection. Ockert (1990) confirmed the presence of ameboid cells in the lumen of pinworm eggs and inside larvae encapsulated in the eggs. He further showed that these objects and *Dientamoeba* trophozoites from culture have the same value for isoelectric point after methylene blue staining. However, unequivocal identification of *Dientamoeba* in pinworms is lacking and attempts to isolate this parasite from pinworm eggs or larvae in culture were unsuccessful (Burrows and Swerdlow 1956; Yang and Scholten 1977; Ockert 1990).

Chemotherapy

Symptomatic *Dientamoeba* infections have been successfully treated with iodoquinol, metronidazole, secnidazole, chloramphenicol, tetracycline, and paromomycin (see Johnson et al. 2004 for references). Many authors consider iodoquinol (di-iodohydroxyquinoline) as the drug of choice for adults. Metronidazole and paromomycin have been used with success in children (Preiss et al. 1991; Cuffari et al. 1998).

It is difficult to recommend control measures against *Dientamoeba* infection without full knowledge of the factors involved in its transmission. However, strong indications for pinworm involvement suggest that good personal hygiene, such as that recommended for the prevention of enterobiosis, may be appropriate. Concurrent treatment for both *Enterobius* and *Dientamoeba* should also be considered when a patient infected with *Dientamoeba* belongs to a community where pinworm infections have been diagnosed.

Conclusions

Dientamoeba is a neglected pathogen that deserves higher awareness from diagnostic laboratories, clinicians

and researchers. The infection rate of *Dientamoeba* all over the world appears to be higher than originally thought, but successful diagnosis depends heavily on proper collection and processing of fecal specimens. The enteropathogenic potential of *Dientamoeba* for both children and adults has been documented and efficient chemotherapy is available. Therefore, it would be desirable to consider *D. fragilis* infection more consistently in differential diagnosis of gastrointestinal disorders and laboratories should adapt their diagnostic protocols to ensure dependable diagnosis of the parasite. The unsolved problems of the parasite life cycle and transmission should be revisited, as new techniques are now available to face this challenge.

ENTEROMONAS HOMINIS

Classification

Enteromonas is the type genus of the suborder Enteromonadina, which comprises uninucleate members of the protozoan order Diplomonadida. An ultrastructural homology of enteromonads with higher Diplomonadida such as *Hexamita* is evident (Brugerolle 1975). By topology of cell structures and organelles, *Enteromonas* resembles one half of a typical diplomonad. An evolutionary route from simple enteromonad-like forms to organisms with the double karyomastigont characteristic of higher Diplomonadida has been proposed and supported by a phylogenetic analysis based on ultrastructural characters (Siddal et al. 1992).

Structure and life cycle

Enteromonas hominis da Fonseca, 1915 is a small, cyst forming flagellate, inhabiting the cecum and other parts of the large intestine of humans and primates (Dobell 1935). The broadly oval trophozoites measure approximately 4–8 μm by 3–5 μm and possess three anterior flagella and one recurrent flagellum attached along two-thirds of the body length and continuing thereafter with a free portion. The adherent part of the recurrent flagellum is accompanied by a darkly staining fibril. The oval nucleus is located in the anterior part of the body, with its longer axis perpendicular to the longitudinal axis of the cell (Figures 3a and 4). The cysts are oval or subspherical with one to four nuclei; four nucleate cysts are considered mature.

Electron microscopy (Brugerolle 1975) revealed the presence of a narrow, shallow cytostomal groove on the ventral surface of the cell that follows the attached portion of recurrent flagellum. The lateral margins of the groove are elevated and each reinforced by a microtubular band descending from the kinetosomal area. The recurrent flagellum is lodged inside the cytosomal depression. *Enteromonas* feeds on bacteria that are endocytosed in the bottom of the cytostomal groove all along its length; cytopharyngeal tube is not developed. The cytoplasm contains numerous food vacuoles and rough endoplasmic reticulum, but mitochondria, Golgi apparatus, and microbodies are absent.

Diagnosis

Cultivation on biphasic media of the Dobell-Laidlaw type is the best method for diagnosis of *Enteromonas hominis*. Identification of this organism in stools may be difficult even on permanently stained smears processed by routine techniques. The protargol silver stain can help with accurate determination (Figure 4).

Epidemiology

DISTRIBUTION

Infections with *Enteromonas hominis* occur worldwide, but their incidence is usually low. An increased prevalence in a population (up to 10 percent) indicates poor hygienic standards.

POTENTIAL PATHOGENICITY AND TREATMENT

Enteromonas is generally considered to be a harmless commensal and treatment is not recommended. However, occasional cases of symptomatic infections in the absence of detectable pathogens have been reported (Spriegel et al. 1989; Goldberg 1990). As symptoms resolved when parasites were eliminated by treatment with metronidazole, the authors advocate use of this drug in similar cases.

CHILOMASTIX MESNILI

Classification

The genus *Chilomastix* belongs to the protozoan order Retortamonadida. Besides the human parasite, the genus includes other species that inhabit the intestines of numerous vertebrate and some invertebrate hosts. A free-living species has also been described.

Structure and life cycle

Chilomastix mesnili (Wenyon, 1910) Alexeieff, 1912 is a cyst-forming flagellate living in the cecum and colon of humans. The trophozoites are pyriform with a rounded or flattened anterior end and a tapered posterior projection (Figures 3b and 5). They measure 14 × 6 μm on average, ranging from 6–20 μm in length and 4–7 μm in width. *Chilomastix* possesses three anterior flagella and one recurrent flagellum confined to the cytostomal cavity. The cytostome forms a large cleft in the dorsal cell surface, extending from the anterior end for half the body length. Two cytostomal fibrils support

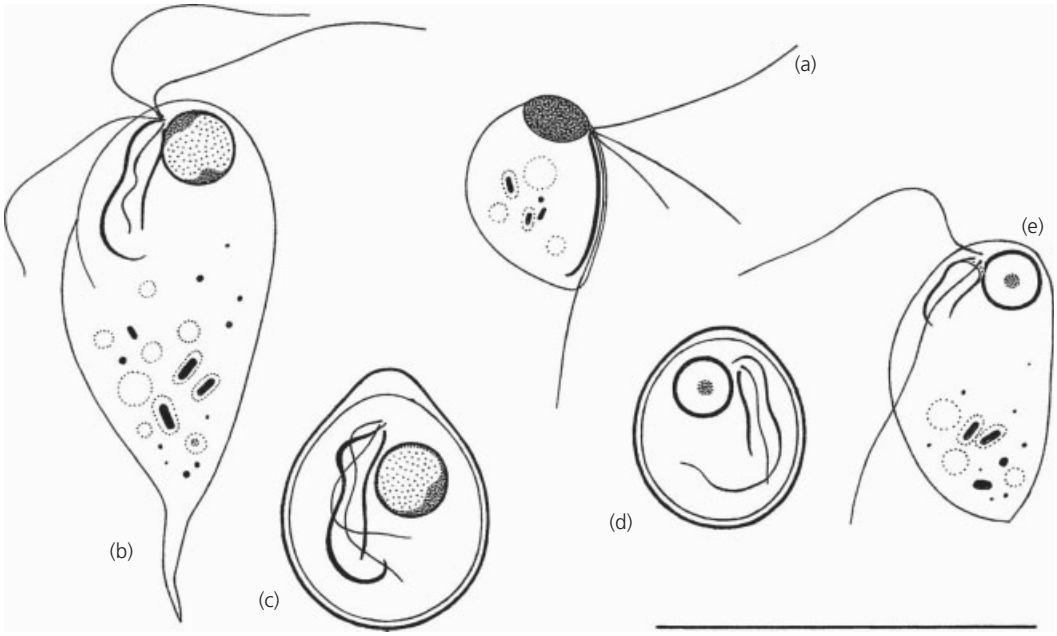

Figure 3 *Flagellates from the large intestine of humans, semidiagrammatic representation based on protargol stained specimens:* **(a)** Enteromonas hominis *trophozoite;* **(b, c)** Chilomastix mesnili*:* **(b)** *trophozoite,* **(c)** *cyst;* **(d, e)** Retortamonas intestinalis*:* **(d)** *cyst,* **(e)** *trophozoite. Bar = 10 μm.*

the edges of the cytostome. The fibril associated with the right cytostomal lip is prominent, characteristically curved, and turned round the posterior end of the cleft toward the tube of cytopharynx. The fibril of the left lip is straight and less conspicuous. The spherical nucleus is located dorsally at the anterior margin of the cell. The cysts are lemon-shaped with a broadly rounded posterior end and narrowed anteriorly. They measure 6–10 by 4–6 μm and contain a single nucleus, internalized flagella and both cytostomal fibrils (Figure 5).

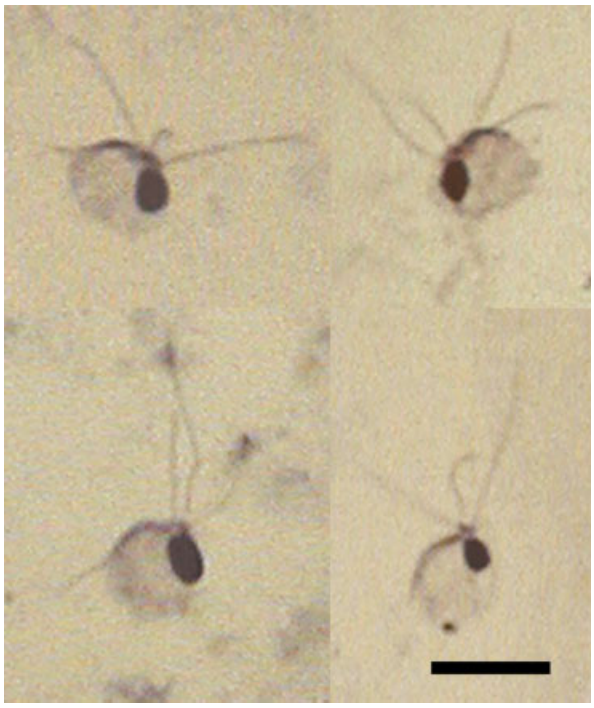

Figure 4 Enteromonas hominis *trophozoites. Protargol silver strain. Bar = 5 μm.*

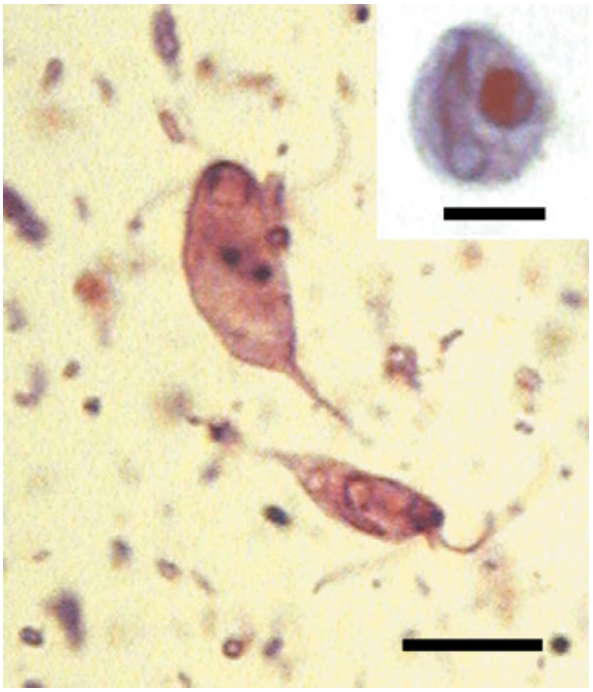

Figure 5 Chilomastix mesnili *trophozoites. Protargol silver stain. Bar = 10 μm. Insert:* Chilomastix mesnili *cyst. Iron-hematoxylin stain. Bar = 5 μm.*

Electron microscopy revealed details of components and the assembly of cytoskeletal structures associated with kinetosomes, cytostome, and cytopharynx, and showed that the cell is completely surrounded by a corset of subpellicular microtubules (Brugerolle 1973). The trophozoite cytoplasm contains numerous food vacuoles and rough endoplasmic reticulum but lacks mitochondria, Golgi apparatus, and any type of microbody-like organelles. Endocytosis of bacterial food takes place in a microtubule-free area of the cytopharynx.

Potential pathogenicity

It is generally accepted that *C. mesnili* is nonpathogenic and its association with diarrhea does not reflect a causal relationship. Therefore, no specific therapy is recommended. Occasional case reports ascribing pathogenic potential to this organism are not very convincing, excepting a single well-documented study. Červa and Větrovská (1958) investigated the etiology of periodic outbreaks of watery diarrhea in a community of institutionalized children aged 1–3 years and concluded that *Chilomastix* was responsible for the disease. Affected children passed four to ten stools in a day; diarrhea was painless but caused a moderate dehydration, decreased weight gain or loss of weight. Infectious origin of the outbreaks was evident and linked to unsatisfactory hygienic conditions in common bathrooms. No bacterial pathogens were found despite repeated examination and symptoms were insensitive to antibacterial chemotherapy. When *Chilomastix* was detected as a potential agent of the infection, the children were treated with carbasone. The treatment resulted in the elimination of parasites and disappearance of symptoms, and marked improvement in weight gain followed. These observations suggest that the possibility of *Chilomastix* occasionally acting as moderate pathogen for small children should not be entirely ignored.

Diagnosis

Trophozoites can be found in soft or liquid stools, cysts predominate in the formed stools. Both trophozoites and cysts can be easily recognized on permanently stained smears by their characteristic morphology – in particular, by the presence of cytostome with the prominent, characteristically curved cytostomal fibril. Cultivation on biphasic media such as Dobell–Laidlaw medium can increase diagnostic recovery.

Epidemiology

DISTRIBUTION AND TRANSMISSION

Distribution of *Chilomastix mesnilli* in humans is cosmopolitan with incidence ranging from 0.2 to 1 percent in standard surveys. No marked differences can be seen in the results of surveys performed in Germany, USA, Mexico, Chile, Indonesia, and Australia. Increased prevalence of the infection has been reported in specific groups with a history of diarrhea; for example, in US troops deployed in Egypt (11 percent, Oyofo et al. 1997), closed communities of institutionalized children (40 percent, Červa and Větrovská 1958) or selected groups of diarrheic patients (12.3 percent, Felsenfeld and Young 1946). No significant differences have been observed in the infestation rate of *Chilomastix* in HIV-positive and HIV-negative groups (Mendez et al. 1994). The species of *Chilomastix* occurring in monkeys and pigs is probably identical with *C. mesnili* as indicated by morphology and by successful experimental transmissions of the human flagellate to these animals. However, natural infections of animals are infrequent; about 1 percent of pigs have been found infected in surveys performed in Europe and the USA.

Interhuman transmission of the *C. mesnili* infection doubtless is of primary importance. Infection of humans by cysts of animal origin cannot be ruled out, but has not been documented. Good sanitation and personal hygiene can prevent the infection.

RETORTAMONAS INTESTINALIS

Classification

Retortamonas is the type genus of the protozoan order Retortamonadida. The genus includes numerous species inhabiting the intestines of vertebrate and invertebrate hosts. A phylogenetic analysis based on sequences of the gene for SSU rRNA (Silberman et al. 2002) showed close relationship of retortamonads to Diplomonadida.

Structure and life cycle

Retortamonas intestinalis (Wenyon and O'Connor, 1917) Wenrich, 1932 is a rare inhabitant of the human cecum. It is a small cyst-forming flagellate with trophozoites of an elongated pyriform shape, measuring 4–8 by 3–4 μm and equipped with a cytostome (Figure 3e). The cytostomal cleft of oval shape is supported by two lateral fibrils of unequal length and covered anteriorly by a membranous roof. In routinely stained specimens, the margin of the roof together with both lateral fibrils may appear as a single fibril rounding the anterior edge of the cytostome and extending posteriorly along each side. A tube-like cytopharynx extends from the cytostomal groove into the cell interior and ingested bacteria are endocytosed at its end. *Retortamonas* possesses two flagella; the anterior flagellum is free, the recurrent flagellum passes through the cytostomal groove and after emerging from it continues as a relatively long free portion. The spherical nucleus is located at the anterior margin of the cell. Mitochondria, Golgi apparatus, and

microbody-like organelles are absent. Arrangements of the *Retortamonas* cytoskeleton and other ultrastructural features of this parasite were described by Brugerolle (1977). The cysts are ovoid with a narrowed anterior end; they measure 4.5–7 by 3–4.5 μm and contain a single nucleus, internalized flagella, and cytostomal fibrils (Figure 3d).

Potential pathogenicity

Although the parasite has also been detected in diarrheic stools, there is no evidence of its pathogenicity. Therefore, no treatment is indicated. Metronidazole has been successfully used to clear the parasite from an unusual localization in the pancreatic juice of a patient with choledocholithiasis (Kawamura et al. 2001).

Diagnosis

Identification of trophozoites and cysts of *Retortamonas* on permanently stained smears stained by standard methods may be difficult but is possible. Small size, presence of the cytostome, and the spherical, apically localized nucleus can help in recognition of the organism even if the flagella are not clearly visible. Protargol silver stain can be used for exact determination. The best method for diagnosis is cultivation on biphasic media for intestinal protozoa and subsequent examination of cultured flagellates by phase contrast microscopy or in fixed and stained preparations.

Epidemiology

DISTRIBUTION AND TRANSMISSION

Infections of humans with *R. intestinalis* are infrequent, with a low prevalence even in groups living under conditions of poor personal hygiene and unsatisfactory sanitation (up to 2 percent). Autochthonous infections have been reported from all continents except Europe. Infection is alimentary, transmitted by cysts disseminated by fecal contamination. Retortamonads of similar morphology live in a variety of mammals, but zoonotic transmission is unlikely. The morphologically identical monkey parasite is considered to be conspecific with *R. intestinalis*, but attempts to infect monkeys by the human flagellate failed (Dobell 1935).

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Balantidium coli

VIQAR ZAMAN AND F.E.G. COX

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HISTORICAL INTRODUCTION

Balantidium coli is the only ciliate known to infect humans and can cause dysentery. Asymptomatic infections are not uncommon, but actual disease is rare and fewer than 1 000 cases of balantidial dysentery have ever been reported. In contrast, infection in pigs is extremely common and it is generally believed that pigs act as the main reservoir for human infection. The subject has been reviewed by Zaman (1993).

Historically, the genus *Balantidium* was first recognized by Claparède and Lachmann (1858–61), who found these ciliates in the rectum of frogs. Since then *Balantidium* species have been found in a variety of animals (Corliss 1979). In 1856 Petr Henrik Malmsten, a Swedish physician, found a ciliate in the dysenteric feces of two Swedish patients which, after wide consultation with Swedish, Norwegian, and Irish colleagues, he called *Paramoecium coli* (Malmsten 1857). This ciliate was indisputably *Balantidium coli* and in 1861 Rudolf Leuckart discovered a similar organism in the large intestine of a pig (Leuckart 1861). Shortly afterwards, Stein pointed out that the human parasite described by Malmsten and the pig ciliate described by Leuckart were morphologically identical and named them both *Balantidium coli* (Stein 1863). Several sightings of other ciliates from humans were made in the 19th and early 20th centuries (see Dobell and O'Connor 1921; Wenyon 1926) but these are no longer regarded as valid. Some early writers have suggested that Leeuwenhoek actually saw this ciliate and was even suffering from balantidial dysentery, suggestions that Dobell (1920, 1932) robustly dismisses. For many years there was some controversy concerning whether or not the species in humans, and pigs are the same and whether *B. coli* from pigs can infect humans, but there now seems to be no doubt that both of these statements are true (see Dobell and O'Connor 1921).

CLASSIFICATION

The genus *Balantidium* belongs to the phylum Ciliophora, class Litostomatea, order Vestibuliferida, and family Balantidiidae (Lee et al. 1985). McDonald (1922) proposed that the pig and human species should be separated as *B. suis* and *B. coli*. This separation has not been accepted, and the parasites in the two hosts continue to be regarded as a single species by most authors.

STRUCTURE AND LIFE CYCLE

Structure

B. coli has a trophozoite and a cyst stage. The trophozoite is actively motile and is the invasive stage; the cyst is the resistant form and the infective stage. The length of the trophozoite varies from 30 to 300 μm and the width from 30 to 100 μm (Sargeaunt 1971). The body shape is also variable, but is generally ovoid and slightly flattened on one side (Figures 1 and 2). At the

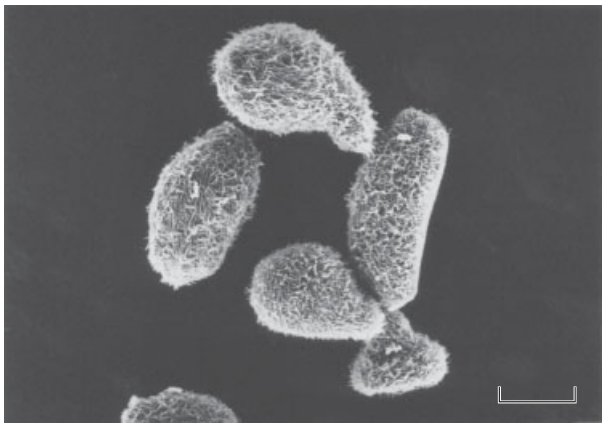

Figure 1 B. coli *trophozoites, showing variability in size and shape. SEM; bar = 30 μm*

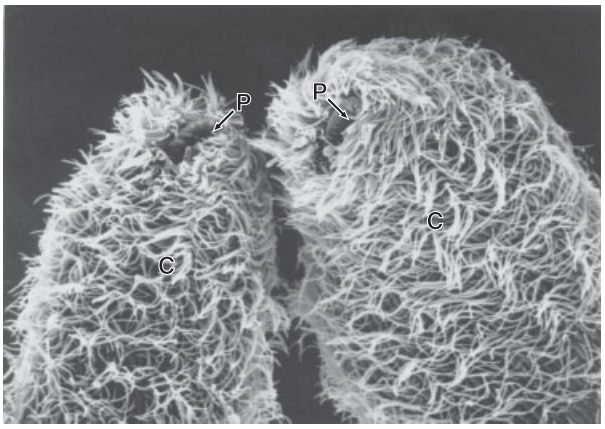

Figure 3 B. coli *trophozoites, showing peristome (P) and cilia (C) covering the body. SEM; bar = 3 μm*

anterior end of the trophozoite there is a funnel-shaped depression or mouth through which food is ingested. The mouth consists of a peristome, cytopharynx, and cytosome. The food passes from the peristome to the elongated cytopharynx and then to the cytosome. The cytopharynx, not visible when the parasite is filled with food, is often small but sometimes reaches about half the length of the parasite. The peristome appears as a rigid structure with striations when observed with the aid of a scanning electron microscope (SEM) (Figures 3 and 4).

The cilia, the organs of locomotion, cover the whole parasite and are embedded in the pellicle in longitudinal rows known as kineties. The number of kineties varies from 36 to 106 (Krascheninnikow 1962). The ciliary movement can be easily observed with the aid of a light microscope and the structure of the cilia is readily seen by SEM (Figures 3 and 4). The peristomal cilia are larger than those on the body (the somatic cilia). The peristomal cilia are used for propelling food into the cytopharynx (Figure 5). The food particles on being ingested become surrounded by a vacuolar membrane and digestion takes place inside the vacuoles. *B. coli* is capable of ingesting a variety of food particles, such as bacteria, starch grains, red blood cells, fat droplets, etc. The parasite has an excretory opening at the posterior end known as the cytopyge, which is circular and much smaller than the peristome (Figure 6).

There are two contractile vacuoles which may lie side by side or one above the other. They are easily visible to an observer using a light microscope with interference or phase-contrast capability (Figure 7). These vacuoles are responsible for maintaining the proper osmotic pressure in the cell by drawing excess water from the cytoplasm and ejecting it to the exterior.

The trophozoite has two nuclei which are clearly visible in stained preparations (Figure 8). The macronucleus, which is large and situated near the middle of the body, may be spherical, curved, elongate, or kidney shaped (Figure 9) and is enclosed in a nuclear membrane from which it can be removed intact by

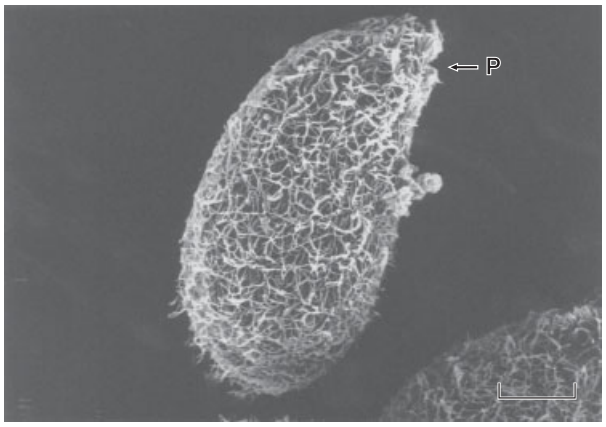

Figure 2 B. coli *trophozoite, showing peristome (P) at the anterior end. SEM; bar = 15 μm*

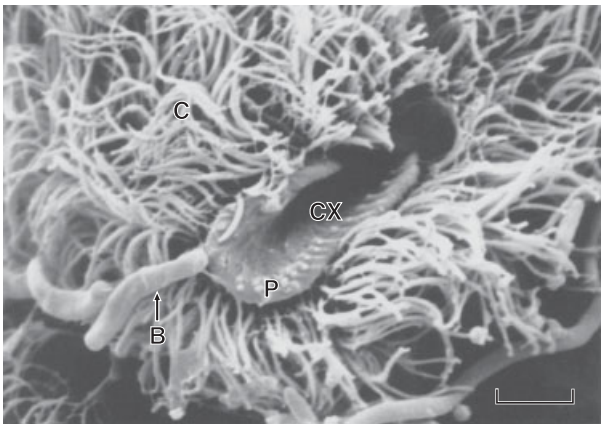

Figure 4 B. coli *trophozoite, showing peristome (P) with striations. A bacterium (B) is lying close to the peristome. CX, cytopharynx. SEM; bar = 1.5 μm*

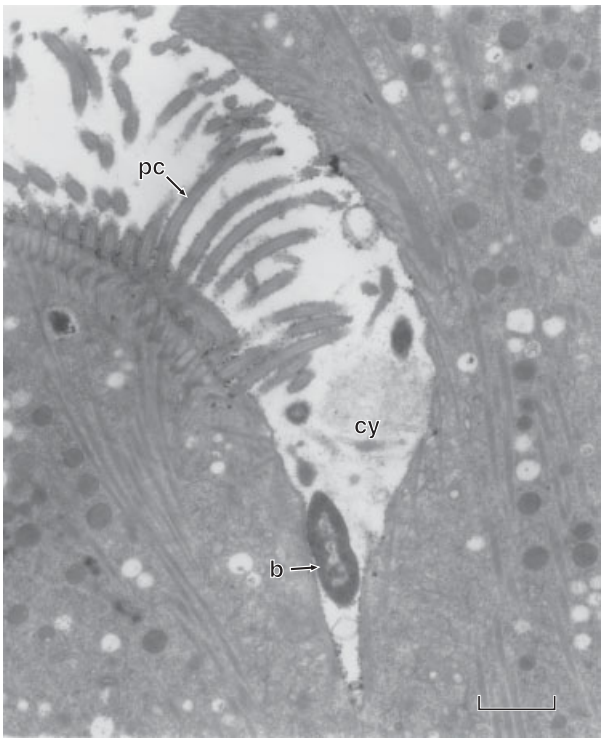

Figure 5 B. coli *trophozoite, showing peristomal cilia (pc) and cytopharynx (cy). A bacterium (b) is lying inside the cytopharynx. TEM; bar = 0.8 μm*

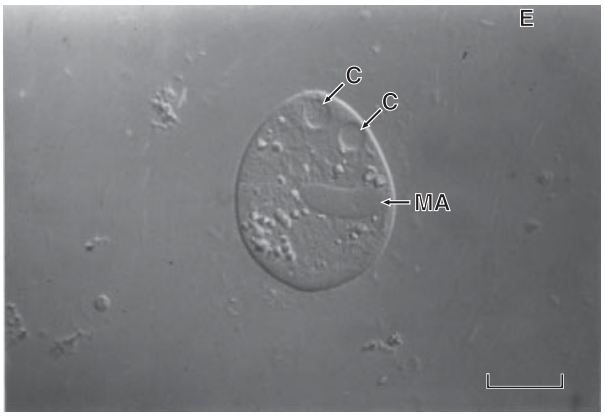

Figure 7 B. coli *trophozoite. Live organism showing macronucleus (MA) and 2 contractile vacuoles (C). Interference contrast; bar = 6 μm*

breaking the cell membrane (Figure 10). At the ultrastructural level numerous small nucleoli are visible inside the macronucleus (Figure 11). The single micronucleus is small and lies in close proximity to the macronucleus (Figures 8 and 9).

The cyst of *B. coli* is spherical to ovoid with a diameter of 40–60 μm (Figure 12). The cyst wall is thick and transparent. In electron microscopic images it is 400–500 nm thick (Figures 11 and 13). The parasite is visible inside the cyst and, in newly formed cysts, shows movement but, as the cyst matures, the cilia are absorbed and the movement ceases. The encysted stage, like the trophozoite, has a macronucleus and a micronucleus.

Life cycle

The parasite is transmitted by the oral–fecal route and the cyst is the infective stage. Excystation probably occurs in the small intestine and multiplication occurs in the large intestine.

Multiplication is by binary fission and the earliest indication of it is the elongation of the organism. Elongation is followed by the formation of a transverse structure through the middle of the body. The body gradually begins to constrict and finally separates into two daughter individuals (Figure 14). The ciliary activity continues during this process; the anterior cell develops a new excretory pore and the posterior cell a new mouth.

Sexual union (syngamy) is an important aspect of this parasite's life cycle and occurs by a process of conjugation, in which two cells come in contact with each other at their anterior ends and exchange nuclear material

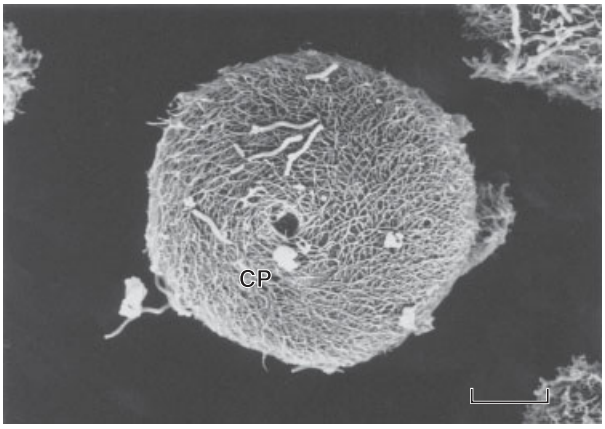

Figure 6 B. coli *trophozoite, showing the cytopyge (CP) at the posterior end. SEM; bar = 1.5 μm*

Figure 8 B. coli *trophozoite, showing macronucleus (MA) and micronucleus (MI). Trichrome stain; bar = 6 μm*

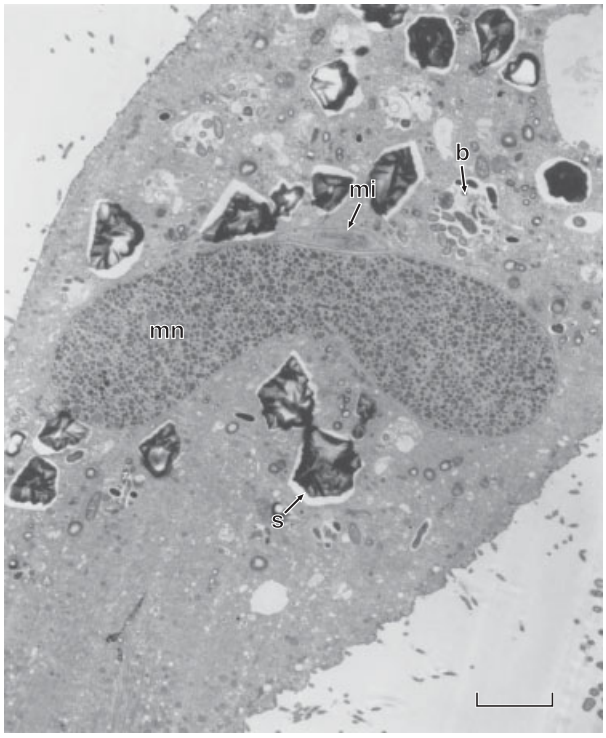

Figure 9 B. coli *trophozoite, showing the macronucleus (mn) and a small spindle-shaped micronucleus (mi). Ingested bacteria (b) and starch (s) are seen in the cytoplasm. TEM; bar = 3 μm*

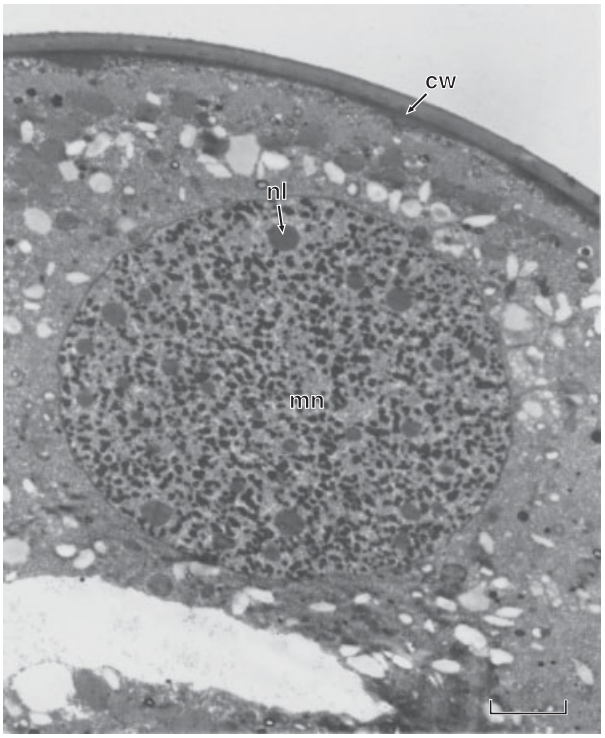

Figure 11 B. coli *cyst, showing the cyst wall (cw), macronucleus (mn) and nucleoli (nl). TEM; bar = 1 μm*

(Figure 15). Conjugation lasts for a few moments, after which the cells detach. There is no increase in numbers as a result of conjugation.

In the infected animal the parasite may be passed in the feces as a trophozoite or a cyst. The trophozoite does not encyst outside the body and disintegrates. The passed cyst survives and may contaminate food and water and may then be passed to other animals or humans. Pig-to-pig transmission is very common, and virtually 100 percent infection occurs in some piggeries where hygienic conditions are poor.

BIOCHEMISTRY

Little is known about the biochemistry of *B. coli*. Agosin and von Brand (1953) found that the ciliate is capable of consuming considerable amounts of oxygen despite the fact that it normally lives in the large intestine where little if any oxygen is available. The parasite prefers anerobic conditions, however, and in the absence of oxygen it produces a large amount of carbon dioxide. Carbohydrates are the main source of energy. Templis and Lysenko (1957) found that *B. coli* can produce hyaluronidase, which probably helps it in its invasion of

Figure 10 B. coli *trophozoite, showing the macronucleus after disruption of the cell membrane. SEM; bar = 3 μm*

Figure 12 B. coli *cyst. The transparent cyst wall (CW) and the macronucleus are visible. Trichrome stain; bar = 15 μm*

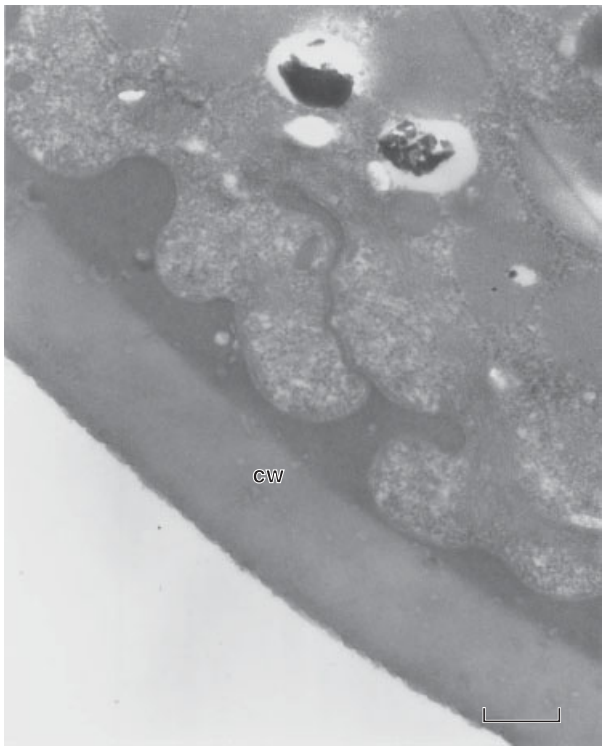

Figure 13 B. coli *cyst, showing the cyst wall (CW) at high magnification. TEM; bar = 0.2 μm*

the host tissues by dissolution of the intracellular ground substance.

CLINICAL ASPECTS

Asymptomatic carrier condition

Asymptomatic carriers harbor the parasites and continue to pass cysts in their feces. They are responsible for spreading infection, especially in insanitary institutional environments, such as long-stay psychiatric units.

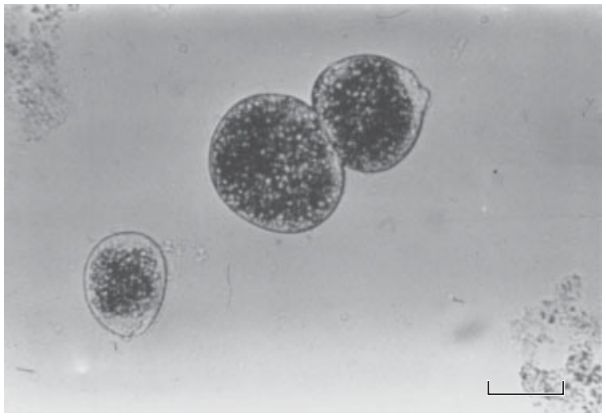

Figure 14 B. coli *undergoing binary fission. Live preparation; bar = 15 μm*

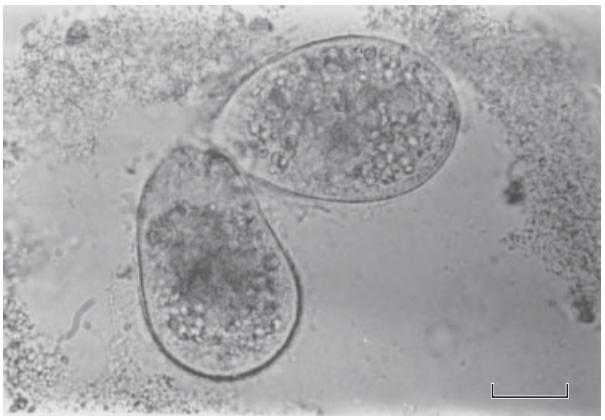

Figure 15 B. coli *undergoing conjugation. Attachment occurs at the anterior end. Live preparation; bar = 15 μm*

Chronic disease

Patients with chronic disease have periods in which frequent bowel movements alternate with periods of constipation. The feces are mucoid and rarely bloody. The organism is not easily seen in the feces and repeated stool examination may be necessary for its detection.

Acute disease

In acute disease, patients have diarrhea and the feces contain a great deal of mucus and blood (Castro et al. 1983). The clinical presentation is identical to that of acute amoebic dysentery. There may be fever and other intestinal symptoms such as anorexia, nausea, epigastric pain, vomiting, and intestinal colic. This may lead to severe dehydration, and sometimes to renal insufficiency. There is usually pain and tenderness in the cecal region. The symptoms may mimic those of appendicitis. In a majority of patients recovery occurs in 3–4 days even without treatment. In some patients, especially immunocompromised and malnourished ones, death may occur due to extensive destruction of the large intestine, involvement of the appendix, perforation, peritonitis, dehydration, and renal failure. In patients with acute infection, extraintestinal involvement such as liver abscess formation, pleuritis, and pneumonia may occur.

Immunology

There is no information about any immune responses to infection with *B. coli*. There is some evidence of age-acquired immunity in rhesus macaques where the parasite counts decrease significantly with age (Knezevich 1998). In some studies only children have been found to be infected (Devera et al. 1999), suggesting the acquisition of immunity, but this could be due to other factors such as changes in behavior. There has been a single

record of balantidial dysentery in an individual with HIV infection, and the authors suggest that the switch from asymptomatic infection to clinical dysentery might be due to immunosuppression resulting from the viral infection (Clyti et al. 1998). In this context it is interesting to note that some rhesus macaques infected with the simian immunodeficiency virus (SIV) were found to be infected with *B. coli* although the cause of diarrhea in these animals was primarily *Escherichia coli* infection (Mansfield et al. 2001). The rarity of severe human infections and the ease with which they can be cured mean that there is no need for a vaccine. The presence of conspicuous and easily identifiable cysts means that there is no need for an immunodiagnostic test.

Pathology

The gross pathologic appearance of the large intestine in patients with *Balantidium* infection is similar to that in patients with amoebiasis. On rare occasions the terminal ileum may also be infected. The gross changes consist of multiple ulcers with necrotic bases and undermined edges. The intervening mucosa may or may not be inflamed. On microscopic examination, parasites are frequently seen in clusters in the submucosa or at the bases of crypts. They can easily be recognized because of the presence of the macronucleus which stains deeply with hematoxylin and eosin (Figure 16). The cellular response is mainly lymphocytic, with some plasma cells being present. Neutrophils are few unless there is a superimposed bacterial infection. Sometimes the parasites may invade the regional lymph nodes and then they may be detected inside the lymphatic tissues. Extraintestinal balantidial infections are rare; there has been one report of *B. coli* in the bladder (Maleky 1998), but this report must be regarded with caution given our knowledge of ciliocytophthoria mentioned below.

The factors that determine whether or not *B. coli* invades the gut wall are unclear. However, in pigs, there is evidence that the presence of *Campylobacter coli* facilitates the invasion of the mucosa and both organisms seem to participate in the colitis associated with the infection (Hosino et al. 1999).

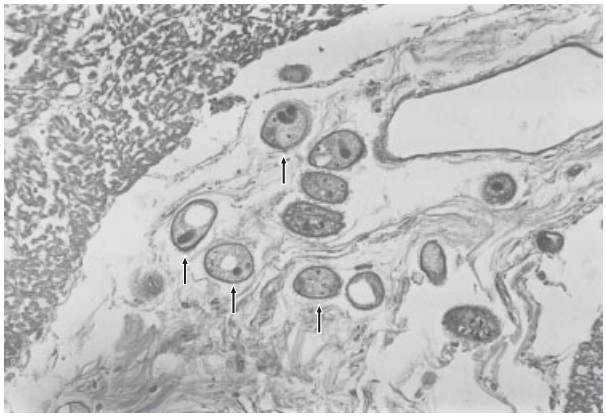

Figure 16 B. coli *in intestine. Many trophozoites (arrows) are lying in the submucosa. Hematoxylin and eosin; bar = 30 μm*

Diagnosis

Diagnosis of balantidiasis on clinical grounds is very difficult because of the nonspecific nature of the infection and possible confusion with other causes of diarrhea. Diagnosis is therefore best based on fecal examination, which reveals mainly trophozoites in acutely infected patients and cysts in patients with chronic infections. Diagnosis can also be made by the examination of biopsy specimens taken with the aid of a sigmoidoscope or by examination of scrapings of an ulcer. *Balantidium* can be cultured in all the media that support the growth of *Entamoeba histolytica* (Clark and Diamond 2002). However, culture is rarely necessary as the parasites are more easily detected in feces by microscopy and in tissues on histological examination (see Orihel and Ash 1995; Gutierrez 2000). One possible cause of confusion is the phenomenon of ciliocytophthoria resulting in normal ciliated cells appearing in unusual circumstances and there have been cases where such specimens have been tentatively identified as *B. coli* (Hadziyannis et al. 2000). These authors advocate the use of the Diff-Quik stain for the diagnosis of ciliocytophthoria.

EPIDEMIOLOGY

B. coli is worldwide in its distribution, particularly in the tropical and subtropical regions of the world including South and Central America, the Philippines, Iran, Central Asia, Papua New Guinea, and some Pacific islands. Although this ciliate is widely regarded as a common parasite of pigs that is transmissible to humans, it actually has a much broader host range and numerous surveys have shown that it occurs in a number of mammal species, both wild and domesticated. In a major survey of 297 Old World primates in Kenya, *B. coli* was detected in 6 out of 7 species with an overall prevalence of 24.9 percent (Muriuki et al. 1998). Also in Kenya, *B. coli* was found in 40 percent of 315 fecal samples from captive and wild-trapped baboons, vervets, and Sykes monkeys (Munene et al. 1998). In the New World *B. coli* was prevalent in 141 rhesus macaques in a free-ranging population (Knezevich 1998). *B. coli* is also common in camels, for example 11.9 percent of 260 fecal samples examined by Tekle and Abebe (2001) in Ethiopia and in young camels suffering from diarrhea in Bahrain (Abubakr et al. 2000). Buffaloes are also known to be infected with *B. coli*, and Patil et al. (1998) have recorded a 17 percent prevalence in 82 buffaloes suffering from diarrhea and dysentery in India. In a major survey of 567 mammalian species belonging to 17

families in Japan *B. coli* was found in 6 species of primates and artiodactyles but not in rodents, cats, or dogs (Nakauchi 1999). However, there have been occasional records of *B. coli* in dogs (Uptal Das 1999). Although there is little doubt that pigs are the main potential reservoirs of human infections, transmission to humans varies from place to place. In a survey of pigs at a Danish research farm, 57 percent of suckling pigs and 100 percent of pigs >4 weeks old were found to be infected with *B. coli*, presumably representative of this major pig-producing country as a whole, but no case of human balantidiasis has been recorded in Denmark (Hindsbo et al. 2000). In contrast, in a rural community in Venezuela, *B. coli* was detected in 33.3 percent of pigs and 12 percent of children, a situation that the authors attribute to deficient sanitation and a low socio-economic level (Devera et al. 1999). In a larger study of 2 124 children in northern Bolivia, Esteban et al. (1998) found that the overall prevalence of *B. coli* was 1.2 percent, again attributed to contact with pigs. In China the parasite appears to be endemic in Yunnan province, with infection rates of up to 4.24 percent in some villages (Yang et al. 1995). However, the highest prevalence (up to 20 percent) has been reported in the mountain districts of West Irian (Indonesia) where there is a close association between humans and pigs. McCarey (1952) has reviewed the prevalence of balantidiasis in Iran – an endemic area of unusual interest, as there is no pig farming there because of the Moslem prohibition on eating pork. The transmission was from human to human and the animal host, if any was not known; the possibility of infection acquired from camels was not considered as it was not known at that time that these acted as potential reservoir hosts.

There have been occasional sporadic outbreaks of balantidiasis in humans. A single outbreak of epidemic proportions was reported from the Pacific island of Truk (Walzer et al. 1973). It involved 110 persons in a short period of time and occurred as a result of contamination of the water supply by pig feces during a typhoon. Balantidiasis has been frequently observed in psychiatric units in the USA and many other countries. Here the transmission occurs by human-to-human contact and is due to a lack of hygienic conditions and to coprophagy.

CHEMOTHERAPY

A number of drugs, including metronidazole, secnidazole, tinidazole, tetracycline, trimethoprim plus sulfamethoxazole, sulfaguanidine, and sulfadimidine, are effective against *B. coli* in vitro (Choudhury et al. 1998), so there is no shortage of potentially useful drugs to treat humans or pigs. At present tetracycline and metronidazole are the drugs of choice for humans. Tetracycline, 500 mg, 4 times a day for 10 days, is recommended although clearance generally occurs in 2–3 days (WHO 1995). Alternatively, metronidazole, 750 mg, 3 times a day for 5–7 days, can be used but, according to WHO, results are less consistent (WHO 1995). No relapse of the infection after treatment and no resistance to these antibiotics have been reported. Treatment should be given to carriers in institutions to prevent the spread of infection to susceptible patients.

INTEGRATED CONTROL

Control consists of hygienic rearing of pigs and preventing the human–pig contact which can lead to human infections. Improved hygiene in psychiatric institutions will prevent human-to-human transmission in these settings, and treatment of humans shedding cysts will prevent human-to-human transmission. The relative lack of host specificity and close contact with other potential reservoir hosts, such as camels and buffalo, suggests that infection with *B. coli* is an ever-present problem and also that individuals working with or coming into contact with primates are at risk.

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Old World leishmaniasis

PAUL A. BATES AND R.W. ASHFORD

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INTRODUCTION

The leishmaniases are a group of human diseases that afflict people in many tropical and sub-tropical regions. They are caused by parasitic protozoa of the genus *Leishmania* and are transmitted to humans by the bite of female phlebotomine sandflies, small blood-feeding insects. Leishmaniasis is not a single entity but a collection of diseases, each with its own clinical manifestations and epidemiology. However, the basic pattern of the life cycle is well conserved among members of the genus, and each species of *Leishmania* tends to cause a certain type of disease within a specific epidemiological context. Therefore, *Leishmania* spp. are most conveniently studied by first considering their general properties before dealing with those specific to individual species; this is the approach adopted here. Five species of *Leishmania* are the medically important agents of Old World leishmaniasis: *L. major*, *L. tropica*, *L. aethiopica*, *L. donovani*, and *L. infantum* (Figures 1 and 2).

The first three of these are predominantly agents of cutaneous leishmaniasis, an infection which is limited to the skin, and the last two are predominantly agents of visceral leishmaniasis, an infection of the liver and spleen that can be fatal. In addition to these five well-established species, a number of other named species can be found in the literature on Old World leishmaniasis. Some have subsequently turned out to be very close, if not identical, to known species; others have not been examined in sufficient detail to reach a firm conclusion with regard to their identity.

CLASSIFICATION

The genus *Leishmania*

Leishmania is one of several genera within the family Trypanosomatidae (class Kinetoplastea, order Trypanosomatida) and, therefore, shares certain properties with other members of this family described elsewhere in this volume (see African trypanosomiasis; New World trypanosomiasis). These common properties include: the possession of a kinetoplast (a unique form of mitochondrial DNA); a single flagellum arising from a flagellar pocket; and an alternation between arthropod and mammalian hosts during the life cycle.

The features that uniquely identify the genus *Leishmania* are the nature of its vertebrate and invertebrate hosts and the developmental cycles, and morphological stages found within each. The vertebrate hosts are all mammals in which the parasites reside within the phagolysosomal system of mononuclear phagocytic cells, typically macrophages (Mauel 1996; Antoine et al. 1998; Rittig and Bogdan 2000). This is a unique location for a eukaryotic parasite and is only used by a handful of prokaryotic parasites (Garcia-del Portillo and Finlay

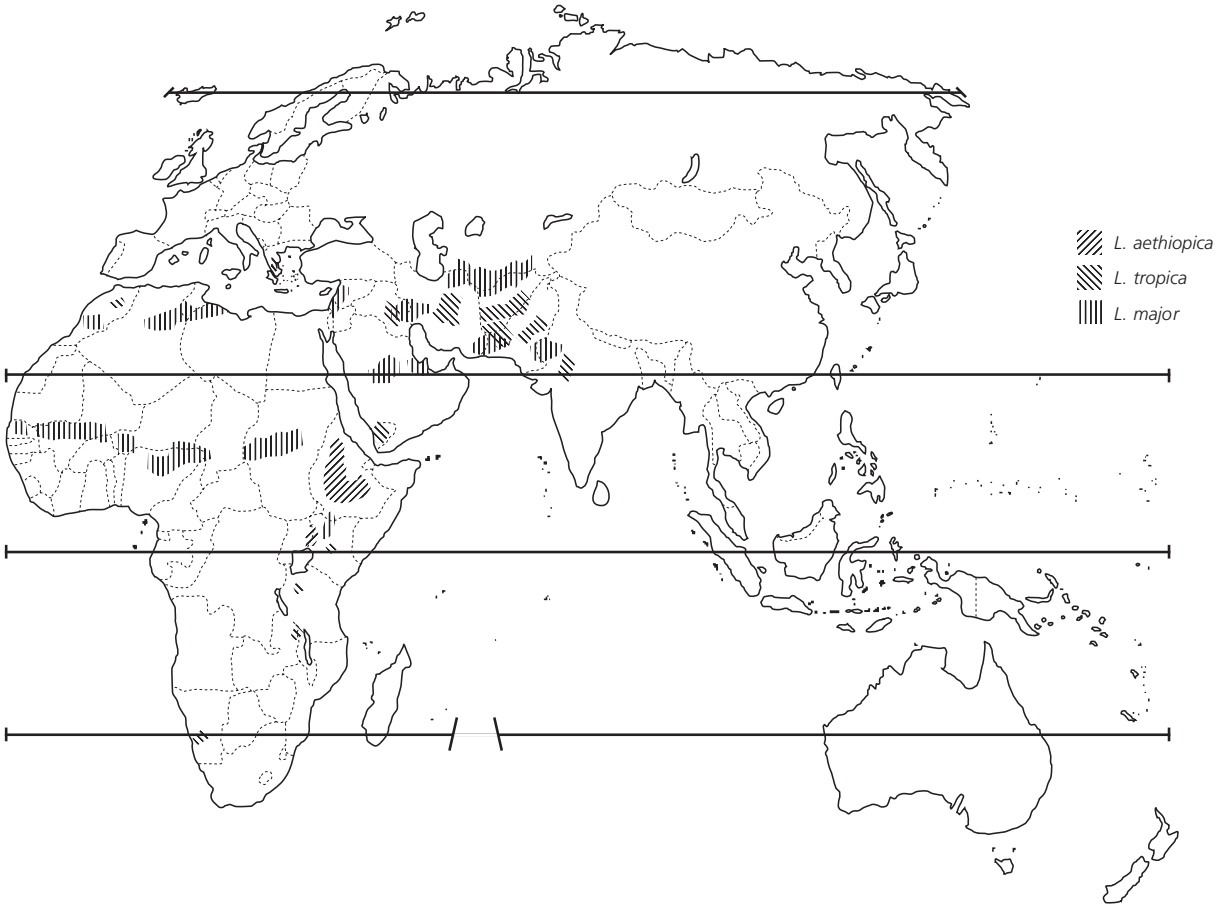

Figure 1 *Geographical distribution of the agents of Old World cutaneous leishmaniasis*

1995). The parasite gains entry to this intracellular residence via host cell phagocytosis. The invertebrate hosts are all phlebotomine sandflies of two genera; *Phlebotomus* in the Old World and *Lutzomyia* in the New World (see New World leishmaniasis). These are small, hairy, dipteran flies of the family Psychodidae, in which only the females feed on blood and transmit disease. In the sandfly, the parasites are extracellular; development occurs exclusively in the gut and transmission is via the mouthparts during blood feeding (reviewed by Schlein 1993; Walters 1993; Bates 1994a; Sacks and Kamhawi 2001).

Various taxonomic distinctions have been proposed at the subgeneric, species, and subspecies levels, but at present there is no generally accepted classification that incorporates all of these elements. Here, we adopt a conservative pragmatic approach, essentially following Lainson and Shaw (1987), but without any subgeneric or subspecific qualifiers. In *Leishmania* the biological species concept of a population of potentially interbreeding individuals cannot be applied, as the genus seems to be largely, if not exclusively, asexual in its mode of reproduction (Victoir and Dujardin 2002). The ideal classification for an asexual organism at the level of the 'species' combines the maximum biological homogeneity within each species, but separates biologically different organisms. Among the techniques available for the classification of *Leishmania*, isoenzyme analysis has been found to describe strains at a level that allows the construction of phenograms and cladograms at an ideal resolution (Rioux et al. 1990). Using a panel of c. 12 enzymes, it is possible to identify and distinguish most, but not all of the currently accepted species (Jamjoom et al. 2004). The group of strains that share a given pattern of enzyme electrophoretic mobilities are known as a 'zymodeme'. A given species may contain a number of zymodemes indicating subpopulations or subspecies. There has been an enormous accumulation of data using this method; it has largely confirmed what was known or expected biologically but has also provided valuable new insights, such as the fact that cutaneous leishmaniasis in Europe is caused by *L. infantum*. An example of a cladogram constructed using isoenzyme data is shown in Figure 3. The main disadvantage of isoenzyme analysis is the lack of standardization between laboratories; the importance of the strictest laboratory protocols to prevent mixing of cultures cannot be overemphasized.

Of the other techniques that have been used, the resolution of restriction fragment length polymorphisms

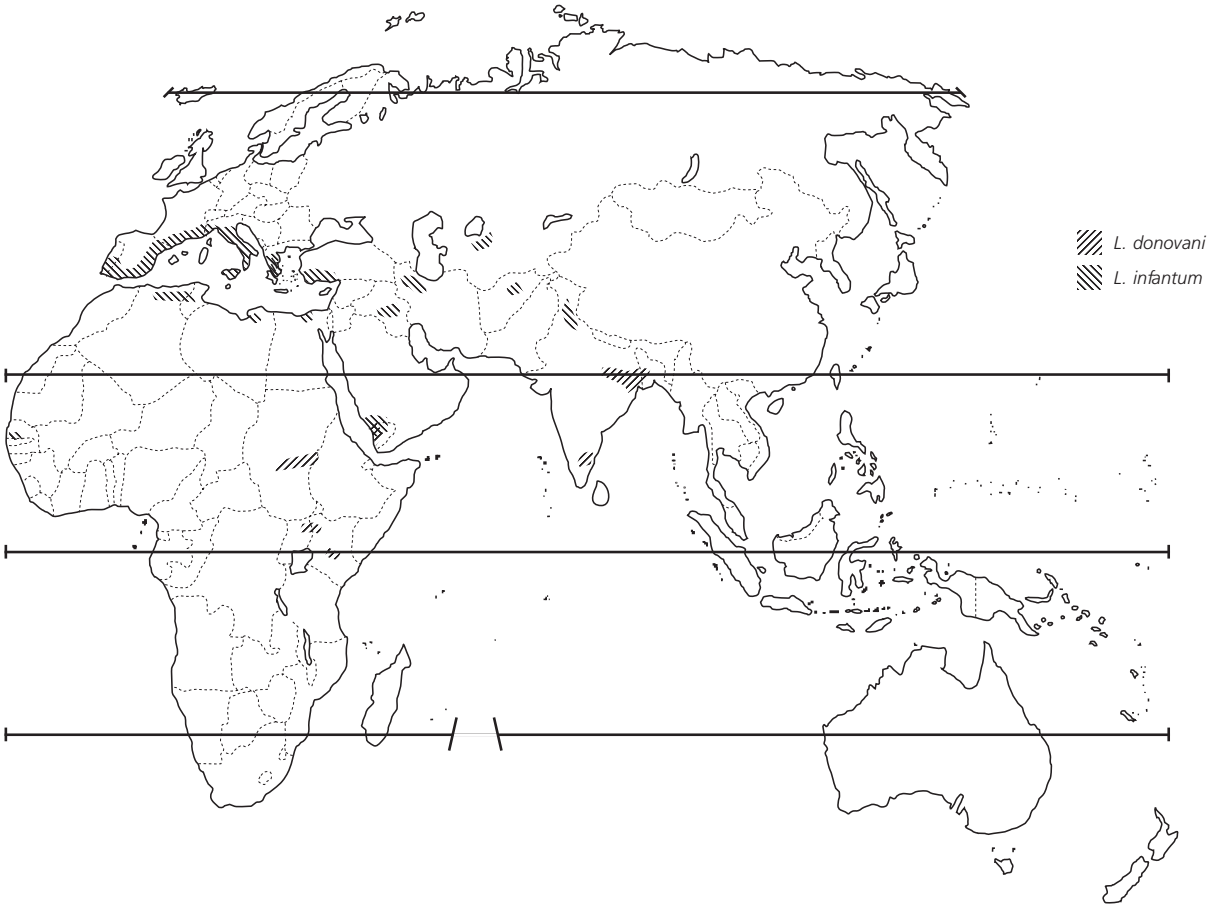

Figure 2 *Geographical distribution of the agents of Old World visceral leishmaniasis*

(RFLP) and karyotype analysis has been found to be too fine except for studies at the population genetic level. Microsatellite DNA analysis is the latest technique being used with *Leishmania* and seems well suited to resolving taxonomic issues at the infraspecific level (Bulle et al. 2002; Jamjoom et al. 2002). DNA and ribosomal RNA sequence information hold much promise and have given valuable results at the generic level and above, but have not been sufficiently widely used yet to contribute to classification at the species level. Monoclonal antibody and DNA probes, with or without polymerase chain reaction (PCR) amplification, are of value in identification of stocks that have been well classified, but provide insufficient information to be of value in constructing a classification. A disadvantage of most current methods is the requirement to culture the parasite in question before identification can be made.

Of the five species considered in this chapter, all are distinct from those found in the New World (see New World leishmaniasis) with one important exception, *L. infantum*. Molecular analysis reveals this to be indistinguishable from *L. chagasi* found in the New World (Mauricio et al. 1999, 2000). Because the former was named first both parasites should be referred to as *L. infantum*. We favor the explanation that '*L. chagasi*' is, in fact, *L. infantum* that was introduced into the New World in post-Columbian times via the domestic dog (Momen et al. 1993; Courtenay et al. 2002). While others may disagree with this interpretation, it is certain that these two parasites are very closely related. The remaining New World parasites have rather different biology, clinical manifestations, and epidemiology to those of the Old World and are dealt with separately in New World leishmaniasis. The main features of the Old World species are summarized in Table 1.

STRUCTURE AND LIFE CYCLE

The parasite exists in two main morphological forms, 'amastigotes' and 'promastigotes', found in the mammalian and sandfly hosts, respectively. Amastigotes are ovoid, non-motile, intracellular stages; promastigotes are elongated, motile, extracellular stages. The form introduced into the skin of the mammalian host is a promastigote, but this soon transforms into an amastigote, and the parasite remains in this form for the duration of the mammalian phase of the life cycle. Similarly, the form taken up by the sandfly is an amastigote, but this soon transforms into a promastigote. The basic life cycle of

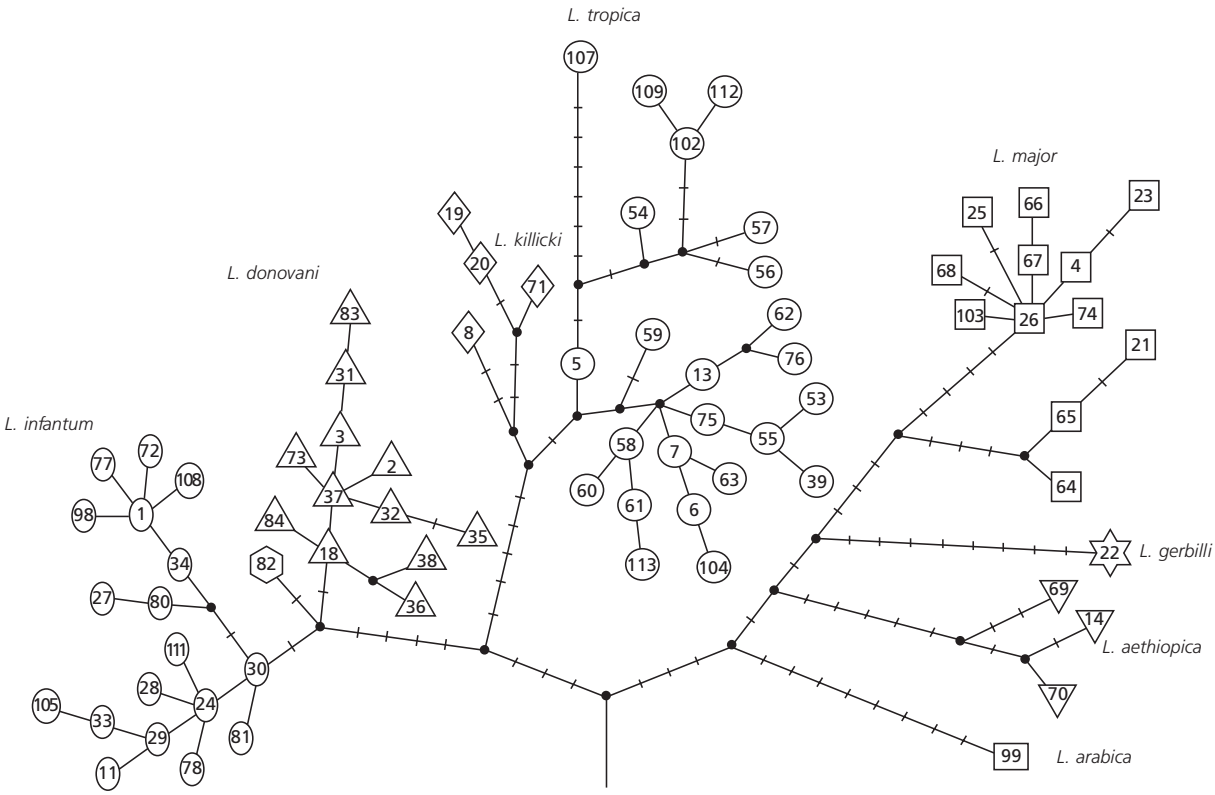

Figure 3 *Cladogram of Old World* Leishmania *zymodemes showing grouping into 'species'.* L. gerbilli *and* L. arabica *are not known from humans. (Modified from Rioux et al. 1990)*

Leishmania is illustrated in Figure 4 (reviewed by Molyneux and Killick-Kendrick 1987).

Amastigote structure

Amastigotes are ovoid cells of 3–5 μm length on the main axis and, therefore, lie at the lower limit of size described for eukaryotic cells. Because of their small size, little internal structure can be discerned in stained preparations at the light microscope level beyond the central round or oval nucleus and adjacent but smaller round or rod-shaped kinetoplast. At the ultrastructural level, more detail is revealed (Figure 5). The surface membrane is a conventional unit membrane under which lies a corset of microtubules, serving as a form of cytoskeleton. These are closely spaced in parallel rows

Table 1 *Summary of the main features of Old World* Leishmania *species*

Species	Disease in humans	Geographical distribution	Important mammalian hosts	Important sandfly hosts
L. major	Rural, zoonotic, cutaneous leishmaniasis, oriental sore	North Africa, Sahel of Africa, Central and West Asia	Great gerbil *Rhombomys opimus*, fat sand rat *Psammomys obesus*	*Phlebotomus papatasi*, *P. dubosqi*, *P. salehi*
L. tropica	Urban, anthroponotic cutaneous leishmaniasis, oriental sore	Central and West Asia	Humans	*Phlebotomus sergenti*
L. aethiopica	Cutaneous leishmaniasis, diffuse cutaneous leishmaniasis	Ethiopia, Kenya	Rock hyraxes *Heterohyrax brucei* and *Procavia* spp.	*Phlebotomus longipes*, *P. pedifer*
L. donovani	Visceral leishmaniasis, kala-azar, post kala-azar dermal leishmaniasis	Indian subcontinent, East Africa	Humans	*Phlebotomus argentipes*, *P. orientalis*, *P. martini*
L. infantum	Infantile visceral leishmaniasis	Mediterranean basin, Central and West Asia	Domestic dog	*Phlebotomus ariasi*, *P. perniciosus*

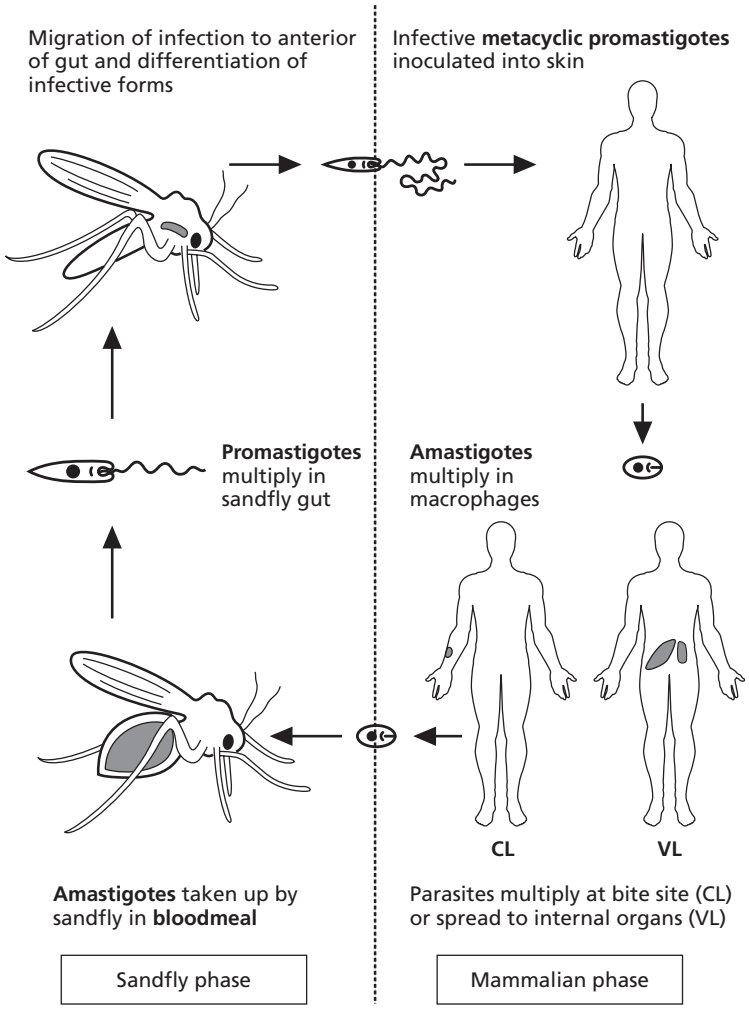

Figure 4 *General life-cycle of* Leishmania. *Parasites alternate between sandfly and mammalian hosts, existing as promastigote and amastigote forms, respectively. Mammalian infection results in cutaneous leishmaniasis (CL) or visceral leishmaniasis (VL).*

which, together with the small size, make the amastigote a very robust eukaryotic cell. One unfortunate consequence of this feature is that it has not proved possible to develop subcellular fractionation techniques for amastigotes that will disrupt the surface membrane without destroying the organelles contained within. On the positive side, fairly vigorous homogenization techniques can be employed for the isolation of amastigotes from infected tissue without destroying their viability or structural integrity.

An infolding of the surface membrane creates an internal space, termed the 'flagellar pocket' (Webster and Russell 1993; Landfear and Ignatushchenko 2001). This is so named because a flagellum emerges from the surface membrane and projects into the pocket. This flagellum is not functional in the amastigote and does not extend beyond the cell body. The flagellar pocket is thus topologically external to the cell although contained within it. In addition to anchoring the flagellum, the main function of the pocket is as a site of endocytosis and exocytosis (McConville et al. 2002; Morgan et al. 2002). Microtubules do not underlie the surface of the flagellar pocket membrane thus allowing access to vesicular traffic. Immediately below the origin of the flagellum lies the kinetoplast, a dense mass of mitochondrial DNA. The kinetoplast DNA is composed of several thousand circular DNA molecules linked together in a catenated network (Shlomai 1994; Shapiro and Englund 1995; Lukes et al. 2002). The DNA circles are of two size classes: each kinetoplast contains 25–50 maxicircles of c. 30 kb, and 5000–10 000 minicircles of c. 2 kb. Together these constitute the mitochondrial genome. Branches of the surrounding mitochondrion extend throughout the cell body and contain plate-like cristae.

The cytoplasm contains both rough and smooth endoplasmic reticulum. The Golgi apparatus is typically found in the vicinity of the flagellar pocket, which probably reflects the role of this organelle in the endocytic and exocytic pathways. Lysosomes are also found in the cytoplasm together with an organelle unique to kinetoplastids, the glycosome. This is so named because a

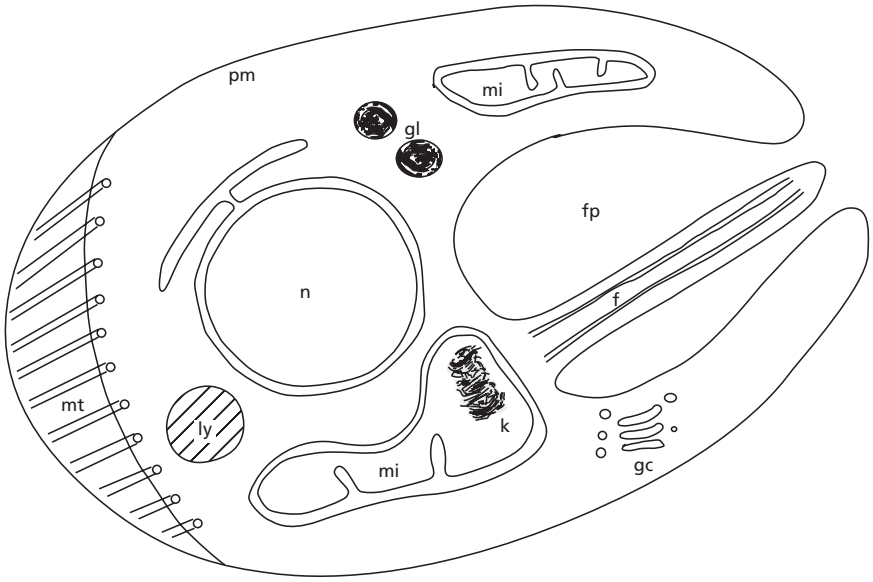

Figure 5 *Ultrastructure of a* Leishmania *amastigote. Amastigotes possess a central nucleus (n) and adjacent kinetoplast (k) within a single branching mitochondrion (mi). The flagellum (f) arises from a flagellar pocket (fp) but does not extend beyond the cell body. Lysosomes (ly), glycosomes (gl) and Golgi complex (gc) are found in the cytoplasm. Rows of microtubules (mt) run just below the plasma membrane (pm).*

number of glycolytic enzymes are specifically located in this organelle, together with some others (Michels et al. 1997; Parsons et al. 2001).

Promastigote structure

In the sandfly host, the parasite is found mainly as a promastigote form (Figure 6). The structural elements of promastigotes are the same as those described for amastigotes although there may be variations in organelle numbers. The main differences from the amastigotes are that the cell body is elongated, 8–15 μm, and that the flagellum emerges from the cell body and is functional thus making these motile cells. The flagellum is found at the anterior end of the cell, i.e. the cell body is trailed behind the flagellum. Desmosomal plaques anchor the flagellum to the cell body as it emerges from the flagellar pocket. The promastigote flagellum has a paraxial rod, a paracrystalline structure running parallel to the microtubules of the axoneme, which plays a role in flagellar motility (Maga et al. 1999). Recently, a novel tubular lysosome that is part of the endocytic pathway has been described in promastigotes (Waller and McConville 2002).

Life cycle

DEVELOPMENT IN THE SANDFLY HOST

The phase of the life cycle in female sandflies is of relatively short duration. Flies that acquire a *Leishmania* infection probably remain infected for life, but this is

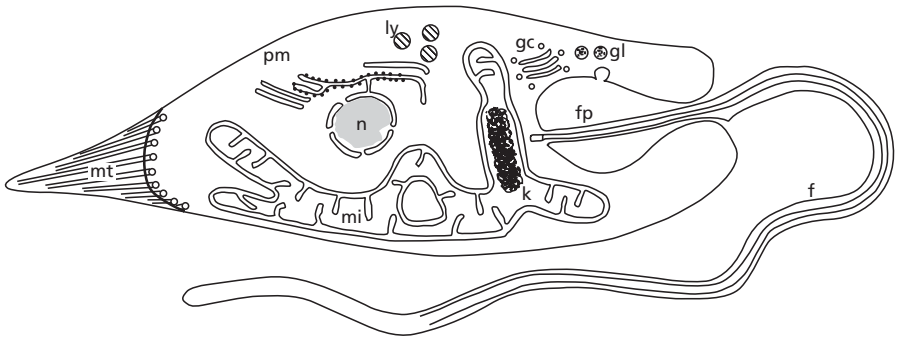

Figure 6 *Ultrastructure of a* Leishmania *promastigote. Many of the features found in the amastigote stage are also found in the promastigotes (abbreviations, see Figure 5). Some differences are that the cell body is elongated, the flagellum extends beyond the cell body and the kinetoplast has a more anterior location relative to the nucleus.*

usually only a matter of weeks. In most parasite–vector combinations that have been studied experimentally, the development of the parasite is sufficiently rapid for mammal-infective promastigotes to be produced by the time the female is ready to take her next bloodmeal. This may be as little as 5–7 days under optimal conditions. Development occurs exclusively in the sandfly gut (Figure 7) and begins with infected macrophages or free amastigotes in the bloodmeal. The macrophages disintegrate over a matter of hours and, as with the mammalian phase, the first event is transformation, in this case to the promastigote form, which takes 24–48 h. Promastigotes of various kinds grow and divide in the sandfly gut. The ultimate products of the developmental cycle are the mammal-infective forms, termed 'metacyclic promastigotes', which accumulate in the anterior midgut and foregut of the sandfly.

The developmental cycle of *Leishmania* in the sandfly host is illustrated in Figure 8 and reviewed in Bates and Rogers (2004). Promastigote development has been studied using a variety of combinations of parasite and vector (Killick-Kendrick 1990a; Walters 1993; Bates 1994a) and two patterns have emerged for those species infective to mammals. Certain species usually include a phase of development in the hindgut of the sandfly, for example the New World species *L. braziliensis*, share certain other features, and form a distinct cluster of species according to isoenzyme and DNA sequence analysis. These exclusively New World species are placed in a separate subgenus, (*Viannia*) and are described in detail in New World leishmaniasis. The majority of *Leishmania* species, including all the Old World parasites, in which there is no hindgut development are placed in another subgenus (*Leishmania*).

There are a variety of different promastigote forms that can be distinguished on morphological grounds and various authors refer to these by different names, often interchanging cultured and sandfly-derived promastigotes. However, the evidence for functional distinction is less complete. In the Old World *Leishmania* species, six developmental forms are recognized during development: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes, paramastigotes, and metacyclic promastigotes (Rogers et al. 2002; Gossage et al. 2003; Figure 9). Other categories and subdivisions can be found in the literature, but at present there is little evidence for any functional distinction between them.

The first major developmental event in the sandfly is the transformation of amastigotes to procyclic promastigotes. However, very few observations on the early events have been made and it is also possible that some amastigotes may divide before transforming to promastigotes. Amastigote to promastigote transformation occurs readily in vitro and is usually completed in 24–48 h when

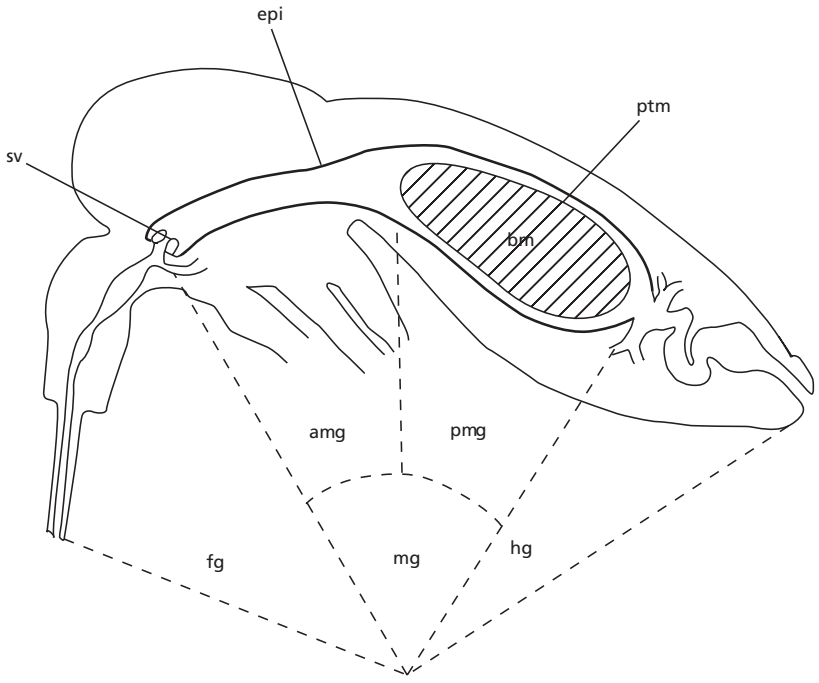

Figure 7 *Structure of the female sandfly gut. The gut can be subdivided into three sections: the foregut (fg), midgut (mg), and hindgut (hg). The midgut is lined by epithelial cells (epi) with microvilli that project into the lumen of the gut and is subdivided itself into the anterior or thoracic midgut (amg) and the posterior or abdominal midgut (pmg). The junction between the anterior midgut and the foregut is formed by the stomodeal valve (sv). The foregut, stomodeal valve and hindgut are lined by a chitinous cuticular layer. Initial development of the parasite occurs within the bloodmeal (bm) encased in a peritrophic matrix (ptm) in the posterior midgut.*

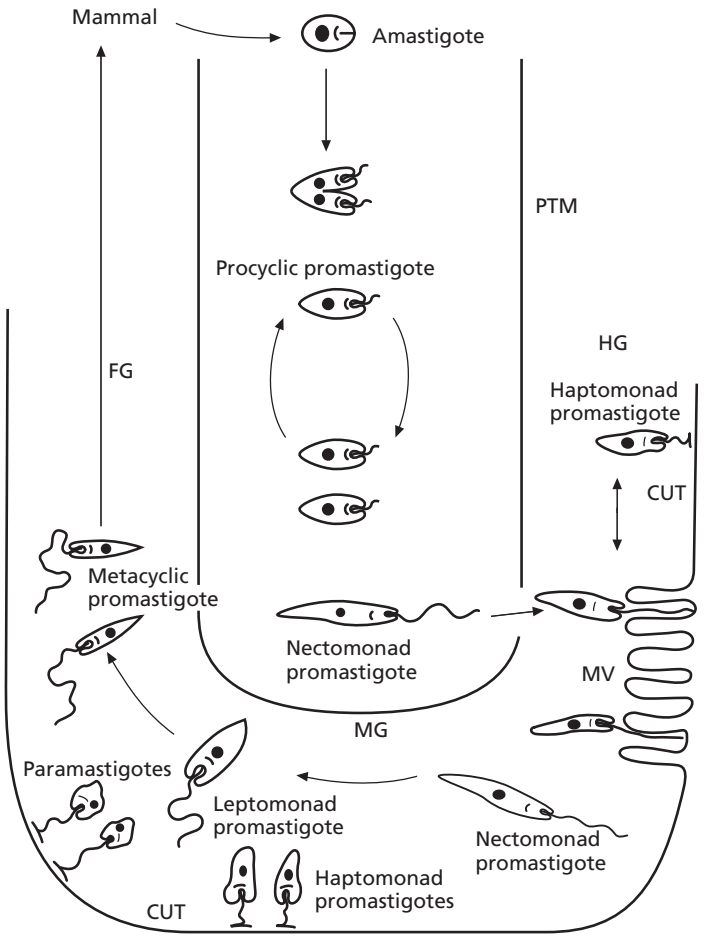

Figure 8 *Developmental cycle of* Leishmania *promastigotes. Amastigotes are ingested by a female sandfly and transform to procyclic promastigotes in the bloodmeal, itself encased in a peritrophic matrix (PTM) in the midgut (MG). Procyclic promastigotes are gradually replaced by nectomonad promastigotes that escape from the ptm; some attach to the microvilli (mv) via their flagella. In some species, haptomonad forms attach to the cuticular lining (cut) in the hindgut (HG), in others they attach in only the foregut (FG) and stomodeal valve. Paramastigotes may also be found in the foregut. Leptomonad promastigotes multiply in the anterior midgut and differentiate into metacyclic promastigotes to complete the developmental cycle.*

a suitable culture medium and an appropriate temperature (about 26°C) is used. The media used vary widely, but the optimal pH for promastigote growth, and that used in culture media, is generally neutral to slightly alkaline (pH 7.0–7.5). In vitro transformation to promastigotes and cell division occur coincidentally, such that, of the first forms observed in division, the majority are partially transformed intermediates (Bates 1994b). The signals initiating both processes may be the same and it is possible that they are mechanistically linked.

In the sandfly, these events occur in the posterior midgut in the bloodmeal that is itself encased in a peritrophic matrix (PTM) secreted by the midgut epithelium. The PTM is a lattice of chitin fibrils, proteins and glycoproteins secreted by the midgut epithelium in response to a bloodmeal (Shao et al. 2001). In phlebotomine sandflies, the PTM is produced at one time and encapsulates the whole bloodmeal offering some protection to the parasite during amastigote to procyclic promastigote transformation by limiting their exposure to trypsin secreted by the midgut epithelium (Pimenta et al. 1997). This temporary vulnerability to trypsin is probably a result of reorganization of the surface glycocalyx during transformation. The procyclics are short, ellipsoid promastigotes generally about 6–8 μm in body length. Multiplication of procyclic promastigotes occurs within the PTM but gradually the promastigotes elongate and transform to nectomonad forms of 15–20 μm body length.

Approximately 3 days after blood feeding, the PTM usually begins to break down and promastigotes begin to

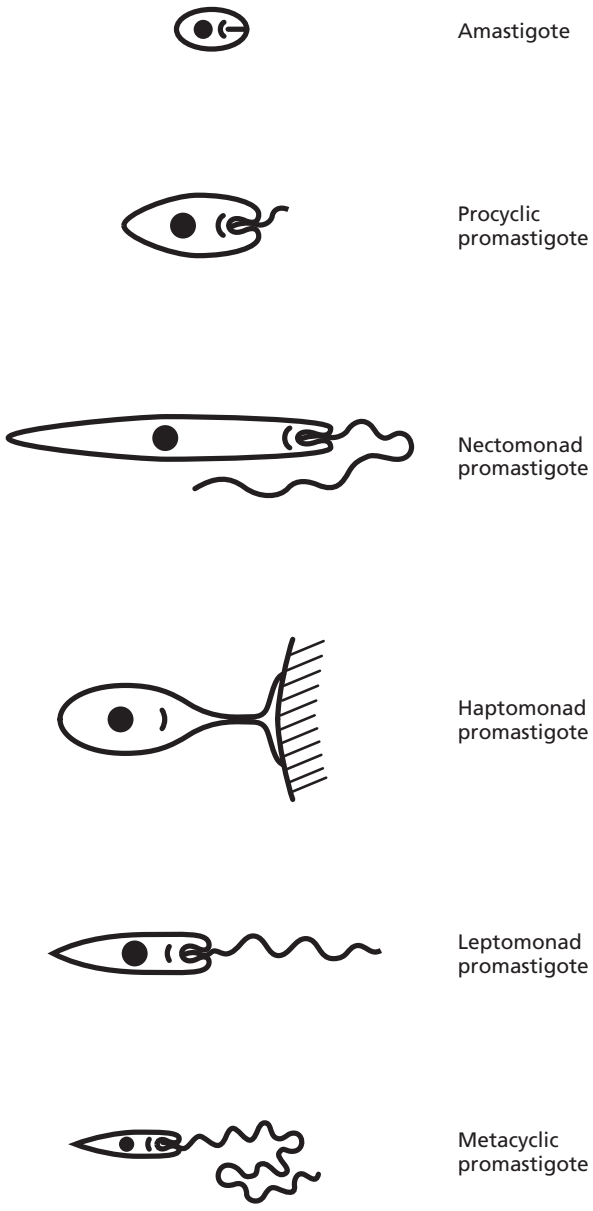

Figure 9 *Promastigote developmental forms (after Rogers et al. 2002). The six main morphological stages found in the life-cycle of* Leishmania *drawn to show their relative sizes and shapes.*

migrate forward to the anterior midgut. There is evidence for an accelerated breakdown of the PTM in infections of *L. infantum* in *Lutzomyia longipalpis* (Walters 1993) and *L. major* in *Phlebotomus papatasi* (Schlein et al. 1991). One explanation for this phenomenon is the presence of a chitinase secreted by promastigotes (Schlein et al. 1991; Shakarian and Dwyer 1998, 2000). This is presumed to have survival value to the parasite as it would permit establishment of a midgut infection outside the PTM earlier than would otherwise be the case. In some vectors, it could be essential for escape from the PTM before defecation of the bloodmeal remnants by the sandfly.

The transformation from procyclics to nectomonads can be mimicked in vitro (Bates 1994b). In this study, lesion amastigotes were allowed to transform, procyclics appeared on day 2 and, after 3 days of growth, a population consisting predominantly of nectomonad forms was obtained. The timing of these events is strongly reminiscent of the situation in sandfly infections and occurs in vitro at a particular growth phase (late exponential phase). The latter suggests that transformation to nectomonads is a density-dependent phenomenon and could be triggered, for example, by a factor such as nutrient depletion. This could be a useful adaptation in vivo as the initial source of nutrition, the bloodmeal, becomes exhausted. Charlab and Ribeiro (1993) found that inhibition of growth by exposure of promastigotes to salivary gland homogenate from *Lu. longipalpis* caused transformation to nectomonads in vitro. Whether parasites are exposed to sandfly saliva during their development in the midgut is uncertain, but is a possibility after escape from the PTM. Flies continue to take sugar meals over the several days that the bloodmeal is being digested and as promastigote development occurs.

Some of the nectomonads attach to the midgut epithelium, inserting their flagella between the microvilli (Killick-Kendrick 1990a). The major surface glycolipid of promastigotes, lipophosphoglycan (LPG) (Turco and Descoteaux 1992), mediates this attachment, which is important for establishment of a mature infection, i.e. one that persists beyond the initial multiplicative phase in the bloodmeal (Lang et al. 1991; Pimenta et al. 1992, 1994). Binding of promastigotes to dissected midguts in vitro can be inhibited by free LPG or its constituents and there is a positive correlation between the binding of LPG isolated from a particular parasite to dissected midguts of sandflies, and the ability of the given vector to transmit that parasite (Pimenta et al. 1994; Sacks et al. 1994; Sacks 2001). Mutants specifically unable to express LPG cannot attach to the midgut and maintain an infection in sandflies (Sacks et al. 2000).

LPG covers the entire surface including the flagellar membrane where it could mediate binding (Lang et al. 1991). However, one unexplained feature of midgut attachment is the striking feature of flagellar insertion, a phenomenon restricted to the subgenus *Leishmania*. The reason may be that the juxtaposition of elongated flagella and microvilli provides a good fit that maximizes the number of binding sites and hence, with time, the promastigotes will adopt this orientation. However, it is premature to discount the possibility that other flagellar-specific molecules could be involved and provide additional specificity.

By 5 days, the infection has usually spread to the anterior midgut and the cuticular surface of the stomodeal valve at the junction with the foregut. Nectomonad forms are responsible for this anterior migration and, upon reaching the anterior midgut, give rise to leptomonad promastigotes and haptomonad promasti-

gotes (Rogers et al. 2002). Leptomonad forms, promastigotes of 6-8 μm, accumulate in the lumen of the midgut producing a gel-like substance, promastigote secretory gel (PSG) (Stierhof et al. 1999; Rogers et al. 2002). Haptomonad forms, broad cells of 5–8 μm, are found attached to the stomodeal valve via hemidesmosomes in expansions of their flagellar membranes (reviewed by Vickerman and Tetley 1990). The PSG produced by leptomonad forms and the attached haptomonads both assist with transmission by helping to create a 'blocked fly' (see below). Attached paramastigotes (nucleus adjacent to kinetoplast) and haptomonads may also be found in the foregut. In these attached forms, the flagellar membrane can be seen at the ultrastructural level to be closely apposed to the surface forming a junctional complex. An electron-dense plaque and associated filaments are located below the flagellar membrane, and thus these attachment organelles bear a superficial resemblance to the hemidesmosomes found at the basal surfaces of vertebrate epithelial cells.

From day 5 onwards, increasing numbers of small (5–8 μm), narrow, highly motile, metacyclic promastigotes, the mammal-infective forms, can be observed in the lumen of the anterior midgut and/or foregut in a position that facilitates their transmission on subsequent blood feeding by the female sandfly. Metacyclics differentiate from leptomonad promastigotes (Rogers et al. 2002), a process that can be induced in vitro by culture at low pH (Bates and Tetley 1993; Zakai et al. 1998), under anaerobic conditions (Mendez et al. 1999), or nutrient depletion (Cunningham et al. 2001). Conversely, both metacyclogenesis and chitinase secretion by *L. major* are inhibited by the inclusion of hemoglobin in the culture medium (Schlein and Jacobson 1994a). Unlike the other transformations described so far, this is more properly described as a differentiation process, as the end-product is in a non-dividing state.

TRANSMISSION FROM SANDFLY TO MAMMALIAN HOST

The opportunity for transmission from an infected sandfly to a mammalian host occurs when the fly is ready to take another bloodmeal. There is debate concerning the precise mechanisms that aid transmission and the extent to which the parasites themselves contribute to this process; these may vary with the specific vector–parasite combination. For the parasites to become lodged in the skin of a mammal, they must travel against the predominant flow of blood, which is into the gut of the fly.

In a sandfly containing a mature infection, the majority of promastigotes are found in the anterior midgut with a smaller population in the foregut. The number of promastigotes required to initiate a mammalian infection varies between hosts and parasite/vector combinations; the numbers inoculated are believed to be in the range 10–1 000 metacyclic promastigotes per infective bite (Rogers et al. 2004).

Under experimental conditions, infected flies show differences in feeding behavior when compared to uninfected flies. Frequently, they are seen to probe the skin an increased number of times and appear to find difficulty in taking a bloodmeal, a behavior that may enhance transmission (reviewed by Molyneux and Jeffries 1986; Molyneux and Killick-Kendrick 1987). Massive infections develop in and block the anterior midgut of both experimentally infected and wild-caught flies, with the promastigotes embedded in PSG (Stierhof et al. 1999; Rogers et al. 2002). The main component of PSG is a mucin-like filamentous proteophosphoglycan (Stierhof et al. 1999; Ilg 2000). Haptomonad forms attached to the stomodeal valve and foregut also contribute to the blockage of the gut. The parasite/gel plug appears to form a physical obstruction to feeding, a so-called 'blocked fly', resulting in an increased frequency of multiple probing and failed attempts to engorge.

During sandfly feeding, infective metacyclic promastigotes are carried out along with sandfly saliva and PSG (Rogers et al. 2004). Both saliva and PSG can enhance the infectivity of the promastigotes in the mammalian host (Titus and Ribeiro 1990; Belkaid et al. 1998; Kamhawi 2000; Rogers et al. 2004). Anti-saliva immune responses may be a useful component of anti-leishmanial vaccines (Kamhawi et al. 2000). Much debate has centered on the position and the types of promastigote found in the foregut and midgut: specifically where the mammal-infective forms are found (Killick-Kendrick 1990a). The two main ideas proposed are that metacyclic promastigotes are directly inoculated from the proboscis (the anterior part of the foregut), or regurgitated forward from more distal parts of the foregut and possibly the midgut. The current consensus favors regurgitation as the main mode of transmission, and this has been recently proven in *L. mexicana*-infected sandflies (Rogers et al. 2004). A variation on simple regurgitation comes from observations that the stomodeal valve can be damaged and remain permanently open as a result of the parasite infection, as has been described with infections of *L. major* in *Phlebotomus papatasi* (Schlein et al. 1992). Where such damage does occur, it may permit mixing of the contents of the anterior midgut with an incoming bloodmeal, such that when the pharynx contracts to deposit blood in the midgut, there is also a forward surge of promastigote-contaminated blood into the wound.

DEVELOPMENT IN THE MAMMALIAN HOST

The developmental cycle of *Leishmania* in the mammalian host is illustrated in Figure 10. This cycle is initi-

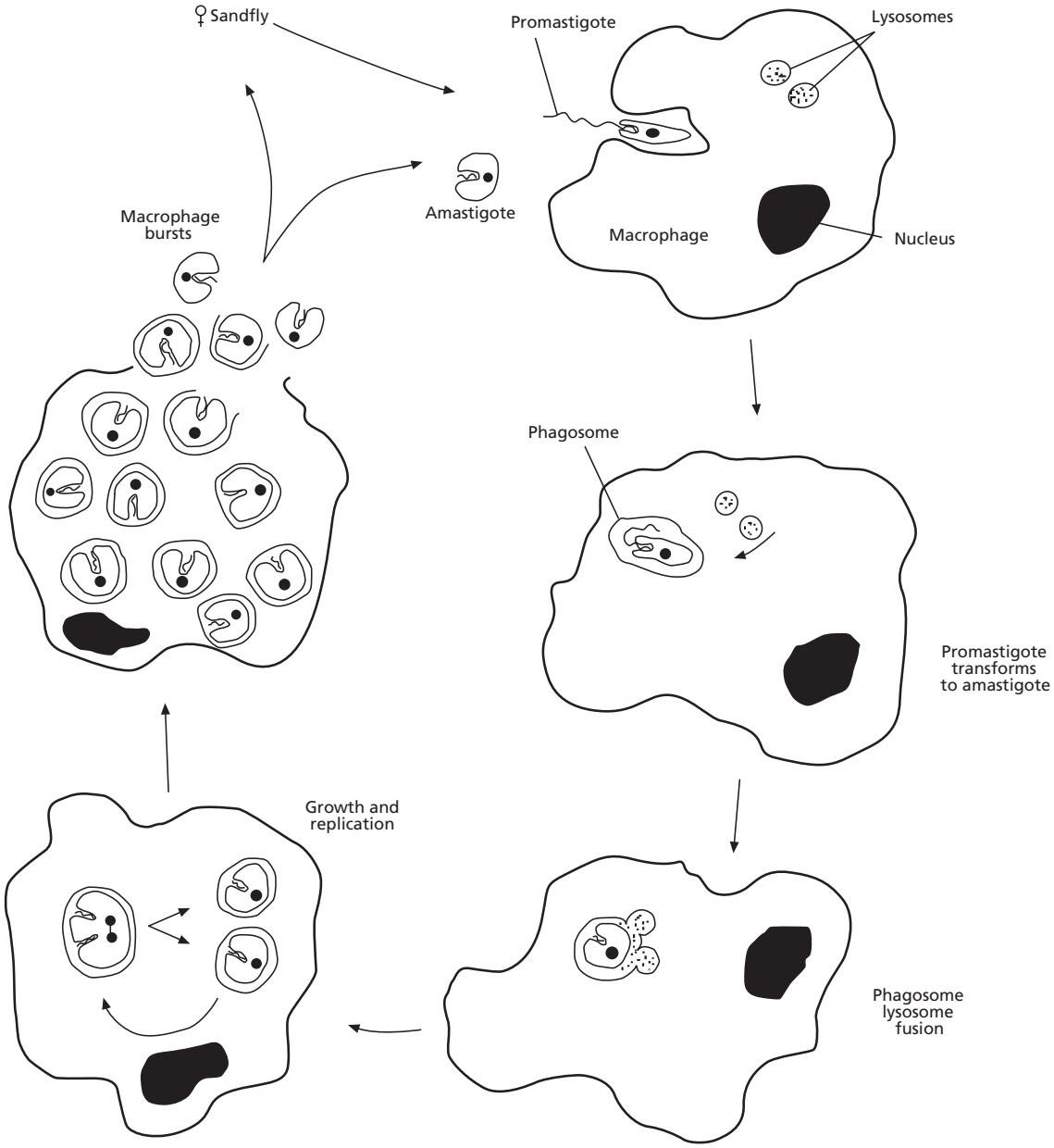

Figure 10 *Developmental cycle of* Leishmania *amastigotes*

ated by the interaction of metacyclic promastigotes with skin macrophages. The precise kinetics of parasite transformation and uptake by skin macrophages is uncertain, but it is generally assumed that uptake occurs soon after inoculation and that the promastigote to amastigote transformation occurs mainly inside the host cell. Direct studies on the binding and uptake of metacyclics by skin macrophages in situ are not possible but this process has been modeled in vitro (Alexander and Russell 1992; Rittig and Bogdan 2000; Handman and Bullen 2002). Two important caveats must be attached to the published reports to date: the types of macrophage used have been either a peritoneal exudate cell or a macrophage-like cell line (inappropriate host cells) and the parasites used have not been sandfly-derived metacyclic promastigotes but cultured forms that are sometimes not properly characterized. Nevertheless, these studies have shown that complement fixation by metacyclic promastigotes is not only an inevitable consequence of exposure to mammalian blood and tissue fluids but is an important adaptation for gaining entry to the host cell (Mosser and Brittingham 1997). Metacyclic promastigotes are relatively resistant to complement-mediated lysis and use the surface-bound complement components as ligands for binding to macrophage complement receptors. This promotes phagocytosis by the macrophage. After uptake and internalization in a phagosome, fusion with lysosomes occurs and the parasite inhabits a

secondary lysosome or phagolysosome (Antoine et al. 1998). During this process, the metacyclic promastigote transforms into an amastigote, a process that takes 12–24 h to complete. Interestingly, the parasite appears able to delay phagosome maturation until this transformation is complete (Swanson and Fernandez-Moreia 2002), a survival strategy mediated by LPG (Descoteaux and Turco 2002). Thereafter, the parasite remains in the amastigote form for the duration of the mammalian phase of the life cycle. Fully transformed amastigotes then continue to grow and divide within the phagolysosomal compartment of the host cell in a parasitophorous vacuole. Different species of parasite inhabit different sized vacuoles. The species causing Old World leishmaniasis all inhabit small vacuoles in which the vacuole membrane is closely opposed to the surface of the parasite itself. As the parasite divides so does the surrounding vacuole and, consequently, the host cell becomes occupied by multiple parasites and vacuoles. Amastigote division is by simple binary fission. Some of the New World parasites, for example *L. mexicana* (see New World leishmaniasis), occupy larger vacuoles, and these have a different effect on host cell endocytic traffic through their vacuoles (Antoine et al. 1998). After division, the parasites remain together in clusters within large vacuoles.

The phagolysosomal compartment presents two threats to amastigotes: the battery of lysosomal enzymes and low pH (4.5–5.5). Defense against the enzymes is mainly passive and relies on the unusual structures of the surface glycolipids and lipophosphoglycans rendering the amastigotes indigestible (Ilgoutz and McConville 2001). Low pH is not a problem as amastigotes are acidophiles: they have effective means of regulating their internal pH in an acidic environment; they are metabolically more active at low pH; and some species can only be cultured axenically in vitro as amastigotes at low pH (Bates 1993; Zilberstein and Shapira 1994). There is no known specific mechanism for escape from the host cell, although recent observations suggest the possibility that amastigotes are shed from peripheral vacuoles (Rittig and Bogdan 2000). The alternative is that parasites simply multiply until the host cell bursts open, making them available for uptake by other macrophages. Amastigotes are usually regarded as obligate intracellular parasites but this may not be strictly true: it is now possible to culture certain species axenically and at least in experimental infections it is possible to find extracellular amastigotes in heavily parasitized tissues. Despite this, the vast majority have an intracellular existence. Different *Leishmania* species infect macrophages at different sites in the body, but it is not known if amastigotes are specifically able to target subpopulations of mononuclear phagocytic cells or if they simply do not have access to or do not survive in macrophages in the wrong site. All amastigotes appear to be equivalent and there is no evidence for functionally distinct subpopulations.

TRANSMISSION FROM MAMMALIAN TO SANDFLY HOST

The opportunity for transmission from an infected mammalian host to a female sandfly occurs when the sandfly feeds. Sandflies are pool feeders, i.e. they possess cutting mouthparts that slice into the skin, and feed from the small pool of blood that seeps into the wound. Thus, despite their small size, 2–3 mm, sandflies give a noticeable and sometimes painful bite. Parasites causing cutaneous infections are acquired by a sandfly along with the bloodmeal if the fly happens to feed on a cutaneous lesion. Tissue damage from the bite releases infected macrophages or free amastigotes into the wound. Visceral parasites are probably acquired with the blood itself. Although the principal pathological events of visceral leishmaniasis occur in the spleen and liver, where the host cells probably remain resident, another site of infection is the bone marrow. This is a major site of hemopoiesis and blood monocytes infected with *Leishmania* amastigotes are released into the peripheral circulation and are thus available to feeding sandflies. It is also possible that infected monocytes leave the circulation and become resident in the skin to act as a source of parasites especially in the case of post kala-azar dermal leishmaniasis or the generalized leishmaniasis typical of canine infections with *L. infantum*. Nevertheless, in most endemic foci the acquisition of *Leishmania* parasites by individual flies is a rare event and the vast majority of flies are uninfected. The few infected flies are, however, efficient vectors.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Surface membrane structure

The surface membrane proteins of *Leishmania* can be subdivided into two groups. Those in the first group are present at high copy numbers per cell and are major structural components of the surface membrane. Members of the second group are present at low copy numbers per cell and have been identified using functional assays, for example, enzymes, transporters, and ion pumps.

Three major surface components have been described: gp63 (or promastigote surface protease (PSP)), lipophosphoglycan and glycosylphosphatidylinositols (Ilgoutz and McConville 2001). gp63 is a major surface protein of c. 63 000 kDa found on the surface of cultured promastigotes in all species of *Leishmania* that have been examined (Chang et al. 1990; Etges and Bouvier 1991) and has also been demonstrated on the surface of sandfly-derived promastigotes. gp63 is a zinc metallopro-

tease, the active site of which is exposed on the external face of the surface membrane (an ectoenzyme). There is some evidence for low level expression of gp63 by amastigotes in some species, but efforts to demonstrate active protease on the amastigote surface have revealed very low or no activity. Potential functions of gp63 in the life cycle of *Leishmania* are not certain, but are likely to be most important in the sandfly or during the brief existence of metacyclic promastigotes in the mammalian host. In the sandfly, gp63 may fulfil a digestive function, helping to provide a supply of amino acids for the parasite. The most abundant substrate would be hemoglobin and by digesting this molecule gp63 could also help to provide pre-formed heme, an essential nutrient for *Leishmania*. It might also help the parasite to penetrate the peritrophic membrane, which is partially proteinaceous in nature. gp63 can also be released from the parasite surface, so it may be able to exert biological effects away from the surface of the parasite (McGwire et al. 2002).

After transmission to a mammalian host, the proteolytic activity of gp63 may help metacyclic promastigotes to defend themselves against lysosomal enzymes while they are transforming to amastigotes in the macrophage. gp63 has also been identified as a significant site of complement fixation on the promastigote surface (Mosser and Brittingham 1997) and has therefore been proposed to be involved in the binding of promastigotes to macrophages via complement receptors. Another potential role is in facilitation of parasite migration through host tissues (McGwire et al. 2003).

LPG is the major surface molecule found on the promastigotes of *Leishmania* species (Turco and Descoteaux 1992; Beverley and Turco 1998). LPG contains four covalently linked structural components: a phospholipid tail that anchors the molecule in the surface membrane; a glycan core of several saccharide residues that lies immediately above the surface membrane; a repeating phosphodisaccharide backbone comprising 15–30 phosphate-galactose-mannose units; and a cap of 2–3 saccharide residues. LPG has been most extensively studied in *L. major* but is assumed to fulfil similar functions in other species. The number of repeated phosphodisaccharide units varies between life cycle stages, approximately doubling in number between *L. major* multiplicative and metacyclic promastigotes. Modeling suggests that the backbone projects away from the surface; it also carries a number of short saccharide side chains which, in *L. major*, terminate mainly in galactose or arabinose residues. LPG is either absent or expressed at low levels on the surface of amastigotes.

LPG has been proposed to fulfil a variety of functions in both the sandfly and mammalian hosts. The unusual structure of LPG may give the parasites general protection against hydrolytic enzymes in both the sandfly midgut and macrophage phagolysosome. One specific function, that of mediating attachment of promastigotes to microvilli on the sandfly midgut epithelium, has been described above. Detachment from the midgut is correlated with developmental modification of the side chains of LPG in *L. major*. In procyclic promastigotes, the side chains terminate in galactose residues that can bind to the midgut epithelium. Subsequently, these are replaced with LPG-carrying side chains that terminate with arabinose residues. These do not bind to the microvilli, allowing the promastigotes to detach, migrate forward to the anterior midgut, and differentiate into metacyclic promastigotes (Pimenta et al. 1992). At the same time, the number of repeat units increases. This latter change is believed to be responsible for the observed resistance to complement-mediated lysis shown by metacyclic promastigotes, a prerequisite for survival in the mammalian host. LPG is the major site of complement fixation on the metacyclic promastigote. Mechanistically, it is proposed that elongated LPG interferes with complement lysis by steric hindrance, the components of a potential membrane attack complex being too far from and lacking access to the plasma membrane to enable insertion. LPG with bound complement components can be released from the surface, which may represent an additional defense mechanism.

LPG is also proposed to have functions in the establishment of the parasite in the macrophage (Descoteaux and Turco 1993, 2002). As with gp63, fixation of complement by LPG provides ligands that can be bound by macrophage complement receptors, stimulating phagocytic uptake of the metacyclic promastigote. LPG has a strong inhibitory effect on macrophage protein kinase C and, consequently, may have the effect of inhibiting signal transduction pathways that trigger microbicidal responses. It is also an efficient scavenger of toxic oxygen radicals.

Glycosylphosphatidylinositols (GPIs) have been found on the surface of both promastigote and amastigotes stages; in the latter, they are a major surface component (McConville and Ferguson 1993; Ferguson 1997). These glycolipids resemble the lipid anchor-glycan core moiety of LPG. The glycocalyx formed by these abundant molecules is assumed to help protect amastigotes against the potentially hostile environment of the phagolysosome.

Surface membrane biochemistry

In addition to gp63, a number of other ectoenzymes have been described on the surface membrane of promastigotes: acid phosphatase, 3′ and 5′ nucleotidases (Bates 1991). Promastigotes, and probably amastigotes, possess a surface membrane proton ATPase (Zilberstein and Shapira 1994) which serves an important function in pH regulation, extruding protons and preventing acid-

ification particularly in the phagolysosomal environment. Transporters for glucose, amino acids, ribose, nucleosides, and folate have also been described (Beck and Ullman 1991; Marr 1991; ter Kuile 1993; Zilberstein and Shapira 1994). The surface membrane has an unusual sterol composition with ergosterol as the major membrane sterol (Haughan and Goad 1991; Chance and Goad 1997). Two enzymatically active secretory molecules have been described in promastigotes; acid phosphatase (Bates and Dwyer 1987) and chitinase (Schlein et al. 1991). In addition, promastigotes and amastigotes secrete various phosphoglycans (Ilg et al. 1994; Ilg 2000).

Molecular biology

Leishmania spp., in common with other kinetoplastids, possesses several unusual features that differentiate them from other eukaryotes described to date, and thus merit brief mention here. The structure of kinetoplast DNA (kDNA) is unique for a mitochondrial genome (Shlomai 1994; Shapiro and Englund 1995; Lukes et al. 2002), but kDNA also exhibits a unique form of post-transcriptional RNA processing termed 'RNA editing' (Stuart and Panigrahi 2002). The 25–50 identical maxicircle DNA molecules of each kinetoplast encode various mitochondrial proteins but the primary RNA transcripts from these are not directly translatable. When compared to the final transcript, the gene sequences are found to contain insertions and deletions of uridine residues. The maxicircle primary transcripts are corrected in an editing process using short sequences, 'guide RNAs', which are themselves encoded by the other component of kDNA, the more numerous minicircles. This process is unique to kinetoplastids and is probably an ancient trait retained since the early divergence of the group from other eukaryotes.

Leishmania and kinetoplastids also exhibit several unusual features in the expression of their nuclear genes (Graham 1995; Stiles et al. 1999). Such genes are usually grouped together in polycistronic transcription units, an arrangement more commonly found in prokaryote organisms. Promoters have not been precisely defined, but there may be as few as one or two transcription start sites per chromosome (McDonagh et al. 2000; Myler and Stuart 2000). Once transcription is initiated, RNA polymerase then proceeds to transcribe in turn many individual genes of the array. Although transcription appears to be continuous, a giant transcript is not produced because, as it is synthesized, each gene is individually processed. A specific 39-nucleotide leader sequence is spliced in *trans* to the 5′ end and a poly(A) tail added to the 3′ end of each gene transcript. These two processes may be mechanistically linked. There are no intervening sequences in the coding regions of nuclear genes. Finally, gene expression itself is rarely, if at all, regulated at the transcriptional level in *Leishmania* (the general rule for eukaryotes) but occurs at a number of post-transcriptional steps depending on the specific gene concerned. These include the RNA processing steps mentioned above, mRNA stability, translational control and post-translational turnover of proteins.

Genetics

Leishmania appear to be essentially diploid organisms (Bastien et al. 1992; Lighthall and Giannini 1992) although occasional examples of aneuploidy have been found. Diploidy has been confirmed for a number of specific genes in which two rounds of gene disruption are required in order to generate null mutants. Chromosomes do not condense during mitosis but can be separated and enumerated by pulsed-field gel electrophoresis. The use of this technique, the identification of genetic markers for specific chromosomes and an ongoing effort to sequence the *Leishmania major* genome have recently established the karyotype of *Leishmania* (Ivens and Blackwell 1999). There are 36 chromosomes in most species, ranging in size from 285 to 2 800 kb. Species related to *L. mexicana* and *L. braziliensis* (both from the New World) possess 35 and 34 chromosomes respectively, a result of possessing fused versions of certain chromosomes that are separate in other species (Britto et al. 1998). However, synteny is well conserved between species examined to date, variation residing mainly in the spacing of genes rather than their order (Britto et al. 1998; Ravel et al. 1999). There is some circumstantial evidence for a sexual cycle in *Leishmania* (Bastien et al. 1992), but so far no one has succeeding in performing a genetic cross (as in African trypanosomes). Population genetic data indicate that *Leishmania* has a predominantly clonal structure and reproduces asexually (Tibayrenc et al. 1990; Victoir and Dujardin 2002). Therefore, sexual reproduction, if present, may be more important on an evolutionary scale than in the routine life cycle progression of the parasite.

CLINICAL ASPECTS

Clinical manifestations

One of the most remarkable features of the leishmaniases is the diversity of diseases caused by morphologically similar parasites living in a single series of cells (Figure 11). This diversity is by no means fully explained by genetic diversity among the parasites and is generated in part by the variety of host responses to the infection and in part by the (largely unexplained) restriction of the parasites to specific parts or organs of the body. One factor restricting parasites to the skin may simply be temperature, to which some species of

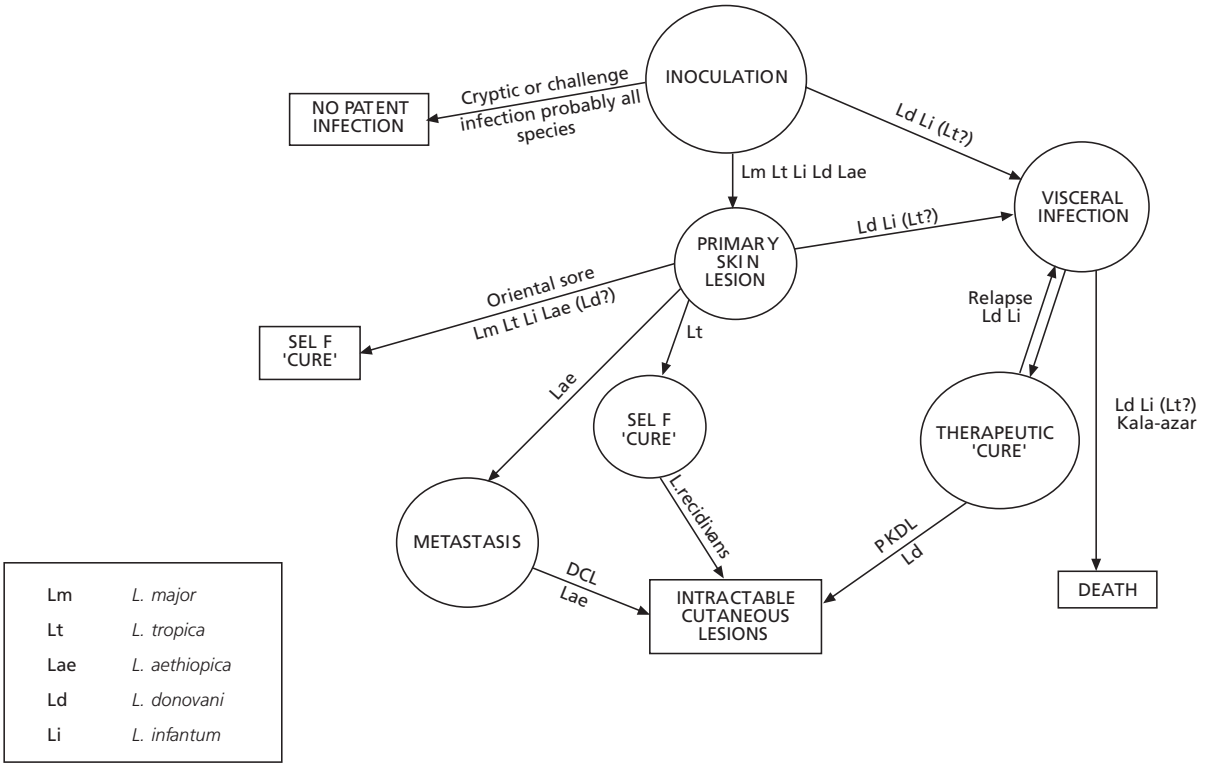

Figure 11 *The 'leishmaniasis spectrum', showing the potential progress and outcome of infection with Old World species. (Modified from Molyneux and Ashford 1983)*

Leishmania are particularly sensitive. In experimental animals, the genetic make-up of the host has been shown to have a great effect on the host response to infection.

There is considerable evidence that even the most virulent *Leishmania* parasites may cause no detectable disease in certain individuals. Wherever transmission occurs, there are numerous people who have positive immunological reactions but no cutaneous scar or history of visceral disease. Sub-clinical infection must be particularly common in foci of *L. infantum*. Here, disease in adults is usually associated with underlying immunosuppressive disease and may then be very common; even among children overt disease may be extremely rare despite intense transmission.

CUTANEOUS LEISHMANIASIS OR ORIENTAL SORE

A single cutaneous lesion, or leishmanioma, at the site of each infective sandfly bite is characteristic of infection with *L. tropica*, *L. major*, *L. aethiopica*, certain strains of *L. infantum* and, rarely, *L. donovani*. Oriental sore, caused by *L. major* or *L. tropica*, is usually painless and self-curing, and is frequently passed off as a trivial matter. Although this may be true of a single lesion in a hidden place, multiple lesions of *L. major* or disfiguring facial lesions due to *L. tropica* may be physically or psychologically crippling.

Generally, all lesions on any one patient have a similar appearance and progress synchronously. In *L. major* infection, the center of the 'wet' lesion becomes necrotic and exudative forming a loose crust of congealed serum above a granulomatous base that eventually produces the characteristic scar. *L. major* lesions may number more than 100. With *L. tropica* the 'dry' lesion is more swollen and less necrotic; the exudate is less profuse and accumulates as a thicker crust. Numerous lesions are rare, the average number being around two. These differences are by no means consistent and are certainly not diagnostic. *L. aethiopica* lesions are even more swollen and less necrotic than those of *L. tropica*; frequently they are barely exudative, with gradual scaling or exfoliation of the dermis at the center. These lesions often last for years before healing. Cutaneous lesions resulting from *L. infantum* resemble those of *L. tropica* but are generally smaller and more indolent.

Oriental sore is not usually associated with systemic manifestations, although there may be enlargement of the draining lymph nodes. The necrotic centers of lesions are heavily contaminated with bacteria but these are not usually invasive, so inflammation, suppuration, and pain are atypical.

LEISHMANIASIS RECIDIVANS

Leishmaniasis recidivans (LR) is an unusual sequel of oriental sore caused by *L. tropica* infection. In this condition, small and usually non-ulcerating lesions appear, mainly on the margin of an apparently healed scar of oriental sore. These may last indefinitely, gradually extending the limits of the initial scar, and causing severe disfigurement. LR is associated with a strong cell-mediated immune response; as it closely resembles cutaneous tuberculosis, both superficially and histologically, the name 'lupoid leishmaniasis' is sometimes used.

DIFFUSE CUTANEOUS LEISHMANIASIS

Diffuse (more correctly 'disseminated') cutaneous leishmaniasis (DCL) is a rare form of disease, caused by *L. aethiopica* in the Old World; just over 100 cases have been recorded. The parasites are restricted to the skin but become widely distributed over much of the surface in large, swollen plaques and nodules. DCL resembles lepromatous leprosy, for which it was initially mistaken, but the abundant parasites in the nodules provide easy distinction. This is an immunologically anergic condition in which neither humoral nor cell-mediated responses are activated; it is very difficult to treat and may last for the rest of the greatly disrupted life of the patient.

VISCERAL LEISHMANIASIS OR KALA-AZAR

Visceral leishmaniasis is characteristic of infection with *L. donovani* and *L. infantum*. There are also suggestions that occasional cases are caused by *L. tropica*. The two main viscerotropic species cause broadly similar diseases although *L. infantum* mainly affects young children and has a greater tendency to cause lymph node enlargement. The general course of the disease is thought to be closely related to the health status of the patient at the time of infection and longitudinal studies have provided good evidence that malnutrition exacerbates *L. infantum* infection (Badaro et al. 1986).

The infection begins at the site of the infective sandfly bite, where there may be an initial ulcerating lesion. This has been best described in dogs, where its occurrence does not reflect the eventual outcome (Vidor et al. 1991). In humans, a leishmanioma is rarely seen and, if present, it may become a self-limiting lesion resembling oriental sore without producing visceral disease.

The clinical features of visceral leishmaniasis are well described by Rees and Kager (1987). Typically, visceral disease develops after an incubation period of 2–6 months and is accompanied by a persistent, irregular, low-grade fever, but onset may be delayed for many years. Once established, subsequent development of disease is quite variable; onset of severe symptoms may be very acute with rapid progression to life-threatening disease within 2 weeks, or progression may be insidious, almost unnoticed, and probably passed off by the patient as malaria, until the abdominal swelling becomes a major concern. Splenomegaly is the most consistent and noticeable sign. The spleen becomes enormously enlarged, extending well below the umbilicus, its size emphasized by an accompanying cachexia. Hepatomegaly is less consistent and less extreme but is usually present in late cases. The hematological picture is greatly altered with anemia and leukopenia being the most manifest changes. The outcome of untreated fully symptomatic visceral leishmaniasis is usually fatal.

POST KALA-AZAR DERMAL LEISHMANIASIS

Post kala-azar dermal leishmaniasis (PKDL) is a relatively common consequence of therapeutic cure from visceral leishmaniasis caused by *L. donovani*. It is not associated with *L. infantum* infection. PKDL is occasionally seen in patients with no history of visceral disease but only in places where *L. donovani* is transmitted; this is probably a sequel to subclinical infection. Sometimes, PKDL develops before the visceral infection has cured but its onset may be delayed for as much as 2 years. The extent to which chemotherapy actually causes PKDL is an interesting conundrum and one that is unlikely to be resolved soon as visceral disease is always treated if drugs are available and untreated cases have a poor prognosis. PKDL has been reported in some 20 percent of cured cases in India and is said to be rare in Africa but a very high incidence has been recorded in a recent Sudanese epidemic (Zijlstra et al. 1994).

PKDL is a variable disease; it may start as a widespread punctate, progressive depigmentation giving the skin a mottled or freckled effect, or may first be noticed as discrete papules, mainly on surfaces exposed to light. It can progress to produce an extensive surface of coalescing papules or large discrete nodules which superficially resemble DCL or lepromatous leprosy. The lesions are delicate, but do not ulcerate unless traumatized. The duration of the untreated condition, the numbers of parasites and the immune responses of patients with PKDL are very variable.

LEISHMANIA AND HUMAN IMMUNODEFICIENCY VIRUS (HIV) COINFECTION

Most of the information on coinfection relates to *L. infantum* acquired in south-western Europe (France, Italy, Portugal, and Spain); few coinfections with other *Leishmania* species have been described to date although they must certainly occur. From 1990–98, 50 percent of visceral leishmaniasis cases presenting in these countries were coinfected with HIV (Dereure et al. 1995; WHO 2000). Visceral leishmaniasis and HIV together are synergistic infections because visceral

leishmaniasis accelerates the development of aquired immune defeciency syndrome (AIDS) and the presence of HIV infection enhances the spread of visceral disease (Wolday et al. 1999). The diversity of strains found is much greater than usual and strains that are normally associated with cutaneous disease may become visceral in coinfection (Pratlong et al. 1995). Serological evidence suggests that HIV may both activate subclinical leishmaniasis and make the patient susceptible to a new infection (Gradoni et al. 1993); serology is of little use in diagnosing coinfection. In a group of patients in Spain, the clinical presentation was characteristic of kala-azar and most were at CDC HIV stage 4 with fewer than 200×10^6 CD4+ lymphocytes per l. Visceral leishmaniasis was the first reported severe infection in 10 of 47 cases (Medrano et al. 1992). Other workers have found a wider diversity of presentations, frequently with infection of the upper alimentary tract (Peters et al. 1990). Many authors have suggested that visceral leishmaniasis should be included in the list of opportunistic infections indicative of AIDS (e.g. Altes et al. 1991). The predominance of intravenous drug users among these patients suggests that syringe transmission may occur (Alvar and Jimenez 1994). Cases of coinfection in Europe are expected to fall as a result of availability of new anti-HIV drugs but the problem is increasing in eastern Africa, the Indian subcontinent, and South America. The true extent is unclear, but it is certainly grossly under-reported, as few of these countries implement active surveillance of *Leishmania*/HIV coinfection.

IMMUNOLOGY

Introduction

The responses of the immune system to *Leishmania* infection are highly complex (reviewed by Mauel and Behin 1987; Liew and O'Donnell 1993; Bogdan and Rollinghoff 1999). They may accelerate cure or exacerbate the disease depending on the particular circumstances. This is partly due to the effects of genetic variation in the mammalian host, partly due to genetic variation in the parasites between species and strains and partly due to chance factors, such as the location, inoculum size, and number of infective bites received.

Host genetic variability

The evidence that genetic variation in humans contributes to variability in their immune responses and clinical outcome is mainly anecdotal and essentially based on the wide clinical spectrum produced by apparently identical parasites. More definitive evidence is now accumulating (see Blackwell 1999), but even under circumstances in which individuals share the same environment and differences exist in incidence or severity of disease, it is impossible to be certain whether this reflects human genetic variation or, for example, different numbers of bites received. The main evidence supporting the importance of genetic variation comes from experimental studies conducted with inbred strains of mice (reviewed by Bradley 1987; Blackwell 1992). It is difficult to determine the extent to which these findings can be extrapolated to human patients. Nevertheless, this experimental approach has proved productive and several genetic loci have been identified in the mouse that are linked to resistance phenotypes. The genes encoding some of these have now been cloned and, although the functions of these are not fully resolved, this opens up the possibility of a search for homologs in the human genome. This should lead to progress in assessing the contribution of human genetic variability to the clinical spectrum of disease.

Human cutaneous leishmaniasis

Spontaneous cure of uncomplicated *L. major* and *L. tropica* infection usually results in a solid immunity. There is a marked development of cell-mediated reactions but a weak antibody response, although specific antibody can be detected. The cell-mediated reactions are responsible for a marked delayed type hypersensitivity (DTH) response to the inoculation of leishmanin (washed promastigotes in 0.5 percent phenol saline) in active and cured cases. The DTH response can often be detected before healing. In persistent *L. tropica* infections that result in LR there is still a strong DTH response, indicating that the parasite can survive despite the high immunological reactivity of the host. In contrast, DCL caused by *L. aethiopica* is characterized by a lack of DTH response but the presence of antibodies thus tending more to resemble response to visceral leishmaniasis.

Human visceral leishmaniasis

Radical cure of *L. donovani* infection by chemotherapy can also generate protective immunity in some individuals. In contrast to cutaneous leishmaniasis, cell-mediated immunity is impaired in active kala-azar patients who consequently lack a DTH response but this can be demonstrated after cure. There is, however, a marked humoral response to visceral leishmaniasis. Polyclonal B cell activation produces mainly immunoglobulin (Ig)G and IgM antibody, most of which is non-specific, but high titers of specific antibody can also be detected. In PKDL, a curious situation arises in which there seems to be immunity in the viscera but not in the skin. Only a proportion of PKDL patients show a DTH response; antibody levels are elevated but are not as high as in active kala-azar.

Experimental leishmaniasis

The relative paucity of information on the immunology of human leishmaniasis is in contrast to an explosion in the literature relating to experimental infections in inbred strains of mice particularly using *L. major* (Liew and O'Donnell 1993; Milon et al.1995; Solbach and Laskay 1995, 2000; Nabors 1997; Alexander et al. 1999; Sacks and Noben-Trauth 2002). This explosion is due not only to the potential impact that such work may eventually have on human medicine, but also to the role that *Leishmania* has played in fundamental immunological research itself. The relatively recent nature of these findings, together with the difficulties of investigating human immunology, means that the extent to which they can be extrapolated to human leishmaniasis is difficult to predict. In all of these experimental studies, it should be remembered that the infection occurs against a specific genetic background, usually in a highly susceptible strain of mouse, and that the interaction between innate genetic factors and specific immune responses is not well understood. Despite these caveats, some of the major findings are likely to be borne out and thus merit brief discussion. The main effector cells in murine cutaneous leishmaniasis are two subsets of CD4+ T cells, T helper 1 (Th1) and T helper 2 (Th2) cells, each associated with a distinct cytokine profile. Resistance and healing in mice is associated with a Th1 type response whereas susceptibility and disease progression is accompanied by a Th2 response. These polarized Th1 or Th2 responses have not been demonstrated in murine *L. donovani* infections which instead are associated with an absence of a Th1 response in susceptible strains (Kaye 1995). Early events in the dermis and epidermis of the skin may play a crucial role influencing the development of resistance or susceptibility (Moll 1993; Solbach and Laskay 1995; Ritter and Korner 2002). A Th1/Th2-like dichotomy in CD4+ T cell clones can be demonstrated in human leishmaniasis (Kemp et al. 1993, 1994; Ghosh et al. 1995). The main effector cell in protective immunity is the macrophage host cell itself, in which the killing mechanism is a cytokine-induced production of nitric oxide (Liew and Cox 1991).

PATHOLOGY

In visceral leishmaniasis, parasites may be found in practically every organ, whereas they are largely limited to the skin in cutaneous leishmaniasis; nevertheless the histological spectrum of the different diseases is surprisingly uniform. Cells of the mononuclear phagocyte system are infected (Ridley 1987), particularly the more actively phagocytic members of the series. Further phagocytic cells are attracted to the site which, in turn, become infected so a colony of infected cells is produced. The parasites may be restricted to the initial cutaneous site or may, after a variable delay, disseminate in the blood rather than the lymphatics to establish metastatic colonies in almost any organ. The temperature dependence of each species may partly be responsible for the ultimate distribution of the lesions. Dissemination is also limited by the histological immune response.

In cutaneous leishmaniasis, a spectrum of histological response to infection, somewhat analogous to that in leprosy, is based on the amount of lymphocyte, plasma cell, and giant cell infiltration constituting the granulomatous inflammation. The minimal, anergic, (Ridley's 'group I') response is best seen in DCL with abundant heavily infected macrophages, few other cells and no giant cells. There is little necrosis and no ulceration. The other extreme, the 'group V' hypersensitive response, is tuberculoid, with an epithelioid cell granuloma, large Langerhans cells, variable plasma cell numbers and scanty lymphocytes. Few parasites are present and, again, there is no necrosis. Langerhans cells may migrate to the draining lymph nodes where they present antigen to T cells initiating the immune response (Moll 1993). Between these two histologically stable extremes, both of which produce long lasting lesions intractable to treatment, are three less well defined categories which, to some extent, represent a progression associated with lymphocyte infiltration, variable amounts of necrosis, and healing. Necrosis is the main feature of an effective immune response and subsequent healing, whereas small numbers of parasites may be destroyed by macrophages and giant cells.

The histological pattern in deep tissues is less fully described but seems to be similar; again, the main feature of healing is the presence of lymphocytic granulomata and necrotic centers. In contrast to cutaneous leishmaniasis, however, natural healing is not the rule and the lesions are more like the anergic, group I type described above with widely disseminated proliferations of infected macrophages and little infiltration. Most of the pathology is caused by this hyperplasia and damage to the organs caused by associated congestion. According to El Hag and Hashim (1994), the liver shows infected Kupffer cells and macrophages together with chronic mononuclear cell infiltration of the portal tracts and lobules, ballooning degeneration of the hepatocytes and fibrosis of the terminal hepatic venules.

DIAGNOSIS

Relative unfamiliarity with the leishmaniases among practitioners in non-endemic areas occasionally leads to serious misdiagnosis and mistreatment. A good travel history may arouse suspicion in those versed in geographical medicine, but reference to a tropical specialist is usually required to confirm the diagnosis. In areas where the disease is better known, clinical diagnosis is frequently regarded as sufficient. Although this may be

justified during epidemics, a number of alternative diagnoses will inevitably be missed if demonstration of the parasites is not attempted on all patients.

Clinical diagnosis

Clinical diagnosis of any of the leishmaniases depends primarily on awareness. None of the signs or symptoms is diagnostic but, when combined with a relevant travel history, or in an endemic area, a good presumptive diagnosis may be made clinically and can be confirmed parasitologically. 'Diagnosis' by therapeutic trial is commonly practiced but is unsatisfactory, particularly in view of the expense and potential toxicity of the drugs.

Visceral leishmaniasis is often mistakenly recorded post-mortem as 'malaria that failed to respond to treatment' (and is probably included in malaria mortality statistics). The typical patient presents at a late stage, with persistent but fluctuating low grade fever, weight loss giving the appearance of severe starvation, and spleno- or hepatosplenomegaly. The patient is usually alert and feels remarkably well, considering his or her condition. The skin is sometimes said to be 'muddy', 'pale' or 'dark', but these are not diagnostically useful descriptions. There is frequently persistent diarrhea. Lymphadenopathy is an irregular feature.

Non-specific laboratory tests will show marked leukopenia (pancytopenia, mainly neutropenia), anemia, raised serum proteins with reversal of the albumin/globulin ratio due to greatly raised IgG levels.

Manson-Bahr (1987) suggests the following differential diagnoses: malaria, relapsing fever, trypanosomiasis, brucellosis, liver abscess, tuberculosis, tropical splenomegaly, myeloid leukemia, lymphoma, cirrhosis of the liver, schistosomiasis, thalassemia, histoplasmosis, and various gammopathies.

Diagnosis of visceral leishmaniasis in the presence of HIV infection is particularly difficult as the presentation may be very atypical and serological tests may be negative.

All forms of cutaneous leishmaniasis usually present without systemic disease. Typical lesions of oriental sore are readily recognized by the raised edges, necrotic, exudative center with granulomatous base, long duration, and painlessness. Less typical lesions take a bewildering array of forms and must be distinguished from basal cell carcinoma, tuberculosis, various mycoses, cheloid and lepromatous leprosy.

LR may be indicated by the presence of a scar and positive skin test, whereas DCL shows no ulceration or scarring and has a negative skin test.

Parasitological diagnosis

A diagnosis of leishmaniasis can be formally confirmed only by the demonstration of the parasites. These are readily seen in smears or touch preparations of infected tissue stained with Giemsa's stain, preferably at pH 7.2 rather than the pH 6.8 normally used in hematology. Sections of tissue stained more conventionally with hematoxylin and eosin are much more difficult to interpret so the diagnosis must be suspected before material is prepared. Preparations from cutaneous lesions may be made from punch biopsies, but it is usually sufficient to take a small quantity of material from the living, but diseased, tissue at the edge of the lesion by a slit smear or other minimally invasive technique.

Deeper tissues may be sampled by needle biopsy. Material may be aspirated from lymph nodes following injection of a small quantity of physiological saline. Bone marrow smears are made using standard hematological techniques. Spleen aspirate is the most reliable material for demonstrating parasites in kala-azar. Although many physicians are reluctant to take spleen aspirates others have no hesitation and even use this method to monitor treatment. It is vital to use the correct technique and equipment with confidence so that the capsule of the spleen is penetrated by a fine needle for only a fraction of a second (see Bryceson 1987a for precise instructions).

Parasites may be scanty and are mostly extracellular in slide preparations so these may have to be examined for at least 15 minutes using a $\times 100$ oil immersion objective before the diagnosis can be confirmed. Amastigotes are identified by their size (3–5 μm) and the possession of a nucleus and kinetoplast; although smaller, the kinetoplast is often easier to detect because the DNA is very densely packed and produces a deep purple spot or rod on staining. It may be possible to provisionally identify the parasites to species level by considering the circumstances and clinical features. *L. major* and *L. tropica* can be separated with some confidence by the abundance of parasites in the latter, their smaller size and the presence of more numerous parasites in each infected cell. Complete identification requires the isolation of parasites in culture.

Whatever material is collected, it is desirable to culture the parasites in blood-agar or insect tissue culture medium so that they can be identified at strain level by isoenzyme analysis. For special purposes, material can be inoculated into a hamster, generally the most susceptible experimental host. Monoclonal antibody and DNA probes, with or without PCR amplification, have been developed for identification of the parasites. These are not yet commercially available but are of great use in epidemiological or ecological investigations.

Two indirect approaches for investigating the presence of parasites in clinical samples are currently under development. There are numerous publications describing the potential application of PCR to *Leishmania* diagnosis (for example, Wiegle et al., 2002; Salotra et al. 2001; Lachaud et al. 2001). There is, as yet,

no general consensus about which sequences are the best targets for diagnosis, but repetitive nuclear genes, such as ribosomal and mini-exon genes, or kDNA are often used. Also, there is no one-step PCR diagnostic test that will identify all species. However, these drawbacks are likely to be solved in the near future. The second area of advance is in antigen detection systems, specifically the detection of antigen in the urine of patients with visceral leishmaniasis (Attar et al. 2001; Sarkari et al. 2002). This is particularly welcome as this is a completely non-invasive technique, a significant advantage compared to bone marrow or spleen biopsy. As yet, these methods have not supplanted direct parasitological diagnosis and require further testing and clinical trials but, given their technical advantages, this seems a matter of time.

Immunological diagnosis

Immunological diagnosis (reviewed by Kar 1995) is of value in screening suspected kala-azar patients, other than those coinfected with HIV, and in epidemiological studies.

In the formol-gel test, a drop of full strength formalin is added to 1 ml of serum. A positive result is indicated by the rapid and complete coagulation of the serum. This simple test merely indicates greatly increased serum proteins and thus is non-specific. Nevertheless, it is still widely used; in order to be acceptable in peripheral clinics in endemic areas, this is the level of technology that any new test will have to approach.

More specific tests, which become positive earlier in kala-azar, include the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) and a direct agglutination test (DAT). This last technique requires minimal equipment, is robust in the most difficult conditions and is exquisitely sensitive. DAT is fast becoming available for use at peripheral-level clinics and the development of freeze-dried antigen (Meredith et al. 1995) promises even wider availability of this test. For active case detection, numerous positive responses to serological tests are found in people who show no disease. To treat all these people as patients would be a mistake; probably the best thing is to examine them carefully, treat those with signs of disease and to monitor the others carefully.

The specific reagents for the above serological tests are not readily available commercially, but they can be prepared relatively easily in the laboratory.

A delayed hypersensitivity reaction to intradermal crude *Leishmania* antigen is produced in healing or cured cases of both cutaneous and visceral leishmaniasis. This leishmanin, or Montenegro skin test is of great value in epidemiological studies, but is of little clinical use as the number of positive reactors is usually much greater than the number with a history of disease suggesting that either the test is non-specific or there is a considerable amount of sub-clinical infection.

EPIDEMIOLOGY

Introduction

Knowledge of the epidemiology of the leishmaniases has benefited in recent years from our growing understanding of the taxonomy of the group and from the possibility of accurate identification of morphologically similar parasites derived from human cases, sandfly vectors or non-human mammalian hosts. Current taxonomy corresponds much better with ecology and epidemiology than it does with clinical manifestations (see Molyneux and Ashford 1983). Anomalies and questions remain and the 'spectral epidemiology' (different diseases caused by ostensibly similar organisms) is largely unexplained.

It has been conservatively estimated that, in the Old World, 150 million people in 40 countries are at risk of cutaneous leishmaniasis and 180 million people in 39 countries are at risk of visceral leishmaniasis. The total number of reported cases has been estimated at 240 000 and 73 000 annually for cutaneous and visceral leishmaniasis respectively but this is doubtless a gross underestimate of the real numbers (Desjeux 1991; Ashford et al. 1992a). The figure of 12 million cases annually (sometimes misquoted as 1.2 million) was estimated some time ago by Walsh and Warren (1979), but although the World Health Organization (WHO 1990) uses the higher figure of 12 million, it is hard to know how it was derived.

Although transmission is possible by contagion, by transfusion or in utero, these routes have little if any known epidemiological significance. Effectively, all transmission is by the bite of a phlebotomine sandfly (Diptera: Phlebotominae) (reviewed by Killick-Kendrick 1990b). The leishmaniases illustrate the entire gamut of zoonotic patterns of transmission.

Epidemiology of *L. major* infection

L. major is widely distributed in the Palaearctic and Aethiopian zoogeographical regions along the northern and southern fringes of the Sahara, extending south into northern Kenya and through Arabia, southern Iraq and Iran, into the central Asian states of Turkmenistan, Uzbekistan, and northern Afghanistan. It also ranges through Iranian and Pakistani Baluchistan to the Indian border in Rajasthan (see Figure 1).

Throughout this wide range, the parasite is restricted to arid and semi-arid zones. Its broad distribution seems to be determined by that of its vectors and its detailed distribution is largely determined by that of its natural mammalian hosts.

The three known vectors belong to the subgenus *Phlebotomus* (*Phlebotomus*) of which *P. papatasi* is the most widespread and abundant. *P. papatasi* transmits *L. major* in all but the sub-Saharan and Indian parts of its distribution; it is replaced by *P. duboscqi* in the former and *P. salehi* in the latter. *P. papatasi* has a much wider distribution than *L. major*, especially in northern India, but here it is largely synanthropic and has no access to suitable reservoir hosts. The efficiency of this species as a vector may be affected by the plant juices on which it feeds, some of which may inhibit growth of the parasites (Schlein and Jacobson 1994b).

The known reservoir hosts of *L. major* are rodents. In the central Asian deserts, the great gerbil *Rhombomys opimus* lives in dense colonies with deep and complex burrow systems that provide shelter and possibly a breeding site for *P. papatasi*. The gerbils breed throughout the warmer months and by the end of the transmission season, in autumn when the rodent population is greatest, almost every animal may be infected. The infection is usually restricted to the ears, which become only slightly swollen and eroded, and there is no indication that the rodent is seriously affected.

Great gerbil colonies are best developed in areas where the soil is deep and of a consistency that allows permanent burrows to be constructed. These conditions are found on the alluvial fans in the valleys of the largest rivers draining the great central Asian loess deposits, such as the Amudarya.

The distribution of the rodent extends much further north in central Asia than that of the vector. In these zones, they are infected with other parasites, *L. gerbilli* and *L. turanica*, which behave similarly in the rodent but do not infect humans and are transmitted by different sandfly vectors (Strelkova et al. 1993).

The other rodent reservoir host of *L. major*, which has very narrow habitat requirements, is the fat sand rat *Psammomys obesus*. This host maintains populations of the parasite in Algeria, Tunisia, Libya, north Sinai, the Jordan Valley, southern Syria, and much of Arabia. This rodent is unique in being able to derive its entire nutrient and water requirements from the succulent, but highly saline, stems and leaves of halophilic plants of the family Chenopodiaceae. These plants dominate the internally draining salt pans, sebchet or playas of much of the desert fringes and it is here that the sand rats are concentrated. *P. obesus* burrows are less deep or extensive than those of *R. opimus* and provide a less favorable habitat for *P. papatasi*. Furthermore, the rodents breed mainly in the winter when there is no transmission so the young are not exposed to infection until they are a few months old. Nevertheless, all the evidence supports the conclusion that *P. obesus* is responsible for the maintenance of *L. major* in most of this part of its range.

Some foci of *L. major* in the Palaearctic occur in the absence of both *P. obesus* and *R. opimus* and have been attributed to various rodent hosts such as jirds, *Meriones* spp. (Rioux et al. 1982) or the mole rat *Nesokia indica*. The structure of these foci, notably that on the Iraq–Iran border, remain to be fully understood.

In its Aethiopian range, where *L. major* is usually transmitted by *P. duboscqi*, various rodents have been found infected, notably the grass rats *Arvicanthis* spp. and the multimammate rats *Mastomys* spp., in which the parasites are found in the viscera but do not seem to cause serious pathology. These rodent populations fluctuate wildly and do not have narrow habitat requirements so they are not good candidates for the maintenance of vector-borne infections. Possibly there is no individual rodent species maintaining the parasite but a diversity of hosts may be required.

L. major infection in humans is most conspicuous in epidemics in groups of people entering sparsely inhabited zoonotic foci. Major outbreaks have occurred in military groups and in workers on development projects. Development frequently alters the environment sufficiently so that, following a brief epidemic period, the infection disappears as has been seen particularly in irrigation schemes. Residents in zoonotic foci usually become infected at an early age and, being familiar with the infection, are less concerned.

The dynamics of human infection are largely governed by the rate of exposure to infection and the almost complete resistance to reinfection. Beljaev and Lysenko (1977) have modeled the stable-state system, making minimal, realistic assumptions; they show that at low rates of exposure (fewer than 0.05 exposures per person per year) incidence is proportional to this rate. They term locations where these conditions prevail 'hypoendemic foci'. In contrast, hyperendemic foci are those where the exposure rate is greater than 0.25 exposures per person per year. In such foci, lifetime incidence is largely independent of the exposure rate, change in which alters only the mean age at which people become infected. As most people who live in such hyperendemic foci are infected when young and subsequently become immune, the incidence is dependent on the rate at which non-immune people are recruited into the population, whether by birth or immigration.

In sub-Saharan Africa, outbreaks are much less frequent and human cases occur sporadically in both space and time. In some foci, in Ethiopia and Kenya for example, human infection is extremely rare even in places where infected rodents have been found.

Transmission between humans without a mammalian reservoir host has not been well established, although the possibility cannot be excluded. A series of epidemics in Khartoum and nearby towns in Sudan in the late 1980s occurred in dense, stable human populations that had never previously known the infection (El Safi et al. 1988). The best available explanation is that the parasite was introduced by displaced people from endemic areas to the west and that it was trans-

mitted to residents by the abundant *P. papatasi* living close to the Nile.

Epidemiology of *L. tropica* infection

L. tropica is mainly found in the ancient cities of west and central Asia (see Figure 1). Generally, *L. tropica* inhabits slightly wetter areas than *L. major*. The two species are geographically sympatric in places but are ecologically separated. Vernacular names for the infection frequently refer to endemic cities, for example Balkh sore, Baghdad boil, Aleppo evil, and Delhi boil; this probably reflects the perception of visitors rather than indigenous people.

Urban cutaneous leishmaniasis is transmitted by *Phlebotomus* (*Paraphlebotomus*) *sergenti* throughout its range. This sandfly can reach high populations in crowded cities and suburbs but is not restricted to the peridomestic environment. Dogs have been found to be infected but it is generally thought that the main hosts are humans. Unlike *L. major*, the rate of exposure to infection depends on the number of active human lesions as well as other factors. The human lesion normally lasts around 1 year and people with cured lesions are immune. Therefore, in conditions of potentially high transmission, most people are immune, but few are susceptible leaving even fewer actually infected. The maintenance of stable transmission in a stable human population has been estimated to require some 20 000 sandfly bites per person per year which, in the seasonal climates of central Asia, may mean 100 bites per person per night. Such conditions are rarely, if ever, met and the infection must rely either on movements of people (Ashford et al. 1992b) or on spatial heterogeneity in the immunity of the population, moving from place to place in localized epidemics (Ashford et al. 1993). In this way *L. tropica* resembles a viral infection such as measles rather than most other eukaryotic parasites.

There are reports of *L. tropica* infection occurring sporadically in rural settings, which cannot easily be explained by the above patterns. Furthermore, the finding of *L. tropica* causing visceral leishmaniasis in American soldiers who served in the Gulf region during the first Gulf war in 1991 (Magill et al. 1993, 1994) and in Kenyan and Indian villagers (Mehbratu et al. 1989; Sacks et al. 1995) in the heart of areas of *L. donovani* transmission, pose intriguing epidemiological questions for which no sensible answer can yet be proposed.

It was thought at one time that *L. tropica* did not occur in Africa. Parasites from an ostensibly zoonotic outbreak of cutaneous leishmaniasis in Tunisia were identified as a new species, *L. killicki*, which, on the cladograms of Rioux et al. (1990) (see Figure 3), is close to *L. tropica*. A wide variety of *L. tropica*-like strains are responsible for another apparently zoonotic focus in Morocco (Pratlong et al. 1991). Parasites from sporadic cutaneous cases, hyraxes *Procavia* spp. and from the sandflies *P. rossi* and *P. guggisbergi*, in Namibia and Kenya were found to be intermediate between the original *L. killicki* and *L. tropica*. It is a matter of opinion whether these parasites should be included with *L. tropica* or not (Sang et al. 1992). Occasional aberrant cutaneous, visceral, or mixed infections have been reported elsewhere in Africa, notably in Tanzania, Zambia, and Malawi (Pharoah et al. 1993). These parasites have not been isolated and their identity is unknown but presumably they originated in zoonotic foci that await discovery and description.

Epidemiology of *L. aethiopica* infection

L. aethiopica is restricted to the highlands of East Africa in Ethiopia and Kenya, between c. 1 800 m and 2 700 m above sea level and within the 800 mm isohyet (see Figure 1). In all known foci, it is associated with hyraxes (Hyracoidea) which are the natural reservoir hosts (Ashford et al. 1973). These animals have very poor temperature regulation and live in deep clefts in rocks or trees in which they can find the shelter they require for the maintenance of their body temperature. Despite this restriction, they are found at all altitudes from below sea level in the Danakil desert to above 4 000 m where there is frost every night. Hyraxes are long-lived and live in colonies or family groups so, although their rate of reproduction is low, they are good candidate reservoir hosts. Parasites have been isolated from normal skin and external mucosa showing no sign of the infection which presumably lasts a long time.

The vectors, *P. longipes* and *P. pedifer*, have less specific habitat requirements, being common in houses or associated with cattle, but they are restricted in altitude. The distribution of the parasite is, therefore, governed by the habitat of the reservoir host and the altitude range of the vectors. Wherever they have been adequately studied, human cases can be attributed to the nearby presence of infected hyraxes. Incidence of disease in humans is rarely high and the rare cases of diffuse cutaneous leishmaniasis caused by this parasite have not been explained epidemiologically.

Epidemiology of *L. donovani* infection

L. donovani is surely one of the great scourges of mankind having been responsible for a series of historical deadly epidemics each causing depopulation of affected areas. The geographical distribution of *L. donovani* infection is somewhat labile (see Figure 2). In the Indian subcontinent, it occurs in the valleys of the Ganges and Brahmaputra Rivers, in Bihar, lowland Nepal, West Bengal, Bangladesh, and Assam, as well as further south, around Madras (reviewed by Sanyal

1985). It is often presumed that visceral leishmaniasis in south China was caused by *L. donovani* but this has not been confirmed. In Africa, the infection is largely restricted to Sudan, Ethiopia, and Kenya.

The ecological distribution of *L. donovani* is well known but poorly understood. In Asia, where humans are the only known hosts, the infection occurs in heavily populated rural areas in the rich silty flood-plains of the great rivers. Here, the vector *P. argentipes* is abundant and strictly synanthropic, breeding in the organically rich material on the floors of cow byres, almost the only sandfly whose larval habitat is reasonably well described (Ghosh and Bhattacharya 1991). The only known reservoirs of infection here are humans and persistent cases of PKDL may be particularly important in maintaining the parasite between epidemics (Addy and Nandy 1992).

In Africa, there are two types of habitat, each associated with a specific vector. *P. orientalis* occurs in *Acacia seyal* woodland, which is the dominant natural vegetation of the light, alluvial, montmorillonite, silty clays of eastern Sudan between latitudes 9°N and 14°N, extending into the western Ethiopian lowlands. *P. martini* occurs in less well defined habitats but is commonly associated with eroded termitaria in the heavy laterite clays of the southern borders of Sudan and Ethiopia, northern Uganda, and much of Kenya. *L. donovani* infection occurs either in fairly stable endemic conditions or as violent epidemics throughout the distribution of these two vectors. The Nile grass rat, *Arvicanthis niloticus*, has been found infected in substantial numbers in southern Sudan and may be an important reservoir host, but the shifting distribution of the epidemics and persistence of intensely endemic residual foci, too dangerous to even visit in the transmission season, remain largely unexplained.

Human *L. donovani* infection is endemic in parts of southern Ethiopia, Sudan, and northern Kenya, where most people are exposed to infection at some time during their lives but relatively few become sick (e.g. Zijlstra et al. 1994). An Ethiopian study indicated an exposure rate of c. 11 percent per year, but there were only 2 percent cases of overt disease annually per 100 non-immune individuals (Ali and Ashford 1994). Nearly all adult males and more than half the females showed evidence of previous exposure.

This apparent insusceptibility of most of the people cannot be true of the early epidemics in northwest India where the disease swept through the population like a plague killing a large proportion (and, incidentally, giving rise to the dreadful reputation of malaria: kala-azar was thought to be *the* malignant, lethal form of malaria (Rogers 1908)). The epidemic of the late 1980s in southern Sudan was equally virulent, and was estimated to have killed half of the affected population, some 100 000 people, in 5 years (Seaman et al. 1992).

Epidemics of kala-azar are often associated with natural disasters or social upheavals, although causal relationships are difficult to establish. The work of Sati (1962) documents the spread of an epidemic in Sudan with famine-related displacement. The 1980s Sudan epidemic may have resulted from the introduction of the parasite by people coming from endemic areas on the Ethiopian border to settled areas with potential transmission. These movements were related to the civil war, as was the poor condition of the recipient people, but any precise interpretation is largely informed speculation.

Although epidemics in Africa occur in rural, largely pastoral environments, those in India are in towns and villages with settled agriculture. In the north Bihar epidemic, maximum incidence was estimated at 6 per 1 000 per year, with more than 100 000 cases reported in some years. In 1992, there were 80 000 cases (Sacks et al. 1995). Reported cases are surely only a small fraction of the actual number occurring.

Epidemiology of *L. infantum* infection

L. infantum parasites are difficult to separate objectively from *L. donovani* and the distribution of the two forms is largely allopatric, so there is a good case for regarding them as subspecies. However, it is convenient to treat them separately because they are very distinct in both their ecology and epidemiology.

L. infantum is the only *Leishmania* species that occurs in both the Old and New Worlds (in the Americas it is sometimes called by its junior synonym *L. chagasi*; see New World leishmaniasis for New World features). In the Old World, it is distributed in much of the Mediterranean basin, both in Europe and North Africa (see Figure 2). It extends erratically through Arabia, Turkey, and Iran to the Central Asian republics, Pakistan, and Kashmir (Rab and Evans 1995), as well as into northwest China. It is thought to have been much more widely distributed in China but visceral leishmaniasis has been almost eradicated throughout that country.

Throughout this range, the main host is the domestic dog, which develops an acute or chronic disease. Though eventually fatal, canine visceral leishmaniasis presents abundant parasites in the skin, available for transmission. The distribution of foci of infection depends on the presence of vector sandflies, usually species of the subgenus *Phlebotomus* (*Larroussius*). Although we are ignorant of the mechanisms by which the distribution of sandflies is regulated, each species has clearly defined ecological preferences. *P. ariasi*, for example, is largely restricted to hillsides with mixed oak *Quercus* spp. woodland. A more important vector, *P. perniciosus*, occurs at lower altitudes, in drier, limestone country, where it is particularly abundant in gardens and suburbs with outcrops and dry-stone walls. These are the main vectors in the western Mediterranean; they are replaced

further east by *P. major* and a wide variety of less well studied probable vectors.

In areas where transmission is intense, almost all dogs become infected and kennels become very difficult to keep. Individual dogs vary greatly in their response to infection and there is some evidence that regionally bred dogs are less susceptible than exotic breeds.

Human infection must be very much more common than overt disease. The latter is classically restricted to children, especially those below the age of 2 years. In the post-war years, the incidence in children, though not in dogs, in most of southern Europe has decreased greatly so the small number of adult cases has become a greater proportion of the total (Marty et al. 1994). In fact, adult cases have now actually increased owing to greater susceptibility to disease in association with HIV and immunosuppression associated with transplant surgery.

CHEMOTHERAPY AND TREATMENT

Treatment of leishmaniasis has a chequered history. Few adequate clinical trials have been carried out, especially with cutaneous leishmaniasis; recommended dosages are frequently based on spurious information and definition of the state of the disease is rarely standardized. Patients frequently present at a late stage, having been treated informally with unknown remedies. Courses of treatment are usually prolonged and so are difficult to monitor or even achieve. Despite these misgivings, the various diseases can generally be cured quite effectively, albeit mainly with drugs that come from archaic pharmacopoeia (Chance 1995; Croft and Yardley 2002). In addition to healing the patient, treatment may also reduce the transmission of anthroponotic *L. tropica* or *L. donovani* infection.

Placebo and physical treatment

The first question is whether or not chemical treatment is justified or even required. Simple, single lesions due to *L. major* will heal naturally in a few months and leave the patient immune for life. It is only with multiple or disfiguring lesions that chemotherapy is justified. The same may be said of lesions due to *L. tropica*, though these are of longer duration. With *L. donovani* infection, sick patients have a high risk of death if left untreated, but active case detection may reveal numerous sub-clinical infections. It may be preferable to keep these under observation if possible, especially if, as is frequently the case, resources are very limited.

Cutaneous lesions, whether or not to be treated, should be disinfected and covered, to prevent secondary infection and to avoid infecting sandflies. Heat treatment, with hot compresses or infra-red radiation may accelerate cure. Hot water, in the form of frequent baths or saunas, is beneficial in diffuse cutaneous leishmaniasis, which responds very poorly to chemotherapy. Removal of lesions by cryotherapy has been recommended, but is controversial. For visceral leishmaniasis associated with massive splenomegaly and unresponsiveness to antimonial drugs, surgical removal of the spleen used to be practiced occasionally and has also been undertaken relatively recently (Magill et al. 1994).

Pentavalent antimonials

Pentavalent antimony, currently used in the form of meglumine antimoniate or sodium stibogluconate, replaced tartar emetic many years ago and is still the first line drug of choice (Croft and Yardley 2002). However, increasing incidence of unresponsive infections, now known to be due to genuine parasite drug resistance, means that this may not remain true for much longer (Sundar 2001). Intralesional infiltration is used for simple, single cutaneous lesions but intramuscular injection is required for all other cases. Despite the prolonged period of repeated treatment required for the cure of visceral leishmaniasis, these drugs are remarkably safe if administered in the correct doses; their reputation for toxicity is unjustified and relates rather to the trivalent antimonials used previously (Bryceson 1987b). Antimony is excreted rapidly from the body so doses are repeated daily and treatment is continuous throughout each course.

For visceral leishmaniasis, the World Health Organization (WHO 1995) recommends intramuscular injection of 20 mg Sb^{5+}/kg daily (to a maximum of 850 mg) for a minimum of 20 days, and until no parasites can be seen in spleen or marrow biopsies taken 14 days apart. Doses may be divided to counteract rapid excretion. Relapsing patients should be treated similarly.

For cutaneous leishmaniasis, the World Health Organization (WHO 1995) recommends 1–3 ml of either of the main antimony preparations, to be infiltrated into the base of the lesion, to be repeated once or twice, at intervals of 1–2 days. For parenteral treatment, 10–20 mg Sb^{5+}/kg i.m. is given daily until a few days after clinical cure.

Aromatic diamidines

Pentamidine, as the isethionate or dimethylsulfonate, is used as a second choice in cases that are unresponsive to antimony. This product may, however, have serious toxic effects, due to sensitivity at the site of intramuscular injection, too rapid intravenous injection, accumulation, or overdosage. The drug may disrupt blood-sugar homeostasis and lead to intractable diabetes after prolonged use. The margin between therapeutic and toxic doses is narrow and the drug is only slowly

excreted, so prolonged treatment at well spaced intervals is required.

The World Health Organization (WHO 1995) recommends treatment with 4 mg/kg three times per week for 5–25 weeks, or even longer, for visceral leishmaniasis. For African diffuse cutaneous leishmaniasis, the only other likely use of this drug in Old World leishmaniasis, treatment is once weekly, but continued for at least 4 months.

Monomycin/paromomycin/aminosidine

The antibiotic monomycin was developed in Russia both for intralesional injection and as a topical treatment for cutaneous leishmaniasis. In the latter use, the active ingredient was contained in a collagen pad tightly applied to the lesion. Paromomycin and aminosidine are probably the same as monomycin, and show considerable promise in topical ointments but in current formulations, irritation, and inflammation are too severe for these products to be more than experimental. This antibiotic shows promise as a synergist when used in combination with antimonials, in both visceral and diffuse cutaneous leishmaniasis (Seaman et al. 1993; Teklemariam et al. 1994).

Amphotericin B

In soluble form, this product is too toxic to be used except where all else has failed. It is administered very slowly, by the intravenous route, requires careful monitoring, and the early doses must be reduced in order to check for excessive side-effects. This drug is mainly used to treat cases of mucocutaneous leishmaniasis in South America (see New World leishmaniasis) and is rarely indicated in Old World leishmaniasis. The recommended dose is 0.5–1.0 mg/kg on alternate days (WHO 1995). More recently, however, there have been important developments in the use of amphotericin, with lipid-associated forms of amphotericin (e.g. liposomal encapsulation) showing much reduced toxicity (Chance 1995). These are now commercially available (AmBisome, Amphocil) and, although relatively expensive, are suitable alternatives to antimonials.

Allopurinol

Allopurinol has the attraction that it is a widely used and well-established drug. Furthermore, biochemical and in vitro studies indicate that it should be effective against *Leishmania*. Despite these factors, clinical trials have been disappointing, although there are suggestions that the drug may have a synergistic effect when used together with antimony.

Miltefosine

Miltefosine or hexadecylphosphocholine, is the phosphorylcholine ester of hexadecanol and was originally developed as an anticancer agent but showed activity against experimental visceral leishmaniasis in mice (Kuhlencord et al. 1992). Recent clinical trials using miltefosine against Indian visceral leishmaniasis have been very encouraging (Jha et al. 1999; Sundar et al. 2002). If miltefosine proves to be clinically useful after more extensive testing, it may represent the most significant step forward in leishmanial chemotherapy for some time as, unlike the other treatments for visceral leishmaniasis, the drug is delivered orally. If safe and effective it is likely to become the first line drug of choice in the future.

VACCINATION

Two important factors suggest that vaccination or other immunological intervention holds promise for the protection of people from at least some of the leishmaniases. First, many individuals appear innately not to be susceptible. This is demonstrated by the survival of some people in even the most serious epidemics and by the finding that some individuals, who have never been exposed, produce a lymphocyte proliferation response to antigen (Akuffo and Britton 1992; Kurtzhals et al. 1995). Second, the immunity produced naturally in response to *L. major* infection is usually solid and there is no evidence that it is not sterile. There may even be a cross-immunity between *L. major* and other species. At present, however, no vaccine is available against Old World leishmaniasis.

Immunization against cutaneous leishmaniasis by deliberate infection with virulent parasites is traditional in parts of west Asia and was carried out on a massive scale during the Iran–Iraq war (Nadim 1988). Both the Russian and Israeli armies abandoned this measure as there were an unacceptable number of serious side-effects. Trials are in progress using various crude and recombinant antigen preparations with acceptable adjuvants and it is hoped that vaccination, at least against *L. major* infection, may soon become feasible (Modabber 1995; Handman 1997; McMahon-Pratt et al. 1998; Handman 2001).

INTEGRATED CONTROL

The principles of control, with particular reference to vector control, are discussed in Control of parasites, parasitic infections, and parasitic diseases. Control of infection depends greatly on local information which, in turn, depends on efficient passive case detection and epidemiological surveillance. These are the minimal activities recommended by the World

Health Organization (WHO 1990). Increased knowledge of the structure of foci of leishmaniasis has led to the proposal of numerous potential active control strategies, but few of these have been evaluated or even tried in practice.

Avoidance of areas of risk is the simplest method and could be applied to military or other activities involving entry into semi-arid areas where zoonotic *L. major* can be a serious risk. In North Africa and west Asia, barracks for soldiers or laborers should be situated well away from depressions with halophilic vegetation and *P. obesus* colonies. People infected with HIV should avoid much of southern Europe in summer, although the coast itself is generally free from *L. infantum* infection. The few known residual foci of intense *L. donovani* transmission in Africa should be avoided. Some of the national parks of Sudan are particularly notorious and are rarely visited in the transmission season.

Environmental management, such as the destruction of desert rodent colonies in inhabited areas, has been attempted with great success in central Asian irrigation schemes, the designs of which were modified to prevent reinvasion by *R. opimus*. Pilot schemes to clear land around affected towns in Tunisia (Ben Ismail 1994) and Jordan (Kamhawi et al. 1993) were inconclusive. The incidence of infection can vary greatly from year to year, so experimental controls are hard to establish. In Ethiopia, it has been suggested that villages affected by *L. aethiopica* infection could be protected by shooting the hyraxes in the vicinity, but this intervention has yet to be tried.

In areas where transmission is peridomestic, as with *L. tropica* and *L. donovani* in Asia, sandfly numbers can be greatly reduced by insecticides. In fact, antimalarial campaigns were credited with the near-eradication of kala-azar in India in the 1950s. Active case detection, treatment and insecticide spraying eliminated *L. tropica* in Azerbaijan in the 1960s. A mass campaign in China in the 1950s, using case detection and treatment, insecticides and elimination of dogs, reduced the number of cases from an estimated 500 000 in 1951 to almost zero (Wang 1985; Guan 1991). Experiments with insecticide-impregnated bednets or curtains have given promising results (Maroli and Majori 1991).

Where transmission is sylvatic, as in the *L. donovani* epidemics in southern Sudan, the economic and political problems that prevent effective control elsewhere are compounded by technological problems. There is no realistic way to alter the environment, we know of no way to reduce sandfly numbers, and the reservoir hosts maintaining residual foci have not been identified. Little can be done other than the provision of active case detection and treatment or the promotion of impregnated bednets, with no guarantee that the latter will be effective. These measures are almost impracticable in such a poor, isolated, and war-torn country. Much of northern Sudan has undergone massive environmental change with the replacement of woodland by mechanized agricultural projects. This elimination of much of the *P. orientalis* habitat has incidentally greatly reduced the area in which kala-azar epidemics can be expected to occur.

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New World leishmaniasis

RALPH LAINSON AND JEFFREY J. SHAW

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INTRODUCTION

Neotropical cutaneous leishmaniasis (NCL) seems to be of great antiquity. Pottery from Peru and Ecuador, dated c. AD 400–900, commonly depicted human faces with mutilations that are remarkably similar to those caused by present day cutaneous and mucosal leishmaniasis (Figure 1) and, at the time of the conquistadores, Spanish historians wrote about ugly facial lesions that frequently afflicted the local Amerindians.

The earliest traceable clinical description of the disease is probably that of a certain Dr L. Villar who, in 1859, wrote 'the disease (Peruvian ''uta'') is very like the Aleppo button' (i.e. oriental sore due to *Leishmania tropica*) (Matta 1918).

The first suspicions that phlebotomine sandflies might be involved in the transmission of NCL seem to have been those of Cosme Bueno who, in 1764, implicated these insects in uta in endemic areas; uta is caused by *L. (Viannia) peruviana* in the Peruvian highlands (Herrer and Christensen 1975). Final proof that NCL was due to infection with *Leishmania* was to await publications by Lindenberg (1909) and Carini and Paranhos (1909), who independently demonstrated 'Leishman-Donovan bodies' in the skin lesions of patients from the State of São Paulo, Brazil. It was left to another Brazilian, Gaspar Vianna (1911) to give the name of *Leishmania brazilienses* to the parasite, later corrected to *L. braziliensis* by Matta (1916).

It is quite extraordinary that until 1972, all cases of NCL in Brazil and a number of neighboring countries were attributed solely to this parasite, although specific species names were attributed in different regions: Velez (1913) had given the name *L. peruviana* to the causative agent of Peruvian uta; Biagi (1953) had named the parasite of Mexican chiclero's ulcer as *L. tropica mexicana,* emended to *L. mexicana* by Garnham (1962); and Floch (1954) considered pian-bois in French Guyana to be due to *L. tropica guyanensis,* later emended to *L. braziliensis guyanensis* by the Brazilian parasitologist Pessôa (1961). During the past 30 years, intensive ecological and epidemiological studies in the Americas have revolutionized previous ideas regarding the etiology of NCL and the classification of its causative parasites. No less than 21 species of *Leishmania* are now recognized within the neotropical region, of which 15 are known to cause either cutaneous or mucocutaneous leishmaniasis or both in humans. There is little doubt that others remain to be discovered and described, particularly in the rich sandfly and mammalian faunas of the great South American forests.

Cutaneous leishmaniasis is widespread in Latin America, and the only two countries that seem to be free of the disease are Chile and Uruguay: cases have even been recorded in the extreme south of the USA in Texas, and rodents naturally infected with *L. (L.) mexicana* have been found in Arizona, USA. A reliable figure for the incidence of the human disease in the Americas is virtually impossible to obtain: it is not a notifiable disease in most of the countries where it occurs. Most of these countries also have poor facilities for unequivocal diagnosis so heavy reliance is placed on

Figure 1 *Peruvian pottery (huaco) showing facial mutilations thought to represent mucosal leishmaniasis. (From Pessôa and Barretto 1948)*

clinical aspects, which are often misleading. In Costa Rica, a small country, but with very good communications and medical assistance, the impressive figure has been given of more than 2 000 cases a year in a population of 2 000 000 (Walton 1987). In Brazil, the Ministry of Health reported an increase in the number of cases from 2 856 in 1977 to 4 821 in 1982, (Walton 1987). Lacerda (1994) suggested the considerably higher figure of 154 103 recorded cases between the years 1980 and 1990, with an incidence of c. 5 people in 1 000 infected between the years 1980 and 1984, rising to 25 per 1 000 during 1987–1990.

In all known instances, NCL is a zoonosis: that is, the causative parasites are primarily those of wild animals. When the various sandfly vectors also feed on humans there may be transmission of a number of species of *Leishmania*; in this 'unnatural' host, they usually provoke an intense reaction and the eventual development of a skin lesion at the site of the bite. Some 7–10 days later, a tiny papule appears which, although usually painless, may itch considerably. In most cases, the papule eventually ulcerates, producing a steadily growing and crater-like lesion with a characteristically inflamed, elevated border (Figure 2). A lesion may remain single, but in some cases infected macrophages may transport the parasites to other parts of the body and establish secondary lesions. One parasite in particular, *L. (V.) braziliensis,* tends to produce

Figure 2 *Simple skin lesion of the arm, due to* Leishmania (Viannia) braziliensis*; Pará, Brazil*

such metastatic lesions in the nasal, pharyngeal and laryngeal mucosae. These may arise within a few months of the original skin lesion, or years later when the patient has supposedly been cured of the initial infection; they may be extremely mutilating (Figures 3 and 4).

Other neotropical *Leishmania* species may produce an even more serious and incurable condition in individuals who fail to mount a fully functional cell-mediated immune reaction against the parasite. Such patients, who develop large numbers of nodular lesions scattered over almost the whole skin surface (Figure 5), have been referred to as cases of diffuse cutaneous leishmaniasis (DCL). This is a misleading term as other perfectly curable cases of cutaneous leishmaniasis in immunologically competent patients may have numerous lesions

Figure 3 *Mucocutaneous leishmaniasis, due to* L. (V.) braziliensis*; Pará, Brazil*

Figure 4 *Destruction of the palate, due to* L. (V.) braziliensis*; Para, Brazil*

scattered over the body surface (Figure 6; see also *Leishmania (Viannia) guyanensis* Floch, 1954, below); the term anergic diffuse cutaneous leishmaniasis (ADCL) is more appropriate. In the Americas, this condition has been found associated only with infection by members of the *mexicana* complex, e.g. *L. (Leishmania) mexicana, L. (L.) pifanoi*, and *L. (L.) amazonensis.* In the Old World, another member of the subgenus *Leishmania*, *L. (L.) aethiopica,* causes a similar incurable disease in immunologically incompetent patients (see Old World leishmaniasis).

The fact that a number of *Leishmania* species cause disease in humans in the neotropics is reflected in variations in chemotherapeutic responses. Thus, drugs that work well in one region may not be so efficient in another because the species of the parasite infecting the patients are different. In Guatemala, for example, individuals infected with *L. (L.) mexicana* responded to treatment with ketoconazole better than those infected with *L. (V.) braziliensis* s.l., but the reverse applied when patients were treated with sodium stibogluconate (Navin et al. 1992).

Unlike humans, the natural sylvatic hosts of the various neotropical *Leishmania* species rarely suffer disease from the infection, which is usually of a benign, inapparent nature. Under certain circumstances (see *Leishmania (Viannia) braziliensis* (Vianna, 1911) Matta, 1916), domestic animals such as dogs, mules, and horses may be found with extensive skin ulcers due to *Leishmania:* an indication that they, like humans, are unnatural and unaccustomed hosts.

The known history of American visceral leishmaniasis (AVL) is comparatively short compared with that of the Old World (Old World leishmaniasis). The first record was probably that of Migone (1913), who saw 'corpuscles', which he was convinced were amastigotes of *Leishmania,* in the blood of a sick man in Paraguay. The patient's symptoms were highly indicative of visceral leishmaniasis and, failing to respond to antimalarial treatment, he died. Prior to his illness, the man had been working on the construction of the São Paulo–Corumbá railway in Brazil, where he most probably became infected. The first undoubted cases to be registered in Latin America were documented by Mazza and Cornejo (1926) in two Argentinian children.

Penna (1934) used the viscerotome to examine liver samples from patients suspected to have died from yellow fever in various parts of Brazil. He diagnosed 41 of them as being cases of visceral leishmaniasis, the largest number coming from the north-east of that country. Sporadic cases began to be recorded in Bolivia, Colombia, Guatemala, Paraguay, and El Salvador, but the full importance of the disease as a public health problem was not realized until as recently as 1953, when a dramatic outbreak was estimated to have been responsible for more than 100 deaths in the small country town of Sobral, in the State of Ceará, north-east Brazil (Deane 1956). Clearly, the history of AVL goes back much further than this and deaths from this highly lethal infection must have long been attributed to other causes, including malaria and yellow fever. To this day, AVL remains second in importance only to malaria among the Latin American tropical diseases, with the very conservative number of over 6 000 cases recorded in Brazil alone, up to 1980 (Deane and Grimaldi 1985).

Although a wide variety of *Leishmania* species have been recorded in humans, the prevalence of each of these will clearly depend on just how anthropophilic their respective sandfly vectors are. Thus, by far the

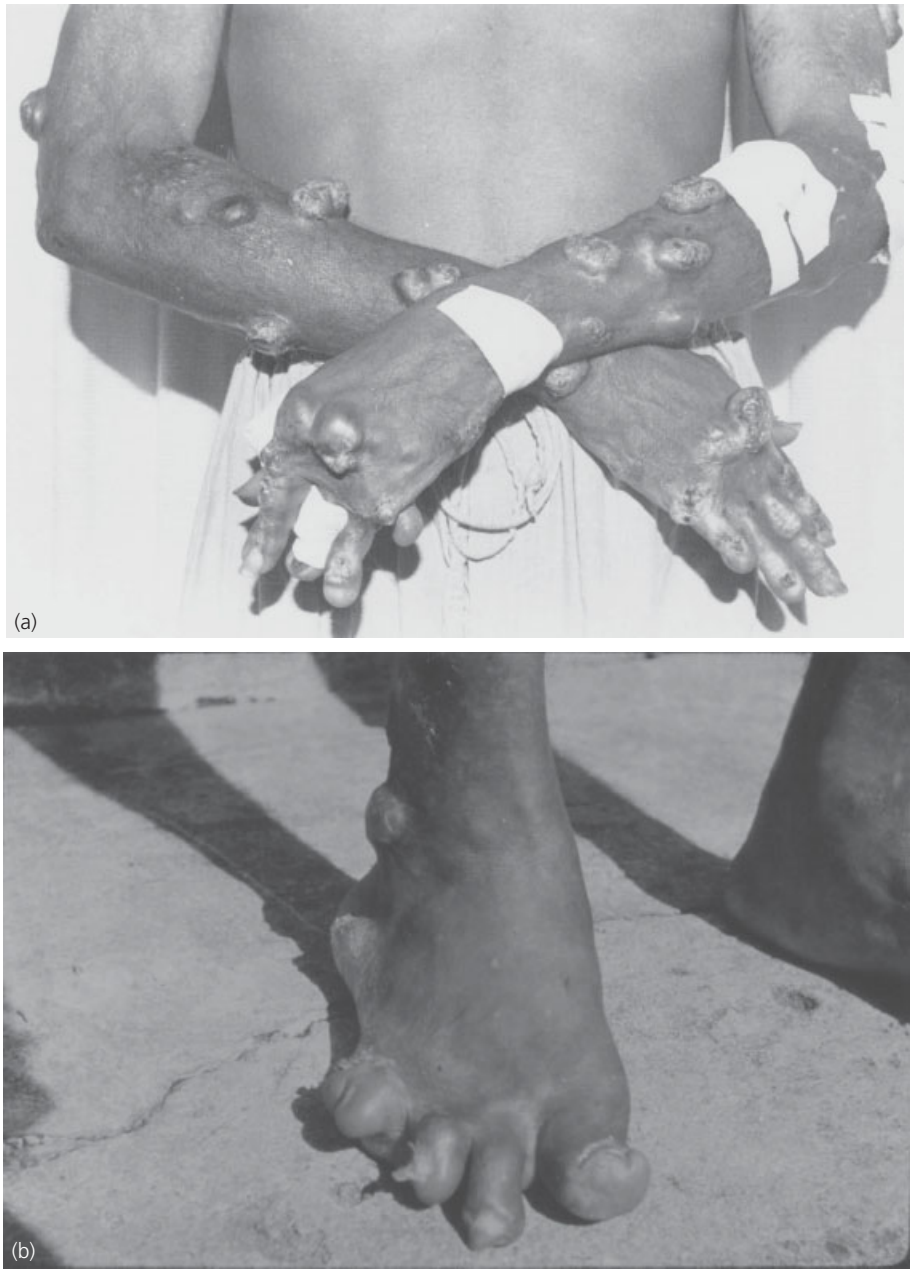

Figure 5 *ADCL due to* L. (L.) amazonensis. *Note the amputation of some fingers* **(a)** *and toes* **(b)**, *simulating lepromatous leprosy, with which disease ADCL is often confused; Pará, Brazil. (Figure 5a from Lainson 1982b)*

largest proportion of NCL cases in Brazil are due to *L. (V.) braziliensis* and *L. (V.) guyanensis*, both of which have sandfly vectors that feed avidly on humans. On the other hand, human infection with *L. (V.) lainsoni* and *L. (V.) naiffi* is relatively rare, as their vectors are disinclined to bite humans. Some neotropical *Leishmania* species, like *L. (L.) hertigi* and *L. (L.) deanei* of porcupines, are unknown in humans, possibly due to their inability to survive in human tissues, but more probably because their sandfly vectors never feed on humans.

It is likely that all neotropical *Leishmania* species once shared a sylvatic ecology, as is still the case in the remaining areas of extensive primary and secondary forest in the Amazon Region. Following the Iberian colonization and ensuing destruction of the forests, some phlebotomine sandfly species adapted to a peridomestic habitat. The persistence of cutaneous leishmaniasis in such ecologically disturbed, although still essentially rural, areas has been taken to indicate the evolution of a secondary, peridomestic transmission of the causative parasite; the occurrence of leishmanial skin lesions in village dogs, mules, and horses suggests that such domestic animals might have now become secondary reservoir hosts. Their development of extensive skin

Figure 6 *Multiple skin lesions due to* L. (V.) guyanensis. *Similar lesions were present on this man's legs, arms and face; Pará, Brazil. (From Lainson 1982b)*

lesions tends to suggest, however, that, like humans, these animals still remain unnatural and unaccustomed 'victim' hosts. Firm evidence is required to show that infected humans, dogs, and equines are capable of infecting sandflies fed on them and thus maintaining the parasite in the absence of another source of infection in wild animals still existing in nearby surviving pockets of woodland. Reithinger and Davies (1999), in a review of the literature, concluded that evidence suggesting the domestic dog as a reservoir for human American cutaneous leishmaniasis was largely circumstantial. This conclusion was supported by Savani et al. (1999) who found no serological evidence of infection in 973 stray dogs examined in an endemic area of cutaneous leishmaniasis in São Paulo State, Brazil. Tolezano et al. (1998) carried out serological surveys of dogs in two endemic areas of the Ribeira River Valley, São Paulo State. It was suggested that dogs played only a minor role in maintaining the parasite population in one area, with only six of 77 (7.8 percent) animals positive to the skin test: in the other area, a positivity of 10 out of 56 (17.9 percent) led to their conclusion that dogs are involved in the maintenance of *L. (V.) braziliensis* to different degrees in different regions of Brazil. Positive skin tests are no indication, however, that the dogs are a source of parasites for the sandfly vector(s).

The role of humans as a source of infection for sandfly vectors of cutaneous leishmaniasis in the Americas remains controversial. The Brazilian parasitologist Aragão (1922) succeeded in infecting *Lu. intermedia* on the skin lesions of patients, and Strangways-Dixon and Lainson (1966) infected six out of eight sandflies *(Lu. cruciata* and *Lu. ylephilator*) by feeding them on the indurated margin of lesions due to *L. (L.) mexicana* in Belize. The fact that sandflies fed on healthy skin of the same patients consistently failed to become infected and that the insects showed a marked reluctance to feed on old, scabbed lesions, led them to conclude that only on rare occasions were humans likely to serve as a reservoir of infection for the sandfly vector. In a similar experiment, Montoya-Lerma et al. (1998) obtained two infected *Lu. longipalpis* out of 103 that took a bloodmeal on the edges of lesions due to *L. (V.) braziliensis*, but none among those that had fed on normal skin of the same patients. Understandably, the systemic nature of visceral leishmaniasis provides a more ready source of amastigotes in the blood of persons with active infections. Costa et al. (2000) fed 3 747 laboratory-bred *Lu. longipalpis* on persons with active, asymptomatic and apparently cured infections, in an area endemic for AVL: 26 flies acquired infection from 11 of 44 persons with active infections, but none of those fed on 137 asymptomatic individuals became infected. This supports the observation of Deane (1956) that man is a poor source of infection compared with the acutely infected dog.

Although *Lutzomyia longipalpis*, the major vector of AVL, is better known as a peridomestic or intra-domiciliary sandfly, its origin is sylvatic (Lainson 1989; Lainson et al. 1990a). In Amazonian Brazil, for example, it has been captured in primary rain forest far from human habitation and when crude roads are cut through such forest, newly constructed houses and animal sheds are soon invaded by this insect. It follows that whether the source of the disease is local foxes (*Cerdocyon thous*), in which the infection rate may be more than 50 percent, or infected dogs brought to the area from distant endemic foci of AVL, transmission of the parasite will ensue and a new focus of the canine and human disease will be established.

Studies on the ecology of the New World leishmanial parasites have strongly suggested the existence of environmental barriers that limit the different species of *Leishmania* to specific sandfly species that transmit to certain mammalian hosts in distinct ecotopes. Extreme care and considerable field research is needed, therefore, before conclusions can be reached as to the principal sandfly vectors of the different parasites. The mere presence of a *Leishmania* in a specimen of a given sandfly species does not necessarily mean that this insect is the vector of that parasite. Before such a conclusion can be reached, the organism must be found with frequency in that particular sandfly and must show

abundant proliferation in the alimentary tract, with migration to the foregut and mouthparts. Ideally, a definite association with the wild mammalian host should be shown to exist and, if the parasite is a cause of human leishmaniasis, the fly must be shown to bite humans.

Similarly, the efficiency of a given mammal in maintaining a *Leishmania* species in nature and in serving as a source of infection for the sandfly vector must be considered. Determining a significant infection rate in the mammalian host and providing experimental proof that the sandfly vector can be infected by feeding on it are prerequisites in labeling the animal as a reservoir host.

A number of the neotropical *Leishmania* species were first described in their wild mammalian or sandfly hosts, considerable time elapsing before they were incriminated as a cause of human leishmaniasis. For this reason, this chapter discusses all the known New World species, regardless of the fact that some have yet to be found in humans. Old World leishmaniasis, deals with the systematic position of the genus *Leishmania*, morphology and development of the organism in the mammalian and sandfly hosts, the application of biochemistry and molecular biology to identification of the different species of the parasite, genetics and immunology, clinical features and the treatment of human cutaneous and visceral leishmaniasis. Many features of these topics are common to both Old World and New World leishmanial parasites and this chapter discusses only those that seem to be peculiar to the American leishmaniases and their causative agents. Lengthy discussions on the evolution, ecology, epidemiology, and classification of the parasites have been given elsewhere (Lainson and Shaw 1979, 1987; Lainson 1983; Lainson et al. 1994; Shaw and Lainson 1987; Shaw 1994), and reviews on the history of the neotropical leishmaniases have also been dealt with in other publications (Lainson and Shaw 1992; Lainson 1996). For methods in the laboratory diagnosis of these diseases, see Lainson and Shaw (1981) and Old World leishmaniasis.

CLASSIFICATION OF THE RECOGNIZED NEOTROPICAL *LEISHMANIA* SPECIES

The genus *Leishmania* has been subdivided into two subgenera, as follows.

The subgenus *Leishmania* Ross, 1903

This subgenus possesses the characteristics of the genus (see Old World leishmaniasis). The life-cycle in the natural sandfly vector is limited to the midgut and foregut of the alimentary tract. Species occur in both the Old World and the New World (type species *Leishmania (Leishmania) donovani* (Laveran and Mesnil, 1903) Ross, 1903 of the Old World). The recorded neotropical species are:

1 *Leishmania (Leishmania) infantum chagasi** Cunha and Chagas, 1937
2 *L. (L.) enriettii* Muniz and Medina, 1948
3 *L. (L.) mexicana** (Biagi, 1953) Garnham, 1962
4 *L. (L.) pifanoi** (Medina and Romero, 1959) Medina and Romero, 1962
5 *L. (L.) hertigi* Herrer, 1971
6 *L. (L.) amazonensis** Lainson and Shaw, 1972
7 *L. (L.) deanei* Lainson and Shaw, 1977
8 *L. (L.) aristidesi* Lainson and Shaw, 1979
9 *L. (L.) garnhami** Scorza et al., 1979
10 *L. (L.) venezuelensis** Bonfante-Garrido, 1980
11 *L. (L.) forattinii* Yoshida et al., 1993.

*Recorded from humans. For distribution, see Table 1.

The subgenus *Viannia* Lainson and Shaw, 1987

This subgenus possesses the characteristics of the genus (see Old World leishmaniasis). The developmental cycle in the natural sandfly vector includes a prolific and prolonged phase of division of rounded or ovoid paramastigotes and promastigotes attached to the wall of the hindgut (pylorus and ileum, see Figure 7) by flagellar hemidesmosomes, followed by migration of free promastigotes to the midgut and foregut. Members of this subgenus are known only in the neotropical region and the recorded species are as follows:

1 *Leishmania (Viannia) braziliensis** (Vianna, 1911) Matta, 1916; type species
2 *L. (V.) peruviana** Velez, 1913
3 *L. (V.) guyanensis** Floch, 1954
4 *L. (V.) panamensis** Lainson and Shaw, 1972
5 *L. (V.) lainsoni** Silveira et al., 1987
6 *L. (V.) shawi** Lainson et al., 1989
7 *L. (V.) naiffi** Lainson and Shaw, 1989
8 *L. (V.) colombiensis** Kreutzer et al., 1991
9 *L. (V.) equatorensis* Grimaldi et al., 1992
10 *L. (V.) lindenbergi** Silveira et al., 2002
11 *L. (V.) utingensis* Braga et al., 2003

*Recorded from humans. For distribution, see Table 1.

Species within the subgenus *Leishmania* Ross, 1903

LEISHMANIA (LEISHMANIA) INFANTUM CHAGASI CUNHA AND CHAGAS, 1937

Known geographical distribution

Distribution has been noted throughout most of the Latin American continent: Argentina, Bolivia, Brazil,

Table 1 *A country-by-country list of the neotropical* Leishmania *species recorded in humans and the resultant pathologies*

Country	Species	Disease forms recorded[g]
Argentina	*L. (L.) infantum chagasi*	VL
	L. (V.) braziliensis s.l.	CL
Belize	*L. (L.) mexicana*	CL
	L. (V.) braziliensis s.l.	CL
Bolivia	*L. (L.) amazonensis*	CL, ADCL
	L. (L.) infantum chagasi	VL
	L. (L.) sp.	CL
	L. (V.) braziliensis s.l.	CL, MCL
	L.(V.) lainsoni[o]	CL
Brazil	*L. (L.) amazonensis*	CL, ADCL, MCL[h], VL[i]
	L. (L.) infantum chagasi	VL, (CL[j])
	L. (L.) sp.	CL[k]
	L. (V.) braziliensis	CL, MCL
	L. (V.) guyanensis	CL, MCL
	L. (V.) lainsoni	CL
	L. (V.) naiffi	CL
	L.(V) shawi	CL
	L.(V.) lindenbergi[p]	CL
Colombia	*L. (L.) amazonensis*	CL, ADCL
	L. (L.) infantum chagasi	VL
	L. (L.) mexicana	CL, ADCL
	L. (V.) braziliensis s.l.	CL, MCL
	L. (V.) colombiensis	CL
	L. (V.) guyanensis	CL
	L. (V.) panamensis	CL, MCL
Costa Rica	*L. (L.) infantum chagasi*	CL[l], VL[q]
	L. (L.) mexicana	CL
	L. (V.) braziliensis s.l.	CL, MCL
	L. (V.) panamensis	CL
Dominican Republic	*L. (L.) mexicana*-like	ADCL
Ecuador	*L. (L.)* sp.[a]	CL
	L. (L.) mexicana[a]	CL
	L. (V.) braziliensis s.l.	CL, MCL
	L. (V.) braziliensis / L.(V.) panamensis?	CL
	L. (V.) panamensis / L.(V.) guyanensis?	CL
El Salvador	*L. (L.) infantum chagasi*	VL
	L. (L.) mexicana	CL
French Guyana	*L. (L.) amazonensis*	CL, ADCL
	L. (V.) braziliensis s.l.[b]	CL, MCL
	L. (V.) guyanensis	CL
	L. (V.) naiffi[c]	CL
Guadeloupe	*L. (L.) infantum chagasi*	VL
	Leishmania sp.	CL
Guatemala	*L. (L.) chagasi*	VL
	L. (L.) mexicana	CL
	L. (V.) braziliensis s.l.	CL
Guyana	*L. (V.) guyanensis*	CL
	Leishmania sp.	MCL
Honduras	*L. (L.) infantum chagasi*	VL, CL[l]
	L. (L.) mexicana	CL, ADCL
	L. (V.) braziliensis s.l.	CL, MCL
	L. (V.) panamensis	CL, MCL
Martinique	*L. (L.)* sp.	CL

(*Continued over*)

Table 1 *A country-by-country list of the neotropical* Leishmania species recorded in humans and the resultant pathologies (*Continued*)

Country	Species	Disease forms recorded[g]
Mexico	*L. (L.) infantum* chagasi	VL
	L. (L.) mexicana	CL, ADCL
	L. (L.) sp.	CL, ADCL[m]
	L. (V.) braziliensis	CL
Nicaragua	*L. (L.) infantum chagasi*	VL, CL[r]
	L. (V.) braziliensis s.l.	CL, MCL
	L. (V.) panamensis	CL, MCL
	L. (V.) braziliensis / panamensis[d]	CL
Panama	*L. (V.) braziliensis* s.l.	CL
	L. (V.) panamensis	CL
	Leishmania sp.	MCL
Paraguay	*L. (L.) amazonensis*	CL, ADCL
	L. (L.) infantum chagasi	VL
	L. (V.) braziliensis s.l.	CL, MCL
Peru	*L. (V.) braziliensis* s.l.	CL, MCL
	L. (V.) peruviana	CL, MCL
	L. (V.) braziliensis / L. (V.) peruviana[e]	CL
	L. (V.) lainsoni[s]	CL
Surinam	*Leishmania* sp.	CL
USA	*L. (L.) mexicana*	CL, ADCL[m]
Venezuela	*L. (L.) infantum chagasi*	VL
	L. (L.) garnhami	CL
	L. (L.) pifanoi	CL, ADCL
	L. (L.) venezuelensis	CL
	L. (V.) braziliensis s.l.	CL, MCL
	L. (V.) colombiensis	VL[n]
	L. (V.) braziliensis / L. (V.) guyanensis[f]	CL

Data from the review articles of Lainson and Shaw 1979, 1987; Shaw and Lainson 1987; Grimaldi et al. 1989 except where otherwise indicated.
a) Katakura et al. 1993. b) Raccurt et al. 1995. c) Darie et al. 1995. d) Darce et al. 1991. e) Dujardin et al. 1995. f) Bonfante-Garrido et al.1992. g) ADCL, anergic diffuse cutaneous leishmaniasis; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis. h) In cases of ADCL. i) Only recorded in a small region of Bahia State, BR. j) Only in Rio de Janeiro, BR. k) Localized in Minas Gerais, BR. l) Zelodón et al. 1989; Ponce et al. 1991. m) In Texas, southern USA. n) Bone marrow only. o) Martinez et al. (2001). p) Silveira et al. (2002). q) Carrillo et al. (1999). r) Belli et al. (1999). s) Lucas et al. (1994).

Colombia, Ecuador, El Salvador, Guadeloupe, Guatemala, Honduras, Martinique, Mexico, Nicaragua, Paraguay, Surinam, and Venezuela.

Known mammalian hosts

Known mammalian hosts include humans and the domestic dog, *Canis familiaris*. Among wild animals the fox, *Cerdocyon thous*, appears to be an important natural reservoir in Brazil in the northeast (Ceará), north (Pará), and southeast (Mato Grosso do Sul) (Deane 1956; Lainson et al. 1987; Mello et al. 1988; respectively). Evidence has been presented suggesting that the infected foxes identified by Deane (1956) in Ceará as *Lycalopex vetulus* were most probably *C. thous* (Courtenay et al. 1996).

There are reports of isolates of the parasite from domestic rats in Honduras (Walton, personal communication) and, on rare occasions, from opossums of the genus *Didelphis* in Bahia, northeast Brazil (Sherlock et al. 1984). In Colombia, *D. marsupialis* is considered to be an important reservoir host (Corredor et al. 1989; Travi et al. 1994).

The latter authors (Travi et al. 1998) obtained positive results using the polymerase chain reaction (PCR)-hybridization test for three out of 34 specimens of the 'spiny-rat', *Proechimys canicollis* from forest in Colombia and suggested that these animals had active infections, although no parasites were isolated from them. Continuing their studies on the epidemiology of visceral leishmaniasis in Colombia, Travi et al. (2002) tested the susceptibility of another species of *Proechimys* (*P. semispinosus*) to *L. infantum chagasi* following inoculation of promastigotes by the intracardial and intradermal routes. No parasites could be detected in smears of the viscera from all of the 10 animals when they were sacrificed 7 months later, but positive cultures were obtained from splenic material of two of five animals inoculated by the intracardial route and three of five inoculated by the intradermal route. Attempts to infect sandflies (*Lu. longipalpis*) after two or three xenodiagnoses on each animal, during a period of 1–6 months, all failed: PCR-

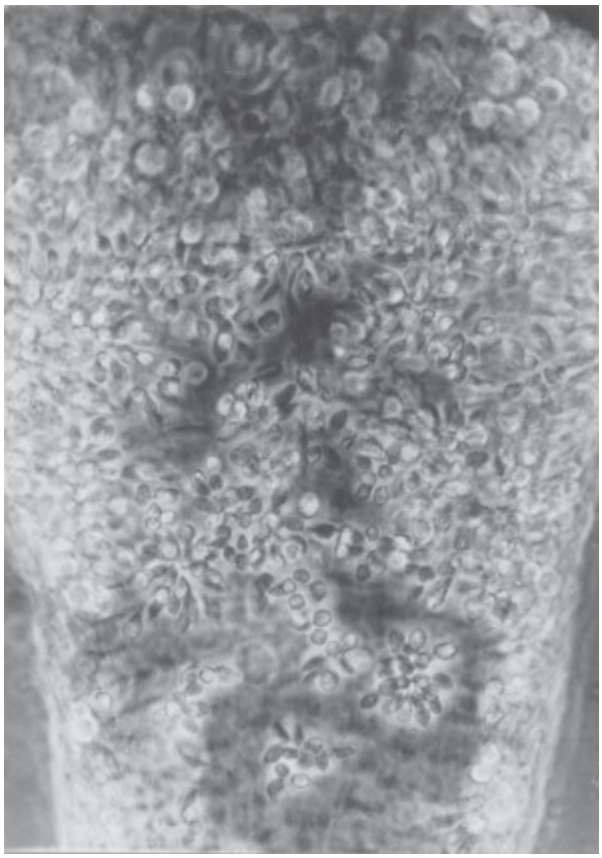

Figure 7 *The pylorus ('hindgut triangle') of a sandfly infected with* L. (V.) braziliensis. *Prolific division of rounded or ovoid promastigotes and paramastigotes attached by flagellar hemidesmosomes to the gut wall, a characteristic of* Leishmania *species within the subgenus* Viannia.

hybridization tests gave negative results. It was concluded that the infection in *P. canicollis* 'is contained and compartmentalized', but that there may be differing degrees of susceptibility among different species of *Proechimys*.

Lainson et al. (2002) attempted to infect 12 laboratory-bred *Proechimys guyanensis* with a strain of *L. i. chagasi* from Amazonian Brazil, by massive intraperitoneal inoculation of amastigotes or promastigotes Although control hamsters succumbed to acute infection 6 months p.i., no infection could be registered in any of the *P. guyanensis* when sacrificed at 6 or 12 months p.i., by stained smears of liver and spleen, culture of these tissues and their inoculation into hamsters, and the PCR-hybridization test. Furthermore, during the experiment laboratory-bred *Lu. longipalpis* showed a complete reluctance to feed on the experimental animals. Based on these results, previous unsuccessful attempts to capture *Lu. longipalpis* in Disney-traps baited with *P. guyanensis* and placed in the backyards of sandfly-infested houses, and a failure to isolate *L. i. chagasi* from large numbers of this rodent captured in foci of AVL in northern Brazil (Lainson et al. 1987), the authors concluded that *P. guyanensis* is an unsuitable host for this parasite and plays no important part in the eco-epidemiology of the disease in north Brazil.

Recorded sandfly hosts

Lutzomyia (Lutzomyia) longipalpis is the major vector of *L. (L.) infantum chagasi* throughout its geographic range (Deane and Grimaldi 1985; Lainson 1989) and the parasite has been transmitted experimentally by the bite of this sandfly, using both laboratory-infected and naturally infected flies (Lainson et al. 1977, 1985). Strong evidence was provided, however, that *Lu. longipalpis* represents a species complex of at least two taxa (Ward et al. 1988), based on slight morphological differences (males with pale spots on terga 3 and 4, versus others with a similar spotted pheromonal gland on only the 4th tergum). Attempts to cross-breed the two forms failed, adding support to this suggestion (Ward et al. 1983). Following a study of population genetics and phylogenetic analyses of *Lu. longipalpis* from Central and South America, Soto et al. (2001) suggested that specific allopatric populations had differentiated to the extent of forming sibling species, with four distinct lineages corresponding to central and northern South America, Brazil and an isolated population in Colombia.

Lanzaro et al. (1993) compared populations of *Lu. longipalpis* from Costa Rica, Colombia and Brazil, using isoenzyme electrophoresis and cross-breeding experiments. They concluded that the three populations represented three different species. This finding has helped to explain why different clinical manifestations of *L. (L.) infantum chagasi* infection in humans occur in some geographic regions (see Clinical features).

In one particular region endemic for visceral leishmaniasis, in the Córdoba Department of Colombia, 87 percent of the sandflies captured were found to be *Lutzomyia evansi* and as one of these flies was infected with *L. (L.) chagasi* it was suggested that this sandfly might be acting as an alternative vector (Travi et al. 1990). In later studies (Travi et al. 1996) infections were found in nine more specimens of *Lu. evansi*, and, taking into account the apparent absence of *Lu. longipalpis* and the peridomestic and intradomiciliary habits of *Lu. evansi*, these authors concluded that this species was the principal vector in that part of Colombia. *Lu. evansi* had long been suspected as an alternative vector of *L. (L.) infantum chagasi* in Venezuela, following failure to find *Lu. longipalpis* in some foci of AVL where *Lu. evansi* was abundant (Potenza and Anduze 1942; Pifano and Romero 1964). The conclusions of these authors have gained strong support from the recent isolation of promastigotes from this sandfly in Venezuela and the finding that k-DNA restriction analysis showed high homologies between the cultures and *L. (L.) infantum chagasi* (Feliciangeli et al. 1999).

In the State of Mato Grosso do Sul, Brazil, suspicions have been raised that *Lu. cruzi* might be another alter-

native vector (dos Santos et al. 1998). Unfortunately, the females of this sandfly are morphologically indistinguishable from those of *Lu. longipalpis* and conclusions were based on an infection in a single female sandfly (*Lu. cruzi* or *Lu. longipalpis*?) and the apparent absence of *Lu. longipalpis* males. The limited geographical distribution of these two 'alternative' vectors compared with that of *Lu. longipalpis*, however, leaves no doubt regarding the overwhelming importance of the latter as the principal sandfly host of *L. (L.) infantum chagasi*.

Disease caused by the parasite in humans

The parasite predominantly causes visceral leishmaniasis, commonly with a fatal outcome unless treated (see Old World leishmaniasis). On rare occasions, the visceral disease may be preceded by a cutaneous lesion, and in some geographical regions the parasite is responsible for an almost exclusively cutaneous disease (see Clinical features). Until recently, only cutaneous lesions were recorded in Costa Rica, but a single case of visceral leishmaniasis has now been recorded (Carrillo et al. 1999). Common names for the visceral disease include kala-azar or calazar but it is more appropriate to reserve these terms for Indian visceral leishmaniasis and to use the name American visceral leishmaniasis or AVL.

Opinions have been divided as to whether the causative agent of AVL is indigenous to the Americas, or whether it is simply *L. (L.) infantum*, which was introduced into the New World by immigrants from the Iberian peninsula in post-Columbian times. Points in favor of the first hypothesis and retention of the specific name *L. (L.) chagasi*, originally given to the parasite, are as follows:

- There is a high incidence of infection in the native fox, *Cerdocyon thous*, in relatively remote areas of Amazonian Brazil and the benign and inapparent nature of these infections is indicative of an ancient host–parasite relationship.
- Wild canids are considered to be the source from which members of the *L. (L.) donovani* complex originated (Lysenko 197l) and canids were present in the Americas as long ago as the Pleistocene era, some 2–3 million years ago.
- *Lu. longipalpis* is a vector of *L. (L.) chagasi* throughout almost the entire geographic range of the parasite, but is not known to transmit any other neotropical species of *Leishmania*; the vectors of *L. (L.) infantum* belong to a different sandfly genus. The host specificity of *Leishmania* species in nature is most pronounced among their sandfly vectors, thus it seems unlikely that introduced *L. (L.) infantum* could have made the relatively sudden jump from one phlebotomine genus to another.
- There are differences in both the kinetoplast DNA fragment patterns and the radio-respirometry profiles of *L. (L.) infantum* and *L. (L.) chagasi* (Jackson et al. 1982, 1984; Decker-Jackson and Tang 1982) and the two parasites are antigenically different (Santoro et al. 1986). The hypothesis that American visceral leishmaniasis is the result of imported *L. (L.) infantum* is based on the similarity of those isoenzyme profiles of the two parasites that have been studied (Rioux et al. 1990), the dual role of the domestic dog as an amplification host in their respective epidemiologies (Killick-Kendrick 1985) and recent studies on the genomic diversity in the *Leishmania donovani* complex (Maurício et al. 1999). The authors of this chapter have long used the name *L. (L.) chagasi*. From the close biochemical and molecular similarities that have now been shown, however, we feel it best to separate the two parasites only at subspecific level, with the names *L. (L.) infantum infantum* and *L. (L.) infantum chagasi*.

LEISHMANIA (LEISHMANIA) ENRIETTII MUNIZ AND MEDINA, 1948

Known geographical distribution

To date, this parasite has been found only in the States of Paraná and São Paulo, Brazil.

Known mammalian hosts

Natural infections have, until now, only been found in domestic guinea-pigs (*Cavia porcellus*). When this strange parasite was first discovered, producing large tumor-like lesions on the ears of two laboratory guinea-pigs (Medina 1946), the origin of the infected animals was obscure. As phlebotomine sandflies are the only known vectors of *Leishmania* species, however, it is difficult to imagine that domestic guinea-pigs are the principal natural hosts of *L. (L.) enriettii*, and it can only be assumed that the animals had spent some time in or near a rural area where transmission was occurring among the true, wild mammalian hosts. There have been two further spontaneous reappearances of the parasite in domestic guinea-pigs, again in Curitiba, Paraná (Luz et al. 1967) and more recently in a rural district of São Paulo State (Machado et al. 1994). Although the exact locality of the animals was on these occasions well documented, attempts to discover the wild animal source of the parasite were not made.

Recorded sandfly hosts

The natural vector of *L. (L.) enriettii* remains to be discovered. Luz et al. (1967) examined the sandfly population of neighboring forest around the site of isolation, where *Lu. monticola* and *Lu. correalimai* were the only species encountered. *Lu. monticola* was caught on tree trunks, in the nests of opossums (*Didelphis*) and from human bait, and six of 10 specimens fed on the lesions of guinea-pigs became heavily infected.

Absence of infection in humans

Human infection has not yet been reported. Muniz and Medina (1948) attempted to infect human volunteers, rhesus monkeys, dogs, mice, and the wild guinea-pig (preá) by the intradermal inoculation of promastigotes from in vitro cultures, without success. Out of eight hamsters inoculated, only one developed an inconspicuous lesion containing scanty amastigotes. Failure to infect the closely related wild guinea-pig (*Cavia aperea*) at first seems surprising. Lainson and Shaw (1979) suggested, however, that if this animal is the natural host of *L. (L.) enriettii* the inoculated preá may well have developed an inapparent infection that went unnoticed.

LEISHMANIA (LEISHMANIA) MEXICANA (BIAGI, 1953) GARNHAM, 1962

Known geographical distribution

The areas of known geographical distribution are Southern USA (Arizona and Texas), Mexico, Belize, Guatemala, Honduras, and Costa Rica. In view of extensive geographic separation from the type locality of *L. (L.) mexicana* and considerable differences in the mammalian and phlebotomine sandfly faunas, reports of this parasite in Panama and some South American countries must be viewed with caution.

Known mammalian hosts

The known mammalian hosts are humans and the forest rodents *Ototylomys phyllotis* (primary host), *Nyctomys sumichrasti*, *Heteromys desmarestianus* and *Sigmodon hispidus* (secondary hosts). In southern USA, the rodent *Neotoma albigula* has been found infected in Pima County, Arizona.

Recorded sandfly hosts

Lutzomyia olmeca olmeca is the only proven vector, but *Lu. (Lu.) diabolica* has been suspected as a vector in foci of cutaneous leishmaniasis due to *L. (L.) mexicana* in southern Texas and northern Mexico, and *Lu. anthophora* in Arizona.

Disease caused by the parasite in humans

This parasite causes cutaneous leishmaniasis, with a pronounced tendency towards long-lasting and destructive lesions of the external ear (Figure 8). Relatively rare cases of ADCL have been recorded, principally from southern Texas and northern Mexico. Local names include chiclero's ulcer, chiclero's ear, and bay-sore.

Parasites clearly related to *L. (L.) mexicana* have been reported from humans and wild animals in Panama, Colombia, Venezuela, Peru, and parts of Brazil. In the absence of the vector, *Lu. olmeca olmeca,* it is perhaps unlikely that any of these are true *L. (L.) mexicana*.

Figure 8 *Chiclero's ulcer, due to* L. (L.) mexicana. *Almost total destruction of the the external ear in a chiclero from Belize with an infection of many years' duration. (From Lainson and Strangways-Dixon 1963)*

Sandflies of the *Lu. flaviscutellata* complex, to which subspecies of *Lu. olmeca* belong, range through these South American countries, however, and are probably the vectors of a number of closely related parasites within the *mexicana* complex: at present this includes *L. (L.) pifanoi*, *L. (L.) amazonensis*, *L. (L.) aristidesi*, *L. (L.) garnhami*, *L. (L.) venezuelensis* and *L. (L.) forattinii*.

LEISHMANIA (LEISHMANIA) PIFANOI (MEDINA AND ROMERO, 1959) MEDINA AND ROMERO, 1962

Known geographical distribution

Known distribution is limited to Venezuela, specifically in the States of Yaracuy, Lara and Miranda.

Known mammalian hosts

Humans are the only known mammalian host: the wild mammalian hosts of the parasite have yet to be discovered. A parasite isolated from the forest rodent *Heteromys anomalus* was shown to behave in hamsters in the same way as *L. (L.) pifanoi* isolated from humans, but its conclusive identification remains in doubt.

Recorded sandfly hosts

A parasite with similar characteristics was also found in a specimen of *Lu. flaviscutellata* but, once again, has not been conclusively identified. This sandfly is the proven vector of *L. (L.) amazonensis*, another member of the *mexicana* complex often found in rodents in South American forests and it remains likely that the Venezuelan workers were dealing with this parasite.

Disease caused by the parasite in humans

All isolates found to date have been from cases of ADCL. It is most likely, however, that simple, curable lesions also occur in immunologically competent

individuals. There has been much controversy regarding the validity of this member of the *mexicana* complex, some authors regarding it merely as an enzymic variant of either *L. (L.) amazonensis* or *L. (L.) mexicana*. Much confusion has certainly resulted from the laboratory mix-up of parasites. Although there are relatively few isolates of *L. (L.) pifanoi*, interest in the clinical features of the human infection has resulted in a wide distribution of the parasite to laboratories throughout the world.

LEISHMANIA (LEISHMANIA) AMAZONENSIS LAINSON AND SHAW, 1972

Known geographical distribution

The parasite has been recorded in Bolivia, Brazil, Colombia, French Guyana, and Paraguay. It is also very likely to occur in other South American countries where the sandfly vector is found.

Known mammalian hosts

Known mammalian hosts are the forest rodents *Proechimys* (principal host), *Oryzomys*, *Neacomys*, *Nectomys*, and *Dasyprocta*; the marsupials *Marmosa*, *Metachirus*, *Didelphis*, and *Philander*; and the fox *Cerdocyon*.

Recorded sandfly hosts

The principal vector of the parasite is *Lutzomyia (Nyssomyia) flaviscutellata*. Occasional infections have been found in the closely related flies *Lu. (N.) olmeca nociva* and *Lu. (N.) reducta* but, if these are capable of transmitting the parasite, they probably play a small role in its ecology and epidemiology. In Bolivia, a parasite identified as *L. (L.) amazonensis* has been isolated from 16 of 1 715 *Lu. nuneztovari* dissected (Martinez et al. 1999).

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, usually of the single sore type and ADCL in individuals with a defective cell-mediated immune system (Figures 5 and 9). If it does occur, classical mucocutaneous leishmaniasis following metastasis to the nasopharyngeal mucosae from a simple cutaneous lesion is extremely rare. In advanced cases of ADCL, however, the disseminated infection may also include those tissues.

Typical visceral leishmaniasis in patients from one particular region of Bahia State Brazil, has been attributed to *L. (L.) amazonensis* (Barral et al. 1986). Conversely, no records exist to confirm this anywhere else in the geographical range of the parasite and cases of ADCL of very long duration show no signs or symptoms of visceral involvement, in spite of their defective immune system. Silveira examined the tissues from a patient who suffered from ADCL due to *L. (L.) amazonensis* from the age of 5 years until his death at the age of 57 years (Figure 5a) (F.T. Silveira, unpublished observations). At the time of his death, scarcely any of his body surface remained unaffected and the cutaneous lesions contained enormous numbers of parasites (Figure 10). No macroscopic or microscopic pathological changes were seen in the viscera and no amastigotes were found in stained impression smears prepared from the spleen, liver and lungs, and bone marrow. Finally, hamsters inoculated intradermally with triturates of these visceral tissues failed to become infected.

Figure 9 *ADCL due to* L. (L.) amazonensis *in a young woman from Pará, Brazil, showing active lesions and extensive scarring of the legs in an infection of some 15 years' duration.*

LEISHMANIA (LEISHMANIA) ARISTIDESI LAINSON AND SHAW, 1979

Known geographical distribution

The parasite is known to occur in the Sasardi forest, San Blas Territory, Eastern Panama.

Known mammalian hosts

Known mammalian hosts are the rodents *Oryzomys capito*, *Proechimys semispinosus*, and *Dasyprocta punctata*; and the marsupial *Marmosa robinsoni*.

Recorded sandfly hosts

The species most suspected is *Lutzomyia (Nyssomyia) olmeca bicolor*. Christensen et al. (1972) showed it to be

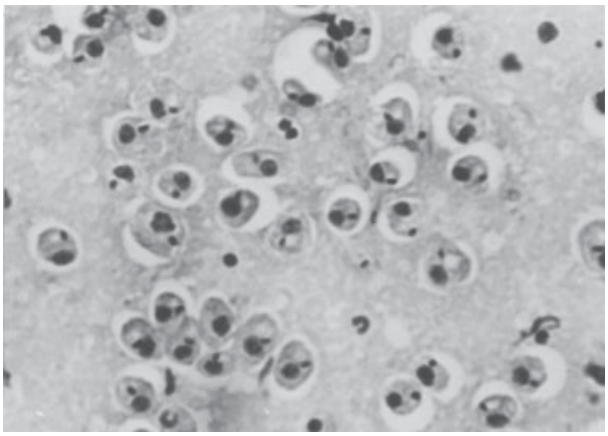

Figure 10 *Amastigotes of* L. (L.) amazonensis *in a Giemsa-stained smear from one of the nodular lesions of the patient shown in Figure 5a.*

the dominant fly on Disney-traps baited with rodents and opossums in the area where infected animals had been captured and the most common species collected among leaf litter on the forest floor.

Possibility of infection in humans

Human infection has not yet been reported. *Lu. olmeca bicolor* does bite humans on rare occasions and it is likely that the parasite may eventually be found infecting a human. In this respect, it should be remembered that following the discovery of *L. (V.) naiffi* in armadillos in 1979, 11 years were to pass before cases of human cutaneous leishmaniasis due to this parasite were diagnosed (Lainson et al. 1979, 1990b; Naiff et al. 1989).

LEISHMANIA (LEISHMANIA) GARNHAMI SCORZA ET AL., 1979

Difference of opinion exists regarding the validity of *L. (L.) garnhami*, which is indistinguishable from *L. (L.) amazonensis* on isoenzyme profiles (Rioux et al. 1990). Guevara et al. (1992), however, noted clear differences between the nontranscribed rDNA intergenic spacer sequences of these two parasites and, in our own laboratory, they have been separated by monoclonal antibodies (Shaw, Ishikawa, and Lainson, unpublished observations).

Known geographical distribution

The parasite has been found only in the Venezuelan Andes.

Known mammalian hosts

Humans are known hosts and a single infection has been recorded in the marsupial *Didelphis marsupialis*.

Recorded sandfly hosts

Experimental infections in the sandfly *Lu. youngi* (*verrucarum* group) in Venezuela have led some authors to suggest this species to be the vector (Grimaldi et al. 1989; Young and Duncan 1994; Killick-Kendrick 1990). In addition, Scorza (in Màrquez and Scorza 1982) reported that he had found natural infections with flagellates in *Lu. youngi* (at the time identified as *Lu. townsendi*) that, on inoculation into the skin of hamsters, produced 'amastigotes de *Leishmania garnhami*'. Unfortunately, the parasite was not specifically identified and the role of *Lu. youngi* as the vector still remains in doubt.

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, but there are no recorded cases of mucocutaneous leishmaniasis or ADCL.

LEISHMANIA (LEISHMANIA) VENEZUELENSIS BONFANTE-GARRIDO, 1980

Known geographical distribution

The parasite has been found in the Lara and Yaracuy States, Venezuela.

Known mammalian hosts

The known mammalian hosts are humans, equines, and the domestic cat. These are best regarded as 'victim' hosts and the wild animal source of infection has yet to be ascertained.

Recorded sandfly hosts

Lu. olmeca bicolor and *Lu. rangeliana* are suspected as possible vectors.

Disease caused by the parasite in humans

The parasite causes single or multiple skin lesions, sometimes of a disseminated, nodular type simulating ADCL but curable by the current method of antimonial treatment.

LEISHMANIA (LEISHMANIA) FORATTINII YOSHIDA ET AL., 1993

Known geographical distribution

The parasite has been found in the States of São Paulo, Bahia and Espirito Santo, Brazil.

Known mammalian hosts

The opossum *Didelphis marsupialis aurita* (São Paulo) and the rodent *Proechimys iheringi denigratus* (Bahia

and Espirito Santo, Brazil) are the known mammalian hosts.

Recorded sandfly hosts

The sandfly vector is unknown. Barretto et al. (1985) showed experimentally that the parasite was capable of development in the sandflies *Psychodopygus ayrozai* and *Lutzomyia yuilli* from the Três Braços area, Bahia, where the two insects are very common.

Possibility of infection in humans

The parasite has not yet been reported to cause human disease. Both *Ps. ayrozai* and *Lu. yuilli* occasionally feed on humans and if one or other of these sandflies is indeed the vector among the wild animal hosts, human infection with *L. (L.) forattini* may well occur.

Species within the subgenus *Viannia* Lainson and Shaw, 1987

These parasites have been separated by their isoenzymatic profiles, the use of monoclonal antibodies, and their biological characteristics. As is clear from Supplement 1 to volume 96 of *Trans R Soc Trop Med Hyg*, molecular biology techniques are now important tools in the field of parasitology and a set of microsatellite markers has recently been developed that enables separation of all known species of *Leishmania* within the subgenus *Viannia* and also indicates possible species hybrids (Russell et al. 1999). It will be particularly useful in the detection and identification of the more closely related pair *L. (V.) braziliensis* and *L. (V.) peruviana*, and the trio *L. (V.) panamensis*, *L. (V.) guyanensis* and *L. (V.) shawi*. A rapid method for the identification of *Leishmania* species using formalin-fixed biopsy samples and PCR is another promising new development (Mimori et al. 1998), as is the claim that these parasites can be reliably detected and identified, at least to the *L. (V.) braziliensis* complex level, by PCR using boiled dermal scrapings instead of skin biopsies (Belli et al. 1998).

LEISHMANIA (VIANNIA) BRAZILIENSIS (VIANNA, 1911) MATTA, 1916

Known geographical distribution

The distribution of this important parasite is badly defined, due to inadequate methods of identification used in the past. Parasites variously described as '*L. braziliensis*', '*L. braziliensis braziliensis*' or '*L. braziliensis* sensu lato' have been reported from most Latin American countries, including Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, French Guyana, Guatemala, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Surinam, and Venezuela.

Environmental factors seem to govern the combinations of sandfly vector and wild mammalian host in the natural history of the different species of *Leishmania*. It seems unlikely, therefore, that *L. (V.) braziliensis* sensu stricto can have such an enormous geographic range and the parasites recorded in many of these regions may represent related, but different, parasites of the *braziliensis* complex. The situation is aggravated by the fact that the type material of *L. (V.) braziliensis* Vianna, 1911 from Além Paraiba, Minas Gerais, Brazil, is no longer available for comparison. It is further complicated by distinctly different ecological and epidemiological features of cutaneous and mucocutaneous leishmaniasis due to *L. (V.) braziliensis* s.l. in different regions, sometimes within the same country, due to human destruction of the sylvatic habitat. Finally, a high degree of enzymic polymorphism has been shown to exist in isolates of *L. (V.) braziliensis* s.l. from Central and South America, with as many as 44 zymodemes obtained in a total of 137 isolates examined by 10 enzyme systems (Chouicha et al. 1997). In a similar manner, a variety of different serodemes have been shown to exist in the parasite from Brazil (Shaw et al. 1986).

Known mammalian hosts

In primary forest in the Serra dos Carajás, Pará State, Brazil, the low-flying habit and high attraction of the principal sandfly vector to both humans and rodent-baited traps led to the conclusion that terrestrial mammals, such as rodents, were probably hosts of *L. (V.) braziliensis* (Ward et al. 1973; Lainson et al. 1973). Subsequent records of parasites considered as this species in the Brazilian rodents *Oryzomys concolor, O. capito*, *O. nigripes*, *Akodon arviculoides*, *Proechimys* spp., *Rattus rattus* and *Rhipidomys leucodactylus*, and the opossum *Didelphis marsupialis* did much to support this suggestion (Forattini et al. 1972, 1973; Lainson and Shaw 1970, 1979; Lainson et al. 1981a; Rocha et al. 1988). In Venezuela, records have been given for the rodents *R. rattus* and *Sigmodon hispidus* (De Lima et al. 2002). In most cases, identification of the parasite was based on only biological features and the isolates are no longer available for confirmation by modern biochemical methods, monoclonal antibodies or molecular techniques. Recently, however, a definitive identification of isolates from the Brazilian rodents *Bolomys lasiurus* and *R. rattus* as *L. (V.) braziliensis* by multilocus enzyme electrophoresis (Brandão-Filho et al. 2003) does suggest that the earlier records of rodent hosts of *L. (V.) braziliensis* were probably correct. It now remains to show that these animals serve as an efficient source of infection for the sandfly vectors.

Domestic animals such as dogs, mules, horses, and (very rarely) cats have been found with skin lesions produced by parasites regarded as *L. (V.) braziliensis* s.l. or simply recorded as leishmanias of the *braziliensis*

complex. These reports, in areas of suspected peridomestic transmission of the parasite, come principally from localities of extensive deforestation in Argentina, southern Brazil, Bolivia, Colombia, and Venezuela.

Recorded sandfly hosts

Uncertainties regarding the exact distribution of *L. (V.) braziliensis* make it difficult to indicate its vector (or vectors). An isolate of the parasite responsible for human cutaneous leishmaniasis in primary Amazonian rain forest in the Carajás highlands of Pará State, Brazil, has been used as a reference strain of *L. (V.) braziliensis* (MHOM/BR/1975/M2903) and in this area the vector is undoubtedly *Psychodopygus wellcomei*. This sandfly, referred to by some as *Lutzomyia (Psychodopygus) wellcomei*, has been found heavily infected on numerous occasions (Lainson et al. 1973) and *L. (V.) braziliensis* has been experimentally transmitted to a hamster by the bite of a naturally infected specimen (Ryan et al. 1987).

Ps. wellcomei is essentially sylvatic and avidly feeds on humans, not only at night but also in the daylight hours during overcast weather. It is extremely abundant in the rainy season (November–April), when it may represent c. 65 percent of the total catch of some 25 different species of sandflies taken off human bait. Captures of sandflies from humans stationed at different heights on tree-ladders have shown that *Ps. wellcomei* has a vertical flight range of only 1–2 meters above ground level: this, and the insect's abundance (25.5 percent) in catches of different sandflies taken from rodent-baited traps, suggest that the principal wild mammalian hosts of *L. (V.) braziliensis*, in the area in question, are likely to be terrestrial animals, probably rodents.

In the lowland regions of Pará State, a parasite identified as *L. (V.) braziliensis* was isolated from a single specimen of a closely related and highly anthropophilic sandfly, *Ps. complexus* (de Souza et al. 1996). The females of this species are morphologically indistinguishable from those of *Ps. wellcomei*, but the males of each species are quite distinct.

In the State of Amazonas another highly anthrophilic species, *Ps. carrerai*, has been found infected with *L. (V.) braziliensis* s.l. (Grimaldi et al. 1989). The strain was biochemically similar to *L. (V.) braziliensis* of humans from the same region but antigenically different from those from other areas of Brazil, including the lower Amazon region.

In other parts of Brazil, occasional isolates of *L. (V.) braziliensis* s.l. have been made from the sandfly *Lutzomyia (Nyssomyia) whitmani* sensu stricto, caught in and around houses in rural parts of Bahia and Ceará States, north-east Brazil (Hoch et al. 1986; Ryan et al. 1990; de Queiroz et al. 1994) and in the States of São Paulo and Minas Gerais (south-east Brazil) it is again suspected as a vector due to its highly anthropophilic feeding habits and its high density in and around human dwelling places and animal sheds in the endemic areas of cutaneous leishmaniasis. In the northern part of Paraná *Lu. whitmani* was shown to form 62 percent of the sandfly population captured, and to be highly anthropophilic. Of 1 628 specimens dissected, three were infected (0.2 percent) and in each case the isolated parasite was identified as *L. (V.) braziliensis* on enzyme profiles using the standard World Health Organization (WHO) reference strains for comparison (Luz et al. 2000).

In the State of Rondônia, Brazil, a parasite identified as *L. (V.) braziliensis* has been isolated from three specimens of *Lutzomyia davisi* (Grimaldi et al. 1991). This sandfly is also highly anthropophilic.

Lutzomyia (Nyssomyia) intermedia is another highly anthropophilic sandfly that, although originally sylvatic, has now adapted well to a peridomestic habitat in deforested, rural areas. It is highly suspected as a vector of *L. (V.) braziliensis* s.l. in the State of Rio de Janeiro and some parts of the State of São Paulo, Brazil and certain regions of Argentina. Flagellates thought to have been promastigotes of *Leishmania* were on one occasion seen in histological sections of the intestines of the sandflies *Lutzomyia migonei* and *Lu. (Pintomyia) pessoai* caught in endemic areas of cutaneous leishmaniasis in São Paulo State (Pessôa and Barretto 1948). Their true nature, however, remains obscure.

In Bolivia *L. (V.) braziliensis* s.l. has been isolated from *Ps. carrerai carrerai* (Le Pont et al. 1988), *Ps. llanosmartini*, and *Ps. yucumensis* (Le Pont and Desjeux 1986), whereas in Colombia (Young et al. 1987) and Venezuela (Young and Duncan 1994) similar parasites have been found in *Lu. spinicrassa*. In north central Venezuela, parasites isolated from *Lu. ovallesi* and *Lu. gomezi* have been typed as *L. (V.) braziliensis* and these sandflies are considered as primary and secondary vectors, respectively, on epidemiological grounds (Feliciangeli et al. 1994). In the Andean region, there is strong evidence that *Lu. youngi* is the vector of local cutaneous leishmaniasis, but the parasites encountered in this sandfly require precise identification. *Lu. spinicrassa* may be a vector throughout areas bordering the endemic Colombian foci.

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, usually with one or few lesions and also mucocutaneous and mucosal leishmaniasis (Figures 2–4). Common names include:
úlcera de Bauru, ferida brava, ferida sêca, bouba, buba, nariz de anta (tapir nose), and espundia.

The various zymodemes of *L. (V.) braziliensis* s.l. are placed in the *braziliensis* complex, together with the closely related parasite *L. (V.) peruviana*.

LEISHMANIA (VIANNIA) PERUVIANA VELEZ, 1913

Known geographical distribution

The parasite has been noted in Peru, on the western slopes of the Andes and in the inter-Andean valleys. Its range may possibly extend into the Argentinian highlands and it is probably more widely distributed in the Andean countries than previously suspected. Transmission seems to take place in relatively barren, mountainous areas with scant vegetation and a relatively restricted wild mammalian fauna.

Known mammalian hosts

Until recently humans and dogs (*Canis familiaris*) were the only known mammalian hosts (Herrer 1951) The role of the dog in the epidemiology of the human disease has remained obscure, however, and it has long been postulated that both humans and dogs are merely 'victim hosts' of a parasite maintained in wild animals of the Peruvian Andes. Recent studies (Llanos-Cuentas et al. 1999) report that isolations of a parasite from two specimens of the rodent *Phyllotis andinum* and an opossum, *Didelphis marsupialis* have been characterized as *L. (V.) braziliensis* by their isoenzyme profiles. Five other isolates from the rodent *Akodon* sp., were identified only to the subgenus *Viannia*. That the prevalence in the wild animals is much the same as in dogs, supports the existence of enzootic disease in wild animals.

Recorded sandfly hosts

Lutzomyia (Helcocyrtomyia) peruensis and *Lutzomyia verrucarum* have long been suspected as probable vectors, in view of their anthropophilic feeding habits. Isolation of a parasite with the biological characteristics of *L. (V.) peruviana* from the former fly, captured in an endemic area, implies its involvement in transmission (Herrer 1982), but much research in the field is needed before significant evidence can be obtained.

Disease caused by the parasite in humans

The parasite causes simple cutaneous leishmaniasis, not associated with the mucocutaneous disease. It is particularly frequent in school children, commonly resulting in extensive facial scars. Ulcers are usually self-healing and a firm immunity to reinfection with the same parasite is usually imparted. Common names include uta, tiaccaraña and llaga.

LEISHMANIA (VIANNIA) GUYANENSIS FLOCH, 1954

Known geographical distribution

This is an essentially sylvatic species that is an extremely common cause of human cutaneous leishmaniasis, particularly in Brazil, north of the Amazon river and the Guyanas. Its range is reported to also extend into Colombia, Ecuador, Venezuela, and the lowland forests of Peru.

Known mammalian hosts

Known mammalian hosts include humans and, in primary forest, the major reservoir hosts are the sloth *Choloepus didactylus* and the lesser anteater *Tamandua tetradactyla* (Xenarthra) (Lainson et al. 1981b; Gentile et al. 1981) with occasional infections found in rodents and opossums (Lainson et al. 1981a; Gentile et al. 1981) The infection is always inapparent, with parasites located in apparently normal skin and in viscera such as the spleen and liver.

Recorded sandfly hosts

The principal vector among wild animals and to humans is the sandfly *Lutzomyia (Nyssomyia) umbratilis*, with infections relatively infrequently found in a closely related fly, *Lu. (N.) anduzei* and *Lu. (N.) whitmani* s.l. Some early records of the parasite in the latter sandfly in Amazonian Brazil, possibly refer to *Leishmania (V.) shawi.*

Lu. (N.) umbratilis is a sandfly that dwells in the forest canopy and on the larger tree trunks. During the early hours of daylight, it may be found in large numbers resting on the larger tree trunks from which the flies will readily fly off and attack humans when disturbed. Although infection of this sandfly clearly takes place when it feeds on the reservoir hosts at night in the canopy, transmission to humans is principally during the day (early morning), when gangs of forest laborers are engaged in their work, particularly deforestation. Others at risk include the collectors of Brazil nuts and other fruits, topographers, visiting botanists and zoologists, and even the occasional tourist.

The enzootic of *L. (V.) guyanensis*, as studied in primary rain forest, is unlikely to survive in secondary forests or man-made plantations of non-indigenous trees. The small girth of young trees provides a microhabitat that is unsuitable for resting sandflies due to the low surface humidity of the smooth trunks. In addition, such immature trees are an equally unsuitable environment for relatively large and heavy animals, such as sloths and anteaters. Finally, in monoculture plantations (e.g. pine and gmelina) sloths are deprived of their normal diet of indigenous fruits and foliage.

Cutaneous leishmaniasis due to *L. (V.) guyanensis* may reach high prevalence in human communities situated in or very near primary forest, leading to an erroneous impression that the sandfly vector, *Lu. (N.) umbratilis*, has adapted to a peridomestic habitat. There is, as yet, no evidence that this occurs and peridomestic acquisition of the disease is doubtless due to infected flies that have been attracted to the lights of houses at night, from nearby forest. Esterre et al. (1986) showed

experimentally that clearing forest to c. 500 meters from a village situated in primary forest in French Guyana completely interrupted the transmission of *L. (V.) guyanensis* among its inhabitants.

The marsupial *Didelphis marsupialis* has rarely been found infected with *L. (V.) guyanensis* in primary forest where there is intensive transmission of the parasite among sloths and anteaters and to humans. Strangely, however, a high rate of infection has been recorded in the abnormally large populations of this opossum that are attracted to human refuse in villages on the borders of virgin forest (Arias and Naiff 1981). Reasons for this are not clear, nor is it certain whether opossums serve as a source of *L. (V.) guyanensis* for the sandfly vector, or if they merely represent dead-ends in the life-cycle of the parasite.

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, very frequently with multiple skin lesions (Figure 6). Cases of mucocutaneous disease appear to be rare. Common names for the disease include pian-bois, bosch-yaws, and forest-yaws.

Multiplicity of the skin lesions arises in two very different ways. First, sloths are rather sedentary animals and an infected animal may remain in a given spot for a considerable period. The infection rate of *Lu. (N.) umbratilis* resting on neighboring tree trunks will thus tend to rise to a high level, with levels as high as 25 percent found among many hundreds of specimens taken from a single tree. It follows that people attacked by sandflies in the area may receive numerous infective bites at the same time on all exposed parts of the body. Forest workers tend to be shirtless and frequently wear shorts: for this reason many patients present with lesions scattered over the face, trunk, arms, and legs (Figure 6). The developing multiple lesions of such individuals tend to be of a similar size and evolution. Secondly, there is abundant clinical evidence indicating the formation of metastatic lesions in persons originally presenting with a single skin lesion. These lesions tend to follow a distinct migratory course along the lymphatics and because of this evolution they are frequently nodular and, when ulcerated, of very unequal size.

L. (V.) guyanensis gives its name to the *guyanensis* complex of closely related parasites including *L. (V.) panamensis* and *L. (V.) shawi*.

LEISHMANIA (VIANNIA) PANAMENSIS LAINSON AND SHAW, 1972

Known geographical distribution

As the name suggests, most information on this parasite comes from Panama and the Canal Zone, where the very frequent acquisition of cutaneous leishmaniasis by American military personnel prompted intensive eco-epidemiological studies. It is also recorded in west and central Colombia, Ecuador, Venezuela, Costa Rica, Honduras, and Nicaragua.

Known mammalian hosts

Humans are a host to the parasite. The eco-epidemiology of *L. (V.) panamensis* follows a very similar pattern to that of *L. (V.) guyanensis*, which is not surprising in view of the close biological and biochemical relationship of the two parasites. The major host is the two-toed sloth *Choloepus hoffmanni* with occasional infections reported in the three-toed sloth *Bradypus infuscatus* and *B. griseus* (Herrer et al. 1973). More rarely, infections have been registered in other sylvatic animals such as *Bassaricyon gabbi*, *Nasua nasua* and *Potos flavus* (Carnivora: Procyonidae), *Aotus trivirgatus*, and *Saguinus geoffroyi* (Primates: Cebidae and Callitrichidae) and *Heteromys* (Rodentia). Hunting dogs occasionally develop skin lesions due to *L.(V.) panamensis*: like humans they are 'victim hosts' that rarely, if ever, serve as a source of parasites for the sandfly vector(s), or as a means of maintaining the enzootic.

Recorded sandfly hosts

The major sandfly vector is considered to be *Lu. (N.) trapidoi*, whereas *Lu.(N.) ylephiletor*, *Lu. (Lu.) gomezi*, and *Psychodopygus panamensis* may act as secondary vectors (Johnson et al. 1963; Christensen et al. 1969)

Disease caused by the parasite in humans

The parasite usually causes single or a limited number of skin lesions. Rare cases of mucocutaneous leishmaniasis have been attributed to *L. (V.) panamensis*.

Studies on populations of *Leishmania* in Ecuador have shown a cluster in which the isoenzyme profile of 6-phosphate gluconate dehydrogenase (6PGD) showed considerable variation (Bañuls et al. 1999). As it is this enzyme that has been extensively used to separate *L. (V.) panamensis* and *L. (V.) guyanensis*, these authors suggested that these parasites are not separate species. Within the cluster of these species in eastern Amazonia, however, *L. (V.) guyanensis* strains all grouped together, while isolates from Ecuador grouped with *L. (V.) panamensis* markers. For this reason, and taking eco-epidemiological and clinical data into consideration, we prefer to maintain specific separation of the two parasites.

LEISHMANIA (VIANNIA) LAINSONI SILVEIRA ET AL., 1987

Known geographical distribution

Until recently, this parasite was recorded only in the State of Pará, north Brazil. Its presence has now been noted, however, in forested areas of Peru and Bolivia (Lucas et al. 1994; Martinez et al. 2001). It probably exists in other regions where the known mammalian and sandfly hosts coexist.

Known mammalian hosts

Humans are a known mammalian host. The only known host among wild animals is the rodent *Agouti paca* (Rodentia: Dasyproctidae) (Silveira et al. 1991a).

Recorded sandfly hosts

Lu. (Trichophoromyia) ubiquitalis is a known host and the first representative of the subgenus *Trichophoromyia* to be incriminated as a vector of a *Leishmania* species.

L. (V.) lainsoni was isolated only from this sandfly, among many other species dissected in forested areas where patients had become infected with this parasite (Silveira et al. 1991b). The puzzling fact remained that *Lu. (T.) ubiquitalis* had not been caught biting humans in the forest. It was found, however, that this sandfly would feed avidly on humans if maintained for some hours in the laboratory after capture and this prompted the conclusion that under certain conditions it must also feed on humans in its natural habitat. Continuing field studies confirmed this (Lainson et al. 1992), although the factors influencing the sandfly's biting habits remain obscure. *Lu. (T.) ubiquitalis* is clearly not particularly fond of human blood, which accounts for the relatively low rate of infection with *L. (V.) lainsoni* in humans, compared with other species of *Leishmania*, such as *L. (V.) braziliensis* and *L. (V.) guyanensis*; these have highly anthropophilic sandfly vectors. In Bolivia, promastigote infections have been recorded in the sandfly *Lu. velascoi*, but the parasite was not isolated for characterization. As this fly is the only species of the subgenus *Trichophoromyia* in a region where *L.(V.) lainsoni* was encountered infecting man, it remains highly likely that it is the local vector of this parasite (Martinez et al. 2001).

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, usually with a single ulcerating skin lesion. Cases of mucocutaneous leishmaniasis due to this parasite have not yet been encountered.

LEISHMANIA (VIANNIA) SHAWI LAINSON ET AL., 1989

Geographical distribution

To date, the parasite has been found in various localities in the Amazon Region of north Brazil, south of the Amazon river.

Known mammalian hosts

Humans are commonly infected. Hosts among the forest animals include the monkeys, *Cebus apella* and *Chiropotes satanas* (Cebidae); the sloths *Choloepus didactylus* and *Bradypus tridactylus* (Xenarthra); and the coatimundi *Nasua nasua* (Procyonidae) (Lainson et al. 1988, 1989). It remains likely that other arboreal animals harbor the parasite.

Recorded sandfly hosts

Infections have so far been recorded in only one species of sandfly, which was provisionally identified as *Lutzomyia (Nyssomyia) whitmani* (Lainson et al. 1989). Morphometric differences have been noted, however, between this insect and the type material of *Lu. (N.) whitmani* sensu stricto from Bahia, northeast Brazil. These, and separation by DNA probes, suggested the vector to be a 'cryptic' species of a *Lu. (N.) whitmani* complex (Rangel et al. 1996). A recent phylogenetic analysis of the described mitochondrial (cytochrome b) haplotypes of *Lu. whitmani*, however, disputes this hypothesis, and suggests the existence of clades of haplotypes and a continuum of interbreeding populations of *Lu. whitmani* in the rain-forest regions of Brazil (Ishikawa et al. 1999). The differences in behavior of the fly in the type locality in Bahia, northeast Brazil, and that in Pará State, north Brazil are nevertheless striking. The former is highly anthropophilic, abundant in human dwelling places, and transmits *L. (V.) braziliensis*; the latter rarely bites man, until now has not been recorded in houses even when these are situated very close to the fly's normal habitat in primary forest, and is a vector of *L. (V.) shawi*.

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, usually with a single ulcerating skin lesion, but cases of multiple lesions of varying pathologies have been observed (Figure 11). Cases of mucocutaneous leishmaniasis due to this parasite have not yet been encountered.

LEISHMANIA (VIANNIA) NAIFFI LAINSON AND SHAW, 1989

Known geographical distribution

Isolates of the parasite are registered in the Brazilian States of Pará and Amazonas and in French Guyana. The range of this parasite will almost certainly extend into other parts of Latin America where the wild animal host and the sandfly vector coexist.

Known mammalian hosts

Humans are a known mammalian host (Lainson et al. 1990b). To date, the only known wild animal host is the nine-banded armadillo, *Dasypus novemcinctus* (Xenarthra: Dasypodidae), in which there is a high infection rate in apparently normal skin and viscera.

Figure 11 *Strange multiple nodules on the foot due to* L. (V.) shawi*, clinically resembling mycotic disease; Pará, Brazil*

Recorded sandfly hosts

Most recorded sandfly infections (Arias et al. 1985; Naiff et al. 1989) involve the sandfly *Psychodopygus ayrozai* which is, therefore, most suspected as the vector among armadillos. This fly is not highly anthropophilic, which possibly accounts for the paucity of human infection with *L. (V.) naiffi*. On the other hand, the parasite has, on rarer occasions, also been found in *Ps. paraensis* and *Ps. s. squamiventris*, both of which are highly anthropophilic and possibly involved in transmission to humans. Further studies are clearly necessary to establish the respective roles of these three sandflies in the eco-epidemiology of *L. (V.) naiffi*.

Disease caused by the parasite in humans

The parasite causes cutaneous leishmaniasis, usually with single, small, ulcerating lesions. No case of mucocutaneous leishmaniasis has yet been attributed to this parasite.

Unlike most species of *Leishmania*, *L. (V.) naiffi* rarely produces a visible lesion when inoculated into the skin of hamsters, although the parasite may be re-isolated following culture of skin from the inoculation site in blood agar media after at least 1 year. For this reason, human infection with this parasite may have remained undiagnosed in the past, when inoculation of hamsters has been the sole method of isolation attempted from human skin lesions. In addition, it may be that *L. (V.) naiffi* can also produce an occult, benign infection in the skin of humans, as it does in the hamster and that transmission to humans is more frequent than has been suspected.

LEISHMANIA (VIANNIA) COLOMBIENSIS KREUTZER ET AL., 1991

Known geographical distribution

The parasite exists in Colombia, Panama, and Venezuela, probably extending into the neighboring forests of Brazil, the Peruvian lowlands, and other Latin American countries where the wild mammalian and sandfly hosts coexist.

Known mammalian hosts

The parasite has been recorded in humans and the sloth, *Choloepus hoffmanni* (Panama).

Recorded sandfly hosts

Lutzomyia (Helcocyrtomyia) hartmanni has been found infected in Colombia and *Lu. (Lutzomyia) gomezi* and *Psychodopygus panamensis* in Panama (Kreutzer et al. 1991).

Disease caused by the parasite in humans

The parasite causes single to multiple ulcerating skin lesions. Cases of mucocutaneous leishmaniasis caused by the parasite have not yet been seen. The strain identified from Venezuela by Delgado et al. (1993) was isolated from a bone marrow aspirate of a patient with visceral leishmaniasis. It is, however, uncertain whether or not this was the parasite responsible for the clinical symptoms observed.

LEISHMANIA (VIANNIA) EQUATORENSIS GRIMALDI ET AL., 1992

Known geographical distribution

The parasite exists along the Pacific coast of Ecuador.

Known mammalian hosts

The sloth, *Choloepus hoffmanni*, and the squirrel, *Sciurus granatensis* have been identified as hosts. In both animals, the parasite was isolated from the liver and spleen but was not found in the skin.

Recorded sandfly hosts

Lutzomyia hartmanni has been found infected in Ecuador (Furuya et al. 1998).

Disease caused by the parasite in humans

As yet, the parasite has not been found in humans.

LEISHMANIA (VIANNIA) LINDENBERGI SILVEIRA ET AL., 2002

Known geographical distribution

At present, this recently described parasite has been recorded only from degraded forest on the outskirts of Belém, Pará State, north Brazil, with isolates made from a number of soldiers who acquired their infections while undertaking maneuvers in this forest at night.

Known mammalian hosts

Humans are the only recorded host so far. From the behavior of the suspected vector and the circumstances in which the soldiers became infected, the wild animal host(s) is (are) most likely terrestrial.

Recorded sandfly hosts

Lutzomyia (Nyssomyia) antunesi is by far the most common sandfly avidly biting man in the type locality. It is of particular interest that the infected soldiers spent much of their time in slit-trenches, so that their heads and resting arms were at ground level: most of their lesions were on these parts of the body. Previous workers recorded heavy promastigote infections in three specimens of *Lu. (N.) antunesi* from Marajó island, near Belém (Ryan et al. 1984), but they failed to identify the parasite. Its development in the sandfly gut was described as being 'suprapylarian in nature' (i.e. characteristic of the subgenus *Leishmania*), whereas Silveira et al. (2002) clearly showed the development of *L. (V.) lindenbergi* to be peripylarian in experimentally infected laboratory-bred *Lu. longipalpis*.

Disease caused by the parasite in humans

Localized cutaneous lesions: as yet, no cases of mucosal lesions have been recorded. *L. (V.) lindenbergi* is distinguished from other members of the subgenus *Viannia* by its enzyme profiles and the use of monoclonal antibodies. It is clearly closely related to *L. (V.) naiffi* and also produces an inapparent infection in the skin of hamsters.

LEISHMANIA (VIANNIA) UTINGENSIS BRAGA ET AL., 2003

Known geographical distribution

As yet, this parasite is recorded only from the Utinga forest, on the outskirts of Belém, Pará State, north Brazil.

Known mammalian hosts

At present, mammalian hosts are unknown, but given the habits of the vector are likely to be arboreal.

Recorded sandfly hosts

The parasite was isolated from a single specimen of the sandfly *Lutzomyia tuberculata*. This sandfly is commonly found on the larger tree-trunks of primary and secondary forests.

Disease caused by the parasite in humans

Not yet found infecting humans. As the vector shows no tendency to bite man, human infections with *L. (V.) utingensis* are unlikely to occur by way of this insect. If any anthropophilic sandflies are also hosts of the parasite, however, human infection may well be found in the future.

'Hybrid' *Leishmania* of the subgenus *Viannia*

Strains with phenotypic and genotypic characters of two species have been recorded in different geographical areas of Latin America. There are two possible interpretations for such strains: either they represent strains that originated directly from a common ancestor or they are the result of genetic exchange.

L. (V.) BRAZILIENSIS/*L. (V.) PANAMENSIS* HYBRID

Known geographical distribution

The parasite has been found in northern Nicaragua (Darce et al. 1991) close to the border with Honduras. To the south, the strains are *L. (V.) panamensis* and to the north, *L. (V.) braziliensis*. Bañuls et al. (1997) recorded what appears to be the result of hybridization between *L.(V.) braziliensis*/*L.(V.) panamensis* and *L. (V.) panamensis*/*L. (V.) guyanensis* in isolates from human infections in Ecuador.

Known mammalian hosts

So far, the parasite is only known in humans.

Recorded sandfly hosts

No sandfly host has been recorded to date.

Disease caused by the parasite in humans

The parasite causes ulcerating skin lesions. Cases of mucocutaneous infections have not yet been seen.

L. (V.) BRAZILIENSIS/*L. (V.) GUYANENSIS* HYBRIDS

Known geographical distribution

In Venezuela, the parasite has been found in Lara State; Tachira; DF, El Junquito; and Miranda, Guarenas. The isolates from the last three localities were similar, and their hybrid alleles differed from those of the isolate previously made in Lara (Bonfante-Garrido et al. 1992; Delgado et al. 1997).

Known mammalian hosts

So far, the parasite has only been recorded from humans.

Recorded sandfly hosts

No sandfly hosts have yet been found.

Disease caused by the parasite in humans

The parasite causes ulcerating skin lesions. Cases of mucocutaneous leishmaniasis caused by this parasite have not yet been seen.

L. (V.) BRAZILIENSIS/*L. (V.) PERUVIANA* HYBRID

Known geographical distribution

To date, four strains have been isolated from patients in the Limapampa region of the Huanuco Valley, Peru (Dujardin et al. 1995).

Known mammalian hosts

Humans are the only known mammalian host to date.

Recorded sandfly hosts

No sandfly vectors have been recorded.

Disease caused by the parasite in humans

Ulcerating skin lesions were noted in all patients and one also had a mucosal lesion.

Leishmania-like parasites of uncertain taxonomic position

Molecular studies suggest that the following parasites are more closely related to *Endotrypanum* (an intra-erythrocytic flagellate of sloths) than they are to *Leishmania*. Until more information is available, however, we feel it best to retain their present names.

LEISHMANIA (LEISHMANIA) HERTIGI HERRER, 1971

Known geographical distribution

The known distribution is limited to Panama and Costa Rica.

Known mammalian hosts

The tree-porcupine *Coendou rothschildi* is the only known mammalian host.

Recorded sandfly hosts

The sandfly vector of the parasite has still to be discovered. The remarkably high infection rate of 88 percent found in the porcupines studied suggests a close association of the vector with the mammalian host, possibly in the hollow trees where these animals live.

Absence of infection in humans

Human infection has not been recorded. *L. (L.) hertigi* seems to be specific to *Coendou rothschildi*, as the parasite has not been found in a wide variety of other forest mammals studied in the Panamanian forests. The apparent absence of human infection may be due to the failure of the parasite to survive in human tissues, or because the sandfly vector does not bite humans.

L. (L.) hertigi is included in the *hertigi* complex, together with another closely related parasite of porcupines, *L. (L.) deanei*.

LEISHMANIA (LEISHMANIA) DEANEI LAINSON AND SHAW, 1977

Known geographical distribution

The parasite has been noted only in Amazonian Brazil.

Known mammalian hosts

The known mammalian hosts are the tree-porcupine *Coendou p. prehensilis* and another, as yet unnamed, species of *Coendou*.

Recorded sandfly hosts

The vector of *L. (L.) deanei* remains to be discovered. As with *L. (L.) hertigi*, the infection rate in Brazilian porcupines is very high, again suggesting a close association of the vector with the mammalian host, probably in the animal's home in hollow trees. A tree-inhabiting sandfly, *Lu. (Viannamyia) furcata*, taken from a tree-hole in which an infected porcupine was living, was shown to have promastigotes of *L. (L.) deanei* in its midgut (Miles et al. 1980). There was, however, no evidence of migration of the parasites to the anterior station of the gut and, in subsequent experimental infections of this sandfly, it was shown that the promastigotes

disappeared following complete digestion of the blood-meal (Lainson and Shaw 1987).

Absence of infection in humans

Human infection has not been recorded. As with *L. (L.) hertigi*, this could be because the sandfly vector is not attracted to humans or because the parasite cannot survive in human tissues.

L. (L.) deanei, like *L. (L.) hertigi*, seems to be restricted to species of the porcupine *Coendou* and an exhaustive examination of other animals in areas of forest where porcupines are commonly infected failed to indicate any other mammalian hosts (Lainson and Shaw, unpublished observations). Although both parasites seem to be peculiar to porcupines, they are readily distinguishable by their isoenzyme profiles and the morphology of their amastigote stages; those of *L. (L.) hertigi* are strangely elongated and measure from 3.5 × 1.2 to 4.8 × 2.5 μm. The amastigotes of *L. (L.) deanei* are the largest of all known species of *Leishmania*, measuring from 5.l × 3.1 to 6.8 × 3.7 μm.

Working with 12 isolates of *L. (L.) deanei* from Pará, north Brazil, Miles et al. (1980) found that they were separable into groups by the enzyme profiles of:

- malate dehydrogenase (oxaloacetate-decarboxylating) (NADP) E.C.1.1.1.40 (ME)
- phosphoglucomutase E.C.2.7.5.1. (PGM)
- malate dehydrogenase E.C.1.1.1.37 (MDH).

Whether or not this is indicative of a third species of *Leishmania* in the *hertigi* complex is debatable and further study is indicated on the biology, biochemistry and molecular biology of the two zymodemes.

Questionable leishmanial parasites

LEISHMANIA HERRERI ZELODÓN ET AL. 1979

This parasite was isolated in Costa Rica from the sloth *Bradypus griseus* and regarded as a new species of *Leishmania*. Mention was made that the DNA buoyant densities and enzyme profiles of the organism were 'totally different from other known hemoflagellates', but no details of the biochemistry were given and, until further isolates of the parasite are available, there must remain considerable doubt as to its true nature.

Infection of human skin by nonleishmanial amastigotes

Amastigotes found in skin lesions of individuals infected with human immunodeficiency virus (HIV) and non-infected individuals on the Island of Martinique have been considered to be those of monoxenous insect flagellates (Boisseau-Garsaud et al. 2000). It is likely that some such lesions have, in the past, been wrongly attributed to *Leishmania*.

GENETICS

The New World *Leishmania* have 20–25 chromosomes that exhibit high degrees of intra- and interspecies size polymorphism. This is thought to reflect a high level of genomic plasticity. Leishmanial genomic DNA is composed of repetitive, moderately repetitive and unique sequences that, respectively, form ca. 25, 13, and 60 percent of the total. Repeated sequences are useful for identification regardless of their distribution, function or copy numbers. Fernandes et al. (1994) found that the mini-exon repeat units of four *Viannia* species were different. Considerable genomic differences have been noted between the two subgenera, but they appear greater for species of *Viannia*. Mendoza-León et al. (1995) found that the β-tubulin gene regions showed a much greater degree of heterogeneity within and between parasites of the *braziliensis* complex than those of the *mexicana* complex. This suggests that there are more random mutations in the former than in the latter. The same gene may be located in more than one chromosome. This may be the result of gene duplication and transposition to another chromosome or chromosomal duplication followed by size divergence (Spithill and Samaras 1985). A useful summary of the recent developments from the *Leishmania* genome project have been given by Myler and Stuart (2000).

Genetic exchange between neotropical *Leishmania* has not been demonstrated experimentally, but the finding of apparent hybrids among *Viannia* species suggests that it may occur. Hybrids only occur in some localities and it is possible that ecological conditions may be important in determining the contact between the species involved. No hybrids have been noted among neotropical species of the subgenus *Leishmania*. Extreme care is needed in the characterization of flagellates from sandflies, as they may sometimes harbor more than one parasite. Monoxenous flagellates, species of *Trypanosoma*, and *Endotrypanum* will contribute to confusion, in addition to the possible coexistence of two species of *Leishmania* in the same insect, as recorded by Barrios et al. (1994). Ideally, all isolates should be carefully cloned, especially if showing unusual characteristics by current methods of identification.

The possible role in humans of genes and chromosome regions suggested by previous studies in mice to be linked to disease resistance or susceptibility to visceral leishmaniasis has been investigated in 638 individuals in 89 families with multiple cases of AVL in northeast Brazil, using complex segregation analyses (Blackwell 1998). Preliminary results indicated that the major histocompatibility complex showed only weak association with AVL; that there is no evidence for linkage between *nramp1* (the positionally cloned candidate for a murine

macrophage resistance gene) and susceptibility to AVL; and that the T helper 2 cytokine gene cluster is not linked to human susceptibility for this disease. It is suggested that the mouse model, together with knowledge of human response to infection, may lead to identification of important candidate gene regions in humans. Feitosa et al. (1999), used complex segregation analyses in a study of the intradermic reaction to antigens derived from *L. i. chagasi* in 502 individuals from 94 families in Bahia State, northeast Brazil. They concluded that the results gave evidence of a major genetic mechanism, with a frequency of a recessive susceptibility gene (q) of approximately 0.45, and that a small multifactorial component (H = 0.29), acting in combination with a major recessive gene (q = 0.37) could be a concomitant factor.

Viral infections in *Leishmania*

Virus-like particles have been described in the cytoplasm of cultured promastigotes of five isolates of *L. (L.) hertigi* and three of *L. (L.) deanei* (Molyneux and Killick-Kendrick 1987). Although unidentified, they were shown to be associated with cytopathological changes in the mitochondrion of the promastigote. The number of virus-like particles was drastically reduced when *L. (L.) hertigi* were grown as amastigotes in mouse peritoneal macrophages and dog sarcoma cell lines incubated at 32°C. The particles were not transmissible to other *Leishmania* species, nor to cell lines susceptible to viruses.

RNA virus particles belonging to the family Totaviridae were described by Tarr et al. (1988) in cultured promastigotes of a strain of *L. (V.) guyanensis* from French Guyana. Subsequent studies revealed the presence of similar viruses in promastigotes of 11 *Leishmania* strains isolated from humans, including both *L. (V.) guyanensis* and *L. (V.) braziliensis* from the Amazonian region of Brazil and Peru (Guilbride et al. 1992). All the viral isolates are considered to be different and are classified within a single genus, *Leishmaniavirus*. None, however, has been given specific status within this genus. The virus-bearing promastigotes were those of *Leishmania* isolated from uncomplicated cases of cutaneous leishmaniasis, but so far the viral particles have not been demonstrated in amastigotes from the skin lesions. The effect that such viruses may have on the *Leishmania* is not known but in experimental infections of *Leishmania major* in mice, it was noted that an infected line was less pathogenic than an uninfected one.

When human biopsy material from some cases of cutaneous leishmaniasis from Peru were examined for the presence of *Leishmaniavirus* RNA by the reverse transcription polymerase chain reaction (RT-PCR), two samples showed RT-PCR bands of the expected size. Sequence analysis indicated them to share approximately 90 percent sequence with the neotropical strain LRV 1-4 of that virus (Saiz et al. 1998).

CLINICAL FEATURES

American visceral leishmaniasis

Throughout most of its geographical range, the clinical features of AVL closely resemble those of infantile visceral leishmaniasis caused by *L. (L.) infantum* of the Old World (see Old World leishmaniasis) and this similarity extends to the fact that the disease is seen mainly in children (Figure 12). However, It has been shown that the same parasite may produce almost exclusively nonulcerative cutaneous lesions in Costa Rica (Zelodón et al. 1989), and may cause both visceral and cutaneous disease in the same focus of AVL in Honduras and Nicaragua (Ponce et al. 1991; Belli et al. 1999).

The saliva of *Lu. longipalpis* has been shown to contain a potent vasodilatory peptide, maxadilan (Lerner et al. 1991). It was found that when sandflies from Brazilian, Colombian, and Costa Rican populations were fed on the arms of volunteers, the degree of erythema was highest for the Brazilian flies, slightly less for the Colombian, and very low for the Costa Rican examples (Warburg et al. 1994). These authors considered this difference to be due to a variation in the *potency* of the peptide but similar recent experiments have suggested it to be due to differences in the *amount* of maxadilan in the saliva of the three populations (Yin et al. 2000). This is in agreement with the distribution of atypical cutaneous leishmaniasis due to *L. i. chagasi*, and lends credence to the involvement of maxadilan in visceralization of the parasite.

There is evidence that *L. (L.) infantum chagasi* may produce a benign, inapparent infection in some individuals and that severity of the disease depends to some extent on the nutritional state of the infected person (Badaró et al. 1986). Differential diagnosis has to be made principally from malaria, schistosomiasis, cirrhosis of the liver, visceral syphilis and other causes of hepatosplenomegaly.

Although serological methods such as the indirect fluorescent antibody, dot-enzyme-linked immunosorbent assay (ELISA) and direct agglutination tests are useful indicators, the novel use of a dipstick (InBios International, Inc) based on recombinant RK 39 antigen for the differential diagnosis of AVL from other sympatric endemic diseases, has been shown to be rapid and highly specific (Delgado et al. 2001). Also, new latex agglutination test (KATEX) is claimed to detect leishmanial antigen in the urine of patients with a 100 percent specificity (Attar et al. 2001). Unequivocal diagnosis of the disease, however, depends on the demonstration of amastigotes in stained smears of aspirates from the spleen, bone marrow or lymph glands. Usually, such smears contain abundant parasites; if not, the material can be cultured in a suitable blood agar medium (varieties of Novy, MacNeal, Nicolle (NNN) medium).

Figure 12 *AVL, due to* L. (L.) infantum chagasi, *in a boy from Marajó island, Pará, Brazil. Note the greatly distended abdomen resulting from hepatosplenomegaly.*

However, some difficulty may be experienced in culturing the parasite, especially when the aspirates contain scanty amastigotes. The intraperitoneal inoculation of aspirates into hamsters is by far the most reliable method of isolating *L. (L.) infantum chagasi* for further study but the long delay before parasites are detectable in these animals makes the method impractical for quick diagnosis. The PCR, together with specific hybridization techniques, will probably be the method of choice in the future (Noyes et al. 1996; Rodriguez et al. 1997; Breniere et al. 1999), at least in the larger and better-equipped hospitals, clinics, and research laboratories. Traditional methods of demonstrating the presence of the parasite will remain vitally important for many more years to come, however, particularly in the more remote rural areas where the majority of cases are concentrated.

Cutaneous leishmaniasis

Simple cutaneous lesions are produced by 12 of the 14 neotropical species of *Leishmania* known to infect humans. It is not possible, however, to diagnose the causative species by the appearance of these lesions, which are in many cases indistinguishable from the classical leishmanial lesion of the Old World, forming a rounded, crater-like ulcer with a raised border (see Old World leishmaniasis, and Figure 2).

In addition, the simple lesion may vary greatly in appearance (Figures 11 and 13–15), evoking such descriptions as framboesiform, lichenoid, lupoid, nodular, vegetative, verrucose, ulcerative, etc. from clinicians and dermatologists. All of these forms have in common that they are painless; unfortunately, this means that the infected person often fails to seek medical advice until the lesion has reached large proportions. Differential diagnosis needs to consider tropical ulcer (painful and suppurative), sporotrichosis, cutaneous tuberculosis, yaws, blastomycosis, lupus, and tertiary syphilis. Bacterial infections of insect bites or skin abrasions are frequently misdiagnosed as early lesions due to *Leishmania* and are particularly common in children living in rural areas with an abundance of biting flies, such as *Simulium* and *Culicoides*.

Although patients under examination may show a positive Montenegro (leishmanin) skin-test, this may be due to a previous, subsequently eliminated infection with *Leishmania* and may be unrelated to present skin lesions. The demonstration of amastigotes in stained smears prepared from the border of the lesions and

Figure 13 *Infection with* L. (L.) amazonensis, *on the elbow; Pará, Brazil*

Figure 14 *A large number of small, papular satellite lesions surrounding the larger, primary lesions in a case of pian-bois due to* L. (V.) guyanensis*; Pará, Brazil*

isolation of the parasite in blood agar culture medium are prerequisite for diagnosis.

Vacuum aspiration of amastigotes in material from human lymph nodes, first used in the examination of patients with visceral leishmaniasis, has been used in cases of cutaneous leishmaniasis resulting from *L. (V.) braziliensis* infection in Brazil (Marzochi et al. 1993; Romero et al. 1999). Aspiration directly into the culture medium, through the rubber cap, greatly reduces the risk of bacterial or fungal contamination.

Figure 15 *Another atypical lesion, due to the same parasite; Pará, Brazil*

Mucocutaneous and mucosal leishmaniasis

For excellent general reading on this unique form of leishmaniasis, reference should be made to Pessôa and Barretto (1948), Marsden (1986), and Walton (1987).

Although the disease has, on rare occasions, been attributed to *L. (V.) guyanensis* and *L. (V.) panamensis*, the great villain is undoubtedly *L. (V.) braziliensis* s.l. The exact percentage of individuals infected with this parasite who develop mucosal lesions is difficult to calculate. In what was almost certainly a hospital series in São Paulo State, southern Brazil, Pessôa (1941) produced figures indicating that among 171 patients with cutaneous lesions of less than 1 year's duration, 105 lacked mucosal lesions and 66 (38.5 percent) had mucosal involvement. Of 110 individuals with cutaneous lesions of more than 1 year's duration, 21 had no mucosal lesions, and 89 (80.9 percent) had developed them. Marsden (1986), however, described Pessôa's figure of 80.9 percent as 'widely quoted out of text ... giving the impression that the great majority (of cases) will proceed to mucosal metastasis'.

In field studies in an endemic area in Três Braços, Bahia State, north-east Brazil, Marsden and his colleagues found only 2.7 percent of 371 patients examined to have both cutaneous and mucosal lesions. Mucosal infection seems to be much more common in some Latin American countries (e.g. Bolivia and Ecuador) than in others, suggesting that certain parasites referred to as *L. (V.) braziliensis* have a greater propensity for producing mucosal lesions than others (these may, in fact, represent different subspecies, or even species, of the *braziliensis* complex).

In spite of its antiquity, the most detailed study of the mucosal lesions remains that of Klotz and Lindenberg (1923), but a wealth of clinical and pathological data is also available in the works mentioned above, and that of Ridley (1987). It is generally agreed that involvement of the mouth, nose, and throat is always the result of metastases from a simple skin lesion elsewhere on the body (Figure 2). Apparently, migration of the parasites to the mucosae, by way of the lymphatics or the bloodstream, may take place quite early in the infection, for amastigotes have been demonstrated in scrapings of the apparently normal nasal mucosae of 12 patients with skin lesions of only 1–11 months' evolution (Villela et al. 1939). The nose is the major site of the metastases (Figure 3) and it remains a mystery as to why this occurs in some individuals and not in others. From the observations of Villela et al. and the fact that years may elapse between the disappearance of the primary skin lesion and the onset of mucosal disease, it seems that the parasite remains dormant in the mucosae for a variable period. Exactly what triggers this occult infection into destructive activity is another mystery, although minor

injury to the mucosae may be one cause. The following account of the developing pathology of the mucosal disease is summarized from Ridley (1987).

When a nasal lesion develops, it is initiated in the deep mucosa of the nose, an accumulation of plasma cells and lymphocytes forming around the small blood vessels. A few amastigotes may appear in the endothelial cells. The major part of the lesion stays in the deep mucosa, accompanied by congestion and edema, a pronounced infiltration of plasma cells and a characteristic proliferation of the vascular endothelial cells, which contain variable numbers of amastigotes. Inflammatory foci proceed towards the mucosal surface, resulting in a patchy desquamation, followed by hyalinization and necrosis of the exposed tissue, accompanied by polymorph infiltration. It should be stressed that the ulcer is due to the desquamation resulting from the inflammatory process and not to the necrosis. In the area of deep inflammation, the endothelial nodule, with its perivascular inflammatory cells, undergoes central necrosis or, in the case of large nodules, hyalinization. Scanty amastigotes may still be found, but they are absent in the necrotic area. Considerable endarteritis may be associated with thrombosis which, together with subsequent fibrosis, deforms and erodes the nasal septum. Liquefaction of cartilage continues, even some distance from the leishmanial nodule and the vascular supply is so reduced that only coarse fibrous tissue can survive.

Blockage of the nasal passages due to the developing lesion usually results in respiratory distress, mouth breathing, and a high frequency of pulmonary infection that may lead to death. Palatal (Figure 4), laryngeal and tracheal lesions are less common; for first-hand accounts of these, reference should be made to Marsden (1986) and Walton (1987).

Differential diagnosis of mucosal leishmaniasis must be made from nasal syphilis (no destruction of the septum), gangosa (ulcerating yaws), blastomycosis, rhinosporidiosis, midline granuloma, carcinoma, and cancrum oris.

Sudanese and Ethiopian oro-nasal (mucosal) leishmaniasis bears a superficial resemblance to American espundia (see El-Hassan et al. 1995, for review). Due largely to *L. (L.) donovani* s.l. and, more rarely, *L. (L.) major*, it is not preceded by a cutaneous lesion. In addition, unlike patients with the South American disease, advanced cases of Sudanese mucosal leishmaniasis respond readily to treatment with pentavalent antimonials and ketoconazole.

Anergic, diffuse cutaneous leishmaniasis

In Venezuela, Convit and Lapenta (1948) described a bizarre form of cutaneous leishmaniasis characterized by nodular lesions scattered all over the body and containing vast numbers of rather large amastigotes; a negative Montenegro skin-test reaction; and almost total resistance to chemotherapy. Further cases were recorded in that country and subsequently in Bolivia, Brazil, Colombia, the Dominican Republic, Honduras, northern Mexico, Texas, USA, and Peru. The causative parasites in Venezuela, Mexico, Texas, and Brazil are *L. (L.) pifanoi*, *L. (L.) mexicana* and *L. (L.) amazonensis* respectively and it is likely that this form of leishmaniasis elsewhere in the Americas is also due to members of the *mexicana* complex within the subgenus *Leishmania*. The disease, as its name suggests, is the outcome of infection by this group of parasites in individuals with little or no cell-mediated immunity. Curiously, parasites of the subgenus *Viannia* do not seem to possess the potential to produce this strange disease.

At one time it appeared that ADCL was an irreversible condition and that the best treatment merely kept the disease in check, without eliminating the parasite. There is some evidence to suggest, however, that the prognosis is not always so grim. One of the several ADCL patients studied in the authors' laboratory acquired her infection with *L. (L.) amazonensis* when she was less than 5 years old and, in spite of constant chemotherapy, her condition had not improved greatly by the age of 28 years. However, the lesions faded away and the patient made a complete recovery following immunochemotherapy. We had noted that, unlike the lesions of other cases of ADCL, those of this young woman had occasionally ulcerated and healed, leaving her with very unsightly scars on her face and legs (Figure 9). This and the final recovery, suggests that there is a gradation in the degree of anergic condition, from total to partial. In many patients, the nodular lesions remain unulcerated, whereas in others, there may be ulceration and even amputation of fingers and toes, resembling that seen in leprosy (Figure 5).

Usually, ADCL forms a minute proportion of the total number of cases of cutaneous leishmaniasis caused by parasites of the *mexicana* complex but an exception is found in the Dominican Republic, where ADCL occurs in the apparent absence of simple, curable cutaneous leishmaniasis. The causative parasite is clearly a member of the *mexicana* complex, but is, as yet, unnamed and its animal reservoir and sandfly vector are unknown. The occurrence of three cases in a single family in the Dominican Republic suggests involvement of an hereditary component (Walton 1987); Petersen et al. (1982) demonstrated a population of specific suppressor cells in four other patients. The deficient immune response in ADCL in general is considered to be related to a thymus-dependent system in both the New World and the Old World diseases (Convit et al. 1971; Bryceson 1970). The only other disease with which ADCL has frequently been confused is lepromatous leprosy; this mistake is unpardonable if Giemsa-stained smears of the skin lesions have been examined (Figure 10).

In a recent paper, Silveira et al. (2004) discussed the clinical and immunopathological spectrum of American

cutaneous leishmaniasis, with particular reference to the disease in Amazonian Brazil. They emphasized the necessity of accurately identifying the species of *Leishmania* stimulating the patient's immune response, together with the quality and magnitude of this response. At the diagramatic center of the authors' clinical spectrum are the most commonly seen localized forms of cutaneous leishmaniasis (LCL), which may be caused by members of both subgenera, *Leishmania* and *Viannia*, and which usually respond well to conventional chemotherapy. The two pathogenicity poles of the spectrum generally recognized are the hyposensitivity pole, occupied by cases of ADCL, and the hypersensitivity pole represented by mucocutaneous leishmaniasis. They proposed the term borderline disseminated cutaneous leishmaniasis (BDCL) for the cases showing disseminated lesions due to some parasites of both the subgenera where there is a partial failure of the cellular immune response (unlike the complete failure seen in ADCL), enabling eventual cure of the patient provided that treatment is promptly given. If this is not undertaken, however, these infections may develop into either ADCL (due, for example, to *L. (L.) amazonensis*) or mucocutaneous leishmaniasis (due mainly to *L. (V.) braziliensis*).

Leishmania infections and the immunosuppressed patient

Cutaneous and visceral leishmaniasis are now firmly established in the list of infections that the clinician must consider when dealing with immunosuppressed patients, in particular those with acquired immune deficiency syndrome (AIDS). Visceral leishmaniasis is the clinical form most commonly associated with HIV/AIDS and has become a public health problem of considerable proportions in south-western Europe. Strangely, although visceral leishmaniasis is far more common in many parts of Latin America (e.g. Brazil) than it is in Europe, cases of AVL/HIV infection are, until now, less common (WHO 1998). Possibly this is because, in the Americas, HIV is predominantly found in the larger cities; the situation will doubtless change as HIV spreads to the more rural areas where AVL is highly endemic or with the migration of leishmaniasis carriers into the large cities and their acquisition of HIV. One of the most striking examples of such migration is that of individuals from the rural areas of northeast Brazil, where AVL is very common, into São Paulo city in search of work. Co-infection of HIV and *Leishmania* species responsible for cutaneous leishmaniasis seems to be less frequent in the Americas, but may result in serious complications, ranging from disseminated skin lesions and mucosal lesions (Coura et al. 1987; Da-Cruz et al. 1999) to rectal involvement (Hernández et al. 1995). *L. (V.) guyanensis* has been isolated from the lesions of an HIV patient presenting with mucocutaneous leishmaniasis in the State of Amazonas, Brazil (de Souza e Souza et al. 1998), and visceral leishmaniasis due to *L. (L.) mexicana* has been recorded in a patient with HIV in Mexico (Ramos-Santos et al. 2000).

PREVENTION AND CONTROL

Visceral leishmaniasis

Campaigns are of fundamental importance, with the distribution of illustrated pamphlets to alert the populations as to the early symptoms of the disease, the signs of infection in the dog and the appearance and habits of the sandfly vector. The staff of small, rural clinics must be trained to recognize visceral leishmaniasis and should have means of reporting suspected cases to centers where more conclusive diagnosis can be made. In view of the impecunious situation of many inhabitants of the rural districts of developing countries, the staff of such centers may need to travel to the patient's village, where the possibility of other cases must be investigated. Periodic surveillance of populations at risk may detect cases of early infection either clinically or, more effectively, by serological methods that can easily be carried out under field conditions, such as the direct agglutination test (DAT) or the dot-ELISA.

In areas of high endemicity, such as the states of Ceará and Bahia in northeast Brazil, past control measures (i.e. destroying infected dogs, regular insecticide spraying of houses and animal sheds, and the early treatment of patients) resulted in a dramatic drop in the number of cases of AVL. A problem arises, however, in maintaining such a control program, which is costly and inevitably meets with considerable opposition on the part of dog owners, who fail to understand why their apparently healthy (but serologically positive) animals must be killed. Many deliberately conceal their dogs, and many strays are never examined. A critical evaluation of the cost-effectiveness of this dog-slaughtering policy (Akhavan 1996) points out the vast amount of work and expense expended in surveying dog populations, and the apparent failure of this method in control programs in some parts of Brazil has led to the question as to what extent the dog population needs to be reduced in order to eliminate AVL or to bring it under control. It has been suggested that it may be preferable to find improved methods of eliminating or controlling the sandfly vector.

Insecticide (deltamethrin)-impregnated plastic dog collars have been found to provide an efficient and prolonged protection of dogs against the bites of sandfly vectors of *L. i. infantum*, thus breaking transmission of the parasite in its major reservoir host (Killick-Kendrick et al. 1997). Such collars have been shown to offer similar protection against the bites of *Lu. longipalpis* in Brazil (David et al. 2001), and preliminary results

following field-trials in three regions of Brazil have been discussed in the 2002 *Proceedings of the Second International Cannine Leishmaniasis Forum, Seville, Spain*. Only after the completion of more lengthy field-trials, however, will it be possible to assess both the cost-efficiency of deltamethrin-impregnated dog collars, or other methods of insecticide application, and the effects they may have on the incidence of human AVL. In the meantime, the development and use of an effective and long-lasting vaccine, preferably in a well-supervised, governmental program similar to that of antirabies campaigns, remains of great importance. Encouraging results have been obtained after the inoculation of dogs with a mixture of promastigotes of *L. (V.) braziliensis*, disrupted by ultrasound, and bacille Camette Guerin (BCG). Of 10 vaccinated dogs, only one succumbed to subsequent challenge with 2.3×10^6 infective promastigotes of *L. i. chagasi*, whereas all of nine nonvaccinated dogs became infected (Mayrink et al. 1996). In addition, field trials using a fucose–mannose ligand (FML)-vaccine prepared from *L. donovani* have afforded a 92 percent protection among dogs in an endemic area of AVL in Rio Grande do Norte, Brazil, 2 years after their vaccination. The 8 percent of dogs that did acquire infection showed only mild signs of disease with no deaths and, significantly, the number of human cases of AVL in the area decreased from 15 in 1996 to six in 1997 and none in 1998 (da Silva et al. 2001).

Cutaneous and mucosal leishmaniasis

Most inhabitants of the endemic areas are very familiar with the dermal leishmaniases, under their wide variety of local names, but ignorance and negligence are all too frequently to blame for allowing these diseases to reach debilitating or mutilating proportions. The painless nature of the lesions makes them of no great inconvenience in their early stages and this encourages the tendency to wait and see if they will cure spontaneously. Again, as these diseases are predominantly zoonotic, most infections are acquired by those living in rural areas, often long distances from the simplest of medical attention. As a result, a high proportion of cases only seek help when the infection is well advanced and, in regions where the causal agent is frequently *L. (V.) braziliensis*, this may prove disastrous. As for visceral leishmaniasis, health education campaigns can help to indicate the importance of early treatment.

Personal avoidance of cutaneous leishmaniasis is, at present, limited to the use of insect repellents, protective clothing and the avoidance of danger areas, particularly at night when the sandfly vectors are most active. These are precautions that may be feasible for the visiting tourist, but they are not very practical for the shirtless forest-worker who can ill afford to be constantly purchasing insect repellents, who is most comfortable wearing shorts (far less expensive than trousers) and short-sleeved shirts and who has to eke out his living by hunting in the forest at night.

The prevention or control of sylvatic leishmaniasis among gangs of laborers, topographers, and other forestry workers can be effective on a small scale by the following measures: placing the encampments of such men in adequate clearings; spraying the bases of the larger, nearby tree trunks with insecticides (e.g. in areas where the vectors are known to be arboreal – see *Leishmania* (*Viannia*) *guyanensis* Floch, 1954 above); and prohibiting night-time hunting. Destruction of the wild animal reservoirs of sylvatic leishmaniasis is clearly neither practical nor desirable. Finally, knowledge of the ecology of a vector can sometimes help in preventing acquisition of the disease under certain circumstances. Thus, *Psychodopygus wellcomei*, an important vector of *L. (V.) braziliensis* s.l. in the highland forests of Pará, north Brazil, is highly anthropophilic and attacks humans not only at night but also frequently during the day. Field studies have shown, however, that this sandfly is only active for about 6 months of the year, during the rainy season (November–April) and that it enters into diapause in the dry season during the rest of the year, when adult flies are rarely seen. The area in question is one of intense human activity due to the mining of iron ore and other minerals and the incidence of cutaneous leishmaniasis has been very high among those clearing the primary forest. Planning such work for the dry season clearly avoids contact with the sandfly vector.

Although drastic ecological changes such as deforestation and the planting of non-indigenous pine, gmelina, and eucalyptus trees might lead to unfavorable conditions for the enzootics of some species of *Leishmania*, it can actually encourage others. Thus, the creation of vast monoculture plantations of non-indigenous trees for paper pulp production in north Brazil may eliminate cutaneous leishmaniasis due to *L. (V.) guyanensis* in the immediate areas, as neither the major reservoir host (the two-toed sloth) nor the sandfly vector (*Lu. umbratilis*) find this new environment suitable. On the other hand, the wild rodent and marsupial hosts of *L. (L.) amazonensis* and the sandfly vector, *Lu. flaviscutellata*, find it ideal.

In those parts of Latin America where vector species have adapted to a peridomestic or domiciliary habitat, the use of insecticides is clearly indicated. In the absence of firm evidence that domestic animals with leishmanial skin lesions offer a source of infection to sandflies, it would seem unwise to recommend their destruction, even in the unlikely event of their owners' consent. Equines respond well to antimonial treatment.

Direct transmission risks

Cases of congenital transmission of visceral leishmaniasis have been reported (Bialek and Knobloch 1999; Knobloch et al. 2001) but would appear to be rare.

It has been estimated that in some endemic areas infection with leishmanial parasites may be as high as 70 percent of the population, and data on patients with *Leishmania*/HIV co-infection have indicated that viable parasites may persist in individuals long after initial infection, irrespective of the presence or absence of primary disease (WHO 1998). Leishmanial antibodies have been detected in 9 percent of a group of blood donors in the city of Natal, Rio Grande do Norte, Brazil, and in 37 percent of patients undergoing hemodialysis in the same locality (Luz et al. 1997); the latter figure was significantly higher than in any other group, including inhabitants in AVL foci on the outskirts of the city! The importance of transfusion transmission of visceral leishmaniasis in Europe has been clearly indicated in non-endemic regions where the sandfly vector is absent (Mauny et al. 1993). With increases in HIV infection and organ transplant surgery involving prolonged use of immunosuppressants, occult infections and contaminated blood or organs become life-threatening risks necessitating special precautions (Schulman 1994).

TREATMENT

Of the various drugs discussed for the treatment of leishmaniasis (see Old World leishmaniasis; Lainson 1982a; Amato et al. 1996; de Carvalho et al. 2000), the pentavalent antimonials are those of first choice in dealing with the neotropical leishmaniases. It is of historical interest that the first use of antimony to treat leishmaniasis was in Brazil. A young clinician, Gaspar Vianna, impressed by the effectiveness of tartar emetic (antimony potassium tartrate) in the treatment of African trypanosomiasis (see African trypanosomiasis), realized its potential against the related organism *Leishmania* and, in 1912, published his spectacular results obtained with this drug in advanced cases of mucocutaneous leishmaniasis (Vianna 1912). There followed development of the somewhat less toxic trivalent antimonials, which still produced unpleasant side-effects, and then the much better-tolerated pentavalent antimonials which, some five decades later, are still the clinician's principal armaments. Even so, these latter, more refined antimonials may sometimes show some cardiotoxicity (Ribeiro et al. 1999).

American visceral leishmaniasis

Treatment usually follows that given in Old world leishmaniasis. Unresponsiveness to the recommended antimonial dosage schedule, however, has been noted in some AVL patients from Bahia, Brazil. In such cases, there is little alternative other than elevating the dosage or using second-line drugs such as pentamidine. In this manner, Bryceson et al. (1985) cured four of ten unresponsive patients with Kenyan kala azar, but all ten suffered serious side-effects from both stibogluconate and pentamidine at the dosage levels used. Liposomal amphotericin B (AmBisome®) is recommended for the treatment of visceral leishmaniasis by the US Food and Drug Administration (Meyerhoff 1999).

Cutaneous leishmaniasis

Once again, the treatment is much the same as that for Old World cutaneous leishmaniasis, using the pentavalent antimonials. Many clinicians prefer, however, to use the intravenous route for both pentostam and Glucantime, rather than intramuscular inoculation.

In general, lesions due to the neotropical leishmanias tend to much greater chronicity than those of the Old World and there is not such a ready response to treatment. There is no justification in delaying treatment in anticipation of an early spontaneous cure, as recommended with *L. (L.) major* or *L. (L.) tropica* infections, or limiting treatment to intralesional injection of drugs or the topical application of these in ointments. The following hazards must be considered: subsequent mucosal disease due to *L. (V.) braziliensis*; ADCL due to parasites of the *mexicana* complex; and multiple lesions following lymphatic or hematogenous spread on the part of *L. (V.) guyanensis*. Treatment should, therefore, be systemic and immediate and identification of the causative parasite is most important. If it proves to be *L. (V.) braziliensis*, treatment should be particularly intensive.

Effectiveness of the pentavalents Pentostam® and Glucantime may vary considerably, not only when dealing with different parasites, but in treating different patients infected with the same organism. Some patients with simple lesions due to *L. (V.) guyanensis*, for example, may be cured by a single course of treatment, whereas others may require three or four. In some cases of poor response, recourse must be made to other drugs, or combinations of drugs, in spite of their greater toxicity. The most commonly used second-line drug is pentamidine, which has been used routinely by French workers in treating cases due to *L. (V.) guyanensis* in French Guyana.

Treatment of patients with the antimalarial drug mefloquine has produced conflicting results (Gómez et al. 1996; Hendrickx et al. 1998; Laguna-Torres et al. 1999).

Mucocutaneous and mucosal leishmaniasis

Advanced cases are difficult to treat, with slow response to the pentavalent antimonials. The high dosage recommended (20 mg Sb^{5+} per kg body weight, given in a single daily injection for a mean of 30 days, or until no evidence of activity of the lesion has been noted for a week) may sometimes produce pronounced side-effects, so that treatment has to be suspended (Marsden 1986).

The considerably more toxic amphotericin B and pentamidine are used only when there is failure to respond to the pentavalent antimonials. In some patients, the early treatment provokes a severe inflammation around the lesion, presumed to be caused by antigen released from killed parasites. Although this reaction is regarded as a favorable prognostic sign, it may prove highly dangerous in patients with laryngeal or tracheal lesions. It is recommended that corticosteroids be used as a prophylactic measure when treating such cases and that there should be a gradual elevation of the drug dose (Marsden 1986). Successful treatment of mucosal leishmaniasis in patients unresponsive to Glucantime has been achieved with liposomal amphotericin B (Sampaio and Marsden 1997).

Another difficulty confronting the clinician is in evaluating the effectiveness of treatment. What are the criteria of cure? Serological monitoring has used complement fixation and indirect fluorescent antibody tests, and it has been suggested that cure is indicated by decline and disappearance of antibody titers (Walton 1987). This method is likely to prove the most useful, provided that measurements of leishmanial antibody can be standardized. Both parasitological and histological examination entail biopsy trauma to the treated lesion and may reactivate a lesion that seems to be healed but still contains parasites. Serological follow-up should accompany testing for antigen: a negative antigen test together with reduced titer in serological tests indicates a lower likelihood of relapse (Amato et al. 1998), but an inflammatory process may persist, even in these patients, and could indicate continued presence of amastigotes.

Mutilation following the mucosal lesions may be so extreme that even after cure the patient is ostracized and unable to lead a normal life. Plastic surgery is helpful, but only when there is no doubt of cure. Walton (1987) cites the case of one patient, with apparently complete healing, who underwent cosmetic surgery to reconstruct his nose: 'The results were disastrous, with widespread reactivation along the surgical wounds and, in the patient's words – the new nose fell off!'.

Immunotherapy, utilizing vaccines with or without BCG (Convit et al. 1987, 1989; Mayrink et al. 1991) has proved effective, but has been shown to give much the same cure rate as conventional chemotherapy (Convit et al. 1987). As pointed out by the latter authors, however, it is considerably cheaper and safer.

Anergic diffuse cutaneous leishmaniasis

A smooth, fleshy, unulcerated lesion containing very abundant, large amastigotes in a patient with a negative Montenegro skin-test reaction is a danger signal that should prompt immediate, high-dosage antimonial treatment. In the authors' laboratory in Amazonian Brazil, this is virtually diagnostic of an early lesion due to *L. (L.) amazonensis* which, in an individual with a defective cell-mediated immune response (suggested by his negative skin-test), will proceed to ADCL unless adequately treated. Fortunately, although simple, curable skin lesions due to this parasite are quite common, ADCL is relatively rare.

Of all the forms of leishmaniasis, this disease undoubtedly represents the clinician's greatest challenge, because the patient is unable to offer the all-important immunological collaboration necessary for successful drug treatment. Advanced cases of ADCL, with multiple nodular lesions, may respond dramatically to the first antimonial treatment, with complete disappearance of the lesions. Unfortunately, this may be taken to indicate cure, but with the cessation of treatment the nodules reappear some time later, usually more abundantly. Repeated treatment with the same drug may again give good results, but the patient will relapse again and the response steadily diminishes until the treatment is virtually ineffective. Similar results may be obtained with a variety of other drugs, until the list is exhausted and the patient's situation becomes desperate.

In patients with ADCL due to *L. (L.) amazonensis*, very hot baths taken daily, together with periodic chemotherapy, have been found to reduce the size of the nodules substantially, giving the patient a much improved appearance over periods of many years (Lainson 1982a). More recently, immunochemotherapy has been used with considerable success in the treatment of persons suffering from ADCL in Venezuela (Convit et al. 1989). 'Marked clinical improvement' was observed in nine out of ten patients given intradermal injections of a mixture of heat-killed promastigotes of '*L. mexicana amazonensis*' (*L. (L.) pifanoi*?) isolated from a Venezuelan case of ADCL, plus 'variable amounts' of BCG, together with the standard Glucantime treatment. A similar treatment of Brazilian ADCL patients infected with *L. (L.) amazonensis*, however, has met with limited success (F.T. Silveira, unpublished observations). Treatment with an association of Glucantime and paramomycin (Gabbrox, via oral) has been found useful (Costa et al. 1999).

VACCINATION

Immunology of infectious diseases in general is discussed in Immunology and immunopathology of human parasitic infections. With regards to leishmaniasis, this is just as well, because a detailed review of the extensive literature on this subject over the past few years would fill many more pages here than space allows.

The pioneer work of Mayrink and collaborators in Brazil, on the production of a vaccine against American cutaneous and mucocutaneous leishmaniasis (reviewed by Genaro et al. 1996), and that of Convit and coworkers in Venezuela on the use of vaccines in immu-

notherapy (reviewed by Convit 1996) have triggered a veritable explosion of studies directed towards the production of vaccines

In early trials, the Mayrink vaccine was a mixture of killed, sonicated promastigotes of *L. (L.) amazonensis*, *L. (V.) braziliensis*, and a *Leishmania* of doubtful identity, without the use of an adjuvant. More recent versions have been a cocktail of promastigotes of *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (V.) guyanensis*, and *L. (V.) braziliensis*, with or without *Corynebacterium parvum* as an adjuvant. While apparently still some way from offering complete protection, the vaccine at least has the distinction of being the only one commercially available (Leishvacin®), and it is to be hoped that continuing studies will improve its efficacy. The protective efficacy of a similar vaccine against cutaneous leishmaniasis in Ecuador is claimed to be 72.9 percent (Armijos et al. 1998).

The immune response of volunteers vaccinated with BCG plus killed promastigotes of *L. (L.) mexicana* in Venezuela indicated the vaccine to be potentially protective for the majority of the vaccinees (Sharples et al. 1994), and follow-up studies showed an immune response in more than 85 percent of the vaccines as indicated either by skin-test conversion, lymphocyte proliferation or interferon-γ production.

Kenney et al. (1999) used rhesus monkeys to assess the safety, immunogenicity, and efficacy of a vaccine prepared from heat-killed *L. (L.) amazonensis* promastigotes combined with recombinant human interleukin-12 (rhIL-12) and aluminum hydroxide gel as adjuvants. Challenge with 10^7 metacyclic *L. (L.) amazonensis* promastigotes 4 weeks after vaccination demonstrated complete protection in 12 monkeys that received 2 μg rhIL-12 with alum/antigen. The safety and efficacy of this vaccine in monkeys suggest a basis for human trials.

Handman (2001) gives a useful update of work on killed and live, attenuated vaccines; recombinant vaccines; synthetic peptides; nonprotein antigens; and 'naked' DNA vaccine. Transmission-blocking vaccines are discussed by Tonui (1999).

CONCLUDING REMARKS

We have discussed the possible origin of the parasitic Kinetoplastida, the family Trypanosomatidae, and the genus *Leishmania* elsewhere (Lainson and Shaw 1987). It is the general opinion that the trypanosomatids have their origin in monogenetic intestinal flagellates of invertebrates and that they subsequently adapted to spend a part of their life-cycle in vertebrates. Thus, it is more correct to consider the phlebotomine sandfly as the primary host of *Leishmania* species, rather than the vertebrate hosts that merely function as reservoirs of infection for the sandfly (Lainson 1997). The finding that promastigotes may undergo a form of conjugation (Lanotte and Rioux 1990), with possible exchange of nuclear material, supports this hypothesis on the reasonable assumption that such a process is more likely to take place in the definitive or primary host of a heteroxenous parasite.

It is difficult to assess the specificity of the *Leishmania* species in their sandfly hosts and unwise to base one's conclusions on the results of laboratory experiments, when unnaturally large numbers of amastigotes or promastigotes, fed to laboratory-bred flies, may well overwhelm the natural resistance of a nonvector species. In nature, however, there is considerable evidence suggesting the limitation of the life-cycle of most leishmanial parasites to specific sandfly vectors. Thus, *Lu. longipalpis* is the major vector of *L. (L.) chagasi* throughout the whole geographical range of this parasite; the closely related *Lu. olmeca olmeca* and *Lu. flaviscutellata* are the only confirmed vectors of *L. (L.) mexicana* and *L. (L.) amazonensis* respectively, in Central and South America, in spite of the presence of many other species of sandflies known to feed on rodents in the endemic areas of these two parasites. As far as is known, *Ps. wellcomei* is the sole vector of *L. (V.) braziliensis* s.l. in the Carajás highlands of Pará and *Lu. umbratilis* is the major vector of *L. (V.) guyanensis* throughout its geographical distribution, again in spite of the presence of a large number of other species of sandflies. On the other hand, because some sandfly vectors feed on a variety of mammalian hosts in nature, the *Leishmania* of a given species of sandfly may sometimes be isolated from a variety of mammalian hosts sharing the same habitat. *Lu. flaviscutellata,* for example, is a low-flying sandfly and transmits *L. (L.) amazonensis* to a number of predominantly terrestrial rodents and marsupials; canopy and tree trunk-dwelling sandflies such as *Lu. umbratilis* and *Lu. whitmani* s.l. transmit *L. (V.) guyanensis* and *L. (V.) shawi*, respectively, to arboreal animals such as sloths, anteaters, monkeys, and procyonids.

The number of *Leishmania* species in a given locality will largely be governed by the number of sandfly species, although some sandflies seem to be resistant to infection with this parasite. Thus, we (Lainson and Shaw, unpublished observations) have failed to infect the sandfly *Lu. carmelinoi* with any neotropical *Leishmania* species. The multiplicity of species within the genus is a relatively recent realization and much research is still needed to further our knowledge regarding the diversity, ecology, and taxonomy of the neotropical leishmanias. For the clinician, the continued isolation and characterization of parasites from cases of human leishmaniasis is sufficient to indicate the spectrum of *Leishmania* species commonly infecting humans in a given area. The parasitologist's interests, however, are much wider and include the wild mammalian reservoir and sandfly vectors of those parasites infecting humans, as well as the possible existence of other *Leishmania* species that rarely, if ever, infect humans.

The number of *Leishmania* species in the neotropical region is anybody's guess and some idea of it will only be gained when sufficient numbers of mammalian and sandfly species have been examined. Clearly, it is easier and more economical to concentrate simply on the sandfly population, a truly gigantic task in itself considering that nearly 400 different species of these insects have been identified in the Americas (Young and Duncan 1994). The Amazon Region has already provided us with almost half of the recognized species of neotropical leishmanias and doubtless this great forest will continue to provide us with many more!

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African trypanosomiasis

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INTRODUCTION

African trypanosomiasis, or sleeping sickness, is a human disease caused by *Trypanosoma brucei rhodesiense* and *T. b. gambiense* that inhabit the fluid compartments of blood, lymph, and interstitial fluids of their human hosts (Figure 1). The parasites belong to:

Kingdom Protozoa
Phylum Euglenozoa
Class Kinetoplastea
Order Trypanosomatida
Genus *Trypanosoma* Gruby, 1843
Section *Salivaria*
Subgenus *Trypanozoon*
Species *Trypanosoma brucei*

This pedigree, distilled from more than one and a half centuries of observation by systematists, signifies that in addition to causing sleeping sickness in people, the parasites are single-celled eukaryotes with a single flagellum, no chloroplast, a conspicuous aggregate of DNA located near the flagellar kinetosomes in a single mitochondrion, and that they are transmitted from the anterior station, i.e. the proboscis, of tsetse flies.

Sleeping sickness is endemic to sub-Saharan Africa and arises within the habitat of tsetse flies (Genus *Glossina*, Order Diptera), which are the primary vectors of African trypanosomes and in which trypanosomes undergo cyclic development. Tsetse flies are constrained by temperature and humidity boundaries that are met within vast swaths of the humid and semi-humid zones of Africa. They infest a land mass of about 10 million square kilometers ranging from the southern boundary

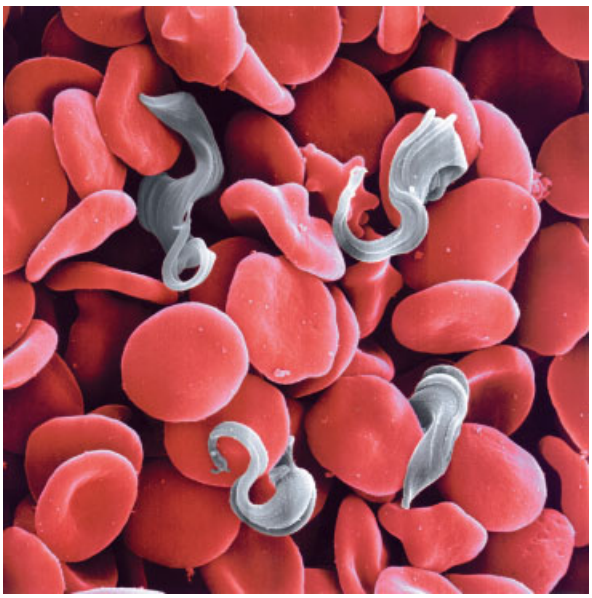

Figure 1 Trypanosoma brucei *in a typical habitat within a mammal. The parasites inhabit the extracellular compartment of blood and tissue fluids and are shown here among red blood cells in a smear of infected blood. (Courtesy of Jürgen Berger and Dr Peter Overath, Max Planck Institute for Developmental Biology, Tübingen)*

of the Saharan desert to around 20° south of the Equator. Within this region, the *Morsitans* subgenus inhabits the savanna, the *Palpalis* subgenus inhabits riverine valleys, the *Austeni* subgenus inhabits thickets, and the *Fusca* subgenus inhabits the forests. All subgenera of *Glossina* transmit African trypanosomes but *T. b. gambiense* is mainly transmitted by the *Palpalis* subgenus and *T. b. rhodesiense* by the *Morsitans* subgenus.

Some species of trypanosomes (see below) infect cattle and other domesticated animals causing a wasting disease, nagana, which costs producers about US$3 billion each year in lost productivity. Some environmentalists see nagana as a disease barrier that protects Central Africa from becoming home to monocultures of grassland and cattle. To others, it is a garrote strangling the capacity of sedentary agriculturists in Central Africa to establish profitable integrated agricultural systems. Both points of view have merit. Overgrazing and environmental degradation are prevalent in some grazed regions abutting the Sahara and could spread south if nagana was no longer a problem. On the other hand, the inability to keep cattle in Central Africa because of trypanosomiasis prevents that region from following successful models of agrarian development established elsewhere.

The importance of trypanosomiasis to human and livestock health in Africa was recognized early in the period of European colonial expansion in Africa (Ford 1971). Various historical accounts of the early work on the epidemiology, the control measures, and the social and economic consequences of trypanosomiasis are available (Duggan 1970; Ford 1971). By the late-1950s, human trypanosomiasis had been contained through vector control, vector avoidance, and a combination of diagnosis and treatment through chemotherapy. Despite this early success, the disease is now a serious public health problem in much of sub-Saharan Africa. The breakdown of public health programs and, as a consequence, a reduction of disease surveillance and treatment, appear to be key factors in disease resurgence. The incidence of sleeping sickness has now reached epidemic proportions in Angola and the Democratic Republic of Congo (formerly the Republic of Zaire) and is high and increasing in Uganda and Sudan. Only about 10 percent of people at risk are screened for trypanosomiasis and of these only a small proportion are treated. It is estimated that 300 000–500 000 people have sleeping sickness, and in some areas the incidence of disease exceeds that of acquired immune deficiency syndrome (AIDS). The incidence of trypanosomiasis is unlikely to decline in the near future unless aggressive diagnosis and treatment regimes are re-established and efforts made to reduce the prevalence of tsetse flies and to limit their contact with people. The World Health Organization is working to achieve these goals by re-enacting earlier successful control programs. These include:

- the assembly of mobile medical teams equipped with the latest diagnostic capability and treatment drugs
- survey of the population at risk with subsequent provision of chemotherapy
- establishment of local health centers to provide long-term care and post-therapy follow up
- motivation of rural communities to assume responsibility for vector control programs and health care initiatives.

This is all timely but vulnerable to unstable sociopolitical conditions. Unfortunately, there are no alternatives: neither long-lived chemoprophylactic treatments nor effective vaccines have been developed.

CLASSIFICATION

There are several species of African trypanosomes distributed through four subgenera: subgenus *Duttonella* (*D*), *Nannomonas* (*N*), *Trypanozoon* (*T*) and *Pycnomonas* (*P*).

Order Trypanosomatida
  Family Trypanosomatidae
    Genus *Trypanosoma*
    Section Stercoraria
    Subgenus *Schizotrypanum*
      Species *T.* (*S*) *cruzi*
    Section Salivaria
    Subgenus *Duttonella*
      Species *T.* (*D*) *vivax*
    Subgenus *Nannomonas*
      Species *T.* (*N*) *congolense*
      Species *T.* (*N*) *simiae*
    Subgenus *Trypanozoon*
      Species *T.* (*T*) *brucei*
      Subspecies *T.* (*T*) *b. brucei*
      Subspecies *T.* (*T*) *b. rhodesiense*
      Subspecies *T.* (*T*) *b. gambiense*
      Subspecies *T.* (*T*) *equiperdum*
      Subspecies *T.* (*T*) *evansi*
    Subgenus *Pycnomonas*
      Species *T.* (*P*) *suis*

The subgenera and species are distinguished on morphologic, isoenzyme, molecular diagnostic, and host-specificity criteria. The sub-genus *Trypanozoon* contains the human infective trypanosomes and is considered to be 'in the midst of a period of rapid evolution at the present time, which makes it difficult for systematists to divide it up neatly into distinct species' (Kreier and Baker 1987). There are two groups of *Trypanozoon* organisms. The first, referred to as the *T.* (*T*) *brucei* group, includes the human infective pathogens *T. b. gambiense* and *T. b. rhodesiense*, and a morphologically identical subspecies, *T. b. brucei*, that does not infect humans. All members of the *T.* (*T*) *brucei* group are transmitted by tsetse flies. Their taxonomic relationships are further discussed below based on molecular characterization and sensitivity to human serum. Kreier and

Baker (1987) use the term *T. brucei* s.l. (= sensu lato, in the broad sense) to describe the *T.* (*T*) *brucei* group. This usage is awkward and in the rest of this chapter when we refer to *T. brucei* without any further qualification it should be taken to mean *T. brucei* s.l.

The second group of *Trypanozoon* species is comprised of *T. evansi* and *T. equiperdum*. These parasites are not cyclically transmitted by tsetse flies. Their taxonomic relationship with each other and with their putative parent species *T. brucei* is unclear. It is thought that they evolved from *T. brucei* organisms that had been carried beyond the northern boundary of the tsetse habitat by infected camels and subsequently 'adapted to noncyclical transmission by biting Diptera and so survived and underwent speciation on their own' (Kreier and Baker 1987). *T. evansi* is widely spread through North Africa, Asia, and South America. Camels, cattle, water buffalo, horses, and dogs are common hosts. *T. equiperdum*, a parasite of horses and donkeys, is morphologically identical to *T. evansi*. It is not dependent on an invertebrate vector but accumulates in inflammatory sites on the skin, including the genitalia that are abraded during coitus thus permitting transfer of the parasites to uninfected breeding partners.

Trypanosoma b. brucei, which is morphologically indistinguishable from the human-infective *T. brucei* subspecies, does not infect humans but does infect cattle and, together with *T.* (*D*) *vivax* and *T.* (*N*) *congolense*, causes nagana, a fatal disease of these livestock. *T. b. brucei*, *T. congolense*, and *T. vivax* are widely distributed throughout the tsetse fly habitat and their presence excludes cattle from all but the fringes of it.

The *T.* (*T*) *brucei* complex and human infectivity

Members of the *T.* (*T*) *brucei* complex, *T. b. brucei*, *T. b. rhodesiense*, and *T. b. gambiense*, share phenotypic characteristics including their morphology, life cycle, and major biochemical features. However, there is significant intraspecific variation evidenced by differences in isoenzyme electrophoretic patterns and in restriction fragment length polymorphisms (RFLP). Cluster analysis of data on isoenzyme variation show that the African trypanosomes can be placed into a number of different principal zymodeme groups. *T. b. gambiense* trypanosomes are fairly homogenous by zymodeme analysis and can be distinguished by this method from *T. b. rhodesiense* and *T. b. brucei* (Stevens and Godfrey 1992). *T. b. gambiense*, *T. b. rhodesiense*, and *T. b. brucei* can be distinguished on the basis of RFLP fingerprints (Hide et al. 1994, Agbo et al. 2002) but intra-subspecies variability exists reducing the usefulness of this test. Evaluation of *Trypanozoon* organisms using a repeated coding sequence and microsatellite markers (Biteau et al. 2000) has revealed great heterogeneity in genotype related to these sequences; however, group-specific genotypes, or alleles, were evident and a *T. b. gambiense* group could be distinguished on the basis of their pattern of reactivity with the probes. Many workers have concluded that the African trypanosomes are evolving rapidly with respect to their population genetics and epidemiology, accounting for the complex variations in isoenzyme and RFLP patterns found among various isolates from tsetse, different host groups, and distinct geographical locations.

T. b. gambiense and *T. b. rhodesiense*, but no other African trypanosomes, are infective for humans. Infections initiated with *T. b. rhodesiense* are more acute than those initiated with *T. b. gambiense* although both are fatal if left untreated. *T. b. rhodesiense* and *T. b. gambiense* also have differing geographic distributions: the former are prevalent in the Eastern half of the African continent within the tsetse habitat, and the latter are prevalent in West and Central Africa. The two groups meet and overlap around the Northern and Eastern shores of Lake Tanganyika.

T. b. gambiense and *T. b. rhodesiense* can be incubated in human serum with no ill effects. In contrast, *T. b. brucei* is lysed by incubation in human serum and is not infective to humans. *T. vivax* and *T. congolense*, which also are not infective for humans, are similarly lysed when incubated in human serum (Black et al. 2001) and it is likely that the other trypanosome species that do not infect humans will be equally sensitive. Lysis in human serum provides a simple, although only partly reliable, method of distinguishing *T. b. brucei* from the human infective *T. brucei* subspecies (Rickman and Robson 1970; Robson and Rickman 1978). The test can record false negatives because human-infective *T. brucei* can reversibly give rise to forms sensitive to human serum when freed from the selective pressure of human serum. Furthermore, individual human serum samples vary with respect to their lytic activity on sensitive *T. brucei* subspecies and a given sample can have higher activity against one or another isolate.

Two species of trypanolytic material have been isolated from human serum, a very high density lipoprotein (VHDL) subfraction containing apoliproteins A-I, A-II, C-I, C-II, C-III, haptoglobin-related protein (hpr), and paraoxonase-arylesterase (Smith et al. 1995), and a non-lipoprotein component that contains apo AI, hpr, and IgM (Raper et al. 1999). *T. b. brucei* that are incubated with human serum undergo characteristic changes in their morphology, starting with pronounced swelling around the flagellar pocket region. It is considered that uptake of the lytic material results in disruption of an endocytic and most likely a lysosomal compartment. The hpr component of the trypanolytic factors has been proposed as the targeting device, allowing the lytic complexes to bind to, and be endocytosed by, the parasites. However, hpr alone, or in combination with mouse serum components, does not harm the parasites (Hatada

et al. 2002). In a stunning series of experiments, Pays and his group have now shown that apolipoprotein L-1 is the lytic factor in human serum and that this minor component of high density lipoprotein exerts its effect in the presence or absence of hpr (Vanhamme et al. 2003). The apolipoprotein L-1 is endocytosed by the parasites and processed in a lysosome. The mechanism through which endocytosed apolipoprotein L-1 destabilizes the *T. b. brucei* lysosomal membrane has not been resolved.

T. b. rhodesiense express a human serum resistance associated (SRA) gene (De Greef et al. 1989), which, upon transfection into *T. b. brucei*, confers resistance to lysis by human serum (Xong et al. 1998) and which can be used as a molecular confirmation of *T. b. rhodesiense* (Welburn et al. 2001; Gibson 2001a). SRA has been shown by Pays and colleagues (Vanhamme et al. 2003) to encode a lysosomal protein that most likely confers resistance to the lytic activity of apolipoprotein L-1 by reacting with the protein in the lysosome. Unlike *T. b. rhodesiense*, *T. b. gambiense* do not express SRA and the mechanism used by these parasites to resist lysis in human serum has not been resolved. Although many crucial details remain to be resolved as to how human serum lyses *T. b. brucei* and how this process is prevented in other *T. brucei* subspecies, the basic finding that apolipoprotein L-1 is the lytic factor of human serum is of huge significance. This finding may lead to novel transgenic solutions to nagana as well as to the development of therapeutic peptides for *T. b. rhodesiense*-sleeping sickness.

STRUCTURE AND LIFE CYCLE

Kinetoplast

Trypanosoma brucei are spindle shaped protozoa about 20 μm in length and 1–2 μm in width. The parasites have the common features of eukaryotic cells, namely a lipid bilayer plasma membrane that encloses a number of membrane-bound organelles including the nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, and endosomal vesicles (Figure 2). They have a single flagellum that emerges from an invagination in the plasma membrane called the flagellar pocket. African trypanosomes also have a number of special organelles. The most notable of these is a mass of mitochondrial DNA called the kinetoplast from which the Class Kinetoplastea gains its name and, as a consequence, the group as a whole is frequently referred to as kinetoplastid flagellates. The kinetoplast is a network of DNA arranged as a flask-shaped body. It is located within the organism's single mitochondrion at the posterior end of the parasite, close to its flagellar pocket. The kinetoplast is composed of a few identical maxicircles each of 20–40 kb intertwined with thousands of minicircles of 0.5–3 kb. The interlinked nature of maxicircle and minicircle DNA in the kinetoplast presents unique problems with respect to replication of this body and has been the subject of extensive investigation (reviewed in Morris et al. 2001). The kinetoplast maxicircles house mitochondrial genes, some of which require extensive editing at the mRNA level (discussed later) to yield functional proteins.

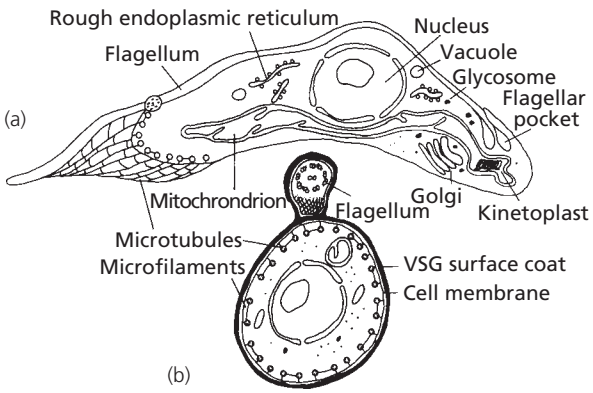

Figure 2 *Ultrastructure of an African trypanosome:* **(a)** *longitudinal section of an intermediate-to-short stumpy blood trypanosome;* **(b)** *cross-sectional view (Redrawn from Seed and Hall 1992, with permission of Academic Press)*

Glycosome

African trypanosomes and related organisms have glycosomes that are membrane-bound assemblies of enzymes that support glycolysis, purine salvage, ether-lipid synthesis, and beta-oxidation of fatty acids. Sequence similarities between genes involved in glycosome and peroxisome biogenesis suggest that these organelles share a common evolutionary origin (Parsons et al. 2001). Enzyme function in the glycosome is considered later in the text.

Variable surface glycoprotein coat and the VSG–glycosylphosphatidylinositiol-specific phospholipase C

African trypanosomes inhabit the blood plasma and, in the case of *T. brucei*, also the interstitial fluids, the lymph, and cerebrospinal fluid. Their extracellular lifestyle presents two unique challenges. First, the parasites must avoid lysis by serum complement factors, a group of enzymes that can assemble into a donut-shaped protein complex on permissive lipid bilayer membranes, leading to loss of the membrane permeability barrier. Secondly, the parasites must evade immune elimination to establish chronic infections. Protection against complement activation and immune elimination is mediated by the variable surface glycoprotein (VSG) coat. In electron micrographs, the coat appears as an amorphous layer 12–15 nm thick on the outer lipid leaflet of the

plasma membrane. On each trypanosome, it comprises about 10^7 VSG molecules encoded by a single VSG gene. The VSG molecules assemble as non-covalent homodimers that stand proud of the membrane (Figure 3). Each VSG monomer is attached to the outer lipid leaflet of the parasites plasma membrane by a glycosylphosphatidylinositiol (GPI) group containing C14 fatty acid tails (Mehlert et al. 1998; Ferguson 1999). The GPI is covalently bound to the VSG C-terminal carboxyl group and the GPI-anchored VSG is free to diffuse in the lateral plain of the membrane. Immune evasion is achieved by antigenic variation of the VSG coat, a process that entails switching among any of a thousand VSG genes and their recombinants of which only one is expressed at a time. Analysis of crystallized VSGs that shared only 16 percent amino acid sequence identity showed that they have highly conserved secondary and tertiary structure (Blum et al. 1993).

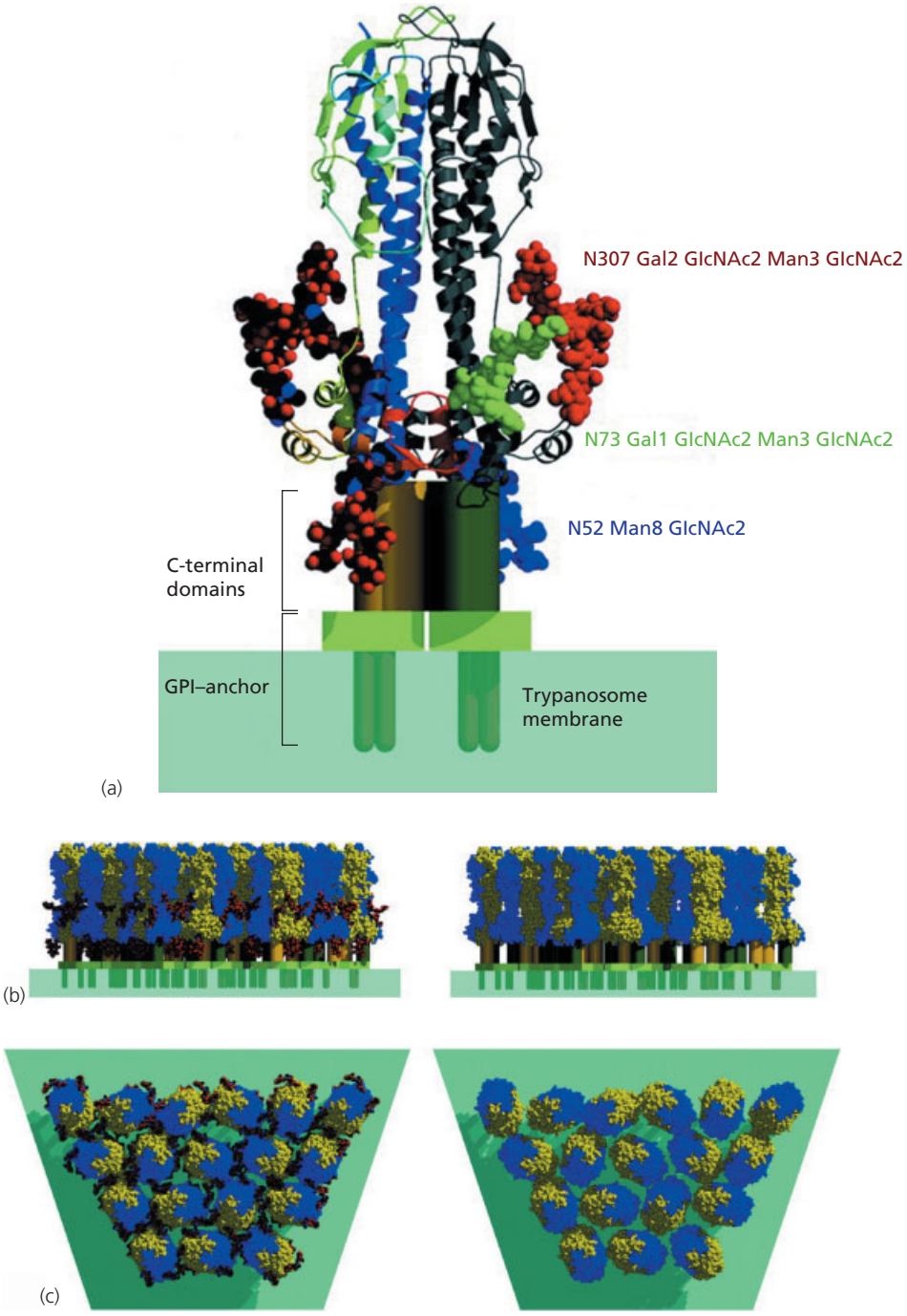

Figure 3 *Molecular models of:* **(a)** *VSG MITat 1.5 with attached, energy minimized, N-linked oligosaccharides;* **(b and c)** *the cell surface coat, viewed from the side and above without (right) and with (left) attached N-linked oligosaccharides (Redrawn from an image by Dr Charles Bond, from Mehlert et al. 2002)*

Consequently, antigenically distinct VSGs assemble into very similar surface coats.

Trypanosomes that are subjected to hypotonic and other stresses release their VSG coat. The released coat lacks its GPI anchor and has an exposed conserved carbohydrate side chain called the cross-reacting determinant (CRD). Release of the VSG is mediated by a VSG–GPI-specific phospholipase (PLC), which is reported to be localized on the cytoplasmic face of vesicles distinct from the endoplasmic reticulum and where it does not normally encounter VSG–GPI. The VSG–GPI-PLC encounters VSG–GPI only under appropriate stress conditions, a process that is thought to involve flipping of VSG from the outer to the inner leaflet of the plasma membrane (Cardoso de Almeida et al. 1999). Conservation of the VSG–GPI-PLC among trypanosomes suggests that it plays an important role in trypanosome biology and/or the parasite–host relationship. However, *T. b. brucei* organisms that are genetically engineered to lack the VSG–GPI-PLC infect and grow in laboratory rodents and tsetse flies. Further studies showed that the organisms do not have a duplicate PLC, suggesting that the VSG–GPI-PLC is not essential for normal *T. b. brucei* function and differentiation events (reviewed in Carrington et al. 1998). However, mice that were infected with the mutant trypanosomes lived for twice as long as those infected with control trypanosomes indicating a role for the enzyme, or for soluble VSG with exposed CRD, in the mammal–trypanosome relationship. The laboratory mouse is not a natural host for *T. brucei*, hence, additional insights into the roles of the VSG–GPI-PLC and released VSG might be gained by studying the behavior of mutant parasites lacking the enzyme in trypanosomiasis-resistant African wildlife species, extant representatives of the hosts in which trypanosomes were selected through evolution.

Additional components of the trypanosome surface

The trypanosome plasma membrane is equipped with transporters for the facilitated diffusion of glucose, pyruvate, and purines. It also has a number of invariant surface glycoproteins (ISG) of unknown function. These are present at about 1 percent of the density of VSG. In addition, the parasite has receptors for host-derived holo- (i.e. iron-loaded) transferrin and serum, and low- and high-density lipoproteins (reviewed by Borst and Fairlamb 1998). The transferrin receptor is encoded by VSG gene expression site associated genes (ESAG) 6 and 7 for which there are several alleles (Steverding 2000). As with VSGs, only one form of the transferrin receptor is expressed at a time by trypanosomes. Although isolation of a receptor for serum low-density lipoprotein has been reported, this was most likely an erroneous assignment (reviewed in Black et al. 2001) and at this time the trypanosome receptors for serum low and high density are not known.

Flagellum and flagellar pocket

African trypanosomes are highly motile. They have a single flagellum, which emerges from an invagination at the posterior end of the cell called the flagellar pocket (Balber 1990) (Figure 4). The structural elements that cause the invagination to form have not been defined. The flagellum comprises an axoneme, a paraflagellar rod, and an attachment zone filament that mediates adhesion to the trypanosome plasma membrane. The flagellum lies in a shallow groove on the cell surface. When the flagellum beats, the attached portion of the cell surface is thrown into folds and gives rise to the undulating membrane visible in the light microscope. The body of the trypanosome has an underlying corset of microtubules that prevent any vesicular exchange with the plasma membrane. This microtubular array is open in the region of the flagellar pocket and at the posterior end of the cell. Although vesicles could transit to or from the cell membrane at either of these zones, endocytosis and exocytosis are restricted to the flagellar pocket region. Bloodstream stage *T. brucei* have much more endocytic activity than other differentiative stages. This is associated with massive upregulation of a clathrin-like heavy chain that is involved in the formation of endocytic vesicles (Morgan et al. 2001).

Trypanosome receptors for host transferrin and serum lipoproteins are thought to be restricted to the flagellar pocket, and to bind ligand that diffuses into the pocket. Indeed, the transferrin receptor has been shown to be restricted to the pocket, at least in organisms that were incubated in the presence of high-affinity transferrin (i.e. donor transferrin) that could bind with high affinity to the receptor. When these same organisms were incubated for 48 hours with low-affinity transferrin, which elevates receptor gene expression, the transferrin receptor was detected over the surface of the parasite. A similar result was obtained using organisms that were engineered to overexpress transferrin receptors and that were incubated in the presence of high-affinity transferrin (Mussmann et al. 2003). These observations were taken to show that the transferrin receptor is held in the flagellar pocket by a saturable mechanism. The transferrin receptor has also been shown to be present in the pocket matrix associated with a gel-like substance. The significance of this dual location is not known. Trypanosome endocytic pathways, endocytic compartments, and sorting signals are the subject of on-going study (Pal et al. 2002). Figure 5 presents a comparison of secretory and endocytic pathways in *T. brucei* and other trypanosomatids. In the case of *T. brucei*, the flagellar pocket and endosomal glycoproteins have been shown to have an attached linear poly-*N*-acetyllactosamine

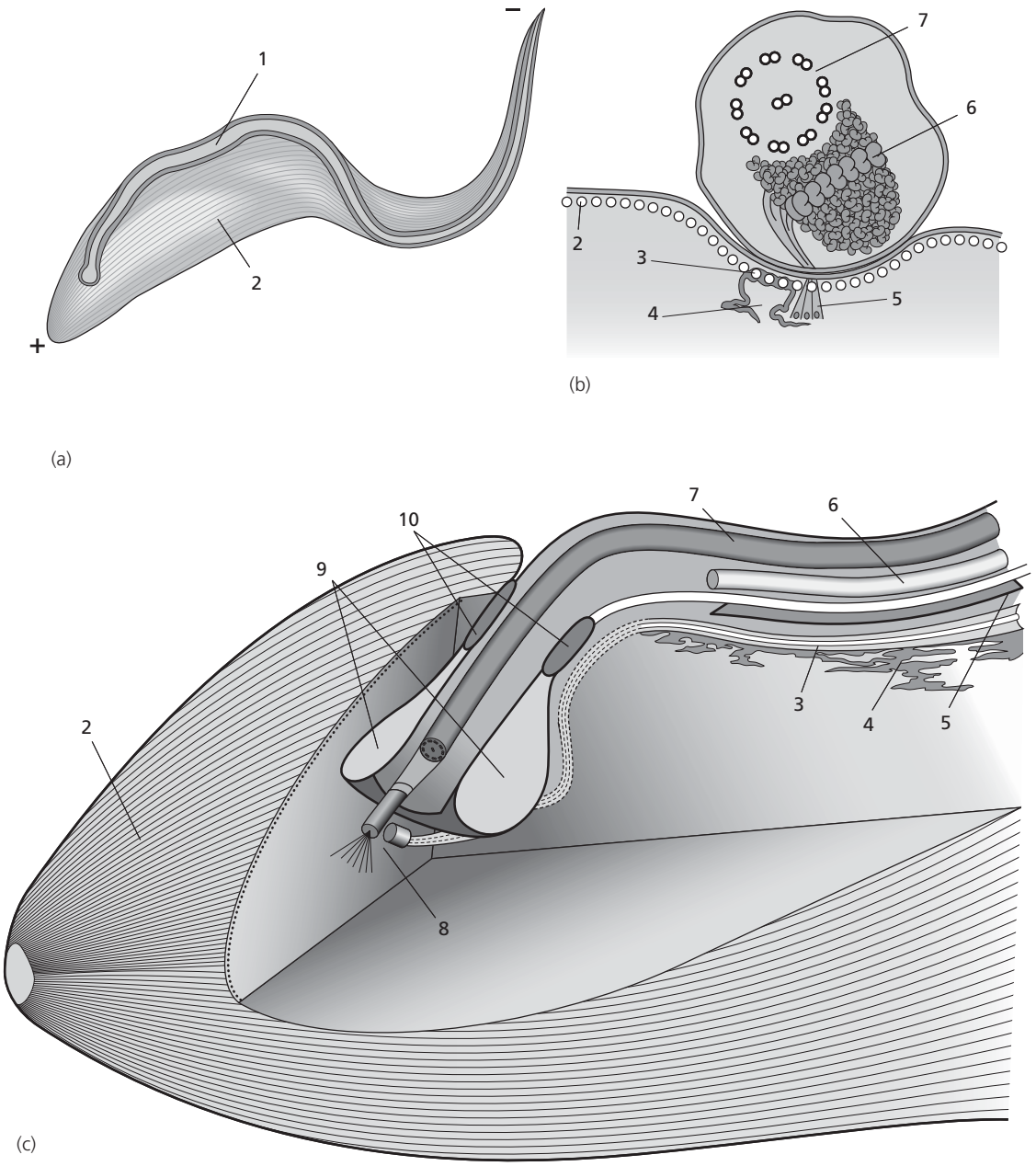

Figure 4 *Schematic illustrations of a trypanosome cell emphasizing the structural components of the cytoskeletion:* **(a)** *detergent-extracted cytoskeleton;* **(b)** *idealized transverse section taken through the midpoint of the cell looking from posterior to anterior;* **(c)** *cut-out view of the flagellar pocket area. Flagellum (1), sub-pellicular microtubules (2), special quartet of reticulum-associated microtubules (3), microtubule-associated reticulum (4), flagellum attachment zone filament (5), paraflagellar rod (6), axoneme (7), basal body complex (8), flagellar pocket (9), adhesion zones (10) (From Hill et al. 2000)*

moiety, a unique carbohydrate that is developmentally regulated and may be required for endocytosis (Nolan et al. 1999).

Pleomorphism

Bloodstream stage *T. brucei* undergo morphological and biochemical differentiation to non-replicating stumpy forms on reaching peak parasitemia. Stumpy forms have a very truncated flagellum and are greater in diameter than the replicating bloodstream forms. This transition plays a significant role in setting the level of parasitemic waves in infected mammals (Black et al. 1985) and is thought to be directed by endogenous quorum-sensing factors and host-derived growth-inhibitors (Murphy and Olijhoek 2001). The slender to stumpy transition occurs via a series of intermediate forms and *T. brucei* strains that undergo this differentiation are referred to as pleomorphic. Strains that are maintained by passage in

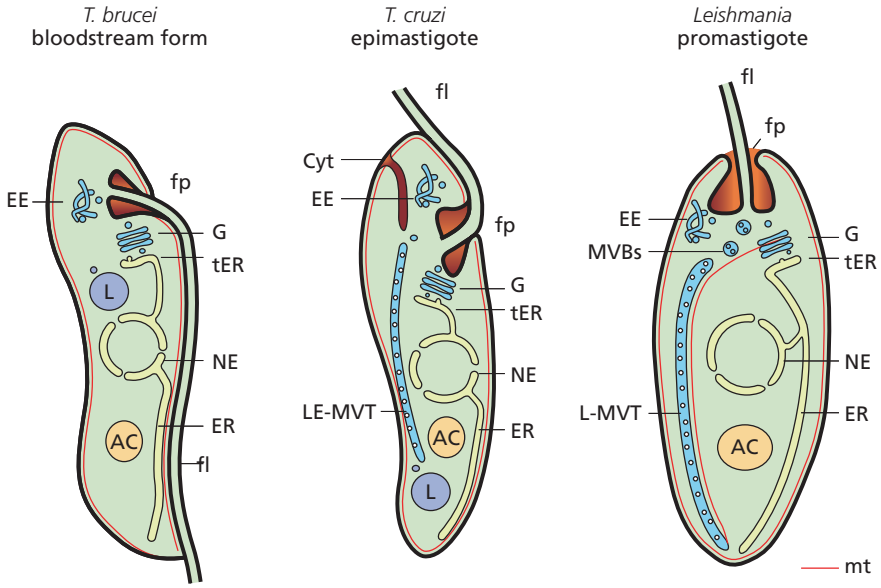

Figure 5 *Schematic representations of the secretory and endocytic organelles of* T. brucei *bloodstream form,* T. cruzi *epimastiogote and* Leishmania *promastigote. Endoplasmic reticulum (ER), transitional endoplasmic reticulum (tER), multivesicular bodies (MVBs), nuclear envelope (NE), Golgi apparatus (G), flagellar pocket (fp), early endosomes (EE), multi vesicular tubule (MVT), lysosome (L), late endosome (LE), cytostome (a second invagination that is specialized for endocytosis, Cyt) flagellum (fl), microtubules (mt) (From McConville et al. 2002)*

laboratory rodents or in tissue culture in the absence of high molecular weight agarose supplements often lose the property of pleomorphism at least with respect to infections that they give rise to in laboratory rodents, i.e. they remain as replicating slender forms in these hosts. These so-called monomorphic *T. brucei* are typically very virulent and poorly or non-infective for tsetse flies.

Slender form *T. brucei* derive energy solely from glycolysis. In contrast, stumpy form *T. brucei* express α-ketoglutarate dehydrogenase, a Kreb's cycle enzyme, and obtain additional ATP by converting α-ketoglutarate, if supplied to them, to succinate. The partial acquisition of mitochondrial function by stumpy form *T. brucei* is considered to be in preparation for establishing an infection in the tsetse vector.

Life cycle

When an uninfected tsetse bites an infected vertebrate, trypanosomes acquired with ingested blood pass through the esophagus, the crop, and the proventriculus until they reach the midgut. In the fly's midgut, the short stumpy forms complete their morphological and biochemical differentiation into procyclic stages. They complete the development of their mitochondrion, cease synthesizing VSG and express a new coat glycoprotein called procyclin (Mehlert et al. 1998), which facilitates survival and growth in the tsetse fly by mechanisms that are not understood (Ruepp et al. 1997). The procyclic form has a fully functional mitochondrion and consequently differs from the slender bloodstream forms in metabolic activity. The procyclic forms are also elongated with respect to the bloodstream-stage organisms and differ from these in the relative position of the kinetoplast and nucleus.

The trypanosomes have a complex life cycle and a low infection rate in their vectors. It has recently been shown that trypanosomes evoke relatively poor protective immune responses in tsetse flies and other factors are being sought with respect to identifying those that influence infectivity (Aksoy et al. 2002). It is known that age, sex, and genotype of the vector, the type of bloodmeal taken by the vector, and many environmental factors can all influence the susceptibility of the tsetse to the trypanosomes (Maudlin and Ellis 1985; Molyneux and Ashford 1983; Otieno et al. 1983; Vickerman et al. 1988).

After a period of growth in the midgut, the trypanosomes undergo a further series of morphological changes, migrate from the midgut into the endoperitrophic space and finally into the tsetse's salivary glands where they develop into epimastigotes. The epimastigote form is not infective for the mammalian host. The epimastigotes attach to the cells of the salivary gland. They divide repeatedly and then transform into nondividing metacyclic forms, which are small, highly motile, short, and stumpy (Figure 6). They have a terminally located kinetoplast but no free flagellum. When mature, the metacyclic forms detach from the salivary gland cells, synthesize a surface coat of the type found on the bloodstream forms, and become infective to the vertebrate host (Vickerman et al. 1988; Vickerman 1989). The signals for these morphological and

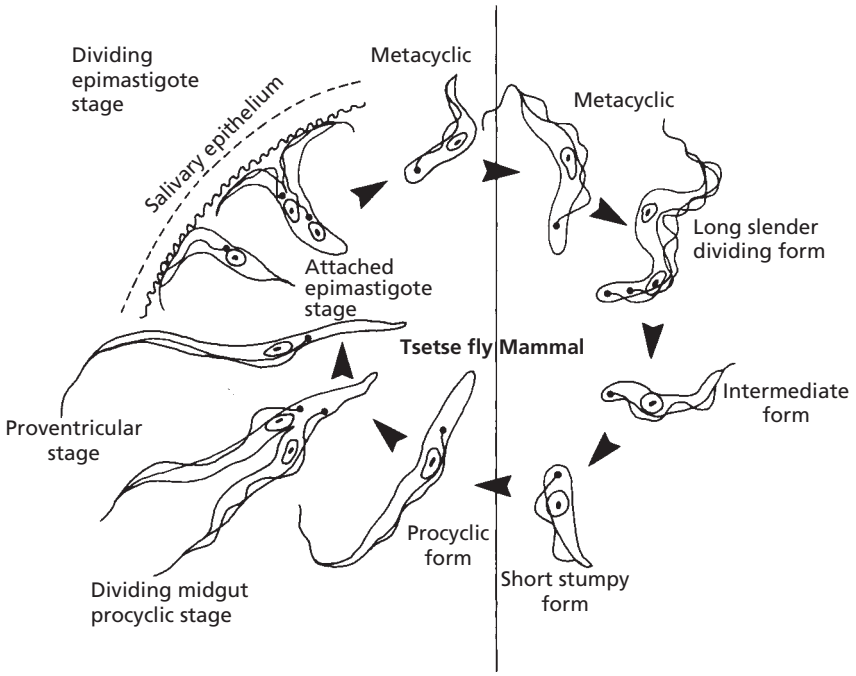

Figure 6 *Life cycle of trypanosomes of the* Trypanosoma brucei *group (Redrawn from Seed and Hall 1992, with permission of Academic Press)*

physiological changes in the trypanosome in its vector are not known. The time required for the trypanosomes ingested by the fly to complete their development in the tsetse and to regain infectivity is approximately 3–4 weeks. However, the length of time will vary depending on the external environmental conditions of humidity and temperature and on the age, sex, and other factors in the tsetse (Molyneux and Ashford 1983). The infective metacyclic forms of the trypanosome are injected into the mammalian host by the biting fly. They transform in the vertebrate host into the long slender trypanomastigote form and increase in number reinitiating the cycle.

BIOCHEMISTRY

In vitro cultivation

The in vitro cultivation of any organism is a prerequisite for any biochemical study and trypanosomes are no exception. Cultivation of procyclic-like *T. brucei* organisms in blood agar medium was achieved early in the 20th century. By the 1970s, semi-defined and defined procyclic culture media had been developed, and by the 1980s, methods were developed for the efficient transformation of bloodstream-stage *T. brucei* into procyclic forms in vitro. Typically, incubations are at 27°C in serum-supplemented medium further supplemented with citrate and *cis*-aconitate (Brun and Jenni 1985; Kaminsky et al. 1988). The signal pathways that are regulated by the drop in temperature and presence of citrate and *cis*-aconitate are not resolved. Procyclic form *T. brucei* can undergo further differentiation in vitro to give rise to mammal-infective metacyclic form trypanosomes (Hirumi et al. 1992). The signaling systems that regulate this differentiation are also not known. Propagation of bloodstream-form *T. brucei* in vitro was achieved in 1977 following a heroic checkerboard comparison involving several media, sera, and fibroblast feeder-layer cells with respect to their *T. b. brucei* growth-supporting capacity (Hirumi et al. 1977). Axenic culture conditions were subsequently established by supplementing culture medium with cysteine or a reducing agent, β2-mercaptoethanol. It is now known that cysteine is an essential growth factor for the parasites (Duszenko et al. 1992). Cysteine is readily oxidized to cystine in the presence of copper, and survival and growth of bloodstream-stage *T. brucei* in vitro is enhanced by inclusion of the copper chelating agent bathocuproine in culture medium. The ability to grow trypanosomes in axenic cultures has greatly facilitated analysis of cell function and biochemistry both by providing controlled experimental conditions, and by expediting the development of mutant organisms through the use of genetic engineering techniques. Gene disruption and addition mutants have been developed, as have organisms expressing inhibitory RNA. These mutant cell lines are a valuable resource in elucidating pathways that regulate endocytosis and exocytosis, VSG switching, and other critical processes.

Not all strains of *T. brucei* grow as bloodstream-form organisms in tissue culture medium, and among those that do there is a high tendency to lose the pleomorphic

phenotype. However, it has recently been found that the inclusion of high molecular weight agarose in the medium facilitates both growth of the parasites in vitro and retention of pleomorphism (Vassella and Boshart 1996). The mechanism underlying the effect is unknown. A serum-free culture medium has also been described for *T. b. brucei* (Hirumi et al. 1997). This medium is supplemented with fatty acid-free albumin and serum lipoproteins, components that are required for progression of the parasites through G_1 of their cell division cycle (Morgan et al. 1996) as well as bovine alpha 2 macroglobulin, which has an undefined function with respect to *T. brucei*. Serum lipoproteins are complexes of apolipoproteins, phospholipids of several types, cholesteryl esters, triglycerides, and cholesterol. It is not known which, or how many, of these components are required to drive progression of the parasites through G_1 of their cell division cycle.

Energy metabolism

The blood stage trypanosomes have a high rate of glucose catabolism. As mentioned above, the glycolytic enzymes carrying out this process are present in a unique microbody-like organelle called the glycosome. An outline of glucose metabolism is shown in Figure 7. Under aerobic conditions, glycerol-3-phosphate is produced from dihydroxy-acetone-phosphate during which process NADH is oxidized to NAD. The glycerol-3-phosphate moves from the glycosome to the parasite's mitochondrion where it is converted back to dihydroxy-acetone-phosphate by glycerol-3-phosphate oxidase. The dihydroxy-acetone phosphate re-enters the glycosome and is further metabolized. Under aerobic conditions, glucose is exclusively converted to pyruvate, which is exported by a pyruvate transporter or transaminated and exported as alanine or utilized in protein synthesis. Under anaerobic conditions, glycerol-3-phosphate cannot be converted back to dihydroxy-acetone-phosphate, hence glucose metabolism results in equal portions of pyruvate and glycerol being produced and exported. In bloodstream *T. brucei* the nine enzymes involved in the conversion of glucose into phosphoglycerate or glycerol account for more than 90 percent of the protein in the glycosome (Hannaert and Michels 1994; Opperdoes 1987; Opperdoes et al. 1990).

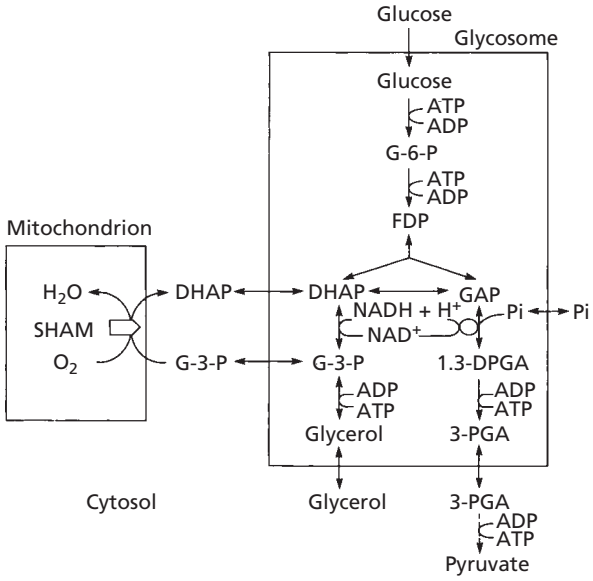

Figure 7 *Glucose metabolism in the bloodstream form of African trypanosomes (From Opperdoes 1987)*

The compartmentalization of the trypanosome's glycolytic enzymes in the glycosomes is a pattern different from that occuring in other eukaryotic organisms where the enzymes involved in glycolysis are present in the cytoplasm. It has been suggested that the close physical association of the glycolytic enzymes in the glycosome and their high concentration there account for the very high rate of glucose catabolism by the blood-stage parasites (Hannaert and Michels 1994; Sommer and Wang 1994). However, model data obtained using a kinetic computer replica of glycolysis in *T. brucei* did not support this idea. Rather, the model suggests that compartmentalization may serve to prevent glycolytic intermediates accumulating to dangerous levels in the trypanosome cytosol, and to facilitate recovery from glucose deprivation (Bakker et al. 2000).

The procyclic forms are adapted to the environment of the vector, which is entirely different from the environment that the blood-forms inhabit. In the procyclic forms, the mitochondrion becomes functional; it has cristae, and contains the tricarboxylic acid cycle enzymes, cytochromes, and cytochrome-dependent respiratory chain enzymes, all of which are absent from the slender bloodstream-forms. The procyclic form has a much lower rate of glucose catabolism than does the slender bloodstream-form. Phosphoenolpyruvate is carboxylated by procyclics to form oxaloacetic acid with subsequent conversion to malate, fumarate, and succinate. The major end products of glucose utilization by the procyclic forms are succinate, acetate, carbon dioxide, and alanine. ATP is generated primarily by oxidative phosphorylation. Amino acids, particularly proline, are the primary substrates used by the procyclics to obtain energy.

Both bloodstream-stage and procyclic *T. brucei* lack key enzymes necessary for classical glyconeogenesis, and both lack the enzymes of the Entner–Douderoff pathway (Cronin et al. 1989).

Redox-regulation

Trypanosomes, like other eukaryotic cells, use NADPH as their major source of reducing power. This is produced in the pentose–phosphate pathway. All the

enzymes of this pathway have been found in *T. b. brucei* (Cronin et al. 1989). Glucose-6-phosphate dehydrogenase, the sentinel that sits at the gateway of the pentose–phosphate pathway has been demonstrated to localize to the glycosome, and the gene encoding the downstream enzyme 6-phospho-gluconolactonase contains a C-terminal peroxisome targeting signal consistent with a likely localization in the glycosome (Duffieux et al. 2000). The trypanosomes possess the enzymes required to produce d-ribose-5-phosphate for nucleic acid synthesis and NADPH for other synthetic reactions. However, there are differences between the procyclic forms and blood-forms in the enzymes of the non-oxidative segment of the pentose–phosphate pathway. Bloodstream-stage *T. brucei* suppress expression of genes encoding ribulose-5-phosphate 3′-epimerase and transketolase (Cronin et al. 1989) and thus terminate their NADPH-yielding pentose–phosphate pathway at ribulose-5-phosphate instead of recycling this product back into the glycolytic pathway. Consequently, the glucose metabolic pathways in bloodstream-stage *T. brucei* appear to be configured to yield ATP or NADPH but not both at the same time. The evolutionary advantage accrued by the trypanosomes through adopting this unusual arrangement is not known.

Trypanosomes spontaneously generate hydrogen peroxide as a result of energy metabolism. They are also exposed to hydrogen peroxide and superoxide anion that are produced by their mammal hosts. The parasites have an Fe-superoxide dismutase that catalizes the reduction of superoxide anion to hydrogen peroxide. However, they lack catalase and rely on coupled redox reactions fueled by the reducing power of NADPH to inactivate hydrogen peroxide (Fairlamb and Cerami 1992; Flohe et al. 1999). The utilization of NADPH deflects glucose-6-phosphate from the glycolytic to the pentose–phosphate pathway by unleashing glucose-6-phosphate dehydrogenase. This may account for the almost instantaneous depletion of the ATP content of bloodstream-stage trypanosomes that occurs when the parasites are placed under oxidative stress (Muranjan et al. 1997). Hydrogen peroxide metabolism by *T. brucei* and other kinetoplastid flagellates involves 'a cascade of three enzymes, trypanothione reductase, tryparedoxin, and tryparedoxin peroxidase… The terminal peroxidase of the system belongs to the protein family of peroxiredoxins, which is also represented in Entamoeba and a variety of metazoan parasites' (Flohe et al. 1999).

Amino acid synthesis

There has been relatively little study of amino acid synthesis and utilization by trypanosomes, but it appears that the organisms have limited ability to synthesize amino acids de novo. A majority of the amino acids required by trypanosomes must either be formed by a salvage pathway such as that bringing about the transamination of pyruvate, oxaloacetic, and α-ketoglutarate to alanine, aspartic, and glutamic acids, respectively, or be directly supplied by the host (Gutteridge and Coombs 1977).

Some amino acids are used for purposes other than protein synthesis. In the procyclic forms, proline is used as a respiratory substrate, and threonine may serve as the preferred substrate for formation of acetyl coenzyme A, which is required for the elongation of fatty acid chains. The only enzymes involved in amino acid metabolism that have received extensive study are those bringing about aromatic amino acid catabolism and those for the use of ornithine in the synthesis of polyamines. The polyamines are known to be important in the proliferation and differentiation of the blood-stage trypanosomes. Inhibition of putrescine and spermidine synthesis blocks trypanosome division and causes changes in cellular morphology (Pegg and McCann 1988).

Purine synthesis

Neither the blood-form nor the procyclic form of the trypanosomes synthesize purines de novo; they require salvage mechanisms to obtain them (Hammond and Gutteridge 1984). Most of the enzymes of the salvage pathway are located in the cytoplasm, and hypoxanthine–guanine phosphoribosyl transferase is found in the glycosome. The trypanosomes can synthesize pyrimidine by a pathway which appears similar to that used by mammalian cells, although there are two differences: (i) in mammals, the enzyme dihydroorotate dehydrogenase is present in the mitochondria but in trypanosomes it is present in the cytosol; (ii) in the trypanosomes, the last two enzymes of the pathway (orotate phosphoribosyl transferase and orotidine 5-phosphate decarboxylase) are in the glycosome rather than in the cytosol as in mammals (Fairlamb 1989).

Lipids

Bloodstream-stage *T. b. brucei* have a limited ability to synthesize lipids and generally depend on the host for fatty acids, phospholipids, cholesterol, and other lipids (reviewed in Mellors and Samad 1989). They obtain phospholipids, lysolipids, cholesterol, and cholesteryl esters from serum low- and high-density lipoproteins (Vandeweerd and Black 1989) and remodel the phospholipids by removing and replacing fatty acids in the sn1 and sn2 positions (Mellors and Samad 1989). The bloodstream-stage parasites also synthesize myristate (Morita et al. 2000), which they preferentially use to remodel the glycosyl-phosphatidylinositol anchor that

holds their glycoprotein coat in the outer leaflet of the plasma membrane.

The trypanosome plasma membrane contains domains with detergent extraction properties shared with the lipid rafts of higher eukaryotic cells. Most of the VSG may be associated with these putative rafts (Denny et al. 2001) and there is evidence that the putative rafts are enriched in the flagellar membrane of the parasites (Toriello et al. 2002).

MOLECULAR BIOLOGY AND GENETICS

The general organization of trypanosome nuclear DNA has been succinctly described by Donelson (2001).

> *Trypanosoma brucei* is a diploid organism with a nuclear haploid DNA content of 35 ± 9 megabase (Mb) pairs depending on the trypanosome isolate. About 15 percent of total cellular DNA is in the kinetoplast... The remaining 85 percent occurs in the nucleus as linear DNA molecules ranging in size from 50 kb to 6 Mb. At least 11 pairs of megabase chromosomes of 1–6 Mb exist that are numbered I–XI from smallest to largest. The two homologues of a megabase chromosome pair can differ in size by as much as 4-fold. Several intermediate-sized chromosomes of 0.2–0.9 Mb and uncertain ploidy are also present... [and there are about 100 minichromosomes]. About 50 percent of the nuclear genome is coding sequence. To date, only one tRNA gene and one protein-encoding gene have been found to contain an intron. [Significant portions of the genome have now been sequenced]... Based on analogy with the *Leishmania* genome, much of the African trypanosome nuclear genome is likely to be arrayed as long transcription units of 50 or more intronless genes.

Chromosomal DNA of all eukaryotes is folded with nucleosomes that are composed of core histones H2A, H2b, H3, and H4. These are highly conserved from yeast to humans but are much more diverse in trypanosomatids. However, there is a degree of sequence conservation suggesting that the overall structure of the *T. brucei* nucleosome is probably similar to that of other eukaryotes (reviewed in Horn 2001). For example, lysine residues are conserved in the N-terminal tails of the core histones of *T. brucei* and higher eukaryotes and may be targets for covalent modifications that affect chromatin structure. Acetylation, phosphorylation, ubiquitination, and methylation of the N-terminal tails of core histones are known to regulate chromatin structure in higher eukaryotes. Nevertheless, chromatin remodeling in *T. brucei* does not fully mimic that in higher eukaryotes. For example, phosphorylation of serine 10 in histone H3 is involved in chromosome condensation in many eukaryotes. However, this amino acid residue is absent from the N-terminal tail of *T. brucei* histone, which may account for the absence of chromosome condensation during trypanosome mitosis.

In higher eukaryotes, nucleosomes form bundles as a result of the binding of linker histone H1 to linker DNA flanking the nucleosome core. *T. brucei* organisms also have linker histone H1 but this has a simple repetitive sequence and is not precisely analogous to that of higher eukaryotes (reviewed in Horn 2001). The level of histone H1 is higher in the bloodstream-forms than in procyclic *T. brucei*, a larger number of H1 variants is expressed and the H1–chromatin association is stronger in the bloodstream-forms (Schlimme et al. 1993) possibly associated with a greater need to stabilize chromatin at the higher temperature encountered in the mammal host.

Sequence information in the *T. brucei* genome database indicates that the parasites also have deacetylases, acetyltransferases, and bromodomain proteins (reviewed in Horn 2001). These proteins affect chromatin by removing groups that covalently modify histones and DNA, and there is evidence that they act as transcription regulators in higher eukaryotes. Perhaps they perform a similar function in trypanosomes. There is evidence that differences in chromatin accessibility may account for developmentally regulated gene expression in *T. brucei* (Navarro et al. 1999).

There is considerable evidence that exchange of genetic information can occur in the African trypanosomes, and that this exchange occurs in the vector's salivary glands, but not in the tsetse gut form or in the mammalian host. The evidence suggests that genetic exchange involves a meiotic division. However, a haploid trypanosome stage has not been identified (reviewed in Gibson 2001b). Genetic exchange has been demonstrated between the various *T. brucei* subspecies, but in the case of *T. b. gambiense* was observed with the relatively virulent so-called group 2 parasites that transmit through the tsetse fly. The less virulent group 1 *T. b. gambiense* do not transmit readily through the fly. The relevance of genetic exchange to the survival and function of *Trypanozoon* organisms is far from clear. In this regard, group 1 *T. gambiense* and *T. evansi*, neither of which are tsetse transmitted, survive quite well without it. Nevertheless, it is apparent from the analysis of isoenzyme patterns, as well as RFLP among different *T. brucei* stocks, that sexual recombination does occur among trypanosomes in the field.

There is debate as to the ploidy of the trypanosomes. The results of isoenzyme and RFLP analysis of genetic crosses between trypanosome clones suggest that the trypanosomes are generally diploid. However, there is evidence to suggest that some hybrids may be triploid and that changes in chromosome size may occur during genetic exchange (Kooy et al. 1989; Gibson et al. 1992). Indeed, 'hybrids with a DNA content of either 2*n* or 3*n*

(but not intermediate amounts) have been found in five of six crosses for which DNA contents have been measured, suggesting that trypanosome DNA is inherited in packets of *n*' (reviewed in Gibson 2001b).

Cell division

In the African trypanosomes, the nuclear membrane does not break down during cell division and chromosomes do not condense. However, as would be expected, chromosomal reorganization does occur as determined by the location of chromosome ends, i.e. telomeres. During interphase, telomeres are located around the periphery of the nucleus in nondividing stumpy form *T. brucei*, and the insect procyclic form, but in the center of the nucleus in dividing bloodstream-forms. The distinct orientation of telomeres in bloodstream-stage trypanosomes may be required for expression of VSG genes as the expression sites are telomeric. At the onset of mitosis, in all trypanosome life cycle stages the telomeres congregate in a central zone of the nucleus and split into two clusters that migrate to opposite nuclear poles. The double-ended nucleus then divides and the telomeres reorganize to the foci present during interphase. During migration, the telomeres are located at the free end of the mitotic spindle, which is contained within the nuclear membrane. The mechanism of chromosome segregation is discussed by Ogbadoyi et al. (2000) and Perez-Morga et al. (2001) and readers are referred to these papers for further information.

RNA editing

RNA editing (i.e. 'the alteration of RNA sequences by base modifications, substitutions, insertions, and deletions') was first described in trypanosomes and has since been identified in other eukaryotes including humans (reviewed in Horton and Landweber 2002). RNA editing in trypanosomes is mediated by guide RNAs that are encoded in the kinetoplast minicircles. These direct the insertion and deletion of U, thus converting mRNA transcribed from so-called 'cryptogenes' into functional gene products. RNA editing can be a cumbersome process sometimes involving multiple guide RNAs, sequentially applied, to edit a single mRNA, and presumably had selective value to be retained. Possible advantageous properties of the process include the stable maintenance of otherwise deleterious mutations in DNA genomes, improved storage of information in crowded genomes, and novel posttranscriptional modes of genetic regulation.

Transplicing

All trypanosome mRNAs are capped with a 39-nucleotide structure that is added at the 5′ end of pre-mRNA by trans-splicing. The totally conserved 39-nucleotide structure, called the spliced leader or mini-exon, is trimmed from an approximately 100-nucleotide nontranslated RNA that is transcribed from a set of nonidentical genes in the trypanosome genome. The regulation of this process is not known. The spliced leader is subsequently attached to each protein-coding sequence present in long polycistronic precursors. This process is an essential step in the biosynthesis of trypanosome mRNAs.

VSG antigenic variation

Trypanosomes contain within their genome over 1 000 different VSG genes. In addition, new variant antigen types (VAT) are created by VSG gene mutations and recombination. With a few special exceptions, each trypanosome expresses only a single VSG on its surface at any given time. This is encoded by a single VSG gene that is expressed at a telomeric expression site. Unexpressed VSG genes are found throughout the trypanosome genome including on minichromosomes and in unexpressed VSG-gene expression sites. There are 20 expression sites for bloodstream-stage trypanosome VSG genes present in the genome, of which only one is active at a time. The promoter for the expressed VSG gene is upstream of and separated from the expressed VSG gene by at least eight ESAGs. Analyses carried out using trypanosomes expressing the AnTat 1.3 VSG show that the ESAGs and expressed VSG are transcribed as one polycistronic unit (Vanhamme et al. 2001). Although only one of the possible 20 bloodstream-stage trypanosome VSG-gene expression sites is fully expressed at a time, transcripts of ESAG 6 from unexpressed expression sites has been recorded (Ansorge et al. 1999). Switching of the expressed VSG gene requires exchange of VSG genes within an active expression site, or silencing of an active expression site and activation of another. Mechanisms of VSG gene switching and expression site silencing are reviewed by Cross et al. (1998) and Borst and Ulbert (2001) and readers are referred to these reviews for further information.

Metacyclic *T. brucei*, i.e. the infective form transmitted from tsetse to the mammal host, are also coated with VSG (Barry et al. 1998). The genes that encode metacyclic VSGs are distinct from those of bloodstream-form organisms and only 27 have been identified. Each metacyclic VSG gene is located at the end of a large chromosome and expression does not require DNA rearrangements. The diversity of metacyclic VSG genes may have evolved to permit multiple reinfection of hosts. Although there are many fewer metacyclic VSG genes than bloodstream-form VSGs, it is unlikely that a metacyclic VSG vaccine will be effective. The expressed metacyclic VSG repertoire has been observed to change over time (Barry et al. 1983).

Transferrin receptor variants

Transferrin is a required growth factor for trypanosomes and is acquired by receptor-mediated endocytosis using receptors that are distinct from mammalian counterparts and are encoded by genes ESAG 6 and 7, which are upstream of the VSG-gene expression site. ESAG 6 and 7 gene products are structurally similar to the N-terminus of VSGs suggesting that they arose from a VSG gene. ESAG 7 encodes a polypeptide of 340 amino acids while ESAG 6 encodes one of 401 amino acids. Only the latter has a carboxy-terminal GPI-addition signal. Hence, the assembled heterodimeric receptor is held in the membrane by only a single GPI. This dimer binds transferrin. It is about 75 percent of the size of a VSG dimer and may be buried in the VSG layer in such a way that its ligand-binding site is accessible to transferrin but the rest of the receptor is not accessible to antibody. The affinity of binding depends on the donor origin of the transferrin, and on the bloodstream-form trypanosome VSG expression site used. Different transferrin receptor alleles associated with different VSG-gene expression sites encode similar but not identical receptors resulting in variation in affinity for transferrins from different mammal host species. This flexibility may allow trypanosomes to escape binding-site-specific antibodies and to meet the needs of the parasites for transferrin acquisition in a particular host. In this regard, antibody specific for the transferrin receptor selectively affected the in vitro growth of parasites expressing receptors with low affinity for the transferrin type present in the culture medium (Gerrits et al. 2002). While discouraging from the point of view of vaccinology, this result suggests that the receptor is directly accessible to antibody, supporting the view that not all molecules that are critical for trypanosome function are obscured by VSG.

CLINICAL ASPECTS

The cell and molecular biology of African trypanosomes is so fascinating that it is sometimes possible to forget that the organisms are devastating pathogens. However, this is not a luxury afforded to the victims of sleeping sickness. *T. b. rhodesiense* and *T. b. gambiense* both cause fatal disease. However, there are differences in the clinical manifestations of the diseases they cause and in their epidemiology (Table 1). The disease produced by *T. b. gambiense* (also called West African trypanosomiasis) is chronic in nature lasting up to 4 years, whereas the disease produced by *T. b. rhodesiense* is more acute, rarely lasting more than 9 months before death occurs. The molecular basis for this difference in virulence is not known.

As noted earlier, one of the key aspects of African trypanosomiasis is the phenomenon of antigenic variation that results in waves of parasitemia occurring in the blood of infected people and animals (Figure 8). The clinical aspects of trypanosomiasis and the chronic nature of the disease can be attributed to these waves of parasitemia.

Phases of the disease

African trypanosomiasis in humans can be separated into three phases (Table 2).

PHASE 1

The first phase occurs just after the inoculation of the metacyclic trypomastigotes by biting tsetse flies. A chancre develops at the site of the bite, and the trypanosomes remain at this site for a short time. There is inflammation of the subdermal tissue at the site, with edema, erythema, tenderness, and heat. The chancre is

Table 1 *A comparison of the biology of the subspecies of African trypanosomes,* T. b. gambiense *and* T. b. rhodesiense [a]

Characteristic	*T. b. gambiense*	*T. b. rhodesiense*
Disease	Chronic	Acute
	Low parasitemia	High parasitemia
	Incubation period is months to years	Incubation period is days to weeks
Main tsetse vector	*G. palpalis* group	*G. morsitans* group
	G. palpalis	*G. morsitans*
	G. fuscipes	*G. pallidipas*
	G. tachinoides	*G. swynnertoni*
Transmission	Human reservoir	Animal reservoir
	– (primary)	– (primary)
	Riverine tsetse	Savanna and woodland tsetse
	– (secondary)	– (secondary)
	Animal reservoir	Human reservoir
Reservoir hosts	Possibly kob, hartebeest, domestic pigs, dogs	Bushbuck, other antelope, hartebeest, hyena, lion, domestic cattle; possibly warthog and giraffe
Geographical range	West Africa, western, and northern Central Africa	East Africa and north Central Africa

a) Reviewed in Seed and Hall (1992)

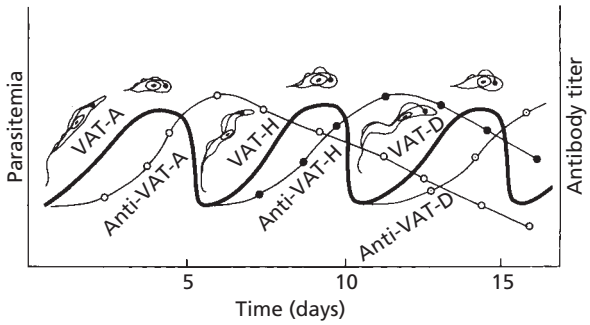

Figure 8 *Waves of parasitemia and their relationship to the humoral response in a mammalian host infected with the African trypanosomes. The first wave consists of a trypanosome population in which the major variant antigen type is VAT-A. This wave of parasitemia is followed by an antibody response (antiVAT-A) whose peak titer is observed shortly after the wave of parasitemia is cleared. A minor VAT (VAT-H) present on some trypanosomes from the preceding population gives rise to the next wave of parasitemia. This population is later cleared by host antiVAT-H. The rise and fall of trypanosome populations continues throughout the life of the infected host. In experimentally infected rodents or rabbits, a peak in parasitemia is observed every 5–7 days. Infected rabbits may live for as long as 6 months, during which time they may experience as many as 30 waves of parasitemia. (From Seed and Hall 1992, with permission of Academic Press)*

more readily observed in white people than in black people and is most frequently found in individuals infected with *T. b. rhodesiense*.

PHASE 2

During the second phase the trypanosomes spread throughout the entire body. They move through the blood and lymphatic vessels and multiply rapidly. Clinical symptoms during this stage appear to be associated with the waves of parasitemia. The high parasitemias are accompanied by fever, headache, joint pain, and malaise (Molyneux et al. 1984). These clinical symptoms may decrease in intensity as the disease becomes chronic. Splenomegaly and lymphadenopathy are major features of this stage of the infection. Histopathological examination of the lesions shows vasculitis with edema and perivascular infiltration of leukocytes.

PHASE 3

Humans infected with the African trypanosomes, if untreated, eventually develop central nervous system (CNS) disorders. They may develop a wide array of behavioral changes ranging from aggressiveness to sleep-like states. The final stage involves complete somnolence (sleeping sickness). The common clinical symptoms of third-phase infection result from damage to the heart, nervous system, and other organs.

IMMUNOLOGY AND PATHOLOGY

Trypanosomiasis-susceptible hosts

Sequential microscopic and serologic analyses of blood from infected patients and experimental animals have shown that African trypanosomiasis is characterized by recurring waves of parasitemia. Each rise in parasitemia is paralleled by an increase in antibody specific for major variant antigenic types in the wave. The variant specific antibody is involved in the removal or clearance of a major portion of the trypanosome population from the blood and other body fluids, a process that ends each wave of parasitemia. Although this specific antibody clears a large majority of the trypanosomes present during a given wave of parasitemia, there pre-exists within that population a minor population of trypanosomes with a different VSG. This minor population gives rise to the next wave of parasitemia. New surface coat variants appear at frequencies of 1 in 10^6 cells in syringe-passaged populations and an estimated 1 in 100 cells in fly-passaged field isolates. Antibody therefore acts as a selecting agent removing the major VSG popu-

Table 2 *Some common clinical symptoms of African trypanosomiasis of humans*

Stage of infection	Symptoms [a]
Fly bite, chancre	Localized tenderness and erythema at site of fly bite
Early parasitemic stage	Headache, joint pain, fever, lymphadenopathy, weight erythema, itching, anemia, dizziness
Later stages – symptoms associated with particular organ systems	
Edema	Peripheral ascites, lung edema, pericardial effusion
Nervous system	Waking EEG changes and sleep–wake cycles[b,c], insomnia/somnolence, mental disorders, slurred speech, paralysis, brisk reflexes, epileptiform fits
Cardiac system	ECG changes, congestive heart failure
Endocrine system	Amenorrhea and impotence with hypogonadism[d], altered thyroid function[e], adrenocortical function[f], and changes in the circadian rhythms of plasma cortisol and prolactin[g]
Other	Puffy facial appearance, diarrhea, anorexia, splenomegaly

a) For further details, see Seed and Hall (1992), b) Buguet et al. (1993), c) Hamon et al. (1993), d) Soudan et al. (1993), e) Reincke et al. (1993), f) Reincke et al. (1994), g) Radomski et al. (1995).

lation and allowing for other VATs that exist in the population to increase in number. VSG gene switching and antibody selection of variants results in the appearance of new peaks in parasitemia every 5–10 days (Figure 8). There is evidence that tumor necrosis factor, which is elaborated by macrophages in infected animals, can also kill trypanosomes, but this action appears to be restricted to one or a few isolates and may therefore be of limited significance (Kitani et al. 2002).

Trypanosome-infected mammals first make VSG-specific antibodies of the IgM class. These facilitate trypanosome clearance in two ways:

1. by promoting assembly of complement components on the parasite surface to yield a membrane attack complex, which lyses the parasites
2. by causing opsonins, namely, complement fragments C3b and iC3b, to be deposited on the parasite surface resulting in their binding to complement receptors on macrophages and neutrophils and their phagocytosis.

It has been shown experimentally that the VSG-specific IgM immune response is primarily a B-cell response of a type that, in mice, does not require T-cell help. However, T-cell-dependent IgG responses also arise in infected animals. VSG-specific antibodies of the IgG class are thought to play an important role in clearing trypanosomes from tissue fluids because IgG is smaller than IgM and can diffuse more readily into the tissues.

The development of broad, and possibly polyclonal, B-cell activation and macroglobulinemia are among the defining characteristics of trypanosomiasis. Both antihost and antitrypanosome antibodies are present in the sera of infected patients and contribute to the macroglobulinemia. After the disease has persisted for some time, the lymph nodes and spleen become enlarged. There is also CNS involvement. Large numbers of white cells are found in the cerebrospinal fluid, which contains high levels of IgM and other proteins, especially during the late stages of the disease (Haller et al. 1986; Molyneux et al. 1984). The presence in brain tissue of 'Morula cells of Mott', which are plasma cells with highly distended sacs of endoplasmic reticulum, was once considered pathognomic of sleeping sickness.

Many signs of disease appear in infected trypanosomiasis-susceptible hosts. There are inflammatory lesions in the heart and brain with perivascular infiltration by lymphocytes, plasma cells, and monocytes. In chronically ill patients, there may be chronic inflammation of muscle and nerve fibers. In patients with advanced disease, these changes lead to edema and fibrosis. Pathology, visible by microscopic examination, has also been seen in the lungs, spleen, and liver. In the blood, there is a reduction in the numbers of erythrocytes and platelets, and a reduction in hemoglobulin content. Edema and hemorrhage develop, due to changes in the vascular beds of the heart and brain. In the CNS, there is demyelination and neuronal damage that may extend into the white matter. The perivascular cuffing and inflammatory cell infiltration extend deep into the choroid plexus. As the disease progresses, chronic meningoencephalitis occurs. Changes in electroencephalograms and diurnal sleep patterns have been detected in chronically infected humans (Bentivoglio et al. 1994; Buguet et al. 1993; Hamon et al. 1993).

Experimental data from studies of infected laboratory animals suggest that the encephalopathy observed in patients with advanced trypanosomiasis has an immunological basis (Jennings et al. 1989). It can be observed, for example, that many aspects of the pathology of African trypanosomiasis are similar to those present in animals undergoing a progressive Arthus-type reaction. For example, high immunoglobulin levels and large quantities of immune complexes are present in the blood and the CNS of experimentally infected animals and infected humans. In addition, high kinin levels with changes in prothrombin activity as well as changes in the levels of fibrin, fibrinogen, and complement have been reported to occur in patients as well as in experimental animals suffering from trypanosomiasis (reviewed in Seed and Hall 1992). These last changes are not only consistent with those occurring during an Arthus reaction, but also with those induced by the activation of the coagulation system as occurs in patients with the disseminated intravascular coagulation often present in patients with the Rhodesian form of this disease. Furthermore, antiCNS components (galactocerebrosides, neurofilaments, tryptophane) have been described in sera and cerebrospinal fluid (CSF) of sleeping sickness patients (Dumas and Bouteille 1996). These may arise as a result of cross reactions with common epitopes between host and trypanosomes or from a breakdown in immune regulation leading to autoimmunity. All of these changes, and others including the changes reported to occur in cytokine levels of patients, are suggestive of an immunologically mediated pathology in trypanosomiasis. Furthermore, the reactive arsenical encephalopathy that occurs in 3–5 percent of late-stage infection patients treated with melarsoprol may also have an immunological basis (Adams et al. 1986; Haller et al. 1986), which is the rationale for the use of anti-inflammatory corticosteroids in the treatment of these patients (Jennings et al. 1989).

Although immunoglobulin levels are increased in humans with trypanosomiasis and their immunological system is very active, it has been found that infected humans respond to secondary infections as if they were immunosuppressed. Such immunosuppression in patients with trypanosomiasis is well documented (Greenwood et al. 1973). The actual mechanisms involved are unknown but there are a number of reasonable suggestions based on data from animal studies. Impaired B-cell responses may result from overactivation and clonal depletion, and from a loss of immune system follicular

structure during infections. It is unclear whether unique trypanosome components are the cause of this suppression or if it results from massive antigenic overstimulation. T-cell suppression is thought to result from the development of immunosuppressive macrophages responding to the combined effects of trypanosome products and interferon-γ with production of immunosuppressive levels of nitric oxide and prostaglandins. In this regard, *T. brucei* have been shown to directly stimulate murine T cells to produce interferon-γ. Trypanosomes have also been recently shown to have an endogenous prostaglandin F(2alpha) synthase and generation of this prostaglandin by the parasites may contribute to host pathology (Kubata et al. 2000).

In addition to the microscopic pathology and the immunopathology that the infection induces, there are extensive changes in host physiology in persons with trypanosomiasis. For example, the reproductive capacity of infected individuals is low. Low estradiol levels are reported to occur in 65 percent of infected females and low testosterone levels in 50 percent of infected males (Ikede et al. 1988; Boersma et al. 1989; Soudan et al. 1993). Abnormalities in thyroid hormones have also been observed (Reincke et al. 1993). These observations suggest that all organ systems and physiological functions are affected by the infection. A summary of some of the physiological changes that occur in people with African trypanosomiasis is shown in Table 2.

Trypanosomiasis-resistant hosts

Several African wildlife species are found throughout areas where trypanosomiasis is endemic. These animals were presumably selected by co-evolution with trypanosomes and tsetse flies to have developed methods to limit the growth of African trypanosomes and the severity of trypanosome-induced pathology. The little information available on mechanisms of resistance expressed by these animals was made possible by the pioneering work of Jan Grootenhuis, a Dutch veterinarian who established a breeding herd of Cape buffalo and eland in Nairobi, Kenya, outside of the tsetse habitat. There, several generations of the wild bovids were raised in the complete absence of trypanosomiasis. Experimental challenge with trypanosome-infected tsetse flies and needle inoculation with cloned bloodstream-form organisms showed that the bovids could be infected but showed few or no signs of disease. In both cases, only one or a few waves of parasitemia developed after which infections became cryptic being characterized by the presence of between one and ten mammal-infective trypanosome(s) per ml blood. (Reduth et al. 1994). Eventually, the infections were cleared. During the period of cryptic parasitemia, the wild bovids could not be super-infected with different trypanosome isolates.

Infected Cape buffalo were shown to develop broad-acting trypanocidal activity in their blood plasma. This was mediated by production of a trypanocidal concentration of hydrogen peroxide as a result of catabolism of endogenous plasma purine by plasma xanthine oxidase (reviewed in Wang et al. 2002), and was contingent on an infection-induced decline in blood catalase (Wang et al. 1999). However, the change in blood chemistry was not responsible for the long-term suppression of parasitemia in the infected Cape buffalo. By a month after infection, blood catalase was restored to preinfection levels but parasitemia remained cryptic (Wang et al. 2002) Consequently, a second mchanism must be responsible for control of parasite population growth at this time. Possible mechanisms include the development of trypanosome growth-inhibitory antibodies directed against conserved receptors for required macromolecules, and the priming of a cell type that can accelerate the development of antibodies specific for trypanosome VSG thus limiting population growth in a VSG-specific manner.

DIAGNOSIS

A primary consideration for diagnosis of African trypanosomiasis should be a history of travel or residence of the patient in an area of Africa where the disease occurs. This said, diagnosis of African trypanosomiasis depends on the microscopic demonstration of trypanosomes in the blood, in lymph node aspirates or in cerebrospinal fluid. The very elevated macroglobulinemia that occurs in patients has been considered diagnostic of African trypanosomiasis but it is only suggestive as other diseases such as leishmaniasis also cause a macroglobulinemia.

Unfortunately, the clinical features of the disease are not sufficiently unique to be diagnostic, except possibly during the late sleeping sickness stage; the number of parasites in the blood and other body fluids is often very low, making detection difficult. Multiple sampling and concentration techniques are often required to find the parasites. In *T. b. gambiense*-infected people in particular, the parasitemias can be extremely low and make infection with this trypanosome particularly difficult to diagnose (DeRaadt and Seed 1977). It should be noted that there have been attempts to develop a list of symptoms (enlarged lymph nodes, CNS-associated signs, etc.) that are common to sleeping sickness patients, which could be used by rural health personnel to make a tentative diagnosis of a *T. b. rhodesiense* infection (Boatin et al. 1986).

Examination of stained thin or thick blood films for trypanosomes is still a good diagnostic technique, and repeated daily examination can increase the probability of detecting the parasite. The use of concentration methods such as miniature anion exchange columns (Lanham and Godfrey 1970; Lumsden et al. 1979, 1981),

or hematocrit tube centrifugation (Woo 1971; WHO 1986; Woo and Hauck 1987; Levine et al. 1989) coupled with microscopic examination can increase the sensitivity of methods for detection of trypanosomes several fold. A modification of the hematocrit tube centrifugation test is the acridine orange quantitative buffy coat (QBC) technique (Bailey and Smith 1992). This test is simple, quick, and sensitive. Since it detects trypanosomes microscopically, it does not produce false positives. The concentration techniques have been modified for use in field surveys, but they are still difficult to use in field settings because of the need for a centrifuge and electrical power (WHO 1986).

If trypanosomes cannot be detected in the blood of a suspected trypanosomiasis patient, the microscopic examination of a wet preparation from an enlarged cervical lymph node may confirm the diagnosis. Once the trypanosomes are detected in the blood and lymph fluids, the CSF must also be examined in order to determine the stage of the disease. Distinguishing between the acute blood stage of the infection and the later chronic neurologic stage is important in determining the appropriate chemotherapeutic regime. Centrifuged sediments of the CSF should be examined for trypanosomes. The fluid should also be examined to determine if there are elevated white cell counts and increases in protein concentration (DeRaadt and Seed 1977). Both CSF and blood from patients should be examined periodically after completion of chemotherapy in order to ensure that the patient is cured.

The use of in vitro cultivation of trypanosomes or the inoculation of suspected samples into experimental animals is not currently practical for routine diagnosis, although both procedures are used extensively for research purposes. A kit for in vitro isolation (KIVI) of trypanosomes from sleeping sickness patients in the field has been developed (Aerts et al. 1992; Truc et al. 1992). This test is of value in isolating trypanosomes from infected individuals; however, because KIVI requires days for the cultures to produce detectable numbers of trypanosomes, it will probably have limited diagnostic value. Its main importance will be as a standard for evaluating other quicker diagnostic tests.

Immunodiagnostic tests can be especially useful for mass field surveys and for other epidemiological investigations as well as for the detection of latent infections. A number of serological tests have been developed and many are currently being tested in the field. Immunofluorescence, complement fixation test, and card agglutination test for trypanosomes (CATT) have all been utilized in epidemiological studies. Several of these procedures are available in the form of commercial kits for field use. These tests are based on the detection of antibody in the sera of infected individuals and utilize antigens from blood-stage trypanosomes. For example, the CATT test employs a fixed, stained suspension of intact trypanosomes containing a mixture of various antigenic types. The various types selected are based on the frequency with which they appear in the human population in a broad geographic area. This test is easy to perform, inexpensive, and convenient for field work. However, like many indirect diagnostic tests, it is not perfect. For example, it is not equally effective in all geographic areas, nor is it equally effective for detection of the two forms of human trypanosomiasis. This test is also reported to give positive reactions with antibodies to species of trypanosomes infecting animals but not humans (Penchenier et al. 1991).

Tests that use monoclonal antibodies to detect trypanosome antigens in serum are now being developed (Komba et al. 1992; Nantulya et al. 1992). Antigen detection tests would be valuable since they are based on detection of trypanosome antigens in the blood and, therefore, on active infection. Tests that detect antibody cannot distinguish between a past and a present infection, but those that detect antigen can. The antigens produced by modern biotechnology have not yet been used for diagnostic purposes. Although molecular probes have been used successfully in epidemiological studies, hybridization and polymerase chain reaction (PCR) technologies are not currently used for routine diagnosis because tests based on them are still too slow and cumbersome.

In a recent comparison of the CATT, the hematocrit centrifuge tube assay, the minianion exchange column technique, the QBC, the KIVI, and the thick blood film (TBF) procedure, the TBF was found to be as sensitive as any of the others. It also has the advantage of being the simplest and cheapest of all the diagnostic methods. It is rapid and simple, requiring only the staining and examination of the slides (Truc et al. 1994).

EPIDEMIOLOGY AND CONTROL

As mentioned at the start of this chapter, African trypanosomiasis is restricted to Central Africa because of the ecology of the insect vector (*Glossina*, tsetse flies). The tsetse habitat is limited by the Sahara Desert to the north and the cool, dry areas of Southern Africa to the south. The tsetse habitat is approximately the size of the USA, and it is estimated that within this region about 60 million people are at risk. However, this figure is based on a number of suppositions and there is no way to truly know the number of people at risk. A small portion of people in trypanosomiasis endemic areas has been evaluated for the disease and based on this evaluation it is estimated that 300 000–500 000 people presently have sleeping sickness. Of these, only a few will be detected by the screening systems in place and even less will be treated. Sleeping sickness now occurs in 36 countries south of the Sahara and there are approximately 200 known disease foci (Kuzoe 1991, 1993).

Epidemiology

The epidemiology of infection caused by *T. b. gambiense* differs from that of infection caused by *T. b. rhodesiense* in many aspects. These include the species of tsetse involved, the type of tsetse habitat, and the number and type of reservoir hosts (Table 1). *T. b. gambiense* is transmitted by flies of the *Glossina palpalis* group, which live primarily in the vegetation along river banks and in moist forests. Tsetse of this group feed frequently on humans in areas where contact is common, such as at river crossings and water holes. There are animals that serve as reservoir hosts of *T. b. gambiense* but people with chronic infection who have relatively mild symptoms provide the major contribution to the persistence of the Gambian type of infection (Jordan 1986; Molyneux and Ashford 1983). *T. b. rhodesiense* is transmitted by tsetse of the *G. morsitans* group. These flies inhabit low woodlands and thickets on lake shores and much of the savanna of Central and East Africa. This group of flies feeds primarily on nonhuman hosts, and humans are only a secondary food source (Seed and Hall 1992). Fishermen, game wardens, and others who enter areas with high tsetse numbers are particularly at risk (Wyatt et al. 1985). In addition, epidemics of the Rhodesian infection occur when there is an increase in tsetse numbers in close association with villages, a development causing an increase in fly–human contact. The areas in which the West African infection occurs overlap the areas of infection caused by *T. b. rhodesiense* in countries such as Zaire and Uganda.

Control

In the past, control of trypanosomiasis was partly based on the removal of reservoir hosts from areas near human settlements. This involved the fencing out and killing of game animals. Other early attempts at control of trypanosomiasis involved the destruction of tsetse habitats. Both the elimination of reservoir hosts and the destruction of tsetse habitats are now considered ecologically unsound. In addition, game animals are a tourist attraction and consequently an important source of revenue for African countries with managed National Parks and Safari operations including lodges.

The epidemiology of human African trypanosomiasis is shaped by many factors (Table 3). They include the type and density of the vector, the type and density of the reservoir host population, and the density of the human population. The densities of the fly population and of the host population are dependent on environmental conditions such as temperature, humidity, water availability, and other factors of the habitat. It is a general truth that any condition that increases the contact between tsetse, the reservoir hosts, and humans, including growth of population, will increase the risk of human infection. The chronic nature of the infection in both the vector and mammalian host makes continued surveillance and treatment of the human population, and vector control necessities if low prevalence rates are to be maintained in the human population. Current control of human African trypanosomiasis is therefore based on three arms (Seed 2000). The first is the continued surveillance in individuals by mobile field teams for the detection of trypanosomiasis. It is believed that every individual in an endemic area should be screened at least once a year. The second arm of a control program is the treatment of the infected individuals. These two phases are essential for a successful control program because African trypanosomiasis is a chronic disease and ambulatory individuals can act as reservoirs of the infection. This is especially important for the West African

Table 3 *Some factors involved in the epidemiology of African trypanosomiasis of humans* [a]

Fly	Mammal	Environmental	Parasite
Fly numbers	Host numbers	Temperature	Parasite numbers
Sex	Host species	Humidity	Genetics
Age at infection	Habitat	Water sources	Growth rates, virulence, drug resistance, human serum resistance, sub-species, strain
Symbiotic infections, viral, bacterial	Attractiveness to fly	Vegetation	
Fly behavior, habitat selection, host preference	Genetics	Wind direction and current	
Physiological status	Immune response		
Genetic factors	Non-specific factors		
Species, subspecies	Race		
Stage of infection in fly	Trypanocidal factors		
	Degree of immunosuppression		
	Intercurrent infections		

a) From Molyneux et al. (1984), Jordan (1986), Seed and Hall (1992), Vale (1993)

form of the infection. Since East African trypanosomiasis is a zoonotic disease, the treatment of infected individuals has less effect on the incidence of human infections. In East Africa, cattle are an important reservoir of the disease. It has been shown that cattle infected with *T. b. rhodesiense* and their movement can significantly contribute to the epidemiology of the human infection especially in the vicinity of cattle markets. It has, therefore, been suggested that the surveillance of cattle for human-infective *T. b. rhodesiense* and the targeted treatment of these cattle would be an important addition to the control of sleeping sickness (Hutchinson et al. 2003). The third arm of current control programs involves vector control.

All species of *Glossina* are blood feeders and as adults are totally dependent on this food source. The different species of *Glossina* have different host preferences, and both sexes take bloodmeals. In addition, tsetse are larviparous and a female produces only 8–12 larvae during her limited life span of 3–5 months. The larvae are deposited on the ground and immediately burrow into patches of moist soil or sand such as occur in shaded areas under fallen logs, rocks or bushes. The need for frequent bloodmeals and the reproduction dynamics of tsetse are important factors in shaping the epidemiology of trypanosomiasis (Molyneux and Ashford 1983).

The environmental requirements of the flies and their low reproductive rates make then vulnerable to control by a variety of techniques. The techniques used are based on our knowledge of tsetse behavior and other aspects of epidemiology of trypanosomiasis. Control programs using insecticide application and traps are in wide use. Traps and insecticides such as the chlorinated hydrocarbons and synthetic pyrethroids, when properly used, cause limited environmental damage (Molyneux and Ashford 1983; Jordan 1986). Depending on the environmental conditions, application may be by spraying with individually carried sprayers or from aircraft. The latter technique is used when broad coverage is needed. Technical factors that are considered important for achieving good results in spraying programs include appropriate formulation of the spray to obtain proper droplet size and the proper selection of insecticide. Either a residual or a nonresidual insecticide may be selected, depending on the retention time desired. Selection of appropriate sites for spraying is also important. Many of the requirements for successful use of insecticides for control of tsetse are reviewed by Jordan (1986) and Molyneux and Ashford (1983). Although control by insecticide application is effective, it is also expensive and repeated application is required if results are to last.

The use of fly traps and screens or rags impregnated with insecticides is more economical than environmental spraying and has been demonstrated to lower tsetse numbers dramatically in limited areas (Molyneux and Ashford 1983; Jordan 1986; Vale 1993). The biconical trap (Figure 9) has been shown to be effective in reducing tsetse numbers in West Africa. Detailed knowledge of fly behavior in the area where control is attempted is required for proper design and placement of traps and screens.

There are chemicals to which tsetse are known to be attracted. These include carbon dioxide and volatile compounds in the breath and urine of cattle (Vale and Hall 1985a, b; Vale et al. 1988). Some of these compounds have been identified: they include acetone, 1-octenol-3-ol, and the phenolic compounds 4-methylphenol and 3-n-propylphenol. The baiting of traps with these synthetic compounds and with carbon dioxide has been shown to increase the numbers of flies caught. There are also substances from humans that are repellent to some tsetse (Vale 1993), and these must not be permitted to contaminate traps. In addition, color and design of traps or screens are important factors in attracting and inducing the landing of flies. In the Congo Republic, a modified biconical trap is used for the control of tsetse in areas where trypanosomiasis occurs (Lancien 1981). The traps are relatively inexpensive and can be constructed and maintained by the villagers themselves if the necessary materials are provided (Okoth 1986).

In Zambia, disposable screens impregnated with insecticide have replaced traps (Figure 9b). The screens are 2×1 m and have a central black area of 1×1 m surrounded by two 0.5×1 m rectangular blue areas. The screens are supported by wooden frames that can be stuck into the ground. The insecticide used, Deltamethrin, persists on the screens for months (Vale 1993). The screens are inexpensive, require no maintenance, and can be utilized with or without the use of chemoattractive odors (Vale 1993). These screens are extremely effective in killing tsetse and in some areas are believed to have reduced or even eradicated tsetse populations. Similar screens have been used to control *G. pallidipes* in Kenya (Opiyo et al. 1990). The use of traps and screens in the control of tsetse has been extensively discussed by Jordan (1986), Laveissiere et al. (1990), and Vale (1993). They hold great promise for future control of tsetse populations and, therefore, African trypanosomiasis in an environmentally friendly way. However, although both traps and disposable screens have been successfully used, they require continued maintenance and replacement due to environmental damage, theft, and animal damage. They are not cost-free and require a strong infrastructure for maintenance.

An approach related to the use of screens and traps in tsetse control has been the use of cattle dipped in insecticide, or on which insecticides have been poured or sprayed. This technique has been shown to lower tsetse numbers (Fox et al. 1993). This procedure is relatively inexpensive and has the additional advantage that it also reduces the populations of ticks and other biting insects.

There have been attempts to control tsetse by the release of sterile flies. *Glossina austeni* was successfully

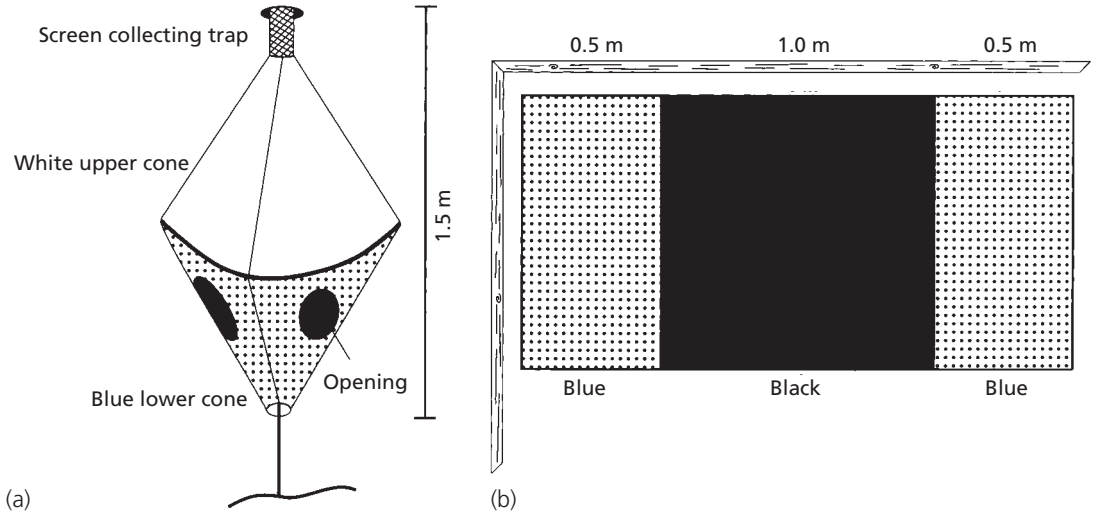

Figure 9 (a) *Biconical tsetse trap and* **(b)** *insecticide impregnated screen or target.*

eradicated from the Island of Zanzibar (Vreysen et al. 2000). Based on this success are programs currently being initiated to attempt to eradicate tsetse using sterile fly technology in combination with other control activities. However, there is considerable debate as to the validity of this approach because of the high cost required to eliminate tsetse from Zanzibar (Molyneux 2001). In addition, the fact that Zanzibar is a small, isolated island that tsetse cannot reinvade has suggested that Zanzibar is not a satisfactory model for the tsetse–African trypanosomiasis situation in mainland Africa (Rogers and Randolph 2002). There have also been attempts to find tsetse pathogens, and to identify pheromones for use in programs to reduce fly populations (Molyneux and Ashford 1983; Langley et al. 1988). An approach using biotechnology to develop a transmissible vector that would cause male sterility has been proposed, as has an approach to control tsetse fly susceptibility to infection that is based on the release of antitrypanosome peptides by symbiotic prokaryotes (reviewed by Aksoy 2001; Aksoy et al. 2002). However, although this is promising, control based on these techniques has not reached a stage of development sufficiently advanced to even permit testing.

The lack of economic resources is a major factor limiting effective control of trypanosomiasis. In many of the endemic countries, the per capita gross national product is less than US$500, and there may be an expenditure of less than US$10 per individual for total health care. Several years ago, the cost of surveillance alone was estimated at approximately US$1 per individual at risk, and the cost of treatment at US$35 per patient in the early stage and US$135 per patient in the late stage of disease. These costs are likely to have increased. In addition, there are costs for vector surveillance and control (Kuzoe 1991; WHO 1986). It is obvious that the necessary resources for control of sleeping sickness are very limited in most areas of Africa, and any change in government priorities, e.g. to control political unrest, will decrease the amount of money available for long-term control activities. Civil unrest also disrupts the flow of external aid required to supplement local funds for control activities.

CHEMOTHERAPY

Chemotherapeutic treatment of African trypanosomiasis is important in reducing the incidence of the infection especially in the West African form of the disease. However, surveillance and early detection of both forms of the disease are essential because the treatment protocol is shorter for patients in the early stages of the disease and drug-induced morbidity and mortality are lower than in patients in the late stages of the disease. Cure rates are also higher when infected individuals are treated early in the disease rather than during the late secondary stages. Control of both forms of sleeping sickness, therefore, works best if early detection and treatment are used together.

Suramin is used to treat patients with primary stage infections that do not involve the CNS. This drug is effective against both the Gambian and Rhodesian form of the disease but because it does not cross the blood–brain barrier, it is not effective against the secondary CNS stages. Suramin is a polysulfonated naphthylamine derivative of trypan red and was first found to have trypanolytic activity by Ehrlich. It is relatively toxic and may cause optic atrophy, blindness, nephrotoxicity, and adrenal insufficiency in some patients (Pepin and Milord 1994). Its exact mode of action is not known, although it inhibits a wide variety of trypanosome enzymes including glycolytic enzymes and the mitochondrial glycerol phosphate oxidase enzymes.

Pentamidine isethionate and other aromatic diamidines were first examined for trypanocidal activity in the 1930s. Like suramin, the dicationic pentamidine isethionate does not cross the blood–brain barrier, and is therefore used in patients with CNS involvement only to clear the blood of trypanosomes prior to treatment with melarsoprol. Pentamidine isethionate may produce nephrotoxicity, hepatotoxicity, and pancreatic toxicity in some patients (Sands et al. 1985; WHO 1986; Goa and Campoli-Richards 1987; Kapusnik and Mills 1988; Pepin and Milord 1994). The exact mode of action of this drug is unknown, but the strongly basic dicationic molecule binds to many cellular components, including a variety of trypanosome enzymes, causing their inhibition. It also binds to DNA, preferentially to adenosine–thymidine-rich regions in the minor groove. It has recently been shown that pentamidine isethionate is actively metabolized by the mammalian cytochrome P450 drug-metabolizing system (Berger et al. 1990).

In contrast to suramin and pentamidine isethionate, melarsoprol does cross the blood–brain barrier and is used to treat patients in the late secondary CNS stages of trypanosomiasis. Until very recently, melarsoprol has been the only drug available for treatment in this stage of the disease. Melarsoprol failures have been reported in several areas of sub-Saharan Africa, where sleeping sickness cases have a high prevalence. At this stage, it is unclear whether the treatment failures are a result of drug metabolism by the patient, or development of resistance in the parasites, or both. melarsoprol is an organic arsenical, first developed by Friedheim (1949). As with pentamidine isethionate and suramin, its exact mechanism of action is unknown, although it has been shown to inhibit a variety of trypanosome enzymes and functions in vitro. It is toxic, and in 1–10 percent of treated patients there is encephalopathy with mortality rates of 1–5 percent. Other adverse side-effects include fever, headache, joint pain, gastrointestinal disturbances, renal damage, and hypertension (Pepin and Milord 1994; WHO 1986). As noted above (see Trypanosomiasis-susceptible hosts) the encephalopathy may have an immunological basis (Jennings et al. 1989). It has been possible to reduce the development of meningoencephalitis and perivascular cuffing in infected mice by treatment with the immunosuppressive drug azathioprine (Jennings et al. 1989).

Suramin, pentamidine isethionate, and melarsoprol are still the primary drugs used to treat patients with African trypanosomiasis. The only new drug that has been developed is difluoromethylornithine (DFMO), also known as eflornithine and Ornidyl) a specific inhibitor of ornithine decarboxylase, which is the first enzyme in the polyamine biosynthetic pathway (Bacchi and McCann 1987). Currently, it is the only drug other than melarsoprol available for treatment of late-stage trypanosomiasis (Schechter et al. 1987). In the trypanosomes, exposure to DFMO leads to rapid depletion of putrescine and to low spermidine levels. It induces transformation of the replicating long slender form to the nonreplicating short stumpy form and, therefore, causes an inhibition of trypanosome growth. DFMO is relatively non-toxic but adverse reactions can include anemia, thrombocytopenia, and gastrointestinal disturbances, all of which are reversible. It is an extremely valuable chemotherapeutic agent for the treatment of late-stage patients who do not respond to melarsoprol. *T. b. gambiense* seems to be more susceptible to DFMO than is *T. b. rhodesiense*, although the reason for this difference is not clear (Bacchi et al. 1990).

Because DFMO is a specific inhibitor of ornithine decarboxylase with no other known inhibitory activity, rapid selection for drug-resistant trypanosomes has been possible in experimental studies. Resistance has also been observed in trypanosomes isolated from humans treated with DFMO. Considerable research is being conducted in an effort to find other compounds that inhibit the polyamine pathway. Treatments using combinations of drugs are also being investigated in an effort to decrease the probability of selecting for drug resistance.

The current treatment regime recommended by the WHO for reducing parasitemia in patients with *T. b. gambiense* infection is the use of pentamidine isethionate or, where there is resistance to this drug, with suramin. This is done in patients in both early (or primary) and late (or secondary) stages of the disease. In the patients in the late stages of the disease who have CNS involvement, this treatment is followed by treating with multiple injections of melarsoprol. The treatment of patients in the early stages of a *T. b. rhodesiense* infection is with suramin. In patients with the late stages of a *T. b. rhodesiense* infection, suramin is used to clear the blood and lymph and then multiple injections of melarsoprol are given (WHO 1986). The details of the treatment regimes are outlined in Table 4 (WHO 1986).

Currently, the number of drugs available for the treatment of African trypanosomiasis is limited and all except DFMO are quite toxic to humans. Researchers are currently searching for better analogues of existing drugs, investigating the use of drugs in combinations, and trying to develop compounds that inhibit biochemical pathways unique to trypanosomes. Although there are new leads in this field, further research is critically required.

VACCINATION

Because of the phenomenon of antigenic variation no vaccine is available.

CONCLUSION

Control of trypanosomiasis is possible, but it requires continuous surveillance of the human population and

Table 4 *The protocol used in the Cote d'Ivoire for the treatment of* T. b. gambiense *in infected patients*[a]

Time (days)	Drug used	Dose (mg/kg)	Route
1 and 2	Pentamidine isethionate	4.0	IM
4	Melarsoprol	1.2	IV
5	Melarsoprol	2.4	IV
6	Melarsoprol	3.6	IV
17	Melarsoprol	1.2	IV
18	Melarsoprol	2.4	IV
19	Melarsoprol	3.6	IV
20	Melarsoprol	3.6	IV
30	Melarsoprol	1.2	IV
31	Melarsoprol	2.4	IV
32	Melarsoprol	3.6	IV
33	Melarsoprol	3.6	IV

a) Abstracted from WHO (1986). For full details on treatment regimens for *T. b. gambiense* and *T. b. rhodesiense* infections, see Annex 5, pp. 118–21 of that publication.

the treatment of all infected individuals. It also requires control of tsetse flies through the use of traps and insecticide-impregnated screens and the judicious application of insecticides to the environment. It is possible that in the future insecticides will be required only in limited circumstances. The application of any control measure is currently limited in many areas of Africa owing to political instability as well as the lack of resources to initiate and maintain them adequately (Williams et al. 1993). While an enormous amount of information on trypanosome cellular and molecular biology and host immune responses has been gathered using modern research approaches and tools, this knowledge has not yet, with the exception of the introduction of DFMO for chemotherapy, resulted in new or improved methods to control trypanosomiasis. However, the discovery that lysis of *T. b. brucei* by human serum is due to the presence of apolipoprotein L-1 (Vanhamme et al. 2003), and the likelihood that this protein is also responsible for the lysis of *T. congolense* and *T. vivax* in human serum, may result in translational research leading to novel prophylactic regimes against nagana and therapeutic regimes against *T. b. rhodesiense*-sleeping sickness.

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New World trypanosomiasis

MICHAEL A. MILES

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INTRODUCTION

In 1907, the Brazilian scientist Carlos Chagas left the city of Rio de Janeiro to work as a malaria control officer at Lassance in the state of Minas Gerais. Chagas noted that poor houses in the area were infested by a large blood-sucking insect, the triatomine bug (Hemiptera, Reduviidae) (Figure 1). He was aware that blood-sucking insects transmitted human diseases such as malaria and immediately suspected that triatomine bugs might also carry infectious agents. He examined bug feces and found a flagellated protozoan parasite. At the Manguinhos Institute in Rio de Janeiro, marmosets exposed to infected bugs developed blood parasitemias of a new trypanosome, which Chagas named *Trypanosoma cruzi* after his mentor Oswaldo Cruz.

Back in Lassance, *T. cruzi* was found in the blood of sick children living in bug-infested houses (Figure 2). Chagas and his distinguished colleagues from what is now the Instituto Oswaldo Cruz, went on to describe clinical aspects of the disease, the life cycle, experimental animal models, and to discover natural mammalian hosts such as the armadillo. These discoveries were remarkable, not least because *T. cruzi* was found first in its insect vector. Chagas' early work was initially controversial, and it was not until some years later that the public health importance of Chagas disease became apparent when scientists in other Latin American countries reported its widespread distribution. The true route of transmission, through contamination with infected bug feces, was conclusively demonstrated in 1912 by Emile Brumpt. The history of the discovery and early investigations of Chagas disease is described by Miles (1996, 2004) and Lewinsohn (2003).

Chagas disease, for those who survive the acute phase of infection, primarily affects the heart and alimentary tract. Its pathogenesis remains enigmatic in that many infected people remain healthy for life and the precise reasons for a poor prognosis are not fully understood. There has been an intense expansion of interest in *T. cruzi*, driven largely by a desire to understand the disease process, how the organism survives for life in the infected mammalian host, and the unique features of trypanosome molecular biology. *T. cruzi* is also now known to be an opportunistic infection: in addition to being transmissible by blood and organ donors, it also relapses in immunocompromised individuals. South American trypanosomiasis is predominantly a disease of poverty. As such it is amenable to control by public health interventions to manage the insect vectors, supplemented by measures to eliminate transmission by blood transfusion.

T. cruzi is one of many trypanosome species in the New World, but only one other species is known to infect humans. Like *T. cruzi*, *Trypanosoma rangeli* was first found in triatomine bugs and later in children, but it is not considered to be pathogenic and its medical importance lies in the need to distinguish *T. cruzi* and *T. rangeli* infections during diagnosis, by isolation of the

Figure 1 *An adult female triatomine bug (*Panstrongylus megistus*) (Courtesy of T.V. Barrett)*

parasite. Unlike *T. cruzi*, *T. rangeli* is transmitted by the bite of the vector, through infection of the triatomine bug salivary glands.

Hoare's classic monograph (1972) still contains an excellent introduction to the New World human trypanosomiases; Tyler and Miles (2003) gives a recent review on research and WHO (2002) and Maudlin et al. (2004) provide texts on clinical aspects, diagnosis, and disease control.

Figure 2 *Carlos Chagas with one of the first discovered infant cases of* Trypanosoma cruzi *infection*

CLASSIFICATION

T. cruzi is a kinetoplastid protozoan parasite in the family Trypanosomatidae, which includes the disease agents of both the leishmaniases and the trypanosomiases. The genus *Trypanosoma* includes two species responsible for major public health problems in South America and Africa (*T. cruzi* and *Trypanosoma brucei*, respectively) and others that are not pathogenic (e.g. *T. rangeli*). *T. cruzi* falls into the section Stercoraria (as distinct from the section Salivaria of *T. brucei*; see African trypanosomiasis). The Stercoraria are characterized as trypomastigotes with a free flagellum, a large kinetoplast that is not terminal, pointed posterior end, discontinuous reproduction in the mammalian host and contaminative transmission (except *T. rangeli*) and nonpathogenic (except *T. cruzi*). The stercorarian trypanosomes, with the notable exception of *T. rangeli*, are transmitted from the vector by contamination with the insect's feces. Reproduction in the mammalian host is typically discontinuous – that is, it is confined to particular life cycle stages or tissue sites. *T. cruzi* is the type species of the subgenus *Schizotrypanum*, an assemblage of morphologically indistinguishable species parasitic in diverse mammals, mostly restricted to New World and comprising *T. cruzi* plus *T. cruzi*-like organisms in the Americas, and a number of cosmopolitan bat trypanosomes. The subgenus *Schizotrypanum* is characterized by trypomastigotes that are small, typically C-shaped with a large kinetoplast near the short, pointed posterior end, and have intracellular reproduction as amastigotes in the mammalian host.

As described below, chemical taxonomic methods, especially phenotypic comparisons by isoenzyme electrophoresis and, more recently, comparative DNA analyses, have demonstrated a remarkable diversity within the *T. cruzi* species and the existence of at least two principal subdivisions named *T. cruzi* I and *T. cruzi* II (Campbell et al. 2004).

STRUCTURE AND LIFE CYCLE

T. cruzi is a eukaryote with a nucleus; it has chromosomes that do not condense during cell division and can only be resolved by electrophoretic methods devised to separate large molecules of DNA. Extranuclear DNA is present in the form of a discrete, visible organelle, the kinetoplast, containing minicircle and maxicircle DNA and associated with a large single-branched cristate mitochondrion. The kinetoplast lies adjacent to a basal body and flagellar pocket from which a flagellum emerges (except in the amastigote stage of the life cycle) having nine peripheral pairs of microtubules, a central doublet and a parallel paraxial rod. The flagellum runs alongside the main body of the organism, in a posterior-to-anterior direction, and adhering to it to

form an undulating membrane leading to an anterior free flagellum. Interior organelles include an endoplasmic reticulum and a Golgi apparatus, and a specialized glycolytic organelle (the glycosome). The organism is bound by a complex network of subpellicular microtubules. Hoare (1972) gives ranges of dimensions for proven *T. cruzi* trypanosomes from humans as: length 11.7–30.4 μm; free flagellum 2.0–11.2 μm; breadth 0.7–5.9 μm. Further details of structure can be found in elegant electron microscopical studies of *T. brucei*, with which *T. cruzi* shares many features, and supplementary studies of *T. cruzi* (Tetley and Vickerman 1991).

There are three principal stages in the *T. cruzi* life cycle. The amastigote stage multiplies within nonphagocytic and phagocytic cells by binary fission. The epimastigotes, which have a kinetoplast adjacent to the nucleus and an undulating membrane that runs along approximately the anterior half of the organism, divide by binary fission in the hindgut of the triatomine bug vector. Trypomastigotes, with the kinetoplast at the posterior end, do not divide and are the forms found circulating in the blood of the mammalian host. Trypomastigotes are also the infective (metacyclic) stage that occurs in the rectum of the vector and in bug feces deposited during feeding. There are, therefore, some similarities between the life cycle stages of *T. cruzi* and those of both *Leishmania* and *T. brucei* in that *Leishmania* also has amastigotes that divide intracellularly (see Old World leishmaniasis and 17, New World leishmaniasis) and *T. brucei* has circulating trypanosomes but, in contrast to *T. cruzi*, the latter divide in the blood by binary fission (see African trypanosomiasis).

The life cycle is summarized in Figure 3. Metacyclic trypomastigotes deposited on the mammalian host in bug feces have the capacity to penetrate abraded skin or the wound made by the bite of the bug. They can also cross the oral and nasal mucosae, or the conjunctiva if bug feces get into the eye. These metacyclic forms are

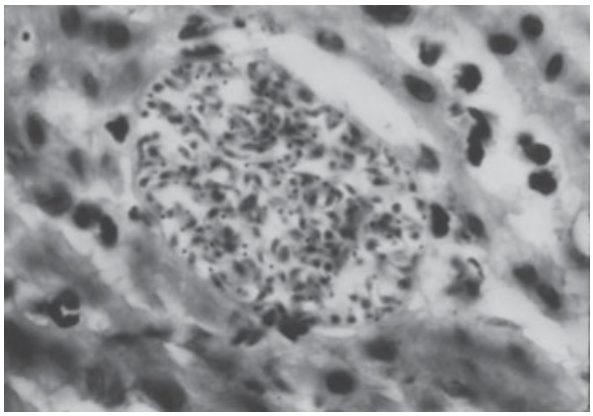

Figure 4 *Pseudocyst of* Trypanosoma cruzi *in umbilical cord (Courtesy of Dr Hipolito de Almeida)*

slender, highly motile organisms, which often rapidly traverse the field of view when seen by light microscopy. Once inside the mammalian host, they can penetrate phagocytic or nonphagocytic cells to form a local cutaneous or ocular lesion. Within the cell, the vacuole containing the *T. cruzi* trypomastigote fuses with lysosomes to form a phagolysosome, from which the organism then escapes to lie free in the cytosol. The trypomastigote transforms to an amastigote which divides by binary fission forming a pseudocyst (false cyst), so called because it has no true cyst wall but is simply bounded by the membrane of the host cell. About 5 days later, there may be up to 500 amastigotes transforming to small motile C-shaped trypomastigotes within the pseudocyst (Figure 4). The trypomastigotes are released into the surrounding tissue, either to infect other cells and repeat the intracellular cycle or to circulate in the blood (Figure 5). When the pseudocyst ruptures, not all the amastigote forms may have transformed to trypomastigotes. Amastigotes released when the cell bursts are thought to be destroyed locally but are occasionally found circulating in the blood of mice with fulminating experimental infections.

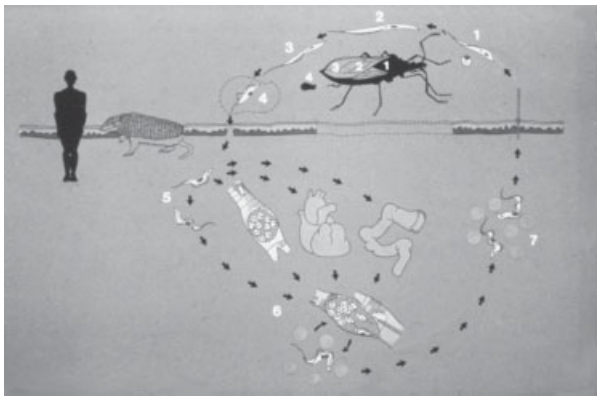

Figure 3 *A summary of the life cycle of* Trypanosoma cruzi *(see text) (Courtesy of Meddia)*

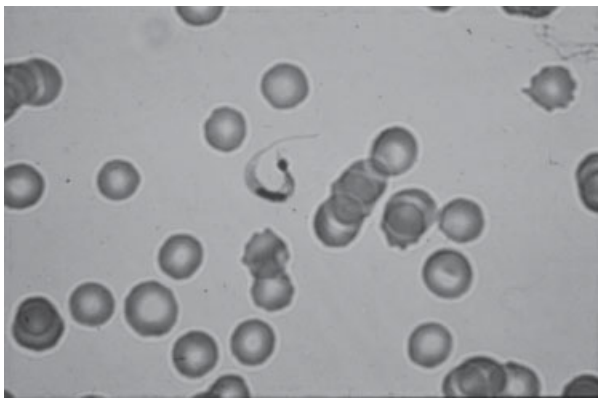

Figure 5 Trypanosoma cruzi*: C-shaped trypomastigote in circulating blood (see text)*

Two types of transformation from amastigote to trypomastigote have been described within the pseudocyst. The first, fusiform transformation, involves elongation of the body and migration of the kinetoplast. The second, orbicular cycle transformation is by unrolling of sphaeromastigotes (flagellated amastigote forms with a flagellum that encircles the body without producing an undulating membrane) that have a central vacuole: this orbicular transformation may, however, be an artefact of dry fixation and staining of tissue smears (Hoare 1972).

The released trypomastigotes do not all have the same morphology and behavior. There are two types: slender highly motile trypomastigotes, reminiscent of the infective metacyclic forms found in bug feces, and smaller, broader less motile forms. The slender forms tend to traverse the field rapidly when seen microscopically, whereas the broader forms, although motile, remain for a longer time in the same field of view. The slender motile form has an elongated nucleus, a subterminal kinetoplast and a short free flagellum, whereas the broad form has an oval nucleus, almost terminal kinetoplast and a long free flagellum. The slender forms are generally only seen in the blood of experimental animals, such as mice, during initial acute or fulminating infections (Risso et al. 2004). It has been proposed, although not proven, that the slender forms are pre-adapted to penetrating cells and renewing the intracellular cycle, whereas the shorter, broader forms may persist in the blood to be taken up by the vector with the bloodmeal. Unlike *T. brucei*, however, both slender and broad forms are thought to have active mitochondria (in *T. brucei* dividing slender forms depend on glycolysis and the mitochondria are only fully active in the stumpy forms, to prepare them for life in the tsetse fly).

Except in experimental models with *T. cruzi* strains that are highly virulent to mice, the number of circulating trypomastigotes in the blood is generally quite low (less than one per field at $\times$400 magnification in a fresh blood film) and the blood parasitemia rapidly becomes subpatent to microscopy. Low levels of circulating organisms, which may remain for the life of the host, can then be detected only by more sensitive methods.

The development of *T. cruzi* in the triatomine bug is confined to the alimentary tract. Bugs acquire infection by feeding on an infected mammalian host (or rarely by cannibalizing other recently fed bugs) and there is no transovarial transmission from adult female to egg. Once infected, triatomine bugs retain infection throughout the molting cycles. The development of *T. cruzi* in the vector, dependent on the stage of the insect, in general takes around 10–15 days. Trypomastigotes in the bloodmeal transform to amastigotes and sphaeromastigotes in the foregut, and multiply by binary fission. In the midgut, division is by binary fission in the epimastigote stage. In the rectum, epimastigotes attach to the epithelium and transformation to metacyclic trypomastigotes begins. More detailed descriptions of the life-cycle stages can be found in Hoare (1972) and Brack (1968).

Contamination with infected bug feces during or just after the bloodmeal is not the only route of transmission. Blood transfusion transmission is commonplace in endemic areas where blood is not screened for the presence of *T. cruzi* antibodies or not pretreated with gentian violet (WHO 2002). All blood donors who have resided in endemic areas and been exposed to triatomine bugs should be screened for antibodies, as should all organ donors or organ recipients with relevant histories. Antibody testing of blood donors is mandatory in some countries. It has been shown experimentally that transmission may also occur by the oral route, by consumption either of triatomine bugs or of food contaminated with bug feces, and possibly by eating uncooked blood and tissues from infected reservoir hosts. Oral transmission may be important in sustaining prevalence rates in insectivorous mammals, particularly those that live in burrows infested with bugs, and several small outbreaks of simultaneous acute cases within families are almost certainly due to oral transmission of *T. cruzi* (see below). Congenital transmission also occurs in a small proportion of children born to seropositive mothers (WHO 2002). Sexual transmission is thought to be extremely rare, as is transmission through the milk from mother to suckling infant. Accidental transmission in the laboratory is not uncommon, usually through inoculation or ingestion. There are no other important insect vectors, although *T. cruzi* may survive in cimicid bugs and produce metacyclic trypomastigotes in the hindgut, which are thought to have given rise to infections in primate colonies, possibly by the oral route. Persistent *T. cruzi* infections may also occur experimentally in ticks (*Ornithodorus moubata*) (Hoare 1972).

All stages of the life cycle of *T. cruzi* are reproducible in culture. Thus, epimastigotes are easily grown on blood agar overlays. A wide range of mammalian cell lines can be infected to produce mature pseudocysts with motile trypomastigotes, which are seen as 'boiling cells' by phase microscopy. Emergent trypomastigotes re-enter cells in vitro such that several intracellular cycles can occur on a single cell monolayer maintained for 20 days or more (Miles 1993).

The life cycle of *T. rangeli* in the arthropod vector is quite distinct from that of *T. cruzi* in that, in bugs belonging to the genus *Rhodnius*, the hemolymph is often invaded, usually more than 40 days after the infective bloodmeal. Salivary gland infections become established in a proportion of bugs that have hemolymph infections. *T. rangeli* is pathogenic to its insect vector and infected bugs may die or not molt successfully. The development of *T. rangeli* in the lumen of the bug gut and in the hemolymph is indistinguishable. Multiplication occurs in the mammalian host but blood parasitemias are scanty. *T. rangeli* is capable of infecting an histiocytic cell line in vitro and surviving as nondividing amastigote-like

forms, but it is not clear whether such intracellular stages contribute to survival in the mammalian host (Eger-Mangrich et al. 2001). The insect vector stages can be grown in vitro. The importance of *T. rangeli* as a human infective agent is secondary, in that it has to be distinguished from *T. cruzi* during diagnostic procedures.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Biochemistry

Drug development is often quoted as the incentive for studying the biochemistry of infectious disease agents such as *T. cruzi*. Thus, the absence of some metabolic pathways may make *T. cruzi* vulnerable to drug action that is circumvented by normal mammalian metabolism and, therefore, not toxic to humans. Alternatively, unique features of trypanosome metabolism may expose the organism to drugs that have no equivalent target in humans. An example of absence of a metabolic pathway is the inactive mitochondrion in the blood-stage form of *T. brucei*, mentioned above (and see African trypanosomiasis), although this is not the case for *T. cruzi*. Examples of prominent differences between the biochemistry of trypanosomatids and mammals have been found in purine salvage pathways, trypanothione biosynthesis and catabolism, sterol synthesis, and glycosomal metabolism. Several enzymes are reminiscent of those found in plants, suggesting that such enzymes are prospective drug targets, vulnerable to drugs based on herbicides (Martin and Borst 2003; Wilkinson et al. 2002b). In addition, other unusual features of the molecular biology of trypanosomes have excited great interest among molecular biologists and encouraged their use as model organisms. These include the presence of the kinetoplast and radical editing of mitochondrial mRNA, discontinuous transcription and *trans*-splicing, polycistronic transcription and antigenic variation with epigenetic mechanisms of controlling gene expression.

T. cruzi utilizes sugars, especially glucose, through active transport into the cell; nevertheless, it is not dependent on glucose and can survive by catabolism of amino acids and proteins – there appear to be no major reserve polysaccharides. Glucose catabolism, incomplete even in the presence of oxygen, produces succinic acids and acetic acid; anaerobic catabolism leads mainly to succinate and L-alanine. Enzymes of the glycolytic pathway, all of which have been reported, mostly occur in a specialized organelle, the glycosome, related to the peroxisome, which assists fatty acid metabolism in eukaryotes. The pentose phosphate pathway of glucose catabolism is also present. Amastigote, epimastigote, and trypomastigote stages have almost all enzymes of the tricarboxylic acid cycle (Cazzulo 1994).

Glycosomes lack catalase and oxidases, which regulate hydrogen peroxide in peroxisomes, but contain enzymes for conversion of glucose and glycerol to phosphoglycerate, as well as enzymes for β-oxidation of fatty acids and for ether–lipid biosynthesis; other activities include pyrimidine biosynthesis, purine salvage and carbon dioxide fixation. Enzymes found in the glycosome of *T. cruzi* include hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, glycerol 3-phosphate dehydrogenase, glycerol kinase, malate dehydrogenase, adenylate kinase, and phosphoenolpyruvate carboxykinase; some of these enzymes are also present in the cytosol. Transport of enzymes into the glycosome does not involve larger cytosolic precursors and cleavage of signal sequences. Peptide recognition signals govern importation, as is the case for peroxisomes (Cáceres et al. 2003; Wilkinson et al. 2002a).

Glutathione (GSH) is the thiol of low molecular weight that is largely responsible for protection against free radicals and reactive oxygen in most aerobic organisms. In trypanosomatids, GSH is replaced by trypanothione, a glutathione dimer conjugated to spermidine. In *T. cruzi* glutathione reductase (GR) is largely replaced by trypanothione reductase (TR), which reduces trypanothione disulfide ($T[S]_2$) to dihydrotrypanothione ($T[SH]_2$) with NADPH as cofactor. Substrate utilization of TR is distinct from that of GR but catalytic mechanism is conserved (Wilkinson et al. 2002a). The two negatively charged carboxyl groups on the glycyl termini of the GR substrate, glutathione disulfide (GSSG), are replaced by internal amide groups in $T[S]_2$ and an amino group carrying a positive charge on the spermidine. Also, the two glutathione chains in GSSG can rotate freely about the disulfide bridge whereas the $T[S_2]$ is more rigid due to the linkage by spermidine. There are distinct distributions of hydrophobic and charged residues in GR and TR adjacent to the glycyl regions of the substrate. The R_{37} and R_{347} amino acids of GR, which are thought to bind the carboxyl groups of GSSG, are replaced by tryptophan and alanine (Taylor et al. 1994). The structural consequences of an engineered change in substrate specificity have been resolved by crystallography (Stoll et al. 1997). Studies of trypanothione metabolism in *T. cruzi* have been an interesting model for the development of rational chemotherapy (Bonse et al. 2000). Other aspects of oxidative defense, such as the presence of an ascorbate hemoperoxidase, may be therapeutically exploitable (Wilkinson et al. 2002b).

Parasitic protozoa such as *T. cruzi* rely on the salvage of free purines or re-use of purines from nucleic acids or nucleotides, whereas mammals can synthesize purines de novo. The hypoxanthine analogue allopurinol has been used as an inhibitor of purine salvage but its value as a therapeutic drug is still greatly in doubt (Stoppani 1999).

There has been much research interest in proteinases of *T. cruzi* with the idea that they may be important for invasion of mammalian host cells, and targets for chemotherapy. Cysteine proteinases are the most prominent of the four main groups (aspartic, cysteine, metallo- and serine) in *T. cruzi* although metalloproteinases and serine proteinase are also present (Cazzulo 2002). There may be several other roles for *T. cruzi* proteinases, such as protection against antibody attack, or adaptation to breakdown of the bloodmeal in the insect vector. Proteinase activities can be demonstrated by using electrophoresis in gels with gelatin or fluorogenic peptides as substrates. A 60 kDa cysteine proteinase (cruzipain) resembles cathepsin and papain, and is also known as the GP57/51 antigen, which is recognized by sera from patients infected with *T. cruzi*. The enzyme produces a C-terminal extension domain by self-proteolysis (Alvarez et al. 2002).

Glycosylphosphatidylinositol (GPI) and GPI-related glycolipids are abundant in trypanosomatids. They serve to anchor surface glycoproteins and, although *T. cruzi* does not have the variant surface glycoprotein (VSG) coat characteristic of the African trypanosomes (see African trypanosomiasis), several glycoproteins are bound to the surface of *T. cruzi* by GPI anchors, including the Ssp-4 antigen of amastigotes and the 90 kDa antigen of trypomastigotes. Ferguson and his collaborators have devoted much effort to determining the GPI biosynthetic pathway in African trypanosomes, not only for the intrinsic biochemical interest, but also because this pathway might present a target for trypanosome-specific chemotherapy (Crossman et al. 2002; Lillico et al. 2003).

Another biochemical feature of *T. cruzi*, distinct from the vertebrate host, is the requirement for specific endogenous sterols such as egosterol, rather than cholesterol. This has led to promising new inhibitors of sterol biosynthesis in *T. cruzi* (Urbina 2002; Urbina et al. 2003).

Molecular biology

There is a plethora of recent advances in understanding the basic molecular biology of trypanosomes, fuelled by an international interest in their unusual features and their role as models for other studies. Two molecular processes that have received detailed attention are discontinuous transcription, or *trans*-splicing, and RNA editing of transcripts from the maxicircle mitochondrial genome.

Studies of antigenic variation in African trypanosomes revealed that each primary mRNA transcript was processed by addition of a 39-nucleotide spliced leader sequence transcribed from a separate chromosomal location – the mini-exon genes. The spliced leader is added by *trans*-splicing via Y-shaped intermediates that consist of the 3′-leader precursor linked to the 5′-portion of the main transcript. *Trans*-splicing is assisted by small nuclear RNAs (snRNAs) complexed to proteins, as is intron splicing in higher eukaryotes. *Cis*-splicing introns have also been discovered in *T. cruzi* (Mair et al. 2000). Many trypanosome genes are polycistronic, with transcripts of several genes in tandem on an initial primary transcript. The mini-exon derived RNA (medRNA) is thought to be functionally equivalent to the U1-snRNA present in other eukaryotes. Mature mRNAs of *T. cruzi* are polyadenylated. Pyrimidine (C,T)-rich regions of downstream splice acceptor sites apparently participate in the control of polyadenylation of upstream portions of the primary transcript. This may have a role in gene regulation (Matthews et al. 1994).

For many years, the function of trypanosome kinetoplast minicircles was obscure. It was finally discovered that some mitochondrial maxicircle genes are condensed and that guide RNAs (gRNAs) transcribed from minicircles carry information for editing maxicircle transcripts, by the insertion (and deletion) of uridines. It is not clear why such a complex RNA editing process is required by organisms such as *T. cruzi*, but it may be linked to developmental regulation during movement between host, vector and life cycle stages (Simpson et al. 2003).

Other research on the molecular biology of *T. cruzi* has concerned the cloning of genes encoding products involved in cell invasion or in gene expression during transformation to the infective form (metacyclogenesis), the structural and functional analysis of genes encoding antigens recognized by the host immune response, and cloning portions of the genome identified by probing with heterologous sequences from other organisms.

T. cruzi carries a surface *trans*-sialidase which transfers sialic acid from host components to mucin-like glycoproteins on the surface of the parasite. In this way, *trans*-sialidase is thought to influence adhesion and penetration of host cells and may also have a role in trypomastigote escape from the phagolysosome into the cytoplasm of the host cell (Tan and Andrews 2002; Buschiazzo et al. 2002). There is a large family of putative mucin genes in *T. cruzi*, with hundreds of copies per haploid genome, which share a signal peptide on the N-terminus and a presumed GPI anchoring sequence on the C-terminus, with hypervariable central regions (Argibay et al. 2002; Buscaglia et al. 2004). Mucins isolated from non-infective epimastigote and infective metacyclic trypomastigotes differ in lipid structure. Metacyclogenesis may be induced in the triatomine bug gut by products of hemoglobin digestion: synthetic peptides corresponding to amino acids 30–49 and 35–73 of αD-globin have stimulated differentiation of epimastigotes into metacyclic trypomastigotes (Garcia et al. 1995). There is a large heterogeneous *trans*-sialidase multi-gene family in *T. cruzi* which, among other members, includes genes for *T. cruzi* neuraminidase

(TCNA), shared acute phase antigen (SAPA), and the GP85 surface glycoproteins. The gene family has been classified into four groups. It has been proposed that changes in expression of such multigene families may provide a mechanism by which *T. cruzi* evades the host immune response, analogous to but distinct from antigenic variation in African trypanosomes (Frasch 2003).

A major technological breakthrough in research on the molecular biology of *T. cruzi* has been the development of genetic transformation systems. New plasmid and cosmid shuttle vectors allow *T. cruzi* DNA to be propagated in bacteria and reintroduced into *T. cruzi*, either episomally, or integrated into the nuclear genome by homologous recombination. In this way, *T. cruzi* genes can be deleted or overexpressed so that gene structure and function can be analyzed. Functional complementation of deletion mutants allows portions of the genome encoding a particular function to be identified. RNA inhibition (RNAi) (Lillico et al. 2003) and chromosomal fragmentation provide alternative approaches to identifying gene function although, at the time of writing, RNAi has not yet been achieved for *T. cruzi*. The wide interest in *T. cruzi* molecular biology has led to the establishment of a World Health Organization-sponsored international collaboration to sequence the entire *T. cruzi* genome, which is currently underway (Andersson et al. 1998; Aguero et al. 2000).

Genetic diversity

Early experimental work on *T. cruzi*, its wide range of mammalian hosts, and the large number of triatomine bug vector species suggested that *T. cruzi* might be genetically diverse. *T. cruzi* strains were reported to differ in their virulence and histotropism in experimental animals, in their infectivity to triatomine bug species, in their susceptibility to drug treatment, and antigenically. In addition, outcome of chronic Chagas disease appeared to show marked regional variation (Miles 1979).

In the 1970s, isoenzyme electrophoresis began to be applied to study the genetic diversity and population genetics of trypanosomatids (Figure 6). Isoenzyme profiles of *T. cruzi* isolates from domestic and silvatic transmission cycles in a single endemic locality (Sao Felipe, Brazil) showed that domestic and silvatic strains were very different phenotypically and, by implication, genotypically. Thus, more enzyme characters separated these *T. cruzi* strains than distinguished species of *Leishmania*. *T. cruzi* isolates were subsequently characterized from many Latin American countries. Analysis of isoenzyme profiles confirmed the extensive diversity within the species, and initially indicated that there were three main strain groups, which were named principal zymodemes Z1, Z2 and Z3 (Miles 1983). Z1 appeared to be associated with marsupials, especially the genus *Didelphis* and Z3 with the armadillo, *Dasypus novemcinctus*. Host associations for Z2 were less obvious but may be linked to caviamorph rodents in the silvatic cycle in Bolivia, from which *Triatoma infestans* was proposed to have spread to the six southern cone countries of South America (i.e. Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay) and to southern Peru. The zymodeme concept contributed to the description of *T. cruzi* transmission cycles as:

- non-overlapping or discontinuous, as in Sao Felipe where distinct *T. cruzi* strains were transmitted by domestic bugs (Z2, *Panstrongylus megistus*) and silvatic bugs (Z1, *Triatoma tibiamaculata*)
- overlapping or continuous, as in Venezuela where the same principal zymodeme (Z1) was believed to be transmitted by a single bug species (*Rhodnius prolixus*) considered to be common to domestic and silvatic transmission cycles
- enzootic, as in the Amazon basin and the USA where triatomine bugs do not colonize houses and silvatic *T. cruzi* strains rarely caused human infection (Miles 1979).

One reason for such interest in the heterogeneity of *T. cruzi* is to assess whether different strains are responsible for the diverse clinical outcomes of Chagas disease. The high prevalence of *T. cruzi* Z1 in the north of South America, where mega-esophagus and megacolon are rare, and the abundance of Z2 in central and eastern Brazil, where megasyndromes are common, suggested that some *T. cruzi* strains were more likely to cause chronic Chagas disease (Miles et al. 1981c). Both Z1 and Z2 have, however, been isolated from symptomatic acute phase infections and both can recrudesce after unsuccessful treatment (Luquetti et al. 1986). Although only Z2 was isolated by the same authors from symptomatic chronic Chagas disease, there is no proof that Z1 was not (also) present earlier in the infection and responsible, at least in part, for the poor prognosis. Mixed zymodeme infections have been reported from both mammalian hosts and triatomine bugs.

Identification of *T. cruzi* strains by isoenzyme profiles led to selection of biological clones representing different epidemiologies in Latin America for use as reference strains in experimental studies (together with some traditional uncloned laboratory strains). The

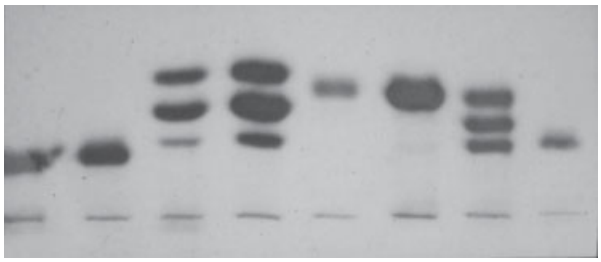

Figure 6 *Isoenzyme profiles of* Trypanosoma cruzi *isolates by starch-gel electrophoresis*

World Health Organization *Trypanosoma cruzi* reference strains are as follows (* = derived from clonal populations):

- M/HOM/PE/00/Peru
- M/HOM/BR/00/12 SF
- M/HOM/CO/00/Colombia
- M/HOM/BR/00/Y strain
- M/HOM/BR/00/CL strain
- M/HOM/CH/00/Tulahuen
- M/HOM/AR/74/CA-I
- M/HOM/AR/74/CA-I/72*
- M/HOM/AR/00/CA-I/78*
- M/HOM/AR/00/Miranda 83*
- M/HOM/AR/00/Miranda 88*
- M/HOM/BR/82/Dm 28c*
- M/HOM/BR/78?/Sylvio-X10-CL1*
- M/HOM/BR/Sylvio/X-10-CL4*
- M/HOM/BR/77/Esmeraldo CL3*
- M/HOM/BR/68/CAN III CL1*
- M/HOM/BR/68/CAN III CL2*
- M/HOM/BO/80/CNT/92: 80 CL1*
- I/INF/B0/80/SC43 CL1*
- I/INF/PY/81/P63 CL*

The use of reference strains encouraged detailed characterization by other methods, particularly by Dvorak and his collaborators (Dvorak 1984). It was found that clones of different principal *T. cruzi* zymodemes differed radically in DNA content, in kinetoplast DNA minicircle fragment patterns (schizodemes), in elemental composition of iron, zinc, and potassium, in oxidative metabolism, in antigenic profiles, in extracellular and intracellular growth rates in vitro, in virulence and tropism in experimental animals, and in response to experimental chemotherapy (Nozaki and Dvorak 1993). There is broad agreement between the zymodeme and schizodeme groupings and a correlation with abundance of a particular surface antigen (Carreno et al. 1987; Chapman et al. 1984). Polymerase chain reaction (PCR)-based analyses of polymorphisms in ribosomal RNA genes and in mini-exon genes have confirmed the presence of at least two major lineages, one that corresponds with Z1 and a second that incorporates Z2 (see below) (Stothard et al. 1998). Functional analysis of gene promoters also partitions *T. cruzi* into two major groups (Nunes et al. 1997).

Tibayrenc and colleagues subdivided the Z1, Z2 groups into many zymodemes, performed extensive further studies on their distribution in South America and elaborated a 'clonal hypothesis' which proposes that distinct *T. cruzi* clones are propagated asexually and largely disseminated separately (Tibayrenc et al. 1993; Brisse et al. 2000). PCR has been used to isolate kinetoplast DNA probes specific to widespread 'major clones' of *T. cruzi*. Most allozyme frequencies and measures of linkage disequilibrium have supported clonal propagation, and accord with single or small number of bugs invading houses and introducing a restricted number of populations of *T. cruzi*. Still further methods, including microsatellite analysis (Macedo et al. 2001) confirmed the broad validity of the principal subdivisions of *T. cruzi*.

The two major subdivisions of *T. cruzi* have now been named by international consensus as *T. cruzi* I (Z1) and *T. cruzi* II (incorporating Z2) (Anon. 1999). It has been proposed that *T. cruzi* I evolved in an arboreal palm-tree habitat with the triatomine tribe Rhodniini, in association with the opossum *Didelphis* (Gaunt and Miles 2000, 2002). *T. cruzi* II may have evolved in a terrestrial habitat with the triatomine tribe Triatomini, in association with edentates or ground-dwelling marsupials or, alternatively, by more recent host transfer of *T. cruzi* I into primates (Fernandes et al. 1999; Gaunt and Miles 2000). *T. cruzi* II has been further divided by into five subgroups, *T. cruzi* IIa–e, all previously recognized by isoenzyme profiles, and of which *T. cruzi* IIa corresponds with Z3 (Miles et al. 2003a). The affinities of *T. cruzi* IIa (Z3), whether with *T. cruzi* I or *T. cruzi* II, are still somewhat controversial (Brisse et al. 2000; Robello et al. 2000; Sturm et al. 2003; Campbell et al. 2004).

Contemporary studies of allozyme frequencies in African trypanosomes suggested the presence of genetic exchange in what hitherto had been assumed to be an asexual organism. Although the genetic mechanisms are not fully understood, experimental crosses with drug-resistance markers have shown that *T. brucei* undergoes recombination in the tsetse fly vector, probably in the salivary glands. Genetic exchange in *T. cruzi* is most likely to occur, as with African trypanosomes, among undisturbed silvatic cycles of transmission. Allozymes of phosphoglucomutase (PGM) for silvatic isolates of *T. cruzi* from the Amazon basin suggested occurrence of sympatric hybrid and parental strains. This is in accord with random amplification of polymorphic DNA (RAPD), which shows sharing of fragments between putative hybrids and one or other of the putative parents (Carrasco et al. 1996). Further evidence of genetic recombination has come from restriction fragment length polymorphisms (RFLP) studies with gene probes for three *T. cruzi* enzyme genes, and suggests that homozygote and heterozygote loci for these genes occur sympatrically among clinical isolates (Bogliolo et al. 1996).

The presence of an extant capacity of genetic exchange in *T. cruzi* has recently been proven by the production of hybrid clones in the laboratory (Gaunt et al. 2003). Two biological clones of *T. cruzi*, from silvatic transmission in the Amazon basin (Carrasco et al. 1996), were transfected to carry different drug-resistant markers and then passaged through the entire life cycle. Six double-drug-resistant progeny clones were recovered from the mammalian stage of the life cycle. The progeny clones were analyzed by isoenzyme, karyotype, RAPD and microsatellite markers and by sequencing of nuclear

and maxicircle DNA targets. The progeny showed fusion of parental genotypes, loss of alleles, apparent uniparental inheritance of maxicircle kDNA and evidence of homologous recombination, although the frequency and precise mechanisms of recombination have not been determined. There were strong genetic parallels between the experimental hybrids and the genotypes among natural isolates of *T. cruzi*. These data (Gaunt et al. 2003) and independent phylogenetic evidence (Machado and Ayala 2001) suggest that *T. cruzi* IId and IIe are hybrid strains, in accord with earlier isoenzyme profiles, and that they are derived from strains similar to *T. cruzi* IIb and IIc. Thus *T. cruzi* IId and IIe haplotypes are split across the IIb and IIc phylogenetic clades. Sequencing of intergenic targets further suggests that *T. cruzi* IIa and IIc may also have some hybrid characters, with *T. cruzi* I and *T. cruzi* IIb being less complicated lineages (Sturm et al. 2003; Campbell et al. 2004). The CL Brewer strain selected for the genome sequence project (www.TcruziDB.org), is a IIe hybrid, which complicates assembly of the sequence.

An absolute link between *T. cruzi* genotypes and clinical outcome of infection remains unproven, and few attempts have yet been made to examine the role of genotypic and phenotypic diversity of the human host in influencing clinical prognosis (Campbell et al. 2004). Multiple human genetic markers can now be used to compare genetic susceptibilities to chronic Chagas disease, although this may have little direct impact on Chagas disease control (Fae et al. 2000). In support of the hypothesis that severity of outcome is determined by the infecting *T. cruzi* strain, Di Noia et al. (2002) report that serological recognition of the *T. cruzi* II specific epitope of a GPI-anchored mucin-like protein is associated with chronic Chagas disease. However, this is not surprising as the sera examined were entirely from the southern cone region of South America, where *T. cruzi* II appears to be overwhelmingly predominant in human populations. Nevertheless, using individual kinetoplast DNA signatures, it has been shown that genetically diverse *T. cruzi* populations may occur in tissues of the same patient, and with a differential distribution between heart and esophagus (Vago et al. 2000).

CLINICAL ASPECTS

Shortly after his pioneering discoveries, Carlos Chagas was once described as a man who searched for diseases that did not exist. Initial acute phase Chagas disease is not frequently noted and is easily confused with other clinical conditions: if present, acute symptoms usually subside within 2 months of their appearance. Patients in certain geographical regions may suffer mega-esophagus and/or megacolon, usually with associated heart disease, but abnormalities in the elecrocardiograph (ECG) and myocardiopathy of chronic Chagas disease may also be mistaken for other forms of heart problem. At least one-third of the 12 million or so people thought to be infected with *T. cruzi* and surviving the initial stage of infection appear to be clinically compromised.

Clinical manifestations

The initial phase of *T. cruzi* infection may lack specific signs and symptoms. In some cases, there is a lesion at the entry portal of the organism, giving rise, in the case of entry through the skin, to a cutaneous chagoma. If metacyclic trypomastigotes cross the conjunctiva, the resultant painless but inflamed, periopthalmic, unilateral edema and conjunctivitis is known as Romana's sign (Figure 7). There may be local infiltration of lymphocytes and monocytes, and regional lymphadenopathy adjacent to these lesions. Rarely, multiple chagomas have been described during acute infections in infants. Acute infections are more common and more severe in children. Less than 10 percent of children die during the acute stage. General clinical changes at this stage may include fever, hepatosplenomegaly, generalized lymphadenopathy, facial or generalized edema, rash, vomiting, diarrhea, and anorexia. Early ECG changes may be sinus tachycardia, increased P–R interval, T-wave changes, and low QRS voltage, and death may be due to

Figure 7 *Romana's sign*

acute myocarditis. Meningoencephalitis is infrequent, mainly found in infants and carries a very poor prognosis. In patients suffering recrudescent *T. cruzi* infection associated with acquired immune deficiency syndrome (AIDS), the organism commonly crosses the blood–brain barrier and causes fatal meningoencephalitis (WHO 2002).

Incubation period may be as short as 2 weeks, or several months if infection is acquired by blood transfusion. Prolonged incubation period in recipients given contaminated blood is thought to be due to the poor capacity of circulating broad blood forms to invade cells. Splenomegaly and general lymphadenopathy are frequent in such cases.

In a small proportion of seropositive mothers, *T. cruzi* crosses the placenta, giving rise to abortion or premature birth. Hepatosplenomegaly is common in congenital infections and there may be fever, edema, metastatic chagomas, and neurological signs, such as convulsions, tremors, weak reflexes, and apnea. Signs of cardiac involvement are rare; ECG is usually unchanged but can show low voltage complexes, decreased T-wave height, and increased atrioventricular (AV) conduction time. Premature birth associated with congenital Chagas disease carries a poor prognosis: symptoms are generally less severe if birth is not premature (WHO 2002).

Patients surviving the acute infection enter the indeterminate phase, in which there are no symptoms or signs of Chagas disease. Around 30 percent of these patients, however, develop chronic disease in which cardiac changes are most common, with arrhythmias, palpitations, chest pain, edema, dizziness, syncope, and dyspnea. The most frequent ECG abnormalities are right bundle branch block (RBBB) and left anterior hemiblock (LAH) but there may also be AV conduction abnormalities, sometimes complete AV block. Many different arrhythmias may occur including sinus bradycardia, sinoatrial block, ventricular tachycardia, primary T-wave changes, and abnormal Q-waves. Enlargement of the heart may be seen by chest radiography. Complications include embolism. Death is often sudden (WHO 2002).

The regions of the alimentary tract most often affected in the digestive form of chronic Chagas disease are the esophagus and colon, with loss of peristalsis, regurgitation and dysphagia, or severe constipation, fecaloma, and progressive dilatation of these organs.

Further details of clinical aspects of chagasic heart disease, mega-esophagus and megacolon can be found in a PAHO Expert Committee Report (PAHO 1994).

IMMUNOLOGY

In view of the intense interest in the immune response to *T. cruzi* infection, it is surprising how little is understood about either the protective mechanisms or the autoimmune involvement in the pathogenesis of Chagas disease. Individuals surviving acute infection are able to mount an immune response that suppresses parasitemia but is unable to eradicate infection. In the absence of specific treatment, infection is normally maintained for life. *T. cruzi*-specific antibodies may be detectable within the first 2 weeks, and seropositivity usually remains throughout life in untreated patients. All immunoglobulin (Ig) subclasses are elevated. IgM titers rise early, with increased concentrations, followed by rising IgG titers. Unlike African trypanosomiasis, in which antigenic variation stimulates a sustained increase in IgM, in Chagas disease IgM levels fall as the acute infection subsides. As anticipated for an organism dividing intracellularly, the suppressive immune response also involves cell-mediated immunity. Partial protection by artificial immunization with purified antigens in mice has been shown to be T-cell dependent (Wrightsman et al. 2002).

Numerous studies have been performed on the immune response to *T. cruzi* infection in mice, recently aided by transgenic mouse technology, which allows the course of infection to be observed in knockout mice that are deficient in particular components of the immune response (Tarleton et al. 2000). Hypergammaglobulinemia (of IgG2a, IgM, IgE) persists in mice until 13 weeks post infection and then IgG2a and IgM levels fall. Although mouse strains vary considerably in their antibody response and resistance to *T. cruzi*, IgG1, IgG2a, IgG2b and IgM titers are sustained throughout infection, possibly with a more dominant IgG2b isotope response in a resistant strain of mouse (Rowland et al. 1992). During the acute phase of infection in mice, production of the cytokine interleukin (IL)-2 is severely suppressed, possibly by repression of transcription of the IL-2 gene. Killing in macrophages is dependent on production of interferon-gamma (INF-γ), synergistic with tumor necrosis factor-alpha (TNF-α), triggering macrophage activation, and nitric oxide production (Silva et al. 1995). Nevertheless, studies in mice suggest that *T. cruzi* infection does not necessarily lead to a dominance of T helper (Th)1 patterns of cytokine production. Th1 cells may be a requirement of immunological control of infection and Th2 cells may contribute to parasite persistence and severity of disease (Kumar and Tarleton 2001).

It is not clear how *T. cruzi* survives for life in the infected mammalian host. Immunohistochemical staining suggests that *T. cruzi* antigen can be detected in chronic, chagasic cardiomyopathy, indicating that residual pseudocysts are present. *T. cruzi* amastigotes could be found at autopsy in the hearts of 84 of 556 patients with diffuse, chronic, chagasic myocarditis (Brener 1994). Nests of *T. cruzi* have also been found in the smooth muscle cells of the central vein of the adrenal gland at autopsy in around 50 percent of patients with chronic Chagas disease (Teixeira et al. 1997). Several methods have been proposed by which *T. cruzi* escapes action of antibody, including binding of the third component of

complement by a specific trypomastigote surface glycoprotein to inhibit activation of the alternative complement pathway and binding of human C4b to restrict the classical pathway (Beucher et al. 2003). The histopathological picture in chronic Chagas disease suggests some autoimmune involvement: antibodies to host epitopes on specific proteins such as myosin and ribosomal P proteins have been reported.

PATHOLOGY

The pathology at the portal of entry is similar whether the chagoma is cutaneous or a Romana's sign, with infiltration of predominantly lymphocytes and monocytes. In the heart, no inflammatory response is said to be directed towards unruptured pseudocysts, but rupture is followed by infiltration of lymphocytes, monocytes and/or polymorphonuclear cells. The degree of parasitism is highly variable in clinical cases and in experimental infections in mice – in the latter, it is dependent on strain of infective organism and strain of mouse (Marinho et al. 2004; Risso et al. 2004). In congenital Chagas disease, amastigotes may be very widespread but are most common in cardiac and skeletal muscle or reticuloendothelial cells. If there is meningoencephalitis, parasites can be found in perivascular spaces or in glial and neuronal cells with an histopathology typical of acute meningoencephalitis (WHO 2002).

In patients surviving the acute infection, the inflammatory response in the heart subsides. Two principal forms of pathology have been described in chronic Chagas disease. In the first or neurogenic form, the heart remains free from any progressive myocarditis, but focal lesions are found in the conducting system of the heart and there is a loss of ganglion cells, particularly from the parasympathetic nervous system. Experimental studies in dogs, in which focal cardiac lesions have been reconstructed from histology of tissue sections taken at autopsy, show a good correlation between ECG abnormalities and histopathology. Longitudinal studies of ECG abnormalities in patients with chronic chagasic heart disease followed up by postmortem histology show a similar correlation between ECG abnormalities and lesions of the conducting system. The right bundle branch is found to be most frequently affected, consistent with the frequency of RBBB in chronic Chagas disease. The left conducting system is considered to be less vulnerable to focal lesions because of its more diffuse or bifascicular course. In a proportion of patients, the inflammatory response appears to be reactivated resulting in a renewed myogenic form of chronic Chagas disease and a progressive myocarditis with more diffuse lesions, a slower decline in cardiac function, and less sudden death. Lymphocytes and macrophages infiltrate the heart and mycocardial fibers are replaced by interstitial fibrosis (Brener and Andrade 1979).

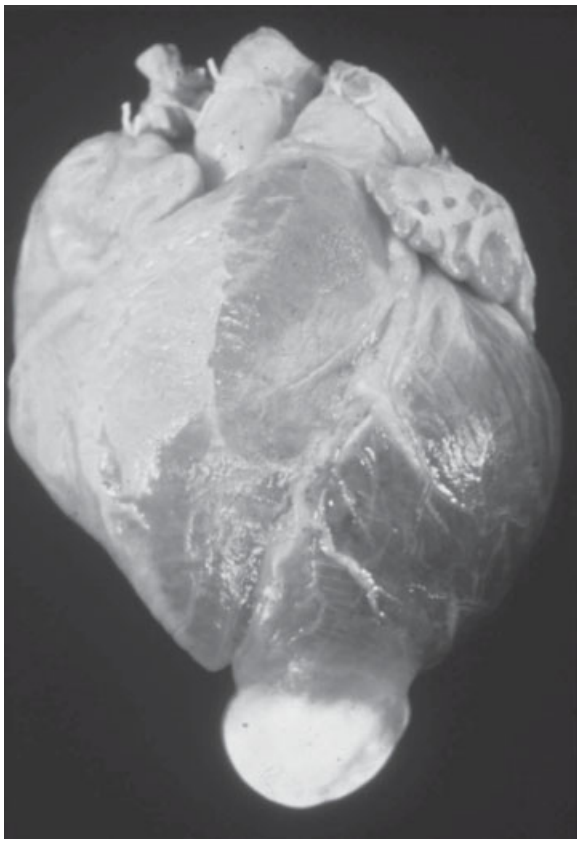

Figure 8 *Apical aneurysm of the left ventricle (Courtesy of Dr J.S. de Oliveira)*

At the gross level, the heart may be enlarged (megacardia) with focal thinning of the myocardium, which may be particularly frequent and pronounced at the apex of the left ventricle, in the form of an apical aneurysm (Figure 8). The presence of apical aneurysm is considered to be a pathognomonic sign of chronic chagasic heart disease. Of the intestinal sequelae chagasic mega-esophagus (Figure 9) is generally more common than chagasic megacolon (Figure 10); both may occur in the same individual and each is frequently associated with chagasic cardiopathy. A moderate or severe inflammatory response may be seen in the smooth muscle of the esophagus or colon and in the myenteric or Auerbach plexus during acute infection; in chronic Chagas disease the inflammatory response in these organs is usually mild.

Qualitative observations of neurological damage in Chagas disease were recorded as early as the 1930s. Köberle and his colleagues made painstaking quantitative studies of the number of ganglion cells present in normal and chagasic sections of heart, esophagus, and colon, and demonstrated widespread and significant ganglion cell loss, especially from the parasympathetic autonomic system (Köberle 1974). Neuron loss was found in many different organs but was most frequent in the heart and alimentary tract. The variable onset of

Figure 9 *Mega-esophagus:* **(a)** *postmortem;* **(b)** *on radiograph (Courtesy of Dr J.S. de Oliveira)*

ECG abnormalities, mega-esophagus and megacolon was understandable in terms of age-related neuron loss exacerbating the neurological damage attributable to *T. cruzi* infection. In addition, it was proposed that different hollow organs varied in the degree to which they could tolerate neuron loss; beyond these organ-specific thresholds, functional abnormalities and dilatation would begin to appear. Several authors have confirmed neuron cell loss in chronic Chagas disease (Lázzari 1994) and experimental studies in animals have shown that this neurological damage can occur early in the acute infection. Thus, the extent of *T. cruzi* infection in the acute phase and concomitant neurological damage may govern long-term prognosis, at least as far as the neurogenic form of chronic Chagas disease is concerned. Although some nerve cells may be parasitized, it is generally considered that ganglion cells are not destroyed as a direct result of parasitism. Early ganglion cell destruction led Köberle to the conclusion that a neurotoxin was produced by *T. cruzi*. Subsequent studies in experimental animals, however, have showed adsorption of *T. cruzi* antigens to uninfected tissue following pseudocyst rupture, with the implication that a T cell-mediated immune response could give rise to focal destruction of uninfected cells. Some of the physiopathological signs of chronic Chagas disease are compatible with dominance of the sympathetic system, predictable if the parasympathetic system is more exposed to damage from *T. cruzi* infection. Indeed, Oliveira demonstrated that the pathognomonic sign of apical aneurysm could be induced in rats, in the absence of *T. cruzi* infection, by inoculation of catecholamines (Oliveira 1969; Lázzari 1994). A sympathetic dominance is also apparently associated with sudden heart failure. Histochemical quantification of sympathetic and parasympathetic innervation has shown more severe parasympathetic denervation (Machado et al. 2000).

The trigger that leads some patients to the renewed inflammatory response and progressive decline of chronic myogenic Chagas disease is not clear, but the histopathological picture is suggestive of an autoimmune process. Release of sequestered antigens by destruction of normal cells to which *T. cruzi* antigens are adsorbed could lead to an autoimmune pathology with an unpredictable onset. Much research has been done in an attempt to elucidate the autoimmune pathogenesis of chronic Chagas disease. It seems clear that autoantibodies appear in experimental animals later than significant neuron destruction. Monoclonal antibodies to rat ganglia cross-react with *T. cruzi*; conversely, some monoclonal antibodies raised to *T. cruzi* cross-react with mammalian tissues. Numerous tissue cross-reactive antibodies have also been reported from patients with chronic Chagas disease. A possible candidate for a cross-reactive epitope between *T. cruzi* and normal host components is the C-terminus of the *T. cruzi* ribosomal P protein (Mahler et al. 2001). IgG fractions of chagasic sera contain antibodies to a motif shared by the C-term-

Figure 10 *Megacolon (Courtesy of Dr J.S. de Oliveira)*

inal region of the P0 ribosomal protein, the β_1-adrenoreceptor and the M_2 muscarinic receptor, yet are absent from control sera, and antibodies affinity-purified from chagasic sera modulated receptor function (Chiale et al. 2001). Putative cross-reactive epitopes have been described from mammalian proteins such as myosin: recognition of *T. cruzi* B-13 protein antigen and a corresponding epitope in human myosin heavy chain is said to be associated with chronic chagasic cardiopathy (Cunha-Neto and Kalil 2001). Several attempts have been made to implicate cell-mediated autoimmune pathology by studies in vitro, or by adoptive transfer of immunopathology with cells from infected donors to naive recipients: granulomata around the sciatic nerve have been induced experimentally by cell transfer and newborn syngeneic grafts were rejected by *T. cruzi* infected mice, but not by control mice (Ribeiro dos Santos et al. 1992). The CD4 T cell-dependent transplantation model of syngeneic mouse hearts grafted onto chronic chagasic recipients has suggested that anti-laminin antibodies might be used to suppress the process of autoimmune rejection (Silva-Barbosa and Savino 2000). In contrast, there is chronic persistence of the parasite (Schijman et al. 2004), and survival of syngeneic heart transplants in mice, with no signs of autoimmune rejection in the absence of direct parasitization of heart tissue (Zhang and Tarleton 1999; Tarleton 2001).

The pathogenesis of Chagas disease remains far from understood. A tenuous unifying hypothesis emerges from these diverse considerations: in the acute phase, neuron loss is precipitated by destruction of normal cells that have adsorbed *T. cruzi* antigens, which, dependent on the degree and extent of damage, is followed by a benign or catastrophic neurogenic clinical outcome; a relapse with progressive inflammation, and putative cell-mediated autoimmune involvement, occurs in an unfortunate minority of patients. Girones and Fresno (2003) review evidence for autoimmunity in chronic Chagas cardiomyopathy.

For a review in English of Chagas disease and the nervous system, see PAHO (1994); for an authoritative review on pathogenesis, see Lopes and Chapadeiro (2004).

DIAGNOSIS

In some acute *T. cruzi* infections, trypanosomes may be found transiently in the peripheral blood by direct microscopy of unstained, wet blood films. More scanty parasitemias can be detected by microscopy of Giemsa-stained thick blood films or by microscopy after concentration of trypomastigotes from blood. Concentration methods include:

- hematocrit centrifugation (with caution to avoid tube breakage and exposure to infection) followed by searching for trypomastigotes in or immediately above the Buffy coat layer
- allowing the blood to coagulate and searching for trypomastigotes in centrifuged serum (Strout's method)
- lysis of red cells with 0.85 percent ammonium chloride and centrifugation to sediment trypomastigotes.

In the indeterminate and chronic phases of the infection, *T. cruzi* is present in such low numbers in the peripheral blood that parasitemia is not patent to microscopy even after the above concentration methods. Xenodiagnosis, in which colony-bred uninfected triatomine bugs are fed on a suspect host and dissected about 20–25 days later to detect epimastigotes, or blood culture, are employed for parasitological diagnosis of chronic infections. Xenodiagnosis is generally favored for routine use in Latin America because, unlike blood culture, it does not require stringent aseptic precautions. Colonies of triatomine bugs are usually maintained by feeding on chicken blood, as birds are not susceptible to *T. cruzi* infection. If *T. infestans* is used, colonies must be examined periodically for presence of the monogenetic kinetoplastid *Blastocrithidia triatomae* as this may be confused with *T. cruzi* when bugs are dissected. Triatomine nymphs can survive for several months

without feeding and xenodiagnosis may thus be used in rural locations, as long as the bugs are protected from high temperatures. It is advisable to use a local species of domestic vector; *R. prolixus* and *T. infestans* are favored, *R. prolixus* feeds most avidly but may give rise to hypersensitivity skin reactions in individuals sensitized to bug bites. Precautions must be taken in dissecting infected bugs to avoid exposure to infection. Sterile diluents for bug feces should be used so that results are not confused by motile contaminants, such as ciliates. Under ideal conditions, blood culture on to a blood agar base with physiological saline overlay can be as sensitive as xenodiagnosis; cultures are inoculated 'through the cap' to minimize contamination (Miles 1993).

In central and northern South America *T. cruzi* must be distinguished from nonpathogenic *T. rangeli*, although in practice *T. rangeli* is almost never seen in human blood. *T. rangeli* and *T. cruzi* can both multiply in bugs used for xenodiagnosis. Long epimastigote forms with a small kinetoplast and pointed posterior end indicate the presence of *T. rangeli*, but such forms are not always present. In a proportion of infected bugs *T. rangeli* infections will progress to hemolymph and salivary gland infections (Hoare 1972).

Detection of parasite DNA is theoretically an attractive alternative to demonstration of the whole organism. PCR protocols have been developed that can show the presence of *T. cruzi* infection in the chronic phase. Although extremely sensitive after DNA extraction of blood samples, the PCR approach is not yet simple enough for routine diagnosis. In the long term, if adaptable to a low-cost rapid assay, PCR might be applicable to screening of transfusion blood or monitoring the success of chemotherapy (Castro et al. 2002).

In principle, serology detects exposure to infection rather than an active infection. In *T. cruzi* infection, however, both the infection and seropositivity are generally maintained for life unless specific antiparasite chemotherapy has been given. Only rarely have untreated individuals become seronegative (and presumably lost their infections). A sensitive and specific complement fixation text (CFT) was developed in 1913, but is now rarely used due to its complexity. Favored serological tests are the indirect fluorescent antibody test (IFAT), the enzyme linked immunosorbent assay (ELISA) and the indirect hemagglutination test (IHAT). Care is required with the IFAT to exclude nonspecific antibody binding (usually by selecting a high cut-off serum dilution between positive and negative sera, such as 1 in 80 or above). The ELISA requires careful standardization of conjugate batches, duplicate test wells, and positive and negative serum controls should be run on every plate. The IHAT is commercially available and convenient but said to be somewhat less sensitive than IFAT or ELISA, which is also commercially available (Oelemann et al. 1998). Serum or blood spots collected on filter paper can be used as the source of antibodies but serum provides a more accurate estimate of antibody titer. Cross-reactions may occur with other infectious diseases, notably visceral leishmaniasis and cutaneous leishmaniasis, which may be sympatric with Chagas disease. The advent of recombinant DNA technology has led to the testing of a series of *T. cruzi*-specific proteins selected from expression libraries (Umezawa et al. 2003). Detection of IgM antibodies (with IgM-specific conjugates) in newborn infants suspected of Chagas disease provides a means of helping to distinguish congenital infection from trans-placental transfer of IgG from mother to child.

In all cases, and especially in travelers returning from Latin America, clinical background should be explored thoroughly for a history of exposure to triatomine bites, blood transfusion or other sources of infection. Parasitological diagnosis is usually performed only if serology is positive. *T. cruzi* is isolated from only about 50 percent of seropositive individuals by xenodiagnosis, with 20 or more triatomine bugs, or by blood culture. Some clinical signs, such as right bundle branch block (RBBB), may be pathognomonic or highly indicative if associated with a supportive history. Hirschsprung's megacolon is seldom confused with chagasic megacolon because the former is rare in adults; when necessary, electromanometry or radiography can help with the differential diagnosis (Miles 1994)

EPIDEMIOLOGY AND CONTROL

Chagas disease is essentially a public health problem that is sustained by poor housing and poverty. The later nymphal stages and adult triatomine bugs are quite large (adults of some species may be more than 4 cm long) and, although they are reclusive insects, bug infestation is a considerable nuisance to householders. Heavy bug infestations would not be tolerated if there were lesser economic and health burdens in the affected communities. It follows that economic development, access to insecticides, improved housing, and health education should interrupt vector-borne transmission of *T. cruzi*, and this has proven to be the case in several successful control campaigns (Wanderley 1993). This is, however, of little comfort to the 11 or 12 million people who are already infected with *T. cruzi*.

A single (unsatisfactory) drug is now readily available for the specific treatment of Chagas disease; access to it may be life-saving in the initial stage of infection. Yet acute cases are seldom seen and recognized, and it is still debated whether the continued presence of the organism in the chronic stage, or repeated infection in endemic areas, has an affect on the long-term prognosis, which might largely be dictated by the severity of early infection. Treatment of chronic cases thus relies on alleviation of symptoms either by drugs or by installation of pacemakers, or by elegant surgical procedures

that have been pioneered in Brazil. Although new drugs are highly desirable, their epidemiological impact depends on their ability to eradicate infection from the reservoir of humans already carrying *T. cruzi*: this demands drugs that are inexpensive and without side-effects (Urbina 2002).

The prospects for vaccination against *T. cruzi* are remote, even though immunization has been shown to protect animals against fatal, acute phase infections, and will remain remote until autoimmune involvement in pathogenesis is fully understood and can be excluded as a complication of the antigen administration. Thus, little research is directed towards vaccine development, and it is difficult to justify in the face of successful campaigns to eliminate domestic triatomine bugs.

Elimination of domestic bug populations is an attainable goal, although eradication of *T. cruzi* is beyond reach as it is a widespread zoonosis, with numerous silvatic reservoir and vector species. The distribution of human Chagas disease is somewhat enigmatic, and changing as both the spread of domestic vectors and destruction of forest vector habitats may lead to the emergence of new endemic areas. Much remains to be learned about the complexities of the epidemiology of Chagas disease in the context of parasite, vectors and reservoir hosts.

Epidemiology (including reservoir hosts)

The blood-sucking insect vectors of Chagas disease are a subfamily (Triatominae) of the Reduviidae in the order Hemiptera. Five tribes, 14 genera, and 130 species of triatomine have been described based on morphological characteristics (Lent and Wygodzinsky 1979; Carcavallo et al. 1997). Thirteen species occur in the Old World, and the remaining 105 are confined to the Americas. Seven of the 14 Old World species are related to *Triatoma rubrofasciata*, which occurs worldwide in ports with its host the rat (*Rattus rattus*), to which it transmits *Trypanosoma conorhini*; the remaining six Old World species are grouped into the unusual Indian genus *Linshcosteus* (Galvao et al. 2002).

Most New World triatomine species have silvatic ecotopes, such as palm trees, burrows, hollow trees, rock crevices or caves, where they feed from mammals, birds or reptiles, and many of the New World species have been reported as infected with *T. cruzi*. Five species, of three genera, have adapted to colonize domestic habitats and are responsible for abundant and widespread household infestations; they are: *Triatoma infestans*, *Rhodnius prolixus*, *Panstrongylus megistus*, *Triatoma brasiliensis*, and *Triatoma dimidiata*.

T. infestans is the main vector in the southern cone countries of South America (Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay) and in southern Peru; it is thought to have spread from the Cochabamba region of Bolivia, where it occurs in rocky silvatic ecotopes: outside Bolivia *T. infestans* is said to be restricted to domestic and peridomestic habitats. *R. prolixus* is the most important vector in northern countries of South America and in central America, with *T. dimidiata* a significant secondary vector. *P. megistus* occurs along the eastern seaboard of Brazil, which was formerly covered by a continuous zone of Atlantic forest, and in central-eastern Brazil. *T. brasiliensis* is the vector in the arid northeastern region of Brazil. *Triatoma sordida*, although extremely widespread in central and eastern South America and often described as an important domestic vector, is usually associated with chickens. Many other species are reported from houses but usually as small, infrequent colonies, or as adult bugs flying into houses attracted to light.

Triatomines are considered to be obligatory blood feeders; most species take mammalian or bird blood but some feed on reptiles. At least one genus (*Eratyrus*) is known to take blood also from large invertebrates that co-inhabit its natural hollow tree ecotope (Miles et al. 1981a). Each nymphal stage must take at least one bloodmeal before molting to the next. Bug pheromones in feces may lead to aggregation of vectors at feeding sites, such as roosting chickens. As there is no transovarial transmission of *T. cruzi*, older bugs are more likely to have fed from an infected host and are, therefore, more likely to be infected.

Adults of all species are winged, with the exception of the Chilean *Triatoma (Mepraia) spinolai*, which has wingless females and either winged or wingless males. Nevertheless, adult triatomines are not frequent flyers; they are almost never observed to fly in laboratory colonies unless artificially induced to do so, but may be seen flying in infested houses at dusk, particularly after heavy rain. Flight is known to be an important method of dispersion since adult bugs of some species commonly fly into houses. Adhesive eggs aid passive dispersion of *Rhodnius*, and nymphal stages can move from host to host by walking. The few natural predators and parasites of triatomines include wasps (*Telonomus*, *Gryon*) that develop in triatomine bug eggs, spiders, lizards, chickens, rodents and various other insectivores, parasitic nematodes, and fungi. Immunological analysis of insect food sources can be used both to determine sources of blood in bugs and to identify their natural predators by detecting ingested bug proteins. When alarmed some triatomines produce the distinctive smell of isobutyric acid, and make sounds by stridulation, in which the proboscis is rubbed in a ridged ventral groove. *Rhodnius* has climbing organs and can scale very smooth surfaces, possibly due to the need to climb smooth-trunked palms and palm-frond stems in its natural arboreal habitats. The distribution of domestic vector species within houses may reflect silvatic habitat preferences: thus *R. prolixus* heavily infests roofs made of palm fronds, and palm crowns are the principal

habitat of the genus; *P. megistus* is most frequently found in compressed mud and wooden framed walls, and its most frequent natural habitats are hollow trees or burrows among tree roots; *T. infestans* can be found in quite good quality housing living in the walls or among tiles at the edges of roofs.

More than 150 species of 24 families of mammals have been reported as infected with *T. cruzi*, and all mammal species are considered to be susceptible. Although birds and reptiles are insusceptible to *T. cruzi* infection, domestic chickens are extremely important epidemiologically because they sustain heavy triatomine bug infestations either at roosting sites inside houses, or in neighboring chicken houses, which may be overlooked during spraying campaigns. Important domestic hosts, apart from humans, are dogs, guinea pigs, cats, rats, mice, and any other mammals sleeping inside houses and closely associated with humans. Dogs are particularly important because of high prevalence rates of infection and as they are often abundant in endemic areas (Cohen and Gurtler 2001). Cats, although less frequently studied, also have high prevalence rates and possibly acquire infection via the oral route, by eating either triatomine bugs or house mice that have fed on them. Although smaller hosts, rodents may be abundant and provide an important source of infective bloodmeals. Pigs, goats, cattle, and equines usually have indirect contact with houses and very low prevalence rates of infection, although their enclosures may be infested with bugs.

The most ubiquitous silvatic reservoir host of *T. cruzi* is the opossum, *Didelphis*, which occurs throughout much of the range of *T. cruzi* in the Americas. Multiple nesting or resting sites of *Didelphis* encompass many types of triatomine habitat. High *T. cruzi* prevalence rates may be in part due to the fact that opossums will eat triatomines and might also transmit infection via anal gland secretions, in which infective metacyclic forms typical of the vector stage of the *T. cruzi* life cycle can occur. *Didelphis* also provides an important potential link between silvatic and domestic habitats as it occasionally nests around or inside houses. Edentates (armadillos and anteaters), especially the armadillo *Dasypus novemcinctus*, are commonly infected with *T. cruzi* and their epidemiological importance requires further investigation; they have contact with human households through hunting of armadillos and their consumption for food. *P. geniculatus*, a silvatic triatomine bug frequently found in armadillo burrows, is one species that is attracted to light and often flies into houses. Rodents are thought to have an important epidemiological role as in times of food scarcity or drought they might introduce triatomines and *T. cruzi* strains into new peridomestic nesting sites. Similarly, bats may roost in domestic habitats, encouraging triatomine infestation and bringing in *T. cruzi* strains. The numerous carnivore and primate species that may harbor *T. cruzi* have tenuous links with domestic transmission cycles.

Silvatic cycles of *T. cruzi* transmission extend approximately from southern Argentina and Chile (latitude 46°S) to northern California (latitude 42°N). Human infection is, however, much less widespread, from 44° 45′S in Argentina to the southern states of the USA. Prevalence estimates for human infection are based on rates of seropositivity (Miles 1982). National surveillance by serology indicates that Brazil has the largest number of infected individuals (about 5 million) in accordance with its land mass and population. Bolivia is reported to have the highest prevalence, with seropositivity rates of greater than 70 percent in some localities, and heavy *T. infestans* infestation of rural communities that are difficult to access. The World Health Organization has provided an outline of geographical distribution of *T. cruzi* infection within the affected countries (WHO 2002). As described above, endemic areas can be classed as having either separate domestic and silvatic transmission cycles, with separate vector species (e.g. eastern Brazil) or overlapping domestic and silvatic transmission cycles (e.g. parts of Venezuela and northern Brazil). Enzootic regions have rare human infections with sympatric (e.g. USA) and sometimes abundant (e.g. Amazon basin) silvatic transmission cycles (Miles et al. 1981a). Locally acquired Chagas disease is rare in the USA, partly because of higher living standards and better housing. Although bugs are occasionally found in and around houses with dogs (Beard et al. 2003) they infrequently attack humans, and transmission of *T. cruzi* is less likely as the local bug species tend to defecate after feeding. Occasionally adult bugs are attracted by light into camp sites. Silvatic bugs are widespread in the USA and *T. cruzi* infections are common in opossums, raccoons, and wood rats (*Neotoma*). Nevertheless, transmission might also rarely occur in the USA due to transfusion of blood from infected immigrant Latin American donors (Schmunis et al. 2001).

Around 300 autochthonous human *T. cruzi* infections have been reported from the vast forested Amazon basin, even though there are extensive roadside and riverine communities with houses ideally suited to colonization by triatomine bugs and *T. cruzi* infections abound among species of forest mammals. Tracing of trapped mammals to their nesting sites with a spool-and-line mammal-tracking device (Miles et al. 1981b) has helped to describe the ecotopes of silvatic triatomines, and show that the 13 known species of the central Amazon basin do not readily adapt to household infestation (Miles et al. 1981b). *R. brethesi*, which infests the piacaba palm (*Leopoldinia piacaba*), is reported to attack forest workers harvesting the palm fronds and sleeping nearby, some of whom acquire infection. *P. geniculatus*, normally associated with high humidity within armadillo burrows and difficult to adapt to laboratory colonies, has recently been reported infesting domestic pigsties in Pará state, Brazil and attacking the inhabitants of adjoining houses (Valente et al. 1998).

The destruction of natural habitats around houses in the Amazon may well encourage triatomine infestation, especially as wood and palm fronds are often used in house construction, inevitably bringing some silvatic vectors into houses (Coura et al. 2002).

Nonvectorial transmission of *T. cruzi* that is epidemiologically important includes transmission by blood transfusion, congenital transmission, and oral transmission. Where prevalence is high, seropositivity rates among blood donors may reach 20 percent (endemic localities in Brazil and Argentina) or even higher in Bolivia (60 percent) (WHO 2002). In large cities where there are many migrants from rural areas and many blood transfusions, a significant proportion of recipients will become infected in the absence of serological surveillance of donors or treatment of transfusion blood to destroy *T. cruzi*; there may be thousands of blood-borne infections each year. Control of blood-borne transmission is thus an essential part of campaigns directed primarily at the elimination of domestic triatomine bugs. In endemic areas where prevalence is high, many pregnant women will carry *T. cruzi*. Estimates vary widely but a low percentage (less than 5 percent) of infants born to seropositive mothers may acquire congenital infection (WHO 2002). If resources allow, serological screening of pregnant women, serological and parasitological follow-up of the newborn, and prompt chemotherapy of infected infants, can reduce mortality due to congenital infections (Blanco et al. 2000). Determination of rates of transplacental acquisition of infection are complicated by passive transfer of maternal IgG antibodies to *T. cruzi*, which may cause infants to be seropositive for several months after birth even in the absence of infection. There is convincing evidence of oral transmission and a high proportion of the few human cases in the Amazon basin are thought to have been acquired by this route (Coura et al. 2002). The most likely sources in such outbreaks are contamination of food with triatomine bugs, or with vector-like forms from opossum anal gland secretions, or through consumption of the uncooked meat or blood of an infected reservoir host. An increasing problem is the transfer of *T. cruzi* to organ recipients from infected donors, who must therefore be routinely screened for seropositivity. If already infected with *T. cruzi*, recipients risk a recrudescent acute phase infection in response to immunosuppression. Similarly, human immunodeficiency virus (HIV)-positive patients who develop AIDS may reactivate *T. cruzi* infection and develop meningoencephalitis (Lazo et al. 1998). *T. cruzi* infections in the immunocompromised will become epidemiologically more important as human migration to South American cities continues and urban HIV transmission spreads to rural areas that have endemic Chagas disease (de Gorgolas and Miles 1994).

TREATMENT

Chemotherapy

Two drugs have been used for specific chemotherapy of *T. cruzi* infection, nifurtimox and benznidazole (WHO 2002). Nifurtimox, a synthetic nitrofuran given by mouth and absorbed through the gastrointestinal tract, is no longer readily available. Dose rates employed were 8–10 mg per kg orally in three divided daily doses for 90 days, using tablets of 30, 20, or 250 mg. Higher doses of 15–20 mg per kg in four divided daily doses for the same period were recommended for infected children. Successful treatment thus required either hospitalization or careful monitoring to ensure compliance. Side-effects could include anorexia, nausea, vomiting, gastric pain, insomnia, headache, vertigo, excitability, myalgia, arthralgia, convulsions, and peripheral polyneuritis, and could lead to interruption of treatment. Nifurtimox is thought to act against *T. cruzi* by increasing oxidative stress through the production of free oxygen radicals. Benznidazole is a nitroimidazol which is also absorbed from the alimentary tract. Doses are 5–7 mg per kg orally in two divided doses for adults for 60 days with 100 mg tablets, and 10 mg per kg similarly for children. Adverse affects may include rashes, fever, nausea, peripheral polyneuritis, leukopenia, and, rarely, agranulocytosis. Benznidazole is thought to act by interaction of drug metabolites with DNA but not via oxidative stress.

Cure rates with both nifurtimox and benznidazole are not total and vary regionally. Suppression of parasitemia during acute infection may be life-saving and treatment is, therefore, recommended during the acute phase. The chronic phase is not always treated because the influence of persistent infection on long-term prognosis is uncertain, and there is a significant failure rate, such that up to half of patients may remain infected after treatment. Nevertheless, treatment is better tolerated in children, leading to the suggestion that, as well as acute cases, chronic cases in children might also always be treated (Estani et al. 1998). Treatment is essential for immunocompromised patients, especially if meningoencephalitis is present, in which case double or even higher dose rates may be recommended. Pre-emptive therapy has been described as safe and effective for preventing reactivation of *T. cruzi* infection during bone marrow transplantation (Dictar et al. 1998). In congenital cases, either drug has been used, nifurtimox at 8–25 mg per kg daily for 30 days and benznidazole at 5–10 mg per kg daily for 30–60 days (WHO 2002). Indicators of elimination of the parasite are negative xenodiagnosis together with, in acute cases, serological reversion within a year. However, negative parasitology is not sufficiently sensitive to prove absence of infection and, for chronic

cases, reversion of serology may take decades (Rassi and Luquetti 2003).

Allopurinol and itraconazole have been suggested as low cost, nontoxic alternatives for treatment of *T. cruzi* infection, but their efficacy is in doubt. Allopurinol, a structural analogue of hypoxanthine that is metabolized via the purine salvage pathway, prevents formation of ATP, and also interferes with protein synthesis by its incorporation into RNA. Experimental drugs thought to act against the enzyme sterol methyl transferase are under development, have shown promising activities in treatment of animal infection, and may proceed to clinical trial (Urbina 2002; Urbina et al. 2003).

Additional chemotherapy is an important part of supportive treatment in both acute and chronic Chagas disease (WHO 2002). Severe acute infections may require treatment for fever, vomiting, diarrhea, and convulsions; if heart failure is present, sodium intake is restricted; diuretics and digitalis may be recommended; anticonvulsants, sedatives, and intravenous mannitol may assist management of acute meningoencephalitis. Chronic chagasic heart disease may require treatment of heart failure through reduced activity, limitation of sodium, diuretics, vasodilation (angiotensin converting enzyme inhibitors), and maintenance of normal serum potassium; digitalis is only advisable as a last resort for treatment because it may aggravate arrhythmias by inducing premature ventricular beats or impeding AV conduction. A pacemaker may be required to manage bradycardia that does not respond well to atropine, or atrial fibrillation with a slow ventricular response in which vagolytic drugs are not effective, or complete AV block.

Life expectancy of patients with ventricular arrhythmias may be prolonged by treatment to prevent ventricular tachycardia or ventricular fibrillation: lidocaine, mexiletine, propafenone, flecainide, β-adrenoreceptor antagonists, and amiodarone are effective for treatment of ventricular extrasystoles; amiodarone is the most effective drug to treat arrhythmias, but in all cases drug treatment of arrhythmias may have an aggravating effect and patient management may be a complex combination of the use of drugs and a pacemaker. Lidocaine may be used intravenously in emergencies prior to drug administration orally. Surgical resection of arrhythmic endocardial regions may be considered, and surgical resection of ventricular aneurysms has been suggested but not extensively used. Expert reports (WHO 2002) and physicians who have specialized in management of Chagas disease should be consulted before case management can be embarked upon with confidence.

Surgical treatment

Elegant surgical procedures have been developed in Brazil to treat severe chagasic megacolon and mega-esophagus. Laxatives, colonic lavage or manual evacuation may be used to relieve symptoms of megacolon, with laparotomy if the fecaloma cannot be reached. Sigmoid volvulus can be relieved by decompressing intubation or surgery. Sigmoidostomy close to the rectosigmoid junction may simplify subsequent surgery to correct megacolon as it allows more of the colon to be retained.

Surgical treatment of megacolon is often based on the operation of Duhamel. The modified Duhamel–Haddad procedure consists of resection of the sigmoid loop, closing of the rectal stump and insertion of the descending colon through the rear wall of the rectum. The Haddad improvements introduced a two-stage operation, in which the lowered colon is first exteriorized through the retrorectal stump as a perineal colostomy and, secondly, with peridural anesthesia, the stump of the colon is sectioned into anterior and posterior halves, the anterior wall of the colon and posterior wall of the rectum held together in an inverted V, and then cut and sutured together to make a wide join between the colon and rectal stump (Figure 11). A series of 624 Duhamel–Haddad operations between 1966 and 1981 had an overall associated mortality of 6.6 percent (Moreira et al. 1985).

Mega-esophagus can be treated by dilatation of the cardiac sphincter or the Heller–Vasconcelos surgical procedure in which a portion of the muscle is removed from the junction of the esophagus and stomach without disturbing the muscular control of the wall of the stomach. More severe mega-esophagus may require partial removal of the distal esophagus and replacement with alternative portion of the alimentary tract such as jejunum. These and other surgical procedures are described in detail in the Brazilian literature (Raia 1983).

VACCINATION

Administration of crude *T. cruzi* lysates to experimental animals has been known for many years to suppress parasitemia, to prevent mortality from a subsequent

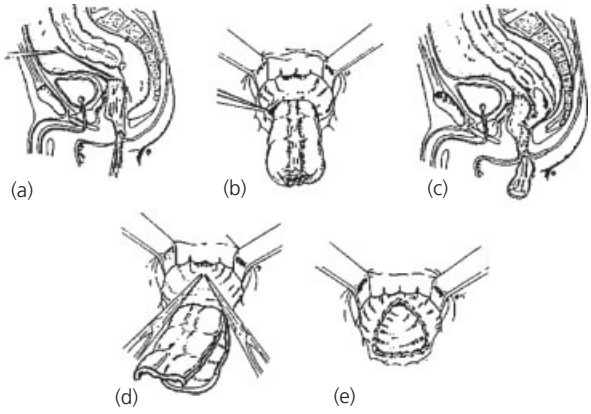

Figure 11 *The modified Duhamel–Haddad procedure for surgical correction of megacolon*

challenge infection, and yet not to prevent re-infection. 'Attenuated' *T. cruzi* strains have been used as experimental vaccines in human trials with small numbers of individuals, but organisms were not necessarily entirely non-infective, and are not known to have conferred resistance to infection (or superinfection). Various semi-purified or purified antigen preparations have similarly been shown in experimental models to confer protection against mortality on challenge but not to provide a sterile immunity (Taibi et al. 1993). An autoimmune response may have a role in the pathogenesis of Chagas disease, and this does not encourage research on candidate vaccines. Without a full understanding of the potential autoimmune involvement in pathogenesis it is difficult to envisage clinical trials of vaccines: subjects could not be given a live challenge, and would have to be followed for many years for the appearance of vaccine-induced pathology in the absence of infection. These aspects of Chagas disease suggest that resources would be most cost-effectively directed to further improvement of vector control campaigns rather than vaccine development. Immunotherapeutic modulation of progressive autoimmune damage in chronic Chagas disease has been proposed: if the intricacies of immune responses in the pathogenesis of Chagas disease can be convincingly unraveled, this may become possible for a small number of privileged patients.

INTEGRATED CONTROL

In 1948, successful trials of the organochlorine insecticide BHC for control of domestic triatomine bug infestation by Emmanuel Dias and Jose Pellegrino in Minas Gerais, Brazil, prompted their telegram to the Brazilian Ministry of Health suggesting that the end to vector-borne *T. cruzi* transmission was in sight (Miles 1996). The fundamental value of insecticides for control of triatomine bugs has never since been in doubt. Nevertheless, much research has been devoted to developing new methods of eliminating domestic triatomines, including the use of parasitic wasps, nematodes, and fungi; sophisticated traps for catching bugs; and juvenile hormones for preventing development of nymphal instars into adults. These alternative weapons have failed to have significant impact on vector populations.

A combination of insecticide spraying, health education or community participation and housing improvement has been the basis of successful national control programs, a superb example of which is the elimination of domestic *T. infestans* populations from São Paulo State in Brazil (Wanderley 1993). João Carlos Pinto Dias, the son of Emmanuel Dias, has led the elaboration of a systematic approach to triatomine bug control (Dias 1987).

- In the preparatory phase, all houses in the endemic area are mapped and sampled manually with flashlight and forceps for evidence of triatomine infestation: sampling may be assisted by spraying irritant pyrethroids on to suspect wall areas to dislodge bugs hiding in crevices. This mapping and sampling phase allows for the cost of insecticides, personnel, transport and equipment to be calculated, and for detailed planning of operations.
- In the attack phase, all houses and outbuildings are sprayed, irrespective of whether they are known to be bug infested. Failure to spray all infested domestic and peridomestic habitats may lead to rapid local re-emergence of bug populations. The entire spraying may be repeated during the next 3 months if live bugs can still be found in more than 5 percent of houses (except where there is heavy peridomestic infestation and a close link with adjacent silvatic cycles).
- In the vigilance phase, houses are sampled periodically through a surveillance system established with the local community and supported by health education. Thus, householders can report any residual infestation to a local volunteer who mobilizes an immediate insecticide spraying response. Simple surveillance kits consist of a sheet of paper pinned to walls suspected of infestation, and a plastic bag. Periodic examination of the paper may reveal either bug feces or bugs concealed between the paper and wall; the plastic bag is used by householders to collect any live bugs, with care to avoid contamination with bug feces. The vigilance phase is also supported by continuing contact with public health personnel. Although triatomines can sometimes be found in quite good quality housing, for example in tiled roofs (*T. infestans*) or in beds that are not regularly dismantled and cleaned, poor housing is most vulnerable to bug infestation. Encouraging and supporting the community to improve local housing, for example by plastering cracked walls, can contribute to the success of a control campaign. In houses infested by *R. prolixus* it is advisable to replace roofs of palm fronds by alternative materials.

In some localities, for example with domestic and silvatic *Triatoma brasiliensis* in northeastern Brazil, there may be heavy peridomestic infestation and a close link with silvatic habitats, such that rapid re-invasion can occur. In such cases, surveillance (rather than a complete respraying program of all houses and outhouses) may be implemented when the overall infestation rate is greater than 5 percent. Similarly, modification to control strategies, or measures to deal with silvatic foci, may be required at other sites with interaction between silvatic and domestic transmission cycles.

Early spraying campaigns used chlorinated hydrocarbons but their short residual activity (30–180 days) required repeated applications. Carbonates and organophosphates have also been used but the former are expensive and the latter are not well received by

communities because of their strong smell. Synthetic pyrethroids have been adopted for triatomine control since 1980 as they have a long residual activity and low toxicity, and several formulations are now available (Schofield 1994). Insecticide resistance (to dieldrin) is known only from small areas of Venezuela and has not interfered with control campaigns.

In 1991, an initiative was launched to use established triatomine control methods in a cooperative international campaign, referred to as the Southern Cone Programme, and designed to eliminate domestic *T. infestans* populations from the Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay, with spraying of infested regions that spanned national frontiers intended to prevent re-infestation (Miles 1992). The strategy for this coordinated international program incorporated World Bank recommendations on the need for clear lines of responsibility, regional integration, and pragmatic local autonomy. The Southern Cone Programme has attracted new financial support and stimulated a vigorous and sustained effort. The overall cost of the programme is estimated to be a fraction of that of dealing with the burden of new cases of Chagas disease. An international review of the programme indicates a huge reduction in the geographical area infested by *T. infestans* and a determination to deal with those regions still affected. Miles et al. (2003b) summarize key features governing the success of the programme. Further intergovernmental control collaborations are being established in Central America, the Andean region and Mexico (Schofield and Dias 1998; Dias et al. 2002). Although the Amazon Basin is free of domestic bug populations, it is under threat from migration of species such as *T. infestans* and *R. prolixus* and from adaptation of local forest species to houses (Coura et al. 2002). A network of collaborating centers has recently been established to train local health workers in the recognition of triatomine bugs and acute cases of Chagas disease, and to plan responses to the appearance of domestic bug populations. In the past, lack of funds and poor continuity frequently interrupted control campaigns, in part because resources were diverted to resurgent malaria or outbreaks of dengue. It is essential that campaigns against different arthropod vectors are integrated, at both local and regional levels.

T. cruzi is one of several disease agents, including HIV infection, hepatitis, syphilis, and malaria, that can be transmitted by contaminated transfusion blood. Screening of donors is an essential component of control campaigns and in almost all South American countries serological testing is mandatory. Effective serological tests include the IFAT, which can be used to distinguish maternal transfer of IgG from IgM due to congenital infections, the ELISA, the IHAT, and the CFT, although the latter is now seldom used. A single standardized test may be adequate, but simultaneous use of two tests is often recommended, and some cross-reactions with diseases such as visceral leishmaniasis are inevitable. It has been shown that CFT, IFAT, IHAT, and ELISA can give comparable results. The IFAT has been employed in large scale surveys in Brazil; ELISA has distinct advantages for survey work as it is highly sensitive, relatively low cost, and does not require microscopy. In some countries, such as Chile, cross-reactions with leishmaniasis are not a problem as visceral and cutaneous leishmaniasis are not endemic. Rigorous internal and external quality control of assay performance are required with repetition of a proportion of tests in a central laboratory and in collaborating external laboratories. Sample collection and record systems must be highly efficient and integrated with spraying operations, health education, and community participation aspects of control. The importance of screening blood for HIV infection in major urban areas is likely to improve access to screening for *T. cruzi* infection. Where seropositive rates are very high, positive transfusion blood may be treated with 125 mg of crystal violet per 500 ml and the blood stored at 4°C for 24 hours to destroy trypomastigotes. Side-effects are blue coloration of mucosae and skin in recipients of blood treated with gentian violet.

Serology is a vital and highly effective method of monitoring the success of control programs. In its simplest form, success is demonstrable by zero serological incidence in children born after the initiation of intervention measures. Thus, for a 10-year campaign, 5 years and 10 years after the beginning of vector control no vector-borne infections should occur in children aged 0–5 and 0–10, respectively. Pre-school children and school children obtaining basic or primary and junior schooling (e.g. ages 1–12 years) provide the ideal populations for serological surveillance. Baseline serological data are useful but not essential to planning serological surveillance of control: if interventions are effective, no significant increase in prevalence of seropositivity should occur for any age group after the campaign begins.

Ideally, all children in populations possibly exposed to vector-borne transmission should be tested as serology is a sensitive independent aid for the detection of residual triatomine infestation. Thus, one seropositive case, if congenital transmission and blood transfusion transmission are excluded, may lead to the identification of a single house that has escaped elimination of bugs. In countries such as Chile, which have a relatively small population, good infrastructure, preschool rural health clinics, regional hospitals, demographic data that include individual identification codes, and clearly defined areas of disease transmission, it is feasible to monitor the entire population exposed to transmission. In other countries, such as Brazil, with a very large population in endemic areas, it may not be possible or appropriate to survey the entire population of preschool and school children. There are two obvious alternatives for selection of sub-populations: the first, on a random sample

basis, and the second, where effort is concentrated on highly endemic localities. The second alternative is preferred as only in areas with relatively high seroprevalence will a detectable number of the very young acquire infection. Baseline serological data are available for Bolivia, Brazil, Peru, Argentina, Paraguay, and Uruguay to allow such sentinel sites of high endemicity to be selected. It might also be appropriate to survey localities at the margins of endemic areas where explosive outbreaks of disease, transmission might occur in previously unexposed populations.

Social and economic development in Latin America should, in time, eliminate vector-borne and blood-borne transmission of *T. cruzi* as a major public health problem. *T. cruzi* will, however, remain as a widely distributed enzootic infection, with the threat of re-emergence and spread to new areas of human settlement. The burden of management of chronic disease will be a problem for several decades. Further research on immunological evasion and molecular pathogenesis might improve prognosis for those already infected, and a low-cost, nontoxic drug may help eliminate *T. cruzi* from the human reservoir of infection. It is debatable whether other aspects of advanced research will have a significant direct impact on the control of Chagas disease, but they will lead to a fuller understanding of the fascinating epidemiology and biology of trypanosomatid infections.

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Intestinal coccidia: cryptosporidiosis, isosporiasis, cyclosporiasis

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CRYPTOSPORIDIOSIS

Introduction

The first description of the genus *Cryptosporidium* was by the American parasitologist E. E. Tyzzer (1907) who obtained, in considerable detail, the life cycle of *C. muris* present in the gastric glands of the common mouse. An unusual feature of development was that the parasite was located at the apical surface of the epithelium. The only major modification that has been made to Tyzzer's description of the life cycle is that the developing stages are intracellular. However, this observation had to wait until electron microscopy was available. The genus name refers to the absence of sporocysts that normally enclose the sporozoites of coccidia (from Greek 'kruptos' meaning 'hidden'). Tyzzer (1912) later identified the smaller parasite *C. parvum*, in the intestinal villi of mice, that is now recognized as a major cause of mammalian cryptosporidiosis. The same worker subsequently reported cryptosporidial infection of turkeys (Tyzzer 1929). Over many years, *Cryptosporidium* infection was described in different host types and numerous new species were named accordingly. In the early 1980s, however, studies of transmission from one host-type to another indicated host-specificity was less stringent than for some other coccidia, and that there was only a small number of legitimate species of *Cryptosporidium* including *C. parvum* and *C. muris* of mammals plus a few others that infect either birds, reptiles or fish (Fayer et al. 1997). More recent genomic analyses combined with limited animal infectivity studies, however, have suggested that the taxonomy is more complicated and so the number of species is likely to increase in future (Morgan et al. 1999).

Until the 1950s, it was not appreciated that *Cryptosporidium* could be pathogenic but even then, a report from Slavin (19z55) of severe diarrhea and some deaths in a turkey flock infected with the species named *C. meleagridis* attracted little attention. The situation gradually altered starting in the 1970s when a few cases were identified first in cattle (Panciera et al. 1971) and then in humans (Meisel et al. 1976; Nime et al. 1976). With the development of simple diagnostic methods in the 1980s, it became clear that *C. parvum* is a common cause of diarrhea in humans worldwide and also in neonatal domestic mammals such as bovine calves and lambs (Tzipori 1983). In human cryptosporidiosis, diarrhea and other symptoms normally last for 1–2 weeks but infection of hosts with impaired T-cell-mediated immunity, such as individuals with acquired immune deficiency syndrome (AIDS), may be life-threatening and chronic (Current and Garcia 1991). The significance of *Cryptosporidium* as a public health problem has been increased by the lack of an effective chemotherapeutic agent and the transmission of infection through public water supplies (Coombs 1999; Rose et al. 2002).

Classification

The protozoan genus *Cryptosporidium* belongs to the phylum Apicomplexa, which comprises several thousand species that possess an apical complex: a specifalized assembly of organelles believed to be involved in host cell invasion (Levine 1984). *Cryptosporidium* spp. are contained in the suborder Eimeriorina along with other

coccidia that cause human or animal disease, *Cyclospora*, *Isospora*, *Toxoplasma*, and *Eimeria*. The oocysts of these latter genera have sporocysts that enclose sporozoites, whereas oocysts of cryptosporidia lack sporocysts and have 'naked' sporozoites; *Cryptosporidium* has, therefore, been placed within a new family, Cryptosporidiidae.

Cryptosporidium spp. were originally designated according to oocyst morphology, site of development, and host specificity. The selection of the latter criterion was presumably based on the assumption that the genus had the restricted host range of related coccidia such as *Eimeria* and *Isospora*. Beginning in the 1980s, the results of cross-transmission studies challenged this view and the number of species was eventually truncated to eight: *C. parvum* and *C. muris* of mammals, *C. wrairi* of guinea-pigs, *C. felis* of cats, *C. baileyi* and *C. meleagridis* of birds, *C. serpentis* of reptiles, and *C. nasorum* of fish (reviewed in Morgan et al. 1999). Cryptosporidiosis of mammals has until recently almost always been associated with *C. parvum*, which has small oocysts (4–5 μm) and infects mainly the intestine, while *C. muris* has larger oocysts and infects the stomach of mice – and perhaps cattle – and is not considered to be a serious pathogen (Upton and Current 1985)

However, evidence from investigations of isoenzyme and antigenic composition of oocysts as well as restriction fragment length polymorphism (RFLP) analysis suggested that *C. parvum* may not be a single species. These studies could clearly distinguish human from animal – mainly bovine – isolates (Awad-El-Kariem et al. 1995; Nichols et al. 1991; Ortega et al. 1991). Furthermore, there are differences between human and bovine isolates in their ability to infect separate host species: bovine isolates are able to infect cattle, mice or humans whereas human isolates usually cannot infect animals (Awad-El-Kariem et al. 1998; Widmer et al. 2000). More recent evidence from gene sequencing (e.g. 18S rDNA, cryptosporidial oocyst wall protein (COWP) and thrombospondin-related adhesion protein (TRAP) and phylogenetic analyses have confirmed this distinctiveness of human and animal isolates, which are now designated Type 1 and Type 2, respectively (Peng et al. 1997; reviewed in Morgan et al. 1999). Additionally, unique *Cryptosporidium* genotypes have been found in oocysts from other mammals, including pigs, dogs, and ferrets (Morgan et al. 1999).

Life cycle

The general features of the life cycle of *C. parvum* are similar to those of other coccidia that infect mammals (Tyzzer 1912; Current and Reese 1986). A unique feature of intracellular development, however, is that the parasite is anchored at the luminal surface and isolated from the host cell cytoplasm. The entire development of the parasite takes place within the same host and begins following ingestion of oocysts. Sporozoite excystation takes place in the intestine under the influence of numerous factors, including temperature and bile salts (Sundermann et al. 1987) and the parasites attach by the apical pole to the brush border of epithelial cells. The attached sporozoite induces local cytoskeletal rearrangement in the host cell and the brush border membrane envelops the sporozoite forming the parasitophorous vacuole (Chen et al. 1998). Parasite antigens are expressed on the parasitophorous vacuole and on part of the outer host cell membrane surrounding the parasite (McDonald et al. 1995). At the site of attachment, interactions between the parasite and host cell components result in the formation of the so-called feeder organelle, which expands as the parasite grows and acts as a barrier between the host and parasite cytoplasms.

The intracellular sporozoite soon rounds up into a trophozoite and, following a period of growth, the parasite undergoes asexual multiplication by merogony (schizogony). The parasite development induces activation of the transcription factor NF-κB in the host cell which inhibits the cell from entering apoptosis (Chen et al. 2001). Eight merozoites are produced which continue the reproductive cycle following invasion of other host cells (Figure 1). Eventually meronts with four merozoites develop and the latter may initiate the development of microgametes (Figure 2) and macrogametes (Current and Reese 1986). Fertilization of the gametes follows and two types of oocyst are formed that undergo sporogony within host cells. The first oocyst type, representing about 20 percent of oocysts, has a thin unit membrane and is able to re-initiate the life cycle in the host. This stage may play an important role in maintaining the infection, particularly in immunocompromised hosts who fail to eliminate the parasite. The second type of oocyst, which has a double-layered or thick wall, accounts for about 80 percent of oocysts and is passed in feces (Current and Reese 1986). The development of an autoinfective stage and maturation of oocysts within the host cell are unusual characteristics for coccidia. Oocysts of *Eimeria* and *Isospora* are of a single type and normally have to escape the host before they undergo sporulation, aided by a reduced temperature and increased amount of oxygen.

Parasite cell and molecular studies

At present, there is no consistently effective chemotherapy for cryptosporidiosis and the formulation of novel drugs may depend on rationale design based on targets identified from studies of parasite molecular functions. Studies in cell biology, biochemistry, metabolism, and molecular genetics have been, for the most part, limited to the examination of oocysts and sporozoites, which can be obtained in copious amounts from large animal infections. The biology of intracellular

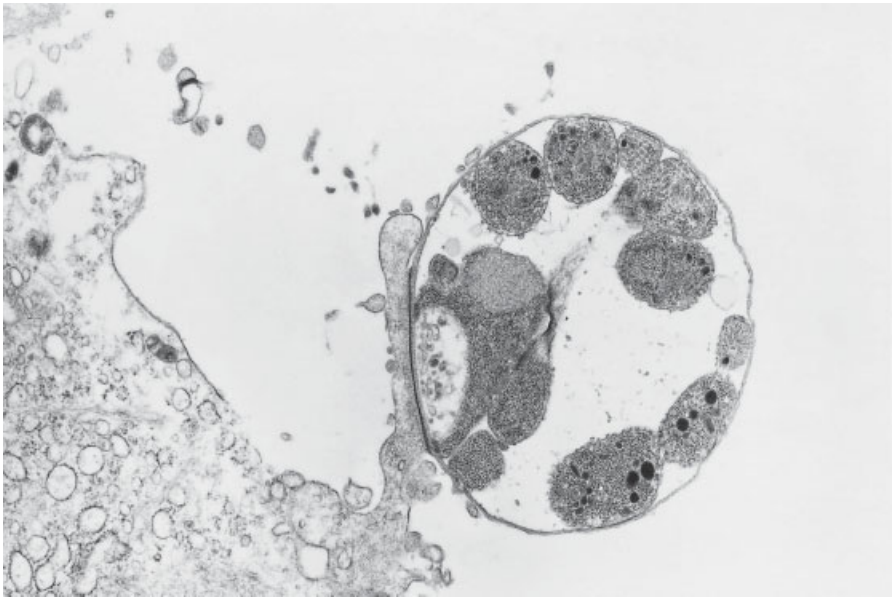

Figure 1 *Electronmicrograph of* Cryptosporidium parvum *development in human intestine. The field shows a maturing meront, probably Type 1 with numerous merozoites around the periphery. The parasite's outer membrane and host's cell membrane surrounding the meront are in close apposition. Note the electron-dense band at the interface between the parasite and the host cell. Magnification ×20 000.*

stages has been poorly studied, partly because parasite development in vitro is relatively poor and there is no transfection technology. Although *C. parvum* reproduction may be maintained in vitro for many days (Hijjawi et al. 2001), the development is not yet sufficient for maintenance of parasite isolates. Furthermore, no reliable method for cryopreservation has been described. From the relatively small body of information available on the biology of *Cryptosporidium*, however, it is clear that the parasite has unique features.

CELL BIOLOGY

The robustness of the cryptosporidial oocyst wall is the key to survival of the parasite outside of the host. Little is known about the molecular structure of the wall or how the components gel to provide such an effective barrier against environmental stresses. Ultrastructural studies indicate that the oocyst wall of *Cryptosporidium* comprises of two layers (Reduker et al. 1985) and contains numerous polypeptides (Tilley and Upton

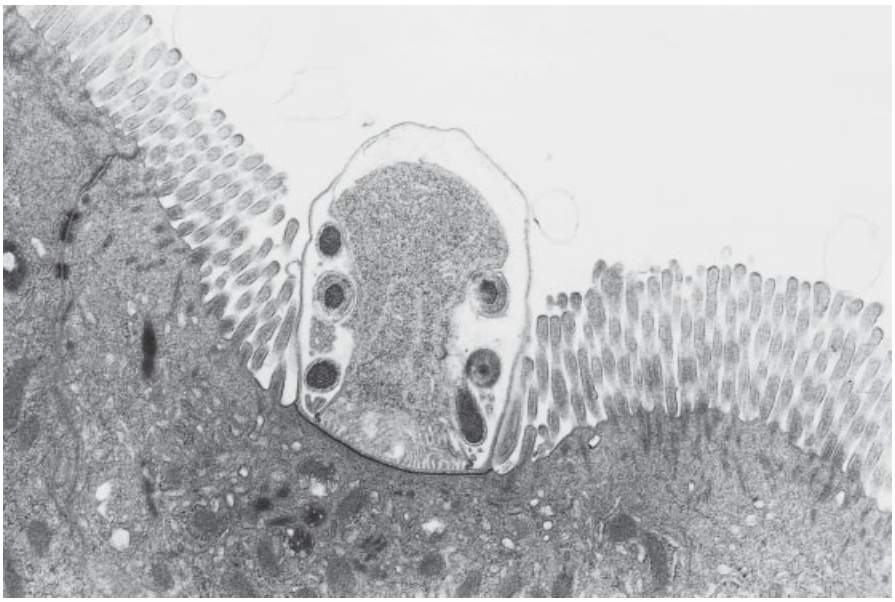

Figure 2 *Electron micrograph showing a developing microgametocyte of* Cryptosporidium parvum. *Microgametes are budding from the periphery of the parasite body. Note the absence of an epithelial cell brush border around the parasite. Magnification ×20 000.*

1997). One 190-kDa COWP has a high cysteine content and two types of amino acid repeat motifs that may provide periodic spacing and appropriate conformation to allow a large number of disulfide bridges between the cysteine residues (Ranucci et al. 1993). The outer oocyst wall is highly glycosylated and its polypeptide composition differs from that of the inner wall (Tilley and Upton 1997). The inner surface contains a filamentous array and sonicated protein K-digested walls fragment into small linear sheets indicating there is an integral highly complex lattice structure (Harris and Petry 1999). Ultrastructural studies of *C. parvum* show that the sporozoite morphology is similar to that of other coccidia, including a typical banana-like shape of the organism. Parasite actin helps to maintain the shape of the sporozoite and is also important in motility (Forney et al. 1998).

The apical complex organelles comprising rhoptry, micronemes, and dense granules are believed to discharge contents that are required for invasion and establishment of apicomplexans inside host cells (Lumb et al. 1988). There appears to be only a single rhoptry in *Cryptosporidium*, whereas various other apicomplexan parasites have at least two and sometimes many more (Tetley et al. 1998). Isolated micronemes of *C. parvum* were demonstrated to contain three major proteins of 30, 120, and 200 kDa, and dense granules held five major proteins in the range 120–180 kDa (Petry and Harris 1999). Coccidia normally possess a mitochondrion but the presence of this organelle in *Cryptosporidium* has been disputed. Numerous studies reported the absence of mitochondria (Current and Reese 1986; Tetley et al. 1998) but such a structure was identified in the merozoites of *C. muris* (Uni et al. 1987) and in sporozoites of *C. parvum* (Riordan et al. 1999). A single plastid-like body of unknown function, probably of green algae origin, has been found in a number of apicomplexans and may also be present in *C. parvum* (Tetley et al. 1998).

How the invasive stages attach to and invade epithelial cells is poorly understood. A number of parasite proteins that may be involved have been characterized in recent years, and possible ligands on the host cell surface have also been identified. *Cryptosporidium parvum* micronemes contain a number of these, including TRAP-C1 which may play an important role in attachment or invasion (Naitza et al. 1998). Related proteins of other coccidia (e.g. MIC2 of *Toxoplasma*) have been shown to be involved in zoite locomotion and also attachment and invasion of cells (Soldati et al. 2001). Another micronemal molecule of *C. parvum*, the mucin-like glycoprotein gp900 with a large N-linked oligosaccharide core region and a cysteine-rich region, is shed onto the sporozoite surface during invasion and antibodies to gp900 inhibit infection in vitro (Petersen et al. 1997). Another glycoprotein, gp60, which shares epitopes with gp900 also has mucin-like domains with a terminal peptide specifying attachment of a glycosyl-phosphatidylinositol (GPI) anchor; this protein is cleaved to gp40 and gp15 (Strong et al. 2000). The gp40 was shown to be shed from sporozoites gliding over epithelial cells and is involved in cell attachment. The circumsporozoite-like antigen (CSL) is a 1 300 kDa glycoprotein found in micronemes and on the surface of sporozoites and merozoites. A monoclonal antibody to a repetitive carbohydrate epitope induces a CSL reaction whereby the antigen moves in a posterior direction along the sporozoite pellicle (Langer et al. 2001). The findings that antibody to CSL inhibit infection of cells and that CSL has been shown to bind to an 85 kDa receptor on intestinal epithelial cells suggests that CSL is an important ligand for attachment and invasion. Another protein designated CP47 and associated with the sporozoite membrane has been found to bind to human and animal ileal cells and a putative receptor of 57 kDa was identified (Nesterenko et al. 1999).

During invasion, the host cell cytoskeleton undergoes rearrangement and filamentous actin is assembled into a plaque-like structure at the host–parasite interface (Elliott and Clark 2000). Infection has been demonstrated to be dependent on actin polymerization and the actin polymerizing proteins ARP 2/3, vasodilator-stimulated phosphoprotein, and neural Wiskott Aldrich syndrome protein have been shown to be present at the site of infection (Elliott et al. 2001). This cytoskeletal remodeling appeared to involve host phosphoinositide 3-kinase activity, suggesting that host signaling pathways were induced to elicit actin rearrangement (Forney et al. 1999). As invasion begins, the host cell membrane moves along the length of the sporozoite pellicle to enclose the parasite in a parasitophorous vacuole (Chen et al. 1998). The parasitophorous vacuole membrane is of mixed host and parasite origin as parasite-derived antigens have been located in the membrane by immunogold electron microscopy (McDonald et al. 1995). During invasion, a dense band appears at the interface between the parasite and the host cell (see Figures 1 and 2) and is retained throughout development (Lumb et al. 1988). Following discharge of the apical organelles, a new parasite vacuole forms at the anterior pole of the sporozoite. This anterior vacuole forms the precursor of the feeder organelle membrane and at the attachment site becomes fused with the parasite pellicular and parasitophorous vacuole membranes (Lumb et al. 1988).

METABOLISM

Years of research have failed to provide adequate chemotherapy for cryptosporidiosis. It may be necessary to adopt a structure-based design approach in which possible molecular targets of the infectious agent are characterized and specific inhibitors constructed (Coombs 1999). Undoubtedly, this process would be facilitated by a better understanding of the biochemical pathways of the parasite, particularly those which are

unique or distinct from host equivalents. Little is known about the metabolism of *Cryptosporidium* in comparison with other coccidia such as *Eimeria* or *Toxoplasma*. The significant features of biochemical pathways in *Cryptosporidium* are reviewed elsewhere (Coombs 1999).

Enzymes of the glycolytic pathway have been identified in oocysts of *C. parvum* (Entrala and Mascaro 1997).With the exception of adenosine triphosphate (ATP)-dependent phosphofructokinase, all the classic glycolytic activities leading to the formation of phosphoenolpyruvate were found. A Type I phosphofructokinase specific for inorganic phosphate has been characterized in a number of coccidia, including *C. parvum* (Denton et al. 1996). The use of this enzyme increases the energy efficiency of glycolysis by 50 percent and may represent adaptation towards anaerobiosis. Indeed, two key observations indicate that energy metabolism in *C. parvum* is similar to that of anaerobes, making glycolysis the main energy source. First, sporozoites were not killed by respiratory inhibitors such as potassium cyanide or sodium azide (Brown et al. 1996) and, secondly, enzymes of the Krebs tricarboxylic acid cycle could not be found in oocysts (Denton et al. 1996; Entrala and Mascaro 1997). The reported absence of a mitochondrion in *Cryptosporidium* (Current and Reese 1986; Tetley et al. 1998) is consistent with anaerobic development but later observation of this organelle accompanied by evidence for the presence of various mitochondrial proteins and susceptibility to drugs which target mitochondrial ubiquinones suggested that there are mitochondria (Riordan et al. 1999).

Energy in coccidia may also be obtained via a mannitol cycle. Mannitol synthesis has been shown to occur in *Eimeria* during development of sexual stages (Schmatz 1989) and two of the enzymes involved in this cycle have been found in *Cryptosporidium*. However, the enzyme which initiates mannitol synthesis, mannitol-1-phosphate dehydrogenase, appears to be regulated by a specific inhibitor during asexual development and so mannitol utilization may be relevant only in oocysts (Schmatz 1997). In support of this view, excysted *C. parvum* sporozoites show little hexokinase activity that is necessary for mannitol utilization, suggesting mannitol is not required for establishing infection (Entrala and Mascaro 1997). Furthermore, *Eimeria* hexokinase activity was observed to be greater at environmental temperature than at body temperature (Schmatz 1997).

Polyamines are involved in protein synthesis, stability of ribosomes and cell membranes, and also in regulation of gene expression. Most eukaryotic organisms, including *Eimeria*, initiate polyamine synthesis by decarboxylation of ornithine to give putrescine with ornithine decarboxylase, subsequently leading to the production of spermine. Keithly et al. (1997), however, demonstrated that in *C. parvum*, a quite distinctive route was involved in synthesizing putrescine. Ornithine decarboxylase was undetectable and putrescine was synthesized from arginine via arginine decarboxylase to provide agmatine which was converted to putrescine by agmatine iminohydrolase. This pathway is usually associated with some bacteria and plants, suggesting polyamine synthesis would be a suitable target for development of anti-cryptosporidial drugs. Another possible target in coccidia is the shikimate pathway which produces chorismate, an essential substrate for the synthesis of *p*-aminobenzoate, folate, ubiquinone, and aromatic amino acids (Roberts et al. 1998). This pathway is absent in mammals but present in plant plastids, algae, bacteria, and fungi. A number of the enzymes involved have also been observed in apicomplexan species which have plastid-like organelles. Also, the herbicide glyphosphate, which inhibits one of the enzymes in the pathway (5-enolpyruvyl shimikate 3-phosphatesynthase), inhibited in vitro development of apicomplexans including *Cryptosporidium* (Roberts et al. 1998). This effect was reduced by addition of *p*-aminobenzoic acid in the study of *T. gondii* and *Plasmodium falciparum*, but development of *C. parvum* was unaffected.

GENETICS

Nucleic acid studies of *Cryptosporidium* have played an important part in building our current understanding of the parasite's molecular cell biology, biochemistry, and taxonomy. In addition, nucleic acid probes have had enormous practical value in molecular epidemiology and as highly sensitive tools for detecting parasite material in tissue.

A difficulty in investigating the karyotypes of *Cryptosporidium* is the absence of chromosome condensation in the course of cell division. However, chromosomal bodies can be separated by pulsed field gel electrophoresis and this method, combined with densitometry analysis, has established that there are at least five, and probably eight, chromosomes in the range 1.03–1.54 Mb and a total genome size of approximately 10.4 Mb (Mead et al. 1988; Blunt et al. 1997). Mead et al. (1988) were able to differentiate between two species, *C. parvum* and *C. baileyi*, each of which demonstrated distinctive banding patterns.

To date, only about 30 genes of *C. parvum* have been fully sequenced and characterized (Spano and Crisanti 2000). One example was the sequencing of the bifunctional enzyme dihydrofolate reductase (DHFR)–thymithymidylate synthase (TS), which was isolated from genomic DNA libraries by hybridization with a probe amplified from *C. parvum* genomic DNA using generic TS primers in the polymerase chain reaction (PCR) (Vasquez et al. 1996). Two significant findings emerged from the sequence data. First, the DHFR active site was found to contain novel residues at several positions where point mutations occurring in other parasites have been demonstrated to produce antifolate resistance in DHFR. This may explain the inherent refractoriness of

C. parvum to treatment with some common antifolates used against other protozoa. Secondly, like other genes that have been characterized, the sequence data for DHFR–TS of different parasite isolates demonstrated polymorphisms that could be employed to examine the genotypic diversity of *C. parvum* isolates.

The adenine, thymine (AT) content for Type 1 and Type 2 *C. parvum* has been estimated at 65 and 68 percent, respectively (Widmer et al. 2002) and there are few introns (e.g. see Vasquez et al. 1996). Telomeres, the repetitive nucleotide sequences at the ends of eukaryotic chromosomes, have been detected in *C. parvum*. A reputed telomeric clone containing the hexanucleotide TTTAGG sequence, similar to a telomeric repeat of *Plasmodium*, was reported to be present in all five separated chromosomal bands of *C. parvum* and a probe containing the repeat hybridized with chromosome VI-specific *Eag*I restriction fragments corresponding to the chromosomal ends (Liu et al. 1998; Putignani et al. 1999). Microsatellite DNAs, which are tandem repeats of short nucleotide sequences have been described in *Cryptosporidium* (reviewed by Spano and Crisanti 2000). Microsatellites are useful genetic tools for intraspecific variation as they are usually highly polymorphic. For example, *C. parvum* Types 1 and 2 have been differentiated by microsatellite analysis (Spano and Crisanti 2000). More than 200 microsatellites have been identified in *C. parvum* DNA and these are usually AT-rich, thus conforming with the overall AT-richness in nucleotide composition of this species. In other eukaryotic species, microsatellites are densely distributed throughout the genome, making them suitable markers for high-resolution genetic mapping (Spano and Crisanti 2000).

Comprehensive genomic maps of *C. parvum* isolates will facilitate identification and characterization of genes that are crucial to the parasite's development, answer questions about the taxonomy of the genus, and provide suitable targets for chemotherapy. Separate approaches have been taken to map the *C. parvum* genome (Spano and Crisanti 2000; Widmer et al. 2002). In one, sequence analysis has been obtained from sporozoite cDNA cloned in a phage vector (see www.ebi.ac.uk/parasites/cparv.html). This method allows only characterization of genes expressed in the sporozoite, however. Other systems have employed sheared genomic DNA inserts derived from a plasmid library or genomic DNA cut with restriction enzymes and cloned into a phage vector. Analysis of sequenced DNA for translated parts of the *C. parvum* genome has indicated that the DNA contains approximately 85 percent coding sequences and about 5 000 genes (Widmer et al. 2002).

A major benefit of molecular genetic studies has been the provision of molecular markers for taxonomic evaluation of the parasite. Use of DNA sequencing and PCR with parasite-specific primers combined with RFLP has been valuable in the differentiation of *Cryptosporidium* spp. and in configuring *C. parvum* isolates into groups. Early evidence of phenotypic variation between isolates of *C. parvum* relating to parasite virulence, drug sensitivity, isoenzyme analysis, and western blotting has been supported by DNA studies (reviewed by Morgan et al. 1999). Phylogenetic analysis of *Cryptosporidium* at different loci indicates that *Cryptosporidium* is not closely related to other apicomplexans such as *Toxoplasma* (Char et al. 1996). Sequence data indicate that there are several distinct species; for example, the similarity between the 18S rDNA of two bovine isolates of *C. parvum* (99.94 percent) was greater than that between one of these isolates and *C. baileyi* (96.26 percent) (Morgan et al. 1999). Importantly, genomic studies based on gene sequences for proteins, rDNA and microsatellite DNA point to the possibility that human Type 1 and animal Type 2 isolates may represent separate species of *Cryptosporidium*. In support of this, cross-transmission studies have shown that human Type 1 isolates rarely infected animals (Awad-El-Kariem et al. 1998; Widmer et al. 2000) and a multilocus study found no evidence of recombination between Type 1 and Type 2 isolates of *C. parvum* (Spano et al. 1998). The widespread prevalence of identical genotypes implies that there is a clonal population structure (Morgan et al. 1997) as there is in *Toxoplasma* (Howe and Sibley 1995). However, using microsatellite loci as genetic markers, sexual recombination was reported to occur during mixed laboratory infections of two distinct Type 2 isolates in interferon-gamma (IFN-γ) knockout mice (Feng et al. 2002). The whole question of whether there is significant genetic recombination in *Cryptosporidium* is still under debate.

A significant practical benefit of studies in molecular genetics is the development of probes for identifying isolates involved in outbreaks of cryptosporidiosis. For example, a Type 1 parasite was likely to have been the cause of a major waterborne outbreak in Milwaukee in the 1990s (Peng et al. 1997).

Clinical aspects

GENERAL

The commonest manifestation of infection is diarrhea. This may be indistinguishable from any other acute nonbloody diarrheal illness, but cryptosporidiosis is more likely to become persistent and this usually leads to weight loss. Other less common clinical features include abdominal pain, low-grade fever, malaise, and other nonspecific symptoms. As cryptosporidiosis is caused by an intracellular parasite, abnormalities of T-cell immunology seriously impair the host capacity to clear infection, leading to persistent infection. Human immunodeficiency virus (HIV) infection and AIDS lead to a greatly increased risk of cryptosporidiosis, as do other primary and secondary immunodeficiencies. These

include severe combined immunodeficiency (SCID), other T-cell deficiencies, deficiencies of mannose-binding lectin (Jacyna et al. 1990; Kelly et al. 2000), and X-linked immunodeficiency with hyper-immunoglobulin (Ig)M caused by mutations in the CD40 ligand gene (Hayward et al. 1997). Children with acute leukemia also have increased susceptibility to cryptosporidiosis (Hunter and Nichols 2002).

There is an important interaction between cryptosporidiosis and nutritional impairment. This is true of children with and without HIV infection (Amadi et al. 2001), probably because persistent cryptosporidiosis causes severe anorexia, and mucosal damage also leads to malabsorption of micronutrients. In children, cryptosporidiosis seems to lead to an increased risk of nutritional problems. Phillips et al. (1992) showed an association between cryptosporidiosis and nutritional impairment in immunocompetent children living in London. A series of studies on children in Guinea-Bissau has revealed that children who were undernourished were not more likely to develop the infection (Molbak et al. 1993), but children with cryptosporidiosis are more likely to go on to lose weight after the infection (Molbak et al. 1997). In a series of studies in Brazil, cryptosporidiosis was associated with persistent diarrhea (Newman et al. 1999) and this is associated with nutritional shortfalls (Lima et al. 2000) and with diminished cognitive function (Niehaus et al. 2002). In adults, the great majority of cases of persistent cryptosporidiosis are related to HIV infection and, again, undernutrition is common but there is no evidence that cryptosporidiosis in this setting is associated with more severe nutritional problems than other causes of HIV-related persistent diarrhea.

AIDS

In many countries, the problem of cryptosporidiosis in AIDS patients is now much less severe than it was because of the widespread use of antiretroviral therapy, which has revolutionized the care of patients with HIV infection. Information on clinical illness is largely based on older studies. However, some cases are still seen because of late presentation or failure of therapy due to resistance of the virus.

The severity of illness in AIDS patients is variable, ranging from asymptomatic infection to fulminant disease in a minority. Fulminant disease is characterized by a high-volume diarrhea and high mortality (Blanshard et al. 1992). Generally, there is a relationship between the severity of symptoms and the CD4 cell count. As the CD4 cell count falls and HIV infection progresses, the severity of the illness associated with cryptosporidial infection increases (Flanigan et al. 1992). The majority of patients in a study reported by Connolly et al. (1988) had stool volumes of 500–1 500 ml per 24 h, with stool frequency of 2–10 per day. The natural history of persistent cryptosporidiosis is a remitting and relapsing diarrhea. In one study, 11 of 38 patients with HIV-related cryptosporidiosis underwent spontaneous remission lasting over 2 months. These patients had higher peripheral blood CD4 cell counts than those who did not undergo remission (McGowan et al. 1993). In a series of patients studied in Lusaka, Zambia (Kelly, unpublished data) the median duration of diarrhea at presentation with cryptosporidiosis was 5 months, with 60 percent admitting to intermittent diarrhea. Among Congolese patients, 89 percent had intermittent diarrhea and the mean duration was 9 months; no difference was demonstrated between patients with cryptosporidiosis and those with other forms of HIV-related diarrhea (Colebunders et al. 1987).

BILIARY DISEASE

The situation here has also changed due to the use of antiretroviral therapy, and this manifestation is now much less common. Some patients with AIDS develop a sclerosing cholangitis, sometimes associated with cholecystitis (Teixidor et al. 1991). This may be associated with cryptosporidiosis, microsporidiosis (see below), cytomegalovirus, or it may be impossible to identify a cause. The disorder usually, but not always, occurs in patients with chronic diarrhea, and it leads to a progressive right-upper-quadrant abdominal pain. Biochemical tests of hepatic damage usually show elevated serum alkaline phosphatase and γ-glutamyl transferase levels in the absence of jaundice. Transaminases may or may not be elevated. Ultrasound examination of the liver may show irregularly dilated intrahepatic bile ducts. The definitive test is endoscopic retrograde cholangio-pancreatography (ERCP), which shows this distortion of the biliary anatomy, with or without papillary stenosis. Forbes et al. (1993) found cryptosporidia in 13 of 20 cases, and estimated that up to one in six of all cases of AIDS-related cryptosporidiosis may also have sclerosing cholangitis.

Immunology

ANIMAL MODELS

As attempts to treat cryptosporidiosis with drugs usually have disappointing results, resolution of infection depends on the ability of the host's immune system to bring the infection under control. When the immune system is defective, as for example in AIDS, the infection may persist, spread to extraintestinal sites, and cause severe morbidity and mortality. The ultimate outcome of infection depends on adaptive immune responses but innate immunity also appears to have a protective role. Mechanisms of parasite killing are still poorly understood, however.

Investigations with animal-infection models have played an important part in the development of our understanding of immune responses to *Cryptosporidium*.

A limitation of animal models is that susceptibility to *C. parvum* infection decreases within a few weeks of birth and adult animals are usually strongly resistant to infection (Sherwood et al. 1982). This age-related alteration in susceptibility to infection appears to be absent in human infection (Current and Garcia 1991). One of the most commonly used models of infection for experimentation involves conventional neonatal mice (Sherwood et al. 1982). Adult immunocompromised mice such as athymic nude mice or SCID mice are susceptible to infection and have often been used in immunological studies (reviewed in McDonald et al. 2000). Drug-immunosuppressed rodents may also be susceptible to *C. parvum* infection and although such a model may be useful in drug studies it is of little value for immunological investigation (Brasseur et al. 1988). The gastric parasite *C. muris* readily produces intense acute infections in adult mice and this model has been used to study mechanisms of T-cell-mediated immunity (McDonald et al. 1992).

Another important tool in the study of host–parasite interactions is in vitro parasite culture in enterocyte cell lines. *C. parvum* infection of human cell lines stimulates the proinflammatory transcription factor NF-κB which activates production of chemokines (Laurent et al. 1997) that may play an important part in initiating an early inflammatory response. Infection also stimulates production of prostaglandins that may regulate immunity (Laurent et al. 1998) and in bovine calf infections upregulation of antimicrobial β-defensin expression by intestinal epithelial cells has been reported (Tarver et al. 1998). Proinflammatory cytokines, particularly IFN-γ, have been shown to activate enterocyte cell lines to inhibit *C. parvum* development (Pollok et al. 2001).

IFN-γ has been demonstrated to be a crucial factor in protection conferred by innate immunity. Infections in nude mice and SCID mice, which are T-cell deficient and T + B-cell deficient, respectively, were exacerbated by administration of anti-IFN-γ-neutralizing antibodies (Ungar et al. 1991). The most likely source of IFN-γ, apart from T cells, are natual killer (NK) cells activated by cytokines including interleukin (IL)-12 and tumor necrosis factor (TNF-α) from macrophages (Tripp et al. 1993) and it was shown in vitro that sporozoites of *C. muris* could stimulate NK cells to produce IFN-γ under regulation by these cytokines (McDonald et al. 2000). Injection of neonatal SCID mice with IL-12 prior to inoculation with *C. parvum* oocysts conferred complete resistance to infection (Urban et al. 1996). However, treatment of SCID mice with either anti-asialo-GM1 antibodies that deplete NK cells or anti-TNF-α antibodies had no effect on *C. parvum* reproduction (McDonald and Bancroft 1994). Nude or SCID mice infected with *C. parvum* survive for several weeks due at least in part to the innate IFN-γ response, but the infection gradually increases in magnitude, spreading to extra-intestinal sites, and the animals usually develop signs of illness and die (reviewed in McDonald et al. 2000).

Clearance of cryptosporidial infection is dependent on T-cell activity and specifically the CD4 T cell subpopulation. Conventional neonatal mice develop acute *C. parvum* infections which disappear around day 14 of infection. The development of chronic cryptosporidial infections in T-cell deficient nude and SCID mice indicates the requirement for T cells to resolve infection (Heine et al. 1984; Mead et al. 1991). Little is known about the role of T-cell costimulatory molecules in immunity to this parasite, but CD40/CD40L interaction, required for T-cell activation and antibody isotype switching, appear to be necessary since both CD40 and CD40L knockout mice develop fulminant infections of *C. parvum* (Cosyns et al. 1998). Mice lacking major histocompatibility complex (MHC) class II and hence CD4 T cells are highly susceptible to cryptosporidial infection and fail to recover, whereas animals deficient in MHC class I and therefore CD8 T cells can control the infection normally (Aguirre et al. 1994). Similarly, reconstitution of the SCID mouse immune system with conventional lymphoid cells confers resistance against cryptosporidial infection, but if the CD4 T cells are depleted this effect is lost; depletion of CD8 T cells affects immunity only marginally (reviewed in Theodos 1998). Intra-epithelial lymphocytes may be important effector cells in controlling infection as the transfer of these cells from *C. muris*-infected mice to SCID mice allowed the recipients to recover from infection (Culshaw et al. 1997).

The protective adaptive response appears to involve a cell-mediated – T helper (Th)1 – response since IFN-γ and IL-12 activity is required. *C. parvum* infection levels increased in mice injected with neutralizing antibodies to these cytokines and IFN-γ knockout mice were more susceptible to infection (Ungar et al. 1991; Urban et al. 1996; Theodos 1998). However, there is also evidence that the allergic or antibody-mediated (Th2) cytokines IL-4 and IL-5 play a part in immunological control. *C. parvum* infection was more intense in mice treated with anti-IL-4 or anti-IL-5 neutralizing antibodies, or in IL-4 knockout mice, than in control animals (Enriquez and Sterling 1993; Aguirre et al. 1998). In one study with C57BL/6 mice, the protective effect of IL-4 did not become apparent until after the peak of infection, suggesting the cytokine was involved in removing parasites surviving the early Th1 response infection (Aguirre et al. 1998). However, in another study, neonatal BALB/c IL-4 knockout mice were more susceptible to *C. parvum* infection than wild-type mice during the acute phase of infection and injection of BALB/c neonates with IL-4 prior to oocyst inoculation increased resistance to infection (McDonald et al., in preparation).

Parasite-specific antibodies develop in the serum and intestine of animals infected with *C. parvum* (Peeters et al. 1992). The peaks of production of intestinal IgA

and IgM were found at the time of recovery from infection, suggesting antibodies could have a role in protection. In a rodent study, bile with anticryptosporidial antibodies derived from rats infected with *C. parvum* reduced the level of infection in nude mice (Albert et al. 1994). However, neonatal mice depleted of B cells following anti-μ chain antibody treatment were no more susceptible to infection than control mice (Takhi-Kilani et al. 1990). This implies that although antibodies may have a protective role, they are not essential for elimination of infection. Monoclonal antibodies developed against a variety of sporozoite antigens or polyclonal antibodies produced in bovine colostrum after immunization with oocyst antigens have been shown to passively transfer immunity to *C. parvum* infection either in mice after oral ingestion or in cell culture (Bjorneby et al. 1990).

HUMAN INFECTION

The human immune response to *C. parvum* is not well understood. T-cell activity is probably necessary for early control of infection since cryptosporidiosis may be more severe in individuals undergoing immunosuppressive chemotherapy or who are immunosuppressed as a result of other infections or malnutrition (Current and Garcia 1991). It is also likely that the CD4 T-cell subpopulation is required for control of infection since, in AIDS, the incidence of infection and severity of disease increases as the CD4 T cell count decreases (Blanshard et al. 1992). Treatment of HIV infection with highly active antiretroviral therapy (HAART) has been found to be highly beneficial in the control of cryptosporidiosis, probably as a result of recovery of CD4 cell counts (Miller 1998). There is a requirement for ligation of CD40/CD40L involved in T-cell activation by dendritic cells as boys with hyper-IgM syndrome caused by a mutation in CD40L have a high incidence of infection (Hayward et al. 1997). The precise role of CD4 T cells in immunity is unclear but, as with animals, resistance to infection appears to be associated with IFN-γ. Studies with human volunteers have shown that individuals who clear *C. parvum* infection express IFN-γ mRNA in the intestine while those with chronic infection as a result of AIDS express little or no IFN-γ mRNA (White et al. 2000). Increased intestinal expression of transforming growth factor-beta (TGF-β) has been demonstrated and this anti-inflammatory cytokine may have an important role in regulating the activities of inflammatory cells and repairing epithelial barrier function (Robinson et al. 2000).

Infection has been shown to induce production of parasite-specific antibodies in the serum and secretory IgA in the gastrointestinal tract (Ungar et al. 1986; Cozon et al. 1994). Persistent infection has been reported in individuals with hypogammaglobulinemia, although this condition may be associated with other immunodeficiencies (Tzipori et al. 1986). A number of studies in developing countries showed a lower incidence of cryptosporidiosis in breast-fed children, but environmental factors such as extent of use of contaminated water may have contributed to these findings (reviewed in Fayer and Ungar 1986). High titers of secretory IgA have been measured in AIDS patients with chronic infection (Cozon et al. 1994), which may indicate that antibody in general is unimportant in control of infection or that these individuals are producing the wrong type of antibodies. Alternatively, it is possible that these antibodies are protective but cannot fully compensate for a defective cell-mediated response.

Pathology and pathological physiology

DISTRIBUTION OF INFECTION IN THE GUT

Studies in piglets suggest that the infection moves along the gut in a caudal direction (Vitovec and Koudela 1992). The heaviest infections were seen in the distal jejunum and ileum, moving to the colon as time passed; they were associated with partial villous atrophy, crypt hyperplasia, and an inflammatory infiltrate in the lamina propria.

Human studies on the distribution of infection are rather limited. Blanshard et al. (1992) found the parasite in 40 percent of duodenal biopsies and 52 percent of rectal biopsies from patients with AIDS-related cryptosporidiosis. In children with normal immune function, Phillips et al. (1992) found trophozoites in jejunal biopsies by light or scanning electron microscopy in seven out of nine children with chronic cryptosporidiosis. Goodgame et al. (1993) found parasites in 8 of 12 patients with AIDS-related cryptosporidiosis. Clayton et al. (1994) found two patterns of disease: one characterized by proximal small intestinal infection and one by distal infection. Patients with proximal infection had more severe disease. Using PCR to detect parasites in different anatomical regions of the intestine in 83 AIDS patients, the most frequently colonized site was the ileo–cecal region (Kelly et al. 1998).

MUCOSAL ABNORMALITIES

Electron microscopy of the infection shows that the presence of the trophozoites leads to destruction of the microvilli comprising the brush border (Figure 2). It seems likely from the appearance of these biopsies that brush border enzyme activity must be reduced, and this has been verified for disaccharidases in children (Phillips et al. 1992).

In germ-free lambs, the villus architecture was normal in the jejunum and ileum during colonization, until the appearance of sexual stages of the life cycle. At this point, the villi shrank and became fused, and an inflammatory infiltrate appeared in the lamina propria. Simultaneously, diarrhea supervened (Angus 1990). This change, however, probably coincides with the beginning

of a heavy parasite burden consisting of both sexual and asexual stages.

In human infections, histopathological abnormalities can be severe even when the patient does not have AIDS (Meisel et al. 1976). In AIDS patients in the USA, the jejunal mucosa from patients with cryptosporidiosis was associated with more severe enteropathy than mucosa from patients in whom no pathogen was detected (Kotler et al. 1990), but this was not found in an African series (Kelly et al. 1997a).

Cryptosporidial infection has been associated with increased numbers of mitotic figures in the crypts and villous atrophy. In the normal human intestine, cell maturation takes place during migration up the crypt and along the length of the villus. This process is accompanied by the change from a crypt cell generating net water and salt secretion to a villus cell with net salt and water absorption. Maturation is also accompanied by the synthesis of a normal complement of brush border enzymes, including disaccharidases, lipases, and alkaline phosphatase. According to the 'enterocyte immaturity hypothesis' (Buret et al. 1990), accelerated cell turnover populates the villus with immature enterocytes, contributing to the maldigestion and malabsorption seen in many protozoal small-bowel parasitoses. An increased apoptosis rate has also been observed in cultured epithelial cells, but anti-apoptotic effects have also been observed (McCole et al. 2000).

DISTURBANCES OF PHYSIOLOGY

It has been assumed that, because some conspicuous individuals have very high salt and water losses in stool, often quoted as up to 20 l per day, the principal mechanism of diarrhea must be a cholera-like jejunal secretory state. However, as noted above, this severe secretory diarrhea is not the commonest manifestation. Also, animal perfusion studies carried out in a neonatal piglet model of cryptosporidiosis do not support this hypothesis (Argenzio et al. 1990). Neither do studies carried out in humans using a jejunal perfusion technique (Kelly et al. 1996). However, there is some preliminary evidence suggesting that *C. parvum* might elaborate a secretory enterotoxin. A protein extract of supernatant from calves with cryptosporidial diarrhea induced a short circuit current in human jejunum mounted in Ussing chambers (Guarino et al. 1994). The same group later showed that this as yet unidentified enterotoxic factor was only present in stool supernatants from patients with an osmotic gap consistent with secretory diarrhea (Guarino et al. 1995). However, it is not clear whether this enterotoxic factor could be parasite derived or an inflammatory mediator secreted by the host.

There is ample evidence that in cryptosporidiosis there is malabsorption of disaccharides (Phillips et al. 1992), and in AIDS patients there is malabsorption of bile acids, and vitamin B_{12} in patients with cryptosporidiosis and other causes of AIDS-related diarrhea (Bjarnason et al. 1996). In pigs, cryptosporidiosis is associated with impaired salt and water absorption (Argenzio et al. 1990), partly brought about by prostanoid action on the enteric nervous system (Argenzio et al. 1996). Glutamine can overcome this effect (Blikslager et al. 2001). At a cellular level, *C. parvum* penetration and intracellular growth causes several signaling effects within the enterocyte, leading to cytoskeletal rearrangements especially of β-actin (Elliott and Clark 2000) and to increased paracellular permeability in vitro (Adams et al. 1994), though there is as yet no direct evidence linking these two events through, for example, β-catenin. In the piglet model, macromolecular permeability was not increased (Moore et al. 1995), but studies in children found increased permeability (Zhang et al. 2000), and in AIDS patients, many of whom had cryptosporidiosis, permeability was increased (Keating et al. 1995). There is evidence for host-derived phosphoinositide 3-kinase participation in the cytoskeletal changes (Forney et al. 1999).

Diagnosis

Cryptosporidiosis is usually diagnosed by the detection of stained oocysts in fecal smears using acid-fast methods such as modified Ziehl-Neelsen (Figure 3) and phenol auramine. The oocysts are 4.5–5.0 μm in size, and round or slightly oval in shape. Oocysts may be concentrated beforehand by centrifugation in Sheather's sugar solution if necessary or saturated sodium chloride (Casemore et al. 1985). Monoclonal antibodies against oocyst-wall antigens have been employed in *Cryptosporidium*-specific immunofluoresence antibody tests with fecal specimens (Arrowood and Sterling 1989). Enzyme-linked immunoassay (ELISA) may also be used for detection of cryptosporidial antibodies in serum and has been used in sero-epidemiological investigations (Ungar et al. 1986). Sensitive ELISA methods for detection of cryptosporidial antigens in fecal samples have been

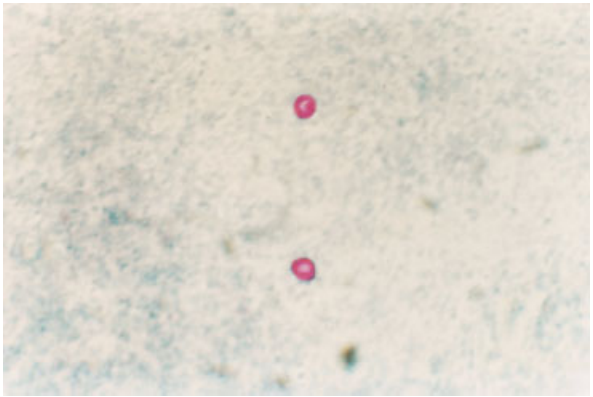

Figure 3 *Light microscopy field showing acid-fast staining of two oocysts of* Cryptosporidium parvum *in a fecal smear. These oocysts are almost spherical with a diameter of approximately 5 μm. Magnification ×1 000.*

described and commercial diagnostic ELISA as well as monoclonal antibody kits are available (reviewed by Petry 2000). The immunological techniques are more sensitive than direct observation or histological staining methods but even more sensitive detection of parasites has been achieved using PCR. Many protocols are now available to target *C. parvum* DNA sequences from genes or unknown DNA sequences (Petry 2000). PCR has been shown to be more sensitive and specific than microscopy in detecting oocysts in fecal specimens (Morgan et al. 1998). Ultimately, however, the method used for diagnosis will usually be determined by cost.

Epidemiology

TRANSMISSION

C. parvum is a highly infectious organism. In infectivity studies in healthy volunteers, the median infective dose was 132 oocysts (DuPont et al. 1995). Transmission of *C. parvum* is seasonal in children (Casemore 1990; Perch et al. 2001) and in adults with AIDS (Blanshard et al. 1992). There is some evidence that transmission may relate to waterborne oocyst contamination (Kelly et al. 1997b; Nchito et al. 1998)

Exposure to *C. parvum* oocysts occurs commonly in childhood in developing countries, and as immunity to this organism is strong, cryptosporidiosis is a relatively uncommon cause of diarrhea in adults with a healthy immune system. *C. parvum* causes diarrhea in adults in such populations principally when there is a problem with T-cell function, as in AIDS.

The pathogenicity of human and bovine genotypes may be host-specific in that bovine genotype (Type 2) *C. parvum* produced more severe disease than human genotype (Type 1) infections in pigs (Pereira et al. 2002). As molecular tools (see above) are now beginning to be applied to analysis of transmission, the next few years will see a rapid expansion of our understanding of routes of spread between human populations and animal reservoirs, and directly and indirectly from person to person. In a recent example, application of these markers demonstrated diversity of *C. parvum* genotypes in AIDS patients in Thailand (Gatei et al. 2002). These authors also found other *Cryptosporidium* species in these patients, and this somewhat surprising finding has also emerged in other studies despite uncertainty regarding species definition within the genus (see above under Classification).

EPIDEMIOLOGY IN THE IMMUNOCOMPETENT

Over the last two decades, there has been a large number of outbreaks of waterborne cryptosporidiosis. The largest of these affected over 400 000 people in Milwaukee, Wisconsin (MacKenzie et al. 1994). In response to this and other outbreaks, water supply companies around the world are concerned with how to prevent waterborne cryptosporidiosis and the UK has introduced a statutory requirement to monitor water supplies for oocysts (Fairley et al. 1999). Chlorination of water at usual levels fails to inactivate oocysts, so outbreaks seem to occur when filtration systems fail (MacKenzie et al. 1994).

Cryptosporidiosis is also a major cause of endemic childhood diarrhea among the poor of the Third World, and now in much of Africa and South and South-East Asia cryptosporidiosis occurs in children with and without HIV infection. In the industrialized world, outbreaks of cryptosporidiosis have often originated in day care nurseries. Traveler's diarrhea may also be caused by this protozoan (Katelaris and Farthing 1992).

The prevalence among children with diarrhea in the industrialized world ranges from 1.4 percent in the UK (Hart et al. 1984) to 4 percent in Ireland (Carson 1989), 4.6 percent in Switzerland (Egger et al. 1990), and 6 percent in another study in the UK (Thomson et al. 1987). In underdeveloped countries, prevalences among children with diarrhea are higher, for example ranging from 2.4 percent in Malaysia (Ludin et al. 1991) to 8.4 percent in Rwanda (Bogaerts et al. 1987), and 15 percent in Nigeria (Nwabuisi 2001). In studies around China, the prevalence ranged from 1 percent to 13 percent (Zu et al. 1992). These estimates are, of course, approximate, and the studies vary in their selection of children in hospitals or in the community. In population-based studies in Lusaka, 17 percent of diarrheal episodes in 222 children were associated with this infection (Nchito et al. 1998). In malnourished children with diarrhea admitted to the University Teaching Hospital, Lusaka, the prevalence was 25 percent, and the prevalence in HIV-seropositive children was similar to that in HIV-seronegative children (Amadi et al. 2001).

Serological studies suggest that even in low-transmission countries, a majority of children are exposed before their 21st birthday (Kuhls et al. 1994).

EPIDEMIOLOGY IN IMMUNOCOMPROMISED INDIVIDUALS

An interesting insight into the transmission of the infection was obtained during an outbreak of cryptosporidiosis in Denmark (Ravn et al. 1991). The setting was an infectious disease ward with a mixed population of HIV-seropositive and HIV-seronegative patients; the index case was a demented HIV-seropositive man with cryptosporidial diarrhea who contaminated an ice machine with feces. Among 73 HIV-seronegative inpatients, the attack rate was zero, but among 57 HIV-positives, the attack rate was 18 (32 percent); 17 of these had AIDS. The mean incubation time was at least 13 days.

Cryptosporidiosis was first recognized as a problem in the context of AIDS to the extent that chronic cryptosporidial diarrhea is a case-defining diagnosis for AIDS.

The prevalence of cryptosporidiosis in patients with AIDS-related diarrhea in industrialized countries ranges from 7 percent in Germany (Ullrich et al. 1992) and 8 percent in the USA (Antony et al. 1988) to 21 percent in France (Rene et al. 1989), but its frequency is now much less following the widespread adoption of antiretroviral therapy. In underdeveloped countries, AIDS-related diarrhea has become a significant public health problem and cryptosporidiosis is still a major contributor to morbidity and mortality. Prevalence ranges from 7 percent in Zambia (Zulu et al. 2002) and 12 percent in Thailand (Saksirisampant et al. 2002) to 22 percent in Zaire (Colebunders et al. 1987) and 46 percent in Haiti (DeHovitz et al. 1986). All of these estimates are, of course, approximate and subject to the same biases as discussed above. Among HIV-2-infected adults in Guinea-Bissau, the prevalence (25 percent) was similar to the studies in HIV-1-infected people (Lebbad et al. 2001).

It is likely that patients with AIDS-related cryptosporidiosis act as reservoirs of the parasite and transmit it to other adults and children in poor communities, as has already been suggested for children with cryptosporidiosis (Newman et al. 1994).

Chemotherapy

C. parvum has proved to be one of the most difficult protozoal infections to treat. Over 100 compounds have been tried without success (O'Donohue 1995). Earlier hopes that spiramycin, letrazuril, and now paromomycin (Hewitt et al. 2000) would be effective have faded. In AIDS patients, effective antiretroviral therapy is a very important component of management (Miao et al. 2000), but for most of the world's AIDS patients this approach is unaffordable.

Nitazoxanide, a nitrothiazolyl–salicylamide derivative, is a broad-spectrum antimicrobial agent with activity against protozoa, nematodes, cestodes, trematodes, and bacteria (Gilles and Hoffman 2002). It has demonstrated activity against *C. parvum* in cell culture and in animal models (Theodos et al. 1998; Gargala et al. 2000). In randomized controlled trials, nitazoxanide was found to have significant benefit in HIV-seronegative children with cryptosporidiosis in Zambia (Amadi et al. 2002), in immunocompetent adults and children in Egypt (Rossignol et al. 2001), and in AIDS patients in Mexico (Rossignol et al. 1998).

Early data suggest intriguingly that rifabutin may prevent cryptosporidiosis in AIDS patients (Fichtenbaum et al. 2000).

IMMUNOTHERAPY

Experimental studies and some case reports suggest that systematic immunotherapy against unrelenting cryptosporidiosis may be an achievable goal. The most studied approach has involved colostral antibodies. The evidence available from animal studies suggests that parasite-specific antibodies developed during infection and present in colostrum are unable to protect offspring from *C. parvum* infection (Moon et al. 1988). However, signs of disease and levels of infection could be shown to be diminished in colostrum from cows immunized mucosally with oocyst antigens and adjuvant (Tzipori et al. 1986). The bovine colostral antibodies that recognize sporozoite antigens were demonstrated to be an important protective element in a murine infection model (Fayer et al. 1990). In a similar approach, antibodies produced in chicken egg yolk following immunization of hens with oocyst antigen provided a degree of protection against infection in mice (Cama and Sterling 1991). Murine monoclonal antibodies directed against particular sporozoite surface or apical complex antigens involved in host cell invasion may potentially have a more potent protective effect. Such antibodies were able to inhibit parasite reproduction in vitro or in neonatal mice (reviewed in Riggs 1997).

A small number of studies have examined the possibility of cytokine therapy. There has been one report of successful treatment of chronic cryptosporidiosis in an immunocompromised child using recombinant human IFN-γ (Gooi 1994) and another demonstrating that treatment of AIDS patients with IL-2 could ameliorate the symptoms of cryptosporidiosis (Connolly et al. 1989).

ISOSPORIASIS

Introduction

Isospora is characterized by the morphology of the oocyst stage and is differentiated from its relative *Eimeria*, a parasite of animals, by the quantity of sporocysts found in the oocyst and number of sporozoites within each sporocyst. Many species of *Isospora* from animals have been described and some hosts are susceptible to more than one species of the parasite. The first identification of human intestinal coccidiosis was by Virchow (1860) and this may have been isosporiasis. Human infection by *Isospora* was first described in a survey of intestinal pathogens found in British troops returning from Turkey during the First World War suffering from dysentery (Ledingham et al. 1915). The parasitologist for the study, H. M. Woodcock, observed large unsporulated and sporulating oocysts with two sporoblasts but no sporulated oocysts were observed. In the same year, Wenyon (1915), with knowledge of Woodcock's findings, described further cases of infection with this parasite and, furthermore, found that oocysts would sporulate when left at room temperature for 3–4 days. The excreted oocysts matured from having a single sporoblast to two sporoblasts from which two oval

sporocysts with four sporozoites developed. Recognizing the distinctive features of an *Isospora* sp., Wenyon suggested that as animal species of *Isospora* could cause severe enteritis, this human parasite might be pathogenic (Wenyon 1915). Wenyon later named the parasite *I. belli* and made a comparative study of *I. belli* and other species from domestic animals (Wenyon 1923). Matsubayashi and Nozawa (1948) demonstrated that the oocysts were infectious, using volunteers who developed symptoms including diarrhea. The development of the capability to obtain intestinal biopsies allowed detailed characterization of the endogenous asexual and sexual stages by light and electron microscopy (Brandborg et al. 1970; Trier et al. 1974) and confirmed the parasite taxonomy.

For many years, *I. belli* infection was considered to be uncommon and cases were normally located in tropical countries (Jarpa Gana 1966), although some cases in the USA were reported (Faust et al. 1961); generally, isosporiasis in the western hemisphere was associated with foreign travel (Godiwala and Yaeger 1987). An important development in the early 1980s was the increasing number of cases of chronic, and sometimes life-threatening, diarrhea in AIDS patients associated with *I. belli* infection. In some surveys, particularly in developing countries, the incidence of *I. belli* infection in AIDS-related diarrhea was high (Lindsay et al. 1997). This problem also arose in western countries, particularly the USA, although many of the individuals were from Latin America. Unlike *Cryptosporidium*, *I. belli* infection is readily treatable, although the infection is often recurrent in immunocompromised individuals (Pape et al. 1989). Infection is usually transient in immunocompetent persons but chronic and even fatal cases have been described in these hosts (Brandborg et al. 1970; Liebman et al. 1980).

Classification

Isospora belongs to the Apicomplexa and the suborder Eimeriorina (Levine 1987). It has been placed in the family Eimeriidae, which includes the animal parasite *Eimeria* and also *Cyclospora*. However, a recent phylogenetic study of the sequences of small-subunit ribosomal RNA of *I. belli* and other coccidia suggests that *Isospora* should belong to the family Sarcocystidae (Franzen et al. 2000). An important characteristic of the genus *Isospora* is that, as in *Eimeria*, the entire endogenous development occurs within a single host. One further defining feature of *Isospora* is the presence in mature oocysts of two sporocysts that contain four sporozoites, whereas *Eimeria* has four sporocysts that have two sporozoites. Another member of the suborder, *Toxoplasma*, has a similar type of development and oocyst morphology but this genus normally has two hosts, one of which is the cat, in which sexual development occurs. Many organisms that were formerly classified as *Isospora* spp. have been renamed as species of *Sarcocystis* (see *Sarcocystis*). The development of the latter occurs in two hosts, a prey (asexual) and a predator (sexual), and the oocysts mature within the predator (Levine 1987). Currently, *I. belli* is the only species of *Isospora* known to infect humans, although historically several others were described. One in particular, *I. hominis*, was at one time commonly reported but its morphology suggests it was probably *Sarcocystis suihominis* (Lindsay et al. 1997). Attempts to infect numerous species of domestic animals with *I. belli* have failed (reviewed by Lindsay et al. 1997), so humans may be the only significant reservoir of infection.

Life cycle

I. belli is transmitted by oocysts in a fecal–oral manner. Following ingestion, oocysts release their sporozoites in the intestine. Excystation of *Isospora* in vitro is induced by body temperature in the presence of bile or bile salts and trypsin (McKenna and Charleston 1982). The sporozoites become motile within the sporocyst and eventually emerge through an opening that appears in the sporocyst wall. The oocyst wall also becomes fractured which allows the sporozoites to escape. *I. belli* develops within epithelial cells of the small intestine and intracellular parasites are rarely observed in the large intestine. The zoites of *I. belli* probably multiply by endodyogeny to form meronts and the merozoite progeny will infect and multiply in other enterocytes. Eventually, some merozoites form micro- or macrogametes that, on maturation, fuse to form a zygote and then an immature oocyst. The asexual and sexual stages have been identified by light microscopy (Brandborg et al. 1970), and ultrastructural study by electron microscopy (Figure 4) demonstrates coccidian structures such as micronemes, conoid, and subpellicular microtubules in merozoites (Trier et al. 1974). In immunocompromised individuals, extraintestinal development of the parasite has been observed in biliary epithelial cells (Benator et al. 1994).

Parasite cysts comprising undeveloped sporozoites or merozoites within a wall rich in polysaccharide have been observed at extraintestinal sites such as lymph nodes, liver, and spleen (Michiels et al. 1994). Zoites were found in lymphatic channels suggesting a route of transport from the intestine. It is possible that recurrence of infection in AIDS patients following successful chemotherapy is caused by activation of these cyst stages. Some animal species of *Isospora* have paratenic nondefinitive hosts in which sporozoites form cysts in extraintestinal tissues (Lindsay et al. 1997). The sporozoites do not undergo development in this host but infection takes place in the definitive host following

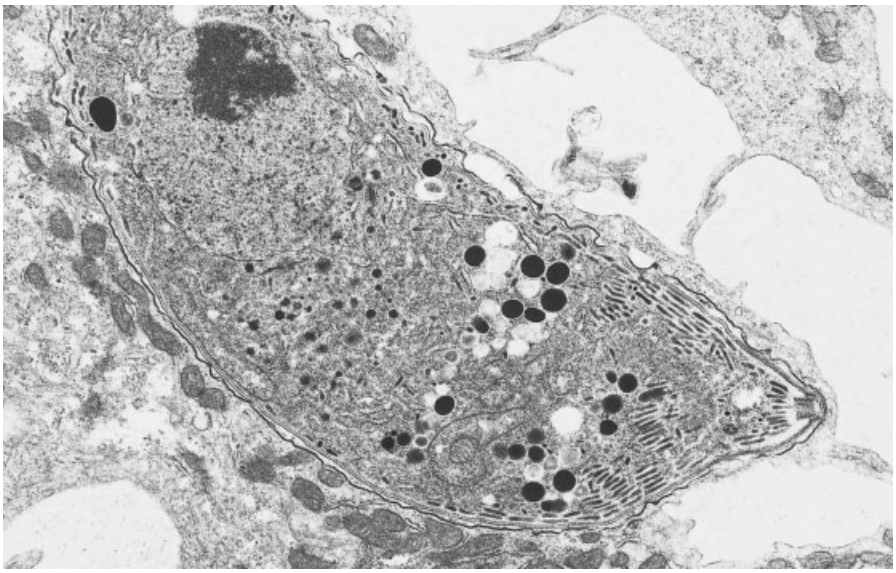

Figure 4 *Electronmicrograph with a merozoite of* Isospora belli *demonstrating some typical apicomplexan organelles including conoid at the apical pole, micronemes extending along the periphery from the apical pole and many dense granules in the cytoplasm. Magnification ×20 000.*

ingestion of the paratenic host. It is not known if there are paratenic hosts for *I. belli*.

Cell and molecular biology

Little is known about the biology of *I. belli*, probably in part because infection is not common in developed countries and parasite material is not easily obtained. Some *Isospora* species such as *I. suis* are able to grow in cell culture but development is limited and no sexual stages or oocysts are produced (Lindsay and Blagburn 1987). Complete development from sporozoites to oocysts of *I. suis* has also been obtained in the chick embryo chorioallantoic membrane but the oocysts do not sporulate (Lindsay and Current 1984).

Clinical aspects

I. belli infects both immunocompetent and immunocompromised adults and children. Infection may be asymptomatic in immunocompetent individuals or it may lead to a mild, self-limiting diarrhea lasting from 6 weeks to 6 months. The most extensive experience of human isosporiasis is in Chile. The most common manifestations reported were diarrhea (98 percent), weight loss (86 percent), and abdominal pain (61 percent). The stools contained fat and Charcot–Leyden crystals, and, most interestingly for a protozoal infection, a peripheral eosinophilia was seen in 54 percent of cases (Brandborg et al. 1970). Vomiting, steatorrhea, headache, fever, and malaise may also be present, and dehydration follows when diarrhea is severe. Persistent nonbloody diarrhea, indistinguishable from that caused by microsporidia and *Cryptosporidium parvum*, is the major manifestation in immunocompromised individuals. Persistent diarrhea may occur in immunocompetent persons as well, but clinical features are usually less severe (Lindsay et al. 1997). Manifestations in children can sometimes be severe.

In AIDS patients, extra-intestinal infections can occur, though they are rare. Necropsy occasionally reveals infection of mesenteric lymph nodes, liver, and spleen. Biliary disease has been described.

Immunology

There is a paucity of data on mechanisms of immunity against *I. belli* and extrapolation from studies with related parasites give only a possible picture of the main elements of the adaptive immune system involved in clearance of the infection. It is likely that *I. belli* infection induces immunity against reinfection as piglets recovered from *I. suis* infection were shown to exhibit strong resistance to reinfection (Stuart et al. 1982). Colostral parasite-specific antibodies did not protect piglets against *I. suis* infection (Lindsay et al. 1997). Presumably CD4 T cells are involved in immunity to *I. belli* as infection in AIDS patients is more severe than in immunocompetent individuals (Soave and Johnson 1988). Immunity to parasites of the related genus *Eimeria*, which has a similar life cycle to *Isospora*, has been studied extensively in rodents and chickens (reviewed in Lillehoj and Trout 1996; Smith and Hayday 1998). The significant features of immunity are that CD4 αβTCR T cells are involved, developing a Th1-cell-mediated response with IFN-γ production. Although parasite-

specific antibody, including secretory IgA, is produced during *Eimeria* infection, it does not appear to play a major role in recovery.

Pathology

There have been few publications on human isosporiasis, possibly fewer than on any other common human infection. Brandborg et al. (1970) observed moderate-to-severe villous atrophy and 'spectacular' collagen deposition in the lamina propria, with eosinophil infiltration which was extreme in two of the six cases analyzed. Ultrastructural work shows vacuolation of the enterocytes, microvillous shortening, and lipid deposits (Trier et al. 1974). Sexual and asexual stages of the parasite life-cycle are seen in the epithelium, enclosed by a parasitophorous vacuole (Comin and Santucci 1994).

There are no data on the disturbed physiology of human isosporiasis, but it is likely that there would be similarities with disturbances found in cryptosporidiosis.

Diagnosis

Examination of a fecal specimen for oocysts would normally allow diagnosis of infection with *I. belli* as the oocysts are large and oval (23–36 × 12–17 μm) (Lindsay et al. 1997). The oocysts may not be as numerous as in cryptosporidiosis and concentration by centrifugal flotation methods are sometimes employed (Whiteside et al. 1984). Oocysts can also be detected in fecal smears following acid-fast staining (Figure 5) or staining with auramine-rhodamine (Forthal and Guest 1984). Use of the latter technique should be employed in conjunction with another method for confirmation, however. Unstained oocysts are autofluorescent, appearing violet under ultraviolet light and green under violet or blue-violet light (Varea et al. 1998). Sometimes infection is diagnosed from stained intestinal biopsy material showing parasites in enterocytes when oocysts are not found by fecal examination (Whiteside et al. 1984). A highly sensitive and specific method for diagnosis has employed PCR with primers for the small-subunit rRNA sequences of *I. belli* (Muller et al. 2000); no amplification was seen with template DNA extracted from other parasites.

Epidemiology

Isospora belli causes human infection in every continent, but transmission is most frequent in tropical zones, predominantly subequatorial Africa, the Caribbean, and some parts of South America. Isosporiasis is emerging as a major problem in populations heavily infected by HIV. The prevalence in AIDS patients with persistent diarrhea is very variable. Isosporiasis is rarely detected in industrialized countries, the highest reported figure being 3 percent in the USA (Antony et al. 1988). Most cases of isosporiasis in Los Angeles were in AIDS patients from Latin America (Sorvillo et al. 1995). This is clearly different from the epidemiology of cryptosporidiosis. Estimates of prevalence in tropical countries range from 3 percent in Mali (Pichard et al. 1990) to 10 percent in Brazil (Sauda

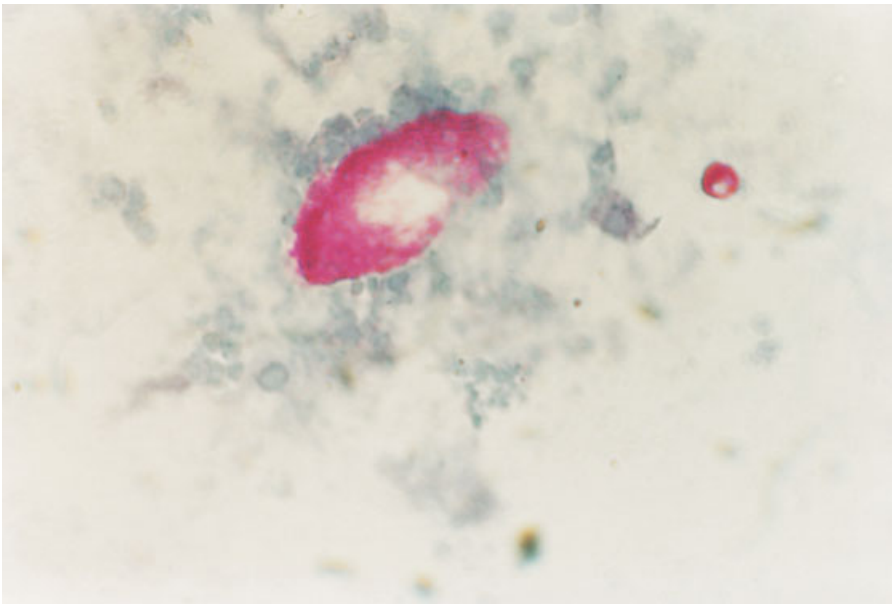

Figure 5 *Light microscopy examination of a fecal smear showing acid-fast staining of the large oval-shaped oocyst of* Isospora belli *(approximately 30 μm in length). An oocyst of* Cryptosporidium parvum *is also present which is almost spherical and much smaller (5 μm in diameter). Magnification ×1 000.*

et al. 1993), 20 percent in Burundi (Floch et al. 1989), and 37 percent in Zambia (Zulu et al. 2002). Isosporiasis seems to be relatively uncommon in AIDS patients in East Africa.

Another important difference between the epidemiology of isosporiasis and that of cryptosporidiosis is the rarity of finding *I. belli* in HIV-infected children. In Zambia, the prevalence was 2 percent in children with persistent diarrhea and malnutrition irrespective of HIV status (Amadi et al. 2001). Isosporiasis is rare in Indian children, being detected in seven of 10 126 children with diarrhea (Mirdha et al. 2002). It was found in 2.8 percent of malnourished Egyptian children (Rizk and Soliman 2001). In Zambia, isosporiasis is not seasonal (author, unpublished observations).

Chemotherapy

The first anecdotal report, to our knowledge, of successful treatment was with nitrofurantoin (Brandborg et al. 1970), and the second success was with pyrimethamine–sulfadiazine (Trier et al. 1974). DeHovitz et al. (1986) obtained good responses with trimethoprim–sulfamethoxazole (960 mg four times daily for 10 days, then 960 mg twice daily for 21 days) in all of 20 patients, but 47 percent relapsed within a few weeks. This regimen was further evaluated in Haitian AIDS patients (Pape et al. 1989) and is now the standard therapy, with initial therapy usually given for 10 days. Secondary prophylaxis (to prevent relapse) is usually required, suggesting that true eradication is not achieved. The dose required is 960 mg orally three times per week. Pyrimethamine–sulfonamide combinations (e.g. Fansidar) are also effective (Trier et al. 1974; Pape et al. 1989). In those intolerant of sulfonamides, pyrimethamine alone has been used (Weiss et al. 1988). Other compounds, including primaquine and diclazuril have also been found useful (Lindsay et al. 1997), and a combination of albendazole and ornidazole has been reported to be effective in sulfonamide-intolerant patients (Dionisio et al. 1996). There is conflicting evidence as to the efficacy of nitazoxanide in human isosporiasis.

CYCLOSPORIASIS

Introduction

Cyclospora cayetanensis is the most recently identified member of the coccidia that is a human intestinal pathogen. The genus was created in 1881 by Schneider for a parasite of a myriapod, *Cyclospora glomerica* and other species have been found in rodents, moles, and snakes (reviewed by Ortega et al. 1994) and in three species of monkey (Eberhard et al. 1999). Probably the first report of a human infection was by Ashford (1979) who described oocysts of a new coccidian from fecal samples of three individuals in Papua New Guinea. From the mid-1980s onwards, similar descriptions were provided in cases of traveler's diarrhea or AIDS-related diarrhea (Soave et al. 1986; Hart et al. 1990; Shlim et al. 1991; Pollok et al. 1992). Cases in the western hemisphere usually involved travelers returning from developing countries in different continents and the symptoms were similar to those associated with cryptosporidiosis, but sometimes lasting longer. The oocysts were approximately 8–10 μm in diameter and stained red with the modified Ziehl-Neelsen method. If the parasite was a coccidian, the oocysts were too small to be from *I. belli* and too large to be from *C. parvum*. Early morphological and ultrastructural studies suggested that the organism was cyanobacterium-like (Long et al. 1990). The parasite was identified as a coccidian and a species of *Cyclospora* following the demonstration of sporulation of the oocysts and sporozoite excystation (Ortega et al. 1993). The mature oocyst had a sporocyst and sporozoite composition characteristic of this genus and distinct from that present in *Cryptosporidium* and *Isospora*. Shortly afterwards, it was reported that *C. cayetanensis* infection was common in Haitians with advanced HIV infection suffering chronic or intermittent diarrhea (Pape et al. 1994). Biliary infection with accompanying symptoms has also been observed in AIDS patients (Sifuentes-Osornio et al. 1995). Cyclosporiasis patients have been found to respond rapidly to antimicrobial therapy but the symptoms often recurred in individuals with AIDS (Wurtz 1994; Pape et al. 1994). There is evidence that infection is transmitted by contaminated water supplies or food, particularly fruit or vegetables (Wright and Collins 1997; Ortega et al. 1997)

Classification

Cyclospora cayetanensis belongs to the phylum Apicomplexa, suborder Eimeriorina, and family Eimeriidae (Ortega et al. 1994). The genus is defined by the morphology of the oocyst which is spheroidal and 8–10 μm in diameter; it matures outside the host and the sporulated oocyst contains two sporocysts, each of which has two sporozoites. Transmission electron microscopy of mature oocysts has demonstrated typical coccidian structures in oocysts such as Stieda body on the sporocyst and rhoptries and micronemes in the sporozoite (Ortega et al. 1998). (Remember, the oocysts of *I. belli* are significantly larger and oval, and the sporulated forms have four sporozoites in each of the two sporocysts.) The entire development of *C. cayetanensis* occurs within a single host and currently humans are the only known hosts (Eberhard et al. 2000). Phylogenetic analysis of the small-subunit ribosomal DNA of *Cyclospora* suggests that it is closely related to members of genus *Eimeria* that infect animals and have oocysts

with four sporocysts containing two sporozoites (Relman et al. 1996).

Life cycle

Oocysts of *C. cayetanensis* mature outside the human host over a period of several days. A single sporoblast in the excreted oocyst divides into two and each develops into a sporocyst with two sporozoites (Ortega et al. 1994). *Cyclospora* oocysts have been shown to have maximal sporulation at temperatures of 22–30°C (Smith et al. 1997). It has been demonstrated that after mechanical disruption of oocysts and in the presence of trypsin and sodium taurocholate, sporozoites will excyst. Endogenous development has been observed in small intestinal biopsies and trophozoites, schizonts, merozoites, and sexual stages have been identified at the luminal end of enterocytes (Bendall et al. 1993; Sun et al. 1996; Ortega et al. 1998). Merozoites of different sizes have also been described suggesting that there might be more than one type of meront (Ortega et al. 1998). Parasite development may spread to epithelium of the biliary system in AIDS patients (Zar et al. 2001). The presence of all life cycle stages in the human host indicates that the parasite is monoxenous. Attempts to infect other species including mice, rats, rabbits, girds, hamsters, ferrets, pigs, dogs, and monkeys have failed suggesting that humans may be the only host for *C. cayetanensis* (Eberhard et al. 2000), unless higher primates are susceptible to infection.

Clinical aspects

In common with the other coccidia described in this chapter, *Cyclospora cayetanensis* causes acute and persistent diarrhea in immunocompetent and immunocompromised adults and children. In two studies of Peruvian children, the proportions of episodes of cyclosporiasis which were accompanied by diarrhea were 11 and 28 percent, and the clinical disease produced was indistinguishable from cryptosporidiosis (Ortega et al. 1993).

In Haiti, cyclosporiasis was responsible for 11 percent of AIDS-related diarrhea (Pape et al. 1994). Again, the clinical features were indistinguishable from cryptosporidiosis or isosporiasis.

Pathology

C. cayetanensis occupies an intracellular position in intestinal epithelial cells, and this is accompanied by increased numbers of intra-epithelial lymphocytes and a variable degree of villous blunting (Bendall et al. 1993). There are no significant data on the pathophysiology of human cyclosporiasis.

Diagnosis

Diagnosis of cyclosporiasis is obtained by identification of oocysts in fecal specimens. The 8–10 μm diameter oocysts of *C. cayetanensis* can be readily differentiated microscopically from those of two other human intestinal coccidia, *Cryptosporidium parvum* and *I. belli*, as they are double the size of the former and two to three times smaller than the latter. Under ultraviolet illumination, unstained oocysts of *C. cayetanensis* are autofluorescent, giving a rapid and inexpensive diagnostic method (Eberhard et al. 1997). With normal microscopy, oocysts can be observed by employing acid-fast staining methods such as modified Ziehl-Neelsen or Kinyoun, and also by using safranin (Long et al. 1990; Pape et al. 1994; Wurtz 1994). The organisms can be concentrated by centrifugation in formalin-ethyl acetate or by sucrose flotation. Oocysts may sporulate after several days in potassium dichromate solution at room temperature (Ortega et al. 1998) (Figure 6). PCR with primers specific for *C. cayetanensis* have been employed to identify the presence of the parasite in fecal specimens and this approach could provide a highly sensitive diagnostic tool (Relman et al. 1996).

Epidemiology

The first descriptions indicated that travelers (to India, Pakistan, and Morocco) and the immunocompromised are the principal risk groups (Bendall et al. 1993). In expatriates living in Nepal, the annual attack rate was 32 percent (Shlim et al. 1999). In Nepal, *C. cayetanensis* was not found in children with diarrhea under 18 months of age, but was found in 12 percent of episodes in children between 18 and 60 months of age (Hoge et al. 1995a).

Cyclosporiasis can be foodborne or waterborne. A large outbreak of 1 465 cases of cyclosporiasis in 1996 in the USA was traced to raspberries imported from Guatemala (Herwaldt et al. 1997). Exposure to untreated water was associated with a fourfold increased risk of cyclosporiasis in foreign residents in Nepal (Hoge et al. 1993).

In Africa, *C. cayetanensis* infection has not been found in surveys in Zambia or Guinea-Bissau (authors' unpublished data) despite the fact that the organisms should be easily visible in Ziehl-Neelsen stained smears, but small numbers of cases were reported in Tanzania (Cegielski et al. 1999). It would appear that cyclosporiasis is much less common in Africa than in Central America or South Asia.

Chemotherapy

Chemotherapy with trimethoprim–sulfamethoxazole (960 mg twice daily for 7 days) has been shown to be

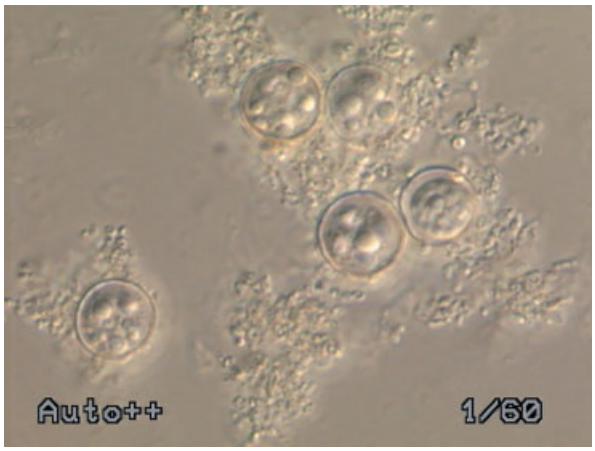

Figure 6 *Light microscopy examination of unstained spherical oocysts of* Cyclospora cayetanensis *that have a diameter of 8–10 μm. These are about X2 the size of oocysts of* Cryptosporidium parvum *but are significantly smaller than the oval-shaped oocysts of* Isospora belli*. (Photograph kindly provided by Monika Kettelhut, Hospital for Tropical Diseases, London, UK)*

effective in immunocompetent adults in Nepal (Hoge et al. 1995b). In AIDS patients, relapse is common, and this can be successfully prevented by 960 mg three times weekly indefinitely (Pape et al. 1994). Ciprofloxacin (500 mg twice daily for 7 days) is effective in patients who are intolerant of sulfonamides (Verdier et al. 2000).

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Toxoplasmosis

J.P. DUBEY

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INTRODUCTIO N

Infection with *Toxoplasma gondii* is one of the most common parasitic infections of humans and other warm-blooded animals. It is found worldwide from Alaska to Australasia and nearly one-third of humanity has been exposed to this parasite. In most adults, it does not cause serious illness, but it can cause blindness and intellectual deficit in congenitally infected children and severe disease in those with depressed immunity. In animals, it is a common cause of abortion in goats and sheep.

T. gondii was discovered by Nicolle and Manceaux (1908, 1909) in Tunisia in a rodent, *Ctenodactylus gundi* and, independently, in a laboratory rabbit by Splendore (1908) in São Paulo, Brazil. It was recognized as a human pathogen when Wolf et al. (1939) reported a confirmed case of congenital toxoplasmosis in a child (see Table 1). The complete life cycle of *T. gondii* was not determined until 1970 (Frenkel et al., 1970; Dubey et al., 1970a, b). The full life cycle clearly shows that this protozoan is a coccidian. Coccidians are very common parasites of animals. Most coccidians are one-host parasites and develop in the intestinal epithelium. An environmentally resistant stage is excreted in feces and the host becomes infected by ingesting feces.

CLASSIFICATION

T. gondii is a coccidian parasite of cats with other warm-blooded animals as intermediate hosts. Coccidiosis is among the most important of parasitic infections of animals. Traditionally, all coccidia of veterinary importance were classified in the family Eimeriidae and were further classified based on the structure of the oocyst. Coccidia having oocysts with four sporocysts each with two sporozoites (total eight sporozoites) are classified as *Eimeria*, and coccidia having oocysts containing two sporocysts each with four sporozoites were classified historically as *Isospora*. After the discovery of the life cycle of *T. gondii*, several other genera (*Sarcocystis*, *Besnoitia*, *Hammondia*, *Frenkelia*, and *Neospora*) were also found to have isosporan oocysts with two sporocysts and eight sporozoites. *T. gondii* and related genera are now classified in the phylum Apicomplexa Levine, 1970, class Coccidea Leuckart, 1879, order Eimeriida Léger, 1911 (see also Classification and introduction to the parasitic protozoa). Opinions differ regarding the placement of *T. gondii* into a family and subfamily: various authorities have placed it in one or the other of the families Eimeriidae Michin, 1903, Sarcocystidae Poche, 1913, or Toxoplasmatidae Biocca, 1956.

STRUCTURE AND LIFE CYCLE

The name *Toxoplasma* (from 'toxon' meaning 'bow' and 'plasma' meaning 'form') relates to the crescent shape of the tachyzoite stage. There are three infectious stages of *T. gondii*: the tachyzoites (in groups), the bradyzoites (in tissue cysts), and the sporozoites (in oocysts) (Frenkel 1973a).

Table 1 *History of* Toxoplasma gondii *and toxoplasmosis*

Contributors and year	Contribution
Nicolle and Manceaux (1908)	Discovered in *gundi*
Splendore (1908)	Discovered in rabbit
Mello (1910)	Disease described in a domestic animal (dog)
Janku (1923)	Identified in human eye at necropsy
Wolf and Cowen (1937)	Congenital transmission documented
Wolf et al. 1939	Congenital transmission coonfirmed
Pinkerton and Weinman (1940)	Fatal disease described in adult humans
Sabin (1942)	Disease characterized in man
Sabin and Feldman (1948)	Dye test described
Siim (1952)	Glandular toxoplasmosis described in man
Weinman and Chandler (1954)	Suggested carnivorous transmission
Hartley and Marshall (1957)	Abortions in sheep recognized
Beverley (1959)	Repeated congenital transmission observed in mice
Jacobs et al. (1960)	Tissue cysts characterized biologically
Hutchison (1965)	Fecal transmission recognized, nematode eggs suspected
Hutchison et al. (1969, 1970, 1971); Frenkel et al. (1970); Dubey et al., (1970a,b); Sheffield and Melton (1970); Overdulve (1970)	Coccidian phase described
Frenkel et al. (1970); Miller et al. (1972)	Definitive and intermediate hosts defined
Dubey and Frenkel (1972)	Five *T. gondii* types described from feline intestinal epithelium
Wallace (1969); Munday (1972)	Confirmation of the epidemiological role of cats from studies on remote islands
Luft et al. (1983)	Toxoplasmosis recognized in AIDS patients
Silveira et al. (1988)	Postnatal opthalmic toxoplasmosis recognized

Reproduced from Dubey 1993; where a complete bibliography may be found.

The tachyzoite is often crescent-shaped and approximately 2×6 μm in smears or globular-to-oval in sections (Figure 1). Its anterior (conoidal) end is pointed and its posterior end is round. It has a pellicle (outer covering), a polar ring, a conoid, rhoptries, micronemes, mitochondria, subpellicular microtubules, endoplasmic reticulum, a Golgi apparatus, ribosomes, rough surfaced endoplasmic reticulum, a micropore, and a well defined nucleus (Figure 2). The nucleus is usually situated toward the posterior end or in the central area of the cell.

The pellicle consists of three membranes. The inner membrane complex is discontinuous at three points: the anterior end (polar ring), the lateral edge (micropore), and toward the posterior end. The polar ring is an osmiophilic thickening of the inner membrane at the anterior end of the tachyzoite. The polar ring encircles a cylindrical, truncated cone (the conoid) which consists of between six and eight fibrillar elements wound like a compressed spring. There are 22 subpellicular microtubules originating from the anterior end and running longitudinally almost the entire length of the cell. Terminating within the conoid are between four and ten club-shaped organelles called rhoptries (Dubey 1977, 1993; Dubey et al. 1998). The rhoptries are glandlike structures, often labyrinthine, with an anterior narrow neck up to 2.5 μm long. Their saclike posterior end terminates anterior to the nucleus. Micronemes are rice grain-like structures, which occur at the anterior (conoidal) end of the parasite.

The functions of the conoid, rhoptries, and micronemes are not fully known. The conoid is probably associated with the penetration of the tachyzoite through the membrane of the host cell. It can rotate, tilt, extend, and retract as the parasite searches for a host cell. *T. gondii* can move by gliding, undulating and rotating. Rhoptries have a secretory function associated with host-cell penetration, secreting their contents through the conoid to the exterior. The microtubules probably provide the cytoskeleton.

The tachyzoite enters the host cell by active penetration of its membrane. After entering the host cell, the tachyzoite assumes an oval shape and becomes surrounded by a parasitophorous vacuole (PV) which, it has been suggested, is derived from both the parasite and the host. Numerous intravacuolar tubules connect the parasitophorous vacuolar membrane (PVM) to the parasite pellicle (Figure 2). *T. gondii* enters host cells by active penetration and the whole event can be completed in 10 seconds, this makes it difficult to study the invasion process. Rhoptries, micronemes, conoid, microtubules, myosin, dense graduals, and other factors are involved in a complex cascade of individual events involving attachment, protrusion of conoid, moving junction formation and exocytosis of contents of rhoptries, micronemes and dense granules (Joiner and

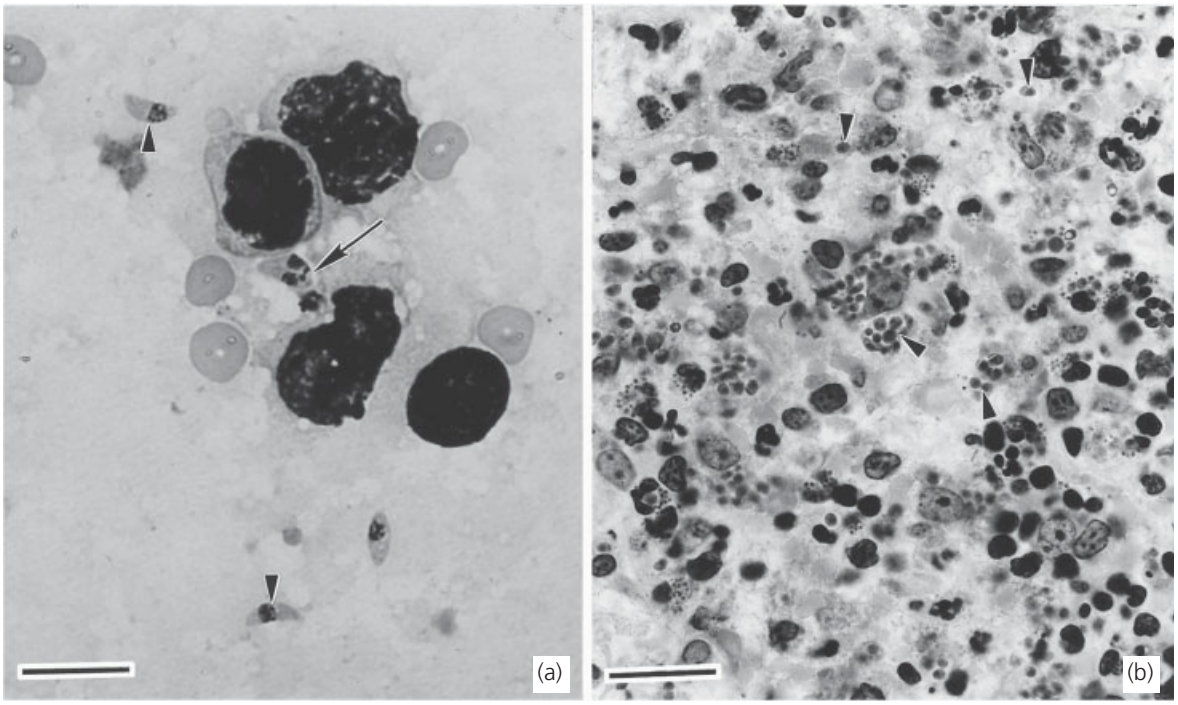

Figure 1 *Tachyzoites of* T. gondii*:* **(a)** *Impression smear. Note individual crescentic (arrowheads) and dividing (arrow) tachyzoites. Giemsa. Bar = 10 μm.* **(b)** *Histological section of mesenteric lymph node. Note numerous oval-to-round tachyzoites (arrowheads). Hematoxylin and eosin (H&E). Bar = 20 μm*

Dubremetz 1993). Parasite proteins P30 and surface lectin-like molecules are involved in finding host cell receptors. Myosin and actin, located at the apical end of the parasite, help in extension of the conoid and formation of a moving junction. The secretions of rhoptries and micronemes probably dissolve the plasma membrane for the parasite invasion. After invasion, the parasite is separated from the host cell cytoplasm by the

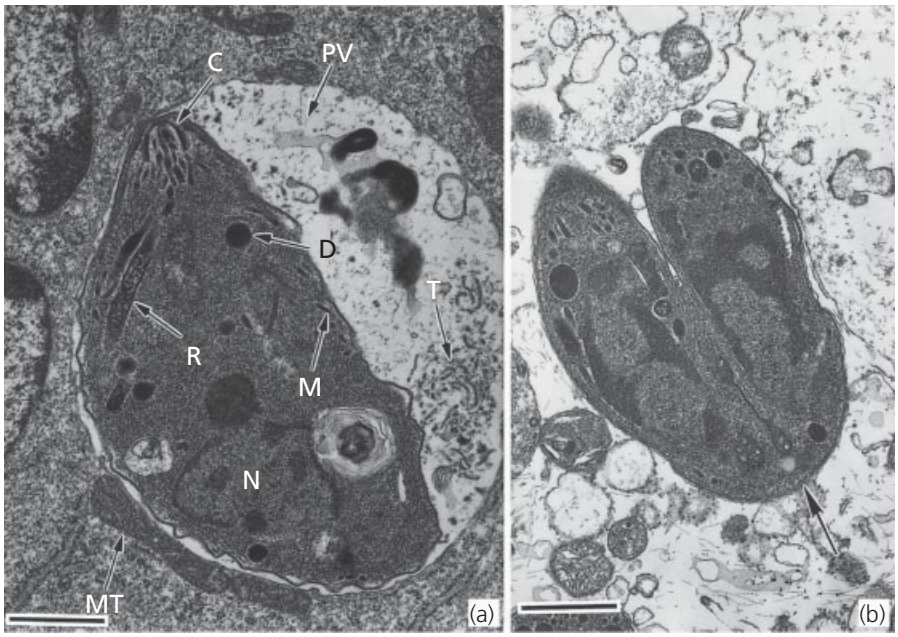

Figure 2 *Transmission electron micrographs of* T. gondii *tachyzoites in cell culture:* **(a)** *Tachyzoite in a parasitophorous vacuole (PV) in the cytoplasm of a host cell. Note conoid (C), rhoptries (R), micronemes (M), nucleus (N), dense granules (D), and intravacuolar tubules (T). The host cell mitochondria (MT) are closely associated with the PV. Bar = 2.1 μm.* **(b)** *Dividing tachyzoites with separated apical ends and the undivided posterior end (arrow). Bar = 1.18 μm*

PVM, which is formed by the invagination of host-cell plasma membrane (Sibley 1995). The proteins derived from the host cell are quickly removed from the PV and a complex membranous tubular network (TMN) is formed in the PV; dense granule secretions contribute to TMN formation. These TMN are connected to the PVM and micropores in the PVM allow diffusion of selective host molecules into the PVM, but otherwise the parasite is independent of the host cell.

The tachyzoite multiplies asexually within the host cell by repeated endodyogeny (from 'endon' meaning 'inside', 'dyo' meaning 'two', and 'genesis' meaning 'birth'), a specialized form of reproduction in which two progenies form within the parent parasite, consuming it (Figure 2). Tachyzoites continue to divide by endodyogeny until the host cell is filled with parasites.

After a few divisions, *T. gondii* encysts to form tissue cysts. Tissue cysts grow and remain intracellular (Figure 3) as the bradyzoites (encysted zoites) divide by endodyogeny. Tissue cysts vary in size (Figure 3). Young tissue cysts may be as small as 5 μm and contain only two bradyzoites, although older ones may contain hundreds of organisms (Figure 3a, b). Tissue cysts in histological sections of brain are often circular and rarely reach a diameter of 60 μm whereas intramuscular cysts are elongated and may reach 100 μm; tissue cysts in unstained live squash preparations vary in size depending on the pressure applied to squash the tissue and the medium of suspension (Figure 3b). Although tissue cysts may develop in visceral organs, including lungs, liver and kidneys, they are more prevalent in the neural and muscular tissues, such as the brain, eye, and skeletal and cardiac muscle. Intact tissue cysts probably do not cause any harm and can persist for the life of the host.

The tissue cyst wall is elastic, thin (less than 0.5 μm) and argyrophilic, and may enclose hundreds of crescent-shaped slender bradyzoites approximately 7 × 1.5 μm in size (Figure 3). Structurally, bradyzoites differ only slightly from tachyzoites. They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located. The contents of rhoptries in bradyzoites in older tissue cysts are electron dense (Figure 4) (Ferguson and Hutchison 1987; Dubey 1993). Bradyzoites contain several amylopectin granules, which stain red with periodic acid–Schiff

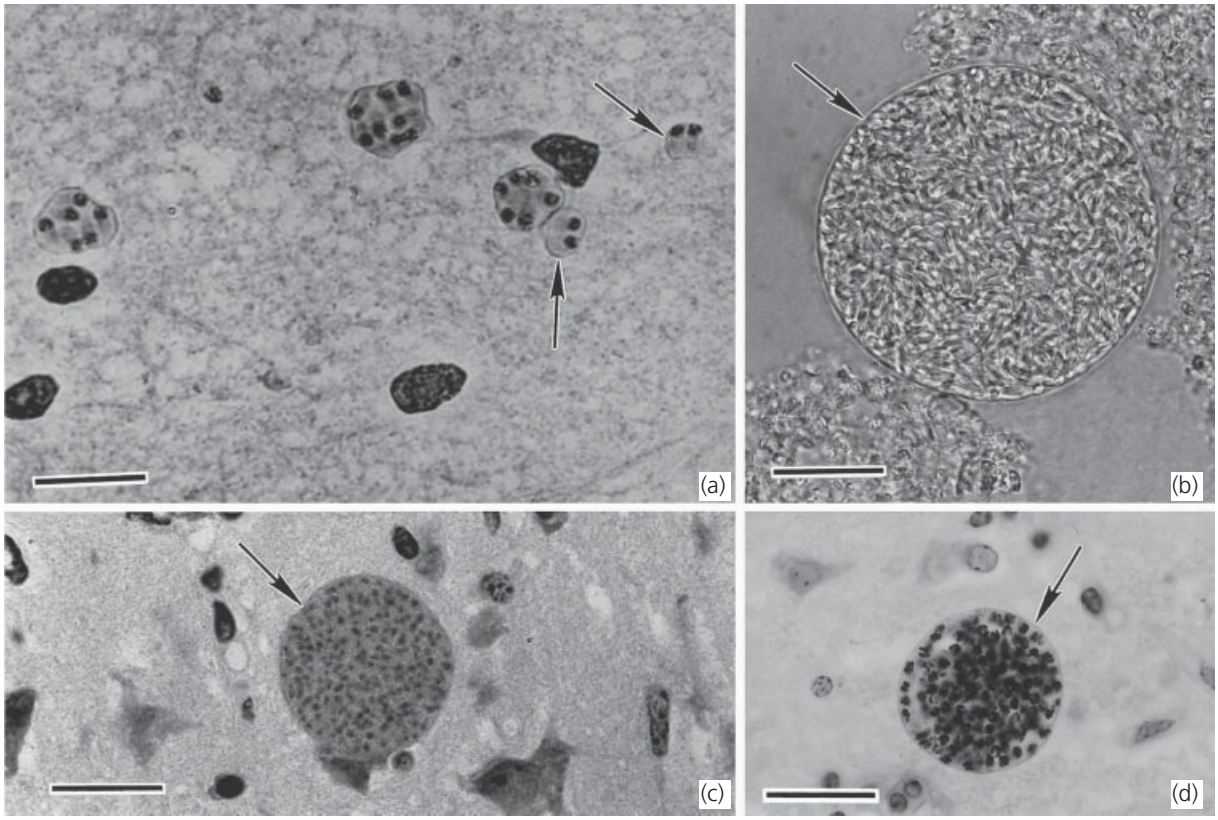

Figure 3 *Tissue cysts of* T. gondii *in brain:* **(a)** *Impression smear. Five young tissue cysts with silver positive cyst walls. Two tissue cysts (arrows) each have two bradyzoites with terminal nuclei. Silver stain. Bar = 10 μm.* **(b)** *Impression smear, unstained. This tissue cyst was freed by grinding a piece of brain in a mortar with a pestle. Note thin, elastic cyst wall (arrow) enclosing hundreds of bradyzoites. Bar = 20 μm.* **(c)** *Histological section. Note only nuclei of bradyzoites are visible. H&E. Bar = 20 μm.* **(d)** *Histological section. Note bradyzoites have PAS-positive red granules that appear black in this micrograph. The tissue cyst wall (arrow) is PAS-negative. PAS–hematoxylin. Bar = 20 μm*

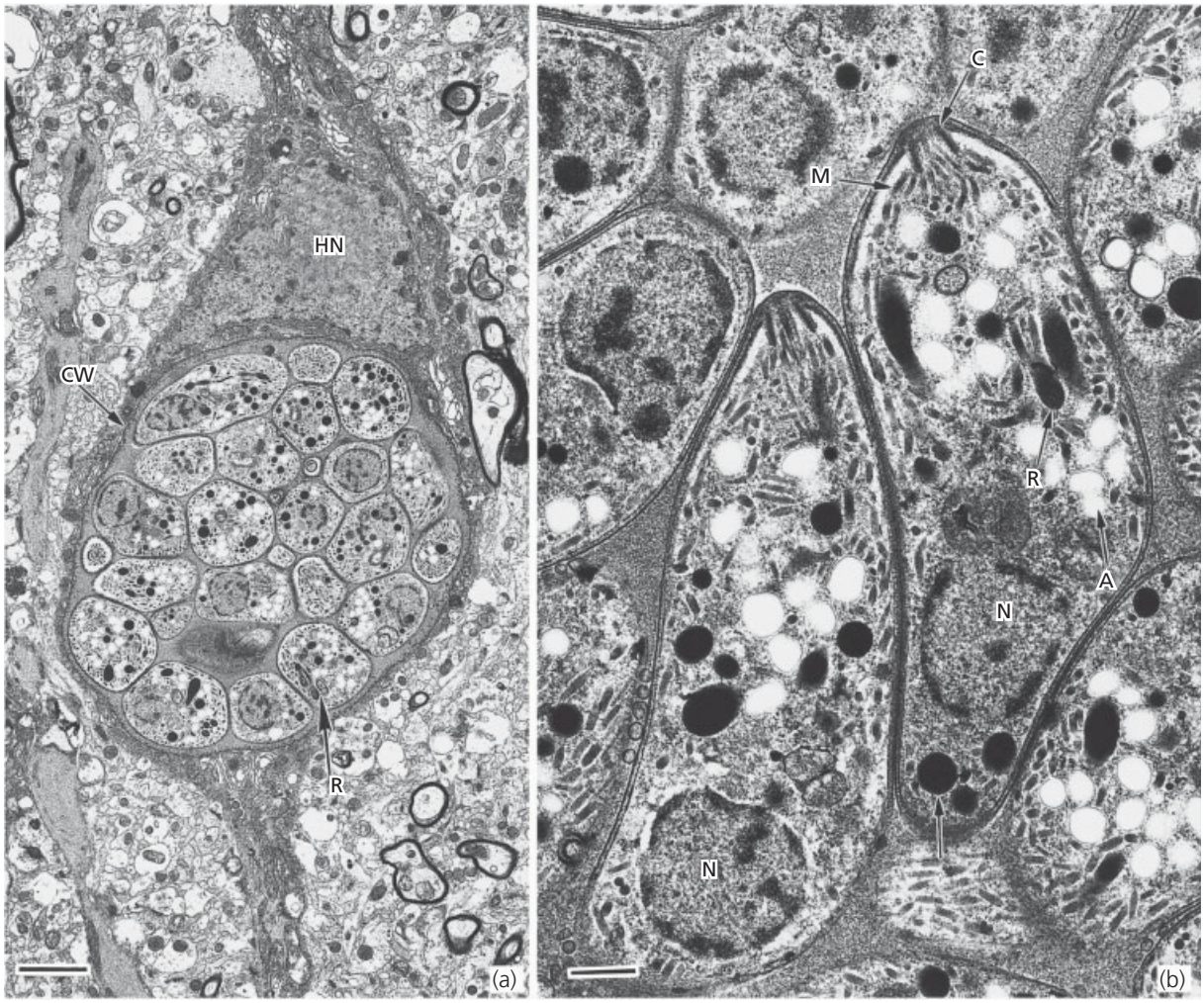

Figure 4 *Transmission electron micrographs of tissue cysts of* T. gondii *in brain.* **(a)** *A young intracellular cyst with well developed cyst wall (CW) is visible. The bradyzoites are plump (dividing or preparing to divide) and the contents of the rhoptries (R) in one* T. gondii *have a honeycomb structure (arrow). HN, host nucleus. Bar = 4.4 μm.* **(b)** *Two longitudinally cut bradyzoites from a large cyst. Note electron-dense contents of rhoptries (R), and the terminal nuclei (N), a conoid (C), numerous micronemes (M), and amylopectin granules (A) that appear as empty spaces here. Bar = 0.77 μm.*

(PAS) reagent; such material is either in discrete particles or absent from tachyzoites. Bradyzoites are more slender than tachyzoites and less susceptible to destruction by gastric juice.

Factors influencing tissue cyst formation are not well known. Tissue cysts are more numerous in animals in the chronic stage of infection, after the host has acquired immunity, than during the acute stage of infection. However, tissue cysts have been found in mice infected for only 3 days and in cells in culture systems devoid of known immune factors. It is possible, therefore, that development of functional immunity and the formation of tissue cysts are coincidental.

Cats shed oocysts (Figure 5) after ingesting any of the three infectious stages of *T. gondii*, (i.e. tachyzoites, bradyzoites or sporozoites) (Frenkel et al. 1970; Dubey et al. 1970a). Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage ingested. Prepatent periods are 3–10 days after ingesting tissue cysts and 18 days or more after ingesting tachyzoites or oocysts (Dubey and Frenkel 1976; Freyre et al. 1989; Dubey 1996, 2002a). Less than 50 percent of cats shed oocysts after ingesting tachyzoites or oocysts, whereas nearly all cats shed oocysts after ingesting tissue cysts.

After the ingestion of tissue cysts by cats, the cyst wall is dissolved by the proteolytic enzymes in the stomach and small intestine. The released bradyzoites penetrate the epithelial cells of the small intestine and initiate development of numerous asexual generations of *T. gondii* (Dubey and Frenkel 1972). Five morphologically distinct types (A–E) of *T. gondii* develop in intestinal epithelial cells before gametogony begins. Types A–E divide asexually by endodyogeny, endopolygeny or schizogony (division into more than two organisms). The origin of gamonts has not been determined; probably the

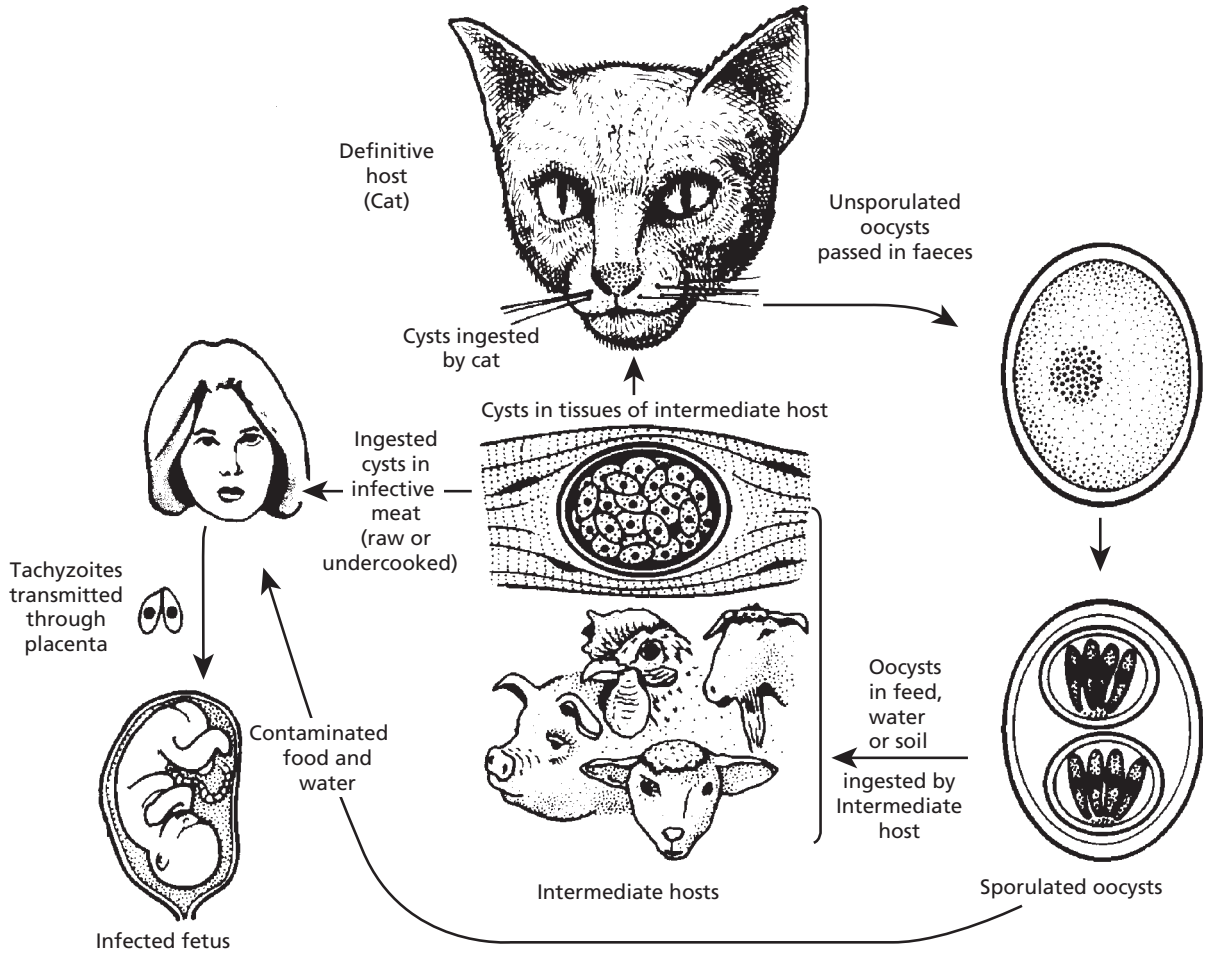

Figure 5 *Life cycle of* T. gondii

merozoites released from meronts of types D and E initiate gamete formation. Gamonts occur throughout the small intestine but most commonly in the ileum (Figure 6). Gamonts and schizonts are located in surface epithelial cells, usually above the host cell nucleus (Figure 7), where they form 3–15 days after infection. The female gamete is subspherical and contains a single centrally located nucleus (Figure 7). Mature male gamonts are ovoid to ellipsoidal in shape (Figure 6). When microgametogenesis takes place, the nucleus of the male gamont divides to produce 10–21 nuclei which move toward the periphery of the parasite entering protuberances formed in the pellicle of the mother parasite. One or two residual bodies are left in the microgamont after division into microgametes. Each microgamete has two flagella (Figure 6) and swims to and penetrates a mature macrogamete. After penetration, oocyst wall formation begins around the fertilized gamete, and, when mature, oocysts are discharged into the intestinal lumen by the rupture of intestinal epithelial cells.

Unsporulated oocysts are subspherical to spherical, 10 × 12 μm in diameter (Figure 8). The oocyst wall contains two layers. The sporont almost fills the oocyst, and sporulation occurs outside the cat within 1–5 days depending on aeration and temperature. Sporulated oocysts are subspherical to ellipsoidal and each sporulated oocyst contains two ellipsoidal sporocysts. These lack a Stieda body (Figure 8). Sporocysts measure 6 × 8 μm. There are four sutures with liplike thickenings in the sporocyst wall (Figure 8) which open during excystation of the sporozoites. A sporocyst residuum is present, but no oocyst residuum. Each sporocyst contains four sporozoites, 2 × 6–8 μm in size with a subterminal to central nucleus and a few PAS-positive granules in the cytoplasm (Dubey et al. 1970a). They have most of the organelles found in other coccidia except a crystalloid body and refractile globules (Figure 8).

As the enteroepithelial cycle progresses, bradyzoites penetrate the lamina propria of the feline intestine and multiply as tachyzoites. Within a few hours after infection of cats, *T. gondii* may disseminate to extraintestinal tissues. *T. gondii* persists in intestinal and extraintestinal tissues of cats for at least several months, if not for the life of the cat.

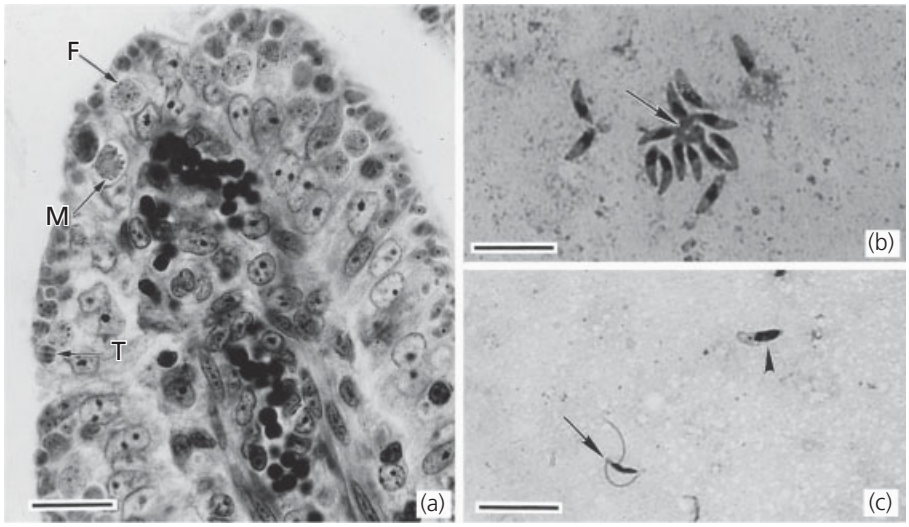

Figure 6 *Enteroepithelial stages of* T. gondii, *6 days after feeding tissue cysts to a cat:* **(a)** *Histological section of a villus in small intestine. Note heavy infection of epithelial cells with* T. gondii *types (T), male gamonts (M), and numerous uninucleate female gamonts (F). Cells in the lamina propria are not infected. H&E. Bar = 15 μm.* **(b)** *Impression smear. Note a ruptured type E schizont with a residual body (arrow). Giemsa. Bar = 10 μm.* **(c)** *Impression smear. A biflagellate microgamete (arrow) and a free merozoite (arrowhead). Giemsa. Bar = 10 μm*

CULTIVATION, ANTIGENS, MOLECULAR BIOLOGY, AND GENETICS

T. gondii has not been grown in cell-free media but can be cultivated in laboratory animals, chick embryos and cell cultures. Mice, hamsters, guinea pigs, and rabbits are all susceptible but mice are generally used as hosts because they are more susceptible than the others and are not naturally infected when raised in the laboratory on commercial dry food that is free from cat feces.

Figure 7 *Electron micrograph of coccidian stages of* T. gondii *in epithelial cells of ileum of a cat 6 days after ingesting tissue cysts. Note two merozoites (M) and a female gamont (G) located just below the microvillus border, and a schizont above the host cell nucleus. Bar = 2.5 μm*

Tachyzoites of some strains of *T. gondii* grow in the peritoneal cavity of mice, sometimes producing ascites; they also grow in most other tissues after intraperitoneal inoculation with any of the three infectious stages of *T. gondii*. Tissue cysts are prominent in the mouse brain about 8 weeks after infection. Virulent strains usually produce illness in mice and sometimes kill them within 1–2 weeks. Most strains of *T. gondii* do not kill mice.

T. gondii tachyzoites will multiply in many cell lines in cell cultures and although most strains can develop tissue cysts in cell cultures, the yield is lower than that produced in mice. The cysts can develop within 3 days of inoculation of tachyzoites in cell culture. Virulent mouse strains rapidly destroy the cells whereas avirulent strains grow slowly, causing minimal cell damage. The mean generation time of tachyzoites of the virulent RH strain is 5 hours. Feline enteroepithelial stages of *T. gondii* have not yet been cultivated in vitro. Oocysts can be obtained by feeding tissue cysts from infected mice to *T. gondii*-free cats. Although there are stage-specific proteins, all infective stages of *T. gondii* also share common proteins. Most of the proteins that have been characterized are from tachyzoites. *T. gondii* tachyzoites have four major surface proteins (of 22, 30, 35, and 43 kDa) of which p30 (SAG1) is the dominant protein and accounts for 5 percent of the total tachyzoite protein. Information about major *T. gondii* antigens and their localization in organelles of *T. gondii* was summarized by Petersen and Dubey (2001).

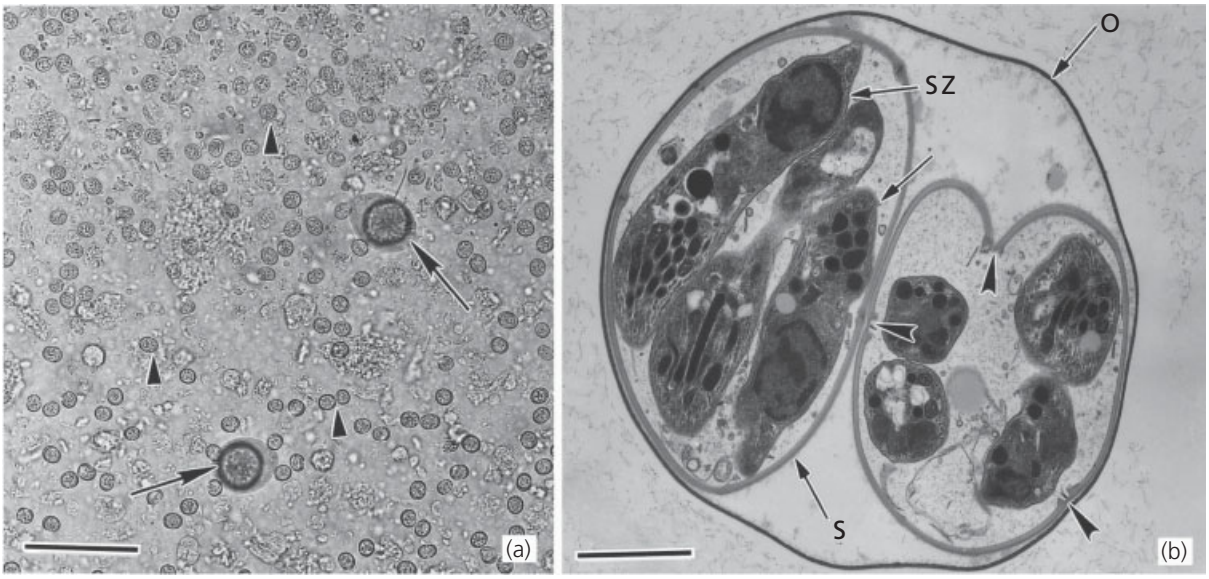

Figure 8 *Oocysts of* T. gondii*:* **(a)** *Unsporulated oocysts (arrowheads) and oocysts of another common feline coccidian,* Isospora felis *(arrows) in a fecal float preparation. Unstained. Bar = 50 μm.* **(b)** *Transmission electron micrograph of a sporulated oocyst. Note thin walled oocyst (O) enclosing the two sporocysts (S) each with four sporozoites (SZ). Each sporocyst has four lip-like thickenings (arrowheads). Bar = 2.8 μm. (Courtesy of Dr D. S. Lindsay)*

The *T. gondii* nucleus is haploid except for the zygote, in the intestine of the cat (Pfefferkorn 1990). Sporozoites result from a miotic division followed by mitotic divisions and genetic segregation seems to follow classical Mendelian laws. The total haploid genome contains approximately 8×10^7 bp and 11 chromosomes. There is also a 36 kb circular mitochondrial DNA that has been partly sequenced. Nine chromosomes have been identified by pulsed field gel electrophoresis. The karyotype has been studied using probes which attach to genes of low copy number (Sibley et al. 1992). Based on a wide host range (all warm blooded animals) and a worldwide distribution, one would expect a great genetic variability among *T. gondii* isolates. Contrary to this expectation, genetic diversity among strains of *T. gondii* so far characterized from humans or animals is low (Howe and Sibley 1995; Lehmann et al. 2000). Evidence suggests that *T. gondii* has a clonal population structure (Howe and Sibley 1995; Grigg et al. 2001a; Su et al. 2002, 2003), which can partly explain the low genetic diversity. Importantly, clonality provides the basis for strain typing using a single locus. Since clonality means that all genes are inherited together, high phenotypic similarity is expected among members of the same type (lineage). *T. gondii* strains have been classified into three genetic types (I, II, III), based on restriction fragment length polymorphism (RFLP). DNA from other sources can be introduced into the *T. gondii* genome (Soldati and Boothroyd 1993).

It has been suggested that type I isolates or recombinants of type I and III are more likely to result in clinical toxoplasmosis (Howe et al. 1997; Grigg et al. 2001a; Fuentes et al. 2001; Aspinall et al. 2003), but genetic characterization has been limited essentially to patients ill with toxoplasmosis. Contrary to the situation in humans, all three types of *T. gondii* have been isolated from asymptomatic animals (Mondragon et al. 1998; Dubey et al., 2002a, b). Little is known of the effect of geography on the genetic variability of *T. gondii* isolates because most of the *T. gondii* isolates genetically typed were from the United States and Europe. Ajzenberg et al. (2002) genotyped 86 isolates of *T. gondii* obtained from suspected or proven cases of congenital toxoplasmosis in Europe. Most of the isolates (73 of 86) were type II, 7 were type I, 2 were type III, and 4 were atypical. Among the 7 type I isolates, 4 were from placenta and the child was not infected. There was no obvious correlation between genotyping and severity of clinical toxoplasmosis. It is noteworthy that in this study all *T. gondii* isolates were genotyped irrespective of the clinical status (Ajzenberg et al. 2002).

T. gondii isolates differ remarkably in their virulence to outbred mice. Type I isolates are usually lethal to mice, irrespective of the dose and virulence is genetically controlled (Howe et al. 1997; Grigg et al. 2001b; Su et al. 2002). Type II and III isolates are less virulent for mice. However, virulence of *T. gondii* in mice should not be equated with virulence in humans or domestic animals. Hundreds of *T. gondii* isolates from aborted ovine fetuses were found to be avirulent for mice.

CLINICAL ASPECTS

T. gondii infection is widespread among humans and its prevalence varies widely from place to place. In the USA and the UK, it is estimated that about

16–40 percent of people are infected whereas in Central and South America and continental Europe infection is estimated to be 50–80 percent (Dubey and Beattie 1988; Tenter et al. 2000; Jones et al. 2001a). Most infections in humans are asymptomatic but at times the parasite can produce devastating disease. Infection may be congenitally or postnatally acquired.

Congenital infection

Congenital infection occurs only when a woman becomes infected during pregnancy; the severity of the disease in the fetus may depend on the stage of pregnancy when the mother becomes infected (Table 2) (Desmonts and Couvreur 1974; Daffos et al. 1988). Although the mother rarely has symptoms of infection, she does have a temporary parasitemia. Focal lesions develop in the placenta and the fetus may become infected. In the fetus, there is generalized infection at first, but later it clears from the visceral tissues and may localize in the central nervous system. A wide spectrum of clinical disease occurs in congenitally infected children (Remington et al. 2001; Jones et al. 2001b). Mild disease may consist of slightly diminished vision; severely diseased children may have the full tetrad of signs: retinochoroiditis, hydrocephalus (Figure 9), convulsions, and intracerebral calcification (Figure 10). Of these, hydrocephalus is the least common but most dramatic lesion of toxoplasmosis. This lesion is unique to congenitally acquired toxoplasmosis in humans and has not been reported in other animals.

By far the most common sequel of congenital toxoplasmosis is ocular disease (Guerina et al. 1994; McAuley et al. 1994; Bosch-Driessen et al. 2002). Except for the occasional involvement of an entire eye, the disease is confined to the posterior chamber (Holland et al. 1996; Lotje et al. 2002) Parasites proliferate in the retina leading to inflammation in the choroid, so the disease is correctly designated as retinochoroiditis. In humans, the characteristic lesions of ocular toxoplasmosis in the acute or subacute stage of inflammation appear as yellowish white, cotton-like patches in the fundus (O'Connor 1975; Dutton 1989). The lesions may be single or multiple and may involve one or both eyes.

Table 2 *The relation of clinical toxoplasmosis in children to the time of infection in the mother*

Trimester infected	Children with toxoplasmosis (%)			Total number
	Serious	Mild	Subclinical	
First	40	50	10	10
Second	17.7	45	37	62
Third	2.7	28.7	68.5	108
Undetermined	16.6	20.6	56.6	30

Reproduced from Couvreur et al. 1984 with permission

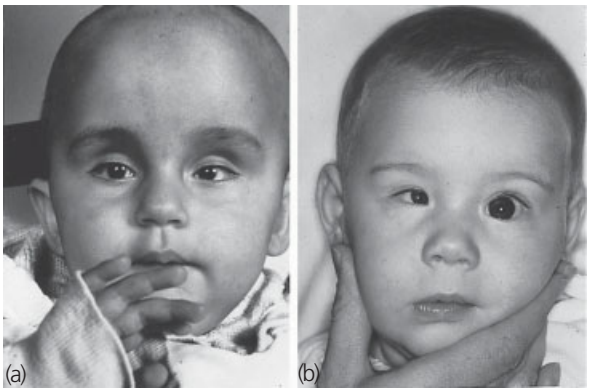

Figure 9 *Congenital toxoplasmosis in children.* **(a)***Hydrocephalus with bulging forehead;* **(b)** *microophthalmia of the left eye. (Courtesy of Dr J. Couvreur)*

During the acute stage, inflammatory exudate may cloud the vitreous fluid and may be so dense as to preclude visualization of the fundus by the examiner using an ophthalmoscope. As the inflammation subsides, the vitreous fluid clears and the diseased retina and choroid can be seen through the ophthalmoscope. Retinal lesions may be single or multifocal small gray areas of active retinitis with minimal edema and reaction in the vitreous humor. The punctate lesions are usually harmless unless they are located in a macular area (Figure 11). Although severe infections may be detected at birth, milder infections may go undetected until they flare up in adulthood.

The socioeconomic impact of toxoplasmosis in human suffering and the cost of care of sick children, especially those with intellectual deficit and blindness, are enormous (Roberts and Frenkel 1990; Roberts et al. 1994). The testing of all pregnant women for *T. gondii* infection is compulsory in France and Austria, and the cost

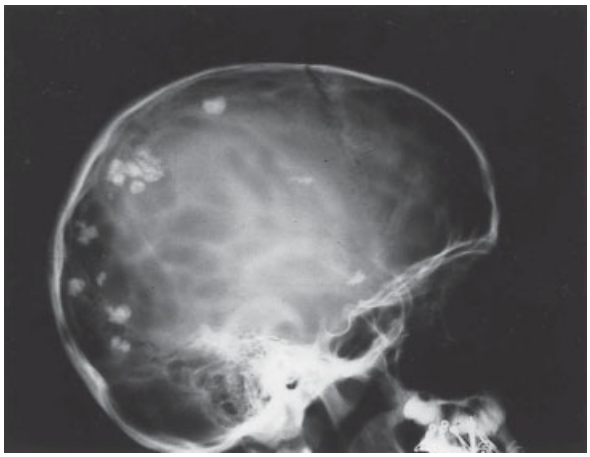

Figure 10 *Intracerebral calcification discovered fortuitously in a 10-year-old girl, on a dental panoramic radiograph asked for by a dentist. The girl had unilateral retinochoroiditis and an IQ of 80. (Courtesy of Dr J. Couvreur)*

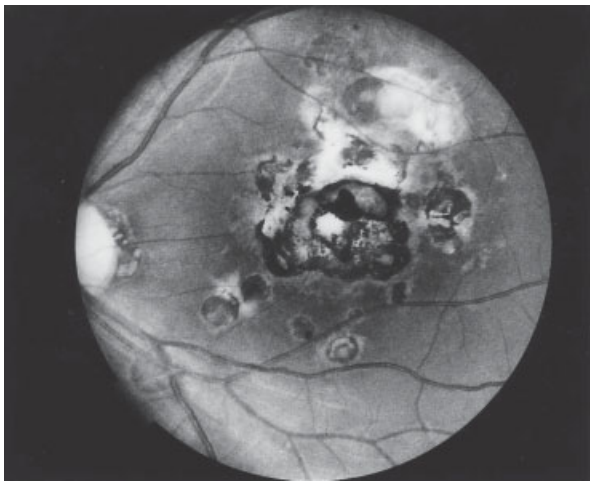

Figure 11 *Congenital toxoplasmosis. Retinochoroiditis in the macula of the left eye. (Courtesy of Dr R. Belfort Jnr)*

benefits of such mass screening are being debated in many countries (Remington et al. 2001). Toxoplasmosis is considered to be a leading cause of food-related illness in humans in the US (Mead et al. 1999).

Postnatally acquired infection

Approximately one-third of human populations has been found to have *T. gondii* antibodies. However, serologic prevalence varies a great deal in a geographical region within a country, and within different ethnic groups. Therefore, no generalizations can be made. The type of the serologic test used to measure antibodies and the cut-off values used for serologic surveys also affect the seroprevalence. Dubey and Beattie (1988) and Tenter et al. (2000) summarized the seroprevalence in different countries. In general, seroprevalence is very high in South America and France as compared with North America. Estimates of serologic prevalences are important for control programs. For example, it is important to know if only a minor proportion of a population in a given country is seropositive and thus at a high risk of acquiring toxoplasmosis, particularly during pregnancy. Recent surveys in the US indicate that about 15 percent of women of child-bearing age have antibodies to *T. gondii* and, based on the tests performed under identical conditions, seroprevalence has remained unchanged in the past decade (Jones et al. 2001a, 2003).

The risk of acquiring *T. gondii* infection during pregnancy has been estimated to be less than 1 to 7 per 1 000 pregnancies based on mass screening (Tenter et al. 2000). The risk of intrauterine infection increases with pregnancy and the placenta is thought to be more permeable to *T. gondii* transmission in the later half of pregnancy than in the first half. The chance of fetal infection is about 50 percent of infections acquired during pregnancy.

Table 3 *Frequency of symptoms in people with postnatally acquired toxoplasmosis*

	Patients with symptoms (%)	
Symptom	Atlanta outbreak[a] (35 patients)	Panama outbreak[b] (35 patients)
Fever	94	90
Lymphadenopathy	88	77
Headache	88	77
Myalgia	63	68
Stiff neck	57	55
Anorexia	57	NR
Sore throat	46	NR
Artharlgia	26	29
Rash	23	0
Confusion	20	NR
Earache	17	NR
Nausea	17	36
Eye pain	14	26
Abdominal pain	11	55

NR, not reported.
a) From Teutsch et al. (1979).
b) From Benenson et al. (1982).

Postnatally acquired infection may be localized or generalized. Oocyst-transmitted infections may be more severe than tissue cyst-induced infections. Lymphadenitis is the most frequently observed clinical form of toxoplasmosis in humans (Table 3). Although any nodes may be involved, the most frequently involved are the deep cervical nodes which, when infected, are tender, discrete but not painful; the infections resolve spontaneously in weeks or months. Lymphadenopathy may be associated with fever, malaise, fatigue, muscle pain, sore throat, and headache (McCabe et al. 1987). Although the condition may be benign, its diagnosis is vital in pregnant women because of the risk to the fetus (Liesenfeld et al. 2001). In tissues from infected nodes examined histologically, reticular cell hyperplasia is usually present whereas necrosis and fibrosis are absent. The node architecture is preserved and usually only a few parasites are present. Diagnosis based on symptoms and histological examination can be confirmed by injection of a homogenate of the lymph node into mice, by immunohistochemical staining of the lymph tissue tagged with *T. gondii* antiserum, or by use of the polymerase chain reaction (PCR) to detect *T. gondii* DNA in tissues.

Until recently, most of toxoplasmic retinochoroiditis was thought to be congenital. Ophthalmologists from Brazil first reported retinochoroiditis in multiple siblings (Silveria et al. 1988). These findings have now been amply confirmed (Couvreur and Thulliez 1996; Nussenblatt and Belfort 1994; Montoya and Remington 1996). The largest outbreak of human toxoplasmosis was epidemiologically linked to drinking water from a municipal

water reservoir in Vancouver, British Columbia, Canada; 20 of 100 patients had acquired retinochoroiditis (Bowie et al. 1997; Burnett et al. 1998). Lymphordenitis was found in 51 of the same 100 patients (Bowie et al. 1997).

Encephalitis is the most important manifestation of toxoplasmosis in immunosuppressed patients as it causes the most severe damage to the patient. Infection may occur in any organ. Patients may have headache, disorientation, drowsiness, hemiparesis, reflex changes, and convulsions, and many become comatose. Diagnosis is aided by serological examination. In immunosuppressed patients, both inflammatory signs and antibody production may be suppressed, thus making the diagnosis very difficult. Encephalitis caused by *T. gondii* is now frequently recognized in patients treated with immunosuppressive agents.

Toxoplasmosis ranks high in the list of diseases which lead to the death of patients with acquired immune deficiency syndrome (AIDS); approximately 10 percent of AIDS patients in the USA and up to 30 percent in Europe are estimated to die from toxoplasmosis (Luft and Remington 1992; Luft et al. 1993; Rabaud et al. 1994) Although any organ may be involved including the testis, dermis, and spinal cord, infection of the brain is most frequently reported in AIDS patients. The reason for the different propensity to develop reactivation of latent *T. gondii* infection is unknown (Mariuz and Steigbigel 2001). Most AIDS patients develop toxoplasmic encephalitis when the CD4 lymphocyte count is les than 100 per mm^3. General symptoms in decreasing order of frequency are headache, fever, psychomotor disturbances, meningeal signs, confusion, lethargy, and coma. Focal signs or symptoms include hemiparesis or hemiplegia, convulsions, cranial nerve palsy, visual field defect, cerebellar disturbances, aphasia, and ataxia (Renold et al. 1992). By using computerized axial tomograohy (CAT) scans, lesions were localized in decreasing order of frequency in cortico-medullary, white matter, basal ganglions, cortex, and posterior fossa (Renold et al. 1992). Macroscopically, unilateral or bilateral areas of discoloration indicative of necrosis and hemorrhage were noticed. Microscopically, encephalitis involving many areas is the predominant lesion and the difference may vary depending on whether the patient has received anti-toxoplasma therapy. In untreated patients, the lesion involves a central area of necrosis with degenerating organisms, surrounded by an inflammatory zone with edema, perivascular infiltration of inflammatory cells and hemorrhage (Bertoli et al. 1995). *T. gondii* are more numerous in this peripheral zone surrounding healthy and inflamed tissue. The lesion may be small or the size of a tennis ball and the contents may vary from fluid to solid. Microabscesses are more common in treated patients. In active lesions, numerous tachyzoites are found destroying host tissue. In subacute cases and treated patients, glial nodules predominate.

Patients should be treated empirically for toxoplasmic encephalitis based on clinical and neurological findings and presence of *T. gondii* antibodies because a specific diagnosis may not be possible without a biopsy, which is now rarely indicated.

Transplantation of infected organs or transfusion of infected leukocytes can initiate fatal infection in a seronegative recipient receiving immunosuppressive therapy. Transplantation of non-infected organs and leukocyte transfusion can also activate latent infection in a seropositive recipient receiving immunotherapy. Ordinary blood transfusion is virtually free from danger, but transfusion of packed leukocytes and transplantation of bone marrow have caused toxoplasmosis. It seems that the danger of transplanting an organ from a seropositive donor into a seronegative recipient is greater than that of transplanting an organ from a seronegative donor into a seropositive recipient. Recipients of heart and heart lung are more likely to have symptomatic infection than kidney or liver transplant patients (Wreghitt and Joynson 2001).

Malignancies or immunosuppressive treatment of malignancies can reactivate latent toxoplasmosis. Toxoplasmosis has been reported most commonly in patients treated for Hodgkin's disease. Untreated Hodgkin's disease is rarely associated with clinical toxoplasmosis. A variety of malignancies including lymphoma, leukemia, and myeloma can reactivate toxoplasmosis but there are rare reports of toxoplasmosis associated with solid tumors (Wreghitt and Joynson 2001).

IMMUNOLOGY

In hosts which develop disease, the host may die of acute toxoplasmosis but much more often recovers with the acquisition of immunity (Frenkel 1973b). In the recovering individual, inflammation usually develops where there was initial necrosis. By about the third week after infection, and as recovery develops, *T. gondii* tachyzoites begin to disappear from the visceral tissues and the parasites localize as tissue cysts in neural and muscular tissues. The tachyzoites may persist longer in the spinal cord and brain than in visceral tissues because immunity there is less effective than in visceral organs; they can persist in the placenta for months after the initial infection of the mother.

T. gondii can multiply in virtually any cell of the body, but how it is destroyed in immune cells is not completely known. All extracellular forms of the parasite are directly affected by antibody but intracellular forms are not. It is believed that cellular factors including lymphocytes and lymphokines are more important than humoral ones in immune-mediated destruction of *T. gondii* (Frenkel 1973b). Interferon-γ is considered by some to be the main factor in cell-mediated immunity to *T. gondii* (Gazzinelli et al. 1993). Under experimental conditions, infection with avirulent strains protects the

host from damage but does not prevent infection with more virulent strains. In most instances, immunity following a natural *T. gondii* infection persists for the life of the host.

Immunity does not eradicate the infection. *T. gondii* tissue cysts persist for several years after acute infection, and their fate is not fully known. Whether bradyzoites can form new tissue cysts directly without transforming into tachyzoites is not known. However, the finding of new tissue cysts adjacent to old ones suggests that it might happen. It has been proposed that tissue cysts may sometimes rupture during the life of the host and the released bradyzoites may be destroyed by the host's immune responses, possibly causing local necrosis accompanied by inflammation. Hypersensitivity plays a major role in such reactions, which afterwards usually subside with no local renewed multiplication of *T. gondii* in the tissue. However, occasionally there may be formation of new tissue cysts.

In immunosuppressed patients, such as those given large doses of immunosuppressive agents in preparation for organ transplants and in those with AIDS, rupture of a tissue cyst may result in transformation of bradyzoites into tachyzoites and renewed multiplication. The immunosuppressed host may die from toxoplasmosis unless treated. It is not known how corticosteroids cause relapse but it is unlikely that they directly cause rupture of the tissue cysts.

Pathogenicity of *T. gondii* is determined by the virulence of the strain and the susceptibility of the host species. Strains of *T. gondii* may vary in their pathogenicity in a given host; for example, certain strains of mice are more susceptible than others and the severity of infection in individual mice within the same strain may also vary. However, mice of any age are susceptible to clinical *T. gondii* infection, whereas adult rats do not become ill although young rats can die of toxoplasmosis. Adult dogs, like adult rats, are resistant, whereas puppies are fully susceptible to clinical toxoplasmosis. Cattle and horses are among the hosts more resistant to clinical toxoplasmosis and certain marsupials and New World monkeys are the most susceptible. Nothing is known concerning genetic-related susceptibility to clinical toxoplasmosis in higher mammals, including humans.

PATHOLOGY

Humans acquire *T. gondii* mostly by ingestion of tissue cysts in infected meat or by ingestion of oocysts in food or water contaminated with cat feces. The bradyzoites from the tissue cysts or sporozoites from the oocyst penetrate the intestinal epithelial cells where they multiply (Figure 12) and spread, first to the mesenteric lymph nodes and then to distant ones by invasion of the lymph and blood. Focal areas of necrosis may develop in many organs. The clinical picture is determined by the extent of injury, especially to vital organs such as the eye, heart, and adrenals. Necrosis is caused

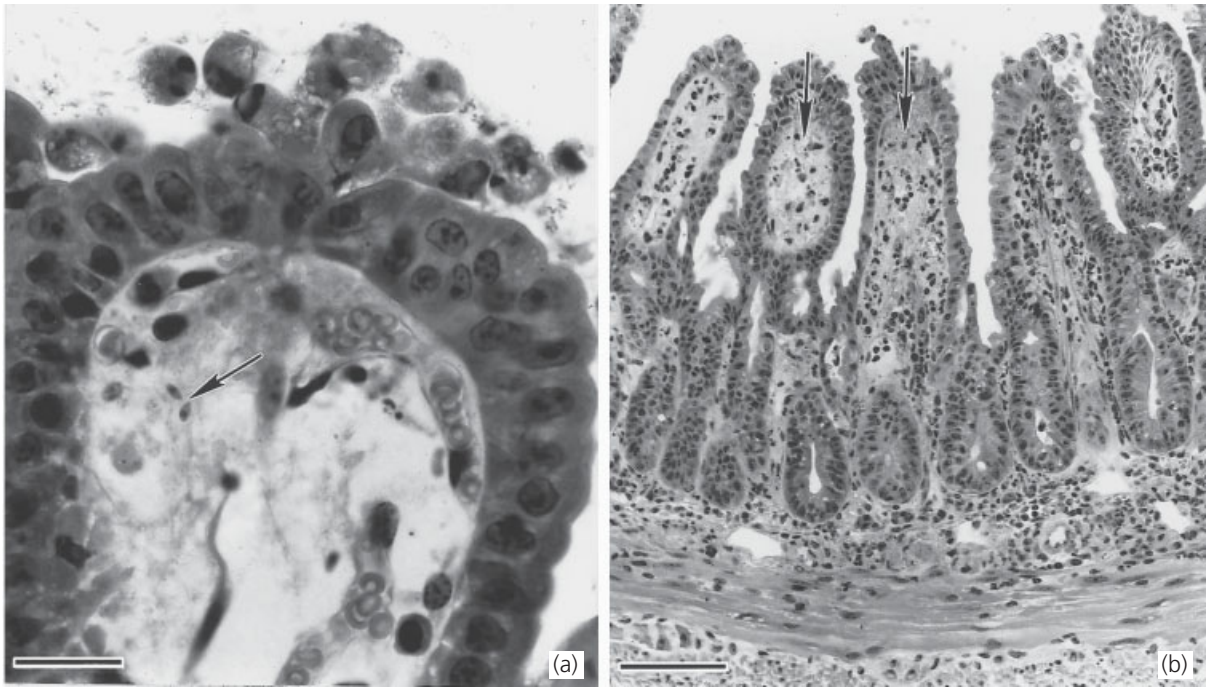

Figure 12 *Histological section of small intestine 6 days after feeding* T. gondii *oocysts to a mouse. H&E:* **(a)** *Intestinal villus showing edema and necrosis of the lamina propria cells associated with tachyzoites (arrow), and extrusion of intestinal cells in the lumen. Bar = 20 μm.* **(b)** *Necrosis of lamina propria (arrows). The surface epithelium is not affected. Bar = 100 μm*

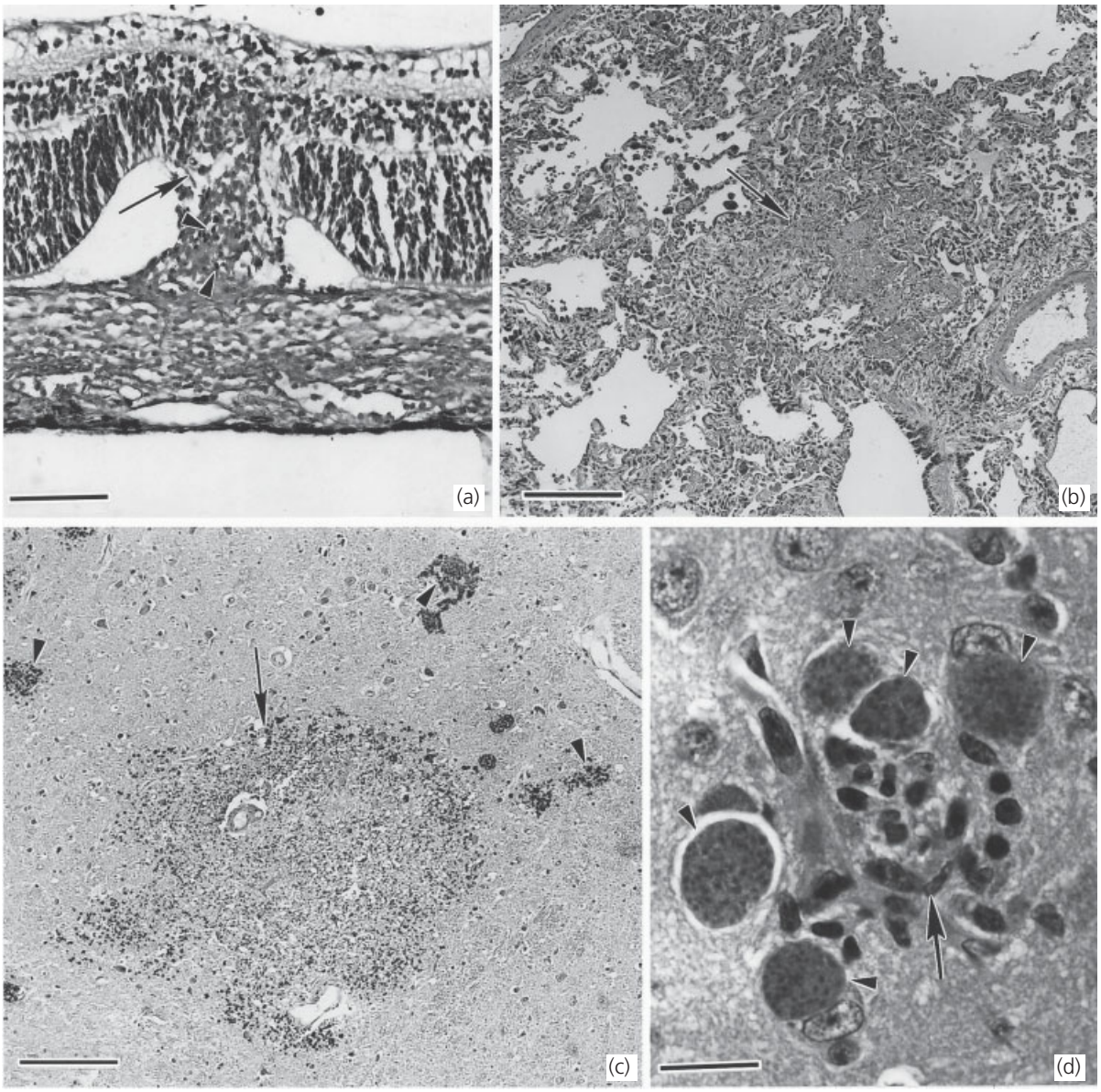

Figure 13 *Lesions of toxoplasmosis:* **(a)** *Focus of retinal detachment (arrow) and inflammation associated with tachyzoites (arrowheads). H&E. Bar = 80 μm.* **(b)** *Necrosis in the lung. H&E. Bar = 100 μm.* **(c)** *A large focus of necrosis (arrow) and several satellite small foci (arrowheads). Enormous numbers of* T. gondii *(all black dots) are present in the brain of an AIDS patient. Immunohistochemical stain with anti-*T. gondii *antibody. Bar = 100 μm.* **(d)** *Five tissue cysts (arrowheads) around a glial nodule (arrow) in the brain. H&E. Bar = 20 μm*

by the intracellular growth of tachyzoites (Figure 13). *T. gondii* does not produce a toxin.

Necrotic foci are the prominent lesions of toxoplasmosis, varying from microscopic (e.g. in the eye) to macroscopic. Macroscopic areas of necrosis cause encephalitis in AIDS patients. Inflammation usually follows the necrosis and is characterized by infiltration of mononuclear cells. By the time the tissue cysts are forming, the inflammation has begun to subside. However, small inflammatory foci (e.g. glial nodules) may persist for months or even years after the primary infection (Figure 13). The hydrocephalus of infected infants is due to a ventriculitis with a blockage of the aqueduct of Sylvius.

DIAGNOSIS

Diagnosis is made by biological, serological, or histological methods or by some combination of these. Clinical signs are nonspecific and insufficiently characteristic for a definite diagnosis because toxoplasmosis mimics several other infectious diseases.

Numerous serological procedures are available for the detection of humoral antibodies, including the

Sabin–Feldman dye test, indirect hemagglutination assay,indirect fluorescent antibody assay (IFA), direct agglutination test, latex agglutination test, enzyme-linked immunosorbent assay (ELISA), and the immunosorbent agglutination assay (ISAGA) (for review see Remington et al. 2001; Roberts et al. 2001; Joynson and Guy 2001; Montoya 2002). The IFA, ISAGA, and ELISA have been modified to detect immunoglobulin (Ig)M antibodies which appear sooner after infection than IgG and disappear faster than IgG after recovery.

The finding of antibodies to *T. gondii* in a single serum sample merely establishes that the host has been infected at some time in the past, so it is best to collect two samples from the same individual, the second 2–4 weeks after the first. A 4–16-fold increase in antibody titer in the second sample indicates an acute infection. A high antibody titer sometimes persists for months after infection. A rise in antibody titer may not be associated with clinical symptoms because most infections in humans are asymptomatic and the fact that titers persist after clinical recovery complicates the interpretation of the results of serological tests. *T. gondii* can be isolated from patients by inoculation into laboratory animals or tissue cultures, of secretions, excretions, body fluids, tissues taken by biopsy, and tissues with macroscopic lesions taken postmortem. Using such specimens, it is possible to attempt isolation of *T. gondii*, to search for *T. gondii* microscopically, and to search for toxoplasmal DNA using PCR (Grover et al. 1990). The PCR and other gene amplification techniques can be designed to target specific regions of the parasite genome. The most commonly used gene of *T. gondii* is B1. By gene amplification, minute quantities of DNA, representing a single organism, can be detected within a few hours. The main drawbacks of these methods are the inability to differentiate between dead and live organisms and the possibility of false positives originating during sample collection and processing. It is advisable not to rely entirely on this test for making vital decisions involving the fetus.

As just noted, diagnosis can be made by finding *T. gondii* in host tissue removed by biopsy or at necropsy. A rapid diagnosis may be made by microscopic examination of impression smears of lesions. After drying for 10–30 minutes, the smears are fixed in methyl alcohol and stained with a Romanowsky stain, Giemsa being very satisfactory. Well preserved *T. gondii* are crescent-shaped (Figure 1a). In sections, the tachyzoites usually appear round to oval (Figure 1b). Electron microscopy can aid diagnosis. *T. gondii* tachyzoites are always located in vacuoles; they have few (usually four) rhoptries and often have a honeycomb structure (Figure 3a). Tissue cysts are usually spherical and lack septa, and the cyst wall stains with silver stains. The bradyzoites are strongly PAS positive. The immunohistochemical staining of parasites with fluorescent or other types of labeled *T. gondii* antiserum can aid in diagnosis (Figure 13).

Serologic screening

Prenatal screening for *T. gondii* infection, to reduce the risk of congenital toxoplasmosis, is routine in Austria and France, and is being considered in several other countries.

Serologic diagnosis in the pregnant woman may be considered under two headings: first, testing of individuals, and second, mass screening. One reason for individual testing is to detect *T. gondii* infection acquired during pregnancy. To do this, the woman's serum is tested early in pregnancy for specific *T. gondii* IgG antibodies by the modified agglutination test (MAT), IgG-ELISA, or Dye test (DT), and for a specific IgM by the double sandwich (DS)-IgM ELISA or IgM-ISAGA tests (Table 4).

- If the IgG test is positive, no matter how low the titer, and the IgM test negative, the woman has been infected before pregnancy, there is no risk to her fetus and no treatment is needed (but see Table 4, footnote a).
- If both the IgG and IgM tests are positive, the tests should be repeated in 3 weeks time. If the titers have not risen, infection may be assumed to have occurred before pregnancy, and there is no risk to the fetus. If the IgG titer has risen, infection has probably taken place about the time of conception and there is a slight risk to the fetus (but see Table 4, footnote b).
- If the IgG and the IgM results are both negative, the woman is susceptible and the tests should be repeated every 4 to 6 weeks to see if either or both become positive. If the woman is infected during pregnancy, the fetus is at high risk, and the woman should be given prophylactic treatment. When she delivers, her child should be examined and, if found to be infected, clinically or subclinically, should be treated.

Another reason for individual testing is the development of lymphadenopathy during pregnancy. In this event, a high IgG titer (greater than 1 000) accompanied by a positive IgM result makes the diagnosis of active *T. gondii* infection probable. If the IgG titer is less than 1 000, a second test should be carried out 2 to 3 weeks later. If infection is proved, the woman should receive prophylactic treatment and her child should also be treated if found to be infected, clinically or subclinically.

EPIDEMIOLOGY AND CONTROL

Prevalence of infection

T. gondii infection in humans is widespread and occurs throughout the world (Dubey and Beattie 1988; Tenter et al. 2000). Infection rates in humans and other animals differ from one geographical area of a country to another. The causes of these variations are not yet

Table 4 *Program for mass screening and prophylactic treatment of pregnant women for* T. gondii

Test 1 (before 2 months of pregnancy)	Test 2 (in second trimester)	Test 3 (in third trimester)	Group
IgG +ve (any titer);	No test;	No test;	I Infection before pregnancy;
IgM –ve	no treatment	No treatment	No risk[a]
IgG +ve;	Repeat IgG after 3 weeks;		II Possible infection soon after conception;
IgM +ve	treat if high or rising titer		slight risk[b]
IgG –ve	Treat if IgG +ve;	Treat if IgG +ve;	III No previous infection;
IgM –ve	Don't treat if IgG –ve	Don't treat if IgG –ve	if seroconversion, high risk[c]

Based on Dubey and Beattie 1988

a) Except in the immunodeficient woman (e.g., with Hodgkin's' disease). In that case, treat the mother if a high or rising IgG titer is observed, and examine the child and treat if found clinically to subclinically infected.

b) In the immunocompetent woman, the slight risk is principally due to infection acquired soon after conception. As only 10 percent of congenital toxoplasmosis arise from maternal infections at this time and as most of these result in abortions or stillbirths, little would be lost by omitting repeated tests.

c) At time of delivery, retest mothers. Examine babies of all mothers who have seroconverted in this pregnancy. Treat the babies if found clinically or subclinically infected.

known. Environmental conditions, cultural habits of the people, and animal fauna are some of the factors that may determine the level of infection, which is more prevalent in hot and humid areas than in dry and cold climates. Only a small proportion (less than 1 percent) of people acquire infection congenitally.

Immunocompetent mothers of congenitally infected children have not been known to give birth to infected children in subsequent pregnancies but repeated congenital infection can occur in mice (Dubey and Beattie 1988). Several litters born to an infected mouse or hamster may be infected even if there is no reinfection from outside sources. A congenitally infected mouse has been shown to be able to produce ten generations of congenitally infected mice. In sheep, congenital infection occurs only when the ewe acquires infection during pregnancy.

Epidemiology

As noted earlier, toxoplasmosis may be acquired by ingestion of oocysts or by ingestion of tissue inhabiting stages of the parasite. The contamination of the environment by oocysts is widespread as oocysts are shed by cats, not only domestic cats but also other members of the Felidae. The domestic cat is probably the major source of contamination as oocyst formation is greatest in this extremely common animal. Widespread natural infection of the environment is possible since a cat can excrete millions of oocysts after ingesting few bradyzoites (Dubey 2001) and these oocysts survive for long periods under most ordinary environmental conditions, for example oocysts in moist soils may survive for months and even years. Oocysts in soil do not always stay there because invertebrates such as flies, cockroaches, dung beetles, and earthworms can spread them mechanically and may even carry them on to food.

Although only a few cats may be shedding *T. gondii* oocysts at any given time (as few as 1 percent) the enormous numbers shed and their resistance to destruction assure widespread contamination. Under experimental conditions, infected cats can shed oocysts after reinoculation with tissue cysts (Dubey 1995) and if this occurs in nature it would greatly facilitate oocyst spread. Congenital infection can also occur in cats, and congenitally infected kittens can excrete oocysts (Dubey and Carpenter 1993), providing another source of contamination. Infection rates in cats are determined by the rate of infection in local avian and rodent populations because cats are thought to become infected by eating these animals. The more oocysts in the environment, the more likely prey animals are to become infected and this, in turn, would increase the infection rate in cats.

Infection in humans is probably most often the result of ingestion of tissue cysts contained in raw or undercooked meat as *T. gondii* is common in many animals used for food, including sheep, pigs, and rabbits. Viable *T. gondii* has not been isolated from beef and the role of cattle in transmission of infection to humans is at best uncertain. Tissue cysts can survive in food animals for years (Dubey and Beattie 1988).

Cultural habits may also affect the acquisition of *T. gondii* infection; for example, in France the prevalence of antibodies to *T. gondii* in humans is very high. In Paris, 84 percent of pregnant women have antibodies to *T. gondii*, comparable figures elsewhere are 32 percent in New York City and 22 percent in London (Dubey and Beattie 1988). The high incidence of *T. gondii* infection in humans in France appears to be related in part to the French habit of eating some of their meat inadequately cooked. In contrast, the high prevalence of the infection in Central and South America is in probably due to high levels of contamination of the environment by oocysts (Teutsch et al. 1979; Bahia-Oliveira et al. 2003). It should be noted, however, that the relative frequency of acquisition of toxoplasmosis from eating

raw meat and that due to ingestion of food contaminated by oocysts from cat feces in the general population is unknown and statements on the subject are at best controversial. At present there are no tests to distinguish between oocyst-acquired infection and meat-acquired infection by *T. gondii*.

In addition to infection as a result of ingestion of oocysts or by eating infected raw meat, transmission of toxoplasmosis can be by semen transfusion, by ingestion of milk or saliva, and by eating eggs. The stages most likely to be involved in these transmissions are tachyzoites, which are not environmentally resistant and are killed by water. However, the probability of transmission of *T. gondii* by these means is rare.

There is little, if any, danger of *T. gondii* infection by drinking cows' milk which, in any case, is generally pasteurized or even boiled, but infection has followed drinking unboiled goats' milk. Raw hens' eggs, although an important source of *Salmonella* infection, are extremely unlikely to harbor *T. gondii*. Transmission by sexual activity including kissing is probably rare and epidemiologically unimportant.

Transmission can also occur through blood transfusions and organ transplants, transplantation being the more important; this is a recent development. In people undergoing transplantation, toxoplasmosis may arise from either:

- implantation of an organ or bone marrow from an infected donor into a non-immune immunocompromised recipient, or
- induction of disease in an immunocompromised latently infected recipient.

The tissue cysts in the transplanted tissue or in the latently infected person are probably the source of the infection. In both cases, cytotoxic and immunosuppressive therapy given to the recipient is the cause of induction of the active infection and disease (Wreghitt et al. 1989; Slavin et al. 1994).

CHEMOTHERAPY

Sulfadiazine and pyrimethamine are widely used for therapy of toxoplasmosis (Guerina et al. 1994; St Georgiev 1994). These drugs act synergistically by blocking the metabolic pathway involving *p*-aminobenzoic acid and the folic–folinic acid cycle respectively. The drugs are usually well tolerated; sometimes thrombocytopenia or leukopenia may develop, but these effects can be overcome by administering folinic acid and yeast without interfering with treatment because the vertebrate host can transport presynthesized folinic acid into its cells whereas *Toxoplasma* cannot. Although these drugs have a beneficial action when given in the acute stage of the disease (when there is active multiplication of the parasite), they will not usually eradicate infection. These drugs appear to have little effect on subclinical infections, but the growth of tissue cysts in mice has been restrained with sulfonamides. Sulfa compounds are excreted within a few hours of administration, so treatment has to be administered in daily divided doses (4 doses of 500 mg each) usually for several weeks or months (WHO 1995). A loading dose (75 mg) of pyrimethamine during the first 3 days has been recommended because it is absorbed slowly and binds to tissues. From the 4th day, the dose of pyrimethamine is reduced to 25 mg, and 2–10 mg of folinic acid plus 5–10 g of baker's yeast are added. Sulfa drugs can cause skin rash and renal toxicity.

The plasma half life of pyrimethamine is 35–139 hours. It has been administered in varying doses and schedules from daily to every 3 or 4 days. Pyrimethamine can cause nausea, headache, dysgeusia, thrombocytopenia, and anemia. Diagnosis and duration of treatment may have to be varied depending on the patient's age and condition (Table 5). For example, prenatally infected children, whether with or without clinical manifestations, should be treated for at least 1 year.

Spiramycin, clindamycin, atovaquone, azithromycin, roxithromycin, clarithromycin, dapsone, and several other less commonly used drugs are available for treatment of toxoplasmosis and these were reviewed by McCabe (2001). Spiramycin is relatively nontoxic to mother and the fetus. It is concentrated in placenta and binds to tissues. Thus, it is used to prevent transmission of *T. gondii* from mother to the fetus.

Clindamycin is absorbed quickly and diffuses well into the central nervous system and, therefore, has been used as alternative to sulfadiazine. It is rarely used to treat the primary maternal infection in pregnancy or congenital infection because it enters fetal blood when given to pregnant women. A major side-effect of clindamycin is ulcerative colitis. McCabe (2001) has discussed in detail treatment of toxoplasmosis in patients with various clinical manifestations.

Prophylactic treatment

IMMUNOSUPPRESSED PATIENTS

Before being given immunosuppressive treatments, patients should be serologically tested and, if devoid of *T. gondii* antibodies, be treated with pyrimethamine and sulfadiazine. This is particularly desirable in the case of patients receiving organ transplants. As it is usually impossible to carry out serologic tests on donors, they must all be considered potentially dangerous. There will, however, be time to test the recipients, and if seronegative, they should certainly be given prophylactic treatment. This strategy appears to have been effective in reducing the incidence of latent *T. gondii* infection in patients given marrow transplants from seronegative donors. Perhaps prophylactic treatment should be given to all recipients, irrespective of their sero-status.

Table 5 *Treatment schedule for toxoplasmosis*

Condition	Treatment	Dose
General toxoplasmosis	Pyrimethamine + sulfadiazine, 21-day course	Pyrimethamine: 0.5–2 mg per kg per day Sulfadiazine: 50–100 mg per kg per day in two divided doses
	Folic acid (leukovorin calcium)	2–20 mg (or 5–10 g bakers' yeast) twice weekly during pyrimethamine treatment
Toxoplasmosis in pregnancy; start prophylaxis as soon as diagnosis is made	Spiramycin *or* Spiramycin before week 20 of pregnancy; pyrimethamine + sulfadiazine thereafter	100 mg per kg per day orally in two divided doses; usually 2–4 g daily
Congenital toxoplasmosis	Pyrimethamine + sulfadiazine + folinic acid	Pyrimethamine: 2 mg per kg orally four times per day for 2 days, then 1 mg per kg per day for 6 months, then thrice weekly Sulfadiazine: 100 mg per kg per day orally in two divided doses Folinic acid: 5–10 mg orally thrice weekly
Ocular toxoplasmosis	Pyrimethamine + sulfadiazine	Pyrimethamine: 75 mg per day Sulfadiazine: 2 g per day
	or	
	Clindamycin	300 mg orally, four times daily
	Corticosteroids (only if inflammation present)	Prednisone or methylprednisone: 1–2 mg per kg per day in two divided doses
	Photocoagulation and cryotherapy around active retinochoroiditis lesion to kill *T. gondii* encysted at periphery of lesions	
AIDS patients		
(i) Acute toxoplasmic encephalitis	Pyrimethamine + sulfadiazine	Pyrimethamine: 200 mg oral initially, then 75–100 mg orally four times daily Sulfadiazine: 1–2 g orally, four times daily
	or	
	Pyrimethamine + clindamycin	Pyrimethamine + clindamycin: 60 mg orally or intravenously 6-hourly
	or	
	Pyrimethamine + azithromycin	Pyrimethamine + azithromycin: 1 200–1 500 mg orally four times daily
	or	
	Pyrimethamine + clarithromycin	Pyrimethamine + clarithromycin: 1 g orally two times daily
(ii) Maintenance treatment	Pyrimethamine + sulfadiazine	Dose reduced to half (or less) that given for acute toxoplasmosis and treatment continued for life
	Folic acid	Daily
(iii) Prophylaxis for AIDS patients with *T. gondii* antibodies.	Trimethoprim–sulfamethoxazole	Widely used for the prophylaxis and treatment of *Pneumocystis carinii* infections, thought to reduce onset of toxoplasmic encephalitis

Modified from Dubey and Beattie (1988) and McCabe (2001)

Prophylactic treatment of all AIDS patients with *T. gondii* antibodies is desirable. Fortunately, some drugs used to prophylactically treat *Pneumocystis* pneumonia and bacterial infections may also prevent onset of clinical toxoplasmosis. The combination of trimethoprim (160 mg) and sulfamethoxazole (800 mg), given twice daily and twice per week, is often used because it is inexpensive, convenient and works against *Pneumocystis* (McCabe 2001).

Cutaneous hypersensitivity, however, can be a problem and then alternative therapies are sought. A combination of dapsone (100 mg) and pyrimethamine (25 mg) orally weekly has also been used effectively. Other combinations of pyrimethamine and sulfonamides

(Fansidar, three tablets every 2 weeks) have also been used (Sabauste et al. 1997).

PROPHYLACTIC TREATMENT DURING PREGNANCY

Prevention of infection of the fetus by prophylactic treatment of the mother depends on the delay occuring between maternal infection and its transmission to the fetus. It is also hoped that if infection is already present in the fetus, such treatment may limit its ill effects. Treatment is initiated as soon as possible during the prenatal incubation period. In Austria, it is by spiramycin before week 20 of pregnancy and thereafter by pyrimethamine and sulfonamide; in France treatment is by spiramycin alone. If these measures are begun sufficiently early, they may be expected to reduce the incidence of congenital toxoplasmosis by 50–70 percent.

In places where it has been carried out with thoroughness, persistence, and determination, as in France, education appears to have contributed to a reduction in the incidence of *T. gondii* infection during pregnancy. Information regarding *T. gondii* infection should be included with the general instructions given in antenatal clinics and by obstetricians and midwives dealing with individual patients. Personal instruction given by word of mouth is likely to be most effective, and should be supplemented by booklets printed in various languages and by videos in the waiting rooms of antenatal clinics.

VACCINATION

The objectives of vaccines against toxoplasmosis include reducing fetal damage, reducing the number of tissue cysts in animals and preventing the formation of oocysts in cats (Araujo 1994; Dubey 1994; Fishback and Frenkel 2001). None of these objectives can be realized by the use of any currently available single vaccine. At present there are no effective subunit or killed vaccines for immunization against *T. gondii* but research is under way in many laboratories (Araujo 1994). There is no vaccine to prevent toxoplasmosis in humans, and none is on the horizon.

Prevention of oocyst shedding by cats is the key to controlling the spread of *T. gondii* and the oral ingestion of live bradyzoites is necessary to induce immunity to oocyst shedding, as the administration of any stage of *T. gondii* parenterally does not induce an immunity capable of inhibiting oocyst shedding (Frenkel and Smith 1982). A proposed vaccine for use in cats contains live bradyzoites from a mutant strain (T-263) of *T. gondii*. After oral administration of T-263 bradyzoites, the cycle of development of *T. gondii* is arrested at the sexual stage because only gamonts of a single sex develop; thus oocysts are not produced (Frenkel et al. 1991). In one trial, 84 percent of cats vaccinated with T-263 bradyzoites failed to shed oocysts following challenge (Frenkel et al. 1991). The duration of immunity in cats induced by the vaccine has not yet been determined. This vaccine is not available commercially.

The objectives of vaccination of farm animals are to reduce the incidence of abortions in sheep and goats resulting from transplacental infection of fetuses, and to reduce the risk of human exposure resulting from ingestion of infected meat (with the subsequent risk of fetal infection). For these purposes, vaccines made of nonpersistent strains of *T. gondii* are under study. One vaccine that contains a strain (S48) of tachyzoites that does not persist in the tissues of sheep is available in Europe and New Zealand, where it is used to reduce fetal losses attributable to toxoplasmosis (Wilkins et al. 1988; Buxton 1993). Ewes vaccinated with the S48 strain vaccine retain immunity for at least 18 months (Buxton 1993). Another strain of *T. gondii* (RH) does not persist in tissues of swine but does induce immunity (Dubey et al. 1994). A mutant (ts-4) of the RH strain is being studied as a vaccine for immunizing hosts other than cats. It grows better at 33°C than it does at 37°C (body temperature). The ts-4 stain is nonpathogenic even to suckling pigs (Lindsay et al. 1993). These strains of *T. gondii* (S48, RH, ts-4) do not induce oocyst shedding in cats.

INTEGRATED CONTROL

To prevent infection of humans by *T. gondii*, the hands of people handling meat should be washed thoroughly with soap and water before they go to other tasks. All cutting boards, sink tops, knives, and other materials coming in contact with uncooked meat should also be washed with soap and water. Washing is effective because the stages of *T. gondii* in meat are killed by contact with soap and water.

Parasites in meat can be killed by exposure to extreme cold or heat; tissue cysts are killed by heating the meat throughout to 67°C (Dubey et al. 1990) or by cooling it to −13°C (Kotula et al. 1991). Tissue cysts are also killed by exposure to 0.5 Gy of γ-irradiation (Dubey and Thayer 1994). All meat should be cooked to 67°C before consumption, and tasting meat while cooking or while seasoning should be avoided. Pregnant women, especially, should avoid contact with cats, soil, and raw meat. Pet cats should be fed only dry, canned or cooked food and the cat litter box should be emptied every day, preferably not by a pregnant woman. Gloves should be worn while gardening and vegetables should be washed thoroughly before eating because of possible contamination with cat feces. Expectant mothers should be aware of the dangers of toxoplasmosis (Foulon et al. 2000; Jones et al. 2001b).

Infection in animals other than humans

T. gondii is capable of causing severe disease in animals other than humans (Dubey and Beattie 1988) and is

responsible for great losses to the livestock industry. In sheep and goats it may cause embryonic death and resorption, fetal death and mummification, abortion, stillbirth, and neonatal death. Disease is more severe in goats than in sheep. Outbreaks of toxoplasmosis in pigs have been reported from several countries, especially Japan, and mortality is more common in young pigs than in adult pigs. Pneumonia, myocarditis, encephalitis and placental necrosis occur in infected pigs. Cattle and horses are more resistant to clinical toxoplasmosis than are other species of livestock. In cats and dogs, the disease is most severe in young animals. Common clinical manifestations of canine toxoplasmosis are respiratory distress, ataxia, and diarrhea. In most infected dogs, pneumonia is caused by a combination of *T. gondii* and distemper virus as the virus is immunosuppressive. Respiratory distress is a common clinical sign in felines with toxoplasmosis. Sporadic and widespread outbreaks of toxoplasmosis occur in rabbits, mink, birds, and other domesticated and wild animals (Tenter et al. 2000; Dubey 2002b). Toxoplasmosis is severe in many species of Australian marsupials and in New World monkeys (Dubey and Beattie 1988). Free-ranging marine mammals have died of acute toxoplasmosis.

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Sarcocystis

J.P. DUBEY

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INTRODUCTION AND CLASSIFICATION

The *Sarcocystis* parasite was first described in the skeletal muscle of a house mouse (*Mus musculus*) in Switzerland in 1843 (Table 1). Before 1972, many of these parasites were given names based on the finding of their cysts in the muscles of a variety of hosts. The true nature of these intramuscular cysts remained unknown until the discovery of the life cycle of *Sarcocystis* in 1972 (Table 1).

Sarcocystis species are coccidian parasites and are classified as follows.

Phylum Apicomplexa Levine, 1979
Class Coccidea Leuckart, 1879
Order Eimeriida Léger 1911
Family Sarcocystidae Poche, 1913
Genus *Sarcocystis* Lankester, 1882

STRUCTURE AND LIFE CYCLE

Sarcocysts (from Greek 'sarkos' meaning 'flesh' and 'kystis' meaning 'bladder'), the terminal asexual stage of development of these parasites, are found primarily in the striated muscles of mammals, birds, marsupials, and poikilothermic animals (Figure 1).

Sarcocystis has an obligatory prey–predator (two-host) life cycle (Figure 2). Asexual stages develop only in the intermediate host, which in nature is often a prey animal, and sexual stages develop only in the definitive host, which is carnivorous. There are different intermediate and definitive hosts for each species of *Sarcocystis*; for example, there are three named species in cattle: *S. cruzi*, *S. hirsuta*, and *S. hominis* (Dubey et al. 1989), the definitive hosts for these species being Canidae, Felidae, and primates, respectively. Species of *Sarcocystis* are generally more specific for their intermediate hosts than for their definitive hosts; for *S. cruzi* for example, ox and bison are the only intermediate hosts whereas dogs, wolves, coyotes, raccoons, jackals, and foxes can act as definitive hosts. In the following description of life cycle and structure, *S. cruzi* serves as the example because its complete life cycle is known.

The intermediate host becomes infected by ingesting sporocysts in food or water. Sporozoites excyst from sporocysts in the small intestine and first generation schizonts are formed in endothelial cells of arteries 7–15 days after inoculation. Second generation schizonts occur 19–46 days after inoculation, predominantly in capillaries throughout the body (Figure 3a). Merozoites are found in mononuclear blood cells 24–46 days after inoculation.

The schizonts divide by endopolygeny (from 'endon' meaning 'inside', 'poly' meaning 'many', and 'genesis' meaning 'birth'). The nucleus becomes lobulated and divides into several nuclei. Merozoites form at the periphery of the schizont. Both first and second generation schizonts are located within the host cytoplasm and are not surrounded by a parasitophorous vacuole. *Sarcocystis* merozoites have much the same organelles as *Toxoplasma gondii* tachyzoites including a conoid, micronemes and a plastid body but no rhoptries (Dubey et al. 1989). Rhoptries are present in *Sarcocystis* bradyzoites.

Merozoites liberated from the terminal vascular generation of the developing parasite initiate sarcocyst

Table 1 *Historical landmarks concerning* Sarcocystis

Year	Findings	Reference
1843	Sarcocysts found in muscles of a mouse	Miesher (1843)
1882	Genus *Sarcocystis* introduced	Lankaster (1882)
1943	*Sarcocystis* not transmitted from sheep to sheep, role of carnivores suspected but not proven	Scott (1943)
1972	Sexual phase cultured in vitro	Fayer (1972)
1972	Two-host life cycle found	Rommel and Heydorn (1972), Rommel et al. (1972)
1973	Vascular phase recognized and pathogenicity demonstrated	Fayer and Johnson (1975)
1975	Multiple *Sarcocystis* species within a given host recognized	Heydorn et al. (1975b)
1975	Chemotherapy demonstrated	Fayer and Johnson (1973)
1976	Abortion due to sarcocytosis recognized	Fayer et al. (1979b)
1981	Protective immunity demonstrated	Dubey (1980a)
1986	Vascular phase cultured in vitro	Speer and Dubey (1986)
2000	Unusual life cycle of *S. neurona* discovered	Dubey et al. (2000)

Modified from Dubey et al. (1989); where a complete bibliography can be found

formation. These merozoites penetrate appropriate host cells. The intracellular merozoite, which is surrounded by a parasitophorous vacuole (PV), becomes round to ovoid (metrocyte) and undergoes repeated binary division eventually producing banana-shaped zoites called bradyzoites (Figure 3b) (also called cystozoites) containing prominent amylopectin granules that stain bright red when treated with the periodic acid–Schiff (PAS) reagent. Some mature sarcocysts may contain some peripherally arranged metrocytes in addition to zoites (Figure 1b). Eventually, the sarcocyst is filled with bradyzoites and it is this stage that is infective for the predator definitive host. Sarcocysts generally become infectious about 75 days after infection, but there is considerable variation between species of *Sarcocystis*. The definitive host becomes infected by ingesting tissues containing mature sarcocysts. Bradyzoites liberated from the sarcocyst by digestion in the stomach and intestine penetrate the mucosa of the small intestine and transform into male (micro-) and female (macro-) gamonts. Within 6 h of ingesting infected tissue, gamonts are found within a PV in goblet cells near the tips of the villi (Figure 3c). Macrogamonts are 10–20 μm in diameter and contain a single nucleus whereas microgamonts are 7 × 5 μm and contain between 3 and 11 slender gametes (Figure 3d). The microgametes,

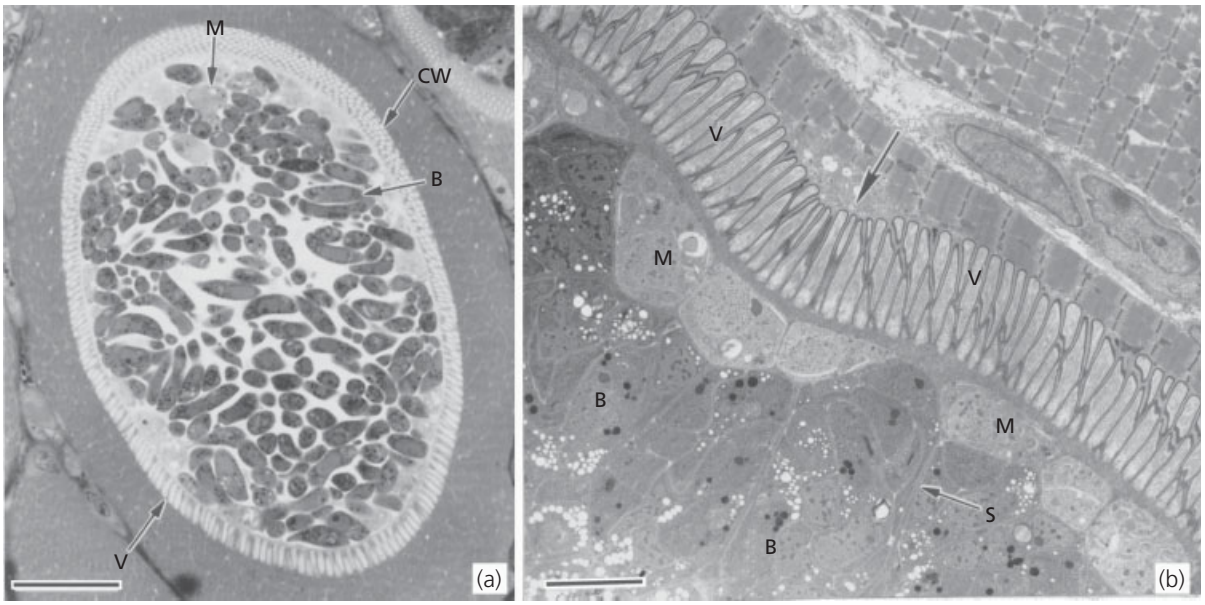

Figure 1 *Intramuscular* Sarcocystis hominis *sarcocysts.* **(a)** *Histological section of a mature sarcocyst. Note finger-like villar protrusions (V) on the cyst wall (CW) enclosing numerous bradyzoites (B) and a few metrocytes (M). Toluidine blue. Bar = 20 μm.* **(b)** *Transmission electron micrograph. Note villar projections (V) on the cyst wall, metrocytes (M), bradyzoites (B) and septa (S). Arrow points to border of the parasite and host cell cytoplasm. Bar = 4.3 μm.*

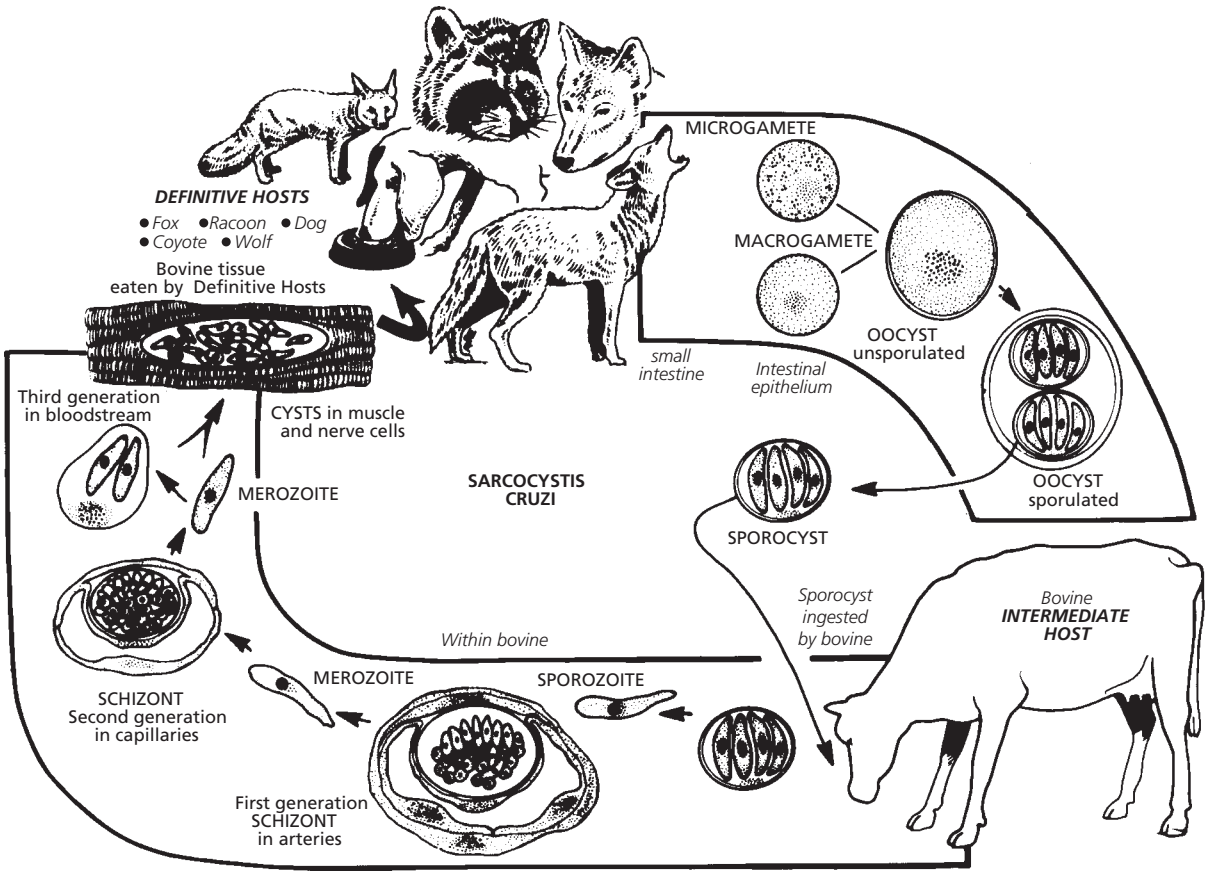

Figure 2 *Life cycle of* Sarcocystis cruzi *(from Dubey et al. 1989, with permission).*

which are about 4 × 0.5 μm, have a compact nucleus, and two flagella. After fertilization of a macrogamete by a microgamete, a wall develops around the zygote and an oocyst is formed. The entire process of gametogony and fertilization can be completed within 24 h. Gamonts and oocysts may be found at the same time in a host (Figure 3).

Oocysts of *Sarcocystis* species sporulate in the lamina propria (Figure 3e). Sporulated oocysts are generally colorless, thin-walled (less than 1 μm), and contain two elongate sporocysts. There is neither an oocyst residuum nor a micropyle. Each sporocyst contains four elongated sporozoites and a granular sporocyst residuum which may be compact or dispersed. There is no Stieda body (a cap-like thickening at one pole). Each sporozoite has a central-to-terminal nucleus, several cytoplasmic granules, and a crystalloid body, but there is no refractile body. The thin oocyst wall often ruptures, releasing the sporocysts into the intestinal lumen from which they are passed in the feces. The prepatent and patent periods vary, but for most *Sarcocystis* species, oocysts are first shed in feces 7–14 days after ingesting sarcocysts.

The number of generations of schizogony and the type of host cell in which schizogony may occur vary with each species of *Sarcocystis*, but trends are apparent. For example, all species of *Sarcocystis* of large domestic animals (sheep, goats, cattle, and pigs) form first and second generation schizonts in the vascular endothelium, whereas only a single precystic generation of schizogony has been found in *Sarcocystis* species of small mammals (mice and deer mice) and this is generally in hepatocytes (Dubey et al. 1989).

Sarcocysts, which are always located within a PV in the host cell cytoplasm, consist of a cyst wall that surrounds the metrocyte or the bradyzoites. The structure and thickness of the cyst wall differs among species of *Sarcocystis* and within each species as the sarcocyst matures (Tadros and Laarman 1978). Histologically, the sarcocyst wall may be smooth, striated or hirsute, or may possess complex branched protrusions. These protrusions are of taxonomic importance (Figure 1). Internally, groups of zoites may be segregated into compartments by septa that originate from the sarcocyst wall or they may not be compartmentalized.

Not all species of *Sarcocystis* cause disease in their host species (Dubey et al. 1989). Generally, species using canids as definitive hosts are more pathogenic than those using felids. For example, of the three species in cattle, *S. cruzi*, for which the dog is the definitive host, is the most pathogenic for cattle, whereas *S. hirsuta* and *S. hominis*, which undergo sexual development in cats and primates respectively, are only mildly pathogenic

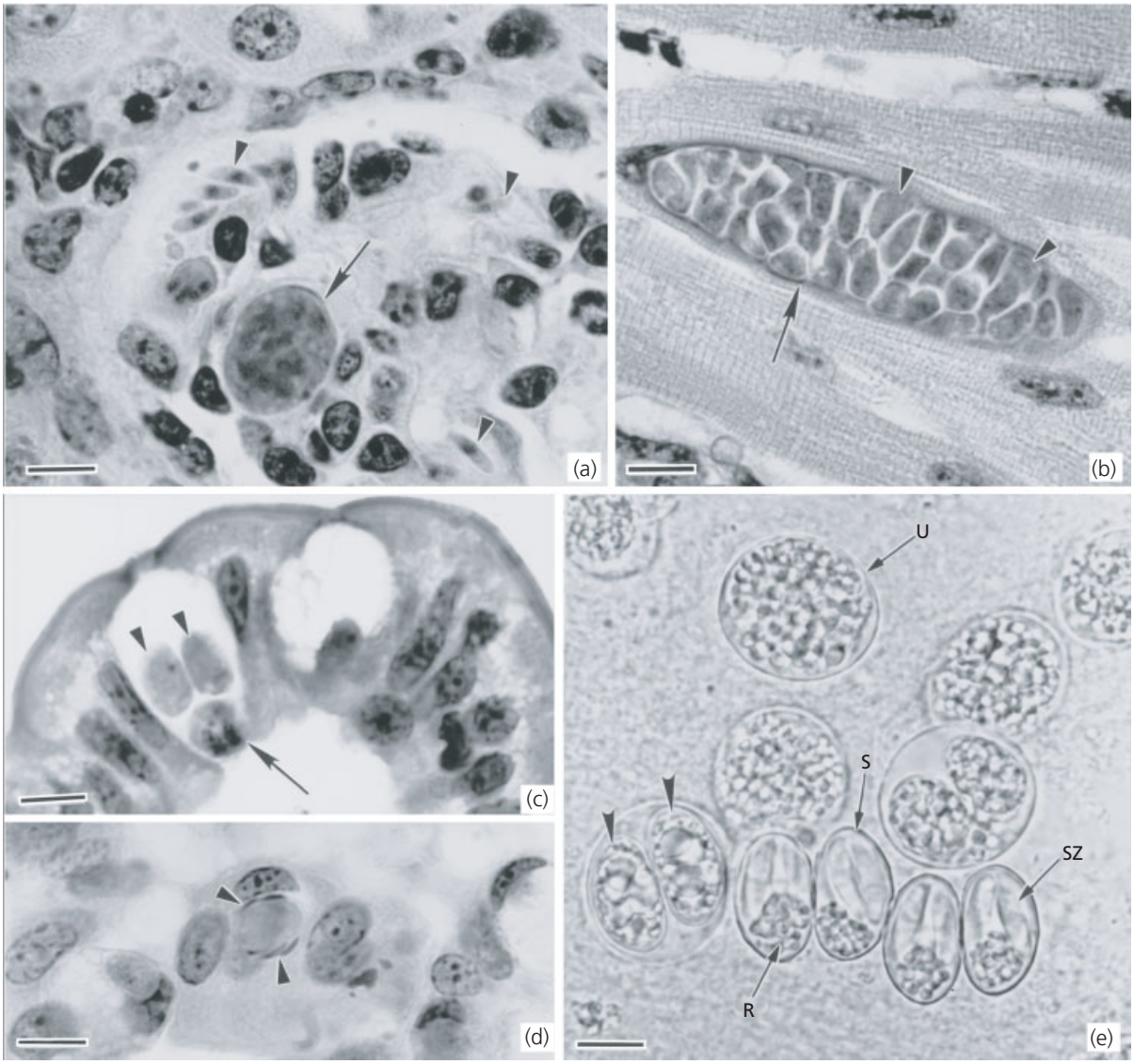

Figure 3 *Asexual and sexual stages of* S. cruzi. **(a)** *Second generation schizont (arrow) and released merozoites (arrowheads) in renal glomerulus of a calf. Hematoxylin and eosin (H&E). Bar = 10 μm.* **(b)** *Intramuscular immature sarcocyst (arrow) containing metrocytes (arrowheads). H&E. Bar = 10 μm.* **(c)** *Two female gamonts (arrowheads) and a male gamont (arrow) in a goblet cell, 6 h after infection of a coyote. Iron hematoxylin. Bar = 10 μm.* **(d)** *Mature microgamont with five microgametes (arrowheads) in an epithelial cell 6 h after infection of a coyote. Iron hematoxylin. Bar = 10 μm.* **(e)** *Sporogony of* S. cruzi *in the intestinal lamina propria of a coyote. Note unsporulated oocyst (U), partially sporulated oocysts with immature sporocysts (arrowheads), an oocyst with two sporocysts (S), and two fully sporulated oocysts containing sporozoites (SZ) and residual body (R). Unstained. Bar = 10 μm (from Dubey et al. 1989, with permission).*

(Table 2). Pathogenicity is manifested in the intermediate host. *Sarcocystis* generally does not cause illness in definitive hosts.

S. neurona is an unusual species of the genus that does not follow the life-cycle pattern of *S. cruzi* outlined above. It is also one of the most pathogenic species of the genus. *S. neurona* is the most frequent cause of a fatal disease in horses called equine protozoal encephalomyelitis (EPM) in the Americas (Dubey et al. 2001). Horses are considered the aberrant host because only schizonts are found in their tissues. Unlike *S. cruzi*, *S. neurona* schizonts occur in neural cells, not in the vascular endothelium, and schizonts may persist in the central nervous system for months. Sarcocysts of *S. neurona* occur in domestic cats, striped skunks, raccoons, sea otters, and armadillos. Opossums (*Didelphis virginianus*, *D. abbreventis*) are its definitive hosts. Only the sexual cycle occurs in the definitive host and it is confined to the small intestine. Encephalomyelitis associated with *S. neurona* has been reported in horses, ponies, zebras, skunks, raccoons, cats, lynx, mink, and marine mammals (Pacific harbor seals and sea otters).

S. canis is another unusual species of the genus with an unknown life cycle. Its sarcocysts, sexual phase, and

Table 2 Sarcocystis *species in livestock*

Intermediate host	*Sarcocystis* sp.	References	Sarcocysts		Pathogenicity	Definitive hosts
			Max length (mm)	Wall type		
Cattle (*Bos taurus*)	*S. cruzi*	(Hasselmann, 1926) Wenyon, 1926	<1	7	++	Dog, coyote, red fox, raccoon, wolf
	S. hirsuta	Moulé, 1888	7	10	±	Cat
	S. hominis	(Railliet and Lucet, 1891) Dubey, 1976	7	10	±	Humans and other primates
Sheep (*Ovis aries*)	*S. tenella*	(Railliet, 1886) Moulé, 1886	0.7	14	++	Dog, coyote, red fox
	S. arieticanis	Heydorn, 1985	0.9	7	+	Dog
	S. gigantea	(Railliet, 1886) Ashford, 1977	10	21	–	Cat
	S. medusiformis	Collins, Atkinson, and Charleston, 1979	8	20	–	Cat
Goat (*Capra hircus*)	*S. capracanis*	Fisher, 1979	1	14	++	Dog, coyote, red fox
	S. hircicanis	Heydorn and Unterhozner, 1983	2.5	7	++	Dog
	S. moule	Nevu-Nemaire, 1912	7.5	7	?	Cat
Pigs (*Sus scrofa*)	*S. miescheriana*	(Künn, 1865) Labbé, 1899	1.5	10(?)	+	Dog, raccoon, wolf, red fox, jackal
	S. porcifelis	Dubey, 1976	?	?	?	Cat
	S. suihominis	(Tadros and Laarman, 1976) Heydorn, 1977	1.5	10	+	Humans and other primates
Horses (*Equus caballus*)	*S. fayeri*	Dubey, Streitel, Stromberg, and Toussant, 1977	1.0	11	±	Dog
	S. equicanis	Rommel and Geisel, 1975	0.35	?	?	Dog
	S. bertrami	Doflein, 1901	12	?	?	Dog
Water buffalo (*Bubalus bubalis*)	*S. levinei*	Dissanike and Kan, 1978; Huong et al; 1997	1.1	7(?)	+	Dog
	S. fusiformis	(Railliet, 1897) Bernard and Bauche, 1912	3	21	–	Cat
	S. dubeyi	Huong and Uggla, 1999	<1 (?)	9	?	?
	S. buffalonis	Huong, Dubey, Nikka and Uggla, 1997				
Came l(*Camelus* spp.)	*S. cameli*	Mason, 1910	0.38	?	?	Dog
	Sarcocystis sp	Mason, 1910	?	?	?	?
Chickens (*Gallus gallus*)	*S. horvathi*	Ratz, 1908	0.98	?	?	?
S. rileyi	*Sarcocystis* sp.	Wenzel, Erber, Boch and Schellner, 1982	?	?	?	Dog, cat
Ducks (*Anas* spp.)		(Stiles, 1893), Michin, 1903	12	23	?	Skunk

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++) very pathogenic; +) pathogenic, mildly pathogenic; ?) unknown or unclassified; –) nonpathogenic; ±) questionable pathogenicity.

definitive hosts are unknown. The schizont is the only stage that is known. *S. canis* has been found associated with fatal hepatitis in sea lions, dogs, black and grizzly bears, a horse, and a dolphin (Dubey and Speer 1991; Resendes et al. 2002). Congenital infection has been documented in dogs.

CLINICAL ASPECTS

Humans serve as the definitive host for *S. hominis* and *S. suihominis* and also serve as accidental intermediate hosts for several unidentified species of *Sarcocystis*. Symptoms vary with the species of *Sarcocystis* causing the infection.

Intestinal sarcocystosis

SARCOCYSTIS HOMINIS (RAILLIET AND LUCET, 1891) DUBEY, 1976

Infection with *S. hominis* is acquired by ingesting uncooked beef containing sarcocysts. *S. hominis* is only mildly pathogenic. A volunteer who ate raw beef from an experimentally infected calf developed nausea, stomach ache, and diarrhea 3–6 h after ingesting the beef; these symptoms lasted 24–36 h and *S. hominis* sporocysts were excreted between 14–18 days after ingestion of the beef, during which time the volunteer had diarrhea and stomach ache (Heydorn 1977). Somewhat similar but milder symptoms were experienced by other volunteers who ate uncooked naturally infected beef (Rommel and Heydorn 1972; Aryeetey and Piekarski 1976; Hiepe et al. 1979). Recently, six of seven human volunteers that ate 128–260 grams of Kibbe (a preparation made from raw beef and spices) obtained from an Arabian restaurant in Saõ Paulo, Brazil, excreted *S. hominis* sporocysts 10–14 days later. Two of these volunteers became ill. One of them had abdominal pain and diarrhea 1–3 days post ingestion and the other had diarrhea 11 days post ingestion (Pena et al. 2001). A patient in Spain developed abdominal pain and loose stools after eating raw beef; the diagnosis was confirmed by finding *S. hominis* sporocysts in feces (Clavel et al. 2001).

A *Sarcocystis* species similar to *S. hominis* named *S. dubeyi* (Huong and Uggla 1999) has been found in water buffaloes. The definitive host for *S. dubeyi* is unknown but suspected to be humans. Although all *Sarcocystis* species capable of infecting domestic livestock are host-specific, researchers from the People's Republic of China believe that *Sarcocystis* species from cattle and water buffaloes (including *S. hominis*) are the same and even suggest that there may be more of them than one species now called *S. hominis* (for review see Yang et al. 2001a, b). However, in my opinion, there is no critical evidence for the hypothesis proposed by Yang et al. (2001a, b) and until proven otherwise, *Sarcocystis* species in cattle, although structurally similar to those in water buffaloes, should be regarded as distinct parasites based on transmission experiments (Dubey et al. 1989).

SARCOCYSTIS SUIHOMINIS (TADROS AND LAARMAN, 1976) HEYDORN, 1977

This species, acquired by eating undercooked pork, is more pathogenic than *S. hominis*. Human volunteers developed hypersensitivity-like symptoms; nausea, vomiting, stomach ache, diarrhea, and dyspnea within 24 h of ingestion of uncooked pork from naturally or experimentally infected pigs. Sporocysts were shed 11–13 days after ingesting the infected pork (Rommel and Heydorn 1972l; Piekarski et al. 1978; Hiepe et al. 1979; Kimmig et al. 1979). Zuo (1991) found sporocysts in stools of 123 of 414 (29.7 percent) people from three villages in Xianguan City in China. He reported that these people ate raw pork but not raw beef.

NATURAL PREVALENCE OF INTESTINAL SARCOCYSTOSIS OF HUMANS

Before the discovery of the life cycle of *Sarcocystis* and recognition of cattle and pigs as sources of human infection, *Sarcocystis* sporocysts in human feces were referred to as *Isospora hominis*. Because of structural similarities between *S. hominis* and *S. suihominis* sporocysts, it is not possible to distinguish between them by microscopic examination. Intestinal sarcocystosis is more common in Europe than in other continents. *Sarcocystis* sporocysts were seen in 2 percent of 3 500 fecal samples in France (Deluol et al. 1980), 1.6 percent of 1 518 samples in Germany (Flentje et al. 1975; Janitschke 1975), and 10.4 percent of 125 fecal samples from 7–18-year-old children in Poland (Plotkowiak 1976). Enteritis was associated with shedding of *Sarcocystis* sporocysts in six cases in Thailand and two cases reported from the People's Republic of China (reviewed in Dubey et al. 1989). Straka et al. (1991) found *Sarcocystis* spp. sporocysts in the stools of 14 of 1 228 students from Vietnam who worked in Slovakia.

Muscular sarcocystosis

Sarcocysts have been found in striated muscles of humans, mostly as incidental findings. Judging from the published reports, sarcocysts in humans are rare (Beaver et al. 1979); most reported cases are from Asia. Of the 40 histologically diagnosed reports that Beaver et al. (1979) reviewed and 6 additional cases since 1982 reviewed by Dubey et al. (1989), 15 were from southeast Asia, 11 from India, 5 from Central and South America, 4 from Europe, 4 from Africa, 3 from the USA, and 1 from China; the source of 2 was undetermined. Of the 46 confirmed cases, sarcocysts were found in skeletal muscles of 35 and in the heart of 11. The clinical significance of sarcocysts and the life cycles of the *Sarcocystis* spp. giving rise to these sarcocysts in humans are

unknown. Recently, 7 of 15 US military men developed acute illness after an army exercise in rural Malaysia (Arness et al. 1999). The illness was characterized by fever, myalgias, bronchspasm, fleeting pruritic rashes, transient lymphadenopathy, and subcutaneous nodules associated with eosinophilia, elevated erythrocyte sedimentation rate, and elevated levels of muscle creatinine kinase. Sarcocysts of an unidentified *Sarcocystis* species were found in skeletal muscle biopsies of the index case. Symptoms in 5 other men were mild to moderate and self-limited, and 1 team member with laboratory abnormalities were asymptomatic. Of eight team members tested for antibody to *Sarcocystis*, six were positive; of four with the eosinophilic myositis syndrome who were tested, all were positive. The illness was considered to be sarcocystosis. Arness et al. (1999) also reviewed other cases of sarcocystosis after 1990.

DIAGNOSIS

The antemortem diagnosis of muscular sarcocystosis can only be made by histological examination of muscle collected by biopsy. The finding of immature sarcocysts with metrocytes suggests recently acquired infection; the finding of mature sarcocysts indicates only past infection.

The diagnosis of intestinal sarcocystosis is easily made by fecal examination. As has been mentioned, sporocysts or oocysts of sarcocystis are shed fully sporulated in feces whereas those of *Isospora belli* are often shed unsporulated. It is not possible to distinguish one species of *Sarcocystis* from another by the examination of sporocysts.

An inflammatory response associated with sarcocystosis may help to distinguish an active disease process from incidental findings of sarcocysts. *Sarcocystis* schizonts have not yet been identified in humans. Although there are several serologic tests and polymerase chain reaction (PCR) techniques developed experimentally to distinguish *Sarcocystis* species in animals, none have been applied to cases of sarcocystosis in humans. Tenter (1995) has reviewed in detail pitfalls of serologic and molecular diagnosis of sarcocystosis in animals.

EPIDEMIOLOGY AND CONTROL

Sarcocystis infection is common in many species of animals worldwide (Dubey et al. 1989). A variety of conditions permit such high prevalence:

- a host may harbor any of several species of *Sarcocystis*
- many definitive hosts are involved in transmission
- large numbers of sporocysts may be shed
- *Sarcocystis* oocysts and sporocysts develop in the lamina propria, and are discharged over a period of many months
- *Sarcocystis* oocysts and sporocysts are resistant to freezing, so they can overwinter on pasture
- *Sarcocystis* sporocysts and oocysts remain viable for many months in the environment and they may be spread by invertebrate transport hosts
- there is little or no immunity to reshedding of sporocysts and, therefore, each meal of infected meat can initiate a new round of sporocyst production
- *Sarcocystis* oocysts, unlike those of many other species of coccidia, are passed in feces in the infective form, this frees them from dependence on weather conditions for maturation and infectivity.

Poor hygiene during handling of meat between slaughter and cooking can be a source of *Sarcocystis* infection. In one survey in India, *S. suihominis* oocysts were found in feces of 14 of 20 3–12-year-old children (Banerjee et al. 1994) indicating that meat was consumed raw at least by some because *S. suihominis* can be transmitted to humans only by the consumption of raw pork. In another study, 3–5-year-old children from a slum area were found to consume meat scraps virtually raw, and many pigs from that area harbored *S. suihominis* sarcocysts (Solanki et al. 1991). In European countries where the frequency of consumption of raw or undercooked meat is relatively high, humans are likely to have intestinal sarcocystosis. In one survey, 60.7 percent of pigs had *S. suihominis* sarcocysts in their muscles (Boch et al. 1978).

CHEMOTHERAPY

There is no treatment for *Sarcocystis* infection of humans. On the basis of results in experimental animals, it is probable that sulfonamides and pyrimethamine are helpful in treating sarcocystosis (Rommel et al. 1981).

There is no vaccine to protect livestock or humans against sarcocystosis. Shedding of *Sarcocystis* oocysts and sporocysts in feces of the definitive hosts is the key factor in the spread of *Sarcocystis* infection; to interrupt this cycle, carnivores should be excluded from animal houses and from feed, water and bedding for livestock. Uncooked meat or offal should never be fed to carnivores. As freezing can drastically reduce or eliminate infectious sarcocysts, meat should be frozen if not cooked. Exposure to heat at 55°C for 20 min kills sarcocysts so only limited cooking or heating is required to kill sporocysts (Fayer 1975). Dead livestock should be buried or incinerated. Dead animals should never be left in the field for vultures and carnivores to eat.

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Babesiosis of humans

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INTRODUCTION

Babesiosis, caused by certain tick-borne intra-erythrocytic sporozoans, has affected human affairs since antiquity. The 'grievous murrain' described in Exodus IX was probably redwater fever of cattle, caused by *Babesia bovis*. Babesiosis occupies a prominent place in the history of biomedical sciences. Smith and Kilborne's landmark discovery (1893) of the role of ticks in the transmission of *B. bigemina*, the Texas cattle fever pathogen, stands as the first proof that hematophagous arthropods can transmit pathogens of vertebrates.

With the exception of the trypanosomes, *Babesia* spp. are the most ubiquitous of the mammalian hemoprotozoa, occurring wherever certain ticks proliferate. Numerous types of mammal serve as hosts for these pathogens, certain of which, particularly *Babesia microti*, have taken a prominent place among the emerging infections of humans. Babesiosis, therefore, spans human history and continues to demand our attention.

Babesias are small sporozoans that cycle between ixodid ticks and vertebrates. They undergo sexual reproduction after ingestion by ticks and replicate one to several times before reaching maturity in the vector's salivary glands. The tick, which is the definitive host, then deposits the infectious sporozoite stage of the *Babesia* within the vertebrate host's dermis. The mode of development at the site of deposition and subsequent path of dissemination remain largely unknown. Ultimately, the parasites appear as characteristic pear-shaped trophozoites (piroplasms) in infected erythrocytes where they multiply, appearing as simple rings, paired or single piroplasms or the pathognomonic tetrads. The parasite's 'accordion-like' intra-erythrocytic gametocyte stage is only identifiable by electron microscopy or staining with DNA-specific stains. Although certain basic elements of the babesial life cycle appear to be well established, much remains to be learned about the biology of this diverse group of parasites.

CLASSIFICATION

The Babesias are protozoa classified within the phylum Sporozoa (= Apicomplexa), order Piroplasmida and family Babesiidae. Those that parasitize mammals have long been considered to form two main lineages, the 'large' and 'small' types of *Babesia*. *Babesia* are divided informally between those with intra-erythrocytic forms that are 1.0–2.5 μm in diameter and those of 2.5–5.0 μm. No pre- or exo-erythrocytic forms have been described for most *Babesia*, which implies that the sporozoites directly invade erythrocytes. Indeed, the absence of a pre-erythrocytic cycle has served as the main feature that distinguishes the Babesiidae from the Theileriidae (Riek 1968), *Theileria* being characterized by a lymphocytic pre-erythrocytic stage whereas *Babesia* lack such a stage in their life cycles. Direct evidence for penetration of erythrocytes by babesial sporozoites, however, is

lacking. The lymphocytic exo-erythrocytic forms that have been shown to occur in the life cycle of one small *Babesia*, *B. equi* (Schein et al. 1981; Mehlhorn and Schein 1984) may also occur in the life cycle of *B. microti* (Mehlhorn and Schein 1984). The existence of lymphocytic stages in the life cycle of some of the small *Babesia* raises a question about the validity of the generally accepted separation of *Babesia*, particularly the small types, from *Theileria*.

Although none of the small *Babesia* so far studied passes from generation to generation within the vector tick (transovarial transmission) (Walter and Weber 1981; Mehlhorn and Schein 1984; Gray et al. 2002), all of the large ones appear to do so. The small *Babesia* share this exclusively intragenerational pattern of development with *Theileria*. The absence of transovarial transmission gives additional biological support to the suggestion that the small *Babesia* should be classified with *Theileria*. Whether all of the small *Babesia* produce pre-erythrocytic stages, and whether all lack the capacity for inherited infection in the tick host remain to be determined.

At least 99 *Babesia* species infecting mammals have been described (Levine 1988), 78 of which infect non-ruminants. The vectors and other details of the life cycles of most of these species, except for those of veterinary or public health importance, remain largely undescribed. Virtually all piroplasm species were described on the basis of their microscopical appearance in Giemsa-stained blood smears (the morphology of the blood forms and the occurrence of binary or tetrad forms), as well as their host range and endemicity. Although it had been expected that many of these species would be synonymized with further study, molecular phylogenetic analysis demonstrates unexpected diversity (Ellis et al., 1992; Thomford et al., 1994; Allsopp et al., 1994; Caccio et al., 2000; Kjemtrup et al., 2000a; Zahler et al., 2000; Goethert and Telford, 2003a). Well-characterized, widely distributed 'species' such as *B. canis* (Carret et al. 1999), *B. gibsoni* (Kjemtrup et al. 2000b) and *B. microti* (Goethert and Telford, 2003a) are now considered to comprise groups of genetically diverse entities based on sequencing of 18S rDNA or other genes. Thus, the genus *Babesia* may be more speciose than previously appreciated, may even comprise several distinct genera. Much work, however, remains to be done prior to naming new species, let alone major taxonomic revision. In particular, a consensus must be formed among protozoologists as to the degree of distinction required to interpret a given difference in nucleotide sequences between samples as representative of distinct species. Such a consensus would require some knowledge of the degree of inter- and intrapopulation variability of the gene being used for classifying the piroplasms, as well as concordance with aspects of their biology such as the identity of their vectors.

STRUCTURE AND LIFE CYCLE

Babesias belong to the sporozoans, the group of protozoa also sometimes referred to as the Apicomplexa, which are characterized by having a variety of apical complex organelles, including rhoptries and micronemes. Mature babesial sporozoites have been described as pear-shaped with the anterior end being the broad end (Kakoma and Mehlhorn 1994). They have one anterior rhoptry and several micronemes but lack conoids, polar rings or subpellicular microtubules. Sporozoites appear to contain free ribosomes, a smooth endoplasmic reticulum, mitochondria-like structures and coiled organelles.

Recently invaded erythrocytes contain merozoites that initially lie within a parasitophorous vacuole (Rudzinska 1981) but this invaginated host-derived membrane soon disintegrates, leaving the maturing trophozoite free in the cytoplasm. Perhaps this unique freedom from a constricting parasitophorous vacuole contributes to the frequently noted pleomorphism of the intra-erythrocytic stage, which often is contorted or ameboid.

As the asexually reproducing intra-erythrocytic trophozoites of *B. microti* mature, new organelles appear, including polar rings, micronemes, larger rhoptries, subpellicular microtubules, and double membrane segments (Rudzinska et al. 1979). Interestingly, no cytostome is apparent; their method for deriving nourishment from a host cell remains undescribed.

Following the ingestion of parasitized erythrocytes by feeding *Ixodes dammini* tick larvae (the main vector for *B. microti* in the northern USA), gametocytes emerge and undergo a process of development into gametes (Strahlenkörper) which then fuse within the tick gut (Rudzinska et al. 1983). The existence of the ray-like Strahlenkörper that Koch associated with sexuality was confirmed by a demonstration of the fusion of the ray bodies emerging from neighboring erythrocytes (Rudzinska et al. 1983). The zygotes resulting from this fusion mature and become ookinetes 14–18 hours after the feeding larval tick becomes replete. The ookinetes subsequently penetrate into the hemolymph and elongate to form the kinetes, which penetrate fat body cells and nephrocytes. A series of divisions within the fat body cells and nephrocytes then results in the production of secondary ookinetes which in some *Babesia* may invade the ovaries (Karakashian et al. 1986). This secondary cycle of division and invasion of the ovaries is characteristic of the large *Babesia* and may facilitate transovarial transmission. Primary ookinetes enter the salivary glands directly, where they have been observed as early as 13 days after the larva became replete.

The salivary acini become hypertrophied when ookinetes invade the salivary glands. The ookinetes in the salivary acini become sporoblasts, which then remain

dormant until the larval tick becomes a nymph and the nymphal tick attaches to a host. In the case of *B. microti*, this state of dormancy in *I. dammini* (the vector) extends naturally through the winter, a period of 9–10 months (Piesman et al. 1987). After the infected nymphal tick attaches, nuclear division occurs, resulting in the formation of about 10 000 sporozoites from each sporoblast. Many thousands of sporozoites appear to be deposited in the dermis around the tick's mouthparts during the final hours of attachment (Mehlhorn and Schein 1984). Some 10 000–25 000 syringe-injected *B. microti* sporozoites are required to infect white-footed mice and hamsters (Piesman and Spielman 1982), but the anti-inflammatory pharmacological activity of tick saliva (Ribeiro 1987) greatly facilitates infection, suggesting that ticks may transmit the infectious agents far more effectively than does a needle.

The events that follow inoculation of babesial sporozoites by the feeding vector tick are poorly understood. In the absence of evidence to the contrary, early reviews of the biology of *Babesia* by Mehlhorn and Schein (1984), Mahoney (1977), and Young and Morzaria (1986) have generally inferred that in *Babesia* no pre-erythrocytic cycle occurs. But in *B. equi*, an agent of equine babesiosis, sporozoites appear to enter lymphocytes directly, both in vitro and in vivo, where they undergo a cycle of merogony before the resulting merozoites emerge to infect erythrocytes (Schein et al. 1981). The exo-erythrocytic cycle of this small *Babesia* was only recently discovered, unlike the exo-erythrocytic stages of *Theileria* which were discovered decades ago and called lymphocytic 'blue bodies' by Robert Koch.

The intra-erythrocytic parasites replicate when a nucleus and other organelles migrate to a location under particular double membrane areas of the parent. These areas then develop and pinch off from the parental piroplasm in a process of budding (Rudzinska 1981). *Babesia* do not undergo synchronous budding (schizogony) as do the plasmodia, but rather one, two or, rarely, three bud off at one time.

In addition to the asexually reproducing trophozoites and their merozoite progeny, there are also non-reproducing accordion-like gametocytes. These latter forms fail to develop the double membrane segments that become the anlage for merozoite formation and do not acquire a rhoptry; rather they just appear to grow larger, folding or coiling themselves within the confines of the erythrocyte (Mehlhorn and Schein 1984; Rudzinska et al. 1979). These accordion-like forms emerge from erythrocytes within the gut of the tick and differentiate into gametes, pairs of which may fuse in a process of syngamy. Unlike the gametocytes of the plasmodia, *Babesia* gametocytes cannot be distinguished from asexual forms by light microscopy; electron microscopy must be used to make the distinction.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

The biochemistry, molecular biology, and genetics of the zoonotic *Babesia* have been inadequately explored. Such work as has been reported has generally had as its goal the discovery of diagnostic antigens, the elucidation of diagnostically useful DNA sequences, or the identification of variation among strains of species. Isoenzymes, for example, were examined in an attempt to distinguish various populations of *B. microti* (Momen et al. 1979). High resolution polyacrylamide gel electrophoresis (Moss et al. 1986) was used to study the degree of polymorphism in the glucose phosphate isomerase locus in low- and high-passage Gray strain *B. microti* (the index strain for human infection) and showed that the Gray strain is heterogeneous, at least in terms of polymorphism at the locus. These preliminary results suggest that study of cloned isolates may be required to detect differences between populations.

Babesia microti infection may only slightly alter the physiology of hamster erythrocytes (Roth et al. 1981). Reduced glutathione levels double, but 2,3-diphosphoglycerate levels remain unchanged. Hamsters in which reticulocytosis is chemically induced experience similar changes, however, suggesting that the observed biochemical changes relate to anemia and not necessarily to the presence of parasites.

The existence of sexuality in piroplasms, initially determined by observation of fusing gametes within the tick with the aid of electron microscopy (Rudzinska et al. 1983), was confirmed by fluorescence microscopy of *Babesia* stained with the DNA-specific bisbenzimide Hoechst 33258. These studies revealed the ploidy of the various life-cycle stages of the cattle piroplasm *B. divergens* (Mackenstedt et al. 1990). Merozoites are haploid, two haploid merozoites are formed during budding within an erythrocyte; zygotes within the tick gut are diploid; kinetes are polyploid, and the polyploid sporoblasts divide to give rise to the uninuclear (haploid) infective sporozoites. Meiosis, however, has not been described.

A putative virulence-associated gene, *Bm13*, was cloned from a mung bean nuclease DNA expression library prepared from *B. microti* DNA (Tetzlaff et al. 1990). Mung bean nuclease, under certain conditions, appears to cleave AT-rich DNA at sites flanking intact genes (McCutchan et al. 1984). Using hyperimmune serum from mice infected by the ATCC 30221 strain of *B. microti*, which kills murine hosts, a clone coding a 54 kDa immunodominant antigen was selected. This sequence did not hybridize with DNA prepared from the less virulent Peabody strain of *B. microti*. Further experiments characterizing the protein that is coded for by the gene, and describing the protein's role in pathogenesis have not yet been published.

CLINICAL ASPECTS

The first convincingly demonstrated case of human babesial infection was reported in 1957 in a splenectomized resident of what was then Yugoslavia. The man died after an acute illness marked by anemia, fever, hemoglobinuria, and renal failure (Skrabalo and Deanovic 1957). Intra-erythrocytic parasites were detected and tentatively identified as *B. bovis*, a piroplasm of cattle. In 1969, an elderly resident of Nantucket Island (USA) with no predisposing immune-compromising factors became infected with a rodent piroplasm, *B. microti* (Western et al. 1970). Since then, infection by *B. microti* has been documented in hundreds of residents of the northeastern and upper Midwestern USA. Molecular phylogenetic methods have demonstrated that several other distinct *Babesia* (designated WA-1, CA-1, M0-1, EU-1) are agents of human babesiosis. Human babesiosis has also been convincingly documented in Taiwan, Japan, and South Africa. The public health significance of zoonotic babesiosis may be greater than previously estimated.

Babesiosis is a malaria-like infection and its pathology, like that of malaria, is caused mainly by the asexually dividing intra-erythrocytic forms. Non-synchronous fevers, chills, myalgia, sweats, and prostration may be observed in patients with acute disease. In such patients, a fulminating parasitemia may lead to anemia, hemoglobinuria, renal failure, disseminated intravascular coagulation, and acute respiratory distress syndrome. Human infection, although occasionally severe, is usually subclinical.

Clinical manifestations

Symptoms of babesiosis caused by *B. microti* may commence 1–4 weeks after a human is bitten by an infected tick. In persons who develop clinical disease, a gradual onset of malaise, anorexia, and fatigue ensues, with subsequent development (within a week) of fever with a temperature as high as 40°C, drenching sweats and myalgia (Ruebush et al. 1977). Nausea, vomiting, headache, shaking chills, emotional lability and depression, hemoglobinuria, and hyperesthesia have also been reported in such patients (Golightly et al. 1989). Pulmonary edema may be a frequent complication in symptomatic persons (Gordon et al. 1984; Boustani et al. 1994). Splenomegaly may occur, but other findings on physical examination are unremarkable. Anemia, thrombocytopenia, and low or generally normal white cell counts may be observed; parasitemias range from 1 to 20 percent in spleen-intact patients and reach 85 percent in asplenic patients (Sun et al. 1983). Lactic dehydrogenase, bilirubin and transaminases may be elevated (Ruebush et al. 1977). The ambiguity of the symptoms of human babesiosis renders clinical diagnosis difficult, particularly in sites where the agents of Lyme disease and human granulocytic ehrlichiosis are co-transmitted (Telford et al. 1995). The presence of fever, malaise, headache, splenomegaly, anemia, emotional lability and, particularly, profound fatigue provide clues useful to the physician.

As noted before, babesial infection in humans is usually subclinical or very mild. A large asymptomatic to symptomatic ratio is evident from serosurveys. For example, 8 percent of residents of Block Island, Rhode Island (USA) have been infected without clinically diagnosed illness (Krause et al. 1994). Even though most infections are subclinical in areas with a high level of infection, moderate to severe disease may be common. Nantucket Island reported 21 cases in 1994, which translates to 280 cases per 100 000 population, placing the community burden of this disease in a category with that of gonorrhea (Wilson 1991). The case fatality rate was estimated at 5 percent in a retrospective study of 136 New York cases (Meldrum et al. 1992). In certain sites, in years of particularly high transmission, babesiosis may constitute a public health burden.

Several factors appear to determine the severity of infection. There appears to be a relationship between the severity of illness and the age of the patient. The average age of seven spleen-intact New York patients with clinically apparent disease was 63 years (Benach and Habicht 1981) as was that of five similar patients from Nantucket (Ruebush et al. 1977). Similarly, infection by human immunodefciency virus (HIV) appears to promote the severe manifestations of babesial infection (Ong et al. 1990; Benezra et al. 1987; Machtinger et al. 1993). Although *B. microti* is enzootic in much of coastal New England, virtually all cases are diagnosed on Nantucket Island, eastern Long Island or in southeastern Connecticut. Parasite strain variation (as yet uncharacterized) may influence the course of infection, as well as the geographical distribution of cases.

Babesial infections acquired in Europe have been attributed to the cattle piroplasm *B. divergens* and tend to be severe. Virtually all European patients had been splenectomized and the disease followed a severe course with the rapid multiplication of parasites (Gorenflot et al. 1990). Infection is usually fulminant; more than half of the 22 recorded patients died. Acute illness appears suddenly and is characterized by hemoglobinuria, which generally serves as the presenting symptom. Jaundice rapidly ensues and is accompanied by persistent non-periodic high fever (40–41°C), shaking chills, intense sweats, headaches, and myalgia, as well as lumbar and abdominal pain. Vomiting and diarrhea may be present. In severe cases, renal failure ensues rapidly (16 of the recorded 22 cases) and is induced by intravascular hemolysis. In fatal cases, the patient loses consciousness and dies in coma. A recent report suggests that the causative agent for reported cases of 'divergens' babesiosis may be uncertain. A *Babesia* sp. that is genetically

more similar to *B. odocoilei* (a parasite of American deer) than to *B. divergens* caused fulminating infection in two Italian patients (Herwaldt et al. 2003). This agent has been designated EU-1; it is morphologically similar to *B. capreoli* from European deer (Enigk and Friedhoff 1962) but could be mistaken for *B. divergens* by less experienced microscopists. *B. divergens* has been definitively identified as a causative agent of babesiosis in Europe by the use of cattle subinoculation and protection of such animals from challenge (e.g. Entrican et al. 1979). Thus, there are at least two agents of 'divergens' babesiosis.

Two splenectomized American patients with no travel history to Europe have been infected by *B. divergens* or something very closely related (Herwaldt et al. 1996; Beattie et al. 2002); one case terminated fatally. The patients presented with fever, headache, and rigors; thrombocytopenia, hemoglobinuria, and proteinuria were noted. Blood smears demonstrated parasites with typical *B. divergens* morphology (accole forms, paired pyriforms), and 18S rDNA sequencing indicated more than 99 percent sequence similarity with the European cattle agent. The same agent is enzootic within cottontail rabbits and their ticks (Goethert and Telford 2003b). Rabbit sera reacts with *B. divergens* (Purnell strain) antigen, more so than to the closely related *B. odocoilei* that is present in deer from the sites where rabbits are found to be infected. All available data suggest that this agent is *B. divergens* regardless of its American location or unusual enzootic host, but final taxonomic assignment awaits definitive cattle inoculation experiments.

Although only a few cases of babesiosis due to the WA-1 *Babesia* have been described, including two transfusion cases (Herwaldt et al. 1997; Kjemtrup et al. 2002) the course of the infection would appear to be similar to that caused by severe *B. microti*. The index case (Quick et al. 1993) was a 41-year-old spleen-intact man from a rural area in south central Washington (USA) with no history of travel. He was hospitalized with a 1-week history of fever, rigors, anorexia, cough, and headache, which progressed to severe rigors and vomiting. Dark-colored urine was noted, but a urine dipstick assay was only weakly positive for occult blood. Intra-erythrocytic parasites were observed, including characteristic tetrad forms. The patient was treated with clindamycin and quinine and recovered uneventfully. An isolate was made by inoculating hamsters with the patient's blood, but unlike *B. microti*, this agent killed these and other rodents within 10 days of infection (unpublished results 1992). Serum reacted weakly with *B. microti* antigen but more intensely with that of *B. gibsoni*. Subsequent molecular analysis confirmed that WA-1 was more closely related to *B. gibsoni* than to other *Babesia* (Thomford et al. 1994; Kjemtrup et al. 2000b).

Since the index case, similar infections have occurred in splenectomized residents of northern California (Persing et al. 1995). One case terminated fatally and another developed disseminated intravascular coagulation and renal insufficiency but recovered. 18S rDNA sequencing demonstrated that the agent was distinct from but related to WA-1 and it has been designated the CA-type babesia. Recent phylogenetic analyses suggest that mule deer and bighorn sheep are infected by parasites that are virtually identical (Kjemtrup et al. 2000a), but no other information is available regarding vectors and reservoirs.

IMMUNOLOGY

The relatively greater severity of human babesiosis in patients lacking a spleen indicates that this organ plays an important role in limiting parasitemia. In particular, early in the infection, parasitized and otherwise altered erythrocytes may be trapped and phagocytosed in the spleen. Absence of the spleen, however, does not necessarily imply that parasitemia may not be eventually limited.

Treatment of splenectomized and intact hamsters with antilymphocyte serum (ALS) demonstrated the importance of cellular immunity because all ALS recipients died upon challenge with *B. microti*, whereas only 20 percent of splenectomized animals died (Wolf 1974). Persistent intense parasitemias in athymic (nude) mice, in contrast to those of their heterozygous (thymus-intact) littermates, suggests the critical role of T cells in regulating the level of parasitemia (Clark and Allison 1974). CD4 gene knockout mice sustained *B. microti* parasitemias for longer periods of time than did congeneic controls (Hemmer et al. 2000). In contrast, humoral immunity appears to be less important than cellular immunity in resolving *B. microti* infection. Passive transfer of immune serum to severe combined immunodeficiency (SCID) mice and to nude mice fails to protect them from *B. microti* infection (Igarashi et al. 1999; Matsubara et al. 1993). T-cell-receptor deficient mice are more easily infected and maintain greater parasitemias than are those that are B-cell deficient (Clawson et al. 2002). Indeed, the severe babesial disease that has been reported to occur in HIV-positive patients would seem to affirm the critical role of T cells in regulating this infection (Machtinger et al. 1993; Falagas and Klempner 1996).

Experiments using inbred mice and murine-adapted *B. microti* have explored the possibility of a genetic basis for susceptibility, defined as the inability of mice of a given strain to regulate and eventually clear parasitemia. Parasitemia was lower in intact BALB/c mice than in intact mice of four other strains inoculated with *B. microti* but was highest in BALB/c mice when they were splenectomized (Ruebush and Hanson, 1979, 1980). Intact C3H mice were most susceptible to infection. Susceptibility does not appear to be related to H-2 (major histocompatibility complex) haplotype, however, because mice of identical haplotypes develop different

maximum parasitemias. The degree of protection of mice against *B. microti* induced by *Propionibacterium acnes* inoculation does not correlate with H-2 haplotype (Wood and Clark 1982). On the other hand, the ability to express this induced protection trait is heritable. Some as yet undefined genetic component appears to influence susceptibility to *B. microti* infection.

Various non-specific factors seem to modify susceptibility to piroplasm infection. The presence of eperythrozoa, for example, may antagonize infection (Peters 1965). Injection of various reagents and coinfection with diverse organisms may non-specifically protect mice against *B. microti* (Clark 1979). The prepatent period is increased and peak parasitemia reduced in mice on a severely restricted protein diet (Tetzlaff et al. 1988). Finally, although old mice experience later and lower peak parasitemias than do younger animals, they fail to clear infection (Habicht et al. 1983). The factors that control susceptibility are complex.

In patients with *B. microti* babesiosis, some antibody responses are generally evident when they are first diagnosed. Seventeen patients with positive blood smears had a median indirect fluorescent antibody test (IFAT) immunoglobulin (Ig)G titer of 1:1 024 (negative, 1:64) with a range of 1:128–1:4 096 in their serum at the time of diagnosis (Telford unpublished). The presence of antibody in the serum when these individuals first became ill indicates that the prepatent period may be prolonged. Titer level is not related to parasitemia, however, consistent with the conclusion from experiments in mice that antibody alone cannot modulate infection.

In one comprehensive study of immunoresponsiveness in humans with *B. microti* babesiosis, lymphocyte responses to non-specific mitogens were markedly suppressed in acutely ill patients (Benach et al. 1982a). The numbers of T cells bearing the IgG Fc receptor were high, as was the relative proportion of B lymphocytes. Diminished levels of complement factors C3 and C4 in the serum of patients with acute babesiosis suggest activation of the classical complement pathway. In addition, the patient's blood contained a high level of circulating immune complexes. These data indicate that *B. microti* infection profoundly alters the cellular immune status of patients.

Reinfection of those who have recovered from babesiosis has not been reported but it would not be easy to prove reinfection because of the difficulty of excluding recrudescence of an earlier infection. The concomitant immunity (or premunition) that characterizes babesiosis in cattle may also occur in humans. Using primers designed from species-specific sequences of the small subunit ribosomal DNA (Persing et al. 1992), the polymerase chain reaction (PCR) assay may facilitate a determination of the prevalence of chronic babesiosis in humans. Detection of non-clinical carriers is important because transfusion-acquired cases are not uncommon (Jacoby et al. 1980; Marcus et al. 1982; Smith et al. 1986).

PATHOLOGY

The development of the pathophysiology of babesial infection is directly related to the development of the parasitemia. Studies on hamsters and laboratory mice infected by syringe with *B. microti* of human origin have provided a wealth of information on host–parasite interactions. Intravascular and extravascular hemolysis develops as the parasitemia rises, resulting in profound anemia; the hematocrit may fall to 20 percent (Lykins et al. 1975). During this acute phase of disease, there is extramedullary hematopoiesis and hyperplasia of the splenic red pulp, giving rise to the virtually pathognomonic gross splenomegaly that accompanies babesiosis in wild rodents (Fay and Rausch 1969). In addition, livers of infected hamsters contain hypertrophied Kupffer cells, many with ingested parasitized erythrocytes but little hemoglobin breakdown products (Cullen and Levine 1987). The proximal convoluted tubules of the kidneys contain abundant hemosiderin, an observation consistent with occurrence of marked intravascular hemolysis (Cullen and Levine 1987).

WA1-infected hamsters die within 2 weeks of infection, and necropsy demonstrates intravascular mononuclear cell aggregates, as well as occlusion of small vessels by parasitised erythrocytes (Dao and Eberhard 1996; Wozniak et al. 1996). Multifocal coagulative necrosis was present in the heart, spleen, lung, and liver, perhaps due to vascular stasis, resultant anoxia, and tissue damage. Mice with a genetic disruption in the tumor necrosis factor-alpha (TNF-α) pathway were less likely to die of fulminating WA1 infection, as were CD8 gene knockout mice, whereas in $\gamma\delta$ T cell knockout mice and control mice WA-1 infection terminated fatally. Thus, CD8 T cells and local expression of TNF-α may contribute to the WA1-induced pathology (Hemmer et al. 2000).

DIAGNOSIS

Diagnosis of babesiosis in human hosts depends mainly on a demonstration of the organism in erythrocytes. Examination of conventional Giemsa-stained thin blood films remains the most generally useful diagnostic procedure. Although the presence of tetrad (Maltese cross) forms is said to be diagnostic, such elements are rarely encountered. Similarly, the absence of parasite hemozoin (malarial pigment) is often considered to be diagnostic for the piroplasms but early ring stages of malaria parasites also lack pigment. Diagnosis of *B. microti* (Figure 1) is made by a combination of criteria, including the presence of an intense parasitemia (1–50 percent), erythrocytes infected by multiple parasites, basket-shaped merozoites which are often extracellular

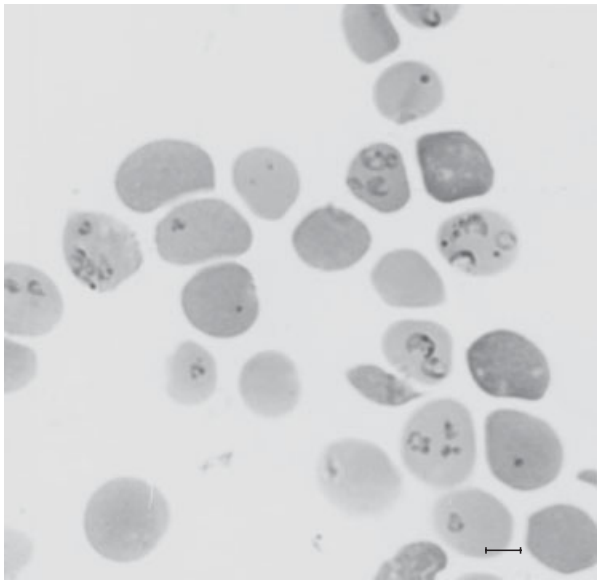

Figure 1 Babesia microti*, hamster blood. Note the varied morphology of the parasites, ranging from that of merozoites to that of mature trophiozoites. The occurrence of multiple infection of single erythrocytes is a characteristic which helps to distinguish these ring-shaped parasites from* Plasmodium falciparum. *Bar = 4 μm*

and which often have a light-colored cytoplasm. The small *Babesia* are difficult to recognize with confidence in a thick film, but the presence of tetrads or paired pyriforms might easily be detected. Parasitemias may sometimes also be exceedingly sparse, and persons with infections with low parasitemias may escape diagnosis when a thin blood film is examined. Inoculation into hamsters of a sample of patient's blood facilitates diagnosis by amplifying the parasitemia but the blood of the hamster should be examined microscopically at weekly intervals for at least 6 weeks before the test is declared negative. Demonstration of the characteristic organisms in the hamster blood proves infection.

Serological testing is useful, particularly in diagnosing chronic *B. microti* infection. The IFAT, using antigen derived from infected hamster red cells (Chisholm et al. 1978), is sensitive and specific and is currently the serological method of choice. A serological reaction at a titer above the cutoff point of 1:64 for IgG is generally considered to be diagnostic (Krause et al. 1994). Examination of paired acute and convalescent serum samples is most useful for a diagnosis of *B. microti* infection. Detection of parasite-specific IgM may indicate that the patient has an acute infection even in the absence of a readily demonstrable parasitemia.

In persons with *B. divergens* babesiosis, specific antibodies do not become detectable until at least 1 week after the onset of illness. Because this infection develops rapidly, serological procedures are not practical for the diagnosis of the infection in its acute form, but serological conversion serves as an aid in making a retrospective diagnosis in survivors. Of course, the high parasitemias usually present in patients with this infection in its acute form are easily detected by examination of blood smears. Paired pyriform and accole parasites are frequently observed in erythrocytes from patients with acute *B. divergens* infection (Figure 2).

Infection with *Babesia* of the WA-1 strain may be readily detected by IFAT and this organism may also be detected by in vitro cultivation (Thomford et al. 1994). Its ease of cultivation facilitates production of a standardized antigen. For unknown reasons, a higher titer in the IFAT (more than 1:160) is required for diagnosis of WA-1 infection than for diagnosis of infection with *B. microti*. Tetrad forms are more frequently seen in blood smears from patients and rodents with WA-1 and CA-type infection (Figure 3) than in those with *B. microti* infections, but otherwise infection with this agent is difficult to distinguish from that with *B. microti*.

The PCR has largely supplanted the hamster inoculation test. In experienced hands, the PCR test detects *Babesia* DNA accurately and usually within a single working day. Hamster inoculation, long thought to be the gold standard for confirmatory diagnosis, has its problems. For example, it requires at least a week and often more than a month for incubation before results are obtained. Blood samples for hamster inoculation must also be taken prior to initiating treatment. An additional problem with use of hamster inoculation for diagnosis is that in many instances infection is transient in hamsters and may be missed if monitoring of blood smears is done only weekly. Finally, an infection due to a *Babesia* other than *B. microti*, *B. divergens* or WA-1, might be missed entirely if it lacks the ability to infect hamsters. These problems are avoided if diagnosis is made with the aid of a genus-specific DNA amplification assay.

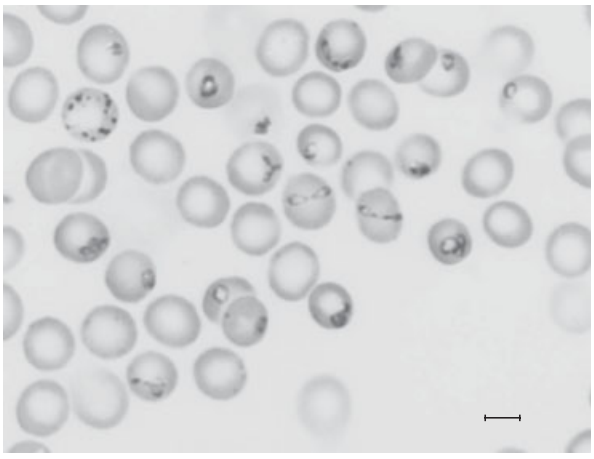

Figure 2 Babesia divergens*, gerbil blood. Pairs of pyriform organisms occur frequently and their presence helps to distinguish* B. divergens *from the other* Babesia *infecting humans. Ring-shaped organisms may also occur, as may tetrad forms. Bar = 4.8 μm*

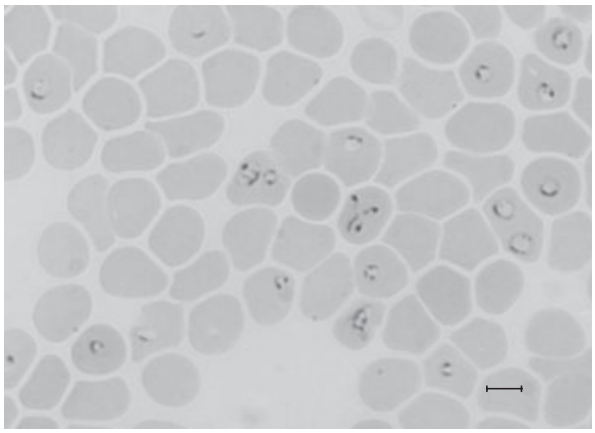

Figure 3 Babesia *sp. (WA-1), hamster blood. Although its morphology is very similar to that of* B. microti*, WA-1 may more frequently occur as tetrads than does* B. microti*; multiple infection of erythrocytes occurs less frequently in blood infected with WA-1 than in blood infected with* B. microti*. Bar = 5 μm*

EPIDEMIOLOGY AND CONTROL

The acquisition of babesiosis due to *B. divergens* or *B. microti* depends on contact with the subadult stages of certain *Ixodes* ticks. The vectors of WA-1, CA-type, or the atypical *B. divergens*-like infections, however, have not yet been described. Environmental disturbance, increasing recreational use of the wilderness and suburbanization promote contact between people, ticks, and the pathogens that they maintain (Spielman 1988).

Two epidemiological patterns are apparent in human babesiosis. The first (pattern I) involves splenectomized or otherwise immunologically compromised people and diverse *Babesia* spp. This pattern includes European 'divergens' babesiosis. The risk of acquiring human babesiosis in the northeastern and northern Midwestern USA does not appear to depend on splenectomy. This second epidemiological pattern (pattern II) involves infection by the rodent piroplasm *B. microti* and being bitten by *I. dammini* ticks. The vectors of *B. microti*-like babesiosis in Asia (Shih et al. 1997; Saito-Ito et al. 2000) remain undescribed.

Although one might predict that the increasing prevalence of HIV infection in many tropical areas of the world may allow the emergence of 'tropical' babesiosis, such cases have not yet been reported. Babesiosis is particularly severe in individuals with HIV and several case reports of babesiosis in patients with acquired immune deficiency syndrome (AIDS) already exist in the literature (Benezra et al. 1987; Ong et al. 1990; Machtinger et al. 1993; Falagas and Klempner 1996). The diversity of *Babesia* species and the ubiquity of ticks suggest a high potential for the emergence of *Babesia* as zoonotic agents. It is at least possible that some chloroquine-resistant malaria parasites may eventually be identified as *Babesia* (Young and Morzaria 1986). Whether or not this will be the case will be determined by careful microscopy, complemented by the use of molecular diagnostic techniques by people aware of the possibility of babesial infection in humans.

Epidemiology and ecology

The ecology of *B. microti* is the best understood of the three known zoonotic piroplasms. The enzootic cycle of *B. microti* clearly depends on the interaction of subadult *I. dammini* and their main host, the white-footed mouse (*Peromyscus leucopus*). Deer (*Odocoileus virginianus*) serve as the host upon which adult ticks commonly feed, but deer are not reservoirs for *B. microti* (Piesman et al. 1979). Adult ticks feed during the autumn, overwinter, feed, and engorge again and lay eggs during the spring (Yuval and Spielman 1990). The eggs hatch synchronously in late July, with larvae feeding on mice mainly during August and September, when they acquire babesial infection; the prevalence of *Babesia* infection in the mice appears to be about 60 percent (Etkind et al. 1980).

Fed larvae overwinter and molt to the nymphal stage during the spring. Thus, non-infected mice resulting from reproduction during May and June are inoculated by nymphs infected as larvae during the preceding fall. About 40 percent of nymphal ticks on Nantucket Island (USA) may contain babesial sporozoites in their salivary glands during June. People become exposed mainly during June (Piesman et al. 1987) when they visit zoonotic sites for recreation or inhabit summer homes located in such sites. The population of Nantucket Island increases from about 7 000 winter residents to over 35 000 summer residents after Memorial Day (at the end of May). The small size of nymphal *I. dammini* makes its detection and prompt removal difficult. Failure to remove a feeding tick promptly determines risk because the probability that the tick will transfer the *Babesia* to the host on which it feeds is directly proportional to the duration of feeding by the tick (Piesman et al. 1986).

The life cycle of the tick is completed when nymphs that have fed on a host molt to the adult stage in the fall. Thus, *I. dammini* ticks develop from egg to egg over a span of at least 2 years (Yuval and Spielman 1990), permitting cohorts of nymphal ticks to overlap, thereby buffering the tick population against years of host scarcity.

The epizootiology of *B. divergens* and the role of its presumed vector, *I. ricinus*, in the spread of infection is poorly understood. In Ireland, a high incidence in cattle is associated with elevated air temperature, which is when there is much tick activity. As in the case of *I. dammini*, nymphs may become active in the spring before the larvae hatch, but incrimination of the vector stage responsible for transmitting infection is confounded by the presence of adult ticks concurrently

with nymphs (Gray 1980). Most cases of babesiosis due to *B. divergens* in humans have occurred in farmers or others who are frequently in contact with cattle (Clarke et al. 1989). Interestingly, although *B. microti* is enzootic in many European sites, human babesiosis due to this parasite has not been observed there, possibly because a uniquely mouse-specific tick, *I. trianguliceps*, maintains *B. microti* in Europe, although the aggressively human-biting *I. ricinus* may densely infest the same sites. Perhaps European strains of *B. microti* are not pathogenic to humans. Recent serosurveys in Germany and Switzerland (Hunfeld et al. 2002; Foppa et al. 2002) suggest exposure, but clinically apparent *B. microti* babesiosis has not been recorded from these European sites.

Neither the vector nor the reservoir of the WA-1 and CA-type *Babesia* are known. Three ticks are prevalent in sites where cases of WA-1 or CA-type babesiosis have occurred: *I. pacificus*, *Dermacentor variabilis*, and *Ornithodoros coriaceus*. Phylogenetic studies (Kjemtrup et al. 2000a) suggest that mule deer or bighorn sheep may be infected by CA-type parasites, and a dog isolate serves as the most closely related agent to WA-1. Identification of candidate reservoir hosts may help focus investigations to incriminate the vectors of these *Babesia* spp.

Residents of few countries outside the USA and Europe appear to have experienced clinical babesiosis. One case has been observed in China (Li and Meng 1984) and two in South Africa (Bush et al. 1990). The species of *Babesia* involved in these cases has not been determined. One fatal case due to infection by *B. caucasica* has been observed in the former Soviet Union (Rabinovich et al. 1978). Unidentified *Babesia* were isolated by hamster inoculation from asymptomatic residents of Mexico (Osorno et al. 1976). A serosurvey conducted in Venezuela revealed that there was a low prevalence of antibody against *B. bovis* in the Venezuelan population (Montenegro-James et al. 1990). In Asia, serosurveys using *B. microti* antigen indicated exposure of a few residents of Taiwan (Hsu and Cross 1977) and three cases of human babesiosis, caused by an agent that infected hamsters and whose 16s rDNA sequence closely resembles *B. microti*, were observed (Shih et al. 1997). An authocthonous Japanese case of *B. microti*-like babesiosis, transmitted by transfusion, has been reported (Tsuji et al. 2001); although *B. microti*-like parasites are prevalent in Japanese rodents (Shiota et al. 1984), the genotype recovered from the patient is apparently only found on a small island, suggesting the hypothesis that only this insular strain of parasite may be zooonotic (Tsuji et al. 2001). Asymptomatic *Babesia* infections have been observed in Africa. A survey conducted in Nigeria indicated that 54 percent of 173 men from the northwestern border may have been infected (Leeflang et al. 1976) and a serological survey in Mozambique suggested that *B. bovis* may infect residents there (Rodriguez et al. 1984).

Two human infections have been ascribed to *Entopolypoides macaci* (Wolf et al. 1978). Organisms in this genus, however, are now generally considered to be *Babesia* (Levine 1988; Bronsdon et al. 1999). This piroplasm (which should now be referred to as *Babesia macaci* although the designation PB-1 was applied by Bronsdon et al. 1999) appears to be commonly found in research primate colonies where ectoparasite control is stringent. It may be that this *Babesia* can be maintained in the absence of tick vectors by transplacental or perinatal transmission or by direct blood contact during aggressive interactions.

CHEMOTHERAPY

Because of its use for malaria, orally administered chloroquine was used in the treatment of the first few Americans in whom *Babesia* infections were observed. Symptomatic improvement was described, but parasitemia tended to continue (Ruebush et al. 1977). Indeed, administration of chloroquine, sulfadiazine, or pyrimethamine fails to reduce parasitemia in hamsters (Miller et al. 1978). Although pentamidine appears to have been useful in several patients who were treated with this drug, such therapy fails to eliminate the parasitemia completely (Francioli et al. 1981). The treatment of choice for babesiosis in humans caused by *B. microti* is quinine and clindamycin, administered in combination (Dammin et al. 1983). Quinine should be administered orally in a regimen of 650 mg, three times daily and clindamycin intravenously at 1200 mg, twice a day. Alternatively, clindamycin can be administered in an oral regimen of 600 mg, three times a day. The treatment should be continued for at least 7 days (Anon 1986) or until the parasitemia is eliminated, whichever comes first. This treatment generally is effective, except in those who are immunosuppressed (Smith et al. 1986) or infected by HIV (Benezra et al. 1987; Ong et al. 1990). Atovaquone (750 mg every 12 hours) and azithromycin (500 mg on day 1 and 250 mg per day thereafter) orally for 7 days appears to be as effective and better tolerated than quinine–clindamycin (Krause et al. 2000). In fulminating cases, exchange transfusion is life-saving (Jacoby et al. 1980; Cahill et al. 1981; Sun et al. 1983).

Any person with babesiosis acquired in Europe should be treated on an emergency basis. In addition to supportive treatment, such patients should receive prompt specific therapy designed to reduce parasitemia and to prevent the extensive hemolysis and consequent renal failure that may follow. Massive exchange transfusion (between two and three blood volumes) should be followed by administration of 600 mg clindamycin intravenously, between three and four times daily, together with quinine (600 mg base) administered orally three times a day (Gorenflot et al. 1990, 1987; Uhnoo et al. 1992). Indeed, because of the rapidly increasing parasitemia characteristic of the disease, exchange

transfusion should be instituted on the first signs of disease due to *B. divergens* infection. The time element becomes crucial because the request for medical attention generally follows the onset of hemoglobinuria, a manifestation of the disease that signals a fulminating parasitemia. Unless blood exchange is promptly undertaken, the prognosis is poor, as the rapidly increasing intravascular hemolysis leads to renal failure.

Babesiosis due to WA-1, CA-type, or atypical *B. divergens* appears to respond to quinine and clindamycin, although too few cases have been reported to gauge the efficacy of this treatment regimen.

VACCINATION

Strong immunity in cattle against bovine babesiosis has been induced by injection of attenuated parasites (Callow 1971) or exoantigen preparations (Smith and Ristic 1981). Less successful vaccines have been prepared from recombinant antigens. Crude parasite lysates serve to immunize hamsters against infection by *B. microti* (Benach et al. 1982b). Culture-derived parasites, crude culture supernatants, and purified exoantigen preparations protect gerbils and cattle against *B. divergens* (Valentin et al. 1993). Immunoscreening of *B. microti* expression libraries have yielded candidate protective antigens such as bmn1-6 and p58 (Homer et al. 2000; Nishisaka et al. 2001), but development of vaccine candidates for human use remains a long-term objective. Other than for those individuals who are splenectomized or otherwise immunocompromised or living in certain high-risk areas, however, the value of an antibabesial vaccine for prevention of babesiosis in humans is doubtful.

INTEGRATED CONTROL

The accepted public health strategies to protect human populations against zoonotic babesial infection depend on reducing the density of ticks. The spraying of acaricidal emulsions (Stafford 1991) has been used to reduce the abundance of ticks on vegetation. Less environmentally intrusive methods of application of acaricides, one particularly based on fiber-formulated permethrin may be more acceptable (Mather et al. 1987). These methods interrupt transmission of *B. microti* infection by depositing acaricide in the nests and on the coats of rodent reservoir hosts. Tick infestations may also be reduced locally by destroying the animals (i.e. deer) that serve as host to the adult stage of the tick (Wilson et al. 1988). An effort to reduce the abundance of the hosts of subadult ticks might be counterproductive because the absence of these hosts might increase the density of ticks seeking alternative hosts (Spielman et al. 1981). Acaricides applied to the coats of cattle could reduce the possible transmission of cattle babesiosis to humans.

Repellents are useful for personal protection, particularly permethrin-based formulations applied to clothing (Schreck et al. 1986). Even volatile compounds such as diethyltoluamide (DEET) may provide a certain measure of protection. Personal protection, based on daily examination of the body surface of a person who has visited a site where transmission is intense, is the most effective means of reducing the risk of contracting babesiosis. All ticks found should be promptly disposed of to prevent their attachment. Ticks already attached should be removed by means of forceps to take advantage of the initial 50–60 hour 'grace period' during which attached ticks rarely transmit infection.

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Malaria

MARCEL HOMMEL AND HERBERT M. GILLES

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INTRODUCTION

Malaria is the most important of all the tropical diseases in terms of morbidity and mortality. Worldwide, some two billion individuals are at risk; 100 million develop overt clinical disease and 1.5 to 2.7 million die every year. Nearly 85 percent of the cases and 90 percent of carriers (many asymptomatic) are found in tropical Africa, where in some countries 20–30 percent of deaths in childhood are attributed to the disease (Defo 1995; Greenwood et al. 1987; Snow et al. 1999, 2003). Human malaria is caused by four species of protozoans belonging to the genus *Plasmodium*, *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* causing malignant tertian, benign tertian, ovale tertian, and quartan malaria, respectively.

Drug and insecticide resistance have aggravated the complexity of the malaria problem worldwide. Thus, among the countries where falciparum malaria persists, only those of Central America have not recorded the resistance of *Plasmodium falciparum* to chloroquine. In contrast, in the Mekong region in Southeast Asia, multidrug resistance to chloroquine, sulfadoxine-pyrimethamine, and even mefloquine, now occurs and the sensitivity to quinine is also diminishing (WHO 1994a).

Maps showing the global distribution of malaria and the countries from which *P. falciparum* resistance to chloroquine and other antimalarial drugs has been reported, are given in Figures 1 and 2. It should be emphasized that degrees of resistance vary within individual countries; for example, in Cambodia multidrug resistance occurs in one part of the country, whereas in another part of the country *P. falciparum* parasites are still sensitive to chloroquine.

CLASSIFICATION

Malaria parasites belong to the genus *Plasmodium* that includes over 125 species infecting reptiles, birds, and mammals. *Plasmodium* is the only genus in the Family Plasmodiidae, Order Haemosporida, Class Coccidea, Phylum Sporozoa (Apicomplexa). The phylum is characterized by the presence, at specific stages of the life cycle, of an apical complex consisting of a number of specialized organelles, which may include a conoid, polar rings, rhoptries, and micronemes.

Morphological characteristics and features of the life cycle, the major criteria used in Garnham's (1966) classification, include the shape of the trophozoite, the gametocyte, and the oocyst, the number of nuclei in the erythrocytic and exo-erythrocytic schizonts, the aspect and distribution of the pigment, and the nature of the damage induced by the parasite in the host cell (e.g. 'Maurer's clefts' or 'Schüffner's dots'). The main biological criteria include the host range, the type of host cell infected, the duration of the different stages in the life cycle, the presence or absence of relapse (with or without recrudescences), the nature of the vector, and

Figure 1 *Global distribution of malaria in 2003 (reproduced by permission of WHO)*

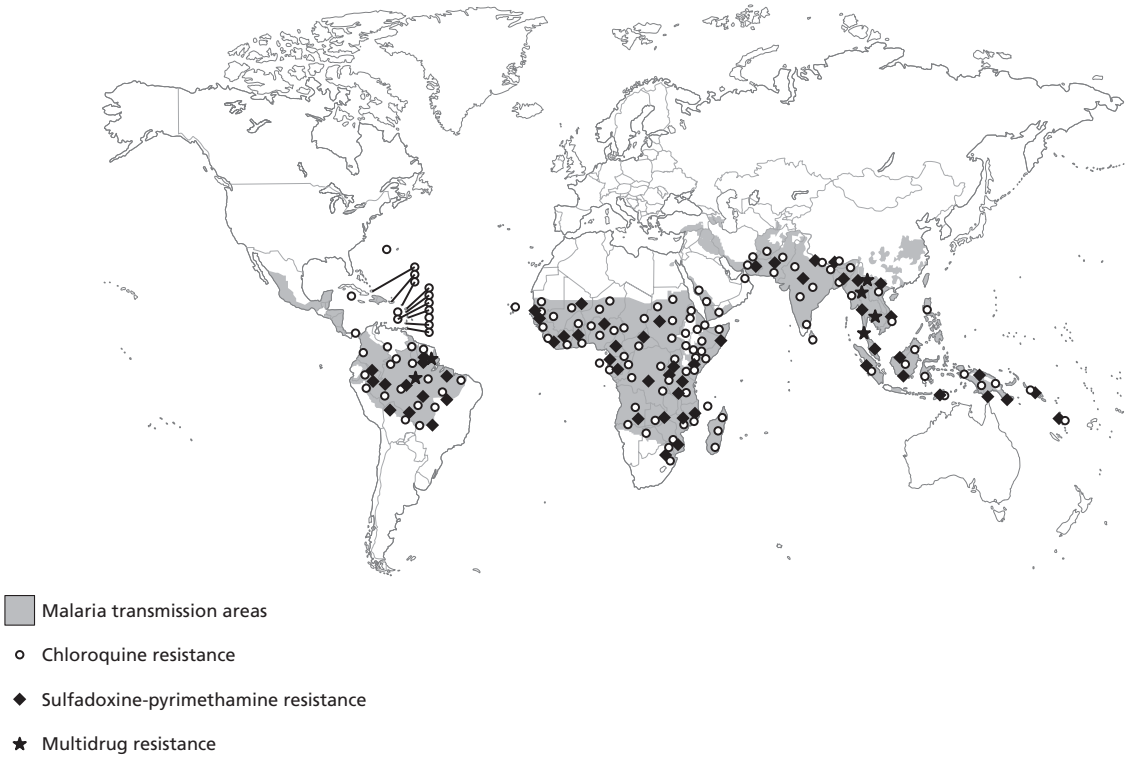

Figure 2 *Malaria transmission areas and reported* Plasmodium falciparum *drug resistance in 2001 (reproduced by permission of WHO)*

the geographical distribution. While none of these criteria are ideal taxonomic markers, in most cases they have been considered broadly adequate to differentiate and classify species.

LIFE CYCLE, DEVELOPMENTAL STAGES, AND TRANSMISSION

Life cycle

Human malaria parasites undergo a complex cycle of development, including stages in a female *Anopheles* mosquito. The infection starts with the bite of an infected mosquito, when the sporozoite is introduced into the skin together with mosquito saliva. The sporozoite rapidly makes its way to the liver, where it invades a hepatocyte. Within the hepatocyte, the parasite undergoes a period of differentiation and multiplication to produce the pre-erythrocytic schizont, containing a few thousand merozoites. The merozoites, when released from the hepatocyte, enter the bloodstream and invade new erythrocytes to start a characteristic periodic phase of differentiation and asexual multiplication leading to the erythrocytic schizont. This contains a small number of merozoites, which in turn invade new erythrocytes. At some stage, some merozoites undergo sexual differentiation and gametocytogenesis. When a new biting mosquito picks up the male and female gametocytes, gametocytes are released from the erythrocytes in the acidic, low temperature environment of the insect midgut, and transform into gametes, which fertilize to form first a zygote, then an ookinete. The motile ookinete crosses the epithelial wall of the midgut before transforming into an oocyst, which divides by schizogony to produce thousands of sporozoites. These undergo a final differentiation into their infective forms while migrating to the salivary glands of the insect, where they stay until the next bite, thus completing the cycle. Figure 3 illustrates the life cycle of *P. falciparum*.

Developmental stages

The various stages of malaria parasites exhibit dramatically different morphological features, which reflect both their very different function and the different microenvironments inhabited. Only the sporozoite, merozoite, and ookinete (which are designed for the invasion of, respectively, the hepatocyte, erythrocyte, or midgut epithelial cell of the mosquito) possess a surface coat and the specialized apical complex. The surface coat is responsible for host cell recognition and adherence, and the invasion organelles allow the parasite to actively enter the target cell. In contrast, other stages of the life cycle, which are designed for growth and development within the host cell, lack these invasion organelles, but possess structures involved in respiration, intake of food or digestion of nutrients (e.g. mitochondrion, cytostome, food vacuoles, and pigment). The sexual stages of the parasite undergo a complex differentiation in order to become equipped for the fertilization process that takes place within the mosquito midgut; the zygote and ookinete are the only parts of the life cycle in which the organism is diploid and where meiosis is observed.

Plasmodium spp. possess a single mitochondrion (Fry 1991), which seems to play a very different function during the vertebrate stage (when it is almost acristate, particularly in mammalian plasmodia) and the invertebrate stage (when it is always fully cristate). A switch of enzymatic activity takes place as the parasite differentiates from one stage to the other. Mitochondria are self-replicating and each stage of the life cycle carries at least one, albeit rudimentary in some cases.

Malaria parasites are unable to ingest particulate food and are entirely dependent upon the intake of soluble cellular macromolecules. Early trophozoites feed by micropinocytosis, pinching off small portions of the erythrocyte cytoplasm and producing small digestive vesicles. Later trophozoites are equipped with a cytostome (i.e. primitive 'mouth'), which becomes functional when micropinocytosis ceases. For most growing stages, the cytostome represents the major route for food intake.

Within the food vacuole, the digestion of hemoglobin leaves an insoluble waste product consisting of hematin and ferriprotoporphyrin coupled to partially degraded globin and plasmodial proteins; this forms malaria pigment or hemozoin. When the mature schizont finally breaks up, liberating merozoites, the pigment is left behind in the 'residual body;' these bodies eventually accumulate and remain within the body, mainly in the liver and spleen, for many years.

The plasma membrane acts as an interface between the parasite and its host and provides both an effective protection against host defense mechanisms (e.g. proteolytic enzymes, cytokines, or antibodies) and the means for a selective permeability and macromolecular exchanges. The parasitophorous vacuole membrane, which separates the intracellular parasite from the host cell cytoplasm, is involved in the complex molecular trafficking events required for macromolecular exchange between the parasite and its host.

Pre-erythrocytic stage

The sporozoite enters the bloodstream and is rapidly carried to the liver, where it invades a hepatocyte and undergoes drastic changes in morphology. It loses its apical complex and surface coat and transforms into a round or oval trophozoite. Development takes place

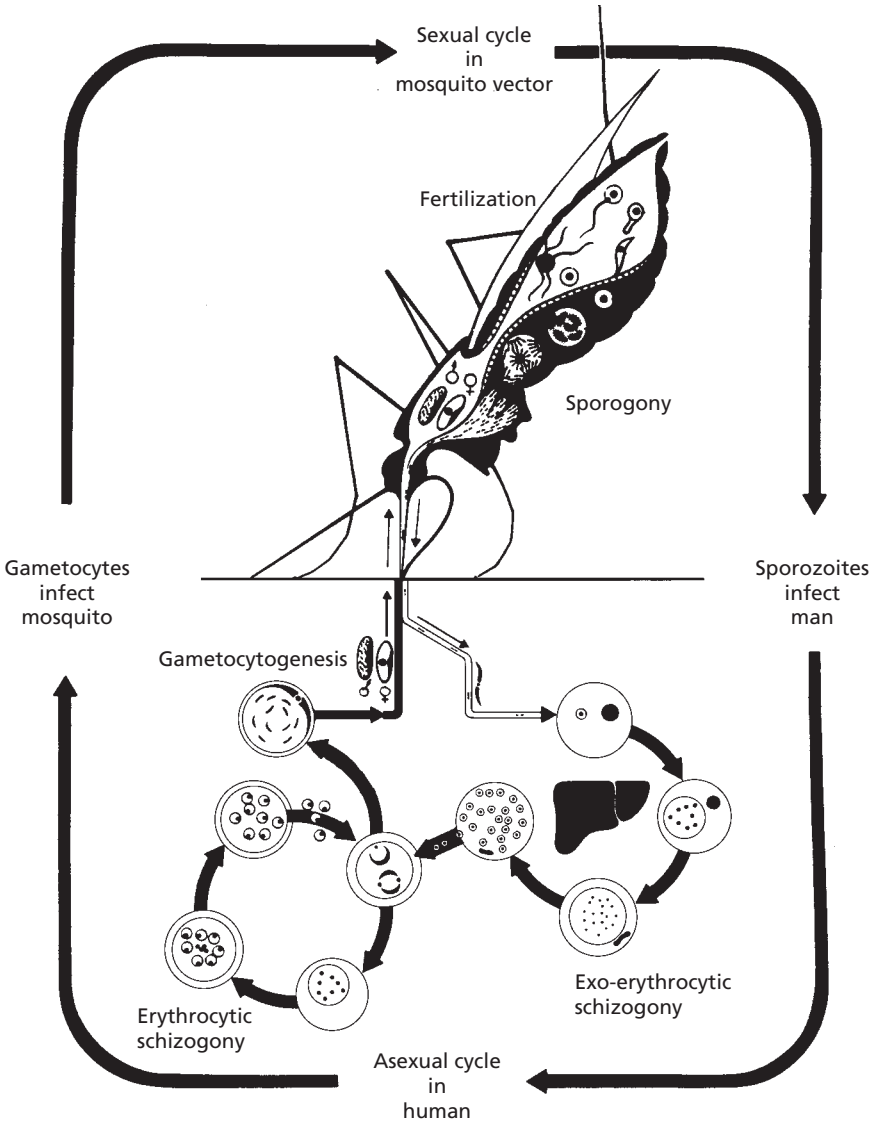

Figure 3 *Life cycle of* Plasmodium falciparum

within a parasitophorous vacuole in the hepatocyte. The nucleus divides many times and the cytoplasmic mass grows substantially; the number of nuclear divisions and the duration of schizogony varies considerably from one species to another, as does the ultimate size of the pre-erythrocytic schizont (which reaches a diameter of 60 μm in *P. falciparum*, and contains more than 30 000 nuclei) (Figure 4).

After completion of nuclear divisions, the cytoplasm segments and individual merozoites are formed. The duration of the pre-erythrocytic schizogony is characteristic for each species; for instance, minimum maturation times of 5.5 days in *P. falciparum* and 15 days in *P. malariae*. In mammalian plasmodia the merozoites arising from pre-erythrocytic schizogony can only invade red cells (i.e. they cannot invade hepatocytes or start a secondary exo-erythrocytic schizogony).

Erythrocytic stage

After invading the erythrocyte, the parasite loses its specific invasion organelles and de-differentiates into a round trophozoite located within a parasitophorous vacuole in the red cell cytoplasm. The young trophozoite (so-called 'ring' stage, because of its morphology on stained blood films) grows substantially before undergoing several nuclear divisions. The number of nuclear divisions and the duration of schizogony vary from one species to another. The timing of the different stages of the erythrocytic schizogony of *P. falciparum* is illustrated in Figure 5

The length of the intra-erythrocytic development is variable; 48 h for *P. falciparum*, *P. vivax*, and *P. ovale* and 72 h for *P. malariae*. At the end of schizogonic development, new merozoites are formed which have a

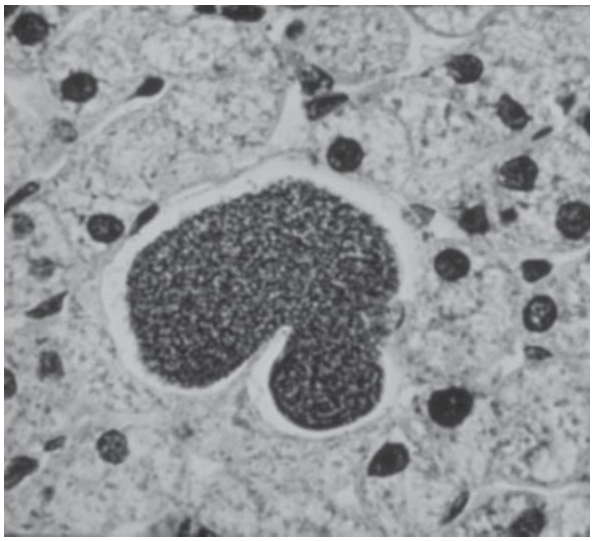

Figure 4 *Liver pre-erythrocytic schizont*

very short extracellular viability (probably less than 30 min) and can only invade erythrocytes.

The development of the parasite within the erythrocyte produces a series of alterations of the host cell, including the presence in the cytoplasm of the red cell of vacuoles or clefts and morphological changes of the erythrocyte membrane (e.g. 'knobs' or 'caveolae'). Some of these alterations are visible in stained parasites and represent useful taxonomic features (e.g. Maurer's clefts in *P. falciparum* or Schüffner's dots in *P. vivax*) (Figure 6).

The metabolism of the malaria parasite is largely dependent on the digestion of red cell hemoglobin, which is transformed into malaria pigment. Pigment is absent in the ring stage and only becomes detectable in the late trophozoite and the schizont. The aspect and distribution of the pigment in the parasite may vary from species to species, and represents another useful distinguishing feature.

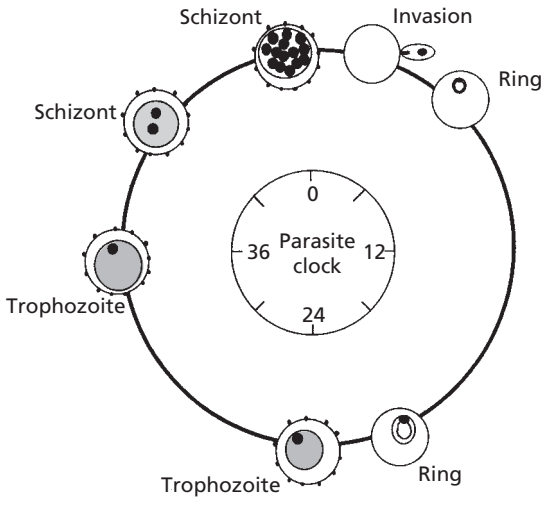

Figure 5 *Timing of the intra-erythrocytic cycle of* Plasmodium falciparum

Gametocytes and gametes

Some merozoites develop either into male or female gametocytes but the factors which induce sexual differentiation, rather than schizogonic development, are essentially unknown. All merozoites emerging from a sexually committed gametocyte transform into gametocytes of the same sex (Smith et al. 2000) and in most *Plasmodium* species the process of gametocytogenesis leads to a female-biased sex ratio. A variety of factors have been shown to stimulate gametocytogenesis, including hyperparasitemia, anemia, and antimalarial drug treatment and it looks as though the parasite is capable of sensing hostile conditions and, by transforming into gametocytes, prepares to escape into a new host. Chloroquine and sulfadoxine-pyrimethamine both stimulate gametocytogenesis, particularly when used in suboptimal doses (Buckling and Read 1999; Sokhna et al. 2001). This may have important implications in the spread of drug resistance, since resistant parasite populations selected by drug treatment have an increased chance of being transmitted (Robert et al. 2000). The effect of anemia on gametocytogenesis is paradoxical since anemia also induces a strongly male-biased sex ratio (Paul et al. 2002).

Mature gametocytes of *P. vivax*, *P. ovale*, and *P. malariae* are round, but the gametocytes of *P. falciparum* are elongated or crescent-shaped (Figure 7). Maturation of *P. falciparum* gametocytes also takes much long than that of other species; 10 days compared to 3 or 4. Although the longevity of mature gametocytes may exceed several weeks, their half-life in the bloodstream may only be 2 or 3 days, while waiting in an arrested state of development to be taken up by a mosquito.

When taken up by a suitable mosquito vector, the gametocytes transform into gametes stimulated by the drop in temperature and the biochemical features of the new environment (Carter and Graves 1988). The maturation of the female gametocyte into a macrogamete takes place without major morphological changes. In contrast, in the male gamete, the nucleus divides three times and each of the eight nuclei formed combines with cytoplasm to form eight thread-like microgametes, a process called 'exflagellation.'

Fertilization and sporogony

Microgametes are motile, highly differentiated stages, which move actively towards the macrogametes to start the process of fertilization and sexual stage of the parasite's life cycle. The haploid nuclei of male and female gametes merge forming the diploid form of the parasite ('zygote'). Meiosis and genetic recombination

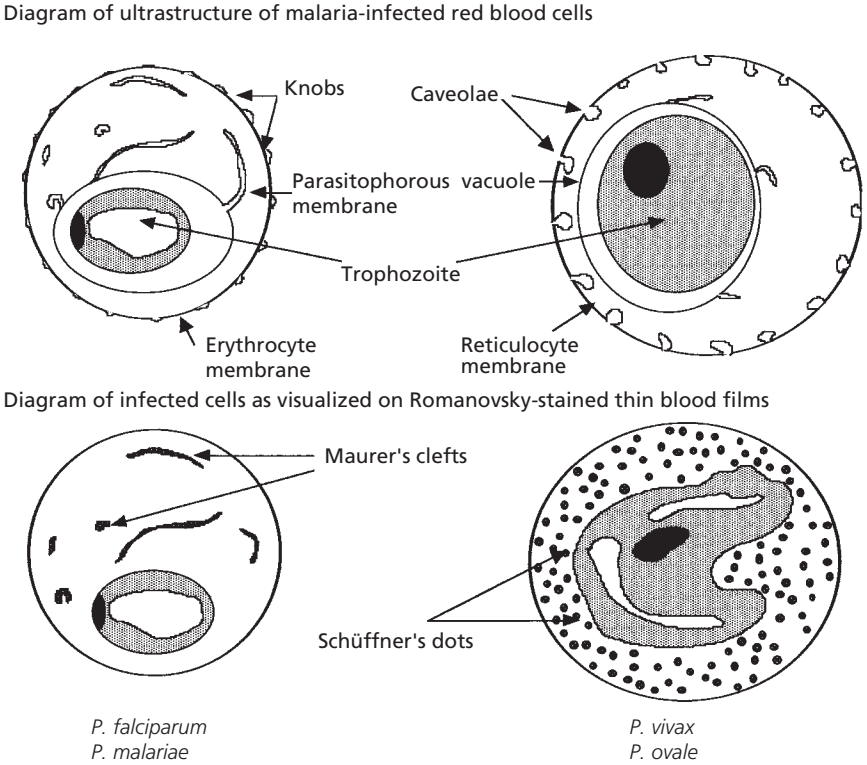

Figure 6 *Diagram describing the difference between Maurer's clefts and Schuffner's dots. The ultrastructure shows that the objects identified as Schüffner's dots in a Romanovsky-stained thin film are caveolae on the surface of infected reticulocytes, while Maurer's clefts correspond to expansions of the parasitophorous vacuole membrane and vesicles derived from this membrane.*

take place while the zygote transforms into a motile form, the ookinete, which crosses the midgut epithelium and settles below the basal lamina to form an oocyst; at this stage, the parasite has returned to a haploid stage. The oocyst grows to reach a diameter of 40–50 μm after 4–21 days and each infected mosquito may carry a few hundred oocysts (as many as 1 600 in some laboratory-infected mosquitoes) (Figure 8), but normally the number of oocysts per mosquito is <100. The size of the mature oocyst and the distribution of pigment are characteristic for each species and may be used as taxonomic markers.

Within the oocyst, the nucleus divides in a schizogonic mode, where multiple, synchronized nuclear divisions take place before individual daughter cells are formed. At the end of the maturation period, sporozoites are formed, the number varying from species to species (e.g. estimated between 1 000 and 20 000 in *P. falciparum*). The immature sporozoites emerge through the oocyst wall into the hemolymph, and migrate to the salivary glands of the mosquito. Infected salivary glands contain between 10 000 and 200 000 sporozoites.

Biology of transmission

The transmission of *Plasmodium* by mosquitoes is driven by the female mosquito's own biological need for regular blood-meals and successful transmission is regulated by a variety of intrinsic and environmental factors. A sequence of blood-feeding, egg maturation, and oviposition is repeated several times throughout the mosquito's life cycle and is referred to as the 'gonotrophic cycle,' the length of which depends on external temperature. In *Anopheles gambiae*, for example, the cycle takes 48 h when the average day–night temperature is 23°C. The average lifespan of the female has direct relevance to its efficiency as a vector: only females that live long enough for the sporogonic cycle to be completed (i.e. approximately 10 days) can successfully transmit *Plasmodium*. It also follows that any intervention to reduce the lifespan of the female (e.g. exposure to insecticides) will affect transmission efficiency. Infected humans are only infective to mosquitoes if they carry mature gametocytes; infection and infectivity generally coincide in *P. vivax*, *P. ovale*, and *P. malariae*, but not always in *P. falciparum* due to the much longer time required for gametocytes to reach maturity.

Within the mosquito, the maturation of the oocyst takes 4–21 days, depending largely on environmental temperature. The sporozoites that emerge from the oocyst are not yet infective and it is during their migration through the hemolymph towards the salivary glands that final differentiation takes place (including the expression of two key binding molecules on their

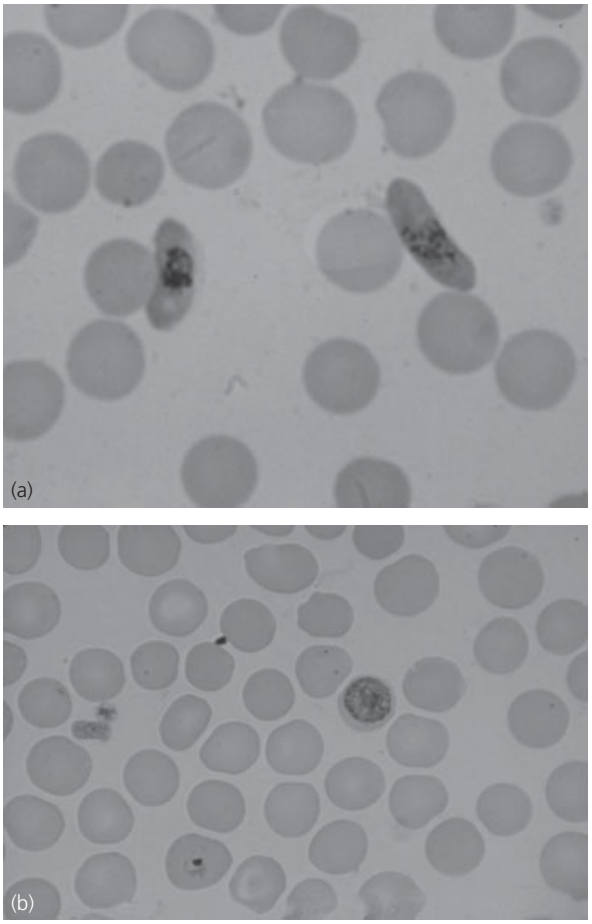

Figure 7 *Gametocytes of* Plasmodium falciparum **(a)** *and* P. vivax **(b)**.

surface, CSP and TRAP/SSP2). Less than 25 percent of the sporozoites produced successfully reach the glands, bind to the cells by means of CSP, then penetrate into the lumen of the glands, from where they are released into droplets of saliva with each subsequent blood-meal (Pimenta et al. 1994).

Only mosquitoes of the genus *Anopheles* support the sporogonic development of human malaria parasites. Of the 460 species of *Anopheles* described worldwide, fewer than 70 are competent vectors and there are substantial differences in vectorial competence, even between subpopulations of a given species. Vectorial competence measures the ability of a mosquito which has ingested mature gametocytes to sustain their development until they again become infectious: this must not be confused with vectorial capacity which measures the ability of a mosquito species to transmit the infection in the field and is a major parameter of malaria epidemiology.

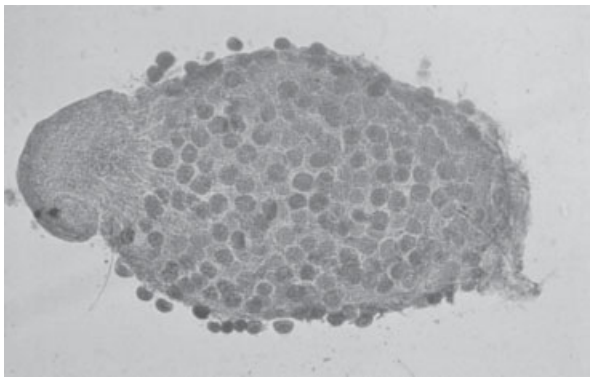

Figure 8 *Oocysts in mosquito*

Female *Anopheles* mosquitoes usually take their blood-meal between dusk and dawn and can feed on a variety of warm-blooded animals, including humans. Some species prefer to feed on humans and are termed anthropophilic, while others prefer to feed on animals, particularly cattle, and are termed zoophilic, but these preferences are by no means absolute. Obviously, species that prefer to feed on humans will be better vectors of human malaria parasites than zoophilic species. Females are attracted to their hosts by a variety of stimuli, including exhaled carbon dioxide and host odor (Braks et al. 1999).

Some species of *Anopheles* bite humans mainly outdoors ('exophagic'), for example *A. albimanus*, a major malaria vector in Central and South America, whereas mosquitoes of the *A. gambiae* complex, the world's most effective malaria vectors bite mostly indoors ('endophagic') and at night. After feeding, females either rest inside houses ('endophilic' species) or outdoors in a variety of natural shelters ('exophilic' species). Many species exhibit a mixture of these extreme behaviors. The time of biting, the biting behavior, and the resting behavior following the blood-meal are important determinants of malaria epidemiology and transmission; it would, for instance, serve no useful purpose to spray the inside of houses with insecticides if the local vectors are exophagic and exophilic.

Because of their nocturnal habits, *Anopheles* females usually feed while their human prey is sleeping, and can thus take a single, uninterrupted blood-meal, which usually lasts for 2–3 min. During the bite, blood is ingested either directly from a capillary or from a micro-hemorrhage resulting from the local damage caused by the tearing action of the toothed mandibles and maxillae. The quantity of blood ingested at a single feed varies from 1 to 2.5 µl representing a larger volume of blood, since *Anopheles* concentrate blood by diuresis (release of a few drops of clear liquid from the anus during feeding).

At the time of mosquito bite, each drop of saliva carries with it a small number of sporozoites which are injected into the skin. An infective bite from a single mosquito will characteristically represent between five and a few hundred sporozoites (Rosenberg et al. 1990), sufficient to induce an infection. Interventions that affect the vector-to-human contact are likely to reduce malaria

transmission, this includes the use of repellents (such as diethyltoluamide (DEET)), special clothing, awareness of risk, use of bednets, or better, insecticide-impregnated bednets, design of housing, and position of housing relative to breeding sites.

Recrudescences and relapses

Some sporozoites develop immediately after entering the hepatocyte and a few days later, when the pre-erythrocytic schizont is mature, produce merozoites that infect red blood cells. This first wave of parasitemia is responsible for the primary attack of malaria and may, in the absence of specific antimalarial treatment, last from a few weeks to a few months in a nonimmune individual (2–3 weeks in some *P. falciparum* infections to 4–6 months in *P. malariae* infections). Further waves of parasitemia may follow a few weeks, months, or years after the primary attack. In the absence of reinfection, there are two possible causes for such secondary attacks:

1. The presence of dormant parasites or 'hypnozoites.' This is a situation in which some of the sporozoites do not immediately start to grow and divide, but remain in a dormant stage for weeks or months (Krotoski et al. 1980). The duration of latency is variable from one hypnozoite to another and the factors that eventually trigger growth are not known; this explains how a single infection can be responsible for a series of waves of parasitemia or 'relapses.' *P. vivax* and *P. ovale* are the only two human malaria parasites which produce hypnozoites and, thus, relapses. Different strains of parasites have their own characteristic pattern of relapse. Lengthy prepatencies or delayed relapses appear to be a feature of parasites endemic in temperate rather than tropical areas (Brumpt 1949).
2. Parasites which do not form hypnozoites (*P. malariae* and *P. falciparum*) may, nevertheless, produce secondary waves of parasitemia called 'recrudescences,' either by long-term survival of erythrocytic stages (e.g. within a 'sequestration' site) or the continuation, at a low or undetectable level, of erythrocytic schizogony in the peripheral bloodstream. The basis for this latent survival of parasites has not, so far, been elucidated. In the case of *P. malariae*, recrudescences have been described as long as 60 years after the primary attack (Brumpt 1949); note that a recrudescence occurring more than 24 weeks after the original attack is sometimes called a 'recurrence.' Drug resistance creates a situation in which the initial peak of parasitemia is only partially controlled and a recrudescence of resistant parasites occurs shortly thereafter. Figure 9 illustrates the differences between reinfection, recrudescence, and relapse.

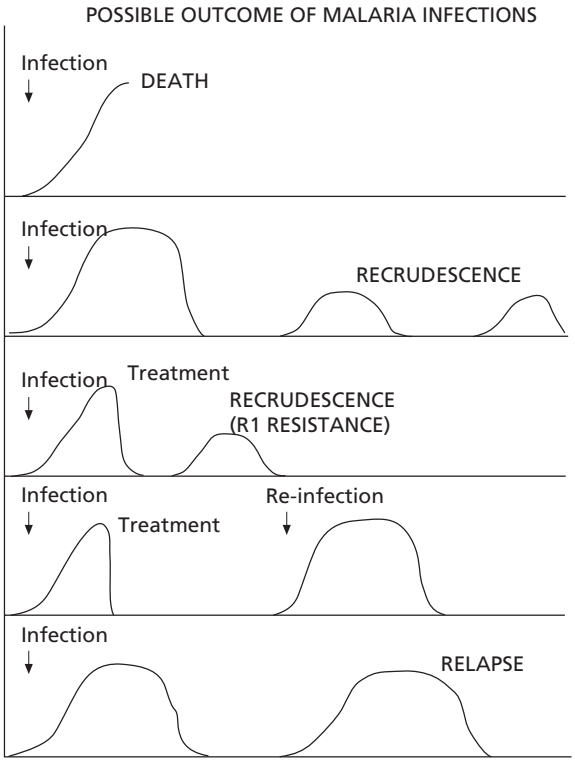

Figure 9 *Relapse, recrudescence, reinfection, and drug resistance*

The main practical difference between relapse and recrudescence is that, whereas latent erythrocytic stages are susceptible to standard chemotherapy, hypnozoites (being cells of low metabolic activity) require a specific treatment (e.g. with primaquine). A true relapse is, therefore, always exo-erythrocytic in nature and may be defined as the 'reappearance of parasitemia in a sporozoite-induced infection following adequate blood schizonticidal therapy' (Cogswell 1992). This also means that a blood-induced infection (e.g. by blood transfusion) cannot relapse.

Synchronicity

One of the striking features of the erythrocytic cycle of mammalian malaria parasites is the fact that the parasites tend to grow in synchrony. The consequence is that parasites examined on a patient's blood film are frequently all at the same developmental stage and that clinical symptoms (e.g. fever paroxysms) often tend to occur at regular intervals of time (e.g. 48 h in tertian malaria or 72 h in quartan malaria). This synchronous life cycle of the parasite is determined, at least in part, by host factors. In *P. falciparum*, a parasite in which synchronicity is particularly striking, it has been suggested that it is the paroxysms of fever themselves which sharpen the level of synchronicity (Kwiatkowski and Greenwood 1989), in consequence of which the

periodicity of fever becomes more regular as the malaria infection progresses. The host's circadian rhythm is also a determining factor in controlling the timing of parasite development. This very accurate 'biological clock' of the parasite is believed to represent an adaptation designed to facilitate the transmission of the parasite by night-biting mosquitoes (Hawking 1975).

Sequestration

The overall distribution of both asexual forms and gametocytes in the blood is not always random and there are circumstances in which parasites may be removed from the peripheral bloodstream and be retained or 'sequestered' in various host tissues.

In *P. falciparum*, only the early trophozoites ('rings') are present in the peripheral circulation, while later developmental stages are sequestered within the capillaries of various organs. This sequestration is caused by the adherence of infected erythrocytes to capillary endothelial cells by means of a specific interaction between a parasite-derived molecule present at the surface of the malaria-infected red cell and specific host cell receptors (Hommel 1997). Adherent malaria-infected red cells can be found in various tissues, including the heart, the liver, and the brain (Figure 10).

Sequestration has important consequences for the diagnosis of falciparum malaria, because it means that parasites may not be found on a blood film at a time when the clinical picture is most suggestive. During pregnancy, infected erythrocytes are preferentially retained in the placenta. This is believed to be of a mechanical nature, due to the sluggish blood flow of the placenta rather than any interaction between infected erythrocytes and specific receptors on the surface of syncytiotrophoblasts (Galbraith et al. 1980).

A radically different form of sequestration is responsible for the removal of immature gametocytes of *P. falciparum* from the peripheral circulation. As early as the first 24 h of gametocytogenesis, the immature gametocytes are retained within the bone marrow and the spleen (Smalley et al. 1980), and are released into the peripheral circulation only after reaching maturity, 8–10 days later. In contrast to the sequestration of immature gametocytes, which is unique to *P. falciparum*, mature gametocytes of all species may be submitted to intermittent sequestration, controlled by the diurnal rhythm of the host. It is conceivable that this sequestration may take place in the capillaries of the upper layer of the skin, more accessible to the feeding mosquito. This would explain why the number of gametocytes in the blood-meal may be far greater than that present in the peripheral circulation of the host (Carter and Graves 1988).

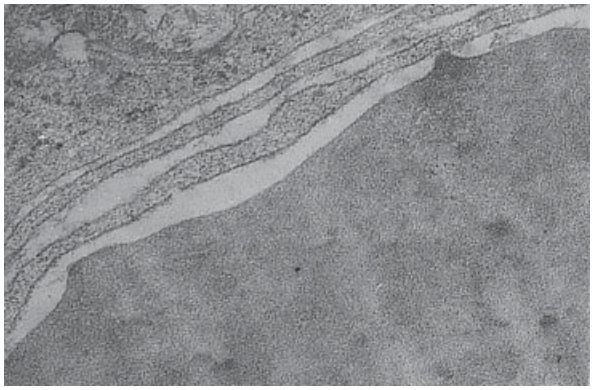

Figure 10 *Cytoadherence of* Plasmodium falciparum

Interaction of parasite and host cell

INVASION OF THE ERYTHROCYTE

In order to invade the erythrocyte, the merozoite has to recognize and attach to its surface components. The nature of the erythrocyte receptor involved varies from one merozoite to another, because each parasite species has specific host cell preferences (Hadley and Miller 1988; Bannister and Dluzewski 1990). In *P. falciparum*, a parasite which can invade erythrocytes of all ages, the receptor varies from one group of isolates to another (Perkins and Holt 1988) and optional receptors include sialic acid (Facer 1983), glycophorins (Pasvol et al. 1982), and band 3 (Okoye and Bennett 1985). In *P. vivax*, a parasite which is restricted to immature red blood cells (reticulocytes), attachment to two receptors is required: one is reticulocyte-specific, the other has been shown to be associated with a glycoprotein serologically defined by the Duffy (FyFy) blood group determinant (Wertheimer and Barnwell 1989). The red cell receptor preferences of *P. ovale* and *P. malariae* are presumed to be different from those of *P. falciparum* and *P. vivax*. *P. ovale* can infect Duffy-negative red cells, which is compatible with the fact that the species is found in West Africa (where the Duffy-negative phenotype is highly prevalent) while *P. malariae* is said to have a predilection for senescent red cells.

INVASION OF THE HEPATOCYTE

After a sporozoite has entered the bloodstream, it rapidly 'homes in' towards liver sinusoids and establishes itself within a hepatocyte; this is supported by the finding that injected CSP is selectively targeted to the liver (Cerami et al. 1994). The membrane of the sporozoite is entirely covered by two molecules (CSP and TRAP/SSP2, see Table 3), which are responsible for the initial attachment to the hepatocyte surface. Heparan sulfate protein glycoconjugates (HSPG) and low-density lipoprotein receptor-related (LPR) protein have both been incriminated as the host receptors for sporozoite invasion (Frevert et al. 1996). Whether sporozoites enter the hepatocytes directly (e.g. into the space

of Disse through gaps in the endothelial lining) or whether they first cross a Kupffer cell, has been a controversial issue for years (Verhave and Meis 1984). Recent findings suggest that sporozoites enter Kupffer cells, manage to survive the passage through these phagocytic cells by blocking phago-lysosomal fusion, then exit from the Kupffer cell and enter a hepatocyte; the binding ligands of the surface molecules, TRAP and CSP, have such a broad specificity that recognition of different cell types is possible (Barnwell 2001; Pradel et al. 2002). Furthermore, it appears that the passage through Kupffer cells is an essential step in the sporozoite maturation, without which the apical end organelles cannot exocytose and enable invasion with the formation of a parasitophorous vacuole. It has been suggested that the sporozoite may, in some circumstances, have to cross more than one cell before this maturation is achieved (Mota et al. 2002). Once inside the hepatocyte, the sporozoite de-differentiates, losing its apical organelles, and starts to grow.

ALTERATIONS OF HOST CELLS

After infection by the malaria parasite, the host cell undergoes a variety of structural changes, which may substantially alter its function, appearance or antigenicity. The nature of the alterations induced varies from one species to another. These host cell alterations have been studied most comprehensively in the erythrocytic stage of the parasite (Hommel and Semoff 1988; Sherman et al. 2003), but it is conceivable that they may also exist in the other intracellular stages.

The alterations identified so far in the membrane of malaria-infected erythrocytes include:

- a visible change of shape and reduced deformability;
- the presence of electron-dense protrusions or 'knobs' (in *P. falciparum* and *P. malariae*) (Figure 11);
- the presence of small depressions, or 'caveolae,' at the surface of the erythrocyte, connected by a network of small vesicles and clefts (in *P. vivax* or *P. ovale*) (Aikawa 1988);

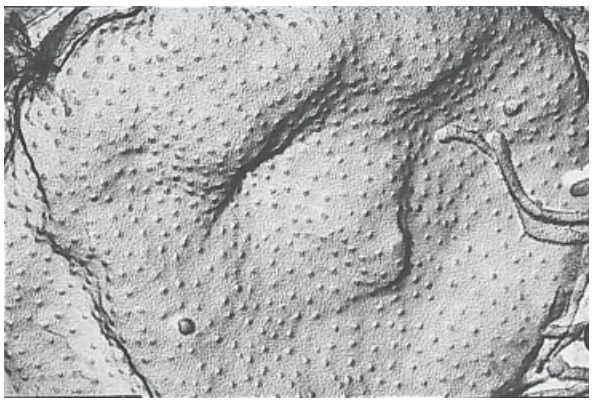

Figure 11 *Knobs on* Plasmodium falciparum*-infected erythrocytes*

- the expression of new sugar moieties, particularly galactose (David et al. 1981);
- the cytoadherence to endothelial cells or rosetting with normal erythrocytes (Wahlgren et al. 1987);
- the presence of new metabolic channels (Ginsburg et al. 1985);
- the evidence of new parasite-specific antigens associated with the red cell membrane (Hommel et al. 1983); and
- the reorganization of normal erythrocyte components (e.g. disruption of spectrin or conformational changes of band 3) (Yuthavong et al. 1979; Winograd and Sherman 1989).

Characteristics of human malaria parasites

The main features of human malaria parasites, as observed on Romanovsky-stained thin blood films, are shown in Figure 12.

PLASMODIUM FALCIPARUM

Of all the human malaria parasites, *P. falciparum* is the most highly pathogenic and responsible for 'malignant tertian malaria,' a form of the disease which runs an acute course in nonimmune patients and is frequently fatal if untreated. This parasite represents the major cause of malaria in tropical Africa and is also responsible for the great regional epidemics that sometimes occur in north-west India or Sri Lanka; it is generally confined to tropical or subtropical areas because its development in the mosquito is greatly retarded when the temperature falls below 20°C. The most characteristic biological features of *P. falciparum* are as follows:

- it infects mature and young erythrocytes;
- the surface of erythrocytes infected with late-stage trophozoites or schizonts is altered so they stick to endothelial cells in various tissues (this cytoadherence is responsible for the sequestration of the parasite);
- the pre-erythrocytic cycle starts immediately after injection of sporozoites by the mosquito, without the production of hypnozoites (i.e. there are no relapses);
- schizogony is particularly prolific in all stages (pre-erythrocytic schizogony produces up to 30 000 merozoites; erythrocytic schizogony produces 16–18 merozoites and sporogony produces up to 20 000 sporozoites) which may be the cause of its success as a species and its virulence;
- infection in the peripheral blood is characterized by the predominant presence of ring forms and gametocytes, whereas late trophozoites and schizonts are only seen exceptionally;
- the level of parasitemia may be high and multiple infection in a single erythrocyte is common; and
- the gametocytes are characteristically crescent-shaped and, unlike the gametocytes of other species, are very

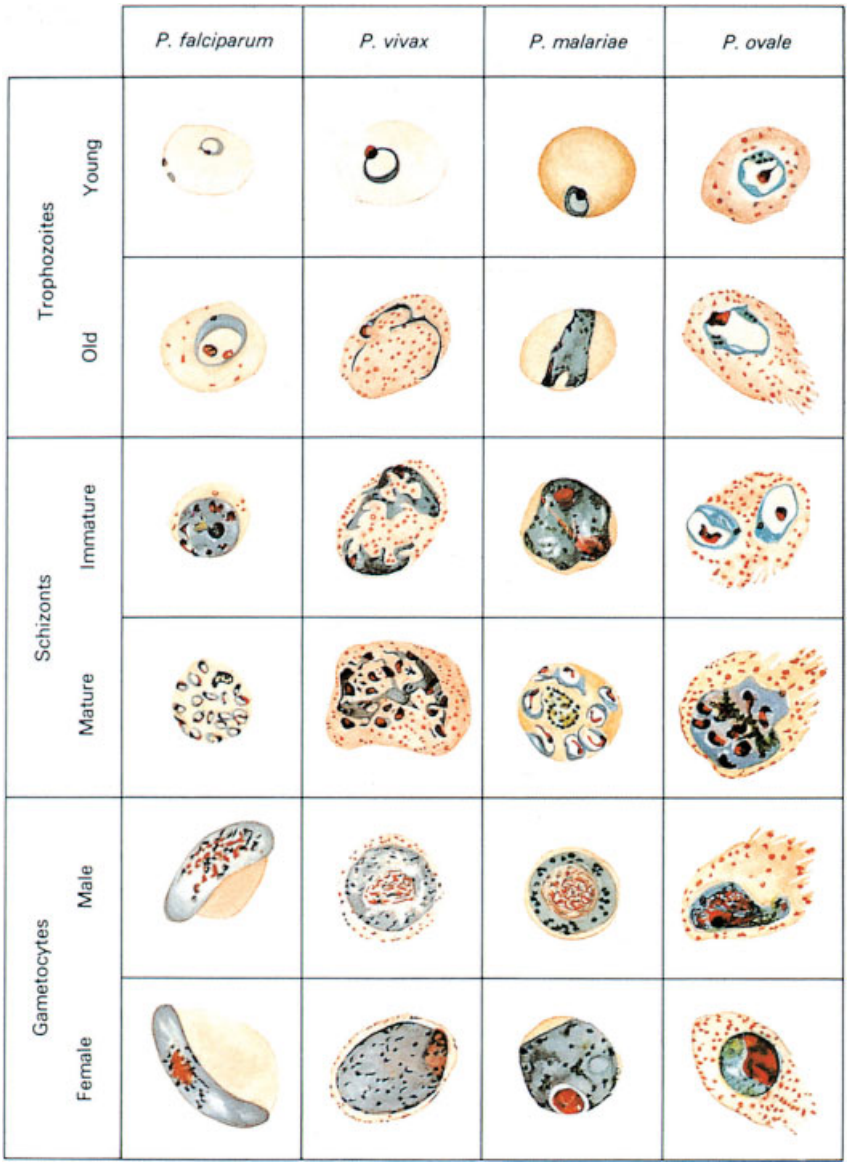

Figure 12 *Morphological characteristics of human malaria parasites*

slow to reach maturity (up to 10 days) and early forms of gametocytes are sequestered.

PLASMODIUM VIVAX

Plasmodium vivax occurs throughout most of the temperate zones as well as large areas of the tropics (but is mainly absent from tropical West Africa). It causes 'benign' tertian malaria, the pattern of which varies with different parasite strains. As a species, *P. vivax* is particularly polymorphic and the subspecies status proposed for some strains may be justified either on morphological grounds (e.g. *Plasmodium vivax multinucleatum* found in China, appears to have a characteristic oocyst), on biological grounds (e.g. *Plasmodium vivax hibernans* found in Russia, whose sporozoites produce only hypnozoites), or molecular grounds (e.g. a series of *P. vivax* isolates with substantial differences in their genetic make-up have been identified, including a *P. vivax*-like parasite, found in humans in Papua New Guinea, Brazil, and Madagascar, indistinguishable from the simian parasite *Plasmodium simiovale*) (Qari et al. 1993). The distinctive biological features are: (1) a restriction of erythrocyte invasion to reticulocytes bearing Duffy blood group determinants, a feature which explains why red blood cells infected with trophozoites of *P. vivax* are sometimes described as 'larger' than normal; (2) the presence of caveolar structures on the surface of the infected erythrocyte membrane, which communicate with underlying cytoplasmic vesicles; these structures readily take up the Romanovsky stain and, to the microscopist, look like a reddish cytoplasmic stippling or 'Schüffner's dots;' and (3) after invading the hepatocyte some, or all, of the sporozoites may transform into

hypnozoites, then remain latent for months or years and be responsible for subsequent relapses.

PLASMODIUM OVALE

Plasmodium ovale is a species truly distinct from *P. vivax*, not only on minor morphological differences (e.g. oval shape of infected red cell with ragged or fimbriated margin, smaller number of merozoites in schizont, aspect of gametocytes), but also presenting antigenic and molecular differences. Nevertheless, most of the biological and clinical features are identical. The major biological difference is that *P. ovale* can infect Duffy-negative reticulocytes, whereas *P. vivax* cannot.

PLASMODIUM MALARIAE

Plasmodium malariae differs from the other three human malaria parasites in its slow development and its longer asexual cycle. Development is slow in both the vector and the human host, because of less efficient schizogony (pre-erythrocytic schizonts have 6 000 merozoites, erythrocytic schizonts have 6–12 merozoites). The asexual cycle is 72 h instead of 48 h, and this feature has given the clinical form of *P. malariae* its name, quartan malaria, because fever paroxysms occur every fourth day according to the Roman custom of regarding day 0 as day 1. The biological characteristics of the parasite are:

- an apparent preference for old erythrocytes, explaining why infected cells are often described as 'smaller' by microscopists (this feature is poorly documented in view of the absence of an in vitro culture system);
- the presence of 'knobs' at the surface of infected erythrocytes which are similar to *P. falciparum*, but the cells do not exhibit any cytoadherence (and therefore no sequestration);
- the surface of infected cells does not exhibit any caveolar/vesicle complexes and, consequently, Schüffner's dots are absent; and
- sporozoites of *P. malariae* do not transform into hypnozoites and, hence, there are no relapses. However, *P. malariae* can survive for a very long time in the peripheral blood (10 years or more) at a very low level of parasitemia, occasionally producing detectable peaks with a recrudescence of clinical symptoms.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Metabolic pathways

Much of what is known about the metabolism of malaria parasites has in the past been derived from studies of blood stages, either isolated from their vertebrate host or produced in culture. However, the availability of the complete genome sequence of *P. falciparum* (Gardner et al. 2002) has generated valuable information regarding malarial enzymes, which classical biochemical methods had failed to achieve. As a result, many metabolic pathways can now be reconstructed in their entirety using similarity-searching methods. Since many of the enzymes are different from the host enzymes, this can potentially lead to the finding of novel antimalarial compounds, probably the most important achievement of parasite genome sequencing.

ACCESS TO NUTRIENTS

The passage of nutrients from the extracellular space to the parasite involves a transfer across the host cell membrane, the parasitophorous membrane, and the parasite's own membrane. Transfer across membranes may be achieved by a variety of mechanisms including diffusion along a gradient of substrate and carrier-mediated transport. Because of rapid parasite growth and replication, the normal transport mechanisms of the red cell are not sufficient to cope with the increased demand (e.g. an infected red cell utilizes almost 100 times more glucose than a normal red cell) (Roth et al. 1982). Increased traffic across the membrane is achieved not only by an increase in the transmembrane gradient of substrate (created by increased consumption of nutrients by the growing parasite) but also by new permeation pathways introduced by the parasite into the host cells (Sherman 1988; Ginsburg and Kirk 1998). The need for such new pathways is probably greater for parasites restricted to mature erythrocytes, since these cells are relatively impermeable to many of the nutrients needed for parasite growth (e.g. L-glutamine) (Elford 1986) and the presence of such pathways may represent a target for new antimalarials (e.g. the derivatives of the Chinese antimalarial qinhaosu are potent inhibitors of the L-glutamine influx).

Most nutrients required by the parasite originate from the extracellular space, e.g. the host plasma. These include glucose, purines and pyrimidines, amino acids, anions, cations, iron, zinc, vitamins, fatty acids, and phospholipids. Some of these nutrients may also be obtained from the host cytoplasm either by transport across the membranes or by uptake through the cytostome, e.g. the uptake of hemoglobin and its breakdown to form malarial pigment. The uptake of antimalarial drugs follows the same pathways as the transport of nutrients and, to some extent, these pathways may also function in the opposite direction for the disposal of waste products such as lactate. Genome analysis has revealed that *P. falciparum* possesses only a very limited repertoire of membrane transporters compared to other eukaryotes, which probably reflects its obligate intracellular parasite status (Gardner et al. 2002).

Secretion pathways in higher eukaryotes normally include a translocation across the endoplasmic reticulum followed by the transfer, by means of transport vesicles, first to the stacked Golgi cisternae then to the trans-

Golgi network. In *Plasmodium*, the understanding of the fate of proteins synthesized by the parasite and destined for secretion raises interesting questions, since it appears to lack 'classical' Golgi stacks. A comparison between two secreted proteins, the glycophorin-binding protein (GBP) and the serine-rich protein (SERP), proved the existence of secretory pathways and showed that different molecules may behave in different ways. SERP is released into the space between the parasite and the parasitophorous vacuole membrane (PVM), while GBP goes through a two step process, being first released into the vacuolar space then actively translocated through the PVM into the erythrocyte cytosol (Lingelbach 1997).

CARBOHYDRATE METABOLISM

The intra-erythrocytic malaria parasites lack reserves of glycogen and of other polysaccharides and are, consequently, dependent on glucose as their primary source of energy. Almost all the glucose utilized by the parasite is used by anaerobic glycolysis, a relatively inefficient process since oxidization stops at the lactate stage; as a result, this generates a large amount of lactate, which reduces the internal pH and has to be efficiently disposed of. All the malarial enzymes of the glycolytic pathway have been identified and found to be isoenzymes different from host enzymes (Roth 1990). Although the hexose monophosphate shunt pathway is operative and represents an essential source of nicotinamide adenine dinucleotide phosphate (reduced) oxidase (NADPH), the activity of the parasite-encoded glucose-6-phosphate dehydrogenase (G6PD) is only about 5 percent of that of the host cell enzyme (Ling and Wilson 1988). This suggests that the host enzyme normally initiates the pathway and that the parasite enzyme may be up-regulated only when the parasite is in a G6PD-deficient red cell; this would explain why the in vitro growth of *P. falciparum* in G6PD-deficient cells is initially impaired but may be overcome after a few cycles of growth.

There was, until recently, considerable doubt concerning the presence or absence of a functional tricarboxylic acid (TCA) cycle in malaria parasites, since the only TCA cycle enzyme identified with any certainty in blood stage plasmodia was malate dehydrogenase (Sherman 1979). Although all the enzymes of the TCA cycle have been identified in the genome of *P. falciparum*, some doubts remain regarding their functionality. It has been suggested that, rather than using the TCA cycle for glycolysis, the parasite may use it to synthesize succinylCoA, which can in turn be used in the heme biosynthesis pathway. The localization of some enzymes of the TCA pathway in the mitochondrion and the apicoplast, rather than the cytoplasm, support this hypothesis.

All malaria parasites appear capable of fixing CO_2, with the production of α-ketoglutarate and oxaloacetate as first intermediates. It has been suggested that a branched pathway from the oxaloacetate thus produced may be preferred by malaria parasites to the Krebs cycle for the production of aspartate, glutamate, malate, and citrate (Scheibel 1988). Like bacteria and plants, the malaria parasite has to replenish its oxaloacetate pool from phosphoenolpyruvate.

PROTEIN AND POLYAMINE SYNTHESIS

The molecular mechanisms involved in plasmodial protein synthesis are typically eukaryotic. The parasite obtains most of the amino acids it needs either from the digestion of red cell proteins (particularly hemoglobin) or by salvage from the free amino acid pool in the plasma; some amino acids, e.g. glutamate, aspartate, alanine, and leucine, are biosynthesized by the parasite itself from glucose and CO_2. The genome sequence has confirmed that few of the enzymes required for amino acid synthesis are present in the parasite genome, but has identified the enzymes required for the interconversion of glycine-serine, proline-ornithine, cysteine-alanine, and glutamine-glutamate (Gardner et al. 2002). The minimal amino acid requirements of the parasite are still not known.

Hemoglobin is broken down within the parasite food vacuoles, into heme (the major component of hemozoin, the malarial pigment) and globin, which is further hydrolyzed to free amino acids by a series of parasite proteases (including serine, aspartic, and cysteine proteases) (Schrével et al. 1990; Rosenthal and Meshnick 1998); up to 75 percent of the hemoglobin of an infected cell may be degraded by the parasite.

Three steps are critical in the biosynthesis of polyamines: the decarboxylation of ornithine to putrescine via ornithine decarboxylase (ODC), the formation of S-adenosylmethionine (AdoMet) from L-methionine and adenosine triphosphate (ATP), and the decarboxylation of AdoMet which provides the aminopropyl groups required for the synthesis of spermidine and spermine (Assaraf et al. 1984). All three steps represent potential targets for chemotherapy.

NUCLEIC ACID METABOLISM

The amount of DNA in a malaria parasite is approximately 10^{-13} g and there is about two to five times more RNA than DNA (Gutteridge and Trigg 1970). DNA synthesis occurs mostly in the late trophozoite to the early part of schizogony (29–44 h in the *P. falciparum* cycle). The nuclear DNA base composition of malaria parasites is characteristically adenine and thymidine (A+T) rich. Because of its rapid schizogonic development (e.g. a merozoite may generate 8–20 new merozoites in 48 h), the parasite needs to possess an effective nucleic acid synthesis, and this implies access to nucleic acid precursors. The mature human erythrocyte has no requirements for pyrimidine and, lacking the

capacity for purine synthesis de novo, relies on the salvage of preformed purines (mostly for its ATP synthesis). Nucleosides are transported into erythrocytes by means of a specific transport protein in the membrane, and adenosine, hypoxanthine, and guanine can thus be salvaged from the plasma. In infected erythrocytes, the influx of purines into the red cell is increased and there is evidence to suggest that the erythrocyte's ATP is broken down to adenosine monophosphate (AMP) and hypoxanthine; although hypoxanthine appears to be the major purine incorporated into the parasite, the parasite has a range of purine salvage pathway enzymes (Reyes et al. 1982).

Malaria parasites must synthesize pyrimidines de novo, because the erythrocyte cannot supply them; the small amount of uridine and thymidine taken up by the erythrocyte does not appear to be incorporated by the parasite. The parasite has all the enzymes required for the synthesis of uridine monophosphate (UMP) from glutamine, ATP, and CO_2 (Gero and O'Sullivan 1990); some of these enzymes have been found to be functionally different from the corresponding host enzymes.

The synthesis of pyrimidines is intimately linked to folate metabolism. All the enzymes of the folate cycle have been studied in malaria parasites, with particular emphasis on dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), which are the targets of two different classes of antifolates used as antimalarials. It seems that the parasite relies more on the de novo folate pathway than folate salvage, which explains the synergy of the antimalarial activity of sulfa drugs (which inhibit de novo synthesis) and pyrimethamine (which inhibits the parasite DHFR, an enzyme which is considerably more sensitive to the drug than the host enzyme). The synthesis of pyrimidines also involves the vitamin *para*-amino benzoic acid (*p*ABA) and several authors have shown that the growth of plasmodia in experimental hosts is affected by the absence of *p*ABA in the diet (Ferone 1977).

LIPID METABOLISM

The growth of the parasite within the host erythrocyte requires a substantial increase of its total membranes; lipids generally constitute about 50 percent of the membrane mass. The lipid content of infected erythrocytes is higher than in normal ones (Homewood and Neame 1980) and the relative distribution of various lipids reflects the separate contributions that are made to erythrocyte membrane, parasitophorous vacuole membrane, external parasite membrane, and parasite organelles membranes.

During the erythrocytic cycle of the parasite, there is a 500–700 percent increase in phospholipid levels and the four major types of phospholipids are found in infected cells (Vial et al. 1990). Although infected cells have the capacity to incorporate some intact phospholipids from the plasma (probably by means of a specific phospholipid transfer molecule), it is thought that de novo synthesis and conversion from one phospholipid species to another represent the two major sources of new phospholipids. Malaria parasites appear to be incapable of producing cholesterol de novo from acetate or mevalonic acid. While malaria parasites do not synthesize their own sterols, they make use of the enzymes of the mevalonate pathway for the synthesis of nonsteroid isoprenoids, which in turn are involved in many different cell functions. The mevalonate pathway has been recognized as an important new target for chemotherapy (Grellier et al. 1994).

As there appears to be no fatty acid synthesis in infected cells, nor the capacity for retailoring available molecules (by elongation or desaturation processes) (Holz 1977), the host plasma must represent the main source of fatty acids. Infected cells incorporate considerable amounts of palmitic, stearic, oleic, arachidonic, and linoleic acid. Parasites have a high acyl-CoA synthetase activity (20 times more active than in normal erythrocytes), which produces fatty acyl-CoA, the usual fatty acid donor molecule in biosynthesis pathways. Choline enters the infected cell by means of a transport-mediated process, similar to that of normal erythrocytes.

ION METABOLISM

The uptake of extracellular calcium (Ca^{2+}) is essential for the growth of malaria parasites. The in vitro growth of *P. falciparum* trophozoites and merozoite invasion is inhibited after depletion of Ca^{2+} by ethyleneglycol-bis(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). This is consistent with the finding of calmodulin, one of the Ca^{2+}-binding proteins, both in the parasite cytoplasm and in apical organelles (Scheibel et al. 1987). The Ca^{2+} content of infected cells increases as the parasite matures due to an increased permeability of infected cells to external Ca^{2+} (Tanabe 1990). Since malaria-infected cells actively incorporate extracellular Ca^{2+}, it is not surprising that blockers of Ca^{2+} channels (e.g. verapamil) or antagonists of calmodulin (e.g. diltiazem or calmidazolium) may arrest parasite development.

Malaria parasites, like most eukaryotic cells, are capable of maintaining a high level of K^+ and a low level of Na^+ in their cytoplasm by means of a Na-, K^+-ATPase, and at the expense of the host cell ionic environment. It is interesting that the ATPase has been identified in the parasitophorous vacuole membrane (not in the parasite membrane) and this implies that the parasite is living in a low Na^+, high K^+ extracellular environment. This unusual feature is consistent with the observation that it is necessary to use a low Na^+, high K^+ medium to grow trophozoites extracellularly in vitro (Trager and Williams 1992).

The requirement for iron in a number of metabolic pathways, including the synthesis of DNA, explains why the parasite may be inhibited by relatively low amounts of the iron chelator, desferrioxamine (Raventos-Suarez et al. 1982). Although hemoglobin is degraded within its food vacuoles, most of the heme released is transformed into crystalline hemozoin and does not represent a usable source of free iron. Older studies suggested that extracellular iron may be taken up by the binding of ferrotransferrin (from serum transferrin) to a parasite transferrin receptor in the infected erythrocyte membrane but a transferrin-independent mechanism is now thought to be more likely (Sanchez-Lopez and Haldar 1992). The role of iron in the viability of malaria parasites is a matter of controversy, since iron deficiency protects mice against *P. chabaudi*, while people with iron deficiency are still susceptible to malaria, but may have an increased parasitemia if given iron supplementation (Murray et al. 1978a). While the excess of heme produced in the food vacuole by hemoglobin degradation is toxic if not transformed into hemozoin, there is evidence that the parasite has its own de novo pathway for the synthesis of the heme required for the production of its iron-containing proteins in the mitochondrion and the apicoplast (Bonday et al. 2000).

OXYGEN UPTAKE AND REDOX STATUS

Low oxygen levels have a beneficial effect on the in vitro growth of *P. falciparum* and increased oxygen uptake is observed in infected erythrocytes in the presence of glucose, suggesting that malaria parasites are essentially microaerophilic (Scheibel et al. 1979). For many years, there was little tangible evidence of the existence of respiration or the presence of an electron transport chain in the parasite. The isolation of purified mitochondria from *P. falciparum* has allowed an unambiguous measurement of the cytochrome content of these organelles and confirmed the existence of a classical respiratory chain including the presence of cytochromes (Fry 1991). Ubiquinone cycling plays a crucial role in mitochondrial physiology and its selective inhibition by hydroxyquinones has provided a target for one of the most potent antimalarials discovered in recent years (Atovaquone®) (Fry and Pudney 1992).

The status of reduction-oxidation ('redox') reactions of the infected red cell is complex since it involves a combination of the oxidative stress exerted by the parasite on the host cell, the ability of the host cell to mount anti-oxidant defenses to avoid oxidative damage, oxidative stress exerted by the host on the parasite (which may include the effect of immune responses and antimalarial drugs) and, finally, the ability of the parasite to mount its own anti-oxidant defenses (Hunt and Stocker 1990). The generation of reactive oxygen intermediates (ROI) is the major factor of oxidative stress both in the parasite and in the host cell. Parasites have been shown to be susceptible to various ROIs and hydrogen peroxide, t-butyl-hydroperoxide, xanthine-xanthine oxidase, and alloxan all have antiparasitic activity (Clark et al. 1989b). A number of antimalarial drugs (e.g. primaquine and qinghaosu) also seem to act by means of oxidation. The reduced growth of parasites in erythrocytes with certain genetic abnormalities (sickle-cell disease, α- and β-thalassemia, persistence of hemoglobin E) may be explained either by a reduced ability to mount anti-oxidant defenses or by an increased level of oxidative stress. Among the anti-oxidant mechanisms used both by the red cell and the parasite, detoxication by the glutathione cycle (by means of glutathione reductase and NADPH) plays a crucial role (Fritsch et al. 1987). Vitamins C and E are found in increased amounts in infected cells and are thought to have a protective role, particularly in preventing the peroxidation of membrane lipids and in preventing the formation of methemoglobin. Both host cell and parasite superoxide dismutases (SOD) have been identified in *P. falciparum*-infected cells, the parasite SOD being cyanide insensitive (Fairfield et al. 1988).

Molecular biology and genetics

The genome of malaria parasites is complex, presenting a high degree of diversity not only from species to species, but also from one isolate to another. Diversity is generated either by cross-fertilization during meiosis or by genomic reorganization during mitosis. The genome is haploid throughout most of the life cycle, except for a short time after zygote formation; it consists of 14 chromosomes of various sizes.

GENOME STRUCTURE

Measured sizes of the haploid genome of malaria parasites range between 20 and 25 Mb, values comparable to the genome of yeast but three to four times greater than that of *Escherichia coli*. The base composition of the genome of all malaria parasites is always A+T rich (from 70 to 83 percent A+T, depending on the species) (Weber 1988). Codon usage by malaria parasites is not random and it has been shown that for some genes (e.g. the repeat region of the circumsporozoite, CS protein), the usage of synonymous codons could be highly biased. The entire genome of *P. falciparum* (clone 3D7) has been sequenced and it was shown to contain 5 300 protein-encoding genes (Gardner et al. 2002). The genome of other species of *Plasmodium* are in the process of being sequenced.

The parasite genome shows variation at many levels and the genetic polymorphism of parasite isolates may be used as a tool for molecular epidemiological studies. The extreme diversity of the genomic make-up of malaria isolates has been demonstrated by the use of Southern blot fingerprinting which has revealed a

different fingerprint pattern for almost every single isolate from a given geographical area (Langsley et al. 1988; Hughes et al. 1989).

As well as nuclear DNA, malaria parasites have two other extrachromosomal forms of DNA: a 6-kb element that is related to the mitochondrial DNA of other species and a 35-kb circular element (or 'apicoplast') which seems to be related to the chloroplast element found in algae (Clough and Wilson 2001). It has been suggested that this organelle is the target of antimalarial antibiotics, such as tetracycline, doxycyclin, and clindamycin, which inhibit prokaryotic translation, as well as rifampicin and quinolones which inhibit prokaryotic transcription (Wilson et al. 1996; Fichera and Roos 1997).

MALARIA GENETICS

Genetic recombination in malaria parasites occurs in the mosquito where fertilization of gametes, zygote formation, and meiosis takes place. For recombination to occur, the parasites picked up by the mosquito must consist of more than one population of parasites of the same species. During recombination, members of pairs of chromosomes segregate randomly into the haploid progeny and crossing-over events take place between homologous chromosomes. Such studies provide information on the relationships between various genes (e.g. the presence of linkages) and the different ways in which recombination can occur. In view of the fact that malarial infections observed in humans are rarely clonal, but more often a mixture of between three and nine different parasite clones, it is surprising that the level of recombination in mosquitoes actually observed is lower than expected (one-and-a-half to two on average). This may be explained by the fact that, although different populations are present at the same time, the different populations are rarely synchronized with regard to gametocytogenesis, with the consequence that homozygote crosses are more likely than heterozygote crosses (Paul et al. 1995).

GENETICS OF CHLOROQUINE RESISTANCE

Forty-five years ago, resistance to chloroquine (CQ) spontaneously appeared, almost at the same time in both Indochina and Brazil, then spread throughout Asia and South America, to eventually reach Africa in the late 1970s (Wernsdorfer and Payne 1988); the current situation is illustrated in Figure 2 showing an almost worldwide distribution of resistance, with the need for countries to switch to new first-line antimalarials, when resistance exceeds 25 percent.

The mechanism of action of CQ is complex and remains controversial. CQ, being a lysosomo-tropic drug, accumulates in the parasite food vacuole where it binds to ferriprotoporphyrin IX (Fitch et al. 1982), a toxic metabolite of heme which is normally detoxified by the formation of hemozoin crystals; the accumulation of toxic metabolites of heme kills the parasite. Chloroquine-resistant (CQR) parasites accumulate less CQ than chloroquine-sensitive (CQS) parasites, below the level necessary to inhibit heme polymerization. Different hypotheses have been proposed to explain the reduced accumulation of CQ: (1) an abnormal proton pump in the food vacuole membrane leading to an elevated pH in the normally acid food vacuole would inhibit CQ transport (Ginsburg and Stein 1991); (2) a reduction of the carrier-mediated entry of CQ, in which CQR cells have a constitutively activated Na^+/H^+ exchanger in the vacuole membrane which makes it impossible for CQ to stimulate its own active uptake (Wünsch et al. 1998); or (3) the presence in CQR parasites of a rapid efflux mechanism by means of a P-glycoprotein *mdr* transporter, which would effectively pump CQ out of the cell. The third hypothesis seemed to take into account the observation that verapamil, and other calcium channel blockers, can partially reverse CQ resistance, presumably by blocking the *mdr* transporter, which is overexpressed in resistant parasites (Martin et al. 1987). The finding that the pH of the food vacuole in CQR parasites was actually decreased (rather than increased, as had been assumed in the first two hypotheses) produced yet another possible mechanism of resistance taking into account the fact that the CQ accumulation in the infected red cell relies on binding to heme (Bray et al. 1998; Dzekunov et al. 2000). In this model, the lower pH in the vacuole of CQR parasites is more suitable for hemozoin production, thus leaving less free heme available for forming toxic complexes with CQ.

Linkage analysis experiments suggested that CQ resistance could be associated with a single locus, perhaps a single gene (Wellems et al. 1990). This was an important step forward, since it was believed, until then, that CQ resistance was multigenic. The search for a potential CQR gene identified the *pfcrt* gene as the most likely CQR gene candidate. The *pfcrt* gene product appears to be localized in the food vacuole membrane. Various *pfcrt* alleles are associated with CQR, which is not surprising since multiple mutations are expected, in view of the slow genesis and spread of CQ resistance (Fidock et al. 2000; Djimde et al. 2001).

RESISTANCE TO ANTIFOLATES

The folate metabolic pathway leads to the de novo synthesis of pyrimidines and is, therefore, essential to malaria parasites incapable, as they are, of pyrimidine salvage. In addition, this pathway includes enzymes with no human counterparts. Despite being nearly ideal antimalarial drugs, resistance to antifolates spread very fast after their deployment. When pyrimethamine was introduced to Tanzania in 1957, rates of resistance rose from zero to 37 percent in 5 months (Clyde and Shute 1957).

Genetic crosses have provided crucial information on the linkage between resistance and the *dhfr-ts* gene (Walliker et al. 1987). The molecule involved is a bifunctional enzyme, where different domains of the molecule act as DHFR and thymidylate synthase (TS), both enzymes of the folate pathway. Pyrimethamine, cycloguanil (a metabolite of proguanil), and chlorcycloguanil are DHFR inhibitors. A series of *dhfr* mutations have been reported, each with a single amino acid substitution within the active site of DHFR; in the primary mutation (which is found in all resistant lines), asparagine is substituted for serine at codon 108. Asn-108 occurs first, conferring only modest pyrimethamine resistance, but further mutations at codon 51, 59, and 164 may increase resistance substantially. So far, only triple resistances (Asn-108, Ile-51, Arg-59) have been found in Africa, while quadruple resistances involving Leu-164 in addition have already been found in South East Asia (Wichmann et al. 2003).

Sulfa drugs act on a different enzyme of the folate pathway, dihydropteroate synthase (DHPS): sulfonamides, and sulfones are structural analogs of *p*ABA, the natural substrate of DHPS. A variety of point mutations of DHPS have been described; the triple mutation (Gly-437, Glu-540, Ala-581), which confers most resistance, is prevalent in areas of widespread sulfadoxine-pyrimethamine failure in South America and Asia, but has not yet been reported in Africa (Plowe 2001).

Point mutations affect antifolates selectively: for example, a parasite line with mutation Asn-108 may be resistant to pyrimethamine but remain sensitive to cycloguanil and the triple resistant (Asn-108, Ile-51, Arg-59), which is highly resistant to pyrimethamine, has only moderate resistance to cycloguanil or chlorcycloguanil. This explains why the association of two drugs working in synergy, such as sulfadoxine-pyrimethamine (SP), has remained effective much longer even in areas with high prevalence of DHFR mutations. This also explains why it is conceivable to replace one antifolate combination with another, e.g. to replace SP with the new combination chlorproguanil (Lapudrine®-dapsone (LapDap)) (Winstanley 2001).

Any widespread use of antifolates is likely to exert a selective pressure, which may increase the spread of resistance. Although trimethoprim-sulfamethoxazole (Co-trimoxazole) is not a very good antimalarial, its widespread prophylactic use in areas where human immunodeficiency virus (HIV) and malaria are both highly endemic could shorten the life-span of SP (Jelinek et al. 1999).

MOLECULAR EPIDEMIOLOGY OF DRUG RESISTANCE

Two approaches have been used extensively in the past to test for drug resistance – in vivo tests and in vitro culture assays. Each of these methods has disadvantages. Molecular techniques, which allow the detection of mutations associated with resistance, offer an alternative approach with many advantages. The difficulty of this approach has been the definition of mutations that accurately define resistance or, more specifically, to predict in vivo resistance based on the finding of mutations. The presence of a mutation of the DHFR gene may confer some resistance to pyrimethamine, but this does not necessarily mean that a patient carrying such a parasite can no longer be treated with SP. Only parasites carrying triple mutations for *dhfr* and double mutations for *dhps* are always associated with SP failure and a high prevalence of such parasites in a particular area suggests the need to switch to a new drug for first-line treatment. Such molecular mapping studies are essential for following the spread of resistance at country level and to provide an early warning system for policymakers. Because it is much easier to perform than in vivo and in vitro resistance tests, molecular screening is likely to succeed where other surveillance programs have failed. A number of regional centers have been set up to coordinate information and work towards a rational implementation of coherent policies, e.g. the best example so far is the East African Network for Monitoring Antimalarial Treatment (EANMAT) (Mutabingwa 2001). Figure 13 provides an example of how Africa-wide information can be gathered at a distance by screening parasites isolated from European tourists using molecular drug resistance markers (TropNetEurop) (Jelinek et al. 2002).

CLINICAL ASPECTS

Malaria can mimic many diseases and there are no absolute diagnostic clinical features. The classical periodicity of febrile paroxysms every 48 h for *P. falciparum*, *P. vivax*, and *P. ovale* (days 1 and 3) or every 72 h for *P. malariae* (days 1 and 4) are usually absent at the beginning of the disease. Periodicity develops only as a result of a delay in treatment when a sufficient number of schizonts rupture at the same time and the infection becomes synchronized.

Clinical manifestations

FALCIPARUM MALARIA ('MALIGNANT TERTIAN MALARIA')

In nonimmune people, falciparum malaria is a medical emergency, as there are few conditions in tropical medicine that can change dramatically from a relatively benign illness to a catastrophic and fatal one (Gilles and Phillips 1988; Molyneux and Fox 1993; Warrell 2002). The incubation period is generally 9–14 days, but it can be as short as 7 days. The symptoms are nonspecific with headache, pains in the back and limbs, simulating influenza, anorexia, nausea, and a feeling of chill rather than a distinct cold phase (as in vivax malaria). Fever is very common but not invariable; it is continuous or

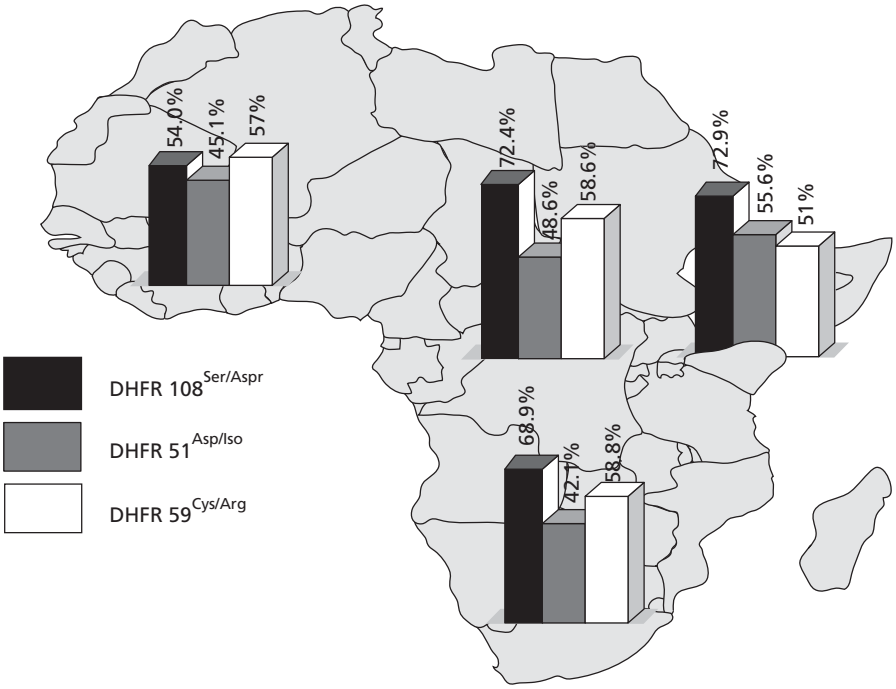

Figure 13 *TropNetEurop a network of travel clinics in Europe acts as sentinel for monitoring the spread of drug resistance in Africa–distribution of DHFR mutants in malaria isolates from European tourists.*

remittent, not tertian. The physical findings include prostration, a tinge of jaundice often misdiagnosed as viral hepatitis, *Herpes simplex* of the lips and tender hepatosplenomegaly. An ophthalmoscopic examination should always be carried out, because multiple retinal hemorrhages (predominantly white-centered) and other retinal changes occur in both children and adults. They are best seen, however, by indirect ophthalmoscopy (Beare et al. 2003). Hyponatremia can occur in both uncomplicated and severe malaria (Ustianowski et al. 2004).

MANIFESTATIONS OF SEVERE FALCIPARUM MALARIA

Cerebral malaria is the most common presentation of severe malaria in adults. They have usually been ill for 4–5 days with fever, slowly lapsing into coma, with or without convulsions. The usual neurological picture is of a symmetrical upper motor neurone lesion with increased muscle tone, brisk, tendon reflexes, ankle clonus, and extensor plantar responses. The abdominal reflexes are absent. A number of bizarre neurological signs can occur; dysconjugate gaze is common. A whole variety of other neurological signs have been described (Warrell 2002). Retinal hemorrhages occur in about 15 percent of adults with cerebral malaria and are associated with low hematocrits and high parasitemias (Looareesuwan et al. 1983) (Figure 14). Even where good standards of care can be provided, the morbidity is about 15–20 percent.

Anemia is common in young children (see below), in pregnant women (see below), and in adults with severe malaria in whom it correlates with parasitemia.

Biochemical evidence of renal dysfunction progressing to oliguria, anuria, and acute renal failure occurs in about 10 percent of cases.

Hypoglycemia is common in patients with hyperparasitemia or as a result of treatment with quinine. The classic symptoms of hypoglycemia may not be present,

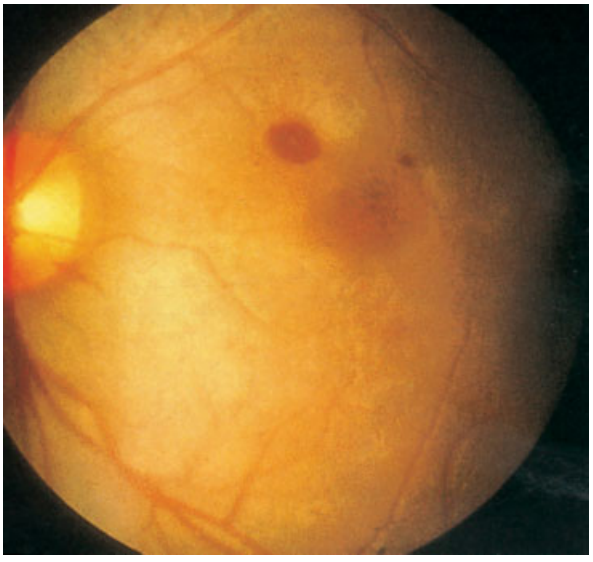

Figure 14 *Retinal hemorrhage*

Table 1 *Complications of severe falciparum malaria*

Complications
Cerebral malaria
Anemia
Renal failure
Hypoglycemia
Hyperparasitemia
Malarial hemoglobinuria
Metabolic acidosis presenting as 'respiratory distress'
Fluid, electrolyte, and acid base disturbances
Pulmonary edema
Circulatory collapse ('Algid malaria')
Bleeding and clotting disturbances
Hyperpyrexia

the only sign being deterioration in the level of consciousness.

Pulmonary edema is the most dreaded complication of malaria with a mortality of over 50 percent. It is sometimes precipitated by excessive parenteral fluid therapy and, as such, avoidable. The earliest signs are an increase in respiratory rate, dyspnea, and crepitations. In most patients, however, it is not iatrogenically produced and the picture is similar to that of the adult respiratory distress syndrome (ARDS) (Charoenpan et al. 1990). The pathogenesis of pulmonary edema in *P. falciparum* is complex and not fully understood.

Malarial hemoglobinuria is now usually associated with hyperparasitemia resulting in severe intravascular hemolysis. Some patients are mistakenly labeled as such when in fact the hemoglobinuria is due to glucose-6-phosphate dehydrogenase deficiency, triggered by a variety of drugs, including the oxidant antimalarial primaquine (Gilles and Ikeme 1960). The known complications of severe falciparum malaria are given in Table 1.

VIVAX AND OVALE MALARIA ('BENIGN TERTIAN MALARIA')

The incubation period of vivax malaria is usually between 12–17 days, but with some strains can be as long as 250–637 days; that of ovale malaria is usually 16–18 days but may also be longer. The clinical manifestations are similar.

After 2–3 days of nonspecific symptoms, similar to those described for falciparum malaria, the classical febrile paroxysm occurs. The cold stage, which lasts for 15–60 min, is characterized by violent rigors, a high core temperature, a cold dry skin due to intense peripheral vasoconstriction, and a rapid, low-volume pulse. The hot stage follows, the patient becoming unbearably hot. The temperature rises to 40–41°C, accompanied by a severe throbbing headache, palpitations, prostration, confusion, and delirium. The skin is flushed, the pulse rapid and full. The patient looks ill, may be anemic, mildly jaundiced, and the liver and spleen are enlarged and tender. The hot stage lasts 2–6 h. It is followed by the sweating stage characterized by drenching sweats, defervescence, and exhausted sleep. Unless treated, the febrile paroxysms recur every other day. Thrombocytopenia is common and the only serious (often fatal) complication is splenic rupture. Untreated or inadequately treated cases relapse after a period of quiescence at intervals varying from 8–40 weeks or more, depending on the strain of the parasite. A few cases of reversible pulmonary edema and neurological manifestations have been described in patients with confirmed vivax malaria (Tanios et al. 2001; Beg et al. 2002).

MALARIAE MALARIA ('QUARTAN MALARIA')

The incubation period of quartan malaria is 18–40 days. The clinical picture is similar to that of benign tertian malaria with the febrile paroxysms occurring every 72 h. Symptomatic recrudescences, which can occur up to 52 years after the last exposure to infection are due to persistent undetectable parasitemia, not to hypnozoites. In several parts of the world there is strong epidemiological evidence that *P. malariae* is an important cause of nephrotic syndrome especially in children under 15 years of age (Gilles and Hendrickse 1963).

Malaria in children

The manifestations of the primary attack of malaria in nonimmune children are very variable, consisting of any of the following symptoms, or combinations thereof: restlessness or drowsiness, refusal to eat or suck, headache, vomiting, loose stools, and cough. In the majority, the temperature is high (40°C), the child is flushed, and febrile convulsions are common, lasting only a few minutes. If the child does not regain consciousness within 30 min following the convulsion, cerebral malaria must be suspected. The liver is often enlarged and tender, splenomegaly develops later. Ocular fundus findings such as papilledema indicate a poor prognosis (Lewallen et al. 1993). Other retinal changes, which are best seen by indirect ophthalmoscopy, include patchy whitening of the peripheral retina and discoloration of the retinal vessels to pale orange or white (Lewallen et al. 2000). Retinal hemorrhages are common.

Children living in highly endemic malarious areas are equally vulnerable until the age of about 5 years, after which they achieve a relative immunity to malaria infection. Many, especially in tropical Africa, are either asymptomatic or have a mild illness, despite a parasitemia of 10–30 percent. Hepatosplenomaly occurs in 80 percent of these individuals.

Many of the clinical features of severe malaria (described in Clinical manifestations above) also occur in children. The commonest and most important complications are cerebral malaria, severe anemia, and

Table 2 *Differences between severe malaria in adults and children*

Sign or symptom	Adults	Children
Cough	Uncommon	Common
Convulsions	Common	Very common
Jaundice	Common	Uncommon
Duration of antecedent illness	5–7 days	1–2 days
Pretreatment hypoglycemia	Uncommon	Common
Resolution of coma	2–4 days	1–2 days
CSF opening pressure	Usually normal	Often raised
Abnormality of brain stem reflexes(e.g. occulovestibular, occulocervical)	Rare	More common
Neurological sequelae	<5%	>10%
Pulmonary edema	Common	Rare
Renal failure	Common	Rare
Bleeding/clotting disturbances	Up to 10%	Rare

Derived from studies in South-East Asian adults and African children (White et al. 1987; Molyneux et al. 1989).

metabolic acidosis. Cerebral malaria is the commonest presentation in the Gambia and in Burkina Faso (Waller et al. 1995; Modiano et al. 1995), whereas anemia is the most frequent presentation in Kenya and in Papua New Guinea (Marsh et al. 1995; Allen et al. 1996).

The differences between severe malaria in adults and in children are given in Table 2. The reasons for these differences have yet to be fully elucidated.

CEREBRAL MALARIA IN CHILDREN

In holoendemic areas of malaria, cerebral malaria occurs in children between 6 months and 5 years, most commonly in those 3–4 years old. The earliest symptom is usually fever (37.5–41°C) followed by failure to eat or drink. Vomiting and cough are common; diarrhea is unusual. The history of symptoms preceding coma may be very brief (1 or 2 days). Hypoglycemia is a particularly common presenting feature in children under 3 years and in those with convulsions, hyperparasitemia, or profound coma (White et al. 1987; Taylor et al. 1988). In the latter, corneal and vestibular-ocular reflexes may be absent. Extreme opisthotonos is sometimes seen (Figure 15) and is easily mistaken for tetanus or meningitis (Taylor and Molyneux 2002).

Some children are in a state of shock. Cerebrospinal fluid (CSF) opening pressure is often raised, sometimes markedly so. Intracranial hypertension and neurological evidence of cerebral herniation has been documented (Newton et al. 1991). Convulsions are common before or after the onset of coma, they are significantly associated with morbidity and sequelae. Neurological sequelae, which occur in about 10 percent of children, include: hemiparesis, cerebellar ataxia, cortical blindness, severe hypotonia, mental retardation, generalized spasticity, and aphasia (Molyneux et al. 1989; Newton et al. 1994). Significative cognitive problems affecting school performance may occur in children who appear to have made a complete neurological recovery (Holding et al. 1999).

Figure 15 *Opisthotonos*

ANEMIA

This is particularly common in children between 6 months and 2 years. It is often the result of repeated untreated or partially treated episodes of uncomplicated malaria. Parasitemia is often scanty, although numerous pigmented monocytes are seen in the peripheral blood. The anemia is normochromic with prominent dyserythropoietic changes in the bone marrow. In older children the anemia is acute and hemolytic and associated with hyperparasitemia. Children with severe anemia may present with tachycardia, dyspnea, respiratory distress, confusion, restlessness, coma, retinal hemorrhages, cardiac failure, and pulmonary edema. A study in Tanzania has shown that treating infants with SP at 2, 3, and 9 months, to coincide with routine immunization, reduces episodes of clinical malaria by 60 percent and severe anemia by 50 percent (Schellenberg et al. 2001).

METABOLIC ACIDOSIS

Metabolic acidosis is an important feature of severe malaria in children. It may present separately or in combination with cerebral malaria or anemia (Taylor et al. 1993; Marsh et al. 1995). Deep breathing ('Kussmaul') is a good clinical indicator of the presence of acidosis. Lactic acidosis is a major contributor to academia (Krishna et al. 1994). Metabolic acidosis is associated with a poor prognosis. The child often presents with other signs of respiratory distress, but is clinically and radiologically clear.

Malaria in pregnancy

Nonimmune pregnant women are susceptible to all the usual manifestations of malaria. Moreover, they have an increased risk of abortion, stillbirth, premature delivery, and low birth-weight of their infant. Mortality from severe malaria is higher than in nonpregnant patients (Wickramasuriya 1935; Menon 1972; Bray and Anderson 1979; Brabin 1983; Looareesuwan et al. 1985; Brabin et al. 1993).

Pregnant women are particularly prone to hypoglycemia which may occur on admission in severe disease, in otherwise uncomplicated malaria and as a complication of quinine therapy. It may be asymptomatic or manifest itself merely as an alteration in conscious level. It is dangerous for the baby causing fetal bradycardia and other signs of fetal distress. In the quinine-induced hyperinsulinemic hypoglycemia, abnormal behavior, sweating, and sudden loss of consciousness usually occur (White et al. 1983). The prevalence and intensity of malaria infection in pregnancy is higher in women who are HIV positive.

Pulmonary edema may occur in pregnant women on admission, may develop suddenly and unexpectedly several days after admission, or may develop immediately after childbirth (see also Figure 16). In holoendemic areas of malaria, partially immune pregnant women, especially primigravidae are susceptible to abortion, stillbirth, premature delivery, and low birth-weight of their infants (Archibald 1956; Bray and Anderson 1979; McGregor et al. 1983; Morgan 1994; Greenwood et al. 1994). Significant increases in parasite rates and densities occur particularly in primigravidae. In the second trimester primiparae are prone to develop a severe hemolytic anemia which bears little relation to their peripheral parasitemia (Gilles et al. 1969; Rougemont et al. 1977; Brabin 1983; McGregor 1984; Fleming et al. 1986; Steketee et al. 1996a). The other complications of severe malaria are, however, rarely encountered.

Figure 16 *Pulmonary edema*

Vertical transmission of malaria parasites across the placenta from mother to fetus occurs and in endemic areas neonates not infrequently have cord-blood and peripheral parasitemia, which disappears within a day or two. Congenital malaria (i.e. symptoms or signs resulting from malarial infection in the neonate) is more common in infants born to nonimmune mothers and during epidemics of malaria. In malaria-endemic regions, the incidence is low, despite a high prevalence of placental infection. A detailed description of malaria in pregnancy is given by Shulman and Dorman (2002).

Hyperactive malarial splenomegaly (tropical splenomegaly syndrome)

In some malaria-endemic regions of the world and particularly in parts of Papua New Guinea and sub-Saharan Africa, adults develop a progressive, sometimes massive enlargement of the spleen (more than 10 cm below the costal margin). The spleen may weigh over 4 kg. The liver is often enlarged with dilated sinuses containing lymphocytes. Hypersplenism can cause a normocytic anemia, leukopenia, and thrombocytopenia. Pregnant women may suffer attacks of acute hemolysis. There is a gradual onset of early lethargy that can progress to an incapacitating weakness. Left upper quadrant pain is common. Physical examination reveals gross splenomegaly with an obvious notch in an afebrile patient, evidence of hypovolemia and moderate anemia (Bates and Bedu-Addo 1997). There are suggestions that hyperactive malarial splenomegaly may, in some patients, lead to the development of a tropical splenic lymphoma (Bates et al. 2001).

The syndrome seems to be an abnormal immune response to recurrent malaria infections with excessive production of IgM. Raised titers of IgM and IgG malaria antibodies and sinusoidal lymphatic infiltration of hepatic sinuses are also often found. Patients respond well, both clinically and immunologically, within 3 months of starting continuous antimalarial chemoprophylaxis (Fakunle 1981; Crane 1986). Recent studies on this syndrome have not been carried out, nor has the response to antimalarial therapy in the light of increasing parasite resistance been recorded. Such a study should be undertaken.

IMMUNOLOGY

Susceptibility to malaria is restricted by a variety of genetic features of the host, generally referred to as 'natural immunity' or 'innate resistance.' When infection does occur, the host immune system responds in a variety of ways, some of which may eventually lead to the clearance of parasites and protection against subsequent infection. In many circumstances, the development of protective immunity is slow and this may be explained by both the extreme antigenic diversity of the parasite and the existence of efficient escape mechanisms.

Innate resistance

The relationship between malaria parasites and their host is a delicately balanced one, which may be affected by a variety of host and parasite features (Miller 1976). When different hosts are exposed to a given malaria parasite, the ensuing infection may range from negligible to fatal. For example, *Plasmodium knowlesi* which produces a chronic but low-level infection in *Macaca fascicularis* (the natural host), a low-level and rapidly self-limiting infection in humans (accidental or experimental host), and a fulminant, fatal infection in *Macaca mulatta* (experimental host). The degree of susceptibility may vary with time, particularly after serial sub-inoculations of the parasite, and it appears that 'adaptation' to an artificial host may occur. When *P. knowlesi* was extensively used for malariotherapy of neurosyphylitic patients in Romania, after a number of sub-inoculations the strain became so highly virulent to humans that it became unmanageable (Coatney et al. 1971).

Although it has often been stated that ethnic groups, which have had a particularly long association with malaria, are less susceptible to infection and disease (e.g. Pygmies in Central Africa, Tharu in Nepal or Orang Asli in Malaysia), the evidence is mostly anecdotal. The best proof of this concept comes from a comparative study of three sympatric ethnic groups in Burkina Faso that showed reduced infection rates and morbidity in Fulanis, a traditionally nomadic tribal group, compared to Mossis and Ribaimas, in a situation where all three groups cohabit in the same villages (Modiano et al. 1996). The follow up of well-characterized family groups seems to implicate a region of chromosome 5 (5q3l-q33) as the locus for susceptibility to *P. falciparum* infection (Flori et al. 2003). Our improved knowledge of human and parasite genomes will open the field for future genetic susceptibility studies.

Susceptibility to infection is primarily determined by the ability of the parasite to invade and survive in appropriate host cells (e.g. hepatocyte and erythrocyte in the mammalian host). With regard to human malaria parasites, specific receptors for hepatocyte invasion are still unknown and there is no evidence to suggest differential susceptibility or resistance in any particular human ethnic group or in association with any specific genetical characteristic of hepatocytes. The maturity of the hepatocyte is crucial, since fetal hepatocytes appear to be resistant to infection.

Susceptibility to erythrocytic infection may be determined at the level of merozoite invasion, intracellular growth, or erythrocytic lysis at the time of merozoite release. *P. vivax* is restricted to reticulocytes and Duffy-positive cells, while *P. falciparum* invasion may be substantially reduced in red cells with glycophorin abnormalities, e.g. the En(a-) or the S-s-U phenotypes (Pasvol and Wilson 1982). Resistance to parasite entry is observed in cells with cytoskeleton abnormalities; ovalocytosis confers resistance to all malarial species while elliptocytosis, due to the absence of band 4.1, may hinder the entry of *P. falciparum* (Nagel 1990). Intra-erythrocytic development of *P. falciparum* is reduced or retarded in red cells presenting various hemoglobinopathies, including HbS, HbE, HbF, HbC, G6PD deficiency or α-thalassemia; the reduced growth of *P. falciparum* in HbC/C cells has been attributed to an increased resistance to red cell lysis and to intracellular degenerescence, compatible with an incapacity of parasites to induce merozoite release. In some cases, reduced growth may be observed only in the initial cycle of development in abnormal cells, as is the case when *P. falciparum* is grown in vitro in G6PD-deficient cells, where the parasite can 'adapt' by switching on its own enzyme.

Resistance to infection may also be caused by the antiparasitic effect of components of the host serum. The serum of adult Sudanese living in an endemic area for malaria may cause intraerythocytic death of *P. falciparum*; the toxic component of serum, named 'crisis forming factor,' being independent of host immunity (Jensen et al. 1983).

In addition to innate resistance to infection, there is considerable diversity in individual susceptibility to severe disease; for instance, it has been estimated that *P. falciparum* leads to cerebral malaria in only a small percentage of cases in an endemic area (estimated at 1:200), and that only a percentage of those are fatal (Greenwood et al. 1991). Many factors can explain this apparently low percentage of disease severity, including the nutritional status and the level of acquired immunity, which is directly related to transmission intensity. A number of genetic susceptibility factors have been identified, the importance of which may vary from one endemic area to another (Hill and Weatherall 1998). For instance, individuals heterozygous for the HLA-B53 haplotype had a significantly decreased susceptibility to severe falciparum disease in the Gambia, but not in Kenya. In contrast, genetic variation in the promoter gene for inducible nitric oxide synthase (iNOS) was found to be responsible for reduced susceptibility to severe malaria in Gabon and increased susceptibility to cerebral malaria in the Gambia (Burgner et al. 2003). The intercellular adhesion molecule-1 (ICAM-1)^{Kilifi} allele was found to be associated with increased suscept-

ibility to cerebral malaria in Kenya, but not in the Gambia and was associated with protection in Gabon (Bellamy et al. 1998). Variable susceptibility to severe malaria was also shown to be significantly linked to the polymorphism of the TNF-α promoter gene (McGuire et al. 1994), the interferon- receptor I gene (Aucan et al. 2003), and the CD36 gene (Aitman et al. 2000). As confusing as such contradictory results may appear to be, they provide a clue to the role of host genetic susceptibility in the complex events leading to different forms of severe malaria. Paradoxically, while the carriers of the α-thalassemia gene are less likely to develop severe falciparum malaria, they have been shown to have an increased susceptibility for *P. vivax* infection, probably because of the preference of this parasite for reticulocytes, which are more abundant in thalassemics (Williams et al. 1996).

Acquired immunity

Even in individuals living in an area endemic for malaria and who are, therefore, exposed to frequent reinfection, an effective immunity against *Plasmodium* takes a long time to develop. The different stages of the development of immunity may be described as follows:

- A child born to an immune mother is protected during the first 6 months of life, as the result of the passive transfer of maternal immunity.
- During that period, the infant is exposed to infection and starts to develop its own immunity. It will, however, take many years before this immunity becomes protective. Once maternal immunity has waned, each subsequent infection will result in clinical malaria and a percentage of children will die in situations where early diagnosis and treatment is not available.
- Once the child reaches school age, malaria paroxysms will progressively become less frequent and less severe: this is the stage of anti-disease immunity (or clinical tolerance). At this stage, the child continues to become infected, sometimes with very high levels of parasitemia, but either develops only mild forms of malaria or remains asymptomatic. Age-dependency is a crucial feature of malaria immunity.
- The state of anti-disease immunity progressively develops into a state of premunition, when the child not only exhibits clinical tolerance but also much reduced parasitemia and shorter episodes of infection.
- A state of complete, sterile immunity may never be reached, even in adults who have never left the endemic zone. At this stage, infections remain mostly asymptomatic, with very low parasitemia and short episodes of infection.
- The state of incomplete immunity (or semi-immunity) is solid, but requires frequent reinfection. Any period spent outside the endemic area may result in a loss of premunition.
- During pregnancy, adult women, who had previously reached a state of premunition, appear to lose it and may again present clinical malaria, sometimes in severe, life-threatening forms.

Important *Plasmodium* antigens

Much of what is known of the antigenic structure of malaria parasites has been derived from studies aimed at identifying protective antigens with a view to the development of an antimalarial vaccine. Table 3 lists the *P. falciparum* antigens which have been considered important in antimalarial immunity, because they are expressed on the surface of extracellular infective forms, on the surface of malaria-infected cells or because their recognition by patient antibodies appears to be consistent with the development of protective immunity. In many cases, these *P. falciparum* molecules are representative of a family of antigens and similar, if not necessarily biochemically identical, antigens may be found in all malarial species.

The antigens of *Plasmodium* spp. present a number of notable features:

1. A relatively small number of molecules actually expressed on the surface of invasive stages and this reflects the fact that these stages are generally covered by a surface coat with crucial biological functions in terms of recognition and adherence to future host cells (CSP-1 and SSP2/TRAP on sporozoites; MSP1, MSP-2, and MSP-4 on merozoites; Pfs230, Pfs45/48, and Pfs25 on gametes or ookinetes).
2. Extracellular stages all possess the characteristic invasion organelles and many immunodominant antigens appear to be associated with these organelles. Some are found in rhoptries (AMA-1, RAP-1-3, Rhop1-3), some in micronemes (EBA-175), and some in dense granules (RESA/Pf155). Although these molecules are not actually on the surface, they seem, nevertheless, to play an important role at the parasite–host cell interface and immune reactions against them interfere with invasion. It is conceivable that the good immunogenicity of these molecules may reflect more their uniqueness (i.e. no similar antigens in the host) than their true biological importance.
3. Some molecules of parasite origin are expressed at the surface of infected erythrocytes or infected hepatocytes, either directly by the parasite in order to fulfill a fundamental parasite function (as is probably the case of PfEMP-1 and rifins, expressed on the surface of host erythrocyte) or by means of a host processing function related to MHC class I expression (e.g. the exo-erythrocytic antigens at the surface of hepatocytes, including CSP-1, hsp70, LSA-1, and LSA-2, STARP, SALSA); and

Table 3 *Important antigens of* Plasmodium falciparum

Antigen	Full name and alternative	Location
Intra-erythrocytic stages		
hsp70	Heat shock protein-1, p75	Parasite cytoplasm
GRP78	Glucose-regulated protein, heat shock protein-2	Parasite cytoplasm
Aldolase	Aldolase	Parasite cytoplasm
HRP-3	Histidine-rich protein-3, SHARP	Parasite cytoplasm
CARP	Clustered asparagine-rich protein	Parasite cytoplasm
RESA	Ring-infected erythrocyte surface antigen, Pf155	RBC internal surface, merozoite dense granule
MESA	Mature-parasite infected surface antigen-2, PfEMP-2	RBC internal surface
AARP	Asparagine- and aspartate rich protein	RBC internal surface
HRP-1	Histidine-rich protein-1, KAHRP, KP	RBC internal surface
HRP-2	Histidine-rich protein-2	RBC internal surface and secreted
Pf332	Pf332, Ag332, giant protein	RBC internal surface
GBP	Glycophorin-binding protein, Pf120-130,	RBC cytoplasm
FIRA	Falciparum interspersed repeat antigen	RBC cytoplasm
S-antigen	S-antigen	Parasitophorous vacuole
SERA	Serine-rich antigen, SERP, p113, p126, Pf140	Parasitophorous vacuole
GLURP	Glutamate-rich protein	Parasitophorous vacuole (erythro, exo-erythro)
ABRA	Acidic basic repeat antigen, p101	Parasitophorous vacuole
SPAM	Secreted polymorphic antigen associated with merozoite, MSP3	Parasitophorous vacuole
PfEMP-1	Erythrocyte membrane-associated malaria protein-1	RBC external surface
Merozoite		
MSP-1	Merozoite surface antigen-1, MSA-1, PMMSA, P190, gp185	Merozoite surface, surface of schizont
MSP-2	Merozoite surface antigen-2, MSA-2, gp56, QF122, GYMMSA	Merozoite surface
MSP-4	Merozoite surface antigen-4, MSA-4	Merozoite surface
MSP-5	Merozoite surface antigen-2, MSA-5	Merozoite surface
AMA-1	Apical membrane antigen-1, Pf83	Apical region, merozoite surface
EBA-175	Erythrocyte-binding antigen, SABP	Micronemes, merozoite surface
RAP-1/-2/-3	Rhoptry-associated protein-1, 2 and 3	Rhoptries
RhopH	Complex of Rhop1, Rhop-2 and Rhop-3	Rhoptries
Ag512	Ag512	Rhoptries
MCP-1	Merozoite cap protein-1	Apical surface
RIMA	Ring-stage membrane antigen	Dense granule
Sporozoite and pre-erythrocytic stages		
CSP-1	Circum sporozoite protein, CSP	Sporozoite surface, infected hepatocyte
SSP-2	Sporozoite surface protein-2, TRAP	Sporozoite surface
LSA-1	Liver-specific antigen-1	Infected hepatocyte
LSA-2	Liver-specific antigen-2	Infected hepatocyte
LSA-3	Liver-specific antigen-3	Infected hepatocyte
STARP	Sporozoite threonine and asparagine-rich protein	Sporozoite surface, infected hepatocyte
SALSA	Sporozoite and liver stage antigen	Sporozoite surface, infected hepatocyte
Sexual stages		
Pfs230	Pfs230	Gametocytes, surface gametes
Pfs45/48	Pfs45/48	Gametocytes, surface gametes
Pfs25	Pfs25	Surface ookinete
Pfs16	Pfs16	Gametes and sporozoite
11.1	11.1	Parasite cytoplasm

4 a large number of antigens are found inside the parasite and, as such, would not be expected to play a role in protective immunity (GLURP, SERA, hsp-70). These molecules are either excreted during schizogony (by means of a transfer from the parasite to the parasitophorous vacuole and then to the outside of the cell) or, more often, released at the time of schizont rupture. They may be highly immunogenic and be responsible for a considerable portion of the total host immune response; some of these molecules are of interest for immunodiagnosis and some may be involved in immunopathological events.

Epidemiological evidence for immunity

The main lesson from epidemiological studies is that malaria is a chronic disease in which immunity is essentially an age-dependent event. Repeated reinfection is necessary to build up protection and this generally takes a few years. The first sign of immunity is a protection against severe forms of the disease, which, according to one study, could occur after only a small number of clinical episodes (Gupta et al. 1999). This is followed by a progressive reduction of the frequency of malarial episodes, without any reduction in the levels of parasitemia; as a result, surveys of malaria prevalence in a malaria-endemic area will show paradoxically high levels of parasitemia in older children in the community (hence the 'clinical threshold' concept discussed under Diagnosis below) (Trape et al. 1994). Even adults living in an area of high endemicity still continue to present asymptomatic infections; in a longitudinal study of adults in a West African community, Bruce-Chwatt (1963) showed that nearly all adults had a patent parasitemia at one time or another during the survey.

This progressive evolution from a state of immunity against 'malaria-disease' to a state of partial immunity against 'malaria-infection' is almost unique among infectious diseases. It has been described as 'premunition' by early malariologists (Sergent et al. 1924) and refers to a form of nonsterile immunity, which has to be maintained by almost continuous exposure to the parasites. This continuous exposure is achieved either as the result of a long-term survival of parasites in the host (*P. falciparum* can survive for over a year and *P. malariae* is said to survive for as long as 25 years in the absence of treatment) or as the result of frequent reinfections. Individuals who temporarily leave the endemic area may lose their premunition and develop clinical paroxysms on their return. This observation does not, however, mean that no immunological memory is associated with premunition. The persistence of protection has best been demonstrated during the 1987 epidemic of falciparum malaria on the Hauts Plateaux of Madagascar. Having been eliminated for over 30 years, malaria returned in the form of an epidemic with many fatalities, but it was noticed that individuals over 40 years of age (who had been exposed to malaria prior to elimination) were significantly less susceptible than younger age groups (Deloron and Chougnet 1992).

Immune effector mechanisms

ANTIBODIES

A rapid increase of immunoglobulin levels is observed in the course of malaria, but only a small percentage of these antibodies is directed against the parasite; most of the characteristic hypergammaglobulinemia observed during acute malaria may be attributed mainly to polyclonal activation (Rosenberg 1978). The role of antibodies in protection has best been demonstrated by the passive transfer of serum from immune adult donors to infected Gambian children (Cohen et al. 1961), in whom falciparum infection was completely cleared 48 h after the serum transfer. Similarly, in the newborn child of an immune mother, the passive transfer of IgG across the placenta protects the child during the early months of life (Edozien et al. 1962).

Individuals living in an area endemic for malaria have high levels of antibodies against many malarial antigens as demonstrated by the use of enzyme-linked immunosorbent assay (ELISA) or immunofluorescence antibody tests (IFAT); these antibodies are generally directed against immunodominant epitopes of the parasite, but are not necessarily protective. Protective antibodies may either act directly on the parasite or parasitized cells, or act in synergy with various effector cells. The possible targets of immune effector mechanisms in the life cycle of malaria parasites are illustrated in Figure 17.

Antibodies may interfere with parasite development at different sites: (a) by blockage of merozoite dispersion; (b) by inhibition of invasion (whether of merozoites into red cells or of sporozoites into hepatocytes); (c) by intracellular killing of erythrocytic stages (presumably by a form of metabolic poisoning induced by interaction of antibodies with specific 'pumps' introduced by the parasite into the red cell membrane); or, (d) by the inhibition or reversal of cytoadherence, which obliges the infected erythrocyte to undergo, 20 times every hour, the perilous journey around the peripheral bloodstream of the host, including passage through the spleen, instead of remaining safely sequestered in capillary venules. Antibodies to sexual stages of the parasite may be picked up, together with gametocytes, by a feeding mosquito and thus block gamete fertilization, a feature which forms the rational basis for the development of transmission-blocking immunity. Finally, antibodies may act in co-operation with various cells (including macrophages and T cells) to increase cell-mediated killing.

Depending on the expected mode of action of the protective antibodies and the target, various in vitro correlates

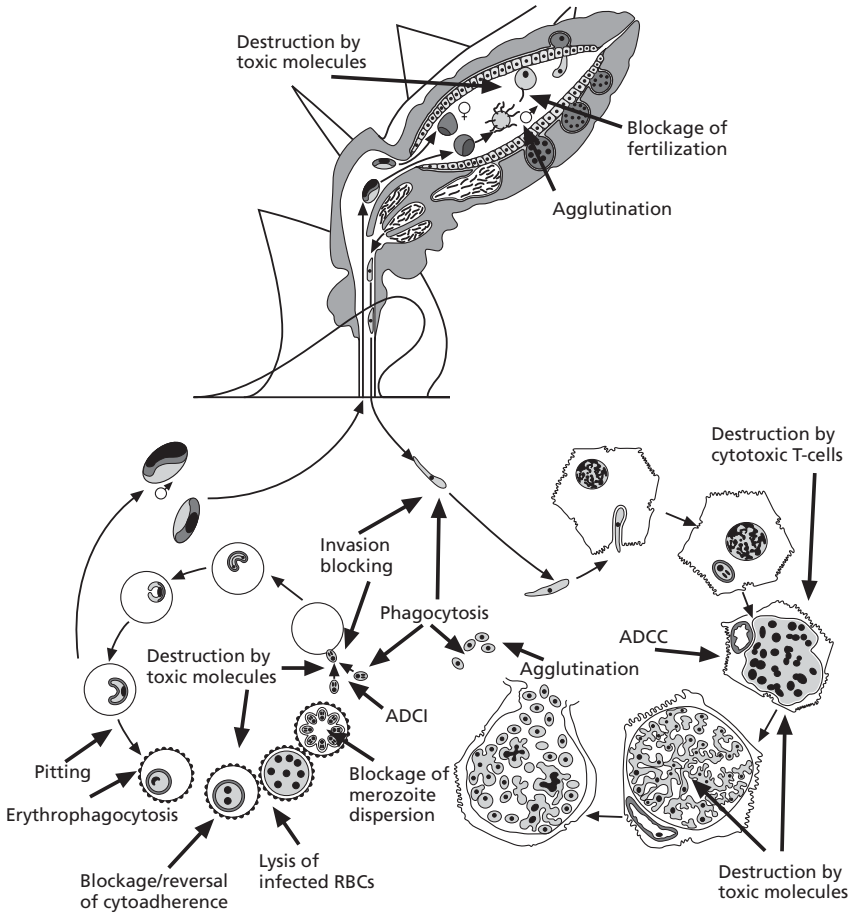

Figure 17 *Targets of immune effector mechanisms in the life cycle of* Plasmodium

for protection have been described including circumsporozoite precipitation (Spitalny and Nussenzweig 1973), inhibition of merozoite reinvasion (Butcher et al. 1978), inhibition of merozoite dispersal from schizonts (Green et al. 1981), recognition of ring-infected erythrocyte surface antigen (RESA) (Perlmann et al. 1984), recognition of the surface of infected erythrocytes by surface immunofluorescence, agglutination or immunogold staining (Hommel et al. 1991; Marsh and Howard 1986), inhibition/reversal of cytoadherence (Singh et al. 1988) or inhibition of rosette formation (Carlson et al. 1990). All these in vitro tests may have a degree of correlation with protection but none is perfect. When a number of such tests was compared and related to clinical outcome in a study of Gambian children, it was shown that, while high antibodies titers had a predictable age distribution, none of the individual tests had a significant correlation with protection (Marsh et al. 1989).

The activity of antibodies may be a consequence of opsonization, since the passive transfer of immune serum is more effective in intact than in splenectomized recipients (Brown and Phillips 1974). While it has been convincingly demonstrated that immune phagocytosis is an important mechanism for the removal of dead parasites (Shear et al. 1979), the true role of opsonization in parasite killing and elimination remains unclear. Cytophilic antibodies (IgG1 and IgG3) may be crucial and this may explain why antibody-dependent cellular inhibition (ADCI), an assay that measures cooperation between antibodies and monocytes, appears to have a better correlation with protection than other in vitro antibody assays (Bouharoun-Tayoun et al. 1995).

MACROPHAGES

The role of macrophages in malaria was recognized by early pathologists, who observed an increased number of macrophages in the spleen, liver, and bone marrow of infected animals (Taliaferro and Cannon 1936). The increased number of macrophages in the spleen has been related to the release of T cell cytokines, which appear to trigger a major cellular influx, leading to splenomegaly (Wozencraft et al. 1984); both macrophage influx and splenomegaly are absent in T-cell-deficient mice (Roberts and Weidanz 1979). During parasitic 'crisis' (when parasitemia starts to decrease under the influence of acquired immunity), there is a considerable increase in phagocytosis of malaria parasites, infected erythrocytes and malarial debris (particularly residual bodies and pigment); in rodent malaria, the phagocytic

activity of splenic macrophages has been estimated to be 20–50-fold greater than that of normal macrophages (Zuckerman 1977). This increase in phagocytic activity has been linked both to a specific activation of macrophages and to humoral factors, particularly opsonizing antibodies, which act in synergy with macrophages to enhance the destruction of parasites. Despite this massive deployment of defense mechanisms against the parasite, the process is not very effective in eliminating the infection; this may be explained by an impairment of some macrophage functions during malarial infection. The antigen-presenting function of macrophages, which is crucial to T-cell activation, may be impaired as the result of the suppressive effect of the malarial pigment ingested (Schwarzer et al. 1998; Taramelli et al. 2000). It has been suggested that cytoadherence of *P. falciparum*-infected erythrocytes to cells of the reticulo-endothelial lineage, particularly dendritic cells, may directly switch off their immunoregulatory function (Urban and Roberts 2002).

In addition to their phagocytic activity, macrophages and monocytes can produce a variety of toxic substances, which may damage or destroy malaria parasites; the production of these toxic molecules is generally enhanced in activated macrophages. Effector functions of macrophages include the release of reactive oxygen intermediates (hydrogen peroxide and hydroxyl radicals) (Clark and Hunt 1983), the production of tumor necrosis factor (TNF-α) (Taverne et al. 1990), and the production of many other cytokines and enzymes, which may adversely affect the parasite (e.g. polyamine oxidase which can kill *P. falciparum* in vitro) (Kumaratilake and Ferrante 1994). While activated macrophage-derived toxic products may damage parasites within erythrocytes (producing 'crisis forms'), these products may also damage the parasite in its exo-erythrocytic cycle, either directly or by means of the production of nitric oxide (Green et al. 1990). The infectivity of gametocytes may also be reduced as a result of the presence of macrophage products (particularly TNF and nitric oxide (NO)) (Naotunne et al. 1991).

T CELLS

It is well documented that T cells are crucial for malaria immunity (Weidanz and Long 1988; Ho and Webster 1989). As far as immunity to the erythrocytic stage of the parasite is concerned, the major functions of T cells seem to be to provide help for the production of antibodies and to activate macrophages. Studies of the development of immunity in murine malaria models, as well as a small number of studies in humans, suggest a slow progression from an initial Th1 response to a Th2 response. The Th1 response may be sufficient to maintain the infection in check (avoiding the kind of fulminant, rapidly lethal infection which occurs in T-deficient mice) but not sufficient to eliminate parasites altogether; clinical protection only occurs when the immune response has switched to an essentially Th2 mode, in which antibody-mediated mechanisms play the major role in the elimination of parasites (Taylor-Robinson et al. 1993; Langhorne et al. 2002).

While neither cytotoxic $CD4^+$ or $CD8^+$ T cells (CTLs) nor antibody-dependent cellular cytotoxicity (ADCC) have ever been conclusively incriminated in immunity to erythrocytic parasites, the situation is very different with exo-erythrocytic parasites since these stages reside within hepatocytes, which bear the MHC class I antigens required for CTL activity. In experimental models, CTLs from mice immunized with x-irradiated sporozoites can kill malaria parasites grown in vitro in mouse hepatocytes and appear to be involved in the destruction of exo-erythrocytic parasites in the livers of immunized mice (Hoffman et al. 1989). C-reactive protein, interferon (IFN)-γ, IL-1, IL-6, TNF, and nitric oxide have all been incriminated in the killing of exo-erythrocytic stages, probably in the form of a cascade of events triggered by T-cell recognition of sporozoite or liver stage antigens and involving both hepatocytes and Küpffer cells (Suhrbier 1991). There is now a reasonable body of evidence to incriminate CTLs in protection against the exo-erythrocytic stages of malaria parasites in humans (Lalvani et al. 1994).

γδ T CELLS

The γδ T cell receptor is normally only expressed in a small percentage of peripheral lymphocytes and, while the number of γδ T cells may be increased in certain infections, the role of these cells in immunity is still imperfectly understood. A substantial increase in the number of γδ cells has been reported both in experimental mouse malaria (Langhorne et al. 1993) and during the acute stage of infection in humans (Ho et al. 1994). Activated γδ cells from malaria-naive donors inhibit parasite growth in vitro, which confirms their role in the innate defense against infection (Troye-Blomberg et al. 1999). The presence of high levels of activated γδ cells contributes to the increased production of cytokines, particularly TNF-α, and may, thus, contribute to the development of a Th1 environment which would, in turn, facilitate the pathophysiological events leading to disease (Grau and Behr 1994).

SOLUBLE MEDIATORS AND CYTOKINES

In malaria-naive individuals, the first contact with the parasites triggers nonspecific defense mechanisms which include the production of IFN-γ by γδ-T cells and the production of interleukin (IL)-1 and TNF-α by macrophages; these will, in turn, stimulate phagocytosis and the production of oxygen-derived reactive species (ODRS) and NO by neutrophils and macrophages. A cascade of reactions leading to the production of NO is responsible for the killing of infected hepatocytes

(Green et al. 1990). As the infection progresses, the host response develops to a typical Th1 response, with the production of pro-inflammatory cytokines (including IFN-γ, TNF-α, lymphotoxin, IL-12, and IL-18) by T cells and activated macrophages) (Torre et al. 2001; Malaguarnera et al. 2002). At this stage, the ability of the host to produce IFN-γ is crucial: peripheral blood mononuclear cells taken from African children with mild malaria are significantly better at producing IFN-γ in response to malarial antigens in vitro, than cells taken from children with severe disease (Luty et al. 1999). It is now also well established that TNF, together with NO, can kill intra-erythrocytic parasites and contribute to the sudden decrease of parasitemia observed during 'crisis' (Naotunne et al. 1991).

As in other infectious diseases, there is only a fine line between the host-beneficial effects and the pathological consequences of immunity. Cytokines may be beneficial and contribute to avoid the development of an overwhelming parasitemia; increased pro-inflammatory cytokine levels also play a crucial role in the pathophysiology of malaria and the evolution towards severe forms of the disease. The outcome hinges on the ability of the host to regulate the inflammatory response.

After repeated infection, the immune response progressively transforms to a Th2 response, with the production of anti-inflammatory cytokines (including IL-4, IL-10, and TNF-β), which enable a more effective production of antibodies and down-regulate the inflammatory events leading to pathology (Troye-Blomberg et al. 1990; Kurtzhals et al. 1998; Omer et al. 2000).

Evasion of host immunity

Protective immunity against malaria parasites is slow to develop and a child born in a malaria endemic zone may need to be exposed to successive infections for years before developing any resistance; complete protection may never be achieved. This 'real-life' situation is in sharp contrast to what is observed in experimental situations where solid, sterile immunity can be achieved. The apparent inefficiency of antimalarial immunity may be explained by the ability of the malaria parasite to evade host immunity by the intrinsic poor immunogenicity of its antigens, by sequestration, by antigenic diversity and variation, or by an alteration of the host immune response (Mercereau-Puijalon et al. 1991).

POOR IMMUNOGENICITY

Crucial antigens involved in protection may be poorly immunogenic either because of their intrinsic molecular structure or their analogy to host molecules or because of immune restriction, which may impair recognition. The presence of multiple repeat sequences on a number of highly immunogenic erythrocytic stage antigens (e.g. RESA, S-antigens, or FIRA) has been interpreted as an evasion mechanism, since such immunodominant structures may act as a 'smoke screen' preventing the development of effective immunity to more relevant epitopes. The existence of cross-reactivity between repetitive epitopes of different malarial antigens (e.g. between RESA, FIRA, Pf11.1, Ag332, and S-antigens, see Table 3) has been interpreted as a further cause for poor immunogenicity since the presence of these epitopes may interfere with the normal maturation of the immune response towards a progressively increasing percentage of high affinity antibodies (Anders 1986).

SEQUESTRATION

The ability of parasites to remain sequestered by cytoadherence to the capillary lining of certain tissues must be regarded as a selected advantage since such parasites can avoid frequent passage through the spleen and thus exposure to immune effector mechanisms. *P. falciparum* isolates are able to switch rapidly from one endothelial receptor to another and this may be part of a parasite survival strategy, particularly since the change of receptor requirements may be in response to a changing environment of the host (acute malaria infection induces cytokine production which in turn up-regulates certain endothelial cell surface antigens) (Hommel 1997). Sequestration does not exist in the other human malaria parasites and this is considered the main reason for the difference in disease severity.

ALTERATION OF THE IMMUNE RESPONSE

Several reports demonstrate that malaria infection can induce a suppression of host immune responsiveness, including increased severity of *Salmonella* infections (Bennett and Hook 1959), decreased efficiency of tetanus vaccination (McGregor and Barr 1962), and the strong association between malaria and Burkitt's lymphoma (Marsh and Greenwood 1986). Various mechanisms of immunosuppression have been proposed, including polyclonal activation, macrophage dysfunction, abnormal antigenic presentation, disruption of lymphatic and splenic tissue architecture, activation of suppressor cells, and antigenic competition. While these mechanisms may affect the outcome of concurrent infections, they may also affect the ability of the host to mount an effective immune response to the malaria parasite itself.

ANTIGENIC DIVERSITY

In addition to the marked species- and stage-specificity of immunity to malaria parasites, there are also marked differences between isolates of the same species. Studies of S-antigens were the first to suggest that field isolates

of *P. falciparum* may vary in their antigenic structure (Wilson et al. 1975). The use of finer methods of analysis (including isoenzymes, two-dimensional gel electrophoresis, monoclonal antibodies, or restriction fragment length polymorphism (RFLP)) has provided further evidence of considerable diversity of both the phenotypes and genotypes of field isolates. Markers of diversity used for the typing of field isolates are mostly polymerase chain reaction (PCR)-based (or PCR combined with RFLP). The most frequently used primers are those derived from MSP-1, MSP-2, CSP-1, TRAP, GLURP, and the Pf60.1 multigene family (Contamin et al. 1995; Carcy et al. 1995). The polymorphism between isolates is usually considerable. In one African village, the study of isolates collected at a single point in time from 19 children revealed 35 different genotypes. The multiplicity of infection (i.e. when isolates consist of more than one genotype) has been linked to age, parasite density, and clinical severity; in a study in Tanzanian children, a greater multiplicity was observed in asymptomatic than in clinical cases (Beck et al. 1997).

The considerable diversity between field isolates may be the major reason why a long time is required to develop immunity, since an individual living in an endemic area would need to be exposed to a vast repertoire of local strains (Day and Marsh 1991). However, before accepting that antigenic diversity is a likely explanation for the slow development of immunity, it is necessary to assume that strain-specific immunity can ultimately be transcended by a broader species-specific immunity. In the absence of such a transcending immunity, it would be difficult to understand why infections become rarer in adults, since adults continue to be exposed to new isolates. The constant fluctuation of isolates 'in circulation' in a given geographical area has best been demonstrated in a study of parasite genotypes over time in villages of Papua New Guinea (Bruce et al. 2000).

ANTIGENIC VARIATION

Malaria parasites, as many other micro-organisms, are capable of periodically changing the expression of their antigens. This provides the parasite with a powerful means for evading host immunity particularly when antigenic variation occurs in conditions where a selective pressure is exerted. In all examples of antigenic variation, the common feature is the presence of successive peaks of infection with each new peak antigenically distinct from the previous one (the latter having been eliminated by host immunity) (Hommel 1985). Surface antigens, which are most exposed to immune pressure, are obviously most likely to exhibit antigenic variation.

In malaria, antigenic variation was first described in the *P. knowlesi*/rhesus monkey model (Brown and Brown 1965) and has since been shown to exist in *P. falciparum*. Antigenic variation has not yet been demonstrated for *P. malariae* or *P. vivax* but the long chronicity of the former and the presence of successive waves of recrudescence in the latter suggest the existence of either a fluctuating immunity or antigenic variation.

Many of the studies on antigenic variation in *P. falciparum* have concentrated on the molecules expressed on the surface of infected erythrocytes (e.g. PfEMP-1). In one study, a single isolate of *P. falciparum* was shown to be able to express ten different variant surface antigens when sub-inoculated in squirrel monkeys. For reasons that remain unclear, the set of variant antigens observed was different in intact and splenectomized animals (Hommel et al. 1991). The rate of switching from one set of antigens to another is thought to be fast; in an in vitro study of *P. falciparum*, 2 percent of parasites switched to a new antigen in every erythrocytic cycle (Roberts et al. 1992).

Antigenic variation of *P. falciparum* has been linked to a large gene family (the *var* genes), which appear to be differentially expressed in different individual parasites (Su et al. 1995; Smith et al. 1995). It has been calculated that the genome of a given clone of malaria parasites has between 50 and 60 different *var* genes (i.e. approximately 5 percent of the total genome), that *var* genes are scattered over most of the malarial chromosomes, and that only one *var* gene may be expressed at a time in a given parasite. Mathematical modeling of switching rates, based on the number of *var* genes that can be transcribed in vitro in one cycle of division, suggests that switching may be as fast as 18 percent per generation (i.e. nine times higher than estimates based on biological observations) (Gatton et al. 2003). A group of similar molecules exists in other *Plamodium* species, e.g. *vir* genes in *P. vivax* (Del Portillo et al. 2001) or *SICAvar* in *P. knowlesi* (encoding for what was previously known as the schizont-infected cell agglutination antigen (SICA), the first variant malarial variant antigen described) (Al-Khedery et al. 1999).

ANTIDISEASE IMMUNITY

Acquired immunity to malaria in humans is a mixture of 'antidisease' immunity, which results in decreased clinical manifestations despite infection, and of 'antiparasite' immunity, which results in a control of the infection itself. The mechanisms of these two types of immunity are clearly distinct since antidisease immunity usually occurs much before antiparasite immunity. The consequence of this is that an individual living in a highly endemic area of the world may harbor very heavy parasite loads and be clinically well (McGregor et al. 1956; Miller 1958). Little is known of the mechanisms leading to antidisease immunity but the most attractive hypothesis so far is that host immunity interrupts a cascade of events, initially triggered by the production of a series of cytokines (including TNF and IL-6) in

response to malarial 'endotoxins' (Playfair et al. 1990); the nature of malarial endotoxins may be either lipidic or carbohydrate in nature, which suggests that antidisease immunity may be controled by T-cell-independent mechanisms and consequently requires frequent boosting to be sustained.

The term 'premunition' refers to a situation where malarial infection is only partially controled by the host (i.e. a state of 'relative immunity') with elements of both antidisease immunity (e.g. the existence of asymptomatic infections) and antiparasite immunity (e.g. an age-dependent reduction of parasitemia), hence the confusion the use of the term has generated in the literature. Recent studies in Indonesia have suggested that, in contrast to established dogma, a state of premunition may be more easily reached by adult migrants to an endemic area rather than by young children with life-long residence in such an area (Baird 1995).

Whether antidisease immunity is specifically linked to a response to the so-called malarial toxins has not yet been conclusively established. *Plasmodium* glycosylphosphatidylinositol (GPI), which serves as a membrane anchor for a number of parasite surface antigens, is the most likely candidate molecule, as it has been shown to trigger a variety of pathways involved in the pathophysiology of malaria, including the induction of TNF-α and NO (Schofield et al. 1996). In a study in Papua New Guinea, antibodies to GPI were inversely associated with increased tolerance to high parasite loads in local residents (Boutlis et al. 2002), but the same association between anti-GPI antibodies and protection against mild or severe disease was not found in a study in the Gambia, showing that the acquisition of anti-GPI antibodies was age- and exposure-dependent (De Souza et al. 2002).

Immunity and nutrition

The nutritional status of the host plays an important role in parasite growth and the outcome of malarial infections. This was first demonstrated in mice fed on a milk diet (lacking the crucial vitamin *p*ABA), which were partially protected from infection (Ferone 1977). Similarly, riboflavin (vitamin B2) deficiency confers a degree of protection against malaria infection (Das et al. 1988). Malnutrition and kwashiorkor are also said to reduce susceptibility and, in famine-relief situations, outbreaks of clinical malaria frequently occur during refeeding (termed 'refeeding malaria') where the improved nutritional status allows previously suppressed malaria to develop (Edington 1967; Murray et al. 1978b). This classical concept has recently been challenged after a careful re-analysis of published studies (reviewed by Shankar 2000), which showed that malnutrition was actually more often associated with an increased risk of malaria, suggesting a more complex relationship between nutrition and malaria than previously supposed. Malnutrition in Kenyan children has been shown to be an important risk factor of severe malaria (Marsh et al. 1995) and a cohort study in Colombia showed that malnourished children had lower antimalarial antibody levels, suggesting an impaired immune response. A variety of specific deficiencies (including vitamin A, thiamin, and zinc deficiencies) has been found to be more frequent in individuals with severe malaria than individuals with mild forms of the disease (Gibson and Huddle 1998; Krishna et al. 1999). Micronutrient deficiencies are of particular interest since their mode of action is more often on host response to infection and immunity than direct effect on parasite development. Both zinc and vitamin A are, for instance, essential for effective immune responses. Intervention campaigns using micronutrient supplementation (particularly iron, vitamin A, and zinc) have had beneficial effects on the whole, showing reduced risk and reduced intensity of infection, but their impact was variable from one population to another. For example, a study in Papua New Guinea has shown that vitamin A supplementation (200 000 IU every 3 months) significantly reduced the number of clinical episodes and that zinc supplementation reduced both the number and the severity of clinical episodes (Shankar et al. 1999). By contrast, a randomized trial of zinc supplementation in children in Burkina Faso did not show any significant effect on malaria (Müller et al. 2001).

Immunity and chemoprophylaxis

It seems well established that appropriate chemoprophylaxis, taken regularly (as is done by expatriates residing in an endemic area) interferes with the development of immunity. The situation is more confused when the effects of targeted mass chemoprophylaxis are studied, as in the Garki Project in Nigeria or, more recently, in the Gambia. Young children receiving chemoprophylaxis are not fully protected from malaria infections, but infections are less heavy, less frequent, and there is a substantially reduced mortality and morbidity (Greenwood 1991). In these children, antibody levels are generally significantly reduced, but reduction is less marked the longer the chemoprophylaxis is given; in contrast, lymphocyte proliferation or IFN-γ responses are increased suggesting that reduced infection levels generate more effective cell-mediated responses. Once chemoprophylaxis is interrupted, there does not appear to be a 'rebound' effect in clinical attacks, morbidity, or mortality, which suggests that children, having received some form of chemoprophylaxis over the first few years of life and thus suffered less from their malaria, have nevertheless developed an antimalarial immunity comparable to that of children without chemoprophylaxis. In any situation where mass chemoprophylaxis is

associated with other effective means of prophylaxis (e.g. impregnated bednets), the chance of infection may be so substantially reduced that the development of immunity can be impaired (Alonso et al. 1993). Intermittent presumptive treatment (IPT), whether in pregnant women or in infants, is theoretically less likely to interfere with the development of immunity, but extensive studies of the immune response have not yet been performed during IPT interventions.

Antimalarial drugs may act much more effectively in semi-immune than in naive individuals, due to a synergy between the effect of drugs and immunity. A consequence of this is, in areas where there is resistance to a given drug, the clinical response may be very different in different age groups. For example, in a holoendemic area, there may appear to be a lower degree of drug resistance in school age children than in infants. Another consequence of this synergy is that, in hyper- or holoendemic areas, where most of the population reaches a state of semi-immunity from a relatively early age, drugs like CQ may continue to be of benefit to the local population even when a relatively high degree of parasite resistance has been reported. This has implications in the interpretation of in vivo drug susceptibility tests and explains some of the discrepancies observed between in vitro and in vivo tests.

Malaria immunity and acquired immunodeficiency syndrome

The question of whether the two major causes of mortality in sub-Saharan Africa, malaria, and acquired immunodeficiency syndrome (AIDS), interact with each other still remains controversial. This is surprising since even minor synergy between the two would be considered of public health importance. While none of the early hospital-based studies in Zaire, Rwanda, or Zambia showed any obvious interaction during co-infections in adults (Nguyen-Dinh et al. 1987; Simooya et al. 1988; Allen et al. 1991), a cohort study did show increased levels of parasitemia in Ugandan adults with HIV, with a progressively increasing incidence of clinical malaria associated with falling CD4 counts (Whitworth et al. 2000). The situation is much clearer during pregnancy. A number of studies in Malawi and Kenya have demonstrated a synergy between HIV infection and malaria, leading to higher maternal parasitemia and increased fetal mortality in co-infections (Bloland et al. 1995; Steketee et al. 1996b; Verhoeff et al. 1999; Van Eijk et al. 2003).

Does HIV-induced immunosuppression affect immunity to malaria? HIV is well known to influence infection by a variety of intracellular parasites (e.g. *Leishmania*, *Toxoplasma gondii*, or *Cryptosporidium parvum*) but this is always explained by a decrease in the Th1 response and would, therefore, not be expected to have a substantial impact on protection against malaria in semi-immune adults, since immunity is believed to be mostly Th2/antibody-dependent. Antimalarial antibody levels are not significantly reduced in African adults with HIV and only a few other parameters of malaria immunity have so far been examined in HIV-co-infected patients. The Th1 cytokine response to MSP1 was, for instance, predictably reduced in HIV-infected adults in Burkina Faso (Migot et al. 1996). A loss of pathogen-specific $CD4^+$ T cell immunity would mostly affect immunity to exo-erythrocytic parasites and the increase in parasitemia, observed in the Ugandan study, could thus be explained. However, increased levels of parasitemia were not observed in another Ugandan cohort study, in which the incidence of malarial fever was significantly higher in HIV-positive individuals (French et al. 2001), suggesting that HIV-positives may be more responsive to parasites or parasite-derived toxins. This is surprising since fever is usually induced by the pro-inflammatory cytokines produced in response to parasite toxins and this would be less effective in a Th1-depressed environment. Clearly, HIV infection raises unresolved issues in our understanding of acquired immunity to malaria. Even the reports which indicate increased clinical malaria in HIV-co-infected individuals do not suggest a significant increase in malaria mortality and, in particular, no increased incidence of cerebral malaria. This is in keeping with existing hypotheses regarding the pathophysiology of cerebral malaria, which involve the up-regulation of endothelial cell cytoadherence receptors (e.g. ICAM-1) which occurs only in a Th1 environment; in support of this are the results obtained in murine malaria models, where the immunosuppression induced by murine AIDS or in IFN-γ knock-out mice, prevents the development of cerebral malaria in the *P. berghei* ANKA model (Eckwalanga et al. 1994).

In pregnancy, HIV infection clearly acts in synergy with malaria to increase both maternal and fetal morbidity/mortality (e.g. a 3.4-fold increased infant mortality has been reported) (Bloland et al. 1995). The most striking finding is the fact that this increased severity of malaria in HIV-co-infected women has been reported to equally affect primi- and multigravidae. This observation contradicts current hypotheses regarding the pathophysiology of malaria in pregnancy, where the crucial event is the difference between primi- and multigravidae, explained by the acquisition of immunity against novel, placenta-specific malaria isolates rather than by the previously accepted belief in pregnancy-associated immunosuppression. It is probable that cytokine dysregulation plays an important part in this. Protection against placental malaria relies on a Th1 environment in the placenta, with increased IFN-γ response to malarial antigens, and this response is impaired in HIV-infected women (Moore et al. 2000).

Does malaria affect the course of HIV infection? Enhanced viral replication and higher viremia have been

reported in association with malaria, which suggest a potentially more rapid evolution and an increased risk of transmission (see review by Rowland-Jones and Lohman 2002). The fact that severe anemia may require blood transfusion is in itself a risk of HIV infection, particularly in children. The suggestion that malariotherapy may be reintroduced in order to treat patients with AIDS is highly controversial, despite the impressive increase in CD4 levels observed during pilot studies performed by the Heimlich Institute in China (Nierengarten 2003).

PATHOLOGY AND PATHOPHYSIOLOGY

Infection by malarial parasites may lead to a variety of clinical syndromes, depending on a combination of different elements, including the 'virulence' of the parasite isolate and a variety of host-related factors such as the status of host immunity and its genetic make up. The development of asexual parasites in the blood plays the central role in disease pathophysiology and, for most malarial species, it is the rupture of the schizont which triggers off the major events leading to the characteristic symptoms of the malarial paroxysm.

Direct effects of the parasite on the host

The rupture of the mature schizont has three consequences:

1. the liberation of merozoites, which leads to further invasion and a sharp increase in parasitemia;
2. the destruction of infected erythrocytes;
3. the liberation of malarial antigens, pigment, and malarial toxins.

While erythrocyte destruction may contribute to the anemia observed in malaria, this is only of importance in chronic infections of semi-immune individuals where high parasitemia (e.g. 5–15 percent) is relatively well tolerated. In nonimmunes, malarial paroxysms occur at very low parasitemia (less than 1 percent) and the effects of massive erythrocyte destruction are negligible at this stage. In contrast, it is the release of malarial antigens, pigment and toxins which are responsible for most of the pathology. Most of these effects are indirect (by means of a cascade of pathological events in the host) (see Indirect effects of the parasite on the host). However, among the parasite products released, two main ones have direct pathological effects: (1) Malarial pigment, which is taken by monocytes, inhibits some of their phagocytic and immunological functions and contributes to the immunosuppression observed (particularly since pigment remains in the reticulo-endothelial system for many years after infection) (Arese et al. 1991); and (2) malarial 'toxins,' including GPI and lipid antigens, may be responsible for the induction of hypoglycemia by acting directly on glucose uptake by adipocytes in synergy with insulin (Taylor et al. 1992; Schofield et al. 1966; Zakeri et al. 2000).

Indirect effects of the parasite on the host

Indirect effects are by far the most important and the production of cytokines, particularly TNF-α induced by the release of parasite products after schizont rupture, appears to play the central role in the pathophysiology of malaria.

TUMOR NECROSIS FACTOR

Several arguments have been put forward to support the hypothesis that TNF-α plays a central role: the experimental injection of TNF-α in humans produces symptoms that closely resemble a malarial paroxysm (Clark et al. 1989a); the rise in temperature during *P. vivax* paroxysms closely follows the rise in circulating levels of TNF-α (Karunaweera et al. 1992); the levels of TNF-α are higher in severe than in mild forms of falciparum malaria and particularly high in fatal cases (Grau et al. 1989); the injection of anti-TNF-α monoclonal antibodies to children with severe malaria abolishes some of the symptoms, particularly fever (Kwiatkowski et al. 1993); there are many similarities between severe malaria and sepsis, and TNF-α and phospholipase-A2 are substantially increased in both (Vadas et al. 1993). The multifaceted effects of TNF-α are illustrated in Figure 18.

At physiological levels, TNF-α may destroy intracellular parasites or reduce the infectivity of gametocytes to mosquitoes. At increased levels, TNF-α induces fever, depresses erythropoiesis, and increases erythro-phagocytosis, thereby contributing to anemia, and directly causes many of the nonspecific symptoms of malaria including nausea, vomiting, and diarrhea.

Experiments in mice suggest that cerebral malaria (CM) may be due to an overproduction of lymphotoxin-α (LT-α) (a cytokine which was previously known as TNF-β) rather than TNF-α. Mice deficient in LT-α were resistant to CM, dying from hyperparasitemia and severe anemia instead, while mice deficient in TNF-α behaved like controls (Engwerda et al. 2002). This study suggests that caution may required in the interpretation of TNF data, since the two molecules share many features and most immunological assays for TNF do not discriminate between TNF-α and LT-α.

Although the circulating level of other 'endogenous pyrogens,' such as IL-1 and IL-6, is also increased during malaria (Kern et al. 1989), they appear to play only a modest pathophysiological role in severe malaria, probably in synergy with TNF-α, as suggested by Rockett and colleagues (1994). IL-6 may play a role in the induction of hypergammaglobulinemia and contribute to some

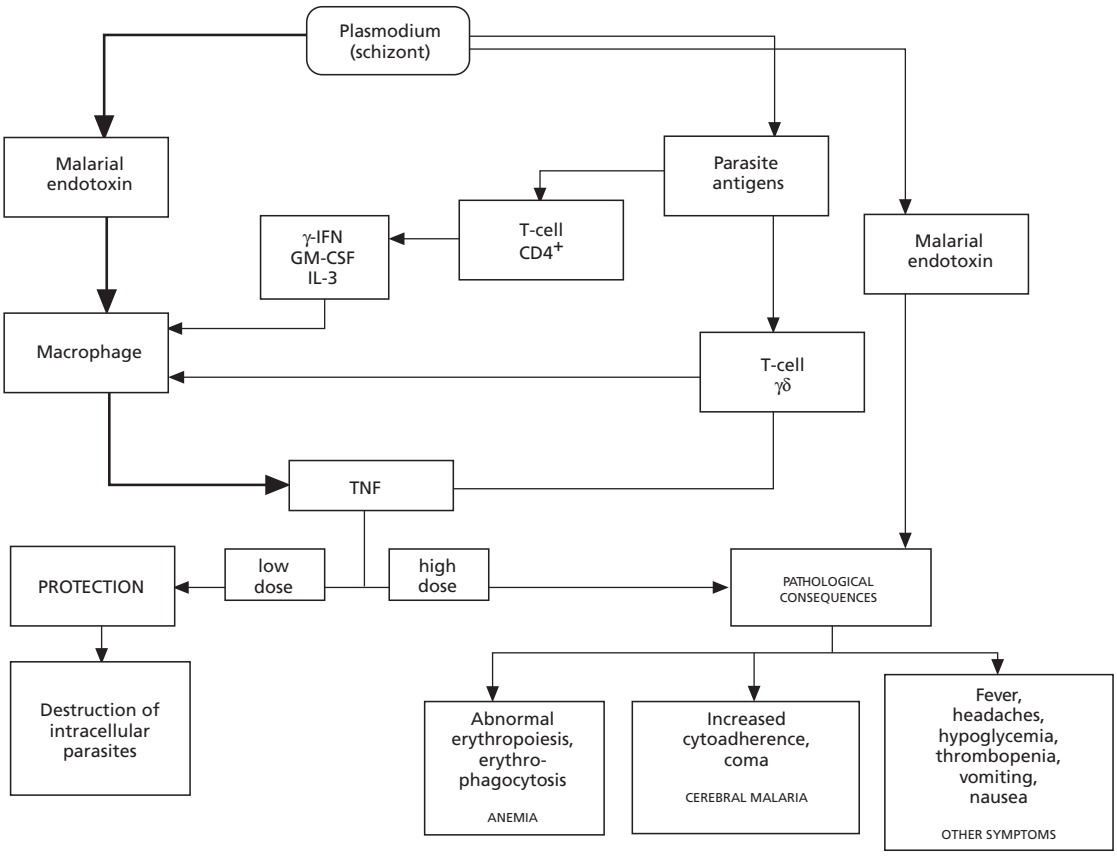

Figure 18 *Role of TNF in the pathophysiology of malaria*

of the malaria complication (e.g. glomerulonephritis and increased frequency of Burkitt's lymphoma) (Grau et al. 1990). One of the major consequences of the inflammatory events is the production of NO as the result of the up-regulation of the synthesis of iNOS. NO is a short-lived, highly reactive molecule with a wide spectrum of biological activities.

INDUCTION OF CYTOKINE PRODUCTION

The induction of inflammatory cytokines is determined by two independent pathways: (1) the direct action of malarial toxins on host macrophages and of malarial superantigens on γδ T cells; and (2) the immune response against malarial antigens and the production of Th1 cytokines (particularly IFN-γ), which up-regulate the production of inflammatory cytokines and act in synergy with them (Clark and Rockett 1994).

The nature of malarial toxins is still controversial, but whether it is hemozoin (Sherry et al. 1995), a proteolipid (Bate and Kwiatkowski 1994), GPI (Schofield et al. 1993), or polar lipids (Zakeri et al. 2000), most authors agree that the toxin is released at the time of schizont rupture, that it is at least partly lipidic in nature, and that it probably is associated with the pigment-containing 'residual body.' Such a toxin can induce TNF-α production by host cells in vitro and this production may be blocked by antitoxic antibodies (Bate et al. 1992). Different isolates of *P. falciparum* can vary considerably in their ability to induce TNF-α and this may be one explanation for the observed differences in the clinical response (Allan et al. 1993). The reader needs to be aware that, since *Mycoplasma* contamination of *P. falciparum* cultures is common (Turrini et al. 1997), the reported results on so-called cytokine-inducing malaria toxins must be carefully disentangled from the effects of potent cytokine-inducing molecules of mycoplasmal origin; this is not always possible from published data. The malarial 'superantigens' responsible for the proliferation of γδ T cells are phosphorylated molecules, similar to isopentenol pyrophosphate from *Mycobacterium tuberculosis* (Behr et al. 1996).

UP-REGULATION OF CYTOADHERENCE RECEPTORS

Cytoadherence of infected erythrocytes to endothelial cells plays a central role in pathophysiology, particularly in the induction of cerebral malaria (MacPherson et al. 1985). Rosetting, which is another form of cytoadherence where each infected erythrocyte binds a number of uninfected erythrocytes, may contribute to this effect. Different isolates of *P. falciparum* are more or less

cytoadherent to a variety of receptor molecules, including CD36, ICAM-1, thrombospondin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), chondroitin sulfate (see reviews by Hommel 1993; Sherman et al. 2003); differences in cytoadherence characteristics may represent a virulence factor and there is some evidence of a correlation between high cytoadherence/rosetting and cerebral malaria (Carlson et al. 1990; Ho et al. 1991).

One of the features of inflammatory cytokines in malaria is to up-regulate the expression of cytoadherence receptors (e.g. ICAM-1 or VCAM-1) in certain tissues; this very localized effect results in an increased sequestration in specific tissues. For example, cerebral malaria may occur when increased cytoadherence takes place preferentially in the brain. There is evidence that receptor up-regulation may also require a Th1 immune response (particularly IFN-γ) (Grau and Behr 1994) and that the development of cerebral complications may be avoided by the injection of Th2 mediators such as IL-10 (Ho et al. 1995).

One of the effects of cytokine-induced up-regulation of cytoadherence receptors is an increased level of the soluble forms of these molecules in the serum of patients. High levels of sICMA-1, sVCAM-1, sELAM-1, or thrombomodulin have not, however, been correlated with increased malaria severity (Hemmer et al. 1994; McGuire et al. 1996).

OXYGEN-DERIVED FREE RADICALS AND NO

The stimulation of pro-inflammatory cytokines leads to a cascade of events, including the release of various mediators, including oxygen-derived free radicals (ODFR) and NO, which are responsible for the pathology observed. The role of ODFR and NO has been extensively reviewed by Clark and Cowden (2003), who suggest that a better understanding of the mode of action of these mediators may open up new avenues for the treatment of severe malaria. For instance, ethyl pyruvate has been shown to be a potent scavenger of ODFRs and can dramatically reduce the effects of septic shock and systemic inflammation in mice (Ulloa et al. 2002); also ethyl pyruvate has not yet been used in severe malaria, desferrioxamine, another scavenger of reactive oxygen, has been shown to reduce the duration of deep coma in African children with cerebral malaria (Gordeuk et al. 1992). Unfortunately, research in this field is difficult because of the evanescent nature of molecules such as NO, as well as the complex interactions between cytokines, secondary mediators, host cells, and parasites. Suggestive experimental data in murine models have often proved hard to confirm in humans, particularly in falciparum malaria where the parasites are sequestered and the interactions occur in inaccessible tissues. In order to measure increased NO synthesis as the result of iNOS stimulation in the brain, the circulating levels of nitrates and nitrites, both NO metabolites, have been measured; the confounding effect of diet and renal impairment often make these measurements difficult to interpret and contradictory results are found in the literature. Al-Yaman et al. (1996) reported an elevation in reactive nitrogen metabolites in children with cerebral malaria in Papua New Guinea, while such an increase was not observed in comparable studies in Tanzania (Anstey et al. 1996) or Ghana (Agbenyega et al. 1997). More direct evidence of the role of NO in pathogenesis is the detection of local iNOS production. This has been found in a small number of autopsy studies by immunocytochemical staining of iNOS in cerebral vascular walls (Maneerat et al. 2000; Clark et al. 2003).

Pathophysiology of different forms of malaria

The cascade of events leading to pathology is a complex one: it is influenced by both parasite and host factors (including host immunity and genetic 'susceptibility' factors). Why do apparently similar events lead to mild malarial paroxysms in some cases and to cerebral malaria or severe anemia in others?

MALARIAL PAROXYSM

The clinical features of uncomplicated malaria, whatever the parasite species responsible, include fever, headache and muscle ache, lethargy, sweating, and shivering; in its more typical form, the 'malarial paroxysm' resembles the endotoxin reactions described in other infections and has a characteristic periodicity (e.g. recurring every 48 h. Classical malarial paroxysms are more often observed during *P. vivax* than *P. falciparum* infections (see review by Karunaweera et al. 2003). The following sequence of events: rupture of schizont, with release of malarial antigens, hemozoin and pigment, which in turn trigger the release of TNF and other pyrogens by host cells, is generally accepted as the starting point of the paroxysm.

The pivotal role of TNF has best been demonstrated by the injection of TNF into humans, which reproduces all the symptoms of a malarial paroxysm. In clinical studies of vivax paroxysms, the rise of body temperature follows the rise of circulating TNF after an interval of 30–45 min, suggesting a direct causal relationship (Karunaweera et al. 1992). The level of parasitemia needs to reach a critical threshold before the rupture of schizonts is able to trigger a clinical paroxysm and, hence, the first few erythrocytic cycles are generally asymptomatic. The threshold of parasitemia is, however, variable from one individual to another: a malaria-naive individual is likely to experience a malarial parasitemia at a barely detectable parasitemia (0.001 percent), while school-age children in a malaria-endemic area may remain asymptomatic at very high parasitemia (5–15 percent). Fever

threshold and antidisease immunity are closely related concepts.

CEREBRAL MALARIA

Cerebral malaria is a severe complication of falciparum malaria and frequently leads to death, even when appropriate therapy has been given. Various hypotheses for the pathophysiology of CM have been proposed over time. While the theories implicating microvascular obstruction by thrombus formation, disseminated coagulation, or immunopathological events due to the deposition of immune complexes have now been generally abandoned, the currently favored hypothesis assumes a central role for intracapillary sequestration of infected erythrocytes by cytoadherence to various endothelial receptors (MacPherson et al. 1985).

Although it has been established that different isolates are more or less cytoadherent to a variety of receptors, it is becoming clear that if 'stickiness' is a necessary requirement, it cannot solely explain the evolution of infection towards CM. The effects of TNF (in synergy with other pro-inflammatory cytokines) in the up-regulation of endothelial cell adherence receptors is another necessary feature in the events that lead to CM in susceptible individuals; immune status is as important as host genetic make up, since receptor up-regulation works best in a Th1 (i.e. during the early stages of malarial immunity) and is reversed in a Th2 environment (for example, by injection of IL-10) (Ho et al. 1995). Genetic susceptibility to CM has been described in various situations (e.g. in certain alleles of iNOS promoter gene, the TNF-α promoter gene or in individuals with the ICAM-1^{Kilifi} allele). It is conceivable that, as a consequence of the presence of Th1 cytokines, other cascades of mediators may be generated (e.g. prostaglandins or tryptophan pathways) which aggravate the situation; it has been shown, in mice, that animals with CM had increased levels of the neurotoxic quinolinic acid, a by-product of tryptophan metabolism (Sanni et al. 1998).

This classical description of the pathophysiology of CM is based on studies of CM in Thailand. More recent studies of CM in children in Malawi and Kenya suggest that this classical picture may not always be found and that CM is, in reality, a wide spectrum of clinical entities. In an autopsy study in Malawi, only 14/32 children had a histological picture of brain tissue compatible with the cascade described above, i.e. significant sequestered parasites, microhemorrhages, intravascular accumulation of monocytes, fibrin, and presence of iNOS staining in vascular walls (Clark et al. 2003). The other cases described in the same study were histologically atypical, many with little or no tissue damage, no detectable iNOS in vascular walls and only minimal sequestration of parasites. The authors suggested that such atypical cases of CM could be the result of concomitant sepsis or metabolic disorders, which had led to coma before the level of parasitemia was sufficient to fill capillaries with sequestered infected erythrocytes. It had previously been established that the risk of mortality was four times greater when metabolic acidosis was associated with CM (Marsh et al. 1996). Histological features, like the disruption of endothelial intercellular junctions (Brown et al. 2001), suggesting an impaired blood–brain barrier, have only rarely been observed and it is generally believed that the blood–brain barrier remains intact in CM (Warrell et al. 1986; Badibanga et al. 1986). This is confirmed by the absence of leakage of radioactive albumin from the serum to the CSF and the lack of detectable cerebral edema on computed tomography (CT) scans.

In view of the different possible pathways leading to CM, coma may be caused by poor oxygen delivery to the brain, due to sluggish blood flow and partial mechanical obstruction or reduced oxygen utilization by brain tissue resulting from an inhibition of mitochondrial functions by the inflammatory process. In both cases, local hypoxia is the crucial factor leading to coma. When hypoxia occurs at the same time as hypoglycemia, or the accumulation of lactate, due either to the high parasitemia in capillaries resulting from sequestration or to the accumulation of glutamate (as a consequence of a reduction of glutamate uptake by astrocytes induced by NO), the resulting neurological damage is always greater than with hypoxia alone.

Neurological damage in CM is usually reversible, with less than 10 percent of African children suffering from neurological sequelae. Raised intracranial pressure is a major cause of poor outcome in encephalopathies, because of the risk of transtentorial herniation or a reduction in cerebral perfusion pressure. This may happen during severe forms of CM and is considered an indicator of poor outcome (i.e. death or neurological sequelae) (Newton and Krishna 1998). Seizures also reflect intense neurological damage when seen in children with CM; multiple and prolonged seizures during CM are always a sign of bad prognosis (Molyneux et al. 1989).

Figure 19 shows the sequence of events leading to CM, taking into account the relevant features of the parasite, the ability to cytoadhere, and the ability of parasite toxins to induce inflammatory cytokines, as well as the various points in the cascade where host factors may intervene.

ANEMIA

The pathophysiology of anemia is still poorly understood (Menendez et al. 2000). In nonimmunes, the destruction of erythrocytes by growing parasites cannot alone explain the degree of anemia observed, because malarial paroxysms occur at low parasitemia (1 percent or less), insufficient to produce anemia. By contrast, individuals having lived their life in malarious areas

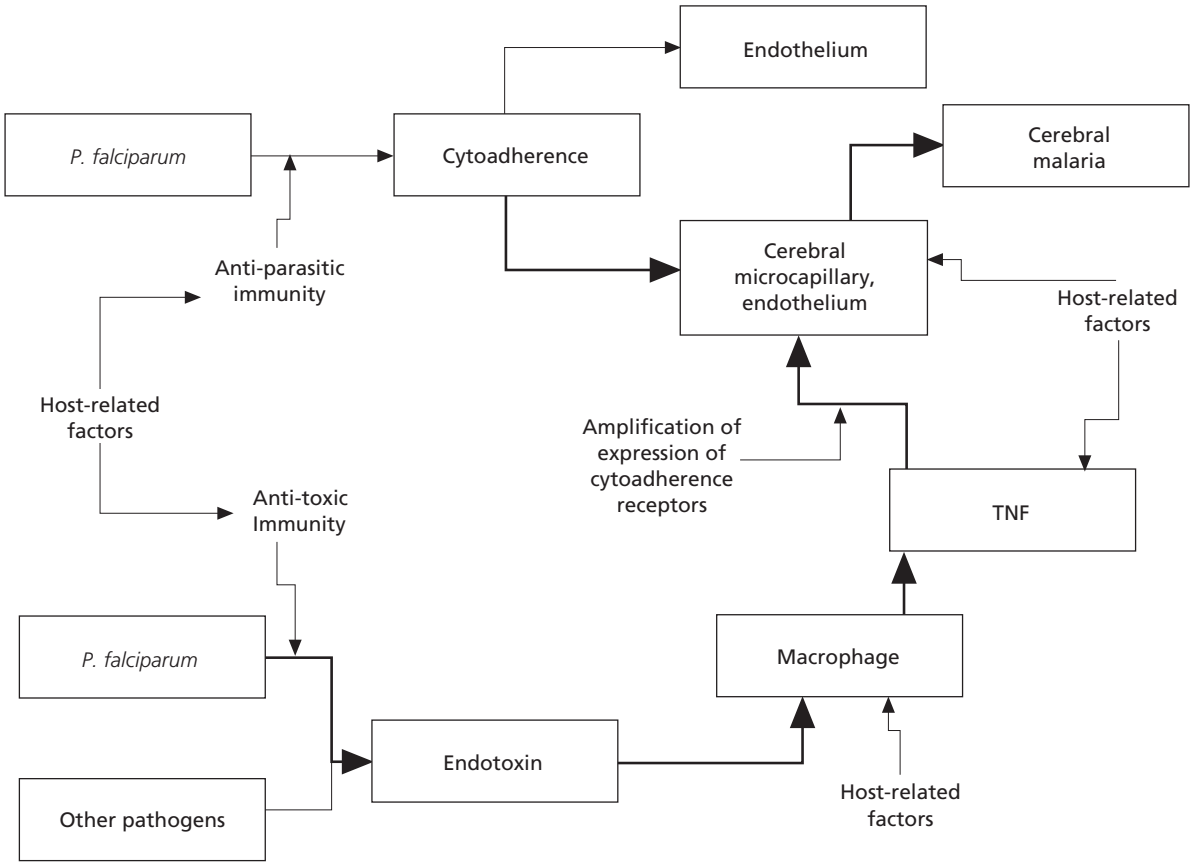

Figure 19 *Sequence of events leading to cerebral malaria*

have acquired a degree of tolerance to high parasitemia and, while such individuals suffer from mild anemia as the direct consequence of important erythrocyte destruction, anemia in older children or adults is not considered a serious health problem.

Severe anemia, such as occurs in early childhood before immunity is effective, is due to the combination of two abnormalities: (1) an increased destruction of normal erythrocytes by erythro-phagocytosis, particularly in the spleen; and (2) impaired production of new erythrocytes in the bone marrow. Dyserythropoiesis, with its accompanying low reticulocyte counts has long been known to be a major cause of malarial anemia (Abdalla et al. 1980). TNF is known to stimulate erythrophagocytosis and depress erythropoiesis in bone marrow (Clark and Chaudri 1988) and an association between high levels of pro-inflammatory cytokines and severe anemia has been observed in African children (Biemba et al. 2000). In addition to TNF, another T-cell cytokine, macrophage inhibitory factor (MIF), has recently been shown to play a role in bone marrow failure in a murine malaria model (Martiney et al. 2000), but this has not yet been confirmed in human malaria. Some authors believe that cytokine-mediated suppression alone could not explain the gross morphological bone marrow abnormalities described in malaria and that other contributing factors need to be looked for. The observation that a high proportion of hemozoin-containing monocytes in the peripheral blood (over 1/500 monocytes) correlates well with the severity of anemia, suggests that hemozoin, or toxic metabolites, induced by the presence of hemozoin, may contribute to malarial dyserythropoiesis. In addition, nutritional factors, particularly severe iron deficiency, may contribute to the pathophysiology of severe anemia, as was observed in adults in Thailand (Phillips et al. 1986), but not confirmed in children in Kenya (Newton et al. 1997).

Of interest is the reported difference in age prevalence of severe anemia (in early childhood) and CM (in children over 2 years old). This is consistent with the view that the pathogenesis of CM has a greater immunological component than anemia, such as a synergy between TNF and IFN-γ. It has been suggested that the priming of T cells in early life may lead to excessive IFN-γ production on reinfection, predisposing the individual to over-production of TNF and resulting in an increased risk of severe pathology (Riley 1999).

MALARIA IN PREGNANCY

A paradoxical situation prevails during pregnancy in women with lifelong residence in a malarious area where

a state of semi-immunity has been reached. Despite an apparent loss of immunity during pregnancy, these women are able to transfer a fully effective protective immunity to their infant. This phenomenon is particularly pronounced in primigravidae and becomes less of a problem during later pregnancies.

The features of falciparum malaria in a primigravida are the following: infection and parasite development take place in the placenta, while remaining at a low level in the peripheral circulation. In the absence of treatment, an infection acquired during the early stages of pregnancy will be maintained throughout pregnancy in the placenta, often with a heavy parasite load. Severe anemia in the mother and a low birth weight in the infant are the two major consequences. In extreme cases, there may be maternal death or abortion of the fetus. It is noteworthy that other forms of severe malaria (such as CM) do not occur in this situation.

The classical explanation of what happens during malaria in first pregnancies is that a reduction of acquired immunity takes place, consistent with the immunosuppression generally observed in all pregnancies. This is in line with the observation of reduced lymphoproliferative responses in pregnant women as compared to age-matched nonpregnant individuals (Rasheed et al. 1993). However, crucial studies, such as the longitudinal follow up of a cohort of women throughout their first pregnancy, have never been performed. The suggested loss of immunity does not account for the ability to transfer immunity to the infant, even if we accept that the described reduction of immune response is mostly in the cell-mediated response, while passive transfer refers mostly to antibodies.

Two alternative explanations for malaria in pregnancy have been proposed. The first takes account of the fact that the placenta is a 'new' organ in primigravidae, which could by-pass existing immune responses in the host, without actual loss of immunity, or allowing for the development of unusual phenotypes of *P. falciparum*. This would be compatible with the observation that parasites which cytoadhere to chondroitin sulfate are unusually found in the general population, but may be common in the placenta (Fried and Duffy 1996). The acquisition of a placenta-specific immunity or previous exposure to new malaria phenotypes in an earlier pregnancy would explain decreased susceptibility to infection in multigravidae. The second alternative suggests that what happens in pregnancy is a change in the balance of the local placental environment from an essentially Th2 environment, as normally prevails in the placenta, to a Th1 environment during acute malaria, a feature which is consistent with the presence of a large number of monocytes in an infected placenta.

Further details on the pathogenesis of malaria may be found in one of the many excellent reviews recently published on the subject (White 2003b; Newton and Krishna 1998; Miller et al. 2002; Clark and Cowden 2003; Clark et al. 2004). The histopathological changes of falciparum malaria have been described in detail on numerous previous occasions (Edington 1967; Edington and Gilles 1976; Lucas 1992; Warrell et al. 2002).

Immunopathology

The fact that the host immune system appears to have difficulty controling the malarial infection is probably the reason why so many aberrant responses have been described. Aberrant responses are generally caused by an over-reaction of the host, where an immunologically inappropriate response has no effect on the parasite but may have immunopathological consequences of various degrees of severity. Table 4 gives a list of immunopathological complications of malaria, some of which have already been briefly described; detailed descriptions of these complications may be found in reviews by Marsh and Greenwood (1986) and by Ho and Sexton (1995).

DIAGNOSIS

Clinical diagnosis

The most important aspect in clinical diagnosis of malaria is a high index of suspicion (Doherty et al. 1995). In these days of widespread travel, a history of travel should be elicited in all patients, particularly febrile ones. It is also important to remember that population movements are common within countries where malaria is not uniformly endemic.

Malaria can mimic many diseases and the differential diagnosis is an extensive one. The golden rule is to always exclude malaria, irrespective of the clinical presentation, if a history of exposure is elicited, even if the patient has been on chemoprophylaxis. The commonest misdiagnoses in nonimmune subjects have been influenza, viral hepatitis, viral encephalitis, meningitis, psychosis, and viral hemorrhagic fever.

Clinical diagnosis can be further guided by epidemiological considerations, e.g. acute febrile illness in high risk groups, such as pregnant women or children under 5 years of age in holoendemic areas. Association with particular occupations, such as those working in forest or forest fringe areas, may be strongly correlated with malaria infection and may serve as a pointer to diagnosis.

The discovery of parasitemia provides an explanation for symptoms and signs in a nonimmune patient, this is not necessarily the case in semi-immune individuals in whom parasitemia may be incidental. In these patients, the diagnosis is more difficult and no 'gold standard' exists. A reasonable set of guidelines is: (1) a clinical illness compatible with malaria, including objective evidence of fever; (2) exclusion of any other likely cause

Table 4 *Immunopathology of malaria*

Pathology	Possible mechanisms
Hypergammaglobulinemia	Antigen induced cytokine production (IL-6)
	Antigenic variation
	Polyclonal activation
Immunosuppression	Antigenic competition
	Structural disruption of germinal centers
	Disruption of spleen function
	Macrophage dysfunction
	Polyclonal activation and immune 'exhaustion'
Nephrotic syndrome	Immune complex deposition
	Auto-immunity
Autoimmunity	Auto-antibodies
	Anti-nuclear antibodies
Anemia	Anti-erythrocyte antibodies
	Dyserythropoiesis (e.g. effect of TNF)
	Excessive erythro-phagocytosis
Thrombocytopenia	Excessive removal of platelets
	Coating of platelets with malaria antigen
Hyperreactive malarial splenomegaly	Genetic predisposition
	Hypergammaglobulinemia
	Chronic increase of lymphocyte proliferation
Burkitt's lymphoma	Co-endemicity with Epstein–Barr virus
	Polyclonal activation
	Antigen induced cytokine production

of fever following physical examination; (3) malaria parasitemia above 10 000/μl; and, (4) appropriate response to treatment.

Parasitological methods

Despite the wide range of serological, immunological, and molecular techniques currently available, the only certain means of diagnosing all four species of human malaria is the detection of the *Plasmodium* spp. by microscopic examination of the blood (see Figure 20).
Both thick and thin blood films should be made. The thick film method, which concentrates layers of red blood cells on a small surface by a factor of 20–30, is the most sensitive and by far the best for clinical use. Diagnostic characteristics of human malaria parasites, as seen in a well-stained thick or thin film are given in Table 5.

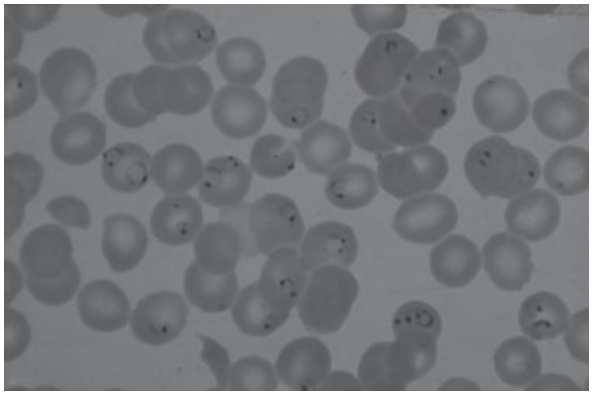

Figure 20 *Blood film of* P. falciparum*-infected blood showing the presence of ring-stage parasites*

Various methods have been designed to enhance the examination of blood films in order to reduce the time spent reading the slides or to enable less well-trained personnel to achieve equally reliable results. Staining with acridine orange, which can be read either on a fluorescence microscope or a microscope equipped with an interference filter system (Kawamoto 1991) allows a quicker screening of films, because parasites are more readily recognized and a lower power microscope objective may be used. The quantitative buffy coat (QBC®) method is also based on acridine-orange staining, but in this case the blood is centrifuged in a specially designed and patented microcapillary tube fitted with a plastic float. The float spreads the buffy coat against the edge of the tube; parasites and leukocytes take up the dye, which is fluorescent when examined under ultraviolet light (Figure 21). This elegant method is easy to perform, fast, and easy to read, but requires specialized equipment (a microcentrifuge and a fluorescence microscope) and the purchase of expensive QBC capillary tubes (Spielman and Perrone 1989; Petersen and Marbiah 1994). The sensitivity of QBC, when used in field conditions, is comparable to, or marginally better than that of thick films. Both acridine-orange staining techniques are inferior to Romanovsky staining for the

Table 5 *Diagnostic characteristics of human malaria*

	P. falciparum	*P. malariae*	*P. vivax*	*P. ovale*
Appearance of infected red blood cells (size and shape)	Both normal	Normal shape; size normal or smaller	1.5–2 times larger than normal; shape normal or oval	As for *P. vivax*, but some have irregular frayed edges
Schüffner's dots (eosinophilic stippling)	None (but presence of occasional comma-like red Maurer's dots)	None	Present in all stages, except early ring form	As for *P. vivax*
Red cells with multiple parasites per cell	Common	Rare	Occasional	As for *P. vivax*
Stages present in peripheral blood	Rings and gametocytes Schizonts rarely seen	All stages	All stages	All stages
Ring form (young trophozoite)	Delicate, small ring; scanty cytoplasm; sometimes at edge of cell ('accole' form)	Ring 1/3 diameter of cell; heavy chromatin dots; vacuole sometimes 'filled in'	Ring 1/3 to 1/2 diameter of red cell; heavy chromatin dots	As for *P. vivax*
Schizont	Rarely seen in peripheral blood; 16–18 merozoites	6–12 merozoites in rosette; coarse pigment clump in center	12–24 merozoites in rosette filling the entire RBC; central pigment	8–12 merozoites in rosette
Gametocyte	'Crescent' shape characteristic	Round or oval; coarse pigment	Round or oval	Round or oval (smaller than *P. vivax*)
Main criteria	Only rings and crescent shaped gametocytes in blood; multiple infection; level of infection high; normal RBC s xhape; no Schüffner dots	All stages in blood; trophozoites compact and intensly stained; band forms suggestive; coarse pigment; normal/small RBC; no Schüffner dots	Large pale RBC; presence of Schüffner dots; round gametocytes; large amoeboid trophozoite with pale pigment	Generally like *P. vivax*, oval RBC with fimbriated edges characteristic but not always present

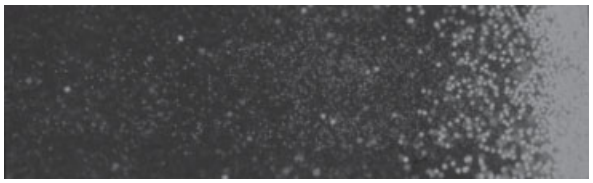

Figure 21 *Quantitative buffy coat (QBC). Blood infected with* P. falciparum *as seen in QBC capillary tubes. The large fluorescent cells are white blood cells, the small fluorescent dots in the dark area are ring-stage parasites within non-fluorescing erythrocytes (courtesy of Becton-Dickinson Tropical Disease Diagnostics, USA).*

precise identification of malaria species or their accurate enumeration. Where it is necessary to identify parasite species with precision, the use of blood films stained by immunocytochemical methods using species monoclonal antibodies may be envisaged (Lindergard 1995; Perez et al. 1995).

Despite its shortcomings, microscopy has qualitative and quantitative features that are not associated with most other techniques. For example, when carefully examined by an experienced microscopist, a thin film with *P. falciparum* can provide clues regarding the degree of disease severity, which include not only the high level of parasitemia but also the presence of more 'mature' ring-stage parasites (linked to the existence of a greater sequestered biomass of parasites) (Silamut and White 1993) or the presence of an unusually high number of circulating schizonts. In addition to the observation of the parasites themselves, the overall examination of a blood film may provide other clues. The presence of visible malarial pigment in neutrophils or monocytes may be used as a criterion of poor prognosis; a fatal outcome being associated with pigment in more than 5 percent of neutrophils (Nguyen et al. 1995). One must, however, be careful not to overinterpret such observations, since pigmented monocytes are not uncommon in asymptomatic African children.

Immunological methods

Instead of identifying the parasite itself, immunological methods provide the means for detecting either the parasite antigens or the host antibodies directed against the parasite. The detection of antigens may be an acceptable alternative to parasite detection, particularly if the assay is robust, inexpensive, easy to use in field conditions, and does not require a microscope, but the detection of antibodies merely provides information on past malaria experience and is of limited use for individual diagnosis.

SEROLOGY

Serological methods have been in use since the early 1960s, when IFAT and indirect hemagglutination assays (IHA) were described. Since such tests detect anti-malarial antibodies they cannot distinguish between current or past infection and are therefore of limited value as a guide to the treatment or management of the disease. At best, a negative serological assay may help to eliminate the possibility of malaria, since it has been shown that antibody levels become detectable a few days after the blood is invaded. A detailed description of the various methods may be found elsewhere (Voller 1988; Hommel 2002).

IFAT is the main method for routine serodiagnosis, because it is relatively easy to make antigen slides for all human malarial parasites (commercial IFAT slides for malaria are also available from various sources) (Figure 22). The disadvantages are the need for a fluorescence microscope, the subjectivity of the reading, and the labor-intensive nature of the method, which limits its application to specialized centers with a relatively small through-put of samples.

ELISA uses a soluble malarial antigen, generally prepared from asexual stages of *P. falciparum*, which can be readily grown in vitro (Trager and Jensen 1976), coated on the wells of a microtiter plate. A large number of samples can thus be processed at the same time and produce quantitative results, when the assay is read on a spectrophotometer (portable, battery-operated ELISA readers are available for field use). In addition to crude extracts of malarial antigens, ELISA has been applied to a variety of defined, synthetic, or recombinant malarial antigens (e.g. MSP-1, RESA, or CSP) (Zavala et al. 1986; Del Giudice et al. 1987; Riley et al. 1992); such studies have been useful in elucidating the role of target malarial antigen in immunity and protection.

A variety of other serological test formats have been explored, including radioimmunoassays, latex agglutination, indirect hemagglutination, solid-phase dipstick, and membrane dot-blot, which may have specific advantages in given situations.

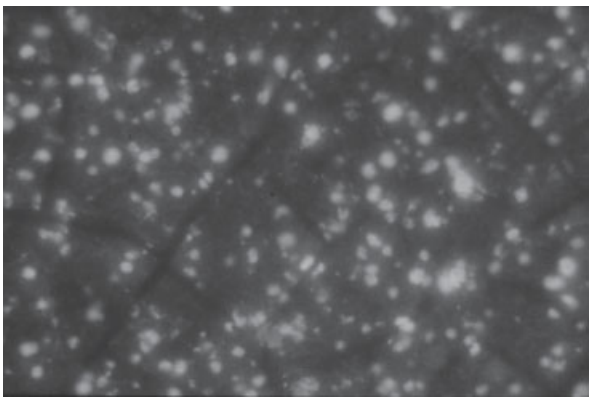

Figure 22 *Positive immunofluorescence antibody test with malaria antigen (courtesy of Dr. W. Bailey, Liverpool School of Tropical Medicine)*

ANTIGEN DETECTION

In contrast to serology, a positive antigen detection assay should only detect a current infection. Experimental tests for detecting malarial antigens are based on either an antigen-capture or an antigen-competition format, and often use ELISA or the radioimmunoassay (RIA) methodology. Once optimal reagents have been identified (i.e. monoclonal or polyclonal antibodies to specific malarial antigens), the assay may be simplified to a straightforward agglutination or immunochromatography ('dipstick') format. The best antigen detection assays described have a maximum sensitivity of 0.01 to 0.001 percent parasitemia and are five to ten times inferior to good quality microscopy (Mackey et al. 1982; Khusmith et al. 1987; Fortier et al. 1987; Taylor and Voller 1993).

ParaSight^{TM}-F (Shiff et al. 1993) was the first commercial detection test in dipstick format (Figure 23), in which a monoclonal antibody captures a specific antigen of *P. falciparum*, PfHRP-2, present in the parasite throughout the erythrocytic cycle. If antigen is present, the positivity of the test is visualized by a second anti-HRP2 antibody labeled with a colored marker, which produces a visible line on the dipstick. The whole test takes only 10 min and gives a sensitivity almost comparable to that of thick films. This simple, robust assay requires no equipment and could be taught to village health workers, since its reading was a straightforward positive/negative assessment (Premij et al. 1994). Its high cost and the fact that the assay was not quantitative are serious limitations to its use in the very field situations for which it was designed. Many other versions of the test are now commercially available.

The detection of parasite lactate dehydrogenase (pLDH) was originally developed as a way to monitor in vitro drug susceptibility assays (Makler and Hinrichs 1993). The principle of the assay is that pLDH has different biochemical characteristics from human lactate dehydrogenase (LDH) and may, therefore, be differentially measured using a simple colorimetric assay. The principle of OptiMAL® (Diamed, Switzerland) is based on the detection of pLDH using a series of monoclonal and polyclonal antibodies; it is the most recently developed immunochromatographic rapid malaria strip tests (Piper et al. 1999). Differentiation of malaria species in the OptiMAL test is based on antigenic differences between pLDH isoforms. Unlike HRP-2, pLDH does not persist in the blood but clears at about the same time as the parasites, following successful treatment. The test is, therefore, useful for monitoring responses to drug therapy and detecting drug-resistant malaria because the level of pLDH correlates with the number of viable malaria parasites in the blood. If less sensitive than HPR-2 tests (with a threshold of 100–200 parasites/μl), the test produces fewer false-positives in patients with rheumatoid factor. Evaluations published so far indicate that the test is sensitive and able to distinguish between *P. vivax* and *P. falciparum* (Palmer et al. 1998).

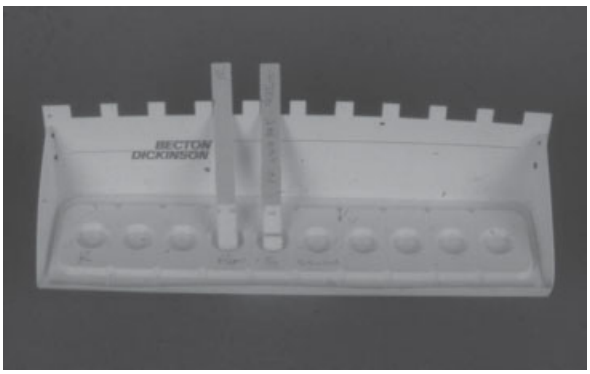

Figure 23 *Positive and negative malarial antigenemia using the ParaSight F dipstick (courtesy of Dr. W. Bailey, Liverpool School of Tropical Medicine)*

MOLECULAR METHODS

The application of DNA or RNA hybridization to malaria diagnosis has several advantages over traditional methods as a research tool to monitor malaria control programs, to perform quality control checks on microscopic diagnosis or to determine the distribution of important genes (e.g. genes associated with drug resistance).

Various methods based on the principle of nucleic acid hybridization have been developed in order to detect parasite DNA and RNA. A known sequence of nucleic acid (oligonucleotide) is synthesized and labeled either with radioactive ^{32}P or a nonradioactive colorimetric reagent and this 'probe' is used to detect parasite nucleic acid taking advantage of the fact that complementary sequences will hybridize. The simplest version of this technique is the use of DNA probes to detect parasites directly in a drop of patient's blood immobilized on filter paper (Franzen et al. 1984). In this test format, the sensitivity and specificity of the technique depends largely upon the choice of nucleic acid sequence. In order to achieve a higher degree of sensitivity, most of the first-generation DNA probes were directed towards repetitive sequences of parasite genes. Since the availability of amplification methods, such as the PCR, the sensitivity of nucleic acid probes has increased exponentially. This has made it possible to use probes against nonrepetitive sequences, which have been useful, for example, to examine whether *pfmdr* genes are or are not associated with CQ resistance (Foote et al. 1989; Wellems et al. 1990) or to use species-specific small subunit ribosomal RNA sequences to differentiate between *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae* (Snounou et al. 1993) (Figure 24).

Many technical refinements of the PCR method have been described, some of which have been applied to

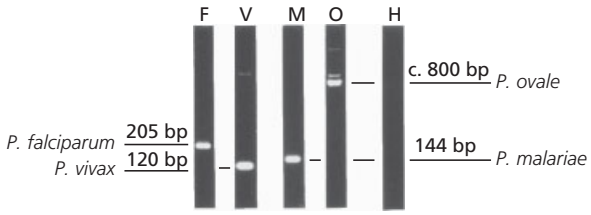

Figure 24 *Polymerase chain reaction (PCR) detection of human* Plasmodium *species (courtesy of Dr. G. Snounou, Institut Pasteur, Paris)*

malaria diagnosis. The use of methods for the detection of immobilized amplified nucleic acid or liquid phase hybridization provides a test format akin to ELISA methods (Oliveira et al. 1995). Various ways have also been described to make the PCR technique more user-friendly under field conditions by reducing the manipulation of the samples and the likely contamination of specimens (e.g. by collection of blood on filter paper) (Long et al. 1995).

The PCR technique has been reported to be capable of detecting parasitemias of less than 0.00002 percent, when used in the best possible conditions. It is theoretically capable of detecting the presence of a single parasite in the sample, although this is rarely achieved, partly because the blood contains poorly defined products which may inhibit the PCR reaction. The detection of a parasitemia of 0.00002 percent, which corresponds to one parasite per mm^3 or five parasites per 5-µl sample of blood, is a detection threshold at least five times lower than the detection threshold achieved by means of a thick film performed in optimal conditions (i.e. 0.0001). A sensitivity of one parasite per 20 µl of blood, as reported by Tirasophon and colleagues (1991) would, therefore, correspond to a sensitivity 100 times higher than a thick film. In reality, the sensitivity of PCR is rarely as good, particularly when performed in field conditions, and may be considered comparable or only marginally better than that of the microscopic examination of a thick film (Laserson et al. 1994). In contrast, the specificity of PCR is generally considered to be better than microscopy.

Appraisal of the relative value of diagnostic methods

Whatever the method used, a diagnostic test should be able to correctly differentiate between individuals that are infected and those who are not; consequently, the validity of a test is usually determined by its sensitivity (i.e. the test with the highest sensitivity has the lowest number of false-negatives) and its specificity (i.e. the test with the highest specificity has the lowest number of false-positives). In reality, it is generally more important to know the capability of a positive assay to predict the probability of infection, i.e. its positive predictive value.

Individual diagnosis

In most situations, the 'gold standard' for individual diagnosis is the microscopic examination of thick and thin films. There are, however, situations where this may not apply. In areas of high endemicity, clinical diagnosis alone is usually the only feasible and cost-effective method for recommending the first-line treatment (CQ, Fansidar® or Mefloquine®). When microscopy is not available, a variety of clinical features may be used as predictive markers of disease (Genton et al. 1994).

The ideal use of methods like QBC, PCR, or ParaSight is in sophisticated laboratories in developed countries that can afford the cost of the assays and have expertise in complex technologies but have been unable to maintain competency in malaria microscopy. Ironically, in these situations, the need for a quantitative evaluation of the level of parasitemia to monitor treatment efficacy may make it necessary to perform a microscopic examination of blood films as a second-line test. In contrast, in hyperendemic areas, in situations where microscopy is not available, one of the most important applications of ParaSight or OptiMal is to identify clinical cases that are not attributable to malaria, since such cases would otherwise be wrongly assumed to be malaria and given antimalarials when the real bacteriological etiology would, for example, require antibiotics. PCR is generally considered unsuitable for routine diagnosis in field conditions (Hänscheid and Grobusch 2002).

When microscopy is used in areas of high endemicity, it is usually necessary to redefine the critical threshold for each situation, i.e. a level above which parasitemia may have a clinical significance. A parasitemia above 5 000 to 10 000 parasites/µl is usually suggested as a guideline, but precise counting may not always be feasible; Coosemans et al. (1994) have proposed the sensible 'rule-of-thumb' that a 100 percent field positivity on a thick film may be used as a reliable morbidity indicator.

Epidemiological surveillance

Active or passive surveillance of malaria prevalence is an important tool for malaria control, particularly when the efficacy of control measures is being evaluated. Microscopy and, by extension, antigen detection or PCR, have serious limitations for such an evaluation, because these techniques only measure a 'point prevalence' of the infection at the time of the survey and is particularly unhelpful in situations where transmission is seasonal. In contrast, sero-epidemiology may help to delineate those areas where there is transmission, may also provide information on species-prevalence or age-related prevalence, and may chart the changes that are taking place as a result of the control intervention.

Another issue in diagnosis for epidemiological purposes is the follow up of the distribution in a community of isolates with specific features, such as antigens of interest, in the context of a vaccination program or drug resistance markers, for the definition of treatment policies. PCR, whose specificity can be changed at will by changing the primer sequences used, is ideally suited for this purpose and is increasingly being used for isolate-specific surveys (Contamin et al. 1995).

Transfusion malaria

The transmission of malaria parasites by blood transfusion is a serious risk, since the diagnosis of malaria in the recipient, being unexpected, is often missed. Microscopic examination of donor blood is highly unsatisfactory since most donor infections are at a sub-microscopic level. Outside endemic areas, the policy for screening donor history for known episodes of clinical malaria or for tropical travel in the past 5 years is generally sufficient. With increasing frequency of tropical travel, the policy in some countries is to reject only donors whose malaria serology is positive, generally using IFAT with homologous antigens for *P. falciparum*, *P. vivax*, and *P. malariae* for maximum assay sensitivity. This screening method is not perfect, but a negative serology gives a high probability of freedom from infection. The use of PCR (recently explored by the Blood Transfusion Center of Ho Chi Minh City in Vietnam) is not, despite its much increased sensitivity, a complete guarantee of safe blood, since the absence of parasites in a 20 µl sample does not exclude the possibility of infection in the remaining 450 ml of the blood unit (Vu thi Ty Hang et al. 1995). In endemic areas, the only safe prevention of transfusion malaria is appropriate preventive antimalarial therapy of the recipient. Malaria transmission has occurred as a result of organ transplantation, e.g. kidney, heart, and bone marrow.

CHEMOTHERAPY AND CHEMOPROPHYLAXIS

Chemotherapy

Although chemotherapy is mandatory in the treatment of malaria (White 2003a; Greenwood 2004), correct management of the patient and the complications that occur with severe malaria is also vital (Warrell 2002; Molyneux and Fox 1993; Wilkinson et al. 1994). A summary of the management of severe and complicated malaria is given in Table 6 (WHO 2000).

For uncomplicated falciparum malaria, oral therapy is the administration of choice and parenteral therapy should only be considered if the patient presents with severe nausea and vomiting. For severe and complicated falciparum malaria parenteral therapy is always indicated, but reversal to oral therapy should be instituted as soon as the patient's condition has improved sufficiently to take the drugs by mouth.

The antimalarial drugs in general use are chloroquine (CQ), amodiaquine, quinine, mefloquine, halofantrine, sulfadoxine-pyrimethamine, atovaquone-proguanil (Malarone®), lapudrine-dapsone (LapDap®), pyronaridine, and artemisinin derivatives singly or in combination, e.g. artesunate, artemether, artemether-benflumetol (Riamet®, Coartem®) and primaquine. Tafenoquine is likely to be on the market soon.

When choosing a treatment it is important to be aware of the regimen recommended for the specific geographical area, both by the national malaria control program and by WHO.

The treatment of uncomplicated *P. falciparum* malaria is given in Table 7; severe malaria in Table 8; *P. vivax* and *P. ovale* malaria in Table 9, and *P. malariae* in Table 10.

Although the proposed regimen for *P. malariae* is that which is usually recommended, the authors feel that a 4-day treatment would be more logical for this parasite, in view of its longer intra-erythrocytic cycle of 72 h instead of 48 h for the other human malaria species. This approach may help to deal with a 'lesser susceptibility' of this parasite to CQ treatment which has sometimes been reported but has not been thoroughly documented.

In the greater Mekong region (i.e. the region including Yunam Province, Thailand, Vietnam, Laos PDR, Cambodia, and Myanmar), where multidrug resistance has been recognized, the treatment of choice is with artemisinin and its analogs (Hien and White 1993; Jing-Bo-Jiang et al. 1982; Looareesuwan et al. 1992; Karbwang and Harinasuta 1992; Bunnag et al. 1995).

In pregnancy, artemisinins should only be used in the second and third trimesters when other treatments, like quinine with clindamycin, are not available. They are not recommended for treatment in the first trimester, unless considered life-saving and when no other suitable antimalarial, e.g. quinine, is available (Nosten et al. 2003).

Combination therapy with antimalarial drugs

The concept of combination therapy is based on the synergistic or additive potential of two or more antimalarial drugs (WHO 2001). In Southeast Asia, artemisinin-based combinations have been successfully used to improve efficiency of treatment and control resistance (White and Olliaro 1996).

The following combinations are possible options:

Amodiaquine + sulfadoxine-pyrimethamine (SP)
Artesunate + amodiaquine
Artesunate + SP (providing levels of resistance to SP less than 15%)
Artesunate + LapDap

Table 6 *Summary of the management of severe falciparum malaria (WHO 2000)*

Summary of management
In all cases, an appropriate antimalarial drug should be started immediately and complications managed appropriately as below.
1. **Coma (cerebral malaria)**
Maintain airway; nurse on side; exclude other treatable causes of coma (e.g. hypoglycemia, bacterial meningoencephalitis). Avoid harmful adjuvant treatments such as corticosteroids, heparin, and epinephrine (adrenaline).
2. **Convulsions**
Maintain airway; treat with diazepam given intravenously (0.15 mg/kg of body weight) or intrarectally (0.5 mg/kg of body weight), or intramuscular paraldehyde injection (0.1 ml/kg of body weight). Paraldehyde should, if possible, be given from a glass syringe. A disposable plastic syringe may be used, provided that the injection is given immediately the paraldehyde is drawn up and that the syringe is never reused.
3. **Severe anemia**
Transfuse screened fresh whole blood or packed cells.
4. **Acute renal failure**
Exclude dehydration; maintain strict fluid balance; carry out dialysis if indicated.
5. **Hypoglycemia**
Measure blood glucose, give 50% dextrose injection 50 ml (1 ml/kg of body weight for children) followed by 5% or 10% dextrose infusion.
6. **Metabolic acidosis**
Exclude or treat hypoglycemia, hypovolemia, and gram-negative septicemia. Give isotonic saline 10 ml/kg of body weight rapidly or screened whole blood 10 ml/kg of body weight over 30 min if hemoglobin is <5 g/dl.
7. **Acute pulmonary edema**
Prevent by avoiding excessive rehydration. Prop patient up; give oxygen. If pulmonary edema is due to overhydration, stop intravenous fluids, give a diuretic (furosemide (frusemide) 40 mg intravenously), and withdraw 3 ml/kg of blood by venesection into a donor bag.
8. **Shock, algid malaria**
Suspect gram-negative septicemia; take blood samples for culture. Give parenteral antimicrobials; correct hemodynamic disturbances.
9. **Spontaneous bleeding and coagulopathy**
Transfuse screened fresh whole blood or clotting factors; give vitamin K, 10 mg intravenously.
10. **Hyperpyrexia**
Give antipyretic (paracetamol 15 mg/kg of body weight) and use tepid sponging and fanning.
11. **Hyperparasitemia**
Give initial dose of parenteral antimalarial therapy; consider exchange transfusion if there are other signs of severity.
12. **Malarial hemoglobinuria**
Continue antimalarial treatment; transfuse screened fresh blood if needed.
13. **Aspiration pneumonia**
Give parenteral antimicrobials; change position of patient; give physiotherapy; give oxygen.
14. **Fluid and electrolyte balance**
Replacement of water and salt with isotonic saline. Monitor fluid therapy by measurement of jugular or central venous pressure.

Table 7 *Antimalarial chemotherapy for adults and children who can swallow tablets (Warrell et al. 2002)*

Chloroquine-resistant *P. falciparum* or origin of species unknown	Chloroquine-sensitive *P. falciparum* or *P. vivax*, *P. ovale*, *P. malariae*
1. Mefloquine Adults: 15–25 mg base/kg[a] given as 2 doses 6–8 h apart Children: 25 mg base/kg given as 2 doses 6–8 h apart	1. Chloroquine[b] Adults: 600 mg base on the 1st and 2nd days; 300 mg on the 3rd day Children: approximately 10 mg base/kg on the 1st and 2nd days; 5 mg base/kg on the 3rd day For radical cure of vivax/ovale add
or	
2. Proguanil *with* atovaquone (Malarone) Adults: 4 tablets (each containing 100 mg proguanil and 250 mg atovaquone) once daily for 3 days Children: 11–20 kg, 1 tablet; 21–30 kg, 2 tablets; 31–40 kg, 3 tablets, all once daily for 3 days	2. Primaquine Adults (except pregnant and lactating women and G6PD-deficient patients): 15 mg base/day on days 4–17 *or* 45 mg/week for 8 weeks[c] Children: 0.25 mg/kg per day on days 4–17 *or* 0.75 mg/kg per week for 8 weeks[c]
or	
3. Artemether *with* lumefantrine (Riamet) Adults: 4 tablets (each containing 20 mg artemether and 120 mg lumefantrine) twice daily for 3 days Children: <15 kg, 1 tablet; 15–<25 kg, 2 tablets; 25–<35 kg, 3 tablets, all twice daily for 3 days	
or	
4. *Quinine* Adults: 600 mg salt 3 times daily for 7 days[d] Children: approximately 10 mg salt/kg 3 times daily for 7 days	
or	
5. Chloroproguanil *with* dapsone (Lapdap) Adults and children: chlorproguanil 2.0 mg/kg with dapsone 2.5 mg/kg once daily for 3 days	
or	
6. Sulphonamide-pyrimethamine[e] Sulfadoxine (500 mg per tablet) or sulfalene (500 mg) plus pyrimethamine (25 mg) Adults: 3 tablets as single dose Children: <5 years, 1/2 tablet; <9 years, 1 tablet; <15 years, 2 tablets, all as single doses	

a) For chloroquine-resistant *P. vivax*, repeat the course.
b) For Chesson-type strains (SE Asia, W. Pacific), use double dose or double duration up to a total dose of 6 mg base/kg in daily doses of 15–22.5 mg in adults.
c) Depending on geographical area and presumed immunity.
d) In areas where 7 days of quinine is not curative (e.g. Thailand), add tetracycline 250 mg four times each day or doxycycline 100 mg daily for 7 days except for children under 8 years and pregnant women or add clindamycin 1 mg/kg twice daily for 3–7 days.
e) Sulfadoxine + pyrimethamine (Fansidar); sulfalene + pyrimethamine (Metakelfin). Contraindicated if patient has known sulfonamide hypersensitivity.
Note (1) Combination therapy is now advocated by WHO; (2) Suppositories of artemisinin and its derivatives can be used if severe vomiting prevents oral therapy and parenteral therapy is unavailable, e.g. in peripheral health centers.

Artesunate + pyronaridine
Artesunate + lumefantrine (Coartem®)

Constraints to the use of combination therapy, especially in Africa, are:

- scanty information on the safety and efficacy of the proposed combinations, particularly in pregnant women and children;
- the present high cost of artemisinin-based compounds. In this context, however, WHO has negotiated an agreement with a major pharmaceutical company to make a co-formulated artemisinin-containing combination therapy for public sector use in malaria-endemic countries at cost price, resulting in a reduction of the cost for a full treatment course for a child to US$ 1.

The optimal time to use combination therapy is before resistance has developed to either of the individual drugs chosen, or when resistance is at a low level – below 15 percent (Adjuik et al. 2004).

Table 8 *Severe falciparum malaria[a]: antimalarial chemotherapy in adults and children (WHO 2000)*

Chloroquine-resistant, or sensitivity unknown	Chloroquine-sensitive
1. Quinine	1. Chloroquine
Adults: 20 mg dihydrochloride salt/kg (loading dose)[b,c] diluted in 10 ml/kg isotonic fluid by intravenous infusion over 4 h then, 8 h after the start of the loading dose, 10 mg/kg[d] over 4 h every 8 h until the patient can swallow, then quinine tablets approximately 10 mg quinine salt/kg (maximum 600 mg) every 8–12 h to complete 7 d treatment[e] or give a single dose of 25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine (maximum 1500 mg sulfadoxine/75 mg pyrimethamine). Children: 20 mg dihydrochloride salt/kg (loading dose)[b,c] diluted in 10 ml/kg isotonic fluid by intravenous infusion over 4 h then, 12 h after the start of the loading dose, 10 mg/kg[d] over 2 h every 12 h until the patient can swallow, then quinine tablets approximately 10 mg quinine salt/kg (maximum 600 mg) every 8 h to complete 7 d treatment[e] or give a single dose of 25 mg/kg sufadoxine and 1.25 mg/kg pyrimethamine	10 mg base/kg in isotonic fluid by constant rate intravenous infusion over 8 h, followed by 15 mg/kg over 24 h
or	or
2. Artesunate[f]	2. Chloroquine
2.4 mg/kg intravenously on the first day followed by 1.2 mg/kg daily for a minimum of 3 d until the patient can take oral therapy or another effective antimalarial	5 mg base/kg in isotonic fluid by constant rate infusion over 6 h, every 6 h, to a total dose of 25 mg/kg over 30 h
or	or
3. Artemether	3. Quinine, artemether or quinidine
1.2 mg/kg intramuscularly on the first day, followed by 1.6 mg/kg daily for a minimum of 3 d until the patient can take oral reatment or another effective antimalarial. In children the use of a 1 ml tuberculin syringe is advisable since the injection volumes will be small.	(see left-hand column)

a) For definition see text.
b) Loading dose should not be used if the patient received quinine, quinidine, or mefloquine within the preceding 12 h.
c) Alternatively, the loading dose can be administered as 7 mg salt/kg by intravenous infusion (or pump) over 30 min followed immediately by 10 mg/kg diluted in 10 ml/kg isotonic fluid by intravenous infusion over 4 h.
d) If there is no clinical improvement after 48 h of parenteral therapy, reduce the maintenance dose to 5–7 mg quinine dihydrochloride/kg or to 3.75–5 mg quinidine base/kg every 8 h.
e) In areas where a 7 d course of quinine is not curative (e.g Thailand) add a course of oral tetracycline 4 mg/kg daily or doxycycline 3 mg/kg once daily except for children under 8 years of age and pregnant women, or clindamycin 10 mg/kg twice daily for 3–7 d.
f) Artesunic acid 60 mg is dissolved in 0.6 ml of 5% sodium bicarbonate diluted to 3–5 ml with 5% (W/v) dextrose and given immediately by intravenous ('push') bolus injection. Note (1) Combination therapy is now advocated by WHO; (2) Suppositories of artemisinin and its derivatives can be used if severe vomiting prevents oral therapy and parenteral therapy is unavailable, e.g. in peripheral health centers.

Drug resistance

Monitoring the spread of drug resistance is one of the most important functions of malaria control programs and such monitoring provides the basis for a national antimalarial treatment policy on evidence-based recommendations (WHO 1994a). The protocol for the assessment of therapeutic efficacy of antimalarial drugs has recently been updated (WHO 2001; White 2002). It takes into consideration the important role of immunity in high transmission areas with more emphasis on parasitological clearance and different transmission intensity (Table 11).

The mechanism of drug resistance of malaria parasites is only well understood for antifolate drugs and CQ. The four groups of antifolates, sulfonamides, sulfones, pyrimethamine, and proguanil, act at different points of the parasite pathway for folate production, with two major target enzymes DHPS and DHFR. Resistance to antifolates is generally induced by one or more mutations on either of these genes and these mutations generally occur in a limited number of sites on the molecules (Foote et al. 1990). Resistance to CQ is associated with mutations in the *pfcrt* gene, but may also correlate with mutations on the pfmdr genes (Fidock et al. 1999). It is now feasible to use molecular techniques, such as PCR, as an alternative to in vitro assays for monitoring the spread of such mutations in an area where antifolate or CQ drug resistance has been reported. The higher the prevalence of the mutations, the lower the efficacy of the drug (Watkins et al. 2005).

Whereas CQ resistance of *P. falciparum* (see Figure 2) is widespread, resistance to CQ by *P. vivax*

Table 9 *Antimalarial chemotherapy for vivax- and ovale-malaria*

Treatment	
1. Chloroquinine	10 mg base/kg on days 1 and 25 mg base/kg on day 3
Plus for radical cure	
2. Primaquinine[a]	0.25–0.33 mg[b] base/kg daily on days 4-17

a) Contraindicated for pregnant and lactating women. For G6PD-deficient patients daily doses of primaquine are also contraindicated, but radical cure can be obtained using 0.75 mg/kg once weekly for 8 weeks, with minimal risk of hemolysis.
b) For Oceania and South East Asia strains.

Table 10 *Antimalarial chemotherapy for malariae-malaria*

Antimalarial chemotherapy	
Chloroquine	10 mg base/kg on days 1 and 2 5 mg base/kg on day 3 and 4

is still limited to Papua New Guinea, parts of India, Indonesia, Myanmar, Brazil, and Guatemala. For *P. vivax*, there is only a single definition of treatment failure namely:

- clinical deterioration requiring hospitalization in the presence of parasitemia;
- presence of parasitemia and axillary temperature >37.5°C anytime between days 3 and 28;
- presence of parasitemia on any day between days 7 and 28, irrespective of clinical condition.

Four basic methods have been routinely used to assess antimalarial drug resistance: in vivo, in vitro, animal models, and molecular characterization. In general, resistance occurs through spontaneous mutations – single or multiple – that result in reduced sensitivity to drugs. The biochemical mechanism of resistance has been described for CQ, the antifolate combination drugs and atovaquone (Bloland 2001).

Chemoprophylaxis of malaria

Drug resistance of *P. falciparum* to CQ and other antimalarial drugs continues to increase in both intensity and geographical distribution. The situation is a dynamic one and advice on the current chemoprophylaxis recommendations for travelers should be sought from the various specialized centers in respective countries (Steffen et al. 1993; Lobel et al. 1995; Bradley and Bannister 2001). To date, resistance of *P. vivax* to CQ has been reported only occasionally from Indonesia, Myanmar, parts of India, Brazil, Guatemala, Papua New Guinea, and Vanuatu, and these reports do not yet justify a recommendation for alternative chemoprophylaxis for these areas.

The principles of prevention that should be borne in mind are:

- Awareness of the malaria risk coupled with a high index of suspicion if fever occurs while in a malarious country or within 3–12 months of return even if all recommended precautions have been taken.
- Personal protection to diminish contact with the mosquito vector is very important. This includes (1) sleeping in screened rooms and spraying the room with a knock-down insecticide before sleeping to kill any mosquitoes that have entered during the day; (2) using nets impregnated with pyrethroids; (3) using an electric mat to vaporize synthetic pyrethroids, or burning mosquito coils; (4) wearing long-sleeved clothing and trousers after sunset; and (5) application of repellents containing diethyltaluanide to exposed skin.
- Strict compliance with the chemoprophylaxis regimens recommended (Bradley and Bannister 2001).

Chemoprophylaxis at community level is a contentious issue, which is not recommended by WHO at present for a variety of reasons: potential dangers of drug resistance; poor compliance in areas of perennial transmission; possible delay in the development of natural immunity; cost; toxicity of drugs; regularity of supply and logistics of delivery. Nevertheless, there may be a place for community chemoprophylaxis targeted to young children and pregnant women in areas where transmission is seasonal and short and where the population is co-operative and well-educated with regard to malaria (as is the case in the Gambia) (Greenwood 1991).

Chemoprophylaxis in pregnancy has long been recommended, particularly in primigravidae (Gilles et al. 1969; Fleming et al. 1986; Greenwood et al. 1989; WHO 1994b, Steketee et al. 1996a). The advent of drug resistance, increasing evidence of poor compliance, unacceptable adverse drug reactions, and economic considerations have resulted in alternative strategies being sought. For instance, in Malawi and Kenya, a two-dose regimen of sulfadoxine-pyrimethamine given at first attendance in the second trimester of pregnancy and repeated at the beginning of the third trimester (IPT), was found to be a cost-effective intervention to reduce the incidence of low birth-weight infants, placental parasitemia, and anemia (Schultz et al. 1995; Parise et al. 2003). This regimen is generally recommended. Meanwhile, a curative treatment with CQ given at first attendance, followed by a prophylaxis continued throughout pregnancy is recommended for Central America where *P. falciparum* is still predominantly sensitive to CQ (WHO 1994a). The spread of SP resistance presents a challenge in areas of high transmission for which no clear cut solution is readily available. In areas of low to moderate transmission, prompt treatment with quinine and clindamycin is an option.

Table 11 *WHO definitions of treatment failures*

Adequate clinical response (ACR)	Early treatment failure (ETF)	Late treatment failure (LTF)
Absence of parasitemia on day 14, irrespective of axillary temperature, without previously meeting any of the criteria of early or late treatment failure	Development of danger signs or severe malaria on day 1, 2, or 3 after drug treatment in the presence of parasitemia	Development of danger signs or severe malaria in the presence of parasitemia on any day from day 4 to 14 after treatment, without previously meeting any of the criteria of ETF
Axillary temperature <37.5°C, irrespective of the presence of parasitemia, without previously meeting any of the criteria of early or late treatment failure	Axillary temperature ⩾37.5°C on day 2 with parasitemia greater than that of day 0 count, irrespective of temperature	In areas of intense transmission, axillary temperature ⩾37.5°C in the presence of parasitemia on any day from day 4 to 14, without previously meeting any of the criteria of ETF
	Axillary temperature ⩾37.5°C on day 3 in the presence of parasitemia. In areas with low to moderate transmission, there must be a measured increase in axillary temperature on day 3	In areas of low to moderate transmission, presence of parasitemia on any day from Day 4 to 28, and a measured axillary temperature ⩾37.5°C, without previously meeting any of the criteria of ETF. If a history of fever, rather than measured fever, was accepted as an entry criterion, then parasitemia with history of fever suffices for LTF

The 2002 WHO Consultancy has modified the definition of cure to adequate clinical and parasitological response (ACPR). In a high-transmission setting, ACPR is defined as an adequate clinical response, but in low to moderate transmission settings, the assessment is made on day 28. The consultancy has also included the definition of late parasitological failure (LPF). In high-transmission settings, LPF is defined as 'presence of parasitemia on day 14 and a measured axillary temperature of <37.5°C, without previously meeting any of the criteria of ETF or LTF.' In low to moderate transmission settings, the definition is the same, except that parasitemia at any time from day 7 to 28 qualifies.

EPIDEMIOLOGY

The epidemiology of malaria can be described in at least four different ways, each of which is complementary: (1) the classical approach; (2) the ecological classification with eight paradigms; (3) a clinico-epidemiological approach; and (4) a conceptual diagram which merely elaborates on the above three aspects.

In addition to transmission by various species of *Anopheles* mosquitoes, malaria parasites can also be transmitted by blood transfusion, by infected needles among drug users, and by organ transplantation. Mosquitoes surviving an aircraft journey or taxi journey from an endemic country may infect persons in a nonendemic area ('airport malaria,' 'baggage malaria,' or 'taxi-rank malaria'). Malaria can be contracted during a short refueling stop 'runway malaria.' Animal reservoirs are of no importance in the epidemiology of human malaria.

The classical macro-epidemiology approach

Two epidemiological extremes are described: stable and unstable malaria. The salient differences are shown in Table 12. In between these two extremes, variable degrees of transmission occur. Moreover, the prevalence of malaria varies considerably within the same country. In Bangladesh, holoendemic falciparum malaria occurs in one area, whilst unstable malaria (or epidemic prone) occurs in another, whereas in most of the country transmission is low and predominantly due to *P. vivax*.

Four major determinants relevant to the dynamics of malaria transmission are: (1) the parasite, (2) the vector, (3) the human host, and (4) the environment.

THE PARASITE

Differences in the biology of the different species of *Plasmodium* are important factors in the epidemiology of malaria. Thus, the prepatency period is shortest in *P. falciparum* (6–25 days) and longest in *P. malariae* (18–27 days) while the incubation period for *P. falciparum* is 7–27 days, in contrast to that for *P. malariae* which is 23–69 days. The parasite life span is usually only 1 year for *P. falciparum* while for *P. malariae* it is many decades. The appearance of gametocytes occurs simultaneously with the asexual parasitemia in *P. vivax*, *P. ovale*, and *P. malariae* is in contrast to *P. falciparum* in which viable gametocytes appear in the circulation only 8–15 days after blood infection. Genetic diversity in *P. falciparum* antigens is well documented and has already been discussed. The potential for multiplication and the ability to relapse in the human host also varies with the different species of parasite.

Table 12 *Epidemiological features of stable and unstable malaria*

	Stable malaria	Unstable malaria
Transmission pattern	Transmission occurs throughout the year. Fairly uniform intensity. Pattern repeats annually	Seasonal transmission variable in intensity. Liable to flare up in dramatic epidemics
Immunity	Potent resistance in the community due to prevailing intense transmission	General lack of communal immunity due to low level and variable intensity of transmission
Impact	Mainly young children	All age groups
Control	Difficult	Much easier than stable malaria
Occurrence	Rural West Africa and East Africa, coastal areas of PNG	Plateau of Ethiopia, Plateau of Madagascar, Sri Lanka, north west India

THE VECTOR

Many factors affect the susceptibility of anopheline mosquitoes (of which around 70 species can transmit malaria) to specific *Plasmodium* species. Air temperature, relative humidity, and types of breeding places can affect the longevity of the adult mosquito and the development of the aquatic stages. The density of the vectors in relation to humans, their parous rates, the frequency of mosquitoes feeding on humans (anthropophilic or zoophilic), the duration of sporogony, the sporozoite rates, the peak biting time, the preference for biting indoors or outdoors (endophagy, exophagy), and the choice of resting place (endophily, exophily), are important determinants which affect the transmission dynamics and are usually measured to quantify the malaria risk (Bockarie et al. 1994; Service and Townson 2002).

THE HOST

Human biological factors, human–parasite interactions, and human behavior all influence the epidemiology of malaria.

Several genetic factors prevent parasite development in the red cell and conclusive evidence of protection against *P. falciparum* malaria is now available for the inherited genes for hemoglobin S, alpha- and β-thalassemia, HbC, G6PD deficiency, and ovalocytosis (Allison 1954; Gilles et al. 1967; Edington 1967; Willcox 1975; Cattani 1987; Ruwende et al. 1995; Agarwal et al. 2000).

Miller et al. (1979), demonstrated natural selection to *P. vivax* malaria in individuals in whom the Duffy blood group antigen is absent (Fy^{a-b-} phenotype). More recently, it has been shown that two HLA types, HLA-BW53 and a haplotype bearing the DRW13.02 antigen, and R111 gene confer a degree of protection against cerebral malaria and malarial anemia in the Gambia (Hill et al. 1991). In contrast, in the same population, homozygotes for the TNF2 allele, a variant of the TNF-γh gene promoter region and H121 phenotype have a relatively higher risk of death or severe neurological sequelae due to cerebral malaria (McGuire et al. 1994; Cooke et al. 2004). Acquired immunity has already been discussed. Other human variables such as pregnancy (especially in primigravidae) modify malaria prevalence and density, as well as the birth-weight of infants and the incidence of anemia (Brabin et al. 1993). Nutritional factors seem to have a paradoxical effect. Severe malaria is seldom evident at necropsy in children who died with marasmus or Kwashiorkor in West Africa (Edington 1967). In Kenya, however, poor nutritional status was found to be a risk factor for severe malaria (Marsh et al. 1995). Human behavior, e.g. sleeping habits, types of housing, occupational activities, and political and social factors can all be responsible for changes in the epidemiology of malaria.

THE ENVIRONMENT

The physical environment has a direct effect on both the parasite and on the vector. Thus, altitude, temperature, rainfall, the rate of flow of rivers and streams, collections of water, and availability of animals all influence transmission.

The effect of global warming could present a risk of epidemics in the highland areas of tropical Africa, by pushing malaria transmission uphill into these populated areas (Bradley 1995). It is, however, quite another matter to attribute recent resurgences of malaria in the highlands of East Africa to climatic changes (Hay et al. 2002). The following can all have a significant impact on malaria endemicity: increases in agricultural colonization; the construction of large economic projects such as dams, irrigation schemes, and highways; mining; deforestation; drug resistance; poor vector control; deterioration of health services; and mobilization of large migrant labor forces. The cultivation of rice and cotton are often associated with increased malaria risk. The creation of new microclimates, following deforestation in urban areas of West Africa, seems to have favored the establishment of *A. arabiensis*, a savannah species, at the expense of *A. gambiae* (Coluzzi et al. 1979). Environmental factors may interact with social, political, and economic pressures, e.g. movements of thousands of refugees, urbanization, smuggling, illegal logging and gem mining, and the existence of ethnic and marginalized communities. The Garki Project is probably the best example of a study where many of the parameters of

malaria epidemiology were critically examined (Molineaux and Grammivcia 1980).

Geographic information systems (GIS) provide a powerful means of capturing large amounts of information and modeling spatial risks. Applications of GIS using meteorological data have been used to develop provisional malaria risk maps in Africa (Craig et al. 1999).

The ecological classification

A pragmatic approach to the epidemiological stratification of malaria has been developed by Najera et al. (1991), defining eight major ecological prototypes:

1 African savannah;
2 plains and valleys outside Africa (areas of traditional agriculture);
3 forest and forest fringe;
4 desert and highland fringe;
5 coastal and marshland;
6 urban slums;
7 agricultural developments;
8 sociopolitical disturbances.

For each paradigm, a description of the circumstances that are responsible for the transmission of malaria is given and the salient features of the impact of the disease are described.

A clinico-epidemiological approach

The natural history of 'stable' malaria infection has been extensively documented. The salient features are as follows: infants born from semi-immune mothers are protected for the first 3–6 months of life, predominantly by passive immunity acquired from their mothers and possibly other factors such as a high concentration of fetal hemoglobin. Although parasitemia occurs during this period, severe and complicated malaria is seldom encountered. From 6 months to 5 years or more (depending on the level of transmission and other factors such as the availability of antimalarial drugs, impregnated bednets, etc.), the child is susceptible to severe attacks of malaria resulting in death in a proportion of children exposed. In the Gambia it has been estimated that 25 percent of all childhood deaths under 5 years were due to malaria (Greenwood et al. 1987). After this danger period, when the cause of death is usually either malarial anemia or cerebral malaria, immunity is gradually acquired and clinical disease is rare except in primigravidae. This sequence of events is graphically presented in Figure 25.

A conceptual diagram of the epidemiology of malaria

A conceptual diagram of the epidemiology of malaria is given in Figure 26. Various factors are known to determine the relative sizes of the circles in various communities. For example, in 'stable' areas of malaria, a substantial proportion of the population will have asymptomatic parasitemia at any one period in time, whereas comparatively few will have clinical malaria and fewer still will have severe malaria (approximately 0.3 percent); this will occur mainly in children and a small proportion (estimated at 0.1 percent) will die. Nevertheless, this amounts to the alarming figure of one million children under 5 years of age in Africa alone. In contrast, in 'unstable' areas the largest circle will be that for uncomplicated malaria, since most of those who get infected usually develop clinical illness.

We have only just begun to identify the factors that might determine which circle an individual patient falls into. For example, some genetic factors that protect against developing severe disease have already been identified but many questions remain unanswered. Some genetic traits that increase the susceptibility to severe disease have also been identified (Knight et al. 1999).

HIV and malaria

The prevalence and intensity of malaria in pregnancy are higher in women who are HIV-positive. In areas of moderate or high transmission, HIV renders multigravidae as susceptible to malaria as primigravidae (Steketee et al. 1996b). Both HIV infection and malaria are independent risk factors. In lower birth weight and maternal anemia, a common occurrence especially in sub-Saharan Africa, the risks for both mother and baby are high.

While most studies have stated that, paradoxically, HIV does not seem to increase the severity of the disease nor significantly enhance the intensity of parasitemia in non-pregnant women and all others exposed to malaria, a study in Uganda (French et al. 2001) has shown a marked inverse relationship between malarial fever and CD4 T-cell counts. In situations where co-infection is a problem, there is some evidence to suggest that antimalarial drugs might be less effective and carry a greater risk of adverse drug reactions (Rowland-Jones and Lohman 2002).

Epidemic malaria

Severe epidemics have occurred in Africa and elsewhere as recently as 2002. They are often the result of exceptional meteorological conditions or massive destruction as a consequence of war or natural disasters followed by population movements. The following risk factors have been identified: (1) abnormally prolonged rains; (2) extensive floods; (3) global warming; (4) colonization of tropical jungle areas by agricultural settlers; (5) explosive growth of urban areas; (6) open-cast mining; (7) arrival of a nonimmune population into a malarious area (e.g. refugees); (8) the introduction of a number of infected individuals into a malaria-free area where both

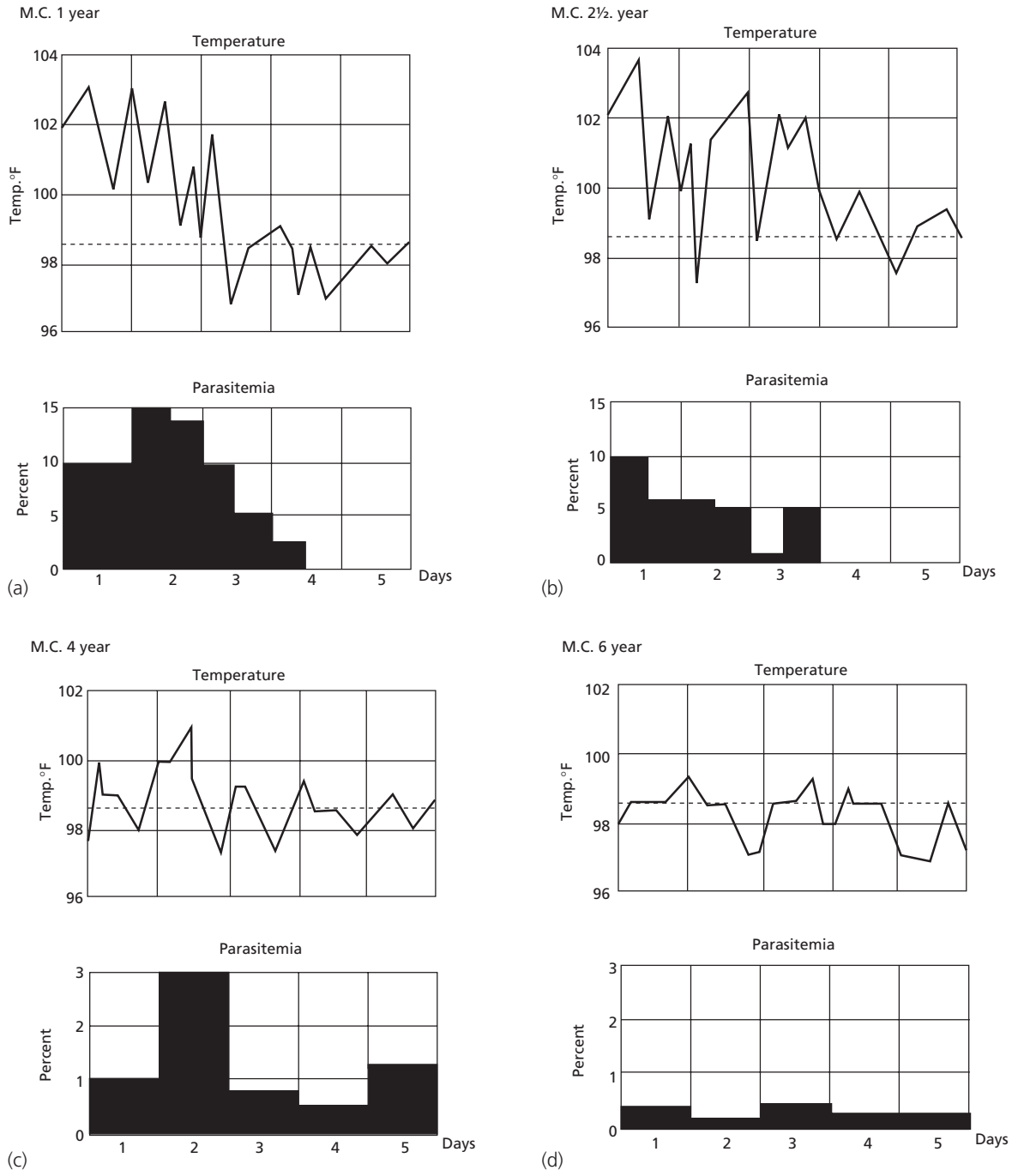

Figure 25 *Natural history of* Plasmodium falciparum *malaria in a 'stable' area showing how clinical immunity develops in a child. With each subsequent phase there is first a decline in the febrile response to infection and later in parasitemia.* **(a)** *Child looks ill, has high temperature and heavy parasitemia.* **(b)** *Child does not look ill, despite high temperature and heavy parasitemia.* **(c)** *Child does not look ill, has mild temperature despite appreciable parasitemia, but may develop anemia insidiously.* **(d)** *Child does not look ill, has no temperature and light parasitemia.*

the *Anopheles* vector and conditions for transmission exist; (9) admixtures of large numbers of immunes and nonimmunes living under primitive conditions (e.g. labor camps); (10) sudden increase in *Anopheles* densities; (11) agricultural development schemes; (12) failure to maintain previous control; (13) breakdown of health services (e.g. Afghanistan); and (14) progressive spread of drug resistance to antimalarials (Snow and Gilles 2002).

Imported malaria

International travel has grown exponentially in the last 50 years and with it imported malaria. This growth has

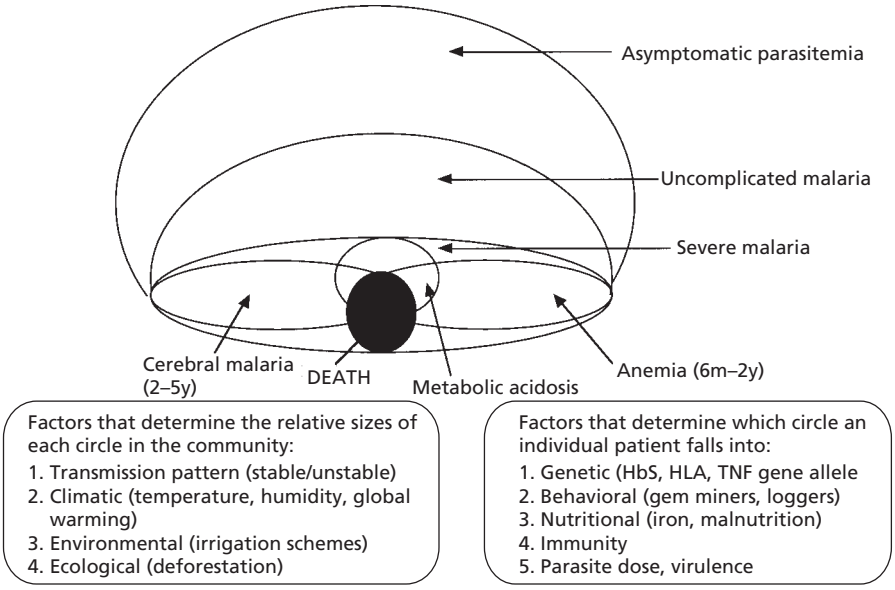

Figure 26 *Conceptual diagram of epidemiology of malaria*

been compounded by the emergence and spread of drug resistance. Labels such as 'airport malaria,' 'baggage malaria' or 'taxi rank malaria' are the result of infected vectors transported from endemic areas in aircraft, suitcases, and taxis, biting nonimmune individuals who had not journeyed to malarious areas.

In the UK, around 1 200 cases of *P. falciparum* are notified yearly; in 2003, the number of cases of *P. falciparum* was 847. Deaths have varied from four in 1989 to 15 in 2000 (PHLS Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine) (Figure 27).

VACCINATION

The prevention of malaria by vaccination is a conceivable approach, which may eventually have a place in malaria control. Unfortunately, no effective malaria vaccine is as yet available and none is likely to be available at an operational level for many years. The topic of malaria vaccines is vast and complex and has been reviewed at length elsewhere (Mendis 1991; Phillips 1994; Hoffman 1996; Facer and Tanner 1997; Engers and Godal 1998; Richie and Saul 2002; Greenwood and Alonso 2002).

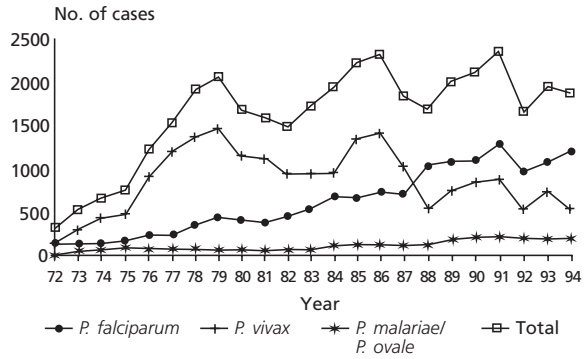

Figure 27 *Annual number of imported cases of malaria in the United Kingdom (based on data from the Malaria Reference Laboratory)*

The aim of a vaccine is to reduce morbidity/mortality due to malaria and this can be achieved in one of two different ways: either by interrupting the infection at one or other of the stages of the parasite life cycle, or by means of an 'anti-disease' vaccine, which would reduce the pathophysiological effects triggered by the release of malarial toxins as proposed by Playfair et al. (1990). *Plasmodium* GPI has been suggested as a potential toxin candidate and the first results of vaccination with this molecule have been reported (Schofield 2002; Schofield et al. 2002).

The interruption of the life cycle may take place at the time of entry of the sporozoite into the host, at the level of schizogony in the hepatocyte, at the time of erythrocyte invasion and intra-erythrocytic parasite development, or by interruption of the sexual development within the mosquito. Figure 28 illustrates potential vaccine targets and indicates which possible parasite molecules may be used as a vaccine in each situation.

The methods by which host immunity will control the infection are variable depending on the target and this will have an implication on the choice of immunogen. For example, a pre-erythrocytic vaccine is likely to rely more on the production of CTLs than antibodies, while a transmission-blocking vaccine is likely to rely essentially on antibodies to sexual stages. It follows that new methods for immunization using DNA injection are likely to be particularly useful for pre-erythrocytic

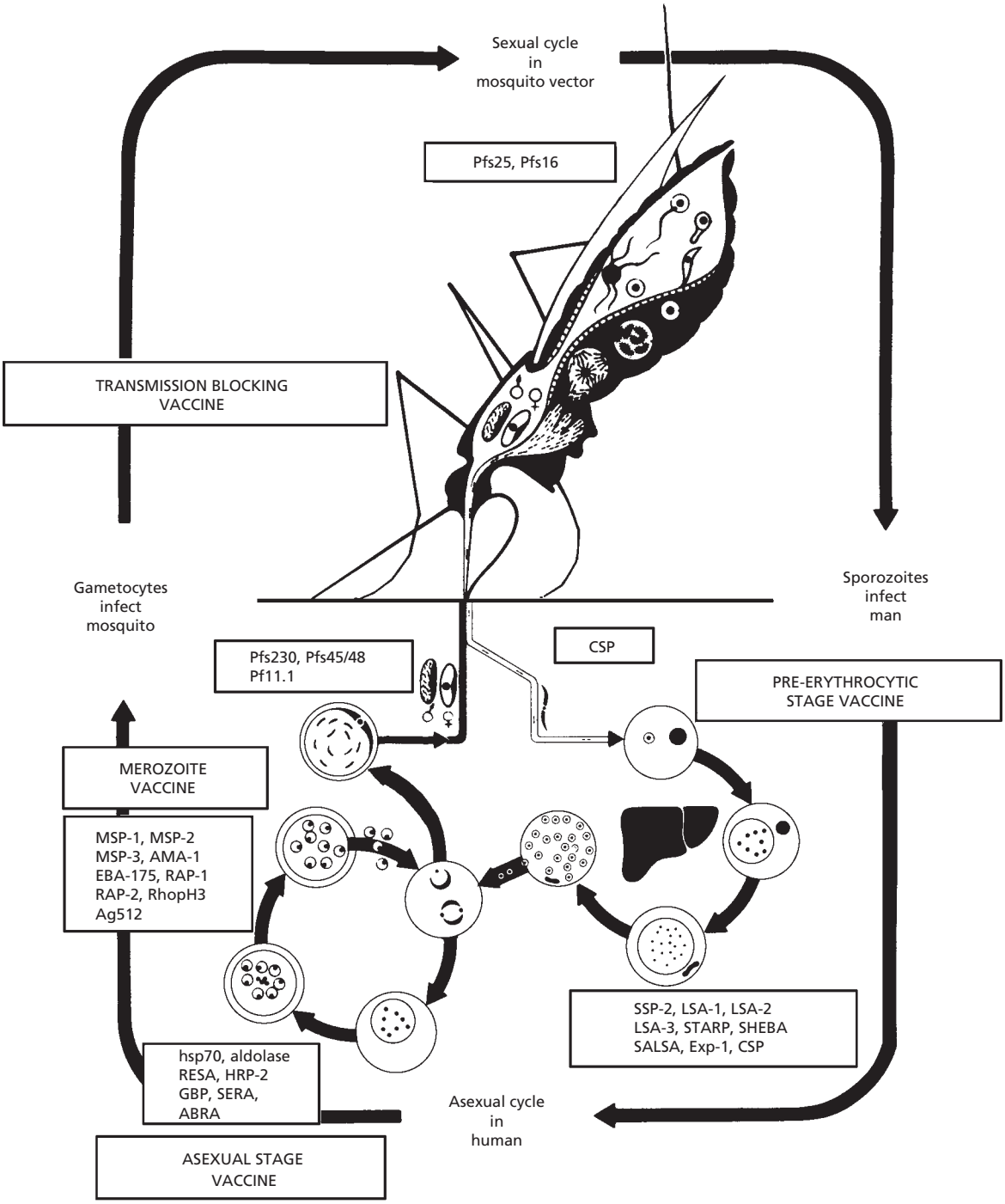

Figure 28 *Possible targets of a malaria vaccine*

stages, since they are good for producing CTLs, and perhaps less useful for transmission-blocking vaccines.

A number of vaccine candidates have been tested in human volunteers with variable success. The best results were obtained with x-irradiated sporozoites (Clyde et al. 1973), while synthetic and recombinant antigens have so far given disappointing results: 1/34 volunteers were protected in a trial using a recombinant CS epitope (Ballou et al. 1987); 0/20 volunteers vaccinated with a recombinant CS co-expressed in yeast with hepatitis B surface antigen were protected (Gordon et al. 1995); a 31 percent efficacy was reported for the synthetic 'Spf66' vaccine, when tested in Tanzania (Alonso et al. 1994), but only a 3 percent efficacy for the same molecule, when tested in the Gambia (D'Alessandro et al. 1995). Current research trends in vaccine development are geared towards 'cocktail' vaccines, which include a combination of multiple epitopes from different malarial

antigens and from different stages of the parasite life cycle. These may include a combination of CSP, LSA-1/3, MSP-1/2, SERA, AMA-1, Pfs48/45, Pfs25, STARP, SSP-2. A synthetic vaccine consisting of part of the CSP fused to the S-antigen of hepatitis B (RTS, S/ASOZA) has produced encouraging results in Mozambique in which 57.5 percent of children were protected (Alonso et al. 2004; Doolan and Hoffman 1997).

The predicted impact of a malaria vaccine in controling the disease has been discussed and analyzed with a variety of mathematical models and with various degrees of optimism (Halloran et al. 1989; Saul 1992).

INTEGRATED CONTROL

Between 1898 and 1940, malaria control was effected by a combination of the use of quinine (prophylactically as well as therapeutically) and mosquito larval control.

The Second World War (1940–45) revolutionized malaria control through the development of the synthetic antimalarials (atebrin, CQ, and proguanil) for effective prophylaxis and treatment, and the first residual insecticide (dichlorodiphenyltrichloroethane (DDT)) which made possible the control of adult mosquitoes by house spraying. Between 1945 and 1955, house spraying with DDT combined with CQ treatment of cases, eliminated malaria from several countries in Europe and gave rise to the concept of malaria eradication as opposed to malaria control. Thus in 1956, the WHO World Health Assembly recommended that WHO should implement a program that would eliminate malaria from the world and this policy was pursued until 1975 when it was abandoned. The reasons for failure can be listed as follows:

1. eradication was an unrealistic objective, particularly in tropical Africa;
2. inflationary price increases of equipment, fuel, and insecticides;
3. political instability;
4. financial and administrative shortcomings;
5. vector resistance;
6. vector-exophily;
7. resistance to antimalarial drugs;
8. household objections to regular spraying; and
9. uncontroled population movements.

In 1976, the WHO World Health Assembly adopted an integrated malaria control strategy, the elements of which are shown in Figure 29. Even this reduced goal proved to be overambitious and unachievable in most of the endemic malaria countries, especially those in tropical Africa, and in 1985 the World Health Assembly recommended that malaria control should be developed as an integrated part of national primary health care systems (see Control of parasites, parasitic infections, and parasitic diseases). The malaria control strategy now being promoted by WHO (Ministerial Conference on Malaria 1992) recognizes four basic technical elements: (1) to provide early diagnosis and prompt treatment; (2) to plan and implement selective and sustainable preventive measures against the parasite, as well as the vector; (3) to detect early, contain or prevent epidemics; and, (4) to reassess regularly a country's malaria situation, in particular the ecological, social, and economic determinants of the disease. This strategy involves a radical shift from the primary emphasis on transmission control of the parasite to the present focus of reduction of malarial disease.

In this context, the use of impregnated bednets is of particular interest. Pyrethroid-treated bednets have been shown to reduce malarial morbidity in certain areas, e.g. China and the Gambia. In areas of intense perennial malarial transmission, there is evidence of reduction of *P. falciparum* parasitemia, reduction in transmission, and reduction in rate of reinfection. In some trials but not in others, widespread community use of insecticide-treated bednets protect even non-users of nets (Greenwood and Baker 1993; Smith et al. 1993; Bockarie et al. 1994; Somboon et al. 1995; WHO 1995). Sustained utilization of nets is clearly important and remains an unsolved challenge (Meek 1995); sustainability is likely to be improved by the development of nets that do not have to be reimpregnated frequently or possibly not at all for many years despite frequent washing (Kroeger et al. 2004), e.g. Olyset® and PermaNet®. The existing vector control tools can still be effective, but they must be selectively deployed, cost-effective, and sustainable.

In the long term, the impact of any malaria control measure (including early diagnosis and treatment, indoor residual spraying, personal protection, or environmental management) depends on the importance given to these measures by the community at risk and on their understanding of and involvement in their application (WHO 1995). The long-neglected social, behavioral, and economic aspects of malaria and its control are being increasingly recognized (Combie 1994; Ruiz and Kroeger 1994; Manderson 1994; Agyepong et al. 1995; Heggenhougen et al. 2004).

It would be wrong to assume that the involvement of primary health care in malaria control in endemic countries is an easy way of solving the difficulties related to administrative, social, or economic obstacles. The following must all be achieved: a proper degree of cooperation between the different levels of the health care system; inter-sectoral coordination; substantial political commitment; adequate human and financial resources; and the full partnership of communities. In addition to these obstacles, the appropriate choice of antimalarial in areas of increasing parasite resistance must also be made if the relatively modest goal of reducing malaria morbidity and mortality is to be achieved (Bryce et al. 1994; Steketee et al. 1996a). Treatment uptake needs to be increased (von Seidlein et al.

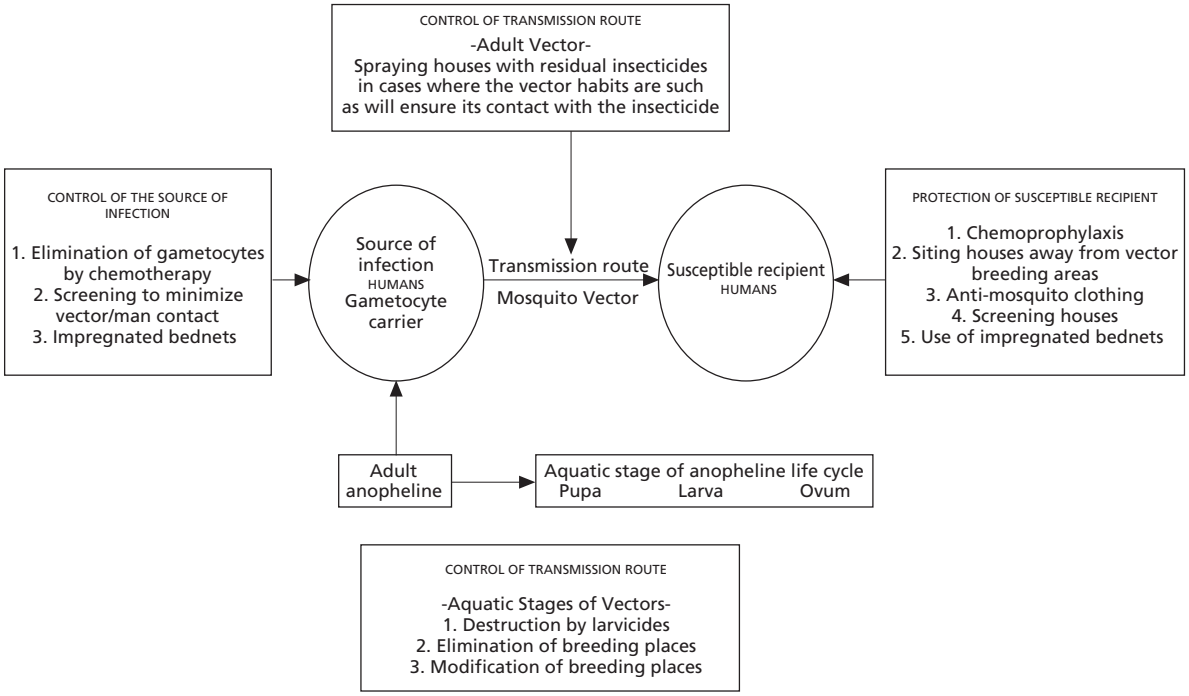

Figure 29 *Integrated malaria control strategy*

2002) and the private sector needs to be educated since it cannot easily be controled especially as it is thegreater provider of antimalarials in most endemic countries.

Roll back malaria

In June 1997, African heads of state met in Harare (Zimbabwe) and issued a declaration on malaria prevention and control in the context of African economic recovery and development. This political commitment was endorsed by the leaders of the industrialized G8 nations. In 1978, this led to a new WHO initiative to roll back malaria (RBM). The goals of RBM include:

1 support to endemic countries in developing their national health systems as a major strategy for controling malaria;
2 developing the broader health sector, i.e. all providers of health care to the community, including the private sector (drug vendors and traditional healers, pharmacists and others); and
3 encouraging the human and financial investments necessary for health system development, nationally and internationally.

The elements of the RBM strategy are:

1 early diagnosis;
2 prompt treatment;
3 multiple prevention;
4 well-coordinated action;
5 dynamic global movement;
6 focussed research.

A functioning partnership with a range of organizations at global, regional, and country levels is being established to ensure sustained capacity to address malaria and other priority health problems. The partners in RBM include malaria-endemic countries, other UN organizations, development banks including the World Bank, bilateral development agencies, nongovernmental organizations, and the private sector.

The RBM campaign focuses first on Africa, where the impact of malaria morbidity and mortality is greatest. It will build upon existing initiatives, such as the African Initiative on Malaria (AIM), the WHO Special Fund for Africa, Medicines for Malaria Venture (MMV), and the Multilateral Initiative on Malaria (MIM). Although the need for new drugs, insecticides, and vaccines still remains, major gains can still be made through better application of current knowledge and best practice.

There are a number of evidence-based, cost-effective interventions available to RBM. These are:

1 insecticide-treated nets (ITN) that are long-lasting (effective up to 4 years) and wash-resistant;
2 intermittent preventive treatment;
3 antimalarial drug combinations;
4 improved access to antimalarial drugs and improving compliance by the use of blister packs and simple dose combination;
5 emergency treatment in the home, at village level, or in rural health centers using either artemisinin suppositories or intra-rectal quinine.

The most difficult hurdle facing RBM is to ensure that sufficient effective drugs are available at the right

time, in the right place, and where they are most needed. New strategies to ensure this is the case must be developed if RBM is to reach its goal of significantly reducing mortality within the next 10 years. A promising strategy entitled 'home management of malaria' is vigorously being pursued (WHO 2004), and may turn out to be the breakthrough we have been awaiting for years, combined with ACT or artesunate suppositories.

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Microsporidiosis

ALAN CURRY

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INTRODUCTION

Microsporidians are all obligate intracellular parasites with a unique mode of entering host cells via a polar tube within a spore. Microsporidians are among the most successful and widespread groups of intracellular parasites in animals (Sprague and Vávra 1977; Canning and Lom 1986; Weidner 1991), infecting other protozoa (Cali 1991; Foissner and Foissner 1995), bryozoans, arthropods, fish, amphibians, reptiles, birds, and mammals. Some are hyperparasites (Hussey 1971). Microsporidians are eukaryotes of ancient origin (Canning and Lom 1986; Vossbrinck et al. 1987); about 100 genera and about 1 000 species are currently recognized worldwide (Sprague et al. 1992). Microsporidians have had a chequered taxonomic history and have nearly always been classified with or alongside the protozoa. At one time, they were classified with the Sporozoa, later they were grouped with the myxosporidians as cnidosporidians and, more recently, elevated to the status of phylum of their own, the Microspora. The Microspora are now thought to be more closely related to fungi than to protozoa (Cavalier-Smith 2001; Hirt et al. 1999), but are retained in this volume because they are traditionally considered together with the protozoa and are more likely to be of interest to parasitologists than mycologists. Relatively few microsporidian species infect homeothermic vertebrates (birds and mammals) and before the recognition of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS), only a handful of human infections were reported in the literature. However, with the spread of HIV infection and AIDS, microsporidia are becoming increasingly recognized as important causes of opportunistic disease in infected individuals.

STRUCTURE AND LIFE CYCLE

The most familiar stage of microsporidians is the small, highly resistant, gram-positive staining spore (Figure 1). Spores contain a coiled filament (polar filament or polar tube) and an infective sporoplasm. The polar tube and its associated organelles are responsible for the unique mode of entering host cells exhibited by microsporidia. Several reviews of the ultrastructure and general biology of the Microsporidia have been published (Canning and Lom 1986; Cali 1991; Perkins 1991; Canning 1993; Bigliardi and Sacchi 2001).

Microsporidian spores are small, more or less ovoid, with a double-layered spore wall. The outer layer, or exospore, is proteinaceous and electron-dense and, according to Weidner and Halonen (1993), is partially stabilized by keratins. The inner layer, or endospore, is chitinous and electron-lucent. The plasma membrane

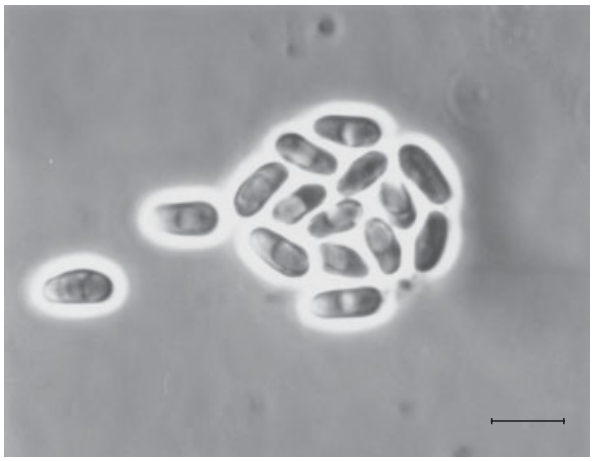

Figure 1 *Light micrograph of spores of* Trachipleistophora hominis *showing shape and posterior vacuole. Bar = 4 μm. (Reproduced with permission from W.S. Hollister and E.U. Canning)*

lines the inside of the spore wall. Organelles within the cytoplasm are the polar sac, polar tube, polaroplast, nucleus, and posterior vacuole (Figures 2 and 3).

All forms lack mitochondria, centrioles, peroxisomes, and a classical Golgi apparatus (Canning 1988; Cali 1991). However, there is molecular evidence that microsporidians possessed mitochondria in their early evolutionary history (Hirt et al. 1997) and that a newly recognized organelle (the multilayered interlaced network) found in the spore and sporoplasm of *Brachiola algerae* may be of Golgi origin (Cali et al. 2002). Ribosomes are

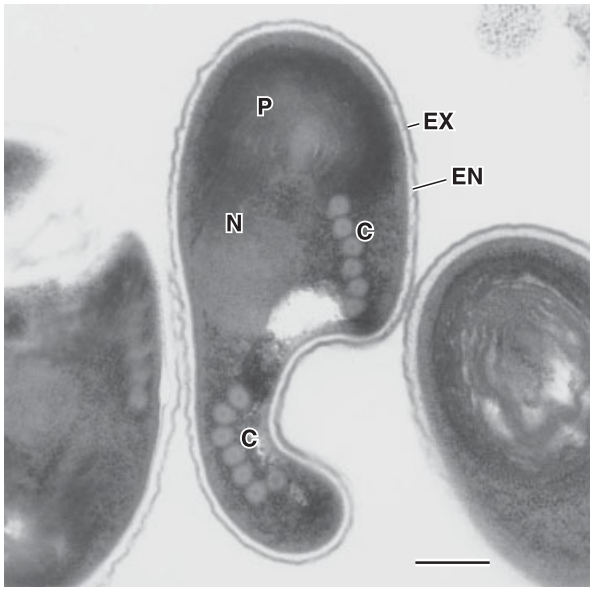

Figure 2 *Electron micrograph of a section through a spore of* Encephalitozoon hellem *showing the coiled polar tube (C), electron-dense exospore (EX), electron-lucent endospore (EN), nucleus (N), polaroplast (P). The indentation in the spore marks the position of the posterior vacuole which collapses during processing for electron microscopy. Bar = 0.18 μm.*

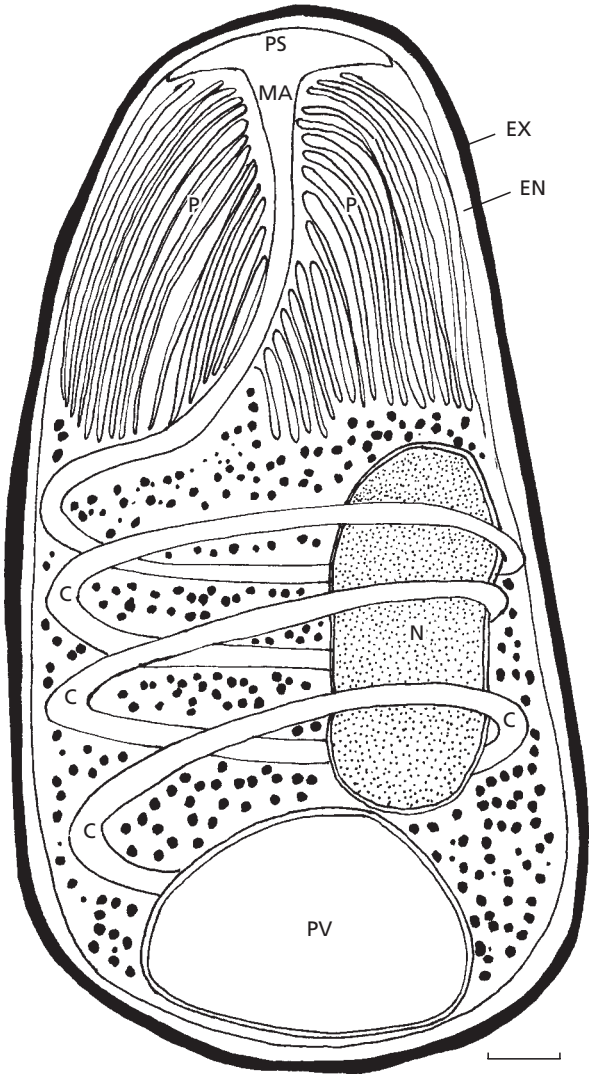

Figure 3 *Diagram of the general features of a microsporidian spore showing coils of polar tube (C), endospore (EN), exospore (EX), manubrium (MA), nucleus (N), polaroplast (P), polar sac (PS), posterior vacuole (PV)*

of prokaryotic size. The polar sac (or anchoring disc), shaped like the cap of a mushroom, is continuous with the polar tube, the base of which appears like the stalk of the mushroom. A thin layer of cytoplasm separates the polar sac from the spore wall. The endospore layer immediately above the polar sac is thinned. From its insertion in the polar sac, the polar tube is initially straight (the manubrium) before coiling into loops around the inside of the spore wall (Figure 4). This polar tube is a complex organelle with intricate anatomical relationships to the other structures of the mature spore (Jensen and Wellings 1972). The structure of the polar tube in the ungerminated spore appears to be tubular, with a filled lumen and concentric rings (Weidner 1982), and was thought to be composed of a single polypeptide of low molecular weight (Weidner 1976) but is now considered to be composed of several

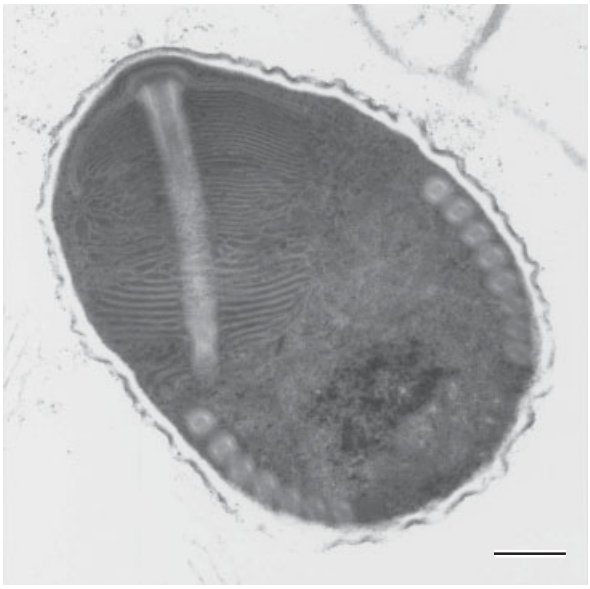

Figure 4 *Electron micrograph of a mature spore of* Trachipleistophora hominis *showing manubrium, polaroplast and coiled polar tube. Bar = 0.48 μm. (Reproduced with permission from Andrew S. Field)*

proteins (Keohane et al. 1999). It is surrounded by a membrane. Around the manubrium is located the polaroplast, which is a component of the extrusion apparatus. Ultrastructurally, the polaroplast is composed of a stack of closely apposed membranes. By light microscopy, the polaroplast region appears transparent and is often referred to as the anterior vacuole. The nucleus is surrounded by a nuclear envelope. In some microsporidial species, nuclei are paired with apposed membranes flattened against one another in a diplokaryon arrangement (Figure 16). The posterior region of the spore is occupied by a membrane-bound posterior vacuole.

On entering a suitable host, the emergence of the polar tube is initiated within a fraction of a second (Weidner 1976). The extruded tube can be very long compared with the size of the spore and is a flexible structure, the purpose of which is to penetrate a host cell and allow entry of the infective sporoplasm. The extrusion process appears to be initiated by swelling of the spore stimulated by a rise in pH and the presence of calcium ions (Weidner and Byrd 1982). Swelling of the polar sac and polaroplast causes the thin anterior area of the spore wall to rupture, a prerequisite for polar tube eversion. The polar tube is believed to be extruded as a solid cylinder, the interior of which flows outward at the growing tip to form a hollow cylinder (Weidner 1982). Once fully everted, the migration of the sporoplasm commences, driven by processes associated with the posterior vacuole. The multilamellar nature of the polaroplast diminishes during extrusion and provides the plasma membrane surrounding the sporoplasm as it arrives within the host cell cytoplasm (Weidner 1982). The migration of the sporoplasm through the lumen of the extruded polar tube occurs within 5–30 s (Weidner 1976) and causes some slight distension of the tube (Figure 5).

Discharged spores, devoid of the sporoplasm, appear empty and show that the proximal part of the polar tube is funnel-shaped, perhaps to guide the sporoplasm cytoplasm into the tube lumen. Little remains of the polaroplast membranes and the externalized polar tube has a flaccid appearance after passage of the sporoplasm cytoplasm.

In some species, penetration into a host cell occurs by the polar tube punching a hole in the host-cell plasma membrane without loss of cytoplasm: an example is *Enterocytozoon bieneusi*. In other species, penetration appears to induce host-cell plasma membrane expansion to cover the emerging sporoplasm, which is itself covered by a membrane thought to be derived from the polaroplast (Figure 6): an example is *Encephalitozoon* (Canning et al. 1992).

Inside the host cell, proliferation begins and two major phases are recognized: merogony and sporogony (Figure 7). In both phases, the parasite nuclei divide without breakdown of the nuclear envelope. Nutrients are absorbed from the host cell to fuel parasite development (Canning and Lom 1986).

Meronts are rounded, irregular or elongated cells with little differentiation of the cytoplasm and are surrounded by a plasma membrane (Figure 8). In most species, the meronts are in direct contact with the host cell cytoplasm (e.g. *Enterocytozoon bieneusi*). However, meronts of *Encephalitozoon* are surrounded by a membrane derived from the host cell that

Figure 5 *Discharged polar tube of* Encephalitozoon hellem *showing slight expansion (arrowheads) due to passage of dense sporoplasm cytoplasm. Bar = 0.13 μm.*

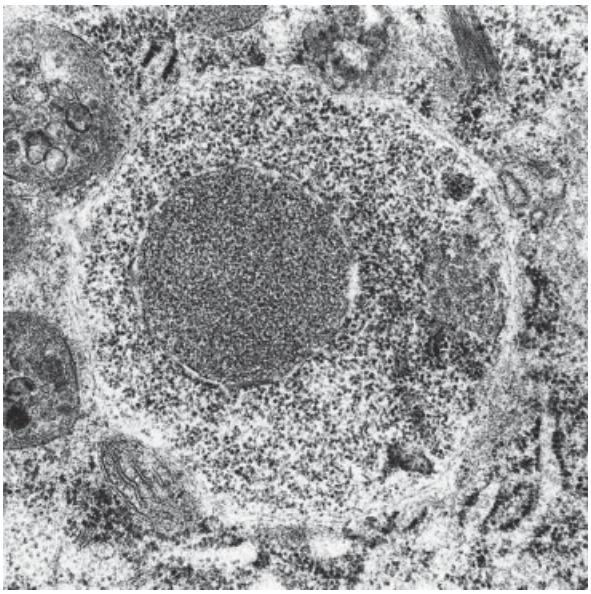

Figure 6 *Possible sporoplasm of* Encephalitozoon hellem *in a nasal epithelial cell. The parasite is surrounded by two membranes: the inner one is the plasma membrane of the parasite, and the outer one a membrane derived from the plasma membrane of the host cell. This latter membrane will ultimately form the margin of the parasitophorous vacuolar membrane seen in the later stages of development. Parasite is about 1 μm in diameter.*

ultimately forms the margin of a parasitophorous vacuole seen in the later stages of parasite development. Meronts may divide by binary fission, multiple fission of a multinucleate meront, or plasmotomy (fission of multinucleate parasite to form multinucleate offspring by division of the cytoplasm without relation to that of the nuclei).

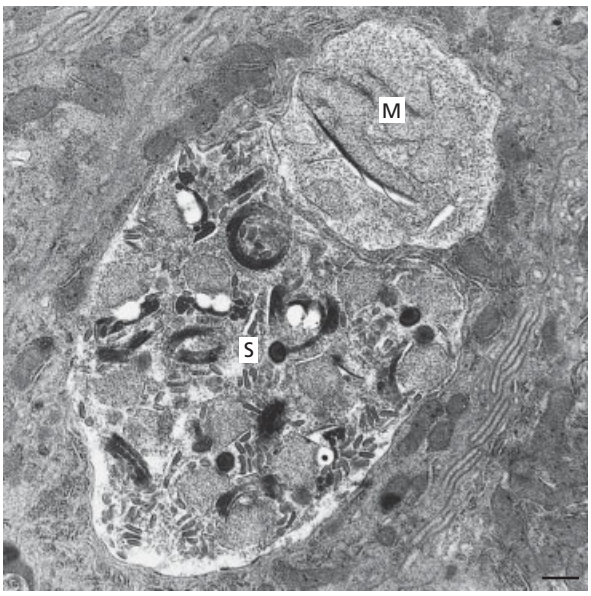

Figure 7 *Duodenal enterocyte infected with* Enterocytozoon bieneusi. *Here, a merogonic (M) and a sporogonic (S) stage lie adjacent to one another within the same host cell. Bar = 0.5 μm*

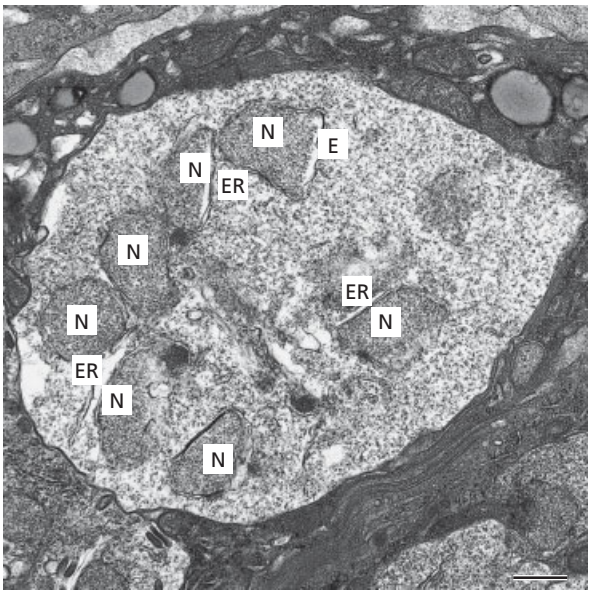

Figure 8 *Meront of* Enterocytozoon bieneusi *showing numerous nuclei (N) and associated cisternae of endoplasmic reticulum (ER). The expanded endoplasmic reticulum can sometimes be seen by optical microscopy as 'cytoplasmic slits' within the host small intestinal epithelial cells. Bar = 0.48 μm*

Meronts develop into sporonts, characterized by the presence of an electron-dense surface coat, on the outside of the plasma membrane, which will ultimately become the exospore layer of the mature spore wall (Figure 9). Sporonts may divide by binary fission directly into sporoblasts (cells that differentiate into spores without further division) or may become multi-

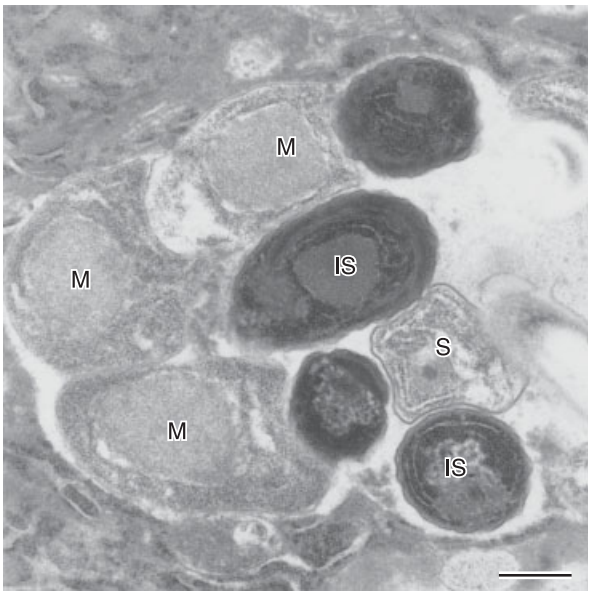

Figure 9 Encephalitozoon hellem *from nasal epithelium. Electron micrograph showing parasitophorous vacuole containing meronts (M), a sporont (S) and immature spores (IS). Bar = 0.37 μm*

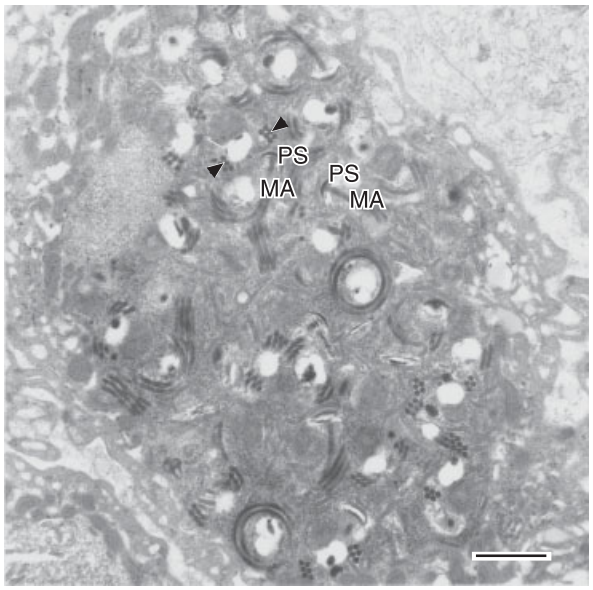

Figure 10 *Late sporogonial sporoplasm of* Enterocytozoon bieneusi *showing many copies of spore organelles. Multiple fission will produce sporoblasts that will mature into spores. Note manubrium (MA), polar sac (PS) and six coils in two rows of a polar tube (arrowheads). Bar = 0.64 μm*

nucleate and form sporogonial plasmodia (Figures 10 and 25.11). Sporogonial plasmodia undergo multiple (sequential) fission to produce sporoblasts that ultimately develop into spores. In some genera, such as *Trachipleistophora* and *Pleistophora*, spores are packaged within sporophorous vesicles (SPOV) (Figure 12). The endospore layer of the spore wall is

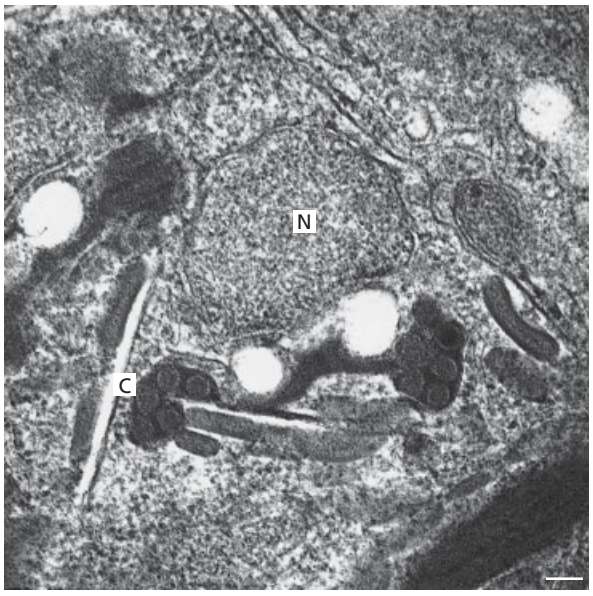

Figure 11 *High magnification micrograph of part of the cytoplasm of a sporogonial plasmodium of* Enterocytozoon bieneusi *showing individual spore organelles. Note nucleus (N) and associated profiles of the coiled polar tube (C). Bar = 0.12 μm*

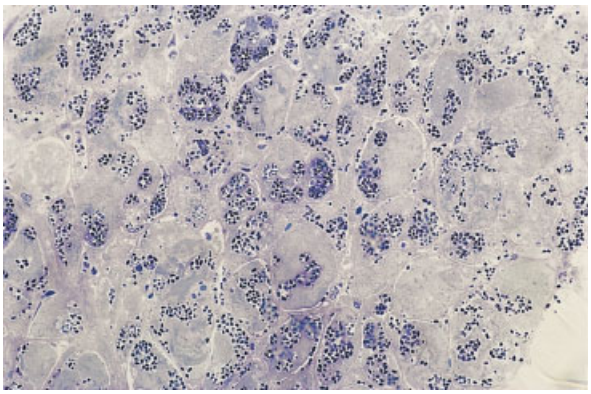

Figure 12 *Light micrograph of* Trachipleistophora hominis *in skeletal muscle of an AIDS patient. Note that the spores are in packets (sporophorous vesicles).*

synthesized between the dense exospore layer and the plasma membrane. Spores may be liberated by lysis of the host cell, although in some species, such as *Encephalitozoon hellem*, mature spores may germinate and infect other neighboring cells without lysis of the host cell (Canning et al. 1992) (Figure 13).

An intranuclear spindle facilitates chromosomal separation during mitosis. The mitotic apparatus consists of two centriolar plaques (electron-dense regions associated with nuclear pores) at the spindle apices (Figure 14), on which the spindle microtubules converge (Canning 1988). Sexual processes, as indicated by synaptonemal complexes, have been reported in some genera parasitizing invertebrates (Andreadis and Vossbrinck 2002). Among the genera that infect vertebrates, meiosis is either unknown or unconfirmed.

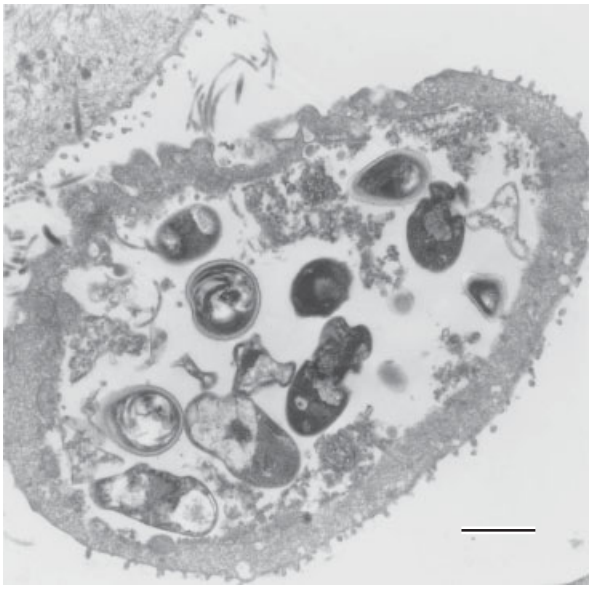

Figure 13 Encephalitozoon hellem *from conjunctival epithelium. Parasitophorous vacuole containing spores. Note that some have germinated as indicated by the presence of profiles of polar tubes between and around the spores. Bar = 1 μm*

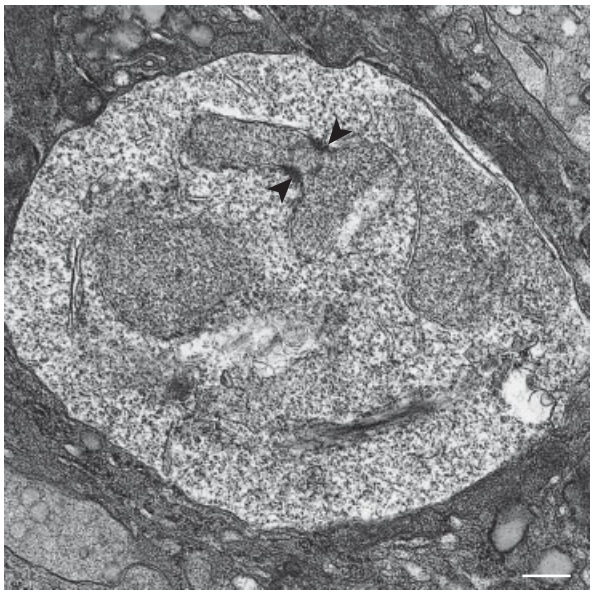

Figure 14 *A meront of* Enterocytozoon bieneusi. *Note dense nuclear plaques (arrowheads) indicating nuclear division. Bar = 0.33 μm*

GENERA, SPECIES, AND TAXONOMIC CHARACTERISTICS

Much of the current taxonomy is based on light microscopy, ultrastructural features, host species and organ or tissue specificity. Important ultrastructural features include nuclear arrangement (mono- or diplokaryotic), mode of division, development of proliferative forms in direct contact with host cell cytoplasm or within a parasitophorous vacuole, sporogony producing spores dispersed or aggregated in a SPOV and spore structure. Many amendments and revisions to the current classification are likely as extensive molecular studies are undertaken.

Encephalitozoon

See Canning and Lom (1986). Unpaired (isolated) nuclei are seen in all stages of development. Development occurs in parasitophorous vacuoles, which contain a finely granular matrix. Meronts lie attached to the vacuolar membrane, whereas sporogony, which is generally disporoblastic, occurs within the lumen of the parasitophorous vacuole. Merogony, typically by binary fission, supplies abundant potential sporonts and the vacuole concomitantly expands to accommodate the growing numbers of parasites. Vacuoles contain mature and maturing spores. Spores are small with a thick endospore layer and a rugose exospore.

Three species of *Encephalitozoon* have been recognized in humans: *E. cuniculi* (type species), *E. hellem*, and *E. intestinalis* (synonym for *Septata intestinalis*). Spores of *E. cuniculi* measure about 2.5–3.2 × 1.2–1.6 μm, with 4–6 coils of the polar tube (Canning and Lom 1986). Spores of *E. hellem* measure 2–2.5 × 1–1.5 μm, with 6–8 coils of the polar tube (Didier E.S. et al. 1991). Spores of *E. intestinalis* measure 2.2 × 1.2 μm, with 5–7 coils of the polar tube (Canning et al. 1994).

Enterocytozoon

See Desportes et al. (1985); Cali and Owen (1990). Unpaired nuclei are seen in all stages of development. Merogonic and sporogonic stages lie in direct contact with the host cell cytoplasm. Proliferative stages develop into sporogonial plasmodia, which produce sporoblasts by multiple fission. Membrane-bound electron-lucent clefts are conspicuous in both meronts and sporonts. In the multinucleated sporogonial plasmodia, polar tube precursors form as electron-dense discs adjacent to the clefts. A unique characteristic of this genus is the complete differentiation of the polar tube within the sporogonial plasmodium, prior to fission (Wongtavatchai et al. 1995). On division into sporoblasts and maturation of these into spores, each becomes dispersed within the host cell cytoplasm.

One species has been recorded from humans, the type species *Enterocytozoon bieneusi*. Spores are very small (about 1.5 × 0.5 μm), with 4–6 coils of the polar tube in two rows. The endospore layer is thin.

Trachipleistophora

See Hollister et al. (1996). Unpaired nuclei are seen in all stages of development. The plasma membrane of meronts is overlain by a dense surface coat, which extends outwards as branched and anastomosing processes. Meronts divide by binary fission or plasmotomy. At the onset of sporogony, the surface coat separates and becomes the thick envelope of the SPOV. Sporonts divide repeatedly by binary fission of binucleate stages to give a variable number of uninucleate sporoblasts and spores. Multinucleate sporogonial plasmodia are not formed.

Two species have been recorded from humans, the type species *T. hominis* (Hollister et al. 1996) and a newly recognized species, *T. anthropophthera* (Vávra et al. 1998). Spores of *T. hominis* measure 5.2 × 2.4 μm, with about 11 coils of the polar tube. The polar tube shows an anisofilar arrangement with 8–11 larger diameter coils and 1–3 smaller diameter coils (Canning et al. 1998). The endospore is thick. *T. anthropophthera* has two types of SPOV: type I SPOV contains usually eight spores measuring 3.7 × 2 μm, with an anisofilar polar tube arrangement (7 larger diameter coils in a single row with 2 posterior coils of smaller diameter shifted inwards towards the centre of the spore); type II SPOV contains two spores, each measuring 2.5 × 1.4 μm, with about 4–5 coils of the polar tube.

Pleistophora

See Canning and Lom (1986). Unpaired nuclei are seen in all stages of development. Meronts form as multinucleate plasmodia that possess a thick amorphous electron-dense surface coat external to the plasma membrane. Division into smaller segments occurs. The surface coat becomes the wall of the SPOV (pansporoblast membranes). Sporogony is polysporoblastic. Division of the sporogonial plasmodium is by repeated segmentation, finally producing uninucleate sporoblasts, which mature into spores. The number of spores produced within the sporophorous vesicles is large and variable (polysporoblastic).

A few cases of human infection have been ascribed to parasites belonging to the genus *Pleistophora*, but only in one case has the organism been specifically named (as *P. ronneafiei* – see Cali and Takvorian (2003). Some of these parasites may not be true species of Pleistophora as their full development has not been adequately described and some may have to be reclassified within the genus *Trachipleistophora* (Hollister et al. 1996). Spores from human infections measure 3.2–3.4 × 2.8 μm, with 11 coils of the polar tube (Ledford et al. 1985) or about 4 × 2 μm, with 9–12 coils of the polar tube (Chupp et al. 1993).

Vittaforma

See Silveira and Canning (1995). Nuclei are seen in diplokaryotic arrangement throughout the life cycle. All stages are individually enveloped by cisternae of the host cell rough endoplasmic reticulum. Merogony is by binary fission and sporogony is polysporoblastic, giving rise to 4–8 linearly arranged sporoblasts.

One species has been recorded from humans, the type species *V. corneae*. Spores, still enveloped by host endoplasmic reticulum, measure 3.8 × 1.2 μm, with 5–7 coils of the polar tube.

Nosema

See Canning and Lom (1986). Nuclei are found in diplokaryotic arrangement throughout the life cycle. Parasites are in direct contact with the host cell cytoplasm. Merogony is by binary fission and sporogony is disporoblastic giving rise to spores, which are dispersed in host cell cytoplasm.

One species is currently recorded from humans, *N. ocularum* (Cali et al. 1991a). Spores measures 5 × 3 μm, with between 9 and 12 coils of the polar tube. Three former species of *Nosema* from humans have now been reclassified: *Nosema corneum* (Shadduck et al. 1990) is a synonym for *V. corneae*; *N. algerae*, a mosquite parasite (Visvesvara et al. 1999), has been renamed *Brachiola algerae* (Lowman et al. 2000) and *N. connori* has been reclassified as *B. connori* (Cali et al. 1998).

Brachiola

See Cali et al. (1998). Nuclei are found in diplokaryotic arrangement throughout the life cycle. Merogonic and sporogonic stages lie in direct contact with the host cell cytoplasm. No plasmodial stages develop. All stages possess electron-dense secretions external to the plasma membrane. Abundant tubulovesicular structures embedded in the secreted coat often extend like appendages into surrounding host cell cytoplasm. Merogony is by binary fission and sporogony is disporoblastic.

Three species have been recorded from humans. The type species is *Brachiola vesicularum* with spores which measure 2.5–2.9 × 1.9–2.0 μm and contain 7–10 coils of the polar tube arranged in two rows. The last 2–3 coils of the polar tube are smaller in diameter than the rest (anisofilar arrangement). The other species are *B. connori*, formerly *Nosema connori* (see Margileth et al. 1973; Sprague 1974) and *B. algerae*, formerly *N. algerae* (Visvesvara et al. 1999; Lowman et al. 2000).

'Microsporidium'

See Canning and Lom (1986). This is an assemblage of identifiable species for which the generic positions are uncertain because of insufficient taxonomic detail. They include *M. ceylonensis* (Ashton and Wirasinha 1973) in which the spores measure 3.5 × 1.5 μm and with about 9 coils of the polar tube and *M. africanum* (Pinnolis et al. 1981) 4.5–5 × 2.5–3 μm, with 11–13 coils of the polar tube.

HISTORICAL ASPECTS OF HUMAN INFECTION

The first authenticated report of human microsporidial infection was by Matsubayashi et al. (1959) who described a case of microsporidial infection in a 9-year-old Japanese boy suffering a severe convulsive illness admitted to hospital unconscious and with a fever. Organisms resembling *Encephalitozoon cuniculi* were isolated on day 5 of the illness from the cerebrospinal fluid, and on days 13–15 spores were identified from the urine. On regaining consciousness, headache and vomiting were recurrent symptoms, but the boy is reported to have made a full recovery.

A Colombian child suffered a similar convulsive illness (Bergquist et al. 1984) and spores of *E. cuniculi* were isolated from the urine. After anticonvulsive therapy, the child made a full recovery. Ashton and Wirasinha (1973) described a case of corneal infection in an 11-year-old Sri Lankan boy. Although the original authors did not specifically identify it, Canning and Lom

(1986) transferred this organism to the collective group *Microsporidium* and named it *Microsporidium ceylonensis*. In 1981, another microsporidian was described from the eye of a Botswanan woman (Pinnolis et al. 1981) and this was later also transferred to the collective group *Microsporidium* as *M. africanum* (Canning and Lom 1986). Margileth et al. (1973) described a case history of an overwhelming disseminated infection in a 4-month-old infant with a defective lymphoid system. After an illness lasting 4 months, characterized by diarrhea and malabsorption and concurrent infection with *Pneumocystis carinii*, the child died. Microsporidian spores were seen in cardiac and smooth muscle, diaphragm, myocardium, kidney tubules, liver, lungs, and adrenal cortex and within the walls of arteries in many organs. On the basis of the cytoplasmic and ultrastructural features of the sporoblasts, immature and mature spores, particularly the diplokarya and about 11 coils of the polar tube, the organism was tentatively classified as *Nosema connori* (Sprague 1974; Shadduck et al. 1979; Canning and Lom 1986). This organism has since been allocated to a newly established genus *Brachiola*, as *Brachiola connori* (Cali et al. 1998).

Since the recognition of HIV and AIDS in the late 1970s and early 1980s, several previously rare or unknown human parasitic protozoa have been recognized in this immunocompromised group (Curry et al. 1991). In 1985, a new microsporidian, *Enterocytozoon bieneusi*, was identified in the duodeno-jejunal enterocytes of an AIDS patient with a history of diarrhea and weight loss (Desportes et al. 1985; Modigliani et al. 1985). Since this description, more microsporidian species have been described. Fifteen species are currently recognized from humans and many of these were previously unknown.

SEROLOGICAL SURVEYS OF MICROSPORIDIAL INFECTION IN HUMANS

Before our recent awareness of human microsporidial infections, one of the few species known to infect homeothermic vertebrates was *E. cuniculi* (Levine 1985; Canning and Lom 1986). It has a worldwide distribution in many mammals including rodents, lagomorphs, carnivores and primates. It is a multiorgan pathogen that can be found in the brain, kidneys, liver, spleen, and other organs. In the past *E. cuniculi* caused many problems in animal houses, and serological tests are now available to test for such infections (Canning and Lom 1986). Considering its wide host range in homeothermic vertebrates, it is not surprising that encephalitozoonosis has also been reported in humans. Bergquist et al. (1984) reported the results of a serological survey for *E. cuniculi* in 22 serum samples from human patients with disorders of the central nervous system (all of uncertain origin); one sample gave clearly positive results. The patient was an apparently healthy Colombian boy, adopted when just over 1 year old by a Swedish family. He was admitted to hospital a year later with generalized convulsive seizures. Based on an indirect immunofluorescence technique, a serum sample taken from the boy about the time of adoption (a year before initial admission to hospital) showed a positive immunoglobulin IgM antibody response to *E. cuniculi*. This original serum sample and those taken during his subsequent admissions to hospital also showed high titers of IgG against *E. cuniculi*. Sedimented urine, obtained during his first stay in hospital, contained gram-positive spores measuring 1.5×2.5 μm, which reacted with a fluorescent anti-*E. cuniculi* conjugate. Urine-derived organisms were injected intraperitoneally into five mice known to be free of encephalitozoonosis and, after 3 weeks, two became infected with parasites indistinguishable from *E. cuniculi*. Hematological testing revealed that the child had a lymphocyte abnormality. The child was given prophylactic anticonvulsive therapy and recovered.

A later serological survey for antibodies to *E. cuniculi* using enzyme-linked immunosorbent assay (ELISA) and utilizing spores harvested from cell culture, demonstrated antibodies to *E. cuniculi* in some patients with psychiatric disorders and also some of those previously exposed to schistosomiasis and malaria (Hollister and Canning 1987).

A more comprehensive survey of human sera (Hollister et al. 1991) utilizing ELISA and also immunofluorescence, immunoperoxidase and Western blots of sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) protein profiles of *E. cuniculi* spores concluded that human *E. cuniculi* infections were common in the tropics and that reactivation of such infections could account for the occurrence of some infections in AIDS patients. Hollister et al. (1993b), in a retrospective analysis of stored serum samples from an AIDS patient, detected antibodies to *Encephalitozoon* sp. 32 months before signs of keratoconjunctivitis developed and 3 years before nasal obstruction became a problem (Hollister et al. 1993b).

Another serological survey in Europe has shown a high seroprevalence to *Encephalitozoon* species in the immunocompetent population (Van Gool et al. 1997): 8 percent of Dutch blood donors and 5 percent of pregnant French women had antibodies. It was concluded that subclinical infection, or infection involving mild transient symptoms may be common. Such transient episodes may be followed by latency (Hollister et al. 1991), which only becomes problematical if the individual subsequently becomes immunosuppressed.

HUMAN MICROSPORIDIAL INFECTIONS AND IMMUNE STATUS

Current information indicates that patients with severe cellular immunodeficiency are at the greatest risk of

developing microsporidial disease (Weber and Bryan 1994). Experimental evidence in mice supports this view (Schmidt and Shadduck 1983) and that resistance to lethal disease appears to be T-cell dependent. *E. cuniculi* causes chronic but not life-threatening infections in euthymic BALB mice, whereas athymic mice succumb to infection. Serum antibodies are not protective.

With the increase in knowledge about microsporidial infections, we now know that they are not restricted to those infected with HIV. Other immunodeficient states, such as those associated with organ transplantation, can make such patients susceptible to microsporidial infection. Indeed, microsporidial infection should be considered for any individual with symptoms who is known to be immunodeficient from any cause. In addition, microsporidial infections are also being reported from individuals with normal immune function and from the elderly in whom the immune system goes into decline.

The clinical spectrum of microsporidial infection includes ocular, intestinal, muscular, and systemic aspects. Most, but by no means all, are associated with HIV infection. The most common microsporidian-associated disease is chronic non-bloody diarrhea and wasting in HIV-infected patients.

Enteric infections

Diarrhea, malabsorption, and wasting are frequently encountered in AIDS patients (Modigliani et al. 1985) and many microbiological causes have been identified for these enteric symptoms; among the parasitic protozoa, both *Cryptosporidium parvum* (see

Intestinal coccidia: cryptosporidiosis, isosporiasis, cyclosporiasis) and microsporidial infections are significant causes of morbidity.

ENTEROCYTOZOON BIENEUSI INFECTIONS

The first reported case of enteric microsporidial infection in an AIDS patient was from France by Desportes et al. (1985). The patient was a 29-year-old nonhomosexual Haitian patient with AIDS, who had severe diarrhea and *Giardia* in stool specimens. Electron microscopic investigations of duodeno–jejunal and ileal biopsies revealed the presence of developmental stages of a microsporidian parasite in the enterocytes (Figures 8, 10, and 11). The ultrastructural details of the development of this microsporidian supported the view that the microsporidial species involved was distinctive enough to merit classification as a new species within a new genus. The name allocated was *Enterocytozoon bieneusi* (in order to avoid confusion with species of *Encephalitozoon*, *Enterocytozoon bieneusi* will subsequently be referred to as *Ent. bieneusi*, as used in Canning et al. 1993). Further details of the intracellular development of this species were reported from cases in the USA (Cali and Owen 1990). Fever is not associated with such infection, but abdominal cramping is (Asmuth et al. 1994). Malabsorption of fat and D-xylose, and low levels of zinc have been noted in such patients (Asmuth et al. 1994).

Ent. bieneusi is likely to have a worldwide distribution as it has been reported from many countries around the world including the UK (Curry et al. 1988), the Netherlands (Rijpstra et al. 1988), Africa (Lucas et al. 1989), USA (Cali and Owen 1990), and Australia (Field et al. 1993b). It is now recognized that the majority of microsporidial infections found in patients infected with HIV are attributable to this species (Weber and Bryan 1994). *Ent. bieneusi* infection in the small bowel can show histological changes such as villous blunting or ballooning (Orenstein et al. 1990a; Kotler et al. 1993; Asmuth et al. 1994), although some biopsies show more or less normal villous architecture (Eeftinck Schattenkerk et al. 1991). Crypt hyperplasia may also be found (Field et al. 1993c). Inflammation in infected tissues is often minimal (Lucas 1989; Orenstein 1991). Such a variability of changes seen in biopsies may reflect the degree of infection, with heavy infections causing the most cellular and histological changes. *Ent. bieneusi* infection is generally restricted to the enterocytes of the small bowel but infection in other sites has been reported. The biliary tract can be infected, leading to cholangitis (Beaugerie et al. 1992). Such infection may be caused by spread along the intestinal–bile duct epithelium. The gall bladder can also be infected causing acalculous cholecystitis. In addition, there are reports of *Ent. bieneusi* infection in nasal (Hollister and Canning unpublished results, quoted in Canning et al. 1993) and bronchial (Weber et al. 1992b) epithelia, respiratory samples (del Aguila et al. 1997) and the liver (Pol et al. 1993). Such non-small-intestinal sites of infection involving *Ent. bieneusi* appear to be uncommon, and the mechanism of spread is uncertain.

Association of *Enterocytozoon bieneusi* with symptoms of diarrhea

A significant number of patients with intestinal microsporidiosis are co-infected with other potential enteric pathogens (Weber and Bryan 1994) making the pathogenic role of microsporidia difficult to assess. However, most prevalence studies of intestinal microsporidiosis in HIV-positive patients have shown a strong association with diarrhea (Greenson et al. 1991; Weber et al. 1992a; Field et al. 1993b; Eeftinck Schattenkerk et al. 1991; Van Gool et al. 1993). In contrast, Rabeneck and colleagues (1993) found that the association between enteric microsporidial infection and diarrhea was not as strong as previous studies had suggested but this is the only study not to link microsporidial infection to symptoms of chronic diarrhea in HIV disease.

Prevalence of *Enterocytozoon bieneusi* infection in Africa

In developing countries, such as those in central Africa, diarrhea and weight loss are important indicators for the clinical diagnosis of AIDS (Kelly et al. 1994). These common manifestations are often referred to as enteropathic AIDS or 'slim disease' (Serwadda et al. 1985; Lucas et al. 1993). Lucas et al. (1989) identified a lower prevalence of microsporidial infection from Uganda and Zambia than in developed countries; 6.5 percent (5 of 77 patients with chronic diarrhea and wasting) had *Ent. bieneusi* infection. This low prevalence suggested that patients were dying from other causes (particularly tuberculosis) before their CD4 lymphocyte count had dropped to the critical level of about 100×10^6/l, where microsporidial infection becomes significant (Canning et al. 1993).

Bretagne et al. (1993), in a survey of children of unknown HIV status from an area of Africa (Niamey, Niger) with a low occurrence of HIV, found that in 990 stool samples tested, 6 of 593 (1 percent) with diarrhea and 2 of 397 (0.5 percent) without diarrhea contained *Ent. bieneusi* spores.

ENCEPHALITOZOON (=*SEPTATA*) *INTESTINALIS* INFECTIONS

Although originally described as resembling *E. cuniculi* (Orenstein et al. 1992a,b), this microsporidian was subsequently assigned to a new genus and named *Septata intestinalis* (Cali et al. 1993). Unlike *Ent. bieneusi*, intracellular development takes place within a lobed parasitophorous vacuole bounded by a membrane (Figure 15). Within the vacuole, parasites are separated by granular septa secreted by the parasite (Cali et al. 1993). However, Hartskeerl et al. (1995) considered that this organism should be classified as *Encephalitozoon intestinalis*, on the basis of restriction fragment length polymorphism analysis of amplified ribosomal RNA sequences which showed about 90 percent identity with *E. cuniculi* and *E. hellem*. In addition, Western blots reveal a significant cross-reactivity between these three species. Hartskeerl and colleagues concluded that *S. intestinalis* should be regarded as a species within the genus *Encephalitozoon* and this is what is accepted in this chapter.

E. intestinalis is not confined to epithelial cells as it is also found in macrophages in the lamina propria. This species appears to have a primary infection site in the small bowel, but can disseminate to the viscera (see below) and has been found in epithelial enterocytes and the lamina propria of the duodenum, jejunum (Orenstein et al. 1992b), ileum, colon (Field et al. 1993a), kidney (Orenstein et al. 1992a; Field et al. 1993a), liver, and gallbladder (Orenstein et al. 1992a) and other sites, including the lower airways (Schwartz et al. 1993b). The prevalence of *E. intestinalis* in small-intestinal biopsies

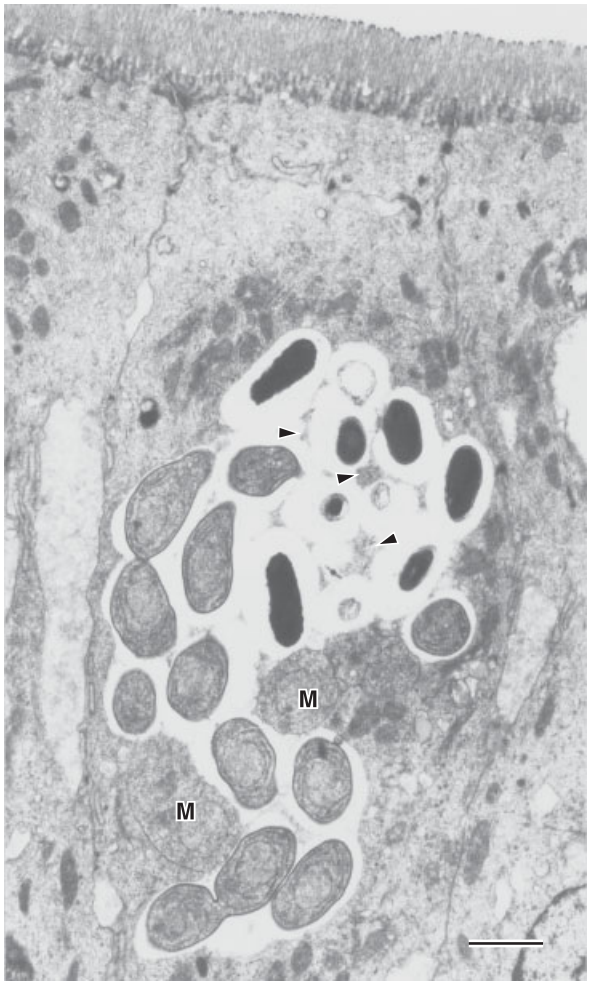

Figure 15 *Electron micrograph of a duodenal enterocyte with a parasitophorous vacuole containing* Encephalitozoon intestinalis. *Note meronts (M) apposed to vacuolar membrane and granular septal material between parasites (arrowheads). Bar = 0.53 μm. (Reproduced with permission from Canning et al. 1994)*

appears to be significantly less than *Ent. bieneusi* infection to which there are now numerous references (Canning et al. 1993). Kelly et al. (1994) found 2 of 75 (3 percent) patients with HIV-related diarrhea infected with *E. intestinalis* in an African (Zambia) study and the prevalence is similar to that found in other studies elsewhere.

Concurrent infections involving both *E. intestinalis* and *Ent. bieneusi* have been reported (Blanshard et al. 1992b; Orenstein et al. 1992a; Cali et al. 1991c). Van Gool et al. (1994), in an attempt to culture *Ent. bieneusi* from stool samples from four AIDS patients with biopsy-proven *Ent. bieneusi* infections, established cultures, but the organism grown was identified as *E. intestinalis*. A possible explanation for this somewhat unexpected result is that *Ent. bieneusi* and *E. intestinalis* are dimorphic forms of the same species. As no evidence of *E. intestinalis* infection had been found in the original biopsies, Van Gool and colleagues dismissed this

explanation and suggested that all the patients from whom stool samples were obtained had heavy infections of *Ent. bieneusi* and light infections of *E. intestinalis*. Critical re-examination of the stool samples revealed abundant *Ent. bieneusi* spores and rarely seen, larger spores, which could possibly have been *E. intestinalis*. These results suggest that *E. intestinalis* can occur as an unapparent infection and that its true prevalence may be much higher than previously thought.

ENCEPHALITOZOON CUNICULI INFECTIONS

It is also possible that *E. cuniculi* infects the gut as it has also been reported from the intestinal tract of an AIDS patient with disseminated *E. cuniculi* infection (Franzen et al. 1995). However, the authors point out that the intestinal infection was not confirmed and was presumed to be *E. cuniculi*, as *E. cuniculi* infection had been confirmed from other sites. In this case, the possibility exists that the enteric infection involved *E. intestinalis* rather than *E. cuniculi*.

CLINICAL FEATURES OF ENTERIC MICROSPORIDIAL INFECTIONS IN AIDS

Asmuth et al. (1994) retrospectively reviewed 20 patients who had small-intestinal microsporidiosis. Fever was not associated with enteric microsporidial infection, but abdominal cramping was. Mean CD4 counts were $35 \pm 29 \times 10^6$/l and most had been lower than 100×10^6/l for at least 16 months. Mean duration of diarrheal symptoms was 8.5 ± 6.9 months, with stool frequency at the time of diagnosis 5.7 ± 2 per day. Within this group of 20 patients, 18 had their microsporidial parasites identified to species level: 14 (78 percent) were infected with *Ent. bieneusi* and 4 (22 percent) with *E. intestinalis*. Asmuth et al. (1994) also showed malabsorption of fat and D-xylose and low levels of zinc, and suggested that the enteric microsporidia have the ability to turn on secretory processes within the small intestine and to induce diarrhea.

Fecal tumor necrosis factor-alpha (TNF-α) has been found to be elevated in cases of HIV-related diarrhea due to enteric microsporidial infection (Sharpstone et al. 1997).

Normally, *Ent. bieneusi* infects enterocytes of the small bowel, but this species can occasionally be found in the lamina propria. Less commonly, it can invade the biliary tree causing cholangitis, and the gall bladder causing acalculous cholecystitis. *E. intestinalis* can also cause cholecystitis (French et al. 1995) and cholangitis (Orenstein et al. 1992a).

ENT. BIENEUSI AND *E. INTESTINALIS* IN IMMUNOCOMPETENT INDIVIDUALS (TRAVELER'S DIARRHEA)

Microsporidia are increasingly recognized among individuals not infected with HIV. *Ent. bieneusi* and *E. intestinalis* have been reported as a cause of traveler's diarrhea (Sandfort et al. 1994; Sobottka et al. 1995a; Wanke et al. 1996; Fournier et al. 1998; Gainzarain et al. 1998; Raynaud et al. 1998; Muller et al. 2001). In such cases, without underlying immunodeficiency, microsporidial infection appears to be self-limiting.

A report from Zimbabwe suggests that *Ent. bieneusi* infection may be a more common cause of diarrhea than previously suspected and that larger prospective studies are needed to assess the true incidence of this infection in patients without evidence of immunosuppression (Gumbo et al. 2000).

ENT. BIENEUSI INFECTION IN THE ELDERLY

Chronically ill, elderly individuals may be an additional group at risk of acquiring microsporidial infection as a result of the diminishment in immune response. A Spanish study has shown significant rates of *Ent. bieneusi* infection in elderly individuals (mean age 75 years) with chronic and nonchronic diarrhea (Lores et al. 2002).

MICROSPORIDIAL INFECTION IN ORGAN TRANSPLANT RECIPIENTS

There are now several reports of microsporidial infection in organ transplant recipients undergoing immunosuppressive therapy (Sax et al. 1995; Rabodonirina et al. 1996; Kelkar et al. 1997). *Ent. bieneusi* infection was diagnosed from diarrheal samples from a heart–lung recipient (Rabodonirina et al. 1996) and an allogenic marrow recipient (Kelkar et al. 1997). In a liver recipient (Sax et al. 1995), the species involved was not identified but, as the spores were measured at 1 μm in length, it is likely that the species involved was *Ent. bieneusi*. Clinical presentation and evolution of the infection were identical to those observed in patients with AIDS.

Ocular microsporidioses

Two types of ocular infection have been described: the first involves the conjunctival and corneal epithelium and occurs concomitantly with HIV. The second involves the corneal stroma and leads to ulceration and suppurative keratitis, and occurs in immunocompetent individuals, free of HIV infection (Shadduck et al. 1990).

CORNEAL STROMAL INFECTIONS

Pinnolis et al. (1981) published a report of a 26-year-old Botswanan woman with a perforated corneal ulcer, who became blind and underwent enucleation. Microsporidial spores were identified mainly in the cytoplasm of corneal histiocytes. Canning and Lom (1986) placed this species in the collective group *Microsporidium*, as *M. africanum*. Canning et al. (1998) drew attention to the similarities between *M. ceylonensis* and *M. africanum*, but it is unlikely that further taxonomically significant details of these microsporidians will become available. Shadduck et al. (1990) reported the case of a

45-year-old HIV-seronegative man with an 18-month history of central disciform keratitis, recurrent patchy infiltration of the anterior stroma and iritis. At biopsy, microsporidia were seen in the corneal stroma by light and electron microscopy. He was treated with topical steroids and broad spectrum antibiotics but ultimately required a corneal transplant. During the transplantation, part of the explanted cornea was inoculated into cell cultures and an infection was established. The organism contained diplokarya, division was by binary fission and the parasite was in intimate contact with the host cell cytoplasm for all stages of the life cycle. Because of these features, it was named *Nosema corneum* and differentiated from *N. connori* (now *B. conneri*) (Margileth et al. 1973) by having 6 coils of the polar tube, compared with 10–11 coils in *B. connori*.

Cali et al. (1991a) described the case of a 39-year-old man with irritation and blurred vision of his left eye. A foreign body was removed but visual problems and a corneal ulcer persisted. A biopsy revealed microsporidia in direct contact with host cell cytoplasm. The nuclei of the parasites were in a diplokaryotic arrangement. The spore size was 3 × 5 μm, with 9–12 coils of the polar tube. The organism was placed in the genus *Nosema* and named *Nosema ocularum*.

Silveira et al. (1993) established an infection of *N. corneum* in athymic mice. Subsequently, Silveira and Canning (1995) revised the classification of this microsporidian using the ultrastructural development of the organism in the mouse hepatocytes, and concluded that it was not a species of *Nosema*, as only the diplokaryotic arrangement of the nuclei was consistent with that genus (Figure 16). Sporogony was polysporoblastic and sporonts were ribbon-shaped, dividing to produce linear arrays of sporoblasts. Each parasite was also completely enveloped by endoplasmic reticulum derived from the host cell (Figure 16). Based on these new taxonomically significant features, the organism was placed in a new genus, *Vittaforma*, and named *Vittaforma corneae*.

PREDOMINANTLY OCULAR INFECTIONS IN HIV DISEASE

In the USA in 1990, five homosexual men with AIDS were diagnosed as having bilateral microsporidial keratoconjunctivitis (Friedberg et al. 1990; Lowder et al. 1990; Orenstein et al. 1990b; Yee et al. 1991). Histological sections of corneal or conjunctival scrapings contained numerous oval spores, confirmed as being microsporidial by electron microscopy. Further cases of ocular microsporidiosis have subsequently been reported

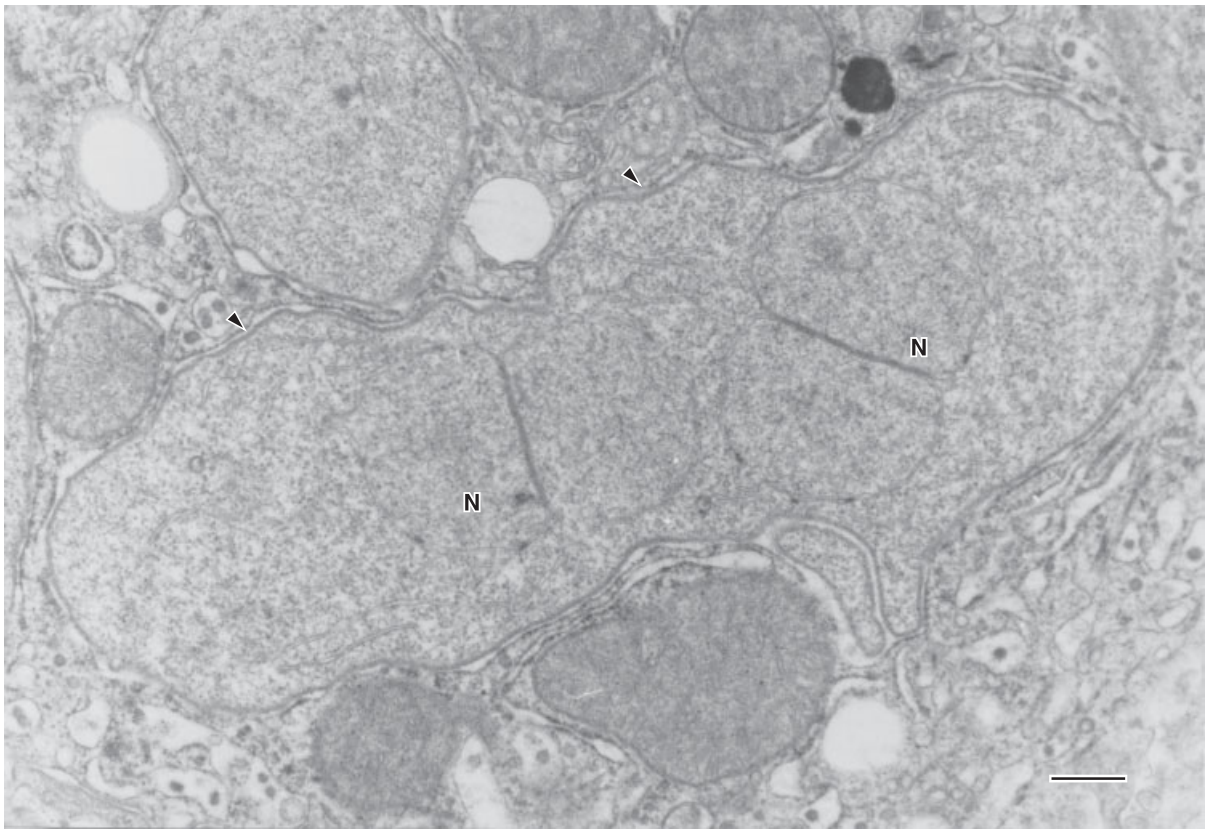

Figure 16 *Electron micrograph of a meront of* Vittaforma corneae *with two diplokaryotic nuclei (N). Note that parasite is enveloped by host endoplasmic reticulum (arrowheads) Bar = 0.26 μm. (Reproduced with permission of the authors and publisher from Silveira and Canning 1995)*

from the USA (Schwartz et al. 1993a). An *Encephalitozoon*-like species was involved in all of the above cases.

Additional cases of ocular infection from other parts of the world have followed these initial reports. Metcalfe et al. (1992) reported a case of keratoconjunctivitis from the UK and McCluskey et al. (1993) reported one from Australia. An *Encephalitozoon*-like species was thought likely to be the microsporidian involved.

Isolates from conjunctival or corneal scrapings obtained from three American AIDS patients, grown in cultures of Madin–Darby canine kidney cells (MDCK cells) by Didier E.S et al. (1991), were all identical by SDS–PAGE analysis but were different from a well-characterized isolate of *E. cuniculi*. Identical banding patterns on Western immunoblotting were obtained from each patient's serum, whereas murine antisera to *E. cuniculi* reacted to only some antigens from the tissue culture-grown isolates. The differences were sufficient for Didier E.S. et al. (1991) to propose that the ocular isolates from the AIDS patients studied should be redesignated as a new species, *E. hellem*. Subsequently, the UK isolate was also found to be *E. hellem* (Hollister et al. 1993b).

In addition to *E. hellem* another microsporidian, *Trachipleistophora hominis* can infect the cornea and was first described from an AIDS patient with muscle, ocular, and respiratory symptoms (Field et al. 1996). Although *V. corneae* was originally described from the eyes of a non HIV infected patient (see above), this species has now been identified in the urinary tract of a patient with AIDS (Deplazes et al. 1998) and must, therefore, be considered as having the potential to infect ocular sites in patients infected with HIV.

CLINICAL FEATURES OF OCULAR INFECTIONS IN HIV DISEASE

Clinical manifestations of ocular infections include conjunctivitis, foreign-body sensation, blurred vision, and photophobia. Concomitant unilateral cytomegalovirus retinitis was noted in two patients. Ophthalmic examination revealed a diffuse punctate keratopathy (Friedberg et al. 1990; Lowder et al. 1990; Orenstein et al. 1990b; Yee et al. 1991). In patients for whom data were available, CD4 lymphocytes were profoundly decreased (mean 26×10^6/l; range 2–50) (Schwartz et al. 1993a). The Australian case was a homosexual male with a CD4 count of 83×10^6/l (McCluskey et al. 1993).

The case reported from the UK involved a married man who had had homosexual activity before marriage. In contrast to the other published cases, his symptoms of ocular infection had been noted 2 years before diagnosis of microsporidial infection, when his CD4 count was of the order of 200×10^6/l. In this individual, both corneas were diffusely covered with fine punctate epithelial opacities and there was also some punctate staining of the interpalpetral bulbar conjunctiva of both eyes. Conjunctival scrapings established the microsporidial nature of the infection (Figure 13). The taxonomic features of this parasite seen by electron microscopy were consistent with a species of the genus *Encephalitozoon*. Further study confirmed the species involved to be *E. hellem* (Hollister et al. 1993b). The same patient also had nasal obstruction and discharge (Lacey et al. 1992). Ear, nose and throat (ENT) examination showed multiple nasal polyps and a computed tomography (CT) scan showed extensive opacities in the maxillary antra, and ethmoid and sphenoid sinuses, as well as minor cerebral atrophy. A nasal polypectomy was performed and on both histological and electron microscopical examination, many superficial epithelial cells were found to contain microsporidial spores, identical to those found in the corneal and conjunctival scrapings. An interesting feature of the ultrastructural examination was the presence of discharged (or germinated) spores within intact parasitophorous vacuoles (Canning et al. 1992). These would probably have contributed to the chronic nature of this infection (Lacey et al. 1992) if spores discharged their sporogonial contents into adjacent epithelial cells without release from the cell in which they developed. Cali and colleagues have also noted this premature germination (Cali et al. 1991b).

It is possible that in the UK case, infection was initially caused by inoculation of spores into corneal abrasions and infection could have spread from the corneal epithelium through the lacrimal canaliculi and nasolacrimal ducts that drain secretions from the eyes into the nasal sinuses (Curry and Canning 1993). However, ocular infection may possibly be acquired by reverse passage from a respiratory source, as microsporidial infection has been identified within the tracheobronchial epithelium (Weber et al. 1992b; Schwartz et al. 1993b).

Other sites of infection and other species

Encephalitozoon cuniculi has been reported from a small number of AIDS cases. A case of peritonitis was reported by Zender et al. (1989) and a case of hepatitis by Terada et al. (1987). Several cases of renal infection involving species of *Encephalitozoon* have been reported (Orenstein et al. 1992a; Cali et al. 1993; Aarons et al. 1994; Dore et al. 1995), as have respiratory tract (Lacey et al. 1992; Schwartz et al. 1993b; Molina et al. 1995) and urinary tract infections (Corcoran et al. 1996; Birthistle et al. 1996).

The genus *Pleistophora* is normally associated with infections of fish (Canning and Lom 1986) but a few cases of myositis caused by *Pleistophora* sp. have been reported in humans (Chupp et al. 1993; Ledford et al. 1985; Macher et al. 1988). In the case reported by Ledford and colleagues, the patient was immunodeficient but HIV seronegative. Further work on this organism has classified it as a new species, *P. ronneafiei*

(Cali and Takvorian 2003). The other reported cases were from AIDS patients. In the case described by Chupp and colleagues, the patient was a 33-year-old Haitian man with AIDS who had pain and weakness spreading to the posterior thighs and upper extremities and daily fevers. Electromyography findings were consistent with a diffuse, active myopathic process including denervation characteristic of inflammatory myopathy. The patient died without the cause of death being immediately known but a muscle biopsy taken before death showed, in one area, that all the fibers were atrophic and contained intracellular basophilic organisms. Electron microscopy revealed all the developmental stages of a microsporidian, which was ascribed to the genus *Pleistophora*.

In a case originating from Australia, *Pleistophora*-like organisms were diagnosed in a muscle biopsy but were also present in corneal epithelium, urine, and nasopharyngeal washings, suggesting disseminated infection (Hollister et al. 1996). Studies of this microsporidian in culture and in athymic mice (Figure 17), indicated that the parasite differed from established species of *Pleistophora* and it has therefore been reclassified as a new species, *Trachipleistophora hominis*.

A second species of *Trachipleistophora*, *Trachipleistophora anthropophthera*, has been reported from the brain and other organs of two AIDS patients (Vávra et al. 1998). This species has two sporogonial development sequences which give rise to two types of SPOV, containing either approximately eight spores (type I SPOV) or containing two thin-walled spores (type II SPOV).

Another species of microsporidian, *Brachiola vesicularum*, has been described from skeletal muscle of an AIDS patient (Cali et al. 1998). Key ultrastructural features of this organism are:

- a diplokaryotic arrangement of nuclei
- all stages in intimate contact with the host cell cytoplasm
- surface appendages that reach into the surrounding host cell cytoplasm
- an anisofilar polar tube arrangement within the spore (posterior coils of a smaller diameter compared to anterior coils of the polar tube).

Disseminated infections

There are now several reports of multiorgan infection (Gunnarsson et al. 1995) or systemic dissemination (Orenstein et al. 1992a; Cali et al. 1993; Doultree et al. 1995). In patients presenting with symptoms localized to one organ system, consideration must be given to the possibility of systemic infection, particularly if species of *Encephalitozoon* are involved (Schwartz et al. 1993b). Dore et al. (1995) suggest that a distinguishing feature of disseminated infection with *E. intestinalis* is the prevalence of symptoms of chronic rhinosinusitis. De Groote and colleagues (1995) describe a case of disseminated *E. cuniculi* infection in which the patient had advanced renal failure. The organism was found in urine and sputum and many tests, including the polymerase chain reaction (PCR) and indirect immunofluorescence, confirmed the presence of *E. cuniculi*. Treatment with albendazole, a broad-spectrum antiparasitic agent with activity against protozoa resulted in improvement of symptoms.

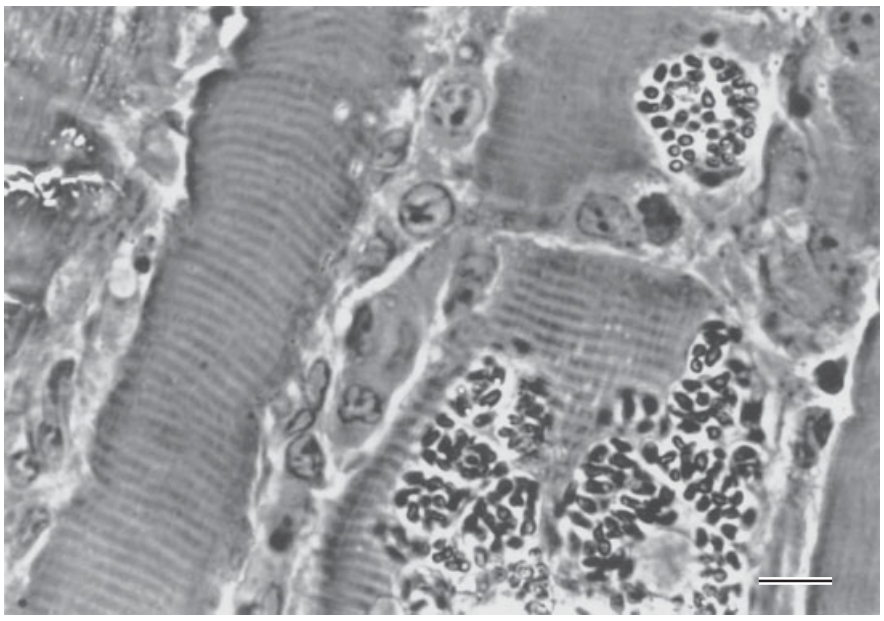

Figure 17 *Light micrograph of* Trachipleistophora hominis *in skeletal muscle fibers of an athymic mouse. Note that muscle fibre is packed with spores. Bar = 10 μm. (Reproduced with permission from W.S. Hollister and E.U. Canning)*

Schwartz et al. (1993b) describe a case of a patient previously diagnosed as having keratoconjunctivitis involving *E. hellem* infection, but who subsequently presented with respiratory problems and fever. A chest radiograph revealed a left lower lobe interstitial infiltrate. Bronchoscopy with biopsy and bronchoalveolar lavage revealed abundant *E. hellem* spores within epithelial cells. Sputum and urine samples also contained the same microsporidian.

Molina et al. (1995) describe five cases of disseminated infection due to *E. intestinalis*. Symptoms included chronic diarrhea, fever, cholangitis, sinusitis, and bronchitis. Albendazole cleared the stool samples of all five patients but the urine of only three patients. Other disseminated infections involving *E. intestinalis* have been reported (Orenstein et al. 1992a; Cali et al. 1993).

The reports outlined above strongly suggest that AIDS patients with low CD4 counts presenting with infections involving any species of *Encephalitozoon* may have unsuspected disseminated infection. Early treatment with albendazole can result in significant improvement of symptoms in most cases. The mechanism of dissemination of microsporidia is uncertain, but infected macrophages may play a significant role in this process (Doultree et al. 1995).

Although only known from a few patients, other species are capable of dissemination and include *T. hominis* (Field et al. 1996) and *T. anthropophthera* (Vávra et al. 1998). *T. hominis* has been demonstrated in corneal scrapings, skeletal muscle and nasal discharge (Field et al. 1996) and *T. anthropophthera* in brain, kidneys, heart, pancreas, thyroid, parathyroid, liver, spleen, and bone marrow (Vávra et al. 1998).

V. corneae was first described from a localized corneal infection in an immunocompetent individual (Shadduck et al. 1990), but experimental infection of athymic mice suggested that it could spread systemically in immunocompromised humans and not be restricted to the eyes (Silveira et al. 1993). *V. corneae* has subsequently been identified from the urinary tract of an AIDS patient (Deplazes et al. 1998).

Dissemination should be seriously considered particularly if the patient is diagnosed with *Encephalitozoon*, *Trachipleistophora* or *V. corneae* infection.

Progression of infection

In an experimental investigation of the route and progression of infection with *E. cuniculi* in adult rabbits, it was found that administration of spores either orally or intratracheally induced serum antibodies within 3 weeks and excretion of spores in urine by 6 weeks (Cox et al. 1979). Kondova et al. (1998) challenged gnotobiotic piglets with *Ent. bieneusi* from human and primate sources. All challenged animals became infected, but immunosuppressed piglets had an earlier onset of excretion and considerably increased spore shedding in feces. Immunosuppressed animals started to excrete spores 5–8 days after challenge compared to 9–12 days for nonimmunosuppressed control piglets. The piglets showed little or no symptoms of diarrhea or wasting and this, the researchers believed, was due to the relatively short period of immunosuppression and was comparable to individuals with AIDS who usually only show symptoms when their CD4 T-cell count drops below 100×10^6/l. *Ent. bieneusi* spores from a human AIDS patient were orally transmitted to two simian immunodeficiency-infected rhesus monkeys and the animals started to excrete spores within 7 and 8 days of inoculation respectively (Tzipori et al. 1997). Shedding of spores continued until the rhesus monkeys were euthanatized 7 and 8 months later, respectively. Jejunal infection was found to be sparse and this was thought to be due to either the number of circulating CD4 cells or that rhesus monkeys are less susceptible than humans to *Ent. bieneusi* infection. Similar information is not available for human microsporidial infections, but the incubation period may be similar to that seen in primate infection.

EPIDEMIOLOGY AND POSSIBLE ANIMAL RESERVOIRS

Microsporidial infections have only become well recognized in humans since the advent of HIV and AIDS. The open question is whether the organisms involved are solely human and primate parasites or may, in some cases, have a zoonotic origin. Any explanation of the current situation, where microsporidial infections are becoming more commonly recognized, must consider possible zoonotic transmission (Glaser et al. 1994). Curry (1999) has reviewed human microsporidial infection and possible animal sources.

Most human microsporidial infections are transmitted (horizontally) by spores (either by ingestion, inhalation, inoculation into abrasions or sexually), although there is some evidence of vertical transmission in other vertebrates such as lagomorphs and rodents (Hunt et al. 1972; Innes et al. 1962). As microsporidia are ubiquitous in the wild, environmental contamination must be common. Spores from infected animals must be widespread in the environment because of urination, defecation or decay after death (Canning 2001). Human exposure to microsporidia is inevitable. A French analysis of risk factors for microsporidiosis showed that swimming in pools was significant, but, surprisingly, that pet contact was not (Hutin et al. 1998). Microsporidian spores (*Ent. bieneusi*, *E. intestinalis* and *V. corneae*) have been identified in water, but the source or sources of this contamination remain unknown (Sparfel et al. 1997; Dowd et al. 1998).

High prevalence rates of *Encephalitozoon* and *Enterocytozoon* in humans may indicate natural human-to-human transmission, but several studies have found

these parasites in animals that are associated with or farmed by humans, thus allowing the possibility that some human infections may be zoonotic.

Domestic animals, such as dogs, could be a source of some human infections. *E. cuniculi*, in particular, is known to occur in dogs (Didier et al. 1996) and a wide range of mammals. Three strains of *E. cuniculi* have been identified so far (Biderre et al. 1999). A culture of *E. cuniculi* from an AIDS patient has been found to be identical to *E. cuniculi* strain III, which had previously only been isolated from domestic dogs (Didier et al. 1996; Snowden et al. 1999). Small mammals such as rats and mice, which are pests of human habitation, can also harbor *E. cuniculi* (Canning and Lom 1986) and may contaminate the environment with spores. Ingestion of spores by severely immunocompromised individuals could result in disease. However, even with our improved diagnostic capabilities, only a few cases of *E. cuniculi* infection have been reported in humans (Franzen et al. 1995; Canning 1998).

The other two species of *Encephalitozoon*, *E. intestinalis* and *E. hellem*, are more commonly reported from human infections and these species are now being identified from animals. Stool samples from domestic animals were collected in two rural villages in Mexico and examined by microscopy (Gram-chromotrope and specific immunofluorescence) and PCR (Bornay-Llinares et al. 1998). *E. intestinalis* was identified in dogs and donkeys and in some animals reared for food (cow, pig, and goat). Bornay-Llinares and colleagues discuss the possibility of transmission to humans via contaminated food or water or via an aerosol route, but such routes of transmission remain to be demonstrated.

The finding of *E. hellem* in birds has highlighted possible avian sources of this parasite. Black et al. (1997) and Pulparampil et al. (1998) have identified *E. hellem* in psittacine birds and Snowden et al. (2000) have also identified *E. hellem* in lovebirds. As birds (particularly psittacine birds) are often kept as pets, this raises the possibility that these may be sources of human disease. In the above studies, the intestinal epithelium, kidneys, cloaca or droppings were found to contain spores. Droppings are dry or become dry quickly and can produce dust (Curry 1999). Inhalation into the respiratory tract of dust containing viable spores may initiate infection in humans, particularly if the pet owner is immunosuppressed.

Infected fish muscle is also emerging as a possible source of human infection. Species of *Pleistophora* are common fish and crustacean pathogens (Cheney et al. 2000). A small number of cases of *Pleistophora* infection in humans have now been described, all involving skeletal muscle infection. Inadequately cooked infected fish or crustaceans may have a role in these infections, particularly in the immunosuppressed. Some evidence for this comes from the incidental finding of microsporidial spores associated with muscle fibers in a human stool sample from an AIDS patient with diarrhea (McDougall et al. 1993) suggesting that infected fish had been consumed and that spores had passed through the gut largely intact.

A molecular study by Cheney et al. (2000) has shown that *T. hominis* has similarities to organisms within the microsporidian genus *Vavraia*, which are mosquito parasites, thus raising the possibility of transmission to humans from biting insects. The likelihood of microsporidial species from poikilothermic hosts being able to infect homeothermic humans, particularly if severely immunosuppressed (as in HIV disease) needs further study. Evidence for this possibility is emerging. *Nosema algerae*, a mosquito parasite was successfully transmitted to mice (Trammer et al. 1997) and has been diagnosed from an ocular lesion in an immunocompetent human (Visvesvara et al. 1999). This mosquito parasite has now been renamed *Brachiola algerae* (Lowman et al. 2000).

Enterocytozoon bieneusi, the commonest microsporidian species to infect humans, has currently been identified in macaques, pigs, rabbits, dogs, cattle, a llama, and a cat (Kondova et al. 1998; Breitenmoser et al. 1999; del Aguila et al. 1999; Mathis et al. 1999; Dengjel et al. 2001). Molecular methods have shown a number of genotypes (Rinder et al. 1997; Dengjel et al. 2001) or genetically distinct strains (Liguory et al. 1998) of *Ent. bieneusi* in humans. In the study of Liguory et al. (1998), four strains were identified, one of which (type IV strain) was similar to a porcine isolate (Deplazes et al. 1996). A study from Sadler et al. (2002) has shown that of 13 human *Ent. bieneusi*-positive samples tested, 11 were of a genotype (genotype B) so far only found in humans, one had been reported from humans and a laboratory macaque (genotype D) and one (genotype K) had previously only been identified from a cat. Sadler et al. concluded that a possible zoonotic link with cats had been demonstrated, but that the origin of the majority of human cases was unknown and this may indicate that genotype B is solely a human pathogen. Dengjel et al. (2001) examined fecal samples from humans and animals and identified 14 different genotypes of *Ent. bieneusi*, 6 of them previously undescribed. Phylogenetic analysis of these genotypes revealed the lack of a transmission barrier between *Ent. bieneusi* from humans and cats, pigs and cattle and they conclude that *Ent. bieneusi* appears to be a zoonotic pathogen.

Mansfield et al. (1998) found simian immunodeficiency virus (SIV)-infected and non-SIV-infected (normal) rhesus macaques to be infected with *Ent bieneusi*, but infection rates were significantly higher in the SIV-infected group (33.9 percent compared to 16.7 percent). The SIV-infected animals also showed more symptoms of diarrhea and *Ent. bieneusi* was detected in the small intestine. In normal animals, *Ent. bieneusi* was not detectable in the small intestine, but was found in bile. *Ent. bieneusi* has been successfully transmitted from humans and rhesus macaques to gnoto-

biotic piglets and propagated serially (Kondova et al. 1998). More studies are needed to determine whether *Ent. bieneusi* is a primate parasite or a widespread pathogen of mammals, particularly primates.

Whether animal infections with *Ent. bieneusi* and *E. intestinalis* are natural or from human handlers remains to be determined. Although still unproven and with firm evidence of transmission currently lacking, clues are emerging as to possible animal links with human microsporidial disease. We must remain open-minded to the possibility that some human microsporidial infection is from animal sources.

DIAGNOSIS

Microsporidiosis in AIDS patients is probably significantly under-reported because of diagnostic difficulties (Aldras et al. 1994). Diagnosing microsporidial infections depends on recognizing that the patient could be infected with microsporidia, particularly when the CD4 lymphocyte count drops below 100×10^6/l, thus allowing the most appropriate specimens to be taken and the laboratory to choose the most appropriate methods of testing. Laboratory diagnosis of microsporidial infections in tissues is not easily accomplished because of the intracellular nature of these parasites, and their small size and poor staining properties (particularly of proliferative stages) with routinely used histological stains. In addition, the histopathologist requires experience in detecting such organisms. To date, 15 microsporidian species have been identified from humans (see Genera, species and taxonomic characteristics). Given the number of microsporidian species now detected, electron microscopy should be considered, particularly if the patient is immunocompromised and a biopsy is to be taken as an infection is suspected.

Microbiological examination of a fecal sample must include appropriate stains for identifying microsporidial spores. Laboratory diagnosis of microsporidial infections initially requires light microscopy (either bright field or epifluorescence, depending on stains used) and putative positives should, ideally, be confirmed by either electron microscopy or molecular techniques.

Small-intestinal biopsies

Enteric infection is the most common type of microsporidial infection. Until about 1990, diagnosis required both light-microscopic and electron-microscopic examination of small-intestinal biopsy sections (Orenstein et al. 1990a) or touch preparations of biopsies (Rijpstra et al. 1988). Microsporidia stain poorly with conventional histopathology stains, but diagnosis can still be accomplished with experience (Peacock et al. 1991). Appropriate special stains should be chosen for the tissue sections, although these are not always considered. Diagnostic workers interested in microsporidial detection have tried many alternative staining methods and often advocate their own particular favorite. Stains used include Giemsa, tissue Gram stain (Rabeneck et al. 1993) and periodic acid–Schiff reagent (Field et al. 1993b). A tissue stain that shows great promise is Warthin–Starry (Field et al. 1993 a, b, c), which stains some sporogonic stages and spores (Figure 18).

Microsporidial infections can be misdiagnosed in tissues. Furuta et al. (1991) thought that pulmonary lesions found in autopsy material from leprosy patients involved microsporidia, but after immunohistochemical staining it was concluded that these lesions were, in fact, caused by *Cryptococcus neoformans*. Resin-embedded tissue stained with toluidine blue can also be useful, but mucus granules in goblet cells also take up stain and can cause confusion. The excellent preservation and thin tissue sections (1 μm) enhance the resolution of the cellular detail compared with wax-embedded material. This intermediate step of resin embedding naturally leads to possible electron-microscopic examination of ultrathin tissue sections, and electron microscopy remains the gold standard for confirmation of microsporidial infection in tissues (Weber et al. 1994; Rabeneck et al. 1993; Asmuth et al. 1994; Field et al. 1993b). Demonstration of the coiled polar tube within spores is pathognomic of microsporidial infection (Figure 2) and identification to generic and sometimes species level can be made from ultrastructural features (Cali 1991). The identification of the species involved in new patients is important, as effective drug therapies can then be instigated. Some workers regard electron microscopy as cumbersome and time-consuming (Visvesvara et al. 1994), but this technique has been involved in the detection and description of most new human microsporidial infections. Molecular detection has usually followed after the detection and description by electron microscopy. In the expanding field of human microsporidial infection, both electron microscopy and molecular methods have their merits.

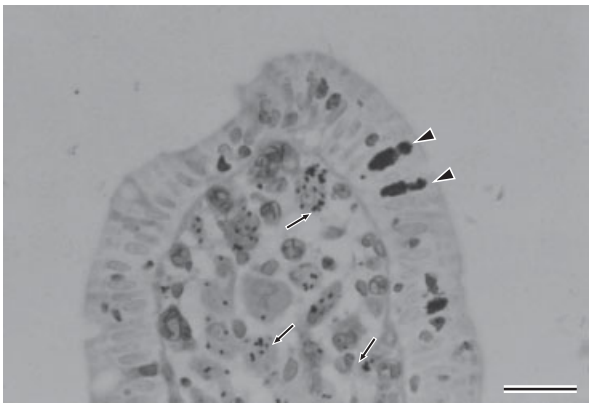

Figure 18 *Light micrograph of a tip of a duodenal villus showing aggregates of* Encephalitozoon intestinalis *in enterocytes (arrowheads) and macrophages in the lamina propria (arrows). Warthin–Starry stain. (Reproduced with permission of the authors and publisher from Field et al. 1993b)*

Immunofluorescent or immunoperoxidase antibody staining of microsporidia in tissue sections would be useful diagnostically if such antibodies to species or genera were commercially available. Visvesvara et al. (1994) produced both polyclonal and monoclonal antibodies to *E. hellem* that successfully differentiated this organism from other species in clinical specimens and in established cultures. Other studies (Schwartz et al. 1993a) have shown that, using the indirect fluorescent antibody test (IFAT) with species-specific antibodies, it is possible to identify microsporidial infection in sections of corneal biopsies.

Because *E. cuniculi*, *E. hellem*, and *E. intestinalis* can be grown in culture (Schwartz et al. 1993a; Hollister et al. 1993b; Beauvais et al. 1994; Van Gool et al. 1994; Visvesvara et al. 1995; Doultree et al. 1995), purified spore preparations can be harvested for inoculation into rabbits or mice. Aldras et al. (1994) describe the production of monoclonal antibodies raised against *E. hellem* and polyclonal antibodies raised against *E. cuniculi* and *E. hellem* which, when used in IFAT, were able to identify specifically the presence of microsporidial spores, including those of *Ent. bieneusi*, in stool samples.

The use of a commercially available fluorescein-labeled monoclonal antibody against species of *Encephalitozoon* has now been reported (Enriquez et al. 1998) and was used to demonstrate the prevalence of *Encephalitozoon* (7.84 percent) in two rural villages in Mexico.

Microsporidial spores in stool samples

Noninvasive techniques have been developed for the detection of microsporidial spores in, for example, stool samples. The contents of the duodenum–jejunum can also be examined for microsporidial spores using the Entero-test (HDC Corporation) method, which is often used to obtain luminal contents for detection of *Giardia* (Hamour et al. personal communication 1995). The staining properties of the microsporidian spore wall differentiate microsporidians from most other microorganisms. Spore-staining methods utilizing a modified Trichrome stain (chromotrope 2R stain) (Weber et al. 1992a), Giemsa (Van Gool et al. 1990) or fluorescent dyes (optical brightening agents) (Van Gool et al. 1993; Vávra et al. 1993) have been described; all these microscopical methods require adequate illumination, high magnification objectives (oil immersion) (Weber et al. 1994), examination of multiple stool samples and an adequate examination time (minimum 10 minutes) (Wuhib et al. 1994) or examination of at least 100 high power fields (Bendall and Chiodini 1993) for reliable diagnosis.

Weber's method (Weber et al. 1992a) utilizing chromotrope 2R has become a routine diagnostic staining method in many laboratories, particularly in the USA. Microsporidial spores stain pink and surrounding bacteria light green in thin stool smears. A number of modifications to Weber's method have been published (Ryan et al. 1993; Kokoskin et al. 1994; Sianongo et al. 2001).

Microsporidial spores are of the same order of size as bacteria and any diagnostic staining method must allow differentiation between spores and fecal bacteria. In addition, the microscopist must be able to differentiate between microsporidial spores and those of fungi and yeasts, as well as oocysts of coccidian protozoal parasites. Of the fluorescent dyes, Uvitex 2B (Ciba-Geigy 48) and Calcofluor (Sigma) are useful for fecal screening as they both stain the chitin in the endospore layer of microsporidial spores. They are quick to use, but require an epi-illumination, fluorescence microscope fitted with a 350–380 nm excitation filter and a light source that emits such wavelengths (e.g. a mercury vapor lamp). Spores are identified by their size, shape, and brilliant blue-white fluorescence staining properties (Figures 19 and 25.20). As fungal and yeast spores also contain chitin, adding Formalin fixative to a fresh fecal sample as quickly as possible reduces contamination due to continued fungal growth. In addition, putative positive microsporidial samples should ideally be confirmed and the species determined by another method such as PCR or electron microscopy (Corcoran et al. 1995). If such confirmatory techniques are not readily available, it is sometimes possible to determine the species involved by careful measurement of any spores detected. *Ent. bieneusi* has very small spores that measure about 1.5 μm in length, compared to spores of *Encephalitozoon* species that are about 2.5 μm in length. Experience is required for reliable results.

Some caution may be needed in ascribing the finding of microsporidial spores in stool samples to patient symptoms. McDougall et al. (1993) report the finding of microsporidian spores in a stool sample from an AIDS patient with chronic diarrhea, anorexia, and lethargy. Further investigations suggested that the microsporidia found had been ingested as food in heavily infected muscle and were, therefore, an incidental finding. This situation is, perhaps, not uncommon. Levine (1985) has reported that oocysts of a number of animal coccidian parasites ingested in food and found in human feces have been mistaken for parasites of humans.

Concurrent infections involving both microsporidia and *Cryptosporidium parvum* may also be common. Garcia et al. (1994) showed that 17 of 60 (28 percent) mainly immunocompromised patients with diarrhea had simultaneous *Cryptosporidium* and microsporidial infections. The finding of one parasite should not preclude further examination by a different diagnostic method for other parasites. In addition, enteric infection with both *Ent. bieneusi* and *E. intestinalis* may not be unusual (Blanshard et al. 1992b; Van Gool et al. 1994) and this may have important implications for treatment, as *E.*

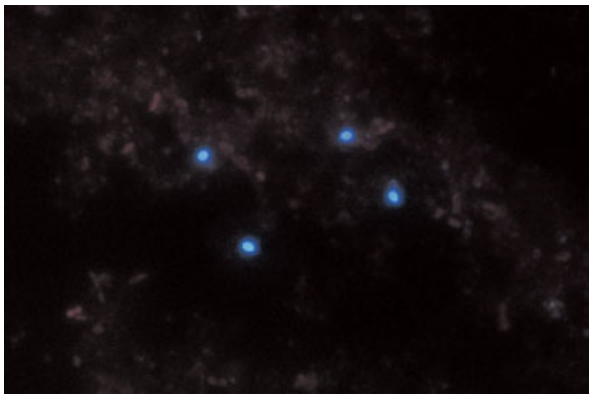

Figure 19 *Spores of* Enterocytozoon bieneusi *from a stool sample stained with calcofluor and examined with 350–380 nm wavelength illumination under an epi-illumination fluorescence microscope. Note ovoid shape of spores, which are shorter than those of* E. intestinalis *(c.f. Figure 20).*

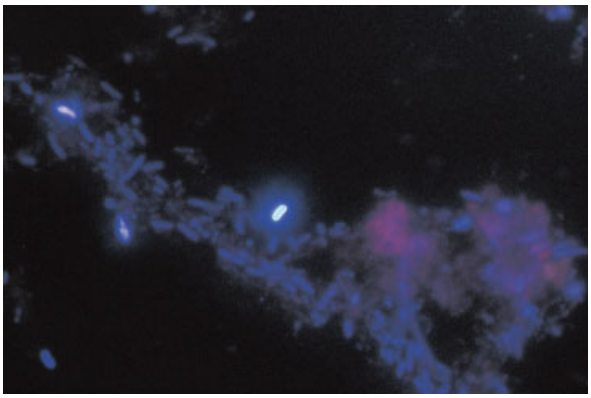

Figure 20 *Spores of* Encephalitozoon intestinalis *from a stool sample stained with calcofluor. Note that the spores of this species are longer than those of* Enterocytozoon bieneusi *(c.f. Figure 19)*

intestinalis appears to be fully susceptible to treatment with albendazole, whereas the response of *Ent. bieneusi* is variable.

Molecular methods of detection

It is now possible to generate sequence data rapidly by direct sequencing of amplified ribosomal DNA using the PCR technique. This technique has identified sequence variation and shows considerable promise for the specific identification of microsporidian species as well as for increasing our knowledge of the epidemiology of these organisms. Molecular techniques for identification of the commoner species of microsporidian infecting humans using either stool or intestinal biopsy specimens are available (Kock et al. 1997).

Sequences have been produced mainly from the small subunit ribosomal RNA gene of microsporidia and from these it is possible to identify genus or species.

Microsporidian DNA can be extracted fairly easily from infected tissues or cells by procedures such as proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation, but DNA extraction from spores is more difficult. Mechanical disruption of the spores in addition to digestion is required (Franzen and Müller 1999; da Silva et al. 1999).

Vossbrinck et al. (1993) sequenced a segment of ribosomal DNA about 1350 bp long and demonstrated that both *E. cuniculi* and *E. hellem* could be specifically identified. A comparison of the sequence data showed relatively high sequence homology, justifying the same generic status of these organisms but also confirmed that the isolates of *E. hellem* and *E. cuniculi* were not of the same species. Restriction digests of the amplified region of ribosomal DNA provides the potential for a rapid method of distinguishing between *E. hellem* and *E. cuniculi*. Visvesvara et al. (1994) using direct sequencing of PCR amplified small-subunit ribosomal RNA (SSU-rRNA) demonstrated similar specific differences between these two species. Initially, PCR techniques were largely based on cultured organisms, but amplification directly from clinical specimens has improved the speed of diagnosis (Visvesvara et al. 1994; Zhu et al. 1994).

A PCR assay of stool samples from AIDS patients known to be infected with microsporidia (identified by chromotrope 2R stain) not only detected microsporidial infection but also identified the enteric species involved (Fedorko et al. 1995). However, this assay required a laborious 4-day extraction procedure and was strongly inhibited by fresh stools, although this could be overcome with the addition of sodium hypochlorite. Current extraction methods are much faster (Franzen and Müller 1999; da Silva et al. 1999). Cultured spores of *E. intestinalis* were added to a stool specimen positive for *Ent. bieneusi* to simulate a dual infection. A single primer (PMP2) complementary to conserved sequences of the SSU-rRNA enabled amplification of DNA from both species. An important observation was that whereas *Ent. bieneusi* lacks a Pst1 restriction site in the amplicon, that of *E. intestinalis* cut into two distinct fragments, thus enabling differentiation of the two species. The authors suggest that PCR may become the method of choice for identification of microsporidia in clinical specimens.

Canning et al. (1993) have also demonstrated differences between *V. corneae*, *E. cuniculi* and *E. hellem* using a random amplified polymorphic DNA (RAPD) polymerase chain reaction.

Vossbrink et al. (1993) suggested that sequence information appropriate for comparison with other microsporidial species should be included in future species descriptions for the specific identification and phylogenetic comparisons of microsporidia.

There is a comprehensive review of molecular techniques applied to microsporidia by (Franzen and Müller (1999).

Culture of microsporidia from clinical samples

Microsporidial spores from clinical samples such as urine (Visvesvara et al. 1991; Bocket et al. 1992; Hollister et al. 1993a; Visvesvara et al. 1995; Hollister et al. 1995; Deplazes et al. 1998), nasal polyps (Hollister et al. 1993b), nasopharyngeal aspirates (Doultree et al. 1995), feces (Van Gool et al. 1994), and muscle biopsies (Hollister et al. 1995) can sometimes be cultured in various cell lines. Once established in culture, enough spores can be produced from the original human sample to allow further tests, such as SDS–PAGE or PCR, to be carried out, which can help to establish the species involved. Ultrastructural studies of the organisms in culture can also be undertaken (Didier P.J. et al. 1991) Of the microsporidia that infect humans, *E. cuniculi*, *E. intestinalis*, *E. hellem*, *T. hominis*, *V. corneae* and *B. algerae* have been successfully cultured (Visvesvara 2002). Once established, activity of anti-microsporidial agents, such as albendazole, can be examined using the culture system (Colbourn et al. 1994; Lafranchi-Tristem et al. 2001). However, *Ent. bieneusi*, the commonest human microsporidial infection, has yet to be successfully cultured and this has hampered research into effective treatments against this species.

CHEMOTHERAPY

Problems of treatment

Microsporidial infections are difficult to treat because of their intracellular habitat and the resistant nature of the spores. Enteric infections have been treated with varying degrees of success with several drugs, but carefully controlled comparative treatment trials are lacking.

Treatment of enteric microsporidial infections

The most frequent microsporidial infections of humans are enteric, *Ent bieneusi* being more commonly detected than *E. intestinalis*. *Encephalitozoon intestinalis* appears to be more susceptible to treatment than *Ent. bieneusi*.

OCTREOTIDE

Octreotide produced a partial remission in one patient infected by *Ent. bieneusi* (Simon et al. 1991) and metronidazole caused some remission in 10 of 13 HIV seropositive patients with mild microsporidial diarrhea caused by *Ent. bieneusi* (Eeftinck Schattenkerk et al. 1991). Blanshard et al. (1992a) treated six AIDS patients with diarrhea and biopsy confirmed infection with *Ent. bieneusi*, but no other identified cause of symptoms.

ALBENDAZOLE

Albendazole was found to be well tolerated. Within 7 days of treatment, there was a marked reduction in stool frequency, volume, and incontinence, with all patients either gaining weight or weight loss being arrested. Four patients relapsed within a month of cessation of treatment, three of whom responded to a further course of albendazole treatment. It was concluded that albendazole had a partial parasitostatic effect, rather than a parasiticidal action. Further studies by Blanshard et al. (1993) confirmed this conclusion. In biopsy material taken from patients being treated with albendazole, damage to developmental stages of *Ent. bieneusi*, causing a partial inhibition of parasite replication, was apparent. Dieterich et al. (1994) showed a similar reduction of symptoms in patients treated with albendazole, but again concluded that *Ent. bieneusi* was not eliminated.

In contrast to *Ent. bieneusi*, *E. intestinalis* appears to be more susceptible to treatment with albendazole, as demonstrated by the disappearance of parasites from the intestine of a case of disseminated infection (Orenstein et al. 1993). Gunnarsson et al. (1995) used albendazole to treat five patients with evidence of multiorgan infection with *E. intestinalis*. After treatment, most clinical samples were negative for microsporidia and there was a significant improvement of symptoms.

The direct effect of albendazole on cell cultures infected with *E. cuniculi* has been examined by Colbourn et al. (1994). Many of the proliferative stages of the parasite became grossly enlarged and devoid of nuclei. As albendazole prevents the polymerization of microtubules, which are known to occur only within the intranuclear spindles of dividing nuclei in microsporidia, parasite growth continues in the absence of nuclear division.

Albendazole was administered to five AIDS patients (400 mg orally twice a day) with evidence of disseminated microsporidiosis caused by *E. intestinalis* (Molina et al. 1995). Spores of *E. intestinalis* were detected in stool and urine samples and confirmed by transmission electron microscopy of duodenal biopsies. There was a rapid clinical response to therapy, with spores being cleared from feces of all patients and from the urine of three. However, during follow-up, spores were detected in feces and mild diarrhea recurred in two patients. It was concluded that albendazole has a significant effect on *E. intestinalis* infection, but that its effects were transient.

Not all AIDS patients relapse. Parasitological cure following albendazole therapy of disseminated *E. intestinalis* infection has been verified at autopsy in two AIDS patients (Sobottka et al. 1995b; Joste et al. 1996). Both patients died of bacterial pneumonia and thorough examination of tissue samples taken at autopsy failed to reveal the presence of microsporidia.

A randomized, double-blind, placebo-controlled trial of albendazole treatment of microsporidiosis due to *E. intestinalis* has been reported by Molina et al. (1998). All four patients in the albendazole treatment group showed clearance of infection compared to none of the four in the control group. The control patients were then given albendazole treatment and the infection cleared. Subsequently, the effect of albendazole prophylaxis was investigated in these eight patients. They were randomly assigned to receive maintenance albendazole therapy (three patients) or no treatment (five patients) for a 12-month period. None of the three patients receiving prophylaxis had a recurrence, but three relapses were recorded among the patients not receiving albendazole. It was concluded that albendazole has both parasitological and clinical efficacy and reduces the risk of relapse.

NITAZOXANIDE

One AIDS patient with chronic diarrhea caused by *Ent. bieneusi* was successfully treated with nitazoxanide twice a day for 60 consecutive days (Bicart-See et al. 2000). At the time of treatment, the patient was not receiving any antiretroviral therapy and his CD4 count was 85×10^6/l. Fecal examination by microscopy was negative for microsporidian spores after 11 days and post-treatment examination (including PCR) did not detect any microsporidia.

FUMAGILLIN

Although reported as toxic to humans (see below), purified fumagillin (98 percent pure) has been administered to a group of four patients infected with *Ent. bieneusi* to test its efficacy (Molina et al. 1997). Other drug regimens were also used on small groups of patients infected with *Ent. bieneusi* in the same study, but only those treated with the purified fumagillin showed clearance of this parasite (mean follow-up period of 10 months) but the drug also induced thrombocytopenia that was reversed when treatment was discontinued. A semi-synthetic analog of fumagillin, TNP-470, has also shown its effectiveness against the microsporidian *E. cuniculi* in infected rabbit kidney (RK) 13 cells and an athymic nude mouse model (Coyle et al. 1998).

THALIDOMIDE

Thalidomide also appears to have anti-microsporidial activity (Sharpstone et al. 1997). Fecal TNF-α was found to be elevated in cases of HIV-related diarrhea due to enteric microsporidial infection. Thalidomide, an anti-TNF-α agent, was given to 18 patients with AIDS and chronic diarrhea due to *Ent. bieneusi*. Seven of these patients had a complete clinical response and three a partial response to thalidomide therapy. These new treatments may prove to be an effective therapy against *Ent. bieneusi* infection.

NUTRITIONAL THERAPY

Asmuth et al. (1994) also suggested that benefit could be derived from nutritional therapy directed at minimizing malabsorption. Low-fat diets and introduction of simple carbohydrates could also induce an improvement of symptoms.

Treatment of keratoconjunctivitis

Various topical antimicrobial, lubricating and anti-inflammatory agents have been used to treat eyes infected with microsporidia. Itraconazole (Yee et al. 1991) was responsible, at least in part, for the resolution of a corneal infection with *Encephalitozoon* sp. (later identified as *E. hellem* by Didier E.S. et al. (1991). Treatment with propamidine isethionate resolved symptoms in a UK patient infected with *Encephalitozoon* sp. (Metcalfe et al. 1992) (also later identified as *E. hellem* by Hollister et al. 1993b). The symptoms returned when treatment was discontinued (Metcalfe et al. 1992) but albendazole treatment improved the nasal infection, with significant regression of sinus opacification. The patient remained free from nasal symptoms until his death from AIDS dementia complex (Lacey et al. 1992). An Australian case of keratoconjunctivitis was also treated with propamidine isethionate eye ointment, with similar resolution of symptoms (McCluskey et al. 1993).

Fumagillin has been known for some time to control *Nosema* infection in honeybees, where infection causes severe economic loss (Katznelson and Jamieson 1952). Shadduck (1980) observed the in vitro effects of fumagillin on *E. cuniculi* and noted that it protected noninfected cells but did not eliminate the parasite from infected ones. This anti-microsporidial agent is toxic to humans (McCowen et al. 1951), but has been used topically to resolve ocular infection by *E. hellem*. Fumagillin is insoluble in water, but a derivative, Fumidil B, is soluble. A Fumidil B preparation applied topically reduced the keratoconjunctivitis in two AIDS patients (Diesenhouse et al. 1993). No toxic side-effects were observed but infection recurred when treatment was stopped. Both Fumidil B and propamidine isethionate, when used topically to treat microsporidial infections of the eye, appear to be inhibitory rather than parasiticidal in action. Rosberger et al. (1993) has also reported successful treatment of keratoconjunctivitis with topical fumagillin.

Treatment of muscle and other microsporidial infections

Albendazole, sometimes in combination with other chemotherapeutic agents, appears to be the treatment of choice for many microsporidial infections.

In one patient with AIDS, myositis due to infection with *T. hominis* was successfully treated with albendazole,

sulfadiazine, and pyrimethamine, with resolution of clinical symptoms (Field et al. 1996). Another AIDS patient with myositis due to *B. vesicularum* infection was treated with albendazole and itraconazole, which successfully cleared the infection both clinically and histologically (Cali et al. 1998).

An AIDS patient with respiratory, abdominal, and urogenital symptoms was found to be infected with two different species of microsporidian, *E. hellem* and *V. corneae*. Treatment with albendazole resulted in abatement of symptoms. Microsporidian spores disappeared from respiratory secretions and there was a reduction in the urinary excretion of spores (Deplazes et al. 1998).

Dunand et al. (1997) reported five cases of parasitic sinusitis and otitis, three of which had *E. hellem* infection of the sinuses. Two of these patients were treated with albendazole. In one patient, the sinus symptoms decreased moderately, whereas in the second, the sinus symptoms initially decreased but because the patient did not receive maintenance (suppressive) therapy, the symptoms recurred within 5 months.

Aarons et al. (1994) describe a case of renal failure in an HIV-seropositive patient, caused by a species of *Encephalitozoon*, which was reversible on treatment with albendazole and correlated with the disappearance of the parasite from the urine. In another AIDS patient with disseminated *E. intestinalis* infection and evidence of impairment of renal function, renal function returned to normal after a course of treatment with albendazole (Dore et al. 1995).

Birthistle et al. (1996) have reported a case of urethritis caused by microsporidial infection, which was successfully cleared after albendazole treatment. The authors believe that this patient had acquired microsporidial infection sexually from his partner who, at the same time, had diarrhea due to intestinal microsporidiosis.

Reconstitution of the immune system

In developed countries, highly active antiretroviral therapy (HAART) is available to AIDS patients and this induces progressive reconstitution of the immune system (Montaner et al. 1998). Remission of intestinal microsporidiosis occurs in patients given HAART and this appears to correlate with an increase in CD4 and CD8 lymphocyte counts (Goguel et al. 1997; Kartalija and Sande 1999).

DISINFECTION AND SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

Disinfection of surfaces contaminated with microsporidia has received little attention. That spores are highly resistant has been demonstrated in the laboratory, where they have been found to be viable after storage for up to 10 years in distilled water (Sprague and Vávra 1977). In the experimental infection of gnotobiotic piglets, Kondova et al. (1998) found that spores of *Ent bieneusi* could remain infectious for at least 2 years when stored at +4°C, although the extent, if any, of the loss of viability over this period remains unknown. Shadduck and Polley (1978) used a rabbit isolate of *E. cuniculi* grown in a rabbit choroid plexus cell line to evaluate various factors influencing the infectivity and replication of this organism. *E. cuniculi* was not affected by penicillin, streptomycin or gentamicin, nor was it affected by sonication, freezing and thawing or distilled water. Organisms survived for 60 min but not 120 min at 56°C. They were, however, killed after 10 min of autoclaving at 120°C, or exposure to 2 percent (v/v) lysol, 10 percent (v/v) formalin or 70 percent (v/v) ethyl alcohol for 10 min. Whether all microsporidian species are affected by these physical conditions to the same degree as *E. cuniculi* is not known.

Undeen and Vander Meer (1990) subjected spores of *N. algerae* to ultraviolet radiation and found that very high dosages (3.8 J/cm^2) were required to inhibit germination. Similar results had been obtained with γ-radiation (Undeen et al. 1984) and it was concluded that spore germination did not involve nuclear function. Significantly, after receiving γ-radiation above 0.5 kGy, this microsporidian was unable to infect its mosquito host, but spore germination rate remained unaffected up to 10 kGy.

In work undertaken by Leitch et al. (1993), on the inhibition of polar tube extrusion in purified spores derived from cell cultures infected with *E. hellem*, germination was found to be pH dependent and was enhanced in the presence of calcium in the medium. Under experimental conditions, four agents inhibited polar tube extrusion: cytochalasin D disrupts microfilaments, demecolcine disrupts microtubules, nifedipine is a calcium channel blocker, and itraconazole an antifungal agent. As a result of this work, it was concluded that future clinical trials employing drug combinations might be useful in treating microsporidiosis.

CONCLUSION

Much remains to be learned about microsporidians, particularly in relation to human infection (Current and Blagburn 1991) and little is known about the epidemiology or pathogenesis of these parasites in humans. Whether or not there are animal sources of human infection will become clearer as epidemiological and experimental studies become better established. Molecular techniques are becoming more commonly available and may provide incontrovertible evidence of zoonotic transmission and also establish the phylogenetic and taxonomic relationships of the microsporidian species involved. Further developments in treatment are needed, particularly in relation to *Ent. bieneusi* infec-

tion. Developments in the culture and of animal models of this species would aid improvements in treatment and understanding.

In the developed world, because of improved treatment against HIV infection and corresponding partial recovery of cellular immunity, human microsporidiosis is now relatively infrequently diagnosed. This contrasts with the situation in the developing world, where microsporidial infections are much more commonly involved in human disease, particularly in those suffering from AIDS. Because of such inequalities in treatment, further study of these parasites is warranted to further improve our knowledge of these emerging pathogens.

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Blastocystis hominis

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INTRODUCTION

Blastocystis hominis is an enigmatic human parasite that has puzzled microbiologists since the early part of the last century. The genus *Blastocystis* was first described by Alexeieff (1911). The following year Brumpt (1912) proposed *B. hominis* for organisms found in human feces. Controversy has surrounded both the taxonomy and pathogenicity of *B. hominis* since its discovery. Although the taxonomy of *B. hominis* has only recently been resolved (Silberman et al. 1996), debate continues on the pathogenic potential of this organism (Zierdt 1991; Stenzel and Boreham 1996; Tan et al. 2002). Indeed, conflicting scientific evidence continues to be published, and it has been reported in both symptomatic and asymptomatic individuals. Although a wealth of information is available on the morphology of *B. hominis* (Boreham and Stenzel 1993; Stenzel and Boreham 1996), relatively little is currently known regarding the mode of transmission and life cycle of this fascinating parasite. *B. hominis* has a worldwide distribution and has been described as probably the most common human gut protozoan (Clark 2000). Prevalence rates of more than 50 percent have been reported in developing countries (Stenzel and Boreham 1996).

CLASSIFICATION

See Zierdt (1991) and Stenzel and Boreham (1996) for a complete history of *B. hominis* classification. The earliest workers regarded *B. hominis* as a harmless intestinal yeast (Alexeieff 1911; Brumpt 1912). Barret (1921) regarded *B. hominis* as of plant origin. Subsequent workers thought *B. hominis* to be a degenerate or cyst form of a flagellate, while others regarded it as a sporozoan or an ameba (Swellengrebel 1917; Zierdt 1991; Stenzel and Boreham 1996). There is no doubt that the presence of multiple morphological forms aided the misclassification of *B. hominis* (Zierdt 1991; Boreham and Stenzel 1993; Stenzel and Boreham 1996). Zierdt and colleagues (1967) described many protozoan-like characteristics of *B. hominis*, which resulted in the organism being reclassified and placed in the class Sporozoa (Zierdt 1978). Silberman and colleagues (1996), using phylogenetic analysis of ribosomal RNAs from *B. hominis*, classified it as the only member of the Stramenopiles found to infect humans. This diverse group of eukaryotes also includes diatoms, kelp, water molds, and slime nets. In Cavalier-Smith's (1998) six-kingdom classification of life, *B. hominis* is placed in Kingdom Chromista, Subkingdom Chromobiota, Infrakingdom Heterokonta (synonymous with Stramenopiles), Subphylum Opalinata, and (new) Class Blastocystea. More recently, Arisue and colleagues (2002) examined the phylogenetic position of *B. hominis* using individual phylogenies based on four molecules and confirmed its position within the Stramenopiles. Furthermore, *Blastocystis*/Stramenopiles were found to be the closest relatives of alveolates. Alveolates are another diverse group of protists and include ciliates, sporozoa, and dinoflagellates.

Blastocystis is a ubiquitous parasite that is found throughout the animal kingdom (Tan et al. 2002), and has been described in many different species including, mammals, birds, reptiles, and insects (Boreham and Stenzel 1993; Abe et al. 2002; Tan et al. 2002). Molecular typing has revealed that some isolates of *Blastocystis* from animal sources share very similar typing patterns to *B. hominis* (Yoshikawa et al. 1996; Clark 1997a). In addition, Snowden and colleagues (2000) described restriction fragment length polymorphism (RFLP) analysis of *Blastocystis* small subunit (SSU) rRNA genes isolated from animal species and found that different animal species shared a single-genotype of *Blastocystis*. Therefore, the potential for possible zoonotic transmission exists and, although not proven conclusively to date, it is plausible that some strains of *Blastocystis* may cross the species barrier.

STRUCTURE

B. hominis shows great morphological diversity. Four main morphological types have been described: vacuolar, granular, ameboid, and cystic (Zierdt 1991; Stenzel and Boreham 1996). *B. hominis* is a strict anaerobe (Zierdt 1983) and exposure to air may adversely affect its morphology (Zierdt 1991). *B. hominis* morphology also differs between fresh fecal specimens and cultures. Discussion continues on whether these different morphologic types are all involved in the life cycle or whether some are merely artifactual or degenerative forms.

Vacuolar form

This form is also termed the central body form and is perhaps the most common form recognized in diagnostic laboratories (Figure 1). The vacuolar form is seen in fecal specimens and is the predominant form in culture material (Stenzel and Boreham 1996). Vdovenko (2000) considered vacuolar and granular forms of *B. hominis* to be degenerative and due to environmental exposure. Vacuolar forms are usually spherical with a large size variation from 2 μm to more than 200 μm (average size between 4 and 15 μm) (Zierdt 1991). Transmission electron microscopy (TEM) studies of the vacuolar form reveal a thin band of cytoplasm around a large vacuole that occupies at least 90 percent of the cell volume (Zierdt 1983) (Figures 2 and 3). The central vacuole is composed of unevenly distributed, finely granular material, the function of which is unknown (Stenzel and Boreham 1996). Mitochondrion-like organelles are found in expanded areas of the cytoplasm and are surrounded by a bilaminar outer membrane, with cristae-like structures on the inner membrane (Stenzel and Boreham 1996) (Figure 4). As *B. hominis* is anaerobic, it has been hypothesized that these mitochondrion-like structures may, in fact, be hydrogenosomes (Boreham and Stenzel 1993; Stenzel and Boreham 1996). The nucleus shows a typical crescent-shaped band of condensed chromatin on one side. *B. hominis* often has a surface slime layer or capsule surrounding the cell membrane (Figure 4).

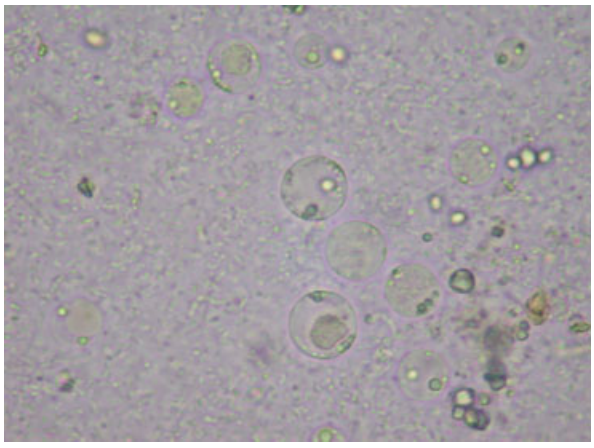

Figure 1 *Vacuolar form of* Blastocystis hominis *(original magnification ×400)*

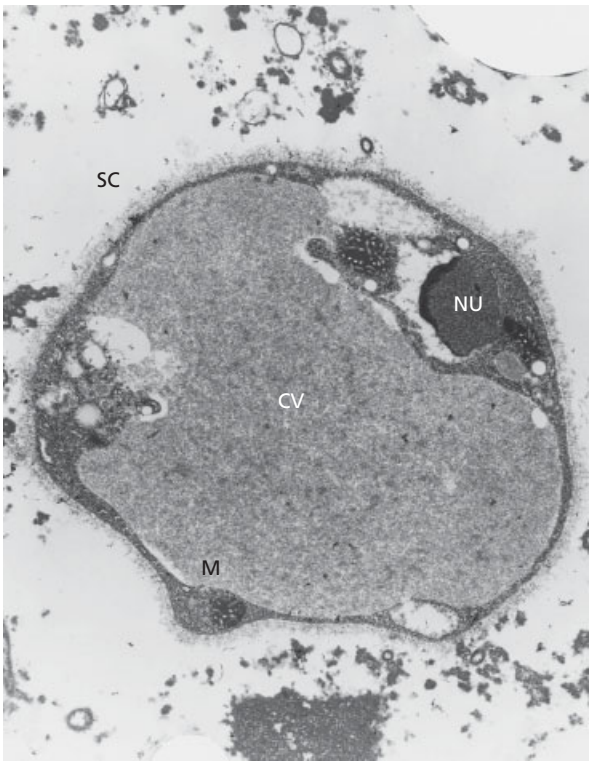

Figure 2 *Transmission electron micrograph of* Blastocystis hominis *from culture. Vacuolated form showing nucleus (NU), central vacuole (CV) surrounded by a thin cytoplasm, mitochondrion-like organelles (M), and a surface coat (SC) (original magnification ×5 200). (Reproduced with permission from A. Curry and J. J. Windsor)*

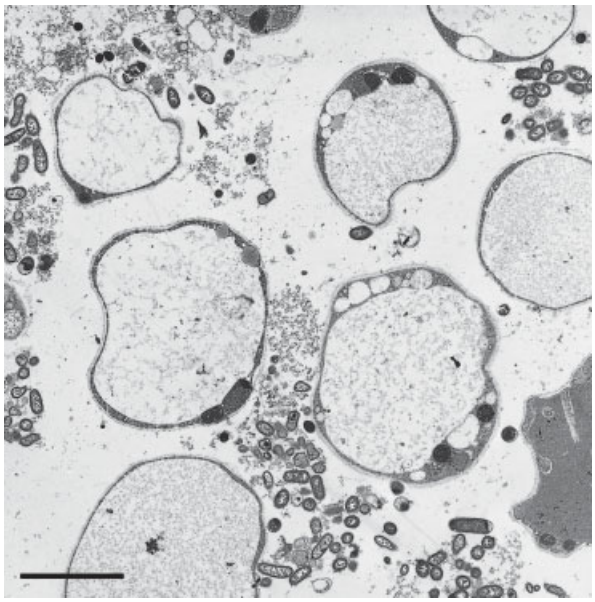

Figure 3 *Transmission electron micrograph of numerous vacuolar forms of* Blastocystis hominis *from culture. Bar = 5 μm. (Reproduced with permission from D. J. Stenzel)*

Granular form

This morphological form is similar to the vacuolar type, and can be regarded simply as a vacuolar form with granules in the central vacuole (Stenzel and Boreham 1996). The size range is similar and the granules are visible in unstained preparations (Figures 5 and 6).

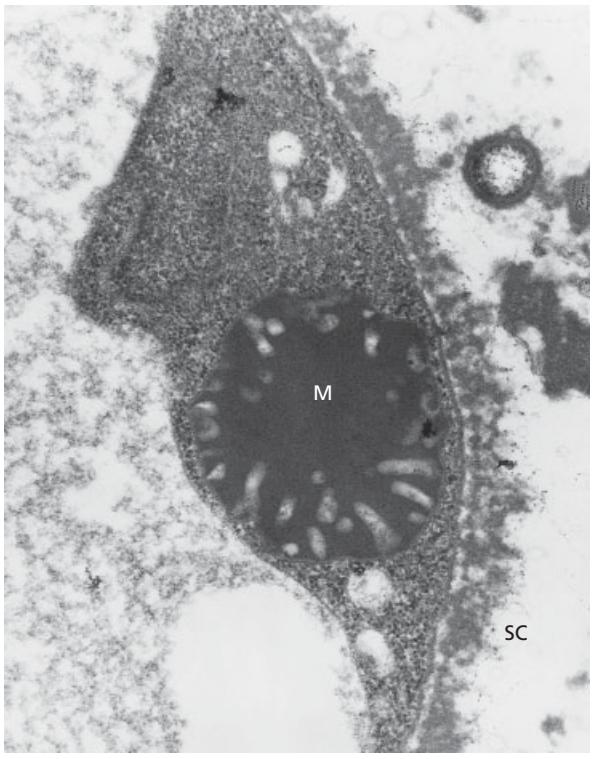

Figure 4 *Electron micrograph of* Blastocystis hominis *from cultured material. Vacuolar form showing mitochondrion-like organelle (M), and a surface coat (SC) (original magnification ×21 000). (Reproduced with permission from A. Curry and J. J. Windsor)*

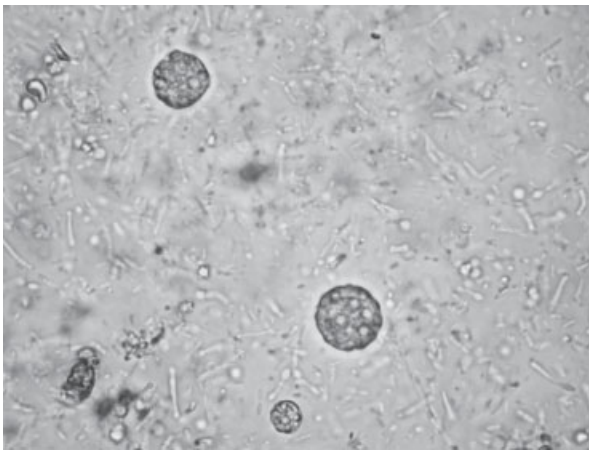

Figure 5 *Granular form of* Blastocystis hominis *(original magnification ×400)*

Many functions have been proposed for the different types of granule, but it is likely that they are involved in metabolism and storage (Stenzel and Boreham 1996). In older cultures, the granular form is often the most frequently found morphotype (Zierdt et al. 1967). Granular forms can be induced in culture by the use of antibiotics, axenic culture, increased serum concentration, and transfer to a different culture medium (Zierdt 1973; Stenzel and Boreham 1996).

Ameboid form

The ameboid form of *B. hominis* is only rarely encountered and there have been inconsistencies in the published data describing this form (Tan and Zierdt 1973;

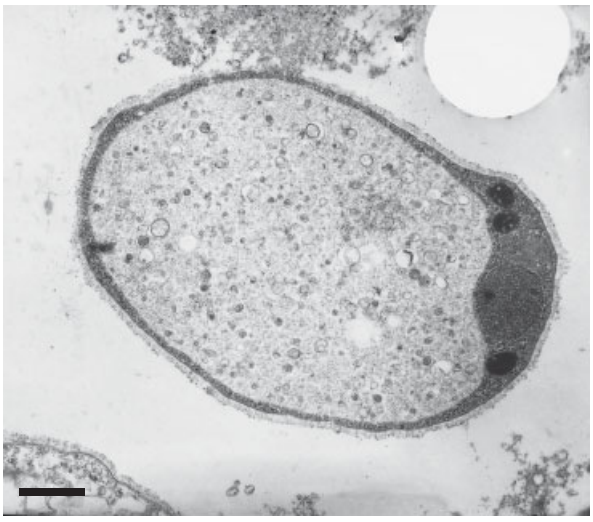

Figure 6 *Granular form of* Blastocystis hominis. *Note the presence of clumps of granules in the central vacuole. Bar = 1 μm. (Reproduced with permission from D. J. Stenzel)*

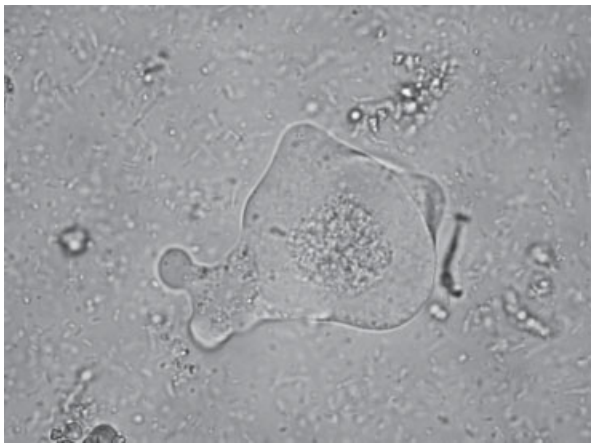

Figure 7 *Ameboid form of* Blastocystis hominis *from culture. Note the irregular shape (original magnification ×400)*

Zierdt 1991; Stenzel and Boreham 1996). Extended pseudopodia are often seen in ameboid forms of *B. hominis* (Tan and Zierdt 1973; Zierdt 1973) (Figures 7 and 26.10b). The ameboid form has been reported to feed on bacteria (Zierdt 1973; Boreham and Stenzel 1993) and cellular debris from dead cells (Tan et al. 2002). It has been postulated that this might provide nutrition for subsequent encystations (Singh et al. 1995). Tan and colleagues (2002) agreed, and concluded that the ameboid form of *B. hominis* is likely to be involved in a nutritional or a regulatory role in programmed cell death.

Cyst form

Mehlhorn (1988) is credited with the first report of a cyst-like form of *B. hominis* in the stool of an HIV-positive patient. Three years later, Stenzel and Boreham (1991) provided a detailed description of the cyst stage. The cysts were often found in stored fecal samples and were small (3.7–5.0 μm), round or ovoid. TEM of the cysts showed a thick wall and a bi-layered membrane between the wall and the cytoplasm (Figures 8 and 9). An external capsular layer was present on some cells. Only one nucleus per cell was noted, though in a subsequent publication, the same group described multinucleated cysts (Boreham et al. 1996). Cysts were also occasionally found in long-term cultures. Zaman and colleagues (1997) reported variations in *B. hominis* cyst morphology. They described two types of cyst, with or without the capsular layer. The surface coat was shed as the cyst matured. Although Mehlhorn (1988) did not see mitochondria in his original study, they have been reported by other workers (Stenzel and Boreham 1991; Boreham et al. 1996; Moe et al. 1996; Zaman and Zaki 1996).

Avacuolar and multivacuolar forms

There is evidence that the morphology of *B. hominis* in vivo may be different than that normally found in stool

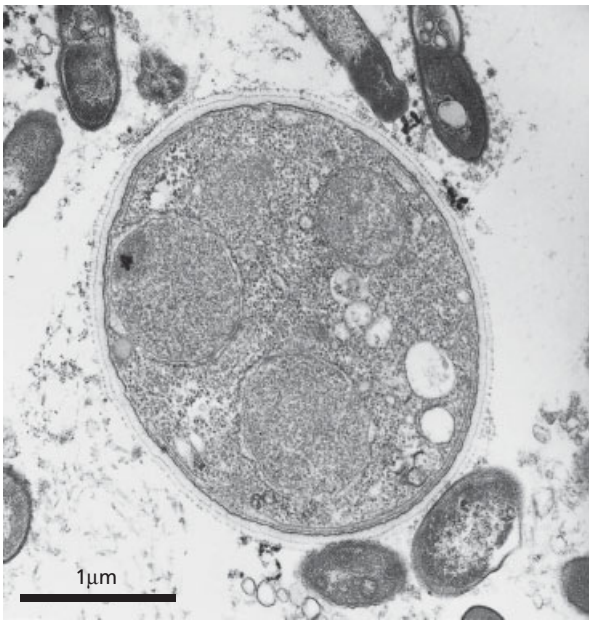

Figure 8 *Cyst of* Blastocystis hominis *showing the multilayered wall. Bar = 1 μm. (Reproduced with permission from D. J. Stenzel)*

samples and fecal cultures. Zierdt and Tan (1976) reported an unusual form of *B. hominis* in a patient with severe gastrointestinal symptoms, producing large volumes of diarrheal fluid. This form did not have a vacuole with numerous mitochondria, and they termed it the trophozoite form. In a later report, Stenzel and colleagues (1991) described a similar form from a colono-

Figure 9 *Higher magnification of cyst of* Blastocystis hominis *showing inner layer (I), electron-dense layer (D) and outer layer (O). Bar = 0.5 μm. (Reproduced with permission from D. J. Stenzel)*

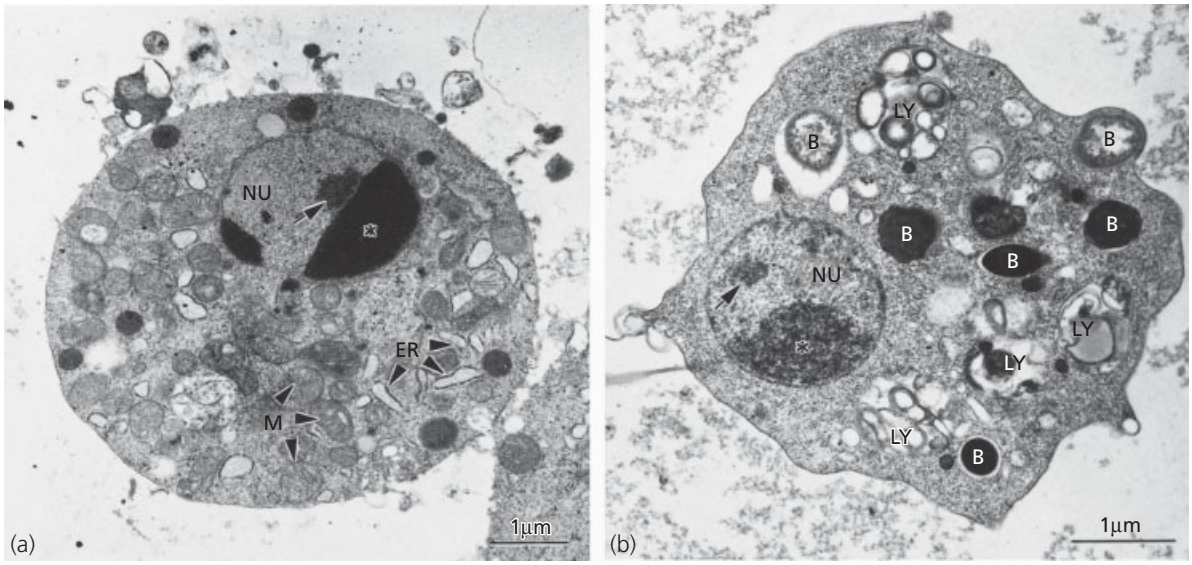

Figure 10 *Transmission electron micrographs of* B. hominis. **(a)** *Avacuolar form obtained at colonoscopy. In addition to a crescentic band (asterisk), the nucleus (NU) contains electron-opaque material (arrow). Mitochondrion-like organelles (M) and rough endoplasmic reticulum (ER) are abundant in the cytoplasm.* **(b)** *Ameboid form showing numerous lysosome-like bodies (LY) in the cytoplasm. Bacteria are seen within these bodies and in other membrane-bounded structures. The nucleus contains a compact band (asterisk) and clumps (arrow) of electron-opaque material. (Reproduced from Stenzel and Boreham 1996, with permission from author and publisher)*

scopy specimen and called it avacuolar. Avacuolar forms lack a central vacuole and contain one or two nuclei (Figure 10a). They are smaller than vacuolar forms (approximately 5 μm as compared to 4–15 μm) and, like the ameboid form, are not surrounded by a surface coat (Stenzel and Boreham 1996).

Multivacuolar forms of *B. hominis* have many small vacuoles of different sizes within the cytoplasm, and are found in fecal material. They are smaller (5–8 μm) than the vacuolar form of *B. hominis* seen in feces and have a surface coat (Figures 11 and 12). Many studies of *B. hominis* concentrated on the forms seen in culture (Boreham and Stenzel 1993). This might explain why the multivacuolar form has not been widely reported. However, Stenzel and colleagues (1991) found the multivacuolar form to be the predominant form in human fecal material, when using TEM. Indeed, they considered the multivacuolar and avacuolar forms more likely to be representative of the parasite in vivo.

LIFE CYCLE

Conflicting reports have been published regarding the life cycle of *B. hominis* and these may reflect the use of light microscopy alone, which has limitations and can lead to inaccurate interpretations (Tan et al. 2002). A number of different life cycles have been proposed, dependent on the division process of *B. hominis*. Some workers support the theory that *B. hominis* divides by either plasmotomy, endodygeny or schizogony (Zierdt and Tan 1976; Zierdt 1988, 1991; Jiang and He 1993; Singh et al. 1995; Zaman et al. 1999), in addition to binary fission. However, TEM has failed to support these hypotheses and it is possible that the schizonts filled with progeny described by light microscopy (Zierdt 1991; Singh et al. 1995) might be granules or inclusions within the cells (Stenzel and Boreham 1996). The most plausible life cycle proposed to date is by Stenzel and Boreham (1996), based on binary fission as the only

Figure 11 *Multivacuolar form of* Blastocystis hominis *from fresh fecal sample showing nucleus (NU), vacuoles (V), mitochondrion-like oroganelles (M), and a thick fibrillar surface coat (SC). Bar = 1 μm. (Reproduced with permission from D. J. Stenzel)*

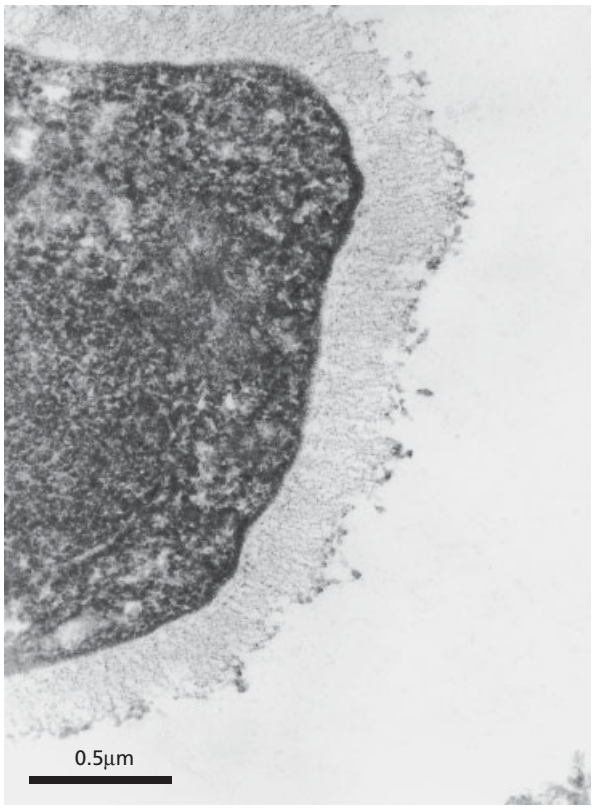

Figure 12 *Higher magnification of multivacuolar form of* Blastocystis hominis *showing surface coat. Bar = 0.5 μm. (Reproduced with permission from D. J. Stenzel)*

method of division (Figure 13). The cyst is regarded as the infective stage, although the role of the ameboid and granular forms remains poorly defined. There is still much to be elucidated, and the factors responsible for excystation and encystation are currently unknown (Stenzel and Boreham 2001).

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

Data regarding the biochemical cycles and cytochemistry of *B. hominis* is somewhat limited and comprehensive studies have not been performed. Conflicting data has been published on many aspects of basic *B. hominis* biochemistry. For a more detailed review see Stenzel and Boreham (1996).

Although early studies indicated otherwise (Zierdt et al. 1967; Zierdt 1973), carbohydrates have been detected in *B. hominis* using periodic acid-Schiff (PAS) and alcian blue (AB) stains (Yoshikawa et al. 1995). Positive reactions were found in the central vacuole and the cytoplasm; however, the intensity varied suggesting the distribution of carbohydrates may be different in individual cells. At the electron microscopic level, using periodic acid methenamine silver, positive reactions were found in the Golgi apparatus, cytoplasmic vesicles, external filamentous layer, and the central vacuole. Fluorescein isothiocyanate (FITC)-labeled lectins have been used to elucidate the surface-coat carbohydrates of *B. hominis* (Lanuza et al. 1996). Although the surface coat was found to be complex, they all contained α-D-mannose, α-D-glucose, *N*-acetyl-α-D-glucosamine, α-L-fucose, chitin and sialic acid. By contrast, other workers, using lectin localization at the ultrastructural level, have found that carbohydrates are not a major component of the surface coat (Stenzel and Boreham 1996).

B. hominis may accumulate lipid in the central vacuole and cytoplasm, as demonstrated with Nile blue and Sudan III staining (Dunn et al. 1989). Lipid analysis of axenic *B. hominis* strains has revealed a variety of neutral lipids, phospholipids, and polar lipids (Keenan et al. 1992). Cardiolipin was also detected, which is almost exclusively found in the inner mitochondrial membrane. Paradoxically, although cardiolipin plays a pivotal role in the cytochrome oxidase complex in mammalian cells, cytochrome oxidase has not been found in *B. hominis* (Zierdt 1988). Other enzymes normally associated with mitochondrial activity were also absent from *B. hominis* isolates (Zierdt 1986; Zierdt 1988). Boreham and Stenzel (1993) suggested that these organelles may be hydrogenosomes. Hydrogenosomes lack catalase and cytochromes and were first described in trichomonads (Landmark and Müller 1973). The mitochondrion-like organelles of *B. hominis* stain positively with the vital stains rhodamine 123 and Janus green, indicating that they are functional and not vestigial remnants (Zierdt 1988). Chitin has been demonstrated in the cyst stage of *B. hominis* (Stenzel and Boreham 1996).

Isoenzyme electrophoretic analysis of *B. hominis* isolates revealed malic enzyme, phospholucomatase, glucose phosphate isomerase, 6-phosphogluconate dehydrogenase, and hexokinase (Mansour et al. 1995). Two variants or zymodemes were found, although the study size was small and only 11 isolates were investigated. Analysis of the polypeptide patterns and immunological characteristics of axenic strains of *B. hominis* revealed at least two different variants (Kukoschke and Müller 1991; Mansour et al. 1995). The existence of biochemically and immunologically different strains of *B. hominis* supported the theory that some types of *B. hominis* may have differing pathogenic potential (Kukoschke et al. 1990). Protein and DNA analysis of ten Australian *B. hominis* isolates also revealed two different demes (Boreham et al. 1992). Lanuza and colleagues (1999) described the soluble-protein profile and antigenic cross-reactivity of *B. hominis* isolates and found three protein groups (I–III) and two antigenic groups (1 and 2). The isolates from patients with chronic diarrhea were in protein patterns I and II and antigenic group 1. Isolates from patients with acute diarrhea were clustered in protein pattern III and antigenic group 2.

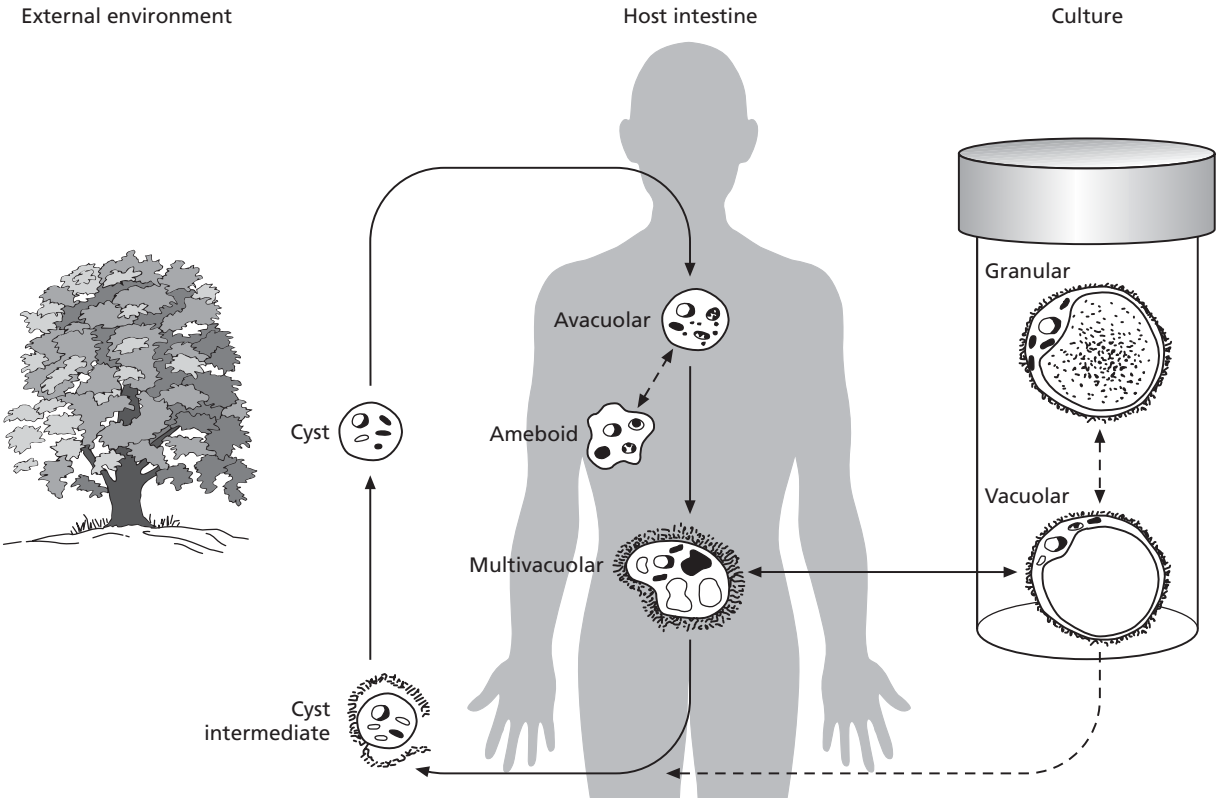

Figure 13 *Proposed life cycle for* Blastocystis hominis *(Redrawn from Stenzel and Boreham 1996, with permission from author and publisher)*

Conflicting data has been reported when karyotyping *B. hominis* isolates. Some workers (Upcroft et al. 1989; Carbajal et al. 1997) found genetic heterogeneity whereas others have reported significant homology (Teow et al. 1991; Ho et al. 1994). Discrepancies may be due to different investigation procedures or may reflect genetic changes in axenic cultures or laboratory stock cultures (Ho et al. 1994).

Various molecular methods have confirmed that *B. hominis* exhibits extensive genetic diversity. The methods used include RFLP analysis (Böhm-Gloning et al. 1997; Clark 1997a; Hoevers et al. 2000; Yoshikawa et al. 2000; Kaneda et al. 2001) and random amplified polymorphic DNA (RAPD) (Yoshikawa et al. 1998).

Clark (1997a) amplified SSU rDNA and analyzed the products using restriction enzyme analysis. Some areas of rDNA are highly conserved in all eukaryotes, but are distinct from bacterial rDNA. An SSU rRNA gene amplicon of approximately 1800 bp was produced. He termed this method riboprinting (Clark 1997b) and obtained seven different ribodemes (distinct ribotypes) after using 11 restriction enzymes. The restriction enzymes *Hin*fI and *Rsa*I, however, could be used to identify all seven ribodemes. Three human *B. hominis* isolates were identical to a guinea-pig isolate, indicating the possibility of cross-species infection. Phylogenetic analysis of the seven ribodemes revealed two distinct lineages; ribodemes 1 and 6 were closely related, but very distant from the others. Using the same methodology, other workers subsequently described a further three ribodemes: 8, 9 and 10 (Yoshikawa et al. 2000; Kaneda et al. 2001). In one of these studies, the results provided the first molecular evidence of possible human-to-human transmission of *B. hominis* (Yoshikawa et al. 2000). Restriction site analysis of polymerase chain reaction (PCR)-amplified 16S-like rDNA of *B. hominis* from symptomatic and asymptomatic individuals revealed five subgroups (Böhm-Gloning et al. 1997). However, not all the SSU rDNA product was examined (approx. 850 bp) and only three restriction enzymes were used. Although it appeared that subgroup II was more prevalent in symptomatic patients, none of the groups were statistically correlated with disease. Analysis of DNA polymorphism of *Blastocystis* strains from humans, a chicken, and a reptile, using an arbitrary primer PCR method, found similar products with human and avian isolates, suggesting that the chicken isolate was a zoonotic strain (Yoshikawa et al. 1996).

Ho and colleagues (2001) used a PCR-based RFLP technique to analyze the elongation factor gene (EF-1α) of *B. hominis*. They termed this technique elfaprinting and the different subtypes elfatypes. Again, extensive genetic diversity was found with *Blastocystis* species. As the EF-1α is highly conserved, elfaprinting has potential

as an epidemiological tool. However, it was used only on axenic isolates of *B. hominis* and the authors concede that, in future, it would be necessary to amplify directly from direct (xenic) cultures.

There is a need to optimize and standardize the molecular methods and terms used to characterize *B. hominis* strains (Hoevers et al. 2000). The terms subgroup, genotype, ribodeme, and elfatype have all been used to describe *B. hominis* strains. Similar problems are found with the use of different primers and restriction enzymes. It has been suggested that future studies should use reference strains and a standard panel of restriction enzymes (Tan et al. 2002). See Tan et al. (2002) for a detailed review of *B. hominis* typing methods.

CLINICAL ASPECTS

Before describing the clinical spectrum associated with *B. hominis*, it must be stressed that the organism has also been frequently found in asymptomatic individuals (Udkow and Markell 1993; Hellard et al. 2000; Tan et al. 2002). In addition, many of the reported cases of *B. hominis* linked with symptomatology are anecdotal and are uncontrolled, making evaluation of its pathogenicity problematical. Moreover, some studies have failed to eliminate all other causes and infections that may produce intestinal symptoms.

The most common symptoms reported with *B. hominis* infection include diarrhea, nausea, abdominal pain, cramps and discomfort, and flatulence (Sheehan et al. 1986; Diaczok and Rival 1987; Guirges and Al-Waili 1987; Kain et al. 1987; Stenzel and Boreham 1996). Other, less frequently reported, symptoms include fever, constipation, vomiting, eosinophilia, anorexia, fatigue, and nonspecific gastrointestinal effects (Sheehan et al. 1986; Stenzel and Boreham 1996).

B. hominis has been reported in patients who had an intestinal blockage due to carcinoma (Horiki et al. 1999). *B. hominis* was considered coincidental rather than related to the neoplastic growth, although it appeared to take advantage of the altered physiology of the intestine. Two studies have found an association between *B. hominis* and irritable bowel syndrome (IBS) (Hussain et al. 1997; Giacometti et al. 1999). In addition, an earlier study by Markell and Udkow (1986) described five patients with pure *B. hominis* infection who fulfilled the medical criteria for IBS. Giacometti et al. (1999) detected *B. hominis* in 18.1 percent of IBS patients versus 6.9 percent of IBS-negative patients. They speculated that *B. hominis* might be an indicator of intestinal dysfunction. Significantly increased immunoglobulin (Ig)G2 antibody titers against *B. hominis* have been described in IBS patients when compared with asymptomatic controls (Hussain et al. 1997). Increased levels of IgG2 were also found in IBS patients without *B. hominis* in their stools, although this might be explained by prior treatment of *B. hominis*, resulting in elimination of the parasite. Indeed, 18 out of 22 (85 percent) patients in one center had received previous antibiotic treatment. It is presently unknown whether *B. hominis* is actively involved in the pathogenesis of IBS or simply takes advantage of the disruption in the microbial flora.

B. hominis has been reported in traveler's diarrhea in some studies (Sheehan et al. 1986; Jelinek et al. 1997), but not in others (Shlim et al. 1995). Jelinek et al. (1997) reported the association of *B. hominis* with diarrhea in German travelers returning from the tropics. In contrast, Shlim et al. (1995) conducted a prospective controlled study of traveler's diarrhea in Nepal and concluded that *B. hominis* did not cause diarrhea. However, this study would seem to be weakened by design flaws as, once other known pathogens were excluded, only 19 patients with diarrhea and 26 controls were infected with *B. hominis* (Keystone 1995). These relatively small numbers reduced the power of the study. Furthermore, only one stool per person was examined and stains to exclude cryptosporidia and *Cyclospora* were not performed.

In 1989, Rolston et al. reported nine cases of symptomatic gastroenteritis in patients with pure *B. hominis* infection and underlying pathology. Three patients had acquired immune deficiency syndrome (AIDS) and the other conditions included Hodgkin's disease, leukemia, bone marrow transplant (BMT), lung and bladder cancer, and pelvic inflammatory disease (PID). In 1990, Garavelli et al. described five cases of *B. hominis* infection in symptomatic human immunodeficiency virus (HIV)-positive patients that responded to treatment with metronidazole. In both of these anecdotal studies, little data is available on laboratory methodology and neither states which pathogens were excluded. Cirioni et al. (1999) described the prevalence and relevance of *B. hominis* in different patient cohorts (immunocompromised patients, elderly subjects, psychiatric in-patients, immigrants from developing countries, travelers to tropical countries, and controls) and found it to be the most common parasite in all groups. Symptoms were found only in patients with *B. hominis* when severe immunosuppression was present and these workers concluded that immunodepression was an important factor in pathogenicity of the organism. Other studies have failed to find a link between *B. hominis* and clinical symptoms in AIDS patients, despite a high carriage rate (Albrecht et al. 1995).

IMMUNOLOGY

Relatively little is known regarding the host immune response to *B. hominis* and serological techniques have been used with limited success. Chen et al. (1987) failed to detect a humoral response in four patients using an IgG immunoblot assay. In contrast, Zierdt et al. (1995)

detected IgG antibodies to *B. hominis* in 28 out of 30 patients using an enzyme-linked immunosorbent assay (ELISA). The patients in this latter study were symptomatic and other protozoa and bacterial pathogens were excluded. Although the cut-off was low (1:50), nineteen titers were 1:100 or greater and all the controls (blood bank sera) were negative. In another study, levels of *B. hominis* IgG antibodies were significantly raised in patients with IBS when compared to asymptomatic controls (Hussain et al. 1997). When the IgG antibodies were further subdivided, a selective increase was found in the IgG2 subclass. IgG2 antibodies are primarily directed against carbohydrate antigens and it was thought that these antibodies might be produced in response to the fibrillar surface coat surrounding *B. hominis*. The discrepancies between studies might be due to differences in antigen preparation, one study used a sonicated antigen that was likely to contain carbohydrates (Zierdt et al. 1995), whereas the other used a protein antigen (Chen et al. 1987). The usefulness of serological testing may be limited however, as antibodies have been reported in 70 percent of asymptomatic individuals infected with *B. hominis* (Kaneda et al. 2000). Using immunoblotting analysis, it was found that the antibody response was directed against a 12 kDa surface protein of *B. hominis*.

PATHOLOGY

The general consensus is that *B. hominis* does not usually invade the intestinal mucosa, but may be associated with inflammation and edema (Stenzel and Boreham 1996). Kain et al. (1987) reported that endoscopic, histopathologic, and radiologic findings were normal in symptomatic patients with *B. hominis* infection. However, Russo et al. (1988) described a case of biopsy-proven colitis with large numbers of *B. hominis* present in the stool sample. The patient in this case report became asymptomatic following treatment for *B. hominis* infection with metronidazole. An invasive *B. hominis* infection has been reported in a child, presenting as diffuse superficial ulcers and pseudomembranes on endoscopy (Al-Tawil et al. 1994). Histological examination revealed numerous *B. hominis* present over the surface and within the superficial lamina propria in the region of ulceration. All known viruses, bacteria, and parasites were excluded, including *Clostridium difficile* toxins. A case of hemorrhagic proctosigmoiditis has also been described in association with *B. hominis* (Carrascosa et al. 1996). Acute inflammation was found in rectal and sigmoid biopsy specimens, and sigmoidoscopy revealed marked diffuse erythema. These findings should be interpreted with caution however, as the stool sample was not examined for viruses or toxins. From the above studies, it would seem that in certain circumstances *B. hominis* might be responsible for causing atypical pathology.

Pathogenicity

Difficulties in proving the pathogenicity of *B. hominis* include the lack of a suitable animal model, failure to exclude all other possible causes of symptoms, and the inability to fulfill even the extended Koch's postulates (Stenzel and Boreham 1996; Anon. 1991). Some workers have proposed that *B. hominis* is only pathogenic when present in large numbers ($\geqslant$ 5 organisms per $\times$400 or $\times$100 field) (Zierdt 1983; Miller and Minshew 1988; Sheehan et al. 1986). This hypothesis has been disputed by others (Shlim et al. 1995; Stenzel and Boreham 1996), and it seems somewhat implausible given that no other human intestinal protozoan is only pathogenic when found in high numbers.

Phillips and Zierdt (1976) inoculated *B. hominis* together with the intestinal bacterial flora from humans into gnotobiotic guinea pigs. Of the 43 animals inoculated orally, 14 developed *B. hominis* infection. Microscopic examination showed penetration of *B. hominis* into the epithelium, but not the lamina propria. When a monoxenic (with *Proteus vulgaris*) culture was used, only one of eight animals was infected. Axenic cultures failed to produce any infection. This failure of an axenic culture of *B. hominis* to cause disease suggests that the symptoms may have been caused by the presence of the original bacterial flora, the bacteria provide essential cofactors for *B. hominis* pathogenicity, or that some strains of *B. hominis* are more pathogenic than others. Xenic *B. hominis* cultures have been shown to produce cytopathic effects in cell lines, although filtrates in which *B. hominis* failed to grow produced similar effects (Walderich et al. 1998). This would seem to suggest that the cytopathic effect was caused by toxins produced by the co-cultivated bacteria and may also explain the earlier findings of Zierdt (1991).

An axenic culture of *B. hominis* did not produce any cytopathic effect on colonic epithelium cell lines (HT-29 and T84) (Long et al. 2001). However, significant increases in the cytokines interleukin (IL)-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF) were discovered. IL-8 activates neutrophils, monocytes, and T lymphocytes, and GM-CSF is a strong chemoattractant for neutrophils and eosinophils. Consequently, Long et al. (2001) concluded that *B. hominis* induces and modulates the immune response in intestinal epithelial cells. A cytopathic effect was produced when *B. hominis* was inoculated into laboratory mice intramuscularly (Moe et al. 1998). Histological examination revealed that *B. hominis* produced a severe inflammatory reaction and myonecrosis. In addition, *B. hominis* was shown to have a strong chemoattractant activity for neutrophils.

Dagci et al. (2002) described an increase in intestinal permeability in patients infected with *Giardia lamblia* and *B. hominis*, but not in patients harboring

nonpathogenic *Entamoeba coli*. Intestinal permeability can be affected by various factors and demonstrates the intactness of the gut.

DIAGNOSIS

Light microscopy remains the mainstay of *B. hominis* detection (MacPherson and MacQueen 1994), with culture techniques usually reserved for research purposes. However, the variable forms of *B. hominis* can present difficulties in reliable detection of this parasite. Indeed, laboratory awareness and the use of suitable methodologies are of paramount importance when detecting *B. hominis* (Windsor et al. 2001a). Irregular shedding of *B. hominis* has been reported and, therefore, more than one fecal specimen must be taken to exclude this parasite in patients with clinical symptoms (Vennila et al. 1999).

Detection in unstained preparations

The vacuolar form of *B. hominis* is usually easily recognizable in either saline or iodine wet preparations (Figure 1). *B. hominis* can be seen in ethylene-acetate fecal concentrations, but reports on the reliability of this method vary considerably (Stenzel and Boreham 1996). Care must be taken to distinguish *B. hominis* from leukocytes and parasites such as *Dientamoeba fragilis*, *Entamoeba* spp, and *Endolimax nana* (Windsor et al. 2001b). Lee (1991) recommended the use of phase-contrast microscopy for the detection of *B. hominis*. Putative *B. hominis* positives should be confirmed with a suitable staining method (see below), as false-positive detections have occurred when using unstained preparations (Windsor et al. 2002).

Permanent staining techniques

Although the use of permanently stained smears is recommended by parasitologists in North America for the detection and identification of fecal protozoan parasites (Garcia 1990), relatively few laboratories in the UK employ such techniques (Windsor and Johnson 1999). Indeed, such permanently stained smears are considered by many to be the gold standard for detection of *B. hominis* (Garcia et al. 1984; Lee 1991; Stenzel and Boreham 1996; Windsor et al. 2001b). Suitable staining methods include trichrome (Figure 14) (Garcia 1990; Windsor et al. 2002) and iron-hematoxylin (MacPherson and MacQueen 1994), which are usually used in conjunction with a specialized fixative such as polyvinyl alcohol (PVA) (Goldman and Brooke 1953) or sodium-acetate acetic-acid formalin (SAF) (Yang and Scholten 1977). Giemsa stain (Ricci et al. 1984) and Fields stain (Moody and Fleck 1985) can also be used after fixing fecal smears in methanol. Gram's stain has been used

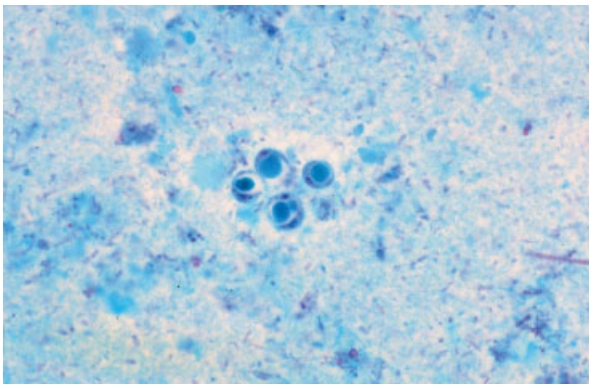

Figure 14 *Trichrome-stained smear of vacuolar forms of* Blastocystis hominis *(original magnification ×600) (Redrawn from Windsor et al. 2002, with permission from author and publisher)*

(Miller and Minshew 1988), but is of limited use as *B. hominis* cells often lyse when using this method (Zierdt 1991). Although *B. hominis* is not acid-fast, it may stain with the counterstain (malachite green or methylene blue) when the modified Ziehl-Neelsen stain is used for the detection of *Cryptosporidium* (Windsor et al. 2001b).

Studies comparing unstained microscopy and permanent stains have shown the latter to be much more sensitive and specific (Windsor et al. 2002). Furthermore, it is likely that some of the smaller multivacuolar and cystic forms of *B. hominis* would only be detected using stained smears. Much variability occurs when using permanent stains, especially with the appearance of the central vacuole (MacPherson and MacQueen 1994; Stenzel and Boreham 1996). However, this should not pose a significant problem to experienced laboratory personnel.

Culture

Zierdt (1991) does not recommend culture of *B. hominis* as a routine procedure, but acknowledges its usefulness when the microscopic diagnosis is uncertain. Clark and Diamond (2002) concur and state that cultivation of intestinal protists plays only a minor role in diagnosis. Kukoschke et al. (1990) compared detection of *B. hominis* by direct microscopy and culture and found almost complete agreement. Zaman and Khan (1994) however, disagreed and found culture to be more sensitive than direct microscopy. They found only 18 positives by microscopy out of 100 patients, whereas 45 were positive for *B. hominis* using culture. Despite these conflicting reports there can be no doubt that culture of *B. hominis* has been of great importance in the study of this organism in the past and will continue to be a useful research tool in the future.

B. hominis will grow in a wide variety of different culture media. In 1921, Barret first described the growth of *B. hominis* in a simple medium of human serum and 0.5 percent salt solution. He was attempting to grow the

ciliate parasite *Balantidium coli*, but found that *Blastocystis hominis* grew very well in this medium. Many of the media designed specifically for culture of *Entamoeba* species also support the growth of *B. hominis* (Jones 1946; Robinson 1968). Indeed, Clark and Diamond (2002) comment that *B. hominis* grows luxuriantly in all xenic media formulated for both *Entamoeba* species and *Dientamoeba fragilis*. Some of the media are monophasic (Jones 1946), whereas others have a solid and a liquid phase (Robinson 1968; Zierdt 1988). Zierdt (1988) favored the use of a modified egg slant with Locke solution and 30 percent horse serum. Antibiotics, such as erythromycin, are often used to suppress unwanted bacterial species (Robinson 1968).

Antibiotics can also be used to produce an axenic culture, which is grown in the absence of bacteria (Zierdt 1991). *B. hominis* was grown axenically for the first time by Zierdt and Williams in 1974. Ho et al. (1993) subsequently produced an axenic growth of *B. hominis* in monophasic Iscove's modified Dulbecco's medium (IMDM). Tan et al. (1996, 2000) took the culture of *B. hominis* to the next level by establishing colonies from single cells, initially in soft agar (Tan et al. 1996) and then on solid agar (Tan et al. 2000). Production of an axenic culture of *B. hominis* is an essential first step for the production of antibodies. However, commercial diagnostic antibodies against vacuolar and cystic forms of *B. hominis* are not currently available (Tan et al. 2002).

Fluorescent staining

Very little has been published on the use of fluorescent staining techniques for the detection of *B. hominis*. Zierdt (1991) reported previously unpublished data on the use of rabbit antisera to unheated whole-cell *B. hominis* antigens. Reactions were clear-cut at a dilution of 1:200, and immunostaining detected vacuolar, ameboid, and granular forms of *B. hominis* from both feces and culture. Antigenic diversity is extensive among isolates of *B. hominis*, and may be one reason why appropriate antibodies are still not available for diagnostic work (Kukoschke and Müller 1991; Lanuza et al. 1999; Tan et al. 2001).

EPIDEMIOLOGY

Stenzel and Boreham (1996) commented that the epidemiology of *B. hominis* is totally unknown. They attributed this to the confusion over the status of the organism. When suitable methodologies are used, *B. hominis* is often the most frequent intestinal protozoan found in humans worldwide (Stenzel and Boreham 1996; Clark 2000). Unfortunately, some studies of intestinal infection do not report potential pathogens such as *B. hominis* and *Dientamoeba fragilis* (Tompkins et al. 1999). Nevertheless, in developed countries, prevalences of *B. hominis* range from 1.5 to 23 percent (Garcia et al. 1984; Kain et al. 1987; Doyle et al. 1990; Senay and MacPherson 1990; O'Gorman et al. 1993; Logar et al. 1994; Amin 2002; Windsor et al. 2002). Higher rates are seen in developing countries, ranging from 25 to 50 percent (Nimri 1993; Nimri and Batchoun 1994; Stenzel and Boreham 1996). Hellard et al. (2000) reported *B. hominis* in 6 percent of asymptomatic individuals in Australia. Martín-Sánchez et al. (1992) also described *B. hominis* in a high number of asymptomatic cases in children from different population groups in Spain (5.3–19.4 percent). Increased prevalences of *B. hominis* may be found in lower socioeconomic groups and in individuals with poor standards of hygiene (Stenzel and Boreham 1996).

In the UK, little has been published on the prevalence of *B. hominis*. Pakianathan and McMillan (1999) described an incidence rate of 26 percent in homosexual men in Edinburgh, though they did not correlate carriage of *B. hominis* with diarrhea. This population subgroup usually has a high carriage rate of intestinal protozoa per se and, therefore, is not representative of the general population. Casemore et al. (1984), while examining 2 000 specimens in North Wales for *Cryptosporidium* species, only found one sample containing large numbers of *B. hominis*. They concluded that *B. hominis* does not contribute significantly to the etiology of gastroenteritis in North Wales. However, small numbers of *B. hominis* may have been overlooked. Furthermore, optimal staining techniques for *B. hominis* detection were not employed. When trichrome-stained smears were examined, *B. hominis* was found in 6.9 percent of unselected fecal samples in mid-Wales (Windsor et al. 2002).

CHEMOTHERAPY

Anecdotal reports of successful treatment of *B. hominis* infection abound in the published literature. As with proving pathogenicity, a similar question arises when assessing successful treatment of *B. hominis* infection – are all other causes of gastrointestinal symptoms excluded? Furthermore, many of the drugs used for treatment of *B. hominis* are also active against a wide range of gram-positive and gram-negative bacteria (Stenzel and Boreham 2001). This makes it difficult to accurately interpret treatment regimens. There is a dearth of experimental data to confirm the efficacy of treatment for *B. hominis* infections.

Guirges and Al-Waili (1987) used metronidazole therapy to treat 103 patients with pure *B. hominis* infection (i.e. they were not knowingly co-infected with another enteropathogen). The main symptoms were diarrhea with abdominal pain and flatulence with abdominal discomfort. They found metronidazole to be effective and concluded that *B. hominis* was a patho-

genic parasite that could be successfully eradicated with this antibiotic. O'Gorman et al. (1993) studied *B. hominis* infections in children and found that gastrointestinal symptoms (abdominal pain, diarrhea, vomiting, and weight loss) resolved in 90 percent of patients receiving a month of antimicrobial therapy, but only in 58 percent of those who received no treatment. Metronidazole was the recommended drug, although furazolidone, mebendazole, and iodoquinol were used for treatment of concurrent *B. hominis* infection with other parasites.

Dunn and Boreham (1991) tested the in vitro activity of various antimicrobials against *B. hominis*. Ketaconazole and iodoquinol were less active than metronidazole, three times and twenty-five times less respectively. Metronidazole resistance was reported by Haresh et al. (1999), with Malaysian strains of *B. hominis* being resistant to 0.01 mg per ml and Indonesian isolates surviving 1.0 mg per ml. Metronidazole has also been shown to have no effect on *B. hominis* cysts (Zaman and Zaki 1996).

Cabello et al. (1997) evaluated nitazoxanide, a 5-nitrothiazol derivative, for the treatment of both intestinal protozoan and helminthic infections in Mexico. It was well tolerated and had a 'cure rate' of 100 percent in ten cases of *B. hominis* infection.

Ok et al. (1999) evaluated the use of trimethoprim–sulfamethaxazole for the treatment of *B. hominis* infection in children and adults. Fifty-three patients (38 children and 15 adults) were treated. Children received 6 mg per kg trimethoprim and 30 mg per kg sulfamethaxazole, and adults received 320 mg per kg trimethoprim, and 1600 mg per kg sulfamethaxazole daily. *B. hominis* was eradicated in 36 of 38 (94.7 percent) children and 14 of 15 (93.3 percent) adults. Clinical symptoms disappeared in 39 (73.6 percent) patients, decreased in 10 (18.9 percent), with one patient showing no change. In 3 (5.7 percent) patients, symptoms persisted and *B. hominis* could not be eradicated.

Crude extracts of traditional Chinese medicinal herbs have been evaluated for activity against *B. hominis* in vitro, and *Coptis chinensis* and *Brucea javanica* were active in concentrations of 100 μg per ml and 500 μg per ml respectively (Yang et al. 1996). These concentrations were not as effective as metronidazole, which was used as a standard and was active at 10 μg per ml. The active ingredients may prove to be more active if identified and purified before evaluating (Stenzel and Boreham 2001). Interestingly, emulsified oil of oregano has been used with good effect to treat 11 patients infected with *B. hominis* (Force et al. 2000). In eight cases *B. hominis* was eradicated and in the other three cases the parasite load declined.

Treatment is not indicated in either asymptomatic individuals or patients with mild or transient symptoms (Stenzel and Boreham 1996). In addition, there is evidence that some *B. hominis* infections may be self-limiting (Boreham and Stenzel 1993). As doubt still remains with regard to its pathogenic potential, *B. hominis* should be treated as a pathogen by exclusion. Consequently, treatment should only be considered when all other causes of gastrointestinal symptoms have been excluded.

CONCLUSION

Ninety years after its first description in humans, many questions remain unanswered regarding the pathogenicity of *B. hominis*. Despite growing anecdotal evidence that *B. hominis* may be pathogenic under certain conditions, scientific studies have largely failed to elucidate its pathogenesis. However, it is difficult to properly evaluate the pathogenic potential of *B. hominis* as elimination of all causes of gastrointestinal symptoms (infectious and non-infectious) in case studies is problematical. Diagnosis has been hampered by the wide variation in morphology, and this is often compounded by unsuitable methodologies and lack of awareness. Consequently, the epidemiology of *B. hominis* is unknown. Molecular techniques have revealed extensive genetic diversity, suggesting that some strains of *B. hominis* may be more pathogenic than others. Some animal isolates of *Blastocystis* have been indistinguishable from human isolates, raising the possibility of zoonotic transmission. Future molecular studies are needed to compare *B. hominis* isolates from differing clinical groups, including symptomatic and asymptomatic individuals.

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Nature and classification of parasitic helminths

DAVID I. GIBSON

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GENERAL INTRODUCTION

The parasitic worms or helminths, which occur in humans, comprise four major groups:

- two groups of flatworms (phylum Platyhelminthes), the trematodes or digeneans (flukes) and the cestodes (tapeworms)
- the nematodes (roundworms, phylum Nematoda)
- the acanthocephalans (thorny-headed worms; phylum Acanthocephala).

Other groups have been reported as parasites of humans, for example free-living flatworms (turbellarians), hairworms (gordiids, Phylum Nematomorpha) free-living nematodes (such as mermithids) and even earthworms (oligochaete annelids). Although these groups may be seen at the site of a toilet (in WCs, after defecation or urination on the ground, etc.) they are very unlikely to have passed through the human digestive system.

Nomenclature

The more important levels used in the classification of parasites can often be recognized by commonly used suffixes:

Phylum (e.g. Platyhelminthes) suffix varies
  Class (e.g. Trematoda) suffix commonly -a
    Subclass (e.g. Digenea) suffixes -ea, -a
      Order (e.g. Ascaridida) suffixes -ida, -idea, -iformes
        Superfamily (e.g. Diplostomoidea) suffix -oidea
        Family (e.g. Diplostomidae) suffix -idae
        Subfamily (e.g. Diplostominae) suffix -inae
          Genus (e.g. *Schistosoma*) suffix varies, if used
            Species (e.g. *mansoni*) suffix varies but depends on derivation and certain rules.

Levels above the family level vary because they are not covered by the International Code for Zoological Nomenclature. Vernacular versions are derived from some groups by the addition of -s or -es to the root, e.g. platyhelminths, diplostomids, diplostomines. The genus and species together, e.g. *Schistosoma mansoni*, form the scientific name of an organism. Any personal name and date following this name indicate the original authority for the name.

A direct life cycle is one in which the final host is reinfected directly without the involvement of intermediate hosts. An indirect life cycle is one in which intermediate hosts or paratenic hosts harbor one or more life-history stages. The final or definitive host harbors the adult (sexual adult in the case of the Digenea). Intermediate hosts are those in which one or more larval stages develop as a necessary part of the life cycle. A paratenic host is one which larval stages may survive but do not normally develop; they are often not a necessary part of the life cycle.

Identification and morphometric analysis

The identification of parasitic worms is possible in certain cases using a variety of techniques, such as:

- the identification of eggs found during coprological examination (e.g. Thienpont et al. 1986)
- the recognition of worm sections in histological investigations (e.g. Chitwood and Lichtenfels 1972)
- immunodiagnostic technique (e.g. Lightowlers and Gottstein 1995)
- enzyme electrophoresis (e.g. Wright and Ross 1980)
- molecular biological approaches (e.g. de Clercq et al. 1994).

However, in the majority of cases the most effective method is still morphometrics. Although this may well change in the future, helminth classification, whether it involves the intuitive approach of the specialist or the more rigorous protocols of cladistics (a method based on the use of derived characters as indicators of evolutionary events), still relies mainly on morphological features.

FIXATION

Morphometric analysis relies on having material in good condition; in order to achieve this, it is best if specimens are fixed live. In many cases, for example when worms are voided after the administration of anthelmintics, this is not possible, and any conventional fixative, such as 10 percent formalin or 70–80 percent alcohol, may be used. Live material is best fixed in Berland's fluid (19 parts glacial acetic acid to 1 part pure formalin) or (especially in the case of cestodes) a very hot conventional fixative or boiling water. After fixation for a minute or so, the specimens are best stored in 80 percent alcohol. Specimens should not be fixed under the pressure (e.g. a coverslip, glass slide or other device) except in the case of large tapeworms and acanthocephlans; some pressure or prior soaking in tap water may help evert the proboscis of live acanthocephalans.

MOUNTING

Nematodes and acanthocephalans are best examined as temporary mounts between a glass slide and a coverslip in a clearing agent, such as beechwood creosote, lactophenol or glycerine; the latter is really only useful for smaller worms (shorter than 15 mm in length). Trematodes and cestodes (or fragments of the latter, usually the head and mature segments) need to be mounted on slides permanently. This requires staining of the worms in a good carmine-based stain such as Mayer's paracarmine for 1–20 min (depending on size), destaining in acid alcohol until the worm is a pale pink color, dehydrating, and clearing and mounting in Canada balsam or some other mountant.

HISTOLOGY

In some cases, serial histological sections of specimens are required for identification. In this procedure, orientation is usually critical, so it must be done manually. The technique is given in detail by Cooper (1988).

SPECIALIST HELP

Help may be obtained from specialist laboratories, which are usually in museums or similar institutes and where there are helminth taxonomists. Contact should be made before sending material. Any material sent should be very well packed: well dried in the case of slidemounts, and in strong plastic (not glass) containers in the case of wet material.

The helminth groups

An account of the helminth groups that might occur as parasites in humans is given below. The higher taxa dealt with tend to be those generally considered important in the systematics of the group, and most information is given at the family level. The account is presented in the form of a summary of the gross morphological features by which a group is recognized, detailed information on many organs and organ systems is omitted. This is followed by a section listing the genera involved and some idea of the basic recognition features of the group; information on the life history, how humans become infected and any control measures relating to the life history; and information on the pathogenicity of the worm which might relate to its morphology, mode of existence or life history. The information was gathered from a variety of sources, but Muller (2001), Coombs and Crompton (1991), and Garcia and Bruckner (1993) were useful references for nonsystematic data.

PHYLUM PLATYHELMINTHES

The platyhelminths or flatworms are bilaterally symmetrical, dorsoventrally flattened worms with a definite head end and lacking a body cavity. They include a variety of free-living turbellarians, which occur in aquatic and terrestrial conditions (a small number are parasitic), and a number of entirely parasitic groups. There are three classes:

1. the Monogenea (mainly ectoparasites of fishes)
2. the Cestoidea (tapeworms; endoparasites)
3. the Trematoda (endoparasitic flukes; mainly digeneans).

Only the latter two classes infect humans.

Class Cestoidea Rudolphi, 1808

Main features Platyhelminthes; primarily intestinal parasites of vertebrates; usually single generation in life cycle

and sexual adult in vertebrate, rarely with asexual reproduction in intermediate host; life cycle indirect (one exception), wide variety of invertebrates and vertebrates used as intermediate hosts; adult segmented or not with duplication of reproductive organs along body (polyzoic) or not (monozoic); segmented forms polyzoic, unsegmented forms monozoic or polyzoic; distinct scolex (head) present or absent; syncytial tegument usually but not always unarmed (at light microscope level); gut absent (Figure 1).

The cestodes, or tapeworms, occur as intestinal parasites of all groups of vertebrates. Their closest relatives are the monogeneans, ectoparasitic flukes mainly of fishes, from which they are likely to have been derived. There are two subclasses, the Cestodaria, which are monozoic forms lacking a scolex and parasitic in fishes and turtles, and the Eucestoda, the majority of which are segmented and polyzoic. Only the latter subclass parasitizes humans.

The higher classification of the cestodes is controversial and complex (see, for example, Brooks 1989).

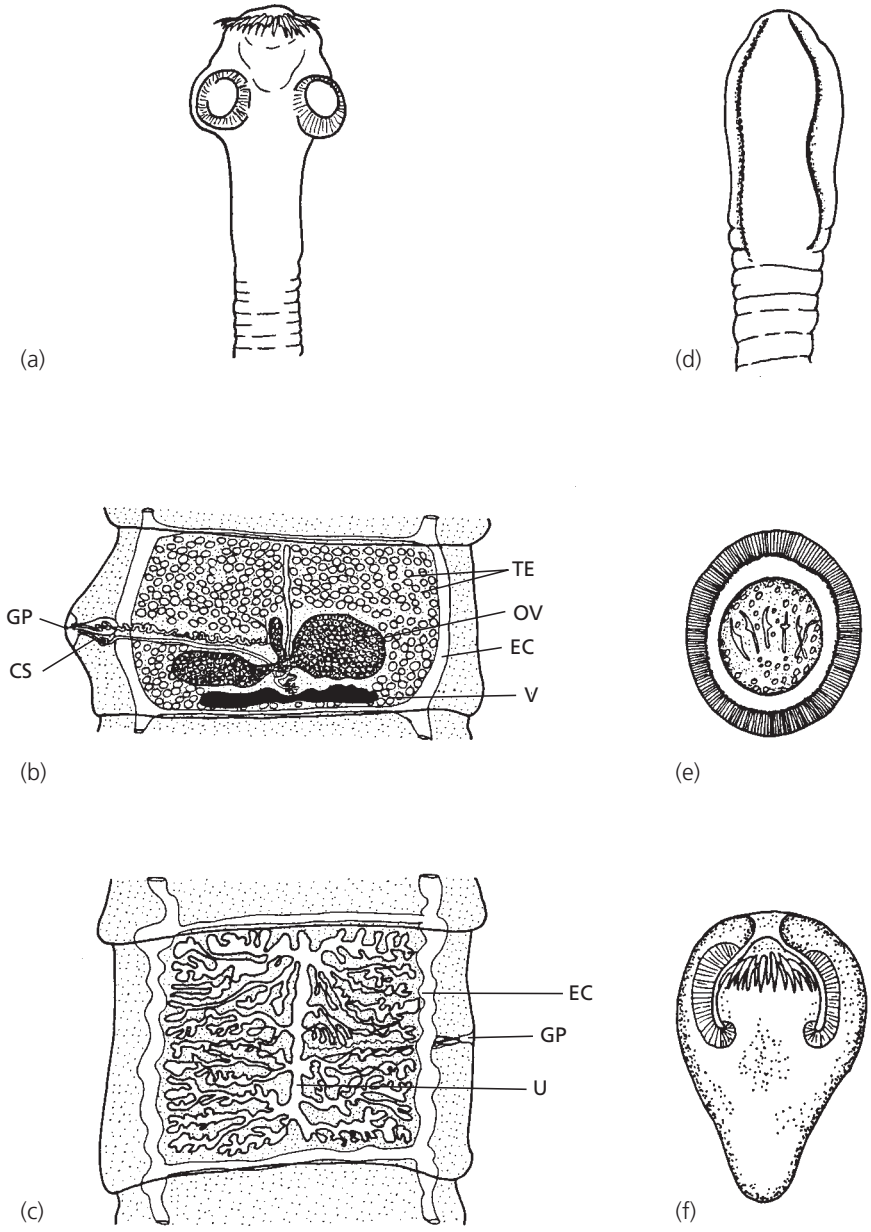

Figure 1 *Cestodes:* **(a)** *scolex (head) of* Taenia *(Taeniidae) with armed rostellum and four suckers;* **(b)** *mature segment of* Taenia*;* **(c)** *gravid segment of* Taenia*;* **(d)** *scolex of* Diphyllobothrium *(Diphyllobothriidae);* **(e)** *egg of* Taenia *containing hexacanth larva;* **(f)** *taeniid cysticercus larva (these can occur singly or, in certain genera, in large numbers within a cyst following asexual multiplication). Cirrus sac (CS), excretory canal (EC), genital pore (GP), ovary (OV), testes (TE), uterus (U), vitellarium (V). Adapted from (a–d) Khalil et al. 1994;* **(e)** *Abuladze 1964;* **(f)** *Fuhrmann 1931.*

The classification of cestodes found in humans used here at ordinal and subordinal taxonomic levels follows that of Khalil et al. (1994):

Order Pseudophyllidea
  Family Diphyllobothriidae
Order Cyclophyllidea
  Family Anoplocephalidae
  Family Davaineidae
  Family Dipylidiidae
  Family Hymenolepididae
  Family Mesocestoididae
  Family Taeniidae.

Subclass Eucestoda Southwell, 1930

Main features Cestoidea; usually segmented, usually polyzoic with one or more sets of reproductive organs per segment; distinct scolex normally present; invariably parasitic in intestine of vertebrates.

The tapeworms of this subclass that infect humans are all segmented as adults (Figure 1) with one or more copies of the reproductive system in each segment (proglottid) along the body (strobila). There are two orders involved:

- the Pseudophyllidea, recognizable by the fact that the scolex lacks suckers and hooks, the attachment organ being a pair of bothria (dorsal and ventral longitudinal grooves on the scolex)
- the Cyclophyllidea, where the scolex is armed with a ring of four suckers and sometimes one or more rings of apical hooks.

In some cases, humans can become infected with the larval stages of tapeworms; these normally reside in the tissues (see below, sections on Diphyllobothriidae and Taeniidae).

Cestodes generally have a life cycle involving one or two intermediate hosts. Only one species (*Hymenolepis*) is capable of autoinfection without the use of intermediate hosts. Since adult cestodes are intestinal parasites of vertebrates, the eggs or gravid segments containing eggs pass out with the feces. In the cyclophyllideans, the eggs must be eaten by a suitable intermediate host, which may be a terrestrial invertebrate (commonly an arthropod) or a vertebrate, in order to hatch and release a hexacanth (six-hooked) larva, called an onchosphere. In the case of the pseudophyllideans, the eggs hatch in water to release a ciliated, motile hexacanth, called a coracidium, which is eaten by an aquatic arthropod, such as a copepod. Once in the intermediate host, the hexacanth usually penetrates the gut wall and develops in the body cavity into a procercoid. This develops further, either in the same host or in a second intermediate host if the first host is eaten, into a resting, normally encysted, stage that takes on a variety of names depending on its form (e.g. cysticercus, cysticercoid, plerocercoid). The final host acquires the parasite when it feeds on the host harboring the encysted stage. In a few instances (see below, Taeniidae) some asexual multiplication of larval heads (protoscoleces) can occur when vertebrates act as an intermediate host.

The form of the attachment organ on the scolex differs markedly in different forms and can be readily used to distinguish the various orders. The form and armament of the scolex distinguishes genera, and the number and morphometrics of the hooks that make up the armature are useful at the specific level. Other important characters relate to the shape of the segments and the arrangement and form of the reproductive system(s) within the segments, e.g. the position of the genital pore, the nature of the vitellarium, the size of the cirrus sac, the shape of the ovary, and the nature of the uterus.

Since adult tapeworms are intestinal parasites that absorb nutrients though their tegument rather than browse, except perhaps at the point of attachment, the worms tend to do little physical damage to their host, apart from the possibility of bowel obstruction in the case of large infections. Their presence in humans, when apparent, is often accompanied by diarrhea and loss of appetite. In some cases, the absorption of certain nutrients is a factor (see below, *Diphyllobothrium* for selective absorption), especially in the malnourished. The presence of encysted larval tapeworms in the tissues is much more serious, notably in certain organs, such as the brain, liver, and lungs, since the cysts can, in some cases, reach a large size. Control is dependent on the life cycle of the worm, but it in most cases it can be effected by the cooking or freezing of meat (especially that of game, fish or other wild animals) and insects intended for ingestion, or the removal of insects from salad and other plant material to be eaten.

ORDER PSEUDOPHYLLIDEA CARUS, 1863

Main features Eucestoda; without suckers or hooks on scolex but normally with pair of longitudinal grooves (bothria) which aid attachment; polyzoic (segmented); first larval stage in crustaceans, second usually in fish; adults in all vertebrate groups, especially fish.

Family Diphyllobothriidae Lühe, 1910

Main features Pseudophyllidea; strobila medium to large; scolex occasionally poorly developed; bothria usually well developed; set of reproductive organs in each segment usually single, occasionally double or multiple; second larval stage (plerocercoid) usually in fish, occasionally in reptiles and mammals; adults in reptiles, birds, and mammals.

Diphyllobothriids are usually parasitic in piscivorous higher vertebrates. One genus, *Diphyllobothrium* Cobbold, 1858, regularly occurs as an adult in humans, *Diplogonoporus* Lönnberg, 1892 occurs occasionally,

and other genera on rare occasions. These may be huge worms, reaching many meters in length. Diphyllobothriids in humans are recognizable by the absence of suckers and hooks on the scolex (head). One genus, *Spirometra* Faust, Campbell and Kellog, 1929, occurs in humans as a plerocercoid larva (called a sparganum).

Adult diphyllobothriids live in the intestine of their final host, producing huge numbers of eggs that pass out with the feces and hatch in water to release a ciliated larva (coracidium). If the coracidium is eaten by a suitable copepod, it develops in the hemocoel as a procercoid larva. If the infected copepod is eaten by a suitable vertebrate host, usually a fish, the larva penetrates the tissues and develops (sometimes encysting) in the body cavity, muscles or other tissues as a plerocercoid larva. If this host is then eaten by a suitable higher vertebrate, the plerocercoid develops to an adult in the intestine. Humans become infected with adult diphyllobothriids by feeding on raw, lightly marinated or inadequately cooked freshwater or, less frequently, marine fishes. Since these parasites are more usually parasitic in other mammals, little can be done to control the presence of larvae in fishes, but the freezing or cooking of fish and fish products kills the worms. Larval forms (*Spirometra*) are acquired by humans through drinking water containing infected copepods, by eating raw or poorly cooked amphibians, reptiles or mammals and, apparently, by using the flesh of reptiles or other species as a poultice for wounds, etc. Control is effected by filtering water and avoiding consumption of or contact with raw flesh that might harbor the plerocercoid larva.

The adult worms are large and absorb nutrients from the gut, notably vitamin B_{12}, absorption of which results in pernicious anemia. Large infections can also result in obstruction of the bowel. Infections with plerocercoids (spargana) are more of a problem, as these larvae wander through the tissues before becoming encysted in a fibrous nodule reaching about 2 cm in diameter. These can be painful in subcutaneous regions, but can be much more serious if they end up in the eye, lymphatic system, brain, etc. (see Intestinal tapeworms).

ORDER CYCLOPHYLLIDEA VAN BENEDEN IN BRAUN, 1900

Main features Eucestoda; scolex normally with four suckers; rostellum (apical protrusion on scolex) usually present but sometimes absent, armed with hooks or not; polyzoic (segmented); parasitic as adults in all vertebrate groups except fish; normally in intestine.

Family Anoplocephalidae Cholodkowsky, 1902

Main features Cyclophyllidea; small to large worms; scolex without rostellum; suckers unarmed; segments craspedote (posterior border overlaps anterior border of next segment) or not; single or double set of reproductive organs per segment; in mammals, birds, and reptiles.

Several genera have been recorded as occasional or accidental parasites of humans, including *Bertiella* Stiles and Hassall, 1902 (species normally occurring in primates), *Inermicapsifer* Janicki, 1910 and *Mathevotaenia* Akhumyan, 1946 (species normally occurring in rodents) and *Moniezia* Blanchard, 1891 (species normally parasitic in domesticated ruminants). A general, but not infallible, recognition feature of anoplocephalids is the absence of both hooks and a rostellum (muscular apical organ) on the scolex.

The eggs of anoplocephalids leave with the feces either freely or in the form of gravid segments. If eaten by a suitable terrestrial arthropod, such as a soil mite, the egg hatches and the embryo (onchosphere) develops into a cysticercoid larva in the hemocoel. Herbivorous vertebrates become infected when they feed on infected arthropods or accidentally ingest them with vegetation. Humans acquire infections by accidentally ingesting mites with raw vegetation (*Bertiella* and *Moniezia*) or by eating larger insects, e.g. beetles, either as food or as medical remedies (*Mathevotaenia* and probably *Inermicapsifer*). Control measures include the careful washing of salad and other items of vegetation intended to be eaten raw and the avoidance or cooking or freezing of insects intended to be eaten.

Little is known regarding the pathogenicity of anoplocephalid infections in humans (diarrhea has been reported), but it is likely that these unarmed worms cause little harm.

Family Davaineidae Braun, 1900

Main features Cyclophyllidea; body small to large; rostellum usually present, rarely rudimentary, armed with crown of hooks usually but not always in two rows; crown of hooks round, oval or undulating, interrupted or not; hooks characteristically numerous, small, hammer-shaped; suckers normally present, armed with small spines or not; segments normally numerous; one or two sets of reproductive organs per segment; adults in birds and mammals.

Several species of *Raillietina* Furhmann, 1920, normally parasites of rodents, have been recorded as occasional parasites of humans. Davaineids are usually recognizable by the presence of a rostellum on the scolex armed with two rings of many minute, hammer-shaped hooks.

The adult worms release gravid segments which pass out in the feces. Eggs eaten by suitable terrestrial insects develop in the hemocoel into a cysticercoid larva. The vertebrate host picks up the parasite by feeding on the infected intermediate host. Humans are thought to acquire *Raillietina* by ingesting insects, such as ants, beetles or cockroaches, either accidentally with food or as part of the diet. Control can be effected by the

careful washing of raw and cold food, keeping cold food in a meat safe or refrigerator, and cooking or freezing of insects intended for ingestion.

Family Dipylidiidae Stiles, 1896

Main features Cyclophyllidea; body small to medium-sized; scolex with protrusible rostellum armed with several rows of hooks; hooks usually rose-thorn-shaped; rostellar sac absent; suckers unarmed; segments numerous, mature and gravid segments longer than wide; two sets of reproductive organs per segment; larval stages in insects, amphibians or reptiles; adults in carnivorous mammals.

One genus, *Dipylidium* Leuckart, 1863, occurs in humans. Dipylidiids are recognizable by the absence of a rostellar sac and the presence of two sets of reproductive organs in each segment.

Dipylidiid tapeworms normally occur in the intestine of carnivorous mammals. The gravid segments break off and leave with the feces, releasing large numbers of eggs. This group of tapeworms appears to have two life-cycle strategies. It seems likely that all use insects as intermediate hosts in which the larval cysticercoid stage develops. In some cases, the mammalian host becomes infected when these insects are ingested; in other cases, insectivorous amphibians or reptiles act as second intermediate or paratenic hosts. In the case of *Dipylidium*, dogs, cats, humans (usually children), etc. become infected by feeding on fleas harboring the cysticercoid larvae. Control relates to improvements in hygiene and the regular de-worming and de-fleaing of pets.

Although the rostellum of these worms is armed with hooks, they appear not to be very pathogenic, causing only slight indigestion and loss of appetite. Infection may only become apparent when segments are observed in stools.

Family Hymenolepididae Perrier, 1897

Main features Cyclophyllidea; rostellum usually present but occasionally lacking or rudimentary, unarmed or armed with single, rarely double, crown of hooks (hooks may be very small); suckers armed or not; segments broader than long, craspedote; single (rarely double) set of reproductive organs in each segment; normally one to three (usually three) testes per segment; adults in birds and mammals.

Two genera, *Hymenolepis* Weinland, 1858 and *Rodentolepis* Spasskii, 1954, occur relatively commonly in humans; both normally parasitize rodents. Although previously considered as *Hymenolepis*, species of *Rodentolepis* differ from *Hymenolepis* in that the rostellum is armed with a crown of hooks. Another genus, *Drepanidotaenia* Railliet, 1892, a parasite of anseriform birds, has also been reported in humans. Hymenolepidids are relatively small tapeworms generally recognizable by the fact that each segment has only three testes.

Hymenolepidids are intestinal parasites. Their eggs leave with the feces and are ingested by arthropods, within which develops a cysticercoid larva. The vertebrate host normally becomes infected by swallowing arthropods harboring this stage. In terrestrial forms (e.g. *Hymenolepis* and *Rodentolepis*) the usual intermediate hosts are beetles and fleas; whereas in aquatic forms (e.g. *Drepanidotaenia*) they are copepods. One species of *Rodentolepis* (*R. nana*), which occurs in humans, is unique for a tapeworm in that direct infection is possible, either by reinfection (autoinfection) of the intestinal mucosa by eggs in the gut or by swallowing eggs that have passed in the feces. Humans also become infected with hymenolepidids by accidentally eating beetles and fleas and, in the rare cases of *Depanidotaenia*, by drinking water containing infected copepods. Control is effected by rodent control, personal hygiene, care when handling laboratory animals, especially where these parasites are used as laboratory models, de-fleaing laboratory rodents, and filtering drinking water.

These worms cause few symptoms, although heavy infections of *R. nana* caused by autoinfection may result in enteritis. This is thought to be caused more by the waste-products of these worms than damage at the site of attachment resulting from the armed scolex (see Intestinal tapeworms.)

Family Mesocestoididae Lühe, 1894

Main features Cyclophyllidea; body narrow; with numerous segments; rostellum absent; mature segments wider than long, gravid segments may be longer than wide; single set of reproductive organs in each segment with median genital pore; larval stage (tetrathyridium) in amphibians, reptiles, birds, and mammals; adults in mammals, rarely birds.

One genus, *Mesocestoides* Lühe, 1894, has been reported from humans, the species concerned being natural parasites of carnivorous mammals, such as foxes, racoons, skunks, etc. Mesocestoidids are recognizable by the absence of a rostellum on the scolex and the median position of the genital pore on the segments.

Eggs pass out with the feces in gravid segments. If these are eaten by a suitable arthropod, probably an insect, the onchosphere develops in the hemocoel as a procercoid larva. If the arthropod is eaten by a vertebrate such as an amphibian, reptile, bird or mammal, the worm develops in the tissue as a type of plerocercoid referred to as a tetrathyridium. The final host acquires the parasite by feeding on the vertebrate intermediate host. Infections in humans appear to have been caused by people feeding on poorly cooked game, raw birds and, especially, raw snake livers. Control is by the proper cooking or freezing of meat of all sorts obtained from the wild.

These unarmed worms appear not to be very pathogenic, but diarrhea, poor appetite, and some anemia has been reported in relation to human infections.

Family Taeniidae Ludwig, 1886

Main features Cyclophyllidea; strobila ribbon-like, usually with many segments; rostellum usually well developed and usually bears two rows of hooks; anterior row of hooks usually larger and alternating with second row; single set of reproductive organs in each segment; larval stage either cysticercus or large cystic structure producing multiple protoscoleces asexually within brood capsules; larval stage and adult in mammals.

Taeniid tapeworms may occur in humans in both larval and adult form. Two genera frequently parasitize humans, *Taenia* Linnaeus, 1758 (now includes *Multiceps* Goeze, 1782 and *Taeniarhynchus* Weinland, 1858) and *Echinococcus* Rudolphi, 1801, the latter only in its larval form.

Taeniids normally occur as adults in the intestine of carnivorous mammals, such as canids and felids. Eggs or segments containing eggs leave with the feces. If the eggs fall on grass or other vegetation and are eaten by herbivorous mammals, the egg hatches in the gut and releases a larva (the onchosphere), which penetrates the gut wall and enters the blood system. These larvae are transported via the circulation until they reach the body muscles, where they encyst and develop as cysticercus larve which have invaginated scoleces (protoscoleces). Carnivores become infected with the adult parasite when they feed on herbivorous mammals. Similarly, humans become infected by feeding on raw or poorly cooked meat, especially pork and beef. Humans can also harbor the larval stage (cysticercus), when the eggs passed by infected humans or carnivores are accidentally ingested. In some species this larva (a coenurus) produces within its body many small protoscoleces (heads), each one of which can develop into an adult worm if eaten by a suitable host; whereas the hydatid cyst of *Echinococcus* contains huge number of 'brood capsules' within each of which develop numerous protoscoleces. In the latter case, the cysts may be huge, reaching 30 cm in diameter. A consequence of this asexual reproduction is that the adult worms are much smaller, have fewer segments, and produce fewer eggs than more conventional taeniids. Control includes the proper cooking or freezing of meat, regular de-worming of dogs especially farm dogs, not feeding raw offal and other parts of slaughtered animals to dogs, keeping dog (and human) feces away from farm animals and vegetable crops, and the proper washing of raw vegetation intended as food.

Adult worms in the intestine cause few symptoms, although diarrhea, bowel discomfort, weight loss, irritation at the site of attachment in the case of species armed with hooks, and bowel obstruction have been reported. The presence of segments in the stools or crawling out of the anus may be the only evidence of infection. The presence of larval stages (cysticercosis, hydatid disease, coenurosis) in the tissues is another matter as, depending on size and location, the cysts can be very dangerous. These larvae may live for many years and may have to be removed surgically. Hydatid cysts can occur in many parts of the body; the commonest site is the liver and another frequent site is the lungs. Symptoms are similar to those of a slow-growing tumor. The cysticerci and coenurus larvae of *Taenia* spp. (including *Multiceps*) frequently occur in the brain in humans, sometimes in the eye or under the skin. Symptoms of brain infections are similar to those caused by any space-filling lesion, including epileptic seizures, paraplegia, etc. (see Intestinal tapeworms and Larval cestodes).

Class Trematoda Rudolphi, 1808

Main features Platyhelminthes; primarily permanent parasites of tissues of mollusks and have single or multiple generations in life history, life cycle may or may not involve other additional hosts; mollusk normally harbors asexual generations or single sexual generation; additional host usually a vertebrate, harbors adult sexual stage, especially in gut and associated organs; sexual adult usually with two organs of attachment organs (normally suckers or sucker-like structures), one generally anterior and one ventral or posterior; syncytial tegument armed with spines or smooth; gut or its vestige always present.

The trematodes or 'flukes' are flatworms that originally evolved as parasites of mollusks; virtually all species retain a molluskan element in the their life history. There are two subgroups, the Digenea and the Aspidogastrea, but only the former occurs in humans and other mammals.

There is no modern accepted classification of the Trematoda or the Digenea. Brooks et al. (1985, 1989) presented a version based on a cladistic analysis, but this has been heavily criticized by, among others, Pearson (1992). For keys to genera, see Gibson et al. (2002) and subsequent volumes. The classification below for forms occurring in humans follows the general outline indicated by Gibson and Bray (1994):

Order Strigeida
- Superfamily Diplostomoidea
  - Family Diplostomidae
- Superfamily Gymnophalloidea
- Family Gymnophallidae
- Superfamily Schistosomatoidea
  - Family Schistosomatidae

Order Echinostomida
- Superfamily Echinostomatoidea
  - Family Echinostomatidae
  - Family Fasciolidae

Superfamily Paramphistomatoidea
Family Zygocotylidae
Order Plagiorchiida
Superfamily Dicrocoelioidea
Family Dicrocoeliidea
Superfamily Opsithorchioidea
Family Heterophyidae
Family Opsithorchiidae
Superfamily Plagiorchioidea
Family Lecithodendriidae
Family Paragonimidae
Family Plagiorchiidae
Family Troglotrematidae.

Subclass Digenea Carus, 1863

Main features Trematoda; alternation of generations, normally two (sometimes more) asexual generations in mollusk and single sexual generation in vertebrate (occasionally invertebrate); normally hermaphroditic, occasionally partly or entirely dioecious; generally small elongate-oval to tubular worms, but various other forms, including round and filamentous, occur; body smooth or armed with spines, usually with two muscular suckers, sometimes one, occasionally none; form of testes variable, one to many, often two; muscular sac (cirrus sac) often envelopes all or part of male terminal genitalia; genital atrium (chamber into which male and female ducts open, opens via genital pore) present or absent; common genital pore normally present, position variable, usually ventral on anterior body, occasionally terminal; ovary normally single, occasionally multiple, position variable; uterus variable in size, form and distribution; eggs normally oval and operculate, normally tanned, occasionally with spine or filament(s); vitellarium (yolk gland) variable in shape and distribution, exhibiting all forms between follicular and single compact mass; parasitic as sexual adult in all vertebrate groups, occasionally in invertebrates, usually present in gut or other body cavities, occasionally in blood or other tissues, rarely ectoparasitic.

Those trematodes (i.e. digeneans) that occur in humans are readily recognized by the fact that they possess two suckers, which act as attachment organs; they have only a single set of reproductive organs and they have a functional gut (Figure 2). They are endoparasites occurring in humans mainly in the gut, liver, bile ducts, lungs or blood system.

Digeneans have a unique life cycle that usually involves three generations: two asexual generations in a mollusk and a sexual generation in a vertebrate. The eggs leave the vertebrate, normally with the feces, and hatch to release a ciliated larva, the miracidium, which penetrates a particular molluskan host; sometimes the egg is eaten by this host prior to release of the miracidium. Within the mollusk, the miracidium develops into a sac-like parthenogenetic adult, the mother sporocyst. Within this stage develop daughter stages called daughter sporocysts or, if a sac-like gut is present, rediae. Within the second parthenogenetic generation develop short-lived, usually tailed, larvae, the cercariae, often in huge numbers (Figure 2). These larvae usually escape from the mollusk and swim or crawl to the next host. Some cercariae penetrate the final vertebrate host directly, but the majority penetrate or settle on its food as a long-lived resting stage called the metacercaria. Metacercariae are normally encysted within invertebrates or other vertebrates, which act as intermediate hosts, or on vegetation. The final vertebrate hosts become infected by feeding on prey or vegetation harboring metacercariae. Digeneans tend to be very specific in relation to their molluskan host, relatively unspecific for intermediate hosts and exhibit various levels of specificity for their final vertebrate host.

Some digeneans differ considerably in shape and morphology, but the majority are similar in overall appearance. Features used to distinguish groups include the presence or absence of spines on the tegument, the position and number of suckers, the form and arrangement of the reproductive organs, and the shape of the gut and excretory vesicle. At the specific level, details such as sucker ratio and egg size are important.

Although, like tapeworms, digeneans can take in nutrients through their body surface, they also tend to feed by browsing. In the gut, this is usually not a great problem especially in light infestations, but in other sites the damage, irritation, and toxins produced can result in inflammation, tissue reactions, fibrosis, obstruction, etc. The body spines of some worms, which help them maintain position, may also cause irritation. In view of the nature of the life cycle, obvious targets for the control of digenean infections are the molluskan host and the development of sewage systems and standards of hygiene that prevent contact between worm eggs and mollusks. Similarly, transmission to humans can be pre-empted by avoidance of contact between bare skin and water containing cercariae, where these penetrate the skin directly, or by changes in food preparation and eating habits.

SUPERFAMILY DIPLOSTOMOIDEA POIRIER, 1886

Family Diplostomidae Poirier, 1886

Main features Diplostomoidea; body usually divided into two regions; anterior region wide, foliate with ventral depression; hindbody cylindrical; oral and ventral suckers well developed, sometimes small; ventral suckers in base of ventral concavity; pseudosuckers often present lateral to oral sucker; cirrus sac absent; metacercaria in fishes or amphibians; adults in intestine of birds and mammals.

One species of the genus, *Neodiplostomum* Railliet, 1919, which is more commonly a parasite of rats, has been recorded in the intestine of humans in Korea on several occasions. Diplostomids are recognizable by the bipartite nature of the body and wide, ventrally concave forebody.

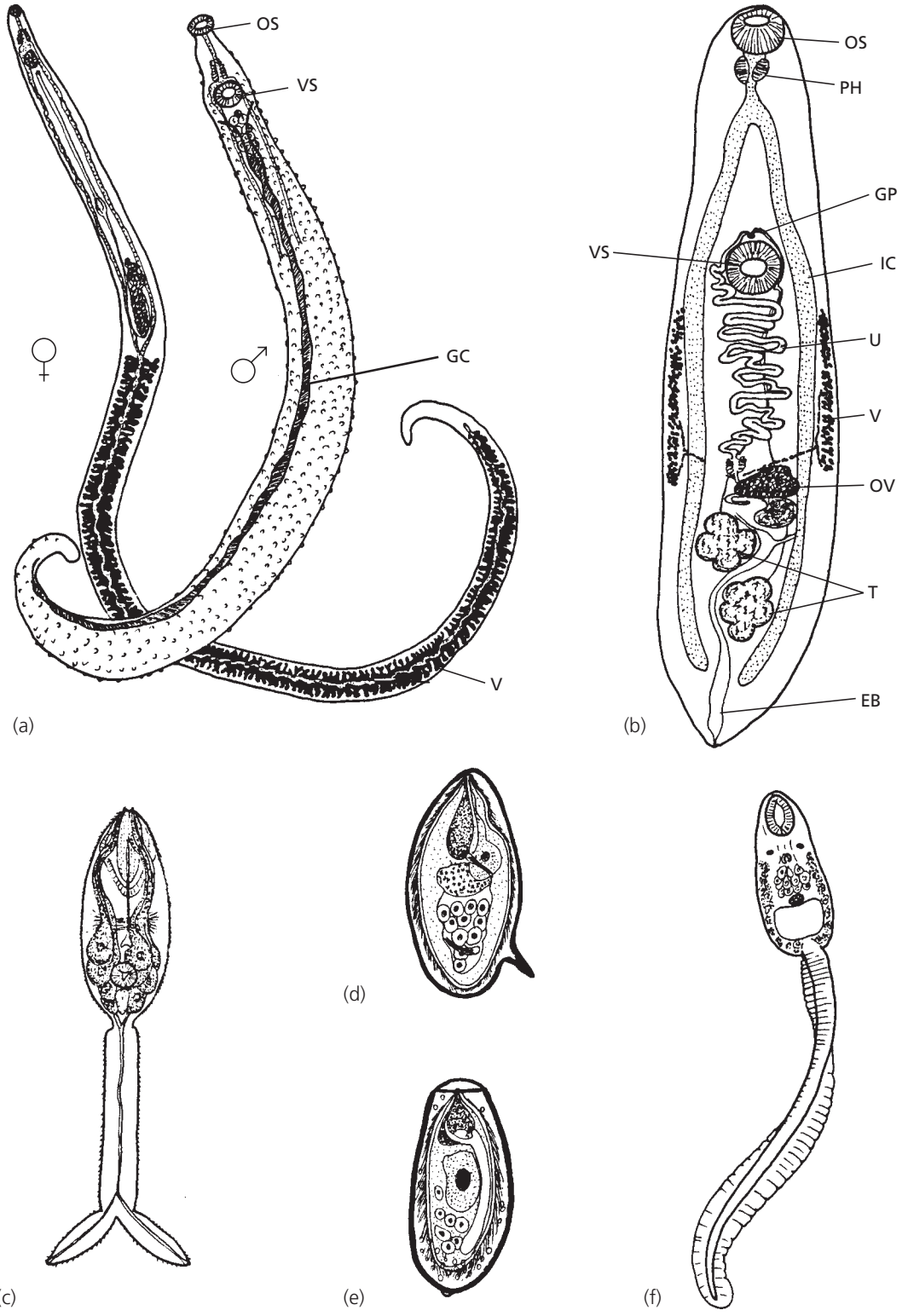

Figure 2 *Trematodes:* **(a)** *male and female* Schistosoma *(Schistosomatidae) (the presence of separate sexes is very unusual for a flatworm);* **(b)** Opisthorchis *(Opisthorchiidae) with more conventional trematode morphology;* **(c)** *cercaria (larva) of* Schistosoma *with bifid tail;* **(d)** *egg of* Schistosoma *containing miracidium (ciliated larva);* **(e)** *egg of* Opisthorchis *with lid (operculum) through which miracidium escapes;* **(f)** *cercaria of* Opisthorchis*. Excretory bladder (EB), gynecophoric canal within which female lies (GC), genital pore (GP), intestinal cecum (IC), oral sucker (OS), ovary (OV), pharynx (PH), testes (T), uterus (U), vitellarium (V), ventral sucker (VS). Adapted from* **(a)** *Schell (1985); (b, e, f) Vogel (1934); (c, d) Cort (1919)*

Eggs of diplostomids leave with the feces. If they enter water, the miracidium is released; after penetrating an aquatic snail, it transforms into a mother sporocyst. The mother sporocyst produces daughter sporocysts that release fork-tailed cercariae. The latter leave the snail and encyst as metacercariae, usually in fishes but in the case of *Neodiplostomum*, in frogs. Snakes, which acquire the parasites by feeding on frogs, act as paratenic hosts. Humans may become infected by feeding on raw or poorly cooked frogs or snakes. Control can be effected by the proper cooking of amphibians or reptiles used as food. Little is known about the pathogenicity of these worms in humans.

SUPERFAMILY GYMNOPHALLOIDEA ODHNER, 1905

Family Gymnophallidae Odhner, 1905

Main features Gymnophalloidea; body small, oval to pear-shaped, flattened; tegument armed with small spines; oral sucker large, often very large, may support pair of lateral papillae; ventral sucker small, in anterior or posterior half of body (posterior for *Gymnophalloides*); cirrus sac absent; in intestine, bursa of Fabricius or gallbladder of birds (mainly) and mammals.

Gymnophallids are usually parasites of birds. Only one genus, *Gymnophalloides* Fujita, 1925, is parasitic in humans; this is a very small worm that can occur in the intestine, or possibly the biliary system, in huge numbers in Korea. An obvious recognition feature is that the small ventral sucker occurs in the posterior quarter of the body. It is readily distinguished from paramphistomoids by its smaller size, spiny tegument, and smaller, nonterminal ventral sucker.

The full life cycle is not known, but the intermediate host is presumed to be a marine oyster or clam. The asexual generations of gymnophallids usually occur in bivalves; but there is no free-living cercarial stage, as the metacercariae encyst directly inside the daughter sporocyst. Consequently, the final host acquires the parasite by feeding on bivalve mollusks. Control can, therefore, be implemented by the avoidance of feeding on raw bivalves or at least those which have not been treated by freezing to kill the parasite.

Little is known about this parasite, but it appears to cause gastric discomfort, indigestion, and diarrhea, and possibly, pancreatitis or cholecystitis. Presumably, when they are present in large numbers, the spined tegument of these worms can cause local irritation to the tissues.

SUPERFAMILY SCHISTOSOMATOIDEA STILES AND HASSALL, 1898

Family Schistosomatidae Stiles and Hassall, 1898

Main features Schistosomatoidea; dioecious; elongate worms, females narrower and longer than males; female clasped in gynecophoric canal (longitudinal groove) of male; oral and ventral suckers normally present; eggs nonoperculate with or without spine; parasitic in blood system of birds and mammals.

Only one genus of schistosome, i.e. *Schistosoma* Weinland, 1858, occurs as an adult in humans. The fact that the sexes are separate and their presence in the blood system makes the group readily recognizable (Figure 2).

Eggs leave the body with the feces or urine. The miracidium hatches from the egg, penetrates an aquatic snail, and transforms into a mother sporocyst. This gives rise to daughter sporocysts, which produce cercariae. The life cycle in this group is different from the majority of digeneans in that there is no metacercarial stage and no intermediate host between the molluskan and vertebrate hosts: the fork-tailed cercariae leave the molluskan host, swim and penetrate the skin of the final host, and enter the blood system. The mode of transmission means that avoidance of contact between the skin and water containing cercariae is important in preventing infection, as are control of the relevant species of mollusks, general sanitation, and hygiene.

Since schistosomes occur in the blood system, there is no direct route for the voiding of eggs, which become lodged in small blood vessels in various parts of the body, including the liver and lungs, causing blockages, local granulomata, and abscesses that form around the eggs and aid their passage into the lumen of the gut or bladder. These worms are, therefore, very pathogenic and cause a variety of symptoms. In addition, the fact that the cercariae penetrate the skin means that local immune responses are elicited, with a resulting skin reaction known as cercarial dermatitis: this reaction is caused not only by species of *Schistosoma* of humans and other mammals but also by avian schistosomes of the genera *Trichobilharzia* Skrjabin and Zakharov, 1920 and *Ornithobilharzia* Odhner, 1912 where the reaction is often more pronounced (see Schistosomes: general, African, and Asian).

SUPERFAMILY ECHINOSTOMATOIDEA LOOSS, 1899

Family Echinostomatidae Looss, 1899

Main features Echinostomatoidea; body oval to elongate; tegument armed with small spines; head collar present, usually armed with single or double crown of spines; suckers well developed; encysted as metacercariae in or on mollusks, annelids, fishes, tadpoles, etc.; eggs relatively large; adults mainly in intestine, occasionally other organs, of birds, mammals, and occasionally reptiles.

Several echinostomatid genera, the most common of which is *Echinostoma* Rudolphi, 1809, occur in humans especially in parts of southeast Asia. Other genera include *Echinochasmus* Dietz, 1909, *Echinoparyphium*

Dietz, 1909 and *Hypoderaeum* Dietz, 1909; these are primarily parasites of birds and mammals and infect humans only accidentally. Echinostomatids, which occur in the intestine, are readily recognized by the single or double crown of large circumoral spines; but worms must be in good condition when they are fixed, since the spines are readily lost in frozen or poorly preserved material.

The life cycle involves two intermediate hosts, both of which may be snails. The eggs pass out from the vertebrate host with the feces and hatch to release the ciliated miracidium. This penetrates a snail and develops into a mother sporocyst. Within this develops a brood of rediae. When these are released into the mollusk's tissues, they produce cercariae that leave the mollusk and then encyst in or on the same snail or neighboring snails. Therefore, the metacercarial stage of echinostomatids tends to occur in or on snails but some species also encyst in or on annelids, fish, and tadpoles. Humans acquire this parasite by feeding on uncooked snails or, more likely, on poorly washed salad or other raw vegetation harboring the snails. Control is, therefore, possible by avoiding the ingestion of snails, by the careful washing or cooking of vegetation intended as food, and by limiting the use of human feces as a fertilizer. *Echinostoma* is not specific to humans and occurs in other mammals; rats and dogs are especially noted for carrying infections.

These worms attach to the wall of the intestine and cause local inflammation, presumably due in part to the large oral spines and general body spination. The oral spines are used by the worm for abrading the gut wall. Heavy infections can cause ulceration of the bowel, diarrhea, and abdominal pain.

Family Fasciolidae Railliet, 1895

Main features Echinostomatoidea; body large, flattened, often broad, sometimes narrow; forebody short; tegument normally armed with small spines; head collar and circumoral crown of spines absent; intestinal ceca with or without dendritic lateral branches; testes usually deeply lobed, sometimes unlobed; in intestine and biliary system of mammals.

Two genera occur in humans, *Fasciola* Linnaeus, 1758 and *Fasciolopsis* Looss, 1899. These are large, broad, leaf-like worms, recognizable by the presence of deeply lobed gonads, the lobes being so deep that the gonads appear dendritic. *Fasciola,* the common liver fluke of sheep, occurs in the biliary system, whereas *Fasciolopisis* occurs in the intestine and is restricted to southeast Asia. The two genera are readily distinguished as *Fasciola* has branched intestinal ceca.

The eggs of fasciolids, whether they be in the intestine or biliary system, pass out with the feces. The miracidia infect freshwater snails, within which the mother sporocyst develops and produces rediae. Accounts of the life history within the molluskan host vary but, apparently, within the rediae may develop a second or even third generation of rediae and any generation of rediae may produce cercariae. The latter leave the snail and encyst as metacercariae usually on vegetation. Encysted metacercariae are especially common on grass close to water and on plants such as watercress and water chestnut. The final hosts, normally herbivorores but occasionally humans, become infected by feeding on vegetation harboring the encysted metacercariae. Metacercariae of *Fasciola* migrate through the liver en route for the bile ducts, where the adults live. These parasites are very common in domesticated animals, such as sheep, pigs, and cattle, which form huge reservoirs of infection. Control is, therefore, facilitated by the avoidance of raw (or unpeeled) vegetables or other plant material which has been associated with water. Similarly, control can be assisted by avoidance where possible of the links between human and animal feces and bodies of water, e.g. by the use of drinking troughs and the draining of pasture land.

Fasciolids cause inflammation at the site of attachment, which may lead to ulceration and hemorrhage, as these spine-covered worms browse on the tissues. The amount of damage caused depends on the worm burden. In the liver, the presence of these worms can cause traumatic damage resulting in necrosis ('liver rot'); in the bile ducts, mechanical irritation can cause hyperplasia and fibrosis. When flukes are present in large numbers, they can result in blockages of the bile ducts or even the intestine (see Lung and liver flukes).

SUPERFAMILY PARAMPHISTOMOIDEA FISCHOEDER, 1901

Family Zygocotylidae Ward, 1917

Main features Paramphistomoidea; body stout, often large, pear-shaped to elongate-oval, ventrally flattened; tegument unarmed, tegumentary papillae present or absent; appears to have suckers at each end of body; oral sucker absent; pharynx resembles oral sucker, terminal at anterior extremity, opening via mouth, with one or two posterolateral sacs; posterior (ventral) sucker normally occurs ventroterminally at posterior end of body, well developed, often very large; eggs very large, numerous; in intestine of mammals and birds (mainly herbivores).

Two genera of zygocotylid paramphistomoids occur in humans, *Gastrodiscoides* Leiper, 1913 and *Watsonius* Näsmark, 1937. These relatively large worms are readily recognizable by the apparent presence of a sucker at each end of the body, hence their vernacular name, 'amphistomes.' The two genera can be distinguished by shape; in *Gastrodiscoides* the body consists of a posterior discoid region and a narrower anterior region.

The zygocotylid life cycle commences with eggs passed out with the feces. When an egg reaches water, it

hatches and the released larva, the miracidium, penetrates an aquatic gastropod mollusk. Within the mollusk, the miracidium transforms into a mother sporocyst, within which develop rediae (some authors maintain that only a single mother redia derives from the mother sporocyst and this produces a second generation of daughter rediae). Cercariae, which develop within the rediae, are released from the mollusk and encyst as metacercariae on the substratum, especially on vegetation and the snail host. The final host, a bird or mammal, normally becomes infected by feeding on vegetation or snails harboring the metacercarial stage.

The general similarity in transmission between the zygocotylids and the fasciolids means that the method of human infection and control measures linked to the worms' life cycles are essentially the same, i.e. avoiding the consumption of uncooked vegetation which has originated from or close to water. There is a difference, however, in that primates rather than domesticated herbivores tend to act as reservoir hosts, although pigs, rats, and other rodents may also be involved. Human feces should be kept away from bodies of water.

Little pathogenicity is observed in light infections, but inflammation of the intestinal mucosa may occur where the large posterior sucker attaches. Heavy infestations may cause edema of the intestinal wall and diarrhea.

SUPERFAMILY OPISTHORCHIOIDEA LOOSS, 1899

Family Heterophyidae Leiper, 1909

Main features Opisthorchioidea; body small, oval to elongate; tegument spined; oral and ventral suckers present; ventral sucker occasionally small or submedian, often associated with genital atrium forming ventrogenital complex; cirrus sac absent; gonotyl(s) (genital sucker, a muscular structure associated with genital atrium) may or may not be developed, armed or unarmed; eggs small; metacercariae on skin and fins and in tissues of fishes; adults in piscivorous birds and mammals.

Two heterophyid genera occur relatively commonly in humans, *Heterophyes* Cobbold, 1886 in lands bordering the western Mediterranean and both *Heterophyes* and *Metagonimus* Katsurada, 1913 in China, Korea, Japan, and neighboring regions. Other genera, such as *Centrocestus* Looss, 1899, *Stellantchasmus* Onji and Nishio, 1915, and *Haplorchis* Looss, 1899, may also occur on rare occasions. *Heterophyes* and *Metagonimus* can be distinguished by the fact that in the latter the mouth of the ventrogenital sac is directed anteriorly, there is no muscular gonotyl and the genital pore is anterior to the ventral sucker. Heterophyids are small worms recognizable by the fact that the genital atrium is associated with the ventral sucker to form a ventrogenital complex.

Heterophyids in humans are intestinal parasites. Their eggs pass out with the feces and hatch to release miracidia which penetrate aquatic snails. Within the snail, there are two generations of rediae. Cercariae produced by the rediae leave the mollusk and encyst under the scales and in the superficial muscles of freshwater fishes. Humans and other piscivorous mammals obtain the infection by feeding on raw, pickled or poorly cooked fish harboring the metacercaria. Infection can be avoided by ensuring that fish intended as food is well-cooked or frozen prior to ingestion. A reduction in the likelihood of infection may also occur if fish in the food of reservoir hosts, such as cats, dogs, and pigs, is treated in the same way. Improvements in sanitation also help disrupt the life cycle.

In small numbers, these small, spined worms cause little damage to the intestine apart from local inflammation at the site of attachment; but more serious damage, including ulceration, may occur when large numbers are present. Occasionally the worms may penetrate the gut wall and eggs may end up in various parts of the body, including the heart and brain.

Family Opisthorchiidae Looss, 1899

Main features Opisthorchioidea; body small to medium-sized, flattened, elongate-oval to fusiform, often translucent; tegument spined; suckers well developed; cirrus sac absent; gonotyl and ventrogenital sac (see above, Heterophyidae) absent; metacercariae present in tissues of freshwater fishes; adults in piscivorous mammals and birds.

Two genera of opisthorchiids occur in humans, *Opisthorchis* Blanchard, 1895 and *Clonorchis* Looss, 1907. They can be distinguished by the fact that in *Clonorchis* the testes are tandem and have long, branched lobes that overlap the intestinal ceca. Both are parasitic in the biliary system and are most common in southeast Asia. They can be distinguished from heterophyids by their greater size, more elongate shape, and the absence of a ventrogenital complex. Body spines are very small and commonly lost in preserved specimens.

Worms in the bile ducts release eggs that pass into the intestine to be voided with the feces. If an egg finds its way to water and is ingested by a suitable aquatic gastropod snail, it hatches in the gut and releases the miracidium, which penetrates the mollusk and develops into a mother sporocyst. Rediae develop within the latter stage, which in turn give rise to cercariae. These leave the snail and swim and encyst as metacercariae under the scales or in the superficial tissues of freshwater fishes. Piscivorous mammals and birds become infected by feeding on fishes harboring the metacercarial stage. Control measures are similar to those outlined for heterophyids, i.e. primarily the avoidance of raw, pickled or poorly cooked fishes. Opisthorchiids are not primarily parasites of humans, as those infecting humans also occur commonly in many piscivorous mammals, including cats, foxes, dogs, pigs, etc., which act as reser-

voir hosts. Avoidance of the link between both human and animal feces and bodies of water is needed to break the life cycle.

Light infections are not pathogenic, but heavier infections of these spine-covered worms cause abdominal discomfort, diarrhea, and even acute pain. This results from inflammation of the biliary epithelium and thickening of the bile ducts as a consequence of mechanical irritation and their browsing activity. The presence of worms may cause gallstones, cirrhosis of the liver, and perhaps even carcinoma of the bile ducts (see

Lung and liver flukes).

SUPERFAMILY DICROCOELIOIDEA LOOSS, 1899

Family Dicrocoeliidae Looss, 1899

Main features Dicrocoelioidea; body oval, lanceolate or filiform, flattened, often translucent; tegument usually spined; oral sucker subterminal; ventral sucker in anterior third of body, occasionally very reduced or absent; cirrus sac present close to ventral sucker; metacercaria occurs in terrestrial arthropods; in biliary system or pancreatic ducts of reptiles, birds, and mammals.

Only one dicrocoeliid genus, *Dicrocoelium* Dujardin, 1845, occurs in humans and then only rarely. It normally parasitizes a wide range of mammals and even birds, but is very common in domesticated ruminants, especially sheep. Dicrocoeliids can be distinguished from other digeneans occurring in the biliary system of humans in that the testes occur close to the ventral sucker and the vitelline fields are in the post-testicular region of the hindbody.

As in the case of other parasites of the biliary system, the eggs pass out with the feces, but, in contrast to other digeneans occurring in humans, the snail host is a terrestrial gastropod. Inside the mollusk, the miracidium transforms into a mother sporocyst within which form daughter sporocysts. The latter give rise to cercariae which end up in slime balls produced by the snail. When the slime balls are eaten by ants, the parasite encysts as a metacercaria in the insect. Herbivores, and humans, become infected with the parasite when ants are eaten accidentally with vegetation. Human infection can, therefore, be avoided by careful washing of salad material and other items of vegetation eaten raw.

Little is known regarding the pathogenicity of these worms in humans, but, since infections are likely to be light, they are presumably similar to mild infections of fasciolids or opisthorchiids.

SUPERFAMILY PLAGIORCHIOIDEA LÜHE, 1901

Family Lecithodendriidae Lühe, 1901

Main features Plagiorchioidea; body spherical to elongate; tegument usually spined; oral sucker well developed; ventral sucker often small, near middle of body; testes usually symmetrical and widely separated near middle of body; cirrus sac usually present; vitelline follicles in forebody, occasionally hindbody; eggs small; metacercariae in aquatic insects; in gut of amphibians, reptiles, birds, and mammals (especially bats).

Lecithodendriids occur in a wide variety of insectivorous vertebrates and are noted parasites of bats. Only one genus, *Phaneropsolus* Looss, 1899, regularly parasitizes humans, although others, such as *Paralecithodendrium* Odhner, 1910 and *Prosthodendrium* Dollfus, 1931 have also been reported. These minute parasites are recognizable by the fact that the gonads and vitelline follicles are in the forebody or at least level with the ventral sucker.

Lecithodendriid eggs pass into water with the host's feces. The miracidium enters the tissue of an aquatic snail and transforms into a mother sporocyst. It is likely that these produce daughter sporocysts, which in turn produce cercariae. The cercariae leave the snail and some are eaten by or penetrate the larvae of aquatic insects, within which they encyst as cercariae. The final vertebrate host acquires the parasite by feeding on larval or adult insects harboring the parasite. It is not clear how people in southeast Asia (Thailand and Indonesia) become infected with lecithodendriids, but it is presumably by feeding on larval or adult insects in regions where these are local delicacies or by accidentally ingesting insect larvae with aquatic vegetation or water. Control measures include the filtering or heating of water, the cleaning of vegetable material, and the cooking or freezing of insects intended as food.

Virtually nothing is known concerning the pathogenicity of lecithodendriids in humans, but it seems likely that some local irritation occurs, similar to that caused by mild infections of other small, intestinal digeneans armed with spines, such as heterophyids.

Family Paragonimidae Dollfus, 1939

Main features Plagiorchioidea; body oval to fusiform; tegument spinose; suckers relatively small, ventral sucker near middle of body; testes symmetrical, lobed, in hindbody; cirrus sac absent; genital pore closely posterior to ventral sucker; metacercariae in tissues of crustaceans; in lungs of mammals.

Numerous species of the genus *Paragonimus* Braun, 1899 occur in the lungs of humans in parts of Africa, South America and especially southeast Asia. They are medium-sized worms readily recognizable by their site in the lungs and the position of the genital pore posterior to the ventral sucker. They can be distinguished from the closely related troglotrematids by the absence of a cirrus sac. Paragonimids are usually parasitic in carnivores, especially felids and others likely to feed on freshwater crabs and other large crustaceans.

The eggs pass out of the lungs with mucus, are swallowed, and then voided with the feces or in sputum. The hatched miracidium penetrates a freshwater snail and

develops into a mother sporocyst. Two generations of rediae are then produced. The second generation gives rise to cercariae, which leave the snail and encyst in the tissues (especially muscles and gills) of crustaceans (usually crabs or crayfish); the latter may apparently also become infected by feeding directly on infected snails. Humans acquire the parasite by eating raw or inadequately cooked crustaceans; the young flukes penetrate the gut wall and find their way to the lungs via the abdominal cavity, diaphragm, and pleural cavity. Control is effected by the cooking of crustaceans intended as food and sanitary measures which break the link between human and, where possible, carnivore feces and bodies of water.

Paragonimids are covered with small spines and presumably browse on the walls of the bronchi, so their presence in the lungs causes local inflammation, resulting in a tissue reaction, cyst formation, and fibrosis. The net result is coughing, bloodstained sputum, chest pains, and bronchitis, which occur when the cysts rupture. The migration of the young worms through the body tissues does not cause any serious problems; but occasionally worms reach other parts of the body where problems may arise, especially when worms reach the brain where they may cause paralysis or even death (see Lung and liver flukes).

Family Plagiorchiidae Lühe, 1901

Main features Plagiorchioidea; body oval, pyriform, lanceolate or elongate; tegument armed with spines; suckers well developed; ventral sucker normally in anterior half of body; testes in hindbody, usually diagonal; cirrus sac present, usually long; genital pore anterior to ventral sucker; ovary pretesticular; uterus in hindbod; in intestine, occasionally biliary system, of vertebrates, especially birds and mammals.

Species of the genus *Plagiorchis* Lühe, 1899 have occasionally been recorded from the intestine of humans in parts of southeast Asia. Plagiorchiids are parasites of insectivorous vertebrates, especially mammals. Although some nominal species are known only from humans, it is not known whether they are zoonotic infections or specifically human parasites. The worms differ from other plagiorchioids found in humans in that the testes tend to be diagonal to tandem rather than symmetrical.

Plagiorchiid eggs leave with the feces and the miracidium enters an aquatic snail and develops into a mother sporocyst. These give rise to daughter sporocysts from which cercariae emerge. These leave the snail and find their way into the larval stages of aquatic insects which act as intermediate hosts. The vertebrate host becomes infected by feeding on larval or adult insects harboring cercariae. Humans are thought to become infected by eating insect grubs.

Little is known regarding the pathogenicity of these worms, but infections are likely to be light and effect similar local responses to those of other small, spined digeneans parasitic in the gut, such as heterophyids.

Family Troglotrematidae Odhner, 1914

Main features Plagiorchioidea; body oval; tegument spinose; suckers well developed, often similar in size; ventral sucker in middle or anterior half of body; testes symmetrical; cirrus sac present; genital pore usually closely posterior to ventral sucker; metacercaria in tissues of fish or crustaceans; in intestine, body sinuses, kidney, liver, etc. of mammals.

One genus of troglotrematid, *Nanophyetus* Chapin, 1927, occasionally occurs in humans. These worms normally occur as parasites of piscivorous carnivores in North America. This group appears closely related to the paragonimids and, like the latter group, tends not to be parasitic in the alimentary system (though *Nanophyetus* is an intestinal parasite) and be recognizable by the position of the genital pore posterior to the ventral sucker.

Eggs leave with the feces. The miracidium enters aquatic snails within which a generation of rediae produce more rediae and cercariae. The cercariae leave the snail and become attached to fish encysting under the scales as metacercariae (some troglotrematid cercariae encyst in crustaceans). Carnivores become infected by feeding on fish carrying the metacercariae. Similarly, humans become infected by eating raw, smoked or poorly cooked freshwater fish. Obvious control measures involve the skinning, cooking or freezing of fish prior to consumption.

Little is known concerning the pathogenicity of these worms, but, as they have body spines and may be acquired in some numbers, some irritation and inflammation of the bowel is to be expected.

PHYLUM NEMATODA

Nematodes are probably the most abundant and widespread animal group, often occurring in huge numbers in environments ranging from the polar regions to hot springs. In addition to free-living marine and freshwater forms, there are free-living forms in the soil and parasitic forms in both animals and plants. Nematodes are symmetrically bilateral, unsegmented, normally dioecious worms that are usually filiform; they have a body cavity with a high hydrostatic pressure, a straight digestive tract with an anteriorly terminal mouth and posteriorly subterminal anus, no circulatory system, a simple excretory system, and a body wall consisting of an outer layer of cuticle and an inner layer of longitudinal muscles (Figure 3). As animal parasites, they occur in virtually all groups, both invertebrate and vertebrate. The phylum is divided into two classes, the Adenophora and the Secernentea; both groups have evolved parasitic members, although the majority of

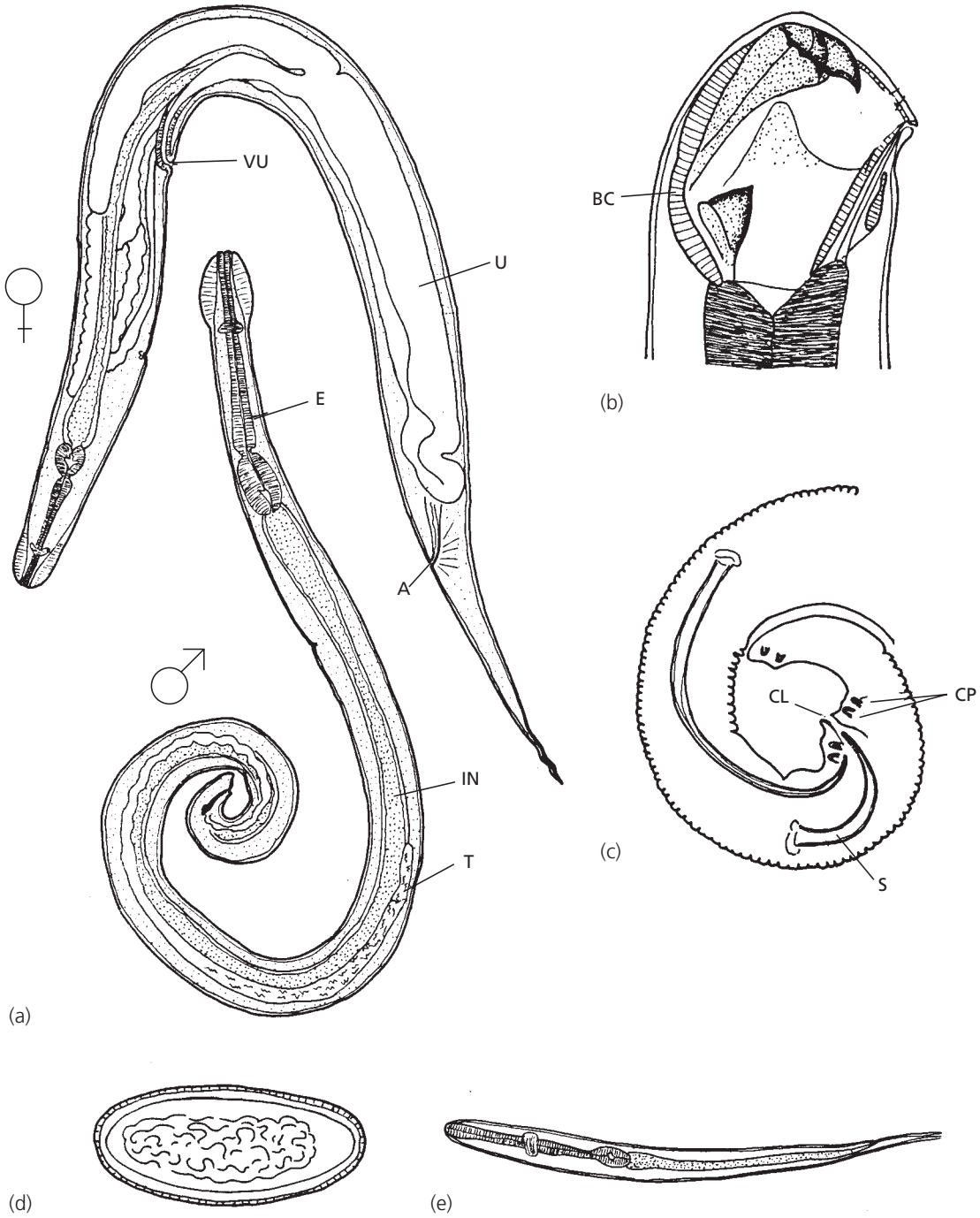

Figure 3 *Nematodes:* **(a)** *male and female* Strongyloides *(Strongyloididae);* **(b)** *head of* Ancylostoma *(Ancylostomatidae) (hookworm) with cuticularized buccal capsule armed with teeth;* **(c)** *caudal end of male* Onchocerca *(Onchocercidae);* **(d)** *egg of* Strongyloides*;* **(e)** *third-stage larva of* Strongyloides*. Anus (A), buccal capsule (BC), cloaca (CL) (in the male, the gut and reproductive system open through the same aperture), caudal papillae (CP), esophagus (E), intestine (IN), spicule (S), testis (T), uterus (U), vulva (VU). Adapted from (a, d, e) Hugot and Tourte-Schaefer (1985);* **(b)** *Yorke and Maplestone (1926);* **(c)** *Caballero y Caballero 1944)*

animal parasites belong to the latter group. The differences between the groups reflect the presence and absence of small sensory structures (phasmids) on the tail and the nature of the excretory system; but there is a fundamental biological difference in the parasitic members, since in the Adenophora the first-stage larva is infective to the definitive (final) host, whereas in the Secernentea it is the third-stage larva.

All nematodes have five life history stages, four larval and one adult, which are separated by a molt of the cuticle. It is common for the first one or two molts to occur within the egg. The life cycle may be direct or

indirect. Except in the case of the Adenophora, the stage infective to the final host is the third-stage larva. Direct life cycles can involve the ingestion of eggs or larvae with food or, in some cases, direct penetration of larvae through the skin. Indirect life cycles usually utilize invertebrate intermediate hosts but sometimes vertebrates (or other invertebrates) may act as intermediate or paratenic hosts. The larvae normally reside, often encysted, in the tissues of intermediate hosts. The majority of nematode parasites of vertebrates occur in the alimentary canal; those in other parts of the body often require the migration of larvae through the body to reach these sites. Some groups also have a larval migration from the gut and into the tissues and back to the gut; this represents the vestige of an indirect life cycle in its evolutionary past. Whichever mode of transmission is utilized, the chance of a particular egg or larva developing into an adult worm is very slight; this is compensated for in many cases by a huge output of eggs, which in some cases reaches 200 000 per female per day.

The morphological features by which nematodes are recognized vary from group to group, but at higher taxonomic levels, the nature of the anterior regions of the alimentary canal, e.g. the esophagus, the form of the head (presence, number of lips, teeth, etc.), and the form of the male tail are usually important. At the specific level, details of the male tail, such as the arrangement of caudal papillae (sensory structures used during copulation) and the length and shape of the spicule(s) (sclerotized copulatory aids) are important. In the majority of cases, males carry more taxonomically useful information than females, the latter often being unidentifiable at the specific level in the absence of males.

The pathogenicity of nematodes in their final host varies considerably, usually being dependent on the size of the infection. Those such as hookworm, which are heavily armed with teeth or other sclerotized mouthparts and browse on the gut wall, can cause considerable damage. Similarly, forms that migrate around the body, both adults in the tissues and larvae (termed larva migrans), can cause serious problems, especially if they reach sensitive regions such as the brain, liver or eyes (see *Toxocara*, and *Trichinella*).

The classification of the nematodes below is based on that of Anderson et al. (1974–83); it includes most of the forms that have been recorded from humans regularly, but accidental infections of other forms do occur infrequently:

Class Adenophorea
  Superfamily Trichinelloidea
    Family Trichinellidae
    Family Trichuridae
Class Secernentea
  Superfamily Ancylostomatoidea
    Family Ancylostomatidae
  Superfamily Ascaridoidea
    Family Anisakidae
    Family Ascarididae
  Superfamily Dracunculoidea
    Family Dracunculidae
  Superfamily Filarioidea
    Family Onchocercidae
  Superfamily Gnathostomatoidea
    Family Gnathostomatidae
  Superfamily Metastrongyloidea
    Family Angiostrongylidae
  Superfamily Oxyuroidea
    Family Oxyuridae
  Superfamily Physalopteroidea
    Family Physalopteridae
  Superfamily Rhabditoidea
    Family Rhabditidae
    Family Strongyloididae
  Superfamily Spiruroidea
    Family Gongylonematidae
  Superfamily Strongyloidea
    Family Chabertiidae
    Family Syngamidae
  Superfamily Thelazioidea
    Family Thelaziidae
  Superfamily Trichostrongyloidea
    Family Trichostrongylidae.

Class Adenophorea Dougherty, 1958

Main features Nematoda; tail occasionally modified to form sucker; esophagus with normal appearance or modified, with esophageal gland cells forming row of large gland cells (stichocytes) or reserve organ (trophosome); oviparous or viviparous, eggs with plug at either end; excretory system without lateral canals; caudal papillae absent or few; phasmids absent; first larval stage usually infective to final host.

SUPERFAMILY TRICHINELLOIDEA WARD, 1907

Family Trichinellidae Ward, 1907

Main features Trichinelloidea; modified esophagus with one row of stichocytes; intestine unmodified, anus present; vulva of female close to middle of esophagus; viviparous; spicule in male absent; larval stage in muscles of mammals; adults usually in intestine of carnivorous mammals.

One genus, *Trichinella* Railliet, 1895, occurs in humans both as an adult in the intestine (usually gut wall) and as a larval stage in the tissues (usually muscles). The adult worms are very small, thread-like, and recognizable by the row of large gland cells (stichocytes) surrounding the esophagus and the absence of spicules in the male.

Adult worms occur in the intestine and intestinal mucosa. Females are viviparous and release first stage larvae into the intestinal mucosa. These larvae migrate via the blood and lymphatic system to muscles where

they encyst in a coiled form. Transmission to another host occurs when the host is eaten by a carnivore or omnivore; here the larvae molt four times in the gut prior to reaching the adult stage. The same animal may serve, therefore, as both intermediate and definitive host. Humans usually become infected by feeding on raw or inadequately cooked meat, especially pork, but also wild carnivores, such as bears. Control is effected by the cooking or freezing of meat, preventing pigs eating raw meat and offal, and getting rid of rats (which may harbor the infection) on pig farms.

The effects of *Trichinella* infection range from diarrhea and nausea during the intestinal phase to eosinophilia, general weakness due to muscle damage, and difficulties in breathing and myocarditis caused by larvae in the muscles. Heavy infection can result in death, especially in cases where the heart or brain is involved (see *Trichinella*).

Family Trichuridae Ransom, 1911

Main features Trichinelloidea; modified esophagus with one to three rows of stichocytes; intestine unmodified; vulva of female close to posterior end of esophagus; oviparous, eggs with plug at each end; single spicule and spicule sheath usually present; in alimentary canal, tissues, and various organs of vertebrates.

Several trichurid genera are known to infect humans, including *Trichuris* Roederer, 1761, *Aonchotheca* Lopez-Neyra, 1947, *Calodium* Dujardin, 1845, and *Eucoleus* Dujardin, 1845; the latter three genera are still commonly referred to as *Capillaria* Zeder, 1800, when the latter is used in its wide sense (see Moravec 1982 for generic differences). *Trichuris* is a common parasite of humans, whereas the capillariines are normally parasites of wild animals which occasionally infect humans. Like trichinellids, these worms have a row of stichocytes around the esophagus, but they can be distinguished by the presence of thick-shelled eggs with terminal plugs in the females and a spicule in the male.

Trichurids inhabit the intestine, intestinal mucosa, liver, lungs, and other tissues of vertebrates, depending on the species involved. Eggs pass out with the feces, or remain in the body until the host dies or is eaten by a predator or scavenger and are voided with its feces. Again, depending on the species involved, the life cycle may be direct (*Trichuris, Aonchotheca, Eucoleus*), in which case the definitive host becomes infected by feeding on eggs, or indirect (e.g. *Calodium*) and involve an intermediate host that has swallowed the eggs. Humans become infected by ingesting eggs in soil (especially children) or on vegetation or, in the case of *Calodium*, by ingesting raw fish harboring the larva. Control is effected by modern sewage disposal and improvements in personal hygiene, the proper cooking of fish (*Calodium*), and the control of rats and other rodents which act as reservoirs (*Aonchotheca*).

Symptoms of infection vary according to the species but in the case of *Aonchotheca* in the liver, they resemble hepatitis as eggs are deposited in the liver parenchyma. Intestinal forms may cause diarrhea, dysentery, and weight loss as the worms penetrate the mucosa, but the severity depends on the size of the infection. In extreme cases rectal prolapse, muscle wasting, and death can occur (see Gastrointestinal nematodes and *Strongyloides* and *Capillaria*).

Class Secernentea Dougherty, 1958

Main features Nematoda; esophagus without stichocytes or reserve organ (trophosome); oviparous or viviparous, eggs without plug at either end; excretory system with lateral canals; caudal papillae numerous; phasmids present; third larval stage infective to final host.

SUPERFAMILY ANCYLOSTOMATOIDEA LOOSS, 1905

Family Ancylostomatidae Looss, 1905

Main features Ancylostomatoidea; buccal cavity large; cuticularized; subglobular; mouth not surrounded by lips or comb-like leaf-crown(s), unarmed or armed with teeth or cutting plates; esophagus claviform, not divided into two regions; male with caudal bursa normally supported by rays; in alimentary canal of mammals.

Two genera of ancylostomatids ('hookworms'), *Ancylostoma* Dubini, 1843 and *Necator* Stiles, 1903, occur in humans mainly in the tropics. These are intestinal parasites recognizable by their large globular, cuticularized buccal cavity, mouth armed with teeth or cutting plates, and dorsally curved anterior extremity. Other genera may occur as accidental parasites on rare occasions.

The life cycle is direct. Eggs laid by female worms in the intestine are voided with the feces and hatch in the soil. The larva feeds on bacteria in the soil and molts twice. The third-stage larvae penetrate the skin of a suitable mammalian host and then migrate via the blood system to the lungs where they pass from the blood system into the alveoli, ascend the bronchi and trachea, and are then swallowed. Once in the intestine, they molt twice and mature. Humans become infected via their bare feet, the skin of which is penetrated by the larvae. Human infection is also possible from larvae swallowed with salad or other raw vegetation. Control is effected by sewage management, the wearing of shoes, and the careful washing of salad material.

The penetration of the skin by larvae can cause an itching and the migrating larvae can result in coughing and bronchitis. The heavy armature of the mouth parts of adults, as they feed on the tissues and blood of the host, damages the intestinal wall, resulting in blood loss, abdominal pain, general weakness, and sometimes even death (see Gastrointestinal nematodes).

SUPERFAMILY ASCARIDOIDEA BAIRD, 1853

Family Ascarididae Baird, 1853

Main features Ascaridoidea; head with three well-defined lips; lips sometimes separated by interlabia, unadorned by spines or cuticular embellishments posteriorly, single row of denticles on anterior margin present or absent; buccal cavity absent or weakly developed; esophagus cylindrical, occasionally divided into two regions (when posterior glandular ventriculus is present), without posterior muscular bulb; excretory system symmetrical, 2-sided and tubular, excretory pore at level of nerve ring; in all vertebrate groups, mainly but not always terrestrial forms.

Three genera of ascaridid nematode, *Ascaris* Linnaeus, 1758, *Toxocara* Stiles, 1905, and *Toxascaris* Leiper, 1907, regularly occur as parasites of humans although only *Ascaris* is a true human parasite, the other two being common parasites of dogs or cats. Other genera occurring in wild animals, such as *Baylisascaris* Sprent, 1968 and *Lagochilascaris* Leiper, 1909, have also been recorded from humans on rare occasions. Only *Ascaris* normally occurs in humans as an adult; the others are present in the tissues in larval form only. Ascaridids are related to anisakids, and like that group have three distinct lips; they differ in that (with the exception of the subfamily Heterocheilinae) their life cycle is linked to terrestrial rather than aquatic conditions.

Ascaridids occur in all vertebrate groups and have a variety of life cycles. Most involve invertebrate or vertebrate intermediate hosts or both, but those occurring in humans normally have a direct life cycle. Nevertheless, even in these forms the vestige of an indirect life cycle is apparent in the form of a larval migration through the tissues of the host prior to maturation in the intestine. In these cases, eggs pass out with the feces and the first two larval molts occur within the egg. If the eggs are eaten by a suitable host, the third-stage larvae hatch in the gut, penetrate the gut wall, and are transported by the blood to the lungs. After a short period in the lungs where they molt twice, they escape into the alveoli, pass up the bronchi and trachea with mucus and are swallowed. Once they reach the intestine, they mature. There are variations: in some genera developing in mammals, migrating larvae in the blood can result in transplacental or transmammary infections of young; and occasionally insects and rodents may act as intermediate hosts. Humans, especially children, become infected by accidentally ingesting soil or food contaminated with eggs. Control is effected by careful sewage management, avoiding the use of human feces as manure, improvements in personal hygiene, keeping children away from the feces of domesticated animals, especially dogs, the regular de-worming of pets, and the careful washing of salad material, fruit, etc.

Despite the fact that *Ascaris* is a large worm, its presence in the intestine does not cause many symptoms unless the worm burden is high. Light infections can cause nausea and other bowel disorders; heavy infections can result in bowel obstruction or peritonitis and even death. The migration of the larval stage of *Ascaris* and especially the other ascaridids mentioned above can be more serious. Larvae passing through the lungs cause some pneumonitis, but larva migrans that lodge in other tissues, such as the liver, spleen, kidneys, placenta, and especially the eye and CNS (e.g. *Toxocara*), can represent a danger, producing inflammation and granulomata in these sensitive areas (see Gastrointestinal nematodes and *Toxocara*).

Family Anisakidae Railliet and Henry, 1912

Main features Ascaridoidea; head with three well-defined lips; lips sometimes separated by interlabia, unadorned by spines or cuticular embellishments posteriorly, single row of denticles on anterior margin present or absent; buccal cavity absent or weakly developed; esophagus with posterior ventriculus; gut ceca present or absent; excretory system asymmetrical, entirely or mainly one-sided, tubular or ribbon-like, excretory pore at level of nerve ring or ventral interlabium; larval stages in fishes or invertebrates or both; in piscivorous vertebrates.

Anisakids capable of accidentally infecting humans are normally parasites in the stomach of marine mammals. The group occurs in all vertebrate groups, but those parasitic as adults in other hosts, such as fishes, are not capable of infecting humans. There are two main genera involved, *Anisakis* Dujardin, 1845 and *Pseudoterranova* Mozgovoi, 1950; the former is the more widely reported. Since humans do not provide a suitable environment for the development of these parasites, they do not reach maturity and usually attempt to penetrate the gut wall in order to re-encyst as they would in any other unsuitable host. Like ascaridids, anisakids have three distinct lips at the anterior end, but differ in the configuration of the excretory system.

All anisakids have an indirect life cycle involving aquatic intermediate hosts. Eggs leave with the feces. The first one or two (accounts vary) larval molts occur within the egg. If eggs released into water are eaten by small planktonic invertebrates, such as copepods, the third-stage larvae develop in the hemocoel. If this host is eaten by a larger crustacean, squid or bony fish, the larvae are transferred and re-encyst in the body cavity or muscles of the new host. The larvae can be transferred to new hosts several times. If a third-stage larva within its intermediate host is consumed by a suitable (usually piscivorous) vertebrate host, the larva molts twice and matures in the stomach or intestine. Humans usually acquire infections of anisakids by feeding on raw,

lightly marinated or inadequately cooked marine bony fishes (infection by consuming raw squid and raw marine crustaceans is also possible). Infection can be prevented by cooking fish properly or by the freezing of fish or fish products intended for consumption raw or marinated. Fish inspection may eliminate heavily infected fish and evisceration immediately following netting may prevent the migration of larvae to the muscles, but it is not possible to prevent the infection of wild marine fishes without a heavy culling of marine mammals.

Although the worms may molt once in humans, they tend to penetrate the gut wall in an attempt to re-encyst; whether this is in the stomach or intestine may depend on the size of the food bolus. Symptoms of infection can mimic gastric or duodenal ulcers. Repeated infections are more serious, and worms may have to be removed by surgery. In Japan, where this has been an important parasite of humans because of local fish-eating habits, the presence of these worms has been linked to cases of gastric and intestinal cancer (see *Angiostrongylus* (*Parastrongylus*) and less common nematodes).

SUPERFAMILY DRACUNCULOIDEA STILES, 1907

Family Dracunculidae Stiles, 1907

Main features Dracunculoidea; considerable sexual dimorphism; head-end rounded, bilaterally symmetrical; lips and pseudolabia absent; buccal capsule weakly developed, reduced to cuticular ring; esophagus apparently undivided; esophageal glands usually uninucleate; anus atrophied in adult; male tail without caudal bursa; body of female long, cylindrical; vulva normally in middle of body; viviparous; transmission indirect, copepods act as intermediate hosts; usually in tissues and tissue spaces of reptiles, birds, and mammals.

One genus, *Dracunculus* Reichard, 1759, infects the subcutaneous tissues of humans, the species involved also occurrs in a variety of mammals, especially dogs. These worms resemble filarioids in being tissue parasites, but they have a different life cycle, which involves an aquatic intermediate host, and they do not release microfilariae into the blood.

Fully developed female worms migrate to the skin and produce a blister that bursts, permitting the rupture of the worm and its uterus when contact is made with water. First-stage larvae eaten by copepods enter the hemocoel and molt twice. Vertebrates become infected with these parasites when they drink water containing copepods harboring the third-stage larva. The larvae penetrate the gut wall and enter connective tissue within the body, where they molt twice. After mating, the females develop in the subcutaneous regions. Humans become infected by drinking water containing infected copepods. The parasite is readily controlled by the filtering of drinking water or by using water sources, such as springs or deep wells, where contact with animal skin is not possible and copepods are absent.

Migrating worms in the subcutaneous regions may cause some tenderness, but the presence of the parasite may not be apparent until the blister forms. The blister may be painful and cause considerable inflammation, a large ulcer and, when secondary infections are involved, an abscess (see Dracunculiasis).

SUPERFAMILY FILARIOIDEA WEINLAND, 1858

Family Onchocercidae Leiper, 1911

Main features Filarioidea; body long, cylindrical; head bilaterally symmetrical; lips and pseudolabia absent; buccal cavity usually relatively small, not greatly cuticularized; esophagus usually with anterior muscular region and posterior glandular region, sometimes regions difficult or impossible to differentiate; male tail without caudal bursa, caudal alae usually narrow or apparently absent; vulva in anterior half of body but posterior to nerve ring; ovoviviparous or viviparous, eggs thin-shelled, poorly differentiated larvae (microfilariae); microfilariae appear in blood, lymph or skin of host; transmission indirect, with blood-feeding insects acting as intermediate hosts; in tissues and tissue spaces of amphibians, reptiles, birds, and mammals.

Several genera of filarioids are parasitic in humans, the more important of which are *Brugia* Buckley, 1958, *Dipetalonema* Diesing, 1861, *Dirofilaria* Railliet and Henry, 1910, *Loa* Stiles, 1905, *Mansonella* Faust, 1929, *Onchocerca* Diesing, 1841, and *Wuchereria* Silva Araujo, 1877. These tissue parasites are usually found in the lymphatic system, subcutaneous connective tissue or other connective tissues. They differ from dracunculids in that their life cycle is terrestrial and they produce larvae (microfilariae) that are found in the blood, skin, etc. A generic key to microfilariae, which are more likely to be seen than the adult worms, was produced in French by Bain and Chabaud (1986).

The life cycle is indirect, involving a biting insect as an intermediate host. Mature female worms release first- or second-stage larvae surrounded by their first-stage cuticle into the blood. These often exhibit a diurnal periodicity that brings them into the peripheral circulation during the period when blood-feeding insects, such as mosquitoes and blackfly, are likely to feed. When taken in by a suitable insect, they pass into the hemocoel, grow, molt into the infective third stage and migrate to the region of the insect's mouthparts. The worm is then injected into a new host when the insect next feeds. After entering a suitable host, the larvae migrate to the lymphatic system or connective tissue, molt twice, and mature. Humans, therefore, become infected when they are bitten by insects, and some control can be achieved by reducing the numbers of vectors by the drainage of marshes, use of insecticides, etc.

Human infection (filariasis) causes various symptoms, depending on the species and site of the parasite; elephantiasis caused by inflammation in the lymphatic

system in various parts of the body is the most extreme. In some cases, symptoms are few; in others, subcutaneous swellings or nodules occur that can result in abscesses if the worm dies. Such infections are often accompanied by rashes and intense itching. Microfilariae may also cause problems when they become lodged in regions such as the central nervous system or eyes (see Lymphatic filariasis and Onchocerciasis).

SUPERFAMILY GNATHOSTOMATOIDEA RAILLIET, 1895

Family Gnathostomatidae Railliet, 1895

Main features Gnathostomatoidea; body long, cylindrical or stout often armed with spines; head bilaterally symmetrical with two large, trilobed, lateral pseudolabia; collar region may be enlarged to form cephalic bulb, which may be covered with transverse rows of recurved hooks; inner face of pseudolabia folded into rounded, tooth-like formations that interlock with those on opposite interlabium; buccal cavity normally relatively uncuticularized; esophagus with anterior muscular region and longer posterior glandular region; male tail without caudal bursa; transmission indirect with copepods and vertebrates acting as intermediate hosts; in anterior gut of vertebrates.

Several species of the genus, *Gnathostoma* Owen, 1836, occur occasionally as accidental parasites of humans in their larval form. These worms normally occur as adults in the stomach, usually in nodules in the stomach wall, of carnivorous mammals and pigs. They are recognizable by the presence of regular rows of spines on the body; on an inflated cephalic bulb these spines are larger and flattened.

The life cycle normally involves two intermediate hosts. Eggs leave the body in the feces. In water, these develop and hatch to release a larva that has molted once. When eaten by a suitable copepod, the larva penetrates the hemocoel; if the copepod is then eaten by a suitable freshwater fish or frog, the larva molts into the infective third stage. Fish, snakes, rodents, chickens, and humans may eat these hosts and act as paratenic hosts. The definitive host becomes infected by feeding on the second intermediate or a paratenic host. Worms found in humans, normally the third-stage larva, are obtained through eating raw or poorly cooked fish (occasionally, perhaps, chicken). Prevention involves the cooking or freezing of freshwater fish and fish products.

In humans, larvae penetrate the gut wall and migrate via the liver to the subcutaneous regions. Their penetration of the gut wall can cause some pain and presence in the subcutaneous regions which results in swelling and symptoms similar to the cuticular larva migrans of some ascaridids. More serious problems and even death can be caused if worms reach more sensitive areas of the body, such as the eyes or central nervous system (see *Angiostrongylus* (*Parastrongylus*) and less common nematodes).

SUPERFAMILY METASTRONGYLOIDEA LEIPER, 1908

Family Angiostrongylidae Böhm and Gebauer, 1934

Main features Metastrongyloidea; longitudinal cuticular ridges on body absent; cephalic vesicle (cuticular inflation) absent; buccal cavity absent or reduced and weakly cuticularized; mouth unarmed, not surrounded by lips or comb-like leaf-crown(s); esophagus cylindrical and not divided into two regions; male with caudal bursa supported by rays, bursa often reduced; vulva near anus; larvae in mollusks; adults in respiratory system and, occasionally, blood system and other organs of marsupials, insectivores, rodents, and carnivores.

One metastrongyloid genus, *Angiostrongylus* Kamensky, 1905 (includes *Parastrongylus* Baylis, 1928) occurs in humans. Metastrongyloids are commonly called lungworms. Angiostrongylids capable of infecting humans normally parasitize the lungs or blood system of rodents. They are recognizable by the presence in the male of a caudal bursa supported by rays, in conjunction with their site of infection, i.e. the respiratory or blood systems.

Eggs produced by worms in the pulmonary or mesenteric vessels of rodents, etc., release first-stage larvae into the intestine (those in the lungs are swallowed with mucus), which are voided with the feces. If these larvae penetrate or are eaten by certain snails, slugs or, less frequently, crabs and land planarians, they penetrate the tissues and molt twice. When an infected mollusk is eaten by a suitable mammal, the third-stage larvae penetrate the gut wall and migrate in the blood system or lymphatic system, molting twice (sometimes in the central nervous system) prior to maturing in the pulmonary or mesenteric arteries. Humans presumably become infected with these worms by eating raw or poorly cooked snails, or accidentally consuming mollusks on salad and other raw plant material. Prevention involves the cooking or freezing of snails, the careful washing of salad material and rodent control.

The migration of these worms to the central nervous system is potentially serious, producing symptoms resembling meningitis, paralysis, and blindness; occasionally this results in death. Pulmonary symptoms may not be apparent, but worms in vessels associated with the intestine can cause inflammation, granulomata, thrombosis, and necrosis, especially in the region of the appendix (see *Angiostrongylus* (*Parastrongylus*) and less common nematodes).

SUPERFAMILY OXYUROIDEA COBBOLD, 1864

Family Oxyuridae Cobbold, 1864

Main features Oxyuroidea; body short and stout; buccal cavity absent or weakly developed; esophagus relatively

short, not divided into anterior muscular and posterior glandular regions but with posterior muscular bulb; male tail with wide caudal alae which may resemble caudal bursa but supported by arrangement of usually similar pedunculate papillae rather than assortment of rays; usually one spicule; eggs often flattened on one side with relatively thick shells, usually embryonated on deposition, two larval molts occur within egg; life cycle direct; in intestine of mammals.

Only one genus, *Enterobius* Leach, 1853 (commonly known as 'threadworm' or 'pinworm'), regularly occurs in humans and other primates, although *Syphacia* Seurat, 1916, a rodent parasite, has been reported as an accidental infection in humans. Oxyurids are small, intestinal parasites with a direct life cycle and recognizable by the presence of a large posterior bulb on the esophagus.

Gravid female worms leave the body through the anus, depositing eggs in the perianal region. As their bodies dehydrate and burst, they eject eggs in huge numbers, which cause an itch. Two larval molts occur within the egg. Humans are reinfected by swallowing the eggs, which become accidentally attached to food or lodged under fingernails following scratching of the perianal region. Once swallowed, the eggs hatch and the third-stage larvae molt and develop into adults in the intestine. Autoinfection of humans is common. Prevention involves improvements in personal hygiene, in terms of both individuals and the entire population, and especial care in relation to food preparation.

The presence of these worms generally does little harm, apart from discomfort related to itching of the perianal region, although penetration of the gut wall has been observed, resulting in nonproven links with appendicitis and other complaints. Gravid females that migrate into the vagina may cause a mucoid discharge (see Gastrointestinal nematodes).

SUPERFAMILY PHYSALOPTEROIDEA RAILLIET, 1893

Family Physalopteridae Railliet, 1893

Main features Physalopteroidea; body cylindrical without lateral rows of spines; head bilaterally symmetrical; two pseudolabia, unlobed, armed with denticles (teeth) on free border; cuticle posterior to pseudolabia often expanded to form cephalic collar; buccal cavity usually relatively small, not greatly cuticularized; esophagus with anterior muscular region and longer posterior glandular region; male tail without caudal bursa, but caudal alae often wide; transmission indirect with arthropods acting as intermediate hosts; adults in gut of all vertebrate groups and occasionally crustaceans.

Only one physalopteroid, a species of the genus *Physaloptera* Rudolphi, 1819, which normally occurs in the intestine of primates, has been reported from humans. Other species of *Physaloptera* occur in birds of prey and insectivorous or carnivorous mammals. Important diagnostic features of physalopterids include the presence of two large, symmetrical, unlobed pseudolips armed with teeth and a cephalic collar (an expansion of the cuticle immediately posterior to the pseudolips).

Physalopterids have an indirect life cycle, normally involving an arthropod intermediate host and often a vertebrate paratenic host. Eggs of these gut parasites leave with the feces; if eaten by insects, such as cockroaches, other beetles, and crickets, the third-stage larva develops. The final host becomes infected by feeding on insects or, especially in the case of carnivores, on insectivorous vertebrates such as frogs and rodents. Humans presumably become infected by feeding (usually accidentally) on insects. Accidental infection can presumably be avoided by keeping food in a meat safe, refrigerator, etc.

Little is known about the pathogenicity of the worm, but infections are likely to be light. Some damage to the intestinal wall may be caused by the armed mouthparts, as species in animals do appear to feed on the gut wall and erode the mucosa, leaving bleeding wounds behind them as they browse.

SUPERFAMILY RHABDITOIDEA ÖRLEY, 1880

Family Rhabditidae Örley, 1880

Main features Rhabditoidea; small, slender worms; head with six lips (or three double lips); buccal cavity long, cylindrical with chitinized thickenings in wall; esophagus long, narrow, with prominent posterior valved bulb; caudal alae of male form bursa; mainly free-living saprophagous forms, occasionally accidental parasites of vertebrates.

Rhabditids are free-living worms that are often swallowed accidentally or become associated with human feces; the most frequently recorded examples are from the genus *Rhabditis* Dujardin, 1844. Some species of the genus *Pelodera* Schneider, 1866 are known to infect human skin in rare cases, producing symptoms similar to cutaneous larva migrans. Humans presumably become infected by soil contamination of existing wounds. The occurrence of these parasites in the skin is so rare that preventative measures are not worthwhile.

Family Strongyloididae Chitwood and McIntosh, 1928

Main features Rhabditoidea; parasitic stage usually a parthenogenetic female (alternates with free-living sexual generation with esophagus bearing two swellings); small, slender worms; buccal cavity reduced or well developed, but not long; esophagus long, narrow, lacking prominent valved bulb posteriorly; vulva in posterior quarter of body; in alimentary canal of terrestrial vertebrates.

This is a group with an irregular alternation of generations, a parasitic parthenogenetic female alternating with a free-living sexual generation. One genus, *Strongyloides* Grassi, 1879, occurs in humans. The parasitic females found in humans are small, slender worms that live in the intestinal mucosa. They can be confused with trichinellids in gross morphology, but the long, cylindrical esophagus is quite different, lacking the large gland cells of the latter.

Several life cycle pathways are possible, both direct and indirect, the latter involving alternation of generations. Eggs laid in the mucosa or submucosa of the intestine release first-stage larvae that escape into the intestine and are voided with the feces. These larva feed in the soil and molt twice. The third-stage larvae *either* continue to develop into male and female worms capable of continuing to produce free-living generations (and thus more third-stage larvae), especially in warmer climates *or* penetrate the skin of a terrestrial vertebrate, migrate via the blood stream to the lungs, escape into the alveoli where they molt twice to become a parthenogenetic female, pass up the trachea, and are swallowed. Variations on the pulmonary migration route may be possible.

Once in the intestine, the worms enter the mucosa and submucosa. Humans become infected by contamination with infected soil or feces; autoinfection is also possible as, in some cases, larvae can reinfect the same host be reinvading the gut wall and migrating via the pulmonary route or penetrating the skin in the perianal region. Prevention involves improvements in sewage disposal and personal hygiene.

Symptoms of intestinal infection vary from none to severe enteritis, although diarrhea is most common. Very heavy infections may result in damage to the mucosa. Penetration of the skin by larvae may cause a rash and symptoms similar to a cutaneous larva migrans, and the passage through the lungs may result in a cough or pneumonia. Autoinfection may cause the disease to last for many years. Hyperinfestation can occur in immunosuppressed patients (see *Strongyloides* and *Capillaria*).

SUPERFAMILY SPIRUROIDEA ÖRLEY, 1885

Family Gongylonematidae Hall, 1916

Main features Spiruroidea; body covered with large verruciform thickenings; head bilaterally symmetrical; mouth octagonal; pseudolabia absent; buccal cavity cuticularized, short; esophagus divided into anterior muscular and posterior glandular regions; caudal alae present in male; adult in mucosa of anterior alimentary canal of birds and mammals.

A species of *Gongylonema* Molin, 1957, the 'gullet worm' of domestic ruminants, has been recorded in humans as an accidental parasite. These worms usually occur in the esophagus or stomach of various mammals embedded in the mucosa or submucosa. They are readily recognized by the verruciform cuticular thickenings on the surface of the body.

The life cycle is indirect. Eggs of these worms are voided with the feces and hatch once they have been swallowed by an insect, such as a coprophagous beetle. The final host becomes infected by feeding on beetles harboring the third-stage larva. Once in the new host, the worms molt twice and penetrate the surface layers of the anterior parts of the alimentary canal, where they reside. Humans, in whom infections are usually sited in the mucosa and submucosa of the buccal cavity, presumably become infected by accidentally eating beetles. Prevention can be helped by regular de-worming of farm animals and careful washing of food items.

Although the worms may be quite large, their presence tends to cause little in the way of symptoms apart from some local irritation and inflammation (see *Angiostrongylus* (*Parastrongylus*) and less common nematodes).

SUPERFAMILY STRONGYLOIDEA BAIRD, 1853

Family Chabertiidae Popova, 1952

Main features Strongyloidea; mouth round or oval, usually surrounded by well developed comb-like leaf-crown (single or double) or lips; buccal cavity cuticularized; esophagus claviform, not divided into two regions; male with caudal bursa supported by rays, dorsal ray usually with two branches on each side of median fissure; in alimentary canal of mammals.

Strongyloid nematodes are occasional accidental parasites of humans. Chabertiids usually occur in the intestine of various herbivorous mammals, including farm animals, where they often cause nodules in the intestinal wall. Species of the genera *Oesophagostomum* Molin, 1861 and, less frequently, *Ternidens* Railliet and Henry, 1909 that parasitize primates, have been reported in humans. They are recognized by the presence of a double crown of leaf-like spines which surround the mouth, a cuticular buccal capsule and by the wide caudal bursa of the male which is supported by rays.

The life cycle is direct. Eggs laid in the intestine pass out with the feces; the first-stage larva emerges from the egg and feeds, developing into an infective third-stage larva. The host, usually a herbivore, acquires the worms when feeding on vegetation; the larvae penetrate the intestinal mucosa, grow and molt, prior to returning to the intestine, molting once again, and maturing. Humans presumably become infected by accidentally ingesting larvae with vegetation from the vicinity of primate populations. In some situations, the parasite can be passed between humans.

When the larvae penetrate the gut wall, they become encapsulated in a cyst that forms a fibrous nodule. Rupture of the nodule, which occurs when the worm returns to the lumen, may result in dysentery or even peritonitis. These

nodules are sometimes misinterpreted as other diseases, such as carcinomas. The worms can also occur in subcutaneous cysts in humans, but how they arrive in this position is not known (see *Angiostrongylus* (*Parastrongylus*) and less common nematodes).

Family Syngamidae Leiper, 1912

Main features Strongyloidea; mouth hexagonal, leaf-crown and lips rudimentary or absent; buccal cavity cuticularized, hexagonal in cross-section, with teeth at base; esophagus claviform, not divided into two regions; male with caudal bursa supported by rays; vulva usually near mid-body; usually in respiratory system of birds and mammals or urinary system of pigs.

One genus of syngamid, *Mammomonogamus* Ryzhikov, 1948, is occasionally reported from humans in the tropics. Syngamids are usually parasitic in the respiratory system (trachea and bronchi) of domesticated ruminants and infect humans only accidentally. Syngamids are recognizable in that the male and larger female are usually joined together in a Y formation; they differ considerably from other lungworms (the metastrongyloids) in the presence of a large, cuticular buccal cavity.

The life cycle of syngamids may be direct, similar to that of the chabertiid strongyloids, or indirect and involve paratenic hosts. Eggs produced in the respiratory tract are swallowed with mucus and voided with the feces. Development in the soil is similar to that of chabertiids, except that the larvae may be eaten by a variety of invertebrate hosts, such as earthworms, snails, slugs, and arthropods, which act as paratenic hosts. If the third-stage larva or its paratenic host is eaten by a suitable vertebrate, the larvae penetrate the gut lining and migrate to the lungs via the blood system. Here they molt and develop to maturity in the respiratory tract. Humans become infected by accidentally ingesting the larvae or its paratenic host. Some prevention is possible by the careful washing of salad material and other vegetation.

Worms in the trachea, larynx, and bronchi may cause irritation and a cough.

SUPERFAMILY THELAZIOIDEA SKRJABIN, 1915

Family Thelaziidae Skrjabin, 1915

Main features Thelazioidea; body often small, cylindrical, unspined; head bilaterally symmetrical; pseudolabia absent; mouth hexagonal or oval; buccal cavity cuticularized, variable, sometimes long, and cylindrical; esophagus may or may not be visibly divided into anterior muscular region and longer posterior glandular region; male tail without caudal bursa, caudal alae usually absent; transmission indirect with invertebrates (but not copepods) and vertebrates acting as intermediate hosts; in orbit of birds and mammals.

One thelaziid genus, *Thelazia* Travassos, 1918, normally parasitic in domesticated animals such as dogs and farm animals, has occasionally been reported in the eye of humans. Thelaziids can be recognized by their location in the eye, the possession of a cuticularized buccal cavity, and the absence of pseudolabia.

Thelaziid worms usually occur in the orbit, conjunctival sac or lachrymal glands. First-stage larvae occur in eye secretions. If ingested by a fly, they molt twice in its tissues, migrate to the mouth parts, and are transmitted to the mammalian host, and presumably to humans, when the fly feeds on eye secretions. Two final molts occur in the eye. There seem to be no practical control measures.

Symptoms caused by the presence of these worms include excessive tear formation, itching, and some discomfort. In animals, conjunctivitis and some damage to the eye tissues may occur, but the worms are not generally very pathogenic.

SUPERFAMILY TRICHOSTRONGYLOIDEA LEIPER, 1908

Family Trichostrongylidae Leiper, 1908

Main features Trichostrongyloidea; body usually not coiled; longitudinal cuticular ridges present or absent; cephalic vesicle absent; male with caudal bursa supported by rays, dorsal ray usually short and not deeply divided; life cycle direct; in mammals such as lagomorphs and ruminants, occasionally in birds.

Numerous trichostrongyloid genera, common stomach parasites of domesticated ruminants, have been reported as accidental parasites of humans. These include *Ostertagia* Ransom, 1907, *Nematodirus* Ransom, 1907, *Haemonchus* Cobb, 1898, *Marshallagia* Orloff, 1933, and, by far the most frequently, *Trichostrongylus* Looss, 1905; with the exception of the molineid *Nematodirus*, these are all trichostrongylids. Trichostrongylids are generally recognizable by the presence of a series of longitudinal cuticular ridges which run along the body, a caudal bursa supported by rays in the male and their presence in the stomach or intestine.

The life cycle is direct. Eggs are passed out with the feces, hatch on the ground, and release a first-stage larva that feeds and develops in the soil. After two molts, the infective third-stage larva can be transmitted to a suitable herbivore on vegetation. Within the gut, the worms molt twice prior to maturing as adults. Humans presumably become infected by feeding on raw vegetation harboring the larvae. Some level of prevention is possible by the cooking or careful washing of vegetables and other plant material.

Light infections pose no problem, but diarrhea and some abdominal pain have been reported. Limited damage at the site of attachment may occur as the head of the worms tend to penetrate the mucosa (see

Angiostrongylus (*Parastrongylus*) and less common nematodes).

PHYLUM ACANTHOCEPHALA

Acanthocephalans, or 'thorny-headed worms', represent a phylum of parasites of uncertain affinities. Like the tapeworms, members of this group are so well adapted to parasitism that they have lost their digestive system and reduced their muscular, nervous, and excretory systems, but they differ in that they are dioecious worms with a body cavity. The body is composed of a large trunk and a retractable anterior proboscis armed with a regular array of hooks used for attachment to the gut wall (Figure 4). Like tapeworms, they are intestinal parasites and absorb nutrients through their body-wall. Movement of the proboscis and its armature is complex, involving retractor muscles, a muscular proboscis receptacle, and fluid-filled sacs (lemnisci). The body wall is a syncytium with large, often fragmented nuclei; it comprises a complex outer tegument and an inner layer of muscles, and contains a lacunar system of interconnecting canals that may serve as a circulatory system. The males contain two testes and a series of accessory glands called cement glands. In females, the ovary breaks down early in development to form ovarian balls; the body cavity of mature females is usually full of eggs.

The life history of acanthocephalans is remarkably constant relative to that of other helminth groups. All have the same larval stages and all utilize arthropods as intermediate hosts. Essentially, female worms in the intestine produce eggs that are voided with the feces. If eaten by a suitable arthropod, the egg releases a larva armed with hooks, called an acanthor, which enters the hemocoel. This develops into a stage called an acanthella within which the internal organs of the adult begin to develop. When the proboscis develops, it usually invaginates into the body and the larva becomes encysted; this resting stage is called a cystacanth (Figure 4). The final host becomes infected either by feeding directly on an arthropod harboring the cystacanth larva or a vertebrate paratenic host harboring a juvenile form which is basically a re-encysted cystacanth. Once in the intestine of a suitable final host, the parasites attach to the gut wall and develop to maturity. In some cases, where the final host does not normally feed on arthropods, the paratenic host becomes a necessary part of the life history.

The different classes of acanthocephalans are not readily distinguished using gross morphology, their systematics are instead based on internal details that are often difficult to elucidate. At the specific level, the armature of the proboscis, in terms of the number of rows of hooks and the number of hooks per row, is the most important criterion.

No acanthocephalans are primarily human parasites. Data on their pathogenicity are limited, as records in humans tend to be scarce. However, since the proboscis with its armature of hooks does penetrate the gut wall,

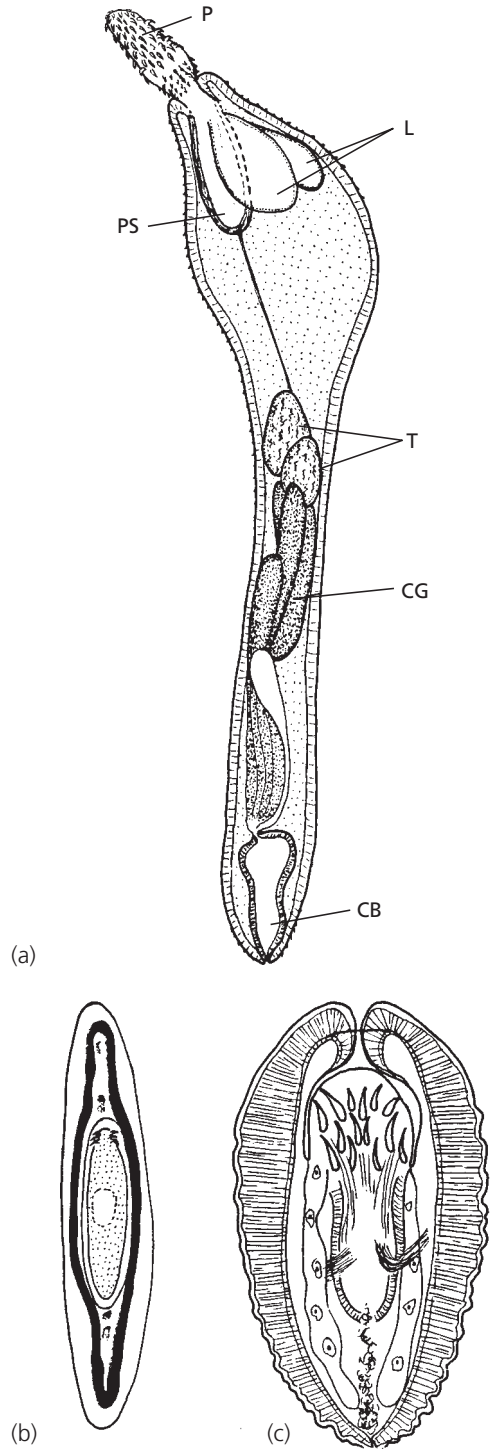

Figure 4 *Acanthocephalans:* **(a)** *male* Corynosoma *(Polymorphidae) (the body cavity of the female is usually full of eggs, apart from which it contains no other useful taxonomic features not present in the male);* **(b)** *egg of* Bolbosoma *(Polymorphidae) containing acanthor larva;* **(c)** *cystacanth larva of* Macracanthorhynchus *(Oligacanthorhynchidae) with withdrawn proboscis. Copulatory bursa (CB), cement glands (CG), lemnisci (L), proboscis (P), proboscis sac (PS), testes (T). Adapted from* **(a)** *Yamaguti 1963;* **(b)** *Meyer 1933;* **(c)** *Van Cleave 1947.*

some local inflammation and abdominal pain is to be expected; but, as the worms do not feed on the gut wall and their location tends to be semi-permanent, this may not persist. Nevertheless, in some cases, especially when the juvenile parasite finds the human gut unsuitable for development, it may pass right through and perforate the intestine with a consequent possibility of causing peritonitis.

The classification of forms recorded from humans below is based on Schmidt (1972) and Amin (1985):

Class Archaeacanthocephala
  Order Moniliformida
    Family Moniliformidae
  Order Oligacanthorhynchida
    Family Oligacanthorhynchidae
Class Palaeacanthocephala
  Order Echinorhynchida
    Family Echinorhynchidae
  Order Polymorphida
    Family Polymorphidae.

Class Archiacanthocephala Meyer, 1931

Main features Acanthocephala; relatively large worms; spines on body trunk absent; main longitudinal vessels of lacunar system dorsal or dorsal and ventral; subcuticular nuclei few, elongate, branched or with residual fragments situated close together; cement glands of male usually eight, uninucleate; insects (and millipedes) act as intermediate hosts; adults in intestine of terrestrial vertebrates.

ORDER MONILIFORMIDA SCHMIDT, 1972

Family Moniliformidae Van Cleave, 1924

Main features Moniliformida; body medium-sized to long, usually with pseudosegmented wall; proboscis cylindrical with long, straight rows of hooks; proboscis receptacle with double-layered wall, retractor muscles piercing its posterior end and cerebral ganglion positioned posteriorly; protonephridial organs absent; eggs oval, with sculptured surface; in birds and mammals.

The order Moniliformida has only one family containing a single genus, *Moniliformis* Travassos, 1915. This genus, which normally occurs in rodents, has been reported on rare occasions in humans. It is recognizable by a pseudosegmentation of the body (i.e. a series of annular thickenings of the body wall) and its cylindrical proboscis. The larger females can reach more than 20 cm in length.

Eggs of these intestinal worms are passed in the feces. When eaten by an insect, normally a beetle (especially a grain beetle) or a cockroach, the cystacanth larva develops in the hemocoel. If the arthropod host is eaten by a rodent, the worms excyst in the gut, the proboscis evaginates, and the worm attaches to the intestinal wall and develops to maturity. Humans become infected by accidentally ingesting beetles with food. Prevention can presumably be effected by rodent control and keeping food intended to be eaten cold in beetle-proof containers.

Since the proboscis of these worms does penetrate the gut lining, it is likely to produce some local inflammation. Symptoms such as abdominal pain and diarrhea have been reported, but data on the effect of these parasites in humans are few.

ORDER OLIGACANTHORHYNCHIDA PETROCHENKO, 1956

Family Oligacanthorhynchidae Southwell and MacFie, 1925

Main features Oligacanthorhynchida; Body medium-sized to very long; proboscis subspherical with short, straight rows of small numbers of hooks; sensory papilla present at apex of proboscis and each side of neck; proboscis receptacle with thick, single-layered wall, retractor muscles piercing it dorsally and cerebral ganglion positioned on ventral inner surface; protonephridial organs present; in birds and mammals.

These parasites are especially common in pigs, but also occur in a wide range of terrestrial mammals. They are commonly covered in transverse wrinkles but lack any regular pseudosegmentation; the females especially reach a large size, sometimes more than 40 cm. One genus, *Macracanthorhynchus* Travassos, 1917, occurs on rare occasions in humans.

The life cycle is similar to that of moniliformids except that, although a range of beetles, cockroaches, etc. can harbor the parasite, the main intermediate hosts would appear to be dung beetles that become infected when they feed on manure. Humans acquire the parasite by ingesting beetles as food, accidentally with food or as a cure for ailments. The keeping of food intended to be eaten cold in beetle-proof containers should help prevent accidental infection.

The worms cause inflammation and a granuloma at the site of attachment; but abdominal pain and perforation of the bowel have been reported and, in pigs, death from peritonitis does occasionally occur.

Class Palaeacanthocephala Meyer, 1931

Main features Acanthocephala; small to medium-sized worms; spines present or absent on trunk; proboscis shape variable; main longitudinal canals of lacunar system lateral; subcuticular nuclei numerous, fragmented, occasionally limited to anterior region of trunk; cement glands of male separate, tubular to globular; in intestine of aquatic vertebrates.

ORDER ECHINORHYNCHIDA SOUTHWELL AND MACFIE, 1925

Family Echinorhynchidae Cobbold, 1876

Main features Echinorhynchida; body not spined; normally in intestine of bony fish and amphibians.

Two echinorhynchid genera, *Acanthocephalus* Koelreuter, 1771 and *Pseudoacanthocephalus* Petrochenko, 1956, have been reported as occurring accidentally in humans. These genera normally occur in the intestine of fishes or amphibians. Humans presumably acquire the parasite by feeding on freshwater crustacean intermediate hosts harboring the cystacanth larvae or a vertebrate host that had ingested the cystacanth. They are unlikely to have any great pathogenic effect, although there is one report of a young worm having penetrated the gut wall and ended up on the peritoneum.

ORDER POLYMORPHIDA PETROCHENKO, 1956

Family Polymorphidae Meyer, 1931

Main features Polymorphida; body spined; normally in intestine of aquatic birds and marine mammals.

Two polymorphid genera, *Corynosoma* Lühe, 1904 and *Bolbosoma* Porta, 1908, are occasionally reported from humans. These are normally parasitic in marine mammals, usually whales and dolphins in the case of *Bolbosoma* and seals in the case of *Corynosoma*. In addition to their marine link, these worms are recognizable by the presence of spines on the trunk of the body and, in the case of *Bolbosoma*, by a large expansion of the neck region posterior to the proboscis.

The basic life history of these marine polymorphids is similar to that of the terrestrial forms, but the eggs are voided with the feces into water and the arthropod host harboring the infective cystacanth larva is a crustacean rather than an insect. Furthermore, bony fishes, which feed on infected crustaceans, act as paratenic hosts, the cystacanth larva passing through the fish's gut wall and re-encysting as a juvenile worm. Marine mammals become infected by feeding on these fishes, which, in cases where the definitive host does not feed on crustaceans, represent a necessary step in the life cycle. Humans become infected by feeding on raw or poorly cooked marine fish. It seems unlikely that the worms can mature in humans. The proper cooking or freezing of fish would kill the parasite.

There is little information on the pathogenicity of these worms; but they are known to begin to develop in humans, so penetration of the gut wall by the proboscis will elicit local inflammation and symptoms of abdominal pain (which is sometimes acute). There is one reported case of perforation of the intestine.

NOTE ADDED BY THE EDITORS FOR THIS EDITION

The classification of helminths as described in the 9th edition has remained essentially unchanged, and therefore that chapter has been repeated in this edition. It still provides an excellent and systematic introduction to the variety of helminths and provides the basis for accurate identification and taxonomic work.

During the last five years there has been an increasing interest in molecular methods of identification and classification to supplement the more traditional morphological approach. An important element of this work has been the development of genetic markers, such as mitochondrial DNA, that are valuable not only in taxonomy, but also for studies relevant to epidemiology, population biology, and parasite control. Although genetic studies with helminths are less complete than those carried out with some of the protozoan parasites, there are a number of programmes designed to provide genomic maps of species of major interest in human medicine, and extensive computer databases of sequences are available. The further reading section at the end of this chapter lists some recent references that will be useful for readers wishing to extend their knowledge of these areas of classification and parasite biology.

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Schistosomes: general

SOMEI KOJIMA AND ANDREW S. MACDONALD

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THE PARASITES

Schistosomes are the causative agents of the disease schistosomiasis. Over 190 million people in 76 countries and territories in the world are affected and about 652 million are at risk, according to recent calculations applied to the 1995 world population estimates (Engels et al. 2002). In terms of global clinical impact, an estimated 85 percent of people infected with schistosomes are thought to be located in the African continent (Engels et al. 2002).

Classification

The human schistosomes or blood flukes are digenetic trematodes belonging to the superfamily Schistosomatoidea of the suborder Strigeata. They differ from other trematodes in that (1) the adults are dioecious, being either male or female; (2) the adult worms parasitize blood vessels; (3) they lack a muscular pharynx; (4) they produce nonoperculate eggs; and (5) the cercaria, with a bifurcated tail, invades the final host percutaneously. Several species can infect humans (Table 1). Four, *S. japonicum*, *S. mekongi*, *S. mansoni*, and *S. haematobium*, are important agents of human schistosomiasis. *S. intercalatum* has a very restricted distribution in humans; *S. bovis* and *S. mattheei*, although primarily parasitic in other mammalian hosts, may produce infections in humans. In addition, human cases with *S. malayensis* have been described among aborigines in Peninsular Malaysia (Murugasu and Por 1973; Murugasu et al. 1978). The advent of modern molecular techniques should make it possible to construct a detailed map of genetic diversity both between species, and within populations, of schistosomes (Rollinson et al. 1997; Oliveira and Johnston 2001).

Morphology and structure

The human-infective schistosome species appear similar morphologically. Near the anterior end of the body there are two suckers, oral and ventral. Following a short esophagus, the intestine divides at the level of the ventral sucker into two parallel gut ceca that rejoin behind the gonads to form a blind cecum ending near the posterior end of the body. In the male, between four and nine testes (depending upon the species) are usually present on the dorsal side just below the ventral sucker. The vas efferens from each of the testes leads to the common vas deferens which runs via a small seminal vesicle to the genital pore situated posterior to the ventral sucker. On the ventral side, the body is flattened and folded to form the gynecophoric canal (Figure 1), in which the longer and more slender female is held firmly (Figure 2). In the females, the ovary is located near the posterior union of the lateral gut ceca about halfway along the body. The vitelline glands surround the cecum from the extreme end of the body up to the level of the posterior end of the ovary. The oviduct runs forward from the end of the ovary and joins the vitelline duct to form the ootype, where ova are fertilized, provided with vitelline materials and shells, and then passed into the uterus. The uterus is a long straight tube and opens behind the ventral sucker; 50–150 eggs

Table 1 *Schistosomes that may parasitize humans*

Schistosome species	Final hosts	Distribution
S. haematobium	Human, monkey, baboon, chimpanzee	Africa, Middle East
S. japonicum	Human, dog, cat, rodent, pig, sheep, goat, water buffalo, horse	China, Philippines, Celebes
S. malayensis	Human, rodent	Malaysia
S. mansoni	Human, monkey, baboon, chimpanzee, dog, rodent	Africa, South America, Caribbean
S. mekongi	Human, dog	Laos, Cambodia, Thailand
S. intercalatum	Human	West and Central Africa
S. bovis	Cattle, sheep, goat, equine, baboon, human	Southern Europe, Africa, Iraq
S. mattheei	Horse, sheep, cow, zebra, antelopes, baboon, human	Southern Africa

develop in utero at one time. Ova deposited in tissues develop to the larval stage, the miracidium, within a week. Passage of the eggs through the vein wall and tissues to the lumen of the intestine is aided by the release through micropores in the shell of histolytic enzymes secreted by the miracidium.

In comparison to both *S. mansoni* and *S. haematobium*, the *S. japonicum* tegumental surface appears smooth under light microscopy, rather than overtly tuberculated. Although the structure of adult worms has been studied mainly using *S. mansoni*, it is considered basically common among other schistosomes in that the worm body is covered by a syncytial tegument approximately 4 μm in thickness, in which surface pits form deep, tortuous channels which may be branched and interconnected, thereby increasing the effective surface by a factor of as much as 10 (Hockley 1973). The physiological role of the pits is as yet unknown. The external surface of the tegument is a plasma membrane composed of two lipid bilayers (Hockley and McLaren 1973), of which the outer may fuse with foreign lipid membranes of host neutrophils and eosinophils (Caulfield et al. 1980), probably as a result of the production of monopalmitoylphosphatidylcholine by the parasite (Furlong and Caulfield 1989). Since the parasite cannot synthesize essential lipids de novo, lipids, particularly free fatty acids, bound to serum proteins such as albumin, provide potential sources for the parasite. The parasite also has receptors for serum lipoproteins on the outer bilipid membrane, by which the parasite may acquire another source of lipids from the host (Rumjanek et al. 1983; Rogers et al. 1990). The outer bilipid membrane is shed continuously and replaced by products of organelles transported through a channel originated from the tegumental cells that are located under the circular and longitudinal muscle layers.

Schistosomes absorb glucose from the host plasma through the tegument (Fripps 1967; Rogers and Beuding

Figure 1 *Scanning electron micrograph of a male worm of* Schistosoma japonicum, *showing the gynecophoric canal*

Figure 2 *Adult worms of* Schistosoma mansoni

1975; Uglem and Read 1975). Glucose must be transported to internal cells across several membranes such as the two lipid bilayers and the basal membrane of the tegument, as well as the plasma membranes of internal cells, a transport system that is unique to schistosomes. Indeed, two cDNA clones encoding the proteins SGTP1 and SGTP4, with features conserved in the facilitated diffusion glucose transporter family, have been identified by screening an adult *S. mansoni* cDNA library (Skelly et al. 1994). Immunolocalization studies have revealed the presence of SGTP4 in both of the lipid bilayers that cover the tegumental surface of adults and schistosomula, and also in the discoid and multilamellar bodies in adults, as well as in the membranous bodies in schistosomula (Jiang et al. 1996), while SGTP1 is present in the basal membrane of the tegument (Zhong et al. 1995). These results suggest that SGTP4 may be responsible for transporting glucose from plasma into the tegument, whereas SGTP1 may function to transport free glucose from the tegument into the extracellular matrix beneath the tegument, as well as into the muscle (Jiang et al. 1996; Zhong et al. 1995). These proteins are homologs of GLUT-1, the human glucose transporter protein (Mueckler et al. 1985).

Absorbed glucose is metabolized through the Embden–Meyerhof pathway and the Krebs cycle. Studies on the mode of action of anti-schistosomal drugs have demonstrated the importance of the key enzymes involved in these pathways as the target of chemotherapy. For example, compounds of antimony, though no longer used for treatment because of their side effects, inhibit phosphofructokinase activity at concentrations 70–80 times lower than those inhibiting the isozyme of the host, while praziquantel, the most effective drug currently available, affects the transport of calcium ions across cell membranes, which may result in tegumental vacuolization and disruption (Mehlhorn et al. 1981, 1983).

Adult worms also ingest red blood cells through the mouth and utilize hemoglobins, as well as serum globulins as nutrients after cleaving by proteolytic enzymes. Binding of the proteolytic enzymes to hemoglobin is optimal at pH 4.0 in the lumen of the gut. Since their digestive system ends in a blind cecum, residual black hematin is regurgitated together with the proteolytic enzyme to the circulation, from where it is taken up by phagocytic cells in the liver and spleen of the host. Meanwhile, the enzyme–hemoglobin complex dissociates owing to the change of pH, resulting in release of the enzyme in the plasma as a circulating antigen that in turn sensitizes hosts, inducing immediate-type hypersensitivity reactions to the enzyme (Senft and Maddison 1975).

It is likely that the female may obtain supplementary nutrients through the intimate contact with the male ventral tegument. This may be related to the fact that unisexual infections usually fail to induce sexual maturation of females, although certain lipid metabolites, such as ecdysteroids and dolichols, have been suggested to act as promotors for sexual development and egg production by adult worms, respectively (Furlong 1991; Fried and Haseeb 1991).

Life cycle

The life cycle of all species follows a common pathway from the sexual generation of adult schistosomes within the vascular system of the definitive host to asexual generations in the intermediate host of freshwater snails, and a return to the definitive host by skin penetration of infective larval stages (Figure 3).

When embryonated eggs discharged in feces or urine enter fresh water, miracidia hatch from the eggs, rapidly swim by means of cilia that cover the body surface except the apical papilla, and penetrate the exposed surface of the body of snail intermediate hosts. The attachment and penetration of the miracidium are assisted by secretions from the anterior penetration glands which open anterolaterally via ducts to the base of the apical papilla. The genera of snails that act as intermediate hosts differ from species to species of schistosome (Table 2). Moreover, differences in host specificity may exist among species or strains of the snail intermediate hosts in terms of infectivity of geographically different strains of schistosomes.

After penetration into the intermediate host, the miracidium loses the ciliated surface and develops into mother and daughter sporocysts, and then produces fork-tailed cercariae in the course of several weeks (4 weeks in the case of *S. mansoni* under favorable conditions but longer in other species, especially in *S. japonicum*) (Figure 4). As a result of asexual multiplication within mother and daughter sporocysts, thousands of cercariae, all of the same sex, are produced from a single miracidium. The cercariae measure around 400 μm in length including the tail, the terminal one-third of which is bifurcated. Their surface and internal structures are essentially identical among schistosome species. They have a muscular oral sucker occupying

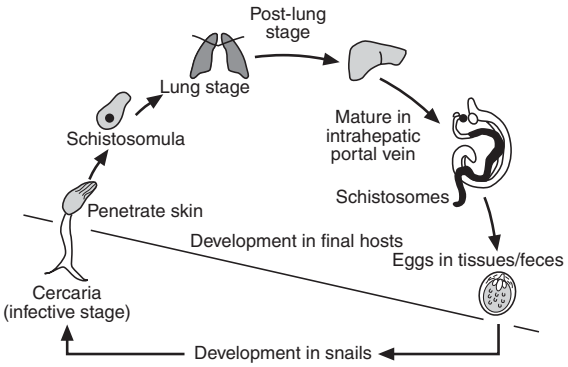

Figure 3 *Life cycle of* Schistosoma japonicum

Table 2 *Genera of snails acting as intermediate hosts for the human schistosomes*

Schistosome species	Intermediate snail hosts
S. haematobium	*Bulinus* and *Physopsis* spp.
S. japonicum	*Oncomelania* spp.
S. malayensis	*Robertsiella*
S. mansoni	*Biomphalaria* spp.
S. mekongi	*Neotricula aperta*

about one-third of the body and a small ventral sucker or acetabulum. The cercariae infect the definitive host by penetrating the skin, aided by proteolytic enzymes secreted from the penetration glands, losing their tails and transforming to the next larval stage, the schistosomulum. During the whole penetration process, drastic morphological and physiological changes take place in the larvae. Thus, the cercarial glycocalyx is lost and replaced by a double bilayer; this is marked by a change from a trilaminate appearance to a heptalaminate membrane.

Schistosomula migrate into the lungs via the venous circulation. This migration route seems to be essential for their development and is associated with the acquisition of biological differences, since schistosomula recovered from the lungs are not only elongated but are more resistant than newly transformed schistosomula to antibody-dependent cell-mediated cytotoxicity, although the mechanisms are as yet unknown. The schistosomula break out from the lungs through the pulmonary capillaries and are carried through the left heart into the systemic circulation, finally reaching the portal system. It takes 3–4 days for *S. japonicum* schistosomula to migrate into the lungs after skin penetration, followed by another 3–4 days stay in the lungs; *S. mansoni* reaches the lungs and leaves for the final destination at least 2 days earlier than *S. japonicum* (Usawattanakul et al. 1982). Some schistosomula may take another route through the diaphragm to reach the liver. In the intrahepatic portal circulation, feeding begins and further growth occurs. The lateral gut ceca grow posteriorly and eventually unite behind the ventral sucker and the developing gonads. Within several weeks, pairing of the worms takes place on sexual maturation, and they migrate to the mesenteric veins, where the females lay eggs. For *S. mansoni* and *S. japonicum*, the prepatent period between penetration by cercariae and the first appearance of eggs in the excreta is around 35 days.

Figure 4 *Cercariae, the infective larval stage*

Once the eggs have been released by the female within the vasculature, they cross the endothelium and basement membrane of the vein, traverse the intervening tissue, basement membrane, and epithelium of the intestine (*S. mansoni* and *S. japonicum*) or bladder (*S. haematobium*) en route to the exterior. While it is unclear precisely how this process occurs, there may be an immunological component since egg excretion is reduced in immunocompromised mice, but can be increased by transfer of sera or lymphocytes from infected animals (Doenhoff 1997). Further supporting this idea, *S. mansoni*-infected Human immunodeficiency virus (HIV)-positive individuals, who have significantly reduced $CD4^+$ T cell counts, display significantly diminished egg excretion in comparison to HIV-negative controls (Karanja et al. 1997).

Transmission and epidemiology

As is obvious from the life cycle of schistosomes, human infections depend absolutely on the presence of intermediate snail hosts in bodies of water which may be contaminated with human feces and excreta as a result of insanitary habits or polluted with excreta from reservoir hosts including livestock, which have to be considered in disease control, especially in the case of *S. japonicum* infection. Thus, transmission is influenced by biological, economic, and sociocultural factors, such as (1) the distribution, biology and population dynamics of the intermediate hosts; (2) the patterns and extent of environmental contamination with human excreta through unsanitary conditions and utilization as fertilizer, or with excreta from domestic animals in the case of *S. japonicum*; (3) human water contact activities, patterns, and duration; (4) water development projects including extension of irrigation systems and construction of dams; and (5) most seriously, people's knowledge of the disease itself and their attitudes towards, or practices for, avoiding the infection.

Despite its public health importance in many tropical and subtropical areas, which is second only to that of malaria, schistosomiasis has been largely ignored and/or given a low priority in health policy mainly because of the low socioeconomic status of developing countries, and the tendency to give economic development priority over health and welfare. Thus, water development projects, particularly construction of dams, artificial lakes, and irrigation canals, often associated with

dynamic population resettlement, have become a major factor in the spread and intensification of the disease.

Schistosomiasis may also be categorized as one of the emerging and re-emerging diseases. A typical example is an outbreak of intestinal schistosomiasis in the delta of the Senegal river basin, where the presence of the disease had not been reported before the mid-1980s. In the 1980s the Diama dam was constructed at the mouth of the river to avoid a backward flow from the sea. A year and a half after it became operational (August 1986), a first case of *S. mansoni* infection was detected at Richard-Toll. The prevalence of *S. mansoni* infection rapidly increased from 1.9 percent in 1988 to 71.5 percent in late 1989, with an accumulative total of 1935 positive cases out of 3 926 stool examinations. *Biomphalaria pfeifferi* was found to be the major intermediate host in this area (Talla et al. 1990). Another example of schistosomiasis as a re-emerging disease occurred in Japan where endemic schistosomiasis was observed among dairy cows pastured along the bank of the Tone River in Chiba Prefecture, and also involved some inhabitants in the infection. This was 15 years after the disease was no longer considered to be endemic (Yokogawa et al. 1971, 1973).

Community-based epidemiological studies of schistosomiasis demonstrate that prevalence (expressed as the percentage of the population found infected at a given point of time) and intensity of infection (expressed in terms of egg output in excreta) are usually higher among younger age groups than in older age groups. A typical example obtained from a study of urinary schistosomiasis in a defined area of southern Ghana is shown in Figure 5, indicating that the peak prevalence and intensity occur in children aged 10–14 years, accompanied by a gradual decline in prevalence and intensity in older age groups (Aryeetey et al. unpublished data). This phenomenon is more marked in *S. haematobium* than in *S. mansoni*, but in *S. japonicum* infection the reduction in prevalence and intensity in older age groups may not be observed. The decline in egg output in community-based studies may reflect the development of immunity to re-infection, or alternatively the possibility of reduced water contact in the older age groups (see Immune responses).

Thus, the control of schistosomiasis apparently involves many factors and requires a long-term effort with a complex of strategies. So far, there are only three countries – Japan, Montserrat, and Tunisia – where the transmission of the disease is considered to have ceased (WHO 1993).

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations, pathogenesis, and pathology

Clinical symptoms and pathological changes caused by schistosome infections may reflect migratory routes and host responses to toxic or antigenic substances derived from developmental stages of the parasites after their penetration of the skin. By far the predominant cause of pathology resulting from infection is the host reaction to parasite eggs that are laid in the vasculature, and subsequently trapped within the host tissues. The specific pathological changes associated with the African and the Asian species are described in detail in.

In general, acute infections are not accompanied by symptoms among people living in endemic areas, unless they are exposed to a massive infection for a specific reason, such as flooding. In these individuals, pathological changes are caused mainly by schistosome eggs deposited in various tissues. The host immune response to antigens excreted from embryonated eggs, which is

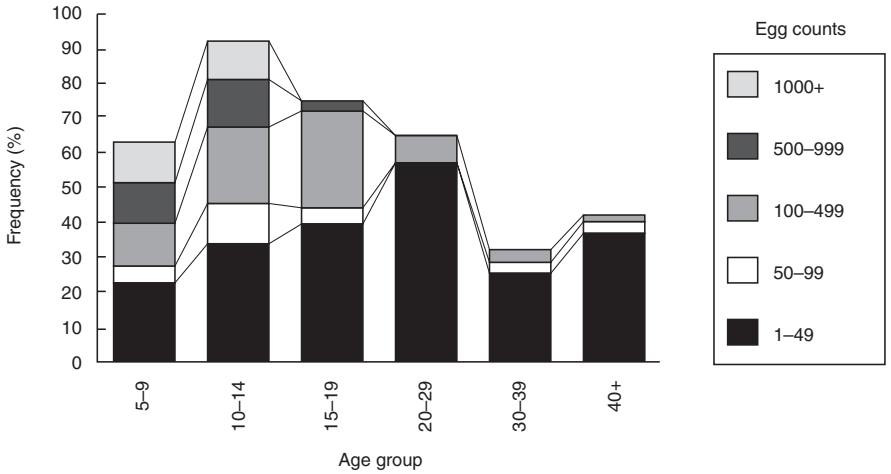

Figure 5 *Prevalence and intensity of* Schistosoma haematobium *infection in southern Ghana (Aryeetey et al. unpublished data)*

now the focus of cytokine studies, results in formation of localized inflammatory reactions to the eggs, which are termed granulomas. These granulomas appear to be simultaneously beneficial and detrimental for both host and parasite. In terms of the parasite, the granuloma may promote the process of egg excretion (Doenhoff 1997). In terms of the host, by effectively isolating the inflammatory reaction to eggs, they may protect host tissue adjacent to the granuloma (Sandor et al. 2003), while also sequestering potentially damaging toxins secreted by the eggs (von Lichtenberg 1964). However, the granulomas themselves are ultimately pathogenic, as they induce fibrosis, increased portal blood pressure, and development of portal systemic shunts.

In primary infections, the granuloma is composed of aggregations of mononuclear phagocytes, neutrophils, lymphocytes, plasma cells, and fibroblasts (Figure 6). Giant cells are also frequently observed in the granulomas. Granulomas may vary in size and cellular components with the immune status of the host; in experimental infections in immunized animals, a dominant cellular infiltration of eosinophils and lymphocytes is observed around the eggs, and the egg granuloma is smaller (Satoh et al. 1997).

Granuloma formation around schistosome eggs has been considered to be the result of delayed-type hypersensitivity reactions mediated through a T-cell mediated immune response to soluble egg antigens (Warren et al. 1967). However, recent studies have demonstrated that there exist at least two subsets of T helper cells with a $CD4^+$ phenotype, termed Th1 and Th2 cells, which can be distinguished from each other by their cytokine productions (Mosmann and Coffman 1989). The cytokines derived from Th1 cells, such as IL-2, interferon-γ, and tumor necrosis factor (TNF)-β, may be responsible for activation of macrophages and cell-mediated immunity, whereas IL-4 and IL-5, cytokines produced by Th2 cells, stimulate IgE production and eosinophilia, respectively (Mosmann and Coffman 1989; Takatsu et al. 1994) (see Immunology and immunopathology of human parasitic infections). The most detailed studies of pathogenesis have been carried out with *S. mansoni*, and these are described in Schistosomes: African.

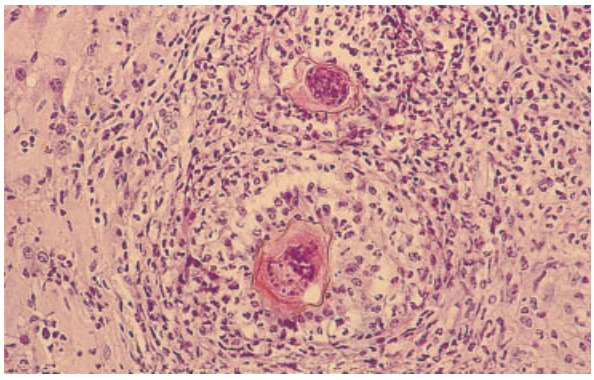

Figure 6 *Granuloma formation around newly deposited eggs of* Schistosoma japonicum

Perhaps a less appreciated consequence of schistosomiasis is that the physical effects of infection can result in attention deficits, learning difficulties, and absenteeism. Unfortunately, such 'downstream' effects can particularly affect children, who often harbor the highest levels of infection.

IMMUNE RESPONSES

It has been assumed from epidemiological studies in endemic areas that age-dependent immunity may develop against infection, or against re-infection after treatment, with *S. mansoni* (Butterworth et al. 1985; Dessein et al. 1988) and *S. haematobium* (Hagan et al. 1991) (for review, see Butterworth 1994; Gryseels 1994). Prevalence and intensity of infection are usually higher among younger age groups than in older age groups. Using a mathematical model, it has also been shown that predicted patterns of variation in age-related changes in the intensity and prevalence of *S. haematobium* infection are consistent with the epidemiological effects of acquired immunity (Woolhouse et al. 1991).

In *S. japonicum* infection, however, the reduction in prevalence and intensity in older age groups may not be observed. Prevalence by age and sex of *S. japonicum* infection in China based on national surveys carried out in eight endemic provinces in 1989 and 1995 revealed that the infection rates peaked at around the age of 35 years and remained high thereafter, especially in males, and that males were twice as likely to become infected as females (Ross et al. 2001), which might be due to male-dominated exposure to cercariae-contaminated water while fishing during the time coinciding with peak cercarial shedding, at least in the Dongting Lake region (Ross et al. 1998). Despite these observations, another study carried out in a well-defined population in Leyte, Philippines, was able to identify individuals resistant and susceptible to *S. japonicum* infection and to demonstrate that the latter individuals, who were most vulnerable to rapid re-infection, belonged to a younger age group of 5–14-year-old children, whereas the former, who belonged to the 15–19 year age group or older, showed decreased incidence and intensity (Acosta et al. 2002a). Cellular immune responses of these two groups to defined vaccine candidate antigens (see below) suggested that a Th1 type of response appeared to be important in predicting resistance in this population (Acosta et al. 2002b).

To better understand effector mechanisms in immunity to schistosomiasis, animal models may be useful at least for the initial steps of analysis, although results observed in animals do not always parallel those in humans (Cheever et al. 2002). There are two experimental models for the induction of immunity to schistosome infections in vivo: the concomitant immunity

model and the attenuated vaccine model. Concomitant immunity is so called after the immunity developed to a second tumor graft in animals that already carry the same tumor; thus animals (originally rhesus monkeys) carrying an initial infection of adult schistosomes show partial but significant protection to a cercarial challenge infection (Smithers and Terry 1967). Although this model may reflect more closely the immunity observed in humans, it has been replaced by the attenuated vaccine model, in which animals are immunized with irradiated cercariae that can migrate into the lungs or liver, but cannot mature. In the former model much of the parasite attrition appears to be closely linked to the pathology induced by the previous infection (Dean et al. 1978; Wilson et al. 1983; von Lichtenberg 1985) rather than to immunologically specific anti-parasite mechanisms.

As in other parasitic infections, cellular and humoral immune responses are observed in schistosomiasis against antigens derived from various developmental stages of schistosomes. Responses may be observed against stage-specific antigens or alternatively cross-reactive antigens common to various stages. Immune responses may vary due to host genetic differences, and a number of studies have identified particular genes regulating both protective immunity and pathology (Marquet et al. 1996; Dessein et al. 1999). In general, $CD4^+$ T helper cells (Th cells) play a major role in responses to schistosome infection through their release of cytokines (Wynn and Cheever 1995; Pearce and MacDonald 2002). Recent work using murine models to address the involvement of Th cells and their cytokines in immune response development during infection is covered in the following chapters.

Worm development and the immune response

Evidence is accumulating that host immune factors may actually act as developmental cues for schistosomes (Davies and McKerrow 2003). While it has been known for some time that parasite fecundity can be severely reduced in immunocompromised animals, there is continued debate about the identity of the host immune factors that are responsible for influencing schistosome development, with IL-7 (Wolowczuk et al. 1999), TNF (Amiri et al. 1992), and TGFβ (Beall and Pearce 2001) all being possible candidates.

Vaccination

Reflecting the complexity of the host–parasite relationship and underscoring the need to more fully understand both immune-response development and parasite biology, an effective rationally designed anti-schistosome vaccine has yet to be produced (Bergquist and Colley 1998; Wilson and Coulson 1998; Capron et al. 2001; Pearce 2003). Because there is a complex of cytokine networks and pathways of signal transduction, the development of vaccines against schistosomiasis must be carefully designed in order to focus particular effectors against specific target stages or molecules. In addition, further studies are urgently required in humans to elucidate immune effector mechanisms. It should be noted that chemotherapy with praziquantel may induce immunological changes (by burst destruction of adult worms and miracidia in eggs) in terms of antigen recognition, antibody classes, and cytokine responses, and that age- and sex-difference of the host may affect immune responses after treatment (Mutapi 2001). Vaccines that can reduce schistosomiasis morbidity and mortality by lowering intensity of the infection should be adopted for practical use, when they become available, even if they are not effective in inducing the complete elimination of parasites. Factors such as the technical feasibility of vaccine production, the prospects of passage through existing regulatory bodies, and the ease of incorporation into existing immunization programs, must also be taken into account (Bergquist et al. 2002). Compared to the other major schistosome infections, schistosomiasis japonica has a characteristic nature as a zoonosis, giving serious economic problems for livestock. However, this characteristic may assist the development of a schistosomiasis vaccine for livestock as the first step, then followed by further application to humans.

Details of vaccines for African and Asian schistosomes are given in and respectively.

DIAGNOSIS

Specific diagnosis of schistosomiasis can be made by detection of the characteristic eggs in the stools or urine under microscopic examination. Biopsy at rectoscopy or cystoscopy may reveal eggs in the mucosa. For quantitative determination, which is necessary for evaluation of the success of chemotherapy or control operations, the Kato-Katz smear technique (though semiquantitative) or urine filtration technique may be used for intestinal or urinary schistosomiasis, respectively.

Indirect methods for diagnosis depend on clinical symptoms and signs, and biochemical or immunological analyses. Immunodiagnosis may be useful for demonstration of active or chronic schistosomiasis. A unique immunological method for the diagnosis of schistosomiasis is the circum oval precipitin (COP) test in which precipitate is formed around the eggs containing live miracidia after incubation in the serum of infected individuals (Figure 7). The enzyme-linked immunosorbent assay (ELISA) is also widely used in diagnosis.

The selection and application of any diagnostic method used for field studies must correspond to the type of information sought by the public health officer or epidemiologist, based on operational and financial

Figure 7 *Circum oval precipitin (COP) test*

constraints, and such constraints and drawbacks must be taken into consideration for interpretation of test results (Feldmeier and Poggensee 1993).

CHEMOTHERAPY, PREVENTION, AND CONTROL

The drug of choice is praziquantel, which is effective against all species of schistosomes, although the precise mechanism by which it kills the parasites remains to be clarified (Redman et al. 1996). In vitro treatment of *S. japonicum* with praziquantel resulted in contraction of the parasite and the vacuolization of the tegument within 5 min after exposure to 1 μg/ml praziquantel in medium TC199 (Mehlhorn et al. 1983). Praziquantel induces an influx of calcium ions across the tegument, causing an immediate muscular contraction (Mehlhorn et al. 1981). Praziquantel-induced contraction was found to be biphasic in normal parasites when incubated in high-magnesium medium, although only the larger, second contraction was observed in worms of which the tegument had been removed (Blair et al. 1994). These observations may suggest the presence of praziquantel-sensitive sites in the tegument (Redman et al. 1996).

Since immature (2–4 week old) worms are less susceptible to praziquantel than are larval (1–2 week old) worms or adult (5 or more weeks old) worms (Fallon et al. 1996) and some isolates of *S. mansoni* have been shown to be resistant to praziquantel, it is important to find new drugs for schistosomiasis (Doenhoff et al. 2002). Quite recently, it has been reported that artemether, the methyl ether derivative of artemisinin, is not only effective against malaria but also therapeutically effective against schistosomes, and is more effective against 7-day-old schistosomules than other developmental stages of schistosomes (Xiao and Catto 1989).

Thus, artemether was shown to be effective for the early phase of infection with *S. mansoni* and *S. haematobium* and morphological alterations, such as swelling and fusion of tegumental ridges, vesiculation, peeling, and erosion were observed for schistsomula, and also for adult worms, with this compound (Utzinger et al. 2001b). Field trials carried out in endemic areas with a single oral dose of 6 mg/kg of artemether administered repeatedly between four and ten times every 15 days resulted in successful prophylactic effects on at least *S. japonicum* and *S. mansoni* infections (Xiao et al. 2002) and this was also confirmed later for *S. haematobium* infection by a randomized, double-blind, placebo-controlled trial, although the protective efficacy was considerably lower (N'Goran et al. 2003). Combination therapy with praziquantel plus arthemether may be recommended since it is more effective than each drug given separately (Xiao et al. 2000, 2002; Utzinger et al. 2001a). Long-term toxicity tests carried out in the rat with up to 11.7-fold the dose recommended for humans revealed no adverse effect on any organs so far examined, except rapidly reversible reduction of the reticulocyte count and rapidly reversible increase in hemoglobin levels (Xiao et al. 2002).

It is clear that a number of factors must be addressed to enable effective national control programs for schistosomiasis. Long-term provision of sufficient clinical care facilities, community-based treatment programs, adequate health education to reduce the chances of transmission and encourage local recognition of schistosomiasis as a public health problem, implementation of environmental management measures, and development of enhanced levels of sanitation and hygiene, are all required to sustain prevention of the emergence or spread of schistosomiasis in endemic areas (Engels et al. 2002).

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Schistosomes: African

SOMEI KOJIMA AND ANDREW S. MACDONALD

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THE PARASITES

Approximately 85 percent of the nearly 200 million individuals estimated to be infected with schistosomes worldwide are thought to live on the African continent (Engels et al. 2002). Three species occur there, *S. intercalatum*, *S. haematobium*, and *S. mansoni* (see Table 1). *S. intercalatum* has a restricted distribution, being found only in parts of West Africa (Cameroon, Equatorial Guinea, Gabon, Nigeria, Democratic Republic of Congo, and Sao Tomé – Tchuente et al. 2003). *S. mansoni* and *S. haematobium* are the main species that cause human schistosomiasis in Africa, although both species can also be found in the Middle East, and *S. mansoni* can be found in regions of South America and the Caribbean. Schistosomiasis is an important public health concern in these regions, with the estimated mortality rates attributed to *S. mansoni* and *S. haematobium* in sub-Saharan Africa alone being some 280 000 per year (van der Werf et al. 2003). For this reason, this chapter will focus on these two species.

Since ancient times there has been an awareness of schistosome infection in endemic areas of Africa. Hierographics in the papyrus of Kahun refer to hematuria (bloody urine), a typical sign of urinary schistosomiasis, which is mentioned 50 times in various medical papyri (Contis and David 1996). In Cairo, in 1851, Theodor Bilharz found a trematode at autopsy in the blood of mesenteric veins of a young man. It was not hermaphrodite, as were other flukes known at that time, but had separate sexes. He named the worm *Distomum haematobium* and described this as the cause of hematuria, based on the presence of terminal-spined eggs in the urine, although he considered lateral-spined eggs to belong to the same species. In 1859, Cobbold used a new generic name *Bilharzia* for the worm he found in the portal vein of a sooty monkey, although the name *Schistosoma* (meaning split body) had already been used by Weinland in 1858. In 1864, Harley found eggs with a terminal spine, but not those with a lateral spine, in the urine of patients with hematuria living in South Africa. In 1902, Manson found lateral-spined ova in a stool sample of a British patient with anemia instead of the hookworm eggs that he had expected, but no ova were detected in the urine of this patient, who had spent 15 years in the West Indies. He suggested the possible existence of two species of *Bilharzia*, one with terminal-spined eggs which were found in the urine, the other depositing lateral-spined eggs in the rectum; in 1907 Sambon designated the latter as *Schistosoma mansoni*.

Classification

Gene discovery and gene mapping projects, which have so far mainly focused on *S. mansoni*, generally agree with original classifications of schistosome species based on morphological or life history characteristics, with a high level of identity observed within species (Rollinson et al. 1997; Oliveira and Johnston 2001). It is hoped that continued work in this important area should provide illuminating information about the evolution and biology of the parasites and prove invaluable for the rational design of control measures.

Table 1 *Intermediate hosts of* S. mansoni *and* S. haematobium[a]

Schistosome species	Intermediate hosts	Geographical distribution
S. mansoni	*BIOMPHALARIA*	
	B. glabrata	Caribbean islands, Venezuela, Brazil, Argentina
	B. straminea	Caribbean islands, Central and South America
	B. tenagophila	Southeast Brazil
	B. pfeifferi group	
	B. pfeifferi	All sub-Saharan Africa
	B. rhodesiensis	East/Central to South Africa
	B. ruepelli	Yemen to East Africa
	B. arabica	Arabian peninsula
	B. choanomphala group	
	B. choanomphala	Central African lakes
	B. alexandrina group	
	B. alexandrina	Nile valley from North Sudan
	B. angulosa	Mountain areas from East to South Africa
	B. sudanica group	
	B. sudanica	Eastern tropical Africa
	B. camerunensis	Western tropical Africa
	B. salinarum	Southwest Africa
S. haematobium	*BULINUS*	
	B. africanus group	
	B. abyssinicus	Somalia, Ethiopia
	B. africanus	East, Central and Southern Africa
	B. globosus	Most of subsaharan Africa
	B. jousseaumei	West Africa
	B. nasutus	East Africa
	B. obtusispira	Madagascar
	B. forskali group	
	B. beccarii	Aden
	B. camerunensis	West Cameroon crater lakes
	B. cernicus	Mauritius
	B. forskali	Most of tropical Africa
	B. senegalensis	Mauritania, Senegal, Gambia
	B. reticulatus group	
	B. reticulatus[b]	Focal from Ethiopia to South Africa
	B. wrighti	Saudi Arabia, Oman, Yemen
	B. truncatus/tropics complex	
	B. guernei	West Africa
	B. liratus	Madagascar
	B. rohlfsi	West Africa
	B. truncatus	Mediterranean, south to Mauritania and Malawi, east to Iran

a) Adapted from Sturrock (1993)
b) Experimentally infected with a strain of *S. haematobium*

Morphology and structure

SCHISTOSOMA MANSONI

The surface of adult male *S. mansoni* is covered with characteristic coarse tubercles. Elliptical eggs (110–175 × 45–70 μm), hundreds of which are produced each day by the sexually mature *S. mansoni* female, are discharged mainly in the feces. They are distinguished from the other species of schistosome by the presence of a lateral spine (Figure 1).

SCHISTOSOMA HAEMATOBIUM

Adult *S. haematobium* worms resemble *S. mansoni* in that their surface tegument is coated in tubercles, rather than appearing smooth as in *S. japonicum*, although these tubercles are smaller than those present on the surface of *S. mansoni*. The elliptical eggs (110–170 × 40–70 μm) produced by sexually mature females are discharged mainly in the urine, and are characterized by the presence of a terminal spine (Figure 2). As with *S. mansoni*, hundreds of eggs can be produced daily by each female.

Figure 1 Schistosoma mansoni *ovum*

Location in the host

Adult worms of *S. mansoni* inhabit the mesenteric veins of the portal circulation, whereas *S. haematobium* adults generally remain in the systemic circulation, maturing in the blood vessels of the ureteric and vesical plexus surrounding the urinary bladder.

Life cycle

The life cycles of both *S. mansoni* and *S. haematobium* follow the common pathway outlined in Schistosomes: general. Intermediate hosts for *S. haematobium* are provided by several species of *Bulinus* (Figure 3) and *Physopsis* snail, and possibly also *Planorbarius*. The main intermediate hosts for *S. mansoni* in Africa and the western hemisphere are *Biomphalaria* snails: *B. alexandrina* in northern Africa, Saudi Arabia, and Yemen; *B. sudanica*, *B. pfeifferi*, and *B. rupellii* in other parts of Africa; *B. glabrata* (Figure 4), *B. tenagophila*, and *B. straminea* in the Americas (Paraense 2001). Intermediate hosts are listed in Table 1.

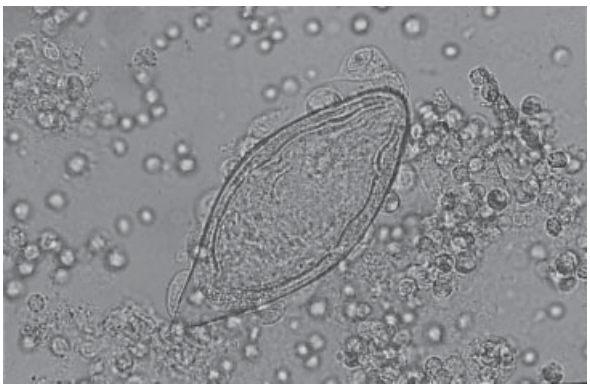

Figure 2 Schistosoma haematobium *ovum*

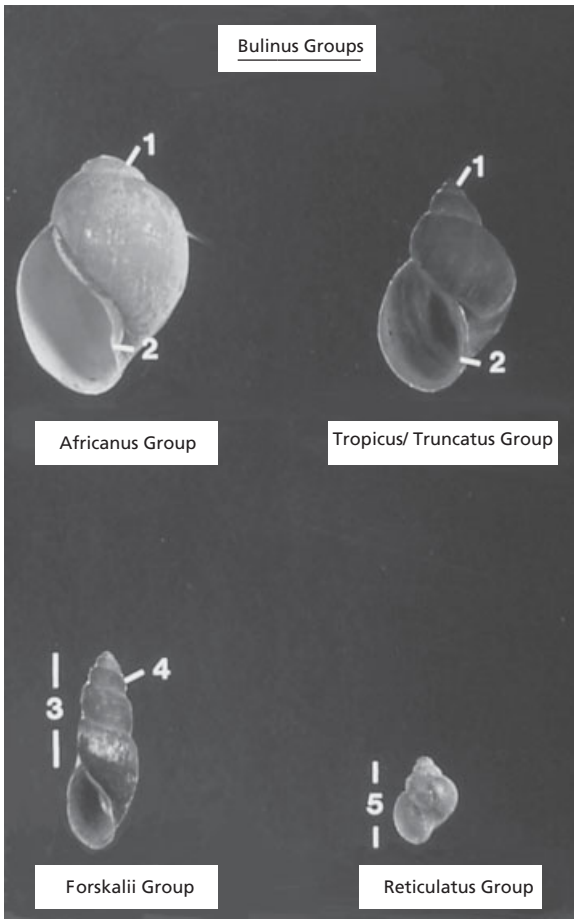

Figure 3 *Four species groups of the intermediate snail hosts of* Schistosoma haematobium *(WHO Slide Set Series; snail hosts, Schistosomiasis)*

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations, pathogenesis, and pathology

Symptoms characteristic of chronic infection with *S. mansoni* include abdominal pain, diarrhea, blood in the stool, and, more severely, hematemesis. Mortality caused by *S. mansoni* is generally associated with the development of liver fibrosis and hepatosplenomegaly, with many individuals dying each year from hematemesis or liver failure as a result of portal hypertension (van der Werf et al. 2003). However, it is not yet clear whether the development of hepatosplenic disease actually requires the development of fibrosis (Dessein et al. 1999b). During *S. haematobium* infection, the most common symptom is hematuria, as well as polyuria and dysuria. A significant proportion of those affected will additionally suffer from major bladder wall pathology and hydronephrosis (Figure 5). Mortality is primarily due to bladder cancer and kidney failure (van der Werf

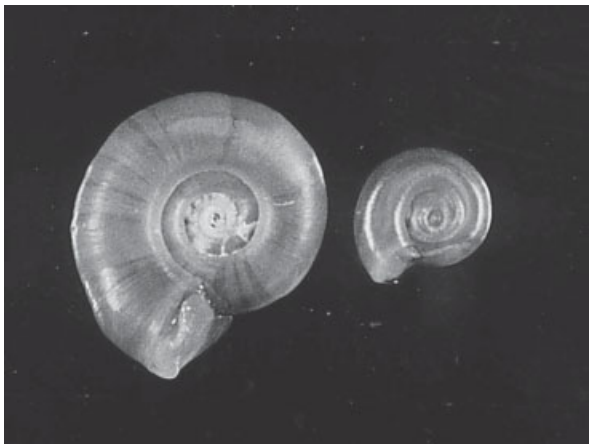

Figure 4 Biomphalaria glabrata*, an important intermediate host of* Schistosoma mansoni

et al. 2003). In addition to the fact that lesions due to *S. haematobium* eggs are found in the vulva, vagina, cervix, and less commonly the ovaries, Fallopian tubes, or uterus (Wright et al. 1982), *S. haematobium* may migrate through the network of female pelvic vasculature, and adaptive changes in the vasculature during puberty and especially during pregnancy make 'ectopic' localization of the parasites possible (Feldmeier et al. 1995). Thus, it has been pointed out that female genital schistosomiasis may be an important risk factor for transmission of Human immunodeficiency virus (HIV), as it is known that lesions in the genital mucosa, associated with sexually-transmitted diseases, increase the probability of viral uptake (Feldmeier et al. 1994). From published data there seems to be pathophysiological, immunological, and epidemiological evidence for an association between genital ulcer due to *S. haematobium* and HIV infection in women (Feldmeier et al. 1995). If so, control of female genital schistosomiasis should be tackled seriously to prevent further widespread dissemination of HIV infection.

Figure 5 *Hydronephrosis due to* Schistosoma haematobium *infection (courtesy of Dr Yukiko Wagatsuma).*

Immunopathology

In recent years, cytokine production from T helper subsets has been intensively studied to gain new insights into granuloma formation (see Schistosomes: general, Figure 6) and development of immunopathology, with the focus of research primarily being murine schistosomiasis mansoni (Hoffmann et al. 2002; Pearce and MacDonald 2002). Initial studies revealed that the production of Th2 cytokines was found to correspond to the onset of egg deposition (Grzych et al. 1991; Pearce et al. 1991). Lymphocytes from mice vaccinated with irradiated cercariae or from infected animals were compared for their ability to produce interferon-γ and IL-2, or IL-4 and IL-5. After stimulation with specific antigen or mitogen, T cells from vaccinated mice or prepatently infected animals responded primarily with Th1 cytokines, whereas lymphocytes from patently infected mice instead produced Th2 cytokines. The Th2 response in infected animals was shown to be induced by schistosome eggs and directed largely against egg antigens, whereas the Th1 reactivity in vaccinated mice was triggered primarily by larval antigens. Interestingly, Th1 responses in mice carrying egg-producing infections were found to be profoundly down-regulated. Moreover, the injection of eggs into vaccinated mice resulted in a reduction of antigen and mitogen-stimulated Th1 function accompanied by a coincident expression of Th2 responses (Pearce et al. 1991). Such studies were instrumental in establishing that it is the egg stage of the schistosome that is the major Th2 stimulus during infection, and in defining those components of egg antigens that are responsible. Th2 response development is an area of active research, with current evidence suggesting that both lipid (Faveeuw et al. 2002) and carbohydrate (Okano et al. 1999) may be involved. Interestingly, IL-4 production has recently been found to be initiated in Kupffer cells in the liver by adolescent *S. mansoni* worms before the females become mature and produce eggs, although the physiological relevance of the levels of cytokine produced has yet to be determined (Hayashi et al. 1999).

Cheever et al. (1992a) were able to demonstrate that in vivo treatment of infected mice with anti-IL-2 antibodies significantly diminished the size of circumoval granulomas in the liver, resulting in a decrease of hepatic fibrosis to half of that in untreated mice. Antibody-treated animals also displayed a marked reduction in both peripheral blood and tissue eosinophilia, while IgE levels were unchanged or increased. Spleen cell cytokine production in response to antigen or mitogen stimulation was selectively altered by in vivo anti-IL-2 administration. IL-5 responses were dramatically reduced, whereas IL-4, IL-2, and interferon-γ responses were not consistently changed. These findings suggest a role for IL-2 in egg-induced pathology, but indicate that the primary function of this cytokine in schistosome-infected mice may be in the generation of Th2- rather than Th1-associated responses (Cheever et al. 1992a).

By using a synchronized granuloma development model in the lung (von Lichtenberg 1962), it has been demonstrated that although the effects of neutralization of interferon-γ or IL-2 were variable, in vivo treatment of egg-injected mice with either anti-IL-2 or anti-IL-4 antibodies dramatically diminished the size of egg granulomas in the lungs (Chensue et al. 1992; Wynn et al. 1993). Both groups of antibody-treated animals displayed a marked reduction in IL-4, as well as IL-5 mRNA expression, although interferon-γ and IL-2 mRNA levels were unchanged or slightly increased. The up-regulation of mRNA expression of the IL-4 and IL-5 genes was also shown to be greater than that of Th1 cytokine genes in the granulomatous liver at 8 weeks after infection (Henderson et al. 1991; Wynn et al. 1993).

Further studies, in which mice were treated with anti-IL-4 before egg deposition, demonstrated decreased IL-4, IL-5, and IL-10 production in response to in vitro antigenic stimulation, as well as decreased IL-5 and IL-13 mRNA levels in the liver (Cheever et al. 1994). It was found that non-B, non-T cells were a major source of IL-4 in infected mice treated with control monoclonal antibody, and that the diminished IL-4 response in anti-IL-4-treated animals was caused, at least in part, by a reduction in the number of these cells, as well as by decreased secretion of IL-4 per cell. In contrast, production of the Th1 cytokines, IL-2 and interferon-γ was elevated in anti-IL-4-treated infected mice in vitro, and the corresponding mRNAs in the liver increased. Anti-IL-4 treatment did not consistently reduce the size of hepatic granulomas around *S. mansoni* eggs, but markedly inhibited granuloma formation in the lungs of the same animals after intravenous egg injection. Nevertheless, anti-IL-4-treatment showed consistent and marked reductions in hepatic collagen deposition at 8 weeks in *S. mansoni*-infected mice (Cheever et al. 1994). Similarly, hepatic fibrosis was markedly diminished in anti-IL-4-treated mice at 10 weeks after infection with *S. japonicum* (Cheever et al. 1995). These findings suggested that IL-4 plays a major role in the development of the Th2 response in murine schistosomiasis and contributes to the pathogenesis of hepatic fibrosis. Moreover, the administration of cytokines themselves into infected mice has also been shown to modulate granuloma formation. According to Yamashita and Boros (1992), chronically infected mice treated with 10–1 000 U of recombinant IL-4 showed significantly enhanced liver granulomatous responses compared with untreated animals and the augmented granulomas contained more enlarged macrophages and connective tissue matrix. However, when mice in which the IL-4 gene had been deleted (IL-$4^{-/-}$ mice) were infected with *S. mansoni* at 8 and 16 weeks after infection, liver pathology was similar to that in wild-type animals in terms of the size, cellularity, cellular composition, and collagen content of granulomas. When compared to normal mice, smaller granulomatous responses were observed in IL-$4^{-/-}$ mice only when eggs were injected intravenously to form granulomas in the lungs, despite the fact that Th1 cytokine production seemed to be dominant in lymphoid cells of IL-4-deficient mice stimulated with soluble egg antigen (Pearce et al. 1996). These results suggest that IL-4 is not an absolute requirement for hepatic granuloma formation. Nevertheless, subsequent investigation of disease progression in Th2-impaired (IL-$4^{-/-}$) mice has shown that a failure to develop a Th2 response has lethal consequences, resulting from a failure to regulate the initial pro-inflammatory response associated with acute infection (Brunet et al. 1997; Fallon et al. 2000). Increased disease severity and mortality in these studies was not due to increased parasite burden, but rather appeared to be a direct consequence of the failure to regulate the production and effector function of inflammatory mediators during infection. Thus, somewhat paradoxically, the dominant polarized Th2 immune response that is induced by schistosomes is intimately involved in many of the pathological changes that accompany infection, but is also required to allow host survival while infected. Whether such a clear requirement for Th2 development to avoid overt pathology is also the case in active human infection remains debatable.

Following these initial studies investigating the role of IL-4 in Th2 induction during schistosome infection, it has been shown that neither IL-6 nor IL-13 cytokines, that have both been implicated in Th2 development in other systems, appear to be critical for the development of this type of response during schistosomiasis. Although it may be involved at some level for regulation of IFN-γ and IL-12 production in the initial response to schistosome eggs (La Flamme et al. 2000), IL-6 is not an absolute requirement for Th2 development to the same stimulus (La Flamme and Pearce 1999). IL-13, a cytokine which is in many ways closely related to IL-4, is not required for Th2 development, but appears to play a key role in granuloma formation and fibrosis during

infection (Chiaramonte et al. 1999; Fallon et al. 2000). Indeed, it appears that the ability of both IL-4 and IL-13 to promote fibrosis is linked to the ability of these cytokines to 'alternatively' activate macrophages (Hesse et al. 2001). An interesting recent study suggests that liver macrophages (Kupffer cells) can provide a source of both IL-4 and IL-13 during the early stages of Th2 development (Hayashi et al. 1999), highlighting the fact that these key cytokines can originate from a number of sources. Additional possible sources of early IL-4 during schistosomiasis include naive T cells (Schmitz et al. 1994; Reiner 2001), basophils (Falcone et al. 1996), the so-called non-B, non-T cells (Williams et al. 1993) (which are most likely mast cells or basophils), and eosinophils (Sabin et al. 1996).

Administration of recombinant interferon-γ or IL-12 can also inhibit granuloma formation (Wynn et al. 1994). IL-12 is a key cytokine that promotes NK cell activity and Th1 responses. It has been demonstrated that the Th1-related cytokine, interferon-γ, augments endotoxin-stimulated IL-12 production in oil-elicited macrophages, whereas IL-4 and especially IL-10, are profoundly inhibitory (Wynn et al. 1994). Further experiments have revealed that sensitization with eggs plus IL-12 partly inhibits granuloma formation and dramatically reduces the tissue fibrosis induced by natural infection with *S. mansoni*. These results may provide an example of a vaccine against parasites which acts by preventing pathology rather than infection (Wynn et al. 1995). However, it has been shown recently that immunization with soluble egg antigens (SEA) plus complete Freund's adjuvant induces a pronounced Th1-shifted response in the low-pathology C57BL/6 mice, which results in a dramatic enhancement of hepatic egg-induced immunopathology, manifested by a marked increase in granuloma size and parenchymal inflammation, thereby leading to early death (Rutitzky et al. 2001). This raises the concern that predisposing individuals to make an extreme Th1 response during schistosome infection may in fact exacerbate disease, and so caution must be exercised in following this kind of immune deviation-based therapeutic approach.

IL-10, a cytokine often associated with Th2 response development and which is produced by a range of cell types, including Th1 cells (Sornasse et al. 1996), has been shown to down-regulate Th1 responses by working synergistically with IL-4 (Oswald et al. 1992). It has long been known that IL-10 is produced rapidly after schistosome egg production, with levels remaining high through the chronic stages of infection (Sher et al. 1991). In the past few years, renewed interest in the immunoregulatory role of IL-10 has revealed a number of interesting functions for this cytokine during infection. IL-10 decreases egg antigen-specific Th cell responses by down-regulating MHC class II, as well as B7 costimulatory molecule expression, on accessory cells (Flores-Villanueva et al. 1994). Systemic administration of IL-10 significantly inhibited delayed-type hypersensitivity reactions to egg antigen as well as primary and secondary granuloma formation to eggs embolized in the lung. However, significant inhibition of hepatic granuloma formation associated with the natural infection required the use of an IL-10/Fc fusion protein with a prolonged in vivo half-life. Lymph node cells from IL-10/Fc-treated mice produced less IL-2 and interferon-γ and more IL-4 and IL-10 than control cells, suggesting that reduced egg granuloma formation resulted primarily from down-regulation of Th1 responses. These results indicate that suitable administration of exogenous IL-10 can be effective in ameliorating immunopathologic damage associated with schistosomiasis (Flores-Villanueva et al. 1996). An elegant series of experiments recently highlighted the importance of IL-10 in maintaining a balanced immune response during schistosome infection. Mice that were doubly deficient for IL-4 and IL-10, IL-12 and IL-10, or IFNγ and IL-10, all developed severe disease (Hoffmann et al. 2000; Vaillant et al. 2001). Infected 'Th1 polarized' IL-4/10-deficient animals, while displaying minimal signs of fibrosis, developed an exaggerated form of the hepatotoxic disease that is seen in IL-$4^{-/-}$ animals which resulted in their death by around 8 weeks post-infection. Interestingly, in contrast to this, infected 'Th2-polarized' IL-12/IL-10 or IFN-γ/IL-10 doubly deficient mice displayed elevated levels of fibrosis, that led to the death of around 50 percent of the IL-12/IL-10-deficient animals by 12 weeks post-infection. These important studies indicate that extreme polarization of the immune response towards either Th1 or Th2 can have fatal consequences during infection with *S. mansoni*.

An additional unexpected role for IL-10 produced in response to schistosomes has become apparent as a result of several recent studies, which have reported reduced allergen responsiveness during *S. mansoni* (Araujo et al. 2000) and *S. haematobium* (van den Biggelaar et al. 2000) infections. The suggestion is that production of IL-10 (and perhaps TGFβ) during infection with schistosomes, and perhaps other helminths, may help prevent or reduce inflammation associated with conditions such as allergy and atopy, providing a new interpretation of the so-called 'hygiene hypothesis' (Yazdanbakhsh et al. 2002).

Granulomatous inflammation in schistosomiasis mansoni seems to be a complexly regulated consequence of T cell-mediated hypersensitivity to egg antigens. Three consecutive independent chromatographic procedures have been carried out to fractionate and identify soluble egg antigens (SEA) recognized by schistosome-specific cloned murine, $CD4^+$ Th1-type lymphocytes, which had previously been shown to be capable of mediating granuloma formation in vivo when adoptively transferred to normal syngeneic hosts challenged with an intravenous injection of eggs. The stimulatory activity resided in two acidic egg molecules, with apparent mole-

cular masses of 64–68 kDa and 38–42 kDa, each of which ran as a single band on SDS-PAGE after purification. Fast performance liquid chromatography and SDS-PAGE performed under reducing conditions suggested that the two molecules are related and that the 38–42 kDa molecule is a subunit of the 64–68 kDa molecule. Polyclonal lymphoid cells from schistosome-infected mice were stimulated by both the purified 64–68 kDa and 38–42 kDa molecules, implying that these are sensitizing antigens in the natural disease (Chikunguwo et al. 1993). This kind of identification of the molecules among crude SEA seems to be important for further studies on the identification of vaccine candidates that might regulate granuloma formation.

Indeed, further studies using SEA-specific monoclonal or hybridoma T cells have revealed that at least three important molecules in SEA are involved in the granuloma formation mediated by MHC class-II-restricted $CD4^+$ Th lymphocytes (Stadecker et al. 2001). Firstly, the most abundant egg component of 40 kDa protein was sequenced as a molecule of 354 amino acids with substantial homology to alpha-crystallins and heat shock proteins (Nene et al. 1986) and this Sm-p40 was found remarkably immunodominant in the large granuloma-forming $H\text{-}2^k$ mouse strains, such as C3H and CBA, whereas none of the SEA-specific T cell hybridomas derived from the small granuloma-forming strain (C57BL/6) responded to Sm-p40 (Hernandez et al. 1997a). Within the immunodominant 13mer peptide 234PKSDNQIKAVPAS246, D237 has been revealed as the MHC class II ($I\text{-}A^k$)-anchoring residue, while N238, Q239, and K241 have been shown as the principal T cell receptor (TCR)-contact residues (Hernandez and Stadecker 1999). Second, with the aid of a specific C57BL/6 T-cell hybridoma (4E6), a 62 kDa egg component was isolated and identified as *S. mansoni* phosphoenolpyruvate carboxykinase (Sm-PEPCK) (Asahi et al. 1999). The recombinant Sm-PEPCK of 626 amino acids stimulates $CD4^+$ Th cells from schistosome-infected or SEA-immunized mice (Asahi et al. 2000), although the response is distinctly polarized towards the Th1 type despite the fact that the native Sm-PEPCK induces a more balanced Th1 and Th2 type response in infected C57BL/6 mice (Asahi et al. 1999). A 12-amino-acid region of 398DKSKDPKAHPNS409 contained a T cell epitope (Asahi et al. 2000). Third, another immunogenic component of 26 kDa was identified as thioredoxin peroxidase-1 (TPx-1) (Williams et al. 2001). Interestingly, TPx-1 is secreted by the eggs and has been localized to the region between the miracidium and the eggshell (von Lichtenberg's envelop), stimulating antibody responses in all mouse strains so far examined (Williams et al. 2001), and thereby producing the circum oval precipitin (COP) reaction (Alger et al. 2002).

Antibody-mediated modulation of granulomas may occur in cases of schistosomiasis japonica, in which IgG1 antibodies are involved (Olds and Stavitsky 1986). Mitchell et al. (1991) have discussed similarities and differences between *S. mansoni* and *S. japonicum* infections from various aspects of biology, as well as immune responses, and also suggested that differences may exist even among geographical isolates or strains of the latter species.

Another important aspect of granuloma formation is the nature of the cellular components. Infiltration of eosinophils is one of the conspicuous cellular responses observed. IL-5 is essential for granuloma eosinophilia, since treatment of mice with anti-IL-5 monoclonal antibody suppressed the eosinophil response (Cheever et al. 1992b). The granuloma eosinophils make substance P, a cytokine with immunoregulatory properties, which belongs to a family of hormones called tachykinins. This substance may modulate interferon-γ production through interaction with a substance P-like receptor expressed on $CD4^+$ granuloma T lymphocytes (Cook et al. 1994). Other prominent cellular components in granulomas are macrophages and fibroblasts. Macrophages produce macrophage inflammatory protein 1α (MIP-1α), a 6–8 kDa protein which is lipopolysaccharide-inducible and monocyte- and neutrophil-chemotactic, and it has been shown that this protein contributes to cellular recruitment during schistosome egg granuloma formation (Lukacs et al. 1993). Moreover, IL-1, interferon-γ, and IL-10, cytokines found within the granuloma were able to induce significant production of MIP-1 by granuloma fibroblasts, whereas the constitutive expression of monocyte chemoattractant protein-1 (MCP-1) was demonstrated in both unstimulated and cytokine-stimulated granuloma fibroblasts. Interestingly, normal noninflammatory fibroblasts from uninfected mice showed no significant production of MIP-1 or MCP-1 in response to these cytokines. These results suggest that granuloma fibroblasts may be phenotypically altered compared with normal fibroblasts and have a significant role in leukocyte recruitment, granuloma growth, and maintenance of the egg-induced lesions (Lukacs et al. 1994a).

Further studies have demonstrated a crucial role for tumor necrosis factor (TNF) during inflammatory granuloma formation. In addition, TNF has been shown to up-regulate adhesion molecules that participate in cellular recruitment and lymphocyte activation. The mechanism of TNF activation during *S. mansoni* egg granuloma formation and its relationship to the expression of ICAM-1 have been studied in some detail. Firstly, high affinity human soluble TNF receptor (TNFR) coupled to the Fc portion of an Ig (sTNFR:Fc construct) could effectively diminish granuloma formation and lymphocyte activation in vivo. Second, increased steady state ICAM-1 mRNA expression was observed in primary egg granulomas when compared with normal lung and foreign body (Sephadex bead) granulomas, which suggests a role for ICAM-1 in

antigen-induced lesion formation. Subsequent studies have demonstrated that sTNFR:Fc treatment down-regulated granuloma formation and ICAM-1 expression, whereas anti-ICAM-1 decreased SEA-specific T cell proliferation in vitro. In addition, passive immunization of mice with anti-ICAM-1 monoclonal antibody during primary granuloma formation resulted in attenuation of lesion development as compared with lesion development in a control antibody-treated group. The proliferative response to SEA was also significantly reduced in ex vivo experiments that used spleen cells from the anti-ICAM-1 treated mice. These data demonstrate that both TNF and ICAM-1 participate in lymphocyte activation and granuloma formation and suggest that one mechanism of TNF in granuloma development is through TNF-induced ICAM-1 expression (Lukacs et al. 1994b).

In addition to cytokines, it is becoming increasingly clear that antigen presenting cells expressing costimulatory molecules participate in the induction of the Th2 response. Both macrophages and dendritic cells have been shown to have the ability potently to activate T cells during schistosomiasis (Hernandez et al. 1997b; Hayashi et al. 1999; MacDonald et al. 2001), although such a role for B cells remains less clear (Hernandez et al. 1997b; Jankovic et al. 1998). It seems that schistosomes might affect Th2 response development by influencing dendritic cell activation (MacDonald et al. 2001; Sher et al. 2003). Schistosome antigens, in contrast to most microbial pathogens, do not seem to activate DC conventionally. In particular, they fail to stimulate IL-12 production, while also failing to up-regulate the expression of surface markers normally associated with the activation or maturation of dendritic cells (MacDonald et al. 2001; Zaccone et al. 2003). Expression of the costimulatory molecules CD40 (MacDonald et al. 2002b) and OX-40L (de Jong et al. 2002) by dendritic cells exposed to schistosomal antigens seems to be important for Th2 skewing. In relation to this, several costimulatory partnerships (including CD40/CD40L (MacDonald et al. 2002a), B7/CD28 (Hernandez et al. 1999), and B7RP-1/ICOS (Rutitzky et al. 2003)) have been implicated in Th2 development and appropriate granuloma formation during active murine infection with *S. mansoni*.

Thus, it is evident from the observations described above that cell–cell interactions through cytokine production and their responses to these cytokines, including expression of costimulatory and adhesion molecules, are essential for appropriate immune response and egg granuloma development during schistosome infection. In future, it might therefore be possible that immunomodulation, using cytokines or antibodies to influence key molecules involved in response development and granuloma formation, may be applied for clinical use in the prevention or treatment of tissue damage due to schistosome eggs.

IMMUNE RESPONSES

In humans, epidemiological surveys carried out in areas endemic for schistosomiasis mansoni or urinary schistosomiasis have suggested that there is a good correlation between the development of IgE antibodies, resulting from Th2 responses, and age-dependent resistance to reinfection (Hagan et al. 1991; Hagan 1992; Butterworth 1994).

Effector cells

In human and rat systems, eosinophils have been shown to be the effector cells involved in antibody- or complement-dependent damage to various parasites including *S. mansoni* (Mackenzie et al. 1977; Capron et al. 1978; Ramalho-Pinto et al. 1978; Anwar et al. 1979). Similar results have not been obtained during murine infection, perhaps as a result of the fact that murine eosinophils, in contrast to human and rat, express no IgE receptor (Dombrowicz and Capron 2001). In the rat system, Capron et al. (1978) demonstrated that IgG2a is the isotype responsible for promoting the adherence of these cells. Adherence of eosinophils to schistosomula, followed by movement of secretion granules toward the basal region of the cell interacting with the parasite surface, is a critical step for mediating the killing activity, the fusion of granules forming small vacuoles, the contents of which are released on to the surface of schistosomula (McLaren 1980). Using a slow-motion movie camera, these steps were also observed in antibody-dependent cell-mediated cytotoxicity (ADCC) against schistosomula of *S. japonicum* by a human eosinophilic leukemia cell line, EoL-3, when the activity of the cells was enhanced by pretreatment with recombinant TNF (Figure 6) (Janecharut et al. 1992). This was consistent with previous results demonstrating that

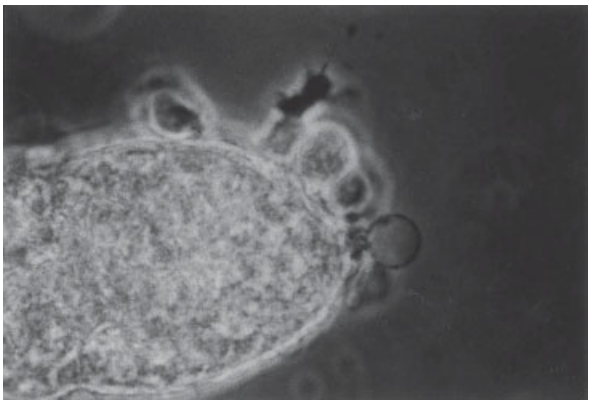

Figure 6 *Antibody-dependent cytotoxicity of EoL-3 cells against schistosomula of* Schistosoma japonicum. *An observation with a slow-motion camera, showing that granules and other contents of the cells are concentrated toward the surface of the schistosomula (Janecharut et al. 1992).*

TNF has no direct effect on the parasite, but enhances human eosinophil cytotoxicity to *S. mansoni* larvae in a dose-dependent fashion (Silberstein and David 1986). In another experiment, TNF was reported to exhibit direct toxicity to schistosomula at high concentrations or at lower concentrations in the presence of interferon-γ (James et al. 1990).

Antigens

Paramyosin is a muscle protein of invertebrates, and Pearce et al. (1988) have demonstrated that vaccination of mice with this molecule is effective in induction of resistance to *S. mansoni* infection. When administered intradermally with BCG at total doses of only 4–40 μg per mouse, both the native molecule and a recombinant expression product containing approximately 50 percent of the whole paramyosin were found to confer significant resistance (26–33 percent) against challenge infection, whereas 2 mg of an unfractionated complex soluble worm antigen preparation (SWAP) was required to induce similar levels of protection. In addition, paramyosin was shown to stimulate T lymphocytes from vaccinated mice to produce lymphokines such as interferon-γ that activate macrophages to kill schistosomula. Neither schistosome myosin nor a heterologous paramyosin from a different invertebrate genus was protective, indicating a requirement for specific epitopes in the immunization. That the protection induced by paramyosin involves a T-cell-mediated mechanism was supported by the failure of anti-paramyosin antibodies to transfer significant resistance to infection in recipient mice. Lymphocytes from mice vaccinated with paramyosin were found to produce interferon-γ in response to living schistosomula, suggesting that, during challenge infection of vaccinated hosts, paramyosin (a non-surface antigen according to Pearce et al. (1988), but now known as the molecule either localized in the tegument or even secreted from larval schistosomes) may elicit a protective T-cell response as a consequence of its release from migrating parasite larvae. These results suggest that the induction of T-cell-dependent, cell-mediated immunity against soluble non-surface antigens may be an effective strategy for immunization against multicellular parasites and, in the case of schistosomes, identify paramyosin as a candidate vaccine immunogen (Pearce et al. 1988). However, it is known that paramyosin is recognized by IgG antibodies in sera from patients with chronic schistosomiasis japonica (Kojima et al. 1987) and elevated levels of antibody responses to paramyosin have been reported in *S. mansoni* infection among cured individuals after treatment with praziquantel (Correa-Oliveira et al. 1989). Therefore, although it may be possible to induce an antibody-dependent cell-mediated immunity to human schistosomiasis by immunization with paramyosin, as is shown by resistance to infection to recipient mice (Kojima et al. 1998), at present, the critical role of induction of Th1 responses in protection to human schistosomiasis cannot be excluded.

GENETIC CONTROL

Genetic segregation analyses carried out on *S. mansoni* infected individuals in the Sudan have associated hepatic fibrosis and portal hypertension with a major gene located in the genetic region 6q22-q23, referred to as *SM2* (Dessein et al. 1999a). A similar approach in Brazil has associated infection intensity with a different major codominant gene located in the genetic region 5q31-q33, termed *SM1* (Marquet et al. 1996). These studies provide compelling evidence that human disease progression and infection intensity may be under distinct major gene control (*SM2* and *SM1*, respectively).

Vaccination

In addition to structural molecules, such as paramyosin, parasite enzymes have also been identified as vaccine candidates (see Figure 7 and Table 2). The triose-phosphate isomerase (TPI) of *S. mansoni* was first described as a 28 kDa target antigen recognized by a monoclonal antibody (M.1) generated from mice immunized with membrane enriched extracts of mechanically transformed schistosomula (Harn et al. 1985). The monoclonal antibody M.1 passively transfers partial resistance (41–49 percent) to cercarial challenge in naive mice. Thus, the 28 kDa antigen recognized by M.1 is a putative vaccine candidate. Purified native 28 kDa antigen from adult parasites was shown to function enzymatically in a manner analogous to yeast and mammalian TPI. Addition of M.1 antibody to the enzyme reaction altered the catalytic activity of schistosome TPI. To determine the immunologic cross-reactivity of this vaccine candidate with mammalian TPI, Western blot analysis was performed and demonstrated that M.1 was immunologically specific for the schistosome enzyme (Harn et al. 1992). Amino acid sequence analysis of the purified native antigen revealed a significant homology to the human glycolytic enzyme, triose-phosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1). The complete coding DNA for *S. mansoni* TPI was isolated and it was confirmed that this cDNA encodes the 28 kDa antigen recognized by M.1. The complete cDNA has been expressed within *Escherichia coli* to produce high levels of soluble recombinant *S. mansoni* TPI protein. The product was purified by the M.1 antibody and a functional TPI was obtained with an intrinsic specific activity comparable to that of rabbit and yeast TPI (Shoemaker et al. 1992). Further studies by Reynolds et al. (1994) have demonstrated that schistosome TPI is a potent inducer of IL-2 and interferon-γ production, driving production of these cytokines in the

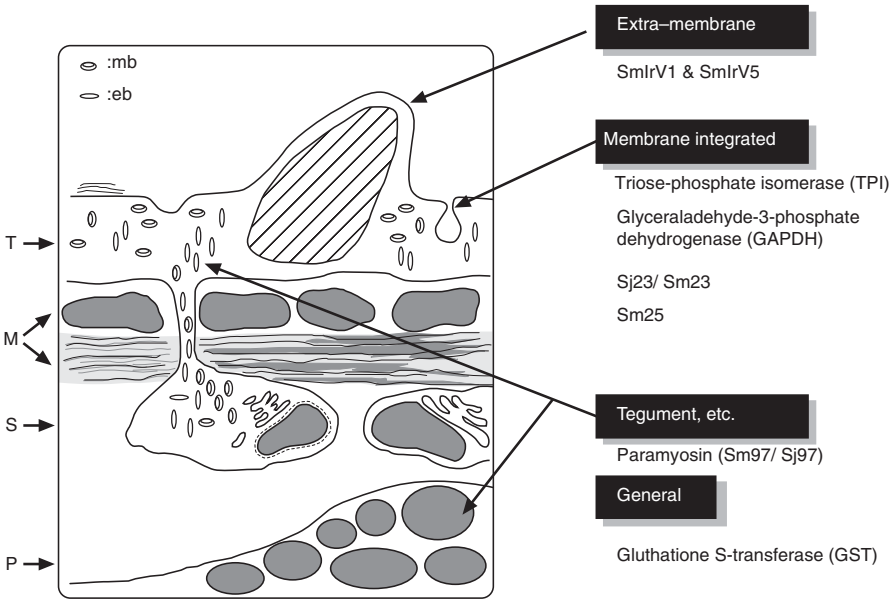

Figure 7 *Localization of vaccine candidates for schistosomiasis. M, muscle layers; P, postacetabular gland; S, subtegumental tissues; T, tegument.*

same cell populations of infected animals that have high Th2 responses directed at other SEA. With the goal of synthetic peptide vaccine design, recombinant TPI was used to determine specific T-cell and B-cell epitopes recognized by two strains of mice representing high and moderate responders (C57Bl/6J and CBA/J). All epitopes were selected from non-conserved regions of TPI and were thus parasite-specific. The investigators defined minimal size immunoreactive epitopes and synthesized four-armed multiple antigenic peptides (MAP) consisting of T-cell and B-cell epitopes that could be recognized by both strains of mice in the same molecule. Characterization of the immunoreactivity of the MAP showed that higher antibody recognition of the MAP was attained when the B-cell epitope was placed on the N-termini relative to the T-cell epitope, whereas T-cell immunoreactivity was equivalent in either position. Most interesting was the finding that one of the minimal T-cell epitopes, when incorporated into the MAP, required enlargement to retain immunoreactivity. Finally, both the full-length TPI molecule and the final version of the MAP were found to be immunogenic to T cells in naive animals, and these molecules induced crossrecognition reflected in IL-2 and interferon-γ production (Reynolds et al. 1994).

Table 2 *Vaccine candidates for schistosomiasis*

Identity of antigen	Size (kDa)	Stage	Function/localization	Protection (%) (mouse)	Reference
Paramyosin (Sm97) (Sj97)	97	Schistosomula Adult worms Schistosomula adult worms cercariae	Muscle protein Muscle, tegument, postacetabular gland	30 20–60	Pearce et al. (1988) Kojima et al. (1987, 1998), Nara et al. (1994)
Sj23/Sm23	23	All stages	Tegument		Reynolds et al. (1992)
TPI	28	All stages	Enzyme	30–60	Shoemaker et al. (1992), Reynolds et al. (1994)
GAPDH	37	Schistosomula Adult worms	Enzyme		Dessein et al. (1988)
GST (Sj26/Sj28) (Sm26/Sm28)	26/28	Schistosomula Adult worms	Enzyme	30–70	Balloul et al. (1987b)
IrV-5	200	Schistosomula	Muscle	32–75	Soisson et al. (1992)
IrV-1	90	All stages	Tegument		Hawn et al. (1993)
FABP (Sm14)	14	Schistosomula	Tegument	67	Tendler et al. (1996)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was first identified as a 37 kDa molecule of schistosomula recognized by Brazilian children resistant to *S. mansoni* infection (Dessein et al. 1988). The cDNA for this antigen was cloned by screening a schistosome cDNA expression library with antibodies against the purified protein. The amino acid sequence of the encoded polypeptide shows 72.5 percent positional identity with human GAPDH. Antibodies against the recombinant protein identified the 37 kDa molecule on the larvae (Goudot-Crozel et al. 1989). Since a number of conserved proteins have been found to be major targets of host-protective immunity against *S. mansoni*, it is interesting to note, as suggested by these authors, that genetic restriction of the immune response to these antigens may occur in heterogeneous human populations because of the limited number of T-cell epitopes carried by these host-like proteins and that such genetic effects might allow parasite transmission through nonresponder (susceptible) individuals.

Other vaccine candidates of an enzymatic nature are the glutathione *S*-transferases (GST), which consist of at least two isoenzymes of 26 and 28 kDa for both *S. japonicum* and *S. mansoni* (Sj26, Sj28, Sm26, and Sm28) (Tiu et al. 1988). Immunization of rats and hamsters with Sm28 resulted in significant protection against a natural challenge infection with live cercariae (Balloul et al. 1987a). Moreover, vaccination of Fisher rats and BALB/c mice with purified 28 kDa protein resulted in a marked decrease (up to 70 percent) in the parasite burden in both experimental infection models, and the antibody raised against this protein was able to kill *S. mansoni* larvae in the eosinophil-mediated cytotoxicity assay (Balloul et al. 1987b). *S. haematobium* GST has successfully passed the industrial scale-up and safety testing and is now undergoing phase II clinical trials (Capron et al. 2001); phase III field trials of this vaccine candidate are being planned (Bergquist et al. 2002).

An interesting molecule has been identified as a schistosome vaccine candidate by Strand and colleagues (Soisson et al. 1992). Mice exposed to radiation-attenuated cercariae of *S. mansoni* were highly resistant to challenge infection, and sera from these mice could confer partial resistance when transferred to naive recipients. These sera recognized antigens present in schistosomula and adult worms, among them particularly a 200 kDa antigen. A cDNA encoding a 62 kDa portion of this antigen was cloned and the deduced amino acid sequence of this cDNA clone was found to share homology with myosins of other species. To assess the immunoprophylactic potential, vaccination trials were carried out in mice using the recombinant polypeptide expressed as a fusion protein with β-galactosidase presented in the form of proteosome complexes with the outer membrane protein of meningococcus. The level of protection achieved was 32 percent, and this could be increased to 75 percent by removal of those amino acids included in the fusion protein that were derived from the vector to yield a polypeptide, designated rIrV-5. A similar level of protection was achieved when mice were immunized with the same dose of rIrV-5 in the form of protein complexes but without outer membrane protein, suggesting that protection did not require the use of adjuvant. However, at least three immunizations were necessary to achieve protection. Using monoclonal antibodies and sera from mice vaccinated with rIrV-5, the native protein recognized by antibodies against rIrV-5 was found to be a 200 kDa protein that was expressed on the surface of newly transformed schistosomula (Soisson et al. 1992). A further vaccination trial has been carried out in baboons with the rIrV-5 or radiation-attenuated cercariae. rIrV-5 was presented either in the form of protein micelles or complexed with the outer membrane protein of meningococcus to form proteosomes. The level of protection achieved in these groups ranged from 0 to 54 percent, with a mean of 27.7 percent, whereas in baboons exposed to radiation-attenuated cercariae the level of protection was very high, with a mean of 84 percent. The resistance observed after vaccination with rIrV-5 or radiation-attenuated cercariae was reflected in the overall histopathology. Vaccination of baboons with rIrV-5 or radiation-attenuated cercariae elicited an antibody response against epitopes exposed on the surface of newly transformed schistosomula. Analysis of individual baboon sera by enzyme linked immunosorbent assay (ELISA) demonstrated that there was a direct correlation between the anti-rIrV-5 titer and resistance to challenge worm burden, suggesting that the immunoprotective mechanism is antibody-dependent (Soisson et al. 1993).

Furthermore, in relation to SmIrV5, Hawn et al. (1993) have carried out molecular cloning and sequencing of SmIrV1, another candidate antigen recognized by immune sera raised in mice vaccinated with irradiated cercariae. SmIrV1 contained a deduced amino acid sequence of 582 residues with similarity to three proteins: calnexin, calreticulin, and OvRal1 (a surface antigen of the filarial nematode *Onchocerca volvulus*). SmIrV1 was divided into three regions: a neutral N-terminal region with a putative signal sequence, followed by a proline- and tryptophan-rich P region in which two sets of sequences are repeated four times and a C-terminal region which is highly acidic with an isoelectric point of 4.7. The P and C regions of SmIrV1 reacted with sera of immunized, as well as chronically infected, mice.

Immunoprecipitation studies with antibodies raised against a portion of recombinant IrV1 demonstrated its presence in cercariae, schistosomula, and adult worms with an apparent molecular mass on SDS-PAGE of 90 kDa. There was an approximate six-fold increase in protein expression level during the transformation from cercariae to schistosomula. Consistent with a potential role as a molecular chaperone, IrV1 was associated with

several metabolically labeled proteins in co-immunoprecipitation studies with the adult worm tegumental fraction. Similar to calnexin, IrV1 was metabolically labeled with phosphorus-32 on serine and threonine residues in adult worms and was one of the major phosphoproteins of this stage. This phosphorylation was developmentally regulated and coincided with the transformation of cercariae into schistosomula. The localization was also stage-specific, as IrV1 was transported from internal regions of cercariae to the outer tegumental layer of schistosomula (Hawn and Strand 1994). Cloning of a cDNA encoding SjIrV1 has also been carried out and shown that the molecule was 83 percent identical to SmIrV1 at the predicted amino acid level. Recombinant, full-length SjIrV1 was expressed with a hexahistidine tag in *E. coli* and the recombinant protein isolated by nickel-chelate chromatography. The recombinant molecule was shown to exhibit calcium-dependent, differential electrophoretic migration and to bind ruthenium red in the absence, but not in the presence, of calcium ions. That SjIrV1 was a functional calcium-binding protein was confirmed by the presence of conserved Ca^{2+}-binding motifs predicted from the primary sequence, together with the Ca^{2+}-dependent electrophoretic mobility of recombinant SjIrV1 (Hooker and Brindley 1999).

A new approach has been carried out to identify a vaccine candidate (Jankovic et al. 1996). A $CD4^+$ clone (clone B) was established from C57BL/6 mice protectively immunized with SWAP plus BCG and this T cell clone was characterized as Th1, based on its selective production of interferon-γ and IL-2. Transfer of the clone to syngeneic mice could activate antigen-elicited peritoneal macrophages to kill schistosomula in vitro. Recipients of the clone also displayed siginificant resistance against cercarial challenge. By screening a battery of λgt11 clones from an adult worm cDNA library, one recombinant (25B) was identified that stimulated clone B specifically. Analysis of the 25B cDNA insert revealed a nucleotide sequence identical with that of the large subunit of schistosome calpain, a Ca^{2+}-activated neutral proteinase. By expressing the products of polymerase chain reaction (PCR) subcloning, the authors identified a 146-amino acid region of the 25B gene containing immunologic activity equivalent to the whole polypeptide. Overlapping peptides spanning this region were synthesized, and a core epitope was identified with sequence EWKGAWCDGS. Since clone B responded to supernatants from cultured schistosomula, it was postulated that the recognition of calpain released by invading larvae and the resulting induction of Th1 cytokines accounted for the protection mediated by the adoptively transferred clone. Thus, calpain was implicated as a target of protective immunity in schistosomes. This report provided the first example of a candidate vaccine antigen for this parasite identified on the basis of T cell reactivity.

A fatty acid binding protein (Sm14) was shown to form the basis of the protective immune crossreactivity between the parasitic trematode worms *Fasciola hepatica* and *S. mansoni* (Tendler et al. 1995). A recombinant form of the *S. mansoni* antigen, rSm14, protected outbred Swiss mice by up to 67 percent against challenge with *S. mansoni* cercariae in the absence of adjuvant and without provoking any observable autoimmune response. The same antigen also provided complete protection against challenge with *F. hepatica* metacercariae in the same animal model. The results suggest that it is possible to produce a single vaccine that would be effective against at least two parasites, *F. hepatica* and *S. mansoni*, which are of veterinary and human importance, respectively (Tendler et al. 1996). Moreover, this vaccine candidate (recombinant Sm14) has been shown to induce proliferative responses of peripheral blood mononuclear cells (PBMC) of individuals living in an endemic area for schistsosomiasis (Brito et al. 2000). The highest proliferation index to rSm14 was detected in uninfected endemic normal (EN) individuals who are naturally resistant to schistosomiasis. Regarding the cytokine produced, the levels of Th2 cytokines IL-5 and IL-10 were not statistically different between chronic, treated patients and EN individuals. In contrast, IFN-γ and TNF-α were produced in significantly higher amounts by PBMC of EN individuals following rSm14 stimulation. Flow cytometry demonstrated that $CD4^+$ T cells from these individuals were the main lymphocyte subpopulation producing IFN-γ and TNF-α. Exogenous rIL-10 suppressed T-cell proliferation, neutralization of endogenous IL-10 with a monoclonal antibody to this cytokine restored lymphocyte activation, and enhanced IFN-γ and TNF-α production in chronically infected patients. In contrast, the addition of anti-IFN-γ totally abrogated the PMNC proliferation within the EN group. These results suggest that Th1 type of immune response induced in EN individuals to a specific schistosome antigen might be associated with resistance to infection and also highlighted the importance of Sm14 as a potential vaccine candidate (Brito et al. 2000).

DIAGNOSIS

In addition to the use of classical parasitological techniques for detection of eggs in urine (*S. haematobium*) or stools (*S. mansoni*), ELISAs are widely used for diagnosis of infection. ELISAs for the detection of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine have been developed and applied as an epidemiologic tool in a recent, intense focus of *S. mansoni* in Senegal (Polman et al. 1995). CAA and CCA in serum and CCA in urine were found in 94, 83, and 95 percent, respectively, of the population, of which 91 percent were positive on stool examination. Circulating antigens were also detectable in sera and urine of most egg-negative individuals. The sensitivities

of the urine CCA and serum CAA ELISA were substantially higher than that of a single egg count, and increased with egg output. The CAA and CCA levels correlated well with egg counts and with each other. The age-related evolution of antigen levels followed a similar pattern to egg counts, providing supplementary evidence for a genuine reduction of worm burden in adults, in spite of the supposed absence of acquired immunity in this recently exposed community.

CHEMOTHERAPY, PREVENTION, AND CONTROL

Praziquantel is the drug of choice, being safe and effective against all of the major human-infective schistosomes (Harder 2002). Further, there is currently little evidence of emergence of drug resistant parasites against this treatment, although this of course remains a potential future problem (Kusel and Hagan 1999). A single oral dose of praziquantel (40 mg/kg body weight) is prescribed for the treatment of *S. mansoni* and *S. haematobium*. Control programs based on praziquantel treatment to target the parasites, coupled with use of chemical molluskicides to control snail populations, have been successful in regions such as South America, Egypt, and the Phillipines (Engels et al. 2002; Fenwick et al. 2003). However, in areas of socio-economic deprivation, these programs have faltered and proved to be difficult to sustain, even though the cost of praziquantel has significantly reduced in recent years. New initiatives, aimed primarily at providing resources to enable sustained control strategies to be implemented in the most needy areas (particularly sub-Saharan Africa) are currently underway (Engels et al. 2002; Fenwick et al. 2003). Prior to the discovery of praziquantel, oxamniquine was used to treat *S. mansoni*, and metrifonate for *S. haematobium*. Oxamniquine is a miracil derivative that has an anticholinergic effect, which is thought to increase parasite motility and inhibit nucleic acid synthesis. It does not appear to affect any of the other schistosome species that cause human disease. Metrifonate is an organophosphate, the mode of action of which may be to target acetylcholinesterase on the parasite surface (Camacho and Agnew 1995). Neither oxamniquine nor metrifonate are currently in widespread use, probably due to a combination of their species-specificity, toxicity (in comparison to praziquantel), lack of availability, and the fact that they are less effective than praziquantel (Ferrari et al. 2003). As has been found for *S. japonicum* (see Schistosomes: Asian), there is potential in the use of artemether, a derivative of the antimalarial artemesinin, for the treatment of *S. mansoni* and *S. haematobium* infection (Utzinger et al. 2001). It is thought that artemether acts primarily on the juvenile stages of schistosomes. This limitation, together with potential long-term toxicity and the possibility that treatment in areas where schistosomes and malaria are co-endemic might select for drug-resistant malaria parasites, somewhat restricts its potential for schistosome control, particularly in sub-Saharan Africa.

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Schistosomes: Asian

SOMEI KOJIMA

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THE PARASITES

In Asia, human schistosomiasis is caused by *Schistosoma japonicum*, *S. mekongi*, or *S. malayensis* (Table 1), while zoophilic species such as *S. sinensium*, *S. incognitum*, and *S. spindale* have also been described from this region. *S. japonicum* affects over 800 000 people in the area along the Yangtze River basin in China, 200 000 people in various islands in the Philippines, and a rather limited population around Lake Lindo in Sulawesi, Indonesia. Control measures have been successfully introduced in some endemic areas, thereby resulting in a significant decrease in the prevalence and intensity of the disease. In other areas, however, the endemic situation has changed little, with severe infections even today, as was the case half a century ago.

Although schistosomiasis has a long history of more than 2 100 years in China, which is evident from the fact that eggs of *S. japonicum* were identified in two ancient corpses excavated from Hunan and Hubei (Chen and Feng 1999), it was in Japan that clinical symptoms were clearly described. In the middle of the nineteenth century Daijiro (Yoshinao) Fujii, a physician of the late Edo era in Japan, gave precise descriptions of clinical symptoms, which clearly refer to schistosomiasis, observed among peasants in Katayama District, although the disease was then of unknown etiology. In 1888, Majima detected a peculiar parasite ovum at autopsy in the liver of a patient who had ascites and systemic edema. On 26 May 1904, Fujiro Katsurada discovered the anterior half of a male worm in the portal vein of a female cat kept for more than 10 years as a pet of a physician in Kofu Valley, Yamanashi Prefecture, where a serious endemic disease with abdominal swelling or abdominal fluid had been noted. Two months later, Katsurada found 24 males and eight females from another cat and described the worms as *Schistosomum japonicum* (Katsurada 1904). Following autopsy of a farmer from Katayama District carried out on 30 May of the same year, Akira Fujinami confirmed the fact that this was a human parasite (Fujinami 1904). It was also in 1904, in Singapore, that Catto independently discovered worms of the same type in a Chinese man from Fukien who had died from cholera, and Blanchard named the parasite *Schistosoma cattoi*. This work was published in 1905, but later the parasite was found to be identical with one described by Katsurada. Using cows for experimental infections, Fujinami and Nakamura (1909) found that the infection was acquired through the percutaneous route. Miyairi and Suzuki (1913) were able to determine that an amphibious snail, *Oncomelania nosophora*, was the intermediate host of this parasite. This discovery had a strong influence on the work of Leiper (1916), who was able to find the snail intermediate hosts of schistosomiasis in Egypt and to differentiate definitively between *S. haematobium* and *S. mansoni* on the infectivity to snails of miracidia derived, respectively, from terminal-spined or lateral-spined eggs.

Meanwhile, the presence of human schistosomiasis has been known in the lower Mekong river basin since the late 1950s, and direct evidence of endemicity in Khong Island, Laos, was obtained by Iijima and Garcia (1967) (cited in Iijima et al. 1971) who demonstrated that 8.6 percent of 547 inhabitants of the island were positive

Table 1 *Human schistosomes distributed in Asia*

Schistosome species	Final hosts	Intermediate hosts	Geographical distribution
S. japonicum	Human, dog, cat, rodent, cow, pig, sheep, goat, horse	*Oncomelania hupensis hupensis*	China
		O. h. nosophora	Japan
		O. h. quadrasi	Philippines
		O. h. lindoensis	Sulawesi
		(*O. formosana*)[a]	(Taiwan)[a]
S. mekongi	Human, dog, pig	*Neotricula aperta*	Laos, Cambodia, Thailand
S. malayensis	Human, rodent	*Robertsiella*	Malaysia

a) Zoophilic strain, not infective to humans.

for *S. japonicum*-like eggs. Iijima et al. (1971) reported that the Mekong schistosome differed from the Japanese strain of *S. japonicum*: the ovary was larger, the variation in number of testes was greater, and the eggs were smaller. Harinasuta et al. (1972) identified an aquatic snail, now known as *Tricula* (currently *Neotricula*) *aperta* (Davis and Greer 1980), as the intermediate host. Voge et al. (1978) concluded that the Mekong schistosome was a new species, *S. mekongi*. In addition, human cases with *S. malayensis* infection have been reported from Malaysia.

Classification

Recent studies on genetic variation among Asian schistosomes have revealed that all of the *S. japonicum* isolates obtained from different localities in mainland China (two from Sichuan and one from Hunan) and the Philippines (Sorsogon) showed limited variation (less than 1 percent) in both the nucleotide and amino acid sequences in mitochondrial DNA (4.9 kbp), whereas *S. mekongi* (obtained from Khong Island, Laos) and *S. malayensis* (Baling, Malaysia), both being recognized as separate but closely related, differed from each other by 10 percent, and differed by about 25 percent from *S. japonicum* (Le et al. 2002). Similarly, a high level of identity (87–94 percent at both the nucleotide and amino acid levels) was observed for all protein-encoding mitochondrial genes of *S. mekongi* and *S. malayensis*, while lesser identity was observed between genes of these two species and those of *S. japonicum* (Le et al. 2001).

Morphology and structure

The adult forms of all schistosomes infective to humans basically resemble one another. In particular, schistosomes distributed in Asia belong to the *S. japonicum* species complex because of their morphological similarities.

SCHISTOSOMA JAPONICUM

The male of *S. japonicum* measures about 15 mm in length by 0.5 mm in breadth, and possesses seven testes; the female is long and slender like a nematode, measuring about 22 × 0.3 mm. The body surface of the male looks smooth under light microscopy, but scanning electron microscopy demonstrates folded protrusions with semi-spherical sensory organs that possess a small process in the center (Sakamoto and Ishii 1977). The surface of the female is covered with minute spines. The eggs discharged in feces are ovoid (70–100 × 50–65 μm) with a small spinose process near one end, but without an operculum (Figure 1).

SCHISTOSOMA MEKONGI

Adult worms of this schistosome closely resemble those of *S. japonicum* in all aspects of size, shape, internal structure, and body surface, except that the ovary is larger (Iijima et al. 1971). Embryonated eggs of *S. mekongi* are also quite similar in shape and structure to those of *S. japonicum*, but are smaller, measuring 61.7 × 51.2 μm (Iijima et al. 1971).

SCHISTOSOMA MALAYENSIS

Eggs of this species are similar to those of *S. mekongi*, with both of them being smaller than those of *S. japonicum*, although only minor morphometric differences were observed among adult worms (Greer et al. 1988).

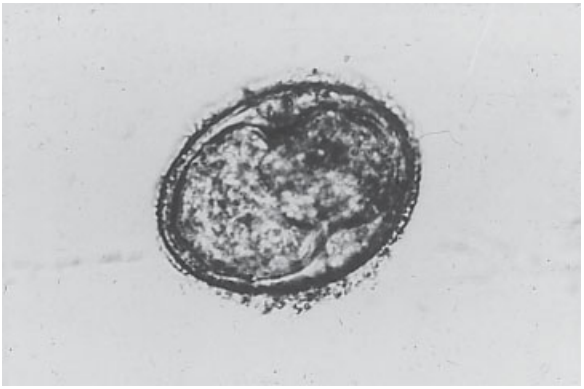

Figure 1 Schistosoma japonicum *ovum*

Location in host and general biology

Adult worms of *S. japonicum* and *S. mekongi* inhabit the mesenteric veins.

Life cycle

The life cycle of all Asian species follows the common pathway described in Schistosomes: general, Figure 3. The intermediate hosts of *S. japonicum* are a complex of amphibious snails of *Oncomelania hupensis* (Figure 2), of which there are six subspecies: *O. h. hupensis* in mainland China; *O. h. quadrasi* in the Philippines; *O. h. nosophora* in Japan; *O. h. lindoensis* in Sulawesi, Indonesia; *O. h. formosana* in Taiwan where a zoophilic strain of *S. japonicum* exists; finally, experimental infection with the strain has been demonstrated in *O. h. chiui*, another subspecies found in Taiwan. Recent studies suggest that these subspecies should be elevated to specific status like *O. hupensis* or *O. quadrasi* (Sturrock 1993). These oncomelanid snails have conical or subconical dextral shells of four to eight whorls with a height of less than 10 mm. *O. quadrasi* is more aquatic than the other species, which may crawl out of water on nearby vegetation and mud surfaces and can survive for at least a few months of a dry season in natural conditions. *S. mekongi* is transmitted by the α, β, and γ races of *Neotricula aperta* (Sornmani 1976), of which the γ race is the most susceptible to infection under laboratory conditions (Liang and Kitikoon 1980), although only the γ race was found to be infected naturally (Sornmani 1976). The intermediate host of *S. malayensis* was described as *Robertsiella*, a sister genus to *Neotricula* (Davis and Greer 1980).

Transmission and epidemiology

From 1947, in Japan, a program to control the disease in previously known endemic areas was undertaken by the Japanese Ministry of Health and Welfare and other institutions in collaboration with the United States 406th Medical General Laboratory. Voluntary organizations, composed of staff from local governments, physicians' associations, teachers' associations, and representatives from endemic communities, made a major contribution to the intensive control program, which included snail control and improvement of environmental and sanitary conditions, since they had been established before the Second World War. Based on these activities, the government carried out massive surveys in 1973 and 1978 on the inhabitants, *Oncomelania* snails and the reservoir hosts throughout endemic areas. Although two snail colonies still exist, including a new habitat found recently in a limited area that has no relation to former endemic areas (Kojima et al. 1988), schistosomiasis japonica is considered to have been eradicated from Japan since 1978 (Hunter and Yokogawa 1984; Tanaka and Tsuji 1997).

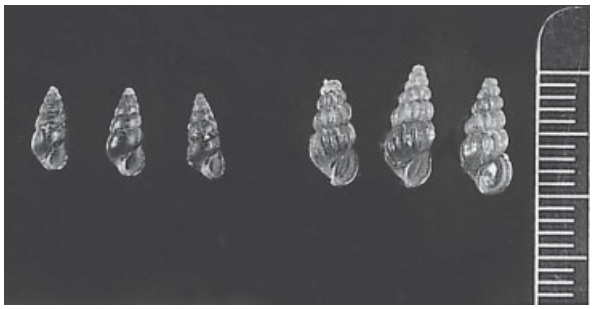

Figure 2 *Intermediate snail hosts of* Schistosoma japonicum*: left,* Oncomelania hupensis nosophora *collected from Yamanashi, Japan; right,* Oncomelania hupensis hupensis *collected from Jian-xi Province, China*

In China, around the time of the founding of the People's Republic in 1949, schistosomiasis was raging in many areas. For example, in 1954 in Jiangxi Province, only two persons were left alive in one particular township which had had about 1 000 households in the mid-nineteenth century. In another township in Jiansu Province, 4 019 people out of about 7 000 villagers acquired acute infection during a flood season in 1950 and 1 335 patients died of schistosomiasis (Chen and Feng 1999). Large-scale national epidemiological surveys carried out in the mid-1950s revealed that schistosomiasis was endemic in 380 counties in 12 provinces along the Yangtze River, with 12 million infected people among approximately 100 million at risk of infection (Li et al. 2000). Thus, schistosomiasis control was given high priority by the Chinese Government and various measures were taken in an integrated approach, including health education through schools, hand-outs, and mass media, as well as principal measures such as snail control, environmental modification, and large-scale chemotherapy, both for infected persons and cattle, molluskiciding activities, sanitation, etc., to control the disease over the last four decades. A World Bank loan was also implemented to strengthen operational reseach capabilities from 1992 to 1998. As a result, the number of infected persons reduced from 1 638 103 in 1989 to 865 084 in 1995, and the prevalence of infected bovines also reduced from 13.3 to 9.1 percent (100 251 cattle and buffalo are estimated to be infected) (Chen and Feng 1999). According to another report, the number of reported cases of schistosomiasis further reduced to 694 788 in the year of 2000, which included 664 acute cases, 671 400 chronic cases and 22 786 advanced cases (Jiang et al. 2002). However, most of the 118 counties and cities still endemic for schistosomiasis are distributed in the swamp and lake regions and also in mountainous regions, where snail elimination is quite difficult, and therefore the only control strategy is morbidity control by repeated chemotherapy (Chen and Feng 1999).

Similarly, the government of the Philippines has adopted the objective of schistosomiasis control from a comprehensive approach that includes treatment of

cases, environmental sanitation, snail control, and health education to morbidity or disease reduction by chemotherapy supplemented with environmental sanitation, health education, and molluskiciding (Leonardo et al. 2002). However, schistosomiasis continues to remain as a public health problem affecting ten out of 16 regions, with 6.7 million people at risk. Many factors may account for this problem: (1) detrimental integration of the schistosomiasis control program with other health services causing confusion in coordination of services, implementation of plans, leadership among related personnel, etc.; (2) low disease awareness among people at risk because of their poverty, resulting in avoidance of participation in control activities, refusal of treatment for fear of drug toxicity if treatment is done without prior examination and laboratory evidence of infection, and refusal of water-sealed toilets; and (3) aggravated security problems in some areas that diminish access of people to the health delivery system (Leonardo et al. 2002). Thus, prevalence of schistosomiasis remains at 4.7 percent in the whole country, with an infection rate nearly as high as 19 percent in some provinces.

In Indonesia, schistosomiasis is limited to two isolated areas in central Sulawesi. A recent survey carried out in 1999 revealed that 11 (0.46 percent) out of 2 387 villagers examined were positive for schistosome eggs in Lindu valley, while 67 (1.55 percent) out of 4 312 persons were positive in Napu valley, indicating a great decrease in the prevalence if compared to previous data in the 1970s (Izhar et al. 2002). However, it seems likely that the life cycle of *S. japonicum* is still maintained among snails (about 7 percent positive for cercariae) and wild rats (5.6 percent infected) in the natural environment.

Meanwhile, transmission of mekongi schistosomiasis occurs in rocky banks of the Mekong River due to the characteristics of the snail host, which is aquatic and usually attaches to small rocks, stones, or old tree branches under slow-running shallow water. According to a recent report, about 140 000 people are estimated to be at risk of infection in Laos and Cambodia, despite universal treatment campaigns (Urbani et al. 2002). Daily water contact activities such as fishing, laundering, bathing, swimming, etc., are risk factors, although the role of dogs and pigs, which harbor the parasite, in transmission (Sornmani et al. 1971; Strandgaard et al. 2001) is not clear.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations, pathogenesis, and pathology

The initial manifestations may appear following penetration of cercariae and migration of schistosomula from the skin to lungs. Table 2 summarizes the symptoms of Chinese and Japanese patients who were infected with *S. japonicum* in the floods that occurred in the Yangtze river in 1945 and the Tone river in 1926, indicating that cercariae may induce dermatitis with slight exanthema and itch upon their penetration. Dermatitis usually disappears within a week and migration of schistosomula into the lungs provokes a cough with association of a mild fever and dullness. Anemia may be observed among one-fifth of patients (Table 2).

Table 2 *Symptoms observed among people with acute schistomiasis japonica (figures are percentages)*

Symptoms	Chinese cases (200 cases)[a]	Japanese cases (75 cases)[b]
Initial phase		
Dermatitis	44.0	67.0
Prepatent phase		
Malaise/fatigue		67.0
Cough	63.0	
Bloody sputum	5.5	
Anorexia	88.0	38.6
Abdominal pain	41.0	(+)
Anaemia	23.0	24.0
Oviposition phase		
Chill	78.0	77.3
Fever	100.0	77.3
Sweat	72.0	73.3
Diarrhea	51.0	5.3
Dysentery	10.0	(–)
Hepatomegaly	96.5	85.3
Splenomegaly	62.5	22.7
Ascites	8.5	9.3

a) Ling et al. (1949)
b) Kashiwado et al. (1927)

After a latent period of about a month, the acute phase of disease may suddenly start with a high fever accompanied by rigors, termed Katayama fever after the Katayama District in Hiroshima Prefecture, Japan, where Yoshinao Fujii originally described the acute onset of the illness (see The parasites). Among the Japanese patients described in Table 2, the majority had such an onset 31–50 days after the infection. The onset corresponds to the start of oviposition by mature female worms. *S. japonicum* female worms lay eggs in the mesenteric branches of the portal vein along the intestinal wall and although a relatively large proportion of the eggs are carried into the liver and other organs by the blood flow, the remainder of them may stay in the small venules until the embryos they contain develop to miracidia within 10 days. Antigenic substances excreted from miracidia diffuse out through submicroscopic pores in the eggshell and elicit an acute inflammation in the surrounding tissues, resulting in the rupture of the vascular wall and escape of the eggs from the venules through the intestinal submucosa and mucosa into the intestinal lumen. Typical egg granulomas formed in

the liver are shown in Schistosomes: general, Figure 28.6. The inflammation causes recurrent daily fever, abdominal pain, and enlarged tender liver and spleen, and discharge of eggs into the intestinal canal is accompanied by dysentery or diarrhea (Table 2). Blood chemistry may reveal a transient elevation of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, and alkaline phosphatase 5–6 weeks after infection. Eosinophilia may be observed in most of the patients with or without increase of leukocyte counts. Serum levels of IgE may increase as observed in other helminth infections (Kojima et al. 1972). These symptoms characterize intestinal schistosomiasis.

The chronic phase of manifestations in *S. japonicum* infection is characterized by hepatosplenomegaly and may therefore be called hepatosplenic schistosomiasis, although development of polyps or mucosal proliferation of the intestine may also be observed in most cases. Egg granulomas are replaced by fibrotic tissues, which are prominent in the periportal areas and lead to the development of pipestem fibrosis (Figure 30.3) similarly to that described in cases with *S. mansoni* infection (von Lichtenberg et al. 1971). In association with liver dysfunction, diagnostic techniques such as computed tomography or ultrasonography may reveal pathological changes with characteristic reticulate or hexagonal patterns in patients with chronic schistosomiasis (Figure 30.4a, b). The liver gradually decreases in size, but increases in hardness as fibrosis is gradually extended into the parenchyma, resulting eventually in liver cirrhosis in severe cases (Figure 30.5). However, it has been pointed out that the use of the term 'schistosomal cirrhosis' may not be appropriate because of the lack of the essential features of cirrhosis, such as disruption of the lobular architecture (Ross et al. 2001). Even though schistosomiasis cases with cirrhosis are observed, it might be difficult to determine the pathogenesis unless there is evidence that alcoholic and/or postviral cirrhosis are not involved. Despite the possible presence of such coincident factors, post-schistosomal hepatic fibrosis has been suggested to be under the control of HLA class II genes, since associations of HLA-DR-DQ alleles or HLA-DP alleles were observed with protection from the early or late phase of fibrosis, respectively, in a study performed on patients with schistosomiasis japonica in China (Hirayama et al. 1999). However, further studies, using defined antigens, for example, will be necessary to clarify the immunogenetic regulation of fibrosis in humans as a result of responses to soluble egg antigens

Figure 30.3 *Pipestem fibrosis in a Japanese patient with schistosomiasis japonica*

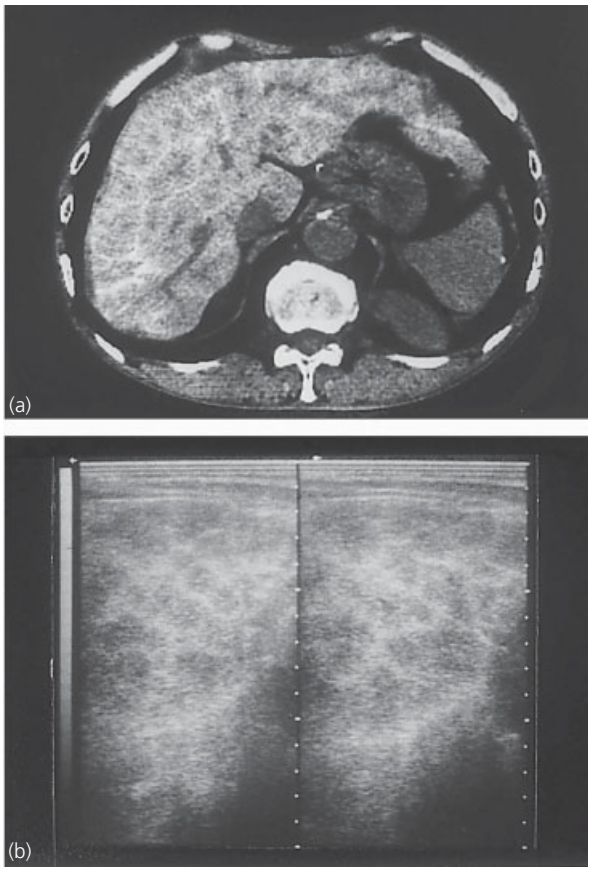

Figure 30.4 *Hepatic fibrosis in a patient with chronic schistosomiasis japonica:* **(a)** *computed tomography;* **(b)***ultrasonography. Note the characteristic reticulate or hexagonal patterns.*

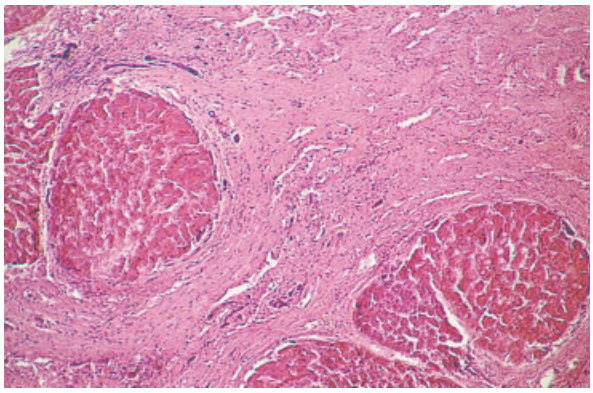

Figure 30.5 *Liver cirrhosis due to* Schistosoma japonicum *infection. A case found in Yamanashi Prefecture, Japan*

(SEA) (see below). In experimental schistosomiasis japonica, it was shown that killing the eggs deposited in the liver by chemotherapy might result in recovery of fibrotic tissues to normal (Kojima 1970). The enlarged spleen may reach the level of the umbilicus or even at times expand to fill most of the abdomen. Portal hypertension and hypoalbuminemia may induce ascites (Figure 30.6). Dilatation of abdominal collateral veins (Figure 30.6) and esophagogastric varices are observed in advanced cases, and bleeding from the varices may cause sudden death. Anemia is more noticeable than in the previous stages of the disease. Acute or chronic cerebral involvement may occur in schistosomiasis japonica due to embolism of eggs or heterotopic parasitism of female worms, causing headache, Jacksonian epileptic seizures, paresthesia, and poor vision (Ariizumi 1963). Hepatic coma may occur as a complication, although its frequency is far less than that of liver cirrhosis.

Similar chronic symptoms and signs, including cachexia, hepatosplenomegaly, stunting, retardation of puberty, and decompensation of portal hypertension with ascites, may be observed in cases of mekongi schistosomiasis and rupture of esophageal varices is usually the cause of death (Urbani et al. 2002).

Figure 30.6 *The abdomen of a patient with chronic schistosomiasis japonica, showing ascites and dilatation of abdominal collateral veins*

In addition, it has been reported that female genital tract diseases may occur in schistosomiasis japonica, although the involvement of gynecological organs is more usually observed in *S. haematobium* infection. A case-control study carried out in 2 106 women aged 18–50 years revealed that cases with chronic cervicitis and uterine enlargement were observed more frequently in the experimental group of 244 women with schistosomiasis japonica than in the control group of 236 age-matched healthy women (Liu et al. 2000). Menstrual disorder and shorter stature and lighter weight of the first newborn also occurred more frequently in the experimental group. It has been pointed out that female genital schistosomiasis may be an important risk factor for transmission of Human immunodeficiency virus (HIV), since sexually transmitted diseases increase the probability of HIV transmission, presumably through lesions in the genital mucosa (Feldmeier et al. 1994). Further studies are necessary for cases with schistosomiasis japonica.

IMMUNE RESPONSES

A hybridoma that produces a monoclonal IgE antibody to this species was established (Kojima et al. 1987) to examine the roles of IgE antibodies in protection against *S. japonicum*. This monoclonal antibody (SJ18ϵ.1) recognized a 97 kDa antigen and the antibody was shown to be protective when it was passively transferred into mice during the early phase of the infection or used in antibody dependent cell-mediated cytotoxicity (ADCC) in the presence of macrophages or rat eosinophils (Kojima et al. 1987; Janecharut et al. 1991, 1992). The antibody reacted with a single protein of the same size extracted from *S. mansoni* that had been identified as paramyosin (Figure 30.7), a muscle protein unique for invertebrates, by gene cloning and analysis of the deduced amino acid sequence (Laclette et al. 1991). By using SJ18ϵ.1 to screen a cDNA library obtained from adult *S. japonicum*, the gene coding for the target molecule has been cloned to confirm that the target of this monoclonal antibody is paramyosin (Nara et al. 1994). It has also been demonstrated that paramyosin localizes in the tegument and postacetabular glands, as well as in the muscle tissues of schistosomula (Nara et al. 1994) (Figure 30.8), although the localization of the molecule in tissues other than the muscles, even in isolated teguments, is questionable (Schmidt et al. 1996). However, a comparative immunolocalization study using electron microscopy among schistosome adults, cercariae, and lung schistosomula has revealed that paramyosin is localized within the muscle layer of all three developmental stages, within granules of the post-acetabular glands of cercariae, and within the tegument matrix and surface of lung schistosomula (Gobert et al. 1997). *S. japonicum* paramyosin is composed of some 866 amino acids and is thought to have an α-helical conformation. If compared with the amino acid sequences of paramyosin demonstrated for other helminths,

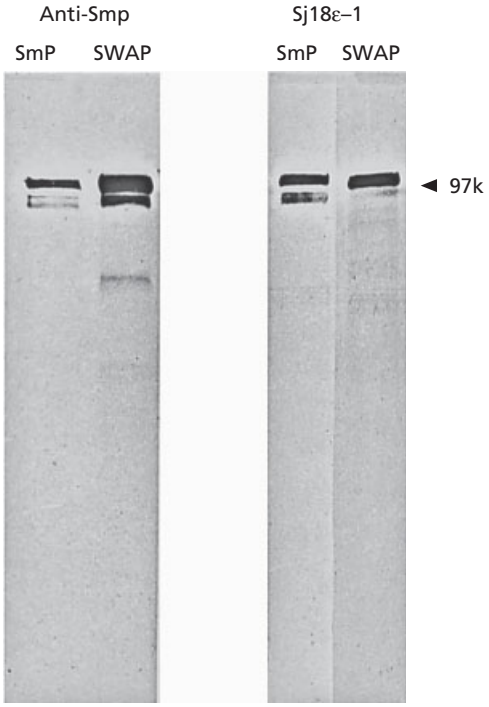

Figure 30.7 *SJ18ε.1 recognizes paramyosin of* Schistosoma mansoni. *SWAP, soluble worm antigen preparation of* S. mansoni*; SmP, paramyosin purified from SWAP; Anti-SmP, rabbit antiserum specific to SmP; SJ18ε.1, a mouse monoclonal IgE antibody (in collaboration with Dr Isabella Oswald and Dr Alan Sher)*

S. japonicum shares 96 percent homology with *S. mansoni* (Laclette et al. 1991), and 72 percent with *Echinococcus granulosus* (Muhlschlegel et al. 1993), but only around 35 percent with a parasitic or non-parasitic nematode, *Dirofilaria immitis* (Limberger and McReynolds 1990) or *Caenorhabditis elegans* (Kagawa et al. 1989), indicating that the sequence of paramyosin is well conserved among platyhelminths.

Since ADCC in the presence of IgE antibodies has been shown to be involved in immunity to schistosome infections (Kojima et al. 1987; Janecharut et al. 1991, 1992; Capron and Capron 1994), determination of the B-cell epitope of paramyosin responsible for protection is important for the development of a peptide vaccine for schistosomiasis. The epitope recognized by SJ18ε.1 was examined using a series of deletion mutants expressed in *Escherichia coli* and it was found that the antibody reacted with recombinant paramyosin containing 113 amino acids (Glu^{301}–Ala^{413}), but not with a shorter peptide (Glu^{301}–Asp^{343}). Thus, the epitope recognized by this antibody was expected to exist within 71 amino acid residues (Asp^{343}–Ala^{413}). Further analysis was carried out by a multi-pin system using heptameric peptides synthesized sequentially from these 71 amino acids of paramyosin and the result demonstrated significant binding of SJ18ε.1 to a sequence consisting of four amino acid residues (Ile-Arg-Arg-Ala^{359}). The target epitope of SJ18ε.1 was common to paramyosins of *S. mansoni*, *Taenia solium*, and *Echinococcus granulosus*, but not to nematode paramyosins, suggesting that the epitope is specific for platyhelminths (Nara et al. 1997).

It is known that paramyosin is recognized by IgG antibodies in sera from patients with chronic schistosomiasis japonica (Kojima et al. 1987). Therefore, it may be possible to induce an antibody-dependent cell-mediated immunity to human schistosomiasis by immunization with this molecule. With regard to this, Hernandez et al. (1999) reported that titers of IgA antibodies to soluble worm antigen preparation (SWAP) correlated with age-dependent resistance to *S. japonicum* infection among Filipinos and that the antibodies from the majority of high IgA responders recognized 97 kDa molecules. Based on these observations, the authors proposed that IgA antibodies to paramyosin might contribute to parasite attrition during migration in the lungs.

Vaccination

The efficacy of vaccination with native or recombinant paramyosin has been examined in various animal models with different regimes including adjuvants and route of immunization. Promising results were obtained against *S. japonicum* infections in mice (Kojima et al. 1998;

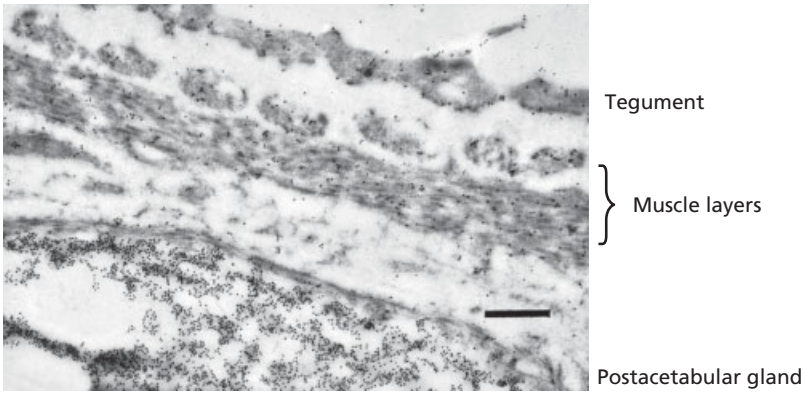

Figure 30.8 *Localization of paramyosin shown by immunoelectromicroscopy (Nara et al. 1994)*

McManus et al. 1998), sheep (Taylor et al. 1998), pigs (Chen et al. 2000), and water buffalo (McManus et al. 2001). Domestic pigs were used in China for evaluation of vaccination with recombinant *S. japonicum* paramyosin (rSJPM) (Chen et al. 2000). As a positive control, 16-week-old pigs were vaccinated with 400 UV attenuated cercariae and they showed a significant reduction of worm recovery (53 percent, $p < 0.001$, if compared with non-immunized controls). The experimental groups were immunized intradermally with rSJPM and alum or TiterMax and they were partially but significantly protected against the challenge infection (32–35 percent reduction) (Chen et al. 2000). Among three water buffalo immunized with rSJPM plus Quil A adjuvant, a significant reduction in liver eggs was recorded in two buffalo and all of them showed high levels of specific anti-paramyosin IgG antibodies. There was no evidence of any toxic effects and the vaccine preparations and Quil A were well tolerated (McManus et al. 2001). Thus, the unique character of schistosomiasis japonica as a zoonosis may provide a useful tool applicable to development of vaccines for the other major human schistosomiasis.

The other vaccine candidates for schistosomiasis are summarized in Table 3 and Figure 9). To avoid duplication with the preceding chapter, however, only molecules related to Asian schistosomes will be discussed below. Among them, the 23 kDa molecules of *S. japonicum* (Sj23) (or *S. mansoni*, Sm23) are integral membrane proteins (Rogers et al. 1988) and the molecules share an 84 percent amino acid homology (Davern et al. 1991). Hydrophobicity analyses for both molecules suggest the presence of transmembrane regions arranged as three consecutive regions at the N-terminus followed by a relatively hydrophilic domain with a fourth domain at the C-terminus (Wright et al. 1990). Using antibodies, this protein has been detected in all stages of the parasite found in the human host, notably the lung stage, and therefore is of interest as a vaccine candidate. It is of particular significance that Sm23 (composed of 218 amino acids) was strikingly similar, with respect to both amino acid sequence (36 percent identity) and putative domain structure, to ME491, a human stage-specific melanoma-associated antigen (Wright et al. 1990). Sm23 and Sj23 also show a strong homology with a family of membrane proteins including the lymphocyte-expressed surface molecules CD37 and TAPA-1 and have therefore been suggested to play a role in cellular proliferation and parasite growth, because these membrane proteins are considered to be involved in cell proliferation (Davern et al. 1991). Thus, Sm23 and Sj23 have been shown to be members of a proposed new superfamily of membrane proteins whose structures do not conform to the previously known classifications. To date, the other members include CD9 (p23), CD53, MRC OX-44, CO-029, MRP-1, L6, the gene product of TI-1, the target of monoclonal antibody AD-1. Most of these molecules, except for Sm23 and Sj23, are found in membranes of hemopoietic and/or malignant cells, although their function is not yet clear. With regard to schistosomes dwelling in the blood, however, this homology may be considered as an example of molecular mimicry, in which the parasite might have evolved a host-like molecule in an attempt to escape from host immune responses. Nevertheless, Sm23 has been shown to be a major antigen recognized by sera from animals vaccinated with irradiated cercariae (Richter and Harn 1993). The two predicted external hydrophilic domains were found to be highly immunogenic and contained several B-cell epitopes. There were at least four T-cell

Table 3 *Vaccine candidates for schistosomiasis*

Identity of antigen	Size (kDa)	Stage	Function/ localization	Protection (%) (mouse)	Reference
Paramyosin (Sm97)	97	Schistosomula Adult worms	Muscle protein	30	Pearce et al. (1988)
(Sj97)		Schistosomula adult worms cercariae	Muscle, tegument, postacetabular gland	20–60	Kojima et al. (1987, 1998), Nara et al. (1994)
Sj23/Sm23	23	All stages	Tegument		Reynolds et al. (1992)
TPI	28	All stages	Enzyme	30–60	Shoemaker et al. (1992), Reynolds et al. (1994)
GAPDH	37	Schistosomula Adult worms	Enzyme		Dessein et al. (1988)
GST (Sj26/Sj28) (Sm26/Sm28)	26/28	Schistosomula Adult worms	Enzyme	30–70	Balloul et al. (1987b)
IrV-5	200	Schistosomula	Muscle	32–75	Soisson et al. (1992)
IrV-1	90	All stages	Tegument		Hawn et al. (1993)
FABP (Sm14)	14	Schistosomula	Tegument	67	Tendler et al. (1996)

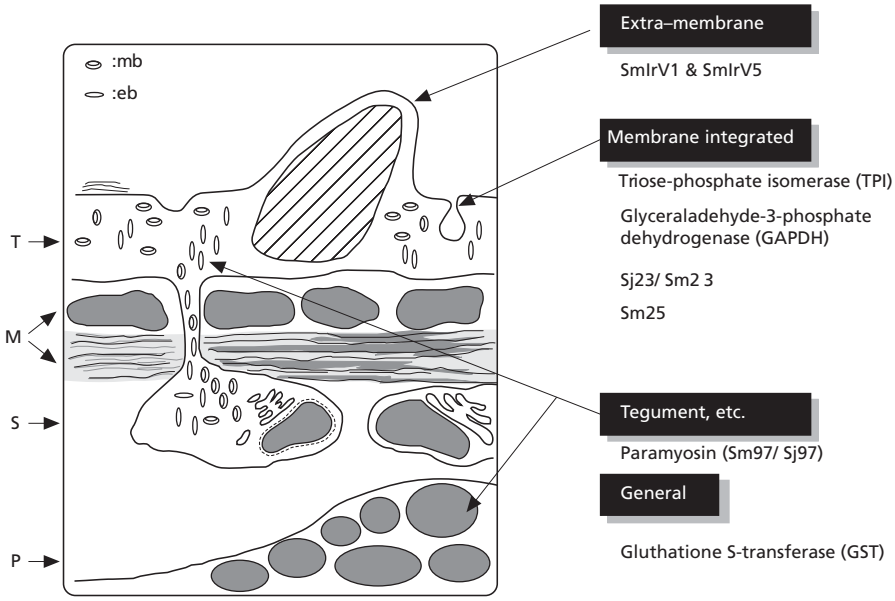

Figure 30.9 *Localization of vaccine candidates for schistosomiasis. M, muscle layers; P, postacetabular gland; S, subtegumental tissues; T, tegument*

epitopes in the large hydrophilic domain. One segment of 23 amino acids contained both a T-cell and a B-cell epitope, as well as the putative glycosylation site. This particular segment was recognized by immune sera and cells of every mouse strain tested. The elucidation of these epitopes demonstrates the immunogenic nature of this molecule and raises questions as to the role of Sm23 in the host–parasite relationship (Reynolds et al. 1992). However, it is still obscure whether these molecules are able to induce protection to human schistosomiasis, although a monoclonal antibody to Sj23 has been successfully used in immunodiagnostic assays to detect *S. japonicum* infection in Filipino patients (Davern et al. 1991). By using full-length Sm23 prepared with the baculovirus expression system, and the N-terminal 133 amino acids and the 85 C-terminal amino acids expressed by means of the prokaryotic vector pGEX, a total of 70 sera from patients from Sudan and Egypt were examined by enzyme linked immunosorbent assay (ELISA) or Western blot analysis (Koster et al. 1993). The anti-Sm23 antibody titers in infected patients varied widely and were not correlated with egg counts or age of the individuals. Most of the seroreactivity was directed against the C-terminal polypeptide.

Parasite enzymes have also been identified as vaccine candidates. Triose-phosphate isomerase (TPI) was isolated from a Chinese strain of *S. japonicum* and this native protein was shown to be functional with enzyme activity of 177 IU/mg of protein. Interestingly, when examined for protective activity, the number of eggs in the liver of immunized mice was significantly reduced compared with adjuvant control animals, whereas a modest reduction in worm burdens was observed in immunized animals (Miao et al. 1998). Further studies made it possible to obtain recombinant TPI with a specific enzyme activity as high as 7687 IU/mg of protein (Hooker and Brindley 1996; Sun et al. 1999).

Other candidates of an enzymatic nature are the glutathione S-transferases (GST) that consist of at least two isoenzymes of 26 and 28 kDa for both *S. japonicum* and *S. mansoni* (Sj26, Sj28, Sm26, and Sm28) (Tiu et al. 1988). Mice of the inbred strain 129/J (WEHI 129/J) relatively resistant to chronic infection with *S. japonicum* were shown to be high responders to Sj26 (Smith et al. 1986). The cDNA sequence encoding Sj26, Sj28, Sm26, and Sm28 has been isolated and expressed in *E. coli* (Smith et al. 1986; Balloul et al. 1987a, b; Henkle et al. 1990; Trottein et al. 1990). Despite their immunological cross-reactivity using rabbit antisera, Sj28 is weakly immunogenic relative to Sm28 in mouse immunization experiments using GSTs purified from adult worms. The difference in immunogenicity is also observed during schistosome infection in mice. Using surface-labeled living *S. japonicum* worms, evidence was obtained for a surface location of Sj28 comparable to that reported for the *S. mansoni* molecule. The nucleotide and deduced amino acid sequences of cDNA clones corresponding to Sj28 and Sm28 have been compared. Despite obvious homology (77 percent identity), differences were found in regions known to contain T-cell epitopes in the *S. mansoni* protein, which may be an explanation for the striking differences in immunogenicity in regard to antibody production in mice. The 26 kDa GSTs (Sj26 and Sm26) are also closely related on the basis of nucleotide and deduced amino acid sequences, with 82 percent identity in the putative coding regions. When the amino acid sequences of Sj28 and Sm28 were compared with those of Sj26 and Sm26,

the overall sequence identity was approximately 20 percent. However, a relatively conserved region was identified in otherwise structurally different molecules which may participate in common properties of these enzymes (Henkle et al. 1990). A study of the tissue distribution of the cloned Sm26 by immunoelectron microscopy demonstrated similarities to Sm28 in that they are present in the tegument and in subtegumentary parenchymal cells. However, a major difference was observed in the protonephridial region in which Sm26 was present in the cytoplasmic digitations localized in the apical chamber, delineated by the flame cell body, suggesting that Sm26 may be actively excreted by adult worms (Trottein et al. 1990). Experimental vaccination of rats, hamsters, and monkeys with a recombinant fusion protein induced a strongly cytotoxic antibody response. Immunization of rats and hamsters with Sm28 resulted in significant protection against a natural challenge infection with live cercariae (Balloul et al. 1987a, b). Moreover, the antibody response raised against this protein was able to kill *S. mansoni* schistosomula in in vitro cytotoxicity assays in the presence of rat eosinophils. The inhibition of this cytotoxic activity by an aggregated myeloma IgG2a indicated that one of the major isotypes involved in this in vitro model is IgG2a. The passive transfer of Sm28 antisera induced a significant level of protection against experimental infection. However, immunization with recombinant Sj26 was not sufficient to induce consistent immunity and maximum resistance was approximately 50 percent, while no protection was obtained in BALB/c mice, known low responders to Sj26. Although only Freund's complete adjuvant was used, the data indicated that satisfactory levels of resistance to *S. japonicum* might not be attained by vaccination with Sj26 alone. The requirement for other antigens, including the additional GST isoenzyme of Sj28, has been suggested to establish whether Sj26 will be an important component of a defined multivalent vaccine against schistosomiasis japonica (Mitchell et al. 1988). Complete cloning of Sj28 has been carried out recently and GST active recombinant proteins were used for examination of vaccine efficacy in various strains of mice. Unfortunately, however, consistent results were not obtained in terms of reduction in the worm burden, liver eggs, or hatched miracidia numbers (Scott and McManus 2000).

A cDNA coding for *S. japonicum* calpain has been cloned from two isolates, Yamanashi and Hunan strains (Zhang et al. 2000). Nucleotide sequence and amino acid sequence revealed 99.1 and 99.8 percent identity between the two calpains, respectively. Both calpains were considered to be translated as a preproenzyme, and a 746-amino acid mature enzyme contained eight motifs without a signal peptide at the N-terminal based on the deduced amino acid sequences. Although mRNA for calpain was detectable in different developmental stages, sera obtained from mice immunized with recombinant calpain showed enhanced binding to cercarial antigen. Human sera from *S. japonicum*-individuals recognized the large subunit, and sera of individuals with light infection showed stronger reactivity than those obtained from moderate or high infection cases. When tested with synthetic peptides, human B-cell epitopes on *S. japonicum* calpain were observed to be common with those of *S. mansoni* calpain. From these results, the authors suggested that calpain of *S. japonicum* has a diagnostic value in addition to being a vaccine candidate.

DIAGNOSIS

Detection of circulating antigens with ELISA kits has been developed for *S. japonicum* infection with high sensitivity and specificity (Wang et al. 1999). Interestingly, it has been reported that keyhole limpet hemocyanin (KLH), extracted from the marine mollusk *Megathura crenulata*, may be utilized as a diagnostic antigen just as *S. japonicum* SEA is used for the dot ELISA (Li et al. 1997). Moreover, if serum samples were diluted at 1:500, the sensitivity of the KLH dot–ELISA for diagnosis of acute and chronic schistosomiasis was 82 and 15 percent, respectively, suggesting possible serological differentiation of both forms.

CHEMOTHERAPY, PREVENTION, AND CONTROL

For schistosomiasis japonica, oral administration of praziquantel in three 20 mg/kg^{-1} doses at 4-h intervals may result in 80 percent cure. At this dosage, about a half of the individuals receiving the treatment may complain of side effects, such as abdominal discomfort, drowsiness, headache, backache, fever, sweating, and dizziness, although these are usually mild and transient. Recent observations using mice heavily infected with *S. japonicum* demonstrated that praziquantel administration induced typical signs of systemic anaphylaxis until half of them died shortly after the treatment (Matsumoto 2002). Autopsy of these mice revealed remarkable intestinal alterations characterized by increased mucosal permeability, mucosal edema, petechial hemorrhage, and histologically, degranulation of intestinal mast cells, which was particularly remarkable around eggs hatched as an effect of praziquantel. Thus, the author suggested that the adverse effects of praziquantel treatment characterized by abdominal signs might result from anaphylactic reactions to antigens released from hatched eggs (Matsumoto 2002).

Artemether, the methyl ether derivative of artemisinin has been tested against *S. japonicum*. When administered intragastrically to mice on day 7 after infection with *S. japonicum* cercariae at a single dose of 300 mg/kg body weight, and the same dose of artemether was repeated at 1–3-week intervals between one and four times after the first dosing, most of the female worms

were killed before oviposition, with female worm reduction rates of 70–90 percent, resulting in protection of the host from damage induced by schistosome eggs. When rabbits and dogs were treated intragastrically with artemether at 10 mg/kg on day 7 after infection, followed by between one and four repeated weekly doses, the worm reduction rates were 85–99 percent. Moreover, some parameters related to acute schistosomiasis, such as temperature, eosinophil count, and eggs in the feces, were negative, and low specific antigen and antibody levels in serum were seen. Further study showed that the appropriate regimens of artemether were also effective in early treatment of re-infection with cercariae. Histopathological examination of the livers showed that early treatment with artemether exhibited a promising protective effect in dogs and rabbits. Based on these results, these authors concluded that early treatment with artemether could be recommended for field trials for controlling acute schistosomiasis, reducing infection rate and intensity of infection (Xiao et al. 1995). Control of schistosomiasis is not an easy task. Even after successful treatment, re-infection easily takes place in most endemic areas, unless transmission is cut off somewhere between the intermediate hosts and the final hosts in the life cycle of the parasites. Control of schistosomiasis japonica is complicated by the existence of reservoir hosts, mainly domestic animals such as cows, pigs, sheep, and goats in China, and dogs, cats, and water buffalo in the Philippines, in addition to wild rats. Use of human night soil for fertilizer should be avoided in endemic areas. The snail hosts constitute a key factor in the control of schistosomiasis. In Japan, a periodical distribution of molluskicides, such as sodium pentachlorophenate, Yurimin and later B-2 (sodium 2,5-dichloro-4-bromophenol), was applied in combination with cement lining of irrigation ditches in endemic areas for control of *Oncomelania* snails (Figure 30.10). However, these measures can hardly be applied in such huge endemic areas as in China, and molluskicides are not suitable for application in water sources where aquatic snail hosts are abundant and on which people's daily life depends. Indeed, even if limited only to the Dongting Lake region, snail habitats are estimated at 1 768 km^2 in 1996, increasing at a rate of 34.7 km^2 annually due to high silt deposition from the Yangtze River and from connecting rivers in that area, and construction of embankments in the lake. In addition, it is anticipated that the construction of the Three Gorges superdam may result in substantial expansion of snail habitats and increase the number of new schistosomiasis cases (Li et al. 2000). The positive impact on the control of schistosomiasis may be that floods in the Yangtze River will occur to a lesser degree, thereby decreasing the chances of snail dispersal and infection of humans and animals, whereas the negative impact should also be taken into consideration in that flushed beaches and migratory settlements may create snail habitats at certain altitudes (Zheng et al. 2002). In addition, the chances of infection may increase in the Dongting Lake beach because of an increase in the likelihood of human and reservoir animal water exposure due to water regression that occurs ahead of time in the autumn, while the impact of the dam construction on the transmission of schistosomiasis was not significant in the Poyang Lake and other areas on the lower reach of the Lake (Zheng et al. 2002). Although remarkable progress has been made in the control of the disease after intensive efforts over four decades in China (Li et al. 2000), the construction of the Three Gorges superdam may make it possible to utilize riverbeds for pasture for a whole year, otherwise impossible during the rainy season, increasing the risk of schistosome infection among livestock and local people.

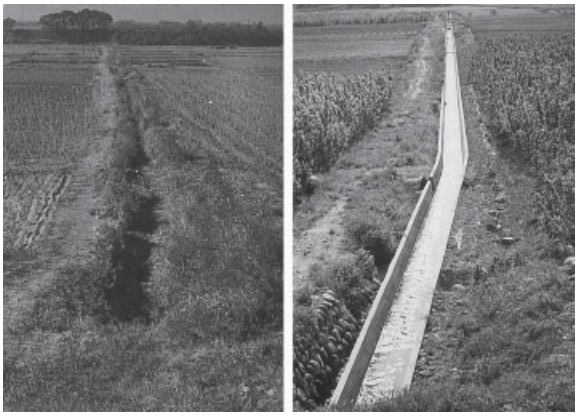

Figure 30.10 *Cement lining of irrigation ditches for control of* Oncomelania *snails (courtesy of Yamanashi Prefectural Institute of Public Health)*

Thus, improvements in environmental sanitation and safety of supply water are essential for control. The successful control accomplished in Japan by 1978 indicates that to achieve the goal of control, the organization of voluntary associations including mothers' groups, and groups of medical doctors and workers, in cooperation with national and local government, may be as important as the education and motivation of the people themselves. Japan has undergone rapid socioeconomic development resulting in drastic changes in agriculture or living styles, land reclamation projects, and increased awareness of schistosomiasis (Hunter and Yokogawa 1984). It should be noted that to overcome a complex of issues involved in the control of schistosomiasis, strengthening of operational research on cost-effective methods of disease control proved to be useful from the experiences in China under the World Bank Schistosomiasis Research Initiative (Yuan et al. 2000).

To control mekongi schistosomiasis, universal treatment campaigns were organized in 1989 in Laos and later in 1996 in Cambodia under the guidance and support of the World Health Organization, in collaboration with several agencies and international organizations. A single

dose of praziquantel of 40 mg/kg was distributed to whole populations in endemic areas where the prevalence was above 50 percent, except children under 2 years of age and pregnant women, while below this prevalence only children between 2 and 14 years were treated. The impact of this campaign was apparent in a remarkable reduction in hepatosplenomegaly in association with decreasing infection rates and improvement of liver pathology monitored with abdominal ultrasonography in a cohort study of an endemic village in north-eastern Cambodia (Hatz 2001). In addition, a decrease in the number of in- and out-patients suffering from schistosomiasis in hospitals in the endemic province of Kratie, Cambodia, was noted, although further ultrasound surveys suggested that repeated treatment might not prevent or reverse advanced periportal fibrosis and development of portal hypertension (Urbani et al. 2002). The ecology of the intermediate host makes it difficult to complete the control of mekongi schistosomiasis transmission by taking measures such as snail control and environmental modification that have been applied to other endemic areas of schistosomiasis japonica as mentioned above. Health education and people's motivation to better manage biological waste are apparently important, but it seems difficult for local people to construct latrines and washing facilities without external financial support (Urbani et al. 2002). Due to the low sensitivity of the Kato-Katz stool examination and high costs of available immunological tests, focusing on school-aged children, in combination with the soil-transmitted helminthiases control program, may be the cost-effective way to control mekongi schistosomiasis.

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Lung and liver flukes

MELISSA R. HASWELL-ELKINS

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THE PARASITES

An estimated 40 million people harbor food-borne trematodes globally (WHO 1995). Although previous estimates of numbers infected may have been inaccurately high, there is evidence that the pattern of human infections with these parasites is in a state of change regarding number, geographic range, and economic and clinical significance (Xu et al. 1995; Eckert 1996). In many areas, their prevalence has decreased as a result of control efforts using drug treatment and health education (Jongsuksuntigul and Imsomboon 1997; Ooi et al. 1997). However, in other places, infections have persisted or increased because of environmental changes increasing snail habitats, the free-market economy with wider distribution networks for food, lack of food inspections, poor sanitation, human migration, and declining economic conditions (Maurice 1994; Mas-Coma et al. 1999; Anantaphruti 2001). Many people also continue to enjoy raw or undercooked foods, often in spite of knowledge about its potential health hazards.

This large group of parasites includes the liver flukes, *Opisthorchis viverrini*, *Clonorchis sinensis*, *O. felineus*, *Fasciola hepatica*, and *F. gigantica*, and nine or more species of the lung fluke, *Paragonimus* (Beaver et al. 1984; Toscano et al. 1995). A third group of food-borne trematodes found in humans, not detailed here, are the intestinal flukes (e.g. *Fasciolopsis*, *Echinostoma*, and *Heterophyes*). Schistosomes, or blood flukes, are also trematodes, but are acquired by skin penetration, not via contaminated food.

Fasciola hepatica (Figure 1a) was first referred to by de Brie in 1379 in sheep with 'liver rot,' 500 years before the discovery and formal description of trematodes that infect humans. The parasite was named by Linnaeus in 1758, just before its discovery in a human by Pallas in 1760. *F. gigantica* was found in a giraffe and named by Cobbold in 1855. The life cycle of *Fasciola* was the first to be elucidated for a trematode; this was achieved concurrently but independently by Leuckart

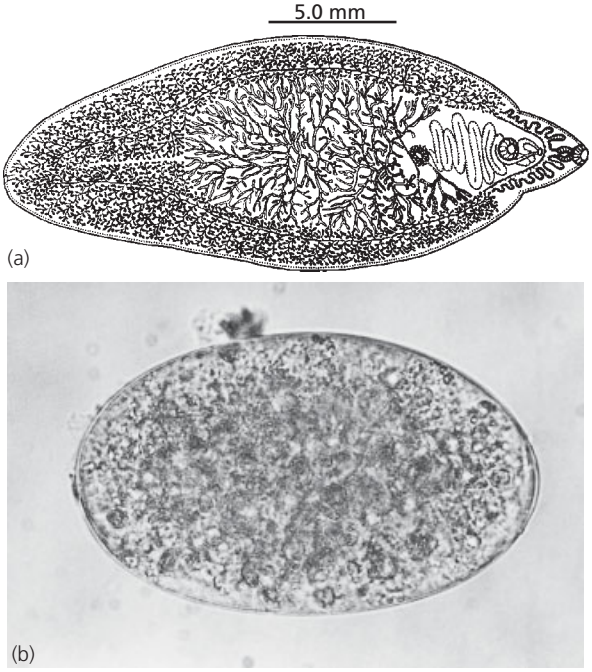

Figure 1 *The adult* **(a)** *and egg* **(b)** *of the liver fluke,* Fasciola hepatica. *(Drawn by S. Kaewkes, reproduced with permission)*

and Thomas in the 1880s and Lutz in 1892 (Beaver et al. 1984).

F. hepatica and *F. gigantica* are parasites of global veterinary importance, causing huge productivity losses in sheep, goats, and cattle. The two species are difficult to differentiate morphologically, although they utilize different snail hosts. Their relative importance to veterinary and human health is debated. The less studied *F. gigantica* may be the more important species in the tropics, while *F. hepatica* predominates in cooler regions. Human fascioliasis is uncommon but distributed globally (Chen and Mott 1990).

Paragonimus westermani (Figure 2a) was first discovered in the lungs of Bengal tigers by Kerbert in 1878. Shortly after, the worm was found at human autopsy and eggs were found in sputum by Ringer, Manson, and Baelz in Taiwan and Japan. Kobayashi, S. Yokogawa, and other Japanese investigators revealed the complete life cycle. There is debate regarding the number and relative importance of individual *Paragonimus* species infecting humans. Until recently, *P. westermani* was thought to be the only agent of human infection, but at least nine species are now known to be involved (Miyazaki 1982; Yokogawa 1982; Toscano et al. 1995; Velez et al. 2002). Considerable work has been carried out to attempt to characterize similarities and differences in their karyotypes and nuclear and mitochondrial genomes (see Blair et al. 1999; Blair 2000).

C. sinensis was discovered by McConnel in 1874 at the autopsy of a Chinese carpenter in Calcutta, and the parasite was subsequently named by Cobbold in 1875. *O. felineus* was first reported in a cat, while in 1892 Winogradoff reported the first human infection in Siberia. *O. viverrini* (Figure 3a) was first described in cats in 1886 by Poirier, then infection was discovered in a resident of Chiangmai, northern Thailand by Kerr. Leiper identified the worms as a species distinct from *O. felineus* in 1915. These parasites have lived with humans for millennia; *Clonorchis* eggs have been found in a corpse from the West Han dynasty in China dated 278BC (cited in Chen et al. 1994).

The liver flukes have attracted considerable interest because of their close association with bile duct cancer. *O. viverrini* infection was classified as a human carcinogen by the International Agency for Research on Cancer, and *C. sinensis* infection was judged a probable carcinogen (IARC 1994). The current understanding of the link between liver fluke infection and cancer has recently been reviewed (Okuda et al. 2002; Watanapa and Watanapa 2002).

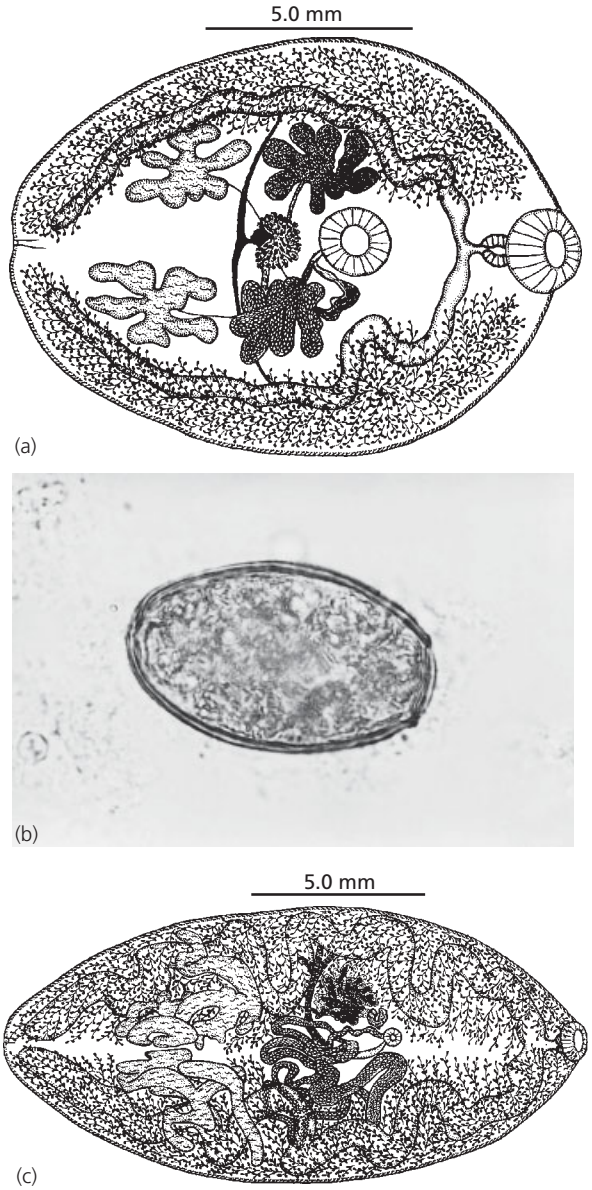

Figure 2 *The adult* **(a)** *and egg* **(b)** *of the lung fluke,* Paragonimus westermani *and adult worm of* P. heterotremus **(c)** *(Drawn by S. Kaewkes, reproduced with permission)*

Classification

The liver and lung flukes belong to the Phylum Platyhelminths (flatworms), Class Trematoda, and Subclass Digenea (Beaver et al. 1984). The latter term (digenetic trematodes) is often used to indicate the indirect life cycle involving several morphological stages and at least one intermediate host. The medically important foodborne flukes were once classified under a single genus, *Distoma*, referring to their two conspicuous suckers. Later it became clear that distomate flukes comprised a large and complex group of parasites, as indicated by today's classification into several superfamilies.

Fasciola, along with intestinal flukes *Fasciolopsis* and *Echinostoma*, belongs to the superfamily Echinostomatoidea. *Paragonimus* is a member of the superfamily Plagiorchioidea; whereas *Opisthorchis* and *Clonorchis* fall within the superfamily Opisthorchioidea. These

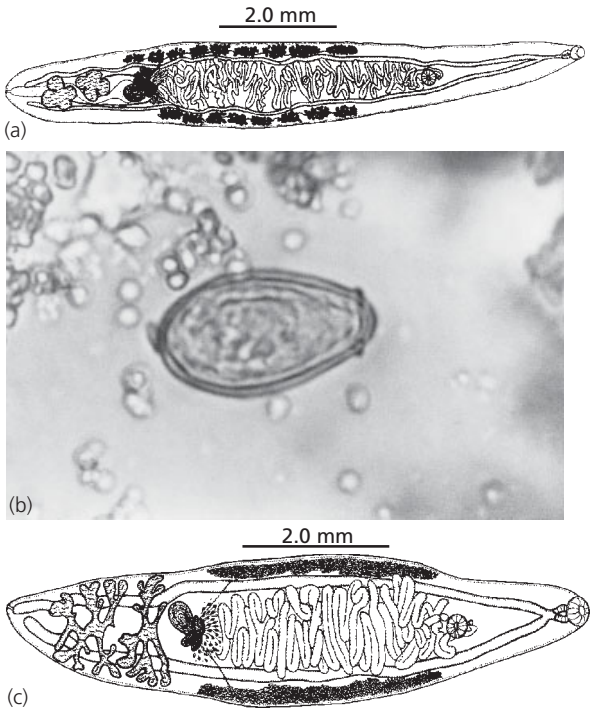

Figure 3 *The adult* **(a)** *and egg* **(b)** *of the liver fluke,* Opisthorchis viverrini *and adult worm of* Clonorchis sinensis **(c)** *(Drawn by S. Kaewkes, reproduced with permission)*

orders are differentiated on the basis of life cycle (intermediate hosts), morphological structure (especially of the excretory bladder), the suckers in the adult worm and spines in intermediate stages, and size and maturity of eggs when laid.

Most food-borne trematodes are zoonotic, i.e. usual parasites of nonhuman animals which 'accidentally' infect people. Several species, for example of *Dicrocelium, Opisthorchis*, and *Eurytrema*, infect birds, livestock, and wildlife and are reported rarely in humans (Beaver et al. 1984).

Morphology and structure

The adult flukes are elongated, often described as 'leaf shaped' and bilaterally symmetrical with three body layers, but have no true body cavity (Schmidt and Roberts 1994; Rim 1986). They are covered with a nonciliated integument which is not simply a secreted sheath: it is composed of complex, living tissue often containing invaginations, spines, and lectin-binding carbohydrate residues (Apinhasmit et al. 2000). *Fasciola* and *Paragonimus* species are covered with large spines. Muscles lie subcutaneously below the integument enveloping the body, while specialized structures, such as the esophagus and suckers, possess radial muscle fibers.

As mentioned above, the oral (surrounding the mouth) and ventral suckers are located in the anterior end, while the digestive system ends blindly with no rectum or anus. A specialized group of cells called solenocytes, or flame cells, join into an excretory bladder that exits through an excretory pore at the posterior end. This system probably carries out excretory functions and controls water balance. There is no circulatory system. The nervous system comprises an esophageal commissure with ladder-like pairs of nerve trunks running in each plane of the body ending in sensory processes. Eyespots may occur on free-swimming larval stages, but not on the adults.

A special feature of food-borne trematodes is their hermaphroditic (monoecious) reproductive system, that is, each individual worm produces both eggs and sperm. This contrasts with the schistosome trematodes which are dioecious (the sexes are separate) but in very close physical association. Most of the reproductive organs are positioned posteriorly to the ventral sucker, with their external opening (genital pore) often in close proximity to the ventral sucker.

The largest of the human liver and lung flukes is *Fasciola gigantica* (up to 75 mm long by 12 mm wide), followed by *F. hepatica* (30 mm × 13 mm) (see Figures 1a and 4). Both possess 'shoulders' and a conical anterior end. No single morphological feature definitively separates the two species, although there are genetic differences (Blair et al. 1999). *F. gigantica* tends to be more oblong with a longer, rounded posterior end, as compared to the short and more angular posterior end of *F. hepatica*. The testes of *F. gigantica* are located closer toward the anterior end, and its ventral sucker is larger than that of *F. hepatica*.

The intestinal caeca, testes, and vitelline follicles of *Fasciola* are extensively branched. The eggs (Figure 1b) are large, ovoid, yellowish brown with a small operculum, and contain an immature larva, the miracidium. The eggs of *F. gigantica* (190 μm) tend to be consistently larger than those of *F. hepatica*, which are 140–150 μm long (Beaver et al. 1984).

Paragonimus (see Figure 2a) is roughly half the size of *Fasciola*, measuring up to 16 mm long and 8 mm wide, oval-shaped, and reddish brown in color with an integument covered with scale-like spines (Miyazaki 1982). The ventral sucker is located toward the middle of the body and is of similar size to the oral sucker on the anterior end. The fluke possesses a large excretory bladder. Various morphological and life cycle differences in the adult worms distinguish the many species capable of infecting humans (e.g. *P. heterotremus*, Figure 2c). The yellowish brown *Paragonimus* eggs are oval, measure 90 × 55 μm, and have a flattened operculum (Figure 2b). They are unembryonated when laid.

In humans, *Clonorchis* measures up to 20 mm long and 5 mm wide (see Figure 3c), whereas the *Opisthorchis* species are somewhat smaller (see Figures 3a and 31.5; Komiya 1966; Rim 1986; Sadun 1955). The

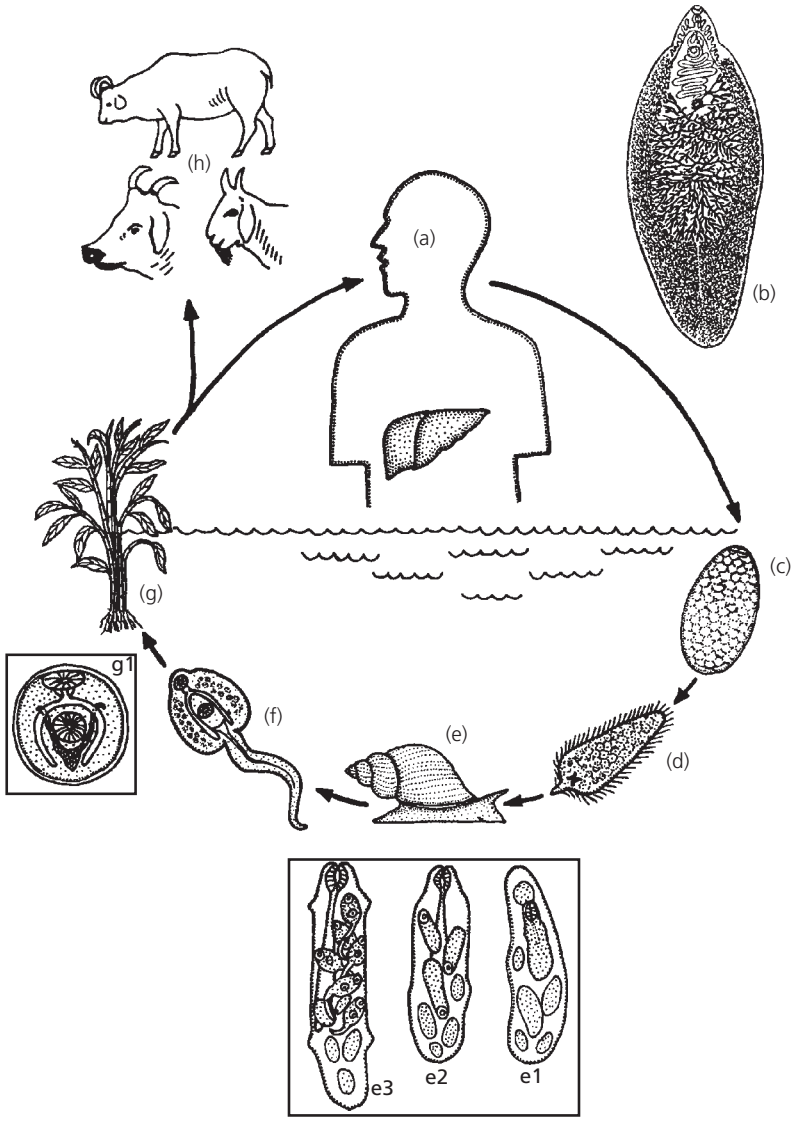

Figure 4 *The life cycle of* Fasciola hepatica. **(a)** *Definitive host: human;* **(b)** *The adult worm;* **(c)** *Unembryonated egg;* **(d)** *Miracidium;* **(e)** *First intermediate host:* Lymnaea *sp. Intramolluscan stages: el, sporocyst; e2, mother redia; e3, daughter redia;* **(f)** *Cercaria;* **(g)** *Second intermediate host: aquatic plants. Aquatic plant stage: gl, metacercaria;* **(h)** *Reservoir hosts: sheep, cattle, goat. (Drawn by S. Kaewkes, reproduced with permission)*

ventral sucker is smaller than the oral and located within the anterior half of the worm. Like *Fasciola*, the adult worm morphologies do not provide definitive separation, but the flame cell patterns on cercariae are distinct (Wykoff et al. 1965). The number and shape of testicular lobes, their location and the appearance of vitelline glands vary between, but also within, the species.

The genital pore through which eggs pass is located near the ventral sucker, with the large, coiled uterine glands filling most of the body. Eggs (Figure 3b) are small (approximately 25×15 μm), yellowish brown, contain fully developed miracidia when laid, and have a knob at the posterior end and a distinct operculum on the anterior end. Measurements and shape vary within and between species (Sadun 1955; Kaewkes et al. 1991; Ditrich et al. 1992).

General biology

Movement is a very important feature of trematodes. Their complex life cycle requires finding and entering appropriate hosts and extensive migration from the duodenum to the appropriate site of maturation within the final host (Schmidt and Roberts 1994). Flukes also undergo remarkable physiological adjustment in order to tolerate and function in their highly varied environments, from fresh water to invertebrate host to mammalian tissue. The fact that trematodes successfully utilize

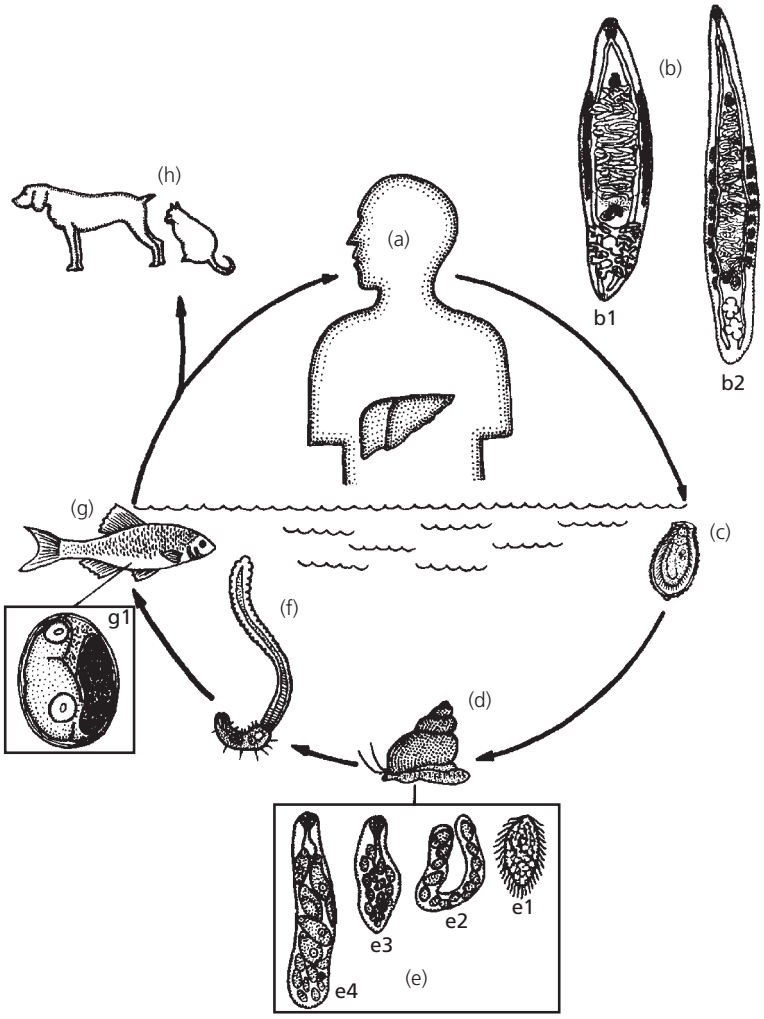

Figure 5 *The life cycle of human liver flukes:* **(a)** *Definitive host: human;* **(b)** *The adult worm. b1,* Clonorchis sinensis*; b2,* Opisthorchis viverrini*;* **(c)** *Embryonated egg;* **(d)** *First intermediate host:* Bithynia *sp.;* **(e)** *Intramolluscan stages. e1, miracidium; e2, sporocyst; e3, mother redia; e4, daughter redia;* **(f)** *Cercaria;* **(g)** *Second intermediate host: cyprinoid fish. Stage in fish: g1, metacercaria;* **(h)** *Reservoir hosts: dog, cat, and other mammals. (Drawn by S. Kaewkes, reproduced with permission)*

these extremely complex routes to achieve adulthood and reproduction reflects complex evolutionary development to maximize transmission.

Hou (1955) described the movement of *Clonorchis* by means of attachment and detachment of its two suckers combined with extension and contraction of the body. The two suckers and a collar of spines on the immature worm are presumably used to migrate up the biliary tract, against the flow of bile (Apinhasmit et al. 1993). Mature worms probably move short distances within the ducts. Attachment is secured by the ventral sucker adhering to the biliary epithelium, leaving the oral sucker free for feeding.

The nutritional supply of human trematodes is not precisely understood. Initially it was postulated that *Clonorchis* feeds on red blood cells, bile, and epithelial cells. Hou (1955), however, suggested that the parasite lives on protein, oxygen, and glycogen derived from mucin and tissue fluid entering the lumen following desquamation. *Fasciola* feeds on blood and hepatic tissue as it transverses the parenchyma during the migration phase, and its food supply in the bile duct may be similar to that of *Clonorchis*. The biliary flukes live in conditions of very low oxygen tension, and anaerobic glycolysis of glycogen to glucose is the major energy pathway (Schmidt and Roberts 1994). A glycolytic enzyme, phosphoglycerate kinase, is found in the tegument of *Clonorchis sinensis* and probably assists in the utilization of external glucose for its metabolism (Hong et al. 2000). Ammonia, urea, and amino acids are excreted. Among the amino acids released by *Fasciola* are remarkably high levels of proline (Wolf-Spengler and Isseroff 1983).

Life cycle

The life cycles of food-borne trematodes are complex, involving one or more intermediate hosts (the first

always a snail), several morphological stages, and distinct generations. These stages are similar between liver and lung flukes, and although intermediate host species vary, all are freshwater not marine.

DETAILS OF THE PARASITES IN HUMANS AND OTHER DEFINITIVE HOSTS

The infective stages of these flukes are called metacercariae. Humans become infected with *Opisthorchis* and *Clonorchis* by consuming raw or undercooked fish containing encysted metacercariae (Figure 5). *Paragonimus* is acquired by eating metacercariae in raw crabs (Figure 6), whereas *Fasciola* metacercariae encyst on aquatic plants (see Figure 4). Ingested metacercariae excyst in the duodenum, releasing larvae.

Newly excysted *Opisthorchis* and *Clonorchis* larvae migrate through the ampulla of Vater and the extrahepatic bile ducts to the smaller, intrahepatic ducts where they mature. Within 1 month, adult worms begin producing an average of 10 000 eggs per day which exit the bile ducts and are excreted in the feces (Sithithaworn et al. 1991).

Paragonimus larvae undergo systemic migration. Upon ingestion, the larvae are released in the duodenum, penetrate the intestine, and enter into the abdominal cavity. The parasites enter and remain in the abdominal wall for some days, then continue migration through the diaphragm into the pleural cavity and lungs. When they reach the terminal alveoli or under the pleura, they become encapsulated by the host's inflammatory response and produce eggs. Eggs are expelled in the sputum or may be dislodged by coughing, are then swallowed and excreted in the feces.

Fasciola metacercariae excyst in the duodenum, and the immature worms migrate through the duodenal wall,

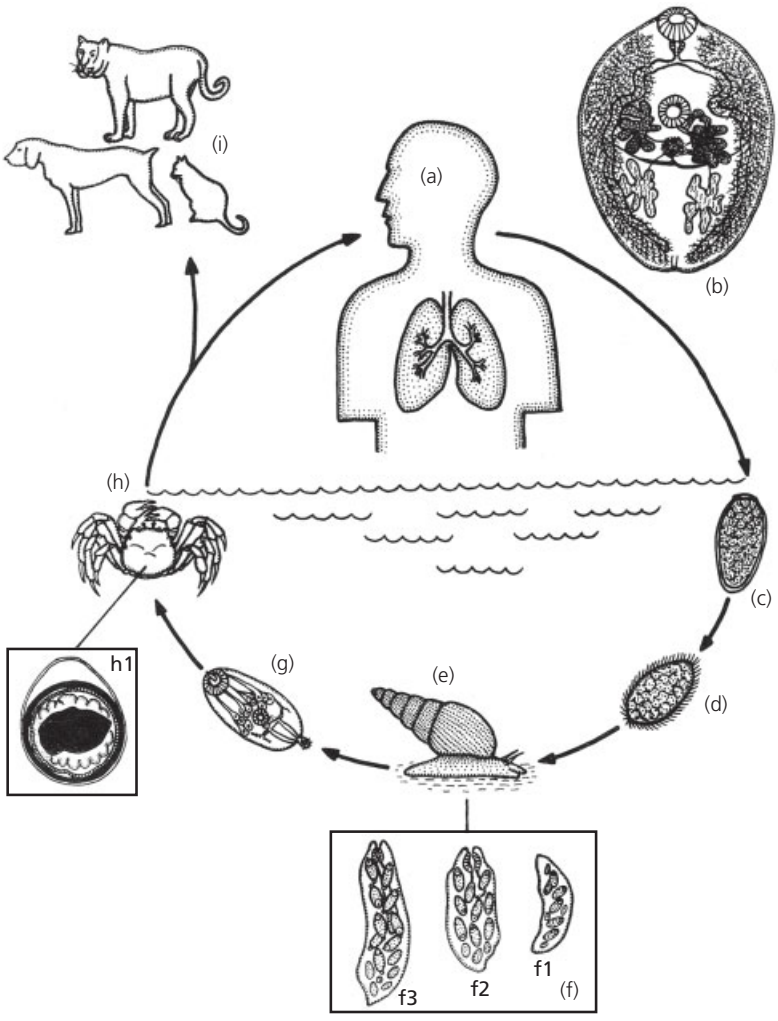

Figure 6 *The life cycle of* Paragonimus westermani*:* **(a)** *Definitive host: human;* **(b)** *The adult worm;* **(c)** *Unembryonated egg;* **(d)** *Miracidium;* **(e)** *First intermediate host: Thiarid snail;* **(f)** *Intramolluscan stages. f1, sporocyst; f2, mother redia; f3, daughter redia;* **(g)** *Cercaria;* **(h)** *Second intermediate host: crustaceans. Stage in crustacean: h1, metacercaria;* **(i)** *Reservoir hosts: dog, cat, tiger. (Drawn by S. Kaewkes, reproduced with permission)*

into the body cavity. They then burrow through Glisson's capsule and across the hepatic parenchyma to the proximal bile ducts and gallbladder where they mature. This migration takes 6–7 weeks. The adult worms begin producing eggs that exit in the feces 3–4 months after ingestion of metacercariae.

The worms may live many years. The maximum reported life span of *Clonorchis* in the absence of reinfection is 26 years from a Chinese emigrant, and average life span in endemic areas is about 10 years (Attwood and Chou 1978; Chen et al. 1994). Similarly *Paragonimus* is thought to live a maximum of 20 years and an average of 6 years; and *Fasciola* probably lives around 10 years (Chen and Mott 1990).

O. viverrini, *C. sinensis*, and *O. felineus* infect pigs, cats, civets, rats, dogs, foxes, and other mammals. *Fasciola* infections are maintained in domestic livestock, e.g. sheep, goats, camels, buffaloes, and cattle. Wild rabbits, hares, and rodents are thought to maintain infection in uncultivated watercress beds in France (Rondelaud et al. 2001). *Paragonimus* infection is found in a range of reservoir hosts such as tigers and cats, rats, wolves, dogs, and foxes. Although infection cannot occur by consuming adult worms or eggs within livers of infected animals, *Paragonimus* may establish through consumption of incompletely cooked pig or wild boar meat containing lung fluke larvae (Miyazaki 1982).

Patterns of infection in reservoir hosts are not always closely linked with those in humans, since these are determined by distributions of intermediate hosts, whereas human infection is further limited by eating behavior (Sadun 1955; Rim 1986). However, reservoir hosts may be particularly important where human infections are uncommon or sporadic, and human egg excretion is inefficient (e.g. *Fasciola*, *Paragonimus*), or blocked by sanitation or anthelmintic treatment (*Opisthorchis* and *Clonorchis*).

DEVELOPMENTAL STAGES OF THE PARASITES

Freshwater bodies (small ponds, streams and rivers, flooded rice fields, and large reservoirs) or (for *Fasciola hepatica*) submerged grass become contaminated with eggs from the feces and sputum (for *Paragonimus*) of infected people or reservoir hosts. The parasites must enter an appropriate snail. Species of *Bithynia, Melanoides, Parafossarulus*, and *Assiminea* are important first intermediate hosts of *Opisthorchis* and *Clonorchis*. These snails become infected by ingesting eggs which contain fully mature miracidial larvae. In contrast, *Paragonimus* eggs develop and hatch in water after approximately 3 weeks. The miracidia must find and penetrate a permissible species of snail; the major ones are of the genera *Semisulcospira, Thiara*, and *Oncomelania*. *F. hepatica* miracidia develop and hatch within 2 weeks and swim until finding an appropriate snail of the genus *Lymnaea*. *F. gigantica* requires aquatic species of *Lymnaea*. Both the snails and the developmental stages of the fluke appear to have made adaptations that provide a greater survival capacity in the high altitudes of the Altiplano of Bolivia and Peru (Mas-Coma et al. 2001).

Inside the snail, the miracidia develop into sporocysts and rediae, which undergo parthenogenetic reproduction, finally giving birth to cercariae. Cercariae emerge and swim to find an appropriate site for transformation into metacercariae. Nearly 100 species of cyprinoid fish serve as the second intermediate host of *Opisthorchis* or *Clonorchis* (Komiya 1966; Waikagul 1998; Fattakhov 2002). *Paragonimus* cercariae penetrate gills and muscles of freshwater mitten crabs (*Potamon*, *Sesarma*, *Eriocher*) or crayfish (*Astacus*). Fifty three crustacean species have been reported to serve as second intermediate hosts of *Paragonimus* spp. (Toscano et al. 1995). The species of *Paragonimus* present appears to be related to water flow and suitability of the aquatic environment for various crab and snail species (Cheng 1999). The cercariae transform into metacercariae in the viscera, muscles, or gills. *Fasciola* encysts on green water vegetation, e.g. watercress; it does not enter a second intermediate host.

These life cycles require at least 4 months to complete and may be prolonged by winter hibernation of snails (Rim 1986). Seasonal variation in transmission, water temperature, densities of intermediate and reservoir hosts, and human sanitation and eating behavior govern life cycle completion and the prevalence of human infection.

TRANSMISSION AND EPIDEMIOLOGY

Geographic distribution

A crude estimate of the global number of human liver fluke infections is in the order of 17 million, made up of 7 million with *Clonorchis*, 9 million with *O. viverrini*, and 1.2 million with *O. felineus* infections (Iarotski and Be'er 1993; WHO 1995). Countries with endemic human liver fluke infection are Thailand, Laos, and possibly Cambodia (*O. viverrini*) (Lee et al. 2002), Korea, China, Taiwan, (formerly Japan), Hong Kong, Macao, and Vietnam (*C. sinensis*), and Russia, Kazakhstan, Ukraine, and possibly some eastern Europe countries (*O. felineus*). Encouraging news has come from northeast Thailand, where the regional prevalence of infection appears to have dropped from 34 percent in 1981, to 24–30 percent in 1992 to 18.6 percent in 1994 (Jongsuksuntigul and Imsomboon 1997). There also seems to be a decline in the frequency of *Opisthorchis* metacercariae found in fish over the same time period (Waikagul 1998). Prevalences remain high in neighboring Laos (Vannachone et al. 1998; Kobayashi et al. 2000), however, and may be increasing in central and northern Thailand. Research in Northern Thailand collecting adult worms post-

treatment and examining metacercariae in fish has suggested this increase may be partly a result of misdiagnosis of minute intestinal fluke infections (Radomyos et al. 1998; Sukontason et al. 2001), which are much less dangerous than liver flukes. Infections in China are also ethnically and geographically associated. Cantonese, notably Hakka people, are most frequently infected in the southern provinces of Guangdong and Guangxi, while infections in northeast China occur among the Korean national minority who migrated there (Chen et al. 1994). Infection in Hong Kong is probably acquired from eating fish imported alive from southern China. A study conducted in Ninh Binh Province in Vietnam revealed a high prevalence of *Clonorchis* metacercariae among fish farmed in ponds (Kino et al. 1998).

The human lung flukes, which comprise approximately nine species of *Paragonimus*, infect an estimated 22 million people globally (Miyazaki 1982; Yokogawa 1982; Toscano et al. 1995; Velez et al. 2002); with approximately 10 million in China alone. China harbors three species infecting humans, *P. westermani*, *P. heterotremus*, and *P. skrjabini*. Other Asian countries with endemic *Paragonimus* infection include Philippines, Korea, India, Thailand, and Laos. Peru, Ecuador, and Colombia report large areas endemic for *P. mexicanus*, while Cameroon and Nigeria report *P. africanus* and *P. uterobilateralis*. Scattered reports have come from parts of India, Japan, Latin America, Liberia, Guinea, and some Pacific countries. In the United States, there are occasional human infections with the lung fluke, *Paragonimus kellicotti*, which normally parasitize wildlife (DeFrain and Hooker 2002).

Fasciola infections number approximately 2.4 million and are more globally widespread than *Paragonimus*. The World Health Organization identified 2 594 human cases of *Fasciolia hepatica* in 42 countries from the scientific literature and public health reports between 1970 and 1990 (Chen and Mott 1990). Countries that reported the most cases were France, the UK, Portugal, Spain, the former USSR (Tadzhikstan), Peru, Cuba, Ecuador, Egypt, and Iran. Community-based studies of *Fasciola* infection using improved diagnostic methods have demonstrated a very high prevalence and intensity of infection in the high altitude regions of Bolivia and Peru (Esteban et al. 1997; Mas-Coma et al. 2001; Haseeb et al. 2002) and Egypt (Hassan et al. 1995). Some argue that *Fasciola* should no longer be considered an accidental zoonosis because it is an important human parasite (Mas-Coma et al. 1999). Less is known about the extent of *F. gigantica* infection, except that it has been reported in Africa, Asia, Hawaii, the former USSR, and Iraq. Although *Fasciola* was previously considered rare in Vietnam, 500 cases have been reported since 1997 (Tran et al. 2001).

In addition to the endemic areas for all of these flukes, regional and global migration of people has sometimes broadened their distribution and returned travelers seek medical treatment in areas well outside the endemic zones. Since life cycles usually do not become established, this may have limited epidemiological relevance. Given the potential severity of resulting disease, however, it is of clinical importance that physicians are better informed to recognize these infections (Chan and Lam 1987; LaPook et al. 2000). The World Health Organization is concerned about the spreading of human trematode infections via the increasing international trade in fresh aquatic foods from countries in which such infections are endemic (Maurice 1994).

Social factors related to transmission

Raw or undercooked freshwater fish and crab are prepared in many different ways and these dishes are often of considerable cultural, medicinal, and nutritional significance, making change difficult. This is aggravated by frequently held beliefs that marination in various sauces or consumption with alcohol kills the parasites. Transmission can also occur via contamination of utensils, hands, and surfaces used first to prepare crabs, fish or vegetables for cooking, and then for other foods taken raw. *Fasciola* and *Paragonimus* infection through contaminated water may be possible, but is generally of lesser importance than through food.

Traditionally in southern China and among the Cantonese of Hong Kong, raw fish containing *Clonorchis* metacercariae is eaten by dipping in rice porridge or kongee (Chen et al. 1994). Large fish may be sliced and eaten raw with ginger and garlic. In some areas, children become infected by catching and incompletely roasting fish during play. Koreans eat raw fish soaked in vinegar, red-pepper mash or hot bean paste with rice wine at social gatherings (Choi 1984; Rim 1986). Vietnamese people, particularly men, reportedly eat raw fish from contaminated fish ponds in salads in the hot season (Chen et al. 1994; Kino et al. 1998). Infection in Japan, which is now very rare, came from eating slices of large raw fish with vinegar or soya bean paste (Komiya 1966). Sushi and other uncooked sea fish eaten in Japan today do not carry *Clonorchis*.

In northeast Thailand and Laos, preparations may contain fresh (*koi pla)*, partially fermented *(som pla)* or fully fermented (*pla ra)* uncooked fish which contain *Opisthorchis* metacercariae. *Koi pla* is probably the most important source of infection (Bunnag and Harinasuta 1984). People in western Siberia and other areas of the former USSR, endemic for *O. felineus*, enjoy eating uncooked fish frozen, salted, or smoked with condiments. In addition to some groups of aboriginal people, migrants to endemic areas also become infected (Iarotski and Be'er 1993).

Raw crabs which may transmit *Paragonimus* infection also carry important social significance in some areas. For example in Korea, raw crayfish juice was used as a treatment for childhood measles (Choi 1990; Cho 1994),

and young Bakossi women in Cameroon ate raw crabs to increase fertility (Kum and Nchinda 1982). Both practices have apparently become rare. Crabs and crayfish are also caught and eaten raw or incompletely roasted by children and workers in rice fields (Cabrera 1984). Traditional dishes often involve raw crab or crayfish meat or juice mixed with lemon juice (*ceviche* in Peru), brine, soya sauce (Korean *ke-jang*), alcohol (drunken crabs, China), or vinegar (Laos).

The consumption of watercress has been implicated in most human *F. hepatica* infections, especially in Europe (Hardman et al. 1970; Rondelaud et al. 2000). In the high altitudes of the Bolivian Altiplano region, people (especially children) consume kjosco (raw water plant salad) while tending their animals in the fields. However, inhabitants of the Peruvian Altiplano who also have a high prevalence and intensity of *Fasciola* infection do not consume aquatic vegetables. Exposure in this group appears to be through contaminated irrigation water used for drinking (Esteban et al. 2002). Morning glory and other water plants are important vehicles of *F. gigantica* infection in Asia (Tesana et al. 1989).

Distribution of infection in human communities

Local patterns of infection within endemic communities are partially determined by social customs and attitudes toward raw foods. This is especially true where these foods are thought to have medicinal or fertility-enhancing properties, where catching and eating raw foods is part of children's play, or where the foods accompany certain social activities, such as drinking parties. Infection patterns may also reflect differential effectiveness of health education efforts in endemic countries.

Levels of *Opisthorchis* and *Clonorchis* infection vary greatly between communities, but age-related patterns are generally similar. The youngest ages show low prevalence and intensity, while these increase in the pre- and early teens and often reach a plateau in the late teen age groups (e.g. 15–19 years) or continue to rise. Prevalence and intensity either do not differ, or are higher, among males compared to females; sex differences tend to be greater in areas endemic for *Clonorchis* (Upatham et al., 1984; Rim 1986; Haswell-Elkins et al. 1991; Kino et al. 1998). The population of *O. viverrini*, and probably of all the liver and lung flukes, is highly aggregated within a small minority of heavily infected people.

There have been very few community-based studies of *Paragonimus* infection, so that its distribution is not well characterized. Studies in the 1950s and 1960s in Korea revealed prevalences of *Paragonimus* infection of up to 45 percent (according to skin testing) in endemic communities and as high as 13 percent nationwide. Recently, however, the occurrence of areas of high prevalence has dropped dramatically (Choi 1990; Cho 1994). In other endemic countries, e.g. China, Thailand, Laos, Cameroon, Nigeria, Peru, and China, infections tend to occur at low prevalences (under 10 percent) in defined areas. Mountainous areas with unpolluted water are most favorable to *Paragonimus* transmission.

Fasciola infection occurs mainly in rural areas, with sheep and cattle herders and people living in small villages typically being most at risk of infection. In France, however, an increase in cases from people living in larger towns within cattle-rearing areas has been observed (Rondelaud et al. 2000). Very high prevalences (up to 75 percent in children) and intensities of infection (>1 000 eggs/g of feces) with *Fasciola hepatica* are found in communities of the Bolivian Altiplano region, where transmission occurs year round, especially from December to March (Esteban et al. 1997; Mas-Coma et al. 1999). A study of schoolchildren in Egypt revealed 11 percent prevalence of infection with *Fasciola* (Hassan et al. 1995). In addition to chronic infection, fascioliasis sometimes occurs in acute outbreaks within communities and households where food from a common source of contaminated aquatic plants is consumed.

Location in host

Opisthorchis and *Clonorchis* locate within the small, intrahepatic bile ducts; in heavy infections, adult worms may also be found in the extrahepatic bile ducts, pancreatic ducts, and gallbladder (Hou 1955; Sithithaworn et al. 1991). Infection is confined to the hepatobiliary tract lumen; there is no tissue migration phase (Sun et al. 1968). *Fasciola* also finally dwells in the bile duct, but its migration out of the duodenum and through the hepatic parenchyma causes much more tissue trauma and acute inflammation than that of *Clonorchis* and *Opisthorchis*. *Paragonimus* flukes are found within fibrotic capsules in the terminal alveoli or under the pleura in the lungs after going through a similarly complex migratory process.

In addition to these usual migratory routes, some worms of *Fasciola* and *Paragonimus* may become lodged in ectopic sites. Ectopic sites of *Paragonimus* include the liver, brain, mesenteric lymph nodes, intestinal wall, and subcutaneous tissue of the groin. *Fasciola* may locate in the liver parenchyma, blood vessels, lungs, subcutaneous tissue, and the brain. Thus, as discussed below, clinical manifestations of this infection may be due to their accumulation in their 'normal' site or due to one worm that becomes encapsulated in a sensitive, ectopic site.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Although these infections are not considered numerous enough to be among the top tropical parasitic diseases according to the World Health Organization, there is no question that they are highly pathogenic and cause

significant human disease. The very important clinical aspect of *Opisthorchis viverrini*, and to a lesser extent of *Clonorchis* infection, is the extreme susceptibility of infected people to bile duct cancer (IARC 1994). This cancer has a very poor prognosis, and few patients live longer than 1 or 2 years after diagnosis and/or surgery (Watanapa and Watanapa 2002). Thus, mortality due to complications of this fluke infection is very high in the endemic area of northeast Thailand, which reports the world's highest incidence of liver cancer (Vatanasapt et al. 1993).

Very importantly, in endemic areas, these infections are usually asymptomatic or may contribute to a high background level of nonspecific illness that is difficult to distinguish from other causes. For example, the hemoptysis of *Paragonimus* requires parasitological and bacteriological differentiation from that of the more prevalent tuberculosis in co-endemic areas (Toscano et al. 1995). Similarly, the abdominal pain reportedly occurring in *Opisthorchis* infection is difficult to differentiate from that due to other causes. However, particularly in the case of the liver flukes associated with cholangiocarcinoma, the absence of symptoms does not indicate a medically unimportant infection. In fact, the typical lack of specific clinical signs and symptoms (or pain and discomfort) makes treatment and prevention even more difficult.

Clinical manifestations

The likelihood and types of clinical manifestations associated with these fluke infections are dependent on the location of the parasite in the liver, lungs, or ectopic site, the species of fluke, number of worms harbored, duration of infection, and, possibly, nutritional status and the individual's pattern of immune responses to parasite antigens. Chronic trematode infection may also play a role in promoting chronic renal disease in tropical countries (Boonpucknavig and Soontornniyomkij 2003).

O. VIVERRINI, *C. SINENSIS*, AND *O. FELINEUS*

The frequency and types of clinical disease seem to differ between the three closely related species. Most notably, there are many reports in the *O. felineus* and *C. sinensis* literature detailing specific signs and symptoms accompanying well-defined clinical stages of opisthorchiasis, from acute to chronic (Bronshtein 1986; Chen et al. 1994). Acute infection, characterized by chills and high fever, abdominal pain and distension, hepatitis-like symptoms, and eosinophilia, is frequently reported in *O. felineus*. In contrast, there are fewer reports of acute clonorchiasis (Koenigstein 1949; Chen et al. 1994) or opisthorchiasis viverrini. This difference may be due to the large number of migrants entering endemic areas of *O. felineus* who first become infected as adults (Iarotski and Be'er 1993), which may be rare for the other two species.

Studies in two communities with very high levels of *O. viverrini* infection reported significantly increased frequencies of hepatomegaly, abdominal pain in the right upper quadrant, flatulence, dyspepsia, and weakness associated with increasing intensity of infection (Upatham et al. 1984). An estimated 5–10 percent of the community had mild symptoms attributable to the infection. Hospital-based studies on patients with *Opisthorchis* and *Clonorchis* infection (lacking comparison with uninfected controls) have reported much higher frequencies of signs and symptoms. In addition to the above-mentioned symptoms, eosinophilia, anorexia, dizziness, weight loss, diarrhea, anemia, edema, neuropsychiatric symptoms, and retardation of growth and sexual maturity have been reported (Bunnag and Harinasuta 1984; Chen et al. 1994).

Stones in the gallbladder, liver, and bile ducts have been linked to liver fluke infection. Eggs or worm fragments are often found in the nidus (Teoh 1963; Riganti et al. 1988). Hepatolithiasis is considered a major clinical manifestation of *Clonorchis* infection (Leung and Yu 1997). An increase in gallbladder stone frequency with increasing intensity of *Clonorchis* infection was found by ultrasonography among Hakka people in Taiwan, from 4.2 percent in uninfected subjects to over 14 percent of those with heavy infections (Hou et al. 1989). Epidemiological associations with stones, however, are weaker for *O. viverrini* infection.

Ascending cholangitis, obstructive jaundice, portal hypertension, ascites, and gastrointestinal bleeding are severe complications of liver fluke infection. However, only 88 cases of severe disease were reported among 15 243 infected people who presented to a Bangkok hospital for praziquantel treatment (Pungpak et al. 1985). Furthermore, these disease presentations are also typical of cholangiocarcinoma that may have been present in some patients.

Studies using ultrasonography have shown very strong relationships between gallbladder enlargement, wall irregularities, and sludge and intensity of infection (Lim 1990; Mairiang et al. 1992). These abnormalities are not accompanied by clinical signs and symptoms and are reversible following praziquantel treatment (Mairiang et al. 1993; Pungpak et al. 1997; Richter et al. 1999).

As mentioned above, the most important clinical manifestation of opisthorchiasis, also occurring in clonorchiasis, is bile duct cancer. People with heavy *O. viverrini* infection face at least a 14-fold increased risk of cholangiocarcinoma over uninfected people from the same communities (Haswell-Elkins et al. 1994a). This cancer is a major cause of death in the endemic area of northeast Thailand, where age-standardized incidences are 84.6 and 36.8 per 100 000 males and females, respectively (Vatanasapt et al. 1993). This compares to two to four cases per 100 000 typically arising in non-endemic

areas. Areas endemic for *Clonorchis* infection also report higher frequencies of bile duct cancer (Rim 1986; IARC 1994). In a study of liver cancer in Korea, *Clonorchis* infection was found to carry a relative risk of 2.7 (95 percent confidence intervals 1.1–6.3) for cholangiocarcinoma (Shin et al. 1996). A number of case studies have recently been published describing extrahepatic cholangiocarcinoma and gall bladder carcinoma associated with *Clonorchis* infection (Kim 2003a, b). The average age of patients is 50 years, with a higher frequency of males and, due to an absence of early symptoms, those affected present to hospital in the late stages of the disease. Prognosis with or without surgery is poor.

FASCIOLA HEPATICA AND *F. GIGANTICA*

As with opisthorchiasis and clonorchiasis, fascioliasis is often asymptomatic, especially in light or chronic infection. Reported symptoms are grouped into three phases, depending on where the parasites are in their migration (Chen and Mott 1990).

First, the acute or invasive stage occurring when the flukes are in migration through the liver parenchyma may be accompanied by fever and night sweats, mild to severe abdominal pain, gastrointestinal disturbances, urticaria, malaise, weight loss, dermatographia, and cough (Hardman et al. 1970; Chen and Mott 1990; Patrick and Isaac-Renton 1992). Clinical signs may include hepatomegaly and splenomegaly, ascites containing eosinophils and other leukocytes, mild to moderate anemia with its accompanying symptoms, pulmonary signs, eosinophilia, and jaundice. Biochemical abnormalities are also observed, for example, elevation of acute phase proteins (Basha et al. 1998), raised serum bile acids (Osman et al. 1999), and sometimes increased liver enzymes (e.g. alkaline phosphatase, although not GGT and SAP).

The latent phase of infection, during which the flukes are in the bile ducts, is generally asymptomatic, perhaps involving gastrointestinal symptoms, and can last for years. Some individuals progress to the obstructive phase of infection with chronic cholecystitis and cholangitis, which may be accompanied by biliary colic, epigastric pain, jaundice, nausea, pruritis, and right upper quadrant pain precipitated by fatty foods. Gallbladder enlargement and stones are frequently found in these patients. Anemia and ascites may occur as a result of blood loss and deaths, although rare, can occur through hemorrhaging in the bile duct.

Ultrasonography, ERCP, and MRI may reveal the worms themselves in the liver or on the gallbladder wall, bile duct dilatation and filling defects. *Fasciola* has been implicated as a carrier for *Salmonella typhimurium* as the bacteria preferentially bind to the metacercariae, thus treatment for both infections may be required (El Zawawy et al. 2002).

Ectopic *Fasciola* infections leading to pulmonary, cardiac, gastric, cecal, cerebral, and neurological disorders have all been reported. These appear to be rare, although the frequency with which parasitic origins of such diseases are missed is unknown.

PARAGONIMUS WESTERMANI AND OTHER LUNG SPECIES

Because of interspecies differences in migratory behavior and extent of pulmonary pathology, clinical manifestations vary between the various *Paragonimus* species infecting humans (Miyazaki 1982).

In general, however, the clinical manifestations of *Paragonimus* infection are often misdiagnosed as tuberculosis or lung cancer (Beaver et al. 1984; Toscano et al. 1995; Nagakura et al. 2002). Nonspecific symptoms, e.g. diarrhea, abdominal and chest pain, allergic reactions, fever and chills, may be present during the migration phase. Once the worms establish, the most common symptoms are cough and hemoptysis which may be accompanied by night sweats and general malaise (Yokogawa 1982). Up to 50 ml of gelatinous, rusty brown sputum containing traces of blood and parasite eggs may be expectorated daily during paroxysmal coughing. Severe infections might progress to pleurisy, persistent rales, clubbed fingers, and pneumothorax. The severity of pulmonary lesions viewed by chest roentgenograms is associated with intensity of egg output and duration of infection with *P. heterotremus* in Thailand (Vanijanonta et al. 1984).

Ectopic infection with *Paragonimus* can occur in the brain, omentum, skin, and liver (Miyazaki 1982; Sasaki et al. 2002; Hughes and Biggs 2002). If parasites lodge in the brain or spinal cord, which happens more frequently in children, severe disease may result (Miyazaki 1982; Bunnag and Harinasuta 1984; Jaroonvesama 1988). Symptoms may include headache, fever, paralysis, visual disturbances, and (sometimes fatal) convulsive seizures. Recovery may be spontaneous, but symptoms may recur. Lesions are visualized by computed tomography or cerebral angiography.

Migration of worms under the skin leads to the formation of migratory swellings containing the parasites and eggs. Intestinal paragonimiasis which manifests as multiple granulomatous nodules or ulcers can be mistaken for gastric or abdominal tumors.

Pathogenesis and pathology

Although the role of immune responses in protection against infection is debated in all of these parasites, it is clear that these play an important role in pathogenesis and may determine whether clinically significant disease results.

PARAGONIMUS INFECTIONS

Juvenile *Paragonimus* which migrate successfully to the lungs elicit insignificant pathology. However, adult

worms and trapped eggs in the pulmonary parenchyma stimulate inflammation and granuloma formation, consisting mainly of eosinophils and neutrophils (Miyazaki 1982; Matsumoto et al. 2002). These granulomas develop into fibrous capsules, 1.5–5 cm in diameter, usually surrounding a pair or triplet of worms, eggs, and blood-streaked fluid. The pleural fluid of patients recently infected with *Paragonimus westermani* may contain large numbers of eosinophils and IgM and high concentrations of IL-5, thymus, and activation-related chemokine and eotaxin which may be involved in the acute pathogenesis of infection (Matsumoto et al. 2002). Leakage of fluid into the bronchioles causes paroxysmal coughing, hemorrhage, and blood in the sputum. Later in infection, parasite-specific IgG dominates and nodular, cavitating lesions develop in the lung parenchyma (Nakamura-Uchiyama et al. 2001). Flukes which lodge in ectopic sites, e.g. the brain, spinal cord, intestine, and heart, invoke similar inflammatory responses leading to ulcerations and abscesses which cause severe damage in sensitive sites.

O. VIVERRINI , *O. FELINEUS*, AND *C. SINENSIS*

The acute phase of disease appears to result from acute inflammatory reactions to parasite antigens met for the first time (Chen et al. 1994). Typically light, chronic infections show minimal pathological change. In heavier infections, the liver may be enlarged with localized dilatation of slightly thickened, fibrosed peripheral bile ducts (Hou 1955; Rim 1986). Histopathological changes (see Figure 7) include proliferation and desquamation of bile duct epithelial cells, glandular formation, goblet cell metaplasia, inflammatory infiltration by lymphocytes, monocytes and eosinophils and severe fibrosis (Pairojkul et al. 1991). Although pathological changes are most frequent in the small ducts where the flukes reside, the gallbladder is commonly enlarged and its function affected, leading to bile stasis. A 24 kDa antigen of *Clonorchis* is thought to be a major cytotoxic protease causing direct pathology (Park et al. 1995), while Sripa and Kaewkes (2000, 2002) found inflammatory changes associated with excretory and secretory antigens throughout intrahepatic and extrahepatic bile ducts in experimental *O. viverrini* infection. Eosinophils were found to be the major inflammatory cell in both *Opisthorchis*- and *Clonorchis*-associated cholecystitis (Kim 1999; Sripa and Kaewkes 2000). In some cases, the extrahepatic bile ducts may also become inflamed and fibrosed, leading to strictures and stagnant bile. This might facilitate bacterial infection, leading to abscess formation and acute cholangitis (Chen et al. 1994). Increased levels of various biochemical indices of hepatic function suggest that chronic liver fluke infection enhances cytolysis of hepatocytes, cholestasis, and hepatic cell insufficiency (Bakshtanovskaia et al. 2002).

Figure 7 *Pathology of liver flukes: mature liver fluke,* Opisthorchis viverrini*, residing in the lumen of the human intrahepatic bile duct. Epithelial desquamation, adenomatous hyperplasia, periductal fibrosis and inflammatory infiltration are seen in the bile duct wall. Bar = 1 mm. (Photograph by B. Sripa, reproduced with permission)*

Parasite products, mechanical or obstructive changes, and the immune response, all appear to play a role in chronic pathogenesis, as well as in the carcinogenesis associated with infection. Based on experimental evidence, some authors suggest that oval cell or bile duct epithelial cell proliferation during chronic infection increases their susceptibility to carcinogens, especially *N*-nitrosodimethylamine, in the diet (Thamavit et al. 1996; Migasena 1982; Lee et al. 1997). High levels of nitrosamines have been found in some traditional Thai foods and Thai brand cigarettes (Migasena 1982; Mitacek et al. 1999a, b). The work of others has suggested that chronic inflammatory responses to *O. viverrini* infection increase the generation of nitric oxide, which combines with amines (Haswell-Elkins et al. 1994b; Satarug et al. 1996a) to form nitrosamines endogenously within the inflamed tissue (Srivatanakul et al. 1991; Satarug and Haswell-Elkins 1998). Specific T-cell responses to particular *Opisthorchis* antigens are closely associated with increased levels of endogenous nitrate and nitrosamine production in humans. Infection and chronic fibrosis of the small bile ducts also appear to stimulate the levels of cytochrome P450 2A6, which can activate dimethylnitrosamine to its highly toxic form (Kirby et al. 1994; Satarug et al. 1996b). All of these reactions are reversed after elimination of the fluke with praziquantel treatment.

FASCIOLA HEPATICA AND *F. GIGANTICA*

Although *Fasciola* inhabits the bile duct and stimulates chronic inflammation and fibrosis, like *Opisthorchis* and *Clonorchis*, it has not been associated with human biliary carcinoma. The pathological lesions are more similar to those of *Paragonimus*. This includes granulomatous inflammation encompassing eggs and abscess formation around the adult worms appearing grossly as multiple yellow nodules. Track-like inflammatory lesions appear along the migration route of the worms (Chen

and Mott 1990). The cells initially involved are mainly polymorphonuclear leukocytes, eosinophils, histiocytes, and lymphocytes. Later lesions contain lymphocytes and plasmocytes with fibrosis and calcification. Similarly, ectopically located parasites, mostly found in subcutaneous tissue, may form abscesses.

Monocytes tested during the acute phase of infection in humans actively produce higher levels of pro-inflammatory cytokines (GM-CSF, interleukin 8, and interleukin 6) in vitro, compared to those taken during the chronic phase (Khalil et al. 1999). Immunoproliferative responses and nitric oxide production appears to be suppressed within a week of infection in experimental animals through production of interleukin 4, gamma interferon, and interleukin 10 (Osman and Abo-El-Nazar 1999; Cervi et al. 2001). Suppression of inflammatory responses may limit the degree of immunopathology during chronic infection but may also inhibit otherwise effective parasiticidal responses.

The pathogenesis of *Fasciola*, therefore, involves acute and chronic immune responses leading to granulomas, abscess, and hepatic fibrosis. Direct parasite products and movement play a well-defined role in pathology as the parasites eat their way through the liver to the bile ducts undergoing considerable growth. In addition, large amounts of proline produced and excreted by the flukes may be directly responsible for bile duct hyperplasia and dilatation and collagen deposition (Wolf-Spengler and Isseroff 1983).

Immune responses, nature, and source of antigen

Whether the immune responses which develop in humans in response to liver and lung fluke infections confer protective immunity against the currently held parasites or newly acquired flukes remains unclear. Little direct progress has been made towards vaccine development for humans, however, a great deal of work has been carried out to characterize certain enzymes shared by these flukes, particularly the cysteine proteases, cathepsin L-like proteases, and glutathione S-transferases. These enzymes are abundant in the tegument and/or excretory and secretory products and play important roles in parasite survival (Kofta et al. 2000). While glutathione S-transferases protect flukes from bioreactive compounds (Sobhon et al. 1998; Hong and Yun Kim 2002), the proteases appear to play several roles in immune evasion (Trap and Boireau 2000; Shin et al. 2001; Park et al. 2002).

Because of the economic and veterinary importance of fascioliasis, the immunology of *Fasciola* has been extensively studied in sheep, cattle, and rodents. Differing degrees of acquired resistance are seen among these animals. Cattle and most rodents develop resistance to reinfection, but sheep and goats do not and may die in the face of continued exposure and accumulation of worms. Human *Fasciola* infections are usually self-limiting and only few migrating larvae achieve establishment. However, this may not be related to immune responses.

Experimental vaccine trials against *Fasciola* in livestock have demonstrated that significant protection (up to 98 percent) can be conferred with irradiated larval vaccines (Spithill 1992). Vaccines targeting important individual antigens have been partially successful. For example, immunization with a recombinant glutathione *S*-transferase of *F. hepatica* has conferred over 50 percent protection against worm establishment in sheep. A detailed review of the elegant and promising work on understanding the immune response in animals and the progress towards an effective vaccine for animals is available (Spithill et al. 1997).

Epidemiological patterns reveal little evidence of, but also do not rule out, protective immunity in humans exposed to *Opisthorchis* and *Clonorchis*. There is no decline in prevalence or intensity of infection among individuals exposed to decades of infection, and reinfection may occur rapidly following treatment in areas of heavy infection (Upatham et al. 1984, 1988). The parasites clearly survive in the face of high levels of parasite-specific IgG, IgA, and IgE in both serum and bile (Wongratanacheewin et al. 1988), and of T-cell reactivity to parasite antigens as demonstrated by delayed-type hypersensitivity responses following skin testing (Rim 1986). Recent studies by Tesana et al. (2000) has suggested that immunization of experimental animals with *Opisthorchis viverrini* antigens prior to live infection and administration of carcinogen, dimethylnitrosamine, may accelerate carcinogenesis.

Even less is known about acquired immunity in human *Paragonimus* infection. The use of skin testing and antibody tests as diagnostic tools (see below) indicates that people mount strong cellular and humoral immune responses to parasite antigens. However, these responses do not appear to be protective, possibly due to the action of secreted cysteine proteases which appear to destroy IgG bound to the parasite surface, reduce eosinophil survival, IL-8 production and degranulation, and inhibit superoxide production by granulocytes (Shin et al. 2001).

Diagnosis

Similarities in egg morphology and cross-reactive antigens between intestinal flukes and liver flukes complicate both parasitological and immunological diagnosis. *Fasciola* eggs are indistinguishable from those of human *Fasciolopsis* and *Echinostoma* species, and *Opisthorchis* and *Clonorchis* eggs look similar to minute intestinal fluke eggs. Food-borne parasites often present in mixed infections and have overlapping endemic areas (Lee

et al. 1984; Radomyos et al. 1984; Kaewkes et al. 1991). Nevertheless, diagnosis of lung and liver fluke infections is reliably and frequently based on the observation of eggs in the stool (all of the parasites) or sputum (*Paragonimus* only). Stoll's dilution method, formalin-ether (or formalin-ethyl acetate) concentration method (FECT) and AMS III are the most sensitive methods for processing specimens for egg examination (Chen and Mott 1990; Sithithaworn et al. 1991; Toscano et al. 1995). Rapid staining with potassium permanganate can help differentiate *O. viverrini* eggs from those of common minute intestinal flukes (Sukontason et al. 2001). *Fasciola* and *Paragonimus* infection pose a problem of diagnosis in the migratory phase, or in ectopic infection, when no eggs are passed.

Peripheral blood eosinophilia and elevated serum IgE levels often accompany parasite infections, including those of lung and liver flukes. Specific serological diagnostic methods, particularly for fascioliasis and paragonimiasis, have improved. Many authors report very good results with enzyme linked immunosorbent assay (ELISA) detecting circulating parasite antigens (Shehab et al. 1999) or specific antibodies to adult worm, excretory, and secretory or metacercarial preparations (Osman et al. 1995; Silva et al. 1996; Maher et al. 1999). Various modifications can enhance assay performance, for example IgM for early infections (Osman et al. 1995) or IgG4 subclass (O'Neill et al. 1999; Hong et al. 1999). The use of single purified antigens (Maleewong et al. 1997; Mansour et al. 1998) or using cystatin or Protein A-treated plates for capture ELISA and the detection of cysteine protease activity (Ikeda 2001) can increase specificity. It has become very clear that serological and antigen detection ELISAs are far superior to egg detection in *Fasciola*, especially in the prepatent, acute phase of infection and are also very useful in assessing treatment efficacy (Hassan et al. 1995; Hammouda et al. 1997; Chaithirayanon et al. 2002).

Potentially promising diagnostic antigens for *Clonorchis* and *Opisthorchis* are being explored, some in isotype-specific assays. An IgG4-specific assay to detect 7–8 kDa antigen (Kim 1998) and an IgE-detection assay detecting antibodies to a 28 kDa glutathione *S*-transferase (Yong et al. 1999; Na et al. 2002) of *Clonorchis* have been shown to be highly specific. However, a mix of antigens appears to be needed to increase sensitivity (Hong and Yun Kim 2002). Excretory and secretory antigens have been the most studied and generate the dominant antibody responses to infection with *Opisthorchis*. Most antigens and antibodies that have been studied have been nonspecific and persistent after treatment (Akai et al. 1995); however, high sensitivity and specificity has been reported using affinity purified oval antigens from adult worms (Wongsaroj et al. 2001) and Bithynia snail antigens (Waikagul et al. 2002). Sirisinha et al. (1995) and Wongratanacheewin et al. (2001) have developed sensitive and specific diagnostic tests that detect *Opisthorchis* material in feces using monoclonal-antibody capture ELISAs and polymerase chain reaction (PCR)-based assay. The PCR assay is also able to detect cercaria and metacercaria in artificially inoculated snails and fish (Maleewong and Intapan 2003). These tests can be easily used in large-scale field studies and control programs.

Clinical, pathological, radiological, and potential exposure information clearly plays an important contributing role in addition to parasitological and immunological tests in diagnosis as well as in determining the extent of pathological change that has occurred. Evidence of *Paragonimus* infection may be shown on radiographs as patchy foci of fibrotic change, with a characteristic 'ring shadow' in the lung (Bunnag and Harinasuta 1984; Toscano et al. 1995) which may be mistaken for tuberculosis (Nagakura et al. 2002). Ultrasound diagnosis reveals abnormalities associated with liver flukes, e.g. gallbladder enlargement, stones and bile duct tumors, and sometimes the parasites themselves (van Beers et al. 1990; Lim 1990; Mairiang et al. 1992; Richter et al. 1999). Endoscopic retrograde cholangiopancreatography (ECRP) may reveal filamentous or elliptic filling defects of the biliary tract associated with clonorchiasis and fascioliasis (Chan et al. 2002). Parasite fragments and eggs can sometimes be seen in surgical tissue or biopsies, revealing the unsuspected cause of the disease.

CONTROL

The main tools for control of liver and lung flukes have been anthelmintic treatment, sanitation improvement, and health education. The rationale is that treatment is required to eliminate the long-lived parasites immediately, sanitation interrupts transmission from human feces to snails, and health education stops people from eating raw foods and becoming reinfected after treatment. Alternative risk reduction strategies to kill metacercaria, such as freezing or irradiating fish and soaking leafy greens in vinegar and potassium permanganate, may be effective if widely promoted (Song 1987; Iarotski and Be'er 1993). Improving sanitation through provision of latrines, stopping night-soil use on fields and in fish ponds, and moving pig farming from lakesides have been widely implemented and useful in preventing liver fluke infection (Jongsuksuntigul and Imsomboon 1997; Ooi et al. 1997). Hazard analysis critical control point procedures are increasingly being used in food safety programs and have been found to be helpful in decreasing contamination of farmed fish (Khamboonruang et al. 1997). Alternative strategies, such as freezing and irradiating fish (Song 1987; Iarotski and Be'er 1993; Park and Yong 2003), biological agents to destroy cercariae (Intapan et al. 1992), and treating reservoir hosts have been suggested but not widely implemented. Improving sanitation through latrines and stopping night-soil use on fields and fish ponds has been

widely implemented to prevent liver fluke infection. Furthermore, environmental changes, the use of pesticides and pollution of river systems by industrial effluents, have also reduced intermediate host populations (Komiya 1966; Cho 1994).

A single dose of praziquantel is generally used at 40 mg/kg body weight for *O. viverrini* and *C. sinensis* in Korea, while higher, multiple doses (3×25 mg/kg for 1–3 days) are used in China. Praziquantel appears to induce an influx of calcium causing depolymerization of the tubular networks and detachment of the parasite tegument (Apinhasmit and Sobhon 1996). *Paragonimus* is also effectively killed by praziquantel at a dose of 25 mg/kg, three times a day for 3 days. Side effects are transient and relatively minor. Published efficacy of praziquantel at these doses is over 90 percent (Chen et al. 1983; Sui et al. 1988). Treatment with *Fasciola* is more problematic as it is not particularly sensitive to praziquantel, unless with multiple treatments at very high doses (Queneau et al. 1997). Recent literature reports good outcomes and low side-effects with the use of triclabendazole at 10 or 20 mg/kg (Apt et al. 1995; Lecaillon et al. 1998). Repeated treatment is frequently required, but generally effective the second time. Recent reports of triclabendazole resistance developing in livestock animals has led to the search for new drugs against *Fasciola* infection with nitazoxanide looking promising in a randomized control trial in Peru (Favennec et al. 2003).

Despite the value of treatment, reinfection can and does occur in the case of *Opisthorchis* and *Clonorchis*. Complete success in control has been hampered by the difficulty in changing human behavior. Health education campaigns have focused primarily on providing information about the disease, rather than on prevention. Since raw food consumption is often culturally specific, it often holds great importance for the identity of minorities, e.g. the Laos-descendent Thais of the northeastern region, the Korean minority of northeastern China, the aboriginal people of Siberia. Community participation and culturally appropriate health education messages are a vital element of long-term success (Sornmani 1987; Jongsuksuntigul and Imsomboon 1997).

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Intestinal tapeworms

JØRN ANDREASSEN

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THE PARASITES

Introduction and historical perspective

Intestinal tapeworms are among the earliest known human parasites. They have been known since prehistoric times and are referred to in the Papyrus Ebers from about 1500BC (Hoeppli 1956). As early as the second century, Galen recognized *Taenia*, but it was not until 1592 that Dunas discovered *Diphyllobothrium*, the broad tapeworm. The first illustration of a tapeworm (*Taenia*) was the *pittoresce* picture made by Andry in 1700. In 1782, the connection between tapeworms and hydatids was found by Goeze, who also found differences between the two human species of *Taenia*. In 1790, Abildgaard demonstrated that plerocercoid larvae from sticklebacks became adult tapeworms in ducks to which they were fed, but it was not until 1845 that Dujardin showed adult *Taenia* in humans to arise from cysticerci in meat. Six years later, the life cycle of *Taenia saginata* was established by Küchenmeister, but it was not until 1917 that the complex life cycle of *D. latum* was found by Janicki (Grove 1990; Smyth 1990; Saklatvala 1993). As late as 1993, a new human species, *Taenia asiatica*, was described (Eom and Rim 1993), although it is now questioned whether this is a separate species or a subspecies of *T. saginata* (McManus and Bowles 1994) (see section on *Taenia saginata saginata* (beef tapeworm) below).

Until the early 1960s, tapeworms were said to have a more or less inert outer cuticle thought to prevent the worm being digested by the host's intestinal enzymes. However, at the same time, the worms had to absorb all nutrients through this surface because tapeworms have no gut. By electron microscopy, it was then shown that the surface was a living structure, a tegument (see section on Morphology and structure), which at the same time could absorb nutrients (see section on Feeding) and have mechanisms to inhibit host enzymes from digesting the worms (see section on Physiology).

The exact prevalence of human intestinal tapeworms is not known, but it has been estimated that as many as 100 million people worldwide may be infected with either *T. solium* or *T. saginata* (FAO 1991), and about 50–70 million with the dwarf tapeworm, *Hymenolepis nana* (Pawlowski 1984; Crompton 1999). None of the intestinal tapeworm infections are really life-threatening, but the presence of the adult pig tapeworm, *T. solium*, increases the risk of acquiring cysticercosis by the uptake of infective eggs, and an infection with the broad tapeworm, *D. latum*, may give rise to pernicious anemia (see section on Clinical and pathological aspects of infection).

The geographical distribution of intestinal tapeworm infections in humans corresponds to the distribution of their intermediate hosts. Since the cyclophyllid tapeworms, such as species of *Taenia* and *Hymenolepis*, have intermediate hosts living in close contact to humans, their distribution is worldwide, while the pseudophyllid species of *Diphyllobothrium* are more restricted, because they need certain freshwater copepods and fishes as intermediate hosts.

Classification

All tapeworms belong to the class Cestoda and the subclass Eucestoda, which is made up of a number of orders. Tapeworm species which may infect humans belong to six families from two orders (Khalil et al. 1994). (See Table 1.)

Morphology and structure

Human adult tapeworms are flat worms (Platyhelminthes) with a length ranging from a few centimeters (*Hymenolepis nana*) up to several meters (e.g. *Taenia* species). They are white or grayish to yellow. Calcareous corpuscles are present in tissues filling the spaces between internal organs. Yellow coloration comes from eggs in the posterior part of the worms.

Tapeworms found in the small intestine of humans are adults in different developmental stages. The adult worm always consists of a head (scolex) and a neck region (the growth region). If time and conditions allow, the neck generates a series of proglottides (segments), which together form a strobila. The scolex has muscular suckers which function as holdfast organs and are used in locomotion. There are either four round suckers (acetabula), as in the cyclophyllids, or two elongated grooves (bothria), as in the pseudophyllids. On the scolex, some species also have small hooks, which can be inserted into the intestinal epithelium and thereby help the worm to maintain its position in the gut of the host. The hooks are often in one or two circles (two in *T. solium* and one in *H. nana*) on a retractable rostellum (cone) between the suckers on the anterior end of the scolex. From the narrow neck region, the width of the worm increases and folds of the body wall are made at regular intervals along its length, forming the proglottides, giving the tapeworm the appearance of a segmented worm (Figure 1) (Mehlhorn et al. 1981).

The body wall (see Figure 2) is called a tegument. This consists of a distal syncytial cytoplasm, with cytoplasmic connections to a perinuclear cytoplasm lying beneath the basal plasma membrane, and circular and longitudinal muscles. The surface of the tegument is made up of microvillus-like structures called microtriches, microthrix in the singular (see Arme 1984). A microthrix consists of a shaft and an electron-dense bent spine and is, like a microvillus on most other absorptive cells, covered by a thin layer of glycoproteins and mucopolysaccharides called a glycocalyx.

Beneath the tegumental basal plasma membrane lie two layers of muscles: an outer circular layer and an inner longitudinal one, allowing the worms to move. Special muscles run to the suckers, where bands of criss-cross fibers help improve the function of the suckers. Radial muscles may go to inner organs such as the cirrus, uterus, and vagina.

The nervous system consists of a brain-like structure in the scolex built up of a central ganglion, and lateral and rostellar ganglia connected by a central nerve ring. From the ganglia, nerves run to the suckers and rostellum. Lateral and median longitudinal nerves run from the ring all the way back to the posterior end of the tapeworm. There are transverse commissures between the longitudinal nerves and connections to inner organs and sensory organs in the body wall.

The excretory system is a typical protonephridial system, as in other platyhelminths, consisting of two lateral canals in each side (dorsal and ventral) connected in the scolex. The two ventral, lateral canals are connected with a transverse canal in the posterior of each proglottid. The excretory canals are built up of terminal cells (flame cells) connected to canal cells; flow through the system is generated by a bundle of cilia in these terminal cells. The excretory fluid is carried to the scolex in the dorsal canals and back through the ventral canals that open to the exterior at the terminal proglottis. Around the cilia in the flame cells is a lattice-like system of projections from the flame cell and the

Table 1 *Classification of human tapeworms*

Order	Family	Species
Cyclophyllidea Scolex with four round suckers and mostly armed with hooks. Development through two larval stages	Taeniidae	*Taenia saginata saginata*, *T. saginata asiatica*, *T. solium*
	Hymenolepididae	*Hymenolepis nana*, *H. diminuta*
	Dipylididae	*Dipylidium caninum*
	Mesocestoididae	*Mesocestoides corti*
	Anoplocephalidae	*Bertiella* e.g. *studeri, Inermicapsifer madagascariensis*
Pseudophyllidea Scolex has two elongated slit-like suckers. Development through three larval stages	Diphyllobothridae	*Diphyllobothrium latum*. A number of zoonotic animal species of *Diphyllobothrium* may sporadically infect humans, e.g. *D. dendriticum*, *D. nihonkaiense*, *D. pacificum*, *D. cordatum*, *D. hians*, *D. houghtoni*, *D. orcini*, *D. scoticum*, *D. ursi*, *D. yonagoense*

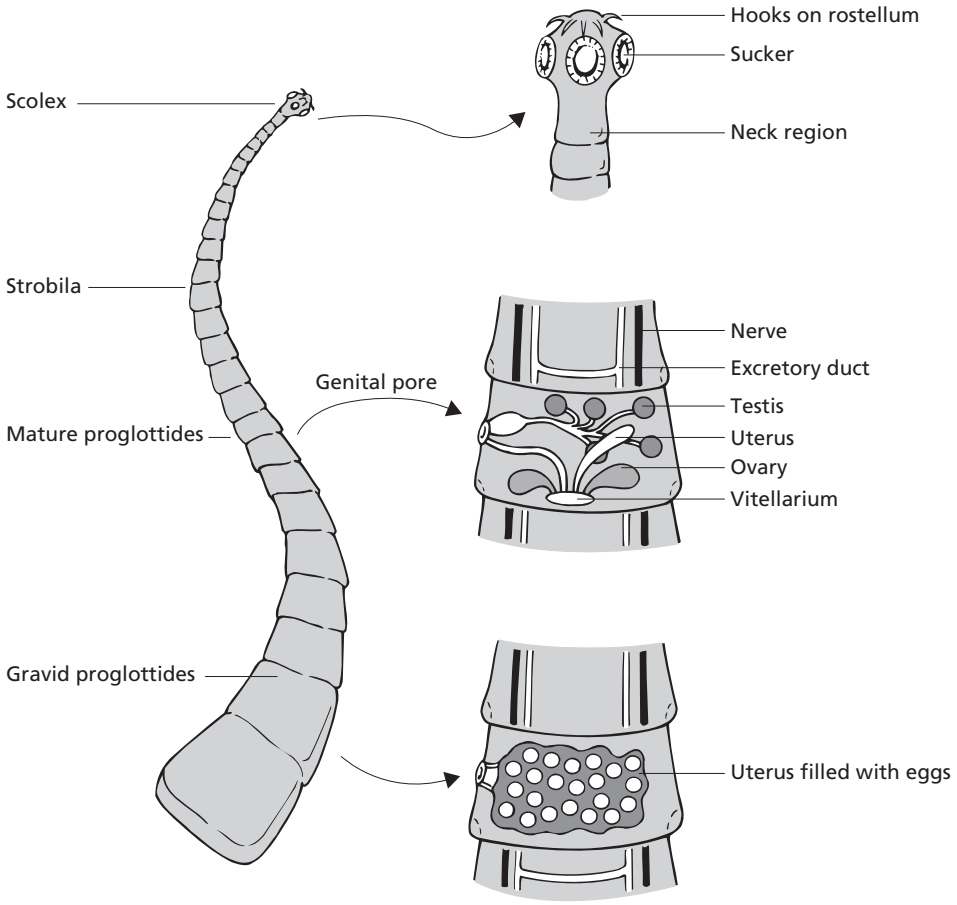

Figure 1 *The organization of an adult tapeworm. (Based on* Taenia*)*

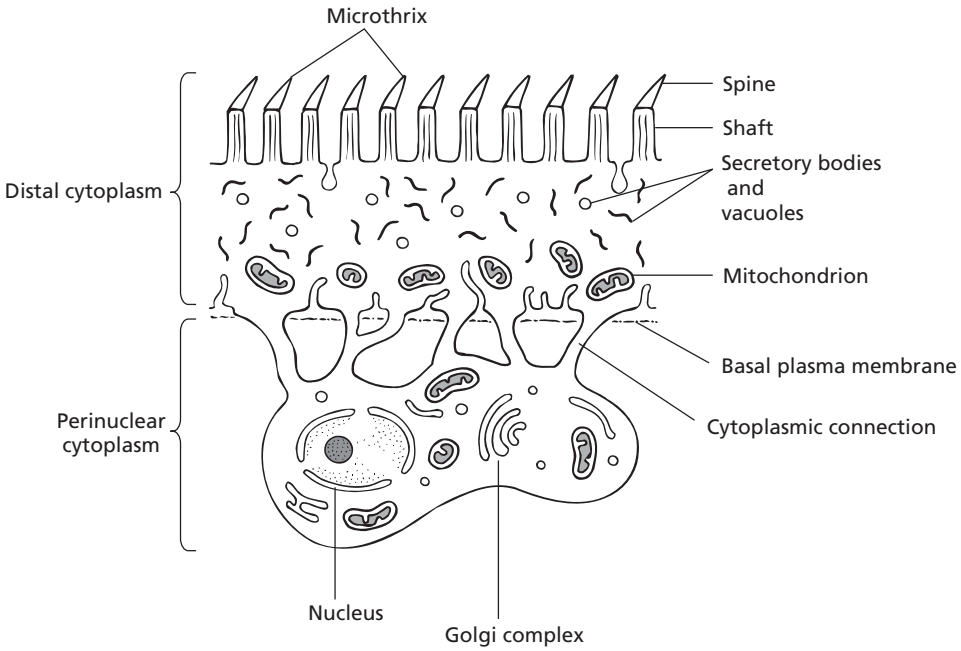

Figure 2 *The tegument of an adult tapeworm*

canal cells. Ultrafiltration takes place at the basal lamina through gaps in the lattice-like system.

Tapeworms are acoelomate, lacking not only a body cavity (coelom) but also a circulatory system.

The reproductive system is a typical hermaphroditic system, where the male and female organs differentiate progressively from the anterior region. The reproductive organs are repeated, with one set in each of the proglottides. Proglottides are considered mature when the reproductive organs found in them are sexually mature. Copulation then takes place and fertilized eggs are formed. When the eggs are mature, i.e. contain the first stage larva, the proglottid is said to be gravid. Male organs typically consist of a few large, or many small, testes from which spermatozoa are passed via vasa efferentia to a common vas deferens and then via a seminal vesicle to a long muscular and protrusible cirrus surrounded by a cirrus sac. The female organs normally consist of a single bilobed ovary, an ootype, where egg composition and wall formation start, a vagina leading to a common genital pore, and a compact or dispersed vitellarium from which vitelline cells are brought to the ootype to participate in the formation of a capsule (normally called an eggshell), as in the case of pseudophyllids, or in nourishment of the fertilized egg cell. In the cyclophyllids, the capsule is absent or poorly developed, while the embryophore (the inner envelope) forms a shell. In the cyclophyllid *Hymenolepis*, only one vitelline cell takes part in egg formation. Eggs are then passed into the uterus, which may or may not extend to the common genital pore. In some species, such as *Diphyllobothrium*, the eggs are operculate, as in most digeneans.

A fertilized egg cell develops to the first larval stage, called either an oncosphere (ball with hooks) or hexacanth (larva with six hooks), inside an eggshell (see Figure 3).

General biology

Usually, only one or a few of the large human tapeworms are present in a patient, but up to 28 worms (*T. saginata*) have been recorded, indicating an aggregated distribution of the parasite in the human population. In infections of the dwarf tapeworm (*H. nana*), many worms may be present in a human intestine. Individuals heavily infected with intestinal nematodes, often called wormy people, have been shown to be predisposed to this condition (Anderson 1986). Whether this situation also exists in humans infected with tapeworms is not known.

The large human tapeworms are capable of living for many years, in some cases up to 20 years (Wright 1984), but perhaps as long as the host. It has been shown that the rat tapeworm, *H. diminuta*, can live as long as the host, and, if repeatedly transplanted into the duodenum of young rats, can survive for at least 14 years (Read 1967).

When only a single tapeworm is present, it has been shown that self-insemination, i.e. transfer of sperm within the same segment or between segments in the same strobila, takes place. When multiple tapeworms are present, both self-insemination and cross-insemination take place (Nollen 1983).

MOVEMENT

The tapeworm utilizes its suckers and, if present, the hooks found on the scolex, to hold on to the villi of the intestine. Movement about the intestine is carried out by means of the suckers and the subtegumental musculature. Prepatent and diurnal movements are discussed below (see section entitled Location in host).

FEEDING

Since tapeworms have no mouth, all food has to be absorbed through the tegument. The microtriches help to increase the absorptive area of the tegument. A functional amplification factor for tapeworms has been shown to vary from slightly less than two to about 12, compared to about 26 for a mouse small intestine (Threadgold and Robinson 1984).

Tapeworms are normally in close contact to the intestinal mucosa (see section Location in host), which may be important for absorption of food. Small molecules,

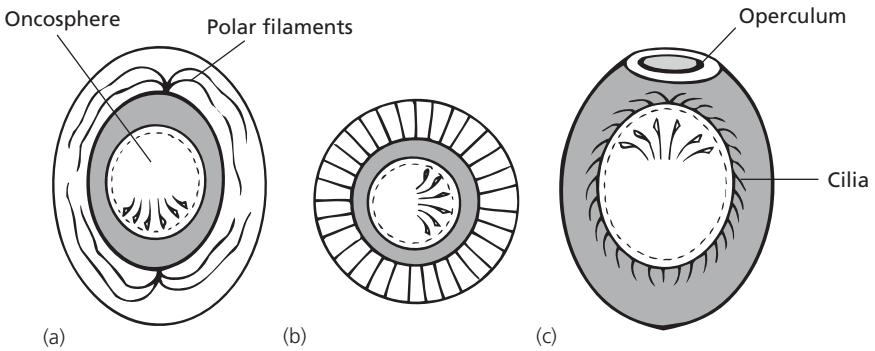

Figure 3 *Representative tapeworm eggs (not to scale):* **(a)** *hymenolepid;* **(b)** *taeniid;* **(c)** Diphyllobothrium

such as amino acids and glucose, have been shown to be taken up through the tegument via diffusion or active transport, but pinocytosis (endocytosis) of larger molecules such as proteins is still controversial (see Siles-Lucas and Hemphill 2002).

Although the energy metabolism of tapeworms mainly involves carbohydrates, amino acids play a major role in the high protein synthesis necessary for the large egg production. Since the worms cannot synthesize long chain fatty acids and lipids such as cholesterol, the absorption of these compounds is essential. It has been shown that bile salts are required for this transport across the tegument. The uptake of purine and pyrimidine is highly complex. For further details see Pappas and Read (1975).

PHYSIOLOGY

Excretion of waste products must take place through the tegument by exocytosis or through the excretory system.

Tapeworms have no special breathing organs and no vascular system: all exchange of oxygen and carbon dioxide has to take place over the tegument. The mitochondria in the tegument have few cristae, indicating that the metabolism of tapeworms is largely anaerobic.

There were few investigations of the physiology and biochemistry of the nervous system of cestodes until the development of immunocytochemical techniques. These techniques have demonstrated a number of neuropeptides which probably function as either neurotransmitters or neuromodulators and are responsible for coordinating behavioral responses (see reviews by Halton et al. 1990 and Siles-Lucas and Hemphill 2002).

BIOCHEMISTRY

Cestodes normally show a rather high level of carbohydrates (especially glycogen) and lipids and a relatively low level of proteins.

Mucopolysaccharides or complexes with proteins, such as mucoproteins or glycoproteins, are the main components of the surface glycocalyx. It has been suggested that this surface glycocalyx protects the worms from being digested by the host's proteolytic enzymes (Robertson and Cain 1984). On the other hand, digestion of nutrients to a state available for absorption can be made by enzymes bound to the glycocalyx, which has a turnover time of about 6 h. These enzymes may be of parasitic origin (intrinsic) or derived from their host (extrinsic).

Several neurotransmitters have been found in cestodes, e.g. acetylcholine, serotonin (5-hydroxytryptamine), noradrenaline (norepinephrine), dopamine, and octopamine. Serotonin is regarded as the main excitatory neurotransmitter of motor activity and acetylcholine as the main inhibitory neurotransmitter.

Life cycle

Not only a final host (a human being) but also at least one intermediate host is necessary for the completion of the life cycle of a cestode. For pseudophyllids, at least two intermediate hosts are necessary. Only one human tapeworm, *H. nana*, can complete its life cycle without an intermediate host. All larval stages are passed on to the next host via the oral route (for details see under the individual species). Humans become infected with an adult tapeworm by eating an infected intermediate host or infected organs from an intermediate host. When an infective larva is eaten by a human, excystation occurs if the larva is encysted, and, if the scolex is 'retracted' it will be 'inverted;' growth then commences from the neck region. In pseudophyllids, growth does not commence until everything except the scolex and neck has been shed from the plerocercoid larva. This active process takes about 60 h in golden hamsters.

Transmission and epidemiology

Human tapeworm infections caused by *T. saginata* and *T. solium*, commonly called taeniosis or taeniasis, are cosmopolitan. Prevalence is normally low but very much dependent on cultural factors and the level of hygiene and veterinary control. In some African countries, prevalences above 10 percent have been reported. In contrast to these low levels in the final host, the prevalence of cysticercus infections in the intermediate hosts may be very high in some regions.

The total production of tapeworm eggs per infected human is an important epidemiological factor, which, for the large human tapeworms, is probably negatively correlated with the total number of tapeworms present per host, so that the fewer tapeworms per person, the more eggs are produced.

Location in host

All adult human tapeworms live in the lumen of the small intestine, but, dependent on the species, in different parts of the small intestine. The two species of *Taenia* have their scoleces in the upper part of the jejunum, while those of *Diphyllobothrium* and *H. nana* normally lie in the upper two-thirds of the ileum. The scolex is found deeply embedded in the villi, while the strobila lies with its flat side close to the mucosa, sometimes in a spiral along the intestine.

The rare human tapeworm, *Hymenolepis diminuta*, which normally has rats as final hosts, has been shown to migrate in the small intestine of rats both during its prepatent period and diurnally. The newly excysted worm establishes itself about 30–40 percent down the small intestine. During the second week, the worm migrates anteriorly, stopping in the upper 10–20 percent

of the small intestine. If many worms are present, they can be found spread over a larger part of the small intestine. Circadian migratory behavior has also been found for *H. diminuta* in rats, where the worms are found more anteriorly in the small intestine in the morning and more posteriorly in the evening. This migration of the worms has been shown to be dependent on the host's feeding rhythm (see review by Arai 1980). Whether these migrations take place for the large human tapeworms is not known, but it is likely.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Generally, intestinal tapeworms are considered to be minimally pathogenic, causing no or very slight symptoms as compared to many other human parasitic diseases. However, they do utilize some of the food consumed by the host. Normally, sufficient food supplies are available and the host is not affected. In cases of malnutrition, tapeworm infections may have a deleterious effect on the infected person. The amount of energy taken from the host by tapeworms has been investigated for *Hymenolepis diminuta* in rats and shown to be 0.8 percent, which is comparable to carnivore predation on a prey population (Bailey 1975).

The effect of the broad tapeworm (*Diphyllobothrium latum*) on vitamin B_{12} and cobalamin absorption is discussed later (see section entitled Clinical and pathological aspects of infection).

Clinical manifestations

The large human tapeworms may disturb gastrointestinal motility. Although no specific symptoms are known for any of the human intestinal tapeworms, patients may experience abdominal pain, nausea, weakness, loss of weight, increased appetite, headache, constipation, dizziness, diarrhea, pruritis ani, excitation, and even vomiting and anorexia. Both nausea and abdominal pain may be explained as a result of extension or spasm of the intestine caused by the movement of the tapeworms. The mechanisms of the other symptoms mentioned are obscure.

Pathogenesis and pathology

Our knowledge of specific changes in the human intestinal environment and intestinal absorption caused by tapeworms is very limited (see review by Rees 1967). However, in rats infected with *H. diminuta*, the worms cause a decrease in pH (perhaps because they excrete fatty acids), an increase in pCO_2 and pO_2, and a reduction to about half the number of intestinal microorganisms, when compared to uninfected rats (Mettrick 1971).

Blood eosinophilia, commonly found in many other human helminth infections, has also been described in some cases, as have inflammatory reactions in the intestinal mucosa. However, only in experimental models with rats and mice have changes in the small intestine in the number of eosinophils, mast cells, and goblet cells been followed during a tapeworm infection. Peripheral eosinophilia, combined with pruritis, urticaria, or asthma, has been described in human taeniasis and is indicative of an allergic reaction stimulated by the tapeworms. Human self-infections with the tapeworm *H. diminuta* also showed pronounced eosinophilia, along with an increase in plasma viscosity, but circulating IgE was not detected (Turton et al. 1975).

Complications may occur when proglottides move into new locations, such as the appendix or the uterine cavity or, after vomiting, into the cavities connecting with the oral cavity.

Immune responses

Although antibodies in sera from humans infected with adult tapeworms have been demonstrated in many cases, almost nothing is known about functional immunity to adult tapeworms in humans; our knowledge is mainly confined to animal models. Turton et al. (1975) made self-infections with *H. diminuta* and found increases in specific IgG and IgM, but not IgE. However, IgE is produced against intestinal tapeworm infections in dogs (Williams and Perez-Esandi 1971), mice (Moss 1971), and rats (Harris and Turton 1973), as in human intestinal nematode infections. It has also been shown, using the macrophage migration inhibition test, that humans infected with adult *Taenia saginata* develop a delayed-type hypersensitivity reaction (Boro'n-Kaczmarska et al. 1978).

In few cases, spontaneous cure has been observed in *T. saginata* infections, but it is not known whether this is caused by a direct immunological reaction or through a nonspecific cross-reaction caused by another parasite, e.g. as a result of increased plasma leakage to the gut lumen caused by hookworms.

The reason why only one or a few of the large human tapeworms are found at a time is not known, but the possibility exists that it is a result of concomitant immunity (previously called premunition), i.e. the host cannot get rid of existing worms but at the same time is in some way immune to new infections. The resistance to new infections is not a result of crowding effects from the present worms, as has been shown in rats infected with *H. diminuta*. The greater the biomass of primary worms present in the intestine, the greater the biomass of the superimposed worms. This indicates that the adverse effect on the superimposed worms comes from the host, i.e. it is immunologically mediated and not just an effect caused by the present primary worms (Andreassen 1991).

Human taeniid tapeworms are found not only together with other tapeworm species, but also with other intestinal worms, especially nematodes (large roundworms and hookworms) and protozoans. The interspecific interactions between these species are not known, but it is obvious that the presence of other parasites in the intestine will interfere with the presence of tapeworms and with the host's reactions against them. It is known from experimental infections in mice and rats with the tapeworm *H. diminuta* that the immunologically mediated expulsion of existing intestinal nematodes will, at the same time, expel a new infection of the tapeworm and destrobilate an existing tapeworm population (Behnke et al. 1977; Christie et al. 1979). These experiments indicate that adult tapeworms are more resistant to nonspecific host reactions than newly excysted worms. In vitro experiments have indeed shown that newly excysted cysticercoids of *H. diminuta* are completely lysed in fresh normal rat serum, while the scolex and neck region of older worms are more resistant (Christensen et al. 1986).

Protozoan infections, such as trypanosomes in the blood and flagellates such as *Giardia* in the gut, are often immunosuppressive and may increase the survival time of tapeworms in humans. Other immunosuppressive infections, special situations such as pregnancy and lactation, and immunosuppressive treatments will probably be favorable to a human tapeworm infection.

The antigens stimulating immune reactions in the final host probably come from the surface of the tapeworm and from gland secretions. It has been suggested (Elowni 1982) that the scolex and the germinative (neck) region of intestinal tapeworms are the main source of protective antigens. This is very likely, because these parts of the tapeworm are in the closest contact with the host's mucosa. Furthermore, the scolex has a rostellar gland, and antibodies have been shown to be produced in the host against secretions from this gland (Hoole et al. 1994).

Diagnosis

An intestinal tapeworm infection as such can be diagnosed by finding proglottides or cestode eggs in the patient's feces. If only eggs are present, it is not possible to diagnose *T. saginata* from *T. solium*. To diagnose the correct infective species, the scolex or those stages of proglottides showing species characteristics (see sections dealing with individual parasites) are needed. Several techniques for detecting eggs in feces are available, e.g. thick and thin smears and concentration techniques using sedimentation or flotation. Anal swabs may also be effective. However, free eggs in feces may be difficult to find and often multiple samples are needed. To help species identification, patients should therefore be asked to look for and collect proglottides.

Serological diagnosis of human intestinal tapeworms has been carried out using different antigen preparations in precipitation, complement fixation, skin hypersensitivity, agglutination, and enzyme-linked immunosorbent assay (ELISA) tests, but with variable results. A single serological test can determine the presence of antibodies, but cannot differentiate between a present and a past infection.

Methods have been developed to detect coproantigens of *Taenia* species in human fecal samples by means of a coproantigen ELISA (Allan et al. 1992). Diagnostic identification of a tapeworm proglottid or a fraction of it has been carried out with *T. saginata* using the polymerase chain reaction to amplify extracted genomic DNA and electrophoresis to identify a 0.55-kb DNA fragment (Gottstein et al. 1991). This is a highly sensitive, specific, and easy technique for identification of *T. saginata* proglottides and eggs; a single egg should be enough to make a species identification (Gottstein and Mowatt 1991). Recently, mitochondrial DNA analyses of human taeniids have shown unique profiles of *T. saginata*, the subspecies *T. saginata asiatica*, and two types of *T. solium* (Yamasaki et al. 2002).

Control

From a human health point of view, the tapeworms *T. solium* and *D. latum* are particularly important: *T. solium* because of the risk of cysticercosis (see

Larval cestodes) and *D. latum* because of the risk of pernicious anemia due to vitamin B_{12} deficiency.

CHEMOTHERAPY

Two compounds, the older niclosamide and the newer praziquantel, act against adult intestinal worms (WHO 1995), but other drugs have been or still are in use, including extracts of certain seeds from locally grown plants. Niclosamide is a very safe drug because minimal amounts are absorbed in the gastrointestinal tract; in contrast, praziquantel is extensively absorbed by the host. Praziquantel has been shown also to have some effects against cysticerci of *T. solium*. Normally, a single dose is highly effective (at least 80 percent). Niclosamide functions by blocking glucose absorption by the adult tapeworm. Adult worms treated with praziquantel rapidly contract and disintegrate in the intestine.

PROPHYLAXIS

For human consumers, prophylaxis depends on the tapeworm species concerned (see descriptions of individual parasites and the section entitled Sporadic zoonotic animal tapeworms that may infect humans). Generally speaking, humans can avoid tapeworm contamination by avoiding food that has not been properly prepared. Meat or fish should be heated to a minimum of 57°C at the center, deep frozen at −10°C for 10 days, or pickled in a

25 percent salt solution for 5 days before being eaten. To reduce the risk of infected meat being eaten by humans, it is important to have an adequate veterinary meat inspection before meat is released for consumption, although a veterinary inspection can never be 100 percent effective. The dwarf tapeworm, *H. nana*, needs special control measures (see section entitled *Hymenolepis diminuta*).

TRANSMISSION

To avoid infection of intermediate hosts, it is important that human fecal material is kept away from these animals. Safe disposal of sewage is important in minimizing the transmission; water from sewage plants should not be used to water grasslands grazed by cattle or pigs. Viable tapeworm eggs can also reach grasslands and cattle grazing along rivers and lakes via effluent from sewage plants. Viable eggs in proglottides taken up by birds can pass through them and be dispersed on to fields, where the intermediate host can be infected or its feed contaminated. Under natural conditions, desiccation is normally the most important factor reducing the survival of the eggs: hot, dry summers will kill more eggs than cold, wet winters. Health education, not only of consumers, but also of people involved in every step in the life cycle, is important to prevent transmission of human tapeworms.

TAENIA SAGINATA SAGINATA

T. saginata (beef tapeworm) has a cosmopolitan distribution wherever inadequately cooked beef is eaten. The name 'beef tapeworm' comes from the fact that beef is the main source of infection.

Stoll (1947) estimated that about 39 million people were infected. Although present data are not sufficient to make a new estimate, it seems justified to multiply Stoll's number by the increase in the total global population, giving about 60 million people currently infected with *T. saginata*.

The economic loss due to taeniasis or cysticercosis in cattle is very high in some countries. The cost due to medical treatment of people is only a small proportion of the loss due to cysticercosis, which varies from region to region, not only in total loss, but also in loss per animal.

Morphology and structure

The mature tapeworm, which can reach a length of 4–6 m or more, has 1 000–2 000 proglottides. In the proglottides the genital pore is seen laterally but irregularly alternating from one side to the other. The scolex has four suckers but no hooks. Because of the lack of hooks, the species has also been named *Taeniarhynchus saginatus*.

General biology

The maximum size of the worm is reached when only one tapeworm is present. When two or more are present the individual length decreases to about 2 m when eight worms are present (Tesfa-Yohannes 1990), and as little as 50–80 cm when 16 worms are present (Altmann and Bubis 1959). An increasing number of worms present in the small intestine of humans coincides with a decrease in the number of proglottides; an increase from one to eight worms shows a decrease in the number of proglottides from about 600 to 350. This is a typical example of the effect of crowding, but, in parasites (unlike free-living animals), not only do the worms themselves interfere with each other and compete for space and food, but also the immunological reactions from the host have an influence on the growth of the worms.

LIFE CYCLE

In the life cycle (see Figure 4a) humans are the only final host and infections are acquired by eating raw or undercooked meat, e.g. by eating a 'rare' steak containing the cysticercus stage in a host capsule. When the cysticercus or bladder worm (previously named as a separate species, *Cysticercus bovis*) reaches the stomach, proteolytic enzymes start dissolving the capsule. In the small intestine, the cysticercus is stimulated to evaginate. The scolex attaches to the intestinal mucosa by means of the four suckers and starts growing into a mature tapeworm. Maturity is reached after about 10–14 weeks. The gravid proglottides contain up to about 80 000 mature eggs each and an average of six to nine proglottides may be expelled in 24 h, giving a total daily egg output of about 600 000 eggs. Meat from domestic cattle is the main source, but water buffaloes are also known as intermediate hosts. Unhooked cysticerci have also been reported in meat and livers from wild ruminants in Africa, but the significance of these observations needs further investigation. Llamas in South America and reindeer in the northern hemisphere have also been reported as intermediate hosts. A dozen cases of hookless cysticerci found in humans and described as *T. saginata* cysticercosis have been reported, but these require confirmation. The intermediate hosts become infected by eating mature eggs or gravid proglottides filled with eggs. The proglottides, which are quite mobile after being deposited, may migrate out of the fecal pat and thereby be more easily eaten by cattle, although this is not proven to have any significance for transmission. During the migrations, most eggs leave the proglottid. The eggs hatch in the small intestine and the free oncospheres then penetrate the gut wall and reach the muscles via the blood. In the intramuscular connective tissue they develop to a cysticercus, which, in the mature stage after about 10–15 weeks, is a grayish white bladder about 5–9 mm with an opaque invaginated scolex and neck.

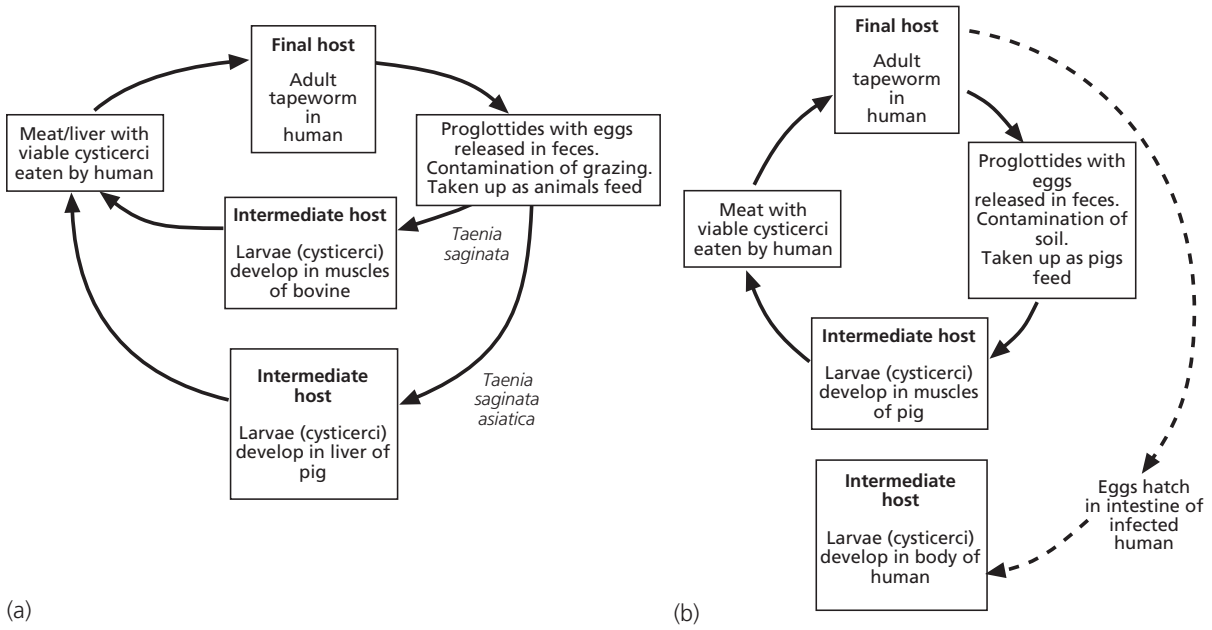

Figure 4 *Life cycles of the human tapeworms:* **(a)** T. saginata *and* T. saginata asiatica*;* **(b)** T. solium

TRANSMISSION AND EPIDEMIOLOGY

The beef tapeworm is an important infection of both humans and cattle throughout the beef-eating world, but especially in the tropics, where cattle-grazing areas are more often contaminated with human feces. It is important from the point of view both of human health and of economics, especially in potentially beef-exporting African countries with a high incidence of cysticercosis in cattle (see section entitled Control above). Under ideal conditions, the eggs may survive in the field for up to 6 months.

Gravid proglottides from infected humans living in areas with water closets accumulate in sewage plants. When sewage sludge is used as fertilizer on grazing pastures, cattle may become infected. Pastures may also be contaminated with eggs from sewage plants when treated effluent (still containing viable cestode eggs) is released into rivers running through grazing areas. Cattle may then become infected by drinking or eating along the rivers or by farmers watering pastures with water from the contaminated rivers. A more 'sophisticated' way to contaminate pastures is through droppings from gulls which have eaten proglottides at open sewage plants or at places where human feces are deposited directly in water. Cattle may also be infected through human activities such as camping, where human feces are deposited on grassland. A farmer may also risk a cysticercus 'storm' in his cattle if he empties septic tanks, mixes the content with slurry, and sprays it over the pastures as a fertilizer.

Another parameter of special epidemiological interest is the longevity of infective cysticerci. This is quite variable, even in the same host. Cysts in some organs are killed, yet remain alive in others. Cysticerci have been shown to be viable for a maximum of 3 years in their intermediate host.

LOCATION IN HOST

The mature tapeworm has been shown to attach mainly to the upper jejunum, with the posterior part of the body down in the ileum. However, the tapeworm is mobile in the intestinal lumen (Prévot et al. 1952).

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations

Infected humans are not always aware of having a beef tapeworm, but the observation of moving proglottides in the anal region, in the underclothing, or on the stool normally brings the patient to the doctor. Infected persons often complain of having abdominal discomfort (epigastric pain), nausea, weakness, loss of weight, increased or decreased appetite, and headache. Constipation, dizziness, diarrhea, pruritis ani, and excitation are also noted, while vomiting is an infrequent symptom.

Pathogenesis and pathology

Occasionally, obstructive appendicitis or cholangitis occurs in *T. saginata* infections due to aberrant migration of segments, which have also been found in the uterine cavity. After vomiting, proglottides may, in very rare cases, obstruct the respiratory tract, enter the middle ear through the eustachian tube or localize in the adenoid tissue of the nasopharynx. Symptoms such as abdominal pain and nausea suggest that the tapeworm

has an irritative action, and biopsies of the intestinal mucosa taken from infected patients often show slight inflammatory reactions. Moderate eosinophilia has been reported in varying percentages of infected persons, but an increase in IgE has not yet been demonstrated.

Immunity

Acquired immunity against *T. saginata* infections in humans after elimination of an infection has not been demonstrated and is probably of little importance; in contrast, concomitant immunity may be important.

Diagnosis

If only gravid proglottides are present for diagnosis, normally as a result of apolytic discharge in feces, the presence of a vaginal sphincter muscle, which can be seen on cleared specimens, can identify the specimens as *T. saginata* and distinguish them from *T. solium* (Figure 5a).

The presence of two ovarian lobes is also species specific for *T. saginata*. If the scolex is recovered after therapy, the absence of hooks is a characteristic of *T. saginata*, while the pork tapeworm (*T. solium*) has hooks on the rostellum (see Verster 1967). The two species overlap in the number of uterine branches in gravid proglottides, which therefore can only be used when the number on each side of the stem is greater than 16 (*T. saginata*) or less than ten (*T. solium*).

For detecting eggs (Figure 3), anal swabs are superior to methods using feces (thick smears and concentration or flotation methods). However, eggs cannot be used for species determination, and more feces samples are often necessary to find eggs.

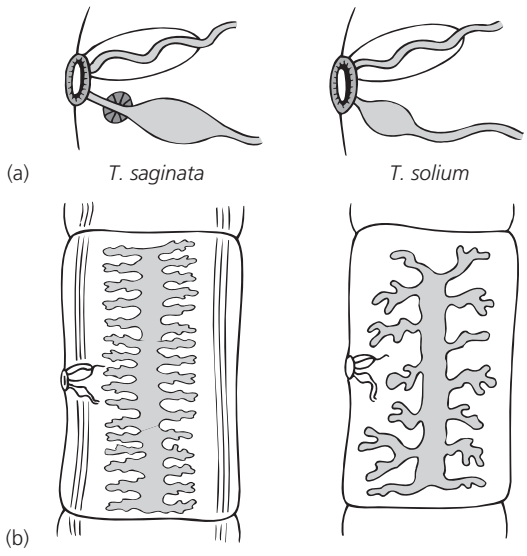

Figure 5 *Diagnostic feature of* T. saginata *and* T. solium*:* **(a)** *genital atria;* **(b)** *gravid proglottides*

Control

Details of prophylaxis and chemotherapy are given in Table 2 (see also section entitled Chemotherapy). Although it seems simple to break the life cycle of *T. saginata* (humans being the only final host), both human and animal infections are in fact very difficult to control. To control cattle infections, contact with human feces should be avoided. Cattle should be kept away from pastures where there is risk of contamination. Humans should not eat undercooked or raw meat unless it has been properly frozen, meat production should be controlled by veterinarians and infected people should be treated in order to break the parasite life cycle.

Eggs of *T. saginata* can survive for a long period of time, depending on temperature and moisture. Higher temperatures and lower humidity result in shorter egg survival times. To prevent establishment of viable cysticerci in cattle, immunization may be a possibility. Although an effective vaccine has been produced, no commercial vaccine is available as yet.

TAENIA SAGINATA ASIATICA

This human tapeworm was described by Eom and Rim (1993) as a new species, *T. asiatica* (Asian tapeworm). Some authors still regard it as a separate species (Galán-Puchades and Mas-Coma, 1996; Queiroz et al. 1998; Hoberg et al. 2000; Eom et al. 2002), while others consider it as a subspecies of *T. saginata* (McManus and Bowles 1994; Fan and Chung 1998; Ito et al. 2002a). It has been characterized genetically by Bowles and McManus (1994). According to Fan (1988), it has been present in Taiwan since at least 1915. It is now found in Korea, Taiwan, Indonesia, China, Malaysia, Philippines, and Thailand, but probably also occurs in other Asian countries (Ito et al. 2002a).

Morphology and structure

The morphology of the adult tapeworm is very similar to that of *T. saginata*, but the scolex generally has a larger diameter and the cysticercus – named *C. viscerotropica* – is different in morphology, development, and species of intermediate host; for further details see Fan (1988), Eom and Rim (1993), Fan and Chung (1998), and Ito et al. (2002a).

LIFE CYCLE

The life cycle differs from that of *T. saginata* in that the intermediate hosts are pigs rather than cattle, and the preferred location in the pig is the liver, rarely the omentum, serosa or muscles (Fan and Chung 1998). Pigs, calves, goats, and monkeys have been successfully infected experimentally with eggs of *T. saginata asiatica*. Fully mature cysticerci are developed within 4 weeks, whereas it takes 10–12 weeks for *T. saginata* in cattle

Table 2 *Control measures against taeniasis (taeniosis)*

Measure	Details
Prophylaxis	
Reduction of human consumption of raw infected meat	Cook properly and/or freeze meat from cattle and pigs and other intermediate hosts
	Veterinary meat inspection
Reduction of intermediate hosts becoming infected	Keep human feces away from cattle and pigs and other intermediate hosts
Therapy	
Chemotherapy (drugs)	
Against intestinal tapeworms in humans	Praziquantel, niclosamide
Against larval stages in the intermediate host	(see Larval cestodes)
Vaccination	
Against adult intestinal tapeworms	Not yet possible
Against larval stages in the tapeworms' intermediate hosts	(see Larval cestodes)

(Figure 4a). Normal and immunosuppressed mice have been shown experimentally to function as intermediate hosts (Wang et al. 1999).

TRANSMISSION AND EPIDEMIOLOGY

To understand the transmission and epidemiology of this new strain of *T. saginata* we require more knowledge of the prevalence and egg production of the adult tapeworms in the human population. Furthermore, it is important to know not only which animals are confirmed intermediate hosts, along with the corresponding prevalence and infection intensity, but also the potential intermediate hosts in the area concerned (see Galan-Puchades and Fuentes (2000) and Eom and Rim (2001)).

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

See under *T. saginata*.

DIAGNOSIS

When a human tapeworm infection is diagnosed as *T. saginata* (see section on *Taenia saginata saginata*), only identification of the source of infection, i.e. whether the patient has eaten undercooked beef or raw pig liver, may indicate if the infection is caused by the Asian subspecies *T. saginata asiatica*.

TAENIA SOLIUM

Taenia solium is known as pork tapeworm because pork is the main source of human infection. It has a worldwide distribution except among Muslims and Jews, who do not eat pork, or where strict veterinary inspection and treatment of human cases have caused its eradication. *T. solium* is especially common in Latin America.

Morphology and structure

The mature tapeworm normally reaches a length of about 2–4 m (sometimes more), and contains about 800–1 000 proglottides. The scolex has four suckers and a small rostellum with a double crown of 25–30 small hooks (see Larval cestodes, Figure 1).

LIFE CYCLE

Humans can act not only as the final host, as for *T. saginata*, but also as an intermediate host (Figure 4b), which is the more serious situation (see Larval cestodes). When humans act as final hosts the source of infection is meat from domestic pigs, wild boar, or dogs containing the cysticercus stage, previously named as a separate species (*Cysticercus cellulosae*). When this is eaten, development to the adult stage is as described for *T. saginata* (see section on *Taenia saginata saginata*). Pigs, wild boar, and occasionally other mammals become infected by eating mature eggs or gravid proglottides filled with up to 40 000 eggs. The proglottides, which, unlike those of *T. saginata*, are only slightly mobile after being deposited in human feces, are in certain places easily eaten by pigs, which will quite happily eat human feces. In pigs, the development to an infectious cysticercus is essentially as for *T. saginata* in cattle, except that the invaginated scolex has hooks on the rostellum and the developmental time is shorter, 7–9 weeks. Chinchillas have been shown to function as definitive hosts for *T. solium* (Maravilla et al. 1998).

TRANSMISSION AND EPIDEMIOLOGY

In some areas, the prevalence in pigs may reach 25 percent, and in heavily infected pigs (so-called measly pork) the infection can be detected by the presence of cysts in the tongue of the living pig. Such levels of infection are not uncommon in countries where pigs live in

close proximity to humans who defecate in places where the pigs can eat the feces. Dogs are intermediate hosts in Irian Jaya (Ito et al. 2002b), where prevalences of human intestinal *T. solium* ranges from 8 to 51 percent (Singh et al. 2002).

LOCATION IN HOST

The mature tapeworm has been shown to attach mainly to the upper jejunum, as in *T. saginata*.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations

Unlike those of *T. saginata*, the gravid proglottides of *T. solium* are released in groups and are not as active as those of *T. saginata*. Therefore, infected persons do not recognize the infection as easily. Otherwise the clinical manifestations are as described for *T. saginata* (see section on *Taenia saginata saginata*).

Pathogenesis and pathology

See Pathogenesis and pathology section and *T. saginata*.

Immunity

See Immune responses section and *T. saginata*.

Diagnosis

Adult *T. solium* worms can be identified by the presence of hooks on the scolex, unlike *T. saginata*. For species characteristics in the proglottides, see *T. saginata*. An immunoblot assay that is 95 percent sensitive and 100 percent specific has been developed for serological diagnosis of *T. solium* from *T. saginata* (Wilkins et al. 1999). Two genotypes of *T. solium* have been found, one from Asia and one from Latin America and Africa (Nakao et al. 2002)

CONTROL

See Table 2 and Pawlowski (1990) for more details and references.

Prophylaxis

To prevent infection, veterinary inspection and thorough cooking of pork are essential. In most parts of the world these methods are easier and cheaper than the measures necessary for prohibiting porcine access to human feces or food contaminated with eggs of *T. solium*.

Chemotherapy

Praziquantel, at a minimum dose of 5 mg/kg body weight, is the drug of choice for *T. solium* because it not only kills the adult tapeworm in a single dose, but, when taken in high doses over 3–7 days, also kills the cysticerci. Other drugs may also help cure human cysticercosis (see Larval cestodes). As for *T. saginata*, treatment with a single dose of four tablets (each of 500 mg) of niclosamide chewed thoroughly after a small meal is also effective against the adult worm in the intestine.

When treating patients, it is important that nausea, and especially vomiting, is avoided; should proglottides with eggs or eggs alone enter the stomach and later the small intestine, the patient may acquire cysticercosis. A purgative should therefore be given 1–2 h after the anthelmintic treatment.

Transmission

Theoretically, transmission to humans may also be inhibited by vaccination of pigs against oncospheres from *T. solium* eggs. However, a commercial vaccine is not yet available (see Larval cestodes).

HYMENOLEPIS NANA

Hymenolepis nana (dwarf tapeworm) infects not only humans but also rodents such as mice and rats. Pawlowski (1984) and Crompton (1999) estimated that about 50–75 million people worldwide are infected. In rodents, the tapeworm is regarded by some as a special strain (*H. nana* var. *fraterna*), but cross-infections in both directions are possible. This means that *H. nana* is a zoonosis, where the infection can be maintained in animals and accidentally infect humans. However, Macnish et al. (2002) failed to infect laboratory rodents with human isolates of *H. nana* from Australia. Some taxonomists (e.g. Czaplinski and Vaucher (1994)) consider *H. nana* to belong to a separate genus *Rodentolepis*.

Morphology and structure

The adult tapeworm is small, as the name indicates, reaching only 4–5 cm in length. The scolex has four cup-shaped suckers and a retractable, prominent, and movable rostellum with a ring of small hooks.

LIFE CYCLE

This tapeworm has an indirect life cycle with an insect as the intermediate host. It is exceptional among tapeworms in also being able to use the final host for development directly from the egg containing the oncosphere larva (Figure 6).

Intermediate hosts include grain- and flour-eating beetles such as species of *Tribolium* and *Tenebrio*, fleas such as *Pulex irritans, Xenopsylla cheopis*, and *Ctenocephalides canis*, and moths. The indirect life cycle begins when these insects or their larvae eat an infective *H. nana* egg. They crush the eggshell, and enzymes in the gut then stimulate the oncosphere to free itself from the enclosing membranes. When free in the gut lumen,

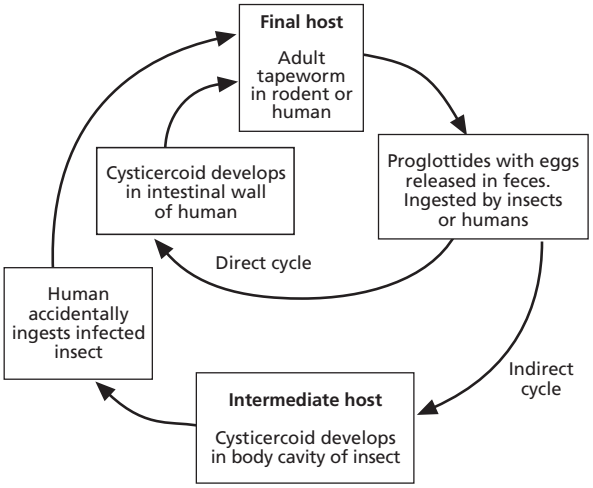

Figure 6 *Life cycle of the human dwarf tapeworm,* Hymenolepis nana

the oncosphere penetrates the gut wall by means of its six hooks and glandular secretions. In the body cavity of the insect, the oncospheres transform and grow into the second larval stage, the cysticercoid, which is infective to the final host. After an infected insect is eaten by humans (chiefly children), bile salts in the small intestine stimulate the emergence and liberation of the young tapeworm from the cysticercoid. In humans the tapeworm grows to patency within 3–4 weeks. The direct life cycle of *H. nana* takes place when infective eggs are ingested. In the lumen of the small intestine a free oncosphere penetrates an intestinal villus and grows to the cysticercoid stage in about 96 h. Thereafter, the villus ruptures, the cysticercoid becomes free in the lumen of the small intestine and grows to an adult tapeworm. It is said that in heavy infections eggs may hatch in the intestine before passing out with the feces resulting in autoinfection, but the importance of this is questionable.

TRANSMISSION AND EPIDEMIOLOGY

Transmission takes place directly from hand to mouth, by contaminated food and drinking water, or in rare cases, by infected insects. Infection is most prevalent where hygiene is insufficient. e.g. in young children and inhabitants of institutions. Although humans are the main source of infection, infected rodents are always a potential source. This tapeworm is present throughout the world, with overall prevalences ranging from nearly zero to about 4 percent in some countries and as high as 16 percent in children.

LOCATION IN HOST

In humans the adult tapeworms are said to be found in the upper two-thirds of the ileum, whereas in mice they are found in the posterior part of the ileum.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations

The presence of low numbers of these tapeworms causes no symptoms, but, if as many as 1 000–2 000 worms are present in children, there may be abdominal pain, lack of appetite, eventual diarrhea or dizziness.

Pathogenesis, pathology, and immunity

In heavy infections, enteritis may be produced. Nothing seems to be known about immune reactions in human infections, although there is considerable knowledge of experimental infections in mice and rats (see review by Ito and Smyth 1987).

Diagnosis

Free eggs or proglottides filled with eggs may be found in feces. The characteristic two groups of polar filaments in the egg is species-specific.

CONTROL

Chemotherapy

The drug of choice is praziquantel, of which a single dose is highly effective. A second choice is niclosamide. When a person is found to be infected, treatment should include the entire household.

Prophylaxis

Sanitary improvements, uncontaminated food supplies, and rodent control in houses and nearby surroundings are important in prevention of this infection, but the hygienic status of children and inhabitants of institutions is the most important factor in control. Because of the direct life cycle and the zoonotic status of this infection, this tapeworm is difficult to control and impossible to eradicate.

HYMENOLEPIS DIMINUTA

Morphology and structure

The scolex of this species has no hooks on its rostellum, unlike *H. nana*. Furthermore, *H. diminuta* is larger than *H. nana*, reaching a length of at least 1 m in single infections in rats, and perhaps more in human infections.

LIFE CYCLE

The life cycle of this tapeworm is identical to the indirect life cycle of *H. nana* (see section Hymenolepis nana) and always needs an intermediate host. The prepatent period in man is about 3 weeks. It has been shown in rats that, when only a few tapeworms are

present, they may live as long as the host (see section entitled General biology) In a self-induced infection with 30 cysticercoids and a superimposed infection with 100 cysticercoids on day 22, Turton et al. (1975) did not find any eggs in feces and concluded that the worms were expelled before patency.

TRANSMISSION AND EPIDEMIOLOGY

In order to be infected with an adult *H. diminuta*, humans have to ingest larvae (cysticercoids) from the body cavity of an insect. Since the tapeworm is very rare in humans, the source of infection must come from infected rats expelling gravid proglottides in their feces, from where the insects eat infective eggs. This infection is therefore a true zoonosis: infected rats must be present in order to infect insects, which in turn are consumed by humans. Poor sanitary conditions are a requirement for occurrence of this parasite.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Since infected rats and insects, functioning as final and intermediate hosts, respectively, may live worldwide, this is a cosmopolitan zoonosis. However, few (200–300) cases have been reported (Levi et al. 1987; Millon et al. 1994). It is found mainly in children under 3 years of age, but surveys have also found infected adults. Because of the few human cases reported and minor pathological effects of the adult tapeworms, this parasite is of little medical importance.

Clinical manifestations

Although diarrhea, anorexia, nausea, headache, and dizziness have been reported, no clinical symptoms were observed in the only experimental human infection (Turton et al. 1975). However, this was in a healthy adult, and a different picture might possibly be detected in children naturally infected with this tapeworm.

Pathogenesis, pathology, and immune responses

In the experimental infection referred to above, eosinophilia was observed, but there was no evidence of anemia or change in serum transaminases, indicating an absence of tissue damage, although there was an increase in plasma viscosity (Turton et al. 1975). Parasite-specific antibodies were detected using a fluorescent antibody technique at a titer of 1:160 on day 21 after an infection with 30 cysticercoids. On day 22, another 100 cysticercoids were ingested. The antibody titer remained at 160 after the second infection and, since no tapeworm eggs were found using saline flotation techniques, the authors concluded that the infection was established but the tapeworms were expelled before reaching maturity. The reason for this spontaneous cure could be host immune reactions caused by the rather high infection doses. A year later, the same person infected himself with 200 cysticercoids on days 0, 6, 13, 19, and 21 and was treated with niclosamide seven times between days 24 and 31. These infections caused pronounced eosinophilia and an increase in parasite-specific IgM and IgG, but not IgE (see Andreassen et al. 1998).

Diagnosis

In fecal samples, this species can easily be differentiated from *H. nana* by the larger size of proglottides or the absence of polar filaments on the inner membrane of the eggs.

CONTROL

Chemotherapy and prophylaxis

Treatment is as for *H. nana*. Good sanitary conditions, reduced rodent populations (especially rats), and avoidance of close rodent contact with humans or human food supplies will reduce the risk of infection.

DIPHYLLOBOTHRIUM LATUM

Diphyllobothrium latum is also known as 'human broad' or 'fish' tapeworm. The name fish tapeworm relates to the fact that a fish is the source of infection to humans, and the name broad (*latum*) to the fact that the proglottides are much wider than they are long, unlike the *Taenia* species.

This tapeworm is found globally, mainly in temperate zones. It has been very common in parts of Europe where fresh water fishes are a common part of the diet; especially where certain lightly salted fish are eaten as delicacies. In Europe, the Baltic countries, Russia, and especially Finland have been pointed out as having a high prevalence of human fish tapeworms. However, it is also present in tropical Africa and parts of Asia. In North and South America it is believed to have been introduced by immigrants. *Diphyllobothrium latum* is the subject of a monograph by Von Bonsdorff (1977).

Morphology and structure

D. latum is the longest tapeworm found in man, reaching up to 10 m or more, with over 3 000 proglottides.

LIFE CYCLE

Fertilized eggs (50 × 60–70 μm in size) are released from mature proglottides through the uterus pore and can be found free in the feces. After the liberation of the eggs, groups of proglottides then detach and degenerate; this is known as pseudoapolysis. The fertilized egg starts development in a suitable environment outside the host and time of embryonation is dependent on the temperature. When the first larva, the coracidium, has developed (after 8–12 days at 16–20°C), it is ciliated, containing an oncosphere. It hatches from the operculated egg only when exposed to light. When freely

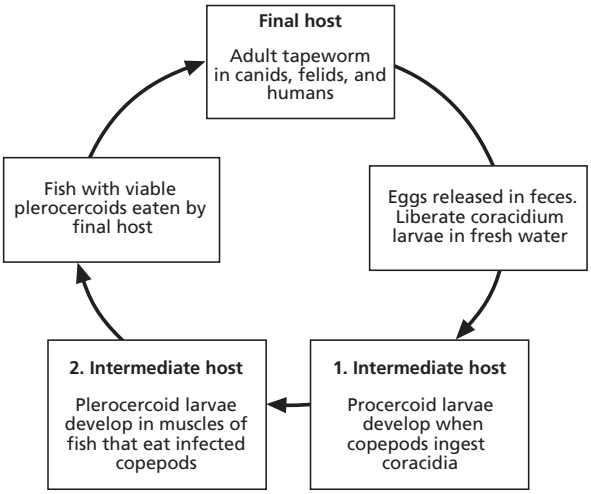

Figure 7 *Life cycle of the human fish tapeworm,* Diphyllobothrium latum

suspended in fresh water, its food reserves enable it to swim around for about 12 h or until eaten. In order to complete its life cycle (Figure 7), the coracidium must be eaten by suitable species and stages of small copepods, mainly of the genera *Diaptomus*, *Eudiaptomus*, and *Cyclops*. In the intestine of the copepod, the ciliated embryophore is shed and the oncosphere (hexacanth) penetrates the intestine into the hemocoele. Here it develops to an infective procercoid larva, where the six hooks of the hexacanth are confined to a small constricted posterior part called a cercomer. When copepods with infective procercoid larvae about 0.5 mm in length are eaten by a suitable second intermediate host (a freshwater fish) the procercoid penetrates the intestine of the fish and grows to the final larval stage, the plerocercoid. This lies freely in the body cavity or in the gut wall, or migrates to the muscles or the ovaries. The elongated plerocercoid (10–20 × 2–3 mm), which is not encysted or encapsulated, has a well-developed and normally contracted, partly invaginated scolex. Only certain species of freshwater fishes are suitable intermediate hosts, and they vary from location to location. If a small infected fish is eaten by a larger suitable fish host, the plerocercoids are, to some degree, able to penetrate the intestine of this second host and survive without further development; i.e. this host is a paratenic host in which the infective stage may accumulate. Secretions of enzymes from glands in the scolex are probably assisting the penetration of the intestinal wall. Humans become infected when they eat undercooked, raw, or lightly salted meat or roe from infected freshwater fishes. The adult, egg-producing stage is reached after about 4 weeks.

TRANSMISSION AND EPIDEMIOLOGY

Humans are the main final host and contribute most to the spread of the infection, but dogs, cats, and foxes and, in some places, bears and pigs, may be possible final hosts. Depending on the geographical location, several different species of both copepods and freshwater fishes may act as first and second intermediate hosts, respectively. In Europe, the most important second intermediate hosts for human infections are pike (*Esox lucius*), perch (*Perca fluviatilis*), and burbot (*Lota lota*). Ruffs (*Acerina cernua*) and small specimens of the other three species are important prey for the larger specimens of the first three species. The pike is also an important intermediate host in North America, where another species of burbot (*Lota maculosa*), yellow perch (*Perca flavescens*), wall-eyed pike (*Stizostedeon vitreum*), and sand pike (*Stizostedeon canadense*) are important species. On the Pacific coast of the Russian Far East, some Russian authors claim that *D. latum* is not present and a previous synonym of *D. latum*, namely *D. luxi*, is a valid species and the principal agent of human diphyllobothriasis, with *D. giljacicum*, *D. nihonkaiense*, and *D. klebanovskii* as synonyms (Dovgalev and Valovaya 1996). Investigations using DNA techniques seem to be necessary to solve this problem.

LOCATION IN HOST

The tapeworm is usually found in the ileum or jejunum.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

The number of human carriers of *D. latum*, which was estimated as about 10 million by Stoll (1947), has decreased in countries like Finland, where it is endemic and has caused many cases of pernicious anemia. Although there are no recent data on the prevalence of *D. latum* infections, this infection is probably still frequent in countries where much freshwater fish is eaten.

Clinical manifestations

Most human infections are with only one tapeworm and cause no, or very vague, ill effects. Minor clinical manifestations such as fatigue, weakness, diarrhea, and numbness of the extremities may occur. It has been shown that worm carriers have a significantly higher frequency of these symptoms than controls (Saarni et al. 1963). In a few cases, pernicious anemia may develop due to manifest vitamin B_{12} deficiency (deficiency of cobalamins in general). Clinical diagnosis of this tapeworm-induced pernicious anemia is made not only by a decreased vitamin B_{12} level in serum (around 100 pg/ml), but also by the presence of macrocytic megaloblastic anemia in conjunction with leukopenia, thrombocytopenia, and increased hemolysis. Furthermore, symptoms involving the central nervous system, such as paraesthesia, disturbances of motility and co-ordination, and impairment of deep sensibility, are also clinical criteria of tapeworm-induced pernicious anemia.

Pathogenesis and pathology

D. latum, which contains more than 50 times as much vitamin B_{12} as *T. saginata*, has been shown to absorb as much as 80–100 percent of a single oral dose of vitamin B_{12}, thereby competing with the host for this important vitamin. The size of the tapeworm and its proximity to the stomach will influence the amount of B_{12} absorption by the parasite: large worms in close proximity to the stomach reduce B_{12} availability to the host, greatly enhancing the probability of parasite-induced pernicious anemia. In fact, it has been indicated that the anemia may be relieved, if the tapeworm is forced further back in the intestine. However, other factors such as a relative deficiency in intrinsic factor due to endogenous or exogenous damage to the gastric mucosa, an inadequate supply of vitamin B_{12} in the diet or an increased requirement of vitamin B_{12} may contribute to the development of tapeworm-induced pernicious anemia.

Immunity

Practically nothing is known about immune reactions in humans against *D. latum*, and there is no evidence of acquired immunity.

Diagnosis

Diagnosis of diphyllobothriasis is impossible from purely clinical symptoms. Confirmation of pernicious anemia in a patient coming from an endemic area and known to have a diet of raw fish are relatively good indications. Evidence of the characteristic eggs of *D. latum* in fecal examinations is a reliable diagnosis, although eggs may be overlooked at routine examinations in about 5 percent of worm carriers. In suspected cases, several fecal specimens must be examined.

CONTROL

Chemotherapy

The broad tapeworm is more easily expelled than the *Taenia* species. Several drugs have been used, but niclosamide or praziquantel are the drugs of choice. Since the cure is not always 100 percent, examination for eggs in fecal samples should be performed 3 weeks after treatment.

Prophylaxis

It is very difficult, and in practice impossible, for sewage plants to remove 100 percent of eggs present in waste water. Adequate heating of fish dishes for a minimum of 10 min at 50°C (bone deep), or deep freezing (at least −10°C for 24 h) of fish or roe intended to be eaten raw or semi-raw is another prophylactic control measure. Brining of infected fish or roe should be very strong (10–12 percent NaCl) in order to kill plerocercoid larvae. However, this does not generally suit the taste of people eating the fish. In certain endemic areas, education of the public about the danger, and how to avoid it, should be given high priority.

Transmission

Because of uncontrolled defecation, many *D. latum* eggs are often transferred directly to freshwater lakes, where first and second intermediate hosts are present. Therefore, control of this infection must cover chemotherapy of infected humans (removal of adult worms) and control of human feces (reducing risk of freshwater contamination). Even if infected persons are found and treated and the egg-removal efficacy of sewage plants is improved to 100 percent, fish infections may still be maintained through infection of wild mammals.

SPORADIC ZOONOTIC ANIMAL TAPEWORMS THAT MAY INFECT HUMANS

Species are listed in alphabetical order of genus and species name.

Bertiella spp.

Since the first report of human infection in 1913, about 60 cases of *Bertiella* in man have been published. Species of *Bertiella* (*mucronata* and *studeri)* are found in non-human primates and have been described as incidental zoonotic infections primarily in children and young people in the tropics. The life cycle of these tapeworms is not yet completely known but mites are suspected to be the infective intermediate host. Human cases of *B. studeri* have been described in Sri Lanka (Karunaweera et al. 2001), India (Panda and Panda 1994), and Indonesia (Kagei et al. 1992). A review of human cases has been published by Denegri and Perez-Serrano (1997).

Diphyllobothrium dendriticum

This tapeworm, which is found in adult form in the intestine of many fish-eating birds, has also been shown to be infective to humans. The plerocercoid is found encysted on the entrails of many salmonid fishes. Because these are normally removed from the fish before preparation for human consumption, it is a rare human infection.

Diphyllobothrium nihonkaiense

This tapeworm, a close relative of *D. latum*, is described in Japan (Ohnishi and Murata 1994), where the number of cases has increased (Nishiyama 1994). Its epide-

miology in Japan has been described by Yamane et al. (1998).

Diphyllobothrium pacificum

This species, which is adult in sea lions, has been described as a human tapeworm acquired from marine fishes. It has been found in Peru (Baer et al. 1967; Baer 1969), Japan (Tsuboi et al. 1993), Chile (Gonzalez et al. 1999), and Equador (Gallegos and Brousselle 1991).

Other *Diphyllobothrium* species

A number of *Diphyllobothrium* species naturally occurring in free-living animals have been reported in a single or very few human cases. *Diphyllobothrium cordatum*, which occurs in seals, walruses, and dogs, has been reported in humans in Greenland and Iceland; *D. hians* has been reported from Japan; *D. houghtoni*, a canine and feline tapeworm, has been found in humans in China; *D. orcini* and *D. scoticum* in Japan; *D. ursi*, a tapeworm of North American bears, has infected one person in British Columbia, Canada; *D. yonagoense* has been described from Japan. Some of these species may not be identified correctly or may be synonyms.

Dipylidium caninum (the double-pored tapeworm)

This is a very common tapeworm in dogs and cats. Human infections are rare and normally restricted to young children, because infected fleas from cats or dogs must be ingested in order to acquire the infective cysticercoid stage (Gleason 1962). However, it has been suggested that infected fleas may be crushed in the dog's mouth, thereby releasing the cysticercoids, and these may be transmitted to children in the dog's saliva (Chappell 1991). This infection, which seldom causes any symptoms, can easily be diagnosed by the presence of active barrel-shaped proglottides. Furthermore, packets of about 15 eggs can be seen microscopically in the proglottides. Cases have been described in Austria (Brandstetter and Auer 1994), USA (Raitiere 1992), and Japan (Watanabe et al. 1993).

Mesocestoides spp.

Species of this tapeworm are common in carnivorous mammals, such as foxes. Mites are probably the infective intermediate host. Incidental intake of infected mites has resulted in a few human cases published in Japan and the United States (Schultz et al. 1992). *Mesocestoides lineatus* has also been found in humans in China (Jin et al. 1991) and Korea (Eom et al. 1992).

Inermicapsifer madagascariensis

This small (about 5 cm long) tapeworm, has been reported in humans from the eastern hemisphere, but also in children in Cuba and South America. It is common in rodents and hyraxes, and human cases have been reported from Madagascar and Mauritius and several countries in South and East Africa, where it is suspected to be more common than records indicate (Nelson et al. 1965). It was found in a 10-month-old girl in Kenya (Chunge et al. 1987).

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Larval cestodes

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THE PARASITES

Introduction, classification, and history

Larval cestodes belong to the phylum Platyhelminthes, which are acoelomate metazoa with an elongated dorsoventrally flattened body in their adult stage and a vesicular bladder in their larval stage. The most important human parasites of this group belong to the class Cestoda, subclass Eucestoda, order Cyclophyllidea, families Taeniidae, Hymenolepididae, Diphylobothriidae and genera *Taenia*, *Hymenolepis*, *Diphyllobothrium*, and *Echinococcus*. Except for *Echinococcus*, these parasites live in the human intestine throughout the adult stage. *Taenia solium*, *Echinococcus granulosus*, *E. multilocularis*, and *E. vogeli* also parasitize humans in their larval stage. The metacestode of *T. solium* is known as a cysticercus and that of *Echinococcus* as a hydatid cyst. The general term for the disease caused by the cysticercus is cysticercosis and, depending upon the site of infection, is also known as neuro, ocular, muscular or subcutaneous cysticercosis. In the case of hydatid cyst, the disease used to be called hydatidosis, but now it is usually termed echinococcosis: there are three types: cystic echinococcosis due to *E. granulosus*, alveolar echinococcosis due to *E. multilocularis*, and polycystic echinococcosis due to *E. vogeli* and *E. oligarthrus*. The first is the most prevalent of the four species, the second one is the most pathogenic, and the last two species are rare zoonoses restricted to Latin America.

Human cysticercosis is considered a public health problem in many developing countries of Latin America, non-Islamic Asia, and Africa, and it is clearly associated with lack of public health education and proper sanitary conditions (Flisser 1988, 1994, 2002a). In addition, immigration from Mexico and other Latin American countries to the United States has increased markedly in each of the last three decades. It is estimated that movement in both directions across the US–Mexico border exceeds 200 million persons per year. Opportunities for acquiring and transporting *T. solium* infections are abundant (Schantz et al. 1998). Cystic echinococcosis is cosmopolitan in its distribution but concentrated in the major sheep-raising and pastoral areas especially of South America, the Mediterranean, the Middle and Near East, East Africa, Russia, Central Asia, and China. Alveolar echinococcosis has been reported in Alaska, China, Japan, parts of Europe, the Near East, and Russia, but since the life cycle involves sylvatic animals such as foxes, rodents, and other small mammals, exposure to humans is uncommon. Polycystic echinococcosis has been identified in a total of 100 cases from Brazil, Colombia, Ecuador, Panama, and Venezuela, because *E. vogeli* is native to the humid tropical forests of South America (D'Alessandro et al. 1979; Kammerer and Schantz 1993; Rausch 1997). To date, only three human infections with *E. oligarthrus* have been reported from Venezuela, Brazil, and Suriname (Basset et al. 1998).

Adult tapeworms in human feces were first recognized by the Egyptians. The species was probably *Taenia saginata* since Egyptians did not eat pork. Hippocrates, Aristotle, and Threophrastus called them 'flatworms' meaning band or ribbon worm, whereas Romans, such

as Celsus, Pliny the Elder, and Galen, named them 'lumbricus latus', meaning broad or wide worm. Only many centuries later were cysticerci discovered in human beings. They were first reported by Rumler in 1558, but the disease was not identified as parasitic until in 1697 Malpighi discovered the animal nature of cysticerci. Goeze recognized their helminthic essence in 1784 and Leuckart (1886) defined them in great detail. Tyson, in 1683, detailed the head of tapeworms and later in 1691 described the hydatides that he found in 'rotten sheep'. Goeze in 1782 described the scolices of echinococcal cysts and indicated their similarity to the heads of tapeworms; he concluded that echinococci were alive, verminous in nature, and related to tapeworms.

The life cycle of *T. solium* was defined by van Beneden in 1854 who fed a pig with eggs from a human *T. solium* and found numerous cysts in muscles, and by Kuchenmeister, who in 1855 discovered adult tapeworms in the intestines of convicts who ate pork infected with cysticerci some time before execution (Grove 1990). In 1933, Yoshino ingested swine cysticerci in order to obtain a 'reliable source' of eggs to infect pigs and studied, with great histological detail, the early development of cysticerci. He also reported that, following his own infection, between one and five gravid proglottids were expelled per day for 2 years (Yoshino 1933). The name '*Cysticercus cellulosae*' was given by Zeder and by Rudolphi at the beginning of last century, but was abolished when cysticerci were shown to be the larval stage of *Taenia*.

The life cycle of *Echinococcus* was demonstrated by von Siebold in 1853 when he infected dogs with hydatid cysts from sheep and 27 days later discovered intestinal worms, consisting of a 'head' and three segments containing reproductive organs and eggs, which he called *Taenia echinococcus*. Leuckart, in 1867, infected suckling pigs with ova of *T. echinococcus* and was able to study the resulting cysts at intervals after infection. Naunyn, in 1863, infected dogs with hundreds of scolices obtained after autopsy of a patient with a large hepatic cyst. The dog, when killed after 5 weeks, had mature small tapeworms identical to *Taenia echinococcus*. Naunyn not only confirmed that hydatid cysts of human origin undergo the same development as cysts of animal origin but, in demonstrating that the adult worms obtained from the two sources were similar, also showed that there was only a single species. Naunyn concluded that the *Echinococcus* from man is the bladder worm stage of *T. echinococcus* living in the intestine of the dog. These results were confirmed soon afterwards by Krabbe and Finsen in Iceland in 1866 and by Thomas in Australia in 1883, all of whom used hydatid cysts obtained from humans (Grove 1990). Until the 1950s however, controversy, surrounded whether human *Echinococcus* infection was due to one species, *E. granulosus*, or two, the second being the cause of multi-vesicular or multi-locular lesions. Parasitological and ecological studies in Alaska by Rausch showed that human infections in Eskimos were the same as those described in Europe, and that similar larval cestode infections occurred in tundra voles. Experimental infections of dogs by Vogel using human isolates confirmed that it was a separate species and it was designated *Echinococcus multilocularis* (Rausch 1954; Vogel 1957).

Morphology and structure

The body of an adult tapeworm has a head (scolex), a neck, and a chain of segments (strobila). The scolex is the size of a pinhead, while the strobila may be several meters long, as in *Taenia*, containing more than 1 000 proglottids. In *Echinococcus* there are only between two and five segments and the strobila measures only a few millimeters. The scolex has four suckers and a rostellum with a double crown of hooks (Figure 1a–c). The development of adult *Echinococcus* may not be a limiting factor for its study, since several canids are definitive hosts, but in the case of *Taenia solium* only humans are definitive hosts. Experimental rodent models for *Taenia solium* have been developed (Maravilla et al. 1998) in which the characteristics of the implantation sites have been described (Merchant et al. 1998) and other studies can now be performed. Adult *E. multilocularis* tapeworms have also been grown successfully in immunosuppressed rodents (Kamiya and Sato 1990).

The most conspicuous features of tapeworms are the lack of a mouth or digestive cavity and the presence of repeated units or proglottids at different developmental stages. The proximal proglottids are immature (i.e. have not yet developed sexual organs), the mature proglottids contain functional hermaphroditic sexual organs, where fertilization occurs, and the terminal proglottids are gravid, being little more than sacs full of eggs (see Intestinal tapeworms).

Eggs are spherical and range in size from 20 to 50 mm. They have a radial appearance under light microcopy because the embryophore that surrounds the oncosphere is formed by contiguous keratinaceous blocks. The eggs of *Taenia* and *Echinococcus* are morphologically indistinguishable at the light microscope level. The embryophore protects the oncosphere, while the egg is in the external environment, making eggs extremely resistant and enabling them to disperse and withstand a wide range of environmental temperatures (Gemmell and Lawson 1986; Torgerson et al. 1995; Veit et al. 1995). When the eggs are released from the definitive host they are, or quickly become, fully embryonated and infective to a suitable host. When eggs are ingested by the intermediate host, the cementing substance that joins the embryophoric blocks is digested and the oncosphere is released in the small intestine (Flisser 1994). Experimental hatching methods include pepsin/trypsin

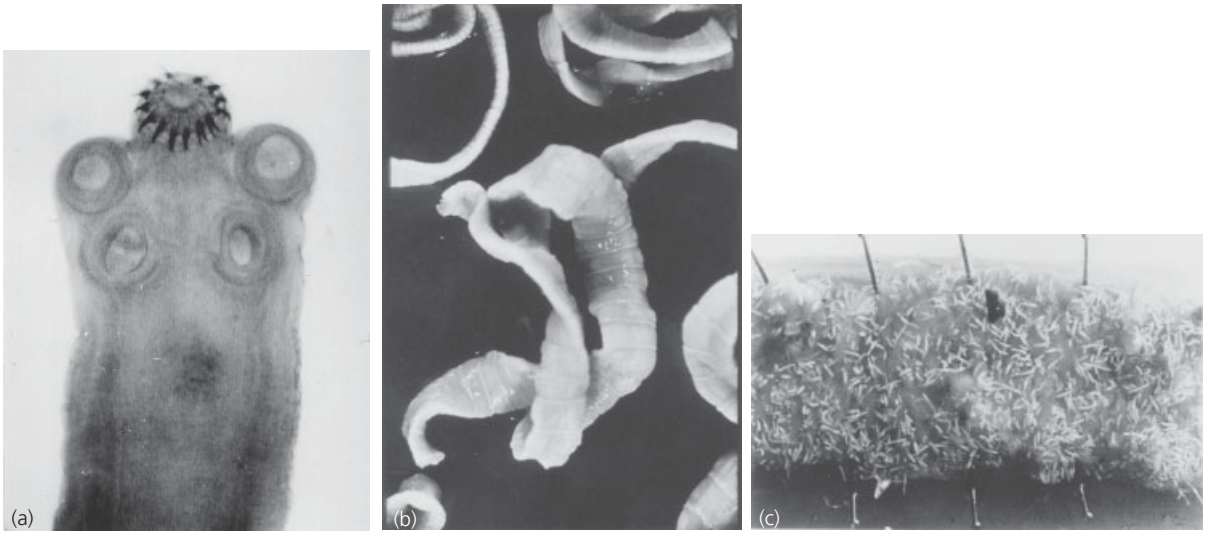

Figure 1 (a) Taenia solium *scolex showing the double crown of hooks and one sucker;* **(b)** Taenia solium *strobila with immature proglottids (upper left) and mature proglottids;* **(c)** *Multiple* Echinoccocus granulosus *adult parasites in the intestine of a dog*

or sodium hypochlorite, the latter being more efficient (Heath 1982; Wang et al. 1997).

The liberated and activated oncosphere is mobile and uses its six hooks to penetrate into the mucosa, reaching the lamina propria within 3–120 min after hatching. Penetration is presumably assisted by secretions from the paired penetration glands, but may be purely mechanical, involving hook and body movements (Thompson 1995; Pawlowski 2002). After penetration, larvae are transported through blood vessels into the tissues, where they develop into the metacestode, specifically named cysticercus (*Taenia*) or hydatid cyst (*Echinococcus*). Macroscopic *T. solium* cysticerci measuring around 0.3 mm were seen in liver, brain, and skeletal muscles of pigs 6 days after infection, and after 60–70 days cysticerci had fully developed scolices and measured 6–9 mm. Full development of cysticerci (to a size of 8–15 mm) takes 2–3 months in pigs (Yoshino 1933). Cysticerci are formed of two chambers; the inner one contains the scolex and the spiral canal and is surrounded by the outer compartment that contains the vesicular fluid, usually less than 0.5 ml. When a living cysticercus is ingested by the definitive host, the first event that takes place is the widening of the pore of the bladder wall for the scolex and neck to emerge, leaving the bladder wall and vesicular fluid to disintegrate in the digestive tract (Rabiela et al. 2000). Several in vivo and in vitro studies have demonstrated that *Echinococcus* oncospheres rapidly undergo a series of re-organizational events during the first 14 days, involving cellular proliferation, degeneration of oncospheral hooks, muscular atrophy, vesicularization, central cavity formation, and development of both germinal and laminated layers (Rausch 1954; Heath and Lawrence 1976). The minimum time required for the development of protoscolices of *E. granulosus* inside cysts within the human host is not known, in pigs it takes 10–12 months, and in sheep 10 months to 4 years, although protoscolices can be formed in hydatid cysts that measure 0.5–2 cm. For *E. multilocularis* in rodent intermediate hosts protoscoleces may develop within 2–4 months. The cysticercus of *T. solium* is formed by one scolex, while all species of hydatid cyst contain multiple protoscolices (often within brood capsules) formed by asexual budding processes of the metacestode germinal layer. All metacestodes and adult worm stages are macroscopic and have a similar scolex, but eggs and oncospheres are microscopic.

There are two morphological types of cysticerci: cellulose and racemose (Figure 2). Cellulose cysticerci are small, spherical or oval, white or yellow, with vesicles that measure 0.5–1.5 cm and have a translucent bladder wall, through which the scolex can be seen as a small solid eccentric granule. Usually this stage grows no further. This type of cysticercus is frequently separated from the host tissue by a thin collagenous capsule, within which it remains alive (Rabiela et al. 1982). The racemose cysticercus either appears as a large, round or lobulated bladder circumscribed by a delicate wall, or resembles a cluster of grapes. It measures up to 10 or even 20 cm and may contain 60 ml fluid. The scolex cannot be seen; in some cases only detailed histological studies will reveal its remains. Neuropathological observations of human brain suggest that parasites lodged in spacious areas are able to grow and transform into racemose cysticerci, since a parasite with a bilobulated aspect was found (Rabiela et al. 1989). In support of this hypothesis is the finding that experimental infections with *T. serialis* in mice demonstrated an aberrant large cysticercus devoid of scolex in the peritoneal cavity (Lachberg et al. 1990). Alternatively, growth of cysticerci might be controlled by the immune response of the host, as suggested by in vitro experiments that showed

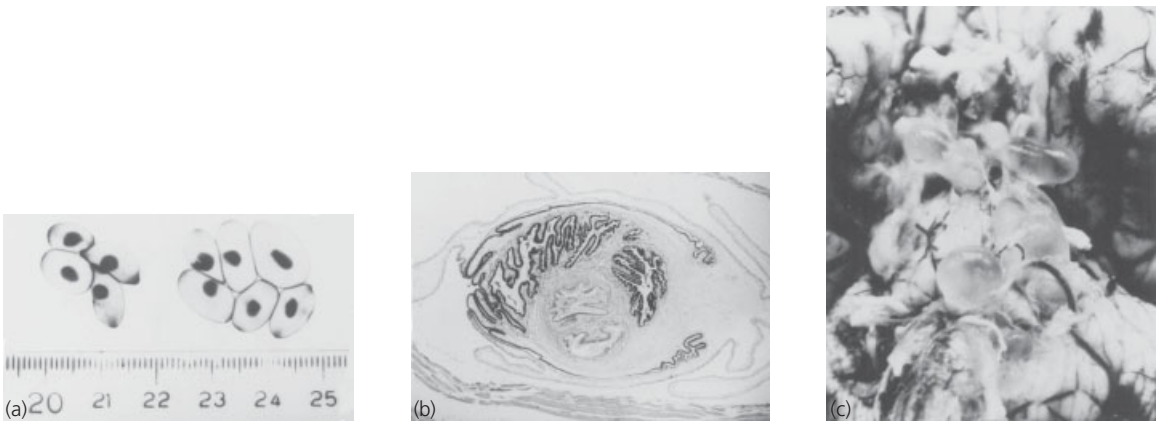

Figure 2 *Cellulose type* Taenia solium *cysticerci from pig muscle (by courtesy of Dr D. Correa);* **(b)** *Histological section of a cellulose type cysticercus in host muscle showing the spiral canal and the bladder wall (by courtesy of Dr A.S. de Aluja);* **(c)** *Racemose type* Taenia solium *cysticerci in the base of a human brain (by courtesy of Dr M.T. Rabiela)*

that the presence of the inflammatory capsule that surrounds *T. solium* cysticerci inhibits their evagination (Ostrosky et al. 1991). A recent study documented the events of evagination employing light and electron microscopy. The authors show that the scolex uncoils during evagination, but does not turn inside out, and that the scolex and the neck comprise a different structure from the bladder wall, although they are contiguous (Rabiela et al. 2000). An autoradiographic analysis of the germinative tissue in evaginated *T. solium* cysticerci identified stem cells that proliferate continuously, differentiate, and migrate to the tegument, constituting the main process by which these worms develop from metacestode to adult stage (Merchant et al. 1997).

The fully developed *E. granulosus* hydatid cyst is typically unilocular, subspherical in shape and fluid filled. It may range in size from a few mm to >30 cm. The cyst consists of an inner germinal nucleated layer supported externally by a tough, elastic, glycan rich, acellular laminated layer of variable thickness, surrounded by a host-produced fibrous adventitial layer. Asexual proliferation takes place in the germinal layer and brood capsule formation is entirely endogenous; within each capsule several protoscolices are found. Occasionally cysts may adjoin and coalesce, forming groups of clusters of small cysts of different size. In humans, the slowly growing hydatid cysts may attain a volume of many liters and contain many tens of thousands of protoscolices. With time, internal septae and daughter cysts may form within the primary cyst, most probably from vesiculating protoscolices, disrupting the unilocular pattern (Schantz 1994; Thompson 1995) (Figure 3).

The metacestode of *E. multilocularis* is the most complex and develops quite differently from that of *E. granulosus*. The size of the parasitic focus varies from less than 1 mm to >20 cm, as lesions caused by the metacestodes vary from minor foci (a few millimeters in diameter) up to large areas of infiltration. The metacestode is a multi-vesicular infiltrating structure with no limiting host–parasite barrier (adventitial layer). In human infections the larval mass usually contains a semisolid matrix rather than fluid and is formed by numerous small vesicles embedded in a dense stroma of connective tissue. The metacestode consists of a network of filamentous solid cellular protrusions of the germinal layer that are responsible for infiltrating growth, transforming into tube-like and cystic structures where proliferation occurs both endogenously and exogenously (Figure 4).

Detachment of germinal cells or microvesicles from infiltrating cellular protrusions and their subsequent distribution via the lymph or blood can give rise to the distant metastatic foci characteristic of alveolar echinococcosis. Protoscolices of *E. multilocularis* appear not to be responsible for secondary spread of the metacestode. In rodents, the natural intermediate hosts, the larval mass proliferates rapidly by exogenous budding of germinative tissue and produces an alveolar-like pattern of microvesicles filled with protoscolices, but in humans the larval mass resembles a malignancy in appearance and behavior because it proliferates continuously by exogenous budding and invades the surrounding tissues. Protoscolices are rarely observed in infections of humans (Wilson and Rausch 1980; Ali-Khan et al. 1983).

The metacestode of *E. vogeli* exhibits developmental and structural characteristics considered intermediate to those of *E. granulosus* and *E. multilocularis*, with sizes that range from 2 to 80 mm. It may occur singly, in small groups, or occasionally in dense aggregations in which each cyst is enclosed by its separate adventitial layer (polycystic development). It has internal division of fluid-filled cysts to form multi-chambered growths due to endogenous proliferation and convolution of both germinal and laminated layers. This leads to the formation of secondary subdivisions of the primary vesicle

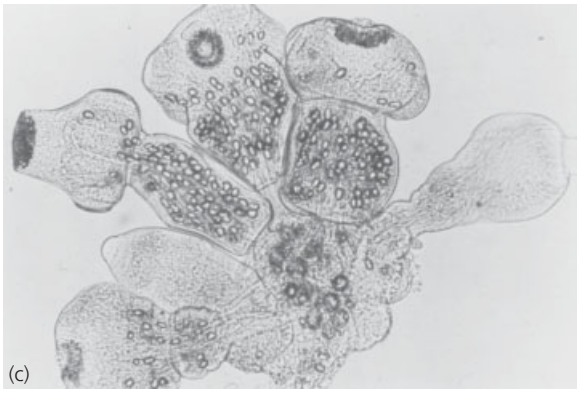

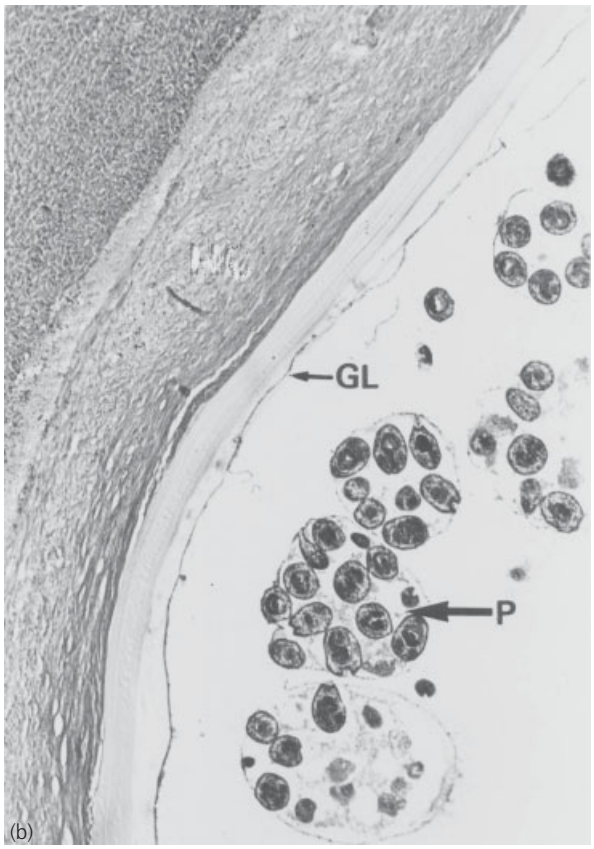

Figure 3 (a) Echinococcus *cyst from the liver of a patient in Urumqi, China;* **(b)** *Histologic section of an* Echinococcus granulosus *cyst with many protoscolices (P) and germinal layer (GL) (by courtesy of Dr P. Schantz);* **(c)** Echinococcus granulosus *protoscolices seen under light microscopy, partly evaginated*

with production of brood capsules and protoscolices in the resultant chambers, which are often interconnected (D'Alessandro et al. 1979; Rausch et al. 1981).

Hydatid cysts due to *E. granulosus* develop in 6 months in mice, 10–18 months in sheep, and 1 year in swine. Alveolar cysts due to *E. multilocularis* develop in 2–4 months in laboratory mice, but faster in experimental voles. Development of any of these metacestodes in humans is not known because it is impossible to define the time of infection. The life span of these parasites can be as long as 4 or more years in sheep, up to 16 years in horses and, impressively, several decades in humans (Dixon and Lipscomb 1961; Spruance 1974).

General biology

Studies on the biochemistry of Platyhelminthes are sparse. The first contributions dealing with tapeworms, from Read (1952), related to anaerobic metabolism in the rat tapeworm and the role of carbohydrates in cestode biology, and these stimulated work on the biochemistry of protoscolices of *E. granulosus*. More recently it was shown that cysticerci use either aerobic or anaerobic pathways according to oxygen availability in the environment (Cervantes-Vazquez et al. 1990), oxygen uptake, and evagination are useful measurements of their viability (Correa et al. 1987; Flisser et al. 1990a; Maravilla et al. 1998).

Larval cestodes obtain nutrients by diffusion through their bladder wall that bears plasma-membrane bounded microvilli covered by a loose glycocalyx. Two facilitated diffusion glucose transporters (TGPT1 and TGTP2) have been identified, the first being abundant in structures underlying the tegument in adult and larval parasites, whereas the latter appears to be localized only in the tegumentary surface of the larvae. None of the glucose transporters were recognized by antibodies in the sera from patients and pigs with cysticercosis (Rodriguez-Contreras et al. 1998, 2002). The syncytial tegument is filled with ellipsoidal vesicles of different sizes and is connected by cytoplasmic processes to the underlying nucleated cell bodies. The excretory or protonephridial system consists of flame cells which form a dense network attached to excretory ducts. The bladder wall also contains a network of nerve-like cells. Immunohistochemical studies on protoscolices of *E. granulosus* have identified serotonin in nerve cell bodies in the lateral ganglia and in association with the lateral longitudinal nerve cords, as well as in the central nerve ring, the rostellar nerves and the nerve plexus of the suckers; some peptidergic nerve elements were also detected

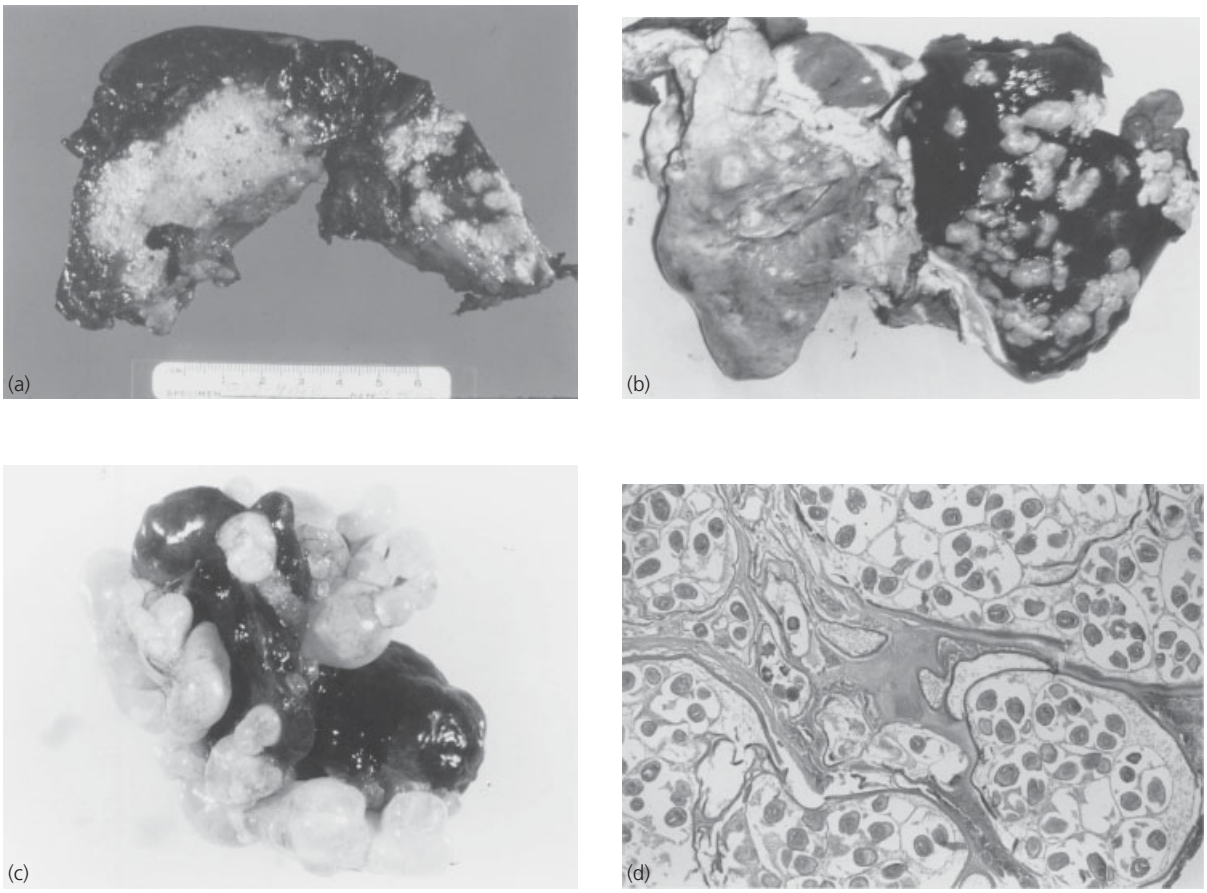

Figure 4 Echinococcus multilocularis *in the liver of a 61-year-old female from Minnesota, USA (by courtesy of Dr P. Schantz);* **(b)** Echinococcus multilocularis *in the liver and lungs of a sheep (by courtesy of Professor R.C.A. Thompson);* **(c)** Echinococcus multilocularis *in the liver of a cotton rat 4 months after infection;* **(d)** *Histological section of* Echinococcus multilocularis *showing multiple vesicle filled with protoscolices (by courtesy of Dr P. Schantz)*

(Fairweather et al. 1994). Platyhelminthes produce mineral concretions which in cestodes are referred to as calcareous corpuscles. In contrast to the intracellular origin formation described for other cestodes, in cysticerci of *Taenia solium*, calcareous corpuscles occur extra-cellularly in the lumen of protonephridial ducts in a way similar to that proposed for trematodes (Vargas-Parada et al. 1999). Their role in larval physiology has been reviewed (Vargas-Parada and Laclette 1999). Formation of calcareous corpuscles in *E. multilocularis* has been reported on the brood capsule and on the germinal layer (Ohnishii and Kutsumi 1991).

Several glycoproteins have been detected on the tegumentary surface of the bladder wall of *T. solium* cysticerci: the most abundant (of 55 kDa) was found to be the heavy chain of the host's IgG and one of 180 kDa was also present on proglottid surface of several taeniids (Landa et al. 1994). Surfaces also contain cytoskeleton proteins, such as myosin, which in *T. solium* cysticerci and tapeworms is of the conventional type II and can also be found in supernatants of parasite cultures and in feces of experimentally infected rodents (Ambrosio et al. 1997). The excretory-secretory molecule designated *T. solium* antigen B, initially identified as an immunodominant antigen (Flisser et al. 1980), has been purified and characterized. It is a glycoprotein (Guerra et al. 1982) with significant homology to the paramyosin of *Schistosoma mansoni* (Laclette et al. 1991). Functionally, it has been shown to inhibit the C1 component of the complement system (Laclette et al. 1992). Recent studies are focused in defining the immunological reactivity of the different regions of paramyosin (Gazarian et al., 2000; Vazquez-Talavera et al. 2001a, b). The gene for paramyosin has been expressed and sequenced. The entire gene is 6 106 bp (from its start to the stop translation codons) and contains 13 introns that have higher A + T content than its exons. A tendency towards decreasing the number of introns during evolution was inferred from gene comparison with the other two genes for which the complete structure is known: *Caenorhabditis elegans* and *Drosophila melanogaster*. No evidence of alternative splicing sites was found, excluding the possibility that *T. solium* expresses a mini-paramyosin, as is the case for *D. melangaster* (Vargas-Parada and Laclette 2003). Cholinesterases, cysteine, and aspartic, but not

serine, protease activities have been identified in *T. solium* cysticerci (White et al. 1992), as well as type II myosin (Ambrosio et al. 1997). Recently, actin was identified and characterized in *T. solium* cysticerci and seven isoforms were resolved, pointing to a family of actin genes (Ambrosio et al. 2003). *Taenia solium* cysticerci respond to temperature stress through two major stress proteins: HSP80 and HSP70; stress proteins were also found in the excretory-secretory products. Patients with neurocysticercosis recognized at least two of the excreted-secreted products, one of which appears to belong to the HSP60 family (Vargas-Parada et al. 2001). Paramyosin from *E. granulosus* shows 71 percent identity with *S. mansoni* paramyosin and a significant homology with *T. solium* paramyosin (Mühlschlegel et al. 1993). In *E. granulosus* several enzymes have been demonstrated in the tegument that may have a digestive or absorptive function (McManus and Bryant 1995). At least five proteolytic enzymes have been detected in the cyst fluid (Marco and Nieto 1991) and gluthathione S-transferase has been isolated from protoscolices (Fernandez and Hormaeche 1994). A characteristic feature of the metacestodes of *Echinococcus* spp. is the formation of an acellular laminated layer which in *E. multilocularis* consists of mucin type glycosylated proteins that appear to have structural and immunoregulatory roles (Gottstein et al. 2002; Hulsmeier et al. 2002). The chemical composition of hydatid cysts from different geographic and host origins has been studied in detail (Sanchez and Sanchez 1971; Frayha and Haddad 1980) and has revealed basic differences between the UK horse and sheep strains of *E. granulosus* and between these and *E. multilocularis* (McManus and Smyth 1978). A subsequent study of the biochemical composition of *E. granulosus* in Kenya indicates differences between protoscolices of cattle, goat, camel, and sheep origin, but similarities between the sheep and human parasites (McManus 1981).

An interesting recent finding in a rural village of central Mexico, where natural conditions favor *Taenia solium* transmission, was that castration of male pigs increased prevalence of cysticercosis from 23 to 50 percent, and pregnancy in sows also increased prevalence from 28 to 59 percent. Endocrinological conditions characterized by low levels of androgens or high levels of female hormones probably influence the susceptibility of pigs to cysticercosis. Therefore, delaying castration of male pigs and confinement of sows during pregnancy might significantly decrease the prevalence of the infection and help curb transmission without much cost or difficulty (Morales et al. 2002).

The use of various molecular techniques for clarification of the taxonomic status within cestodes generated knowledge related to the evolution, ecology, and population genetics of these parasites. Intra-specific DNA variability occurs in *T. solium* isolates from India, Mexico, and Zimbabwe (McManus et al. 1989), while the analysis of the sequence variation within a 366 nucleotide for cytochrome C oxidase I (COI) from *T. solium* isolates recovered from India, China, and Zimbabwe showed only slight differences in three conservative changes in the third base position of codons of the isolate from Zimbabwe (Bowles and McManus 1994). Also, low variability in *T. solium* DNA coding for COI, ITS1, and the diagnostic antigen Ts 14 has been found (Hancock et al. 2001). In contrast, the sequence of the entire COI and cytochrome b from 13 isolates of *T. solium* from various regions showed 1.7 and 2.9 percent variant nucleotide positions for COI and cytochrome b among all isolates and the phylogenies obtained show that the isolates from Asia (China, India, Irian Jaya, and Thailand) formed a single cluster, whereas the isolates from Latin America (Bolivia, Brazil, Ecuador, Mexico, and Peru) combined with those from Africa (Cameroon, Mozambique, and Tanzania) form a second cluster (Nakao et al. 2002). Furthermore, recent studies on the variability of individual *T. solium* cysticerci from Mexico, Honduras, and Africa by random amplified polymorphic DNA analysis (RAPD) showed several alleles fixed and the presence of a clonal structure, suggesting the existence of local lineages with events of genetic recombination within them (Maravilla et al. 2003; Vega et al. 2003).

Also, many different molecular biological techniques have been applied with great success to the characterization of *E. granulosus* host isolates or strains. Biological criteria indicate that distinct horse-dog and sheep-dog forms of *E. granulosus* occur in UK (reviewed by Thompson and Lymbery 1988) and this has been confirmed by a variety of molecular techniques (Bowles and McManus 1993). These studies also indicate that the sheep strain is genetically uniform and cosmopolitan in its geographical distribution and that the horse strain or genotype is genetically similar to the one that infects equines in other countries. DNA sequence data show that the sheep and horse genotypes do not interbreed despite the fact that they use the same definitive host and occur sympatrically. Based on this and complete mitochondrial sequence data, it has been suggested that the sheep and horse genotypes of *E. granulosus* could probably be regarded as different species (McManus and Bryant 1995; Le et al. 2002). In total, nine distinct genotypes or strains of *E. granulosus* have been described using DNA sequence data and have been designated G1–G9. Apart from the sheep and horse strains, genotypes characteristic for cattle, buffalo, camel, pig, and cervid (deer) hosts have been described, as well as a second sheep strain in Australia and a pig-related isolate from Poland (Bowles et al. 1995). The majority of human isolates of *E. granulosus* have been shown to be the common sheep strain, however surgical biopsy material has also indicated that infection with cattle, pig, or camel genotypes is possible (Rozenzvit et al. 1999; Bardonnet et al. 2002).

Life cycle

The life cycle of taeniid larval cestodes involves two mammalian hosts, one definitive and one intermediate, and three developmental stages: the adult tapeworm in the definitive host, eggs in the environment, and the metacestode in the intermediate host (Figures 5 and 33.6). The cycle is simple in principle: the adult stage develops in the intestine after the host ingests the metacestode lodged in the tissue of the intermediate host. The metacestode develops in muscle, liver, lungs, or elsewhere after the intermediate host ingests eggs from the environment. Eggs or proglottids are released into the environment from the adult intestinal parasite. The adult stage of *T. solium* occurs only in human beings, whereas canids and felids are the hosts of adult *Echinococcus* spp. Intermediate hosts for *T. solium* are mainly pigs; however, hydatid cysts can occur in a great variety of mammals. Cysticerci can develop in other mammals but have no epidemiological relevance. The importance of *T. solium* and *Echinococcus* spp. is that humans can also harbor the larval stage that may cause severe disease (see Figures 5 and 6). A recent cladistic analysis of the genus *Taenia* indicates that there has been an extensive host-switching among definitive carnivorous hosts, in contrast there has been a more pervasive co-evolution with intermediate herbivorous hosts, and rodents are postulated as ancestral for both *Taenia* and *Echinococcus* (Hoberg et al. 2000).

Depending on the geographical location and the intermediate hosts, two biological forms of *E. granulosus* have been recognized, the so-called northern and the European biotypes. The northern biotype is maintained in the tundra and taiga biomes by a predator–prey relationship between the wolf (*Canis lupus*) and large deer (moose, reindeer, and other cervids), but may also occur in humans where reindeer are domesticated, as in parts of western Alaska and Eurasia, being acquired from dogs used in herding or from eggs dispersed by wolves. Coyotes (*Canis latrans*) may also become infected from scavenging. The intermediate hosts of the European biotype include camels, cattle, goat, horses, pigs, and sheep; while buffalo, wild boar, llama, kangaroo, wallaby, gazelle, giraffe, and impala, among others, have also been reported to have hydatid cysts. The European biotype of *E. granulosus* is almost cosmopolitan, as a result of the introduction of domestic animals and their helminths by Europeans in colonizing other regions from the early sixteenth century onwards. The adult stage occurs mainly in dogs but also in foxes, dingoes, jackals, hyenas, and lions (Rausch 1995).

The adult stage of *E. multilocularis* is found mainly in foxes (principally the red fox *Vulpes vulpes*), rarely in wolves, regionally in coyotes, and also in dogs and cats. At least eight families of rodents and small mammals have been reported to be infected with the metacestodes, these include voles and lemmings, hamsters and gerbils, rats and mice, squirrels, shrews, muskrats, and pikas. Cattle and swine, as well as captive non-human primates, may also acquire the infection accidentally but are not important epidemiologically.

Echinococcus vogeli is a neotropical species maintained in the bush dog (*Speothus venaticus*) and forest rodents like the paca (*Cunniculus paca*). Other

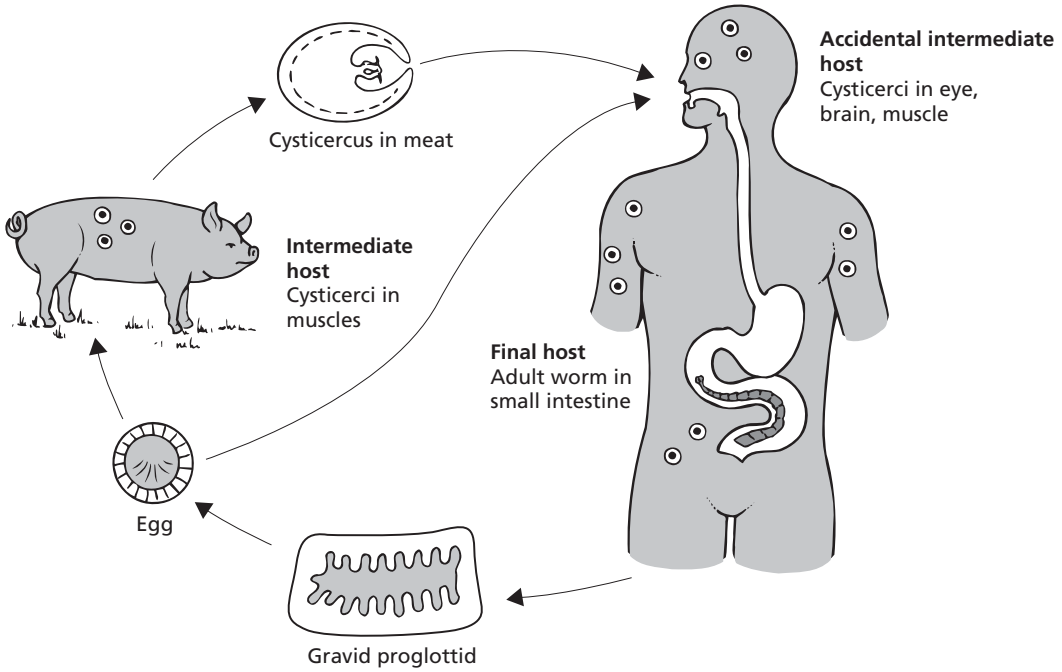

Figure 5 Taenia solium *life cycle*

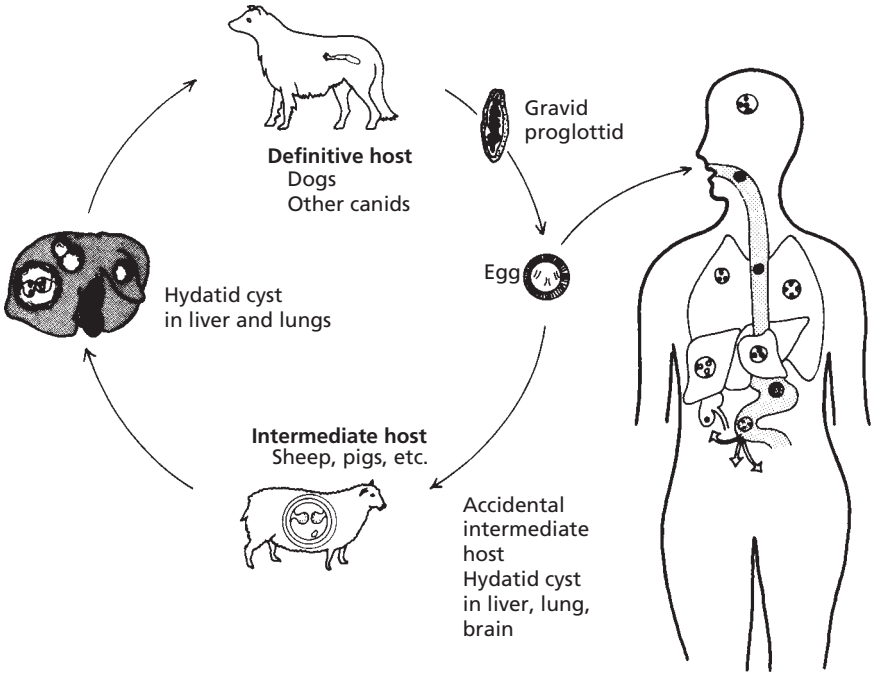

Figure 6 Echinococcus *life cycle*

mammals can be easily infected, as was seen in Los Angeles Zoo where several young animals were housed near a bush dog captured in Colombia. During the following 10 years, seven orangutans, three chimpanzees, two gibbons, and a siamang died or were killed with terminal disease; thereafter three gorillas died and two survivors had advanced disease (Howard and Gendron 1980). The second neotropical species *E. oligarthrus* has been recorded in six species of indigenous felids including the jaguar (*Panthera onca*) in South America and also the bobcat (*Lynx rufus*) in northern Mexico, and in several rodent intermediate hosts including the agouti (*Dasyprocta* spp.) (Rausch and D'Alessandro 2002).

Location in the host

Characteristically, *T. solium* cysticerci lodge in the central nervous system, the eye, striated muscle, and subcutaneous tissue (Figure 7). It is known that cysticerci are able to evaginate in the eye (Figure 7b). Unexpectedly, an evaginated cysticercus was seen by magnetic resonance in the fourth ventricle of the brain (Flisser and Madrazo 1996). These data support experimental ones that indicate that the host's inflammatory capsule, and not physical restraint, inhibits spontaneous evagination of cysticerci (Ostrosky et al. 1991). Parasites lodged in eye chambers or brain ventricles, as opposed to those found in muscle or brain parenchyma, are usually not surrounded by the host's capsule (Rabiela et al. 1982). It is not known why cysticerci develop primarily in these tissues; cysticerci have exceptionally been found in other organs and tissues, most probably as a result of severe immunosuppression. The location of parasites in 2 188 cases of cysticercosis collected from several Latin American countries was: central nervous system 82 percent, eye and annexes 17 percent, subcutaneous tissue 7 percent, muscle 5 percent, other organs 6 percent, generalized 1 percent. In the central nervous system cysticerci are more frequently found in subarachnoidal spaces, and less often in parenchyma and ventricles (Rabiela et al. 1982; Escobar 1983). From 161 neurosurgical cases, 89 had cysticerci in the fourth ventricle, 20 in the third ventricle, 17 in the lateral ventricle, 27 were subarachnoidal, and eight were spinal (Madrazo and Flisser 1992). Cysticerci found in basal ganglia are related to the total number of parasites, ranging from 5 percent in patients with a single lesion to 60 percent in patients with more than five parasites; putamen and caudate nuclei were the most frequent sites of lesions (Cosentino et al. 2002). The cellulose type is the most frequent cysticercus in human brain, although in 9–13 percent of studied cases, cysticerci of both types coexisted in the same brain (Rabiela et al. 1982). Racemose cysticerci are only found in spacious areas of the brain such as ventricular cavities and some subarachnoidal spaces, mainly the basal meningeal cisternae (Rabiela et al. 1989); they are found only in human brain and not in swine. Studies of ocular infections indicate that 46 percent of cysticerci are found in vitreous humor, 37 percent are subretinal, 12 percent subconjuntival, 2 percent occur in the orbit, 2 percent are subcutaneous, and 1 percent occurs in the anterior

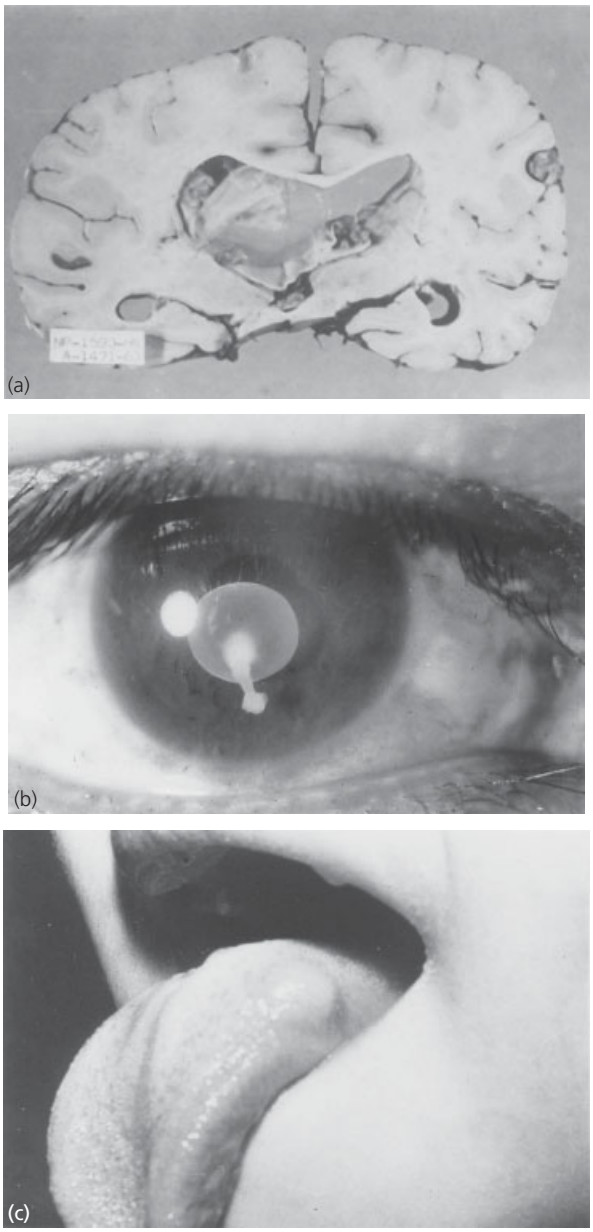

Figure 7 (a) *Brain section showing a huge ventricular cysticercus and a small subarachnoidal parasite (by courtesy of Dr J. Olvera);* **(b)** Taenia solium *cysticercus in the anterior chamber of the eye: interestingly, it is evaginated (by courtesy of Dr D. Lozano);* **(c)** *Tongue of child with a cysticercus in the muscle (by courtesy of Dr A. Martuscelli)*

chamber (Puig-Solanes 1974; Gomez-Leal 1989). In ocular, muscular, and subcutaneous cysticercosis only cellulose cysticerci are found.

Most primary infections of *E. granulosus* in humans consist of a single hydatid cyst; however, up to 20–40 percent of patients have multiple cysts or multiple organ involvement. The liver is the most common site, followed by the lungs. The frequency of *E. granulosus* cysts recorded in the Australian Hydatid Registry was: liver 63 percent, lungs 25 percent, muscles 5 percent, bones 3 percent, kidney 2 percent, spleen 1 percent, brain 1 percent, and exceptionally other organs. Similar figures were recorded in Switzerland. In the Xinjiang region of China, of a series of 15 289 surgical cases, 70 percent were in the liver, 19 percent in the lungs, 3 percent liver and lungs, 3 percent in the abdominal cavity, 0.4 percent spleen, 0.4 percent brain, 0.3 percent kidney, 0.3 percent pelvic cavity, and 0.3 percent bone (National Hydatid Disease Center of China 1993). Cysts may be very big (one reported in 1928 contained 48 liters of fluid), but at the time of diagnosis most cysts measure 1–10 cm. The average size of 304 hepatic hydatid cysts in 212 patients treated in China was 10.1 cm with a range of 4–22 cm, while in 65 hepatic cases detected by ultrasound based mass screening (therefore largely asymptomatic), the average cyst size was 7.2 cm, with a range of 2–13 cm (Wang et al. 2003). In contrast to cysticercosis, where parasites do not change in size, except when racemose cysts are formed, *E. granulosus* hydatid cysts can continue to grow. A study performed in 265 Turkana patients showed that average growth was 9 cm per year, 16 percent of cysts did not expand, 30 percent grew slowly (1–5 mm per year), 42 percent showed a moderate growth rate (6–15 mm per year), and 11 percent increased rapidly in size (31 mm on average per year), with a maximum growth in one case of 160 mm per year (Romig et al. 1986).

The primary location of alveolar echinococcosis is the liver (approximately 98 percent), but the capacity for tumor-like proliferation and the potential for metastasis means that development can also occur in adjascent tissues and structures, and in lung, brain, bone, or other organs (Eckert et al. 1983; Ammann and Eckert 1995). Distant metastases were found in 13 percent of 70 Swiss patients and in 10 percent of 152 Japanese patients (Mesarina-Wicki 1991; Sato et al. 1993). Polycystic echinococcosis has characteristics intermediate between the cystic and alveolar forms (D'Alessandro et al. 1979; Meneghelli et al. 1992). The relatively large cysts are filled with liquid and contain brood capsules with numerous protoscolices. The primary location is the liver, but cysts may spread to contiguous sites.

THE DISEASES

Clinical manifestations and pathology

Neurocysticercosis is characterized by its great diversity of signs and symptoms. It is a complex disease with major manifestations depending on the number, location, and type of parasites lodged in the central nervous system (CNS) and meninges, as well as by the extent of the inflammatory response (Earnest et al. 1987; Takayanagui and Jardim 1983; del Brutto and Sotelo 1988, Sotelo and Del Brutto 2000). Seizures is the most

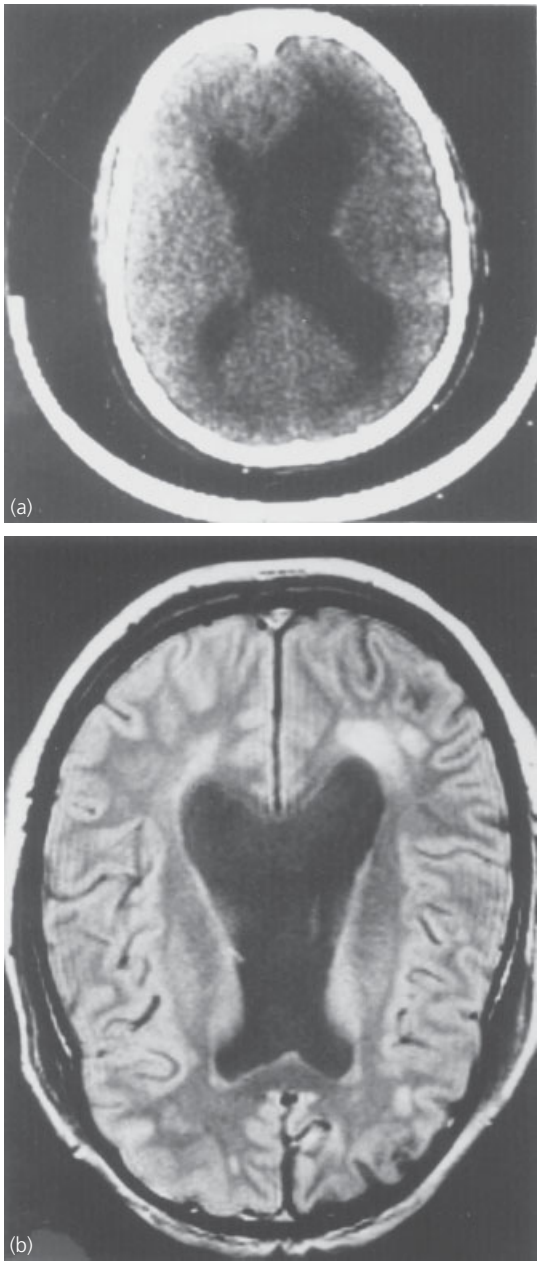

Figure 8 *Hydrocephalus due to CSF blockage caused by a racemose type cysticercus seen in CT* **(a)**, *MR* **(b)**, *and during surgery* **(c)** *(by courtesy of Dr I. Madrazo)*

frequent clinical manifestation, occurring on average in 70 percent of cases (Flisser 1994; Garcia et al. 1995; Palacio et al. 1998) and late onset epilepsy shows a strong association with cysticercosis (Vazquez and Sotelo 1992; Garcia et al. 1993; Sanchez et al. 1999). Patients with seizures caused by parenchymal calcified or cellulose single cysticerci have a mild disease as compared to those that have hydrocephalus as a consequence of meningeal arachnoiditis, which is frequently fatal (Estanol et al. 1986; Sotelo et al., 1985a; Figure 8). Perilesional edema has been observed in patients with only calcified cysticerci and is associated with seizures and neurological morbidity, being more frequent in enhancing calcified lesions (Nash et al. 2001). The glial fibrillary acidic protein was shown to be elevated in the cerebrospinal fluid (CSF) of patients with neurocysticercosis, thus it can be used as a marker of glial damage (Quintanar et al. 2003).

Racemose cysticerci elicit a more intense inflammatory reaction and frequently produce severe mass effects. Progressive inflammation is elicited when the parasite is in contact with the meninges and causes scar tissue proliferation that gives rise to mechanical obstruction of CSF circulation and intracranial hypertension due to hydrocephalus. Acute stages of the disease produce intracranial hypertension due to intense edema induced by numerous developing cysticerci (most commonly found in young patients), ependymitis or arachnoiditis (Rabiela et al. 1982; Escobar 1983; Rabiela et al. 1989; Puri et al. 1991; Madrazo and Flisser 1992; Sotelo and Del Brutto 2000). Idiopathic epilepsy, multiple intracranial space-occupying lesions, chronic meningitis, some bacterial and parasitic infections (including cystic echinococcosis), as well as primary and secondary malignancies affecting the central nervous system can mimic neurocysticercosis (Rodriguez-Carbajal and Boleaga-Duran 1982; Almeida-Pinto et al. 1988; Puri et al. 1991). A similar picture of clinical heterogeneity is also found in ocular cysticercosis (Puig-Solanes 1974; Gomez-Leal 1989). Cysticerci in the

subcutaneous tissue and muscles are usually asymptomatic or well tolerated (Botero et al. 1993; Cruz et al. 1994), probably because they are found in small numbers. Muscular pseudo-hypertrophy due to cysticercosis has been reported in cases that have many parasites and marked muscle enlargement and pain (Zhipiao et al. 1980; Rim and Joo 1989). In 2 188 patients with neurocysticercosis from several Latin American countries, 68 percent were between 20 and 49 years of age, only 19 percent were detected below 20 years, and 13 percent were above 50 years. In contrast, in 30 Mexican cases of ocular cysticercosis, 38 percent were under 20 years, 58 percent were between 20 and 49 years and 4 percent were above 50 years (Cardenas et al. 1989), probably indicating that cysticerci in the eyes are easily seen and thus diagnosed at a younger age, and that neurological symptoms start several years after infection.

Symptoms due to cystic echinococcosis (CE) is correlated with the site of infection and parasite burden or biomass. Because of the slowly growing nature of echinococcal cysts, most cases of liver and lung cysts are diagnosed in adult patients and symptoms are related to space occupying or tumor-like lesions. Only 10–20 percent of CE cases are diagnosed at hospital in patients less than 16 years, primarily where cysts are located in the brain or eye, the latter being similar to cysticercosis. Clinical manifestations are variable and determined by the site, size, and condition of the cysts, for example, 67 percent of 297 CE patients with cysts >5 cm diameter had symptoms, as compared to 48 percent of 123 cases with cysts <5 cm; overall, 38 percent were asymptomatic. Mass screening programs using portable ultrasound scanners have shed light on the natural history of pathology of hepatic cystic echinococcosis in endemic communities (Craig et al. 1996). Studies in North Africa and South America found that simple univesicular or hyaline hydatid cysts, called type 1, occurred in 17–28 percent of asymptomatic persons, and were considered to be an early or evolutive developmental stage. Cysts with a clear laminated membrane and/or presence of daughter cysts (types 2 and 3) comprised 11–55 percent of community detected cases, while hydatid cysts showing signs of involution, such as infiltration and calcification (types 4 and 5) made up 18–45 percent of cases (Cohen et al. 1998; Shambesh et al. 1999). These and other similar studies indicate that many cases of cystic echinococcosis are asymptomatic and that spontaneous regression and healing occurs in a significant proportion of infected people in endemic communities. These features are also similar to cysticercosis. The signs and symptoms of hepatic echinococcosis may include hepatic enlargement, with or without a palpable mass, liver abscesses, calcified lesions, epigastric pain, nausea and vomiting, portal hypertension, inferior vena cava compression or thrombosis, secondary biliary cirrhosis, biliary colic-like symptoms, biliary peritonitis or fistula formation. If a cyst ruptures, the sudden release of its contents may precipitate allergic reactions ranging from mild to fatal anaphylaxis, bacterial infection may occur and there is spread of protoscolices, which may result in a multiple secondary echinococcosis disease (Kammerer and Schantz 1993; Ammann and Eckert 1995). Clinical symptoms of pulmonary cystic echinococcosis are tumor-like chest pain, chronic cough, fever, hemoptysis pneumothorax, pleuritis, lung abscess, eosinophilic pneumonitis, lung embolism, and sometimes expectoration of cyst content (Schantz and Okelo 1990). Nearly 40 percent of patients with pulmonary hydatidosis have liver involvement as well (Little 1976). Cysts in other locations such as the heart, spine, brain, and even bone also generate tumor-like symptomatology (Ammann and Eckert 1995). In that respect bone infection may also resemble an alveolar echinococcosis lesion (Bonifacino et al. 1997). Brain hydatid cysts may be confused with neurocysticercosis because of similar images in tomography. In these cases, the epidemiological history is the best way of confirming diagnosis, together with specific immunological tests (Flisser 1988).

Infection of the liver with *E. multilocularis*, the primary location in humans, closely mimics hepatic carcinoma or cirrhosis. Clinical cases are characterized by a chronic course of the disease lasting for weeks, months, or years. Advanced lesions often consist of a central necrotic cavity surrounded by a white amorphous material that is covered with a thin peripheral layer of dense fibrous tissue (Wilson and Rausch 1980; Rausch et al. 1987). Focal areas of calcification exist, as does extensive infiltration by proliferating vesicles. The initial symptoms of alveolar echinococcosis (AE) are usually vague. Mild upper quadrant and epigastric pain with hepatomegaly may progress to obstructive jaundice. Occasionally, the initial manifestations are related to metastases to the lungs or brain. Symptoms at diagnosis in 40 Turkish AE patients included cholestatic jaundice (45 percent), dyspepsia (40 percent), and epigastric pain (35 percent), the disease was detected incidentally during a check-up in 38 percent of the cases (Polat et al. 2002). The mortality in progressive, clinically manifest AE cases may be 50 to 75 percent (Wilson and Rausch 1980; Kammerer and Schantz 1993) and much higher figures (up to 100 percent) have been recorded in untreated or inadequately treated patients. Radiographic or serologic screening of high-risk patients results in detection of early-stage disease, improving prognosis through earlier application of therapy (Kammerer and Schantz 1993; WHO 1996). Spontaneous death of alveolar hydatid cysts was clearly documented in five asymptomatic individuals in Alaska; lesions were circumscribed and calcified with a mineralized wall and a cavity filled with amorphous necrotic material, in some cases also with folded parasite membranes, suggesting that death at early stages is probably not uncommon

(Rausch et al. 1987). Community mass screening studies in France and China also identified seropositive individuals with calcified lesions >2 cm which were considered to be abortive infections (Bresson-Hadni et al. 1994; Bartholomot et al. 2002). Alveolar echinococcosis is typically seen clinically in persons of advanced age, the average age being >50 years as compared to <40 in cystic echinococcosis.

The clinical characteristics of polycystic echinococcosis, like that for cystic echinococcosis, depend on the location and larval biomass, but, as for alveolar echinococcosis, also on the degree of tissue infiltration by exogenous proliferation. It is less organ specific than *E. multilocularis* and proliferates more rapidly. Up to 78 percent of cases due to *E. vogeli* infection involved the liver alone or in association with the lungs and abdominal organs, while pulmonary, mesentery, or stomach locations have also been described (D'Alessandro 1997). Three cases of human infection with *E. oligarthrus* involved the orbit or the heart. Mean age of 51 cases of polycystic echinococcosis was 44 years (range 6–78 years) of which 40 percent survived at least 1.5 years after initial diagnosis.

IMMUNE RESPONSE

Immune responses in patients with neurocysticercosis have been studied mainly because of the need to standardize immunological methods for diagnosis (Flisser et al. 1980; Flisser and Larralde 1986; Richards and Schantz 1991; Dorny et al. 2003). Anti-cysticercus antibodies of several immunoglobulin isotypes have been detected, although the most frequently found are IgG antibodies in serum, cerebrospinal fluid, and saliva. Interestingly, IgG anti-cysticercus antibodies are not found in all compartments of the same patient (Cho et al. 1986; Espinoza et al. 1986; Feldman et al. 1990; Wilson et al. 1991). The presence of IgG confirms that the disease is usually chronic and long-term. A few reports indicate an increase in total or specific IgE (Goldberg et al. 1981; Gorodezky et al. 1987; Short et al. 1991), IgM is less frequently found in CSF than IgG, and IgA antibodies have been reported in few cases, although not in saliva. Antibodies have been detected in 94 percent of cases with two or more parasites, but only 28 percent of cases with single lesions, and in most cases with undamaged parasites, but in only 44 percent of cases with calficied cysticerci (Espinoza et al. 1986; Baily et al. 1988; Chang et al. 1988; Michault et al. 1990; Michel et al. 1990; Wilson et al. 1991). The humoral immune response in patients with neurocysticercosis is quite heterogeneous as evidenced by the number of antigens recognized: patients' antibodies may react with one to eight antigens in immunoelectrophoresis and up to 30 antigens in Western blot (Flisser and Larralde 1986; Correa et al. 1985, 1989a; Larralde et al. 1989; Proaño-Narvaez et al. 2002). Differences have also been found between benign and malignant cysticercosis, the later state being more antigenic. It has also been shown that the immune response may be transient in households of neurocysticercosis patients and in apparently healthy individuals in the open population (Garcia et al. 2001; Meza-Lucas et al. 2003).

As in cysticercosis, the humoral immune response in patients with hydatid disease has been used widely to diagnose infection or disease and the main emphasis has been characterization of parasite antigens (Lightowlers et al. 1993; Lightowlers and Gottstein 1995). Experimental studies indicate that antibodies to antigen 5 are among the first detected following infection with *E. granulosus* (Conder et al. 1980), but are also found in patients with neurocysticercosis or alveolar echinococcosis (Varela-Díaz et al. 1978; Schantz and Gottstein 1986; Moro et al. 1992). ELISA and Western blot are usually performed with anti-human IgG as this is the main isotype in human hydatid disease. Antibodies of the subclass IgG4 are particularly evident in the serum of advanced CE and AE patients (Wen and Craig 1994; Shambesh et al., 1997; Ortona et al. 2002). The demonstration of parasite-specific IgE in cystic echinococcosis has attracted particular attention but, as in human neurocysticercosis, appears to have no significant diagnostic advantage. However, high levels of IgE antibody may correlate with allergic reactions to *E. granulosus* antigens with homology to cyclophilin, and low levels with beneficial post-treatment chemotherapy (Ortona et al. 2002; Rigano et al. 2002). In contrast, 68 percent of patients with alveolar echinococcosis tested positive for parasite-specific IgE by ELISA and between 50 and 88 percent by the radioallergosorbent test (Vuitton et al. 1988; Gottstein 1992; Liu et al. 1992; Moro et al. 1992; Lightowlers and Gottstein 1995). Strain variation in the parasite, geographical origin of the patient, and differences in the host–parasite relationship may significantly affect the humoral responses (Rafiei and Craig 2002).

The main antigen recognized by patients in both diseases, though different, has unfortunately been designated the same, i.e. antigen B. However, *T. solium* antigen B is a different molecule from *E. granulosus* antigen B. The first is a glycoprotein of 110 kDa (Guerra et al. 1982), while that of *E. granulosus* is a lipoprotein of 120–160 kDa (Oriol and Oriol 1975). Both are immunodominant; 85 percent of patients with neurocysticercosis react with *T. solium* antigen B (Flisser et al. 1980; Espinoza et al. 1986) and 80–90 percent of patients with advanced cystic hydatid disease recognize *E. granulosus* antigen B (Williams et al. 1971; Wen and Craig 1994). Recognition of antigen B in asymptomatic cystic hydatid patients is, however, variable with greater responses in patients with evolutive disease (Shambesh et al. 1999; Daeki et al. 2000; Rigano et al. 2002). Immunological and biochemical assays have demonstrated that for echinococcus immunodominant antigen B is

highly genus specific. *T. solium* antigen B, or as recently termed, paramyosin, (Laclette et al. 1991) is non-specific; it is found in many platyhelminthes, and sera from patients with hydatid disease cross-react with this antigen (Olivo et al. 1988). *E. granulosus* antigen B, and antigen 5, another large lipoprotein, have been extensively characterized (reviewed in Lightowlers and Gottstein 1995), and, as these authors suggest, 'it would be valuable for these antigens to be compared between the different laboratories involved, in order to clarify whether the molecules are indeed one and the same'. It is known that antigen B of *E. granulosus* consists of at least three components with molecular weights of 8, 16, and 24 kDa, suggesting that this antigen is a polymer of an 8 kDa subunit (Lightowlers et al. 1989). The 8 kDa subunit is considered to be the most specific for *E. granulosus* and has been cloned (Shepherd et al. 1991; Frosch et al. 1994a) and more recently shown in fact to comprise two 8 kDa subunits (AgB8/1 and AgB8/2) with approximately 50 percent amino acid homology between them (Gonzalez et al. 1996). Both native and recombinant antigen B of *E. granulosus* preferentially bind IgG4 subclass antibodies which is most pronounced in the active or evolutive phase of cystic hydatidosis (Wen and Craig 1994; Rigano et al. 2002).

For the diagnosis of cysticercosis, antigenic extracts are usually prepared from *T. solium* cysticerci from infected pigs (Flisser and Larralde 1986). Alternatively, antigens from other sources such as *T. crassiceps*, *T. hydatigena*, and *T. saginata*, or those obtained by genetic engineering (McManus et al. 1989) have also been used. Cyst fluid, protoscolices, and cyst membranes of *E. granulosus* from a variety of hosts are the sources of antigen for diagnostic tests of hydatid disease. Fluid from fertile cysts contains higher concentrations of antigens than from sterile cysts, although viable non-fertile cysts also contain suitable antigens, while fluid from sheep and human liver hydatid cysts also contain higher concentration of the two major antigens (antigen B and antigen 5) than that of cattle and pig cysts. In general, hydatid cyst fluid has been a better source of *E. granulosus* antigens than protoscolices or cyst membranes; however, both these also contain dominant antigens (Rogan and Craig 1997; Rafiei and Craig 2002). *T. hydatigena* and *T. ovis* have also been evaluated as source of antigens (Yong et al. 1984), enabling successful antigen preparation from heterologous taeniid parasite species. Oncospheres have also been used as a source of antigens: antibodies to high molecular weight antigens were detected in pig sera after 1 month of infection (Garcia-Allan et al. 1996), in 20 percent of serum samples from patients with active neurocysticercosis, and in 95 percent of tapeworm carriers (Verastegui et al. 2003).

Because crude antigen extracts generate cross-reactions, the use of purified or semi-purified antigens will increase specificity. Characterization of *T. solium* and *E. granulosus* antigens has changed from gel diffusion and immunoelectrophoresis to Western blot, lentil-lectin chromatography, immunoprecipitation, monoclonal antibodies, chromatofocusing, and recombinant DNA technology (reviewed in Flisser (1994) for cysticercosis and in Lightowlers and Gottstein (1995) for hydatid disease). In cysticercosis the use of a crude electrophoretic extract gave no clear specific band patterns in Western blots (Espinoza and Flisser 1986; Larralde et al. 1989). However, a similar assay using a semi-purified antigenic fraction identified seven specific glycoproteins (Gp50, Gp42-39, GP24, GP21, GP18, GP14, and GP13), the three with highest molecular weights reacting most frequently with sera from clinical cases (Tsang et al. 1989; Feldman et al. 1990; Wilson et al. 1991) and population samples (Schantz et al. 1994; Thais et al. 1994). In contrast, low molecular weight antigens have also been identified as immunodominant in human cysticercosis (Chung et al. 1999; Plancarte et al., 1999b; Restrepo et al. 2000, 2001; Sako et al. 2000; Dorny et al. 2003). Further progress has been made by recombinant DNA techniques, using *E. granulosus* clones that react with sera from patients with cystic hydatid disease (see for example, Ferreira and Zaha 1994), and clones from *E. multilocularis* immunoreactive with sera from patients with alveolar hydatid disease (see for example, Hemmings and McManus (1989) and Muller et al. (1989), and review by Siles-Lucas and Gottstein (2001)).

Data on cellular immune responses are limited (Flisser et al. 2003), but, interestingly, show similarities in both larval cestode diseases: an increase in $CD8^+$ lymphocytes (Allan et al. 1981; Flisser et al. 1986; Vuitton et al. 1989; Molinari et al. 1990; Craig 1994), polyclonal activation of B lymphocytes (Craig 1994), and a decrease in γ-interferon (IFN) (Suntsov et al. 1990a, b; Ostrosky-Zeichner et al. 1996), although γ-IFN was increased in a group of patients who had not received previous treatment of any kind (Medina-Escutia et al. 2001), and tumor necrosis factor α was increased in children with active neurocysticercosis (Aguilar-Rebolledo et al. 2001). Parasite molecules with immunosuppressive effects have also been reported (Molinari et al. 1990). Nevertheless, a systematic and focused analysis of the relationship between cytokine effects and the outcome of larval cestode diseases is still needed. The role of Th1/Th2 balance upon resolution of the disease is still not clear. Increased levels of IL-1 and IL-6 in CSF of patients with inflammatory neurocysticercosis were found (Ostrosky-Zeichner et al. 1996). Serum levels of IL-5 and eotaxin were also increased in patients as compared to controls in one study (Evans et al. 1998), but were not detected in another (Aguilar-Rebolledo et al. 2001). IL-2 was synthesized by peripheral blood cells in half of patients with neurocysticercosis (Medina-Escutia et al. 2001) and a Th1/Th2 mixed pattern of interleukins were found at the host–parasite interface (Restrepo et al. 1998) and in the CSF (Rolfs et al. 1995).

The mechanisms by which established larval cestodes survive in hosts resistant to re-infection remain unknown. Evidence exists for a variety of immune-evasion mechanisms. Several have been described in cysticercosis (Flisser 1989; White et al. 1997; Flisser et al. 2003): survival of parasites lodged in 'immunologically privileged sites', masking of cysticerci by host immunoglobulins, suppression of host responses by taeniaestatin, paramyosin, sulfated polysaccharides, enzymes that detoxify reactive oxygen intermediates, low molecular RNA, cysteine proteinases, and PGE2. In hydatid disease, studies in experimental models have demonstrated the influence of the parasite on the specific and non-specific immune responses of the host, such as pathological alterations in the architecture of lymphoid organs, inhibition of cellular immune responses to specific antigens and of leukocyte chemotaxis, reduction in macrophage activity, and reduced blastoid transformation and autoantibody production (Dixon and Jenkins 1995; Rogan and Craig 1997; Vuitton 2003). The impact of acquired immune deficiency syndrome (AIDS) on the susceptibility of endemic populations for cysticercosis and hydatid disease could result in a significant terminal disease (Heath 1995). As an example, huge brain cysticerci have been found in two HIV patients (Soto-Hernandez et al. 1996). Few studies have been undertaken to determine factors that regulate innate susceptibility to larval cestode infections. Host age, sex, strain, and physiological state have marked influences in determining innate resistance to infection (reviewed by Flisser 1994). Intermediate hosts develop specific humoral and cellular responses to the parasites that confer a significant level of resistance to reinfection. The contribution of these immune responses to the destruction of the parasite after initial establishment is less clear. A proportion of cysticerci and of hydatid cysts die some time after initial establishment, so that calcified lesions can be observed together with viable and with dead parasites. Although it is not known whether immunity is responsible for the death of these parasites, there is evidence, for example, that the inflammatory response is associated with restricting the growth and metastasis of the cyst mass in hosts refractory to infection with *E. multilocularis* (Ali-Khan and Siboo 1980) and that in experimental treatment of pigs with cysticercosis, cestocidal drugs damage cysticerci and eosinophils, lymphocytes, and macrophages destroy them (see Figure 9) (Flisser et al. 1990b; Torres et al. 1992). Nevertheless, despite the development of specific immune responses, viable cysts frequently persist for long periods (Rogan and Craig 1997). Production of a T-helper cell type 2 (Th2) response in human cystic echinococcosis, was characterized by low γ-IFN, and high IL4 and IL10 when patients had a poor prognosis after albendazole chemotherapy. In contrast, a Th1 type response characterized by production of γ-IFN and low IL4 and IL10 levels was correlated with beneficial therapeutic response to chemotherapy (Rigano et al. 1995). High counts of $CD4^+$ T cells in hepatic granuloma was correlated in persons with abortive AE lesions, while $CD8^+$ T cells were invariably present in chronic active AE disease (Vuitton 2003). Evidence for a genetic susceptibility to human AE has also been demonstrated to be associated with the HLA DR3/4 gene with a possible phenotypic outcome of increased IL10 production (Godot et al. 2000).

Several aspects of the immunobiology of larval cestode infections in the intermediate host have

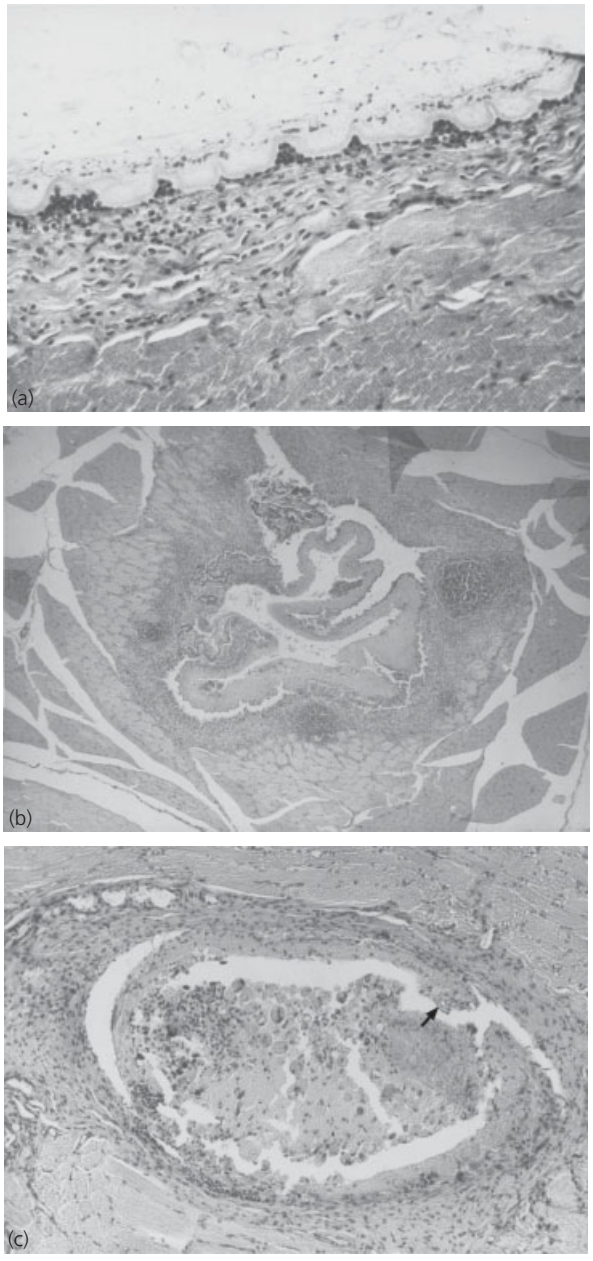

Figure 9 *Effect of treatment of pigs with praziquantel on cysticerci: eosinophils* **(a)** *and lymphocytes* **(b)** *surround the parasite and macrophages phagocytose cell debris* **(c)**. *The arrow in* **(c)** *indicates a larval hook (by courtesy of Dr A.S. de Aluja)*

provided the framework for research on vaccination. Many studies have been performed in order to evaluate protection against cysticercosis and against hydatid disease (Mitchell 1990; Rickard et al. 1995; Lightowlers 1996; Lightowlers et al. 2000). The immune response against larval cestodes may be divided into two phases, the first directed against recently hatched oncospheres attempting to penetrate the gut mucosa and establish themselves in host tissues, and the second aimed at the established metacestode. Immune effector mechanisms of the first phase are more successful in destroying the parasites than those of the second phase because established metacestodes have evolved highly effective physical and biochemical mechanisms to evade the host's defenses, some mentioned above. Early oncospheres are highly vulnerable to antibody-dependent complement mediated immune attack, but only briefly, and thus there is a race between parasite development and the generation of the host immune response in the critical first few days of infection (Ito 1997). Antigens from various sources have been used as vaccines (Flisser 1994) and immunological studies are being undertaken (Toledo et al. 1999; Flisser and Lightowlers 2001; Verastegui et al. 2002). Recombinant oncosphere antigen-based vaccines were first used successfully against ovine cysticercosis caused by *Taenia ovis* (Johnson et al. 1989) and most recently against *T. solium* in pigs and *E. granulosus* in sheep (Manoutcharian et al. 1996; Lightowlers et al. 1996; Plancarte et al. 1999a). A successful field trial with a synthetic peptide vaccine in pigs raised and kept in their original rural environment was reported (Huerta et al. 2001). Furthermore, experimental vaccination of rustically raised pigs was more effective if performed when pigs were 70 days of age as compared to 40 days (Huerta et al. 2000), and cestocidal treatment of pigs protected them for at least 3 months against cysticercosis acquired naturally in hyperendemic areas (Gonzalez et al. 2001). The 45W protective antigen from *Taenia ovis* shows high homology to *T. solium* in studies of genes and transcripts of this antigen in both species, making it a vaccine candidate for the latter species (Gauci and Lightowlers 2001). A cDNA library immunization, based on the use of a large number of pathogens' cDNA clones, induced a cellular protective immune response (Manoutcharian et al. 1998, 1999, 2000). A high degree of immune protection in sheep (96–100 percent) against cystic echinococcosis was obtained with an *E. granulosus* oncosphere sub-unit recombinant vaccine (EG95) (Lightowlers et al. 1996), which was antibody mediated and directed at conformational epitopes of a 16.9 kDa protein expressing a fibronectin type domain (Chow et al. 2001; Woolard et al. 2001). The EG95 homolog was also identified in an *E. multilocularis* cDNA library and purified as an experimental vaccine which gave 78–83 percent protection in mice against an *E. multilocularis* egg challenge (Gauci et al. 2002). An unrelated metacestode protein of *E. multilocularis* called 14-3-3, has also been cloned and provided >95 percent protection of mice against an egg challenge, but not secondary infection and therefore must have shared epitopes with the oncosphere (Siles-Lucas et al. 2003).

DIAGNOSIS

In the past, the pleomorphic symptomatology of neurocysticercosis has required the use of a variety of diagnostic procedures. Most of these have now been replaced by imaging techniques, such as computed tomography (CT) and magnetic resonance (MR) (Suss et al. 1986; Almeida-Pinto et al. 1988; Jena et al. 1988; Rodacki et al. 1989; Teitelbaum et al. 1989, Flisser et al. 1988; Rajshekhar 1991; Rajshekhar and Chandy 1997; Sotelo and Del Brutto 2000; Garcia and Del Brutto 2003). As alternative or complementary diagnostic procedures, immunological assays are used to detect anti-cysticercus antibodies in CSF or serum. Enzyme-linked immunosorbent assays (ELISA) and Western blot (WB) are currently used for clinical support of symptomatic patients in several countries (Espinoza et al. 1986; Gottstein et al. 1986; Pammenter et al. 1987; Michault et al. 1989; Tsang et al. 1989; Zini et al. 1990; Wilson et al. 1991; Sanchez et al. 1999; Dorny et al. 2003). Their high positivity facilitates diagnosis of neurocysticercosis, especially when CT or MR is not available or not conclusive. Diagnostic critera for neurocysticercosis have been proposed recently by a panel of experts that are based on objective clinical, imaging, immunologic, and epidemiologic data. These include four categories of criteria stratified on the basis of their diagnostic strength: absolute, major, minor, and epidemiologic. Interpretation of these criteria permits two degrees of diagnostic certainty: definitive and probable diagnosis (Del Brutto et al. 2001).

CT and MR are used to identify cysticerci in the brain and thus confirm the etiology of the disease and define the number, stage, location, and extent of lesions. Because of their vesicular structure, living cysticerci are seen as hypodense or low signal intensity images, the scolex giving a hyperdense or high signal intensity, usually eccentric, inside the vesicle (see Figure 10).

Isolated cysticerci detected in brain parenchyma by imaging techniques are frequently seronegative, whereas multiple or subarachnoidal cysticerci are usually seropositive (Wilson et al. 1991). Cysticerci in ventricles can also be detected, especially by MR. Imaging techniques also show vasculitis, inflammation, and edema surrounding the parasite, ring-like enhancement being associated with an acute infection (cysticercotic encephalitis) and indicating an active inflammatory process. Hydrocephalus is a common finding associated with intraventricular and/or basal cysticerci, and imaging techniques are very helpful non-invasive techniques for following the outcome, especially after a shunt has been introduced. In long-term disease, parasites of different

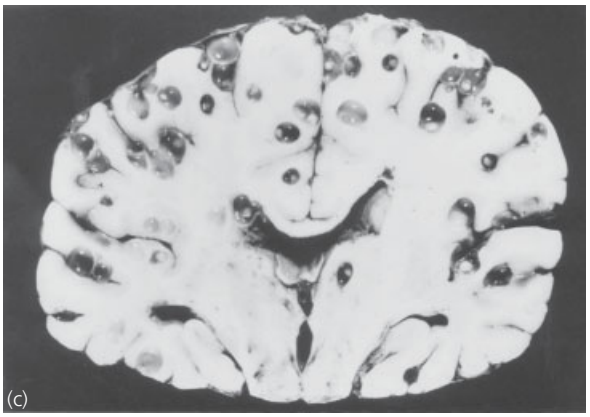

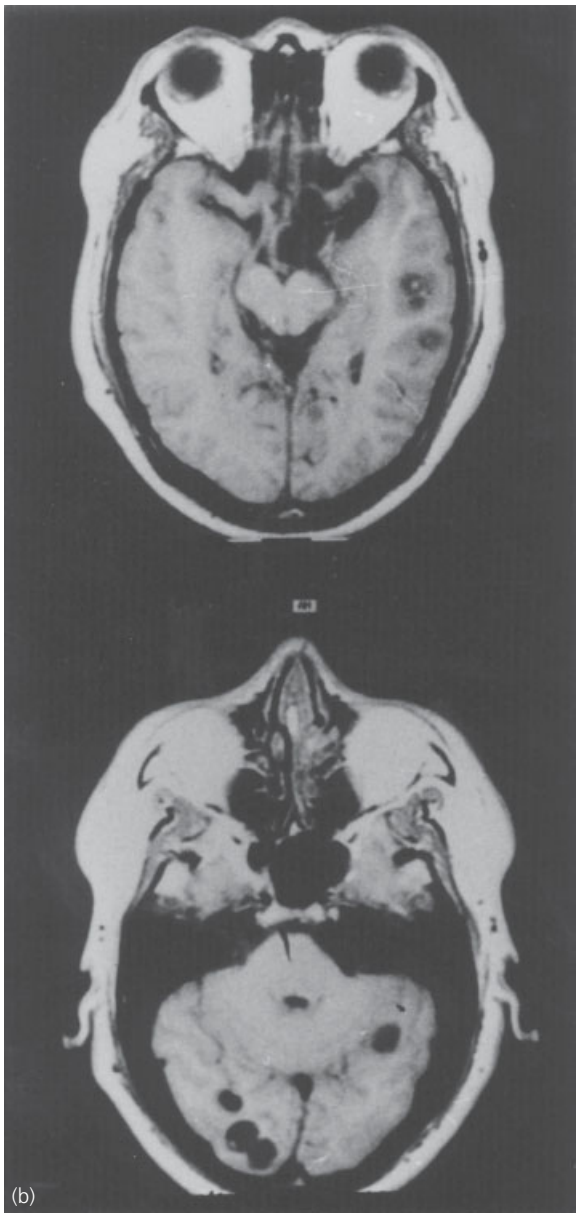

Figure 10 *Multiple small cysticerci seen by* **(a)** *CT,* **(b)** *MR and* **(c)** *in anatomical section of the brain. The scolex can be seen in some parasites (by courtesy of Drs I. Madrazo and J. Olvera)*

sizes can be seen with diverse tomographic pictures of brain response (Flisser et al. 1988). Calcified cysticerci are seen in CT as hyperdense dots, but are usually not detected by MR because of the lack of signal from calcifications. In countries where cysticercosis is endemic, clinicians usually consider this etiology in the initial diagnosis of cases that have mass occupying lesions, enhancing nodules, hydrocephalus, or other images that might be associated with neurocysticercosis. Solitary cerebral granulomas causing seizures can be due to cysticerci or to tuberculomas, and are not only common in developing countries but also in developed ones. Diagnostic criteria have been defined for accurately and specifically identifying solitary cerebral cysticercus granulomas (Rajshekhar and Chandy 1997). More consideration should be given to it in developed countries, where cases of neurocysticercosis are currently being diagnosed or misdiagnosed (Michael et al. 1990). Cysticerci in eyes are easily diagnosed when parasites are viable, but can easily be misdiagnosed when there are inflammatory reactions or involution of the parasite (Gomez-Leal 1989). Subcutaneous and muscle cysticerci may be detected by palpation and diagnosed by biopsy.

Immunodiagnosis has the great advantage of lower cost than CT or MR, and the presence of specific anti-cysticercus antibodies may confirm the disease. ELISA has clearly improved immunodiagnosis and is being used routinely for confirmation of clinical diagnosis when imaging techniques are not available or are not conclusive. It has a high sensitivity, between 75 and 90 percent with serum, and, seemingly, lumbar CSF gives better results. The main disadvantage of ELISA is cross-reactivity with other helminth infections since the antigen employed is a crude homogenate of cysticerci or vesicular fluid. Western blot overcomes cross-reactivity because it employs an enriched fraction of glycoproteins (Gp). In addition the technique separates the antigens present in the Gp fraction during electrophoresis prior to the antigen–antibody reaction. The presence of between one and seven specific Gp bands is considered

diagnostic of *T. solium* infections. When two or more cysticerci are present, the blot is 100 percent specific and has 100 percent sensitivity with serum samples and 95 percent with CSF. Antigens can be detected in CSF by ELISA using monoclonal antibodies (Correa et al. 1989b; Choromanski et al. 1990; Chen et al. 1991; Garcia et al. 1998a, 2000, 2002c). Although the sensitivity of antigen detection is lower than that of antibody detection, positive cases confirm the presence of the parasite without the need for other diagnostic procedures.

Improvement of immunodiagnosis of human neurocysticercosis, specifically regarding the characterization and purification of antigens (Garcia-Allan et al. 1996; Rodriguez-Canul et al. 1999; Vaz et al. 1997; Ito et al. 1998; Ko and Ng 1998; Yang et al. 1998; Greene et al. 1999; Plancarte et al. 1999a, b; Casagranda et al. 2000; Hernandez et al. 2000; Chung et al. 1999; Restrepo et al. 2000, 2001; Sako et al. 2000; Gazarian et al. 2001; Quintanar et al. 2003; Dorny et al. 2003), the follow-up of patients (Rajshekhar 1991; García et al. 1997a, 2000) and the diagnosis of swine cysticercosis (Pinto et al. 2000a, b; Rodriguez-Canul et al. 1998; Sciutto et al. 1998a, b) has obvious priority in cysticercosis, others relate to taeniasis regarding the distinction of *T. solium* from *T. saginata* by DNA analysis (Chapman et al. 1995; Gasser et al. 1995; Mayta et al. 2000; Gonzalez et al. 2002; Yamasaki et al. 2002) and the identification of serum antibodies (Wilkins et al. 1999).

The presence of a cyst-like mass in a person with a history of exposure to dogs in areas in which *E. granulosus* is endemic supports the diagnosis of cystic echinococcosis. However, hydatid cysts must be differentiated from benign cysts, cavitary tuberculosis, mycoses, abscesses (microbial or parasitic), racemose cysticercosis, and benign or malignant neoplasms. A non-invasive confirmation of the diagnosis can usually be accomplished by the use of ultrasonography (US), CT, MR, or radiography (Ammann and Eckert 1995). The liver is the most frequently involved organ and 60–85 percent of the cysts are located in the right lobe. Ultrasonography, CT, and MR are useful in diagnosing deep-seated lesions in all organs and defining the extent and condition of avascular fluid-filled cysts (Figure 11) (von Sinner 1991; Choji et al. 1992; Ammann and Eckert 1995). Images of *E. granulosus* hydatid disease typically show round, solitary or multiple, sharply contoured cysts, measuring from 1 to over 15 cm, these represent about 38–48 percent of all cysts. In 29–46 percent of all cysts the presence of internal daughter cysts produces structures comparable to a cartwheel. Thin, crescent or ring-shaped calcifications of variable degree located in the cyst wall occur in about 10 percent of the cysts, giving an eggshell pattern. These require about 5–10 years to develop (Figure 11a, b). Since Gharbi used ultrasound images to classify *E. granulosus* cyst presentation into five basic types (types 1–5), several similar hydatid cyst pathology profiles have been described (Gharbi et al. 1981; Perdomo et al. 1997; Larrieu et al. 2000). Recently, a revised US classification for human cystic echinococcosis (CE) was published by WHO in order to try and simplify and combine elements of several similar classifications (WHO/OIE 2001). CT allows measurement of the size of the parasites, which is useful for chemotherapeutic follow-up and gives a correct diagnosis in a high proportion of cases (60–90 percent). Serology for cystic hydatid disease is useful in confirmation of radiographic images, but may be variable dependent on site, type, number, and condition of the cysts.

The usual CT image of *E. multilocularis* infection is that of indistinct solid tumors seen as heterogeneous hypodense masses often associated with central necrotic areas and calcifications, lesion contours are irregular without a well-defined wall (Figure 11c, d). Frequently, the lesion extends beyond the liver, which can cause compression or obstruction of other structures, such as the inferior vena cava, hepatic veins, and portal branches (Ammann and Eckert 1995). Clusters of micro-calcifications or irregular plaque-like calcified foci are often found in central or peripheral parts of the lesions. In a study of 30 AE patients, CT was considered the method of choice for hepatic lesions followed by MR and US; MR was less effective for calcified lesions though superior for extra-hepatic sites (Reuter et al. 2001). Ultrasound, as for CE, has been demonstrated to be effective in mass screening for hepatic AE in endemic communities (Bartholomot et al. 2002). The lungs may be involved either by direct extension of the liver process or by parasite metastases seen as multiple small solid foci located usually eccentrically at the periphery of the lobes. Serologic tests are usually positive at high titers; purified *E. multilocularis* antigens are highly specific, and comparing a patient's titers to both purified-specific and shared antigens allows serologic discrimination between patients infected with *E. multilocularis* and those infected with *E. granulosus* (Ito et al. 2002).

Diagnostic puncture should usually be avoided because of the risk of secondary echinococcosis due to spillage of viable protoscolices or of metastases in the site of injection from germinative tissue adhering to the needle. There is also the risk of anaphylactic reactions from leakage of cyst fluid especially from *E. granulosus* cysts (Ammann and Eckert 1995). The availability of immunodiagnostic tests for confirmation of US, CT, or MR images reduces the need of puncture for diagnosis, although puncture is presently being used successfully as an alternative therapeutic treatment for cystic hydatidosis (PAIR; see section on Therapy), and has been used in a diagnostic biopsy capacity for specific antigen detection (Paul and Stefaniak 1997).

Virtually every serodiagnostic technique devised for any disease has been evaluated for diagnosis of cystic echinococcosis (CE), often with considerable

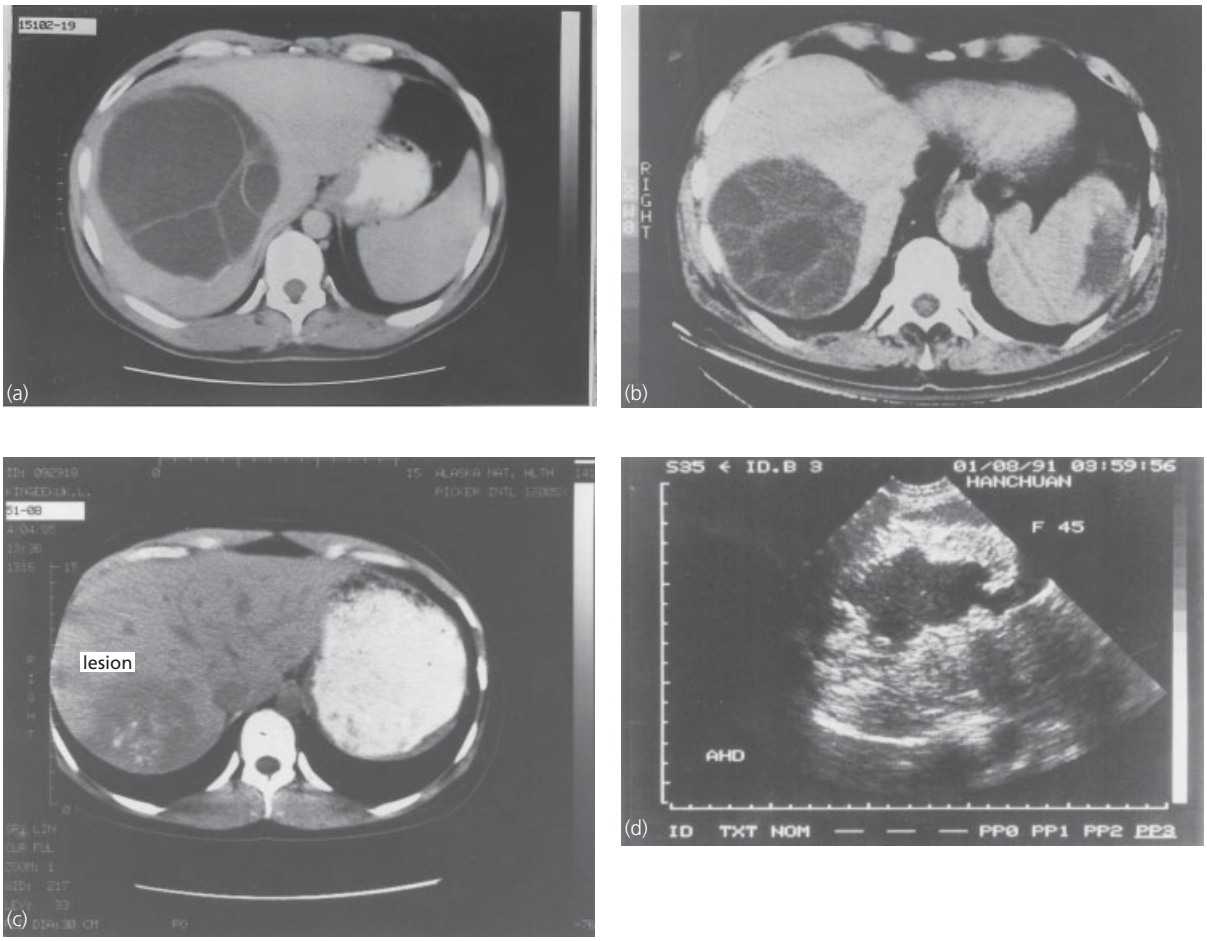

Figure 11 (a) *CT showing a solitary Echinococcus granulosus human liver cyst (by courtesy of Dr P. Schantz);* **(b)** *CT showing an* Echinococcus granulosus *human liver cyst with a cartwheel appearance (by courtesy of Dr P Schantz);* **(c)** *CT showing an Echinococcus multilocularis liver cyst showing plaque-like calicified foci (by courtesy of Dr P. Schantz);* **(d)** *Ultrasonography of a liver from a patient from Gansu, China with alveolar echinococcosis (by courtesy of Professor P. Craig)*

discrepancies between laboratories (Lightowlers and Gottstein 1995). As Schantz and Gottstein (1986) discuss, selection of a particular immunodiagnostic test involves consideration of the sensitivity and specificity of the techniques, the purpose for which they will be used, available technical expertise, and cost. Sensitivity and specificity vary according to the technique used, the quality of antigens and the characteristics of the groups of CE patients and controls used in the study. ELISA, indirect hemagglutination, latex agglutination, and indirect immunofluorescence have a high sensitivity (60–90 percent), although some patients do not develop a detectable immune response. Hepatic CE cysts are more likely to elicit an immune response than pulmonary cysts, but about 10 percent of patients with hepatic cysts and 40 percent with pulmonary cysts do not produce detectable serum antibodies and give false-negative results, which may result in a dangerous puncture of a hydatid cyst (Ammann and Eckert 1995; Paul and Stefaniak 1997). ELISA, which is now used routinely, has a similar sensitivity to the indirect hemagglutination test and thus both are procedures of choice for the initial screening of serum. Specific confirmation of reactivity for CE can be obtained by demonstrating antibodies to antigen 5 (arc 5) by immunodiffusion or immunoelectrophoresis or more specifically to the 8–12 kDa bands (= antigen B) in Western blot (Madisson et al. 1989). Antigen B of *E. granulosus* has been cloned by several groups (Siles-Lucas and Gottstein 2001), and both native and recombinant antigens have the propensity to bind specific IgG4 antibodies in CE patients with advanced disease (Wen and Craig 1994; McVie et al. 1997). Antigen B is an antigen of choice for seroepidemiological studies and in support of ultrasound for CE mass screening surveys (Cohen et al. 1998; Wang et al., 2001). Eosinophilia is present in fewer than 25 percent of infected patients and hypergammaglobulinemia in about 30 percent of the patients with CE (Ammann and Eckert 1995). Sero-conversion is usually higher in human alveolar echinococcosis (AE) compared to human CE. About 95 percent or more of patients suffering from advanced AE exhibit antibodies against

the laminated layer derived glycan Em2 antigen (Siles-Lucas and Gottstein 2001) and also the recombinant EM10 antigen, a 65 kDa molecule which is 99 percent specific in *E. multilocularis* (Helbig et al. 1993; Frosch et al. 1994b). An *E. multilocularis* 18 kDa native antigen (Em18) was identified using immunoblotting to be highly species specific and sensitive for AE, and a recombinant Em18 was also useful in differential immunodiagnosis of alveolar and cystic echinococcosis (Ito et al. 1999, 2002). Serological tests have also proved useful in confirmation of AE cases in screening surveys and for epidemiological studies in endemic areas (Romig et al. 1999; Bartholomot et al. 2002). Techniques useful for diagnosing cystic or alveolar echinococcosis are also of value in diagnosing polycystic echinococcosis due to *E. vogeli* infection, since these parasites have common antigens (Gottstein et al. 1995).

THERAPY

Treatment is based on palliative drugs to control symptoms and inflammation, placement of ventricular shunts in patients with neurocysticercosis that drain CSF to the peritoneal cavity, bile shunts in AE and CE, or surgery to remove brain cysticerci or hydatid cysts/lesions from liver, lungs or other organs; the advent of cestocidal drugs has improved prognosis of many cases (Earnest et al. 1987; Madrazo and Flisser 1992; Kammerer and Schantz, 1993; Ammann and Eckert 1995; Escobedo 2000; Sotelo and Del Brutto 2000; White 2000, Garcia and Del Brutto 2000; Garcia et al. 2002a). Recently a panel of experts published current consensus guidelines for treatment of neurocysticercosis, where a basic set of principles to follow is clearly described. They advise that treatment of neurocysticercosis should not be generalized; instead, each case should be approached and assessed individually. Furthermore, the selection of the treatment option must include in the consideration of risks and benefits, the economic situation of the patient. Also, the current controversy of the benefits of anti-parasitic therapy is thoroughly discussed (Garcia et al. 2002b).

Anti-epileptic drugs are the treatment of choice in patients with neurocysticercosis that have calcifications, in which seizures are the only manifestation of the disease and there is no imaging evidence of living parasites. Ventricular shunting is the most common neurosurgical approach to control hydrocephalus caused by cysticerci in the ventricles, periventricular or basal cisternae, or by ependimitis, inflammatory scarring, arachnoiditis or adhesive basilar meningitis. Patients with hydrocephalus always require a ventricular shunt before other measures are attempted. CSF shunting is a relatively simple surgical technique used to treat intracranial hypertension due to blockage of CSF circulation, but complications may arise from the high protein levels in CSF (which can cause blockage), from bacterial infections or from surgical inexperience (Colli et al. 1986; Madrazo and Flisser 1992). A ventriculoperitoneal shunt with a new design based on the rate of CSF production and shunt resistance, rather than on ventricular pressure, has recently been evaluated with promising results (Sotelo et al. 1995). In a hospital study of 632 cases subjected to ventriculoperitoneal shunting, 77 percent were resolved with one operation, but 11 percent needed three or more shunt placements (Mateos and Zenteno 1987). In another study, 68 percent of 69 cases were re-admitted to the hospital one or more times for shunt revision (Colli et al. 1986). Removal of solitary brain cysticerci is often followed by prompt improvement and excellent recovery (Colli et al. 1994). In a series of 27 cases with cysticerci in the fourth ventricle submitted for direct surgical removal of the parasite, 81 percent had excellent results, but the remaining cases had poor results owing to severe arachnoiditis found at surgery (Loyo et al. 1980) (Figure 12). Recently, endoscopic excision of a cysticercus in the fourth ventricle via a frontal burr hole was performed. The patient had an uneventful recovery and remained asymptomatic and shunt free 1 year after the procedure. Follow-up MRI showed no signs of hydrocephalus and a normal fourth ventricle (Zymberg et al. 2003). The neuroendoscopic management of intra-ventricular cysticercosis should be considered as the primary treatment whenever possible since it is safe, effective, and provides a definitive treatment for this disorder. In addition to avoiding a CSF shunt in many cases, removal of the cyst(s) offers a reduced risk of inflammatory sequelae. Neurosurgeons with familiarity and experience with flexible neuroendoscopes should find these cases straightforward and highly gratifying (Bergsneider and Nieto 2003).

Parasites in the intraspinal subarachnoidal space require surgery because the compression causes spinal cord dysfunction. Neurocysticercosis can involve any cranial nerve; surgery is the most frequent indication for optic nerve involvement and removal of arachnoiditic tissue surrounding nerves will produce different degrees of clinical benefit (Madrazo and Flisser 1992; Escobedo 2000). Treatment of ocular cysticercosis usually involves removal of parasites from vitreous or subretinal locations. In some cases photocoagulation with a laser beam is used and in severe inflammation extrusion of the eye is recommended (Santos et al. 1979; Kruger-Leite et al. 1985; Cardenas et al. 1992). Muscle and subcutaneous cysticerci are efficaciously eliminated by cestocidal treatment (Rim and Joo 1989).

Surgical removal of single hydatid cysts due to *E. granulosus*, which can be performed in about 90 percent of patients, may have few complications and the best prognosis (WHO 1996). It is the preferred treatment when cysts are large (>10 cm diameter), secondarily infected, or located in the brain or the heart. The aim of surgery is total removal of the cyst carefully avoiding the

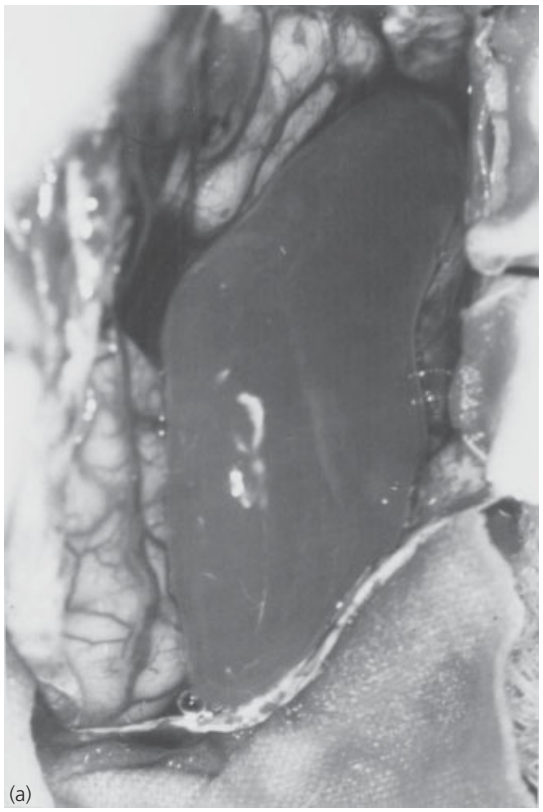

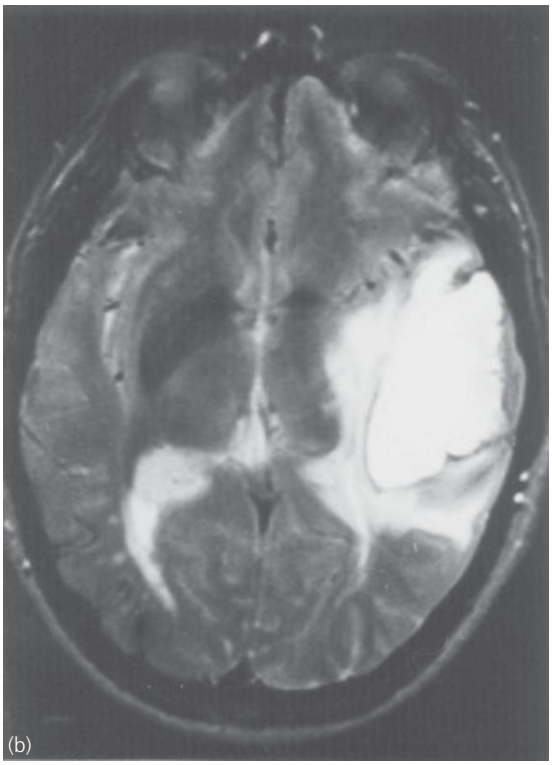

Figure 12 *Surgical removal of a huge brain cysticercus* **(a)** *that can be seen to have a similar size in MR* **(b)** *and as the end of the vesicle surrounded by an inflammatory reaction in CT* **(c)**. *(by courtesy of Dr G. Zenteno)*

adverse consequences of spilling its contents. Pericystectomy is the usual procedure, but resection of the involved organ may be used depending on the location and condition of the cyst. Although surgery provides cure in a high percentage of cases there may be 2–25 percent recurrence (Kammerer and Schantz 1993; Ammann and Eckert 1995). Postoperative complications may occur in 10 to 25 percent of cases and mortality is around 2 percent, but may increase considerably with further operations. Surgical resection of the infected liver segment and of alveolar echinococcosis lesions from other affected organs is indicated in all operable cases even though it is impossible to know whether an operation has removed all parasite tissue. Liver transplantation has been performed in French AE cases with end stage liver failure; however, re-colonization of the donor liver in immunosuppressed patients was a significant problem (WHO 1996). It is now common practice for postoperative chemotherapy to be routinely carried out for at least 2 years after radical surgery, with careful monitoring of the patient over a minimum of 10 years for possible recurrence (Ammann and Eckert 1995). Because alveolar hydatid disease is often not diagnosed until the disease is advanced, the lesion is then inoperable and albendazole chemotherapy is the recommended treatment; however, efficacy may be variable (Polat et al. 2002).

Cestocidal treatment of cysticercosis is based on praziquantel (a synthetic acylated isoquinoline-pyrazine) and albendazole (a benzimidazole derivative). Pharmacokinetic and toxicological studies of both drugs indicate rapid absorption and, in general, no toxicological effects (Marriner et al. 1986; Frohberg 1989). Both drugs are useful for eliminating living cysticerci located in brain

parenchyma and the subarachnoidal space (Cruz and Cruz 1991; Takayanagui and Jardim 1992), but the proper dosages have not yet been precisely defined. Praziquantel is usually used at 50 mg/kg body weight in three daily doses given with meals over 15 days with improvement in 70–96 percent of patients with cysticerci in brain parenchyma (Earnest et al. 1987; Madrazo and Flisser 1992; Kammerer and Schantz 1993; Garcia and Del Brutto 2000; Sotelo and Del Brutto 2000; White 2000). One-day treatment has also been successful (Bittencourt et al. 1990; Corona et al. 1996; Sotelo and Flisser 1997; Pretell et al. 2001a), especially in patients with a single or few viable brain cysticerci, but is poorly effective for multiple cysts, it can be optimized with a high carbohydrate diet (Lopez-Gómez et al. 2001; Pretell et al. 2001b). Albendazole was initially used at 15 mg/kg for 1 month, but in later protocols this was reduced to 7–8 days (García et al. 1997b). Efficacy of cestocidal treatment is measured by the reduction in number and/or size of cysticerci seen by imaging techniques, clinical improvement, withdrawal of corticoid or anti-epileptic treatment, and disappearance of ventricular dilatation. A case of myositis in disseminated muscular cysticercosis induced by praziquantel therapy (Takayanagui and Chimelli 1998) demonstrates that these effects are seemingly due to a strong inflammatory reaction and can be considered as a reliable indicator of drug effectiveness. Steroids should be used in this type of patients who show exacerbation of neurological symptoms or develop adverse reactions not related to drug toxicity. Headache, nausea, and seizures are common during treatment, but are usually transient and can be ameliorated with analgesic, anti-emetic, or antiepileptic drugs when reactions are mild, or with steroids when severe (Sotelo et al. 1985b; Del Brutto and Sotelo 1988). Pharmacokinetics of albendazole, in combination with dexamethasone or cimetidine, indicates that their presence decreases the rate of elimination of albendazole (Jung et al. 1990; Takayanagui et al. 1997). Albendazole therapy results in significantly faster and increased resolution of solitary cysticercus lesions and appears to reduce the risk of late onset epilepsy recurrences (Baranwal et al. 1998).

The need for cestocidal drugs has been questioned mainly because children appear not to warrant antiparasitic drug therapy (Mitchell and Snodgrass 1985; Mitchell and Crawford 1988). The controversy for treatment depends on the associated inflammatory reaction, as well as clinical and pathological features (Evans et al. 1997). As Evans and colleagues (1997) state: 'Although recommendations cannot yet be definitive, available evidence suggests that viable, intact cysticerci which cause epilepsy or other symptoms can be treated with cestocidal therapy, especially if they are causing mass effect.' Furthermore, there are several reasons why cestocidal treatment should always be considered: intraparenchymal single lesions can mimic neoplastic or other infectious brain diseases; drugs are efficacious and well tolerated, side effects can be controlled, and some cases are seronegative even with the highly sensitive Western blot. Subarachnoidal cysticerci in the cerebral convexity or in the cisternas of the skull base should be surgically treated only when cestocidal or anti-inflammatory treatment has failed, especially when there is secondary hydrocephalus or diagnostic doubt (Madrazo and Flisser 1992; Sotelo and Del Brutto 2000). A recent study showed that children with neurocysticercosis who were only under antiepileptic treatment and had more than five lesions in active or transitional stages, showed a high seizure frequency, predicting a worse short-term prognosis (Ferreira et al. 2002). Even today, there are several controversies with regard to the medical treatment of cysticercosis. Most importantly, debate continues over the usefulness of anticysticercal drugs. Other issues, where opinion varies, include the specific drug – praziquantel versus albendazole – to be used; the drug dosage and duration of treatment; the specific role, indications, and duration of corticosteroid co-medication and antiepileptic drugs (Singhal and Salinas 2003).

Albendazole and mebendazole, in relatively high doses and over prolonged periods, can severely damage or kill protoscolices and cysts of *E. granulosus* in animals (Pérez-Serrano et al. 1994; Taylor and Morris 1988). When surgery is not recommended in CE because of the patient's condition and the extent and location of the cysts, cestocidal treatment should be used. Both albendazole (10 mg/kg body weight per day) and mebendazole (40–50 mg/kg) are effective. However, because of its superior intestinal absorption and penetration into the cysts, albendazole is slightly more efficacious. Adverse reactions (neutropenia, liver toxicity, alopecia, and others), reversible upon cessation of treatment, have been noted with both drugs (WHO 1996). A minimum period of treatment is 3 months, but the long-term prognosis in individual patients is difficult to predict, therefore, prolonged follow-up with ultrasound or other imaging procedures is needed to determine the eventual outcome. Recent trials have shown that, with both drugs, success rates for CE are variable. Between 20 and 70 percent of patients improved, but this included high proportions of inoperable and severe cases (Davis et al. 1989; Todorov et al. 1992; WHO 1996). In general, approximately one-third of CE patients treated with benzimidazole drugs have been cured of their disease, as seen by complete and permanent disappearance of cysts, and an even higher proportion have responded with significant regression of cyst size and alleviation of symptoms. Small (<7 mm diameter) isolated cysts, surrounded by minimal adventitial reaction, respond best, whereas complicated cysts with multiple compartments and daughter cysts, or with thick or calcified surrounding adventitial reactions, are relatively refractory to treatment. Difficult presentations such as bone cysts, however, may also respond favorably

for individual patients (Bonifacino et al. 1997). CE disease responds more readily to chemotherapy in children than it does in adults (Goccmen et al. 1993; Kammerer and Schantz 1993; Ammann and Eckert 1995). The few available results of praziquantel treatment of cystic echinococcosis do not yet allow conclusions to be drawn (Craig 1994; Ammann and Eckert 1995), but the potential efficacy of praziquantel in association with albendazole needs critical assessment for cystic hydatid disease (Bonifacino et al. 1997). The use of cyclosporin A for postoperative control of secondary cystic echinococcosis has been suggested (Hurd et al. 1993). Recently hydatid cysts in infected gerbils were injected by a laparotomic procedure with ivermectin and found severely damaged and with no viable protoscolices between 44 and 58 days after injection (Ochieng-Mitula and Burt 1996). Co-administration of albendazole with cimetidine in Chinese CE patients significantly increased bile and cyst fluid concentrations of the active metabolite, albendazole sulfoxide, and resulted in improved therapeutic benefit (Wen et al. 1994).

Long-term treatment with mebendazole (50 mg/kg per day) or albendazole (10 mg/kg) inhibits growth of larval *E. multilocularis*, reduces metastasis, and enhances both the quality and length of survival; prolonged therapy may eventually be larvicidal in some patients, but recurrence of the disease also occurs (Ammann et al. 1993). In one study of 23 inoperable Turkish AE patients, a beneficial outcome was observed in eight cases, while nine others showed lesion progression, and none were considered cured (Polat et al. 2002). Liver transplantation has been employed successfully on otherwise terminal AE cases (WHO 1996). Because the lesions due to *E. vogeli* are so extensive, surgical resection is always difficult and usually incomplete and a combination of surgery with albendazole is most likely to be successful (Kammerer and Schantz 1993). The principles of management of cystic and alveolar echinococcosis also apply to polycystic echinococcosis.

Combinations of cyst puncture, aspiration, and drainage, with or without injection of chemicals (called percutaneous aspiration–injection–re-aspiration (PAIR)), have also been evaluated. Percutaneous puncture is performed under sonographic guidance, followed by aspiration of the liquid contents, instillation of a protoscolicidal agent (95 percent ethanol or 20 percent sodium chloride solution) and re-aspiration (Filice et al. 1990; Khuroo et al. 1993). To avoid sclerosing cholangitis, this procedure must not be performed in patients whose cysts have biliary communication. The presence of the latter can be determined by testing the cyst fluid for presence of bilirubin or by intraoperative cholangiogram. Mild anaphylaxis has been reported using PAIR, and care is required to prevent secondary microbial contamination when drainage tubes are temporarily left in place (WHO 1996; Wang et al. 2003). The possibility of secondary echinococcosis resulting from accidental spillage during this procedure can be minimized by concurrent treatment with albendazole. Indeed, combining PAIR and chemotherapy may improve the results of either treatment alone. PAIR may have advantages over surgery particularly in less well equipped hospitals (WHO 1996; Filice and Brunetti 1997).

TRANSMISSION, EPIDEMIOLOGY, AND CONTROL

Human cysticercosis is a disease related to underdevelopment. It is present in countries that lack proper sanitary infrastructure and hygiene, as well as insufficient health education (Gemmell et al. 1983; Schantz et al. 1998), as exemplified by the emergence of neurocysticercosis in 1978 in West New Guinea where it became a disaster among the Ekari population, to whom the disease was unknown prior to the entrance of cysticercotic pigs as official gifts. Some 18–20 percent of the population acquired cysticercosis. The disease was detected by an epidemic of severe burns resulting from convulsions manifested while the people were sleeping around house fires. Individuals also had subcutaneous nodules (Muller et al. 1987). Between 1991 and 1995, a rapid increase in the number of cases of epileptic seizures and burns in Irian Jaya was found, 67 and 65 percent of persons with epileptic seizures and with subcutaneous nodules, respectively, showed antibody responses highly specific to cysticercosis, indicating that most cases of epilepsy and burns were associated with cysticercosis (Wandra et al. 2000). Furthermore, serological analysis of pigs in this region appears to be a good indicator of infection (Subahar et al. 2001). Human cysticercosis is still highly endemic in Brazil, Colombia, Ecuador, Guatemala, Mexico, Peru, China, India, Indonesia, New Guinea, Benin, Mozambique, South Africa, West Africa, Zimbabwe, Ile de la Reunion, and Madagascar. Cases are also reported in the USA, Honduras, Panama, Venezuela, Nepal, Portugal, Spain, and the UK, though these include imported cases (Flisser 1994; Chimelli et al. 1998; Ferrer et al. 2002; García et al. 1995, 1999; Garcia-Noval et al. 1996; Garcia and Del Brutto 2000; Takayanagui et al. 1996; Houinato et al. 1998; Schantz et al. 1998, Vilhena et al. 1999; Wandra et al. 2000).

Many epidemiological studies have shown a correlation between human cysticercosis, taeniasis, and epilepsy and between seropositive people, infected pigs, and disposal of feces (Diaz-Camacho et al. 1990; Michault et al. 1990; Sarti et al. 1992, 1994; Aranda-Alvarez et al. 1995; Cruz et al. 1995; García et al. 1995, 1997c, 1998b; Correa et al. 1999; Garcia-Garcia et al. 1999; Rodriguez-Canul et al. 1997; Sakai et al. 2001; Widdowson et al. 2000). The results of surveys have identified community, behavioral, and environmental practices that must be

modified to prevent continued transmission of cysticercosis and taeniasis. Most importantly, these studies have shown that the main risk factor is the presence of a Taenia carrier in the immediate environment. Strawberries and flies were considered of high importance in the transmission of *Taenia* eggs, but recent publications demonstrated that both potential vectors did not carry *Taenia* eggs (Spindola-Felix et al. 1996; Martinez et al. 2000). A proposal to declare neurocysticercosis an international reportable disease has been published (Roman et al. 2000). This proposal, if taken into account, could be helpful in the control of cysticercosis, since, if cases of cysticercosis and of taeniosis are reported in all countries, it will provide accurate quantification of the incidence and prevalence of neurocysticercosis at regional level, thus permitting the rational use of resources in eradication campaigns.

Recent studies suggest that neurocysticercosis may be a risk factor for human cancer, 1 271 autopsy files were reviewed, 113 of which had a malignant hematological disease, of these 6 percent also had neurocysticercosis, while only 1 percent of those with other neoplasias ($n = 448$) and 2 percent of those that did not have any malignant neoplasia ($n = 710$) had neurocysticercosis (Herrera et al. 1999). The authors conclude that 'while it will be difficult to demonstrate that neurocysticercosis is a causal agent of malignant hematological diseases, it should be considered a potential risk factor for cancer induction in countries where cysticercosis remains a public health problem'. Several data support this hypothesis: chromosome aberrations induced in peripheral blood lymphocytes were higher in human patients with neurocysticercosis and in cysticercotic pigs as compared to those observed in the same cases after cestocidal treatment and in healthy controls (Flisser et al. 1990a; Montero et al. 1994; Herrera et al. 2000). Also, 17 percent of 43 patients with glioma, but only 3 percent of 172 controls had neurocysticercosis (Del Brutto et al. 1997). The apparent association of neurocysticercosis and oncogenesis was reviewed recently (Del Brutto and Dolezail 2000; Herrera and Ostrosky-Wegman 2000), that the parasite is capable of immunosuppressing the host's immune response has long ago been proposed, and recently data have been compiled (Flisser 1989; White et al. 1997).

Taeniosis, the infection with the adult stage of a tapeworm, is the main element that maintains the life cycle of *Taenia* in the environment, since tapeworms release permanently thousands of eggs inside gravid proglottids. Two tapeworms infect human beings: *Taenia saginata* and *Taenia solium*. It is known that only the second species causes human cysticercosis. The first one is, even nowadays, a cosmopolitan parasite. Its biological characteristics and life cycle have been thoroughly studied (Pawlowski and Schultz 1972), many of these studies referring to *T. solium*. Nevertheless, it is remarkably difficult to find human *T. solium* carriers, while those of *T. saginata* are easily detected. Coproparasitoscopic studies are generally used to identify carriers in epidemiological studies, but unless gravid proglottids or the scolex are found, it is impossible to differentiate between the species, unless molecular biology techniques are used (Chapman et al. 1995; Gasser et al. 1995; Gonzalez et al. 2002; Mayta et al. 2000). The establishment of experimental models (Maravilla et al. 1998) will hopefully support the knowledge of the host–parasite relationship in *T. solium* for example the understanding of the characteristics of the cellular and humoral immune response in the intestinal mucosa (Avila et al. 2002, 2003), but of special relevance is the understanding the life span of adult *T. solium* in field conditions for its control. Field characteristics of this parasite were studied in rural Guatemala showing a high prevalence of tapeworm carriers (Allan et al. 1996). A higher prevalence of taeniasis than previously reported, among patients with neurocysticercosis, was found in Peru. In addition, a clear association between the presence of taeniasis and the severity of neurocysticercosis was found. Most massive cerebral infections probably resulted from a maintained infective source in patients harboring the adult tapeworm in the intestine. Therefore, the perception that *Taenia* are silent guests causing no harm to humans is erroneous, and tapeworm carriers should be regarded as potential sources of contagion to themselves and to those living in their close environment (Gilman et al. 2000). A recent study performed in 68 asymptomatic individuals from Amerindians living in the Venezuelan state of Amazonas showed that 65 percent had circulating *T. solium* antigens and 77 percent had specific IgM antibodies, suggesting that recent exposure has occurred and active recent infections are present (Ferrer et al. 2002), therefore a control program adapted to local conditions, knowledge, and practices should be implemented.

The knowledge of the main risk factors has led to the evaluation of control measures (Flisser 2002b, 2003). Mass treatment against intestinal tapeworms provides quite good results (Cruz et al. 1989; Pawlowski 1990; Diaz-Camacho et al. 1991; Allan et al. 1997; Sarti et al. 2000), although in one study, a case of occult neurocysticercosis was exacerbated (Flisser et al. 1994). Targeted treatment, instead of mass drug administration, can be used since potential tapeworm carriers are identified by direct questioning or by detection of parasite antigens or eggs, as well as by association with late onset epilepsy (Sarti et al. 1992, 1994, 2000; Garcia-Noval et al. 1996). One intervention study based on a specially prepared health education strategy was very successful since 4 years and 6 months after the project ended no pigs with cysticercosis were identified in the whole community (Sarti et al. 1997). Apparently an economic factor facilitated the success, since people learned that by having pigs restrained in certain areas without access to human feces or garbage they would not acquire the

disease and thus could be sold at a higher price. A third alternative for the control of cysticercosis is vaccination, especially because pigs are the only intermediate hosts that participate in the maintenance of the parasite in the environment. Many studies have been performed to evaluate the effectiveness of vaccination against larval cestodes (Lightowlers 1994, 1996; Lightowlers et al. 2000; Huerta et al. 2000; Flisser and Lightowlers 2001). Results show that it is possible to protect pigs from acquiring cysticercosis. The present challenge is to identify the optimal vaccination protocol and carriers for the vaccine. Meanwhile cysticercotic pigs can receive 1-day treatment with cestocidal drugs (Torres et al. 1992; Gonzalez et al. 1998) to interrupt transmission of *T. solium*.

Neurocysticercosis has been recognized in recent years as a significant public health problem due to the fact that the tapeworm carrier can migrate to countries where *Taenia solium* was previously absent. This has generated various review articles and books of interest for the international scientific community and governments (Del Brutto 1997; Flisser 1995; Flisser et al. 1998; San Esteban et al. 1997; Del Brutto and Sotelo 1988; Del Brutto et al. 1998; Schantz et al. 1998; Garcia and Martinez 1999; Garcia and Del Brutto 2000; Passos-Barbosa et al. 2000; Roman et al. 2000; Sciutto et al. 2000; Sotelo and Del Brutto 2000; White 2000; Singh and Prabhakar 2002; Craig and Pawlowski 2002), as well as special issues of scientific journals (Van der Kaay and Overbosch 1989; Murrell 2003).

The greatest prevalence of cystic echinococcosis is in temperate countries, including southern South America, the entire Mediterranean littoral, Eastern Europe, the Middle East, southern and central parts of the former Soviet Union, Central Asia, India, Nepal, China including Tibet, Australia, and parts of Africa. In the USA, most infections are seen in immigrants from countries in which the disease is highly endemic. Sporadic autochthonous transmission is currently recognized in Alaska, California, Utah, Arizona, and New Mexico. Geographic strains or genotypes of *E. granulosus* exist with different host affinities. The northern or sylvatic strain is maintained in wolves and wild cervids (moose and reindeer) in northern Alaska, Canada, Scandinavia, and Eurasia. Pastoral strains are maintained in dogs and domestic ungulates throughout the world. Populations of *E. granulosus* in different assemblages of domestic hosts (dog-sheep, dog-horse, dog-pig, dog-camel and dog-cattle) may differ morphologically, developmentally, biochemically, and possibly in infectivity and pathogenicity to humans. For example, the dog-sheep strain, the most widespread of the variants, is relatively pathogenic in humans in comparison to the northern sylvatic strain that occurs in wolf-wild cervid hosts. There is much evidence that the strain adapted to the dog-horse cycle, occurring in parts of Europe and the Middle East, rarely, if ever, infects humans. Probes characterizing the nuclear and mitochondrial DNA of the variant populations provide reliable genetic markers to distinguish them; nine genotypes (G1–G9) have been identified. Globally, sheep are the most important intermediate hosts, but swine, cattle, buffalo, horses, and camels are more important in certain regions. Apart from the sheep strain (G1), human CE cases that have been DNA tested after surgical biopsy have been identified infected with *E. granulosus* of bovid, camel, or cervid genotypes (Pearson et al. 2002). Certain human activities, such as the widespread rural practice of feeding the viscera of home-butchered sheep to dogs, facilitate transmission of the sheep strain and consequently increase the risk that humans will become infected. People who practice seasonal movement of sheep between summer and winter pastures and other nomadic lifestyles appear to be at greater risk of transmission of *E. granulosus* (Macpherson and Craig 2000; Wang et al. 2001).

Genetic heterogeneity is much less evident in *E. multilocularis*, although some differences in nuclear and mitochondrial genes have suggested separation into the traditional view of Alaskan and Eurasian types (Rinder et al. 1997). Despite this, it is known that a great variety of small mammal species are susceptible to metacestode development (WHO/OIE 2001). Those small mammal species that are prone to rapid population increases either seasonally and/or pluri-annually (e.g. the vole species *Arvicola terrestris,* and *Microtus arvalis* in Europe, and *Microtus oeconomus* in Alaska, or the lagomorph *Ochotona curzonae* on the Tibetan plateau) appear to contribute most significantly to the parasitic life-cycle of *E. multilocularis*. That is because they become preferential prey for the fox definitive host (red fox in Europe, arctic fox in Alaska, and the Tibetan fox in China), and also may be eaten by domestic dogs if they occur in endemic communities (WHO/OIE 2001; Giraudoux et al. 2002). The availability of suitable habitats for small mammal hosts within upland temperate and holarctic landscapes also appears to be important in the ecology of transmission of *E. multilocularis* in sylvatic host cycles. Application of remote sensing and geographic information system (GIS) to quantify landscape cover appears to be useful in development of risk models for human AE at village level in China (Danson et al. 2002).

Dogs infected with *Echinococcus* spp. tapeworms pass proglottids and eggs in their feces, and humans become infected through fecal–oral contact, particularly in the course of playful and intimate contact between people and dogs. Eggs adhere to hairs around the infected dog's anus and are also found on the muzzle and paws. Indirect transfer of eggs, either through contaminated water and uncooked food or through the intermediary of flies and other arthropods, may also result in infection of humans. In *E. granulosus* endemic areas, personal preventive measures include careful hygiene, strict dietary regulation of pet dogs to preclude ingestion of

livestock offal, and avoidance of dogs that are not so regulated. Periodic prophylactic treatment of pet dogs for intestinal echinococcosis may sometimes be necessary. Control measures applicable in communities include health education, regulation of livestock slaughtering in abattoirs and on farms, control of dogs, and periodic or regular mass treatments of dogs with praziquantel (5 mg/kg) to reduce the prevalence of *E. granulosus* below levels necessary for continued transmission (reviewed in Gemmell and Roberts 1998). A targeted campaign of education and surveillance based on specific antibody detection in dogs as an indicator of exposure to *E. granulosus* has been used in Western Australia (Thompson et al. 1993). The most successful national or provincial control programs for *E. granulosus* have, however, focused on dog registration and regular dosing of dogs at 6–12-week intervals using arecoline or preferably praziquantel in the 'attack phase', followed by slaughter-house surveillance of transmission using sheep hydatid prevalence rates. This approach at a national or regional level in Chile, Argentina, Falkland Islands (Islas Malvinas), New Zealand, and Tasmania has resulted in significant reduction in the incidence of human cystic echinococcosis within 10 years in some programs (Gemmell and Roberts 1998).

The life cycle of *E. multilocularis* involves foxes (principally the red fox and the arctic fox) and their small mammal prey, particularly microtine voles, in ecosystems generally separate from humans. There is often, however, an ecological overlap with humans, because domestic dogs or cats may become infected when they eat infected wild rodents. As a result, exposure of humans to *E. multilocularis* is relatively less common than exposure to *E. granulosus*. Alveolar echinococcosis has been reported in parts of central Europe, Turkey, and Iran, much of eastern Russia, the Central Asian Republics, and western China, Japan, the northwestern portion of Canada, and western Alaska. Wildlife transmission, involving red foxes, coyotes, and prairie voles, appears to be increasing in central North America. Hunters, trappers, and persons who work with fox fur may be exposed to AE, but the main risk factors appear to be farming or agricultural occupations and a history of dog ownership (Stehr-Green et al. 1988; Eckert and Deplazes 1999; Craig et al. 2000; Kern et al. 2003). Hyperendemic foci have been described in some Inuit villages of the Alaskan tundra and also in China, where both communities owned dogs regularly fed on infected commensal rodents. The epidemiology of alveolar echinococcosis in China remains one of the major challenges in temperate areas with human prevalences of 2–15 percent in some communities (Craig et al. 1992, 2000). Eliminating *E. multilocularis* from its wild animal hosts is virtually impossible; therefore, contact with dogs and foxes in areas where the infection is endemic should be avoided. Preventing infections in humans depends on education to improve hygiene and sanitation. Infections in dogs and cats prone to eat infected rodents can be prevented by monthly treatments with praziquantel (Kammerer and Schantz 1993; Rausch 1995). Use of fox baits containing praziquantel has had some success in reducing fox prevalence rates of *E. multilocularis* in focal endemic areas of Germany (Tackmann et al. 2001).

Very little is known about the circumstances associated with polycystic hydatid disease due to *E. vogeli*. Bush dogs (*Speothus venaticus*) are rare and avoid man, and therefore probably play little role in transmission to humans. In endemic areas, infections are probably acquired from the feces of domestic dogs that have been fed on rodent viscera of infected bush rodents (e.g. pacas), a practice that has been reported commonly by patients (Meneghelli et al. 1992; D'Alessandro 1997).

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Gastrointestinal nematodes – *Ascaris*, hookworm, *Trichuris*, and *Enterobius*

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Nearly 200 species of helminth have been found associated with the human alimentary tract (Coombs and Crompton 1991), many of these probably being the result of accidental or spurious infections. Of the habitual parasites, the pinworm or threadworm *Enterobius vermicularis* (L, 1758) and four species of soil-transmitted nematodes, the roundworm *Ascaris lumbricoides* L, 1758, hookworms *Ancylostoma duodenale* (Dubini, 1843) Creplin, 1845 and *Necator americanus* (Stiles, 1902) Stiles, 1903 and the whipworm *Trichuris trichiura* (L, 1771) Stiles, 1901 are some of the commonest infections on earth (Table 1).

The origins of these human–helminth relations are intriguing and might perhaps be traced to human activities leading to the domestication of animals. A qualitative survey indicates that humans share surprisingly few intestinal helminth infections with the other 180 or so species of primate (see Coombs and Crompton 1991; Macdonald 1984). *E. vermicularis* may well have evolved with humans from our primate ancestors, but the soil-transmitted species might perhaps be traced to human activities leading to the domestication of animals. Humans do, however, seem to have the same or similar types of infection to those found in domesticated animals, especially dogs (Beaver 1954) and pigs (Bell et al. 1988). Perhaps human *A. lumbricoides*, if it really is a species in its own right (see Crompton 1989), might have been acquired from porcine *A. suum* or its ancestor after the last ice age. Pigs were probably domesticated in several places (Clutton-Brock 1987), but China is likely to have been one of the first and there remains an intimate relationship today between Chinese people and their pigs (Peng et al. 1995).

The domestication of the very few species of mammal that humans now rely on for food, clothing, and transport began about 9 000 years BC (Clutton-Brock 1987) when the human population was about 10 million (Lewin 1989). Evidence from early writings (Bryan 1930; Ebbell 1937), from mummified bodies (see Cockburn and Cockburn 1980) and from archaeological digs (Fry and Moore 1969; Bundy and Cooper 1989) indicates that pinworm, roundworm, hookworm, and whipworm were

Table 1 *Features of* Ascaris, *hookworms and* Trichuris *in human hosts*

Feature	*Ascaris*	*Ancylostoma*	*Necator*	*Trichuris*
No. species/subspecies in genus[a]	16	23	8	71
No. species infecting humans[b]	2	7	3	3
Global no. of infections[c]	1470×10^6	135×10^6	735×10^6	1300×10^6
Estimate of cases of morbidity[d]	$120–215 \times 10^6$	$27–39 \times 10^6$	$63–91 \times 10^6$	$90–130 \times 10^6$

a) Yamaguti (1961).
b) See Table 2 and Coombs and Crompton (1991).
c) Chan (1997).
d) Chan et al. (1994). The estimates for the separate species of hookworm are based on the assumption that 30 percent are due to *A. duodenale* and 70 percent to *N. americanus* (Crompton and Stephenson 1990).

well established in humans a few thousand years ago. Today, the human population is over 6.3 billion and by the year 2010 is likely to be 7 billion with around 80 percent of the people living in the countries currently identified as less developed (Bulatao et al. 1990). Pinworms are the only species common in temperate countries; pinworms and the soil-transmitted species are widely distributed in countries with warmer climates, often occurring concurrently in individuals. It has been estimated that around a billion people are infected with *A. lumbricoides*, *A. duodenale*, *N. americanus*, and *T. trichiura,* often concurrently (Crompton 1989); this means that four out of every ten people in much of Africa, Asia, and South America harbor worms.

The notion that humans may have acquired the soil-transmitted nematodes during the domestication of a few species of wild mammal over a period of a few thousand years should not be discarded too lightly without further consideration. The human host has undergone about 400 generations and a vast social and technical evolution during the last 10 000 years, while *A. lumbricoides* has undergone about 20 000 generations which, coupled with its prodigious fecundity, could have created the circumstances for an explosive evolution.

The history of human helminth infections has been carefully documented by Grove (1990). Although three of these intestinal species (*Ascaris*, *Enterobius*, and *Trichuris*) were known to Linnaeus in the 18th century, and *Ancylostoma* was identified in the early 19th century, details of their life histories did not become known until much later. A fundamental observation in the case of *A. lumbricoides* was made by Koino (1922) who courageously swallowed about 2 000 eggs and subsequently passed 667 worms after taking an anthelmintic drug. He was extremely ill during the course of this experimental infection at a time when the larvae would have been occupying his lungs. Looss (1901) published the results of an experiment which proved that hookworm larvae penetrate skin. He placed a drop of water containing infective larvae of *A. duodenale* on the skin of a leg awaiting amputation. An hour later, when the surgery had been completed, Looss retrieved samples of skin and found the larvae beneath the skin. Calandrucci in 1886, having confirmed over a period of 6 months that he was free from infection with *T. trichiura*, swallowed infected eggs of the helminth and found eggs in his stools 27 days later. This result was published by Grassi (1887) who may not have given Calandrucci sufficient credit for his effort (Grove 1990). These studies are of great importance because they established the nature of the helminths' life cycles and the routes of transmission to human hosts.

CLASSIFICATION

In this chapter, the five species of helminth are assumed to belong to the phylum Nematoda, as advocated by Maggenti (1982), rather than to a class of the phylum Aschelminthes, as adhered to by Hyman (1951). Much work needs to be done before we have a secure understanding of the phylogeny of these ubiquitous animals, which thrive as free-living, plant-parasitic and animal-parasitic organisms (Andrassy 1976; Maggenti 1982; Poinar 1983). Here is a simple classification for soil-transmitted nematodes, based mainly on Anderson et al. (1974):

Phylum Nematoda
  Class Secernentea
    Order Ascaridida
      Family Ascarididae
        Genus *Ascaris*
          Species *lumbricoides*
          Trivial name roundworm
    Order Strongylida
      Family Ancylostomatidae
        Genus *Ancylostoma*
          Species *duodenale*
          Trivial name hookworm
      Family Ancylostomatidae
        Genus *Necator*
          Species *americanus*
          Trivial name hookworm
  Class Adenophorea
    Order Enoplida
        Family Trichuridae
        Genus *Trichuris*
          Species *trichiura*
          Trivial name whipworm

Table 2 *Zoonotic infections of humans with species of* Ascaris, Ancylostoma, Necator *and* Trichuris

Genus and species	Nonhuman hosts	Observations on infection in human hosts	References
Ascaris suum	Pig	Sexually mature; in small intestine	Davies and Goldsmid (1978)
Ancylostoma brasiliense	Cat, dog	Cutaneous larva migrans (CLM)[a]	Yoshida et al. (1974)
A. caninum	Dog	CLM; adult in small intestine	Croese et al. (1994)
A. ceylanicum	Cat, dog	Adult in small intestine	Carroll and Grove (1986)
A. japonica	Unresolved	Reported once from human in Japan	Barriga (1982a)
A. malayanum	Bear	No information	Yorke and Maplestone (1926)
A. tubaeforme	Cat	CLM	Muller (1975)
Necator argentinus	Unresolved	Reported once from human	Yoshida (1973)
N. suillus	Pig	Reported at least once from human	Barriga (1982a)
Trichuris suis	Pig	Adult in large intestine	Barriga (1982b)
T. vulpis	Dog	Sexually mature adult in appendix	Kenney and Eveland (1978)

a) For a full discussion of CLM, see Beaver (1956).

Although the ecology and general biology of *A. lumbricoides*, *A. duodenale*, *E. vermicularis*, *N. americanus* and *T. trichiura* have much in common, the latter species has major morphological differences from the others. Most noticeably, it lacks phasmids (paired, glandular sensory organs) at its caudal end and is equipped with a stichosome esophagus. Taxonomists have used the presence or absence of phasmids as a basic feature in the systematic organization of nematodes.

If adult worms (either alive or in a good state of preservation) are available, an investigator should have no difficulty in identifying the genera under discussion. The keys prepared by Yamaguti (1961) and Anderson et al. (1974) are recommended together with the biological and clinical descriptions published by Beaver and Jung (1985). If only morphological evidence is available, problems are likely to be encountered in trying to distinguish from each other, for example, all the seven species of *Ancylostoma* reported from humans (Tables 1 and 34.2). Adult *A. lumbricoides* and adult *A. suum* remain virtually indistinguishable on morphological grounds (see Crompton 1989) as are *T. trichiura* and the other species of *Trichuris* found in non-human primates and pigs.

MORPHOLOGY AND STRUCTURE

Detailed accounts of the anatomy, functional morphology, and structure of animal-parasitic nematodes, including the species considered here, *Trichuris*, have been published by Chitwood and Chitwood (1974), Gibbons (1986), and Bird and Bird (1991) among others. Each of these treatises deals comparatively with different features such as the cephalic region and the nervous system rather than presenting descriptions of individual species. The brief accounts given below for the four species of soil-transmitted nematode under review in this chapter cover features not highlighted in the definitive works cited above. Inevitably, some of the details are based on extrapolation from the results of studies on *A. suum*, *Anc. caninum*, and *T. suis* from animal hosts.

Ascaris lumbricoides

The eggs of *A. lumbricoides* are seen in human stool samples in two general forms: unfertilized eggs (Figure 1a) and fertilized eggs which may or may not possess a cortical layer (Figure 1b, c). Typically eggs in stools appear to be brown in color, probably because they become stained by bile pigments while in the gastrointestinal tract. The eggshell of a fertilized egg (50–70 × 40–50 μm) consists of an inner lipid layer responsible for selective permeability (Perry and Clark 1982), a chitin–protein layer responsible for structural strength and an outer vitelline layer (Foor 1967; Wharton 1980). The inner layer contains a remarkably resilient lipoprotein, known as ascaroside, which explains how the eggs with enclosed infective larvae can survive formaldehyde, disinfectants and other destructive chemicals. The fertilized egg is frequently observed to have an uneven deposit of mucopolysaccharide (the cortical layer) on its outer surface (Figure 1b). This deposit is obtained when the egg is passing through the uterus of the female worm (Foor 1967) and is responsible for its adhesive properties (Kagei 1983).

The first-stage larva of *A. lumbricoides* develops inside the eggshell, molts there, and forms the second-stage larva. Soon after hatching, second-stage larvae have a typical filariform appearance and measure about 250 × 14 μm (Nichols 1956). Just before the second molt in the lungs, they measure about 560 × 28 μm in length. After the fourth (final) molt in the small intestine, growth is rapid. Freshly recovered adult male and female *A. lumbricoides* are often observed to be a pinkish cream in color and to measure 200–300 mm in length. A male, which is usually smaller than a female of

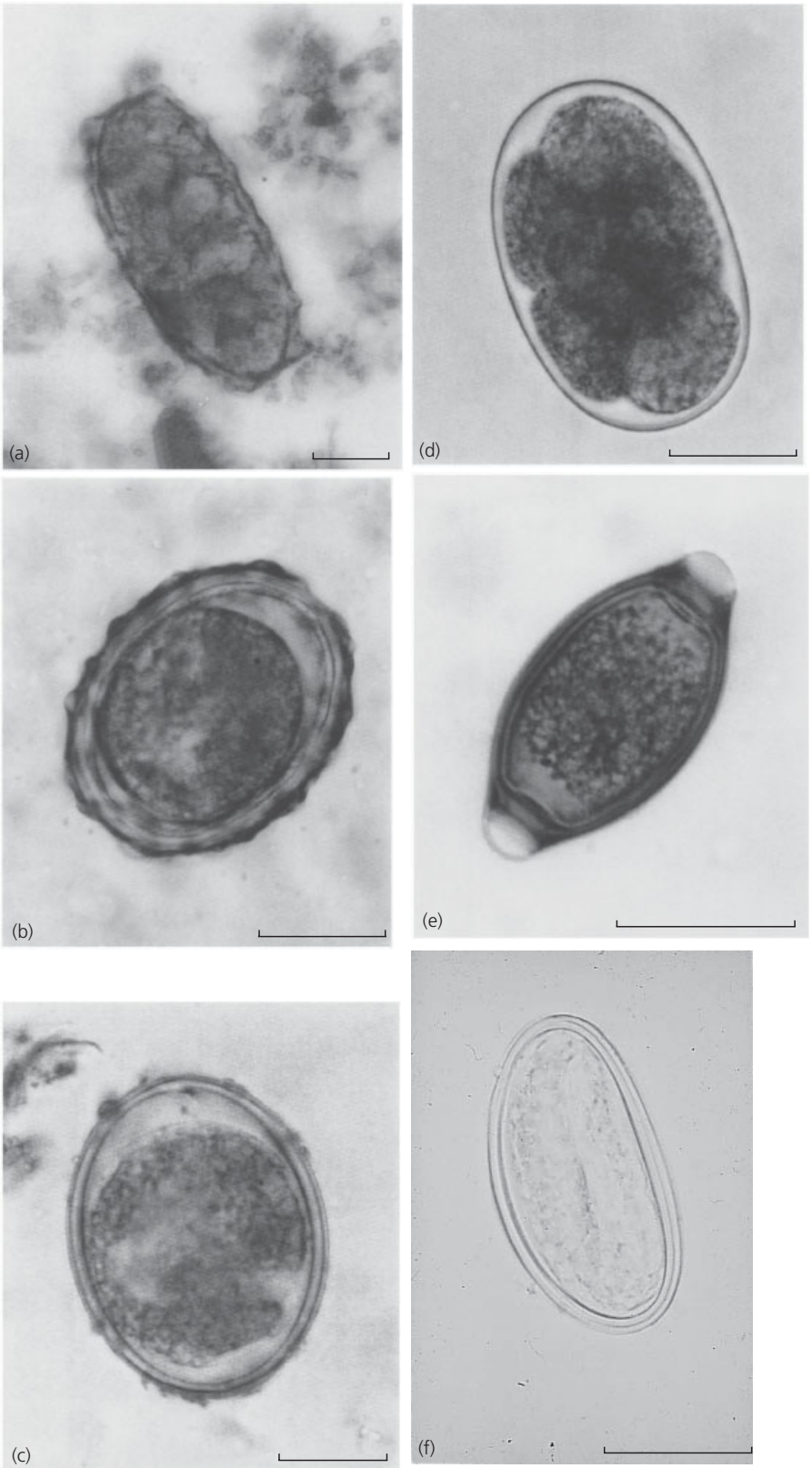

Figure 1 *Photomicrographs of eggs of soil-transmitted nematodes as seen in human stool samples:* **(a)** *unfertilized egg of* Ascaris lumbricoides*;* **(b)** *fertilized egg of* A. lumbricoides *with cortical layer;* **(c)** *fertilized egg of* A. lumbricoides *without cortical layer;* **(d)** *egg of a hookworm;* **(e)** *egg of* Trichuris trichiura*;* **(f)** *egg of* Enterobius vermicularis*. Bar = 25 µm. (Photomicrographs (a–e) from WHO 1994;* **(f)** *courtesy of R. Muller)*

the same age, has a curved posterior tail that accommodates the copulatory apparatus. Skryabin et al. (1991) describe adult *A. lumbricoides* as having a mouth surrounded by three well-developed lips, rows of small 'teeth' (i.e. denticles of Sprent (1952)) on the lips, a cuticle with transverse striations, a didelphic uterus and the vulva in the anterior half of the body. The morphology of these denticles is a character widely used by workers who seek to distinguish *A. lumbricoides* from *A. suum* (Maung 1973).

The cuticle of *A. lumbricoides* under natural circumstances is only ever exposed to the conditions prevailing in the host. Collagen, stabilized by disulfide links, forms the main structural protein of the adult cuticle, which becomes more complex with each molt (Bird and Bird 1991). Collagen is now known to be the principal protein in the cuticles of all adult nematode species investigated to date. An investigation into the composition of the cuticles of developmental stages of *A. suum* from pigs revealed that the proteins extracted with 2-mercaptoethanol from third- and fourth-stage larvae and from adult worms were readily digested on incubation with bacterial collagenase (Fetterer et al. 1990). A significant amount of the cuticle from second-stage larvae was not affected by 2-mercaptoethanol and was not digested by bacterial collagenase. Presumably, the same results may apply to *A. lumbricoides*, with the differences detected in the second-stage larval cuticle being an adaptation either to survival outside the host or the infection process or both events.

Ancylostoma duodenale and *Necator americanus*

When seen in human stools (Figure 1d), the eggs of these two species are indistinguishable, being characteristically barrel-shaped with a thin shell and measuring about 60 × 75 × 36–40 μm (WHO 1994). If hookworm eggs alone are available, identification of the species cannot be made without first using the Harada–Mori technique to culture the newly-hatched larvae to the third stage when morphological differences become apparent (WHO 1981).

Descriptions of the morphology of the larval stages of hookworms are available through the experimental studies of Nichols (1956) and observations summarized by Pawlowski et al. (1991). The first-stage larvae, which escape from the eggs, measure about 200 μm in length. They feed on organic debris, then molt and the resulting second-stage larvae measure up to 500 μm. After another molt, the infective third-stage larvae measure from 500–700 μm, those of *Ancylostoma* being generally longer than those of *Necator* (WHO 1981).

The most detailed description of the morphology of adult *A. duodenale* is to be found in the classic account by Looss (1905). For both species, male worms (5–11 mm) are shorter than females (9–13 mm) and *A. duodenale* is generally longer and more sturdily built than *N. americanus*. Proof of identity is obtained by comparing the morphologies of the buccal capsule (Figure 2). The term 'hookworm' derives from the prominent curve to the anterior end of specimens of *N. americanus* (Pawlowski et al. 1991).

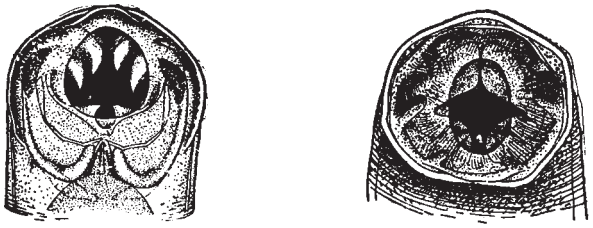

(a) (b)

Figure 2 *Buccal capsules:* **(a)** *adult* Ancylostoma duodenale*;* **(b)** *adult* Necator americanus

Enterobius vermicularis

Pinworm eggs are ovoid, measuring 50–54 μm × 20–27 μm. They are asymmetrically flattened on one side, and appear colorless when recovered from the perianal skin (Figure 1f). The outer layer of the eggshell is albuminous and sticky, enabling the egg to adhere readily. When laid, the egg contains an immature first-stage larva, but this develops rapidly. When swallowed, the eggs hatch in the small bowel and the larvae pass down into the large bowel, where they complete their molting and development. Adult worms, which measure about 10 × 0.5 mm (female) and 2.5 × 0.2 mm (male), live primarily in the cecum, in close contact with the mucosa. Males are rarely seen, but the gravid females can sometimes be seen as white or yellowish worms around the anus. Such worms have prominent uteri filled with eggs.

Trichuris trichiura

The eggs of *T. trichiura* are lemon-shaped with a characteristic plug at each end (Figure 1e). They are usually brown in a human stool sample and measure from 57–58 × 26–30 μm. As with eggs of *Ascaris* spp. and hookworms, it is virtually impossible to distinguish eggs of *T. trichiura* from those of *T. suis* and *T. vulpis* (Kenney and Yermakov 1980; Yoshikawa et al. 1989). According to studies by Wharton and Jenkins (1978), the eggs of *T. suis* have a shell similar in structure to that of *A. lumbricoides*, an observation that explains the survival of the enclosed infective larva under adverse environmental conditions (see Bundy and Cooper 1989). Apparently, there is a higher proportion of chitin in the plugs at the poles of an egg of *T. trichiura* than elsewhere in the eggshell. Wharton and Jenkins have

suggested that this may facilitate the hatching process if the activated larva releases chitinase (see Rogers 1960). On hatching in the intestine, the second-stage larval *T. trichiura* measures about 260 × 15 μm in length (Beck and Beverley-Burton 1968). These larvae, if they behave as do those of other species of *Trichuris*, burrow into the intestinal mucosa, and it is Bundy and Cooper's view that *Trichuris* spp. are essentially tissue parasites. There is some confusion about where the intestinal penetration takes place and whether or not the larvae migrate along the intestinal tissues to reach the large intestine. During this prepatent phase (Table 3), *T. trichiura* must complete the series of four molts and is found as an adult intimately associated with the wall of the large intestine.

Adult whipworms have a highly characteristic shape from which the trivial name is derived. The long, thin anterior end lies in a burrow in the mucosa while the thicker end, which contains the reproductive tract, extends into the intestinal lumen. The worms are white in color and the males (30–45 mm) are not only shorter than the females (30–50 mm), but also have a coiled posterior end when viewed in vitro. The most characteristic morphological feature of species of *Trichuris* and their relatives is the stichosome, which is a glandular structure encircling the slender esophagus in the thin, anterior half of the worms (Beck and Beverley-Burton 1968).

Although *Enterobius* occurs throughout the world, it is usually perceived as important only in temperate countries, primarily because it is the only common intestinal nematode there. Many characteristics of pinworms (e.g. their relatively low pathogenicity and their biology and mode of transmission, which is directly contaminative and not dependent on initial fecal dissemination into the environment) differentiate them from the other parasites covered in this chapter. *Ascaris*, hookworms and *Trichuris* are more pathogenic, they often occur together, their modes of transmission are facilitated by similar environmental and socio-economic factors, and they are studied epidemiologically as a coherent group. For these reasons, the soil-transmitted species will be considered both together and separately.

SOIL-TRANSMITTED NEMATODES: BIOLOGY AND FEEDING

Comprehensive summaries of current knowledge and assumed knowledge of specific aspects of the biology of *A. lumbricoides*, *A. duodenale*, *N. americanus*, and *T. trichiura* are available in reviews by Crompton (1989, 1994, 2001), Bundy and Cooper (1989), Schad and Warren (1990) and Misegna and Gilles (1987). We may assume that molting, energy metabolism, excretion and reproduction occur as described for animal-parasitic nematodes generally by Bird and Bird (1991), Barrett (1981), Lee and Atkinson (1976), and Adiyodi and Adiyodi (1983). Their feeding activities merit special consideration, partly because little is understood about how they obtain nutrients and energy while living as larval parasites and partly because of the role of the feeding of the adult stages in pathogenesis and chronic disease. Feeding is based on the development of the muscular esophagus to pump fluids or materials in suspension along a simple gut (Bennet-Clark 1976).

Ascaris lumbricoides

To ensure development of the eggs, the parent worm must have supplied the zygote with nutrients sufficient to

Table 3 *Some features of the life histories of* Ascaris, *hookworms and* Trichuris[a]

Feature	*Ascaris lumbricoides*	*Ancylostoma duodenale*	*Necator americanus*	*Trichuris trichiura*
Longevity in host (years)	1–2	1	3–5	1–3
Location of adult worms	Jejunal lumen	Jejunal mucosa	Jejunal mucosa	Tissue of large intestine
Reproductive strategy	Dioecious	Dioecious	Dioecious	Dioecious
Fecundity (eggs/female/day)	c. 240 000	10 000–25 000	5 000–10 000	14 000–20 000
Prepatent period (days)	67–76	53	49–56	60–90
Patent period (days)	c. 300–650	c. 300	c. 1 050–1 775	c. 300–1 000
Embryonation (days)	12 ± 2, at 31°C			28, at 25°C
Infective stage	L2 in eggshell[b]	Free L3	Free L3	L2 in eggshell
Max. survival of infective stage	Up to 15 years			Up to 6 years[c]
Usual transmission route	Oral	Skin/oral	Skin	Oral
Tissue migration	Liver and lungs	Not necessary	Lungs	
Arrested development		+[d]		

a) Abstracted mainly from Hoagland and Schad (1978); Bundy and Cooper (1989); Crompton (1994).
b) L, larva; L1, L2, L3, L4 refer to the first, second, third and fourth larval (juvenile) stages, there being a molt after each stage has completed its contribution to the life history.
c) Based on observations made on *T. suis* by Hill (1957).
d) Discovered by Schad et al. (1973).

ensure progression to the infective second-stage larva and subsequent survival. After hatching in the human gut, the larvae must either carry with them or obtain, through direct feeding on host tissues and metabolites, enough food and energy to ensure a successful migration back to the gut and a series of development molts. The tissue migrations of parasitic larvae have remained an enigma: are the parasites reliving their life cycles in a once intermediate but now definitive host? (Smyth 1994). Given that *A. lumbricoides* starts its association with its host in the gut and then returns there, perhaps natural selection would have been expected to have eliminated the migratory phase, with its exposure to the host's full array of immunological mechanisms, unless there were some compensatory benefits for the parasite (Read and Skorping 1995). Read and Skorping found that helminths that migrated grew relatively larger than those that did not. Nematode fecundity is linked to worm size, so a benefit for the survivors of the tissue migration becomes apparent.

Once *A. lumbricoides* is established in the lumen of the host's small intestine, it feeds on digestion products by pumping chyme through its own gut. In doing so, adult *A. lumbricoides* are likely to swallow bacteria including some that may be pathogenic to the worms, such as *E. coli* and *S. aureus* (Adedeja and Ogunba 1991). Wardlaw et al. (1994) found that the pseudocoelomic fluid of *A. suum* contained potent bactericidal activity that was not due to lysozyme. Antibacterial defense mechanisms would be expected to be present in an organism adapted to live in the intestine and feed on its intestinal contents.

Davey (1964), after examining *A. suum* obtained within half an hour from freshly slaughtered pigs, observed many pig intestinal epithelial cells in the parasites' guts. He estimated that whereas the concentration of free epithelial cells in the pig's gut would be expected to be about $6 \times 10^4/mm^3$, the concentration in the anterior part of the gut of the worms was about $4 \times 10^6/mm^3$. This finding suggests that *A. suum* may either selectively feed on discharged epithelial cells or even browse on the mucosa in addition to ingesting chyme. There is no doubt that the mucosal surface of a pig harboring intestinal stages of *A. suum* shows histological damage (Martin et al. 1984), a finding commensurate with the observed maldigestion of lactose in *Ascaris*-infected pigs (Forsum et al. 1981) and children (Carrera et al. 1984).

Ancylostoma duodenale and *Necator americanus*

Hatched larval hookworms feed voraciously on bacterial and organic matter until the non-feeding third-stage larvae are formed. Presumably, these contain sufficient stored energy to support skin penetration. It is not known whether newly penetrated third-stage larvae feed in the host or whether the next molt must occur and then the fourth-stage larva will feed actively. Third-stage larvae of *A. duodenale* entering the host by the oral route appear not to undergo a tissue migration (Pawlowski et al. 1991). Those larvae of *A. duodenale* that penetrate skin and migrate through tissue must need much more food and energy than those entering the oral route. The third-stage larvae of *N. americanus* must always penetrate skin and undergo tissue migration to reach the gut.

The intestinal stages of hookworms feed on blood obtained by puncturing the capillary network in the mucosa. Host blood is then pumped along the worm's gut and nutrient molecules absorbed from the host's plasma. Feeding by both *A. caninum* and *A. duodenale* involves the release of an anticoagulant secreted at the time of feeding (Hotez and Cerami 1983). Probably much of the blood taken by a hookworm as it feeds passes through its body into the host's gut lumen (Crompton and Whitehead 1993). When the worm bites into the mucosa at a second site, the lacerations at the first site, together with residual anticoagulant activity, mean that further blood loss occurs. From an experimental study of *A. caninum* in dogs, Wang et al. (1983) concluded that an individual female worm pumped about 0.043 ± 0.04 ml of host blood per day and that a further 0.046 ± 0.019 ml was lost as a hemorrhage from the abandoned feeding site. Female worms cause more blood loss than males.

Trichuris trichiura

Despite current interest in the biology of whipworms, largely stimulated by the work of Bundy and colleagues (see Bundy and Cooper 1989), there remains some controversy about their feeding activities. Do the worms suck blood, a view supported by Beck and Beverley-Burton (1968), or do they digest host tissues by releasing proteolytic enzymes (see Watson 1960)? Burrows and Lillis (1964) clearly demonstrated that *T. vulpis* feeds on dog blood. They found dog blood in the gut of *T. vulpis* and by direct observation described how the worms, through a series of rapier-like slashing movements, could lacerate capillaries and then pump released blood into their guts. They also found blood in the gut of *T. trichiura*. The structure of the buccal apparatus with its piercing stylet also suggests that blood-feeding is its probable purpose. Furthermore, rectal bleeding and iron-deficiency anemia are characteristics of some patients suffering from trichiuriasis (Fisher and Cremin 1970; Lotero et al. 1974; Bundy and Cooper 1989). The etiology of iron deficiency anemia is complex and may not arise directly from the feeding activity of *T. trichiura*; the nutritional status of the hosts and polyparasitism are two of the factors which must be accounted for. Nevertheless, whipworms undoubtedly

ingest host blood obtained from the mucosa of the large intestine.

SOIL-TRANSMITTED NEMATODES: LIFE HISTORIES

The direct life-history patterns of *A. lumbricoides*, *A. duodenale*, *N. americanus* and *T. trichiura* are well understood in general terms and can be represented diagrammatically in the form of a flow chart (Figure 3). This scheme is important because it focuses attention on the fact that a soil-transmitted nematode exists in two populations: a free-living cohort based on the eggs and related early larval stages, and an endoparasitic cohort based on the later larval stages and the adult worms in the gut. More detailed information about the life histories is summarized in Table 3, and much recent information is available in articles edited by Crompton et al. (1985, 1989) and Schad and Warren (1990), and in the review by Bundy and Cooper (1989). Aspects of the life histories are treated in some detail in the sections dealing with transmission and with epidemiology.

SOIL-TRANSMITTED NEMATODES: TRANSMISSION AND ESTABLISHMENT OF INFECTION

Ascaris lumbricoides

Infective second-stage larvae of *A. lumbricoides* must usually be swallowed by the human host in order to initiate the establishment of an infection. The larvae escape from the egg, presumably in response to the same stimuli identified by Rogers (1960) for the hatching in vitro of eggs of *A. suum*. Experimental work by Murrell et al. (1997) has shown that *A. suum* L_2 in the pig host almost exclusively invade the wall of the cecum and colon en route to the liver, rather than doing so into the intestine as was generally thought. The findings of these authors raise important questions as to whether *A. suum* and *A. lumbricoides* share a similar migratory pathway and may suggest a greater potential for liver damage in human hosts. If the time course for *A. lumbricoides* in humans is the same as that for *A. suum* in pigs, mice, and rabbits, the larval stages spend about 4 days in the liver and about 14 days in the lungs and then begin to re-enter the gut via the bronchi and trachea. At least 65 days must pass after infection before eggs are first detected in the stools (Table 3; Takata 1951).

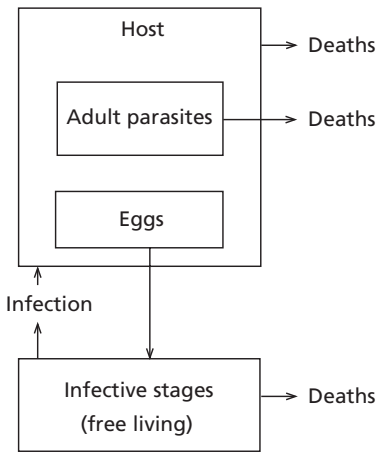

Figure 3 *Life-history pattern of soil-transmitted nematodes*

The eggs of *A. lumbricoides* are produced in vast numbers (Table 3) and, although many will either perish during embryonation due to exposure to ultraviolet radiation or become inaccessible to humans through the activities of earthworms or pass into the soil during rainfall, enough survive to ensure the persistence of the infections. In a recent experimental study carried out with eggs of *A. suum* contaminating soil in Poland, Mizgajska (1993) found that 0.1–4.5 percent of the eggs retained infectivity (observed larval movements) for 17 months and most of these had remained within the top 50 mm of the soil. During this period, some eggs had reached a depth of 210 mm in the soil. Any community lacking facilities for the safe disposal of human feces will remain vulnerable to infection with *A. lumbricoides*, and use of untreated night-soil as a fertilizer for vegetables will probably increase the risk of infection. The complex eggshells of *A. lumbricoides* (Wharton 1980) protect the larvae from mechanical, chemical, and physical damage, and the external mucopolysaccharide ensures that the eggs adhere to objects. In areas where infection is endemic, infective eggs have been found adhering to cooking and eating utensils, money, furniture, door handles, fruit, vegetables, and fingers (Kagei 1983), as well as contaminating the soil in households, gardens, and public parks (Morishita 1972; Yadav and Tandon 1989; Wong and Bundy 1990). Some evidence suggests that the eggs can become airborne and be inhaled in dust, thus gaining access to the alimentary tract (Bidinger et al. 1981; Kroeger et al. 1992).

Although there need be no doubt that transmission nearly always depends on swallowing eggs from a contaminated environment, other routes of transmission may also exist. For example, three case studies indicate that *A. lumbricoides* may occasionally pass the placenta (Chu et al. 1972; Rathi et al. 1981; da Costa-Macedo and Rey 1990) and, since larval *Ascaris* can be transplanted between hosts experimentally, there is even the slight chance that infections may be established inadvertently through organ-transplant surgery.

Ancylostoma duodenale and *Necator americanus*

The third-stage larvae of hookworms are responsible for transmission and the establishment of infections.

Hoagland and Schad (1978) have concluded that *A. duodenale* is an opportunistic species, partly because the third larval stages (Table 3) can penetrate skin or enter via the oral route. Recent evidence from China indicates that larval *A. duodenale* may pass from the mother to infect the fetus in utero; transmammary infection with *A. duodenale* cannot be ignored (see Banwell and Schad 1978).

Under favorable conditions, first-stage larvae hatch from hookworm eggs within about 24 hours of feces being deposited on the soil. One of the factors which explains why *A. duodenale* is not confined to tropical and subtropical countries is the ability of its eggs to survive at 7°C even if embryonation is not occurring (Beaver and Jung 1985). Hookworms flourish particularly in rural communities where there are long-established traditions of the daily use of defecation fields. The rhabditiform larvae feed on bacteria and organic debris, molt and continue to feed as second-stage larvae. These molt in turn to form third-stage filariform larvae, which no longer feed, but wait for the conditions facilitating skin contact and penetration. Hookworm larvae that fail to make contact with a susceptible host probably die from desiccation within a few days. According to Schad, even under the most favorable survival conditions of shade, moisture, and soil texture, probably less than 1 percent of a batch of infective larvae will live for more than a month (see Banwell and Schad 1978).

Skin penetration by hookworms has been studied in most detail using a hamster-adapted strain of *N. americanus*. Based on earlier work which showed that larval enzymes were involved (Matthews 1982), Salafsky et al. (1990) developed an artificial membrane that the larvae would attack and penetrate. They found that the presence of human essential fatty acids in the membrane significantly increased the ability of the larvae to penetrate in vitro and that these acids, especially linoleate, influenced the secretion of eicosanoid compounds by the larvae. Eicosanoids affect T and B cells and so may modulate the host's immune response during skin penetration (Salafsky et al. 1990).

Hoagland and Schad (1978) took the view that *N. americanus* was less of an opportunist than *A. duodenale* with less flexibility in the infection process. *Ancylostoma duodenale* can function as a food-borne infection (Schad et al. 1983).

Trichuris trichiura

The transmission and establishment of an infection of *T. trichiura* has much in common with that of *A. lumbricoides*, being dependent on the ingestion of an infective egg from a contaminated environment. The overall success of the process depends on the numbers of infective eggs, their accessibility to humans and their survival characteristics, which are related to climatic and soil conditions (see Bundy and Cooper 1989). Geophagia was demonstrated to be a significant risk factor for exposure to *Trichuris* among 10- to 18-year-old Kenyans, with a threefold higher intensity of reinfection with *T. trichiura* 11 months after treatment in geophagic individuals compared to those who did not exhibit geophagia (Geissler et al. 1998).

SOIL-TRANSMITTED NEMATODES: EPIDEMIOLOGY AND POPULATION BIOLOGY

Knowledge of the epidemiology and population biology of soil-transmitted nematodes has advanced rapidly during the last decade. Recognition of the need to quantify the public health significance of the infections and the development of a mathematical approach to study how these infections survive have given impetus to this work. The theoretical aspects of the population biology of the helminths have also led to the development of models for:

- the control of soil-transmitted helminth infections by means of chemotherapy (Anderson 1989)
- evaluating the effects of control measures (Medley et al. 1993)
- estimating the morbidity attributable to the infections (Guyatt and Bundy 1991; Lwambo et al. 1992; Chan et al. 1994)
- simulating host immune responses (Anderson 1994).

Some workers have expressed concern about the lack of validation of some of the models and the fact that they are generally based on limited data (Goodman 1994). This view does, however, not detract from their value in helping to understand the relationships between humans, roundworms, hookworms, and whipworms.

The four common species of soil-transmitted nematode are particularly amenable to study and the following epidemiological generalizations apply.

- Accurate diagnosis can be made by the detection of eggs in the stools.
- Counts of eggs in defined quantities of stool can give a useful comparative indirect measure of the intensity of infection, assuming that the egg count increases as the number of female worms in the gut increases.
- In practice, density-dependent constraints apply to the individual fecundity of female worms; after a certain worm burden has been reached, individual egg production will decline.
- The life histories are direct and the population of worms in a host does not increase unless new infective stages are acquired.
- The distribution of numbers of worms per host is over-dispersed or aggregated so that in a given population of hosts a few will tend to harbor most of the worms.

- Individual hosts appear to be predisposed to a particular infection intensity.

Detailed treatments of the principles underlying the epidemiology and population biology of soil-transmitted nematodes are documented and developed by Bundy (1986, 1988), Anderson and May (1985, 1991), and Scott and Smith (1994). Perhaps Croll and Ghadirian's (1981) research on 'wormy persons', which dealt with infections on *A. lumbricoides*, *A. duodenale*, *N. americanus* and *T. trichiura* was the catalyst for much of the endeavor that has been applied to understanding soil-transmitted nematodes. In this section of this chapter, the four species are treated together since their population biology has so much in common and they often occur together in the same community or individual.

Global distribution and abundance

Crompton (1989) reckoned that human infections with *A. lumbricoides* were occurring in 150 of the world's countries; some national situations were trivial but others were probably of considerable public health significance. The same global pattern of infection probably applies to the hookworms and to *T. trichiura*. Of the hookworms, *N. americanus* prevails in tropical and subtropical regions, whereas *A. duodenale* tends to occur in the cooler and somewhat drier regions (Pawlowski et al. 1991). Mixed infections of hookworms also exist, especially in northern India and the middle of China. Bundy and Cooper (1989) pointed out that while *T. trichiura* overlaps with *A. lumbricoides* in its distribution, it is also still a problem in some temperate countries. Estimates of the numbers of cases of infection with the four species of soil-transmitted helminth are given in Table 1. Overall, at least a fifth of the world's population is infected. The recent collaboration of scientists between China and other countries has enabled new estimates to be made of the numbers of cases of *A. lumbricoides* in that country. Chan et al. (1994) estimated 568 million cases and Peng et al. (1995) have calculated the number to be 538 million, indicating that the national prevalence of *A. lumbricoides* infection in China is about 47 percent. A national prevalence figure, however, belies the fact that infections like *A. lumbricoides* in humans are not distributed evenly between communities (Crompton 1989; Yu 1994; Peng et al. 1995). Climate, local economies, social customs, and ethnicity all influence the distribution.

Prevalence

The results of surveys to estimate the prevalence of these infections are of limited usefulness. They identify locations where infections occur and provide information about the numbers of cases; they contribute little to assessments of the extent of morbidity. Apart from providing information about the number of cases, the most useful aspect of prevalence data is to examine the relationship between prevalence and host age. Typical patterns are shown diagrammatically for *A. lumbricoides*, hookworm, and *T. trichiura* (Figure 4). These relationships have been detected in many surveys carried out in many countries (Bundy and Cooper 1989; Anderson and May 1991; Crompton 1994). The data are generally obtained by examining stool samples for the presence of helminth eggs, the stools having been obtained during cross-sectional surveys. The conduct of a survey is often complex and the reader is recommended to consult the protocol described by Thein Hlaing (1989).

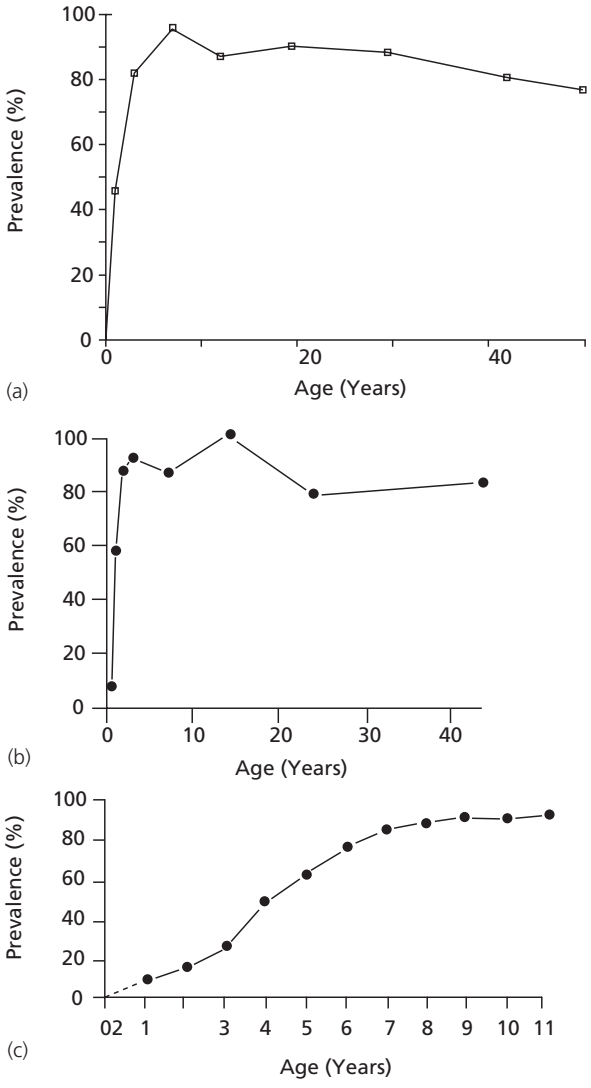

Figure 4 *Diagrammatic representations of the age–prevalence pattern between human hosts and soil-transmitted nematodes:* **(a)** Ascaris lumbricoides*;* **(b)** Trichuris trichiura*;* **(c)** *hookworm (Redrawn from:* **(a)** *Elkins et al. 1986;* **(b)** *Bundy 1986;* **(c)** *Nawalinski et al. 1978)*

The relationship between prevalence and age reveals that *A. lumbricoides* and *T. trichiura* become established in infants, soon after weaning (Figure 4a, b). Maximum prevalence values are attained in most cases when children reach the age of 10 years and then a gradual decline with age is observed. The age–prevalence pattern is somewhat different in the case of hookworm infections, there being a more gradual rise in the number of cases with plateau values observed as people reach young adulthood (Figure 4c).

Intensity

The population biology and public health significance of soil-transmitted nematodes cannot be understood, and strategies for their control cannot be developed, without reliable information about the intensities of infection. Intensity in this context is defined as the number of worms per infected host and it can be measured directly by counting worms expelled with stools after anthelmintic chemotherapy or indirectly by counting eggs in known amounts of stool.

Provided there is good compliance, expulsion chemotherapy is the preferred method for measuring intensity, but it relies on highly effective anthelmintic drugs. Ideally, all the worms present in the host's intestine should be expelled within 24 hours so that relatively few stools have to be examined. This is generally the case for *A. lumbricoides*. Hookworms and *T. trichiura* are much smaller than *A. lumbricoides* and are much harder to find in the stools. Furthermore, more than one dose of drug and more than one stool collection may be required to release and recover all the *T. trichiura* from the large intestine (Bundy and Cooper 1989). Although quantitative egg counts are much easier to perform and many more subjects can be investigated in an intensity survey as a result, the information is inevitably less accurate than that obtained from the expulsion method. Egg counts may fail to give information about intensity if the infection consists of male or immature worms only or if there are density-dependent constraints on female worm fecundity (Thein Hlaing et al. 1984). Generally, egg counts using the Kato Katz method (WHO 1994) do provide a useful estimate of intensity with the number of eggs per gram (epg) of stool being observed to increase as the worm burden increases (Forrester and Scott 1990).

The typical relationship between infection intensity and host age is shown diagrammatically in Figure 5 for *A. lumbricoides*, hookworms, and *T. trichiura*. During childhood, the age–intensity profile for *A. lumbricoides* and *T. trichiura* mirrors that for the age–prevalence profile, there being a rapid acquisition of worms. In later life, the intensity of these two species declines (Figure 5a, b); due to the development of some degree of immunity or to changes in behavior or

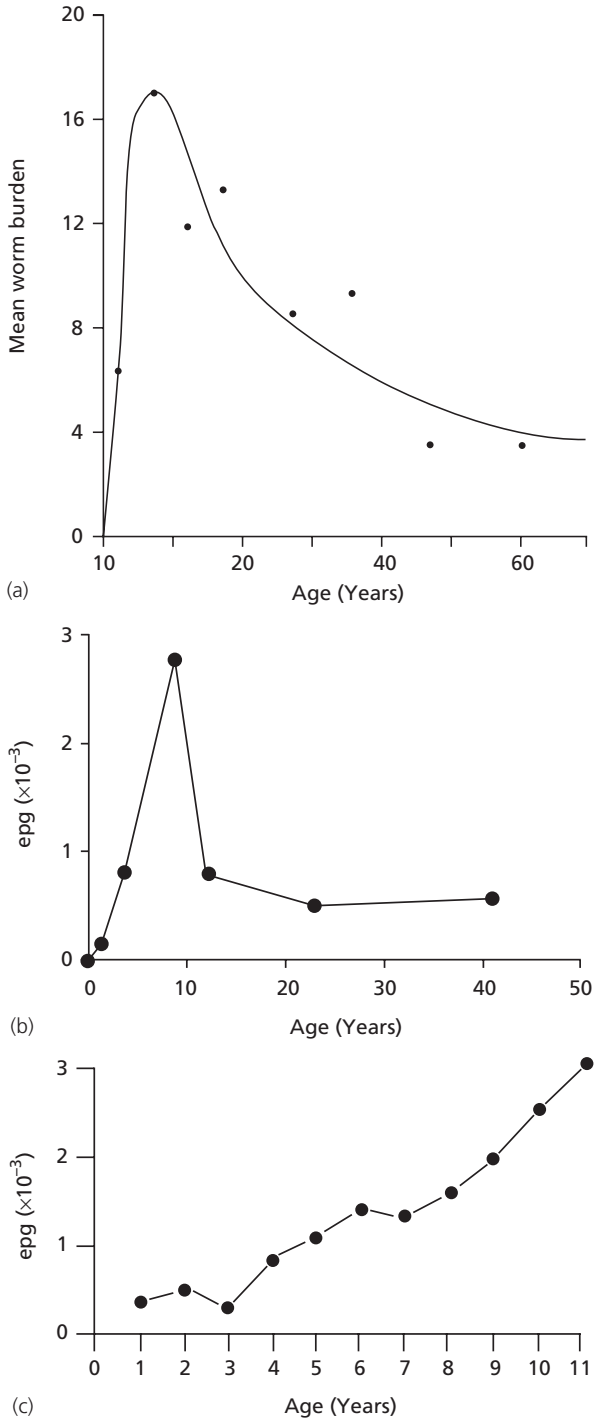

Figure 5 *Diagrammatic representations of the age–intensity patterns between human hosts and soil-transmitted nematodes:* **(a)** Ascaris lumbricoides*;* **(b)** Trichuris trichiura*;* **(c)** *hookworm (Redrawn from:* **(a)** *Thein-Hlang 1985;* **(b)** *Bundy 1986;* **(c)** *Schad and Anderson 1985)*

to a combination of these factors. The intensity of hookworm infections follows the same trend as the age–prevalence relationships (Figure 5b) with adults tending to carry the greater worm burdens. These age–intensity relationships determine which sections of the

population will be most at risk of morbidity, which will be responsible for most contamination of the environment with transmission stages, and which will be most in need of treatment if resources become available.

Frequency distribution of numbers of worm per host

Prevalence and intensity data reveal that soil-transmitted nematodes are not distributed randomly in host populations; most infected hosts harbor a few worms each, while a few hosts harbor most of the worms (Anderson 1986; Bundy 1986; Crompton 1994). The observed pattern (Figure 6) is best described by the negative binomial distribution and the worms are said to be aggregated or over-dispersed. When the negative binomial model is fitted to worm frequency distribution data, the aggregation constant (k) is usually found to be <1, indicating aggregation (Anderson 1986). Similarly, if the ratio of the variance to mean number of worms in the population under study ($S^2/\bar{x}$) is >1, aggregation or over-dispersion is also confirmed (Anderson and Gordon 1982).

Predisposition to intensity of infection

Predisposition, in the context of soil-transmitted nematodes, is the term used to describe the observation that after anthelmintic chemotherapy, individuals seem to acquire worm burdens similar to those they harbored before treatment. The observation appears to be secure whether expelled numbers of worms or egg counts are used as measures of intensity.

Predisposition is now a well-established epidemiological feature of *Ascaris* (Elkins et al. 1986), *Trichuris* (Bundy et al. 1987), and hookworm infection (Schad and Anderson 1985). Evidence for multiple species predisposition (*Ascaris*, *Trichuris*, hookworm, and *Enterobius*) was also provided by Haswell-Elkins et al. (1987a). The factors that contribute to the observed predisposition and their relative importance have been the subject of review and debate by a number of authors (Bundy 1988; Keymer and Pagal 1990; Bundy and Medley 1992; Holland and Boes 2002). Whether predisposition is a feature of long-term causal factors, such as host genetics and host socioeconomic status, or short-term factors, such as the host acquired immune response, is obviously important for the design of appropriate control strategies. Recently, Quinnell et al. (2001) assessed reinfection and predisposition to *N. americanus* in a rural village in Papua New Guinea over an 8-year period. Interestingly, predisposition could be detected 6–8 years after a single round of chemotherapy but was not detectable after repeated chemotherapy. The authors concluded that differences in susceptibility are likely to influence predisposition but that longer-term variation in either exposure or susceptibility limits the period over which significant predisposition can be detected. Holland and Boes (2002) reviewed the evidence available from field-based studies concerning the relationship between measures of host exposure or susceptibility and reinfection or predisposition to geohelminth infection. Two studies concerning the involvement of the immune response illustrate the difficulties experienced in trying to unravel this complex phenomenon.

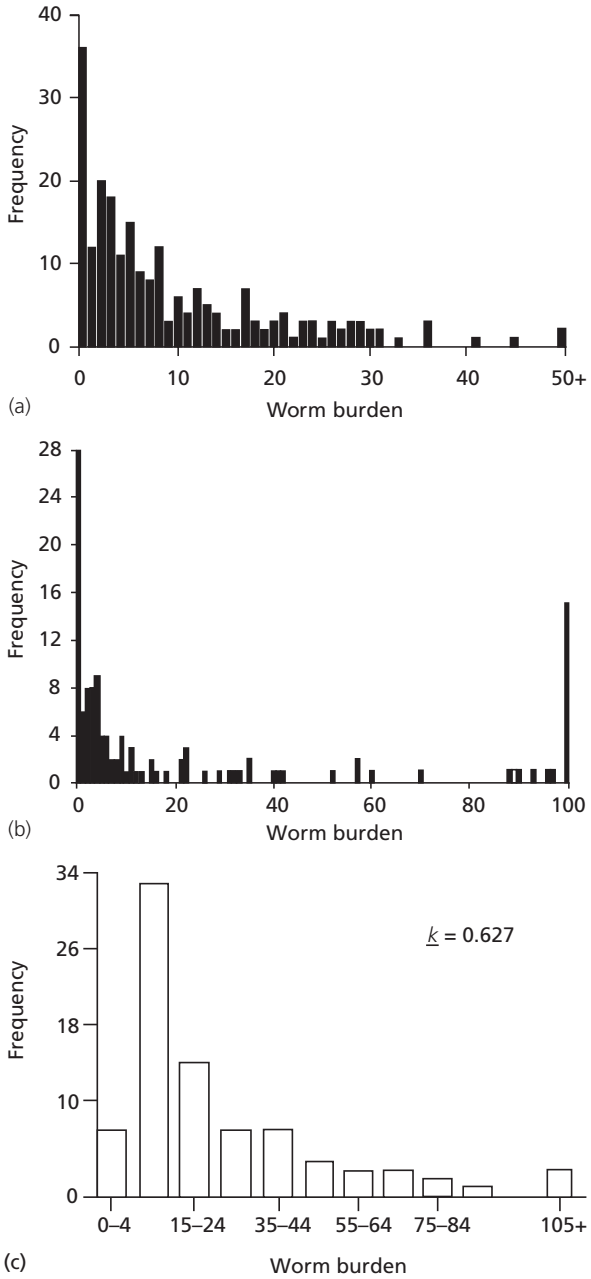

Figure 6 *Diagrammatic representations of the frequency distribution of numbers of worms per host:* **(a)** Ascaris lumbricoides*;* **(b)** Trichuris trichiura*;* **(c)** *hookworm (Redrawn from:* **(a)** *Elkins et al. 1986;* **(b)** *Bundy 1986;* **(c)** *Schad and Anderson 1985)*

Palmer et al. (1995), in a carefully designed case-control study, compared consistently lightly infected subjects with those consistently heavily infected. A range of antibody isotypes were measured, including total immunoglobulin (Ig)G, IgG1, IgG2, IgG3, IgG4, IgA, total IgE, and parasite-specific IgE. Children who were predisposed to heavy infection showed higher concentrations of antibody isotypes compared to children predisposed to light infections. In contrast to the findings of Hagel et al. (1993), the concentrations of total IgE and parasite-specific IgE in this study mirrored the infection intensity of the subjects. The authors do not rule out an effector role for these antibodies, but suggest that the utilization of more specific antigens may rule out polyspecific responses to numerous antigens that may mask any epitope-specific protective responses.

This point was borne out in a study by McSharry et al. (1999) who compared a range of serum factors in children predisposed to remain uninfected, lightly infected, and heavily infected. These groups of children showed few differences in measures of socioeconomic status and lived in environments where samples of soil contained eggs of *Ascaris*, assumed to be those of *A. lumbricoides*. Three different sources of *Ascaris* antigen were used but only the most defined allergen, *Ascaris* rABA-1, provided evidence for a significant relationship between predisposition status and parasite-specific IgE. A subgroup of children, who responded to the ABA-1 allergen, was selected and a relationship between reduced rABA-1 specific IgE titer and increasing parasite load was detected. Subjects were further divided according to high or low levels of IgE antibody using a threshold median value, and a distinct pattern emerged. The putatively immune group tended to have higher levels of rABA-1 specific IgE and the susceptible groups had low levels. Significantly higher levels of inflammatory indicators – such as serum ferritin, eosinophil cationic protein and C-reactive protein – were detected in the putatively immune group. The authors concluded that IgE responses, in conjunction with innate inflammatory responses, are associated with natural immunity to ascariasis.

Remarkably little work has been performed on the relationship between susceptibility to human helminths and host genetics (in contrast to genetic studies in laboratory and other animals), but recent contributions by Williams-Blangero and colleagues have provided important evidence for the role of host genetics in helminth aggregation and predisposition. In a large-scale study, which involved 1 261 subjects, all of whom belonged to the same pedigree, these authors demonstrated a strong genetic component accounting for 30–50 percent variation in *Ascaris* worm burden. Sharing a household accounted for only 3–13 percent of the total phenotypic variance (Williams-Blangero et al. 1999). Furthermore, a variance components linkage analysis resulted in the localization of two genes (one on chromosome 1 and another on chromosome 13) with clear, significant effects on susceptibility to *Ascaris* infection (Williams-Blangero et al. 2002).

SOIL-TRANSMITTED NEMATODES: CLINICAL MANIFESTATIONS, MORTALITY, AND MORBIDITY

Many of the millions of people infected with soil-transmitted nematodes may show no signs or symptoms of any ill health. Clinical problems are generally related to the intensity of infection and so only the relatively few hosts who carry the heavy worm burdens will be expected to be ill. Nevertheless, diseased individuals will not be numerically rare (Bundy and Cooper 1989). Chan et al. (1994) have estimated that there may be 120–215 million cases of morbidity due to *A. lumbricoides*, 90–130 million due to hookworm, and 60–100 million due to *T. trichiura*.

The difficulties encountered in estimating morbidity rates include:

- the problem of deciding what constitutes a clinical case (see Pawlowski 1982)
- the possibility that the infections exacerbate existing problems rather than cause them
- the scarcity of accurate records and reporting, particularly in the regions where the infections are highly endemic.

In such regions, growth stunting should be considered as a feature of chronic ascariasis, hookworm disease, and trichuriasis; iron-deficiency anemia as a feature of hookworm disease and sometimes of trichuriasis; and rectal prolapse and chronic dysentery as features of trichuriasis. These conditions, the mechanisms by which they arise and the related pathology have been summarized by Holland (1987a, b), Stephenson (1987), Bundy and Cooper (1989), and Tomkins and Watson (1989). The impact of each infection invariably has its main effect on a different section of the population and the threshold burden responsible for causing disease may vary depending on local conditions.

Mortality rates are even more difficult to estimate although people regularly die as a result of soil-transmitted nematode infections. Pawlowski and Davis (1989) considered that perhaps 100 000 people die annually from ascariasis and Pawlowski et al. (1991) suggested that 60 000 die from hookworm disease.

Ascariasis

The illness associated with *A. lumbricoides* infection occurs in acute and chronic forms (Table 4). The acute forms may arise from the larval migration through the lungs, from allergic responses or from complications involving the adult worms.

Table 4 *Features of morbidity due to ascariasis (based on Stephenson 1987)*

Stage	Event	Clinical features	Outcome
Larval migration	Migration of larvae through liver and lungs	Pneumonitis, asthma, dyspnea, cough, substernal pain	?Decrease food intake
Maturation, oviposition	Presence of juveniles and patent adult worms in small intestine	Abdominal pain, abdominal distention, colic, nausea, vomiting, intermittent diarrhea, anorexia, restlessness, anal itching, enterocolitis; disordered small bowel pattern, jejunal mucosal abnormalities	Decrease food intake; increase nutrient loss; malabsorption of protein, fat, d-xylose, lactose, and vitamin A
Allergic reaction	Exposure to *Ascaris* allergen at any stage of life cycle[a]	Hypersensitivity reactions including asthma, conjunctivitis, facial edema, urticaria abdominal pain, heartburn, diarrhea	Decrease food intake; increase nutrient loss
Complications	Migration or aggregation of adult *Ascaris* in intestine	Intestinal obstruction, intussusception, volvulus; invasion of bile duct (producing obstructive jaundice, gallstones, cholangitis, or liver abscesses); acute pancreatitis, acute appendicitis, intestinal perforation, peritonitis, upper respiratory tract obstruction	Life-threatening illnesses that all decrease food intake and may increase nutrient requirements (due to fever) and nutrient losses (due to diarrhea)

a) Uninfected individuals can also develop *Ascaris* allergy.

Larval migration leading to acute pulmonary ascariasis is associated with fever, skin rash, pneumonitis, substernal pain, and eosinophilia (Stephenson 1987). The problem appears to be more serious in regions like Saudi Arabia where climatic conditions ensure that *A. lumbricoides* is transmitted seasonally (Gelphi and Mustafa 1967). Stephenson pointed out that larval *A. lumbricoides* will remain associated with respiratory complications rather than being identified as a causative agent. However, the deliberate self-infection by Koino (1922) and the malicious contamination of a festive meal with infective eggs of *A. suum* described by Warren (1972) indicate that the larval stages initiate acute pulmonary disease.

Larval and adult *A. lumbricoides* and *A. suum* secrete allergens that elicit the production of IgE by the host leading to hypersensitivity and histamine release (Pawlowski 1982; Coles 1985). Hypersensitivity may be induced in uninfected laboratory workers studying the worms. With its most serious form, a sensitized person will collapse with bronchospasms. There is little quantitative information about the extent of this aspect of the biology of *A. lumbricoides*. In both infected and uninfected people, the presence of *Ascaris* appears to either stimulate or complicate asthma but more research is needed to clarify this problem. Lynch and colleagues have undertaken a number of studies on the relationship between *Ascaris* and asthma in Venezuelan patients (Lynch et al. 1992a, 1992b, 1997) and provided some evidence for the amelioration of asthmatic symptoms for a period of up to 2 years following anthelminthic treatment (Lynch et al. 1997). Experimental infections of rhesus monkeys with eggs of *A. suum* induces an IgE-mediated pathology in the lungs (Patterson and Harris 1985).

Acute ascariasis involving adults presents as a variety of obstructions and surgical complications (Table 5). Among children, intestinal obstruction caused by a bolus of worms appears to be the commonest problem and may lead to tissue necrosis and perforation of the intestinal wall. From the data summarized in Table 5, it would appear that girls suffer more intestinal obstructions than boys. Louw (1966) drew attention to the importance of *A. lumbricoides* as a cause of abdominal complications by recording that 100 (12.8 percent) of the 731 abdominal emergencies in children (aged 1–12

Table 5 *Complications of acute ascariasis related to adult* Ascaris lumbricoides *in relation to patient age and sex (Crompton 1994)*

		Age		Sex		Deaths	
Complication	All cases	Child	Adult	Male	Female	Child	Adult
Biliary system	1 124	380	374	92	117	4	23
Gastrointestinal tract	3 408	2 149	73	572	716	85	9
Hepatic abscess	100	52	6	17	15	13	1
Pancreatitis	67	28	26	11	15	1	2
Miscellaneous complications	94	77	15	50	41	9	3
Totals	4 793	2 686	494	742	964	112	38

The cases recorded in this table were abstracted from 230 reports published between 1971 and 1992. The cases described were from 54 countries. Often reports were found to be incomplete, with no information given about the age or gender of the patients.

years) admitted between 1958 and 1962 to a hospital in Cape Town, South Africa, were due to *A. lumbricoides*. Of the 100 cases, 68 proved to be intestinal obstructions. Among adults, complications involving the biliary system appear to be the commonest acute complication due to *A. lumbricoides* (Table 5). Presumably, the adult worms migrate from small intestine up the common bile duct where they may remain or may move on to the liver or to the pancreas. It is not known why adult worms leave the intestine; in some cases, larval worms may have reached unusual sites during the natural tissue migratory phase. That might explain how *A. lumbricoides* have been recovered from sites such as the middle ear (Berkowitz et al. 1980). Details of the diagnosis and management of cases of acute ascariasis due to adult worms have been given by Pinus (1985) and Erdener et al. (1992) among others.

Chronic ascariasis (Table 4) has been extensively studied in children in many locations during the past 20 years (Crompton 1992; Thein Hlaing 1993). Given that the study design is adequate and there is good compliance in the study population (Crompton and Stephenson 1985), convincing circumstantial evidence can be obtained to show that infection with *A. lumbricoides* interferes with the growth and development of children, especially during the period of 2–10 years of age. This conclusion is based on a study design in which the nutritional status (height and weight for age) was compared before and after intervention with an effective anthelmintic drug. The adverse contribution of chronic ascariasis to childhood nutritional status has been demonstrated in various countries worldwide (Gupta et al. 1977; Stephenson et al. 1980a; Thein Hlaing et al. 1992; see Tomkins and Watson 1989), although not every investigation has detected this effect.

Various experimental studies of ascariasis in pigs and clinical investigations of ascariasis in humans have shown that the presence of adult worms in the small intestine is associated with some degree of crypt hyperplasia and villous atrophy (Tripathy et al. 1972; Stephenson et al. 1980b; Martin et al. 1984). Under certain conditions, maldigestion and malabsorption occur (Forsum et al. 1981; Carrera et al. 1984) with recovery being observed following anthelmintic treatment (Taren et al. 1987). Probably the most serious aspect of an *Ascaris* infection is a reduction in food intake, which has been well documented for *A. suum* infections in pigs (see Nesheim 1985) and has been demonstrated more recently by Jalal (1991) in rural children in Indonesia.

Currently, ascariasis (and hookworm disease and trichuriasis) should be considered as contributors to different degrees of childhood malnutrition. Infections with soil-transmitted nematodes have also been convincingly linked to impaired cognitive performance in young children (Nokes et al. 1992; Nokes and Bundy 1994; Simeon et al. 1994). It may be wiser to view these infections as one of the many interacting socioeconomic determinants that impair cognitive performance (see Connolly and Kvalsvig 1993).

Hookworm disease

Not all the people infected with hookworms suffer from iron-deficiency anemia, but many do and many of them are at risk because the intestinal stages feed on blood and cause a loss of blood into the intestinal lumen (Roche and Layrisse 1966). Anemia is defined as a reduction in blood hemoglobin concentration below expected values for age and sex (WHO 1972). In physiological terms, in an anemic individual, the circulating number of red blood cells becomes insufficient to meet the oxygen needs of the body and the symptoms of a severe hookworm infection mimic those of anemia: breathlessness, lassitude, headache, palpitations, apathy and depression. Often occult blood is found in the stools (Holland 1987a). Studies from many countries including India, Kenya, Thailand, and Venezuela have detected the relationship depicted in Figure 7 (Hill and Andrews 1942). Blood hemoglobin concentration in adults is observed to fall in a nonlinear manner in relation to increasing hookworm infection intensity. A value of 100 mg/ml in an adult is judged to indicate anemia (WHO 1972).

Whether anemia develops or not during the course of a hookworm infection depends not only on the intensity of the infection and species of hookworm (*A. duodenale* takes more blood than *N. americanus*), but also on the iron status and physiological needs of the host, the

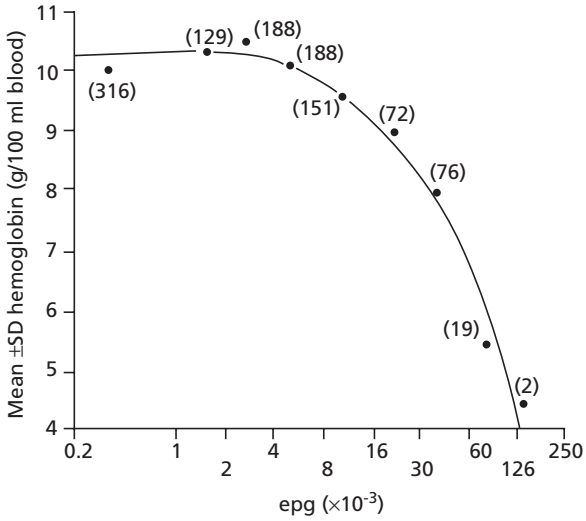

Figure 7 *The relationship between declining blood hemoglobin concentration and increasing hookworm intensity, expressed as egg-count classes, obtained from a study of 1 141 residents of South Georgia, USA. The egg counts have been corrected to a formed-stool basis (Data from Hill and Andrews 1942)*

quality and quantity of the daily iron intake and the bioavailability of the iron for absorption from the small intestine. This complex relationship has been discussed in detail by Holland (1987a), Crompton and Stephenson (1990), and Crompton and Whitehead (1993). Maintaining the required number of circulating red blood cells probably has a high priority for iron metabolism, so hookworm-infected individuals who are not clinically anemic may have a deteriorating iron status and latent iron deficiency. Measurements of plasma ferritin concentration, transferrin saturation, and erythrocyte protoporphyin would probably indicate that clinically nonanemic individuals with hookworm infections are under iron stress, as has been demonstrated by Mansour et al. (1985) for people suffering from schistosomiasis mansoni. Stoltzfus and colleagues studied the impact of hookworms on the iron status of Zanzibar schoolchildren (Stoltzfus et al. 1997, 1998). They demonstrated that in the absence of iron supplementation, anthelminthic treatment of varying frequency significantly reduced the risk of these children developing both moderate-to-severe and severe anemia (Stoltzfus et al. 1998).

Since many features of hookworm disease indicate iron-deficiency anemia, it is to be expected that women and girls of child-bearing age, pregnant women, and people engaged in heavy manual labor will be most at risk from the adverse effects of infection. On average, menstruating females lose about 1.5 mg iron per day, about the same amount as is absorbed from the gut under optimal conditions (Crompton and Whitehead 1993). Although there is no menstrual loss during pregnancy and lactation, the mother must meet all the iron requirements of the developing fetus and the suckling infant. H. Torlesse (personal communication 1994) has recently estimated that at any given time about 30 million women are currently pregnant and infected with hookworm. Many of these women must be experiencing acute anemia exacerbated by the demands of pregnancy and the blood loss due to hookworms. The birth of underweight children is a major consequence of anemia during pregnancy, and, in developing countries, the deaths of about 200 000 women can be ascribed to anemia as a complication of childbirth or the early postpartum period (Viteri 1994). As many as 1 in 25 pregnant women die in some developing countries due to complications associated with the pregnancy and birth (WHO 1995).

Worker productivity in relation to anemia has attracted considerable research attention. Study designs have sought to examine the cost–benefit ratios in financial terms of expenditure to relieve anemia (iron supplements and antiparasite drugs) compared with the increased revenue from increased productivity by the nonanemic workers. The results have shown that anemic sugar cane cutters (Viteri and Torun 1974), road builders (Brookes et al. 1979; Wolgemuth et al. 1982), rubber tappers (Basta et al. 1979), and tea pickers (Gardner et al. 1977) do not have the same work output as either nonanemic controls or those given treatment to relieve the anemia. Vigorous physical work is difficult to carry out and sustain once blood hemoglobin concentration has fallen to a value of 7 mg/ml (see Figure 7). In developing countries, where productivity depends largely on manual labor, widespread hookworm infections may make a substantial contribution to a depressed economy (Holland 1987a).

Iron deficiency anemia is a factor that reduces cognitive performance: on measures of achievement at school, anemic children usually show lower scores than their nonanemic counterparts (see Connolly and Kvalsvig 1993). According to Pollitt (1990) there is sufficient evidence to conclude that iron-deficiency anemia is linked to impaired educational performance. Again hookworm infections, with their contribution to iron loss, must contribute to this problem. It should be noted that reduced appetite and loss of nutrients including zinc (Migasena et al. 1984) through plasma leakage into the gut, together with malabsorption, which also occurs in some cases, are likely to affect school performance, as well as growth (Holland 1987a) and physical fitness (Stephenson et al. 1993).

Trichuriasis

The pathology of an infection with *T. trichiura* involves chronic inflammation of the mucosa of the large intestine associated with the intimate contact that the worms make with the mucosa. The degree of inflammation may extend from the distal part of the small intestine to the rectum depending on the intensity of the infection (Bundy and Cooper 1989). Since the infection is confined largely to the large intestine, malabsorption of digestion products is not likely to be a serious consequence. However, the lacerations caused by the feeding activities of the worms may enable secondary bacterial infections to become established. Secondary bacterial establishment has been demonstrated in experimental infections of *T. suis* in pigs (Hall et al. 1976), which developed dysentery. It is frequently observed that children with heavy infections of *T. trichiura* often suffer from chronic dysentery (see Holland 1987b).

Rectal prolapse is the most striking lesion associated with trichuriasis. Apparently, the surface tissue of the rectum becomes extremely edematous and the prolapsing occurs as the patient strains to defecate. Bundy and Cooper (1989) suggest that the mechanism of the prolapse is similar to that of intussusception and that remission often occurs within a few days of effective anthelmintic chemotherapy.

Rectal bleeding occurs during trichuriasis (Fisher and Cremin 1970; Lotero et al. 1974) and this has prompted a vigorous debate about the contribution of the worms

to the development of iron-deficiency anemia. Infected people undoubtedly lose blood during an infection from both the feeding activities of the worms and the extensive damage to the mucosa of the large intestine. Iron lost into the gut lumen from this tissue would essentially represent a net loss to the host. After a critical review of an extensive literature, Holland (1987b) and Bundy and Cooper (1989) independently concluded that trichuriasis contributed significantly to iron-deficiency anemia. Recently, Ramdath et al. (1995) compared the blood picture of 264 *Trichuris*-infected children with that of 157 matched, uninfected children. Children judged to have heavy infections (more than 10 000 eggs/g) had significantly lower ($p < 0.05$) blood hemoglobin concentrations and mean cell volumes than uninfected children or those with lower infection intensities. Also, 33 percent of the heavily infected children were diagnosed as anemic compared with 11 percent of the rest of those in the study.

Several studies have shown that trichuriasis, like ascariasis and often in combination with it, is a factor involved in growth stunting in children (Bowie et al. 1978; Holland 1987b; Simeon et al. 1994). For example, Gilman et al. (1983) found that the nutritional status of children increased significantly following anthelmintic expulsion of *T. trichiura* ($p < 0.05$). The children in this study who were judged to be heavily infected also showed blood in their stools and some degree of anemia and dysentery. Twelve percent of the children presented with finger clubbing; this lesion is a curious thickening at the ends of the fingers and toes and is associated with other illnesses besides trichuriasis. In a cross-sectional survey of 260 children in St Lucia, Cooper and Bundy (1987) found a strong association between measures of impaired rates of growth and infection intensities greater than 20 000 eggs/g. More recently, Cooper et al. (1990) have linked the observed growth depression in children with heavy infections to the dysentery syndrome that is a feature of trichuriasis. Nineteen Jamaican children, ranging in age from 2 to 10 years and known to be suffering from heavy *T. trichiura* infections, were studied. Nutritional status was assessed and blood hemoglobin concentrations ($\bar{x} = 7.0 \pm 2.5$ mg/ml) measured. The growth rates of 11 of these children were calculated, following anthelmintic treatment, for a period of 6 months, and a remarkable rate of catch-up growth was detected despite the absence of any food supplementation other than the provision of oral ferrous sulfate to correct the iron-deficiency anemia. Cooper et al. (1990) proposed that the chronic inflammation of the large intestine which persists during *T. trichiura* infections was in some way linked to the growth deficits detected in these heavily infected children. Recent developments concerning the pathophysiology of *Trichuris* dysentery syndrome are reviewed by Stephenson et al. (2001).

SOIL-TRANSMITTED NEMATODES: IMMUNITY

The human response to *A. lumbricoides* is characterized by prominent antibody and cellular responses that are directed at the larval stages of infection (Cooper 2002). The most consistent finding relates to the production of large amounts of total and parasite-specific IgE, which is most likely to be stimulated by the tissue migratory stage of the life cycle (Hagel et al. 1993; Lynch et al. 1993; McSharry et al. 1999). There is a paucity of data on cellular responses to geohelminths in humans in general, but a recent paper by Cooper et al. (2000) demonstrated a polarized T helper 2 (Th2) response accompanied by interleukin (IL)-4 and IL-5 production in *Ascaris*-infected subjects. Furthermore, for this parasite there is more convincing evidence that differential immune responses both humoral and cellular play a part in the observed predisposition as discussed in the previous section.

In contrast to *Ascaris*, studies on immunity to trichuriasis have been greatly assisted by the provision of an entirely appropriate animal model – *T. muris* in the mouse (Faulkner and Bradley 2002). Evidence for the importance of host genetic background has been provided from the identification of resistant and susceptible strains of mice (Else and Wakelin 1988) and cytokines produced by Th2 cells such as IL-4, Il-3, and Il-9 have been identified as important for worm expulsion. Studies of immune responses in humans have produced less clear-cut results and evidence for protective responses remains uncertain. *Trichuris*-specific IgG appears to reflect current infection status, whereas IgA persists at high levels in adulthood (Needham et al. 1992). Faulkner and Bradley (2002) advocate the use of more defined antigens in order to detect key responses mirroring the experience of McSharry et al. (1999) working with *Ascaris*.

In a series of field-based studies based in Papua New Guinea, Pritchard and co-workers (Pritchard et al. 1992, 1995; Quinnell et al. 1995) reported the relationship between *N. americanus* infection and humoral antibody responses in subjects who experienced reinfection after chemotherapy. After controlling for the effects of age, the authors demonstrated that correlation coefficients between levels of anti-adult worm ES IgG and worm burden declined significantly with age and did not persist after reinfection. The trend was the same for antilarval IgG response, but the pattern persisted after reinfection. The switch from positive to negative correlation in adults appears consistent with a protective role (Quinnell et al. 1995). Furthermore, the effect of the humoral immune response on the weight and fecundity of the parasite was investigated in the same subjects. After controlling for the effects of age and parasite burden, a significant negative correlation between total and specific IgE and the weight and fecundity of *Necator* worms was

detected at initial treatment and after reinfection (Pritchard et al. 1995), which the authors suggest may reflect a Th2 response. For a recent update on approaches to vaccination for hookworm including the identification of novel vaccine candidates, see Pritchard et al. (2002).

Important evidence is now accumulating that helminth infections can modify the host response to secondary infections or vaccination (see Bentwich et al. 1995, 1999; reviewed in Borkow and Bentwich 2002). This may be particularly important with regard to populations in developing countries severely affected by epidemics of human immunodeficiency virus (HIV) and tuberculosis (TB). Disease progression and susceptibility appears to accelerate in individuals co-infected with helminths; one explanation for this may be an influence on the cellular immune response. Helminth infections are associated with a strong Th2-type responses that can down-regulate production of T helper 1 (Th1)-type cytokines important in the control of microparasite infections (Urban et al. 1992).

SOIL-TRANSMITTED NEMATODES: DIAGNOSIS

For epidemiological surveys, operational research programs and control activities, the diagnosis of soil-transmitted nematode infections continues to rely on the collection and examination of stool samples for helminth eggs or larvae (Figure 1) and the method to be used depends on the purpose of the study or survey (Thein Hlaing 1989). A useful summary of those available is given by Theinpont et al. (1986). The procedure now recommended by the World Health Organization (WHO 1994) is that known universally as the Kato Katz method, which gives reliable information about infection intensities. Despite the efforts to develop serological tests to aid diagnosis and techniques to detect antigens and metabolites in feces, coprological examination remains the method best suited to the resources and skills available in developing countries where the infections are endemic (Wakelin et al. 1993).

In modern hospitals and clinics, other diagnostic techniques are available, but not for mass application. For example, in the case of acute ascariasis, real-time ultrasound (Cremlin 1982), radiography (Choudhuri et al. 1986), and endoscopic retrograde cholangiopancreatography (Khan et al. 1993) are regularly employed. Fiber optics may be used; Cooper and Bundy (1987) describe the use of a flexible fiber optic colonoscope to study infections of *T. trichiura*.

SOIL-TRANSMITTED NEMATODES: PROSPECTS OF PREVENTION AND CONTROL

Geohelminth infections represent a serious public health problem in countries where sanitation is poor and access to safe, effective anthelmintic drugs is minimal (Savioli et al. 2002). The World Bank has ranked intestinal helminth infections first as the main cause of morbidity in children aged 5 to 14 years in developing countries and has also identified these infections as being targets for efficient control by cost-effective intervention (World Bank 1993). It is now agreed that one of the best available means of reducing morbidity and mortality due to these infections is to treat high-risk groups, particularly school-age children, through the regular administration of single dose, oral anthelmintic drugs (WHO 2001). Schools can serve as a gathering point for drug delivery and administration, which can be undertaken by teachers; schools can further act as a focus for education in the community so that health education can be reinforced. A successful example of this approach is taking place in Zanzibar where, in addition to the treatment of school-enrolled pupils, an outreach program for non-enrolled but school-age children was also put in place (Savioli et al. 2002; Montresor et al. 2001). Recently, attention has been focused on preschool children and the impact that soil-transmitted helminths may be having on their health and well being (Stoltzfus et al. 2001). Furthermore, in a recent informal consultation the WHO has been considering the issues surrounding treatment of these younger children (Montresor et al. 2002; WHO, 2003). For a recent review of the economics of worm control see Guyatt (2002).

Geohelminth infections are also very suitable diseases to incorporate into integrated control efforts. This has been successfully employed with family planning and nutrition programs by the Japanese Organization for International Co-operation in Family planning (Kunii 1983) and is now the focus of an important initiative concerning the eradication of lymphatic filariasis. The provision of two-drug regimens, namely albendazole-plus-ivermectin or albendazole-plus-diethycarbamazine designed principally to interrupt the transmission of lymphatic filariasis through mass drug administration to at-risk populations, will also have a major impact on intestinal helminth infections (Stephenson et al. 2000).

ENTEROBIUS: BIOLOGY, TRANSMISSION, AND EPIDEMIOLOGY

Biology

Infection occurs as a result of ingesting fully developed, infective eggs. After hatching in the small bowel, subsequent development takes place entirely within the intestine, there being no systemic migration similar to that undertaken by *Ascaris*, and worms mature within about 5 weeks. The adults are found primarily in the large bowel, lying closely applied to the mucosal surface. On the basis of studies made with rodent pinworm, it can be assumed that the worms feed on material present in the

lumen and possibly on the cellular debris that accumulates on the epithelium and in the crypts. The worms are mobile and move within the large bowel, the females also migrate at night down the rectum to the anus to release eggs. Males live for some 7 weeks, females 5–13 weeks (Cook 1995).

Unlike the soil-transmitted species considered above, the eggs of *Enterobius* are not released into the intestinal lumen, but are released on the perianal skin after the females have migrated to the anal region. Hence, diagnosis of pinworm infection cannot be made reliably through fecal smears or similar techniques. Egg release occurs through the female reproductive opening or when the worm dies and disintegrates on the surface of the skin, each female releasing an estimated 10 000 eggs (Faust et al. 1970). Movement to the anal region causes local irritation and itching. When laid, the eggs already contain immature larvae and these complete development very rapidly, maturing within 6 hours at body temperature (Cook 1995). The eggs are 'sticky' and readily adhere but, being light, are also easily dispersed. If the local environmental conditions are not too dry, they can survive for considerable periods.

Transmission

The characteristics of the eggs, and the way in which they are released from females favors several routes of transmission. In children, the itching associated with the presence of female worms on the perianal skin prompts scratching, as a result of which eggs adhere to the hands and fingers, from where they are easily transferred to the mouth. Contamination of nightclothes or bedding can also result in eggs being transferred to the hands. The lightness of the eggs results in a wide dispersal in bedrooms and houses, and accidental transmission to other family members can occur from these sources. In one school, counts of eggs present on the wall of the lavatory showed some 5 000 per square foot (0.093 m^2) (Br Med J 1974). Eggs can become airborne when clothes, bedding or dust are disturbed. They may then contaminate food or the hands of people in the vicinity, but it has also been suggested that airborne eggs can be inhaled and then swallowed.

Epidemiology

Pinworm infections are most frequent in school-age children, but can occur in any age group. They are the commonest worm parasites in temperate, developed countries, where prevalence figures in young children can reach 80–90 percent, but infections are distributed globally, and *Enterobius* often accompanies the soil-transmitted nematodes in the warmer, less developed parts of the world (reviewed by Haswell-Elkins et al. 1987b). Data on prevalence can be misleading, as some figures have been based on detection of eggs in stool samples, which frequently fails to detect infections. Haswell-Elkins et al. (1987b) determined prevalence in an Indian village by recovering worms after chemotherapy and recorded overall figures of 70.8 percent (74.3 percent in males, 67.5 percent in females) with a mean intensity of 25.8 worms. By the age of 4 years, 60 percent of children were infected. Eleven months after initial treatment, the population was sampled again and prevalence and intensity were found to be significantly higher (87.1 percent and 40.2 worms). Infections were markedly aggregated in the host population: for example, in the second study the heaviest infection recorded was 2 152 worms.

ENTEROBIUS: CLINICAL MANIFESTATIONS

In most cases, *Enterobius* infections are asymptomatic. The commonest symptom is the itching associated with movement of the female worms to the anal regions (anal and perineal pruritus), although it has been suggested that these symptoms reflect the existence of other dermatological problems (Ganor 1987). Infected children may show irritability, enuresis, and weight loss. The worms normally cause only minor pathology in the intestine. Unlike the other intestinal nematodes, *Enterobius* infection is not associated with a pronounced eosinophilia. or with elevated IgE (Jarrett and Kerr 1973). In girls, worms may migrate into the vagina, causing a vaginitis. Urinary infections in children have been associated with such migratory behavior and adult women may also be affected in this way. The presence of *Enterobius* in the appendix (2.7 percent of 1 419 appendices removed during a 5-year period in Bristol, UK (Budd and Armstrong 1987), 2.5 percent of 2 921 appendices in India (Gupta et al. 1989)) has suggested a link with appendicitis, but this is considered rare.

Infections can lead occasionally to more severe complications, if the worms find their way into the peritoneum, either via intestinal perforations or following migration through the female reproductive tract. Dead worms and eggs have been identified in granulomata in several sites (vagina, cervix, fallopian tubes, omentum, peritoneum, even the liver, kidneys, and lungs (Cook 1995).

ENTEROBIUS: DIAGNOSIS AND CONTROL

Infection can be diagnosed directly by observing worms in the perianal region or on the surface of the stool. More commonly, diagnosis requires the detection of eggs. These occur on the perianal skin and can be collected on to the surface of an adhesive transparent tape, pressed on to the skin. The tape can then be transferred to a slide, with a clearing agent (e.g. toluene,

cedar oil) for microscopic examination. Such examination is best performed in the morning when the patient first wakes, and repeated examination (3–6 tests) may be necessary to ensure positive diagnosis. Commercial detection kits are available. Eggs can sometimes be found in stools, but diagnosis is very unreliable. If infection is confirmed in one individual in a household, other family members should also be examined and treated.

Pinworms are easily removed by anthelmintic treatment. The use of piperazine compounds has been standard for many years but requires treatment daily for 7 days. Pyrantel compounds are effective in a single (10 mg/kg) dose, as are the benzimidazoles mebendazole (100 mg tablet) and albendazole (400 mg tablet). Where *Enterobius* occurs with other intestinal nematodes, use of the benzimadazoles has the additional benefit of eliminating the other species as well.

Although treatment is effective, reinfection occurs readily unless steps are taken to minimize contact with infective eggs. Personal hygiene is important, particularly hand washing, fingernail cleaning, and regular bathing. Infected children can be made to wear gloves in bed to prevent contamination of their hands. Heavily infected areas such as bedrooms must be kept scrupulously clean and dust free, and bed linen and night clothes should be changed and laundered frequently. In practice, elimination of eggs in this way is very difficult and to be effective these approaches must be accompanied by repeated chemotherapy to remove the adult worms.

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Stronglyloides and *Capillaria*

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STRONGYLOIDES

THE PARASITE

Nematodes belonging to the genus *Strongyloides* were first identified as a cause of intestinal disease by Normand in 1876, who discovered these worms in the feces of French troops in Cochin-China. At autopsy, morphologically distinct worms were identified within the intestinal mucosa of those suffering from overwhelming disease. Bavay (1876) subsequently named the intestinal forms *Anguillula intestinalis* and the fecal forms *A. stercoralis*. Soon after, it was determined that these represented different stages of development of the same parasite. The existence of an autoinfective cycle was suggested by Fuelleborn (1914), and later work by Nishigori (1928) demonstrated that first stage larvae could indeed develop into infective filariform larvae and invade the colonic mucosa, thereby initiating development within the same host.

Classification

Strongyloides belongs to the phylum Nematoda and the class Phasmidia, i.e. nematodes containing phasmids or caudal chemoreceptors. Within this class, they are assigned to the order Rhabditida and the superfamily Rhabditoidea, which is composed of both free-living and parasitic worms. They are distinguished from the superfamily Rhabdiasoidea, also within the suborder Rhabditina, by the presence of a distinct esophageal bulb. Two species cause disease in humans, *S. stercoralis* and *S. fuelleborni*.

Morphology and structure

Parasitic females, which reside in the mucosa of the small intestine, are small and thin, measuring approximately 2–3 mm in length and 30–50 μm in width (Figure 1). The anterior portion is thicker than the posterior, and contains the esophagus. The triradiate pharynx contains two subventral and one dorsal gland, which deposit their secretions into the intestinal lumen. The reproductive system contains a midline vulva and paired uteri leading anteriorly and posteriorly from the vagina. The spirally wound ovaries of *S. fuelleborni* help

Figure 1 Strongyloides stercoralis *adult parasitic female (courtesy of Ash and Orihel (1997))*

to distinguish it morphologically from *S. stercoralis*, whose ovaries are linearly aligned within the body of the worm.

The existence of parasitic males has been debated over many years. Early observations by Faust (1933) that parasitic males exist, and that sexual reproduction may represent one route by which *Strongyloides* reproduces within the host, have not been confirmed by others. Part of the reason for the skepticism surrounding his findings may be the close morphological resemblance of the putative adult males to the free-living rhabditiform stage (Schad 1990).

The free-living adult female is approximately half the length of its parasitic counterpart (approximately 1.0 mm), although it is nearly twice as thick (80 μm). While the reproductive systems are morphologically similar, the uteri in the free-living adult female contain significantly more eggs. The free-living male is slightly smaller than the female, measuring approximately 50 μm in width. The reproductive system is composed of a tubular structure containing the testis, vas deferens, and seminal vesicle. The copulatory spicules, which penetrate the female during copulation, are located on each side of the gubernaculum.

First-stage rhabditiform larvae of *S. stercoralis* measure approximately 250 × 15 μm (Figure 2). They are characterized by a muscular esophagus, which comprises the anterior third of the body, and a short buccal cavity that distinguishes them from other nematodes, such as hookworms, which possess a more developed buccal tube. Third-stage filariform, or infective, larvae are long and thin compared to the rhabditiform stages (Figure 3). Paired alae on both sides of the worm offer structural stability during its movements along the ground. The mouth opening is small and closed to the external environment, as the filariform stage is probably non-feeding. Recent work using electron microscopy with three-dimensional reconstruction has delineated the location and structure of the amphidial neurons, highly specialized organs that may represent a means through which larvae sense environmental stimuli (Ashton et al. 1995; Lopez et al. 2000).

The eggs of the parasitic female are deposited within the mucosa of the small intestine and usually hatch before reaching the lumen. As a result, they are rarely excreted in the feces. Eggs from the free-living adult female are partially embryonated, and measure approximately 50–70 μm in length with an oval shape.

General biology

Perhaps the most intriguing aspect of the biology of *Strongyloides* is its ability to alternate parasitic (homogonic) and free-living (heterogonic) life cycles. The factors that determine which route will be chosen have been studied for some species, and it appears that both internal, i.e. host specific, and external, i.e. environmental, conditions play a role in influencing developmental outcome (Moncol and Triantaphyllou 1978). In addition, certain geographic strains of *Strongyloides* may have a particular predilection for developing into one generation or the other (Neva 1986). It has also been observed that the free-living cycle develops most frequently in tropical climates with moist soil, while the parasitic life cycle may predominate in more temperate regions.

Rhabditiform first-stage larvae deposited with feces feed on bacteria and organic debris found in the

Figure 2 Strongyloides stercoralis *first stage (L1) rhabditiform larva, with* **(a)** *and without* **(b)** *iodine stain (courtesy of Ash and Orihel (1997))*

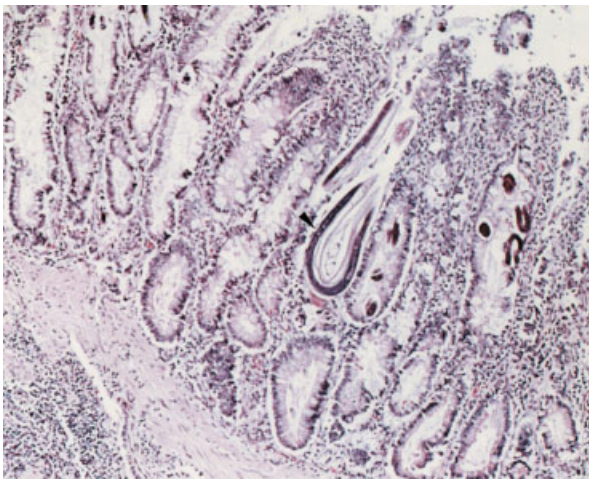

Figure 3 *Adult female* Strongyloides stercoralis *worm (see arrow) in biopsy of small intestine (hematoxylin and eosin stain) (courtesy of Orihel and Ash (1995))*

surrounding soil. Those that develop into free-living adults continue to survive in this environment, whereas those that become filariform (i.e. infective third-stage larvae) will ultimately cease to feed, while engaging in activities that might facilitate contact with a susceptible host. Filariform larvae have been shown to cluster in groups and stand on their tails, a behavior called 'questing,' which is characteristic of the infective larval stage of parasitic nematodes.

Life cycle

Adult parasitic females, which reside within the crypts of the small intestine, deposit eggs that hatch prior to migrating into the lumen. The resulting first-stage larvae are transported through the bowel, and excreted with the feces. Larvae that are deposited on to moist, warm soil will develop either directly into infective filariform larvae or into free-living adult males and females, whose progeny will ultimately molt to become infective larvae. These infect their host by penetrating the skin, a process associated with the release of hydrolytic enzymes (Brindley et al. 1995; Gallego et al. 1998) that may function to degrade the extracellular dermal matrix. The traditionally accepted view of the life cycle of *S. stercoralis* maintains that, once within the dermis, larvae invade the venous circulation and are carried passively to the lungs, where they become trapped within capillaries and break through to the alveolar space. After migrating up the respiratory tree into the pharynx, the larvae are swallowed and travel to the small intestine. During this process, they molt to the fourth stage, probably during passage through the lungs. Work by Schad et al. (1989), however, has raised the possibility that this 'canonical route' represents only one of many potential pathways by which larvae may reach the small intestine.

Only females develop into adults within the intestine, where they reside and deposit eggs that have been produced by parthenogenesis. These eggs normally hatch within the epithelium, liberating first-stage larvae into the lumen of the intestine. As a result, *Strongyloides* eggs are not routinely detected in the feces of infected individuals. If the first-stage larvae molt to the filariform stage during passage through the bowel, they may penetrate colonic mucosa or perianal skin, where they once again locate the venous circulation and begin the infective process anew. This autoinfection provides a means by which the parasite can multiply within the host, and explains how infections can be maintained for years after an individual leaves an endemic area. What regulates larval development in the intestine is not known, nor are the factors that sometimes trigger the explosive amplification of *Strongyloides* within the host called hyperinfection (see section entitled Clinical manifestations), which can lead to the dissemination of parasites throughout various tissues.

Transmission and epidemiology

The predominant route of transmission of *S. stercoralis* is by skin contact with fecally contaminated soil. As a result, communities where close living conditions and poor sanitation facilities exist are frequently characterized by high prevalence rates of strongyloidiasis. These include both rural and urban areas in the developing world, as well as closed communities, such as institutions for the mentally handicapped (Braun et al. 1989). Zoonotic transmission of strongyloidiasis from asymptomatic dogs to animal workers has also been reported (Georgi and Sprinkle 1974). Although transmammary transmission of *S. stercoralis* has been demonstrated in laboratory animals infected experimentally (Shoop et al. 2002), this has not been confirmed to occur in humans.

The geographic distribution of *Strongyloides* is quite broad, and includes temperate as well as tropical climates. This undoubtedly is attributable to the remarkable phenotypic flexibility of the parasite, as it is able to adapt its life cycle and development to suit environmental conditions. Moreover, internal autoinfection allows for maintenance of the parasite within the host for years following initial exposure. Surveys of prisoners of war from World War II conducted more than 40 years later succeeded in identifying patent infections in up to 37 percent of those soldiers who were held captive in various parts of the Pacific (Hill 1988; Genta 1989). Since all infected individuals are at risk of hyperinfection and disseminated disease, the identification of those who may have acquired their infection in the distant past is of significant clinical importance (Link and Orenstein 1999). This group includes not just former military personnel, but also immigrants and refugees from highly endemic areas (De Silva et al. 2002; Gyorkos et al. 1992). Strongyloidiasis remains a chronic infection among people from certain parts of the United States, particularly Appalachia (Kitchen et al. 2000; Siddiqui and Berk 2001), as well as throughout Europe (Sanchez et al. 2001).

Strongyloides hyperinfection is most frequently seen in those with underlying immune defects such as hematological malignancy, autoimmune disease or severe protein malnutrition. In addition, immunosuppressive therapy, particularly glucocorticoids, associated with any of the above conditions, as well as organ transplantation, may also precipitate hyperinfection and dissemination. Whether this is due to immunosuppression or a direct effect of steroids on the reproductive and developmental patterns of *Strongyloides* remains to be determined (Genta 1992). A cDNA encoding a putative steroid hormone receptor has recently been cloned from *S. stercoralis*, suggestive of the possibility that host hormones may, in fact, influence the behavior of the parasite in vivo (Siddiqui et al. 2000). Coinfection with human immunodeficiency virus (HIV), although reported in

association with severe strongyloidiasis, is less common than might have been expected, given the significant overlap in the geographic distribution of these two diseases (Celedon et al. 1994; Gompels et al. 1991). In contrast to HIV, there is evidence that coinfection with human T-lymphotropic virus type 1 (HTLV-1) may be associated with more severe strongyloidiasis than that which is seen in HTLV-1 seronegative individuals (Robinson et al. 1994; Gotuzzo et al. 1999; Satoh et al. 2002).

S. fuelleborni is also transmitted primarily by skin contact. However, epidemiological data from parts of Africa and Papua New Guinea suggest that this may not be the only route of infection. Investigations into the high prevalence of *S. fuelleborni* in African children led to the postpartum identification of larvae in the breast milk of a woman from Zaire (Brown and Girardeau 1977). In Papua New Guinea, high rates of infection (up to 65 percent) in children up to 3 months of age suggest that transmammary infection might also occur in this endemic area (Barnish and Ashford 1989; Ashford and Barnish 1990). In addition, *S. fuelleborni* has been identified as the causative agent of 'swollen belly syndrome,' a fulminant and fatal enteritis that has been well documented among infants less than 6 months of age born in the highly endemic areas of Papua New Guinea (see Clinical manifestations). It has been suggested that transmammary infection followed by early external autoinfection from soiled nappies may result in the rapid multiplication of worms in these very young children (Ashford and Barnish 1990).

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Strongyloidiasis can be divided into the following clinical categories: (1) asymptomatic carriage; (2) intestinal disease; and (3) hyperinfection with or without dissemination. Symptomatology is somewhat dependent on worm burden, which appears to be regulated by a delicate balance between the immune status of the host and the developmental tendencies of the parasite. In chronic strongyloidiasis, egg output remains relatively low and constant, proportionally few larvae undergoing internal autoinfection. In contrast, hyperinfection is associated with both an increase in the number of eggs produced as well as the proportion of larvae that develop internally to the filariform stage and reinvade the host.

Clinical manifestations

Chronic infection with *S. stercoralis* is most frequently asymptomatic (Milder et al. 1981; Neva 1986; Berk et al. 1987). When symptoms are present, they usually involve the gastrointestinal tract. Intermittent abdominal pain, distention, bloating, and diarrhea alternating with constipation are characteristic of intestinal disease. Children may develop a malabsorption syndrome caused by infection with *S. stercoralis*, characterized by growth stunting and failure to thrive (O'Brien 1975; Burke 1978). Pruritus and urticaria, particularly involving the perianal skin and buttocks, are also common symptoms of chronic *Strongyloides* infection. A serpigenous, pruritic eruption usually found on the trunk or buttocks, named larva currens (racing larva) by Arthur and Shelley (1958), may represent skin penetration by autoinfective filariform larvae in this area. This pathognomonic rash tends to progress and subside over a period of hours, and its intermittent nature often hinders the diagnosis of strongyloidiasis. Other dermatoses, including prurigo and lichen simplex chronicus, have also been associated with chronic strongyloidiasis (Albanese et al. 2001; Jacob and Patten 1999).

The most serious clinical manifestations of strongyloidiasis occur in the setting of hyperinfection, where the dramatic increase in the number of parasites within the host results in dissemination of larvae to various tissues. The increase in worm burden results from an increase in both the number of eggs produced by the female parasites in the intestine and the proportion of those larvae that undergo autoinfection. Usually associated with some underlying host immune defect, hyperinfection is most frequently associated with immunosuppressive therapy, particularly glucocorticoids, which are often used as part of the treatment of such conditions as hematological malignancies and autoimmune diseases. Coinfection with HTLV-1 may also predispose to *Strongyloides* hyperinfection, perhaps due to a defect in effector IgE responses (Dixon et al. 1989; Robinson et al. 1994; Satoh et al. 2002).

Patients with *Strongyloides* hyperinfection generally experience a worsening in abdominal symptoms, often accompanied by paralytic ileus (Cookson et al. 1990), gastrointestinal bleeding (Bhatt et al. 1990; Dees et al. 1990; Daubenton et al. 1998), and even perforation. In addition, the increase in the number of worms migrating through the lungs often results in wheezing, dyspnea, and occasionally pulmonary hemorrhage. Patchy infiltrates, diffuse interstitial pneumonitis, and bronchopneumonia may be detected by chest radiography or CT scan (Meltzer et al. 1979; Berenson et al. 1987; Chu et al. 1990; Kramer et al. 1990; Upadhyay et al. 2001). This can occasionally be interpreted as a relapse in underlying malignancy (Sandlund et al. 1997).

During hyperinfection, filariform larvae may gain access to the arterial circulation, thereby allowing dissemination to numerous tissues and solid organs. In fact, filariform (and rarely rhabditiform) larvae have been recovered from biopsies of lymph nodes (Adam et al. 1973), endocardium (Neefe et al. 1973), pancreas (Kuberski et al. 1975), liver (Neefe et al. 1973), kidney (Civantos and Robinson 1969), and brain (Neefe et al. 1976), and have even been isolated from peripheral blood (Onuigbo and Ibeachum 1991). Involvement of

the central nervous system can be associated with seizures and mental status changes (Igra-Siegman et al. 1981; Genta and Walzer 1989; Cappello and Hotez 1993), and is frequently accompanied by pyogenic abscess or meningitis caused by gram-negative bacteria, which may be carried from the gut by migrating larvae (Link and Orenstein 1999). Cutaneous manifestations of disseminated strongyloidiasis include petechiae and sometimes severe purpura (Kalb and Grossman 1986; Berenson et al. 1987; Genta and von Kuster 1988; Ronan et al. 1989). Unlike the rash of chronic strongyloidiasis (larva currens), skin biopsies in disseminated disease often reveal filariform larvae.

Children suffering from swollen belly syndrome caused by *S. fuelleborni* usually present early in life (8–10 weeks of age) with protein-losing enteropathy, abdominal distention, and respiratory distress (Ashford and Barnish 1990). Diarrhea is generally not pronounced and fever is absent. Severe hypoproteinemia (without proteinuria) is a characteristic finding and leads to the development of peripheral edema and ascites. Egg counts are often greater than 100 000/g feces. Untreated, the condition is associated with significant mortality.

Pathogenesis and pathology

The intestinal pathology associated with *Strongyloides* infection is indicated by a variety of histological findings. The spectrum of damage, ranging from scattered petechiae with mild edema to ulcerated, atrophic, and fibrotic mucosa can occasionally coexist in a single patient. Parasites may be found in all layers of the intestinal wall, and infiltrates of mononuclear cells and neutrophils may be identified (Genta and Walzer 1989). A recent histologic evaluation of small bowel biopsy specimens demonstrated no significant inflammatory response in 19 individuals with mild chronic infections, compared to uninfected controls (Trajman et al. 2000).

In disseminated disease, migrating larvae cause extensive tissue damage to the colon, lungs, and other organs. Because of the association with immunosuppression, biopsy specimens containing these aberrantly migrating larvae may reveal only a mild inflammatory response surrounding the parasite. Invasion of the central nervous system can be associated with meningitis characterized by a predominance of either neutrophils or lymphocytes.

Immune responses

The immune responses that accompany *Strongyloides* infection are not well understood. To date, there have been reports of elevated serum IgE in chronic strongyloidiasis. However, the diagnostic significance of this observation is unclear, since at least one study reported elevated serum IgE levels in less than 10 percent of chronically infected individuals (Willis and Nwokolo 1966). Moreover, it appears that IgE levels may wane over time, thus allowing for the maintenance of a chronically infected state (Atkins et al. 1997). An association between HTLV-1 infection and fecal excretion of *Strongyloides* larvae has been reported from Jamaica (Robinson et al. 1994). The authors found that HTLV-1-infected individuals with strongyloidiasis showed an age-related depression in serum IgE levels when compared to those seronegative for HTLV-1. These data suggest that serum IgE may confer some degree of protection against heavy infection with *Strongyloides*, and that this immune response is somehow inhibited by HTLV-1. More recent studies have shown that HTLV-1-infected patients with strongyloidiasis produce elevated levels of the cytokines IFN-γ and TGF-β1 compared to those infected with *S. stercoralis* alone. Moreover, coinfected individuals had reduced circulating levels of IgE and IgG4, which was associated with lower cure rates following anthelminthic treatment (Satoh et al. 2002).

Diagnosis

The diagnosis of strongyloidiasis, particularly chronic or asymptomatic infection, can be extremely challenging (Siddiqui and Berk 2001). A definitive diagnosis is most reliably made by identifying rhabditiform (or rarely filariform) larvae in the feces of an infected individual. However, the excretion of eggs in uncomplicated intestinal infection can be quite erratic, and multiple stool examinations may be required. Various stool concentration techniques and use of the Baermann method (by which motile larvae are extracted from stool specimens) may increase the diagnostic yield of fecal examination (Genta et al. 1987). An agar plate method that relies on the use of inverted microscopy to identify tracks made by motile *S. stercoralis* larvae may be more sensitive than some of these other techniques (Sukhavat et al. 1994; Iwamoto et al. 1998).

Strongyloides larvae can also be detected in duodenal aspirates, either by endoscopy or by the use of the string test (Beal et al. 1970; Goldsmid and Davies 1978). The latter involves swallowing a gelatin capsule containing a coiled piece of string. The proximal portion of the string is attached externally, and approximately 4 h later, the string is removed. The duodenal fluid adhering to the string is then examined microscopically for the presence of larvae. The sensitivity of the string test is probably slightly higher than that of routine stool examination. Adult worms may be identified in small bowel biopsy specimens (Figure 3).

Serological tests for strongyloidiasis are available through certain reference laboratories. Multiple enzyme-linked immunosorbent assays (ELISA) have been developed, some of which are sensitive and relatively specific (Loutfy et al. 2002; Ramachandran et al. 1998; Carroll et al. 1981; Genta et al. 1987). An indirect

immunofluorescence antibody test (IFAT), which utilizes antigens from the rodent species *S. venezuelensis* has also been reported to be sensitive for diagnosing human infections (Machado et al. 2001). Recent identification of immunoreactive recombinant antigens from *S. stercoralis* should provide for standardization of testing for strongyloidiasis, particularly as a means of screening patients who are at risk of hyperinfection and disseminated disease (Ravi et al. 2002). Anyone with a positive serological test for *Strongyloides* should have a thorough stool examination to identify the presence of larvae and confirm the diagnosis.

During hyperinfection with dissemination, *Strongyloides* larvae can be detected in a variety of body fluids and tissues. The massive migration of parasites through the pulmonary circulation and into the lungs results in the frequent identification of larvae in sputum or bronchoalveolar lavage specimens (Berenson et al. 1987; Chu et al. 1990; Kramer et al. 1990) (Figure 4). Strongyloides larvae have also been identified in pericardial fluid (Lai et al. 2002), cerebrospinal fluid (Cappello and Hotez 1993), and brain biopsies (Neefe et al. 1976). *Strongyloides* eggs have even been isolated from a urethral smear from a patient following bone marrow transplant (Steiner et al. 2002). Eosinophilia, which is a frequent finding in chronic strongyloidiasis, is often absent in hyperinfection, in many cases because of exogenous corticosteroid therapy.

In contrast to *S. stercoralis*, the diagnosis of *S. fuelleborni* infection is made by identifying eggs in the feces, rather than larvae. Children with swollen belly syndrome tend to have extremely heavy infections, and excrete large numbers of eggs daily.

Control

For many years, thiabendazole was the drug of choice for the treatment of chronic strongyloidiasis. Although cure rates of 60–100 percent have been reported (Schaffel et al. 2000), its use is associated with an extremely high occurrence of adverse side effects. These include nausea, dizziness, and neuropsychiatric symptoms, which have been reported in up to 89 percent of patients taking this drug (Grove 1982). It has recently been shown that ivermectin, a broad spectrum anthelmintic, is at least as effective and better tolerated than thiabendazole for the treatment of chronic strongyloidiasis (Naquira et al. 1989; Lyagoubi et al. 1992; Gann et al. 1994; Adenusi 1997; Zaha et al. 2002). Ivermectin, therefore, is now considered the drug of choice for chronic strongyloidiasis, with a two-dose regimen of 200 μg/kg given on successive days or at an interval of 2 weeks demonstrated to cure nearly all chronic infections (Gann et al. 1994; Zaha et al. 2002; Medical Letter 2002). In addition, albendazole has also been suggested as an alternative therapy (Datry et al. 1994; Sato et al. 2000), although this agent appears to be less efficacious than ivermectin.

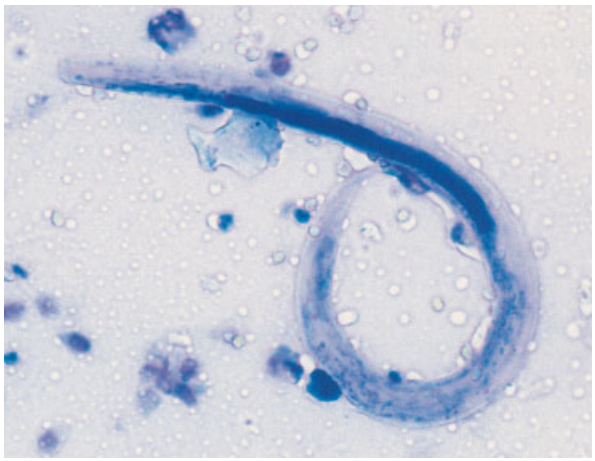

Figure 4 Strongyloides stercoralis *third-stage (L3) filariform larvae detected in sputum specimen (courtesy of Orihel and Ash (1995))*

Follow up of patients after treatment for chronic strongyloidiasis should include a follow-up eosinophil count and serologies. It has been reported that both eosinophil counts and *Strongyloides* specific IgG should return to baseline within 6–9 months following effective treatment (Lindo et al. 1996; Loutfy et al. 2002). Because the cure rate in patients coinfected with HTLV-1 appears to be lower, these patients should be monitored closely for evidence of treatment failure (Satoh et al. 2002; Terashima et al. 2002).

Successful treatment of *Strongyloides* hyperinfection requires prompt diagnosis and initiation of anthelmintic therapy. Treatment with ivermectin (Torres et al. 1994) or thiabendazole (25 mg/kg twice a day) should be continued indefinitely, pending both clinical response and the eradication of larvae from stool, sputum, and other body fluids. Although no intravenous formulations are currently available, veterinary preparations of ivermectin have been successfully used to treat severe human infections (Chiodini et al. 2000). Concurrent bacterial infections, e.g. bronchopneumonia and meningitis, should be treated aggressively with antibiotics. Even with appropriate therapy, the mortality from disseminated strongyloidiasis approaches 50–75 percent (Genta and Walzer 1989). In light of this, appropriate screening of high risk individuals should be carried out prior to the initiation of therapy that is associated with immunosuppression, particularly glucocorticoids (Avery 2002).

Thiabendazole treatment of children with swollen belly syndrome caused by *S. fuelleborni* appears to be effective, although reinfection is likely to occur in endemic areas (Ashford and Barnish 1990; Barnish and Barker 1987).

Because *Strongyloides* larvae can survive in warm, moist environments for days, control of transmission within an endemic area requires proper disposal of human waste. In addition, outbreaks documented among

animal care workers underline the importance of exercising caution when handling specimens from laboratory animals. Despite the overwhelming numbers of larvae excreted by individuals experiencing hyperinfection syndrome, the risk of person to person transmission appears to be low (Maraha et al. 2001).

CAPILLARIA

THE PARASITE

Introduction and historical perspective

Human parasitic zoonoses caused by members of the genus *Capillaria* are uncommon, except in the Philippines and Thailand, where epidemics caused by the intestinal species *C. philippinensis* have been described. Since 1964, intestinal capillariasis has been recognized as an important cause of morbidity and mortality in these areas, with impressive outbreaks in Northern Luzon, Southern Leyte (Cross 1995), and the Compestela Valley (Belizario et al. 2000). Outbreaks have also been described elsewhere in Asia, and more recently, case reports of *C. philippinensis* infection have been reported in Egypt (Anis et al. 1998; Ahmed et al. 1999) and Colombia, South America (Dronda et al. 1993). Unlike most other intestinal nematodes, *C. philippinensis* is associated with substantial mortality during epidemics (Detels et al. 1969), and shares with *Strongyloides stercoralis* the unusual ability to undergo autoinfection and hyperinfection in humans. Much of our existing knowledge about human intestinal capillariasis derives from the earlier investigative work of Professor John H. Cross during his tenure at the US Naval Medical Research Unit in the Philippines (Cross 1992).

In contrast to intestinal capillariasis, extraintestinal zoonotic capillariasis, caused by other members of the genus *Capillaria* (*C. hepatica*, *C. aerophila*, and *C. plica*) is probably rare. However, with improved detection methods (Juncker-Voss et al. 2000), the number of actual human cases may greatly exceed those reported to date.

Classification

The Capillariinae is one of three subfamilies belonging to the superfamily Trichinelloidea. The Trichurinae (whipworms) and the Trichosomoidinae comprise the other two subfamilies. All members of the Trichinelloidea have a unique esophageal structure that distinguishes them from other nematodes (see below). Anderson (1992) points out that the 'classification of the Capillariinae is one of the most difficult and unsatisfactory in the Nematoda.' Many investigators including Skrjabin et al. (1957) have assigned the four human parasites of the genus *Capillaria*, namely *C. philippinensis*, *C. hepatica*, *C. aerophilus*, and *C. plica*, to other genera, including *Calodium*, *Eucoleus,* *Pearsonema* (Anderson 1992), *Paracapillarias*, and *Crossicapillaria* (Moravec 2001). Because these terms are usually unfamiliar to medical parasitologists and clinicians we will adopt the common usage term *Capillaria*.

Morphology and structure

Like all members of the Trichinelloidea, the adult *Capillaria* have a modified esophageal gland called a stichosome comprised of unique gland cells called stichocytes. Each stichocyte communicates with the lumen of the esophagus by a single pore (Wright et al. 1985; Anderson 1992). Generally speaking, the Trichinelloidea have a long narrow anterior end containing the stichosome, which broadens into a thickened posterior end. In the *Capillaria*, however, the differences in width between the anterior and posterior regions are much less exaggerated. The adult Capillariinae are also more delicate and smaller than the other Trichinelloidea subfamilies. Adult males of *C. philippinensis* measure 1.5–3.9 mm in length and females measure 2.3–5.3 mm in length (Chitwood et al. 1968; Cross 1995). Because it is difficult to recover and measure intact *C. hepatica* and *C. aerophila* from human tissue, there are no precise estimates of their length. From nonhuman hosts, lengths of 4–12 mm have been reported for *C. hepatica*, and 30–40 mm for *C. aerophila*.

The uterus of the female adult *C. philippinensis* contains thick-shelled eggs, thin-shelled eggs, and larvae (Cross 1992). Eggs passed in the feces are bipolar 'lemon-shaped' or 'barrel-shaped' and bear a superficial resemblance to those of the human whipworm *Trichuris trichiura* (see Figure 5). They are, however, smaller (36–45 × 20 μm for *C. philippinensis* vs 50–54 × 22–23 μm for *T. trichiura*), more cylindrical in shape and have less prominent bipolar plugs which are somewhat flattened (Cross 1992). In contrast, the eggs of *C. aerophila* are large, measuring 59–80 × 30–40 μm (Soulsby 1982).

General biology

Humans are accidental hosts for all members of the genus *Capillaria*.

C. PHILIPPINENSIS

There is evidence to suggest that fish-eating birds are the natural definitive hosts of *C. philippinensis* (Cross and Basaca-Sevilla 1983). Humans become infected when they accidentally interrupt the fish–bird life cycle by eating infected, uncooked freshwater fish. *C. philippinensis* and *Strongyloides stercoralis* are the two major nematodes that can undergo autoinfection and hyperinfection in the human host.

Figure 5 *Eggs of* Capillaria philippinensis **(a)** *and* C. hepatica **(b)**. *Included for comparison is an egg from* Trichuris trichiura **(c)** *(courtesy of Dickson D. Despommier, Columbia University School of Public Health)*

C. HEPATICA

C. hepatica occurs in the liver of numerous rodent species worldwide, but will also infect at least 20 other mammals, including squirrel, muskrat, opossum, porcupines, and, rarely, humans (Borucinska and Nielsen 1993; Hamir and Rupprecht 2000). *C. hepatica* has the unusual feature that all of its mammalian hosts are 'dead end' in that the eggs must be released from the liver by a predation (including cannibalism and scavenging).

C. AEROPHILA

C. aerophila is a worldwide parasite of wild carnivores where it lives in the upper and lower respiratory tree and parenchyma.

Life cycle

C. PHILIPPINENSIS

As Cross (1995) points out, the *C. philippinensis* life cycle is unique among human nematode parasites and was elucidated primarily by laboratory experimentation. Eggs from the feces of infected patients embryonate in water at ambient temperatures in 5–10 days and develop further only if swallowed by small freshwater and brackish water fish. The bagsit (*Hypseleotris bipartita*) is a major intermediate fish host in the Philippines (Cross 1992). Upon ingestion the eggs hatch, giving rise to larval stages that grow in size. Humans become infected when they ingest larvae contained within the uncooked fish. The larvae become adults in the lumen and mucosa of the intestine. The length of time during which ingested larvae develop into adult male and oviparous adult female worms in humans is not known, although in

experimental gerbil infections, the larvae develop into adults in 10–11 days (Cross 1992, 1995).

An unusual feature of *C. philippinensis* is its ability to cause autoinfection and hyperinfection in humans. The initial evidence for hyperinfection arose from studies with monkeys (*Macaca* spp.), in which 10 000– 30 000 worms were recovered from monkeys infected with 30–50 larvae contained within fish (Cross et al. 1972; Cross 1992). This observation explains the extreme morbidity and relatively high mortality of intestinal capillariasis in humans. Autoinfection arises because the female adult worm is sometimes larviparous or can produce thin-walled eggs that hatch within the intestinal tract; these newborn larvae presumably can molt and reinvade the intestinal mucosa. Very heavy infections can develop as a result, as many as 200 000 worms having been recovered from 1 l of human bowel fluid at autopsy (Cross 1992).

C. HEPATICA

The adult female resides in the liver, where it deposits groups of eggs. These develop larvae but remain encapsulated in the liver (Wright 1974). Therefore, for transmission to occur, the embryonated eggs contained within infected livers must be eaten by animals or humans during cannibalism, predation, or scavenging (Anderson 1992). In contrast, the unembryonated eggs will pass through the gut of so-called 'disseminator animals' and are then dispersed into the environment with the feces (Anderson 1992). Ingestion of nonembryonated eggs does not lead to hepatic capillariasis (Juncker-Voss et al. 2000). When infective eggs are ingested accidentally by humans, they hatch in the small intestine and produce larvae that migrate through the portal system to the liver (Gutierrez 1990). After approximately 4 weeks, the female adult worm begins to release eggs.

C. AEROPHILA

The adult female lives in the upper and lower respiratory tree (under the mucosa), where it produces eggs that are coughed and swallowed. Eggs passing out with the feces will embryonate and enter a new host via contaminated food or water (Gutierrez 1990).

Transmission and epidemiology

C. PHILIPPINENSIS

The diet and eating habits of indigenous populations living in areas such as the Philippines and Thailand have been implicated in the transmission of intestinal capillariasis. For example, the Ilocanos of Northern Luzon use the intestinal juices from animals to season rice and typically consume whole freshwater fish uncooked (Cross 1992). Since the original reports of widespread intestinal capillariasis in the Philippines and in Thailand (Pradatsundarasar et al. 1973), infection with *C. philippinensis* has been subsequently reported from other parts of Asia, including Japan (Nawa 1988), Indonesia (Chichino et al. 1992; Bangs et al. 1994) and India (Kang et al. 1994), as well as from nonendemic areas such as Iran, Egypt, and South America (Dronda et al. 1993).

C. HEPATICA

Probably because of its broad host specificity, there is a wide distribution of animal and human hepatic capillariasis. Cases have been reported from the Americas, Europe, Asia, and Africa. Humans may be at high risk for *C. hepatica* infection in Zaire, Nigeria, and other parts of West Africa where they consume the Gambian rat or cricetoma (Malekani et al. 1994).

C. AEROPHILA

Like *C. hepatica*, *C. aerophila* is a worldwide parasite of wild carnivores. Human infections have been reported primarily from Russia, although other cases have been reported from Morocco and Iran.

Location in host

C. PHILIPPINENSIS

In heavy infections, all stages of *C. philippinensis* can be found in the small intestine, particularly the jejunum. Extraintestinal *C. philippinensis* infection is a rare occurrence.

C. HEPATICA

The adult worms live in a host-derived capsule within the liver where they feed on cytoplasmic debris.

C. AEROPHILA

The adult worms live beneath the mucosa of the upper and lower respiratory tree.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

C. philippinensis can be one of the most virulent helminthic pathogens of humans, with extremely high mortality rates during epidemics of intestinal capillariasis. Parasite virulence can be largely ascribed to the ability of the worm to cause hyperinfection.

Extraintestinal infections caused by other members of the genus *Capillaria* are relatively rare. All together, only 37 cases of human hepatic capillariasis caused by *C. hepatica* have been reported between 1924 and 1996 (Juncker-Voss et al. 2000), although the potential for transmission of this zoonosis may be high in some areas (Malekani et al. 1994). It has been postulated that many subclinical cases of *C. hepatica* may also go undetected (Juncker-Voss et al. 2000). Zoonotic transmissions of *C. aerophila* and *C. plica* are probably very rare, as

reflected by only a handful of case reports in the medical literature.

Clinical manifestations

C. PHILIPPINENSIS (INTESTINAL CAPILLARIASIS)

There is no evidence for asymptomatic intestinal capillariasis. Patients usually develop diarrhea and abdominal pain that may progress in intensity. Borborygmi is a common presenting sign of intestinal capillariasis. Dehydration resulting from intestinal fluid losses is exacerbated by vomiting and anorexia. Infections lasting 2–3 months result in a severe protein-losing enteropathy characterized by cachexia, dehydration, and anasarca. Death is often the outcome in patients who do not receive adequate fluid resuscitation, nutritional supplementation, and anthelmintic chemotherapy. These patients often die from electrolyte disturbances that contribute to heart malfunction, as well as secondary bacterial superinfections and sepsis (Cross 1995).

C. HEPATICA (HEPATIC CAPILLARIASIS)

Heavy infections may cause an acute or subacute hepatitis accompanied by peritonitis, ascites, and eosinophilia. A triad of persistent fever, hepatomegaly, and leukocytosis with eosinophilia has been described (Juncker-Voss et al. 2000). Anemia, pulmonary symptoms, splenomegaly, kidney enlargement, and cachexia may also occur (Juncker-Voss et al. 2000).

C. AEROPHILA (PULMONARY CAPILLARIASIS)

Heavy infections cause a tracheobronchitis accompanied by dyspnea, dry cough, mild hemoptysis, and pulmonary infiltrates (Gutierrez 1990).

Pathogenesis and pathology

C. PHILIPPINENSIS

The protein-losing enteropathy of intestinal capillariasis is a result of direct mucosal invasion by the adult worms, which is sometimes exacerbated by the host inflammatory response. Although the intestinal tract frequently has a gross normal appearance, a histopathological examination of the intestine from human autopsies reveals villus flattening and dilated crypts, with occasional inflammatory infiltrates (Cross 1995); numerous adult and larval worms can be identified in the intestinal mucosa where they are associated with ulcerative and degenerative changes. Nothing is known about the molecular and cellular events associated with *C. philippinensis* invasion, although the parasite may secrete bioactive molecules similar to those of *Trichuris*, which releases a proteolytic enzyme and a pore-forming protein from their stichosome (Drake et al. 1994).

C. HEPATICA

In hepatic capillariasis there is a remarkable degree of fibrosis in the liver (Attah et al. 1983). Histopathological examination reveals areas of focal destruction and numerous granulomata consisting of mononuclear cells and eosinophils. Many of these inflammatory changes occur at the site of worms and eggs. The adult worm is frequently identified by the characteristic presence of the stichosome on histological section. The eggs frequently appear in clusters.

C. AEROPHILA

A single case report of a biopsy in pulmonary capillariasis demonstrated numerous granulomas within the bronchiolar wall, producing marked airway destruction (Aftandelians et al. 1977). The parasite was identified in tissue sections (Gutierrez 1990).

Immune responses

Very little has been published on the immune responses to human capillariasis. Patients infected with *C. philippinensis* acquire humoral antibodies, including circulating immunoglobulin E (Rosenberg et al. 1970).

Diagnosis

C. PHILIPPINENSIS

In epidemic situations, patients who present with diarrhea, borborygmi, and abdominal pain are presumed to have intestinal capillariasis. The definitive diagnosis is usually made by identifying the characteristic-shaped eggs in the stool, although in severe hyperinfection it is not uncommon to also find larvae, or even adult worms (Cross 1995). For lightly infected patients a number of concentration techniques are available in order to increase the sensitivity of fecal examination.

C. HEPATICA

The clinical manifestations of human *C. hepatica* infection can resemble those of visceral larva migrans caused by *Toxocara canis*. A definitive diagnosis can be made by demonstrating the presence of eggs, larvae or adults in liver biopsy specimens. Usually, there is extensive accompanying hepatic fibrosis (Gutierrez 1990). More recently, an indirect immunofluorescence assay based on human serum samples has been developed (Juncker-Voss et al. 2000).

C. AEROPHILA

Conceivably, parasite eggs may be demonstrated in sputum or feces, although a pulmonary biopsy may be required to establish the diagnosis definitively (Aftandelians et al. 1977).

Control

C. PHILIPPINENSIS

Anthelmintics of the benzimidazole class, namely albendazole and mebendazole, are the treatments of choice for intestinal capillariasis. Mebendazole treatment usually requires 200 mg twice daily for 20 days, or albendazole 400 mg daily for 10 days (Medical Letter 2002). Relapses can occur with either agent, so that the patient must be followed and retreated for longer periods if necessary. Mebendazole is thought to be less active against the larval stages of the parasite and therefore may require longer treatment regimens (Cross 1995). Outpatient management of patients with intestinal capillariasis is often unsatisfactory because of the need for supportive therapy and the possibility of relapse following treatment. Cross (1995) points out that patients receiving specific anthelmintic therapy are often poorly compliant as outpatients, particularly with regard to receiving a full treatment course. Therapy must therefore be closely monitored, preferably in an in-patient setting. Patients need to be educated on the dangers of eating uncooked small fish, especially in endemic areas. The sanitary disposal of feces will also reduce parasite transmission.

C. HEPATICA AND *C. AEROPHILA*

Little is known about the efficacy of specific anthelmintic therapy for human infections with either of these two parasites. Because mebendazole is poorly absorbed outside the gastrointestinal tract, high doses would presumably be needed to achieve a therapeutic effect. Albendazole or thiabendazole may also be effective.

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Toxocara

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THE PARASITES

The discovery of a larval nematode within the retinal granuloma of a child by Wilder in 1950 (Wilder 1950) led to the description of the condition known today as visceral larva migrans (VLM). Two years later, Beaver and colleagues (Beaver 1952) described a number of infections, again in children, in which eosinophilia and severe multisystem disease were caused by migrating larvae of *Toxocara canis* and *Toxocara cati*, parasites whose definitive hosts are dogs and cats, respectively.

MORPHOLOGY AND STRUCTURE

Toxocara adult parasites are large, pink roundworms, measuring 7–10 cm × 5–6 mm (Figure 1). The male has a curved posterior end, which distinguishes it from the straight-tailed female (Figure 2a). Females lay large quantities of unembryonated eggs (Figure 2b) into the lumen of the small intestine. These eggs need to pass into the external environment in order to embryonate (Figure 2c). The larvae of *T. canis* are 290–350 × 18–21 μm (Figure 2d). The diameter of *T. cati* larvae is somewhat smaller (65–75 μm) (Ash and Orihel 1984). The eggs of *T. canis* measure approximately 85 × 75 μm and the eggs of *T. cati* are approximately 75 × 65 μm (Beaver 1984).

GENERAL BIOLOGY

Adult worms occupy the lumen of the small intestine and live unattached to the host. They maintain their position in the lumen by serpentine movement against the flow of peristalsis. Worms ingest partially digested food of the host and process it through their gut. Little is known about the digestive physiology, but it is likely to be similar to that of *Ascaris lumbricoides*, i.e. to have the capability of digesting most foodstuffs and absorbing the resulting small molecular weight nutrients across the midgut microvilli. *Toxocara* is a facultative anaerobe and derives most of its energy from glycogen degradation (Gopinath and Keystone 1995). The pseudocoelom

Figure 1 *Adult male and female* Toxocara canis. *The female is larger than the male and has a straight tail. Unstained, formalin-fixed specimens*

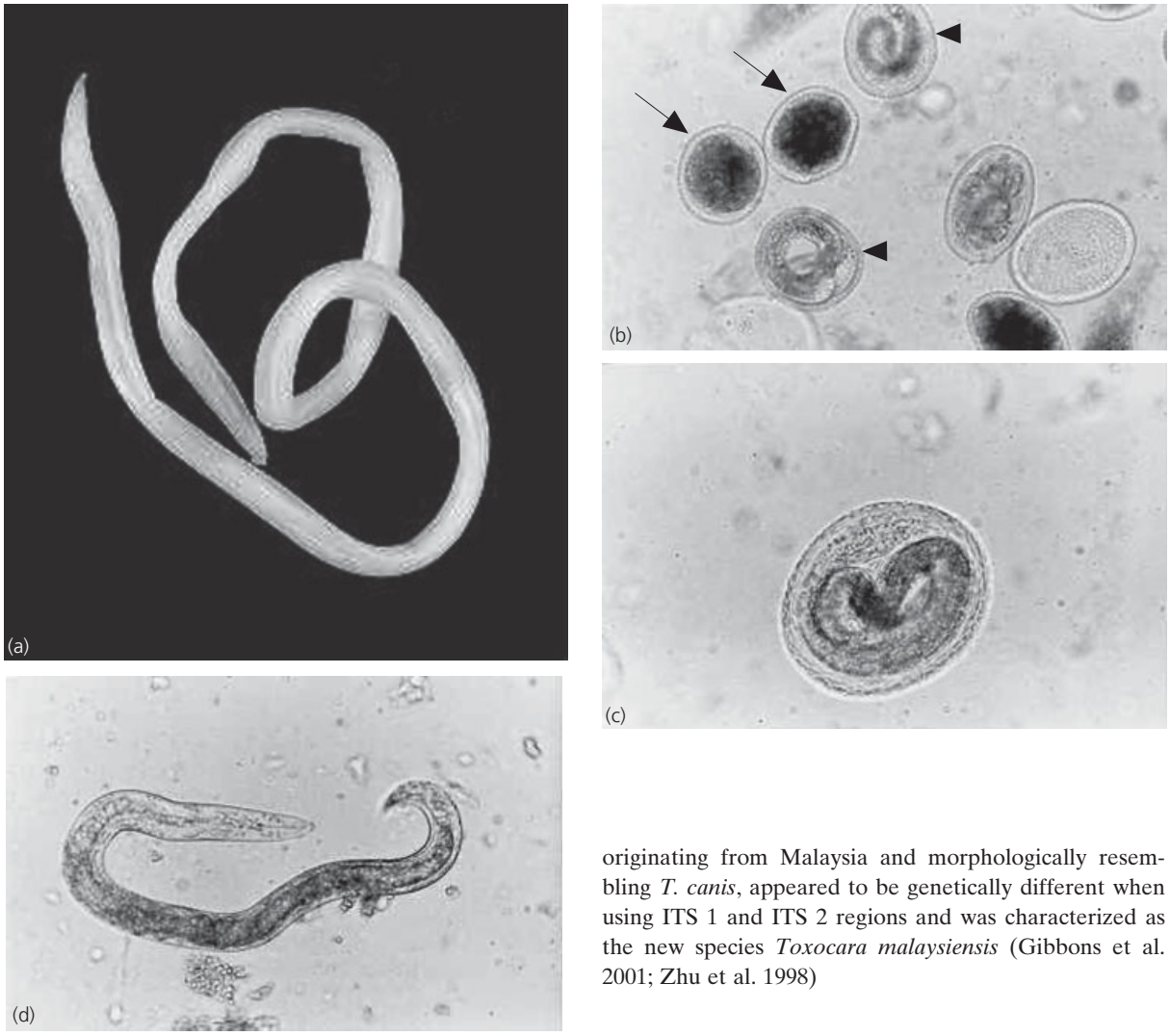

Figure 2 *Adult worm, eggs and larvae of* Toxocara canis*:* **(a)** *female worms measuring 7–10 cm shed* **(b)** *unembryonated eggs (arrows) which are passed with the feces into the environment. Eggs embryonate (arrow heads) within a period of 3–6 weeks (×200);* **(c)** *embryonated eggs containing the L2 larvae (×400).* **(d)** *L2 larvae (×100) hatch in the intestine, invade the intestinal mucosa, and migrate through the body causing the visceral larval migrans (VLM) syndrome.*

contains a special hemoglobin, which presumably binds oxygen thus augmenting energy metabolism. The worms' pink color is due to the presence of this substance.

MOLECULAR BIOLOGY

Ribosomal DNA (rDNA) markers and, in particular, the internal transcribed spacer (ITS) regions can be used to characterize and to identify the different *Toxocara* species. Polymerase chain reaction (PCR) derived strategies based on ribosomal DNA can be used as diagnostic tools where animal fecal material, as well as animal and human tissues, could be used (Jacobs et al. 1997; Zhu et al. 2001). Recently, a *Toxocara* variant in cats, originating from Malaysia and morphologically resembling *T. canis*, appeared to be genetically different when using ITS 1 and ITS 2 regions and was characterized as the new species *Toxocara malaysiensis* (Gibbons et al. 2001; Zhu et al. 1998)

LIFE CYCLE

In their definitive hosts, *T. canis* and *T. cati* have similar life cycles, which resemble that of *Ascaris lumbricoides* in humans (Figure 3). Nonembryonated eggs are passed with the feces into the environment and incubate in soil. Within 2 weeks, the eggs contain an infectious second-stage larva that, if eaten by a dog (*T. canis*) or cat (*T. cati*, *T. malaysiensis*), initiates infection by hatching in the small intestine. The freed larvae penetrate the small intestine and enter the general circulation. In the liver, they lodge in the presinusoidal capillaries because of their large diameter, and are stimulated to enter the parenchymal tissue where they feed on liver cells. They molt in the liver to the third stage, re-enter the general circulation, and are carried to the lungs. Trapped by the alveolar capillaries, again because of their size, the larvae penetrate into the alveolar space, crawl up the bronchioles into the trachea, bypass the epiglottis and are swallowed. In the small intestine, larvae molt for a fourth time, transforming into adult worms. Mating ensues soon after and the

females begin to pass unembryonated eggs, thus completing the life cycle in their definitive hosts.

Toxocara spp. cannot complete their life cycle in humans. Infection is initiated, as in the dogs or cats, by the ingestion of embryonated eggs. Larvae hatch in the small intestine but, as they do not receive the proper environmental signals (Castro 1982), begin their odyssey, wandering throughout the body, invading all organs and damaging tissue wherever they go. The larvae never mature and eventually die in situ. Death may occur soon after infection, but many larvae can survive for several months, even up to years (Smith and Beaver 1953). In experimentally infected rhesus monkeys, *Toxocara* larvae have been reported to remain viable and infective in tissues for at least 9 years (Schantz 1989).

TRANSMISSION AND EPIDEMIOLOGY

Transmission of *Toxocara* occurs by ingestion of embryonated eggs present in soil either directly by geophagia or indirectly by consumption of unwashed contaminated fresh vegetables. Human infections with larvae have also been described and can take place by eating undercooked tissues of a paratenic host (Schantz 1989).

Toxocara spp. are found throughout the world but the recorded seroprevalence varies among countries or even within countries. In Ireland, *Toxocara* seroprevalence in schoolchildren is high (31 percent) and rises with age (Holland et al. 1995). The seroprevalence in children in the state of Connecticut, USA, varied from 6.1 percent in New Haven to 27.9 percent in Bridgeport (Sharghi et al. 2001). Children are most at risk of infection, especially when there is a history of pica. Several risk factors have been reported, which differ between countries. In the USA, an association with living in an urban area, race, and family income has been described. In this study, no association was found with owning dogs or puppies and gender (Sharghi et al. 2001). In Ireland however, males were significantly more infected than females, as well as children attending rural schools versus urban schools (Holland et al. 1995).

The prevalence of ocular toxocariasis is still largely unknown. In a 1-year survey in Alabama, USA, the estimated prevalence was 1 per 1 000 population (Maetz et al. 1987). There are no accurate data on the number of patients with ocular lava migrans (OLM) who seek medical attention. Patients with OLM are usually older than VLM patients, probably because they lack systemic signs and do not have a history of pica (Schantz 1989).

Concerning the definitive hosts, infections with *Toxocara* spp. are not only prevalent in dog and cat populations but several epidemiological studies around the world showed a high prevalence of *Toxocara* spp. in wild carnivores like foxes and wolves. In the Netherlands, the high prevalence of *Toxocara* spp. in domestic and wild carnivores ranges from 4.7 percent in domestic cats and 2.9 percent in domestic dogs to 21 percent in stray cats (Overgaauw 1997). In Poland, the prevalence has been reported to be 39 percent in cats, 32 percent in dogs, and 16 percent in foxes (Luty 2001); in Spain, 6.4 percent has been reported in wolves (Segovia et al. 2001). Because of the reservoir in wild carnivores, eggs are not only found in high densities in public and private places but also in more remote areas around the world. The numbers of eggs detected in soil depends on season, presence of earthworms, and depth of the soil (Weller et al. 1983). Children's sandpits can be contaminated by *T. canis* and *T. cati*, depending on their maintenance (Jansen et al. 1993).

CLINICAL SYNDROMES AND MANIFESTATIONS

Three clinical syndromes of human toxocariasis are recognized: VLM, OLM, and covert toxocariasis (CT).

VLM is mainly a disease of young children and its symptoms include fever, respiratory distress, and hepatosplenomegaly (Gutierrez 1990; reviewed in Despommier 2003). Other symptoms include an eosinophilic pneumonitis (Loeffler's pneumonia) that bears a clinical resemblance to the pulmonary inflammatory responses observed in asthmatic patients. Myocarditis, nephritis, and central nervous system (CNS) symptoms are not uncommon. CNS involvement may lead to seizures, psychiatric manifestations, or encephalopathy (Fortenberry et al. 1991). The different manifestations of VLM and OLM have been related to the infective dose. This has been clearly shown in murine models for toxocariasis. After infection of mice with large numbers of *T. canis* embryonated eggs, larvae can be recovered from brain tissue. The presence of larvae in the brain has been associated with behavioral changes observed in these infected animals (Holland and Cox 2001).

OLM occurs primarily in older children and usually manifests as a unilateral vision disorder often accompanied by strabismus (Dinning et al. 1988). However, the age of onset of ocular toxocariasis has been reported to range from 2 to 50 years (Taylor 2001). Invasion of the retina leading to granuloma formation is the most serious consequence of the infection (Gillespie et al. 1993) and occurs peripherally or in the posterior pole. These granulomata distort the retina creating heteropia, or macular detachment (Small et al. 1989). Diffuse uniocular endophthalmitis or papillitis with secondary glaucoma are other complications of larval migration and death. Blindness is common, but the degree of damage depends on the area affected. It is still not clear what role *T. cati* might play in causing ocular lesions (Taylor 2001).

Patients with toxocariasis whose symptoms do not fit into the categories of VLM or OLM are described as having CT. The symptoms are nonspecific but when grouped together form a recognizable syndrome. These

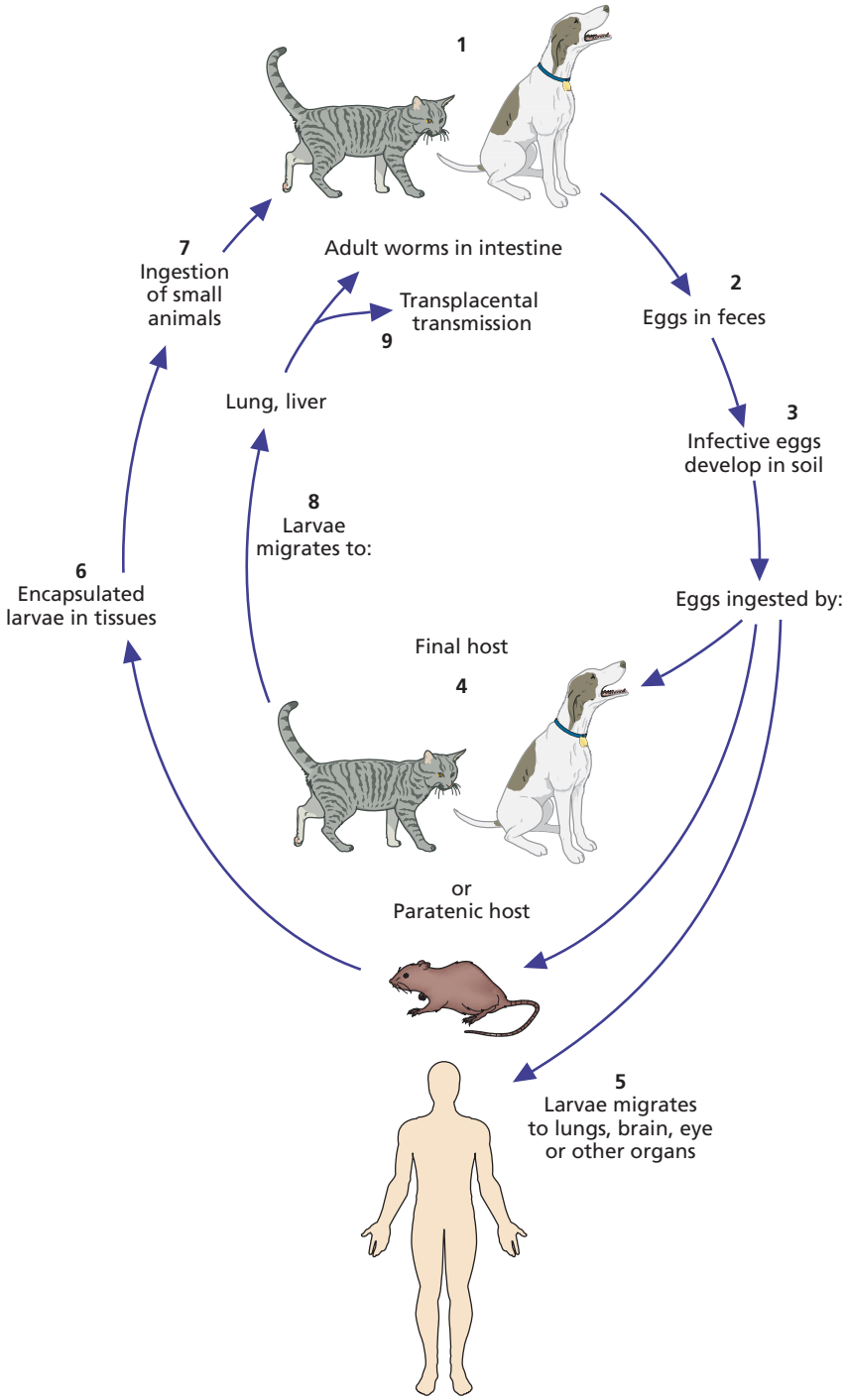

Figure 3 *Life cycle of* Toxocara canis *and* Toxocara cati. *Adult worms present in the intestinal lumen of dogs or cats (1) shed a large number of unembryonated eggs that are passed into the environment via the feces (2). These eggs are usually found in soil contaminated with feces of these animals. In the soil, the first and second larval stages (L1, L2) are formed within the eggshell under favorable conditions (3). The infective eggs can be ingested by final (4) or paratenic (5) hosts. In the intestinal lumen of the paratenic host, the L2 larvae hatch from the egg, penetrate through the intestinal wall and migrate through the body, but there is no further development. Such larvae may become included in granulomas, remain in tissues (6) for a long time and are infective to final hosts (7). In the final host, the L2 larvae hatch from the egg inside the intestinal lumen, penetrate the intestinal wall and are transported via the bloodstream to the liver and lung (8). The larvae molt twice (L3, L4) in the lungs. The L4 larvae migrate up the trachea and are swallowed, reaching the intestinal lumen where they mature. After mating, females release fertilized eggs and the cycle starts again. In the majority of adult final hosts, the larvae do not complete the lung migration and remain 'dormant'. During pregnancy, larvae are activated, re-enter the circulatory system, are carried to the placenta, and penetrate the fetus (9).*

symptoms include abdominal pain, anorexia, behavior disturbances, cervical adenitis, wheezing, limb pains, and fever (Schantz 1989; Taylor et al. 1988).

Asymptomatic cases, only detected by positive serology, do occur. However, there are no data available on the extent of these cases (Schantz 1989).

PATHOGENESIS AND PATHOLOGY

Damage depends on the tissue invaded; the lungs, eyes, and CNS are the most seriously affected (Figure 4). Although tissue damage due to the migration of worms is a major cause of cell death, it is less harmful than the eosinophilic granulomata that are the hallmark of worm death. When worm death occurs in the eye, the resulting damage often resembles retinoblastoma (Despommier et al. 1994). In the past, enucleation was performed in some unfortunate cases because of the unavailability of modern diagnostic tests. Granulomata induced by dying worms can be sufficiently intense to be responsible for the loss of sight in the affected eye.

Infection with this parasite can also result in asthmatic manifestations associated with the hypereosinophilic syndrome (Feldman and Parker 1992). Studies in the murine model for toxocariasis have shown that *T. canis* larvae migrate to the lungs resulting in pulmonary inflammation. The cells that infiltrate the lung include eosinophils, macrophages, and lymphocytes (Buijs et al. 1995). Additional changes after infection include hypertrophy of goblet cells, indicative of mucus production, and increased total immunoglobulin (Ig)E in serum (Pinelli et al. 2001).

Epidemiological studies have suggested that allergic manifestations occur more often in *Toxocara*-seropositive patients (Buijs et al. 1997; Chan et al. 2001). However, this remains a point of discussion since other authors have not found any significant association between *Toxocara* infection and asthma (Sharghi et al. 2001).

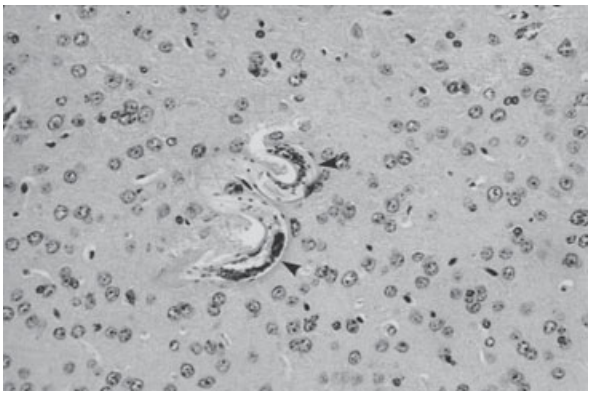

Figure 4 *A larva of* Toxocara canis *in the brain tissue of an experimentally infected mouse. Note the lack of any inflammatory response around the area of damage. The worm is approximately 20 μm in diameter. Hematoxylin and eosin-stained section of paraffin-embedded tissue.*

IMMUNE RESPONSES

Immunological features of infection with *Toxocara* spp. are increased serum IgE levels and eosinophilia (Brunello et al. 1983; Kayes 1997; Sugane and Ohshima 1984). The death of migrating larvae is accompanied by striking delayed-type and immediate-type hypersensitivity. Humoral responses include an elevation in levels of IgG, IgM, and IgE only some of which is specific for parasite components (Schantz et al. 1979). Most antibody responses are against a unique cuticular-associated trisaccharide epitope that is shed by the worm during its migration throughout the tissues. This antigen, which can be collected in vitro, is useful in the immunodiagnosis of the infection. However, immune responses against this antigen appear not to be protective. Nothing is known regarding the immune mechanisms responsible for the death of the worm.

Infection with helminths is generally associated with the induction of a T-helper 2 (Th2) type of immune response. Th2 cells produce cytokines such as interleukin (IL)-4/IL-13 and IL-5 that are required for the production of IgE and the maturation of eosinophils respectively (Mosmann and Coffman 1989). Infection with *Toxocara* spp. results in elevated circulating levels of eosinophils, which target dying worms in tissues. Granulomata consist of collections of eosinophils, lymphocytes, fibroblasts, epithelioid cells, and giant cells. Remnants of larval tissue may be present in the center of each granuloma. The nature of the role of eosinophils during infection is not clear. There is a limited number of studies in vivo to support a protective role for eosinophils against helminth infections (Weiss 2000). Experiments in vitro, however, have demonstrated that eosinophils can adhere in an antibody-dependent manner and kill larvae of *Schistosoma mansoni* (Butterworth et al. 1979), newborn larvae of *Trichinella spiralis* (Kazura and Aikawa 1980), and microfilariae of *Onchocerca volvulus* (Greene et al. 1981). For *Toxocara canis* however, eosinophils do not appear to play a major role in the killing of larvae (Fattah et al. 1986; Badley et al. 1987).

DIAGNOSIS

Biopsy of liver or other tissues in which larvae are suspected is rarely positive, hence diagnosis depends primarily on indirect measures of the presence of the worm, such as immunological tests, particularly enzyme-linked immunosorbent assay (ELISA) and Western blotting (Speiser and Gottstein 1984; Taylor et al. 1988). Using antigens secreted by the second-stage larvae and ELISA, sufficient specificity (91–93 percent) and sensitivity (78–91 percent) has been reported (Jacquier et al.

1991; Speiser and Gottstein 1984; Taylor et al. 1988). The specificity depends on the threshold used. A titer greater than 1:32 is considered to be indicative for the diagnosis (Smith et al. 1983; Taylor et al. 1988). Higher titers, with optical denisty (OD) values greater than 1.8, are common in well expressed toxocariasis (Luzna-Lyskov et al. 2000). Elevated gammaglobulin and an elevated isohemagglutinin titer (Despommier et al. 1994) can also help the clinician to confirm toxocariasis. Extreme eosinophilia is rarely seen in toxocariasis cases, although it can be an important indicator (Taylor et al. 1988).

OLM is diagnosed on clinical criteria during an ophthalmologic examination. Serological tests for antibodies are not as reliable for OLM as they are for VLM. In a study with OLM patients, only 45 percent of the patients had antibody levels that correlated with the infection (Schantz et al. 1979). A positive titer may be a diagnostic aid, but a negative titer cannot exclude the diagnosis. Western blotting with specific anti-*Toxocara* IgE detection appears to be an accurate procedure for the immunodiagnosis of OLM, provided that the testing is performed simultaneously on serum and ocular fluid (Magnaval et al. 2002).

CONTROL (THERAPY, PROPHYLAXIS, AND TRANSMISSION)

Although diethylcarbamazine (DEC) is listed in some references as an effective drug, its use is not recommended. Patients treated with DEC reported a significantly higher rate of adverse reactions than patients treated with mebendazole. Mebendazole in a dose of 20–25 mg/kg for 21 days was effective in treating an adult case of VLM (Magnaval 1995). Albendazole (15 mg/kg, 5 days) may also be effective (Pawlowski 2001). Corticosteroids suppress intense inflammation and thus relieve some of the more serious symptoms associated with worm migration and worm death. Ivermectin given as a single oral dose of 12 mg was less effective in clinical improvement and reduction of eosinophilia (Magnaval 1998). Liposome-incorporated benzimidazole carbamates have been shown to be effective in a murine model. Fenbendazole was more effective in muscles, whereas albendazole was more effective in the brain (Hrckova and Velebny 2001; Okada et al. 1996).

Treatments for OLM include surgery (vitrectomy), anthelmintic chemotherapy, and corticosteroids. In an Austrian study, albendazole (adults: 800 mg b.i.d.; children: 400 mg b.i.d.), in combination with systemic steroids, was shown to be a useful regimen to treat OLM syndrome (Barisani-Asenbauer et al. 2001).

Education of patients and healthcare workers on the importance of avoiding contact with contaminated environments is essential. The most important preventive measures are:

- regular de-worming of dogs and cats, beginning at 2 weeks of age (pups), 4, 6, and 8 weeks (pups and cats), followed by treatment every 2 months until 6 months of age and thereafter twice a year
- preventing contamination of soil by removing cat and dog feces in places immediately adjacent to houses and children's playgrounds
- covering children's sandpits
- regular washing of hands after handling soil and before eating
- teaching children not to put dirty objects into their mouths (Chin and Asher 2000).

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Trichinella

DICKSON D. DESPOMMIER

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THE PARASITE

Trichinella spiralis was described in 1835, first by James Paget, then by Richard Owen from independent observations on muscle tissue from the same cadaver. Paget viewed the encapsulated worms through a compound microscope borrowed from Robert Brawn, a botanist at the British Museum (Campbell 1983a). The infected muscle tissue which harbored the parasites was from a 51-year-old male who had died that day from the ravages of tuberculosis. He had apparently been coincidentally infected with *T. spiralis* some years prior to his death. Following this landmark discovery, work began on deciphering its life cycle and the worm's relationship to various clinical manifestations. By 1859, the major components of its life cycle had been described (Virchow 1859). Much of its natural history and basic biological features are now known (see Campbell 1983b).

All nematodes within the genus *Trichinella* are dependent upon carnivorism in order to move from one host to another, and they infect an extremely wide range of vertebrate host species (birds, reptiles, and mammals). All mammals exhibit some degree of susceptibility to infection with *T. spiralis* and its relatives, ensuring that the parasite's geographic distribution is both widespread and its incidence of infection is extensive. Several new species have been described which will undoubtedly lead to a better understanding regarding the evolutionary history of the genus (Pozio 2001b). There are now eight recognized species, with more surely to be discovered over the next few years. They are: *Trichinella spiralis*, *T. britovi*, *T. nelsoni*, *T. nativa*, *T. murrelli* (Pozio and La Rosa 2000), *T. pseudospiralis*, *T. papuae* (Pozio et al. 1999), and *T. zimbabwensis* (Pozio 2001a; Pozio et al. 2002). Clinically, *Trichinella spiralis* still remains the dominant cause of trichinellosis in humans throughout the world. In addition, the vast majority of experimental work employs *T. spiralis*. Thus, what follows will pertain mainly to this single species.

MORPHOLOGY AND STRUCTURE

T. spiralis adult male and female worms are dimorphic (Figures 1 and 2).

The female measures 3 mm in length by 36 μm in diameter, and the male measures 1.5 mm in length by 36 μm in diameter. Besides its length, the male can be distinguished from the female by its obvious reproductive organs at its tail end, termed claspers, that it employs to hold on to the female worm while mating. The newborn larva (Figure 3) measures 80 μm in length by 7–8 μm in diameter. Its esophagus has a stylet, a spear-like organ (Figure 4), which it uses to enter cells (Figure 5). The infective muscle larva (Figure 6) measures 1 mm in length by 36 μm in diameter, and is an obligate intracellular parasite (Figure 7). This enables all species of trichinella to share in the world title as holders of the record for being the largest examples of this kind of parasitism.

GENERAL BIOLOGY

Adult worms of both sexes also live as intracellular parasites (more accurately, intramulticellular), occupying

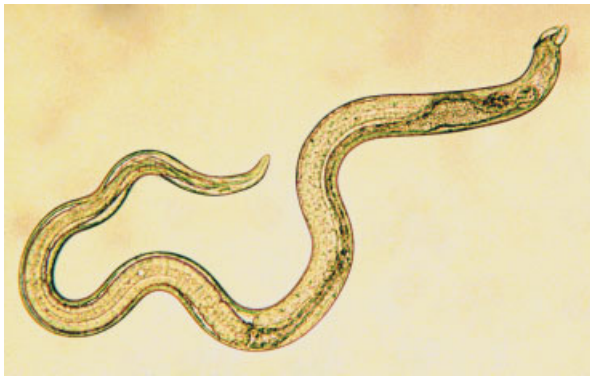

Figure 1 *The adult male* Trichinella spiralis *measures 1.5 mm × 36 μm.*

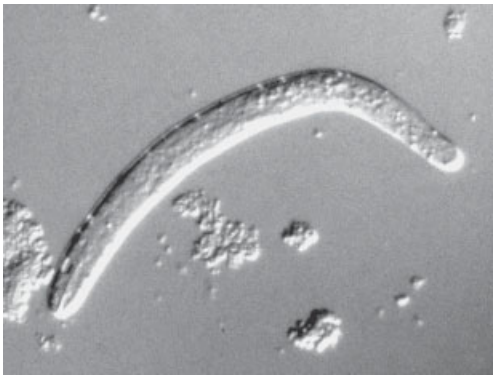

Figure 3 *The newborn larva of* Trichinella spiralis *measures 80 × 7 μm. Nomarski interference.*

a row of columnar cells in the upper part of the small intestine (Figure 8). In contrast to the larva, their metabolism is aerobic, deriving energy from the surrounding cytoplasmic milieu by an unknown mechanism(s). Host tissue is not directly ingested as evidenced by electron microscopy (Wright 1979). Most likely, the worms actively transport small molecular weight substances across their cuticular surfaces, or perhaps employ the pores of the hypodermal gland cells as an entry point for nutrients (Figure 9). Apparently, all stages have the ability to metabolize low molecular weight substrates, synthesizing protein, lipid, DNA, and RNA from them.

The infectious stage is the muscle larva (L1). It occupies a special niche which it creates during its development: the Nurse cell (Despommier 1998) (see Figure 10). Its metabolism is strictly anaerobic, despite the fact that the Nurse cell–parasite complex is surrounded by a circulatory rete consisting of a branching network of sinusoidal-like blood vessels (Figure 11). Some details of its intermediary metabolism are known, as the larvae are easy to obtain in large numbers (Stewart 1983).

Figure 2 *The adult female* Trichinella spiralis *measures 3 mm × 36 μm.*

As with all other nematodes, this worm's developmental cycle includes four larval stages. However, trichinella progresses rapidly through them – L1 to L4 in just 28 h after ingestion (another world record?) – all the while remaining in situ within the columnar epithelium (Figure 12). Adults can be demonstrated in the gut of experimentally infected rodents as early as 30 h after oral infection. Also, unlike most other nematode species, they do not change their overall dimensions until they achieve adulthood, when they grow longer, but thereafter remain at the same diameter.

The newborn larva migrates from the small intestine to the muscle tissue via the lymphatic circulation and/or the bloodstream, invading tissues, aided by its stylet. This stage is thus the most pathogenic form of trichinella (Capo and Despommier 1996), killing all cells it enters, with one notable exception: the striated skeletal muscle cell. Like the full-grown muscle larva, its metabolism is anaerobic, but few studies on this aspect have been carried out, probably owing to the difficulty and expense in obtaining sufficiently large numbers of this stage on which to work.

LIFE CYCLE

Ingestion by the host of the Nurse cell–parasite complex within infected muscle tissue initiates infection (Figure 13). The larvae are immediately freed from their intracellular niche by digestive enzymes and are transported by peristalsis to the upper two-thirds of the small intestinal tract. A small portion of epicuticular material (Figure 14) becomes altered by trypsin and perhaps by other proteases as well (Despommier 1983), allowing the parasite to receive external environmental cues within the lumen of the small intestine, resulting in alterations of its behavior (Stewart et al. 1987). Ultimately, these stimuli cause it to penetrate the columnar epithelium, perhaps aided in part by its own complement of proteases (Lun et al. 2003). After undergoing four molts in rapid succession during a 30-h period, the parasite matures to adulthood. Mating

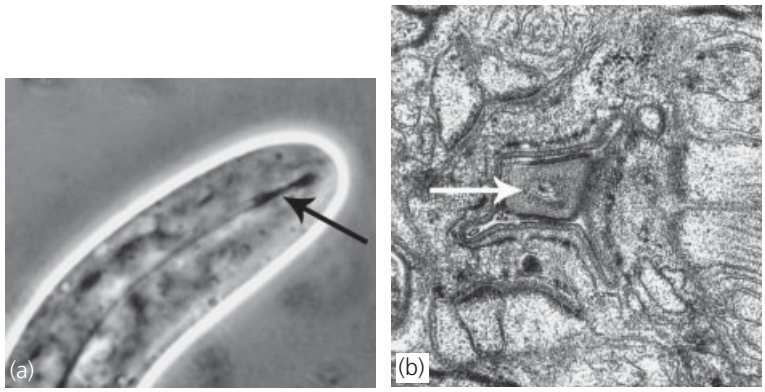

Figure 4 (a) *Stylet of the newborn larva of* Trichinella spiralis *(arrow).* **(b)** *Stylet of the newborn larva of* Trichinella spiralis *(arrow). Transmission electron micrograph*

ensues over the next few hours. The gestation period is 5 days, after which females begin depositing live newborn larvae into the lamina propria. Larvae are born continuously over the next several days, depending upon a variety of factors including the development of host immunity. Thus, the total number of newborns produced varies greatly from host to host, and also differs between males and females, and young and old members of the same host species (Despommier 1983). The intestinal phase of the infection in most mammalian hosts, including humans, routinely lasts up to 2–3 weeks, but ultimately adult worms are expelled by immune responses.

The route taken by the newborn larva from the gut to the muscle cell involves traversing the lymphatics as well as the general circulation. As a result, newborns invade all tissues. The migration phase is associated with bacteremia caused by the larvae dragging in members of the enteric flora along with themselves, and deaths in humans due to sepsis may occur during heavy infection. Larvae not finding striated skeletal muscle cells emerge from capillaries and penetrate other cell types, killing them in the process. Only skeletal muscle cells offer the prospect of a suitable niche in which the parasite is able to grow and develop (Despommier 1990; Jasmer 1995). When the newborn larva penetrates this cell type, it remains in place and begins the next phase of its life, now as an intracellular organism. Within 20 days, the larva has re-engineered that environment, forcing host cytoplasm to transform from that of a contractile cell to one that aids the worm in its quest to achieve infectivity. The mechanisms by which this intriguing outcome of infection occurs are currently only hinted at from an extensive set of data extracted from the experimental literature. The Nurse cell thus becomes the life support system for the parasite, and can sustain the parasite for long periods of time – months to years (Despommier 1993). This ecologically sound strategy enables the parasite to travel great distances in some cases, ensuring the wide distribution of this most unique group of nematode parasites.

Nurse cell formation is thought to be the direct result of host–parasite interactions between the stichosome-derived secretions of the developing worm and host cell DNA. To date, no other nematode group has been shown to be this intimately connected metabolically with its mammalian host (Despommier et al. 1990). The vast majority of larvae eventually die and the remnant Nurse cell–parasite complexes calcify (Gerwel et al. 1970).

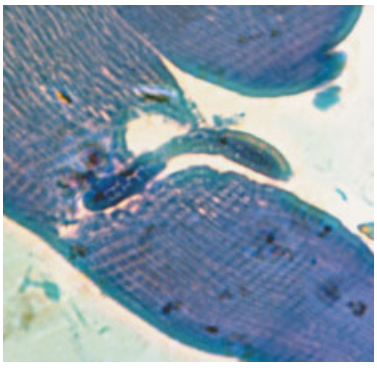

Figure 5 *Newborn larva of* Trichinella spiralis *entering a muscle cell. Note collateral damage to surrounding cytoplasm.*

Figure 6 *The infective muscle larva of* Trichinella spiralis *measures 1 mm × 37 μm. Phase contrast*

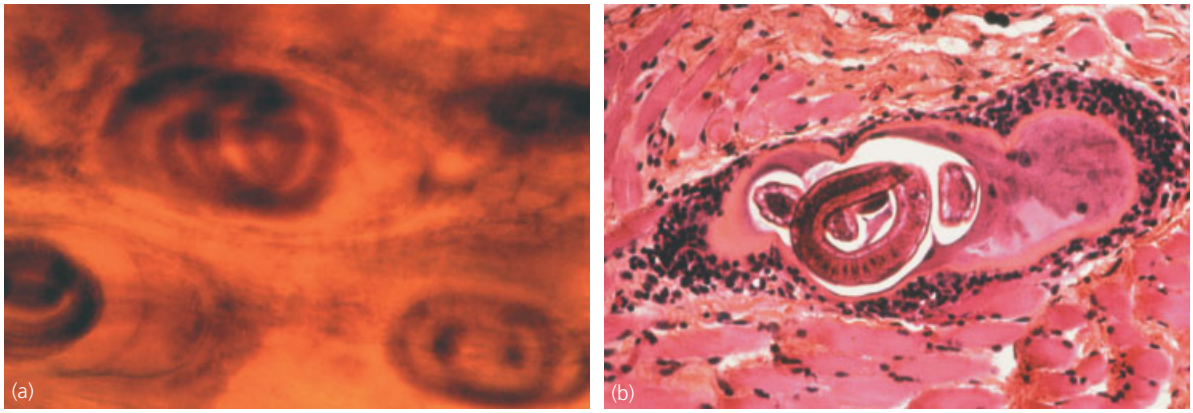

Figure 7 (a) *Whole mounted, unstained preparation of infected muscle tissue harboring numerous Nurse cell–parasite complexes.* **(b)** *Histological section of infected muscle tissue shown in* **(a)** *with Nurse cell–parasite complex of* Trichinella spiralis. *Note the intense cellular infiltrate surrounding the Nurse cell capsule.*

TRANSMISSION AND EPIDEMIOLOGY

As already stated, eating meat is the typical way of acquiring infection with *Trichinella spiralis* (Campbell 1983b). From an ecological standpoint, meat is an energy-rich, valued commodity in nature and rarely goes unconsumed when available, either as prey or as a scavenged carcass. Therefore, a wide variety of animals harbor one or more species of trichinella, many of which most would consider to be strict herbivores. The current world prevalence of infection with *T. spiralis* cannot be estimated because there have been no recent global surveys conducted. Several new reports document the prevalence and incidence of infection in some parts of Europe and China. The loss of infrastructure in the war-torn regions of Central Europe (i.e. lack of government-sponsored meat inspection programs, etc.) (Pozio and Marrucci 2003) have ushered in a significant increase in the number of new human cases (Geerts et al. 2002) and a dramatic increase in prevalence in pigs, particularly in those raised on small farms. In China, the incidence of infection in some domestic animals (e.g. dog, cat, pig) (Cui and Wang 2001) and subsequent increase in the rate of human infection in some southern provinces (e.g. Hunan, Szechwan) is substantially higher than previous estimates (Liu and Boireau 2002). Sporadic epidemics continue to occur with regularity (Capo and Despommier 1996; Bruschi and Murrell 2002). Within the last 10–15 years, in addition to those referenced above, outbreaks have occurred in Japan (Takahashi et al. 2000) and the Middle East (Olaison and Ljungstrom 1992). Apparently, Puerto Rico and mainland Australia (but not Tasmania) are among the few places in the world that have remained trichinella-free. *Trichinella pseudospiralis*, a non-capsule forming species, is thought mainly to infect birds, but can also occasionally infect mammals. It has been isolated from the Tasmanian Devil (Obendorf et al. 1990). Human cases have occurred in Tasmania (Andrews et al. 1994) and Thailand (Jonwutiwes et al. 1998). Apparently, the clinical manifestations of infection with this species are more prolonged than for *T. spiralis* (Bruschi and Murrell 2002). Four additional species of trichinella are potentially of medical importance. *T. nativa* occurs sylvatically in the Arctic where the reservoir hosts are polar bears and walruses. Eskimo populations are at high risk for infection due to their penchant for eating raw meats. *T. nelsoni* is also sylvatic and causes trichinellosis in equatorial Africa. There, hyenas and large cats serve as reservoirs. Infection in both wild and domestic pigs with *T. papuae*, another non-encapsulated species, occurs in Western Province, New Guinea (Owen et al. 2000). Human infections may also occur there, as indicated by the presence of *Taenia solium* infection (Margono et al. 2003), since native populations continue the habit of eating undercooked pork. *T. zimbabwensis*, the third species that does not form a capsule as a larva, has recently been described from Central Africa as an infection of domestic crocodiles. Infection in humans with this species has yet to be detected.

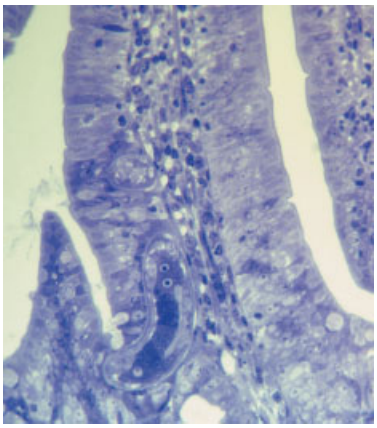

Figure 8 *A single adult worm of* Trichinella spiralis *in situ in a row of enterocytes in the small intestine of an experimentally infected mouse*

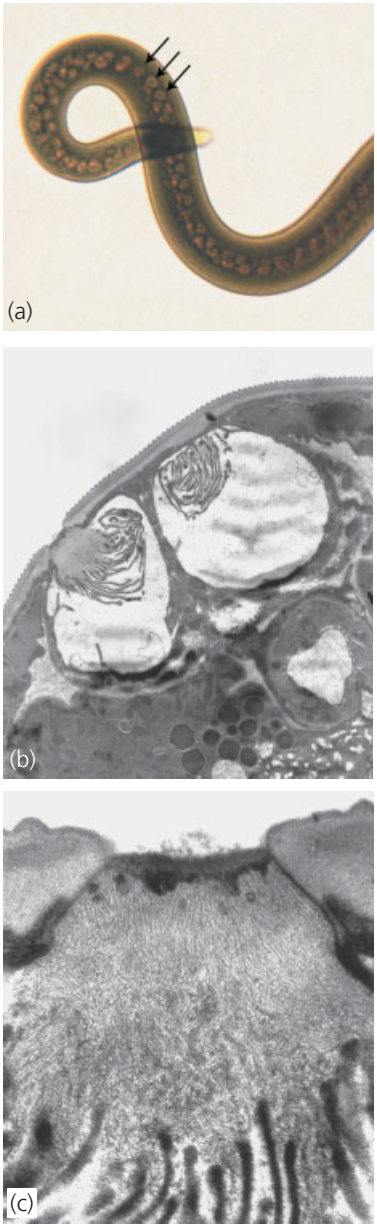

Figure 9 (a) *Whole mount of an adult female* Trichinella spiralis. *Note double row of hypodermal gland cells (arrows).* **(b)** *Two hypodermal gland cells of an adult female* Trichinella spiralis. *Transmission electron micrograph.* **(c)** *A portion of a hypodermal gland cell, high magnification. Transmission electron micrograph*

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION

Clinical manifestations

ENTERIC STAGES

Symptoms resulting from infection with *Trichinella spiralis* are often first ascribed to other illnesses until laboratory tests come back negative for them. Then physician attention shifts to the more 'exotic' diagnoses. Despite the lack of physician awareness of this worldwide pathogen, keeping in mind specific signs and symptoms can greatly enhance the chances for making a correct diagnosis prior to running a battery of expensive assays, even in the early stages of the disease. For the duration of the 3-week period of the intestinal infection, patients can suffer many effects related to the damage that occurs there. Development of larvae to adults in the columnar epithelium is associated with enteritis. As newborn larvae are shed (i.e. 5–21 days after infection), mucosal inflammation intensifies, with inflammation consisting of eosinophils, neutrophils, and lymphocytes. Antigen–antibody complexes develop in the surrounding tissues and probably also contribute to intestinal disease experienced by patients who have ingested large numbers of larvae.

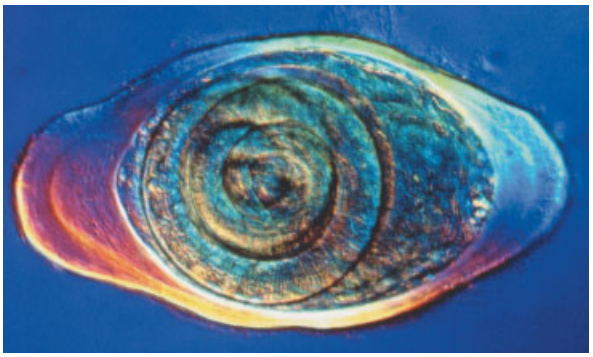

Figure 10 *Nurse cell–parasite complex of* Trichinella spiralis. *Nomarski-phase interference. Photo courtesy of Eric Grave*

PARENTERAL STAGES

Damage caused by larvae penetrating cells becomes serious when this occurs in cardiac and central nervous system (CNS) tissues. Myocarditis, sometimes severe enough to cause death, is transient as Nurse cells cannot form in heart tissue. In the CNS, larvae tend to stay and wander about, frequently causing significant damage even in mild infection.

In skeletal muscle fibers, parasite-secreted proteins and other antigens induce progressive infiltration of inflammatory cells. Myositis and tissue edema develop on or about 14 days after penetration of the fiber. The amount of pathological change caused by the larvae is directly proportional to the number shed in the small intestine. However, there is much variation in the clinical presentation, related mostly to the type of muscle affected and the species of trichinella causing the damage. Lethal infection for most adults is of the order of 15 *T. spiralis* larvae per gram of diaphragmatic muscle, whereas as many as 1 000 *T. nelsoni* parasites per gram of muscle are still well tolerated.

Moderate to heavy trichinellosis presents signs and symptoms in the early (intestinal), middle (systemic and tissue invasion), and late (convalescent) phases (Murrell

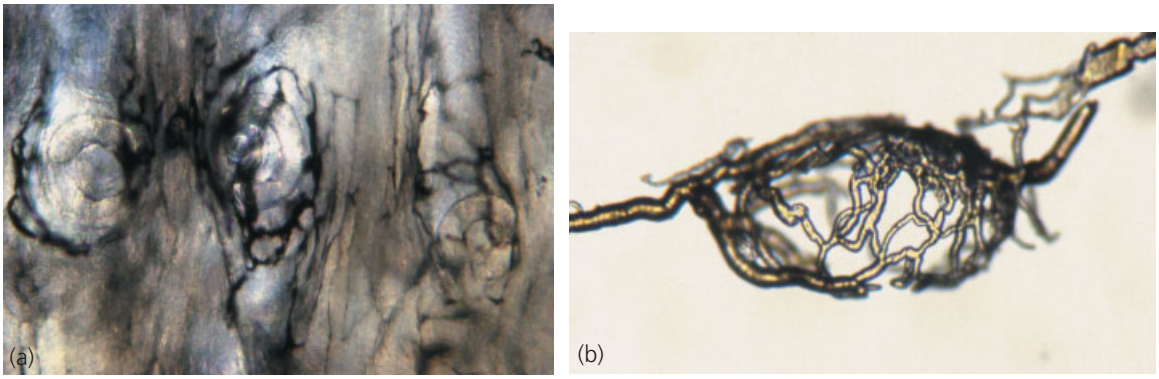

Figure 11 (a) *Three circulatory rete surrounding Nurse cell–parasite complexes in a whole mount preparation of an experimentally infected mouse. India ink was injected into the circulatory system immediately after administration of a lethal injection of Nembutal to reveal the blood vessels.* **(b)** *Anatomic plastic cast of the circulatory rete of an experimentally infected mouse*

and Bruschi 1994; Capo and Despommier 1996). Early intestinal distress (in weeks 1–3) is associated with diarrhea, abdominal pain, and vomiting. The systemic and tissue invasion phase (weeks 3–8) is accompanied by fever and myalgia, periorbital edema, and hemorrhages in the nail beds and sclera of the eye, but can also seen in the mucous membranes. Edema leads to muscle tenderness and pain. Photophobia is another consequence of heavy infection.

Progressive infection, in which larvae penetrate a variety of tissues, gives rise to even more serious and often confusing sequelae. Myocarditis occurs in nearly 20 percent of seriously ill patients and electrocardiographic changes are frequently noted during this phase. Dyspnea is another complication of myositis. Neurological signs and symptoms vary greatly, but, coupled with other laboratory findings and a history of raw meat eating, should lead the alert physician to suspect infection with trichinella.

There is no evidence that long-term sequelae occur following even severe infection, at least with *Trichinella spiralis* (Feldmeier et al. 1991). However, from the worm's perspective there are long-term consequences, since numerous Nurse cells calcify and, as a result, the larvae inside them perish. This process usually takes place over several months to years following infection. Whereas disease caused by other species of *Trichinella* present somewhat differently (Murrell and Bruschi 1994; Capo and Despommier 1996), details are unwarranted because of their low prevalence.

Infection with *T. pseudospiralis* apparently runs a longer course as compared to that typically reported from outbreaks due to infection with *T. spiralis* (Bruschi and Murrell 2002).

Pathogenesis and pathology

The migrating newborn larvae cause the vast majority of pathological consequences which occur mainly during the first 3–5 weeks after ingesting infected meat. The use of their stylet to enter and leave cells accounts for most

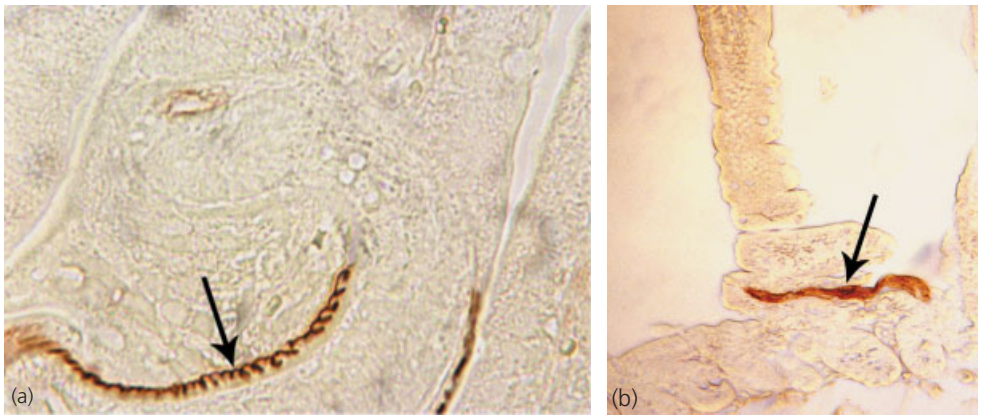

Figure 12 (a) *Immunoperoxidase-stained infected small intestine of an experimentally infected mouse. The L2 larva of* Trichinella spiralis *is in the process of molting and is crawling out of its L1 cuticle (arrow). The monoclonal antibody employed recognized only tyvelose epitopes.* **(b)** *Remnant of L1 cuticle (arrow) in enterocytes of a mouse experimentally infected with* Trichinella spiralis*. The same monoclonal antibody was used here as described in* **(a)**.

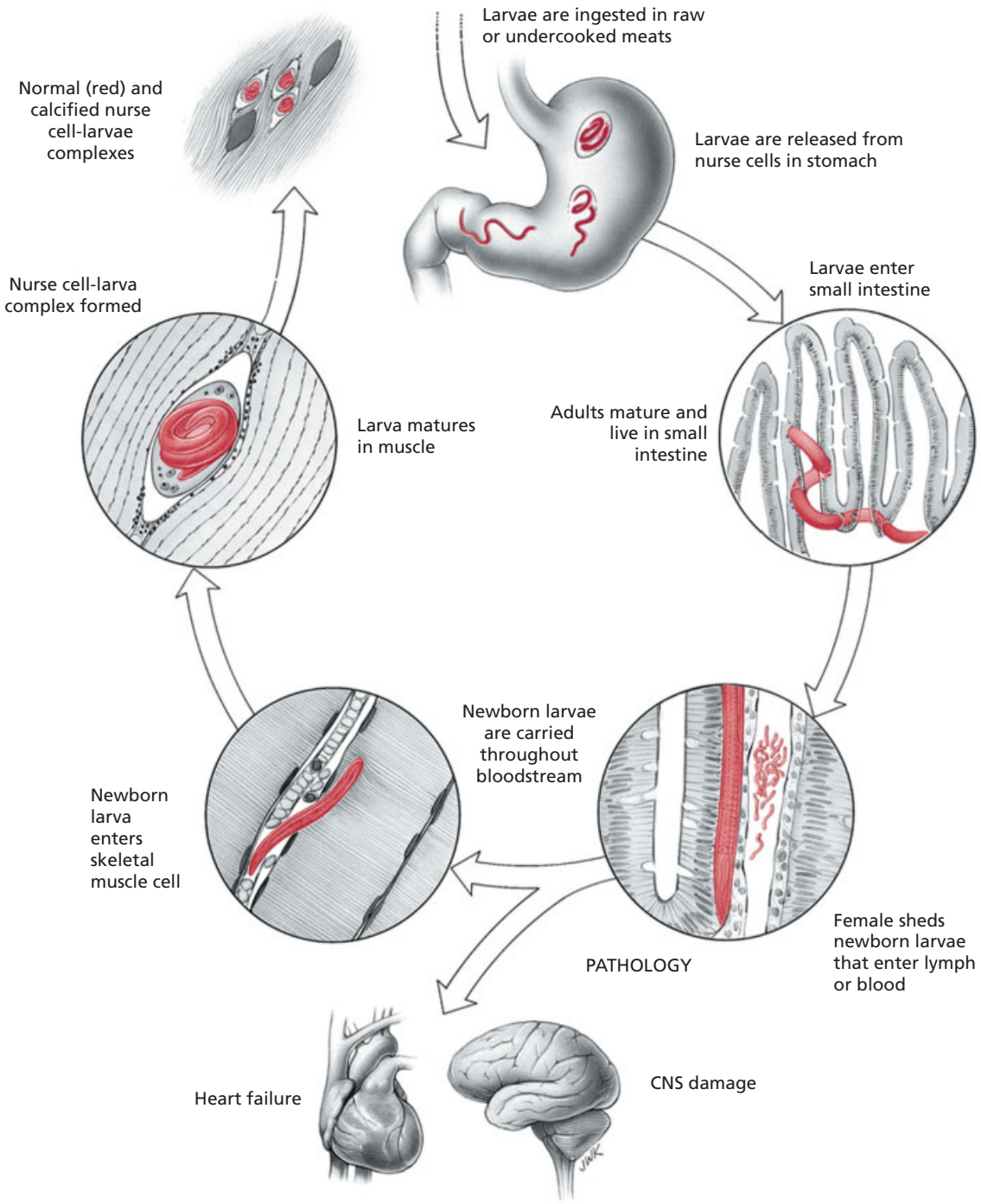

Figure 13 *Life cycle of* Trichinella spiralis. *Illustration by John Karapelou*

of the cell death and is associated with inflammation and subsequent granuloma formation. These are the most significant features of clinical trichinellosis. Granulomata are composed of macrophages, neutrophils, and eosinophils, and lymphocytes of various subsets (Wakelin and Denham 1983). The presence of these cells concentrated in a given region of a histological section of tissue should lead the pathologist to the parasite, even if the offending organism is not seen in that particular section of muscle (Figure 15).

Symptoms attributable to the enteric stages (L1–L4 and the adult worm) result from exposure of the host to the secretions of those stages and presumably to the damage they induce in the invaded enterocytes (Castro and Powell 1994). The pharmacological properties of the stichosomal secretions of the enteral stages remain

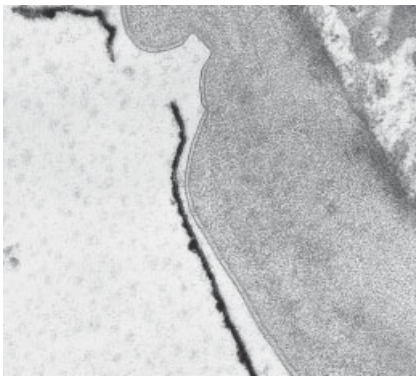

Figure 14 *Epicuticular and cuticular surfaces of the L1 larva of* Trichinella spiralis *after exposure for 1 h to trypsin digestion at pH 8.2. Note the break in the epicuticle. This treatment resulted in a marked change in behavior – from coiling–uncoiling to serpentine-like movement – when treated worms were subsequently placed in a mammalian tissue culture medium at pH 7.0. Transmission electron micrograph*

largely undefined, while a few have been characterized in terms of their putative biochemical functions (e.g. metalloproteases, nucleotidases) (Gounaris 2002; Lun et al. 2003). This area of research continues to represent a fertile area for investigation, and eventually may lead to a biochemical description of how this parasite re-models its niche into a Nurse cell. Damage to the enterocytes of the small intestine lasts only for the time the parasites occupy this niche, yet some effects last well beyond the presence of the parasite, at least in experimental infections. Experimentally infected animals lose their wheat germ agglutinin binding sites along the brush border of the entire epithelium and never regain them (Harari and Castro 1988), whereas other changes reverse after the parasites have been eliminated by host immune responses (e.g. myenteric electric potential, peristalsis, and secretory dysfunction (Castro and Powell 1994)). Hypermotility of the small intestine induced by the enteral stages is well documented, and is ablated by inhibiting nitric oxide synthase (Torrents et al. 2003). The enteral phase of the infection also induces nerve growth factor. Increased numbers of nerve cells in the area of infection accelerates the gut transit time in response to parasite products (Torrents et al. 2002). Ablating the growth of new nerve cells with anti-NGF antibodies eliminates hypermotility, but not the inflammation.

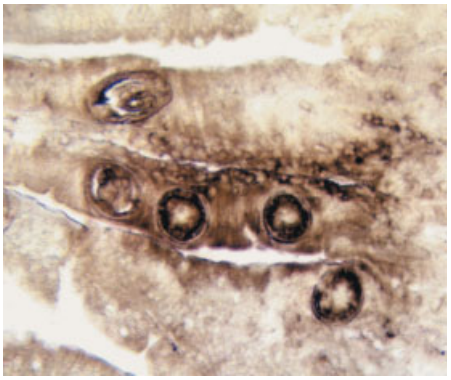

Figure 15 *Immunoperoxidase stain for tyvelose epitope in small intestine of an experimentally infected mouse. Note the presence of the antigenic determinant in surrounding non-infected enterocytes. Numerous sections of the parasite are visible in this section.*

Immune responses and protection

Most immune responses elicited during infection with trichinella, regardless of the host species, are non-protective in nature, and some even result in pathological changes and tissue damage to the host (Garside and Grencis 1992; Wakelin 1997). The vast majority of antigens are thought largely to be contained within the secretions of the parasite's stiochosome cells (Despommier 1998; Wu et al. 1998). Each stage possesses a unique set of these secretory proteins (2-D gel analysis indicates there may be hundreds), most of which are antigenic to the host during the infection (Silberstein 1983; Wakelin and Denham 1983; Gold et al. 1990). This is due to the presence of an unusual sugar residue on most of them, namely tyvelose (Wisenewski et al. 1993). This antigen is also found on the cuticle as well (Despommier et al. 1967; Philipp et al. 1980). Interestingly, although it is one of the most immunodominant of the antigenic determinants the worm secretes (Appleton and Romaris 2001), tyvelose does not elicit protection by itself (Goyal et al. 2002). Thus, the role of tyvelose in the development of protective responses and the epitopes that elicit them remains undefined (Romaris et al. 2002).

Protective immune responses are of the Th2 type (Wakelin and Denham 1983; Despommier 1988; Curman et al. 1992; Murrell and Bruschi 1994; Capo and Despommier 1996). In consequence, many valid immunodiagnostic tests exist for this pathogen (Ljungstrom 1983). Recent evidence suggests that IL-4, IL-5, IL-9, IL-10, IL-13, and IL-18 are important interleukins for mediating protection (Vallance and Matthaei 2000; Urban et al. 2001; Akiho et al. 2002; Helmby and Grencis 2002, 2003; Khan et al. 2003). Inflammation plays a central role in worm expulsion during a primary infection, with IL-4 and IL-13 being the most significant contributors to it (Urban et al. 2001). Reducing tissue mast cells by depletion of IL-10 enables the parasite to establish larger than normal numbers in the small intestine on first challenge due to reduced mast cell production (Helmby and Grencis 2003). IL-4 and IL-10 are down-regulated by IL-18, so when IL-18 is knocked out, mast cell production is increased during infection. The reverse is true when rIL-18 is given, inhibiting the production of mast cells and prolonging the intestinal phase of the infection. However, reducing the IL-10

response allowed the host to act against the larval stages in muscle tissue much more efficiently, by producing larger amounts of INF gamma (Helmby and Grencis 2002).

A monoclonal antibody of the IgA class reacted with various tissues of the infective L1 larva, but not with any other stage of the infection, and conferred high levels of protection (95 percent reduction) when administered prior to primary challenge with L1 larvae (Inaba et al. 2003). The same monoclonal antibody did not interfere with infection if given at the same time as oral challenge with L1, indicating that the epitope(s) of interest were specific to the L1 larva only. Identification of this parasite antigen(s) will undoubtedly shed much needed light on the overall subject of acquired immunity to trichinella.

Diagnosis

If the physician suspects that their patient is infected with any species of trichinella, a positive diagnosis can be made as follows:

- Muscle biopsy (Figure 15). This is the most direct means of detecting the infection, but larvae that are newly arrived in muscle tissue are hard to distinguish from muscle fibers and may be missed on biopsy. Low numbers of worms can also escape detection by this method (Figure 16). Removal of muscle tissue by digestion in 1 percent pepsin–1 percent HCl makes larvae easier to identify, but young ones may become digested, as well.
- Eliciting a history of eating raw or undercooked meat of any kind, but particularly pork or pork products.
- Laboratory findings consistent with the diagnosis of trichinellosis, such as an elevated circulating eosinophilia (Murrell and Bruschi 1994; Capo and Despommier 1996; Bruschi and Murrell 2002) and antibody detected by enzyme-linked immunosorbent assay (ELISA) (Ljungstrom 1983).

Immunological tests may be positive within 2 weeks after infection. Counterimmunoelectrophoresis and ELISA occasionally detect antibodies within 12 days after infection (Ljungstrom 1983). Both tests stay positive for long periods of time following recovery from infection (i.e. months to years), so a positive test does not necessarily rule in this parasite as the cause of the present illness. In the case of moderate to severe disease, muscle enzymes such as creatine phosphokinase and lactic dehydrogenase are released into the circulation causing an increase in serum levels, and their presence may be another clue to the presence of trichinella (Murrell and Bruschi 1994; Capo and Despommier 1996).

A laboratory finding that is nearly always associated with the infection is circulating eosinophilia (Gould 1970) that shows a pattern of increasing (week 2–5), plateauing (week 4–8), then decreasing (week 9–12) levels. Eosinophilia may reach levels of 80–95 percent in severe cases (Kaljus 1936). Total white blood cell count is slightly elevated (i.e. 12 000–15 000 cells/mm^3). Admission of having eaten raw or undercooked pork, or meat derived from game animals (Schellenberg et al. 2003) 2 weeks or so earlier, accompanied by a recent bout of gastroenteritis or 'flu-like' illness gives additional useful hints to the diagnosis, and should alert the physician as to the possibility of trichinellosis (Figures 17–21).

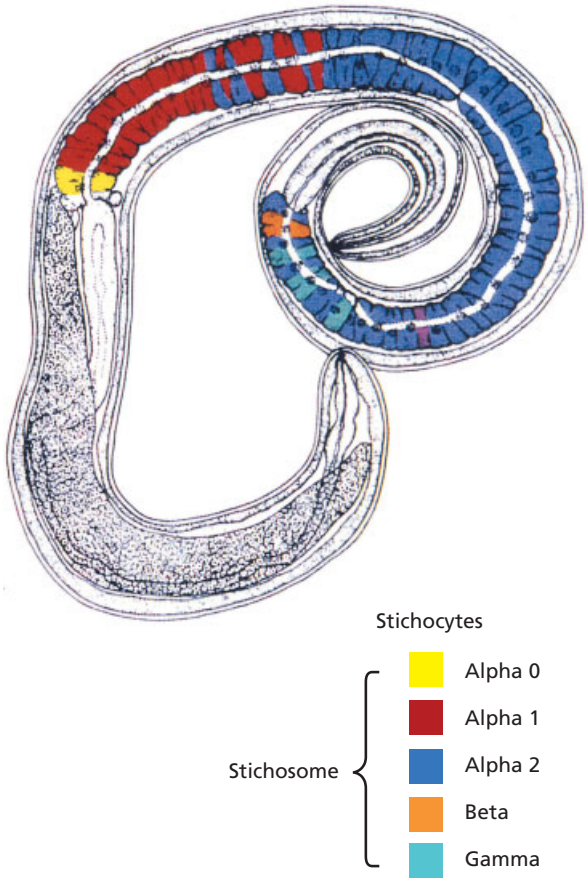

Figure 16 *Schematic drawing of an L1 infective larva of* Trichinella spiralis *color-coded for the five types of stichocytes (Wu 1955)*

Control (therapy, prophylaxis, transmission)

Clinically, there are no recommended specific therapies once the diagnosis is made. Removal of adult worms from the small intestine with thiabendazole (Gerwel et al. 1974) or mebendazole may reduce the number of newborn larvae that reach the skeletal muscle, but diagnosis is almost invariably determined after most of the larvae have been produced (i.e. weeks 3–6 after infection). Mebendazole and albendazole reduce the worm burden in the muscle tissue in experimental animals, but

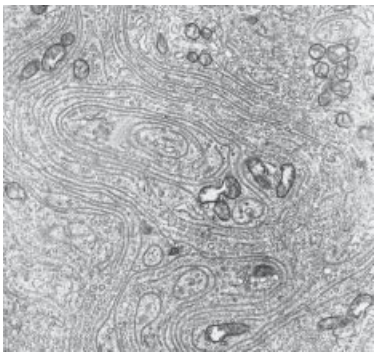

Figure 17 *Cytoplasm of a portion of Nurse cell 30 days after infection. Transmission electron micrograph*

this stage of the infection does not cause illness once it is in its intracellular niche.

Prevention of infection with any species of trichinella involves cooking all meat products at 59°C (137°F) for 10 min or freezing them at −20°C for 3 days. The one exception is *T. nativa* that survives freezing (Dick 1983; Kapel et al. 1999). Most outbreaks due to infection with *Trichinella spiralis* occur as the result of eating commercially available pork products (Noeckler et al. 2001). Inspection of meat products at the slaughter house is a proven strategy for controlling the incidence of infection in human populations (Blancou 2001), and the breakdown of such programs due to civil unrest and war has led to increases of this infection in those regions of Europe which recently experienced such activity (Djordjevic et al. 2003). In other parts of the world, the United States and Asia, for example, meat is not inspected for *Trichinella*. Spread of infection into commercial meat supplies is by contamination of animal food with infected meat scraps. Outbreaks within the USA are infrequent, and are usually associated with the ingestion

Figure 18 *Two-dimensional gel (stained with silver) of the secretions of the L1 larva of* Trichinella spiralis

Figure 19 *Splinter hemorrhages due to infection with* Trichinella spiralis

of infected game animal meats. These outbreaks are more difficult to control because few hunters are aware of the risk of acquiring this infection from their kill.

Herbivores are usually not infected with trichinella but occasionally may acquire it by accidentally eating a meal contaminated with meat from an infected carcass (Bellani et al. 1978). An outbreak of trichinellosis in

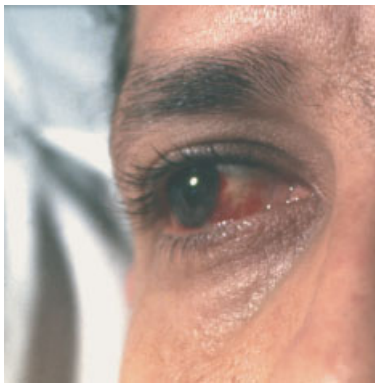

Figure 20 *'Conjuntivitis' due to infection with* Trichinella spiralis

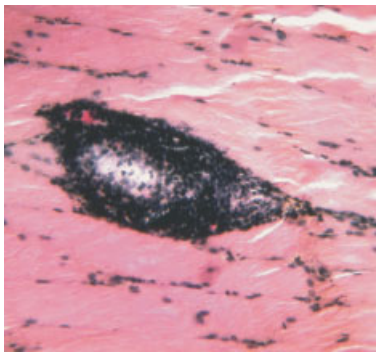

Figure 21 *Histological section of muscle tissue infected with* Trichinella spiralis. *No parasite is visible in this section. Note the intense infiltration of cells surrounding the capsule of the Nurse cell.*

France, involving thousands of people (Ancelle et al. 1985), was traced back to a single horse imported from the United States and sold in Paris as 'steak' tartare. Horsemeat continues to be a source of infection in Europe (Tamburrini et al. 2001).

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Lymphatic filariasis

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OVERVIEW

The term 'lymphatic filariasis' encompasses infection with three closely related nematode worms – *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. All three parasites are transmitted by the bites of infective mosquitoes and have quite similar life cycles in humans (Figure 1) with the adult worms living in the afferent lymphatic vessels while their offspring, the microfilariae, circulate in the peripheral blood and are available to infect mosquito vectors when they feed. The majority of infected individuals develop patent infection, with circulating microfilariae, that is subclinical (or clinically asymptomatic). The disease manifestations vary from acute adenolymphangitis to those associated with chronic infection such as hydrocele, lymphatic obstruction, and elephantiasis. Filarial infection can rarely manifest as tropical pulmonary eosinophilia, or more atypical manifestations such as filarial arthritis can also occur (WHO 1992). Though not fatal, the disease is responsible for considerable suffering, deformity, and disability and is the second leading parasitic cause of disability (Ottesen et al. 1997) with disability-adjusted life years (DALY) estimated to be 5.644 million (WHO 2003).

Lymphatic filariasis is a global health problem. At a recent estimate, it has been determined that over two billion people are at risk and at least 129 million people are actually infected (Michael et al. 1996). *W. bancrofti* accounts for nearly 90 percent of these cases. *W. bancrofti* has the widest geographical distribution and is present in Africa, Asia, the Caribbean, Latin America, and many islands of the western and south Pacific Ocean (Figure 2). *B. malayi* is geographically more restricted, being found in southwest India, China, Indonesia, Malaysia, Korea, the Philippines, and Vietnam (Michael et al. 1996). *B. timori* is found in Timor, Flores, Alor, Roti, and southeast Indonesia (WHO 1992) (Table 1).

HISTORICAL PERSPECTIVE

The existence of lymphatic filariasis has been recorded in ancient Chinese, Indian, Persian, and Arabic writings. The ancient Indian physician/surgeon Sushruta was the first to describe cases of elephantiasis, which he called *slipada* (*sli* elephant; *pada* leg). The microfilariae of *W. bancrofti* were first discovered by Demarquay in hydrocele fluid from a patient in Cuba in 1863 (Demarquay 1863). Wucherer in 1868 (Wucherer 1868) and Lewis in 1872 (Lewis 1872) were the first to discover microfilariae in the urine and blood of infected patients, respectively. Manson described the periodicity of microfilariae in the peripheral blood (Manson 1899) and also demonstrated that mosquitoes transmit the parasite (Manson 1877). Bancroft was the first to describe the adult female worm (Cobbold 1877), and this was followed by the discovery of the adult male worm by Bourne (Bourne 1888).

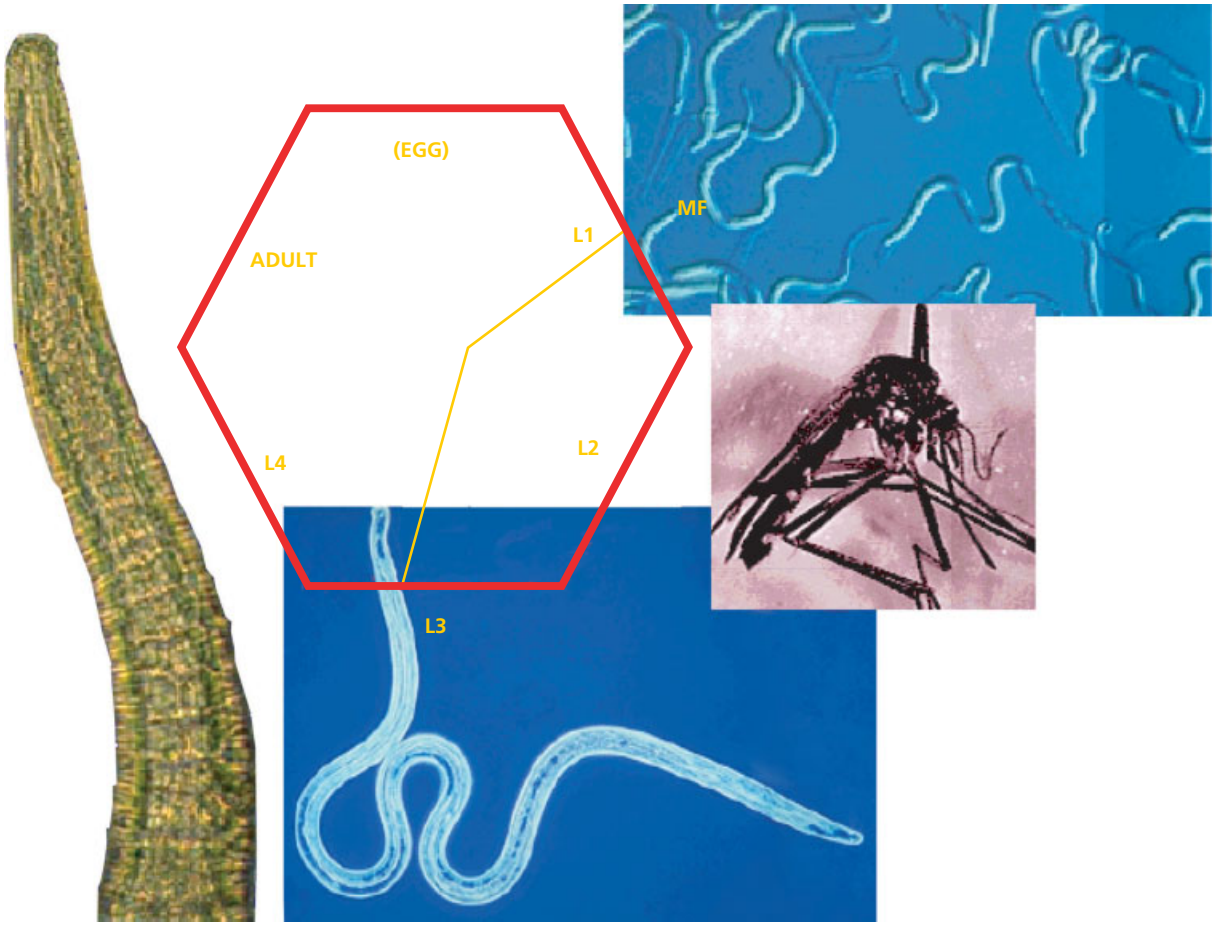

Figure 1 *Life cycle of the filarial parasites showing the microfilarial stages (MF), the mosquito, the infective stage (L3) larvae, and adult worm*

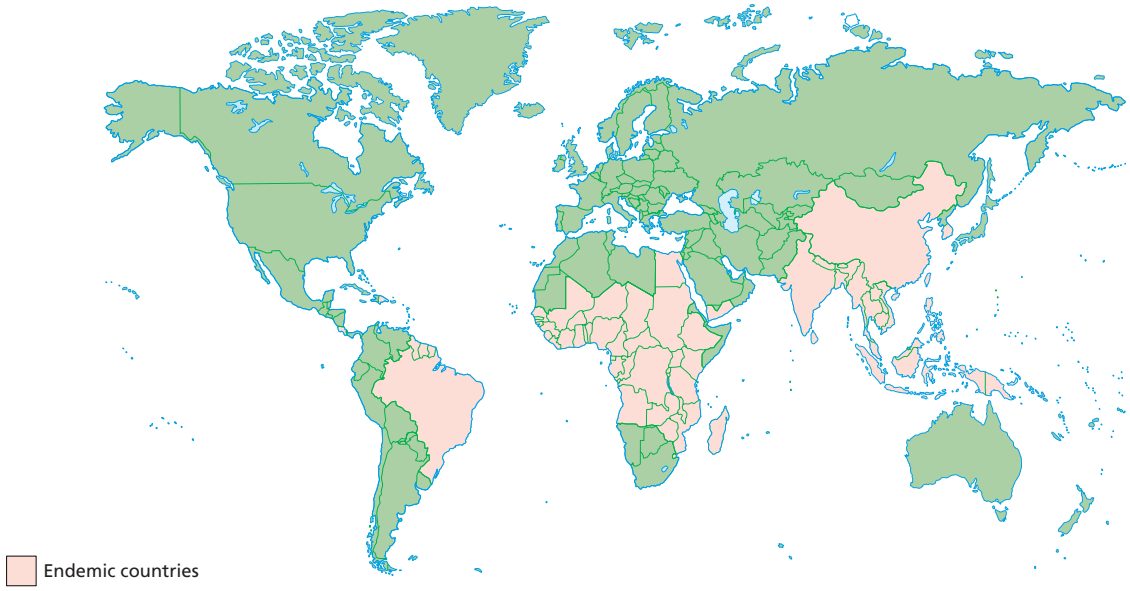

Figure 2 *Geographical distribution of lymphatic filariasis. Lymphatic filariasis is endemic in Asia, Africa, Latin America, and Pacific islands (courtesy of World Health Organization, June 2002)*

Table 1 *Lymphatic-dwelling filarial parasites infecting humans*

	Mosquito vectors	Periodicity	Distribution	Primary pathology	Morphology
Wuchereria bancroft	Culex, Anopheles, Aedes	Periodic and non-periodic	Asia, Africa, Australia, Pacific, South America	Lymphatic and lung	Sheathed Mf with tail containing no nuclei
Brugia malayi	Mansonia, Anopheles, Aedes	Periodic and non-periodic	South-East Asia	Lymphatic and lung	Sheathed Mf with tail containing two nuclei
Brugia timori	Anopheles	Periodic	Indonesia and East Timor	Lymphatic and lung	Sheathed Mf with tail containing two nuclei

Bancroft in 1899 and Low in 1900 (Low 1900) established the mode of transmission of the parasite. Lichenstein, Brug, and Buckley were primarily responsible for the identification of the Brugian parasites (Buckley 1960).

MORPHOLOGY

Filarial nematodes belong to the phylum Nematoda, class Secernentea, and superfamily Filarioidea. Adult *W. bancrofti* and *B. malayi* worms are long, slender, tapered, and cylindrical. The males (4 cm × 0.1 mm for *W. bancrofti* and 3.5 cm × 0.1 mm for *B. malayi*) are strikingly smaller than the females (6–10 cm × 0.2–0.3 mm for *W. bancrofti* and 5–6 cm × 0.1 mm for *B. malayi*). The males can be distinguished from the females by their smaller size, as well as the presence of a corkscrew-like tail and the presence of two spicules that serve as organs of copulation (Ash and Orihel 1987). The microfilariae of *W. bancrofti* are ensheathed and measure about 245–300 μm by 7.5–10 μm (Figure 3). The tail is pointed and the terminal nuclei do not extend to the tip. The microfilariae of *B. malayi* are ensheathed and measure 175–230 μm by 5–6 μm. The tail is pointed with two terminal nuclei. Finally, the microfilariae of *B. timori* are also ensheathed and have terminal nuclei in the tip but are longer than those of *B. malayi* (265–325 × 4.5–7 μm) (Partono et al. 1977; Eberhard and Lammie 1991). In the mosquito, the filarial larvae measure between 0.8 and 1.5 mm. Infective L3 larvae are between 1.2 and 1.6 mm long and newly formed L4 larvae are 3–6 mm in length (Ash and Schacher 1971).

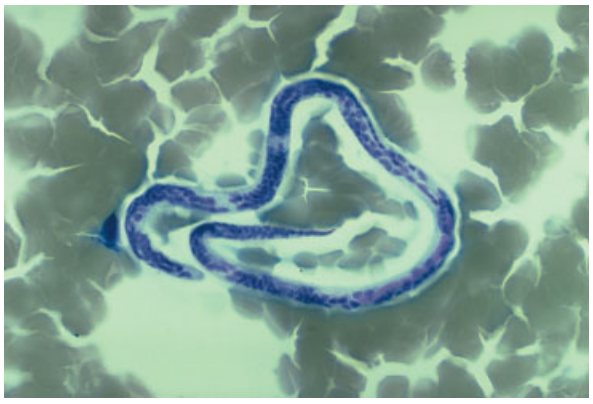

Figure 3 *Microfilaria of* W. bancrofti *in a blood film stained with Giemsa. The stain reveals the nuclei and the sheath.*

LIFE CYCLE

Humans are the definitive host and mosquitoes the intermediate hosts of *W. bancrofti* and *Brugia* spp. The life cycle of filarial parasites involves four larval stages and an adult stage. Infection begins with the deposition of infective stage larvae (L3) on the skin near the site of puncture during a mosquito bite. The larvae then pass through the puncture wound and reach the lymphatic system. The host or parasite factors dictating this tropism are entirely unknown. The minimum number of L3 larvae required to successfully transmit infection is also not known. However, it is estimated that an average resident in an endemic area is exposed to 50–300 L3 larvae per year (Hati et al. 1989). Within the lymphatics and lymph nodes, the L3 larvae undergo molting and development to form L4 larvae. This takes about 7–10 days for both *W. bancrofti* and *B. malayi*. The L4 larvae undergo a subsequent molting/developmental step to form adult worms. This occurs about 4–6 weeks after L3 entry in the case of *B. malayi* and after several months in the case of *W. bancrofti* (Cross et al. 1979; Ash and Schacher 1971). The adult worms take permanent residence in afferent lymphatics or the cortical sinuses of lymph nodes and generate microscopic live progeny called 'microfilariae.' The female worms can give birth to as many as 50 000 microfilariae per day, which find their way into the blood circulation from the lymphatics. The adult worms are estimated to survive for a period of 5–10 years (Vanamail et al. 1989), although longer durations have been recorded (Carme and Laigret 1979). The time interval between inoculation of infective larvae and the initial appearance of detectable microfilariae in the blood is known as the 'prepatent period' and the development of microfilariae from adult worms is referred to as 'patency.' Information on the duration of the prepatent period in human infection based on experimental infection of human volunteers suggest that it is between 80 and 150 days (Nutman 1991) depending on the species.

The major vectors of *W. bancrofti* are culicine mosquitoes in most urban and semi-urban areas, anophelines in the more rural areas of Africa and elsewhere, and *Aedes* species in many of the endemic Pacific islands. For the Brugian parasites, *Mansonia* species serve as the main vector, but in some areas, anopheline mosquitoes can transmit infection as well. *Culex quinquefasciatus* is the most important vector of *W. bancrofti* and is responsible for more than half of all lymphatic filarial infections. The microfilariae of *W. bancrofti* and *B. malayi*, for the large part, exhibit a phenomenon called nocturnal periodicity (i.e. they appear in larger numbers in the peripheral circulation at night and retreat during the day). This may be a biological and evolutionary co-adaptation to the feeding habits of the mosquito vectors (Manson 1899). Subperiodic or nonperiodic *W. bancrofti* and *B. malayi* are also found in certain parts of the world.

The life cycle in the mosquito begins when the vector ingests microfilariae during a blood meal. The following stages of development have been described in the mosquito (Fuhrman et al. 1987; Devaney and Howells 1979; Agudelo-Silva and Spielman 1985):

1. Exsheathing: the larva comes out of the sheath in which it is enclosed, within 1–2 h of ingestion;
2. First stage (L1) larva: after exsheathing, the larva penetrates the stomach wall within 6–12 h and migrates to the thoracic muscles wherein it grows and develops into a sausage-shaped (short, thick) form;
3. Second stage (L2) larva: the larva molts and increases in length (long, thick form) and is relatively inactive;
4. Third stage (L3) larva: a final molt results in the third stage or infective larva (long, thin form), which may be found in any part of the insect. The L3 is highly active and motile, and, when it migrates to the proboscis of the mosquito, it is ready to be transmitted to a new host.

Under optimum conditions, the mosquito cycle (otherwise known as extrinsic incubation period) is between 10 and 14 days. Although filarial infections occur in animals, human filariasis is not usually a zoonosis (subperiodic form of *B. malayi* may be an exception). Animal reservoirs of Brugia are present in monkeys, cats, and dogs, but these animals are not regarded as important sources of human infection.

GENOMICS AND THE ENDOSYMBIONT

The filarial genome is estimated to be about 85–95 Mb based on work done on *B. malayi*. The genome is AT rich (78 percent) and is organized in five pairs of chromosomes (4 chromosome pairs and one XY or XX sex chromosome pair). Within the parasite, two other genomes have been discovered – the mitochondrial genome (14Kb) and the genome of a bacterial endosymbiont (Williams et al. 2000). Most pathogenic human filarial parasites are infected with a bacterial endosymbiont called *Wolbachia*. It is an alphaproteobacteria, related to *Rickettsia*, *Erlichia*, and *Anaplasma* and is maternally inherited. It is widespread in arthropods, and, in filarial parasites, the genome is 1.1 Mb in length and the *Brugia* endosymbiont has been fully sequenced (Ware et al. 2002). It has been detected in all life cycle stages of the parasite and found to be essential for adult worm viability, normal fertility, and larval development (Taylor and Hoerauf 1999).

An expressed sequence tag (EST) sequencing of the *B. malayi* genome was initiated in 1995 under the auspices of a WHO-sponsored Filarial Genome Project (1995–2000); genomic sequencing of *B. malayi* has now been performed and completion is expected in 2004. The filarial specific databases can be accessed at NCBI (dbEST) (www.ncbi.nlm.nih.gov/dbEST/index.html), TIGR (*B. malayi* Genomic Database) (www.tigr.org/tdb/e2k1/bma1/) and Nembase (Bm cluster database) (www.nematodes.org/nematodeESTs/nembase.html).

EPIDEMIOLOGY AND OCCURRENCE

Lymphatic filariasis is endemic in 80 countries, and more than two billion people worldwide are estimated to be at risk. Approximately 129 million people in tropical and subtropical areas of the world are infected. Of these infections, 90 percent are caused by *Wuchereria bancrofti*, and 10 percent by *Brugia malayi* (limited to Asia and parts of the Pacific). The largest numbers of infected people are present in India (45.5 million) and subSaharan Africa (40 million) with prevalences of 5 and 8 percent, respectively. Another region of importance is the Pacific Islands where high prevalences are found (Papua New Guinea (72 percent) and Republic of Tonga (48 percent)). Some of the low prevalence areas are Egypt and Latin America (Michael et al. 1996). Almost 25 million men suffer from genital disease (most commonly hydrocele); an estimated 15 million people have lymphedema or elephantiasis. Another 76 million have preclinical damage to the lymphatic and renal systems (Michael et al. 1996). In addition, millions of people suffer from acute attacks of adenolymphangitis that can be quite debilitating (WHO 1992) and extract a great socioeconomic and psychological burden.

Men are more susceptible to infection with filarial parasites than women (Brabin 1990). The gender of the individual has an important influence on the incidence of chronic pathology in lymphatic filariasis; males are more commonly susceptible to chronic sequelae than women (Brabin 1990). Interestingly, the incidence of acute filarial infection is not significantly different between males and females. The basis for this gender difference could be both environmental (men are more exposed to mosquito bites than women) and genetic factors (sex hormonal influence on innate and adaptive immunity).

The age dynamics of filarial infection reveals that the frequency of infection increases as a function of age up

to about 20–30 years of life, after which it reaches a plateau (Dondero et al. 1976; Day et al. 1991). All infected individuals tend to acquire infection by the third decade of life. Previously, lymphatic filariasis had always been regarded as a debilitating disease of adults, but recent studies using sensitive antigen detection techniques have revealed that infection is first acquired in childhood, with as many as one-third of children infected before 5 years of age (Lammie et al. 1994; Simonsen et al. 1996; Steel et al. 2001). Lymphatic damage begins early in childhood but remains asymptomatic until the development of pathology later in life (Witt and Ottesen 2001). The earliest age when bancroftian microfilariae has been detected in the blood of children is 7–10 months, and the earliest reported case of brugian microfilaremia is 3.5 months.

CLINICAL MANIFESTATIONS

Lymphatic filariasis can manifest itself in a variety of clinical and subclinical conditions.

Subclinical (or asymptomatic) microfilaremia

In areas endemic for lymphatic filariasis, many individuals exhibit no symptoms of filarial infection and yet, on routine blood examinations, demonstrate the presence of significant numbers of parasites. These individuals are carriers of infection (and the reservoir for ongoing transmission) and have commonly been referred to as asymptomatic microfilaremics. The parasite burdens in these individuals can reach dramatically high numbers, exceeding 10 000 microfilariae in 1 ml of blood. With the advent of newer imaging techniques, it has become apparent that virtually all persons with microfilaremia have some degree of subclinical disease. Lymphoscintigraphy has revealed that the lymphatics of these individuals show profound changes including marked dilatation and tortuosity of lymph vessels with collateral channeling, increased flow, and abnormal patterns of lymph flow (Freedman et al. 1994, 1995). Ultrasound examination of scrotal lymphatics in microfilaremic men has revealed that approximately half of the men have nests of motile adult worms in their lymphatics: commonly known as the 'filarial dance sign' (Noroes et al. 1996). In parallel, a considerable degree of scrotal lymphangiectasia has been noted in these individuals. Mirroring the imaging studies, the examination of superficial skin punch biopsies reveal abnormally dilated lymphatic vessels in the limbs of patients with asymptomatic microfilaremia. In addition, approximately 40 percent of microfilaremic individuals suffer from microscopic hematuria and/or proteinuria that are indicative of low-grade renal damage (Dreyer et al. 1992). Thus, while apparently free of overt symptomatology, the asymptomatic microfilaremic individuals clearly are subject to subtle pathological changes.

Acute clinical disease

The acute manifestations of lymphatic filariasis are characterized by recurrent attacks of fever associated with the inflammation of lymph nodes (lymphadenitis) and lymphatics (lymphangitis) (Partono 1987). In brugian filariasis, episodes of fever, lymphadenitis, and lymphangitis are common, while bancroftian filariasis presents more insidiously with fewer overt acute episodes (Pani et al. 1990). The lymph nodes commonly involved are the inguinal, axillary, and epitrochlear nodes and, in addition, the lymphatic system of the male genitals are frequently affected in *W. bancrofti* infection leading to funiculitis, epididymitis, and/or orchitis (Pani and Srividya 1995).

It has been proposed that there are at least two distinct mechanisms involved in the pathogenesis of acute attacks. The more classical is acute filarial adenolymphangitis, which is felt to reflect an immune-mediated inflammatory response to dead or dying adult worms. The striking manifestation is a distinct well-circumscribed nodule or cord along with lymphadenitis and retrograde lymphangitis. Funiculo-epididymo-orchitis is the usual presenting feature when the attacks involve the male genitalia. Fever is not usually present, but pain and tenderness at the affected site is common (Dreyer et al. 1999a).

The other has been termed acute dermatolymphangitis, a process characterized by development of a plaque-like lesion of cutaneous or subcutaneous inflammation and accompanied by ascending lymphangitis and regional lymphadenitis. There may or may not be edema of the affected limbs. These pathological features are accompanied by systemic signs of inflammation including fever and chills. This manifestation is thought to result primarily from bacterial and fungal super-infections of the affected limbs (Dreyer et al. 1999a).

Acute filarial attacks have been described in infants as young as 3 months of age, but are usually more common in older children and continue throughout life (Dasgupta 1984; Nanduri and Kazura 1989). They can occur in both Mf+ and Mf− individuals and are frequently associated with chronic pathology (Dreyer et al. 1999a). Acute filariasis is not confined to residents in an endemic area but has also been observed in filarial-infected expatriates (Wartman 1947; McQuay 1967; Melrose et al. 2000), although this may reflect early, prepatent infection and responses to L3 and L4 larvae.

Manifestations of chronic infection

The chronic sequelae of filariasis are postulated to develop approximately 10–15 years after initial infection

Figure 4 *Chronic manifestations of lymphatic filariasis.* **(a)** *Early lymphedema (grade 1) of both the lower limbs;* **(b)** *Advanced lymphedema (grade 3) of both the lower limbs with mild skin changes;* **(c)** *Elephantiasis of the lower limb with verrucous and fibrotic skin changes;* **(d)** *Hydrocele in a male patient.*

(Partono 1987). In bancroftian filariasis, the main clinical features are hydrocele, lymphedema, elephantiasis, and chyluria. The manifestations in descending order of occurrence are hydrocele and swelling of the testis, followed by elephantiasis of the entire lower limb, the scrotum, the entire arm, the vulva, and the breast (Pani and Srividya 1995). In Brugian filariasis, the leg below the knee and the arm below the elbow are commonly involved but rarely the genitals (Pani et al. 1990). Lymphedema can be classified or graded, a scheme proven very useful in clinical trials:

Grade 1: Pitting edema reversible on limb elevation (Figure 4a)
Grade 2: Pitting/non-pitting edema not reversible on limb elevation and normal skin
Grade 3: Non-pitting edema of the limb, not reversible on elevation with skin thickening (Figure 4b)
Grade 4: Non-pitting edema with fibrotic and verrucous skin changes (elephantiasis) (Figure 4c).

In men, scrotal hydrocele is the most common chronic clinical manifestation of bancroftian filariasis (Wijers 1977; Gyapong et al. 1994). Hydroceles are due to accumulation of edematous fluid in the cavity of the tunica vaginalis testis (Figure 4d). Chronic epididymitis and funiculitis can also occur. Chyloceles can also occur. The prevalence of chyluria (excretion of chyle, a milky white fluid in the urine) is very low.

Tropical pulmonary eosinophilia

Tropical pulmonary eosinophilia (TPE) is a distinct syndrome that develops in some individuals infected with *W. bancrofti* and *B. malayi* (Ottesen and Nutman

1992; Ong and Doyle 1998). This syndrome affects males and females at a ratio of 4:1, often during the third decade of life. The majority of cases have been reported from India, Pakistan, Sri Lanka, Brazil, Guyana, and Southeast Asia. The main clinical features include paroxysmal cough and wheezing that are usually nocturnal (and probably related to the nocturnal periodicity of microfilariae), weight loss, low-grade fever, adenopathy, and pronounced blood eosinophilia (>3 000 eosinophils/ml). Chest X-rays may be normal but generally show increased bronchovascular markings; diffuse miliary lesions or mottled opacities may be present in the middle and lower lung fields (Khoo and Danaraj 1960). Tests of pulmonary function show restrictive abnormalities in most cases and obstructive defects in half (Nesarajah 1972). Total serum IgE levels (10 000–100 000 ng/ml) and antifilarial antibody titers are characteristically elevated (Neva and Ottesen 1978). In TPE there is rapid clearance of microfilariae and parasite antigens from the bloodstream by the lungs, and the clinical symptoms result from allergic and inflammatory reactions elicited by the cleared parasites (Ottesen et al. 1979; Pinkston et al. 1987; O'Bryan et al. 2003). In some subjects, trapping of microfilariae in other reticuloendothelial organs can cause hepatomegaly, splenomegaly, or lymphadenopathy (Neva and Ottesen 1978). In the absence of successful treatment, interstitial fibrosis can lead to progressive pulmonary damage. Fortunately, TPE responds rapidly to anti-filarial chemotherapy (diethylcarbamazine (DEC)).

Other manifestations

Lymphatic filariasis has been associated with a variety of renal abnormalities including hematuria, proteinuria, nephrotic syndrome, and glomerulonephritis (Melrose 2002). Circulating immune complexes containing filarial antigens have been implicated in the renal damage. Lymphatic filariasis may also present as a mono-arthritis of the knee or ankle joint (Adebajo 1996).

Uninfected, but exposed individuals (asymptomatic amicrofilaremia or endemic normals)

In endemic areas, a proportion of the population remains uninfected despite exposure to the parasite to the same degree as the rest of the population (WHO 1992). This group has been termed endemic normal. The prevalence of endemic normals in a population ranges from zero (Day et al. 1991) to 50 percent (Weil et al. 1987; Ramzy et al. 1991; Steel et al. 2001) in different endemic areas. However, the true prevalence of endemic normal individuals in any population needs to be re-examined with the advent of newer techniques to diagnose subclinical infection. There could be several mechanisms by which an endemic normal phenotype is achieved: insufficient exposure, low-grade (occult) infections not detected by current techniques, prepatent infections at the time of study, 'burning out' of infections naturally, and finally, genuine immunological resistance to the parasite (Beaver 1970).

PATHOGENESIS AND PATHOLOGY

The main pathological features in chronic lymphatic filariasis are the consequence of inflammatory damage to the lymphatics. The damage to lymphatics is probably the summation of the effect of tissue alterations related to live adult parasites, tissue alterations related to dead adult parasites, the host inflammatory response to living and dead parasites, the host inflammatory response to the endosymbiont *Wolbachia*, and tissue alterations due to secondary bacterial or fungal infections.

The adult worms reside in the lymphatics and lymph nodes and induce changes that result in dilatation of the lymphatics and thickening of the vessel walls. This is characterized by infiltration of plasma cells, eosinophils, and macrophages in the lymphatic vessels. This is often accompanied by proliferation of endothelial and connective tissue and tortuosity of the lymphatics and damaged lymph valves, lymphedema, and chronic stasis with brawny edema of the overlying skin (Figueredo-Silva et al. 2002).

It is postulated that as long as the adult worms are alive, the lymphatic vessels, though damaged, still remain patent. Death of the worm, however, leads to progressive fibrosis, obliteration of vessels by granuloma, and thrombi formation, extensive perilymphangitis resulting in irreversible lymphatic damage. In addition, secondary bacterial or fungal infections of limbs of affected patients exacerbate the chronic obstructive changes (Dreyer et al. 2000). One of the newer concepts to emerge in the field of pathogenesis is the contribution of the filarial endosymbiont *Wolbachia*. Several studies have shown that the potent inflammatory response engendered by filarial parasites is due to the endotoxin-like activity of *Wolbachia*. This response resembles a classical endotoxin response and involves the pattern recognition receptors, CD14 and TLR4 (Taylor et al. 2000, 2001).

IMMUNOLOGY

It has been postulated that the intensity and type of host immune response to the parasite or parasite products reflect the range of clinical manifestations of filariasis. Prepatent (or early) filarial infections are characterized by the presence of parasite-specific delayed-type hypersensitivity responses, parasite-specific T cell proliferation, cytokine production with a mixed phenotype (both Th1 cytokines (IL-2, IFNγ) and Th2 cytokines (IL-4 and IL-5)), profound eosinophilia and high titers of IgE anti-

body. When the infection progresses to patency (development of microfilaremia), the immune responses are profoundly altered. The marked change is the diminished parasite-specific T cell proliferation, IL-2 and IFNγ production, and an increase in IL-10 production, and class switching to IgG4 antibody production (Mahanty et al. 1992, 1993; Dimock et al. 1996). The mechanisms for this selective immune tolerance have been postulated to include diminished frequency of T and B cell precursors (King et al. 1996), suppressor T cells (Piessens et al. 1982), altered immune responses due to in utero exposure (Steel et al. 1994), increased expression of down-regulatory molecules like CTLA-4 (Steel and Nutman 2003), altered antigen-presenting cell function or apoptosis (Semnani et al. 2003), and the presence of regulatory cytokines such as IL-10 and TGFβ (King et al. 1993; Maizels and Yazdanbakhsh 2003).

One of the hallmarks of chronic filarial infection is the classical Th2 response characterized by the production of IL-4, IL-5, and IL-13 to parasite stimulation (Mahanty et al. 1993, de Boer et al. 1998a). In addition, two other key cytokines produced in chronic infection are IL-10 and TGFβ, which have regulatory functions (King et al. 1993). The Th2 cytokine profile is responsible for the high levels of IgE and eosinophilia that are associated with chronic helminth infections (King et al. 1990, 1991). However, the production of IgG4 in filarial infections is independent of IL-4 and IL-13 (de Boer et al. 1998b) but surprisingly influenced by IL-12 (de Boer et al. 1997). The intensity of transmission in endemic areas (King et al. 2001) and the in utero exposure to filarial antigens (Malhotra et al. 2003) also plays a role in influencing this cytokine bias in filarial infections.

In contrast to Mf-positive patients, patients with chronic pathologic conditions such as elephantiasis exhibit a different type of immune response. They display IgG1, IgG2, and IgG3 antibody responses to parasite antigens (Maizels et al. 1987), mount vigorous IL-2 and IFNγ responses, and good T cell proliferation to parasite antigens (Mahanty et al. 1996). Finally, patients with tropical pulmonary eosinophilia mount vigorous systemic and local immune responses characterized by high levels of IgE and IgG as well as T cells producing IL-4 and IL-5 (Ottesen and Nutman 1992; Nutman and Kumaraswami 2001).

The regulation of the human immune response to filarial parasites is complex with effects on both innate and adaptive immunity. Filarial parasites have been shown to induce apoptosis in dendritic cells, impair their capacity to produce IL-12 and IL-10 and to activate T cells (Semnani et al. 2003). In addition, filarial parasites have also been shown to downregulate the activity of macrophages (Chaussabel et al. 2003). Filarial parasites secrete certain immunoregulatory molecules including homologs of human macrophage migration inhibitory factor (MIF) (Pastrana et al. 1998) and human transforming growth factor-beta (TGFβ) (Gomez-Escobar et al. 1997), which may be partly responsible for the downregulatory effect on professional antigen-presenting cells. Filarial parasites are also capable of inducing pro-inflammatory cytokine expression in T cells early following exposure (Babu and Nutman 2003).

Experimental animal models of filariasis have been used for identifying the kinetics and type of immune response as well as the factors necessary for resistance to the parasite. L3 larvae, as well as adult worms, evoke predominantly Th2 responses (IL-4 and IL-5) in mice, while microfilariae evoke Th1 responses (Pearlman et al. 1993; Lawrence et al. 1994). Wild-type mice with an intact immune system are resistant to infection (Chong and Wong 1967; Carlow and Philipp 1987) with *B. malayi* (*W. bancrofti* has never been observed to survive in any small animal model), whereas mice lacking T cells (nude mice) and mice lacking T and B cells (SCID mice) are susceptible (Vincent et al. 1980; Suswillo et al. 1980; Nelson et al. 1991). Thus, the adaptive immune system is absolutely necessary for the elimination of the parasite. The exact mediators in this resistance phenotype remain to be determined although IL-4 (Babu et al. 2000), B cells (Babu et al. 1999; Paciorkowski et al. 2000), IgE (Spencer et al. 2003), and eosinophils (Martin et al. 2000) have all been postulated to be involved.

DIAGNOSIS

The traditional method of diagnosing lymphatic filarial infections has been the detection of microfilariae in the peripheral blood collected during the night in areas of nocturnal periodicity and during the day in areas of subperiodic lymphatic filariae. The simplest method is a thick blood film of capillary blood stained with Giemsa stain (reviewed in Melrose (2002)), its disadvantage being poor sensitivity. The sensitivity of detection can be augmented by the use of concentration techniques such as the Knott's concentration method, in which 1 ml of whole blood is added to 9 ml of a 2 percent formalin solution, centrifuged, and the sediment examined for microfilariae. Another widely used concentration technique is the membrane filtration technique whereby 1–5 ml of blood is passed through a 3 or 5 μM polycarbonate membrane which retains the microfilariae (Bell 1967).

For bancroftian filariasis, assays for the detection of circulating parasite antigens have been developed based on one of two well-characterized monoclonal antibodies, Og4C3 or AD12 (Weil et al. 1987; More and Copeman 1990). The commercial Og4C3 ELISA (Trop Bio Og4C3 Antigen test, produced by Trop Bio) has a sensitivity approaching 100 percent and specificity of 99–100 percent. The ICT filarial antigen test (Binax) is a rapid format card test with a sensitivity of 96–100 percent and

specificity of 95–100 percent. It utilizes capillary or venous blood and is simple enough for field use (Weil et al. 1997). There are currently, however, no antigen tests for Brugian filariasis.

Antibody-based assays for diagnosing filarial infection have typically used crude parasite extracts and have suffered from poor specificity. Improvements have been made by the use of detection of anti-filarial IgG4 antibodies in that they are produced in relative abundance during chronic infection. IgG4 antibodies correlate well with the intensity and duration of filarial exposure and the level of microfilaremia; these IgG4 antibodies also have very little cross-reactivity to non-filarial helminths (Lal and Ottesen 1988; Mahanty et al. 1994). In addition, IgG4 antibodies are also useful in the diagnosis of Brugian infections (Rahmah et al. 1998a; Haarbrink et al. 1999); indeed a diagnostic dipstick test has been used in areas endemic for Brugian filariasis based on a recombinant Brugian antigen (Rahmah et al. 2001).

Polymerase chain reaction (PCR)-based methods have been developed for the detection of *W. bancrofti* DNA in blood, plasma, paraffin-embedded tissue sections (McCarthy et al. 1996), sputum (Abbasi et al. 1996), urine (Lucena et al. 1998), and in infected mosquitoes (Chanteau et al. 1994). For *B. malayi*, DNA in blood (Lizotte et al. 1994; Rahmah et al. 1998b) and in mosquitoes (Vythilingam et al. 1998) has been detected.

Finally, the examination of scrotum (Amaral et al. 1994) and breast (Dreyer et al. 1996) using ultrasonography in conjunction with pulse wave Doppler techniques can identify motile adult worms within the lymphatics. The adult worms exhibit a characteristic pattern of movement known as the filarial dance sign (Amaral et al. 1994) and the location of these adult worm nests remains remarkably stable (Dreyer et al. 1994). On rare occasions, living adult worms reside in the lymphatics of inguinal crural, axillary, and epitrochlear lymph nodes (Dreyer et al. 1999b).

TREATMENT

With better definitions of clinical syndromes in lymphatic filariasis and new tools to assess clinical status (e.g. ultrasound, lymphoscintigraphy, circulating filarial antigen assays, PCR), approaches to treatment based on infection status can be considered. DEC (6 mg/kg daily for 12 days), which has both macro- and microfilaricidal properties, remains the treatment of choice for the individual with active lymphatic filariasis (microfilaremia, antigen positivity, or adult worms on ultrasound), although albendazole (400 mg twice daily for 21 days) has also demonstrated macrofilaricidal efficacy. Ivermectine (400 μg/kg) is another drug with efficacy against filarial parasites.

A growing body of evidence indicates that, although they may be asymptomatic, virtually all people with *W. bancrofti* or *B. malayi* microfilaremia have some degree of subclinical disease (hematuria, proteinuria, abnormalities on lymphoscintigraphy). Thus, early treatment of asymptomatic persons is recommended to prevent further lymphatic damage. Because lymphatic disease is associated with the presence of adult worms, treatment with DEC is recommended for microfilaria-negative adult-worm carriers.

In persons with chronic manifestations of lymphatic filariasis, treatment regimens which emphasize hygiene, prevention of secondary bacterial infections, and physiotherapy have gained wide acceptance for morbidity control. Hydroceles can be drained repeatedly or managed surgically. In patients with chronic manifestations of lymphatic filariasis, drug treatment should be reserved for individuals with evidence of active infection as therapy has been associated with clinical improvement and, in some, reversal of lymphedema.

The recommended course of DEC treatment (12 days; total dose, 72 mg/kg) has remained standard for many years; however, data indicate that single-dose DEC treatment with 6 mg/kg may be equally efficacious (Kimura et al. 1992; Dreyer et al. 1995; Kazura et al. 1993). The 12-day course provides more rapid short-term microfilarial suppression. Regimens that utilize single-dose DEC or ivermectin or combinations of single doses of albendazole and either DEC or ivermectin have all been demonstrated to have a sustained microfilaricidal effect (Horton et al. 2000).

In the past few years, the use of antibiotics such as doxycycline, which have an effect on *Wolbachia*, to treat lymphatic filariasis has been investigated. Preliminary data suggest that 200 mg of doxycycline for 6 weeks is effective in eliminating microfilaremia (Hoerauf et al. 2003).

PREVENTION AND CONTROL

DEC has the ability to kill developing forms of filarial parasites (Ewert and Emerson 1975; Fujimaki et al. 1988) and has been shown to be useful as a prophylactic agent in humans (Lagraulet 1973). DEC has also been shown to reduce community levels of microfilaria so as to interrupt transmission. To this end, DEC has even been used as an additive to common table salt. The combination of community-wide distribution of a yearly single dose of DEC/ivermectin or DEC/albendazole is being touted as a major mechanism of control of the infection in that there is a theoretical basis for interruption of transmission providing the community drug coverage is large.

Like the integrated control programs used for onchocerciasis, long-term microfilarial suppression using mass, annual distribution of single-dose combinations of albendazole with either DEC or ivermectin is underway in many parts of the world. These strategies have as their basis the microfilarial suppression of >1 year using single-dose combinations of albendazole/ivermectin

or albendazole/DEC. An added benefit of these combinations is their secondary salutary effects on gastrointestinal helminth infections.

Vector control measures such as the use of insecticides, the use of polystyrene beads in infested pits, the use of *Bacillus sphaericus* as a larvicide, the use of larvivorous fish, and the use of insecticide-treated bed nets have all been advocated as adjunct measures for control of filariasis (Maxwell et al. 1999).

Lymphatic filariasis is one of six potentially eradicable diseases (WHO International Task Force 1993) and the development of a global program to eliminate filariasis (GPELF) came about following a resolution by the WHO Assembly in 1997 (Molyneux et al. 2000; Ottesen 2000). The principal aims of the program are to interrupt transmission of infection and to alleviate and/or prevent disability. The recent advances in immunology, molecular biology, and imaging technology have helped pave the way for the realization of the goal to eliminate lymphatic filariasis.

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Onchocerciasis

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THE PARASITE

Introduction and historical perspective

Onchocerciasis is commonly called river blindness and is caused by infection with the parasitic nematode *Onchocerca volvulus*, which occurs naturally mainly in humans and occasionally in gorillas, but can be transmitted experimentally to chimpanzees. Other species of *Onchocerca* are found in ungulates, particularly bovines. Human infections with the cattle parasite *O. gutturosa* have occasionally been documented. These have presented as fibrous nodules containing infertile adult worms.

Adult worms, which have an average estimated lifespan of 10 years, are typically found in subcutaneous nodules where they are usually benign. Female worms produce millions of embryos (microfilariae) throughout their lives and a heavily infected carrier may harbor 50–200 million of them. Microfilariae live for about 1 year in the skin and must be ingested by the correct species of the insect vector (the *Simulium* blackfly) if they are to develop further. Microfilariae that are taken up by a blackfly migrate to its flight muscles, where they mature over the course of about 1 week into infective larvae. These enter another human via the bite wound made when the insect next feeds.

Those microfilariae not taken up by a vector die within skin and eye tissues and provoke an immune response which, it is thought, leads to the inflammatory lesions and subsequent fibrosis that underlie the disease of onchocerciasis. Recent studies have shown that endotoxin-like molecules released from the endosymbiotic rickettsial bacteria *Wolbachia* living in onchocercal worms and many other filarial nematodes generate inflammatory responses that may cause the adverse reactions after chemotherapy and be involved in the pathogenesis of the disease (Saint André et al. 2002). This is a chronic disease characterized by itchy and unsightly skin lesions and progressive eye damage, sometimes leading to blindness.

O. volvulus can only be transmitted by blackflies of the genus *Simulium*. *Onchocerca* species that parasitize other animals may also be transmitted by blackflies or by biting midges of the genus *Culicoides*. It is only the female blackflies that bite humans, in order to obtain the blood necessary for egg development. They use their short, broad, rasping mouthparts, which cause a painful bite but which are unable to penetrate clothing. The flies only bite during daylight hours. Because of the feeding and metabolic requirements of the larvae, blackfly eggs are laid in well-oxygenated waters. Transmission of infection, therefore, usually occurs close to blackfly breeding sites in fast flowing rivers, giving rise to the apt term 'river blindness' for this disease.

The first account of the microfilariae of *O. volvulus* was probably made by O'Neill (1875), who found them in the skin of six West African natives with papular skin rashes. He described the microfilaria as 'easily detectable … by its violent contortions. Thread-like in form, at one time undulating, and now twisted as if into an inexplicable knot, then, having rapidly untwined itself, it curls and coils into many loops'. This accurate and evocative description is readily recognized by those who

have examined microfilariae emerging from skin fragments under a microscope. O'Neill also described the pointed tail, and reported the size of the microfilariae to be about 250 × 12 μm. As this is too long for *Mansonella perstans* and too wide for *M. streptocerca*, there can be little doubt that O'Neill was describing *O. volvulus*.

Prout (1901) described worms from a subcutaneous nodule removed from a Sierra Leone frontier policeman with vague rheumatic pains. He described the adult male and female worms and microfilariae (unsheathed with a sharp tail, central granular appearance, and a size of 250 × 5 μm), which he tentatively called *Filaria volvulus* after Leuckart's 1893 description. Parsons (1908) also gave a description of nodules and commented that he suspected that the disease was more common than generally recognized and probably spread by a blood-sucking insect.

Robles (1917), working in Guatemala, demonstrated an association between nodules and the skin lesions and anterior ocular features of onchocerciasis, and suggested that blackflies could transmit the infection. However, it was Blacklock, working in Sierra Leone, who provided definite evidence of transmission of onchocerciasis by *Simulium damnosum* (Blacklock 1926a, 1926b). He found microfilariae in 45 percent of skin snips taken from subjects with nodules. No definite evidence of skin or eye disease was noted. Blacklock showed that onchocercal larvae developed first in the gut of simuliids, then the thorax, subsequently invading the head and escaping from the proboscis through the membranous labrum.

The use of skin snips for diagnosis was first recorded in 1922 (Macfie and Corson 1922) when a needle and scissors were used to remove a 0.25 cm piece of skin from the lower back. The recognition of ocular onchocerciasis in Africa came from the work of Hissette (1932) in Zaire, who described punctate keratitis, sclerosing keratitis, iritis, retrobulbar neuritis, and retinal lesions, whereas Bryant (1935) noted diffuse retinochoroiditis associated with optic atrophy in the Sudan. The definitive description of ocular onchocerciasis was published as a monograph in 1945 by Ridley, who was working in Ghana (Ridley 1945).

Classification

O. volvulus is a spirurid nematode within the superfamily Filarioidea, and family Onchocercidae. Adult female *O. volvulus* worms, at more than 35 cm, are exceptionally long for a member of the Filarioidea, which usually measure only 2–10 cm in length. Adult *O. volvulus* are normally found in characteristic fibrous nodules, distinguishing them from *Dracunculus medinensis* (superfamily Dracunculoidea), another long (about 100 cm) filarial human parasite found free in subcutaneous tissues (see Dracunculiasis)

Morphology and structure

The adult worms are long and thin, tapering at both ends, although rounded at the anterior end. Both sexes exhibit sluggish movements. Microscopically, the cuticle is raised in prominent transverse ridges and annular and oblique thickenings, which are more distinct posteriorly. In the male, the cuticular annulations are more closely spaced and less conspicuous, and the cuticle contains more layers and is thinner than in females. The presence of two striae in the inner layer of the cuticle is a helpful diagnostic feature of *Onchocerca* in tissue sections. In females, the prominence of the cuticular ridges diminishes with age as the thickness of the cuticle increases. As worms become older, they become thicker and discolored, changing from transparent white to yellowish to brown. They also exhibit more patches of calcification and cytoplasmic inclusions of iron granules, vacuoles and lipid droplets as they age.

Adult male worms lengthen throughout their life and usually measure 2–4 × 0.15–0.20 cm. Morphologically, male worms are typical nematodes and considerably more mobile than females. Within nodules, males are usually found on the outside of the worm bundle, coiled around the anterior end of a female. The nerve ring is 140 μm from the anterior end. The alimentary canal is straight, ending close to the tip of the tail at the cloaca. The tail ends in a spiral and has a bulbous tip. There are two pairs of preanal and two postanal papillae, an intermediate large papilla and two unequal copulatory spicules that can protrude from the cloaca. The single reproductive tract runs centrally for almost the entire length of the worm. It consists of an anterior testis, a vas deferens, and an ejaculatory duct leading to the cloaca. Spermatogenesis is continuous and synchronized, so that only a single developmental stage is found in a particular transverse section.

Adult female worms are highly modified nematodes, notable for their extreme length, wide lateral hypodermal chords, and their reduced muscle cells and intestinal lumen. They measure 35–70 cm in length by about 400 μm in breadth. The nerve ring is 170 μm from the anterior end, and the anus is about 210 μm from the posterior end of the worm. The female reproductive tract runs from the posterior to the anterior end of the worm and consists of:

- paired ovaries, one lying more posteriorly than the other
- oviducts
- seminal receptacles and uteri
- a single short vagina and vulva, found close to the anterior extremity.

O. volvulus eggs are 30–50 μm in diameter and have a striated shell with a pointed process at each end.

The unsheathed, nonperiodic microfilariae occur mainly in the skin (90 percent) but are also found in

nodules. They are occasionally found in blood specimens, in most cases probably by dislodgement from the skin. They are also found in the tissues and chambers of the eye in heavy infections, and have been reported in urine, sputum, cerebrospinal fluid, and ascitic fluid, particularly after treatment with diethylcarbamazine.

Microfilariae are 200–360 × 5–9 μm. They have a clear, swollen head with a cephalic space 7–13 μm long. The nuclei are well marked, with the anterior nuclei in rows and elongated terminal nuclei. The tail tapers to a sharp point with no nuclei in the caudal space, which measures 9–15 μm from the terminal nucleus to the tip of the tail. These features distinguish microfilariae of *O. volvulus* from those of the following unsheathed microfilariae that are possible sources of misdiagnosis of onchocerciasis in endemic areas:

- *M. perstans* (<200 μm long and no caudal space)
- *M. streptocerca* (180–240 μm long, a curved tail and no caudal space)
- *M. ozzardi* (200–230 μm long and a fine attenuated hooked tail).

General biology

The nodules (onchocercomata) containing the adult worms are usually less than 2 cm in diameter, firm, mobile, and well defined, and neither tender nor painful. Each nodule typically contains one or two male worms and one or two female worms coiled in a mass, lying within a rim composed of vascularized and hyalinized scar tissue derived from the host. Invading infective larvae seem to be attracted to existing nodules (Duerr et al. 2001) and nodules may occasionally contain ten or more adult worms. Nodules are also found that contain liquefied or calcified necrotic worms. Nodules are well vascularized with fine vessels in close contact with worm coils. Interspersed among the worm bundle is material indicative of chronic inflammation: fibrin, plasma cells, neutrophilic and eosinophilic granulocytes, lymphocytes, and giant cells. Nodules lie subcutaneously in association with bony prominences, sometimes attached to the skin or, occasionally, more deeply. The majority of nodules in African patients occur on the pelvic girdle (Albiez et al. 1988). In young children in Africa, relatively more nodules are found on the upper part of the body, especially the head. In Guatemala and Mexico, nodules are more frequent on the head in all age groups, (Figure 1) while in South America nodules are mostly found on the lower half of the body with the exception of the Amazonian focus. It is recognized that not all nodules are palpable and that not all adult worms are found within such nodules (Albiez 1983).

From the evidence that nodules normally contain more female worms than males (ratio 1:1.1 or 1.2) and that some nodules contain fertilized females, but no male worms, it is thought that the males are migratory,

Figure 1 *Nodules being excised from the head of a young child in Guatemala (Courtesy of J. Bradley)*

moving from nodule to nodule (Schulz-Key and Albiez 1977), whereas the female worms are generally sessile. Females have a cyclical reproductive pattern, requiring fertilization by males for each successive brood of embryos (Schulz-Key and Karam 1986). Primary oocytes mature as they pass down the length of the ovary and are released as individual cells into the oviduct and seminal receptacles where they are fertilized and begin dividing. Multicellular stages develop along the length of the uteri until, finally, they escape from the vulva as elongated microfilariae. Each reproductive cycle lasts about 2–4 months, development of an oocyte into a microfilaria taking about 3 weeks.

It has been suggested that the majority of microfilariae normally reside in the lymph vessels of dermal papillae, at which site they usually cause no host reaction, and that it is only extralymphatic microfilariae, particularly those that are dead or dying, which elicit the host immune response thought to be responsible for the pathological features of onchocerciasis (Vuong et al. 1988). Adult worms do not directly contribute to the pathological features although cephalic location of nodules is a risk factor for ocular disease (Anderson et al. 1975).

Life-cycle

The life-cycle of *O. volvulus* is shown in Figure 2. Infective third-stage (L3) larvae are introduced into the human host by biting female *Simulium damnosum* or other blackfly vectors. There is no significant zoonotic cycle. Larvae undergo two molts before developing into adult worms. The first molt occurs close to the point of entry after 3–10 days and the second molt probably occurs about 1–2 months after the infective bite

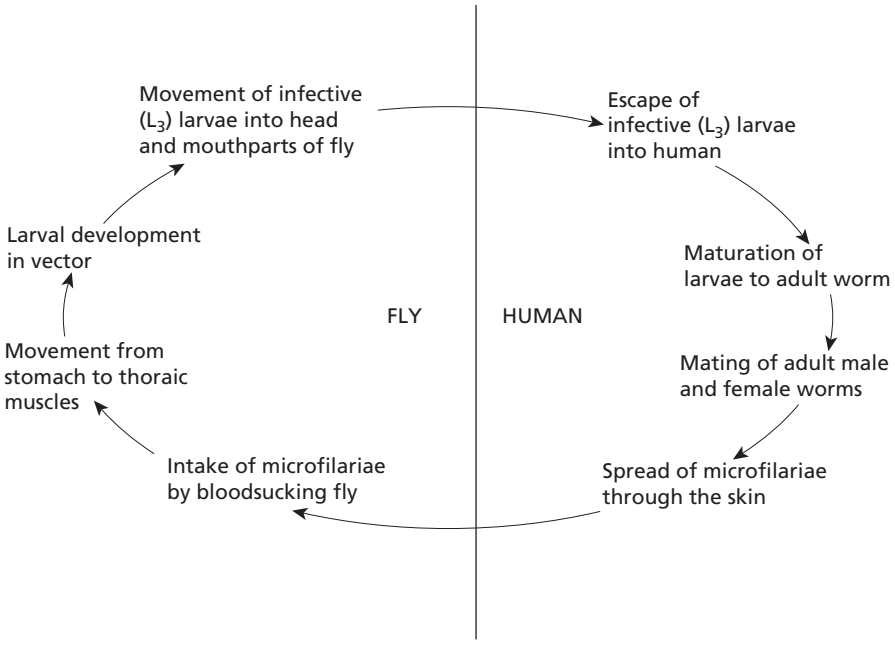

Figure 2 *Life cycle of* O. volvulus

(Lok et al. 1984; Strote 1987; Bianco et al. 1989; Duke 1991). The prepatent period from infection to production of detectable microfilariae is usually 12–15 months, with a range of 7–34 months (Prost 1980). Adult worms are long-lived: the average life span of female worms is estimated to be about 10 years, but can be as long as 15 years (Plaisier et al. 1991; Roberts et al. 1967).

Microfilariae are produced by the female worms in large numbers (500–1 500 per day) and migrate to the skin and eyes of infected subjects. They can survive in the body for 1–2 years (Eberhard 1986). When microfilariae are taken up with the bloodmeal by biting simuliids, some migrate from the gut of the vector into the thoracic muscles. There, they molt twice and develop into infective larvae over a period of 6–8 days, increasing in length to 440–700 μm. These larvae then migrate to the head of the blackfly where they may be transmitted to humans at the next bloodmeal by emerging through the membranous labrum of the mouthparts and penetrating into the wound.

TRANSMISSION, EPIDEMIOLOGY, AND CONTROL

Vectors of *O. volvulus*

Human onchocerciasis is transmitted by biting flies of the genus *Simulium*, commonly known as blackflies. Adult blackflies are small (under 4 mm long) Diptera found in all parts of the world, with the exception of a few islands. They are squat, heavy-bodied flies with a pronounced humped thorax. They are usually black and may have black, white or silvery hairs on the body. The wings are short and broad, with well developed anterior veins but otherwise membranous. In males, the compound eyes occupy almost all of the head and meet anteriorly and dorsally (holoptic), whereas in females, the eyes are separated on top (dichoptic). Only females feed on blood (hematophagic) and can transmit the parasite (Crosskey 1990).

The species of blackflies acting as the main vectors of onchocerciasis in Africa and Arabia belong to the *Simulium damnosum sensu lato* (*s.l.*) species complex. A species complex comprises closely related species that are nearly identical morphologically (sibling species), but that can be distinguished by chromosomal (cytogenetic) techniques (Rothfels 1981). The diversity of simuliid species complexes acting as vectors of human onchocerciasis in the Americas is far greater than in Africa. In the Americas, the main vectors in each of the geographical areas are:

- *S. ochraceum s.l.* in southern Mexico and Guatemala (Dalmat 1955; Ortega and Oliver 1985)
- *S. metallicum s.l.* in northern Venezuela (Duke 1970; Grillet et al. 1994)
- *S. exiguum s.l.* in Colombia and Ecuador (Procunier et al. 1986; Shelley et al. 1990)
- *S. guianense s.l.* in the Amazonian focus between southern Venezuela and northwestern Brazil (Takaoka et al. 1984; Basáñez et al. 1988).

Blackflies lay their eggs in batches on trailing vegetation in fast-flowing water. Each batch may contain 100–900 eggs, which are laid with a secretion of mucus that is immediately wetted, thus cementing the eggs to the

substrate. Eggs hatch after 1–2 days; the emerging larvae remain attached to the vegetation and filter the water for food. Larvae develop into pupae after 5–10 days, and adult flies emerge to surface in a gas bubble 2 days later. Swarming and mating usually occur soon after emergence. Female blackflies need mate only once during their life.

All blackflies feed on plant juices and sugar solutions. Only the female feeds on blood and this process has to be repeated for each ovarian cycle, so the female fly lays a batch of eggs as a result of each bloodmeal. Host-seeking and blackfly feeding occurs in daylight, and in *S. damnosum* biting activity peaks soon after sunrise, followed by a lesser peak in the late afternoon (Garms 1973), a pattern not uncommon among other species. Host location is probably related to odor, CO_2 output, movement, color or outline (Wenk 1981). In West Africa, most blackfly bites on humans are on the ankles and calves, with few bites more than 50 cm above the ground (Renz and Wenk 1983). In Central America, most bites by *S. ochraceum* are on the head and shoulders (Dalmat 1955), and in South America, *S. guianense* bites are most frequently on the lower half of the body (Basáñez et al. 1988). If these areas of the body are not exposed, the blackfly may seek an alternative host. The relative efficiency of a species of blackfly as a vector for *O. volvulus* thus depends partly on the degree to which a blackfly will seek a bloodmeal from a human, rather than from an animal. *S. damnosum* normally takes 5 min to feed to full engorgement on humans (Crosskey 1962). The fly's rasp-like mouthparts tear a ragged hole in the host's skin, to a depth of about 400 μm, blood from ruptured capillaries is then sucked up. The egg-laying cycle of the fly takes usually about 3–6 days (gonotrophic cycle), but development of *O. volvulus* to the infective (L3) stage takes 6–8 days under tropical conditions. Thus, a female blackfly infected with microfilariae at her first bloodmeal cannot transmit infective larvae until her third meal. The longevity of blackflies in the wild was originally estimated at about 15 days (Duke 1968a), with a theoretical maximum of four infective bites, but subsequent reports estimate the maximum life span at 4 weeks (WHO 1987) and the average life expectancy at about a week (Basáñez et al. 1996). Studies of the movement of waves of migrating flies suggest that survival of individual blackflies is possible for up to 7–10 weeks (Baker et al. 1990). Dalmat (1955) reported a maximum longevity of up to 85 days for *S. metallicum* in Guatemala.

There are normally major differences in the sizes of blackfly populations in the dry and wet seasons related to the height of rivers and availability of breeding sites (Renz 1987; Grillet et al. 2001). Dispersal of blackflies away from their riverine breeding sites does occur, especially in forested areas but, in general, the risk of infection is highest close to rivers (Renz and Wenk 1987). Migration, as distinct from dispersal, of blackflies is probably a mainly windborne phenomenon. In West Africa, this may occur over long distances, up to 500 km (Baker et al. 1990), and may be crucial for the survival of *S. damnosum* populations during the dry season. Under field conditions, only a small percentage of blackfly populations is infected. Near to breeding sites, the figure averages 3–5 percent of the total population and 6–10 percent of the parous population.

The blackflies belonging to each species complex differ in their ability to transmit the parasite – such differences in transmission ability are referred to as differences in competence. A good illustration is found in the very different vectorial roles played by members of *S. exiguum s.l.* along parts of its distributional range in South America. Members of this complex include the highly anthropophagic and efficient *O. volvulus* vectors in the Esmeraldas province of Ecuador (Santiago focus); blackflies that yield lower numbers of L3 larvae in the San Antonio (River Micay) focus of Colombia; blackflies that are mainly zoophagic in the Amazonian focus (Shelley 1988a, b; Shelley et al. 1990); and even those that are refractory to parasite development in the Colombian–Venezuelan (Orinoco river) border (Basáñez et al. 2000).

Epidemiology

Onchocerciasis is generally a cumulative infection, the severity of clinical features depending on the length of exposure to blackfly bites and the density of microfilariae in the skin. Because of this, the disease tends to affect mainly the rural poor in sub-Saharan Africa, particularly West Africa. It is also endemic in Yemen and in parts of Central and South America. Onchocerciasis is an unpleasant disease, lasting many years and causing ever-increasing disability to those affected. It has serious socioeconomic consequences for the most heavily affected communities in endemic areas. In hyperendemic communities, more than 60 percent of the population are infected with microfilariae, and about half of these will have symptoms. About 15 percent will have serious skin or eye disease, and up to about 5 percent will be blind. Onchocerciasis is, therefore, one of the major causes of infectious blindness in the world, second only to trachoma (Figure 3a and b).

The most important determinants of the burden of infection in a community are the infective density of the vector and, more crucially, the number of L3 larvae inoculated per person per year by the biting blackfly vectors. The former is known as the infective biting rate and the latter as the annual transmission potential (ATP). The biting density of blackflies can be measured by regular dawn-to-dusk catches using human collectors at selected sites. This allows the calculation of the monthly biting rate (MBR), a theoretical estimate of the total number of bites an individual could receive if maximally exposed to blackfly bites at that site during

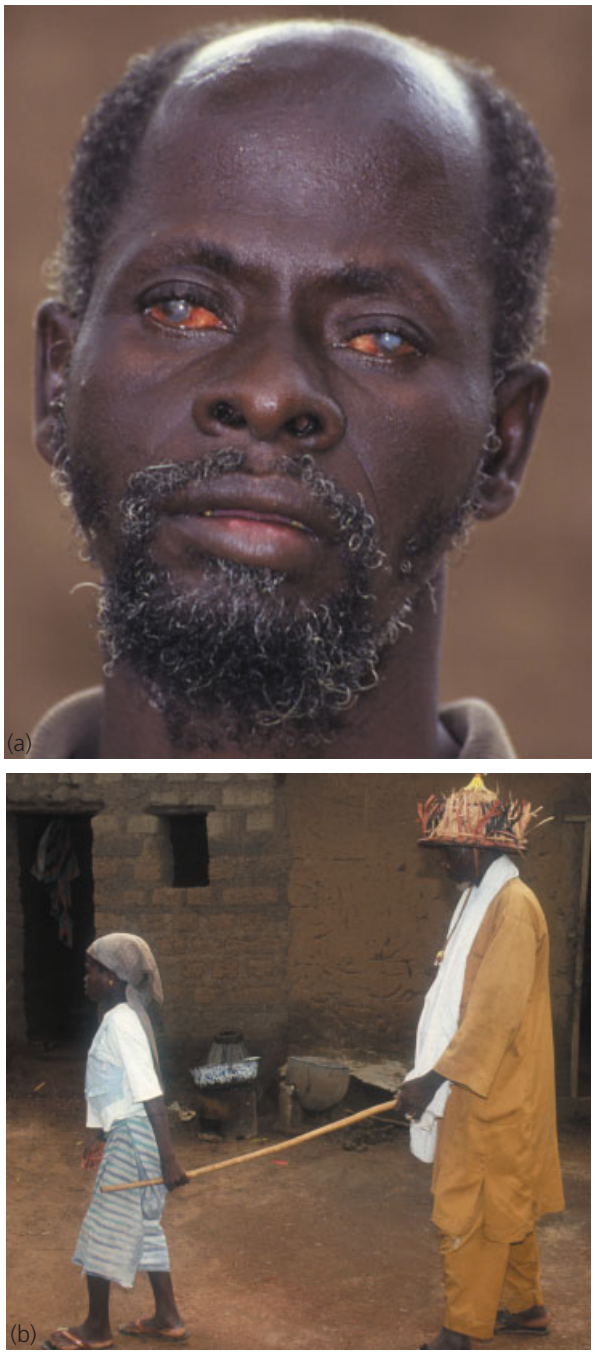

Figure 3 (a) *Anterior eye damage caused by onchocerciasis;* **(b)** *a blind man being led to work in West Africa (Courtesy of J. Bradley)*

a month (Duke 1968b; Walsh et al. 1978).

$$\text{MBR} = \frac{\text{number of blackflies caught} \times \text{number of days in month}}{\text{number of catching days in month}}$$

The annual biting rate (ABR) is the sum of 12 consecutive MBRs.

The number of third-stage infective larvae can be estimated by fly dissection. This permits the calculation of the monthly transmission potential (MTP), a theoretical estimate of the number of infective larvae that could be transmitted to a maximally exposed individual during a month (Duke 1968b; Walsh et al. 1978).

$$\text{MTP} = \frac{\text{MBR} \times \text{number of } O.\ volvulus \text{ L3 larvae detected}}{\text{number of flies dissected}}$$

The ATP is the sum of 12 consecutive MTPs.

More recently, the proportion of infective flies has been ascertained in pools of flies, rather than by dissecting each individual fly, by a combination of *O. volvulus*-specific DNA probes and the polymerase chain reaction (PCR) assay (Yaméogo et al. 1999; Rodríguez-Pérez et al. 1999).

In practice, these indices give only a rough guide to the level of transmission because:

- not all third stage larvae are truly infective
- only about 50–80 percent of L3 larvae are transmitted during a bloodmeal
- no single individual is likely to be maximally exposed to bites
- the infective larvae of other *Onchocerca* species present in manually dissected blackflies may be hard to distinguish from *O. volvulus*
- there is considerable heterogeneity in individual exposure to vector bites (Renz et al. 1987).

Vector biology is also an important determinant of transmission. Vectorial capacity depends on the density of vectors in relation to that of hosts (vector to human ratio), the biting frequency, and the proportion of bites on humans (Dietz 1982; Basáñez and Boussinesq 1999; Basáñez et al. 2002). Vector species is also of importance. For example, the critical biting rates necessary for the infection to become endemic are roughly ten times higher for *S. ochraceum s.l.* than for *S. damnosum s.l.* In Latin America, altitudinal variation in vector composition and abundance is clearly related to the prevalence and endemicity of onchocerciasis, with lower competence vectors (*S. oyapockense s.l.*) being associated with lower altitudes and with areas of lower endemicity, while highly competent vectors (*S. guianense s.l.*) are mostly responsible for hyperendemic transmission in the highlands (Vivas-Martínez et al. 1998; Grillet et al. 2001). Therefore, distance from breeding sites and blackfly species composition and abundance (Vivas-Martínez et al. 1998) have been used in Africa and Latin America, respectively, as entomological indicators for rapid epidemiological mapping of onchocerciasis (REMO) to set priorities for mass treatment with ivermectin (see Control, below).

Nevertheless, when averaged over long time-periods, it has been shown that the prevalence and intensity of infection and clinical features in humans are related to the numbers of biting flies and measured transmission potentials in both West Africa (Duke et al, 1972, 1975), and Latin America (Basáñez et al. 2002).

It is thought that microfilariae show some geographical strain differences throughout their range. The

pattern of clinical features of the disease shows geographical variation, which may be related to differences in parasite strain and vector parasite relationships. In West Africa, there are three major strains of *O. volvulus*: a forest strain with low ocular pathogenicity associated with high nodule numbers and severe skin disease; a dry savannah strain with high ocular pathogenesis and an associated high rate of blindness; and a humid savannah strain with an intermediate pattern (Anderson et al. 1974b; Dadzie et al. 1989). Microfilariae are generally morphologically indistinguishable throughout their range, although significant differences have been reported between savannah and forest microfilariae from West Africa, and between these and microfilariae from South America. In West Africa, at least four different patterns of acid phosphatase staining are found, supporting the hypothesis that a number of biological strains exist (Omar 1978). Differences in the relative frequency of such patterns have also been found between microfilarial populations from West Africa, South America, and the Yemen (Yarzábal et al. 1983). It has been reported that strain-specific DNA probes can distinguish *O. volvulus* parasites originating from forest and savannah areas of West Africa (Erttmann et al. 1987; Zimmerman et al. 1992). A possible mechanism to explain the evolution of these strains is that local populations of microfilariae became adapted to the specific (homologous) sibling species of vector in their area. This could then lead to incompatibility between parasite strains and vectors from different areas. In fact, various degrees of incompatibility have been shown between different strains of *O. volvulus* and heterologous sibling species of *S. damnosum s.l.* from different areas (Duke et al. 1966; WHO 1981). Strong local vector adaptation has also been demonstrated in the Americas (Basáñez et al. 2000). However, recent field studies using the West African forest-specific and savannah-specific DNA probes on vector species from different areas showed that savannah-dwelling vectors carried forest-strain parasites, and vice versa (Toé et al. 1997).

Control

The control of onchocerciasis can be approached either by treating infected patients or by reducing transmission of infection and thereby preventing new cases (Table 1). Currently, control is *either* by reducing morbidity in the human through decreasing transmission from human to vector with the drug ivermectin and, to a lesser extent, nodulectomy, *or* by interrupting transmission from vector to human by killing the larval blackflies. Other possibilities for control remain at the development stage; they include the production of a vaccine and chemotherapy by treating the obligate endosymbiotic bacteria *Wolbachia*. An integral part of control has to be effective diagnosis both in the human and the blackfly.

Table 1 *Methods of control of onchocerciasis*

Control method	Means of achievement
Reduce human/vector contact	Environmental means Protective clothing Insect repellents
Vector control	Regular larviciding at blackfly breeding sites
Vaccine	A possibility for the future
Nodulectomy	Especially in central America (possibly for head nodules in African children)
Drugs	
(a) Microfilaricides	Ivermectin Diethylcarbamazine (obsolete) Doxycycline (anti-embryogenic)
(b) Macrofilaricides	Suramin (obsolete)
(c) Anti-*Wolbachia* therapy	A future possibility

There are three control programs that have been set up to organize regional control strategies for onchocerciasis: The Onchocerciais Control Programme (OCP) in West Africa, The African Programme for Onchocerciasis Control (APOC) and the Onchocerciasis Elimination Program for the Americas (OEPA). All have employed slightly different control strategies, reviewed in Richards et al. (2001) and Hoerauf et al. (2003), based on the timing of available tools at the time of instigation and local logistical considerations. The OCP primarily concentrated on vector control, APOC on chemotherapy, and OEPA employed nodulectomy in addition to chemotherapy.

The OCP

The first report of successful vector control was made by Buckley (1951) who achieved control of *S. naevei* in a small focus in Kenya. Garnham and McMahon (1947) successfully eliminated *S. naevei*, and eventually eliminated onchocerciasis from four isolated foci in Kenya, by using large doses of DDT at a few selected sites at 10–14 daily intervals. The insecticide was carried downstream, killing simuliid larvae over long stretches of river.

Several attempts to control *S. damnosum* were made in West Africa during the 1950s and 1960s (Taufflieb 1955; Davies et al. 1962; Le Berre 1968). The promising results obtained led to the inception of the OCP in 1974 by the World Health Organization and the World Bank. The original area included Benin, Burkina Faso, Côte D'Ivoire, Ghana, Mali, Niger, and Togo. This area was expanded to include Guinea, Guinea Bissau, Senegal, and Sierra Leone by 1986. The two objectives were to combat a disease that was widespread and severe in West Africa, and to remove a major obstacle to economic development (Walsh et al. 1981). The OCP employed vector control as its primary strategy. This was achieved by helicopter distribution of the larvicides

Figure 4 *Ground application of larvicide to a river system (Courtesy of J. Bradley)*

at weekly intervals with supplementary ground applications wherever possible (Figure 4), The success of the programs was monitored by teams visiting the treated breeding sites weekly and recording the presence of nulliparous females, pupae, and late instar larvae. This strategy proved highly successful with *S. damnosum* virtually eliminated from large areas, and transmission generally being reduced to very low levels, almost always to less than 10 percent of pre-control figures. However, the program has faced problems. First, the development of widespread insecticide resistance has necessitated the introduction of more expensive and toxic insecticides. Five insecticidal compounds were used in rotation to combat resistance, including the biocide *Bacillus thuringensis*, which has no side-effects on other invertebrates and fish. In addition, the OCP benefited from the use of chemotherapy, as ivermectin was used in conjunction for vector control (see Chemotherapy, below).

The intention of the OCP was to devolve responsibility for maintaining onchocerciasis control to the participating countries. The program was officially closed in December 2002 by which time, it was estimated, between 100 000 and 200 000 people have been prevented from going blind and 30 million people have been protected (Molyneux 1995). The strategy for using vector control in this area has been proved valid (Hougard et al. 2001) and the aim of 'eliminating onchocerciasis as a public health problem in West Africa' (WHO 2002) has been achieved.

APOC

Although vector control proved extremely effective in controlling onchocerciasis in much of West Africa, large parts of Africa remained where vector control was neither cost-effective nor technically feasible. The provision of ivermectin at no cost from the manufacturers provoked the institution of the APOC, which has as its objective 'to establish, within a period of 12 years, effective and self-sustainable community-based ivermectin treatment throughout the remaining endemic areas in Africa' (Remme 1995). It did aim for total elimination of transmission but its objective was to control serious onchocerciasis and reduce transmission. It was, however, suggested that increasing ivermectin distribution from annually to six monthly might increase the possibility of long-term elimination of the parasite (Richards et al. 2000). Simulations using the onchocerciasis simulation model (ONCHOSIM) indicated that if the dosing regime were to be increased from annually to six monthly in areas with medium to high levels of infection, the time required to eliminate infections would be halved from around 25–30 years; however, the simulations also concluded that it was unrealistic to expect the high level of coverage required to be sustained (Winnen et al. 2002). Additional funds for the accelerated elimination of river blindness in Africa were approved at the G8 summit meeting in Kanaskis in 2002.

OEPA

There are six endemic countries in the Americas: Brazil, Colombia, Ecuador, Guatemala, Mexico, and Venezuela. The characteristics of the foci are very different: although ATPs in some of these areas can be as high as those in West African savanna (Basáñez et al., 2002), they lead to less blindness, and transmission appears to

occur only in restricted areas that are not rapidly spreading. These factors make elimination of onchocerciasis a viable aim in the Americas. The major strategy employed for the control of onchocerciasis in the Americas is the use of ivermectin. The strategy is for the total elimination of the disease spurred on by the success achieved in isolated foci in Guatemala and Mexico (Cupp 1992; Cupp et al. 1992; Collins et al. 1992; Guderian et al. 1997) when coverage levels were very high. The strategy was originally based on systematic nodulectomy campaigns stimulated by the work of Robles in Guatemala and Mexico. In Guatemala, nodulectomy campaigns have been carried out since 1935 with over 250 000 nodules being removed. During this time, the nodule carrier rate has fallen from 24 to 9 percent. It is not clear if this reduction has been wholly due to the campaign or if other factors have contributed. Nevertheless, in one area with hyperendemic onchocerciasis, the prevalence of blindness fell from 7 percent in 1934 to 0.5 percent in 1979 (WHO 1987).

Nodulectomy has been less popular in Africa with no systematic campaigns. However, since nodules on the head are associated with ocular complications (Anderson et al. 1975; Fuglsang and Anderson 1977), it may be reasonable to remove head nodules, particularly from children.

CHEMOTHERAPY

The drug of choice for the treatment of onchocerciasis is ivermectin. Mass treatment campaigns using this drug are also the cornerstone of the current control programs. Prior to the discovery of ivermectin, diethylcarbazine (DEC) was the only realistic drug available. The filaricidal action of DEC was first reported by Hewitt et al. (1947) and it remains the drug of choice to treat lymphatic filariasis. There was much hope that the drug would be useful for onchocerciasis, but, while DEC is an effective microfilaricidal drug, it does not kill adult *O. volvulus* and produces serious side-effects (Mazzotti 1948). In the past, it was often used in combination with corticosteroids, aspirin or antihistamines to control the side-effects. Treatment with DEC has now been superseded by ivermectin.

Ivermectin is a semi-synthetic product, being an 80:20 mixture of avermectins B_{1a} and B_{1b}, which are macrocyclic lactones synthesized by the actinomycete *Streptomyces avermectilis*. It is formulated as 6 mg tablets and given orally. The recommended dose is 150 μg/kg bodyweight taken annually. The manufacturer's exclusion criteria are as follows: children under 5 years of age or under 15 kg bodyweight; pregnant women; breastfeeding mothers within 1 week of delivery; and individuals with neurological disorders or severe intercurrent disease. The drug reaches a peak plasma concentration about 4 hours after administration, is highly bound to albumin, and is reported to have a wide tissue distribution. The precise mechanism of action is unknown, but the drug can act as an agonist of the neurotransmitter γ-aminobutyric acid (GABA), resulting in paralysis of microfilariae (Goa et al. 1991).

Ivermectin was developed initially as a wide-spectrum veterinary anthelminthic agent. It was first studied in humans in Senegal by Aziz et al. (1982) and subsequent studies have found that ivermectin was as effective as DEC in reducing microfilarial counts, but had significantly fewer side-effects (Greene et al. 1985b; Larivière et al. 1985; Diallo et al. 1986). The suppression of microfilarial levels lasts longer after ivermectin than DEC although it is not complete; 20 percent of pretreatment burdens will have reappeared a year after treatment (Awadzi et al. 1986, 1995; Taylor et al. 1986). Ivermectin does not kill adult worms but inhibits microfilarial release from female worms, with a consequent intrauterine accumulation of degenerate microfilariae (Schulz-Key et al. 1985, 1986). Since ivermectin is only microfilaricidal, treatment is suppressive rather than curative and it may be necessary to take the drug for at least the life span of adult worms, i.e. up to 15 years (Whitworth 1992).

Community trials have shown that ivermectin is safe and well accepted and has important effects on the clinical morbidity of the disease, particularly on the ocular lesions (Whitworth et al. 1991b; Abiose et al. 1993). Ivermectin also has some useful activity against other nematode parasites including *Wuchereria bancrofti*, *Ascaris lumbricoides*, *Strongyloides stercoralis* (see Gastrointestinal nematodes; *Strongyloides* and *Capillaria*; and Lymphatic Filariasis), and ectoparasites including head lice (Dunne et al. 1991; Whitworth et al. 1991a). Care is required where *Loa loa* and *O. volvulus* coexist because ivermectin has been shown to cause serious side-effects including encephalopathy in heavy infections of loiasis (Gardon et al. 1997; Boussinesq et al. 1998). The risk of adverse reactions to ivermectin is related to the severity of infection. These have been reported in approximately 20 percent of individuals in hyperendemic areas, but the vast majority are mild and self-limiting. About 5 percent of patients have more severe reactions that affect the ability to work for a day or more. Commonly seen adverse reactions include increased skin rash and itching, nontender soft tissue swelling, musculoskeletal pains, fever, lymphgland pain, and swelling. More serious reactions, which include severe postural hypotension, bronchospasm in asthmatics, abscesses, and bullous eruptions, are rare. All of these respond to symptomatic treatment. Adverse reactions are much less common after subsequent doses of treatment, presumably because the microfilarial load has been reduced (Whitworth et al. 1991c).

Ivermectin is provided free by the manufacturers, and the cost of population-based distribution in endemic countries ranges from US$0.29 per person to more than US$2 per person, depending on the precise system that is used. There are now about 20 million people being

treated under community-directed treatment schemes, which are likely to be necessary for at least 10 years (www.who.int/tds/about/products/comdt.htm). In nonendemic areas, where infections are usually light and patients are not exposed to further infection, ivermectin may be given at 3–6 monthly intervals as necessary, depending on the reappearance of skin microfilariae or recurrence of symptoms. About two-thirds of patients can be expected to relapse within 6 months of each dose of treatment (Churchill et al. 1994).

For morbidity control, ivermectin is a good drug but it has limitations in its use as a tool for control mostly due to the fact it has little effect on adult worms. The reappearance of microfilariae after treatment appears to be at a sufficient density for transmission to continue (Alley et al. 2001). Studies using albendazole in addition to ivermectin were performed in the hope that it might have additive effects in reducing embryogenesis but these proved to be transient and insufficient to stop transmission (Awadzi et al. 1995). Recent studies using higher doses of ivermectin at more frequent periods has shown some effects on the embryogenesis of microfilariae (Gardon et al. 2002), but the need for an effective macrofilaricidal drug or one that has a sustained effect on the production of microfilariae remains an urgent requirement.

Suramin was the first successful drug for the treatment of onchocerciasis and, although no longer used, it remains the only true macrofilaricide. A dose of 6 g is sufficient to kill all adult worms, but significant side-effects were reported in 10–30 percent of patients. This drug had to be given in a course of intravenous injections over several weeks and so treatment was restricted to hospital in-patients. It is clearly too toxic for general use, with treatment being occasionally fatal.

Recently, it has been realized that *Wolbachia*, the endosymbiotic bacteria of most filarial parasites (see Pathology and pathogenesis of clinical features, below and Figure 5a and b), are a good target for chemotherapy. They appear to be essential for worm fertility and reproduction as depletion by chemotherapy disrupts embryogenesis (Taylor and Hoerauf 2001; Bandi et al. 2001) and may even cause worm death (Langworthy et al. 2000). These rickettsial bacteria are sensitive to a range of antibiotics such as tetracyclines, rifampicin, and chloramphenicol (Taylor and Hoerauf 2001; Bandi et al. 2001). Phase II drug trials have been carried out using doxycycline at 100 mg/day for 6 weeks with excellent results. This regime resulted in depletion of the *Wolbachia* and interruption of embryogenesis for the 18-month period of the trial (Hoerauf et al. 2000, 2001). Preliminary data on other treatment regimes have suggested that the treatment period required to deplete the endosymbionts is between 2 and 4 weeks (Hoerauf et al. 2003). The requirement for a long treatment regime is a disadvantage for control strategies because of the logistics of ensuring compliance, but it is a viable treatment for anyone with an imported infection.

Figure 5 (a) Wolbachia *(yellow) in adult male* Onchocerca ochengi *(Courtesy of M. Taylor);* **(b)** Wolbachia *(red) in* O. volvulus *(Courtesy of D.W. Büttner, A. Hoerauf, M. Taylor)*

The search continues for an effective and safe macrofilaricide to complement the effects of ivermectin and larviciding on onchocerciasis control. Suramin, the only currently available effective macrofilaricide has serious side-effects and is too toxic for general use. Amocarzine shows some success, but its macrofilaricidal properties are not ideal. Moxidectin, which is widely used as a wide-spectrum anthelminthic in veterinary practice, shows promise as a single dose and either kills or sterilizes adult worms in animal models but it has not been evaluated in humans (Alley et al. 2001).

DIAGNOSIS

Parasitological

The gold standard diagnostic test in the human is the demonstration of microfilariae in skin snips. Bloodless

Figure 6 *A skin snip being taken from the iliac crest using a corneoscleral punch (Courtesy of J. Bradley)*

skin snips (typically, two to six) 2–3 mm in diameter and 0.5–1 mm deep are taken from both iliac crests, and sometimes the calves in African, Yemeni, and South American cases and the shoulders in Mesoamerican cases. The skin can be raised with a needle and the tip sliced off with a razor blade, or a corneoscleral punch (an obsolete ophthalmologic surgical instrument) can be used (Figure 6). These punches enable snips to be taken easily and rapidly, but they are expensive, need regular setting and sharpening, and should be sterilized in cold glutaraldehyde between patients. Microfilariae will emerge from the piece of skin after incubation in normal saline. About 60 percent of microfilariae will emerge after 30 min, rising to over 75 percent after 24 hours. Live microfilariae can be seen moving vigorously in the medium by direct microscopy. They can be distinguished from other species of microfilariae after staining with Giemsa or Mayer's hemalum.

In detailed community-based surveys in endemic areas, the numbers of microfilariae are counted and the skin snip weighed. The community microfilarial load (CMFL) can then be derived as the geometric mean microfilarial count (per skin snip or per mg of skin) for a cohort of adults aged 20 years or older. This is an accurate measure of the endemicity of onchocerciasis in a community (Remme et al. 1986).

For monitoring control programs, the main requirement is to monitor for recrudescence of infection. The requirements of a diagnostic assay for such purposes are totally different from those of an assay used on an individual to determine treatment. The former assay is necessarily specific, but need not be 100 percent sensitive as results will be assessed at a community level. Parasitological diagnosis is not ideal for this purpose, particularly where the microfilaricidal drug ivermectin is employed.

Serological diagnosis

Serological diagnosis can be by the detection of circulating antigen or by the detection of specific antibodies to the parasite. Tests based on the detection of circulating antigen are more desirable as they define the current infection status of an individual. However, in contrast to the success in developing the rapid format card test for the detection of antigen for lymphatic filariasis (Weil et al. 1997), it has not been possible to develop a reliable test for the detection of antigen for onchocerciasis. This could be because the anatomical locations of the microfilariae and adults in onchocerciasis (in the skin and subcutaneous tissues, respectively) do not allow reliable release of antigen into the circulation. Tests based on the detection of antibody have the problem that it is difficult to distinguish between current and historical infection; moreover, early tests using parasite preparations suffered from poor specificity due to the extensive cross-reacting antigens between nematodes (Karam and Weiss 1985). However, recombinant antigen-based tests have been developed that are able to specifically detect exposure to *O. volvulus* (Lucius et al. 1992; Lobos et al. 1991; Bradley et al. 1998). The use of individual recombinant antigens was shown to have poor sensitivity, but this could be overcome by using a cocktail of antigens (Ramachandran 1993; Bradley et al. 1998). The problem of detecting current infection has at least been partially overcome by using anti-immunoglobuin (Ig)G4 reagents to detect the reactivity in the serum because this antibody isotype has been shown to be related to active infection (Weil et al. 1990; Lucius et al. 1992). As mentioned above, to monitor control programs it is important to have a rapid test where specificity is critical but sensitivity is less so. For this purpose, a dipstick is ideal, and such a test based on the use of one of the specific diagnostic antigens used in the cocktail described above that detects IgG4 antibodies in the serum of infected individuals has been developed (Weil et al. 2000)

DNA-based detection

DNA-based detection methods have been developed that are able to distinguish between *Onchocerca* species, which is of particular importance for monitoring transmission in the blackfly vector, and strains of *O. volvulus*, which will define if an individual is infected with blinding or nonblinding parasites (reviewed in Harnett 2002).

DIAGNOSIS IN FLIES

There have been a number of DNA probes developed that can distinguish *O. volvulus* from the other species of *Onchocerca* that infect the blackfly vector (Perler and Karam 1986; Shah et al. 1987; Harnett et al. 1989;

Meredith et al. 1989). These probes are all members of a 150 bp repeat that makes up about 1 percent of the genome (Meredith et al. 1989). Early attempts to detect *O. volvulus* larvae in the blackflies was by DNA hybridization in slot blots but this proved to be a technically demanding procedure and PCR-based assays were developed in which the 150 bp repeat was amplified and then probed with appropriate labeled probes (Meredith et al. 1991). The technique has subsequently been developed to have the sensitivity to detect a single larva in a pool of around 100 blackflies (Katholi et al. 1995; Oskam et al. 1996; Rodríguez-Pérez et al. 1999). It is possible, by the use of DNA probes, to detect the strain of parasite infecting the blackfly (Toé et al. 1997; Fischer et al. 1997).

DIAGNOSIS IN HUMANS

PCR-based diagnostic tests on skin snips (Zimmerman et al. 1994) and, more recently, superficial skin scratches (Toé et al. 1998) have provided an alternative to the skin-snipping-and-microscopy technique and have also proved to be more sensitive (Elson et al. 1994; Freedman et al. 1994). The use of DNA probes can distinguish between forest and savannah (or blinding/nonblinding) isolates of the parasite strains (Erttmann et al. 1987; Zimmerman et al. 1992) and the use of such probes has shown good correlation with the epidemiology and pathogenicity of a strain in a given area (Erttmann et al. 1987; Meredith et al. 1989; Ogunrinade et al. 1999; Zimmerman et al. 1992).

Other tests

None of the available tests is totally ideal for the purposes of monitoring the recrudescence of infection in the post-OCP era because they are either insensitive or technically demanding. The use of a noninvasive patch skin test using DEC has shown promising results (Stingle et al. 1984). This provokes a local reaction due to the death of the microfilariae in the skin and has shown to have better sensitivity than conventional skin snipping and the PCR skin-scratch test (Boatin et al. 2002).

Rapid mapping techniques

A different challenge was posed to APOC in deciding where the mass distribution of ivermectin was required. The approach taken was to use the rapid field assessment process (REMO) to define areas of high risk (Ngoumou et al. 1993). The information provided from REMO was integrated into a geographical information system (GIS) which has allowed delineation of the areas of endemicity and definition of their thresholds for community treatment programs (Noma et al. 2002)

CLINICAL, PATHOLOGICAL, AND IMMUNOLOGICAL ASPECTS OF INFECTION

Introduction

Onchocerciasis causes ocular disease, including blindness in long-standing severe cases, and dermatological pathology. The disease is found in 36 countries of West, East and Central Africa, the Arabian Peninsula and parts of South and Central America (Figure 7). It is predominantly a disease of Africa; of 120 million people at risk of infection, 96 percent are in Africa; of the 18 million infected, 99 percent are Africans and of these 270 000 people are blind (WHO 1995).

The communities affected often suffer from socio-economic depression, with the effects of the disease further exacerbating poverty. Visual damage is also significantly associated with blindness, poor nutritional status, and increased risk of premature death (Prost and Vaugelade 1981; Kirkwood et al. 1983b; Pion et al. 2002). Studies from villages in West African savanna

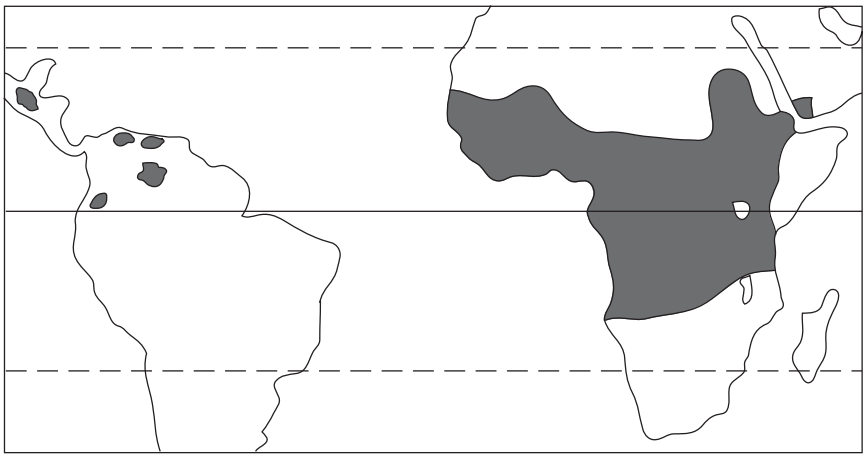

Figure 7 *Geographical distribution of onchocerciasis*

regions prior to the instigation of the OCP suggested that blindness from onchocerciasis reduced life expectancy by at least 13 years, and that if the prevalence of blindness in a community is 5 percent, almost half of adult males and one-third of females would become blind before they died (WHO 1987). It has recently been shown that onchocerciasis infection per se is associated with excess human mortality, i.e. not only through its effect on blindness (Little et al. 2004).

The symptoms of onchocerciasis are associated with the presence of the microfilariae in the eye and the skin. Ocular disease is characterized by slowly progressive inflammatory change in most parts of the eye leading to visual impairment and blindness in those most severely affected. Skin disease often starts as an itchy papular dermatitis leading to irreversible skin changes in long-term infection. The pathogenesis has long been thought to be associated with the death of microfilariae (Ottesen 1992; Pearlman 1996). Recently, however, it has been suggested that the release of products from the symbiotic bacteria (*Wolbachia* sp.) of many filarial nematodes may be involved in the pathogenesis of both eye and skin lesions (Saint André et al. 2002).

Clinical features of onchocerciasis

The pattern and frequency of the clinical features of onchocerciasis vary according to duration and frequency of exposure, geographical location, and individual variation (Cook and Zumla 2003). The most frequent symptom is itching. This may be of any degree of severity and is sometimes incapacitating. It is usually generalized and often associated with excoriations. Other acute reactive dermal lesions include papular eruptions anywhere on the body (reflecting intraepithelial abscesses), transient localized intradermal edema, and lymphadenopathy (particularly of the inguinal and femoral glands). These lesions are typically firm and nontender.

In a small minority of individuals, there may be an acute hyperreactive form of the disease known as sowda, in which it appears that there is killing of the microfilariae and severe inflammatory skin lesions occur. Later skin lesions give the appearance of premature aging associated with:

- lichenoid change, hyperkeratosis, and exaggerated wrinkling of the skin
- atrophy of the epidermis, with loose, redundant, thin, and shiny skin (Anderson et al. 1974a)
- depigmentation, often initially hyperpigmented macules, but more typically a spotty depigmentation of the shins that represents islands of repigmentation around hair follicles in areas of depigmentation; this is often termed 'leopard skin' (Buck 1974).

Chronically enlarged lymph nodes and the surrounding fluid may become dependent leading to 'hanging groin' (Nelson 1958) and predisposing to hernia formation, lymphatic obstruction, and mild elephantiasis. Some of these features are shown in Figures 8–10.

A careful characterization of onchocercal skin pathology has been created for Africa where skin lesions are defined as acute or chronic and graded according to severity (Murdoch et al. 1993).

The subcutaneous nodules containing adult worms are often visible and palpable, but they rarely cause any symptoms. Occasionally they may spontaneously rupture through the skin, cause local pressure symptoms or the contents may necrose, leading to abscess formation.

Visual damage is the most serious clinical feature of onchocerciasis and may affect all tissues of the eye. Ocular lesions are usually seen only in those with moderate or heavy microfilarial loads. Two types of lesion affect the cornea: punctate keratitis and sclerosing keratitis (Anderson et al. 1974a). Punctate keratitis is caused by an acute inflammatory exudate surrounding dead and dying microfilariae in the cornea. These give rise to 'snowflake opacities' and resolve without sequelae. Sclerosing keratitis is a progressive exudative process with fibrovascular pannus formation that starts at the inferior or medial and lateral margins of the cornea and slowly becomes confluent. This may lead to irreversible visual damage and blindness if it encroaches on the visual axis. Anterior uveitis and iridocyclitis are usually mild, chronic, and nongranulomatous conditions, but may cause anterior and posterior synechiae, leading to seclusio or oclusio pupillae and serious, potentially blinding, complications such as secondary cataract and glaucoma. Characteristic chorioretinal lesions are atrophy or hyperplasia of the retinal pigment epithelium,

Figure 8 *Papular eruption (onchodermatitis) on the thighs of an adolescent girl in West Africa; note the asymmetrical distribution (Courtesy of Dr D. Morgan)*

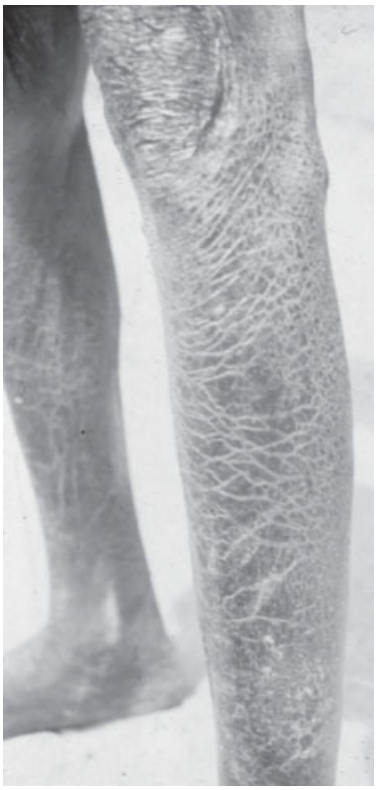

Figure 9 *Presbydermia (atrophic skin changes) on the leg of a young man (Courtesy of Dr D. Morgan)*

chronic nongranulomatous chorioretinitis and chorioretinal atrophy. These are typically widespread, unlike toxoplasmosis, and are usually first seen temporal to the macula. Postneuritic optic atrophy with constriction of visual fields is common in advanced disease, often associated with dense sheathing of retinal vessels.

Other clinical features such as weight loss, musculoskeletal pains and dizziness have been associated with onchocerciasis (Lamp 1967; Pearson et al. 1985; Burnham 1991), but the main clinical burden is due to the skin and eye lesions. In West Africa, the prevalence of infection, as measured by the presence of skin microfilariae, increases with age up to 25 years and is generally higher in males than in females (Kirkwood et al. 1983a). The degree of visual damage has a linear relationship to microfilarial density in savanna regions and the prevalence of visual loss increases with age (Remme et al. 1989). The relationship of blindness to intensity of infection does not hold true for parasites from forest regions, which may be explained by the microfilaria from the nonblinding strains being less eye invasive (Dadzie et al 1989, 1990). Males are 1.5 times more likely to be blind than females of the same age and microfilarial density. Blindness strikes mainly at economically active adults in the prime of life. The development of onchocercal eye disease in an individual is, therefore, both a catastrophe for the individual and a burden for the family. At the community level, visual impairment can have serious socioeconomic consequences for the community, especially if villages are in an area of high transmission. Under these conditions, heavy infections are acquired relatively early in life, so that there may be a considerable proportion of young, productive individuals, especially men, with visual loss. It was in such situations that villages in fertile river valleys were abandoned in Burkina Faso and northern Ghana prior to the start of the OCP in West Africa.

Figure 10 *Dispigmentation (leopard skin) on the shins (Courtesy of Dr D. Morgan)*

Pathology and pathogenesis of clinical features

Although nodules cause very few symptoms, there are cellular infiltrates around the adult worms in the nodules. There appears to be little or no reaction around live microfilariae, which are characteristically found in the upper dermis but lesions are found around dying microfilariae. Such lesions are characterized by an infiltration of eosinophils and neutrophils and these cells, along with macrophages, have been shown to attack microfilariae (Greene et al. 1981; Medina de la Garza et al. 1990). Early skin changes include:

- perivascular inflammatory infiltrates of eosinophils, neutrophils, plasma cells, histiocytes, and lymphocytes
- hyperkeratosis
- acanthosis with increased melanin in the upper dermis
- dilated tortuous lymph and blood capillaries.

Prolonged and heavy infections of the skin eventually lead to loss of elasticity with fibrosis, scarring of the

papillae, replacement of dermal collagen by hyalinized scar tissue and, eventually, atrophy of the epidermis. Although the recruitment and activation of eosinophils has been shown to be a response to parasite products, recent evidence has indicated that neutrophils are attracted to the nodule by products of the endosymbiotic bacteria *Wolbachia* (Brattig et al. 2001). The hyperreactive form of the disease (sowda) is associated with high IgE levels, and a much increased infiltration of lymphocytes and eosonophils around the parasites (Brattig et al. 1987, 1994). Recently, a polymorphism in the T helper 2 (Th2) cytokine interleukin (IL)-13 has been associated with this form of onchocerciasis (Hoerauf et al. 2002).

Lymph node pathology in Africa tends to show scarring of lymphoid tissue with histological evidence of atrophy and fibrosis, chronic inflammatory infiltration and sinus histiocytosis (Gibson and Connor 1978). In the Yemen, follicular hyperplasia is a more common sign of the disease.

Ocular lesions are also thought to be related to the local death of microfilariae. The snowflake opacities of punctate keratitis are focal collections of lymphocytes, eosinophils and neutrophils. Sclerosing keratitis, in contrast, consists of an inflammatory exudate mainly of lymphocytes and eosinophils, scarring, and a fibrovascular pannus formation. Uveitis and chorioretinitis are usually low-grade indolent inflammatory processes. Studies in experimental models have shown that there is an immunological basis of the eye lesion pathology with a role for T cells, neutrophils, parasite-specific antibody, and immune complexes (Pearlman 1997; Pearlman et al. 1995; Hall et al. 1999; Kaifi et al. 2001). It has also been shown that a component of the pathology is regulated by Th2 cytokines: IL-4 is required, as lesions do not develop after immunization in IL-4 gene knockout mice (Hall et al. 2002) and IL-13 is required for eosinophil recruitment into the eye (Berger et al. 2002). Recently, endotoxin-like products of *Wolbachia* have also been shown to provide a major pro-inflammatory stimulus for the induction of eye pathology in the mouse model. Parasites containing *Wolbachia* induced the characteristic infiltrate of neutrophils and keratitis when they were injected, whereas such effects were absent when worms that had been depleted of their *Wolbachia* by chemotherapy were used (Saint André et al. 2002).

Molecular mimicry has been suggested as having a role in the development of onchocercal eye pathology, indeed an *O. volvulus* antigen termed Ov39 was found to cross-react with an antigen of retinal pigment epithelium (Braun et al. 1991). Autoantibodies to this antigen were found in patients with both onchocerciasis and lymphatic filariasis, so it was suggested that intraocular presentation of this antigen by microfilariae is required for the development of disease (McKechnie et al. 1993). Subsequent studies in a rat model showed that immunization with Ov39 can induce ocular inflammation that can be transferred by antigen specific T cell lines (McKechnie et al. 1997, 2002).

Post-treatment adverse reactions can also be attributed to inflammatory responses to *Wolbachia* bacteria. The Mazzotti reaction after treatment with DEC often gave very severe side-effects, and this was originally thought to be due to parasite products. However, recent studies have indicated that there is a link between the side-effects and the concentration of *Wolbachia* DNA (Keiser et al. 2002).

Immunity and immune responses

There is no direct evidence for acquired immunity in individuals exposed to or infected with *O. volvulus*. The fact that worm burdens plateau or decline with age has been suggested as evidence for concomitant immunity, i.e. protection from reinfection despite having a current infection. This phenomenon could equally be explained by exposure levels declining with age. However, in many endemic areas, microfilarial levels appear to continue to rise throughout life. The occurrence of uninfected individuals within endemic areas despite evidence of exposure, has also been cited as evidence for resistance to infection, but as diagnostic techniques are not 100 percent sensitive, it is difficult to prove absence of infection.

Onchocerciasis is a spectral disease with many individuals in a hyperendemic area having high numbers of microfilariae in the skin. Such individuals are often asymptomatic and have suppressed cellular responses to parasite antigens, although they have significant antibody responses (Greene et al. 1985a; King and Nutman 1991; Elson et al. 1994, 1995; Ottesen 1985; Gallin et al. 1988). The characteristics of the cytokines and antibodies produced by these individuals to parasite antigens suggest they are polarized towards type 2 responses as they have high levels of IL-4 and IL-5, and produce significant amounts of IgG4 and IgE.

Increased titers of specific and nonspecific antibodies, particularly IgG, IgM, and IgE, are found in symptomatic subjects. Some are specific for microfilariae, infective larvae and adult worms (Greene et al. 1985a; Ottesen 1985). There are also increased levels of circulating immune complexes of unknown significance. Cell-mediated immune responses are generally suppressed, at least in adults with patent infection (Gallin et al. 1988). Immune responsiveness appears to change with time of exposure to infection (Karam and Weiss 1985), with the IgG–IgE balance being an important determinant of microfilarial levels and clinical disease and, like the suppression of cell-mediated immunity, altering between the ages of 10 and 19 years.

In populations exposed to infection, there are always individuals who appear to be free from infection despite evidence of exposure; these have been suggested to be

immune to infection. Such putative immune individuals have immune responses that confirm exposure to the parasite. They have vigorous proliferative responses to parasite antigen (Ward et al. 1988; Elson et al. 1995; Lüder et al. 1996; Brattig et al. 2002), but there are conflicting reports about the types of cytokines produced. Several reports have shown cytokine production indicative of a type 1 response with high interferon gamma (IFN-γ) levels (Ward et al. 1988; Elson et al. 1995; Lüder et al. 1996). Other studies, however, have shown either high Th2 cytokines (IL-5 and IL-13) levels to parasite antigens in individuals with either no parasites or low parasite burdens (Brattig et al. 2002) or heterogeneity in cytokine production particularly to larval and male antigens (IFN-γ and IL-5) (Turaga et al. 2000).

Cross-sectional studies such as those described above do not accurately reflect the fact that onchocerciasis is a dynamic, progressive disease, amicrofilaridermic individuals may in fact be prepatent or postpatent and symptoms may appear late in infection. Longitudinal studies are difficult because of the time scale of infection but age-profile studies can be performed to reflect the accumulated years of exposure in older subjects (Faulkner et al. 2001; MacDonald et al. 2002). If concomitant immunity does exist, it appears to be related to increasing levels of Th2 cytokines to larval antigens and IgG3 and IgE to infective larvae (MacDonald et al. 2002). Studies on migrant populations can also give an indication of the early response to infection and it seems that early infection is associated with a vigorous immune response with elevated IFN-γ and IL-5. In comparison with chronically infected individuals, it seems that this early response is down-regulated partly through an IL-10 dependent mechanism (Cooper et al. 2001).

Immunoregulation

The intriguing features of immunoregulation in onchocerciasis are marked increases in polyclonal IgE and eosinophils, but the persistence of skin microfilariae with minimal host responsiveness (immunotolerance). There is some evidence of active and specific immune suppression by parasite antigens. Most people in endemic areas, although microfilaridermic, are asymptomatic, show reduced specific antibody levels, and have depressed B- and T-cell responses when these are tested in vitro (King and Nutman 1991). One suggestion is that these observations can be explained by a balance between T helper 1 (Th1) and Th2 phenotypes, the asymptomatic microfilaridermics having elevated Th2 cytokine production and Th2 controlled effectors, but depressed proliferative responses and IFN-γ production, the converse being true in exposed uninfected individuals (Soboslay et al. 1999). However, more recent data suggest that the situation is more complex. There is strong evidence that the cellular response is down-regulated by IL-10 and transforming growth factor beta (TGF-β) which are produced by T regulatory (Tr) cells (Soboslay et al. 1999; Cooper et al. 2001; Doetze et al. 2000). Cells that bear characteristics of Tr1 cells have been cloned from individuals with chronic onchocerciasis. These cells produce substantial amounts of IL-10 and are able to inhibit other T cells in coculture (Satoguina et al. 2002) The presence of the microfilariae is essential for the maintenance of the down-regulation in immune responses as chemotherapy restores responsiveness, at least until microfilarial levels return (Steel et al. 1994).

FUTURE DIRECTIONS

Between 1985 and 1999, a targeted approach to develop a vaccine for onchocerciasis was funded by the Edna McConnell Clark foundation (Cook et al. 2001). Although to date the development of a product to protect against infection by the parasite remains in the future, our understanding of the mechanisms of acquired immunity to parasitic helminths such as *Onchocerca* have been enormously extended because of this program. Major advances have been made in the development of animal model systems for onchocerciasis (Abraham et al. 2002) and a large number of potentially protective antigens have been identified about 14 of which reduce larval survival (Lustigman et al. 2002).

Although there is considerable progress in controlling the worst pathological consequences of onchocerciasis, it is clear that elimination of infection is not currently an achievable goal. Without additional tools such as a vaccine or effective macrofilaricides to combat transmission, it will be necessary to have long-term control and surveillance programs.

With advances in genomic technologies, and as the techniques of transfection and gene knock out become as available for parasitic nematodes as they are for the free-living nematode *Caenorhabditis elegans*, we will be able to increase our knowledge of the biology of these organisms exponentially. Already there are a growing number of *Onchocerca volvulus* expression sequence tag (EST) sequences (Williams et al. 2002) that can be exploited, and with the complete genome sequences for *C. elegans* and the proposed sequencing of the *B. malayi* genome, it is certain that many more vaccine targets will be identified.

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Angiostrongylus (*Parastrongylus*) and less common nematodes

KENTARO YOSHIMURA

Angiostrongylus cantonensis Dougherty, 1946 1
Angiostrongylus costaricensis Morera and Céspedes, 1971 7
Ternidens deminutus Railliet and Henry, 1909 9
Oesophagostomum species 10
Trichostrongylus species 11
Anisakis and related species 12
Gnathostoma species 17
Gnathostoma spinigerum Owen, 1836 17
Gnathostoma hispidum Fedtschenko, 1872 21
Gnathostoma doloresi Tubangui, 1925 22
Gnathostoma nipponicum Yamaguti, 1941 23
Spiruroid type X larva 23
References 24

ANGIOSTRONGYLUS CANTONENSIS DOUGHERTY, 1946

Introduction

The genus *Angiostrongylus* includes approximately 20 species infective to small mammals, of which two are definitively known to be pathogenic to humans. *A. cantonensis* is inherently neurotropic, causing eosinophilic meningoencephalitis, whereas *A. costaricensis* causes abdominal angiostrongyliasis. *A. mackerrase* may also cause eosinophilic meningitis in Australia. *A. malaysiensis* occurs in South East Asia and three cases of eosinophilic meningoencephalitis might have involved this species (Cross 1987). A comparative study of *A. cantonensis* and *A. malaysiensis* in monkeys revealed that the former was more pathogenic causing fatal infections.

A. cantonensis was first found in rats, a discovery made in China in 1935 (Chen 1935) and in Taiwan 2 years later. Human infection was first confirmed in Taiwan, but its public health importance was not recognized until the parasite was found in the brain of a Filipino patient in Hawaii (Rosen et al. 1962).

Classification and morphology

The genus *Angiostrongylus* belongs to the order Strongylida and the superfamily Metastrongyloidea. Although the genus has now been divided into five, and *A. cantonensis*, *A. costaricensis*, *A. malaysiensis*, and *A. mackerrase* placed in the new genus *Parastrongylus*, *Angiostrongylus* is used for classification here, because most parasitologists still use this terminology.

Adult males measure 20–25 × 0.32–0.42 mm. The buccal cavity has two lateral teeth, but no lips. The esophagus is club-shaped, and measures 0.31–0.32 mm long (Figure 1). There is an excretory pore just posterior to the esophagus. Spicules are slightly subequal in length, and range between 1.02 and 1.25 mm long. The gubernaculum is 0.095 mm long. The bursa and rays are well developed (Figure 1).

Adult females measure 22–34 × 0.34–0.56 mm. The esophagus is 0.35–0.46 mm long. The blood-filled intestine and white uterine tubules are spirally wound in a characteristic 'barber's pole' pattern. The vulva and anus open at distances of 0.19–0.27 and 0.04–0.06 mm, respectively, from the tip of the tail (Figure 1). Eggs measure approximately 70 × 30 μm.

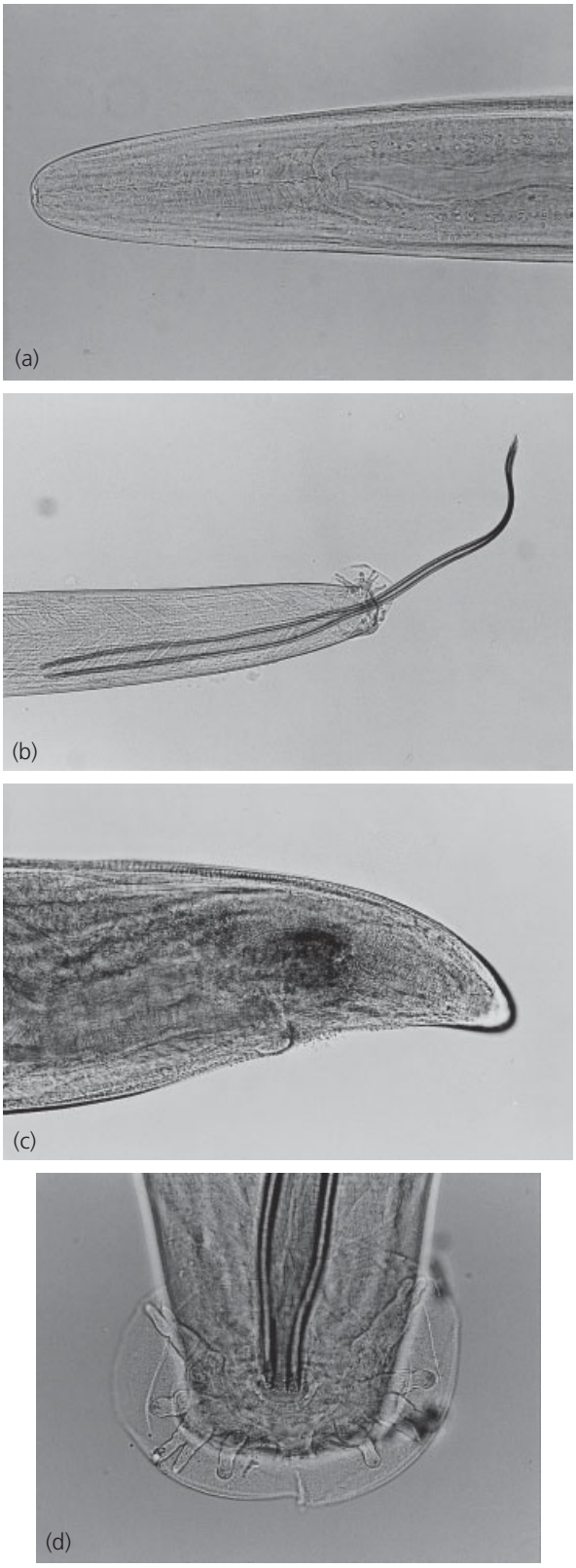

Figure 1 Angiostrongylus cantonensis. **(a)** *Anterior end of male (×123).* **(b)** *Posterior end of male, showing copulatory bursa and spicules (×61).* **(c)** *Posterior end of female, showing rectum and vulva (×123).* **(d)** *Copulatory bursa of male, dorsal view (×248).*

Life cycle

The life cycle in the rat was first described by Mackerras and Sandars (1955). Adult worms live and lay eggs mainly in branches of the pulmonary artery, but sometimes in the right ventricle. Eggs are carried to the lung capillaries where they form emboli. They embryonate and hatch after about 1 week. First-stage (L1) larvae enter alveoli, then migrate up the trachea. They are subsequently swallowed and passed out with feces. In the intermediate host, larvae undergo two molts to the second (L2) and third (L3) stage, after 7–9 and 12–16 days respectively. The L3 larvae are 460–510 μm long and, in molluscan intermediate hosts, remain enclosed in the L1 and L2 sheaths.

Many terrestrial and aquatic snails and slugs can serve as intermediate hosts (Chen 1979; Otsuru 1979). Important snails for transmission are the giant African snail, *Achatina fulica* and *Pila* spp. More recently, another edible snail, *Ampullarium canaliculatus*, was found to be naturally infected in Japan and Taiwan.

Animals that acquire L3 larvae by eating infected mollusks may act as paratenic (transport) hosts and may transmit *A. cantonensis* to humans. Known paratenic hosts are toads, frogs, freshwater prawns, land planarians, and land crabs. The yellow tree monitor has also been described as an epidemiologically important paratenic host in Thailand.

When ingested by rats, L3 larvae exsheathe and penetrate into the intestinal wall, reaching the heart via the portal system and the inferior vena cava. Larvae then reach the left ventricle through the pulmonary circulation. Some migrate to the brain directly, whereas others migrate first through other organs or tissues. In either case, L3 larvae reach the central nervous system (CNS) within 2–3 days, after which they migrate within the brain and grow. The larvae molt twice, to become young adults that migrate through brain tissue to the subarachnoid space. Approximately 26–29 days after infection, the worms, now 11.5–13.0 mm long, penetrate cerebral veins and return to the pulmonary arteries via the heart. The worms now grow rapidly and females lay eggs from around day 35. L1 larvae appear in the feces 40–42 days after infection.

In non-permissive hosts, including man, L3 larvae reach the CNS and molt twice, developing into young adults. They die in the brain without returning to the heart and lungs, causing serious eosinophilic meningoencephalitis.

Although *A. cantonensis* has been recovered from the lungs of four patients, it seems unlikely that it can develop to sexual maturity in immunocompetent humans. Among these four, a fatal case reported by Cooke-Yarborough et al. (1999), was an 11-month-old boy who did not show any cerebrospinal fluid (CSF) eosinophilia and harbored adult *A. cantonensis* in the

lung after having been treated with intravenous methylpredonisolone. Experimentally, young adult worms have been recovered from the heart and lungs of immunosuppressed mice (Yoshimura et al. 1982; Sugaya and Yoshimura 1988; Sasaki et al. 1993), but it is still unclear whether full maturity is reached. The pulmonary migration and maturation of *A. cantonensis* in non-permissive hosts may be associated with the immunocompromised state of the host.

Transmission and epidemiology

A. cantonensis infection can occur at any age, even in young infants (Shih et al. 1992). Humans are infected by the following routes: (1) ingestion of L3 larvae in raw or undercooked intermediate or paratenic hosts; (2) by drinking infected water; and (3) oral contact with hands contaminated with larvae released from mollusks. Infections can be established in rodents by various routes including lacerated, abraded, or intact skin. Theoretically, therefore, humans may also acquire infections through the skin.

In Taiwan, most infections occur in aboriginal children, and are acquired by eating infected giant African snails; 80 percent of cases are in children under 14 years old (Chen 1979). The overall snail infection rate is 28 percent, but snails of size >60 mm show 65 percent infection. Infections also occur in infants in the islands of Reunion and Mayotte (Graber et al. 1997; Edmar et al. 1999). In contrast, infections in Thailand are commonly seen in adult males, who often eat chopped raw *Pila* snails with vegetables as an accompaniment to alcoholic drinks. The overall snail infection rate has been calculated as 20 percent, but it was as high as 73 percent in some areas (Teekhasaenee et al. 1986). Infection generally occurs during the rainy months.

Most of the cases reported in Japan have originated from the Ryukyu Islands, where patients have become infected by eating fresh toad liver or raw slugs as medicines (Otsuru 1979). Freshwater prawns, or sauces containing them, are responsible for infections acquired in the Pacific Islands, while in New Caledonia, infections are linked to accidental ingestion of infected small planarians (or fragments of these) on vegetables (Ash 1976). In Micronesia, land crabs and mangrove crabs act as paratenic hosts (Alicata 1965). L3 larvae shed in slug mucus on lettuce may be a source of human infection in Malaysia (Heyneman and Lim 1967). Initial reports of *A. cantonensis* in rodents and mollusks were restricted to tropical and subtropical areas (Alicata and Jindrak 1970), but it has since been found in many other countries.

Cerebral and ocular infections in humans have been confirmed in Hawaii, Japan, China, Taiwan, Thailand, Vietnam, Malaysia, Indonesia, Vanuatu, American Samoa, and the Ivory Coast (Cross 1987). Confirmed or suspected angiostrongyliasis has also been reported in Australia, Fiji, New Orleans, Sri Lanka, Egypt, Cuba, and Madagascar. Cases are common in Taiwan, Ponape island, Tahiti island, and New Caledonia and thousands of cases occur every year in Thailand. Recently, an outbreak of *A. cantonensis*-associated eosinophilic meningitis was recorded in US tourists returning from Jamaica (Slom et al. 2002) and the enzootic *A. cantonensis* infection in rats and snails was noted in Jamaica (Lindo et al. 2002).

It was previously thought that the spread of *A. cantonensis* was linked to dissemination of the giant African snail (Alicata and Jindrak 1970), but this snail does not occur in many of the new endemic areas. This suggests that if infected rats are present, the life cycle of this parasite may easily become adapted to indigenous mollusks (Cross 1987).

Clinical manifestations

These are primarily associated with meningeal irritation and cerebral damage (Table 1). Symptoms develop 3–36 days after infection.

Severe headache is the most common symptom (occurring in 86–99 percent of patients). It initially occurs intermittently, but later becomes more frequent. Nausea or vomiting, fever (<38°C), constipation, malaise, and anorexia are frequent (Yii 1976). Less common symptoms are cough, neck stiffness, paresthesia, weakness of extremities, muscle twitching, diplopia, strabismus, and facial paralysis. Patients often present with abnormal (usually decreased) tendon reflexes, Kernig's sign, Brudzinski's sign, sometimes with absence of abdominal reflexes and eye muscle paralysis.

The disease is self-limiting and recovery usually occurs within 4 weeks. Serious cases sometimes result in coma, paralysis of the extremities, urinary incontinence or retention, profuse salivation, convulsions, and the persistence of symptoms (Prociv and Tiernan 1987). Fatality ranges from 0.5 percent (Thailand) to 3 percent (Taiwan).

Eosinophilic radiculomyeloencephalitis has been reported, characterized by peripheral and spinal fluid eosinophilia, severe radicular pain, weakness, and hyporeflexia of the legs and dysfunction of the bladder and bowels (Kliks et al. 1982). Patients may exhibit transient hypertension and lethargy and some become comatose. In eosinophilic myelomeningoencephalitis, patients develop a generalized maculopapular rash followed by myalgia, marked paresthesia, fever, and headache, with progressive weakness, particularly of the legs rather than the arms. Some patients may progress to coma and die (Witoonpanich et al. 1991). A long-lasting, chronic intractable pain, refractory to usual pain management, has also been noted in some patients, the pain of whom

Table 1 *Frequency of symptoms/signs among patients with eosinophilic meningitis or meningoencephalitis due to* A. cantonensis *infection*

Symptom/sign	Punyagupta et al. (1975) (484 Thailand cases) (%)	Yii (1976) (114 Taiwanese cases) (%)
Headache	477 (99)	98 (86)
Sensory impairment (lethargy, coma, and confusion)	30 (6)	104 (91)
Vomiting or nausea	425 (88)	94 (83)
Fever	177 (37)	91 (80)
Constipation	–	87 (76)
Malaise	–	81 (71)
Anorexia	–	73 (64)
Neck stiffness	312 (64)	45 (40)
Cough	–	62 (54)
Paraesthesia	181 (37)	32 (28)
Abdominal pain	–	39 (34)
Weakness or paralysis of extremity	4 (1)	26 (23)
Diplopia	184 (38)	11 (10)
Strabismus	–	11 (10)
Muscle twitching	–	15 (13)
Irritability	–	9 (8)
Aching of body and extremities	30 (6)	–
Urinary incontinence or retention	2 (1)	7 (6)
Facial paralysis	20 (4)	–
Convulsion	17 (4)	3 (3)

was successfully relieved by an implantable spinal cord stimulator (Crump et al. 1999).

Infected mice usually develop paralysis of the extremities, ataxia, circling, and torticollis at around day 20 after infection. They develop degenerative or necrotic Purkinje cells that subsequently disappear from the cerebellum, suggesting a Gordon-like phenomenon (Figure 2) (Yoshimura et al. 1988, 1994). Eosinophils in the spinal fluid and those around degenerating worms exhibit degranulative changes (Figure 3). Eosinophil peroxidase has been detected in the spinal fluid of infected guinea pigs (Perez et al. 1989). Therefore, neurological disorders in eosinophilic meningoencephalitis are probably due not only to mechanical damage caused by migrating worms, but also to the neurotoxicity of eosinophil-derived basic proteins.

Ocular angiostrongyliasis has been reported from Thailand, Taiwan, Vietnam, Indonesia, Japan, Papua New Guinea, and Sri Lanka, worms being found in the anterior chamber, retina, and other sites. This form is rarely accompanied by meningoencephalitis (Nelson et al. 1988). L3 larvae migrate to the base of the brain, move anteriorly into the orbit and finally penetrate the eye via the cribriform plate (Teekhasaenee et al. 1986). Intracerebral migration may also cause optic nerve injury. Worms measuring 500 μm–18.6 mm have been found in the anterior chamber, vitreous humor, and posterior pole of the eye, provoking a variety of symptoms (Scrimgeour et al. 1982; Teekhasaenee et al. 1986). Blindness may develop in rare cases.

Pathogenesis and pathology

Parasites, usually young adult worms, are found mostly in the medulla, pons, and cerebellum and in the adjacent leptomeninges (Jindrak 1975) (Figure 4). Some worms are alive at post-mortem, whereas others are disintegrating. The number of worms varies, but may reach several hundreds. Reactions are rarely seen around live worms, but dead worms are often surrounded by infiltrates of polymorphs and eosinophils, or by a zone of suppurative necrosis or granulomatous tissues. Migrating tracks (infiltrates of macrophages, foreign-body giant cells, plasma cells, eosinophils) are found near to worms and at some distance away. Nerve cells near tracks show central chromatolysis and cytoplasmic axonal swelling and blood vessels show perivascular cuffs of eosinophils, lymphocytes, and plasma cells.

The leptomeninges are infiltrated with leukocytes, infiltration being marked over the cerebellum, pons, and medulla. Worms are commonly seen within the underlying nerve tissue and the vessels, particularly veins, are dilated. The subarachnoid space is widened. Worms are found on the surface of the pia mater and foreign-body giant cells are found in the subarachnoid space near to parasites, as well as more remotely. Cases of eosinophilic radiculomyeloencephalitis show a markedly swollen brain, intensely engorged meningeal vessels, and diffuse subarachnoid hemorrhage. Numerous worms are found in sections of the spinal cord, but not the brain

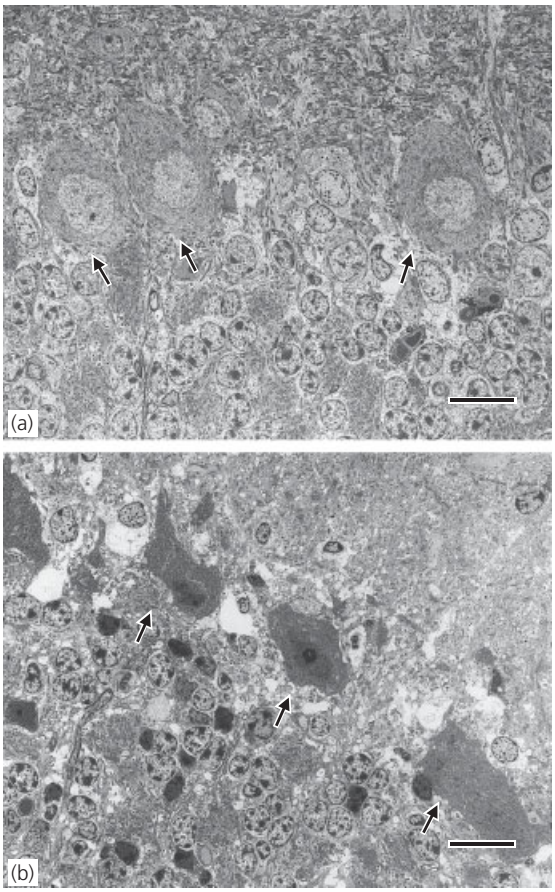

Figure 2 *Purkinje cells (arrows) of the cerebellum from a normal ddY mouse* **(a)** *and from an infected ddY mouse at 25 days after infection* **(b)**. *Purkinjé cells in the infected mouse are small and irregular shaped, with pyknotic nuclei. Bar, 10 μm*

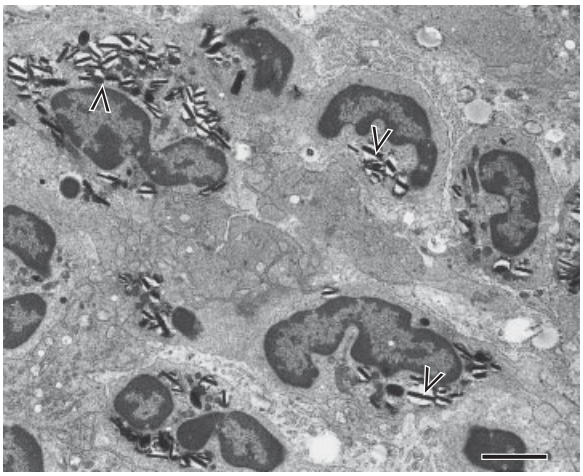

Figure 3 *Electron micrograph of eosinophils in a granulomatous reaction around degenerative* A. cantonensis *worms in a cerebellar fissure of a ddY mouse at 25 days after infection. Eosinophils show marked degranulative changes, i.e. loss of matrix material from specific granules (arrowheads). Bar, 2 μm*

(Kliks et al. 1982). The most striking findings are multiple hemorrhage tracks and cavities caused by worms migrating in the brain parenchyma and spinal cord (Witoonpanich et al. 1991).

Diagnosis

Eosinophilic pleocytosis (Table 2) and a history of eating mollusks are indicative. Spinal fluid eosinophilia develops at around day 12 after infection, peaking at days 25–30 (Punyagupta et al. 1975). Opening pressure is raised to >200 mm H_2O. Spinal fluid is usually clear or slightly cloudy, but not xanthochromic (Table 2). The white blood cell count in the fluid range from 150 to 2 000/μl, of which eosinophils exceed 10 percent in 95 percent of patients and usually represent 20–70 percent of the total white cells (Weller 1993). Protein is slightly elevated, but glucose is normal (Table 2).

Diagnosis should not depend on the identification of larvae in spinal fluid; among 257 cases in Taiwan, worms were found in only 25 patients (Chen 1979). Worm recovery is improved if patients sit upright for some time before sampling (Cross 1987).

According to Punyagupta et al. (1975), most patients had peripheral eosinophilia >10 percent and 43 percent showed eosinophilia of 21–50 percent. Blood eosinophilia did not correlate with cerebrospinal fluid eosinophilia or with the clinical course. Over half of patients (56 percent) showed leukocytosis >10 000/mm^2. Chest X-ray findings are normal (Koo et al. 1988). Recently, Graber et al. (1999) noticed the presence of enlarged ventricles and cerebral subarachnoid spaces in computed tomography findings of severe eosinophilic meningitis in infants. Meanwhile, Kanpittaya et al. (2000) suggest a diagnostic value of magnetic resonance imaging (MRI); abnormal findings on MRI of eosinophilic meningoencephalitis include dilated perivascular space, subcortical enhancing lesions, and abnormal high T2 signal lesions in the periventricular regions. Another MRI study showed multiple small high intensity areas on contrast-enhanced T1-weighted images, suggesting tissue reactions to dead or dying worms, and local vasodilatation associated with minimal thrombus formation (Ogawa et al. 1998).

Many serological tests have been used to support the diagnosis (Koo et al. 1988). With partially purified antigens, enzyme-linked immunosorbent assay (ELISA) detects significantly higher IgM and IgE levels in serum than in the spinal fluid (Yen and Chen 1991). Immunoblot analyses for detecting serum antibodies to 31 and 29 kDa female worm antigens are also useful (Akao et al. 1992). More recently, the detection of IgG4 antibody to 29 kDa antigen from young adult worms by immunoblotting was found to be most reliable for discriminating between human angiostrongyliasis cantonensis, gnathostomiasis, and cysticercosis (Intapan et al. 2003). Tests are sometimes complicated by possible cross-reactions between *A. cantonensis* and other nematodes. A

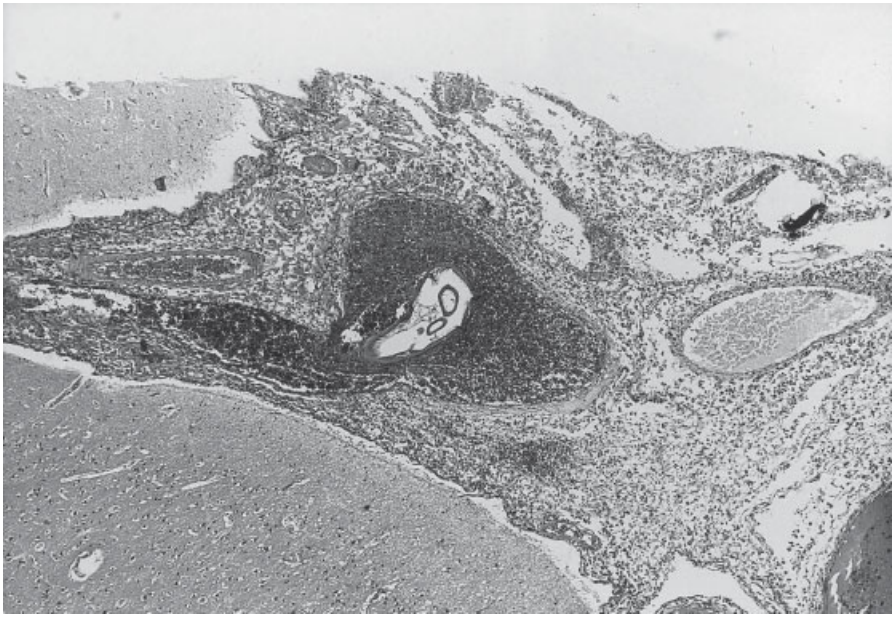

Figure 4 *Cross-section of* Angiostrongylus cantonensis *in a blood vessel of the subarachnoid space of a human patient (×47).*

purified 204-kDa (Chye et al. 2000) and 31-kDa (Eamsobhana et al. 2001) antigen with high specificity and sensitivity improved the accuracy of specific immunodiagnosis by ELISA. Shih and Chen (1991) described two monoclonal antibodies that recognize a 91 kDa antigen in excretory-secretory (E-S) products of L3 larvae, and they used an enzyme-linked fluorescent assay to detect circulating antigen in the serum and cerebrospinal fluid. A simple agglutination test using gelatin particles as an antigen carrier is also useful for detecting *A. cantonensis* antibodies in routine immunodiagnosis (Eamsobhana et al. 1999).

Cerebrospinal fluid eosinophilia often occurs in other helminth infections. In Thailand, it is especially important to distinguish between cerebral and ocular angiostrongyliasis, gnathostomiasis, and cysticercosis, all of which are endemic (Teekhasaenee et al. 1986).

Control

Thiabendazole and levamisole have been used to treat eosinophilic meningitis, but their effectiveness is still doubtful. Symptoms may worsen during treatment (Bowden 1981) and anthelminthics should be used with caution, as most of the pathogenesis is ascribed to dead or dying worms (Cross 1987). Corticosteroids may alleviate some symptoms of increased intracranial pressure and relieve allergic reactions. Lumbar puncture may

Table 2 *Differential diagnosis of cerebrospinal fluid from patients with angiostrongyliasis cantonensis or gnathostomiasis spinigera*

	Angiostrongyliasis (five cases)	Gnathostomiasis (39 cases)
Color	Clear or slightly cloudy	Slightly turbid, xanthochromic, and bloody
Leukocytes/μl	Increased 840–3750 (2 039)[a]	Increased 110–3 000 (920)
Eosinophils (%)	Very high 35–85 (71)	High 15–90 (54)
Protein (g/l)	Slightly increased 0.45–1.25 (0.68)	Increased Not bloody, *n* = 29: 0.43–1.80 (0.80) Bloody, *n* = 6: 3.45–9.00 (4.96) Xanthochromic, *n* = 4: 1.15–3.60 (2.17)
Glucose (g/l)	Normal 0.32–0.62 (0.49)	Normal 0.018–1.00 (0.51)
Opening pressure (mm)	Increased 210–360 (280)	Increased 90–350 (200)

Data from Schmutzhard et al. (1988)
a) Range (mean)

relieve severe headache. Treatment of ocular angiostrongyliasis depends on surgical removal of the parasite. A 2 percent isoptocarpin solution may prevent the worm from entering the deeper parts of the eye (Widagdo et al. 1977).

In order to prevent disease, ingestion of infected hosts and water or vegetables contaminated with L3 larvae must be avoided. Larvae are killed by freezing at −15°C for >12 h or by boiling for 2–3 min (Alicata 1967). The practice of eating toads or slugs for medical purposes (Otsuru 1979) should be prohibited. Controlling the spread of infected rats and mollusks to areas currently free of *A. cantonensis* will restrict further spread of the parasite. Trends towards increased travel, importation of food, and movement of refugees from endemic areas are all factors which suggest that awareness of this infection must be raised.

ANGIOSTRONGYLUS COSTARICENSIS MORERA AND CÉSPEDES, 1971

Introduction

A. costaricensis was discovered in the mesenteric arteries of patients in Costa Rica by Morera and Céspedes (1971), who established it as the cause of abdominal angiostrongyliasis. It has subsequently been reported from Latin America, USA and, more recently, from Africa (Baird et al. 1987). In humans, adult worms lay eggs that can embryonate, but not hatch. *A. costaricensis* therefore seems to be more adapted to humans than *A. cantonensis*, because it attains sexual maturity (Morera and Céspedes 1971).

Morphology

A. costaricensis lacks a buccal capsule and the excretory pore is located slightly posterior to the esophago-intestinal junction (Morera 1973). Adult males measure 17.4–22.2 × 0.28–0.31 mm. The club-shaped esophagus is 0.182–0.225 mm long. Spicules are of equal length, measuring 0.318–0.330 mm. The gubernaculum has two branches, the bursa is symmetrical and well developed. Behind the cloaca are three papillae (Figure 5). Adult females measure 28.2–42.0 × 0.322–0.35 mm and the esophagus is 0.23–0.26 mm long. The anus measures 0.060–0.065 mm and the vulva is situated 0.24–0.29 mm from the tip of the tail. The uterine tubules arise near the esophago-intestinal junction and spiral around the intestine.

Life cycle

Although the cotton rat, *Sigmodon hispidus*, is the most important natural final host in Costa Rica, 11 additional rodents can also carry infection. Coati-mundi, marmosets, and dogs can also serve as final hosts (Morera 1985; Brack and Schröpel 1995). In Brazil, small rodents other than cotton rats are important final hosts (Graeff-Teixeira et al. 1990). L1 larvae from the final hosts enter slugs, develop to the L3 stage, and are frequently shed into the mucus (Morera 1973). The most important intermediate host in Costa Rica, Ecuador, and Nicaragua is the slug *Vaginulus plebeius*, with infection rates reaching 85 percent (Morera 1985; Duarte et al. 1992). Other mollusks are also susceptible; for instance, in southern Brazil, *Sarasinula linguaeformis* shows a high

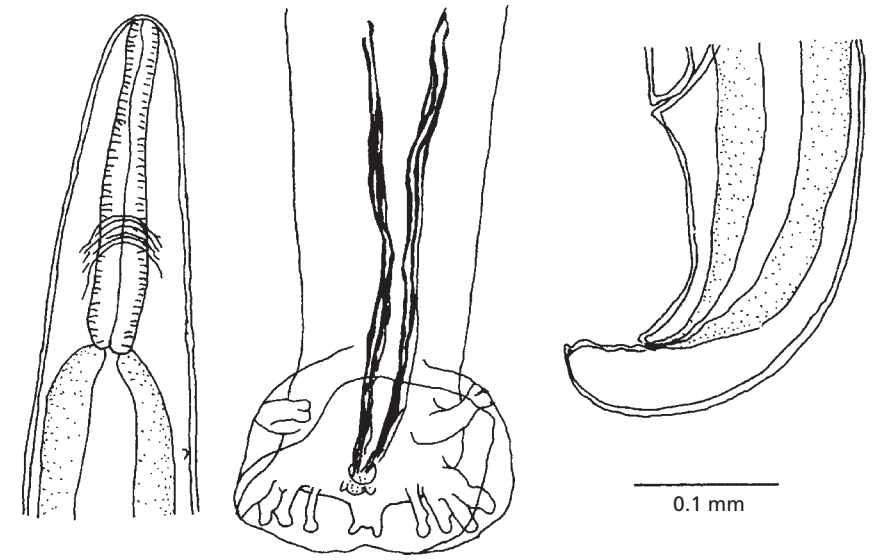

Figure 5 Angiostrongylus costaricensis. **(a)** *Anterior end of the body.* **(b)** *Posterior end of male, showing spicules and bursa.* **(c)** *Posterior end of female, showing rectum and vulva (adapted from Ohbayashi 1979)*

prevalence (86 percent) and individual parasitic burdens (Laitano et al. 2001).

When cotton rats ingest infected slugs, the larvae penetrate the intestinal wall and migrate to the lymphatics. They complete their molts within 7 days. Young adult worms (measuring 3.8–4.4 mm long) migrate to the mesenteric arteries by day 10 and reach maturity and release eggs from day 18. Eggs deposited in the intestinal wall develop and hatch, the L1 larvae appearing in feces 24 days after infection (Morera 1973).

More recently, a new migratory pathway of *A. costaricensis* has been proposed, using the mouse as a definitive host (Mota and Lenzi 1995). The pathway consists of two vascular routes: one is the lymphatic/venous/arterial pathway and the other is the venous portal pathway. The presence of larvae in the lung and liver, especially in the portal veins, of human patients can be accounted for by this new pathway. Moreover, the dog may be a potential reservoir host for *A. costaricensis*, since an experimentally infected dog shed L1 larvae into the feces (Rodriguez et al. 2002).

Transmission and epidemiology

Transmission to humans occurs by ingesting infected slugs, either intentionally or by the accidental ingestion of slugs hidden on vegetables. Larvae shed by slugs can also contaminate hands and food items. L3 larvae can enter abraded (but not unabraded) skin of the cotton rat (Ubelaker et al. 1981).

A. costaricensis is widespread in the American continent, from the USA to northern Argentina. Human infections have been reported from southern Mexico to Argentina. Incidence is particularly high in Costa Rica, numbering some 650 cases annually, or 18 cases/100 000 inhabitants (Morera 1994). Infection is twice as frequent in males as females and occurs predominantly in children under 13 years (99 percent of cases), especially in wet months (Loría-Cortés and Lobo-Sanahuja 1980). In Brazil, the incidence of infection is still increasing.

Clinical manifestations

Symptoms are localized in the abdominal regions, the parasite usually being present in the ileo-cecocolic branches of the anterior mesenteric artery (Morera 1985). Clinical manifestations appear approximately 14 days after infection. Most patients complain of abdominal pain in the right iliac fossa and right flank, and palpation and rectal examinations are painful. Fever lasting for 2–4 weeks is common. Occasionally anorexia, vomiting, diarrhea, and constipation are seen. Patients usually show palpable tumor-like masses in the lower right quadrant and these are often confused with malignant tumors. Hematologically, there is significant leukocytosis and eosinophilia (Table 3). The fatality rate ranges from 1.7 to 7.4 percent (Loría-Cortés and Lobo-Sanahuja 1980; Graeff-Teixeira et al. 1991).

Radiology usually reveals abnormalities in the terminal ileum, cecum, and ascending colon. Contrast medium demonstrates spasticity, filling defects, and irritability at the cecum and ascending colon. Fluoroscopy shows Stierlin's sign. The intestinal lumen becomes narrow. When the liver is affected, pain is localized in the upper right quadrant and hepatomegaly is noticeable. Leukocytosis and eosinophilia are usually higher when there is liver involvement than in the intestinal form of the disease, and liver enzymes may be elevated.

Table 3 *Frequency of clinical symptoms and signs in patients with abdominal angiostrongyliasis*

	No. of cases (%)	
	(116 Costa-Rican cases)	(13 Brazilian cases)
Symptom/sign	Loría-Cortés and Lobo-Sanahuja (1980)	Graeff-Teixeira et al. (1991)
Abdominal pain	98 (85)	13 (100)
Fever	93 (80)	10 (77)
Abdominal tenderness	79 (68)	–
Anorexia	71 (61)	8 (61)
Malaise	–	7 (58)
Abdominal tumor	59 (51)	4 (31)
Vomiting	52 (45)	5 (38)
Abdominal rigidity	51 (44)	–
Painful rectal examination	50 (43)	–
Weight loss	–	5 (38)
Diarrhea	40 (34)	1 (8)
Nausea	–	4 (31)
Constipation	16 (13)	5 (38)
Hepatomegaly	3 (3)	–
Testicular tumor	1 (1)	–
Jaundice	1 (1)	–

Pathogenesis and pathology

Pathogenesis is attributed either to adult worms in the mesenteric arteries or eggs in the intestinal wall (Morera 1985). Worms damage arterial endothelia, causing thrombosis and distal necrosis. In humans, eggs fail to hatch, but provoke local inflammatory reactions with numerous eosinophilic granulomas and ulceration, causing necrotic lesions and thickening of the intestinal wall. The intestinal lumen becomes narrow and restricts passage of fecal material. Additionally, gangrenous ischemic enterocolitis (Vazquez et al. 1993), eosinophilic ileitis with perforation (Wu et al. 1997), and more rarely, jejunal perforation (Waisberg et al. 1999) have been described.

Hepatic lesions are produced by larvae that invade the peritoneal cavity and migrate to reach branches of the hepatic artery. Larvae cause thromboses and the subsequent inflammation destroys the arterial walls (Vazquez et al. 1994). Ectopic infection in the liver may also occur (Morera 1985). Adult worms may block the testicular arteries and cause extensive necrosis (Ruiz and Morera 1983).

Diagnosis

Diagnosis is difficult, because L1 larvae are not discharged in stools. In endemic areas, physicians must pay special attention to appendicitis-like symptoms in children with prominent eosinophilia. Serological tests have been employed, including ELISA and latex agglutination. More recently, a parasite-specific IgG ELISA has been shown to be useful for the diagnosis of the acute phase of the disease (Geiger et al. 2001). Radiological findings of a mass in the right lower quadrant, thickening of the intestinal wall at the ileo-cecal region, and a narrowed intestinal lumen are also suggestive (Loría-Cortés and Lobo-Sanahuja 1980). Definitive diagnosis depends on the examination of biopsy specimens or surgical resections.

Control

Surgical treatments are generally required for definitive cure, but mild disease may resolve spontaneously (Graeff-Teixeira et al. 1987). The following chemotherapeutic regimens are recommended: (1) thiabendazole in combination with diethylcarbamazine; (2) thiabendazole alone; or (3) high-dose mebendazole.

Prevention is the best management. Children in endemic areas must wash their hands before eating. Inspection and thorough washing of vegetables and related foods before consumption are recommended.

In Guatemala, raw mint, frequently eaten separately or as an ingredient of ceviche, is the most likely vehicle of infection (Kramer et al. 1998). Meanwhile, infective larvae become significantly inactive when they are incubated at 5°C for 12 h in solutions of saturated sodium chloride, vinegar or 1.5 percent sodium hypochlorite (Zanini and Graeff-Teixeira 1995). When the larvae are incubated in tap water in a refrigerator at 5°C, however, it will take 80 days to inactivate all the larvae (Richinitti et al. 1999).

TERNIDENS DEMINUTUS RAILLIET AND HENRY, 1909

Introduction

Ternidens deminutus is found in southern Africa, where it infects the large intestines of primates (baboon, vervet monkey, humans), and in parts of Asia where it has been reported only in monkeys.

Classification and morphology

This genus belongs to the order Strongylida, and the superfamily Strongyloidea. Adult males measure 6–13 mm long, and adult females are 9–17 mm. There is a buccal capsule with three deep-set teeth and a mouth with a mouth-collar. The anterior end has four submedian papillae and two lateral amphids. Males have cup-shaped copulatory bursa, two spicules, and a gubernaculum. Females have protuberant vulva slightly anterior to the anus. The eggs are frequently misdiagnosed as hookworm eggs (and hence are known as 'false hookworm'), but their measurements (81.5 × 51.8 μm) are slightly larger (Goldsmid 1968). The L3 larva has paired sphincter cells at the esophageal-intestinal junction.

Life cycle

Eggs hatch into L1 larvae in 2–3 days. Subsequently, they molt to L2 larvae in 2–3 days and to L3 filariform larvae in 8–10 days (Goldsmid 1971).

Transmission and epidemiology

Infection is by oral ingestion of L3 larvae. Cases have been noted in Comoros, Mauritius, and southern Africa, from the Congo to the Republic of South Africa. A maximum prevalence of 87 percent was recorded in Rhodesia (Rogers and Goldsmid 1977). Fecocultures of Bush-negroes in Suriname revealed that two (0.2 percent) of 431 positive fecal cultures were positive for *Ternidens* sp. L3 (Jozefzoon and Oostburg 1994).

Clinical manifestations and pathology

L3 larvae penetrate the mucosa of the large intestine and molt, the L4 larvae producing nodules or ulceration. L4 larvae then molt to the adult stage. If worm loads are

heavy, anemia might result, although *T. deminutus* was not considered to be a blood sucker by von Boch (1956).

Diagnosis

T. deminutus and hookworm L3 can be differentiated morphologically, or by egg volume (Goldsmid 1968). An indirect fluorescent antibody test, using frozen sections of adult worms has been used to distinguish *T. deminutus* from hookworm infections (Rogers and Goldsmid 1977).

Control

Thiabendazole and albendazole give cure rates of >90 percent (Goldsmid 1972; Bradley 1990). Accidental ingestion of the third stage infective larvae should be avoided.

OESOPHAGOSTOMUM SPECIES

Introduction

Although principally parasitic in the large intestines of ruminants and swine, some species of *Oesophagostomum* parasitize monkeys and apes. Larvae produce nodules in the intestinal wall and cause diarrhea, anemia, and intussusception. Human esophagostomiasis occurs predominantly in Africa, with some cases reported in Indonesia, China, and South America. Five species are known in humans (Polderman et al. 1991), *O. bifurcum* being the most common in Africa and Asia (Blotkamp et al. 1993). *O. aculeatum* occurs in South East Asia.

Classification and morphology

This genus belongs to the order Strongylida and the superfamily Strongyloidea. *O. bifurcum* has a mouth surrounded by an oral collar. The cephalic end has a ventral groove and an excretory pore and the club-shaped esophagus is 0.58–0.63 mm long. Adult males measure 8.6–15.1 mm long. The copulatory bursa is trilobate, the dorsal lobe small. Spicules are 1.02 mm long, and a gubernaculum is present. Adult females are 11.0–16.8 mm long. The vulva and anus are located 0.36–0.62 mm and 0.17–0.26 mm, respectively, from the tip of the tail. Eggs measure 58–69 × 39–47 μm, and are indistinguishable from *Necator americanus*.

Life cycle

Eggs develop to the L1 stage in 1 or 2 days at 30°C. The larvae molt twice in 7 days to produce the L3 stage (712–950 μm) which has triangular intestinal cells and a very long, finely tapered tail of the sheath.

Infection occurs by oral ingestion of L3 larvae, which produce nodules in the large intestine wall. After further development, they return to the intestinal lumen, attach to the mucosa, and develop into adult worms. Eggs are discharged in the feces.

Transmission and epidemiology

Oral infection is the most likely route, but infection through the skin is also possible (Ross et al. 1989). *O. bifurcum* L3 are resistant to desiccation and freezing (−15°C for 24 h), implicating the possibility of dust-borne infections in sub-Sahelian areas like northern Togo and Ghana (Pit et al. 2000). Nevertheless, the transmission of infection appears to be limited to the rainy season.

The prevalence of *O. bifurcum* in northern Togo and Ghana has been calculated as 14.2 percent in males and 20.1 percent in females (Polderman et al. 1991). Infection was epidemic in 38/43 villages and prevalence was high (up to 59 percent) in small isolated villages. Infection was rare in children <5 years old, but high levels were found in individuals >5 years old (Krepel et al. 1992). Pit et al. (1999) estimated that more than 100 000 people in Togo were infected with *O. bifurcum*, and actually, infections were more prevalent in women than in men. Likewise, in northern Ghana and Togo, *O. bifurcum* infects an estimated 250 000 people, as determined by cultures of stool samples (Storey et al. 2001).

Clinical manifestations and pathology

L3 larvae penetrate the intestinal wall and produce a solitary, tumor-like inflammatory mass or abscess (helminthoma), measuring one to 2 cm. This is usually located in the ileo-cecal region, but can be present in other organs and in the abdominal wall. The presence of a helminthoma and abdominal pain are major symptoms of the disease. Multiple abdominal masses or nodules containing grayish-green pus and a single immature worm may be present. Intense rectal bleeding and subsequent anemia have been recognized but most cases are totally asymptomatic (Ross et al. 1989).

The clinicopathology has two distinct forms. The multinodular form comprises hundreds of small nodules within a thickened, edematous wall of the large intestine, presenting with weight loss, persistent mucus diarrhea, diffuse abdominal pain, and occasionally, rectal bleeding. Urgent surgery may be required for luminal narrowing of the colon, or bowel obstruction secondary to inflammatory adhesions. On the other hand, the uninodular form, called the Dapaong tumor (Figure 6), presents as a painful 30–60 mm granulomatous mass in the abdominal wall or within the abdominal cavity, often associated with fever (Polderman and Blotkamp 1995; Storey et al. 2000a). Ultrasonography is helpful for diagnosis of these two forms; multinodular disease shows nodular ‘target’ and ‘pseudokidney’ colonic lesions,

Figure 6 *Dapaong tumor resulting from* Oesophagostomum bifurcum *infection (courtesy of Mr P.A. Storey and* Oesophagostomum *research group of Parasitology Department, Leiden University Medical Center)*

whereas the Dapaong tumor is an echo-free ovoid lumen enveloped within a well-defined, poorly reflective wall (Storey et al. 2000a). An epidemiological study in northern Ghana indicates that 13 percent of the cases were multinodular disease, evenly distributed throughout all age-groups, while 87 percent were Dapaong tumor, especially predominant in 5–9-year-old children (Storey et al. 2000b). Oesophagostomes occasionally produce subcutaneous nodules caused by direct skin penetration of L3 larvae or by vascular dissemination of larvae from the bowel (Ross et al. 1989).

Diagnosis

Clinical diagnosis is not necessarily easy and oesophagostomiasis has frequently been misdiagnosed as carcinoma, appendicitis, amoeboma, and other etiologies. Although principally a self-limiting disease, unnecessary radical surgery has frequently been performed. Leukocytosis and blood eosinophilia are not very useful and diagnosis can often be made only by laparotomy (Barrowclough and Crome 1979).

Barium enema examination is useful for detecting a filling defect on the mucosal surface of the large intestine. Harada-Mori test-tube culture technique is used to obtain L3 larvae for morphological identification. A two-step, semi-nested polymerase chain reaction (PCR) method for the specific amplification of minute amounts (fg) of *O. bifurcum* DNA from human fecal samples has been established using genetic markers in the second internal transcribed spacer of ribosomal DNA (Verweij et al. 2000).

ELISA for worm-specific IgG4 antibody has a specificity of >95 percent (Polderman et al. 1993). Body wall and internal structures help to identify worms in histological sections.

Control

Albendazole and pyrantel pamoate give cure rates >80 percent (Krepel et al. 1993). The effect of the drugs on the tissue-dwelling stages is unknown. Accidental oral ingestion of L3 larvae should be avoided.

TRICHOSTRONGYLUS SPECIES

Introduction

At least ten trichostrongylid species are capable of parasitizing humans. Most species are normally found in herbivores, human infections being accidental, but most *T. orientalis* infections have been recorded in humans. Human infections are prevalent in Iran.

Classification and morphology

This genus belongs to the order Strongylida, superfamily Trichostrongyloidea. The worms are minute and delicate, measuring approximately 4–8 mm long. The head is unarmed and lacks a distinct buccal capsule. The male bursa has long lateral dorsal rays that are poorly developed. Spicules are yellowish-brown, stout, and their chrysanthemum-flower-leaf shape is useful for species differentiation. A gubernaculum is present. The female vulva is situated midway along the body. Eggs resemble hookworm ova, but are longer and narrower (measuring 75–91 × 39–47 μm), with one end more pointed than the other. They are discharged at the 16–32 cell stage (Figure 7).

Life cycle

Eggs develop in 1–2 days and then hatch. L1 larvae molt to L2 larvae in 2–3 days and to the ensheathed filariform L3 larvae in 7–8 days. L3 larvae are resistant to desiccation. When ingested by humans with vegetables or drinking water the larvae develop in the intestinal mucosa, molting twice and developing into adult worms in approximately 4 weeks. Percutaneous infection may occur.

Transmission and epidemiology

Trichostrongylus infections have been reported from every continent. Infective larvae are relatively resistant to environmental pressures, and infections are thus prevalent even in relatively cold areas. In Iran, nine species have been recorded from humans; in central Iran, the incidence of *T. orientalis* and *T. colubriformis* has been recorded as 67 percent (Ghadirian and Arfaa 1975). In this region of the world, it is common for

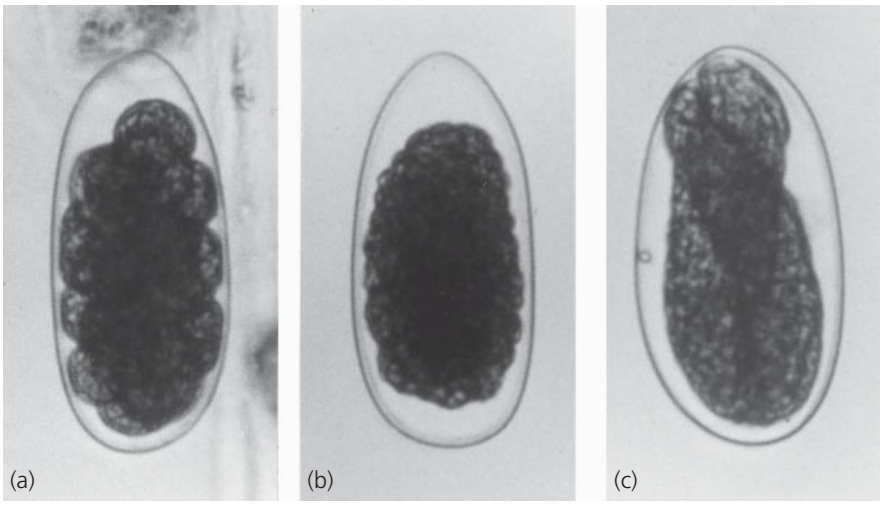

Figure 7 Trichostrongylus orientalis *eggs* **(a)** *as seen in fresh feces;* **(b)** *at the morula stage of embryonation;* **(c)** *with developed rhabdtiform larva (×600)*

women to mold animal dung into solid masses, to be dried and burnt as fuel. This practice may be important in the transmission of animal *Trichostrongylus* to humans. Human infections with *T. orientalis* are principally acquired by eating vegetables contaminated with night soil. In Japan, *T. orientalis* infection was previously common in cold areas, the prevalence in Aomori Prefecture in 1975 being approximately 19 percent, but the current figure is estimated to be very low (unpublished data).

Clinical manifestations and pathology

Trichostrongylids penetrate into the intestinal mucosa, producing redness, erosion, and local bleeding. Patients present with epigastric pain, diarrhea, anorexia, nausea, dizziness, and generalized fatigue or malaise (Otsuru and Ito 1972). If worm loads are light patients may be asymptomatic, but heavy worm loads are associated with the development of anemia, and the entry of worms into the biliary tract, provoking cholecystitis.

Diagnosis

Stool examinations are used to detect ova. Harada-Mori cultures are used to obtain L3 larvae for morphological differentiation. Several methods for detecting ova or larvae should be used, because females discharge only a small number of eggs daily.

Control

Pyrantel pamoate, bephenium hydroxynaphthoate, and 1-bromo-naphthol are effective. Ingestion of fresh vegetables or pickles contaminated with L3 larvae should be avoided. Caution must be used when handling animal dung to prevent infection with animal trichostrongylids.

ANISAKIS AND RELATED SPECIES

Introduction

Anisakids live in the stomach of marine mammals. *Anisakis simplex* was first discovered as a cause of larva migrans by van Thiel et al. (1960) who noted a nematode larva in the intestinal wall of a patient suffering from acute abdomimal pain after eating raw herrings. The larva was identified as *Eustoma rotundatum*, but the disease was later named 'anisakiasis' (van Thiel 1962). Prior to this discovery, cross-sections of a nematode-like parasite had frequently been recognized in Japanese patients suffering from acute terminal ileal enteritis, with prominent eosinophilic infiltration and severe allergic tissue reactions. These specimens were initially identified as *Ascaris* larvae. Later, *Anisakis* larvae were removed under gastric endoscopy (Namiki et al. 1970) and since then, many cases of gastric anisakiasis have been reported in Japan, linked to the habit of eating raw fish (sashimi or sushi). Anisakiasis has most frequently been found in Japan, followed by the Netherlands (where infections are acquired from ingesting raw herrings). The increasing popularity of 'sushi bars' has contributed to the spread of infections in the USA and Europe. Anisakid larvae parasitic to humans known to date are *Anisakis simplex*, *A. physeteris*, *Pseudoterranova decipiens*, *Contracaecum osculatum*, *Hysterothylacium aduncum*, and *Porrocaecum reticulatum*; *A. simplex*, and *P. decipiens* being the most important.

Classification and morphology

The genus *Anisakis* belongs to the order Ascaridida, family Heterocheilidae and subfamily Anisakinae. *P. decipiens* was initially called *Ascaris decipiens*, was

subsequently transferred to the genus *Porrocaecum*, *Terranova* or *Phocanema* and finally to the genus *Pseudoterranova* (Gibson 1983).

A. simplex L3 were initially called *Anisakis* type-I larva. The larvae have a dorsal lip and two subventral lips orally, with a boring tooth on the dorsal lip. The excretory pore opens at the base of the subventral lips. The esophagus is composed of two parts; the preventriculus and a relatively long ventriculus, with an oblique ventriculo-intestinal junction. The tail is short and rounded, with a mucron (Figure 8). Larvae measure 28.40 × 0.45 mm on average. *A. physeteris* was initially called *Anisakis* type-II larva. These measure 27.80 × 0.61 mm on average, have a short ventriculus, a horizontal ventriculo-intestinal junction, and a long tapering tail without a mucron (Figure 8).

L3 larvae of *P. decipiens* measure 32.6 × 0.8 mm on average and are yellowish-brown. The ventriculus partly overlaps the intestinal cecum posterior and there is a mucron at the tail end (Figure 8). In Japan, *P. decipiens* was previously called *Terranova* type A larva.

Anisakis species have a unique morphology in cross-sections; clear Y-shaped lateral cords (Figure 12), cuticular lateral alae absent, 60–90 muscle cells/quadrant, and 60–83 columnar intestinal cells. *P. decipiens* larvae have >100 intestinal cells and butterfly-like lateral cords (Oshima 1972).

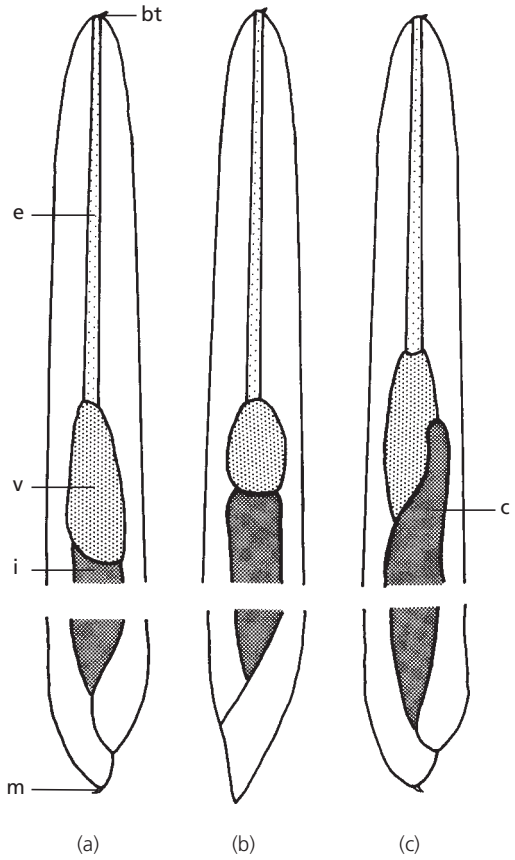

Figure 8 *Diagram of the anterior and posterior end of* **(a)** Anisakis simplex*;* ***(b)*** A. physeteris*; and* **(c)** Pseudoterranova decipiens. *bt, boring tooth; c, cecum; e, esophagus; i, intestine; m, mucron; v, ventriculus (adapted from Koyama et al. 1969)*

Life cycle

Figure 10 summarizes the life cycles of *A. simplex* and *P. decipiens*. Adult worms live in the stomachs of marine mammals. Eggs (40 × 50 μm) are discharged with the feces, then the larvae molt once into the L2 stage, which hatch when ingested by euphausiid krill and develop into the L3 stage. When infected krill are eaten by squid or marine teleosts, e.g. salmon, cod, herring, and mackerel, the larvae encyst in the viscera or muscles (Figure 9), but do not develop further. When these paratenic hosts are eaten by marine mammals, the L3 larvae molt twice in the stomach and develop into adult worms. In Japan, *A. simplex* larvae have been found in 164 species of fish and one squid species, and adult worms have been found in 26 cetacean species and 12 species of pinnipeds. Marine mammals may be infected by ingesting crustacean intermediate hosts. Humans are not suitable hosts; L3 or L4 larvae are found, but never adult worms.

Isopods and mysids serve as intermediate hosts for *P. decipiens*. Cod, haddock, halibut, greenling, pollack, and other fish serve as paratenic hosts (Figure 10). L3 larvae have been found in nine species of fish in Japanese waters and 11 species in Californian waters (Ishikura et al. 1992). Seals, sea lions, and walruses act as definitive hosts (Figure 10). Adult worms have rarely been recovered from humans.

Transmission and epidemiology

Anisakiasis is linked to the ingestion of raw or undercooked marine fish or squid. Many fish are involved in *A. simplex* transmission in Japan (Figure 9) (Oshima and Kliks 1987; Kino et al. 1993). Pacific cod and halibut are important for transmission of *P. decipiens* in Japan. Other important sources of infection include rockfish, salmon, and red snapper in the USA (Kliks 1983; Oshima 1987) and herring in Europe (van Thiel 1976).

Human anisakiasis has been reported from many countries in Asia, Europe, North and South America, and New Zealand. In Japan, a total of 20 582 cases were reported between 1980 and 1993 (Ishikura et al. 1993), but only 660 cases were reported from other countries by the end of 1992. In Japan, 93 percent of cases involved the stomach and only 4.4 percent were intestinal. In other countries intestinal anisakiasis accounts for a much larger percentage of cases. The annual incidence in Japan is >2 000 cases. Changes in dietary habits may account for the escalation of anisakiasis in the USA, where >50 cases have been reported.

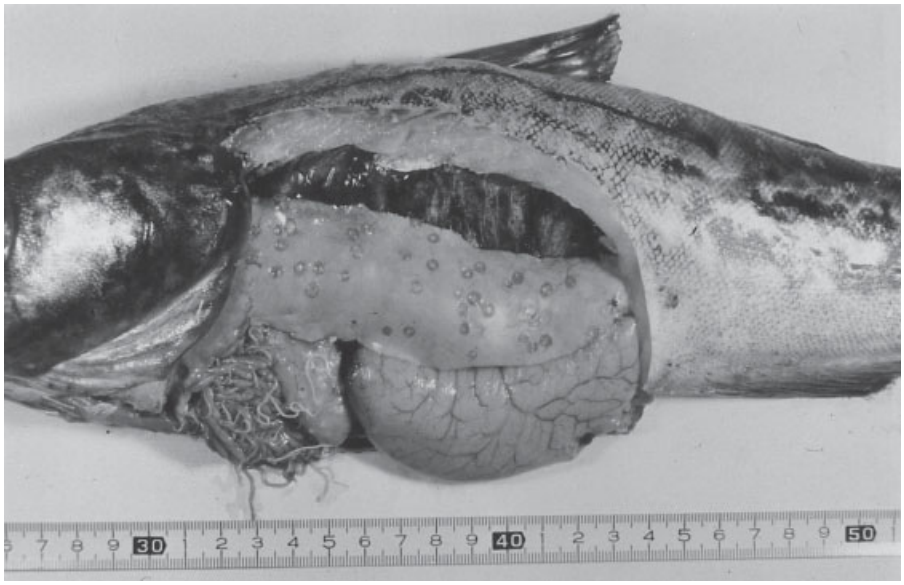

Figure 9 Anisakis simplex *third-stage larvae encapsulated in the liver of the pollack,* Theragra chalcogramma *(courtesy of Dr K. Ishida)*

Clinical manifestations

Clinical manifestations can be severe if a secondary infection induces a strong immediate hypersensitivity reaction around the site of worm penetration. Mild clinical symptoms result from local foreign body formation during a primary infection. The disease is also classified into gastric, intestinal, and extra-gastrointestinal (ectopic) anisakiasis. The last form is caused by larvae that penetrate into the abdominal or pleural cavity, entering abdominal or pleural organs or tissues (e.g. omentum, mesentery, peritoneum, liver, pancreas, ovary,

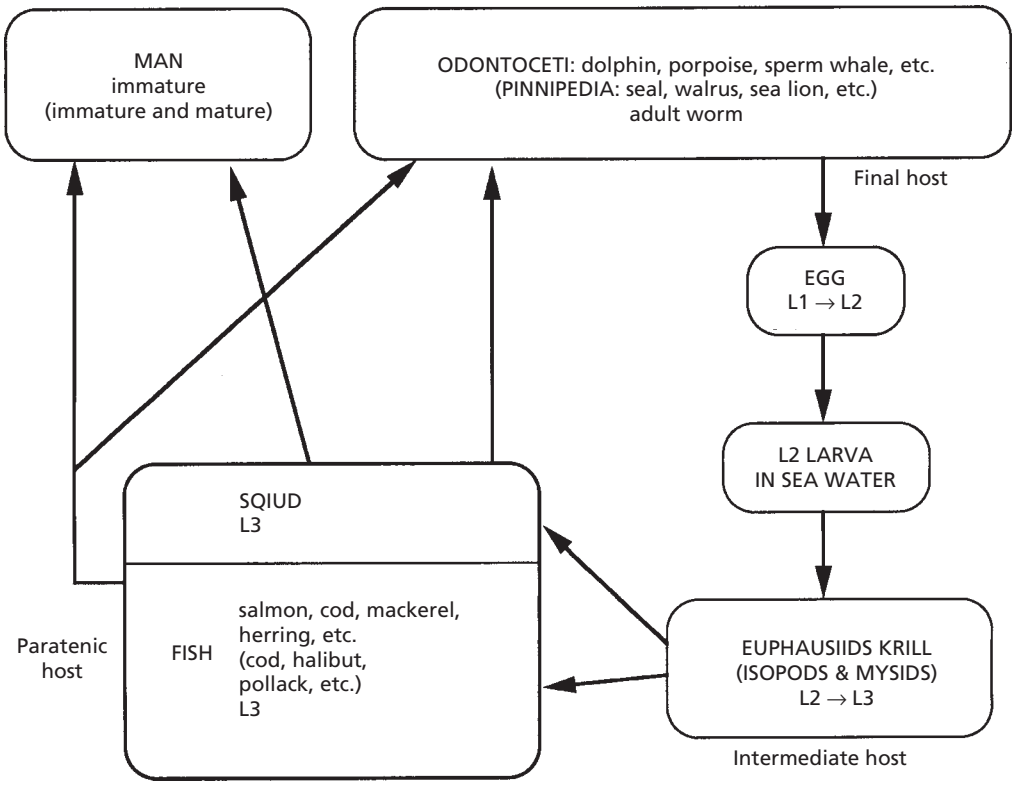

Figure 10 *Life-cycle of* Anisakis simplex *and* Pseudoterranova decipiens. *Names in brackets refer to host of* P. decipiens. *L1, L2, and L3: the first-, second- and third-stage larvae (adapted from Oshima 1987).*

oviduct, utero-cervix, lymph node, lung, and even in the subcutaneous tissue), and provoking inflammatory foci.

Acute gastric anisakiasis occurs approximately 6 h after the ingestion of raw seafood (Deardorff et al. 1991). Larvae normally parasitize the fundus of the stomach (rarely tongue, tonsil, uvula, and esophagus), especially the greater curvature (Figure 11). Symptoms are summarized in Table 4. Rarely, gastric anisakiasis may cause severe chest pain or angina-like pain (Sugano et al. 1993) that is often misdiagnosed as gastric ulcer, cancer, or polyp.

Acute gastric anisakiasis is rare in persons over 60 years of age and in patients who have undergone gastric surgery and this is a result of the low gastric acidity in these people (Muraoka et al. 1996).

Intestinal anisakiasis usually develops within 2 days after infection and occurs in any region between the duodenum and the rectum, most frequently in the ileal region (Matsui et al. 1985). Symptoms (Table 4) last for 1–5 days and then lead to ileus. Acute intestinal anisakiasis may cause ascites. The disease is frequently misdiagnosed. Patients usually have no pyrexia, but develop moderate leukocytosis and, in some cases, eosinophilia (4–41 percent) (Ohtaki and Ohtaki 1989; Ishikura 1990; Deardorff et al. 1991).

P. decipiens larvae are less invasive than *A. simplex*, are frequently expelled by vomiting, and rarely cause intestinal anisakiasis (Oshima 1987). Instead, they often provoke a tingling throat syndrome, associated with larval migration from the stomach to the mouth. In contrast, *A. simplex* larvae readily penetrate the gastrointestinal wall and invade the abdominal cavity, provoking peritonitis and intruding into various organs and tissues. Ectopic anisakiasis is usually mild and most cases are discovered accidentally during surgery (Ishikura et al. 1992).

Single worm infection is common, but multiple infections occur, for example approximately 50 *A. simplex* larvae were recovered from a Japanese patient (Takamuku et al. 1994).

A. simplex present in parasitized fish sometimes cause urticaria/angioedema, anaphylaxis, atopic dermatitis, arthralgias/arthritis associated with urticaria, and occupational asthma or conjunctivitis in persons handling fish (fishermen/fishmongers) or fish flour (Kasuya et al. 1990; de Corres et al. 1996; Anibarro and Seoane 1998; Armentia et al. 1998; Cuende et al. 1998). Patients presenting with dermatitis after handling fish should be suspected of having contracted protein-contact dermatitis caused by *A. simplex* (Anibarro et al. 1997). Patients with these allergic diseases show positive on skin prick test to *A. simplex* antigen, are positive for histamine release, and have both IgE antibody specific to the worm and a higher total IgE level (del Pozo et al. 1996; Cuende et al. 1998). *Anisakis* allergen is considered to be thermostable and thus, the ingestion of safely cooked but parasitized seafood causes an allergy. The high frequency of *Anisakis*-allergy and the severity of some cases are more important than gastrointestinal anisakiasis, and are becoming a public health problem especially in Spain. Asymptomatic gastro-intestinal anisakiasis sometimes accompanies allergic or anaphylactic reactions (Garcia-Labairu et al. 1999).

Hypersensitivity to *A. simplex* is associated with HLA class II DRB1*1502-DQB1*0601 haplotype, suggesting this haplotype is a susceptibility factor for hypersensitivity to *A. simplex* antigens (Sanches-Velasco et al. 2000).

Table 4 *Clinical symptoms of patients with gastric or intestinal anisakiasis*

Symptom/sign	No. of cases (%)	
	Gastric (363 cases)	Intestinal (124 cases)
Epigastric pain	228 (62.8)	16 (12.9)
Generalized abdominal pain	7 (1.9)	12 (9.7)
Lower abdominal pain	14 (3.9)	56 (45.2)
Epigastric distention	19 (5.2)	1 (0.8)
Abdominal bloating	3 (0.8)	13 (10.5)
Nausea	63 (17.4)	26 (21.0)
Vomiting	34 (9.4)	45 (36.3)
Heart burn	9 (2.5)	–
Anorexia	8 (2.2)	–
Tumor	8 (2.2)	8 (6.5)
General fatigue or malaise	7 (1.9)	1 (0.8)
Blood vomitus	7 (1.9)	–
Diarrhea	5 (1.4)	14 (11.3)
Other peritoneal irritation symptoms	1 (0.3)	18 (14.5)
Urticaria	4 (1.1)	–
Asymptomatic	21 (5.8)	6 (4.8)

Data from Totsuka (1974)

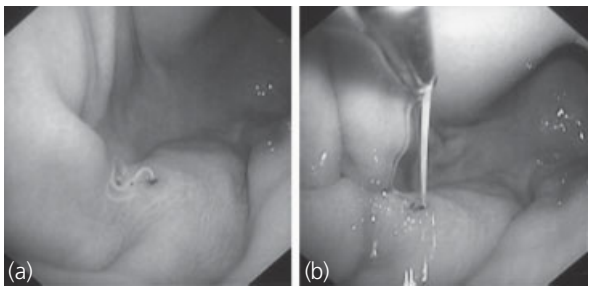

Figure 11 Anisakis *larvae just penetrating into the stomach mucosa* **(a)**. *The larva being removed with biopsy forceps of the endoscope* **(b)**. *(Courtesy of Dr I. Sato)*

Pathology

Initially, lesions are infiltrated mainly by neutrophils, with a few eosinophils and foreign body giant cells.

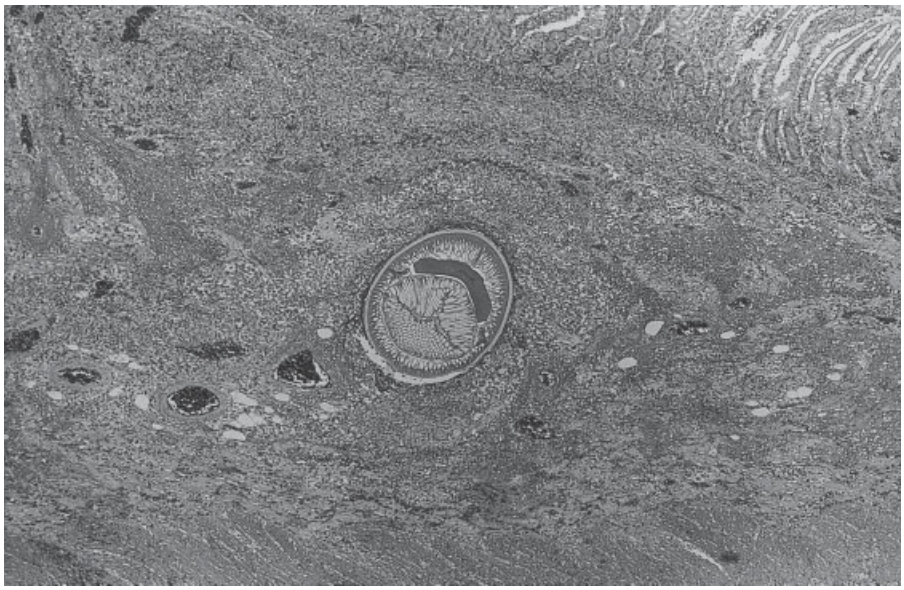

Figure 12 *Cross-section of an* Anisakis *larva invading the submucosa of the ileum. Note the characteristic 'Y-shaped' lateral cords and the rennette cell, and intense cellular infiltration around the larva (×47).*

There is little edema, fibrinous exudation, hemorrhage, or vascular damage. Within the first week there may be edematous thickening of the submucosa and infiltration by numerous eosinophils and other cells (Figure 12).
In chronic gastric and intestinal anisakiasis, necrotic and hemorrhagic abscesses with eosinophilic infiltration may be present. Eosinophilic infiltration becomes less extensive after 6 months and lymphocyte infiltration predominates. Degenerative worms may be surrounded by foreign body giant cells. In more advanced stages (6 months to 1 or more years) the abscess or granulomatous inflammation may be replaced by granulation tissue with some eosinophil infiltration. Fragments of the degenerated worm are usually, but not always, present.

Diagnosis

Clinical manifestations are not specific and therefore diagnosis is difficult, especially with intestinal or extra-gastrointestinal anisakiasis. When patients complain of abdominal pain, nausea, and vomiting, their dietary history immediately prior to the onset of the symptoms should be determined. If raw seafood has been eaten, diagnostic measures must be performed.

ENDOSCOPY

Endoscopy is useful for gastric anisakiasis and sometimes for duodenal anisakiasis (Kliks 1986). The site of larval penetration is edematous, with various degrees of hyperemia, erosion, and bleeding. The penetrating *Anisakis* larva can be easily removed endoscopically with biopsy forceps (Figure 11).

RADIOLOGY

Radiology is effective for diagnosing intestinal anisakiasis. Double-contrast, barium-filled roentgenographic images reveal coiled or thread-like filling defects. Edematous, thickened, narrowed, and obstructed areas in the gastrointestinal walls are present (Sugimachi et al. 1985; Matsui et al. 1985). *Anisakis* infection may also cause a vanishing tumor of the stomach.

Sonographic examination is useful for the diagnosis of intestinal anisakiasis when the combined findings of ascitic fluid, dilation of the small intestine, and localized edema of Kerckring's fold is observed in the patients with acute abdomen after recent ingestion of seafood (Ido et al. 1998).

IMMUNODIAGNOSIS

Although several serological tests are used, their application has limitations because of cross-reactions with other nematodes and because low levels of specific antibody may be present in acute anisakiasis. Nevertheless, immunodiagnosis is essential for chronic infections, intestinal, and extra-gastrointestinal anisakiasis. Takahashi et al. (1986) established two monoclonal antibodies, one of which (An2) recognized a specific epitope in the intestine, muscle cells, and E-S products of the larvae. A micro-ELISA assay using this antibody has been developed (Yagihashi et al. 1990). Antilarval *A. simplex*-specific IgG, IgA, and IgM antibodies are detectable 4–5 weeks after the onset of disease; specific IgE antibody can be detected as early as 1–7 days. An immunoblot assay, using E-S antigens of *A. simplex* larvae and detecting IgA or IgE antibodies specific for E-S antigens of the larvae, is also useful (Akao et al. 1990).

IgE immunoblotting with *A. simplex* antigen, positive skin prick test to *A. simplex* but not to fish, and antigen-capture ELISA using O-deglycosylated antigen bound by the monoclonal antibody UA3 are useful approaches to *Anisakis*-allergy diagnosis (del Pozo et al. 1996; Garcia et al. 1997; Lorenzo et al. 2000). More recently, determination of specific IgE directed to *A. simplex* major allergen (24 kDa and named Ani s 1) by immunoblotting has been shown to be a useful tool for the diagnosis of hypersensitivity and intestinal anisakiasis, whereas measurement of specific IgG to Ani s 1 is only valid for *A. simplex* allergy (Caballero and Moneo 2002).

Control

Gastric endoscopy is effective for locating and removing penetrating larvae. If diagnosis is confirmed and there is no ileus, surgical treatment is not necessary. Instead, supportive treatment is recommended until the larvae die and are absorbed. In experimental animals this occurs within 3 weeks (Jones et al. 1990).

Thorough cooking and adequate freezing of seafood are easy and practical preventive measures. In the Netherlands, legislation (The Green Herring Law) requires fresh herring to be frozen to at least −20°C for 24 h before release to the public. Fish should be cooked so that the internal temperature reaches 60°C or higher for 10 min. Seafood should be frozen at −20°C for 3–5 days (Sakanari and McKerrow 1989; Deardorff et al. 1991). At room temperature, *Anisakis* larvae can survive for many days in vinegar, and for 1 day in soy sauce or Worcester sauce. The larvae may be harmed, but not killed, by gastric fluid. Microwave processing of seafood, for example, arrowtooth flounder fillet, to kill *A. simplex* larvae is effective only when the internal temperature of the fillet reaches higher than 77°C (Adams et al. 1999).

GNATHOSTOMA SPECIES

Owen (1836) collected worms from a stomach wall tumor of a tiger in the London Zoological Gardens and named them *G. spinigerum*. The genus now contains 12 distinct species, four of which (*G. spinigerum*, *G. hispidum*, *G. doloresi*, and *G.nipponicum*) are zoonotic in South East Asia and the Far East.

The number of patients in Mexico has been increasing since the first recorded case in 1970, and the estimated number of patients was more than 1 380 even between 1980 and 1996 (Ogata et al. 1998). Although the causative agent of the human infections in Mexico and Ecuador was identified as *Gnathostoma binucleatum* (Almeyda-Artigas 1991), the validity of *G. binucleatum* and its involvement in human gnathostomiasis was questioned (Akahane et al. 1994; Diaz-Camacho et al. 1998; Ogata et al. 1998). A recent comparative study on ITS-2 rDNA sequences of Mexican *Gnathostoma* spp. indicates that *G. binucleatum* is a valid species and incriminated as an etiologic agent of human gnathostomiasis in Mexico and Ecuador (Almeyda-Artigas et al. 2000). In Sinaloa, Mexico, *Gnathostoma* advanced third-stage larvae have been recovered from ichthyophagous birds, e.g. *Egretta alba*, and several fishes, e.g. blue sea catfish (*Arius guatemalensis*), Pacific fat sleeper (*Dormitator latifrons*), fat sleeper (*Gobiomorus* sp.), Nile tilapia (*Oreochromis* sp.), Sinaloan cichlid or green guapote (*Cichlasoma beani*), and spotted sleeper (*Eleotris picta*) (Diaz-Camacho et al. 2002). Moreover, the first human infection of *Gnathostoma malaysiae* was recently found in two Japanese travelers returning from Myanmar (Nomura et al. 2000). These patients ate raw freshwater shrimp in Myanmar and then showed a creeping eruption and Quincke's edema.

G. spinigerum is the most important species in Asia, causing cutaneous, cerebrospinal, and ocular lesions. *G. hispidum* was first found to cause human cutaneous gnathostomiasis in Japan (Tsushima et al. 1980), and many cases have subsequently been reported. *G. doloresi*, and *G. nipponicum* have also been shown to provoke creeping eruption in humans in Japan (Ogata et al. 1988; Ando et al. 1988). *G. hispidum*, *G. doloresi*, and *G. nipponicum* usually provoke creeping eruption and rarely affect deeper tissues. They differ from *G. spinigerum* in not causing long-term (>10 years) recurrent cutaneous lesions and they have not been recognized outside Japan.

GNATHOSTOMA SPINIGERUM OWEN, 1836

Introduction

Gnathostoma spinigerum was first discovered in 1889 in Thailand and named *Cheiracanthus siamensis* n. sp., later identified as *G. spinigerum*. Subsequently, many human cases have been reported from various South East Asian countries, Japan, and China. In Thailand, eosinophilic-encephalitis, myelitis, radiculitis, and subarachnoid hemorrhage caused by *G. spinigerum* are often confused with eosinophilic meningoencephalitis caused by *Angiostrongylus cantonensis*; differential diagnosis is therefore important in this country.

Classification and morphology

This genus belongs to the order Spirurida, superfamily Gnathostomatoidea. The adult males and females are 12–30 and 15–33 mm long, respectively. The head-bulb bears between eight and 11 rows of cuticular hooklets. The anterior body has toothed spines. The posterior body is naked apart from the presence of minute terminal spines. Males have four pairs of large papillae in the

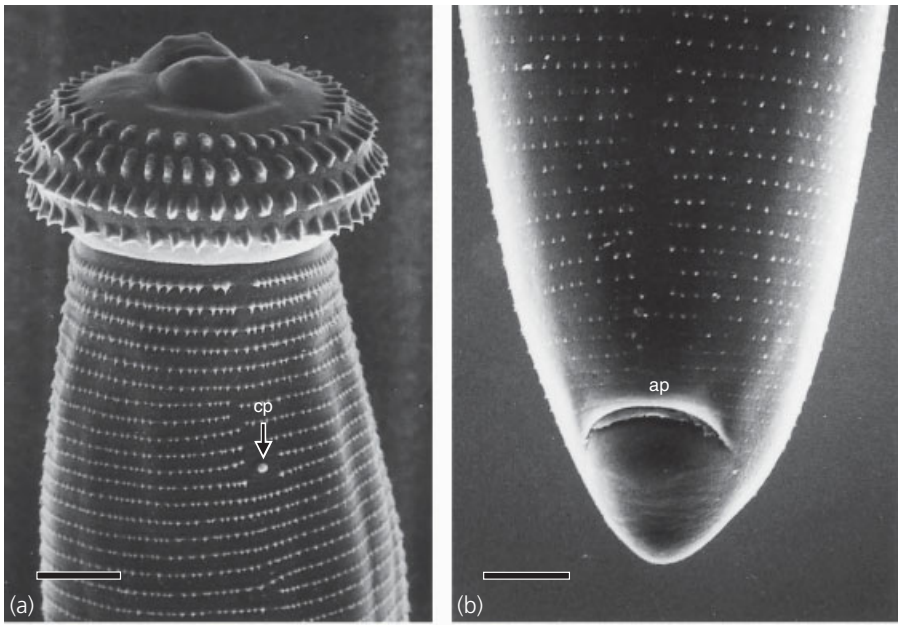

Figure 13 *Advanced third-stage larva of* Gnathostoma spinigerum. **(a)** *Anterior end. cp, cervical papilla. Bar, 30 μm;* **(b)** *ventral surface of the terminal end. ap, Anal pore. Bar, 20 μm (courtesy of Dr M. Koga)*

caudal alae and four pairs of small ventral papillae around the cloaca. Spicules are unequal in length, one being between three and four times longer than the other. The vulva is situated just behind the middle of the body. Eggs (measuring 69.3 × 38.5 μm on average) are oval, brownish, and unsegmented with a transparent knob-like thickening at one pole.

Advanced L3 larvae are 3–4 mm long (Figure 13 and Table 5). The body is entirely covered with cuticular spines. The morphology of the intestinal epithelial cells of the larvae is shown in Table 6.

Life cycle

G. spinigerum adult worms occur naturally in the cat, dog, and many other carnivores. In northern Thailand, 4.1 percent of 2 940 dogs examined had adult worms in stomach nodules (Maleewong et al. 1992). Eggs laid in these nodules pass into the gastric lumen through a small aperture and are discharged into the feces. L1 larvae develop in the eggs and molt to the L2 stage (Figure 14) after which the eggs hatch. L2 larvae are ingested by an aquatic crustacean (*Cyclops*), after which they develop in the hemocoele and molt into the early L3 stage in 6–10 days.

When early L3 larvae are ingested by freshwater fish, they penetrate into the gut, reaching the muscle and developing into the advanced L3 larvae, measuring 2.8–5.2 × 0.3–0.8 mm. These lie coiled and encysted by connective tissues (Daengsvang 1982). If hosts with L3 larvae are eaten by paratenic hosts (reptiles, birds, or mammals), the larvae encyst again. Advanced L3 larvae have been found in 44 species of vertebrates in Thailand and in 36 species of animals in Japan. Rusnak and Lucey (1993) have listed the many species that can serve as natural and experimental hosts.

When advanced L3 larvae are ingested by a final host they excyst in the stomach, then penetrate the gastric wall to the peritoneal cavity to reach the liver. Subsequently, they migrate through the host and 4 weeks later, they return to the stomach, penetrating the gastric wall from the outside to form a tumor that connects with the stomach lumen through a small aperture. The adult worm develops within 4.5–6 months after infection. Although the adult stage can develop in humans, the worms cannot return to the stomach (Miyazaki 1991).

Transmission and epidemiology

Infections are normally acquired by ingestion of second intermediate or paratenic hosts or, possibly, by drinking water containing infected *Cyclops* or free L3 larvae. Although the most important source of infections is snake-headed fish in both Thailand and Japan, many other species of fish, amphibia, reptiles, birds, and mammals are possible sources. People from Thailand often become infected by eating raw or fermented fish flesh, or raw or undercooked chicken flesh. Freshwater fish and chickens are major sources of human infections in Japan. The incidence of infection in snake-headed fish varies, but overall ranges from 44 to 99 percent (Daengsvang 1982). In Thailand, the incidence in freshwater eel has been reported to reach 80–100 percent, thus both the eel and snake-headed fish are epidemiologically important. Three possible human cases of prenatal infections have been documented (Rusnak and Lucey 1993).

Table 5 *Differential diagnosis of advanced third-stage larvae of four species of* Gnathostoma

Species	No. of hooklets on head-bulb				Remarks	Reference
	1st row	2nd row	3rd row	4th row		
G. spinigerum	43.2	44.8	46.7	52.3	The number of hooklets in each row is more than 40, increasing posteriorly	Miyazaki (1960)
G. hispidum	38.3	40.5	41.8	46.0	The number of hooklets gradually increases posteriorly. The first row of hooklets are smaller than the others	Akahane et al. (1982)
G. doloresi	38.3	37.9	35.6	35.7	The number of hooklets in each row is fewer than 40, decreasing posteriorly. The hooklets in the first row are especially small	Mako and Akahane (1985)
G. nipponicum	37.0	37.1	41.0	–	Lack the fourth row of hooklets	Ando et al. (1988)

Table 6 *Differential diagnosis in cross-section of the abdominal region of advanced third-stage larvae of four species of* Gnathostoma

Species	No. of muscle cells/ quadrant	Number	Intestinal cell shape	No. of nuclei
G. spinigerum	10–15	21–29	Columnar	0–7 (mostly 3–7)
G. hispidum	11–15	19–31	Spherical	0–2 (mostly 1)
G. doloresi	11–15	18–28	Spherical	0–3 (mostly 2)
G. nipponicum	10–14	10–14	Columnar	0–4 (1 in 50% of cells)

After Ando et al. (1991).

Advanced L3 larvae can penetrate the intact skin of mice, rats, and cats, with complete penetration occurring within 30 min. Human infections may also be acquired by this route (Rusnak and Lucey 1993).

Human gnathostomiasis has been recorded from 19 countries, predominantly in Asia, especially Thailand and Japan. In Thailand, infections mainly occur in the rainy season (Punyagupta et al. 1990) and are most prevalent in females, aged 20–25 years, whereas in Japan, they are most prevalent in males in their twenties (Daengsvang 1982). The youngest recorded case in Japan was an infant less than 2 years old.

Clinical manifestations and pathology

Gnathostomiasis spinigera has cutaneous and visceral forms and the latter is divided into pulmonary, gastrointestinal, urogenital, ocular, otorhinolaryngeal, and cerebral forms (Rusnak and Lucey 1993). Patients develop various prodromes, e.g. general malaise, anorexia, urticaria, vomiting, epigastric pain, diarrhea, and fever in about the first 5 days after infection, i.e. when L3 larvae excyst, penetrate into the peritoneal cavity, and migrate through the liver. Right upper quadrant pain may be experienced. The larvae then migrate to various parts of the body, most frequently to muscle and subcutaneous tissues. A specific cutaneous symptom, i.e. migrating intermittent swelling, usually develops within 1 month after infection, but may sometimes occur 1 or 2 years later. During this process, tissues or organs are mechanically injured by the migrating worms and probably also by the toxins they release (Miyazaki 1960). The lesions thus become enlarged, with allergic inflammation around infected foci. Worms in human tissues are not encysted.

In cutaneous gnathostomiasis, migrating swelling frequently appears first in the abdominal area, after which the lesion migrates at random. Worms move at about 1 cm/h in human skin. Swellings are rather hard, variable in size, and mostly erythematous. Skin lesions are normally painless apart from light or moderate itch. Swellings last 1–2 weeks and then disappear, another swelling appearing elsewhere. Migrating swellings may recur intermittently for up to 10–12 years. Worms migrating near the body surface may cause creeping eruption, cutaneous abscesses, or cutaneous nodules, from which the worms can often be resected. Inflammatory foci with profound eosinophilic infiltration are commonly seen in the skin lesions.

Migrating worms in deeper tissues produce visceral gnathostomiasis and serious symptoms may develop, depending on the organs or tissues affected (Rusnak and Lucey 1993). An acute or chronic inflammatory reaction occurs around invading worms and local hemorrhage, necrosis, edema, fibrosis, and tumor formation may occur. When the lungs are affected,

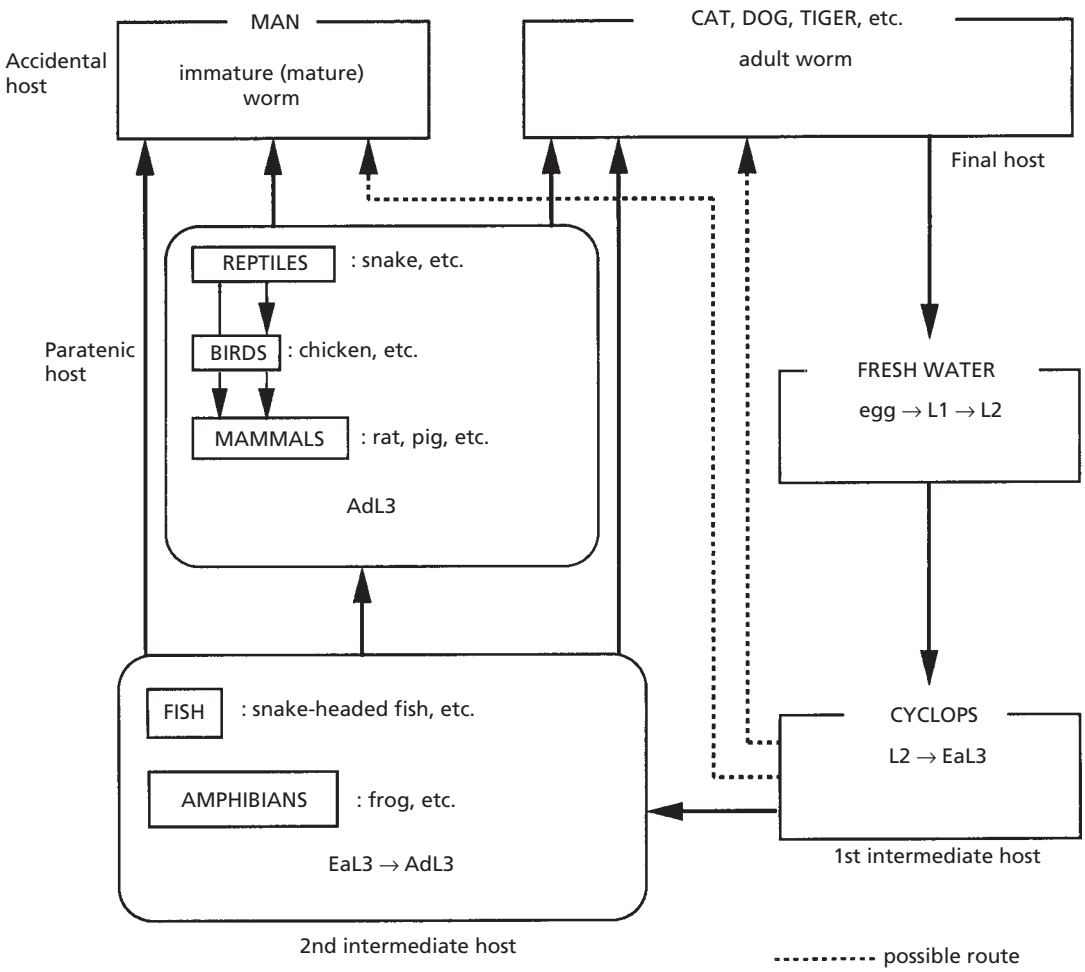

Figure 14 *Life-cycle of* Gnathostoma spinigerum. *L1 and L2, the first- and second-stage larvae; EaL3, early third-stage larva; AdL3, advanced third-stage larva.*

respiratory symptoms occur. Involvement of the gastrointestinal tract is rare and usually asymptomatic, only being found incidentally at surgery. Urogenital involvement is also rare. Ocular involvement presents as pain and visual impairment, with a variety of signs (Teekhasaenee et al. 1986). Iris holes, uveitis, and subretinal hemorrhage with subretinal tract are characteristic features of intraocular gnathostomiasis (Biswas et al. 1994). Involvement of the ear, nose, and throat is always preceded by facial swellings; worms may extrude from tissues and there may be dyspnea and difficulty in swallowing. The prognosis of gnathostomiasis with CNS involvement is poor and the infection is sometimes fatal (Punyagupta et al. 1990). A common clinical symptom in this form is radiculomyelitis, typically characterized by excruciating radicular pain associated with a burning sensation, which may last for 1–5 days as the worms migrate from the nerve root. When a cranial nerve or a cervical nerve is affected, the patient develops severe headache, convulsion, vomiting, paraesthesia, and unconsciousness, followed by paralysis or weakness of the extremities. In Thailand, differential diagnosis between cerebral angiostrongyliasis and gnathostomiasis is required, because both involve marked eosinophilia in the spinal fluid (Table 2).

Initially, patients show peripheral leukocytosis and 5–96 percent eosinophilia, but both these signs later gradually decrease. Patients with ocular gnathostomiasis show normal hematology. Cerebral gnathostomiasis usually shows xanthochromic cerebrospinal fluid with numerous leukocytes, especially lymphocytes and eosinophils (8–98 percent), and some erythrocytes. Protein in the spinal fluid is unchanged or slightly elevated, whereas glucose is normal (Table 2). Computed tomography of the head is useful for diagnosis of intracranial hemorrhage or obstructive hydrocephalus. Death is usually due to direct involvement of the brain stem and massive hemorrhage in this area; mortality ranges from 8 to 25 percent (Rusnak and Lucey 1993).

Diagnosis

If biopsy specimens or sections containing worms are available, diagnosis can be made morphologically

(Tables 5 and 6). Recovery of worms is not always possible and therefore serological tests have also been employed. An ELISA test using crude extract of advanced L3 larvae or their E-S products is used for detecting antigen specific IgE (Soesatyo et al. 1987) or IgG antibody (Tuntipopipat et al. 1989) in sera or spinal fluid. A 24-kDa glycoprotein antigen from advanced L3 larvae is specific to *G. spinigerum* and is thus useful for immunodiagnosis (Nopparatana et al. 1991; Tapchaisri et al. 1991). E-S antigens from advanced L3 larvae give a better diagnostic result than crude somatic extracts. Furthermore, an ELISA assay with low molecular weight E-S or somatic antigens (less than 29 kDa), purified by a simple two-step fractionation process, is sensitive and specific (Tuntipopipat et al. 1993). Detection of circulating antigens seems to be unreliable (Tuntipopipat et al. 1989).

Control

Until recently, there were no effective routine therapeutic measures other than surgical removal of worms, which is not always feasible; supportive, symptomatic, and anti-inflammatory treatments were recommended. Recently, however, it has been shown that cutaneous gnathostomiasis can be treated successfully with a single 3-week course of albendazole (400 mg) either once or twice daily (Kraivichian et al. 1992; Crowley and Kim 1995). Ivermectin is also effective and more convenient in a single-dose treatment (Nontasut et al. 2000). Preventive measures include avoidance of raw or inadequately cooked hosts that may contain L3 larvae and avoidance of drinking water that may contain infected *Cyclops*. L3 larvae are killed by boiling for 5 min or freezing at −20°C for 3–5 days (Schantz 1989; Rusnak and Lucey 1993). Use of gloves or frequent washing of hands while handling food is recommended to prevent possible larval penetration of the skin. Relatively recent increases in world travel and importation of food mean that greater awareness of gnathostomiasis spinigera is required, even in the USA and European countries.

GNATHOSTOMA HISPIDUM FEDTSCHENKO, 1872

Introduction

Gnathostoma hispidum was discovered in 1872 in wild pigs in Turkestan and in domestic pigs in Hungary. Since then it has been found in pigs in Germany and many Asian countries (Daengsvang 1982). Two doubtful human cases were described in 1924 and 1949, and four cases of creeping disease were more recently found in Japan (Tsushima et al. 1980). Subsequently, a further 79 cases of cutaneous gnathostomiasis due to *G. hispidum* have been recognized in Japan (Ando 1992).

Morphology

Adult worms live in the stomach wall, singly or in groups of a few worms, without producing a tumor. Males are 20 mm long and females are 25 mm long. Fresh specimens are red. The head-bulb bears between nine and 12 rows of cephalic hooklets and the entire body surface is covered with cuticular spines. Eight pairs of papillae are located caudally in the male worm, whereas the female has one pair of papillae. Eggs resemble those of *G. spinigerum*, but are slightly larger (72 × 40 μm). Advanced L3 larvae from humans measure approximately 900 μm (Tables 5 and 6).

Life cycle

Eggs shed in swine feces release L2 larvae in approximately 2 weeks. If ingested by *Cyclops* they molt into early L3 larvae in approximately 8 days. These develop into advanced L3 larvae in second intermediate or paratenic hosts (e.g. fish, amphibians, and rodents) and become encysted in their muscles. Prevalence in loaches from the Beijing and Nanjing areas has been estimated at 12 and 6 percent, respectively (Akahane et al. 1982). In China, advanced L3 larvae have been found in 14 species of freshwater fish, frogs, snakes, chickens, and rats, the incidence in snakes being 100 percent (Chen and Lin 1991). The prepatent period in pigs varies from 3 to 6 months. Unlike *G. spinigerum*, *G. hispidum* cannot mature in humans. Experimentally, cats and rats can be infected by skin penetration of advanced L3 larvae.

Transmission and epidemiology

Humans acquire infections by eating raw loaches containing early L3 larvae or by consuming various vertebrates containing advanced L3 larvae. All human infections in Japan have come from loaches imported from China, Korea, and Taiwan.

Clinical manifestations and pathology

Clinical symptoms develop 3 weeks to 3 months after infection. Initially, patients complain of anorexia, diarrhea, and abdominal pain. All cases in Japan showed creeping eruption or mobile speckled erythema and a few also showed migratory intermittent swellings. Creeping eruption with erythema and itch normally occurs on the trunk or extremities. Morita et al. (1984) classified the disease into five types: (1) skin creeping eruption, on trunk and extremities; (2) migrating tracks on the liver; (3) migrating speckled erythema; (4) cerebrospinal form; and (5) Löffler's syndrome revealed by transient pulmonary infiltration on X-ray. Blood leukocytosis and eosinophilia (up to 84 percent) commonly

occur, peaking 1–2 months after infection. Serum IgE levels are highly elevated. Clinical manifestations normally disappear within 3 months and never recur.

Monkeys experimentally infected with larvae from loaches yielded 64 percent encysted advanced L3 larvae after 466 days (Koga et al. 1988). This suggests that encysted larvae may survive in human musculature for long periods.

Diagnosis

Morphological diagnosis (Table 6) is possible using resected whole worm specimens or sections. Patients with creeping eruption and peripheral eosinophilia, and a history of eating raw loaches from endemic areas, should be examined for antignathostome antibody. Crude *G. spinigerum* antigens are commonly used in Japan, because *G. hispidum* antigens are unavailable.

Control

No effective drugs are available. Ingestion of raw loaches from endemic areas or flesh from chickens fed with loaches should be avoided.

GNATHOSTOMA DOLORESI TUBANGUI, 1925

Introduction

Gnathostoma doloresi was first discovered by Tubangui (1925) in the stomach wall of a pig in the Philippines. Later, it was also noted in pigs in India by Maplestone (1930) and it has subsequently been recovered from domestic pigs and wild boars in many Asian countries. The first human infections (with creeping eruption) were found by Ogata et al. (1988) in Japan. All eight patients had a history of eating local freshwater fish. Larvae were found in the skin of three out of eight patients. As of 2001, 58 human cases have been noted from Japan.

Morphology

Adult worms occur singly or in groups of two to three worms in the gastric walls of domestic pigs or wild boar, inserting the anterior body into the thickened gastric wall. Males are 20 mm long and females are 34 mm long. The posterior half of the body is thicker than the rest. Worms are covered with cuticular spines, except for a small area at the tail end of the male. Males have eight pairs of caudal papillae. Eggs are 61.3 × 30.9 μm on average, with transparent knob-like bulges at both ends.

Advanced L3 larvae are approximately 3 mm long. The head-bulb has four rows of cephalic hooklets, those in the first row being extremely small. The average number in each row is <40 (Table 5). The morphological characteristics of cross-sections are presented in Table 6.

Life cycle

The recent incidence of infection in wild boars examined at two locations in Miyazaki Prefecture, Japan was 95 and 100 percent with an average of 20 and 57 worms per boar, respectively (Ishiwata et al. 1998); in Thailand the incidence in pigs was 2.1–8.7 percent (Daengsvang 1982).

Eggs shed into the feces develop to the L2 stage. These develop in *Cyclops* to the early L3 stage. If *Cyclops* are eaten by fish or amphibians, advanced L3 larvae develop. Snakes that feed on these second intermediate hosts can act as paratenic hosts. The prevalence in the poisonous snake *Agkistrodon halys* has been recorded as 100 percent (Imai et al. 1988). A pig infected with advanced L3 larvae from *A. halys* shed eggs 58 days after infection (Imai et al. 1989).

Transmission and epidemiology

In Japan, freshwater fish are probably the major source of human infections, although several infections occurred by ingesting of the flesh of *A. halys*. *G. doloresi* advanced L3 have been found in a freshwater fish, *Lepomis macrochirus* (common name: blue-gill), which had been ingested by some past patients (Nawa et al. 1993).

Clinical manifestations and pathology

Before cutaneous symptoms develop, patients may experience prodromes (such as fever, 'cold-like' symptoms, vomiting, abdominal pain, weakness, and malaise). Infections normally provoke creeping eruption with local pain and itch on the skin, particularly the trunk, and migratory swellings sometimes develop (Quincke's edema). Creeping eruption leaves pigmentation in the skin. Histopathologically, there is prominent eosinophil infiltration. Total serum IgE may be elevated and patients may show peripheral eosinophilia (6–67 percent).

Miyamoto et al. (1994) reported a case of pulmonary involvement in a patient with right chest pain and high fever. Clinical examination showed eosinophilia (18 percent), elevated IgE, a nodular lesion on r-S4 as revealed by plain X-ray and computed tomography, and anti-*G. doloresi* antibody. No cutaneous lesions developed. A case with an ileus due to an eosinophilic nodular lesion around migrating larvae in the colonic subserosa (Seguchi et al. 1995) and an intraocular case (uveitis) (Sasano et al. 1994) have also been reported. *G. doloresi* larvae can migrate to almost any location in the human body, e.g. the liver and CNS, and give rise to a variety of clinical manifestations.

Diagnosis

Various serological tests employ *G. doloresi* crude extracts as an antigen. If excised skin containing migrating worms is available, the species may be identified by the morphology of whole worms or cross-sections (Tables 5 and 6).

Control

No effective drugs are available. The best treatment is to excise worms from the skin lesions, if this is possible. No fatal cases have been reported. It is advisable to avoid eating raw, freshwater fish from local rivers in endemic areas.

GNATHOSTOMA NIPPONICUM YAMAGUTI, 1941

Introduction

This species was first found in the esophagus of weasels in Japan, and was mistakenly identified as *G. spinigerum*. It was subsequently renamed *G. nipponicum* by Yamaguti (1941) and has since been found in weasels in various regions of Japan (incidence of 40 percent). The species may also exist in China.

Ando et al. (1988) reported patients in Japan with creeping eruption after eating raw loaches; a *G. nipponicum* larva was found in a skin biopsy specimen. Since then, more than 15 patients with similar gnathostomiasis have been noted.

Morphology

Adult worms live singly or in groups in a hard tumor of the esophageal wall of the weasel, located 2–3 cm from the stomach. The anterior body is embedded and the posterior lies free in the lumen. Adult males are 20–23 mm long and females are 29–34 mm long. The anterior body is covered with cuticular spines, as in *G. spinigerum*, but there are no spines caudally in the female. There are also differences in the shape of the spines of *G. nipponicum* and *G. spinigerum* (Miyazaki 1960, 1991). The caudal alae of the male have small cuticular spines and eight pairs of papillae. Eggs measure 72.3 × 42.1 μm on average, with a transparent knob-like bulge at one end.

Advanced L3 larvae are found encysted in the muscles of the second intermediate or paratenic hosts. They have only three rows of hooklets on the head-bulb (Table 5). Morphological features of the cross-sections of the larvae are presented in Table 6.

Life cycle

L2 larvae released from fully developed eggs are eaten by *Cyclops* and molt to early L3 larvae. When cyclops are eaten by loaches, the larvae develop to the advanced L3 stage and encyst. Several species of freshwater fish, amphibians, and mammals can act as second intermediate hosts and several species of reptiles, birds, and mammals act as paratenic hosts (Ando et al. 1992). Weasels experimentally infected with advanced L3 larvae shed eggs 3 months after infection. Ferret and mink also serve as experimental hosts.

Transmission and epidemiology

Humans acquire infections by ingesting raw or inadequately cooked loaches or catfish. Recently, a human infection occurred through ingestion of raw, largemouth, black bass (*Micropterus salmoides*) in Akita, Japan (Ishida et al. 2003).

Clinical manifestations and pathology

All 15 cases studied to date developed creeping eruption on the abdomen, waist, and hip areas. The patients had all ingested loaches or various freshwater fish 3 to 4 weeks before the onset of symptoms. Some had peripheral eosinophilia (maximum 29 percent); leukocytosis was not always seen.

Diagnosis

Excised worms or resected skin specimens can be used for morphological identification (Tables 5 and 6).
Some patients show a positive serological reactions, but data are still scanty.

Control

Excision of worms is the best therapeutic measure if feasible. Mebendazole was effective for stopping the creeping eruption (Taniguchi et al. 1991). Eating raw freshwater fish must be avoided in endemic areas.

SPIRUROID TYPE X LARVA

Since 1991, many human cases have been reported from Japan of a creeping disease (most often with blister formations) caused by the type X larva of suborder Spirurina. This larva was first described by Hasegawa (1978) and has subsequently been recovered from human skin, the intestinal wall (especially ileum), and the anterior chamber of the eye. Sometimes, the larvae show intrahepatic erratic parasitism and provoke intestinal perforation. The adult stage has not been identified. Kagei (1991) suggested that the larvae of Spiruroid nematodes

other than the Spiruroid type X larva might cause cutaneous or visceral larva migrans.

Spiruroid type X larvae measure 6.7–8.0 × 0.08–0.10 mm, with two tubercles at the tail and two pseudolabia at the head. Larvae detected in sections of resected skin are 80–100 μm in diameter with characteristic cuticular annulations and intracuticular striae. The muscle layer is polymyarian-coelomyarian. Lateral cords are large and Y-shaped. The esophagus has muscular and glandular parts, the latter filled with minute granules that resemble secretory granules or vacuoles. The intestine has between five and six columnar intestinal cells.

These larvae have been found in marine fish which are therefore possible sources of human infections. Some species of squid also harbor type X larvae. Firefly squid are probably the most important source of human infections, because they are the type most frequently eaten raw in Japan. Spiruroid type X infections in humans occur predominantly, especially during March to June, and as of 2001, there are more than 70 cases in Japan.

The disease is classified into ocular, cutaneous, and intestinal (ileus) forms. Cutaneous symptoms develop as early as 2–9 days after eating raw squid (Shinozaki et al. 1993). There is usually only a transient, linear, or meandering erythema, without significant leukocytosis or eosinophilia. Some cases showed a significant rise of serum total IgE levels. Testing with immunofluorescence assay (IFA) or ELISA is recommended for the immunodiagnosis of the disease.

Effective therapeutic measures are unavailable. Most patients with ileus undergo surgical resection of the intestinal lesions and confirmed diagnosis is then made. It is recommended that firefly squid are frozen at −32°C for 30 min or more (in practice at −40°C for more than 40 min) to prevent infection (Akao et al. 1995).

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Dracunculiasis

RALPH MULLER

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Dracunculiasis is an ancient disease quoted by many classical authors and possibly mentioned in the Old Testament. For the last few years, there has been a world eradication campaign and this chapter has been written in the light of this campaign (Chippaux 1994; Cairncross et al. 2002). It is hoped that it will be superfluous in the next edition.

MORPHOLOGY

The mature female of the dracunculoid nematode *Dracunculus medinensis* (Linnaeus 1758) Gallandant, 1773 measures 500–800 × 1.0–2.0 mm. The mouth has a triangular oval opening surrounded by a quadrangular cuticularized plate, and an internal circle of four double papillae. The vulva opens halfway down the body but is nonfunctional in the mature worm. The uterus has an anterior and a posterior branch; it is filled with 1–3 million embryos and occupies the entire body cavity (pseudocoel), the gut being completely flattened (Cairncross et al. 2002).

Males recovered from experimental infections in animals measure 15–40 × 0.4 mm. The tail has 4(3–6) pairs of preanal and 4–6 pairs of postanal papillae; the subequal spicules are 490–750 μm long with a gubernaculum measuring about 117 μm. The males probably die in the tissues before the females mature but have only doubtfully been seen in human infections.

LIFE CYCLE

In the human body, each female worm takes about one year to mature and moves to the surface of the skin. It provokes a blister at the anterior end which bursts and leaves an ulcer through which about 5 cm of the worm is extruded, particularly after immersion in water (Figure 41.1a). Many thousands of larvae are released into the water where they can live for only a few days; for further development they have to be ingested by small freshwater crustacea (cyclops). The larvae molt twice in the body cavity and are infective in about 2 weeks. If cyclops are ingested in drinking water, released larvae penetrate the human intestinal wall and male and female worms mate in the connective tissues in about 3 months.

TRANSMISSION AND EPIDEMIOLOGY

Dracunculus infection still occurs in 12 countries of West and Central Africa: principally in Sudan, Ghana, and Nigeria but also in Benin, Burkina Faso, Cote d'Ivoire, Ethiopia, Mali, Mauritania, Niger, Togo, and Uganda (Cairncross et al. 2002) and with a recent isolated outbreak in Libya. The disease has been eliminated or has died out in Cameroon, Central African Republic, Chad, Guinea Bissau, India, Iran, Pakistan, Saudi Arabia, Senegal, and Yemen in recent years.

Dracunculiasis is typically a disease of rural communities that obtain their drinking water from ponds (or, formerly, in India, from large step wells) where cyclops can breed (Figure 41.2). In all areas, transmission is markedly seasonal and, in most parts of Africa, the maximum incidence coincides with the planting season, resulting in great economic hardship. In semi-desert (Sahel) areas of Africa (Burkina Faso, Mauritania, Niger, northern Nigeria, and Sudan), drinking water is

Figure 41.1 *Dracunculiasis:* **(a)** *foot of child with three worms emerging and being wound out on sticks (Courtesy of Dr Ahmed Tayeh);* **(b)** *secondarily infected and swollen knee of girl with guinea worm emerging*

obtained from ponds during the rainy season but from deep (safe) wells for the rest of the year when the ponds are dry. However, in the humid (Guinea) savanna regions of West Africa where rainfall exceeds 150 cm per year (Benin, Cote d'Ivoire, Ghana, southern Nigeria, and Togo) there is almost no transmission during the rainy season (July–September) when ponds turn into streams and cyclops densities are low because of the large volume and turbidity of the water. The life history of the parasite is well adapted to provide the maximum chances of transmission as the female takes almost exactly a year to mature and release its larvae.

Figure 41.2 *One of many large artificial ponds constructed in Nigeria for drinking water and agriculture and which are transmitting both dracunculiasis and schistosomiasis*

LOCATION IN HOST

One to many adult female worms emerge from the subcutaneous tissues, usually of the foot or lower limbs, but sometimes from any part of the body.

CLINICAL MANIFESTATIONS AND PATHOLOGY

In most patients, the first physical sign is the local lesion, accompanied by an intense burning pain usually relieved by immersion of the affected limb in water. The blister fluid is bacteriologically sterile and contains numerous white cells and larvae. In uncomplicated cases, the worm emerges from the subsequent ulcer over a few weeks and then the lesion rapidly heals. Thus, if there is only one worm present, patency will last for only 4–6 weeks. Unfortunately, secondary infection along the track of the worm in the tissues is very common, often with spreading cellulitis, and approximately 40 percent of patients will be totally incapacitated for an average of 6

weeks (Figure 41.1b). More serious and permanent damage can follow the bursting of a worm in the tissues or as the result of bacterial infection, e.g. ankylosis of joints in about 1 percent of cases or, occasionally, tetanus.

Dracunculiasis is unusual among infectious diseases in that the parasite does not appear to stimulate a protective response, so that the same individual can be reinfected year after year.

DIAGNOSIS

Clinical and parasitological diagnosis

Patients in an endemic area usually have no doubt of the diagnosis as soon as or even before the first signs appear. Local itching, urticaria, and a burning pain at the site of a small blister are usually the first signs of infection. The blister bursts in about 4 days and active larvae, obtained by placing cold water on the resulting small ulcer, can be recognized under a low-powered microscope.

Immunological diagnosis

Immunological methods are not useful in practice. Enzyme-linked immunosorbent assay (ELISA) and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS PAGE)/western blotting worked well in one trial for patent infections. Detection of parasite-specific immunoglobulin (Ig) G4 might be able to diagnose prepatent infections possibly up to 6 months before emergence (Bapna and Renapurkar 1996).

TREATMENT

Surgery

Guinea worms have been wound out on sticks since antiquity (e.g. in the Rig Veda of about 1350 BC). Provided that bacterial infection or other complications have not occurred, regular winding out of the worm on a small stick, combined with sterile dressing and acriflavine cream, usually results in complete removal in about 4 weeks with little loss of mobility. Treatment should be commenced as soon after emergence as possible (Magnussen et al. 1994). Sometimes, worms can be seen and surgically removed before emergence while there is no tissue reaction against them.

Chemotherapy

There is no evidence that any chemotherapeutic agent has a direct action against guinea worms. However, many compounds, including thiabendazole, niridazole, metronidazole, mebendazole, and albendazole, have been reported as hastening the expulsion of worms and may act as anti-inflammatory agents (Muller 1971). Ivermectin had no action against pre-emergent worms (Issaka-Tinorgah et al. 1994).

THE ERADICATION CAMPAIGN

The eradication of dracunculiasis from the world was adopted as a sub-goal of the Clean Drinking Water Supply and Sanitation Decade (1981–90) and was formally endorsed by the United Nations World Health Assembly in 1986 and 1989. If the original aim of complete eradication by the end of 1995 had been achieved, this chapter would have been superfluous. However, the task is likely to take quite a while longer, particularly in the Sudan. Nonetheless, the prevalence and distribution of infection have diminished markedly in the last few years: worldwide there were about 3.5 million cases in 1986, 1 million in 1989 but only 46 000 in 2002. Disease has been eliminated from Asia with no cases reported from Pakistan since 1993, nor from India since 1997 (Cairncross et al. 2002). The situation in African countries is not so far advanced but active campaigns are being carried out in almost all endemic countries and some are at the stage of case containment (Figure 41.3). In Nigeria and Ghana for instance, new cases have reduced markedly since 1988, and in all African countries on average 56 percent of cases were contained in 2002 (i.e. all larvae were prevented from reaching ponds). In 2002, only 3 000 cases were reported from Nigeria and 4 900 from Ghana. In 37 countries where the disease used to be present in historical times, applications for certification of absence have been made.

The first priority has been to have accurate figures on the distribution and prevalence in each endemic country. Because infected persons rarely report to health clinics, passive surveillance is almost useless (identifying less than 3 percent of cases) and trained teams of community workers are required to visit each village and obtain the necessary information. These teams have to be carefully monitored and, when trained, can also be associated with other primary healthcare initiatives, such as control of malaria and diarrhea diseases and immunization campaigns.

Once active surveillance measures have been initiated, there are various possible interventions based on knowledge of the life cycle of the parasite.

The provision by governments and aid agencies of safe drinking water from tubewells is a priority in rural areas of Africa and Asia. The United Nations (International) Children's (Emergency) Fund (UNICEF) aimed to provide one borehole for every 200 persons in suitable geological regions by the year 2000. In some areas, traditional (usually hand built) draw wells can be equally effective and in all areas,

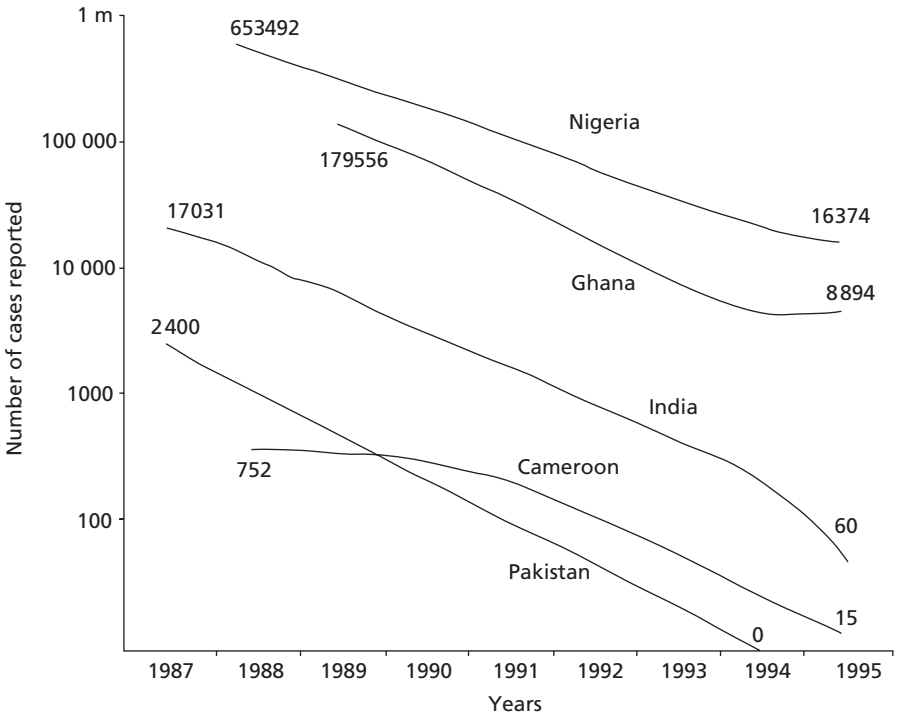

Figure 41.3 *Decrease in numbers of cases of guinea worm infection reported each year in five countries that have active campaigns (note log scale). The campaign in India started in 1980 (Sudan is not included).*

cisterns can be constructed for storing rainwater (Figure 41.4).

Health education interventions by local health workers form a very important component of any control or eradication campaign. Interventions being made include the following:

- Filtering or boiling all drinking water. Boiling water is not usually feasible because fuel is a scarce commodity but filters are playing an important role in all countries now mounting campaigns. The donation by the manufacturer of monofilament nylon nets, which are long lasting, have a regular pore size, are

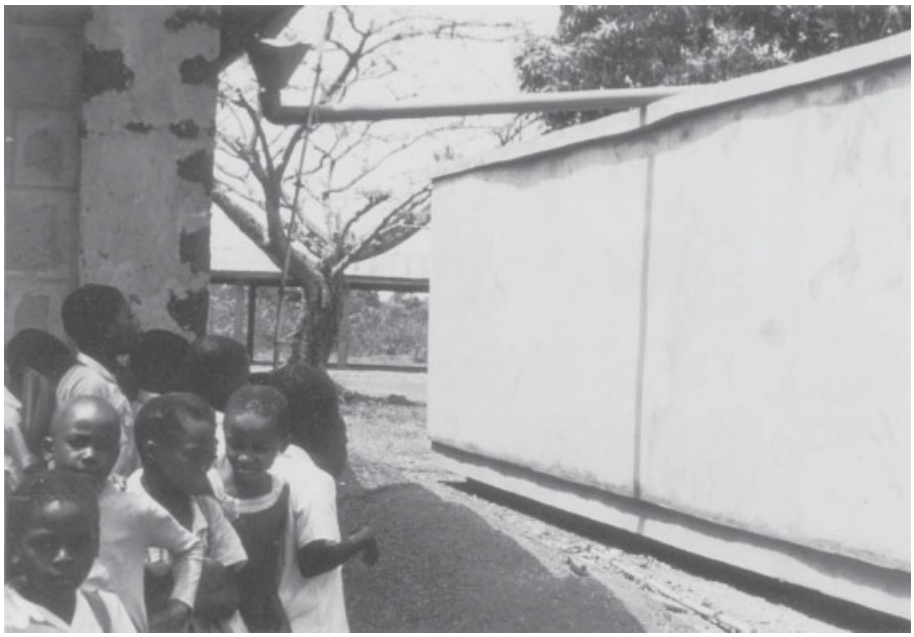

Figure 41.4 *Cistern built at a school in Nigeria to store rainwater for use during the dry season.*

easily washed and dry quickly, is proving of great help in sieving out cyclops. Health educators in all countries have been very successful in promoting the use of filters.

- Persuading or preventing infected persons with an emerging worm from entering the water source (containment). This is particularly important when prevalence has been greatly reduced (the current motto is 'detect every case, contain every worm'). Bandaging very early lesions can help to prevent subsequent immersion in the water.
- Treating water sources. The chemical treatment of ponds can prove a useful adjunct to other measures, particularly when there is only a low level of transmission remaining. The insecticide temephos added to ponds at a concentration of 1 ppm will kill cyclops for 5–6 weeks and has low toxicity to mammals and fishes. In areas with a long transmission season, it has to be added a few times a year. The amount of temephos estimated to be needed for total eradication in Africa has been donated by the manufacturer. The main problem is the amount of time that needs to be expended by trained technicians.

The disease was eliminated from Kenya and Senegal in 1996, and from Cameroon, Chad, and Yemen in 1999. There are very few cases left in all African countries apart from Ghana, Nigeria, and Sudan (49 000 cases in 2001 and provisionally 40 000 in 2002). The number of cases in Ghana and Nigeria is decreasing greatly each year but the future in Sudan is more uncertain (target eradication date of 2009).

ZOONOTIC ASPECTS

Female worms of the genus *Dracunculus* have been reported as emerging from a wide range of mammals and reptiles from many parts of the world, both endemic and nonendemic for the human disease. Those found in reptiles clearly belong to other species but the situation in regard to those in mammals is not clear. For instance, guinea worm is common in wild carnivores in North America and the species was named *D. insignis* by Leidy in 1858 but there is very little morphological evidence for separating this from the human species.

There have been two documented cases of clearly zoonotic infections, from Japan in 1986 and from Korea in 1926. In both cases, the patients had eaten raw freshwater fish that have been proved experimentally to be capable of acting as paratenic hosts.

In most highly endemic areas, occasional infections in dogs and donkeys with what is presumably the human parasite have been reported but there is no evidence that they have any part in maintaining transmission. The parasite can still be found in dogs in the formerly endemic areas of Tamil Nadu in India and the central Asian republics of the former Soviet Union but no new human cases have been reported, so it is not thought that this will be a problem once world eradication has been achieved.

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A short history of research on viruses

LESLIE COLLIER

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INTRODUCTION

Virus (Latin, from Greek φιοσ): a poisonous or slimy fluid. I have not called this chapter 'The history of virology' because it pertains almost entirely to viruses themselves, and not to the whole subject, which includes epidemiology, immune responses, pathogenicity, and many other topics. Such extended coverage would have demanded a chapter of inappropriate length. The survey deals mainly with the period from the end of the nineteenth century to about the end of the twentieth, and includes references to papers that are now seen to have provided points of departure for major advances.

THE FOUNDATIONS

The discovery of viruses

Bacteria were seen and cultivated before their association with disease was determined. By contrast, the nature of viruses was not elucidated until well after it was realized that certain diseases of plants and animals could be transmitted by an invisible infective principle that differed considerably from that of the known parasites. This discovery was made amid the ferment of ideas and experimentation on microbes that took place in the closing decades of the nineteenth century; the technical advance on which it was based was the invention by Charles Chamberland (1884), a colleague of Louis Pasteur, of a porcelain filter originally designed to sterilize drinking water. In 1892, Ivanowski in Russia showed that such a filter allowed passage of the agent that caused mosaic disease of tobacco. Somewhat diffidently, he suggested that the infective principle might be a toxin elaborated by bacteria, but did not pursue the matter. In 1898, Beijerinck independently made similar observations on this infection, but extended them much further (Beijerinck 1899, cited by Waterson and Wilkinson 1978: 27). To exclude the possibility that his porcelain filter was letting through a very small bacterium he used the then novel device of diffusing sap through agar gel, after which it still retained infectivity. He also observed that the agent multiplied only in dividing cells, and that it withstood desiccation but was inactivated by boiling. Having failed with the means at his disposal to demonstrate its particulate nature, he termed it a *contagium vivum fluidum*. In the same year, Loeffler and Frosch (1898), both associates of Robert Koch, reported passage of the agent of foot-and-mouth disease through a bacteria-retaining filter. They ruled out the idea of an inert toxin on the basis of the dilution factors involved, and concluded first, that the agent must be able to replicate, and second, that it must be smaller than the smallest bacterium then known, and thus beyond the resolving power of the best available microscopes. Although the great majority of viruses were beyond the bounds of resolution of the light microscope, Buist (1887) visualized one of the largest, vaccinia virus, after staining it with aniline methyl violet. He also assigned it the remarkably accurate measurement of 100–500 nm.

Propagation in tissue culture

By the end of the nineteenth century, viruses had been defined solely in terms of their infectivity, filterability,

and, as a result of Beijerinck's percipience, their requirement for living cells as a substrate for growth. Hitherto, viruses had been propagated only in intact animals or plants. Further progress demanded a much more simple and controllable system, the foundation of which was laid by Harrison (1906–7), who devised the first tissue culture, not for propagating viruses but for studying the growth of frog nervous tissue in clotted lymph. Steinhardt et al. (1913) exploited Harrison's technique to grow vaccinia in fragments of guinea-pig cornea embedded in clotted plasma. They could not demonstrate the virus directly in these preparations, but concluded from the results of serial subcultures that replication had taken place.

In 1928, Maitland and Maitland propagated vaccinia in suspensions of minced hens' kidneys. Their method was not widely used and its potential was not fully realized until a quarter of a century later, when flask cultures of trypsinized fragments of monkey kidney were used to grow poliovirus on a large scale for vaccine production.

Quantification

Guérin (1905) found that the number of vesicles produced by a suspension of vaccinia inoculated into scarified rabbit skin was roughly proportional to its dilution. This method was used by Steinhardt et al. (1913) in their tissue culture experiments, and was the precursor of many later and more accurate methods of assaying viral infectivity.

Host range and pathogenicity: effects on cells

The discovery of animal and plant viruses was soon followed by the finding of others that affected insects and bacteria. Oncogenic viruses were discovered by Ellerman and Bang (1908) and by Rous (1911), who respectively showed that a leukemia and a sarcoma of fowls could be transmitted by filterable agents. Another category, bacterial viruses (Twort 1915; d'Herelle 1917), was also to prove of the first importance, particularly for the study of viral replication and bacterial genetics.

In the early years of this century various workers described intracellular inclusion bodies in the tissues of animals and humans infected with viruses (e.g. rabies, *Cytomegalovirus*, and vaccinia). As the only obvious manifestation of viral activity within cells, inclusions were long the objects of intense study and speculation; they were categorized in terms of staining properties (eosinophilic or basophilic), location (intranuclear or cytoplasmic), and morphology (Cowdry 1934). They are now largely of academic interest.

Immune responses

It was long ago appreciated that certain infectious illnesses were followed by resistance to a second attack. This observation prompted the inoculation of material from cases of smallpox (variolation), which was for centuries practiced in eastern countries. The practice was associated with significant mortality (approximately 2 percent) and was eventually superseded by the use of cowpox as the result of astute observations in the eighteenth and early nineteenth centuries by Edward Jenner (1801) and by lesser-known figures such as Benjamin Jesty, a farmer in Dorset. The last two decades of the nineteenth century saw the pioneering work of Louis Pasteur and his associates on immunization against both bacterial and viral infections, the most dramatic example being rabies. Meanwhile, the mechanisms of immunity underlying these somewhat empirical observations were being unraveled in the laboratory. Elias Metchnikoff (1891) demonstrated the importance of phagocytes in combating certain bacterial infections, and thus founded the concept of cell-mediated immunity. This line of investigation was long overshadowed by work on humoral factors. Around the turn of the century, the quantitative and qualitative aspects of antigen–antibody reactions and their specificity were under intensive study by von Behring, Kitasato, Ehrlich, Landsteiner, and others; and Nuttall and Bordet discovered a heat-labile factor in blood, later to be designated complement, that was bactericidal in the presence of specific antibody. For a more detailed account of early research on immunology, see History, in the Immunology volume of this series.

The end of the beginning

By the end of the First World War, therefore, the foundations of virology had been laid. It had been established that viruses are much smaller than bacteria, and appeared to be capable of growing only in living cells, sometimes leaving evidence of their presence in the form of inclusion bodies. The main categories of viruses affecting respectively vertebrates, invertebrates, bacteria, and plants had been identified, the existence of oncogenic viruses was established, and the outlines of the immune response to viruses and other microbes were beginning to take shape.

PROPAGATION OF VIRUSES IN THE LABORATORY

Before discussing work on the properties of viruses, mention must be made of their propagation outside the intact animal, because such techniques are essential for studying them and most other microorganisms.

Chick embryos

Before the advent of antibiotics, tissue culture methods depended mostly on the growth of explants in clotted

plasma, and freedom from contamination was difficult to maintain. The removal of these constraints, which impeded both quantitative work and large-scale cultivation, was signaled in 1931 with the finding by Woodruff and Goodpasture that fowlpox virus inoculated on to the chorioallantoic membrane of 10–15-day-old embryos produced discrete lesions (pocks). Within the next few years other viruses, including herpes simplex, Newcastle disease virus of fowls, and louping ill virus, had been propagated in this way and infectivity titrations by the pock-counting method were being carried out (Burnet 1936). Later, the allantoic and amniotic routes of inoculation were used to grow a range of viruses (Beveridge and Burnet 1946), and some, notably yellow fever virus and influenza, were grown in large quantities for vaccine production.

Cell cultures

The tedious method of growing tissue fragments in plasma clot was quite unsuited to the propagation of viruses in any quantity. In the early 1950s, it was largely replaced by true cell cultures as a result of several technical advances, given impetus by the celebrated discovery of Enders et al. (1949) that poliomyelitis virus would grow in nonneural tissues. The technical advances were: (1) the introduction of antibiotics to control bacterial and fungal contamination; (2) the use of trypsin to obtain suspensions of single cells that could then be grown as monolayers on glass or plastic surfaces; and (3) the production of lines of cells that could be propagated by serial passage, which fell into two main groups. Some were derived either from malignant tumors, for example the HeLa line (Scherer et al. 1953), or from cells that had acquired malignant characteristics, including aneuploidy, during serial transfer, or after infection with certain viruses; these 'continuous' cell lines would grow indefinitely. Others were derived from normal tissues and retained their diploid karyotype for a limited number of transfers. Monolayer cultures had the great advantage that the effects of viruses in terms of cell destruction (cytopathic effect) could be readily observed by low-power microscopy.

Quantitative methods

The use of cell cultures greatly improved the accuracy of infectivity titrations, for which two principal methods were developed. The first was analogous to the counting of bacterial colonies obtained from a known dilution of suspension and, as we have seen, was applied to infectivity titrations of viruses that produced discrete lesions on the chick embryo chorioallantoic membrane. Suitably diluted suspensions of bacteriophage produced cleared areas ('plaques') in confluent lawns of bacteria on solid media, and, likewise, some animal viruses produced foci of destruction in cell monolayers overlaid with gel (Dulbecco and Vogt 1954). Because, in theory at least, each plaque resulted from the replication of one virus particle, the infectivity of the suspension could be accurately assayed as the mean of a number of replicate counts.

The second method was useful when plaque counting was impracticable. It depended on ascertaining the highest dilution of suspension producing a certain endpoint, for example the death of an animal or a cytopathic effect in a cell culture. The precision of the method was much enhanced by estimating the dilution producing the endpoint in 50 percent of the animals or cultures inoculated. The earliest and perhaps best known method for doing so was that of Reed and Muench (1938).

From about 1970 onwards, developments in rapid methods of nucleic acid and protein analysis contributed greatly to fundamental knowledge of the properties of viruses and to the diagnosis of viral infections. During the same period, the introduction of micromethods and automation made possible the use of techniques such as enzyme-linked immunosorbent assays (ELISA) for analyzing large numbers of specimens. Such methods progressively replaced the more cumbersome and less informative assays using, for example, complement fixation.

THE PROPERTIES OF VIRUSES

Physical characteristics

SIZE

Probably because of the difficulty of propagating them in the laboratory, research on the sizing of viruses by filtration and centrifugation was an important focus of attention until well into the 1940s.

In 1929, Elford described a method of making series of collodion membrane filters of known average pore diameter (APD); with the proviso that the conditions of filtration had to be carefully controlled, their availability greatly assisted the measurement of viruses. Thus, by 1940, the sizes of about 25 animal viruses, ranging from the enteroviruses (approximately 20 nm) to poxviruses (approximately 250 nm) had been measured with fair accuracy (see van Rooyen and Rhodes, 1940a). Such studies were further advanced by the use of high-speed centrifuges, notably of the Sharples and Svedberg varieties (reviewed by van Rooyen and Rhodes 1940b), to estimate size as a function of the sedimentation characteristics.

Centrifugation was also useful for purifying viruses and became more so with the introduction by Brakke (1953) of sucrose density gradients, in which particles within a mixture can be separated according to their

sedimentation rates. Later, cesium chloride and other salts were used with great success in high-speed centrifuges to separate viruses from contaminating material on the basis of their differing buoyant densities.

MORPHOLOGY

Until 1939, next to nothing was known about the structure of viruses because of the constraints imposed by the limited resolving power of light, even of short wavelengths in the ultraviolet range. In that year Kausche, Pfannkuch, and Ruska visualized a virus with the newly invented electron microscope (EM), in which beams of electrons travelling in a vacuum are focused on the object with electromagnetic fields. Given the then current interest in crystalline tobacco mosaic virus (TMV) (see Chemical composition: the role of the nucleic acids), it is not surprising that this was the first virus to be examined. Kausche and his colleagues (Kausche et al. 1939) employed the technique of shadow-casting, whereby the objects on the specimen grid were coated with gold particles generated by heating the metal in a vacuum; they defined the size of the crystals as about 25 × 300 nm.

After the interruption of the Second World War, new techniques were introduced to exploit the potential of the EM; they are well reviewed by Almeida (1984). In brief, shadow-casting revealed important morphological features of viruses, including the fact that some have an icosahedral structure. Negative staining, in which the background, but not the virus, is stained with an electron-dense salt of tungsten, uranium, or osmium, found wide application, not least in the diagnostic laboratory, and demonstrated that the outer surfaces of virus particles are composed of subunits. Methods were developed for cutting ultrathin sections in the 2-nm range which, alone or treated with labeled antibodies, yielded much information about virus–host-cell relationships.

These techniques, together with X-ray diffraction studies, showed that most viruses fall into one of two groups: the icosahedral viruses with so-called cubic symmetry; and those with helical symmetry, in which the outer subunits are arranged like the treads in a spiral staircase.

Chemical composition: the role of the nucleic acids

The demonstration by Stanley (1935), that TMV could be obtained in an apparently crystalline form, further stimulated the active, but ultimately sterile, debate on whether viruses are living entities. More importantly, it prompted study of the chemical composition of such preparations; and in 1937, Bawden and Pirie showed that they were not, as Stanley at first thought, pure proteins but also contained a nucleic acid, probably RNA. From this observation stemmed the realization that viruses consist of a nucleic acid contained within a protein coat; by the end of the decade, it was also clear that some viruses contain DNA rather than RNA. Following the demonstration by Avery et al. (1944) that the genetic information determining the type specificity of pneumococci resides in their DNA, Hershey and Chase (1952) confirmed the crucial role of nucleic acid in viral replication by showing that, after the attachment of bacteriophage T2, the DNA, but not the protein coat, enters the bacterial cell. A further major step was the demonstration by Gierer and Schramm (1956) and Fraenkel-Conrat (1956) that RNA in infective form could be extracted from TMV.

THE CONCEPT OF THE VIRION

Not long after Crick and Watson had solved the structure of DNA, they suggested that viral nucleic acid is protected by a shell of identical protein subunits (Crick and Watson 1956). This notion, based on the limited coding capacity of viral nucleic acid, proved essentially correct. All viruses were found to have this basic architecture (Klug and Caspar 1960; Caspar and Klug 1962), the nucleic acid being, with few exceptions, double-stranded DNA or single-stranded RNA. Caspar et al. (1962) proposed the following terminology: the complex of protein shell (capsid) and nucleic acid is the nucleocapsid; the mature nucleocapsid, surrounded in some viruses by an outer envelope, is referred to as the virion.

Replication

For well over half a century after their discovery, nothing was known of the way in which viruses reproduce themselves. From about 1940 onwards, however, research by Delbrück, Luria, Hershey, Lwoff, and many others elucidated the basic mechanisms. Much of the work was done with bacteriophages, which had the advantages over animal viruses of easy propagation in vitro, accurate quantification, and growth cycles measured in minutes rather than hours; considerable use was made of radiolabeling to trace the synthesis of nucleic acids and proteins. The mechanisms of viral attachment, penetration, and uncoating were defined, as was that of the coding of enzymic and structural proteins by messenger RNA.

By 1957, André Lwoff was able to crystallize the essential features that distinguish viruses from all other organisms: they are strictly intracellular and potentially pathogenic entities with an infectious phase, and (1) possess only one type of nucleic acid; (2) multiply in the form of their genetic material; (3) are unable to grow and undergo binary fission; and (4) are devoid of a Lipmann system of enzymes for energy production.

'Unconventional' viruses

Two groups of viruses or virus-like agents stand apart from the generality of animal viruses: these are the retroviruses and the prions.

RETROVIRUSES

The finding by Ellerman and Bang (1908) and Rous (1911) that certain malignancies of fowl are caused by a transmissible agent was followed by the observation that many others in birds and animals, both of the solid tissues and of blood, can be transmitted vertically, from parents to offspring, as well as horizontally. The oncogenic effects of the viruses concerned are characterized by unusually long incubation periods. Temin argued for many years that the persistence of these RNA-containing viruses was due to a DNA 'provirus' that had become integrated into infected cells. This was finally confirmed when it was discovered that their RNA is transcribed to a DNA copy by means of an enzyme, reverse transcriptase (Baltimore 1970; Temin and Mituzani 1970), so called because it reverses the usual flow of genetic information from DNA to RNA.

Fatal infections, also with long incubation periods but otherwise dissimilar, appeared among sheep in Iceland following the importation of Karakul sheep from Germany in 1933; known as maedi and visna, they respectively affected the lungs and central nervous system (Sigurdsson 1954a). Other viruses, the so-called 'foamy agents,' were first detected in monkey kidney cells used for polio vaccine production during the 1950s and later in the kidneys of other species, including humans; these agents appear not to cause disease.

The viruses concerned have now been grouped into the *Retroviridae* family. The insertion and integration of viral DNA (provirus) into the host-cell genome accounts both for the chronicity of these infections and for the oncogenic potential of many of the viruses in the family. Later, retroviruses were discovered that cause infections of humans, notably leukemias and acquired immunodeficiency syndrome, and viruses of other families, notably hepatitis B, were found to utilize reverse transcription during replication; but these developments are outside the scope of this chapter.

PRION DISEASES

For the past 200 years, a widespread disease of sheep and goats has been recognized that affects the central nervous system, has an incubation period of several years, and is always fatal; it is known as scrapie because the severe itching impels affected animals to rub against posts and fences. The disease could be transferred between sheep by injection of brain tissue (Cuillé and Chelle 1936); its transfer to mice (Chandler 1961) and hamsters, with an incubation period of less than a year, greatly facilitated experimental work. A similar disease, rida, was reported in sheep in Iceland (Sigurdsson 1954b), and such infections were later identified in other animals, notably cattle in the UK. All were characterized by, inter alia, severe neuronal degeneration and vacuolation (spongiform encephalopathy), and by the presence in the brain of tangles of fibrils (scrapie-associated fibrils (SAF)). Analogous infections were found in humans, in the form of Creutzfeldt–Jakob disease (Creutzfeldt, 1920; Jakob, 1921), Gerstmann–Sträussler–Scheinker syndrome, and kuru (Gajdusek and Zigas 1957), a now extinct disease of the Fore tribe in Papua New Guinea spread by endocannibalism.

The year 1986 saw the start of a major epizootic of bovine spongiform encephalopathy (BSE), first in the UK, and later in other countries, involving many thousands of cattle infected by meat and bone-meal (MBM) given as a dietary supplement; it was eventually brought under control by extensive slaughter and the banning of suspect foodstuffs. In 1995, two adolescents in the UK, and subsequently others, were diagnosed with a form of Creutzfeldt–Jakob disease whose agent was indistinguishable from that of BSE. These cases were characterized by their early onset, longer than usual time to death, amyloid plaques, and absence of the usual electroencephalographic changes. This syndrome is termed new variant CJD (nvCJD). The number of cases ran into three figures, but is now declining.

The causal agents of these infections differ in many respects from 'conventional' viruses and cannot be classified on similar criteria. They are very small and highly resistant to heat and ionizing radiation. Even so, they can reproduce themselves, and some, at least, can undergo genetic variation. Prusiner (1982) marshalled the evidence suggesting that these agents are protein and devoid of nucleic acid; he termed them prions (proteinaceous infectious particles). His view that they are an aberrant form (PrP^{Sc}) of a protein (PrP) normally found in the brain has now gained wide acceptance. The mode of reproduction is probably explained by repetitive conformational changes induced by PrP^{Sc} in the normal protein. (Weissmann 1991).

CLASSIFICATION OF VIRUSES

Now that the story of research on the properties of viruses has been described, it is appropriate to consider how they have been applied to viral classification. The early history of this topic was reviewed by Brown (1984); here, there is space to refer only to the main events.

The earliest attempts at grouping viruses were necessarily based on what they do rather than on what they are: the criteria included clinical effects, pathological changes, and tissue tropisms. The inadequacies of such systems were apparent, and in 1950 a Subcommittee on Virus Nomenclature under the chairmanship

of C.H. Andrewes was set up at the International Congress of Microbiology held at Rio de Janeiro; it recommended use of a system based on the physical, chemical, and antigenic properties of the viruses themselves, and allotted names to some of the better known (e.g. poxvirus, herpesvirus, myxovirus). In 1961, Cooper proposed, inter alia, the fundamentally important grouping by type of nucleic acid. Soon afterwards, Lwoff et al. (1962) put forward a universal system that has contributed much to the modern classification. The criteria were:

1 the type of nucleic acid (DNA or RNA);
2 the symmetry of the virus;
3 the presence or absence of an envelope;
4 the diameter of the nucleocapsid for helical viruses or the number of capsomeres for cubic viruses.

On the basis of these and other criteria, viruses are assigned to families (and sometimes subfamilies), genera, and species. Baltimore (1971) provided a basis for further refining classification that takes into account genome conformation and strategies for mRNA synthesis, characteristics that are being used on an increasing scale (van Regenmortel et al. 2000.

IMMUNIZATION AND ANTIVIRAL THERAPY

At the beginning of this chapter, I stated that it would not be possible to cover applied virology. Nevertheless, at a time when major successes are being scored by both virus vaccines and antiviral drugs, it is only right to include mentions of these topics, brief though they must be.

Viral vaccines

Smallpox vaccine was the first to come into general use and the first to eradicate an infectious disease worldwide (Fenner and Henderson 1988), the last natural case having occurred in Somalia in 1977. The conquest of smallpox is set fair to be repeated with other vaccines, notably poliomyelitis and measles. The development of viral vaccines reflects the advances in virological techniques over the past two centuries, ranging successively from animal inoculation to the use of chick embryos, cell cultures, and finally recombinant DNA technology (Table 1).

Antiviral therapy

Because of the intimate association between viruses and their host cells, the development of antiviral agents lagged behind that of compounds active against other microbes. Because the replication of viruses was not well understood, its basis was at first purely empirical and depended largely on random screening of many potentially useful compounds; the devising of compounds directed against specific target activities, such as attachment to the host cell, nucleic acid synthesis, and the release of mature virus, is of more recent origin.

An early development in antiviral therapy was the discovery by Isaacs and Lindenmann (1957) of interferon (IFN), a low molecular weight protein produced by cells in response to infection with viruses, some bacteria, double-stranded nucleic acids, and other biological compounds (see also Isaacs et al. 1957). Three classes of IFN (α, β, and γ) with differing properties are now recognized. The early finding that IFN inhibited a

Table 1 *Vaccines for human use developed in the past two centuries*

Decade in which introduced	Vaccine	Live (L) or inactivated (I)	Derivation
1800–1809	Smallpox	L	Animals
1880–1889	Rabies	I	
1940–1949	Yellow fever	L	Chick embryos
	Influenza	I	
1950–1959	Polio (Salk type)	I	Cell cultures
1960–1969	Rabies	I	Cell cultures
	Polio (Sabin type)	L	
	Measles	L	
	Mumps	L	
	Rubella	L	
1980–1989	Hepatitis B	I	Human plasma
	Hepatitis B	I	Yeast[a]
	Japanese B encephalitis	I	Mice or cell cultures
	Varicella	L	Cell cultures
1990–1999	Hepatitis A	I	Cell cultures

a) Genetically engineered antigen.

Table 2 *Examples of antiviral compounds developed since 1960*

Year[a]	Compound	Target	Viruses inhibited
1960	Methisazone	Ribonucleoside reductase	Poxviruses
1963	Amantadine/Rimantadine	Inhibits virus uncoating	Influenza A
1972	Ribavirin	Not yet fully defined	Respiratory syncytial virus; Lassa fever
1978	Acyclovir	DNA synthesis	Some herpesviruses
1985	Azidothymidine	DNA synthesis	HIV
1990	Saquinavir	Protease inhibitor	HIV (and probably others)

a) Approximate only. Indicates an early description rather than when the compound was first licensed for clinical use.

wide range of viruses raised hopes that it would be in the nature of a universal panacea for viral infections. This expectation was not fulfilled, but more recently IFN have proved useful in treating some cases of chronic hepatitis B. The large doses required can be produced by recombinant DNA techniques.

The precursor of all synthetic antiviral compounds was *p*-amino-benzaldehyde, 3-thiosemicarbazone (Brownlee and Hamre 1951), which inhibited vaccinia virus. A related compound, methisazone, appeared to have a prophylactic effect in smallpox contacts, but was rendered superfluous by the success of the vaccine eradication campaign. However, a more profitable approach was becoming apparent. By 1955, Matthews and Smith were able to write: 'There is a growing body of evidence suggesting that the genetic properties of viruses may reside largely in their nucleic acids, and a number of recent observations show that virus multiplication can be delayed by compounds which interfere with nucleic acid metabolism. As a class, analogues of the purine and pyrimidine bases may inhibit growth by a variety of mechanisms' (Matthews and Smith 1955). This prediction was fulfilled; the greatest achievements have in fact been the therapy and prophylaxis of herpesvirus infections with nucleoside analogs, at first idoxuridine (5-iodo-2′-deoxyuridine) and latterly acyclovir (9-(2-hydroxyethoxymethyl) guanine) (Table 2). Another such compound, zidovudine, has had a limited measure of success against infection with human immunodeficiency virus. It is, however, clear that our successors will regard this era as still part of the early history of antiviral therapy. The benefits of molecules designed to block specific receptors, drugs accurately aimed at defined targets within the replication cycle, synthetic antigens, DNA vaccines, and novel delivery systems lie still in the future.

CONCLUSION

The virologist will readily appreciate how much has had to be omitted from this brief account, but I hope that it will convey to the nonspecialist something of the many and complex strands that have combined to make the study of viruses such a leading influence in microbiology. From its beginnings, virology has provided a high degree of intellectual challenge that has attracted many leading minds, not least through its contributions to our knowledge of biological processes at the molecular level. The reader who wishes to study the fascinating story in more detail will find good accounts in the books by Lechevalier and Solotorovsky (1965) and Waterson and Wilkinson (1978).

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The origin and evolution of viruses

ESTEBAN DOMINGO AND JOHN J. HOLLAND

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THEORIES ON THE ORIGIN OF VIRUSES

Some fundamental questions such as the origin of life or the origin of viruses cannot be answered with any precision because the critical events occurred eons ago, and the questions do not lend themselves to direct experimental studies. There is no fossil record of viral sequences and even if molecular techniques enabled recovery of putative viral sequences from well-preserved fossils, it is likely that such sequences would be cryptic and difficult to relate to modern viruses because of their rapid evolution. Thus questions such as if there were (and how they were) viruses during the Cambrian explosion (a critical period from around 550 until about 485 million years ago in which a relatively rapid diversification of animal life occurred) are unlikely ever to be answered.

There have been several theories of the origin of viruses (reviewed by Joklik 1974; Luria et al. 1978; Botstein 1981; Atkins 1993):

- Viruses may be the descendants of primitive, precellular replicons that existed before the first cellular forms appeared on earth. These primitive replicons evolved to become dependent on cells for replication. Reverse transcription, which at some stage must have generated DNA from RNA, must have been active in producing primitive DNA viruses. According to this theory, the viruses we know today are the result of a long coevolutionary process of early viruses and cells which underwent a process of differentiation.
- In a completely different theory, viruses have never been descendants of simple replicons but, rather, they were the result of regressive evolution of complex microbial forms that displayed cellular organization and metabolism. A virus would be a parasitic, subgenomic form of a formerly free-living organism.
- Viruses originated from cellular DNA or RNA, or from subcellular organelles (such as mitochondria or chloroplasts). Part of the DNA or RNA could evolve to become decreasingly dependent on the cell, and eventually could include an extracellular step in its replication cycle.
- Viruses are as ancient as early cellular forms with which they have shared functional modules. The growing list of complete genomic nucleotide sequences of viral genomes and many unicellular and differentiated organisms (Mount 2001; Bushman 2002) suggests that all life forms and subcellular replicons share some basic structural and functional motifs. There are similarities between viral and cellular RNA structures and proteins involved in genome replication, proteolytic enzymes, and general patterns of genome organization and expression. No viral functions outstandingly different from similar cellular functions have been characterized. These findings of molecular genetics support the view that exchanges of functional modules, mediated by several forms of genetic recombination, together with mutation, contributed to coevolution of cells and a number of autonomous replicons, including viruses (Botstein 1981; Zimmern 1988; Gorbalenya 1995; Holland and Domingo 1998; Baranowski et al. 2001; DeFilippis and Villarreal 2001; Brown 2003).

Regardless of the details of early evolution of life on earth, and of the earliest origins of viruses, it is clear that cellular nucleic acids interact with, exchange with, and coevolve with, the DNA and RNA of viruses, plasmids, transposons, retrotransposons, and other mobile genetic elements (Hayes 1968; Monroe and Schlesinger 1983; Boeke 1988; Berg and Howe 1989; Harrison et al. 1991; Coffin 1992; Doolittle and Feng 1992; Syvanen 1994; Lodish et al. 1995; Bushman 2002). Novel viruses are among the 'new' life forms that evolve from such interactions, and the 'origination' of viruses is therefore a never-ending process (van Regenmortel et al. 2000). Their ubiquity, mobility, and capacity to recombine with cellular and other viral genetic elements put viruses (and other mobile elements) in the mainstream of all evolution on earth.

DNA viruses and RNA viruses may have had different origins or even if they shared a common ancestor they may have coevolved with cells in substantially different patterns. Examination of some distinctive features of DNA and RNA viruses may provide some insight into their evolutionary origins.

DNA viruses

DNA viruses may have arisen from combinations of cellular genes or by reverse transcription of RNA molecules. It is not yet clear, for example, whether a hepadnavirus (such as hepatitis B virus) was formerly a DNA virus that acquired a reverse transcriptase to allow replication via RNA transcripts, or if it was a retrovirus that evolved to encapsidate DNA replication intermediates instead of the RNA 'genome' (Doolittle and Feng 1992). Regardless of ultimate origins, any DNA virus has the potential to capture useful genes from cell chromosomes via DNA recombination (Cooper 2000).

DNA viruses as mobile DNA replicons

The distinction between DNA viruses and other autonomously replicating DNA replicons is sometimes tenuous. Plasmids, insertion sequences, transposons, and retrotransposons are mobile autonomous DNA replicons (Boeke 1988; Berg and Howe 1989; Coffin 1992; Doolittle and Feng 1992; Syvanen 1994; Bushman 2002) that resemble viruses in many respects, but they lack the capsid protein coats (and lipid/glycoprotein envelopes) that package viral genomes, protect them, and deliver them to appropriate receptors on susceptible host cells. It is this extracellular infectious stage that distinguishes viruses from other mobile DNA elements. Bacteriophage Mu is an example of a virus that is also a transposon (Berg and Howe 1989; Bushman 2002). It is a temperate phage, which, unlike phage lambda, can insert its DNA into numerous sites in the host bacterial chromosome. Copies of Mu DNA then transpose to many thousands of other chromosomal sites, thereby causing an enormous increase in mutation frequency. Mu therefore differs from the more common transposons because it encodes phage coat proteins which enable efficient transfer as a virus. As first suggested by Botstein (1981), it is likely that virus genomes undergo 'modular evolution' in which 'new' viruses can be created by a combination of genes or gene clusters derived from multiple sources, including chromosomes, defective viruses, plasmids, and mobile elements (Zimmern 1988; Baranowski et al. 2001).

Viroids and circular satellite RNAs

Of all the infectious viruslike mobile genetic elements, the viroids and related circular satellite RNAs (Maramorosch 1991; Robertson 1992; Semancik and Duran-Vila 1999) are the most primitive, and the most likely to have descended directly from primordial RNAs during the early evolution of life on earth. The viroids are small, naked RNA molecules several hundred nucleotides in length. They are covalently closed circular strands of RNA with extensive secondary structure, which replicate autonomously when introduced into susceptible plants, frequently causing characteristic signs of disease. They have intrinsic ribozyme self-splicing activity related to group 1 introns, and apparently replicate via a rolling-circle mechanism by interaction with plant cell transcription components. They do not require a protein coat for transmission and do not encode capsid proteins, so genome transmission and disease progression can be very slow, but effective. However, there are related satellite viruses (sometimes termed 'virusoids') that depend on coinfection of plants with an infectious helper virus to provide them with capsid proteins for efficient transmission (Vogt and Jackson 1999).

As yet, there are no known viroids (or viroid diseases) of animals or humans. Robertson (1992) has, however, pointed out the close similarity between human hepatitis D virus (delta (δ) agent) and viroids. Delta agent contains a self-cleavage viroid domain joined to a protein-coding domain that encodes the δ antigen. Furthermore, the δ agent RNA is a defective circular 'satellite' RNA that depends on coinfection with hepatitis B virus as a helper virus to provide capsid protein. This striking similarity to viroids or circular satellite RNAs led Robertson (1992) to postulate mechanisms of evolution of early self-replicating RNA genomes to form various 'conjoined' RNA replicons that ultimately evolved into mosaic DNA systems. These plausible speculations envisage early RNA replicons as progenitors of both present-day viroids and the intricate DNA-based life forms which now predominate. It should be noted that naked viroid RNAs and circular satellite RNAs thereby bear a relationship to RNA viruses similar to that of naked DNA replicons, including plasmids and

transposons, to DNA viruses. In both cases, viruses are distinguished as autonomously replicating nucleic acid mobile elements with the capacity to encode capsid proteins.

RNA viruses

RNA viruses are the most ubiquitous and diverse viruses, and they include two major groups, the riboviruses and the retroviruses. Both have very high mutation rates, and even clones (derived from a single parental genome) rapidly form diverse 'quasispecies' populations or 'mutant swarms.' The extreme mutability of RNA viruses and its biological consequences are discussed in detail in the sections on Mutation of virus genomes and Biological significance of virus quasi species.

RIBOVIRUSES

The ordinary non-retrovirus RNA viruses that replicate their RNA genomes entirely via RNA templates are called riboviruses. Their ultimate origins are uncertain, and are probably quite varied (see section on Theories on the origin of viruses). Some may be directly descended from early primordial RNA replicons, whereas others may have originated more recently by recombination, reassortment, or mutation of cellular RNAs. Regardless of their ultimate origins, it is likely that most riboviruses have exchanged RNA sequences with cellular RNAs (and other virus RNAs) and so their genomes are mosaics of sequences from multiple sources. 'New' riboviruses may frequently arise by recombination of genome segments from two or more different riboviruses. One example is western equine encephalomyelitis virus, which is a recombinant virus derived from parental genomes of eastern equine encephalomyelitis virus and from New World relatives of sindbis virus (Hahn et al. 1988; Weaver et al. 1994). Genetic recombination in all riboviruses may be common on evolutionary time scales (Strauss and Strauss 1988; Zimmern 1988). A striking laboratory example of the creation of a new virus by recombination of unrelated riboviruses was reported. Rolls et al. 1994, using recombinant DNA intermediates, created a remarkable infectious virus in which the surface glycoprotein of vesicular stomatitis virus (VSV) was expressed from a replicon of Semliki Forest virus (SFV). The SFV replicon encoded only the SFV replicase, and no structural proteins. When the VSV glycoprotein gene was inserted by molecular genetic techniques, infectious virus particles were formed by budding of plasma membrane vesicles containing the VSV glycoproteins enclosing RNA replicons. Because VSV is a negative-strand ribovirus and SFV is a positive-strand ribovirus, a highly unexpected 'hybrid virus' was created. This demonstrates not only that novel viral genome matings can be productive but also that enveloped infectious viruses can be much simpler than virologists had expected.

Chance may have played a role in the way functional modules have been linked together to form viable forms of viruses. Recently, G. Wertz and colleagues have altered the gene order of VSV and found that virtually all gene combinations tested yielded viable virus (Ball et al. 1999; Wertz et al. 2002). Therefore, founder events in processes of gene transfer and ligation may have exerted a definitive influence on the organization patterns of the viruses we see today.

RETROVIRUSES

Retroviruses, hepadnaviruses, and caulimoviruses share a unique capacity of their genomes to participate regularly as part of both the RNA and the DNA worlds. Although their ultimate origins are uncertain, it is probable that they have evolved from retroid transposable elements, as originally proposed in Temin's (1970) 'protovirus' hypothesis. There is a wide variety of 'viral' and 'nonviral' retrotransposons and related elements, including Gypsy, Copia, LINES and LINE-like elements, mitochondrial introns, mitochondrial retroplasmids, and bacterial reverse transcriptases (Boeke 1988; Temin 1989; Coffin 1992; Doolittle and Feng 1992; Bushman 2002; Brown 2003). The so-called 'viral' retrotransposons more closely resemble retrovirus genomes. All these elements have in common the presence of reverse transcriptase, but most lack envelope genes which allow cell-to-cell and organism-to-organism transmission as mature virus particles (virions). Other retroviral genes and domains such as long-terminal repeat (LTR), capsid protein, ribonuclease, integrase, and protease are variably present in various 'viral' and 'nonviral' retroid elements (Boeke 1988; Temin 1989; Coffin 1992; Doolittle and Feng 1992; Bushman 2002). Most of these retroid elements may represent little more than 'selfish' jumping genes which sometimes cause useful rearrangements of host cell DNA sequences and sometimes damage or kill their hosts via DNA alterations. At least some of them may, however, perform essential functions. For example, a LINE-like retrotransposon, TART in *Drosophila*, apparently preferentially retrotransposes to the termini of chromosomes in an essential process by which *Drosophila* telomeres are maintained (Sheen and Levis 1994). The telomerases involve reverse transcription to elongate chromosome termini (telomeres), a process required for indefinite periods of cell division in mammals (Kim et al. 1994; Cooper 2000).

Transposons have been found in the eukaryotic genomes sequenced to date such as the human genome, yeast, *Drosophila*, the worm *Caenorhabditis elegans*, mice, and several plant species (reviewed in Bushman 2002). Remarkably, 44 percent of the human genome is

made up of transposons or transposonlike elements. It is suspected that much of the 50 percent of the human genome that has not been assigned to a function, was likely contributed by mobile elements.

These findings provide strong evidence that retroid elements have been around for eons, that they can acquire additional genes, and that they can become retroviruses only if they acquire envelope genes to confer infectivity. Even now, 'new' retroviruses are continuously born (or reborn) by recombinational acquisition of cellular genes (e.g. proto-oncogenes in the case of some transforming retroviruses). Another source is the acquisition of genes from other retroviruses (e.g. LTR sequences, or altered *env* genes from proviruses in the case of mouse lymphoma virus) (Coffin 1992).

The origin of viruses is likely to remain a mystery for a long time. New observations may suggest one of the proposed mechanisms (condensed in the section on Theories on the origin of viruses) over another. In fact, multiple mechanisms may have participated in several origins of viruses at different stages of the evolution of life on earth. While it is plausible that viroids and other simple RNA elements may have evolved from primitive replicons (Semancik and Duran-Vila 1999; Vogt and Jackson 1999) it is hard to conceive the origin of complex DNA viruses (such as poxviruses, iridoviruses or herpesviruses with genomes of 130 000 up to 370 000 bp) as not being related to cells or cell organelles, either as a source of functional modules or as the starting point of a degenerative process. An interesting outcome of the development of tools to probe cellular and viral genome structures and their replication strategies is that they offer a number of plausible mechanisms (based on gene transfers, recombination, and mutation) for virus origins. Remarkably, these same mechanisms are acting on present-day viruses, and they can be observed experimentally as affecting not only virus evolution but also disease manifestations on the hosts they parasitize.

VIRUS EVOLUTION AND MECHANISMS INVOLVED

Because they can replicate rapidly with high yields of progeny, and readily mutate, recombine, and reassort their genomes, most viruses can exhibit great genetic plasticity and adaptability. This provides the possibility for extremely rapid evolution, but does not dictate constant rapid genome evolution. Viruses, like other living things, can have periods of relative evolutionary stasis, punctuated by periods of evolutionary disequilibrium and rapid genetic change (Holland 1992).

Recombination

Recombination affords rapid, major rearrangements of viral genomes, and this mechanism for evolution is widespread among DNA viruses and RNA viruses. Both homologous and nonhomologous recombination are very common, and the latter has the potential to produce quite extensive genome alterations and bizarre phenotypes.

DNA VIRUS RECOMBINATION

Mechanisms of DNA recombination and transposition have been widely studied, are well characterized (Kucherlapati and Smith 1988; Cooper 2000; Bushman 2002) and will not be reviewed here. Recombination or transposition allows DNA viruses to acquire new genes from other viruses; to integrate into, and excise from, host-cell chromosomes; to generate defective virus genomes and to rescue genes from them; and to capture host-cell genes or to transfer them to new host cells. In short, DNA virus genomes have available to them all the recombinatorial mechanisms extant in the DNA-based world, and this gives them the potential for profound genome rearrangements and for extensive genetic interactions with DNA-based hosts. Many DNA virus genomes contain genes that have been captured (or rescued) from earlier hosts. For example, the thymidine kinase (TK) genes of herpesviruses exhibit broad substrate specificity, and have apparently evolved from cellular deoxycytidine kinase enzymes (Harrison et al. 1991), whereas poxvirus TK genes were apparently derived from cellular TK genes (Boyle et al. 1987). Defective viruses produced by recombination are ubiquitous among DNA viruses and may often help shape their evolution by maintaining functional (and rescuable) gene modules. Botstein (1981) suggested that the product of virus evolution 'is not a given virus but a family of interchangeable genetic elements (modules) each of which carries out a particular biological function. Each virus encountered in nature is a favorable combination of modules. . . .' Exchange of modules allows the evolution of viral genes to occur within differing viral genomes, host chromosomes, defective viruses, plasmids, transposons, etc. Viral genes can not only associate with plasmids and episomes and other replicons, but they can also become episomes or plasmids (autonomous, noninfectious replicons). Examples are bacteriophage F1 selected for 'benevolent' interactions with host *Escherichia coli* (Bull and Molineux 1992) and Epstein–Barr virus (EBV) virus 'episomal' circular genomes within transformed (immortalized) B lymphocytes (Lindahl et al. 1976; Lawrence et al. 1988; Middleton et al. 1991; Kirchmaier and Sugden 1995). The 'benevolent' F1 plasmids eventually lost viral genes and infectivity, but replicated efficiently within host bacteria, and the EBV episome genomes in EBV-immortalized B cell lines are generally non-infectious or poorly infectious unless 'induced' (Weigel and Miller 1983).

RETROVIRUS RECOMBINATION

Retroviruses, of course, have the potential to recombine at the proviral DNA level (as for any other DNA).

However, they have the additional capacity to undergo recombination as a result of the tendency for reverse transcriptases to switch from one template to another (copy choice) during DNA chain elongation. This occurs at an extremely high frequency, is responsible for most retrovirus recombination events, and can play a major role in retrovirus evolution (Coffin 1992). Recent quantifications have estimated a minimum rate of 2.8 crossovers per replication cycle of a HIV-1-based vector system (Zhuang et al. 2002).

One of the most striking developments during the acquired immunodeficiency syndrome (AIDS) epidemic has been the discovery of the contribution of recombination to generate new variant forms of HIV-1 (Thomson et al. 2002). An increase of the number of circulating HIV-1 recombinants is likely to be influenced by higher frequencies of re-infections with different HIV-1 viruses as the AIDS epidemic advances, and geographical boundaries for HIV-1 subtypes become gradually fuzzier. Also, HIV-1 evolution is associated with an expanded mutant repertoire which increases the probability of producing recombinants with sufficient relative fitness (a measure of the relative replication capacity of a virus; see section on Biological significance of virus quasi species) to become dominant in infected individuals and to be transmitted.

RIBOVIRUS RECOMBINATION

Riboviruses can also exhibit very high rates of genome recombination due to frequent copy choice switching from one template RNA strand to another by RNA replicase. The RNA replicase carries the growing RNA chain to another RNA template and can align it precisely by base pairing. Therefore, most recombination is homologous ('legitimate') for positive-strand RNA viruses, which replicate their genomes as naked RNA (King 1988; Lai 1992; Wimmer et al. 1993; Nagy and Simon 1997). Such homologous recombination is clearly important for RNA virus evolution. For example, recombinants between different serotypes of poliovirus vaccine strains occur frequently in vaccinated humans and can be rather strongly selected in their intestinal tract (Kew et al. 1981; Minor et al. 1986; Minor 1992; Agol 2002; Dahourou et al. 2002). During picornavirus infections, average recombination frequencies in cell culture using selectable markers have been estimated in 10–20 percent of the progeny; recombination frequencies tend to decrease with the genetic distance between the parental genomes (King 1988; Lai 1992). Frequent homologous recombination helps to shape the evolution of numerous riboviruses such as coronaviruses (Lai 1992) and brome mosaic virus of plants (Rao and Hall 1990; Nagy and Bujarski 1995; Nagy and Simon 1997). Even though copy choice recombination is much more common than non-homologous ('illegitimate') recombination among positive-strand riboviruses (and retroviruses), non-homologous recombination can occasionally produce major genome changes of great evolutionary importance. For example, the coronavirus mouse hepatitis virus appears to have acquired its hemagglutinin-esterase gene by recombination with influenza C virus, a negative-strand virus (Luytjes et al. 1988). Mucosal disease variants of bovine viral diarrhea virus (BVDV), a positive-strand pestivirus, are cytopathogenic viruses often generated either by multiple mutations or by recombination of parental, nondefective, noncytopathogenic BVDV genomes with cellular mRNAs (Meyers et al. 1991; Tautz et al. 1994; Kummerer et al. 2000).

Negative-strand riboviruses, which replicate their genomes as ribonucleoprotein strands rather than naked RNAs, generally undergo homologous recombination much less frequently than positive-strand RNA viruses. Replicase template switching occurs frequently, but this nearly always produces nonhomologous recombination because the growing RNA strands, being ribonucleoprotein, are not able to anneal and align the growing RNA chain precisely on homologous segments of the new template (Barrett and Dimmock 1986; Roux et al. 1991). Recently, homologous recombination of the negative-strand RNA Tula virus has been described (Plyusnin et al. 2002). To what extent homologous recombination in nature occurs with negative-strand RNA viruses is not known, but the study by Plyusnin et al. (2002) has certainly opened prospects for interesting research. Recombination of negative-strand riboviruses regularly produces biologically important variant genomes, particularly defective interfering (DI) particles. These can exert significant modulatory effects on parental virus infections (Barrett and Dimmock 1986; Huang 1988; Roux et al. 1991). Occasionally, quite bizarre recombinants between cellular RNAs and viral RNA genomes arise. For example, when a 54 nucleotide segment of 28S ribosomal RNA was inserted into the hemagglutinin gene segment of influenza A virus (by recombination during growth in chick embryo cells), the resulting recombinant virus was much more virulent, causing systemic lethal infections in chickens (Khatchikian et al. 1989). Similarly, a number of isolates of potato leafroll virus have acquired a 119 nucleotide segment from plant chloroplast DNA (Mayo and Jolly 1991), and cell culture replication of well-characterized strains of poliovirus (Charini et al. 1994) and sindbis virus (Monroe and Schlesinger 1983) led to acquisition of a short ribosomal RNA segment and a tRNA, respectively. Thus, although major nonhomologous recombination events are generally infrequent in ribovirus evolution, they occur sufficiently often to have significant evolutionary effects. They might even influence the results of biotechnological manipulations in which resistance to virus is conferred on transgenic plants expressing viral mRNA segments. For example, recombination between a defective plant virus genome and a viral

mRNA transcript expressed in transgenic plants restored infectivity to the defective viral genome (Greene and Allison 1994).

Recombination may have two disparate effects in evolution. It may produce entirely new genome combinations from distant parental molecules such as the heterologous recombination events that led to influenza viruses with a fragment of ribosomal RNA (Khatchikian et al. 1989). This is an exploratory activity that may produce novel genome combinations, and only an extremely small fraction out of many unsuccessful trials are likely to produce high fitness virus (see section on Biological significance of virus quasi species). For example, the influenza recombinant was very virulent for chickens, but did not survive to represent an epidemiologically relevant threat for other hosts. This unique recombinant probably occupied a very high fitness peak surrounded by deep, low fitness valleys in other environments (Wright 1982; Domingo et al. 2001a). Recombination may have an opposite effect in the rescuing of viable gene combinations from debilitated parents. This conservative activity will often be mediated by homologous recombination, and it is one of the possible mechanisms to counteract fitness loss in RNA virus due to Muller's ratchet (see section on Biological significance of virus quasi species).

Reassortment of ribovirus genome segments

A number of riboviruses have segmented genomes either of single-stranded RNA (influenza viruses, arenaviruses, bunyaviruses, several plant viruses) or double-stranded RNA (reoviruses, rotaviruses). Their genomes consist of a number of specific segments, each of which encodes one or more gene products. Each specific segment must be included in a virion together with all other segments in order for that virion to be infectious and to replicate infectious progeny. As expected, the various genomic segments from genetically different (but related) viruses can reassort to produce reassortant viruses, a process equivalent to the reassortment of maternal and paternal chromosomes that occurs during meiotic gametogenesis in diploid eukaryotic organisms. Ribovirus gene reassortment occurs when two distinct viruses simultaneously infect the same cell(s). Gene reassortment can cause major genome alterations during evolution of segmented riboviruses. The most striking examples are the influenza A viruses. Both the 1957 Asian and the 1968 Hong Kong influenza pandemics began in south China. These 'new' human viruses arose by reassortment of gene segments apparently derived from circulating avian viruses and from circulating human virus sources. Domestic pigs were suggested as the probable hosts in which the reassortment events took place ('mixing vessels') (Gorman et al. 1992; Webster et al. 1992; Webster 1999; Wuethrich 2003). It is probable that the next major influenza A pandemic will emerge some time during the next few decades, and that it will be due to reassortment events occurring in swine or humans or another 'mixing vessel.' Influenza C viruses, which usually circulate as multiple, independently evolving lineages in humans, have also undergone gene reassortment in man (Peng et al. 1994).

Major gene reassortment events between avian and human influenza A viruses are rare. This is undoubtedly because the currently circulating gene segments are co-evolving (and selected) for optimal interactions with each other. Therefore, only rare events could create a new virus with optimally interacting gene segments derived from widely divergent sources. Reassortment is common; it is the low probability for efficient interactions among diversely derived gene segments that greatly restricts the emergence of new influenza A pandemics. Reoviruses and many other segmented riboviruses also readily undergo genetic reassortment of segments from related lineages, and similar principles apply (Ramig et al. 1983; Desselberger 1996). There is, for example, evidence for segment reassortment in nature among viruses related to hantavirus pulmonary syndrome (Li et al. 1995), and for interspecific segment reassortment in the evolution of cucumoviruses of plants (White et al. 1995).

Mutation of virus genomes

Mutation is the primary driving force for all evolution, and it occurs inevitably and inexorably in all life forms, including viruses. In the RNA viruses (both riboviruses and retroviruses), a combination of small genome size, high mutation rates, high virus yields and very rapid replication, can produce spectacular rates of evolution. DNA viruses apparently mutate and evolve at lower rates, but they can nevertheless be quite mutable and biologically adaptable.

MUTATION AND EVOLUTION OF DNA AND RNA VIRUSES

The mutation rates of DNA bacteriophages have been characterized much more thoroughly than have those of the DNA viruses of humans and animals. Drake 1969 estimated the mutation rate of *E. coli* bacteriophages λ and T4 to be about 2×10^{-8} substitutions per nucleotide, two orders of magnitude higher than that of the host bacterium. Although mutation rates vary widely at individual genome sites, Drake (1991) has shown that there is a rather constant rate of spontaneous mutations per genome per replication in DNA-based microbes (including bacteria, yeasts, molds, and three DNA bacteriophages). Their genomic mutation rates per replication varied only about 2.5-fold around an average value of 0.0033 mutations per genome per replication round. Because the genome sizes of these diverse

DNA-based microbes vary by thousands-fold, their average mutation rates per base pair also vary by thousands-fold. Drake (1993) later calculated the genomic rates of spontaneous mutation among RNA viruses (the only known life forms with RNA genomes) and found them to be consistently higher. Animal lytic viruses (riboviruses) and RNA bacteriophages have an average genomic mutation rate per replication in the neighborhood of one, and retroviruses average slightly (about ten-fold) lower genomic mutation rates (Drake 1993; Drake and Holland 1999). Over all, these calculations tell us that genomic mutation rates do not vary markedly. They are remarkably fixed for DNA-based organisms, and are also fairly constant, but higher for RNA-based life forms (riboviruses and retroviruses). Obviously, as Drake (1993) pointed out, evolutionary forces must be shaping genomic mutation rates.

Despite the quite constant nature of genomic mutation rates, there is enormous variation in the mutation rates per nucleotide, per genome replication. This difference arises from the huge variation in genome sizes among living cells and subcellular replicons (Drake 1991; Drake 1993). Complex DNA viruses such as the herpesviruses are replicated by a viral-coded DNA polymerase endowed with a $3'\rightarrow5'$ exonuclease proofreading activity to increase DNA copying fidelity (Hwang et al. 1997). Proofreading and postreplicative misincorporation-correcting activities have evolved to ensure the copying fidelity needed for maintenance of genetic information of complex DNA genomes. By contrast, RNA replicases and reverse transcriptases lack exonuclease proofreading activities, as evidenced by biochemical tests (Steinhauer et al. 1992; Cameron et al. 2002). RNA viruses have mutation rates in the range of 10^{-3} to 10^{-5} substitutions per nucleotide copied, expected from their small genome size (Batschelet et al. 1976; Eigen and Biebricher 1988; Drake and Holland 1999). These values exceed by several million-fold the base misincorporations rates of their eukaryotic hosts. A clear example of this was given by Gojobori and Yokoyama (1985), who showed that the Moloney murine sarcoma virus, a retrovirus of mice, evolves at a rate a millionfold higher than the rate of its integrated cellular homologue, c-*mos*. This allows (but does not necessitate) rates of RNA virus evolution to proceed millions of times faster than that of their animal and human hosts (Holland et al. 1982; Smith and Inglis 1987; Domingo et al. 1988, 1992, 2001a; Eigen and Biebricher 1988; Domingo and Holland 1994; Duarte et al. 1994).

Extremely high mutation rates generate 'mutant swarms' or 'quasispecies' populations of RNA viruses, and can provide great adaptability. This factor must be balanced against the inevitable high levels of lethality when the rates are too high. Eigen and Biebricher (1988) have reviewed results that show by computer simulation that when mutation rates increase they suddenly exceed an 'error threshold' at which there is a 'melting' of genetic information or a transition into 'error catastrophe.' They have proposed that RNA virus quasispecies generally adapt to the error threshold at which the misincorporations rate per nucleotide per replication will be in the neighborhood of $1/\nu$ where ν is the length (in bases) of the genome. This figure can vary somewhat, depending on a selectively factor for the most fit 'master' sequences within a quasispecies population (Eigen and Biebricher 1988). This threshold limit dictates that genomic mutation rates generally cannot exceed values of about one. Drake's (1993) calculations indicate that most RNA virus genomic mutation rates are in fact close to one. Strong chemical mutagenesis of RNA viruses (inducing high lethality in progeny) only slightly increases mutation rates in viable progeny (Holland et al. 1990). This indicates that RNA viruses are at or near their error threshold. As Eigen and Biebricher (1988) have pointed out, occasional, short duration violations of the error threshold can provide brief evolutionary advantages, but persistent violations are lethal for viral genomes.

One mechanism for radical mutational changes in RNA viruses is biased hypermutation (Cattaneo et al. 1988; Wain-Hobson 1992; Cattaneo 1994; Martínez et al. 1994; Meyerhans and Vartanian 1999). Biased hypermutation can produce long segments of viral genome in which certain bases are substituted in a regular, recurring pattern. There are two major mechanisms, one of which involves deamination mediated by cellular enzymes (Cattaneo 1994; Harris et al. 2003). The other mechanism involves reverse transcriptase errors in retrovirus replication due to biased deoxyribonucleotide triphosphate pool concentration (Wain-Hobson 1992; Martínez et al. 1994). Either can produce sudden, massive genome changes.

In general, most RNA viruses are life forms that replicate and evolve near the extreme limits of mutability and adaptability. Genomic mutation rates in the range around 0.1–1 dictate that even clones of riboviruses and retroviruses are quasispecies, or mutant swarms, rather than collections of identical virus genomes. This quasispecies nature of RNA virus populations has numerous biological consequences, some of which are outlined below.

BIOLOGICAL SIGNIFICANCE OF VIRUS QUASI SPECIES

First and foremost, quasispecies populations endow RNA viruses with enormous environmental adaptability and capacity for extremely rapid evolution (Holland et al. 1982, Holland et al. 1992; Domingo et al. 2001a). Mutations are immediately seen when an RNA virus starts replicating in a new host, as documented with recent emergences such as HIV-1 and the severe acute respiratory syndrome (SARS) coronavirus (Holmes and Enjuanes 2003; Marra et al. 2003; Rota et al. 2003).

Rates of evolution for RNA viruses are often in the range of 10^{-2}–10^{-4} substitutions per nucleotide site per year (Domingo and Holland 1994). During persistent infections of cattle with biological clones of foot-and-mouth disease virus (FMDV), rates of evolution approached 10^{-1} substitutions per site and year in the genomic region encoding capsid protein VP1. This is one of the largest evolutionary rates recorded and demonstrates the capacity for rapid genetic change of viral quasi species evolving in their natural hosts (Gebauer et al. 1988). In sharp contrast, rates of evolution for cellular genes have been estimated in 10^{-8}–10^{-9} substitutions per nucleotide and year (reviewed by Domingo and Holland 1994).

Certain regions of an RNA virus genome (and certain single-base sites) may be much less variable (more highly conserved) than others and therefore evolve at much slower rates. In any currently circulating human strain of influenza A virus, evolution is gradual, and virus surface antigens evolve much more rapidly than do internal proteins (Webster et al. 1992). Sequence comparisons among all influenza viruses, all rhabdoviruses or all viral reverse transcriptases show that, eventually, nearly all sites can undergo viable mutations. Influenza A virus strains apparently evolve much more slowly in birds than in humans, even though these human strains have been derived from avian sources (Webster et al. 1992). Similarly, many arthropod-borne RNA viruses such as alphaviruses evolve about an order of magnitude more slowly in nature than do most other RNA viruses (Weaver et al. 1992, 1994; Strauss and Strauss 1994). Presumably, adaptive constraints necessary for efficient replication in two quite different environments (animal and insect tissues) can limit the rate of arbovirus evolution. But it is the evolutionary adaptability of RNA viruses that allows insect vector–host transmission cycles to evolve in the first instance. With very few exceptions, viruses that infect both insects and animals (or insects and plants) are RNA viruses. Generally, only RNA virus quasi species have the diversity and adaptability to move readily back and forth between quite diverse selective environments. In a number of cases of evolutionary stasis of RNA viruses, evolutionary rates are in the range of 10^{-4} substitutions per nucleotide per year, still 10^4–10^5-fold more rapid than cellular genes (Domingo et al. 2001a). RNA virus populations can reach adaptive equilibrium with host species, and relative evolutionary stasis results, whereas the same virus genes and replication enzyme in a new host can be driven to disequilibrium and rapid evolution when introduced into different selective environments (Holland et al. 1982; Domingo et al. 1988, 2001a; Domingo and Holland 1994).

Wide variations in evolutionary rates (among different genomic regions or within the same region depending on epidemiologic features and time intervals between sequence determinations) are expected from quasi species dynamics. This is because perturbations of mutant spectra that lead to modifications of dominant sequences are unpredictable. Therefore, a 'molecular clock' will rarely operate during evolution of an RNA virus (Domingo et al. 2001a). Minimal requirements for the measurements of evolutionary rates are that a founder sequence and its time of appearance in nature be identified and that there is a proper reconstruction of the evolutionary pathways of its progeny (Hillis et al. 1996). But even these conditions will rarely yield a constant rate of RNA virus evolution in nature because the response of individual hosts to the infection will not be identical (Holland et al. 1992) and dominant sequences will not be renewed in a time-dependent manner but rather in an environment-dependent manner.

Quasi-species dynamics: fitness variations and molecular memory

Because of their rapid rate of replication, high mutation rates, frequently large population sizes, and their limited genome length, RNA viruses have a great capacity for exploration of sequence space. This refers to the total number of possible sequences theoretically available to a genome (Eigen and Biebricher 1988; Domingo et al. 2001a). A numerical approximation illustrates this point. A viral genome of 10 000 residues has a total of 3×10^4 possible single mutants, which is below the population size of many viral populations. By contrast, in a mammalian genome the number of possible single mutants is about 10^{10}, a value which is well above the population size of any mammalian species. An essential feature of sequence space is the high connectivity among its different points which facilitates 'walks' to high fitness points. Fitness is a measure of the relative replication capacity of a virus in a given environment. Fitness differences have been quantitated in vivo by infecting animals with two viruses which compete for replication (Carrillo et al. 1998) and ex vivo, by infection-competition experiments in cell culture using variant viruses from in vivo infections (Quiñones-Mateu and Arts 2002; Ball et al. 2003). Model studies in cell culture have shown that serial passages of large populations of viruses result in fitness gains when measured in that same environment (Novella et al. 1995a; Escarmís et al. 1999). By contrast, repeated plaque-to-plaque transfers (serial bottleneck events) of virus in cell culture result in fitness losses (Chao 1990; Duarte et al. 1992; Escarmís et al. 1996; Yuste et al. 1999). During large population passages, selection of the best adapted mutant distributions can occur, while upon plaque-to-plaque transfers genetic drift prevails with the random accumulation of mutations, and little opportunity for compensation of their deleterious effects. In this case Muller's ratchet operates (Muller 1964). However, the virus population size that leads to fitness gain or fitness loss depends on the initial fitness of the population. Low

fitness virus tends to gain fitness upon passage of modest population numbers while for high fitness viruses the usual large population passages may not be sufficient for fitness gain (Novella et al. 1995b, 1999).

One of the consequences of quasispecies dynamics as it affects fitness variations is that viruses may maintain a memory of their past evolutionary history, as documented with independent evolutionary lineages and genetic markers of FMDV in cell culture (Ruíz-Jarabo et al. 2000, 2002; Arias et al. 2001). When FMDV genomes that were dominant in a population were outcompeted either by revertants or superior variants, the proportion of the outcompeted genomes did not return to a basal level dictated by mutational pressure but, instead, remained at a significantly higher level termed memory level. Memory levels were dependent on the initial fitness of the dominant genomes destined to become memory, and this is in agreement with theoretical predictions of quasispecies dynamics (Ruíz-Jarabo et al. 2002). Genetic bottlenecks erase memory as expected from memory being deposited in minority components of an evolving quasispecies. Memory reflects the behavior of viral quasispecies as complex adaptive systems (Frank 1996), and may allow viral quasispecies to respond more readily to a selective constraint that has been previously experienced by the same population, provided no genetic bottlenecks intervened. The presence of memory shows that mutant spectra of viral populations may not be only a random distribution of variants but may also be structured according to evolutionary history. This has a number of implications for viral diagnosis, now under investigation (Domingo et al. 2003).

EFFECT OF MUTATIONS ON VIRAL PHENOTYPES

Very often only one, or a few mutational changes can allow a virus to gain virulence, change its tissue tropism, or become resistant to an antiviral drug, or a monoclonal antibody, etc. (Holland et al. 1982; Smith and Inglis 1987; Domingo et al. 1988, 1992, 2001b; Domingo and Holland 1992, 1994; Duarte et al. 1994). Compared with the average (consensus) genome sequence, a large RNA virus quasispecies population will potentially contain almost every possible single-base change (see section on Quasi-species dynamics: fitness variations and molecular memory) and many double- and triple-base change combinations required for significant biological changes, so population selection rather than further mutation will often be sufficient. This is clearly illustrated by a study of HIV-infected individuals in whom antiretroviral inhibitor-resistant mutants were already present in the complete absence of current or prior drug treatment (Nájera et al. 1995). Following antiviral drug treatment of influenza-infected (Hayden and Hay 1992) or HIV-infected (Richman 1992) individuals, a substantial proportion of drug-resistant variants can emerge within days or a few weeks. Use of additional antiretroviral inhibitors has not ceased to enlarge a list of single and multiple mutations in the reverse transcriptase and protease of HIV that confer various levels of resistance to one or several inhibitors due primarily to mutation but also to recombination. This manifestation of adaptability of HIV constitutes a major drawback for the control of AIDS (Coffin 1995; Cabana et al. 1999; Menéndez-Arias 2002). Inhibitor-resistant mutants of other RNA viruses are also readily selected, as expected from high mutation rates and quasispecies dynamics (Domingo et al. 2001b).

Mutations in structural and nonstructural viral proteins may alter host-cell tropism and host range, with implications for viral disease emergence and re-emergence (Baranowski et al. 2001). Because of a frequent overlap between receptor-recognition sites and antigenic sites located on the surface of viral capsids and envelopes, a coevolution of host-cell tropism and antigenicity can take place (Stewart and Nemerow 1997; Baranowski et al. 2001), a concept first described with influenza virus (review by Skehel and Wiley 2000).

Large population passages of FMDV in cell culture offer a striking example of phenotypic modification resulting in expansion of host-cell tropism, with the use of integrins, heparan sulfate, and a third unidentified entry pathway to infect even the same cell type (reviewed by Baranowski et al. 2001; Airaksinen et al. 2003). Recent evidence suggests that antigenic variation that affects integrin recognition may be operating in vivo, again a demonstration of the adaptive potential of viral quasispecies within natural hosts (Tami et al. 2003; Zhao et al. 2003).

VIRUS EVOLUTION AND NEW ANTIVIRAL STRATEGIES

The Darwinian principles of genetic variation, competition, and selection that dominate biological evolution underlie several problems of disease control (antibiotic resistance in bacteria, drug resistance in eukaryotic pathogens, heterogeneity of tumor cells and metastasis, etc.) (review by Domingo et al. 2001a). Difficulties are even greater for the control of diseases associated with some genetically and antigenically highly variable viruses, and no effective preventive vaccines or therapeutic designs have yet been developed for important diseases such as AIDS or hepatitis C infections.

A new antiviral design based on the concept of error catastrophe (Eigen and Biebricher 1988; Eigen 2002) is under intensive investigation. For any complexity (amount of genetic information) hereditarily transmitted, there is a maximum error rate compatible with the maintenance of genetic information. Experimental evidence for the existence of an error threshold has been obtained with a number of RNA viruses including

poliovirus (Holland et al. 1990; Crotty et al. 2000, 2001), VSV (Holland et al. 1990; Lee et al. 1997), FMDV (Sierra et al. 2000; Pariente et al. 2001, 2003; Airaksinen et al. 2003), hepatitis GBV-B virus (Lanford et al. 2001), hantavirus (Severson et al. 2003), lymphocytic choriomeningitis virus (LCMV) (Grande-Pérez et al. 2002; Ruiz-Jarabo et al. 2003; Baranowski et al. 2003), and HIV (Loeb et al. 1999; Loeb and Mullins 2000). Interestingly, the antiviral nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to be mutagenic for a number of RNA viruses (Crotty et al. 2000, 2001; Lanford et al. 2001; Airaksinen et al. 2003; Severson et al. 2003). Its pharmacologically active ribavirin triphosphate form is a substrate for some viral RNA-dependent RNA polymerases (Crotty et al. 2000; Maag et al. 2001; Graci and Cameron 2002). Ribavirin is used clinically to treat some viral infections, including hepatitis C virus infections in combination with interferon. Therefore, it is possible that some of the antiviral effects already documented for ribavirin in clinical practice were exerted through the transition of viral replication into error catastrophe.

An encouraging result regarding the potential application of an error catastrophe-based antiviral approach has been obtained by de la Torre and his colleagues (Ruiz-Jarabo et al. 2003) by showing that administration of the mutagenic base analogue 5-fluorouracil to mice prevented the establishment of a persistent infection with LCMV in mice. This constitutes a proof of principle of the feasibility of an error catastrophe-based approach as an antiviral strategy.

This chapter has shown that in addition to the fascinations and mysteries of virus origins and their evolution that we can observe today, a knowledge of the mechanisms underlying virus evolution has important implications for the understanding of viral disease and for the design of better strategies for their control. This is an enlightening demonstration on how basic research may lead to practical applications in the most unexpected ways.

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The nature and classification of viruses

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THE FILTERABILITY OF VIRUSES

The first evidence that viruses are infectious, disease-causing agents different from pathogenic bacteria was obtained by filtration experiments with Chamberland filters. These bacteria-retaining filters which had been used by Pasteur to remove pathogenic microorganisms from water were used by Dmitri Ivanovsky in his classical studies of tobacco mosaic disease published in 1892. Ivanovsky showed that when sap from a diseased tobacco plant was passed through the filter, the filtrate remained infectious and could be used to infect other plants. The same experiment was repeated in 1898 by Beijerinck who again showed that the filtered tobacco sap was still infectious. Beijerinck went further and established that the infectious agent was able to diffuse through several millimeters of an agar gel, from which he concluded that the infection was caused not by a microbe but by what he called a *contagium vivum fluidum* or contagious living liquid. He called the agent which reproduced itself in the plant, a virus. During the same year, Loeffler and Frosch who were investigating foot-and-mouth disease reported that although the causative agent passed through a Chamberland-type filter, it did not go through a Kitasato filter which had a finer grain than the Chamberland filter. From this result, Loeffler concluded that the virus was a corpuscular particle and not a soluble agent as claimed by Beijerinck.

Although all historical accounts of the beginnings of virology refer to the work of Ivanovsky, Beijerinck, and Loeffler, there is disagreement among various authors about who should be credited with the discovery that viruses were a new type of infectious agent (Lvov 1993; Bos 1995; Witz 1998). This is an interesting debate since it concerns the question of what is a scientific discovery. Although Ivanovsky was clearly the first one to show that the agent causing the tobacco mosaic disease passed through a bacteria-retaining filter, all his publications show that he did not grasp the significance of his observation. He actually believed that the filter he used might have had fine cracks and that small spores of a microbe might have passed through the filter. More than 10 years after his initial observations he remained convinced that he was dealing with a bacterium rather than with a new type of infectious agent (Bos 1995). Beijerinck on the other hand, realized he was dealing with something different from a microbe but he thought that the virus was an infectious liquid and not a corpuscular particle as claimed by Loeffler (Witz 1998). Only Loeffler correctly concluded that the virus causing foot-and-mouth disease was a small particle stopped by a fine-grain Kitasato filter. The debate about who should be considered the founder of virology may be settled only if it is accepted that, in order to make a discovery, it is not sufficient to make a novel observation (i.e. the filterability of an infectious agent) but that, in addition, it is also necessary to interpret the observation correctly (Root-Bernstein

1989). Good science does not consist only in making new observations or collecting new data but it requires also unbiased, imaginative thinking which enables the scientist to arrive at the correct interpretation of his experimental findings. Loeffler's interpretation of his filtration experiments came the closest to the modern concept of a virus. However, it was only the work of William Elford with graded collodion membranes, done more than 30 years later, which established that different viruses had particle diameters of 20–200 nm (Grafe, 1991). The actual morphology of virus particles was finally elucidated only when the particles could be visualized by electron microscopy.

THE NATURE OF VIRUSES

At the beginning of the last century, viruses were characterized only by negative features, i.e. they were not identifiable by light microscopy, they could not be cultured on conventional bacteriological media, and they were not retained by filters used for sterilization (Grafe 1991). The perception of what is a virus changed dramatically in 1935 when Wendel Stanley showed that tobacco mosaic virus could be crystallized. This seemed to indicate that an infectious virus was a chemical molecule rather than an organism and it stimulated an intense interest in viruses as entities at the borderline between chemistry and biology. Many scientists became fascinated with viruses 'as living molecules.' This led physicists like Max Delbrück to turn to the study of bacteriophages because these viruses were more suitable for the quantitative analysis of virus multiplication. A reductionist, chemical approach to the study of biology was also stimulated by Erwin Schrödinger's book *What is life* published in 1945. Viruses were increasingly studied by physicochemical techniques and these studies contributed significantly to the development of molecular biology (Grafe 1991).

As our knowledge of the biochemistry of virus multiplication increased, it became clear that viruses depended for their multiplication on the protein-synthesizing and energy-producing systems of the host cell. Viruses were recognized to be subcellular infectious agents that, at one stage of their growth cycle in the infected cell, were reduced to their nucleic acid component. The virus particle or virion appeared simply as one stage of the virus life cycle. The virion was seen to protect the viral genome from degradation by nucleases and ultraviolet radiation and to allow the infectious agent to be transferred to a new host.

Whether or not viruses should be regarded as living organisms was regarded by some to be only a matter of taste. The question was sidestepped by Lwoff (1957) who declared that 'viruses should be considered as viruses because viruses are viruses.' However, it was generally accepted that viruses belong to biology since they possessed some of the properties of living systems, such as having a genome and being able to adapt to particular hosts and biotic habitats. On the other hand, viruses do not possess some of the essential attributes of living systems, such as the ability to capture and store free energy, and as a result they lack the characteristic autonomy that arises from the presence of a set of integrated, metabolic activities. Viruses are replicated through the metabolic activities of infected cells and they become part of a living system only after they have infected a host cell. The consensus among biologists at present is that the simplest system that can be said to be alive is a cell. The individual macromolecules and organelles found in a cell are not considered to be alive and there is indeed little justification for regarding viruses as living microorganisms. They are nonliving, infectious entities and they can be said, at best, to lead a kind of borrowed life (Van Regenmortel 2000a). According to D.J. McGeoch, viruses can be described as 'mistletoe on the Tree of Life' (Calisher et al. 1995).

The distinction between viruses and various types of organisms is highlighted when the functional roles of proteins present in viruses and in unicellular and multicellular organisms are compared. When proteins are classified in three broad, functional categories corresponding to energy utilization, carriers of information, and communication mediators, the proportion of each protein class found in viruses differs markedly from that found in living organisms (Tamames et al. 1996; Patthy 1999). Viruses have the highest proportion of proteins involved in information processes, i.e. in the control and expression of genetic information, and they have very few proteins of the energy and communication classes (Figure 1). This distribution obviously reflects the fact that viruses utilize the host cell's metabolic machinery and rely entirely on the energy supply system of the host they infect. By contrast, bacteria have the highest proportion of proteins of the energy

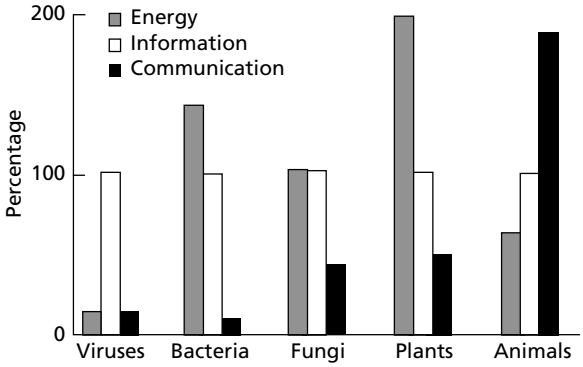

Figure 1 *The proteins of viruses, microorganisms, plants, and animals have different functional roles. The vertical bars represent the proportion of proteins in the categories of energy utilization and carrier of information relative to those in the category of mediator of information. Viruses have the highest proportion of proteins involved in information processes, i.e. in the control and expression of genetic information (from Patthy 1999).*

class involved in small molecule transformations whereas animals have a high proportion of proteins associated with intra- and intercellular communication (Figure 1).

VIRUSES SHOULD NOT BE CONFUSED WITH VIRUS PARTICLES OR VIRIONS

A virus is an elementary biosystem that replicates as an intracellular parasite in its host cell. During its multiplication cycle it takes on various forms and manifestations, for instance as a replicating nucleic acid in the host cell. One stage in the so-called 'life cycle' of a virus corresponds to the virus particle or virion which can be characterized by various intrinsic, structural, and chemical properties such as size, mass, chemical composition, etc. A virus, on the other hand, possesses, in addition, various relational properties that exist only by virtue of a relation with other objects such as a host or a vector. Some of these properties, for instance the replication cycle, are emergent properties that are possessed only by the system as a whole and are not present in its constituent parts. A virus should thus not be reduced to the physical constituents and chemical composition of a virion and it is necessary to include in its description also the functional activities that become actualized only inside the host as well as a variety of other biotic interactions (Van Regenmortel 2000a). Confusing the entity 'virus' with virion is similar to confusing the entity 'insect' with either a pupa, a caterpillar or a flying moth or butterfly. It is unfortunate that, in the scientific literature, virions are often referred to as the virus, for instance when one talks of 'the picture of the virus' or of 'the process of purifying the virus.'

VIRUS CLASSIFICATION AND NOMENCLATURE

The earliest attempts to classify viruses were based on the type of diseases they caused and the names given to the causative viruses were derived from the symptoms of the diseases and the hosts and organs that were infected. By the end of the 1950s, enough information on some types of virus particles had been gathered to allow the recognition of groups like the herpesviruses, the myxoviruses, and the poxviruses as well as several groups of elongated plant viruses. Following the development of the negative staining technique for observing virions in the electron microscope (Brenner and Horne 1959), it became relatively easy to establish the morphology of virus particles also in crude extracts. This in turn enabled the placement of viruses in genera and families according to virion morphology and stimulated the development of the Lwoff et al. (1962) scheme of virus classification. This hierarchical system was based on the assumption that the most important characteristics for classifying viruses were nucleic acid type and virion morphology. Although this scheme was initially criticized because the choice of criteria was deemed to be arbitrary, the attempt to replace it by an Adansonian system that gives equal weight to a large number of different characteristics was short-lived (Matthews 1983). Properties such as the size of the genome and the strandedness and polarity of the nucleic acid are stable properties since they determine the viral genome expression strategy and cannot be altered without being lethal for the virus. Likewise, the structural elements that build up a virion cannot undergo major changes without resulting in lethal instability of the virion.

The 63 virus families recognized at present (Van Regenmortel et al. 2000b) were demarcated on the basis of the nature of the viral genome, the strategy of replication used by the virus, and the structure of the virion. The family level in the taxonomic hierarchy has changed very little over the years, the main changes being the addition of more families. As new types of viruses were discovered in the families *Poxviridae*, *Herpesviridae*, *Parvoviridae*, and *Paramyxoviridae*, subfamilies have been introduced that group genera possessing certain common features.

In recent years, three virus orders representing groupings of families of viruses that share common characteristics have been created (Fauquet 1999). The order *Mononegavirales* comprises the families *Bornaviridae*, *Filoviridae*, *Paraxymoviridae*, and *Rhabdoviridae* that contain viruses with similar replication strategy. The order *Caudovirales* comprises the families *Myoviridae*, *Podoviridae*, and *Siphoviridae*, consisting of tailed, double-stranded DNA bacteriophages. The third order *Nidovirales* comprises the families *Coronaviridae*, *Arteriviridae*, and *Roniviridae*.

Viral families are subdivided into genera on the basis of characteristics that differ from one family to another, for instance differences in genome size and organization, virus replication strategy, vector transmission, etc. In some cases, when it is not clear whether a group of viruses placed in a genus should be included in an existing family or not, the corresponding genus is given the status of unassigned or floating genus.

The names of orders, families, subfamilies, and genera are latinized and written in italics with a capital initial letter and with the endings – *virales* for orders, *viridae* for families, *virinae* for subfamilies and *virus* for genera.

The families and unassigned genera of viruses that have been recognized up to July 2002 are listed in Table 1.

THE INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES

The authority on matters concerning virus classification and nomenclature is the International Committee on Taxonomy of Viruses (ICTV), which is a Committee of

Table 1 *Families and unassigned genera of viruses*[a]

Family or unassigned genus	Morphology of virus particle	Host
The dsDNA viruses		
Myoviridae	Tailed phage	Bacteria
Siphoviridae	Tailed phage	Bacteria
Podoviridae	Tailed phage	Bacteria
Tectiviridae	Isometric	Bacteria
Corticoviridae	Isometric	Bacteria
Plasmaviridae	Pleomorphic	Mycoplasma
Lipothrixviridae	Rod-shaped	Archaea
Rudiviridae	Rod-shaped	Archaea
Fuselloviridae	Lemon-shaped	Archaea
'SNDV-like viruses'	Droplet-shaped	Archaea
Poxviridae	Pleomorphic	Vertebrates/Invertebrates
Asfarviridae	Spherical	Vertebrates
Iridoviridae	Isometric	Vertebrates/Invertebrates
Phycodnaviridae	Isometric	Algae
Baculoviridae	Bacilliform	Invertebrates
Nimaviridae	Rod-shaped	Invertebrates
Herpesviridae	Isometric	Vertebrates
Adenoviridae	Isometric	Vertebrates
Rhizidiovirus	Isometric	Fungi
Polyomaviridae	Isometric	Vertebrates
Papillomaviridae	Isometric	Vertebrates
Polydnaviridae	Rod, fusiform	Invertebrates
Ascoviridae	Reniform	Invertebrates
The ssDNA viruses		
Inoviridae	Filamentous	Bacteria
Microviridae	Isometric	Bacteria
Geminiviridae	Isometic	Plants
Circoviridae	Isometric	Vertebrates
Anellovirus	Spherical	Vertebrates
Nanoviridae	Isometric	Plants
Parvoviridae	Isometric	Vertebrates/invertebrates
The DNA and RNA reverse-transcribing viruses		
Hepadnaviridae	Spherical	Vertebrates
Caulimoviridae	Isometric, bacilliform	Plants
Pseudoviridae	Spherical	Fungi, plants, invertebrates
Metaviridae	Spherical	Fungi, plants, invertebrates
Retroviridae	Spherical	Vertebrates
The dsRNA viruses		
Cystoviridae	Spherical	Bacteria
Reoviridae	Isometric	Vertebrates/invertebrates, plants
Birnaviridae	Isometric	Vertebrates/invertebrates
Totiviridae	Isometric	Fungi, protozoa
Partitiviridae	Isometric	Fungi, plants
Chrysoviridae	Spherical	Fungi
Hypoviridae	Pleomorphic	Fungi
The negative-strand ssRNA viruses		
Bornaviridae	Spherical	Vertebrates
Filoviridae	Bacilliform	Vertebrates
Paramyxoviridae	Pleomorphic	Vertebrates
Rhabdoviridae	Bullet-shaped	Vertebrates/invertebrates, plants
Varicosavirus	Rod-shaped	Plants
Ophiovirus	Filamentous	Plants

(*Continued over*)

Table 1 *Families and unassigned genera of viruses*[a] *(Continued)*

Family or unassigned genus	Morphology of virus particle	Host
The negative-strand ssRNA viruses (continued)		
Orthomyxoviridae	Pleomorphic	Vertebrates
Bunyaviridae	Spherical	Vertebrates, invertebrates, plants
Tenuivirus	Filamentous	Plants
Arenaviridae	Spherical	Vertebrates
Deltavirus	Spherical	Vertebrates
The positive-strand ssRNA viruses		
Leviviridae	Isometric	Bacteria
Narnaviridae	Rnp complex	Fungi
Picornaviridae	Isometric	Vertebrates
Dicistroviridae	Spherical	Invertebrates
Sequiviridae	Isometric	Plants
Comoviridae	Isometric	Plants
Potyviridae	Filamentous	Plants
Caliciviridae	Isometric	Vertebrates
Astroviridae	Isometric	Vertebrates
Nodaviridae	Isometric	Vertebrates/invertebrates
Tetraviridae	Isometric	Invertebrates
Sobemovirus	Isometric	Plants
Luteoviridae	Isometric	Plants
Umbravirus	Rnp complex	Plants
Tombusviridae	Isometric	Plants
Coronaviridae	Isometric	Vertebrates
Arteriviridae	Isometric	Vertebrates
Roniviridae	Bacilliform	Invertebrates
Flaviviridae	Isometric	Vertebrates
Togaviridae	Isometric	Vertebrates
Tobamovirus	Rod-shaped	Plants
Tobravirus	Rod-shaped	Plants
Hordeivirus	Rod-shaped	Plants
Furovirus	Rod-shaped	Plants
Pomovirus	Rod-shaped	Plants
Pecluvirus	Rod-shaped	Plants
Benyvirus	Rod-shaped	Plants
Bromoviridae	Isometric	Plants
Ourmiavirus	Isometric	Plants
Idaeovirus	Isometric	Plants
Closteroviridae	Filamentous	Plants
Capillovirus	Filamentous	Plants
Tymoviridae	Isometric	Plants
Carlavirus	Filamentous	Plants
Potexvirus	Filamentous	Plants
Allexivirus	Filamentous	Plants
Foveavirus	Filamentous	Plants
Trichovirus	Filamentous	Plants
Vitivirus	Filamentous	Plants
Barnaviridae	Bacilliform	Fungi

a) Van Regenmortel et al. 2000a.

the Virology Division of the International Union of Microbiological Societies (IUMS). This Committee was established in 1966 at the International Congress for Microbiology held in Moscow and was initially called the International Committee on Nomenclature of Viruses. A few years later, the name of the Committee was changed to International Committee on Taxonomy of Viruses. The membership of ICTV comprises the 18 members of its Executive Committee, a number of Life members, over 40 National members and the members

of its specialist subcommittees (Mayo and Fauquet 2000). The ICTV operates through six subcommittees responsible for Bacterial Viruses, Fungal Viruses, Invertebrate Viruses, Plant Viruses, Vertebrate Viruses, and an electronic Virus Database [the ICTV database (ICTVdB)]. The subcommittees coordinate the work of about 60 Study Groups comprising more than 500 virologists representing all areas of virology. The current membership of the ICTV is listed at the ICTV web site (www.ncbi.nlm.nih/ICTV).

The activities of ICTV are regulated by statutes approved by the Virology Division of IUMS and by an International Code of Virus Classification and Nomenclature (Mayo and Horzinek 1998). The International Code requires that virus classification and nomenclature must be international and uniformly applicable to all viruses infecting animals, plants, and microorganisms.

Although the virus classification system employs the hierarchical levels of order, family, subfamily, genus, and species, it is not obligatory to use all levels of the taxonomic hierarchy. Most virus species are classified into genera and most genera are classified into families. When it is uncertain to which genus a virus species should be allocated, even though its classification in a family is clear, the species is classified as an unassigned species of that family. If it is not clear to which family a genus belongs, it remains an unassigned genus. In its effort to develop an internationally agreed taxonomy for all viruses, the ICTV relies on guidance from individual Study Groups composed of recognized experts in each area of virology. However, any recommendations made by Study Groups are first evaluated by the subcommittees and Executive Committee of ICTV for their acceptability to the virological community in general. The ICTV Executive Committee then prepares taxonomic proposals for ratification by the full membership of the ICTV, either by post or at plenary sessions held during the triennial International Congresses of Virology. Recently the Executive Committee of ICTV has started to use the ICTVnet to allow more extensive consideration of proposals by Executive Committee members prior to an Executive Committee meeting. This procedure was extended to allow a wider consultation with the virological community than was possible previously. The new procedure goes through several stages of discussion, modifications, and public scrutiny and has been described in detail (Mayo et al. 2003).

At regular intervals, the ICTV publishes reports which represent the state of virus taxonomy and nomenclature at the time of publication. Seven reports have so far been published (Wildy 1971; Fenner 1976; Matthews 1979, 1982; Francki et al. 1991; Murphy et al. 1995; Van Regenmortel et al. 2000a). The 7th ICTV Report published in 2000 lists three orders, 63 families, 240 genera, and 1 550 viral species.

An important change in the 7th Report (Van Regenmortel et al. 2000a) was that the criteria used for demarcating species within many genera were defined for the first time. In the 6th Report, more than 3 600 viral 'entities' were listed, without, in many cases, a clear indication of their status as species, strains, serotypes, or isolates. In the 7th Report, only 1 550 viral species were officially recognized as distinct taxonomic entities. The major changes in the classification scheme introduced in the 7th Report have been summarized by Fauquet and Mayo (2001). The 7th ICTV Report needs to be consulted to ascertain the current status of virus taxonomy. This information is also available on the ICTVnet (www.danforthcenter.org/ITAB/ICTVnet). Subsequent changes introduced up to July 2002 and approved by the ICTV are listed in Mayo (2002). The 8th ICTV Report is currently under preparation and is likely to be published in 2005.

The activities of the ICTV are publicized in the 'Virology Division News' section of *Archives of Virology*, which is the official journal of the Virology Division of IUMS (Mayo and Van Regenmortel 2000).

THE SPECIES ISSUE IN VIROLOGY

All classifications are conceptual constructions made up of classes (hence their name) and the categories used for building them are not found in nature but arise in human minds. The categories of species, genus, family, and order are abstract concepts which, like all abstractions, have no location in space and time. The categories should therefore not be confused with groups of real objects or organisms which obviously are located in space and time. The distinction is readily accepted in the case of genera, families, and orders, and virologists have no difficulty in regarding these categories as abstract constructions that they will never physically encounter in their handling of viruses. It is indeed impossible to centrifuge a virus family, i.e. a concept, or to visualize it in an electron microscope. It is, therefore, rather odd that in the case of the species category, the distinction tends to be blurred with the result that species are often considered to have a real spatiotemporal existence. Part of the problem lies in the fact that the term species has been given different meanings. In addition to referring to a class or category, which is an abstract concept, the term species is sometimes used to refer to a collection of concrete objects, or real organisms localized in space and time (Ghiselin 1997). On the other hand, when a species is viewed, correctly, as an abstract class, it is impossible for concrete individuals to be part of such a species since the part–whole relationship does not apply to entities of different logical type. The entities or organisms belonging to such a species can only take part in a relation known as class membership (Mahner and Bunge 1997). An organism can only be a **member** of the species and not a **part** of it because it is logically impossible for a concrete object to be part of an abstract entity, i.e. of an entity of different logical type

(Van Regenmortel 2003). It is of course impossible for a virus species to cause a disease since abstractions do not cause diseases. On the other hand, a concrete virus entity that causes a disease can be a member of an abstract virus species. Unfortunately, many authors are inclined to use shorthand, sloppy expressions and will write that the species *Mus musculus* has been inoculated with one or other virus species, instead of saying correctly that a mouse (a member of the species *Mus musculus*) has been inoculated with a member of a certain virus species (i.e. with particles of an isolate of a certain species).

Another difficulty arises because classes like genera and families are so-called universal or Aristotelian classes, i.e. classes defined in terms of properties that are collectively necessary and sufficient for membership in the class. A virus family, for instance, is a universal class that consists of members, all of which share a number of defining properties that allow unambiguous identification of any member. For instance, all members of the family *Herpesviridae* are enveloped viruses that contain an icosahedral particle and double-stranded DNA and it is possible to allocate a virus to this family by considering only a few morphological and chemical features. By contrast, a virus species is not a universal class because its members undergo continual evolutionary changes and always show considerable variability. In practice, this means that it is not possible to rely on a single discriminating property for differentiating between two species. It is for this reason that although the species category is the most fundamental one in any biological classification, it has been the most difficult one to apply in virus classification.

Species are fuzzy sets

The difficulty of defining species is not a problem restricted to virus classification but is prevalent in all biological classifications. It often comes as a surprise that more than a century after the publication of Darwin's *On the origin of species* there is no general agreement about what constitutes a plant, animal, or microbial species. As many as 22 different species concepts have been used in various fields in biology (Mayden 1997).

The traditional view of species is that they correspond to groups of similar organisms that can breed among themselves and produce fertile offspring. A classical definition of biological species states that 'species are groups of interbreeding natural populations that are reproductively isolated from other such species' (Mayr 1963). One problem with this definition is that it can only be applied to organisms that reproduce sexually; furthermore, it also does not take into account the phenomenon of interspecies hybridization which is very common in the plant kingdom. In order to make it applicable to asexual organisms for which the criterion of interbreeding breaks down, Mayr (1982) modified his definition to: 'a species is a reproductive community of populations, reproductively isolated from others, that occupies a specific niche in nature.' Another species concept is that of evolutionary species which has been defined as 'a single lineage of ancestor–descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate' (Wiley 1981). This concept emphasizes the historical dimension but does not provide any guidance on how far back in time a species can be traced. Life on earth is a biological and historical continuum and it is equally difficult to demarcate boundaries in time for identifying evolutionary species as it is to define clearcut breeding discontinuities in order to identify biological species. These difficulties led to the view that species are fuzzy sets with hazy boundaries and that it is not possible to draw sharp boundaries between them as is done with universal classes and classical sets. The use of fuzziness or vagueness for describing reality has a long history in Western thinking. Vagueness stems from the existence of a continuum with innumerable steps and is illustrated by the sorites paradox of the heap described by Greek philosophers. This paradox arises because it is impossible to say how many grains of sand can be removed from a heap before it stops being a heap. Clearly, the concept of a heap cannot be defined in a precise manner and it would be counterproductive to try to impose an artificial precision on such a term.

Fuzzy logic is a method that has been developed to handle concepts and entities that do not possess sharp boundaries (McNeill and Freiberger 1993). Fuzzy logic as a method for handling fuzzy sets accepts the inherent imprecision of terms and categories used in both ordinary language and in science. In ordinary language, a glass can be said to be full when it is only 95 or 98 percent full and the statement 100 percent full is rarely relevant. In classical logic, on the other hand, it will be maintained that a glass is necessarily either full or not full. In the real world of empirical and scientific observations this dichotomy is absent since fullness admits of degrees. Since fuzzy sets have no sharp boundaries, membership of a fuzzy set is not an all-or-nothing matter. This means that the two pillars of classical logic, the law of excluded middle (a swan is either white or nonwhite) and the law of noncontradiction (a swan cannot be both white and nonwhite) do not apply to fuzzy sets. Handling fuzzy sets falls outside the scope of classical, bivalent logic. When fuzziness is accepted as an unavoidable ingredient of the species concept, it becomes possible to describe species in terms of continuums devoid of artificial sharp edges (Van Regenmortel 1998). In a biological classification, it is often necessary to allocate an organism to a particular species purely as a matter of convenience and expediency rather than logical necessity. In a similar way, colors can be distinguished conceptually in spite of the continuous

nature of the spectrum of electromagnetic waves and mountain peaks can be identified and are given names in spite of the absence of sharp boundaries in geological rock formations (Van Regenmortel 2000a).

What is a virus species?

The question of what is a virus species is related to the general problem of deciding if one virus is the same as another. This in turn requires that an answer be given to the identity question: how different must two virus isolates be in order to be considered different types of virus rather than the same virus? Although mutants or pathogenic variants may be clearly distinguishable from the wild-type virus, virologists usually have no difficulty in recognizing them as being the same type of virus. This ability to pass judgment on the significance of the extent of difference observed between individual objects is a typically human faculty that in turn gives rise to our urge to classify and order the world around us. In biology, the hierarchical categories of family, genus, and species are used to classify organisms, the species class being the most difficult one to define. Since viruses are biological entities, albeit not living organisms, the ICTV has attempted to use the same categories for classifying viruses. Whereas the categories of family and genus were readily accepted by most virologists, it took many years before the species category gained acceptance by the virological community as a whole. As recounted in detail elsewhere (Van Regenmortel 1989), there was considerable opposition among plant virologists to the idea that the concept of species could be applied to viruses (Milne 1984; Harrison 1985). The unwillingness of some plant virologists to accept the species category was due to two erroneous assumptions on their part (Milne 1984). They assumed that the only legitimate species concept was that of biological species defined by gene pools and reproductive isolation, thereby ignoring the many other definitions of species some of which may have been applicable to entities such as viruses (Ghiselin 1997; Mayden 1997). They also assumed that the acceptance of virus species would inevitably lead to the latinization of virus names which they strongly opposed (Matthews 1983; Van Regenmortel 1989).

The battle against the use of the species category in virology was very much an argument over semantics. For many years some of the plant virologists who objected strongly to the term species had been engaged in a most successful enterprise of delineating separate plant viruses known as the *CMI/AAB Descriptions of Plant Viruses* (Matthews 1985). These *Descriptions* of more than 300 plant viruses were widely accepted as the standard demarcation of separate viruses. Instead of calling them separate species, however, the editors of the *Descriptions* preferred to call them 'viruses' where the inverted commas were meant to convey the message that these entities corresponded to a separate taxon and not merely to a particular viral strain or isolate. These 'viruses' really represented de facto species and plant virologists were in fact ahead of vertebrate virologists in delineating viral species, but without actually calling them species (for a review, see Van Regenmortel 1989).

The arguments against the use of the term species were also reinforced by the absence of an appropriate definition of the concept of virus species. In 1981, the ICTV had approved the following definition: 'A virus species is a concept that will normally be represented by a cluster of strains from a variety of sources, or a population of strains from a particular source, which have in common a set of correlating stable properties that separate the cluster from other clusters of strains' (Matthews 1982). Since this rather unhelpful definition did not explain what a strain was and did not indicate that viruses possess genes, Kingsbury (1985) suggested that it should be replaced by the following definition: 'A virus species is a population of viruses sharing a pool of genes that is normally maintained distinct from the gene pools of other viruses.' One difficulty with this definition is that many viruses reproduce entirely by clonal means and do not possess gene pools.

In order to emphasize the cohesive forces present in ancestral descendant clones that share a common biotic niche, the following definition was proposed in 1989: 'A virus species is a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche' (Van Regenmortel 1989). This is the definition which the ICTV endorsed in 1991 (Pringle 1991).

Virus species as polythetic classes

The concept of polythetic class was introduced in biology by Beckner (1959) who initially called this class polytypic to differentiate it from classical, universal classes defined by properties that are both necessary and sufficient for membership in the class. A polythetic class is characterized by a combination of properties, each of which might also occur outside the given class and could also be absent in a particular member of the class. Although the members of a polythetic class always have several properties in common, there is no single attribute absent in other classes which they all share (Figure 2). This means that no single property can be used as a defining property of a polythetic species on the basis that it is universally present in all the members of the species, and at the same time, always absent in the members of other species. It is for this reason that a single discriminating character, such as a certain percentage of genome sequence dissimilarity or a particular host reaction, cannot be used as a valid criterion for differentiating two virus species within the same genus. It is the inherent variability of the members of a species that prevents a single discriminating character from

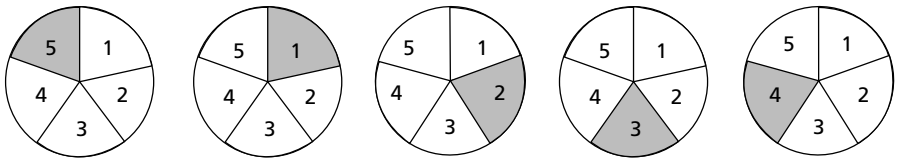

Figure 2 *Schematic representation of five members of a polythetic class characterized by five properties, 1–5. Each member possesses several of these properties, but no single property is present in all the members of the class. The missing property in each case is represented by the gray sector.*

being used for species differentiation (Van Regenmortel 2000a; 2003). It is always a combination of properties that must provide the justification for considering that two virus species are different.

The advantage of defining virus species as polythetic classes is that this makes it possible to accommodate individual viruses that lack one or other character that would normally be considered typical of the species. Polythetic classes are thus well-suited for dealing with biological entities like viruses which undergo continual evolutionary changes and show considerable variability.

Since there are mostly no sharp boundaries between different virus species, they are best treated as fuzzy sets (see section on Species are fuzzy sets). Species are thus very different from the other taxonomic categories used in virus classification, such as genera and families which can easily be defined in an unambiguous manner by a few morphological and chemical characteristics. The difficulties inherent in defining virus species are demonstrated by the fact that it was only in the 7th ICTV Report published in 2000 that the criteria used for differentiating many of the virus species were clearly stated for the first time.

Virus species as replicating lineages

The membership of a virus species varies over time and since all the members share descent from a common ancestor, they represent an evolving lineage. Since shared descent is a property that also links different species and genera, phylogeny is not a useful criterion for species demarcation. The same shortcoming applies to the evolutionary species concept since there are no clearcut criteria for deciding how far back in time a species can be traced (Wiley 1978; Lovtrup 1979). Virus species undergo continuous variation in time and transition from one species to another during evolution occurs within the continuity of gene duplication. As variations accumulate, a point will be reached where the importance of phenotypic and genotypic differences will lead an observer to conclude that he is dealing with a different virus species. Although increasing dissimilarity in genome sequence may provide some guidance, it must be stressed that there is no precise degree of genome difference which can be used as a cutoff point to differentiate between two species. The reason for this is that there is no direct quantitative relationship between the extent of genome sequence similarity and the similarity in biological or phenotypic characteristics of a virus. Since the biological properties of viruses are the ultimate reason why virologists engage in species demarcation, it would be unrealistic to differentiate between viral species only on the basis of genome properties. Unfortunately, there is at present an increasing tendency to want to rely only on genomic data for virus classification (Calisher et al. 1995; Zanotto et al. 1996). It should be borne in mind that classifying viral genomes is not the same as classifying viruses (Van Regenmortel et al. 1997) and that a viral nucleic acid sequence is not the same as a virus (Drebot et al. 2002). Genome comparisons cannot by themselves justify taxonomic placements that would disregard the biotic and phenotypic characteristics of viruses which remain the most important justification for developing a practical viral taxonomy.

Ecological niche occupancy

The ecological niche refers to the biotic properties of members of a virus species such as the host range, tissue tropism, and vector. The ecological niche does not only refer to a location in space but is a functional concept based on the relational properties of the virus (see Viruses should not be confused with virus particles or virions) which involves aspects such as host responses, pathogenesis, and virulence. The niche is not a property of the environment but a property of the virus related to its biotic habitat. There are, therefore, no vacant or empty niches but only unoccupied habitats or geographic spaces. In the absence of the virus, its ecological niche property is also absent and the notion of a vacant niche is thus meaningless (Colwell 1992). A niche provides the needs that must be met for the virus to replicate and to survive.

What is a type species?

Although the term 'type species' appears in the *International Code of Virus Classification and Nomenclature* (Mayo and Horzinek 1998), a precise definition of the concept has been proposed only recently: 'A Type Species is a species whose name is linked to the use of a particular genus name. The genus so typified will always contain the Type Species' (Mayo et al. 2002).

A type species is thus a nomenclatural or name-bearing type corresponding to a class which itself is included in a particular genus class. A type species, therefore, only typifies the use of the genus name and is not typical in the sense of possessing the greatest number of characteristics of the genus. Some confusion may arise if the concept of virus species as an abstract class is not clearly differentiated from that of the concrete infectious entity called a virus. A virus species may have as one its members a reference type which could for instance be a culture collection isolate that can be exchanged between laboratories and is useful for making comparisons with any new isolate. Such a reference type is actually a type strain and should not be confused with a type species, which being a species, is an abstraction and not a virus culture (Mayo et al. 2002).

The quasi-species misnomer

In recent years it has become fashionable to refer to RNA viruses as quasi-species populations (Smith et al. 1997). RNA viruses have genomes that replicate in the absence of repair mechanisms and they possess a mutation frequency per nucleotide site of 10^{-3}–10^{-5}. A clone of an RNA virus will, therefore, always generate many thousands of different genomes, all of which compete during replication of the clone (Holland et al. 1992). This population consists of a master sequence corresponding to the most fit genome sequence with respect to a given environment, together with a very large number of competing virus mutants. Such a population is often referred to as a quasi species. This is an unfortunate term since it implies that the virus corresponds to some sort of imperfect species, as opposed to a true or genuine species that would possess a single, invariant genome sequence (Domingo et al. 1995). Such an idealized virus species consisting of members that all have the same sequence of course does not exist (Van Regenmortel et al. 1997). The confusion arises because a chemical concept, i.e. that of a molecular species consisting of molecules that are all identical, is applied to a biological entity that is not defined by chemical homogeneity but by its inherent variability. Like all biological entities that possess the ability to reproduce themselves or to replicate, viruses are endowed with an intrinsic variability that results from the error-prone process of nucleic acid duplication.

The term quasi species was introduced by Manfred Eigen and his colleagues to describe self-replicating RNA molecules which because of mutation do not consist of a unique molecular species (Eigen 1993). The members of a chemical species are all identical molecules whereas the members of a virus species are not. The biological and taxonomic concept of virus species, therefore, cannot be reduced to that of molecular or chemical species since it would amount to the reductionist fallacy of attempting to reduce biology to chemistry. Taxonomic species are always quasi-species in the molecular sense and no additional clarification is provided by giving the label quasi species to viral species (Smith et al. 1997). It has been proposed that a 'virus quasi-species corresponds to a dynamic distribution of non-identical but closely related mutants and recombinant viral genomes subjected to a continuous process of genetic variation, competition and selection, and which act as a unit of selection' (Domingo 1999). However, such a definition reduces viruses to genome sequences. The term quasi species is, indeed, used appropriately for describing a population of genome sequences rather than a population of viruses since the implicit reference to a unique molecular species then applies to a chemical species consisting of a single nucleotide sequence.

Virus species demarcation

Although the acceptance of a definition of virus species by the virological community was an important step for establishing a virus classification based on traditional taxonomic categories, it should be stressed that such a definition did not solve the problem of demarcating individual virus species. Defining the virus species concept amounted to defining the category of species classes and it did not do away with the need to delineate individual virus species in the classification scheme.

Demarcating individual species is an attempt to introduce some order into the bewildering variety of viruses and this task requires the input of individuals with extensive experience and expertise with the viruses in a particular taxon (Van Regenmortel et al. 1997). This is the reason why the task of demarcating individual species was assigned to the ICTV Study Groups composed of virologists who possess in-depth knowledge of particular areas of virology. Only specialists are aware of the facts, issues, and nuances about viruses and their biotic interactions and of the importance of making certain distinctions, which may not be the same in all virus genera, for achieving a convenient and useful classification (Van Regenmortel et al. 1997).

In order to differentiate between individual species, it is necessary to rely on properties that are not present in all the members of a genus or family, since such properties obviously will not permit species demarcation. Characters such as virion morphology, genome organization, method of replication, and number and size of structural and non-structural proteins are properties that are shared by all the members of a genus or family and which, therefore, cannot be used for demarcating individual species within a genus. The properties that are useful for discriminating between individual virus species are characters that are not necessarily constant within the species and which can often change as a result of only few mutations. This situation is responsible for

much of the difficulty of demarcating species. Another difficulty, discussed in the section Species are fuzzy sets is that species are fuzzy sets, which means that there is in most cases no logical necessity in any particular species demarcation. Taxonomy at the level of species tends to be mostly a matter of opinion. Properties that are useful for demarcating species are the natural host range, cell and tissue tropism, pathogenicity and cytopathology, mode of transmission, certain physicochemical properties, antigenic properties, and genome sequence similarities (Van Regenmortel et al. 1997). All these characters are not equally important for demarcating species in different viral genera and families. This is often deemed to be unsatisfactory, although there is really no need to harmonize discriminating criteria across all species, genera, and families. A classification scheme has to answer the practical needs of people who deal with viruses and these needs are not the same in all areas of virology. From a human perspective, all the organisms infected by viruses are not equally relevant and it is to be expected that human pathogens or pathogens that infect animals and plants of economic importance will be studied more intensely than, for example, the viruses that infect the myriad species of insects. Fine distinctions based on relatively minor differences in host range, pathogenicity, or antigenicity may thus seem to be more relevant for viruses that are of particular interest to humans (Van Regenmortel and Mahy 2004).

Since virus species are polythetic, the ICTV Study Groups, in order to demarcate the 1 550 species that are listed in the 7th ICTV Report, had to rely on a combination of properties rather than on the presence or absence of a single key feature. Once a species has been established in this manner it is possible, however, to identify a virus isolate as a member of that species by considering only a few of the properties of the species. For instance, if a virus isolate reacts with a panel of monoclonal antibodies in the same way as an established member of a given species, the virus will be considered a member of that species. This apparent contradiction between the need for many characters to define a virus species and the fact that a single property may suffice to identify a member of a species disappears when it is realized that species definition and demarcation is a different endeavor from virus identification (Van Regenmortel 2003).

VIRUS IDENTIFICATION

There is a common misconception that the existence of a definition of the concept of virus species should make it an easy matter to decide if a particular virus isolate is a member of an established virus species or not. One reason for this misconception is the failure to appreciate that it is only the abstract concept of species, as a class of classes, that can be defined by explaining the meaning of the concept. Individual viruses like any other concrete entities that are located in space and time cannot be defined but can only be named (Kitts 1984; Van Regenmortel 1990) and identified by the use of so-called diagnostic properties (Ghiselin 1984). A definition only explains the meaning of a concept but it does not provide the means to decide which concrete entities are members of an abstract class. What is required is a set of characters and diagnostic properties that can be used for identifying individual members of a particular species. For different virus species, different types of properties may have to be used (Van Regenmortel et al. 1997).

The identification of a virus isolate is a comparative process based on a limited number of diagnostic properties which clarifies the extent of the relationship of the isolate with known members of an established species. Practical aspects of the virus identification process have been discussed by Murphy (1996). For unambiguous identification it is often convenient to compare new isolates with virus strains available in culture collections such as the American Type Culture Collection. These strains and reference types, which should not be confused with type species (see section on What is a type species?), are chosen and designated not by the ICTV but by international specialty groups, some of which operate under the auspices of the World Health Organization and other agencies. Levels of classification below the level of species, such as strain, serotype, and isolate, are not the remit of the ICTV, although such entities may be listed in ICTV Reports, admittedly without being defined (Drebot et al. 2002). A viral strain is a biological variant of a given virus that is recognizable because it possess some unique characteristics that remain stable under natural conditions. A viral serotype is a strain that is recognizable because it possess unique antigenic properties that remain constant under natural conditions. In the 7th ICTV Report, human polioviruses 1, 2, and 3 are considered serotypes of the species *Poliovirus* and they clearly constitute stable replicating lineages, a feature which allowed them to remain distinct over time (Drebot et al. 2002). The fact that these three serotypes do not cross-protect against each other is sufficient reason for epidemiologists to want to consider them as separate, clinically relevant entities. This important differentiation is clearly feasible even if the three serotypes are classified as members of the same virus species. In human and animal virology, individual viral serotypes are recognized only if they do not cross-protect against each other. In plant virology, the cross-protection criterion is not relevant and the label serotype is given to any antigenic variant that can be recognized as such. A viral isolate, on the other hand, is simply one particular virus culture that is being studied and it may correspond to a member of a species, a strain, or an entity of unknown taxonomic status.

Deciding whether viral isolates correspond to strains or serotypes of an established species or belong to separate species remains in many cases one of the most difficult challenges that virologists are faced with. The ICTV

Study Groups continue to address this issue by providing species demarcation criteria that can serve as guidelines for identifying which properties of viral isolates will be most useful for diagnostic purposes.

THE ICTV DATABASE (ICTVdB)

Although only about 1 600 virus species are currently recognized by the ICTV, it has been estimated that about 30 000 different viruses, strains, and subtypes are kept in specialized laboratories, reference centers, and culture collections. One of the aims of ICTV is to develop a universal virus database, the ICTVdB, which will store primary descriptive data of virus isolates formatted for the DELTA Computer System (DEscriptive Language for Taxonomy). The ICTVdB intends to be the main repository for descriptive information on viruses at the strain and isolate level and it will therefore be a valuable resource for virus researchers or anyone who needs information on currently known viruses (Büchen-Osmond et al. 2000). The ICTVdB uses a decimal numbering system similar to that used for enzymes (Büchen-Osmond and Dallwitz 1996). The database has been constructed using a descriptor list of several hundred characters which have been translated into single property statements which can be recognized by the search facilities of the database and can be used for virus identification. The ICTVdB also provides links with genome sequence and protein sequence databases (GenBank and SWISS-PROT).

Information on the ICTVdB is available from the US website www.ncbi.nlm.nih.gov/ICTVdb/ or the UK site www.ictvdb.iacr.ac.uk/

Information on individual viruses stored in the ICTVdB should in future be a useful tool for ICTV Study Groups when they need to make decisions regarding species demarcation and other taxonomic assignments.

NAMES AND TYPOGRAPHY OF VIRUS SPECIES

In the 6th ICTV report (Murphy et al. 1995), names of viral orders, families, subfamilies, and genera were written in italics with a capital initial letter. Following the 1998 revision of the International Code (Mayo and Horzinek 1998), the same typography was used for the names of virus species in the 7th Report. This typography provides a visible sign that species correspond to viral taxa, just like genera and families. For most viruses, the English common names of viruses became the species names and these were written in italics with the initial letter capitalized (Van Regenmortel 1999). This made it possible to differentiate virus species officially recognized by the ICTV from other viral entities such as tentative species, viral strains, and serotypes written in Roman characters. This new typography has met with some criticism (Bos 2000; Gibbs 2000) and corresponding rebuttals (Van Regenmortel 2000b; Van Regenmortel et al. 2000b) and is now generally applied in most scientific publications (Mahy 2001). The use of italicized English names for virus species reflects the emergence of English as the international language of scientific communication and is in line with the general dislike of virologists for Latin virus names (Van Regenmortel 2000b; 2003).

It should be emphasized that italicized virus species names are not meant to replace existing vernacular or common names of viruses written in Roman characters (Van Regenmortel and Fauquet 2002). The viruses studied by virologists are concrete, disease-causing entities and not abstract classes and they should be referred to by their common, non-italicized names. It is only the names of viral taxonomic classes that are written in italics, not the names of viruses (Drebot et al. 2000). In a scientific paper it will be necessary to refer only once, for instance in the Materials and Methods section, to the official, taxonomic species name, while in the remainder of the text reference will be made to the virus as a physical entity, denoted by its common name written in Roman characters (Van Regenmortel and Mahy 2004).

The claim that common virus names can be used in an abstract, taxonomic sense (Bos 2002) is based on the erroneous assumption that a virus is an abstraction rather than a concrete entity. A virus name is a sign or label that designates a real, concrete object located in space and time, and the word virus corresponds to what logicians call a general term which denotes any number of concrete objects or entities. The word 'virus' itself as a term referring to an infectious entity and the picture of a 'virion' should not be confused with the real objects to which they refer and which are the entities studied by virologists. The painter Magritte reminded us of that distinction in his well-known painting of a pipe that included the caption 'this is not a pipe.' A pipe is a general term denoting concrete objects and is no more an abstraction than a virus or a virus-infected plant (Van Regenmortel 2003). Only confusion results if a virus is considered to be an abstraction (Bos 2000; Van Regenmortel 2000b). Abstractions are concepts that are not located in space and time; examples are concepts like beauty, generosity, disease, and class (Mahner and Bunge 1997). Taxonomic classes are abstractions and the use of italics for the names of viral classes is a useful reminder of their abstract nature. Common names of viruses actually refer to concrete objects and not to abstract taxonomic categories, and this distinction is a crucial one when constructing a virus classification.

A POSSIBLE FUTURE BINOMIAL NOMENCLATURE FOR VIRUS SPECIES

For many years, some plant virologists have been using an unofficial binomial system for referring to virus species. In this system, the word 'virus' appearing at the

end of the current species name is replaced by the genus name, which also ends in 'virus;' *Tobacco mosaic virus* then becomes *Tobacco mosaic tobamovirus*, *Bluetongue virus* would become *Bluetongue orbivirus* and *Measles virus* would become *Measles morbillivirus*. The advantage of such as system is that inclusion of the genus name in the species name provides additional information about the properties of the viruses. To non-specialists, it would become immediately obvious that hepatitis A, B, and C viruses are very different entities belonging to separate genera if their species names would be *Hepatitis A hepatovirus*, *Hepatitis B orthohepadnavirus* and *Hepatitis C hepacivirus*. If such names became official names recognized by the ICTV, they should, of course, be written in italics, as they are in fact simply a contraction of the current species and genus names written in italics.

A binomial system for species names would have the additional advantage that there would then be a clear distinction between the species name written in italics (*Measles morbillivirus*) and the common non-italicized, virus name, measles virus. At present, the distinction between the name of the virus and that of the species relies in most cases only on typography (measles virus and *Measles virus*), which can lead to confusion (Van Regenmortel and Fauquet 2002).

The issue of whether nonlatinized binomial names should become the official species names of viruses has been hotly debated for many years (Bos 2000; Gibbs 2000; Van Regenmortel 2000b, 2001) and the ICTV has been criticized by some plant virologists for not making binomial names the official names of all virus species. However, the ICTV has moved cautiously since it was not clear whether a majority of virologists would be in favor of a binomial system that would lead to a change of all the current, official names of virus species. Efforts were made in 2002 to canvass the opinion of virologists regarding the acceptability of binomial names. The results of two ballots showed that a sizeable majority (80–85 percent) of the virologists (about 250) who expressed an opinion were in favor of a binomial system of species names (Mayo 2002; Van Regenmortel and Fauquet 2002; Van Regenmortel and Mahy 2004). This is the first time that the ICTV has obtained some indication about the way virologists feel about a particular taxonomic issue. Those who oppose a binomial system may still want to argue that the number of virologists who expressed an opinion is too small to justify a change in species names. Published taxonomic debates are usually conducted by a very small number of strongly opinionated individuals and the ICTV has always found it difficult to implement some sort of democratic decision-making since very few virologists bother to express their views on matters of taxonomy (Matthews 1983; Van Regenmortel 2000b; Van Regenmortel et al. 2000b). In the case of binomial species names there is now at least some indication on how the virological community thinks about the issue. At present, it is not clear whether the ICTV will change the International Code of Virus Classification and Nomenclature and will introduce new binomial names for all virus species.

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The classification of vertebrate viruses

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INTRODUCTION

A formal taxonomy of viruses, known now as The Universal System of Virus Taxonomy, has developed from the operational need for a uniform system of nomenclature of viruses to facilitate the accurate identification and control of nonbacterial disease-producing agents. The concept of virus originated from the discovery that tobacco mosaic disease of plants could be transmitted by sap that had been rendered free of bacteria by passage through a Chamberland filter. Subsequently, the particulate nature of viruses was established by Loeffler who observed that foot-and-mouth disease of cattle was transmitted by a nonbacterial agent that could pass through Kieselguhr filters, but was retained by finer-grained Kitasato filters (see Witz 1998). Following the discovery of these viruses at the turn of the century, several other diseases were recognized as virus-associated. The physical nature of viruses remained unknown for several decades, however, and viruses were named and grouped according to their biological attributes, for example, pathogenicity, host range, tissue and organ specificity, transmission routes, etc. As knowledge of the chemical and physical properties of viruses accrued, it became apparent that the disease-producing potential of viruses was an unsound basis for virus classification. With the rapid expansion of virology from the 1950s onwards, the requirement for a uniform system of nomenclature became compelling. Several competing classification schemes were proposed based on a variety of criteria, but none gained wide acceptance. Systems of nomenclature at the extremes ranged from a rigid hierarchical system embracing subphyla, classes, orders, and families, based on an arbitrary set of characteristics (Lwoff et al. 1962), to a nonhierarchical system whereby any virus could be identified by a unique cryptogram defined by eight virus properties (Gibbs et al. 1966). To introduce order into this undertaking, an International Committee on Nomenclature of Viruses (ICNV) was established at the International Congress of Virology in Moscow in 1966. This committee was renamed the International Committee on Taxonomy of Viruses (ICTV) in 1973, and now operates under the auspices of the Virology Division of the International Union of Microbiological Societies (IUMS). The Universal System of Virus Taxonomy has been developed to its present state through a series of seven reports, the most recent of which was published in 2000 (Wildy 1971; Fenner 1976; Matthews 1979, 1982; Francki et al. 1991; Murphy et al. 1995; van Regenmortel et al. 2000). The classification presented in this chapter is a condensed version of the Universal System of Taxonomy (Pringle 1999), which was published in full as the Seventh Report of the ICTV in 2000 (van Regenmortel et al. 2000), and includes all revisions that have been approved by the ICTV up to the time of its plenary meeting in Paris in July 2002 (Mayo 2002). It is inherent in the design of the Universal Taxonomy that it can accommodate change in the nomenclature of viruses, as knowledge accrues, without obscuring previous usage. An Eighth Report is nearing completion and will be published under the title *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*, edited by C.M. Fauquet et al., London: Elsevier Academic Press. The overall structure of the higher level of the taxonomy remains largely unaltered, the principal

difference being that the number of designated virus species has almost doubled, and is likely to continue to increase exponentially in future years.

The ICTV operates through six subcommittees, which coordinate the work of approximately 50 study groups, with a total involvement of some 450 virologists representing all areas of virology. Five of these six subcommittees have responsibility for bacterial viruses, fungal viruses, plant viruses, invertebrate viruses, and vertebrate viruses, while the sixth is concerned with the development of an electronic virus database (the ICTVdB). An executive committee oversees the activities of the subcommittees and their study groups, and prepares all taxonomic proposals for ratification by the full membership of the ICTV, which comprises the executive committee, the life members, and the national members (nominated by countries supporting a national microbiological society). Normally a new taxonomic proposal should be directed to the chair of the appropriate study group, who becomes responsible for its progress through the evaluation procedure. An updated list of the names and addresses of study group chairs and the membership of the ICTV can be accessed at the ICTV website (www.ncbi.nlm.nih.gov/ICTV). Taxonomic proposals under consideration can also be viewed at the ICTV website on an interactive basis.

The system of taxonomy adopted by the ICTV is sufficiently flexible to withstand the stress of the continual discovery of new viruses from an ever-increasing diversity of host organisms. A hierarchy of five taxa, ranked as orders, families, subfamilies, genera, and species, has been developed. Taxonomic categories below the species level, such as subspecies, strains, variants, genotypes, etc., are excluded from the taxonomy and are defined at the discretion of working virologists. The concept and definition of the category of virus species is central to the structure of the taxonomy, and the nature of the virus species is discussed separately under The concept of the virus species. Artificially constructed hybrid viruses, genetically engineered and re-engineered viruses, mutants, recombinants, and reassortants are not included in the Universal Taxonomy; their status is a matter for consideration by interested specialist groups. Satellite viruses, satellite genomes, viroids, and prions are included, but not dealt with in detail in this chapter.

DEFINITION OF TAXA

The order

The highest taxon, the order, comprises a group of families that have certain features in common. Currently only three orders are recognized. It is anticipated that the number of orders will increase in step with refinement of the methods of characterization of viruses. Orders are designated by names with the suffix *-virales*. For example, the order embracing all the monopartite negative-sense single-stranded RNA viruses is the *Mononegavirales*.

The family

The taxon below the order is the family and 68 families are now recognized. Families consist of groups of genera exhibiting common properties not present in other genera, and they are identified by the ending *-viridae*, for example, *Herpesviridae*. In some instances subfamilies have been introduced to accommodate new knowledge regarding the relationships of genera within families. Subfamilies are indicated by the suffix *-virinae*, for example, *Alphaherpesvirinae*. In general, however, the policy is to avoid subdivision of primary taxa, except for reasons of expediency. The family is the bedrock of the universal taxonomy and most have been reinforced rather than undermined by the accumulation of knowledge. The members of the same family have similar morphology, genome organization, and replication strategy. However, since virology has no fossil record to fall back on, proof of common phylogeny is not a defining property. Of the 68 virus families currently recognized, only 28 (38 percent) contain viruses with vertebrate hosts.

The genus

Virus genera are assemblages of species of viruses that share common properties that distinguish them from the members of other genera. Genera are designated by the suffix *-virus*, for example, *Simplexvirus*. Currently 250 genera have been designated, 222 of which have been assigned to families. The remaining 28 genera, mostly positive-sense single-stranded RNA viruses of plants, are referred to as unassigned or 'floating' genera. There are 99 genera of viruses infecting vertebrates, four of which remain 'unassigned.' The genera occupy the most stable level of the hierarchy and establish the point of departure from which other taxa are defined. It is inherent in the taxonomy at its present stage of development that there is a lack of equivalence in different branches, and the criteria used for definition of genera differ from family to family. Evidence of common phylogeny is a desirable but not an exclusive criterion for definition of a genus. The horizontal transfer of genetic information during virus evolution and the occasional incorporation of host-derived genes can rapidly obscure or mimic phylogenetic relationship. It is implicit in the designation of a genus, however, that its constituent species will not exhibit conflicting phylogenetic relationships.

The species

The species is the most important category in any system of biological taxonomy. The concept of species in

clonally related organisms is discussed under The concept of the virus species. The definition of virus species finally adopted by the ICTV is the one formulated by van Regenmortel (1990). This definition states that: 'A virus species is defined as a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche.' A polythetic class is a class that is defined by more than one property, and no single property is necessary or sufficient to confer membership. The adjectival phrase 'ecological niche' refers to the total environment as distinct from its restrictive usage in contemporary environmental philosophies. The principal advantage of the van Regenmortel definition is that it takes account of the inherent variability of viruses and is not reliant on a single diagnostic characteristic. This definition is sufficiently elastic to accommodate the differing perceptions of virus species by virologists working in different fields of virology. One of the most difficult tasks facing virus taxonomists currently is to establish some uniformity in the nature of the phenotypic properties employed in the definition of species (van Regenmortel et al. 1997). The seventh report of the ICTV (van Regenmortel et al. 2000) should be consulted for detailed information concerning the criteria used for species designation in each family or floating genus.

The International Code of Virus Classification and Nomenclature

The naming of species has been codified (see Mayo and Horzinek 1998). A species name must be unambiguous and should consist of as few words as practicable, for example, *Human herpesvirus 1*. It must not consist solely of a host name with the word virus appended. A recent change in practice approved by the ICTV is that, in common with other biological taxonomies, virus species names will be rendered in italics with the initial letter capitalized. Tentative species, i.e. those with unconfirmed taxonomic status and awaiting approval are not italicized but do have the initial letter capitalized. Each species is also identifiable by a unique acronym (for example, see Fauquet and Martelli 1995; Fauquet and Pringle 1999a, b, 2000), which can be used unitalicized in text subsequent to the first mention of the species name, for example, ASFV is the approved acronym for *African swine fever virus*.

In future, all taxonomic categories from orders down to species should be rendered in italics with the initial letter capitalized. The name of the taxon should precede its actual name, for example, the genus *Simplexvirus*, rather than the *Simplexvirus* genus. This is the convention in the formal identification of a taxon, for example, in designation of the species *Tobacco mosaic virus*. In other circumstances, such as when used in an adjectival form, italicization and capitalization of the initial letter are not required; e.g. in the adjectival statement, the tobacco mosaic virus polymerase.

The use of vernacular nomenclature can be a source of ambiguity where a common root name is employed in designation of both family and genus. A pertinent example is the confusion generated by the use of the vernacular term 'paramyxovirus.' This is ambiguous because the vernacular term could refer either to viruses belonging to the genus *Paramyxovirus,* or to constituent members of the family *Paramyxoviridae*. This usage lacks precision because the family *Paramyxoviridae* comprises five uniquely named genera. To deal with this problem the Paramyxovirus Study Group of the ICTV recently decided that the genus *Paramyxovirus* should be renamed the genus *Respirovirus*, notwithstanding the familiarity and historical associations of the original name. Although the International Code of Virus Classification and Nomenclature (see Mayo and Horzinek 1998) states that existing names should be retained wherever feasible, priority is not a factor that is given precedence. To increase precision and to avoid ambiguity it is the intention in the longer term to eliminate all vernacular names from the Universal Taxonomy and to replace them with international names, for example, the species formerly designated as lambda phage is now designated the species *Enterobacteria phage λ*.

The International Code also requires that the names of taxa should be easy to use, euphonious, and easy to remember. No person's name should be used in designation of a new taxon. Nor should subscripts, superscripts, hyphens, oblique bars, and Greek letters be used in the designation of a new taxon, notwithstanding the fact that examples of these remain in the existing taxonomy. Sigla may be accepted as names of taxa, provided that their meaning is obvious to the members of the relevant study group and to general virologists. For example, the name of the genus *Reovirus* is a siglum compounded from 'r' for 'respiratory', 'e' for 'enteric', and 'o' for 'orphan'.

THE UNIVERSAL SYSTEM OF VIRUS TAXONOMY

Strategy of the viral genome

The order of presentation of taxa adopted by the ICTV is based on the four criteria that define the strategy of the viral genome (see The genetics of vertebrate viruses). The virus taxa are grouped according to (1) the nature of the viral genome; (2) the strandedness of the nucleic acid; (3) the facility for reverse transcription; and (4) the polarity of the genome. There are seven categories: single-stranded DNA viruses (Table 1), double-stranded DNA viruses (Table 2), reverse-transcribing viruses (Table 3), double-stranded RNA viruses (Table 4), negative-sense single-stranded RNA viruses (Table 5), positive-sense single-stranded

Table 1 *The taxonomy of single-stranded DNA viruses*

Order	Family [Subfamily]	Genus	Type species
–	*Inoviridae*	*Inovirus*	*Enterobacteria phage M13*
		Plectrovirus	*Acholeplasma phage MV-L51*
–	*Microviridae*	*Microvirus*	*Enterobacteria phage ϕX174*
		Spiromicrovirus	*Spiroplasma phage 4*
		Bdellomicrovirus	*Bdellovibrio phage MAC1*
		Chlamydiamicrovirus	*Chlamydia phage 1*
–	*Geminiviridae*	*Mastrevirus*	*Maize streak virus*
		Curtovirus	*Beet curly top virus*
		Topocuvirus	*Tomato pseudo curly top virus*
		Begomovirus	*Bean golden mosaic virus*
	Nanoviridae	*Nanovirus*	*Subterranean clover stunt virus*
		Babuvirus	*Banana bunchy top virus*
–	*Circoviridae*	*Circovirus*	*Porcine circovirus 1*
		Gyrovirus	*Chicken anaemia virus*
–	(Unassigned)	*Anellovirus*	*Torque teno virus*
–	*Parvoviridae* [*Parvovirinae*]	*Parvovirus*	*Murine minute virus*
		Erythrovirus	*B19 virus*
		Dependovirus	*Adeno-associated virus 2*
	[*Densovirinae*]	*Densovirus*	*Junonia coenia densovirus*
		Iteravirus	*Bombyx mori densovirus*
		Brevidensovirus	*Aedes aegypti densovirus*

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned'.

RNA viruses (Table 6), and unconventional agents, comprising naked RNA viruses, viroids, and subviral agents (Table 7). The order of presentation does not imply hierarchical or phylogenetic relationship. In each table the viruses that infect vertebrates are indicated by the absence of shading. It will be obvious that no single strategy is typical of vertebrate viruses. The only unique strategy is exhibited by the wasp-infecting viruses of the family *Polydnaviridae*, which are the only viruses to possess segmented double-stranded DNA genomes. Otherwise segmentation is absent in DNA-containing viruses, although not uncommon among RNA-containing viruses, particularly those infecting plants. Segmentation of the genome may contribute to control of gene expression, but the consequences are different in the case of animal and plant viruses. The different segments of the genome of viruses infecting plants are encapsidated separately, possibly as an adaptation to movement within the host, and an infectious unit is a complement of particles. As far as is known in viruses infecting vertebrates the full complement of segments is encapsidated in each infectious particle.

The viruses infecting bacteria are predominantly DNA viruses, whereas the viruses infecting plants are either RNA viruses, viruses with a reverse-transcription stage in their multiplication cycle, or single-stranded DNA viruses. The viruses infecting fungi, invertebrates, and vertebrates may have either RNA or DNA genomes, and reverse-transcribing viruses infect all types of hosts except bacteria. The viruses of vertebrates and plants may also infect and multiply in invertebrates, but so far no virus is known that can infect all three types of host, and no vertebrate virus can multiply in plant hosts and vice versa.

The single-stranded DNA viruses

There are six families and one unassigned genus of viruses with single-stranded DNA genomes (Table 1). Viruses of two of the families and the unassigned genus infect vertebrates, and the remaining families embrace viruses found in bacteria, mycoplasmae, plants, and invertebrates.

THE FAMILY *CIRCOVIRIDAE*

The family *Circoviridae* comprises two genera, *Circovirus* and *Gyrovirus*, the type species of which are *Porcine circovirus 1* and *Chicken anemia virus*, respectively. The name of the family derives from the circular form of the approximately 2 kb genome. The vertebrate circoviruses resemble the plant nanoviruses, which have similar particle architecture and circular single-stranded DNA genomes. Indeed, the nanoviruses were initially classified as members of the family *Circoviridae* before achieving separate family status. *Porcine circovirus 1*, the type species of the genus *Circovirus*, resembles plant and bacterial viruses of the families *Geminiviridae* and *Microviridae* respectively, in terms of nucleic acid and protein homologies related to rolling circle DNA replication (Meehan et al. 1997).

The name *Anellovirus* has been proposed for an unassigned genus to include the recently discovered 'TT virus' and 'TT virus-like mini virus', which contain circular single-stranded DNA genomes of about 3.8 and 2.9 kb respectively. Despite their delayed recognition, these viruses are globally distributed in human and some nonhuman populations and may prove to be the most abundant of all viruses. The anelloviruses share similarities with the circoviruses regarding genome organization and expression, but all isolates possess negative sense genomes like chicken anemia virus (the sole member of the genus *Gyrovirus*) in contrast to the ambisense genomes of the circoviruses belonging to the genus *Circovirus*. The anelloviruses are unusual among DNA viruses in displaying extreme genetic heterogeneity.

THE FAMILY *PARVOVIRIDAE*

The family *Parvoviridae* comprises two subfamilies, the *Parvovirinae* comprising three genera of viruses infecting vertebrates and the *Densovirinae* comprising three genera of viruses infecting invertebrates. Viruses of both subfamilies possess linear single-stranded DNA genomes of 4–6 kb, unlike the other families of single-stranded DNA viruses where the DNA is circularized. The polarity of the single-stranded DNA encapsidated in virions varies according to species. *Murine minute virus* (genus *Parvovirus*) preferentially incorporates negative strands, whereas *Adeno-associated virus* (genus *Dependovirus*) incorporates strands of either polarity in quasi-equivalent amounts. The principal human pathogen in the single-stranded DNA virus category is *B19 virus*, which is classified as the type species of the third vertebrate-infecting genus, the genus *Erythrovirus*.

A feature distinguishing some viruses of the genus *Densovirus* from the vertebrate parvoviruses is encoding of the replicative function gene (REP) and the capsid protein gene (CP) on complementary strands. The processing of capsid proteins from the CP ORF involves splicing, which is mediated by an alternative splice donor in the case of viruses belonging to the genus *Parvovirus*, and an alternative splice acceptor in the case of viruses belonging to the genus *Densovirus*. Phylogenetic analyses support the formal taxonomy of the *Parvoviridae* (see *Virus Taxonomy, the Seventh Report of the ICTV*, van Regenmortel et al. 2000).

The double-stranded DNA viruses

There are 20 families and three unassigned genera of viruses with double-stranded DNA genomes (Table 2). Nine of the families and one of the unassigned genera contain viruses infecting bacteria, archaea or mycoplasmae, one family contains viruses infecting algae, four families contain viruses infecting invertebrates, and four families and one unassigned genus contain viruses infecting vertebrates. Two other families contain viruses with either vertebrate or invertebrate hosts. The unassigned fungal virus genus *Rhizidiovirus* was previously associated with the adenoviruses. There are no viruses infecting plants in this category.

The three families of bacterial viruses characterized by possession of complex contractile (*Myoviridae*) or noncontractile (*Siphoviridae* and *Podoviridae*) tail structures include 96 percent of the more than 4 500 bacterial viruses described in the literature. These three families are now grouped together as the order *Caudovirales* (Maniloff and Ackermann 1998). The species classified in these families infect hosts on most branches of the bacterial phylogenetic tree, and two of the three families include species infecting *Bacteria* and species infecting *Archaea*, suggesting that tailed viruses originated before the divergence of these hosts. The rationale for establishment of the order *Caudovirales* differs from that for the two orders of vertebrate viruses, *Mononegavirales* and *Nidovirale*s (see The negative-sense single-stranded RNA viruses and The positive-sense single-stranded RNA viruses), where there is evidence of common phylogeny and genome organization. Evidence of common phylogeny of the constituent members of the order *Caudovirales* cannot be expected on account of the time scale of evolution, the enormous population sizes, and the ecological diversity of the tailed bacterial viruses. The occurrence of horizontal gene transfer is another complicating factor. The criteria for creation of the order *Caudovirales* are the following: a highly conserved complex morphology; a common replication pattern, involving injection of DNA, formation of concatamers and cleavage to unit length progeny genomes; the insertion of DNA into preformed proheads fabricated on scaffolding proteins; and the release of the tailed particle by virally encoded lytic enzymes. This example illustrates that a logical and useful taxonomy of viruses can be constructed in the absence of knowledge of phylogenetic relationships.

THE FAMILY *POXVIRIDAE*

The family *Poxviridae* comprises species of virus infecting either vertebrate or invertebrate hosts. The members of the family are distinguished from the other double-stranded DNA viruses by their cytoplasmic site of multiplication. The genome is a single, linear molecule of covalently closed double-stranded DNA, 139–375 kbp in size. The subfamily *Chordopoxvirinae* consists of eight genera of viruses infecting vertebrates generally with restricted host ranges. The subfamily *Entompoxvirinae* embraces three genera of viruses infecting distinct insect hosts. The two subfamilies are also distinguished by a different linear arrangement of core genes. Phylogenetic analyses of poxvirus DNA polymerases, NTPases, thymidine kinases, and DNA uracyl glycosulases support the formal taxonomy in

Table 2 *The taxonomy of double-stranded DNA viruses*

Order	Family [Subfamily]	Genus	Type species
Caudovirales	*Myoviridae*	'T4-like viruses'	*Enterobacteria phage T4*
		'P1-like viruses'	*Enterobacteria phage P1*
		'P2-like viruses'	*Enterobacteria phage P2*
		'Mu-like viruses'	*Enterobacteria phage Mu*
		'SPO1-like viruses'	*Bacillus phage SPO1*
		'ϕH-like viruses'	*Halobacterium virus ϕH*
	Siphoviridae	'λ-like viruses'	*Enterobacteria phage λ*
		'T1-like viruses'	*Enterobacteria phage T1*
		'T5-like viruses'	*Enterobacteria phage T5*
		'c2-like viruses'	*Lactococcus phage c2*
		'L5-like viruses'	*Mycobacterium phage L5*
		'ψM1- like viruses'	*Methanobacterium ψM1*
	Podoviridae	'T7-like viruses'	*Enterobacteria phage T7*
		'ψ29-like viruses'	*Bacillus phage ψ29*
		'P22-like viruses'	*Enterobacteria phage P22*
–	*Rudiviridae*	*Rudivirus*	*Sulfolobus virus SIRV1*
–	*Tectiviridae*	*Tectivirus*	*Enterobacteria phage PRD1*
–	*Corticoviridae*	*Corticovirus*	*Alteromonas phage PM2*
–	*Lipothrixviridae*	*Lipothrixvirus*	*Thermoproteus virus 1*
–	*Plasmaviridae*	*Plasmavirus*	*Acholeplasma phage L2*
–	*Fuselloviridae*	*Fusellovirus*	*Sulfolobus virus SSV1*
–	*Phycodnaviridae*	*Chlorovirus*	*Paramecium bursaria Chlorella virus 1*
		Prasinovirus	*Micromonas pusilla virus SP1*
		Prymnesiovirus	*Chrysochromulina brevifilum virus PW1*
		Phaeovirus	*Ectocarpus siliculosis virus 1*
–	–	'Sulfolobus SNDV-like viruses'	*Sulfolobus virus SNDV*
–	*Poxviridae* [*Chordopoxvirinae*]	*Orthopoxvirus*	*Vaccinia virus*
		Parapoxvirus	*Orf virus*
		Avipoxvirus	*Fowlpox virus*
		Capripoxvirus	*Sheeppox virus*
		Leporipoxvirus	*Myxoma virus*
		Suipoxvirus	*Swinepox virus*
		Molluscipoxvirus	*Molluscum contagiosum virus*
		Yatapoxvirus	*Yaba monkey tumor virus*
	[*Entomopoxvirinae*]	*Entomopoxvirus A*	*Melolontha melolontha entomopoxvirus*
		Entomopoxvirus B	*Amsacta moorei entomopoxvirus*
		Entomopoxvirus C	*Chironomus luridus entomopoxvirus*
–	*Iridoviridae*	*Ranavirus*	*Frog virus 3*
		Lymphocystivirus	*Lymphocystis disease virus 1*
		Itidovirus	*Invertebrate iridescent virus 6*
		Chloriridovirus	*Invertebrate iridescent virus 3*
–	*Polydnaviridae*	*Ichnovirus*	*Campoletis sonorensis ichnovirus*
		Bracovirus	*Cotesia melanoscela bracovirus*
–	*Herpesviridae [Alphaherpesvirinae]*	*Simplexvirus*	*Human herpesvirus 1*
		Varicellovirus	*Human herpesvirus 3*
		Mardivirus	*Gallid herpesvirus 2*
		Iltovirus	*Gallid herpesvirus 1*
	[Betaherpesvirinae]	*Cytomegalovirus*	*Human herpesvirus 5*
		Muromegalovirus	*Murid herpesvirus 1*
		Roseolovirus	*Human herpesvirus 6*

(*Continued over*)

Table 2 *The taxonomy of double-stranded DNA viruses (Continued)*

Order	Family [Subfamily]	Genus	Type species
	[*Gammaherpesvirinae*]	*Lymphocryptovirus*	*Human herpesvirus 4*
		Rhadinovirus	*Saimirine herpesvirus 2*
	(Unassigned)	*Ictalurivirus*	*Ictalurid herpesvirus 1*
–	*Polyomaviridae*	*Polyomavirus*	*Simian virus 40*
–	*Papillomaviridae*	*Papillomavirus*	*Cottontail rabbit papillomavirus*
–	*Adenoviridae*	*Mastadenovirus*	*Human adenovirus C*
		Atadenovirus	*Ovine adenovirus D*
		Aviadenovirus	*Fowl adenovirus A*
		Siadenovirus	*Frog adenovirus*
	(Unassigned)	*Rhizidiovirus*	*Rhyzidiomyces virus*
–	*Ascoviridae*	*Ascovirus*	*Spodoptera frugiperda ascovirus*
–	*Baculoviridae*	*Nucleopolyhedrovirus*	*Autographa californica nucleopolyhedrovirus*
		Granulovirus	*Cydia pomonella granulovirus*
–	*Asfarviridae*	*Asfivirus*	*African swine fever virus*
–	*Nimaviridae*	*Whispovirus*	*White spot syndrome virus 1*

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned'.

Table 2. The major human pathogen, the species *Variola virus*, no longer exists in nature.

THE FAMILY *IRIDOVIRIDAE*

The family *Iridoviridae* comprises large enveloped icosahedral viruses with linear double-stranded DNA genomes of 140–300 kbp. Two of the four genera in the family are viruses with vertebrate hosts; that is, the genus *Ranavirus*, infecting amphibians, and the genus *Lymphocystivirus* infecting fish. The DNA of the vertebrate viruses is highly methylated, whereas the DNA of the invertebrate viruses is not. The vertebrate viruses possess an additional outer envelope derived by budding through the plasma membrane, which may also be present in invertebrate viruses grown in tissue culture, but this is not essential for infectivity. Phylogenetic analysis of major capsid protein sequences confirms the formal taxonomy, which is based on a variety of phenotypic properties.

The major capsid protein exhibits detectable amino acid sequence homology with the corresponding proteins of both *Paramecium bursaria Chlorella virus 1* of the family *Phycodnaviridae* and *African swine fever virus* of the family *Asfarviridae*. There are no recognized human pathogens in the famly *Iridoviridae.*

THE FAMILY *ASFARVIRIDAE*

The family *Asfarviridae* contains a single genus *Asfivirus*. The taxonomic position of the species *African swine fever virus* has been problematic in view of the distant homology with iridoviruses referred to above. Furthermore, there is resemblance to the *Poxviridae* in the intricate morphology, genome structure, and replication pattern of *African swine fever virus*. Originally this species was listed as a member of the family *Iridoviridae,* then later designated as the solitary member of an unassigned and unnamed genus. In the revised taxonomy of the seventh report it has been elevated to the status of type species of the genus *Asfivirus* of the family *Asfarviridae*. The name of the family is a siglum compounded from the phrase African swine fever and related viruses.

THE FAMILY *HERPESVIRIDAE*

The family *Herpesviridae* was one of the first taxonomic groups to be defined on the basis of virion properties rather than by disease association (Andrewes 1954). It now comprises three subfamilies and an unassigned genus. The order of the genes is largely conserved within subfamilies, but extensive gene rearrangement is observed between subfamilies. Each of the three subfamilies contains two or more genera, with important human pathogens in each subfamily. Related herpesviruses are classified as distinct species on the basis of quantifiable sequence differences across the whole genome and possession of distinct phenotypes with respect to host, epidemiology, or pathogenesis. For example the two serotypes of herpes simplex virus are now designated as the species *Human herpesvirus 1* and *Human herpesvirus 2* because these viruses exhibit sequence differences across the genome and infect different tissues with distinctive epidemiological consequences. The ability of these two species to undergo recombination in cultured cells is not considered relevant since no natural recombinants have been isolated despite the apparent parallel evolution of these viruses over millions of years.

The formal taxonomy of three subfamilies derived initially on the basis of phenotypic properties has been substantiated by phylogenetic analyses of eight

well-conserved genes. The viral phylogenies fit well with the host phylogenies derived from the fossil record. On the assumption of the existence of a constant molecular clock and cospeciation of virus and host, the divergence of the three subfamilies is estimated to have occurred 180–220 million years ago. Major sublineages discernible within each subfamily were probably established before the radiation of the *Mammalia* 60 to 80 million years ago, with speculation within these sublineages occurring subsequently (McGeoch et al. 1995).

The fish herpesviruses are anomalous and have been consigned temporarily to the unassigned genus *Ictalurivirus*. The species *Ictalurid herpesvirus 1* (which includes channel catfish virus) is classified as a herpesvirus on the basis of virion morphology, and indeed this virus was instrumental initially in deriving the structure of the mammalian alphaherpesvirus genome. However, nucleotide sequence analysis has revealed no specific relationship with mammalian herpesviruses at the amino acid sequence level, other than with respect to ubiquitous enzymes not unique to mammalian herpesviruses (Davison 1998). Consequently the fish and mammalian herpesviruses may have arisen independently and may have acquired similar morphologies by convergent evolution. Alternatively they may have evolved by divergence from a common ancestral herpesvirus and their phylogenetic relationship has been obscured by 400 million years of progressive covariation with their hosts. These findings illustrate again that taxonomy cannot be reduced solely to the classification of viruses by extent of sequence homology. It has been suggested recently that two additional families should be created: one to accommodate the fish and amphibian herpesviruses and the other to embrace some recently discovered herpesviruses from molluscs. These viruses exhibit common structural and physical organization but appear to have separate evolutionary histories from the avian and mammalian herpesviruses. It was further suggested that to resolve the taxonomic difficulties the three families should together constitute a new virus order, the *Herpesvirales* (Davison 2002).

THE FAMILIES *POLYOMAVIRIDAE* AND *PAPILLOMAVIRIDAE*

As a result of continuing molecular and genetic characterization of the small oncogenic DNA viruses, the family *Papovaviridae* has disappeared to be replaced by two new families, the *Polyomaviridae* and the *Papillomaviridae*. Both families include human pathogens. *Simian virus 40* now becomes the type species of the single genus *Polyomavirus* of the family *Polyomaviridae*, and *Cottontail rabbit papilloma virus* becomes the type species of the single genus *Papillomavirus* of the family *Papillomaviridae*. This situation illustrates an unresolved problem in virus taxonomy. The assignment of taxonomic levels is peculiar to each group of viruses, and as yet there are no rules or criteria for achieving parallelism or equivalence between different taxonomic lineages. These two families of small oncogenic DNA viruses may be the next candidates for inclusion in an order.

THE FAMILY *ADENOVIRIDAE*

The taxonomy of the family *Adenoviridae* has been revised as a result of synthesis of the extensive literature on the molecular and genetic properties of these viruses. The serotypes of the traditional classification of adenoviruses have now been grouped into species. Species are designated on the basis of several properties in accordance with the van Regenmortel definition. For example, lack of cross-neutralization, phylogenetic difference exceeding 10 percent, etc. The criteria are flexible so that where phylogenetic distance is <1 percent, additional criteria may be used, such as ability to recombine, degree of oncogencity, GC content, etc. The type species of the genus *Mastadenovirus* is now designated *Human adenovirus C*, which includes human adenovirus types 1, 2, 5, and 6, bovine adenovirus type 9, and simian adenovirus types 13 and 26. As a consequence of this revision the diversity of mammalian adenoviruses has been reduced to about 20 species.

The avian adenoviruses are serologically distinct from the mammalian adenoviruses and are included in the genus *Aviadenovirus*. The type species is *Fowl adenovirus A*. The avian adenoviruses lack the genes of mammalian adenoviruses that encode proteins V and IX, and also lack homologues of early regions E1 and E3. The avian adenoviruses have been grouped into five species in the same manner as the mammalian adenoviruses.

Since publication of the seventh report, two new genera have been added to the *Adenoviridae* as a result of characterization of adenoviruses from nonhuman hosts. The genus *Siadenovirus* includes only two species: a virus isolated from a leopard frog and an avian virus distinguishable antigenically and genetically from the other avian adenoviruses. The genus *Atadenovirus* embraces a larger group of adenoviruses originating from several different hosts, and which includes several serotypes of bovine adenoviruses and adenoviruses from a wide range of reptiles.

The reverse-transcribing viruses

There are five families of viruses that have a reverse transcription stage in their replication cycle (Table 3). Reverse transcribing viruses have been isolated from fungi, plants, invertebrates, and vertebrates, but not from bacteria. The families differ with respect to the stage of the replication cycle sequestered in the extracellular particle. Consequently some viruses encapsidate DNA in the virion, whereas others encapsidate RNA. Remarkably all of the known double-stranded DNA-

Table 3 *The taxonomy of reverse-transcribing viruses*

Order	Family	Genus	Type species
–	*Pseudoviridae*	*Pseudovirus*	*Saccharomyces cerevisiae Ty1 virus*
		Hemivirus	*Drosophila melanogaster copia virus*
–	*Metaviridae*	*Metavirus*	*Saccharomyces cerevisiae Ty3 virus*
		Errantivirus	*Drosophila melanogaster gypsy virus*
–	*Hepadnaviridae*	*Orthohepadnavirus*	*Hepatitis B virus*
		Avihepadnavirus	*Duck hepatitis B virus*
–	*Caulimoviridae*	*Badnavirus*	*Commelina yellow mottle virus*
		Caulimovirus	*Cauliflower mosaic virus*
		Tungrovirus	*Rice tungro bacilliform virus*
		Soymovirus	*Soybean chlorotic mottle virus*
		Cavemovirus	*Cassava vein mosaic virus*
		Petuvirus	*Petunia vein clearing virus*
–	*Retroviridae* [*Orthoretrovirinae*]	*Alpharetrovirus*	*Avian leukosis virus*
		Betaretrovirus	*Mouse mammary tumour virus*
		Gammaretrovirus	*Murine leukemia virus*
		Deltaretrovirus	*Bovine leukemia virus*
		Epsilonretrovirus	*Walleye dermal sarcoma virus*
		Lentivirus	*Human immunodeficiency virus 1*
	[*Spumaretrovirinae*]	*Spumavirus*	*Simian foamy virus*

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned'.

containing viruses of plants replicate via reverse transcription and have been grouped into the single family *Caulimoviridae.* Of the other four families, the *Hepadnaviridae* and the *Retroviridae* comprise viruses infecting vertebrate hosts only, and the *Metaviridae* and the *Pseudoviridae* contain viruses infecting either fungal or invertebrate hosts, according to genus.

THE FAMILY *RETROVIRIDAE*

The taxonomy of the retroviruses has been simplified in the latest revision, and there are now seven genera designated as *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Lentivirus,* and *Spumavirus*. The genus *Alpharetrovirus* comprises the viruses previously designated as Avian type C retroviruses, and the genus *Betaretrovirus* embraces the viruses previously classified as the Mammalian type B retroviruses and the type D retroviruses. The genus *Gammaretrovirus* replaces the former Mammalian type C retroviruses and in addition includes the reptilian type C oncoviruses and the *Reticuloendotheliosis viruses*. The genus *Deltaretrovirus* includes the former 'BLV-HTLV-like retroviruses.' A new genus, *Epsilonretrovirus*, has been introduced to accommodate several complex retroviruses isolated from fish. The genus names *Lentivirus* and *Spumavirus* have been retained from earlier classifications on the principle inherent in the nomenclature code that 'existing names of taxa should be retained wherever feasible.'

The family *Retroviridae* embraces vertebrate viruses with genomes consisting of a dimer of linear positive-sense single-stranded RNA. The monomers range in size from 7 to 12.8 kb and are held together by hydrogen bonding. Purified virion RNA is not infectious. Each monomer is polyadenylated at the 3′ end, and has a type 1 cap structure at the 5′ end. A specific tRNA is base-paired to a primer-binding site near the 5′ end. Infectious virus has four major genes in the order 5′–gag–pro–pol–env-3′. The *pro* gene encodes a specific protease, and the *pol* gene encodes the reverse transcriptase and an integrase. Additional cell-derived genes may be present as additions or substitutions in retroviruses classified in the genera *Alpharetrovirus*, *Betaretrovirus*, and *Gammaretrovirus*, with important pathogenic consequences. Viruses may become helper-dependent as a result of gene substitution. Retroviruses classified in the genera *Deltaretrovirus*, *Epsilonretrovirus*, *Lentivirus*, and *Spumavirus* contain genes encoding additional nonstructural proteins, which play a role in control of gene expression and viral replication. Integration into cellular DNA is an obligatory stage in the multiplication cycle. As a consequence retroviruses exist as both exogenous infectious agents of vertebrates, and as endogenous proviruses inherited as Mendelian genes as a result of infection of germ line cells. The latter are not part of the universal taxonomy at present. Phylogenetic analysis of the pol genes of retroviruses produces trees consistent with the formal taxonomy (see *Virus Taxonomy, the Seventh Report of the ICTV*, van Regenmortel et al. 2000).

THE FAMILY *HEPADNAVIRIDAE*

The family *Hepadnaviridae* contains two genera of DNA viruses. The genome of hepadnaviruses consists of a

single molecule of circular DNA, 3.0–3.3 kb in size, which is not covalently closed. The DNA may be partially single-stranded in the virion, the extent of this differing in the two genera. The reverse-transcription stage in the replication cycle occurs in the cytoplasm and consequently integration into cellular DNA is not an obligatory or usual part of the cycle. *Hepatitis B virus* is the type species of the genus *Orthohepadnavirus* and *Duck hepatitis B virus* is the type species of the genus *Avihepadnavirus*. Host range is the primary determinant of assignment of species to genus.

THE FAMILIES *CAULIMOVIRIDAE*, *METAVIRIDAE*, AND *PSEUDOVIRIDAE*, THE NONVERTEBRATE FAMILIES OF REVERSE-TRANSCRIBING VIRUSES

The family *Caulimoviridae* contains five genera of plant-infecting DNA viruses. The genome is a single molecule of circular DNA, 7.2–8.1 kbp in size. The genome contains between two and seven ORFs, depending on the genus. The organization of the genome is one of the principal distinguishing features of the individual genera. The functions of the virus-encoded proteins common to all genera are the following: the capsid protein gene, an aspartate protease, a reverse transcriptase, and ribonuclease H. Additional gene functions relate to movement in the plant host and vector transmissibility. The replication cycle is episomal and does not involve an integration phase.

The family *Metaviridae* includes species of retrotransposons infecting yeast (the genus *Metavirus*) or invertebrates (the genus *Errantivirus*), and the family *Pseudoviridae* includes the retroelements known as LTR-retrotransposons infecting yeast (the genus *Pseudovirus*) and invertebrates (the genus *Hemivirus*).

The double-stranded RNA viruses

Double-stranded RNA viruses have been isolated from bacteria, fungi, plants, invertebrates, and vertebrates (Table 4). These viruses are classified into six families and one unassigned genus. The double-stranded RNA viruses are predominantly segmented genome viruses. Four of the six families and the unassigned genus contain viruses with segmented genomes. Two of the families, the *Reoviridae* and the *Birnaviridae*, contain viruses infecting vertebrate hosts, although not exclusively.

THE FAMILY *REOVIRIDAE*

The family *Reoviridae* comprises ten genera: six (*Aquareovirus*, *Coltivirus*, *Orbivirus*, *Orthoreovirus, Rotavirus*, and *Seadornavirus*) contain vertebrate-infecting viruses, three (*Fijivirus*, *Phytoreovirus*, and *Oryzavirus*) plant-

Table 4 *The taxonomy of double-stranded RNA viruses*

Order	Family	Genus	Type species
–	*Cystoviridae*	*Cystovirus*	*Pseudomonas phage (6*
–	*Reoviridae*	*Orthoreovirus*	*Mammalian orthoreovirus*
		Orbivirus	*Bluetongue virus*
		Rotavirus	*Rotavirus A*
		Coltivirus	*Colorado tick fever virus*
		Seadornavirus	*Kodipiro virus*
		Aquareovirus	*Aquareovirus A*
		Cypovirus	*Cypovirus 1*
		Fijivirus	*Fiji disease virus*
		Phytoreovirus	*Rice dwarf virus*
		Oryzavirus	*Rice ragged stunt virus*
–	*Birnaviridae*	*Aquabirnavirus*	*Infectious pancreatic necrosis virus*
		Avibirnavirus	*Infectious bursal disease virus*
		Entomobirnavirus	*Drosophila X virus*
–	*Totiviridae*	*Totivirus*	*Saccharomyces cerevisiae virus L-A*
		Giardiavirus	*Giardia lamblia virus*
		Leishmaniavirus	*Leishmania RNA virus 1-1*
–	*Partitiviridae*	*Partitivirus*	*Gaeumannomyces graminis virus 019/6-A*
		Alphacryptovirus	*White clover cryptic virus 1*
		Betacryptovirus	*White clover cryptic virus 2*
–	*Chrysoviridae*	*Chrysovirus*	*Penicillium chrysogenum virus*
–	*Hypoviridae*	*Hypovirus*	*Cryphonectria hypovirus 1-EP713*
–	(Unassigned)	*Varicosavirus*	*Lettuce big-vein virus*

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned.'

infecting viruses, and one (*Cypovirus*) invertebrate-infecting viruses. Reoviruses contain 10, 11, or 12 segments, depending on the genus, of linear double-stranded RNA. The cypoviruses share a property in common with the DNA-containing baculoviruses. Namely, possession of a gene encoding a polyhedrin protein which provides a protective matrix to enhance the survival of virus released into the environment after excretion from the gut or on death of the host.

In this family the prime determinant for classification of viruses into species is the Darwinian criterion of ability to exchange (reassort) genome segments. However, such information is rarely available and the genetic compatibility of isolates is estimated indirectly from serological, physical, and sequence data. The electropheretic migration patterns of the double-stranded RNA genome segments isolates (the electropherotype) may also be employed on account of their high resolution and reproducibility, particularly in the case of rotaviruses many of which cannot be propagated in vitro.

THE FAMILY *BIRNAVIRIDAE*

The family *Birnaviridae* comprises three genera of viruses with bisegmented linear double-stranded RNA genomes. The larger genome segment is variable around 3 kbp in size, and the smaller more homogeneous around 2.7 kbp. There are minor differences in the processing of the viral genes between genera, and the taxonomy is based on serology, host range, and pathology. Two of the three genera contain vertebrate-infecting viruses: species of the genus *Aquabirnavirus* are major pathogens of salmonid fish, and *Infectious bursal disease virus*, the type species of the genus *Avibirnavirus*, is an important pathogen in the poultry industry. The natural host of *Drosophila X virus*, the type species of the genus *Entomobirnavirus*, is probably *Culicoides* spp., with infection rendering the host sensitive to CO_2.

THE NONVERTEBRATE FAMILIES OF DOUBLE-STRANDED RNA VIRUSES

The bacterial viruses of the family *Cystoviridae* have tripartite genomes of some 13 kbp in total. The fungal and plant viruses of the famly *Partitiviridae* have bipartite genomes of 2.8–6.0 kbp total. The unassigned genus *Varicosavirus* also has a bipartite linear genome of 13.5 kbp total. The *Totiviridae* and *Hypoviridae*, on the other hand, have nonsegmented linear double-stranded RNA genomes of some 4.6–7 kbp and 9–13 kbp total, respectively.

The negative-sense single-stranded RNA viruses

Vertebrate-infecting viruses are classified in all seven families of negative-sense single-stranded RNA viruses (Table 5). Plant viruses are included in two of the seven families and are the sole representatives of the two unassigned genera, *Ophiovirus* and *Tenuivirus*. *Deltavirus* is formally included with the negative-sense RNA viruses as an unassigned genus, although the single species *Hepatitis D virus* exists only as a satellite of the species *Hepatitis B virus* (family *Hepadnaviridae*). At this time no negative-sense single-stranded RNA virus has been isolated from bacteria, fungi, or protozoa.

THE MONOPARTITE NEGATIVE-SENSE GENOME VIRUSES – THE ORDER *MONONEGAVIRALES*; COMPRISING THE FAMILIES *BORNAVIRIDAE*, *FILOVIRIDAE*, *PARAMYXOVIRIDAE*, AND *RHABDOVIRIDAE*

The first order in viral taxonomy was created to embrace the three families, *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*, on the basis of similar genome structure and individual gene functions, and the apparent absence of genetic recombination. The latter property suggests that evolutionary relationships are not obscured by horizontal transfer of genetic information. Phylogenetic analysis has supported the taxonomic association of these families (Pringle and Easton 1997; see The order *Mononegavirales*). Subsequently the order *Mononegavirales* was extended to include the family *Bornaviridae* (see Borna disease virus, and Pringle 1997). The bornaviruses are vertebrate viruses with monopartite negative-stranded RNA genomes, which have features in common with the other three families in the order. They are substantially different in other properties, however, such as their intracellular location and limited processing of mRNA by splicing. Some disputed evidence suggests that bornaviruses may be associated with human psychiatric disease.

The two genera of viruses, *Ebolavirus* and *Marburgvirus*, classified in the family *Filoviridae* are enigmatic human pathogens thought to be exclusively vertebrate viruses. The classification of these viruses remains in a fluid state due to the hazardous nature of these viruses, which restricts their characterization.

The viruses classified in the family *Paramyxoviridae* are predominantly associated with respiratory disease and childhood illnesses. Two subfamilies have been designated. The subfamily *Paramyxovirinae* comprises the three genera *Respirovirus*, *Morbillivirus*, and *Rubulavirus* described in the Seventh Report, and two newly designated genera; the genus *Avulavirus* accommodating *Newcastle disease virus*, a species now recognized to possess unique properties; and the genus *Henipavirus*, created to accommodate *Hendra virus* and *Nipah virus*, two recently discovered bat paramyxoviruses associated with outbreaks of life-threatening human disease. The subfamily *Pneumovirinae* contains the genera *Pneumovirus* and *Metapneumovirus*, both of which

Table 5 *The taxonomy of negative-sense single-stranded RNA viruses*

Order	Family [Subfamily]	Genus	Type species
Mononegavirales			
	Paramyxoviridae		
	[Paramyxovirinae]	*Respirovirus*	*Sendai virus 1*
		Morbillivirus	*Measles virus*
		Rubulavirus	*Mumps virus*
		Avulavirus	*Newcastle disease virus*
		Henipavirus	*Hendra virus*
	[Pneumovirinae]	*Pneumovirus*	*Human respiratory syncytial virus*
		Metapneumovirus	*Turkey rhinotracheitis virus*
	Rhabdoviridae	*Vesiculovirus*	*Vesicular stomatitis Indiana virus*
		Lyssavirus	*Rabies virus*
		Ephemerovirus	*Bovine ephemeral fever virus*
		Cytorhabdovirus	*Lettuce necrotic yellows virus*
		Nucleorhabdovirus	*Potato yellow dwarf virus*
		Novirhabdovirus	*Infectious hematopoietic necrosis virus*
	Filoviridae	*Marburgvirus*	*Lake Victoria marburgvirus*
		Ebolavirus	*Zaire ebolavirus*
	Bornaviridae	*Bornavirus*	*Borna disease virus*
–	*Orthomyxoviridae*	*Influenzavirus A*	*Influenza A virus*
		Influenzavirus B	*Influenza B virus*
		Influenzavirus C	*Influenza C virus*
		Thogotovirus	*Thogoto virus*
		Isavirus	*Infectious salmon anemia virus*
–	*Bunyaviridae*	*Orthobunyavirus*	*Bunyamwera virus*
		Hantavirus	*Hantaan virus*
		Nairovirus	*Dugbe virus*
		Phlebovirus	*Rift Valley fever virus*
		Tospovirus	*Tomato spotted wilt virus*
–	*Arenaviridae*	*Arenavirus*	*Lymphocytic choriomeningitis virus*
–	–	*Ophiovirus*	*Citrus psorosis virus*
–	–	*Tenuivirus*	*Rice stripe virus*
–	(Unassigned)	*Delta virus*	*Hepatitis delta virus*

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned.'

contain viruses uniquely associated with respiratory disease of human infants. These two subfamilies are separated by differences in gene number, gene order, and gene size. Unexpected sequence homologies between pneumoviruses and members of the other families of monopartitite negative-sense RNA viruses was one of the initiating factors in the establishment of the taxon of order in virology.

The family *Rhabdoviridae* embraces one of the largest and most catholic of virus families. Four of the six genera include viruses infecting vertebrates. The hosts of rhabdoviruses include plants, invertebrates, and vertebrates, and different rhabdoviruses may multiply in invertebrates and plants, or invertebrates and vertebrates, but none is known which will multiply in vertebrates and plants. The plant rhabdoviruses classified in the genus *Nucleorhabdovirus* are exceptional, being located in the nuclei of infected cells. All other rhabdoviruses are cytoplasmic. Because of their distinctive morphology many rhabdovirsues have been named on evidence from electron microscopy alone. The status of these viruses is unknown since they do not exist as isolates and as a consequence they are not considered in the current taxonomy.

THE SEGMENTED GENOME VIRUSES – THE FAMILIES *ORTHOMYXOVIRIDAE*, *BUNYAVIRIDAE*, AND *ARENAVIRIDAE*

Thee three families of segmented genome viruses have diverse properties and exhibit no common link other than segmentation of the genome. Consequently an order has not been created to embrace these three families. Reassortment of segments may occur among serologically related viruses, a property which can be employed in taxonomy at the species level. Reassortment of segments has been demonstrated in all three families, but in the absence of comprehensive genetic data, the definition of species within a genus is usually

based on serological properties, host range, geographical distribution, and disease association.

The family *Orthomyxoviridae* includes three genera of vertebrate viruses (*Influenzavirus A*, *B*, and *C*) and the genus *Thogotovirus* containing a vertebrate-infecting species and an invertebrate-infecting species. The hemagglutinin-esterase (HE) protein of *Influenzavirus C* exhibits 30 percent amino acid identity with the HE protein of the positive-sense toroviruses, suggesting that genetic information may have passed horizontally by heterologous RNA recombination. Viruses in the genus *Thogotovirus* have six or seven segments and some of their genes exhibit homologies to influenza virus core protein genes. *Thogoto virus*, the type species of the genus *Thogotovirus*, is a biological enigma. The single glycoprotein gene has no homology with any influenza virus gene but does show similarity to the glycoprotein (*gp64*) gene of baculoviruses. The enigma is compounded by the fact that thogotoviruses are tick-borne viruses, whereas baculoviruses are DNA viruses predominantly parasitizing lepidoptera. Since publication of the Seventh Report, a fifth genus, the genus *Isavirus*, has been added to accommodate *Infectious salmon anemia virus*, a pathogen of marine teleosts. Like the mammalian influenza viruses *Infectious salmon anemia virus* possesses an eight-segmented negative-sense single-stranded RNA genome of approximately 14.5 kb. Low sequence homology is exhibited at the amino acid level with the polymerase gene (*PB1*) of *Influenza C virus* (note: the type species of the genus *Influenzavirus C* is designated *Influenza C virus*, etc).

The family *Bunyaviridae* is possibly the most diverse of all the families of viruses, and many trisegmented bunya-like viruses have yet to be classified. Five genera are differentiated at present. Three of the genera, *Orthobunyavirus*, *Hantavirus*, and *Nairovirus*, are regular negative-sense RNA viruses, whereas the remaining two genera, *Phlebovirus* and *Tospovirus*, have one or more segments of ambisense RNA (see). The genera are defined on the basis of phylogenetic divergence, host range and transmission characteristics. Viruses of the genera *Orthobunyavirus*, *Nairovirus*, and *Phlebovirus* are arthropod-transmitted viruses of vertebrates. Viruses of the genus *Hantavirus* are vertebrate viruses with no known invertebrate hosts, and viruses of the genus *Tospovirus* are plant viruses transmitted by arthropods.

The family *Arenaviridae* comprises a single genus of vertebrate viruses with bipartite RNA genomes. Although included in the negative-sense RNA virus category, both segments of the genome have information encoded in an ambisense orientation. Variable amounts of viral complementary RNA, subgenomic mRNA, and cellular ribosomal RNA may be present in purified arenavirus particles.

The positive-sense single-stranded RNA viruses

These viruses have the most expansive taxonomy (Table 6). So far 22 families and 20 unassigned genera have been designated. Plant virus taxa are the most abundant with eight families and 18 unassigned genera, followed by vertebrate viruses with seven families and one unassigned genus. There are three families and one unassigned genus of invertebrate viruses, one family embracing both vertebrate and invertebrate viruses, two families of fungal viruses and one family of bacterial viruses. No positive-sense single-stranded RNA viruses have been isolated from protozoa.

The existence of three 'supergroups' of positive-sense single-stranded RNA viruses has been hypothesized on the basis of common features of genome organization and expression, reinforced by sequence analyses of conserved motifs within the replicase gene (Goldbach and de Haan 1994; Koonin and Dolja 1993; Ward 1993). Families have been clustered into supergroups of 'picorna-like viruses,' 'toga-like viruses' and 'flavi-like viruses,' and variously designated as higher taxa. The 'flavi-like' supergroup, however, is less homogeneous than the other two. None of these proposals has been ratified by the ICTV. The phylogenetic significance of these observations has been questioned recently and is discussed in the section Phylogenetic analysis and taxonomy.

THE FAMILY *PICORNAVIRIDAE*

The family *Picornaviridae* comprises nine genera of vertebrate viruses. The genome is a single molecule of positive-sense single-stranded RNA, 7.0–8.5 kb in size. The overall sequence identity between viruses of different genera is typically less than 40 percent. Most picornaviruses are specific for one or a few hosts, with the exception of the two species, *Foot-and-mouth disease virus* and *Encephalomyocarditis virus*.

The picornavirus species has been defined by the Picornavirus Study Group as a polythetic class of phylogenetically related serotypes or strains which would normally be expected to share: (1) a limited range of hosts and cell receptors; (2) a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination; and (3) essentially identical genome maps (see, *Virus Taxonomy, the Seventh Report of the ICTV*, van Regenmortel et al. 2000). As a consequence, the familiar serotypes of picornaviruses have been condensed into a limited number of species. For example, the type species *Poliovirus* of the genus *Enterovirus* includes serotypes 1, 2, and 3, and the type species *Foot-and-mouth disease virus* of the genus *Aphthovirus* includes the serotypes, A, C, O, SAT 1, SAT 2, SAT 3, and Asia 1. The genus *Parechovirus* has been created to accommodate two viruses formerly

Table 6 *The taxonomy of positive-sense single-stranded RNA viruses*

Order	Family [Subfamily]	Genus	Type species
–	*Narnaviridae*	*Narnavirus*	*Saccharomyces cerevisiae namavirus 20S*
		Mitovirus	*Chryphonectria parasitica mitovirus 1-NB631*
–	*Leviviridae*	*Levivirus*	*Enterobacteria phage MS2*
		Allolevivirus	*Enterobacteria phage Qβ*
–	*Picornaviridae*	*Enterovirus*	*Poliovirus*
		Rhinovirus	*Human rhinovirus A*
		Hepatovirus	*Hepatitis A virus*
		Cardiovirus	*Encephalomyocarditis virus*
		Aphthovirus	*Foot-and-mouth disease virus*
		Parechovirus	*Human parechovirus*
		Erbovirus	*Equine rhinitis B virus*
		Kobuvirus	*Aichi virus*
		Teschovirus	*Porcine teschovirus*
–	*Dicistroviridae*	*Cripavirus*	*Cricket paralysis virus*
	(Unclassified)	*Iflavirus*	*Infectious flacherie virus*
–	*Sequiviridae*	*Sequivirus*	*Parsnip yellow fleck virus*
		Waikavirus	*Rice tungro spherical virus*
–	*Comoviridae*	*Comovirus*	*Cowpea mosaic virus*
		Fabavirus	*Broad bean wilt virus 1*
		Nepovirus	*Tobacco ringspot virus*
–	*Potyviridae*	*Potyvirus*	*Potato virus Y*
		Rymovirus	*Ryegrass mosaic virus*
		Bymovirus	*Barley yellow mosaic virus*
		Macluravirus	*Maclura mosaic virus*
		Ipomovirus	*Sweet potato mild mottle virus*
		Tritimovirus	*Wheat streak mosaic virus*
–	*Caliciviridae*	*Vesivirus*	*Vesicular exanthema of swine virus*
		Lagovirus	*Rabbit hemorrhagic disease virus*
		Norovirus	*Norwalk virus*
		Sapovirus	*Sapporo virus*
		Hepevirus	*Hepatitis E virus*
–	*Astroviridae*	*Astrovirus*	*Human astrovirus 1*
		Avastrovirus	*Turkey astrovirus*
–	*Nodaviridae*	*Alphanodavirus*	*Nodamura virus*
		Betanodavirus	*Striped jack nervous necrosis virus*
–	*Tetraviridae*	*Betatetravirus*	*Nudaurelia capensis β virus*
		Omegatetravirus	*Nudaurelia capensis ω virus*
–	*Tombusviridae*	*Tombusvirus*	*Tomato bushy stunt virus*
		Carmovirus	*Carnation mottle virus*
		Necrovirus	*Tobacco necrosis virus A*
		Dianthovirus	*Carnation ringspot virus*
		Machlomovirus	*Maize chlorotic mottle virus*
		Avenavirus	*Oat chlorotic stunt virus*
		Aureusvirus	*Pothos latent virus*
		Panicovirus	*Panicum mosaic virus*
Nidovirales	*Coronaviridae*	*Coronavirus*	*Infectious bronchitis virus*
		Torovirus	*Equine torovirus*
	Arteriviridae	*Arterivirus*	*Equine arteritis virus*
	Roniviridae	*Okavirus*	*Gill-associated virus*
–	*Togaviridae*	*Alphavirus*	*Sindbis virus*
		Rubivirus	*Rubella virus*
–	*Flaviviridae*	*Flavivirus*	*Yellow fever virus*
		Pestivirus	*Bovine viral diarrhea virus*
		Hepacivirus	*Hepatitis C virus*

(*Continued over*)

Table 6 *The taxonomy of positive-sense single-stranded RNA viruses (Continued)*

Order	Family [Subfamily]	Genus	Type species
–	*Bromoviridae*	*Alfamovirus*	*Alfalfa mosaic virus*
		Ilarvirus	*Tobacco streak virus*
		Bromovirus	*Brome mosaic virus*
		Cucumovirus	*Cucumber mosaic virus*
		Oleavirus	*Olive latent virus 2*
–	*Closteroviridae*	*Closterovirus*	*Beet yellows virus*
		Crinivirus	*Lettuce infectious yellows virus*
		Ampelovirus	*Grapevine leafroll associated virus 3*
–	*Barnaviridae*	*Barnavirus*	*Mushroom bacilliform virus*
–	*Luteoviridae*	*Luteovirus*	*Barley yellow dwarf virus-PAV*
		Polerovirus	*Potato leafroll virus*
		Enamovirus	*Pea enation mosaic virus-1*
–	–	*Tobamovirus*	*Tobacco mosaic virus*
–	–	*Tobravirus*	*Tobacco rattle virus*
	–	*Hordeivirus*	*Barley stripe mosaic virus*
	–	*Furovirus*	*Soil-borne wheat mosaic virus*
–	–	*Pomovirus*	*Potato mop-top virus*
	–	*Pecluvirus*	*Peanut clump virus*
–	–	*Benyvirus*	*Beet necrotic yellow vein virus*
–	–	*Idaeovirus*	*Raspberry bushy dwarf virus*
–	–	*Capillovirus*	*Apple stem grooving virus*
–	–	*Trichovirus*	*Apple chlorotic leaf spot virus*
–	–	*Sobemovirus*	*Southern bean mosaic virus*
–	–	*Umbravirus*	*Carrot mottle virus*
–	*Tymoviridae*	*Tymovirus*	*Turnip yellow mosaic virus*
		Maculavirus	*Grapevine fleck virus*
		Marafivirus	*Maize rayadofino virus*
–	–	*Carlavirus*	*Carnation latent virus*
–	–	*Potexvirus*	*Potato virus X*
–	–	*Allexivirus*	*Shallot virus X*
–	–	*Foveavirus*	*Apple stem pitting virus*
–	–	*Vitivirus*	*Grapevine virus A*
–	–	*Ourmiavirus*	*Ourmia melon virus*

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned.'

classified as enteroviruses and known as echovirus 22 and 23. These viruses have predicted protein sequences that have less than 30 percent identity with any other picornavirus. They are now ranked as members of the type species *Human parechovirus*. Since publication of the Seventh ICTV Report, three additional genera have been designated: the genus. *Erbovirus* to accommodate *Equine rhinitis B virus*, which is significantly different from the human rhinoviruses, the genus *Kobuvirus* to accommodate *Aichi virus*, which unlike other picornaviruses exhibits icosahedral surface structure by electron microscopy; and the genus *Teschovirus* to accommodate *Porcine teschovirus*.

The 'picornavirus supergroup' comprises the family *Picornaviridae* and the three families of plant viruses, *Comoviridae, Potyviridae*, and *Sequiviridae*. These families share common features of genetic organization (5′-VPg, 3′-polyA, post-translational cleavage of a polyprotein), similar capsid structure, and sequence similarity of the nonstructural proteins. However, there is no biological overlap and the apparent relationship of these families could equally be a consequence of convergent evolution, modular evolution, or divergence from a common ancestor.

THE FAMILY *CALICIVIRIDAE*

The family *Caliciviridae* includes four genera of vertebrate viruses. The assignment of genera is supported by phylogenetic analysis, and species are distinguished by differences in gene sequence, gene product sequences and serology. The caliciviruses have some properties in common with the vertebrate picornaviruses and the plant comoviruses and potyviruses, for example, a VPg at the 5′ end and a poly(A) tract at the 3′end. The putative viral replicase of the caliciviruses shares sequence homology with that of picornaviruses.

THE UNASSIGNED GENUS *HEPEVIRUS*

Hepatitis E virus, which was loosely associated previously with the caliciviruses on morphological criteria, is now ranked as the type species of an unassigned genus. Phylogenetic analysis of polymerase and helicase regions justifies the separate status of the genus *Hepevirus*. Weak homology is evident with members of the *Togaviridae* (e.g. *Rubella virus*) and the plant furoviruses.

THE FAMILY *ASTROVIRIDAE*

The family *Astroviridae* comprises two genera: *Mamastrovirus* and *Avastrovirus*. The genus *Mamastrovirus* contains four species of viruses infecting mammals, and the genus *Avastrovirus* contains two species infecting avian hosts. Each species includes a range of serotypes; e.g. the seven serotypes of human astroviruses, as defined by immune electron microscopy and neutralization tests, are all included in the type species *Human astrovirus 1*.

THE FAMILY *NODAVIRIDAE*

The family *Nodaviridae* comprises two genera of viruses with bipartitite genomes; the *Alphanodavirus* infecting insects and the *Betanodavirus* infecting vertebrates. *Nodamura virus*, the type species of the genus *Alphanodavirus*, may occasionally infect vertebrates. Seven species are defined within this genus on the basis of a complex of molecular and biological characteristics. The genus *Betanodavirus* comprises seven species of viruses infecting different species of marine fish. There is 10 percent or less amino acid homology between the coat proteins of viruses in the two genera, whereas the level of identity is >80 percent within the genus *Betanodavirus*.

THE NESTED SET GENOME VIRUSES – THE ORDER *NIDOVIRALES*; COMPRISING THE FAMILIES *ARTERIVIRIDAE*, *CORONAVIRIDAE*, AND *RONIVIRIDAE*

The two families of vertebrate viruses, *Coronaviridae* and *Arteriviridae,* and the invertebrate (decapod crustacean) virus family *Roniviridae* are grouped as the order *Nidovirales* by virtue of their distinctive genome organization and similar morphology in the case of the vertebrate viruses (Cavanagh 1997; see The order *Nidovirales*). The mRNAs are processed as a 3′ coterminal (nested) set of subgenomic RNAs. Although the mRNAs are structurally polycistronic, translation is restricted with a few exceptions to the unique 5′ region, which is absent from the next smallest mRNA. Another unique feature of the order *Nidovirales* is the configuration of the replicase gene as two open reading frames with expression mediated by ribosomal frameshifting. The viruses of the two families of vertebrate viruses are not serologically related, but sequence identity may reach 30 percent in selected regions.

The family *Coronaviridae* comprises two genera, *Coronavirus* and *Torovirus*, with little sequence similarity. There is a structural distinction also in that the coronaviruses have a helical nucleocapsid protected by a core shell, whereas the toroviruses have a tubular nucleocapsid. The coronavirus genome is the largest monopartite RNA genome at 27–31 kb in size. Species are distinguished on the basis of serology and sequence comparison. Phylogenetic analyses of gene sequences support the formal taxonomy, even though very high frequency recombination is an attribute of coronaviruses.

The family *Arteriviridae* comprises a single genus, *Arterivirus*. Macrophages are the primary target cells for all arteriviruses and the genus includes the type species *Equine arteritis virus*, and the species *Lactate dehydrogenase-elevating virus*, *Porcine respiratory and reproductive syndrome virus*, and *Simian hemorrhagic fever virus*. Genome strategy and replication pattern are similar to those of the *Coronaviridae*, but the genomes and virions of arteriviruses are about half the size of those of coronaviruses.

THE FAMILY *TOGAVIRIDAE*

The family *Togaviridae* comprises two disparate genera, *Alphavirus* and *Rubivirus*, associated by similar morphology and genome strategy. The species included in the genus *Alphavirus* are serologically related viruses infecting vertebrates and transmitted by mosquitoes. They exhibit a high level of sequence identity (>60 percent in the case of structural protein genes). The single species of the genus *Rubivirus* is a human pathogen with no arthropod vector and no sequence relationship with the alphaviruses.

The replicases of alphaviruses show some sequence homology with the nonstructural proteins of *Hepatitis E virus* and those of several groups of plant viruses, including the families *Bromoviridae* and *Closteroviridae*, and the unassigned genera *Hordeivirus*, *Tobamovirus*, and *Tobravirus*. These constitute the 'toga-like supergroup,' and transcription from genomic and subgenomic RNA is a feature of their multiplication cycle. The closteroviruses possess a toga-like polymerase, but their replication strategy more closely resembles that of the coronaviruses, although the latter have a picorna-like polymerase. These relationships may be a consequence of past recombination events and modular evolution, or merely fortuitous.

THE FAMILY *FLAVIVIRIDAE*

The family *Flaviviridae* comprises three biologically distinct genera. Most viruses of the genus *Flavivirus* are transmitted to vertebrate hosts by arthropod vectors. All

flaviviruses are serologically related. The type species is *Yellow fever virus*. Species are defined on the basis of sequence data, antigenic characteristics, and various biological criteria, and more than 50 species have been designated. Viruses of the genus *Pestivirus* infect ruminants and pigs, have no arthropod vectors, and encode two gene products unique to this genus. Some cytopathogenic isolates of the type species, *Bovine viral diarrhea virus*, have a small insertion of host-derived nucleic acid integrated at a specific site, whereas others have gene duplications or deletions. The genus *Hepacivirus* contains a single species, *Hepatitis C virus*. This virus is transmitted between humans mainly by exposure to contaminated blood. No arthropod vectors are known. Three related viruses isolated from monkeys, *GB virus A*, *GB virus B*, and *GB virus C/Hepatitis G virus* are ranked for the present as unassigned species in the family. Only the latter is associated with hepatitis in humans.

Other categories

The Universal Taxonomy also covers several other agents (Table 7). The family *Narnaviridae* comprises two genera of mycoelements, which exist as ribonucleoprotein complexes but have no virion stage in their multiplication cycle. These have been listed here and also together with the positive-sense single-stranded RNA viruses, because some regard their status as viruses to be dubious. The viroids of plants are now classified as two families, using the suffix *-viroidae* to differentiate them from the true viruses. Finally, consistent nomenclatures for subviral satellites and prions are under consideration.

THE CONCEPT OF THE VIRUS SPECIES

The species is the basic taxonomic group in biological systematics, and because viruses are biological entities the species concept can be extended to virology. Viruses exhibit intrinsic genetic variability due to the error prone processes of nucleic acid duplication. Genetic variability enables biological systems to adapt and survive. As a consequence, unlike chemical compounds, viruses cannot be grouped into universal classes (e.g. species) defined by a single property or set of properties that are both necessary and sufficient for class membership (van Regenmortel et al. 1991). A virus with a positive-sense single-stranded RNA genome is per se a member of a class of positive-sense single-stranded RNA viruses. It could also be a member of several other universal classes defined by morphology, host, antigens, gene order, etc. It follows, therefore, that although viruses can be members of several universal classes, where each class is defined by a single property necessary and sufficient for class membership, the taxonomic grouping designated as a species is not a universal class definable by a single property. Members of a species can only be accommodated in a different type of class, the polythetic class, definable exclusively by several independent properties. In a polythetic class each member possesses most but not all of the properties of the set, and each property in the set is possessed by the majority but not all of the members. The polythetic class definition of a viral species implies that there can be no single defining property for class membership, and search for such a property is unrewarding. Such a property would have to differ from a common property defining a higher category in the taxonomic hierarchy. Common

Table 7 *Other categories*

Order	Family	Genus	Type species
Naked RNA viruses			
–	*Narnaviridae*	*Narnavirus*	*Saccharomyces cerevisiae 20SRNA narnavirus*
		Mitovirus	*Cryphonectria parasitica mitovirus 1-NB631*
Viroids			
–	*Pospiviroidae*	*Pospiviroid*	*Potato spindle tuber viroid*
		Hostuviroid	*Hop stunt viroid*
		Cocadviroid	*Coconut cadang-cadang viroid*
		Apscaviroid	*Apple scar skin viroid*
		Coleviroid	*Coleus blumei viroid 1*
–	*Avsunviroidae*	*Avsunviroid*	*Avocado sunblotch viroid*
		Pelamoviroid	*Peach latent mosaic viroid*
Subviral agents			
Agents	Genus		Example
Satellites	–		Tobacco necrosis virus satellite
Prions	–		Scrapie agent

Vertebrate virus taxa are indicated by absence of shading.
The dashes signify 'unassigned.'

properties shared by the members of different species, such as are used to designate higher tax (genera, families, and orders), cannot be species-defining properties.

The polythetic definition of virus species (see Definition of taxa) emphasizes the biological nature of viruses and has dispensed with the reference to 'gene pools', which featured in earlier definitions, because most viruses replicate clonally and do not share gene pools. The concept of the replicating lineage, which replaces it, indicates an inherited genealogy extending over many generations, unified by a common descent, and evolving in response to natural selection. The reference to ecological niche occupancy in the definition emphasizes the role of environmental factors (host, tissue tropism, vector specificity, etc.) in determining the identity of species.

This species definition establishes the meaning of the categories used in classification, but it does not provide rules for demarcating the boundaries between species. Nor does it establish equivalence between the diagnostic properties used for delineation of species in different parts of the taxonomy. The phenotypic properties available to define different polythetic classes of viral species are variable in quantity and quality, and progress towards equivalence will be a gradual process (see van Regenmortel et al. 1997).

The high mutability of replicating RNA viruses has led to the development of the 'quasi-species' concept. A quasi-species distribution is the result of self-replication and corresponds to a mutant distribution consisting of a master sequence and an attendant swarm of mutants derived from it. Implicit in the concept of quasi species, however, is the assumption that the species is a unique sequence. This is at variance with the polythetic species concept, which incorporates variability into the definition. Quasi-species distributions have been demonstrated in most RNA viruses at the genotypic level, but nonetheless many RNA viruses exhibit remarkable stability at the phenotypic level, reflecting the existence of powerful selective forces, which maintain an average sequence and genetic stability.

PHYLOGENETIC ANALYSIS AND TAXONOMY

The ascendancy of molecular virology has fostered the conception that viral taxonomy may eventually be reduced to a cataloging and ranking of genome sequences. This is a receding prospect (Mayo and Pringle 1998). Phylogenetic trees derived from nucleotide or inferred amino acid sequences are generally concordant with existing taxonomies over short ranges as noted throughout the section on The Universal System of Virus Taxonomy in this chapter. Furthermore, in monopartite negative-sense single-stranded RNA viruses, where horizontal transfer of genetic information by genetic recombination is rare or nonexistent, sequence relationships can be used to substantiate taxonomic groups up to the level of the order. In particular situations useful inferences can be made about the evolution and origins of viruses (see Virus evolution below), and concerning the mechanics of epidemic diseases. Phylogenetic analysis is less successful in the analysis of more distantly related groups. Polymerase-based phylogenetic trees have suggested previously unsuspected putative links between the polymerases of plant and animal viruses (see The positive-sense single-stranded RNA viruses). However, such data establish relationships between individual genes only and should not be extrapolated to indicate phylogenetic relationships between complete genomes. The genomes of many viruses, particularly RNA viruses, often appear to have evolved in a 'modular' fashion acquiring genes or blocks of genes from the genomes of other viruses or even from their host (for examples, see Mayo and Pringle 1998).

More instructive is the statistical re-evaluation of the worth of RNA-dependent RNA polymerases of RNA viruses as a phylogenetic marker undertaken by Zanotto et al. (1996). The results of a Monte Carlo stimulation assay of sequence similarity among 40 RNA-dependent RNA polymerase and 40 RNA-dependent DNA polymerases were transformed to a gray scale density plot that allowed the significance of the 1 600 possible comparisons to be scanned visually and evaluated statistically by densometric scanning. The concentration of white and light gray squares along the diagonal in the density plots demonstrated that significant values were only obtained between viruses known to be similar on other criteria (i.e. within families). Overall the data do not provide statistical support for proposals for the existence of evolutionary groupings of RNA viruses above the family level (Ward 1993, Koonin and Dolja 1993, Goldbach and de Haan 1994), although they did not negate them. Similarly, this type of comprehensive analysis provided no statistical support for a presumptive common ancestry of viral RNA-dependent RNA polymerases and viral reverse transcriptases.

VIRUS EVOLUTION

The origin and evolution of viruses is too broad a question to consider here. The section on The Universal System of Virus Taxonomy in this chapter indicates that there is no genome strategy or molecular mechanism of processing genetic information that is peculiar to viruses infecting a specific type of host. Viruses leave no fossil record. Where there is evidence of co-speciation of virus and host, however, persuasive estimates of the origin and antiquity of particular viruses can be made (McGeoch and Cook 1994). In general, phylogenetic analysis is hampered by horizontal gene transfer and the absence of isolates from much before the present. The phylogenetic trees for some viruses are merely a

reflection of isolate abundance rather than lineal descent. Where there are obvious relationships but no phylogenetic support, the direction of evolution may be difficult to discern, as for example in the case of the order *Caudovirales* cited in the section on The double-stranded DNA viruses, where convergent or divergent evolution cannot be distinguished (see also Genetics of vertebrate viruses).

More problematic and challenging are the results of recent reverse genetic experiments involving the reengineering of viral genomes. Throughout the order *Mononegavirales* the order of genes is highly conserved. Nonetheless, several gene order variants of vesicular stomatitis virus have been produced by a reverse genetics. Surprisingly, most can be propagated in cultured cells and experimental animals without rearrangement or restoration of the wild-type conserved sequence (Wertz et al. 1997, 2002). Indeed several of the variants slightly exceed the wild-type virus in reproductive potential. It is clear that in the case of RNA viruses at least, strong selective forces maintain an average genome sequence. Until the nature of these selective forces is defined, it will be difficult to arrive at any sound conclusions regarding virus evolution.

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The morphology and structure of viruses

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INTRODUCTION

Viruses are small quantities of genetic information. This information codes for tools for their multiplication and for a vehicle to go from one site of multiplication to the next. The information can be coded in RNA or DNA. In fact, the information may even be present in the conformation of a particular protein, as in 'prion' proteins, for example, but these are not really viruses. The nature of the viral nucleic acid leads to particular trade-offs that are fundamental to the observed virus morphologies. For the replication of viruses, the genetic information has to be copied by an RNA- or DNA-dependent polymerase. All these polymerases make, on average, one mistake in 10 000 copying steps. This is probably due to the chemical nature of the nucleotide bases, which can exist in two forms, the keto- (lactame-) form and the enol- (lactime-) form (tautomere) (Figure 1a). The equilibrium between these two forms is far towards the keto-form, with an equilibrium constant of 10^{-4}, meaning that there is a chance of 10^{-4} that the nucleotide is in the enol-form when the polymerase passes to place the complementary base. Normally, guanines pair with cytidines but not with thymidines (as shown in Figure 1b). However, a thymidine in the enol-form can base pair with a keto-guanine and form three hydrogen bonds (Figure 1c). The same holds for the other bases: keto-A can pair with enol-C, keto-C with enol-A, and keto-T with enol-G. Therefore, the polymerase will add a wrong base when the template base is in the enol-form. DNA-dependent polymerases have a proofreading activity that corrects these errors in base pairing, leading to error frequencies in DNA organisms of only about 10^{-7} per copied base. This means that DNA virus genomes are rather stable and the genomes of DNA viruses can be large, ranging from about 5 000 (polyomavirus) to 200 000 base pairs (herpes- and poxviruses), the largest genomes belonging to the phycodnaviridae, algal DNA viruses with genomes up to 560 kbp (Yan et al. 2000; Sandaa et al. 2001; van Etten et al. 2002), certain bacteriophages, such as phage G of *Bacillus megaterium*, which packages ~670 kbp (Hutson et al. 1995), and the latest champion, a virus found in ameba with a genome size of about 800 kbp (La Scola et al. 2003). These large viral genomes are bigger than those of the smallest bacteria, such as mycoplasma (580 kbp, Fraser et al. 1995). However, RNA-dependent RNA polymerases usually do not have a proofreading activity (or only a rudimentary form of it). This means that for an RNA virus with a genome of 10 000 nucleotides, on average every offspring virus will have one mutation. Because selection mechanisms cannot function with more than one mutation per offspring virus, this limits the genome size of RNA viruses to about 10–20 000 nucleotides, the only exception being the coronaviruses that have a positive-strand RNA genome of between 27 and 32 kb (Lai and Holmes 2001). Note that the amount of information space that a virus can attribute to coding for its transport vehicle depends on the total amount of information space. Therefore, RNA viruses have capsids with, in general, simpler structures with fewer structural proteins than DNA viruses. Also, because of the genome size, the space inside a DNA virus capsid has to be larger than that for an RNA virus, leading to more elaborate capsids for DNA viruses.

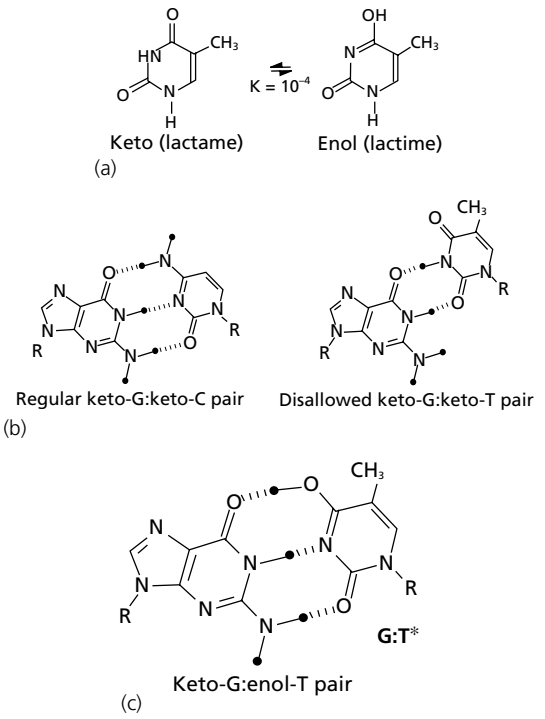

Figure 1 (a) *Keto–enol tautomerism of nucleotide bases.* **(b)** *Allowed and disallowed nucleotide pairs.* **(c)** *Allowed keto-G:enol-T pair.*

RNA viruses usually only code for the necessary transport and replication proteins, plus often for a protein that counters or avoids the effects of innate immunity. Apart from proteins analogous to those encoded by the RNA viruses, the larger DNA virus genomes also code for a considerable number of ancillary proteins that modify the host metabolism, and also for immunity-modifying factors.

The replication component of RNA viruses may consist of the naked nucleic acid. When positive-strand RNA viruses enter the cell, the vRNA is directly translated into viral proteins. For this effect, the vRNA contains a specific three-dimensional (3D) RNA structure, the internal ribosome entry site (IRES), to which host translation factors bind in order to start viral protein production (Kieft et al. 2001; Sarnow 2003). However, sometimes protein components are needed for the first steps in the replication process. Retroviruses need reverse transcriptase and integrase for insertion of the transcribed DNA into the genome of the host cell, and negative-strand RNA viruses have a viral RNA that does not directly code for proteins but which has to be transcribed into mRNA first. For this function, negative-strand RNA viruses assume a unique structural composite in which the viral RNA is stoichiometrically bound to the nucleoprotein. This helical nucleocapsid, rather than the naked vRNA, is the template for transcription and replication. The viral RNA-dependent RNA polymerase is bound to this helical template, either directly (influenza virus) or with the help of a phosphoprotein (all nonsegmented negative-strand RNA viruses). This complex structure enters the infected cell and is directly active in transcription. Thus, if a virus needs a virus-specific process at the start of its replication cycle, the proteins needed for this process have to be packed inside the virus particle.

The transport component protects the virus when it is outside a cell. This component contains a receptor-binding protein (or proteins) and sometimes also proteins needed for release from the infected cell. The surface of the virus is also involved in cell entry by membrane fusion (for the membrane or enveloped viruses) or by some other membrane penetration mechanism (for nonenveloped viruses). Because of the limited genome size for encoding protein subunits, viral transport components often exploit symmetry in order to make a 3D structure from many copies of only a few building blocks. There are two major types of symmetry used by viruses: helical symmetry (mainly plant viruses and bacteriophages, bacteriophage tails and the above-mentioned nucleocapsids of negative-strand RNA viruses) and icosahedral symmetry (many small positive-strand RNA viruses, small and large DNA viruses, and bacteriophage heads). The small icosahedral viruses have very simple capsids with only one type of protein, or with three or four closely related proteins. The large icosahedral viruses, such as adenoviruses and herpesviruses, have more complex capsids with several major capsid proteins arranged into topological units, usually pentameric and hexameric, called capsomers, plus a number of small proteins that glue the capsomers together. There are icosahedral viruses with a single protein capsid layer (rhinovirus, hepatitis B virus) and a double protein layer (reoviruses), and there are also icosahedral viruses that have a membrane with proteins both outside and inside the membrane (togaviruses and alphaviruses).

However, many enveloped viruses do not have icosahedral symmetry and formation of the virus particles takes place in a different manner. All negative-strand RNA viruses, the positive-strand coronaviruses and, most likely, the retroviruses (although there is still some debate about this point), are not icosahedral.

An interesting feature of the transport components of certain viruses is that they must be stable enough to form inside the infected cell and to resist attacks in the extra cellular environment, but that they have to fall apart when the virus enters a new cell. This implies that the conditions present during virus formation (encapsidation) must be different from those during decapsidation. Different viruses solve this problem in different ways. Viruses such as HIV undergo cleavage of the GAG polyprotein inside the virion by a virus-encoded protease for maturation. It is the immature, intact GAG polyprotein that is assembled in the infected cell and the mature, cleaved capsid that will fall apart upon cell

entry. Influenza viruses code for a membrane channel that allows acidification of the virus interior when it passes through the acidic endosome upon entry. Acidification not only leads to membrane fusion by the hemagglutinin, but it also loosens the interactions between the matrix proteins inside the virus (Hay et al. 1985; Martin and Helenius 1991). Adenoviruses are uncoated in a stepwise fashion: the viral protease is activated by two sequential events, first interaction of the receptor-binding protein with the cellular receptor (an integrin), and then activation of the protease by the reducing environment inside the cytoplasm. This leads to sequential loss of viral proteins and degradation of the core necessary to release the viral DNA for transport through the nuclear pore (Greber et al. 1996).

HELICAL VIRUSES AND HELICAL VIRAL COMPONENTS

One of the symmetries used by viruses in order to build a capsid from repeating units is helical symmetry. The characteristics of a helix are defined mathematically by two parameters: u corresponds to the number of subunits per turn (often not an integral number) and p is the axial rise per unit (Figure 2). The pitch (P) of a helix corresponds to the distance along its length that has to be traveled to reach the next subunit, i.e. $P = u \times p$. Helical structures can have a rotation axis coincident with the helix axis, like the tail of bacteriophage T4, which has sixfold symmetry and is made by stacked rings with a rotation angle of 60° between subsequent rings. If a helix is perfect, then a single view of the helix will give all the 3D information of the protein and nucleic acid components that make up the helix, because the same protein can be observed along the helix but in different orientations. Different types of helices can exist in virus particles:

- The nucleic acid can be associated with and protected by many copies of a single protein and form an infectious virus particle. In these helices the protein serves to protect the nucleic acid. Examples are the tobamoviruses (tobacco mosaic virus (TMV)); Figure 3), tobraviruses (tobacco rattle virus), hordeiviruses (poa semilatent virus (PSLV) and lychnis ringspot virus (LRSV)), furoviruses (potato mop-top virus (PMTV)) and pecluviruses (peanut clump virus). These viruses are all rod-like positive-strand RNA viruses. Other examples are the badnaviruses, bacilliform double-stranded DNA plant viruses such as sugarcane bacilliform virus (SCBV-IM). Sometimes, proteins with specific functions are added at one or both ends, as is found with single-stranded filamentous DNA bacteriophages such as Pf1 and M13.
- The nucleic acid can be associated with many copies of the same protein and form the matrix for transcription and replication of the nucleic acid, but other proteins and a lipid membrane are needed to complete the virus particle. Good examples of this kind of structure are the nucleocapsids of negative-strand RNA viruses (see under Nonsymmetrical viruses ; Figure 9, p. 15). However, under physiological conditions these helical structures are very flexible and do not have defined helical parameters.
- The helix is not associated with the nucleic acid but is part of the virus structure, like the tail of double-stranded bacterial DNA viruses such as bacteriophage T4 that is used for injecting the nucleic acid into the bacterial host cell (Figure 4).

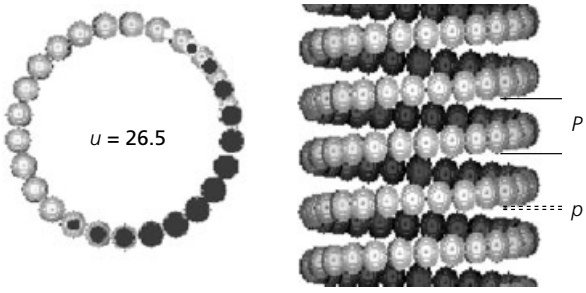

Figure 2 *Schematic representation of a helix.* P *is helical pitch,* p *corresponds to the axial rise per unit, and* u *is the number of subunits per helical turn.*

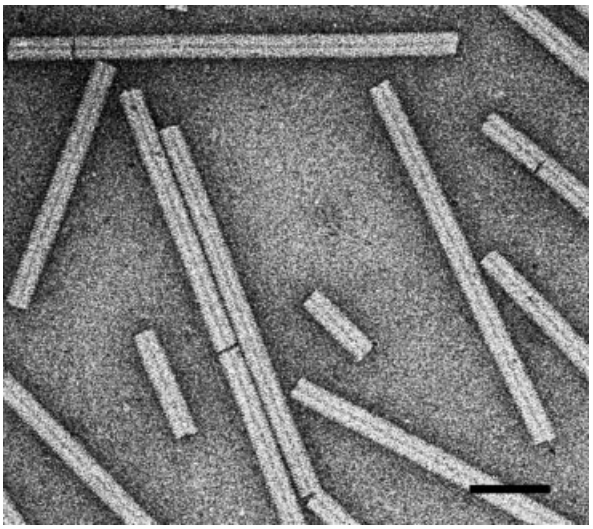

Figure 3 *Electron micrograph of tobacco mosaic virus negatively stained with 1% uranyl acetate. The bar corresponds to 100 nm.*

In principle a helix can have more than one start (like microtubules), but in the case of a helix associated with nucleic acid the helix has always a single start because as there is only a single nucleic acid molecule.

Methods to study helical virus structures

Electron microscopy (EM) is probably the best method to visualize helical viruses or viral components. TMV

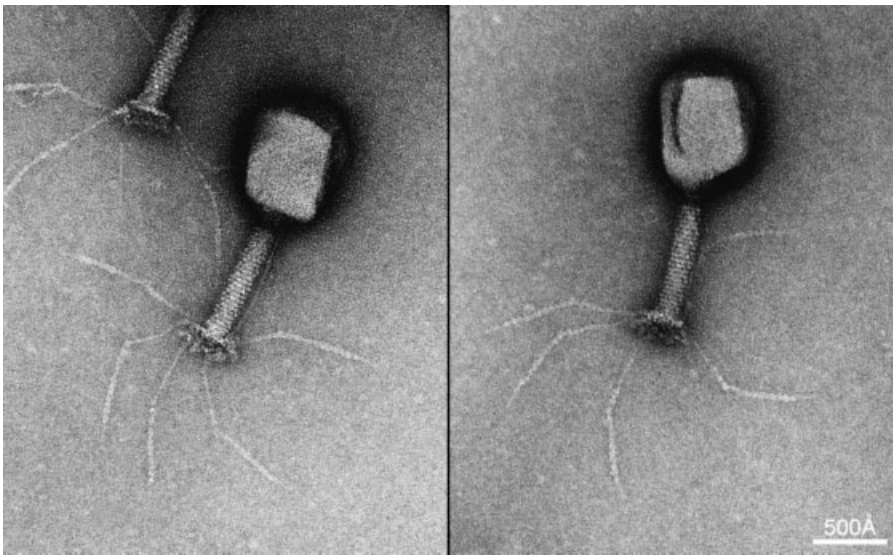

Figure 4 *Phage T4 visualized by negative-stain EM. The extended icosahedral head is crumpled by dehydration but the thick contractile tail is well preserved, as are the long tail fibers that extend from the baseplate. Short tail fibers are also visible underneath the baseplate. Figure courtesy of M.E. Cerritelli, N. Cheng, and A.C. Steven, LSBR-NIAMS-NIH.*

was one of the first biological objects to be observed in EM (Horne et al. 1939). For negative-stain EM, virus specimens are first absorbed on to a support film that is transparent to electrons, for example a thin film of evaporated carbon. The embedding of the helix in a layer of stain gives high contrast and makes it possible to visualize the pitch of the helix (Figures 3 and 4). If the stain stays low on the carbon only one side of the helix is visualized, but the stain may also cover the side of the helix that does not touch the support film. Another method of preparing virus for EM is to evaporate a thin film of metal at an angle over a dried sample on support film. Differences in sample height will lead to differences in metal deposit and, hence, in contrast. Because metal shadowing outlines only one side of the helix, this method gives information on the handedness of the helix (right or left turning). The virus can also be prepared by fast freezing of the virus in a very thin film of buffer – so-called cryo-electron microscopy (cryo-EM), leading to a state of the virus particle that is close to its native form in solution. The picture obtained from cryo-EM is a two-dimensional (2D) projection of the 3D structure of the virus. The disadvantage of this technique is the very low image contrast that makes it necessary to use image analysis in order to enhance the signal-to-noise ratio. In principle, cryo-EM may lead to subnanometer structural information on viruses.

The Fourier transform of the 2D projection of a helix gives a characteristic diffraction pattern in which the information is situated on lines, the layer lines. Analysis of the diffraction pattern leads to total definition of the helical parameters and can be used to recalculate a 3D model of the helical structure. For this approach the cryo-method is the most useful way to prepare the virus sample for EM. However, this analysis method is difficult and time-consuming. Recently, new algorithms have been developed for the reconstruction of helical filaments using single-particle methods (Egelman 2000; Li et al. 2002). This technique uses iterative real-space reconstruction and is able to overcome most of the problems that can be encountered in helical reconstruction using Fourier analysis, which is more dependent on long-range order.

Another method for the analysis of helical structures is fiber diffraction, for which a sample has to be prepared in which the helices are all aligned in the same direction. Well-aligned fibers can be prepared by drying a gel of purified helical virus under controlled humidity and temperature conditions in a strong magnetic field (Nave et al. 1979, 1981), which is then analyzed by X-ray diffraction. The structure of TMV was solved to a resolution of 3.6 Å with this technique (Namba and Stubbs 1986). TMV contains a single molecule of single-stranded positive-strand RNA plus 2 130 copies of a single coat protein of 17.5 kDa. The pitch of the helix is 23.0 Å, with 16.33 subunits per turn and three nucleotides per protein subunit. The RNA is squeezed in a groove between protein subunits of subsequent helical turns and follows the helical path at a radius of 40 Å.

ICOSAHEDRAL VIRUSES

The high symmetry of the icosahedron offers similar advantages to the helix for capsid architecture, including maximizing packaging volume for the viral genome at a minimal cost in coding space by making use of repeated protein subunits (Crick and Watson 1956). In a simple icosahedron 60 identical subunits are arranged in a

closed shell with fivefold axes of symmetry at the 12 vertices, threefold axes on the 20 facets, and twofold axes on the 30 edges (Figure 5). Each subunit has equal contacts with its neighbors. However, most icosahedral viruses use elaborate variations on this scheme to construct larger and multifunctional capsids by coding flexibility into the subunits that allow non-identical interactions, as described below, which introduce additional sites of local hexameric symmetry. Consequently, these structures are useful model systems for studying protein folding and assembly, and the flexibility of subunit interfaces.

Viruses from all walks of life have icosahedral capsids, although a spectacular range of variations is observed in their structural details. General principles about assembly, maturation and function of these viruses might best be listed as a menu of strategies from which a combination may be chosen to describe particular cases. For example, the nature of the genome may be RNA or DNA; single- or double-stranded; its recruitment by packaging into a preformed capsid through a specialized vertex, or capsid assembly around a genomic or pregenomic complex; delivery by capsid entry into the cell or by some membrane-penetrating mechanism from an external membrane-anchored location. Structural variations include possible use of a scaffold for capsid assembly, which may be internal or external; proteolysis of structural proteins during capsid maturation; reorganization of the capsid proteins, which may lead to a change in the capsid diameter; the possible incorporation of a membrane, which may be external to the capsid or internal (or sandwiched between protein shells); host recognition by fibers protruding from the capsid surface or by the capsid surface itself; and so on.

One element remains central to the many aspects of the virus lifecycle, and that is the symmetric capsid. This icosahedral protein complex serves a variety of functions, the most obvious of which is as a protective container for the transport of the viral genome from one cell to another. However, various roles are observed for capsids, including interaction with other virus proteins (connector-tail complex, fibers, tegument, membrane-

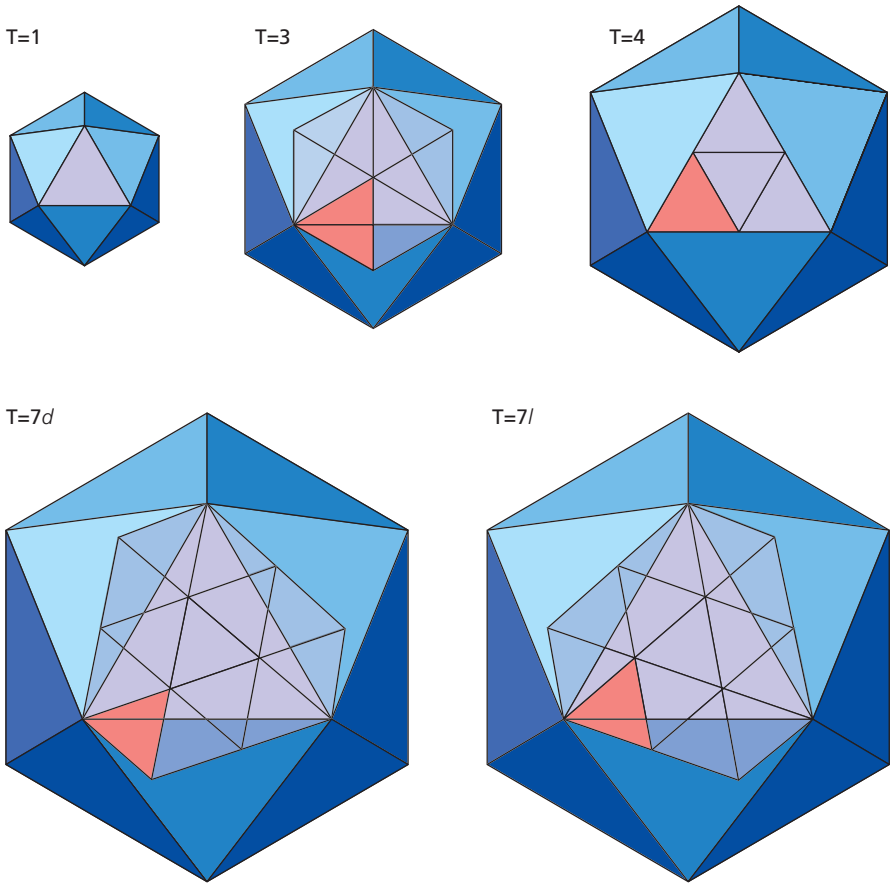

Figure 5 *Icosahedral geometry. An icosahedron has 20 triangular facets bounded by the five-fold symmetrical vertices of the icosahedron. By tiling the icosahedral surface with triangles, as shown for different Triangulation numbers 3–7, larger volumes may be enclosed. Additional hexavalent sites are created where triangular vertices meet. Skew symmetries, such as T = 7, may be 'handed' and both laevo* **(l)** *and dextro* **(d)** *forms are observed in viral capsids (see Figure 6, p. 8). The largest T-number observed to date is in the capsid of PBCV-1 (Yan et al. 2000) with T = 169.*

bound glycoproteins), transcriptional compartment, receptor recognition, and genome release through disassembly. The capsid itself may be as simple as a single-species assembly (e.g. hepatitis B virus (HBV)) or include a number of structural components (e.g. herpes simplex virus (HSV)), and generally the complexity is observed to increase with size.

Recent work has revealed some particular elements of capsid structure and function that are common to seemingly quite unrelated viruses. One example is the remarkable similarity in structure between phage PRD1 and the mammalian adenovirus, which extends from capsid architecture to the structures of the major capsid proteins, P3 and hexon, respectively (Benson et al., 1999, 2002). Another example is the similarity between phage T4 and HSV, including the recent discovery of a specialized vertex on the capsid of HSV-1 that is implicated in uptake of the dsDNA genome (Newcomb et al. 2001), analogous to the way in which tailed dsDNA phage capsids package their genome through a unique 'connector' complex prior to attachment of their tail structure. The fold of the capsid protein is also conserved in viruses from a wide variety of sources – for example, the 'jelly roll,' an eight-strand β-barrel, is found in RNA and DNA viruses from bacteria, plants, insects, and animals. In such cases, the study of structure has led not only to an understanding of virus function, but also to the possibility of extensive evolutionary relationships.

An outline of icosahedral architecture is given below, including examples of contrasting systems to highlight connections between structural features and function.

A note on methodology

Electron microscopy has played an important role in visualizing virus structure, starting from the development of the negative-stain technique (e.g. Brenner and Horne 1959), to which the methodology of image reconstruction was subsequently applied (Crowther et al. 1970) for extending structural analyses of icosahedral viruses into three dimensions. Cryo-EM now substitutes for negative staining and avoids dehydration, fixation, and staining artifacts (Adrian et al. 1984). Although EM has been important in studying virus structure, it is equally true that the highly symmetric structure of icosahedral viruses has made them invaluable for the development of 3D structural analysis methods for cryo-EM data. This synergy has led to achievements of subnanometer resolution for several icosahedral capsids (Bottcher et al. 1997; Conway et al. 1997; Mancini et al. 2000; Zhang et al. 2002; Zhou et al. 2000), with the expectation of higher resolution in the future (see Baker et al. 1999b for a recent review of such structural analyses). X-ray crystallography has also been applied to whole capsids (Hogle et al. 1985; Johnson and Chiu 2000), although this kind of analysis remains challenging owing to the large size of the particles leading to large unit cells and, thus, very close packing of diffraction spots. A combination of these two techniques is often employed wherein subunit atomic models from crystallography are placed into a lower-resolution structure of the complex from cryo-EM (Chiu and Rixon 2002; Modis et al. 2002). This hybrid approach, although not atomic in resolution, is nonetheless valuable for situations where crystallization is difficult or impossible. Examples include capsids decorated with antibodies or Fabs (Prasad et al. 1990; Porta et al. 1994; Conway et al. 2003), capsids decorated with receptor fragments (Hewat et al. 2000; Rossmann et al. 1994), and even capsids alone based on subunit models (Speir et al. 1995; Martin et al. 2001; Modis et al. 2002). Another hybrid approach is the use of cryo-EM density maps as phasing models to initiate crystallographic analyses (e.g. Speir et al. 1995; Grimes et al. 1998).

The nonsymmetric components of a virus, such as membranes and nucleic acid, are generally not visualized by these methods and so less is known about the structures they adopt. The problem is one of low signal and high noise; information from many identical copies must be averaged together to yield an interpretable structure by cryo-EM or X-ray crystallography, and this is clearly impossible to do with the nonuniform pieces. Several exceptions have been observed, however, although these are consequences of association with the icosahedral capsid. One is regions of packaged RNA that contact the capsid and which are visualized by crystallography, for example at icosahedral twofold axes of the nodamura viruses, Flock House virus (Fisher and Johnson 1993), and pariacoto virus (Tang et al. 2001), where they appear to influence the interface between capsid proteins and determine in part the $T = 3$ capsid geometry, and at the transcribing vertices of bluetongue virus cores (Gouet et al. 1999). Another is the highly concentrated dsDNA of bacteriophages, which appears as organized layers spaced ~25 Å apart in cryo-EM images, and as concentric shells of DNA in image reconstructions, which, although somewhat artificial owing to the imposition of symmetry, nonetheless appears to have some bearing on the genome organization within the capsid (Cerritelli et al. 1997; Olson et al. 2001). A similar spacing has been observed for herpes virus DNA (Booy et al. 1991), although the ordering does not appear on the global scale of the phage DNA.

The icosahedral capsid

Icosahedrally symmetric capsids come in various degrees of complexity, from the small and simple, such as capsids of parvoviruses that are built from 60 copies of (essentially) a single protein, to the large and complex, including those of herpesviruses, which comprise 960

copies of the major capsid protein and hundreds of copies of several other structural proteins. Capsids may be multilayered, such as those from rotaviruses and reoviruses, with different copy numbers of the constituent proteins in each layer. How is this variation consistent with icosahedral symmetry, which strictly demands 60 identical asymmetric units? And how might the 960 copies of the major capsid protein of HSV, for example, be subdivided into 60 asymmetric units of 16 copies each and arranged identically to form a closed shell? The prevailing explanation is the concept of 'quasi equivalence' (Caspar and Klug, 1962; Johnson and Speir, 1997), in which identical subunits are placed in slightly different chemical environments according to their position within the asymmetric unit. The different numbers of subunits may be described by a 'triangulation number,' T, which is a count of the number of triangles that may be used to tile an icosahedral facet so that each triangle accounts for the positions of three protein subunits (Figure 5). A triangulation number T = 1 corresponds to perfect icosahedral symmetry with each subunit in an identical environment. A selection rule determines the allowable triangulation numbers as follows: $T = h^2 + hk + k^2$, where h and k are integers 0, 1, 2, ... The total number of subunits is then 60T. The capsid surface is triangulated by pentavalent positions at the vertices and hexavalent positions elsewhere (except in the case of T = 1). Certain T numbers are forbidden, such as T = 2, although this number has been used to describe protein shells from dsRNA viruses (yeast L-A virus – Cheng et al. 1994; Caston et al. 1997, and the cores of some multilayer capsids; see below) with 120 copies of a capsid protein subunit arranged as 60 asymmetric dimers. Some extensions to this simple geometric descriptor have been developed to account for more complex structures. One is the pseudo-T number, where the subunits are non-identical, for example in the case of picornaviruses, which have three similar but non-identical capsid subunits arranged in a pseudo-T = 3 lattice (Arnold and Rossmann, 1990), and adenovirus, which has pseudo-T = 25, where the hexavalent positions are occupied by trimers and the pentamer subunits on the vertices are a different protein. Another is the addition of a second triangulation number, Q, that is used to describe the geometry resulting from one or more additional 'bands' of subunits around the equator in elongated capsids such as that of bacteriophage T4 (T = 13/Q = 21) (Aebi et al. 1976).

The concept of quasi equivalence has therefore had to adapt to the various ways in which flexibility between subunits contacts is expressed in virus capsids. In some cases this flexibility involves rather larger departures from symmetry than those originally envisaged. The 'forbidden' T = 2 capsid described above is one example. Another is the capsid geometry of SV40, a *Polyomavirus* in which pentameric capsomers occupy both hexavalent and pentavalent sites in a T = 7 geometry (Liddington et al. 1991). This architecture, common for all polyoma and papilloma viruses, utilizes just 360 copies of the capsid protein (corresponding in quantity to the forbidden T = 6) instead of the expected 420, and involves different conformations for the C-terminal arms that interface with (and invade) neighboring capsomers (Figure 6a). A third kind of departure is observed in the hexameric capsomers of some T = 7 dsDNA bacteriophages, such as λ, T7, P22, and HK97, which obey 'classic' hexamer and pentamer capsomer structure (Figure 6b, c). The hexamers of the early assembly forms of these capsids exhibit a marked departure from local sixfold symmetry, but this is 'cured' upon maturation (Dokland and Murialdo 1993; Conway et al. 1995; Lata et al. 2000). Recently a structure of the procapsid form of an animal virus, HSV-1, has been solved and this shows similar, although less pronounced, changes for the hexameric capsomers during maturation (Heymann et al. 2003; Trus et al. 1996). Yet another example of low quasi equivalence comes from the geminiviruses, plant viruses with double heads of partial T = 1 icosahedra fused at a ring of five vertices (Zhang et al. 2001).

The flexibility of capsid proteins at subunit interfaces must also, in some cases, accommodate mismatches in symmetry. A well-known example of this is from tailed bacteriophages, where a 12-subunit connector complex occupies one of the vertices of the capsid, a site of icosahedral fivefold symmetry. The connector is thought to nucleate assembly of the capsid, after which DNA is packaged through it followed by attachment of the tail. The mismatch has been proposed to allow rotation of the 12-fold connector within its fivefold 'bearing' as part of the DNA-packaging (Simpson et al. 2000), in line with rotational functions implicated in other symmetry mismatches (Beuron et al. 1998; Junge et al. 2001; Thomas et al. 1999). However, rotation is not always implicated in symmetry mismatches: for example, it has no obvious role in the mismatch between the penton base of adenovirus and the trimeric fiber that binds to it.

Connectorless capsids may also exhibit large-scale symmetry mismatches, as in the case of some dsRNA viruses which have an inner core that exhibits the forbidden T = 2 geometry and is surrounded by an outer layer with T = 13 geometry (reovirus – Reinisch et al. 2000; bluetongue virus – Grimes et al., 1998; *Rotavirus* – Lawton et al., 1997; phi6 – Butcher et al. 1997). The subunit interactions between the layers involve non-equivalent contacts that are additional to those within each layer.

Icosahedral capsid protein folds

The most commonly observed fold for capsid proteins is the eight-strand β-barrel mentioned above, first seen in tomato bushy stunt virus (Harrison et al. 1978) and later

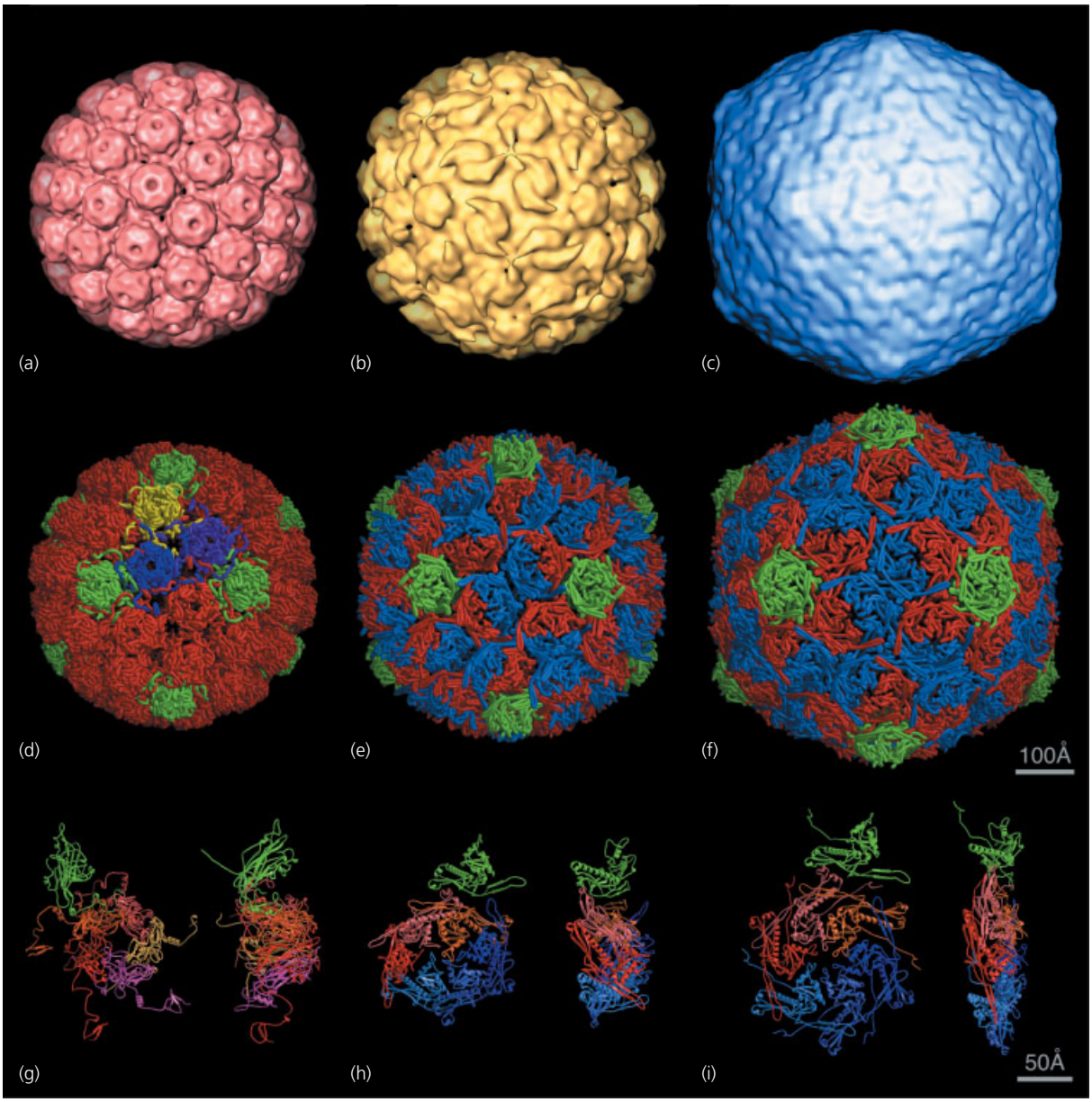

Figure 6 *Capsids with T = 7 geometry include polyomaviruses and lambdoid bacteriophages. The atomic model for SV40 (Stehle et al. 1996; PDB Id: 1SVA) is rendered as a surface at 16 Å resolution in panel* **(a)**, *showing the capsomers as protruding knobs in either pentavalent or hexavalent positions and with the right-handed, or T = 7*d, *configuration. Cryo-EM reconstructions of the procapsid (16 Å – Conway et al. 2001) and mature capsid (24 Å – Conway et al. 1995) of bacteriophage HK97 are shown in panels* **(b)** *and* **(c)**, *respectively. In this case the surface lattice has opposite hand, T = 7*l, *and the capsomer appearance, although similar to SV40 at the vertices (pentavalent positions), is quite different elsewhere. In particular, the HK97 procapsid shows only local twofold symmetry at the hexavalent sites, whereas the mature capsid shows good six-fold symmetry. The procapsid expands into the mature form during packaging of the dsDNA genome: this remarkable transformation involves a coordinated change in subunit interactions between the 420 copies of the capsid protein. When rendered as tubes, the SV40 capsid* **(d)** *reveals that all capsomers are pentamers, unlike many T = 7 capsids, such as that of HK97, which has a combination of pentamers and hexamers. In* **(d)**, *pentamers at the vertices are colored green, and the rest are red, except for three on a frontmost facet of the icosahedron that are colored blue, purple, and yellow to distinguish them. The HK97 procapsid model* **(e)** *is pseudoatomic, resulting from fitting an atomic model of the mature capsid protein into a 12 Å resolution cryo-EM model of the procapsid (Conway et al. 2001). As for SV40, capsomers at the vertices are green, but the hexameric capsomers are colored as one trimer in red and the other in blue to highlight the local two-fold symmetry. The mature capsid* **(f)** *is from a crystallographic model (Wikoff et al. 2000) and is colored as for the procapsid. Ribbon diagrams of the asymmetric units are shown at the bottom in each case – a top-view at left and a side-view at right – including one subunit from pentavalent capsomers in green and the others in shades to match the views in the middle row. For SV40* **(g)** *the extended carboxy termini are involved in interactions between capsomers. The views of HK97 procapsid* **(h)** *and mature capsid* **(i)** *show the more regular hexon in the mature form, as well as flattening and thinning of the capsomers.*

in picornaviruses and other positive-strand RNA viruses (Rossmann and Johnson 1989) from animals, plants, and insects. It has been seen subsequently in dsDNA viruses, including hexons of adenovirus (Roberts et al. 1986) and phage PRD1 (Benson et al. 1999), and in the enzymatically active cores of dsRNA reoviruses (Grimes et al. 1998; Reinisch et al. 2000), although these also have additional domains that include significant amounts of α-helix. Perhaps surprisingly, the architecturally related yeast L-A virus, which adopts the same arrangement of 60 asymmetric dimers (Cheng et al. 1994; Caston et al. 1997) with similar overall shapes and dimer interface contacts (Naitow et al. 2002), does not, however, share the same fold as the reovirus cores.

Folds with predominantly α-structures are less commonly observed. One example is the capsid of HBV, where the protein dimer interface is a four-helix bundle (Bottcher et al. 1997; Conway et al. 1997; Wynne et al. 1999), including a helix–turn–helix contributed by each of the two subunits (Figure 7). A similar motif has also been observed in HIV-1 CA protein dimers (Gamble et al. 1997; Zlotnick et al. 1998), although HIV does not conform to icosahedral symmetry. A recently solved 65 kDa fragment of the 150-kDa major capsid protein of HSV-1 and located in the upper domains of the capsomers is also predominantly α-helical (Bowman et al. 2003). Few structures are available for the capsid proteins of the widespread dsDNA phages, but those for HK97 (Wikoff et al. 2000) (Figure 6i) and P22 (Jiang et al. 2003) are similar, although they have no structural homology with any other proteins solved to date.

Capsid assembly

From the descriptions above of icosahedral symmetry and the variety of departures from it, we must suppose that capsid assembly is directed so as to achieve the correct structure in each case (Dokland 2000). Overexpression of capsid proteins sometimes results in multiple and deviant assembly forms, including tubes (e.g. Lepault et al. 2001), and closed shells of various sizes (e.g. Salunke et al. 1989), clearly the result of non-native conditions and elevated concentration. Assembly of 60-fold subunits to form a specific capsid structure is a complex process involving at least the following steps: onset (e.g. nucleation); accumulation of identical subunits into non-identical positions within a specific architecture; and completion of the capsid by closing the shell. At some stage in this process the non-structural elements, including the genome, must be packaged, and additional maturational events may also occur, including proteolysis of the capsid protein, reorganization of the subunits in the completed capsid, and additional stabilization of the capsid through binding of accessory proteins or some other modification of the subunit interactions. Assembly is generally not well understood, particularly the stages before completion of the capsid.

Innate curvature between subunits offers a framework for constructing a container, although it may be insufficient for specifying a particular geometry or even producing closed surfaces. Smaller icosahedral viruses generally have one capsid protein (or several but with largely similar folds), and these are often capable of self-assembly into virus-like particles in expression systems. Larger capsids, such as that of HSV (Nicholson et al. 1994), assemble with the aid of a scaffold that does not itself obey icosahedral symmetry but which presumably enforces the correct curvature on the assembling subunits and is later removed. Likewise, the outer T = 13 shells of dsRNA viruses are organized by self-assembled T = 2 cores, although these cores are essential to the virus and remain in place. For the *Alphavirus* Sindbis the capsid proteins do not appear to interact strongly with each other, but instead the association of the amino-terminal domain with the genomic ssRNA is the organizing force for assembly (Zhang et al. 2002). In this case, the capsid is surrounded by a membrane penetrated by coiled-coil domains of heterodimeric E1 and E2 glycoproteins that conform to the T = 4 geometry of the underlying capsid. These may also be involved in specifying the correct assembly state of the virus. Similar organization is possible in other alphaviruses, such as Ross River virus and Semliki Forest virus, as well as the more distantly related *Flavivirus*, tick-borne encephalitis virus, which shares structural homology in parts of the glycoprotein.

The necessity for a scaffold appears to be related to capsid diameter, with the limit being approximately 500 Å. Some dsDNA phages of this size, such as λ and P22, require a scaffold, whereas others do not, as is the case for HK97. In the absence of the scaffold protein, phage P22 capsid protein assembles mostly into aberrant particles with T = 4 geometry, as well as some with the normal T = 7 (Thuman-Commike et al. 1998), demonstrating that the flexibility between protein subunit interactions, which serves to allow non-equivalent bonding arrangements in the capsid, must also be controlled to avoid non-productive assembly.

Any internal scaffolding must be expelled to allow space for the genome. In some cases the scaffolding protein is proteolysed, presumably to aid its egress, and often by a virally encoded protease. In the case of HSV, the scaffold and protease genes overlap, ensuring colocalization of protease with the substrate scaffold protein. Phage HK97 apparently has no distinct scaffold protein – one of the largest icosahedral virus to forgo this assembly component – but the capsid protein includes an amino-terminal ~100 residue domain that is proteolysed after assembly and which is proposed to contain the scaffolding function (Conway et al. 1995). In other cases, such as phage P22, the scaffold protein is not proteolysed but none the less exits through holes in the procapsid surface and is recycled to construct new capsids (King and Casjens 1974).

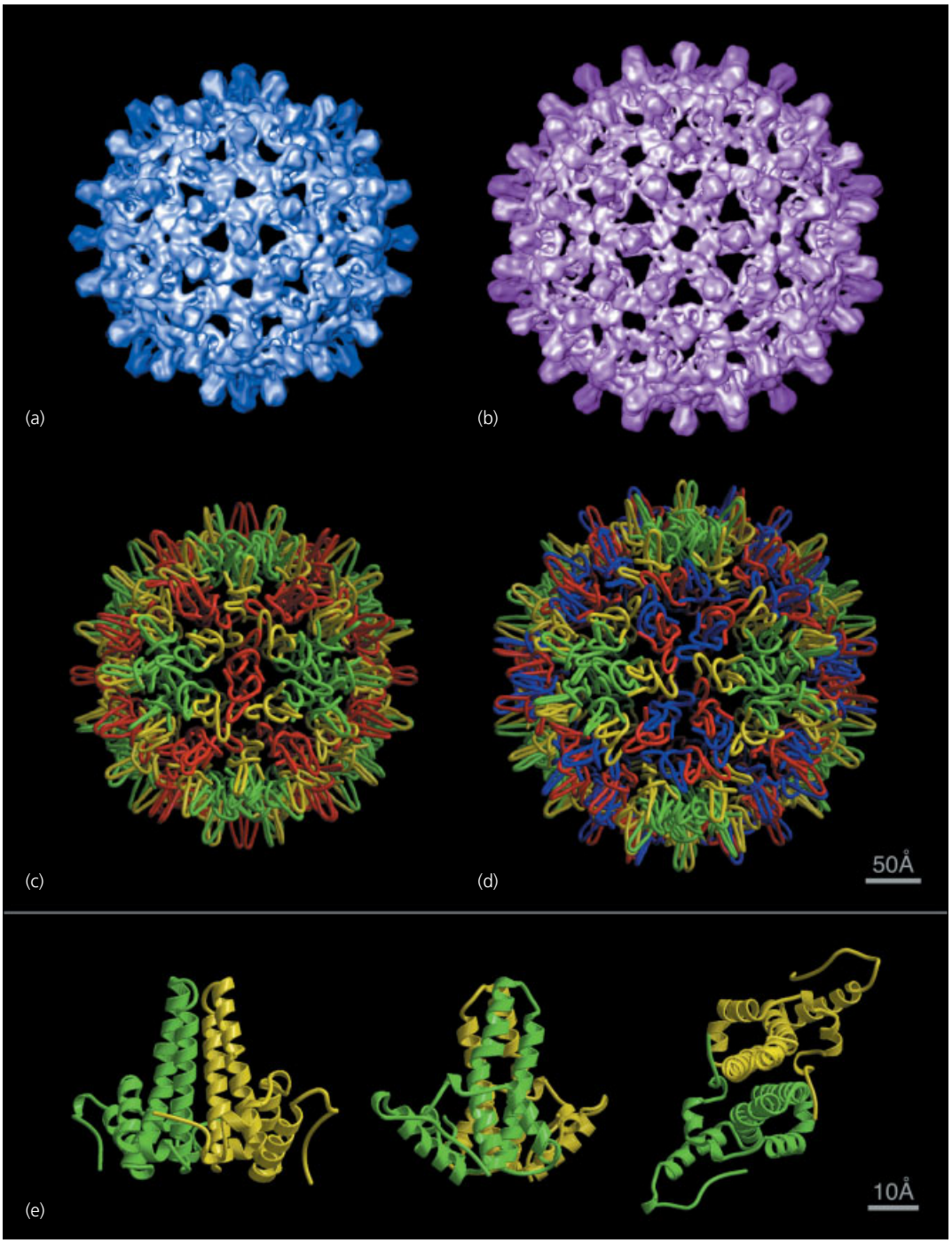

Figure 7 *Hepatitis B virus capsids come in two sizes: the smaller T = 3 capsid* **(a)** *and* **(c)** *with 180 subunits, and the larger T = 4 capsid* **(b)** *and* **(d)** *including 240 subunits. Surface views from cryo-EM reconstructions are shown in the top row at resolutions of 10 and 9 Å for* **(a)** *and* **(b)**, *respectively (Conway et al. 1997). The spikes rising from the capsid surface are four-helix bundles, with each of two capsid protein subunits contributing a helix–turn–helix motif to form a tight dimer interface. Unlike the SV40 and HK97 capsids of Figure 6, HBV does not have capsomers located on pentavalent and hexavalent sites, but instead the dimers cluster around these positions. The large holes in the capsid are presumably to allow nucleotides to diffuse inwards during reverse transcription of the RNA pregenome, following which the capsid will be enveloped on exiting from the host cell. The middle row shows tube renditions of atomic models of the capsids, based on the crystal structure of the T = 4 capsid (Wynne et al. 1999; PDB Id: 1QGT) but modified to fit the cryo-EM models (Watts et al. 2002). The monomer has three quasiequivalent positions in the T = 3 capsid* **(c)**, *color-coded as a green–yellow dimer and as a red–red dimer that lies on an icosahedral two-fold symmetry axis. For the T = 4 capsid* **(d)** *there are four different positions, coded as green–yellow and red–blue dimers. Views of a dimer are shown in* **(e)**, *where the four-helix bundle is evident. At left and center are two side views, and at right is a top view.*

Smaller capsids assemble around the viral genome or pregenome, whereas larger capsids are assembled first and then the genome is packaged through a specialized vertex, or portal. Packaging into a preformed capsid is driven by an ATP-dependent motor that works against a steadily increasing pressure gradient as the capsid fills. For dsDNA phages such as λ and HK97, the onset of packaging triggers a large-scale irreversible rearrangement of the capsid protein subunits, termed expansion (Figure 6b,c). For HK97 this trigger is proposed to be the repulsive force between the incoming DNA and the net negatively charged interior surface of the capsid (Conway et al. 2001). As a consequence of expansion, the HK97 subunits are displaced outwardly by 40 Å and rotate by 40° on average, and the internal volume doubles. Even with this additional volume to fill, the DNA is at high concentrations in these particles, of the order of 500 mg/ml. The capsid, which is round in the precursor form and highly contoured with protruding hexameric and pentameric capsomers, becomes planar and thin walled after expansion. Despite this thinning of the capsid wall, the buried surface area between subunits is estimated to be ~50 percent greater after expansion than before, suggesting that the larger subunit interfaces confer additional stability on the capsid, allowing it to withstand both the internal pressure of the packaged genome and external environmental stress.

Although this expansion transition is common among dsDNA phages, not all capsids that undergo a maturational reshaping necessarily expand. Recent work on HSV capsids indicates a number of conformational rearrangements, which serve to enhance contacts between subunits by closing gaps in the capsid, but with no significant change in its internal volume (Heymann et al. 2003). Another example is Nudaurelia capensis omega virus, a nonenveloped T = 4 RNA virus that infects insects, from which precursor virus-like particles are 16 percent larger than the mature virus, suggesting a maturational shrinkage (Canady et al. 2000). Large-scale structural rearrangements have not been seen in the assembly pathways of other icosahedral RNA viruses, although some plant viruses are observed to have a pH-dependent swollen state that is considered to be a disassembly transition during infection, much as is seen for poliovirus and other picornaviruses as a consequence of receptor binding (Fricks and Hogle 1990; Belnap et al. 2000; Hewat et al. 2002).

Tailed phages often bind one or more accessory proteins to the exterior surface of the capsid after expansion that confer additional stability. Trimers of the viral gpD protein bind to sites of local threefold symmetry on the surface of the T = 7 phage λ capsid, and these are essential for withstanding the internal pressure of packaging the full-length genome (Sternberg and Weisberg 1977). Phage T4 similarly binds the small outer capsid (Soc) protein at most local threefold sites, although it does not form a tight trimeric molecule (Iwasaki et al. 2000; Olson et al. 2001) as does λ's gpD (Yang et al. 2000). T4 also binds a second accessory protein (highly antigenic outer capsid (Hoc)), which populates local sixfold symmetry positions as a monomer (Iwasaki et al. 2000; Olson et al. 2001) but whose role is unknown. Somewhat akin to both Soc and Hoc is the vp26 protein of herpesvirus, which is dispensable for capsid formation and binds to the tips of hexamers of the major capsid protein vp5, but not to pentamers (Booy et al. 1994). The function of vp26 is unknown. Given its location, it may be expected to interact with a nonsymmetric protein layer called tegument that is between the capsid and the viral envelope (Trus et al. 1999), but this is not supported experimentally (Chen et al. 2001a). Phages HK97 and (probably) HK022 follow a different strategy to that of stabilizing the expanded capsid with accessory proteins. Covalent crosslinks between capsid proteins form autocatalytically as a consequence of expansion (Duda et al. 1995), generating a highly robust shell of interlinked protein rings (Duda 1998; Wikoff et al. 2000). This strategy of rigidifying the capsid is only feasible when the genome is to be released through a prebuilt channel, such as the connector-tail complex, rather than by capsid disassembly.

Different species, similar structures

Several observations of conserved architecture between viruses from quite different sources suggest far-reaching evolutionary relationships. The common features may be at the level of the capsid protein fold, as in the eight-strand β-barrel visualized in bacteriophages, plant, insect, and animal viruses (see Icosahedral capsid protein folds), to the larger-scale morphology of capsid geometry, including the recent intriguing identification of a special DNA packaging vertex on the HSV capsid that is reminiscent of the phage connector (Newcomb et al. 2001). For HSV the parallels with bacteriophages, particularly T4, have been drawn before. Common features include scaffold-assisted assembly, virus-encoded protease to process the immature capsid, maturational reorganization, the binding of accessory proteins, and the passage of the viral genome through a specialized vertex. There are, of course, important differences: the T4 tail, the HSV tegumentation and membrane, different proteins for hexameric and pentameric capsomers in T4, and so on. Possibly the similarities at the gross morphological scale are the results of convergent solutions to similar problems that the viruses face in packaging and delivering their genomes to new host cells. However, in another example, that of adenovirus and phage PRD1, the similarities on the gross scale are matched at the atomic level (Figure 8). Both viruses are unusual in the trimeric organization of their major capsid protein at hexavalent positions on a T=25 lattice, and they both employ a different protein for the

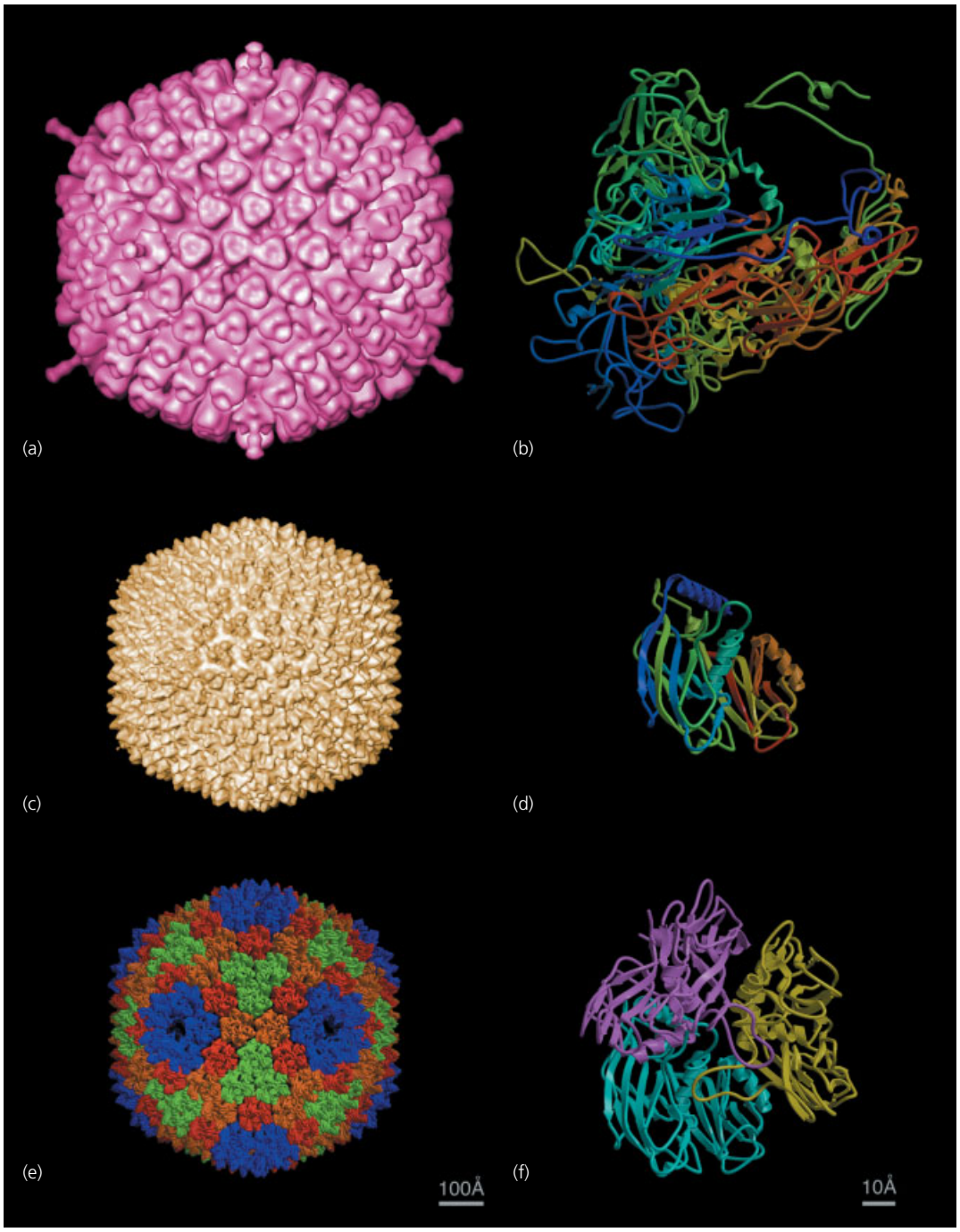

Figure 8 *Comparison of capsids from the mammalian adenovirus and bacteriophage PRD1. Despite differences in host-cell types, adenovirus and PRD1 share important structural similarities. Surface views of capsids from cryo-EM reconstructions are shown in panel* **(a)**, *adenovirus 2 at 25 Å resolution (Stewart et al. 1993; EMDS accession code 1016 – note that full icosahedral symmetry was not evident in the online density map, but has been imposed on the structure viewed here), and panel* **(c)**, *PRD1 at 16 Å resolution (San Martin et al. 2002; EMDS accession code 1011). These show the trimeric clustering of subunits on a T = 25 lattice. The adenovirus hexon trimers appear to have considerably more bulk than those of PRD1, which is a smaller capsid that also encloses a membrane. Trimeric spikes are partially visualized in the adenovirus structure, but not in that of PRD1. Atomic models of the major capsid proteins, colored from blue at the amino termini to red at the carboxy termini, are shown in panel* **(b)** *adenovirus hexon (Athappilly et al. 1994; PDB Id: 1DHX), and panel* **(d)** *PRD1 P3 protein (Benson et al. 2002; PDB Id: 1GW7). The adenovirus hexon is considerably larger (967 amino acids) than the PRD1 P3 protein (394 residues), with additional loops that extend upwards, but the core β-barrel is similar. An atomic model of the capsid, built from trimers of P3, is shown in panel* **(e)**, *where four adjacent trimers comprising the asymmetric units are colored purple, orange, red, and green. No structures are available yet for the pentameric vertex proteins, which are missing from this image. A single trimer of P3 is rendered as a ribbon diagram in panel* **(f)**.

pentameric vertices. Also in common is the binding of their receptor recognition molecule, a trimeric fiber, to the respective vertex pentons. The recent crystallographic structure of the PRD1 major capsid protein (Benson et al. 1999) has revealed a fold that is largely identical to that of the equivalent 'hexon' protein of adenovirus, with the exception of the protruding upper domain that is missing in PRD1. This remarkable set of similarities at all levels of structural detail suggests a common ancestry for these two virus proteins, which is further reinforced by a similar protein-primed mechanism of DNA replication initiation (Caldentey et al. 1992). Nonetheless, and not surprisingly, several striking differences are evident between these viruses, in particular the incorporation of a lipid bilayer on the interior of the PRD1 capsid and within which the viral DNA is enclosed. Structural comparisons of other important components, such as the pentameric vertex and trimeric fiber proteins, will be interesting to make when possible. A recent addendum to this fascinating story is the structural analysis of a large algal virus, PBCV1. This virus also incorporates an internal membrane, as does PRD1, but with a diameter of 1900 Å and triangulation number T = 169 its capsid is immensely larger than those of PRD1 and adenovirus (Yan et al. 2000). Nonetheless, the outer surface at 27 Å resolution appears to be populated by trimeric knobs that resemble the adenovirus hexon, and the recently solved structure of its major capsid protein is similar to the PRD1/adenovirus hexon fold (Nandhagopal et al. 2002).

NONSYMMETRICAL VIRUSES

Nonsymmetrical viruses do not have unique structures and cannot be crystallized. As mentioned above, cryo-EM leads to images with rather low signal-to-noise ratios, which can, in principle, be raised by averaging many virus particles if these are identical and can be aligned with each other. This is not possible for viruses that do not have unique structures. For these viruses the negative-staining technique is still often used.

All nonsymmetrical viruses are RNA viruses that have lipid membranes with membrane-embedded glycoprotein spikes. In most cases these spikes are associated with special lipid structures, called rafts (for reviews see Simons and Ehehalt 2002; Schmitt and Lamb 2004; Briggs et al. 2003). Lipid rafts are rich in cholesterol and sphingolipids and are not soluble in Triton X-100 or CHAPS at 4°C (London and Brown 2000), hence their alternative name: detergent-resistant membranes (DRM). The glycoprotein spikes associate with the lipid rafts during maturation in the Golgi apparatus and are transported in the membrane domains to the cell surface. The nonsymmetrical viruses usually also have a small matrix protein (20–30 kDa) that associates with the rafts and the cytoplasmic tails of the membrane-embedded glycoproteins and polymerizes at the interior of the membrane, bringing the rafts together and excluding cellular membrane-embedded proteins. The (encapsidated) genome binds to the layer of matrix protein to form an infectious virus particle. It seems that for the majority of nonsymmetrical viruses, much of the energy for building and maintaining the structure of the virus particle is due to these various and extensive interactions between membranes and proteins.

The glycoproteins are necessary for receptor binding, membrane fusion and, in some cases, for destroying the cell-surface receptors for release of newly formed virus from the infected cell (for those viruses that use sialic acid as receptor, such as influenza and parainfluenza viruses). The proteins that are active in fusion need to undergo a conformational change during which membrane fusion takes place. The trigger for the conformational change can be either low pH, if the virus is internalized through endocytosis (influenza virus and rhabdoviruses), or the trigger can be binding to a virus-specific cell-surface receptor (HIV, paramyxoviruses). The only structures solved for native fusion proteins are those of the hemagglutinins (HA) of influenza A virus and influenza C virus (Wilson et al. 1981; Rosenthal et al. 1998) and that of the fusion protein of Newcastle disease virus (Chen et al. 2001b). There exist now many structures of fusion proteins that correspond to the state of the protein after the conformational change related to membrane fusion. All these structures consist of triple coiled coils with a second layer of helices outside the inner coil (Skehel and Wiley 1998). This probably means that the fusion mechanism for all these viruses is similar, and it may also indicate that the structures before the change have similar features. The only exception may be the fusion-related conformational change of the rhabdovirus glycoproteins. The conformational change of VSV and rabies virus G is reversible, and after the change the fusion peptide is not inserted into the viral membrane, as is the case for influenza virus HA (Gaudin et al. 1995b, 1999).

As mentioned above, there exist neither atomic structures nor 3D EM models for the nonsymmetrical viruses. However, many proteins from these viruses have been crystallized and their structures solved. Table 1 gives a list of these structures per virus family. For the retroviruses we have only given the structures of the HIV proteins, although many other protein structures are also known, for example those of SIV proteins.

Negative-strand RNA viruses

The viruses in this group have a genome in the opposite sense to that of mRNA, and for this reason transcription is the first event that takes place after the virus enters the cell. All these viruses have a nucleocapsid in which the viral RNA is tightly and stoichiometrically bound to a nucleoprotein. The interactions between the

Table 1 *Structures of proteins of nonsymmetrical viruses*

Protein structure	Nonsymmetrical virus	Reference
Orthomyxoviruses		
Influenza A virus	Native HA (H3)	Wilson et al. 1981
	Uncleaved HA (H3)	Chen et al. 1998
	Native HA (H5, H9)	Ha et al. 2002
	HA after fusion	Bullough et al. 1994
	NA	Varghese et al. 1983
	NS1 (X-ray)	Liu et al. 1997
	NS1 (NMR)	Chien et al. 1997
	M1 pH 5	Sha and Luo 1997
	M1 pH 7	Arzt et al. 2001
Influenza B virus	NA	Burmeister et al. 1992
Influenza C virus	Hemagglutinin-esterase	Rosenthal et al. 1998
Paramyxoviruses		
NDV	HN	Crennell et al. 2000
NDV	Native fusion protein	Chen et al. 2001b
SV5	Fusion protein after fusion	Baker et al. 1999a
hRSV	Fusion protein after fusion	Zhao et al. 2000
Sendai virus	Phosphoprotein polymerization domain	Tarbouriech et al. 2000
Rhabdoviruses		
VSV	Matrix protein	Gaudier et al. 2002
Filoviruses		
Ebola virus	Matrix protein (VP40)	Dessen et al. 2000
	VP40 octamer	Gomis-Rüth et al. 2003
	Glycoprotein (after fusion)	Weissenhorn et al. 1998; Malashkevich et al. 1999
Retroviruses		
HIV	GP120	Kwong et al. 1998
	GP41 after fusion	Weissenhorn et al. 1997; Chan et al. 1997; Tan et al. 1997
	Matrix	Massiah et al. 1994; Matthews et al. 1994; Hill et al. 1996
	Capsid, N-terminal	Gitti et al. 1996; Gamble et al. 1996; Momany et al. 1996
	Capsid, C-terminal	Gamble et al. 1997
	Capsid, intact	Berthet-Colominas et al. 1999
	Nucleocapsid	Morellet et al. 1992; Summers et al. 1992
	Protease	Miller et al. 1989; Navia et al. 1989; Wlodawer et al. 1989
	Reverse transcriptase	Kohlstaedt et al. 1992
	Integrase	Dyda et al. 1994; Eijkelenboom et al. 1995; Lodi et al. 1995; Cai et al. 1997

nucleoprotein molecules determine the helical parameters of the nucleocapsid (Ruigrok and Baudin 1995); see Figure 9. They also have matrix proteins with affinities for lipid membranes and for nucleocapsids. All the matrix proteins have a tendency to polymerize, either into well-defined oligomers or into large polymers (Heggeness et al. 1982; Gaudin et al. 1995a, 1997; Ruigrok et al. 2000b; Baudin et al. 2001; Timmins et al. 2003). Polymerization of the matrix proteins probably pulls the glycoproteins in their lipid rafts together to form the virus particle. Interestingly, there are now three structures known of negative-strand RNA virus M proteins, from influenza virus, Ebola virus, and VSV (see Table 1), and all are different, suggesting that they are not evolutionarily related. Because the polymerization of the matrix proteins drives the assembly of the negative-strand RNA viruses, the existence of evolutionarily unrelated matrix proteins could imply that the blueprints of viruses from the various families are different.

ORTHOMYXOVIRUSES

We will use the influenza A virus to explain in detail the structure of negative-strand RNA viruses. A schematic

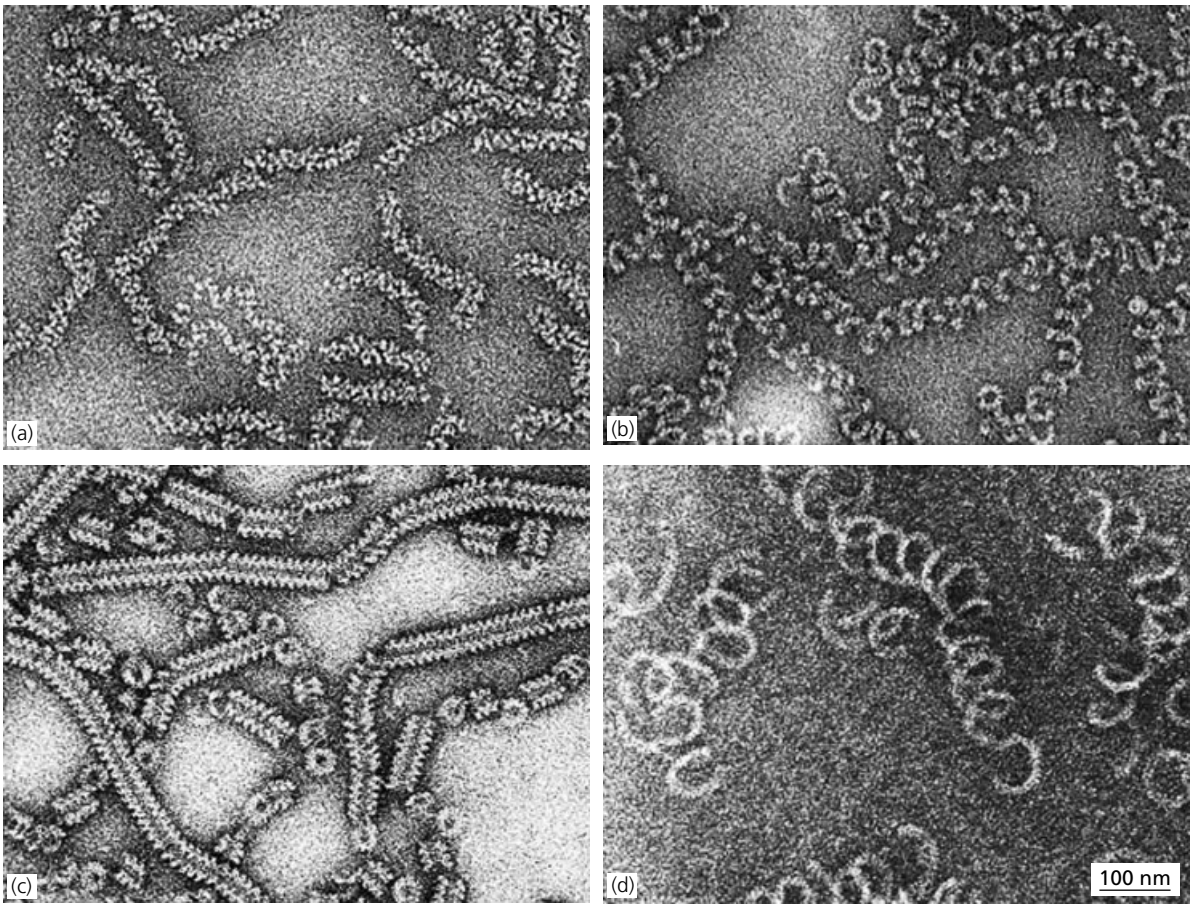

Figure 9 *Electron micrographs of negatively stained nucleocapsids of* **(a)** *influenza,* **(b)** *rabies,* **(c)** *measles, and* **(d)** *Marburg viruses. Scale bar = 100 nm.*

drawing of influenza A virus is shown in Figure 10 and various electron micrographs in Figure 11, p. 17. Influenza virus has a segmented RNA genome consisting of eight segments (seven for influenza C virus), each individually bound to a polymerase complex and each encapsidated by the nucleoprotein (Martín-Benito et al. 2001).

Transport component

Influenza virus has three transmembrane proteins: hemagglutinin (HA), neuraminidase (NA), and the M2 protein. HA is a homotrimer with a stalk part consisting of HA_2, which carries the membrane fusion activity, and a more globular head part made up by HA_1, which carries the receptor-binding function (Wilson et al. 1981; Skehel and Wiley 2000). NA forms tetramers and consists of a boxlike head that contains the enzymatic receptor-destroying activity, on a long and thin stalk (Varghese et al. 1983; Burmeister et al. 1992). M2 forms a tetrameric membrane channel (Sugrue and Hay 1991; Pinto et al. 1992; Shuck et al. 2000) that is needed for acidification of the interior of the virus as it passes through the endosomal system. Acidification is needed for depolymerization of the matrix protein so that the individual RNPs can enter the nucleus (Martin and Helenius 1991). Both HA and NA are embedded into lipid rafts, but M2 is not (Scheiffele et al. 1999; Ali et al. 2000; Zhang et al. 2000a, b; Barman et al. 2001).

Replication component

Each RNA segment is individually bound to a heterotrimeric polymerase complex and each is encapsidated by the nucleoprotein (Figure 9). The polymerase binds directly to the 3′ and 5′ ends of the RNA, and this complex RNA element forms the promoter for transcription and replication (Hagen et al., 1994; Cianci et al. 1995; Li et al. 2001). Martín-Benito et al. (2001) have made a 3D reconstruction from recombinant and short RNPs that lacked the extensive flexibility and diversity in shape of real viral RNP. The nucleoprotein molecules touch at one extreme side, as was already suggested from negatively stained nucleoprotein polymers (Ruigrok and Baudin 1995). Interestingly, the 3D model also shows extensive contacts between NP molecules and two of the polymerase subunits. Early work suggested that each NP monomer was associated with 20–26 nucleotides (nt) (Compans et al. 1972; Jennings

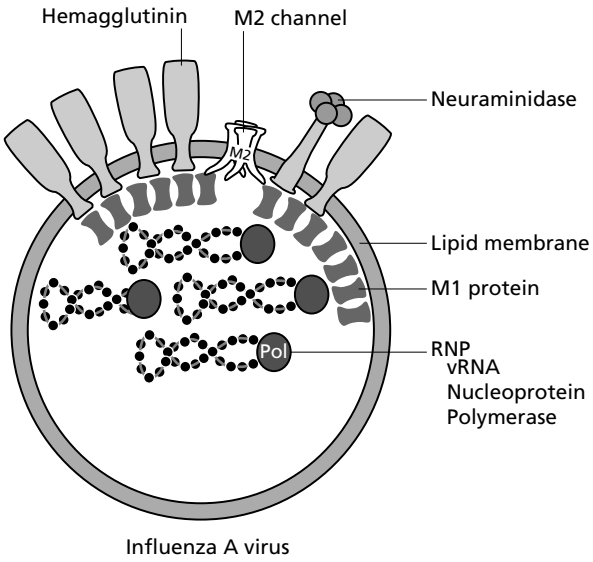

Figure 10 *Schematic drawing of an influenza A virus particle.*

et al. 1983). The same group that made the 3D EM reconstruction found that certain lengths of RNA produced recombinant RNP that replicated better than other lengths. These results suggested that the optimal unit of influenza virus RNA that can be encapsidated is not 24 nt per NP monomer, but 48 nt per NP dimer (Ortega et al. 2000). NP binds to the phosphate sugar backbone of the RNA in a sequence-nonspecific manner and, in doing so, prevents RNA secondary structure formation and exposes the nucleotide bases to the outside. In this way, the polymerase can probably read the vRNA without the need for dissociation of NP from the RNA (Baudin et al. 1994; Klumpp et al. 1997).

Connection between replication and transport components

The matrix protein (M1) binds the two components together in the virus particle. M1 consists of two domains, an N-terminal domain that has affinity for membranes and a C-terminal domain that has affinity for the RNP (Baudin et al. 2001). The affinity for membranes appears to be electrostatic and M1 in the virus does not seem to insert into the viral membrane (Ruigrok et al. 2000a). M1 binds to the cytoplasmic tails of the glycoproteins (Jin et al. 1997) and binds also to the M2 membrane channel (Zebedee and Lamb 1989). It is likely that the polymerization of M1 pulls together the fields of lipid rafts with their embedded glycoproteins, and that these fields are subsequently glued together through the M1–M2 interactions. If this model were correct, it would imply that the virus contains more or less rigid and curved protein-membrane sections stuck together at hinge points containing M2 protein. This kind of organization could explain some of the particle shapes of frozen hydrated virus (Figure 11a) and of virus negatively stained with tungstate salts such as sodium silicotungstate (SST) and phosphotungstate (PTA) (Figure 11c). A similar model has been proposed for immature HIV particles (Fuller et al. 1997). Such a model also implies that all particles will be slightly different in size and shape. Interestingly, there seem to be about as many copies of M1 protein in a virus particle as there are monomers of the glycoproteins (Ruigrok 1998).

Influenza B and C viruses

The overall shape of influenza B virus seems to be similar to that of the A virus, albeit more spherical than the ellipsoid A virus (Cusack et al. 1985). An interesting detail was observed for the structure of the influenza C virus glycoprotein (Rosenthal et al. 1998). This protein is a trimer of identical subunits. Each subunit consists of two parts (obtained after maturation cleavage by a host protease): a fibrous stalk containing the fusion activity and a more globular head domain carrying the receptor-binding activity, just as in influenza A virus HA (Wilson et al. 1981). However, the influenza C glycoprotein has an extra domain inserted into the receptor-binding domain. This extra domain contains the receptor-destroying activity, and as the influenza C virus does not have a special gene for this function it has not eight but seven gene segments (Compans et al. 1977; Herrler et al. 1981; Desselberger et al. 1980). This single influenza C virus glycoprotein can be organized into a hexagonal surface lattice (Flewett and Apostolov 1967). The influenza C virus glycoprotein is not associated with DRM (Zhang et al. 2000b). Apparently, the structure of the glycoproteins can accommodate additional domains and the different viruses can have their various functions associated with different glycoproteins.

PARAMYXOVIRUSES

The viruses in this large group are all spherical particles that easily deform when negatively stained for EM observation (Figure 12, p. 18). The main difference with the influenza viruses is that the genome is not segmented.

Transport component

The paramyxoviruses have trimeric fusion proteins that do not carry the receptor-binding function. This function is found on the H or HN (hemagglutinin–neuraminidase) protein, which has a similar shape to that of influenza virus neuraminidase. Those viruses that use sialic acid as receptor also have a sialidase with receptor-destroying function associated with the HN protein. However, those viruses that bind to other receptors, such as measles virus, do not have a receptor-destroying activity. The glycoproteins of the viruses in this group are also associated with lipid rafts (Manie et al. 2000; Brown et al. 2002; Henderson et al. 2002).

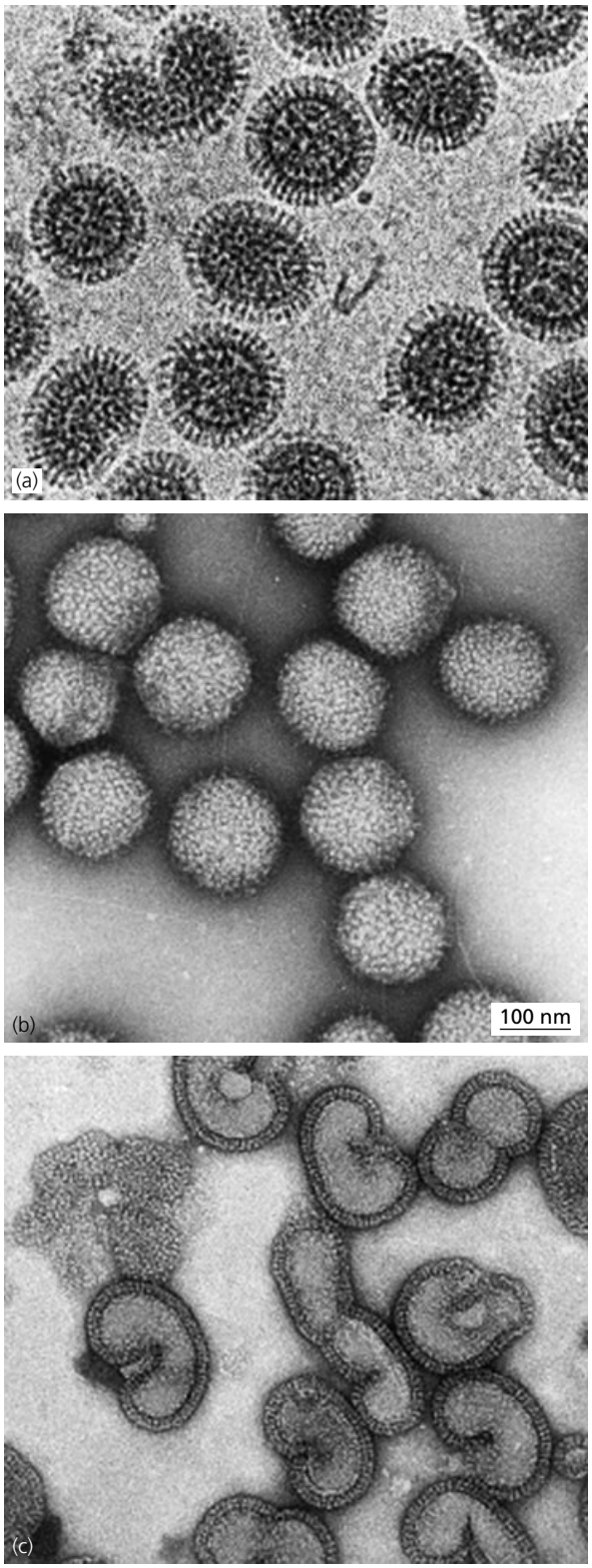

Figure 11 *Electron micrographs of influenza B virus (orthomyxoviruses prepared by* **(a)** *fast freezing (cryo-EM),* **(b)** *negative staining with 1% uranyl acetate, and* **(c)** *negative staining with 1% sodium silicotungstate. Scale bar = 100 nm.*

Replication component

The viral RNA is bound to the nucleoprotein with a stoichiometry of six nucleotides per nucleoprotein monomer (Egelman et al. 1989). This N-RNA forms the characteristic herringbone structure that is often used for the identification of paramyxoviruses (Figures 9 and 12). For some of the viruses in this group this stoichiometry even translates into a 'rule of six': these viruses must have a total number of nucleotides that is a multiple of six (Calain and Roux 1993; Kolakofsky et al. 1998). The proper positioning of the nucleoprotein on to the RNA has an influence on how the polymerase reads the various transcription signals, i.e. on how the polymerase recognizes the nucleotide bases (Iseni et al. 2002). The helical parameters for the nucleocapsids (NC) of the different viruses in this group are not the same: Sendai virus and measles virus have about 13 subunits per helical turn; simian parainfluenza virus 5 (SV-5) NC have 14 subunits; and respiratory syncytial virus (RSV) nucleocapsids contain predominantly ten subunits (Bhella et al. 2002).

The paramyxovirus polymerase is a monomer with polymerase activity that also produces a 5′ cap structure plus the poly-A tail of the viral mRNA. The polymerase is fixed on to the N-RNA template by a tetrameric phosphoprotein (Tarbouriech et al. 2000). The phosphoprotein binds with its C-terminal domain to the C-terminal domain of the nucleoprotein, and the polymerase binds to the oligomerization domain of the phosphoprotein (Curran et al. 1995).

Connection between replication and transport components

As with the influenza viruses, the viral matrix protein makes the connection between the membrane with its embedded spikes and the replication component. Isolated M polymerizes into helical structures depending on the salt concentration (Hewitt and Nermut 1977) and forms orthogonal arrays at the inside of the viral membrane and at the inside of the plasma membrane of infected cells (Büechi and Bächi 1982). The nucleocapsid packs against these arrays to form new virus particles.

RHABDOVIRUSES

This family contains two major groups of bullet-shaped viruses (Figure 13), the vesiculoviruses and the lyssaviruses. The viruses in these groups have similar structures, although there are also differences.

Transport component

These viruses have a single, trimeric glycoprotein G that contains the receptor-binding and the fusion activities. The viruses do not have a receptor-destroying activity. Although Brown and Lyles (2003) show interaction of

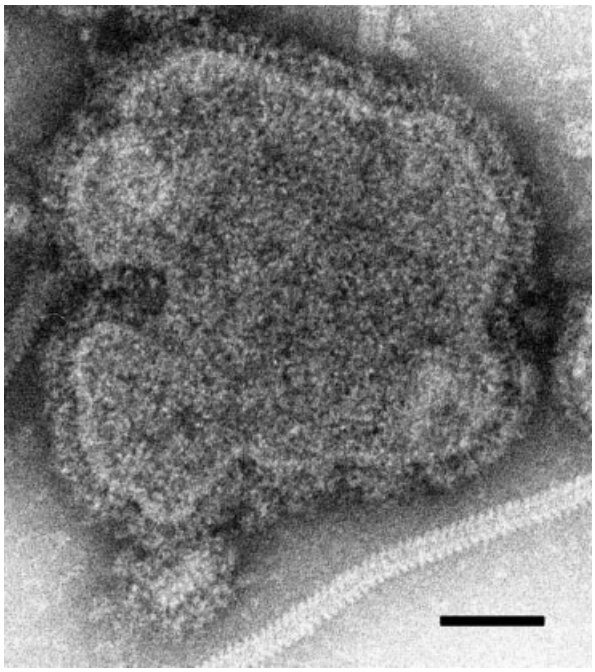

Figure 12 *Electron micrograph of measles virus (paramyxoviruses) negatively stained with 1% sodium silicotungstate. Note the helical nucleocapsid in the background. The bar corresponds to 100 nm.*

VSV G with membrane microdomains, there is still debate about whether or not the protein is associated with DRM (discussed in Briggs et al. 2003). It is possible that the membrane domains with which G associates do not have the same structure and composition as detergent-resistant membranes (Owens and Rose 1993; Johnson et al. 1998). For rabies virus, there is also no proof for DRM association from detergent resistance and membrane-floatation experiments (Y. Gaudin, personal communication). The trimeric nature of the rabies virus glycoprotein is clearly visible on EM under slightly acidic conditions (Gaudin et al. 1992). Under these conditions G can also self-associate to form a honeycomb structure, thought to be an intermediate for membrane fusion (Gaudin et al. 1996). Trimerization of the glycoprotein of VSV is also considerably more stable at low pH (Doms et al. 1987).

Replication component

The NC of rabies virus and VSV are loose coils (Figure 9) where each N monomer is bound to nine nucleotides (Thomas et al. 1985; Iseni et al. 1998). As for influenza virus and Sendai virus, the nucleoprotein is bound to the sugar phosphate backbone of the RNA and the nucleotide bases are exposed to the outside (Iseni et al. 2000). The polymerase is bound to the N-RNA by the phosphoprotein, which is also an oligomer, but the exact oligomeric state has not yet been clarified (Gigant et al. 2000).

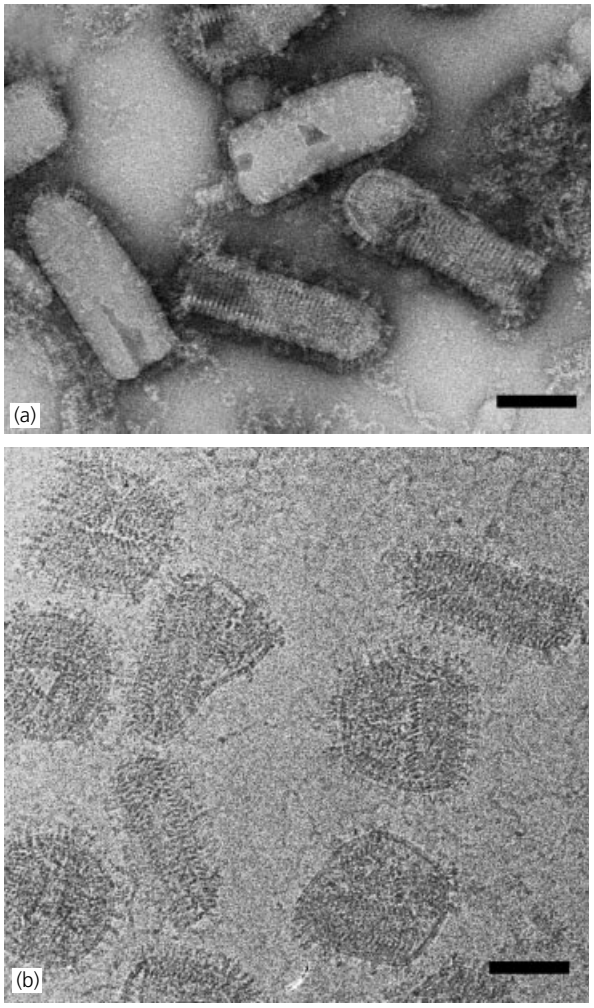

Figure 13 *Electron micrographs of VSV (rhabdoviruses) prepared by* **(a)** *negative staining with 1% sodium silicotungstate and* **(b)** *fast freezing (cryo-EM). Note in* **(b)** *that the nucleocapsid is sometimes doubled up to form a more spherical particle. The bar corresponds to 100 nm.*

Connection between replication and transport components

The formation of rhabdovirus particles is different from that of the myxoviruses described above. The viral matrix protein associates with the nucleocapsid in the cytoplasm and forms a tightly coiled M–NC complex (Newcomb and Brown 1981; Newcomb et al. 1982; Odenwald et al. 1986). There is a significant increase in the diameter of the helical nucleocapsid upon association with M, owing to slight changes in the angle between N subunits and the tilt angle with respect to the helical axis (Schoehn et al. 2001). The M–NC complex then binds to the cell membrane and, while pushing through it, associates with the glycoprotein spikes (Odenwald et al. 1986). The exact structure of the M–NC complex and the position of the matrix protein is not known. For VSV it has been observed that there is

mass inside the nucleocapsid coil, and it has been proposed that at least some of the matrix protein forms a scaffold around which the nucleocapsid is coiled (Brown and Newcomb 1987; Barge et al. 1993). However, this hypothesis on the positioning of the matrix protein is contested by claims that all of M is in contact with the membrane (Chong and Rose 1993). In any case, in contrast to the viruses described above, where the polymerization of the matrix protein leads to membrane curvature and particle shape, for the rhabdoviruses it is the preformed M–NC structure that gives the bullet shape to the rhabdovirus particles.

The VSV particle contains some 1 200 monomers of G, 1 260 monomers of N, and 1 800 monomers of M (Thomas et al. 1985). The rabies virus particle contains much less M – only about 700 copies – whereas it was estimated that there are as many N as G monomers, i.e. some 1 330 copies (Dietzschold et al. 1979; Flamand et al. 1993). Therefore, it would be possible that, in rhabdoviral particles, G associates with N rather than with M. The difference in M protein content between rabies and VSV could mean that there is a difference in structure between the two viruses.

FILOVIRUSES

Filoviruses are long and thin (Figure 14), much longer than rhabdoviruses (Feldmann and Klenk 1996; Feldmann and Kiley 1998). There are two groups of filoviruses, Ebola and Marburg. Although many different shapes, such as comma and laureate, have been described, only long and nonbranched particles are seen budding from infected cells (Feldman and Klenk 1996). Because of the high pathogenicity of the virus, only extensively fixed infected cells or purified virus have been used for electron microscopy.

Transport component

The filoviruses have a single glycoprotein with receptor-binding and membrane-fusion activity. This protein is associated with lipid rafts (Bavari et al. 2002).

Replication component

The image of the isolated nucleocapsids in Figure 9 was made from nucleoprotein expressed in insect cells. This protein binds to cellular RNA with a stoichiometry of about one N monomer per 12 or 15 nucleotides (Mavrakis et al. 2002). The shape of the N monomers looks somewhat like that of rhabdovirus N, as does the overall appearance of the N RNA. Apart from the nucleoprotein, the polymerase and the phosphoprotein (probably VP35), there is a fourth protein associated with the nucleocapsid of filoviruses, the VP30 protein (Becker et al. 1998; Modrof et al. 2001).

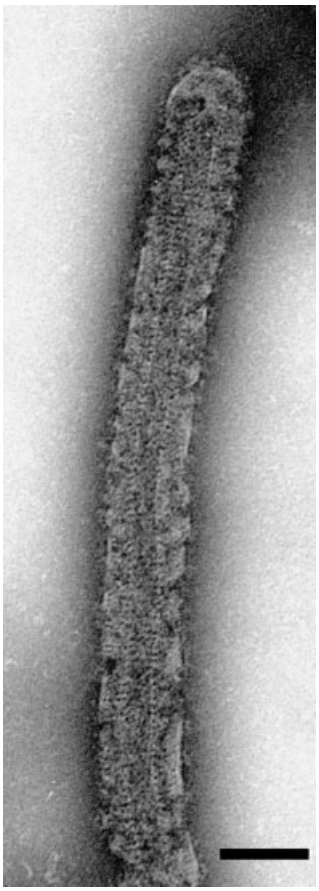

Figure 14 *Electron micrograph of an extensively fixed Marburg virus particle (filoviruses) negatively stained with 1% uranyl acetate. The bar corresponds to 100 nm.*

Connection between replication and transport components

The filoviruses have two matrix proteins, VP40 and VP24. VP40 is a two-domain protein (Dessen et al. 2000). The two domains can be dissociated by mutations that destabilize the domain interface, by treatment with low concentrations of urea or by putting the protein in contact with negatively charged lipid membranes (Ruigrok et al. 2000b). When the domains separate, the N-terminal domain associates into ringlike structures and the C-terminal domain binds to membranes (Ruigrok et al. 2000b; Timmins et al. 2003). It is thought that such a conformational change is needed for binding of VP40 to the cell membrane to start particle formation. The diameter of the free Marburg virus nucleocapsid is about 40 nm, whereas the diameter of this structure inside the virus is only 27 nm (Mavrakis et al. 2002). Therefore, whereas the coil diameter of the rhabdovirus nucleocapsid increases as it condenses into the M–NC complex, reducing the length of the structure, for filoviruses the NC diameter decreases, leading to an increase in length. This may be the reason why filoviruses are so long. The *Filovirus* nucleocapsid is also condensed in the cytoplasm, like that of the

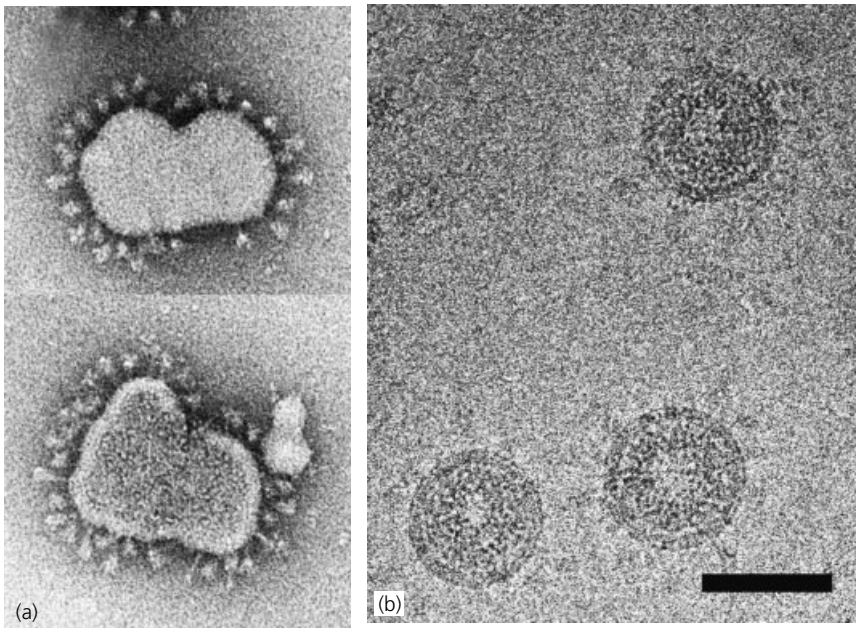

Figure 15 *Electron micrographs of Porcine respiratory coronavirus (PRCV) after* **(a)** *negative staining with 1 percent sodium silicotungstate, and* **(b)** *fast freezing (cryo-EM). Note the petalshaped spikes in* **(a)** *and the spherical shape of the particles in* **(b)**. *The bar corresponds to 100 nm.*

rhabdoviruses, before it goes into the virus particle (Kolesnikova et al. 2000), and it also seems to be this preformed structure that gives the shape to the virus particle. Between the nucleocapsid and the viral membrane there is an extensive and regular network or scaffold of proteins (Mavrakis et al. 2002; see Figure 14). This network is probably made up by the VP40 rings, with or without a contribution of VP24.

CORONAVIRUSES

The coronaviruses, together with the toroviruses and the arteriviruses, form the order of the *Nidovirales*.

Transport component

The name of this group of viruses is derived from its image in negative-stain EM of a spherical particle crowned with petalshaped glycoproteins spikes (S) (Figure 15a). S is a trimer (Delmas and Laude 1990).
There are two major subgroups of mammalian coronavirus. It seems that S of viruses of subgroup II is cleaved into two parts (like influenza virus HA) and that the trigger for fusion is low pH; and that S of subgroup I viruses is not cleaved and that fusion takes place at neutral pH after interaction with a specific receptor (Hansen et al. 1998; Zelus et al. 2003). However, Gallagher et al. (1991) have isolated different mutants from the same strain of murine hepatitis virus type 4 (group II) with very different pH requirements for fusion, with only a limited number of amino acid substitutions between the mutants. It is possible that the conformational changes that accompany fusion for the *Coronavirus* S glycoprotein are similar to those of the majority of other viral fusion proteins, because the predicted secondary structure of S also shows two amphiphylic helices per monomer (de Groot et al. 1987; Bosch et al. 2003). The terminal S1 domain is involved in receptor binding (Suzuki and Taguchi 1996), whereas the S2 subunit is involved in membrane fusion (de Groot et al. 1989; Yoo et al. 1991). The *Coronavirus* membrane has at least one other glycoprotein embedded: the small M protein that traverses the membrane three times (Machamer and Rose 1987). For the arteriviruses it has been shown that the membrane can contain several other minor proteins (hemagglutinin esterase (HE), small envelope protein (E), GP(3), and GP(4); Wieringa et al. 2002). It is not known yet whether the coronavirus surface glycoproteins are associated with membrane rafts.

Replication component

Coronaviruses are positive-strand RNA viruses with large genomes of between 27 and 32 kb. This genome size is one of the reasons for the high production of mutant viruses. The viral RNA is capped and polyadenylated (Schochetman et al. 1977; Lomniczi 1977), and upon cell entry the first open reading frame coding for the polymerase is translated. The polymerase then produces subgenomic (sg) copies of the RNA that code for the rest of the viral proteins (Lai and Cavanagh, 1997; van Marle et al. 1999; van Vliet et al. 2002).

Connection between replication and transport components

There is a disagreement in the literature about the function and position of the internal 'nucleocapsid protein' (N). For the arteriviruses it is assumed that N forms a spherical (icosahedral?) shell around the viral RNA. However, no icosahedral reconstructions of these viruses exist, and electron micrographs of thin-sectioned virus particles do not show spherical but rather ellipsoid shapes (Brinton 1999), thereby throwing doubt on the existence of icosahedral symmetry. Older literature on coronaviruses describes N forming helical complexes with vRNA (Macnaughton et al. 1978; Davies et al. 1981) and, because of its positively charged nature, N has been compared with influenza virus nucleoprotein (Laude and Masters 1995). Later work on coronaviruses has suggested that N forms a spherical structure beneath the viral membrane, and that maturation of this structure takes place upon transport of the virus towards the outside of the cell (Risco et al. 1998; Salanueva et al. 1999). The diameter of coronaviruses embedded in vitreous ice is very variable, with no specific size classes (Schoehn, unpublished results), suggesting that the virus does not have icosahedral symmetry.

A number of the characteristics of *Coronavirus* N correspond also to those of the matrix proteins of the negative strand RNA viruses that are also strongly positively charged.

- *Coronavirus* N does not only bind to RNA but also to lipid membranes (Stohlman et al. 1983), a property shared with the positively charged M1 protein of influenza virus that binds to membranes and to naked nucleic acids (Baudin et al. 2001).
- *Coronavirus* N has a tendency to form trimeric complexes (Laude and Masters 1995), a property shared with the MA protein of the retroviruses.
- Detergent-treated influenza virus particles also release helical structures that were initially interpreted to be formed of ribonucleoprotein but which are now thought to consist of oligomeric M1 complexes (Murti et al., 1980, 1992; Ruigrok et al., 1989).
- Experiments with plasmid-expressed proteins suggest that the viral glycoproteins alone can form VLP and leave the cell, but N expressed alone can also form VLP (Vennema et al. 1996), like the matrix proteins of negative-strand RNA viruses and retroviruses.

We only make these comments in order to indicate that, in our opinion, the fine structure of coronaviruses is not yet known in detail.

RETROVIRUSES

As a group, retroviruses are perhaps as variable as the negative-strand RNA viruses. This part of the review will discuss the general principles of retrovirus structures; for more detailed information the reader is referred to Goff (2001) and subsequent chapters in *Fields' Virology*. The structures of virtually all HIV proteins (Table 1) and many proteins of other retroviruses have been determined by either crystallography or NMR, but no intact virus or capsid structure has been determined because of a lack of defined symmetry.

Transport component

Retroviruses have a single trimeric glycoprotein (GP160) that, at least for HIV, is associated with lipid rafts (Manes et al. 2000; Ono and Freed 2001) (see Figure 16 for a negatively stained SIV particle showing end-on views of the triangular spikes). As for influenza virus HA, GP160 is posttranslationally cleaved into two parts: N-terminal GP120, which is responsible for binding to primary and secondary receptors, and C-terminal GP41, which is membrane anchored and responsible for membrane fusion. GP120 is placed upon GP41, much like the organization of influenza virus HA. GP41 has the same triple coiled coil structure as the fusion proteins of the segmented and nonsegmented negative-strand RNA viruses, implying that the fusion proteins are evolution related and that the fusion processes of all these viruses follow the same mechanism.

Replication component

The replication component of retroviruses consists of the viral RNA bound to the nucleocapsid (NC) protein plus copies of the reverse transcriptase and integrase (see below), necessary for the production of retrotranscribed viral DNA and its integration into the host genome. NC forms a complex with vRNA and facilitates annealing of vRNA with the tRNA primer for reverse transcription (Barat et al. 1993), and prevents the reverse transcriptase stopping at stable stem-loops in the RNA (Wu et al. 1996). In this respect NC has

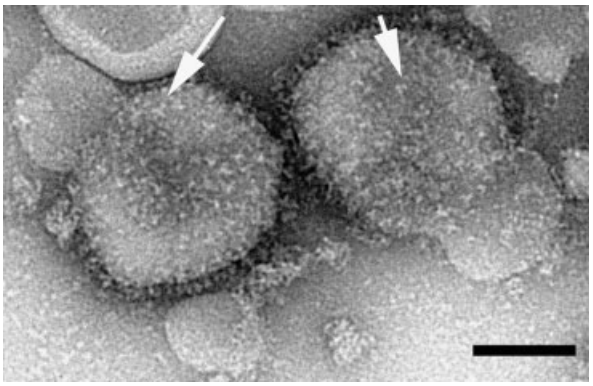

Figure 16 *Electron micrograph of simian immunodeficiency virus (SIV, retroviruses) particles negatively stained with 1% uranyl acetate. The arrowheads point out triangular shapes of the trimeric surface glycoprotein. The bar corresponds to 100 nm.*

been named an RNA chaperone (Feng et al. 1999). Although probably structurally different, the function of the retroviral NC protein may be similar to that of the negative strand RNA virus nucleoprotein (see above).

Virus assembly and maturation

The most simple retroviruses code for two polyproteins, Gag and Pol, and a small percentage (about 10 percent) of Gag–Pol proteins when the ribosome reads through the stop codon or when a frame shift occurs at the Gag–Pol boundary, depending on the virus family. More complex viruses have a series of smaller proteins that modulate cellular processes (Goff 2001). The Gag polyprotein consists of the matrix (MA), capsid (CA), and the NC proteins, and Pol consists of the protease (PR), integrase (IN), and the reverse transcriptase (RT). The structure of immature virus was studied from Gag expressed in insect cells or bacteria, which forms virus-like particles (VLP) (Nermut et al. 1994, 2002; Fuller et al. 1997), and from virus with a mutated and inactive protease (Stewart et al. 1990). In immature virus or VLP Gag binds to membranes through the matrix protein (see Figure 17) and it forms locally ordered hexagonal lattices (Nermut et al., 1994, 2002; Yeager et al. 1998; Forster et al. 2000; Mayo et al. 2003). However, the spherical structures that are formed are not icosahedral (Fuller et al. 1997; Yeager et al. 1998). Rather, it appears that the Gag polymers bind to membrane patches, leading to shells with local curvature. Somehow, these shells are then joined to form VLP with various diameters (Fuller et al. 1997). The presence of local organization of Gag and curved membrane elements could be similar to what is observed for influenza viruses, where curved M1 membrane shells may be joined by the transmembrane M2 protein (see above). The Gag–Pol proteins are included in the viral bud because the Gag of the Gag–Pol polymerizes with the rest of Gag into the virus. Inside the Gag polymer, nucleic acid (random for recombinant VLP and viral RNA for intact or PR virus) is bound to the NC part of Gag.

When the virus has budded from the plasma membrane and left the cell, the protease cleaves Gag into MA, CA, and NC, and Pol into PR, IN, and RT. NC remains associated with the viral RNA, MA remains at the membrane and there is no indication that it rearranges at the membrane, but CA forms a new structure inside the virus particle, the capsid (Figures 17 and 5.18). The capsid is a closed structure for all the retroviruses, but its form differs by family. As described above for the icosahedral viruses, closed capsids may consist of 12 pentavalent capsomers and a variable number of hexavalent capsomers. The retroviral capsid is also built from CA monomers that are organized into 12 pentavalent and a variable amount of hexavalent units. However, the pentavalent units can be placed at various positions (Ganser et al. 1999; Li et al. 2000). If six pentavalent units are placed close together at one end, six at the opposite end and the rest of the structure consists of hexavalent units, a hollow tube is formed that is indeed sometimes observed in structures formed by purified CA protein in vitro, and which are also observed inside mature HIV (Li et al. 2000; Briggs et al. 2003). Other conical forms are made when the pentavalent units are distributed in an unequal fashion, with five at one end and seven at the other, or other variations (Ganser et al. 1999). All these forms are observed in mature HIV (Briggs et al. 2003). Other retroviruses, such as murine leukemia virus, form rather spherical capsids with a more equal distribution of the pentavalent

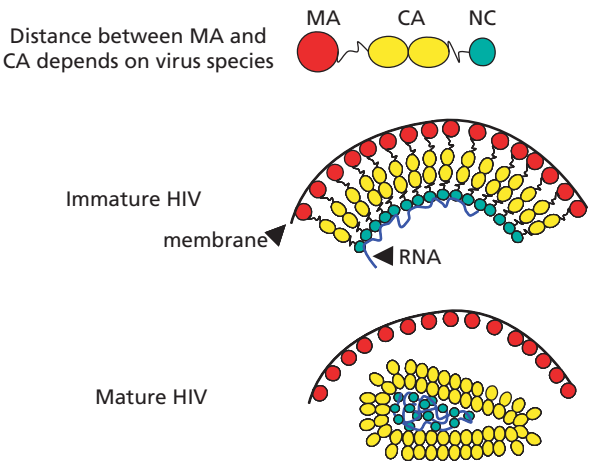

Figure 17 *Schematic representation of the changes in HIV capsid structure upon maturation.*

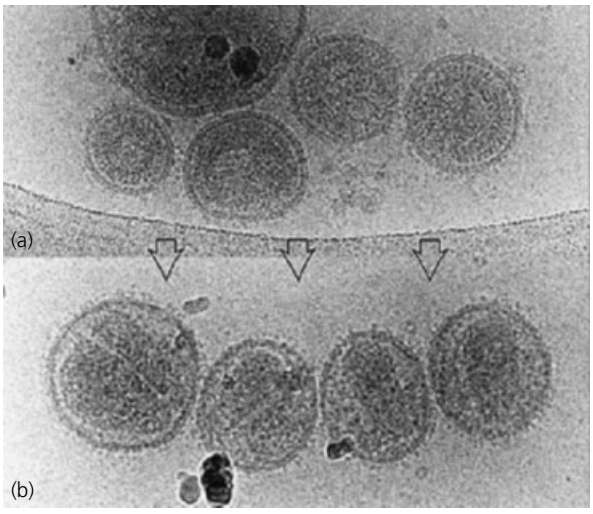

Figure 18 *Cryo-electron micrographs of* **(a)** *immature and* **(b)** *mature Human immunodeficiency virus (HIV, retroviruses) going from immature virus above the arrows to mature virus below. Note the change in shape of the capsid inside the membrane. Photograph courtesy of Stephen Fuller. Reprinted from Fuller, S.D., Wilk, T., et al. Cryo-electron microscopy reveals ordered domains in the immature HIV-1 particle. Current Biology, Vol. 7, pp. 729–38. Copyright (1997), with permission from Elsevier.*

units on a spherical surface, but these capsids are also not all identical and are not icosahedral (Yeager et al. 1998; Kingston et al. 2001). Another observation which goes against the idea that retroviruses have icosahedral structures is that mature and immature viruses do not have diameters with defined values but with rather wide distributions (Fuller et al. 1997; Yeager et al. 1998; Kingston et al. 2001; Briggs et al. 2003). This size variation suggests that smaller viruses can include less nucleic acid than larger viruses, and for HIV it was indeed found that the larger viruses had more than one capsid cone (Briggs et al. 2003).

Some retroviruses do not assemble at the cell membrane but form an immature Gag sphere in the cytoplasm, which subsequently associates with the membrane and the glycoproteins. One could imagine that these viruses are icosahedral, but careful analysis of many different retrovirus families has failed to reveal icosahedral symmetry (S.D. Fuller, personal communication). Another indication that the structures of viruses that assemble at the plasma membrane and those that assemble in the cytoplasm are not essentially different is that a single mutation in MA can change a virus that normally assembles in the cytoplasm into a membrane assembler (Rhee and Hunter 1990).

FINAL COMMENTS

This chapter on virus structures is necessarily incomplete owing to the vastness of the topic. One may look into *Fields' Virology* or similar monumental tomes for additional information. Viruses and virus families may not have been treated here, mainly because none of the authors has worked with them: for example, the nonsymmetrical negative-strand RNA bunya and arenaviruses. However, what has become clear to us during writing this text is that even viruses of the same family can show significant differences in structure, and recent studies also demonstrate that structure may be shared between apparently distant families. We expect that the continuing development of biophysical techniques, especially X-ray crystallography and electron microscopy, will continue to play an important role in defining more details about virus morphology and connecting that information to the dynamics of protein and protein complexes, and their functions. Although we focus on virus structure here, this field also has widespread application to protein structure and function in general, as well as to the burgeoning technologies of nanoparticles and nanomachines.

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Propagation and identification of viruses

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This chapter provides an overview of the main techniques used for propagating and identifying viruses. Detailed descriptions can be found in the appropriate journal citations. In some instances, more general references are made to textbooks of laboratory methods, without necessarily citing specific sections.

SYSTEMS FOR PROPAGATING VIRUSES

Viruses can only replicate in living cells. Therefore, an essential first step in propagating viruses in the laboratory is the provision of a suitable culture system. Cell culture is the most widely used method for propagating viruses, but older techniques, such as tissue or organ culture, may still be used for specialized purposes, as may chick embryo and animal inoculation.

Cell culture

GENERAL

Cell cultures are prepared either as single layers (monolayers) on a glass or plastic surface, or as suspensions, the choice depending on their proposed use. Another choice to be made is the type of cell needed for a particular study. Several international repositories can serve as the starting point for reference cell lines and for microbiological reagents. They include the European Collection of Animal Cell Cultures (www.ecacc.org.uk), the American Type Culture Collection (ATCC) (www.atcc.org), Statens Seruminstitut (www.ssi.dk/sw162.asp), and Institut Pasteur (www.pasteur.fr/externe). Detailed information on culture media and specialized techniques is also available from these centers, and in other reference works (Lennette et al. 1988, 1995; Schmidt 1989; Freshney 2000; Murray and Baron 1995).

SAFETY

All procedures involving cell lines should be performed in a class II biological safety cabinet under strict aseptic conditions, both to minimize the risk of contaminating cells with bacteria, yeasts, and mycoplasmas and to protect the worker. Cell lines should be considered potentially hazardous. Although continuous (transformed) cell lines are assumed to be free of infectious agents, they may in fact harbor latent viruses that could infect the worker (Barkley 1979). Safety precautions should include the general measures reviewed by Fleming and Richardson (1995), Lennette et al. (1995), Sewell (1995) and in The laboratory diagnosis of viral infections.

CELL CULTURE MEDIA

Most of the basic culture media used today are chemically defined, but must be supplemented with 1–20 percent serum. This is usually obtained from calf fetuses and must be carefully checked for contamination by viruses, mycoplasma, bacteria, and specific viral antibodies. Serum-free media is also commercially available

for specialized applications (e.g. vaccine development). Cells generally grow well at pH 7.0–7.4. Phenol red is often used as an indicator in the medium, changing progressively from purple at pH 7.8 through red at pH 7.4 to yellow at pH 6.5 (Freshney 2000).

Culture media require careful buffering to stabilize the pH under all conditions. In plastic dishes with unsealed lids, in which exposure to oxygen causes the pH to rise, exogenous CO_2 is required by cell lines, particularly at low cell concentrations, to prevent total loss of dissolved CO_2 and bicarbonate from the medium. In tubes or flasks with tight caps, pH can be adjusted by loosening the caps, changing the medium or placing the vessels in a CO_2 incubator with a gas-permeable cap, as dictated by the indicator. The CO_2 level is critical and can be monitored by a gas analyzer. As little as a 1percent increase can result in cell death.

Medium components should be prepared with endotoxin-free water. Water systems are available to produce type I reagent grade water, which minimizes metal and organic ions leaching from storage containers and prevents bacterial and yeast contamination by being used immediately.

Media, media components, and reagents are sterilized by autoclaving if they are heat-stable (e.g. water, salt solutions, amino acid hydrolysates) or by membrane filtration if they are not (e.g. protein or sugar solutions). The type of membrane is important. Cellulose acetate membranes are best for applications involving low protein binding, and cellulose nitrate membranes for general purpose filtration. Cotton pads, membranes of larger pore size, or commercial prefilters should be used for prefiltering, and membranes of 0.22 μm average pore diameter (APD), for sterilization.

TYPES OF CELL CULTURE

Cell cultures may be either primary or continuous. Primary cultures contain mixtures of cells freshly derived from the tissue of origin, which may be obtained either from laboratory animals or from human material procured in the course of surgery. Some specimens (e.g. foreskin) are not derived from pathological tissues. Tissues from young or embryonic animals give better results than those from adults. The tissues are first minced into 3-mm pieces and the cells disaggregated by one or more treatments with a solution of 0.22 percent trypsin and 0.02 percent versene. The cells are then filtered through sterile gauze, washed by centrifugation in cold growth medium (GM), and finally suspended in GM to the desired concentration. The concentration of viable cells is determined by counting a sample diluted in trypan blue, which selectively stains dead or damaged cells. Tubes or flasks are seeded at approximately 3×10^5 viable cells/ml.

Cell lines are also derived from primary cells, but can be repeatedly subcultured. They fall into two categories. Euploid cells, such as normal human fibroblasts (e.g. MRC-5, WI-38, HFL1), retain their normal karyotype throughout their culture lifespan, rarely give rise to continuous lines and, after approximately 50 generations, stop dividing (Hayflick 1973). They are sometimes called semicontinuous lines. By contrast, aneuploid (or heteroploid) cells, such as those from many human and animal tumors, may give rise to true continuous cell lines (e.g. HEp-2, HeLa, LLC-MK2). Aneuploid cell lines have a chromosome number that is not an exact multiple of the haploid number.

Insect cell lines are continuous cultures derived from, for example, the SF9 ovary (fall armyworm, *Spodoptera frugiperda*) and from mosquitoes (e.g. *Aedes aegypti* and *Ae. albopictus*). The SF9 line, which is grown in special media, is highly susceptible to infection with baculoviruses and can be used for baculovirus expression vectors and other needs. Mosquito lines are used as suspension cultures for the replication of many mosquito-borne viruses. They have special media and subculturing requirements (Lennette et al. 1995).

Suspension cultures can be prepared from many mouse and human leukemias and ascites tumors. They are incubated on shakers or in roller drums or allowed to settle to the bottom of a large culture flask, such as a roller bottle. Alternatively, a spinner apparatus is available that forces normally adherent cells to grow in suspension in a special medium.

Shell vial cultures contain a cell monolayer grown on a coverslip in a 1-dram vial. The vial is then inoculated with the specimen and centrifuged at low speed (700 *g* for 45 min), which apparently distorts the cell surface and renders it more susceptible to viral attachment. After 1–3 days of incubation, irrespective of any cytopathic effects (CPE), the coverslip is washed briefly, and tested by immunofluorescence assay (IFA), nucleic acid hybridization, or other tests for whichever viruses are suspected. Shell vial cultures have been successfully used as a rapid test for adenovirus (Espy et al. 1987), Cytomegalovirus (CMV) (Gleaves et al. 1989; Buller et al. 1992), varicella-zoster virus (VZV) (Schirm et al. 1989), respiratory syncytial virus (RSV) (Smith et al. 1991), and other respiratory viruses (Matthey et al. 1992; Schirm et al. 1992; Olsen et al. 1993).

Microcultures are cost-efficient systems of growing popularity, having overcome earlier problems with cross-contamination between wells, over-oxygenation, and toxic plastics. Microcultures in 24- to 48- and 96-well plates with sealable lids (or tightly fitting lids for CO_2 incubators) are now commonly used in a variety of viral assays.

In 96-well microtiter plates with flat well bottoms, microcultures are ideal for neutralization tests, tissue culture enzyme immunoassay (EIA), monoclonal antibody testing, and many screening assays (Hierholzer and Bingham 1978; Anderson et al. 1985; Lennette et al. 1995; Murray and Baron 1995). Macrocultures in

standard tubes, however, are still used for primary virus isolation because of the ease of setting up stationary or roller cultures in ordinary incubators, of reading the monolayers for CPE and of obtaining sufficient volume of virus culture to use for subpassaging, identification tests, viral genetic analyses, and storage.

Specialized culture systems have been devised to satisfy particular needs. For example, suspension cultures of human peripheral blood lymphocytes are sensitive to the Epstein–Barr herpesvirus, where it is detected by a transformation assay, and, under cocultivation procedures, to the human T-cell leukemia viruses (HTLV) and human immunodeficiency viruses (HIV) (Lennette et al. 1988, 1995; Fields and Knipe 1990; Isenberg 1992). Immortalized human microvascular endothelial cell cultures derived from foreskin are sensitive to a wide variety of viruses under special culture conditions, and have potential use as a model to study the biology of these important cells (Ades et al. 1992). Extracellular matrix systems support the three-dimensional growth of cells as opposed to monolayer cultures. The Matrigel invasion chamber (Collaborative Biomedical Products, Bedford, MA, USA), for instance, can support fastidious cell types and is currently being explored for its use in viral culture and alterations in cell physiology following viral infection (Bissell et al. 1990; Thompson et al. 1991). Finally, large-volume culture systems, such as suspension culture vats, cells on the surfaces of Sephadex beads or other microcarriers, and cells on spirals of Sterilin or other plastic film are used extensively in industry to prepare vaccines, interferons, and monoclonal antibodies (White and Ades 1990).

Mixed cultures of different cell types combined in a single vial have been used successfully to grow a broad range of viruses (Huang and Turchek 2000; Navarro-Marí et al. 1999). A commercial product, Mixed *Fresh-Cells*, available from Diagnostic Hybrids, Inc. (Athens, OH, USA), combines two compatible cell lines optimized for growing particular groups of viruses, e.g. R-Mix for respiratory viruses, E-Mix for enteroviruses and H&V-Mix for herpesviruses. For example, R-Mix consists of a mixed monolayer of mink lung cells (Mv1Lu) and human adenocarcinoma cells (A549) that have been shown in clinical studies to support the detection of influenza A and B viruses, RSV, adenovirus, and parainfluenza viruses 1, 2, and 3 (Barenfanger et al. 2001; St George et al. 2002).

Genetically engineered cell lines offer potential improvements in our ability to grow viruses and to rapidly detect virus infection. Stable transformation of specific virus receptors for HIV-1 (Chesebro and Wehrly 1988) and EBV (Li et al. 1992) into previously nonpermissive cell lines has been shown to permit growth of these viruses. Genetically modified cells that express an easily measured reporter enzyme after infection with a specific virus have also been developed. For example, a transgenic baby hamster kidney cell line (BHKIC-P6LacZ) that contains multiple copies of a virus-inducible HSV promoter sequence linked to the reporter gene *LacZ* developed by Stabell and Olivo (1992) has been used successfully for diagnosis of infections with HSV types 1 and 2 (LaRocco 2000). An FDA-approved diagnostic assay for HSV, based on these cells, is commercially available (ELVIS HSV test; Diagnostic Hybrids, Inc.). For a more comprehensive review of the potential benefits of genetic engineering of cell lines for diagnostic virology, see Olivo (1999).

QUALITY CONTROL

Contamination by bacteria and fungi is an inherent problem of cell culture work. Contamination can be visually detected by appearance of turbidity or color changes due to changes in the pH of the culture medium, or more definitively by microscopic examination of the culture. Detailed protocols for the detection of most bacteria and fungi that would be expected to survive in cell cultures in the presence of the customary antibiotics may be found elsewhere (ATCC 1992). These methods should be incorporated into the quality control program of the media production laboratory to preclude the contamination of cells by detecting organisms in the culture GM.

Contamination by mycoplasmas and viruses is more difficult to detect, because these organisms usually do not cause turbidity or pH changes, may not cause a CPE in the cells, and are able to pass through 0.2 μm filters commonly used for sterile filtration of media and media components. Thus, these agents may be passaged with the cells indefinitely without detection unless specific testing is performed. Primary rhesus monkey kidney (MK), primary African green monkey kidney (AGMK), and primary bovine embryonic kidney cell cultures are particularly notorious for adventitious virus contamination (Hull 1968; Crandell et al. 1978; Schmidt 1989; Eberle and Hilliard 1995). A variety of methods have been described for detection of the mycoplasma species most commonly found in mammalian cell culture, including microbiological culture, DNA staining with fluorescent dyes, mycoplasmal enzymatic assays, IFA, and EIA, hybridization probes, and polymerase chain reaction (PCR) assay. Commercial testing services are widely available for mycoplasmas and viruses and commercial kits, such as the MycoTect test (Invitrogen), MycoSensor PCR Assay (Stratagene), and Mycoplasma Detection Kit (Roche Applied Science) are available that can be incorporated into a quality control program.

Contamination by other cell lines can also occur, the intrusion of HeLa cells into many other lines giving the most problems. Preventative measures that can be taken include working with only one cell line at a time; keeping bottles of medium, trypsin/versene, etc., separate for each cell line; allowing a ‘resting’ period of approximately 30 min between manipulation of different

cell lines in a biological safety cabinet; and decontaminating cabinet surfaces before introducing another cell line to the work area. The species of origin of a cell line can be determined by various immunological, isoenzyme, and cytogenetic tests (ATCC 1992) that are available commercially. Thus, quality control programs for cell culture laboratories must incorporate periodic searches for contaminants of microbiological and cellular origin, because the contaminants may cause irreversible changes in the cell cultures and can completely confound the interpretation of diagnostic tests.

PROPAGATION OF CELLS

Propagating cells by scraping is a physical method of removing adherent cells from the surface of a culture vessel, and is used when enzymatic removal (see below) may be toxic to the cells or may destroy receptors or other important cell surface molecules. The GM is discarded, the cells are physically scraped from the vessel surface with a disposable cell scraper into fresh GM, and the cells are then dispersed by vigorous pipetting to obtain a suspension of single cells. The cells are counted and appropriately diluted for passage to new flasks or other vessels.

Cell counts are used to quantify viable cells for passaging and seeding. They are best performed in the presence of a vital stain, which is excluded by living cell membranes and is incorporated only into dead cells. In the trypan blue vital stain procedure, a sample of the cell suspension is mixed with 0.4 percent dye in physiological saline, placed in the counting chamber of a hemocytometer, and the cells counted at $\times 100$ magnification. The stained (dead) cells and the unstained (living) cells can be counted separately to determine percentage viability if this is desired. Trypan blue and erythrosin B are the dyes most commonly used for dye exclusion tests. Others (e.g. methylene blue, acridine orange, eosin, nigrosin, and safranin) have also been used (Evans et al. 1975). Dyes, such as crystal violet, stain both living and dead cells and are best used to clarify morphology.

Cells can also be propagated by enzyme treatment, a chemical method of detaching adherent cells from the culture vessel with enzymes, such as trypsin, pronase, or collagenase, together with chelating agents, such as versene (tetrasodium ethylenediaminetetraacetic acid (EDTA)). After washing twice with phosphate-buffered saline (PBS), the monolayer is covered with a solution of 0.05 percent trypsin and 0.53 mM versene and placed at room temperature with cell surface down until the cells detach. After vigorously pipetting to break up clumps, the cells are resuspended at the desired concentration in fresh GM.

Cells in suspension cultures are propagated by adding a sample to sufficient GM to give the desired concentration.

For lymphocyte cultures, fresh mononuclear cells can be isolated from whole blood by Ficoll–Hypaque gradient centrifugation and cultured in suspension. Their uses for diagnosis, evaluation of immunity, and detection of disease are numerous, as are the procedures for isolating particular subpopulations of cells from peripheral blood or lymphoid tissues (Markham and Salahuddin 1987; Castro et al. 1988; Coligan and Kruisbeek 1991).

MAINTENANCE OF CULTURES

Once cells have been passaged, routine medium changes are necessary to encourage their viability by feeding them with fresh amino acids, vitamins, glucose, and other metabolic constituents. The initial cell concentration and the metabolic rate of the cells will determine the feeding schedule. Feeding is done with a maintenance medium (MM), which is the regular medium with reduced serum content (1–2 percent serum vs. 10–20 percent used in GM). MM may hold untransformed cell lines at a single cell layer for an extended period, even 2–3 weeks, because it tends not to stimulate mitosis. Diploid cells, with a finite lifespan, can thus be maintained for extended periods without using up the limited number of cell generations available to them.

Cells can also be maintained by subculturing, particularly important for transformed cells, which continue to divide under MM and thus cannot be successfully fed. Transformed (heteroploid) epithelial cells, such as HeLa and HEp-2, metabolize rapidly and should be subcultured twice a week. Alternatively, the seeding concentration of such rapidly proliferating cells may be lowered to reduce subculturing to once a week. Diploid fibroblast cells, such as MRC-5 and WI-38, metabolize slowly and require subculturing only once a week, with a feeding at least once during that week. Suspension cultures must be subcultured, or the volume of medium increased as the cell population reaches approximately 2×10^6 cells/ml, because the viability of suspended cells drops rapidly at higher concentrations.

STORAGE OF CELLS

Cells may be stored by cryopreservation, which provides a ready stock if any is lost through contamination, incubator malfunction, natural senescence, or a genetic drift that may affect their ability to exhibit the expected response in routine assays. Cells are packed by low-speed centrifugation and resuspended in a freeze medium containing serum and dimethylsulfoxide (DMSO) (Schmidt 1989; Mahy and Kangro 1996; Freshney 2000). One-ml volumes are dispensed into cryovials, frozen slowly at approximately 1°C per min, preferably in a controlled-rate freezer, and stored in liquid nitrogen (−165°C).

Cells are recovered from storage by rapidly thawing in a 37°C water bath, diluting the cells with the appropriate

medium, and placing them in a cell culture flask. The medium is replaced after 24 h to remove all traces of DMSO. The medium exchange for a suspension culture requires centrifugation and resuspension in fresh medium.

Tissue and organ cultures

Laboratory cultures of pieces of human embryonic lung, kidney, intestine, and other organs have proven essential to the discovery of many viruses, such as the respiratory coronaviruses and rhinoviruses. Organ cultures are explants of whole tissue (approximately 1 mm^3), so many cell types are present in their natural form, and feeding medium must be perfused through the explants to maintain viability. Once inoculated with a clinical specimen, virus may be detected by cessation of ciliary movement (in lung) or by specific tests for the viral products accumulated in the medium (McIntosh et al. 1967; Tyrrell and Blamire 1967; Caul and Egglestone 1977).

Embryonated eggs

The chick embryo is a versatile host system in diagnostic virology, although its use has been diminished by the development of alternate culture and direct detection methods. It is still vital to the isolation of some influenza viruses, and the size and appearance of pocks on the chorioallantoic membrane (CAM) afford a quick diagnostic criterion for poxviruses. The advantages of eggs include ease of handling, production of large amounts of infectious amniotic or allantoic fluids, and lack of antibody production by the host which might hinder virus identification. Disadvantages include the need to obtain sterile eggs of known age (certified germ-free eggs can be virtually unobtainable in some countries) and the need to eliminate all viable bacteria, yeasts, and mycoplasmas from the patient's specimen before inoculation.

Fertile eggs must be incubated at 37°C in 40–70 percent humidity and rotated about every 4 h to keep the embryo floating in the center of the egg. For influenza virus isolation in amniotic or allantoic cavities, 9- to 11-day-old eggs are needed. For poxvirus isolation in the CAM, 12-day-old eggs are best.

Laboratory animals

Many animal systems have been developed to serve as models of human infection and to produce high-titer antisera for diagnostic tests. Others have been developed for propagating viruses that do not grow in cell cultures. Suckling mice (see Gould and Clegg 1985) are required for the recovery of some coxsackie A enteroviruses, and suckling mice, suckling hamsters, rabbits, mosquitoes, or ticks are needed for some arboviruses. Primates are the most sensitive systems for the hepatitis viruses, with ducks, woodchucks, and mice as alternatives for hepatitis B virus and mice and piglets for hepatitis E virus. In addition, newborn mice or hamsters, young adult guinea-pigs, and primates are required for some of the biosafety level (BSL)-4 agents (e.g. filoviruses, arenaviruses). These highly specialized and dangerous systems are beyond the reach of most laboratories, and the reader should consult other works for details (Lennette et al. 1988, 1995; Isenberg 1992; Murray and Baron 1995).

Animals should be purchased from companies specializing in the breeding of laboratory animals, so that the strain of the species is documented, immunizations for known adventitious organisms are given, and the quality of care (housing, temperature, air filtraton, food, and water) are within the appropriate national guidelines for animal care. The strains of mice and guinea-pigs may be important; often, only a single strain is suitable for a particular virus. Housing of animals in the receiving laboratory is also critical, not only to conform with national regulations on the humane use of animals, but also to minimize the introduction of exogenous microorganisms and cross-contamination.

PREPARATION OF REAGENTS FOR VIRUS IDENTIFICATION

Preparation of viral antigens

Viral antigens may be structural or nonstructural proteins or glycoproteins produced during viral replication, and may be located internally in the virion or be part of the external capsid components. Many are produced in excess during replication and are released into the supernatant fluid as 'soluble' antigens. For some tests, the antigen may simply be the supernatant fluid from the cell culture in which the virus was propagated. For others, intact virus is required at the highest titers achievable, so several freeze–thaw or sonication cycles are applied to the virus culture to disrupt any remaining cells. Again, a particular viral product may need to be enriched in proportion to other products, but not purified in the biochemical sense. For still others, only a specific viral protein can be used in the test, so it must be purified, concentrated, and preserved after removing interfering viral products and medium components. Alternatively, it may be specifically produced by a eukaryotic or prokaryotic gene expression system.

The two most basic concepts are microbiological purity of the starting viral culture and laboratory safety. For purity, starting cultures and final virus products should be proven free of viable bacterial, fungal, and mycoplasmal contamination by appropriate culturing in thioglycolate broth, trypticase soy broth, neopeptone infusion agar streak/pour plates with 5 percent

defibrinated sheep blood, Sabouraud glucose agar slants for yeasts, and Hayflick's biphasic medium for mycoplasma. Depending on the test and the day-to-day usage, many antigens can be preserved by adding thiomersal (thimerosal) or sodium azide to the final product to final concentrations of 0.01 and 0.1 percent, respectively. For safety, the routine laboratory precautions discussed in The laboratory diagnosis of viral infections, must be followed throughout.

For every virus antigen, a parallel 'dummy antigen' should be prepared as a negative control. Dummy antigens are prepared by sham-inoculating cell cultures, eggs, or animals with normal medium and thereafter treating them exactly as for the active preparation.

RELEASING VIRUS FROM CULTURE CELLS

Starting with the highest titers of infectious virus achievable is essential for antigen preparations. Freeze–thaw cycles, sonication and chemical treatments are the most widely used methods for liberating virus from the host cells and for disrupting virions to expose particular antigens (Mahy and Kangro 1996).

For freeze–thawing, cultures with maximal CPE are frozen at −70°C or below, then thawed to a slush, and agitated to dislodge the cells. The procedure is repeated twice and the final material is clarified by low-speed centrifugation.

For sonication, either the cultures may be freeze–thawed but not centrifuged, or the cells may be scraped off the vessel surface with a rubber 'policeman.' The energy level and time are determined in pilot tests; 30–70 watts output for 1–3 min with multiple pulses per minute will generally disrupt the cells without denaturing the antigen. The sonicated material is clarified by low-speed centrifugation.

Chemical treatments may also be used. Alkaline glycine extraction exposes intracellular antigens useful for complement fixation (CF), indirect hemagglutination (IHA), and EIA for many viruses, particularly those that are membrane-associated (Mahy and Kangro 1996). Treating with trypsin or trypsin–versene, followed by sonicating and centrifuging, produces high-titer antigens useful for EIA and hemagglutination (HA) tests for many viruses. Antigens for use in immunofluorescence tests may be prepared from infected cells disaggregated with trypsin and washed.

VIRUS INACTIVATION PROCEDURES

Viruses can be inactivated to permit safe handling. Choice of inactivation strategy is dictated by the lability of the particular virus and the stability of the viral antigens. Three principal methods used for virus inactivation include sonication, β-propiolactone (BPL) treatment, and radiation (Mahy and Kangro 1996).

Sonication can disrupt virus particles and cleave macromolecules if sufficient energy is applied. The optimum power, number of cycles and length of time must be determined for each virus and test.

BPL treatment is widely used to inactivate viruses because it generally does not interfere with antigen test systems. Cultures are clarified by centrifugation and mixed with BPL in the presence of tris(hydroxymethyl)amino-methane (TRIS) buffer, the final BPL concentration and incubation time having been previously determined in pilot tests. They are then clarified again, adjusted to pH 7.3–7.4 as needed and preserved with thiomersal or sodium azide.

Radiation is a very effective means for inactivating viruses, but must be carried out by people trained and certified for such procedures. The clarified virus/antigen is exposed in an unbreakable container in an ice bath to a ^{60}Co γ source. The dosage required for complete inactivation of the virus (Table 1) is estimated from dose–response curves. The product is tested for residual infectivity and for potency (Gamble et al. 1980).

Other virus inactivation methods are useful for specific purposes, but should not be relied upon to insure complete inactivation for some viruses. For immunoperoxidase assays (IPA) and IFA, infected cells may be fixed with acetone for 10 min at 4°C, which stabilizes viral proteins by partial denaturation and preserves morphological structure and immunological reactivity. Alternatively, viruses can be inactivated with 10 percent formalin at pH 7.0; methanol or ethanol (4 min at 4°C); acetone/methanol 50:50 (Lennette et al. 1995); 80 percent acetone (Anderson et al. 1985); or photochemically with psoralen and related compounds (Hanson et al. 1978). Copper-catalyzed sodium ascorbate preserves CF and IHA antigens of herpes simplex virus (HSV) (White et al. 1986); certain detergents (e.g. 1 percent Nonidet P-40, 0.1–0.5 percent sodium lauryl sulfate, Tween-20, or Tween-80) can break up virus envelopes without destroying antigenic activity.

ANTIGEN EXTRACTION PROCEDURES

Extraction procedures can free antigens of nonspecific inhibitors, anticomplementary reactions, competing proteins and lipoproteins, and undesired reactions related to the viral host system. For example, HA anti-

Table 1 *Dosage of ^{60}Co γ-irradiation required for complete inactivation of viruses*

Mrad[a]	Viruses inactivated
0.5	Cytomegalovirus
1.5	Influenzavirus
1.6	Herpes simplex virus 1, 2
1.7	Respiratory syncytial virus
2.0	Newcastle disease virus, Rabies virus
2.2	Rotavirus
3.4	Lymphocytic choriomeningitis virus

a) Megarad, or 10^6 rad (rad, radiation absorbed dose).

gens must be free of serum antibodies, host-cell components and microbial contaminants that may agglutinate the erythrocytes used in the assay. Some antigens are enhanced in reactivity by treatment with freeze–thaw cycles, sonication, Tween-80, fluorocarbon, ether, or other organic solvents (Norrby 1962; Chappell et al. 1984). The Tween-80–ether extraction method is found in Mahy and Kangro (1996). The sucrose–acetone–BPL and fluorocarbon methods are briefly described here, by way of example. Alternatively, nonionic detergents, such as Nonidet P-40, high concentrations of salts in the presence of EDTA and β-mercaptoethanol, and other extracting agents can be used to prepare viral antigens (Isenberg 1992; Mahy and Kangro 1996).

The sucrose–acetone–BPL extraction method is useful for the CF and HA antigens of some viruses, including togaviruses (Venezuelan equine encephalomyelitis virus) and flaviviruses (Dengue virus, Japanese B encephalitis virus, Rocio virus, St. Louis encephalitis virus, Yellow fever virus and other formerly group B arboviruses) (Schmidt and Lennette 1971; Chappell et al. 1984). A 40 percent suspension of infected tissue (e.g. mouse brain) is homogenized in borate-saline, pH 9.0 in ice; 1 M TRIS/borate-saline equal to 1/10 the volume of suspension is added, followed by cold BPL drop-wise to a final concentration of 0.1–0.3 percent. After mixing for 1 day in the cold, an equal volume of 17 percent sucrose in distilled water is added, together with 20 volumes of cold acetone chilled to −20°C per volume of homogenate, still in ice and with mixing. After vigorous shaking the tissue is allowed to settle out at –20°C for 30 min, and the supernatant acetone is aspirated. The preparation is again extracted with acetone, keeping the 20:1 ratio. The antigen may be preserved best as a freeze-dried powder, but should be reconstituted in borate-saline and centrifuged at 10 000 *g* for 1 h before use.

For fluorocarbon extraction, the centrifuged starting material is placed in a stainless steel vessel in an ice bath for high-speed homogenization. Sufficient fluorocarbon (e.g. Genetron; CF_3CCl_3) is added to obtain a 2:1 ratio of antigen to fluorocarbon and the mixture is homogenized at full speed for 4–6 min. The aerosol is allowed to settle in the closed container for 1 h in ice. The suspension is then decanted into bottles or tubes for layer separation at 1000 *g* for 20 min at 4°C; the supernatant is the treated antigen (Gessler et al. 1956).

Viral antigens can be partially purified by adsorption/elution from erythrocytes or from calcium phosphate gels (Mahy and Kangro 1996). More effective purification is achieved by various types of solid-phase immunological adsorption/elution techniques and by affinity chromatography. Antigens may be immunoaffinity-purified by passing viral cultures (usually after SLS or enzyme treatment to disrupt virions and liberate proteins) through columns containing gel beds coated with specific antibody, or beds of monoclonal antibody-bound protein A–sepharose beads, etc. Other types of affinity chromatography use enzyme- or substrate-bound matrices. The reader is referred to other sources for detailed methods of affinity chromatography (Eisenberg et al. 1982; Wilchek et al. 1984; Chong and Gillam 1985; Harlow and Lane 1988).

SYNTHETIC AND EXPRESSED ANTIGENS

Polypeptides representing discrete viral antigens can be chemically synthesized for use in diagnostic assay and special studies. However, synthetic peptides are typically restricted to single linear epitopes that do not possess the secondary and tertiary structure of more complex antigens. Alternatively, various commercially available recombinant gene expression systems can be used to construct complex viral antigens that may be more suitable for viral diagnostic assays. Prokaryotic expression systems are convenient and inexpensive, but possess different processing pathways which prevent the amino terminal modifications, disulfide bond formation, and glycosylation required for many viral antigens to be active. Eukaryotic expression systems are more complex and expensive, but are closer to the natural mode of replication of the virus. Thus, post-translational modifications necessary for antigenicity are more likely to take place. Many viruses can serve as expression vectors in eukaryotic systems. For example, baculovirus is particularly useful because it can be 'engineered' to incorporate various tag genes which make it possible to purify and identify the virus or its expressed products. Once the gene for the desired antigen has been inserted into the recombinant baculovirus, the virus is grown at 27°C in SF9 or SF21 fall armyworm ovary cells and the desired antigen identified by IFA, EIA, or western blot. The reader is referred to other sources for the details and methods of gene expression systems (Smith and Johnson 1988; Bennink and Yewdell 1990; Vialard et al. 1990; Ausubel and Brent 1991; Jarvis 1991; Taylor et al. 1992; Cox et al. 1993; Alberts et al. 1994).

Preparation of high-titer antisera

High-titer antisera to viruses and viral antigens are readily produced in animals for use in diagnostic assays. Animal-derived antisera were the backbone of early virology, being widely used to distinguish between strains and serotypes and to study the antigenic make up of viruses. Polyclonal antisera of high titer ('hyperimmune sera') is prepared by repeated injection of virus or viral antigen into, for example, rabbits, mice, and guinea-pigs, or larger animals, such as goats and horses. The general procedure is to clarify the preparation by centrifugation, mix it with an equal volume of Freund's incomplete adjuvant or a synthetic muramyl dipeptide adjuvant, inject the mixture into suitable sites between two and four times biweekly, and obtain a blood sample

10–14 days after the final injection. The pre- and post-immunization sera are then tested for the desired antibody. Use of other adjuvants, injection in multiple sites with or without adjuvant, techniques to minimize the development of host-cell antibodies, and preparation of antigen/antibody complexes on solid matrices, such as staphylococcal protein A, are described in other sources (Hierholzer et al. 1991; Lennette et al. 1995; Murray and Baron 1995; Mahy and Kangro 1996).

Preparation of monoclonal antibodies

Monoclonal antibodies have become a vital immunological reagent since their inception in 1975 (Scharff et al. 1981; Peter and Baumgarten 1992; Lennette et al. 1995; Murray and Baron 1995). Many variations in their production have been described, including immunization in vitro and EBV-transformation techniques (Seigneurin et al. 1983; Boss 1986; Foung et al. 1986; Treanor et al. 1988). Suitable methods are described by Goding (1996).

ISOLATION AND IDENTIFICATION OF VIRUSES FROM CLINICAL SPECIMENS

Specimen collection and treatment

To avoid misleading results, it is of the utmost importance that adequate specimens are taken from sites appropriate to the clinical findings and then correctly stored and processed. The type of specimen and the manner of collection depend upon the laboratory methods to be used and on the sites clinically affected (Johnson 1990; Hierholzer 1991, 1993; Lennette et al. 1995). Specimens should ideally be collected within a few days of the onset of symptoms, because most viruses are shed only in the initial stages of illness. Swab specimens are placed in 2 ml of transport medium (e.g. tryptose phosphate broth with 0.5 percent gelatin, veal heart infusion broth or trypticase soy broth), preferably without antibiotics to allow the culture of bacteria and mycoplasmas. Nasopharyngeal aspirates are excellent specimens for respiratory viruses and are collected with a neonatal mucus extractor and mucus trap to which transport medium is added (Waris et al. 1990). According to the circumstances, other specimens may include urine, stool, whole blood, serum, cerebrospinal fluid, biopsy and autopsy specimens, and swabs or scrapings of vesicular lesions. Specimens are placed on wet ice and transported to the laboratory for immediate culturing. If testing is not possible within 5 days of collection, they are frozen on dry ice and stored at –70°C until they can be processed, although this may reduce the amount of viable virus. Before inoculation into the appropriate host system, specimens are treated with antibiotics, mixed vigorously, and clarified by centrifuging at 1000 *g* for 3 min at 4°C to remove cell debris and bacteria. Many viruses require one or more subpassages for recovery.

Procedures for isolating viruses

CELL CULTURES

Cell cultures should include a continuous human epithelial line (e.g. HEp-2 (Johnston and Siegel 1990); KB, A549 (Woods and Young 1988), HeLa); a human embryonic lung diploid fibroblast cell strain (e.g. HLF, HELF, MRC-5, WI-38); human lung mucoepidermoid cells (NCI-H292) to replace MK cells for most applications (Castells et al. 1990; Hierholzer et al. 1993b); and human rhabdomyosarcoma cells (RD) for the broadest coverage of viruses within practical limitations (Matthey et al. 1992). Treated specimens are adsorbed on to cell monolayers whose GM has been decanted. MM is added and cultures are incubated at 35–36°C up to several weeks, with subpassaging as required. Toxic specimens (urine, stool, tissue homogenates) should be decanted before MM is added.

Some cell types and certain viruses require roller cultures, whereas others do best as stationary cultures during their incubation period. In general, all tube cultures of MK, AGMK, and their derivative cell lines (Vero, BSC-1, LLC-MK2, etc.), all diploid fibroblast cell cultures, and NCI-H292 cells should be rolled in roller drums or agitated on rocker platforms to remove toxic byproducts from the cell surface and to replenish critical nutrients to the cells more quickly. Cultures of human embryonic kidney (HEK), HEp-2, KB, A549, and HeLa do not need to be rolled, except for the isolation of RSV and measles virus.

Small variations in media composition and incubation temperature are also important for the isolation of certain viruses. For instance, viruses may require prior treatment with trypsin (Almeida et al. 1978; Sanchez-Fauquier et al. 1994), or the cells may require trypsin in the MM to render them sensitive to certain viruses (Itoh et al. 1970; Frank et al. 1979; Agbalika et al. 1984; Castells et al. 1990). Cooler incubation temperatures (33–35°C) are best for less invasive viruses, such as coronaviruses, rhinoviruses, and the enteroviruses that cause hemorrhagic conjunctivitis. Finally, whenever MK or AGMK cells are used (e.g. for influenza, measles, or rubella), one should presume that simian adventitious agents are present and may confound the isolation and identification of human viruses (Hull 1968; Eberle and Hilliard 1995).

Viral growth is usually detected as CPE in the infected cell monolayer by scanning at ×40–100 magnification and observing in greater detail at ×200–400 magnification (Figure 1). The type of CPE and the time required for it to appear in a particular cell type are suggestive of the type of virus present. Thus, careful

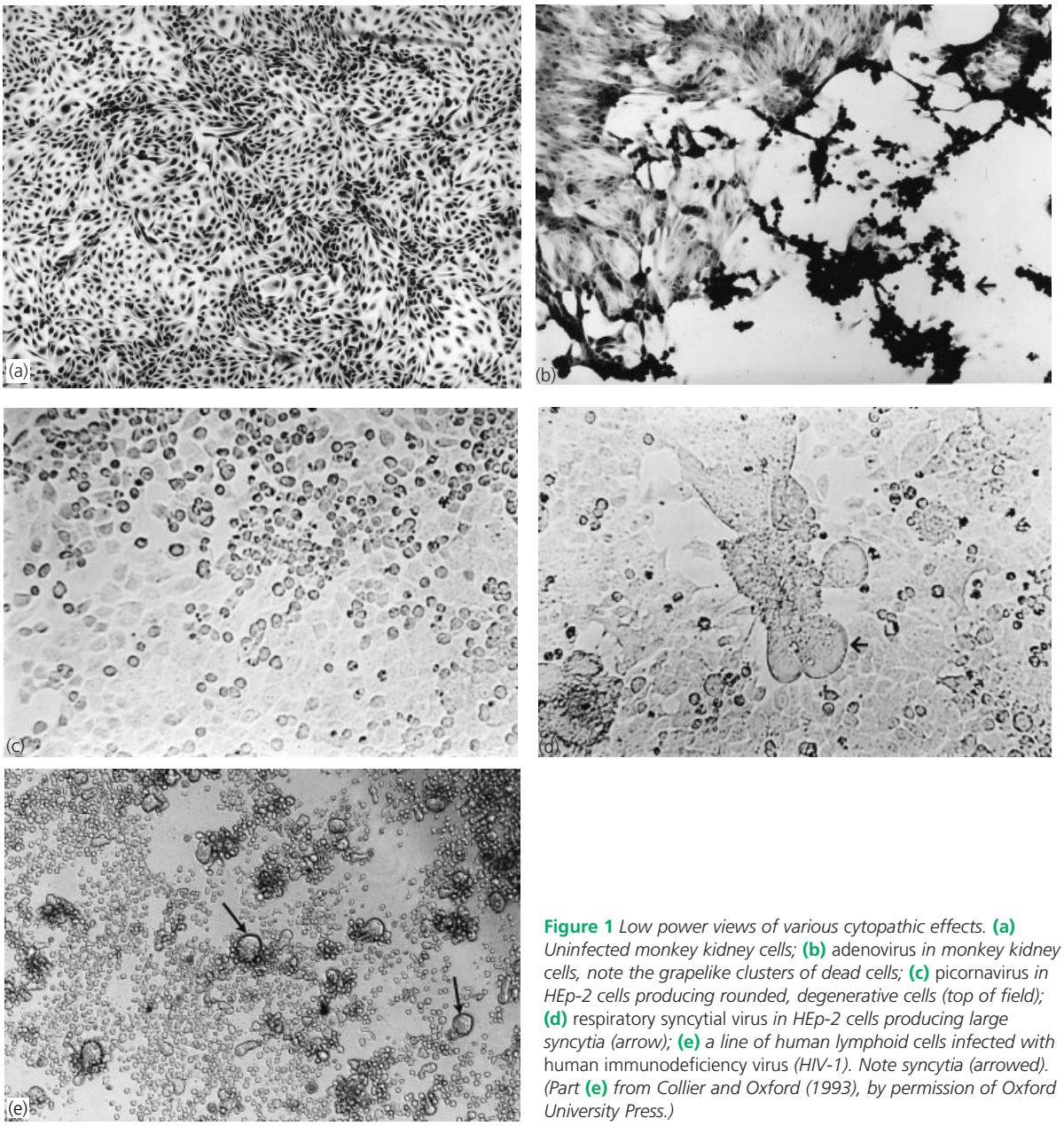

Figure 1 *Low power views of various cytopathic effects.* **(a)** *Uninfected monkey kidney cells;* **(b)** adenovirus *in monkey kidney cells, note the grapelike clusters of dead cells;* **(c)** picornavirus *in HEp-2 cells producing rounded, degenerative cells (top of field);* **(d)** respiratory syncytial virus *in HEp-2 cells producing large syncytia (arrow);* **(e)** *a line of human lymphoid cells infected with* human immunodeficiency virus *(HIV-1). Note syncytia (arrowed). (Part* **(e)** *from Collier and Oxford (1993), by permission of Oxford University Press.)*

observation of the monolayers is important for applying the proper identification tests.

EMBRYONATED EGGS

Except for influenza and poxviruses, chick embryos have been largely superseded by cell cultures for virus isolation and identification.

The amniotic route of inoculation is used mainly for primary isolation of influenza viruses, and the allantoic route for serial passages and for obtaining large quantities of virus. Embryos (approximated 10 days old) are inoculated by well-established methods and the fluids are harvested 2–4 days later.

The chorioallantoic membrane (CAM) method is used mainly for poxviruses. The membranes of 12-day-old embryos are inoculated through an opening in the shell about halfway along the long axis of the egg, the CAM having first been pulled away from the shell by applying negative pressure to a second hole drilled over the natural air sac. The CAM is inspected for the appearance of pocks after further incubation for 2–4 days.

For a detailed account of chick embryo methods, see Barrett and Inglis (1985) and Lennette et al. (1995).

LABORATORY ANIMALS

Like chick embryos, animals have largely been superseded by other systems, although they are occasionally

used for special purposes, such as the isolation and partial characterization of coxsackieviruses in suckling mice, which can be inoculated both intracerebrally and intraperitoneally (Gould and Clegg 1985).

For use of other animal systems, the reader is referred to chapters dealing with the relevant virus groups in this volume and elsewhere (Lennette et al. 1988, 1995; Murray and Baron 1995).

Recovery and identification of viruses

Table 2 summarizes the relative value of a range of methods for isolating and propagating human viruses. Further details may be found in Isenberg (1992), Lennette et al. (1995), and Murray and Baron (1995).

ADENOVIRUSES

Adenoviruses (see Adenoviruses) are associated with diverse clinical syndromes. They are spread by droplets, fomites, and the fecal–oral route; some serotypes are also spread venereally. Most serotypes replicate readily in HEK, A549, HEp-2, KB, and NCI-H292 cells, with or without rolling, producing grapelike clusters of round, refractile, enlarged cells. Adenoviruses grow in most of the same cells as herpes simplex viruses, but more slowly (3–14 days), depending on serotype, and make more discrete, irregularly shaped cell clusters. Adenoviruses can be adapted to replicate in fibroblast cells, but the CPE develops slowly and infectivity titers are low. Types 40 and 41, associated with infant gastroenteritis, grow best in an Ad5-transformed HEK cell line (Graham-293 cells) or in HEp-2 under a fortified Opti-MEM medium containing 0.4 percent fetal calf serum and 0.1 percent 2-mercaptoethanol. The adenoviruses are among the easier viruses to identify because they are unique in producing in cell culture very large quantities of soluble antigens with many type- and group-specific properties that lend themselves to a wide variety of diagnostic tests. Adenoviruses are distinguished from other viruses by electron microscopy (EM), latex agglutination (LA), IFA, IPA, EIA, time-resolved fluoroimmunoassay (TR-FIA) (Hierholzer et al. 1987; Brown et al. 1990), DNA probes, restriction enzyme analysis (REA) (Adrian et al. 1986), PCR assay (Hierholzer et al. 1993c), and cytological and inclusion-body staining methods. Adenoviruses are serotyped by HI and serum neutralization (SN) tests (Hierholzer 1992).

'ARBOVIRUSES'

The togaviruses (see Togaviruses), flaviviruses (see Flaviviruses), arenaviruses (Arenaviruses), rhabdoviruses, filoviruses (Filoviruses), and bunyaviruses (Bunyaviridae) contain most of the agents covered by the term 'arboviruses' and many rare and unusual viruses that cause disease in geographically distinct parts of the world. Arboviruses often have special isolation requirements. Certain arboviruses can be isolated in primary hamster kidney cells, primary chick or duck embryo cells, or derivative cell lines such as AP61 mosquito cells, BHK-21 hamster kidney cells, Vero (African green monkey kidney cells), and LLC-MK2 rhesus monkey kidney cells, after 2–10 days of culture. For example, the Sin Nombre hantavirus is a bunyavirus that can be propagated in the Vero E6 cell line after growth in deer mouse lungs (Elliott et al. 1994). The CPE may not be apparent at first, but becomes generalized in subpassages or forms plaques under agarose. Other arboviruses grow best in mosquito suspension-cell cultures at 20–30°C, in which replication is detected by subpassaging to monolayer cultures, in which CPE may be evident, or by HA/HI, EIA, IFA, CF, SN, or nucleic acid tests for the specific viruses suspected. Still other arboviruses can be recovered only in whole animal systems such as chick embryos, suckling mice or hamsters, or mosquitoes. Fortunately, most diagnostic laboratories will not encounter these viruses, either because of their rarity in most parts of the world or because of their requirement for BSL-3 or -4 containment facilities. The reader is therefore referred to other works that detail the isolation requirements and systems, the specific identification tests and the laboratory precautions for these viruses (Fields and Knipe 1990; Lennette et al. 1988, 1995; Murray and Baron 1995).

CORONAVIRUSES

The human coronaviruses (see Coronaviruses, toroviruses, and arteriviruses) cause a significant proportion of 'common colds' and are spread by droplet route. Because these viruses are extremely labile and difficult to recover by culture, they are best identified directly in nasal and throat specimens by IFA, EIA, TR-FIA, or reverse transcription (RT)-PCR. The viruses growing in diploid fibroblast cells (229E virus) or in RD cells (OC43 virus) do not cause discernible CPE during their replication phase. Only when viral replication is complete and the virus titer has peaked (at approximately 10^7 tissue culture infective doses ($TCID_{50}$/0.1 ml), after 26–30 h of roller incubation, do changes begin to be evident in the monolayer. For the next several days, CPE develops as a uniform degeneration across the monolayer. The CPE becomes complete concomitant with autolysis of the newly formed virions. By this time, little infectious virus is left in the culture (Hierholzer 1976; Schmidt et al. 1979). The peplomers constitute the primary antigen detected in all tests, including the HI test for strain OC43 (Schmidt et al. 1979; Hierholzer et al. 1994a). Alternatively, the viral RNA can be identified by RT-PCR (Vabret et al. 2001).

The recently identified severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV)

Table 2 *Isolation and propagation systems for human viruses*

	Cell culture						Embryonated eggs		Laboratory animals		
Virus family/group	**Primary**	**Epithelial**	**Fibroblast**	**Vero**	**Other**	**Organ culture**	**Amn/All**	**CAM**	**Rodents**	**Primates**	**Other**
Adenoviridae											
Adenovirus 1-51	+	+	±	+	+	+	–	–	–	–	–
Arenaviridae											
LCM, Lassa virus, others	–	–	±	+	±	–	–	–	+	+	–
Astroviridae											
Astrovirus	±	±	–	–	–	–	–	–	–	–	–
Bunyaviridae											
CE, La Crosse virus	±	–	–	±	+	–	+	+	+	±	+
Hantaviruses	–	–	–	±	–	–	–	–	+	–	–
Other arboviruses	±	–	–	±	+	–	+	+	+	±	+
Caliciviridae											
Norovirus, Sapovirus	–	–	–	–	–	–	–	–	–	±	–
Hepatitis E virus	±	±	–	–	–	–	–	–	+	+	+
Coronaviridae											
Coronaviruses 229E, OC43	±	–	±	–	+	+	–	–	–	–	–
SARS associated coronavirus	+	–	–	+	–	–	–	–	+	+	–
Filoviridae											
Marburg virus, Ebola virus	–	–	±	+	+	–	–	–	+	+	–
Flaviviridae											
Dengue viruses 1–4, JE virus, SLE virus, West Nile virus, Yellow Fever virus, and other arboviruses	+	–	–	+	+	–	+	+	+	±	+
Hepatitis C virus	–	–	–	–	–	–	–	–	–	+	–
Hepadnaviridae											
Hepatitis B virus	±	–	–	–	±	–	–	–	±	+	+
Herpesviridae											
Herpes simplex 1, 2	+	+	+	+	+	+	–	+	+	–	–
Human herpes 6A, 6B, 7, 8	+	–	–	–	–	–	–	–	–	–	–
Varicella-zoster virus (VZV)	+	+	+	±	±	–	–	–	–	–	–
Cytomegalovirus (CMV)	–	–	+	–	–	–	–	–	–	–	–
Epstein–Barr virus (EBV)	–	–	–	–	+	–	–	–	–	–	–
Orthomyxoviridae											
Influenza A, B, C viruses	+	–	–	–	+	+	+	–	±	–	+
Papovaviridae											
Papillomaviruses	–	–	–	–	–	–	–	–	–	–	–
Polyomaviruses BK, JC	+	–	±	±	–	–	–	–	–	–	–

(*Continued over*)

Table 2 *Isolation and propagation systems for human viruses (Continued)*

	Cell culture						Embryonated eggs		Laboratory animals		
Virus family/group	**Primary**	**Epithelial**	**Fibroblast**	**Vero**	**Other**	**Organ culture**	**Amn/All**	**CAM**	**Rodents**	**Primates**	**Other**
Paramyxoviridae											
Parainfluenza viruses 1–3, 4A, 4B	+	+	−	±	+	±	±	−	±	−	+
Mumps virus	+	±	−	+	+	+	+	−	+	±	−
Measles virus	+	−	±	+	+	−	−	−	−	+	−
Respiratory syncytial virus (RSV)	+	+	±	±	+	+	−	−	+	+	+
Human metapneumovirus	+	+	−	+	−	−	−	−	+	+	+
Parvoviridae											
Parvovirus B19	−	−	−	−	±	±	−	−	−	−	−
Picornaviridae											
Polioviruses 1, 2, 3	+	+	+	+	+	+	−	−	−	+	−
Coxsackie A virus (23 types)	±	−	±	−	+	±	−	−	+	±	−
Coxsackie B virus (6 types)	+	+	+	+	+	+	−	−	+	−	−
Echovirus (31 types)	+	±	±	±	+	+	−	−	−	−	−
Enterovirus 68-71	+	±	±	±	+	−	−	−	−	±	−
Hepatitis A virus	±	−	±	±	±	−	−	−	−	+	−
Rhinoviruses (>100 types)	+	±	+	−	+	+	−	−	−	+	−
Poxviridae											
Vaccinia virus	+	+	+	+	+	−	−	+	+	−	+
Smallpox virus	+	+	+	+	+	−	−	+	−	+	−
Parapoxviruses	+	−	±	−	+	−	−	−	−	−	+
Molluscum contagiosum virus	−	−	−	−	±	−	−	−	−	−	−
Reoviridae											
Reovirus 1, 2, 3	+	±	±	±	+	+	−	−	+	+	+
Colorado tick fever virus	−	−	−	−	±	−	−	−	+	−	−
Rotaviruses	+	−	−	−	+	−	−	−	−	±	±
Retroviridae											
HIV, HTLV	−	−	−	−	+	−	−	−	−	±	−
Rhabdoviridae											
Rabies virus	+	−	−	±	+	−	±	±	+	+	+
VSV, other arboviruses	±	−	−	±	+	−	±	+	+	+	+
Togaviridae											
Sindbis virus, EEE virus, WEE virus, Semliki Forest virus, and other arboviruses	+	−	−	+	+	−	+	+	+	±	+
Rubella virus	+	−	−	+	+	−	−	−	±	±	±

+, Suitable for growth of virus; ±, partially suitable, i.e. some strains do not grow, or titers are low; −, unsuitable; Amn., amniotic cavity; All., allantoic cavity; CAM, chorioallantoic membrane; CE, California encephalitis; EEE, Eastern equine encephalitis; Embry., embryonated; JE, Japanese encephalitis; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia/lymphoma virus; LCM, lymphocytic choriomeningitis; SARS, severe acute respiratory syndrome; SLE, St. Louis encephalitis; VSV, vesiculovirus; WEE, Western equine encephalitis.

is distinct from the other human coronaviruses in that it readily grows in MK, Vero, or Vero E6 cells, but does not grow or grows poorly in most other cell lines (Ksiazek et al. 2003). CPE is focal, with cells giving a rounded, refractile appearance, and is rapidly progressive, leading to detachment of the cell monolayer. Because culture requires BSL-3 procedures and facilities, diagnosis of SARS coronavirus infection is best made by serology (EIA, IFA) or detection of viral RNA by RT-PCR assay.

GASTROENTERITIS VIRUSES

These viruses (see Human enteric RNA viruses: Astroviruses; and Reoviruses, Orbiviruses, and Coltiviruses) also belong to diverse families and have unique requirements for culture. Rotaviruses will replicate in BSC-1, MA104, and AGMK cells under roller conditions with trypsin treatment. Astroviruses replicate in human CaCo-2 and PLC/PRF/5 cells under a fortified medium with trypsin (Taylor et al. 1997; Brinker et al. 2000). The noroviruses and sapoviruses cannot be isolated in the laboratory (Koopmans and Horzinek 1994; Lennette et al. 1995). The gastroenteritis viruses can be distinguished by EM, EIA, and RT-PCR (Erdman et al. 1989; Coulson et al. 1999; Rabenau et al. 2003).

HEPATITIS VIRUSES

Hepatitis A virus, a picornavirus (see Hepatitis A and E), replicates in MK cultures in 1–4 weeks, but is noncytolytic. It is detected by EIA or RT-PCR. The hepatitis B virus, hepatitis C virus, hepatitis D virus, and hepatitis E virus do not grow in routine cell cultures and must be diagnosed by antigen (EIA or TR-FIA), or nucleic acid detection assays (Siitari et al. 1983; Balayan 1993; Wilber 1993; Kurstak et al. 1995).

HERPESVIRUSES

The herpesviruses are spread by aerosolized droplets, fomites, and direct contact, and cause a wide range of ocular, oropharyngeal, genital, and generalized disease in humans. They have three types of CPE in cell culture. HSV-1 and -2 grow well in many cell types, producing grapelike clusters of round, refractile, enlarged cells often identical to adenovirus CPE in HEK, HEp-2, KB, HeLa, A549, HLF, NCI-H292, primary rabbit kidney, and other cells. At complete CPE, all cells become lysed and detached from the vessel surface. HSV-1 grows fast (1–2 days), makes fewer clusters, and may produce ballooned, multinucleate giant cells with granulated cytoplasm. HSV-2, usually a genital isolate, grows more slowly than type 1. HSV does not require roller cultures, but HEp-2, NCI-H292, and fibroblast cells will probably be on roller or rocker apparatus for the benefit of other viruses to which these cells are susceptible.

CMV is notably labile. The only herpesvirus that is shed in great amounts in the urine, it replicates slowly (12–30 days) in roller cultures of diploid fibroblast cells. CMV produces giant-cell CPE resembling elongated foci of refractile, swollen cells, somewhat akin to measles CPE.

Varicella-zoster virus (VZV), the cause of chickenpox and herpes zoster (shingles), also grows slowly in fibroblasts on roller culture. It produces a CPE characterized by enlarged, round, glassy cells in small foci in 2–10 days, which is thus distinct from that caused by CMV. VZV can produce large foci of multinucleate giant cells when the individual foci coalesce.

Epstein–Barr virus (EBV) is associated with infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma; human herpesvirus 6 (HHV-6), the cause of roseola infantum (exanthema subitum or 'fourth disease') in children; human herpesvirus 7 (HHV-7), the probable cause of some roseola cases; and human herpesvirus 8 (HHV-8), the cause of AIDS-associated Kaposi's sarcoma (Chang et al. 1994). EBV requires special conditions and cells for successful cultivation in the laboratory. In culture, it infects both B lymphocytes and epithelial cells with the CD21 receptor; HHV-6 grows best in primary $CD4^+$ T lymphocytes rendered more susceptible by the presence of antibody to CD3 (Pellett et al. 1992; Yasukawa et al. 1993; Hall et al. 1994); HHV-7 also replicates in $CD4^+$ T-lymphocytes (Black and Pellett 1993; Lusso et al. 1994; Tanaka et al. 1994). HHV-8 has been difficult to isolate from cell culture, although limited infections of CD19 B lymphocytes, macrophages, and certain endothelial cells has been demonstrated (Blackbourn et al. 2000).

Herpesviruses are readily visualized by EM, the icosahedral nucleocapsid and the baggy envelope being prominent. The viruses are commonly speciated by IFA, EIA, LA, and SN tests and by DNA hybridization and PCR assays (Buller et al. 1992; Drew 1992; Perez et al. 1995).

ORTHOMYXOVIRUSES

Influenza A, B, and C viruses (see Orthomyxoviruses: influenza) are spread by aerosol droplets and fomites, and are best recovered in roller cultures of primary MK and MDCK cells and in intact chick embryos. Chick embryo cells, MDCK, and other cells require rolling and a fortified medium containing trypsin 2 μg/ml for optimal sensitivity. The CPE may be seen as a combination of syncytia, rounding, and degeneration. The syncytia caused by influenza B virus may be accompanied by vacuolation. Influenza CPE may develop in 4–7 days, but cultures should be blind-passaged and held an additional week. Because the cells rarely become detached and may not have an obvious CPE, virus detection in MK cultures is verified by hemadsorption with chicken or guinea-pig erythrocytes, and in MDCK

cells by HA. Some strains of influenza A and B can be isolated only in 9- to 11-day-old chick embryos. The viruses are then typed by HI, IFA, EIA, TR-FIA or RT-PCR assay (Frank et al. 1979; Grandien et al. 1985; Walls et al. 1986; Bucher et al. 1991; Ziegler et al. 1995; Stockton et al. 1998).

Hemadsorption is a fast and convenient method for detecting orthomyxoviruses (influenza A, B, C) and non-RSV paramyxoviruses (parainfluenza 1, 2, 3, 4A, 4B; mumps; measles) in cell cultures. It is even used in the presence of CPE to obtain a quick distinction from other virus groups that may cause similar CPE in the same types of cultures. The supernatant fluid from the cell culture is decanted into a sterile tube at the end of the incubation period (7–10 days), to conserve virus and viral antigens for passaging and testing. The monolayer is washed twice with 2–3 ml of plain Hank's balanced salt solution (HBSS) at room temperature; 1 ml of fresh HBSS is added, followed by 0.2 ml of 0.4 percent mammalian erythrocyte suspension from the appropriate species. The tube is incubated stationary with the fluid covering the monolayer. The test is read three times at 20-min intervals by agitating the tube in a sideways motion and then observing at ×40–100 magnification for erythrocytes firmly attached to the cultured cells (positive) or floating free in the fluid (negative).

PAPOVAVIRUSES

The papovaviruses (see Papillomavirus; and 24, Polyomaviruses) consist of two genera (*Papillomavirus* and *Polyomavirus*), but only *Polyomavirus* can be isolated in cell culture. The BK polyomavirus can be recovered in NCI-H292 and diploid fibroblast cells, and the JC polyomavirus replicates in primary human fetal glial cells, but both viruses may be missed because their CPE develops slowly and may be ill-defined. It is a granular, degenerative, slowly lytic CPE, similar to that caused by reoviruses. BK virus is identified by hemagglutination inhibition (HI) tests at 4°C with human 'O' erythrocytes and specific antiserum; BK and JC viruses are identified by EIA, SN, hybridization probes or PCR assay. The other genus, papillomaviruses, cannot be propagated in cell culture so are identified in biopsied tissues by antipeptide EIAs and by hybridization probes and PCR (Eklund and Dillner 1995; Ylitalo et al. 1995).

PARAMYXOVIRUSES

The human paramyxoviruses (General properties of the paramyxoviruses) are parainfluenza types 1, 2, 3, 4A, 4B, mumps, measles (rubeola), RSV and the recently identified human metapneumovirus (hMPV) (van den Hoogen et al. 2001). The parainfluenza and mumps viruses replicate well in roller cultures of NCI-H292 cells under a fortified medium containing trypsin 1.5 μg/ml for optimal sensitivity, and in primary MK cells without trypsin (Meguro et al. 1979; Castells et al. 1990). The CPE induced by these viruses may develop in 4–7 days, and is a combination of syncytia and rounding. The syncytia may be accompanied by a granular degeneration with parainfluenzavirus types 1–4. Mumps virus often gives rise to large syncytia. The cultures must generally be blind-passaged and held an additional week to ensure viral growth. The cells rarely become detached, and may not show obvious CPE at all, so NCI-H292 or MK cultures for these viruses must be hemadsorbed with guinea-pig, human, or monkey erythrocytes at the end of the culture period and then typed by HI, IFA, EIA, TR-FIA or RT-PCR assay (Grandien et al., 1985; Van Tiel et al. 1988; Hierholzer et al. 1989, 1993a; Takimoto et al. 1991; Echevarria et al. 1998).

Measles virus can be grown in a wide range of cell culture systems, but is most successfully isolated in the Epstein–Barr virus-transformed marmoset B lymphoblastoid cell line, B95a (Kobune et al. 1990). CPE is characterized by presence of classical multinucleate giant cells resulting from fusion of adjacent cells. Because the CPE may develop slowly and not be recognized, the monolayers should be screened to ensure detection of the virus. Measles can be identified by HI tests with vervet monkey erythrocytes, IFA, EIA, SN, or RT-PCR assay (Hummel et al. 1992; Nakayama et al. 1995).

RSV produces a distinct syncytial CPE in HEp-2, HeLa, and NCI-H292 cells in 5–12 days in roller cultures. The balled-up syncytia usually become detached from the glass surface and float freely in the medium. Some strains of RSV, particularly of group B, produce more cellular degeneration than syncytia in HEp-2 and NCI-H292 cells. Group A and B strains of RSV are readily identified by IFA, EIA, TR-FIA, or RT-PCR (Freymuth et al. 1991; Waris 1991; Cane et al. 1992; Hierholzer et al. 1994b). Details on paramyxovirus typing are found elsewhere (Popow-Kraupp et al. 1986; Ahluwalia et al. 1987; Akerlind et al. 1988; Anderson et al. 1991).

hMPV produces a delayed cellular degeneration with round and refractile cells and occasional syncytial formation in tertiary MK and LLC-MK2 cells supplemented with 1.5 μg/ml trypsin. The appearance of CPE can take up to 2 to 3 weeks from inoculation of the specimen, or may not be recognized, and therefore cultures should be screened to ensure detection of the virus. HMPV is identified by RT-PCR assay (Boivin et al. 2002; Falsey et al. 2003).

PARVOVIRUSES

The human parvovirus B19 (see Parvoviruses) causes 'fifth disease' (erythema infectiosum) and is easily spread by aerosolized droplets and fomites in schools, and vertically to the fetus during the first trimester of pregnancy (see Virus infections in

immunocompromised patients). The virus can be grown in specialized erythropoietic stem cell cultures, but not in standard culture systems. Therefore, B19 infection is established by EIA testing of the patient's acute-phase serum for antiviral IgM antibodies or by PCR assay for viral DNA (Heegaard and Brown 2002).

PICORNAVIRUSES

This large family (see Picornaviruses) includes the enteroviruses (poliovirus 1–3, 23 coxsackie A viruses, six coxsackie B viruses, 31 echoviruses, and five more recent enteroviruses), and the rhinoviruses (approximately 125 serotypes). The enteroviruses are spread by aerosolized droplets, fomites, and the fecal–oral route, whereas the rhinoviruses are spread by aerosols and fomites only (Agbalika et al. 1984; Dick et al. 1987; Mandell et al. 1990). These viruses produce a 'shrunken cell degeneration' CPE in NCI-H292, MK, RD, trypsin-treated MA-104 and diploid fibroblast cells, preferably in roller cultures, although some coxsackie A viruses grow only in the brain of suckling mice. The CPE is often observed as tadpole-shaped, shrunken cells with pyknotic nuclei, beginning in patches at the edges of the monolayer and progressing inward. CPE for polioviruses and some coxsackie B viruses is quite rapid, becoming complete in 1–3 days with all cells detached from the glass. CPE for the remaining enteroviruses and the rhinoviruses generally requires 4–7 days or longer, is often characterized by individual small, rounded, and sometimes refractile cells or by a degenerative appearance across the monolayer, and may never become complete. Subpassaging is helpful. The variable CPE found in enterovirus- or rhinovirus-infected cells is not diagnostically confusing, however, because these viruses do not hemadsorb, rarely hemagglutinate with any species of erythrocytes, and do not interfere with test systems for other viruses. Enteroviruses are distinguished from rhinoviruses by acid- and chloroform-lability tests in which both genera are chloroform-stable, but only enteroviruses are acid-stable, or by group-specific IFA or RT-PCR assay (Hyypia and Stanway 1993; Egger et al. 1995; Halonen et al. 1995; Lipson et al. 2001). The viruses can be serotyped by type-specific SN tests.

POXVIRUSES

Vaccinia and certain other poxviruses (see Poxviruses) can be isolated in MK, Vero, NCI-H292, and diploid fibroblast cells. Their CPE is characterized as cell fusion with plaques. Plaques ranging from 1 to 6 mm in diameter (depending on the virus) are formed in 2–4 days, during which the infected cells fuse, form cytoplasmic bridging, and then disintegrate. Some poxviruses produce plaques on the CAM of 12-day chick embryos. Identification is accomplished from the type and size of plaques formed and by EM, HI, EIA, IFA, REA, and PCR.

REOVIRUSES

Reoviruses 1–3 can be isolated from throat and rectal specimens in NCI-H292, MK, and HeLa cells under stationary or roller conditions after 5–14 days of culture, but, unlike other members of the family *Reoviridae* (see Reoviruses, Orbiviruses and Coltiviruses), have never been clearly associated with human disease. In cell culture they cause a slow lytic degeneration with granulation of the cytoplasm, similar to polyomavirus CPE. MK cultures should be rolled. Reoviruses are easily visualized by EM and serotyped by SN and HI tests with human type 'O' erythrocytes.

RETROVIRUSES

The HIV and HTLV viruses (see Prions of humans and animals) also require specialized systems for isolation and identification, and will not be recovered by standard culture methods. They are identified by EIA, western blot, or RT-PCR assay (Isenberg 1992; Persing and Smith 1993; Signoret et al. 1993).

RUBELLA

Rubella virus, a togavirus (see Rubella), was originally detected because of its ability to prevent another virus from infecting the cells in which it was replicating. Current diagnostic testing for rubella may employ specialized cultures in which CPE may become evident. Rubella can, under certain conditions, cause an ill-defined and variable CPE in rabbit cornea, rabbit kidney, and Vero cells. Usually, however, rubella is detected by the interference test, in which replication of another virus (e.g. echovirus 11 or coxsackie virus A9) is inhibited by prior inoculation of the test material. A positive result may be confirmed by HI, EIA, LA, TR-FIA, or RT-PCR assay (Shankaran et al. 1990).

The interpretation of any culture isolation result or of any test used to identify the isolate depends on the methods employed, but viral growth alone should never be considered pathognomonic. The association of an isolate with disease depends on the clinical data obtained with the specimen and on the known epidemiology of the virus. Thus, careful virus isolation and identification test results, coupled with sound epidemiological data are the key to accurate diagnosis of viral infections.

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The replication of viruses

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INTRODUCTION

An essential feature of all viruses is that they are obligate intracellular parasites and are biochemically inert in the extracellular environment. They have no mechanisms for energy generation, protein translation, or a host of other complex biochemical processes that are essential for the maintenance and growth of living cells. Consequently they are totally dependent on the structure and function of cellular machinery to enable the macromolecular synthesis required during the reproductive phase of their life cycles.

Because they co-opt cellular structures and functions to facilitate their own replication, viruses survive successfully with small genome sizes. The number of genes encoded within different viruses ranges from one in the smallest to a few hundred in the largest and most complex. With the largest viruses many of their genes are not required for replication per se, but are involved in the avoidance of host immune defense mechanisms.

From the perspective of their genetic organization, viruses are the most varied organisms in the biosphere. In addition to the wide range in genome size exhibited by different viruses, every conceivable means of nucleic acid genetic storage mechanism seems to have been adopted within the virological spectrum. Viral genomes can be encoded by DNA or RNA, which can be single-stranded or double-stranded, of positive or negative polarity, and comprise single or multiple components. In addition, retroviruses and hepadnaviruses alternate between DNA and RNA as the carrier of their genetic information at different stages in their life cycles.

Despite the wide variation evidenced between viruses, there are a number of features that are common to the replication of all. To be rescued from their inert state in the environment it is essential that viruses can efficiently recognize and bind to appropriate host cells. Having achieved this specific association, the virus must have mechanisms to enable the genome to be introduced to the interior of the cell. This requires breaching cellular membranes and is achieved by markedly different mechanisms in different viruses. Once internalized into the cytoplasm or nucleus, macromolecular synthesis is initiated from the viral template. In most cases the first event is the synthesis of mRNA, although in some RNA viruses the genomic RNA can act directly as mRNA. The mRNA is transcribed by host or viral enzymes, depending on the virus type, and is usually post-transcriptionally modified, again by host or viral enzymes. Viral protein synthesis is entirely dependent on host-cell machinery, although many viruses have mechanisms to subvert the process to their own advantage. The viral protein products are frequently post-translationally modified, again by host or virally encoded enzymes. Virus genome replication frequently occurs in discrete compartments within the cell that are assembled during the infectious process and often involve massive reorganization of the intracellular structure. The assembly of new virus progeny is the penultimate stage of virus replication. It can occur by the spontaneous association of the structural components of the virus in a relatively simple mass action process or, in the case of the larger viruses such as herpesviruses, may involve complex multistep pathways requiring the assembly of

templates constructed from proteins that are not incorporated into the mature virus particles. Finally, the progeny virus particles must be released from the infected cell. This can occur by a variety of methods ranging from destructive lysis of the cell to the budding and release of new particles at the cell surface.

As an essential accompaniment to the replication process, many viruses utilize a range of mechanisms to divert or inactivate the innate and adaptive immune responses which would otherwise terminate viral replication either directly, or indirectly through the destruction of the infected cell. In the smaller viruses, these functions are often associated with multifunctional proteins that are also involved in viral replication. With the larger DNA viruses a significant proportion of the genome is devoted to the encoding of a wide range of proteins whose roles are specifically concerned with diverting the immune response mechanisms.

INITIATION OF INFECTION

The roles of virus particles

The essential roles of the virus particle, or virion, are to protect the viral genome from damage in the extracellular and extrahost environments, to efficiently bind to new host cells and to facilitate the delivery of the genome into the new host cell. These requirements place important and sometimes apparently conflicting demands on the structure and function of the virion. To protect the genome the particle must resist, as far as possible, the chemical and physical insults it is likely to encounter in the extrahost environment. It must, therefore, be a stable structure. However, this stability must be reversed during the infection process to allow release of the genome and its delivery into the new cell. In order to accommodate these diverse functions, triggering systems are frequently built into virus particles such that conformational changes occur during the initiation of infection that convert the virion from a protective capsule into an efficient delivery machine. Although these principles apply widely across the virological spectrum, there is great variety in the ways in which they are achieved.

Virus attachment

The first event in the infection process is the attachment of the virus particle to a receptor molecule at the surface of the new host cell. This involves the specific recognition between specialized receptor-binding domains on the virion surface and receptor molecules on the cell. In many cases the specificity of this interaction is a major determinant of cell tropism and can, therefore, be an important factor in defining the pathogenesis of infection. Many cell surface molecules are utilized as receptors for different viruses. In some cases viruses bind to rather nonspecific features of host-cell envelope proteins, for example, sialic acid residues. In other cases the virus/host cell receptor interaction is highly specific and is a major determinant of host and tissue tropism.

For enveloped viruses the first receptor binding protein to be studied in atomic detail was the hemagglutinin (HA) protein of influenza virus (Wiley and Skehel 1987). It is a type 1 envelope protein with a large, highly glycosylated ectodomain exposed at the surface of the virus particle, a single transmembrane domain and a short C terminal domain located on the inside of the viral envelope. The monomeric HA protein trimerizes to form functional spikelike projections from the surface of the virus (Figure 1). Each trimer has a stalklike domain adjacent to the membrane and terminates in a globular domain. This globular head contains the receptor binding pockets, three per HA trimer, which engage sialic acid moieties on cell receptor glycoproteins. The specificity for sialic acid is defined by the amino-acid side chains lining the receptor-binding pocket. Although these amino acid residues are necessarily highly conserved across the influenza subtypes, their location within a pocket renders them invisible to antibody recognition. This feature allows the virus to evolve variants able to escape immune responses directed against antigenic sites distributed around the globular domain of HA, while retaining the ability to utilize sialylated host-receptor glycoproteins. A second envelope glycoprotein of influenza virus is the neuraminidase protein. This removes sialic acid residues from host glycoproteins and is important for the release of progeny virus from infected cells which would otherwise be sequestered to membrane surfaces via their HA proteins.

Another virus receptor-binding protein that has received much attention is that of the human immunodeficiency virus (HIV). As for influenza virus, the HIV receptor-binding protein is a type 1 membrane glycoprotein which binds to a 60-kDa envelope glycoprotein, CD4, present at the surfaces of helper T cells and macrophages. The extreme specificity of this recognition process is one of the major factors responsible for the restriction of HIV infection to the higher primates. Although CD4 acts as the primary receptor for HIV, secondary receptors, the chemokine receptors, CXCR4 and CCR5, are engaged post-CD4 binding and are essential for completion of the infection process. Adaptation to either of these coreceptors dictates the cell tropism of different strains of the virus.

Utilization of multiple receptors during the infection process is seen in a wide range of viruses. Several viruses, herpesviruses for example, bind to heparin sulfate found ubiquitously linked to gangliosides at cell surfaces. This nonspecific initial binding probably functions as a means to concentrate virus particles at cell surfaces prior to engagement of more specific receptors

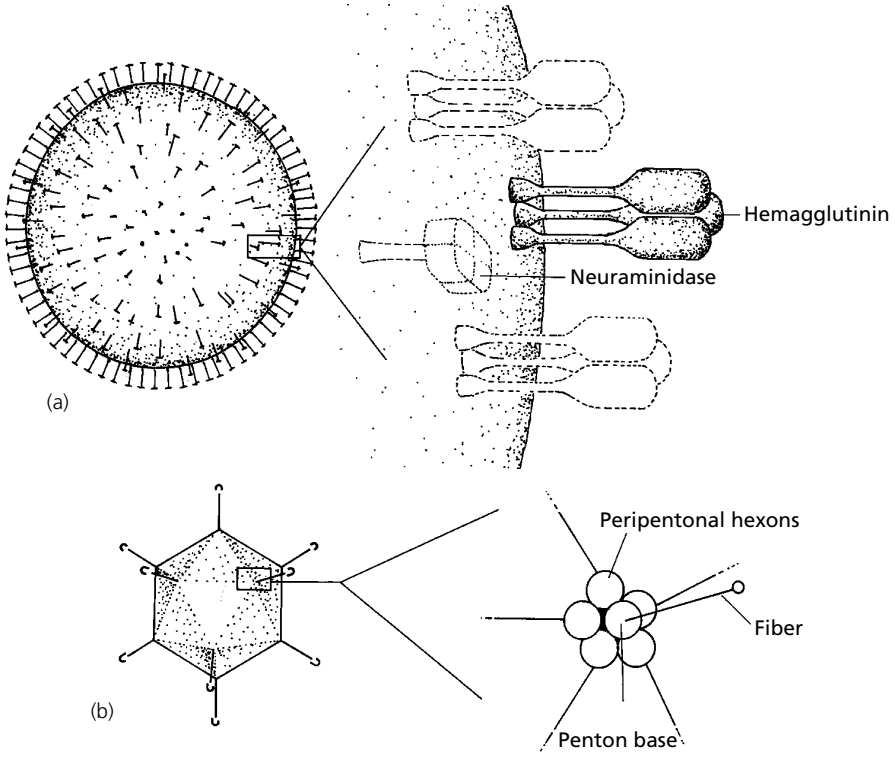

Figure 1 *Attachment of viruses to host cells.* **(a)** *Influenza is shown as a typical example of an enveloped virus. The virus membrane is decorated with the trimeric hemagglutinin protein (HA) and the tetrameric neuraminidase protein (N). The cellular receptor binding region of HA is located at the membrane distal 'head' domain of the molecule and associates with sialic acid residues on cell proteins. The N protein is required for release of virus at the end of the replication cycle.* **(b)** *Adenovirus is shown as an example of a naked, nonenveloped virus. The primary receptor binding domain of the virus is located in the knob protein at the ends of the 12 fibers situated at the vertices of the icosohedral capsid. Following initial attachment, conformational changes in the fiber bring the particle closer to the cell surface where a second receptor, an integrin, engages at a site on the penton base. This second receptor interaction is essential for completion of the infection process. The receptor-binding domains of most viruses protrude from the particle surface but in some cases, poliovirus for example, the receptor-binding region of the virus is buried within a groove or 'canyon' in the capsid.*

necessary to complete the infection process. In a further example, the primary receptor binding feature of the nonenveloped adenoviruses is a knob structure located at the tip of long fibrous proteins projecting from the 12 vertices of the icosahedral virus particle (Figure 1). After binding of the knob to the primary receptor, the fiber distorts to bring the virus particle closer to the cell surface and allows the engagement of a second receptor as a necessary component of the infection process. The secondary receptors are members of the integrin family, a large family of heterodimeric membrane glycoproteins involved in a range of cellular recognition events. The engagement with integrin molecules initiates viral internalization within endosomes, which is a necessary step in the infection process.

VIRUS ENTRY

All viruses must gain entry to the host-cell cytoplasm and/or nucleus to initiate the infection process. In some cases this occurs directly at the plasma membrane, for others it is subsequent to internalization into endosomes or other invaginated vesicles. However, the crucial step is the transport of the viral genome across a cellular membrane, for even within endosomes the virus is still topologically outside the cytoplasm of the cell. For enveloped viruses the problem of gaining entry is conceptually simple. Fusion of the envelope of the virus with the cell membrane will result in delivery of the viral content directly into the cytoplasm. For nonenveloped viruses, the problem is more complex and different viruses have either evolved mechanisms for the disruption of membranes within vesicles, such as endosomes, to allow the virus particle access to the cytoplasm or they can form pores in cell membranes, which allow the nucleic acid alone to enter the cytoplasm.

Influenza is the classic example of entry of an enveloped virus via endocytosis. The binding of the virus to host-cell receptors initiates the process of endocytosis which draws the attached virus particles into clathrin-coated pits. These pits invaginate into the cell and are pinched off to form endosomal vesicles or endosomes. These migrate within the cytoplasm towards the nucleus, becoming increasingly acidified by the action of proton ATPase during the process. The reduced pH within endosomes triggers conformational changes in HA and

facilitate a crucial step in the infection process of enveloped viruses, that of membrane fusion (Weissenhorn et al. 1999). The globular head domains of the HA molecules move away from the central stalk region, which is reorganized into an elongated alpha helical structure terminating in a short hydrophobic region, the fusion peptide. Insertion of this peptide into the endosome membrane and subsequent conformational change brings the viral and cellular membranes into close proximity and initiates the fusion of the two membranes, although the precise details of this process are only now beginning to be understood (Figure 2). A key feature of this process is that the fusion peptide is sequestered within the stalk region of the HA trimer and only becomes exposed to facilitate the fusion process following acid-induced reorganization of the structure of the protein within endosomes. Another important feature of the mechanism is that the HA protein is synthesized as a precursor protein, HA0, which must be activated by a maturation cleavage, carried out by a host cell protease, furin, into HA1 and HA2. This cleavage leaves the fusion peptide at the N terminus of HA2 and allows the molecule to undergo acid-induced rearrangement.

The example of influenza illustrates the basic principles that underlie the infection mechanisms of many viruses that gain entry to the cytoplasm via endocytosis (Klasse et al. 1998; Bentz and Mittal 2000). Quasistable components of the virus undergo acid-induced conformational rearrangements to expose specialized features that interact with and modify cellular membranes. Recently the entry mechanisms of the alphaviruses and flaviviruses, two families of enveloped positive-strand viruses, have been elucidated. Although there are important differences from influenza HA in the details of the process, the underlying principles are similar. The surface glycoproteins of these viruses are highly ordered and their interactions define the icosahedral symmetry of the virus particle, in contrast to influenza in which the HA spikes are randomly distributed in the envelope. In addition, the fusion peptide is located internally within an envelope glycoprotein rather than at the N terminus. Marked rearrangements of the envelope proteins result in the insertion of this peptide into the host membrane and draw the host and virus membranes into close apposition to facilitate their fusion, as in the influenza case.

In the examples quoted above, the switch to a fusogenic form of the virus is triggered by acidification in endosomes. Other viruses achieve the same state following receptor or coreceptor engagement. In these cases the acid pH found in endosomes is not necessarily required for virus entry and fusion may occur at the plasma membrane. HIV is a well-studied example of this mechanism in an enveloped virus. As for influenza virus, the receptor binding glycoprotein of HIV is produced as a type 1 envelope protein, gp160, which is subsequently processed into a membrane-associated C-terminal domain, gp41, and a major receptor binding component, gp120. Engagement of the virus with its primary receptor, CD4, occurs via an interaction with gp120. Subsequent engagement of a secondary or coreceptor, which can be CVCR4 or CCR5 depending on the cell tropism of the virus, triggers the conformational changes in gp41 required to convert the protein into a fusogenic state.

Although membrane fusion per se is clearly not an option for nonenveloped viruses, the principle of acid or receptor-induced triggering of conformational changes

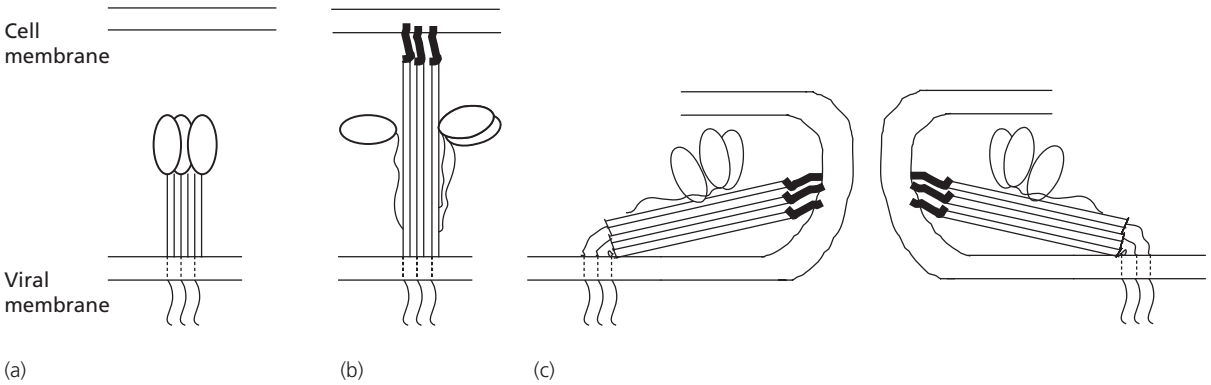

Figure 2 *The cell entry process of enveloped viruses. Enveloped viruses gain entry to host cells via fusion of the viral and cellular membranes, thus making the contents of the viral envelope contiguous with the cytoplasm. The diagram illustrates the mechanism for influenza virus, but the principles appear to be the same for most, if not all, enveloped viruses.* **(a)** *The virus attaches to the cell by interactions between HA and proteins in the plasma membrane.* **(b)** *Conformational changes are induced in the HA molecules and result in the exposure of a hydrophobic 'fusion peptide' sequence, which is projected from the viral membrane and inserts into the cell membrane. In the case of influenza these changes are induced by the reduction in pH in endosomes. Similar conformational changes may be induced by other mechanisms, such as engagement of a second or coreceptor in some viruses.* **(c)** *Further conformational changes result in the cellular and viral membranes being brought into close proximity, the reorganized HA being anchored in the cell membrane by the fusion peptide and the viral membrane by the transmembrane domain. This results in membrane fusion, although the details of the final process are not fully understood.*

associated with the infection process still apply. For some viruses, for example the adenoviruses and the reoviruses, the entry process involves the disruption of endosomal membranes so that the virus particles within the vesicles become confluent with the cytoplasm. Although the mechanisms involved in this membrane disruption process are not fully understood, they are clearly preceded by conformational changes in the virus particles.

In many, if not all, picornaviruses only the viral RNA is introduced into the cytoplasm, the proteins of the virion remaining topologically on the outside of the cell. The mechanisms involved are best understood for poliovirus. The receptor for the virus is a type 1 glycoprotein known as CD155 or PVR (poliovirus receptor) and binds within a groove, known as the canyon, which surrounds the fivefold axis of the icosahedral virus capsid. Engagement of the receptor induces major changes in the virus particle, which results in the externalization of the N termini of the VP1 protein and the loss of the small myristoylated internal protein, VP4. The N-terminal amino acid sequence of VP1 has the potential to form an amphipathic helix and this entry intermediate particle associates with membranes via this sequence. Both the externalized N termini of the VP1 proteins and the released VP4 protein are thought to form channels or pores through which the RNA is transported into the cytoplasm (Hogle 2002). Similar mechanisms probably apply to other picornaviruses but studies with these are less well advanced.

Having breached the membrane and gained entry into the cytoplasm different viruses distribute to different compartments within the cell. Many viruses replicate within the cell nucleus, for example, most DNA viruses and influenza virus. Transport to the nucleus is achieved by association with the microtubule system within the cytoplasm and traveling along this in a retrograde direction to the perinuclear region (Sieczkarski and Whittaker 2002; Greber and Fassati 2003). Either the internal contents of the virus, the genome, and associated proteins, migrate into the nucleus or, as in the case of hepatitis B virus the entire nucleocapsids are transported through nuclear pores.

VIRUS GENE EXPRESSION

As for their strategies of genome organization, viruses have adopted a wide range of mechanisms for gene expression and, of course, the two factors are frequently related. These properties of viruses form the basis of the Baltimore classification of viruses (Baltimore 1971) (Figure 3).

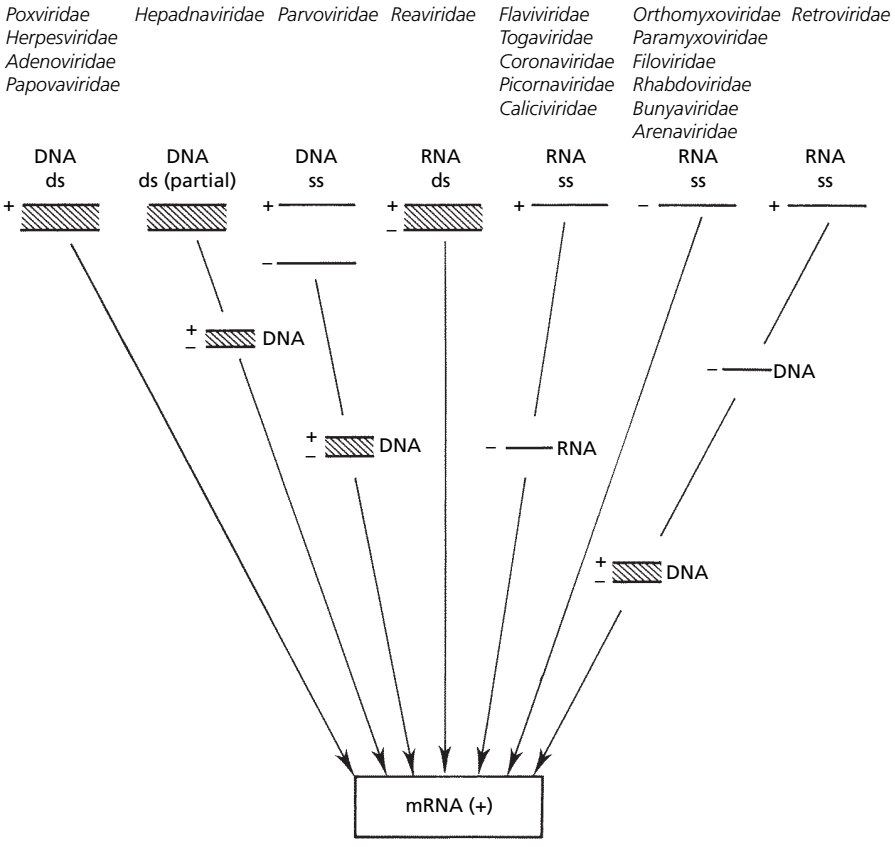

Figure 3 *Grouping of viruses by genome composition and pathway of mRNA synthesis.*

Most DNA viruses utilize the basic cellular enzymes for transcription of their mRNAs, although they usually exert strong controls over the specificity of gene expression to their own advantage. A notable exception to this generality is the case of poxviruses, which replicate within the cell cytoplasm and are thus obliged to incorporate virally encoded transcription enzymes in the virus particle.

RNA viruses, on the other hand, invariably encode their own RNA-dependent RNA polymerases (RdRp), which function both as transcriptases to produce mRNAs and as replicases to produce progeny genomes. This is an absolute requirement for RNA viruses as RdRp enzymes are rare in normal cells. For positive-strand RNA viruses, the viral genome functions directly as a mRNA and is translated to produce the virally encoded proteins, including the RdRp. For this reason the naked RNA of positive-strand viruses is itself infectious if appropriately introduced into cells. As a consequence reverse genetics, the in vitro manipulation of cloned cDNAs of viral genomes to introduce specific mutations and their recovery as virus from in vitro transcribed RNA copies, was first developed for positive-strand viruses. This technique has been an immensely useful research tool for dissecting the molecular details of virus replication. Negative-strand viruses, on the other hand, must incorporate the RdRp within the virus particle. This is essential for the transcription of mRNAs required to initiate viral protein synthesis.

DNA viruses

The majority of double-stranded DNA viruses, for example *Papovaviridae*, *Herpesviridae*, and *Adenoviridae*, use the transcription machinery within the host nucleus to synthesize their mRNAs. The initiation of transcription is strictly controlled, often on a temporal basis, in order to co-ordinate the production of viral proteins and to afford maximum advantage to viral biosynthesis. However, some double-stranded DNA viruses, notably the poxviruses, replicate within the cytoplasm. As the host enzymes responsible for transcription and replication are located entirely within the nucleus, these viruses encode all of the enzymes required to carry out these functions. Another important consequence of the cytoplasmic lifestyle is that the enzymes required for mRNA synthesis must be encapsidated along with the viral genome into virus particles in order to initiate the infection process. Consequently, the naked genomes of these viruses are non-infectious when introduced into cells, in contrast to the genomic DNA of nuclear replicating double-stranded DNA viruses.

Single-stranded DNA viruses, such as the *Parvoviridae* must be converted into a double-stranded form before they can act as templates for the synthesis of mRNA. This is accomplished by host polymerase enzymes.

RNA viruses

The genomes of the single-stranded positive-sense RNA viruses, which include the *Picornaviridae*, *Togaviridae*, *Flaviviridae*, *Caliciviridae*, and *Coronaviridae*, are infectious in the absence of any other viral components. The RNA is translated into viral proteins immediately upon delivery into the host cell and the same molecule must function as template for both protein synthesis and, subsequently, for genome replication. The *Picornaviridae* and *Flaviviridae* RNAs are translated into single polyproteins, which are then proteolytically processed into mature protein products, whereas the other viruses produce subgenomic mRNAs. There is a variety of strategies for the production of subgenomic RNAs and their purpose is to control the relative amounts of viral proteins produced; structural proteins generally being required in greater amounts than nonstructural proteins (Figure 4).

The single-stranded negative-sense RNA viruses replicate in the cytoplasm. The RNA alone is not infectious as the production of mRNA requires the function of the viral RdRp, an enzyme not found in host cells. The RdRp functions as both transcriptase, to produce viral mRNAs, and as a replicase, to produce progeny genomic RNA later in the growth cycle. It is introduced into the cell along with the genomic RNA as a ribonucleoprotein complex. The mRNAs encoding the different viral proteins are separately transcribed, thus allowing for differential control of the relative amount of each that is produced. In the segmented negative-sense RNA viruses most of the viral genes are encoded on separate RNAs and a successful infection requires the simultaneous delivery of all segments. Each functions as a separate template for transcription and replication. The segmented negative-sense RNA viruses, such as the *Orthomyxoviridae* and the *Bunyaviridae* employ a mechanism known as ‘cap snatching’ to initiate transcription of their mRNAs. In this process short oligonucleotides are cleaved from the 5′ ends of host mRNAs, including the 5′ terminal CAP structure, and are used to prime transcription of the viral mRNAs. This process occurs in the host-cell nucleus and all of these viruses require a functional host-cell nucleus for their replication.

The double-stranded RNA viruses all have segmented genomes which range in number from two to 11. The double-stranded genomic RNAs are retained within the nucleocapsids at all times during the replication cycle and the particles function as complex transcription machines, ejecting single-stranded RNAs, which can function both as mRNAs and as templates for new viral progeny. Double-stranded RNA is a powerful inducer of innate immune responses, such as the interferon response, and the strategy of these viruses of sequestering the double-stranded genome within particles has

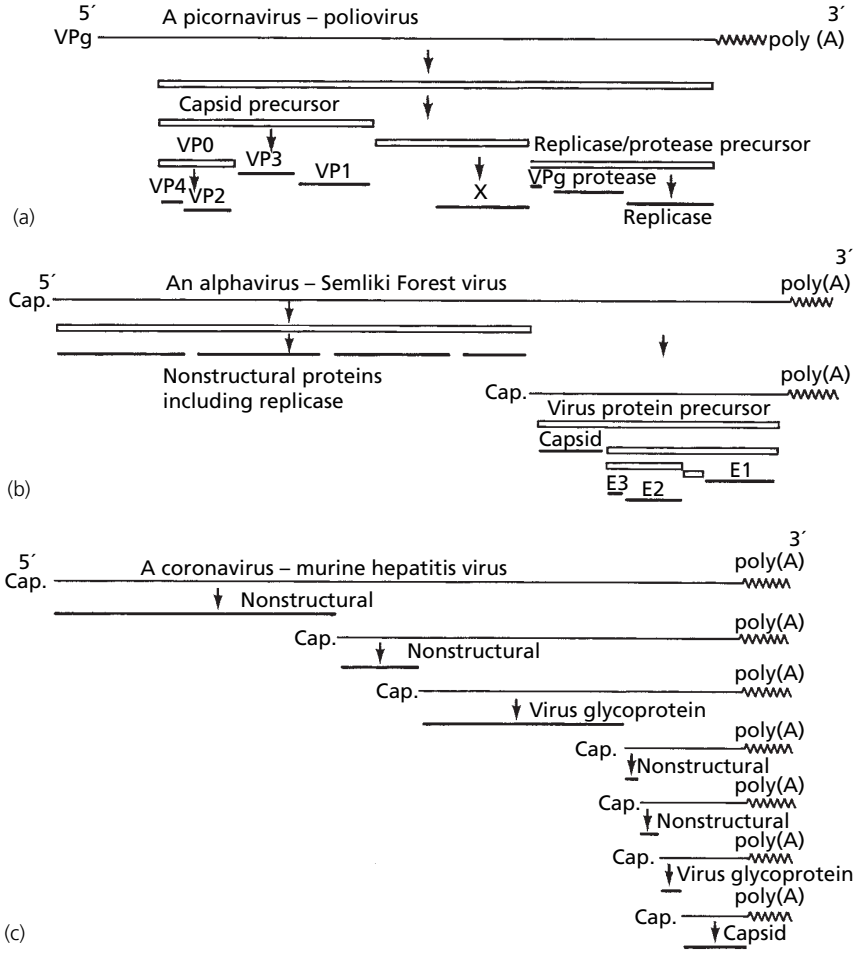

Figure 4 *Gene expression strategies of positive-strand RNA viruses.* **(a)** *The picornaviruses, such as poliovirus, translate their genomes as a contiguous polyprotein, which is subsequently proteolytically processed into a series of intermediate and mature proteins. Many of the intermediate products have functional roles different from those of the final cleavage products. The open reading frame for the polyprotein occupies most of the viral sequence and translation is initiated from a highly structured sequence within the 5′ untranslated region of the genome.* **(b)** *The genomic RNA of alphaviruses also functions as a mRNA to produce a polyprotein, but in this case only the nonstructural proteins responsible for virus replication are derived from the virus RNA. During replication, a subgenic mRNA encoding the structural proteins is produced in abundance. The nonstructural protein precursor is processed by viral protease and the structural proteins are processed by cellular enzymes.* **(c)** *The production of subgenomic mRNAs is most evident in the coronaviruses, which synthesize a nested set of subgenomic mRNAs each of which, with minor exceptions, is translated into a single viral protein encoded in the 5′ portion of the sequence.*

obvious relevance for avoidance of these defense mechanisms. The strategy requires that the viral entry mechanism allows for the transport of the entire nucleocapsids into the host-cell cytoplasm and also that there is an accurate packaging mechanism to ensure the incorporation of a full set of genome segments into at least the majority of virus particles.

Reversiviruses

The identification of reverse transcriptase broke the central dogma that stated that information transfer progressed from DNA through RNA to protein and could not travel back up this chain. The reverse transcriptase polymerase is able to copy an RNA template into DNA and its discovery was of great benefit to the expanding field of molecular biology, as well as being of fundamental importance in understanding the life cycle of many significant viruses. Reverse transcriptase is integral to the replication strategies of both the *Retroviridae* and the *Hepadnaviridae*, but it functions at different stages in the life cycles of these two families of viruses. Retrovirus particles contain genomic RNA and are haploid, each particle containing two template molecules. Immediately after infection the encapsidated reverse transcriptase copies the virion RNA into DNA, using a host tRNA as primer. The resulting double-stranded DNA is integrated into the host genomic DNA from where virus-specific mRNAs are transcribed by host transcription mechanisms. In the hepadnaviruses,

such as hepatitis B virus, the timing of the reverse transcriptase function is reversed. In these viruses a pregenomic RNA molecule is encapsidated along with the viral polymerase. This then copies the RNA into an incomplete double-stranded circular DNA molecule within the nucleocapsid particle. Upon infection of a new host cell the partial circular DNA is completed and functions as a template for host transcriptase, but without the requirement for integration into the host genome.

mRNA synthesis

The majority of viral mRNAs conform to the paradigm for cellular mRNA structure and function. However, there are some notable exceptions, as will be discussed later. Cellular mRNAs are produced in the nucleus where they undergo a number of processing events before they are exported to the cytoplasm to program protein translation. Viruses that replicate in the nucleus have access to the host-cell mRNA modification machinery, whereas cytoplasmic replicating viruses must encode for the required functions within their genomes. Because of their small size and relative ease of manipulation, viruses have been invaluable agents for the elucidation of many of the molecular mechanisms involved in synthesis, processing, and functioning of mRNAs.

The main steps involved in the synthesis and maturation of host-cell mRNAs can be summarized as follows:

- Initiation of transcription by DNA-dependent RNA polymerase at a defined position on the genomic DNA. The location of the initiation site is defined by promoter binding sequences which are crucial for the control of the nature and amounts of mRNA transcripts produced.
- The cap structure, a modified guanosine residue, is added to the 5′ terminal nucleotide of the nascent transcript.
- The 3′ end of the transcript is extended with a polyadenylate (poly(A)) sequence.
- For most mRNAs, the primary transcript is far longer than the final mature mRNA that is exported to the cytoplasm. A complex series of cleavage and ligation events remove parts of the initial transcript known as introns and splice the remaining exons into the final mRNA.

Capping and polyadenylation

All cellular mRNAs terminate at the 5′ end with a modified nucleotide, 7-methylguanylate (m^7G) which is linked to the 5′ terminal encoded nucleotide of the mRNA by an unusual 5′–5′ linkage. Specialized transferases are responsible for the methylation of the guanyl nucleotide and the addition of the modified nucleotide to the mRNA. The cap is important for maintaining mRNA stability in the cytoplasm and in the process of initiation of protein synthesis. It is recognized by the cap binding protein, eIF4E, which is one of the components of the eukaryotic translation initiation factor eIF4. The other members of this complex are eIF4A, which may be an RNA helicase and is involved in the migration of the completed complex along the mRNA and eIF4G, which functions as a scaffolding protein to link the cap, via eIF4E, to further initiation factors including the 40S subunit of the ribosome. Once assembled the entire preinitiation complex migrates in a 3′ direction until a translation initiation codon (AUG) in a favorable sequence context is encountered. At this point some initiation factors are released, the 60S ribosomal subunit is engaged to assemble the full 80S ribosome and translation begins.

The 3′ ends of most mRNAs also possess a common feature, the poly(A) tract. This again is nontemplated and is up to 200–250 nucleotides in length. As for the cap, the poly(A) tract is important for mRNA stability within the cell. It is also recognized by the poly(A) tract binding protein (PABP) which, in addition, binds to regions of the initiation factor eIF4G. This interaction effectively results in the circularization of the mRNA and is thought to increase RNA stability and the efficiency at which reinitiation of translation on the mRNA occurs.

Thus, capping and polyadenylation are very important for mRNA function and, with some notable exceptions discussed below, are features of viral, as well as host mRNAs. Viruses that replicate in the nucleus generally make use of the host biochemical machinery present in that compartment of the cell to carry out the capping and polyadenylation modifications of their mRNAs. However, viruses that replicate within the cytoplasm need to supply these functions in addition to the polymerase activity of their transcriptase. The poxviruses are very large DNA viruses that replicate in the cytoplasm and encode all the enzyme functions required for capping and polyadenylation. The best studied member of the family, vaccinia virus provided much of the earliest detailed information on the biochemistry of the capping process. Interestingly, the capping enzyme of vaccinia has multiple roles in the replication of the virus as it also functions as a transcription termination factor of early mRNA synthesis and as an initiation factor in intermediate gene transcription. This illustrates a common theme in virology that virus products often have multiple functions, presumably in the interests of genomic economy. For most of the cytoplasmic replicating RNA viruses, the capping and polyadenylation functions are additional functions associated with the viral RdRp. The importance of this multifunctional polymerase is reflected in the fact that it is often the largest gene encoded by these viruses and accounts for 20 percent of the genome in picornaviruses and 63 percent in rhabdoviruses viruses.

The poly(A) tract found at the 3′ end of most mRNAs is added by the transcriptase in a stuttering function initiated by specific signal sequences. The most important of these is the conserved consensus sequence AAUAAA situated some 15–30 nucleotides upstream of the polyadenylation site.

Some viruses, such as the *Picornaviridae* and the *Pestivirus* and *Hepacivirus* genera of the *Flaviviridae* have dispensed with the requirement for a cap structure and adopted a different strategy for protein translation as outlined below. In the *Picornaviridae*, the *Flavivirus* genus of the *Flaviviridae* and the *Togaviridae* the poly(A) tract is templated in the genomic RNA while some viruses, the hepaciviruses, pestiviruses, and viruses of the *Reoviridae* family do not have polyadenylated mRNAs.

Control of transcription

The function and nature of every cell is a consequence of it transcription and translation activities, i.e. its gene expression profile. Consequently, the control of transcription and translation are crucial to normal cell function and the same applies, to a greater or lesser extent, to virus gene expression. The three major classes of cellular RNA transcripts, transfer RNAs (tRNA), ribosomal RNAs (rRNA), and messenger RNAs (mRNA) are synthesized by different polymerase enzymes; tRNA by pol I, rRNA by pol III, and mRNA by pol II. It is the profile of mRNA transcripts produced by pol II that defines the gene expression patterns within cells. The rate of transcription of each gene is controlled by specific recognition sequences, termed promoters, which are located at defined distances upstream of the coding sequence for that gene. Promoters are located within 100 base pairs of the start site for transcription of a gene and define the precise position at which transcription of the gene sequence into mRNA is initiated and the frequency of transcription initiation. Within this region two or three short sequences are the most important recognition elements. The most highly conserved of these is the TATA sequence, often referred to as the TATA box. This is located about 30 bases upstream of the initiation site for transcription and artificial movement of this sequence shifts the start site for transcription accordingly. Removal of the TATA box prevents or greatly reduces transcription frequency. Other sequences that are frequently seen in the promoter region are CCAAT and GC boxes. Promoters consist of canonical sequences, like the TATA box, which are involved in DNA-dependent RNA polymerase recognition and specialized sequences that are gene specific and are recognized by highly specific binding proteins, transcription factors, that form complexes with the transcriptase and define the specificity of transcription initiation. Thus the control of gene expression via transcription is dictated by the levels and activities of a range of transcription factors within the cell. The presence and activity of different transcription factors can vary in response to signals received by the cell via a plethora of receptors and transmitted to the transcription machinery in the nucleus by a cascade of biochemical modifications of intermediary signalling molecules. Some of these signalling pathways are initiated by the recognition of viral infection within the cell, e.g. by the presence of double-stranded RNA, and result in the induction of a suite of antiviral response proteins. Many viruses have evolved mechanisms to avoid these responses by either interfering with the cellular signalling pathways or by resisting the inhibitory effects of the induced antiviral response mechanisms.

Another level of transcriptional control is provided by a further series of sequence elements known as enhancers which are present in regions flanking genes. Enhancer sequences are longer than promoters and can be located at considerable distances from the genes whose expression they influence. Moreover they are orientation independent and can be found either upstream or downstream of genes. They are typically 200 nucleotides in length and clearly play important roles in DNA viruses as they are highly conserved and the longest known enhancer sequences have been described in viruses. For example, enhancers in cytomegalovirus range from 400 to 700 nucleotides in length and include the strongest known. They function by altering the structure of DNA to make it more accessible to transcription factors.

DNA viruses that replicate in the nucleus utilize the host pol II-dependent transcriptase activities to express their own genes. DNA viruses that replicate in the cytoplasm, such as the poxviruses, have no access to the host machinery and need to encode their own transcriptase enzymes. In order to initiate the infection process these enzymes must be introduced into the host cell within the virus particle. For this reason the DNA alone of poxviruses is not infectious, in contrast to DNA of herpesviruses and adenoviruses. Sequence analysis of the core polymerase genes of a range of viruses shows considerable conservation between these and host enzymes. In fact, there is significant conservation of certain sequences across the whole biological spectrum from bacteria through to plants and animals. The most striking of these is the triplet GDD which is found in DNA-dependent RNA polymerases, RNA-dependent RNA polymerases, and reverse transcriptases and is involved in the co-ordination of catalytically active magnesium atoms. Other regions of sequence conservation are distributed throughout the polymerase sequences interspersed between more variable regions. Determination of the molecular structures of an ever-increasing range of polymerase enzymes at atomic resolution by X-ray crystallographic techniques is providing insights into their structure/function relationships. From these studies the subdomain components of the enzyme

structures are resolved and clarify the patterns of sequence conservation and variation that characterize polymerases from different sources. In addition to the core RNA polymerase enzyme, the functional transcriptase is a complex of a number of proteins with ancillary roles in the transcription process, such as helicase function for unwinding the template during the copying process. The transcriptase complex of vaccinia virus, for example, comprises a total of seven proteins with a combined molecular weight of 500 kDa.

All RNA viruses are transcribed using virus-encoded enzymes. This is because RdRp activities are not available in normal host cells and must be provided by the virus genome. RNA viruses typically have smaller genomes than most DNA viruses and a significant proportion of their genome encodes the RdRp. For example, 19.5 percent of the poliovirus genome, 49.3 percent of the influenza virus genome, 54.5 percent of the Sendai virus genome, and 62.7 percent of the vesicular stomatitis virus (VSV) genome encode this enzyme. RdRps do not have proofreading functions and the replication of RNA viruses is consequently highly error prone. This is likely to be a reason for the small size of RNA virus genomes. Also, for RNA viruses the same polymerase enzyme functions as a transcriptase to produce viral mRNA and as a replicase to produce progeny virus RNA. A wide range of strategies to differentiate the transcriptase and replicase functions exists in RNA viruses, as will be discussed below. RdRp activity was first described and studied in detail for VSV. This is a single-stranded negative-sense RNA virus and encapsidates its polymerase within the virus particle. Removal of the viral envelope with mild detergent releases the ribonucleoprotein core of the virus particle and this is capable of synthesizing viral mRNAs if provided with the four ribonucleotide triphosphate substrates in appropriate buffer conditions. The RdRp component of this functional complex is known as the L protein and has a molecular weight of 241 kDa, comprising 2 109 amino acids. In addition to its function as a polymerase enzyme it also has the enzymatic activities associated with capping and polyadenylation of the mRNA transcripts. In common with the RdRps of other negative-stranded RNA viruses, the VSV L protein can only transcribe mRNAs from a ribonucleoprotein template and is inactive when presented with naked RNA. Thus the transcriptionally active ribonucleoprotein complex comprises the viral RNA genome of 11 000 nucleotides, approximately 1 200 copies of the N or nucleoprotein polypeptide, 500 copies of a phosphoprotein, NS, and 50 copies of the L protein.

Protein encoding and translation strategies

Collectively, viruses have adopted a wide range of strategies for the translation of their genetic information into proteins. The two functional reasons underlying most of the unusual aspects of information transfer in viruses are to maximize the efficiency of information storage in the viral genome and to facilitate control of the expression levels of viral genes. Hepatitis B virus provides a good example of economic storage of protein coding information within a small genome. The circular DNA genome of the virus comprises only 3.2 kbp, but every nucleotide is involved in coding for proteins, most in more than one reading frame.

Eukaryotic mRNAs typically have a 5′ cap structure followed by a short 5′ nontranslated sequence. A complex of translation initiation factors, including the 40S ribosomal subunit, assemble at the cap and migrate along the 5′ nontranslated region until an AUG in a favorable sequence context is encountered, at which point the 60S ribosomal subunit is engaged, the initiation factors are released and translation commences. The optimal initiation sequence for protein synthesis, known as the Kozak sequence (Kozak 1986), is AxxAUGG. In most cases, the translation initiating AUG is the first one encountered by the initiation complex in its migration along the 5′ nontranslated region. Once initiated protein translation proceeds along the mRNA until the ribosome reaches a termination codon near the 3′ end of the message where translation ceases and the ribosome dissociates. Beyond the termination site there is usually a short 3′ nontranslated region terminating in a poly(A) tract. As discussed earlier, there is increasing evidence that the poly(A) tract noncovalently associates with the 5′ end of the mRNA through protein factor interactions and that this pseudocircularization increases the efficiency of mRNA template function.

Although many viral mRNAs conform to this paradigm, there is a fascinating array of exceptions to the norm. These include cap-independent mechanisms for translation initiation, the use of noncanonical initiation codons, proteins encoded in overlapping reading frames, slippage mechanisms to facilitate change of reading frame, RNA editing, and ribosome 'skipping.' In addition, some viruses utilize the normal cellular process of RNA splicing to produce multiple mRNAs from the same genetic sequence, but to an almost profligate degree.

The assembly of a variety of mRNAs derived from a single genomic sequence by splicing mechanisms is restricted to viruses that replicate in the nucleus. In particular, adenoviruses, papovaviruses, and some retroviruses (the lentiviruses, such as HIV) make extensive use of splicing to maximize the information storage and retrieval potential of their genomes (Figure 5). Analysis of the mRNA transcript profile of adenoviruses provided the first evidence for RNA splicing which was subsequently shown to be a universal mechanism in eukaryotic gene expression. In the splicing process internal regions within the primary RNA transcript,

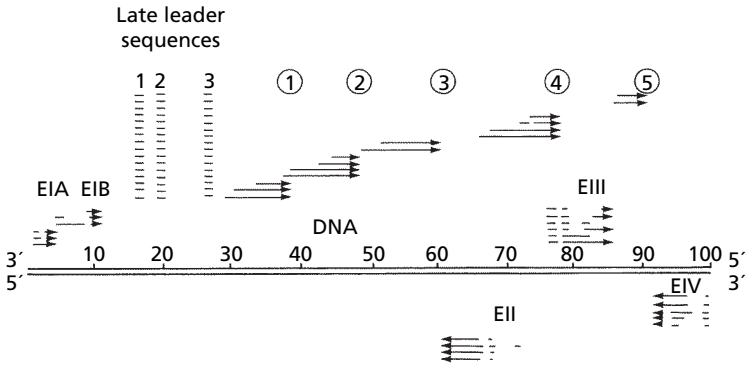

Figure 5 *Gene expression strategy of adenoviruses. Adenoviruses demonstrate complex patterns of gene expression. Different mRNAs are produced at early or late stages of infection and the genetic potential of the genome is amplified by the extensive use of RNA splicing. There are five early gene transcription units (the E transcripts) and another five late gene clusters. The 5′ end of each of the late gene mRNAs has a common tripartite leader sequence comprising three spliced sequences.*

known as introns, are removed and the remaining exon sequences are spliced together by an RNA ligation mechanism. If there are a number of splice donor and acceptor sequences within the primary sequence there is the potential to form an array of mature mRNAs that are recombinant assemblages derived from the same primary sequence. An example of this process is the series of T antigens expressed by viruses of the *Polyomaviridae*. For SV40, early in infection two versions of T antigen, large and small T antigens, are produced from differentially spliced mRNAs. Both mRNAs share a common open reading frame at the start of the transcript. At the end of this common sequence, an intron of approximately 350 nucleotides is removed from transcripts destined to be mRNAs for large T antigen and the open reading frame is maintained through the splice site. The mRNA for the small T antigen is produced by the excision of a smaller intron starting from an alternative splice donor sequence, but terminating at the same splice acceptor site as for the large T antigen. In this case the reading frame is not maintained and translation is terminated at the splice acceptor site. Thus the two transcripts encode unrelated amino acid sequences after the common 5′ region. In other members of the *Polyomaviridae*, further alternative T antigen proteins are produced via the use of additional splice donor/acceptor sites. Most of the RNA viruses replicate in the host cytoplasm and hence do not have access to splicing mechanisms. The *Orthomyxoviridae* are an exception to this and, for example, the mRNAs derived from gene segments 7 and 8 of influenza A virus are produced as spliced variants. In each case one protein is translated from the contiguous unspliced transcript and a different protein is encoded in a spliced version of the transcript. In the spliced mRNA, a sequence a little downstream of the translation start site is removed as an intron and the downstream exon is ligated to the 5′ exon in a different reading frame. Thus, the two proteins share a common short N-terminal sequence, but thereafter are completely different. Additional spliced mRNAs have been demonstrated, but it is unclear whether these are translated into protein products.

Thus, splicing is an important mechanism for deriving the maximum information content from limited genome size. However, this mechanism is not available to cytoplasmic replicating DNA viruses, such as the poxviruses, as no viruses have evolved or acquired the complex enzymatic machinery required to perform the function. In addition, none of the cytoplasmic RNA viruses utilize splicing mechanisms, although these viruses have evolved a number of alternative mechanisms for maximizing genetic storage and control of gene expression and of gaining a competitive advantage over cellular gene expression.

The *Picornaviridae* and the *Pestivirus* and *Hepacivirus* genera of the *Flaviviridae* have evolved a mechanism for the initiation of their protein synthesis that bypasses the normal requirement for recognition of a cap structure by the initiation complex. In these positive-strand RNA viruses the genomic RNA serves as template both for protein translation and for genome replication. They initiate translation via direct association of the initiation complex with a highly conserved RNA sequence located within the 5′ nontranslated region (Figure 6). This sequence comprises about 350 nucleotides in the *Flaviviridae* and is nearer 450 nucleotides in the *Picornaviridae*. In both cases the sequences are predicted to possess a high degree of secondary structure and this has been supported by direct biochemical analysis. In addition there is evidence for a considerable amount of conserved tertiary structure. As these RNA structures are able to initiate translation through their ability to associate directly with the initiation complex and so bypass the requirement for a free, capped 5′ end to the mRNA they have been termed internal ribosome entry sites (IRES). The rational for the possession of IRESs may be that they allow for the presence of highly structured sequences at the 5′ end of the RNA that may be

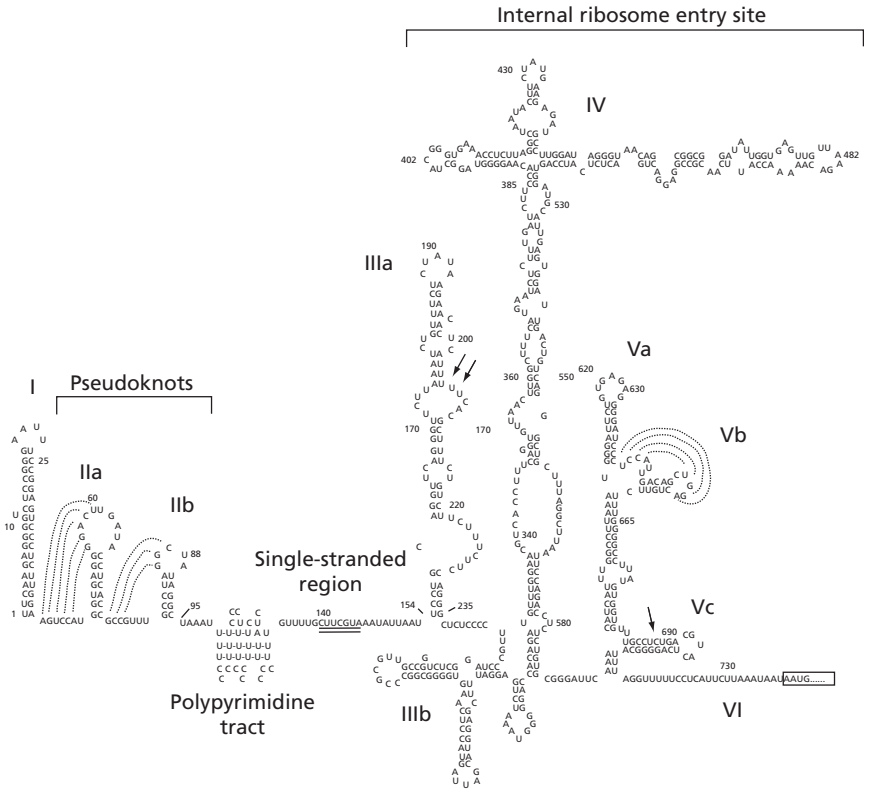

Figure 6 *Proposed RNA structure of the 5′ UTR of HAV. Dotted lines represent potential base-pair interactions in putative RNA pseudoknots; the box indicates the 5′ end of the open reading frame. Stem–loops IIIa–VI (nts 155–735) comprise the IRES. Arrows indicate the site of mutations in cell culture-adapted HAV which facilitate translation. Deletion of the underlined sequence (nts 140–144) leads to a temperature-sensitive phenotype. Figure courtesy of Martin and Lemon (2002).*

important for RNA replication. Such sequences would be inhibitory for the normal process of ribosomal scanning through the 5′ nontranslated region to reach the initiating AUG codon. Whether or not this is the primary raison d'être for IRESs, in several picornaviruses they allow the virus to gain a translation advantage over host mRNAs. The enteroviruses, rhinoviruses, and aphthoviruses encode for proteases that specifically cleave the host translation initiation factor eIF4G. This protein forms a bridge linking the cap recognition protein eIF4E to the rest of the translation initiation complex and its hydrolysis by the viral enzymes effectively negates cap-dependent translation in the cell. Thus, the majority of cell protein translation is inhibited leaving the whole translation apparatus available for the virus. Although the cardioviruses do not cleave eIF4G, their IRESs seem to be highly efficient and can outcompete host mRNA translation and also there is recent evidence that one of the viral proteins, 3A, can associate with nascent ribosomes in the nucleus, resulting in modified organelles that preferentially initiate from the virus IRES. IRESs were first described in viruses but there is increasing evidence that some host mRNAs are translated by the same mechanism. In some cases IRESs have been found to be located within viral mRNAs so as to produce a bicistronic message, translation occurring independently from the 5′ and 3′ portions of the RNA. This has been recognized for some time in certain insect viruses and more recently there is evidence that one of the mRNAs of the herpesvirus, Kaposi's sarcoma virus, is bicistronic, the 3′ open reading frame being translated from an internal IRES.

A number of alternative methods to splicing as a means to maximize information storage have been evolved by viruses that do not have access to splicing machinery. The utilization of alternative translation initiation sites and RNA editing are two methods which are well illustrated by viruses belonging to the *Paramyxoviridae*, for example Sendai virus. Transcripts of the *P* gene of Sendai virus encode three distinct proteins, P, V, and C. The latter is produced as a C terminal nested set of four variant proteins, each initiating at a different in-frame initiation site. The P and C proteins are translated from the primary *P* gene transcript, but initiate at different codons. The frequency at which translation is initiated at different sites is a reflection of their flanking sequence contexts. These include the noncanonical initiation codons ACG and GUG. The *V* gene is produced from a variant transcript in which an extra, nontemplated G residue is added during transcription at a specific RNA editing site. This, of course, alters the reading frame and so the V protein is a hybrid

sequence, the N-terminal part shared with P and the C-terminal region derived from an alternative reading frame. A mechanism of RNA editing is also employed during the replication of hepatitis delta virus. The sole open reading frame of this virus encodes the delta antigen which is produced as a long or short form. The long form is translated from transcripts in which an RNA editing event eliminates a stop codon and allows translation to proceed down the mRNA. The long form has a nuclear location signal not present in the short form and so the two proteins are found in different locations in the cell and this differential distribution is important for the regulation of virus replication and encapsidation to a different stages in the life cycle.

The utilization of alternative reading frames within a single sequence by RNA editing, as illustrated above, requires the transcription of subtly modified mRNA transcripts. Another method of utilizing alternative reading frames has been developed in a number of different viruses and relies on manipulation of the translational rather than the transcriptional machinery. This is ribosome slippage in which specific sequence and structural features within the mRNA induce a slowing and stuttering of translation and result in a proportion of ribosomes changing to a different reading frame, thus producing a hybrid protein as in the case of RNA editing. The normal random error rate in translation of eukaryotic mRNA is in the order of 5×10^{-5} per codon, whereas specific frame-shifting in viral mRNAs can exceed 10 percent. Frame-shifting typically occurs at a homopolymeric sequence of the general structure XXXYYYZ followed a short distance downstream in the mRNA by a pseudoknot (Figure 7). This is a stem–loop structure in which the stem is formed through the hybridization of complementary sequences in the RNA. The sequences within the loop at the top of the stem–loop also hybridize to sequences adjacent to the stem of the stem–loop. This results in the formation of a pseudo-double-stranded structure which impedes the passage of the translating ribosome and facilitates a single nucleotide slippage at the slippery sequence so that a significant proportion of the ribosomes continue translating the message in the −1 reading frame (Deiman and Pleij 1997; Giedroc et al. 2000). For example, ribosome frame-shifting is utilized by viruses in the *Retroviridae* family to control the levels of reverse trancriptase (*pol*) produced in the infected cell. The *gag* and *pol* proteins are encoded by adjacent regions of the viral RNA, but in different reading frames. A frameshifting event occurs at a frequency of about 10 percent to produce a *gag–pol* fusion protein. The mature *pol* protein is then separated from the *gag–pol* fusion protein by a virus-specific proteolytic event.

Another virus-specific variation on the normal process of protein translation has recently been demonstrated for the picornavirus, foot-and-mouth disease virus. This phenomenon has been termed ribosomal skipping and

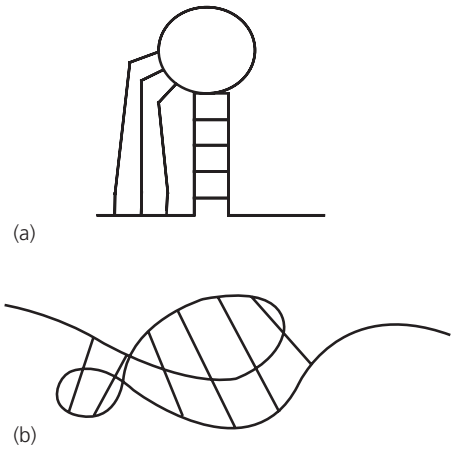

Figure 7 *Ribosome frameshifting: diagrammatic representation of the structure of a pseudoknot. A pseudoknot is formed when there is complementarity between residues in the loop of a stem–loop structure and residues adjacent to the stem–loop* **(a)**. *The actual folding pattern of such a structure is depicted in* **(b)** *and resembles double-stranded RNA. Translating ribosomes pause when encountering such a structure. This, in conjunction with a nearby 'slippery sequence,' leads to a shift in the translational reading frame by a small proportion of the ribosomes.*

occurs when translation continues along the viral RNA, but fails to create a peptide bond at a specific site (Donnelly et al. 2001). This has the same effect as proteolytic processing of the polyprotein translation product of the virus, as occurs at many positions in the sequence, but in addition it permits a level of translational control not otherwise available to viruses that have a single uninterrupted translation product. The skipping event occurs at a position just after the end of the structural protein coding region and involves a 16 amino acid sequence known as protein 2A and is critically dependent on the 2A sequence. It is postulated that termination of translation at this point through a failure to pass through the skipping position could provide a mechanism for excess production of structural proteins at late stages in the virus replication cycle.

As for host mRNAs, the majority of viral mRNAs are monocistronic and in most cases they are subgenomic. In DNA viruses the mRNAs for individual genes are initiated and terminated at specific sequences within the genome sequence and for nuclear replicating viruses the patterns of gene expression can be further complicated by splicing, as discussed above. For the double-stranded and the negative-sense segmented RNA viruses each genome segment generally encodes a single protein. However, in some segmented RNA viruses some genome segments are ambisense, i.e. the virion-sense and complementary-sense RNAs each encode separate proteins. In the single-stranded negative-sense RNA viruses mRNAs for the different gene products are initiated at specific recognition sites within the viral ribonucleoprotein, but are transcribed in sequence from the 3′ end of the viral RNA. The single-strand positive-sense

Togaviridae produce their structural proteins from a subgenomic message representing the 3′ region of the viral genome and in the *Coronaviridae* seven separate subgenomic mRNAs are produced as a 3′ nested set (Figure 4).

Quantitative and temporal control of viral gene expression

DNA VIRUSES

Most viruses have mechanisms to regulate the production of their proteins during infection, but the patterns of control are far more evident in the DNA viruses than the RNA viruses. Some proteins, such as the structural proteins required for assembly of progeny virions, are required in much larger amounts than the enzymatic proteins involved in the replication process and their production is often modulated accordingly. In addition, many viruses produce different proteins at different stages in the infection process. Typically replication proteins and proteins that modulate the host-cell environment to facilitate virus replication are required early in infection while the synthesis of structural proteins is more important at later stages.

The control of protein synthesis in the adenoviruses is well understood and serves as a good example of the principles that apply to many DNA viruses (Figure 5). Transcription is divided into two major phases, early and late. Three of the early genes are transcribed from one of viral DNA strands and two are transcribed from the complementary strand. For all of the adenovirus transcripts multiple alternative initiation, termination, and splicing events result in the production of a variety of proteins from each gene. The first gene expressed when the viral DNA enters the nucleus is E1A. As is often observed with viral proteins the E1A proteins have multiple functions and act as a master gene to both modify the host-cell environment and to orchestrate the transcription of other adenovirus early genes. The E1A proteins have been shown to interact with a large number of host proteins and the functional consequences of all of these interactions are not well understood. However, it is clear that E1A can act as a transactivator to control the transcription of other genes within the early gene cluster via activation of TATA box containing promoters. The activation of these promoters is not through direct interaction with E1A and it does not act as a transcription factor per se. Instead it binds to and modulates the activities of cellular transcription factors and regulatory proteins, such as the TATA box binding protein. It thus facilitates the upregulation of transcription and results in the expression of other early genes. Another important role of E1A is its ability to induce the host cell to progress from a quiescent state into S phase of the cell cycle. This is important for viral replication as the cell environment is far more conducive to DNA replication when in S phase. It achieves this control through its interaction with the cellular protein, retinoblastoma tumor suppressor protein, pRB. This, in turn, controls the activity of E2F cellular transcription factor and when the inhibitory effects of pRB on this transcription factor are released a number of cellular proteins involved in cell cycle progress are produced. Other members of the early gene families of proteins have important roles in negating the antiviral host responses, both innate and adaptive. For example, E1B binds to p53 and inhibits its proapoptotic activity, E3 blocks immune presentation of viral antigens at the cell surface for recognition by cytotoxic T cells and a series of short RNA transcripts, the VA RNAs, inhibit the protein kinase, PKR. This protein is induced as part of the interferon response and in the presence of double-stranded RNA it phosphorylates the translation initiation factor eIF2α, resulting in the inhibition of protein synthesis. The *E2* genes encode the polymerase and terminal protein responsible for viral DNA replication.

Viral DNA synthesis triggers the switch from early to late transcription although the precise mechanism underlying this change is not fully understood. The major late promoter is minimally active before DNA replication and its activity increases enormously thereafter. It has been shown experimentally that this is a *cis*-active phenomenon and so must reflect a change in the physical nature of the DNA template. This may be due to changes in the profile of associated proteins or, more likely, is due to exposure of the promoter region as a consequence of conformational changes during replication. The five late gene families of proteins are all derived from a single long (29 000 nucleotide) transcript through differential polyadenylation site usage and splicing. All of the late transcripts start with a 200 nucleotide spliced region, the tripartite leader. This sequence confers a translational advantage on the viral transcripts at late times in infection. At these times, the eIF4 transcription factor complex is largely dephosphorylated and this results in an inability to scan through mRNAs from the 5′ cap to the translation initiating AUG codon. The tripartite leader allows the ribosome to 'jump' directly from the cap to the initiating AUG without scanning the intervening sequence. An additional translational advantage is gained by the virus late in infection, since at these times newly synthesized host mRNAs are not exported from the nucleus to be translated in the cytoplasm. Viral transcripts are transported to the cytoplasm by a complex of E1B and E4 with cellular factors and this complex may sequester transport factors to the benefit of the viral transcripts and detriment of cellular transcripts.

The general principle of differential expression of viral genes at early and late stages of infection is common to most DNA viruses and many have multifunctional proteins that perform many of the functions outlined above for the E1A protein of adenoviruses. Examples include the T antigens of the papilloma and

papovaviruses and ICP4 of herpes simplex virus, which has a negative effect on the expression of other early genes, but is required for the expression of later genes. Splicing is used minimally by the herpesviruses and not at all by the poxviruses, which lack their own splicing mechanisms and replicate in the cytoplasm away from the cellular systems. These viruses possess some of the largest known viral genomes and express their gene products via a complex cascade of transcriptional events. The series of genes expressed during herpesvirus infection are classified as α, β, and γ proteins and there are further subdivisions of β_1 and β_2 classes. The poxviruses encapsidate both the enzymes required for transcription and the transcription factors required to initiate the process for the early genes. Herpes simplex also encapsidates a protein, VP16, that is involved in the initiation of transcription of its early genes. It forms a complex with cellular proteins Oct1 and HCF-1 and together they promote transcription from viral sequences preceded by the motif TAATGARAT, where R is a purine.

RNA VIRUSES

Positive-strand viruses

The genomes of picornaviruses are single, positive-sense RNA strands of between 7.5 and 8.5 kb in length. The 5′ end is covalently linked to a small protein, VPg, and the 3′ end is polyadenylated. A long 5′ nontranslated region precedes a single open reading frame occupying most of the genomic RNA. This is translated into a single polyprotein product which is cleaved by virus-encoded proteases, both co- and post-translation, into a total of about 12 mature products (Figure 4). The VPg is not required for mRNA function and is normally cleaved from actively translating genomes by a cellular activity. Although no subgenomic mRNAs are produced and there is no mechanism for temporal control of gene expression differential cleavage at the processing sites of the polyprotein are used to increase the complexity of the gene expression profile (Bedard and Semler 2004). Several 'immature' precursor proteins have functions that are different from those of their component 'mature' cleavage products. The genomic RNAs of the flaviviruses are also translated as a continuous polyprotein and, like the picornaviruses the structural proteins are encoded towards the N terminus, the remainder encoding proteins involved in replication. As with the picornaviruses the polymerase protein is located at the C-terminal end of the polyprotein. The flaviviruses are enveloped and their structural proteins are cleaved from the polyprotein by host signal peptidases in the endoplasmic reticulum. The remaining cleavages are carried out by virus-encoded proteases. Although the translation of most flaviviruses is cap-dependent, viruses in the pestivirus and hepacivirus genera use an IRES to initiate translation.

The gene order of the togaviruses differs from the picornaviruses and flaviviruses in that the nonstructural replication proteins are encoded within the 5′ two-thirds of the genomic RNA and the structural proteins are encoded on a separate transcription unit representing the 3′ portion of the genome (Figure 4). The capped and polyadenylated viral RNA is translated on entry into the host cell to produce the polymerase and associated proteins which copy the input RNA into a complementary negative strand. This is transcribed in its entirety to produce progeny viral RNAs or from an internal promoter to produce a subgenomic mRNA representing the 3′ one-third of the genome sequence. This small RNA encodes the structural proteins and is produced in approximately three-fold excess compared with the full length viral RNA. Later in the replication cycle the virion RNA is sequestered by the structural proteins to assemble progeny virions and so the effective concentration of the subgenomic RNA is approximately ten times higher than the viral RNA. As a result, this simple strategy allows for a degree of quantitative and temporal control of the relative synthesis of structural and replication proteins.

The strategy of producing subgenomic mRNAs is carried to its extreme for the positive-stranded viruses in the coronaviruses (Figure 4). A total of six to eight mRNAs, depending on the specific virus, are produced in infected cells and represent a 3′ nested set, the longest being full-length viral RNA. Most of these RNAs are monocistronic and only produce the protein encoded at the 5′ end, the remainder of the RNA remaining translationally silent. Thus, essentially each gene is translated from a separate mRNA. As these are not produced in equivalent amounts this strategy allows for control of the relative amounts of viral proteins produced, although there is no temporal control. Each of the subgenomic RNAs has a common 'leader' sequence at the 5′ end and two mechanisms for their synthesis have been proposed. In one model short RNAs representing the leader sequence are transcribed from the 3′ end of full-length complementary copies of the virion RNA. These then dissociate from the replication apparatus and prime transcription of the individual subgenomic RNAs from conserved sequences present at each intergenic region. In an alternative model, transcription of negative strands is interrupted at the intergenic sequences and resumes at the 5′ leader sequence. Thus a nested set of templates is produced from which the mRNAs are individually transcribed.

Negative-strand viruses

The negative-strand RNA viruses can be broadly divided into those with a continuous genome and those with segmented genomes. Of the single-stranded viruses, VSV is probably the best studied and understood. The obligatory first step in replication is transcription from

the virion ribonucleoprotein by the encapsidated L and P proteins, which together constitute the functional transcriptase, to produce a set of mRNAs. Transcription starts at the extreme 3′ end of the RNA to produce a 47 nucleotide leader RNA terminating in a 5′ triphosphate group. After a short pause, transcription is reinitiated to produce the first mRNA. A pause again occurs at the end of the first transcription unit before transcription is again reinitiated at the start of the next gene on the genome. The order of genes on the genome is *N*, *P*, *M*, *G*, and *L* and this reflects the order of abundance of each protein that is required for virus replication. Each mRNA is capped by the transcription complex and is polyadenylated from a polyadenylation signal present in each short conserved intergenic sequence. Transcriptase complexes dissociate from the template at a frequency of 20–30 percent at each intergenic junction so that the proportion of each mRNA transcript produced is dependent on its position on the genome. The positional frequency of transcription is maintained if the order of genes on the virion RNA is reversed. This strategy allows for a hierarchy of gene expression but not for temporal control of protein synthesis.

Influenza provides a good example of a segmented negative-strand RNA virus. In common with VSV, the viral polymerase is introduced into the cell as part of the ribonucleoprotein viral core. In contrast, it has no capping activity and the viral mRNA transcripts obtain their 5′ caps from host mRNAs by a process known as cap snatching. The polymerase has a nuclease activity that cleaves host mRNAs at a position about 12 downstream of the cap. These heterogeneous fragments are then used to prime the transcription of the influenza virus mRNAs from the individual viral ribonucleoprotein segments. In addition to acting as templates for mRNA synthesis, the input viral RNA segments act as templates for the synthesis of full-length complementary template RNAs. These are subsequently transcribed into progeny vRNA molecules which can act as fresh templates for further mRNA synthesis. Although the production of template RNAs appears not to be, the synthesis of new vRNAs is regulated. This results in inequality in the abundance of different mRNAs and so control of gene expression on a temporal basis.

Double-stranded viruses

The reoviruses are the best studied of the double-stranded RNA viruses. The virus particles are multishelled structures and a core component containing the multisegmented double-stranded RNA genome is introduced intact into the host-cell cytoplasm to initiate infection. At no time during the virus replication cycle is the double-stranded genome exposed to the cytoplasm outside the protein cores. As double-stranded RNA is one of the most potent inducers of host antiviral interferon responses, the sequestering of the genome within particles is probably an important viral defense mechanism. The icosohedral core particles are, in effect, highly ordered transcription machines which synthesize and export RNA transcripts through pores at the fivefold axes of symmetry. The transcripts are capped by the viral transcription apparatus but, unusually, are not polyadenylated at their 3′ ends. The transcripts are full-length copies of the genome segments and function as mRNAs and as templates for progeny double-stranded genome RNAs, when encapsidated within progeny core particles. The genomes of these viruses comprise ten to 12 segments and their high particle:infectivity ratios suggest that there must be very efficient segment sorting in the assembly of progeny virions, but the mechanism for this is not understood. mRNA transcripts are produced in relative abundance according to their segment lengths, thus giving a measure of control of expression levels. In addition, there appears to be differential efficiency of translation of the different mRNA, as well as a general preference for viral gene translation at later times of infection but, again, the mechanisms for this are unclear.

Protein synthesis, transport, and processing

VIRAL EFFECTS ON HOST PROTEIN SYNTHESIS

Cellular and viral mRNAs, alike, are translated into protein by the ribosome, a machine that is too complex to be encoded by viral genomes. In general, ribosomes are not modified in the infected cell, although recent evidence suggests that one of the nonstructural protein products of viruses of the cardiovirus genus of the *Picornaviridae* may associate with nascent ribosomes to specifically facilitate translation of the viral template. However, many viruses have evolved strategies to usurp cell mRNA translation and to avoid some of the cellular antiviral responses at the level of the initiation of protein synthesis. Disruption of the translation of host mRNAs in a way that permits the continuation of viral protein synthesis allows the virus to maximize its own expression and dominate the host machinery. This is a valuable strategy for viruses that induced acute cytolytic infections to produce a maximum progeny burst size and concomitant death of the cell. Some viruses, such as hepatitis C virus, are able to induce persistent infections without cell death. Clearly, disruption of host-cell protein synthesis would be inappropriate for this viral strategy and such viruses must replicate within a competent, surviving cell.

Different viruses have evolved a variety of mechanisms to inhibit cell protein synthesis while allowing their own gene expression to proceed. As mentioned above, many picornaviruses encode proteases that cleave the important scaffolding protein, eIF4G, which connects the cap-binding protein eIF4E to the remainder of the

translation initiation complex. Thus cap-dependent translation is effectively abrogated in the infected cell and the virus overcomes this inhibition by initiating translation via a specialized RNA structure, the IRES. This associates directly with the ribosome and initiation factors without the requirement for cap recognition.

Adenoviruses appear to employ several mechanisms to disrupt cell protein synthesis to their own advantage. Firstly, they can control export of mRNAs from the nucleus. Virus-encoded proteins associate with a cellular RNA transport factor and sequester it in the region of viral replication and transcription. This has the effect of selectively facilitating viral transcript export to the detriment of cellular mRNAs. Secondly, small RNA transcripts known as virus associated (VAI) RNAs are produced in large quantities late in infection and these inhibit the interferon response activated kinase, PKR. Thus the PKR is unable to phosphorylate and inactivate the translation initiation factor eIF2α. As the VAI RNAs are associated with viral transcripts they may selectively protect the virion transcripts from eIF2α phosphorylation. Thirdly, late viral transcripts are able to avoid the inhibition of translation due to phosphorylation of the initiation factor, eIF4F. This is because of a unique property of the so-called tripartite leader sequence common to all late transcripts which allows the translation initiation complex to jump directly from the 5′ cap to the initiating AUG codon without having to scan the intervening sequence. Finally, a late viral protein is able to specifically activate translation of viral transcripts. Rotaviruses produce a protein that interacts with the initiation factor eIF4G to disrupt its interaction with poly(A)-binding protein. This leads to inhibition of translation of polyadenylated cellular transcripts and favoring the nonpolyadenylated viral transcripts.

Infection by a number of viruses leads to reduction in the levels of host mRNAs. In herpesviruses, for example, one of the virus particle-associated proteins that are introduced into the cell during infection seems to be or to activate a nonspecific ribonuclease. This degrades both host and viral mRNAs, but its activity seems to diminish during infection resulting in a selective accumulation of viral transcripts.

PROTEIN TRANSPORT

Viruses make use of the wide range of signalling and transport systems present in host cells to direct their proteins to appropriate compartments. For example, signal sequences are present at the N terminus of proteins destined for secretion into the endoplasmic reticulum, a compartment that is topologically outside the cell. These are relatively short hydrophobic amino acid sequences that associate with the secretory mechanisms and result in the extrusion of the nascent protein through specific pores in the endoplasmic reticulum (ER) membrane. As this occurs concomitantly with translation ribosomes and polysomes that are synthesizing secreted proteins are intimately associated with the ER membrane. The signal sequence is usually cleaved from the nascent polypeptide by proteases resident in the ER lumen. Proteins destined to become membrane proteins are prevented from being secreted completely by stop transfer sequences and remain anchored to the membrane by hydrophobic stretches of sequence and usually have a C-terminal cytoplasmic domain that may be involved in particle assembly or in signal transduction from interactions involving the extracellular domain. Having been inserted into the ER lumen, the proteins are usually glycosylated and can be transported to various cellular locations as dictated by further recognition signals, often located in the transmembrane domain or cytoplasmic tail. Viruses also use many other intracellular transport systems to distribute their proteins to defined destinations. For example, influenza virus ribonucleoprotein cores interact with cellular proteins to facilitate their transport to and through nuclear pores. Many viral proteins have nuclear location signals and others are able to reversibly shuttle between nucleus and cytoplasm to transport viral components between these compartments. Progeny poxvirus particles are able to stimulate the polymerization of actin and this enables them to be physically pushed through and out of the cell.

PROTEIN PROCESSING

Proteolytic cleavage

Proteolytic cleavages are involved in the replication strategies of many viruses. Practical evidence of their importance is furnished by the success of inhibitors of the virus protease in HIV therapy. Proteolytic cleavages can be broadly divided into two classes, those carried out by host cell proteases and those performed by virus encoded enzymes. The most common examples of the former are the normal modifications of secreted glycoproteins by signal peptidases which remove signal sequences in the ER lumen and signal peptide peptidases which can cleave within the transmembrane portion of the signal sequence. In addition, the surface proteins of many viruses are cleaved by cell surface proteases, such as furins, to prime them for subsequent conformational changes involved in the cell entry process, as discussed earlier. A good example is the HA protein of influenza virus which is synthesized as the precursor protein, HA0. This is activated to a cell fusion competent form by cleavage into HA1 and HA2. Mutations at this cleavage site can influence its susceptibility to cleavage, which in turn can have profound effects on the tissue tropism and pathogenicity of the virus.

Many viruses encode their own proteases which are used in the processing of precursor polyproteins. This is especially important for the single-strand positive-sense RNA viruses, such as the *Flaviviridae* and *Picornaviridae*

which translate their entire genome as a single polyprotein (Figure 4). This is processed by a cascade of proteolytic cleavages which function in *cis* or in *trans* to produce the final mature proteins. In the *Picornaviridae*, this strategy serves to increase the functional flexibility of a small genome as many of the intermediate proteins produced in the processing cascade have properties and functions that are distinct from those of the final mature products. In addition, the viral proteases are used to cleave specific cellular proteins, such as the translation initiation factor eIF4G, which results in the shut-off of host protein synthesis. In some cases, particularly with the nonenveloped viruses, virus-induced proteolysis also produces maturation cleavages involved in virion assembly. Proteolysis is also important for controlling the complex series of events in the assembly of herpesviruses. Core particles are initially assembled in the nucleus around scaffolding proteins. The scaffolding proteins are then cleaved by a virus protease and removed from the particles prior to packaging of the viral DNA.

Glycosylation

The great majority of secreted eukaryotic proteins, whether they are shed from the cell or remain tethered to cellular membranes by membrane insertion sequences, are post-translationally modified by the addition of carbohydrate residues. Glycosylation probably serves several functions: involvement in the folding process to produce the correct protein conformation, protection of the protein from extracellular enzymes, intercellular recognition, and, in the case of viral glycoproteins, to help mask the protein from immune recognition. Glycosylation of viral membrane proteins is carried by host-cell enzymes in accordance with the same biochemical rules as host proteins. There are two sorts of glycosylation, *N*-linked or *O*-linked of which the former is the dominant form. The recognition signal sequence Asn–X–Ser/Thr is essential for *N*-linked glycosylation, which occurs in the lumen of the ER. The initial step in *N*-linked glycosylation consists of the addition of a multibranched structure consisting of two residues of *N*-acetylglucosamine, nine of mannose, and three of glucose. The glucose residues report on the folding state of the protein, which is released from the chaperone, calnexin, when all have been removed. Further modifications of the carbohydrate chains are carried out in a cell-specific manner in the Golgi apparatus as the protein migrates through the secretory machinery.

Other post-translational modifications

A number of other modifications of viral proteins have been observed. One of the most important is phosphorylation which is used in many host cell and viral examples as a mechanism for inducing reversible conformational changes in proteins, so enabling them to act as molecular switches. Cascades of phosphorylation and dephosphorylation are the most widely used mechanisms for transducing messages from receptor interactions at cell surfaces to effector molecules in the nucleus. Many virus proteins are also modified by phosphorylation which probably alters their interactions with other proteins and/or nucleic acids, but in most cases their function is poorly understood.

A number of viral proteins are known to be modified by fatty acid acylation, the most common being N-terminal myristoylation. The 14 carbon myristic acid is added to the N-terminal amino acid (after removal of the initiation amino acid, *N*-acetyl methionine) of proteins starting with the sequence Gly–X–X–Ser/Thr. It is thought to be important for the interaction of proteins with membranes and may also be involved in 'molecular switching,' in conjunction with phosphorylation. For example, the small internal protein, VP4, of most picornaviruses is myristoylated and there is evidence that the protein interacts with cell membranes to create pores or channels when it is externalized during the infection process. The Nef protein of HIV is also myristoylated and this modification is probably involved in its ability to interact with intracellular membranes to dictate its intracellular localization. It is possible that conformational changes, perhaps induced by phosphorylation, may sequester the N-terminal region, including the myristate moiety, within the structure of the protein and so reversibly alter its distribution within the cell. Other modifications, such as sulfation, poly(ADP) ribosylation, and palmitoylation have been reported but their functions are not well understood. In some cases, proteins involved in viral replication are modified by the addition of nucleosides to enable them to function as primers to initiate nucleic acid synthesis. For example, the small genome linked protein, VPg, of picornaviruses is modified by the addition of one or two uridine residues on the side chain of a highly conserved Tyr residue, after which modification it can function to initiate replication of the viral RNA.

GENOME REPLICATION

DNA viruses

A problem inherent in cellular DNA replication is that of terminal loss. The priming and replication systems in normal cells lead to the loss of small amounts of sequence at each round of replication of the chromosomal DNA. This is overcome by the action of telomerase that adds back sequence to the termini so that there is no loss of important sequence. Viruses do not use this nonspecific addition system and have overcome

the problem of terminal loss in several interesting ways. One of the best studied viruses from a replication perspective is SV40 which overcomes the problem by having, in effect, no end. The genome is circular and during replication it produces daughter circles. Most of the enzymes involved in the replication are recruited from the host cell, but the process is initiated and controlled by the multifunctional viral protein, TAg. This binds to the origin of replication sequence as a hexameric complex and opens the complementary DNA strands. It has a helicase function which continues to enlarge the separated domain to allow access by the plethora of proteins involved in normal cell DNA replication. As all DNA is copied in the 3′ to 5′ direction, one strand is replicated discontinuously in a series of small steps (Figure 8). These are primed by short RNA sequences synthesized by DNA primase and extended as DNA sequence by Pol α. The RNA primers are excised by RNase H and the single-stranded region thus exposed is filled in by Pol α and joined to the preceding DNA segment by DNA ligase. The opposite strand is extended continuously by Pol δ from a single RNA primers sequence. Thus replication proceeds as two continuously moving forks, one moving in each direction. When they meet, two double-stranded circular DNA molecules have been replicated from one.

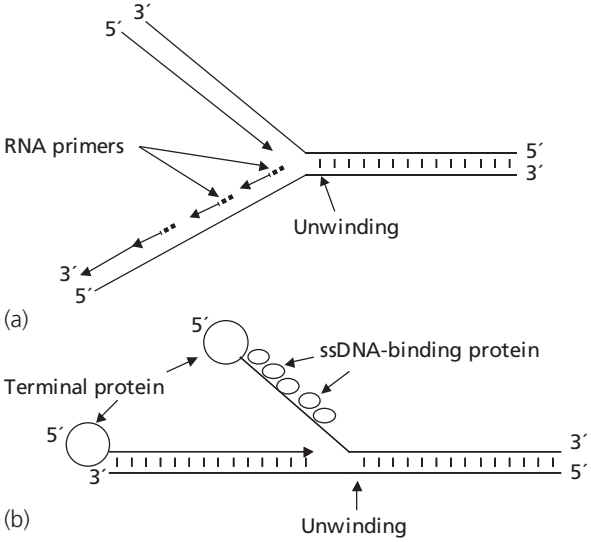

Figure 8 *Genome replication strategies of DNA viruses.* **(a)** *Replication of most double-stranded DNA viral genomes, SV40, for example, proceeds as a replicating fork with each of the parental strands being copied simultaneously. Since DNA polymerase is unidirectional, proceeding from the 3′ end of the template towards the 5′ end, the two parental strands are copied differently, one continuously, the other discontinuously. Discontinuous synthesis is primed by short RNA molecules 9–10 nt long which are subsequently removed when the nascent DNA fragments reach 100–200 nts. The resulting gaps are filled in with deoxyribonucleotides and the discontinuous fragments ligated together.* **(b)** *In the adenoviruses, a single nascent strand displaces one of the parental strands during synthesis. DNA synthesis is primed by terminal protein, which is modified by the addition of nucleotides complementary to the 3′ end of the viral sequence. The progeny molecule is encased in single-stranded DNA binding protein and this complex acts as template for synthesis of a complementary strand to recreate the double-stranded genome. Synthesis of the second strand is also primed by terminal protein.*

Another way of preserving ends is exemplified by the parvoviruses. These single-stranded DNA viruses have inverted repeats at their termini which, therefore, resemble self-priming molecules and are extended to form a hairpin structure. A sequence-specific cleavage occurs near the self complementary loop and the exposed 3′ end is extended to produce a complete double-stranded copy of the genome. Rearrangement of the palendromic end sequences exposes a free 3′ end which is copied back on itself to displace the partner strand as a new single-stranded viral DNA.

Some viruses, such as the adenoviruses, overcome the terminal loss problem by using a protein, to which nucleotides have been covalently attached, as a primer for DNA synthesis. The terminal protein of adenoviruses has a short chain of three nucleotides attached which complement a sequence near the 3′ end of the double-stranded viral DNA. In the presence of the viral polymerase and host factors the end of the DNA is melted and the terminal protein binds via its complementary nucleotides. The complex then jumps back by three nucleotides to bind to a repeat of the recognition sequence located at the extreme 3′ end and then proceeds to copy the entire DNA. Replication proceeds in a single direction, displacing one of the template strands as a single-stranded product (Figure 8). This is converted into a double-stranded molecule by the viral polymerase, again using terminal protein as a primer.

The complementary strands of poxvirus DNA are covalently linked so that the molecule is in effect an unbroken hairpin. The herpes virus replication products are continuous concatenated molecules and are cut into genome length units during the encapsidation process.

‘Reversiviruses’

Retroviruses and hepatitis B viruses alternate between DNA and RNA versions of their genomes at different stages in the replication cycle using reverse transcriptase. In both cases the replication strategies rely on the presence of terminal repeat sequences in the RNA versions of their genomes (Hu and Seeger 1997). Replication starts at a sequence near the 5′ end of the genome RNA. In the case of retroviruses the primer used to initiate replication is a host tRNA, which is extended by the viral reverse trancriptase as a DNA copy. In hepatitis B viruses an N-terminal domain of the polymerase protein acts as a protein primer, recognizing a stem loop structure in the RNA (Figure 9). In both cases the nascent DNA molecule is extended to the end of the template RNA at which stage it is transferred to a

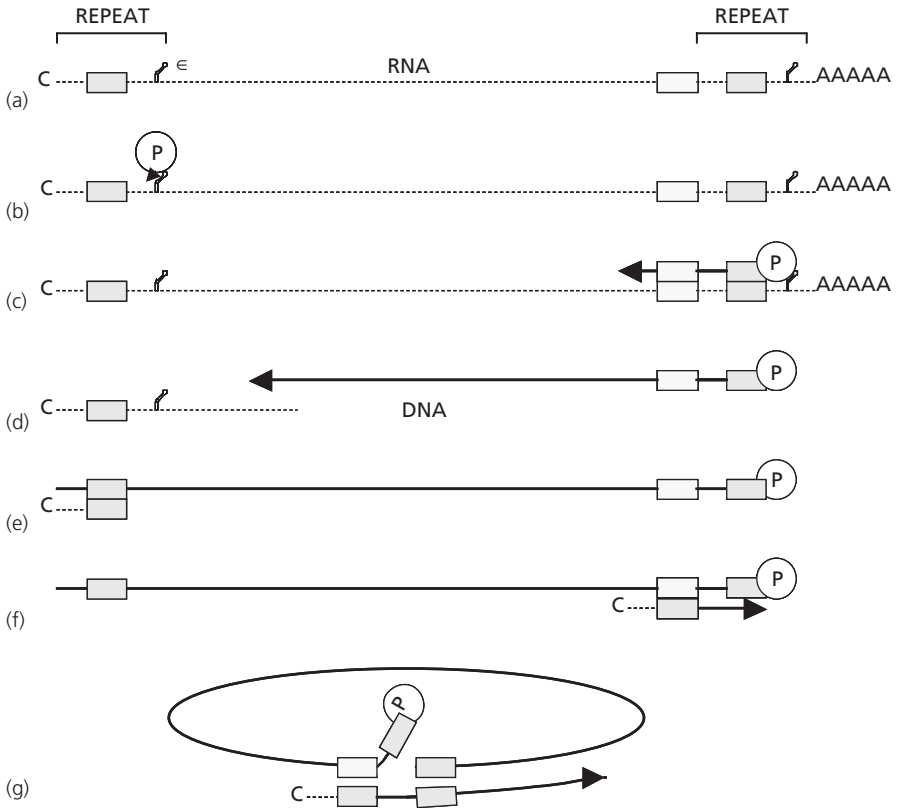

Figure 9 *Replication of the hepadnavirus genome. The genomes of hepadnaviruses (e.g. hepatitis B virus (HBV)) and retroviruses alternate between RNA and DNA forms, but at different stages in their life cycles. In both cases, reverse transcriptase copies RNA into a DNA strand and then functions as a DNA-dependent DNA polymerase to synthesize the complementary DNA strand. Repeated sequences at the ends of the molecules are essential for completion of the process.* **(a)** *The pregenomic RNA showing terminal repeat sequences (boxed) and the ϵ structure at which priming of DNA synthesis occurs.* **(b)** *The polymerase binds to the ϵ sequence and primes DNA synthesis via nucleotides linked to its terminal protein domain.* **(c, d)** *The replication complex with nascent DNA fragment is transferred to a complementary sequence at the 3′ end of the RNA and DNA synthesis proceeds towards the 5′ end of the template. The RNase H function of the polymerase protein digests the RNA template as synthesis proceeds.* **(e)** *A residual RNA fragment remains undigested and this is transferred to a complementary sequence towards the 5′ end of the cDNA to initiate synthesis of the second DNA strand* **(f)**. **(g)** *The molecule is then circularized through interactions of the complementary terminal regions and synthesis of the second strand continues. The replication of retroviral genomes is similar in principle, except that a host tRNA is used as the primer for initiation of DNA synthesis.*

repeat sequences at the 3′ end of the RNA from where it is extended to make a complete DNA copy of the genome. In both cases an RNase H activity is associated with the reverse trancriptase and this removes the RNA template. A small residual RNA sequence is used to prime synthesis of the second strand of DNA using the first strand as template. In the retroviruses, the DNA product is integrated into the host genome whereas the hepatitis B virus DNA remains as an episomal circular molecule in the nucleus. In both cases RNA transcripts, both mRNA and viral RNA are produced by host DNA-dependent RNA polymerase.

RNA viruses

HEPATITIS D VIRUS

Hepatitis D virus is unique amongst animal viruses and has a single-stranded circular RNA genome. It encodes a single viral protein which modifies host-cell polymerase to enable it to make transcripts from the viral genome. The transcript is synthesized as a continuous molecule which is pealed off from the circular template by a ‘rolling circle’ mechanism. An 83 nucleotide sequence within the viral RNA is highly structured and acts as an RNA enzyme or ribozyme (Shih and Been 2002). This is able to cut the RNA at a specific nucleotide to produce genome length segments of RNA and to ligate the ends to regenerate a circular molecule. The ribozyme functions on both the positive- and negative-sense RNAs.

POSITIVE-STRAND RNA VIRUSES

The genomes of the positive-strand RNA viruses must perform two apparently incompatible functions: to act as mRNA for viral protein translation and as templates for viral replication. As these functions start from opposite

ends of the RNA, there is the potential for conformational clashes for molecules performing both functions simultaneously. How this is prevented is only just beginning to be resolved and appears to involve crosstalk between the termini of the RNA by host and protein factors (Gamarnik and Andino 1998). Priming can involve a protein primer, as in the picornaviruses, or the polymerase can initiate synthesis de novo as in the case of hepatitis C virus. Picornavirus RNA replication is primed by the small protein, VPg, which is urydilated by the viral polymerase by a poorly understood mechanism which also involves a conserved stem–loop structure in the RNA, the *cis*-acting replication element (CRE). The primer-independent initiation seen in some viruses appears to require precise structural features of the polymerase to ensure that synthesis starts with the correct nucleotide. Progeny positive-strand RNA is typically produced in large excess ($\times 10$) over negative strand, but the mechanisms governing this imbalance are poorly understood.

NEGATIVE-STRAND RNA VIRUSES

In contrast to positive-strand viruses, the genomic RNA of negative-strand RNA viruses is always found as a ribonucleoprotein complex. This has to function as the template for the transcription of mRNAs and as template for genome replication. The switch from mRNA template to genome template function is determined by the levels of nucleoprotein available in the cell (Portela and Digard 2002). As this increases later in infection so the nascent transcripts are encapsidated and the polymerase functions in an antitermination mode, ignoring the sequence signals important for transcription of individual mRNAs. More recent evidence suggests that for VSV the P and L proteins are important, in addition to the nucleocapsid protein, N, and that different complexes are involved in transcription or replication.

DOUBLE-STRANDED RNA VIRUSES

The replication of the double-stranded RNA viruses occurs entirely within viral core particles. The infecting virus particle is stripped of its outer protein shells to reveal the core particle which contains multiple double-stranded RNA genome segments and the transcription machinery. This transcriptional machine is highly ordered and extrudes nascent positive-strand progeny RNA molecules through pores at the vertices of the icosahedral particle. These act as templates for both translation and for the production of new double-stranded viral genomes. The latter event occurs within newly assembled core particles which can then function to amplify the production of further positive-strand RNAs or to assemble into progeny virus particles. A fascinating and as yet unresolved feature of the double-stranded RNA viruses is that each core particle appears to assemble with a complete set of genome RNA segments. Part of the supporting evidence for this is that these viruses have very high infectivity to particle ratios, implying that all or most particles must contain a complete set of genome segments. How this selection process is controlled is not known.

RNA synthesis does not require a primer and the crystal structure of the polymerase of the double-stranded RNA phage, $\phi 6$, has demonstrated a novel mechanism for the initiation of replication. The structure of the active site pocket of the enzyme is so constructed that the 3′ end of the template strand abuts a structural 'wall' which holds it in place to pair with the first substrate molecules. As these are covalently linked the template strand progresses though the active site, effectively pushing the restraining wall from its path. Interestingly, studies of the crystal structure of the polymerase of hepatitis C virus, which also initiates RNA synthesis in a primer-independent fashion, suggest that a similar mechanism operates in that virus (van Dijk et al. 2004).

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Negative-strand RNA virus replication

RICHARD M. ELLIOTT

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As a group, negative-strand RNA viruses include some of the most dreaded human viruses, such as Ebola virus, Lassa fever virus, Crimean-Congo hemorrhagic fever virus and rabies virus, as well as the causes of many common human infections such as influenza, measles, and mumps. Some negative-strand viruses infect domestic animals or plants, causing substantial disease and crop losses, and thus the overall impact of these viruses on human well-being is immense. Negative-strand viruses are united by the coding strategy of their genomes, though they display great variation in both biological and molecular characteristics. In this chapter, an overview of common features in replication is presented; more detailed reviews are provided by the subsequent virus-specific chapters.

CLASSIFICATION OF NEGATIVE-STRAND RNA VIRUSES

By convention, mRNA is termed the plus or positive strand as it contains protein coding information in the directly translatable form. Baltimore (1971) developed a classification scheme for viruses based on the relationship of viral genomic nucleic acid to viral mRNA (Figure 1). Hence a strand of genomic nucleic acid that is of equivalent polarity to mRNA is also called positive, and the complement of the positive strand is called negative. Thus viruses could be divided into a series of classes, of which class V comprises the negative-strand RNA viruses. Although this classification scheme is little used today, the concept of positive- and negative-strand RNA viruses remains. (Note that the terms 'strand' and 'sense,' as in negative strand or negative sense, can be and are used interchangeably).

Viral classification is now under the auspices of the International Committee on Taxonomy of Viruses, which publishes regularly updated reports on classification in both printed (7th Report; Van Regenmortel et al. 2000) and World Wide Web (www.ncbi.nlm.nih.gov/ICTVdb/index.htm) media. There are seven families of negative-strand RNA viruses and three orphan genera that are not yet incorporated into higher taxons (Table 1, p. 3). The families can be separated into two groups, those whose genomes comprise a single molecule of RNA (these four families make up the order *Mononegavirales*; see The Order Mononegavirales) and those that have segmented genomes. Six of the families contain viruses that cause disease in humans, ranging from respiratory tract disease, through encephalitis to hemorrhagic fever. *Bornavirus*, the sole member of the *Bornaviridae* family, has been implicated in neurological disease in horses, and may be linked to psychiatric disease in humans, though this remains controversial (Staeheli et al. 2000). In addition, some viruses in the families *Rhabdoviridae* and *Bunyaviridae* can infect plants. Viruses in the orphan genera *Ophiovirus* and *Tenuivirus* also infect plants and share some similarities with bunyaviruses (van Regenmortel et al.

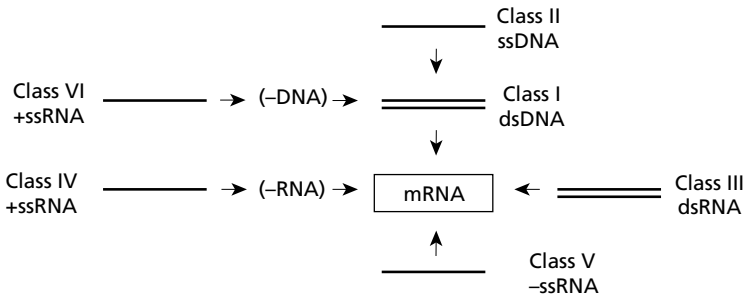

Figure 1 *The Baltimore Classification System. Viruses are classified according to the polarity and strategy of their genomes to produce mRNA. By convention, mRNA is designated the positive strand.*

2000). *Hepatitis delta virus* (HDV), the lone virus of the *Deltavirus* genus, is not usually mentioned when discussing negative-strand viruses, but is included in Table 1 for completeness. HDV has a 1.7 kb circular, negative-strand RNA genome that is heavily base-paired and more akin to a viroid genome. The genome encodes a single known product, the delta antigen, though this exists in two forms (large and small). HDV is obligatorily dependent on coinfection with *Hepatitis B virus* (HBV) for replication, and uses several HBV proteins to form its viral particle (Lai 1995; Gerin et al. 2002). HDV genome replication is quite distinct from that of the 'classical' negative-strand viruses; it occurs in the nucleus by a rolling-circle mechanism driven by cellular RNA polymerase II. The reader is referred to

Hepatitis delta virus for a detailed description.

OVERVIEW OF NEGATIVE-STRAND VIRUS REPLICATION

The implications for a virus possessing a negative-stranded genome are as follows: the genomic RNA itself cannot be translated into proteins directly, but must serve as the template from which positive-sense mRNA is copied. Since eukaryotic cells do not contain the appropriate enzyme to carry out this process – an RNA-dependent RNA polymerase (RdRp) – the infecting virus must encode and bring into the cell its own RdRp. As a consequence, and in contrast to positive-strand RNA viruses (Positive-strand RNA virus replication in vertebrate hosts), the isolated genomic RNA of a negative-strand RNA virus is not itself infectious.

All negative-strand RNA viruses share the property that the genomic RNA (and the antigenomic RNA that is synthesized during replication; Figure 2, p. 4) is encapsidated by the viral nucleoprotein (N or NP) and is associated with the viral polymerase components to form a ribonucleoprotein complex (RNP) or nucleocapsid. A feature of all negative-strand RNA viruses is that the RNP has helical symmetry. Both genomic and antigenomic RNAs are only found in RNPs and never as naked RNA, implying that encapsidation occurs concurrently during RNA synthesis. Furthermore, only RNP is the functional template for both transcription and replication.

In simplistic terms, all negative-strand RNA viruses replicate in a similar manner (Figure 2). After uncoating of the infecting virion, the genomic RNA (as RNP) is transcribed by the viral RdRp to produce viral mRNAs. These are translated to produce viral proteins, and once sufficient levels of protein have accumulated in the cell, the virus switches to replication mode. The same genomic RNP complex is the template for synthesis of an exact complementary copy of the genomic RNA – the antigenome – that is also encapsidated by the nucleoprotein. (Although this molecule is a positive-sense RNA, encapsidation by N protein prevents access to ribosomes and hence the antigenome cannot function as an mRNA). The antigenomic RNP is then the template for repeated synthesis of new genomic RNA molecules. These can be either packaged into progeny virus particles or act as new templates for further mRNA synthesis, termed secondary transcription. However, there are some key differences in the replication processes between nonsegmented and segmented genomes. In addition, the ambisense coding strategy (Bishop 1986; Nguyen and Haenni 2003) adopted by arenaviruses and some bunyaviruses shows further variation on the theme.

NONSEGMENTED NEGATIVE-STRAND RNA VIRUSES

Although the genomic RNA is packaged into helical RNP and the viruses are enveloped, the four families of nonsegmented negative-strand RNA viruses display a range of morphologies from spherical/pleomorphic (bornaviruses and paramyxoviruses), through bullet-shaped (rhabdoviruses) to filamentous (filoviruses), though variation is seen even within a family. Viruses in all four families share a core complement of genes that are arranged on the genome in a similar order (Strauss et al. 1996). The simplest genome structure is shown by rhabdoviruses (Figure 3, p. 4), such as vesicular stomatitis virus (VSV), which has just the core complement: N, P (phosphoprotein, a polymerase subunit), M (matrix), G (surface glycoprotein), and L (RdRp). More

Table 1 *Classification of negative-strand RNA viruses*

Family/subfamily/genus	Genome segments/ Size (kb)[a]	Site of replication	Host	Type virus[b] or virus associated with human disease
Order *Mononegavirales*				
Bornaviridae				
Borna disease virus	1/9	Nucleus	Vertebrate	Bornavirus: neuropsychiatric?
Filoviridae				
'Marburg-like viruses'	1/17	Cytoplasm	Vertebrate	Marburg virus: hemorrhagic fever
'Ebola-like viruses'	1/17	Cytoplasm	Vertebrate	Ebola virus: hemorrhagic fever
Paramyxoviridae				
Paramyxovirinae				
Respirovirus	1/15	Cytoplasm	Vertebrate	Human parainfluenza virus type 1: respiratory disease
Morbillivirus	1/16	Cytoplasm	Vertebrate	Measles virus: measles
Rubulavirus	1/15	Cytoplasm	Vertebrate	Mumps virus: mumps
Henipavirus	1/18	Cytoplasm	Vertebrate	Hendra virus: respiratory disease; Nipah virus: encephalitis
Avulavirus	1/15	Cytoplasm	Vertebrate	(Newcastle disease virus)
Pneumovirinae				
Pneumovirus	1/15	Cytoplasm	Vertebrate	Human respiratory syncytial virus: respiratory disease; croup
Metapneumovirus	1/13	Cytoplasm	Vertebrate	Human metapneumovirus: respiratory disease
Rhabdoviridae				
Vesiculovirus	1/11	Cytoplasm	Vertebrate	(Vesicular stomatitis Indiana virus)
Lyssavirus	1/12	Cytoplasm	Vertebrate	Rabies virus: rabies
Ephemerovirus	1/15	Cytoplasm	Vertebrate	(Bovine ephemeral fever virus)
Cytorhabdovirus	1/nc	Cytoplasm	Plant	(Lettuce necrotic yellows virus)
Nucleorhabdovirus	1/14	Cytoplasm	Plant	(Potato yellow dwarf virus)
Novirhabdovirus	1/11	Cytoplasm	Plant	(Infectious hematopoietic necrosis virus)
Bunyaviridae				
Orthobunyavirus	3/12	Cytoplasm	Invertebrate/ vertebrate	La Crosse virus: encephalitis; Oropouche virus: fever
Hantavirus	3/12	Cytoplasm	Vertebrate	Hantaan virus: hemorrhagic fever with renal syndrome; Sin Nombre virus: hantavirus pulmonary syndrome
Nairovirus	3/19	Cytoplasm	Invertebrate/ vertebrate	Crimean-Congo hemorrhagic fever virus: hemorrhagic fever
Phlebovirus	3/12	Cytoplasm	Invertebrate/ vertebrate	Rift Valley fever virus: hemorrhagic fever, retinitis
Tospovirus	3/18	Cytoplasm	Invertebrate/ plant	(Tomato spotted wilt virus)
Arenaviridae				
Arenavirus	2/11	Cytoplasm	Vertebrate	Lassa virus: hemorrhagic fever
Orthomyxoviridae				
Influenzavirus A	8/15	Nucleus	Vertebrate	Influenza A virus: influenza
Influenzavirus B	8/15	Nucleus	Vertebrate	Influenza B virus: influenza
Influenzavirus C	7/12	Nucleus	Vertebrate	Influenza C virus: respiratory disease
Thogotovirus	6/12	Nucleus	Invertebrate/ vertebrate	(Thogoto virus)
Isavirus	8/nc	Nucleus	Fish	(Infectious salmon anemia virus)
Orphan genera				
Ophiovirus	3/12	Cytoplasm	Plant	(Citrus psorosis virus)
Tenuivirus	4/5/18	Cytoplasm	Plant	(Rice stripe virus)
Deltavirus	1 (circular)/1.7	Nucleus	Vertebrate	Hepatitis delta virus: hepatitis

a) Approximate size; nc, not completed.
b) Nonhuman-type viruses are in parenthesis.

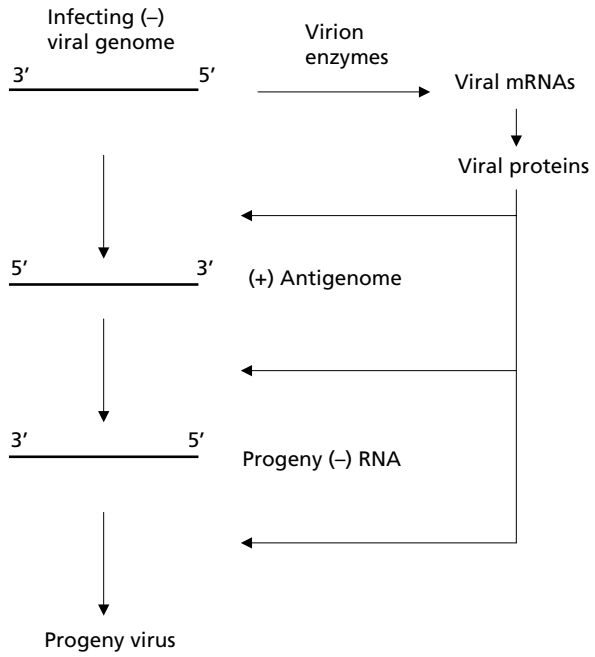

Figure 2 *Basic replication of negative-strand RNA viruses. The genome of the infecting virion is transcribed by the viral RdRp to produce viral mRNAs. The viral mRNAs are translated into viral proteins, that in turn are involved in replicating the infecting genome RNA via a complementary positive-strand RNA (the antigenome) and packaging into progeny virions.*

complex genomes such as paramyxoviruses encode other gene products such as additional glycoproteins and also nonstructural proteins. In all cases the genome is bounded by noncoding leader and trailer sequences, and each gene is flanked by transcriptional start and stop sequences. The internal genes are separated by intergenic sequences. Intergenic sequences range from just 2 nt in VSV to more than 60 nt in some paramyxoviruses. The gene stop sequences contain short tracts of U residues that template, via reiterative copying, the addition of a poly(A) tail at the 3′ end of the viral mRNA (Poon et al., 1999; Figure 3).

The generally accepted stop–start model of transcription (Banerjee et al. 1977; Emerson 1982), based largely on in vitro studies, is that viral RdRp begins RNA copying at a single promoter site at the 3′ end of the genome (Figure 3), and that termination of an upstream gene is required for subsequent transcription of the downstream gene. For VSV, a 47 nt leader RNA is synthesized and then released, and the RdRp initiates synthesis of the first mRNA, for N protein. The RdRp possesses capping activity so that the viral mRNAs have 5′ cap structures (m^7GpppG) similar to host cell mRNAs. After polyadenylation, the mRNA is released and the polymerase reinitiates after the intergenic sequence to synthesize the next mRNA. Transcription of the genome is thus sequential. During transcription, there is attenuation at each initiation step (the RdRp sometimes dissociates from the template and does not reinitiate), such that there is a gradient in the number of copies of each mRNA produced: N mRNA is most abundant and L mRNA the least abundant (Villareal et al. 1976; Iverson and Rose 1981). This is an important regulatory step for the virus in controlling the different amounts of each viral protein needed. For VSV, recombinant viruses have been created by reverse genetics (see later) in which the gene order has been rearranged (Wertz et al. 1998); although viable, such viruses grow less well than wild-type, confirming the importance of mRNA attenuation in viral replication.

Viral proteins are translated from the mRNAs and the amounts of these produced, particularly the N protein,

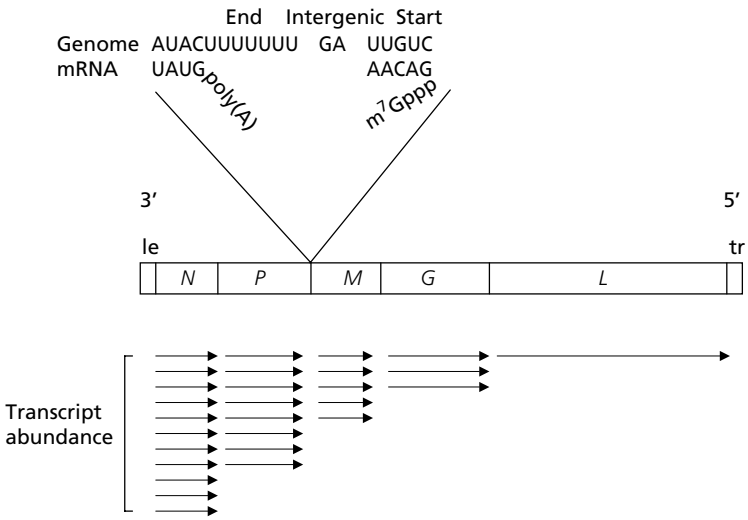

Figure 3 *Transcription of a nonsegmented negative strand RNA genome. The rhabdovirus VSV is used as an example. The five viral genes (*N, P, M, G, *and* L*) are flanked by leader (le) and trailer (tr) sequences. At each gene junction are found transcription termination and polyadenylation signals (end), an intergenic sequence (just 2 nt in the case of VSV) and a transcription initiation signal (start). There is attenuation of the polymerase at each junction, resulting in a gradient of transcript abundance from the 3′ to 5′ direction.*

are thought to signal the switch from transcription to replication. Sufficient N protein must be available to encapsidate the nascent antigenome RNA. Under these conditions the RdRp does not recognize the mRNA termination or polyadenylation signals but produces a full-length positive-strand RNA molecule that is cotranscriptionally encapsidated by N protein to make a positive-strand RNP. This in turn becomes the template for the synthesis of progeny genomic RNA, which again is cotranscriptionally encapsidated into RNP.

Recent in vivo studies (Whelan and Wertz 2002), however, provide evidence that the VSV polymerase can initiate transcription directly on the *N* gene start, and that prior transcription of leader RNA is not required. Thus the authors hypothesize that the regulation of transcription versus replication is through the polymerase binding to alternative sites on the genome: internally at the *N* gene for transcription and at the extreme 3′ end for replication. While this model has attractions, the factors responsible for which initiation site is used (possibly modification of the template or of the RdRp, or role of host-cell proteins, etc.) remain to be determined.

SEGMENTED GENOME NEGATIVE-STRAND RNA VIRUSES

A significant difference between orthomyxoviruses (e.g. influenza virus) and bunya- and arenaviruses is that the former transcribe and replicate their genomes in the nucleus. This presents an additional dimension to viral replication – transport of viral components in and out of the nucleus. After infection, the influenza virus RNPs must enter the nucleus, newly transcribed mRNAs exit the nucleus to be translated, viral proteins involved in RNP formation must travel back into the nucleus, and progeny negative-strand RNPs must transport out of the nucleus for virus assembly and release through the plasma membrane (Portela and Digard 2002). As orthomyxovirus mRNAs are produced in the nucleus, they can avail themselves of the cellular mRNA splicing machinery; for example, influenza A virus synthesizes differently spliced forms of mRNAs derived from RNA segments 7 and 8. These are translated into different proteins, thus increasing the coding capacity of these genome segments (Lamb and Krug 2001).

mRNA synthesis of all groups of segmented negative-strand RNA viruses is a primer-dependent process (Figure 4). The primers are derived from the capped 5′ ends of cellular mRNAs that are cleaved by an endonuclease activity contained within the PB2 protein of influenza virus or the L protein of bunya- and arenaviruses. The capped primers are incorporated into the viral mRNA, such that the viral mRNAs contain short (12–18 nt) nontemplated heterogenous sequences at their 5′ ends (Plotch et al. 1981). Thus these viruses do not need to encode capping functions unlike members of the *Mononegavirales*. This process, termed 'cap-snatching,' occurs in the nucleus for orthomyxoviruses and there-

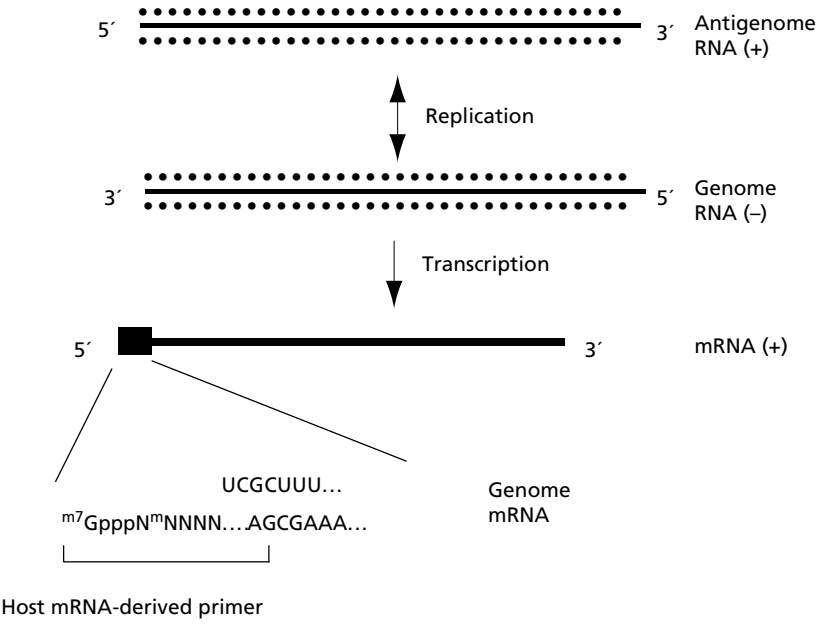

Figure 4 *Transcription and replication of a segmented negative-strand RNA virus. The genome and antigenome RNAs are shown encapsidated by nucleocapsid protein. Transcription to produce mRNA is a primer-dependent process where the capped, 5′ ends of host-cell mRNAs are cleaved by a viral endonuclease and the primers incorporated into the viral mRNA. The primers are usually 12–18 nt in length and heterogenous in sequence, though cleavage usually occurs at a base complementary to the 3′ end of the viral genomic RNA. The example shows the 5′ end of an influenza A virus mRNA. Viral mRNAs are truncated relative to the genome RNA, and are not encapsidated by nucleocapsid protein.*

fore needs ongoing host cell transcription; hence orthomyxovirus replication is inhibited by drugs, such as α-amanitin, which prevent host transcription. By contrast, bunya- and arenaviruses cap-snatch in the cytoplasm from previously synthesized host mRNAs and their replication is not inhibited by α-amanitin. Orthomyxovirus mRNAs are polyadenylated, signaled by a polyU stretch in the genomic RNA. Similar to the RdRp of nonsegmented viruses, the orthomyxovirus polymerase stutters on this sequence to add a poly(A) tail (Poon et al. 1999). By contrast, neither bunya- nor arenavirus mRNAs are polyadenylated (Nguyen and Haenni 2003).

The promoters for mRNA synthesis are located in the terminal untranslated regions of each genome segment. The 3′ and 5′ terminal sequences show a degree of complementarity that permits base-pairing between the ends, creating a panhandle, and accounts for the circular appearance of isolated nucleocapsids (Hsu et al. 1987; Pardigon et al. 1982). The relative strength of each promoter regulates the mRNA transcript abundance from each segment (Barr et al. 2003). Similar to the nonsegmented genome viruses, the concentration of viral N protein is thought to be involved in the switch to replication. RNA replication is a primer-independent event that results in production of full-length positive-strand RNAs (antigenomes) that are assembled into RNPs; these in turn serve as templates for synthesis of progeny genome RNA segments. Like the nonsegmented genome viruses, the newly synthesized genomic RNPs can be packaged into new virions or are involved in secondary transcription. In order to generate an infectious virus particle, one copy of each segment (at least) must be packaged, and there is debate over whether this is a random or specific event. Extraction of viral RNA from purified virions often reveals nonequimolar ratios of RNA, and it could be that virions contain more than just, for example, eight (influenza A virus) or three (bunyavirus) RNA segments. Recent work on influenza A virus has provided evidence for specific packaging signals that span the noncoding/coding region of some segments (Fujii et al. 2003).

AMBISENSE-CONTAINING VIRUSES

A variant of the classic negative-sense coding strategy is displayed by some genome segments of certain bunyaviruses and both segments of arenaviruses (Bishop, 1986; Nguyen and Haenni, 2003; Figure 5). Analysis of the genome RNA segments of these viruses reveals two open reading frames (ORF), one negative-sense and one positive-sense. These are separated by an intergenic region that typically shows secondary structure (often a stem-loop). The negative-sense ORF is translated from a subgenomic mRNA transcribed from the infecting genome in the usual manner, with transcription termination occurring at the intergenic structured RNA sequence.

The positive-sense ORF cannot be translated directly from the infecting genome since it is in an RNP form, but from a second subgenomic mRNA that is transcribed from the antigenomic RNA (Figure 5). Thus the negative-sense ORF is expressed early, whereas the positive-sense ORF is expressed later in infection, once genome replication has begun, giving the virus a degree of temporal regulation of gene expression. In all other regards, however, ambisense viruses replicate similarly to their strictly negative-sense counterparts.

GENETIC VARIATION, RECOMBINATION, AND REASSORTMENT

A particular feature of all RNA viruses is their genetic variability that is much higher than DNA genome viruses (Holmes 2003). This is because viral RdRp do not contain proofreading activity and thus when incorrect nucleotides are incorporated into the nascent RNA

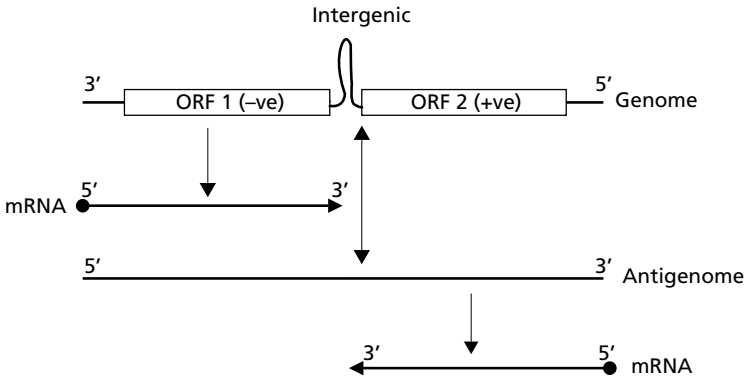

Figure 5 *Transcription and replication of an ambisense RNA. The genome RNA contains ORFs in both negative- and positive-sense orientations, separated by an intergenic sequence that usually shows secondary structure. Each ORF is translated from a subgenomic mRNA (transcribed in a primer-dependent manner), with the mRNA for ORF2 being transcribed from the full-length antigenome RNA. This means that the protein encoded by ORF2 is translated relatively late during the replication cycle, after the onset of genome replication itself.*

strand there is no means to correct them. The error frequency of RNA virus polymerases is in the range 10^{-3}–10^{-4}, resulting in rapid evolution of the virus. In fact, the resulting progeny genomes will each probably contain a nucleotide difference from the master or consensus sequence such that, rather than a homogeneous sequence, the virus consists of a population of related sequences known as a quasispecies. A consequence of this rapid evolution is that many progeny genomes will carry lethal mutations. The implications of this are to limit the size of RNA genomes – the bigger the genome the more chance of incorporating a lethal mutation. The positive-strand coronaviruses have the largest known RNA genomes, up to 32 kb (Coronaviruses, toroviruses, and arteriviruses), and the negative-strand viruses have genomes less than 20 kb. The ability of viruses to evolve and adapt to, for example, host immune pressure is clearly seen with influenza virus where the accumulation of point mutations year on year in the genes encoding the hemagglutinin and neuraminidase surface glycoproteins (antigenic drift) necessitates annual vaccination to maintain protection.

Genetic recombination – the breaking and joining of distinct nucleic acid molecules – has been regarded as a rare occurrence in negative-strand RNA viruses, but evidence is accumulating to suggest that it happens more frequently than previously thought, for instance in hantaviruses, arenaviruses, and paramyxoviruses such as mumps virus and Newcastle disease virus (Chare et al. 2003). However, at present, recombination is not seen as a major evolutionary force in these viruses.

By contrast, genome segment reassortment among the segmented viruses occurs frequently during the course of mixed infection. In this way progeny viruses may contain a genome comprising segments derived from each of the parents, though reasssortment is not entirely random. For as yet unknown reasons, certain genetic constellations are apparently not viable. Reassortment has been studied extensively in influenza viruses, where it is acknowledged as the route by which pandemic influenza virus strains arise – so-called antigenic shift (Reid and Taubenberger 2003). 'New' viruses emerge containing surface antigens not previously experienced by the human population (often the surface genes of an avian influenza virus) and thus the new strains cause havoc among the immunologically naïve population.

MAXIMIZING GENOME CODING POTENTIAL

Although all the viruses discussed in this chapter show the property of a negative-sense coding strategy, the viruses implement this in a number of variations to maximize the protein coding content of their genomes. The genomes encode protein highly efficiently – in general >90 percent of the genome encodes protein. As discussed above, influenza viruses encode different gene products from the same segment by splicing of the transcribed mRNAs. Orthobunyaviruses employ overlapping reading frames to encode two proteins on the S segment, both proteins being translated (unusually for a eukaryotic system) from the same mRNA (Bishop 1996). However, the extreme example of multiple protein expression from a single gene is shown by the paramyxovirus P gene, where up to six distinct proteins may be produced (Curran et al. 1998). Most paramyxoviruses exhibit RNA 'editing' during transcription of their P mRNAs, where nontemplated G residues (usually one, two or four Gs) are inserted, causing a frame-shift in the encoded mRNA. Thus different forms of mRNA are synthesized, encoding different proteins. Coupled with alternate initiation codon usage, including noncanonical AUG codons, a plethora of proteins can be produced from the single *P* gene. RNA editing also occurs in the glycoprotein mRNA of Ebola virus, producing both full-length and truncated glycoprotein products (Sanchez et al. 1996).

THE MODERN APPROACH TO STUDY RNA VIRUS REPLICATION: REVERSE GENETICS

One of the impediments to detailed analysis of RNA virus replication has been the nature of the genetic material itself; RNA is biochemically more labile than DNA, and techniques to produce specific nucleotide alterations directly in an RNA molecule are not available. In the field of RNA virology, 'reverse genetics' is usually described as the ability to recover (rescue) an infectious virus from cloned complementary DNA. Thus the viral genome can be manipulated at the DNA level using the full gamut of recombinant DNA protocols, and then virus recovered containing defined mutations. The phenotype of the mutant virus can then be related to the genotypic changes (hence **reverse** genetics). Reverse genetics of RNA viruses actually began in 1978 with the recovery of an RNA bacteriophage, Q**β**, from cloned cDNA (Taniguchi et al. 1978), and the first animal virus rescued from cDNA was the positive-strand poliovirus in 1981 (Racaniello and Baltimore 1981). However, progress on developing similar methodologies for negative-strand RNA viruses was more difficult, in part because of the requirement to produce a biologically active RNP form isolated components.

A system to manipulate individual genome segments of influenza A virus was reported by Luytjes et al. in 1989, but the first recovery of a negative-strand RNA virus entirely from cDNA was not achieved until 1994: perhaps surprisingly this was rabies virus by Schnell et al. (1994). Since then representatives of paramyxoviruses, filoviruses, bunyaviruses, and orthomyxoviruses have all been rescued entirely from cDNA (Conzelmann 1998; Neumann et al. 2002), and encouraging progress has

been made with bornaviruses and arenaviruses (Lee et al. 2000; Perez et al. 2003) to suggest that these will be rescued soon.

These achievements have led to unprecedented progress in understanding detailed aspects of viral replication, structure–function analyses of viral proteins, possibilities for engineering 'designer' vaccines, and in delineating viral–host interactions. These topics are dealt with in detail in the following chapters.

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Positive-strand RNA virus replication in vertebrate hosts

JO ELLEN BRUNNER AND BERT L. SEMLER

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COMMON CHARACTERISTICS ACROSS FAMILIES

Classification and medical significance

This chapter is organized around the features of the intracellular replicative cycle shared by single-stranded, positive-strand RNA viruses that infect vertebrate hosts. Most of the discussion focuses on specific examples of viruses from the families *Picornaviridae*, *Caliciviridae*, *Flaviviridae*, *Togaviridae*, and *Coronaviridae*. Replication of single-stranded, positive-strand RNA plant viruses, a significant proportion of the viruses in this class, will not be covered here. The term positive-strand RNA refers to the taxonomic criterion proposed by Baltimore (Baltimore 1971) to classify viruses, namely, the relationship between the viral genome and the messenger RNA used for translation during the process of viral gene expression. Accordingly, a positive-strand RNA (class IV) virus has a genome that is identical to messenger RNA and competent for translation once released in the cytoplasm of the host cell. This scheme for virus classification does not necessarily take into account the more current genetic information which would form an additional basis for possible reassignments of viruses into superfamilies.

Poliovirus, the prototypic member of *Picornaviridae*, is arguably the most comprehensively studied mammalian single-stranded, positive-strand RNA virus. Much of the information and many of the mechanisms presented here come from the large body of knowledge relating to poliovirus. The majority of the early research concerning the biology and pathogenesis of the virus was motivated by the desire to diminish the human suffering caused by poliomyelitis, and subsequently led to the development of two effective vaccines. As a direct consequence of the widespread application of the Salk and Sabin vaccines during the last five decades, world health officials envision that poliomyelitis may be eradicated worldwide by the end of the year 2005. Ironically, the same scientific research which led to breakthroughs in the understanding of the molecular biology of poliovirus now creates the possibility that this virus could be reconstructed as an agent of bioterrorism in the post-eradication era (Cello et al. 2002).

Human immunodeficiency virus (HIV) is responsible for the current pandemic of acquired immunodeficiency syndrome (AIDS) and the associated climbing levels of worldwide morbidity and mortality. HIV and the other members of the *Retroviridae* are covered in subsequent chapters due to the fact that several aspects of their intracellular replication cycles are unique among single-stranded, positive-strand RNA viruses. Nonetheless, two other viruses of currently increasing medical importance globally, *West Nile virus* (WNV) (*Flaviviridae*) (Briese et al. 1999; Lanciotti et al. 1999; Jordan et al. 2000; Brinton 2002) and severe acute respiratory syndrome virus (SARS-CoV) (representing a new group of *Coronaviridae*) (Marra et al. 2003; Rota et al. 2003) are introduced.

Limited coding capacity of small genomes

The limited coding capacity of the relatively small genomes typical of most single-stranded, positive-strand

RNA viruses (approximately 4–30 kilobases) necessitates gene expression strategies aimed at generating multiple viral proteins or activities from apparent mono- or oligo-cistronic genomic RNAs (Figure 1). Viruses in the families *Picornaviridae* and *Flaviviridae* contain a single open reading frame (ORF) within their genomic RNAs which encodes a polyprotein that is processed into functional precursors and mature proteins (Agol et al. 1999; Brinton 2002; Leong et al. 2002). The viral structural proteins are encoded within the 5′ region of the genomic ORF, while the nonstructural proteins are encoded within the 3′ region. Human caliciviruses have small genomes that contain either three [Norwalk-like viruses (NLV)] or two [Sapporo-like viruses (SLV)] ORFs. NLVs express ORF1 as a polyprotein precursor that is cleaved by the viral 3C-like (picornaviruses) proteinase into the nonstructural proteins. ORF2 encodes the single major structural protein, and ORF3 encodes a small basic protein of unknown function (Clarke and Lambden 1997; Robinson et al. 2002). Other families within this class, *Togaviridae* and *Coronaviridae*, with their larger, more complex genomes, follow an expression strategy that involves discontinuous RNA synthesis. For togaviruses, production of subenomic positive-strand RNA(s) is templated from a full-length negative-strand intermediate RNA (Sawicki and Sawicki 1998a). A polyprotein processed into the nonstructural proteins is encoded by the 5′ end of the genomic RNA. A single subgenomic positive-strand RNA representing the 3′ end of the genome is translated into a polyprotein that is the precursor to the viral structural proteins.

For coronaviruses, the favored model of gene expression involves production of a set of subgenomic negative-strand RNAs, derived from discontinuous transcription of genomic RNA, which will serve as templates for transcription of subgenomic mRNAs (Makino et al. 1988; Sawicki and Sawicki 1998b). As with togaviruses, a polyprotein processed into the nonstructural proteins is encoded by the 5′ two-thirds of the genomic RNA. Coronaviruses generate a nested set of 3′ coterminal subgenomic positive-strand RNAs corresponding to the 3′ end of the genome. Each of these RNAs has at its 5′ end an approximately 70 nt long, capped leader sequence derived from the 5′ end of the genome (Holmes and Enjuanes 2003). The unique portion of the 5′ end of each mRNA is translated to synthesize an individual viral structural or nonstructural protein even though most of these mRNAs contain more than one ORF. Marra et al. identified nine potential ORFs within the SARS-CoV genome that could encode nonstructural proteins unique to this virus (Marra et al. 2003).

Another strategy which in effect amplifies the information encoded by the genome is the production of viral gene products that are multifunctional. The poliovirus precursor polypeptide, 3CD, is involved in protein

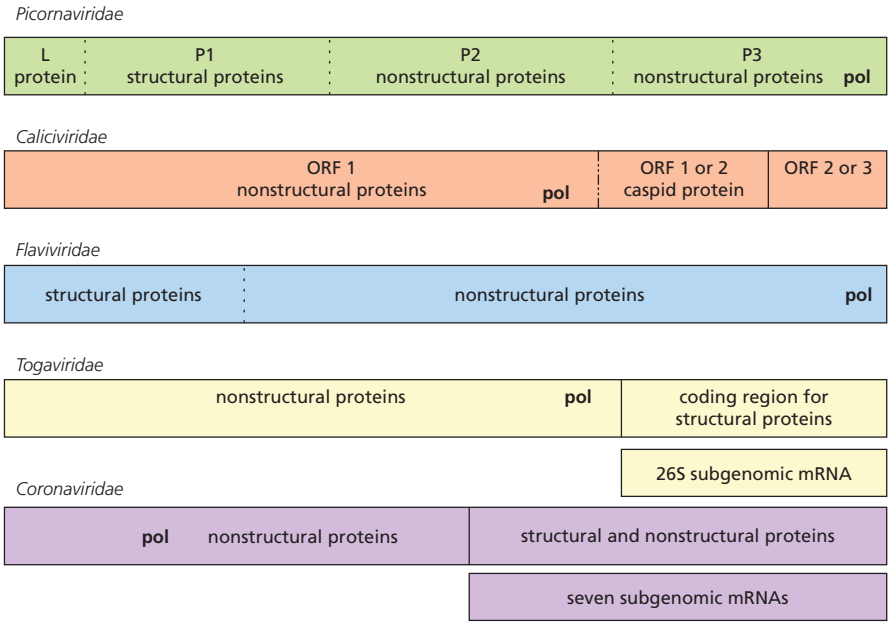

Figure 1 *Comparison of genome organization for five positive-strand RNA virus families. Picornaviruses and flaviviruses have a single ORF within their genomic RNAs. The structural proteins are encoded by the 5′ region of the genome and the nonstructural proteins are encoded by the 3′ region. Human caliciviruses have either two or three ORFs. The capsid protein is encoded by the 3′ terminus of ORF1 or ORF2. For togaviruses, a polyprotein processed into the nonstructural proteins is encoded by the 5′ region of the genomic RNA. A single subgenomic positive-strand RNA corresponding to the 3′ region of the genome is translated into a precursor to the viral structural proteins. For coronaviruses, a polyprotein processed into the nonstructural proteins is encoded by the 5′ two-thirds of the genomic RNA. Seven 3′ coterminal subgenomic positive-strand RNAs encode the structural proteins as well as some of the nonstructural proteins.*

processing as well as RNA replication, by way of replication complex formation with host and viral proteins at the 5′ and 3′ ends of the viral genome (Andino et al. 1990, 1993; Herold and Andino 2001). The NS5 protein of the *Flavivirus*, WNV, has methyltransferase and RNA polymerase activities (Brinton 2002). In fact, although the 11 kb genome of WNV is of intermediate length for a positive-strand RNA virus, most of the nonstructural proteins are multifunctional.

Investigators have long recognized that replication of many RNA viruses is cell-type specific, suggesting that there is a dependence on cell-specific factors (Lai 1998). RNA viruses are known to subvert host proteins that are normal components of cellular RNA processing or translation machineries for their own translation, replication, and packaging, another approach by which these viruses overcome the limited coding potential of their genomes (Table 1). The most frequently observed cellular proteins thought to be involved in viral RNA synthesis are the heterogeneous nuclear ribonucleoprotein (hnRNP) complex proteins. Several of these RNA-binding proteins, normally involved in assembly of the ribonucleosome and mRNA biogenesis, have been proposed to play a role in the life cycles of different viruses by interacting with viral RNAs in functional complexes (Lai 1998). hnRNP A1 binds to the 3′ untranslated region (UTR) and mediates potential 5′–3′ end crosstalk of the coronavirus mouse hepatitis virus (MHV) RNA.

Within the *Flavivirus* hepatitis C virus (HCV) genome, the 5′ UTR has been shown to interact with several cellular factors: the La autoantigen (Ali and Siddiqui 1997; Spangberg et al. 1999), polypyrimidine tract-binding protein (PTB) (Ali and Siddiqui 1995), hnRNP L (Hahm et al. 1998), eukaryotic initiation factor 3 (eIF3) (Sizova et al. 1998), and poly(rC) binding protein 1 (PCBP1) and poly(rC) binding protein 2 (PCBP2), also known as hnRNP E1 and hnRNP E2, respectively (Spangberg and Schwartz 1999). Human RNA-binding protein (HuR) and hnRNP C have been shown to bind to the 3′ ends of HCV RNAs of both polarities (Spangberg et al. 2000). PTB, also known as hnRNP I, interacts with the pyrimidine-rich region within the 3′ UTR of the HCV RNA genome (Gontarek et al. 1999). Eukaryotic elongation factor 1 alpha (EF-1α) interacts with the 3′ stem–loop structure of the genomic RNA of WNV (*Flavivirus*) and appears to play a functional role in replication (Blackwell and Brinton 1997).

Picornavirus investigators have determined that the cellular protein PCBP2 interacts with the major stem–loop structure in the internal ribosome entry site (IRES) of the 5′ noncoding region (NCR) of poliovirus positive-strand RNA and is essential for translation initiation (Blyn et al. 1996; Blyn et al. 1997). In addition, the binding of poly(rC) binding protein (PCBP) to the 5′ positive-strand cloverleaf structure in concert with the viral precursor 3CD is required for initiation of negative-strand RNA synthesis (Gamarnik and Andino 1997, 1998; Parsley et al. 1997; Walter et al. 2002).

Conservation of amino acid sequences, functional domains, and common replication strategies

Comparative analysis of amino acid sequences between positive-strand RNA viruses has revealed relationships between disparate groups of plant, animal, and bacterial viruses. Enzymes mediating the expression and replication of virus genomes contain arrays of conserved sequence motifs despite the potential for rapid evolution of RNA genomes (Koonin and Dolja 1993; Steinhauer and Holland 1987) Proteins that include such motifs are the RNA-dependent RNA polymerase (RdRp), a putative RNA helicase, a chymotrypsin-like proteinase and papain-like proteinase, and a methytransferase. Nonetheless, the RdRp is the only universally conserved protein of positive-strand RNA viruses. Evolutionary virologists are developing a concept of the RNA virus genome as a relatively stable foundation of housekeeping genes with a more flexible collection of genes coding for virion components and accessory proteins (Koonin and Dolja 1993). It is thought that all positive-strand RNA viruses and some related double-stranded RNA viruses could have evolved from a common ancestral virus that contained a gene for a RdRp, a chymo-

Table 1 *Examples of cellular proteins shown to interact with viral RNAs*

Virus	RNA	Cellular proteins
Poliovirus (*Picornaviridae*)	IRES – 5′ NCR of genome	PCBP2 (hnRNP E2), La, unr, PTB (hnRNP I)
	Cloverleaf – 5′ NCR	PCBP (hnRNP E)
	3′ NCR	Nucleolin
West Nile virus (*Flaviviridae*)	3′ Stem–loop of genome	EF-1α
Hepatitis C virus (*Flaviviridae*)	5′ UTR of genome	La, PTB, hnRNP L, eIF3, PCBP1 (hnRNP E1), PCBP2 (hnRNP E2)
	3′ End of + strand RNA	HuR, hnRNP C
	3′ End of − strand RNA	HuR, hnRNP C
	Pyrimidine-rich region of 3′ UTR of genome	PTB (hnRNP I)
Mouse hepatitis virus (*Coronaviridae*)	3′ UTR of genome	hnRNP A1

trypsin-related proteinase that also functioned as the capsid protein, and perhaps an RNA helicase.

Conservation of functional domains exists within the genomes of positive-strand RNA viruses in the form of *cis*-acting sequence motifs and RNA secondary structures necessary for translation, RNA synthesis, and packaging. The 5′ termini of these viruses typically contain such elements known to participate in binding of viral and host cell factors. Interaction of protein factors and RNA structures is necessary to selectively initiate viral-specific processes such as synthesis of negative-strand intermediate RNA. Binding of protein factors to internal sequence motifs can be a prerequisite to genomic RNA synthesis by implementing the release of single-stranded 3′ termini on negative-strand intermediate RNAs or by participating in a complex that initiates or increases the efficiency of genome replication.

Within the intracellular replicative cycle of most positive-strand RNA viruses, we see asymmetric production of excess genomic positive- over negative-strand RNA. Only a single, nascent negative-strand RNA is copied from a positive-strand RNA template at one time and the full-length duplexed RNA molecule that results from this transcription is referred to as the replicative form (RF). Although genomic positive-strand RNA synthesis may initiate on RF, eventually negative-strand RNA serves as the template for simultaneous synthesis of numerous new genomic RNAs by efficient reinitiation (Figure 2). This form of partially duplexed RNA is referred to as the replicative intermediate (RI). The fact that free negative-strand RNA is not recovered from the cytoplasm of the infected cell supports the replication models of RF and RI for picornaviruses, caliciviruses, flaviviruses, and togaviruses. The transition of the RdRp from competence for negative- to positive-strand RNA synthesis often involves the recruitment of additional host cell factors in temporal conjunction with availability of viral replication proteins.

In addition to demonstrating connection with respect to genomic organization, families within the class of positive-strand RNA viruses show similarity in the morphological types of virus particles assembled (Table 2). Viruses with cubic symmetry in the form of naked, icosahedral particles are seen in *Picornaviridae*, *Caliciviridae*, and *Astroviridae* and in the form of enveloped, spherical viruses with icosahedral nucleocapsids in *Togaviridae* and *Flaviviridae*. *Coronaviridae* includes viruses with more complex morphology, that is, spherical viruses with helical nucleocapsids and envelopes with long glycoprotein spikes.

THE INTRACELLULAR REPLICATIVE CYCLE

Initiation of the intracellular replicative cycle

The event that initiates the intracellular replicative cycle is the binding of a viral ligand to a receptor on the surface of a permissive host cell. These receptors, normally bound by cellular ligands, are utilized by virus particles for attachment at the plasma membrane. Host cell receptors thus far identified as the molecules used by positive-strand RNA viruses belong to several groups including the immunoglobulin-related proteins [poliovirus receptor (PVR); intracellular adhesion molecule

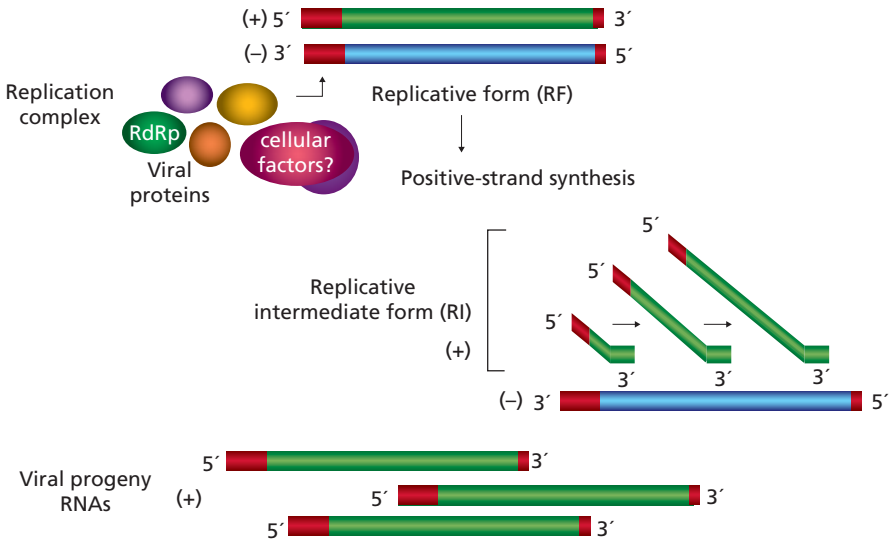

Figure 2 *Schematic representation of positive-strand RNA virus genomic RNA synthesis. A replication initiation complex, composed of the RNA-dependent RNA polymerase (RdRp), other viral proteins, and cellular proteins forms on the 3′ end of the negative-strand intermediate RNA or on the duplex RF. Multiples initiations on a single template produce an excess of positive-strand over negative-strand RNAs.*

Table 2 *Comparison of positive-strand RNA virus features by family*

Family	Enveloped	Polyprotein	Subgenomic mRNAs
Picornaviridae	No	Yes	No
Caliciviridae	No	Yes	No
Flaviviridae	Yes	Yes	No
Togaviridae	Yes	Yes	Yes
Coronaviridae	Yes	Yes	Yes

(ICAM)-1 – picornaviruses; mouse hepatitis virus receptor (MHVR)/biliary glycoprotein 1 (Bgp1) – coronaviruses], low density lipoprotein-related (LDLR) receptors (LDLR/α2MR/LPR – picornaviruses), integrins (αvβ3 – picornaviruses), receptors involved in complement pathways (CD55 – picornaviruses), as well as miscellaneous other receptors including aminopeptidase-N (coronaviruses), laminin receptor (togaviruses), and glycosaminoglycans (flaviviruses) (Young 2001). Viruses that are transmitted between insect and vertebrate hosts during their natural life cycle, such as some of the flaviviruses, are thought to use highly conserved cell receptor proteins (Brinton 2002). It is thought that alphaviruses and flaviviruses may use various receptor/coreceptor combinations to give the diverse host range (birds, mammals, mosquitos) and tropisms seen in vivo.

Viral ligands differ for each family, primarily based on either the presence or absence of an envelope with embedded glycoproteins. Naked viruses, such as members of the *Picornaviridae*, have a groove, or canyon surrounding each fivefold axis of symmetry in the capsid. These canyons have been shown to be the site of interaction with host cell receptors (Belnap et al., 2000b; He et al. 2000; Xing et al. 2000). Flaviviruses attach to host cells through an interaction between various receptors and the major viral surface protein, the E protein. Within the *Togaviridae*, the alphavirus envelope glycoproteins, E1 and E2, mediate binding of virus particles to host cell receptors. With coronaviruses, although the S protein is likely the main determinant of viral tropism due to specific cell surface glycoprotein binding, members containing the HE glycoprotein may bind to the common surface moiety, 9-*O*-acetylated neuraminic acid (Schultze and Herrler 1992).

Our understanding of the actual process whereby the virus particle or genomic RNA enters the cell has increased significantly in recent years. Virus particles such as poliovirus are thought to undergo conformational changes in the capsid proteins as the result of receptor binding. These changes expose hydrophobic domains on two of the capsid proteins which subsequently interact with the plasma membrane or endocytotic vesicle membrane, forming a pore through which the genomic RNA may be extruded and relocated into the cytoplasm of the cell (Hogle 2002). The movements of the capsid proteins that occur during this process have been compared with the movements of tectonic plates in the Earth (Belnap et al. 2000a). Human rhinovirus (HRV), a picornavirus, togaviruses (Smit et al. 1999), flaviviruses (Brinton 2002), and coronaviruses (Hansen et al. 1998) are thought to gain entry into the cytoplasm of host cells via the process of receptor-mediated endocytosis. Briefly, receptor-bound particles accumulate in coated pits on the cell surface, which are then endocytosed to form coated vesicles. These vesicles are subsequently uncoated to form acidified endosomes, which creates the environment that triggers low pH-mediated fusion of the viral capsid/envelope with the endosomal vesicle membrane and releases the nucleocapsid into the cytoplasm (DeTulleo and Kirchhausen 1998).

Translation of viral-specific RNAs and processing of polyproteins

Viral gene expression for positive-strand RNA viruses begins with translation of genomic (messenger) RNA in the cytoplasm. The structure of viral messenger RNAs differs somewhat between families with respect to modifications of the termini and presence or absence of an IRES (Table 3). Picornavirus genomes are uncapped, have a viral protein VP_g(3B), linked to the 5′ end, have either a type I or type II IRES in the 5′ NCR, and are polyadenylated at the 3′ terminus. Caliciviruses also have a VPg, linked to the 5′ end of their 3′ polyadenylated genomes. Togavirus and coronavirus genomes and subgenomic positive-strand RNAs are terminated with a 7-methylguanosine cap at their 5′ end and polyadenylated at the 3′ end. In flaviviruses, two different types of genomic RNAs exist. Viruses in the genus *Flavivirus* are capped with a 7-methylguanosine at the 5′ end but do not contain a 3′ polyadenylate (poly(A)) tract, while hepacivirus and pestivirus genomes are uncapped at the 5′ end, have an IRES in the 5′ NCR, and are not polyadenylated at the 3′ end. *Flaviviridae* is the only family of positive-strand RNA viruses, with members that infect mammalian hosts, that do not have a poly(A) tail at the 3′ end of the genome.

Since most cellular mRNAs are capped at the 5′ end, there are mechanisms which specifically select for translation of viral messages that are capped. In some cases, host-cell factors are involved in the favored recognition of viral mRNAs by the host translational machinery. Each coronavirus subgenomic RNA contains a 5′ leader sequence that enhances translation in lysates from virus-infected cells, suggesting that virus-specific mRNAs may be preferentially translated in the context of decreased host translation, brought about by competition for active ribosomes and degradation of host mRNAs in the infected cell (Tahara et al. 1994).

Although all but the smallest coronavirus mRNAs contain multiple ORFs, only the 5′-most ORF of each

Table 3 *Structure of positive-strand viral (genomic) mRNAs*

Family	5′-Linked protein or cap	IRES	3′-Poly(A)
Picornaviridae	VPg	Yes	Yes
Caliciviridae	VPg	?	Yes
Flaviviridae			
Flavi-	7-Methylguanosine cap	No	No
Hepaci-, Pesti-	Uncapped	Yes	No
Togaviridae	7-Methylguanosine cap	No	Yes
Coronaviridae	7-Methylguanosine cap	Yes (some downstream ORFs)	Yes

mRNA is generally translated by a cap-dependent ribosomal scanning mechanism. However, in several coronavirus mRNAs, the second or third ORF is preceded by an IRES and is efficiently translated by allowing ribosomes to bypass the upstream ORF and bind internally to the IRES.

Initiation of translation by cap-independent mechanisms involves some of the cellular proteins that function in cap-dependent translation. In cells infected by some picornaviruses, eIF4G is cleaved by a viral proteinase. The C-terminal fragment created by this cleavage, which contains binding sites for eIF3 and eIF4A, stimulates IRES-mediated translation of poliovirus RNA (Buckley and Ehrenfeld 1987). Several cellular proteins that bind to the picornavirus IRES have been identified. These include PTB (Hellen et al. 1993), PCBP2 (Blyn et al. 1996), the La autoantigen (Meerovitch et al. 1993), and unr (Hunt et al. 1999). It is thought that these cellular proteins may act as RNA chaperones, maintaining the IRES in a structure that allows direct binding to the translation machinery. Several cellular proteins, including PTB (Ali and Siddiqui 1995), the La autoantigen (Ali and Siddiqui 1997), and hnRNP L (Hahm et al. 1998), have been shown to interact with the 5′ NCR of HCV and function in the process of internal initiation of translation.

Polyprotein processing is required for the generation of functional polypeptides utilized by positive-strand RNA viruses. It is also a means by which they amplify the coding capacity of their relatively small genomes. For picornaviruses, almost all of the proteins necessary for a complete intracellular replicative cycle are found within the single long ORF of the genome in the form of intermediates and mature protein products. The full-length polyprotein product encoded by the ORF is never seen since viral-encoded proteinases begin cleavage cotranslationally. The initial processing events of the picornavirus polyprotein differ among the genera with respect to site and proteinase. Primary cleavage of the enterovirus and rhinovirus nascent polyprotein occurs at the P1/P2 junction and is mediated *in cis* by the viral-encoded 2A proteinase (Toyoda et al. 1986). For aphthoviruses, the primary cleavage at the L/P1 junction is thought to occur by enzymatic activity of L *in cis*, while for cardioviruses, the L protein is cleaved from the P1 region by the 3C proteinase. The 2A proteins of these two genera are not proteinases, but cause their own release at the junction with 2B by an unknown mechanism that appears to be a novel form of peptide bond scission (Palmenberg et al. 1992). In hepatoviruses and parechoviruses, 3C carries out the primary cleavage at the 2A/2B junction (Schultheiss et al. 1994; Stanway and Hyypia 1999). Viral proteinases L, 2A, and 3C (3CD) are active in the nascent picornavirus polypeptide and are involved in their own release by self-cleavage. All subsequent polypeptide cleavages occur *in trans*. For poliovirus, HRV, and coxsackie virus, the precursor, 3CD, is responsible for further processing of P1 following the initial cleavage (Ypma-Wong et al. 1988). The 3D portion of 3CD may be required for recognition of structural motifs in the folded P1 precursor allowing efficient processing by the 3C part of the enzyme (Parsley et al. 1999; Cornell and Semler 2002). The existence of multiple activities in a single proteinase provides another example of maximization of coding capacity of a small RNA genome.

The single large ORF of the flavivirus genome is translated as a polyprotein that is both co- and post-translationally processed by cellular proteases and viral-encoded proteinases. For HCV, the initial processing event of the polyprotein at the NS2/3 junction is mediated by the autoprotease, NS2-3. The NS3 serine protease, with the help of the NS4A cofactor, performs the other cleavages in the nonstructural region of the polyprotein. Cleavages within the structural region of the polyprotein are thought to be effected by the cellular resident endoplasmic reticulum (ER) signal peptidases (Houghton et al. 1994; Reed and Rice 2000).

The togavirus genomic RNA serves as the messenger RNA for translation of the polyprotein that is cleaved by a virally encoded protease (Hardy and Strauss 1989) to form the nonstructural polypeptides. Translation of the polyprotein is initiated at a single AUG near the 5′ end of the genome and proceeds continuously until encountering three termination codons located just downstream of the start of the sequences corresponding to the subgenomic RNA. Several of the alphavirus genomes have an opal stop codon upstream of the nsP4 coding region that is sometimes suppressed to allow read-through and inclusion of nsP4 in the polyprotein.

The final products of translation of the subgenomic mRNA are the capsid protein, released cotranslationally by autoproteolysis, two transmembrane glycoproteins, and a small membrane-embedded protein (Strauss and Strauss 1994) (Figure 3).

For MHV, a coronavirus, the structural proteins are each encoded by a separate subgenomic mRNA. However, two polyproteins translated from the replicase gene (gene 1) are processed by the viral-encoded proteinases, papain-like cysteine proteases (PLP1 and PLP2) and chymotrypsin-picornavirus 3C-like protease (3CLp), into at least 15 nonstructural protein products, including the presumed RNA helicase and the RdRp (Denison et al. 1999).

Alteration of the intracellular environment by viral gene products

Positive-strand RNA virus genomes encode nonstructural proteins that induce biochemical and structural changes in the host cell following infection (Figure 4). These viral proteins are thus indirectly involved in the process of RNA synthesis since they give rise to modifications in the environment where synthesis occurs. Down-regulation of cellular protein synthesis is one such common consequence of infection by nearly all members of the *Picornaviridae*. Cleavage of eIF4GI, eIF4GII, and poly(A) binding protein (PABP) by picornavirus 2A and/or 3C proteinases results in cessation of host translation due to destruction of the eIF4F complex and end-to-end communication in cellular mRNAs (Bushell and Sarnow 2002). For example, the synthesis of poliovirus proteins is not affected since the viral mRNA is translated by internal ribosome binding to the IRES, a mechanism that does not require an intact eIF4F complex. Since optimal function of the poliovirus IRES does require the C-terminal cleavage fragment of eIF4G, the modification of this cellular factor by 2A assists viral replication by inhibiting cellular translation and stimulating IRES-dependent translation. Cleavage of eIF4G in cells infected with foot-and-mouth disease virus (FMDV) is mediated by the viral L protein. Infection of cells with encephalomyocarditis virus and some of the other picornaviruses causes inhibition of cap-dependent translation as well, not by proteolytic cleavage of translation factors but by alteration of the phosphorylation state of two other cellular proteins, 4E-BP1 and 4E-BP2. Dephosphorylation of 4E-BP1, which allows it to bind to eIF4E and thus prevent formation of eIF4F and recognition of capped mRNAs, inhibits translation of cellular messages but does not affect translation of viral mRNA.

Intuitively, in strict consideration of the machinery of RNA synthesis, competition between virus and host may seem minimized by the fact that positive-strand RNA viruses code for their own RdRp. However, down-regulation of cellular transcription mediated by viral gene products would favor the synthesis of viral RNA by increasing the pool of free nucleotides available for the

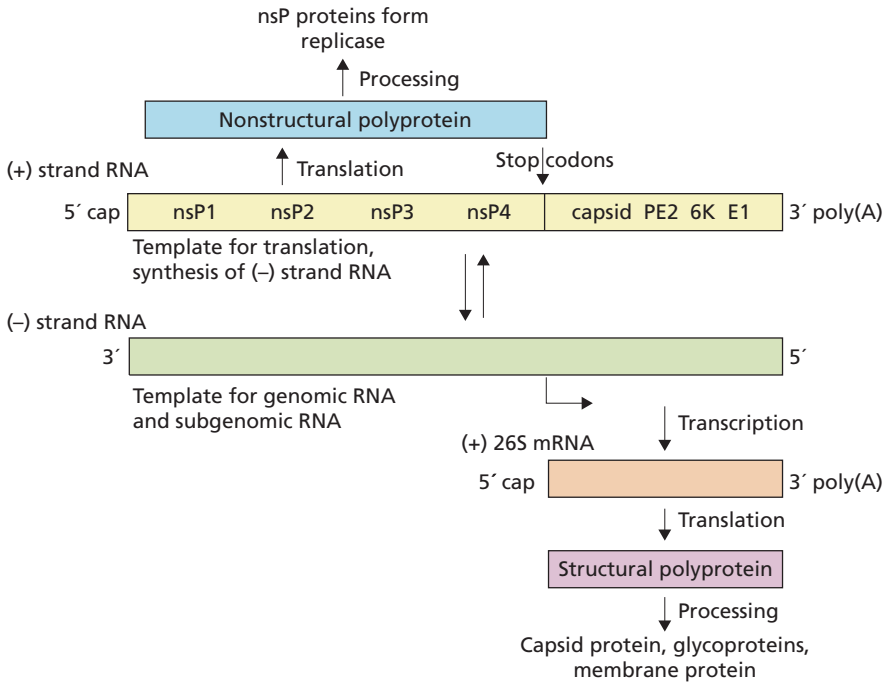

Figure 3 *Transcription and replication of alphavirus RNAs including translation and processing of polyprotein precursors. Positive-strand genomic RNA is the template for a full-length negative-strand intermediate RNA. The 5′ two-thirds of the genomic RNA encodes a polyprotein processed into the nonstructural proteins. The negative-strand RNA is the template for production of more genomic RNAs as well as the template for production of the 26S subgenomic mRNA that encodes a polyprotein processed into capsid protein, envelope glycoproteins, and membrane protein.*

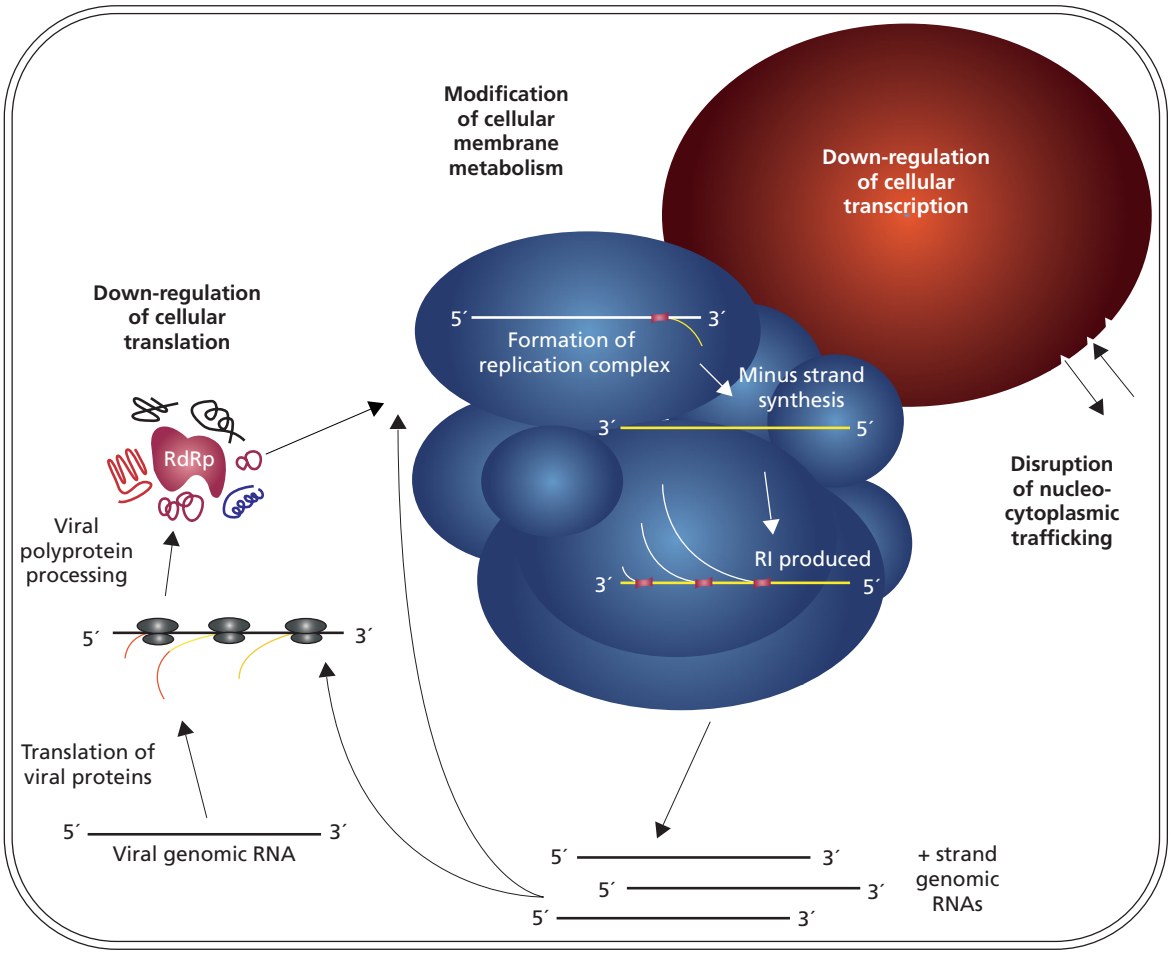

Figure 4 *Biochemical and structural changes in the host cell following infection by positive-strand RNA viruses. Down-regulation of cellular protein synthesis favors translation of viral mRNAs. Cellular transcription is often down-regulated, thereby increasing the pool of free nucleotides available for viral RNA synthesis. Excessive proliferation of intracellular membranes provides a matrix for viral RNA synthesis. Disruption of nucleocytoplasmic transport may result in an abundant pool of nuclear proteins in the cytoplasm that may assist with viral protein synthesis, RNA synthesis, assembly, or packaging. Red oval represents the nucleus; blue ovals represent cytoplasmic membranous vesicles.*

viral polymerase. Interestingly, at an intermediate to late time in the course of infection by most picornaviruses, there is a rapid inhibition of host cell RNA synthesis catalyzed by all three classes of DNA-dependent RNA polymerases. Since pol I, pol II, and pol III isolated from such cells are active, the inhibitory mechanism is thought to involve accessory transcription proteins. In fact, cleavage of TATA-binding protein (TBP) and other transcription factors, by poliovirus proteinase 3C and proteinase 2A, has been mechanistically associated with this transcription inhibition (Dasgupta et al. 2002).

During the intracellular replication cycle of positive-strand RNA viruses, modifications of cellular membrane metabolism and function commonly occur. For example, during flavivirus infection, there is extensive reorganization and proliferation of cytoplasmic, perinuclear endoplasmic reticular membranes (Brinton 2002). In picornavirus infection, there is an accumulation of small membranous vesicles thought to be caused by the inhibition of membrane vesicle transport from the endoplasmic reticulum to the cell surface. For poliovirus, viral proteins 2B, 3A, 2BC, and 2C are believed to be involved in this process (Cho et al. 1994; Doedens and Kirkegaard 1995). Such modifications of the cytoplasmic environment favor viral replication since the abundant, localized membranes not only provide a subcellular compartment which concentrates cellular and viral proteins but also serve as a matrix upon which viral RNA replication complexes can form.

Even though the positive-strand RNA viruses typically complete their replicative cycle strictly in the cytoplasm, there can be a disruption of nucleocytoplasmic trafficking as the result of viral infection (Gustin and Sarnow 2001, 2002). Alteration in nuclear import, export or both can result in an increase in the levels of nuclear proteins in the cytoplasm available for use in viral translation, replication, and/or packaging. In addition, disruption of signaling to the nucleus can attenuate the antiviral response by preventing transcription of the relevant cellular messages.

Genome replication

The process of RNA-dependent RNA synthesis is foreign to most eukaryotic cells, in which RNA synthesis normally occurs from a DNA template. Eukaryotic DNA-dependent RNA polymerases are formed by multiple subunits. Thus, although the RdRp elongation activity must be encoded by the viral genome, typically there is a dependence on cellular proteins to complete the replicase activity or provide specific selection of the viral template RNA. Cellular factors may serve as part of the RdRp holoenzyme by binding directly to the core enzyme, or they may direct the RdRp to the RNA template by binding directly to the template, or they may carry out both functions by forming a bridge between the RdRp and the RNA template (Lai 1998).

The first structure of an RdRp solved was that of poliovirus $3D^{pol}$ and it was found to have the same overall configuration as that of the other three types of polymerases, that is, DNA-dependent DNA polymerases, DNA-dependent RNA polymerases, and RNA-dependent DNA polymerases (reverse transcriptases) (Hansen et al. 1997). The native three-dimensional shape of all these polymerases is that of a cupped right hand, with domains corresponding to the thumb, palm, and fingers. The palm domain contains the active site of the enzyme. RdRps may not only be template dependent, but also primer-dependent enzymes as is the case for picornaviruses. The RdRp of poliovirus, $3D^{pol}$, copies the viral RNA in the presence of the uridylylated VPg (3B) protein primer.

The first step in RNA synthesis of positive-strand RNA viruses is synthesis of the complementary intermediate negative-strand RNA. With respect to a single strand of viral genomic RNA, it is generally assumed that viral translation is terminated prior to the initiation of negative-strand synthesis, since the ribosomes and RdRp would be unable to travel along the same molecule in opposite directions without colliding. In general, at the beginning of the replication cycle, nascent genomic RNAs may alternate between replication and translation until a sufficient pool of nonstructural proteins has accumulated.

Negative-strand RNA synthesis is presumed to be initiated at the 3′ end of the positive-strand RNA and requires proteins of both viral and cellular origin as well as *cis*-acting structural and sequence determinants in the genomic RNA. Since some viral RdRp do not bind directly to *cis*-acting regulatory elements on the viral RNA, initiation of RNA synthesis may depend upon interactions with cellular proteins or viral proteins that bind directly to the viral RNA. It is hypothesized that RNA viruses may assemble RNA transcription or replication complexes which bring noncontiguous regions of viral RNA together, similar to the interaction between enhancer and promoter elements in DNA-dependent RNA synthesis. One end of the viral RNA could function as an enhancer while the other end could serve as a promoter (Lai 1998).

Long-range interactions between the 5′ and 3′ ends of the viral genome mediated through protein–protein contacts have been studied in the context of initiation of negative-strand and/or positive-strand RNA synthesis. For poliovirus, it has been shown that PABP interacts with the cellular protein, PCBP2, and the viral polymerase precursor, 3CD (Herold and Andino 2001). These long-range interactions could bring the two termini of the viral genomic RNA together if PABP were bound to the 3′ poly(A) tail. For HCV, studies indicate that there may be 5′ to 3′ crosstalk between the termini of the RNA of both polarities which may be important for RNA transcription and replication (Huang and Lai 2001).

For single-stranded, positive-strand RNA viruses, RNA synthesis is asymmetric. Genomic RNA synthesis is more efficient than negative-strand synthesis due to multiple initiations on the same negative-strand RNA or RF template. For flaviviruses, the ratio of positive-strand RNA to negative-strand RNA is about 10:1; for poliovirus it is about 40:1.

Initiation of positive-strand synthesis presumably occurs at the 3′ end of the negative-strand RNA. It is not clear if the negative-strand RNA template is single-stranded or in the duplexed RF. Furthermore, it is assumed that the mechanisms for initiation of positive-strand RNA synthesis require *cis*-acting determinants in the RNA and viral and cellular *trans*-acting factors and that these mechanisms and factors are, in part, distinct from those used for initiation of negative-strand synthesis. For togaviruses, negative-strand synthesis requires viral protein precursors in replication complexes (Sawicki and Sawicki 1994). When these precursors accumulate and are partially or fully processed, negative-strand synthesis ceases and positive-strand synthesis is initiated. For poliovirus, replication defects due to mutations in specific replication proteins can be rescued *in trans*, but only when the wild-type viral proteins are supplied in appropriate precursor form (Towner et al. 1999).

Assembly and release of virus particles

Once sufficient viral structural proteins have accumulated, nascent genomic RNAs can function as templates for translation and transcription and as substrates for encapsidation. For Kunjin virus, now considered a subtype of the flavivirus WNV (Scherret et al. 2001), translation is a prerequisite for replication of a nascent RNA, and replication is a prerequisite of encapsidation (Khromykh et al. 2001; Khromykh et al. 1999). The coupling of encapsidation to viral RNA synthesis may account, in part, for the fact that the encapsidation process is highly specific. Selection of a viral RNA for

packaging may also depend on the presence of a packaging signal in the RNA. Genomic RNA is selected for encapsidation even in the presence of a molar excess of subgenomic RNA in togaviruses. Sindbis virus contains a structural packaging signal in the 5′ region of the genome, within the coding region for nsP1. For picornaviruses only VPg-linked positive-strand RNA, and not viral mRNA, negative-strand viral RNA, or any cellular RNA, is packaged.

The morphogenetic pathway for formation of mature virions generally involves both assembly intermediates, or substructures, as well as precursor forms of the structural proteins. The first assembly intermediate in the poliovirus pathway, the 5S protomer, consists of one copy each of VP0, VP1, and VP3. Five protomers then assemble to form a pentamer (14S) and pentamers assemble into empty capsids. One assembly model predicts that the viral RNA is threaded into the empty capsid and the final morphogenetic step, cleavage of VP0 to VP2 and VP4, produces the fully mature, infectious virion. In an alternative model, 14S pentamers assemble with virion RNA to form provirions. This pathway suggests that empty capsids, found in infected cells, serve as storage units for 14S pentamers (Nugent and Kirkegaard 1995). For WNV, immature virions, with precursor forms of structural proteins, accumulate in vesicles and are then transported through the host secretory pathway. These precursor interactions may maintain structural proteins in stable conformations until assembly and release of new virions is complete.

Flavivirus virions are transported to the plasma membrane in vesicles and released by exocytosis. For the alphaviruses of *Togaviridae*, nucleocapsids interact with the host cell plasma membrane at sites occupied by viral transmembrane glycoproteins. This leads to binding of additional viral glycoproteins, wrapping of the membrane around the nucleocapsid (budding) and release of the enveloped particle (Garoff et al. 1998).

Coronavirus assembly initiates with binding of the N (nucleocapsid) protein via a specific signal located in the 3′ end of ORF 1b of the genomic RNA. Interaction between the nucleocapsid and the M protein, in the membranes of the ER and Golgi complex, triggers packaging. Virus budding take place in the budding compartment between the ER and Golgi when the E protein interacts with the M protein. Envelope glycoproteins, S and HE, are incorporated into virions through interactions with the M protein. Virions accumulate in secretory vesicles, which eventually fuse with the plasma membrane to release virus (Lai and Holmes 2001).

CONCLUSIONS

RNA viruses are inherently and uniquely interesting due to the fact that, unlike nearly any other biological agent or organism, their genetic blueprint is stored biochemically in the form of RNA instead of DNA. According to the central dogma of molecular biology, the flow of genetic information normally proceeds from DNA to RNA via transcription and from RNA to protein via translation. For positive-strand RNA viruses, this process is more direct since genomic RNA is messenger RNA and can be translated to protein upon release in the cytoplasm of the host cell. These viruses encode their own RdRp for RNA chain elongation activity, although formation of the replicase complex may include other viral as well as cellular factors.

A viral RNA-dependent RNA polymerase is intrinsically more error-prone than a DNA-dependent DNA polymerase and produces a population of viruses, referred to as a quasi species, characterized by members that are not genetically identical (Domingo and Holland 1997). Mutations in the coding region of viral genomic RNA, due to decreased fidelity of the viral polymerase, may be inconsequential, deleterious or even lethal, or may permit survival of the virus. For example, a random single-point nucleotide substitution in viral genomic RNA could result in one of the following five possibilities:

1. no change in amino acid due to redundancy of the genetic code
2. change to a different amino acid similar enough to be inconsequential to overall folding or function of the protein
3. change to a different amino acid dissimilar enough to diminish function due to alteration in protein conformation or catalytic site
4. change to a different amino acid that randomly confers functional benefit in a specific environment, due to improved conformation and/or catalysis, thus allowing survival of the mutant virus
5. change to create an early stop codon or eliminate an existing stop codon
6. no change or conservative change in amino acid with concomitant change in RNA secondary structure that is neutral, harmful or beneficial to function.

Similarly, mutations in the noncoding region of the viral genomic RNA can result in inconsequential, disadvantageous or advantageous functional modifications through alterations in *cis*-acting signals or specific RNA structures required for RNA–RNA or RNA–protein interactions. Thus, paradoxically, long-term survival of an RNA virus with limited coding capacity in a constantly evolving host is accomplished through accidental adaptive modifications that result from slightly unfaithful viral RNA replication by a viral-encoded error-prone polymerase.

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DNA virus replication

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INTRODUCTION

The DNA viruses exhibit diverse mechanisms of genome replication that are important to study for many reasons. Viruses provide useful models for the study of eukaryotic DNA replication because of their relative simplicity and ease of study; in fact, much of what we know about eukaryotic DNA replication has come from the study of viral systems. For instance, viruses that rely heavily on host-cell machinery for replication have provided experimental systems that have led to the identification of the components of the cellular replication machinery. Furthermore, viral enzymes involved in replication provide important targets for antiviral therapy, and the study of the mechanisms of viral DNA replication will therefore continue to provide vital information in the development of effective antiviral strategies. DNA viruses exhibit considerable diversity with respect to the molecular mechanisms of DNA replication. This is due to differences not only in genome size and structure but also in the location of viral DNA replication in the cell and the reliance on viral versus host enzymes. In this chapter we will consider DNA viruses of the following families: polyomaviruses, papillomaviruses, parvoviruses, adenoviruses, herpesviruses, and pox viruses (Table 1). While the polyoma- and papillomaviruses have circular genomes, the genomes of the other DNA viruses are linear. Among viruses with linear genomes, considerable diversity can be observed with respect to structure: single-stranded for the parvoviruses versus double-stranded for adenoviruses, herpesviruses, and pox viruses. Diversity in size is also apparent from less than 5 bp in the parvoviruses to approximately 200 kbp for the poxviruses. The diversity in size and structure of viral genomes has important implications in terms of mechanisms of DNA replication. For these reasons, in this chapter, replication strategies of each virus family will be considered separately. First, however, some guiding principles concerning DNA replication in general will be considered.

GENERAL PRINCIPLES OF DNA REPLICATION

DNA synthesis in viral as well as cellular systems involves the incorporation of deoxynucleosides in a template-directed process. Incorporation of deoxynucleosides invariably occurs onto the 3′ OH end of a growing DNA chain, resulting in a 5′ to 3′ polarity of elongation on the growing chain (Kornberg and Baker 1992). Replication generally begins at specific sites on viral genomes known as origins of replication, and many viruses encode specific origin binding proteins responsible for initiation of viral DNA synthesis (Stenlund 2003). DNA replication in DNA viruses is catalyzed by DNA-dependent DNA polymerases which in general require a free 3′ OH end in order to add deoxynucleosides. This is in contrast to DNA-dependent RNA polymerases and RNA-dependent RNA polymerases that are capable of initiating template-directed RNA synthesis de novo and do not require the presence of a free 3′ OH. One implication of this requirement is that viral

Table 1 *DNA viruses considered in this chapter*

Virus	Size of viral genome	Structure of viral genome	Location of replication	Replication proteins
Polyomavirus				
Simian virus 40	~5 000 base pairs	Closed circular double-stranded DNA	Nuclear	Viral and host
Papillomavirus				
Human and Bovine papillomaviruses	~8 000 base pairs	Closed circular double-stranded DNA	Nuclear	Viral and host
Parvovirus				
Minute virus of mice, Adeno-Associated Virus	~5 000 bases	Linear single-stranded DNA	Nuclear	Viral and host
Adenovirus				
Human adenovirus	~36 000 base pairs	Linear double-stranded DNA	Nuclear	Viral and host
Herpesvirus				
Herpes simplex virus type 1	~150 000 base pairs	Linear double-stranded DNA	Nuclear	Viral primarily (could be some host protein involvement)
Poxvirus				
Vaccinia virus	~200 000 base pairs	Linear double-stranded DNA	Cytoplasm	Viral

DNA replication is dependent on the presence of a primer; however, it is remarkable how different viruses have evolved novel strategies for priming DNA synthesis. Parvoviruses rely on structural features of the viral genome itself to prime new DNA synthesis whereas adenoviruses utilize a protein priming mechanism. Other DNA viruses utilize a strategy of formation of RNA primers similar to mechanisms used during cellular DNA replication. DNA synthesis for all DNA viruses requires the use of a DNA-dependent DNA polymerase; however, the smaller DNA viruses (polyoma-, papilloma- and parvoviruses) do not have the genetic capacity to encode a large number of viral proteins dedicated to viral replication. These viruses therefore utilize host-cell polymerases, while adenoviruses, herpesviruses, and poxviruses encode their own viral DNA-dependent DNA polymerases as well as additional replication proteins. Poxviruses are unusual in that DNA replication occurs in the cytoplasm and must therefore encode all enzymes and proteins needed for DNA replication.

As mentioned above, DNA replication generally begins at specific sites on viral genomes known as origins of replication with local unwinding or melting of the duplex to provide access to template strands (Stenlund 2003). Origins can increase the efficiency of DNA replication by directly or indirectly recruiting replication machinery leading to the assembly of multiprotein complexes needed for DNA replication. Given the complexity of the environment in which viral genomes replicate and the fact that viral genomes are present as single copy genomes within the host cell, it is important for sufficient concentrations of all of the essential factors to be brought together in one place. It would thus be expected that origin recognition proteins would exhibit highly specific DNA binding. In some systems this specificity is achieved by the initiator protein itself while in others a second viral or cellular protein increases specificity (Table 2).

Many viral origins consist of a core origin and auxiliary regions that are important for optimal viral replication. The core origins generally contain binding sites for viral initiator proteins and AT-rich sequences which facilitate melting during initial unwinding reactions. The auxiliary sequences enhance the efficiency of viral DNA replication and interestingly, often contain binding sites for cellular or viral transcription factors. The importance of auxiliary regions was originally discovered in polyoma viruses where the presence of the transcriptional enhancer stimulates DNA replication up to 1 000-fold; however, this has turned out to be a much more general phenomena (Table 2). The binding of transcription factors at origins of replication in many cases facilitates the rate limiting steps in the initiation process (Table 3). In some cases binding helps to recruit other replication proteins such as polymerases. In other cases, they help to change the activity of initiation proteins. Transcription factors can also cause changes in the global architecture or nucleosome structure of the origin. Thus, specific protein–DNA and protein–protein interactions are very important for initiation and recruitment of replication machinery to occur, and we will see many diverse examples in this chapter.

Table 2 *Viral DNA proteins that initiate and participate in DNA replication*

Virus	Origin binding protein(s)	Polymerase(s)	Other replication proteins
Polyomavirus			
Simian virus 40	Large T antigen (helicase activity)	Host cellular DNA polymerases α and δ	Many cellular: RPA, RF-C, RNase H, PCNA, Fen1, DNA ligase I
Papillomavirus			
Human and Bovine Papillomaviruses	E1 protein (helicase activity)	Host cellular DNA polymerases	Many cellular
Parvovirus			
Minute virus of mice	NS1 (helicase activity)	Host cellular DNA polymerases	RPA, PCNA
Adeno-associated virus	Rep 68, Rep 78 (helicase activity)		
Adenovirus			
Human adenovirus	DNA polymerase, preterminal protein complex	Viral	Viral SSB (DBP), Cellular proteins: transcriptional activators (NF1, Oct-1 and topoisomerase)
Herpesvirus			
Herpes simplex virus type 1	UL9, origin-binding protein (helicase activity)	Viral polymerase (UL30) and accessory protein (UL42)	Viral helicase-primase (UL5, UL8, and UL52), viral SSB ICP8)
Poxvirus			
Vaccinia virus	?	Viral	Several viral, no cellular

The binding of viral and cellular proteins at an origin of replication is followed by a melting or opening of the duplex. Once a region or regions of the origin are melted or distorted, recruitment of helicases and other replication proteins occurs (Waga and Stillman 1994). The function of the helicase is to unwind the DNA in advance of the replication fork (see Figure 1). Helicases translocate along single- or double-stranded DNA in an ATP-dependent fashion. Many DNA and RNA viruses encode helicases (Marintcheva and Weller 2001). The only DNA virus which does not encode its own helicase is adenovirus whose mechanism of replication does not require helicase activity.

In the simplest replication systems, replication proceeds by the formation of two replication forks which synthesize DNA in different directions from the origin (Kornberg and Baker 1992). This requires the use of leading- and lagging-strand DNA replication also known

Table 3 *Viral origin recognition proteins*

Virus	Initiator Protein	Binds specifically to ori	Melts ori	Other activities
SV40	Large T antigen	+	+	DNA-stimulated ATPase and helicase activities; recruits RPA, polα-primase, Topo I, and other cellular proteins
Adenovirus	pTP and DNA pol	+ (stimulated by cellular proteins Oct-1 and Nf-1)	+	Priming of DNA synthesis by protein priming
Herpes simplex virus	UL9	+	?	DNA-stimulated ATPase and helicase; binds ICP8 and the UL8 subunit of the viral helicase-primase
Papilloma viruses	E1 and E2	+	+	DNA-stimulated ATPase and helicase (E1); recruits RPA, polα-primase, Topo I, and other cellular proteins
Parvovirus MVM	NS1	+	–	Site-specific endonuclease; DNA-stimulated ATPase and helicase

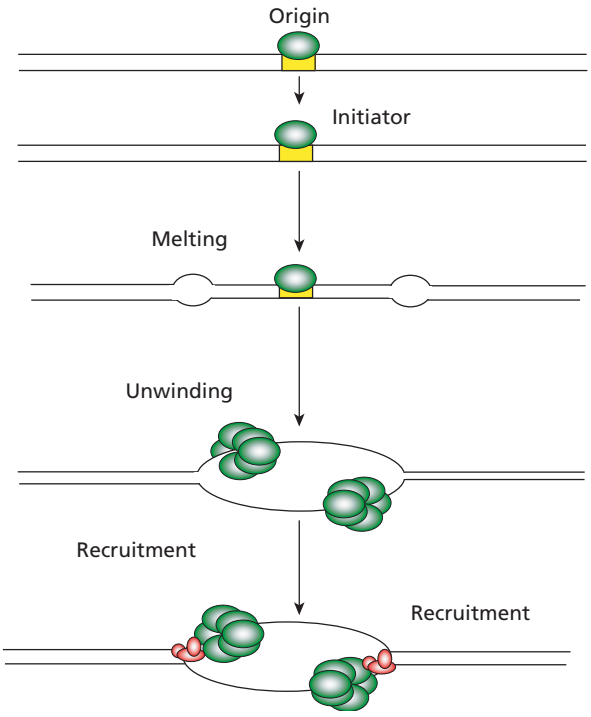

Figure 1 *Initiation of DNA replication. DNA replication generally initiates at an origin of replication in four distinct steps. The first step involves specific binding by an origin binding or initiator protein (initiator). In the example shown, specific binding is carried out by a single viral protein, but in some cases additional viral or cellular proteins are required for this initial binding event. In the second step, the initiator protein causes melting or distortion at the origin. The third step, helicase is loaded at the partially unwound origin and in the fourth step additional replication proteins are loaded. In the polyoma- and papillomaviruses, the initiator protein carries out all of these functions, binding, melting, unwinding, and recruitment. SV40 large T antigen and papilloma E1 proteins both form hexameric helicases which also carry out unwinding during the elongation step of DNA replication. (Adapted from Stenlund 2003)*

as discontinuous DNA synthesis (Figure 2). As mentioned above, all known DNA polymerases must elongate via addition of nucleoside monophosphates to an existing 3′ OH group resulting in DNA synthesis which proceeds in a 5′ to 3′ direction (Kornberg and Baker 1992; Depamphilis 1996). In order for a replication fork to move bidirectionally, both strands must be replicated by a mechanism that involves continuous synthesis on one strand (leading-strand DNA synthesis) and discontinuous synthesis on the other strand (lagging-strand DNA synthesis) (see Kornberg and Baker (1992) for detailed review). Lagging-strand DNA synthesis is accomplished by synthesizing short DNA fragments termed Okazaki fragments. Okazaki fragment synthesis involves first the synthesis of RNA primers; keep in mind that DNA-dependent DNA polymerases are not capable of de novo incorporation of deoxynucleosides into DNA. In order to produce the final DNA product, an enzyme which removes the RNA primers and a ligase

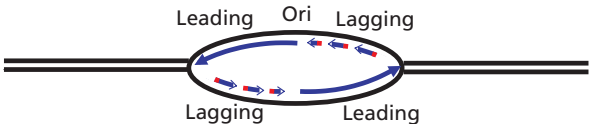

Figure 2 *Leading- and lagging-strand DNA synthesis. Since all DNA polymerases synthesize DNA only in a 5′ to 3′ direction, one strand (the lagging-strand) must elongate in the opposite direction from that of the movement of the fork itself. Lagging-strand DNA synthesis is accomplished by the synthesis of short DNA fragments termed Okazaki fragments. Okazaki fragment synthesis involves first the synthesis of a short RNA primer. In order to produce the final DNA product, an enzyme which removes the RNA primers and a ligase to repair the gaps between adjacent Okasaki fragments are needed. This mode of replication involving leading- and lagging-strand DNA synthesis is utilized by many replication systems including bacterial, eukaryotic, and many viruses. Black lines represent the parental duplex. Solid blue lines represent the nascent DNA strand and the red boxes represent RNA primers.*

to repair the gaps between adjacent Okasaki fragments are needed. This mode of replication involving leading- and lagging-strand DNA synthesis is utilized by many replication systems including bacterial, eukaryotic, and many viruses.

Some eukaryotic viruses have evolved unusual mechanisms for priming DNA synthesis. The parvoviruses have hairpin termini which are self-complementary and the 3′ termini can prime DNA synthesis. In adenoviruses, a protein is used to prime DNA synthesis. The first phosphodiester bond is formed between a nucleotide and a serine residue of the virally encode preterminal protein.

Several DNA viruses will be considered in this chapter including *Polyomaviridae, Papillomaviridae, Parvoviridae, Adenoviridae, Herpesviridae, and Poxviridae*. All of these virus families utilize the general principles of DNA replication, but each virus illustrates variations which serve to underscore the fascinating diversity among different viruses including genome size, strandedness, the use of novel methods for priming DNA polymerases, and intracellular localization.

POLYOMAVIRUSES

Polyomaviruses infect a wide variety of mammals and include four major species. A polyomavirus originally isolated from mice (murine polyomavirus (MPyV), simian vacuolating virus 40 (SV40) isolated from monkey kidney cells, and two human polyomaviruses, BK polyomavirus (BKPyV) (isolated from an immunosuppressed kidney transplant patient) and JC polyoma virus (JCPyV) from a case of progressive multifocal leukoencephalopathy. Because of the central role the polyomaviruses have played in our understanding of both viral and cellular DNA replications, this genus will be considered first. Because of the small size of

their genomes, their oncogenic properties and the existence of in vitro systems, polyomaviruses have been used extensively as models for eukaryotic DNA replication. Polyomavirus replicates lytically in mouse cells and causes abortive infections of rat and hamster cells in culture. SV40 replicates lytically in monkey and human cells but transforms rodent cells in culture.

General features of SV40 DNA replication

The *Polyomaviridae* have small circular genomes and replicate entirely by theta replication, resulting in interlocked circles that must be separated prior to genome encapsidation (Figure 3) (Depamphilis 1996). Polyomaviruses encode only one viral gene product responsible for DNA replication and rely on the host cellular machinery for the rest. SV40 will be discussed here as the best studied example of the polyomavirus family. The development of a reconstituted in vitro replication reaction capable of supporting SV40 DNA replication represented an important breakthrough in the study of chromosomal replication and allowed the identification of cellular proteins required for both SV40 and cellular DNA synthesis (Li and Kelly 1984; Waga and Stillman 1994). The detailed study of SV40 DNA replication has revealed many common elements between polyomavirus DNA synthesis and cellular DNA replication including chromatin structure, a common set of proteins comprising the replication machinery and the formation of replication forks which engage in bi-directional replication. In the early 1970s, electron microscopic analysis of replicating viral genomes revealed that SV40 DNA could initiate replication at a single site and that replication proceeded bidirectionally from this site. Replication bubbles could be observed which consisted of two replication forks moving in opposite directions. Bidirectional replication from a single origin was confirmed by elegant biochemical analysis (Hay and Depamphilis 1982). Another interesting feature of polyomavirus DNA replication is that it is tightly regulated; in fact, the study of how initiation and elongation are controlled provides insight into the mechanisms of how eukaryotic DNA replication is regulated.

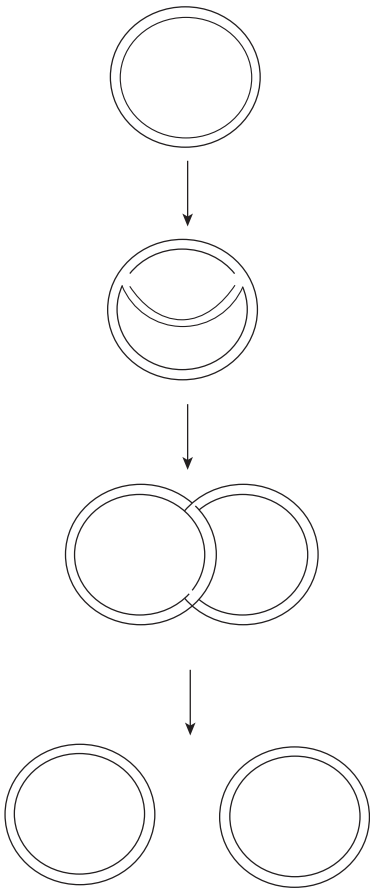

Figure 3 *Theta structure DNA replication. In this mode of viral DNA synthesis, double-stranded circular DNA replicates by bidirectional DNA synthesis in opposite directions from the origin of replication. When the forks meet at a point on the genome diametrically opposite to the origin of replication. This results in the formation of a two interlocking daughter molecules. The catenated circles can be resolved into monomer circles by a topoisomerase activity.*

SV40 GENOME

The SV40 viral genome consists of a single double-stranded circular supercoiled DNA molecule approximately 5 kbp in size (Depamphilis 1996). The genome is complexed with histones both in virions and in infected cells, and the chromatin structure is believed to be similar if not identical to host DNA: four cellular histones H2A, H2B, H3, and H4 are associated with viral DNA. The origin of replication of SV40 is surrounded by noncoding regions which control transcription of early and late genes which are transcribed in opposite directions (Figure 4). The early genes of the SV40 encode 'T antigens' – proteins that can be detected by sera from animals bearing SV40-induced tumors. SV40 encodes large and small T antigen while murine polyomavirus encodes small, middle, and large T antigens. These proteins have common N-terminal regions but unique COOH-termini derived from alternative splicing patterns. The only viral protein believed to play a direct role in viral DNA replication is the large T antigen, a multifunctional protein of approximately 90 kDal known to be involved in regulation of transcription, DNA replication, and morphological transformation. The late genes of SV40 encode structural proteins VP1, VP2, and VP3.

At the earliest stages of infection of human or monkey cells, early transcription results in the synthesis of both large and small T antigens. Large T antigen localizes primarily to the nucleus and acts to induce

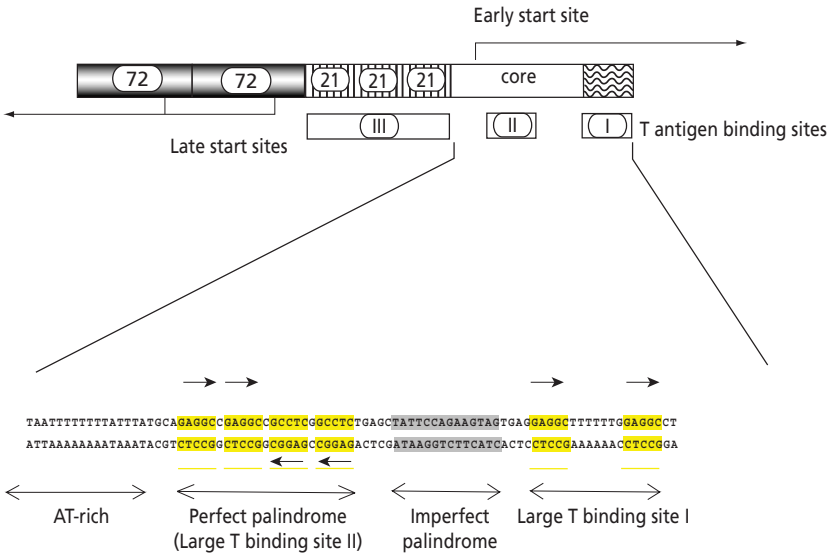

Figure 4 *Regulatory regions of SV40. The control region of gene expression in SV40 is shown here. Early and late genes are transcribed in opposite directions. This region contains an origin of DNA replication as well as binding sites for large T antigen. The 72-bp enhancer sequences and the 21-bp repeats are signals which enhance transcription in this region. The minimal origin region is shown below consisting of an AT-rich region, the large T binding site II, an imperfect palindrome and large T binding site I. The pentameric T antigen binding sites are shown in yellow.*

expression of S phase specific cellular genes thereby causing cells to enter S phase. Large T antigen has several functions including down regulation of its own transcription and initiation of viral DNA replication. Once DNA synthesis has initiated, late viral gene expression and assembly of virions proceeds. The binding of large T antigen to the SV40 origin of replication is a critical step and the subject of intensive study.

Origin of replication

As described above, viral origins of replication are discrete DNA segments containing specific sequences to which viral initiator proteins can bind and generally consist of a core region flanked by surrounding auxiliary sequences that can enhance the efficiency of viral DNA replication (Depamphilis 1996; Stenlund 2003). The importance of auxiliary regions was originally discovered in polyomaviruses, but the utilization of auxiliary regions has now turned out to be a general phenomena.

The SV40 origin of replication consists of a core origin region of 64 bp and an extended region containing an enhancer (72 bp) and a series of Sp1 binding sites (21 bp) (Figure 4) (Depamphilis 1996). The core region contains a 17-bp AT-rich element, four GAGGC pentanucleotides to which large T antigen binds and the early palindrome (EP). The four pentanucleotides (T antigen binding site II) are arranged as inverted pairs that serve as binding sites for T antigen. The binding of T antigen to site II is believed to bend the DNA. The AT-rich region and the 10-bp early imperfect palindrome are also essential for DNA replication. The AT-rich element is known to undergo bending independent of protein binding. The AT track can also bind cellular factors such as the transcription factor Oct1. The flanking regions also contain binding sites for cellular transcriptional activators. A second T antigen binding site to the right, site I, is not essential, but can stimulate the efficiency of viral DNA replication in vivo. Other flanking sequences to the left include three copies of the 21-bp repeats containing binding sites for the cellular transcription factor Sp1 and two copies of the 72-bp enhancer. The start sites for the early and late mRNAs are also shown. As will be seen later in this chapter, origins of replication are often situated between promoters, emphasizing a very close relationship between the regulation of transcription and the initiation of viral DNA replications.

Large T antigen

SV40 large T antigen is a 708-aa phosphoprotein that plays multiple functions during the SV40 life cycle. T antigen localizes primarily in the nucleus and in addition to its role in initiation and elongation of viral DNA synthesis it can drive the infected cell into S phase, cause morphological transformation, down-regulate its own transcription, and stimulate late transcription (Fanning and Knippers 1992; Simmons 2000). During DNA replication, T antigen binds the SV40 origin of DNA replication in a sequence-specific manner and its helicase activity unwinds the duplex in an ATP dependent fashion. It is also capable of recruiting cellular replication proteins to the origin through protein–protein interactions. Numerous post-translational modifications have been reported for T antigen including

phosphorylation, glycosylation, adenylation, ADP ribosylation, and acylation. It is one of the best studied viral proteins and a large database of mutations that alter various activities of T antigen has been compiled (Fanning and Knippers 1992; Simmons 2000; Ott et al. 2002).

Considerable effort has been given to understanding the structure and function of the 708 residues of SV40 large T. The domain structure of this multifunctional protein is now becoming more clear (Fanning and Knippers 1992; Simmons 2000; Ott et al. 2002; Li et al. 2003a). A minimal sequence-specific DNA binding domain lies in the middle of the protein residues 131–260. The solution structure of the DNA-binding domain has been solved (Li et al. 2003a); interestingly, it is similar to the minimal DNA-binding domain of the papillomavirus replication helicase, E1, described below. Of note is that considerable structural similarity exists despite only an approximately 6 percent identity in the primary amino acid sequence between the two initiator proteins (Li et al. 2003a; reviewed in Stenlund 2003). A zinc-binding domain lies downstream of the DNA-binding domain. The ATPase/helicase domain has been mapped to the C terminus (residues 131–616), and other functional domains which have been mapped include the J domain and binding sites for the cellular cell cycle regulatory proteins Rb and p53.

Mechanism of SV40 DNA synthesis

The binding of an initiator protein to a specific origin of replication is a common theme in the replication of DNA viruses (Depamphilis 1996; Stenlund 2003). A generic reaction of this type is shown in Figure 1 in which the initiation process is divided into four separate steps, recognition, melting, unwinding, and recruitment of replication factors.

Initiation At the outset of viral DNA replication, T antigen binds specifically to the SV40 origin of replication as a monomer. In the presence of ATP, individual monomers of T antigen assemble into a single hexamer. In addition to the pentanucleotide binding sites, the EP region is required for hexamer formation. A second hexamer then forms over the origin which causes two types of structural changes: the EP region is melted and the AT track is distorted or twisted (Depamphilis 1996). Following this structural distortion at the origin, T antigen functions as a helicase to unwind the duplex DNA in a reaction which involves ATP hydrolysis. The actual initiation of viral DNA synthesis requires the recruitment of three cellular proteins to the unwound origin: replication protein A (RPA), DNA polymerase α-primase and topoisomerase I (topo I) (Simmons et al. 1996; Gai et al. 2000). T antigen is known to bind RPA and it is believed that DNA unwinding by T antigen produces single-stranded DNA; the interaction between T antigen and RPA results in loading of RPA onto the single-stranded DNA (Figure 5). The DNA polymerase α-primase is responsible for the synthesis of Okazaki fragments. Polymerase α-primase binds specifically to both the RPA and large T antigen assembled at the origin (Ott et al. 2002). Once bound, the primase initiates synthesis of the leading strands by producing RNA primers that can be extended as DNA by polymerase α. Topo I is believed to nick and religate DNA at the origin to relieve torsional strain ahead of the replication fork (Waga and Stillman 1994).

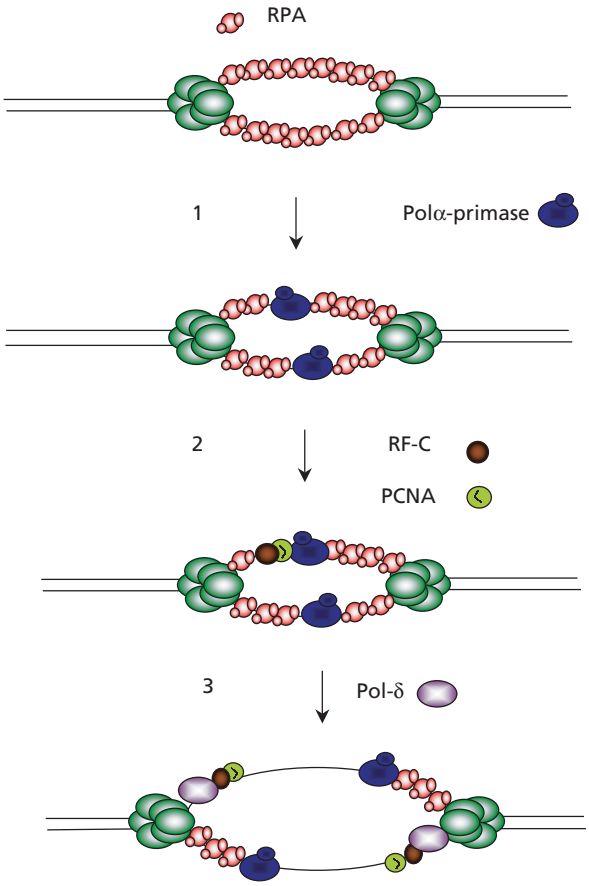

Figure 5 *SV40 DNA synthesis. Large T antigen (also depicted in Figure 1) recruits cellular proteins to the unwound origin of replication. Once RPA and polymerase α-primase are recruited by T antigen, synthesis of the leading strands is initiated by producing RNA primers which can be extended by pol-α creating an RNA-DNA fragment of approximately 30 nucleotides (Step 1). RF-C binds to the 3′ OH of the RNA-DNA fragment in a reaction that requires ATP hydrolysis. RF-C can load PCNA onto the template (Step 2). DNA pol-δ is then recruited and binds to the PCNA–RF-C complex (Step 3). The complex is now able to carry out leading and lagging-strand DNA synthesis.*

Elongation The 3′ OH of the nascent RNA–DNA fragment (about 30 nucleotides) can then be recognized by the replication factor C (RF-C). RF-C promotes the ATP-dependent loading of PCNA onto the template which is followed by the recruitment of DNA polymerase δ. Although pol δ is sufficient for continuous

leading strand DNA synthesis in an in vitro reconstitution experiment, in vivo another cellular polymerase, pol ϵ, appears to be required in addition. Lagging-strand synthesis initiates on the opposite strand again through the action of DNA polymerase α-primase. Thus, leading-strand and lagging-strand replication occurs at each replication fork in the bidirectional origin of replication. The classic problem of how a single replication fork apparatus can synthesize DNA in opposite directions at the same time was solved by the proposal that the template DNA for discontinuous synthesis can be spooled through an immobile replication complex containing all proteins necessary for synthesis of both leading and lagging strands (Wessel et al. 1992; mechanism reviewed in Stenlund 2003). Spooling has been observed by electron microscopic analysis. The DNA fragments produced by lagging-strand synthesis are sealed in a reaction that involves removal of the primers by RNase H (specifically degrades RNA from RNA–DNA hybrids) and the 5′–3′ exonuclease *Fen*1. The gaps can then be repaired by DNA polymerase δ and the ends of the DNA fragments joined by DNA ligase I. When replication gets to a point on the circular molecular opposite from the origin of replication, the reaction is terminated in a reaction which involves topoisomerases I and II. These enzymes remove torsional stress which accumulates through the action of the helicase unwinding DNA ahead of the growing fork. Topoisomerase II mediates the separation of the two intertwined (catenated) daughter molecules by the passage of one through the other. Table 4 lists the functions of each of these cellular proteins. As mentioned above, it was through the analysis of an in vitro reconstitution experiment with SV40 and large T antigen that the components of the cellular machinery were first identified (Li and Kelly 1984).

When these viruses infect permissive hosts, they initiate a productive cycle of infection; however, there are some cell types that do not support productive infection. Instead, in these cells the viral DNA becomes integrated into that of the host cells, in many cases transforming them. This is known as morphological transformation and occurs through the interaction of viral and cellular proteins (Simmons 2000).

PAPILLOMAVIRUSES

The papillomaviruses are a large family of small double-stranded DNA which are closely related to the polyomaviruses but differ in several important ways (Depamphilis 1996). Papillomaviruses infect mucosal or cutaneous epithelia causing warts and cervical carcinoma. Most of the initial work on DNA replication of these viruses was performed with Bovine papillomavirus

Table 4 *Cellular proteins required for SV40 DNA synthesis*

Cellular protein	Molecular mass (kDal)	Function
RPA	70	Binds single-stranded DNA; promotes origin unwinding; stimulates pol-α:primase; cooperates with RF-C and PCNA to stimulate DNA pol-δ
	32	
	12	
DNA pol-α: primase	180	Initiates leading- and lagging-strand synthesis through the synthesis of RNA primers and Okazaki fragments
	70	
	58	
	48	
RF-C	140	Auxiliary factor for DNA pol-δ and -ϵ; loads PCNA onto DNA; DNA-dependent ATPase
	40	
	38	
	37	
	36.5	
PCNA	36	Processivity factor for DNA pol-δ and -ϵ
DNA pol-δ	125	Completes leading- and lagging-strand synthesis; processive when bound to PCNA
	48	
DNA pol-ϵ	255	DNA polymerase which may complete lagging-strand synthesis
	55	
Topoisomerase I	110	Relieves torsional strain in front of replication forks
Fen1	48	5′ to 3′ exonuclease; removal of RNA primers
RNase H1	89	Endonuclease; cleaves RNA primer
DNA ligase I	125	Ligates Okazaki fragments

(BPV) which has served as a prototype for the papillomaviruses. Recent work with human papillomavirus (HPV) has revealed similarities and differences between BPVs and HPV. Examples from both replication systems will be discussed in this chapter.

General features of papillomavirus DNA replication

Papillomaviruses can be distinguished from polyomaviruses by exhibiting a more complex pattern of replication governed by the differentiation stage of the infected cell. In fact, the life cycle of papillomaviruses is known to be tightly coupled to the cellular differentiation program of the epithelium (Longworth and Laimins 2004). DNA replication actually occurs in three different stages. Three different modes of DNA synthesis during different stages of infection of skin have been reported: amplification, maintenance, and productive replication. The virus first infects proliferating basal epithelial cells, and in these cells the genome enters the nucleus and is amplified as a circular genome to a concentration of approximately 50–100 copies per cell. This replication requires the viral proteins E1 and E2 as well as the cellular replication machinery. This stage is believed to occur by theta mechanism and therefore resembles SV40 DNA replication. These circular DNA episomes are then maintained by a regulated process in which the episomes are replicated once per cell cycle again using an SV40-like mechanism. The final productive stage of DNA replication occurs in the outer layers of the epidermis, in differentiated cells. The shift from maintenance to productive replication is not well understood, but may involve a transition from SV40-like theta replication to a rolling circle mechanism of replication (Flores and Lambert 1997). DNA synthesis during the maintenance and the final productive stage of DNA replication also utilizes E1, E2, and the cellular DNA replication machinery (Broker and Chow 2001; Stenlund 2003). The fact that the productive phase of viral DNA replication occurs in terminally differentiated epithelial cells presents an interesting dilemma in that the cellular replication machinery required for viral replication would not be expected to be present in noncycling terminally differentiated cells (Broker and Chow 2001). Cellular replicative proteins are generally turned off in such cells. During the productive phase of the viral life cycle, papillomaviruses are presumed to be able to induce S-phase-specific viral functions. The viral protein E7 is required for productive infection in these differentiated cells and is believed to function by inducing the synthesis of the cellular replication proteins (Cheng et al. 1995; Broker and Chow 2001). Cellular proteins such as polymerase α and PCNA are likely induced in a manner similar to induction of host DNA synthetic machinery by SV40 T antigen.

The ability to induce the expression of S-phase-specific cellular genes is shared by several DNA viruses. The transcription of such cellular genes is tightly regulated by the Rb-E2f complex (Munger et al. 1989). E2f proteins are transcriptional regulators which bind cellular promoter sequences and stimulate transcription of S-phase-specific cellular genes. The presence of Rb in the E2f complex results in repression of these cellular genes. During the progression into S phase, Rb is phosphorylated resulting in its release from E2f and the induction of cellular genes encoding replication proteins such as histones and polymerases. Several viral proteins including adenoviral E1A, SV40 large T, and the E7 proteins of some human papillomaviruses have been shown to bind to the region of Rb that contacts E2f thereby resulting in activation of the E2f transcription factor. Thus, during productive papillomavirus infection, the E7 protein can stimulate the expression of the cellular DNA synthetic machinery. Following the replication of viral genomes, late viral proteins such as capsid proteins are made and progeny virus are assembled and eventually shed. These stages of replication are believed to be tightly regulated.

PAPILLOMAVIRUS GENOME

The papillomavirus genomes are small double-stranded DNA molecules of approximately 8 kbp (larger than the closely related polymaviruses) (Depamphilis 1996). All ten open reading frames are located on one strand of the viral DNA and all transcription is in a clockwise direction. Open reading frames are designated as either early (E) or late (L) based on their location on the genome. The early region encodes regulatory proteins which are expressed in nonproductively infected cells and also in cells transformed by papillomaviruses. Early genes *E1* and *E2* are required for DNA synthesis (reviewed in Stenlund 2003). At least five other early genes expressed from differentially spliced and partially overlapping mRNAs play various roles in regulation of gene expression and transformation. The late genes *L1* and *L2* encode structural proteins for the viral capsid and are only expressed in productively infected cells.

Origin of replication

The papillomavirus origin of replication lies in a small fragment located between the early open reading frames and a region called the long control region which contains regulatory signals for viral transcription (Stenlund 2003). Thus, as with the polyomaviruses, a relationship between the origin and binding sites for transcription factors exists (Depamphilis 1996). In the case of the papilloma viruses, the definition of the core origin is complicated by the apparent redundancies in this region. In the BPV ori, the core ori is contained in a fragment containing an AT-rich region, a binding site for E1 and

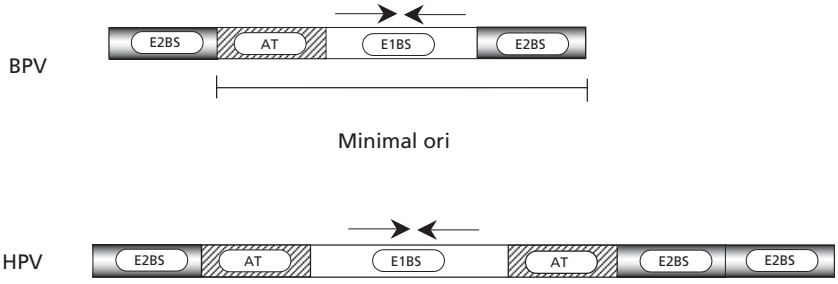

Figure 6 *Origin region of papillomavirus. BPV origin contains an AT rich region and a palindromic binding site for the initiator protein E1. However, these two elements are not sufficient for even minimal origin activity which requires at least one binding site for the transcription factor E2. In this case the E2 activator is required for loading of E1 to the origin (see text). In the HPV type origins, two binding sites for the E2 transcription factor are apparently required.*

a binding site for E2 to the right (Figure 6) (Chen and Stenlund 2002). However, if the right hand E2 binding site is deleted, replication can occur if the left hand E2 binding site is present. The HPV type origin contains a binding site for the viral replication initiator E1, three copies of a binding site for the viral transcriptional activator E2 and two AT-rich sequences. Thus, as with the polyomavirus origins of replication, both the BPV and HPV origins contain core essential elements flanked by auxiliary sequences which appear to enhance replication activity (Depamphilis 1996).

Replication proteins E1 and E2

E1 is the only viral protein that is directly involved in DNA replication. E2 is a transcription protein which greatly stimulates E1 binding at the origin. E1 proteins from both BPV and HPV share similarities with SV40 large T antigen (Enemark et al. 2000; Hickman et al. 2002). The BPV E1 protein is a 68-kDa nuclear phosphoprotein that binds specifically to the origin of replication. E1 has DNA-dependent ATPase and DNA helicase activities and binds to the origin of replication with weak affinity. E1 also interacts with cellular replication proteins such as the large subunit of DNA polymerase α (reviewed in Broker and Chow 2001). E2 interacts with E1 and facilitates E1 binding to the origin. E2 may also stimulate viral DNA replication by interacting with cellular proteins recruited to the origin such as RPA. E2 also plays a role in genome maintenance.

Mechanism of papillomavirus DNA replication

Initiation Much of the analysis of papillomavirus initiation has been performed in the BPV system. The availability of an in vitro system in which viral DNA replication occurs in a cell-free extract has been very valuable in our understanding of events which occur during the initiation of DNA replication. It should be kept in mind, however, that such in vitro reactions do not always accurately mimic events which occur in vivo. E1 and E2 proteins interact with one another and form a complex at the viral origin of replication. E1 is related in sequence and possesses activities analogous to the SV40 large T antigen such as ATPase, helicase, origin binding, and unwinding (Hickman et al. 2002; Stenlund 2003). BPV type 1 E1 binds to an 18-bp inverted-repeat element within the origin DNA with low specificity (Chen and Stenlund 2002). E2 is a dimeric transcription-replication factor that also binds to the origin. E1 and E2 bind cooperatively to the origin of replication to form an active initiation complex. The E2 protein is believed to act as a clamp-loading protein that stabilizes the E1-origin interaction and increases its specificity. A dimer of E1 is first bound together with a dimer of E2 ($E1_2E2_2$) (Chen and Stenlund 2002; Enemark et al. 2000). Binding of a second dimer of E1 occurs by displacing the E2 dimer in an ATP-dependent fashion creating a tetramer of E1. A bend is induced in the DNA by these interactions. Additional monomers of E1 are recruited to the complex eventually forming a hexamer and a double hexamer of E1 at the origin. ATP is required for the formation of the higher order structures. Cell cycle-specific phosphorylation may also be important in this assembly process (Broker and Chow 2001).

The formation of the initiation complex in HPV may differ slightly from BPV in some details; for instance, the HPV-1 E1 appears to be able to bind ori in a stable manner and to initiate replication on its own, at least in vitro. On the other hand, recent evidence suggests that the E1/E2 ratio is important for HPV replication efficiency and that regulation of E1 and E2 protein expression may be important for regulation during various stages of the virus life cycle.

E1 hexamers or double hexamers assemble at the origin, and it is this larger oligomer of E1 that is active in melting or distorting the duplex DNA (Stenlund 2003). This melting step is distinct from the ATP-dependent unwinding which occurs later. Melting is a change in the conformation of the DNA and can be observed by reactivity with single strand-specific agents such as permanganate ions which are able to modify non-base-paired resides. This melting or distortion step is

analogous to that described above for melting at the SV40 origin. Once a small region of single-stranded DNA is generated, RPA is recruited and melting occurs. The actual unwinding step involves the double hexamer which is now believed to form a bidirectional helicase. As with SV40, although the two hexamers are believed to unwind DNA in opposite directions, they appear to be held together. A new model for replication is emerging which suggests that instead of the hexameric helicases moving along the viral DNA template during replication in opposite directions, the template is actually drawn through the double hexamer as the DNA is unwound (reviewed in Stenlund 2003). The notion of a helicase which functions by 'pumping' DNA through a double hexamer is not unique to the E1 protein. Similar mechanisms have been proposed for SV40 T antigen and for the *E. coli* replicative helicase DNA B.

Elongation Little is known about the actual elongation phase of papillomavirus DNA replication. The early stages of DNA replication appear to occur by theta replication, however, it is possible that a switch from theta to rolling circle replication occurs during productive DNA replication (Flores and Lambert 1997). As in SV40 DNA replication, the E1 helicase is presumed to be the replicative helicase (Broker and Chow 2001). E1 also interacts with the polymerase α primase complex. Bidirectional DNA synthesis utilizes the cellular DNA synthetic machinery. In addition to RPA and the polymerase α primase complex, PCNA, RF-C, DNA polymerase δ or ϵ, and topisomerases I and II are presumably needed for papillomavirus DNA replication (reviewed in Broker and Chow 2001).

PARVOVIRUSES

The *Parvoviridae* are the smallest of the eukaryotic DNA viruses with genomes consisting of linear single stranded DNA molecules of 4 500–5 000 nucleotides. These viruses have been classified as either nondefective or autonomous parvoviruses, genus *Parvovirus*, such as the minute virus of mice (MVM), or defective or dependent parvoviruses, genus *Dependovirus*, such as the adeno-associated viruses (AAV) which rely on coinfection with adenovirus or herpesviruses for productive infection. Members of the autonomous *Parvovirus* genus can replicate productively without the aid of a helper virus. Replication of the autonomous parvoviruses requires that the infected cell enter S phase, since this virus utilizes components of the cell DNA replication machinery for its own replication; however, in contrast to SV40, these viruses cannot induce resting cells to enter S phase. As a result, productive infection is restricted to proliferating cells (Berns 1990; Depamphilis 1996).

General features of parvovirus DNA replication

Parvoviral DNA replication is similar to replication strategies of single-stranded coliphage genomes from bacteria (Berns 1990). The general mechanism has been called a 'rolling hairpin.' The incoming single-stranded DNA molecules form secondary structures at the termini which provide a 3′ OH primer for complementary strand synthesis. A linear duplex intermediate with one covalently closed end is generated. The resolution of this end by a site-specific nick results in the regeneration of the genomic termini. Again, the terminal redundancy can lead to another hairpin which can be elongated to make a double-stranded dimer molecule. Viral DNA replication proceeds through a series of concatemeric duplex intermediates by a strand displacement mode of synthesis involving leading-strand synthesis only. The autonomous and dependent parvoviruses share several features, but differ in some important respects. Both AAV and MVM have palindromic ends, but in MVM, the ends are not identical. Another difference is that in MVM, only the minus or noncoding strand is packaged.

MVM GENOME AND ORIGINS OF REPLICATION

The negative-sense MVM genome is approximately 5 200 bp long and exists primarily as a single-stranded DNA with terminal palindromic units which are predicted to form hairpin and other secondary structures. These terminal palindromic regions act as viral origins of replication during the process of regeneration of terminal sequences from long concatamers (termed junction resolution). They also play a unique role in the rolling hairpin replication mechanism used by this virus (described below) (Berns 1990).

MVM GENE PRODUCTS

The genome encodes two structural proteins VP1 and VP2; a third virion protein VP3 is present in infectious particles and derives from a proteolytic cleavage from VP2. The virus also encodes two nonstructural proteins, NS1 and NS2. NS1 is involved in both viral DNA replication and the transactivation of structural protein expression (Cotmore et al. 1995; Christensen and Tattersall 2002). NS1 is a site-specific nickase, recognizing the origin sequences present in the two viral termini and has been shown to nick and resolve these concatemers into unit length molecules suitable for packaging. The NS1 molecule becomes bound at the 5′ end of the viral DNA after nicking. It also comprises a 3′ to 5′ helicase, similar to the SV40 T antigen and papillomavirus E1 (reviewed in Marintcheva and Weller 2001). Alternate splicing events result in the production of three different versions of NS2 which differ in their C-terminal

sequences. NS2 may be involved in DNA replication, although its role is not clear at this time.

Mechanism of replication

Parvoviral DNA replication utilizes a modification of rolling circle replication mechanism dubbed rolling hairpin synthesis (Berns 1990). The genome is replicated through a series of monomeric and concatemeric duplex replicative-form (RF) intermediates. Replication proceeds unidirectionally by leading-strand continuous synthesis of DNA. The incoming viral genome is a linear single-stranded DNA molecule; however, the palindromic origin regions are believed to adopt secondary structures which may be simple hairpins or more complex T- or Y-shaped structures. The ability of the 3′ end to base pair within the terminal palindrome results in the formation of an ideal template-primer structure for the initiation of DNA replication (see Figure 7) (Berns 1990). This molecule is converted into a double-stranded intermediate by extension of the 3′ end by the cellular replication machinery resulting in a linear duplex intermediate, the original right-end hairpin is unfolded and copied to form a duplex terminal palindrome. In this model, the unidirectional replication fork shuttles back and forth along the linear genome. This mechanism involves unfolding and refolding of the two palindromic viral termini in such a way that the advancing replication fork can switch directions backwards and forwards generating a series of dimeric and tetrameric replication intermediates. Duplex copies of the unit-length genome become linked in head-to-head and tail-to-tail configurations.

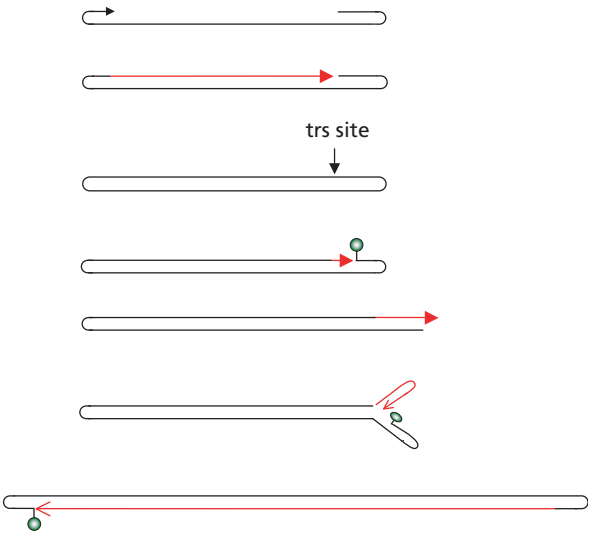

Figure 7 *Model for MVM DNA replication. The 3′ terminus of the virion strand is thought to fold back on itself to provide a primer for initiating synthesis of the complementary strand. When DNA synthesis reaches the right end, it is thought that a ligation event produces a linear duplex molecule with covalently closed termini. The right end can then be nicked by NS1 and the protein becomes covalently associated with the 5′ end, leaving a 3′ OH that copies the right end to a duplex extended form. The right end undergoes a conformational change resulting in the formation of a double hairpinned molecule which can be extended by further DNA synthesis. A dimer molecule is generated in which the monomer viral genomes are inverted with respect to one another. Terminal resolution and strand exchange leads to different orientations of the terminal sequences and eventually one strand is packaged into progeny genomes.*

Initiation The viral NS1 protein plays a vital role in this mechanism by recognizing origin sequences present in the two viral termini (Cotmore et al. 1995). NS1 recognizes a specific site in the origin region and introduces a nick at an adjacent initiation site. The minimal core origin is approximately 50 bp long and contains three distinct recognition sequences. NS1 binds site specifically to the (ACCA)2 repeat motif in an ATP-dependent manner. Although NS1 can bind by itself, nicking does not occur unless a cellular factor, called parvovirus initiation factor (PIF) is also present. Together, PIF and NS1 form a high-affinity ternary complex capable of nicking and subsequently initiating replication at the origin (Christensen and Tattersall 2002). After nicking, NS1 remains covalently attached to the 5′ end of the nicked DNA thereby freeing the 3′ hydroxyl group to act as a primer for DNA synthesis. The NS1 molecule bound at the 5′ end may also serve as a 3′ to 5′ helicase. Interestingly, it has recently been shown that similar to the SV40 T antigen, NS1 can be regulated by post-translational modification such as phosphorylation. Modification by phosphorylation of NS1 regulates the site-specific binding and DNA unwinding activities of the viral polypeptide (Nuesch et al. 2001). These phosphorylation events also determine the ability of NS1 to nick the left-end MVM origin site-specifically and control strand-displacement synthesis through its progressive helicase function. Multiple cellular kinases are believed to be responsible for these phosphorylation events (Nuesch et al. 2001).

Elongation DNA replication occurs through a series of monomeric and concatemeric duplex replicative-form intermediates and DNA synthesis by a unidirectional, leading-strand-specific process. The other cellular proteins required for MVM replication are also consistent with a leading-strand synthesis mechanism of DNA replication including RPA and proliferating cellular nuclear antigen (PCNA) (Christensen and Tattersall 2002). Although the cellular polymerase involved in vivo MVM DNA replication has not been identified, a processive leading-strand synthesis reaction has been reconstituted in vitro on a PIF-NS1 initiated origin by the addition of recombinant RF-C, PCNA, RPA and pol δ (Christensen and Tattersall 2002). RF-C is expected to act as a clamp loading protein recognizing the primer-template junction and loading PCNA onto the template to form a sliding clamp which stabilizes DNA poly-

merase on the template thereby promoting processive chain elongation. Enzymes identified in the SV40 DNA replication system which are involved in the generation or processing of lagging-strand Okazaki fragments (polymerase α-primase, RNase H, ligase I, and MF-I) have not been implicated in parvovirus replication.

ADENO-ASSOCIATED VIRUS (AAV)

In contrast to the MVM autonomous replicating parvovirus, AAV generally requires a co-infecting virus to efficiently replicate. Interestingly however, under conditions of genotoxic stress, AAV is capable of replicating in the absence of helper virus suggesting that whatever functions are provided by the helper viruses can be mimicked in the cell under certain kinds of stress conditions (Yakobson et al. 1989).

AAV genome and origin

Like MVM, AAV is a single-stranded DNA (ssDNA) virus whose genome is approximately 5 kbp and contains inverted terminal repeat (ITR) structures at each end. Trs can self-anneal to form terminal hairpinned structures and serve an important role as primers for DNA synthesis using host-cell synthetic machinery. In addition to containing an origin of replication, TRs also contain the packaging signal and the *cis*-acting elements needed for chromosomal integration (Berns 1990; Im and Muzyczka 1990).

AAV proteins

The 4.7 kbp ssDNA genome of AAV has two open reading frames, rep and cap (Im and Muzyczka 1990). Four viral proteins are encoded by the rep genes, two larger proteins, Rep68 and 78 and two smaller proteins, Rep52 and Rep40. These four gene products are produced by differential use of promoters: Rep68 and 78 are translated from mRNAs initiating at the one viral promoter, and the smaller proteins are translated from mRNAs that originate from another promoter. Differential splicing accounts for the differences between Rep68 and 40 and their longer counterparts, Rep78 and Rep52. Rep 68 and Rep78 are multifunctional proteins involved in DNA replication, transcription, packaging, and proviral integration (Li et al. 2003b). Rep68 and Rep78 exhibit several activities including sequence-specific double-stranded DNA binding, site-specific, single-stranded DNA endonuclease activity and ATP-dependent DNA helicase activities. Interestingly, the smaller Rep proteins 52/40 have been shown to play a role in packaging and this role is inhibited by the larger Reps78/68. In the case of MVM, there are no smaller versions of NS1, and it is not clear how regulation between replication and packaging occurs.

Mechanism of replication During AAV DNA replication, Rep68 and 78 bind viral Trs and induce a site-specific, single-stranded nick. Rep is thought to form oligomeric structures when bound to DNA containing a specific DNA-binding site (RBE) (Li et al. 2003b). Although a dimer of rep is believed to be required for trs nicking activity, it is thought that larger oligomeric structures then form on DNA, the largest of which appears to contain six Rep molecules (Li et al. 2003b). ATP may stimulate the formation of these higher order structures. A higher order structure containing six Rep molecules is reminiscent of other viral replication proteins which form hexamers upon binding their DNA substrates including SV40 large T antigen and papillomavirus E1 protein (Stenlund 2003; Hickman et al. 2003).

Initiation of replication Replication of AAV is initiated from the 3′ hairpin primer of the single-stranded input genome. The hairpin exposes a 3′ hydroxyl primer that can be extended by host–cell polymerase to initiate viral DNA synthesis a linear duplex molecule in which one of the ends is covalently joined (Berns 1990). To replicate the covalently closed terminal sequence, the termini are nicked on one of the two strands, and the newly exposed 3′ OH primer that is generated by the nick is used to repair the terminal sequence. This process is similar to the replication of the autonomous DNA viruses and ensures the integrity of the terminal sequences. The helicase activity of Rep68/Rep78 is thought to be involved in unwinding the covalently closed end of the linear viral DNA after it makes the site-specific nick at the trs site. As described above for T antigen, ATP is thought to stimulate multimerization facilitating the formation of double hexamer structures on the origin of DNA synthesis, and these double hexamers are believed to be active for helicase activity. The linear duplex end is then unwound to reform a terminal hairpin, thus providing a 3′-OH primer for strand-displacement synthesis. Elongation from the hairpin primer then generates a single-stranded genome (which is presumably packaged) and a new RF molecule, which again can undergo terminal resolution.

As with MVM, the cellular proteins involved in leading-strand DNA synthesis have also been implicated in the elongation step of AAV DNA replication including RPA, RF-C, PCNA, and a DNA polymerase (Christensen and Tattersall 2002). No requirement for DNA polymerase α-primase has been demonstrated. In an in vitro replication system, purified cellular factors PCNA, RPA, and RF-C and a partially purified fraction containing cellular DNA polymerases are necessary for reconstituting AAV DNA replication. However, investigators were unable to reconstitute activity in assays using combinations of known polymerases, suggesting that an additional cellular factor, which had not been previously identified in the SV40 system, may be necessary for AAV DNA synthesis

Requirement for helper virus In the absence of helper virus, only limited AAV genome replication can occur, and integration of the virus into a specific region of the host chromosome occurs. Interestingly, some transformed cell lines which have been treated with genotoxic agents (heat shock, hydroxyurea, UV light, or carcinogens) can support AAV DNA replication in the absence of helper virus (Yakobson et al. 1989).

Helper activity can be supplied by adenovirus and herpesviruses (including both HSV and HCMV). In the case of Ad, five genetic regions, the E1A, E1B, E4, E2A, and VA regions, are required for complete helper function (Richardson and Westphal 1981). However, none of these genes appears to be directly involved in AAV DNA replication. Genetic analyses have suggested that the role of Ad co-infection is primarily to maximize the synthesis of AAV-encoded gene products and possibly cellular genes that are required for AAV DNA replication. In the case of HSV, the minimal proteins that support AAV are the single-stranded DNA-binding proteins, ICP8 and the three components of the helicase/primase complex (UL5, UL52, and UL8) (Weindler and Heilbronn 1991). Interestingly however, the helicase and primase activities of the HSV proteins are not required for helper activity (Stracker et al. 2004). It has been suggested that the helper activity of the four proteins may be important as a scaffold which can recruit viral and cellular proteins important for AAV replication. Furthermore, it appears that the localization within the nucleus is important for efficiency of AAV replication. Both adenovirus and herpesviruses replicate at discrete sites within the nucleus and are able to subvert host cellular mechanisms to enhance viral replication (discussed below) (Carrington-Lawrence and Weller 2003; Wilkinson and Weller 2003). As discussed above, several viral replication proteins have been shown to interact directly with RPA, including T antigen and E1 of papillomaviruses. AAV rep and MVM NS1 have also been reported to interact with RPA, suggesting that recruitment of RPA and other cellular proteins may be important for parvoviral replication (Christensen and Tattersall 2002; Stenlund 2003; Stracker et al. 2004).

Summary Parvoviruses replication proteins NS1 for MVM and Rep68/78 for AAV act to nick the viral DNA and also as oligomeric helicases. Strand displacement synthesis is then carried out using cellular replication machinery; however, since only leading-strand synthesis occurs, the cellular machinery needed for lagging-strand DNA synthesis does not appear to be involved in parvovirus DNA replication.

ADENOVIRUSES

Adenoviruses cause respiratory infections and conjunctivitis among other ailments and have been isolated from a wide range of mammalian and avian hosts. Human adenoviruses can also cause morphological transformation in rodent cells. Forty-nine human adenovirus serotypes have been identified. All adenoviruses isolated to date contain a linear, double-stranded DNA genome of approximately 35 kbp packaged in a nonenveloped icosahedral capsid (Depamphilis 1996).

General features of adenoviral DNA replication

The linear genome contains two copies of the viral origin within the terminal inverted repeats. Initiation can occur at either end of the viral genome resulting in the formation of a daughter strand and the displacement of one of the parental strands (reviewed in Depamphilis 1996). The displaced parental strand can then serve as a template for the synthesis of a second daughter strand. The DNA replication strategies of this moderately sized DNA virus exhibit several unusual features. For instance, in contrast to the parvo-, polyoma-, and papillomaviruses, adenovirus encodes its own DNA polymerase (pol) and DNA-binding protein (DBP). Another interesting feature is the covalent attachment of a 55 kDa terminal protein (TP) to the 5′ end of each strand. The covalent attachment of TP results from that fact that adenovirus utilizes an unusual protein priming mechanism for replication initiation. Three cellular proteins have been shown to stimulate viral DNA synthesis: two transcription factors nuclear factor I (NF1) and Oct-1 and a topoisomerase (NF-II) (de Jong and van der Vliet 1999). Thus this virus is intermediate between the smaller DNA viruses which only encode one viral replication protein, and the larger herpes- and poxviruses which encode not only their own polymerases but most or all (in the case of pox) of the other proteins required for DNA replication. Another interesting feature exhibited by adenovirus will be discussed at the end of this chapter and relates to complex virus–host interactions related to intracellular localization and inactivation of cellular response signals.

As with the SV40 system, the identification of viral and cellular proteins involved in DNA replication was facilitated by the development of a soluble cell-free DNA-replication system. These experiments indicated that adenovirus replication requires three virally encoded proteins: precursor TP (pTP), DNA polymerase (pol) and the DBP. The availability of the reconstituted cell free replication system also allowed the identification of two cellular transcription proteins required for efficient initiation, NF1 and Oct-1 and a third cellular protein, the type I topoisomerase NFII required for efficient elongation in the strand displacement synthesis reaction.

ADENOVIRUS GENOME

The linear double-stranded DNA genome consists of five early transcription units, two delayed early units,

and one late unit. Both strands of the viral genome are transcribed although the majority of viral genes are generated from the so-called rightward reading strand. Differential spicing and the use of alternate splicing increase complexity of viral gene expression (Depamphilis 1996).

Origin of replication

The origin of the best studied adenovirus (Ad 2) consists of two distinct regions, a minimal origin region within the first 18 bp and a second region 19–51 which serves to enhance the efficiency of the initiation reaction. The minimal origin (1–18) contains a 10-bp region (bps 9–18) conserved in all adenovirus terminal regions. The minimal origin by itself can only support a limited, basal level of initiation of DNA replication. The auxiliary region contains binding sites for the cellular proteins NF1 and Oct-1. Interestingly, the ori region for another adenovirus, Ad 4, requires only the terminal 18 bp of the viral genome for efficient viral replication. Ad 4 does not seem to require the host factors NF1 and Oct-1 (de Jong and van der Vliet 1999).

Viral proteins involved in DNA synthesis

Adenoviruses encode three viral proteins that are essential for DNA synthesis, the adenovirus polymerase (Ad pol), the pTP and the single-stranded DBP (reviewed in Depamphilis 1996). Sequence analysis reveals that the 140Ad pol is related to the larger family of DNA polymerases. Within this family, the Ad pol is a member of a subfamily of protein-priming DNA polymerases. The polymerase activity of the pol protein is located at the C terminus of the protein (Brenkman et al. 2002). The Ad pol is capable of catalyzing two types of reactions: the covalent addition of dCTP to a hydroxyl residue on a serine in the 80 kDa pTP, and the elongation of a growing DNA chain from the protein primer terminus (de Jong et al. 2003b). It is believed that the switch from a protein- to a DNA-priming polymerization mode may require a conformational change within the polymerase. Biochemical analysis of Ad pol has shown that it replicates DNA in a processive manner and that it has a distributive 3′–5′ exonuclease activity on single-stranded DNA. Both the polymerase and exonuclease activities are decreased when pTP is complexed with Ad pol. This difference in activity depending on association with pTP may explain the switch from protein to DNA-priming mode in that pTP is believed to dissociate from pol before elongation proceeds (de Jong et al. 2003a). Dissociation from pTP is believed to increase processivity of pol. The viral DBP can stimulate the activity of the polymerase and can also increase its processivity.

The 72-kDa single-stranded DBP is essential for DNA replication. This was originally determined by the isolation of temperature sensitive mutations in the DBP gene. DBP participates in the initiation of DNA synthesis as well as the elongation step. DBP stimulates initiation by enhancing the binding of Pol to the origin of replication (van Breukelen et al. 2003). DBP helps to unwind the double-stranded DNA template and enhances the processivity of Pol by the removal of secondary structures. DBP binds single-stranded DNA in a sequence-independent and cooperative manner. Interestingly, DBP can support the movement of the replication fork over large distances without hydrolysis of ATP. This property of DBP abrogates the need for a helicase during DNA synthesis. In addition to its role in DNA synthesis, DBP plays a role in control of transcription, transformation, and virus assembly. The DNA-binding domain resides in the C terminal 174–529 residues. The N-terminal domain contains the nuclear localization signal.

The 671 amino-acid pTP protein makes many contacts with viral DNA and with other proteins during initiation of viral DNA synthesis. The N terminus interacts with DNA and C-terminal residues interact with Ad DNA pol (de Jong et al. 2003a). pTP also appears to harbor multiple binding sites for Oct 1. The most important single amino acid in pTP is Ser 580, which is covalently coupled to the incoming dCTP (de Jong et al. 2003a). Ad5 pTP contains three protease cleavage sites that are recognized by a virally encoded protease. Cleavage at the TP site most likely occurs after packaging into the viral particle, generating a TP-DNA complex which lacks the N-terminal 349 amino acids of pTP. Another interesting feature of the pTP is its apparent ability to attach to the nuclear matrix. This interaction could provide a scaffold for the recruitment of replication proteins that explains the observation of specific replication foci in the nucleus (see below). Thus the important attributes of pTP include multiple DNA and protein contacts, the presence of Ser 580 which becomes covalently attached to the nascent DNA chain and its ability to undergo cleavage.

Cellular proteins

Oct-1 is a human transcription factor that can stimulate both RNA pol II and pol III transcription from a variety of promoters. It consists of a central bipartite POU DNA binding domain surrounded by activation regions. POU domains can recognize a variety of different promoter DNA sequences. Although adenovirus DNA replication can occur in vitro with only the viral genome and the three virally encoded replication proteins, pTP, pol, and DBP, replication is stimulated up to 200-fold by the addition of the cellular transcription factors Oct-1 and NF1 (de Jong and van der Vliet 1999). The Oct-1 protein interacts with pTP directly and the presence of an Oct-1 binding site in the origin stimulates viral replication in vivo. Oct-1 is believed to enhance the DNA binding of pTP–pol by lowering its dissociation rate leading to a model in which Oct-1 tethers pTP–pol to the replication

origin. NF1 is another cellular transcription factor which binds as a dimer to viral and cellular promoters. A type II cellular topoisomerase, nuclear factor II, is also required for adenovirus DNA replication and it thought to relieve overwinding of the template during replication (de Jong and van der Vliet 1999).

Mechanism of viral DNA synthesis

A mechanism for initiation and elongation of viral DNA synthesis has now emerged.

Initiation The first stage of DNA replication involves the formation of a preinitiation complex comprising NF1, Oct-1, pTP, Pol, and DBP at the origin of replication. The recruitment of viral and cellular proteins to origins of replication is a theme repeated in many other viral and cellular replication systems studied. In the first step of initiation a heterodimer of pTP and pol assembles at the origin of replication in a binding reaction stimulated by the binding of cellular transcription proteins NF1 and Oct-1 to adjacent sites (see Figure 8). NF1 and Oct-1 apparently target the pTP-Pol complex to the origin (de Jong and van der Vliet 1999). The DBP can also stimulate the binding of Pol at the origin. The ability of both cellular proteins NF1 and Oct-1 and DBP to stimulate binding of pTP–pol at the origin illustrates the importance of these types of protein–protein interactions in recruitment of the components of a replication machinery to viral origins of replication.

DNA replication is initiated by an unusual protein priming mechanism in which a covalent attachment is formed between the first base and a serine residue in pTP (de Jong et al. 2003b). This reaction is catalyzed by pol, but relies on multiple DNA–protein and protein–protein interactions which act to stabilize the preinitiation complex formed at the adenovirus origin of DNA replication. The pTP and pol must assemble in such a way that the serine-580 in pTP is accessible to the Ad pol catalytic site and the DNA template to react with the first nucleotide to be added, dCTP to give dCMP, in the protein priming reaction (de Jong et al. 2003a). The addition of dCMP is template driven as DNA synthesis is initiated opposite the fourth base of the template strand. In a reaction catalyzed by the Ad polymerase, CMP is covalently bound to the hydroxyl group of a serine residue in the primer pTP. Two more nucleotides are added forming a trinucleotide intermediate (pTP–CAT). Then, in another remarkable reaction, the trinucleotide intermediate jumps back to base pair with template bases 1 to 3 (another CAT triplet). pTP then starts to dissociate from Ad pol (de Jong et al. 2003b).

Elongation and strand displacement synthesis After dissociation, pol replicates the entire adenovirus genome resulting in strand displacement DNA synthesis of the nontemplate strand. Oct-1 dissociates as the polymerase elongates past the Oct-1 binding site. Strand displacement elongation synthesis requires DBP and another cellular protein, type I topoisomerase nuclear factor II. At late times after infection, pTP is processed by a virus-encoded protease into the mature TP. The displaced strand is coated by DBP and by virtue of its inverted terminal repeats it can form a panhandle type molecule which contains a stem reminiscent of the duplex of the initial double-stranded DNA template. The duplex can then be recognized by NF1, Oct-1 and the pTP–pol heterodimer and a new cycle of protein primed DNA synthesis can occur (de Jong et al. 2003b).

Summary

In summary, adenovirus DNA replication is of interest because of its intermediate size and coding potential; it encodes many but not all DNA replication proteins. It is thus a relatively simple replication system compared to SV40 and the other smaller DNA viruses which utilizes the very elaborate cellular replication complex. The protein priming mechanism used by adenovirus and the lack of a requirement for a helicase contribute to the simplicity of this system compared to polyoma- and papillomaviruses that utilize cellular polymerases. Other DNA viruses which encode replication machinery such as the herpesviruses and poxviruses are also much more complex. Also of interest are the complex interactions between adenovirus and the host cell.

HERPESVIRUSES

The family *Herpesviridae* encompasses a wide variety of both human and animal pathogens capable of causing medically and economically important diseases in a broad range of hosts. Eight are known to infect humans including herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), Epstein–Barr virus (EBV), cytomegalovirus (HCMV), varicella-zoster virus (VZV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and Kaposi's sarcoma herpesvirus (KSHV). Herpesviruses, in general, share many aspects of virion structure, genomic organization, and life cycle (described in Herpesviruses: general properties). For the purposes of this chapter, HSV-1 will be discussed as the primary example of herpesvirus DNA replication; HSV has been studied more extensively than the other herpesviruses, perhaps due to the fact that it is amenable to both genetic and biochemical analysis.

General features of DNA replication

The herpesviruses encode a large number of replication proteins, some of which are functional analogues of cellular replication machinery. Furthermore, the virus appears to have evolved a strategy of DNA replication

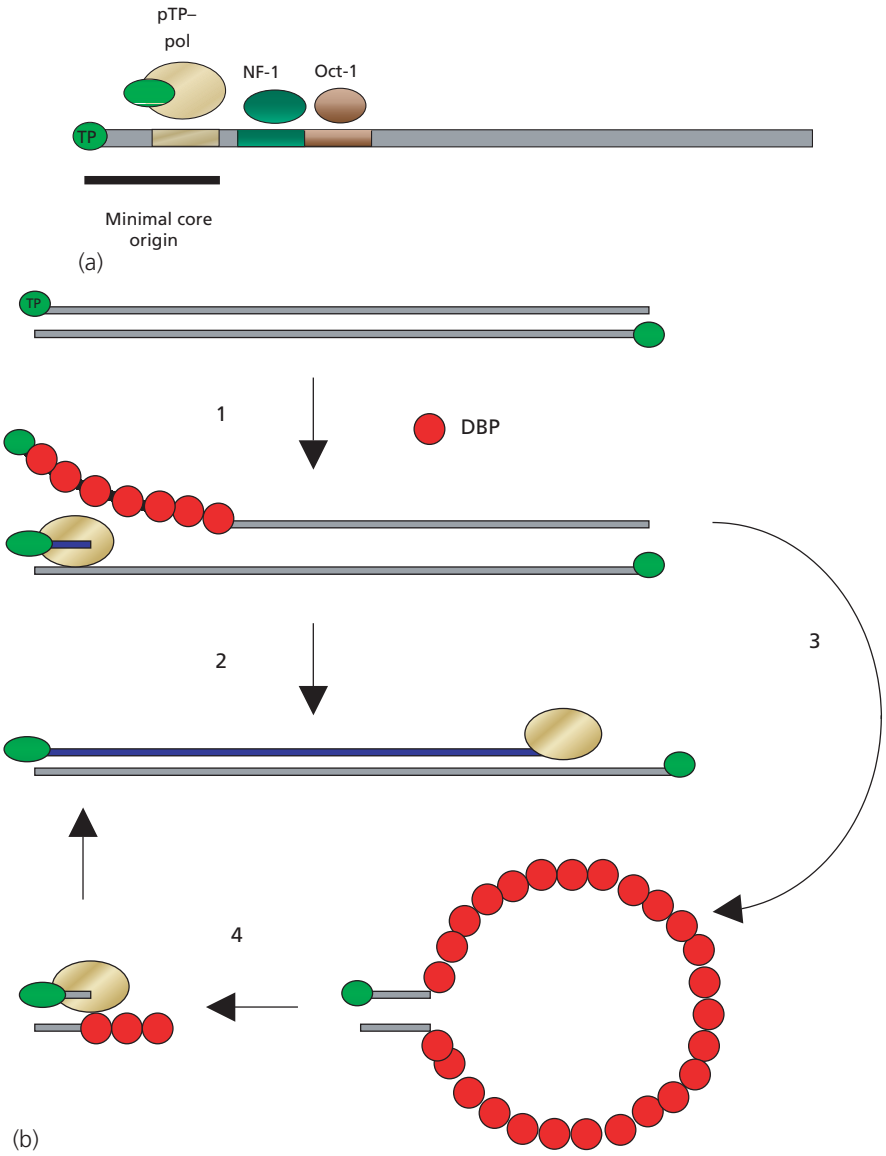

Figure 8 *Replication of adenoviral DNA.* **(a)** *The minimal core adenovirus origin of replication consists of the terminal 18 bp of the viral genome which contain the binding site for the pTP–pol heterodimer (9–18 bp). Adjacent to the 9–18 bp region are binding sites for the cellular transcriptional activators NFI (bind residues 25–39) and Oct-1 (binds 39–48), and these proteins facilitate the initiation reaction by binding at the origin and stimulating the association of the pTP–pol heterodimer to the origin. The pTP–pol interaction with the origin is stabilized by interactions with the TP–DNA, between pTP and Oct-1, and Adpol and NF1.* **(b)** *Step 1.The initiation of adenoviral DNA synthesis involves the assembly of a heterodimer consisting of the preterminal protein (pTP) and DNA polymerase (pol) (light green and gold). This heterodimer forms a preinitiation complex with the minimal core origin of replication. DNA replication is initiated by an pol-catalyzed protein priming mechanism in which a covalent bond is formed between the -phosphoryl group of the terminal residue, dCMP, and the hydroxyl group of a serine residue in pTPB. Step 2. The free 3′ OH group of the pTP–dCMP moiety primes elongation in a leading strand type reaction, resulting in the displacement of the non-template strand which becomes coated with DSB, the single-stranded binding protein. The elongation reaction also requires a cellular topoisomerase. Step 3. The displaced strand can form a panhandle reaction due to the complementarity of the inverted repeats at the termini of the adenoviral genome. The short duplex stem can be used to initiate a new round of viral DNA synthesis, steps 3 and 4. At later times in infection pTP (larger green oval) is processed by a virus-encoded protease into the mature TP (smaller green oval) (not shown).*

which utilizes DNA recombination (Wilkinson and Weller 2003). This feature is reminiscent of several of the large DNA bacteriophages including T4 and lambda. The viral genomes are linear molecules which replicate through longer-than-unit-length concatemers. Monomeric genomic units are packaged by cleavage from concatemers in a reaction that occurs in conjunction with the uptake of genomes into preassembled capsids.

HSV GENOME

The genome of HSV is large (152 kbp) and structurally complex, containing two unique regions (U_L and U_S)

that are flanked by the inverted repeat sequences *b* and *c*, respectively. The *a* sequence is present at both termini and an inverted copy is present at the U_L–U_S junction (Figure 9a). The HSV genome is capable of genomic inversions in which the U_L and U_S components invert their orientation during replication. HSV-1 is believed to encode over 80 open reading frames. Viral genes are classified as immediate–early, early or late and are transcribed from both strands of the viral genome.

Origins of DNA replication

Three origins of replication have been identified within the HSV genome (Figure 9b) (reviewed in Marintcheva and Weller 2001). One origin, OriS, is present in each 'c' component of the short region and thus is present twice in the viral genome and one origin, OriL, is present in the unique long region between the major DNA binding protein (ICP8) and the DNA polymerase. Both origins contain AT-rich sequences and several recognition sites for UL9, the origin binding protein. OriL contains a near perfect palindrome of 144 bp while OriS contains a shorter palindrome. Both origins are situated between transcriptional initiation sites. OriL, and one copy of OriS, can be deleted without affecting the ability of the virus to multiply, suggesting that viral replication can occur in genomes containing only one copy of the genome.

Viral replication proteins

The HSV-1 genome encodes seven essential replication proteins and several nonessential replication proteins (Table 5) (Challberg 1986). These include an origin

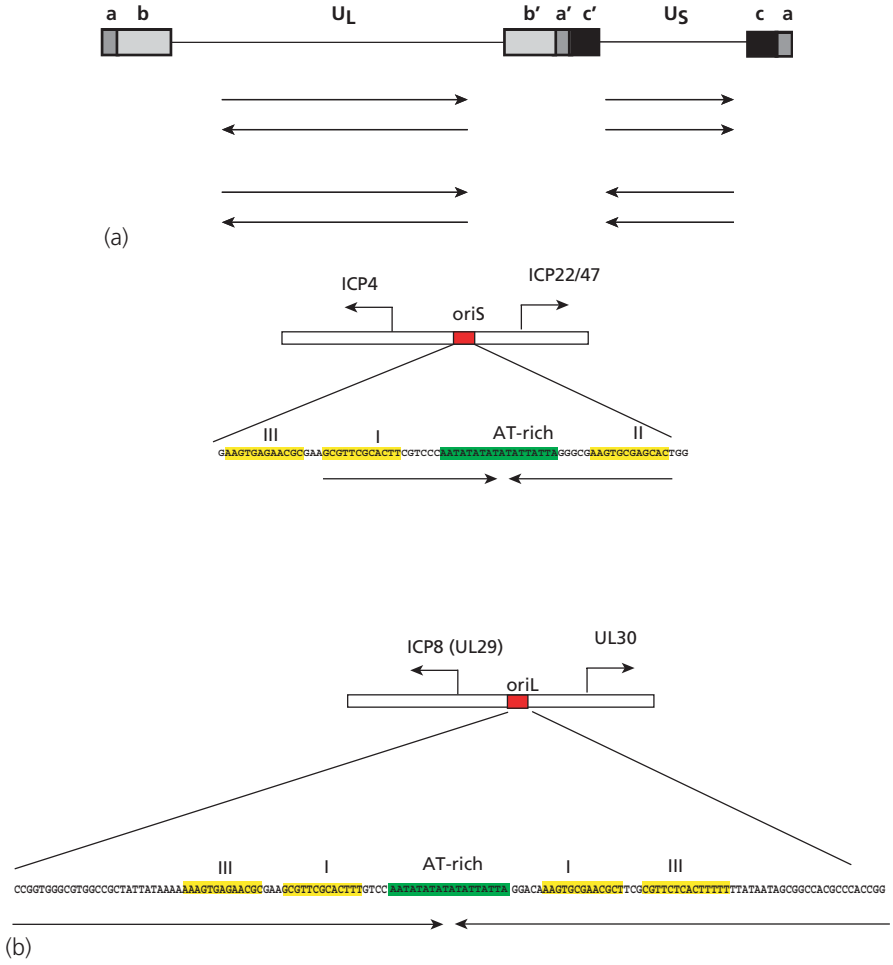

Figure 9 HSV *genome and origins of replication. A. The HSV-1 genome consists of two unique regions* U_L *and* U_S *flanked by repeated sequences.* U_L *is flanked by* ab *and* b'a'*;* *whereas,* U_S *is flanked by* ac *and* c'a'*. During infection, the two unique regions invert relative to each other. The arrows reflect possible orientations of the* U_L *and* U_S *segments as a result of genomic inversion.* **(b)** *HSV-1 Origins of replication. OriS is situated between the immediate early genes ICP4 and ICP22/47. It contains three UL9 binding sites highlighted in yellow (Boxes I, II, and III) and an AT-rich region (highlighted in green). OriS also contains an almost perfect 22-bp palindrome (marked with arrows) OriL is situated between the* ICP8 *gene (*UC29*) and the DNA polymerase gene (*UL30*). It contains four binding sites for the origin recognition protein UL9 highlighted in yellow (Boxes I, II, III, and IV), and AT-rich region (highlighted in green), and a perfect 144-bp palindrome (marked with arrows).*

Table 5 Herpes simplex virus *replication proteins*

Viral Protein	Function
UL30 (DNA pol)	Synthesis of leading- and lagging-strand DNA; associated 3′ to 5′ proofreading exonuclease; target for several antiviral drugs
UL42	HSV DNA pol processivity factor
UL9	Origin binding; DNA-stimulated ATPase and helicase
UL5, UL8, and UL52	Helicase primase complex; UL8 interacts with ICP8 and HSV pol
ICP8 (UL29)	Viral single-strand DNA-binding protein; binds cooperatively to single-stranded DNA; binds UL42 protein
Thymidine kinase	Phosphorylates thymidine; essential in nondividing cells
Ribonucleotide reductase	Reduces ribose to deoxyribose; essential in nondividing cells
dUTPase	Hydrolyzes dUTP to dUMP; prevents uracil incorporation into DNA
Uracil N glycosylase	Removes uracil from DNA; may be important for DNA repair
Alkaline nuclease (UL12)	Interacts with ICP8 and may promote single-stranded annealing during recombination-dependent DNA replication

binding protein (UL9) and six core replication proteins including a two subunit DNA polymerase (UL30 and UL42), a single-stranded DNA-binding protein (known as ICP8 or UL29) and a three subunit helicase/primase complex (UL5, UL8, and UL52) (Challberg 1986; reviewed in Marintcheva and Weller 2001). Among the eight human herpesviruses and dozens of animal herpesviruses, most encode homologues of the six core replication viral proteins involved in DNA replication, and it is likely that the strategy of replication is also conserved.

UL9

UL9 is a 94-kDa protein possessing the following activities: DNA-dependent nucleoside triphosphatase, DNA helicase on partially double-stranded substrates, ability to form dimers in solution, and cooperative origin-specific DNA binding. The ability of UL9 to bind specifically to origin DNA has been localized to the carboxy-terminal one-third (residues 564–832) of UL9. Protein sequence analysis indicates that the N-terminal domain (residues 1–534) contain seven conserved helicase motifs, characteristic of the SF2 family of helicases. The nuclear localization signal (NLS) of UL9 has been mapped to the C terminal 105 amino acid residues (reviewed in Marintcheva and Weller 2001).

UL30/UL42

UL30 encodes the 140 kDal catalytic subunit of the HSV polymerase, and UL42 encodes the 53 kDal processivity factor. Since no viral DNA synthesis can be detected in the presence of drugs which inhibit the viral polymerase or in cells infected with mutants defective for viral polymerase, it is believed that UL30/UL42 is the sole polymerase needed during viral DNA replication and that it functions as both the leading and lagging strand polymerase. The HSV polymerase has been studied extensively and is the target for antiviral drugs.

UL5/UL8/UL52

The HSV-1 helicase/primase is a three protein heterotrimer consisting of the products of the *UL5*, *UL8*, and *UL52* genes. All three genes are essential in cell culture. The *UL5*/*UL8*/*UL52* complex displays DNA-dependent ATPase, primase, and helicase activities (reviewed in Marintcheva and Weller 2001). A subcomplex consisting of UL5/UL52 displays similar activities. The precise function of UL8 within the heterotrimeric complex is not clear. In vitro, the helicase and primase activities do not depend on UL8; however, the ATPase and primase activities of the UL5/UL52 subcomplex can be stimulated in vitro by the addition of UL8. UL8 may also play a role in the proper localization of the UL5/UL8/UL52 complex to the nucleus. Furthermore, since UL8 can interact with other members of the replication machinery including UL9, UL30, and ICP8, it appears that UL8 may act to coordinate complex protein–protein interactions which occur at the replication fork. UL52 contains a motif conserved in many primases which, when altered, abolishes primase but not ATPase and helicase activity. This suggests that the UL52 subunit is likely the primase of the complex; however, recent work indicates that the UL52 subunit may contribute significantly to helicase activity as well (Carrington-Lawrence and Weller 2003). Sequence analysis indicates that UL5 contains seven motifs found in a large superfamily of known and putative helicases, SF1. Thus UL5 is likely to be the helicase of the complex, although it appears that UL52 may also be involved in helicase activity, perhaps by providing at least one DNA binding site.

UL29 or ICP8

ICP8 is the 130 kDa single-stranded binding protein of HSV. ICP8 binds preferentially to ssDNA and exhibits helix-destabilizing activity. ICP8 can also promote strand transfer and is believed to play a role in the high level of recombination exhibited during HSV DNA replication.

Recent work indicates that through its interaction with a viral nuclease (UL12), this two subunit complex may constitute a simple recombinase analogous to the simple red recombinase encoded by bacteriophage lambda (Reuven et al. 2004).

ICP8 is known to interact with other DNA replication proteins such as UL9, UL8, and HSV polymerase, and is believed to be essential for both the initiation and elongation of viral DNA synthesis. ICP8 also plays a role in the localization of viral proteins to replication compartments in infected cell nuclei and in the control of viral gene transcription.

Nonessential viral gene products

In addition to the seven required viral replication proteins, HSV also encodes several nonessential auxilary proteins such as thymidine kinase, deoxyuridine triphosphatase, ribonucleotide reductase, and uracil *N*-glycosylase. Some of these proteins participate in nucleotide biosynthesis and others such as dUTPase and uracil-*N*-glycosylase may be important in preventing misincorporation of uracil residues into the viral genome.

Overall model for HSV DNA replication

Although the exact mechanism of HSV DNA replication is not known, it has long been recognized that the products of DNA replication are larger-than-unit-length concatemers consisting of tandem repeats of the viral genome (reviewed in Marintcheva and Weller 2001). Most of the incoming viral DNA molecules lose their free ends shortly after infection by a process that does not require de novo protein synthesis, and the simplest interpretation of these results is that the viral genome loses its free ends through the formation of a covalently closed circular molecule. It was previously thought that linear virion DNA is rapidly circularized in infected cells and that concatemers were generated by rolling circle replication; however, this model has never been directly confirmed experimentally. Several aspects of this model, including the initial circularization and the rolling circle mechanism, have recently been challenged experimentally, and new models are being considered (Jackson and De Luca 2003). For instance, replicating DNA adopts a complex, possibly branched, structure which could reflect recombination intermediates. It appears that endless viral DNA does not arise as a result of simple end-to-end joining of viral termini by nonhomologous end joining, although it is possible that homologous recombination events may play a role. New models have been proposed suggesting that the viral genome adopts an endless configuration by an intra- or intermolecular homologous recombination events which occur during replication (discussed below) (Wilkinson and Weller 2003).

Initiation Viral DNA synthesis is believed to initiate at one of the three viral origins of replication. UL9 is essential for HSV DNA replication and has been shown to bind the origins of DNA replication in a cooperative and sequence-specific manner; however, many details of this reaction are still unclear. For instance, although UL9 is believed to be an ATP-dependent DNA helicase, in vitro, it has not been shown to unwind duplex DNA containing an origin of replication. Some distortion at this region can be detected but no actual unwinding (reviewed in Marintcheva and Weller 2001). This is in contrast to SV40 large T antigen that is able to unwind duplex DNA in an ATP-dependent fashion. Recent evidence suggests that UL9 binding at the origin may not be as straightforward as binding to its recognition site within duplex DNA. In in vitro experiments, single-stranded oriS has been reported to fold into a unique conformation, OriS*, which is stably bound by UL9. OriS* contains a stable hairpin formed by complementary base pairing between box I and box III in OriS (Aslani et al. 2001). It has been proposed that that UL9, in conjunction with the single-stranded DNA-binding protein ICP8, can convert OriS to its alternate conformation, OriS*, and form an OBP–OriS* complex. This model has not been tested in vivo however.

Elongation Once the origin is distorted by UL9 and ICP8, it is believed that the heterotrimeric helicase/primase complex can be recruited (Figure 10) (Marintcheva and Weller 2001). Several lines of evidence suggest the ordered assembly of viral replication proteins at the origin. Using immunofluorescence, a subassembly, or scaffold, has been identified consisting of five viral proteins: UL9, ICP8, and the helicase/primase. This scaffold can be detected as ICP8-containing foci in cells infected with wild-type HSV (Burkham et al. 2001). The recruitment of polymerase to this scaffold requires the presence of an active primase subunit (Carrington-Lawrence and Weller 2003). These results suggest that polymerase recruitment to replication foci requires primer synthesis and support the model of an ordered assembly of replication proteins at the replication fork leading to leading and lagging strand DNA synthesis.

Once elongation has begun, leading- and lagging-strand DNA synthesis would be expected to proceed until the replication apparatus either reaches the end of the linear DNA molecule or reaches a nick or gap. Since nicks and gaps have been reported to be present in the viral genome, this may occur soon after elongation has begun. It has recently been proposed that such an event could result in a fundamentally different mode of replication which utilizes a recombination strategy to continue DNA synthesis (Wilkinson and Weller 2003). The ability of replication to begin again after such an event may signal the second stage of replication which would be dependent on cellular or viral recombination

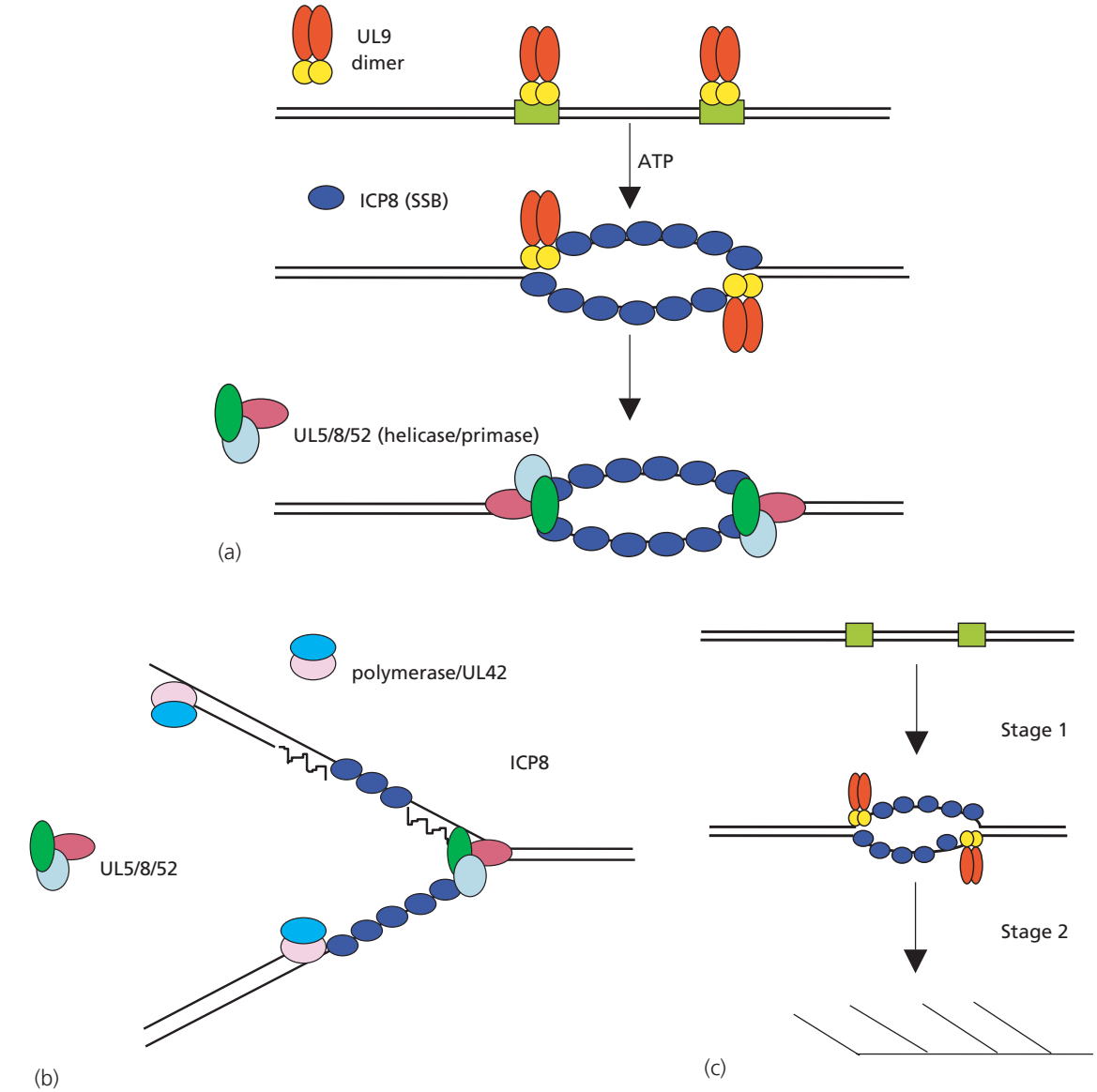

Figure 10 *Model for* HSV *DNA replication.* **(a)** *OriS can be depicted with three recognition sites for UL9, the origin-binding protein (Boxes I, II, and III) and an AT-rich linker positioned between box I and II. According to this model, UL9 dimers bind cooperatively to Boxes I and II and cause either a distortion or an opening of the AT-rich spacer region. In this step UL9 likely acts in conjunction with ICP8. Following the opening, the helicase/primase can be recruited to the complex. UL9, ICP8 and the helicase/primase complex are apparently recruited together during the formation of replication centers [80–82].* **(b)** *An HSV-1 replication fork would be expected to contain the helicase-primase complex (UL5/UL52/UL8) at the fork: UL5 would be expected to unwind duplex DNA ahead of the fork and U52 would be expected to lay down RNA primers which could then be extended by the two subunit DNA polymerase (UL30/UL42). The HSV-1 pol would also be expected to carry out leading-strand synthesis. ICP8 (UL29, SSB) would be expected to bind to ssDNA generated during HSV DNA synthesis. C). In this model, two stages of replication are envisioned, an UL9 dependent step which takes place at origins of replication (Stage 1) and a UL9 independent step which likely involves recombination-dependent replication (Stage 2).*

proteins to 'restart' the replication process. This second stage of replication would not be expected to be dependent on UL9 and, in fact, UL9 is not essential at later times after infection. The later stages of HSV replication are likely to involve recombination although it cannot be ruled out that some rolling circle replication also occurs (Figure 10c). The virus encodes a simple recombinase consisting of ICP8 and the viral nuclease UL12 (Reuven et al. 2003). It is possible that viral and cellular recombination proteins are involved during these later stages of viral DNA replication (Wilkinson and Weller 2003). Details of these reactions are the subject of on going studies.

Summary

HSV appears to be unique in the animal DNA viruses in that it exhibits a complex relationship between DNA

replication and recombination. In this respect it seems more similar to the large DNA bacteriophages, T4 and λ, which replicate via concatemers of tandemly repeated monomeric units using rolling circle replication as well as multiple pathways to carry out homologous recombination. The seven replication proteins of HSV may work in combination with both viral and cellular recombination proteins during this process, the details of which remain unclear. In summary, it appears that HSV DNA replication occurs in two stages. The first stage involves an origin-UL9 dependent step and in the second stage DNA replication may proceed in an origin-independent manner via a recombination-driven and/or rolling circle mechanism (Wilkinson and Weller 2003).

POXVIRUSES

The poxviruses make up a particularly interesting group of pathogens responsible for devastating human diseases such as smallpox. Poxviruses have been isolated from many animal hosts, and cowpox and vaccinia viruses have been used as vaccines to protect humans from the smallpox virus, variola virus. Poxviruses are unique among the DNA viruses in their ability to replicate entirely within the cytoplasm. They are large complex viruses which exhibit considerable independence from the host cells (Depamphilis 1996).

General features of poxvirus DNA replication

Poxviruses have the largest genomes of any known DNA viruses, and they have been shown to encode all of the proteins needed for DNA replication including DNA polymerase, a processivity factor, two protein kinases, a protein phosphatase, and a DNA ligase (Boyle and Traktman 2004). Although the function of the kinases and phosphatase are not entirely clear, their existence suggests that protein phosphorylation may play an important role in regulating either DNA replication or another step in the virus life cycle. DNA replication occurs in the cytoplasm in specialized foci called viral factories. After fusion of viral and cellular membranes, the inner viral core is localized in the cytoplasm, and early gene expression occurs within these cores. Early mRNAs are extruded from the core where they are translated by host ribosomes. Some of these early proteins encode replication proteins. A secondary uncoating event occurs and viral genomes are released and accessible to replication proteins. Replication occurs in two stages: synthesis of genomes as concatemeric intermediates and resolution of these concaters into mature, monomeric genomes (Depamphilis 1996).

POXVIRUS GENOMES

Poxviruses have linear double-stranded DNA genomes which vary in size from 130 kbp to around 230 kbp. The vaccinia genome has a very unusual structure: it is composed of a linear duplex of 192 kbp with covalently closed hairpin termini resembling telomeres. The genomes are AT-rich and the inverted terminal hairpin repeats also contain primarily A and T residues (Du and Traktman 1996).

Origins of replication

The termini are believed to contain the origins of replication. In vaccinia, for instance, the telomeric 65 nucleotides can be replicated in infected cells; however, the efficiency of replication increased with the inclusion of additional adjacent sequences. Maximal efficiency was obtained with plasmids containing the 200 terminal bp (Du and Traktman 1996). This region contains the hairpin loop, a region of unique sequences containing a sequence required for genome resolution and a set of 70-bp repeats. The hairpin loop contains 40 base-paired residues and a four-nucleotide loop at the very terminus. In addition, there are some unpaired residues termed extrahelical bases which are asymmetrically located; one strand has ten and the other two. This unusual genomic structure is conserved in all poxviruses. Two isoforms of the genome are found which represent inverted complements of each other and are called flip and flop. The presence of these extrahelical bases and their position within the termini are believed to be important during genome replication.

Viral proteins involved in DNA replication

Several viral proteins have been implicated in viral DNA replication; however, their roles are not well understood (Table 6). Genetic analysis has revealed three gene products, E9, D5, and B1 as important since mutants with defects in these genes are defective in DNA synthesis. Another gene, *D4*, may also be important. Some genes have been identified by sequence analysis. The *E9* gene encodes the viral DNA polymerase which is related to alpha family of DNA polymerases. This protein exhibits DNA polymerase as well as 3′ to 5′ exonuclease activity. A factor which enhances the processivity of the polymerase has been inferred by the observation that infected cell extracts contain a polymerase activity which is highly processive while the purified *E9* gene product is distributive. The product of the A20 open reading frame has been reported to be the polymerase processivity factor (Ishii and Moss 2001; Klemperer et al. 2001). Vaccinia also encodes an NTPase (*D5* gene) and a protein kinase (*B1* gene) whose precise roles in DNA replication are not clear. An early protein H5 appears to be a substrate for the B1 kinase and these two proteins interact with each other, but its role is unclear. Recent evidence suggests that A20 may interact with three other vaccinia proteins, D4, D5, and H5, and that A20 may play an important role in

Table 6 *Poxviral proteins that participate in genome replication*

Vaccinia Viral Protein	Function	Expression
DNA polymerase (E9)	Synthesis of viral DNA; associated 3′–5′ exonuclease for proofreading	Early
A20	Processivity factor	Early
D5	NTPase	Early
B1	Serine–threonine kinase	Early
D4	Uracil DNA glycosylase	Early
A50	DNA ligase	Early
Type I topoisomerase (H6)	Unknown but essential	Late (in capsid)
Thymidine kinase ((J2)	Phosphorylates thymidine; essential	Early
Thymidylate kinase (A48)	Phosphorylates TMP	Early
Ribonucleotide Reductase (F4, 14)	Reduces ribose in ribonucleotides to deoxyribose; essential in nondividing cells	Early
dUTPase (F2)	Hydrolyzes dUTP to dUMP	Early
DNase (?)	Has nicking; joining activity	Early

forming or stabilizing the DNA replication complex. Other viral proteins include a DNA ligase which is not essential in tissue culture, and enzymes involved in nucleotide biosynthesis including thymidine kinase, thymidylate kinase, and ribonucleotide reductase. The virus also encodes a Topisomerase I which is found in viral capsids and a dUTPase and a uracil DNA glycosylase which may play roles in DNA replication and/or DNA repair (De Silva and Moss 2003). Details about the precise roles of these proteins are unclear.

Mechanism of DNA replication

Although poxvirus DNA replication is not well understood, models have been suggested based on current evidence. The first step in genome replication is believed to be the introduction of a nick in sequence within the genomic terminus generating a 3′ OH which can be extended by the viral DNA polymerase. Strand displacement synthesis would be expected to extend the template to the terminus. Since the terminus itself is self complementary, a duplex hairpin can form providing a 3′ OH group that can be extended back in the other direction resulting in the formation of a dimeric tail-to-tail concatemer. This process could occur again resulting in the generation of tetrameric or even higher order concatemers. Concatemeric intermediates have been detected experimentally. This model is consistent with leading strand DNA synthesis. As with the herpesviruses, recombination is also a frequent event during poxvirus DNA replication, and it is possible that recombination events such as strand invasion may play a role in viral DNA replication.

It is believed that concatemeric intermediates can be resolved into monomeric units prior to packaging. Plasmids containing the unique resolution sequences can be resolved into linear minichromosomes with authentic hairpin termini after transfection into infected cells. The protein(s) which recognize the resolution sequences has not been identified to date.

Summary

The poxviruses provide a fascinating example of a viral DNA replication system which encodes all of the required proteins. This virus poses unique challenges experimentally which have slowed progress in elucidation of the precise roles of the viral factors.

HOST–VIRUS INTERACTIONS

In recent years it has become clear that viruses rely on the host in previously unknown ways both in terms of localization within the host cell and the utilization of host factors. For instance, viral genomes have been shown to localize to specific sites within the nucleus (Ishov and Maul 1996; Tang et al. 2000). This localization is thought to involve specific interactions with cellular proteins. In addition, several viruses exhibit complex interactions with the cell cycle. Infection with SV40 and papillomaviruses can alter the host-cell cycle by inducing the host-cell DNA synthetic machinery (Munger et al. 1989; Cheng et al. 1995). Since these viruses depend on cellular proteins such as DNA polymerases, RPA, PCNA etc., the induction of the S phase of the cell cycle is essential for genome replication. Other types of interactions involve both the utilization of host cell proteins which benefit their reproductive strategies and in some cases inactivation of cellular response mechanisms which are inhibitory.

Interactions between viral and cellular proteins

Although adeno- and herpesviruses encode their own polymerases and are therefore somewhat more indepen-

dent of the cell machinery than are the polyoma- and papillomaviruses, these viruses also exhibit very complex interactions with host cell machinery. For instance it has recently been shown that in cells infected with adenovirus, a multiprotein complex consisting of Mre11, Rad50, and NBS1 appears to be inactivated (Stracker et al. 2002). The Mre11 complex is important for double-strand break repair, and infection with adenovirus alters its activity by two mechanisms: spatial reorganization of this complex within the cell and degradation of certain components of this complex. Mutants of adenovirus which fail to inactivate the Mre11 complex have been shown to form concatemers and to replicate inefficiently (Stracker et al. 2002). The evolution of a strategy to promote replication of monomer length molecules by strand displacement as seen in Figure 8b belies a complex viral–host interaction in which some cellular pathways remain intact and others are inactivated. Recent work with herpes simplex viruses suggests that this virus inactivates a different cellular pathway, the nonhomologous end joining (NHEJ) pathway (Jackson and DeLuca 2003; Wilkinson and Weller 2003). During the earliest stages of infection, the NHEJ pathway is inactivated by spatial reorganization and by degradation. Another fascinating aspect of virus–host interactions involves the regulation of viral proteins by post-translational modification. We have seen several examples of viral proteins regulated by phosphorylation using viral and cellular protein kinases (Fanning and Knippers 1992; Nuesch et al. 2001; Ott et al. 2002). Also of interest is the use of molecular chaperones during the viral life cycle. The use of both host-encoded chaperones and virus-specific chaperones has been reported. For instance, it has been shown that in human papillomavirus, E1 protein binding to the origin can be stimulated by the human Hsp70 and Hsp40 chaperone proteins (Lin et al. 2002). Furthermore, SV40 T antigen itself is a functional molecular chaperone J protein. It is expected that chaperones will be implicated in the assembly and function of the replication machinery of many if not all DNA viruses.

Replication within replication compartments or replication centers

Viral DNA replication often takes place within well-defined regions of the cells. In the case of poxviruses, replication occurs within cytoplasmic viral factories. All other DNA viruses replicate in the nucleus, again within well defined regions. In the case of adenovirus and herpesviruses, DNA replication occurs within replication compartments or centers which contain viral genomes and viral and cellular replication proteins. The formation of such compartments may be important for replication efficiency as cellular and viral factors can be concentrated at these sites. Sequestration in such

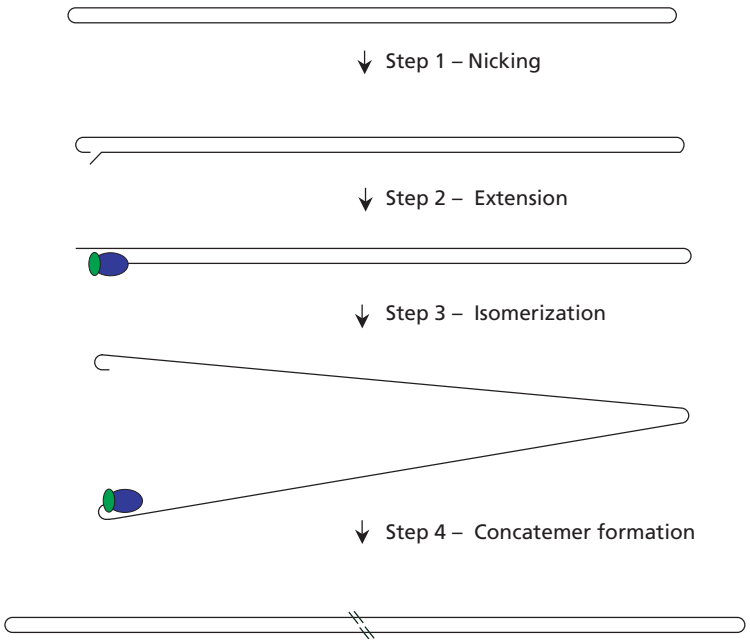

Figure 11 *Poxvirus DNA replication. A model for poxviral DNA replication is shown. The parental genome is a linear duplex with covalently closed termini. Step 1. A nick is introduced into the duplex creating a 3′ OH group which can be extended by the viral DNA polymerase and processivity factor (shown as ovals). Step 2. Replication proceeds by extension of the primer and copies the left end of the genome. Step 3. Because of the terminal redundancy, the nascent DNA can isomerize to form another hairpin. Step 4. The hairpin structure can be elongated along the length of the genome, around the hairpin and along the complementary strand to form a dimer intermediate.*

compartments may also represent a strategy for limiting access to viral genomes during replication for the purpose of inhibiting apoptosis.

Genomes of several DNA viruses which replicate in the nucleus including SV40, adenoviruses, and herpesviruses (at least HSV and HCMV) appear to localize at sites adjacent to ND10 (Ishov and Maul 1996; Tang et al. 2000; Everett 2001). ND10 are subcellular structures or foci which are defined by those which have been identified including PML containing foci known as ND10 or PML nuclear bodies or PML oncogenic domains (PODs) (Burkham et al. 2001). Viral gene expression is also believed to occur at these sites. ND10 are defined by the accumulation of PM, SP100, SUMO, and other cellular proteins involved in growth control, gene expression, and possibly DNA recombination. ND10 are dynamic structures with many of its constituents able to shuttle between the cytoplasm and the nucleus. The common association of viral DNA genomes with ND10 may indicate that proximity to ND10 or some cellular site associated with ND10 may provide a beneficial environment for viral gene expression and viral DNA replication. Cellular proteins involved in DNA recombination and repair have been shown to localize to ND10 or to ND10-related sites. Ionizing radiation induces the increased expression of PML and the relocalization of ND10 proteins to sites of DNA damage as defined by staining for γH2AX, a marker for dsDNA breaks. The relationship of DNA viruses with the repair/recombination machinery of the cell may be important for viral DNA replication (Wilkinson and Weller 2003). Viral DNA molecules which are located at or near ND10 may have access to cellular recombination and repair proteins. This may confer an advantage for DNA replication.

Viral replication proteins and cellular transformation

Several DNA viruses have been associated with human cancers and morphological transformation. The best studied is SV40 which is known to transform rodent cells in culture. SV40 large T antigen is known to deregulate cellular proliferation pathways and can efficiently immortalize primary rodent cells and transform established rodent cell lines to tumorigenicity (Simmons 2000). The transformation ability of T antigen is caused by its specific interactions with host-cell proteins. The tumor suppressor protein p53 was originally identified by its ability to interact with SV40 large T antigen. T antigen also interacts with another tumor suppressor protein, pRB, and the study of these interactions has greatly improved our understanding of the roles of these cellular proteins in the cell cycle and the role of viral oncogenes transformation. As mentioned above, the N terminus of T antigen has also been shown to contain a DnaJ domain which is required for efficient viral replication and perhaps for transformation as well. Recently, the N terminus of T antigen has also been shown to interact with the mitotic spindle checkpoint protein Bub1. Perturbations of this spindle checkpoint may result in aneuploidy and genetic instability leading to cell transformation. Thus viral proteins such as large T antigen continue to provide many insights into the host cell and to responses which lead to oncogenesis and cancer (Munger et al. 1989; Cheng et al. 1995; Simmons 2000; Longworth and Laimins 2004).

SUMMARY

The study of viral DNA replication has been important for many reasons. Replication proteins are still the major targets for antiviral therapy, and thus a thorough understanding of viral replication proteins continues to facilitate the development of novel antiviral strategies. In addition, viruses provide useful windows into the cell: most of what is currently known about mammalian DNA replication strategies came about through studies with SV40. The diversity of strategies exhibited by DNA viruses from SV40 and polyomaviruses which rely almost entirely on host-cell machinery to the poxviruses which encode all the proteins required for genome replication have provided a rich field of study. By comparing viral systems, several important principles have emerged and common mechanisms have been identified. Most viral systems require an origin recognition protein, one or more DNA polymerases, proteins that enhance processive DNA synthesis, origin unwinding and helicase functions and a mechanism for priming DNA synthesis. The diversity in priming is perhaps the most interesting: some viruses use RNA primers and discontinuous synthesis of lagging strands reminiscent of cellular DNA replication strategies, whereas others use protein priming or DNA sequence primers. Each mechanism has implications for how ends are replicated and how progeny genomes are produced during encapsidation. Adenovirus genomes replicate and are packaged primarily as monomers, while the herpesviruses replicate as concatemers which must be processed into monomers during encapsidation.

Because viruses need to replicate many copies of their genomes in relatively short periods of time, most DNA viruses have evolved very efficient replication strategies. The evolution of viral replication proteins which can recruit cellular factors results in the concentration of viral and cellular proteins in specialized regions of the cell leading to enhanced replication efficiency. It is anticipated that better understanding of the complex virus–host interactions leading to efficient viral replication will provide additional insights into eukaryotic cell biology as well. Furthermore, the ability of some viruses to transform cells suggests that important lessons of cellular transformation will be learned from the

continued study of the replication strategies of DNA viruses.

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Genetics of vertebrate viruses

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GENETIC SYSTEMS

The major aim of virus genetics is elucidation of the detailed structure of the genomes of the various types of viruses found in nature and the extent to which the individual components of the virus genome determine the biological and disease-producing potential of viruses. Virus genetics is also concerned with understanding the pattern and origin of the variability of viruses, in terms both of virus evolution and of the temporal changes in antigenicity and pathogenicity of viruses that determine the prevalence and course of virus-associated infectious diseases of humans and animals. Although virus genetics impinges on all aspects of virology, this account will be limited predominantly to a consideration of genome strategy, methods of genetic analysis, and some of the applications of genetic methodology.

Viruses display a diversity of genetic systems unparalleled in any other group of organisms. The viruses considered in this chapter are those that infect humans and higher animals. The genetic systems described here do not include all the known categories, and only occasional reference is made to viruses that infect invertebrates, plants, fungi, protozoa, algae, mycoplasmae, bacteria, and archaea (see The classification of vertebrate viruses). With the determination of the complete sequences of the genomes of many plant and animal viruses, previously unsuspected phylogenetic relationships are being revealed. For example, cowpea mosaic virus, a plant comovirus with a bipartite positive-sense RNA genome, shows organizational similarity and local sequence homology with the monopartite vertebrate picornaviruses. Tobacco mosaic virus, a monopartite positive-sense RNA virus, and alfalfa mosaic virus and brome mosaic virus, both tripartite genome viruses, show sequence relationships with the animal alphaviruses. On the basis of genome organization and comparison of RNA-dependent RNA polymerase sequences, most plant positive-sense RNA viruses can be classified into two supergroups comprising the picornavirus-like plant viruses (como-, nepo-, and potyviruses), and the *Alphavirus*-like plant viruses (tobamo-, bromo-, carla-, clostero-, cucumo-, furo-, gordei-, ilar-, potex-, tymo-, and tobraviruses) (Koonin and Dolja 1993). The picornavirus-like plant viruses and the animal picornaviruses resemble each other in encoding a number of nonstructural proteins that exhibit similar functions and show some degree of amino acid homology. The *Alphavirus*-like plant viruses are more diverse, but again they encode genes in the same relative order that specify proteins exhibiting significant sequence homology to the three nonstructural proteins of the vertebrate Sindbis virus. There is also an apparent genetic link between the potyviruses of plants and the flaviviruses of animals, in that the putative helicase protein of potyviruses exhibits a closer resemblance to the NS3 protein of flaviviruses, which contains a nucleotide-binding motif, than to the helicases of picornaviruses (Goldbach and de Haan 1993).

Although most viruses are restricted in their host range, several groups of viruses are characteristically less restricted by taxonomic barriers and may multiply in hosts of quite different origin. Some of the arthropod-borne bunyaviruses and rhabdoviruses that infect vertebrates or plants are also able to multiply in their invertebrate vector. Despite the apparent phylogenetic relationships described above, however, no contemporary virus exists that is able to multiply in both vertebrates and plants. Nonetheless, it has been demonstrated experimentally that yeast cells infected artificially can support the replication of vertebrate papillomavirus genomes (Angeletti et al. 2002), and that replication-competent chimeras of plant and animal viruses can be constructed that replicate efficiently in plant protoplasts (Peng et al. 2002).

MOLECULAR PHYLOGENY AND VIRUS EVOLUTION

The existence of plant and animal RNA viruses with similarly ordered genomes encoding proteins with similar amino acid sequences suggests a common ancestry. However, the common ancestors from which these viruses diverged probably appeared subsequent to the separation of the plant and animal kingdoms some 10^9 years ago, and divergent evolution alone cannot account for certain features of these relationships. The coupling of common genes to unique genes in several of these genomes suggests a polyphyletic origin mediated by recombination. Many viral genomes can best be regarded as different assemblages of modules of conserved genes that have arisen by a process of modular evolution. From a phylogenetic analysis of the vertebrate circoviruses and the nanoviruses of plants, both of which have small single-stranded circular DNA genomes, Gibbs and Weiller (1999) concluded that these viruses had not arisen by parallel evolution. Rather, the vertebrate circoviruses had evolved from the plant nanoviruses by a complex recombinational event which involved the incorporation of a gene from an RNA virus, possibly a vertebrate calicivirus, into a plant nanovirus DNA genome. Their hypothesis requires that the circoviruses evolved as a consequence of a host-switch whereby a nanovirus genomic DNA was transferred from a plant to a vertebrate host, perhaps as a consequence of exposure of a vertebrate to infected plant sap or via a vector. The replication-competent DNA persisted, and by a subsequent recombinational event became a virus capable of infecting vertebrate hosts. Because caliciviruses are vertebrate viruses, the hypothesis requires that the host-switch preceded the recombinational event. Unlikely as this scenario may seem, involving the hybridization of a DNA and an RNA virus, there is the precedent of Thogoto virus a negative-sense RNA virus that possess a glycoprotein gene resembling that of a DNA baculovirus (Morse et al. 1992).

The application of molecular phylogeny analysis to the large and complex DNA-containing herpesviruses has confirmed the ancient origin of these viruses and provided a timescale for their evolution. The branching pattern of the three subfamilies of the mammalian herpesviruses is congruent with that of their corresponding host lineages (McGeoch et al. 1995). Assuming a constant molecular clock, the *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* subfamilies would have diverged $1.8–2.2 \times 10^8$ years ago, at about the time of emergence of mammals from mammal-like reptiles, and the major sublineages within these subfamilies probably arose before the radiation of placental mammals some $6–8 \times 10^7$ years ago. Paleontological dating of host lineages has suggested that the contemporary virus lineages within the subfamily *Alphaherpesvirinae* have evolved by a process of co-speciation of viruses with their mammalian hosts (McGeoch and Cook 1994). However, several avian herpesviruses are included in the *Alphaherpesvirinae* and, on the basis of similar assumptions, would have evolved independently with their avian hosts for some 80–120 million years (McGeoch and Davison 1999). Such a timescale, however, would place the origin of the avian viruses in too recent an era, as the ancestors of birds and mammals are considered to have diverged some 310 million years ago. This suggests that the avian herpesviruses may not have evolved with their hosts, but may have resulted from ancient cross-species transmissions of ancestral mammalian alphaherpesviruses to birds (McGeoch et al. 2000). Recently two other major herpesvirus lineages have been identified, one embracing fish and amphibian herpesviruses, and the other represented by unique herpesviruses recovered from bivalve molluscs. If these viruses similarly evolved with their hosts, the fish herpesviruses lineage would have been established some 450 million years ago and the invertebrate herpesvirus lineage perhaps a billion years ago. In contrast to the relationships evident between herpesviruses within the same lineage, there is no obvious relationship between herpesviruses belonging to the three different lineages (avian/mammalian, fish/amphibian, and invertebrate) in terms of amino-acid sequence comparisons, other than those functions that are common to all organisms (e.g. DNA polymerase, helicase, and primase). This extreme divergence perhaps suggests that other mechanisms of herpesvirus evolution remain to be identified. A shared ancestral root for all herpesviruses cannot be assumed, because none of the genes common to these three lineages of herpesviruses are confined to herpesviruses, and could have been acquired independently by gene capture rather than by lineal descent (Davison 2002). On the other hand, a member of the fish/amphibian lineage, Channel catfish virus, possesses a gene distantly related to the ATPase subunit of bacteriophage T4

terminase, a complex of proteins responsible for energy-dependent packaging of nascent genomes into preformed capsid. Although this gene may owe its origin to horizontal exchange, it is possible that herpesviruses share their origins with the ancestors of bacteriophage T4 and other icosahedral double-stranded DNA bacterial viruses (Davison 2002).

The ancestral origins of most other viruses cannot be deduced with any certainty from phylogenetic analysis where there is no linkage to the geological record. Generally there are few virus isolates originating from more than a few decades before the present, and even the direction of evolution cannot be discerned in all cases with any degree of certainty. The antiquity of the retroviruses, however, has been established using polymerase chain reaction methodology to screen for the presence of endogenous retroviruses in a wide range of vertebrate hosts, including amphibian, avian, mammalian, piscine, and reptilian. Phylogenetic analyses have demonstrated that viruses resembling (but not necessarily related to) two (the spumaviruses and the murine leukemialike viruses) of the seven recognized genera of retroviruses are widespread and abundant in vertebrate (but not invertebrate) genomes, whereas viruses resembling the other five genera of retroviruses are restricted to mammals and birds (Herniou et al. 1998). Calculation of the integration dates of some of the numerous families of defective human endogenous retroviruses (HERV) identified recently indicates that most of them are likely to have been part of the human lineage since it diverged from that of the Old World monkeys more than 25 million years ago (Tristem 2000). HERVs probably represent the footprints of ancient germline retroviral infections. Furthermore, HERVs may influence genome regulation through expression of retroviral genes, via genomic rearrangements and the involvement of HERV LTRs in the regulation of gene expression (Sverdlov 2000). Analysis of the human genome has revealed that some 45 percent of it consists of various types of transposable elements, with around 8 percent of human DNA derived from retrovirus-like elements (Paces et al. 2002).

THE STRATEGY OF THE VIRUS GENOME

The 'strategy of the virus genome' is a phrase that has been used to describe collectively the organization and function of the genetic information content of viruses. The mammalian viruses fall into three major categories: the DNA-containing viruses, the RNA and DNA reverse-transcribing viruses, and the RNA-containing viruses (Table 1). The DNA viruses range from viruses with small genomes, such as the parvoviruses and the papovaviruses, which depend on host-cell functions for their replication, to large genome viruses such as the nuclear herpesviruses and the cytoplasmic poxviruses, which have acquired a battery of additional genes that function to mediate replication independently of the host cell and to subvert the immune response of the host. The encoding of genetic information in RNA is unique to viruses (animal, plant, and bacterial), and RNA is the predominant molecular form of the genome in animal and plant viruses. The genomes of more than 90 percent of plant viruses consist of single- or double-stranded RNA. The reverse-transcribing viruses alternate between RNA and DNA forms of the genome; in the retroviruses the genome exists as proviral DNA integrated into the host cell genome and as RNA in the extracellular virion. By contrast, in the DNA-containing hepadnaviruses and the plant caulimoviruses (sometimes referred to as pararetroviruses) the DNA form is present as partially or completely double-stranded DNA in the virion, and the RNA form acts as a replicative intermediate in the infected cell. Exceptionally, recent evidence suggests that the replicative cycle of the foamy viruses (members of the genus *Spumavirus*, one of the seven genera of the family *Retroviridae*) resembles that of pararetroviruses in that the extracellular particles contain full-length double-stranded infectious DNA, as well as RNA (Yu et al. 1999). The spumaviruses differ from hepadnaviruses, however, in that they encode proteases and integrases, and integration is an obligate event in the viral replicative cycle.

The coding potential of viruses

The molecular sizes of the genomes of the vertebrate DNA viruses extend from the 1.7 kb of the circular single-stranded genome of porcine circovirus to the 300 kbp genome of fowlpox virus. (The genomes of the some invertebrate iridoviruses and entomopoxviruses are larger and may extend up to 383 kbp.) In contrast, the RNA viruses are more uniform, varying within a 15-fold range from the 1.7 kb of the single-stranded helper-dependent deltavirus to the 31 kb of certain coronaviruses. This circumstance has been interpreted as a reflection of the low fidelity of viral RNA polymerases, which lack proofreading capability. The consequent high mutability of RNA viruses may impose an upper limit on the size of informational RNA molecules.

Table 1 lists the currently recognized vertebrate viruses according to the taxonomy defined in the Seventh Report of the International Committee on Taxonomy of Viruses (ICTV) (Van Regenmortel et al. 2000; see also The classification of vertebrate viruses). The first column lists viruses by family, and the second column gives an indication of the inherent diversity of the group in terms of the number of distinct genera and their constituent species, as currently listed by the individual specialist study groups of the ICTV. This is of course an imperfect measure of genetic diversity, as the extent of knowledge of any particular

Table 1 *The properties of the vertebrate virus genome*

Family		Diversity								
(Order)	Subfamily (unassigned genus)	Genera	Species	Nucleic acid type	Strandedness sense	Configuration	Size	ORF	Particle type	Other features
The DNA viruses										
Parvoviridae		3 (+3 invert)	34 (+5 invert)	DNA	Single (negative or positive)	Linear	4.7–5 kb	2 major	isometric	Nuclear mitosis dependent
Circoviridae		2	4	DNA	Single (negative or ambisense)	Circular	1.8–3.8 kb	3 major (overlaps)	isometric	Nuclear S phase dependent
	(*Anellovirus*)	1	4	DNA	Single (negative)	Circular	2.9–3.8 kb	2 major (overlap)	isometric	High genetic heterogeneity
Papovaviridae		1	12	DNA	Double	Circular	5.2 kb	8 (overlaps)	isometric	Early and late mRNA ex opp. strands
Papillomaviridae		1	7	DNA	Double	Circular	6.8–8.4 kb	9 or 10 (overlaps)	isometric	Early and late mRNA ex same strand
Iridoviridae		1 (+3 invert)	1 (+4 invert)	DNA	Double	Circular (circularly permuted)	150–170 kbp (107 kbp unit)	>40	isometric (enveloped)	Nongenetic Reactivation
Adenoviridae		2	30	DNA	Double	Linear	26–45 kbp	40 (splicing)	Isometric (fibers at vertices)	Covalent-linked protein temporaral gene exp.
Herpesviridae										
	Alphaherpesvirinae	4	23	DNA	Double	Complex linear 4 isomers in HSV	*ca*. 150 kbp	70–100 splicing rare	isometric	Latency in sensory ganglia
	Betaherpesvirinae	3	7	DNA	Double	Linear	*ca*. 235 kbp	*ca*. 200 splicing common	isometric	Latency in lymphoreticular cells
	Gammaherpesvirinae	2	20	DNA	Double	Linear	*ca*. 170 kbp	*ca*. 150 splicing common	isometric	Latency in B lymphocytes
	(Ictalurid herpes-like viruses)		1	DNA	Double	Linear	134 kbp	*ca*. 80	isometric	Phylogeny uncertain
Asfarviridae		1	1	DNA (inverted term rep.)	Double	Linear, Covalently closed	170–190 kbp	*ca*. 150	Complex (NC/isometric/env.)	Perinuclear factories nucleus dependent
Poxviridae										
	Chordopoxvirinae (+1 invert subfamily)	8 (3 invert.)	35 (27 invert.)	DNA (cytoplasmic)	Double	Linear, Covalently closed	130–250 kbp	*ca*. 150–300	complex	Inclusions temporaral expression

(*Continued over*)

Table 1 *The properties of the vertebrate virus genome (Continued)*

Family		Diversity								
(Order)	Subfamily (unassigned genus)	Genera	Species	Nucleic acid type	Strandedness sense	Configuration	Size	ORF	Particle type	Other features
The DNA and RNA reverse-transcribing viruses										
Hepadnaviridae		2	5	DNA (reverse transcription)	Double (incomplete)	Circular (noncovalent link)	3.2 kbp	4 (overlap)	spherical/enveloped	5′-covalent protein
Retroviridae		7	53	RNA* (reverse transcription)	Single (positive sense)	Linear dimer (pseudodiploid)	7–11 kb (monomer)	6–10 (PTC)	spherical/enveloped	tRNA primer high f. recombination
The RNA viruses										
Picornaviridae		6	17	RNA	Single (positive sense)	Linear	7–8 kb	1 (PTC/op)	isometric	5′-covalent VPg IRES and polyC in some
Caliciviridae		4	5	RNA	Single (positive sense)	Linear	7.4–8.3 kb	1, 2 or 3 (PTC/op)	isometric	5′-covalent VPg sgRNA in some
Hepacivirus		1	1	RNA	Single (positive sense)	Linear	7.2	3 (PTC/op)	isometric	Encodes mRNA capping enzyme
Astroviridae		1	7	RNA	Single (positive sense)	Linear	6.8–7.9 kb	3 (PTC/opP)	isometric	sgRNA; polymerase Exp. by ribosomal fr.
Coronaviridae (O. Nidovirales)		2	16	RNA	Single (positive sense)	Linear	28–31 kb	7–10 (nested set)	spherical/enveloped	Amplification of mRNA High fr. recombination
Arteriviridae (O. Nidovirales)		1	4	RNA	Single (positive sense)	Linear	13–16 kb	7–12 (nested set)	spherical/enveloped	as above
Flaviviridae		3	58 (+3?)	RNA	Single (positive sense)	Linear	10–12 kb	1 (PTC)	spherical/enveloped	Most arthropod-borne str. proteins N-terminal
Togaviridae		2	25	RNA	Single (positive sense)	Linear	9–12 kb	1 (and 1 ex sgRNA)	spherical/enveloped	Most arthropod-borne 5′-m7G cap
	(Deltavirus)	1	1	RNA	Single (negative sense)	Circular (forms unbranched rod)	1.7 kb	1	spherical	HBV dependent encodes ribozyme
Bornaviridae (O. *Mononegavirales*)		1	1	RNA	Single (negative sense)	Linear	8.9 kb	6 (spl/opP)	spherical/enveloped	Nuclear Recomb. deficient?
Rhabdoviridae (O. *Mononegavirales*)		4 (and 2 plant)	23 (and 15 plant)	RNA	Single (negative sense)	Linear	11–15 kb	5–10	rod/bacilliform shaped	Recomb. deficient DI mutants common
Paramyxoviridae (O. Mononegavirales)										
	Paramyxovirinae	5	29	RNA	Single (negative sense)	Linear	15–18 kb (n x 6)	9–11 (overlaps)	pleomorphic/env.	Recomb. deficient ‘Rule of six’ applies
	Pneumovirinae	2	5	RNA	Single (negative sense)	Linear	15 n ≠ x 6	8–10	pleomorphic/env.	Recomb. deficient No ‘rule of six’

(*Continued over*)

Table 1 *The properties of the vertebrate virus genome (Continued)*

Family		Diversity								
(Order)	Subfamily (unassigned genus)	Genera	Species	Nucleic acid type	Strandedness sense	Configuration	Size	ORF	Particle type	Other features
Filoviridae (O. Mononegavirales)		2	5	RNA	Single (negative sense)	Linear	19 kb	7 (overlaps)	bacilliform/env.	Recomb. deficient GP may be edited
Arenaviridae		1	19	RNA	Single (ambisense)	Linear ×2 (L and S segments)	L – 7.5 kb S – 3.5 kb	4	pleomorphic/env.	Rodent-borne Subunit reassortment
Bunyaviridae		4 (and 1 plant)	87 (and 8 plant)	RNA	Single (negative and ambisense)	Linear ×3, (L, M, and S segments)	L - 6/12 kb M - 3/5 kb S - 1/1.7 kb	6 (overlaps)	spherical/enveloped	Most arboviruses Subunit reassortment
Orthomyxoviridae		5	6	RNA	Single (negative sense)	Linear ×6, 7 or 8 (n = 900– 2350 kb)	10–14.6 kb (total)	10 (spl/op)	spherical/enveloped	Nuclear phase subunit reassortment
Birnaviridae		2 (+1 invert.)	3 (+1 invert.)	RNA	Double	Linear (n = 2715– 3104 kbp)	6 kbp (total)	5 (overlap)	Isometric (single shell)	Subunit reassortment
Reoviridae		5 (+4 pl/inv)	38 (+27 pl/inv)	RNA	Double	Linear 11, or 12 (three size classes)	19–32 kbp (total)	10+ (overlaps)	Isometic (1, 2, or 3 shells)	Subunit reassortment

env. enveloped; exp. expression; invert, invertebrates; opp, opposite; pl/inv, plant and invertebrate; PTC, post-translational cleavage; recomb. recombination; sgRNA, subgenomic RNA; str. structural; term rep, terminal repeat.

taxonomic group is determined to some degree by the extent of its adverse interactions with human and animal populations.

Vertebrate virus genomes, according to virus family, may be linear or circular, single-, or double-stranded, covalently linked to small terminal proteins, or may exist as a unique complement of subunits. The minimum information content of a viral genome can be estimated from the number of nonoverlapping coding triplets if the complete nucleotide sequence is known, or if not, from the ratio of the molecular mass of the genome and the gene products. As a rough estimate the ratio of the molecular mass of genome and gene products is approximately 10:1 for single-stranded genomes and 20:1 for double-stranded genome viruses. Thus, for example, the 4.2×10^6 Mr of the genome of the rhabdovirus vesicular stomatitis Indiana virus (VSIV) is sufficient to code for polypeptides with a total mass of 400 kDa, which approximates closely to the actual 396 kDa of the five gene products of VSV. This is an extreme case, however, because in the genome of this virus the noncoding regions are limited to dinucleotide intergenic junctions, and short leader and trailer regions.

The relationship between linear length and coding capacity is maintained in the larger DNA viruses, but the coding capacity of smaller virus genomes can be considerably expanded by a number of devices operating at the levels of genome organization, mRNA transcription and translation strategies. At the level of genome organization these devices can include overlapping reading frames (almost ubiquitous), and transcription from both DNA strands (*Polyomavirus*); at the level of transcription, mRNA splicing (most nuclear viruses), the generation of mRNA families by alternate splicing (adenoviruses), accessing of multiple open reading frames by RNA editing (paramyxoviruses), use of alternate start sites (adenoviruses and paramyxoviruses) and alternate termination sites (paramyxoviruses and rhabdoviruses), and nontemplated insertion of nucleotides (paramyxoviruses); and at the level of translation, leaky scanning of mRNA (paramyxovvirus *P* gene products), suppression of termination (retrovirus *gag–pol*), ribosomal frameshifting, (several viruses), posttranslational cleavage of polyprotein precursors (picornaviruses, togaviruses, and retroviruses), the presence of internal ribosome entry sites (picornaviruses and flaviviruses), usurpation of host factors (several viruses), and transactivation of host genes (lentiviruses).

Ribosomal frameshifting is observed predominantly in RNA viruses and is often involved in the expression of replicases (Brierle 1995). Slippage of ribosomes into the –1 reading frame (in a 5′ direction) occurs at a specific site comprising two essential elements: a homopolymeric slippage sequence separated by five to eight bases from an RNA pseudoknot structure. Ribosomal frameshifting may be a strategy to achieve stoichiometry of gene products or to ensure inclusion of replicative enzymes in virions (e.g. by production of *gag–pol* fusions in retroviruses).

The virus classification system known as the Baltimore system (Baltimore 1971) is derived from the obligatory dependence of all viruses on the protein synthesizing machinery of the host cell. Viruses must produce or act as messenger RNA (mRNA) that can be translated by cellular ribosomes. In this classification system the unique relationship of the various forms of the viral genome to mRNA defines virus categories. Class I viruses comprise the double-stranded DNA viruses, class II are the single-stranded DNA viruses, class III are the double-stranded RNA viruses, class IV are the single-stranded positive-sense RNA viruses, and class V are the single-stranded negative-sense RNA viruses. This numbered classification system played a formative role in the development of molecular virology, but is now rarely used. The diverse nature of the viral genome can be rationalized by regarding the genome as an intermediate stage in the cycle of replication of virus nucleic acid that has become sequestered in an extracellular particle. In the Baltimore system a single-stranded RNA molecule is designated as positive sense if it functions as mRNA in protein synthesis, and its complement as the negative-sense strand or anti-message. The retroviruses and some RNA viruses encapsidate the positive-sense RNA strand in the virion, whereas other RNA viruses encapsidate the negative-sense strand or the double-stranded form in the virion. The DNA viruses may be single- or double-stranded, and the positive and negative strands may be encapsidated in separate particles in some of the single-stranded parvoviruses, whereas negative strands are encapsidated exclusively in the recently characterized single-stranded anelloviruses (see

The classification of vertebrate viruses). The only stage in the replication cycle of viral nucleic acids (excluding the replicative intermediates) not represented in known viruses is the RNA:DNA intermediate in the reverse transcription pathway. A small proportion of hepatitis B virus particles, however, do contain DNA/RNA hybrid molecules.

Segmentation of the viral genome

Segmentation of the genome is observed in the arena-, birna-, bunya-, orthomyxo-, and reoviruses. A consequence of segmentation of the genome is the potential for increasing genetic heterogeneity by reassortment of subunits during replication and morphogenesis; the evolution of segmentation in RNA viruses has been equated somewhat fancifully with the evolution of sex, allowing the participation of two, three, or more parents in the production of offspring (Chao 1994). Segmentation and the consequent facility for reassort-

ment may allow the rapid elimination of deleterious mutations and defective genomes. Thus segmentation may be a device evolved to circumvent the assumed limitation on the absolute size of informational RNA molecules imposed by their high mutability. This explanation is difficult to sustain, however, because the total genome sizes of the segmented genome viruses are in the same range as those of nonsegmented genome viruses, and the genome of the largest segmented genome viruses (the 19 kb of the Nairovirus, Crimean–Congo hemorrhagic fever virus) is less than that of the largest nonsegmented genome virus (the 30 kb of murine hepatitis virus, a *Coronavirus*). Segmentation of the genome may more often be a device to facilitate regulation of transcription.

Among mammalian viruses segmentation of the genome is confined to the negative-sense single-stranded RNA viruses and the double-stranded RNA viruses, where the complete complement of genome subunits is contained within a single particle and the infectious unit is a single virion. Segmentation of the genome is common also among the positive-sense single-stranded RNA viruses of plants. These are all multicomponent viruses, however, in which the individual genome subunits are contained in different particles and the infectious unit is a full complement of particles. Genetic reassortment can accompany the maturation of multicomponent viruses and is termed pseudorecombination by plant virologists.

Ambisense encoding of information

Ambisense encoding of genetic information is a phenomenon exhibited by viruses of the family *Arenaviridae*, and by some members of the families *Bunyaviridae* and *Circoviridae*. Ambisense encoding is illustrated diagrammatically in Figure 1. In *Punta Toro virus* (family *Bunyaviridae*, genus *Phlebovirus*) the N polypeptide is encoded in a viral complementary subgenomic RNA corresponding to the 3′ half of the S RNA, whereas the NS protein is encoded in a subgenomic viral sense RNA corresponding to the 5′ half of the genome; thus the S RNA of punta Toro virus is both positive and negative sense. Ambisense encoding of information is common to members of the genus *Phlebovirus*, but is not observed in the other genera of the *Bunyaviridae*. In the family *Arenaviridae* both segments of the bipartite genome exhibit ambisense encoding of information. The large subunit encodes the *L* gene in the 3′ half and the *Z* gene in opposite sense in the 5′ half, and the small subunit encodes the *N* gene in the 3′ half and the *G* gene in opposite sense in the 5′ half. Ambisense encoding may be a device to obtain temporal control of transcription and is not a means of expanding coding capacity.

Reverse transcription, endogenous viruses, and retroid elements

Reverse transcription is not uniquely associated with vertebrate viruses belonging to the families *Retroviridae* and *Hepadnaviridae*. The viruses of plants belonging to the family *Caulimoviridae* also replicate by a reverse transcription route. Comparative analysis of the organization of the RNA associated with vertebrate transposable genetic elements, such as the intracisternal A particles, *VL30* genes and LIMd, and a variety of transposable elements from yeast (Ty), *Drosophila* (copia), *Dictyostelium* (DIRS-1) and maize (B1) revealed structural similarities with the integrated form of the retrovirus genome. Furthermore, the RNA forms of the nucleic acid component of *Drosophila melanogaster copia* transposable element and *Saccharomyces cerevisiae* Ty1 transposable element are present within virus-like particles that have associated reverse transcriptase activity. As a consequence these genetic elements are now ranked as viruses and have been included in the Universal Taxonomy of Viruses as members of a new family – the *Pseudoviridae*

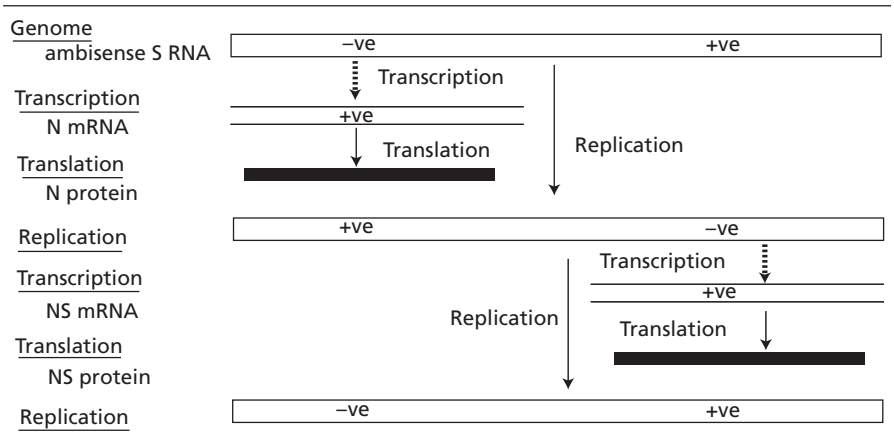

Figure 1 *The ambisense encoding of genetic information in the S RNA of Punta Toro virus,* Phlebovirus *(family* Bunyaviridae*). (After Ihara et al. 1984.)*

(Van Regenmortel et al., 2000; see also The classification of vertebrate viruses).

The common features of the genome maps of these viruses (when linearized in the case of the DNA viruses) are terminal redundancies, which can range from the 17–21 nucleotides of Rous sarcoma virus to the 1123 nucleotides of a human *Spumavirus*, the partial or complete *gag–pol–env* gene order, and the domain structure (protease, reverse transcriptase, integrase) of the polymerase gene. It has been proposed that, during the evolution of these agents, cassettes of genes were added to the basic *gag–pol* core to facilitate adaptation to specific host environments (Hull and Covey 1986). For example, the *env* gene of vertebrate retroviruses is required for protection of the core complex and for cell-to-cell spread, whereas the caulimoviruses have acquired additional genes to facilitate plant-to-plant transmissibility and movement within the plant.

Endogenous retroviruses are defective or nondefective retroviruses transmitted from one generation to the next via the germline, and which probably have evolved in synchrony with their hosts to establish multigenic families. HERVs comprise over 200 distinct groups or subgroups. Although many may have entered the human genome 10–50 million years ago, others appear to have originated from crossinfections at an earlier stage in the evolution of their host. The endogenous feline retrovirus RD114, for example, is thought to have originated as a result of horizontal transfer of an endogenous primate retrovirus, possibly as far back as the Pliocene Age. One view is that HERVs may have played a role in early human evolution by conferring antiviral resistance and altering patterns of gene expression (Sverdlov 2000). Many of the larger mammalian viruses appear to have acquired genes from their host in the course of evolution, but occasionally the reverse can occur. An example is the apparent involvement of a captive retroviral envelope protein, syncytin, in morphogenesis of the human placenta. The syncytin protein is encoded in the *env* gene of a defective endogenous human retrovirus (HERV-W) and its major sites of expression are multinucleate cells in the placenta that originate from fetal trophoblasts. It is presumed that its presence may contribute to normal human placental morphogenesis (Mi et al. 2000).

Infectious nucleic acid

RNA viruses replicate in the cytoplasm, with the exception of the segmented genome orthomyxoviruses and the monopartite negative-sense Bornaviruses (Schneemann et al. 1995), which have nuclear phases. Some others fail to replicate in enucleated cells and are dependent on the presence of a functional nucleus (Pringle 1977). The genome of the positive-sense single-stranded RNA viruses is infectious, because by definition it acts directly on entry as the mRNA for the entire complement of viral proteins, which include the RNA replicase required for replication of the viral genome. The genome of the negative-sense single-stranded RNA viruses, on the other hand, is not infectious because it cannot function as mRNA or be replicated. Mammalian cells do not contain enzymes that can transcribe or replicate RNA templates, and so transcription can be initiated only if the appropriate RNA polymerase is introduced into the cytoplasm of the host cell together with the infecting genome in the form of a nucleocapsid structure.

The DNA of the nuclear DNA viruses is generally infectious, but that of the cytoplasmic poxviruses is not. The poxviruses exhibit the greatest extent of independence of host cell activities of any virus, and the virion contains many enzyme activities, including a viral DNA-dependent RNA polymerase whose presence is essential for infectivity. The discovery of this enzyme by Kates and McAuslan (1967) was crucial to our understanding of the nature of viral genomes and led ultimately to the independent discovery and characterization by Baltimore and Temin and Mitzutani in 1970 of the reverse transcriptase of retroviruses (Baltimore 1971), which is perhaps the greatest single contribution of virology to general biology and which greatly accelerated the exploitation of recombinant DNA technology.

The genomes of retroviruses are also not infectious, probably because preformed virion-associated reverse transcriptase is necessary for initiation of replication. By contrast the proviral DNA form is infectious because it can be transcribed by the host-cell DNA-dependent RNA polymerase to initiate infection. The reverse transcription stage of retrovirus replication takes place in the cytoplasm prior to entry into the nucleus and integration into the host genome.

METHODS OF GENETIC ANALYSIS

Classic genetic analysis of viruses depended on the isolation of spontaneously occurring mutants or the induction of mutations by random mutagenesis followed by nonselective isolation of mutants exhibiting a single conditional lethal phenotype. Further selective screening procedures or systematic characterization of individual mutants were required to identify useful mutations in specific viral genes. Nevertheless, despite the imperfections, many well-defined mutations were identified which played significant roles in the molecular characterization of mammalian viruses, and provisional genetic and physical maps of many viral genomes were obtained by this means (Ramig 1990). The isolation and characterization of revertants is an essential requirement in defining the phenotype of any specific mutant.

Conditional lethal mutants

Conditional lethal phenotypes (temperature sensitivity and host range) have been the favored phenotypes for

the isolation of mutations in essential genes. Other phenotypes, such as plaque morphology, drug resistance, enzyme deficiencies, and spontaneous deletions (sometimes presenting as defective interfering particles), have had application in specific circumstances. The conditional lethal mutants employed in animal virology have been temperature-sensitive mutants, cold-sensitive mutants, or temperature-dependent host range mutants. Host range conditional lethal mutants analogous to the suppressible chain-terminating mutants of bacteriophages (amber or nonsense mutations) have played virtually no role in animal virus genetics, although in principle such mutants allow direct identification of the mutated gene product, because a truncated gene product may be present in the nonpermissive host cell and an intact protein in the permissive cell. Host restriction in animal cells is probably due to the absence of a specific receptor, and permissive mammalian cells naturally able to suppress amber mutations by insertion of an amino acid at an amber stop codon have not been identified. Mammalian cells expressing amber-suppressible tRNAs have been engineered, however, and these have been used successfully to isolate mutations of vesicular stomatitis virus following random mutagenesis (White and McGeoch 1987) and by mutagenesis of poliovirus cDNA in vitro (Sedivy et al. 1987).

Spontaneous mutants and random mutagenesis

Spontaneous mutants occur at low frequency, and a variety of mutagens have been employed to increase the frequency of recovery of mutants. Mutagens that have proved effective with human and animal viruses include base analogues (e.g. 5-fluorouracil and 5-azacytidine for RNA viruses and 5-bromodeoxyuridine for DNA viruses), alkylating agents (e.g. ethylmethane sulfonate, diethyl sulfate), intercalating agents (e.g. proflavine and *N*-nitro-*N*-nitrosoguanidine), deaminating agents (e.g. nitrous acid), hydroxylamine, ultraviolet light irradiation, and miscellaneous agents (e.g. the antiviral agent ribavirin). The base analogues are incorporated into the viral genome and miscoding during replication produces mutations. The other mutagens induce mutations by direct chemical action on the target nucleic acid. The majority of induced mutations seem to be missense mutations whereby substitution of an amino acid modifies the functional activity of a gene product. Frameshift and nonsense mutations of animal viruses are rare. (Practical protocols for random mutagenesis and isolation of mutants are described by Leppard and Pringle (1996).)

Site-specific and directed mutagenesis

These empirical methods described above have been superseded by the adoption of in vitro methods of site-specific mutagenesis. The methodology for constructing such mutants is similar for all DNA and positive-stranded RNA viruses, although there is an operational distinction between viruses whose genome is small enough to be cloned (as a cDNA in the case of RNA viruses) in its entirety (parvoviruses, polyomaviruses, picornaviruses, retroviruses) and those in which mutagenesis is best carried out with a fragment that, after genetic manipulation, is incorporated into a complete genome, generally by recombination in vivo (adenoviruses, herpesviruses, poxviruses).

A prerequisite for directed mutagenesis is a source of molecularly cloned viral nucleic acid. RNA genomes must be first converted to cDNA by reverse transcription and the larger genomes cleaved with an appropriate restriction endonuclease to generate a library of fragments. Once cloned, directed mutagenesis may make use of existing fortuitous restriction sites or employ synthetic oligonucleotides with specific mismatches flanked by regions of complementarity. Then oligonucleotide-directed mutagenesis can be used to obtain point mutations or precisely located deletions. This can be achieved by cloning wildtype genomic DNA into a single-stranded bacteriophage vector (e.g. M13mp19). The single-stranded phage DNA is isolated and annealed to the mutagenizing oligonucleotide that comprises two noncontiguous complementary sequences flanking the region to be looped out to produce the required deletion, or a mismatched base for the production of point mutations. The annealed oligonucleotide acts as a primer for DNA synthesis in vitro and the resulting double-stranded DNA is circularized with DNA ligase. A mutated genome is recovered by repair synthesis on a single-stranded DNA template or, increasingly, by polymerase chain reaction (PCR). (For a generalized protocol for directed mutagenesis, see Leppard and Pringle (1996).)

PCR-based directed mutagenesis has gained the ascendancy in practice because of its greater flexibility. Synthetic primers are designed to locate on opposite strands on either side of the target site. The region targeted for mutagenesis can be further defined by two opposing internal primers, one of which generates the desired mutation while the other eliminates one of two restriction sites flanking the target site. Two-stage amplification yields DNA that can be cleaved at the sites flanking the target site; only mutated DNA should retain the dispensable restriction site (Figure 2). The recovered fragment can then be ligated into an appropriately cleaved plasmid or M13 vector, a process that is facilitated if the restriction sites in the amplified fragment are also unique to the recipient genomic clone. In the case of parvoviruses, polyomaviruses, picornaviruses, and retroviruses, infectious progeny can be generated by direct rescue, i.e. by direct transfection of the reconstructed DNA into susceptible host cells. Transfection of nucleic acid is enhanced variously by

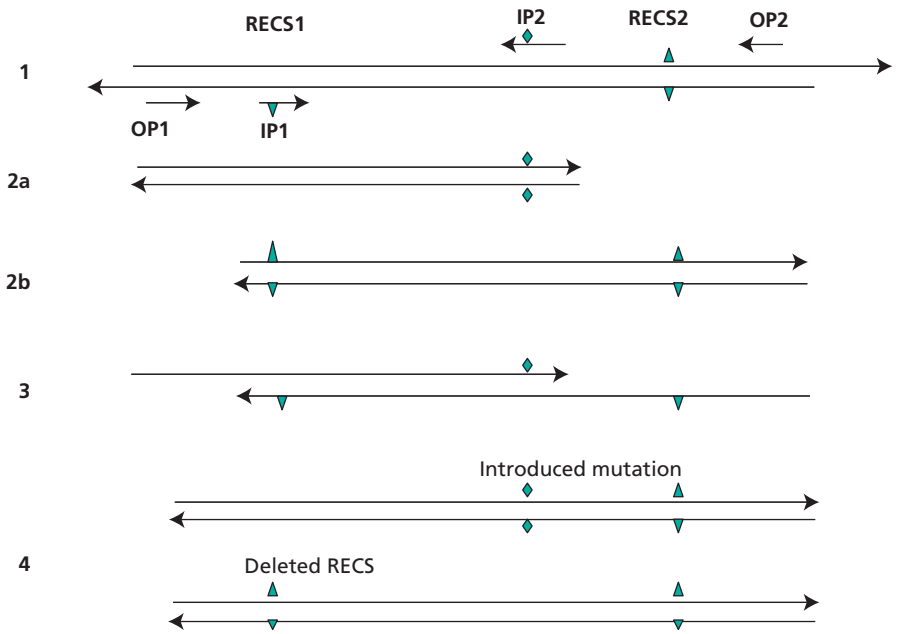

Figure 2 *A generalized two-stage protocol for DNA mutagenesis. OP1, OP2, IP1, and IP2 are oligonucleotide outer (OP) and inner (IP) primers with homology to the target DNA at the positions shown; RECS1 and RECS2 are restriction endonuclease cleavage sites. The inner primer IP2 carries the mutation to be introduced into the target region. All DNA strands are shown with the arrowheads directed towards their 3′ ends. See the text for further explanation. Where only a single mutation is being introduced, a convenient variation of this protocol is to overlap the internal primers, such that each carries the desired mutation (or its homologue). Cleavage of the second-stage PCR product at sites RECS1 and RECS2 will produce a homogeneous mutated DNA fragment.*

calcium phosphate precipitation, lipofection, electroporation etc. Although picornaviruses can be rescued by transfection of cloned DNA, the process is more efficient if RNA transcribed in vitro is transfected by electroporation. For viruses with larger genomes (adeno-, herpes-, and poxviruses), rescue of the mutated fragment into infectious virus is achieved by cotransfection of the mutated sequence and intact viral genomic DNA into permissive cells. Recombination will occur provided the flanking sequences on either side of the mutated site are sufficient to permit homologous recombination. With the intermediate-sized adenoviruses, viral genomic DNA can be inactivated by cleavage with a single-cutting restriction enzyme such that the genome fragments overlap with the mutant-carrying fragment. In this situation infectious virus can be generated only by a process of homologous recombination, and most of the progeny virus will contain the mutation (Figure 3). In the herpesviruses and poxviruses, rescue of directed mutations is mostly confined to genes with phenotypes that are amenable to selection (e.g. enzyme deficiencies, drug resistance, cytopathology) on account of the high background of nonmutated virus in the absence of selection.

Deletion mutants

Operationally, deletion mutants are the simplest to obtain. However, deletion of sequences from a specific protein-coding gene does not always produce a 'null' phenotype. For example, large stretches of the carboxy terminal region of the attachment (G) protein of respiratory syncytial virus can be deleted without affecting the attachment function or the viability of the virus. Similarly, herpesvirus thymidine kinase retains substantial activity despite loss of the first 45 amino acids. Deletion mutant analysis is particularly useful in defining promoter regions. Specific deletions can be engineered by digesting cloned single-stranded DNA from a single-stranded DNA bacteriophage vector with one or two restriction enzymes and recircularizing to generate two circular molecules, the extent and type of deletion obtained depending on the availability of restriction enzyme sites. Sets of deletion mutations can be obtained by restriction enzyme cleavage of a circular DNA molecule followed by progressive extension of a deletion using an exonuclease and recircularization.

Linker scanning mutagenesis

Linker scanning mutagenesis introduces clustered sets of point mutations at desired locations and allows more refined analysis of *cis*-acting control sequences and characterization of protein-binding sites. This procedure allows rapid screening for mutations and their immediate localization as a result of the appearance of a new restriction site (McKnight and Kingsbury 1982). A limitation of deletion mutant analysis is that if the dele-

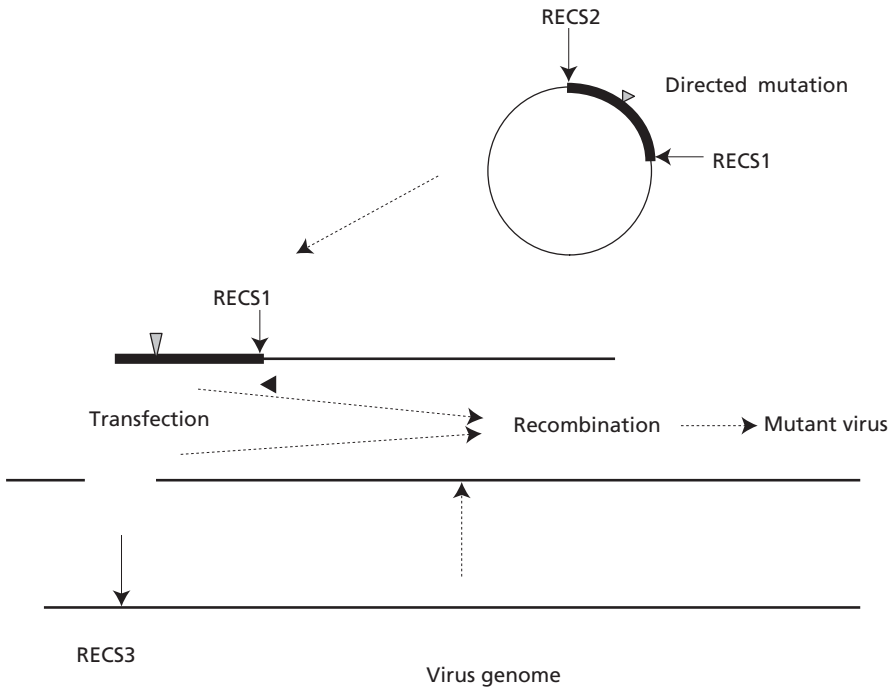

Figure 3 *Rescue of a mutated sequence by recombination. A generalized strategy for introduction of mutations into the terminal region of an adenovirus genome. (Similar strategies can be designed to introduce mutations into other regions of the genome.) The cloned adenovirus terminal genome fragment containing the desired mutation is shown as the thick line. Release of the fragment and cleavage of the target virus genome is achieved by digestion with appropriate restriction endonucleases at sites RECS1 and RESC2, and the products are transfected into appropriate susceptible cells (293 cells in the case of adenovirus). Recombination in vivo results in the generation of infectious virus carrying the desired mutation. (See text for further explanation.)*

tion is large it may cause nonspecific effects, for example steric interactions in proteins, or spacing constraints on promoter function.

Linker insertion mutagenesis

Linker insertion mutagenesis does not involve deletion; a linker is inserted at restriction sites within an open reading frame and many sites can be mutagenized by limiting digestion by a frequent cutting restriction enzyme. A linker of 3 bp (or multiples of 3) will maintain the reading frame beyond the insertion, whereas chain termination can be produced in all three reading frames by insertion of other linkers.

COMPLEMENTATION AND OTHER NONGENETIC INTERACTIONS

Interactions between viruses can be classified broadly as either genetic or nongenetic. Nongenetic interactions include complementation, phenotypic mixing, and interference, and will be considered first.

Complementation

Complementation is the ability of the defective gene product of one virus to be substituted by the normal gene product of another, when both viruses are present in the same host cell. Complementation provides a test for nonidentity, whereby mutants of similar phenotype (e.g. temperature sensitivity) can be assigned to different genes without prior knowledge of the existence or function of those genes. Operationally, complementation is measured in terms of a complementation index (CI). The CI can be used to assess the efficiency of complementation, i.e. in the case of temperature-sensitive mutants, the ratio of yield from a mixed infection (A × B) to the sum of the yields of the single infections (A + B) determined at the restrictive temperature. Progeny virus produced as a consequence of complementation between viruses retains the mutant genotype, because the interaction between viruses occurs at the level of gene products only. Consequently, assay of the progeny from a complementation experiment is carried out at the permissive temperature, and a positive CI indicates nonidentity of function. Complementation is normally intergenic in nature, but in the case of multifunctional gene products (e.g. many viral polymerases) intragenic complementation may be observed.

Complementation analysis has had a prominent role in defining gene function in the papovaviruses, adenoviruses, herpesviruses, poxviruses, retroviruses, alphaviruses, flaviviruses, paramyxoviruses, rhabdoviruses,

arenaviruses, bunyaviruses, orthomyxoviruses, and reoviruses.

Phenotypic mixing and pseudotype formation

Phenotypic mixing is a form of nongenetic interaction between viruses whereby the structural components (capsid or envelope) may be interchanged partially or completely when two different viruses multiply in the same cell. Phenotypic mixing normally occurs between related viruses, but heterologous phenotypic mixing can occur between unrelated viruses. Viruses that are enveloped on exit from the host cell are prone to phenotypic mixing. The mixing of genomes and structural proteins results in the production of progeny virus particles in which the phenotype does not reflect the genotypic properties of the virus. Phenotypic mixing is thus a transient phenomenon.

Pseudotype formation, the complete investment of the core of one virus by the envelope of another, has provided a convenient means of rapid assay of noncytopathic or fastidious viruses. For example, some of the progeny virus released from cells coinfected with the nonfastidious and rapidly cytopathic rhabdoviruses, vesicular stomatitis virus, or Chandipura virus, and a host-restricted and noncytopathic or slowly cytopathic retrovirus will possess the genome of the lytic virus contained within the envelope of the retrovirus. The pseudotype yield, detected as rapidly plaque-forming virus resistant to neutralization by antiserum to the parental lytic rhabdovirus, provides a means of detection and rapid assay of its nonlytic partner. This technique has been used successfully in studies of host range and for detection and assay of HIV-1, HIV-2, HTLV-I, HTLY-II, mouse mammary tumor virus, bovine leukemia virus, and several avian retroviruses.

Many of the oncogenic avian and mammalian retroviruses exist as replication-defective viruses and are dependent on helper leukemia-associated retroviruses. These oncogenic retroviruses often exist as pseudotypes because their envelope glycoproteins are supplied by the helper virus. Other examples of asymmetric complementation or phenotypic mixing are the commensual association of hepatitis D virus and hepatitis B virus and the adenovirus helper dependence of some parvoviruses.

Interference and defective interfering particles

The ubiquity of nongenetic interactions such as complementation and phenotypic mixing is responsible for the prevalence of defective viruses. Defective viruses accumulate in many laboratory-propagated stocks of viruses and are dependent on the presence of their nondefective progenitor for their survival. It is a characteristic of most defective viruses that they specifically interfere with the replication of their helper or closely related viruses. Interference occurs at the level of transcription and replication, and is not interferon-mediated. The mechanism of interference varies between viruses belonging to different families and between different defective viruses of the same virus. For example, the majority of the defective interfering (DI) particles of vesicular stomatitis virus, known as panhandle or hairpin DIs, which contain genomes with complementary 5′ ends and incomplete *L* gene sequences, interfere with the replication of viruses of homologous serotype only, whereas the DI particles that contain internal deletions of the *L* gene interfere with vesicular stomatitis virus of both homologous and heterologous serotypes. In formal terms, a single mutant or nucleotide substitution can constitute a DI genome, and deletion of sequence is not a necessary characteristic. It has been proposed that the generation of DI virus is a natural phenomenon that moderates the course of viral disease and contributes to the self-limiting nature of most viral diseases (Huang and Baltimore 1970). It has been difficult, however, to obtain evidence of the presence of DI virus in natural infections, although in a few instances the administration of DI particles can alter the outcome of an infection. Mice can be protected from an influenza virus-induced immune-mediated pneumonia by the administration of DI particles. However, the DI particles did not reduce the replication of virus in the lung, but seemed to act by preventing the virus-induced T-cell response that is responsible for consolidation (Morgan and Dimmock 1992).

DI particles have been implicated in the initiation, although not necessarily the maintenance, of persistent infection in vivo and in vitro. For example, a DI particle associated with the tsG31 mutant of vesicular stomatitis virus, possessing a 90 percent deletion of the genome, was a good inducer of persistent infection. Indeed, it has been proposed that persistent infection may be an important force in evolution, maintaining genetic variability in those viruses (such as the negative-sense RNA viruses) unable to acquire variation by genetic recombination. The generation of DI virus is a spontaneous mutational event, which in due course forces a mutational response in the parental virus to counteract the DI-mediated interference, and a cyclical phenomenon of alternating generation of new DI and specific DI interference-resistant viruses ensues. Progressive and extensive mutational change over the whole genome was observed to accompany the propagation of the normally lytic vesicular stomatitis virus as a persistent nonlytic infection in cultured cells, whereas by contrast the virus is genetically stable during cycles of lytic infection. The extensive mutation of the measles virus genome observed in the defective viruses recovered from the brain of patients with subacute sclerosing panence-

phalitis (SSPE) may be an indication that this phenomenon plays a role in human disease processes.

GENETIC INTERACTIONS

Genetic interactions include recombination, subunit reassortment, and multiplicity reactivation. Their characterization provides information about the evolutionary potential of viruses as well as means of their experimental manipulation.

Recombination in DNA viruses and positive-sense RNA viruses

Recombination occurs as a consequence of the physical interaction of viral genomes in the infected cell. It is a phenomenon common to all DNA-containing viruses, both DNA-containing and RNA-containing retroviruses, and most positive-strand RNA viruses. Recombination between DNA genomes is probably a conventional intramolecular mechanism involving a breakage and reformation of covalent bonds, whereas recombination between RNA genomes is a process mediated by a template switching ('copy-choice') mechanism during minus-strand synthesis. The probability of occurrence of a recombinational event between two genes is proportional to the physical distance separating them on the genome, and genetic maps can be constructed on the basis of recombination frequencies. Temperature-sensitive (ts) mutants have formed the framework for most genetic maps. The recombination frequency (RF) between any pair of temperature-sensitive mutants, A and B, is measured as the frequency (percentage) of wildtype virus generated in multiple infections, such that the RF between any two temperature-sensitive mutants, A and B, is measured as a frequency (percentage), such that $RF = 2 \times 100 \times \text{yield } (A + B)^{RT} - (\text{yield } A^{RT} + \text{yield } B^{RT})/\text{yield } (A + B)^{PT}$, where RT is the restrictive temperature and PT the permissive temperature. The single and mixed infections are carried out at the permissive temperature and yields assayed at the temperatures indicated by the superscript. The factor 2 is included because only the wildtype recombinant is measured by this assay, and it is assumed that the reciprocal recombinant (the double ts mutant) occurs with equal frequency. Using a quantitative PCR assay, Jarvis and Kirkegaard (1992) confirmed that frequencies of the reciprocal recombinants in poliovirus recombination are equivalent, but only when the parental input multiplicities are identical. Linear genetic maps of several DNA viruses (adeno-, herpes-, pox-, and picornaviruses) have been constructed on the basis of two factor crosses to establish relative proximity and three factor crosses to confirm orientation (for a detailed account, see Ramig 1990).

Mutants classified in the same complementation group cluster in the map, and the greatest distance separating mutants in the cluster defines the minimum size of the gene. In the adenoviruses and herpesviruses intertypic crosses, in which the parental ts mutants are derived from viruses of different serotype, have been employed to confirm the linear order of the genetic map and to show that the genetic and physical maps of the genome, constructed on the basis of restriction endonuclease fragment analysis or direct sequencing, are colinear. Recombinants between herpesvirus types 1 and 2 have been isolated from cultured cells, but natural recombinants have not been recovered from patients, suggesting that there are selective constraints preventing such interactions. Paradoxically, although the frequency of recombination is generally lower, natural recombinants occur more frequently in RNA viruses. For example, intertypic recombinants are prominent in the virus recovered from children after vaccination with the trivalent Sabin vaccine, although none of these recombinants has as yet escaped the immune response of the host to become established in nature. Rare heterologous recombinants also occur: for example, the *Alphavirus* known as Western equine encephalitis virus seems to be a recombinant of another *Alphavirus*, Eastern equine encephalitis virus, and a New World relative of Sindbis virus (Hahn and Lustig 1988).

Recombination in RNA viruses is not universal (reviewed by Lai 1992; Wimmer et al. 1993). Low-frequency genetic recombination (up to 0.9 percent) occurs in viruses with positive-strand genomes, and linear genetic maps were derived for both poliovirus and foot-and-mouth disease virus, which were subsequently found to be colinear with physical maps. Homologous recombination does not seem to be site specific, although intertypic recombination in poliovirus and foot-and-mouth disease virus may be influenced by local structural features. The three poliovirus types differ by about 15 percent in their nucleotide sequences, and the frequency of recombination observed between viruses of different subtypes, as expected by a template switching (copy-choice) mode of recombination, is about 100 times less than between viruses belonging to the same serotype.

Large genome sections can be transposed as a result of natural recombination in vivo, and the characterization of the phenotypic properties of recombinants has played a prominent role in the mapping of attenuating mutations in the Sabin poliovirus vaccine strains. Transposition of segments of any size can now be achieved in a more controlled manner by exchange of segments between existing or engineered restriction sites in infectious cDNA clones. This approach also provides a means of direct verification of the association of particular mutations with a specific phenotype. For example, a poliovirus mutant carrying a four-base insertion at position 70 within the 5′ untranslated leader region has a small plaque ts phenotype and is deficient in RNA synthesis. Revertants of this mutant retained the four-

base insert, and mix-and-match experiments with cDNA located these second site suppressor mutations to a site within the 3C protease gene (Andino et al. 1990). The isolation and characterization of revertants is an essential feature of the characterization of any mutant. The same approach can be used to produce chimeric viruses in order to study the homology and functional compatibility of genetic elements. For example, it has been shown that the 5′ untranslated leader regions of poliovirus and a coxsackie B virus can be interchanged.

The 5′ untranslated region of the genome of picornaviruses contains an internal ribosomal entry site (IRES), and poliovirus can be transformed into a vector by insertion of a foreign gene between tandemly arranged IRES sequences, in which one is derived from a nonhomologous IRES (e.g. from encephalomyocarditis virus (EMCV)) to prevent loss by homologous recombination. A dicistronic virus mimicking the genetic organization of the plant picornavirus cowpea mosaic virus (CPMV) can be created by insertion of the IRES from EMCV between the capsid precursor coding region and the nonstructural protein coding region of the poliovirus genome. Unlike CPMV, however, the two transcriptional units are linked and contained within the same particle.

Exceptionally, high-frequency genetic recombination is observed in mixed infections of coronaviruses, but a linear map of the coronavirus genome has not been produced. This may be a consequence of the complexity of the organization of the *Coronavirus* genome, with its unique 'nested set' configuration of genetic information, whereby subgenomic RNAs with a common 3′ terminus and differential extensions in a 5′ direction are intermediates in mRNA transcription. In addition, the polymerase protein of coronaviruses is encoded in two large open reading frames, from which the gene product is derived by translational frameshifting (ribosomal slippage). The largest *Coronavirus* genome (approximately 31 kb) is much larger than the picornavirus genome (approximately 7.5 kb), but the rate of recombination per nucleotide is similar at approximately 0.1 percent per 100 nucleotides (Lai 1992).

Reverse transcription and recombination

The retrovirus genome is present in the virion in a pseudodiploid form. Two copies of the viral genome are associated head-to-head by hydrogen bonding between short repeats at their 5′ ends. Despite the fact that two genomes are encapsidated in a single virion, only a single provirus is generated under conditions of infection of cells by single virions. The reason for the unique pseudodiploid form of the genome is unknown, except that it does allow for damage repair by recombination during the reverse transcription process. In other viruses replication is generally accompanied by amplification of the genome, whereas in the replication of retroviruses there is no net gain; the process of reverse transcription results in the conversion of a one pair of single-stranded RNA genomes into one double-stranded DNA provirus (the process has been described as destructive replication). Reverse transcription not only generates a linear DNA copy of the retroviral genome but also produces the long terminal repeats (LTR), which contain the signals necessary for transcription of the integrated DNA, and which together are designated the provirus. At the cell–virus DNA junction there is a 5-bp direct repeat of cellular DNA next to a 3-bp inverted repeat of viral DNA, a structure similar to that present at the termini of several bacterial transposons. A major distinction between the retroviruses and other nuclear DNA viruses is that the former maintain productive infections and do not usually kill the host cell, whereas the latter invariably destroy their host cell and can only become stably integrated in a nonproductive form. (The phenomenon of latency in herpesviruses, however, to some extent blurs this distinction.)

High-frequency recombination is an inherent consequence of retrovirus replication. In crosses of exogenous retroviruses, recombination has been detected both between and within genes and between exogenous and endogenous viruses. The pseudodiploid nature of the genome means that heterozygous genomes can exist and may be intermediates in the generation of stable recombinants. In the replication of retroviruses, the location of the tRNA primer for DNA synthesis near the 5′ ends of the tandemly associated monomers means that the reverse transcriptase must transfer from the 5′ end of one monomer to the 3′ end of the same or the other monomer soon after the initiation of synthesis. Thus every round of synthesis is effectively a recombinational event. Furthermore, the process of reverse transcription itself involves synthesis of negative-strand DNA on the genomic positive-strand RNA template, and simultaneous degradation of the RNA template by the ribonuclease H activity of the enzyme complex, followed by synthesis of a positive DNA strand with internal discontinuities on the negative-strand template. There are abundant opportunities for recombination, either during negative-strand synthesis by a copy choice mechanism, or during positive-strand synthesis by strand displacement and assimilation on to replicating DNA from another genome. Comparison of the inferred amino acid sequences of retroviral proteins suggests that recombination has played an important role in the diversification of the envelope proteins of retroviruses, whereas the polymerase gene seems to have been protected from functionally disruptive recombinational events (McClure et al. 1988).

The extreme variability of the surface proteins of HIV-1 is not mediated by recombination alone. Progressive mutational change plays a role in addition to recombinational events, as does the phenomenon of biased

hypermutation (Martinez et al. 1994), see later under Biased hypermutation.

Multiplicity reactivation, marker rescue and cross-reactivation

Multiplicity reactivation occurs when cells are infected with parental viruses which themselves are noninfectious (usually as a result of UV irradiation). At higher multiplicities of infection progeny virus may be released from susceptible cells that contain markers contributed by both parents. Infectivity has been restored by replacement of defective genes by recombination (or reassortment). Multiplicity reactivation is observed only if the inactivating lesions in the two parental viruses are located in different genes. Multiplicity reactivation has been observed between strains of the same virus, and also between viruses of different serotypes. Reactivation between an inactivated parent and an infectious parent is termed crossreactivation, or marker rescue, and is likewise mediated by recombination (or reassortment). Cross-reactivation can be exploited to increase the yield of recombinants (or reassortants) by infecting susceptible cells with a nonplaque-forming virus and a UV-inactivated plaque-forming virus.

Reverse genetics of double-stranded and negative-sense single-stranded RNA viruses

Intermolecular recombination has not been demonstrated unequivocally for any RNA virus with a negative-strand or double-stranded genome, with the exception of the tripartite genome double-stranded RNA phage ϕ6 (Mindich 1995). It is likely that recombination can occur as a rare event; otherwise it is difficult to account for the origin of some of the DI viruses of vesicular stomatitis virus, influenza A virus, and rotavirus which have truncated and juxtaposed genes, or rare phenomena such as the inversion of genes in the mammalian pneumoviruses, which departs from the otherwise invariant gene order found in other paramyxoviruses.

The double-stranded RNA viruses can exchange genes by reassortment of genome subunits. However, the development of a reverse genetics system to allow controlled mutagenesis of individual genome subunits has proved unexpectedly difficult to achieve. Roner and Joklik (2001) have recently described a reverse genetic system for reovirus type 3, which may be generally applicable to other members of the family *Reoviridae*.

The single-stranded negative-sense RNA viruses as a group do not exhibit genetic recombination, apart from the phenomenon of subunit reassortment in those viruses that have segmented genomes. For long this prevented the application of molecular genetic analysis to the negative-sense RNA viruses, which include many important animal and human pathogens. However, reverse genetic systems are now available for many members of the nonsegmented genome families *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*, and the segmented genome families *Bunyaviridae* and *Orthomyxoviridae* (reviewed by Neumann et al. 2002). The genomic RNA of negative-strand RNA viruses is not infectious because the negative strand is not translated. However, Luytjes et al. (1989) achieved amplification, transcription, and rescue of synthetic RNA molecules by reconstitution of a biologically active nucleoprotein complex using synthetic RNA and purified core proteins in the presence of helper influenza virus. The packaging of the larger genomic RNAs of the nonsegmented genome viruses (the order *Mononegavirales*) also presented difficulties. The breakthrough came with the construction of a complete infectious cDNA copy of the genome of rabies virus by Schnell et al. (1994), and infectious cDNA clones for many viruses are now available.

Strategies for rescuing negative-sense RNA virus genomes rely on transfection of the cloned copy of the viral genome (usually as the anti-genome) into susceptible host cells, together with a source of T7 RNA polymerase, usually provided by a vaccinia virus recombinant vector, to direct the synthesis from bacteriophage T7 transcription plasmids of RNAs encoding the components of the viral nucleocapsid complex (Figure 4). The cloned anti-genomic strand is terminated with a ribozyme sequence. Self-cleavage of the primary transcript then removes the ribozyme sequences, leaving a precise 3′ terminus. The newly transcribed nucleocapsid components first replicate the anti-genomic RNA and then transcribe the newly synthesized negative strand. This results in production of the remaining structural proteins, further replication, and packaging as in a normal infection. Initially it was presumed that expression from an anti-genomic template, rather than genomic RNA, was obligatory to avoid sequestration and functional loss of the L, M, and P mRNAs of rabies virus by annealing to genomic-sense RNA. However, this is not an absolute requirement, and Sendai virus has been rescued from genomic DNA, albeit with reduced efficiency.

Reverse genetic systems for segmented genome viruses were more difficult to exploit because of the difficulty of delivering multiple genomic RNAs. The first successful reverse genetic system was achieved with the tripartite Bunyamwera virus, adapting the methodology developed for nonsegmented genome viruses. Later modified procedures were developed for the reverse genetics of the eight-segmented influenza A virus and influenza B virus, which take account of the nuclear phase in the replication cycle of these viruses. Figure 5 illustrates the methodology for the genetic manipulation

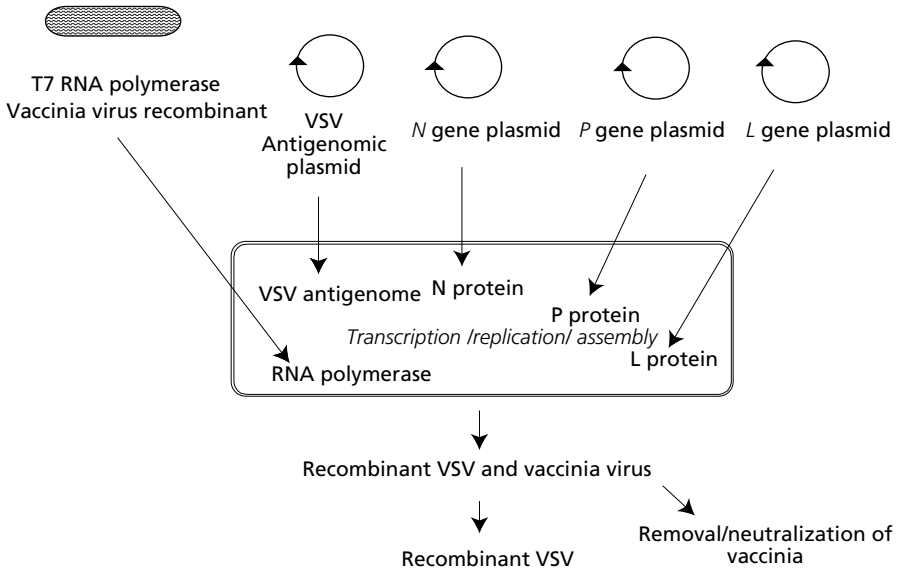

Figure 4 *Reverse genetics of nonsegmented negative-sense RNA viruses. The diagram depicts the strategy used to rescue infectious virus from the cDNA clone of a negative-sense RNA virus with a nonsegmented genome: vesicular stomatitis virus (VSV) is used as the example. Plasmids expressing the N, P, and L constituent proteins of transcribing nucleocapsids, and a plasmid containing a full-length cDNA replica of the viral antigenome, all under the control of the T7 RNA polymerase promoter (→), are transfected into susceptible cells. The T7 RNA polymerase is provided by coinfection of the cell substrate with a recombinant vaccinia virus expressing the T7 RNA polymerase. This is sufficient to initiate synthesis of functional nucleocapsids, followed by transcription, replication, assembly, and release of infectious virus.*

and recovery of negative-sense RNA viruses from cDNA clones of segmented genome viruses.

Recombination with host transcripts

Functions essential to replication and maturation can be attributed to no more than half the genes of the herpes simplex virus genome. It is likely that some of the excess coding capacity of the large DNA viruses represents accretion and modification of host genetic material by recombination. It is clear also that the oncogenic retroviruses have acquired their oncogenic potential by incorporation of cellular oncogenes by a recombinational mechanism. It is perhaps surprising, in view of the obligatory proviral phase in the multiplication cycle of these viruses, that these events have been so rare and restricted to experimental propagation of viruses in an inappropriate host.

Although conventional RNA viruses might not be expected to interact with the host-cell genome, there are isolated instances of such interactions for several positive-sense RNA viruses (poliovirus, Sindbis virus, and bovine viral diarrhea virus) and the negative-sense influenza A virus. The latter is the most exceptional because negative-sense RNA viruses do not exhibit intermolecular recombination. None the less, a variant of influenza A virus exists whose *N* gene subunit has an additional sequence exhibiting homology with host ribosomal RNA sequences. Best documented are the several independently derived isolates of the *Pestivirus*, bovine viral diarrhea virus (BVDV), which have acquired simultaneously a cytopathic (cp) and a virulent phenotype. Molecular analysis of these events suggested that each cp virus evolved from the pre-existing noncytopathic (noncp) virus by a process of RNA recombination. A mechanism based on template switching of the viral polymerase during negative strand synthesis has been proposed to explain this phenomenon. Nonstructural protein NS3 is found exclusively after infection with cp BVDV, whereas uncleaved NS2-3 is characteristic of persistent noncp virus. The mutations that lead to the generation of NS3 from the polyprotein precursor include the precise insertion of cellular sequences, frequently together with large duplications and other genomic rearrangements. Two different kinds of cellular insertion have been identified, either (poly)ubiquitin or part of a cellular polypeptide of unknown function. Cleavage of the polyprotein at the N terminus of NS3 can be mediated by any of the several proteases that cleave ubiquitin in uninfected cells (Meyers and Thiel 1996). The expression of NS3 protein was considered to be strictly correlated with the cp phenotype, but later Qu et al. (2001) isolated a series of non-cp mutants from cp virus that still expressed NS3 protein. Sequence analyses of these noncp revertants uncovered no change in NS3, but all exhibited a single substitution in the NS4B region. Although the mechanism remains to be elucidated, these results illustrate that mutations in NS4B can attenuate BVDV cytopathogenicity despite NS3 production.

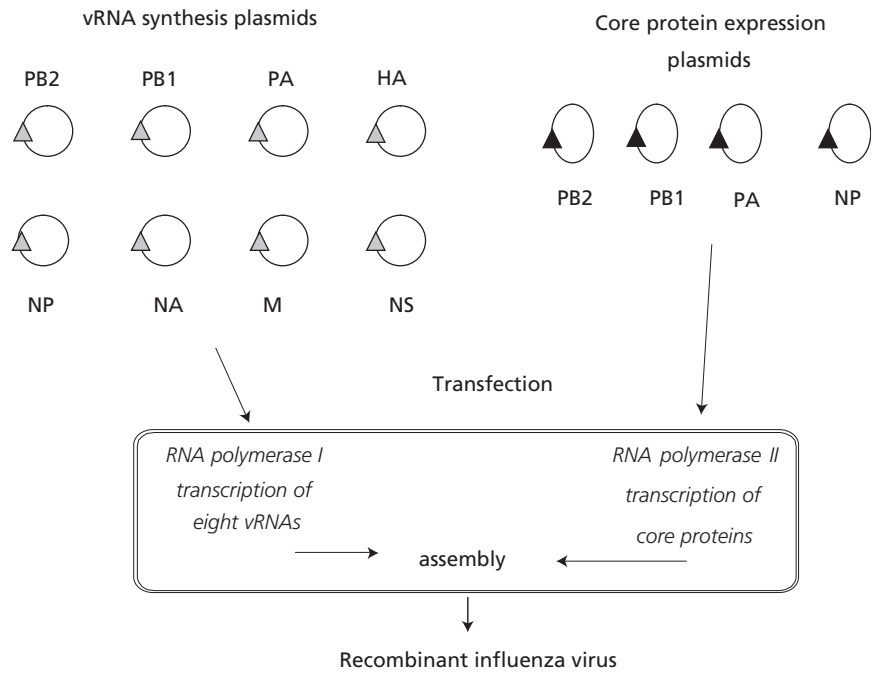

Figure 5 *Reverse genetics of segmented genome negative-sense RNA viruses. The diagram depicts the strategy used to rescue infectious virus from cDNA clones of the genome segments of a negative-sense RNA virus; influenza A virus is used as the example. Susceptible cells are transfected with plasmids encoding all eight components of the negative-sense genome under the control of the RNA polymerase I promoter (gray arrow-heads), together with four plasmids expressing the nucleocapsid components (PB1, PB2, PA, and NP) of influenza virus under the control of the RNA polymerase II promoter (black arrow-heads). The viral genomic RNAs are synthesized by cellular RNA polymerase I, and replication and transcription are initiated by synthesis of the viral polymerase (PB1, PB2, and PA) and the nucleoprotein (NP), followed by assembly and release of infectious virus.*

Interactions between cellular RNAs and replicating viral RNA have also been demonstrated in plant viruses, where a replication-competent deletion mutant of CPMV was rescued by recombination in transgenic plants expressing the deleted sequences (Greene and Allison 1994).

Subunit reassortment

All the segmented genome RNA viruses of animals are able to exchange genetic information by a process of subunit reassortment. Reassortment occurs at high frequency between related viruses; for example, all influenza A viruses are able to exchange genome subunits, but influenza A, B, and C viruses do not interact. In the bunyaviruses and the reoviruses restriction on reassortment of genome subunits closely parallels the serological and sequence relationships of the viruses, and does not correlate with the geographic origin of the isolates. In the orthomyxoviruses it is probable that there is random (i.e. unlinked) association of genome subunits and that the low electron microscope particle/plaque-forming unit ratio is a reflection of the imprecision of the maturation process, whereby only some particles receive the essential complement of eight unique subunits.

In the bunyaviruses, with their tripartite genomes, it is possible to undertake complete progeny analysis, because there are only six (2^3–2) nonparental reassortant genotypes, in contrast to the 254 (2^8–2) of influenza A virus, to be assayed. Reassortment appears not to be random and there are preferred associations of subunits even in the case of closely related viruses. The mechanism of restriction appears to operate at the level of gene products, as the terminal sequences of the subunits are identical and do not exhibit the specificity required for nonrandom reassortment. However, in crosses of Bunyamwera virus and Maguari virus, which belong to the Bunyamwera serogroup, once a parental subunit configuration had been broken by reassortment, the restriction on reassortment among progeny virus genomes was relaxed (Pringle 1996).

In the reoviruses, where maturation is efficient and precise, and particle/infectivity ratios are close to unity, reassortment appears to be random. In sharp contrast, if cells were infected (by lipofection) with reovirus infectious cores rather than virions, the progeny was almost exclusively monoreassortant virus (i.e. virus with nine subunits from one parent and one from the other). Lipofection of cells with naked RNA permitted only assortment (i.e. recovery of parental virus), not reassortment (Joklik and Roner 1995). These observations emphasize the complexity of the reassortment process

and suggest again that protein–protein interactions, rather than RNA-sequence recognition, determine the specificity of reassortment. A complicating factor in the analysis of reassortment, described in rotavirus progeny analysis but probably true for all segmented genome viruses, is that the phenotype of the reassortants recovered may be modified by the genetic background of the recipient (Chen et al. 1989).

In general, reassortment is restricted to taxonomically related viruses: in the family *Orthomyxoviridae* the boundaries are defined by species, and by serogroups within species in other virus families. Conversely, genetic compatibility, as measured by the ability to reassort genome subunits, may be a useful approach to equating taxons in different virus families. All segmented genome viruses have the potential for rapid adaptation to new hosts, for abrupt generation of viruses with new antigenic characteristics, and for sudden extensions of host range and virulence. Transmission by vectors and multiplication in alternate hosts are also common features of these viruses.

Genetic reassortment plays a major role in the epidemiology of human influenza. Mutation accounts for the progressive genetic and antigenic variation (antigenic drift) of influenza A virus during interpandemic periods, but the appearance of new pandemic strains usually requires the introduction of new genes by reassortment (antigenic shift). Avian viruses seem to provide the reservoir of antigenic variation from which new pandemic strains can be generated. Much evidence suggests that the pig provides the 'mixing vessel' in which avian and human viruses can interact, generating progeny with new antigenic properties and the constellations of genes required to allow transmission to and spread within human hosts. In South China the domestic duck may be the intermediate host carrying viruses from feral ducks to the pig, whereas in North America domesticated turkeys may be a more important intermediate host. Reassortment of influenza A virus seems to have been responsible for the major pandemics afflicting the human population since the beginning of the twentieth century. However, a recent outbreak of a lethal antigenically novel avian-like virus in horses in the Far East, and since 2003 the appearance of various avian influenza A viruses in humans (H5N1 in East Asia, H7N7 in the Netherlands, H7N3 in Canada, and H7N2 in the USA), indicate that avian viruses can spread directly to mammalian species without the need for reassortment or passage through an intermediate host.

Superinfection exclusion and gene capture

The three serotypes of reoviruses exchange genome segments freely during mixed infection both in vitro and in vivo, but little is known about the role of reassortment in the biology of these viruses. In some segmented genome viruses (e.g. some bunyaviruses and orbiviruses), but not in others (e.g. rotaviruses), a phenomenon of superinfection exclusion is observed whereby a superinfecting virus is able to participate in subunit reassortment for only a limited period after primary infection. Superinfection exclusion is also host-dependent. For example, reassortment of the genome subunits of the *Orbivirus*, bluetongue virus, is inhibited if the introduction of the superinfecting virus is delayed by more than 4 h, whereas there is no interference for a period of at least 5 days in asynchronously infected midges.

Natural reassortants of rotaviruses are probably frequent in occurrence, and there is ample evidence for gene capture by the interaction of segmented genome viruses present in alternate hosts. For example, the Au-1 strain of human *Rotavirus* has a VP-4-coding subunit encoding a feline *Rotavirus*-like VP-4, suggesting that genetic interaction between feline and human rotaviruses is not infrequent. Other rotaviruses recovered from humans seem to be reassortants of human, bovine, and a third unidentified parental rotavirus. Likewise, it has been implied that the five serotypes of bluetongue virus present in North America are derived from three distinct gene pools, and that serotype 17 has evolved by a combination of reassortment and genetic drift.

VIRUS VARIABILITY

Like all biological entities that posses the ability to replicate and reproduce themselves, viruses are endowed with an intrinsic variability derived from the error-prone process of nucleic acid replication. This inbuilt variability enables viruses to respond to selective forces and ultimately guarantees their survival.

The quasi-species concept

It is generally accepted that mutation frequencies in most RNA viruses are very high, ranging from 10^{-3} to 10^{-6}, and usually in the range 10^{-4} to 10^{-5}. Consequently, a 10-kb RNA genome will on average carry one or more mutations, and any population of RNA viruses will comprise a heterogeneous swarm of diverse mutants. The term quasi-species was introduced to take account of this phenomenon and the consequent difficulty of defining a viral species (Eigen and Biebricher 1988). A single 'master' sequence may predominate at any one time during replication in a defined environment, but its existence will be transient and dependent on the maintenance of strong external selective forces. The high mutation frequencies of RNA viruses are attributed to the inherent error-proneness of RNA-dependent RNA polymerase and reverse transcriptase, and to the lack of a proofreading function.

Paradoxically, some RNA viruses exhibit great sequence stability during replication in vivo or during

propagation in cultured cells. Even the highly variable HIV-1 is more stable during replication in cell culture than the error-proneness of the purified polymerase would predict, indicating the existence of cellular stabilizing factors (Mansky and Temin 1995).

Although the high mutation rates of RNA viruses provide them with great adaptability and may explain why in recent times they have been causes of emerging diseases (e.g. hepatitis C virus, Nipah virus, Hendra virus, severe acute respiratory syndrome (SARS) *Coronavirus*, enterovirus 71, and others) their limited genome size means that they are also subject to major evolutionary constraints. Also gene duplication and lateral gene transfer from their host or other viruses are inherently less likely (Holmes and Rambaut 2004).

Hypermutation

Enhanced frequency mutation mediated by an error-prone polymerase has been described for vesicular stomatitis virus (Pringle 1987). Also, hypermutable sites occur in genomes, such as those generating frameshift and other mutations in the attachment (G) protein gene of human respiratory syncytial virus (Cane et al. 1993). Likewise, in DNA viruses mutations in accessory DNA replicating functions have been shown to be responsible for effects on the mutation frequency of herpes simplex virus type 1 (HSV-1) in cultured cells. Mutational loss of HSV-1-encoded dUTPase activity resulted in a fivefold increase in the relative mutant frequency, whereas thymidine kinase-negative mutants reduced the relative mutation rate up to 40-fold (Pyle and Thompson 1994).

Biased hypermutation

Several instances of biased hypermutation have been described. A phenomenon of G→A hypermutation has been observed in retroviruses and hepadnaviruses. Among negative-sense single-stranded RNA viruses, biased A→I hypermutation has been observed to affect mainly the matrix protein gene of measles virus, possibly as a consequence of posttranscriptional enzymatic misreading of A residues (Billeter and Cattaneo 1991); and multiple A→G transitions occur in the attachment protein gene of human respiratory syncytial virus. The latter are characteristic of neutralization escape mutants selected with monoclonal antibodies against the conserved central region of the G protein (Martinez and Melero 2002). A high frequency of frameshift mutations was observed also in the same gene, mediated by the addition or deletion of adenosine residues in some, but not all, runs of such residues. The fidelity of the viral polymerase appeared to be dependent on the template sequence (Cane et al. 1993).

The biased hypermutatiion in which many U residues were substituted by C residues, predominantly in the *M* (matrix) protein gene of measles virus, was characteristic of isolates of measles virus associated with neurologic conditions such as subacute sclerosing panencephalitis and measles inclusion body encephalitis (MIBE). In the extreme case, 54 percent of U residues were changed to C residues. The phenomenon may be mediated by the presence in the cytoplasm of neural cells of a double-stranded RNA unwindase, an enzyme normally abundant in nuclei. This enzyme (duplex RNA-dependent adenosine deaminase) converts adenosine to inosine ribonucleotide. It is hypothesized that the predominance of U→C misincorporations over A (to I)→G mutations is a consequence of the formation of double-stranded RNA molecules by localized collapse of transcribed mRNA on to the genomic- (negative-) strand template, whereas the collapse of replicating negative-sense RNA on to the positive-sense anti-genome would be less frequent because of the encapsidation of replicating molecules, which is a feature of the synthesis of negative-strand RNA viruses. The phenomenon seems to have clinical significance in that clonal expansion of hypermutated measles viruses was demonstrated throughout the brain of an SSPE patient (Baczko et al. 1993).

Retroviral G→A hypermutation takes place during reverse transcription when the intracellular dTTP concentration greatly exceeds that of dCTP (Vartanian et al. 1997), This results in extensive incorporation of dT opposite rG, leading to G→A substitutions. In vitro the extent of G→A hypermutation can be influenced by manipulation of the dTTP/dCTP ratio. The extreme of such biased hypermutation is observed in the lentiviruses, where misincorporation may reach 60 percent in short stretches. It occurs predominantly during negative-sense DNA strand synthesis, and it has been established that G→A hypermutation can be sustained throughout synthesis of the entire 10-kb negative-sense DNA strand of HIV type 1, resulting in substitution of approximately 31 percent of the G residues (Vartanian et al. 2002).

Gunther et al. (1997) have described two naturally occurring hepatitis B virus genomes from a chronic carrier which exhibited G→A mutations distributed across the whole genome, with a strong dinucleotide preference in the order GpA > GpG > GpC/GpT. The frequency of replacement of guanosine residues by adenosine was 12 and 26 percent. The detection of hypermutation in natural isolates of hepatitis B virus suggested that a minority of hepatocytes might have distorted dNTP pool which, as well as generating hypermutation might be reflected in the fidelity of hepatocyte DNA synthesis and repair.

APPLICATIONS OF GENETIC ANALYSIS IN VIROLOGY

A genetic approach has many actual and potential applications in virology: for example, considered here are the

analysis of antigenic properties (epitope mapping), identification of determinants of virulence and drug resistance, interpretation of molecular epidemiology, and the engineering of live virus vaccines.

Epitope analysis

Neutralization escape mutants can be isolated following exposure to, or growth in the presence of, a specific antiserum or an appropriate monoclonal antibody. Specific epitopes can be identified by analysis of the reactivity of neutralization escape mutants with panels of monoclonal antibodies. These epitopes can then be located by nucleotide sequencing of the escape mutants and physical mapping of the mutational changes. Optimally, these changes can then be located on the three-dimensional structure of viral attachment proteins (e.g. the HA protein of influenza A virus; see Orthomyxoviruses: influenza) or the whole virion (e.g. poliovirus; see Picornaviruses).

Nonneutralizing monoclonal antibody escape mutants can be obtained by a modification of the selection technique. Infected cell monolayers are reacted with an appropriate murine monoclonal antibody and then treated with an enzyme-linked second antibody (e.g. a horseradish peroxidase rabbit anti-mouse polyclonal serum). Plaques that are unstained represent monoclonal antibody-resistant (MAR) mutants, which can be employed in analysing the antigenic properties of nonstructural and internal components of viruses.

Drug resistance

The isolation of drug resistance mutants can identify the site of action of an antiviral agent and assist in analyzing both the mechanism of action of the drug and the function of the target protein. For example, identification of the function of the M2 protein of influenza virus and confirmation of its presence in the virion were direct results of the isolation of amantidine-resistant mutants of influenza A virus (Hayden and Hay 1992).

The isolation of drug resistance mutants can also provide information of relevance in the treatment of viral disease. For example, molecular analysis of mutants of HIV-1 virus resistant to 3′-azido-3′-deoxythymidine (AZT), nucleoside analogues, and other nonnucleoside reverse transcriptase (RT) inhibitors revealed unpredicted interaction between drugs in patients. In particular, suppression of phenotypic AZT resistance was observed in some viruses with mutations in the RT gene, which conferred resistance to other anti-retroviral drugs. Other combinations of mutations conferred co-resistance. A conclusion from such analyses is that certain combinations of antivirals are more likely than others to induce multidrug resistance in the patient, and that it may be possible to design treatment schedules to reduce this possibility, and even perhaps to reverse the loss of efficacy of AZT (Larder 1994).

Identification of virulence determinants

Analysis of the determinants of the virulence of the three Sabin poliovirus vaccine strains, which were produced by a process of adaptation to growth in nonneural tissue in vitro, provides one of the best examples of the potential of genetic analysis.

Determination of the complete sequence of the type 1 Sabin vaccine strain and its Mahoney virulent progenitor revealed that 56 single-site mutations, resulting in 21 amino acid substitutions, differentiated the two strains. The production of intratypic and intertypic recombinants generated by recombination experiments, the construction of in vitro clones from cDNA, and site-directed mutagenesis led to the conclusion that several mutations, mapped to VP1, VP3 , VP4, $3D^{pol}$, and the 3′ noncoding region, contributed to the attenuation and temperature-sensitive phenotypes. Although there was a strong correlation between the rct (temperature sensitivity) phenotype and loss of neurovirulence, assay of these recombinants and mutants in transgenic mice expressing the human poliovirus receptor revealed that the determinants of temperature sensitivity and attenuation were separable genetically (Bouchard et al. 1995).

A similar analysis was not possible with the poliovirus type 2, because the progenitor was already avirulent in monkeys. Recognition of virulence determinants in this case relied on comparison of the vaccine virus with a neurovirulent revertant isolated from a vaccine recipient. It was concluded that two major attenuating mutations resided in the 5′ NTR (noncoding region) and in VP1.

A similar conclusion was derived from analysis of the type 3 vaccine strain, in which the wildtype (Leon) progenitor and a neurovirulent revertant from a patient were available for comparison. Remarkably, only 10 single-site mutations, resulting in four amino acid substitutions, differentiated the vaccine strain from its virulent progenitor. Two mutations, one located at site 472 in the 5′ NTR and the other a ts mutation in the VP3 region, contributed to the attenuation phenotype. Later, a third mutation located in the VP1 region was identified as an additional attenuating factor. The C→U mutation at 472 associated with the attenuated phenotype was observed to revert at high frequency following vaccination. Virus recovered from the gut of a human infant as early as 47 h after vaccination had reverted, and exhibited increased neurovirulence in monkeys. The role of mutation in the 5′ NTR region in attenuation was confirmed by mix-and-match type in vitro recombination experiments. Subsequently, mutations at positions 480 and 481 of poliovirus types 1 and 2, respectively, were similarly shown to be involved in the attenuation phenotype

(Figure 6). The 5′ NTR mutations locate at different bases in the same inferred secondary structure and may act by destabilizing this stem–loop structure, possibly affecting the translation efficiency of the viruses (Macadam et al. 1994). The other mutations affecting attenuation show no common features, and it is considered that 5′ NTR mutations may be responsible for the greater stability of the three Sabin live attenuated vaccines over their rivals. Similarly, a mutation in the 5′ noncoding region of coxsackie B3 virus has been identified as a determinant of cardiovirulence in mice (Tu et al. 1995).

It is likely for any virus that attenuation may be achieved by more than one route: for example, in the case of human respiratory syncytial virus (HRSV) mutations located in the *L* (polymerase) protein gene, the *F* (fusion) protein gene, or the start region of the *M2* protein gene, all have the potential to attenuate virulence. For this reason the most economical and reliable route to the identification of virulence factors, as exemplified in the case of the Sabin attenuated polioviruses, is to undertake a comparison of the genomic sequences of an existing empirically derived attenuated virus, its progenitor virulent parent, and, optimally, a revertant from the attenuated phenotype to a virulent phenotype. Once identified, critical attenuating mutations can be inserted into the genome of other strains of the same or closely related viruses by site-directed mutagenesis and recombinant virus rescued by recombination or reverse genetics according to the size and nature of the viral genome.

As in the case of the Sabin attenuated polioviruses, often remarkably few mutations distinguish virulent parental virus and attenuated progeny virus. Only 13 amino acid substitutions differentiated a candidate respiratory syncytial virus vaccine, derived semi-empirically by sequential selection of temperature-sensitive mutants under conditions of limiting mutagenesis, from its virulent parent. Comparison of the complete genome sequences of parental virus and first-, second-, and third-stage mutants, combined with testing in adult volunteers, enabled identification of a specific site in the respiratory syncytial virus polymerase protein gene that was a major determinant of attenuation (Tolley et al. 1996).

Attenuation by gene expression control

The location of a major attenuation determinant in the 5′ NTR region of poliovirus indicates that attenuation can be mediated at the level of control of mRNA synthesis, as the internal ribosome entry site of the poliovirus genome is located also in the 5′ region. In the case of nonsegmented negative-sense RNA viruses gene expression is controlled by the linear order of the individual genes and their relative distance from the single 3′-terminal transcriptional promoter. Thus the phenotypes of these viruses can be altered by gene rearrangement (Wertz et al. 1998; Ball et al. 1999) (Figure 7).

Flanagan et al. (2001) showed that rearrangement of the genes of vesicular stomatitis virus could eliminate clinical disease in swine, the natural host of the virus, thereby identifying an alternate route to attenuation (Figure 7b). Moving the nucleocapsid protein gene away from it normal location adjacent to the single transcriptional promoter site attenuated in stepwise fashion and finally eliminated the potential of the virus to produce disease in an experimental animal (Figure 7a). Combining this with relocation of the surface glycoprotein gene closer to the promoter site to increase its level of expression produced a virus that protected vaccinated animals against challenge with virulent virus of the same strain (Flanagan et al. 2000). Remarkably, these rearranged genomes could be propagated indefinitely and retained their altered phenotypes; some internal gene arrangements reduced viability, but others did not (Figure 7c). Subsequent work has shown that additional sequences can be inserted and retained in the genome of vesicular stomatitis virus, and that the level of expression of the supernumerary gene is determined by its relative position in the gene order (Wertz et al. 2002).

These results illustrate that negative strand viruses such as vesicular stomatitis virus, which are immune to disruption of gene order by recombination, can serve as stable gene vectors and vaccine delivery agents. The genome of vesicular stomatitis virus will tolerate a 40 percent increase in genome content (Haglund et al. 2002), allowing the insertion of heterologous antigens whose expression can be controlled by their position of insertion, and thereby increasing the scope of this approach to vaccine development. An unresolved question is why the majority of the nonsegmented negative-sense genome viruses (the order *Mononegavirales*) retain a common gene order, rather than any of the several viable permutations of the gene order.

Similar rearrangement of a strictly conserved genome organization without loss of viability has been observed also in the positive-sense RNA coronaviruses (de Haan et al. 2002).

Natural chimeras, transgenes, and genetically engineered vectors

In addition to the natural reassortants and recombinants of influenza viruses, rotaviruses, and picornaviruses encountered in nature, an expanding list of genetically engineered hybrid viruses is being described, e.g. Sindbis/VSV, influenza A/B, and VSV Indiana/VSV New Jersey, among others. The best-documented

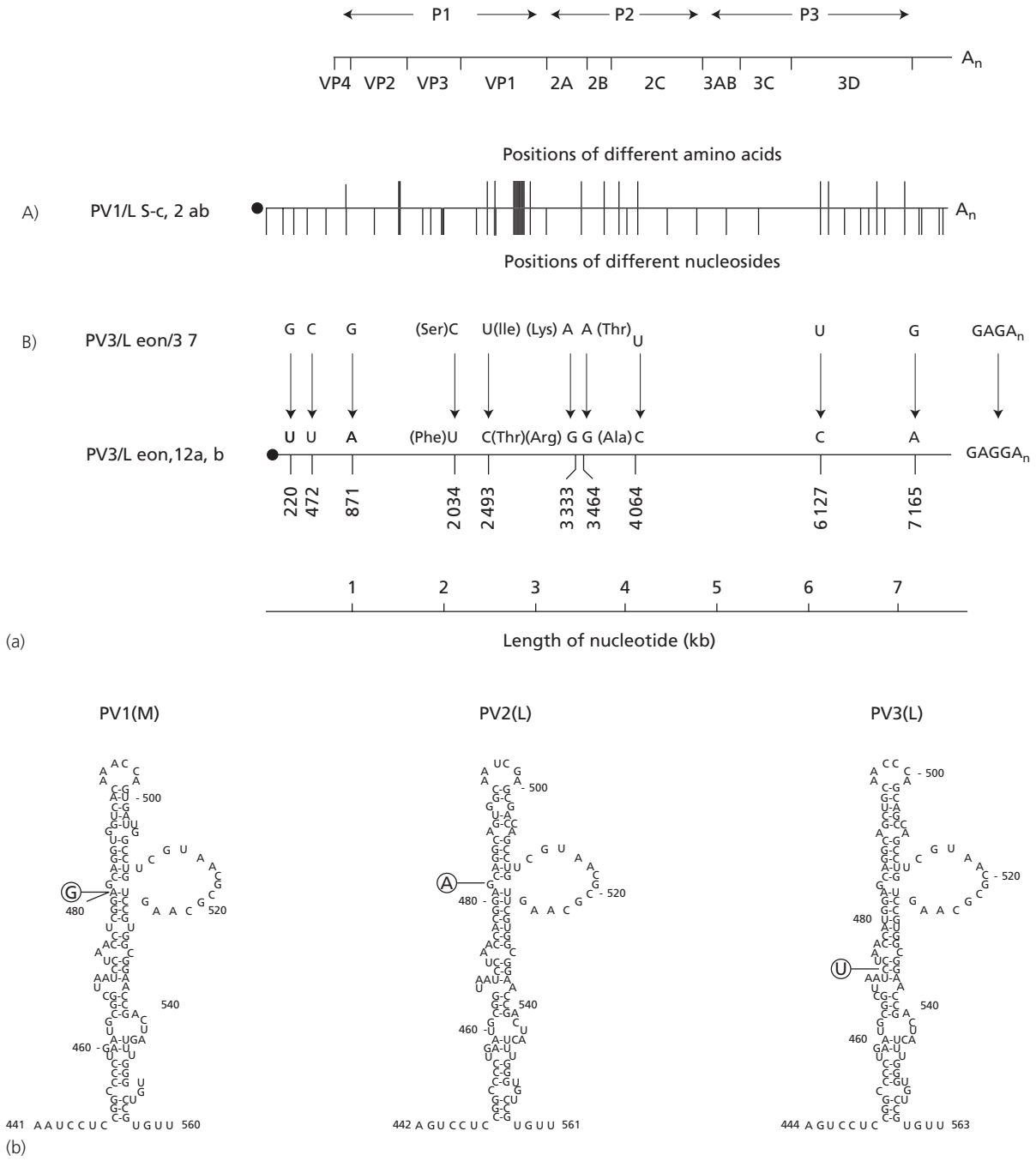

Figure 6 (a) *Location of attenuating mutations in the Sabin strains of poliovirus types 1 and 3: (A) the 21 amino acid and 56 nucleotide differences between the virulent parental (P1/Mahoney/41) and vaccine (P1/LSs, Zab) type 1 strains; (B) the four amino acid and ten nucleotide differences between virulent parental (P3/Leon/37) and vaccine (P3/Leon 12a1b) type 3 strains.* **(b)** *Domain V of the 5′ noncoding region of poliovirus types 1, 2, and 3. The attenuating mutations are circled. (Reproduced with permission from Almond 1987).*

chimeras are the adeno–SV40 hybrid viruses, which were inadvertently isolated during the 'adaptation' of adenovirus type 7 to growth in nonpermissive SV40-contaminated African green monkey kidney cells (Grodzicker 1981). Hybrid viruses were obtained in which adenovirus and SV40 virus genetic material was covalently linked. A few adeno–SV40 hybrids were isolated in which an intact adenovirus or SV40 genome was present, whereas others were incomplete and dependent for their existence on an adenovirus helper. Subsequently, helper-dependent and helper-independent adeno2–SV40 hybrids were produced in the same way. The ad2ND1 hybrid contained a nondefective, though incomplete, adenovirus genome covalently linked to

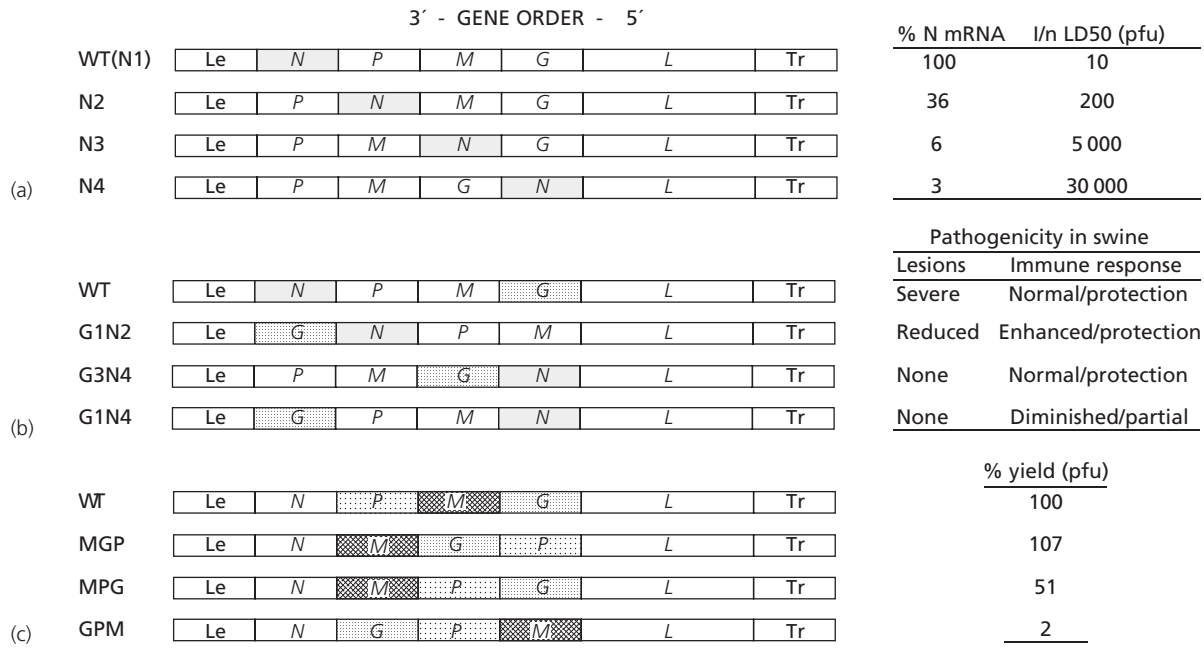

Figure 7 *Consequences of rearrangement of the gene order of vesicular stomatitis virus. The order of the components of the genome of vesicular stomatitis virus is indicated diagrammatically: the wildtype (WT) order is noncoding leader (Le), nucleocapsid* (N) *protein gene, phosphoprotein* (P) *gene, matrix* (M) *protein gene, glycoprotein* (G) *gene, polymerase* (L) *gene, and noncoding trailer (Tr) region. Panel* **(a)** *illustrates that translocation of the nucleoprotein gene down the gene order both attenuates transcription and reduces lethality for mice. Panel* **(b)** *indicates that translocation of the glycoprotein gene to a promoter-proximal position can enhance immunogenicity and eliminate clinical disease in the natural host of the virus. Panel* **(c)** *indicates that rearrangement of the internal genes can both enhance and reduce viability in cell culture.*

SV40 sequence. The SV40 sequence, which conferred the ability to multiply in monkey cells, varied from 7 to 43 percent, and the dispensable amount of adenovirus genome deleted varied from 4.5 to 7.6 percent. In all the adeno2–SV40 hybrids the insertion point was the same, namely at a site 14 percent from one end of the adenovirus genome.

Continuous passage of papovaviruses at high multiplicity of infection generates particles that have lower buoyant density than competent virions and contain less DNA. The DNA in these particles is circular and may have 10–50 percent of the papovavirus genome deleted. The segment deleted may be substituted by reiterated or nonreiterated host-cell DNA sequences, which may be larger than the segment deleted. These phenomena show that the genome of papovaviruses, and perhaps other DNA viruses, can be modified by the incorporation of host DNA sequences, and on rare occasions can contribute DNA sequences, either by homologous recombination or by illegitimate recombination, to other DNA-containing viruses.

An additional dimension has been added to genetic analysis by the ability to introduce foreign DNA into fertilized murine ova and to raise to maturity transgenic animals that have the foreign DNA integrated chromosomally. Among other things, this technique enables the determinants of tissue specificity to be investigated directly. For example, comparison of the pattern of tumor development in mice transgenic for genes from SV40, JC, and BK viruses indicated that tissue specificity is determined by early genes.

The construction of recombinant viruses for use as vaccines and gene vectors is probably the most obvious practical outlet for genetic technology. Vaccines of proven worth and safety (e.g. vaccinia virus, fowlpox virus, simian foamy virus, poliovirus, adenovirus, and others) can be employed as vectors for heterologous antigens. This approach to vaccine development (sometimes called vectored antigen delivery) has the advantages of inherent safety, speed, ease of administration and, in contrast to inactivated vaccines, the likelihood of obtaining a balanced cellular and humoral immune response. The potential limitation of pre-existing or induced immunity to the vector has not been a major problem in experimental trials. Control of rabies in wildlife by oral vaccination of red fox populations in Europe using a bait laced with a vaccinia virus/rabies *G protein* gene recombinant virus was the first successful application of this approach (Aubert et al. 1994).

Attenuated vesicular stomatitis viruses have been developed as vectors which can be delivered intranasally and support higher levels of expression than poxvirus vectors. In experimental animals these recombinants have proved effective in inducing humoral and cell-mediated responses to a variety of viral antigens (HIV gag and env; HCV E1 and E2; bovine viral diarrhea E2;

influenza A virus HA; rabbit papilloma virus L1; and respiratory syncytial virus G and F). Vesicular stomatitis vectors with the *G* (attachment) *protein* gene deleted (and expressing the influenza HA protein, or either G or F of respiratory syncytial virus) were protective and nonpathogenic in mice, and had the additional advantage of not inducing neutralizing antibodies to the vector itself (Kahn et al. 2001).

Gene therapy

Viruses have potential as efficient gene delivery vehicles in gene therapy, provided efficient transduction and sustained transgene expression can be achieved without compromising safety. Retroviruses and adenoassociated viruses are favored as gene delivery vehicles because of their low cytopathogenicity and/or tissue specificity. The strategies employed have ranged from complex to simple. The first successful treatment by gene therapy of a disease affecting multiorgan systems in a large mammal employed one of the simplest approaches. Mucopolysaccharidosis VII is a rare inherited disorder in dogs which manifests as ocular defects, cardiac disease, and bone abnormalities leading to loss of mobility by 6 months. This condition is due to a deficiency in the activity of the hepatic enzyme β-glucuronidase. Several 2–3-day-old dogs with this disorder received intravenous injections of Moloney murine leukemia virus carrying a normal canine *β-glucuronidase* gene under the control of a liver-specific promoter (Ponder et al. 2002). Transduced hepatocytes expanded clonally during normal liver growth and secreted normal levels of β-glucuronidase. Dogs treated in this way achieved normal body weight and were fully active at 17 months.

Molecular epidemiology

Recognition of genetic diversity through genome sequencing and phylogenetic analysis plays an increasingly important role in understanding the epidemiology of infectious disease. For example, a high proportion of patients with vaccine-related poliomyelitis exhibit vaccine-derived interserotype recombinant viruses that have also lost their attenuated phenotype (Cherkasova et al. 2002). Study of these isolates revealed a nonrandom distribution of synonymous mutations, conservation of secondary structural features, a relative dearth of mutations affecting the integrity of antigenic determinants, and a frequent occurrence of tripartite intertypic recombinants with either type 1 or type 3 homotypic genomic ends.

Analysis of all available complete genome sequences of dengue viruses revealed that about 10 percent were intrasubtype recombinants (Worobey et al. 1999), thereby discounting the use of partial genome sequences as markers for the whole genome. This phenomenon is a confounding factor in analysis of the circumstances that contribute to the appearance of the lethal hemorrhagic form of dengue fever. In the absence of recombination this complication does not arise. For example, a study of the genetic variability of human RSV, a virus initially assumed to be associated only with seasonal respiratory disease in infancy in temperate climates, established that RSV is a universal respiratory pathogen evolving in a global milieu. Isolates of RSV originating from many parts of the world could be fitted into a genotype classification defined by analysis of viruses recovered from the catchment area of a single hospital in a suburb of Birmingham (Cane and Pringle 1995).

The developing AIDS epidemic is one of the greatest challenges facing mankind. Phylogenetic analysis has established that the origin of the AIDS syndrome was not a unique event, and that the M, N, and O groups of HIV-1 have each arisen as a consequence of independnent zoonotic transmission of a simian immunodeficiency virus (SIVcpz) from a subspecies of the common chimpanzee (*Pan troglodytes troglodytes*) to humans. Other explanations for the origin of the M, H, and O groups of HIV-1, such as viral diversification within human populations, are inconsistent with existing phylogenetic data (Gao et al. 1999). The closest relatives of HIV-2 have been found in the sooty mangabey (*Cerocebus atys*), and again phylogenetic evidence suggests independent transmissions to humans. A recent survey has established that a substantial proportion of wild monkeys in West Africa are infected with a diversity of simian immunodeficiency viruses, serologically related to HIV to varying extents, representing a reservoir of potential human pathogens. The emergence of the lentivirus immunodeficiency diseases in humans has been correlated with the increased hunting of primates to provide meat for human consumption (Peeters et al. 2002). On the other hand, a putative evolutionary link has been proposed between HIV-1 and a group of endogenous retroviruses (HERV-K) that are present at about 50 proviral copies per haploid genome and which entered the human genome some 30 million years ago. The connection is based on identification of a functional homolog of the HIV-1 Rev protein encoded in the HERV-K genome, and the presence of a target RNA sequence in the HERV-K LTR region recognized by HIV-1 Rev (Yang et al. 1999), perhaps indicative of previous human encounters with such retroviruses.

Immune evasion

Characterization of the genomes of the large DNA viruses has identified a number of genes that interfere with the immune response of the host. The genome of vaccinia virus comprises about 200 genes, about one-third of which are dispensable for propagation of the

virus in cultured cells. These apparently dispensable genes map in the extensive dispensable terminal regions of the vaccinia virus genome, together with host range and virulence markers. Some of these dispensable genes seem to contribute to the survival of the virus in vivo by interfering with specific arms of the immune system, such as complement, interferon, inflammation, cytokines, and cytotoxic T-lymphocyte recognition. The vaccinia virus genome encodes several proteins that have amino acid similarity to components of the complement system, and may prevent complement activation by competitively binding either complement control factors or complement receptors. Analogous genes are present in some herpesviruses.

At least four poxvirus genes interfere with interferon action. However, interference with the interferon response is not restricted to the large genome DNA viruses. Negative-sense RNA viruses also encode proteins that interfere with the interferon response. For example, the *NSs* gene product of bunyamwera virus, identified as a nonessential protein modulating pathogenesis, functions as an interferon induction antagonist to enhance virulence (Weber et al. 2002). Similarly, by construction of deletion mutant and recombinant viruses by reverse genetics it has been established unequivocally that the promoter-proximal *NS1* and *NS2* genes of pneumoviruses, two abundantly transcribed genes of previously unknown function, function as α/β-interferon induction antagonists (Bossert and Konzelmann 2002). Other paramyxoviruses have evolved different mechanisms for counteracting the cellular interferon response. Sendai virus, SV5, and human parainfluenza virus 3 block both type I and type II inferferon responses by targeting the cellular signaling factors (STAT 1 and STAT 2) involved in interferon signaling, an effect which is mediated by one of the alternate products expressed from the virus phosphoprotein gene (Andrejeva et al. 2002).

These are some examples of the continuing value of application of genetic methodology in both fundamental and applied virology.

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Virus–host cell interactions

L. ANDREW BALL

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INTRODUCTION

As obligate intracellular parasites, viruses depend on their host cells at all stages of their infectious cycles. Different viruses impinge on almost every aspect of host-cell metabolism, so virus–host cell interactions are diverse and complex. The details of these interactions have been shaped during the co-evolution of individual viruses and their hosts, and they can be fully understood only within that context. In fact, examination of the defenses mounted by cells and organisms against viral infection, and of the various mechanisms that different viruses have evolved to help them evade these defenses, provides a revealing glimpse into the heart of the host–parasite relationship (Smith 1994).

Experimental studies of virus–cell interactions are usually performed in cell culture, by using purified viruses to infect cultured cells that may be a different type or even a different species from those in which the viruses evolved. Because the consequences of infection can vary greatly from one cell type to another, the link between laboratory studies of virus–cell interactions and the behavior of viruses in their natural state is sometimes tenuous. Nevertheless, experimental studies of virus–host cell interactions have not only enriched our understanding of viral infection but also illuminated many areas of molecular and cellular biology. Among these areas are the mechanisms of receptor function (Nomoto 1992; Haywood 1994; Wimmer 1994) and membrane fusion (White 1992; Bentz 1993; Earp et al. 2005); the pathways and control of macromolecular synthesis, processing, and transport (Nevins 1989; Ehrenfeld 1993; Gebauer and Hentze 2004); the regulation of cell cycling (Levine 1993; Weinberg 1995); and oncogenic transformation (Teich 1991; Jansen-Durr 1996). This chapter presents a survey of this complex and far-reaching subject, and refers the reader to more detailed coverage of specific topics in other chapters. It also contains an extensive bibliography that includes many review articles to guide those interested in reading further.

Cytopathic effects of viral infection

Infection of susceptible cells with an animal virus usually causes cytological changes that are visible by light microscopy and collectively referred to as the cytopathic effect (CPE) of the virus. The nature of the CPE differs between viruses and from one type of virus–cell interaction to another (described in Types of virus–host cell interactions). In the case of a cytocidal virus infecting a cell monolayer, for example, the CPE frequently includes cell rounding and loss of adherence to the tissue-culture plate, visible consequences of the perturbations of cellular metabolism that presage the death of the cell (Figure 1a). In contrast, cells infected with a tumor virus, where the outcome is not cell death but neoplastic transformation, undergo more subtle changes in morphology, although the overall consequences of infection both in cell culture and in animals can be at least as profound (Figure 1c).

Viral CPEs are not due simply to the increased load on cellular protein synthesis (which is usually insignificant)

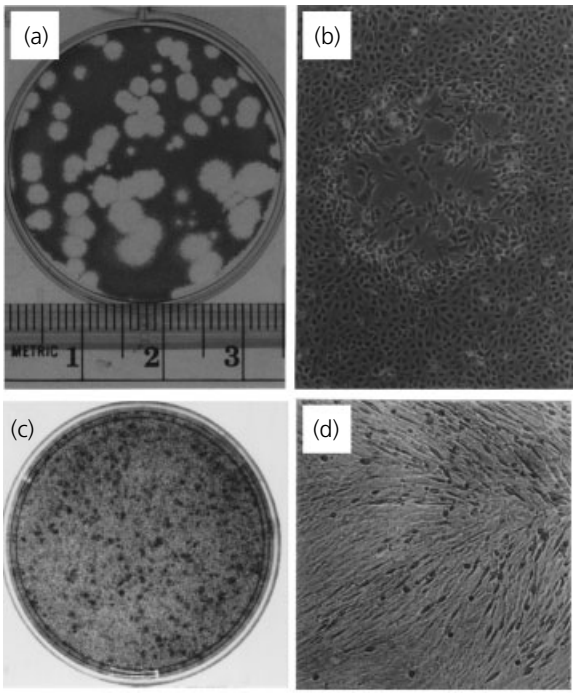

Figure 1 (a) *Plaque assay of a lytic virus (vesicular stomatitis virus).* **(b)** *Microphotograph of the typical cytopathic effect (CPE) of a lytic virus (vaccinia virus).* **(c)** *Focus-forming assay of a transforming retrovirus.* **(d)** *Microphotograph of part of a focus of transformed cells. Thanks to Drs. Donald Blair and Karen Joyce Dunn (NIH, NCI) for the photographs shown in* **(c)** *and* **(d)**.

nor even to its inhibition, because translation in uninfected cells can be blocked by drugs without inducing a CPE. On the contrary, some aspects of viral CPEs are directly due to the action of specific viral proteins: for example, detachment of adenovirus-infected cells from cell-culture plates results from the penton-base protein of the virion binding to cellular integrins during entry and thereby disrupting their interaction with vitronectin that was responsible for cellular adherence (Wickham et al. 1993). Similarly, some retroviral proteases cause cell rounding by disrupting the cytoskeleton (Luftig and Lupo 1994). However, it also seems that cells can respond to viral infection by launching a specific genetic program that leads to cell death (see Apoptosis), which indicates that some of the consequences of infection are self-inflicted (Razvi and Welsh 1995; Steller 1995; Everett and McFadden 1999). The benefit to the organism of thus limiting the spread of infection outweighs the cost in sacrificed cells (Williams 1994).

QUANTITATIVE ASPECTS OF VIRUS–CELL INTERACTIONS

Multiplicity of infection

Infection is an encounter between two populations: the virus particles and the host cells. The average number of infectious virus particles per cell is called the multiplicity of infection (MOI). When the MOI is very low (10^{-4}, say), only a correspondingly small fraction of the cell population will be infected initially, although the infection can then spread through the cell culture as the virus multiplies. If the spread is contained, by an agarose overlay for example, areas of infected cells (plaques) will develop around each initial infection site, allowing the number of infectious particles (or plaque-forming units (pfu)) in the virus inoculum to be calculated. This is the basis of the plaque assay, an example of which is shown in Figure 1a,b. In contrast, when the MOI is high, essentially all the cells will be infected at the outset and the virus will be limited to a single round of synchronous replication. Tumor viruses, which transform cells rather than killing them, produce local foci of transformed cells in culture (Figure 1c,d) and can therefore be quantified by an analogous assay that measures the number of infectious particles (or focus-forming units (ffu)) in a tumor virus preparation.

Poisson distribution

The distribution of virus particles among a population of cultured cells is random and can be described mathematically by the Poisson distribution. This formula relates the average MOI (m) to the fraction of cells (P) that actually receive any given number (k) of infectious particles. It shows that an MOI of 1.0 pfu per cell will result in the infection of only 63.2 percent of the population, and that an MOI of $\geqslant 5$ is required to infect at least 99 percent of the cells (Table 1).

Ratios of physical particles to plaque-forming units

Not all virus particles in a preparation are capable of forming a plaque; indeed, the particle:pfu ratios for most animal viruses are seldom lower than 10 and sometimes much higher. There are several explanations for this surprising and often overlooked result. First, infection is a multistep process in which the probability of successful completion of each step may be less than 100 percent even for an intrinsically infectious particle. Secondly, the apparent homogeneity of a virus preparation can conceal a substantial level of microheterogeneity: differences in the age of individual particles, their protein composition, and even their genome sequences can influence infectivity. Among the RNA viruses, the high polymerase error rate that results from the absence of proofreading during RNA replication creates a population of genomes whose sequences compose a molecular swarm or quasi-species distribution around a consensus sequence (Smith and Inglis 1987; Holland et al. 1992; Drake 1993; Eigen 1993; Duarte et al. 1994; Domingo and Holland 1997). This concept has profound

Table 1 *Poisson distribution: percentage of cells receiving 0, 1, and >1 particles at different MOIs*

Multiplicity of infection (pfu/cell)	Uninfected cells (%)	Singly infected cells (%)	Multiply infected cells (%)
0.0	100.0	0.0	0.0
0.5	60.7	30.3	9.0
1.0	36.8	36.8	26.4
2.0	13.5	27.0	59.5
3.0	5.0	15.0	80.0
5.0	0.67	3.35	96.0
10.0	0.0045	0.045	99.95
$P(k) = (e^{-m}.m^{k}).I/k!$			
$P(0) = e^{-m}$			
$P(1) = m.e^{-m}$			
$P(>1) = 1-e^{-m}(m+1)$			

implications for our understanding of RNA virus evolution (The origin and evolution of viruses).

Noninfectious virions can nevertheless participate in infection under both natural and experimental conditions. Typically, the number of particles that can undergo receptor binding and other early stages of infection will far exceed the infectious titer, a fact that complicates the study of early events in virus–cell interaction because it is difficult to identify the minority of particles that are on the pathway to productive infection (Wilson 1992). In many cases the genomes of noninfectious particles can engage in complementation and recombination with one another and thus contribute to the outcome of infection. Moreover, some of the most important physiological effects of infection, such as killing of the host cell and the induction of interferon (see Induction and actions of interferons and The role of cytokines in viral infections), do not depend on the production of viral progeny and can therefore be accomplished by more virus particles than are needed to demonstrate full infectivity (Marcus and Sekellick 1974). The situation is further complicated by the fact that most animal viruses generate defective particles, particularly on repeated passage at high MOI. As their name implies, defective particles are incapable of replication except when supported by coinfection with their parent virus with which they often interfere, thus earning the name defective interfering (DI) particles. Under experimental conditions DI particles can play critical roles in maintaining persistent infections, modulating viral pathogenesis (Pathogenesis of viral infections), and guiding virus evolution (The origin and evolution of viruses), and it seems likely that they exert similar effects in nature (Dimmock 1985; Barrett and Dimmock 1986; Whitaker-Dowling and Youngner 1987; Roux et al. 1991; Bangham and Kirkwood 1993).

Single-hit relationships

The fact that preparations of animal viruses have high particle:pfu ratios does not mean that more than one particle per cell is required to initiate a productive infection. The quantitative relationship between virus inoculum and infectivity is generally linear rather than exponential, which shows that infection is a single-hit phenomenon. Combining this observation with a demonstration that the inoculum contains only monomeric virus particles proves that under normal conditions a single virion suffices to infect a cell, although infections that depend on complementation or recombination between viral mutants provide exceptions to this rule. For viruses that have naturally segmented genomes, the quantitative dependence of infectivity on inoculum can supply information on the distribution of genome segments among the individual virus particles.

TYPES OF VIRUS–HOST CELL INTERACTIONS

Viral infections fall into two categories, productive and nonproductive, depending on whether or not viral progeny are produced from the infected cells (Table 2). In reality, cellular permissiveness covers a wide spectrum from total nonproductivity, through cases where very low numbers of virions are released, to fully productive situations in which each cell makes hundreds or thousands of infectious particles. Moreover, in the case of latent infections in animals (e.g. by members of the herpesvirus family), infectious virus can re-emerge several years after a nonproductive interaction in one cell type as a fully productive infection in another (Herpesviruses: general properties and Alphaherpesviruses: herpes simplex and varicella-zoster). As far as the host cell is concerned, its survival is determined not simply by whether the infection is productive, but by the interplay between its genetic program and that of the virus, as described in Cellular responses to viral infection (and see Table 2).

Productive infections

The steps in a typical productive viral infection are as follows:

Table 2 *Classification of virus–cell interactions*

Consequences for the:				
Virus	**Cell**	**Classification**	**Specific example**	**Chapter**
Productive	Lethal	Cytolysis	Poliovirus in human cells	40
	Nonlethal	Transformation	Rous sarcoma virus in chicken cells	58
		Persistence	Lymphocytic choriomeningitis virus in mouse cells	49
Nonproductive	Lethal	Apoptosis	Vaccinia virus in Chinese hamster ovary cells	30
	Non lethal	Transformation	Simian virus 40 in mouse cells	24
		Latency	Herpes simplex virus in neural cells	26

1 adsorption of the virus to specific cellular receptors;
2 its penetration across the plasma membrane and into the cell;
3 virus disassembly resulting in partial or complete uncoating of its genome;
4 transcription, translation, and replication of the viral nucleic acid (the order and subcellular location of these steps reflecting the genome strategy of the family to which the virus belongs);
5 assembly of progeny virus particles;
6 their release from the cell.

CYTOCIDAL INFECTIONS

Whether these events kill the host cell depends on the type of virus. For a typical, nonenveloped, cytocidal virus such as poliovirus (Picornaviruses) which is released when the cell membrane disintegrates, cell death coincides with virus release, although some lytic viruses may be released by an unknown mechanism before the cells burst (Tucker and Compans 1993). In contrast, the release of cytocidal enveloped viruses such as vesicular stomatitis virus (VSV) (see Rhabdoviruses: rabies), which mature by budding, occurs in a more prolonged manner and precedes the death of the host cell.

PRODUCTIVE TRANSFORMATION

This ability of enveloped viruses to bud from cells without necessarily killing them is exploited by the RNA tumor viruses (Prions of humans and animals), which establish productive infections in which the host cells undergo neoplastic transformation rather than death (Viral oncogenicity). By integrating their entire genome into that of the host cell and expressing oncogenes, RNA tumor viruses transform cells to a state of uncontrolled cell division and continuous release of infectious viral progeny.

PERSISTENCE

Persistent infections in cell culture provide situations that are intermediate between these two extremes, and several mechanisms of persistence have been identified (de la Torre and Oldstone 1996). These include carrier cultures, in which for some reason only a specific subpopulation of cells is susceptible to infection; cases in which virus spread is restricted because it can occur only by cell-to-cell contact; and steady states in which infected cells coexist with the virus because they have similar rates of multiplication. In cell culture, temperature-sensitive mutants, DI particles, and interferon have all been implicated in maintaining persistence; the situation in animals is even more complex because it also involves interactions with different cell types, cytokines, and the immune system (Oldstone 1991; Levine et al. 1994; ter Meulen 1994). These aspects are described in detail elsewhere (The immune response to viral infections and The role of cytokines in viral infections).

Nonproductive infections

Counterintuitively, nonproductive infections are often more challenging than productive ones, both intellectually and medically. In cells that carry receptors for a virus, the initial stages of a nonproductive infection are the same as those listed above for productive situations: receptor binding, penetration, and uncoating. In contrast, cells that lack appropriate receptors are usually not susceptible to infection, although some exceptions to this rule are described in Antibody-mediated enhancement. After these initial stages, however, nonproductive infections can have several different causes, courses, and consequences (see Table 2).

APOPTOSIS

In some cases, the early steps of infection trigger the onset of the cell's apoptotic response, resulting in prompt cell death without either virus replication or the production of progeny (Razvi and Welsh 1995; Roulston et al. 1999). Infection of Chinese hamster ovary cells by vaccinia virus provides a good example of this. Several viral genes have been identified whose actions delay the onset of apoptosis (White 1993; Clem and Miller 1994; Ink et al. 1995; Benedict et al. 2002), which suggests that anti-apoptotic mechanisms may be widespread among animal viruses.

NONPRODUCTIVE TRANSFORMATION

Replication of the smaller DNA viruses such as those in the polyoma- and papilloma virus families (

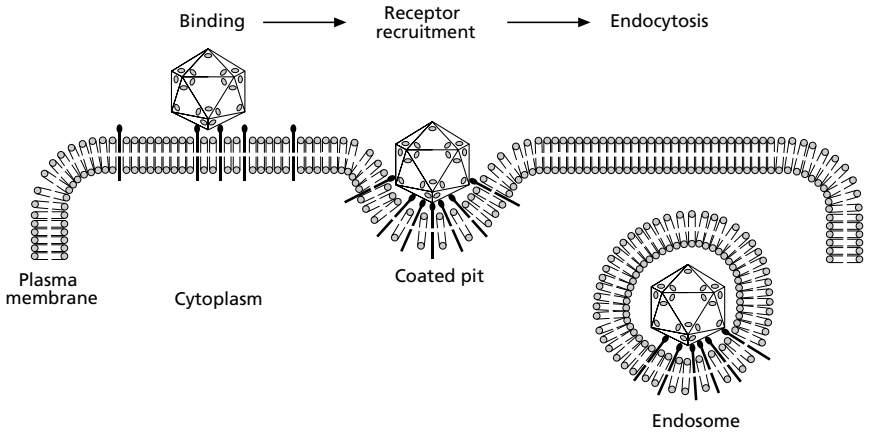

Figure 2 *Receptor-mediated endocytic uptake of a virus particle*

The origin and evolution of viruses) depends on viral oncogenes that coax the infected cell into the S phase of the cell cycle and thus establish conditions that are conducive for DNA synthesis. This is particularly critical for papillomaviruses, which replicate only in terminally differentiated, noncycling cells of the epidermis. In this situation, any block in virus replication that allows the cell to survive the infection can lead to integration of the viral oncogenes and consequent neoplastic transformation of the cell (see Table 2).

LATENCY

Of all the types of virus–host interactions, those that result in latency are the most perplexing (Garcia-Blanco and Cullen 1991). Members of the alphaherpesvirus family (Alphaherpesviruses: herpes simplex and varicella-zoster) typically progress from primary productive interactions, often with epithelial cells, to secondary latent infections, usually in neural cells, from which they can re-emerge periodically throughout the life of the infected individual (Wagner 1994). The viral genome remains intact during latency and is maintained as an episome in the nucleus, with at most a small subset of the viral genes being expressed. This situation is best understood for latent infections established by Epstein–Barr virus in B lymphocytes (Masucci and Ernberg 1994; Farrell 1995; Rowe 1999), which can be examined in cell culture. The general mechanisms by which herpesviruses establish, maintain and re-emerge from latency are subjects of intense investigation, but the scarcity of cell culture systems in which these processes can be duplicated renders them difficult to study.

ADSORPTION OF VIRUSES TO HOST CELLS

The first step in virus–cell interaction is a specific noncovalent binding between attachment sites on the surface of the virion and receptors on the plasma membrane of the host cell. Unlike some bacteriophages, all animal viruses carry multiple copies of their attachment proteins or sites, usually arranged in symmetrical or regular arrays on the surface of the virion (The morphology and structure of viruses). Even the small picornaviruses have 60 receptor-binding sites per virion, and influenza virus has several hundred. What fraction of these sites must be occupied for successful virus binding and uptake is unknown, although it is probably much less than 100 percent and may vary widely among different viruses. Indeed, a current model for the early stages of virus uptake proposes that the initial interaction with one or a few receptor molecules is followed by the recruitment of more receptors that can diffuse laterally in the plasma membrane. This would strengthen the binding, cause invagination of the membrane around the virus particle, and initiate what used to be called 'viropexis,' but is now generally referred to as 'receptor-mediated endocytosis' (Figure 2). See Wimmer (1994) for several reviews on all aspects of this subject.

The receptor–virus interaction can fulfil two distinct functions: positioning the virus particle for uptake by the cell and, at least in some cases, promoting virion disassembly. Virus structures have evolved to serve as metastable intermediates between successive cycles of replication, and some receptors not only bind the incoming virus particle but also act as keys to unlock its structure (see Consequences of receptor binding). Of course, the presence of cell surface components that can perform these functions is not due to natural selection of cells for their ability to be infected, but rather to opportunism by viruses whose potential for faster evolution allows them to take advantage of existing host cell molecules with other functions. A remarkable variety of cell surface molecules have been co-opted by different viruses to enable them to bind and enter cells (Table 3), and these receptors are described further in the section entitled Cellular receptors for viruses.

Table 3 *Some examples of virus receptors*

Virus family	Viruses	Receptors
Coronaviridae	*Murine hepatitis virus*	Carcinoembryonic antigen family member
	Human coronavirus 229E	Aminopeptidase *N*
	Human coronavirus OC43	*N*-acetyl-9-*O*-acetylneuraminic acid
Herpesviridae	Epstein–Barr virus	B lymphocyte C3d complement receptor (CD21)
	Herpes simplex virus	Heparan sulphate moieties of proteoglycans
Orthomyxoviridae	*Influenza A and B viruses*	*N*-acetylneuraminic acid (sialic acid) on glycoproteins and glycolipids
	Influenza C virus	*N*-acetyl-9-*O*-acetylneuraminic acid
Paramyxoviridae	*Sendai virus*	Sialoglycoprotein GP2
	Measles virus	Complement regulator CD46 and SLAM
Picornaviridae	*Poliovirus*	Poliovirus receptor (immunoglobulin superfamily member)
	Rhinoviruses (major group)	Intercellular adhesion molecule 1 (ICAM-1)
	Rhinoviruses (minor group)	LDL receptor
	Foot-and-mouth disease virus	Integrin (RGD-binding protein)
Reoviridae	Reovirus type 3	β-Adrenergic receptor
Retroviridae	*Human immunodeficiency viruses*	T-cell surface glycoprotein CD4, galactosyl ceramide, and chemokine co-receptors
	Ectropic murine leukemia virus	Cationic amino acid transporter
	Gibbon ape leukemia virus	Sodium-dependent phosphate symporter
	Feline leukemia virus	Sodium-dependent phosphate symporter
Rhabdoviridae	Vesicular stomatitis virus	Phosphatidylserine and phosphatidylinositol
Togaviridae	*Sindbis virus*	High-affinity laminin receptor

For references to the original work that described these receptors, see Lentz (1990), Haywood (1994), and Wimmer (1994).

The presence of an appropriate receptor is usually necessary but not always sufficient to allow a particular virus to infect a cell or organism. For example, poliovirus naturally infects only humans and closely related primates because certain cells of these species have a receptor (the poliovirus receptor (PVR)) to which the virus can attach, but the host range of the virus can be extended to mice if they are engineered to express the human gene for PVR (Ren et al. 1990; Nomoto et al. 1994). In contrast, mouse cells expressing CD4, the receptor for human immunodeficiency virus (HIV), can bind the virus but are not infected by it, showing that in some cases there is more to uptake and penetration than simple binding (see Entry accessory factors). Similarly, although receptor distribution is a major determinant of viral tissue tropism, the susceptibility of different tissues in an organism does not simply reflect the tissue distribution of the receptor, because productive infection often requires accessory factors for virus entry (Entry accessory factors), as well as a hospitable intracellular environment for replication. The overall relationship between viral entry mechanisms examined in cell culture and virus spread during a natural infection is a difficult and contentious area of virology.

Viral attachment proteins and sites

In the simplest cases, the task of binding to the host cell has been assigned by evolution to a single species of viral surface protein, such as the hemagglutinin (HA) of influenza virus (Weis et al. 1988) or gp120 of HIV (Weiss 1992), although such attachment proteins usually function as oligomers. In more complex situations such as the picornaviruses, receptor-binding sites on the virion surface are created at the interfaces between two or more viral polypeptides (Colonno 1992; Olson et al. 1993). Larger viruses may show a still higher level of complexity, in which two or more viral surface proteins are involved in a series of progressively tighter interactions with different cell surface components. For example, glycoprotein C of herpes simplex virus (HSV) binds the abundant heparan sulfate moieties of cell surface proteoglycans whereas glycoprotein D binds to mannose-6-phosphate receptors (Spear 1993; Brunetti et al. 1994). Strictly speaking, such downstream components of a multistep entry pathway are best considered 'entry accessory factors' (Entry accessory factors) rather than viral receptors. Nevertheless, the idea of a rapid, relatively low-affinity interaction being followed by a slower, tighter binding, either to a secondary component or to newly recruited primary receptors, is reminiscent of the mechanism by which circulating leukocytes adhere to vascular endothelial cells, by rolling across weak receptors (selectins) before binding irreversibly to integrins (Lawrence and Springer 1991). The speed of the initial binding in three dimensions that is required to capture a passing particle is combined with a slower, two-dimensional search for the interactions that give the overall process its necessary high affinity.

GLYCOPROTEIN SPIKES

Many enveloped viruses display their attachment proteins as surface spikes that project perpendicularly from the viral envelope. These spikes are visible in electron micrographs and consist of oligomers of virus-specified integral membrane glycoproteins. In some, perhaps many, cases, the arrangement of the surface spikes reflects the symmetry of the nucleocapsid underlying the viral envelope (Paredes et al. 1993; Cheng et al. 1995). The three-dimensional structure of one viral attachment glycoprotein, the influenza virus HA, was determined at near-atomic resolution in 1981 and has been influential in shaping ideas of the structure and function of such proteins (Figure 3) (Wiley and Skehel 1987). It consists of a bundle of three identical monomers extending 135 Å from the surface of the viral envelope, arranged around a symmetry axis that is parallel to its length. Each monomer contains two disulfide-linked polypeptides, HA1 and HA2, produced by cleavage of the 550 amino acid primary translation product. The globular top of the trimeric bundle has three concave binding sites for sialic acid, the cellular receptor for influenza virus, as well as several antigenic sites that elicit and react with antibodies that neutralize the infectivity of the virus. At its bottom, HA2 anchors the protein by passing through the viral membrane, although the protein whose structure was determined was first made soluble by removal of the transmembrane domain.

A very different picture is presented by glycoprotein E of the flavivirus tick-borne encephalitis virus (TBEV) (Flaviviruses) (Rey et al. 1995). Like influenza virus HA, this is a type I integral membrane protein that is anchored in the viral envelope by its C terminus. However, rather than forming a protruding spike, the TBEV glycoprotein forms an elongated homodimer that lies flat on the surface of the viral envelope with a symmetry axis that is perpendicular to its length (Figure 4). The receptor-binding domain has an immunoglobulin-like folded structure that presents the binding site on the surface of the virion.

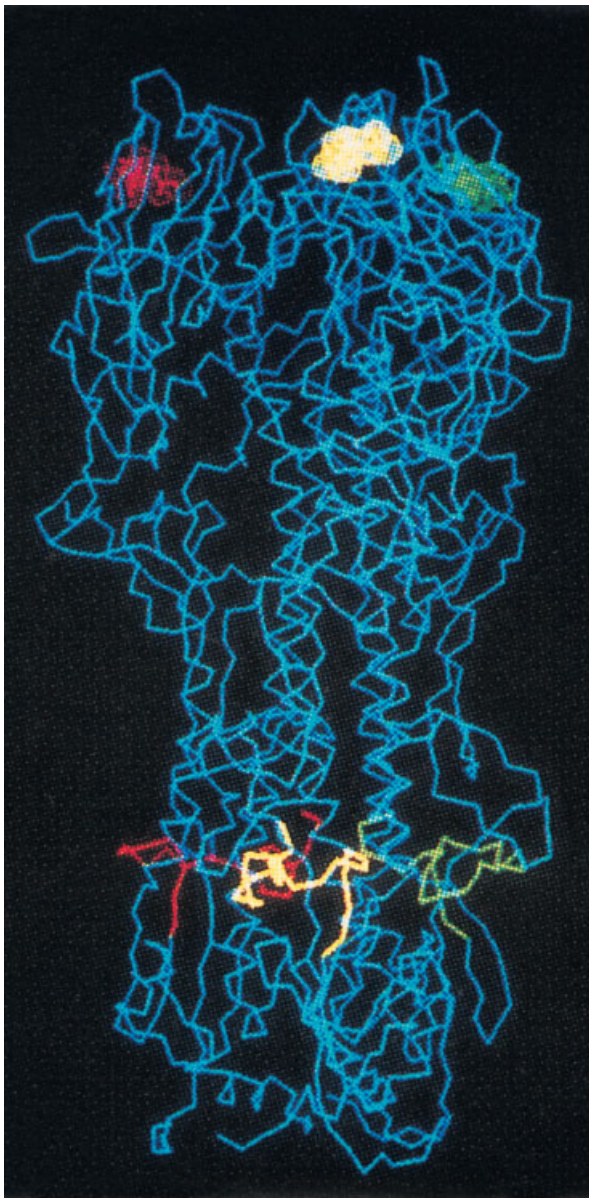

Figure 3 *The influenza virus hemagglutinin (HA). The α-carbon backbone of the HA trimer is displayed in blue with the three fusion peptides, located in the stem of the molecule, highlighted in red, yellow, and green. The molecular surfaces of three sialic acid residues are shown (red, yellow, green) in the receptor-binding sites of the globular head domains. The three monomers would be anchored in the viral membrane at the bottom (from White (1992)).*

CAPSID ATTACHMENT SITES

In nonenveloped viruses, the receptor-binding sites are built into the architecture of the particles and are thus displayed in a symmetry determined by that of the capsid. A technical consequence of this is that individual virus particles with receptors bound at each site can be visualized using cryo-electron microscopy and image reconstruction methods to achieve a resolution of about 25 Å. Figure 5a shows a rhinovirus particle decorated with its receptor, intercellular adhesion molecule 1 (ICAM-1) (Olson et al. 1993; Rossmann et al. 1994). Sixty receptor molecules are bound to each virion, arranged in groups of five around each of the 12 axes of five-fold symmetry in the icosahedral particle. The receptors thus occupy chemically identical binding sites which, in this and some other picornaviruses, lie in a trenchlike depression called the canyon that encircles each fivefold axis. A higher resolution image that shows how a single receptor molecule fits into the canyon has been generated by combining the crystal structure of the rhinovirus particle with the predicted structure of ICAM-1 (Figure 5b). It shows that the binding site is created and protected by the juxtaposition of the capsid proteins VP1, VP2, and VP3, parts of which form the

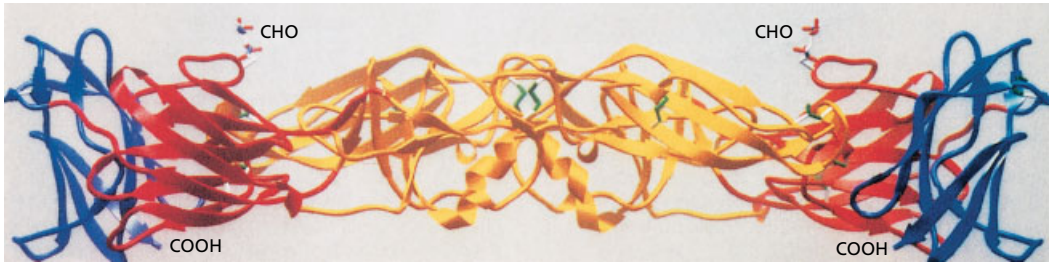

Figure 4 *Three-dimensional structure of glycoprotein E of tick-borne encephalitis virus. The dimer is viewed from the side, as it would lie on the surface of the viral membrane (from Rey et al. (1995)).*

walls of the canyon. Ultimately, it may prove possible to co-crystallize a virus–receptor protein complex and directly examine the molecular contacts at atomic resolution.

The specific contacts by which a viral attachment protein binds to its receptor, and hence the amino acid residues involved, are largely fixed by the requirement that they must create a binding surface with steric, electronic, and hydropathic complementarity for the receptor. It is important, therefore, that these residues avoid immune surveillance, because an antibody directed specifically and exclusively against the receptor-binding site would create a barrier to infection that the virus could not circumvent by mutation. This problem has been solved in different ways by different viruses (Chapman and Rossmann 1993). For example, location of the receptor-binding sites in the canyon of rhino- and polioviruses protects them from reach by antibodies which are much bulkier than the receptors (Rossmann 1989). In contrast, the receptor-binding site in foot-and-mouth disease virus is in a surface loop that may avoid antigenic scrutiny by being structurally mobile. Finally, there may be cases where the receptor-binding site mimics the structure of the natural ligand so precisely that the immune system does not recognize it as foreign.

Cellular receptors for viruses

A wide variety of cell surface molecules can be used as receptors by different viruses (Table 3). In some cases, a ubiquitous carbohydrate modification is recognized, such as sialic acid or heparan sulfate on a glycoprotein or glycolipid, whereas in other cases it is a specific protein that may be present on the surface of only certain cell types (such as CD4 on T lymphocytes), which thus directs and restricts the tropism of the virus (Lentz 1990; Wimmer 1994). The receptors for poliovirus (PVR), rhinovirus (ICAM-1), and HIV (CD4), among others, belong to the immunoglobulin superfamily of proteins, and contain three to five repeats of the 'immunoglobulin fold,' a structural domain that is characteristic of the immunoglobulins (White and Littman 1989). The three-dimensional structure of two such domains in a fragment of CD4 that includes the HIV-binding site is illustrated in Figure 6 (Harrison et al. 1992; Signoret et al. 1993). Since members of this protein superfamily have been naturally selected for cell surface expression and the ability to bind protein ligands with high specificity, it is easy to understand why they should have been commandeered as viral receptors. Indeed, two very different viruses, HIV and human herpesvirus 7, both use CD4 as their receptor, and the malaria parasite also uses ICAM-1 for cell attachment. The versatility of the immunoglobulin fold as a protein-binding domain is further illustrated by the fact that it sometimes occurs in the other partner in the interaction, the viral attachment protein (Rey et al. 1995).

Just as dissimilar viruses can share the same receptor, so can closely related viruses use different receptors. Human rhinoviruses, of which there are over 100 serotypes, divide into a major group that use ICAM-1 and a minor group that use the low density lipoprotein (LDL) receptor. In this case, the use of different receptors does not seem to influence the outcome of infection, but there are examples where the use of alternative receptors by a single virus can have profound consequences. The ability of HIV to use galactosyl ceramide instead of CD4 to enter cells may be responsible for its infection of the brain and the resulting acquired immunodeficiency syndrome (AIDS)-related dementia (Weiss 1992).

Infection of animals is usually initiated in epithelial cells, which are naturally polarized and express different sets of proteins on their apical and basolateral surfaces (Tucker and Compans 1993; Eaton and Simons 1995). Interaction of viruses with polarized cells whose receptors are distributed asymmetrically can occur only at the cell surface that expresses the receptor, because the intercellular tight junctions prevent access of the virus to the other face of the polarized cell layer. For example, SV40 infection can occur only from the apical surface. Since virus release also often occurs in a polarized manner from epithelial cells, asymmetric receptor distribution can greatly influence the course of infection and the pathway of virus spread in an infected animal (Tucker et al. 1994).

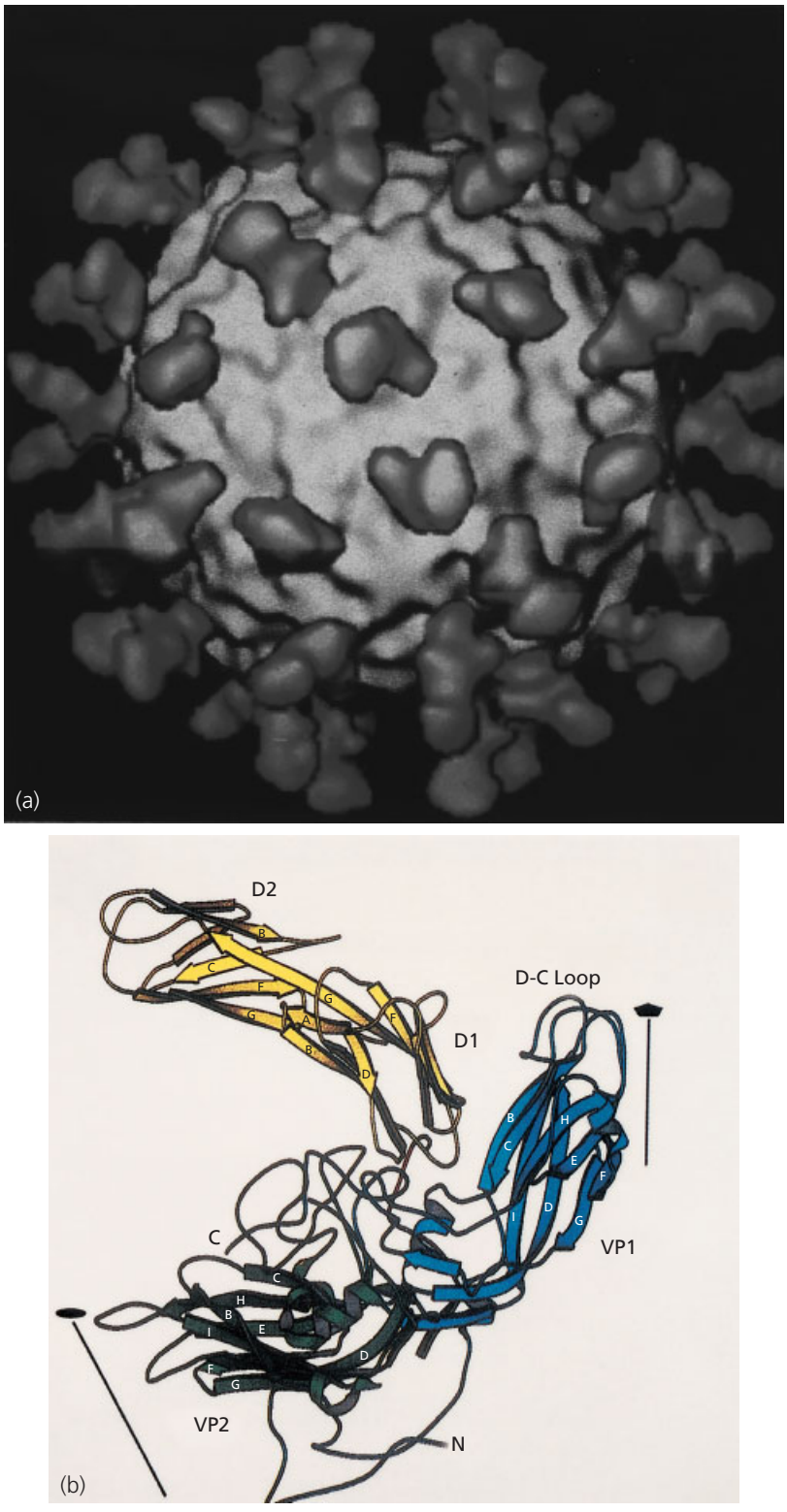

Figure 5 *Interaction of rhinovirus with its receptor ICAM-1.* **(a)** *Cryo-EM reconstruction of a virus particle complexed with 60 receptor molecules.* **(b)** *High resolution image of a single receptor-binding site interaction (from Olson et al. (1993)).*

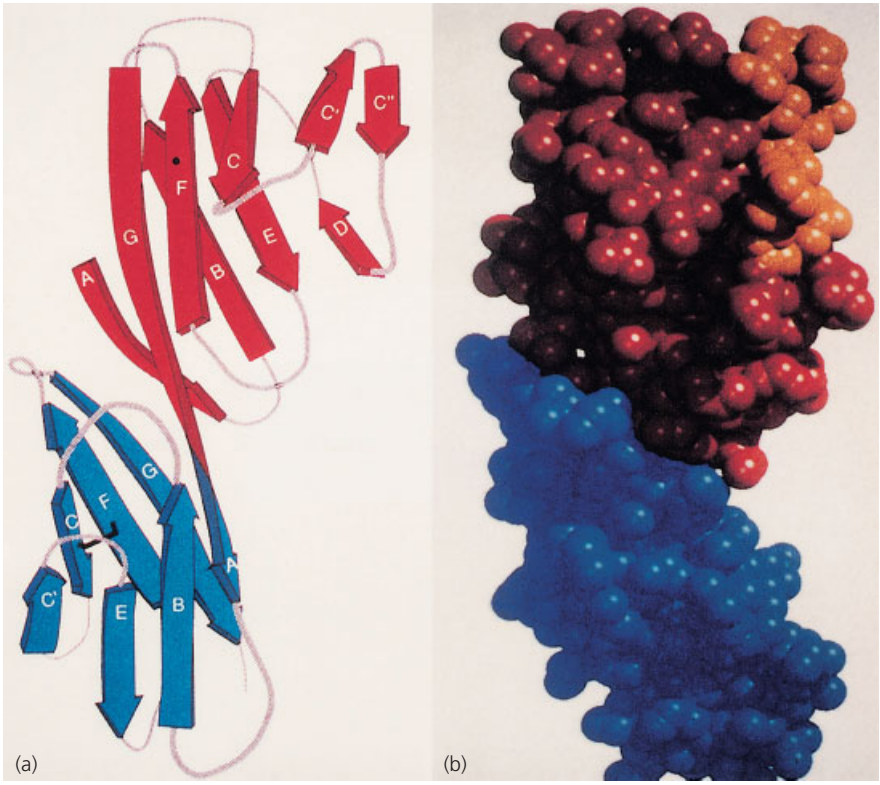

Figure 6 *Backbone* **(a)** *and solid* **(b)** *representations of the three-dimensional structure of CD4 (D1D2 domains). The D1 domain is in red, the D2 domain is in blue, and the region of D1 implicated in the binding HIV gp120 is yellow (from Wang et al. (1990)).*

Antibody-mediated enhancement

Although the presence of a cognate receptor is usually a prerequisite for infection, some virus–cell interactions can occur through intermediate molecules for which both the virus and the cell have affinity. Paradoxically, virus-specific antibodies can play this role and actually promote virus uptake because some cells express a receptor for the Fc portion of the antibody molecule (Porterfield 1986; Halstead 1994; Tirado and Yoon 2003). In this way, the immune system can collaborate in the spread of infection by circumventing the requirement for a virus-specific receptor (Mason et al. 1994). The pathogenesis of infections by both dengue virus and HIV can be exacerbated by antibody-mediated enhancement of infectivity, but the phenomenon is probably irrelevant for viruses such as polio, in which interaction with the cognate receptor mediates an essential structural change in the virion (Consequences of receptor binding).

Consequences of receptor binding

As mentioned at the start of this section, receptor binding initiates virus entry into the cell and, at least in some cases, destabilizes the structure of the virion so that it begins to disassemble. Before binding to the receptor, poliovirus is stable at pH 2 and can therefore enter the body through the strongly acidic environment of the stomach. However, reaction with PVR causes a major conformational change in the capsid that involves swelling, loss of a small internal protein (VP4), and extrusion of the hydrophobic N terminus of VP1 in preparation for its interaction with membranes (Flore et al. 1990; Racaniello 1992; Gomez Yafal et al. 1993). However, only a few of the poliovirus particles that interact with the receptor are internalized. Substantial numbers of these antigenically altered particles, which can no longer bind the receptor and have therefore lost their infectivity, dissociate from cells during poliovirus infection and may play a role in decoying the immune response. In the case of some rhinoviruses, reaction with the receptor is sufficient to release the viral RNA and produce empty capsids, whereas in other cases this requires exposure to mildly acidic conditions such as those encountered during passage through the endocytic pathway into the cytoplasm (Giranda et al. 1992; Greve and Rossmann 1994) (see Penetration and uncoating). These observations suggest that receptor binding and acid pH may co-operate to initiate picornavirus disassembly. In the case of HIV, binding to the receptor CD4 causes the release of the viral attachment protein gp120 from the particle, exposing a hydrophobic fusion peptide whose interaction with membranes

mediates the next step in viral entry (see Penetration and uncoating).

When the natural function of the receptor is transmembrane signaling, virus binding can trigger a physiological response even without internalization. It is thought that Epstein–Barr virus can activate B lymphocytes by binding to the C3d complement receptor (Hutt-Fletcher 1987). Moreover, this type of effect can be widely disseminated because infected cells often secrete soluble forms of the viral attachment protein. Some of the complex effects of HIV on T-lymphocyte function may be mediated directly by the phosphorylation of CD4 and the consequent transmembrane signaling that is caused by the binding of gp120.

Entry accessory factors

There are many instances in which the ability of a virus to bind to the surface of a cell is sufficient for uptake, but there are others where something else is needed. For example, rodent cells that express human CD4 fail to become infected with HIV unless they also express the human membrane protein fusin, which is required for penetration of the virus–receptor complex into the cell (Feng et al. 1996). In the case of adenovirus, initial receptor binding by the fiber protein must be followed by an interaction between the penton base protein of the virus and cellular integrins in order for internalization to occur (Wickham et al. 1993). The distinction between secondary receptors and entry accessory factors, and the roles that each plays in virus uptake are unclear and will probably remain so until more details of this multistep process are known.

Release from receptors

For viral progeny to spread most effectively, some mechanism is needed to ensure release from receptors expressed by the primary infected cells. In general, a combination of virus-mediated receptor down-regulation and the inhibition of protein synthesis contribute to this requirement, but some viruses that bind to sialic acid also carry specific 'receptor-destroying enzymes' that remove terminal sialic acid residues from oligosaccharides. Along with the HA protein described in the section entitled Viral attachment proteins and sites, particles of influenza A virus and influenza B virus have a second surface glycoprotein (NA) that has neuraminidase (sialidase) activity. Although the presence of this enzyme may allow infecting virions to 'browse' the cell surface during entry, its only essential roles in infection are to prevent infected cells from trapping progeny virus particles and to eliminate the self-aggregation that would otherwise result from HA binding to its own oligosaccharides (Liu et al. 1995). Particles of influenza C virus and some coronaviruses, whose attachment proteins bind to *N*-acetyl-9-*O*-acetylneuraminic acid, carry an esterase that likewise mediates destruction of receptors. In the latter case, and in some paramyxoviruses, the receptor-binding and receptor-destroying activities are both present in the same molecule (HE and HN, respectively), raising interesting questions about their interrelationship and regulation.

PENETRATION AND UNCOATING

Virus particles assemble in cells as very stable structures that are often able to withstand harsh extracellular environments, but when re-exposed to intracellular conditions they spontaneously disassemble. In some cases, binding to the receptor contributes to this dramatic change in virion stability (Consequences of receptor binding), but an important role is played by the paths that viruses follow as they enter cells. Depending on the type of virus, its genome must cross one, two, or three lipid bilayers between binding to the receptor at the plasma membrane and reaching its intracellular destination. During infection, all viral genomes must penetrate the plasma membrane; those of enveloped viruses must also cross the viral membrane, and those that replicate in the nucleus also need to traverse the nuclear membrane. Virion proteins play active roles in these transmembrane passages, and have been sculpted by natural selection to transfer the viral genome to the appropriate cellular compartment. Some stages of virion disassembly and genome uncoating invariably accompany membrane penetration (Hoekstra and Kok 1989; Marsh and Helenius 1989; Carrasco 1994; Greber et al. 1994; Lanzrein et al. 1994; Poranen et al. 2002).

Two pathways of virus entry

Although some viral receptors naturally transport small molecules (see Table 3) (Dautry-Varsat and Lodish 1984), it seems that not even the smallest virus is directly carried across the plasma membrane by its receptor. Instead, enveloped viruses transfer their nucleocapsids across membranes by fusing with them, a reaction that achieves simultaneous envelope removal and cell entry. Fusion is mediated by specific virion surface proteins that can fuse lipid bilayers (Viral fusion proteins), and some nonenveloped viruses probably achieve the same result by inserting capsid proteins into a cellular membrane (Uncoating of nonenveloped viruses). For many viruses, both with and without envelopes, activation of the ability to fuse with membranes requires exposure to a mildly acidic environment (pH 5–6); such viruses enter cells by receptor-mediated endocytosis (see Figures 2 and 7) into endosomes whose contents then become acidified by an ATP-driven proton pump. The result is fusion of the virus with the membrane of the endosome and release of the viral

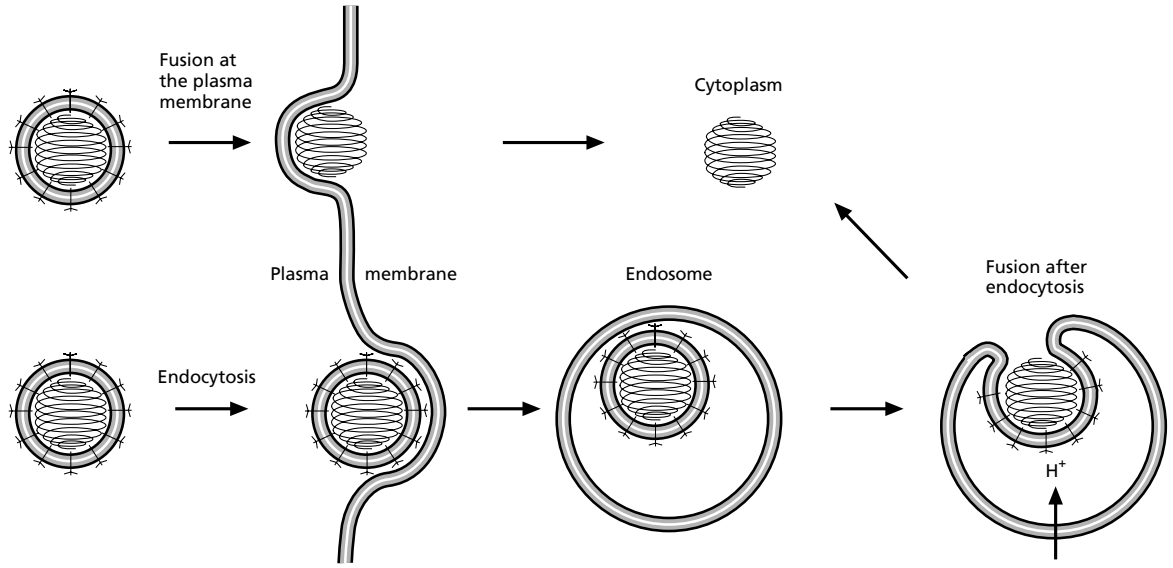

Figure 7 *Two pathways of enveloped virus entry: fusion at the plasma membrane or fusion after endocytosis.*

genome into the cytoplasm. Other viruses can fuse without being exposed to low pH, and can therefore enter the cytoplasm directly by fusion with the plasma membrane (Figure 7).

Infection by viruses that enter through endosomes can be inhibited by lysosomotropic agents such as ammonium chloride or chloroquine which raise the endosomal pH, whereas viruses that enter at the plasma membrane are insensitive to these reagents. The pathway of entry that leads to a productive infection is thus determined largely by the properties of the viral protein responsible for membrane fusion: whether exposure to low pH is required to activate it. In general, viruses in the herpes-, polyoma-, paramyxo-, retro-, and rotavirus families can enter cells by fusion with the plasma membrane in a pH-independent manner, whereas those in the adeno-, alpha-, bunya-, flavi-, orthomyxo-, picorna-, reo-, and rhabdovirus families need to pass through an acidic endosome. However, there are several individual exceptions to these family rules.

The ability of many viruses to fuse with cellular membranes is responsible for a common cytopathic effect of viral infection: the formation of multinucleated syncytia by cell-to-cell fusion. This is due to the abundant expression of the viral proteins that cause membrane fusion (see Viral fusion proteins) on the surface of infected cells, where they can interact with both infected and uninfected neighbors ('fusion from within'). Cell fusion creates a pathway for the local spread of an infection by which a virus can partly elude the immune system. For respiratory syncytial virus, a member of the paramyxovirus family, the extensive syncytium formation that accompanies infection both in the respiratory tract and in cell culture was used in naming the virus and in diagnosing infections. Another paramyxovirus, Sendai virus, has been widely used by cell biologists as a laboratory reagent to induce the formation of heterokaryons by 'fusion from without.'

Viral fusion proteins

Some viruses (e.g. the paramyxoviruses) have separate envelope glycoproteins for receptor binding and membrane fusion; others (e.g. influenza and the retroviruses) combine the two functions into a single viral protein. Despite their variety of structures, however, the fusion proteins of enveloped viruses have features in common. They all contain two hydrophobic regions: a transmembrane domain by which they are anchored in the viral envelope, and a fusion peptide of 16–26 nonpolar amino acids in the ectodomain of the protein that interacts with the target membrane (Schlesinger and Schlesinger 1987; White 1990, 1992; Doms et al. 1993; Smith and Helenius 2004; Earp et al. 2005). Formation of a bridge between the viral and cellular membranes by means of divalent anchoring is thought to mediate membrane apposition (Figure 8), an essential step in fusion (Bentz 1993).

The hydrophobic nature of the fusion peptide must remain masked, both during translation, to allow it to pass through the membrane of the endoplasmic reticulum (ER), and during virus assembly and release, to prevent premature activation of its fusogenic potential. For these reasons, fusion peptide sequences are initially buried, both in the primary sequences and in the oligomeric structure of fusion proteins. A maturational cleavage event, which sometimes occurs after virus release, places the fusion peptide sequence at a newly created N terminus, as in the influenza HA structure where it forms the N terminus of HA2 (see Figure 3) or in the HIV gp120/gp41 structure in which it is the N terminus

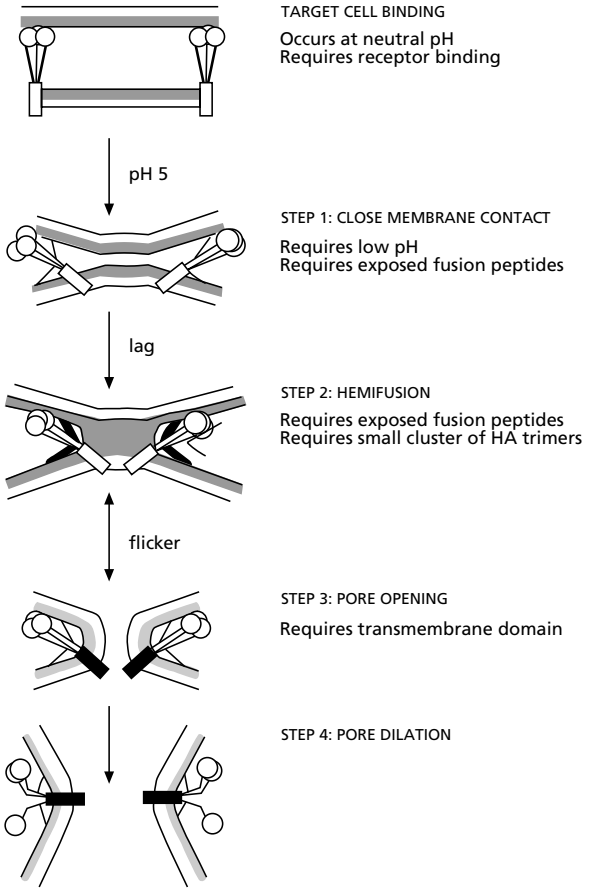

Figure 8 *Steps in HA-mediated membrane fusion (from White (1994)).*

of gp41. However, the two cleavage products remain associated with one another, sometimes by disulfide bonds, in an oligomeric structure that continues to mask the fusion peptide. In the case of influenza HA, rearrangement of the subunits of the trimer is necessary to expose their fusion peptides to the target membrane, and it is likely that a major conformational change of this sort is a common feature of the activation of fusion proteins (see Mechanisms of membrane fusion).

Different viruses prevent premature activation of their fusion proteins in different ways. For Sendai virus, the required cleavage of the fusion protein precursor is mediated by a protease at the membrane of the target cell. For HIV, receptor binding dissociates gp120 and thereby exposes the hydrophobic N terminus of gp41. For Semliki Forest virus, the fusion protein monomer is maintained as an inactive heterodimer with the viral attachment protein until receptor binding and exposure to low pH release it to form the homotrimer that is fusogenic (Kielian 1995). For some paramyxoviruses, fusion requires both the attachment protein HN and the fusion protein F, because binding of HN to its receptor somehow activates the fusion protein, its partner in the viral envelope (Lamb 1993). For influenza A virus, where exposure to low pH triggers the conformational change that activates HA, the protein is chaperoned during its synthesis and maturation by the viral M2 protein, which acts as a transmembrane proton channel to regulate the pH in the cellular compartments through which the maturing HA must pass (Pinto et al. 1992). Another role for the influenza virus M2 protein as an ion channel is described under Transit to the nucleus.

Mechanisms of membrane fusion

The three-dimensional structure of the neutral form of HA (see Figure 3) raised some perplexing questions about how it mediated membrane fusion. Because the receptor-binding site is 135 Å from the transmembrane domain and about 100 Å from the fusion peptide, it was not clear how, after receptor binding, the protein could closely juxtapose the viral envelope in which it was anchored with the target membrane for insertion of the fusion peptide. Some of these problems were solved when it was discovered that a region of HA2 underwent a massive conformational change at the pH of membrane fusion, resulting in a 100 Å relocation of the

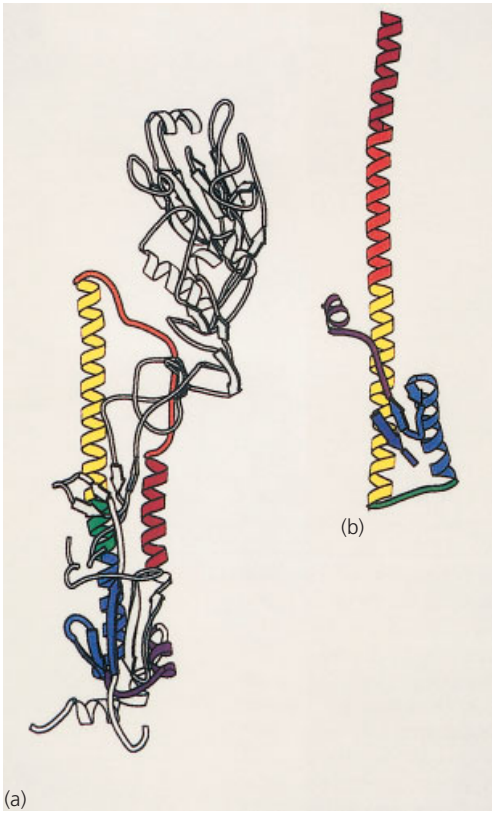

Figure 9 *The conformational change induced by low pH in* influenza virus *HA.* **(a)** *The neutral pH form of the HA1,2 monomer is shown in the same orientation as in the HA trimer shown in Figure 3.* **(b)** *The low pH form of the HA2 monomer. Corresponding regions are colored identically (from Bullough et al. (1994)).*

fusion peptide to one apex of the molecule (Figure 9) (Carr and Kim 1993; Bullough et al. 1994).

In this new location, membrane insertion of the N terminus of HA2 is easy to envisage, although it is not clear whether this and other fusion peptides are inserted into their target membranes vertically, obliquely, or horizontally, as random coils or perhaps as amphipathic α-helices. Unfortunately, the new HA2 structure does not reveal the relative positions of the fusion peptide and the anchor domain, so the way that these regions and the receptor-binding site collaborate to mediate membrane fusion is still unclear (Carr and Kim 1994; Stegman 1994; Hughson 1995; Skehel and Wiley 2000). When last seen in the low pH structure, the C terminus of HA2 was heading in the direction of the fusion peptide (Figure 9), raising the possibility that the acid-induced conformational change brings the anchor and fusion peptides (and hence perhaps the viral and cellular membranes) into close proximity with one another. However, there is much yet to be discovered about the mechanism of membrane fusion and several models have been proposed that involve hemifused intermediates (Figure 8), inverted micelles, proteinaceous pores, or intermembrane stalks (Stegman 1994). It is interesting that an engineered mutant of HA that is anchored only in the outer membrane leaflet rather than traversing the lipid bilayer mediates only hemifusion, showing that the anchor domain plays different roles in mixing the lipids of the two leaflets (Kemble et al. 1994).

Uncoating of nonenveloped viruses

Nonenveloped viruses are also destabilized during cell entry, by interactions with their receptors (see Consequences of receptor binding), and in some cases by exposure to endosomal pH, reducing conditions or low intracellular calcium ion concentrations. Although entry of nonenveloped viruses into the cytosol cannot be achieved by membrane fusion, it is possible that the hydrophobic N terminus of VP1 of some picornaviruses may play the role of a fusion peptide. It is buried in the capsid structure but becomes exposed when the virus binds to its receptor, and may perhaps be inserted into the cell membrane as a pentamer derived from one of the 12 fivefold axes of the icosahedral virion. The internal protein VP4, which exits the virus particle after receptor binding, is myristylated at its N terminus, and could thereby also interact with membranes. Such a complex might form a hydrophilic transmembrane channel through which the viral RNA could pass into the cytosol (Figure 10) (Mosser et al. 1994; Johnson 1996). Despite the external protein symmetry of icosahedral virions and nucleocapsids, it is possible that the asymmetry of the nucleic acid inside confers different properties on some of the capsomers and that these differences are exploited during virion disassembly. Whether the RNA enters the cytosol in a polar manner as depicted in Figure 10 remains to be determined. However, the capsid proteins of infecting alphavirus particles are transferred to 28S ribosomal RNA (Singh and Helenius 1992), thus releasing the viral RNA genome for translation. The disassembly of tobacco mosaic virus and cowpea chlorotic mottle virus in plants also occurs cotranslationally, by ribosomes displacing capsid proteins from the viral RNA during the initial round of protein synthesis (Verduin 1992), and a similar possibility exists for extraction of the RNA from positive-strand RNA animal viruses.

For adeno- and reoviruses, whose genomes do not interact directly with ribosomes, partially disassembled subviral particles exit the endosome by lysis or permeabilization of the vesicle membrane. As in the examples described above, it is the acidic environment, sometimes in conjunction with proteolytic cleavage of viral structural proteins, that activates the virus parti-

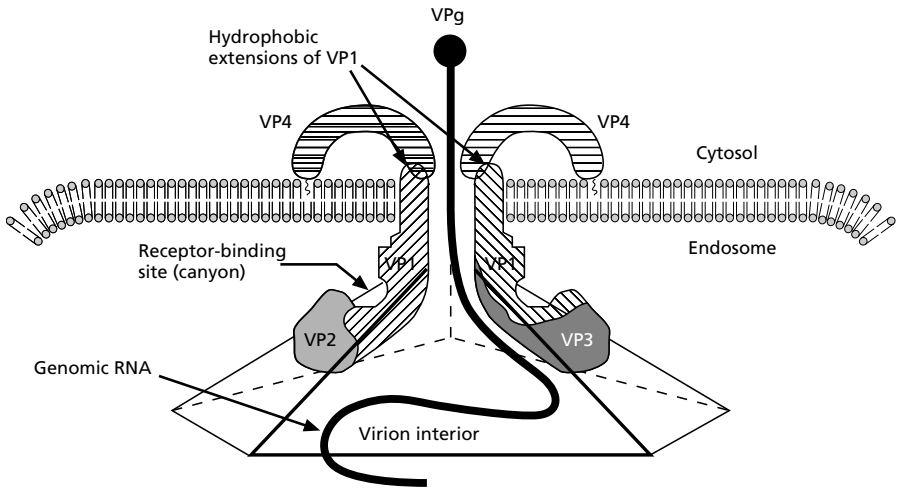

Figure 10 *Possible model for the exit of picornavirus RNA into the cytoplasm (adapted from Mosser et al. (1994)).*

Table 4 *Initial biosynthetic events in animal virus infections*

Initial biosynthetic event	Virus families	Subcellular location	Origin of enzymes
DNA-dependent DNA synthesis	*Parvo*	Nucleus	Cell
	Hepadna	Nucleus	Virion
RNA-dependent DNA synthesis	*Retro*	Cytoplasm	Virion
DNA-dependent RNA synthesis	*Polyoma*	Nucleus	Cell
	Papilloma	Nucleus	Cell
	Adeno	Nucleus	Cell
	Herpes	Nucleus	Cell
	Baculo	Nucleus	Cell
	Irido	Nucleus	Cell
	Pox	Cytoplasm	Virion
RNA-dependent RNA synthesis	*Orthomyxo*	Nucleus	Virion
	Delta agent	Nucleus	Cell (RNA pol II)
	Paramyxo	Cytoplasm	Virion
	Rhabdo	Cytoplasm	Virion
	Filo	Cytoplasm	Virion
	Bunya	Cytoplasm	Virion
	Reo	Cytoplasm	Virion
	Arena	Cytoplasm	Virion
Protein synthesis	*Picorna*	Cytoplasm	Cell (ribosomes)
	Toga	Cytoplasm	Cell (ribosomes)
	Flavi	Cytoplasm	Cell (ribosomes)
	Corona	Cytoplasm	Cell (ribosomes)
	Calici	Cytoplasm	Cell (ribosomes)
	Noda	Cytoplasm	Cell (ribosomes)

cles to disrupt the endosomal membrane (Dryden et al. 1993).

Transit to the nucleus

Viral genomes that replicate in the nucleus need to travel there from where they entered the cytosol. For a herpesvirus particle infecting a neuron, this may be a journey of several centimeters during which the viral nucleocapsid is transported by axonal flow (Herpesviruses: general properties). In less extreme cases, transport mechanisms that are thought to involve interactions with the cytoskeleton deliver the viral genome or nucleocapsid to the cytoplasmic face of pores in the nuclear membrane. For adeno- and herpesviruses, whose nucleocapsids are too large to enter the nucleus intact, a final stage of uncoating occurs at the nuclear pores to release the viral DNA into the nucleus. In contrast, most retroviruses require the natural breakdown of the nuclear membrane that occurs during mitosis, and they are therefore restricted to infection of dividing cells.

Influenza virus replicates in the nucleus but assembles its progeny in the cytoplasm and therefore faces unusual problems of compartmentation. The binding of viral nucleocapsids to the matrix protein (M1), which is responsible for their transport out of the nucleus in preparation for virus assembly, must be disrupted at the start of infection in order to permit the incoming viral genome to migrate into the nucleus. This is another function of the viral M2 protein; it creates acid-activated ion channels in the viral envelope which open at the low pH of the endosome to admit ions into the interior of the virion, thereby releasing the viral nucleocapsids from their association with the matrix protein (Hay 1992; Helenius 1992; Marsh 1992). The anti-influenza drug amantadine inhibits this process by blocking the ion channel activity of the M2 protein.

Poxvirus uncoating requires viral gene expression

In the uncoating mechanisms described above, components of the infecting virions played active and essential roles, but the expression of new viral genes was not necessary. Members of the poxvirus family, however, uncoat by a two-stage process in which the second stage depends on viral gene expression (Poxvirus replication). Vaccinia virus, for example, is taken up by receptor-mediated endocytosis (Hugin and Hauser 1994; Chang et al. 1995). On release of the nucleocapsid core into the cytosol, a viral DNA-dependent RNA polymerase that is part of the core begins to transcribe several viral genes, among them one for a protease that triggers the second stage of uncoating that releases the

viral DNA into the cytosol in preparation for genome replication.

EFFECTS ON HOST-CELL METABOLISM

An incoming virus particle faces a formidable challenge in subverting the metabolism of the host cell to its own ends. Depending on the virus, a single copy of its genetic program dominates one that is 10^4–10^6 times larger and already being expressed. To meet this challenge, viruses have evolved a wide variety of ways to manipulate the intracellular environment so that it can better support their replication. These include changes in intermediary metabolism, particularly of deoxyribonucleotides, as well as effects on the synthesis, processing, transport, and turnover of cellular macromolecules. Viruses achieve dominance by combining mechanisms that enhance the expression of viral genes with those that interfere with the host. Many of the latter perturbations have detrimental effects on the infected cell that result in viral pathogenesis and disease (Pathogenesis of viral infections), but it seems that few if any of them evolved with the primary purpose of causing cellular damage. On the contrary, viruses, like all successful parasites, survive not because they kill the host but because they avoid inducing its premature demise.

The nature of the initial biosynthetic reaction – DNA, RNA, or protein synthesis – and where it occurs in the infected cell depend on the genome strategy of the virus (Table 4). Most DNA genomes replicate in the nucleus, whereas most RNA genomes replicate in the cytoplasm, although the pox- and orthomyxoviruses are notable exceptions to this generalization. The retro- and hepadnaviruses are difficult to classify in this regard because their genomes alternate between RNA and DNA during each replication cycle. Whatever the initial biosynthetic reaction, a mechanism that most viruses use to help them attain dominance over the cell is to differentiate their infectious cycles into an early phase, when the viral genomes are present in low copy-number and direct the synthesis of catalytic amounts of viral replication proteins, and a late, postreplicative phase, when the abundant progeny genomes direct the synthesis of stoichiometric amounts of structural proteins.

DNA metabolism

Host-cell DNA replication occurs only during the S phase of the cell cycle, in a tightly regulated manner that is co-ordinated with the cell cycle as a whole. It requires pools of deoxyribonucleotides, replication enzymes, continuous protein synthesis, and access to appropriate anchor points on the nuclear matrix (Challberg and Kelly 1989; Echols 1990; Challberg 1991; Stillman et al. 1992). Viruses perturb this process to different degrees, depending on how much they rely on it for their own replication. Retroviruses, for example, which integrate DNA copies of their genomes into host chromosomes, ensure their replication by the expression of oncogenes that drive the cell relentlessly through the cell cycle, but do not otherwise affect cellular DNA replication (Prions of humans and animals). At the other end of the spectrum, the elaborate genomes of herpes- and poxviruses replicate with almost complete autonomy, to the extent that infection by HSV blocks cells from progressing to S phase, and infection by vaccinia virus even causes cellular DNA degradation (Kelly et al. 1988).

DEOXYRIBONUCLEOTIDE BIOSYNTHESIS

Most cells in the body continue to make RNA and protein throughout their lives, but many terminally differentiated cells stop dividing and therefore suppress DNA synthesis. For a virus with a DNA genome to be able to replicate in such cells, it must not only circumvent this suppression or provide the necessary enzymes for DNA replication (or both) but also restore the cellular pools of deoxyribonucleoside triphosphates (dNTPs). Even in dividing cells, the metabolic load imposed by viral DNA synthesis can exceed the capacity of the cell and require continuous replenishment of the dNTP pools. This problem is particularly acute for the poxviruses, which replicate their DNA in the cytoplasm (Poxvirus replication).

Two distinct mechanisms have evolved to relieve this constraint: DNA viruses with relatively small genomes, such as the polyoma- and adenoviruses, induce cells to enter S phase, when the cellular genes for the necessary enzymes are naturally expressed. This is the basis for the oncogenic potential of viruses in these families (Jansen-Durr 1996), which is described in Viral oncogenicity. In contrast, larger DNA viruses in the herpes- and poxvirus families have genomes with sufficient capacity to encode viral versions of some of the critical enzymes of nucleotide metabolism. Figure 11 shows the major pathways of dNTP biosynthesis in animal cells, with the enzymes encoded by either herpes- or poxviruses (or both) underlined. It is presumed that the genes for these enzymes were acquired from the host during viral evolution and serve to liberate these viruses from dependence on S phase. However, the genes are usually dispensable for virus replication in cultured cells, which are metabolically active and cycling.

Although these virus-specified enzymes catalyze the same reactions as their cellular counterparts, their substrate specificities and regulatory properties are often somewhat different, which makes them feasible targets for the development of antiviral agents (The emergence and re-emergence of viral diseases). For example, the antiherpetic drugs acyclovir and ganciclovir are analogues of guanosine that can be phosphorylated by the thymidine kinase enzyme of HSV, but not by that

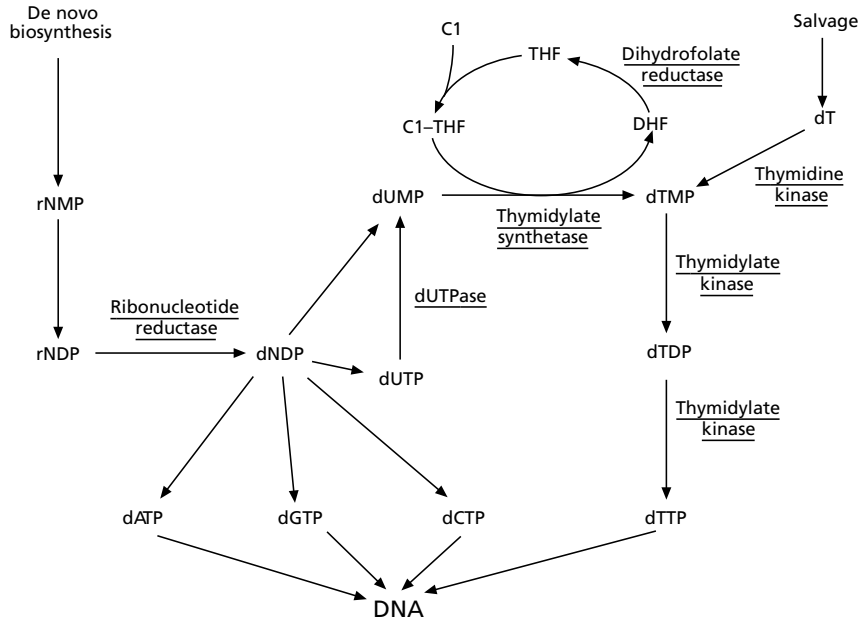

Figure 11 *Pathways of deoxyribonucleotide biosynthesis. Underlined enzymes are encoded by some herpes- or poxviruses.*

of the host. Because the 5′-triphosphates of these drugs act as chain terminators during DNA synthesis, they block DNA replication specifically in HSV-infected cells. However, the propensity of viral genomes for mutation and the facile selection of drug-resistant mutants create problems for the long-term use of antiviral agents that are similar to those encountered with antibiotics. For example, suppression of vaccinia virus replication by hydroxyurea, which inhibits ribonucleotide reductase by a radical scavenging mechanism, readily selects for resistant mutants that contain multiple copies of the gene for one of the subunits of the viral enzyme (Slabaugh et al. 1989). Nevertheless, the virus-specific enzymes of nucleotide metabolism that are encoded by the larger DNA viruses provide versatile genetic tools, as well as possible targets for the development and testing of antiviral agents. The possibility is also being explored of incorporating the HSV thymidine kinase gene into suicide vectors targeted to tumor cells to render them sensitive to acyclovir (Morgan and Anderson 1993).

DNA REPLICATION

As described above, DNA viruses with small genomes stimulate the host cell to provide them with many of the enzymes they need for DNA replication. Polyomaviruses, such as SV40, induce S phase in infected cells by the action of a single viral gene product, large T antigen, which is expressed early in the infectious cycle (Fanning and Knippers 1992). This viral protein binds to the cellular tumor-suppressor protein pRb (Wiman 1993), displacing transcription factors of the E2F family to which it is normally bound (Nevins 1992). These transcription factors switch on several cellular genes involved in cell cycle control and DNA replication, including the genes for DNA polymerase α, the enzyme that replicates the viral genome. T antigen also binds to another tumor-suppressor protein, p53, and relieves the cell cycle block that is normally imposed by this protein (Ludlow 1993; Ludlow and Skuse 1995). Thus, by expressing a single viral protein, SV40 prepares the cell for DNA replication and gains access to the enzymes necessary to catalyze it. During a productive infection, viral DNA replication will ensue, but the properties of large T antigen are those of an oncogene, and expressed on its own it will transform cells and cause tumors (whence it derives the name of Tumor antigen). In the related papillomaviruses, the inactivation of pRb and p53 is delegated to two viral proteins, E7 and E6, respectively (Vousden 1993; Farthing and Vousden 1994).

With DNA genomes three to six times larger than those of the polyomaviruses, adenoviruses (
Adenoviruses) have sufficient genetic capacity to encode three of the proteins necessary for DNA replication: DNA polymerase, a genome-linked terminal protein involved in the initiation of viral DNA synthesis, and a single-stranded DNA binding protein. However, they still need to push the cell into S phase to establish supportive conditions. This push is mediated by two early viral gene products, E1A and E1B, which together perform the functions described above for SV40 T antigen: binding to the tumor-suppressor proteins pRb and p53, respectively (Moran 1993).

In addition to the enzymes of nucleotide metabolism (Figure 11), HSV encodes seven proteins that are directly involved in DNA replication (

Alphaherpesviruses). By providing so much of the replication apparatus itself, HSV gains independence from the cell cycle and attains a degree of autonomy that lets it replicate in highly differentiated cells, such as neurons. Similarly, encoding most or all of their replication machinery enables poxviruses to replicate their DNA in the cytoplasm (Poxvirus replication).

Two families of animal viruses synthesize DNA by a pathway that has no cellular counterpart, that of reverse transcription of RNA. Retro- and hepadnaviruses both encode an RNA-dependent DNA polymerase (reverse transcriptase) that catalyzes this unique reaction which is central to their mechanisms of genome replication (Prions of humans and animals; and 56, Hepatitis delta virus). The enzyme is a structural component of virus particles from both families and it catalyzes the initial biosynthetic reactions of their infectious cycles, the synthesis of viral DNA. Retroviruses also encode an integrase enzyme that recombines the proviral DNA into the genome of the host.

Because cellular DNA replication depends on continuous protein synthesis, it is inhibited by many DNA and RNA viruses as a consequence of their effects on translation (see Protein metabolism). The viruses probably benefit from the increased availability of nucleotides. Inhibition also results from the ability of viral DNA molecules to displace cellular DNA from anchor points on the nuclear matrix that are thought to provide the necessary structural framework for DNA replication. It has been suggested that competition for these sites may modulate the interaction between viral and host cell DNA replication (Cook 1991), and, in accordance with this idea, components of the cell's replication machinery are also redistributed within the nucleus following HSV infection (de Bruyn Kops and Knipe 1988).

DNA MAINTENANCE AND TRANSFER

Viral DNAs can be stably maintained in the host cell either by integration into a cellular chromosome, as with the retroviruses, or in the form of extrachromosomal circular episomes, as with papilloma and hepadnavirus DNAs and during latent infections with herpesviruses. In the integrated state, retroviral DNAs use both the origins and the enzymes of the cell for replication, but episomal DNAs contain their own *cis*-acting replication origins and usually express *trans*-acting viral proteins to assist in their replication and maintenance. In general, the long-term co-existence of viral and host DNAs in the same cellular compartment creates possibilities for the transfer of genetic information in both directions. Transfers from virus to cell are exemplified by the many instances of cellular transformation mediated by the incorporation of oncogenes from DNA tumor viruses (Teich 1991). Transfers from cell to virus are considered to have been responsible for the acquisition of proto-oncogenes during evolution of the RNA tumor viruses and for the integration of a cellular retrotransposon into baculovirus DNA (Friesen and Nissen 1990).

DNA MODIFICATION AND DEGRADATION

Unlike bacteria, eukaryotic cells do not generally contain DNA restriction/modification enzyme systems to protect themselves against invading DNA, although a few examples have been found of such enzymes being induced or encoded by viruses of eukaryotes. A family of DNA viruses that infect the green alga *Chlorella* encode DNA methyltransferases and sequence-specific endonucleases that resemble restriction enzymes of prokaryotes (van Etten et al., 1988, 1991; van Etten and Meints 1999). Cells infected by frog virus 3, an iridovirus, express a cytosine methyltransferase that protects the viral DNA against degradation by a virus-encoded DNase (Murti et al. 1985). Poxviruses, on the other hand, rely on subcellular compartmentation to achieve specific degradation of host DNA. Vaccinia virus particles contain a DNase that enters the host-cell nucleus early in infection and degrades the cellular DNA. The liberated nucleotides are recycled into viral DNA to such an extent that the incorporation of exogenous thymidine via the thymidine kinase pathway is almost completely suppressed during the later stages of viral DNA synthesis. Degradation of cellular DNA also occurs as a result of the induction of apoptosis in response to infection by some viruses (see Apoptosis). During this process, internucleosomal cleavage of chromatin produces a characteristic ladder of DNA fragments that can be resolved by agarose gel electrophoresis. Finally, the genomes of vaccinia virus and HSV encode uracil DNA glycosylases that are thought to be involved in viral DNA base excision repair (Dodson et al. 1994; Millns et al. 1994).

RNA metabolism

Whatever the initial biosynthetic event in infection, sooner or later every virus has to deliver mRNA to the cytoplasm to direct viral protein synthesis. Because the nature of the problem varies with the genetic strategy of the virus, virus families have evolved different mechanisms to ensure efficient transcription of viral genes and to disrupt the expression of host-cell genes (Nevins 1989; Tevethia and Spector 1989).

PREFERENTIAL SYNTHESIS OF VIRAL RNAS

Three general mechanisms have been identified that promote transcription of viral genes at the start of infection:

1. seduction of the cellular RNA polymerase (pol II) by means of a strong promoter/enhancer sequence in the viral DNA (e.g. polyoma- and adenoviruses);
2. import of a new transcription factor that redirects pol II to the viral DNA (e.g. HSV);

3 import of a new RNA polymerase with specificity for the viral genome (e.g. poxviruses and all negative-strand and double-stranded RNA viruses).

These three distinct mechanisms are described below.

Enhancers

Studies of the early SV40 promoter, which directs pol II to transcribe the viral T antigen gene, were the first to discover the *cis*-acting DNA element now known as an enhancer which increases the level of transcription from a minimal promoter on a DNA molecule (Tjian and Maniatis 1994). Enhancers work by recruiting cellular transcription factors which are often differentially expressed in different cell types, so the properties of the viral enhancer can determine which types of cell are able to support replication of the virus (Drapkin et al. 1993). For example, human papillomavirus (Papillomaviruses) is restricted to replication in keratinocytes partly because the enhancer for its E6 and E7 oncogenes must be recognized by a keratinocyte-specific transcription factor.

Transcription factors

In HSV infection, the viral DNA enters the nucleus together with a virion structural protein called α-*trans*-inducing factor (α-TIF) (or VP16) which is necessary for efficient transcription of the α class of viral genes that are expressed immediately after infection. α-TIF works in conjunction with the cellular transcription factor Oct-1 to recruit pol II to the Oct-1-binding sites (ATGCAAAT) that are present in the promoters for the α-genes (Kristie and Sharp 1993). Among the products of α-gene expression are at least two other transcription factors that change the specificity of the cellular polymerase to prepare it for the next stage of viral gene expression, transcription of the β-genes (Herpesviruses: general properties; and Alphaherpesviruses). In general, DNA viruses switch transcription from one temporal class of genes to the next by the synthesis of viral factors that act directly or indirectly on the corresponding RNA polymerases. Some of the properties of the SV40 T antigen and the E1A/E1B proteins of adenovirus that provide examples of this were described in the section entitled DNA metabolism. Transcription factors for immediate–early genes (e.g. HSV α-TIF) are synthesized late in the preceding infectious cycle because they themselves are encoded in late viral genes. It is not yet known what prevents them from becoming active until the start of the next infection.

RNA polymerases

Many virus families have genomes that cannot be transcribed by any cellular polymerase and must therefore be presented to the cell in conjunction with a functional virus-specific transcriptase. For example, although poxvirus genomes are composed of double-stranded DNA, infection delivers them to the cytoplasm, which lacks RNA polymerase activity, and anyway their promoter sequences are unrecognizable by the nuclear RNA polymerases (Poxvirus replication). To ensure transcription of their genes under these conditions, infecting poxvirus particles contain a multisubunit transcription complex that is activated once the viral core reaches the cytoplasm. It is guided specifically to the early viral genes by a viral early transcription factor (VETF) that is bound to early promoters, in conjunction with a polymerase-associated protein (RAP94) that is responsible for recruiting the subunits of the transcription complex during virus assembly in the previous infection (Zhang et al. 1994).

RNA viruses whose genomes consist of either double-stranded RNA or single-stranded negative- or ambisense RNA face the same problem and solve it in the same manner: infection delivers the genome complexed with a virus-encoded RNA-dependent RNA polymerase that transcribes the viral genes (Ishihama and Barbier 1994; O'Reilly and Kao 1998). In many cases, virion-associated enzymes then cap, methylate and polyadenylate the primary transcripts to produce functional mRNAs. The following virus families use this strategy: reo-, orthomyxo-, paramyxo-, rhabdo-, filo-, bunya-, and arenaviruses (see Table 4).

PERTURBATIONS OF CELLULAR RNA METABOLISM

Every step of cellular RNA metabolism is disrupted by one virus family or another: transcription, processing, transport from the nucleus to the cytoplasm, translation, and RNA degradation. These effects usually give the viral genes a competitive advantage, although in some cases the advantage may be too slight to be demonstrable during a single infectious cycle. Effects on translation are described in Protein metabolism, the others below.

RNA synthesis

The expression of oncogenes by tumor viruses results in a stimulation of cellular RNA synthesis as a consequence of cell cycling. In contrast, most nononcogenic viruses inhibit cellular transcription, although the mechanisms may be consequences of the inhibition of protein synthesis or the rearrangement of cellular chromatin, or both. The benefit to the virus is presumed to be decreased competition for ribonucleoside triphosphates, and in some cases the increased availability of pol II. Curiously, VSV, a rhabdovirus that replicates entirely in the cytoplasm (Rhabdoviruses), also inhibits cellular transcription. The 46 nucleotide (nt) VSV leader RNA migrates into the nucleus early in

infection, associates with the cellular La protein and can inhibit pol II. However, the idea that leader RNA is responsible for the inhibition of transcription in infected cells has been challenged, and the physiological significance of these effects is unclear.

Influenza virus disrupts cellular RNA synthesis in an unusual manner that is directly related to its own mechanism of transcription (Orthomyxoviruses: influenza). By endonucleolytic cleavage, the viral RNA polymerase removes 10–13 nt fragments that include the cap from the 5′ ends of newly made cellular transcripts, and uses these fragments to prime the synthesis of viral mRNAs in the nucleus of infected cells (Lamb and Krug, 2001). The effect is to transfer the caps from cellular mRNA precursors to viral messages, thereby labilizing the former and ensuring the translatability of the latter. Bunyaviruses (*Bunyaviridae*) use a similar cap-snatching mechanism to derive the 5′ ends of their mRNAs, except in this case the cap donors are mature cellular messages that are already in the cytoplasm (Kolakofsky and Hacker 1991).

RNA processing and transport

For viruses that replicate in the nucleus, RNA splicing is usually an important control point in gene expression. In the interests of genetic economy, DNA viruses with small genomes frequently splice their primary transcripts in several different ways, both to derive multiple mRNAs from a single promoter and to access two overlapping reading frames in the same region of the viral genome. Retroviruses use alternative splicing patterns too, but they also need a mechanism to allow the export to the cytoplasm of unspliced RNA to encode some of the viral structural proteins and to be encapsidated into progeny virions. Complex retroviruses, such as HIV, achieve this by the interaction between a viral protein rev, itself the translation product of a spliced message, and a specific RNA sequence in the viral genome (the rev-response element (RRE)). This interaction allows the export of viral RNAs that still contain complete introns and would otherwise be retained in the nucleus (Gait and Karn 1993; Cullen 1994). In simpler retroviruses such as Mason–Pfizer monkey virus, the function of the rev/RRE complex is performed instead by a small *cis*-acting constitutive RNA transport element (CTE) that somehow promotes the export of unspliced viral RNAs to the cytoplasm (Bray et al. 1994; Ernst et al. 1997).

The NS1 protein of influenza virus inhibits splicing of both viral and cellular mRNA precursors by binding to their poly(A) tails and blocking spliceosome function, but in this case the unspliced host cell transcripts are retained in the nucleus, where they provide a source of the capped oligonucleotides that are needed as primers for viral transcription. Phosphorylation of NS1 regulates this inhibition to permit the release of viral mRNAs to the cytoplasm (Lamb and Krug 2001). Yet another variation is provided by adenovirus, the system in which splicing was originally discovered (Berget et al. 1977; Chow et al. 1977). Splicing is not inhibited in infected cells, and indeed primary adenovirus transcripts are extensively and differentially spliced to access many different open reading frames from a few promoter sites (Adenoviruses). However, two of the early proteins (E1B and E4) specifically inhibit the export of processed cellular mRNAs from the nucleus and promote that of late viral mRNAs, resulting in the preferential synthesis of viral proteins. It is likely that further studies of the ways that different viruses impinge on the processing and transport of cellular transcripts will deepen our understanding both of virus–cell interactions and of the regulation of mRNA synthesis in uninfected cells (Krug 1993).

RNA degradation

The pathways and control of mRNA degradation in eukaryotic cells are not fully understood (Katze and Agy 1990; Rajagopalan and Malter 1997) but, as with RNA processing and transport, viral genetics provide powerful experimental tools to assist research in this area. Both HSV and vaccinia virus infections increase the rate of host mRNA degradation and thereby inhibit cellular protein synthesis (Rice and Roberts 1983; Strom and Frenkel 1987). The effects are not specific for cellular messages, because viral mRNAs are turned over equally fast, but the greater vigor of viral transcription is better able to compensate for the short mRNA half-lives, which also facilitate the rapid transitions from expression of one temporal class of viral genes to the next. For HSV-1, the ability to induce mRNA labilization has been mapped to the *vhs* gene (for viral host shut-off), which encodes a virus structural protein. The vhs protein in the virion is sufficient to trigger mRNA degradation in some cells, although it is not known whether it works directly as a nuclease or by activating a host cell degradative mechanism. In vaccinia virus, the gene responsible for enhanced mRNA turnover has not been mapped, but mutations that subtly affect the function of the viral RNA polymerase can cause massive nonspecific RNA degradation by producing abundant double-stranded RNA which activates the cellular 2–5A RNase L system for RNA breakdown (Bayliss and Condit 1993) (see Induction and actions of interferons, and

The role of cytokines in viral infections).

Protein metabolism

The synthesis, processing, and transport of viral proteins lie at the heart of the overlap between viral and cellular metabolism, because, whereas most viruses encode specific enzymes or factors that participate in viral transcription and genome replication, they all depend on

the host cell for the entire machinery of protein synthesis and transport. One consequence of this is that translation, which is such an effective target for antibacterial agents, is not available as a target for the development of antiviral drugs.

PROTEIN SYNTHESIS

The rate of protein synthesis from a particular mRNA is influenced by many factors (Lodish 1976). In eukaryotic cells, most translational control is exerted at initiation (Merrick 1990; Gale et al. 2000), and a schematic picture of this multistep reaction is shown in Figure 12. Because of its complexity, the regulation of this process is not yet fully understood, but the following factors all play a part: the abundance of the mRNA, its 5′ cap structure, the local nucleotide sequence context and secondary structure surrounding its initiation codon, whether it is translated on free or membrane-bound polysomes, and the availability and activity of cellular initiation factors (Hershey 1990, 1993; Kozak 1991a; Wek 1994). Two prominent control points among the latter are eIF4F, the cap-binding complex, and eIF2, which binds the initiator met-tRNA to the 40S ribosomal subunit. Both of these initiation factors are altered in some virus-infected cells, as described below (Kozak 1986; Schneider and Shenk 1987).

Initiation in uninfected eukaryotic cells usually involves recognition by eIF4F of the 7-methylguanylate cap at the 5′ end of cellular mRNA, followed by association of the mRNA with a complex that contains the 40S ribosomal subunit, met-tRNA$_i$ and eIF2 (Figure 12). eIF4F is a cap-dependent RNA helicase that is required for the translation of almost all capped messages. On a very small number of cellular mRNAs and on picornaviral RNAs, initiation occurs at an internal ribosome entry site (IRES), in a manner that is independent of the 5′ cap and eIF4F. In both cases, the 40S subunit then scans in a 3′ direction until it encounters the first AUG codon. If the local sequence context of this AUG is favorable for initiation, the 60S ribosomal subunit joins the complex, eIF2 dissociates and the synthesis of the polypeptide chain begins. Initiation efficiency is influenced most by the identity of the nucleotides in the −3 and +4 positions relative to the AUG, the sequence . . . C**R**CCAUG**G** . . . being optimal for initiation, but

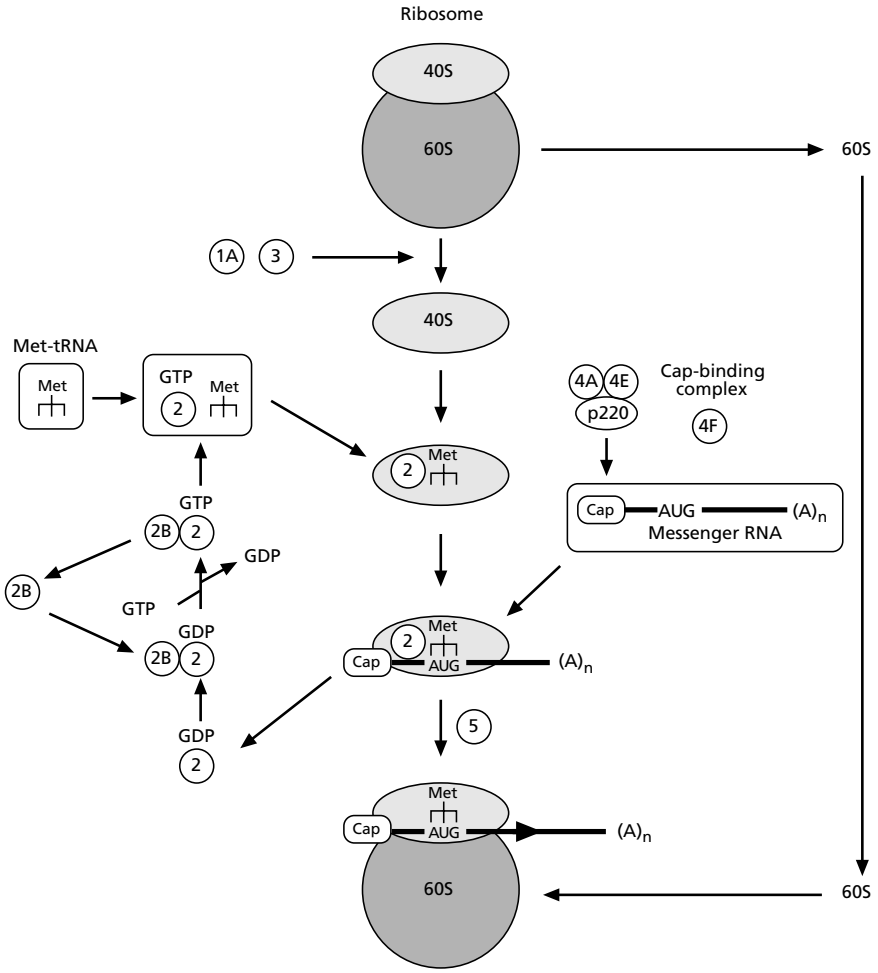

Figure 12 *Mechanism of initiation of protein synthesis in animal cells. The numbered circles represent the initiation factors.*

other features of the mRNA 5′ end including its secondary structure can also exert effects (Kozak 1989, 1991a, b, 1992a, b, 1994). Although eukaryotic protein synthesis usually starts at the first AUG codon, if its context is unfavorable for initiation the 40S subunit can continue scanning until it finds the next and starts there instead. In rare instances, alternative initiation codons, including non-AUG codons, are used to derive more than one translation product from a single mRNA, as in the case of the P mRNA of some paramyxoviruses (Curran et al. 1986) (see General properties of paramyxoviruses). When eIF2 dissociates from the initiation complex it is bound to GDP, but before it can form another met-tRNA-40S subunit complex for another round of initiation, it must exchange this GDP for GTP, a reaction that requires initiation factor eIF2B (Figure 12). In several metabolic situations including viral infection, this exchange reaction is a major site at which protein synthesis is regulated, as described below (Hershey 1993).

Host shut-off and the preferential translation of viral mRNAs

The inhibition of host-cell protein synthesis is one of the most obvious metabolic consequences of viral infection, and it has been the focus of much research using several different virus systems (see Ehrenfeld (1993) for reviews). Unfortunately, although it is easy to demonstrate the phenomenon, it has proved difficult to elucidate its mechanisms, because, somewhat surprisingly, viruses shut off host protein synthesis in different ways and even closely related viruses can use completely different mechanisms (Table 5). Cytocidal viruses such as polio and vaccinia inhibit host translation profoundly, but retroviruses, which depend on the cell's continued growth and survival, scarcely perturb host protein synthesis at all. Many other viruses lie between these two extremes. Some of the conflicts in the literature on this subject may be because some viruses use more than one mechanism or use different mechanisms when infecting different cell types.

Because viral and host mRNAs compete directly for translation, viruses have evolved mechanisms that favor the synthesis of their own proteins, often at the expense of the cell's. However, the total amount of translation occurring in infected cells is almost always less than that in uninfected cells, because infection generally reduces the overall capacity of the cell for protein synthesis. Rather than simply displacing host mRNAs, viral messages are preferentially translated against the background of a progressive inhibition of total protein synthesis to which they are relatively resistant. For this reason, how viruses inhibit host protein synthesis and the mechanisms they use to achieve preferential translation of their own mRNAs are interdependent.

Several such mechanisms have been identified (Table 5). To begin with, the abundance of individual viral mRNAs is usually greater than that of most cellular mRNAs, because of vigorous viral transcription and the high gene copy-number that results from viral genome replication. In the herpes- and poxviruses, this effect is magnified by an increased rate of mRNA breakdown (see RNA metabolism). In addition, many viral mRNAs have a competitive advantage whose structural basis is not fully understood (Garfinkel and Katze 1993; Kozak 1994). Part of the answer lies in the nucleotide sequence context of their initiation codons, but strong contexts, although common, are not ubiquitous in viral mRNAs (Kozak 1986). Their 5′ untranslated regions are usually shorter than those of host mRNAs, and the resulting proximity of the cap to the initiation codon and other undefined structural features of their 5′ ends may also favor translation.

For polio- and rhinoviruses, the mechanism is clear and elegant (Sonenberg 1987; Jackson et al. 1991; Wycoff 1993; Thompson and Sarnow 2000): infection results in the expression of a viral protease (2A), which mediates cleavage of a component (p220 or the γ subunit) of the cellular cap-binding complex, eIF4F (Etchison et al. 1982; Etchison and Fout 1985). The

Table 5 *Mechanisms of inhibition of host-cell protein synthesis*

Virus	Mechanism	Reference
Herpes simplex	Degradation of host (and viral) mRNAs	Strom and Frenkel (1987)
Vaccinia	Degradation of host (and viral) mRNAs	Rice and Roberts (1983)
Polio	Inactivation of eIF4F-γ by cleavage	Etchison et al. (1982)
Rhino	Inactivation of eIF4F-γ by cleavage	Etchison and Fout (1985)
Adeno	Inactivation of eIF4F-α by underphosphorylation	Huang and Schneider (1991)
	Inactivation of eIF2B-β by phosphorylation	O'Malley et al. (1989)
Vesicular stomatitis	mRNA abundance	Lodish and Porter (1981)
	Not mRNA abundance	Schnitzlein et al. (1983)
	Affinity for ribosomes	Nuss et al. (1975)
	Interference with eIF2 function	Centrella and Lucas-Lenard (1982)
	Interference with eIF3/4B function	Thomas and Wagner (1983)

result is inactivation of the initiation factor and consequent inhibition of translation of capped mRNAs. Viral RNA is immune to this inhibition because it is translated from an IRES and naturally lacks a 5′ cap (Meerovitch and Sonenberg 1993). In virions, polio- and rhinovirus RNAs carry a covalently attached viral protein (VPg) at their 5′ ends, while the translated form of the viral RNA starts with a 5′-monophosphate, pUp (Picornaviruses).

It is surprising that cleavage of the cap-binding protein is unique to polio- and rhinoviruses, and does not occur in cells infected with other picornaviruses, despite the fact that all their RNAs contain IRES elements, are translated in a cap-independent manner, and mostly shut-off host protein synthesis (Duncan 1990). Because it is incontrovertible that picornaviruses are all descended from a common ancestor, their use of different shut-off mechanisms suggests either that an ancestral mechanism was replaced in some but not other descendants or that the ancestor did not inhibit host translation and that the present-day mechanisms evolved separately after divergence. Taken with the fact that polio- and herpesvirus mutants that cannot shut off host translation are only partly debilitated for growth (Bernstein et al. 1985; Strom and Frenkel 1987), this evidence suggests that inhibition of host protein synthesis, though beneficial, is not essential for virus replication. Indeed, a general selection method has been developed for the isolation of nonconditional viral mutants that are deficient in host protein shut-off (Francoeur et al. 1987).

The activity of the cap-binding complex is also reduced in adenovirus-infected cells, but in this case it is by reduced phosphorylation of the α subunit (eIF4A, which is the cap-binding protein itself) rather than by cleavage of p220 (Huang and Schneider 1991; Zhang and Schneider 1993). Because adenovirus mRNAs are capped, the rationale for eIF4F inactivation is less obvious than with the picornaviruses. However, it seems that the tripartite leader, a 200-nt RNA segment that is common to the 5′ end of all late adenovirus mRNAs (Adenoviruses), renders them less dependent for translation on the helicase activity of eIF4F than are cellular messages.

Changes in the level of phosphorylation of another initiation factor, eIF2, are responsible for the regulation of protein synthesis under many different conditions of cellular stress, but in this case it is increased phosphorylation that interferes with initiation. As shown in Figure 12, eIF2 needs to exchange its bound GDP for GTP between each round of initiation, and this exchange requires the formation of a complex with eIF2B. Phosphorylation of the α subunit of eIF2 prevents the dissociation of this complex, and, because eIF2B is scarce relative to eIF2, a modest increase in the level of phosphorylation inhibits initiation profoundly. As described in Induction and actions of interferons, this mechanism is part of the cell's response to viral infection that is enhanced by treatment of cells with interferon. Increases in the phosphorylation of eIF2-α occur in cells infected with adeno- and reoviruses, but it is unclear whether these are part of the virus-mediated inhibition of host mRNA translation or the cell-mediated inhibition of the virus. Nevertheless, this regulatory mechanism is a key point in the balance between viral and cellular protein synthesis, as shown by the fact that vaccinia virus encodes a homologue of eIF2-α, and several other viruses perturb the phosphorylation of eIF2-α in other ways (see Viral mechanisms to evade cellular defenses).

Access to alternative open reading frames

In addition to alternative RNA splicing (see RNA metabolism) and transcriptional editing (Cattaneo 1991; Jacques and Kolakofsky 1991), some viruses use translational frameshifting to express open reading frames that would otherwise be inaccessible in an mRNA. Retroviruses in particular use this mechanism to make proteins that they require only in small amounts, like the gag–pol precursor to the reverse transcriptase in Rous sarcoma virus (Jacks and Varmus 1985). Frameshifting is promoted by specific 'slippery' sequences in the mRNA, which, often in conjunction with adjacent 'hungry' codons and strong RNA secondary structure, persuade ribosomes occasionally to switch reading frames during translation (Brierley 1993). Whether viral infection modulates the intrinsic propensity of the ribosomes for frameshifting is not yet clear. In other retroviruses, synthesis of the gag–pol precursor is achieved by suppression of the termination codon at the end of the *gag* gene (Prions of humans and animals).

PROTEIN PROCESSING

During and after their synthesis, viral proteins are processed along many of the same reaction pathways as cellular proteins, and, because they offer such attractive experimental subjects, they have been widely used as model systems for the study of these pathways. There are also several cases in which protein processing reactions are mediated by viral enzymes or modified by viral proteins, and this is a frequent cause of CPEs.

Folding and oligomerization

Viral proteins must adopt the correct tertiary and quaternary structures before they become functional and, in many cases, before they can be transported to their ultimate destinations in the cell. These reactions have been examined in particular detail for viral envelope glycoproteins such as influenza HA, which must undergo glycosylation, folding and trimerization before it can move from the ER, via the compartments of the Golgi apparatus, to the cell surface in preparation for virus assembly (Gething et al. 1986; Doms et al.

1993; Byland and Marsh 2005). Folding is facilitated by interaction with a cellular protein BiP which resides in the lumen of the ER and binds transiently to newly made glycoproteins. BiP belongs to the hsp70 family of cellular proteins that are induced by heat shock and other types of stress, and it functions both to catalyze protein unfolding/refolding and to target misfolded and mutant proteins for degradation (Pelham 1988; Hartl 1996; Paulsson and Wang 2003).

Proteolytic cleavage

Many viral proteins are cleaved during maturation, by either cellular or viral proteases. For example, the activation of viral fusion proteins by cleavage (see Viral fusion proteins) is often catalyzed by members of the furin family of cellular proteases (Klenk and Garten 1994a,b). However, viral proteolysis is used most extensively by the nonsegmented, positive-strand RNA viruses of the picorna-, toga-, and flavivirus families (Picornaviruses; Togaviruses; and Flaviviruses). These viruses synthesize large polyprotein precursors that undergo multiple co- and post-translational cleavages catalyzed by several viral proteases to yield the mature viral proteins (Krausslich and Wimmer 1988; Strauss 1990; Dougherty and Semler 1993; Ryan and Flint 1997; Ryan et al. 1998; ten Dam et al. 1999). They are forced into this strategy of gene expression by a combination of their need to make several different viral proteins from a single mRNA and the reluctance of eukaryotic ribosomes to initiate at more than one site on a message. In contrast, viruses whose transcriptional mechanisms allow them to synthesize individual mRNAs and those with segmented genomes rely less heavily on proteolytic processing.

The assembly of icosahedral and more complex viral structures is also often accompanied by cleavage of structural protein precursors, which imposes a rigid order on the assembly process and can result in large-scale conformational changes in the maturing virion, often coincident with the acquisition of stability and infectivity (Guo 1994). Both in picorna- and in nodaviruses, the final maturational cleavage event occurs inside the assembled virion, and is catalyzed by the interior of the capsid itself. It is interesting that the assembly of helical nucleocapsids generally seems not to involve protein cleavage.

Glycosylation

All enveloped viruses carry surface glycoproteins whose glycosylation is mediated by the normal cellular pathways of carbohydrate attachment and processing. Both *N*- and *O*-linked oligosaccharides are found but viral proteins with the former modification are more common.

Whether glycosylation is required for glycoprotein transport and function varies from one case to another, and probably reflects the different roles that oligosaccharides can play in determining the native structures of individual proteins. In certain cases, however, the influence of glycosylation can be dramatic: the presence or absence of a particular *N*-linked oligosaccharide in influenza HA that partially obstructs the HA1–HA2 cleavage site can have a profound effect on viral virulence because it determines which proteases can cleave the HA precursor, and hence how the virus can spread in the infected animal (Deshpande et al. 1987).

Phosphorylation

Viruses and cells interact with one another by phosphorylation in both directions: the phosphorylation of cellular proteins by viral kinases and the phosphorylation of viral proteins by cellular kinases. Prominent among the former are reactions catalyzed by the tyrosine kinases that are encoded by the oncogenes of Rous sarcoma virus (v-src) and its relatives. In these cases, the major targets of kinase action are cellular proteins involved in cell cycle regulation whose activities are perturbed by phosphorylation. Conversely, cellular kinases can phosphorylate viral proteins and thereby modify their activities. For example, phosphorylation of the P protein of VSV by cellular casein kinase II enables it to form oligomers, which is an essential step in viral transcription (Gao and Lenard 1995).

Other covalent modifications

A wide variety of other covalent modifications of viral proteins have been described in different virus–cell systems, including N-terminal acetylation, acylation (with both palmitate and N-terminal myristate), ADP ribosylation, isoprenylation, methylation, and sulfation. In most cases the cellular enzymes that catalyze these reactions are widely distributed and therefore do not restrict virus replication, but the effects of many of the modifications on the properties of viral proteins have yet to be fully explored.

PROTEIN TRANSPORT

Viral proteins are transported to the appropriate intracellular sites for replication and assembly by interaction with cellular protein trafficking pathways: to the plasma membrane via the ER and Golgi apparatus, to the nucleus, and to other organelles. Despite the disruption of the cytoskeleton and the extensive changes in cell morphology that often accompany infection, these trafficking pathways are generally not disrupted. Many viral proteins contain signals that define their destination in the cell, such as the hydrophobic signal sequences of integral membrane glycoproteins which, by binding to the signal recognition particle, commit the proteins to

ER membrane insertion and transport along the exocytic pathway. Nuclear localization signals, which were first identified in the SV40 T antigen (Kalderon et al. 1984), target the corresponding viral proteins to the nucleus for replication and assembly. Proteins that contain *cis*-acting signals such as these are transported to the appropriate cellular compartment even when they are expressed from a vector in the absence of other viral proteins. In contrast, proteins that lack signals of their own can co-localize by binding with targeted proteins. A striking example of the influence of such signals on virus assembly is provided by the observation that a point mutation in a retroviral matrix protein determines whether the viral nucleocapsids assemble in the cytoplasm or at the plasma membrane (Rhee and Hunter 1990).

PROTEIN DEGRADATION

As described above, viruses in many families encode proteases with new specificities that can cleave cellular as well as viral polypeptides. It is likely that the resulting exposure of new N termini (Varshavsky 1992) to the protein degradation pathways (Hershko and Ciechanover 1992; Rubin and Finley 1995) will enhance the rate of turnover of specific proteins in cells infected with these viruses, although, apart from the cleavage of eIF-4F described above, few specific examples have yet been described.

CELLULAR RESPONSES TO VIRAL INFECTION

Because viruses are a threat to the survival of cells and organisms, it is inevitable that cells and organisms should have evolved defense mechanisms to counteract them. It is similarly inevitable that those viruses with sufficient genetic capacity should have evolved countermeasures to circumvent these defenses. In higher vertebrates, the major defense of organisms against infection is provided by the mucosal, humoral, and cell-mediated arms of the immune system, which are described in The immune response to viral infections. The major defenses at the cellular level are the interferon (IFN) system (see below and The role of cytokines in viral infections), RNA interference (Hannon 2002; Ding et al. 2004), and the mechanisms of stress response and apoptosis. The interplay between the processes of virus replication, these cellular responses and the corresponding viral evasion mechanisms determines the outcome of infection at the cellular level.

Induction and actions of interferons

The IFNs are a family of cytokines that elicit profound physiological changes in the cells with which they interact, including the development of resistance to viral infection. In higher vertebrates, the IFN system acts within a few hours of infection, long before the onset of the immune response, to limit the spread of the infection and thus to diminish its severity. Indeed, many of the familiar early symptoms of a viral infection in humans are attributable to IFN action. IFNs are synthesized and secreted by most cell types in response to infection and some other stimuli, and they exert their effects by inducing the expression of several IFN-responsive genes. Understanding the system as a whole, therefore, requires knowledge of the IFN genes, how they are induced, the IFN proteins themselves, and how they interact with their target cells, how these interactions trigger expression of the IFN-inducible genes, and, finally, how the products of these latter genes render the cell resistant to viral infection and mediate their other effects. Although much remains to be learned about this complex and multifunctional regulatory system, intense research in recent years has clarified many important aspects. Here is presented only an overview of the IFN system; for more details the reader should consult The role of cytokines in viral infections or a review article (Revel and Chebath 1986; Pestka et al. 1987; Samuel 1988, 1991; Staeheli 1990; Sen and Lengyel 1992; Sen and Ransohoff 1993; Johnson et al. 1994; Sen 2001; Malmgaard 2004).

INTERFERONS

Four distinct types of IFNs have been identified and characterized: α, β, γ, and ω. Although their properties overlap to the extent that they are all recognized as IFNs, they are secreted by different cell types in response to different inducers and they exert somewhat different physiological effects on different target cells. In the context of virus–host cell interactions, α, β, and γ IFNs are the most relevant because α and β induce a virus-resistant state in most cell types, whereas IFN-γ primarily modulates aspects of the cell-mediated immune response via its effects on macrophages. Alpha IFNs, which are encoded in a multigene family, are homologous to IFN-β and interact as monomers with the same receptor on the surface of target cells. In contrast, IFN-γ has little homology with the other types and binds as a dimer to two copies of a different cell surface receptor (Walter et al. 1995).

INTERFERON INDUCTION

Alpha and β IFNs are induced and secreted from many different cell types in response to infection by most viruses, as well as some other assaults, and these cytokines act as an early warning system that alerts other cells in the body that infection is imminent. In contrast, IFN-γ is produced only by T lymphocytes and natural killer cells, in response to induction by cognate antigens and other T-cell activators. Induction of IFNs occurs by transcriptional activation of the IFN genes, and,

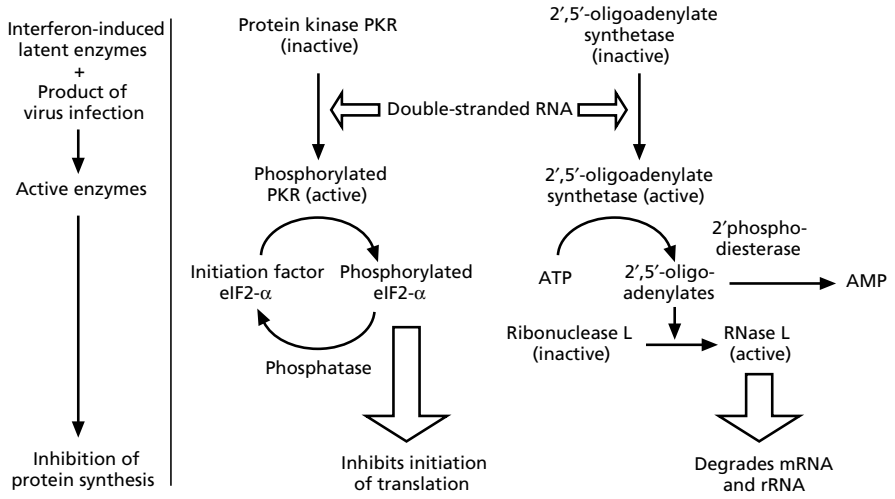

Figure 13 *Mechanisms of the antiviral action of interferons.*

although many of the mechanistic details remain obscure, a satisfying picture is emerging for the induction of IFN-β. It has long been known that IFN-β can be induced by treating cells with double-stranded RNA, and indeed the production of double-stranded RNA may be a general mechanism by which viruses induce IFNs. Among the cellular targets for double-stranded RNA is a cytoplasmic protein kinase called PKR, which is also involved in the mechanism of IFN action (see below). On activation by binding to double-stranded RNA, PKR phosphorylates I-κB, the inhibitory subunit of the transcription factor NF-κB (Karin and Hunter 1995). Phosphorylation of I-κB allows NF-κB to enter the nucleus where it recognizes specific DNA binding sites and thereby activates the transcription of several genes, including that for IFN-β (Proud 1995).

INTERFERON ACTION

The binding of IFNs to their corresponding receptors initiates signals that are transmitted to the nucleus of the IFN-treated cell via the JAK/STAT pathway. Signal transduction occurs by the activation of a family of tyrosine kinases (Janus kinases (JAK)) that are associated with the cytoplasmic domains of the transmembrane IFN receptors and activated when the receptors are occupied by binding to IFNs. The activated JAKs phosphorylate a family of transcription factors (signal transducing activators of transcription (STAT)), which then migrate from the cytoplasm to the nucleus. Here they form active complexes that bind to interferon-specific response elements (ISRE) present in the promoter sites upstream of IFN-inducible genes, thereby activating their transcription (Williams 1991; Pellegrini and Schindler 1993). Many other polypeptide ligands that exert a wide variety of physiological effects on cells also transmit their signals to the nucleus via the JAK/STAT pathway. The specificity of the ligand/response relationship seems to be maintained by subtle quantitative, qualitative, and temporal differences in the spectra of JAK activation and STAT phosphorylation that are elicited by the different ligands, although much more work is needed to define fully how the integrity of different signals is maintained (Schindler and Darnell 1995; Aaronson and Horvarth 2002).

Although the IFN system was first discovered by the observation that IFN-treated cells are resistant to viral infection, it has since become clear that this is only one aspect of their altered physiology. IFNs can also have profound antiproliferative and immunomodulatory effects, so the physiology of the IFN response in whole animals is complex. The multiple effects of IFNs are mediated by the IFN-inducible proteins of which scores have been identified, but the roles that many of them play in the altered physiology of the IFN-treated cell are still unknown. Best understood are two inducible enzymes that are responsible, in part, for the resistance of cells to infection by several different viruses. These are the protein kinase (PKR) that is also implicated in the induction of IFN-β (see above), and a family of enzymes called 2′,5′-oligoadenylate (2–5A) synthetases (Figure 13).

PKR, a double-stranded RNA-dependent protein kinase

The protein kinase now known as PKR is a 68-kDa polypeptide that is induced by IFN treatment of most types of cultured vertebrate cells (Williams 1999). The enzyme is active only when bound to double-stranded RNA, whereupon it phosphorylates and activates itself, and also phosphorylates the translational initiation factor eIF2-α. This interferes with the initiation of protein synthesis (as described under Protein metabolism and Figure 12), which limits viral replication (Hershey 1990, 1993). Untreated cells contain only basal

levels of PKR and the enzyme is largely inactive because of the absence of double-stranded RNA. IFN-treated cells contain substantial levels of PKR, but it remains inactive until viral infection produces double-stranded RNA, either as an intermediate of RNA replication or as a byproduct of overlapping transcription. In this way, the effects of IFN are fully manifested only after viral infection, a mechanism that restricts the major physiological consequences to the cells where they will have the greatest effect. Viruses have evolved numerous ways to counteract the effects of IFN in general and PKR in particular (see Viral mechanisms to evade cellular defenses), implying not only that the IFN response significantly restricts virus replication in nature, but also that PKR is a central component in the mechanism of resistance to many viruses.

2–5A synthetases

The 2–5A synthetases that are also induced by IFN treatment catalyze the template-independent oligomerization of ATP to form a series of 2′–5′-linked oligomers that are referred to collectively as 2–5A (Ball 1982; Player and Torrence 1998; Rebouillat and Hovanessian 1999). Like PKR, the 2–5A synthetases depend on double-stranded RNA for enzyme activity, so full development of this aspect of the IFN response also requires viral infection of IFN-treated cells. The 2–5A oligomers have the general structure $ppp(A^{2'}p^{5'})_nA_{OH}$, and in cells the trimer and tetramer predominate. In general, 2′,5′-phosphodiester bonds are a biochemical rarity, although single such bonds are formed during RNA splicing (Sharp 1994) and also during the priming of cDNA synthesis by bacterial reverse transcriptase (Shimamoto et al. 1995). The only known function of 2–5A oligomers is to activate an endoribonuclease, RNase L, which is present as a constitutive but latent enzyme in most cell types (Bisbal 1997). On activation by 2–5A, RNase L degrades mRNAs and ribosomal RNAs, and thereby inhibits protein synthesis. The degradation of 2–5A itself by a 2′-phosphodiesterase restores RNase L to its latent state. Thus, in IFN-treated virus-infected cells, PKR and 2–5A synthetase collaborate to restrict virus replication by inhibiting both the initiation and the elongation of polypeptides.

Specificity of IFN action

Although IFN treatment generally induces resistance to a wide range of viruses, the induced gene products play quantitatively different roles in combating infection by different viruses. For example, the 2–5A system seems to be particularly important in the resistance to picornavirus infection, but much less so in the resistance to several other virus families. The murine *Mx* gene is a more extreme example of an IFN-induced resistance mechanism that is effective only against a specific virus family because the Mx protein product makes mice and murine cells resistant to infection by influenza virus, but not by other viruses (Staeheli 1990; Samuel 1991; Haller and Kochs 2002). However, a broader and more complex question is whether IFN action can discriminate generally between viral and cellular events and, if so, how this is achieved. IFN treatment clearly enables organisms and cell cultures to survive viral challenges that would otherwise be lethal, and yet the properties of PKR and 2–5A synthetase provide only a partial explanation for this apparent selectivity. The recognition by these enzymes of double-stranded RNA as a signature of viral infection establishes a mechanism for discriminating between infected and uninfected cells rather than between viral and cellular mRNAs, and in some cases the infected cells are sacrificed to prevent viral replication. In other cases, enzyme activation imposes an indiscriminate but transient suppression of protein synthesis, a situation from which the cell can recover but the virus cannot. Finally, in some situations, the IFN-induced enzymes seem to be preferentially activated in cytoplasmic microenvironments surrounding the viral double-stranded RNA molecules that are responsible for their activation, thus achieving selectivity on the basis of compartmentalization.

It seems likely that combinations of these effects enable PKR and 2–5A synthetase/RNase L, which have no demonstrable specificity for viral mRNAs, nevertheless to discriminate between cellular and viral processes.

VIRAL FACILITATION BY INHIBITION OF THE IFN SYSTEM

Viruses differ in their sensitivity to IFNs, in part because some of them have evolved effective countermeasures against one or more component of the IFN system (see Viral mechanisms to evade cellular defenses). Accordingly, a virus such as vaccinia, which is relatively insensitive, can in some circumstances facilitate the replication of a coinfecting IFN-sensitive virus such as VSV by inhibiting the action of IFN. This contrasts with the more common situation in which coinfecting viruses interfere with one another by a variety of mechanisms that are unrelated to the IFN system (Whitaker-Dowling and Youngner 1987).

Induction and actions of cellular stress proteins

Although viral infection suppresses the synthesis of most cellular proteins by the mechanisms described in the section entitled Protein metabolism, some host cell genes are induced by infection and their proteins are relatively resistant to shut-off. These include the genes that encode the IFNs (see Induction and actions of interferons) and also those for a set of proteins that are generally induced by conditions of metabolic stress,

including heat shock (Jindal and Malkovsky 1994; Sarkar and Sen 2004). Some of the cellular stress proteins normally function as molecular chaperones, and certain viruses exploit these properties to assist their own replication and assembly. This is true of some bacteriophages and has also been suggested for several eukaryotic viruses. For example, the cytoplasmic hsp70 protein binds transiently to the poliovirus capsid precursor, perhaps facilitating its folding and assembly. Similarly, BiP protein, which resides in the lumen of the rough endoplasmic reticulum, binds briefly to newly synthesized integral membrane proteins, such as viral glycoproteins, and assists their maturation (Hartl 1996). In addition to these roles, however, the display of virus-induced stress proteins or their peptide fragments on the surface of infected cells may stimulate immune recognition and participate in the activation of the immune response.

Apoptosis

Metazoan organisms have evolved a highly conserved and tightly regulated cellular pathway called 'apoptosis' which, once triggered, leads irreversibly to cell death (Martin et al. 1994; Martin and Green 1995; Steller 1995; Roulston et al. 1999). Apoptosis is the mechanism by which unwanted cells are eliminated during development, but it is also triggered in response to infection by a wide variety of cytocidal viruses (Vaux et al. 1994; Razvi and Welsh 1995; Everett and McFadden 1999). It is characterized by cell rounding, condensation of chromatin, fragmentation of cellular DNA, blebbing of the plasma membrane, and finally break up of the cell contents into membrane-bound apoptotic bodies that are suitable for ingestion by phagocytes. In the context of viral infection, apoptosis serves as an effective defense mechanism for the organism whereby a relatively small number of cells die altruistically to limit virus spread (Williams 1994). This is reminiscent of the self-sacrifice of some IFN-treated infected cells that was described in the section entitled Induction and actions of interferons. The mechanisms by which viruses induce apoptosis have yet to be fully defined, but changes in membrane permeabilities leading to Ca^{2+} influx, the expression of viral proteases with new specificities, and perturbation of the functions of the cellular tumor-suppressor proteins p53 and pRb may all be involved in different situations.

The most persuasive evidence that the effects of apoptosis in virus–host cell interactions are, on balance, detrimental to the virus comes from the observation that several viruses have evolved countermeasures that delay or prevent the onset of apoptosis (Ink et al. 1995; Razvi and Welsh 1995; Gillet and Brun 1996). For example, cowpox virus expresses an inhibitor of an enzyme that is involved in the apoptotic pathway, the interleukin-1β-converting enzyme (ICE) (Kumar 1995), and it thereby blocks both apoptosis and inflammation (Ray et al. 1992). In the absence of an effective apoptotic response, a normally cytocidal virus, such as Sindbis virus, can instead establish a persistent infection (Griffin et al. 1994; Griffin and Hardwick 1997). Conversely, suppression of apoptosis by the adenovirus E1B oncogene is necessary to allow the induction of cell proliferation that is mediated by the E1A oncogene to result in the transformation of adenovirus-infected cells (White 1993).

VIRAL MECHANISMS TO EVADE CELLULAR DEFENSES

During co-evolution with their hosts, different viruses have adopted several different methods of counteracting the various defense mechanisms, an observation that provides compelling evidence for the efficacy of the corresponding host defenses themselves (Smith 1993, 1994; Levy and Garcia-Sastre 2001). As might be expected, inactivation or deletion of the viral genes involved in these evasion mechanisms generally reduces the virulence of the virus. This aspect of the study of virus–host interactions is particularly informative because it provides a window on the relationships as they exist in nature. Moreover, some of the evasion mechanisms have involved the viral acquisition and adaptation of host genes that benefit the virus in much the same way as the acquisition of an oncogene benefits a retrovirus by ensuring cell cycling and concomitant viral replication. These host-derived genes and their products can provide insight into the heart of the virus–host relationship, as well as potential reagents for the experimental and therapeutic manipulation of the host's defense mechanisms (McFadden 1995).

Evasion of immune responses

Many RNA viruses continually evade the immune responses of the host by mutation. The inability of RNA polymerases to correct their mistakes leads to heterogeneous virus populations or quasi species from which antibody-escape mutants are readily selected (Smith and Inglis 1987; Duarte et al. 1994). For viruses that have segmented genomes (e.g. influenza), this effect is reinforced by the potential for reassortment of RNA segments between viruses with different pedigrees (see Orthomyxoviruses: influenza). With HIV, the continuous generation of antigenic variants within each infected individual eventually overwhelms the ability of the immune system to respond and contributes to the onset of AIDS (see Prions of animals and humans). In contrast, viruses with double-stranded DNA genomes are usually replicated with much greater fidelity and have therefore resorted to other methods of immune evasion. For example, herpesviruses are hidden from immune surveillance during latent infections (Masucci and Ernberg 1994; Wagner 1994; Lewin 1995), and during lytic growth they specifically inhibit the intracellular transport mechanism by which viral peptide

fragments are presented to the cell-mediated immune system (York et al. 1994; Fruh et al. 1995). Adeno-, cytomegalo-, and poxviruses also interfere with peptide presentation, by down-regulating the expression of the major histocompatibility complex class I protein (Smith 1993, 1994). In addition, poxviruses encode a variety of soluble cytokine 'receptors' which are secreted from infected cells to intercept immunostimulatory cytokines (Pickup 1994; Spriggs 1994; Lalani and McFadden 1999) (see also The role of cytokines in viral infections).

Evasion of complement

The complement system is a major host defense mechanism which, on activation, attacks and permeabilizes the surface membranes of enveloped viruses and infected cells. Complement is activated by two tightly regulated pathways: the classical pathway, which requires an antibody response to the infecting virus, and the alternative pathway, which is antibody-independent. Several members of the pox- and herpesvirus families express cell surface or secreted proteins that interfere either with the complement activation pathways or with the formation of the membrane attack complex itself, which attests to the potential efficacy of complement against these viruses (Isaacs et al. 1992; Smith 1994).

Evasion of interferon

In contrast to the ease with which antibody-resistant mutants can be isolated, IFN-resistant mutants seldom arise even when they are deliberately selected for (Novella et al. 1996), probably because IFNs induce several distinct and overlapping antiviral mechanisms. Nevertheless, viruses vary considerably in their intrinsic sensitivities to IFNs, not only because they differentially activate the IFN-induced enzymes but also because some of them deploy specific IFN evasion mechanisms (McNair and Kerr 1992). For example, among the soluble cytokine receptors secreted from poxvirus-infected cells are proteins that decoy both α and γ IFNs (Smith 1993; Pickup 1994; Spriggs 1994; Alcami and Smith 1996). Intracellularly, reovirus and vaccinia virus express double-stranded RNA-binding proteins that interfere with the activation of PKR, whereas adenovirus and Epstein–Barr virus (EBV) synthesize abundant small RNAs (called virus-associated (VA) RNAs and EBV-encoded early RNAs (EBERs), respectively) that compete with double-stranded RNA for PKR binding, but which fail to activate the kinase (Mathews and Shenk 1991; Mathews 1993). Yet other mechanisms are used by poliovirus, which degrades the activated PKR, and by influenza virus, which activates a latent cellular inhibitor of PKR (Katze 1993, 1995). Its role in uninfected cells has yet to be determined. Finally, vaccinia virus expresses a homologue of eIF2-α, which decoys the kinase and protects the authentic initiation factor against phosphorylation. The variety of mechanisms that have evolved to circumvent the PKR system argues strongly that it can potentially restrict the replication of a wide range of viruses. Viral mechanisms to evade the 2–5A system also exist, but they remain to be fully characterized.

SUMMARY

Their large population sizes and high mutation rates allow viruses to evolve much faster than the host organisms on which they depend for replication. For this reason, they will continue to threaten the health of the rest of the biosphere and to shape the evolution of their hosts. However, as described in this chapter, examination of the intricacies of virus–host interactions provides unique insights into these competitive relationships and illuminates almost every aspect of the biology of the host organisms. The future promises an even richer harvest of experimental and conceptual advances in this area, both enhancing our ability to control and prevent viral diseases and increasing our understanding of the mechanistic and evolutionary relationships between viruses and their hosts.

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Pathogenesis of viral infections

NEAL NATHANSON AND KENNETH L. TYLER

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INTRODUCTION

Viral pathogenesis deals with the interaction between a virus and its host. Included within the scope of pathogenesis are the stepwise progression of infection from virus entry through dissemination to shedding, the defensive responses of the host, and the mechanisms of virus clearance or persistence. Pathogenesis also encompasses the disease processes that result from infection, variations in viral pathogenicity, and the genetic basis of host resistance to infection or disease. A subject this broad cannot be treated in a single chapter, and this presentation focuses on the dissemination of viruses and their pathogenicity, while other aspects of pathogenesis will be dealt with in. Several detailed reviews of viral pathogenesis have recently been published (Tyler and Nathanson 2001; Nathanson et al. 2002).

SEQUENTIAL STEPS IN VIRAL INFECTION

One of the cardinal differences between viral infection of a simple cell culture and infection of an animal host is the structural complexity of the multicellular organism. The virus must overcome a number of barriers to accomplish the stepwise infection of the host, beginning with entry, followed by dissemination, localization in a few target tissues, shedding, and transmission to other hosts (Figures 1 and 2).

Entry

SKIN AND MUCOUS MEMBRANES

Some viruses (such as papillomaviruses, poxviruses, and herpes simplex viruses) will replicate in cells of the skin or mucous membranes. It is likely that infections with these viruses are initiated at breaks in the epithelium, just as the skin must be scratched to facilitate a take by vaccinia virus. Mucous membranes represent a minimal physical barrier to infection, but are bathed in mucus that contains immunoglobulin and provides protection by virtue of its viscous nature. Following infection of the skin, viruses may spread further by passage of intact virions or virus-infected macrophages and dendritic cells to the regional lymph nodes.

The epidermis consists of several layers of cells including, from the deepest to the most superficial: the basal germinal layer of dividing cells (malpighian stratum), the granular layer of dying cells (stratum granulosum), and the superficial layer of keratin (stratum corneum). Most viruses that replicate in the skin grow in the germinal cells, and some viruses, such as papillomaviruses, have a selective tropism for these cells (Lowy and Howley 2001). Poxviruses and herpes simplex viruses will also replicate in fibroblasts and macrophages in the dermis (Blank et al. 1951; Roberts 1962b). Some adenoviruses, coxsackievirus A24, and enterovirus type 70 are regularly associated with conjunctivitis, and the conjunctiva may represent a primary portal of entry in such instances. Table 1 lists representative viruses that invade via the skin and mucous membranes.

UROGENITAL TRACT

A considerable number of viruses cause sexually transmitted disease, which is usually acquired by exposure to virus-containing secretions. Infection is initiated either

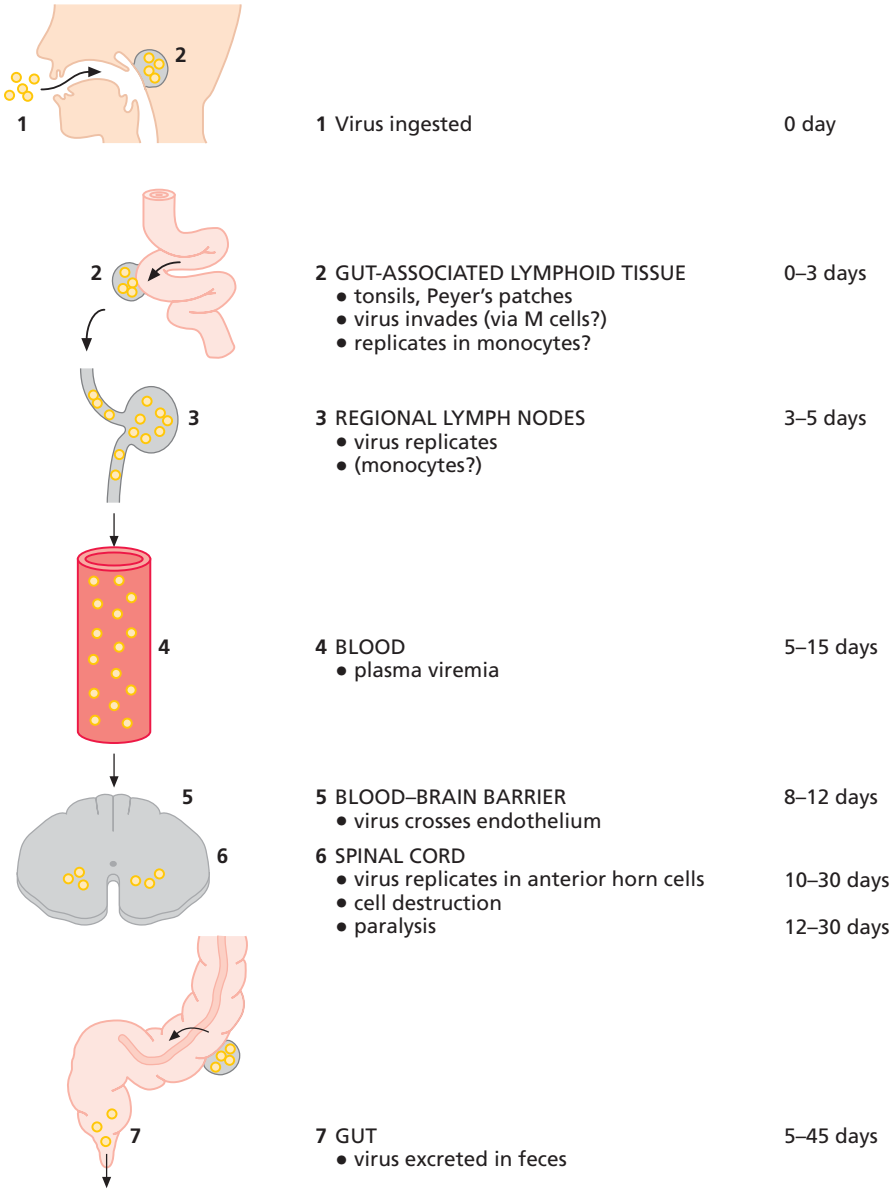

Figure 1 *Spread of* poliovirus*, an example of a virus that can disseminate through the blood (after Nathanson et al. 2002)*

by penetration through breaks in the skin or by direct invasion of the superficial epithelium of the mucous membranes (Pomerantz et al. 1988; Tan et al. 1993).

Some sexually transmitted viruses, such as hepatitis B virus (HBV) and human immunodeficiency virus (HIV), are associated with persistent viremia. Thus, contaminated blood, semen, or secretions may introduce HBV directly into the circulation via breaks in skin and membranes. HIV may be trapped on the surface of dendritic cells (Langerhans cells) in the epidermis and then transported to draining lymph nodes where it infects $CD4^+$ T lymphocytes. Also, HIV may infect T lymphocytes in the submucosal tissues, which are the first infected cells detected in experimental vaginal infection of macaques with simian immunodeficiency virus (SIV).

TRANSCUTANEOUS AND INTRAVENOUS INJECTION

Although the skin is a formidable barrier, mechanical injection that breaches the barrier is a natural route of entry for many viruses.

Arboviruses

Many animal viruses are maintained in nature by a cycle that involves a vector and a vertebrate host (Monath 1988), including over 500 individual viruses mainly in the families *Bunyaviridae*, *Flaviviridae*, *Reoviridae*, *Rhabdoviridae*, and *Togaviridae*. When the infected vector takes a blood-meal, virus contained in the salivary gland is injected. Although most viruses that are transmitted by an insect vector replicate in the vector, there are a few instances in

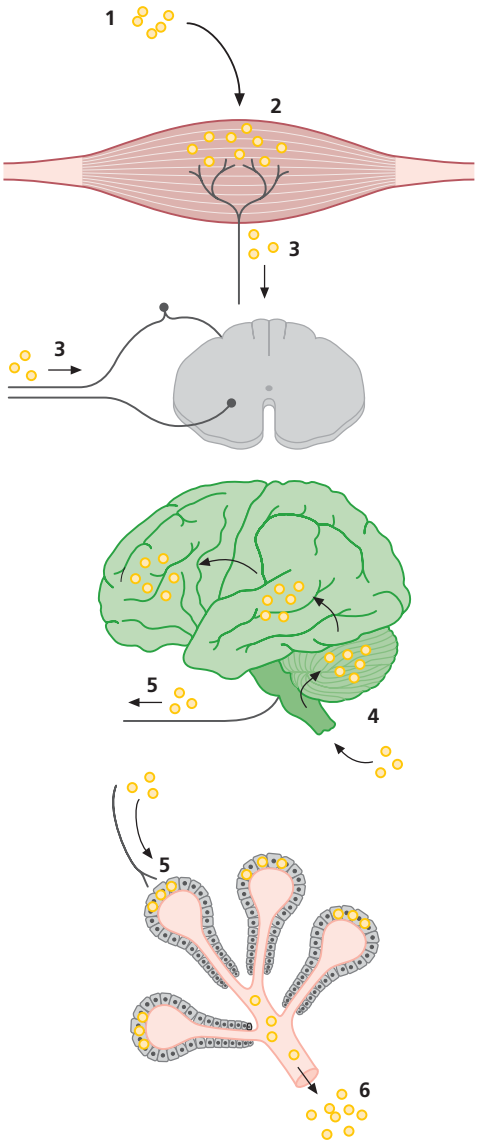

1 VIRUS ENTRY — Day 0
- by bite of rabid animal
- infected saliva is injected

2 STRIATED MUSCLE — Day 1–60
- virus replicates in myocytes

3 PERIPHERAL NERVES — Day 10–60
- virus enters nerve ending
- nucleocapsid carried by fast axoplasmic flow to spinal cord

4 CENTRAL NERVOUS SYSTEM — Day 12–60
- virus travels along neural processes, spreads and replicates
- neuronal dysfunction — Day 50–70
- clinical rabies, death

5 PERIPHERAL NERVES — Day 30–70
- virus travels along peripheral nerves from CNS
- invades salivary gland

6 SALIVARY GLAND — Day 40–70
- virus replicates in acinar cells
- virus is discharged in saliva

Figure 2 *Spread of rabies virus, an example of a virus that spreads through the neural route only (after Nathanson et al. 2002).*

Table 1 *Representative viruses that invade via skin and mucous membranes (modified after Nathanson et al. 2002).*

Site of entry	Route	Virus family	Representative example
Skin	Minor breaks	*Papillomaviridae*	Papillomaviruses
		Herpesviridae	Herpes simplex virus 1
		Poxviridae	Ectromelia virus
Conjunctiva	Contact	*Picornaviridae*	Enterovirus 70
		Adenoviridae	Adenoviruses
Oropharynx	Contact	*Herpesviridae*	Epstein–Barr virus
Genital tract	Contact	*Retroviridae*	HIV
		Papillomaviridae	Papillomaviruses
		Herpesviridae	Herpes simplex virus 2
Rectum	Contact	*Retroviridae*	HIV

which the vector acts only as a flying pin and apparently carries the virus mechanically from one vertebrate host to another. Examples are myxoma virus of rabbits and rabbit papillomatosis virus (Fenner 1983; Dalmat 1958).

Intramuscular injection: rabies

Rabies is the only virus that is maintained in many of its natural cycles by the bite of a sick animal (Baer 1991). Following transcutaneous and intramuscular injection, the virus may either enter the distal processes of peripheral nerves directly or replicate in striated muscle. It can then cross the neuromuscular junction and spread along peripheral nerves to the central nervous system.

Injection or transfusion

Virus may be transmitted accidentally by repeated use of contaminated needles, injection of a virus-contaminated therapeutic, transfusion with virus-contaminated blood or blood products, or tattooing. The agents most frequently involved are those that produce persistent viremias, such as hepatitis B, C, D viruses, cytomegalovirus, and human immunodeficiency virus. Contaminated needles play a major role in transmission, either by parenteral injection of drugs by abusers in developed countries, or by reuse of needles in developing countries. Following parenteral injection, virus appears to be widely disseminated, replicating in those cells for which it has a tropism.

OROPHARYNX AND GASTROINTESTINAL TRACT

The oropharynx and gastrointestinal tract are important portals of entry for many viruses, and enteric viruses may invade the host at a variety of sites from the oral cavity to the colon (Table 2). Some viruses produce localized infections that remain confined to the gastrointestinal tract, whereas others disseminate to produce systemic infection. Most enteric viruses, such as rotaviruses, infect the epithelium of the small or large intestine (Saif 1990; Little and Shadduck 1982) (Figure 3), but some enteroviruses, such as poliovirus, replicate in the lymphoid tissue of the gut and nasopharynx.

There are numerous barriers to infection via the enteric route. First, much of the ingested inoculum will remain trapped in the luminal contents and never reach the wall of the gut. Secondly, the lumen constitutes a hostile environment because of the acidity of the stomach, the alkalinity of the small intestine, the digestive enzymes found in saliva and pancreatic secretions, and the lipolytic action of bile. Thirdly, the mucus that lines the intestinal epithelium presents a physical barrier protecting the intestinal surface. Fourthly, phagocytic scavenger cells and secreted antibodies in the lumen can reduce the titer of infectious virus.

Acid labile viruses, such as rhinoviruses, cannot infect by the intestinal route. Viruses that are successful in using the enteric portal tend to be resistant to acid pH, proteolytic attack, and bile, and some may actually exploit the hostile environment to enhance their infectivity (Clark et al. 1981; Estes et al. 1981). hepatitis A and B viruses illustrate this point – both viruses are excreted in the bile into the intestinal tract, but hepatitis A virus survives and is excreted in an infectious form in the feces while hepatitis B virus is inactivated. As a consequence, only hepatitis A is transmitted as an enterovirus.

Lower gastrointestinal tract

The importance of anal intercourse as a risk factor for hepatitis B and HIV infection has led to the recognition that some viruses can gain entry through the lower gastrointestinal tract. HIV has been detected in bowel epithelium (Levy 1998), but the exact mechanism of HIV infection through the anocolonic portal remains to be determined.

RESPIRATORY TRACT

Respiratory infection may be initiated either by virus contained in aerosols that are inhaled by the recipient

Table 2 *Representative enteric viruses that do and do not cause gastroenteritis (modified after Nathanson et al. 2002).*

Disease	Replicate in the pharynx and/or gastroenteric tract	Virus family	Representative example
Gastroenteritis	Yes	*Astroviridae*	Astroviruses
	Yes	*Caliciviridae*	Norwalk virus
	Yes	*Coronaviridae*	Transmissible gastroenteritis virus of swine
	Yes	*Rotaviridae*	Rotaviruses
	Yes	*Parvoviridae*	Canine parvoviruses
	Yes	*Adenoviridae*	Adenoviruses 40,41
No enteric illness	Yes	*Picornaviridae*	Poliovirus
(± systemic illness)	Yes	*Picornaviridae*	Coxsackieviruses
	Yes	*Picornaviridae*	Enteroviruses
	No	*Picornaviridae*	Hepatitis A virus
	Yes	*Adenoviridae*	Adenoviruses

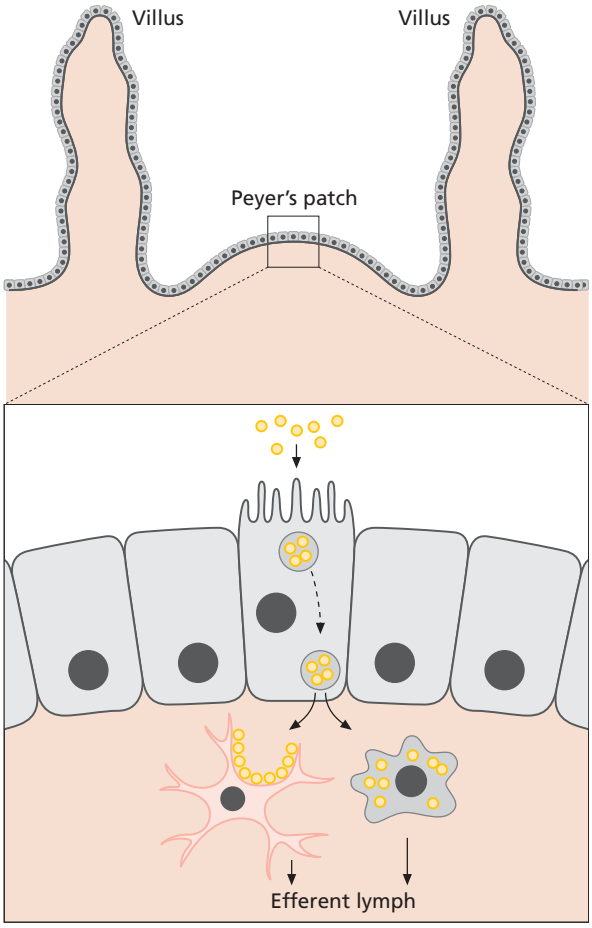

Figure 3 *Virus invasion of the intestine, showing the pathway taken by reovirus in the mouse. The virus binds to M cells, is carried by transcytosis to the basolateral surface, where it infects dendritic cells and macrophages in the lamina propria. This well-studied experimental model probably resembles many natural infections (after Wolf et al. 1981).*

host, or by virus that is contained in nasopharyngeal fluids and is transmitted by hand-to-hand contact. Important groups of viruses transmitted by the respiratory route include rhinoviruses, myxoviruses, adenoviruses, herpesviruses, and poxviruses (Table 3).

Aerosolized droplets are deposited at different levels in the respiratory tract depending upon their size: those over 10 μm diameter are deposited in the nose, those 5–10 μm in the airways, and those <5 μm in the alveoli (Lippmann et al. 1980). Once deposited, the virus must bypass several effective barriers to initiate infection. These barriers include phagocytic cells in the respiratory lumen, a covering layer of mucus, and ciliated epithelial cells that clear the respiratory tree of foreign particles. The temperature of the respiratory tract varies from 33°C in the nasal passages to 37°C (core body temperature) in the alveoli. Viruses (such as rhinoviruses) that can replicate well at 33°C, but not at 37°C, are limited to the upper respiratory tract and, conversely, viruses that replicate well at 37°C, but not at 33°C (such as influenza virus), mainly infect the lower respiratory tract. Most respiratory viruses initiate infection by replicating in epithelial cells lining the alveoli or the respiratory tree, but some viruses will also replicate in phagocytic cells located either in the respiratory lumen or in subepithelial spaces (Douglas et al. 1966; Roberts 1962a).

Spread

Once infection is established at the site of entry, most viruses will spread locally by cell-to-cell transmission of infection. Some viruses remain localized near their site of entry while others spread widely. One determinant of dissemination is the pattern of viral release from polarized epithelial cells. Viruses that are released only at the apical surface of infected cells tend to remain localized, while those that are released at the basal surface tend to disseminate (Tucker and Compans 1992; Jourdan et al. 1997). Usually, the first step in dissemination of the infection is the transport of virus via the efferent lymphatic drainage from the initial site of infection to the regional lymph nodes, either as free virions or in virus-infected phagocytic cells.

VIREMIA

The single most important route of dissemination is the circulation, which can potentially carry viruses to any site in the body. There are several sources of viremia. Virus may enter with the efferent lymphatic flow, or may be shed from infected endothelial cells or circulating mononuclear leukocytes (Charles et al. 1998; Fish et al. 1998). Viruses can circulate either free in the plasma phase of the blood (plasma viremia) or associated with formed elements (cell-associated viremia), and these two types of viremia have quite different characteristics and implications.

Passive viremia is used to describe the entry of a virus inoculum directly into the circulation following injection, without any preceding replication (Figure 4). Passive viremia typically occurs about 3–6 h after injection and disappears within 12–24 h. It has been estimated that about 0.1 percent of the virus in a subcutaneous inoculum enters the bloodstream (Griot et al. 1993b; Pekosz et al. 1995).

Active viremia is used to designate viremia caused by the active replication of virus in the host. In some instances, active viremia may occur in two phases, a primary viremia in which virus spreads from a focal site of entry and a secondary viremia generated after infection has occurred and virus has initially replicated in peripheral tissues, such as endothelium, tissue macrophages, liver, muscle, or other sites (Oaks et al. 1998). An example of an active viremia is shown in Figure 5, in which the virus appears in the plasma a few days after infection and lasts for about 1 week.

Table 3 *Representative viruses that invade via the nasopharynx or respiratory tract, according to localization of disease (modified after Nathanson et al. 2002).*

Localization of disease	Virus family	Representative example
Upper respiratory	*Picornaviridae*	Rhinoviruses
	Adenoviridae	Adenoviruses
Lower respiratory	*Coronaviridae*	Bovine coronaviruses
	Orthomyxoviridae	Influenza viruses
	Paramyxoviridae	Respiratory syncytial virus
	Bunyaviridae	Sin Nombre virus
No respiratory illness	*Togaviridae*	Rubella virus
(± systemic illness)	*Paramyxoviridae*	Mumps virus
	Bunyaviridae	Hantaan virus
	Arenaviridae	Lassa fever virus
	Reoviridae	Reovirus
	Papovaviridae	Murine polyomavirus
	Herpesviridae	Varicella-zoster virus
	Poxviridae	Variola virus

The initial lag period is the interval required for tissue replication and shedding into the circulation. Termination of viremia is often quite abrupt and coincides with the appearance of neutralizing antibody in the serum. When an infected animal is treated with an immunosuppressive regime, the viremia may not be cleared quickly, but persist for longer periods (Nathanson and Cole 1971).

Plasma viremia reflects a dynamic process in which virus continually enters the circulation and is removed. The rate of turnover of virus within the plasma compartment is best expressed as transit time, the average duration of a virion in the blood compartment. Typically, transit times range from 1–60 min and tend to decrease as the size of the virions increases (Mims 1964). Circulating viruses are removed by the phagocytic cells of the reticuloendothelial system, principally in the liver (Kupffer cells), and to a lesser extent in the lung, spleen, and lymph nodes. The fate of viruses that are ingested by Kupffer cells is quite variable and may be an important determinant of pathogenesis. Ingested virions may be degraded, may transit macrophages to underlying parenchyma without replicating, or may replicate in macrophages with or without spread of infection to underlying parenchymal cells. Once the host has developed circulating antibody, plasma virus is very rapidly neutralized in the circulation (Figure 5), and virus–antibody complexes bind to Fc receptors, facilitating removal by sessile macrophages lining the circulation. Under these circumstances, transit time is reduced by several fold (Igarishi et al. 1999; Zhang et al. 1999; Mittler et al. 1999).

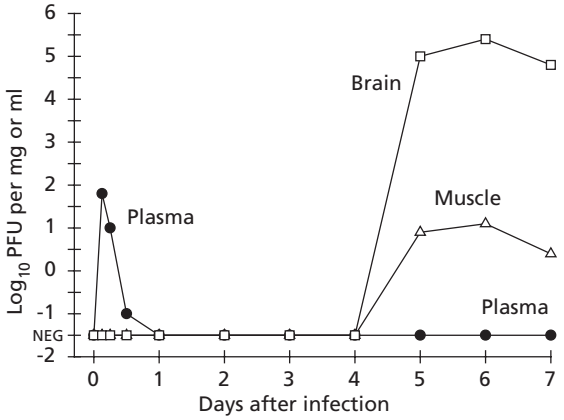

Figure 4 *Passive viremia, after injection of La Crosse virus in weanling mice. Viremia appears as a sharp peak limited to the first 12 h and stands out in profile since there is no active viremia in mice of this age. Even this transient viremia was sufficient to deliver enough virus to the target organ to initiate a lethal encephalitis (data from Pekosz et al. 1995).*

Although plasma viremias are usually shortlived, there are notable exceptions. Some viruses are bound by antibody, but the immune complex retains infectivity. In these instances, such as Aleutian disease virus (a parvovirus) and lactic dehydrogenase virus (a togavirus), plasma infectivity may resist neutralization with exogenous antibody but may be reduced by the addition of anti-Ig antibodies (Notkins et al. 1966; Porter and Larsen 1967). Other exceptions are instances where the infected host is tolerant of the viral antigens and fails to develop a sufficient level of antibody to clear viremia. Examples are hepatitis B virus and lymphocytic choriomeningitis virus (Figure 6).

A number of viruses replicate in cells that are found in the circulation, particularly monocytes, B or T lymphocytes, or (rarely) erythrocytes (Table 4). Under these circumstances, the viremia is cell-associated, although some virus may also be found free in the plasma compartment. Cell-associated viremias may be of short duration, as in the case of ectromelia and other poxviruses. However, in many instances of cell-associated viremia, virus titers are low, virus may be difficult to isolate from the blood, and viremia persists

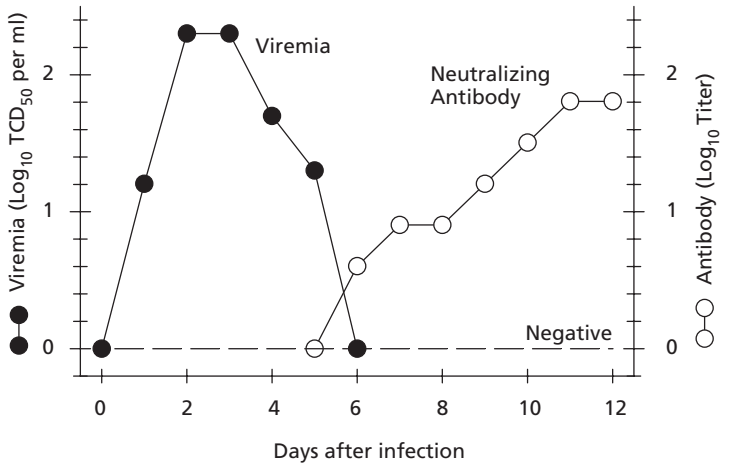

Figure 5 *Active plasma viremia after intramuscular injection of poliovirus in monkeys. Viremia lasts for about 1 week, and the clearance of viremia coincides with the appearance of neutralizing antibody in the serum (data from Nathanson and Bodian 1961).*

for the life of the host rather than terminating when neutralizing antibody appears (Figure 7).

Human immunodeficiency virus provides an important instance of cell-associated viremia (Hsia and Spector 1991; Bagasra et al. 1993; Levy 1998). HIV infects both $CD4^+$ T lymphocytes and monocytes in the blood, but most infected cells are $CD4^+$ T lymphocytes. The frequency of infected peripheral blood $CD4^+$ T lymphocytes varies from 0.2 to 10 percent in asymptomatic persons to between 2 and 60 percent in symptomatic patients, and 50–90 percent of infected T lymphocytes contain the genome in a latent state. In typical asymptomatic individuals, there are about 5 000 infected cells per ml blood. In addition to infected cells, free infectious HIV is also present in the plasma, the amount varying with the stage of disease. In typical asymptomatic individuals, it has been estimated that there are about 100 tissue culture infectious doses (TCD) per ml plasma, considerably fewer than the number of infected cells.

Antibody-dependent enhancement of infection is a special aspect of cell-associated viremia, which has been described mainly for dengue and other flaviviruses (Halstead and O'Rourke 1977; Gollins and Porterfield 1984). Dengue virus replicates primarily in monocytes, but it does not enter these cells readily. However, in the presence of low concentrations of antibody, infection is markedly enhanced, presumably because the virus–antibody complex is bound to Fc receptors on monocytes, enhancing viral entry.

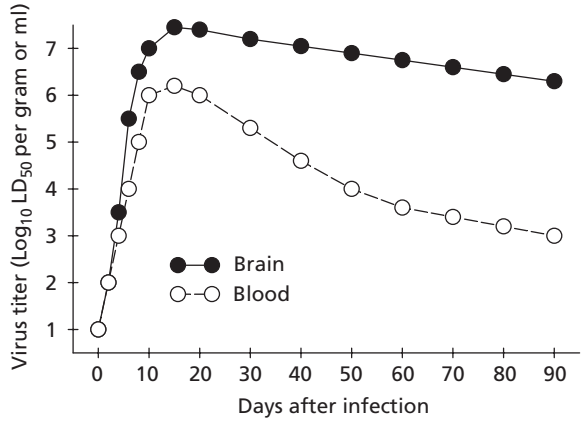

Figure 6 *Persistent plasma viremia associated with immunological hyporesponsiveness to the viral antigens. Adult mice were infected with lymphocytic choriomeningitis virus (LCMV) intracerebrally and were immunosuppressed with aminopterin on days 4 and 6 after infection. Mice infected in the same way but not treated with aminopterin died of acute choriomeningitis about 8 days after infection (data from Hotchin 1962).*

Tissue invasion from the blood

There are several ways in which a virus might cross the vascular wall (Johnson and Mims 1968; Johnson 1998), but the precise mechanism for penetrating the blood–tissue barrier is unknown in most instances.

- In many tissues, the capillary endothelial cells are not joined by tight junctions, and viruses can pass between them. One example is the fenestrated capillaries of the choroid plexus of the central nervous system. Penetration at this site provides access to the epithelial cells of the choroid plexus and can be followed by entry into the cerebrospinal fluid and spread to the ependymal cells lining the ventricles (Herndon et al. 1974).
- Virus may be transported through endothelial cells by a process of endocytosis, translocation of virus-containing vesicles, and subsequent release by exocytosis at the basal surface of the endothelial cell (Johnson and Mims 1968; Pathek and Webb 1974).
- Some viruses are capable of replicating within endothelial cells and are released from these cells into the surrounding tissues. Examples include selected picorna-

Table 4 *Viruses that replicate in circulating blood cells, a representative list*

Cell type	Virus family	Representative virus	Duration viremia	References
Monocytes	*Flaviviridae*	Dengue	acute	Halstead and O'Rourke (1977)
	Togaviridae	Rubella	acute	Chantler and Tingle (1982)
	Paramyxoviridae	Measles	acute	Esolen et al. (1993)
	Arenaviridae	LCMV	persistent	Matloubian et al. (1993)
	Retroviridae	HIV	persistent	Collman et al. (1989)
		Ovine visna maedi	persistent	Gendelman et al. (1985)
		EIAV	persistent	Oaks et al. (1998)
	Herpesviridae	Cytomegalo	persistent	Taylor-Weideman et al. (1991)
	Poxviridae	Mousepox	acute	Fenner (1949)
		Rabbit myxoma	acute	Fenner (1983)
B lymphocytes	*Retroviridae*	Murine leukemia	persistent	Kozak and Ruscetti (1992)
	Herpesviridae	Epstein–Barr	persistent	Ho (1981)
T lymphocytes	*Retroviridae*	HIV	persistent	Bagasra et al. (1993)
		HTLV-I	persistent	Depper et al. (1984)
	Herpesviridae	Human herpes 6, 7	acute	Takahashi et al. (1989)
Erythroblasts	*Reoviridae*	Colorado tick fever	acute	Oshiro et al. (1978)

EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus; LCMV, lymphocytic choriomeningitis virus.

viruses, retroviruses, alphaviruses, and parvoviruses (Johnson 1965; Baringer and Nathanson 1972; Friedman et al. 1981; Pitts et al. 1987).

- Viruses that cause a cell-associated viremia may invade by an alternate mechanism, namely, as passengers in infected lymphocytes or monocytes, a mechanism that has been suggested for tissue invasion of HIV and other lentiviruses (Haase 1986). Infected mononuclear cells can cross the blood–tissue barrier as part of their normal trafficking pattern, or may be actively recruited into sites of inflammation.

NEURAL SPREAD

Some viruses can disseminate by spreading through the axons of peripheral nerves (Table 5). Although less

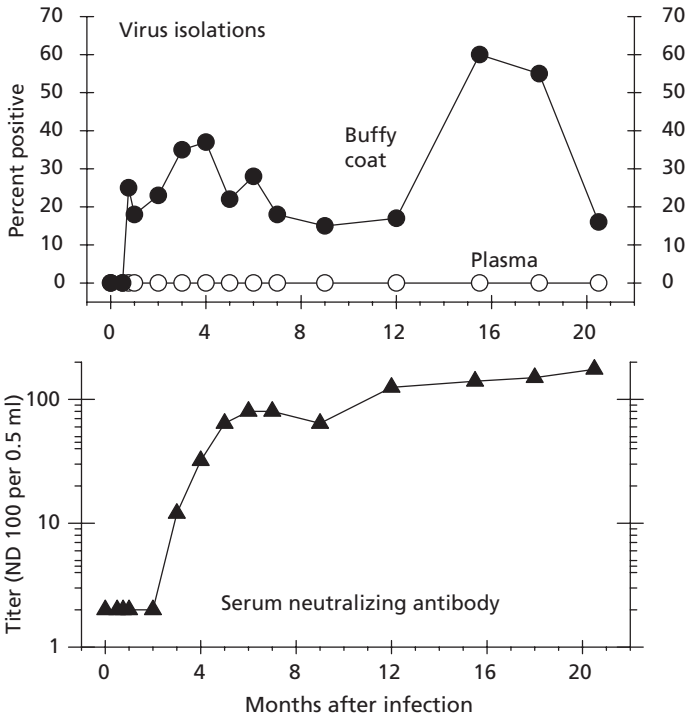

Figure 7 *Cell-associated viremia in sheep infected with visna maedi virus, a* Lentivirus*. Virus was isolated by cocultivation of buffy coat cells with indicator cells and was never isolated from the plasma. The frequency of virus isolations was unrelated to the appearance or titer of neutralizing antibody (data from Petursson et al. 1976).*

Table 5 *Viruses that spread by the neural route, a representative list*

Virus family	Representative virus	Notes	References
Picornaviridae	Poliovirus	Neuroadapted clones only; natural isolates are viremogenic	Nathanson and Bodian (1961); Bodian (1955)
Flaviviridae	Yellow fever	Neuroadapted clones only; natural isolates are viremogenic	Strode (1951)
Alphaviridae	VEE	Hematogenous invasion of peripheral nerves	Charles et al. (1995)
	Semliki Forest	Age-specific olfactory invasion	Oliver and Fazakerley (1998)
Coronaviridae	Mouse hepatitis	Neural spread after intranasal infection	Perlman et al. (1989); Barnett and Perlman (1993)
Rhabdoviridae	Rabies	Exclusively neural	Baer (1991)
Reoviridae	Reo	Type 3 uses neural route	Tyler et al. (1986)
		Type 1 spreads by the hematogenous route	Morrison et al. (1991)
Herpesviridae	Herpes simplex 1, 2	Exclusively neural in adults; pantropic in newborns	Cook and Stevens (1973)
	Pseudorabies	Used for neural pathways	Enquist (1994)

Venezuelan equine encephalitis virus (VEE).

important than viremia, neural spread plays an essential role for certain viruses (such as rabies viruses and several herpesviruses), while other viruses (such as poliovirus and reovirus) can utilize both mechanisms of spread (Johnson 1998). Different strains of the same virus may differ markedly in their ability to use the neural route (Nathanson and Bodian 1961; Tyler et al. 1986; Ren et al. 1990; Barnett and Perlman 1993), as shown in Table 6. Also, different viral variants may utilize different neuronal pathways (Card et al. 1998).

It seems certain that viruses enter neurons by mechanisms similar to those involving entry into other cell types. The uncoated nucleocapsid is then carried passively along axons or dendrites, probably by fast axoplasmic flow (Maratou et al. 1998; Ohka et al. 1998). After the transport process, the virus may replicate in the perikaryon, but this is a much slower process and is not required for neural spread. There are several lines of evidence for this reconstruction of neural spread:

Table 6 *Different mode of dissemination of a neurotropic and a pantropic strain of the same virus*

	Neuroadapted MV strain		Pantropic Mahoney strain	
	Control	Block	Control	Block
Paralysis	25/26	0/11	19/19	18/20
Site of initial paralysis				
Injected leg	24	–	3	5
Other	1	–	16	13
Incubation to paralysis	5 days	–	7 days	7.5 days

Comparison of a neuroadapted and a natural pantropic isolate of poliovirus following intramuscular injection in cynomolgus macaques. The neuroadapted virus spreads only by the neural route, causes initial paralysis in the injected limb and fails to paralyse after a nerve block, while the pantropic strain spreads by viremia, does not cause localised initial paralysis, and is not impeded by nerve block. (Data from Nathanson and Bodian 1961).

- Neural transection will block neural spread (Table 6) and the rate of movement (>5 cm per day) is similar to the rate of axoplasmic transport (>10 cm per day) (Kristensson et al. 1971; Tyler et al. 1986; Tsiang 1993).
- Drugs, such as colchicine, which block fast axoplasmic transport by disrupting microtubules, also block the transport of viral genomes (Tsiang 1979).
- Temperature-sensitive strains of herpesvirus (which cannot replicate at body temperature) are able to move from a peripheral site of inoculation to a sensory ganglion, such as the trigeminal ganglion, and may be recovered by culturing the explanted ganglion at temperatures that are permissive for temperature-sensitive clones (Sederati et al. 1993).
- Several days after initiation of neural spread, the nucleocapsids of viruses, such as rabies virus, herpes simplex virus, and pseudorabies virus (an alphaherpes virus), may be seen within axons (Rabin et al. 1968).

Localization or tropism

One of the salient features that distinguishes viruses is their tropism within the animal host (see Figures 1 and 2). The names of individual viral diseases often reflects the organs or tissues that are involved. Thus, smallpox, poliomyelitis, and hepatitis each have their characteristic features. Localization of infection, or viral tropism, is regulated both by viral dissemination and cellular susceptibility. Disease localization may not correspond to the distribution of infection since it reflects both the spread of the virus and the host response to infection.

VIRAL DISSEMINATION

The localization of a virus is determined at several phases of infection, including penetration at the portal of entry, systemic spread by viremia or the neural route, and the invasion of local organs or tissues.

Portal of entry

The physical characteristics of a virus that influence its ability to survive in the external environment play a role in circumscribing the portal of entry. Thus, many picornaviruses are enteroviruses that are capable of surviving in the hostile environment of the enteric tract, but some picornaviruses, such as the rhinoviruses, are acid labile, cannot survive gastric acidity, and are confined to the respiratory tract. The biological characteristics of the virus also plays a role in its initial localization. Again, the rhinoviruses replicate well at 33°C, the temperature of the nasopharynx, while influenza virus has a replication optimum of 37°C that permits it to infect the lower respiratory tract. These limiting factors are particularly important for the localization of viruses that fail to spread systemically.

Viremia and neural spread

Viremia plays a key role in systemic spread of most viruses (Figures 1 and 2), and the viremogenicity of the specific viral strain determines its likelihood of reaching target organs. Conversely, the inability to initiate viremia explains the local nature of the infections caused by many respiratory and enteric viruses. The importance of viremia in determining localization is illustrated in Table 7, which indicates that in adult mice La Crosse virus (a bunyavirus) has the capability of initiating a lethal encephalitis after intracerebral injection of a minimal dose (approximately 1 plaque forming unit (pfu)), but fails to cause illness after subcutaneous injection of $>10^7$ pfu because no viremia is produced. Likewise, poliovirus, in spite of its high potential to induce paralysis, is estimated to cause only one paralytic case per 150 human infections, presumably because it rarely generates sufficient viremia to reach the spinal cord (Figure 8).

For viruses that spread primarily by the neural route, the ability to disseminate is also an important determinant of localization (Gromeier and Wimmer 1998). For instance, herpes simplex virus is capable of initiating a very severe encephalitis in humans if the virus reaches the central nervous system. However, most infections are either silent, or limited to mucocutaneous lesions, and encephalitis occurs at a rate of less than one case per 1 000 primary infections, indicating that the virus rarely reaches its potential target in sufficient dose to localize in the brain.

CELLULAR RECEPTORS

Most viruses are quite selective in the cell types that are infected in vivo and this selectivity plays a significant role in localization. One major determinant of cellular susceptibility is the presence of viral receptors, namely, molecules on the cellular surface that act as receptors for the specific virus, usually by binding a protein on the virion surface (the viral attachment protein). From studies of many viral receptors (Figure 9), several generalizations can be made (Wimmer 1994a; Dimitrov 2000):

- Although receptors have been identified for only a small proportion of viruses, it is generally thought that specific receptors are necessary for efficient infection by most viruses. Presumably viruses have evolved to utilize, as receptors, cellular surface molecules that subserve other functions required for normal cellular activity.
- A variety of molecules, including glycoproteins, glycolipids, and glycosaminoglycans, can serve as viral receptors. The domain of the receptor that binds the virus may be either a polypeptide sequence or a carbohydrate moiety, often located at the external tip of the receptor molecule.
- Different viruses employ different cellular receptors, although a few receptors are used by more than one virus.
- Many viruses are capable of utilizing more than a single cellular molecule as a receptor.
- Different isolates of the same virus may prefer different receptors, and a specific virus isolate may alter its receptor preference by selection of a mutant viral attachment protein during serial passage in animals or cell cultures (Borza and Hutt-Fletcher 2002).
- In some instances, viral entry requires two or more different coreceptors on the cell surface. Usually, both coreceptors are necessary and neither alone is sufficient.

Table 7 *The relative virulence of viruses depends on variables such as the route of entry and age of the host.*

	PFU per LD_{50}			
Virus clone	IC infection suckling mice[a]	SC infection suckling mice	IC infection adult mice	SC infection adult mice
Wild-type La Crosse original	~1	~1	~1	$>10^7$
Attenuated B.5 clone	~1	$>10^5$	$>10^6$	$>10^7$

Illustrated by two variants of La Crosse virus, a bunyavirus, when injected by different routes in suckling and adult mice. One clone, La Crosse/original, is a wild-type isolate and the other clone, B.5, is a laboratory-derived attenuated strain. Note that the two viruses exhibit striking differences in virulence upon intracerebral injection of adult mice or subcutaneous injection of suckling mice, but that both appear similar in their high virulence for suckling mice inoculated intracerebrally and similar in their low virulence for adult mice inoculated subcutaneously. Virulence is expressed as the ratio of pfu per LD_{50}; the lower the number the more virulent the virus. IC, intracerebral; SC, subcutaneous (data from Endres et al. 1990).

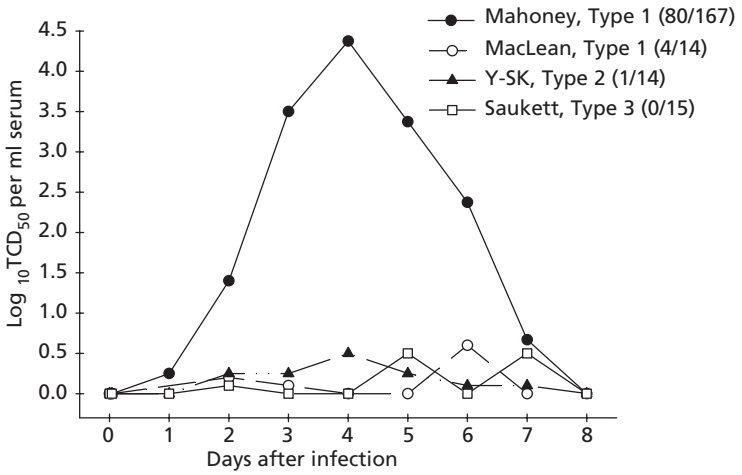

Figure 8 *Viremia as a determinant of viral virulence. Type 1 Mahoney virus produced the highest level of viremia and the highest frequency of paralysis (48%) while the other three (low viremia) strains produced an aggregate paralytic rate of 12% (the frequency of paralysis is shown in parentheses) (data from Bodian 1954).*

- Entry of a virus into a potential host cell is a multistep process in which binding to the receptor is only the initiating event.
- Not all cells that express the viral receptor are capable of supporting the complete cycle of viral replication.

These generalizations are illustrated by human immunodeficiency virus (HIV) (Levy 1998; Weiss 1994). The CD4 molecule is a type I glycoprotein, which is expressed by certain subsets of T lymphocytes and monocytes/macrophages, and subserves signaling between lymphoid and monocytic cells. CD4 has four immunoglobulin-like domains in its extracellular amino terminus, and the outermost of these domains contains a region that binds a specific site on gp120, the surface glycoprotein of HIV. The primary cellular targets of HIV are $CD4^+$ T lymphocytes and

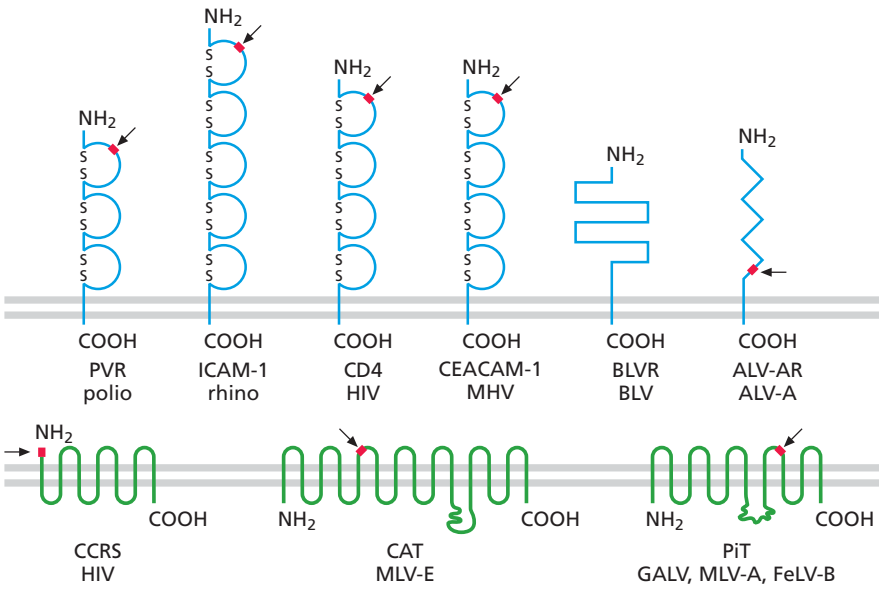

Figure 9 *Molecular backbone cartoons of some glycoprotein viral receptors. Receptors diverge widely in their structure and in their physiological function. The amino and carboxy termini are shown, together with important disulfide bonds and the probable domains that bind virus. ALV-AR, avian leukosis virus A receptor; BLVR, bovine leukemia virus receptor; CAT, cationic amino acid transporter; CCR5, chemokine receptor 5; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; ICAM, intercellular adhesion molecule; PiT, inorganic phosphate transporter; PVR, poliovirus receptor; Viruses: polio, poliovirus; rhino, rhinovirus, major group; HIV, human immunodeficiency virus; MHV, mouse hepatitis virus (a coronavirus); BLV, bovine leukemia virus; ALV-A, avian leukosis virus A; MLV-E, murine leukemia virus E; GALV, gibbon ape leukemia virus; MLV-A: murine leukemia virus A; FeLV-B; feline leukemia virus B (after Wimmer 1994b; Weiss and Tailor 1995; Holmes, 1997).*

monocytes/macrophages and the distribution of CD4 explains this cellular localization.

However, the expression of CD4 on the surface of a cell is not sufficient to assure viral entry. HIV utilizes a coreceptor, several members of the family of chemokine receptors, most frequently CCR5 or CXCR4 (Berger et al. 1999). HIV-1 strains can utilize either CCR5 or CXCR4 or, in some instances, both coreceptors. CCR5 is expressed on macrophages, while CXCR4 is expressed on T-lymphocyte cell lines, explaining why some HIV-1 strains will infect macrophages but not T-cell lines, while the reverse is true for other HIV-1 strains (Figure 10). Furthermore, there are important biological distinctions between HIV-1 isolates that use R5 or X4 coreceptors. Under natural conditions, infections are mainly initiated by R5 viruses, while X4 viruses are more frequent in patients with advanced AIDS. In experimental infections of monkeys, R5 viruses preferentially infect lymph nodes while X4 viruses replicate preferentially in gut-associated lymphoid tissue (Harouse et al. 1999).

Poliovirus offers another example of localization determined by receptors (Wimmer et al. 1994; Freistadt 1994; Evans and Almond 1999). It was long known that poliovirus would only replicate in primate tissues, and it had been deduced that this was because a putative poliovirus receptor (PVR) was expressed only by primate cells, since nonprimate mammalian cells would support poliovirus replication if transfected with viral RNA, thereby bypassing the requirement for a viral receptor. Using this conceptual framework, the PVR was cloned and sequenced, and when transfected into rodent cells it rendered them permissive for the virus (Mendelsohn et al. 1989). Furthermore, when transgenic mice were created that expressed the PVR, the animals developed typical clinical and pathological poliomyelitis after intracerebral injection of human poliovirus (Ren et al. 1990).

However, the relationship of the PVR to viral localization appears to be more complex, since the PVR alone may not confer cellular susceptibility. Thus, the PVR is expressed quite ubiquitously in human tissues, but most of these tissues are not infected by the virus. Also, PVR transgenic mice cannot be readily infected by the oral route (the natural route of infection in primates) and pantropic strains of poliovirus that are viremogenic in primates fail to cause viremia in these mice (Ren and Racaniello 1992).

OTHER MECHANISMS OF CELLULAR LOCALIZATION

Cellular susceptibility may be determined at post-entry steps in the replication cycle. Some viruses are quite fastidious and can replicate only in selected cell types, thus accounting for their selective tropism. For example, some of the murine leukemia viruses appear to require enhancers or promoters that are supplied only in certain cell types (Evans and Morrey 1987). In these instances, genetic studies have mapped the viral determinants of replication to the unique 3′ (U3) noncoding region of the viral genome (Hopkins et al. 1989).

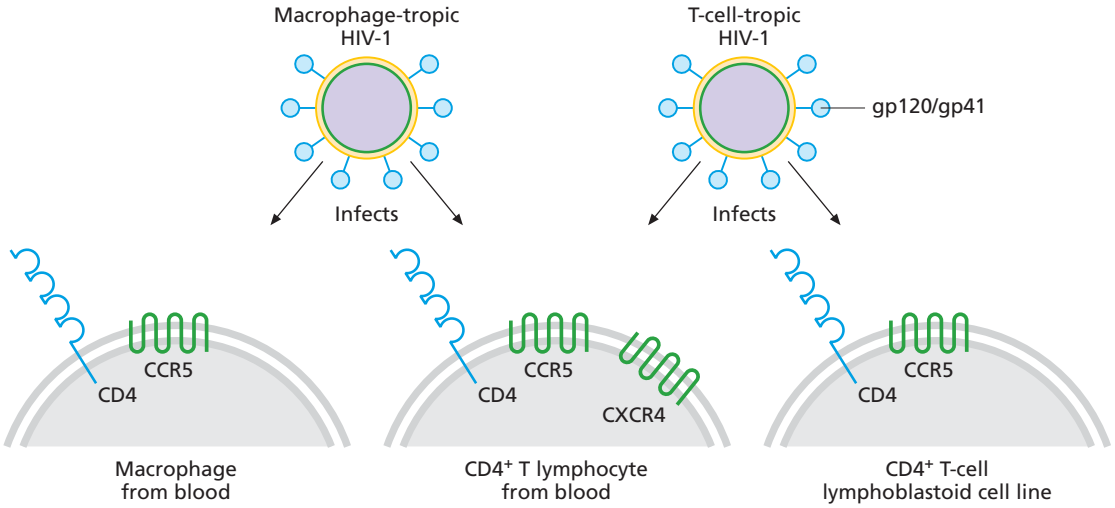

Figure 10 *HIV-1 is an example of a virus that requires a primary receptor and a coreceptor to infect target cells efficiently. The primary receptor is the CD4 molecule, a protein expressed on the surface of certain subsets of lymphocytes (so called CD4⁺ cells). The gp120 spike glycoproteins of all HIV strains can bind to human CD4. In addition, virus entry requires a coreceptor, which is either CCR5 or CXCR4. These proteins are members of a large family of molecules that serve as chemokine receptors on the surface of lymphoid cells or macrophages. Some HIV-1 isolates are macrophage-tropic because their gp120 spikes use CCR5, a chemokine receptor that is expressed on the surface of macrophages, while other isolates are T-cell-tropic because they utilize the CXCR4 molecule, another chemokine receptor expressed on the surface of T-cell lines. Both kinds of viruses can replicate on peripheral blood mononuclear cells (PBMC), lymphocytes freshly cultured from peripheral blood, since these cells express both coreceptors. By the same token, some HIV-1 isolates (not shown) are dual-tropic since they can utilize both coreceptors. (Recent studies have shown that macrophages express low levels of CXCR4 at concentrations insufficient for entry of T-cell-tropic HIV-1.)*

A number of enveloped viruses encode glycoproteins that require cleavage to activate either their viral attachment function or their fusion function. Absent proteolytic cleavage virions are not infectious. An example is Newcastle disease virus, a paramyxovirus of birds. Virulent isolates of Newcastle disease virus encode a fusion protein that is readily cleaved by furin, a proteolytic enzyme present in the Golgi apparatus, so that the protein is activated during maturation prior to reaching the cell surface, and before budding of nascent virions. This makes it possible for virulent strains of the virus to infect many avian cell types, thereby increasing its tissue host range, and causing systemic infections that are often lethal. In contrast, avirulent strains of Newcastle disease virus encode a variant fusion protein that is not cleaved during maturation in the Golgi, so that nascent virus requires activation by an extracellular protease. The required protease is found only in the respiratory or enteric tracts, thereby limiting tropism to surface cells and conferring an attenuated phenotype on the virus (Nagai 1995).

LOCALIZATION OF VIRAL PATHOLOGY

Pathological lesions were traditionally used to trace the spread of viral infections. However, the pathological process initiated by viral infections does not always co-localize at the sites of viral replication. Some viruses kill all cells in which they replicate and, in such instances, viral disease tends to mirror the localization of viral replication. In contrast, certain viruses will destroy some, but not all, of the cells in which they replicate. For instance, viruses that are cytocidal because they trigger a cascade of events leading to cellular apoptosis may replicate in a noncytodical fashion in cells in which apoptosis is blocked by the protective effect of the bcl-2 proto-oncogene (Griffin et al. 1994).

A number of viruses initiate immune-mediated cellular pathology in which $CD8^+$ T lymphocytes attack cells that present viral peptides associated with MHC class I proteins. If the virus infects both cells that do and do not express MHC class I molecules, then the MHC negative cells are not attacked. This phenomenon is thought to explain the sparing of neurons after infection by certain viruses, since neurons do not express class I molecules (Rall et al. 1995). Immune-mediated lesions may be even more indirect, such as those that are caused by antigen–antibody complexes. In this instance, the lesions are seen in locations such as the glomerulus of the kidney, which cannot handle the large accumulations of macromolecular complexes, while the virus has replicated at sites distant from the kidney (Oldstone 1975). Dengue hemorrhagic fever is thought to be initiated by the antiviral immune response that leads to a consumptive coagulopathy (Rothman and Ennis 1999).

For oncogenic viruses, the tumors may represent a distant echo of virus localization (Figure 11).

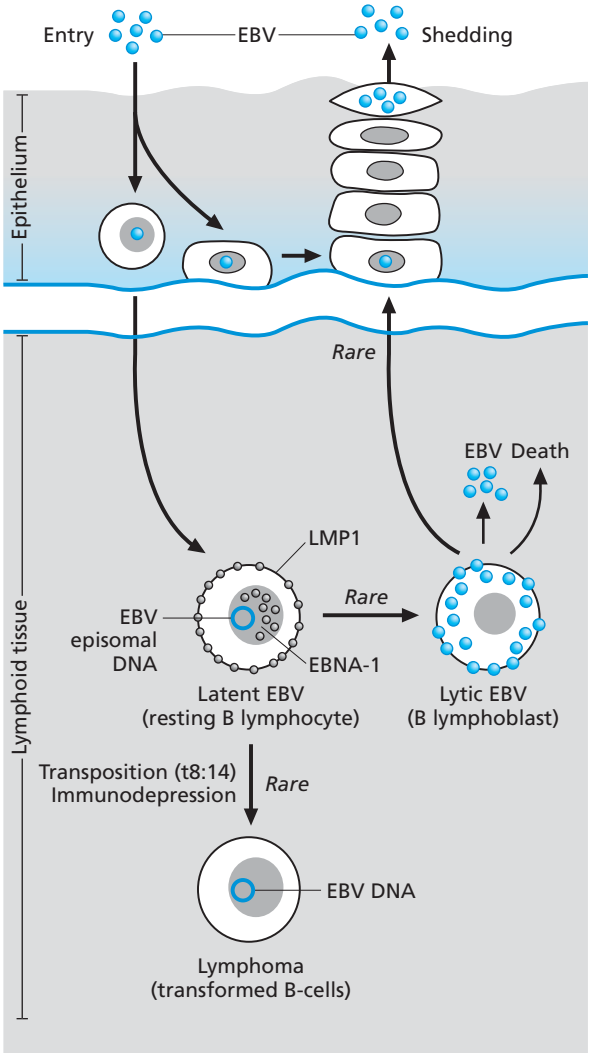

Figure 11 *The localization of pathological lesions may only be a remote echo of the distribution of the causal virus, as exemplified by this diagrammatic summary of possible pathogenesis of EBV and Burkitt's lymphoma. The virus initially infects epithelial cells and B lymphocytes. In epithelial cells, EBV causes both latent and lytic infection, the latter resulting in virus shedding into the oral cavity. EBV persists in resting B cells as a latent infection which is rarely activated to produce new virus. During latent persistence in lymphocytes, several EBV genes are expressed, particularly EBNA-1 (EB nuclear antigen) and LMP1 (latent membrane protein) which immortalizes B cell clones. In a few infected people, persistently infected B cells undergo further transformation into tumor cells, producing a variety of lymphomas, such as Burkitt's lymphoma, which is frequently associated with a chromosomal transposition (t8:14) that up-regulates the* c-myc *oncogene (after Nathanson et al. 2002).*

Epstein–Barr virus (EBV) infects epithelial cells and B cells (Burkitt 1958; Epstein et al. 1964; Hutt-Fletcher 1999; Cohen 2000). EBV causes both a latent and a lytic infection in epithelial cells from which it is shed into the oral cavity. Latently infected B cells are immortalized, and – rarely – these B cells undergo further transforma-

tion into lymphomas by virtue of additional genetic events such as chromosomal transposition (t8:14). Lymphoma cells often carry EBV DNA as nuclear episomes.

Shedding

Acute viral infections are characterized by brief periods of intensive virus shedding into respiratory aerosols, feces, urine, or other bodily secretions or fluids. Persistent viruses are often shed at relatively low titers, but this may be adequate for transmission over the prolonged duration of infection.

GASTROINTESTINAL AND RESPIRATORY TRACT

The course of shedding of enteric viruses can be readily followed. For instance, poliovirus is shed in the pharynx for 2–4 weeks and in the feces for 1–2 months after infection, although viremia only lasts about 1 week (Figure 5). Respiratory viruses may be transmitted by aerosols generated by coughing or sneezing or by virus-containing nasopharyngeal fluids that are transmitted by hand-to-hand contact. Most acute respiratory viruses are excreted over a relatively short period and infected persons are potential transmitters for only about 1 week (Figure 12). Transmission may begin a few days before onset of symptoms (Douglas et al. 1966).

SKIN, MUCOUS MEMBRANES, ORAL AND GENITAL FLUIDS, SEMEN, AND MILK

Although many viruses replicate in the skin, relatively few are spread from skin lesions. Exceptions are herpes simplex (labial transmission), varicella-zoster (rarely spread from zoster to cause chickenpox), papillomaviruses, and Ebola virus, a filovirus which causes hemorrhagic fever (Peters 1997). Those viruses, such as herpes simplex type 2 and papillomaviruses, which cause sexually transmitted disease, are often shed from lesions of the genital mucous membranes. In addition, some viruses, such as rabies virus and mumps virus, replicate in the salivary glands and are discharged into the saliva to enter the oral cavity.

Several viruses are shed in the semen, including hepatitis B and human immunodeficiency virus (Alter et al. 1977; Levy 1998). A number of viruses are excreted in colostrum and milk, including cytomegalovirus, mumps, rubella, and ovine visna maedi virus (de Boer and Houwers 1979) and HIV.

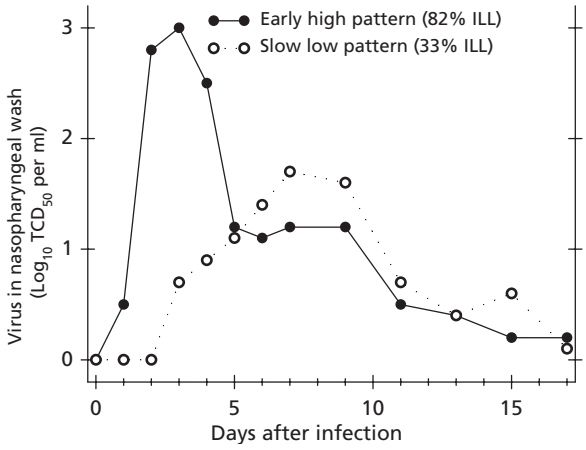

Figure 12 *The excretion of a typical respiratory virus, rhinovirus type 15, following inoculation of human volunteers by the transnasal route. The subjects demonstrated either an early onset high concentration pattern associated with a high frequency of respiratory symptoms, or a late onset low concentration pattern associated with a low frequency of symptoms (data from Douglas et al. 1966).*

BLOOD AND URINE

Blood is also an important source of transmitted virus, particularly for those viruses that produce persistent plasma or cell-associated viremias. Hepatitis B, C, D viruses, human immunodeficiency virus, HTLV-1, HTLV-2, and cytomegalovirus are among the important human pathogens shed in this manner. Arboviruses usually cause short-term acute viremias, and are transmissible only if an appropriate vector ingests a blood meal during this brief interval. In view of the limited amount of blood taken, the viremia must reach considerable levels if it is to be transmitted.

A number of viruses have been isolated from the urine, but viruria is probably not important for transmission of most viruses. A special exception are certain zoonotic viruses, such as the arenaviruses and the hantaviruses, which cause lifelong virurias in their natural rodent hosts, leading to human infection through exposure to aerosolized dried urine (Gonzalez-Scarano and Nathanson 1990). Cytomegalovirus spreads readily among young children in enviroments contaminated by urine (such as creches and play centers).

SURVIVAL IN THE ENVIRONMENT

The probability that a virus will be transmitted depends both upon the intensity and duration of shedding, and upon its ability to survive in the environment. Viruses differ in their ability to survive in aerosols and, somewhat surprisingly, under conditions of low humidity, enveloped viruses appear to be better able to retain viability than do nonenveloped viruses (deJong and Winkler 1968). To be excreted in an infectious form, enteric viruses must survive the harsh environment of the lower gastrointestinal tract.

Transmission

Once shed, there are several means by which a virus is transmitted from host to host in a propagated chain of infection. Probably the most important mechanism

is contamination of the hands of the infected transmitter from feces, oral fluids, or respiratory secretions expelled during coughing or sneezing. The virus is then passed by hand-to-hand contact leading to oral, gastrointestinal, or respiratory infection. A second common route is inhalation of aerosolized virus. A third significant mechanism involves direct person to person contact (oral–oral, genital–genital, oral–genital, or skin–skin). Finally, indirect person-to-person transmission can occur via blood or contaminated needles. Common source transmissions are less frequent but can produce dramatic outbreaks, via contaminated water, food products, or biologicals, such as blood products and vaccines.

The success of a virus, as a life form, can be defined as its ability to be perpetuated over the millenia. In turn, perpetuation of a virus depends in part upon its efficient transmission, particularly for viruses that cause only acute infections. The relative transmissibility of a virus in different populations is reflected in the age-specific profile of immune individuals, as illustrated in Figure 13 for hepatitis A virus. For viruses that can persist in individual hosts, perpetuation is determined by transmission over the lifetime of the host, and in some instances, by the ability to be vertically passed to offspring of the host, either by transplacental or perinatal routes, or as germline genes. If transmissibility is reduced sufficiently, then a given virus will disappear from a defined population. This can happen in nature, when a virus spreads so widely in a specific population that it eliminates most susceptibles and burns out. Transmissibility can also be reduced by immunization which, under certain circumstances, can be used to eradicate a virus from its host population. Smallpox is the seminal example of planned eradication (Fenner et al. 1988), and the elimination of wild poliovirus from the western hemisphere is another important illustration.

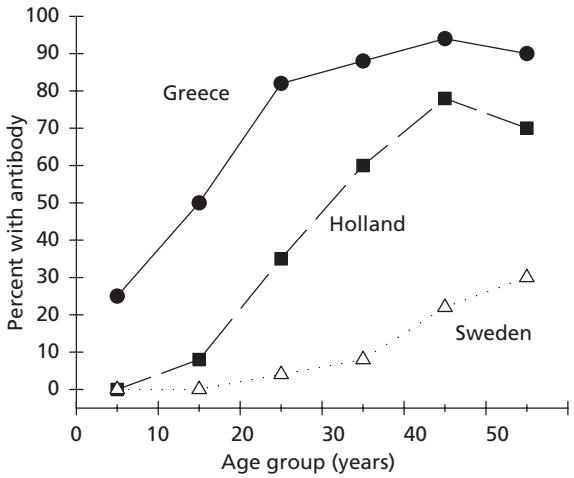

Figure 13 *Differences in transmissibility of hepatitis A virus in three European countries, as reflected in the different age-specific profiles of antibody prevalence (data from Frosner et al. 1979).*

VIRULENCE OF VIRUSES

Virulence refers to the ability of a virus to cause illness or death in an infected host, relative to other isolates or variants of the same agent. From the beginning of experimental virology, it has been recognized that different strains of a given virus may vary in their ability to cause disease. The study of virulence variants can provide important insights into pathogenesis, and variation in viral phenotype must be given consideration in design and interpretation of in vivo studies of the dissemination, localization, and pathological consequences of viral infection. Study of virulence also has practical implications because it carries the potential for development of attenuated live virus variants that can be used as vaccines, a principle that was initially recognized by Jenner in 1798 when he introduced vaccinia virus for the prevention of smallpox (Fenner et al. 1988).

There are two distinct approaches to the study of virulence. If virulence is regarded as a property of the virus, it is possible to map the genetic determinants that underlie the virulence phenotype. The other approach to virulence is through a study of pathogenesis, which describes the differences in infections with viral variants of different virulence. Pathogenic measures of virulence may be quantitative, involving the rate of viral replication or number of cells infected. Alternatively, differences in virulence may be qualitative, since variants of a single virus may differ markedly in their cellular tropism, their mode of dissemination in the infected host, or the disease phenotype that they produce.

Measures of viral virulence

RELATIVE NATURE OF VIRAL VIRULENCE

Virulence is not an absolute property of a virus strain but depends upon the dose of virus, the route of infection, and the age, gender, and genetic susceptibility of the host. Two viruses that differ in virulence in one experimental paradigm may exhibit similar degrees of virulence in another paradigm. This point is illustrated in Table 7, which compares a virulent wild-type clone with a laboratory-derived attenuated clone of California serogroup bunyaviruses. When the two viruses are injected intracerebrally in adult mice, the differences in their virulence are striking. One plaque forming unit (pfu) of the wild-type virus will initiate a fatal encephalitis, whereas 10^6 pfu of attenuated clone B.5 fails to cause any illness. On the other hand, both viruses are highly virulent when injected intracerebrally in suckling mice (approximately 1 plaque-forming unit (pfu) of either virus is lethal) and cannot be distinguished.

CLINICAL, PATHOLOGICAL, AND FUNCTIONAL INDICATORS OF DISEASE

The conventional measure of viral virulence is the production of disease in the host, using as an endpoint either death or some specific constellation of signs and symptoms caused by the infection. This is illustrated in Table 8, which compares the paralytic rate following primary infection with wild-type virus versus the rate following oral poliovirus vaccine. An alternative measure of virulence is an assessment of virus-induced pathological lesions. Also, laboratory tests, that reflect virus-induced pathophysiological changes, can be used as surrogate markers of virulence. For instance, the severity of hepatitis can be assessed by the serum titer of alanine aminotransferase that signals release of hepatocellular proteins, and the severity of human immunodeficiency virus infection by the reduction in the the blood concentration of $CD4^+$ T lymphocytes.

For a precise estimation of virulence, it is important to adjust for virus dose, which can conveniently be done by titrating different virus strains in a permissive cell culture system, as well as in animals. The ratio of plaque forming units per 50 percent lethal dose (pfu per LD_{50}) can then be compared for different virus variants (Figure 14).

Experimental manipulation of virulence

A prerequisite to the study of virulence is the availability of viral variants differing in their virulence phenotype. In some instances natural isolates may differ in virulence, for instance, serotype 1 and serotype 3 reoviruses, which vary remarkably in their biological properties even though reassortants between them are readily constructed. In many instances, laboratory manipulation must be used to obtain virulence variants.

PASSAGE IN ANIMAL HOSTS

In general, passage by a defined route in a particular species enhances the virulence of the virus when assessed under the conditions of passage, but often reduces the virulence in other animals or by alternate routes of infection. Human polioviruses on occasion have been adapted to mice; repeated mouse passage of monkey neurovirulent isolates produces variants that often have high intracerebral and intraspinal virulence for mice, but may exhibit relatively low intracerebral virulence for monkeys (Racaniello 1988).

PASSAGE IN CELL CULTURE

Passage in cell culture frequently leads to the selection of attenuated virus strains. A prominent example was Theiler's successful search for an attenuated variant of yellow fever virus, culminating in the selection of the 17D strain (Theiler and Smith 1937). Experience with cell passage has led to several common observations: (1) apparently identical passage protocols can yield stocks with different degrees of attenuation so that there is an element of unpredictability in this approach to attenuation; (2) viral stocks often represent a swarm of genetically diverse variants that differ in their degree of attenuation; and (3) attenuated variants may be temperature sensitive even when they were not selected for this phenotype.

MUTAGENESIS AND TEMPERATURE-SENSITIVE MUTANTS

A standard strategy for the production of attenuated variants is the treatment of a virus with a mutagen. One convenient way to select mutants is to look for viral variants that are temperature sensitive (ts), and that will replicate well at the permissive temperature of 33–37°C, but not at an elevated nonpermissive temperature, such as 40°C, where the nonmutagenized parent virus will still replicate well. Many ts mutants have reduced virulence in animals. Reduced virulence is not necessarily a direct consequence of temperature sensitivity, since the body temperature of the host is frequently within the range of permissive temperatures. It is more likely that such ts mutants are also host range restricted in certain cell types even at the permissive temperature, thus explaining their in vivo attenuation. Although random mutagenesis is a convenient way to produce attenuated viruses, it has the drawback that there may be mutations in several viral genes that are silent in cell culture, but influence the in vivo phenotype, complicating conclusions about the mechanism of attenuation.

Many viruses can be cold adapted by passage at a reduced temperature (traditionally 25°C) and such variants are often attenuated in animals (Maassab and DeBorde 1985). Cold-adapted variants of influenza virus replicate as well at 37°C and much better at 25°C than wild-type virus, but are usually temperature sensitive at

Table 8 *Virulence expressed as the comparative ability of different strains of a virus to produce clinical illness*

Virus	Paralytic case rate per 1 000 000 primary infections (range)	Relative rates	Study period
Wild-type (mainly type 1)	7 000 (2 000–20 000)	~10 000	1931–1954
OPV (mainly type 3)	0.62	1	1961–1978

The paralytic rate following infection with wild-type polioviruses is compared to the rate following infection with attenuated oral polioviruses (OPV). Data from Schonberger et al. 1976; Nathanson and Martin 1979; Nathanson 1984; Nkowane et al. 1987.

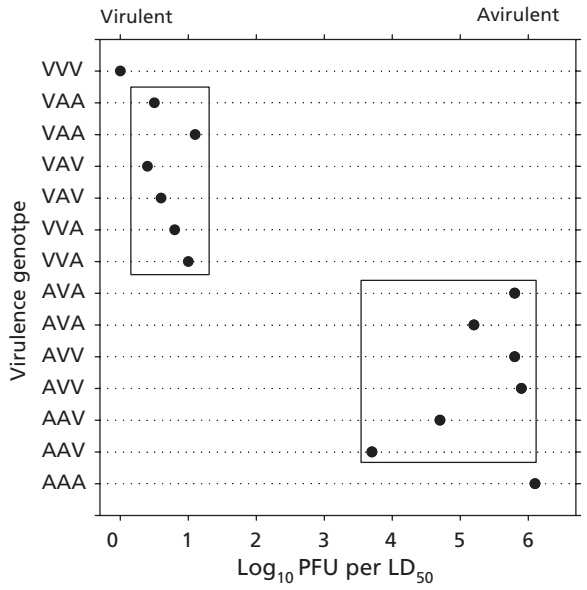

Figure 14 *Quantitative comparison of virulence using a panel of California serogroup viruses. Virulence is expressed as the ratio of plaque forming units per 50% lethal dose (pfu per LD_{50}) plotted on a log scale. Reassortants were made between a virulent (genotype VVV for the large, intermediate, and small gene segments, respectively) and an avirulent clone (genotype AAA). Reassortants bearing a large gene segment derived from the virulent parent had a virulent phenotype and those bearing a large segment derived from the avirulent parent had an avirulent phenotype (data from Griot et al. 1994).*

40°C, and have markedly reduced pneumopathogenicity when tested intranasally in ferrets. Cold-adapted influenza viruses have mutations in many or all of their eight gene segments, but reassortant viruses bearing only a few segments from cold-adapted variants exhibit the cold-adapted attenuated phenotype.

DESIGNED MUTATIONS

With the development of methods for introducing mutations into DNA, it has become possible to design mutants of many DNA and RNA viruses. Several different kinds of mutations can be introduced, including point mutations that result in substitution of a single or several individual amino acids, construction of genetic chimeras between two viral variants by exchange of genetic sequences, or inactivation of an open reading frame either by introduction of a stop codon, insertion of a selectable marker cassette, or deletion of part of the open reading frame. In some instances, noncoding domains of the viral genome carry major determinants of virulence, which can be mapped by similar methods (Almond 1991; Minor 1993; Racaniello et al. 1993).

An example of the use of these methods is provided by human and simian immunodeficiency viruses (HIV and SIV), where genetic modifications have been utilized to address various aspects of virulence (Levy 1998). For instance, chimeric viruses have been used to map macrophage tropism to the *env* gene and point mutations have been used to map tropism within the fine structure of the V3 loop of the gp120 envelope protein. Introduced stop codons and deletions have been used to explore the role of the *nef* gene of SIV, with the finding that *nef* deletions do not affect the ability of the virus to replicate in cell culture, but markedly reduce the ability of a virulent clone, mac 239, to induce AIDS in rhesus monkeys (see below).

SELECTION OF ATTENUATED VARIANTS

In selecting variants of reduced virulence, several points should be kept in mind. First, as noted above, most virus pools consist of a mixture of variants, which may differ markedly in their biological phenotype. Thus, it is necessary to plaque purify and characterize a number of stocks prior to selecting virus variants for study. Secondly, the mutations of greatest interest are those that specifically impair virulence in vivo without affecting the ability of a virus to replicate in a defined reference cell culture system. Such mutants can be considered to be 'host range' variants in contrast to 'global' mutants that impair replication in all cell hosts.

With the development of panels of monoclonal antibodies for most viruses, it has become possible to select variants that escape neutralization. Such variants often represent point mutations and occur naturally at a frequency of about 10^{-5} depending on the virus (Steinhauer and Holland 1987). When a panel of such monoclonal antibody-resistant (MAR) variants are tested in vivo, it is often observed that a few of the escape mutants are attenuated. Variants selected in this manner generally have mutations in the viral attachment protein since this is the typical target of neutralizing monoclonal antibodies.

Pathogenic mechanisms of virulence

There are many sequential steps in a viral infection and a difference in the comparative replication of two virus variants at any one of these steps could explain their relative degrees of virulence. In practice, attenuated variants will usually replicate or spread less briskly in one or several tissues associated with the pathogenic process. Also, variant viruses may differ qualitatively in their cell and tissue tropism or in their mode of dissemination.

VIREMIA

A reduction in viremia can be an important mechanism for the reduction in virulence. An example is shown in Figure 8 that compares poliovirus strains of different virulence. The Mahoney strain of type 1 poliovirus caused a much higher viremia and paralytic rate than did several other strains that were less viremogenic.

NEURAL SPREAD

Rabies viruses are obligatory neurotropes and disseminate only by neural spread. The attenuation of rabies virus has been investigated using MAR mutants. Some of the MAR variants in one epitope group were markedly attenuated, and one attenuating mutation was mapped to a single amino acid residue (at position 333) in the rabies glycoprotein (Tuffereau et al. 1989). After intramuscular injection, an attenuated mutant spreads to the brain at a rate similar to virulent parent CVS virus, but within the central nervous system the attenuated clone spreads to relatively few neurons, while the virulent virus spreads rapidly to many contiguous neurons (Jackson 1991). The difference between virulent and attenuated MAR viruses lay in their differential efficiency of entry into neuronal cells, probably associated with differences in the binding of their glycoproteins to cellular receptors (Dietzschold et al. 1983).

TROPISM AND HOST RANGE

Different variants of a single virus can exhibit differences in their tropism for organs, tissues, and cell types, which confers a multidimensional character upon virulence. Thus, a variant virus may be reduced in its ability to cause one type of pathology but enhanced in its ability to cause another. Human immunodeficiency virus (HIV) presents an example of tropism differences (Table 9). Most strains of HIV can be classified into two categories, according to their ability to replicate in T-cell lines (T-tropic) or in primary macrophages (M-tropic) (Levy 1998). Tropism has been mapped to the V3 domain on the envelope glycoprotein (gp120) and is associated with coreceptor preference. HIV utilizes members of the chemokine receptor family as a co-receptor. M-tropic viruses preferentially utilize CCR5 while T-tropic viruses preferentially utilize CXCR4 as a coreceptor. Most viruses isolated shortly after infection are M-tropic, while most viruses isolated from patients with severe AIDS are T-tropic. It is inferred that M-tropic viruses are preferentially transmitted for reasons yet to be elucidated, while T-tropic viruses replicate preferentially in T lymphocytes, particularly in patients lacking immune defenses.

VIRULENCE FOR TARGET ORGANS

Different variants of a specific virus may show different degrees of virulence for a key target organ. The three attenuated strains of poliovirus used in the oral poliovirus vaccine provide an example (Table 10). The type 3 strain has the greatest virulence since it causes more cases of paralytic poliomyelitis in vaccine recipients than does either the type 1 or type 2 strains. The type 2 strain is clearly the most viremogenic in children. However, after intracerebral injection of monkeys, the type 3 strain produces very severe spinal cord lesions at a higher frequency than do the type 1 or type 2 strains. In this instance, virulence for the target organ (the spinal cord), rather than viremogenicity, appears to be the most important determinant of virulence after virus feeding.

TUMORIGENESIS

When applied to tumor viruses, virulence can be expressed in several different ways. The most common differences among the oncogenic viruses involve either the latent period from infection to development of a neoplasm, or the cell type that is transformed. In general, the transforming retroviruses can be divided into two major classes, according to whether they cause tumors with a long latency or are acutely transforming. The long latency viruses are often replication competent and do not carry a transduced oncogene. They act as insertional mutagens when their proviruses are integrated into the host DNA. On rare occasion, insertion occurs at a site where the promoter, enhancer, or terminator sequences in the viral genome can influence the transcriptional rate of a proto-oncogene. Up-regulation of such a proto-oncogene then leads to transformation of the infected cell, which can result in neoplasia usually after a long latency.

Murine leukemia viruses (MuLV) are replication-competent long latency oncogenic retroviruses. Different MuLV isolates induce different kinds of neoplasms, involving T lymphocytes, B lymphocytes, erythroid, or myeloid precursor cells. Genetic studies, including the production of viral chimeras, has mapped the genetic determinants to the viral U3 region, a

Table 9 *Isolates of a single virus may vary in their host range*

		Growth in each cell type		
Viral biotype	**HIV-1 isolate**	**Peripheral blood T lymphocytes**	**Monocyte-derived macrophages**	**T cell line Sup-T1**
T-tropic	IIIB	++++	+	++++
	DV	++++	++	++++
M-tropic	SF162	++++	+++	–
	89.6	++++	+++	–

HIV (human immunodeficiency virus) strains can be classified into two major biotypes, M-tropic (replicating in macrophages) and T-tropic (replicating in T-lymphocyte cell lines) as shown for several different virus strains.
Data from Collman et al. 1989; Collman and Nathanson 1992.f

Table 10 *Virulence for the target organ appears to be the major determinant of the relative in vivo virulence of the three oral poliovirus vaccine strains.*

	Type 1	Type 2	Type 3
In vivo virulence (poliomyelitis in OPV recipients)	22	13	62
Viremogenicity (frequency of viremia)	0/16	17/19	0/16
Neurovirulence (percent severe lesions)	0.05%		1.15%

Top line. The type 3 vaccine strain causes paralytic poliomyelitis at a rate that is about threefold higher than the type 1 and fivefold higher than the type 2 strain. Cases of paralytic poliomyelitis in recipients of oral poliovirus vaccine (OPV), United States, 1961–1984. Data from Schonberger et al. 1976; Nkowane et al. 1987.
Middle line. The type 2 strain generates viremia more effectively than either of the other strains indicating that it has a much higher potential for invading the spinal cord. Frequency of viremia in infants fed oral poliovirus vaccine. Data from Horstmann et al. 1964.
Bottom line. On direct intraspinal injection of monkeys, the type 3 strain causes severe spinal cord lesions at a rate that is much higher than the rate associated with the type 1 strain. Data from Nathanson and Horn 1992.

noncoding part of the viral genome that is just upstream from the transcriptional start site and has a domain that contains a number of transcriptional enhancer sites (Hopkins et al. 1989). Tropism is thought to correlate with relative ability to replicate in different target cells, and this has been borne out, at least in part, by in vivo studies of the relative replication rates of different MuLVs in different tissues (Evans and Morrey 1987).

Genetic determinants of virulence

Viral virulence is encoded in the viral genome and expressed through structural proteins, nonstructural proteins, or noncoding sequences. A large body of information about virus variants has established several important points:

- The use of mutants has made it possible to identify the role of individual genes and proteins as determinants of the biological behavior of many viral variants.
- There is no 'master' gene or protein that determines virulence, and attenuation may be associated with changes in any of the structural or nonstructural proteins, or in the noncoding regions of the genome.
- The virulence phenotype can be altered by very small changes in the genome, if they occur at 'critical sites.' At such sites, a single point mutation leading to the substitution of a specific amino acid or base is often sufficient to alter virulence. For most viruses with small genomes (<20 kb) only a few discrete 'critical sites' have been discovered, usually fewer than 10 per genome.
- It is possible to create variants with attenuating mutations at several critical sites and these may be more attenuated than single point mutants. Also, reversion to virulence is less frequent in variants with several discrete attenuating mutations.
- Attenuating mutations are often host range mutations that affect replication in some cells, but not in others. This is partly a reflection of the fact that most well characterized mutants have been deliberately selected because they replicate well in a standard cell culture system.
- Although many attenuating mutations have been sequenced, relatively few have been characterized at a biochemical or structural level as to their mechanism of action.

These generalizations may be illustrated with examples from a few selected virus groups.

POLIOVIRUSES

Polioviruses are single-stranded RNA viruses of positive polarity, with a genome that contains a relatively long 5′ and a shorter 3′ nontranslated region (NTR) flanking a single open reading frame of about 7 kb that encodes a single polyprotein that is cleaved into four structural proteins (VP1-VP4) and several nonstructural proteins.

Virulence studies have focused on the three attenuated strains of types 1, 2, and 3 poliovirus that constitute the oral poliovirus vaccine (OPV) originally developed by Sabin (1985). The three vaccine strains are about 10 000-fold less virulent than wild-type poliovirus (Table 8), and they can be compared to the wild-type viruses from which they were derived. However, the genomes of wild-type parental viruses and their vaccine progeny differ at a considerable number of sites (reflecting their complex passage history) and many of these differences are not relevant to the attenuated phenotype. Virulent revertants of the vaccine strains occur on passage in humans or in cell culture and are better suited to genetic analysis, since there are relatively few sequence differences between them and the vaccine strains. Using reverse genetics (Racaniello and Baltimore 1981), infectious DNA clones of poliovirus genomes can be used to construct chimeric viruses and these clones can then be transfected into permissive cells to reconstitute mutated infectious virus.

The 5′ nontranslated region (NTR)

The attenuation of the vaccine strains of poliovirus is due to critical sites, both in the 5′ NTR upstream from the long open reading frame and in selected structural

and nonstructural proteins (Racaniello 1988; Minor 1992, 1993; Racaniello et al. 1993). Each of the three strains of OPV carry point mutations in the 5′ NTR, at positions 480, 481, or 472, in types 1, 2, and 3, respectively. Attenuating mutations at these sites are associated with reduced neurovirulence in monkeys, and reduced ability to replicate in the central nervous system of transgenic mice bearing the poliovirus receptor. Attenuated variants are temperature-sensitive host range mutants that replicate well in primate fibroblast cell lines, such as HeLa cells, but have reduced ability to replicate in neuroblastoma cells (La Monica and Racaniello 1989). The bases at attenuating sites in the 5′ NTR are thought to be involved in an RNA stem loop based on computer models of their predicted secondary structure. It is believed that the stem structure at positions 470–540 is involved in the binding of ribosomal complexes at the initiation of translation. Presumably, initiation factors must differ, either quantitatively or qualitatively, in different cell types to explain the cellular host range restriction of the vaccine strains.

Structural or nonstructural proteins

Each of the OPV strains carries at least one mutation that is associated with an alteration in a viral structural or nonstructural protein and confers temperature sensitivity and reduced neurovirulence, and these mutations involve different proteins for the three OPV strains. For type 1 OPV, mutations associated with increased virulence are found in the virus capsid proteins, and in the nonstructural 3D polymerase (Bouchard et al. 1995; Georgescu et al. 1995). For type 2 OPV, it appears that neurovirulence in the mouse is associated with virus capsid protein VP1 (Ren et al. 1991; Macadam et al. 1993). For type 3 OPV, sites in virus capsid proteins VP3 and VP1 confer temperature sensitivity and neuroattenuation (Tatem et al. 1992). It is not clear how mutations in the structural proteins produce attenuation, but it has been suggested that these are involved in structural transitions that occur either during virion assembly or virion uncoating.

BUNYAVIRUSES

Bunyaviruses are negative-strand (or ambisense) RNA viruses with a trisegmented genome. The large (L) RNA segment encodes the viral polymerase; the middle (M) RNA segment encodes two glycoproteins (G1 and G2) and a nonstructural protein, Ns_m; the small (S) segment encodes the nucleoprotein (N) and a nonstructural protein, Ns_s. Most studies of virulence have utilized members of the California serogroup that will reassort readily with each other. Beginning with two parent viruses, panels of reassortants containing all possible combinations of gene segments can be constructed. In addition, monoclonal antibody-resistant (MAR) mutants have been used to identify variants in the major G1 glycoprotein that are associated with differences in virulence. Attenuation can involve either the ability of the virus to replicate in the central nervous system (neurovirulence) or its ability to cause viremia and reach the central nervous system (neuroinvasiveness).

The middle RNA segment and glycoprotein G1

A detailed comparison has been made of two California serogroup viruses, wild-type La Crosse virus and a laboratory passaged strain of Tahyna virus which exhibited reduced neuroinvasiveness, but high neurovirulence (Griot et al. 1993a). The reduction in neuroinvasiveness of the attenuated virus is associated with low viremogenicity that, in turn, is associated with a reduced ability to replicate in striated muscle, the major extraneural site of replication of this group of viruses. Attenuation maps to the M RNA segment encoding the viral glycoproteins suggesting that there is an alteration in the entry phase of infection of myocytes (but not of neurons). These viruses plaqued equally well on BHK-21 cells. However, on a murine myocyte cell line (C2C12), viral variants bearing the M RNA segment of the attenuated parent failed to plaque while variants bearing the M RNA segment of the virulent parent plaqued well (Griot et al. 1994).

MAR mutants of La Crosse virus are readily obtained, using antibodies directed against the major (G1) glycoprotein, and a mutant at one epitope site (but not at other sites) exhibited reduced neuroinvasiveness with wild-type neurovirulence (Gonzalez-Scarano et al. 1985). Furthermore, the attenuated clone showed strikingly reduced fusion efficiency at acid pH compared to parental wildtype La Crosse virus. These observations suggested that attenuation might be associated with an alteration in glycoprotein G1 that reduced efficiency in the infection of myocytes.

The large RNA segment and the viral polymerase

An attenuated California serogroup virus clone, B.5, obtained by passage in BHK-21 cells, showed a striking reduction in its ability to replicate in the central nervous system of adult mice. Using reassortant analysis (Figure 14) clone B.5 was shown to bear an attenuated L RNA segment (Endres et al. 1990). This clone is a temperature-sensitive host range mutant that replicates poorly in C1300 NA neuroblastoma and other murine cell lines, although it grows well in BHK-21 cells. Presumably, clone B.5 has a mutant polymerase that restricts viral replication in neurons and certain other cell types.

REOVIRUSES

Reoviruses are double-stranded ten-segmented RNA viruses. The gene segments and the proteins that they

encode have been characterized in detail. The large number of segments have made reoviruses an attractive model system for study of the genetic determinants of viral phenotypes (Virgin et al. 1997). These studies have been facilitated by major differences in the biological phenotypes of naturally occurring reovirus serotypes, particularly serotypes 1 and 3. Table 11 illustrates the use of reassortants to map virulence phenotypes to reovirus gene segments.

Biological functions have been ascribed to many of the reovirus genes and proteins. Most of the virulence determinants represent qualitative differences between type 1 Lang and type 3 Dearing (T1L and T3D) in tissue and organ tropism rather than quantitative differences in disease severity.

- The S1 segment encodes the σ1 protein, which is the viral attachment protein that binds to receptors on permissive cells, and is a major target for neutralizing and hemagglination inhibiting (HI) antibodies. T1L and T3D viruses differ markedly in the disease that they cause in suckling mice. T1L disseminates through the blood and causes an ependymitis in the brain, while T3D disseminates through the neural route and causes a neuronotropic encephalitis in the brain. The S1 segment is the major determinant of dissemination and brain cell tropism (Table 11). In addition, the S1 segment is a determinant of replication in cardiac myocytes and myocarditis, of replication levels in the intestine, and of differences in reovirus strains to induce apoptosis and perturb the cell cycle.
- The M1 segment encoding the μ2 protein influences replication in cardiac myocytes and myocarditis.
- The M2 segment encoding the μ1 protein affects the protease sensitivity of the virion, and quantitative neurovirulence.
- The L1 segment encoding λ3 protein influences replication in cardiac myocytes and myocarditis.
- The L2 segment encoding λ2 protein acts as a guanylyl transferase in cell culture and influences replication levels in the intestine, titers of shed virus, and horizontal transmission between mice.

Viral virulence genes of cellular origin

Since 1985 a new class of virus-encoded proteins has been recognized that contribute to the virulence of viruses by mimicking normal cellular proteins (McFadden 1995). This group of 'cell-derived' genes has been identified primarily within the genomes of large DNA viruses that probably have a greater capacity to maintain accessory genes than do viruses with small genomes. Because these genes are homologues of cellular genes, it is hypothesized that they were acquired by recombination and modification. Thus, from an evolutionary viewpoint, they resemble viral oncogenes.

Cell-derived viral genes encode proteins that enhance virulence by many different mechanisms. 'Virokines' are secreted from virus-infected cells and mimic cytokines, thereby perturbing normal host responses. 'Viroceptors' resemble cellular receptors for cytokines (including antibodies or complement components) that are thereby diverted from their normal cellular targets. Some virus-encoded proteins interfere with antigen presentation and

Table 11 *Mapping virulence to a specific viral gene segment*

	Outer capsid gene segments			Core gene segments					Nonstructural gene segments		
Pattern of spread	M2	S1	S4	L2	L1	L3	M1	S2	M3	S3	Clone
Viremia	L	L	L	L	L	L	L	L	L	L	T1L
	D	L	L	L	L	L	L	L	D	D	R
	L	L	L	D	D	D	D	D	L	D	R
	L	L	D	L	L	L	L	L	L	L	R
	D	L	D	D	D	D	D	D	D	D	R
	D	L	L	D	D	D	L	D	D	D	R
	D	L	D	D	D	D	D	D	L	D	R
Neural	D	D	D	D	D	D	D	D	D	D	T3D
	D	D	D	D	D	D	D	D	D	L	R
	L	D	L	L	D	L	L	L	L	L	R
	L	D	L	L	L	L	L	L	L	L	R
	L	D	L	D	L	L	L	L	L	L	R

The *S1* gene segment of reovirus determines whether the virus spreads by viremia or by the neural route, since it was the only gene segment that cosegregated with the mode of spread. Two viruses, type 1 Lang (T1L) and type 3 Dearing (T3D) were used to derive reassortant clones (R) that were genotyped (L or D indicates origin of each gene segment) and were tested for their mode of spread.
Adapted from Tyler et al. 1986.

immune induction (Smith et al. 1997; Lafon 2002; Jackson and Ramsay 2001), while others prevent apoptosis or interrupt intracellular signalling initiated by cytokines or interferons. A brief review of selected cell-derived genes that have been described for pox and herpes viruses will serve as examples of this group of viral genes and illustrate how they contribute to viral virulence.

POXVIRUSES

Tumor induction

Several poxviruses cause either benign or malignant tumors. However, it appears that the mechanism of oncogenesis is different from that associated with classic DNA or RNA tumor viruses, since there is no evidence that viral sequences are incorporated into the genome of transformed cells. Virus strains may gain or lose tumorigenicity quite independent of their ability to replicate, and continued productive replication of the virus appears to be required for tumor growth. It was postulated in the 1960s that these viruses might produce a growth factor that accounted for their induction of tumors (Kato et al. 1963). A large body of research has now documented that several poxviruses encode a protein that can be classified as a member of the epidermal growth factor (EGF)-like family of growth factors (McFadden et al. 1995). Shope fibroma virus and malignant rabbit fibroma virus both encode a very similar protein (Shope fibroma growth factor (SFGF)), while myxoma virus encodes a related myxoma growth factor (MGF). These proteins contain the shared functional domains (six characteristically spaced cysteines) shared by members of the EGF family. Furthermore, when these genes are deleted from the virus genomes, the MGF-negative or SFGF-negative viruses replicated normally in cell culture, but showed much reduced ability to induce tumors in rabbits (Opgenorth et al. 1992).

Receptors for tumor necrosis factor

Tumor necrosis factor (TNF) is a potent pro-inflammatory cytokine that is produced by activated macrophages and T cells. TNF acts by binding to a class of cellular receptors that are type 1 integral membrane proteins characterized by multiple cysteine-rich exodomains. Through their receptors on myeloid and lymphoid cells, the TNFs exert complex pleiotropic effects on immune networks and host responses to infection. A number of poxviruses encode a protein designated T2 that is a soluble form of the cellular p75 TNF receptor (Smith and Goodwin 1995). It is presumed that this protein binds free TNF, thereby modulating TNF-mediated cellular responses to infection. It is postulated that T2 could enhance replication of poxviruses in vivo by down-modulating the antiviral defenses of the infected host, although T2 might simultaneously favor the host by reducing the severity of virus-induced inflammatory lesions.

Vaccinia virus complement control protein (VCP)

The complement system is a complex group of proteins that act in a cascade that forms one of the initial host defenses against microbial pathogens. The system is a potent one that, uncontrolled, has the potential to cause severe damage, and is therefore tightly regulated by several different mechanisms, including C4-BP, a plasma protein that binds C4b2a, one of the complexes formed in the course of complement activation. Vaccinia virus encodes a protein, vaccinia virus complement control protein or VCP, that is homologous to C4-BP (Isaacs and Moss 1995). VCP binds human C4b and a VCP– mutant of vaccinia virus fails to inhibit complement-mediated lysis of sensitized sheep erythrocytes, while wild-type vaccinia virus has an inhibitory effect (Kotwal et al. 1990). When wild-type and VCP– vaccinia viruses were compared in vivo, the VCP– mutant was less virulent upon intracerebral injection of mice and, as shown in Figure 15, produced smaller skin lesions after intradermal inoculation in rabbits (Isaacs et al. 1992).

HERPES SIMPLEX VIRUSES

Herpes simplex virus (HSV) complement receptors

The importance of the complement cascade and its regulation already have been mentioned. C3b, one of the intermediates in the cascade, plays a key role in initiating the formation of the membrane attack complex (the last part of the complement cascade) so that its regulation is important. A number of cell types,

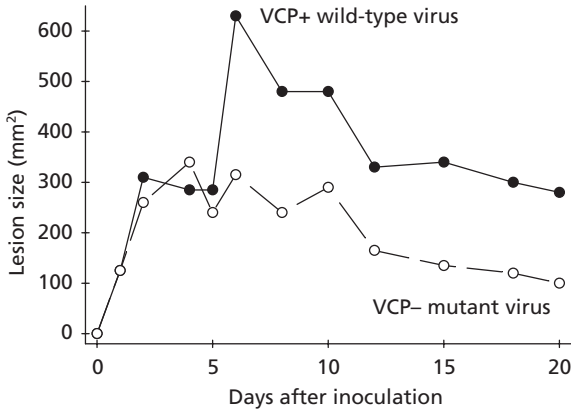

Figure 15 *Cell-derived viral genes can act as virulence factors. The skin lesions (median of three rabbits) produced by a wild-type vaccinia virus expressing the vaccinia complement control gene (VCP+) are more extensive than the lesions caused by the same virus from which the gene had been deleted (VCP–) (data from Isaacs et al. 1992).*

including monocytes/macrophages, neutrophils, and some T cells, express receptors for C3b, that down-modulate the complement cascade by reducing the levels of C3b and enhancing its inactivation by cleavage to iC3b. Glycoprotein C (gC) of HSV-1 binds C3b and iC3b, thereby providing protection against complement-mediated neutralization of HSV, and against complement-mediated lysis of HSV-infected cells (Harris et al. 1990; York and Johnson 1995). Although it has not yet been demonstrated that gC− mutants are less virulent in vivo, the conservation of gC in natural isolates of HSV suggests that this protein (and its complement-binding activity) play a role in HSV survival in nature.

HSV Fc receptors

HSV-infected cells express receptors (FcR) for the Fc domain of the immunoglobulin (IgG) molecule (Baucke and Spear 1979). A considerable body of work (York and Johnson 1995) has identified glycoproteins gE and gI of HSV as the molecules that bind IgG. It has been suggested that the presence of Fc receptors on the HS virion or on the surface of virus-infected cells would lead to bipolar bridging of the IgG molecule that might reduce secondary effects of antibody binding, such as the ability to initiate the complement cascade. Comparison of an FcR-deficient mutant, expressing an altered gE protein, with wild-type HSV, has shown that FcR-deficient virus is more susceptible to complement-mediated neutralization and lysis of virus-infected cells, as shown in Figure 16 (Frank and Friedman 1989; Dubin et al. 1991). Furthermore, gE− HSV exhibits reduced virulence in mice (Rajcani et al. 1990).

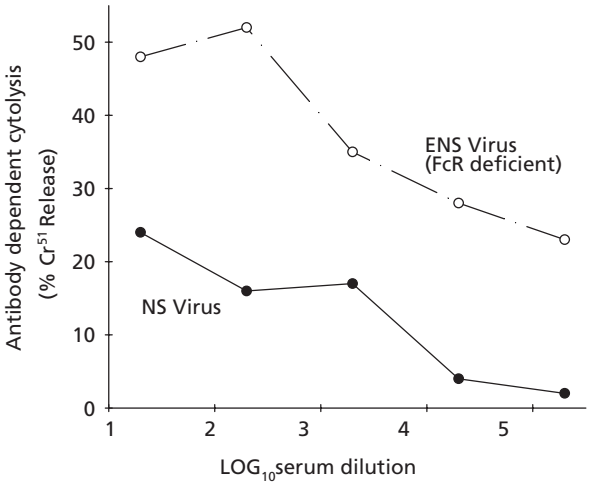

Figure 16 *Cell-derived viral genes can act as virulence factors. Glycoprotein gE of herpes simplex virus, which functions as an Fc receptor (FcR), influences the susceptibility of HSV-infected cells to antibody-dependent cell-mediated cytotoxity (ADCC). Target cells infected with the NS (FcR+) strain of HSV show lower percent lysis than cells infected with an FcR-deficient mutant of NS virus (strain ENS), indicating that the viral-encoded FcR can reduce the susceptibility of HSV-infected cells to ADCC (data from Dubin et al. 1991).*

PERSISTENT INFECTIONS

A number of viruses are capable of producing infections that persist for the lifetime of the host (Ahmed and Chen 1999). In order to persist, a virus must achieve a delicate balance such that it maintains its genome without killing the host. In addition, the virus must evade immune surveillance mechanisms that are sufficiently potent to be able to clear many acute viral infections (Ploegh 1998).

Immune clearance of acute virus infections

If the duration of an acute infection is defined as the time between acquisition of infection to total clearance, then length ranges from about 1 week (for rhinoviruses) to about 6 months (for hepatitis B virus). Before considering immune evasion, it is useful to recapitulate briefly the mechanisms by which the immune response eliminates an acute infection. Effector T lymphocytes with specificity for the antigenic determinants of an infecting virus can destroy virus-infected cells, produce antiviral cytokines, such as interferon, and recruit phagocytic cells to the sites of infection. B lymphocytes differentiate into plasma cells that secrete antiviral antibodies which can neutralize virions, initiate complement-mediated virolysis, and opsonize virus so that it is efficiently phagocytosed (Figure 5) (Binder and Griffin 2001). To persist, a virus must evade these mechanisms (Igarishi et al. 1999).

Mechanisms of persistence

Many different genera of both RNA and DNA viruses include members that can cause persistent infections. Furthermore, the same viruses may cause either acute self-limited or persistent infections, depending upon the circumstances of infection. Lytic viruses that kill the cells in which they replicate tend to use different strategies than nonlytic viruses that can be shed from cells over long periods of time without cell death. The strategies of persistence can be classified into three major categories, which are outlined in Figure 17. For each viral strategy, there is a corresponding strategy for evasion of the immune response.

- *High titer replication* requires that virus either be noncytocidal or that there is rapid replacement by cellular proliferation of target cells. Immune surveillance is unable to eliminate the virus, due to tolerance, immune complex formation, viral mutation, or another mechanism.
- *Latency* requires that the viral genome can persist in a nonreplicating mode, either integrated into the

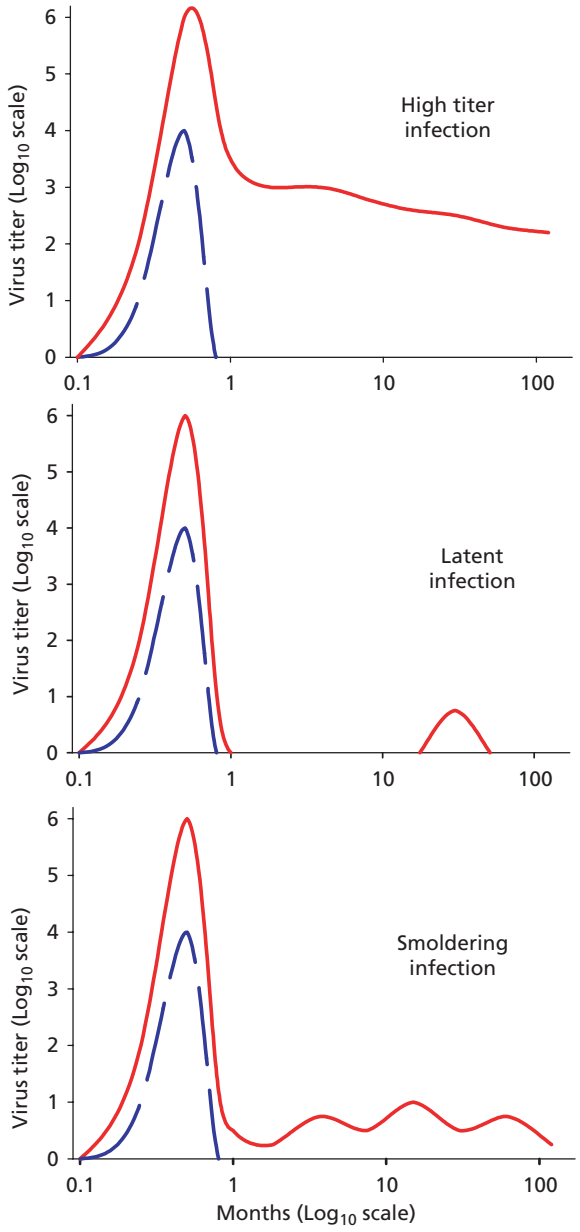

Figure 17 *Patterns of virus persistence. For reference, an acute infection is shown by dashed line in all panels.* High titer infection*: An acute phase is often apparent, following which the titer drops but persists at a high level for a long time.* Latent infection*: An acute infection is followed by disappearance of replicating virus which persists only as a latent genome. Periodic recrudescence occurs, during which replication of the virus may be accompanied by signs of disease, followed by another period of latency. Intervals between recrudescences may last from weeks to many years, and some infected individuals may never experience a recrudescence.* Smoldering infection*: An acute infection of varying severity is followed by marked reduction in overt virus replication, but infectious virus may be frequently recovered indicating that true latency has not occurred (after Nathanson et al. 2002).*

genome of the host cell or as an episome. Immune surveillance may be competent to eliminate the virus which can be activated but can only replicate for brief intervals. Alternately, recurrence may be associated with intermittent reduction of immune defenses.

- *Smoldering infections* involve continuous productive infection and cell-to-cell transmission at a low level. Usually, potentially effective immune surveillance is circumvented by mechanisms such as antigenic variation, infectious immune complexes, or intercellular bridges.

HIGH-TITER PERSISTENT INFECTIONS

Nonlytic viruses

Many viruses can replicate productively without causing cell death, and a number of them can cause persistent infections. In such instances, the initial dynamics resemble those of an acute infection, following which the virus titer decreases somewhat, but then reaches a setpoint that may be maintained indefinitely or gradually decline. Examples of this pattern are hepatitis B virus HBV and lymphocytic choriomeningitis virus (LCMV) of mice (Buchmeier and Zajac 1999).

High-titer persistence is often characterized by tolerance, an apparent absence of virus-specific immunity. The mechanisms by which the tolerance can be induced include deletion of forbidden clones of naive T lymphocytes in the thymus and exhaustion of peripheral virus-specific T lymphocytes in the presence of excess antigen. Tolerance may be limited to specific components of the effector limb of the immune response. For instance, hepatitis B virus persistence is characterized by absence of antiviral antibody against HbsAg while anti-HBcAg may be induced. LCMV persistence is characterized by absence of cellular immune responses while virus-specific antibody is produced. The presence of persistent high titers of virions and viral antigens may overwhelm either antibody or $CD8^+$ lymphocytes, so that assays must be interpreted with caution. Thus, in persistently infected mice, anti-LCMV antibodies circulate as immune complexes and not as free antibody, which originally led to the mistaken view that antibody was not induced in persistently infected mice.

Evidence for the role of immune tolerance in maintaining viral persistence is provided by the experimental termination of persistence by intravenous injection of virus-specific $CD8^+$ cells. Figure 18, summarizing an experiment with LCMV, exemplifies this phenomenon. Similar results have been obtained with HBV, where CTLs specific for HBs epitopes cleared virus from hepatocytes.

Lytic viruses

It is unusual for high titer persistence to be produced by a cytolytic virus, but the primate lentiviruses represent an important exception. Figure 19 shows the persistent viremia produced by simian immunodeficiency virus (SIV). The main target cells for these lentiviruses are

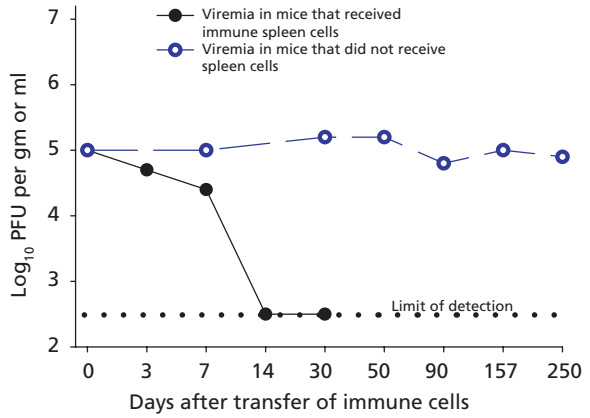

Figure 18 *Immune T lymphocytes can clear a persistent virus infection, implying that (in some instances) persistence involves escape from the cellular arm of the immune response. In this example, LCMV causes persistent lifelong infection of mice. When such mice are treated by adoptive immunization of CTLs from an immune donor ($10^{7.3}$ spleen cells from adult mice immunized by infection 60 days prior to transfer), the virus is cleared. The specificity of the process is shown by the requirement for syngeneic donor lymphocytes and by the ability of clones of CD8$^+$ T lymphocytes specific for an individual viral epitope to clear infection. The rate of clearance differs for different organs and tissues, implying some differences in the action of effector cells and in the mechanisms of immune evasion (after Ahmed et al. 1987).*

CD4$^+$ lymphocytes which undergo lytic infection. It has been calculated that the continuous destruction of CD4$^+$ cells results in a reduction of the average half-life of these cells from 75 to 25 days. However, the bone marrow is able to respond to the abnormal rate of destruction by increasing the production of naive CD4$^+$ cells, at a rate sufficient to maintain a reasonable concentration of circulating CD4$^+$ cells. The compensatory replenishment of target cells permits this generally lytic virus to persist at a high titer for an extended period of time, although eventually the bone marrow is unable to compensate and CD4 levels drop, leading to functional immunodeficiency.

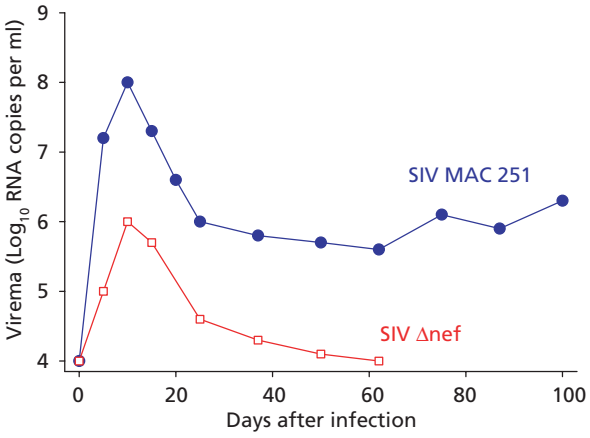

Figure 19 *Viral genes that influence immune clearance can alter in vivo infection. SIV mac 251 wildtype is compared with a mutant engineered to delete the* nef *gene, a regulatory gene that reduces expression of MHC class I molecules on the plasma membrane, permitting escape of virus-infected cells from immune attack. The figure shows the typical course of viremia in individual animals infected with wild-type and Δnef viruses (after Connor et al., 1998).*

In contrast to most high-titer persistent infections, lentiviruses induce immune responses rather than tolerance. The immune response to lentiviruses is quite effective, as judged by its ability to rapidly contain the acute phase of infection, resulting in a reduction from peak viremia at about 6 weeks to a setpoint at about 3–6 months which may be as much as 1 000-fold lower than the peak viremia. Once this setpoint is reached, a dynamic equilibrium is established between virus production and clearance. The half-life of individual SIV virions is <30 min in the absence of immunity and about 10 min in infected animals with an established immune response. It has been calculated that to maintain virus titers of 10^2 to 10^4 infectious virions per ml plasma requires the production of 10^{10} to 10^{12} new infectious virions daily. In this instance, high titer persistence is maintained by an extraordinary rate of virus production that exceeds the rate at which a potent immune response can clear the virus.

LATENCY

Latent infections are produced by a considerable number of herpesviruses, including herpes simplex viruses (HSV), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), and cytomegalovirus (CMV) of humans. There is a characteristic sequence of events following primary infection. Initially the virus replicates in permissive cells at the portal of entry. The virus is lytic and destroys permissive target cells. Once immune induction has occurred, the virus is cleared and appears to be eliminated.

However, the viral genome persists in a latent form. Latency occurs in one or more cell types – such as neurons – that are distinct from the permissive cell types that support productive lytic infection. Neurons appear to be restrictive or permissive, depending upon their physiological state (Stevens 1994). Under conditions of restriction, the virus undergoes the early steps of entry and uncoating, but further steps in replication are blocked. In the absence of active replication, the viral genome is maintained in one of several forms, depending upon the specific virus and host cell. In some instances, the double-stranded DNA genome integrates into the host genome, while in other examples the genome persists as a non-integrated episome, in the nucleus or cytoplasm.

If latency occurs in cell types such as neurons that do not divide, then there is no need to replicate the latent genome. If the latent genome is maintained in dividing cells, then the genome must be replicated or it will be

diluted to extinction. If the genome is integrated into the host genome then it will be automatically replicated during the cell cycle. Episomal DNA can also be replicated by the enzymes involved in copying cellular genomes. However, there are no parallel mechanisms for RNA, so RNA viruses cannot assume a latent state unless they undergo reverse transcription to DNA intermediates. Latent viral genomes can be detected by in situ polymerase chain reaction (PCR) methods that – at their most sensitive – can detect as little as one genome per infected cell.

Latency maintains the viral genome for the lifetime of the infected host. Activation of latent infections occur at irregular intervals, and may never occur in some infected individuals. Activation of latent genomes can be initiated by a number of stimuli, characteristic for each virus. For instance, herpes simplex virus (HSV) can be activated by fever, sunburn, and trigeminal nerve injury. Most of these stimuli appear to act upon the primary sensory neurons in which latent HSV genomes are maintained. However, on occasion, waning of the immune response can serve as a trigger for activation of some herpesviruses.

Following reactivation of HSV, the viral genome may be transported by axoplasmic spread in both centripetal and centrifugal directions. Centrifugal spread, toward the periphery, conducts the virus to the skin where it may replicate and spread, causing herpes labialis (fever blister or cold sore). After spreading for a few days, host defenses prevent further spread, and the skin lesion heals. Centripetal spread from the trigeminal ganglion conducts the HSV genome to the central nervous system, where, in some instances, it can spread to cause an encephalitis that may be devastating.

Typically, viruses that cause latent infections induce a brisk and potent immune response that clears the initial infection. However, latently infected cells do not express viral proteins, permitting escape from immune surveillance. Furthermore, when the latent infection is activated, immune surveillance limits its spread. Latent viruses cannot be spread from host to host, but virus produced during activation may be spread to another host. For instance, activation of latent varicella zoster virus (VZV) produces characteristic skin lesions in older adults. Seronegative children exposed to virus aerosolized from these lesions can develop chicken pox, the primary form of VZV infection.

SMOLDERING INFECTIONS

Smoldering infections fall between the extremes of high titer persistence and latency. Infectious virus is produced, but at minimal levels that may require special methods for detection and isolation. Virus continues to spread from infected to uninfected cells, but often at an indolent tempo. If the virus is pathogenic, it may produce a gradually progressive chronic disease (McCune 2001). There is a detectable immune response to the virus and, in some instances, the response may be hypernormal, due to the continuous presence of viral antigens. The ability of a virus to spread in the presence of a potentially effective immune response is a paradoxical phenomenon, and involves a variety of strategies several of which may operate in any given example (Billeter et al. 1994; Cattaneo et al. 1987). Some of the more important strategies are described below.

Immunologically privileged sites

There are a few organs and tissues that favor virus persistence, particularly the brain and kidney. The brain has classically been considered an immunologically privileged site because immunological effector mechanisms may spare cells bearing foreign antigens if these cells are located in the brain (in contrast to foreign cells in other sites). There are at least two factors that account for virus persistence in the brain. First, the blood–brain barrier limits the trafficking of lymphocytes through the brain and, secondly, neurons express little if any MHC class I molecules rendering them relatively poor targets for virus-specific cytolytic T lymphocytes (CTL).

The kidney is the other tissue that frequently harbors persistent viruses, such as JC and BK polyomaviruses, and cytomegalovirus. Also, LCMV is cleared more slowly from the kidney than from other tissues, even the brain. However, there is no clear explanation why virus in the kidney should be able to evade immunological surveillance, although it has been speculated that lymphocytes may not readily cross the subendothelial basement membrane to access infected glomerular epithelial cells.

Intercellular bridges

In some instances, the process of entry of viruses into cells can be shortcircuited, so that a transient intercellular bridge is formed. Intercellular bridges permit the viral genome to pass from cell to cell without having to survive in the extracellular environment (Figure 20), thus providing a means of avoiding neutralizing antibody. This phenomenon is probably operative in a progressive fatal disease called subacute sclerosing panencephalitis (SSPE). In SSPE a defective variant of measles or rubella virus spreads gradually from neuron to neuron in spite of extraordinarily high titers of neutralizing antibody in the extracellular fluid of the brain parenchyma (Cattaneo et al. 1987; Billeter et al. 1994).

Suppression of MHC class I expression

A number of viruses, including adenoviruses and lentiviruses, encode specific proteins that are capable of down-regulating the expression of MHC class I mole-

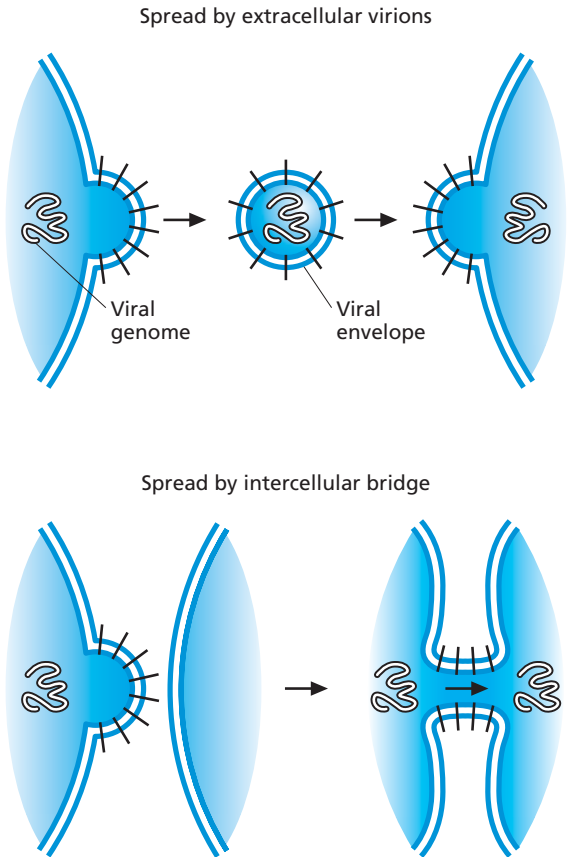

Figure 20 *Cell-to-cell transmission of a virus by intercellular bridges, a hypothetical reconstruction. Top, normal cell-to-cell spread of an enveloped virus as free extracellular virions. Bottom, cell-to-cell spread of the viral genome through a transient intercellular bridge. This phenomenon has been invoked to explain the spread of measles and rubella viruses during persistent infection of the brain.*

cules (Brodsky et al. 1999). Virus-infected target cells are rendered relatively less sensitive to virus-specific CTL attack, permitting them to continue to produce virions for an extended period of time.

Infectious immune complexes

In some instances where a virus persists in the presence of an active immune response, infectivity in the blood circulates in the form of immune complexes that are composed of infectious virions coated by virus-specific antibodies. Immune complexes can be demonstrated by the addition of anti-IgG antisera that will neutralize the infectivity. The molecular mechanism by which an antibody-coated virion can retain its infectivity has never been well elucidated. One possibility is that the complex is bound to Fc receptors on macrophages and internalized in vacuoles in which the complex dissociates, followed by infection of the macrophage. Consistent with this hypothesis, several of the persistent viruses (lymphocytic choriomeningitis virus, lactic dehydrogenase virus, and Aleutian disease virus) for which infectious complexes have been demonstrated, target macrophages as a major host cell.

Antigenic variation

During the course of persistent infection, there may be a selection for viral variants that are able to escape neutralization (Lutley et al. 1983). This phenomenon has been observed with several persistent lentiviruses such visna/maedi virus of sheep and equine infectious anemia virus. Equine infectious anemia virus (EIAV) is a lentivirus that produces a lifelong persistent infection of horses – the virus may be isolated from the blood even though the animals develop neutralizing antibody. Newly infected horses undergo discrete episodes of acute anemia, associated with bursts of viral replication. In each instance, the virus isolated during the episode of illness resists neutralization by serum obtained at the time of virus isolation (Table 12). However, the same serum can neutralize virus isolated at earlier times in infection. In other words, there is sequential replacement of virus with newly emerging variants that can escape neutralization. Ponies that survive repeated episodes of illness finally develop such a broad neutralization response that they can suppress all potential variants, thereby modulating, but not clearing, the smoldering infection. A similar evolution of virus during persistent infection has recently been shown to be a common phenomenon in persistent HIV-1 infections (Richman et al. 2003).

Escape mutants can also be selected by the cellular immune response (Aebischer and Mokophidis 1991; Allen et al. 2000; Ciurea et al. 2001). Most evidence for this phenomenon has been derived from experimental models (Schmitz et al. 1999; Evans et al. 1999; Allen et al. 2000), but it is possible that similar mechanisms play a role in naturally occurring persistent infections. Figure 21 shows an example where a macaque successfully immunized was able to contain a virulent challenge virus until a breakthrough occurred (Barouche et al. 2002). Analysis of viral isolates demonstrated that the breakthrough was associated with the emergence of a point mutation in the immunodominant epitope that was responsible for cellular immune control of infection.

Persistent viral infections often cause chronic diseases (McCune 2001). Some representative examples are set forth in Table 13.

HOST DETERMINANTS OF SUSCEPTIBILITY AND RESISTANCE

The outcome of the virus–host interaction is determined in part by host variables that can be conveniently considered under two categories, physiological factors and genetic determinants.

Table 12 *A smoldering infection associated with antigenic variation of the persistent virus*

Virus isolate (day of infection)	Fever spike (day of infection)	Neutralization index (log_{10}) of serum collected on the indicated day after infection					
		0 days	20 days	44 days	62 days	83 days	155 days
0 days		0	0	0.7	2.5	3.2	3.2
20 days	21	0	0	1.0	1.5	1.5	2.5
44 days	44	0	0	0	3.5	5.4	>5.4
62 days	62	0	0	0	0	2.0	2.0
83 days	83	0	0	0	0	0	3.5
155 days	155	0	0	0	0	0	0

Equine infectious anemia virus (EIAV) infection is associated with periodic febrile episodes. One infected horse experienced several fever spikes each lasting about 5 days, during which virus was isolated from the blood and was subsequently tested for neutralizability. Each virus isolate was neutralized by sera collected after the time of the isolate, but not by sera collected at or prior to the time of isolation. Likewise, each serum neutralized all the virus isolates made prior to the date of the serum, but none of the isolates made thereafter. This is evidence of continual antigenic drift of the virus, which probably explains the burst of replication associated with each fever spike, as well as the persistence of the virus in the face of an active neutralization response. After Kono et al. 1973.

Physiological factors

The host response to a viral infection may be influenced by a variety of physiological variables such as age, sex, stress, and pregnancy, all of which have been shown to influence the outcome of infection with some viruses (Brinton 1996). Age is perhaps the most consistently important variable in this category.

In general, very young animals are more susceptible than adult hosts to acute viral infections, and are more likely to undergo severe or fatal illness (Jubelt et al. 1980; Ogata et al. 1991; Tucker et al. 1993). However, it is important to recognize that in most mammalian species, young animals born of immune mothers receive maternal antibody either in utero by the transplacental route and/or postnatally, in colostrum and milk, or both. Furthermore, newborns have a special transport system that permits them to absorb intact immunoglobulin from the gut lumen into the circulation. The innate high susceptibility of infants born of non-immune mothers is revealed only in those rare instances where a virus has disappeared from an isolated community so that all age groups lack immune protection. A classical example is measles, which can be a devastating disease with a mortality of up to 25 percent in non-immune infants (Panum 1940; Morely 1969), but which is relatively benign in most societies where almost all women of childbearing age have acquired active immunity from natural infection or from measles vaccine. The mechanisms of enhanced susceptibility of newborn animals have been obscure, but recently it has been shown that an alphavirus induces apoptosis in neurons of newborn mice, but does not kill neurons from older mice in which enhanced expression of the cellular oncogene, bcl-2, blocks apoptosis (Levine et al. 1993).

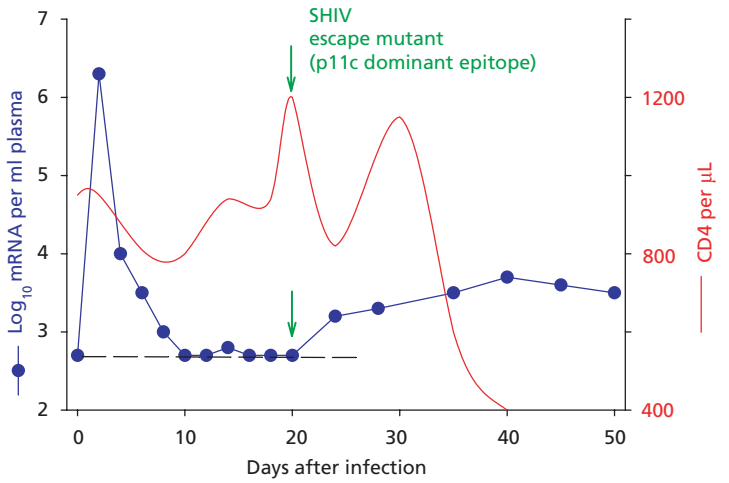

Figure 21 *Selection of viral variants that can escape the cellular immue response is one mechanism of viral persistence. In this example a monkey was immunized with a potent vaccine against SHIV (chimeric SIV–HIV virus) and then infected with a virulent SHIV strain. The monkey contained the infection quite effectively until the SHIV broke through due to emergence of a SHIV variant with a point mutation in an immunodominant epitope (data from Barouche et al. 2002).*

Table 13 *Diseases associated with persistent viral infections, selected examples*

Virus family/genus	Virus	Host	Disease
Oncogenic viruses			
Retroviridae	MuLV	Mice	Hematopoietic, lymphoreticular neoplasms
Hepadnaviridae	HBV	Humans	Hepatocellular carcinoma
Papillomaviridae	HPV	Humans	Cervical carcinoma
Herpesviridae	EBV	Humans	Burkitt's lymphoma
High titer persistence			
Arenaviridae	LCMV	Mice	Glomerulonephritis, vasculitis
Parvoviridae	Aleutian disease	Mink	Glomerulonephritis, vasculitis
Latent infections			
Herpesviridae	HSV	Humans	Cold sores, encephalitis
	CMV	Humans	Pneumonitis, retinitis, encephalitis
	EBV	Humans	Mononucleosis
	VZV	Humans	Herpes zoster
Smoldering infections			
Morbillivirus	Measles virus	Humans	Subacute sclerosing panencephalitis
	CDV	Dogs	Encephalitis, demyelination
Retrovirus	HTLV I	Humans	Tropical spastic paraparesis
Polyomavirus	JC	Humans	Progressive multifocal leukoencephalopathy
Lentivirus	VMV	Sheep	Interstitial pneumonitis, demyelination
	EIAV	Horses	Episodic hemolytic anemia
	HIV	Humans	AIDS

JC, the initials of the patient from whom virus was isolated; EIAV, equine infectious anemia virus; CDV, canine distemper virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HTLV-1, human T cell leukemia virus; LCMV, lymphocytic choriomeningitis virus; MuLV, murine leukemia virus; VMV, visna maedi virus; VZV, varicella zoster virus.

Less commonly, advanced age can also be associated with increased susceptibility to viral infection. St. Louis encephalitis is an example of this phenomenon (Table 14). In one epidemic, the ratio of encephalitis cases to infections was about 0.2 per 1 000 for children aged 0–9 years and about 3.0 per 1 000 for adults aged 60 and over. Another example is varicella zoster virus, which usually produces primary infections in childhood followed by latent infections of the spinal ganglia. Reactivations of latent infections, in the form of herpes zoster, rarely occur before age 50 (except in individuals with other debilitating illnesses), but are much more common over 60 years of age.

Table 14 *Age is one of the physiological variables that can determine host susceptibility*

Age group	Encephalitis rate per 100 000	Case-fatality rate per 100	Percent with SLE antibody
0–9	8	0	41%
10–19	13	0	29%
20–29	15	0	38%
30–39	14	0	36%
40–49	17	0	26%
50–59	28	13%	38%
60+	78	27%	25%

Age distribution of St. Louis encephalitis showing that attack rates increased strikingly in the older age groups, although the frequency of infection was similar for all ages.
Data from Luby et al., 1967; Henderson et al., 1970.

Host genes

Studies with inbred animals have documented that the outcome of infections often varies in different strains of mice (Brinton 1996), and genetic analyses have been conducted to determine the form of inheritance of susceptibility, and also to map the chromosomal location of the responsible genetic loci (Lodmell 1983; Oktsuka and Taguchi 1997). Several generalizations may be made, based on numerous studies of a wide variety of viruses:

- Most genetic loci control susceptibility to a specific family of viruses and not to all viruses.
- Susceptibility can often be mapped to a single autosomal locus, but multiple loci have been identified in some instances.
- When a single genetic locus has been identified, susceptibility may be either dominant or recessive.
- Where loci have been mapped, they are usually distant from the major histocompatibility locus, and in most such instances, the mechanism of susceptibility is not immunological.
- The exact mechanism of susceptibility has not been well defined in most instances, although there are some examples that map to the MHC, implying an

immunological explanation (Miyazawa et al. 1992; Poskophidis et al. 1994).

An example of genetically determined host susceptibility is shown in Table 15, in which there is a single autosomal genetic locus and resistance is dominant (Haller et al. 1980). In this instance, the genetic determinant, called the *Mx* locus, has been shown to influence the response to influenza viruses and is related to differential ability to mount an interferon response (Pavlovic et al. 1992; Staeheli et al. 1993). Thus, treatment with anti-interferon antibody abrogates resistance, and renders all strains of mice equally susceptible.

It is difficult to investigate genetic determinants of host susceptibility in an outbred population. Classical studies of myxoma virus in outbred rabbit populations (Fenner 1983) have clearly demonstrated genetic determinants that became manifest under the selective pressure of a virulent virus. Likewise, individual monkeys exhibit marked differences in susceptibility to Simian immunodeficiency virus (SIV) that correlates with ex vivo infectibility of their T lymphocytes (Goldstein et al. 2000). If it is assumed that there are human homologues for most mouse genes, then it may be inferred that the outcome of viral infections in humans is also influenced by genetic determinants. Consistent with this presumption are observations that human responses to standardized virus inocula may be somewhat variable (Table 10 and Figure 12).

Recent studies of HIV-1 have provided substantial evidence of a number of human genetic determinants of susceptibility to infection or to the rate of progression to overt AIDS (Liu et al. 1996; Kaslow et al. 1996; Migueles et al. 2000; Moore et al. 2002). The most striking of these is a polymorphism in the gene that encodes the chemokine receptor, CCR5, which is the major coreceptor for HIV-1. Some people have a deletion in the gene (Δ32 deletion) such that no CCR5 protein is expressed, and these individuals are at very low risk of HIV infection (Table 16). People who are heterozygous for the Δ32 deletion can be infected but, on average, have prolonged incubation periods to the onset of AIDS by comparison with $CCR5^{+}$ homozygous individuals.

Table 15 *Genetic determinants of host susceptibility can be delineated in inbred strains of mice*

Mouse strain	Genotype	Mortality dead/total	Log_{10} virus titer in blood
Not treated			
A/J	r/r	4/4	6.0
A2G	R/R	0/4	3.7
F1 (A/J × A2G)	R/r	0/4	3.5
Treated with anti-interferon antiserum			
A/J	r/r	4/4	6.0
A2G	R/R	4/4	6.3
F1 (A/J × A2G)	R/r	4/4	6.5

A/J mice are susceptible and A2G mice are resistant to a hepatotropic strain of influenza virus, and the F1 hybrid is also resistant, indicating that a single dominant autosomal gene determines resistance. In addition, treatment with anti-interferon antiserum abrogates resistance, suggesting that the effect is mediated through the action of interferon.
Data from Haller et al. (1980).
R, resistant; r, susceptible.

Table 16 *Differences in host susceptibility have been defined for a few viruses of humans*

HIV-1 antibody	Parameter	Total	CCR5 +/+	CCR5 +/−	CCR5 −/−
Positive	Number	1343	1148	195	0
	Percent	100%	85%	15%	0%
Negative	Number	612	508	87	17
	Percent	100%	83%	14%	3%

In this example, the chemokine receptor CCR5 acts as a coreceptor for most wildtype HIV isolates. A naturally occurring mutation (Δ32) in the CCR5 gene abrogates the expression of CCR5 and homozygous carriers of the mutation are resistant to HIV-1. Subjects at high risk of HIV exposure were divided into those who were HIV-infected and those who were not, and tested for their CCR5 status (CCR5 +/+, +/−, or −/−). There was a striking absence of $CCR5^{-/-}$ persons in the HIV-infected group and an excess of $CCR5^{-/-}$ persons (above the 1% expectancy) in the HIV-uninfected group.
After Dean et al. 1996.

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The immune response to viral infections

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Viruses and the immune system are continually involved in a sophisticated game of hide and seek. Whereas many virus infections are readily controlled by the immune response, some have evolved strategies to evade detection and persist in their host. This balance in the host–pathogen relationship is maintained by the immune system; a breakdown in immune surveillance can lead to recurrent infections and clinical disease.

The immune system employs a variety of strategies to combat virus infection. These can be divided into innate or 'nonspecific' defenses, which include interferon (IFN), natural killer (NK) cells, and macrophages, and adaptive or specific immunity that involves T cells and antibodies. The evolution of T-cell and antibody receptor diversity has probably been critical for the survival of higher vertebrates; it has enabled the host to match evolutionary mutations in structure used by viruses to evade host defenses (Janeway 1992; Zinkernagel 1995; Litman et al. 1999; Janeway and Medzhitov 2002).

Immunological defenses are marshaled at strategic areas in the body where entry of virus is favored. For example, IgA at mucosal surfaces functions by blocking virus adsorption and penetration, macrophages are located throughout the body in various tissues and IFN is produced by most cells in response to viral infection. Once the outer defenses are breached, viruses and viral antigens become channeled into the lymphoid system where they encounter T and B cells. A critical event in this process is the uptake and presentation of antigens by specialized antigen-presenting cells, which include dendritic cells (DC), macrophages, and B lymphocytes. From such encounters, antiviral T cells are generated that target and rapidly destroy infected cells, and antiviral antibodies are produced that protect the host from viremia and reinfection. How the immune response recognizes and kills virus-infected cells, how virus is neutralized by antibody and complement, and how these responses can have pathological outcomes will be discussed. These events will be contrasted with the evasion strategies employed by viruses.

INNATE IMMUNE DEFENSES

The innate defenses restrict early infection and limit spread to other tissues. Because this first line defense is mobilized within minutes or a few hours, it may hold the balance in the host response to virus infection. A key early surveillance mechanism against virus infection is mediated by Toll-like receptors (TLR) found on macrophages, DCs, and polymorphonuclear cells. TLR are a family of receptors with an extracellular domain containing leucine-rich repeats and an intracellular domain homologous to the cytoplasmic tail of the interleukin (IL)-1 receptor (Takeda et al. 2003). Signaling through TLR's leads to activation of NF-κB resulting in the production of pro-inflammatory cytokines. Of the current 11 TLR's, TLR-3 and TLR-7 are of interest here as they recognize dsRNA (TLR-3) and ssRNA (TLR-7) (Diebold et al. 2004). Restricting the early

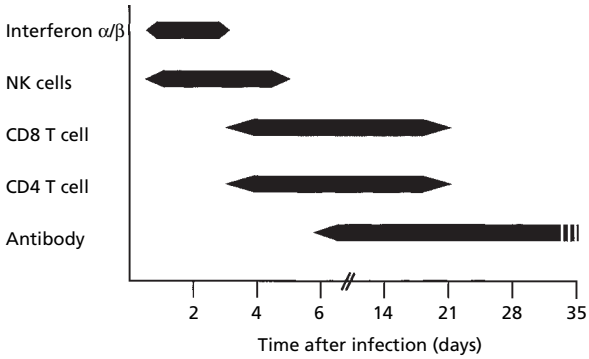

Figure 1 *Time scale of the innate and adaptive immune response.*

infection is the province of IFNs α and β, NK cells, and macrophages. The inflammatory response that follows this early encounter with viruses signals its location to the specific immune system, which becomes effective after a few days to a week. Only then will T cells home to the site of infection and antibody be active in limiting virus spread. A time scale for the evolution of the immune response is shown in Figure 1.

Antiviral properties of IFN

IFN is a first line of defense against viruses. There are two major families of IFN. Type I IFN, which includes IFN-α (leucocyte-type) encoded by 13 genes and IFN-β (fibroblast-type) encoded by a single gene on human chromosome 9 (mouse chromosome 4). Other type I IFN genes include human IFN-ω, IFN-τ/ϵ (trophoblast), and the recently described IFN-κ (LaFleur et al. 2001), and IFN-λ (IL-28/IL-29 family) (Kotenko et al. 2003). IFN-α/β is induced within hours of infection and initiates the antiviral state in neighboring cells via the IFN-α/β receptor. This results in the activation of a number of genes associated with the antiviral response as well as anti-proliferative and immunomodulatory activities. In contrast, type II IFN or IFN-γ is produced following antigenic or mitogenic activation of T cells and NK cells. IFN-γ binds to the type II IFN receptor and mediates a similar cascade of gene activation to type I IFN (for reviews see Vilcek and Sen 1996 and Goodbourn et al. 2000) (see also The role of cytokines in viral infections).

The antiviral response mediated by IFN involves three well described mechanisms, although several others probably exist. Of these protein kinase R (PKR) and 2′–5′ oligo adenylate synthetase are the best characterized. dsRNA-dependent PKR is activated by viral dsRNA and acquires a central role in initiating a number of interactions. PKR phosphorylates the α-subunit of eIF2 preventing the recycling of initiation factors resulting in a block in translation of viral RNA (Clemens and Elia 1997). PKR also mediates apoptosis via Bcl-2 and caspase-dependent mechanisms, thereby thwarting virus infection (Lee et al. 1997). In addition, PKR plays a role in signal transduction via NF-κB, important for activation of the *IFN-β* gene (Williams 1999). Although a key protein in a number of cellular events, PKR is not essential for IFN-mediated antiviral responses, as mice lacking PKR are still able to resist viral infection (Abraham et al. 1999).

2′–5′ oligoadenylate synthetase catalyses the synthesis of short oligomers (2′–5′ A oligomers) from ATP that act as potent activators of the latent endonuclease RNase L. This endonuclease mediates cleavage of ssRNA thereby inhibiting protein synthesis (Silverman 1999). This IFN mechanism is known to be effective against vaccinia virus and reovirus.

Mx proteins are found in all vertebrate species. They are readily induced by type I IFN and function by inhibiting transcription of a range of RNA viruses including orthomyxoviruses and paramyxoviruses.

Other IFN-dependent mechanisms clearly exist, since mice deficient in PKR, RNase L, and Mx (triple gene knock-out) still retain antiviral activity (Zhou et al. 1999). Other likely mechanisms include dsRNA-dependent adenosine deaminase (ADAR) and caspases 1, 3, and 8.

IFNs play a major role in the immunomodulation of innate and adaptive immune responses. IFN-α/β is an important activator of major histocompatibility complex (MHC) class I and NK cell activity. IFN-γ is a powerful inducer of MHC class I and class II expression, as well as the machinery involved in antigen presentation, for example, proteosomes and TAP-1 and TAP-2 peptide transporters. This augments the activity of cytotoxic T cells (CTL) making them more efficient killers of virus infected cells (see section entitled T lymphocytes and immunity to viruses). Other roles for IFN-γ include directing $CD4^+$ T cells towards a Th1 phenotype, and activating the antiviral state of macrophages.

The importance of type I IFNs in antiviral immunity stems from three experimental approaches: (1) the local administration of IFN to sites such as the nasal passage which protects against rhinovirus infection (Merigan et al. 1973); (2) the administration of anti-IFN antibodies to neutralize IFN-α/β in mice which leads to an increased susceptibility to infection with vesicular stomatitis virus (VSV) and herpes simplex virus (HSV) (Gresser et al. 1976); and (3) the use of IFN receptor gene knock-out mice. The last demonstrates, quite dramatically, the importance of IFN in arresting viral replication in vivo. A variety of RNA viruses (lymphocytic choriomeningitis virus (LCMV), VSV, Theiler's murine encephalomyelitis virus), and DNA viruses (vaccinia, herpesviruses) show increased replication and pathogenic effects in IFN-α/β receptor knock-out mice (van den Broek et al. 1995).

NK cells in antiviral immunity

NK cells are large granular lymphocytes distinct from T- and B-cell lineages. Human NK cells are characterized

by the presence of CD16 and CD56 markers. Mouse NK cells have NK1 or NK2 and asialo-GM1 markers that are used to study their function in vivo. A population of NK1.1, $CD8^+$ cells has been described, which have important regulatory functions in the immune response (Vicari and Zlotnik 1996). NK cells produce a number of cytokines involved in antiviral immunity (see The role of cytokines in viral infections), including IFN-γ, tumor necrosis factor α (TNF-α), tumor growth factor β1 (TGF-β1), granulocyte–macrophage colony-stimulating factor (GM-CSF), and IL-1β (Trinchieri 1989; Perussia 1991).

NK cells display a number of receptors involved in the activation and inhibition of NK cell function. Activation receptors include NKp30, NKp44, and NKp46 on human NK cells and Ly49D and Ly49H on mouse NK cells. These receptors are involved in recognition of virus infected cells with NKp46 recognizing influenza hemagglutinin and Ly49H recognizing m157 of murine cytomegalovirus (Mandelboim et al. 2001; Arase et al. 2002). Other activation receptors include LFA-1 and CD2 members. In addition, NK cells have receptors for a number of cytokines known to be important in activation and proliferation, for example, type I IFN, IL-2, IL-12, IL-15, and IL-18.

In order to protect normal cells from overt NK cell activation and damage, NK cells also carry a variety of inhibitory receptors. This includes the killer cell immunoglobulin-like receptors (KIR), immunoglobulin-like inhibitory receptors (ILT), and lectin-like heterodimers CD94–NKG2A. These receptors bind most MHC class I molecules and deliver inhibitory signals to the cells via immunoreceptor tyrosine-based inhibitory motifs (ITIM). This ability to regulate NK-cell activation has been exploited by some viruses by mimicking inhibitory receptors thereby confusing NK cells at sites of infection (see section Interference with MHC function and NK cell recognition, below).

NK cells can also recognize IgG antibody-coated cell surfaces via FcγRIII (CD16) and kill target cells. This process, known as antibody-dependent cell cytotoxicity (ADCC), is a highly efficient mechanism for activating NK cells and precipitating rapid destruction of virus-infected cells. Cell killing is mediated by a perforin-dependent mechanism (Kagi and Lederman 1994).

NK cells seem to be rapidly mobilized following virus infection. They are detected at sites of infection within 2 days of virus entry, and their appearance is related to a rise in type I IFN levels in the blood. Initial encounter of NK cells with virus-infected cells results in a 'bystander' proliferation mediated by cytokines such as IL-15 and type I IFN. The latter is known to induce blastogenesis in NK cells, probably via up-regulation of IL-15 (Nguyen et al. 2002). After the initial phase of nonspecific activation, there appears to be a more selective amplification of NK cells. In murine cytomegalovirus infection there is a preferential selection for Ly49H-expressing NK cells that appear around day 4–6 following recognition of m157 encoded by the virus (Dokun et al. 2001; Arase et al. 2002). This specific expansion of NK cells is only seen during murine cytomegalovirus (MCMV) infection in resistant mouse strains. Conversely, m157 also interacts with Ly49I, an inhibitory molecule on NK cells. This interaction predominates in MCMV-susceptible mouse strains which lack Ly49H. Around day 6 of MCMV infection there follows a contraction of NK-cell expansion at a time when there is a rise in the adaptive immune response.

Mice vary in susceptibility to MCMV. Resistance to infection is controlled by both *H-2* and non-*H-2* genes. The latter was identified as a single autosomal dominant gene called *Cmv-1* (Scalzo et al. 1992). This gene is located on chromosome 6 and is linked to the NK cell complex, which includes *NK1.1* and *Ly49* genes. Depletion of NK cells in mice bearing the *Cmv-1* gene increases viral replication, proving the link between this gene and NK cell activity (Scalzo et al. 1990). The *Cmv-1* gene has now been identified as Ly49H which targets the m157 protein on MCMV infected cells.

An interesting series of experiments involves the role of NK cells in immunity to vaccinia. Athymic nude mice are highly susceptible to vaccinia but are resistant to vaccinia virus expressing the *IL-2* gene. The mechanism of resistance in this instance involves activation of NK cells by IL-2 to produce IFN-γ, which mediates protection, probably via macrophage activation (Karupiah et al. 1990).

In humans, NK cells are considered to play an important role in immunity to herpesvirus infections. A single NK cell-deficient patient has been reported with severe herpesvirus infections, including cytomegalovirus (CMV) and varicella-zoster virus (VZV) (Biron et al. 1989, 1999).

The key role of macrophages and DCs in immunity to viruses

Macrophages are strategically located throughout the body as a first line of defense against infectious agents. Their importance in virus infections is often underestimated; nevertheless, they have potent antiviral activity and serve to limit virus spread. Macrophage antiviral activity can be broadly divided into intracellular and extracellular defense mechanisms. Macrophages are major producers of IFN-α found in the blood stream following infection. Other antiviral molecules produced by macrophages include:

- TNF-α, which functions either by inducing genes coding for 2′–5′A synthetase and Mx, or by selectively killing virus-infected cells, via apoptosis (Wong and Goeddel 1986).
- Arginase, which depletes local arginine concentrations and effectively aborts HSV infection (Bonina et al. 1984; Mistry et al. 2001).

- Nitric oxide synthase, which is induced following IFN-γ activation of macrophages and leads to the generation of nitric oxide which inhibits the replication of vaccinia virus and HSV (Karupiah et al. 1993).

For many viruses, the hostile environment of a phagolysosome leads to a loss of infectivity. Here, both oxygen-dependent and oxygen-independent mechanisms are likely to contribute to the inactivation of certain viruses. Viruses can be targeted for intracellular destruction by phagocytosis of infected cells or by opsonization of virus–antibody complexes via the Fc receptor; the latter is an efficient way of removing virus from the blood stream. Macrophages can also kill virus-infected cells either by direct contact or, more efficiently, by ADCC through recognition of IgG antibody. In herpesvirus infections, neutrophils have also been implicated in the destruction of infected cells via ADCC.

Despite the hostile nature of macrophages, some viruses selectively exploit these cells for growth and survival; they include lactate dehydrogenase virus, maedi-visna virus, human immunodeficiency virus (HIV), and CMV.

DCs play a major role in the induction of the adaptive immune response (see section entitled T lymphocytes and immunity to viruses). However, it is now apparent that multiple subsets of DCs exist, some of which may have specialized functions in the control of virus infection (Shortman and Liu 2002). An example is the plasmacytoid-derived DC-2 which is a major producer of type I IFN (Siegal et al. 1999). They have been found during influenza virus, HSV, and CMV infections where they appear at sites of infection and in lymphoid tissue associated with infection. During influenza virus infection, DC-2 cells make large amounts of IFN-α which results from endosomal recognition of viral RNA by TLR-7 and activation through MyD88 (Diebold et al. 2004). One role for DC-2 cells is to orchestrate the innate immune response through production of IFN-α. This almost certainly involves activation of NK cells and NKT cells. NK cell–DC interactions are known to be important in the response to MCMV infection (Andrews et al. 2003). This interaction is independent of DC-2 cells and favors a subset of DCs in the mouse that are $CD8^+$.

Collectins and the complement system

Collectins are soluble proteins found in serum, lung, and nasal secretions that can act as a first line of defense against infectious agents (Epstein et al. 1996). They include mannose-binding lectin (MBL) (also known as mannose-binding protein), conglutinin, and lung surfactant proteins A and D (SP-A, SP-D). The basic structural unit is composed of a collagen stalk and a globular head with a lectin-binding domain. Each unit exists as a trimer or multimeric complex. These structural features are similar to the first complement protein C1q. The collectins constitute a primitive antibody-like defense able to target diverse carbohydrate structures on pathogens and yet distinguish self from nonself structures. MBL interacts with HIV, influenza virus, and hepatitis B virus (HBV), and is capable of activating the classical complement pathway to neutralize the virus (Malhotra and Sim 1995; Reading et al. 1997; Hakozaki et al. 2002).

Complement proteins can also directly target viruses or virus-infected cells. Human serum is particularly efficient in the neutralization of certain retroviruses (e.g. murine leukemia virus (MLV)). Human C1q binds the p15E protein of MLV, resulting in activation of the complement system and neutralization of the virus by a process termed virolysis, i.e. damage to the virion envelope (Bartholomew et al. 1978). Classical complement pathway is also activated by CMV-infected cells (Spiller and Morgan 1998) and by cell-free humanT-lymphotropic virus type 1 (HTLV-1) (Ikeda et al. 1998). Sindbis virus is also able to activate the classical complement pathway, but can also directly activate the alternative pathway, i.e. via C3. Other important roles for complement involve interaction with antibody: these events are discussed in the section entitled The role of antibodies in immunity to viruses. C3a and C5a are both inflammatory mediators and as such can serve as signals for alerting inflammatory cells to sites of infection. This is probably one of the reasons why some viruses e.g. herpesviruses and poxviruses carry genes able to subvert C3 activation (see section entitled Viral strategies for evading the immune response). A summary of the complement activation pathways and their functions is shown in Figure 2.

The significance of the complement system in antiviral immunity has been difficult to evaluate. In humans and animals, natural deficiencies of complement components do not predispose the host to severe virus infection (Lachmann and Rosen 1979). Experimental approaches have involved the administration of cobra venom factor to exhaust endogenous C3 and hence incapacitate the complement system and the use of complement deficient, i.e. gene knock-out mice. Although most virus infections studied were not affected by depletion of C3, with Sindbis virus an increased viremia was observed in the depleted mice (Hirsch et al. 1980).

Summary of innate immunity

Innate immunity performs an essential role in the early stages of virus infection. Components of the system act rapidly and initiate the cascade of events that focus the specific immune response on the infected area. Factors released in the processes described above lead to an up-regulation of adhesion and MHC molecules on local blood vessels, which enable lymphocytes to extravasate and enter the parenchyma. Chemoattractants produced

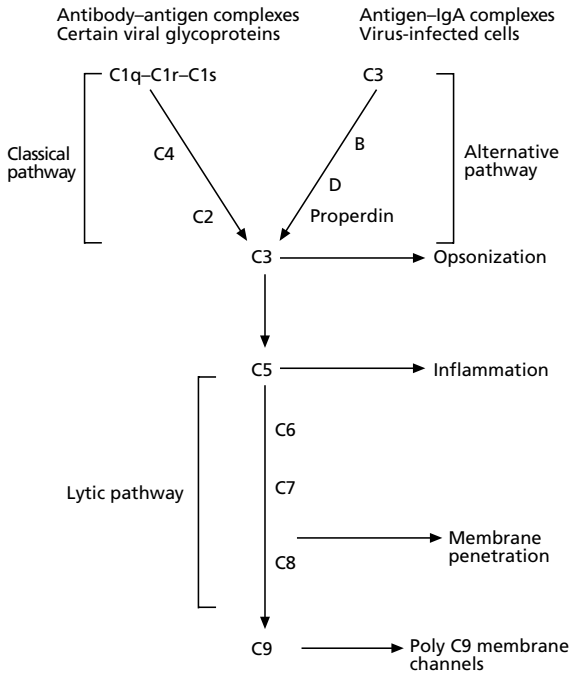

Figure 2 *Activation of the complement cascade by virus infection.*

by mast cells, macrophages, NK cells, and complement components recruit lymphocytes, which can then interact with antigen-presenting cells both at the site of infection and in the local lymph nodes.

T LYMPHOCYTES AND IMMUNITY TO VIRUSES

T lymphocytes are central for inducing immunological responses and, in many instances, for the recognition and destruction of virus-infected cells. A deficiency or complete absence of T cells in a host often predisposes to severe or fatal disease. This is particularly so in patients who are severely immunocompromised during organ transplantation. These individuals are at particular risk from reactivating herpesviruses, such as CMV and Epstein–Barr virus (EBV). The complex interrelationship between HIV and humans is another example of an apparent selective immunodeficiency of $CD4^+$ T cells leading inexorably to opportunistic infection in which CMV becomes a life-threatening infection.

Our understanding of the importance of T cells in viral infections stems from studies in animal models. In mice, selective immunodeficiency states can be 'manufactured' using gene knock-out technology (Doherty 1993a). These enable specific questions to be addressed on the function of different T-cell populations and their products (for example, cytokines and receptors). A similar approach involves the use of monoclonal antibodies to interfere selectively with the function of lymphocytes and other cells and cytokines. The advantage of these techniques is that cell activity can be interrupted before or during an infection, thus permitting examination of the induction and effector capacity of the immune response. The inbred nature of laboratory mice also enables specific T-cell populations and clones to be adoptively transferred to syngeneic recipients and their function monitored. This approach has implications for immunotherapy in humans, in whom protection of high risk transplant patients from CMV disease can be achieved by the transfer of selective anti-CMV T-cell clones derived from the patient prior to immunosuppression (Riddell and Greenberg 1994).

These powerful techniques emphasize the importance of α/β T-cell receptor populations, in particular the role of $CD8^+$ T cells in immunity and immunopathology of virus infections. The significance of γ/δ T cells in antiviral immunity is still unclear although they are found at sites of virus infection, suggesting a role in defense (Born et al. 1991). They have a more restricted receptor repertoire than α/β T cells and are thought to recognize different restriction elements, including CD1 molecules (Sciammas et al. 1994). In this section we focus on the activities of α/β T cells.

Central role for $CD4^+$ T cells in the induction of the immune response to viruses

As with any antigenic challenge, the initial events in the induction of immunity involve the uptake of virus or viral proteins by DCs and processing to peptides for presentation by MHC class II molecules (Watts and Powis 1999). Briefly, antigen is internalized in endosomes and subjected to proteolytic degradation. These endosomal compartments then fuse with vesicles carrying MHC class II molecules to the cell membrane. MHC class II arises in the endoplasmic reticulum (ER) complexed with the invariant chain. The invariant chain serves two important functions: (1) to target MHC class II to the trans-Golgi and (2) to block the MHC class II cleft from binding peptides prematurely in the ER. On fusing with endosomes, a combination of the acidic pH and MHC class II M molecules (a nonclassic MHC class II molecule) dissociates the invariant chain and allows peptides of around 15 amino acids in length to associate with MHC molecules (Busch and Mellins 1996). Recognition of this structure on the antigen-presenting cell is by the T-cell receptor (TCR) and CD4 molecule on T cells. Additional secondary interactions between cell surface receptors, for example B7 and CD28, LFA-1 and ICAM 1/2, LFA-3, and CD2, lead to signal transduction and the production of IL-2 and IL-2 receptors, resulting in clonal expansion of the interacting $CD4^+$ T cell (Dustin and Springer 1991; Montoya et al. 2002). These events initiate the T helper cell response, which catalyses the induction of $CD8^+$ CTLs, activates NK cells and macrophages, and is critical for induction of the

T-dependent antibody response. Many of these amplifying events involve cytokines and the interaction with key cell surface molecules CD40 and CD40L (T-cell ligand) and B7.1 and CD28. B lymphocytes are the other major antigen-presenting cells in the immune system and are particularly effective at presenting to memory T cells. Figure 3 outlines the various helper interactions mediated by CD4 T cells.

Recognition of virus-infected cells by $CD8^+$ T lymphocytes

$CD8^+$ T cells recognize a complex of MHC class I with a foreign (virus) peptide on infected cells. The process by which viral antigens are processed and presented is referred to as the endogenous antigen-presenting pathway (Lehner and Cresswell 1996). Briefly, newly synthesized viral proteins are degraded in part by a complex of proteolytic enzymes called the proteosome. Small peptides generated by this process are actively transported via peptide transporters (TAP1 and 2) to the ER where they associate with the MHC class I heavy chain. Peptides may be trimmed by enzymes in order to fit snugly into the MHC cleft which accommodates peptides of around nine amino acids. The light chain (β2-microglobulin) associates with the heavy chain and the trimolecular complex is transported to the cell surface. The evolutionary advantage of this process is that peptides derived from intracellular pathogens can be processed at an early stage in the infection, well before replication or assembly of new virions has occurred. If they can be recognized by the immune system the infectious agent can be rapidly eliminated. The fact that viral immediate early and early antigens can be recognized by T cells supports this view.

Selection for the relevant peptides can occur at three stages in this processing pathway: (1) the proteosome, i.e. where peptide cleavage occurs according to the enzymatic specificity within the proteosome; (2) the peptide transporters that show a preference for selecting certain C-terminal amino acids; and (3) the MHC molecule for which many allelic forms exist, all differing at the peptide-binding groove/cleft.

Because MHC class I molecules occur on virtually all nucleated cells, viruses will have difficulty avoiding the attentions of cytotoxic CD8 T cells. They have, however, evolved some intriguing strategies to disrupt antigen presentation, discussed below (see section entitled Viral strategies for evading the immune response). One strategy adopted by HIV involves antigenic variation and T-cell receptor antagonism. Mutation in antigenic peptides is a feature of HIV infection. Changes can occur that affect (1) peptide binding to MHC class I molecules, (2) peptide recognition by the TCR, and (3) peptide processing by proteosomes. In (3), alterations to amino acids flanking either side of an antigenic peptide could alter peptide processing. Altered peptides affecting TCR recognition can antagonize the T-cell response to the unmutated antigenic peptides (Klenerman et al. 1996).

T cells in antiviral immunity

The importance of particular T-cell subsets in immunity to viruses depends on several, often interrelated, factors, including the nature of the infecting virus, dose of infecting agent, route of infection, and age of the host.

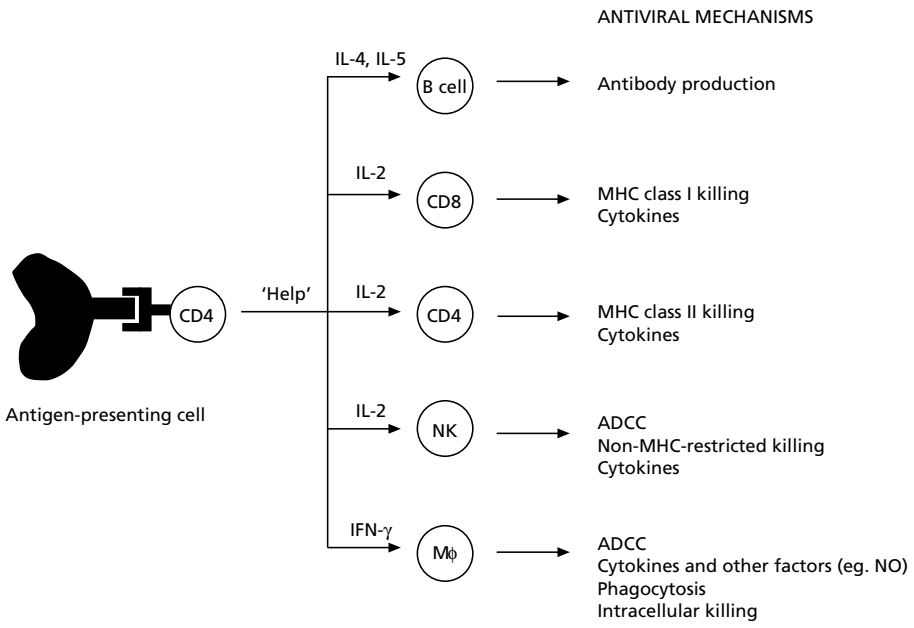

Figure 3 *CD4 T cell 'help' for other arms of the immune response. ADCC, antibody-dependent cell cytotoxicity; IL, interleukin; MHC, major histocompatibility complex; Mϕ, macrophage.*

Since 1976, a vast amount of information has been generated on the role of T cells in a number of virus infections, including the well-studied examples of influenza virus, LCMV, vaccinia virus, HSV, and HBV. A more detailed analysis of the immune response to selected virus families is provided in the section entitled Examples of immune responses to specific viruses. These studies indicate a direct role for CD4 and CD8 T cells in recovery from infection, which is emphasized in studies on mice deficient in MHC class I and II genes.

Targeted disruption of β2-microglobulin resulted in low levels of MHC class I expression. These mice have normal CD4 T cells but are severely lacking in CD8 T cells. Such deficient mice infected with LCMV had an impaired ability to clear the infection, stressing the importance of CD8 T cells in this process. In contrast, when these mice were infected with influenza, Sendai or vaccinia viruses they cleared the infection normally. This was surprising, because previous experiments on the adoptive transfer of CD4 or CD8 T cells from infected mice had indicated that CD8 T cells were central to recovery and that CD4 T cells contributed to pathology of the lung infection (Leung and Ada 1982). Not all antiviral CD4 T-cell responses are pathological in outcome, many play an important role in recovery from infection through interaction with B cells and in activating cell-mediated immune responses. In cutaneous HSV infection of mice, a clear role for CD4 T cells exists in clearing virus from the epidermis. The mechanism of protection involves IFN-γ and macrophage recruitment and activation, and resembles a delayed hypersensitivity response (Nash and Cambouropoulos 1993). Similarly, control of vaccinia virus infection of ovaries and VSV infection of the central nervous system (CNS) is mediated by an infiltrating CD4 Th1 response, but not by a CD4 Th2 response (Maloy et al. 2000).

Mice deficient in MHC class II molecules had a deficient CD4 response but normal numbers of CD8 T cells. Infection of these animals with influenza virus resulted in the generation of a virus-specific CD8 T-cell response and recovery from infection (Bodmer et al. 1993). This and previous observations on HSV infection in CD4 T-cell-deficient mice (Nash et al. 1987) indicate that virus-specific CD8 T cells can be induced in a 'helperless' environment. It is interesting that, in mice with a CD4 disrupted gene, there is a failure to maintain a LCMV-specific CD8 T-cell response, leading to persistence of the virus (Battegay et al. 1994). These data argue in favor of a role for CD4 T cells in the maintenance of a continuing CD8 T-cell response and of T-cell memory. In fact, the number of memory T cells arising from an infection is related to the extent of the CD8 T-cell expansion during the primary response (Marshall et al. 2001). The maintenance of T-cell memory is related to the presence of cytokines such as IL-7 and IL-15 (Becker et al. 2002; Tan et al. 2002) and not the continued presence of antigen (Murali-Krishna et al. 1999).

How do CD4 and CD8 T cells mediate antiviral immunity in vivo? Following recognition of the relevant restriction element (MHC class I or II molecules) and T-cell activation, the effector activity of T cells involves cytolysis of the infected cell or control of infection by cytokines, or both. Cytolysis can occur via perforin and the fragmentins, e.g. granzymes, Fas–Fas ligand interaction and tumor necrosis factor (TNF) (Doherty 1993b). The key cells in cytolysis are the CD8 T cells; 'killer' CD4 T cells have been described in measles and HSV infections but are clearly limited to target cells expressing MHC class II molecules (Yasukawa and Zarling 1984). The major cytokines in antiviral immunity include IL-2, IFN-γ, and TNF.

The importance of cytolytic mechanisms in vivo has been demonstrated by targeted gene disruption. Mice deficient in perforin expression are susceptible to LCMV infection. Susceptibility is correlated with a considerably reduced ability of CD8 T cells to lyse infected target cells in vivo. However, some lytic activity is still detected which is attributed to Fas-mediated cell death (Kagi and Lederman 1994). In murine gammaherpesvirus infection, perforin does not seem to be essential for antiviral immunity mediated by CD8 T cells (Usherwood et al. 1997).

In many virus infections the destruction of certain tissue cells by cytolytic mechanisms may be an unacceptable price for the host to pay. Consequently, nondestructive mechanisms that eliminate the virus but spare the cell are biologically advantageous. Cytokines clearly fulfill this role and are now viewed in a much more positive light as effector molecules in viral infections (Ramshaw et al. 1992; Ramsey et al. 1993) (see also The role of cytokines in viral infections). Perhaps the best characterized is IFN-γ. A striking illustration of cytokine-mediated antiviral immunity is seen in HBV transgenic mice where clearance of virus is mediated in the absence of cell death, thereby 'curing' the infected cell (Guidotti et al. 1999) (see also section entitled Immune response to hepatitis B, below).

THE ROLE OF ANTIBODIES IN IMMUNITY TO VIRUSES

In general, antibodies appear 5–7 days after virus infection. The first antibody class to appear is IgM, which is usually of low affinity. Such antibodies are effective in controlling the spread of virus in the blood stream. As the antibody response evolves and affinity maturation proceeds (a process involving B cell proliferation leading to somatic mutation of immunoglobulin V regions and immunoglobulin class switching) IgG becomes the dominant class in serum and is characterized by increased affinity for viral antigens (Wabl and Steinberg 1996). Such antibodies are the major defense against reinfection by virus, which becomes neutralized by a variety of mechanisms (see section entitled Destruction of infected cells,

below). The main antibody defense at mucosal surfaces involves IgA, which occurs as a dimeric immunoglobulin with a secretory piece which protects them against the hostile environment of the gut and other mucosal sites. As discussed in the section entitled Destruction of infected cells below, antibodies form an important link with the innate immune response where they focus and activate the complement system and interact with a variety of phagocytic cells and lymphocytes via receptors for the Fc region of IgG (FcγR) and complement (CR). This combined response leads to neutralization of virus or lysis of virus-infected cells or phagocytosis.

Mechanisms of antibody-mediated neutralization of virus

The process whereby antibody neutralizes a virus is extremely varied and depends on the nature of the viral epitope, the class of antibody, and the nature of the infected cell (for review, see Dimmock 1995 and Klasse and Sattentau 2002). This process falls into two parts: neutralization at the cell surface and neutralization within the cell. A summary of the various antiviral mechanisms involving antibody is shown in Figure 4.

An obvious mechanism involves the blockade of virus ligand–cell receptor interaction. However, this event is quite rare, because a build-up of antibody on the virion surface is necessary in order to occupy all the available attachment sites, thus making the process inefficient from the viewpoint of the host defenses. Steric hindrance of receptor–anti-receptor interaction occurs with antibody neutralization of rhinoviruses. Here the antibodies attach to the rim of a 'canyon' or pit on the virion which is the site for binding to ICAM-1 on the cell membrane (Rossman 1989). In the early stages of the immune response, when antibodies have a low affinity for viral antigens, activation of the complement system can augment neutralization. This is illustrated with monoclonal antibodies that neutralize only in the presence of complement. The rapid build-up of complement on the virion membrane can lead to steric hindrance of virus–cell interaction or lysis of the virion envelope.

Another form of antibody-mediated neutralization involves inhibition of penetration of the virion. This might involve interfering with the fusion of the virion envelope with the cell membrane, as seen with HIV (Dimmock 1995). This process is probably the main mechanism of neutralization of paramyxoviruses and herpesviruses.

Even when a virus has entered the cell it is still subject to neutralization by antibody. For example, fusion with the endosome is a key mechanism used by influenza virus to enter the cytoplasm. To achieve this, hemagglutinin undergoes a major conformational rearrangement to engage and fuse with the endosomal membrane. Antibodies have been implicated in inhibiting this process (Wharton et al. 1995). Certain nonenveloped viruses enter the cell by adsorptive endocytosis, and changes in local pH lead to acidification and eventual uncoating. Poliovirus undergoes a series of changes resulting in a reduced sedimentation coefficient from 160S to 140S to 80S. Several neutralizing antibodies have been defined that inhibit this progression, blocking uncoating (Vrijsen et al. 1993). A similar process occurs with adenovirus, in which the anti-hexon antibody inhibits the pH-dependent changes required for fusion with the endosome and infectivity (Wohlfart 1988). In rare instances, antibody can neutralize infectivity after uncoating has taken place. With influenza virus, transcription is blocked by a failure of the ribonucleoprotein to function (Armstrong and Dimmock 1992).

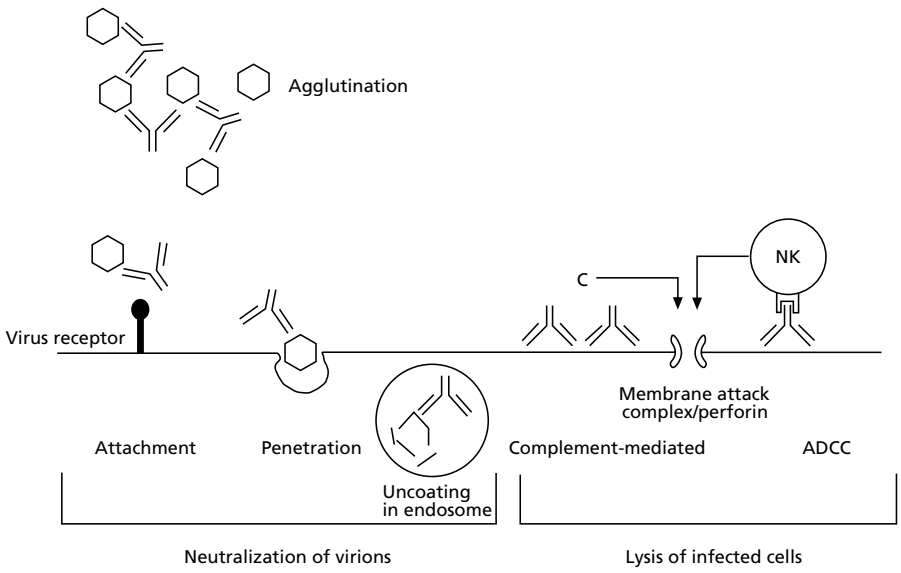

Figure 4 *Antiviral effector mechanisms mediated by antibodies. ADCC, antibody-dependent cell cytotoxicity; C, complement; NK, natural killer.*

Other direct effects of antibody

In neurons infected with Sindbis virus, replication is inhibited following antibody binding to viral glycoproteins on the cell membrane. This occurs with neutralizing and nonneutralizing monoclonal antibodies and is independent of complement and ADCC. The mechanism is unclear but may involve up-regulation of some cytoplasmic defense system (Levine et al. 1991; Levine and Griffin 1992).

Neutralization by IgA is a highly effective method of inhibiting virus invasion at mucosal surfaces. This can take place at two levels: extracellular and intracellular neutralization of virus. The latter is thought to occur in polarized cells in which IgA passes through via the poly-Ig receptor from the luminal to the apical surface. It is speculated that, during this process, vesicles containing IgA interact with those containing virus, leading to neutralization (Mazanec et al. 1992, 1993).

Antibodies can inhibit the release of some viruses from the infected cell. Examples include the herpesviruses in which antibodies to glycoprotein H of HSV-1 can block virus egress. Antibodies are also effective in blocking virus-induced cell fusion, thereby inhibiting syncytial (giant cell) formation.

Destruction of infected cells

Antibody can contribute to the destruction of virus-infected cells in two ways: complement-dependent lysis and ADCC.

Usually, a high density of membrane-bound antibody (calculated to be of the order of 5×10^6 IgG molecules/cell) is required for complement-mediated lysis of measles virus-infected cells. Clearly, such mechanisms are likely to be effective late in the infectious cycle. It is interesting that in most cases of antibody-targeted complement-mediated lysis it is the alternative complement pathway that becomes activated and not the classical pathway as one might have predicted (Sissons 1984).

ADCC is a highly efficient process, requiring only small quantities of bound IgG (10^3 molecules/cell). With HSV-1-infected cells ADCC is effective early on, before new progeny virus is assembled and released (Kohl 1991). The types of cell involved in this process have been discussed in the sections entitled Antiviral properties of IFN and NK cells in antiviral immunity, above.

Significance of antiviral antibody mechanisms in vivo

It is likely that all the antibody mechanisms discussed above contribute to immunity in the host. Studies with monoclonal antibodies have allowed dissection of the role of specific mechanisms. Antibodies that neutralize viruses in vitro are also effective at inhibiting their replication in vivo. This is illustrated by the inactivation of respiratory syncytial virus (RSV) by Fab fragments directed against the F protein of the virus (Crowe et al. 1994). This observation has formed the basis for the therapeutic use of 'humanized' anti-F protein antibodies in the treatment of RSV infections.

Nonneutralizing monoclonals against some viruses (e.g. HSV-1) can also protect against infection when passively administered to mice. The antibodies function in the absence of an active complement system (i.e. in C5-deficient mice), indicating a likely role for ADCC (Kohl 1991).

Passive delivery of antibody is an important natural process in the protection of the newborn. Transplacental transport of IgG antibodies offers an important initial defense against microbial invasion, allowing the infant's immune system time to develop. Likewise, antibodies delivered via colostrum are a major defense against rotavirus infections.

Despite the obvious advantages of antiviral antibodies in preventing infection, individuals with natural deficiencies in antibody are generally not prone to severe virus infections. Exceptions include echovirus infections, in which individuals with X-linked agammaglobulinemia (XLA) are prone to chronic infections of the CNS and muscles that can prove fatal (McKinney et al. 1987).

Summary of T- and B-cell immunity

T and B lymphocytes form an integrated system that tailors the immune response specifically to the relevant virus infection. In addition to specificity, the system retains a memory of the viruses to which it has been exposed, so that the host can be protected better on subsequent encounter. Central to the specific response is the CD4 T cell, which produces growth factors that induce the proliferation and differentiation of both T and B lymphocytes to magnify an immune response. In addition, 'help' from CD4 T cells enables B cells to produce higher affinity antibodies as the response progresses, which are more potent in binding to virus proteins. Such antiviral antibodies can neutralize free virions, and are therefore effective in infections in which there is a cell-free viremia. CTLs then complement antibody by destroying cells that have already been infected. In influenza virus infection, CTLs are necessary to resolve an initial infection; however, antiviral antibody prevents virus spreading in the body and provides subsequent protection from reinfection with the same virus strain.

IMMUNOPATHOLOGY IN VIRUS INFECTION

In any virus infection there is a likelihood of pathological damage to tissues by the immune system. The mechanisms associated with damage are varied and depend on

the nature of the virus, its tissue tropism, and the antigenic load associated with the infection. Examples of virus-induced immunopathology are listed in Table 1.

Antibody-mediated pathology

Antibody-mediated damage can be divided into immune complex diseases and enhancement of virus infectivity. Antibody-dependent enhancement (ADE) of virus infection is a process whereby virus-specific antibodies enhance the entry of virus into macrophages via interaction with Fc and/or complement receptors (Porterfield 1986; Tirado and Yoon 2003). The phenomenon is observed with many diverse virus infections, for example, flaviviruses to poxviruses, with the common theme that they target macrophages as a site of infection, that they preferentially induce a weak neutralizing antibody response in the infected host, and that they are associated with persistent, viremic infections. ADE has been implicated in a number of human and animal viral diseases. The most compelling examples include dengue virus, Aleutian mink disease virus (AMDV), and feline infectious peritonitis virus (FIPV) (Weiss and Scott 1981; Halstead 1982). In dengue virus infection, children aged <1 year are particularly susceptible, probably because of waning protective maternal antibody leading to infectious immune complexes resulting in exacerbation of infection. This enhancement of infectivity can lead to dengue hemorrhagic fever or dengue shock syndrome (Halstead 1988), the latter resulting in excessive procoagulant release by monocytes. ADE can also be seen in individuals subsequently infected with another dengue virus subtype to which there are cross-reactive antibodies. One of the major problems associated with the development of vaccines to a number of viruses, for example FIPV, lentiviruses, dengue virus, AMDV, is the potential for ADE in natural virus challenges. In several situations the induction of antibodies following vaccination resulted in exacerbations of disease following subsequent virus challenge, for example FIPV (Vennema et al. 1990).

In immune complex disease there is usually a large excess of virus antigen which circulates as complexes with antibody and becomes deposited in tissues such as the kidney or blood vessels, leading to complement activation and inflammation. In persistent LCMV infection, only a weak neutralizing antibody response is generated and immune complexes become lodged in the kidney, resulting in glomerulonephritis (Oldstone et al. 1975). Chronic HBV carriers are also prone to glomerulonephritis and polyarteritis nodosa, an inflammatory disease of blood vessels initiated by deposition of virus–antibody complexes (Lai et al. 1991).

T-cell-mediated immune pathology

Damage mediated by T cells can occur via cytolysis of infected cells or by cytokine liberation. The widely used example of T-cell-mediated pathology in virus infection involves LCMV. In adult mice, infection of the CNS

Table 1 *Examples of antibody and T-cell-mediated immune pathology in virus infections*

Virus	Host	Comments
T-cell-mediated effects		
LCMV	Mouse	CD8 T-cell damage during virus infection of CNS
Hepatitis B	Humans	CD8 T-cell destruction of chronically virus-infected hepatocytes
HSV	Mouse	CD4 T-cell-mediated stromal keratitis
EBV	Humans	Infectious mononucleosis
Alcelaphine herpesvirus 1	Cattle	Malignant catarrhal fever
Theiler's murine encephalomyelitis	Mouse	CD4 T-cell-mediated demyelination
Measles	Humans	Rash dependent on T-cell immunity
Immune complex disease		
Aleutian mink disease	Mink	High risk of immune complex disease in kidney and blood vessels
LCMV	Mouse	Nonneutralizing antibody in a persisting virus infection leads to glomerulonephritis
Hepatitis B	Humans	Rash, glomerulonephritis, polyarteritis nodosa
Feline infectious peritonitis	Cat	Disseminated intravascular coagulation triggered by immune complexes
Other antibody effects		
Dengue	Humans	Antibody-dependent enhancement of infection link to dengue hemorrhagic fever
Rabies	Humans/animals	Antibody enhancement linked to early death syndrome

EBV, Epstein–Barr virus; HSV, herpes simplex virus; LCMV, lymphocytic choriomeningitis virus.

results in severe pathological changes mediated by CD8 T cells. Treatment of mice with anti-CD8 antibodies protects against disease. The damage is not related to massive cell destruction, but is attributed to cerebral edema arising from damage to the blood–brain barrier (Buchmeier et al. 1980). CD8 CTLs are thought to underlie the damage to hepatocytes seen in chronic HBV carriers.

Delayed type hypersensitivity mediated by CD4 T cells (Th1 cells) has been associated with several types of virus-triggered immunopathology; for example, lung damage in experimental influenza virus infection (Leung and Ada 1982) and deep stromal disease of the eye in HSV-1 infection (Doymaz and Rouse 1992). CD4 T cells have also been implicated in RSV vaccination pathology. Vaccination of mice with the G protein of RSV induces a CD4 Th2 cell response, which, on challenge with RSV, initiates eosinophilic infiltration in the lung (Alwan et al. 1994). This type of pathology was identified in patients who received an ineffective formalin-inactivated RSV vaccine and subsequently became infected with RSV.

In EBV-induced infectious mononucleosis there is amplification of lymphocytes and an outpouring of cytokines (see section entitled Immune response to herpesviruses, below). An exaggerated form of infectious mononucleosis is malignant catarrhal fever seen in cattle infected with alcelaphine herpesvirus-1. This fatal disease is associated with intense proliferation of T cells resulting from a dysregulation of the cytokine response (Reid et al. 1984).

Immune-mediated pathology is a feature of infection of the CNS by a number of different viruses. Theiler's murine encephalomyelitis virus, a picornavirus, establishes a chronic infection in glial cells and induces a chronic delayed-type hypersensitivity response. Activated macrophages recruited to the CNS are believed to secrete factors such as TNF-α that kill local glial cells and result in areas of demyelination around infected cells (Chamorro et al. 1986; Clatch et al. 1986). Similarly, Semliki Forest virus (an alphavirus) causes demyelinating immunopathology, but in this case the culprit is believed to be virus-specific CD8 T cells that kill infected oligodendrocytes (Subak-Sharpe et al. 1993). Sheep infected with the maedi-visna retrovirus often develop a chronic inflammatory condition that can result in accumulation of mononuclear cells and accompanying pathological changes in various organs, including the CNS, respiratory tract, bones, and joints (Sigurdsson et al. 1962; Oliver et al. 1981).

Summary of immunopathology

The immune system is often likened to a double-edged sword, capable of efficient protection from infectious agents but sometimes causing damage to healthy tissue in the process. When the intricate series of checks and balances that regulate immune control of the host–virus relationship is upset (for example by excessive secretion of a viral protein in a persistent infection), an inappropriate response can often be deleterious. In some virus infections (for example hepatitis B, see below, section entitled Immune response to hepatitis B) the disease has a large immunopathological component, and therapy for such diseases is the focus of much research.

EXAMPLES OF IMMUNE RESPONSES TO SPECIFIC VIRUSES

General mechanisms of immunity to viruses have been outlined above; however, it is useful to consider certain case histories to illustrate how different components of the immune response are integrated. Picornaviruses are chosen as examples of acute virus infection in which antibody plays an important role. The three other virus families cause persistent and chronic infection in which the immune system has failed to clear the virus and the infection continues in the face of a continuing immune response. The outcome of HBV infection can be virus clearance or, in up to 10 percent of cases, a chronic infection with continuing virus production over long periods of time. Here, the continuing immune response causes immunopathological damage manifested as liver disease. Retroviruses and herpesviruses establish persistent infections where the virus cannot be eliminated from the host by the immune system. In the case of herpesviruses, the virus is generally in a latent form with restricted expression of viral genes presenting fewer targets to the immune system. An intact CTL response is crucial in controlling occasional reactivation and productive virus infection but does not clear latent virus. Retroviruses also establish latent infections where they do not need to transcribe viral genes but generally this is accompanied by chronic virus production. Both herpesviruses and retroviruses illustrate an equilibrium between virus replication and antiviral immunity where the virus persists but damage to the host is limited by the immune system. This equilibrium is a compromise which enables both virus and host to survive, but if breached by a breakdown in the immune system, for example in an immunosuppressed host, it can lead to serious disease. These four examples broadly cover the spectrum of viral disease ranging from acute self-limiting viral disease to life-long, chronic conditions.

Immune response to retroviruses

Retroviruses (Retroviruses and associated diseases in humans) have several unique characteristics that make recognition by host defenses particularly difficult. Their life cycle involves reverse transcription of viral RNA into DNA, which is then integrated into the

host genome. Once integrated, the virus may remain transcriptionally inactive and become methylated along with the surrounding cellular DNA. This ability to exist in the host genome without expressing viral protein makes the retrovirus invisible to the immune system. Indeed, it is estimated that 5–10 percent of the mammalian genome consists of elements introduced by reverse transcription, 10 percent of which are retrovirus-like. Most of these endogenous retroviruses cause no harm to their host, but coexist and are propagated along with host DNA and inherited by any offspring.

Vertical transmission via the germ cells is a mode of spread unique to retroviruses, and enables the virus to influence the host's immune system in early development. Retrovirus proteins expressed early in development are recognized as 'self' by the developing immune system and the responding T cells are purged from the immune repertoire. The adult animal does not therefore possess lymphocytes capable of recognizing and eliminating infected cells expressing this antigen. Ronchese and colleagues (1984) showed that mice carrying endogenous Moloney murine leukemia virus (MoMLV) did not develop antibody or cytotoxic T lymphocyte responses to the antigenically cross-reactive murine sarcoma virus. Mice infected with MoMLV had a lower than normal complement of CTL precursor cells, suggesting that clonal deletion of virus-specific cells had taken place.

In a different murine retrovirus system, mouse mammary tumor virus (MMTV) uses superantigens which bind to specific TCRs to delete reactive T cells in infected mice. This virus is transmitted from infected mothers to offspring via milk and, in susceptible mouse strains, virus integration leads to the formation of mammary tumors. The virus targets B lymphocytes and DCs where reverse transcription, integration, and gene expression lead to expression of viral superantigen (SAg) which primes an SAg specific T-cell response. The virus uses the immune system to enhance its infection in two ways – it binds directly to TLR-4 on B cells and DC resulting in the cellular activation necessary for efficient infection of B cells (Rassa et al. 2002) and in up-regulation of its receptor on DC (Burzyn et al. 2004); expression of SAg on infected B cells leads to T-cell-driven proliferation of infected cells. Continued expression of the SAg eventually leads to deletion of reactive T cells. Later in infection, the virus targets epithelial cells of the exocrine secretory organs from where it can be transmitted to offspring. Antibody is important in determining the outcome of infection. Passive immunization of susceptible mice with neutralizing antibody can prevent infection (Mpandi et al. 2003). A strong neutralizing antibody response leads to decreased mammary gland infection, development of tumors, and infection of progeny. There is a correlation between a strong antibody response and resistance to MMTV infection. Antibody plays an important role in maintaining a balance so that infection of exocrine glands, which is necessary for transmission of the virus, occurs but levels of infection are sufficiently controlled to prevent rapid tumor development (Finke et al. 2003).

Much effort has been expended in recent years on the investigation of immune responses to human retroviruses, research that has been driven partly by the HIV epidemic. HIV, a retrovirus of the genus *Lentivirus*, is therefore the most intensively studied retrovirus, although care must be taken when extrapolating from HIV to other retrovirus infections.

There is a growing body of evidence that the interaction of HIV with the immune system represents a unique situation. Acute HIV infection causes cytolytic infection of CD4 T cells, particularly those at mucosal sites, a common point of entry for the virus. The predominant CD4 T cell at those sites is the memory CD4 T cell expressing CCR5, the coreceptor for HIV in most natural infection. This results in a severe depletion of these cells which continues throughout the course of the infection. The tropism of HIV for CD4 T cells also presents particular problems for the immune system. HIV-specific CD4 T cells are particularly good targets for virus infection as activated rapidly expanding T cells are highly susceptible to HIV infection. This activation of the CD4 T-cell response presents the virus with more targets to infect and maintain the chronic infection. (Douek et al. 2003).

HIV-specific CD8 T-cell responses are present throughout the course of infection before the onset of acquired immunodeficiency syndrome (AIDS) but are unable to clear HIV infection in the majority of cases. The major problem for CD8 CTL control of HIV infection lies in the high levels of mutation rate associated with high levels of HIV replication, which results in escape epitope mutation such that at any time the CTL pool may be directed against, and indeed continuously stimulated by, epitopes which represent a very minor portion of the current HIV population. HIV infection leads to down-modulation of MHC class I on infected cells contributing to the failure of CD8 T cells to recognize and kill infected cells. Depletion of the CD4 T-cell numbers leads to loss of available CD4 T-cell help for CTL responses. Another reason for the failure of CD8 T cells to control infection may be inactivation; there is evidence that during chronic HIV infection where there is chronic stimulation of CD8 T cells, the cells loose responsiveness to antigen, for example, they often have TCRs of low functional avidity and inappropriate signaling, even those against well-conserved epitopes on one of the outer loops of gp120. This is also seen with other virus infections such as CMV and EBV. Regulatory CD4 T cells can cause functional impairment of virus specific CD8 CTLs in Friend murine leukemia virus (FrMLV) infection (Dittmer et al. 2004), and it is possible that a similar mechanism may occur in HIV infection.

HTLV-1 also targets CD4 T cells, although in this case CD8 T cells are also infected, albeit at a lower level. However, HTLV-1 is not associated with severe immunodeficiency but with adult T cell leukemia/lymphoma and a number of chronic inflammatory diseases. HTLV-1 varies little in sequence and does not present the immune system with the escape mutation scenario found in HIV infection, rendering CTL control of infection much more efficient.

An important role for antibody can be demonstrated in equine infectious anemia virus (EIAV). Episodes of disease coincide with viremia, which is brought under control by the antibody response. Disease then remits until a new virus variant emerges that cannot be neutralized by the previous antibodies; there follows another disease episode. With time, the episodes of disease become less severe, until the horse enters an asymptomatic carrier state. In this phase the disease is under effective immune control, as demonstrated by the fact that immunosuppression induces relapse.

Evasion of the antibody response is an interesting feature of HIV infection. The receptor-binding protein of HIV, gp120, is a prime target for an antibody response; however, the high degree of glycosylation and the tight conformation of this protein restrict the access of antibodies to the highly conserved receptor binding domains within the protein. In addition, the high degree of variability in the exposed faces of gp120 and the conformation-dependence of most anti-gp120 antibodies render most of the antibodies raised to be ineffective against the vast majority of the circulating virus population. One of the exposed loops, thought to contain an important neutralizing determinant, is exceptionally variable, enabling rapid escape from any effective neutralizing antibody response. Soluble gp120 is secreted in large amounts during infection, and it has been suggested that this may act as a decoy to raise an antibody response, but the conformation of gp120 on the virion is different, so the antibodies are not neutralizing. This may explain why there is a large amount of virus-specific antibody in infected individuals but the vast majority is not neutralizing.

In conclusion, while antibodies can contribute to limiting the disease process in retrovirus infection, they are generally ineffective at clearing the infection. The CTL response seems to be more important than the antibody response in protection against most retroviruses

Immune response to picornaviruses

Picornaviruses are small nonenveloped RNA viruses with a relatively simple structure consisting of an icosahedral capsid comprising four proteins and a single strand of positive-sense RNA. Members of this family are very diverse and can cause a wide range of diseases, which are usually acute infections in which the virus replicates in the host over a short time period and generally does not persist. There are some exceptions: for example, Theiler's murine encephalomyelitis virus can remain in the CNS for the lifetime of the host (Lipton et al. 1984). Others do not generally persist but can do so on rare occasions: for example, some cattle infected with foot-and-mouth disease virus go on to become chronic virus secretors.

Most picornavirus infections occur at mucosal surfaces, so the first line of defense for the host lies in the acidic mucus, which contains a number of proteases. Sensitized B cells may secrete antibody of the IgA isotype, which is transported through mucosal cells and secreted from the luminal surface. Challenge with a virus of a strain previously encountered by the immune system will result in neutralization of the virus by secreted antibody before it is able to gain access to the epithelial surface. Stimulation of IgA is the rationale behind the oral polio vaccine: it is more effective than the inactivated virus given systemically because it stimulated gut immunity directly, leading to an effective mucosal antibody response.

The replication of rhinoviruses is confined to the epithelial surface of the upper respiratory tract, and occurs so quickly that by the time the immune system has raised a response, the infection has already resolved. The object of the immune system here is to prevent reinfection. Rhinoviruses exist in hundreds of different serotypes, so there is little chance of preventing reinfection with viruses other than the initial strain. This explains the high incidence of, and poor protection against, the common cold.

In the course of systemic picornavirus infection, the virus penetrates the mucosal surface and can invade the blood stream. The viremia is not cell-associated, making the virions particularly susceptible to neutralization by serum antibody. Poliovirus infection illustrates this very well. After initial replication in the small intestine and mucosal-associated lymphoid tissue (tonsils, Peyer's patches, and lymph nodes of the neck) poliovirus can be detected in the blood. The relatively rare neurological disease (poliomyelitis) occurs following virus invasion of the CNS via the blood or through retrograde transport inside neurons. The experimental administration of neutralizing antibodies can prevent the paralytic disease in both monkeys and humans.

Both B and T cells are involved in the generation of an effective antibody response, because in the absence of T cell help only low affinity antibodies can be made, and there is no immunological memory. In recent years, the contribution made by T lymphocytes in immunity to picornavirus infections has been studied extensively. CD4 T cells involved in providing immunological 'help' for the antibody response are often cross-reactive between virus serotypes, and sometimes between different members of the picornavirus family.

The importance of T cell help can be illustrated by experiments performed in the mouse poliovirus model. Poliovirus does not normally infect mice, but using transgenic mice expressing the human poliovirus receptor they become susceptible to infection of the CNS. T cell clones have been raised against poliovirus proteins, and the transfer of these cell lines with immune B cells was able to protect the mice from lethal infection (Mahon et al. 1995). Transfer of immune B cells alone had no effect emphasizing the importance of T cells in protection. The T-cell lines secreted interferon-γ and this seemed to stimulate the preferential secretion of the IgG2a isotype antibody.

There have been several reports of CTLs in picornavirus infections (reviewed by Usherwood and Nash 1995). These are usually of the CD8 phenotype, although specific CD4 CTLs are also known to exist. Their role in the protection against picornavirus infection is unclear at present, although CD8 T cells are known to contribute to viral clearance in Theiler's virus in both susceptible and resistant mouse strains (Pullen et al. 1993; Begolka et al. 2001).

Immune responses to coxsackie B virus have been implicated in virus associated myocarditis and diabetes. Molecular mimicry (where an antiviral response cross-reacts with host antigens) is thought to be involved in the etiology of these diseases. Antisera generated against synthetic peptide sequences derived from coxsackie B3 protein and the cellular adenine nucleotide translocator cross reacted with both proteins, and antibodies which reacted with the peptides were present in patients with inflammatory cardiomyopathy. Removal of immunoglobulin from patients has been demonstrated to improve the clinical situation and, in a severe combined immunodeficiency (SCID) mouse model, human PBL have been shown to transfer disease symptoms (Schwimmbeck et al. 2004). Other evidence implicates both CD4 and CD8 T cells in coxsackie virus induced myocarditis (Henke et al. 1995). However, there is also evidence that direct viral damage is involved in disease, and limiting viral replication can limit disease (Horwitz et al. 2000). Similarly, autoreactive T cells clearly are associated with coxsackie B virus induced diabetes but there is evidence that T cells do not initiate the disease process. Phagocytosis of infected pancreatic β cells by macrophages has been observed prior to T-cell infiltration. Subsequent antigen presentation by these cells may drive an autoimmune response (Horwitz et al. 2004).

Because of their small genome (7–8.5 kb) it has been possible to map mutations that affect antibody binding sites and T-cell recognition elements for a number of picornaviruses (Usherwood and Nash 1995). In several members of the family, antibody binding sites are present on all three external capsid proteins (for example poliovirus, human rhinovirus), whereas in others, only two proteins are thought to be involved (for example, hepatitis A virus). There does not seem to be a close association between the position of T and B cell epitopes on a given virus protein.

Immune response to hepatitis B

Over 300 million people worldwide are chronically infected with HBV. HBV infection of adults is often asymptomatic, and in most cases the infection is completely cleared. However, in 5–10 percent of adults, the virus establishes a persistent infection. Neonatal infection also occurs, resulting in a degree of immunological tolerance to the virus; over 90 percent of such children become chronically infected. The persistent infection is associated with liver disease and cirrhosis in 25 percent of carriers and can progress to hepatocellular carcinoma. Reports indicate that the lifetime risk to males infected with HBV at birth of developing hepatocellular carcinoma approaches 40 percent (Beasley et al. 1981). The different outcomes of HBV infection illustrate the varied effects of alterations in the equilibrium between virus replication and the antiviral immune response.

Activation of innate nonspecific mechanisms represents a key step in the control of the initial burst of HBV replication. These mechanisms are highly efficient causing an 80 percent drop in the quantity of HBV DNA prior to the onset of the antigen-specific adaptive immune system. NK and NK-T cells are the major component of this response (Kakimi et al. 2000), but the interferon system and liver Kupffer cells play a role. During acute, self-limiting viral hepatitis, antibodies can be detected that are specific for the viral envelope (HBsAg), core antigens (HBcAg and HBeAg), and the viral polymerase (Chisari and Ferrari 1995). Antibodies to HBsAg in serum are neutralizing and are thought to play an important role in viral clearance by binding to cell free virus and preventing spread to other susceptible cells. Induction of anti-HBsAg alone during prophylactic vaccination is often sufficient to completely prevent infection.

Strong CD4 T-cell responses to HBeAg and HBcAg are seen in the peripheral blood of almost all patients with acute hepatitis. Historically, HBeAg is seen as a marker of acute infection but sensitive assays indicate replicating virus remains after removal of HBeAg. The CD4 response to the envelope is much less vigorous, the reason for which is not understood. The CD4 response in self-limiting acute hepatitis is predominantly Th1 while T-cell clones from chronically infected patients produce mainly Th2 responses (Bertoletti et al. 1997; Penna et al. 1997).

CD8 CTLs are known to be crucial in the control of the infection and strong responses to HBsAg, HBcAg and polymerase are found in patients who control HBV. Multiple epitopes are recognized. Activated HBV-specific CTL can persist long after recovery from active HBV infection, suggesting that low levels of persistent

infection maintain and are controlled by the CTL response. CD8 CTL responses in chronically infected patients are difficult to detect by classical cytotoxicity assays in both peripheral blood and the liver. Clearance in these patients via spontaneous or interferon induced remission is associated with a boost in this response. Use of MHC class I tetramer technology, which allows direct detection of virus-specific CTLs, has shown that CTLs specific for residues 18–27 of HBcAg comprise up to 1.3 percent of circulating CTLs in acutely infected patients. This is accompanied by a CTL response directed against a number of other viral epitopes. In contrast, in chronically infected HBeAg positive patients, these are barely detectable (Jung and Pape 2002). Tetramer staining shows that, in patients with a high rate of HBV replication and liver inflammation, the numbers of intrahepatic HBcAg-specific CTLs are similar to those in patients without disease, but they are accompanied by large numbers of nonspecific CD8 cells meaning that specific CTLs are present at low frequencies. Thus comparable numbers of intrahepatic virus specific CTLs could be associated with either protection or pathology raising the possibility that there is a functional defect in CTLs in chronic HBV infection (Maini et al. 1999).

HBV does not infect mice or rats but lines of transgenic mice which express HBV and can mimic chronic liver infection have been invaluable in understanding the role of the immune response in control of infection. Transfer of virus specific CTLs into these mice showed that, in addition to killing infected cells, these CTLs eliminate virus from many other infected cells noncytolytically by release of IFN-γ and TNF-α (Guidotti and Chisari 2001). Studies in chimpanzees have confirmed the importance of IFN-γ and TNF-α in removing HBV replicative intermediates from the cytoplasm and closed circular form from the nucleus of HBV infected cells (Guidotti et al. 1999). The transgenic mouse model has been used to show the importance of type I interferons in control of infection and to dissect the pathways involved in clearance. Studies show that neutrophils induce matrix metalloproteinase (MMP) which leads to recruitment of antigen nonspecific mononuclear cells. Elimination of MMPs does not affect viral clearance but prevents damage to the liver by nonspecific mechanisms (Sitia et al. 2004).

Current evidence suggests that both T cell and antibody responses to HBV are necessary to clear HBV and that CTLs act not only by lysing infected cells, but also by releasing cytokines such as IFN-γ and TNF-α that reduce the intracellular virus load without killing the infected cell. The reasons why some HBV infections of adults become chronic, while the majority are limited and cleared by the immune system, are not understood. Model systems provide support for the possibility that outcome is determined by the size of the inoculum and replication rate of the infecting virus. If this is high, then even with a fully activated immune response, viral persistence may be favored (Jung and Pape 2002). It is likely that persistence is determined by multiple mechanisms. The immune system also seems to contribute to the chronic liver pathology by causing destruction of hepatocytes, resulting in a high rate of mitosis and therefore an increased risk of damaging mutations.

Immune response to herpesviruses

There are three subfamilies in the *Herpesviridae*, each with different biological and genetic characteristics (see Herpesviruses:general properties; Alphaherpesviruses; Betaherpesviruses; Human herpesvirus 8; and Gammaherpesviruses). A central feature of the biology of these viruses is their ability to establish latent infections in either the nervous or immune system. Herpesviruses commonly have numerous immune evasion strategies which allow them sufficient time to progress from the site of primary infection and establish and maintain latency.

HSV is the prototype of the alphaherpesviruses. The natural history of HSV involves infection at mucosal surfaces followed by transmission to the peripheral nervous system where the virus remains latent in neurons. Periodic reactivation occurs with reinfection of epithelial cells which may present as recrudescent disease. HSV lesions are often more severe in immunocompromised individuals, emphasizing the importance of the immune system in controlling disease.

Components of the innate immune system including NK cells, type I interferon, and complement, play a role in the control of HSV infection in vivo but are not sufficient to prevent symptomatic infection. Antibody has been shown to interfere with virus entry to, and movement from, the nervous system (Simmons and Nash 1985), and the existence of immune evasion mechanisms directed against antibody function emphasizes the importance of antibody in neutralization of infectious virus and in antibody mediated cytoxicity. However, T lymphocytes provide the major control mechanism for both primary and recurrent infection.

DCs play a central role in stimulating the T-cell response to HSV. They are readily infected with the virus which inhibits their function as antigen presenting cells (APC) and their migration from the skin. However, infected DCs are potent producers of IFN-α which may act as a danger signal to stimulate uptake and presentation by surrounding DCs (Pollara et al. 2004). These uninfected DCs from the lesion site cross-present viral antigens and stimulate both CD4 and CD8 T cells.

A clear role for CD4 T cells is identified by their clearance of virus from the mouse epidermis. CD4 T cells are potent producers of IFN-γ which stimulates macrophage function. IFN-γ may also be important in counteracting the action of viral immune evasion molecules. HSV-specific CD4 CTL clones are present in skin

lesions and are capable of killing infected cells in vitro. The human CD4 T-cell response is broadly directed including targets from all stages of the virus life cycle (Koelle and Corey 2003).

CD8 T cells also participate in resolution of peripheral lesions (van Lint et al. 2004), but in the nervous system they assume a more important role and regulate virus replication in neurons without destroying these cells (Simmons and Tscharke 1992). Evidence suggests that within the human trigeminal ganglia, HSV infection is associated with a chronic immune reaction which influences reactivation (Theil et al. 2003). The CD8 T-cell response appears to be narrower than the CD4 response but as with CD4 T cells, the glycoproteins gB and gD are major targets (Koelle and Corey 2003).

The betaherpesviruses are typified by human and murine cytomegaloviruses, but also include human herpesviruses 6 and 7. The initial infection by CMV is via epithelial surfaces and involves a persistent or latent phase in cells of the myeloid lineage. MCMV can also establish latent infection in other cell types, probably endothelial cell or histiocytes. (Reddehase et al. 2002). cytomegaloviruses in general cause asymptomatic infection in immunocompetent hosts but are important pathogens in immunocompromised individuals.

There is a clear role for NK cells in early protection against lethal MCMV infection. An NK cell receptor which confers resistance to MCMV infection by binding directly to a viral protein expressed on the surface of MCMV infected cells and selectively killing the infected cells has been identified (Arase et al. 2002; Smith et al. 2002). The NK-cell response is limited to the first 3 days of infection and thereafter the CD8 T-cell response dominates recovery from primary infection. In MCMV, the CD8 T-cell response is against an immediate–early (IE) antigen IE1. A single antigenic peptide is recognized in the context of H-2L^{d} (MHC class I restriction molecule) of BALB/c mice. Immunization with this peptide is sufficient to confer protection against MCMV infection. Likewise, adoptive transfer of T-cell clones specific for the antigenic peptide also eliminates productive infection in most tissues (Koszinowski et al. 1990). In mice lacking CD8 T cells, CD4 T cells can take over this function. In this case, CD4 T cells derived from CD8-deficient animals can confer protection, unlike those from immunocompetent mice. Thus, the immune system appears to be able to compensate, to some extent, for lack of specific function under certain circumstances, however, this compensation does not appear to be complete. For example, mice depleted of CD4 T cells cannot eliminate virus from the salivary gland. Thus, both CD8 and CD4 cells are essential for an efficient immune response to MCMV (Krmpotic et al. 2003). CTL responses to human cytomegalovirus (HCMV) are also directed against one major target, the tegument protein pp65, although the major IE protein may be important in some individuals. CD8 T-cell responses are critical in control of HCMV infection but, like MCMV, CD4 T cells also play a role (Gamadia et al. 2003). Studies in B cell knock-out mice show that antibody is important in protecting from reactivation while CD8, CD4, and NK cells are important in both primary and recurrent infections.

The principal feature of the gammaherpesviruses is a tropism for either B or T lymphocytes. The best studied member is EBV, which targets and establishes latent infection in B cells. This virus also has the ability to transform B cells in vitro and is associated with lymphoproliferative disease. The main immunological surveillance is by CD8 T cells, which target predominantly B cells expressing the latent viral antigens EBNA-3 and LMP (Rickinson and Kieff 2001). Immunosuppression as seen in transplantation affects this balance by reducing the number of EBV-specific CD8 T cells, which results in the appearance of B cell lymphomas.

Murine gammaherpesvirus 68 (MHV-68) infection of laboratory mice has provided an opportunity for studying the immune response to a gammaherpesvirus in its natural host. This virus establishes a latent infection in B cells, DCs, macrophages, and lung epithelial cells and, like EBV, is associated with lymphoproliferative disease and an infectious mononucleosis-like syndrome (Nash et al. 2001). The availability of gene knock-out mice with targeted deletions in genes affecting immune function has made it possible to study the role of individual aspects of the immune response to MHV-68 infection. Studies in mice which lack a type I interferon response have shown that type I interferons are critical in the early control of infection (Dutia et al. 1999). MHV-68, like Kaposi's sarcoma herpesvirus and herpesvirus saimiri encodes a regulator of complement activation which has been shown to play a role in control of persistent replication and virulence in mice lacking IFN-γ responses (Kapadia et al. 2002).

During the primary infection, the virus replicates in alveolar epithelial cells. CD8 T cells clear the productive infection in the lung. A critical event is splenomegaly. This is dependent on CD4 T cells and is accompanied by a transient high load of latently infected cells. Although CD4 T cells are not essential for clearance of the initial lung infection, they are crucial for long term control of infection. Mice lacking CD4 T cells initially clear the infection, but within 3–4 months there is a recurrence of infectious virus in the lung and mice develop severe lung pathology (Cardin et al. 1996). The critical role of CD4 T cells seems to come not from providing help for antibody production or to CD8 T cells. Infection of mice lacking B cells shows that antibody is not essential for control of acute or latent infection although levels of latency are very low in these mice (Usherwood et al. 1996). Depletion of CD8 T cells and IFN-γ in B-cell-deficient mice has identified a critical role for IFN-γ produced by CD4 T cells in control of latent infection (Christensen et al. 1999). Mice lacking CD4 T cells have normal numbers of

CD8 T cells (Stevenson et al. 1998). The exact role of IFN-γ remains to be determined as mice lacking an IFN-γ response, while exhibiting pathological changes (Dutia et al. 1997), do not succumb to infection.

VIRAL STRATEGIES FOR EVADING THE IMMUNE RESPONSE

In order to survive in the host for a prolonged period a virus must either hide from or evade the antiviral immune response. As mentioned above in the section entitled Immune response to retroviruses, viruses such as MMTV that contain a superantigen, can induce a state of tolerance against themselves. A similar situation has been reported with lymphocytic choriomeningitis virus infection, in which an overwhelming infection is thought to 'exhaust' the immune response; the virus then infects the thymus so that newly emerging T lymphocytes are tolerant to virus antigens (Moskophidis et al. 1993). Some sites in the body are regarded as immunologically privileged, meaning that the ability of the immune system to respond to foreign proteins present in them is impaired. Some viruses have exploited such shelters, and preferentially infect sites such as the CNS (Fazakerley and Buchmeier 1993).

As with any rapidly replicating organism, viruses can evolve quickly in response to a changing environment. In the case of eukaryotic viruses, the immune system is a potent selective pressure, so, in order to survive, many viruses have acquired ways either of evading recognition or of obstructing the effector arms of the immune response. Most RNA viruses have high mutation rates due to errors produced by virus polymerases that lack proof-reading functions. A mutation rate of 10^{-4}–10^{-5} per base per replication cycle is typical (Holland et al. 1992). Any mutant virus that is recognized less efficiently by the immune system will dominate the virus population in the face of a stringent immune response. For example, the retrovirus HIV has a highly variable surface protein, gp120, which is the target for antiviral antibodies. Many serotypes of influenza virus exist, which have been selected by their ability to evade neutralizing antibody responses. Periodically, an antigenic shift occurs, when reassortment between two distinct influenza virus isolates result in antigenically distinct progeny that may give rise to pandemics as the virus replicates in a nonimmune population (Webster et al. 1992).

DNA viruses have less plastic genomes, often of sufficient size to accommodate genes acquired from host cells that may allow them to subvert immune attack strategies. There are also examples of convergent evolution whereby viral genes have apparently evolved to mimic cellular genes involved in immune regulation. As should be clear from the preceding sections of this chapter, many facets of the immune system are involved in the antiviral response, and the following section details how each part of the immune network may be subverted by viruses.

The literature underpinning this field has risen dramatically over the past few years. Consequently, we refer the reader to several excellent reviews that go into far greater detail on the viral genes and their mechanisms of action (Tortorella et al. 2000; Alcami and Koszinowski 2000; Alcami 2003; Benedict et al. 2002; Yewdell and Hill 2002; Goodbourn et al. 2000; Orange et al. 2002; Favoreel et al. 2003)

Inhibitors of inflammation

Inflammation is the first response to virus-induced tissue damage, which releases many cytokines necessary to attract immune cells. Vaccinia virus encodes a steroid-synthesizing enzyme, 3β-hydroxysteroid dehydrogenase, which is transcribed early in infection. It is not known which steroids are synthesized by this enzyme; several glucocorticoids are, however, anti-inflammatory and it is known that this gene contributes to viral virulence. Several poxviruses possess serine protease inhibitors (serpins), which are thought to interfere with the pathways leading to the production of lipoxygenase metabolites and IL-1β production, both of which are important pro-inflammatory factors. African swine fever virus (ASFV) directly inhibits the activation of pro-inflammatory cytokines in pig macrophages through the expression of an IκB homologue (A238L). This protein interferes with cytokine transcription by inhibiting the function of NFκB (see Alcami and Koszinowski 2000 and Tortorella et al. 2000)

Inhibition of interferon

Type I interferon released from infected cells can both induce enzymes that destroy intracellular viral nucleic acid and prevent translation of viral mRNA. Proteins made by reovirus and vaccinia virus can complex with double-stranded RNA (dsRNA) and inhibit dsRNA-dependent protein kinase (PKR) activation. Small RNAs made by adenovirus, HIV-1, and EBV are able to bind to PKR and inhibit its activation. Another protein produced by vaccinia virus acts as an alternative substrate for PKR, preventing it from phosphorylating eIF-2a. The normal cellular control mechanisms for PKR regulation are subverted by influenza virus, resulting in the repression of PKR by another cellular factor, p58. By contrast, poliovirus induces the phosphorylation and degradation of PKR. Another strategy used by viruses to disrupt interferon response involves inhibiting the activation of the JAK/STAT signaling pathway. SV5 specifically targets STAT1 for proteasome-mediated degradation. Other viruses target STAT2 (human parainfluenza virus) or JAK1(murine polyomavirus). Poxviruses have yet another mechanism for inhibiting interferon responses involving the expression

of secreted receptors or binding proteins for IFN-α/β and IFN-γ (see Goodbourn et al. 2000).

Viral interference with apoptosis

For some DNA viruses, survival in the cell is of paramount importance. To this end they have exploited strategies for disrupting apoptotic mechanisms by the use of decoy proteins with anti-apoptotic activity. Bcl-2 homologues are a feature of many herpesviruses and function as a powerful inhibitor of apoptosis. In the process of apoptosis caspase activation is a critical event. The cytokine response modifier A (CrmA) of cowpox virus is a potent inhibitor of caspase 1 and caspase 8 activity, following a variety of death inducing stimuli. Adenovirus also encodes an inhibitor of caspase 8.

Both NK cells and cytotoxic T cells kill target cells through TNF's and FasL interaction with TNF receptor (TNFR) family molecules. Poxviruses counter this by production of secreted viral TNFR which neutralizes cytotoxic cytokines. The gammaherpesviruses, Kaposi's sarcoma associated herpesvirus (KSHV) and herpesvirus saimiri (HSV) encode molecules called vFLIPS that interfere with the Fas-associated death domains (FADD)–caspase interaction blocking induction of apoptosis (see Benedict et al. 2002).

Humoral factors: complement and antibody

As mentioned in the section entitled Collectins and the complement system, above, the complement cascade is activated by a C3-convertase that can be induced by either cross-linked antibody or the surface of particular pathogens, the end result being the deposition of a membrane attack complex that causes lysis of cells or virus. Proteins encoded by some poxviruses and herpesviruses contain short consensus repeats (SCR), which are present in many complement control proteins, for example CD46 and CD55. Viral homologues of CD46, CD55, CR1, and CB4P are known to bind to C3b and C4b, inhibiting the C4b2a C3 convertase. Purified EBV virions have also been reported to accelerate the decay of C3 convertase. The *ORF4* gene of MHV-68 is present as a membrane-bound and secreted protein and functions as a regulator of complement activation. Virus lacking ORF4 is attenuated in infected mice, suggesting this viral gene product functions in vivo as a virulence determinant. Similarly, HVS possesses a protein with four SCRs that is both secreted by, and present on, infected cells and virions. HVS also has a homologue of CD59, a cellular factor that accelerates the decay of the membrane attack complex. Some viruses, HCMV and HIV, actually hijack host CD59 and incorporate this into the viral envelope. A protein present on the infected cell and on the virion of HSV-1, glycoprotein C, can bind C3b and C3bi, inhibiting initiation of the complement cascade.

Two herpesviruses, HCMV and HSV-1, are known to code for Fc receptor homologues. It is thought that these bind antibody molecules in such a way that their Fc portion cannot activate the classical complement pathway or be available for engagement by Fc receptors on phagocytes. The presence of bound immunoglobulin on the cell surface may also hinder the attachment of antibodies specific for virus antigens (see Favoreel et al. 2003; Alcami and Koszinowski 2000).

Interference with MHC function and NK cell recognition

As described in the section entitled Recognition of virus-infected cells by $CD8^+$ T lymphocytes, above, cytotoxic lymphocytes recognizing MHC class I in association with a viral peptide represent an important defense mechanism against viruses. Not surprisingly some viruses have developed ways of manipulating the CTL response, by interfering with normal antigen presentation mechanisms through MHC class I. This can be achieved at various levels in the infected cell including resistance to the catalytic processes of the proteosome, for example EBNA-1 of EBV, blockade of peptide transport by TAP proteins, for example HSV, and inhibition of maturation, assembly and migration of MHC class I molecules, for example human and mouse CMV.

The down-regulation of MHC class I expression by viruses might well disrupt CD8 T-cell recognition. However, NK cells take advantage of reduced MHC class I expression and become activated, killing virus infected cells (see section entitled NK cells in antiviral immunity). In order to thwart the attentions of NK cells, CMV encodes a MHC class I homologue (UL18 HCMV and m144 MCMV) that becomes expressed on the surface of infected cells and serves to engage Ly49 inhibitory molecules or killer inhibitory receptors (KIR) resulting in the inactivation of NK-cell killing. Another strategy used by HCMV involves the induced expression of HLA-E by a peptide generated by the viral protein UL40. HLA-E serves to protect infected cells from NK-cell attack.

Some of the DNA viruses have developed strategies to disrupt MHC class II expression. For example, adenoviruses and some herpesviruses block MHC class II transcription. HCMV US2 protein interferes post-translationally with MHC class II by translocating DRα and the DMα chain to the cytosol for proteosome-mediated degradation. HIV Nef protein interacts with the endocytic mechanisms and initiates the rapid internalization of CD4 and MHC class I molecules from the cell surface. This process brings Nef into contact with the proton pump in MHC class II containing vesicles leading to acidification and interference with peptide presentation. A similar mechanism exists for the E5 protein of papillomavirus (see Yewdell and Hill 2002; Tortorella et al. 2000; Orange et al. 2002).

Subversion of cytokine and chemokine function

The cytokine system represents the communication network coordinating the character and magnitude of the immune response. Viruses target cytokines at two levels. One involves production of viral cytokines homologues that mimic normal cytokine activity. The second involves viral cytokine receptors and binding proteins. KSHV encodes a vIL-6 and EBV produces a vIL-10, both cytokine homologues have the potential to act as growth factors for B cells with implications for the biology of these B cell tropic viruses. Poxviruses encode vTGF-β (fowlpox virus) and vSEMA, a semaphorin homologue (vaccinia virus). However, the function of these cytokine homologues has still to be determined. Both poxviruses and herpesviruses encode vTNF receptors that are secreted from infected cells and act to neutralize soluble TNF. Poxviruses also encode a variety of secreted binding proteins, for example vIL-18BP which can inhibit IL-18 induced IFN-γ and NK cell responses.

A second major immunological network involves chemokines and the role played in trafficking leukocytes to sites of inflammation and in regulating movement in and out of lymphoid compartments. The herpesviruses and poxviruses have an elaborate armory for disrupting or utilizing the chemokine network. One viral strategy involves production of viral chemokine homologues. KSHV encodes vMIP homologues that act as chemokine antagonists that attract Th2 cells and also have angiogenic activity. These activities aid the virus by disrupting the induction of protective Th1 responses and also recruiting cells to sites of infection for the purpose of virus infection.

Many gammaherpesviruses and some poxviruses encode viral chemokine receptors (vCKR). Over expression of these receptors can lead to cell transformation. A more relevant biological role for these receptors could be in transient cell proliferation and cell migration.

A third group of proteins with direct chemokine inhibitory activity are the chemokine-binding proteins. These proteins have no homology to chemokine receptors yet have evolved a high affinity for chemokines. Some binding proteins have a restricted affinity for CC chemokines, whereas the M3 protein of MHV-68 targets C, CC, CXC, CX3C chemokines. M3 functions in vivo by inhibiting lymphocyte migration to sites of infection and also plays a role in the establishment of latency, presumably by interfering with T-cell recognition of latently infected cells (for a review of this section see Alcami 2003)

CONCLUDING REMARKS

Viruses of humans and animals and their immune systems have evolved together over millions of years, each shaping the other. As protective mechanisms become more efficient and sophisticated, so do the strategies used by viruses to favor their survival. Because of their short replication time and their ability to mutate and evolve rapidly, viruses have been able to exploit niches inaccessible to immune cells. Some viruses have appropriated cellular genes coding for proteins that have a role in immune regulation, whereas others synthesize their own mimics to fool the hapless lymphocyte.

In most cases, however, the immune system can limit the damage caused during virus infections. From the perspective of the virus it is a distinct advantage if the host remains healthy, as this will assist its dissemination. Thus, acute infections (for example, rhinoviruses) may replicate quickly, causing a mild, self-limiting disease that can spread to a new host before the immune system eliminates it. The strategy adopted by viruses causing chronic infections is different: the virus can be disseminated over a much longer period of time but it must evade the immune response. Some herpesviruses (for example, HHV-6) seem to have mastered the art of persisting in a large proportion of the population for long periods without causing disease except in special circumstances. Infections such as these can be considered to have reached an amicable compromise with the host's immune system, causing little disease yet enabling the virus to spread. Viruses causing more severe disease may be those that have recently spread to their host species and have not yet evolved a mutually acceptable relationship.

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The role of cytokines in viral infections

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INTRODUCTION

The pathological manifestation of viral disease is the result of complex interactions between the direct cytopathic effect of viral infection and the local and systemic immune responses to infection. Cytokine production is responsible for common manifestations of viral diseases such as fevers, chills, and myalgia, and the cytokine response has been associated with complications secondary to some viral infections, such as immunosuppression and dementia. It is also now clear that cytokines produced either directly in infected cells or indirectly by lymphocytes and macrophages activated by the immune response to viral proteins play an important role both in the outcome of the viral infection and in its virulence. These proteins can inhibit viral replication either directly by inducing the antiviral state in the host cells or indirectly by increasing the activation of macrophage and NK cell responses, expression of major histocompatibility antigens, initiating inflammatory responses, and stimulating the development of cytotoxic T cells, as well as the differentiation of B cells into antibody-producing plasma cells.

Recently, a new dimension of complexity has been added to the virus-mediated immune response by the finding that certain viruses have evolved mechanisms that allow them to overcome some of the components of the host-induced cytokine response by producing proteins that mimic cytokines or their receptors. Other viruses have developed defense mechanisms that enable them to interfere with the transcriptional activation of cytokine genes or with their mechanisms of action. Thus, many of the viral genes that are not required directly for viral replication may be essential for pathogenicity of the virus in vivo. These aspects of viral escape from immune surveillance are discussed elsewhere in this volume (The immune response to viral infections).

Local immune responses seem to be especially important in the development of cell-mediated immunity and antibody production. During the early stages of viral infection, innate cytokines such as interferon (IFN)-α and -β play a critical, but not overlapping role, in the stimulation of NK cell functions. The cytokine expression profile defined as Th1, associated with the production of IFN-γ, interleukin (IL)-2, and IL-12, leads predominantly to cell-mediated immunity. The Th2 profile of cytokine expression, in which IL-4, IL-5, IL-6, and IL-10 are produced, results in the production of virus-specific antibodies. Depending on the type of infection, the T-helper (Th)1 or Th2 response can be local or systemic, as has been suggested for human immunodeficiency virus type 1 (HIV-1) infection. This chapter deals with the role of cytokines in the early inflammatory responses and the outcome of viral infection. Particular emphasis is given to the viral infections of the central nervous system (CNS), HIV-1, herpesvirus and hepatitis virus infections, where there is clear evidence of a close relationship between cytokine production, viral replication, and pathogenicity. The use of recombinant viruses

to examine the effect of a selected cytokine is also discussed. Finally the current knowledge of the immune response triggered by the ligation of Toll receptors is summarized, since there is accumulating evidence that these are recognized not only by bacterial envelopes and double-stranded RNA and DNA, but also by viral proteins.

CYTOKINES INDUCED DURING VIRAL INFECTIONS

Interferons

IFNs have a broad antiviral action against many viruses and play a critical role in innate immunity. They are the earliest defense mounted by the host during viral infection, until immune mechanisms can be fully engaged. An important element of the extensive cytokine network, IFNs also play a crucial role in the modulation of the immune system (de Maeyer and de Maeyer-Guignard 1991). Functionally, IFNs can be divided into two groups: type I IFNs, IFN-α, IFN-β, IFN-ω and IFNκ and type II IFN, IFN-γ (Table 1). Recently, a new group of antiviral proteins that are induced as response to viral infection and share the antiviral properties with type I IFNs, IFNλ (Kotenko et al. 2003; Sheppard et al. 2003) has been identified.

Normally silent, IFN A and B genes are rapidly induced in response to viral infection, while a distinct feature of IFNκ is its constitutive expression. A deficiency in the IFN system can lead to fulminate viral disease (Levin and Hahn 1985). Human type I IFN genes lack introns and map to the short arm of chromosome 9. There are more than 20 IFN-α genes and pseudogenes, five IFN-ω genes, but only one IFN-β and IFNκ gene (Diaz 1995). The single IFN-γ gene contains three introns and maps to the long arm of chromosome 12. Whereas IFN-α is a family of nonglycosylated monomeric proteins (Allan and Fantes 1980), IFN-β, IFN-ω and IFN-γ are *N*-glycosylated. Functionally, IFN-β is a dimer, whereas IFN-γ is a tetramer. Synthesis of IFN-α takes place mainly in cells of lymphoid origin, while IFN-β is synthesized in fibroblasts, epithelial cells, and macrophages. IFN-ω is induced in leukocytes and trophoblasts and IFNκ is expressed in keratinocytes and dendritic cells. Production of IFN-γ is a specialized function of T lymphocytes, although NK cells and macrophages can produce IFN-γ as well. Competitive binding studies (Merlin et al. 1985) indicate that a common receptor for IFN-α, -β and -ω, as well as the receptor for IFN-γ, are encoded by genes mapping to chromosome 21.

IFNs exert their multiple effects through binding to cell-specific receptors and receptor-mediated transduction pathways results in the induction of IFN-stimulated genes (ISG) (Darnell et al. 1994). The type I IFNs, IFN α, β, ω and κ bind to a common receptor consisting of two subunits that both map to human chromosome 21. Type II IFN, IFN-γ binds to a distinct set of receptors that also consists of two subunits. The recently identified IFN λ binds to a heterodimeric receptor that consists of IL-10Rβ and orphan class II receptor chain, IL-28Rα (Sheppard et al. 2003; Kotenko et al. 2003). The gene arrays analysis has shown that the type I IFN binding to its receptor results in a major transcriptional

Table 1 *Human interferons*

Name	Genes	Proteins	Producer cells	Effect
IFN-α	>20	Nonglycosylated	Lymphoid cells	Antiviral state
	No introns	166 aa	Macrophages	ISG induction
	Chromosome 9	Monomer		MHC I induction
IFN-β	1	*N*-glycosylated	Fibroblasts	Antiviral state
	No introns	166 aa	Epithelial cells	ISG induction
	Chromosome 9	Dimer	Macrophages	MHC I induction
IFN-ω	5	*N*-glycosylated	Leucocytes	Similar to IFN-α
	No introns	172 aa	Trophoblasts	
	Chromosome 9	...		
IFN-γ	1	*N*-glycosylated	T cells	2′,5′-OAS induction
	3 introns	146 aa	Macrophages	IL-1 enhancement
	Chromosome 12	Tetramer	NK cells	MHC I induction, MHC II induction
IFNκ	No introns	180 aa	Keratinocytes, DC	Antiviral
	Chromosome 9			Cytokine induction
IFN λ	3	Glycosylated	Most tissues	Antiviral activity
	6 introns			2′,5′-OAS, Mx induction
	Chromosome 19			MHC I induction

aa, amino acid; DC, dendritic cells; IFN, interferon; ISG, interferon-stimulated gene; MHC, major histocompatibility complex; 2′,5′-OAS, 2′,5′-oligoadenylate synthetase.

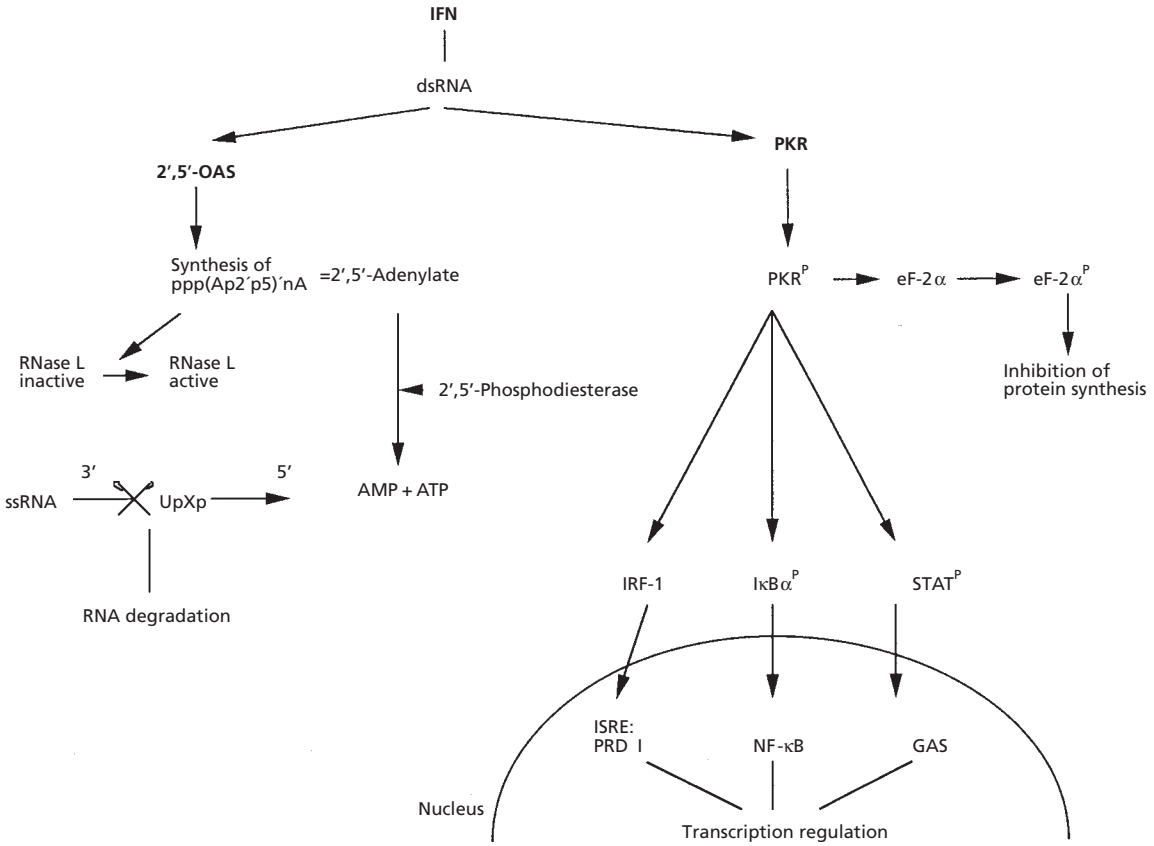

Figure 1 *Double-stranded (ds) RNA-dependent interferon-stimulated antiviral activities. GAS, γ-interferon-activated sequence; IκBα, inhibitor protein binding NF-κB transcription factors; IRF-1, interferon responsive factor 1; ISRE/PRD 1, interferon-sensitive response element/positive regulatory domain 1; P, phosphorylated; STAT, signal transduction activator transducer; UpXp, (by convention) a short RNA sequence in which the phosphorylated base uridine is followed by any other phosphorylated base (A, C, or G).*

activation and expression of more than 100 cellular genes, including genes encoding proteins with antiviral properties, growth-regulating proteins, and components of ubiquitin pathway (Samuel 1991; Malakhova et al. 2003).

The IFN-induced signaling pathway involves activation of several Janns-activated kinase (JAK) kinases that are associated with IFN receptors and by the consequent tyrosine phosphorylation cascade they activate the pre-existing transcriptional factors called signal transducers and activators of transcription (STAT). Phosphorylated STAT proteins, bind to importin and accumulate in the nucleus assembled in multimeric complexes where they interact with interferon-responsive elements present in the 5′ flanking region of interferon-stimulated genes (ISGs) (Levy 1995). Although both IFN-α and -β bind to the same receptors induction of distinct gene(s) by IFN-β was reported suggesting a possible existence of yet unidentified cofactor. Type I and type II IFNs are essential for antiviral defense and they are not functionally redundant (Muller et al. 1994). Both types of IFNs modulate the expression of major histocompatibility complex (MHC) antigens. It is via that mechanism that IFN-α/β increases the susceptibility of fibroblasts infected with vaccinia virus or lymphocytic choriomeningitis virus (LCMV) to cytotoxic T lymphocyte (CTL) lysis (Bukowski and Welsh 1985). Type I and type II IFNs modulate MHC II antigen expression, IFN-γ and tumor necrosis factor (TNF)-α are the most powerful inducers of MHC II antigens. IFN-γ enhances expression of MHC II antigens on accessory cells, stimulates the interaction of these cells with T cells and promotes CTL development. Murine IFN-α and IFN-β can block IFN-γ-mediated class II antigen up-regulation (de Maeyer and de Maeyer-Guignard 1991).

IFNs act via a paracrine loop. Virus-induced IFNs are secreted by the cell and activate neighboring cells into an antiviral state characterized by the expression of a number of ISGs. Several of the proteins encoded by ISGs have direct antiviral activities (Samuel 1991). The antiviral activities of 2′,5′-oligoadenylate synthetase (2′,5′-OAS), PKR protein kinase, and Mx proteins have been well characterized (Figure 1). The 2′,5′-OAS pathway comprises three enzymes. 2′,5′-OAS, upon activation by double-stranded RNA, polymerizes ATP into pppA(2′p5′A)n, (2′,5′A) (Kerr and Brown 1978). 2′,5′A in turn activates a cellular endonuclease, 2′,5′-OAS-dependent RNaseL (Williams and Silverman 1985;

Zhou et al. 1993). RNaseL is present in the cells in an inactive form. When activated by 2′,5′A, it degrades both cellular and viral RNAs at UU or AU nucleotides. 2′,5′-Phosphodiesterase (Lengyel 1982) catalyzes the degradation of 2′,5′A. Expression of 2′,5′-OAS (40 kDa) in cells leads to the establishment of an antiviral state which results in the selective inhibition of the replication of picornaviruses, such as mengovirus and encephalomyocarditis virus (Chebath et al. 1987). The antiviral effect of the 2′,5′-OAS system is specific for picornaviruses and no inhibition of VSV or HSV-1 has been observed.

Activation of the IFN-induced kinase, PKR, depends on the presence of dsRNA. The phosphorylation of the α subunit of protein synthesis initiation factor eF-2 (Samuel 1979; Berry et al. 1985) by PKR has been implicated in the inhibition of viral protein synthesis (Pathak et al. 1988). The crucial role of PKR in the antiviral effect of IFN is further indicated by the observation that a number of viruses have developed mechanisms to negate the function of PKR. Among these adenovirus encodes small RNAs, VA-RNA, which are synthesized in the late stages of the replication cycle and interfere with the activation of PKR and phosphorylation of eF-2a (Davies et al. 1989). Vaccinia virus encodes a specific PKR inhibitory factor (SKIF) (Akkaraju et al. 1989).The reovirus encoded protein G3 binds dsRNA and inhibits the activation of PKR by competing for dsRNA (Imani and Jacobs 1988). Poliovirus, on the other hand, inactivates PKR by inducing the autophosphorylation of PKR and its subsequent degradation. In addition to its antiviral effect PKR is also implicated in the control of cell proliferation (Samuel 1991).

The Mx proteins are GTPases and the molecular mechanism and the antiviral activity of Mx proteins depends on specific MX protein, its cellular localization of the type of viral infection. The antiviral activity of MX is dependent on its oligomerization, GTP binding, and hydrolysis. Athough originally identified as inhibitor of influenza virus infection (Haller et al. 1979; Samuel 1991). It has recently been shown that the Mx protein inhibits also additional RNA viruses, including measles and hantavirus replication (Frese et al. 1995).

The IFN induced RNA-specific adenosine deaminase-ADAR1 catalysis A-I editing and recent studies described an extensive A-I (G) hypermutation editing for several viral RNA.

ISG 20 was recently identified as single-stranded RNA endonuclease and its role in the antiviral effect of IFN against VSV and EMC was implicated (Espert et al. 2003).

These and possibly other IFN-regulated proteins confer on IFN its broad antiviral spectrum. In addition to the above-described IFN-mediated viral inhibition, IFN has been shown to block other steps of the viral life cycle. IFN-α inhibits cytomegalovirus (CMV) at the level of transcription of viral immediate–early (IE) genes, in particular that of the major genes *IE1*, *IE2*, and *IE3* (Martinotti and Gribaudo 1992). Whereas both IFN-α and IFN-γ inhibit adenovirus early gene expression, only IFN-γ inhibits late transcription (Mistchenko et al. 1989). IFN-α inhibits HIV-1 at many steps of its life cycle, but predominantly at the level of reverse transcription in acutely infected T cells and monocytes (Shirazi and Pitha 1992, 1993) and at the level of virus assembly in chronically infected cells (Pitha 1991). Inhibition of virus assembly and maturation is the major mechanism by which IFN blocks the replication of murine retroviruses (Pitha 1980). Stimulation of HLA class 1 expression on measles virus infection is mediated by IFN-β.

The availability of purified and recombinant IFN preparations has made possible the clinical use of IFN-α for the treatment of papillomavirus infections of the larynx, the anogenital regions, and the skin (Fingerote et al. 1995). IFN-α has also become the treatment of choice for chronic hepatitis B and hepatitis C virus infections. Hepatitis virus replication is inhibited by IFN-induced 2′,5′-OAS in mouse peritoneal macrophages. The antiviral effect of 2′,5′-OAS is not sufficient, however, to prevent the hepatocellular injury associated with hepatitis virus infection (Fingerote et al. 1995). Intranasal use of IFN-α for rhinovirus infections causes substantial damage to the nasal mucosa (Finter et al. 1991; Gutterman 1994). The major role of IFN-γ in antiviral defense is to modulate the immune response to viral infection. IFN-γ has, however, a direct antiviral effect, possibly through the induction of 2′,5′-OAS (de Maeyer and de Maeyer-Guignard 1991). In mice, IFN-γ controls T cell-mediated viral clearance in acute cutaneous and ocular infection with herpes simplex (HSV-1) and in poxvirus infection. In macrophages, IFN-γ inhibits HSV-1 and vaccinia virus replication by inducing the production of nitric oxide in the infected cells (Karupiah et al. 1993). The growth of hepatitis virus is restricted in mice by IFN-γ-activated macrophages (Vassao et al. 1994). IFN-γ is also necessary for a normal antigen-specific immunoglobulin G2a response, as demonstrated in mice lacking the IFN-γ receptor (Huang et al. 1993). Intraperitoneal infection with mouse cytomegalovirus or herpes simplex virus (HSV) results in inflammatory infiltrates, consisting mainly of macrophages with increased expression of IFN-γ and TNF-α. CMV titers are increased in IFN-γ-depleted mice and in mice with severe combined immunodeficiency (SCID) (Heise and Virgin 1995). The antiviral effect of IFN-γ has also been demonstrated by the observation that the replication of recombinant vaccinia virus expressing IFN-γ is markedly inhibited, compared to that of wild-type virus (Ramshaw et al. 1992). The virulence of poxvirus infection is increased in mice lacking IFN-γ, and one of the myxoma virus genes implicated in the growth of the virus in vivo encodes the IFN-γ receptor (Upton et al. 1987).

Viruses have, however, evolved mechanisms to evade the antiviral effects of IFN. Many viruses have developed mechanisms by which they negate the function of RNA-activated protein kinase (PKR) (Reich et al. 1988). By contrast, flaviviruses (e.g. West Nile virus and Kunjin virus) induce expression of intercellular adhesion molecule-1 (ICAM-1) by two distinct mechanisms. First, induction is mediated by type I IFNs and occurs only in quiescent fibroblasts in G0 phase; the second induction is cytokine-independent and occurs within hours of viral challenge. The induction of cell–cell contact via ICAM-1 expression early in infection may significantly enhance the ability of the virus to spread before the immune response is triggered (Shen et al. 1995).

Cross-talk between IFNs and other cytokines is an essential component of the immune response

The experimental evidence suggests that both the innate and the acquired immune response involves cytokine-mediated amplification and cross-talk network. Thus the antiviral response seems to undergo the amplification step in which the IFN-β induced during the initial response to infection stimulates the expression of transcription factors that are required for the induction of expression of the IFN-α family of proteins resulting in much robust antiviral response.

In addition to the antiviral function, the IFN-α/β-mediated signaling pathway affects also the immune responses through the modulation of expression of several cytokines. Thus, while IFN-γ promotes expression of IL-12, IFN-α/β down-regulates IL-12 expression in monocytes and dendritic cells. IFN-α/β mediates induction of IL-15 that promotes survival of activated NK cells and their accumulation (Biron 2001). In human cells but not in mice, IFN-α/β activates IFN-γ and Th1 development of $CD4^+$ cells. Altogether these results indicate that IFN-α/β stimulates the signaling pathway for the innate regulation of adaptive immunity.

Tumor necrosis factor

TNF-α (cachectin) and -β (lymphotoxin) are two related peptides encoded by linked genes mapping within the MHC (Spies et al. 1986). Although TNF-α is secreted mainly by macrophages/monocytes and TNF-β by T cells, they compete for a common receptor. TNF-α, like IFN, can establish an antiviral state, and TNF-α-mediated induction of 2′,5′-OAS has been demonstrated in a number of cell lines (Mestan et al. 1988). IFN-γ and IFN-β synergize with TNF-α to increase levels of 2′,5′-OAS and viral inhibition (Mestan et al. 1988). Studies in vivo, with mice as a model for encephalomyocarditis virus (EMCV) infection, have shown that TNF-α alone is not sufficient to inhibit viral replication, but requires combined treatment with IFN-γ (Czarniecki 1993). The same requirement has been observed with murine CMV (Anderson et al. 1993). The direct antiviral effect of TNF-α has been well documented with recombinant vaccinia virus expressing TNF-α (Ramshaw et al. 1992). In contrast, the replication of retroviruses, such as simian immunodeficiency virus (SIV) and HIV, is activated by TNF-α (Lairmore et al. 1991). Although IL-6 mRNA levels are increased in response to TNF-α, IL-6 alone does not seem to contribute to the TNF-α-induced antiviral state (Jacobsen et al. 1989).

Both RNA and DNA viruses can induce TNF-α expression in peripheral blood mononuclear cells (PBMC) and macrophages. Influenza virus increases TNF-α mRNA levels in monocytes (Nain et al. 1990), respiratory syncytial virus (RSV) stimulates TNF-α secretion in macrophages (Franke-Ullman et al. 1995) and human herpesvirus-6 (HHV-6) triggers TNF-α secretion in PBMCs (Flamand et al., 1991). TNF-α secretion by PBMCs from patients with chronic hepatitis B virus infection correlates with the level of virus replication. TNF-α inhibits replication of vesicular stomatitis virus (VSV) in WISH cells and replication of EMCV in HeLa cells. Another mechanism by which TNF-α limits viral spread is by enhancing viral cytotoxicity. TNF-α induces cytolysis of HSV-1 and HSV-2-infected rat embryo cells (Koff and Fann 1986). Cytolysis is also observed with either TNF-α or TNF-β in VSV or adenovirus-2-infected A549 cells. In vivo, however, adenovirus *E1A* gene encodes a 14.7 kDa protein which suppresses TNF-α-mediated cytolysis and thereby allows escape from the antiviral effects of TNF-α (Laster et al. 1994). Furthermore, co-expression of the adenovirus 14.7 kDa protein in a recombinant vaccinia virus expressing TNF-α inhibits the TNF-α-mediated attenuation of vaccinia virus virulence (Tufariello et al. 1994). Also, one of the genes in the terminal repeat of myxoma virus involved in modulating the replication of the virus in vivo encodes a soluble TNF-α receptor. Because induction of TNF-α is an essential part of the cellular defense against myxoma virus infection, the virus is able to neutralize the effect of TNF-α. Induction of TNF-α, as well as IFN-γ and IL-1 during dengue virus infection in macrophages may result in permeability of vascular endothelial cells and plasma leakage, which are characteristic of the hemorrhagic fever secondary to dengue virus infection. Not all viruses, however, can induce TNF-α. HSV-1, for instance, is a strong inducer, whereas Epstein–Barr virus (EBV) down-regulates the levels of TNF-α mRNA and protein in PBMCs (Gosselin et al. 1992).

Interleukin-1

The interleukin-1 (IL-1) family is composed of two cytokines, IL-1α and IL-1β, and one inhibitor, IL-1 receptor antagonist (IL-1ra). Although IL genes are highly

conserved across species, they share little homology with one another and their regulation is different. Both IL-1α and IL-1β are synthesized as membrane-bound precursors of 270 aa, cleaved by proteases to yield circulating soluble proteins. IL-1 is produced mainly by macrophages, but T and B cells, dendritic cells, fibroblasts, epithelial cells, and astrocytes can synthesize IL-1 as well. IL-1 has no direct antiviral activity but, together with IL-2, induces IFN-γ synthesis and thus contributes indirectly to the antiviral immune response. IFN-α has been reported to down-regulate IL-1β in bone marrow stromal cells, while stimulating expression of the IL-1 receptor, thereby contributing to the myelosuppression associated with viral infections (Arman et al. 1994). Not all viral infections result in the induction of IL-1. Whereas influenza virus induces substantial IL-1 activity in macrophages, RSV induces IL-1 inhibitor activity (Roberts et al. 1986). In situ hybridization studies have shown that HIV-1, herpesviruses, EBV and CMV, adenovirus, and chronic hepatitis B virus (HBV) infection induce *IL-1β* and *IL-6* genes, not, however, in the infected cells themselves but in macrophages and epithelial cells adjacent to the infected cells (Devergne et al. 1991). IL-1, together with TNF-α and IL-6, is a key cytokine expressed during inflammation. In mice, cerebral expression of IL-1α and -1β and TNF-α correlates with the onset of neurodegenerative processes secondary to scrapie infection (Campbell et al. 1994). Although both adult T cell leukemia (ATL) and human T-cell lymphotrophic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) are associated with HTLV-1 infection, the difference in virus-induced cytokine expression may underlie the differences in pathogenesis between the two diseases: IFN-γ, TNF-α, and IL-1β expression is induced in HAM/TSP, whereas elevated levels of transforming growth factor β (TGF-β) are secreted by leukemic cells in ATL (Tendler et al. 1991).

Interleukin-6

IL-6 is produced by T and B cells, macrophages, fibroblasts, and endothelial cells. During viral infection, *IL-6* seems to play an essential role in antibody production, since it induces B cells to differentiate into antibody-producing cells. Mice with targeted disruption of the *IL-6* gene have a reduced number of IgA-producing cells. Expression of IL-6 enhances systemic and mucosal antibody responses in mice infected with a recombinant fowlpox virus expressing influenza virus hemagglutinin and IL-6 (Ramsay et al. 1994). In contrast, IFN-γ expression in those animals inhibits antibody responses, but not cell-mediated immunity (Leong et al. 1994). Both RNA and DNA viruses induce IL-6 expression. Thus, HTLV-I transactivator protein, Tax, induces IL-6 expression through the direct activation of the IL-6 NF-κB site (Mori et al. 1994). Chronic virus-producing, but not chronic latent, hepatitis B infection increases serum IL-6 activity, and IL-6 levels are further enhanced during acute exacerbation of the disease (Kakumu et al. 1991). Dengue virus, which is transmitted through mosquito bites, induces a rapid expression of IL-6 in fibroblasts, as well as granulocyte/macrophage colony stimulation factor (GM-CSF) and IFN-β. IFN protects uninfected cells from infection and limits virus spread (Kurane et al. 1992). Elevated serum levels of IL-6, IL-1 and TNF-α are also found in patients with dengue hemorrhagic fever (Hober et al. 1993). HIV-1 transactivating protein tat can induce IL-6 production in uninfected PBMCs in vitro and IL-6-dependent IgG and IgA synthesis (Rautonen et al. 1994). In the presence of IL-4, HIV-1 infection induces an autocrine IL-6 loop in B cells. Acquired immune deficiency syndrome (AIDS)-associated Kaposi's sarcoma positive PEL cells express the IL-6 receptor and use IL-6 as an autocrine growth factor (Masood et al. 1994). Macaque monkeys infected with a lethal variant of SIV (SIVsmm/PBj-14) show a marked increase in both IL-6 mRNA in inflamed tissues and IL-6 protein in serum (Birx et al. 1993).

Virally induced IL-6 participates in inflammatory reactions together with IL-1 and TNF-α. Live or inactivated RSV induces increased levels of IL-6, as well as TNF-α mRNA in alveolar macrophages. The combination of these two cytokines (IL-6 and TNF-α) does not seem, however, to have a direct antiviral effect but modulates the inflammatory response to RSV infection (Becker et al. 1991). In a similar manner, live or UV-irradiated coxsackie virus B3 (CVB3) stimulates the release of IL-6, IL-1, and TNF-α in infected human monocytes, and supernatants from CVB3-infected monocytes are cytotoxic to heart cells, suggesting that these cytokines induced by viral infection may contribute to the pathology of CVB3-induced myocarditis. In patients with cerebrospinal inflammation, however, it is acute bacterial meningitis and not viral meningitis (such as is caused by mumps virus) that is associated with increased IL-6 activity (Torre et al. 1992).

Interleukin-7, 8, and 10

IL-7 is a 177 aa protein secreted by thymic stroma cells and acts as a T-cell growth and differentiation factor and a macrophage activation factor. IL-7 also augments an *env*-specific CTL response in HIV-1 infected individuals in a dose- and time-dependent manner and promotes the maturation of precursor CTLs independently of IL-2. IL-8 is a member of a large family of factors secreted by macrophages and endothelial cells which act as mediators in inflammation. Constitutive expression of IL-8 and IL-6 by tracheobronchial and nasal epithelial cells is significantly increased upon viral infection (Adler et al. 1994). RSV, in particular, induces the release of IL-8 and IL-6 in the human epithelial cell line A549 in a time- and viral load-dependent manner. In addition, proinflammatory cytokines, such as TNF-α and IL-1

Table 2 *Virus-induced modulation of cytokine effects*

Virus	Viral gene	Cellular homologue	Effect
Poxviruses	MGF	EGF/TGF-α	Cell growth
	T2	TNF receptor	TNF inhibition
	T7	IFN-γ receptor	IFN-γ inhibition
	Unmapped	IL-1 receptor	IL-1 inhibition
EBV	BCRFI = vIL-10	IL-10	Escape from immune surveillance
Adenovirus	E1A = 14 ± 7 kDa protein	...	Suppresses TNF lysis
	E1B = 19 kDa protein		Counteracts TNF effects
	VA RNA		Inhibits IFN-α/β

synergistically enhance IL-8 secretion in RSV-infected A549 cells (Arnold et al. 1994). IL-10, appropriately termed cytokine synthesis inhibitory factor, inhibits the production of IFN-γ, and secretion of IL-1, IL-6, and TNF-α by macrophages. An open reading frame from EBV, *BCRFI*, shows extensive homology with IL-10, and it has been suggested that viral IL-10 expression enables infected cells to escape from immune surveillance (Moore et al. 1990) (Table 2).

Interleukin-4 and 12

IL-4 is a 20 kDa glycoprotein, B cell growth and differentiation factor which is secreted by Th2 cells and plays an essential role in the initiation of humoral immunity. IL-12, also known as NK cell stimulatory factor (Kobayashi et al. 1989), is a heterodimer which stimulates IFN-γ production. It has become clear now that IL-12 and IL-4 play pivotal roles in the differentiation of naive T cells into Th1 and Th2 subsets. Although viruses tend to trigger cell-mediated immunity, not all do so. CNS infection with live measles virus induces IL-4 expression and Th2 cell activation (Ward and Griffith 1993). Sindbis virus, which causes acute encephalomyelitis in mice, also triggers a Th2 response in the brain, characterized by increased expression of IL-4, IL-16, IL-10, and TGF-β in the brain (Wesselingh et al. 1994). IL-4 and IL-12 also seem to have an enhancing effect on HIV-1 replication, as is described in detail later under Role of cytokines in specific viral infections. The role of IL-4 and IL-12 in the Th1 and Th2 immune responses is explored more fully later in this chapter (see Th1–Th2 shift, below).

MODULATION OF THE IMMUNE RESPONSE

Th1–Th2 shift

As mentioned earlier, T lymphocytes are required for cell-mediated immune responses and antibody production by B cells. This dual role is controlled by two distinct Th cell subsets. Th1 cells produce IL-2 and IFN-γ and direct cell-mediated immunity, whereas Th2 cells produce IL-4, IL-6, and IL-10 and promote antibody production for humoral immunity. IL-12, secreted by macrophages and B cells, stimulates the production of IFN-γ in T cells and NK cells. IL-12 has been shown to act directly on $CD4^+$ naive T cells derived from T receptor transgenic mice to induce IFN-γ production and T-cell differentiation towards Th1 development (Seder et al. 1993). In contrast, IL-4 has been shown, in the same system, to drive Th2 cell development and Th2-specific cytokine expression. Moreover, Th2-specific cytokines, IL-4 and IL-10, inhibit IL-12 production in human macrophages and, thereby, the Th1 response. As both IL-12 and IL-10 are produced by macrophages, these can either promote or inhibit Th1 cell development, depending on the relative production of either cytokine by these cells. IFN-α stimulates production of Th1 cells and IFN-γ blocks Th2 development (Sher et al. 1992). Th1 and Th2 responses play important roles in viral clearance. In mice, Th1 clones are protective against lethal challenges with influenza virus, whereas Th2 clones are not protective and delay viral clearance (Graham et al. 1994). A switch from Th1 to Th2 responses has been suggested as playing a role in HIV infection and progression to AIDS. According to this model, HIV-specific T cell responses of the Th1 type would keep the infection under control. An increased viremia would result in a Th2 response, antibody production, and progression to AIDS. In this model, any stimulus or pathogen that could promote a switch from Th1 to Th2 would act as a cofactor in the progression to AIDS. If this model holds true, treatment with exogenous IL-12 might support and maintain Th1 cell function and thus delay the progression of the disease. This question is being explored in a number of clinical trials.

ROLE OF CYTOKINES IN SPECIFIC VIRAL INFECTIONS

HIV replication and pathogenicity

Human immunodeficiency virus (HIV-1) is recognized as the etiological agent of the slowly

progressing immune deficiency in humans that culminates in the development of AIDS (Barré-Sinoussi et al. 1983). The rate of progression of this lentiviral infection and the onset of the disease seem to depend on viral determinants (multiplicity of infection, virus strain, or isolate), the interaction of HIV-1 with the host immune system and a number of cofactors, such as secondary viral and bacterial infections, cytokines, or stress factors. The primary targets of HIV-1 infection are $CD4^+$ cells, especially T cells and the cells of the myeloid lineage, monocytes and macrophages.

Initial infection with HIV-1 is followed within 3–5 weeks by an acute phase characterized by influenza-like symptoms, lymphadenopathy, plasma viremia, and a significant decrease in circulating $CD4^+$ cells. During the acute phase, HIV-1 disseminates to other tissues, particularly to the lymph nodes. The generation of an immune response at those sites results in the suppression of HIV-1 levels in the blood and restoration of the $CD4^+$ cells. Infection then enters a state of clinical latency, when little virus can be detected in plasma. However, viral replication takes place in lymph nodes, where it continues to infect the $CD4^+$ cells (Pantaleo et al. 1993). Recent studies from several laboratories have shown that, during these seemingly asymptomatic stages, virus-infected T cells are rapidly cleared from the circulation by the vigorous immune response (Coffin 1995; Ho et al. 1995). The high replication rate of HIV-1 results in the death of lymphocytes that are continuously being replenished by the immune system. This leads to the gradual decrease in $CD4^+$ T cells and the deterioration of the immune response. In the final stages of infection, HIV-1 reappears in the blood as a consequence of a breakdown of lymph node ultrastructure; the paucity of $CD4^+$ cells and the lack of appropriate immune response facilitate numerous opportunistic infections and the development of neoplasia.

Effect of HIV infection on cytokine gene expression

The production of cytokines by macrophages and T cells is part of the inflammatory response to viral infection, which is essential for lymphocyte activation, generation of cytotoxic cellular responses, and antibody production.

Increased levels of TNF-α, IL-1, IL-6, transforming growth factor β (TGF-β), and IFN-α and IFN-β have been found in the sera of AIDS patients (Birx et al. 1990; Breen et al. 1990). However, in vitro, the spontaneous and lipopolysaccharide (LPS)-stimulated production of TNF-α, IL-1, and IL-6 does not seem to be significantly different in peripheral blood lymphocytes (PBL) isolated from AIDS and uninfected donors (D'Addario et al. 1990; Molina et al. 1990; Yamato et al. 1990). In another set of reports, an increase in TNF-α production in PBLs has been observed with the progression of HIV-1 infection (Rossol et al. 1992). An increase in the relative levels of TNF-α, IL-1, and IL-6 mRNAs has also been observed in LPS-stimulated alveolar macrophages isolated from individuals with AIDS, compared with the levels of mRNAs present in macrophages isolated during the early stages of HIV infection. Polyclonal activation of B cells (Reickmann et al. 1991) and increased levels of IL-6 may be associated with the higher frequency of B cell malignancies seen in AIDS patients.

Increased levels of IFN-α and IFN-γ (acid-labile IFN-α) were found both in pre-AIDS and in AIDS patients, but the frequencies and levels were higher in AIDS patients (Künzi and Pitha 1995). Elevated levels of IFN-α serum have been associated with a poor prognosis (Voth et al. 1990). It is not clear, however, what type of cell is responsible for IFN-α production in people infected with HIV-1 because, in vitro, HIV-1 infection of PBL does not result in the induction of IFN gene expression. PBMC isolated from individuals infected with HIV-1 or from AIDS patients has impaired IFN production upon stimulation with double-stranded RNA, NDV or HSV-1 (Fitzgerald-Bocarsky et al. 1989). This impairment in IFN production seems to occur at the transcriptional level (Gendelman et al. 1990).

In contrast, HIV-1 infection in vitro seems to induce cytokine expression. Thus, stimulation of HIV-1-infected monocytes with bacterial antigens from *Pneumocystis carinii* or *Mycobacterium avium* results in enhanced expression of TNF-α, IL-1, IL-6 and IL-8 mRNAs, compared to those present in stimulated but uninfected controls (Newman et al. 1993; Kandil et al. 1994). Both enhanced and unaltered cytokine expression have, however, been reported in HIV-1-infected monocytes stimulated with LPS, and no difference in cytokine production between HIV-1-infected and uninfected monocytes has been observed after stimulation with endotoxin or double-stranded RNA (Gendelman et al. 1990; Molina et al. 1990). A large variability in reported results suggests that inducible expression of cytokine genes may depend both on genetic determinants and on the nature of the inducer.

The molecular mechanism by which HIV-1 infection alters spontaneous and inducible cytokine gene expression is not clear. However, as most of the cytokines (whose expression is up-regulated by HIV-1 infection) contain an NF-κB enhancer in their promoter and NF-κB-specific binding is essential for transcription activation, it is likely that the binding of the HIV-1 or gp120 to the CD4 receptor induces change in the signaling pathways, such as those leading to the activation of NF-κB heterodimers, that alter both the constitutive and the inducible expression of the cytokine genes.

Effect of cytokines on HIV-1 replication

Transcription of HIV-1 is controlled by cellular and viral transactivators that bind to regulatory sequences present

both in the long terminal repeat (LTR) of HIV-1 provirus and in the untranslated leader sequence of viral RNAs. Activation of viral transcription occurs upon binding of virally encoded tat protein to the tat-binding region present in all viral mRNAs (Rosen et al. 1985; Cullen and Greene 1989). In contrast, DNA elements present in the HIV-1 LTR, such as NF-κB enhancer (Nabel and Baltimore 1987), play a major role in the activation of the promoter by cellular and extracellular factors. Additional regulation of the inducible response is mediated through specific interaction between nuclear transactivators and the AP-1, NFAT, LBP-1, HLP-1, and TCF-1 DNA-binding sequences (Jones et al. 1988).

Many inflammatory cytokines stimulate HIV-1 transcription from the LTR and affect HIV-1 replication (Butera 1993; Poli and Fauci 1993). This observation is of biological importance, because cytokines are released during the immune response to HIV-1 infection and increased cytokine expression can be detected in lymph nodes in the vicinity of the focus of infection (Emille et al. 1991). The local environment in the infected lymph nodes allows for extensive interactions between activated B cells and T cells, resulting in the production of a variety of cytokines secreted in both an autocrine and a paracrine fashion. The profile of cytokines induced at the site of infection can then not only determine the nature of the immune response (cellular immunity mediated by CTL response versus antibody response) but also directly affects HIV-1 replication and thus positively or negatively enhances the progression of disease.

Cytokine-mediated up-regulation of HIV-1 replication

Of the stimulatory cytokines, TNF-α probably has the most significant effect on HIV-1 replication and a direct role in the pathogenicity of HIV-1 infection has been attributed to it. In vitro, TNF-α can up-regulate the transcriptional activity of HIV-1 LTR and the effect is mediated by the induction of NF-κB binding in the nucleus (Duh et al. 1989; Osborn et al. 1989). It has been shown that the TNF-α-mediated signal transduction pathway begins with the binding of TNF-α to one of the membrane receptors TNF-Rβ (55 kDa) and proceeds through sphingomyelinase and ceramide (Schutze et al. 1992). The TNF-α-mediated activation is not limited to the HIV-1 LTR. TNF-α can also effectively induce the expression of poorly expressed (latent) HIV-1 provirus in both T cells and monocytes. An interesting feature of TNF-α activation from the point of view of HIV-1 replication is that it is independent of transactivation mediated by the virus-encoded transactuator, tat. TNF-α can effectively rescue transcription and viral replication of a mutant HIV-1 provirus in which the tat region is deleted and therefore transcriptionally inactive (Popik and Pitha 1993). These results point to the existence of an alternative pathway in HIV-1 transcription regulation that is independent of tat. Because elevated levels of TNF-α can be detected in the serum in the later stages of HIV-1 infection, it is assumed that TNF-α is one of the factors that contribute to the progression of the disease and to some of the clinical signs, such as AIDS-associated weight loss (Serwadda et al. 1985). The ability of TNF-α to activate HIV-1 replication in a tat-independent manner has important implications for the antiviral strategies aimed at eliminating tat transactivation. Effective inhibition of HIV-1 replication requires both tat and NF-κB inhibition, and, in the presence of TNF-α, the drug RO5 that blocks tat transactivation is not effective (Popik and Pitha 1994). Thus, the presence of TNF-α in the serum and the ability of TNF-α to transactivate HIV-1 replication in a tat-independent fashion may be one of the reasons why the drug RO5 was ineffective in clinical trials.

Several other cytokines up-regulate HIV-1 transcription and HIV-1 provirus in vitro. In vitro, IL-1 stimulates transcription of a latent HIV-1 provirus in both T cells and monocyte lines (Poli et al. 1994). The induction was associated with the stimulation of NF-κB activities. IL-3 and the proteins of the colony stimulating factor (CSF) family, GM-CSF and M-CSF, up-regulate HIV-1 replication in primary macrophages and in monocyte and myeloid cell lines (Gendelman et al. 1990). It is interesting that CSF-mediated stimulation does not seem to be mediated entirely through NF-κB binding, as the region upstream of the NF-κB enhancer is also required for activation (Zack et al. 1990). IL-6 is another cytokine that has been implicated in the upregulation of HIV-1 replication (Poli et al. 1994). In contrast to the other cytokines, IL-6-mediated enhancement does not seem to take place at the transcriptional level, but occurs at the post-transcriptional level. The exact mechanism of this enhancement is not known. Of importance from a biological point of view is the synergy between IL-6 and TNF-α or IL-1 which leads to a significant enhancement of HIV-1 replication (Poli et al. 1990, 1994).

Cytokine-mediated down-regulation of HIV-1 infection

Recent data indicate that progression to AIDS is accompanied by a shift from a Th1 cytokine response and production of IL-2 and IFN-γ to a Th2 cytokine response, with production of IL-4, IL-6, and IL-10. In vivo, production of IFN-γ during the Th1 response may play a role in the down-regulation of HIV-1 infection. Although in vitro HIV-1 infection in macrophages does not induce IFN-α or IFN-β production, IFN-α can be detected in serum during the later stages of HIV-1 infection. The biological role in vivo of this IFN is not known, but IFN-α, IFN-β, and IFN-γ can effectively inhibit HIV-1 replication in vitro.

In vitro, IFNs inhibit HIV-1 replication both in primary cells and in established T cells and monocytic cell lines (Yamamoto et al. 1986; Kornbluth et al. 1989; Poli et al. 1989; Gendelman et al. 1990). In a single cycle infection in T cells (Shirazi and Pitha 1992) or in IFN-producing cells (Bednarik et al. 1989), IFN inhibits early steps in HIV-1 replication and HIV-1 transcripts cannot be detected in these cells (Shirazi and Pitha 1993). Furthermore, in primary macrophages, Type 1 IFNs decrease the levels of integrated provirus (Kornbluth et al. 1990). Hence, in multiple infection cycles or in chronic infection, IFN inhibited HIV-1 replication at the post-translational level and decreased the levels of virus released into the culture medium, but did not substantially affect the synthesis of viral RNA and proteins in infected cells. The mechanism by which IFN inhibits HIV-1 replication at the preintegrational step is not clear. Inhibition of the early replication steps has been observed in cells constitutively producing low levels of IFN-β, and it has been suggested that inhibition occurs at the level of viral uptake.

IFN also inhibits the activation of HIV-1 provirus by external stimuli in vitro. Poli et al. (1989) have shown that TNF-α-mediated stimulation of HIV-1 provirus is inhibited by IFN-α, and it has been suggested that the IFN-mediated block is at the level of virus maturation and assembly. Activation of a *tat*-defective provirus by 12-*O*-tetradecanylphorbol 13-acetate (TPA) or by HSV-1 is also inhibited in the presence of IFN. However, the inability of these inducers to activate HIV-1 provirus in the presence of IFN occurs at the transcriptional level, and is associated with the alteration of NF-κB-specific binding (Popik and Pitha 1991, 1992).

Several studies have shown that, although HIV-1 infection does not induce IFN type I synthesis in vitro, it can modulate the levels of at least two dsRNA-dependent enzymes, 2′,5′-OAS and RNaseL, and induce expression of Mx protein. It has also been shown that the activation of PKR by tat-binding region (Edery et al. 1989) inhibits the translation of HIV-1 mRNAs and that inhibition of protein synthesis correlates with phosphorylation of eF-2. Yet none of these IFN-induced proteins seems to play a significant role in the IFN-mediated inhibition of HIV-1 replication. Neither small nor large 2′,5′-OAS can be detected in HIV-1-infected cells (Popik and Pitha, unpublished). Productive HIV-1 infection leads to a decrease, not an increase, in cellular PKR activity. It has been suggested that the binding of the cellular protein tat region binding protein (TRBP) to the tat-binding region interferes with recognition of the dsRNA structure and prevents induction of PKR (Park et al. 1994) in HIV-1-infected cells. Together, these results indicate that IFN can inhibit many steps of the HIV-1 replication cycle with differing efficiency and they suggest that, in vivo, IFN may have a broad spectrum of action (Figure 2).

Cytokines with both stimulatory and inhibitory effects on HIV-1 replication

The wide range of biological properties of certain cytokines is often manifested by their bifunctional effects. This is also true of their effects on HIV-1 replication. TGF-β pretreatment can stimulate HIV-1 replication in primary T cells and in myeloid and monocytic cell lines (Lazdins et al. 1991; Poli et al. 1991). However, when TGF-β is applied to infected cultures, it inhibits PMA, TNF, and IL-6-mediated stimulation of HIV-1 replication (Poli et al. 1991). IL-4 is another cytokine with a bifunctional effect on HIV-1 replication: it can either induce or inhibit HIV-1 replication in myeloid cells. In undifferentiated monocytes, IL-4 stimulates HIV-1 replication, as well as the proliferation of the cells (Kazazi et al. 1992; Foli et al. 1995) whereas, in differentiated macrophages, IL-4 suppresses HIV-1 replication (Novak et al. 1990). In T cells, however, IL-4 does not affect HIV transcription. This indicates that differences in host cells and types of infection, as well as the timing of exposure to cytokines, may determine the outcome of the cytokine effects.

Herpesvirus infections

Herpesviruses selectively regulate proinflammatory cytokine synthesis. Human herpesvirus-6 infection induces high levels of IL-1β and TNF-β in wildtype cultures, but inhibits the induction of IL-6. In the same cells, EBV inhibits TNF-α secretion (Gosselin et al. 1992). The pattern of cytokine expression during herpes simplex virus-induced keratitis (HSK) has been well studied in the mouse and human cornea. HSV-1 infection in murine keratinocytes induces transient expression of TNF-α, IL-6, and IL-1, a cytokine profile that is characteristic of a transient inflammatory response (Sprecher and Becker 1992). Exogenous IL-10, a cytokine with inflammatory suppressive activity, also significantly decreases IL-6 levels in HSV-1-infected murine cornea and sharply reduces the incidence of blindness secondary to the HSV-1-induced inflammatory response, even though IL-1α levels are not decreased (Tumpey et al. 1994). At low concentrations, IFN-γ and TNF-α induce a synergistic antiviral effect against HSV-1 in human corneal cells (Chen et al. 1994). Cells isolated from eyes in the active phase of HSK have been found to be of the $CD4^+$ Th1 subset, secreting IL-2, IFN-γ, and TNF-α, but not IL-4 or IL-10 (Niemialtowski and Rouse 1992). Treatment with monoclonal antibodies specific for IFN-γ or IL-2 reduces the severity of HSV-1-induced corneal inflammation in mice, and inhibition of IFN-γ production by UV-irradiation enhances IL-4 production, as well as zosteriform skin lesions (Hendricks et al. 1992; Yasumoto et al. 1994). These findings indicate that Th1 and Th2 responses define the pathogenicity of HSV-1 in

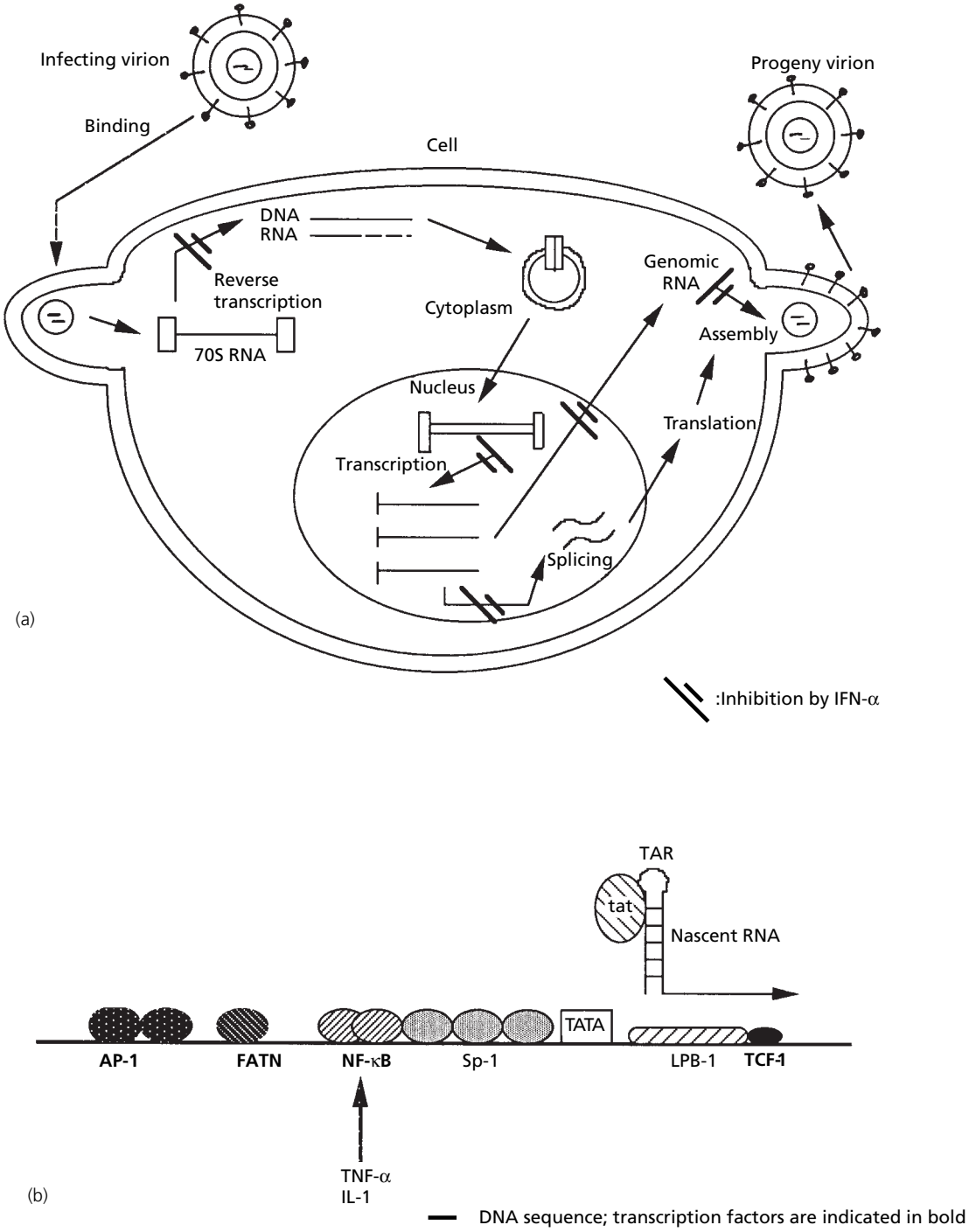

Figure 2 (a) *IFN-α inhibition of the HIV-1 life cycle.* **(b)** *Cytokine activation of* HIV *long terminal repeats.*

the cornea and the skin. HSV-1 and -2 activate macrophages and induce IFN-α/β production, which synergizes with TNF-α in an autocrine manner to protect macrophages from herpetic infection (Ellerman-Eriksen et al. 1994).

EBV infection is associated with acute, self-limiting, infectious mononucleosis, chronic infectious mononucleosis, and lymphoproliferative disease (LPD). In all acute forms of EBV infection, IFN-γ, TNF-α, IL-2, and IL-6 are elevated (Linde et al. 1992). EBV-associated LPDs are a common complication of primary immunodeficiency and are associated with elevated serum levels of IL-4 and, to a lesser extent, IL-5, IL-10, and IFN-γ (Mathur et al. 1994). Cyclosporin A, used in immunosuppressive therapy, increases EBV-associated lymphoproliferative disorders by inducing

IL-6 expression in T cells and by increasing the number of EBV-immortalized B cells expressing viral lytic antigens. Exposure to EBV stimulates IL-6 expression in B cells and in monocytes (Tanner and Menezes 1994).

EBV-infected B cells express IL-10 and B-cell regulatory factor (BCRF) 1, the viral homologue of IL-10, and expression of both viral and cellular cytokines is maintained during transformation. Inhibition of viral IL-10 (vIL-10) expression with antisense oligonucleotides at the time of viral challenge prevents EBV B-cell transformation. Conversely, transformation is rescued by exogenous cellular IL-10. These observations indicate that EBV-encoded *IL-10* is a latency gene involved in B cell transformation (Miyazaki et al. 1993). EBV has been implicated in the etiology of Sjögren's syndrome, a disease characterized by lymphocytic infiltration, squamous cell metaplasia and a decrease in goblet cell numbers in the conjunctival epithelium of the eye. Although the marker for latent EBV infection, EBNA-1 or EBV IL-10 is not expressed at a higher frequency than in the normal eye, HLA-DR, ICAM-1, and IL-6 expression is significantly increased in Sjögren's syndrome (Jones et al. 1994).

Infections with hepatitis viruses

Hepatitis virus clearance and persistence hinge upon the intricate, and as yet not fully understood, modulation of virus-induced cytokines. Monocytic production of IL-6, the mediator of acute-phase protein synthesis in hepatocytes, is decreased in chronic HBV infection, whereas it is normal in chronic hepatitis C virus (HCV) and in acute hepatitis A virus (HAV), HBV and HCV infections. Exacerbation of chronic HBV induces expression of IL-6 and TNF-α which correlates with an increase in HBV DNA levels in serum (Kakumu et al. 1991; Sheran et al. 1991). Moreover, IL-6 contains recognition sites for the segment of the HBV envelope protein through which the virus binds to cell surfaces (Neurath et al. 1992).

HBV is not cytopathic, but it evokes a strong lymphomononuclear inflammatory response in the liver during acute and chronic infections. Transgenic mice whose hepatocytes do not secrete HBV, but retain the antigen in the endoplasmic reticulum, are highly susceptible to liver damage induced by bacterial lipopolysaccharide, a potent inducer of inflammatory cytokines. The effect seems to be mediated by both IFN-γ and TNF-α. Hepatocellular injury and induction of inflammatory cytokines coincide with a reduction in HBV steady-state mRNA content (Gilles et al. 1992). TNF-α induces two other proinflammatory cytokines, IL-8 and IL-6, in patients with chronic HBV. Inflammatory cytokines appear to down-regulate HBV expression by both TNF-α-dependent and TNF-α-independent pathways. Administration of exogenous recombinant TNF-α, IL-2, IFN-α, and IFN-β has been shown to reduce steady-state levels of HBV RNA in transgenic mice. TNF-α antibodies block the effect of TNF-α and IL-2, but not that of IFNs. Administration of exogenous IFN-γ, IL-1, IL-3, IL-6, or TNF-α has no effect on the HBV RNA levels (Guidotti et al. 1994). De-regulation of the inflammatory cascade can have disastrous effects, as illustrated by fulminant hepatic failure, characterized by a striking elevation of IL-6, TNF-α, IL-1Rα, and IL-1β levels (Sekiyama et al. 1994). IFN-γ and HBV X protein increase the rate of transcription of intercellular adhesion molecule 1 (ICAM-1), a molecule critically important to adhesion-dependent leukocyte functions, such as antigen presentation and target cell lysis (Hu et al. 1992). IFN-γ initially also activates HBV-specific class I restricted CTL, suppressing expression of the HBV gene.

Chronic hepatitis B, C, or D may lead to cirrhosis, hepatocellular failure or hepatocellular carcinoma. IFN-α has recently become the treatment of choice for chronic hepatitis, because HBV and HCV infections respond well to treatment with it. Sustained loss of HBV DNA and HBV nucleocapsid and surface antigens leads to the restoration of normal liver function. However, the relatively high prevalence of resistance of HBV to IFN is of concern. It was shown that the terminal domain of HBV polymerase is responsible for the resistance of the virus to IFN-α and IFN-γ. Individuals infected with several HCV quasi species seem to be less responsive to IFN therapy than are those with a single major species (Gutterman 1994; Dusheiko 1995).

CYTOKINE RESPONSES TO VIRAL INFECTIONS OF THE CENTRAL NERVOUS SYSTEM

There is increasing evidence that local cytokine production induced as a response to viral infection of cells of the CNS or infiltrating lymphoid cells plays a crucial role in the pathogenicity and outcome of viral infection in this location (Benveniste 1992).

Local synthesis of TNF-α within the CNS seems to be especially damaging. TNF-α damages glial cells and has a direct toxic effect on neurons. In vitro, TNF-α induces demyelination of oligodendrocytes, whereas the closely related TNF-β kills oligodendrocytes by inducing apoptosis. Production of both TNF-α and TNF-β in the CNS results in the death of myelin-producing oligodendrocytes. TNF-α can also act indirectly by stimulating microglia and astrocytes to produce nitric oxide and the neurotoxic metabolites of arachidonic acid (Benveniste 1995). TNF-α also stimulates synthesis of other cytokines such as GM-CSF, G-CSF, and M-CSF, which enhance inflammatory responses as well as the synthesis of IL-6 (Doherty et al. 1989; Moskophidis et al. 1991; Campbell et al. 1994). IL-6 enhances inflammation

in the CNS, as well as stimulating the proliferation of B cells and their differentiation into antibody-producing cells.

The local immune responses in the CNS and the outcome of the infection are affected by the cytokines induced in situ, whether directly in infected cells (Sebire et al. 1993; Wesselingh et al. 1994), as a response to viral infection (e.g. IFNs, TNF-α or IL-6 in astrocytes or microglia) or by infiltrating T cells and macrophages during local inflammation (Shankar et al. 1992; Gillespie et al. 1993).

It is interesting that the profile of cytokines synthesized in the CNS seems to be determined by the type of infecting virus rather than by the type of host cells. Thus, infection of neurons with LCMV leads to synthesis of TNF-α, IL-1, IL-6, and IFN-γ, generating a strong CTL response (Campbell et al. 1994). The strong Th1 cytokine response and induction of cytotoxic T cells leads ultimately to death, whereas viral replication alone is regulated by the presence of IFNs. The association of LCMV pathogenicity with the immune response provided the first clear evidence that viral pathogenicity may be mediated by the immune response of the host (Rowe 1954). In contrast, the infection of neurons with the Sindbis virus (an alphavirus closely related to Western equine encephalitis virus), which causes acute encephalomyelitis in mice, results in generation of the Th2 cytokine response (Wesselingh et al. 1994). Increases in mRNAs and IL-1, IL-4, IL-6, IL-10, and TNF-α proteins have been observed during the early stages of acute infection in mice, followed by the appearance of antiviral antibodies and clearance of virus from the CNS (Tyor et al. 1989; Levine et al. 1991). Although it has been suggested that the CNS may provide an environment that favors B-cell antibody response, it is difficult at present to generalize this finding. Thus, recovery from acute HSV infection depends on a T-cell response, whereby the MHC class I-restricted $CD8^+$ cytotoxic T lymphocytes control HSV infection in the brain (Schmidt and Rouse 1992; Nash and Cambouropoulos 1993). Secretion of IFN-γ from activated T cells is critical for clearing of herpes virus infection (Raniero et al. 1990; Lucin et al. 1992). Recently, it has been shown that the T cell inflammatory response and production of IFN-γ persist in trigeminal ganglia long after HSV-1 has established the latent phase of infection (Cantin et al. 1995). This observation may indicate that the prolonged production of IFN-γ and possibly also of other cytokines (e.g. TNF-α) (Schmidt and Rouse 1992) may block or suppress HSV-1 replication after reactivation from latency (Cunningham and Merigan 1983; Torseth and Merigan 1986).

De-regulated local production of cytokines in the brain has also been observed in patients with AIDS dementia complex (ADC), which afflicts 30–50 percent of all people infected with HIV-1 (Johnson et al. 1988). In the CNS, HIV-1 can be found primarily in infiltrating monocytes and macrophages, as well as in resident microglia (Koenig et al. 1986; Wiley et al. 1986), but there is evidence for major damage to neurons (Ketzler et al. 1990). There is no explanation at present for the cause of this extensive neurological impairment. It has been suggested that cytokines produced in macrophages and glial cells in response to HIV-1 infection (Gelbard et al. 1994) may either directly damage the neurons or alter the function of astrocytes and, consequently, neuronal function (Benveniste 1992, 1995).

Analysis of the cerebrospinal fluid from ADC patients has shown elevated levels of IL-1, TNF-α, IL-6, and neopterin, an indicator of the presence of IFN-γ (Grimaldi et al. 1991). With the exception of IFN-γ, all these substances are the products of activated macrophages and microglia. IFN-γ is most likely to be produced by infiltrating $CD8^+$ cells (Tyor et al. 1992). The expression of cytokine mRNA in the brain has been analyzed (Wesselingh et al. 1993). The results reveal a strong association between increased levels of TNF-α mRNA and ADC. As discussed above, TNF-α induces many abnormalities in the CNS and so its overproduction may be one of the causes of the neuron dysfunction in ADC. It is interesting that an increased number of TNF-α-producing cells was also observed in patients with other neurological disease, such as multiple sclerosis (Benveniste 1995). These data suggest that TNF-α may play a major role in inflammatory disorders of the CNS.

DIRECTED CYTOKINE EXPRESSION WITH RECOMBINANT VIRUSES

The role of individual cytokines in response to viral infection has been studied by using recombinant cytokines administered systematically. This approach does not mimic well conditions in vivo, in which cytokines are acting locally and presumably in much higher concentrations (Biron et al. 1990). Results from experiments with transgenic animals expressing IL-2 (Ishida et al. 1989), IL-4 (Tepper et al. 1990), IL-5 (Tominaga et al. 1991), or GM-CSF (Lang et al. 1987) have often been difficult to interpret, because of the widely spread expression. More clear-cut results have been obtained with experiments in which animals with a homozygous deletion of a given cytokine or its receptor are used (e.g. IFN-γR nu/nu mice (Huang et al. 1993), IL-6 nu/nu mice) or when production of cytokines is suppressed by monoclonal antibodies (Leist et al. 1989).

Recently, a new technique has been used, whereby a cytokine gene inserted in a nonessential part of a recombinant virus is able to target cytokine production directly to the focus of infection. This makes it possible to determine the effect of local cytokine production on viral replication and the immunological consequences of cytokine overproduction. The most common type of virus for cytokine delivery has been vaccinia virus (Ramshaw

et al. 1992), although HSV-1 (Mester et al. 1995) and mouse cytomegalovirus (Burnes, personal communication, 1995) have also been used.

Vaccinia virus is a complex DNA virus that replicates in the cytoplasm. Foreign DNA can easily be inserted into the virus under the control of a viral promoter (Davidson and Moss 1989), and foreign proteins are processed and transported with fidelity (Moss and Flexner 1987). Furthermore, a large region of this virus DNA genome is not essential for replication. Insertion of the *IL-2* gene into vaccinia virus enhances neither T cell-mediated immunity nor the immune response, but it decreases the virulence of the virus and enables immune-suppressed mice to resolve infection (Ramshaw et al. 1992). Enhancement of NK cell cytotoxic activity is an important factor in the clearance of infection in nude mice, and it has been found that IFN-γ plays an essential role in IL-2-mediated recovery. Since no host response was demonstrated that explained the rapid clearance of IFN-γ-expressing virus, these authors concluded that the observed inhibition in viral replication is caused by the direct antiviral effect of IFN. Similarly, the recombinant virus expressing TNF-α was rapidly cleared without any detectable involvement of the host immune system. Furthermore, local production of TNF-α was not toxic and no pathogenicity induced by TNF-α could be detected. Thus, TNF-α, like IFN-γ, seems to inhibit viral replication by the direct antiviral effect (Sambhi et al. 1991). These results indicate that overproduction of Th1 cytokines inhibits viral replication. Inhibition of viral replication is localized and limited to the recombinant virus and does not affect the coinoculated control virus (Ramshaw et al. 1992).

To determine the effect of Th2 cytokines on the course of viral infection, recombinant viruses expressing Th2 cytokines have been constructed. In normal mice, recombinant virus encoding IL-4 replicates more efficiently than control virus, whereas expression of IL-5 or IL-6 has no apparent antiviral effect. However, vectors encoding IL-5 and IL-6 significantly increase terminal differentiation of IgA-committed B cells and mucosal IgA antibody response in vivo (Ramsay and Kohonen-Corish 1993; Ramsay et al. 1994). Expression of IL-6 augments both the primary systemic antibody and the mucosal antibody responses. In contrast, expression of IFN-γ significantly inhibits antibody responses without affecting the general cell-mediated immune response (Leong et al. 1994). Furthermore, the vectors expressing IL-6 are able to reconstitute the IgA and IgG responses in IL-6-deficient mice (Ramsay et al. 1994). These results indicate that IL-6 plays a critical role in the induction of mucosal immunity.

Herpes simplex virus type 1 (HSV-1) has also been used for targeted delivery of cytokine genes. Weir and Elkins (1993) inserted a human IFN A gene into a replication-incompetent herpesvirus and showed that IFN expressed in infected monocytes is able to inhibit HIV-1 replication. Murine IFN A has been inserted in both replication-competent and incompetent HSV-1. HSV-1-derived IFN A gene expression did not inhibit HSV-1 replication in vitro. However, preinfection of the cells with the nonreplicating HSV–IFN vector provided complete protection against a subsequent viral challenge (Mester et al. 1995). Replication-defective vectors derived from HSV-1 have also been used for delivering genes to neurons (Fink et al. 1992), suggesting their usefulness for analyzing cytokine effects within the nervous system. In summary, these approaches suggest a novel and highly effective approach to the study of local cytokine effects in viral infections and to the effectiveness of virus-derived vectors in vaccine development and cytokine gene therapy.

CONCLUSION

It is evident that viral infection induces a complex host response mediated by the induction of genes encoding various cytokines and lymphokines, as well as stress proteins. This response can be induced directly in the infected cells or indirectly in cells of lymphoid origin tethered to the focus of infection during the immune response. The primary function of these cytokines is either to inhibit viral replication or to activate the cells mediating the cellular and humoral immune responses. However, inflammatory cytokines, produced during the immune response, are not always beneficial to the host and can contribute to the pathogenicity and side-effects of viral infections. The detrimental effect of cytokines can be most clearly demonstrated in conditions in which infection is localized to a specific organ or system such as the CNS. Then, production of even relatively low levels of cytokines, such as TNF-α, can have profoundly toxic effects and result in the death of neurons or the dysfunction of oligodendrocytes.

Several viruses have developed ways to eliminate or overcome the effect of cytokines. This again demonstrates the great plasticity of some viral genomes and their ability to adapt. Thus, HIV-1 has modified its regulatory region to resemble the promoter of some of the inflammatory cytokines, and can therefore use several of the inflammatory cytokines to stimulate its own replication. Other viruses, such as the poxviruses, carry several genes that encode soluble cytokine receptors. Others, such as adenovirus, have developed a specific mechanism that can allow them to inhibit the effects of cytokines. Some of the HIV-1-encoded accessory proteins may carry similar functions. Expression of viral genes that inhibit cytokine induction and function seems to be essential for the replication of many of these viruses in vivo, because the replication and pathogenicity of mutants with 'mimicry' functions deleted are usually considerably diminished. One of the main objects of future research should, therefore, be to characterize these cytokine-inhibiting viral functions. This

would help not only to understand the role that a given cytokine plays in a particular viral infection but also to generate attenuated, nonpathogenic viruses that could be used for vaccine development. However, the real challenge will be to use the mechanisms that viruses have developed to avoid the immune response as selective tools to eliminate viral infections.

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Viral evasion of the host immune response

ANTONIO ALCAMI

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INTRODUCTION: AN INTIMATE RELATIONSHIP BETWEEN VIRUSES AND THEIR HOSTS

Viruses depend on the cell for replication and production of new virus particles, and thus viruses have established a complex interaction with the cellular machinery for nucleic acid replication and protein synthesis, and have 'learned' how to take advantage of cellular compartments to generate virus particles. The viral replication cycle and morphogenesis of virus particles can be observed in vitro in cell culture. However, in real life, viruses replicate in an animal host that is well prepared with a variety of defense mechanisms to prevent viral infection. In turn, viruses have evolved many different strategies to counteract host defenses.

The immune evasion strategies used by different virus families are related to the 'lifestyle' of the virus, which depends on the nature and replication of their genetic material, cell tropism, mechanism of transmission, and ability to persist in the infected host. Some viruses such as human immunodeficiency virus (HIV) infect immune cells and may cause immunosuppression. RNA viruses take advantage of the high mutation rate of the RNA polymerases to generate variants that escape recognition by the host immune system. Herpesviruses establish latency, a viral stage that maintains the viral genome with minimal expression of viral proteins, avoiding recognition by the host immune system and allowing viral persistence for the life of the host. In this case, intermittent phases of reactivation generate newly synthesized virus particles to ensure transmission to other individuals and virus persistence in the host population. Recent investigations during the last few years have shown that viruses not only hide to avoid immune recognition but they take a very active role to interfere with specific molecular mechanisms of immunity. Large DNA viruses, such as poxviruses and herpesviruses, may accommodate between 100 and 200 genes in their genomes and have the capacity to code for a large number of proteins that interfere with the immune antiviral response.

Viral strategies of immune evasion block the development of innate (nonspecific) or adaptive (antigen-specific) immune responses. The innate response constitutes the first line of immune defense since it does not require previous encounters with the viral agent, and is rapidly activated during the first hours after infection. Innate responses include interferons (IFN), the complement system or natural killer (NK) cells. The adaptive immune response consists of antibodies and cytotoxic T cells (CTL) which are directed against specific antigens encoded by the viral invaders. This response requires days or even weeks to develop and induces immunological memory, so that the host will respond more

rapidly and efficiently to viruses that have been experienced previously. The inflammatory response illustrates how innate responses may favor specific immune responses. As a result of viral infection and tissue damage, the inflammatory response is triggered which is characterized by an increased permeability of blood vessels and the production of soluble mediators such as cytokines and chemokines which induce the migration of immune cells into areas of infection. The end result is a massive infiltration of cells and soluble factors that protect the host from viral infection and trigger antigen-specific cellular and humoral responses that will offer further protection mechanisms.

The activation of the immune response requires the cooperation of T helper (Th) lymphocytes which differentiate into Th1 and Th2 cells that produce specific sets of cytokines. Th1 cells produce cytokines such as interleukin (IL)-2 and interferon (IFN)-γ that promote cellular pro-inflammatory responses associated with the maturation of macrophages and CTLs. Other cytokines such as IL-12 play an important role by inducing Th1 and NK cells to produce IFN-γ. By contrast, Th2 cells promote the humoral response inducing the differentiation of B cells to produce antibodies. In addition, Th2 cells produce IL-4 and IL-10 which may reduce cellular inflammatory responses. Whereas cellular immune responses are thought to play a critical role in viral elimination once viral infection has been established, the antibody response plays an important role in preventing the early stages of infection.

We have just started to understand the complex interaction of viruses with their hosts and how viruses achieve an equilibrium leading to the survival of both virus and host. Viruses are fully dependent on the host for their replication and production of progeny virus, and thus they should avoid high virulence that may lead to extermination of the host. Much of the pathology associated with viral diseases may be the result of an unbalanced virus–host interaction, and in some cases it represents uncontrolled attempts of the immune system to destroy the virus that lead to damage of the host. It has become clear that a more profound knowledge of the interaction of viruses with their host will help us to understand the molecular mechanisms of viral pathogenesis.

The study of the interaction of viruses with the host immune system is a research area that has expanded enormously in recent years and it is not possible to cover all aspects in depth in a chapter. Here I provide a general view of the different strategies used by viruses to evade the host immune response. Basic concepts of viral immune evasion are discussed and illustrated with representative examples. More information can be found in review articles (Alcami and Koszinowski 2000; Johnson and Hill 1998; Katze et al. 2002; Seet et al. 2003; Tortorella et al. 2000).

INHIBITION OF HUMORAL IMMUNITY

Genetic variability

The production of antibodies specific to viral antigens that neutralize virus particles is an important mechanism to prevent viral infection. One of the first mechanisms of immune evasion identified in viruses was the accumulation of mutations that translate into antigenic variability, a mechanism that has special relevance in viruses with RNA genomes (Flint et al. 2000). In contrast to DNA polymerases, RNA polymerases have low fidelity and introduce a high rate of mutations as the genetic material is copied. As a consequence, viral RNA genomes consist of a collection of RNA species (quasi species) carrying point mutations. These mutations translate into viral epitopes that are recognized with lower affinity by antibodies that neutralize the infectivity of the viral particles. An example of this strategy is found in rhinoviruses, which exist in the human population in the form of 160 serotypes. Neutralizing antibodies raised after the infection with one particular serotype do not protect from infection by other viral serotypes. Genetic variability may also generate variant peptide sequences that have lost the ability to bind to major histocompatibility complex (MHC) molecules, thus evading recognition by the cellular immune system as well.

Influenza virus is one of the best examples to illustrate genetic variability as a mechanism to escape immune recognition (Flint et al. 2000). The genome of influenza virus consists of eight RNA fragments encoding different proteins, including the virion surface proteins known as hemaglutinin (HA) and neuraminidase (NA). Coinfection of the same host with influenza viruses bearing different subtypes of HA and NA may generate virus particles containing new combinations of genomic fragments. This results in a sudden change of antigenic properties, known as antigenic shift, that facilitates the immediate evasion of neutralizing humoral responses in the majority of the population. This phenomenon may lead to pandemics such as that of 1918 in which more than 20 million people died. At the same time, influenza virus accumulates point mutations continuously due to the low fidelity of its RNA polymerase, a phenomenon known as antigenic drift. These limited changes are responsible for the flu epidemics of each season.

Fc receptors

Some viruses encode receptors for the Fc domain of immunoglobulin G (IgG) to evade the action of antibodies (Johnson and Hill 1998) (Figure 1). The glycoproteins gE and gI from herpes simplex virus (HSV) form a heterodimeric complex that functions as an Fc receptor. It has been proposed that gE–gI interacts with antibodies

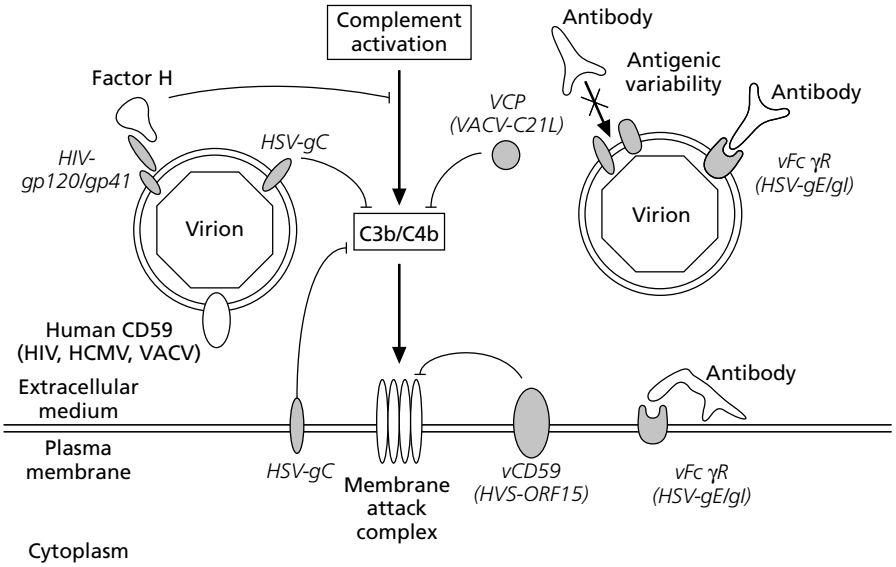

Figure 1 *Viral mechanisms to evade the complement and antibody responses*

that recognize specific HSV antigens and inhibit complement-mediated antibody neutralization and antibody-dependent cellular cytotoxicity by cells carrying Fc receptors (Frank and Friedman 1989; Lehner et al. 1975). The spike protein of the coronaviruses mouse hepatitis virus, bovine coronavirus, and transmissible gastroenteritis virus display Fc receptor activity (Oleszak et al. 1993).

Complement system

The complement system constitutes one of the major defense systems against pathogens. Viral antigens or antigen–antibody complexes may activate the complement cascade, a complex family of proteins that are sequentially activated and culminates with the formation of a membrane attack complex leading to the destruction of infected cells or the envelope of virus particles in those viruses with an outer lipid membrane.

Herpesviruses and poxviruses encode proteins with amino acid sequence motifs found in complement regulatory proteins and bind critical factors in the complement activation pathways, such as C3b and C4b, and inhibit their activity (Favoreel et al. 2003; Johnson and Hill 1998; Kotwal et al. 1990) (Figure 1). The HSV membrane glycoprotein gC binds the complement factor C3 and protects cellfree virus from complement-mediated neutralization and inhibits the lysis of HSV-infected cells by antibody and complement (Lubinski et al. 1999). The poxviruses variola virus, vaccinia virus (VACV), and cowpox virus (CPXV) encode a secreted protein, known as viral complement control protein (VCP), that blocks the classical and alternative pathways of complement activation (Kotwal et al. 1990; Rosengard et al. 2002). Inactivation of the *VCP* gene in VACV causes virus attenuation in animal models, demonstrating an important role of this protein in preventing host defense mechanisms and a role for complement in antiviral immunity (Isaacs et al. 1992). Interestingly, inactivation of the CPXV VCP, known as inflammation modulatory protein (IMP), caused a substantial increase in the inflammatory response in infected mice compared to wild type CPXV infections (Miller et al. 1997). This illustrated that inhibition of the inflammatory response by viral proteins may reduce tissue damage and reduce the pathology associated with viral infection.

The complement system has the potential to cause extensive damage in the host cells and is therefore strictly controlled by both soluble and membrane proteins that block its activity. A strategy used by HIV is to recruit the soluble factor H that inhibits complement activation by interacting with the gp120–gp41 complex (Stoiber et al. 1996). Herpesvirus saimiri (HVS), a virus that infects monkeys, encodes a homologue of the inhibitor of the membrane attack complex CD59 that protects the infected cells from complement-mediated lysis (Rother et al. 1994). An alternative strategy is adopted by human retroviruses, VACV and human cytomegalovirus (HCMV). Instead of encoding homologues of host proteins, these viruses incorporate into the virion envelope host complement regulatory proteins, such as CD56 and CD59, that confer complement resistance to the virus particle (Saifuddin et al. 1995; Spear et al. 1995; Vanderplasschen et al. 1998).

EFFECTS ON SOLUBLE MEDIATORS OF THE IMMUNE SYSTEM

Modulation of IFN functions

IFNs were discovered because of their ability to protect cells from viral infection. IFNs can be classified as type I

(α and β) and type II (γ), and represent one of the first antiviral defense mechanisms to be activated after infection. The finding in recent years of anti-IFN strategies encoded by most viruses highlights the key role of IFNs against viral infection (Figure 2). IFNs are produced and secreted in response to viral infection and bind specific receptors which trigger intracellular pathways that activate antiviral mechanisms (Goodbourn et al. 2000; Katze et al. 2002). Two major pathways that induce an antiviral state in cells are up-regulated by IFN: the double-stranded RNA (dsRNA)-dependent protein kinase (PKR) and the 2′-5′ oligoadenylate system (2′-5′ OAS) that activates RNase L. In addition, IFNs have immunomodulatory activity that can influence the type of immune response mounted in response to infection.

Viruses block the activity of IFN at three different levels: (i) blockade of IFN binding to specific receptors; (ii) inhibition of signal transduction pathways induced by IFN; and (iii) inhibition of antiviral effector functions induced by IFN (Figure 2).

SECRETED VIRAL IFN RECEPTORS (IFNRS) AND IFN BINDING PROTEINS (IFNBPS)

Poxviruses encode proteins that are secreted from infected cells and bind with high affinity type I or type II IFN and prevent their interaction with IFNRs (Alcami 2003; McFadden and Murphy 2000) (Figure 2). This mechanism inhibits the initiation of IFN-mediated biological effects that lead to the establishment of an antiviral state in the cell or the activation of immune functions. Soluble receptors or binding proteins for other cytokines have been identified, mainly in the poxvirus family. Of particular relevance for IFN activity is the poxvirus IL-18 binding protein (vIL-18BP) which blocks the activity

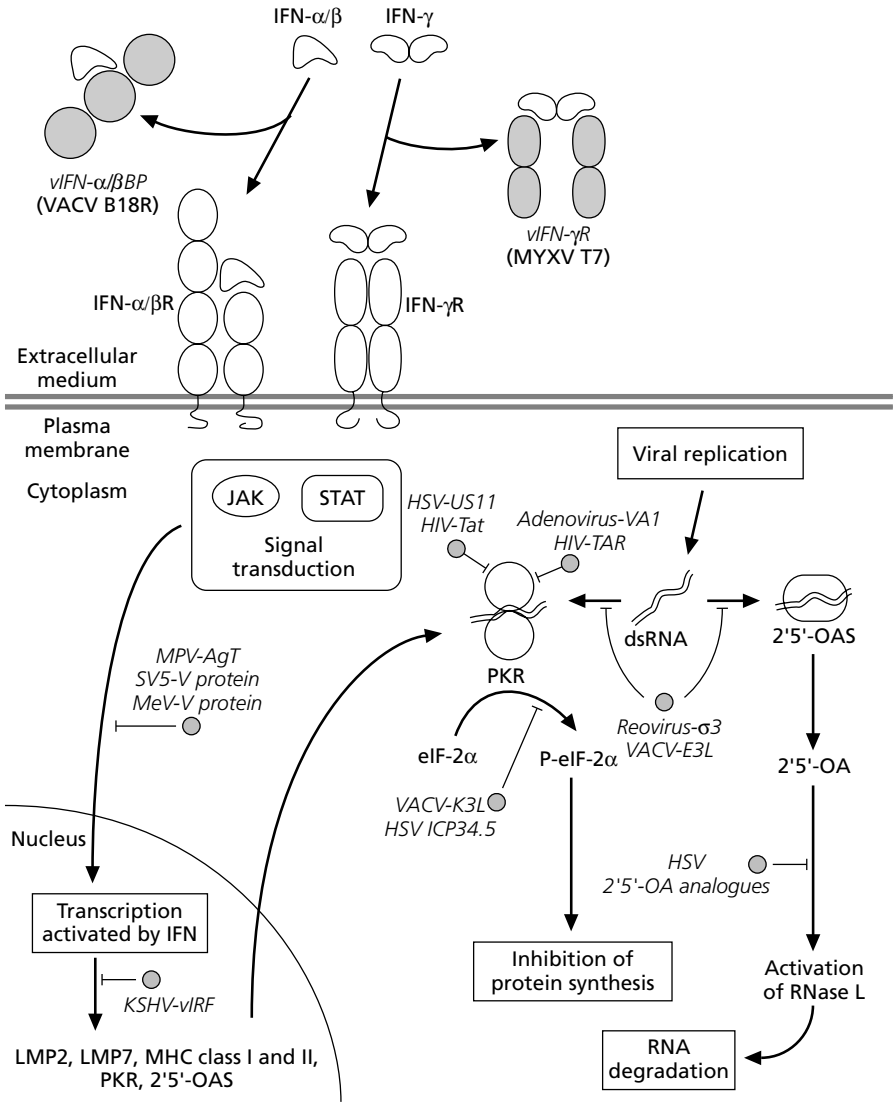

Figure 2 *Viral mechanisms to evade IFN activity*

of IL-18, a cytokine required together with IL-12 for the induction of IFN-γ (see below).

The viral IFN-γR (vIFN-γR) was first identified in myxoma virus (MYXV) and later in other members of the poxvirus family including VACV and CPXV (Alcami and Smith 1995; Mossman et al. 1995; Upton et al. 1992). The vIFN-γR has sequence similarity to the IFN binding domain of the cellular IFN-γR. The role of vIFN-γR during viral infection has been demonstrated by the attenuated phenotype a MYXV mutant lacking this IFN inhibitor (Mossman et al. 1996). A recent report describes a secreted IFN-γBP encoded by fowlpox virus that has no sequence similarity to known cellular IFN-γRs (Puehler et al. 2003). Because the IFN-γR of birds has not yet been characterized at the molecular level, this interesting finding raises the possibility that this virus may have hijacked an avian IFN-γR of different structure to the mammalian counterparts.

VACV and various poxviruses encode a secreted IFN-α/β receptor or binding protein (vIFN-α/βBP) that has very limited sequence similarity to the cellular counterparts (Colamonici et al. 1995; Symons et al. 1995). The vIFN-α/βBP has the unique property of binding to the cell surface after secretion (Alcami et al. 2000). The expression of viral decoy IFN-α/βRs at the cell surface of infected tissues will prevent the activation of IFN-mediated pathways more efficiently. A VACV mutant lacking the *vIFN-α/βBP* gene is attenuated in mice and its ability to grow in tissues and spread to other organs is impaired (Symons et al. 1995).

INTERFERENCE WITH SIGNAL TRANSDUCTION INDUCED BY IFNS

The interaction of IFNs with specific receptors at the cell surface triggers a complex cascade of intracellular pathways through janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathways (Goodbourn et al. 2000; Katze et al. 2002) (Figure 2). These signaling events result in the up-regulation of cellular proteins that limit viral replication in several ways. Some of the genes upregulated by IFN are the low-molecular-weight protein (LMP) 2 and LMP7 subunits of the proteasome, involved in the generation of viral peptides for MHC class I molecules, the molecules that present viral peptides to T cells. Other genes up-regulated by IFNs are protein kinase dsRNA (PKR) and 2′5′-OAS which confer an antiviral state in cells.

Some viral proteins interfere with the IFN signaling cascade (Goodbourn et al. 2000; Katze et al. 2002). For example, the T antigen of murine polyomavirus (MPV) binds to and inactivates JAK1 (Weihua et al. 1998), whereas the protein V from simian virus 5 (SV-5) interacts with STAT1 and induces its degradation by the proteasome (Didcock et al. 1999). Other paramyxovirus V proteins target different components of the IFN signaling pathway. Type II human parainfluenza virus V protein targets STAT2, mumps virus V protein targets both STAT1 and STAT3, and measles virus (MeV) V protein causes a defect in STAT nuclear accumulation and STAT-inducible transcription (Nishio et al. 2002; Palosaari et al. 2003; Parisien et al. 2001; Ulane et al. 2003). Kaposi's sarcoma-associated herpesvirus (KSHV) uses a different mechanism by encoding a homologue of the IFN regulatory factor (IRF) that represses the IFN-mediated transcriptional activation of host genes (Zimring et al. 1998).

VIRAL MODULATION OF IFN EFFECTOR FUNCTIONS

One of the best-characterized antiviral effects of IFN is the induction of the PKR and 2′5′-OAS pathways that lead to the blockade of viral gene expression in the cell (Gale and Katze 1998; Goodbourn et al. 2000; Katze et al. 2002) (Figure 2). The replication and transcription of viral genomes leads to the formation of dsRNA that activates PKR, which phosphorylates the translation initiation factor 2α (eIF-2α) causing its inactivation and the subsequent arrest of protein synthesis in infected cells. Viral mechanisms that prevent the activation of PKR include: (i) proteins encoded by reovirus (σ3) and VACV (E3L) that bind dsRNA; (ii) RNAs encoded by adenovirus (VAI) and HIV (TAR) that bind PKR but do not activate the enzyme; and (iii) proteins encoded by HSV (US11) and HIV (Tat) that bind directly to PKR and inactivate the enzyme. An alternative strategy to block the PKR pathway is to prevent the phosphorylation of eIF-2α. VACV and other poxviruses encode an eIF-2α homologue, the K3L protein in VACV, that acts as a substrate of PKR and prevents phosphorylation of eIF-2α. An alternative strategy is illustrated by the HSV IC34.5 protein which activates protein phosphatase 1α that removes the phosphate groups from eIF-2α.

The formation of dsRNA during viral replication also activates the enzyme 2′5′-OAS, producing 2′5′-OA which in turn activates RNase L and the degradation of RNA within the infected cell, causing the blockade of viral replication. HSV produces analogues of 2′5′-OA which bind RNaseL and inhibit this pathway (Goodbourn et al. 2000). The proteins σ3 from reovirus and E3L from VACV bind dsRNA and thus inhibit the activation of 2′5′-OAS as well as PKR (see above).

Modulation of cytokine networks

Cytokines are a family of molecules that initiate and orchestrate the immune response, and include growth factors, IFNs, tumor necrosis factor (TNF), ILs, and chemokines. Cytokines are normally secreted from cells, although some are expressed at the cell surface, and activate specific responses in cells expressing the appropriate receptors. Immune cells use this complex network of ligands and receptors to orchestrate the immune response. In this way, the production of a particular cytokine after viral infection will determine the type of

immune and inflammatory response that takes place. In addition, some cytokines such as IFN and TNF may act directly on infected cells and restrict virus replication.

The importance of cytokines in antiviral defense is highlighted by the number of mechanisms that viruses encode to block their activity (Alcami and Koszinowski 2000; Tortorella et al. 2000). The purpose of many of the viral anticytokine strategies is to down-regulate the immune responses to infection. However, it is important to consider that some viruses replicate in immune cells and these may be taking advantage of the effects that cytokines have on cell physiology to enhance the ability of cells to support viral replication, thus promoting viral replication rather than evading the immune system. Viruses may block the synthesis and maturation of cytokines or may modulate cytokine signaling in the target cells. Large DNA viruses such as poxviruses and herpesviruses have captured in their genomes genes encoding host cytokines and cytokine receptors (Alcami 2003; McFadden and Murphy 2000; Seet et al. 2003) (Figures 3 and 4).

VIRAL MODULATION OF CYTOKINE EXPRESSION AND ACTIVATION

Mechanisms that modulate the synthesis of cytokines have been described (Figure 3). African swine fever virus (ASFV) replicates in macrophages, one of the major cytokine producers in the immune system, and offers a good example to illustrate the control of cytokine production by viruses. ASFV encodes an IκB homologue that interacts with the transcription factors nuclear factor κB (NF-κB) and nuclear factor activated T cell (NFAT) and prevents the transcription of cytokine genes (Miskin et al. 1998). During virus entry into cells, the interaction of MeV with its cellular receptor CD46, a complement regulatory protein that protects cells from lysis by complement, blocks the production of IL-12, a potent inducer of IFN-γ and cellular responses. The CPXV protein cytokine response modifier A (CrmA) was found to inhibit the IL-1β converting enzyme (ICE) or caspase 1, which proteolytically cleaves the precursor form of proinflammatory cytokine IL-1β (pro-IL-1β) to generate mature, active IL-1β (Ray et al. 1992). CPXV CrmA may also prevent the maturation of IL-18, which is also proteolytically cleaved by ICE, and is an inhibitor of apoptosis (see below).

VIRAL MODULATION OF CYTOKINE SIGNAL TRANSDUCTION

There are several examples of viruses that inhibit cytokine-induced intracellular pathways, such as some of the anti-IFN mechanisms discussed above. The interaction of TNF and Fas ligand (FasL) with their receptors induce signaling that lead to an apoptotic response that some viruses are able to block (see below). Adenoviruses interfere with signaling induced by TNF at different levels (Mahr and Gooding 1999). The poxvirus VACV encodes two intracellular proteins (A52R and A46R) that block signaling mediated by Toll-like and IL-1 receptors by mimicking intracellular regulatory domains of these receptors (Bowie et al. 2000; Harte et al. 2003). The Toll-like receptor pathway is important in NF-κB activation and amplification of inflammatory signals.

Lastly, some viruses may subvert cytokine-mediated signaling for their own benefit. The latent membrane

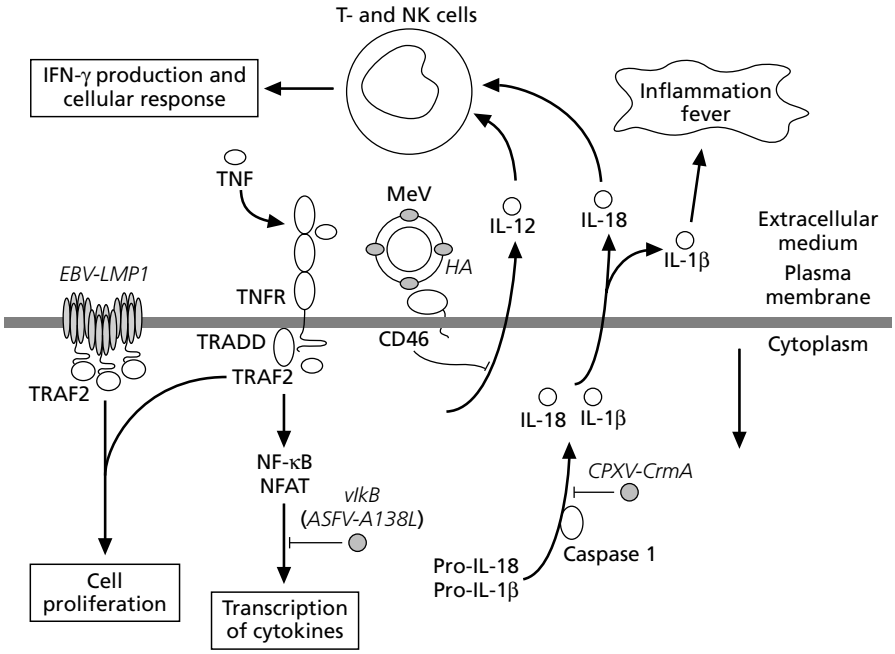

Figure 3 *Viral modulation of cytokine synthesis and signal transduction*

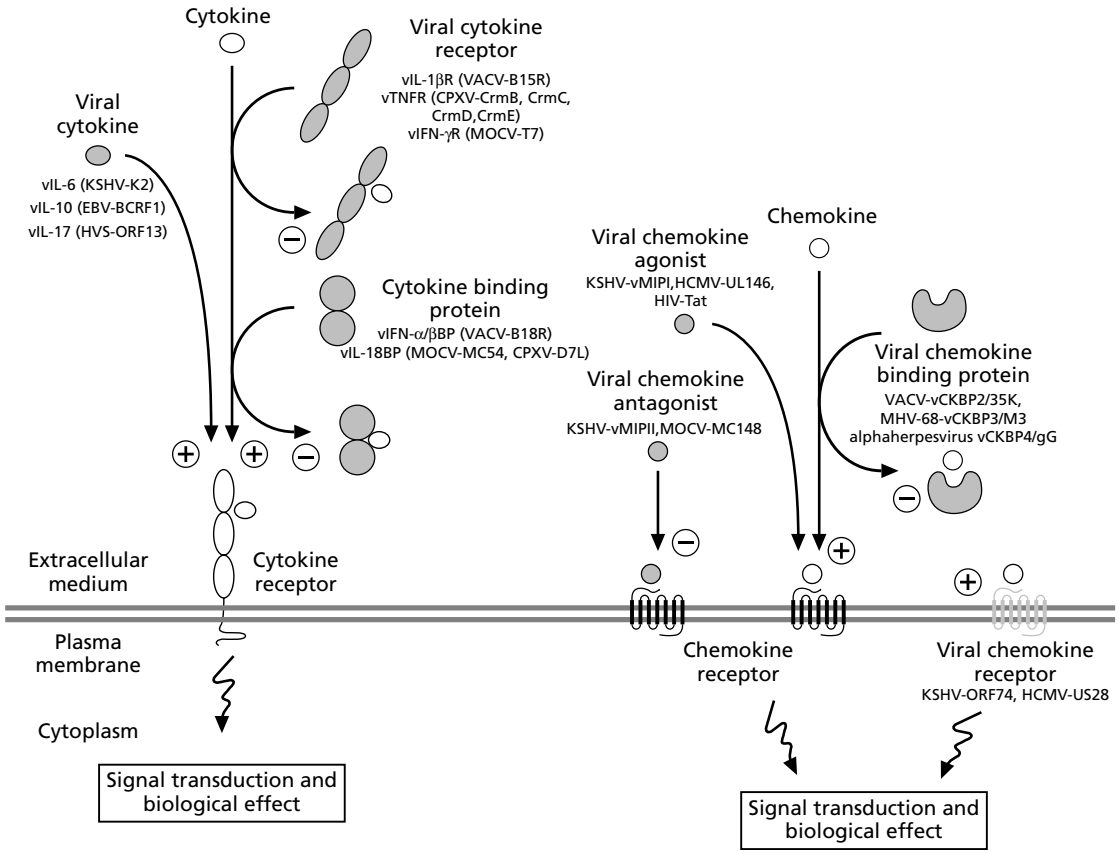

Figure 4 *Cytokines and cytokine receptors encoded by viruses*

protein 1 (LMP1) of Epstein–Barr virus (EBV), a virus that establishes latency in B cells, best illustrates this (Figure 3). LMP1 is a membrane protein that lacks sequence similarity to members of the TNF receptor (TNFR) superfamily but has a short cytoplasmic sequence that binds TNFR activating factors (TRAF), components of the TNFR and CD40 transduction machinery. During the immune response, binding of trimeric TNF to its receptor induces receptors multimerization and recruitment of TRAFs followed by the initiation of a signaling cascade leading to either B cell proliferation or the induction of apoptosis. In EBV-infected cells, the recruitment of TRAFs by LMP1 induces biological responses such as cell proliferation that may enhance virus replication (Farrell 1998). In this way, EBV takes advantage of a B-cell activation pathway to ensure survival of the infected cell that carries the latent viral genome.

Molecular mimicry: viral cytokines and cytokine receptors

The sequence of the genome of large DNA viruses (poxviruses and herpesviruses) showed that some viral proteins have sequence similarity to ligands and receptors of the cytokine family (Alcami 2003; McFadden and Murphy 2000; Seet et al. 2003) (Figure 4). The sequence similarity suggests that, during evolution, viruses may have 'stolen' host genes that play critical roles in the immune system and incorporated them into their genomes. Viruses may have later modified the function of these host proteins for their own benefit since very often the biological properties of the viral proteins differ from their cellular counterparts.

VIRAL CYTOKINES

Viral cytokines act normally as agonists, driving the immune response in a direction that will be more beneficial for the virus (Figure 4). The VACV epidermal growth factor homologue (VGF) was the first viral cytokine identified. VGF activates cell growth and promotes viral replication, but an immune-related function has not been ascribed to it (McFadden et al. 1994). The EBV-encoded IL-10 homologue (vIL-10) was the first viral cytokine identified with immunomodulatory activity (Hsu et al. 1990). Genes encoding related vIL-10s have also been identified in other viruses. It has been shown that the EBV vIL-10 suppresses cellular immunity by inhibiting the production of IFN-γ, but has lost the immunostimulatory properties of the host counterpart. Other viral cytokines are the IL-6 homologue (vIL-6) encoded by KSHV (Moore et al. 1996) and the IL-17

homologue (vIL-17) encoded by HVS (Yao et al. 1995). In addition to their immunomodulatory activity, vIL-6 and vIL-17 may also promote the proliferation of B and T cells, respectively. These are the major host cell types for these viruses and their increased presence in infected tissues may favor viral replication.

The identification of semaphorin homologues (vSEMAs) in poxvirus and herpesvirus genomes was somehow surprising because semaphorins were described as chemoattractants and chemorepellents involved in axonal guidance during nervous system development, and only CD100 had been identified as a semaphorin involved in the immune system (Spriggs 1999). Investigations on the vSEMA encoded by VACV and ectromelia virus (ECTV) led to the identification of a specific receptor in macrophages that triggers expression of cytokines. The pro-inflammatory activity of vSEMA has been observed in mice infected with a VACV expressing an active protein (Gardner et al. 2001). However, the precise role of vSEMA in immune modulation during viral infection remains to be determined.

SECRETED VIRAL CYTOKINE RECEPTORS

Viral genes encoding soluble cytokine receptors have been identified mainly in the poxvirus family and a few examples have been recently described in the herpesvirus family (Figure 4). Viral cytokine receptors lack the transmembrane and cytoplasmic domains of the host counterparts, are secreted in large quantities from infected cells and have the ability to bind with high affinity the cognate cytokine and to block its activity. The poxvirus soluble cytokine receptors were initially identified because of their sequence similarity to the extracellular binding domain of their host counterparts. A second class of cytokine receptors, known as binding proteins, have very limited sequence similarity to the host cytokine receptors and were identified in cytokine binding or activity assays.

The soluble TNFR (vTNFR) M-T2 encoded by MYXV was the first viral cytokine receptor identified (Smith et al. 1991). Four different genes encoding vTNFRs were subsequently identified in other poxviruses such as CPXV, VACV, and ECTV, and were designated CrmB, CrmC, CrmD, and CrmE (Hu et al. 1994; Loparev et al. 1998; Saraiva and Alcami 2001; Smith et al. 1996). These vTNFRs have different molecular size, are produced at different times of infection, and in some cases show distinct binding properties. The reasons for the existence in poxvirus genomes of such a variety of vTNFRs apparently targeting the same ligand are unknown. Further complexity has been added by the recent description of a secreted protein encoded by Tanapox virus that binds TNF but has no sequence similarity to TNFRs (Brunetti et al. 2003). It appears that different poxviruses encode related, but distinct, proteins to block TNF activity extracellularly. Clinical isolates of HCMV, but not laboratory strains, encode a membrane TNFR homologue (UL144) related to herpesvirus entry mediator (HVEM) that is retained intracellularly (Benedict et al. 1999). No ligand has been identified for this protein and its function remains to be determined. Maybe this membrane TNFR subverts TNF-mediated pathways in the infected cell.

Another member of the TNFR superfamily has been recently identified in CPXV and ECTV: a secreted and shorter version of CD30 (vCD30) (Panus et al. 2002; Saraiva et al. 2002). Viral cytokine receptors function as decoy receptors and, accordingly, vCD30 blocks the interaction of CD30 to CD30 ligand (CD30L). However, in this case, the ligand is only expressed at the cell surface and vCD30 induces reverse signaling in CD30L-expressing cells. The function of vCD30 in the context of infection has not been elucidated, but vCD30 has been shown to down-regulate type 1 cytokine-mediated inflammatory responses in mice, a property that may help the virus to evade an efficient antiviral response (Saraiva et al. 2002).

The viral IL-1β receptor (vIL-1βR) is related to the human type II IL-1R and acts as a decoy receptor (Alcami and Smith 1992; Spriggs et al. 1992). In contrast to the membrane IL-1Rs encoded by the host, the vIL-1βR binds exclusively IL-1β and not IL-1α or IL-1 receptor antagonist. Deletion of this gene in VACV causes attenuation when the virus is administered intracranially (Spriggs et al. 1992) but leads to enhanced virulence associated with fever and accelerated death in mice infected through the respiratory route, a natural route of poxvirus transmission (Alcami and Smith 1992; Alcami and Smith 1996). The enhanced virulence observed after deletion of the vIL-1βR from VACV illustrates that the role of some viral immunomodulatory proteins may be to reduce pathology induced by excess production of cytokines, in this case IL-1β, during infection rather than to cause immunosuppression.

VIRAL CYTOKINE-BINDING PROTEINS

IL-18 is a pro-inflammatory cytokine that is required, together with IL-12, for induction of IFN-γ and the generation of an efficient cellular response against viral infection. The viral IL-18 binding protein (vIL-18BP) encoded by several poxviruses is homologue of the human IL-18BP but has no sequence similarity to membrane IL-18 receptors (Figure 4). The vIL-18BP is an effective scavenger of IL-18 and down-regulates IFN-γ production and NK responses in ECTV-infected mice (Born et al. 2000; Smith et al. 2000). Interestingly, the vIL-18BP is also expressed by molluscum contagiosum virus (MOCV), a poxvirus that causes benign skin tumors and persists in the skin for months without inducing an inflammatory response (Smith et al. 2000; Xiang and Moss 1999). The vIL-18BP is the only

cytokine receptor encoded by MOCV and may be responsible in part for the lack of inflammation in MOCV lesions.

The EBV-encoded colony stimulating factor 1 binding protein (vCSF-1BP) has limited sequence similarity to the cellular counterpart and is the only secreted cytokine binding protein identified in herpesvirus (Strockbine et al. 1998). It has been proposed that vCSF-1BP may modulate the response of macrophages during EBV infection. An interesting example of a viral secreted cytokine binding protein that binds ligands that interact with different host receptors is the orf virus-encoded protein that binds granulocyte–macrophage colony stimulating factor (GM-CSF) and IL-2. The role of this protein during infection of Orf virus, which causes skin lesions in sheep, goat, and man, has not been defined (Deane et al. 2000).

Modulation of chemokine networks

Chemokines are a family of small molecular size cytokines that induce cell migration and other biological effects, such as cell differentiation and angiogenesis, and control the trafficking of cells of the immune system throughout the body (Baggiolini 1998). There are more than 40 chemokines identified that can be structurally classified as CC, CXC, C or CX3C, according to the number of amino acids found between the first cysteine residues. Chemokines mediate their biological effects by binding and signaling through G-protein coupled receptors, proteins that have seven transmembrane domains. More than 20 chemokine receptors have been identified. Chemokines also interact with glycosaminoglycans (GAG) to enable their presentation at the surface of the endothelium and the establishment of chemokine gradients in tissues. There is recent evidence that chemokine–GAG interactions are required for the activity of chemokines in vivo (Proudfoot et al. 2003). The immune system makes the decision of which type of immune cell will migrate in response to infection or inflammation depending on the chemokine that is produced and the cell type that expresses the appropriate receptor. Thus the chemokine system plays a critical role in determining the type of immune response initiated after infection.

Viruses, mainly herpesviruses and poxviruses, utilize three mechanisms to modulate chemokine activity: expression of chemokine homologues, of chemokine receptor homologues, and of secreted chemokine binding proteins (Alcami 2003; Lalani et al. 2000; McFadden and Murphy 2000; Murphy 2001) (Figure 4). The function of these viral proteins during the infectious cycle is diverse and is discussed below (Figure 4).

VIRAL CHEMOKINE HOMOLOGUES

Some viruses encode chemokine homologues that are secreted from infected cells and function as agonists or antagonists (Figure 4). The chemokine homologues vMIP-II from KSHV and MC148 from MOCV bind to chemokine receptors but do not transduce signals (Boshoff et al. 1997; Damon et al. 1998; Kledal et al. 1997; Krathwohl et al. 1997). These antagonists block the binding of endogenous chemokines to their specific receptor and inhibit the migration of immune cells into infected tissues. By contrast, some viral chemokines have agonistic activity and induce cell migration into areas of infection, and thus their function may be unrelated to immune evasion. Viruses encoding chemokine agonists take advantage of the chemokine system to recruit to areas of infection immune cells that represent good targets for viral replication in order to enhance viral dissemination. HCMV UL146, known as vCXC-1, induces neutrophil chemotaxis by binding to CXCR2 and may account for the association of neutrophils with HCMV infections (Penfold et al. 1999). Human herpesvirus 6 (HHV-6) U83 induces monocyte migration that may favor the establishment of viral latency and persistence in the infected host (Zou et al. 1999). Another example of chemokine mimetics is the protein Tat from HIV that shows limited sequence similarity to chemokines. HIV Tat is a chemoattractant for monocytes and may enhance replication and spread of HIV in infected individuals (Albini et al. 1998). Direct evidence for a role of viral chemokine agonists in vivo comes from studies with murine cytomegalovirus (MCMV) mutants lacking the chemokine genes M131/129. The MCMV chemokine is encoded by the m131 orf and is expressed as a protein fused to the m129 orf. The isolated synthetic m131 product attracts activated macrophages by engaging a receptor related to human CCR3 (Saederup et al. 1999). A mutant MCMV lacking the function shows reduced spread to salivary glands, indicating that monocyte attraction serves for virus trafficking.

The role of the viral chemokines encoded by KSHV in viral dissemination has not been fully defined but they may play a role in pathogenesis due to their angiogenic properties that may cause the enhanced vascularization observed in Kaposi's sarcoma lesions (Boshoff et al. 1997). In addition, the KSHV chemokines are chemoattractants for Th2 polarized T cells and may influence the type of immune response initiated after viral infection (Dairaghi et al. 1999; Endres et al. 1999; Sozzani et al. 1998; Stine et al. 2000).

An interesting addition to chemokine mimicry by viruses is the glycoprotein G (gG) of respiratory syncytial virus which has partial sequence similarity to fractalkine and chemokine-like activity (Tripp et al. 2001). This property of gG may be used to facilitate virus entry into cells and to modulate the host immune response.

VIRAL CHEMOKINE RECEPTOR HOMOLOGUES

There are many examples of chemokine receptor homologues in members of the herpesvirus family and some

in the poxvirus family (Figure 4). These proteins are expressed at the surface of infected cells and their ability to neutralize large amounts of chemokine may be limited. Some viral chemokine receptors have been found to bind chemokines and to transduce signals, and they may function to modulate the physiological state of the cell by activating chemokine-mediated signaling pathways. The ORF74 protein from KSHV is constitutively active and binding of chemokines can modulate the activation status of the receptor (Arvanitakis et al. 1997; Rosenkilde et al. 1999). Expression of this receptor in cell culture induces cell proliferation and may favor the persistence of KSHV. Transgenic mice expressing ORF74 develop Kaposi's sarcoma-like lesions (Arvanitakis et al. 1997; Yang et al. 2000). The possibility that expression of KSHV ORF74 may contribute to neoplasia through a paracrine effect has been proposed but may be difficult to demonstrate in KSHV-infected individuals. ORF74 is one of the best examples to illustrate the direct effect that viral immunomodulatory proteins may have on the pathology caused by viral infection. Four chemokine receptor homologues are encoded by HCMV: UL78, UL33, US27 and US28. MCMV encodes the viral chemokine receptors M33 and M78, which are homologous to their counterparts in HCMV. HCMV US28 can modify the chemokine environment of infected cells by sequestering and internalizing chemokines and depleting them from the medium (Bodaghi et al. 1998). Similarly, HHV-6 UL51 reduces extracellular accumulation of chemokines by sequestration, but also by a novel mechanism of down-regulation of CCL5 (RANTES) transcription (Milne et al. 2000). The potential contribution of viral chemokine receptors to pathology is also illustrated by the fact that HCMV US28 expression on the cell surface mediates cell adhesion, vascular smooth muscle cell migration, and it is thought to be related to vascular disease (Streblow et al. 1999). Evidence for a role for viral chemokine receptors in virus replication in vivo was demonstrated first for MCMV M33 (Davis-Poynter et al. 1997).

VIRAL CHEMOKINE BINDING PROTEINS (VCKBP)

The third class of viral chemokine inhibitors are the vCKBPs from poxviruses and herpesviruses that sequester chemokines in solution without apparent sequence similarity to host chemokine receptors (Seet and McFadden 2002; Smith et al. 2003) (Figure 4).

The MYXV M-T7 protein (vCKBP-1) is a soluble vIFN-γR with sequence similarity to human IFN receptors that was later found to interact also with chemokines. MYXV M-T7 binds a broad spectrum of chemokines and it has been proposed that it prevents the interaction of chemokines with GAGs and their correct presentation to leukocytes (Lalani et al. 1997). vCKBP-2 was identified in the medium from cultures infected with MYXV, VACV, or CPXV (Alcami et al. 1998; Graham et al. 1997; Smith et al. 1997). In this case, the protein binds CC chemokines with high affinity and neutralizes their effect by preventing the binding of chemokines to specific receptors in leukocytes. The protein M3 (vCKBP-3) encoded by murine gammaherpesvirus 68 (MHV-68) was found to bind a broad range of chemokines, including C, CC, CXC, and CX3C chemokines, and to block their interaction with cellular receptors (Parry et al. 2000; van Berkel et al. 2000). More recently, another family of vCKBPs has been identified in alphaherpesviruses (Bryant et al. 2003). gG is a membrane protein, present in the virions of some alphaherpesviruses, that is proteolytically cleaved and released to the extracellular space. Broad spectrum chemokine binding activity has been found in gG encoded by some alphaherpesviruses. This vCKBP blocks the interaction of chemokines to both specific chemokine receptors and GAGs.

The vCKBPs have amino acid sequences unrelated to chemokine receptors or other vCKBPs. Interestingly, the existing experimental data indicates that vCKBPs mimic the interaction of chemokines to their membrane receptors, in spite of the lack of amino acid sequence similarity (Alexander et al. 2002; Beck et al. 2001; Seet et al. 2001; Webb et al. 2003). The crystal structure of the poxvirus 35 kDa and MHV-68 M3 vCKBPs have been reported (Alexander et al. 2002; Carfi et al. 1999).

The generation of relevant virus mutants lacking specific vCKBPs and the infection of rabbits or mice suggest that these proteins may block chemokine-mediated leukocyte infiltration into infected areas in vivo (Graham et al. 1997; Lalani et al. 1999; Mossman et al. 1996; Reading et al. 2003; van Berkel et al. 2002). Interestingly, expression of the vCKBP-2 from VACV attenuates the virus in a murine intranasal model, probably due to a reduced inflammatory reaction to infection (Reading et al. 2003). In addition, MHV-68 M3 has been proposed to be required by the virus to establish virus latency and to enable persistence of the virus in the host (Bridgeman et al. 2001).

EVASION OF ANTIGEN PRESENTATION AND THE CELLULAR RESPONSE

The variability of viral antigens, discussed above in the context of the humoral response, is also relevant in the evasion of CTL recognition. Peptides derived from mutated viral proteins may lose their ability to bind to MHC molecules and be presented to the immune system. However, those viruses such as herpesviruses that establish infections for the life of the host but have a very low mutation rate must identify alternative mechanisms to persist in the presence of a vigorous immune response (Figure 5).

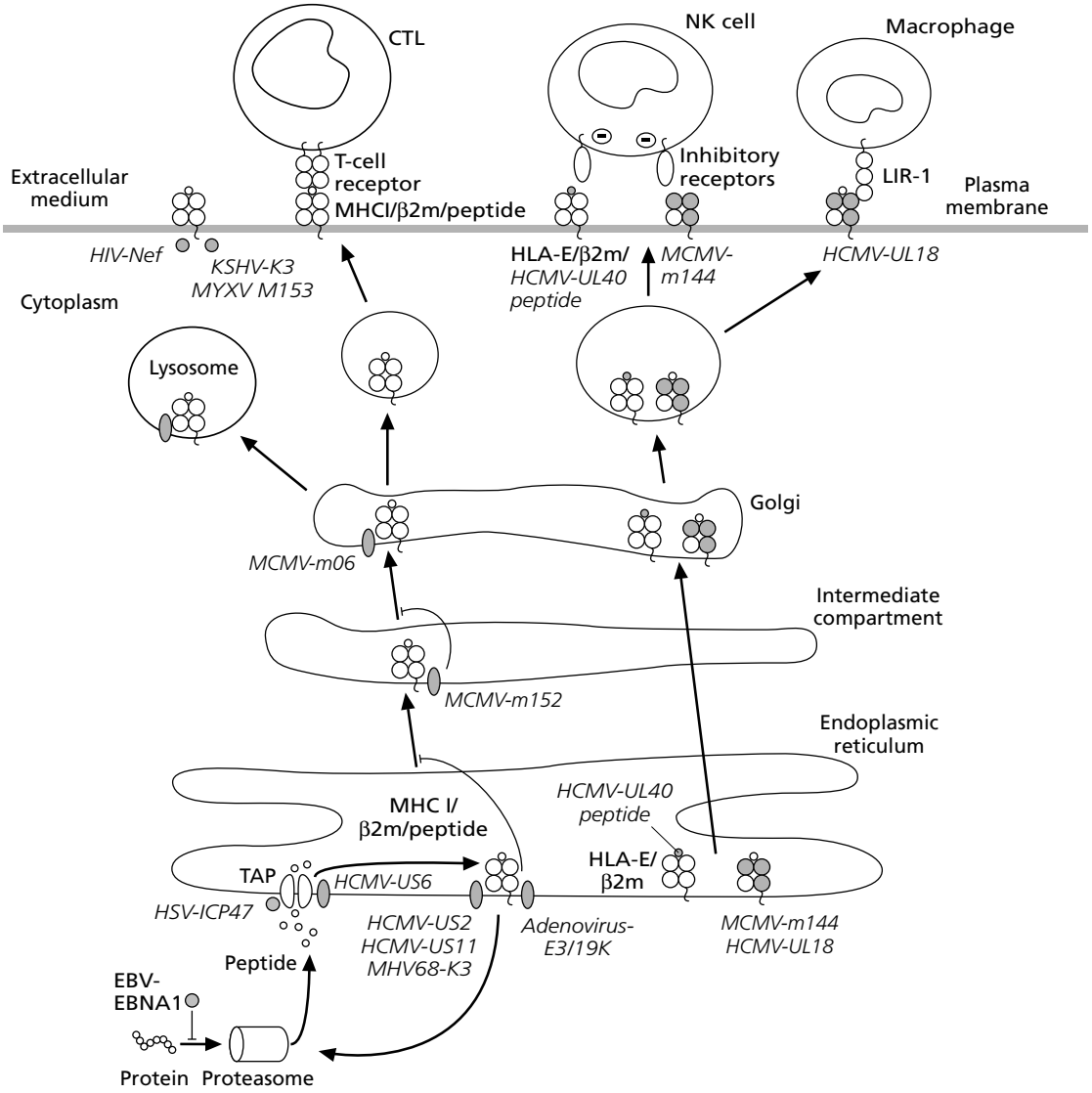

Figure 5 *Viral inhibition of antigen presentation and NK cell response*

Blockade of antigen presentation mediated by MHC class I molecules

MHC class I molecules display peptides at the cell surface derived from proteins expressed in the cell for recognition by the immune system (Figure 5). Viral proteins synthesized in the infected cell are degraded in the cytosol mostly by the proteasome, the major intracellular proteolytic complex, and the peptides generated are translocated to the lumen of the endoplasmic reticulum (ER) by transporters associated with antigen processing (TAP). The peptides associate with MHC class I molecules and β2-microglobulin to form a complex that is transported to the cell surface through the Golgi apparatus. The presentation of peptides derived from viral proteins activate specific CTLs that may destroy the infected cell. Herpesviruses are particularly well prepared with mechanisms that inhibit almost every step of the antigen presentation pathway (Figure 5) (Fruh et al. 1999; Johnson and Hill 1998; Yewdell and Hill 2002).

The nuclear antigen EBNA1 encoded by EBV has a long segment of repeated glycine and alanine residues that prevents protein degradation by the proteasome (Levitskaya et al. 1995). The HSV protein ICP47 and the HCMV US6 bind to TAP and inhibit peptide translocation from the cytosol to the ER lumen (Ahn et al. 1997; Hengel et al. 1997; Lehner et al. 1997; York et al. 1994). While ICP47 is a cytosolic protein, US6 is a transmembrane glycoprotein that binds TAP on the lumenal side of the ER. In the ER, MHC class I molecules associate with TAP as part of a peptide loading complex that includes tapasin. The adenovirus E3/19K protein binds TAP and interferes with the ability of tapasin to bring class I molecules and TAP together (Bennett et al. 1999).

The degradation of newly synthesized MHC class I molecules is an alternative mechanism. The HCMV US2 and US11 proteins are membrane glycoproteins that interact with lumenal domain of MHC class I molecules and cause them to be transported back to the cytosol, where MHC class I molecules are ubiquitinylated and destroyed by the proteasome (Wiertz et al. 1996a; Wiertz et al. 1996b). The molecular mechanism behind this immune evasion strategy has attracted much interest because it sheds light on an important transport system from the ER to the cytosol. The K3 protein of MHV-68 is a ubiquitin ligase that ubiquitylates the cytoplasmic tail of ER-resident class I molecules and targets them for destruction in the cytosol (Boname and Stevenson 2001). The related K3 and K5 proteins encoded by KSHV also targets class I, but in this case the viral proteins target cell surface class I molecules for degradation (see below).

Several viruses block trafficking of class I molecules to the cell surface. The adenovirus E3/19K protein and the m152 protein from MCMV block the transport of peptides complexed to MHC class I molecules and β2-microglobulin (β2m) at different levels (Jefferies and Burgert 1990; Ziegler et al. 1997). The m06 protein from MCMV binds MHC class I molecules and directs them to the lysosome for degradation, due to the presence of a di-leucine motif in its cytoplasmic tail (Reusch et al. 1999). HIV Nef removes MHC class I molecules from the cell surface and directs them to the trans-Golgi network, due to the interaction of Nef with the PACS-1 protein which sorts molecules to the trans-Golgi network (Piguet et al. 2000). The KSHV K3 and K5 proteins ubiquitylate MHC class I and this signals class I internalization from the plasma membrane for endosomal degradation (Hewitt et al. 2002). A related protein encoded by MYXV, named M153, has been shown to target class I molecules both at the cell surface and in the post-Golgi compartment for retention and degradation in the lysosome. MYXV M153 has also been implicated in the depletion from the cell surface of CD4, the coreceptor in MHC class II-restricted antigen-induced T-cell activation, and Fas, which triggers apoptosis after interaction with FasL (Guerin et al. 2002; Mansouri et al. 2003).

The significance of the viral interference with CTL function is not clear in the context of the infected host since most of these mechanisms have been identified in cell cultures in vitro. A modest role for MCMV m152 in CTL escape in vivo has been reported, but no major effect on the virus replication was observed (Krmpotic et al. 2002). In the case of MHV-68, a mutant virus lacking K3 grows normally in the initial lung infection but does not establish latency in the spleen, which is controlled by CTLs (Stevenson et al. 2002). The in vivo advantage of CTL escape mutants in simian immunodeficiency virus (SIV) and HIV expressing mutant CTL epitopes has been established (Allen et al. 2000; Goulder et al. 2001). Also, there is evidence that the ability of SIV Nef to down-regulate class I molecules is important in vivo (Munch et al. 2001).

Blockade of antigen presentation by class II MHC molecules

In contrast to MHC class I molecules, which present peptides derived from newly synthesized proteins, MHC class II molecules present peptides derived from exogenous antigens that are internalized and degraded in the lysosome. Only a few viral proteins have been found to block this pathway of antigen presentation. The US2 protein from HCMV induces the degradation of MHC class II molecules in the proteasome and HIV Nef interferes with the transport of MHC class II molecules to compartments where they associate with peptides before they reach the cell surface (Tomazin et al. 1999). HCMV US3 has also been shown to cause intracellular MHC class I retention (Hegde et al. 2002).

Evasion of the lysis of virus-infected cells by NK cells

NK cells constitute one of the first lines of defense against viruses. These cells posses specific activating receptors that recognize carbohydrate at the cell surface and induce their cytolytic activity. The NK cells also express other receptors, known as inhibitory receptors, that recognize MHC class I molecules and transduce a signal that blocks their cytotoxic machinery. In this way, NK cells recognize healthy cells expressing normal levels of MHC class I molecules and destroy cells that do not express class I molecules. The down-regulation of MHC class I molecules by some viruses prevents the specific recognition of the infected cell by CTLs, but may have dramatic consequences by increasing the susceptibility of virus-infected cells to NK cell lysis (Orange et al. 2002).

To solve this problem, some viruses encode homologues of MHC class I molecules (Figure 5). The MCMV protein m144 forms a complex with β2-microglobulin and is expressed at the cell surface where it interacts with NK cells and induces inhibitory signals that prevent the lysis of the infected cell (Farrell et al. 1997). A virus lacking m144 is attenuated in mice but is similar to wild-type virus in mice depleted of NK cells, suggesting that m144 controls NK activity in vivo. The role of the HCMV protein UL18, a class I homologue, in evasion of NK cell lysis is controversial (Reyburn et al. 1997). The demonstration that HCMV UL18 interacts with a receptor in macrophages suggested that these class I homologues may also modulate the activation of other immune cells that play a critical role against viral infections (Cosman et al. 1997). MOCV, unlike other poxviruses, establishes persistent infections in the skin

and encodes an MHC class I homologue of unknown function (Senkevich and Moss 1998).

HLA-E is an MHC class I molecule that presents peptides derived from signal sequences from other MHC class I molecules, interacts with an inhibitory receptor in NK cells, NKG2A, and prevents lysis mediated by NK cells (Figure 5). In this way, HLA-E 'informs' the NK cells on global levels of MHC class I expression in the cell. To compensate for the low levels of MHC class I molecules in the infected cell, HCMV encodes a signal peptide, present in the UL40 protein, that specifically binds HLA-E. In this way, normal levels of HLA-E-peptide complexes at the cell surface are present in the infected cell and protect it from NK cell attack (Tomasec et al. 2000).

The MCMV protein m152 interferes with class I antigen presentation (see above). In addition, it was found that m152 also reduces the cell surface levels of the class I-like molecules RAE and H60, which serve as ligands for the NK cell activating receptor NKG2D (Krmpotic et al. 2002). An MCMV mutant lacking m152 was more susceptible to NK cell lysis.

A soluble form of HCMV UL16 binds to a family of human glycosylphosphatidylinositol-linked receptors and MHC class I-related proteins know as UL16 binding proteins (ULBP). The NK cell activating receptor NKG2D binds ULBPs, and this interaction is blocked in the presence of soluble UL16 (Cosman et al. 2001). These data suggest a mechanism by which UL16 may inhibit NK cell function.

Viral inhibition of dendritic cell (DC) functions

Antigen presenting cells (APC), usually DCs, capture antigen at the site of infection and migrate to the lymph nodes where they interact and activate naïve T cells. The T cells acquire a mature phenotype and can migrate to infected tissues where they can activate effector functions with antiviral properties, such as cytolytic activity or the release of antiviral cytokines. The inhibition of DC function by viruses is predicted to have a major impact in antiviral immunity.

By infecting DCs viruses may interfere with class I MHC processing and presentation would inhibit the role of DCs in immunity. A number of reports have shown that some viruses interfere with the maturation of DCs, which is necessary for DCs to present antigens to T cells and to activate them. DCs infected by MeV inhibit rather than promote the proliferation of T cells and express the apoptosis inducing ligand TRAIL (Grosjean et al. 1997; Vidalain et al. 2000). The interference of MeV with DC function is thought to be largely responsible for the generalized immunosuppression associated with MeV infection. Inhibition of DC maturation has been reported for HSV, MCMV, and HCMV (Andrews et al. 2001; Moutaftsi et al. 2002; Salio et al. 1999).

APOPTOSIS

Apoptosis or programmed cell death can be activated in several ways, including by TNF superfamily members, irradiation, inhibitors of cell cycle or infectious agents such as viruses. The sequential activation of a family of proteases known as caspases plays a central role in the control of the apoptotic response (Figure 6). Apoptosis can be considered an innate mechanism of defense against viral infection that limits virus replication. In addition, CTLs and NK cells destroy virus-infected cells by inducing apoptosis as a result of the production of cytokines such as TNF, the activation of Fas at the cell surface by FasL or the secretion of perforin that enables other secreted proteins (granzymes) to enter the cell. Although the apoptotic response limits virus replication when activated at early times of infection, the induction of apoptosis may also favor the release of virus particles and viral dissemination within the host under some circumstances. Many cellular proteins involved in the control of apoptosis are targets of viral anti-apoptotic mechanisms (Figure 6) (Benedict et al. 2002; Everett and McFadden 1999).

Blockade of signaling through TNFR and Fas

The soluble vTNFRs encoded by poxviruses, discussed above, prevent the binding of TNF to its receptor and the induction of TNF-mediated apoptosis (Alcami 2003; McFadden and Murphy 2000; Seet et al. 2003) (Figure 6). The interaction of TNFRs and Fas with their respective ligands induces the binding of cytoplasmic factors that interact through death domains (DD) and death effector domains (DED) present in adaptor proteins, such as TRADD (TNF-receptor-activated death domain) and FADD (Fas-associated death domain), and in some caspases. These factors initiate a cascade of interactions that culminate with the activation of caspases and the induction of apoptosis. MOCV and KSHV encode vFLIPs (FLICE or caspase 8-inhibitory proteins) that have death domains and prevent signal transduction through receptors for TNF and FasL (Bertin et al. 1997; Thome and Tschopp 2001). The HCMV *UL36* gene product vICA associates with caspase 8 and blocks its activation, but its sequence is unrelated to FLIPs (Skaletskaya et al. 2001). Adenoviruses produce a number of proteins encoded by the E3 region that inhibit TNF- and Fas-mediated apoptosis (Wold et al. 1999). The adenovirus receptor internalization and degradation (RID) complex, formed by the E3/10.4K and E3/14.5K proteins, induces internalization and subsequence degradation in lysosomes of TNFR and Fas. As a result of this, the infected cell becomes resistant to TNFR- and Fas-mediated apoptosis (Burgert et al. 2002; McNees and Gooding 2002).

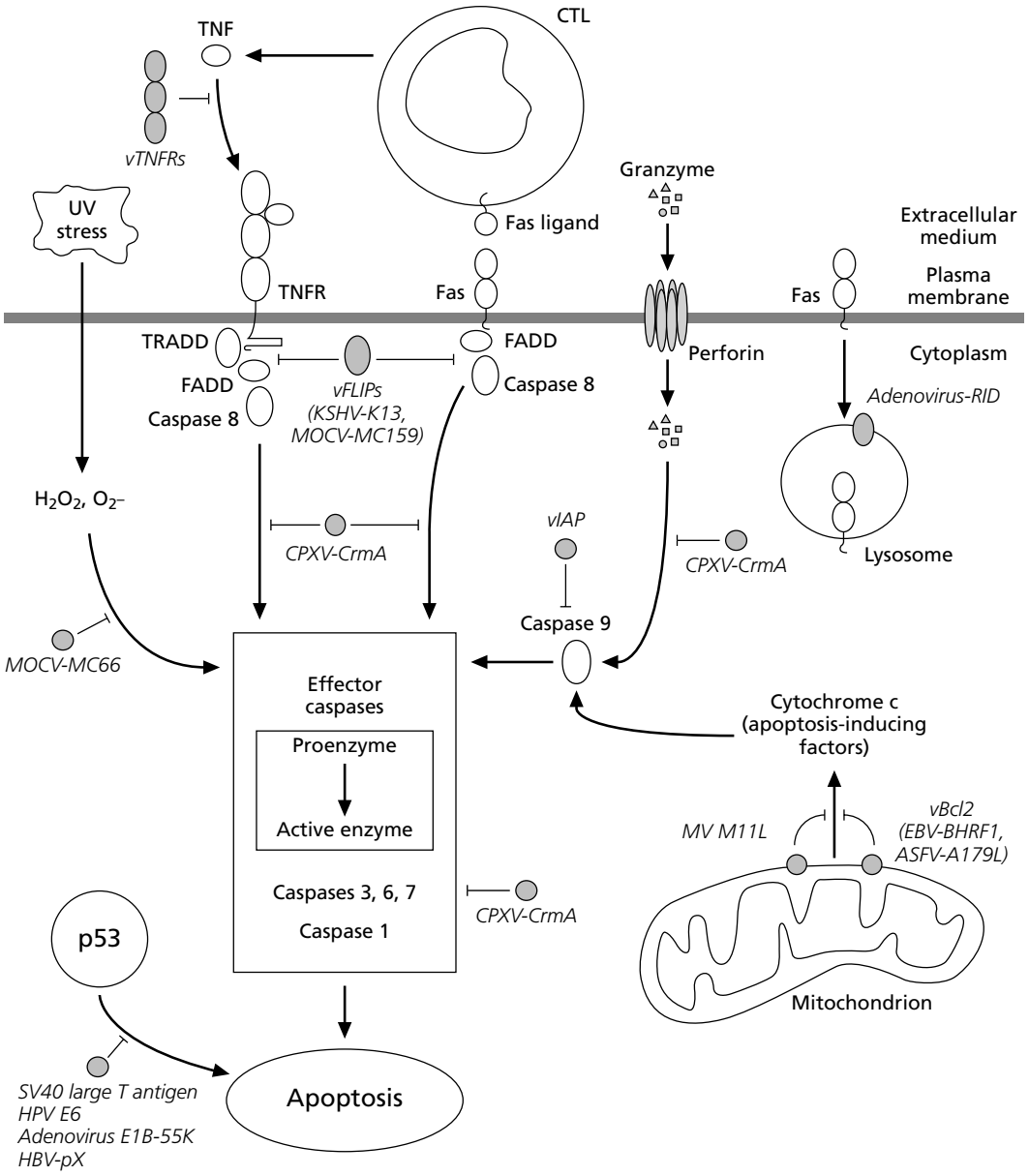

Figure 6 *Viral inhibition of apoptosis*

Inhibition of caspases

Caspases are proteolytic enzymes that are critical for the induction of apoptosis, and many viral proteins have been described to inhibit their activity (Figure 6). The CrmA protein encoded by CPXV, VACV and other poxviruses blocks caspase 1 and caspase 8, which play critical roles in major pro-apoptotic pathways, and prevents apoptosis initiated by several stimuli including TNF, FasL, and granzyme B (Miura et al. 1995; Talley et al. 1995; Tewari et al. 1995). In addition, CrmA inhibits the proteolytic maturation of IL-1β and, presumably, IL-18, cytokines that are cleaved by caspase 1 (Ray et al. 1992). The enzymatic activity of caspases is regulated by a family of inhibitor of apoptosis proteins (IAP) that were originally identified in baculoviruses, a family of insect viruses (Clem and Miller 1994). IAP homologues are not frequently found in mammalian viruses, with the exception of ASFV (Neilan et al. 1993). The adenovirus E3-14.7K protein inhibits caspase 8 through interactions with a small GTPase and dynein (Lukashok et al. 2000).

Inhibition of mitochondrial pathways of caspase activation

Apoptosis can also be controlled at the level of mitochondria, as a result of loss of extracellular viral signals or by signals affecting the cell cycle (Rathmell and Thompson 2002). The release of cytochrome c, which

resides in the space between the inner and outer membranes of the mitochondria, oligomerizes APAF1 and recruits pro-caspase 9 forming a protein complex known as the apoptosome and activates caspase 9 leading to apoptosis. Bcl2 is a cellular protein that resides in the outer mitochondrial membrane and inhibits the release of cytochrome c from the mitochondria. A number of viruses encode Bcl-2 homologues to prevent the induction of apoptosis, including adenovirus, ASFV, and the gammaherpesviruses EBV, KSHV and MHV-68 (Afonso et al. 1996; Cheng et al. 1997; Gangappa et al. 2002; Henderson et al. 1993; Sarid et al. 1997) (Figure 6). The HCMV protein UL37 shares no sequence similarity to Bcl-2 but resides in the mitochondria and is functionally similar to Bcl-2 (Goldmacher et al. 1999). Recently, the MYXV protein M11L has been shown to closely associate with a component of the mitochondrial permeability transition pore and to prevent apoptosis (Everett et al. 2002). A related protection of mitochondrial membrane potential has also been observed with VACV, but the viral protein responsible for it has not been identified (Wasilenko et al. 2001).

Other anti-apoptotic mechanisms

The tumor suppressor p53 limits cell proliferation by inducing apoptosis or cell cycle arrest. p53 stimulates transcription of genes involved in apoptotic pathways, and viruses can disrupt apoptosis by inactivating p53 (Figure 6). The simian virus 40 (SV40) large T antigen sequesters p53 in an inactive complex (Lane and Crawford 1979; Linzer and Levine 1979). The human papillomavirus (HPV) E6 protein and adenovirus E1B-55K protein promote ubiquitylation and degradation of p53 by different mechanisms (Scheffner et al. 1990; Steegenga et al. 1998; Werness et al. 1990). The hepatitis B virus (HBV) pX protein binds p53 and inhibits p53-mediated apoptosis (Wang et al. 1995). An alternative mechanism is represented by the glutathione superoxide MC66 encoded by MOCV which protects infected cells from peroxide radicals and ultraviolet radiation, and perhaps from peroxide radicals induced by TNF, macrophages, and neutrophils (Shisler et al. 1998).

CONCLUSIONS AND FUTURE PERSPECTIVES

In the past, viral immunology studies have characterized the host mechanisms to prevent and eliminate viral infections. The ability of many viruses to evade the host immune response by antigenic variation was shown many years ago. However, it is in the last ten years that we have become more aware of the many viral proteins that have evolved to block host specific mechanisms of antiviral immunity. There are numerous viral proteins without an ascribed function and it is likely that many of these proteins encode new viral immune evasion strategies, which may only be evident in the infected host. The fact that we have not identified these viral functions may reflect our limited knowledge of the molecular mechanisms of immunity.

The reasons why a virus blocks a particular immune mechanism is not clear, and may depend on the replication and transmission mechanisms adopted by the virus. Many questions need to be resolved in the future. We need to determine which viral genes are required for the initial steps of the infection or for efficient transmission to another host in the presence of an active immune system. The apparent complexity and redundancy of these viral proteins may only be understood in the context of viral infection in the host. The lack of good animal models of infection for many viruses is limiting our understanding of the function of viral immune evasion strategies in vivo.

A better knowledge of the interaction of viruses with the host defense mechanisms will help us to understand the mechanisms of viral pathogenesis. Very often, the pathology observed in viral infections is not due to viral replication but a consequence of the immune and inflammatory responses to infection that may cause damage in the host. Moreover, some viral proteins may directly cause the pathological effects during infection. Some human inflammatory and autoimmune diseases are the result of an uncontrolled production of immune mediators such as TNF and IL-1 that constantly activate the immune response. We are finding examples of viral proteins that may enhance these immunopathological responses in the infected host.

The deletion of viral immune evasion proteins from the viral genome causes viral attenuation in most cases due to increased host defenses. However, enhanced viral virulence has been observed in VACV and CPXV when some of these viral functions have been deleted from the genome. These include the soluble vIL-1βR, IMP, and the 35 kDa vCKBP (Alcami and Smith 1992; Miller et al. 1997; Reading et al. 2003). These surprising observations suggest that the function of some of the viral immune evasion proteins is not to promote viral replication but to 'protect' the host from the detrimental effects of an over-reactive immune system, mediated in these examples by IL-1β, complement or chemokines, respectively. An enhanced host survival will be beneficial since the virus fully depends on the host for its replication. The complex interaction of each virus with the immune system is unique, and a better understanding of this interaction will lead to new strategies to control viral replication and the design of more efficient viral vaccines.

The fact that viruses have co-existed with their hosts during evolution and are able to survive in the presence of potent host defense mechanisms suggests that viruses know the mechanisms of immunity better than us. Viruses point at those molecules that play a critical role

in defense against pathogens, one of the primary aims of the immune system, and thus viruses offer us a unique opportunity to identify basic mechanisms of immunity. Moreover, viruses may help us to understand the function of some of the large number of human genes recently identified in the human genome. The study of viral mechanisms of immune evasion is fascinating and will increase our understanding of the complex interaction of viruses with the host immune system.

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Viral oncogenicity

JAMES C. NEIL AND JOHN A. WYKE

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INTRODUCTION

A brief history of tumor virology

The incidence of tumors in animals and humans often shows geographical variation. This clustering sometimes reflects genetic differences in the populations at risk but more often results from environmental factors that vary between populations. Features of the environment that enhance the risk of tumor development (i.e. carcinogens) comprise physical factors (such as ultraviolet and other irradiation that impinges on and penetrates the body), chemical factors (ingested or contaminating the body surface), and infectious agents. Of the last, the most important are viruses. Viruses that produce tumors in animals have been known since the early 1900s, but it is only in recent decades that they have excited great interest. The reasons for this are twofold.

First, as diverse viruses were increasingly implicated as causes of cancer in animals of all vertebrate classes, it was hoped that they might also be important in the etiology of human neoplasia. This concept was attractive, not least because it suggested that, if the causative organisms could be identified and characterized, the incidence of cancer might be reduced by the prophylactic measures so successful against other infectious agents. The development of a tumor is usually a rare outcome of virus infection but it has nevertheless been estimated that up to 20 percent of human tumors have a viral risk factor (zur Hausen 1991) (Figure 1). Only one family of RNA-containing viruses, the retroviruses, has well-established oncogenic members (Table 1), but representatives of all the major subdivisions of DNA-containing viruses (except the parvoviruses) in vertebrates have been implicated in neoplasia. Examples are given in Tables 2 and 3, p. 3, but, because all these agents receive attention elsewhere in this volume, this chapter will concentrate on general principles rather than specific details.

The second reason for an interest in viruses was their promise as tools for studying the basic mechanisms underlying neoplastic change. This idea rested on several earlier developments in laboratory research. Inbreeding of laboratory animals produced some strains with high incidence of various virus-associated neoplasms. In addition, inbred strains permitted transplantation studies, which demonstrated that a single donor cell could produce a tumor in the recipient animal. This finding focused attention on changes that occurred at a subcellular level and provided the rationale for studying oncogenesis in vitro rather than in the whole animal. This switch in emphasis was facilitated by the development of tissue culture techniques and, at the same time, it was found that tissue culture was an ideal way to measure the cytopathic effects of viruses, so that animal virologists could adopt the quantitative approach developed in studies on bacteriophages.

A correlation soon emerged between the ability of viruses readily to cause solid mesenchymal tumors in animals and their induction of morphological transformation of cultured cells (Figure 2, p. 4). This finding had important implications, not widely appreciated at the time. Following Theodor Boveri's proposals in 1914, the notion that tumors were clones of cells whose altered

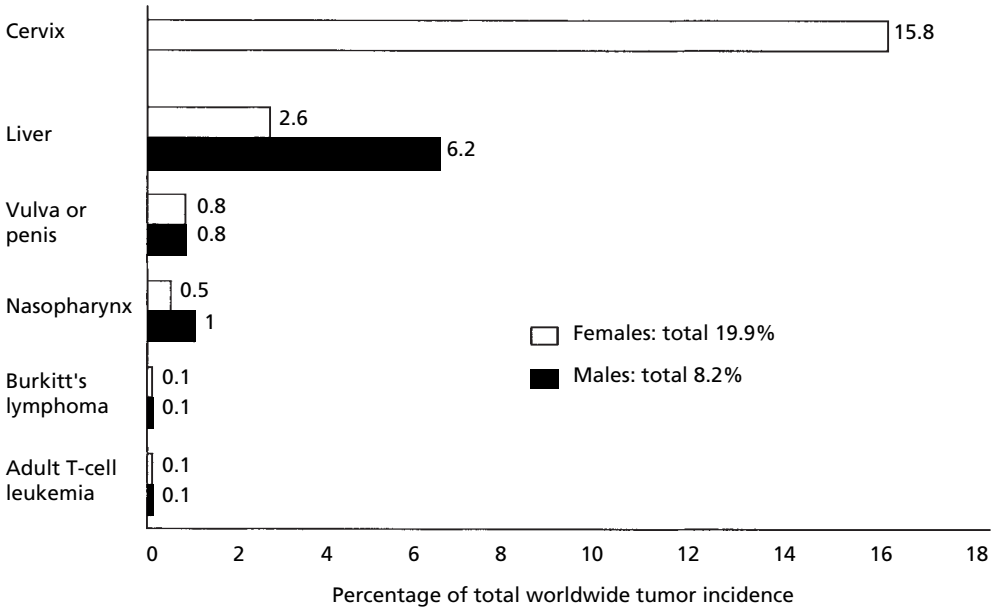

Figure 1 *Incidence of human tumors in which virus infection is a risk factor. The viruses most strongly implicated are papillomaviruses – tumors of cervix and external genitalia (Papillomaviruses); HBV – primary liver tumors (Hepatitis delta virus); EBV – Burkitt's lymphoma and nasopharyngeal carcinoma (Gamma herpesviruses: Epstein–Barr virus); and human T-cell leukemia viruses – adult T-cell leukaemia (Retroviruses and associated diseases in humans). (Modified, with permission, from zur Hausen 1986.) Note that these estimates do not include recent changes in cancer incidence due to HIV infection (Retroviruses and associated diseases in humans and 60, Human immunodeficiency virus).*

properties result from accumulated mutations was accepted by many scientists (Baltzer 1964). However, before molecular genetic technology was developed, they could not conceive ways of identifying, among the thousands of genes in every somatic cell, the putative subsets that were mutated. Tumor viruses seemed to offer a way round this impasse. Some of them possessed only a few genes among which, it was argued, were those that

Table 1 *Some RNA viruses implicated in cancers of animals and humans*

Virus classification	Virus	Associated tumors	Other risk factors
Retroviridae			
Alpharetroviruses	ALVs; Rous sarcoma virus	Various sarcomas, some carcinomas, lymphomas, and leukemias	Genetic susceptibility affecting virus penetration replication and spread
Betaretroviruses	MMTV	Mammary adenocarcinomas, T-cell lymphoma	Pregnancy (altered hormone levels)
	JSRV	Pulmonary adenoma	. . .
	Mason–Pfizer monkey virus	Fibrosarcomas	. . .
Gammaretroviruses	MLV, murine sarcoma virus	Lymphomas, leukemias, and sarcomas	Genetic susceptibility to viral infection and spread
	Reticuloendotheliosis virus (avian)	Lymphomas and leukemias	. . .
	FeLV, feline sarcoma virus	Lymphosarcomas (mainly T cell), myeloid leukemias, fibrosarcomas	Age at exposure, immunosuppression
Deltaretroviruses	HTLV-I, HTLV-II	Adult T-cell leukemia	. . .
	BLV	Lymphosarcoma (B cell)	. . .
Lentiviruses	HIV	B lymphomas, Kaposi's sarcoma	EBV, KSHV
	Simian immunodeficiency virus	B lymphomas	Simian herpesvirus
	Feline immunodeficiency virus	B lymphomas	FeLV
Flaviviridae	HCV	Hepatocellular carcinoma	. . .

ALV, avian leukosis virus; BLV, bovine leukemia virus; BPV, bovine papillomavirus; EBV, Epstein–Barr virus; FeLV, feline leukemia virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; HTLV, human T-cell leukemia virus; HVS, herpesvirus saimiri; JSRV, jaagsiekte sheep retrovirus; MLV, murine leukemia virus; MMTV, mouse mammary tumor virus; SIV, simian immunodeficiency virus; WHV, woodchuck hepatitis virus.

Table 2 *Some oncogenic small DNA viruses of animals and humans*

Virus family	Virus	Host of origin	Associated tumors	Other risk factors
Hepadnaviridae	Hepatitis B group	Humans, apes, rodents, ducks	Primary hepatocellular carcinoma	In humans: alcohol, smoking, fungal toxins, other viruses
Papovaviridae	Polyoma	Mouse	Various carcinomas and sarcomas	. . .
	SV40	Monkey	Sarcomas, mesothelioma (in rodents, man?)	. . .
	BK and JC	Human	None in humans; neural tumors in rodents and monkeys	. . .
	Papilloma	Human	Genital, laryngeal and skin warts; may progress to:	. . .
			Cervical carcinoma	Smoking, herpes simplex viruses, immunosuppression
			Laryngeal carcinoma	X-irradiation, smoking
			Skin carcinoma	Sunlight, genetic disorders
		Cattle	Genital, alimentary, skin warts; may progress to:	. . .
			Alimentary carcinoma	Carcinogens and immunosuppressants in bracken fern
			Skin carcinoma	Sunlight, genetic predisposition (lack of pigmentation)
		Other mammals	Papillomas; may progress to carcinomas	Experimentally, carcinogens such as methylcholanthrene

transformed cells in vitro and induced tumors in vivo. Identification of these viral cancer genes (oncogenes) should help define the molecular basis of cancer, because they might have functional analogues among the cellular genes required for neoplasia. Although naive in hindsight, this concept proved correct for the retroviruses used widely by laboratory workers. Indeed, for the retroviruses, its significance exceeded expectations, for in many cases the viral oncogenes were descended from cellular cognates, the proto-oncogenes. These, in turn, have been convincingly implicated in the genesis of a range of animal and human tumors of both viral and nonviral etiology. The

Table 3 *Some oncogenic large DNA viruses of animals and humans*

Virus family	Virus	Host of origin	Associated tumors	Other risk factors
Adenoviridae	Types 2, 5, 12	Human	None in humans; sarcomas in hamsters	. . .
Herpesviridae	Frog herpesvirus	Leopard frog	Adenocarcinomas	Ambient temperature
	Marek's disease	Fowl	Neurolymphomatosis (T cell)	Genetic predisposition, MHC and non-MHC loci
	Herpesvirus ateles and saimiri	Monkeys	Lymphoma, leukemia	. . .
	EBV	Human	Burkitt's lymphoma, Hodgkin's disease, immunoblastic lymphoma	Malaria, HIV, immunodeficiency
			Nasopharyngeal carcinoma	Salted fish in infancy, HLA type
	Herpes simplex (types 1and 2)	Human	Cervical neoplasia	Papillomaviruses, smoking, immunodeficiency
	Cytomegalovirus	Human	Cervical neoplasia	Immunodeficiency, HLA type
	Kaposi's sarcoma herpesvirus (KSHV/HHV8)	Human	Kaposi's sarcoma, primary effusion lymphoma/ Castleman's disease	HIV
Poxviridae	Shope fibroma	Rabbit	Fibroma	. . .
	Yaba monkey tumor virus	Monkey	Nodular fibromatous hyperplasia	Not relevant; progressive tumors never develop
	Molluscum contagiosum	Human	Nodular epidermal hyperplasia	. . .

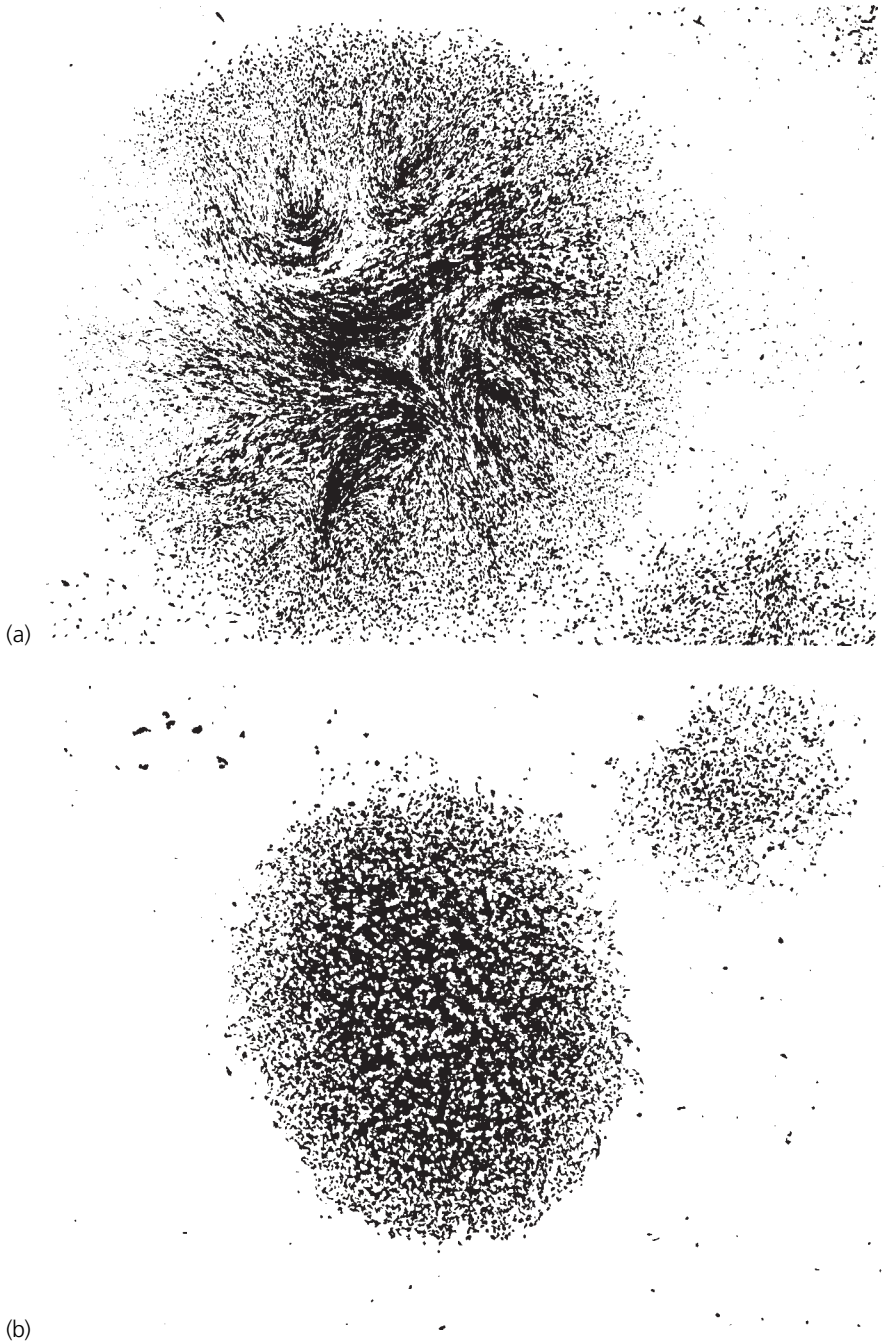

Figure 2 *The morphological appearance of colonies of normal and transformed cells in tissue culture (×20).* **(a)** *A colony of BHK-21/C13, a cell line derived from Syrian hamster kidney. Note that the colony is flat and the cells are lined in parallel array.* **(b)** *The same cell line after transformation by* Polyomavirus. *The cells are piled on one another and lack orientation.*

DNA tumor viruses have also provided vital insights into the cancer process because their oncogenes encode proteins that interfere with cellular checkpoint controls of proliferation and survival which have in a similar fashion proved to be of general relevance in human cancer.

These concepts gave rise to a large amount of work, reviewed elsewhere (Bishop 1991; Vogelstein and Kinzler 1992). This chapter summarizes in turn the two broad divisions of tumor virology:

1. studies on the molecular mechanisms of virus-induced neoplasia
2. attempts to implicate viruses in the aetiology of cancer.

These divisions, however, share concepts and techniques, and are increasingly considered together (Minson et al. 1994). Such a unitary approach is particularly important when attempting to combat virus-associated cancer, as outlined in the third part of this chapter.

Paradoxically, the tumor viruses themselves are now being developed as part of our anticancer armory.

Cancer – a disorder of cellular controls

The development of a fertilized egg into a multicellular organism and the subsequent maintenance of its form and function require complex controls over cell proliferation and cell death, and the expression of differentiated cell functions. These attributes of cells result from an interplay between stimuli from outside the cell and its current program of activity. Many of these stimuli are mediated by chemical signals classified according to their origin; endocrine signals originate from distant cells, paracrine signals from cells in the neighborhood of the recipient, and autocrine signals from the recipient cell itself. Whatever their origin and consequence, these signals have an effect only if the recipient cell has appropriate cell surface or internal receptors for the signal, an appropriate machinery of second messengers to process it and the metabolic capacity to respond.

Perturbations in these controls can lead to local increases in tissue mass. These may be the response of a normal cell to abnormal stimuli, leading to cell hypertrophy or, if the cells can multiply, to hyperplasia. On the other hand, the cells themselves may be altered, so their growth and behavior are no longer controlled in the same way as their normal counterparts and they become neoplastic. We might expect neoplasia to result from a change in any of the components of the system by which the cell responds to external signals, and there is now considerable evidence that in neoplastic cells mutations exist that change the function of various components in the signaling pathway controlling cell proliferation. As we shall see, viral oncogenes frequently act to stimulate growth in this way.

However, the oncogenes represent only one facet of the genetic basis of cancer. The requirement for recessive mutations in cancer was long suspected, both from the occurrence of inherited cancer syndromes (Knudson 1993) and from classic in vitro cell fusion studies where the transformed phenotype was often found to be extinguished in hybrid cells (Harris 1985). It was suspected that the genes underlying these phenomena might play a role in commitment to terminal differentiation, with loss of function leading to a block at an immature, highly proliferative stage. Although there is evidence to support this hypothesis for a number of genes, the most widely implicated tumor suppressor genes appear to play quite different roles in controlling cell cycle progression ('gatekeepers') or in maintaining the integrity of the genome ('caretakers') (Shields and Harris 2000). Given the fundamental nature of these roles, the discovery that the archetypal tumor suppressor, p53, is dispensable for normal development was a surprise to the field. Mice lacking functional p53 are ostensibly normal, but are prone to develop tumors, particularly lymphomas (Donehower et al. 1992). In its normal physiological role, p53 orchestrates cellular responses to genotoxic damage; induction of p53 leads to cell cycle arrest and allows repair synthesis to occur. Whilst this phenomenon is a partial explanation for the protective role of p53, there is another important facet to the story. Cells in which p53 is induced may also undergo programmed cell death or apoptosis as a consequence (Yonish-Rouach et al. 1991). This cell suicide mechanism, which plays vital roles in normal development and morphogenesis, also serves as a brake to cancer development. For cancer to develop, it seems that cells must acquire mutations which block cell death as well as those which drive cellular proliferation (Liebermann et al. 1995).

In practice, the simple binary classification of cancer genes as oncogenes or tumor suppressors is no longer tenable as some genes display facets of both, with gain or loss of function of a single gene contributing to tumor development in different cellular contexts (Lee and Farnham 2000; Wotton et al. 2002). The p53 protein itself is functionally complex, as some mutant forms act as dominant negative inhibitors of the wild-type counterpart, hence acquiring oncogene-like properties (Vogelstein and Kinzler 1992). Also, certain oncogenes (e.g. c-*myc*) actually induce growth stasis or apoptosis instead of proliferation when overexpressed in a cellular environment unfavorable for growth (Evan et al. 1992). Such mechanisms serve to protect multicellular organisms from cancers and explain the need for multiple genetic events to transform normal cells to full malignancy.

THE PATHOGENESIS OF TUMOR VIRUSES

Reasoning from first principles, we can envisage many ways by which features of virus infection might favor the development of tumors. The interactions between viruses and their hosts are such that it is clear that most of these mechanisms exist in one or another naturally occurring cancer. Indeed, the genesis of a given neoplasm may be influenced by more than one mechanism, because they are not mutually exclusive.

Direct oncogenic mechanisms

A first major category of pathogenic mechanism is direct or intrinsic, in which the tumor cells, or at least their ancestral lineage, are infected by the causative virus. Although this mechanism was presumed to be involved in studies of tumor viruses in vitro, only one aspect of it was useful to the experimenter – that in which all or part of the virus genome persisted in the tumor lineage. Genome persistence provides a marker that pinpoints crucial events in tumorigenesis, either because a specific portion of the virus is always present or because the

genome is inserted in a specific region of the host chromosome DNA.

The consistent presence of a viral gene, either free in the cell or integrated randomly in host DNA, suggests that it directly contributes to the neoplastic phenotype. These viral oncogene functions can supersede those of the cell and invariably mediate either oncogenicity by the virus or its presumed counterpart in vitro, cell transformation (see Figure 2).

THE RETROVIRAL ONCOGENES

The oncogenes of most retroviruses play no part in virus replication. Indeed, many replace portions of viral replicative genes in the virus genome. These oncogenes (v-*onc*) are related to sequences in normal host cells from which they are believed to evolve after capture (transduction) of the cellular gene, known as a proto-oncogene (c-*onc*), during some ancestral infection (Table 4). Such transduction is observed in certain naturally occurring tumors; however, because it is sporadic, and because oncogene transduction appears to confer no advantage on the virus for transmission (Onions et al. 1987), it appears to represent an evolutionary dead-end. However, for the 'simple' retroviruses such as the alpha and gammaretroviruses, de novo oncogene transduction can in some circumstances be observed in a high proportion of tumors (Neil et al. 1984; Miles and Robinson 1985; Tsatsanis et al. 1994). Transduction has major implications for understanding the mechanism of neoplasia because, if cell proto-oncogenes can mediate neoplasia when incorporated within a virus genome, they might also be able to do so in other circumstances. A considerable body of evidence now suggests that this is the case, some of the earliest data coming from work on the mode of tumor virus pathogenesis described next.

Since the first glimpses of oncogenes in rapidly transforming retroviruses, it has been apparent that their products can be demonstrated in many different cellular

Table 4 *Examples of genes transduced by retroviruses in cancer*

Viral oncogene	Location, structural features of gene product	Normal function of host-cell gene[a]	Transducing virus[b]	Associated tumors
v-*sis*	Secreted growth factor	B chain PDGF	GaLV/FeLV	Sarcoma
v-*erb*-B	Transmembrane receptors	EGF receptor	ALV	Erythroblastosis
v-*kit*		SCF receptor	FeLV	Fibrosarcoma
v-*fms*		CSF-1 receptor	FeLV	Fibrosarcoma
v-*ros*			ALV	Fibrosarcoma
v-*Notch1*			FeLV	T-cell lymphoma
v-*mpl*		Thrombopoietin receptor	MLV	Myeloproliferative disease
v-*sea*			ALV	Erythroblastosis
v-*tcr*		T-cell receptor β-chain	FeLV	T-cell lymphoma
v-*abl*	Plasma membrane tyrosine kinases		MLV/FeLV	B-cell leukemia, fibrosarcoma
v-*fes*/v-*fps*			FeLV/ALV	Fibrosarcoma
v-*fgr*			FeLV	Fibrosarcoma
v-*ryk*			ALV	. . .
v-*src*			ALV	Fibrosarcoma
v-*yes*			ALV	Fibrosarcoma
v-Ha-*ras*	Plasma membrane G proteins		MLV	Sarcoma
v-Ki-*ras*			MLV	Sarcoma
v-*mos*	Cytoplasmic serine/threonine kinase		MLV	Sarcoma
v-*raf*/v-*mil*			MLV/ALV	Sarcoma, carcinoma
v-*crk*	Cytoplasmic adapter		ALV	Sarcoma
v-*erb*-A	Cytoplasm/nucleus	Thyroid hormone receptor	ALV	Erythroblastosis
v-*maf*	Nuclear transcription factors		ALV	Fibrosarcoma
v-*myb*			ALV	Myeloblastosis
v-*myc*			ALV, FeLV	Myelocytomatosis, lymphoma
v-*qin*			ALV	Fibrosarcoma
v-*rel*		NFκB transcription factor	REV	Reticuloendotheliosis
v-*fos*		AP-1 transcription factor	MLV	Sarcoma
v-*jun*		AP-1 transcription factor	ALV	Sarcoma

a) CSF-1, colony stimulating factor type 1; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; SCF, stem cell factor.
b) ALV, avian leukosis virus; FeLV, feline leukemia virus; GaLV, gibbon ape leukemia virus; MLV, murine leukemia virus; REV, reticuloendotheliosis virus.

sites; their functions, where elucidated, identify these as components of pathways of growth control signaling and response. The captured versions of these cellular genes are released from normal transcriptional and translation controls when expressed as part of the viral genome. Furthermore, if the viral version of the gene is truncated or mutated, functional controls may be lost. Thus, retroviral oncogenes are in some cases altered forms of growth factors (e.g. v-*sis*), transmembrane growth factor receptors (some of which are protein tyrosine kinases such as v-*erb*-B) or 'G proteins' that couple ligand–receptor interactions to intracellular signaling events (e.g. the *ras* gene family). Others are second messengers, such as the intracellular protein tyrosine kinases and serine–threonine kinases. The oncogenes whose products are located in the nucleus can affect DNA synthesis or, more probably, gene expression; examples are v-*jun* and v-*fos*, which encode nuclear proteins that assemble as heterodimers to form a well characterized transcription factor complex (AP-1). It is easy to appreciate that mutations altering the expression of one of these genes, or their products, might short-circuit normal cell controls and lead to unregulated growth and behavior. However, it remains a major challenge to understand the detailed mechanism of many of these perturbations. For a comprehensive listing of the known oncogenes and their properties, the reader is referred elsewhere (Hesketh 1997).

The leukemogenic deltaretroviruses bovine leukemia virus (BLV), human T-cell leukemia viruses (HTLV) (HTLV-I, HTLV-II), and simian T-cell leukemia virus (STLV-I) form a discrete subgroup where a normal constituent of the viral genome is implicated in transformation. They contain a region, called p*X*, which encodes Tax and Rex, along with a series of minor gene products. The Tax protein is the most clearly implicated in the process of in vitro transformation by these viruses. It acts on the long terminal repeat (LTR) of the integrated provirus, usually to increase viral transcription, through interaction with cellular transcription factors (Figure 3); as a result of this activity, it can also modulate the transcription of cellular genes (see Retroviruses and associated diseases in humans). However, it should be noted that tumors are rare and late consequence of HTLV infection, and that most tumors have downregulated the expression of Tax and other viral proteins (Matsuoka 2003). In this context, the deltaretroviruses must be considered as initiating factors, with largely unknown mutations to cellular genes playing a rate-limiting role in the leukemogenic process.

A further example of a retroviral product that plays a direct role in oncogenesis is provided by the Friend spleen focus-forming virus (SFFV), an unusual defective isolate encoding a truncated envelope protein that binds to and activates the erythropoietin receptor in the absence of its natural ligand (Ruscetti 1999). Another is the recently characterized jaagsiekte sheep retrovirus (JSRV), a betaretrovirus which has been shown to be the causal agent of the sporadic epithelial tumor, ovine pulmonary adenomatosis. Transformation of fibroblast cells in vitro is mediated by the viral envelope gene, although it as yet unclear whether this activity is sufficient to explain the malignant potential of JSRV (Chow et al. 2003).

THE ONCOGENES OF DNA TUMOR VIRUSES

The observation that SV40 T antigen binds tightly to a cellular protein, p53 (Lane and Crawford 1979; Linzer and Levine 1979) was the first step towards the elucidation of a transformation mechanism common to many of the DNA tumor viruses. Binding results in the inactivation or degradation of the cellular target protein. Furthermore, T antigen also binds and inactivates a second tumor suppressor, pRb. The p53 and Rb pathways are common targets for many of the DNA virus oncoproteins, although the mechanisms by which their functions are subverted are varied and may be direct or indirect (see Table 5, p. 9).

The transforming genes of SV40 and the other small DNA viruses of the papovavirus group bear no close relationship to cellular gene products, but interact with key factors controlling host cell replication to create a permissive environment for the virus to replicate its own genome. If the viral replication cycle fails to proceed past the expression of these early gene products, the result is a persistently infected, transformed cell. In this abortive mode of replication, the host cell is induced to multiply abnormally and will present few viral antigens to the host immune system, with obvious advantages to the virus in establishing latent or persistent infection. There are other mechanisms by which DNA tumor virus oncogenes can interfere with cell growth regulation, as shown by polyomavirus middle T antigen which interacts in subtle ways to activate nonreceptor tyrosine kinases at the plasma membrane (Kiefer et al. 1994). Moreover, polyomavirus small t antigen interferes with p53 indirectly by uncoupling responses to its upstream activator, Arf (Lomax and Fried 2001). Also, cell lifespan, and hence susceptibility to transformation, can be increased by activation of telomerase as reported for human papillomavirus (HPV) E6 (Klingelhutz et al. 1996). For hepatitis B virus (HBV), analysis of viral oncogenic functions has been hampered by the lack of tractable in vitro systems. However, both p*X* and the large envelope protein preS can transactivate cellular genes (Rossner 1992), while the pX protein has been reported to bind and inactivate p53 (Truant et al. 1995).

While transformation by the papovaviruses is a consequence of abortive infection, the oncogenic herpesviruses have evolved latent infection as an essential feature of the normal life cycle. In the minimal state, the virus expresses only those functions necessary to maintain and ensure replication of its episomal DNA genome

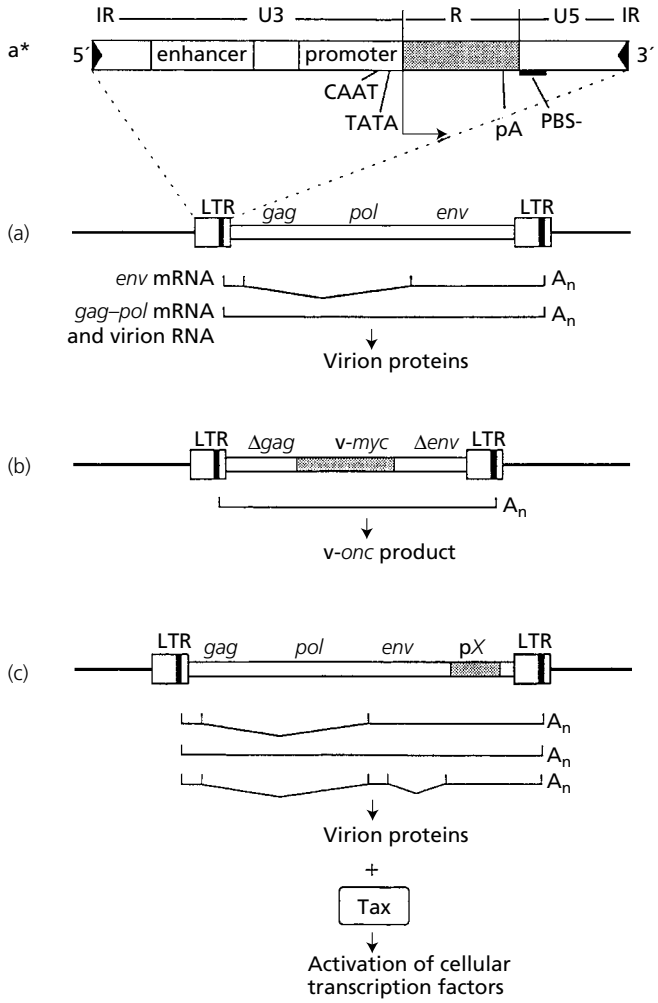

Figure 3 *Retroviruses and associated diseases in humans, considers retroviruses at length. This figure and Figure 4 give only enough detail to appreciate their pathogenesis.* **(a*)** *Detail of a typical retroviral LTR. Each LTR resembles an insertion sequence, having an inverted repeat (IR) at its extremities. A direct repeat (R) derives from either end of the virion RNA, with a cap site at its 5′ end (large arrow) and a polyadenylation signal (pA) close to its 3′ end. The 3′ boundary is marked by a primer binding site (PBS) to which is bound the tRNA primer for reverse transcription of virion RNA. The remainder of the LTR comprises U3 and U5 regions, derived respectively from the 3′ and 5′ ends of viral RNA. U3 contains a promoter region, with characteristic 'CAAT' and 'TATA' boxes that regulate proviral transcription. Upstream (5′) to the promoter is an enhancer with a nonpolar stimulatory effect on gene expression.* **(a)** *A retroviral provirus (boxes) integrated in cell DNA (thick line). LTRs flank and control the expression of coding regions that comprise, in replication-competent retroviruses, at least three genes,* gag, pol, *and* env, *expressed through unspliced or spliced polyadenylated mRNAs.* **(b)** *A retroviral provirus bearing a viral oncogene (v-*onc*), in this case the v-*myc *in the avian myelocytomatosis virus 29. In transducing v-*myc *the virus has lost the* pol *gene and portions of* gag *and* env, *so it can be propagated only if these missing functions are provided* in trans *in the same cell by a 'helper' virus of the sort shown in* **(a)**. **(c)** *A simplified diagram of the human T-cell leukemia virus. The oncogene region of this virus (pX) encodes Tax, Rex, and a series of other minor products (p30, p12), which have no known cellular counterparts. Tax interacts with cellular transcription factors to transactivate a range of cellular genes, including IL-2 and its receptor.*

in the host cell. For the best characterized oncogenic herpesvirus, Epstein–Barr virus (EBV), a subset of six viral gene products has been implicated in the efficient immortalization of B cells, including a series of nuclear proteins (EBNAs) and two membrane proteins (LMP1 and 2), and the same gene products are expressed in EBV-positive immunoblastic lymphomas which arise in immunosuppressed transplant recipients and patients with the acquired immunodeficiency syndrome (AIDS). A key effector of transformation is LMP1 that appears to mimic the action of a constitutively active tumor necrosis factor (TNF) family receptor (Eliopoulos and Young 2001). A more limited subset of EBV proteins (EBNA1, LMP1 and -2) is expressed in nasopharyngeal carcinoma cells, while Burkitt's lymphoma cells express only EBNA1 (Rickinson 1994).

The human adenoviruses are oncogenic only in rodents, but have served as important models of cell transformation. Again, their major effectors (E1A, E1B, E4Orf6) appear to operate by similar principles, by

Table 5 *Selected DNA tumor virus oncogenes and mechanisms of action*

Virus	Effector	Target/mechanism
SV40	Large T antigen	Binds, inactivates p53, Rb
Polyoma	Middle T antigen	Binds activates src family kinases
	Small T antigen	Inhibits Arf/p53 activation
HPV	E6	p53 binding, degradation
	E7	Rb binding
BPV	E6	p53 binding
HBV	pX	Binds inactivates p53
Adenovirus	E1A	Rb
	E1B	Bak, Bax binding; inhibition of apoptosis
	E4Orf6	p53 binding
EBV	LMP1	Activation of NFκB, JNK pathways
	LMP2	Recruit src, syk tyrosine kinases
	EBNA5/LP	cdk6/D2 activation (inactivates Rb)
KSHV	LANA	p53, Rb binding
	v-cyclin	Phosphorylation/ inactivation of Rb

BPV, bovine papillomavirus; HBV, hepatitis B virus; HPV, human papillomavirus; SV40, simian virus 40.

binding to and inactivating p53 (Shen and White 2001; Tauber and Dobner 2001) and Rb (Gallimore and Turnell 2001) or inhibiting their downstream effectors (Shen and White 2001).

The large DNA tumor viruses encode many products that affect growth and survival of the infected cell, and others that allow infected cells to evade the host immune response. These include homologues of cellular genes encoding cytokines as well as regulators of apoptosis and cell-cycle progression. Many of these products have the theoretical potential to contribute to neoplastic transformation, but the term 'oncogene' is usually reserved for those products that have been implicated directly in the initiation or maintenance of oncogenic transformation. Despite their potency in various in vitro transformation assays, most DNA tumor viruses are weakly oncogenic, particularly in their natural hosts. This may be explained in part by the efficiency of the host immune response in controlling virus replication and destroying potential tumor cells that express virus-coded cell surface antigens or T-cell epitopes. However, immunosuppression reveals the malignant potential of these infections. EBV-induced malignancies are a significant complication in patients undergoing organ transplant or in the context of human immunodeficiency virus (HIV)-induced immunosuppression (Holmes and Sokol 2002). In a similar fashion, the indirect effects of HIV potentiate the carcinogenic effects of HPV infection (Hawes et al. 2003) and have increased the prevalence of Kaposi's sarcoma to the point where it is now one of the most common virus-associated malignancies (Boshoff and Weiss 2001).

A summary of the targets and mechanisms of action of DNA tumor virus oncoproteins is given in Table 5. Note, however, that these are in general a small subset of the binding interactions and effects of these multifunctional viral proteins.

INSERTIONAL MUTAGENESIS

Many retroviruses lack an oncogene but induce clonal tumors in which their genome is integrated as a DNA provirus in a specific region of the host chromosome (Figure 4). This insertion is mutagenic, so it changes the sequence of DNA in that region, and can have either of two results: activation of a gene or its ablation. In most cases proviral insertion activates a nearby gene, either by qualitative alteration or by augmentation of its expression (Peters 1990; Kung et al. 1991). In some cases the activated gene proves to be the cellular counterpart of a known retrovirus oncogene. Thus, B-cell lymphomas induced by avian leukosis virus (ALV) and T-cell lymphosarcoma induced by feline leukemia virus (FeLV) often contain a c-*myc* gene activated by insertional mutagenesis. In ALV-induced erythroleukemia, on the other hand, it is the c-*erb*-B proto-oncogene that is activated. These findings support a role for proto-oncogenes in neoplasia, and in the latter case also appear to represent an intermediate stage in the process of oncogene transduction (Nilsen et al. 1985).

By extrapolation from the observed activation of known proto-oncogenes by proviral insertion, it can be argued that, when an inserted virus consistently integrates into a limited region of the host genome in a series of tumors, the affected region harbors a putative oncogene. In this way a large number of loci have been identified, of which the activation is characteristic of certain virus-induced tumors; the available evidence as to the nature of these loci strongly supports the concept that their activation is relevant to neoplasia.

Although the first example of insertional mutagenesis came from avian lymphomas (Neel et al. 1981), much recent work to identify new oncogenes has been in the laboratory mouse. Moreover, the use of this facet of retroviral oncogenesis as a gene discovery tool by 'tagging' of target genes has enjoyed a renaissance since the completion of the draft human and mouse genome sequences (Neil and Cameron 2002). The target gene at a common insertion site can in some cases be difficult to determine, not least because retroviral *cis*-activation of target genes can in some cases operate over a great distance (up to 300 kb) (Lazo et al. 1990; Hanlon et al. 2003).

The strategy of retroviral tagging is complemented by the parallel development of techniques for the manipulation of the mouse genome. Thus, the introduction of

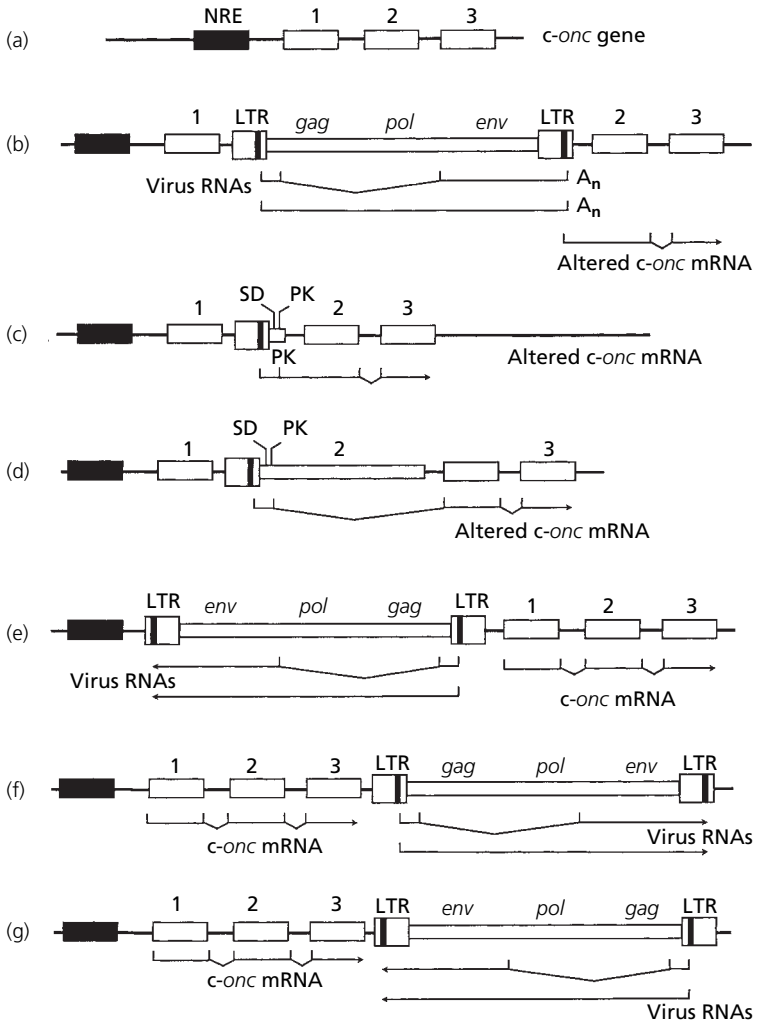

Figure 4 *Modes of insertional mutagenesis. The examples given show insertional mutagenesis by a retroviral provirus, but WHV (Buendia 1994) and others may have comparable effects.* **(a)** *Diagram of a proto-oncogene (*c-onc*) comprising three exons (open boxes), the first of which is largely noncoding, and an upstream negative regulatory element (NRE) (closed box), which helps to ensure that the gene is often poorly transcribed.* **(b)** *Insertion of a provirus between exons 1 and 2 of the* c-onc *gene. Expression of* c-onc *is elevated by 'promoter insertion,' regulatory elements in the 3′ LTR driving transcription of a viral* c-onc *hybrid message.* **(c)** *Insertion of a truncated provirus, with* c-onc *transcription initiating in the 5′ LTR. In this instance the transcript is not spliced at the donor site (SD) for generating subgenomic viral mRNA, so it includes the sequence (PK) 3′ to SD that permits packaging of the RNA into virions. This altered* c-onc *mRNA may thus be incorporated in an infectious virion, and infection leading to reverse transcription and genetic recombination with another viral RNA could generate a virus containing a transduced* onc *gene.* **(d)** *Another example of promoter insertion by a truncated provirus. In this case the packaging signal is spliced out of the mRNA so, like subgenomic viral mRNA, it is unlikely to be incorporated into virions.* **(e)** *Proviral insertion 5′ to* c-onc*, but in the opposite transcriptional orientation, precluding enhancement of* onc *expression by the viral promoter. The virus thus acts either through the effect of its enhancer alone or by distancing* c-onc *from the 'upstream' NRE.* **(f and g)** *Proviral insertion 3′ to* c-onc *in either transcriptional orientation. In these instances it appears that the virus can augment proto-oncogene expression only by virtue of its enhancer.*

mutant proto-oncogenes into the mouse germline has confirmed their oncogenic potential, whilst their inactivation by homologous recombination has provided valuable insights into their normal functions (Meuwissen et al. 2001). Insertional mutagenesis is also a prominent oncogenic mechanism for at least one representative of the hepadnaviruses; woodchuck hepatitis virus (WHV) often activates the c-*myc* or N-*myc* genes in the genesis of liver tumors that arise without the extensive cirrhosis that precedes HBV-associated cancers (Buendia 1994). By contrast, there is only fragmentary evidence for insertional mutagenesis in HBV-associated tumors. The reasons for the marked difference in mode of action between two similar viral agents are unknown.

So far we have focused on the capacity of integrated viruses to activate genes. The converse of this process, gene disruption by retroviral insertion, underlies some developmental mutations in laboratory mice (Lock et al. 1991). In such cases it is possible to reveal the disruptive effects of a single insertion by back-crossing to generate

a homozygous mutation. In somatic cells, gene ablation is an improbable outcome because it requires inactivating insertions at both alleles. Nevertheless, there are instances in which retroviral insertions have been shown to ablate gene function in tumor cells. Examples include the inactivation of p53 by insertion of Friend murine leukemia virus (MLV) in erythroleukemia (Ben-David et al. 1988) and the targeting of the neurofibromin gene (*NF1*) by BXH-2 virus in myeloid leukemia (Largaespada et al. 1995).

At first sight, mutagenic ablation might be thought to be more likely because it is less specific than activation, and so insertion anywhere within the coding region would destroy the integrity of the gene. However, mutational activation seems to operate by such a variety of mechanisms that it, too, can result from insertion at many locations near the activated gene (Figure 4). In the classic example of avian B-cell lymphomas, c-*myc* transcription is driven by the viral promoter in an LTR situated 5′ to the gene (Figure 4b). However, the subsequent discovery of enhancers in retroviral LTRs, their nonpolar action over long distances, and their effects predominantly on the nearest promoters now indicate that, in different circumstances, a viral LTR can augment activity of a gene either 5′ or 3′ to itself in either orientation (Figure 4e–g). Moreover, it is theoretically possible that an inserted provirus may activate a gene by simply separating it from *cis*-acting cellular repressors.

Although the focus of most current research is on the γ-retroviruses that infect the laboratory mouse, the mechanism of insertional mutagenesis is relevant in tumors induced by other members of the γ-retrovirus and β-retrovirus families. While FeLV appears to target a similar set of genes to MLV (Tsatsanis et al. 1994), a very different pattern emerges for mouse mammary tumor virus (MMTV), which frequently targets genes of the *Wnt* and *Fgf* families (Kapoun and Shackleford 1997). The relevance of insertional mutagenesis to human cancer has also come into focus recently with the observation of leukemia in gene therapy subjects which appear to have been induced by integration at the *LMO2* oncogene. This observation has led to a renewed interest in the specificity and consequences of retroviral integration in the host genome (Baum et al. 2003).

OTHER EVIDENCE IMPLICATING PROTO-ONCOGENES IN NEOPLASIA

A detailed consideration of the molecular and cell biology of neoplasia is outside the scope of this chapter, but the following brief survey is intended to provide some understanding of the basis of viral oncogenicity.

Two major lines of investigation have further incriminated proto-oncogenes in cancer.

1 DNA from certain tumor cells, but not DNA from their normal counterparts, can induce morphological transformation of, or confer tumorigenicity upon, appropriate recipient cells in culture. The genes responsible are often mutant members of the *ras* oncogene family, first encountered as the v-*onc* genes of certain murine sarcoma viruses. However, as with insertional mutagenesis by viruses, DNA 'transfection' has revealed further novel putative oncogenes whose properties, where known, are compatible with a role in neoplasia (Bishop 1987).
2 Many cancers display characteristic karyological abnormalities, typically deletions, translocations or amplification of identifiable regions of chromosomes. In some cases the genes affected by translocation and amplification are the cellular relatives of known v-*onc* genes; again a number of novel loci are deemed guilty by association (Rabbitts 1994).

In short, proto-oncogenes have been incriminated in neoplasia by a variety of phenomena, and some genes have been implicated in several ways. The mutations suffered by proto-oncogenes in tumor cells – be they single base changes, transduction or gross disruption by viruses or massive, microscopically visible aberrations in karyotype – have one (or both) of two effects: they change either the expression of the gene or the nature of its product.

'HIT AND RUN' MECHANISMS

The pattern of virus persistence in a tumor may be complex but is amenable to study. The same cannot be said for those instances in which a virus is implicated in tumor formation in vivo or cell transformation in vitro but in which it is impossible to demonstrate either persistence of a specific portion of the viral genome or integration in a limited region of host DNA. The virus in such cases is presumed to act transiently. It may operate by one of the mechanisms detailed above but the genes involved are required only in the early stages of tumor evolution and unknown selective pressures against the virus eliminate it during tumor progression.

The absence of bovine papillomavirus type 4 from the alimentary carcinomas that it causes may reflect such a mechanism, because at least some papillomaviruses are known to carry transforming genes. On the other hand, this virus, as proposed for herpes simplex viruses and cytomegalovirus in cervical cancer, may act as a mutagen, through an ephemeral association of its genome with cell DNA or, possibly, through the action of viral enzymes (Minson 1984; Macnab 1987). The mechanisms in either case are hard to study and investigators have invoked this 'hit and run' explanation only after eliminating other possibilities (McDougall 2001).

Indirect mechanisms

The second major category of pathogenic mechanism is also frustrating for the student of in vitro systems but is clinically significant, whether operating alone or in

concert with mechanisms outlined above. In these indirect, or extrinsic, mechanisms neither the cells of the tumor nor their ancestral lineage need ever have been infected by the causative virus; instead, tumors arise in response to virus infection of other cells, the nature of the response taking several forms.

Cell death or impaired function as a direct or indirect effect of virus infection can have different consequences depending on the type of cell affected. If the immune system is compromised the outgrowth of cells might be permitted from many tissues that had acquired neoplastic potential by various means; in practice, however, immunosuppression leads to increases in the incidences of a relatively small spectrum of tumors, a significant proportion of which are associated with viral risk factors (Figure 1). Cell death, or untoward cell proliferation, among hemopoietic tissues can also upset delicate homoeostatic cellular interrelations, and compensatory proliferation in other lineages may favor independent neoplastic alterations in these reactive cells.

A number of tumor viruses cause immune impairment (Table 6), probably by a variety of mechanisms. Immunosuppression by some viruses may be due to cytotoxicity, reflecting, in the case of avian reticuloendotheliosis viruses, the burden of intracellular unintegrated viral DNA. The immunosuppressive effect of HIV could also result simply from cytopathic effects on $CD4^+$ T cells, but the extent of infection does not seem sufficient to account for the degree of immune impairment. Various theories of HIV immunosuppression have been elaborated, including virus-induced autoimmune attack, T-cell apoptosis following nonspecific immunosuppression, cytokine dysregulation, and cell destruction due to chronic antiviral immune responses (Zinkernagel and Hengartner 1994). Two points should be noted at this juncture:

1. with the possible exception of HIV and its relatives, the tumor viruses that are immunosuppressive also seem to have efficient and direct means of inducing neoplasia
2. immunosuppression is the only indirect mode of viral carcinogenesis that does not necessarily involve cell proliferation as an early stage.

Death or damage to nonhemopoietic cells may likewise favor neoplastic change, but in uninfected cells of the same lineage that are dividing in an attempt at tissue regeneration. This is one way in which chronic hepatitis B or hepatitis C infections may increase the risk of primary liver cancer (Slagle et al. 1994).

Virus infection may also stimulate cells to produce growth factors of various types. In some cases, the viruses themselves carry growth factor-like genes, such as the EBV *BCRF1* gene which has homology with IL-10 (Morein and Merza 1991). When the producing cell itself possesses receptors for these factors, their effects can be autocrine with the virus acting directly, as described above. An example of such a process is the stimulation of IL-2 and its receptor by HTLV-I in infected T cells (Ruben et al. 1988).

A further example of viral interplay with host immunoregulatory systems is provided by the MMTVs, which encode superantigens. Endogenous MMTVs in the mouse genome express these polymorphic proteins that stimulate developing T cells via the Vβ of the αβTCR, leading to clonal deletion of reactive cells and a consequent bias in the Vβ repertoire of mature T cells. This phenomenon may be regarded as an aberrant outcome of viral sequestration in the mouse germline. However, it seems that the expression of these ligands on B cells is an important early event in establishment of infection by MMTV, because this may stimulate mature T cells to release growth factors that drive proliferation of the infected cell. Along the way, the virus usurps the host immune response to favor its own dissemination (Acha-Orbea and MacDonald 1995).

The potential stimulatory effects of viral antigens may also be due to their mimicry of ligands for receptors on a range of cell types. If these receptors modulate normal cell growth and behavior, chronic unscheduled binding of ligand analogues may lead to hyperplasia and then neoplasia. Such effects probably are widespread, and the oncogene of the spleen focus-forming component of Friend murine leukemia retrovirus is perhaps the best characterized example. This oncogene has no cell counterpart, being derived entirely from virus envelope gene sequences, but it appears to interact with the erythropoietin receptor, replacing the requirement for exogenous growth factors (Ruscetti 1999).

IMPLICATION OF VIRUSES IN THE ETIOLOGY OF CANCER

The pathogenic strategies just discussed are clearly crucial to the concepts of the genesis of virus-associated tumors but are only part of this complex process. Experimental, clinical, and epidemiologic observations all suggest that malignancy results from a step-by-step accumulation of stable cellular alterations whose occurrence reflects, and is greatly accelerated by, exposure to multiple risk factors. The age of the animal and the duration, intensity, and frequency of exposure to risk factors are important elements in tumor evolution, and these variables tend to obscure any causal relationship between virus infection and the eventual development of malignancy. We must take account of these other components if we are to understand the etiologic significance of viruses.

The influence of risk factors on viral carcinogenesis can be considered by dividing them into three major, but overlapping, categories:

1. factors associated with the virus infection and its effect on host cells

Table 6 *Viral functions implicated in cancer*

	Virus group (and representatives)								
	Retroviruses					Hepadna	Papova SV40;	Papilloma	Herpes
Function	**α: ALV**	**γ:MLV; FeLV**	**β:MMTV; JSRV**	**δ:HTLV; BLV**	**Lenti:HIV; SIV**	**HBV; WHV**	**polyoma**	**HPV; BPV**	**EBV; KSHV; HVS**
Viral gene product stimulates proliferation	–	+/–	+	+	+/–	+	+	+	+
Insertional mutagenesis	+	+	+ (MMTV)	–	+/–	+ (WHV)	–	+/–	–
Generalized immunosuppression	+	+	–	–	+	–	–	–	–

ALV, avian leukosis virus; BLV, bovine leukemia virus; BPV, bovine papillomavirus; EBV, Epstein–Barr virus; FeLV, feline leukemia virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; HTLV, human T-cell leukemia virus; HVS, herpesvirus saimiri; JSRV, jaagsiekte sheep retrovirus; MLV, murine leukemia virus; MMTV, mouse mammary tumor virus; SIV, simian immunodeficiency virus; WHV, woodchuck hepatitis virus.

2 factors that determine the evolution and properties of neoplastic cells
3 factors that affect the host response to both virus and tumor.

We can consider in turn the interplay of these factors during three arbitrary stages of tumor development:

1 the successful infection of the organism by a virus and, when the virus acts by a direct means, its infection of the tumor cell lineage
2 the conversion within the tumor cell lineage of a normal cell to neoplastic growth and behavior
3 the survival and growth of its descendants to form a detectable tumor.

An assessment of the relative importance of the different risk factors at each stage of tumor development is shown in the histogram in Figure 5. In this diagram the three stages of development are shown on the abscissa, which is thus a time scale axis; it is, however, impossible to draw this to scale. Stage 2 is probably the longest period; important stage 2 events probably often precede those in stage 1.

Virus infection/transmission

An animal becomes infected with an oncogenic virus by one of three routes (Weiss 1984). The first is genetic transmission, by which most individuals, of many species, have acquired endogenous retroviruses during evolution. These viruses seem to be nonessential but innocuous passengers in the genomes of their hosts and they are often xenotropic (i.e. capable of replication

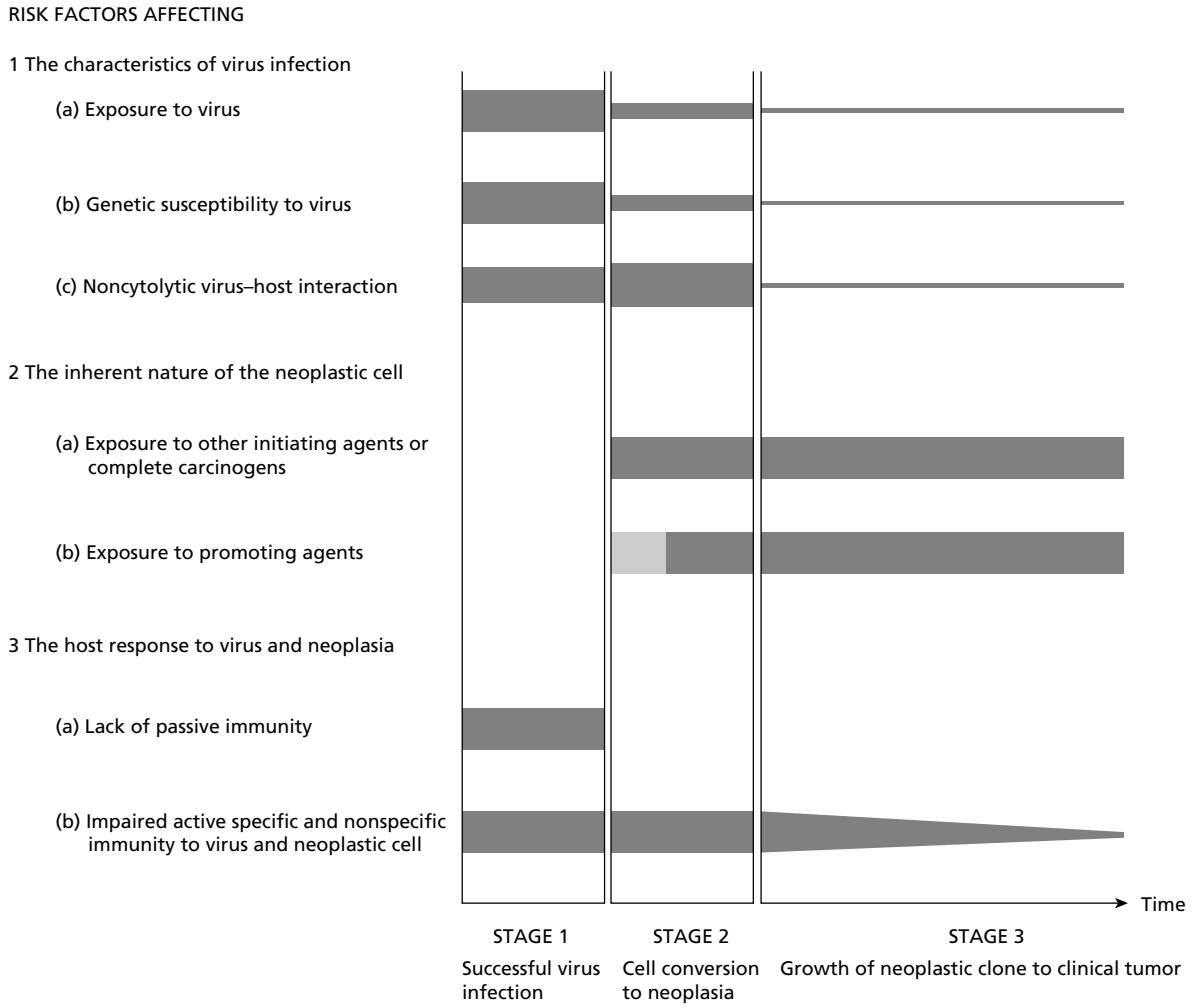

Figure 5 *Risk factors in viral carcinogenesis: the relationship between the arbitrary stages of virus-associated neoplasia, the features of the neoplastic process, and the risk factors that influence each feature. The stages of neoplasia are shown on the abscissa. The breaks in the time axis indicate that these stages vary in length and they are not shown to scale (in general, stage 1 is likely to be relatively short and stage 2 is usually the longest period). The risk factors affecting any of the three features characterizing virus-associated neoplasia are shown on the ordinate and they may operate at different, and often more than one, stages of the disease process. The thickness of the bars indicates the relative importance of each risk factor at each stage of neoplasia, broken lines indicating periods at which the risk factors are of no or uncertain significance. (Modified, with permission, from Wyke and Weiss 1984.)*

only in other species). A clear exception to this harmlessness is that certain strains of mice harbor endogenous viruses that are ecotropic (i.e. they can infect mouse cells). Moreover, these viruses evolve by recombination with other defective endogenous proviruses, generating variants with enhanced leukemogenicity (Stoye et al. 1991). Comparable variants of altered, if not augmented, pathogenicity can also arise by recombination of FeLV with endogenous cat retroviruses (Neil et al. 1991). In both mice and cats the recombinant viruses reveal altered viral envelope glycoproteins, some of which have an increased propensity to bind to and stimulate proliferation of immune-responsive and other cells.

The second route, congenital transmission of exogenous viruses across the placenta or oviduct, is important in the case of leukemogenic avian, feline, and bovine retroviruses. However, for these and many others the third route, horizontal transmission peri- and postnatally is also significant. Whereas the papovaviruses are hardy, the enveloped herpesviruses and retroviruses are far more labile; their horizontal spread is therefore more efficient when animals are grouped together and make close contact with carriers. Moreover, such conditions increase the risk of repeated exposure to high doses of virus early in life, a pattern which, in turn, appears to encourage the eventual appearance of neoplasia.

With regard to the routes of transmission, the pattern of virus spread is also greatly influenced by its site of replication in the animal. Thus, for FeLV and EBV, free virus is shed in the buccal cavity and transmitted in saliva. Marek's disease virus of fowls, on the other hand, replicates in feather follicle epithelium and is spread in dander, whereas BLV and HTLV remain cell-associated and require the transmission of whole cells. In the case of the last two viruses, iatrogenic transfer and 'flying pin' arthropod vectors might be expected to be significant routes of infection, and epidemiologic data support this notion.

Genetic susceptibility to viruses

All viruses require the means to enter a cell, express their genomes, and assemble progeny for spread to other cells. Interspecies variation in the susceptibility of cells to viruses – often associated with severe constraints on the virus life cycle – are taken for granted but there can also be pronounced variation within a species. In cases of virus-associated neoplasia, such variation is indicated by clustering of tumor incidence. Clusters are often due to variation in the other risk factors discussed here, but difference in virus susceptibility is a likely cause when clustering is demonstrably familial. In domestic animals, where breeding is often controlled and one male can sire many dispersed offspring, it is easier to discern familial clusters. The clusters of, for example, bovine leukosis, reflect genetic factors that are not well defined, but in other instances the basis for the underlying enhanced susceptibility is better understood. The species in which this has been studied most comprehensively is, of course, the laboratory mouse (Rosenberg and Jolicoeur 1997).

The need for entry of virus into cells provides a crucial barrier to infection; in many cases, efficient penetration is highly specific, not only for the whole animal but also for particular types of cell within the host. Retroviruses enter cells whose surfaces bear receptors to which the viral envelope glycoprotein can bind. In chickens, receptors are found on a wide range of cell types but their specificity (and hence host susceptibility to virus) reveals genetically determined polymorphisms between individual birds (Payne 1992). Other receptors are of limited distribution within the host: human CD4 lymphocyte antigen is an important component of the HIV-1 receptor, this stringency explaining in part the tropism of HIV-1 for a particular T-cell subset (Dalgleish et al. 1984). These receptors do not of course exist merely to facilitate virus penetration and, indeed, not all binding sites serve this role. They clearly have structural or other functional purposes on the cell surface. For the mammalian type C retroviruses, related families of transmembrane transporter proteins for basic amino acids and phosphate have been identified as mediators of viral entry (Cunningham and Kim 1994). However, the relevance of these to oncogenesis seems to be slight, as there is little evidence of effects on cell growth and viability emanating from virus binding to these receptors.

EBV gains entry to B cells via binding to the complement receptor molecule CR2 (Nemerow et al. 1987). Other oncogenic DNA virus receptors have yet to be definitively identified. Once inside cells, viruses may do some damage, which will, however, be greatly limited by any further restrictions on their replication and spread. For instance, retroviruses are subject to both physiological and genetic constraints after penetration. For the type C retroviruses, stable integration of a complete DNA transcript of the virion RNA genome can occur only in cells that synthesize DNA; on the other hand, the early gene functions of polyomaviruses can themselves stimulate DNA synthesis. The host range restrictions of ectropic MLV mediated by the mouse Fv-1 locus also operate during this early part of the infectious cycle. Recently, the retroviral LTR (see Figure 4) has attracted considerable attention and there is a wealth of evidence to suggest that its potency varies with cell type. Cellular factors interact *in trans* with the LTR enhancer to modulate the level of transcripts mediated by this element in a given cell and hence the extent of viral replication, the oncogenic potential of the virus, and its cell tropism (Tsichlis and Lazo 1991). Thus, the pathogenesis of, for example, retrovirus-induced mouse leukemia is a very complex process in which both the virus and the tumor cell evolve. When virus infection of an appropriate host results in proviral insertion at a specific site, the expression is stimulated by one or more

of a range of cell genes. The amplitude of this expression may depend on the potency of the viral LTR in that cell (Morrison et al. 1995).

Noncytolytic virus–host interactions

Some tumor-associated viruses, such as HBV and many retroviruses, can be released by cells without compromising viability, but for many others cytolysis is necessary for escape of virus. Cytolysis is, of course, incompatible with direct mechanisms of virus oncogenesis, so neoplasia is likely to be a rare and aberrant consequence of infection by these viruses. In such cases, either the host restricts the later stages of virus replication or the virus is defective in directing these events, but, in both instances, viral functions needed to induce neoplasia are retained.

Two other properties of tumor viruses are worth mentioning here: virus integration and latent virus infection. The viruses of all families that include directly oncogenic members can exist as DNA within the cell, inserted in the host chromosome. This may be a rare outcome of infection or, as with retroviruses, an essential part of the virus life cycle. Virus integration is obligatory for oncogenesis by insertional mutagenesis, but there seems to be no reason why it should be required for other forms of oncogenesis. Indeed, as virus genomes are absent from some tumors for which viruses are considered risk factors, integration is presumably not essential – at least in the later stages of tumor evolution. Poxviruses, although not true tumor viruses, need special consideration because they can induce proliferating lesions, probably through the action of viral genes that encode molecules resembling cell growth factors (Opgenorth et al. 1992) but the lesions produced do not progress to full-blown cancer. Nevertheless, infection can be fatal due to concomitant inflammatory disease. It is possible that poxviruses fail to induce tumors because they replicate in the cytoplasm, never integrate, and so do not achieve a sufficiently stable association with the host cell. A number of RNA viruses also achieve persistent infections, yet they rarely, if ever, integrate and only one representative (hepatitis C virus (HCV)) has so far been incriminated in neoplasia. Integration is clearly important in achieving a stable infection but may have additional undefined significance.

Latency is a characteristic of two families of oncogenic viruses: the retroviruses and the herpesviruses. Prolonged latent infection is a common sequel of primary FeLV infection in cats. It is associated with a humoral and cellular immune response and can be abolished by treatment with immunosuppressive agents (Rojko et al. 1982). Without such insults, however, this form of latent infection tends to resolve in recovery with subsequent immunity (Pacitti and Jarrett 1985). Latent infection with BLV has also been described but in this case a non-immunoglobulin plasma protein seems to be involved in virus suppression (Gupta and Ferrer 1982). Like those of many endogenous retroviruses, latent exogenous genomes may be in the configuration of inactive chromatin, which is highly methylated. This has practical significance because tumor cells do not usually express their resident viral genomes unless the animals are immunosuppressed or the cells are cultured, with or without agents intended to activate silent genomes. At the mechanistic level, the role of latency in neoplasia is not obvious, but, like integration, it may serve primarily to ensure persistence of the viral genome, thus facilitating its interactions with the other risk factors germane to neoplasia.

EBV latency is a paradigm for this mode of infection by the γ-herpesviruses and appears to be critical to virus persistence and central to its involvement in tumors (Rickinson and Kieff 1996). Human peripheral blood cells can be transformed and immortalized by EBV infection in vitro. These latently infected cells express a subset of the EBV genome comprising six nuclear proteins (EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, and EBNA3C), as well as two oncogenic membrane proteins (LMP1, 2) and small EBV RNAs (EBER). EBV-positive tumor cells display a restricted subset of these genes. The minimal expression pattern is exemplified by Burkitt's lymphoma, where viral expression is restricted to a EBNA-1 and EBERs. A more complex pattern is observed in nasopharyngeal carcinoma, which includes the oncogenic latent membrane proteins (LMP-1,2) as well as EBERs and EBNA-1. A variation on this theme is seen in EBV-associated lymphoproliferative diseases of transplant recipients and AIDS patients, which express EBNA-2 in addition to the list for nasopharyngeal carcinoma. These distinctive patterns reflect the critical role of the immune system in containing EBV infection and the consequences of its compromise in neoplastic disease and immunosuppressed states. They probably also reflect differences in the relationship of EBV to tumor initiation and maintenance in these diverse neoplastic diseases.

A similar pattern is emerging for Kaposi's sarcoma-associated herpesvirus (KSHV), where HIV appears to be the primary cofactor, revealing its latent oncogenic properties and enabling a wider range of viral products to be expressed in the tumor cell (Schulz and Neil 2002).

Viruses and the multistep development of neoplasia

Many of the viruses that transform cells in vitro carry oncogenes of which the activity is under the control of powerful viral regulatory elements (see Figure 4). Oncogenes – perhaps aided by a spreading virus infection – are very potent carcinogens that rapidly induce tumors in host animals. In some circumstances, however, oncogene-mediated viral carcinogenesis is far

less efficient and the appearance of a neoplastic cell phenotype requires other genetic or epigenetic events such as the loss of tumor suppressor genes or the acquisition of additional, activated oncogenes. There is abundant evidence for this. Some tumor viruses carry more than one oncogene, each of which contributes to their tumor-inducing capacity. In the example of the retrovirus avian erythroblastosis virus, *erb*-B encodes a transmembrane protein [epidermal growth factor (EGF) receptor] and *erb*-A a nuclear protein (thyroid hormone receptor) (see Table 4). Other examples are the presence of the c-*myc* nuclear oncogene and the protein kinase oncogene *mil*/*raf* in avian carcinoma virus Mill Hill virus 2 (ACMHV-2), and the presence of two nuclear oncogenes, *myb* and *ets* in avian myeloblastosis virus (AMV).

Further evidence of the cooperative action of multiple genes in lymphoma development comes from the γ-retroviruses, in which multiple different insertional mutagenic events can be observed in a single tumor (Tsichlis et al. 1985; Tsatsanis et al. 1994). Furthermore, mice carrying an activated oncogene in the germline may show greatly accelerated tumor onset when infected with the virus, and activation of cooperating genes by insertional mutagenesis occurs at high frequency. Further evidence of the cooperative action of these genes is provided by the strongly synergistic effect on tumor development of crossing mice carrying different activated oncogenes (e.g. c-*myc* and *pim*-1) (Jonkers and Berns 1996). Similarly, MMTV appears to be capable of mediating multiple mutational hits by insertion at synergistic oncogenes of the *Wnt* and *Fgf* families (MacArthur et al. 1995). The relatively unrestricted replication of these agents in the persistently infected host may account for their unusual propensity for insertional mutagenesis.

In naturally occurring cancer, the requirement for multiple cellular changes is mirrored in the time- and risk-factor-dependent pattern of tumor development. Many clinically important tumor viruses, particularly those of humans, need the operation of additional chemical, physical, or infectious risk factors to initiate or promote neoplasia (see Tables 1, 2 and 3). Because these viruses are incomplete carcinogens, it is not certain whether they act as initiators or promoters of oncogenesis.

Viruses have often been considered initiators, for a number of reasons. Initiation in experimental systems is a dose-dependent, irreversible event with many hallmarks of mutation (Montesano and Slaga 1983), and direct models of virus oncogenesis either induce or introduce mutant genes. Moreover, virus infection is often observed early in life in the susceptible individual, perhaps before exposure to other likely risk factors. However, aside from instances of possible transient effects, virus-induced cancer is often associated with persistent infections, virus integration, and possibly latency. Infection early in life may simply be a prerequisite for achieving persistence, a prolonged exposure to virus that can be significant in two ways. First, it may increase the chance of crucial, if rare, virus–cell interactions. Second, and this seems particularly relevant to viruses that act indirectly, it may enhance the possibility that other environmental risk factors that may be encountered can be influenced by a chronic viral tumor-promoting activity. Some instances, however, are not clearcut. Consider, for example, tumor induction by EBV and HTLV-I: both seem to stimulate a proliferation of lymphoid cells that is strictly neoplastic (in that it is driven from within the cell and results from a 'mutation') but which resembles hyperplasia in being polyclonal. These viruses seem to behave as tumor promoters, and subsequent clonal events such as c-*myc* translocation in EBV-associated Burkitt's lymphoma are more likely to be the result of initiation. It seems that the concepts of initiation and promotion that have been so fruitful in studying experimental carcinogenesis are not so readily applicable to viral cancer. The consequences of virus infection are, however, clearly capable of contributing to several stages of neoplasia.

The host response

The ability of the infected animal to limit both virus infection and the multiplication of neoplastic cells is of great importance in virus-associated neoplasia, particularly the leukemias in which the tumors themselves are prone to perturb both specific and nonspecific immune regulation. Establishment of virus persistence increases the chance of viral pathogenic mechanisms coming into play; persistence can often be achieved by exposing a young animal, with waning maternal immunity, to large and possibly repeated doses of virus, resulting in impaired ability to limit the extent of the infection. Episodes of immune impairment later in life, due to nutritional or hormonal factors, disease or other physiological stresses, can reinforce this effect or even favor infection of a previously virus-free animal. When the viral risk factor is itself an immunosuppressant (see Figure 5) the problem is exacerbated, and, once a tumor burden develops, it may reinforce the predisposing impairment.

An intimate association with compromised immunity is often seen in the natural history of virus carcinogenesis. Examples from avian, feline, and human hemopoietic neoplasms, in which the virus infection contributes wholly or partially to the immunosuppression, have been given above. In other instances, another risk factor is responsible. Thus, an immunosuppressant in bracken fern seems to be important in the etiology of papillomavirus-associated alimentary carcinomas in cattle (Jarrett 1985; Beniston et al. 2001). In humans, iatrogenic immunosuppression is associated with an increased incidence of certain tumors, many of

which had prior association with a virus risk factor. Indeed, so striking is this that Kinlen has suggested that all human tumors of which the incidence increases in immunosuppressed patients may have a viral component in their cause (Kinlen 1992). In short, the host response seems to be more important in controlling virus infection than other aspects of cell neoplasia.

The search for new viruses

There are three important motifs in naturally occurring viral cancers in animals and humans:

1 virus
2 immune impairment
3 co-carcinogen.

The first two components appear to be particularly important in most animal tumors, whereas the third is of greater importance in humans. These must all be considered in assessing the roles of other potential tumor viruses.

The most profitable tumors for study are those showing clustering that can be familial, geographical, or social/occupational, implying important genetic or environmental risk factors, both of which can point to viruses. Another important clue is any evidence of increased tumor prevalence in immunosuppressed individuals.

Both intact animals and tumors must be examined for characteristics of virus infection. Virus isolation from tumor material may require culture of tumor cells to demonstrate any latent virus, perhaps with addition of hormones, or of chemicals such as bromo- or iododeoxyuridine to activate endogenous viruses, or 5′-azacytidine which inhibits cytosine methylation. Virus can sometimes also be detected by electron microscopy or immunological techniques. It is important to try to discover an appropriate cell type for virus propagation in vitro because the tumor itself is often an unsuitable host. The complexity of the mammalian genome was a barrier to solving this problem by subtractive hybridization methods, but polymerase chain reaction (PCR)-based techniques can now identify small amounts of foreign DNA in tumor cells (representational difference analysis). This strategy led to the discovery of a new human herpesvirus (KSHV) in Kaposi's sarcoma cases (Chang et al. 1994).

Isolation of a candidate virus provides the opportunity for a more detailed study of its association with the tumor. If, however, isolation fails, much can be learned by comparison with known tumor viruses together with the screening of tumor cells for molecular evidence of viral infection and examination of intact animals for epidemiological clues. If infection with a virus is common in a population, features of the infection peculiar to the tumor-bearing hosts should be sought. Is the infection unusually persistent? Is the serological response abnormal? Is the virus in the tumor defective? Are unusual viral antigens expressed? Even when a virus cannot be identified, a specific tumor antigen may be an important clue to a viral etiology.

Epidemiological evidence is crucial in humans, in whom it is impossible to test a causal role for the agent by virus inoculation. Case–control studies can associate the virus with the tumor, and laboratory investigations will establish the features of high- and low-risk groups. Retrospective and, ideally, prospective studies of high-risk groups can establish a temporal relationship between virus infection, other risk factors, and tumor development. This knowledge can then serve as a basis for the management of the tumor.

PRINCIPLES OF THE MANAGEMENT OF VIRUS-ASSOCIATED CANCER

Although little is known of many aspects of the pathogenesis of virus-associated tumors, the knowledge that a virus is implicated can be used to try to manage the disease at any of three arbitrary stages of virus neoplasia described earlier (Figure 5). Two points should be remembered. First, a wider range of prophylactic measures can be applied in veterinary medicine (where the health of the herd can override the survival of the individual) than in human practice. Second, for all tumors in which a virus is implicated, the detection of virus infection at any stage of the disease might be of diagnostic or prognostic use. This is clearly the case in the few diseases in which infection often leads to tumor formation. However, even when neoplasia is rare, detailed knowledge of the role played by the virus might identify features of the infection that are characteristic of hosts at high risk.

Prevention of virus infection

Before infection, steps can be taken to:

- decrease the concentration of virus in the hosts' environment
- increase the natural (genetic) resistance of host populations
- increase the resistance of the hosts by active or passive immunization.

THE SOURCE OF VIRUS

This is determined by the density of symptomatic or asymptomatic carriers in the population, the ability of the virus to survive outside its host, and whether other animals act as natural reservoirs for the virus. Many tumor viruses are species-specific and in practice only the first two factors are amenable to prophylactic measures.

The identification of carriers often requires specialized techniques to detect virus infection. It is thus costly and further action is effective only when the carrier can be killed, cured, or isolated from the susceptible population.

For these reasons, identification of human carriers poses ethical problems but can be important for several groups of patients. The first are those whose habits place them at particular risk, for example intravenous drug abusers in the case of HIV and HBV infections and the sexually promiscuous in the case of papillomavirus and herpesvirus infections. The second are those who have acquired iatrogenic infections, such as HIV and HBV from contaminated blood products. The third are mothers infected with HTLV, where breast milk can be a very important but avoidable means of transmission.

Identification of carriers can be useful in the control of avian, feline, and bovine lymphoid leukemias. A small proportion of cats infected with FeLV do not develop immunity and remain viremic. Such animals can either be removed from colonies or identified before introduction into virus-free groups. Eradication of enzootic bovine leukosis is greatly assisted by tests for virus carriers, whose lineage can be maintained free of virus by the transfer of well-washed embryos (with the zona pellucida intact) to virus-negative recipients.

Improved hygiene to reduce the source of the virus is probably of little significance within susceptible host populations but, in combination with appropriate husbandry, can reduce the dissemination of disease between local concentrations of animals such as the spread of ALV from one poultry farm to another. Many tumor viruses are highly susceptible to chemical disinfectants, drying, and other inactivating agents, but horizontal and congenital spread depend on such intimate contact between hosts that disinfection is impracticable. Papillomas and enzootic bovine leukosis are exceptions to this because the causal viruses can be spread on the instruments of stockmen and veterinarians. Another possible measure, applicable to BLV and HTLV-I, is the control of insects that may spread infection. The social and hygiene practices in many Western countries delay some EBV infections until puberty or later, but these increase the incidence of infectious mononucleosis. A delay in the age of acquisition of EBV infection in Africa and Asia might reduce the incidence of Burkitt's lymphoma or nasopharyngeal carcinoma, but this has not yet been demonstrated. However, one virus-associated human neoplasm for which a change in habits should reduce incidence is carcinoma of the uterine cervix. As with other venereally transmitted infections, the spread of papillomavirus and herpes simplex virus (HSV) type 2 has been favored by promiscuity and changes in contraceptive practices.

THE GENETIC SUSCEPTIBILITY OF HOST ANIMALS

The selective breeding of genetically resistant stock is possible only in animals, and particularly in those that have a short generation time. It has been successful in reducing the incidence of avian lymphoid leukosis; the causal virus exists in the field as two major subtypes, characterized by the presence of particular glycoproteins on the viral envelope. These glycoproteins interact with specific cell surface receptors to allow the viruses to penetrate the host cells. Strains of domestic chicken have been bred that lack the surface receptors for the field strains of ALF and these birds are resistant to infection (Crittenden and Motta 1969). Some strains partly resistant to lymphoid leukosis apparently permit infection but not tumor development. A similar genetic resistance is seen in the production of Marek's disease lymphomas.

IMMUNITY TO VIRUS INFECTION

Although a number of commercial vaccines exist for oncogenic viruses of animals, proposals to produce vaccines against human oncogenic viruses are controversial, for several reasons.

- In many instances the role of the virus in the tumor is uncertain; indeed, proof of a causal role may rely on the prophylactic effect of vaccination. This leads to the dilemma of whether to embark on such measures before they can be shown to be necessary.
- Tumor production may be a rare outcome of infection by a widespread and not very pathogenic virus.
- The presence of many latently infected carriers and the frequent acquisition of infection early in life (e.g. with human herpesviruses) will limit vaccine efficacy.
- Because tumors can result from an aberrant virus–cell interaction, a traditional type of vaccine, based on inactivated or attenuated virus, may itself pose a risk of oncogenicity. This last objection might be overcome by the use of purified immunogens either produced by genetically manipulated portions of viral genomes or synthesized in the laboratory.

However, the first two considerations suggest that the returns (in terms of improved health of the human population) may justify the outlay only if three conditions are fulfilled:

1. the virus causes significant disease in addition to its oncogenic potential (as is the case with HIV and HBV)
2. the populations concerned are clearly defined, small and at high risk
3. the virus is the only clearly defined risk factor in the genesis of a common tumor (as with EBV-associated nasopharyngeal carcinoma).

The first successful commercial vaccines against a neoplasm were produced for Marek's disease and have proved of great value to the poultry industry. Avirulent strains of the virus, either natural or artificially attenuated, have been used as vaccine, as has a related herpesvirus of turkeys; continual modification of the vaccine, however, has been necessary to try to keep up with the emergence of highly virulent strains (Schat

1987). The vaccine virus strains establish a latent infection and induce tumor-associated surface antigens in lymphoid cells, sometimes with minor cytopathic effects and neuritis. There is, however, no immunosuppression and vaccinated birds resist challenge with oncogenic virus (Calnek et al. 1979), apparently by an immunity directed against both viral and tumor antigens.

Approaches to vaccination against human herpesviruses have been more conservative, most efforts being directed to the testing of recombinant subunit vaccines in animal models. Using recombinant subunit vaccines based on viral surface glycoproteins, protection against infectious virus challenge has been achieved against EBV in a cottontop tamarin model, and against HSV in mice (reviewed in Morein and Merza 1991). While it would be desirable to eradicate both these viruses from the human population, the case for large-scale vaccination is not clearcut.

Recombinant subunit vaccines based on HBV surface antigen have been available since 1982 and have proved effective in reducing the incidence of infection in selected at-risk groups. However, these measures have had little impact on the overall incidence of infection in the population, leading to calls for mass vaccination programs to be extended (Rizzetto and Zanetti 2002).

Vaccines made from ground-up wart tissue have long been used for prophylaxis and therapy of papillomas in animals. This empirical approach illustrates the potential for vaccination against papillomavirus infection, but is clearly incompatible with modern quality-control standards for human medicine. More recently, recombinant gene products of bovine papillomavirus, both early and late genes, have been efficacious in preventing warts and in accelerating rejection (Campo 1995). Translation of these results to human vaccination is complicated by the multiplicity of HPV types and our limited knowledge of the natural history of HPV infections (Galloway 1994).

A number of commercial vaccines have been developed for feline leukemia, including inactivated virus, infected cell extracts, and recombinant subunit *env* vaccines (Jarrett 1994), and attempts to produce a BLV vaccine are also under way.

Although prophylactic vaccination is also considered highly desirable as a means of containing the HIV epidemic, trials of candidate subunit vaccines to date have failed to demonstrate efficacy. Experiments with animal lentivirus models such as simian and feline immunodeficiency viruses have shown only weak protection or short-term suppression of virus replication with conventional subunit or inactivated virus vaccines, and the problem of vaccine escape due to strain variation is significant (Mwau and McMichael 2003).

ANTIVIRAL THERAPY

Another approach to the control of oncogenic viruses (one popular with the pharmaceutical industry) is to try to limit the infection – usually after it is clinically evident – with antiviral chemotherapy. This, too, poses problems inherent in the close symbiosis between viruses and their host cells, because drugs must be devised that are selectively toxic for the virus. One approach is to direct research to the development of inhibitors of processes essential to the virus but dispensable by the cell, such as reverse transcriptase. Within these constraints, some widely used and moderately effective drugs have been developed against, for instance, herpesviruses and retroviruses. These drugs may assist in the control of virus replication but in their current modes of use have shown no beneficial effect on tumor development or on established cancers. For example, the most widely used antiretroviral agent, azidothymidine, was first tested as a potential anticancer agent but its long-term use in AIDS patients does not appear to reduce, and might conceivably increase, the incidence of non-Hodgkin's lymphoma (Pluda et al. 1990).

The use of interferons to control virus-associated cancers was considered an exciting prospect in the last decade but clinical trials have, in general, been disappointing. Striking exceptions have been the effects of interferon-α in virus-associated laryngeal and genital papillomas and in hairy cell leukemias. However, effects other than antiviral activity may be involved in the former examples (Gangemi et al. 1994) while in the latter case the ability of interferon to induce cell differentiation seems to be the most likely mechanism (Vedantham et al. 1992).

Prevention of cell conversion to neoplasia

The problems of tackling viral infection suggest that other potentially avoidable risk factors (see Tables 1, 2, and 3), which tend to operate in association, might provide easier targets for preventive measures. Such hopes, however, seem largely misplaced. In humans these risk factors include habits (notably smoking), dietary factors (which may be even harder to eliminate than smoking unless, like aflatoxin contamination, their deleterious effects are obvious), and other diseases. The most striking example of the last is malaria, a risk factor in Burkitt's lymphoma in certain tropical areas but also a major disease problem in its own right in many parts of the world. Indeed, the reduction or elimination of some of these risk factors would have benefits far beyond the postulated decrease in cancer incidence. This is so self-evident that there must be doubt as to whether the incentive of cancer prevention per se will succeed where other imperatives have failed.

Therapy of virus-associated cancers

Once a virus-infected cell has become neoplastic, the prevention of further tumor growth depends on therapy

rather than prophylaxis. But with virus-associated tumors, the detection of infection at any stage of the disease might itself influence prognosis. In cases in which neoplasia is a rare outcome of virus infection, knowledge of the role played by the virus might identify features characteristic of hosts at high risk. Such knowledge has been used, for instance, in attempts at early diagnosis of nasopharyngeal carcinoma by screening populations at risk in China for EBV-specific salivary IgA (Zeng et al. 1982).

When viral etiology is established, immunotherapy might be considered as a means of achieving regression. Indeed, early experimentation demonstrated that most virally induced tumors were quite readily rejected, unlike those without obvious viral etiology (Klein and Klein 1977). However, these experiments involved the inoculation of immunocompetent animals with tumors expressing readily detectable viral antigens. By contrast, the human malignancies most tightly linked to virus infection are seen in the context of obviously impaired immunity (e.g. AIDS or transplant patients) or express very little if any viral antigens [e.g. HTLV-I-associated adult T-cell leukemia (ATL), Burkitt's lymphoma]. Moreover, the latter tumors may have arisen in individuals with specific deficits in their immunological repertoire, as suggested by associations with specific types of major histocompatibility complex. In these contexts, immunotherapy seems much less promising and it may be necessary instead to consider the means of repairing the defective response. One way to achieve this may be to create tumor cell vaccines by transfection with co-receptor or cytokine genes. Ironically, the retroviruses have recently found a role as vectors to mediate this type of gene therapy for cancer (Vieweg and Gilboa 1995).

Papillomavirus-associated neoplasms have long been treated with extracts of tumors. For example, treatment of bovine ocular squamous cell carcinoma (a common neoplasm in light-skinned cattle in areas of high sunlight) with an allogeneic tumor extract resulted in remissions (Hoffmann et al. 1981). However, it is not clear whether viral antigens were targets in the regression of this malignancy. As discussed earlier, the possibility that vaccines against HPV-16 and 18 might be able to cause regression of cervical carcinoma is now under experimental trial (Frazer 2002).

Finally, if viral genes can be shown to play a role in the maintenance of the tumor state, then direct inhibition of their expression or even their downstream effectors may be a route to therapy. For example, antisense inhibition of NF-κB was found to limit the growth of HTLV Tax-transformed cells in a mouse tumor model, while antisense to Tax itself was ineffective (Kitajima et al. 1992).

CONCLUSIONS

In recent years, research on tumor viruses has identified some of the genetic lesions that underlie neoplasia and has led to some remarkable advances in basic cancer research. These studies are now influencing areas of cell biology and biochemistry that seem far removed from their virological origins. At the same time, however, tumor viruses have become more important to the virologist. We now appreciate that viruses are often one of a complex of risk factors that predisposes to a significant proportion of human disease. Although the part they play often results from an insidious and intractable pathogenic process, they are, in theory, avoidable risk factors. As such, they may provide the key to the management of many cancers.

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The epidemiology of viral infections

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INTRODUCTION

Epidemiology describes the occurrence of illness in populations and, by means of specific study designs, facilitates analysis of the risk factors that determine its distribution. Such investigations are often observational and do not involve experimentation or intervention but, rather, they gather and interpret events as they occur in nature. In the process, the occurrence of diseases is quantified according to their characteristics. Hypotheses concerning etiology and other variables are generated and are tested using statistical techniques. Epidemiology also provides experimental approaches for evaluating the efficacy of interventions such as vaccines to prevent or drugs to treat disease. Infectious diseases were the primary concern of epidemiologists for many years, and concepts were developed to evaluate the infectious process in populations. Now, most epidemiologists work on noncommunicable diseases, and methods originally developed to study acute infections are being used to evaluate etiology and risk factors for conditions such as coronary heart disease and cancer.

Whilst similar methods are now employed to study both communicable and noncommunicable diseases, the unique nature of replicating agents makes certain techniques more relevant to infectious illnesses, especially in examining questions of etiology. Debate has raged about the appropriateness of separating causes of diseases into 'necessary' and 'contributing' (Susser 1973). For example, it is impossible to produce a case of polio without the poliovirus, although contributing factors may be involved in determining the consequences of the infection, i.e. whether disease develops and how severe it is. Epidemiological approaches may also be required to evaluate some of the basic characteristics of infectious agents in humans, such as the infectious dose, which is important in terms of both the amount of pathogen required to initiate infection and the ease and likelihood of transmission. When animal models are available, many of these variables can be estimated. Results may or may not be applicable to humans, so epidemiological studies are needed for confirmation. They are essential when no animal model is available.

When dealing with viral infections (as opposed to those caused by other infectious agents), epidemiological principles are modified only because of characteristics of the pathogens. Most reflect differences in the ability of the agents to persist in the environment (typically more limited with viruses) and the comparative lack of the intermediate hosts that exist with animal parasites. Features such as the possibility of persistent infection and induction of chronic disease and malignancies are not unique to viruses, but are certainly more characteristic of infection with some of them than with other

types of agents. In this chapter, the examination of the epidemiology of viral infections emphasizes the elements that are unique to viruses, but the discussion is not limited to them. More general issues are also covered and viral infections serve as illustrations of the principles and methods discussed.

CLASSIFYING MECHANISMS OF VIRAL TRANSMISSION

Direct transmission

In the cycle of infection, the period during transmission of the agent from one host to the next is when the virus is exposed to the environment and therefore most vulnerable. Systems have been developed to divide the transmission mechanisms of infectious agents into different categories and subcategories. There are classification systems based on portals of entry and exit of the agent to and from the host (Fox et al. 1970). That shown in Table 1 is based on duration and route in transit from one host to the next, but some categories are limited to specific portals such as the skin or the respiratory tract (American Public Health Association 1985). The major division is between direct transmission, which is essentially immediate, and indirect transmission, which is delayed. Direct contact generally implies no exposure of the agent to the environment. This type of transmission occurs during sexual intercourse, as with human immunodeficiency virus (HIV) or hepatitis B, or during direct skin contact, as with herpes gladiatorum, a herpes simplex infection of wrestlers transmitted as they rub their skin against that of an opponent (Belongia et al. 1991). In most cases, virus-containing fluids are involved and the viruses are often cell associated. Direct contact includes, by extension, vertical transmission which may take place transplacentally, during the birth process or in breast milk. All these extended mechanisms are documented for HIV, which can be transmitted directly but is not well transmitted when the agent comes in contact with the environment (Friedland and Klein 1987).

Table 1 *Principal mechanisms of transmission*

Principal mechanisms
Direct (essentially immediate)
Contact (touching, kissing, sexual intercourse, etc.)
Large droplet – limited distance
Indirect
Vehicle-borne
Indirect contact – fomites
Other inanimate objects and biological substances (water, milk, urine, stool, etc.)
Vectorborne
Mechanical – without multiplication
Biological – with multiplication
Dusts and airborne droplet nuclei (aerosols)

Droplets of various sizes are expelled as part of respiratory discharges; the larger droplets, greater than 5 μm in diameter, settle out rapidly and do not spread further than about 1 m, rendering this form of transmission essentially immediate. Thus, even though the previously and the newly infected hosts are not in contact with each other, this is also considered to be a form of direct transmission, because of the limited transit time of this class of infected droplets which do not stay in the air for long periods. Agents potentially destroyed by longer exposure to the environment can still be transmitted by this mechanism. Rhinoviruses are spread by large droplets. As a result, rhinovirus disease transmission, which generally peaks in the autumn when schools reopen, is in fact a summation of mini-outbreaks produced by concurrent dissemination of many different serotypes (Monto et al. 1987). Some large droplets evaporate to become aerosols that may stay suspended and remain infectious for long periods. Influenza is spread by both large droplet and aerosol; a single viral subtype is generally responsible for outbreaks (Couch et al. 1966), in contrast to the relatively limited nature of transmission via large droplets alone. The childhood exanthemata, measles, mumps, and chickenpox, are transmitted mainly by large droplets, which explains why outbreaks may take months to run their course in a partially immune population.

Indirect transmission

Indirect transmission covers many diverse mechanisms that have in common only the fact that there can be a delay, often prolonged, in the infectious agent reaching a new host. Indirect transmission is divided into three categories (Table 1). Vehicles are varied nonliving entities, ranging from inanimate objects to water and biological products such as milk, urine, or human tissue on or in which the infectious agent remains in transit to a new host. Personal inanimate objects, such as combs, blankets, and writing materials serving as vehicles for transmission of infective agents, are termed fomites. Many important viruses can be transmitted by fomites, including the agents of measles, chickenpox, and smallpox, but the period in transit cannot be long. Again, the issue is one of survival during exposure to environmental extremes. Because infections known to be transmitted from person to person can also be spread via freshly contaminated fomites, the definition of a contagious disease was extended to include this route, which is sometimes termed 'indirect contact.' Thus, a contagious disease is one transmitted by direct or indirect contact. In reality, contagious agents are transmitted mainly directly, especially by large droplets. Only infrequently are fomites involved,

because this route is a relatively inefficient means of transmission.

Whereas some viruses transmitted by vehicle are spread in water, urine, and milk, this kind of transmission is more common with bacteria that can survive in the environment for prolonged periods. The spread of picornaviruses (Picornaviruses), other enteric viruses (Human enteric RNA viruses: astroviruses, and Human enteric RNA viruses: noroviruses and sapoviruses) and hepatitis A and E viruses (Hepatitis A and E) has been related to exposure to infected water. These viruses, known to be associated with fecal contamination, may be spread not only by drinking water itself but also by ingesting shellfish which filter and concentrate the virus (Hedstrom and Lycke 1964; Portnoy et al. 1975). Transmission from infected urine occurs with a number of the viruses whose primary hosts are sylvatic, such as the arenavirus Machupo (Arenaviruses). Likewise, hantaviruses such as the agents of Korean hemorrhagic fever and the hantavirus pulmonary syndrome (*Bunyaviridae*), recently described in southwestern USA may be transmitted from infected urine or feces (Johnson et al. 1966; Childs et al. 1994). For these, and for other agents similarly transmitted, rodents are the reservoir of infection. They often live close to or in human dwellings and infect humans via their excreta.

Vector transmission is divided into mechanical and biological categories. Mechanical transmission implies that the vector, typically an arthropod, is involved only in providing transport for the agent, i.e. moving it from one site to another without multiplication taking place. This occurs with certain enteric bacteria, in which the pathogen may adhere to the surface of the vector. It rarely takes place with viruses because the agents would be inactivated by the environmental exposure. No multiplication of the agent occurs, so it is not an efficient form of transmission. In contrast, biological transmission is highly efficient. Most of the large group of viruses transmitted by this mechanism were formerly termed the arthropod-borne or arboviruses (Flaviruses; and Togaviruses). They are now divided into different families, such as the flaviviruses and the togaviruses (Westaway et al. 1985a, b). By definition, arboviruses are transmitted by mosquitoes, ticks, or other arthropods, and, in the process, the quantity of virus increases, thus enhancing the probability of infection. Multiplication takes place in the body of the arthropod, and, for example, is released from the salivary glands during a mosquito bite. There has been speculation that nonarboviruses, such as hepatitis B, might be mosquito transmitted, because epidemiological data suggested that distribution was related to mosquito density (Papaevangelou and Kourea-Kremastinou 1974). However, multiplication has never been demonstrated, so the likelihood of a bite producing transmission is low even if infected blood has been taken up (Berquist et al. 1976).

Infected dusts are rarely involved in transmitting viral infections by the airborne route, because desiccation usually inactivates them if they stay exposed to the environment for prolonged periods. Certain respiratory viral infections such as influenza and coxsackie virus A21 are commonly transmitted by small droplets or droplet nuclei (Couch et al. 1966). The droplet nuclei are formed from large droplets as they evaporate while suspended in the air around a spicule of dust. In most cases it has not been possible to demonstrate by air sampling the presence of virus in aerosols, but this mechanism is assumed to operate because of the ability of a virus to infect large numbers. These are the true viral aerosols: the small droplets are able to stay in the air and remain suspended long enough to infect occupants of an entire naval vessel or aircraft with influenza, or to transmit smallpox from one infected person to another through the air-conditioning system of a hospital (Wehrle et al. 1970; Moser et al. 1979). The small droplets also have the ability to reach into the lower respiratory tract, which influences the character of illness that results.

The issue of whether aerosol spread occurs affects control strategies. Natural smallpox was eradicated by ring vaccination, that is, vaccinating those exposed to a case. However, aerosol transmission may rarely occur naturally and could take place in a terrorist incident. The difference in results of mathematical models about how many individuals should be vaccinated in anticipation of an incident is in large part a result of different opinions about how many susceptibles a case can infect (Fauci 2002).

QUANTIFICATION OF CHARACTERISTICS OF VIRAL INFECTION

Infectious dose and secondary attack rate

Epidemiology provides definitions that help to quantify the occurrence of infection and disease in a population. Experimental infection of laboratory animals is always accompanied by quantification; similarly, for work in human infection and disease, quantification is needed. However, because the situation is typically observational and not experimental, different terms and definitions are used. For example, to calculate the infectious dose required to initiate infection in animals, a 50 percent infectious dose (ID_{50}) is determined by inoculation of serial dilutions. Except for certain self-limited infections such as those caused by the rhinoviruses, such experimental studies cannot be carried out in humans (Tyrrell et al. 1960). Even when experimental infection of humans is ethically possible, there are always questions

concerning their comparability with those that occur naturally. Such questions apply to experimental studies of influenza, where large droplet inoculation in the nose does not mimic the aerosol spread of natural infection in which the lower respiratory tract is exposed.

The epidemiological measure historically used to approximate indirectly the infectivity of an agent in humans is the so-called secondary attack rate (SAR). It is more than a measure of infectivity: it describes numerically the communicability of a contagious disease, which is in part related to the ID_{50} of the virus but is also affected by loss of viability during transmission and the susceptibility of the recipient. An index or primary case is identified in a family or other similar semi-closed group. Cases that subsequently occur in the group during the incubation period (the time between inoculation and development of illness) are regarded as secondary; those that occur before are considered co-primary, i.e. a separate introduction into the group. The denominator for the calculation is based on the total number in the group less the index or co-primary cases. Other variations are possible, especially when using laboratory data to identify those truly at risk of infection or illness – i.e. those without prior evidence of infection such as antibody and therefore susceptible to infection. The classic calculation of the SAR is most appropriate for diseases with relatively long incubation periods, such as rubella and mumps. A problem arises with diseases of shorter incubation period (approximately 2–4 days), such as influenza and the common cold, in which it is difficult to distinguish between co-primary and secondary cases because they are only days apart or may overlap. It is possible mathematically to model transmission: if infection rates are known for sufficient numbers of families to be representative of the community, statistical methods can be used to adjust the observed, or so-called apparent, SAR by the probability of acquiring from the community a new infection that had seemed to be a secondary case (Longini et al. 1982). Among infections of high SAR are measles and chickenpox. Rubella is less contagious, thus leaving residual susceptibles among young women entering childbearing age (Sever et al. 1964).

Pathogenicity and virulence

'Pathogenicity' sometimes compares the capacity of different organisms to cause disease, whereas 'virulence' compares the severity of disease caused by different strains of the same organism (see Pathogenesis of viral infections). Here, these terms are specified somewhat differently. In the epidemiological sense, 'pathogenicity' is the proportion of total infections that produce overt disease, which varies from virus to virus and may be affected by host factors (e.g. prior infection with the agent). 'Virulence' here indicates the capacity of a pathogen to produce severe disease. The likelihood of a fatal outcome is calculated as the case-fatality rate or ratio. It is not a true rate, which should cover a specific period, but is in fact a proportion, the denominator being represented by all recognized cases and the numerator by all deaths included in the denominator. Case fatality is high in many hantavirus and filovirus infections; it still is 100 percent in clinical cases of rabies, with rare and often questionable exceptions (Hattwick et al. 1972; Peters et al. 1994). There have been attempts to quantify virulence other than as case fatality, which has the disadvantage of not being a fixed quantity but one that can be reduced in most situations by appropriate treatment. Also, many diseases with the capacity to produce severe illness infrequently cause death; thus the measure is not a good indication of virulence. A persistent problem in quantifying severity is defining exactly what is meant by this term. In contrast, death as an endpoint is unambiguous. Polio is an illustration of the relative independence of pathogenicity and virulence; the disease is potentially very severe or virulent if it occurs, but only 0.1–1 percent of infections are symptomatic. Therefore the virus would be considered of relatively low pathogenicity but of relatively high virulence (Melnick and Ledinko 1951).

OBSERVATIONAL EPIDEMIOLOGY

Viral infections in person, place, and time

Many epidemiological studies of infections involve description of the occurrence of disease and identification of factors that appear to be related to their distribution. Description is the first phase of an epidemiological investigation. It accepts that there is in fact an infection problem and permits development of hypotheses concerning etiology, risk factors, and other variables determining distribution. These relationships may be investigated further in analytical studies. Occurrence of the infectious disease can be described on the basis of a number of criteria. For some conditions, a case definition may be used: a list of signs and symptoms that are required before a case is recognized. Laboratory confirmation may or may not be included in this definition. Sometimes the aim is to identify inapparent infections as well as cases of disease. Here the criteria may be based on laboratory data only, such as virus identification or significant change in antibody titer.

PERSONAL FACTORS

Descriptions of infections or diseases are made in terms of their occurrence in person, place, and time. Whilst temporal, geographical, and personal factors are interrelated, it is useful to examine each separately as a

systematic way of describing the occurrence of an infectious disease. Age is the most important personal determinant of disease because of development and ageing of the immune system, accumulation of exposures over time, and behavioral changes that affect the likelihood of exposure to infectious agents. It is impossible to generalize how age affects pathogenicity or virulence. Hepatitis A and polio are typically mild in young children and become increasingly virulent as children become young adults. Polio emerged as a recognized problem in northern and western Europe only at the end of the nineteenth century. It was called infantile paralysis because it infected mainly the very young, but as time passed it also caused disease in older children. In developing countries the process is being repeated; polio infections often occur in the first 6 months of life, modified by the universal presence of maternal antibody, but the disease is now also present in older children (Nathanson and Martin 1979). Hepatitis A has had a similar progression as the age at the time of infection has changed. In contrast, respiratory viruses such as respiratory syncytial virus (RSV) and parainfluenza produce the most severe disease in young children. In the USA, the median age of infants hospitalized because of RSV is 3 months (Parrott et al. 1973).

The proportion of those infected who develop overt disease also falls with increasing age, in part as the result of repeated exposure to those agents that have the capacity to reinfect with regularity (Monto and Lim 1971). Previous exposures result in a spectrum of outcomes ranging from asymptomatic infection to disease of diminished severity. Other personal factors affecting outcomes of infection are listed in Table 2.

Position in the family affects the occurrence of infections best transmitted in that setting (e.g. the respiratory viruses and enteric agents such as the rotaviruses). An only child in a family will experience lower rates of respiratory infection until other siblings are born (Monto and Ross 1977). Other exposures, such as in day care, may increase the probability of the youngest child acquiring an agent which can then be introduced into and spread within the family (Bartlett et al. 1985). There is evidence that this pattern of occurrence is common with hepatitis A; in fact, community-wide outbreaks of hepatitis A have frequently been traced to day care transmission (Matson et al. 1989). Occupational exposure plays a role in the acquisition of, for example, sylvatic or jungle yellow fever by people working in the forest. The risk from rabies among veterinarians is another type of occupational exposure, but such infections do not result in further dissemination.

Table 2 *Examples of personal factors important in viral illness*

Personal factors
Age and gender
Position in family – birth order
Income
Education
Crowding
Socioeconomic status
Occupation – exposures
Genetic and related factors
Immunity
Nutrition

FACTORS RELATED TO PLACE

Place of occurrence of viral infection may be related to purely local biological or physical factors. Regional characteristics such as climate and resident flora and fauna determine whether transmission can take place. Most arboviruses are found in specific locations, determined by the distribution of the appropriate vectors or animal hosts. Sometimes the range of a particular agent is quite limited, minor ecological variation producing a niche where transmission can take place. Similarly, agents that are transmitted mainly among animals are limited to the range of that particular animal. Humans are often infected only incidentally, having been in the area where transmission is occurring to the animal hosts or vectors. Infections of animals in which humans are not involved in the major cycle of transmission but are occasionally infected are termed zoonoses.

Other physical or biological environmental factors determine whether common source outbreaks, such as those produced by hepatitis A or E from contaminated water, can occur at a particular location. Generally, special situations in the area, such as sewage outflows or substandard wells are responsible. The presence of an individual in certain locations such as a hospital could result in transmission of hepatitis B in those parts of the world where nosocomial transmission still occurs. In certain areas of Africa where the Ebola virus is present, being in a hospital adds greatly to risk (Baron et al. 1981). Events seemingly associated with place are often equally related to person or to time. Clusters of illnesses are sometimes detected, indicating that transmission of infection is taking place in a particular location. For a cluster to be recognized the transmission must occur over a limited period of time. Such a cluster may involve everyone in that location or may be limited to certain individuals. Reports of clusters are an important alert that unusual transmission is taking place. The first cases of acquired immune deficiency syndrome (AIDS) were detected as simultaneous clusters, in several cities, of a disease producing Kaposi's sarcoma or opportunistic infections in young homosexual men (Gottlieb et al. 1981; Friedman-Kien et al. 1982). The existence of these clusters focused attention on an infectious etiology of the disease, which at the time was thought by some to have other causes such as exposure to environmental toxins or to drugs. HIV transmission was later found to be the explanation.

TEMPORAL FACTORS: CLASSIFICATION OF OUTBREAKS

Time of occurrence may be the most systematically evaluated of the three factors. Much of the terminology used in epidemiology has a temporal component. The terms epidemic, endemic, and sporadic do not have any statistical definitions but simply mean, respectively, occurrence of an infection at a higher than expected frequency, at the expected frequency, or only rarely and in seemingly unconnected fashion. Another term frequently used, also without statistical definition, is pandemic, meaning an epidemic appearing in many geographic locations at about the same time. The term has been used for influenza outbreaks occurring on a global scale following appearance of variants with a new hemagglutinin or a new hemagglutinin and new neuraminidase. It is now appropriately applied to HIV/AIDS which has involved all continents in its inexorable spread (World Health Organization/Global Program on AIDS 1994). The years this has taken contrasts with the months needed for an influenza pandemic to involve the whole world. This contrasting pattern is related to differences in transmission characteristics between HIV, spread by direct contact, and influenza, transmitted by droplet and airborne routes.

The characteristics of outbreaks are described according to the occurrence of cases over time. These characteristics are generally studied by creating an epidemic curve, with cases on the ordinate and time (in days or hours, depending on the speed of development) on the abscissa. Figure 1 shows the pattern of a point source outbreak of Norvovirus infection in which all individuals were exposed at the same time and either became infected and ill with diarrheal disease or remained well (Warner et al. 1991). The group exposed was large (3 000 people) and the attack rate, at 48 percent, was high. The times of onset were divided into 4-h intervals. The suspect meal in question was consumed at noon on Monday; illnesses reported earlier are viewed as baseline cases of diarrhea unrelated to the exposure. Such cases are typical in investigations of a common illness such as gastroenteritis, which can be produced by many agents. The time from the exposure to the peak of the outbreak is the mean incubation period of the pathogen, in this case 40 h. The peak occurred during the 4-h period beginning at 5 a.m., Wednesday.

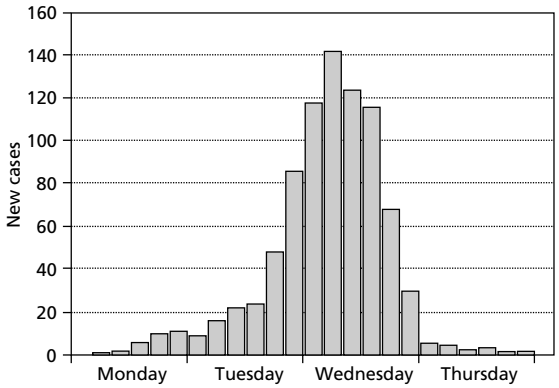

Figure 1 *A point source outbreak of enteritis attributed to Norvovirus after a meal eaten at noon, Monday. Onset of illness (moving means) shown in intervals of 4 h (from Warner et al. 1991).*

If the source remains infectious for a time, the outbreak may continue. The new cases are still being infected from the source and the outbreak may now be better termed common rather than point source. A contaminated well is an example of a common source. With viruses such as hepatitis A or Norwalk-like agents, cases may arise in lower numbers even when the source is no longer infectious. Such continued occurrence is possible only for viruses that can also spread from person to person, a different type of transmission. Pure point source outbreaks assume exposure from one site or at one point in time, the time from exposure to peak being the median incubation period. In contrast, in propagated source outbreaks the infectious agents spread exclusively from person to person. Spread may be by different transmission mechanisms, often large droplet. Even with influenza, which may be transmitted by the more efficient airborne route, one person can infect only two or three individuals, and the outbreak takes 6–10 weeks (or more) to move through a community. It should be remembered that the incubation period of the infection is only 2–4 days, so many generations of spread occur until the outbreak has run its course (Monto and Kioumehr 1975).

Many infectious diseases such as hepatitis A and rubella do not occur at uniform levels in populations but undergo predictable changes in frequency. Certain terms are used to describe these changes. Seasonal variation describes the phenomenon, well recognized with the respiratory viruses, of much higher frequency in colder than in warmer months. Other seasonal variations are less obvious, with rubella generally increasing (before vaccine control) in the spring and hepatitis A in the fall. In temperate zones, transmission of rotavirus takes place almost exclusively during cold weather. Figure 2 shows 5 years of data from the study of young children hospitalized for diarrhea in Washington DC (Brandt et al. 1983). Hospitalizations over all are most common in colder months. Cyclical variation refers to changes of frequency over periods of years, with gradual increases in intensity of transmission. Hepatitis A, for example, has a cycle of 5–7 years. This means that, when intensity of transmission is increasing, illness in the autumn is more frequent than in the previous autumn. The term 'cycle' also implies that there is not only a waxing but also a waning period. In contrast, the term 'secular' is used to indicate long-term trends, which can be unidirectional in nature. The time periods involved must be long, generally more than 10 years.

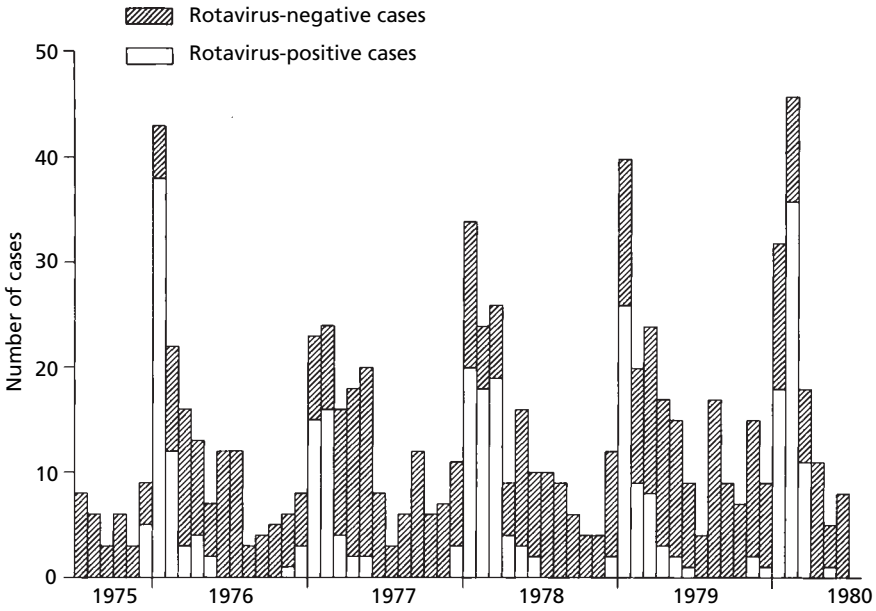

Figure 2 *Distribution by month over a 5-year period of children hospitalized for gastroenteritis and the proportions rotavirus-positive. Children's Hospital National Medical Center, Washington DC (from Brandt et al. 1983).*

Incidence and prevalence

INCIDENCE

Two critical terms used in epidemiology that have temporal definitions are incidence and prevalence. 'Incidence' is the number of new cases of a disease arising during a fixed period in a population of given size; for example, 1 000 or 10 000 individuals. Incidence is sometimes further specified as either a cumulative incidence which expresses incidence as a risk or probability, or as an incidence density which does so as a rate.

These terms, whilst often used interchangeably, provide two types of information (Kleinbaum et al. 1982). Risk is the probability of a disease-free person developing the disease of interest over a defined time and not dying from any other disease during that period. As a probability, risk can have values between zero and one. Risk is measured as cumulative incidence, the proportion of the study population becoming ill in a given period: for example, 'the risk of becoming ill with influenza-like illness in the study population was 12.8/1 000 persons per year.' The need to specify a period can be appreciated by looking at the extreme case of mortality from all causes. If a cohort of 1 000 subjects is followed for 120 years, the risk of dying during this period will be 1 000/1 000 per 120 years. However, during any 1 year of this period, the risk of dying will be much smaller. Thus, when applied to a vectorborne infection, risk of acquiring dengue over a 4-month mosquito season in an endemic area might be 10/100, but only 1.2/100 for a given week. The term attack rate is a cumulative incidence measure used to describe the number in a defined population who become infected or ill during the period of transmission of an agent; for example, the number of children infected with measles in the course of a school outbreak, using the number of susceptible children as the denominator of the proportion. This is more properly termed a risk, and is an example of cumulative incidence. The secondary attack rate, described above, is also an example of such a risk.

In contrast to risk, the incidence rate, sometimes called incidence density, describes the occurrence of new cases in the population per unit of time. Incidence density is most useful for testing epidemiological hypotheses when a long time is needed to follow a cohort to estimate risk, as in most chronic conditions and in some population-based studies of infection. The rate over a period of time is typically estimated using person-time as the denominator of the rate calculation. Person-time is the amount of time each study subject contributes to a study before becoming ill or leaving the study for some reason. Sometimes new individuals may be added to the study population. Figure 3 illustrates the calculation of person-time. Among ten people who were followed over differing periods of time, two cases of disease developed. In the incidence density calculation, eight subjects completed the study, each contributing 12 person-months or a total of 96 person-months. Subjects 3 and 7 contributed 6 and 10 person-months, respectively, before becoming ill. The number of new cases (two) divided by the total number of person-months (112) yields a rate of 2/112 or 1.78/100 person-months. Dividing person-months by 12 would give person-years. Thus, the above translates to a rate of 1.78/8.33 person-months or 21/100 person-years. The same data could be used for an incidence density calculation for ten subjects at risk. The data would give a rate

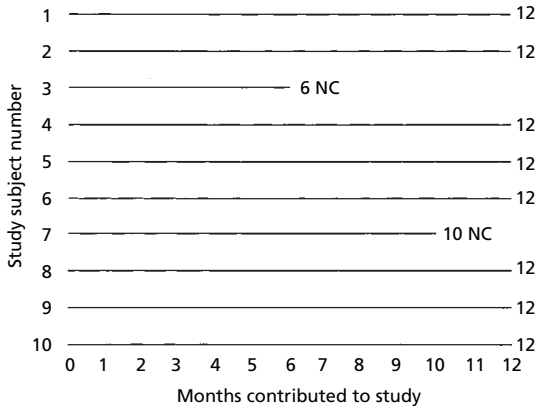

Figure 3 *Hypothetical 1-year study of ten individuals. Two new cases (NC) of disease developed during this period, giving a risk calculation of 2/10 per year and a rate calculation of 2.1/10 person-years.*

of two new cases per ten persons at risk, or 20/100 per year. The differences between the risk and rate calculations, while small, would have been greater with more cases developing or if the study had been carried out for a longer period.

PREVALENCE

The other important term, prevalence, is used less often when dealing with acute illnesses. Most often, the concept is defined as point prevalence, the point here being an arbitrary point in time. In prevalence, it is not new but existing cases that are identified, thus the ideal would be to enumerate them as a simultaneous count. Again the denominator is the population at risk from which the cases are drawn. Enumeration of all cases of AIDS existing in the population of interest at one time, whatever their date of onset, would provide a prevalence rate (Elandt-Johnson 1975).

Prevalence is often used in viral epidemiology when doing serological surveys based on collecting one specimen per person. Serum banks have been established to reflect past experience with infectious diseases in a population. Ideally, the specimens should be representative of the population from which they have been drawn. Antibody present at a specific titer in a population sample collected to represent all age groups will indicate the pattern of past infection. For certain agents, such as arboviruses present intermittently in a geographical area, and for other more common agents such as measles in isolated populations, age-specific prevalence will indicate when infection was last present in that population, the years of birth of the youngest cohort with antibody indicating the last period of spread of the agent (Black 1989). Documenting the occurrence of influenza A subtypes over the last century is another example of use of antibody prevalence. Antibody present in sera, collected before the appearance of a new subtype of type A, has been taken as evidence for the spread of that subtype many years before (Masurel and Marine 1973). When seroepidemiology is carried out with paired sera, change in antibody titer can be sought between two specimens collected from the same individual. The results are the equivalent to incidence, not prevalence, because they indicate that a new infection has occurred in the interval. This is also the case when examining IgM antibody in a single specimen for agents such as hepatitis A, known to produce that antibody for only a short time and thus indicating recent infection.

Monitoring and surveillance

The descriptive variables time, place, and person are employed in monitoring and surveillance of viral disease. For infections that occur regularly the intention is to detect unusual increases. The expected frequency of these conditions, identified either by laboratory tests or on the basis of standard case definitions of illness, can be calculated. An example of the latter involves the use in the USA of reported deaths from pneumonia and influenza (P&I) from 121 cities. The reports are based on a list of specific causes of death, not on the laboratory detection of influenza infection, and are calculated weekly as a proportion of total deaths. Results of the method are shown in Figure 4, with distribution of isolates by type in each of the influenza seasons. The P&I mortality curve has three components: the expected value, the threshold value (1.65 S.D. above the expected) and the actual values observed in the various cities. The expected values are a mathematical expression of past observed values derived from observations of periods without influenza transmission (Serfling 1963). They are higher in winter months than in summer, because of other noninfluenza causes of pneumonia deaths that increase at the same time, such as those caused by primary bacterial infections. As can be seen in Figure 4, in some years the observed mortality considerably exceeds the threshold level for several weeks. Typically, these are years in which there is considerable transmission of type A(H3N2) virus (Eickhoff et al. 1961). In some years (not shown in the figure), excess mortality has also occurred with type B transmission, but this is observed less frequently (Nolan et al. 1980). When examining the data in Figure 4, it is important to realize that the mortality data are the result of clinical diagnoses at the time of death, i.e. without laboratory evidence that the death was related to influenza. Thus the data for laboratory surveillance on which the annual distribution of isolates is based can be viewed as an independent determination. Only influenza produces this characteristic increase in mortality. The phenomenon is remarkable because the data are collected nationally in a country as large as the USA, in which outbreaks do not necessarily occur synchronously in different regions (Glezen et al. 1982). Similar systems

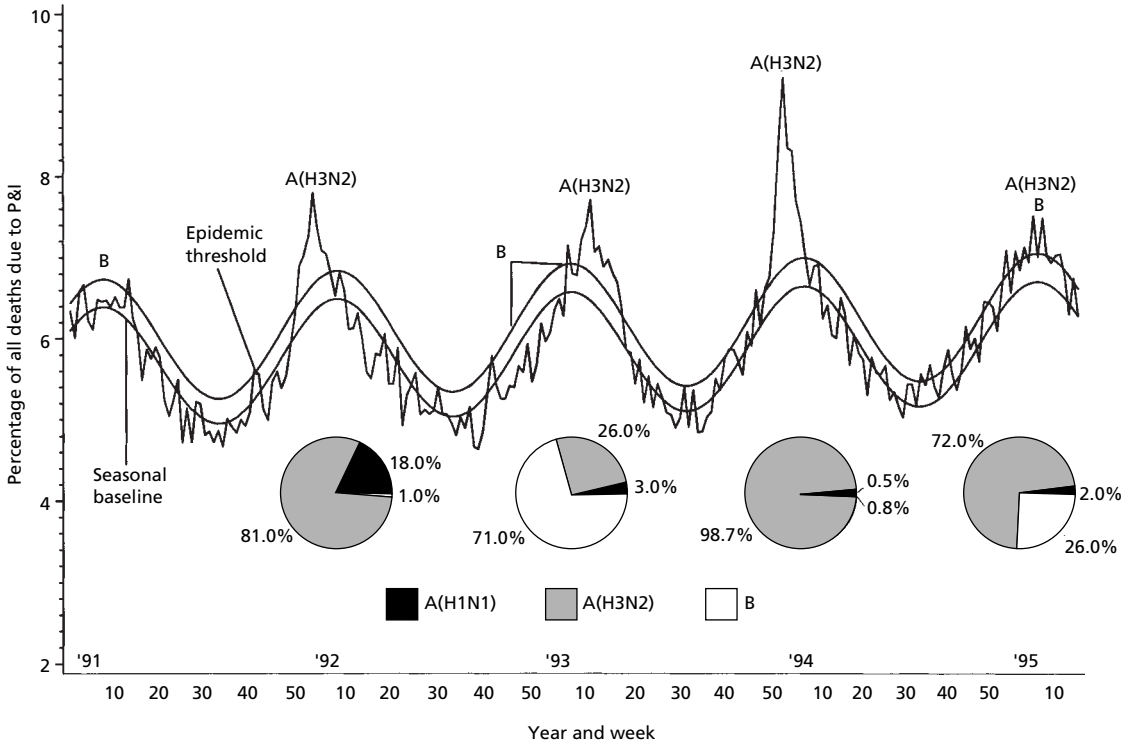

Figure 4 *Data on pneumonia and influenza mortality for 121 US cities, collected by the Centers for Disease Control. The curve indicates the observed mortality data, superimposed on the seasonal baseline and epidemic threshold. Also shown are the distributions of influenza isolates from specimens collected independently during the four seasons.*

are in use in other parts of the world, the data on virus circulation being based on collection of specimens in standard surveillance systems based in physicians' practices (Johnson et al. 1991).

Other diseases only rarely increase in frequency sufficiently to become noticeable in data collected nationally. Most infections are likely to be more localized, and the detection of outbreaks is based on the recognition of the unusual occurrence of cases in time, place or person. Outbreaks are variously defined according to expected occurrence; for some entities a single case may be so unusual as to produce an outbreak investigation. Rabies cases in areas of developed countries where this disease is unusual will be investigated on the basis of a single event. The virus causing hantavirus pulmonary syndrome was recognized and identified as a newly emerging pathogen in southeastern USA on the basis of deaths and severe illnesses clustered in time, place, and, in part, person (Centers for Disease Control 1994). The regional nature of the outbreak contributed to its recognition; had the same number of cases occurred in a wider area, they would have gone unnoticed. As with other similar situations, prospective surveillance initiated after identification of the 'new' virus suggested that it had occurred at low frequency in the past, and that recognition was due to an unusual natural event that produced a cluster of cases; in the case of the hantavirus pulmonary syndrome, the increase in food available for the rodent hosts resulted in their proliferation and an increased likelihood of interfacing with humans. The spread of West Nile virus through much of the United States is an example of a contrary situation. It represented a new introduction into a continent where it had not existed before and was originally recognized because of the clustering of cases. However, the extensive subsequent spread is a result in part because of lack of immunity in the population (Petersen et al. 2002).

Primary analytical methods

CASE–CONTROL STUDIES

The descriptive phases of epidemiological investigations generate hypotheses about factors influencing the occurrence of a disease. Analytical studies then follow, and may involve formal outbreak investigations or more elaborate longitudinal studies. The investigations have in common the use of specific study designs. These designs are divided into case–control and cohort studies. In the first, cases of the disease or outcome being studied are identified and controls without the particular outcome are selected by matching or similar methods to be comparable to the cases in age and other characteristics. Past exposures or risk factors are then examined to determine if they are associated with the disease (Schlesselman 1982). In contrast, in the cohort design indivi-

duals are selected according to characteristics other than presence of the disease or infection of interest. They are followed over time and cases of the disease outcome are observed (Breslow and Day 1987).

Case–control studies can be relatively straightforward in their design and produce results quickly because all the exposures and outcomes have already occurred. The investigation of an outbreak of an infectious disease is generally case–control in nature, one group having the disease and the comparison group not having it. For example, an outbreak of vomiting and diarrhea occurred in a group outing on an American lake where food was served in common and where many had gone swimming (Koopman et al. 1982). Individuals who developed disease according to a case definition were compared with those who did not. The food intake and other exposures of the cases and the controls were then compared. There was no significant difference between cases and controls in terms of foods consumed or other relevant characteristics. However, swimming in the lake, especially with the head submerged, was the risk factor that was more common in those who developed diarrhea compared to those who did not, and the differences were statistically significant. Furthermore, some samples, when available, showed rises in titer for the Norvovirus, suggesting that it was the agent responsible. In this sort of outbreak investigation, the group studied is small. However, it is very important that the cases and controls have similar possibilities of exposure; in this case, people not at the lake would be ineligible.

Larger and more elaborate case–control studies were used to confirm the role of rubella as a cause of congenital anomalies. The original description of clusters of congenital anomalies involving the eyes, and then the heart, ears, and other organs occurring in children whose mothers had experienced rubella in pregnancy allowed development of an etiological hypothesis (Gregg 1941; Swan et al. 1946). The clusters seemed to be related to the epidemic behavior of rubella, since frequency of the abnormality increased cyclically when rubella was widespread (Sever et al. 1965). Many of the initial studies were simply a series of cases. For the analytical studies, cases were children with certain types of abnormalities identified at birth or shortly thereafter and controls were children without abnormalities born in the same locations. Pregnancy histories of mothers of both case and control children were obtained; because rubella and other conditions of interest do not generally result either in a doctor being consulted or in hospitalization, data are generally obtained by interviewing the mothers. This has a disadvantage of introducing potential recall bias: mothers know whether their children had birth defects or were normal and, if the former, are more likely to remember every event that might possibly have been related to the defect (Klemetti and Saxen 1967). Methods can sometimes be developed to try to validate these reported events. If possible, medical or other records collected before the birth are used. By their very nature, case–control studies are nearly always retrospective: cases and controls are identified on the basis of the outcome, and the prior factors determining the development or nondevelopment of the disease are compared. Table 3 lists some of the advantages and disadvantages of the basic study designs. Accurate recall of previous events is an inherent problem with case–control studies because of knowledge of the outcome. The design has other attractive features: it is highly cost efficient, especially for outcomes that are relatively rare. Cases can be gathered easily and, because they have already occurred, the study can be carried out quite quickly. Another inherent problem of the design, however, is the selection of controls. Controls should be similar to the cases in all characteristics except the disease in question. This is often difficult to achieve and questions are always raised about this point. Typically there are two choices of populations from which to draw controls: hospital databases or the community. Each has its own advantages and disadvantages. In the study of infectious diseases, controls must have similar probabilities of exposure to the infections as the cases. In terms of, for example, rubella, cases and controls must have the same opportunity to be exposed and to experience rubella, which varies by both time and place in intensity of transmission. The case–control studies concluded that there was a high level of association between rubella in the first trimester of pregnancy and

Table 3 *Advantages and disadvantages of study designs*

Advantages	Disadvantages
Case–control	
Efficiency – results rapidly available	Selection of comparable controls
Superior for rare outcome	Recall problems
Relatively inexpensive	Validity of recorded data
Cohort studies	
Reduction in potential bias	Duration – may be long
Greater ability to generalize results	Greater expense
No necessity to select healthy comparable controls	Uncertainty of adequate numbers and loss to follow-up
	Rare diseases require large numbers

fetal malformations. That association was very strong and suggested that the risk of fetal malformation was greater than 50 percent in some studies, in one case 80 percent (Pitt 1957; Ingalls et al. 1960) (see also Rubella).

COHORT STUDIES

Cohort studies are generally undertaken after case–control studies indicate the likelihood of an association between a particular exposure and disease outcome. With rubella, these studies began relatively early, because of recognition of biases associated with obtaining histories on the cases. Such investigations are typically prospective in nature, and allow collection of data as new events occur, thus minimizing the possibility of recall bias. However, they must generally be larger with sufficient participants to allow for reasonable numbers of dropouts, and must last long enough to achieve the necessary numbers of events. As a precondition, the outcome of interest must be so frequent that the group followed does not need to be too large or followed for too long. For all these reasons, cohort studies are carried out less frequently than case–control studies because they are more expensive and problematic in terms of producing the required number of observations. However, when feasible, they are powerful evidence for confirming results of case–control studies, as many sources of bias can be avoided and there is not the problem of selecting comparable controls.

In this design the cohort is the population unit selected for study. The only requirement is that the selection cannot be on the basis of disease outcome or anything that might be closely related to the disease. Typically the unit may be individuals in an occupational group, such as nurses or physicians or people living in a certain area. In the case of rubella, two kinds of cohort studies have been carried out (Manson et al. 1960; Sever et al. 1969). In the most powerful design, all children born during specific periods in particular hospitals were studied. Because all children born at the appropriate time were eligible, there was no need to identify controls, and all adverse outcomes of pregnancy could be followed in the cohort. Some mothers would have been exposed to rubella and might therefore give birth to children born with malformations. Most would not.

Another cohort design attempts to minimize the number of nonexposed women who would have to be included. This method identifies an exposure cohort (mother exposed to rubella) and a comparison cohort of mothers not exposed. Note that the selection here is based not on the outcome, congenital abnormalities, as would be the situation in a case–control study, but on exposures, so this remains a cohort study. There is no issue of recall bias because the comparison group is selected on documented lack of exposure. However, it has the potential problem of not being as representative as if all individuals, rather than a selected subgroup, were followed up. Estimates of the risk of maternal rubella in the first trimester in producing malformations was as low as 7–17 percent in some cohort studies, although others felt that, depending on length of follow-up and definitions used it might actually be higher (Jackson and Fisch 1958; Lundstrom 1962). This reduction in risk estimation had public health implications before the vaccine era, because pregnancies were often terminated after maternal rubella. From an epidemiological standpoint, cohort studies are better than case–control studies in providing data and results that can be generalized to the population of interest so that appropriate decisions can be made.

OTHER USES OF ANALYTICAL STUDIES

Although vaccines are usually evaluated by randomized controlled trials, an experimental study design involving an intervention, observational designs can also be employed successfully to evaluate the effectiveness of vaccines. The term ‘efficacy’ is generally employed for results of randomized trials; ‘effectiveness’ is used to indicate the results of observations when the intervention is in general use, especially when the outcome is not a laboratory-confirmed infection but a clinical diagnosis. The latter sort of study is the only type possible when, as is the case in North America for influenza vaccine for the elderly, ethical considerations preclude use of a placebo and questions still exist as to the value of the preparation. The observational design does not require that vaccine be denied to part of the group, as would be the case if a placebo were employed. The purpose of using inactivated influenza vaccine in older people is to prevent serious complications of the disease, especially those requiring hospitalization. However, unless a large population can be followed, these hospitalizations do not occur sufficiently often to allow the use of the cohort design. For this reason, two multi-year case–control studies were carried out, one in the USA and the other in Canada (Foster et al. 1992; Fedson et al. 1993; Ohmit and Monto 1995). The exact designs varied, but in both cases the influenza season was identified and possible influenza complications were defined from hospital records. Recognizing the need for generalization of results, both studies were designed to select their controls not from hospitals but from comparable community residents. In both, the vaccine was found to be significantly effective in preventing hospitalization, and, in the Canadian study, in preventing deaths in the influenza seasons coded as being related to respiratory diseases. Another study used a cohort design to confirm these findings (Nichol et al. 1994). As expected, many people needed to be followed up. The effectiveness of the vaccine was comparable with that in the previous case–control investigations. That several differently designed nonexperimental studies came to similar

conclusions is convincing evidence that the results in a single investigation were not simply a chance occurrence.

Observational studies to confirm etiology

Koch's postulates in their various forms were the first systematic approach for determining that a pathogen caused a disease (Rivers 1937). They were formulated after early observations that simply isolating an agent from an individual with a particular disease did not necessarily mean that the agent caused that disease. The postulates were based on experiments, provided that disease could be reproduced in laboratory animals. They did not allow for asymptomatic infection because they stated that the agent should always produce disease. They also assumed that the potential pathogen had a unique role in producing a disease: only one hepatitis virus could produce hepatitis. We now know that clinical syndromes can frequently be caused by a variety of agents and that clinical manifestations of infection vary greatly in terms of age and other factors, so it might not seem that the same pathogen was involved. In addition, many viruses may not infect experimental animals at all or, if they do, will not produce diseases comparable to those in humans. For this reason, Koch's postulates have been complemented (or modified) by epidemiological studies, usually observational, to confirm etiology. Typically, case–control designs are used, because they are easy to carry out and require smaller numbers of observations. Studies of this sort are particularly necessary when dealing with agents that produce chronic infections.

Another problem occurs when a potential pathogen is shed for prolonged periods from people who have had an illness in the past, or after an asymptomatic infection. Agents may be isolated from individuals who develop another illness, and thought, incorrectly, to cause the current instead of the preceding illness. This misconception can occur with the herpesviruses. At the very least, an agent must be identified significantly more frequently in cases with the disease than in comparable controls. This analytical design assumes that asymptomatic or chronic infection will occur and that some controls will be virus positive, but not as frequently as cases. Etiological association of infectious mononucleosis with Epstein–Barr virus (EBV) was difficult to accomplish, because of continuing shedding of the virus (Hallee et al. 1974). Even more difficult has been the demonstration of the association of hepatitis B virus with primary hepatocellular carcinoma, because of the long period between infection and development of the disease (Beasley 1982).

When observational approaches, rather than experimentation, are used to demonstrate etiology, additional standards are generally applied to assist in deciding that there is a statistically significant etiological relationship between a disease and an agent or other potential precipitation factor. These standards were developed to evaluate the relationship between environmental exposure and chronic diseases, specifically cigarette smoking and lung cancer. They apply equally to viral infection (Hill 1965). There must always be a proper temporal relationship: the putative etiological factor is present before the disease occurs. This point is not particularly difficult to understand; in fact, there is often a tendency to assume an etiological role on the basis of a simple temporal relationship. It is further required that the etiological agent must have a scientifically logical role in the disease it is thought to cause. In other words, hepatitis B virus has a logical role in producing chronic liver disease, whereas it would be difficult to hypothesize such a role for the influenza virus. Strength of the association is also critical; i.e. if the associations are strongly statistically significant, an etiological role is more likely. Most critical, as illustrated by the examples given above, is reproducibility of results. Consistent results from different study designs are strong evidence of an etiological association.

The road to demonstrating etiology involves an initial hypothesis and a series of studies. Such a road can be followed in the demonstration in North America of the role of aspirin in the production of Reye's syndrome. The syndrome is a combination of encephalopathy and acute liver abnormalities involving mitochondrial damage. It was recognized for many years to be an unusual complication of various infections, mainly influenza and chickenpox, occurring a few days after the acute illness. Why it happened in a small proportion of infected children was unknown. Descriptive studies suggested that aspirin ingestion might be involved, but this notion was rejected by many investigators because of lack of a clear dose–response relationship (Starko et al. 1982). However, the characteristics of aspirin intoxication provided a credible scientific basis for the relationship. A series of independent case–control studies demonstrated such a statistically strong and consistent role for aspirin that the last study was suspended in the pilot phase and an educational program was begun (Halpin et al. 1982; Waldman et al. 1982; Hurwitz et al. 1985). Cohort studies were not carried out because Reye's syndrome was too rare to allow use of that design and the associations in the case–control studies were so strong.

EXPERIMENTAL EPIDEMIOLOGY

Simulations

Although most contributions of epidemiology to our understanding of viral infections are observational, experimental approaches are often useful in understanding relationships not defined by other methods. Two will be considered: one involving actual interventions and the other involving simulations. The latter

method has mainly been applied to modeling transmission and epidemics mathematically and identifying the effects of various interventions that might be used to prevent or interrupt the outbreaks of infection. Mathematical models have evolved from rather simple approaches ranging from the use of balls to simulate individuals (Monte Carlo models) to elaborate computer structures designed to resemble a community with families and schools that mix with each other. The two basic types are deterministic models, which use differential equations, and stochastic models, which use random number generation to approximate the manner in which events take place naturally (Abbey 1952; Bailey 1957). The latter type of simulation is now easier with use of powerful computers so that many replications can be carried out. One of the first infections to be studied with these methods was influenza. Simulations were performed to determine the effects of, for example, vaccinating school age children with influenza vaccine (Elveback et al. 1976; Longini et al. 1977). As demonstrated in one experiment, vaccination of school age children increased immunity, sometimes termed herd immunity, in the segment of the population most responsible for introducing the virus into families and spreading it in the community (Monto et al. 1970). The result was not only protection of the vaccinated children but also interruption of transmission, which indirectly protected the rest of the community. Simulation modeling has also been applied to HIV transmission and AIDS forecasting, and has been particularly valuable, given the long-term nature of the infection and the difficulty in predicting spread (Hethcote et al. 1991). Most recently, it has been especially useful in assessing the effects that vaccination could have on the future occurrence of the disease.

Controlled trials

The most accepted intervention study in infectious disease epidemiology is the randomized controlled trial. Traditionally, the intervention has been vaccination, although more recently antivirals have frequently been studied. Methods for randomized clinical trials have been the subject of a number of reviews and comments, and the many issues and approaches will not be discussed here (Spilker 1991). The endpoint for acute illnesses is generally prevention of the disease. The efficacy of hepatitis B vaccine was evaluated by antibody production and prevention of acute disease in populations of homosexual males (Szmuness et al. 1975). The design was a randomized, controlled trial. The group chosen for study was at high risk of infection and the study could be concluded in a short period of time. Potentially eligible participants were screened and only those without antibody were included. The frequencies of infection and disease at various periods after vaccination were compared in those who received the active preparation and those who received placebo. The results demonstrated the value of this vaccine in primary prevention. In addition to protecting against acute infection in adults living in developed countries, the vaccine is now used in developing countries, not only to prevent the acute illness but mainly to prevent primary hepatocellular carcinoma. If this tumor decreases in frequency as a result of vaccination, it will be ultimate proof of etiology. Similarly, with Reye's syndrome, education about the role of aspirin in the disease resulted in decreased use. Reye's syndrome has nearly disappeared in areas where it was previously common, adding further evidence for an etiological connection (Remington et al. 1986). Certain potential etiological relationships have bedeviled investigators for many years. It was thought that certain viral infections trigger attacks of asthma in sensitive children. The data are better for children than for adults, and, in both, rhinoviruses seem to be the virus most frequently involved (Mcintosh et al. 1973; Minor et al. 1974). Development of PCR techniques which can identify rhinoviruses which cannot be cultivated has helped to document this relationship (Johnston et al. 1995a). A similar situation applies to triggering exacerbations of chronic respiratory disease in the elderly although the relationship here is more tenuous. If use of an antiviral to prevent or treat rhinoviral infection also reduces episodes of the chronic condition, it would be the final demonstration of an etiological relationship.

EPIDEMIOLOGICAL CHARACTERISTICS OF RESPIRATORY VIRUSES

Causes of common respiratory illnesses

Many viruses are responsible for the bulk of respiratory infections. Although the agents are very different from a virological standpoint, they do share epidemiological characteristics. Being surface infections, they do not produce long-lasting immunity. Instead they have the capacity to reinfect, even without antigenic change (Glezen et al. 1986). Other than influenza (Orthomyxoviruses: influenza), which can produce severe disease in older children and adults, some viruses such as parainfluenza and RSV (see Respiroviruses: parainfluenza virus), may cause severe disease in infants and young children on initial infection and mild or inapparent infection indistinguishable from the common cold in older people. Thus, older siblings who introduce a particular infection to the family may contract a mild illness, while the younger child may experience bronchiolitis or pneumonia. RSV is the principal cause of severe lower respiratory illness in young children. The mean age for hospitalization, which occurs in a small proportion of infected children, is 3 months

(Parrott et al. 1973). This early age of onset prompted the hypothesis, later proven to be incorrect, that maternal antibody, which is always present to this ubiquitous virus, is responsible for the pathogenesis of the disease. It has now been shown that children born with the highest titers of maternal antibody are those least likely to develop severe disease (Glezen et al. 1981). As a result, maternal immunization is being examined as a way of modifying the initial infantile infection and the use of immune globulin is being examined for prophylaxis and treatment. Parainfluenza virus types 1 and 2 are the major agents of laryngotracheobronchitis (croup). The peak age for these initial, recognizable infections is later than that for RSV, and the protective effect of maternal antibody was never in doubt (Monto 1973).

Rhinoviruses (Picornaviruses) and coronaviruses (Coronaviruses, toroviruses, and arteriviruses), in contrast to RSV and parainfluenzaviruses, produce only coldlike symptoms regardless of whether they infect children or adults. Rhinoviruses, as the principal agents of the common cold, infect individuals many times throughout life. Even in the first years of age, when attention is usually directed to the other agents that may produce lower respiratory infection, the rhinoviruses are more commonly isolated (Monto et al. 1986). Data on the annual incidence of respiratory infections due to all causes were collected from residents of the small community of Tecumseh, Michigan (Monto and Sullivan 1993). These data are shown as curves in Figure 5, with results arranged by gender. As can be seen, there is a suggestion of sparing of infants aged <1 year, because of the presence of maternal antibody. Thereafter the frequencies fall off with increasing age, rising again in young adulthood when individuals start their families and are exposed to young children. The higher frequency of illnesses in females as compared to males is well recognized. In women who work outside the home, the frequency of illness in those aged 20–34 years is higher than in those who do not. Thus continual exposure to children must be a major factor. The age-specific frequency of rhinoviruses roughly parallels these curves. This similarity is a result of the major role of rhinoviruses in the etiology of the common cold and other frequent respiratory illnesses (Monto et al. 1987). Coronaviruses are responsible for illnesses similar to those caused by rhinoviruses. Because they are difficult to isolate and occur much less frequently than the rhinoviruses, much less information is available on their occurrence in populations. Newer tests involving antigen detection may increase our knowledge of their behavior and distribution (MacNaughton 1982).

Respiratory virus infections differ in their seasonality (Monto and Sullivan 1993). Although, overall, viral respiratory infections increase in frequency in cold weather in many geographic areas, isolation of rhinoviruses peaks in the period after schools reopen: children come into contact with each other for long periods and can spread these viruses that require relatively close contact for transmission. Rhinoviruses account for most of the respiratory illnesses in the community at that time. In other geographic areas, although the autumn peak is prominent, another peak of infection in the spring is also recognized (Hendley et al. 1969). Other respiratory viruses have different periods of spread in different regions. In many areas, RSV transmission peaks in winter or early spring and parainfluenza virus in spring and late autumn, the exact occurrence varying by place and by year. Outside the temperate zones, periods of activity are different and must be defined locally. Because person-to-person transmission is always involved, the agents take weeks to move through even a small community.

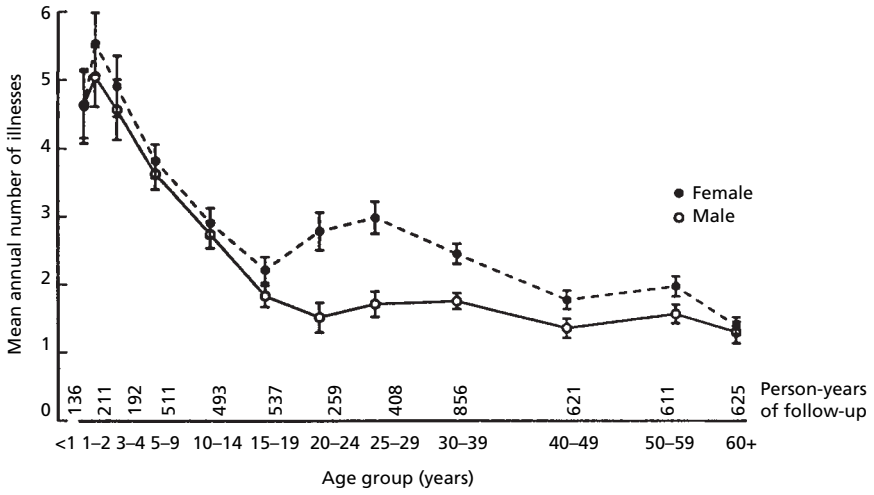

Figure 5 *Mean number of respiratory illnesses per person-year (95 percent confidence intervals) in males and females living in Tecumseh, Michigan (from Monto and Sullivan 1993).*

INFLUENZA

Influenza (Orthomyxoviruses: influenza) is potentially serious in all individuals who develop disease. Infection rates are highest in children and adults who are sufficiently ill to seek medical advice (Monto et al. 1985). However, the elderly and those with chronic illnesses experience the most severe disease, often with complications such as pneumonia (Chin et al. 1960). Reasons for the susceptibility of older individuals are varied, and probably related to depressed cell-mediated immunity (Phair et al. 1978). Because of the danger of illness in older people and those with chronic conditions, vaccination programs in many countries are not intended to prevent infection in the young. Rather, older people are vaccinated as a means of preventing hospitalization or death. As a result, disease in the young, who sustain the major burden of a complicated infection, is not affected, and outbreaks continue. An exception is Japan, where a former policy was designed to limit spread by vaccinating school-age children (Dowdle et al. 1980).

Influenza, like other respiratory agents, is seasonal, no obvious transmission taking place in warmer months of the year in the temperate zones, although asymptomatic infections have been detected serologically, particularly with type A viruses. Outbreaks of influenza in cold seasons can be recognized by various indicators, including increased visits to physicians or school absenteeism. These indicators can be found during major outbreaks of all types and subtypes of influenza; they are a reflection of the high infection frequency in the general population. Transmission of influenza types A(H3N2) and B can also be detected by the ability of these viruses to be fatal in older people and those with high risk conditions. Such deaths, as complications of the primary viral infection, generally occur 2 weeks after the initial episode, so excess mortality is a delayed indication of transmission.

Although seasonal outbreaks of influenza may be caused by one viral type or subtype, mixed or sequential outbreaks of different types may also occur. The viruses appear at various other times in tropical climates, either throughout the year or during the rainy season, and such regions are likely to be the source of new strains. Pandemics of type A influenza take place at irregular intervals following sudden changes in the viral hemagglutinin or the neuraminidase, or both ('antigenic shift'). For a time, it was thought that new viruses with pandemic potential emerged at regular intervals, approximately every 10 years. In retrospect, these conclusions were based on confusion concerning whether a shift in surface antigens had occurred during the period 1918–1956, and observations during only a limited period thereafter. It had always been assumed that, when a type A strain with new surface antigen or antigens emerged, it would totally replace the previous A subtype. In 1977, the A(H1N1) subtype reappeared 20 years after it had been completely replaced in 1957 by type A(H2N2). The type A(H3N2) viruses have been circulating since 1968 and, instead of replacing these viruses, both subtypes have co-circulated since that time. Type A(H1N1) has largely been restricted to the younger segment of the population and type A(H3N2) has occurred in all age groups (Masurel 1976; Kendal et al. 1977).

When pandemics of influenza have followed a shift in type A antigens, the same age-specific morbidity and mortality patterns have been observed as in the annual epidemics of influenza but at much higher frequency. However, in 1918, the pandemic viruses thought, on the basis of serological epidemiology, to be related to swine influenza, produced unusually high mortality in young adults, a group usually experiencing significant morbidity, but little mortality. At least 20 million people died worldwide during this pandemic, which is still a unique and unexplained event, possibly related to unusual bacterial superinfections (Shope 1936). Even with the identification of the hemagglutinin of the 1918 virus, reasons for its higher pathogenesis have not emerged (Taubenberger et al. 1997). Recent pandemic strains of influenza first caused outbreaks of disease in humans in southern China. There, the combination of high population density and animals, especially domestic ducks and pigs, living in close contact with humans, often in the same dwellings, might have been responsible (Kawaoka et al. 1993). Many domestic animals have their own type A influenza viruses, but such infections are not true zoonoses because these viruses are not pathogenic for humans. A recent exception was the spread in Hong Kong of A(H5N1) virus from chickens to humans (Subbarao et al. 1998; Claas et al. 1998).

New pandemic viruses seem to result from reassortment made possible by the segmented genome of the influenza virus. The gene segments coding for the surface antigens of the animal strains replace the comparable segments of the strains pathogenic for humans. The result is a new virus with internal components from the old pathogenic virus but with new hemagglutinin and sometimes a new neuraminidase from the animal strain. Type B influenza viruses do not infect animals, and pandemics of type B do not occur, strengthening the hypothesis of the animal origin of pandemic type A variants. Thus, the only changes seen with type B viruses are gradual year-to-year variations, referred to as 'antigenic drift.' The same drift also occurs with type A strains over the years, in addition to the episodic shift associated with pandemics. This regular drift makes it necessary to incorporate fresh strains in the vaccine, because efficacy drops if there is not a good match between circulating variants and in the vaccine strains. Updating is usually done annually with type A(H3N2) and not as often with type A(H1N1) and type B viruses, reflecting more rapid changes in type A(H3N2).

VIRUSES INFECTING THE GASTROINTESTINAL TRACT

Agents of diarrheal disease

Diarrheal disease in industrialized countries is of varied etiology. Unlike respiratory illnesses, in which viruses are the principal pathogens, enteric illnesses are sometimes of bacterial or parasitic origin, especially when they occur as outbreaks. Much of the disease that occurs more regularly in families is of viral origin and its frequency increases in the cold season, reflecting greater opportunities for transmission (Monto and Koopman 1980). Rotaviruses are the main cause of dehydrating diarrheal disease in young children throughout the world (see Rotaviruses). Although infants and young children in developing countries may die of these infections if they are not rehydrated with salt-containing solutions (orally or sometimes intravenously), the disease is rarely so severe in the developed world, mainly because of such factors as better nutrition (Black et al. 1981). These are surface infections that recur throughout life. Whilst initial infections in infants may be severe, re-infections usually produce mild illness or asymptomatic infection. As with the respiratory viruses, this has implications for spread within families.

Paradoxically, the pattern of occurrence of rotavirus infection is more like that of the respiratory viruses than of other enteric agents, although transmission clearly takes place by the fecal–oral route; whereas large amounts of virus are shed in the stools, none has been detected in the upper respiratory tract. As with respiratory viruses it is possible to examine the role of the family and the community in determining the occurrence of infection. All rotavirus types are seasonal in the temperate zones, most illness occurring in the colder months (Brandt et al. 1982; Vesikari et al. 1992). Rotavirus vaccines, prepared from live-attenuated strains, have been and continue to be developed for use in infants (see Rotaviruses). Because of the need for multiple types, there has been at least a potential problem with interference, a phenomenon common to live attenuated vaccines, with difficulty in producing immunity simultaneously to several types. The purpose of vaccination is not necessarily to prevent infection or illness completely, but to render it more like re-infection, thus preventing severe dehydration.

Rotaviruses are responsible for only some of the enteric illnesses experienced in the community during the cold season. Identifying the etiological agents of other common illnesses has been complicated by the difficulty or impossibility of growing many of them in cell culture. Norvovirus, now recognized to be a calicivirus, is one of a group of viruses causing water-related outbreaks (see Human enteric RNA viruses: norvoviruses and sapoviruses). A variety of exposures are involved, including drinking and swimming in water containing the virus, especially when the head is submerged; contaminated seafood has also been implicated (Gunn et al. 1980; Kaplan et al. 1982). Person-to-person transmission also occurs, increasing the potential for spread in families and the community.

Immune electron microscopy was the basic method for identifying rise in antibody titer and reagents had to be prepared by inoculating human volunteers (Herrmann et al. 1985).

The recent cloning of the Norwalk agent will allow better understanding of its epidemiology as reagents become available for testing sera. Similarly the enteric adenoviruses, types 40 and 41, originally detected only by electron microscopy can now be identified by enzyme immunoassay (EIA) (Johansson et al. 1985). The association of these viruses with enteric disease in infants is an example of use of the case–control design. The respiratory adenoviruses, which could at that time be easily cultivated in cell culture, were isolated in equal numbers from the stools of cases (children hospitalized with diarrhea) and controls (children hospitalized with other conditions). However, adenoviruses that could not be cultivated in routine cell cultures were significantly more commonly detected in children with disease than in controls (Brandt et al. 1985). It has been suggested that an autumnal increase in enteric illness is related to these adenoviruses, but such seasonality could not be demonstrated in hospital-based studies.

Polio and enteric hepatitis viruses

The enteroviruses are generally transmitted by the fecal–oral route. Some exceptions exist, however, such as coxsackie virus A21, an unusual cause of respiratory infection that can spread by aerosol. The dynamics and characteristics of this infection were well studied in the military, in whom it had presented an occasional problem. Polio is the best known of the enteroviruses, producing a potentially serious disease in a small proportion of those infected. Global elimination of polio is underway, by use of the live-attenuated vaccine administered universally and repeatedly to young children. The method is based on the known transmission pattern of the vaccine virus, which resembles that of the wild virus in being shed in large quantities and for long periods in the stool of inoculated children (Hull et al. 1994). The shed virus can be transmitted to contacts, especially other children, and to adults in the family. By inoculating children regularly (annually or more frequently) as part of a mass vaccination program, it is possible to spread the vaccine viruses in the environment so that children who are missed by the vaccine program are indirectly exposed to them. This approach, which combines herd immunity, or increasing the level of antibody in a population so that susceptibles are rare

and transmission is interrupted, together with the special characteristics of transmissibility of the live polio vaccine, has resulted in the elimination of transmission of wild polio virus in the Americas. The procedure is now being applied in other parts of the developing world (Centers for Disease Control 1995).

Hepatitis A is classified as an enterovirus; it shares epidemiological characteristics with other enteroviruses but is unusual in having a relatively long incubation period (median 28 days) (Lemon 1985). Spread in the developed countries typically occurs from person to person in the family or in settings such as day care centers for children, especially when they are not toilet trained. Much of the infection in North America has been traced to these day care outbreaks, from which spread continues in families and older individuals (Hadler and McFarland 1986). In developing countries and in special situations in the industrialized world, spread can often occur through contaminated water or by consuming uncooked shellfish from contaminated waters. Other food transmission is possible whenever there is fecal contamination. In the developing world, infection occurs at an early age and is often asymptomatic. In the industrialized countries, infection is delayed and often will not occur at all, rendering much of the population susceptible if exposed. Travelers to developing countries generally do not have protective antibodies from past exposure, and are thus at increased risk. One solution, before vaccine became available, was use of immune globulin, which gives temporary protection. There are now vaccines that can protect individuals from developed countries who will be exposed in the developing world. Such infection has been a major risk, especially for people exposed for long periods in areas with poor standards of hygiene (Wagner et al. 1993). Hepatitis E has epidemiological characteristics similar to those of hepatitis A, and increasing availability of reagents will allow better understanding of its behavior in populations. Some outbreaks of disease thought to be due to hepatitis A are now recognized to have been, in all probability, caused by hepatitis E (Wong et al. 1980).

VIRAL INFECTIONS WITH BLOODBORNE TRANSMISSION

Hepatitis B and related viruses

Hepatitis B was, before the discovery of HIV, the most extensively studied bloodborne pathogen. During the course of many viral infections, there is often a phase of viremia; however, bloodborne transmission is unlikely when viremia lasts for only a period of hours. The probability of such transmission is increased when viremia persists for days or, as is the case with some infected with hepatitis B, for years. The likelihood that infection will persist decreases with increasing age at time of exposure. Thus, perinatal infection is most likely to result in chronic viremia. The transmission of hepatitis B by blood or blood products was documented well before the virus or its antigens were identified (Sawyer et al. 1944). During the Second World War, yellow fever vaccine used by the US military was stabilized by the addition of human albumin. Those who received the vaccine developed hepatitis and, by epidemiological evidence, it became clear that it was contaminated by what is now known to be hepatitis B virus. On initial study, the cases of hepatitis that were detected did not seem to bear any temporal relationship to an exposure to persons with infection or to a common event. Then it was realized that vaccination might be the common source. Because vaccinations had been administered over a long period, cases were occurring in an apparently unrelated fashion. Plotting of duration since vaccination resulted in a typical epidemic curve, with cases distributed around the long median incubation period of approximately 120 days from exposure.

Hepatitis B is transmissible not only through blood and blood products but also through sexual contact. Simply living in a family with an infected person slightly increases the probability of transmission. It is not clear exactly how this occurs. The virus is present in various body fluids, in addition to blood and semen (Irwin et al. 1975). Transmission requires only very small amounts of blood, so leakage through minor abrasions and tears may be responsible. Hepatitis B was known to be more common in certain professional groups such as dentists, almost certainly because of exposure to small amounts of infected blood commonly lost in dental procedures rather than through virus present in saliva (Mosley et al. 1975). It was especially common among homosexual men, especially those with multiple partners. In fact, the trial of hepatitis B vaccine was conducted in uninfected sexually active homosexual men, because exposure was predictable (Szmuness et al. 1980). The transmission was clearly sexual, but how much of it involved transfer of small quantities of highly infectious blood and how much other infected bodily fluids was difficult to quantify. Various specific sexual practices such as anal intercourse were identified as risk factors even before the study of HIV transmission (Szmuness et al. 1975).

A particular problem with hepatitis B, especially in eastern Asia (including China) and in sub-Saharan Africa, is that transmission is mainly vertical and children become infected at birth from transplacental and direct exposure to maternal blood (Stevens et al. 1975). When infection occurs at this age, it is likely to persist, and thus may be carried to the next generation. Hepatitis C shares many features with hepatitis B, including its transmission by blood and blood products. Risk groups differ somewhat with, for example, less sexual transmission (Alter et al. 1989).

Human immunodeficiency virus

When human immunodeficiency virus (HIV) was first described, its epidemiology was found to be remarkably similar to that of hepatitis B (see Hepatitis B). Differences are related to the natural history of the disease and to the far lower transmissibility of HIV under most conditions. A persistently high level of viremia allows hepatitis B virus transmission to occur with relative ease for long periods. In contrast, transmission of HIV is most likely early in the infectious process when there is viremia of high titer, or later in the development of AIDS itself when the level of viremia increases again. The early period is probably the most important for transmission, because at that time individuals may not know they are infected and remain sexually active (Jacquez et al. 1994). Because of its importance, the epidemiology of HIV has been studied intensively over the period since the virus was identified and many characteristics of its distribution are now well established. Much attention has been given to the role that various behaviours play in transmission, since, in the developed countries, antibody tests have ensured that blood and blood products are safe and transmission by that route of infection no longer occurs. Among homosexual men, receptive anal intercourse is the prime risk factor, but other sexual practices are possibly to blame, if not nearly as risky. Heterosexual intercourse can also transmit the virus efficiently, but male-to-female transmission is accomplished more easily than the reverse (Padian et al. 1991). As with hepatitis B, injecting drug use appears to be an independent risk factor for transmission. Transmission also occurs through breast milk and artificial insemination. In one controversial case, the argument for transmission of virus to the patients of an HIV-infected dentist was strengthened by the similarity of the viral nucleic acids in the various cases, a technique sometimes referred to as molecular epidemiology (Crandall 1995).

In the developed countries, the disease has predominantly been one of males, both homosexual and bisexual men and injecting drug users. Heterosexual transmission has often involved sexual partners of these individuals. In the USA, African-Americans and other minority groups are increasingly involved. In developing countries, the male to female ratio often is 1:1, and the prevalence of infection in the general population, especially in certain countries of Africa, is high. The infection has entered south and southeast Asia, where transmission has been rapid. Mathematical epidemiology has been used to study the relative probability of different transmission mechanisms when several may be involved, and to predict the course of the pandemic. Simulations have been particularly valuable in trying to understand how altering transmission by vaccination or behavior modification will affect the course of the epidemic in different regions.

ZOONOSES AND VECTORBORNE INFECTION

Zoonoses not involving arthropods

Many, but not all, of the vectorborne viral infections are also zoonoses and are discussed together. The zoonoses are human infections in which the principal reservoirs of the viral agent are wild and domestic mammals, birds, and possibly other species such as reptiles. Cycles of infection can be maintained in these reservoirs without the involvement of humans, in whom infection occurs incidentally. Zoonoses not involving arthropod-vector transmission are listed in Table 4, with animal reservoir and route transmission. Rabies (see Rhabdoviruses: rabies) is an example of a zoonosis not involving vectors. In most of the world, rabies is widely distributed in a variety of mammals, the predominant species varying from region to region. In North America, foxes, skunks, and raccoons are mainly involved (Anderson et al. 1984). Of these, only raccoons frequently invade human habitations but in most cases transmission remains within the particular species. Transmission sometimes involves domestic animals such as dogs or cats, who, because of their activities outside the home, will occasionally come in contact with the wildlife hosts. Insectivorous bats also maintain rabies in their own colonies but, because they often live in human dwellings, direct transmission to humans occasionally occurs. Vaccination of domestic pets is used to create a barrier between wildlife rabies and humans; it is highly effective, with the possible exception of preventing direct bat transmission. There have been several years in the USA without a single case of human rabies, even though extensive transmission in animals still occurs. In other parts of the developed world, the species involved in maintaining wildlife rabies differ. In Europe, foxes are often involved, and attempts are now being made to control transmission in these hosts by drops of baited food containing vaccine (Brochier et al. 1991). This is possible only when the geographic areas are relatively limited. In remote areas, many more species are involved and control is impractical.

Some parts of the world, typically islands such as Great Britain and Hawaii, are rabiesfree; because potential hosts are present in the wild, exclusion of animals who might be responsible for importation is a critical part of control activity. In many developing countries, rabies of domestic animals is common, especially of dogs which are barely domesticated and often form feral packs. Transmission from dog to dog does occur, as well as from dog to humans. This is in contrast to the typical situation in the developed world, where the dog, as well as humans who become infected, are ‘dead-end hosts’; that is, they are generally not responsible for any further transmission. The few human cases that occur in indus-

Table 4 *Zoonotic viral infections without arthropod transmission*

	Virus and disease	Animal reservoir	Mode of transfer
Rhabdoviruses	Rabies virus	Foxes, skunks, bats, raccoons, mongooses, dogs, cats, etc.	Bite. Possibly saliva through abraded skin or airborne
Arenaviruses	Lymphocytic choriomeningitis virus	*Mus musculus*	Contact with urine, feces or other infected fluids. Rarely person-to-person or nosocomial spread. Possible airborne distribution
	Junin–Argentine hemorrhagic fever	*Calomys musculinus*	
	Machupo–Bolivian hemorrhagic fever	*Calomys callosus*	
	Guanarito virus disease–Venezuelan hemorrhagic fever	*Sigmodon hispidus*	
	Lassa fever	*Mastomys natalensis*	
Bunyaviruses/ hantaviruses	Hantaan virus		Contact with urine, feces, and saliva. May be aerosolized or enter through abraded skin
	Korean hemorrhagic fever virus	Field mice	
	Seoul virus	Urban rats	
	Sin Nombre virus		
	Hantavirus pulmonary syndrome	Deer mice and other rodents	
Puumulavirus	Nephropathia epidemica virus	Voles	
Filoviruses	Marburg virus	Reservoir not clear	Contact with infected monkeys
	Ebola virus	Reservoir not clear	Blood and other personal contact after infection in humans
Poxviruses	Cowpox virus, Monkeypox virus	Cows, monkeys, sheep	Animal skin lesion to abraded human skin
	Contagious pustular dermatitis (orf)		
Herpesvirus	Herpesvirus B	Rhesus monkeys	Saliva by bite or contact
Orthomyxovirus	Swine influenza virus	Pigs	Close contact
Picornavirus	Encephalomyocarditis	Rodents and monkeys	Possible contamination by urine and feces

trialized countries are often imports from developing countries, where exposure of travelers to infected domestic animals is more likely. Molecular techniques, based on differences in viral nucleic acid structure in rabies viruses transmitted in different parts of the world, are often used to determine the source of exposure (Smith et al. 1992). The few cases and intensive study of those that do occur have allowed recognition of unusual sources of infection, such as a transplanted cornea obtained from a cadaver whose death was caused by undetected rabies (Houff et al. 1979).

Zoonoses not involving arthropods are typical of infections produced by arenaviruses and hantaviruses. With both, rodents and other wildlife hosts are responsible for transmission and maintenance of infection. Sometimes the rodents become diseased, but in most cases the infections are inapparent. Humans become involved incidentally and have no further role in maintenance of the chain of transmission. Viruses such as the arenaviruses (Machupo, the cause of Bolivian hemorrhagic fever, and Junin, the cause of Argentine hemorrhagic fever), as well as the hantaviruses (producing Korean hemorrhagic fever and the hantavirus pulmonary syndrome in North America) are limited geographically by the area ranged by the rodents primarily infected. Human-to-human transmission is rare; most human infection appears to result from exposure to rodent urine heavily contaminated with virus, and possibly other animal products such as feces or saliva (Peters et al. 1974; Mercado 1975). The rodents often live in or near dwellings, and humans may be exposed via aerosol or through abraded skin. Increases in frequency of the disease, which, for example, led to the recognition of the hantavirus pulmonary syndrome in southwestern USA, are related to changes in the environment affecting numbers and activities of the rodent host (Hjelle et al. 1994). It is suggested that an overproduction of pine nuts, the rodents' principal food,

led to the emergence of the disease in the Four Corners area of the USA.

Yellow fever and dengue

The term 'arbovirus' was used for many years to describe a large group of enveloped, lipid-containing viruses that had in common transmission by arthropods (Theiler 1957). This diverse group of viruses has many epidemiological features in common (see Flaviviruses; 47, Togaviruses; and 48, Bunyaviridae). The principal vector is the mosquito, although there are important infections involving ticks and other arthropods. Many are also zoonoses, in which the infection is maintained in wild or domestic animals (the reservoirs), humans being involved only occasionally as dead-end hosts. Because of the nature of the vectors and the reservoirs, particular arboviruses are often geographically limited in distribution. Certain mosquitoes cannot exist outside the tropical or subtropical areas, so climate becomes a major determinant, as does other vector behavior, including preferences in multiplication sites, sources of blood meals, and flight distances. The introduction of West Nile virus from the Middle East and subsequent spread throughout much of North America shows that geographic limitations are not absolute (Petersen et al. 2002).

A list of some important arthropod-borne viral infections, according to the geographical area of their occurrence is given in Table 5. Two important infections, both transmitted preferentially by the mosquito *Aedes aegypti*, which can be spread from human to human by the vector, are yellow fever and dengue. Thus, unlike many other arboviruses, dengue and (in this context) yellow fever are not zoonoses. The mosquito is closely associated with human dwellings in urban areas of the tropics and subtropics, but does not persist in areas with cold winter seasons. Urban outbreaks are typical, because infected humans are ready sources for the transmission of viruses to others. In certain regions, outbreaks of the infections are a regular seasonal occurrence. Control of yellow fever was accomplished in many cities of the Americas before availability of insecticides by eradicating the vector from urban areas. Early control of yellow fever transmission made possible the construction of the Panama Canal. With no urban yellow fever, sylvatic or jungle yellow fever was detected in areas without the usual vectors. The same virus caused the disease, which was found to have a zoonotic host, i.e. subhuman primates. It could be transmitted to humans, especially to people working or living in the forests, by hemagogus mosquitoes. *Aedes aegypti* has recently returned to many cities from which it had been eliminated, and, with it, urban outbreaks of yellow fever.

Several vaccines have been developed to control outbreaks of yellow fever and for primary prevention in endemic areas. The most successful, the 17-D vaccine, is a live-attenuated preparation that confers long-term protection.

Dengue is currently the most important arthropod-borne virus because of its widespread distribution in large parts of the tropics and subtropics and the potential there for extensive outbreaks of life-threatening disease. Urbanization in these regions has led to an

Table 5 *Arthropod-borne viruses of global importance and their vectors*

	Diseases	Family/genus	Vector
Tropics and subtropics	Yellow fever	*Flaviviridae/Flavivirus*	*Aedes* and hemagogus mosquitoes
	Dengue	*Flaviviridae/Flavivirus*	*Aedes aegypti* and *A. albopictus*
	Sandfly fever	*Bunyaviridae/Phlebovirus*	Phlebotomine sandflies
American	Eastern, Western, Venezuelan equine encephalitis	*Togaviridae/Alphaviruses*	*Aedes* and *Culex* mosquitoes
	St. Louis encephalitis	*Flaviviridae/Flavivirus*	*Culex* mosquitoes
	California encephalitis	*Bunyaviridae/Bunyavirus*	*Aedes* mosquitoes
	California tick fever	*Reoviridae/Orbivirus*	*Dermacentor* ticks
Eurasian	Tick-borne encephalitis (Russian spring–summer/central European)	*Flaviviridae/Flavivirus*	Ixodid ticks
	Crimean hemorrhagic fever (Crimean–Congo)	*Bunyaviridae/Nairovirus*	*Hyalomma* ticks
	Omsk haemorrhagic fever	*Flaviviridae/Flavivirus*	*Dermacentor* ticks
	Japanese B encephalitis	*Flaviviridae/Flavivirus*	*Culex* mosquitoes
African	Rift Valley fever	*Bunyaviridae/Phlebovirus*	*Culex* and *Aedes* mosquito
	Chikungunya fever	*Togaviridae/Alphavirus*	*Aedes aegypti* and others
	West Nile fever	*Flaviviridae/Flavivirus*	*Culex* mosquitoes
Australasian	Murray Valley encephalitis	*Flaviviridae/Flavivirus*	*Culex* mosquitoes
	Ross River fever	*Togaviridae/Alphavirus*	*Aedes* and *Culex* mosquitoes

increase in the probability of dengue infection because the virus is transmitted by the vector mosquito from one human host to another without the involvement of a zoonotic reservoir. The high concentration of susceptibles in the sprawling cities results in large-scale outbreaks. The vector *A. aegypti* is well adapted to multiplying in the small pools of water that are frequently found in these areas, especially in seasons with heavy rainfall. In its acute form, dengue is debilitating with high fever and deep pain; and infection can result in dengue hemorrhagic fever/dengue shock syndrome (DHF/DDS). These are life-threatening conditions, which affect mainly children. There are four types of dengue virus, and infection with one type produces only partial immunity to another type. The principal hypothesis to explain the pathogenesis of dengue hemorrhagic fever is based on epidemiological observations (Halstead 1970). The disease is widespread when a second outbreak of infection, typically caused by type 2, follows shortly after another outbreak caused by a different type. Reinfection appears to be the common factor; this occurs mainly in children, and maternal antibody may play a role in infants. A number of immunological characteristics of the disease support this hypothesis, although there is some evidence that the syndrome can be produced on initial infection in individuals without maternal antibody (Barnes and Rosen 1974). In recent years, dengue has been spreading, especially in the Americas. This is of particular concern in the USA because *A. aegypti* is present in the southern states.

Other arboviruses

For arboviruses that are principally zoonoses, disease in humans is generally sporadic or occasional. In North America, eastern equine encephalitis (EEE) is an example of an agent that occurs sporadically, during the summer. Many of the infections are inapparent. Symptomatic infection occurs only occasionally, but may manifest as severe encephalitis with residual mental retardation. The reservoir is wild and domestic birds; maintenance of infection does not require either humans or horses, both of which are dead-end hosts. With arboviruses in the temperate zones there is always the question of persistence through the winter (overwintering) during the period when there is no vector activity. Migrating birds infected by the virus winter in areas where mosquitoes are present and are responsible for the continued transmission of the agent. They return, some being infected, in the spring (Lord and Calisher 1970). With other arboviruses there is some evidence that animals in hibernation may serve to overwinter the virus (Reeves 1974).

In the Eurasian land mass, tick-borne encephalitis (TBE), also called Russian spring–summer encephalitis, is an important, though uncommon, sporadic disease associated with forested areas. The reservoir is small mammals and, to a lesser extent, birds; humans moving into sites where transmission is occurring become infected incidentally. Transovarian transmission can take place in ticks in which the virus is passed to the next generation, further maintaining the infection. In contrast, the most important arbovirus of eastern and southeast Asia, Japanese encephalitis, may be responsible for large numbers of cases of relatively severe encephalitis in humans. This disease is mosquito transmitted and occurs in rice-growing regions which generally have a high mosquito density. The principal reservoir is pigs, also common in the region. Because vector control in these areas has been difficult, emphasis has been placed on vaccination.

VIRUSES AND CHRONIC DISEASES

Persistent infection is well documented for many DNA viruses and some RNA viruses. The herpesvirus group is well known for producing persistent infections, for example recurrent cold sores or genital ulcers caused by herpes simplex virus and herpes zoster due to varicella-zoster virus. The retroviruses, including HIV, are also characterized by long-term chronic infection. It is difficult to establish the role of various viruses to late, seemingly unrelated, events such as the development of cirrhosis and primary hepatocellular carcinoma (PHC) or carcinoma of the cervix. Relating these conditions to viral infections requires the application of epidemiological study designs. For hepatitis B and the production of primary hepatocellular carcinoma, the studies were mainly conducted in Taiwan, where there is a combination of high prevalence of chronic infection and a health care system that allowed a longitudinal study of health care workers. A problem in any of these evaluations is the long time between the infection, which may have been acquired perinatally, and development of the tumor, which can occur many decades later. Demonstration of viral antigen in the tumor is of help, but is not conclusive proof of etiology where the population has a history of widespread infection with the agent. The major epidemiological evidence for etiology has come from a series of case–control and cohort studies that noted a strong association between the persistent infection and primary hepatocellular carcinoma even after controlling for potential confounding variables (Beasley 1988). Similar work is now underway to investigate the link between hepatitis C and PHC.

Epidemiological approaches have also provided evidence of involvement of certain types of papilloma virus in carcinoma of the cervix (Papillomaviruses). Descriptive epidemiological studies have long suggested that an infectious entity, probably sexually transmitted, was involved in etiology. Attention was first focused on behavioral differences and on herpes simplex virus. Case–control studies revealed that herpes anti-

body titers were higher in people with tumors than in those without (Rawls et al. 1970). It was possible to identify viral components in excised tumor, and other factors seemed to confirm the relationship. However, the link was not well sustained in studies controlling for confounding factors, such as sexual activity. When it became easier to work with papillomaviruses, epidemiological studies confirmed the specificity of the association with certain types. Moreover, it was possible to replicate the relationship in different studies in different parts of the world and to demonstrate the strength of the association with the specific papilloma types in question (Reeves et al. 1987). Vaccine control now appears possible (Koutsky et al. 2002).

Research into the long-term consequences of viral infection is very important, because it brings together acute and chronic outcomes affecting the health of populations. Examples include the relationship of EBV to the later development of nasopharyngeal carcinoma and Burkitt's lymphoma. Work in this area is diverse, and ranges from efforts to relate acute respiratory infection to later chronic bronchitis and emphysema to studies attempting to relate infectious agents to the pathogenesis of atherosclerosis. All these investigations must use appropriate epidemiological methods, especially as these techniques are a recognized component of other investigations of risk factors for chronic disease.

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The order *Mononegavirales*

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INTRODUCTION – THE UNIVERSAL SYSTEM OF VIRUS TAXONOMY

The *Universal System of Virus Taxonomy* has been developed to its present state by the International Committee on Taxonomy of Viruses (ICTV) through a series of seven reports (see The classification of vertebrate viruses), the most recent of which was published in 2000 (van Regenmortel et al. 2000) and an eighth report is reaching completion. The system of taxonomy adopted by the ICTV is sufficiently flexible to withstand the stress of the continual discovery of new viruses from an ever-increasing diversity of host organisms. A hierarchy of five taxa, ranked as orders, families, subfamilies, genera, and species, has been developed. Taxonomic categories below the species level, such as subspecies, strains, variants, genotypes, etc., are not included in this taxonomy and are defined according to the practical needs in each particular field. The concept and definition of the category of virus species is central to the structure of the taxonomy, and the nature of the virus species is discussed separately in The classification of vertebrate viruses.

The highest taxon, the order, comprises a group of families that have certain features in common. In the 7th Report of the ICTV only three orders are recognized. The taxon below the order is the family and 68 families are recognized at present. As yet only nine of these 68 families have been grouped into each of the three orders. However, it is anticipated that the number of orders will increase in parallel with refinement of the methods of characterization of viruses. Orders are designated by names with the suffix *-virales,* families by the ending *-viridae*, and sub-families by the ending *-virinae*. The first order described in virus taxonomy was the order *Mononegavirales,* which contains the families of nonsegmented single-stranded negative-sense RNA viruses (Pringle 1991, 1997; Pringle and Easton 1997). The other orders currently recognized are the order *Caudovirales*, comprising the three families of tailed phages – *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Maniloff and Ackermann 1998) and the order *Nidovirales* comprising the positive-sense RNA viruses with nested-set genomes – the families *Coronaviridae* and *Arteriviridae* (see The order *Nidovirales*).

The orders *Mononegavirales* and *Nidovirales* were established to embrace families of viruses exhibiting similar features of genomic organization and replicative strategies. In the case of the order *Caudovirales*, different criteria were required since any preexisting phylogenetic relationships were obscured by a combination of great geological age, large population sizes, and extensive horizontal gene transfer. Nonetheless, relationships are still evident in respect of common aspects of morphology, replication, and assembly, which can be utilized to provide an operationally useful classification. This emphasizes a fundamental aspect of the *Universal System of Virus Taxonomy*; namely, it is hierarchical and consistent with phylogenetic relationships but not

dependent on them. To some extent this is exemplified also in the case of the order *Mononegavirales*, which was established initially to accommodate three families (*Filoviridae*, *Paramyxoviridae, Rhabdoviridae*) with clear phylogenetic links, and subsequently expanded to include a fourth family (*Bornaviridae*) on the basis of similar genomic organization rather than close phylogenetic relationship. The following list presents the current taxonomy of the order *Mononegavirales*. The convention is now that all taxonomic names, including those of virus species, should be rendered in italics with an initial capital letter when used in a taxonomic context. The names can be used without italics in other contexts.

Bornaviridae
- *Bornavirus*
  - *Borna disease virus*

Filoviridae
- *Marburgvirus*
  - *Lake Victoria marburgvirus*
- *Ebolavirus*
  - *Zaire ebolavirus*

Paramyxoviridae
- *Paramyxovirinae*
  - *Avulavirus*
    - *Newcastle disease virus*
  - *Henipavirus*
    - *Hendra virus*
  - *Morbillivirus*
    - *Measles virus*
  - *Respirovirus*
    - *Sendai virus 1*
  - *Rubulavirus*
    - *Mumps virus*
- *Pneumovirinae*
  - *Pneumovirus*
    - *Human respiratory syncytial virus*
  - *Metapneumovirus*
    - *Turkey rhinotracheitis virus*

Rhabdoviridae
- *Cytorhabdovirus*
  - *Lettuce necrotic yellows virus*
- *Ephemerovirus*
  - *Bovine ephemeral fever virus*
- *Lyssavirus*
  - *Rabies virus*
- *Novirhabdovirus*
  - *Infectious hematopoietic necrosis virus*
- *Nucleorhabdovirus*
  - *Potato yellow dwarf virus*
- *Vesiculovirus*
  - *Vesicular stomatitis Indiana virus*

THE ORDER *MONONEGAVIRALES*

Background

The order *Mononegavirales* was established to accommodate all known viruses with nonsegmented negative-sense single-stranded RNA genomes as indicated by the derivation of the name (mono – single, nega – negative-sense, -virales – viruses). Initially that comprised viruses classified in the families *Filoviridae, Paramyxoviridae*, and *Rhabdoviridae*. The features that distinguished these three families were predominantly morphological and biological. Later, molecular characterization of *Borna disease virus* (the type species and sole representative of the family *Bornaviridae*) revealed that this virus possessed a nonsegmented, negative-sense single-stranded RNA virus genome (De la Torre 1994; Schneemann et al. 1995). It differs substantially from the other three families in several respects, the most fundamental being the intranuclear site of transcription and replication. Entry by receptor-mediated endocytosis is followed by transport of viral ribonucleoprotein to the nucleus where transcription and replication occur. The features common to the four families are listed in Table 1, and the characteristics which distinguish each family are listed in Table 2. (Detailed descriptions and references can be found in .)

Table 1 *Common properties of the members of the order* Mononegavirales

Common properties
Negative-sense nonsegmented single-stranded RNA in the virion
Conservation of gene order
Virion-associated RNA polymerase mediates transcription and replication
Genome transcribed into 6–10 discrete mRNAs from a single 5′-terminal promoter, with the exception of *Borna disease virus* where two primary transcripts are post-transcriptionally processed by the host-cell mRNA splicing machinery
Replication occurs by synthesis of a complete positive-sense RNA antigenome
Nucleoprotein is the functional template for the synthesis of replicative and mRNA
Independently assembled nucleocapsids are enveloped at the cell surface at sites containing virus proteins, except in the case of *Borna disease virus*
All cytoplasmic, except viruses in the genera *Bornavirus* and *Nucleorhabdovirus*
Gene boundaries marked by an end/intergenic/start configuration in all except *Borna disease virus*
None exhibit genetic recombination
Occur in invertebrates, plants and vertebrates

Table 2 *Characteristic features of the four families of the order* Mononegavirales

Family *Bornaviridae*
Spherical particles of 90 nm diameter, with 50–60 nm diameter electron dense core, and outer membrane with 7 nm-long spike projections
Nuclear
Spherical 50–60 nm diameter nucleoprotein core
Genome size: 8.9 kb
Six major open reading frames encoding presumptive 3′-NP (p40)-P (p24)/NS? (p10)-M (p16)-G (p56)-L (p180). Complex pattern of mRNA transcription involving 3 initiation sites and 4 termination sites, mRNA splicing at two introns
Wide host range, horses and sheep regarded as main natural hosts. Associated with neurological disease and behavioural abnormalities
Family *Filoviridae*
Filamentous forms with branching, sometimes U-shaped, 6-shaped or ring-shaped. A unique diameter of 80 nm and varying lengths extending up to 14 000 nm, with spike projections of 10 nm approximately. Infectious particle length is 790 nm for Lake Victoria marburgvirus and 970 nm for Zaire ebolavirus
All cytoplasmic
Helical nucleocapsid, 50 nm diameter, with an axial space of 20 nm diameter and a helical periodicity of 5 nm approximately
Genome size: 18.9–19.1 kb
Seven proteins are encoded in the genome in the order 3′-NP (nucleoprotein)-VP35 (phosphoprotein)-VP40 (matrix protein)-GP (glycoprotein)-VP30(?)-VP24(?)-L (polymerase)-5′. Most genes are separated by nonconserved intergenic sequences, but some genes overlap in a highly conserved pentamer junction region
Biology enigmatic: only two antigenically unrelated viruses known; transmitted by the bloodborne route; restricted to primates; reservoir species unknown
Family *Paramyxoviridae*
Pleomorphic, some roughly spherical, 150 nm in diameter, filamentous forms common in some. All cytoplasmic
Helical nucleocapsid; 13–18 nm diameter, 5.5–7 nm pitch
Genome size: all in the range15.4–15.9 kb, with the exception of the 18 kb of the recently discovered *Hendra virus* and *Nipah virus*
The family comprises two phylogenetically distinct subfamilies; the genome size of viruses in the sub-family Paramyxovirinae conforms to 'the rule of six' (i.e. the nucleotide number is divisible by six), whereas this rule does not apply in the subfamily Pneumovirinae
Seven to nine proteins are encoded in the Paramyxovirinae (of which two to four are derived from overlapping reading frames in the *P* gene), and 9 to 11 in the Pneumovirinae, The virion proteins common to all genera are three nucleocapsid–associated proteins (N or NP, P and L), three membrane-associated proteins (M, G or H or HN, and F). Variable proteins include several non-structural proteins (C, NS1, NS2), a cysteine-rich zinc-binding protein (V), a small integral membrane protein (SH), and a transcription processivity protein (M2). Genes non-overlapping except in the case at the M2/L junction in some pneumoviruses
Found only in vertebrates; no arthropod vectors known. Horizontal transmission, mainly by the airborne route. Predominantly associated with respiratory infections
Family *Rhabdoviridae*
Bullet-shaped or bacilliform; 100–430 nm in length and 45–100 nm in diameter. Surface spikes 5–10 nm in length × 3 nm in diameter
Helical nucleocapsid, 50 nm diameter, unwinding to helical structure of 20 × 700 nm (VSV)
Cytoplasmic with the exception of plant viruses of the genus Nucleorhabdovirus, which multiply in the nucleus and form nuclear inclusions
Genome size: 11–15 kb
Generally have five structural proteins (3′-N-P-M-G-L-5′) plus an additional presumptive non-structural protein in some. Exceptionally, members of the genus Ephemerovirus have up to ten genes encoding glycoproteins of uncertain function
Wide host range, different species infect plants, invertebrates and vertebrates. Some multiply in invertebrates and plants, some in invertebrates and vertebrates, but none in plants and vertebrates. May be vertically transmitted in arthropods (Sigman virus), otherwise transmitted horizontally

General properties

Conventional transmission electron microscopic analysis shows that the virions are large enveloped structures with a prominent fringe of peplomers, 5–10 nm long and spaced 7–10 nm apart, in all except the *Bornaviridae*. The morphologies are variable, but in general distinguish the families. Virion Mr is 300–1 000 × 10^6. S_{20W} is 550–1 045 (plant rhabdoviruses have larger S_{20W} values). Virion buoyant density in CsCl is 1.18–1.22 g/cm^3. Virus

infectivity is rapidly inactivated by heat treatment at 56°C, or following UV- or X-irradiation, or exposure to lipid solvents.

Virions contain one molecule of linear, non-infectious, negative-sense, single-stranded RNA, 8.9–19 kb in size, (M_r of 3–5 × 10^6), which comprises about 0.5–2.0 percent of the particle weight. The viral RNA lacks a capped 5′ terminus, or a covalently associated protein. The 3′ terminus of viral RNA lacks a poly(A) tract. The immediate 5′ and 3′ terminal regions exhibit inverse complementarity, and there are conserved motifs in the terminal regions of all four families. Full-length positive-sense (anti-genomic) RNAs are found in infected cells. The genome comprises a linear sequence of genes, with limited overlaps in some viruses, and with short terminal noncoding regions. There are conserved motifs in the transcription start and end signals in all families and the intergenic regions, which lie between the transcribed genes, range from two to several hundred nucleotides. Exceptionally, genetic information may be encoded in additional reading frames in the *P* genes of respiroviruses and morbilliviruses. Splicing of some mRNA and overlapping start/stop signals are characteristic of bornaviruses.

There are a limited number of proteins in relation to the large particle size. The 5–7 structural proteins comprise envelope glycoprotein(s), a matrix protein, a major RNA-binding protein, other nucleocapsid-associated protein(s), and a large molecular weight polymerase protein, plus in some viruses, several additional nonstructural proteins, which may be phosphorylated. The glycoproteins may have a covalently associated fatty acid proximal to the lipid envelope. The matrix protein is nonglycosylated in all except the bornaviruses in which it is *N*-glycosylated and expressed on the surface of virions and may function as an attachment protein. Enzymatic activities associated with the virions may include RNA polymerase, polyadenylate transferase, mRNA transferase, and neuraminidase.

Virions are composed of about 15–25 percent lipids, their composition reflecting that of the region of the host cell membrane where virions bud. Generally, phospholipids represent about 55–60 percent, and sterols and glycolipids about 35–40 percent of the total lipids. Virions are composed of about 3 percent carbohydrates by weight, which are present as *N*- and *O*-linked glycan chains on surface proteins and on glycolipids. When made in mammalian cells the oligosaccharide chains are generally of the complex type, in insect cells they are of noncomplex types.

In the *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*, discrete mRNAs are transcribed by sequential interrupted synthesis. Transcription is polar with stepwise attenuation. Generally genes do not overlap, the exceptions being the *M2* and *L* genes of respiratory syncytial virus (family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Pneumovirus*), the *VP30* and *VP24* genes of *Lake Victoria marburgvirus* (family *Filoviridae* genus *Marburgvirus*) and the *VP35/VP40*, *GP/VP30*, and *VP24/L* genes of *Zaire ebolavirus* (family *Filoviridae*, genus *Ebolavirus*). The site of multiplication is the cytoplasm, with the exception of plant viruses classified in the genus *Nucleorhabdovirus*. The *P* genes of respiroviruses and morbilliviruses are exceptional in that additional open reading frames may be utilized via alternative non-AUG start codons, and mRNA editing occurs by insertion of nontemplated nucleotides to change the reading frame for the expression of *P* gene products. In the filovirus *Zaire ebolavirus*, a nontemplated insertion event during transcription of the glycoprotein gene generates the membrane-inserted glycoprotein seen in virions, whereas the faithfully transcribed mRNA encodes a secreted form of the glycoprotein lacking the membrane anchor. This does not appear to occur in the other filovirus, *Lake Victoria marburgvirus*. In the *Bornaviridae*, the site of multiplication is the nucleus and transcription is complex with splicing of mRNA and overlapping transcriptional stop/start signals.

Replication occurs by synthesis of a complete positive sense anti-genomic RNA. Genomic and anti-genomic RNAs are present only as nucleocapsids. The mRNAs of bornaviruses are capped, but synthesis is not inhibited by α-amanitin suggesting that a cap-snatching mechanism is not involved. In the filoviruses, paramyxoviruses and rhabdoviruses, maturation of the independently assembled helical nucleocapsid occurs by budding through host membranes with investment by a host-derived lipid envelope containing transmembrane proteins. The process of assembly and maturation of bornaviruses is not understood at present.

Membrane glycoproteins are involved in antibody-mediated neutralisation. Virus serotypes are defined by the surface antigens. Filoviruses are an exception in that they are poorly neutralized in vitro. In bornaviruses antibodies to both the glycosylated matrix protein and the gp94 envelope protein neutralize infectivity.

The host ranges vary from restricted to unrestricted. Filoviruses have only been isolated from primates. Paramyxoviruses occur only in vertebrates and no vectors are known. Rhabdoviruses infect invertebrates, vertebrates, and plants. Some rhabdoviruses multiply in both invertebrates and vertebrates, some in invertebrates and plants, but none in all three. In human hosts the pathogenic potential tends to be characteristic of the family: i.e. hemorrhagic fever (*Filoviridae*); respiratory and neurological diseases (*Paramyxoviridae*); mild febrile to fatal neurological diseases (*Rhabdoviridae*). Bornaviruses have been isolated from horses, cattle, sheep, rabbits, rats, cats, ostriches, and man. The pathology associated with virus infection is variable. Infection of animals is associated with conditions ranging from behavioral disturbances to severe nonpurulent encephalomyelitis, and there is some evidence of association of bornavirus

infection with neuropsychiatric illness in man. Cytopathology varies from none (bornaviruses and filoviruses) to rapidly lytic (rhabdoviruses and paramyxoviruses); syncytium formation is common in paramyxoviruses.

BRIEF DESCRIPTIONS OF THE FOUR FAMILIES COMPRISING THE ORDER *MONONEGAVIRALES*

The family *Bornaviridae*

The family *Bornaviridae* is represented by *Borna disease virus*, a species of some antiquity. Borna disease takes its name from the town of Borna in Saxony, Germany, where extensive outbreaks of fatal neurological disease in horses, and occasionally sheep, have occurred at least since 1895. The viral etiology of the disease was established in 1925, but it was not recognized to be a negative-sense RNA virus until 1995. As yet, no other virus with comparable molecular characteristics has been isolated. Borna disease was considered to be sporadically endemic only in Central Europe, but it may have spread worldwide in recent times. In horses, the outcome of infection is often death, and surviving animals may exhibit permanent sensory or motor deficiencies. No data are available that might identify reservoir hosts facilitating the spread of the virus, and there is no evidence for vectorborne transmission. The question of the involvement of Borna disease virus in human neuropsychiatric illness remains unresolved, but experimental infection of a primate (the tree shrew *Tupaia*) appeared to induce aberrant social behaviour and altered response to environmental stimuli (see also: Borna disease virus).

The family *Filoviridae*

The family *Filoviridae* comprises two genera, *Ebolavirus* and *Marburgvirus. Marburgvirus* is represented by a single species, now designated *Lake Victoria marburgvirus*, whereas *Ebolavirus* is represented by four distinct species *Cote d'Ivoire ebolavirus*, *Reston ebolavirus*, *Sudan ebolavirus*, and *Zaire ebolavirus*. All these viruses, except for *Reston ebolavirus*, which does not appear to be pathogenic for humans, have been associated with outbreaks of acute and often fatal hemorrhagic fever in Central Africa. The virions exhibit similar filamentous pleomorphic morphology and virus infectivity is poorly neutralized in vitro. There is virtually no cross-reactivity between the two genera. The amino acid homology of the surface glycoproteins of ebolaviruses and marburgviruses is approximately 30 per cent, compared with 55–66 percent between ebolavirus species. The natural reservoirs of filoviruses are unknown, but no vector appears to be involved in transmission (see also: Filoviruses).

The family *Paramyxoviridae* – subfamily *Paramyxovirinae*

The family *Paramyxoviridae* is divided into two subfamilies; the *Paramyxovirinae* containing the five genera *Avulavirus, Henipavirus, Morbillivirus, Respirovirus*, and *Rubulavirus*; and the *Pneumovirinae* containing the two genera *Pneumovirus* and *Metapneumovirus*. The features exhibited by all members of the *Paramyxovirinae* are the following: identical genome organisation consisting of six (seven in some rubulaviruses) transcriptional elements; nucleocapsid diameter of 18 nm, 5.5 nm pitch; genome length a multiple of six nucleotides; a 3' leader of approximately 55 nucleotides with the first three nucleotides (ACC) absolutely conserved; conserved transcription start and stop signals; RNA editing site in the *P/V* gene; fusion and attachment functions segregated to separate glycoproteins (F and HN or H) that exhibit sequence relatedness across the subfamily.

The avian pathogen *Newcastle disease virus* was long regarded as representative of all paramyxoviruses, but as molecular characterization of other paramyxoviruses progressed it became apparent that *Newcastle disease virus* possessed unique properties. Consequently, it has been reassigned as the sole member of the new genus *Avulavirus* and it is anticipated that other avian paramyxoviruses may be reassigned to this genus when more molecular information is available. The genus *Respirovirus* comprises *Sendai virus*, *Human parainfluenza viruses 1* and *3*, *Bovine parainfluenza virus 3*, and *Simian virus 10*. The genus *Morbillivirus* comprises *Measles virus*, *Canine distemper virus*, *Rinderpest virus*, and *Peste-des-petits-ruminants virus*. In addition, *Cetacean morbillivirus* and *Phocine distemper virus*, two viruses from marine mammals, have been added to the *Morbillivirus* genus recently. The genus *Rubulavirus* comprises *Mumps virus*, *Human parainfluenza viruses 2* and *4*, *Porcine rubulavirus*, *Simian virus 5*, *Simian virus 41*, and three viruses isolated from bats – *Mapuera virus*, Menangle virus, and Tioman virus. The avian paramyxoviruses are currently grouped with the rubulaviruses but this is expected to alter with the advent of molecular characterization of these viruses. Recently two other viruses isolated from bats in South-east Asia and Australia – Hendra virus and Nipah virus – proved to be related to each other but quite distinct from the bat rubulaviruses and indeed other paramyxoviruses. As a consequence these viruses have been assigned to a new genus *Henipavirus*. These viruses are distinguished by their larger genome size (18 kb compared with the uniform 15–16 kb of all other paramyxoviruses). The size of the *Henipavirus* gene products is similar to those of other viruses in the subfamily, with the exception of

the P protein which is approximately 100 amino acids longer than the longest P protein previously characterized. The size, sequence, and domain structure of the Hendra virus L protein are very similar to those of other viruses in the subfamily. Extensive phylogenetic analyses of both full-length L protein and the more conserved domain III sequences confirmed that Hendra virus is a member of the subfamily *Paramyxovirinae* and is more closely related to morbilliviruses and respiroviruses (see below and Figure 1a). However, the L proteins of the two henipaviruses and Tupaia paramyxovirus (an as yet unclassified paramyxovirus isolated from the tree shrew *Tupaia belangeri* in Thailand) differ from the L proteins of other negative-sense RNA viruses in one important respect. The four-amino acid sequence GDNQ, that is absolutely conserved among all known *L* genes of negative-sense RNA viruses is replaced by GDNE (Figure 1b). This sequence resides in the highly conserved and functionally important C motif of domain III of all negative-sense RNA virus polymerases.

The subfamily *Paramyxovirinae* is an ever-expanding taxonomic group; Salem virus, isolated in North America from a horse, and Tupaia paramyxovirus are in the process of characterization and their place in the

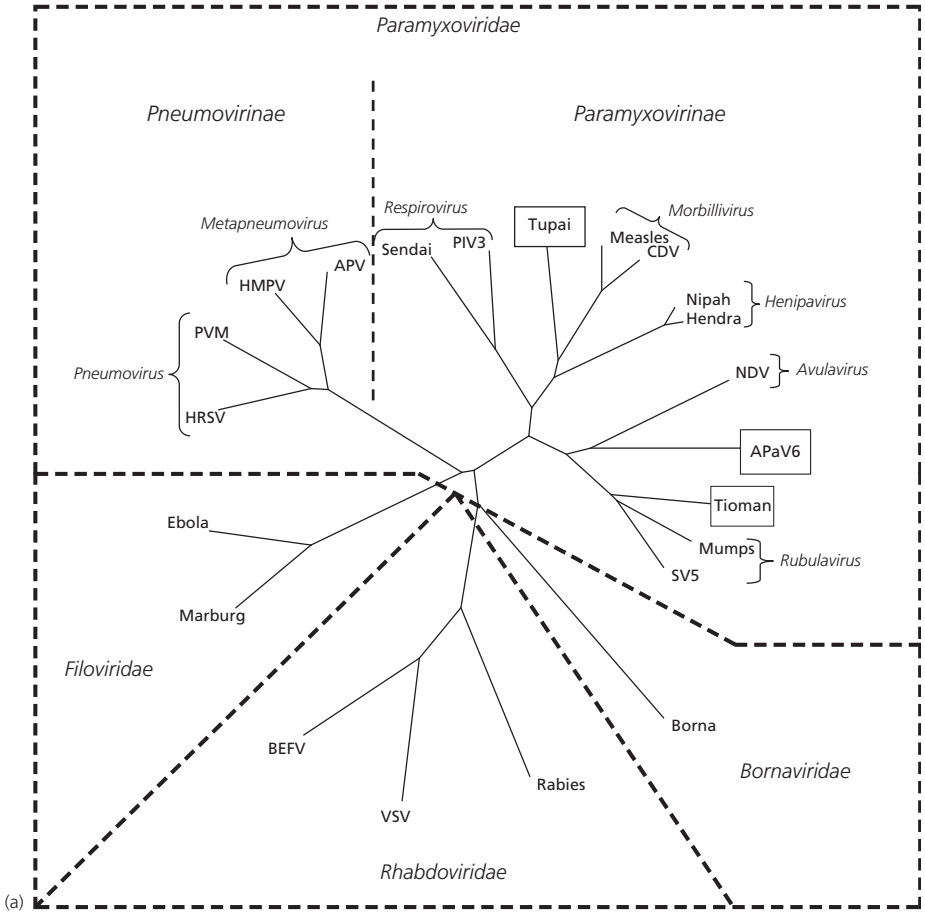

Figure 1 (a) *Unrooted phylogenetic tree of members of the order* Mononegavirales*. The tree was constructed using the CLUSTALX programme with the sequences of the conserved domain III region of the* L *polymerase proteins (Poch et al. 1989, 1990). The ICTV classification designations of the viruses are indicated. For the* Paramyxoviridae*, the two subfamilies and five genera are also indicated. The unclassified Tupaia paramyxovirus (Tupai), avian parainfluenzavirus type 6 (ApaV6) and Tioman virus (Tioman) are indicated.* **(b)** *Alignment of the domain III region sequences of members of the order* Mononegavirales*. The four conserved polymerase motifs (A–D) are indicated. The conserved GDNQ motif is highlighted. The viruses are:* Sendai virus *(Sendai; Middleton et al. 1990),* human parainfluenza virus type 3 *(HPIV-3; Galinski et al. 1988), Tupai paramyxovirus (Tupai; Accession number NP_054697), Measles virus (measles; Bankamp et al. 1999), Canine distemper virus (CDV; Sidhu et al. 1993), Nipah virus (Nipah; Chan et al. 2001), Hendra virus (Hendra; Wang et al. 2000), Newcastle disease virus (NDV; Yusoff et al. 1987), avian paramyxovirus 6 (ApaV6; Accession number NP_150063), Tioman virus (Tioman; Chua et al. 2002), Mumps virus (Mumps; Okazaki et al. 1992), Simian virus 5 (SV5; Higuchi et al. 1992), Human respiratory syncytial virus (HRSV; Tolley et al. 1996), pneumonia virus of mice (PVM; L. Thorpe and A. Easton, unpublished), Human metapneumovirus (HMPV; Accession number AF371337), avian pneumovirus (APV; Randhawa et al. 1996), Marburg virus (Marburg; Muhlberger et al. 1992), Ebola virus, strain Zaire (Ebola; Volchkov et al. 2000), Rabies virus (Rabies; Conzelmann et al. 1990), Vesicular stomatitis Indiana virus (VSV; Schubert et al. 1984), Bovine ephemeral fever virus (BEFV; Walker et al. 1994), Borna disease virus (Borna; Briese et al. 1994). (Adapted from Pringle, C.R. 2004.* Mononegavirales*. In Fauquet, C.M. et al. (eds) Virus Taxonomy, VIII Report of the ICTV. London: Elsevier/Academic Press, pp. 609–14.) (Continued over).*

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Sendai :    FLTTDLKKYCLNWRFESTALFGQRCNEIFGFKTFFNWMHPVLERCTIYVGDPYCPVADRMHR-QLQDHADSGIFIHNPRGGIEGYCQKLWTLISMSAIHLAAVRVGVRVSA
PIV3   :    FLTTDLKKYCLNWRYESTALFGETCNQIFGLNKLFNWLHPRLEGSTIYVGDPYCPPSDKEHI-SLEDHPDSGFYVHNPRGGIEGFCQKLWTLISISAIHLAAVRIGVRVTA
Tupai  :    FLTTDLQKFCLNWRYETSAIYAQRLDEIYGLPNFFEWLHKRLERSTLYVCDPSCPPKLAKHV-DLDTMPNEHIFIKNPMGGIEGYSQKLWTIATIPYLYLSAYEVGVRIAS
Measles:    FITTDLKKYCLNWRYETISLFAQRLNEIYGLPSFFQWLHKRLETSVLYVSDPHCPPDLDAHV-PLCKVPNDQIFIKYPMGGIEGYCQKLWTISTIPYLYLAAYESGVRIAS
CDV    :    FITTDLKKYCLNWRYETISIFAQRLNEIYGLPSFFQWLHRRLEQSILYVSDPHCPPDLDRHV-DLNTAPNSQIFIKYPMGGVEGYCQKLWTISTIPYLYLAAHESGVRIAS
Nipah  :    FLTTDLKKFCLNWRYESMAIFAERLDEIYGLPGFFNWMHKRLERSVIYVADPNCPPNIDKHM-ELEKTPEDDIFIHYPKGGIEGYSQKTWTIATIPFLFLSAYETNTRIAA
Hendra :    FLTTDLKKFCLNWRYESMAIFAERLDEIYGLPGFFNWMHKRLEKSVIYVADPNCPPDIGKHI-NLDDTPEDDIFIHSPKGGIEGYSQKTWTIATIPFLFLSAYETNTRIAA
NDV    :    FITTDLQKYCLNWRYQTIKLFAHAINQLMGLPHFFEWIHLRLMDTTMFVGDPFNPPSDPTDC-DLSRVPNDDIYIVSARGGIEGLCQKLWTMISIAAIQLAAARSHCRVAC
ApaV6  :    FLTTDLQKYCLNWRYTVVKPFAQRLNQLFGIPHGFEWIHLRLMNTTMFVGDPHNVPQFSSTH-DLESQENDGIFIVSPRGGIEGLCQKMWTMISIAAIHLAATESGCRVAS
Tioman :    FLTTDLSKYCLNWRYQSIILFAKSMNQLYGYNHLFEWIHLRLMRSTLYVGDPFNPPRNLTSA-DLDLVENGDIFIVSPRGGIEGLCQKLWTMISIAVIVLSATEAGTRVMS
Mumps  :    FLTTDLTKYCLNWRYQVIIPFARTLNSMYGIPHLFEWIHLRLMRSTLYVGDPFNPPSDPTQL-DLDTALNDDIFIVSPRGGIEGLCQKLWTMISISTIILSATEANTRVMS
SV5    :    FLTTDLKKYCLQWRYQTIIPFAQSLNRMYGYPHLFEWIHLRLMRSTLYVGDPFNPPADTSQF-DLDKVINGDIFIVSPRGGIEGLCQKAWTMISIAVIILSATESGTRVMS
RSV    :    SIITDLSKFNQAFRYETSCICSDVLDELHGVQSLFSWLHLTIPHVTIICTYRHAPPYIRDHIVDLNNVDEQSGLYRYHMGGIEGWCQKLWTIEAISLLDLISLKGKFSITA
PVM    :    SLITDLSKFNQAFRYESSCVCSDLLDELHGTQSLFSWLHLTVPLTTIMCTYRHAPPDTGNNY-NVDDIAEQSGLYRYHMGGIEGWCQKLWTTEAIALLDTVAVKGRFQLTS
HMPV   :    SIVTDLSKFNQAFRYETTAICADVADELHGTQSLFCWLHLIVPMTTMICAYRHAPPETKGEY-DIDKIEEQSGLYRYHMGGIEGWCQKLWTMEAISLLDVVSVKTRCQMTS
APV    :    SIVTDLSKFNQAFRYETTSVCADVADELHGTQSLFCWLHLTVSSTTMICTYRHAPPDTGGIY-DIDQIPEQSGLYRFHMGGIEGWCQKMWTMEAISLLDVVSVRNRVQLTS
Marburg:    SFVTDLEKYNLAFRYEFTRHFIDYCNRCYGVKNLFDWMHFLIPLCYMHVSDFYSPPHCVTED-NRNNPPDCANAYHYHLGGIEGLQQKLWTCISCAQITLVELKTKLKLKS
Ebola  :    SFVTDLEKYNLAFRYEFTAPFIEYCNRCYGVKNVFNWMHYTIPQCYMHVSDYYNPPHNLTLE-NRDNPPEGPSSYRGHMGGIEGLQQKLWTSISCAQISLVEIKTGFKLRS
Rabies :    AFHLDYEKWNNHQRLESTEDVFSVLDQVFGLKRVFSRTHEFFQKAWIYYSDRSDLIGLREDQIYCLDASNGPTCWNGQDGGLEGLRQKGWSLVSLLMIDRESQIRNTRTKI
VSV    :    ANHIDYEKWNNHQRKLSNGPVFRVMGQFLGYPSLIERTHEFFEKSLIYYNGRPDLMRVHNNT--LINSTSQRVCWQGQEGGLEGLRQKGWTILNLLVIQREAKIRNTAVKV
BEFV   :    ANNIDYEKWNNYQRIESNGPVFTVMGRFLGLPNLFTRTHEFFQKSLIYYNQRPDLMMVRGRE--CLNRLGVKVCWEGQKGGLEGLRQKGWSILNYLMIERESRVRNTRVKI
Borna  :    VINLDYSSWCNGFRPELQAPICRQLDQMFNCGYFFRTGCTLPCFTTFIIQDRFNPPYSLSGE----PVEDGVTCAVGTKTMGEGMRQKLWTILTSCWEIIALREINVTFNI

                      A                                                                                   B

Sendai :    MVQ**GDNQ**AIAVTSRVPVAQTYKQKKNHVYEE---ITKYFGALRHVMFDVGHELKLNETIISSKMFVYSKRIYYDGKILPQCLKALTKCVFWSETLVDENRSACSNISTSIA
PIV3   :    MVQ**GDNQ**AIAVTTRVPNNYDYRVKKEIVYKD---VVRFFDSLREVMDDLGHELKLNETIISSKMFIYSKRIYYDGRILPQALKALSRCVFWSETVIDETRSASSNLATSFA
Tupai  :    VVQ**GDNE**VGAITKRVKSSLPYSVKKRMSTQM---ALEFFDRLRWNFSMVGHNLKASETIISSHFFVYSKRIYYDGVCMTQGLKAVARCVFWSETIVDETRSACSNISTSLA
Measles:    LVQ**GDNQ**TIAVTKRVPSTWPYNLKKREAARV---TRDYFVILRQRLHDIGHHLKANETIVSSHFFVYSKGIYYDGLLVSQSLKSIARCVFWSETIVDETRAACSNIATTMA
CDV    :    LVQ**GDNQ**TIAVTKRVPSTWSYALKKSEASRV---TTEYFIALRQRLHDVGHHLKANETIISSHFFVYSKGIYYDGMLISQSLKSIARCVFWSETIVDETRAACSNISTTLA
Nipah  :    IVQ**GDNE**SIAITQKVHPNLPYKVKKEICAKQ---AQLYFERLRMNLRALGHNLKATETIISTHLFIYSKKIHYDGAVLSQALKSMSRCCFWSETLVDETRSACSNISTTIA
Hendra :    IVQ**GDNE**SIAITQKVHPNLPYKVKKEICARQ---AQLYFDRLRMNLRALGHNLKATETIISTHLFVYSKKIHYDGAVLSQALKSMSRCCFWSETLVDETRSACSNISTTIA
NDV    :    MVQ**GDNQ**VIAVTREVRSDDSPEMVLTQLHQA---SDNFFKELIHVNHLIGHNLKDRETIRSDTFFIYSKRIFKDGAILSQVLKNSSKLVMVSGDLSENTVMSCANIASTVA
ApaV6  :    MVQ**GENQ**AIAITTEIEEGEDASVASIRLKEI---SERFFRVFREINRGIGHNLKVQETIHSESFFVYSKRIFFEGKILSQLLKNASRLVLVSETVGENCVGNCSNISSTVA
Tioman :    LVQ**GDNQ**AMAITTMVPRGLPHHEKKRIAYEN---SQTFIRRLRENNLGMGHHLKEQETIVSSEFLIYSKRIIYNGRILNQSLKNVSKLCLIADILGESTQTSCSNLATTVM
Mumps  :    MVQ**GDNQ**AIAITTRVVRSLSHSEKKEQAYKA---SKLFFERLRANNHGIGHHLKEQETILSSDFFIYSKRVFYKGRILTQALKNVSKMCLTADILGDCSQASCSNLATTVM
SV5    :    MVQ**GDNQ**AIAVTTRVPRSLPTLEKKTIAFRS---CNLFFERLKCNNFGLGHHLKEQETIISSHFFVYSKRIFYQGRILTQALKNASKLCLTADVLGECTQSSCSNLATTVM
RSV    :    LIN**GDNQ**SIDISKPVRLMEGQTHAQADYLL----ALNSLKLLYKEYAGIGHKLKGTETYISRDMQFMSKTIQHNGVYYPASIKKVLRVGPWINTILDDFKVSLESIGSLTQ
PVM    :    LIN**GDNQ**SIDISKPTRL-GTRTQSEADYDL----AINSLRLISAAYKGIGHKLKEGETYLSRDMQFMSKTIQHEGVYYPASIKKILRVGPWINTILDDIKTSTESIGSLTQ
HMPV   :    LLN**GDNQ**SIDVSKPVKLSEGLDEVKADYSL----AVKMLKEIRDAYRNIGHKLKEGETYISRDLQFISKVIQSEGVMHPTPIKKILRVGPWINTILDDIKTSAESIGSLCQ
APV    :    LLN**GDNQ**SIDVSKPVRLTGAQTEIQADYSL----AIKMLTAVRDAYYNIGHKLKEGETYVSRDLQFMSKTIQSEGVMYPAAIKKVLRVGPWINTILDDIKTSMEAIGSLCQ
Marburg:    SVM**GDNQ**CITTLSLFPIDAPNDYQENEAELN---AARVAVELAITTGYSGIFLKPEETFVHSGFIYFGKKQYLNGVQLPQSLKTMARCGPLSDSIFDDLQGSLASIGTSFE
Ebola  :    AVM**GDNQ**CITVLSVFPLETDADEQEQSAEDN---AARVAASLAKVTSACGIFLKPDETFVHSGFIYFGKKQYLNGVQLPQSLKTAARMAPLSDAIFDDLQGTLASIGTAFE
Rabies :    LAQ**GDNQ**VLCPTYMLSPGLSQEGLLYELERISRNALSIYRAVEEGASKLGLIIKKEETMCSYDFLIYGKTPLFRGNILVPESKRWARVSCVSNDQIVNLANIMSTVSTNAL
VSV    :    LAQ**GDNQ**VICTQYKTKKSRNVVELQGALNQMVSNNEKIMTAIKIGTGKLGLLINDDETMQSADYLNYGKIPIFRGVIRGLETKRWSRVTCVTNDQIPTCANIMSSVSTNAL
BEFV   :    LAQ**GDNQ**TISMCYKTESWQNEEELDNHIKNMVSNNNQIMQAIINGTEKLGLRINLDETMTSADYINYGKVPIIEGTIKGLPTKRWSRVNFTSNDQLPSTSTVINSSSTNAL
Borna  :    LGQ**GDNQ**TIIIHKSASQNNQLL------------AERALGALYKHARLAGHNLKVEECWVSDCLYEYGKKLFFRGVPVPGCLKQLSRVTDSTGELFPNLYSKLACLTSSCL

(b)                   C                                                                                   D
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Figure 1 *Alignment of the domain III region sequences of members of the order* Mononegavirales *(Continued). For full caption see previous page.*

existing taxonomy is still uncertain. Paramyxoviruses have been isolated frequently from reptiles, including snakes, lizards, and turtles. Preliminary molecular characterization of 18 reptilian viruses, isolated from 18 species of snakes belonging to three different families, suggests that these viruses should be classified in a new genus within the subfamily *Paramyxovirinae* (Franke et al. 2001) .

The family *Paramyxoviridae* – subfamily *Pneumovirinae*

Members of this subfamily are distinguished by the possession of 8 or 10 transcriptional elements, smaller open reading frames, an additional unglycosylated nucleocapsid-associated M2 (sometimes designated 22k) protein, an extensively *O*-linked G (attachment) protein, an unedited *P* gene and in some members, two 3′-terminal nonstructural protein genes (*NS1* and *NS2*). The open reading frames encode proteins with little amino acid sequence homology with those of any of the *Paramyxovirinae* except in the *F* and *L* genes. The nucleocapsid diameter is 13–14 nm and the pitch is 7 nm. The G protein is structurally unrelated to the HN or H proteins of the *Paramyxovirinae* and exhibits a high level of strain variability with as little as 53 percent identity between strains.

The subfamily *Pneumovirinae* comprises two genera: *Pneumovirus* and *Metapneumovirus*. The genus *Pneumovirus* includes three virus species: *Human respiratory syncytial virus*, *Bovine respiratory syncytial virus*, and *Pneumonia virus of mice*. The bovine and human respiratory syncytial viruses share considerable sequence and antigenic relatedness, with the F and HN proteins exhibiting 81 percent identity. They differ predominantly in their hosts. Murine pneumonia virus (sometimes still given as Pneumonia virus of mice) differs from the other two species to a greater extent, with amino acid sequence relationships varying from almost nil in the case of the NS1 and NS2 proteins to 60 percent identity in the case of the nucleoprotein (NP). Pneumonia virus of mice lacks the *M2/L* gene overlap present in the genome of human respiratory syncytial virus and the pneumonia virus of mice *P* gene encodes several additional proteins by using alternative AUG translation initiation codons. Gene transfer experiments have established that the functions of the proteins encoded by the 3′-terminal *NS1* and *NS2* genes, which have no homologues in other paramyxoviruses, are involved in suppression of the antiviral interferon response of infected cells.

The second genus *Metapneumovirus* was originally assigned to accommodate the avian virus *Turkey rhinotracheitis virus* (also known as avian pneumovirus), and has recently been extended by the inclusion of a human virus (designated provisionally as human metapneumovirus), which closely resembles its avian counterpart in molecular properties, but exists in the same biological niche as human respiratory syncytial virus. The metapneumoviruses lack the 3′-terminal *NS1* and *NS2* genes characteristic of other pneumoviruses and exhibit a displacement of the *SH-G* and *F-M2* gene pairs in the gene order. This is rather remarkable since the same relative gene order is conserved throughout the order *Mononegavirales*, except for the two virus species making up the genus *Metapneumovirus* and some genetically engineered recombinant rhabdoviruses (see below). All of the pneumoviruses lack a neuraminidase and all, except pneumonia virus of mice, a hemagglutinating activity. In contrast to the *Paramyxovirinae*, in none of the known pneumoviruses is the genome size a multiple of six nucleotides (see also: Pneumovirus and metapneumovirus).

The family *Rhabdoviridae*

The rhabdoviruses infecting vertebrates are bullet-shaped or cone-shaped, whereas the rhabdoviruses infecting plants appear bacilliform when fixed prior to negative staining or bullet-shaped or pleomorphic if unfixed. The virions are 100–430 nm long and 45–100 nm in diameter. The nucleocapsid of rhabdoviruses contains RNA polymerase activity and is infectious, whereas the naked negative-sense RNA genome is not. Some putative plant viruses, identified solely by electron microscopy appear to lack envelopes. The family consists of six genera: *Vesiculovirus, Lyssavirus, Ephemerovirus, Novirhabdovirus, Cytorhabdovirus*, and *Nucleorhabdovirus*. The genomic RNA has a 5′-terminal phosphate and is not polyadenylated. The ends of the genome have inverted complementary sequences, a feature consistent with the mechanism of replication of rhabdoviruses. Defective RNAs are easily detected because the size of the encapsidated RNA determines the linear dimension of the virus particle. Defective RNAs are dependent on infectious virus but they also interfere specifically with replication, and are responsible for reduction of virus yields by autointerference in virus passaged at high multiplicity.

Currently, nine species and at least 19 tentative species have been assigned to the genus *Vesiculovirus*. These viruses originate from mammals, fish and insects. *Vesicular stomatitis Indiana virus* is the type species of the genus and represents the basic organisational pattern for the entire order *Mononegavirales*. The 11.2 kb genome encodes a 3′-leader sequence of 47 nucleotides that precedes the N, P, M, G, and L open reading frames, which are separated by minimal intergenic dinucleotides, and terminates in a 54 nucleotide trailer region. There is a common 3′-AUACUUUUUUU sequence preceding each intergenic region and a UUGUCNNU sequence at the beginning of each gene.

The leader region is transcribed and a Le RNA may be present in the nuclei of infected cells. The viruses in this genus show varying degrees of amino acid identity in N protein comparisons. *Vesicular stomatitis Indiana virus* has been employed widely as a model system in virus research since all the gene products are structural proteins and can be isolated from purified virions. The genus *Lyssavirus* contains *Rabies virus* and another six rabies-related viruses, four of which originate from bats. Until recently the island continent of Australia was considered to be rabies-free prior to the isolation of *Australian bat lyssavirus* from both pteropid and insectivorous bats. Phylogenetic analysis of the genomes of these viruses suggest that they may have been endemic in Australia prior to European colonization. The 11.9 kb rabies virus genome comprises a 60 nucleotide leader region followed by the five basic rhabdovirus genes, separated by di- or pentanucleotides and followed by a 70 nucleotide trailer. (Exceptionally, the Pasteur strain includes a 423 nucleotide pseudogene region between the *G* and *L* genes which is transcribed within the G mRNA). The rabies viruses are neurotropic and all the viruses in the genus show some antigenic cross-reactivity. The viruses classified in the genus *Ephemerovirus* depart significantly from the standard rhabdovirus pattern. There are three species in the genus: *Bovine ephemeral fever virus*, *Adelaide river virus*, and *Berrimah virus*. The 14.9-kb genome of *Bovine ephemeral fever virus* encodes ten genes in the order 3′-N–P–M–G–Gns–α1–α2–β–γ–L-5′, with intergenic regions of between 26 and 53 nucleotides. Adelaide river virus has intergenic regions of 1 to 4 nucleotides, lacks the *γ* gene and has a β–L overlap. The functions of the products of the *α1*, *α2*, *β*, and *γ* genes have not been identified. Gns is a nonvirion glycoprotein and may be analogous to the soluble G proteins of some paramyxoviruses and ebolaviruses, but it does not induce neutralizing antibodies and is not protective, causes economically important enzootic disease of cattle and is transmitted by, and replicates in, hematophagous arthropods. The genus *Novirhabdovirus* comprises three virus species infecting aquatic hosts: *Infectious hematopoietic necrosis virus*, *Hirame rhabdovirus*, and *Viral hemorrhagic septicemia virus*. These viruses encode the standard rhabdovirus complement of five structural protein genes, plus a sixth nonvirion (NV) protein gene of unknown function located between the *G* and *L* genes. There is a 60 nucleotide leader sequence, a 100 nucleotide trailer, and single nucleotide intergenic regions. The N protein sequence identity between these species is about 34 percent and there is no cross-neutralisation. The genus *Cytorhabdovirus* comprises eight species of plant rhabdoviruses, which multiply in the cytoplasm in association with thread-like structures (viroplasms). They are transmitted by arthropod vectors (predominantly leafhoppers and aphids), and most appear to be able to multiply in the arthropod vector as well as the host plant. The gene order of the type species, *Lettuce necrotic yellows virus*, is 3′-N–P(4a)–4b–M–G–L–5′ with an 84 nucleotide leader and a 187 nucleotide trailer. The *4b* gene is thought to encode a nonstructural protein. The genus *Nucleorhabdovirus* comprises seven species of plant viruses, which multiply in the nucleus of plant cells forming large granular inclusions. Virus morphogenesis occurs at the inner nuclear envelope and enveloped particles accumulate in the perinuclear space. The genome order is similar to that of the cytorhabdoviruses, except that the nonstructural protein gene is designated *SC4*, and there is an additional gene between the *G* and *L* genes in *Rice yellow stunt virus* which encodes a virion-associated protein. There is a 114 nucleotide leader and a 160 nucleotide trailer. Like the cytorhabdoviruses, the nucleorhabdoviruses are predominantly transmitted by leafhoppers and aphids.

In addition to the rhabdoviruses assigned to genera, there at least six serogroups of rhabdoviruses whose taxonomic affiliations are indeterminate at present. There are also more than 37 ungrouped animal viruses that have been propagated and characterized to varying extents, and another 58 plant viruses of uncertain status because their identification is based often on morphological evidence only. Some of the ungrouped animal viruses, such as the arthropod-borne Obodhiang virus and Kotonkan virus previously included in the genus *Lyssavirus*, have been excluded from earlier classifications as knowledge of their uniqueness accrues. Perhaps the most significant of the ungrouped animal rhabdoviruses is Sigma virus, which is transmitted vertically through the germ cells of *Drosophila* spp. conferring CO_2 sensitivity on the infected insect. Both host and viral genes contribute to maintenance of the virus in the host. The Sigma virus genome has an *M–G* gene overlap, variable intergenic regions, and encodes a sixth gene of unknown function, but with motifs related to reverse transcriptase motifs, located between the *P* and *M* genes (see also Rhabdoviruses: rabies).

Table 3 provides a diagrammatic representation of the 3′ to 5′ characteristic arrangement of the transcriptional elements of the genomes of viruses classified in the four families comprising the order *Mononegavirales.*

THE CONSERVATION OF GENE ORDER

The conservation of a preferred gene order is one of the striking and unifying features of the order *Mononegavirales* (Table 3). The order of their genes is the major determinant of the relative levels of gene expression, since genes that are close to the 3′ promoter site are transcribed at higher levels than those that occupy more distal positions. Genetic manipulation of an infectious cDNA clone of vesicular stomatitis Indiana virus resulted in production of viable viruses with rearrangements of gene order, which left the viral nucleotide sequence otherwise unaltered (Ball et al. 1999). The

Table 3 *A diagrammatic representation of the 3′ to 5′ arrangement of the transcriptional units in the genomes of viruses classified in the four families (*Bornaviridae, Filoviridae, Paramyxoviridae, *and* Rhabdoviridae*) comprising the order* Mononegavirales. *Genes encoding polypeptides of presumed homologous function are aligned vertically*

Family	Genus	*VIrus*	3′	Gene order											5′
Subfamily															
Bornaviridae	*Bornavirus*	BDV	le			N	(P)		(M)				(G)			L	tr
Filoviridae	*Ebolavirus*	ZEBOV	le			N	P		(M1)				GP/SP	(?)	(M2)	L	tr
	Marburgvirus	MARV	le			N	P		(M1)				G	(?)	(M2)	L	tr
Rhabdoviridae	*Vesiculovirus*	VSV	le			N	P		M				G			L	tr
	Lyssavirus	RV	le			N	P		M				G	Ps		L	tr
	Cytorhabdovirus	LNYV	le			N	P	4b	M				G			L	tr
	Nucleorhabdovirus	SYNV	le			N	P	Sc4	M				G			L	tr
	Novirhabdovirus	IHNV	le			N	P		M				G	NV		L	tr
	Ephemerovirus	BEFV	le			N	P		M				G	Gns	((1.(2.,(.,()	L	tr
	″	ARV	le			N	P		M				G	Gns	((1,(2,()	L	tr
	Unassigned	SiV	le			N	P	(RT)	M				G			L	tr
Paramyxoviridae																	
Paramyxovirinae	*Avulavirus*	NDV	le			N	P/V		M	F			H			L	tr
	Henipavirus	HeV	le			N	P/C/V		M	F			H			L	tr
	Morbillivirus	MV	le			N	P/C/V		M	F			H			L	tr
	Respirovirus	SeV	le			N	P/C/V		M	F			H/N			L	tr
	Rubulavirus	MuV	le			N	P/V		M	F		SH	H/N			L	tr
Pneumovirinae	*Metapneumovirus*	TRTV	le			N	P		M1	F	M2	SH	G			L	tr
	Pneumovirus	RSV	le	NS1	NS2	N	P		M1	SH	G	F	M2			L	tr
	″	PVM	le	NS1	NS2	N	P/(C)		M1	SH	G	F	M2			L	tr

Abbreviations: viruses; BDV: Borna disease virus; VSV: vesicular stomatitis virus; SYNV: Sonchus yellow net virus; LNYV: lettuce necrotic yellows virus; SiV: Sigma virus; RV: rabies virus; IHNV: infectious hematopoietic virus; BEFV: bovine ephemeral fever virus; ARV: Adelaide river virus; ZEOV: Zaire ebola virus; MARV: Marburg virus; MV: measles virus; SeV: Sendai virus; MuV: mumps virus; TRTV: turkey rhinotracheitis virus; RSV: respiratory syncytial virus; PVM: pneumonia virus of mice; transcriptional units; le: noncoding leader region; NS: nonstructural protein gene; N: nucleoprotein gene; P: phosphoprotein gene; V and C: dispensible nonstructural protein genes; sc4, 4b and (RT): genes of unknown function; M and M1: nonglycosylated matrix protein gene; (M): glycosylated matrix protein gene; F: fusion protein gene; SH: small hydrophobic protein gene; G (or H or HN): glycosylated (or hemagglutinin or hemagglutinin/neuraminidase) attachment protein gene; M2: nonglycosylated (BDV excepted) envelope protein gene; Ps: pseudogene; NV: nonvirion protein gene; Gns: presumptive duplicated G sequence; $\alpha 1$, $\alpha 2$, β, χ: glycoprotein gene products of unknown function; L: large (polymerase) protein gene; tr: noncoding trailer region. (Modified from Pringle and Easton (1997).)

three central genes in the gene order, which encode *P*, *M*, and *G*, were rearranged in all six possible configurations. All six rearrangements were propagated stably as infectious virus and each rearrangement had changed the expression levels of the encoded proteins in accordance with their relative proximity to the 3′ promoter. Similarly, translocation of the *N* gene away from its promoter proximal location to successive positions down the gene order reduced N mRNA and protein expression in a step-wise manner (Wertz et al. 1999). Remarkably, some of the viruses with rearranged genomes replicated as well as or slightly better than the unrearranged wild type virus. Despite the highly conserved gene order of the *Mononegavirales*, gene rearrangement is neither lethal nor necessarily detrimental.

Furthermore, moving the attachment protein gene (*G*) from fourth to first position in the gene order of vesicular stomatitis virus increased expression of the G protein and enhanced the kinetics and level of antibody response in mice infected with this gene order variant (Flanagan et al. 2000). Similar experiments have shown that vesicular stomatitis Indiana virus can tolerate the insertion of duplicated or heterologous genes into the gene order (Wertz et al. 2002), thereby opening up new routes to vaccine development. In general, the nonstandard gene rearrangements and the inserted genes were stably maintained in the modified genomes on repeated passage. The absence of a mechanism for homologous recombination in negative-sense RNA viruses may be responsible for the maintenance of a preferred gene order in evolution, but the reason for choice of the particular gene order adopted by the *Mononegavirales* is an enigma.

PHYLOGENY OF THE ORDER *MONONEGAVIRALES*

The determination of the nucleotide sequences of specific genes, and in many cases the complete genomes, of many members of the order *Mononegavirales* has allowed an analysis of the molecular phylogeny of this group of viruses (Figure 1a). While in principle a phylogenetic analysis can be performed with any of the genes shared by all members of the *Mononegavirales*, it is most appropriate to use sequences of internal proteins as these are least affected by the selective pressure of antibody surveillance in the host. Of the internal protein genes, the most conserved is that encoding the RNA polymerase, the L protein. A comparison of the polymerase protein genes of a number of nonsegmented viruses identified four conserved domains (Poch et al. 1989, 1990; Le Mercier et al. 1997). Domain III contains four conserved motifs, including the GDNQ motif associated with polymerase activity (Figure 1b). A phylogenetic tree constructed using the sequences of the RNA polymerase conserved domain III of a representative selection of viruses is shown in Figure 1a. This analysis shows clearly the phylogenetic divisions of the different virus groups represented in the ICTV classification system. The families *Paramyxoviridae*, *Bornaviridae*, *Rhabdoviridae*, and *Filoviridae* segregate into different lineages, presumably linked by a common ancestor. Within the family *Paramyxoviridae*, the two subfamilies *Paramyxovirinae* and *Pneumovirinae* can be seen to segregate and within each the genera are clearly distinct, though related. Molecular phylogeny has confirmed the relationship of the viruses assigned to the order *Mononegavirales* on the basis of a number of physical and biochemical parameters and now allows a rapid classification of newly isolated viruses. This approach has recently led to the identification of new genera, the genus *Avulavirus* and the genus *Henipavirus*. Figure 1 also shows the phylogenetic relationship of three currently unassigned viruses to the other members of the *Paramyxovirinae*. *Avian paramyxovirus 6* appears to be related most closely to *Newcastle disease virus* (also known as *avian paramyxovirus 1*), whereas Tioman virus and Tupaia paramyxovirus are closest to the rubulaviruses and morbilliviruses, respectively.

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The order *Nidovirales*

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INTRODUCTION

Nidovirus infections in both animals and man result in a variety of diseases that may range from relatively benign to fatal. The order *Nidovirales* (Figure 1) is comprised of the genus *Coronavirus*, which together with the genus *Torovirus* forms the *Coronaviridae* family, and the families *Arteriviridae* and *Roniviridae*. The biological and pathogenic properties of individual coronaviruses, toroviruses, and arteriviruses are summarized in . In this chapter, we present a comparative overview of nidoviruses that has three major goals. First, this broader approach presents our knowledge of human coronaviruses in an evolutionary perspective, thus preparing the ground for further expansion of a cluster of viruses that contains pathogens responsible for emerging infections. Second, it highlights the parallels and homologies amongst nidoviruses and, as such, forms a common framework for studies on animal models and human nidovirus infections. Third, it provides an overview of both unique and conserved nidovirus properties, thus highlighting potential targets for the development of reagents to detect and combat nidovirus infections.

Over the past 20 years, molecular biology and genetics have contributed enormously to the characterization of viruses and, at the same time, changed the face of virus taxonomy. It has become increasingly evident that the comparative analysis of virus genomes provides a better foundation for comprehensive virus taxonomy than more traditional parameters, such as virion morphology or host range. This realization has resulted in the reclassification of specific viruses (or groups of viruses) and also in the introduction of higher order taxa to recognize evolutionary ties that surpass the level of the virus family.

Besides the order *Nidovirales*, only two other virus orders have been recognized thus far. The order *Caudovirales* unites several families of tailed DNA viruses of bacteria (bacteriophages) and the order *Mononegavirales* is comprised of three virus families with a negative-strand RNA genome that consists of one single-stranded RNA segment. The order *Nidovirales* was established by the International Committee on the Taxonomy of Viruses (ICTV) in 1996 (Cavanagh 1997). At that time, it united the newly established family *Arteriviridae* with the pre-existing family *Coronaviridae* (containing the genera *Coronavirus* and *Torovirus*). As will be explained below, the inclusion of these groups of positive-strand RNA viruses in the order *Nidovirales* recognized striking similarities at the level of genome organization and expression, and the intriguing phylogenetic clustering of their giant replicase polyproteins, the precursors to the key enzymes that drive virus replication (Gorbalenya et al. 1989; den Boon et al. 1991). More recently, and on the same grounds, the order *Nidovirales* was expanded to include the family *Roniviridae* (Walker et al. 2004; Spaan et al. 2005), which currently comprises a single genus, *Okavirus*. Whereas other known nidoviruses infect

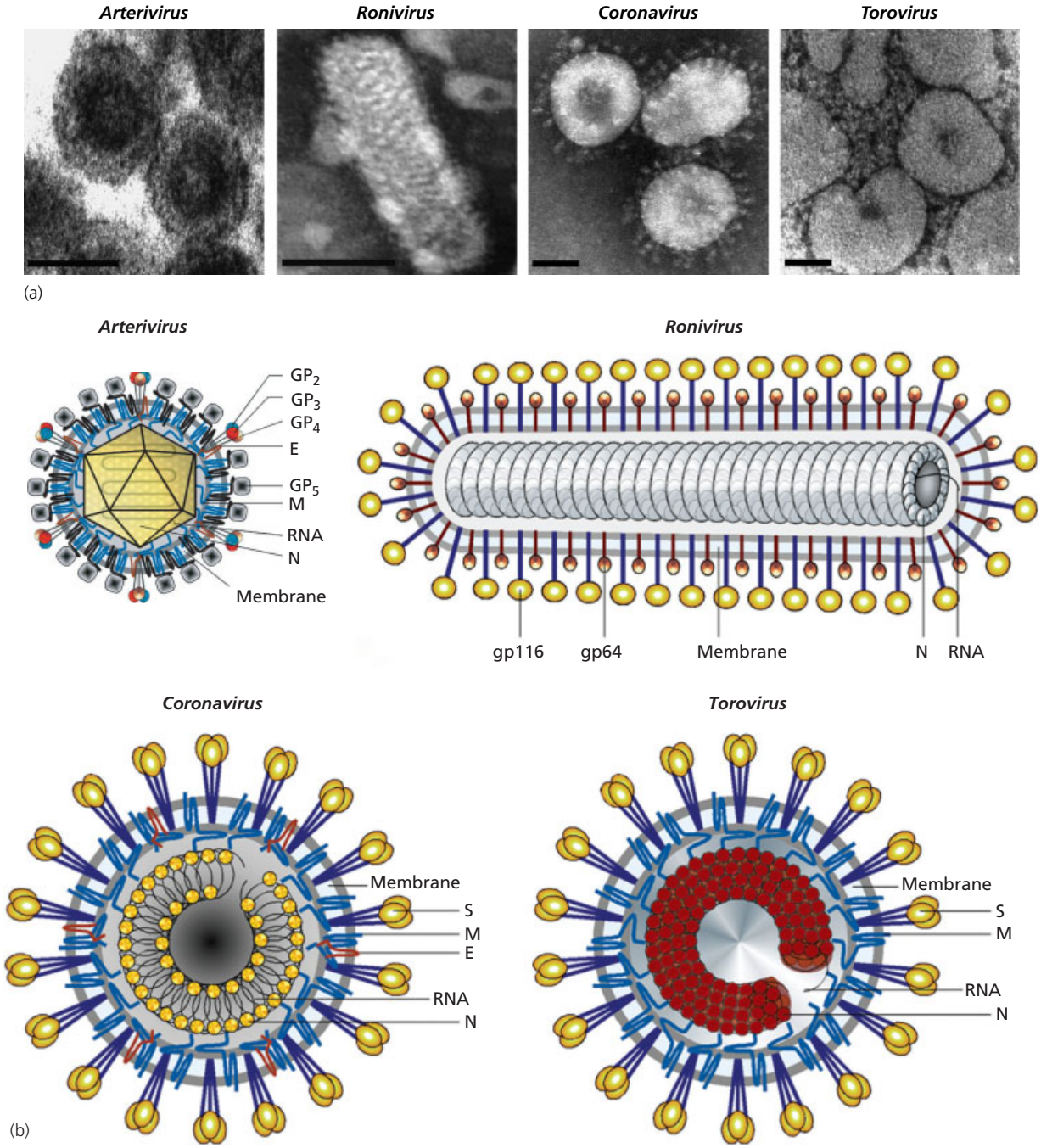

Figure 1 *Electron micrographs* **(a)** *and schematic representations* **(b)** *of nidovirus structure.* **(a)** *Characteristic images of negatively stained arterivirus, ronivirus, coronavirus, and torovirus particles, as captured with a transmission electron microscope. The diverse size and different structural features of representatives of the four main nidovirus branches are evident. Images were reproduced (with permission) from Snijder and Meulenberg (1998), arterivirus; Spann et al. (1995), ronivirus; Dr. Fred Murphy, Centers for Disease Control and Prevention (CDC), Atlanta, USA, coronavirus; and Weiss et al. (1983), torovirus. Bar is 50 nm.* **(b)** *Schematic representation of arterivirus, ronivirus, coronavirus, and torovirus virion structure. The different symmetries of the nucleocapsid structures and virions are indicated (see for more details). Viral membrane proteins present in the virion envelope are depicted. Note that some viruses have been found to contain additional envelope proteins, e.g. the hemagglutinin esterase (HE) protein identified in certain coronaviruses and toroviruses. The stoichiometry of the virion components is shown (more or less) arbitrarily. N, nucleocapsid protein; M, membrane protein; S, spike protein; E, envelope protein; GP or gp, glycoprotein.*

vertebrate hosts (mostly mammals and some avian hosts), roniviruses infect invertebrates, specifically prawns. Nevertheless, their molecular biological features and elements of their genetic plan clearly link them to arteriviruses and coronaviruses (Cowley et al. 2000). The expansion of the *Nidovirales* with new viruses, and the extensive characterization of a few nidoviruses over recent years, have resulted in a refinement and partial

revision of the framework of specific and conserved features that defines this virus order.

MAJOR NIDOVIRUS BRANCHES AND THEIR PROTOTYPE VIRUSES

For obvious reasons, the best-studied nidoviruses are the viruses that either cause significant disease in humans or livestock and/or are most easily studied in cell culture or small animal model systems. In the family *Coronaviridae* (Lai and Cavanagh 1997; Lai and Holmes 2001), the prototype of the genus *Coronavirus* is the avian *Infectious bronchitis virus* (IBV), although *Murine hepatitis virus* (MHV), has probably been more extensively studied. Modest attention has also been paid to the two human common cold viruses, HCoV-229E and HCoV-OC43. The recent emergence of a third human coronavirus, which was identified as the cause of severe acute respiratory syndrome (SARS), has boosted research on human coronaviruses in general (Drosten et al. 2003; Ksiazek et al. 2003; Peiris et al. 2003a). Currently, many aspects of SARS-coronavirus (SARS-CoV) and the disease that it causes are being characterized at an unprecedented rate (Stadler et al. 2003; Peiris et al. 2003b; Berger et al. 2004; Ziebuhr 2004a). The identification of a fourth human coronavirus (NL63 and HKU1) (van der Hoek et al. 2004; Fouchier et al. 2004; Woo et al. 2005), which are relatively closely related to previously identified group 1 and group 2 coronaviruses, respectively, was recently reported, and the detection of additional human coronaviruses can be expected now that the significance of this group of viruses has been recognized in the wake of the SARS outbreak.

Animal coronaviruses that have been studied quite extensively because of the respiratory and/or enteric disease that they cause in livestock or companion animals are IBV, *Bovine coronavirus* (BCoV), porcine *Transmissible gastroenteritis virus* (TGEV), and *Feline coronavirus* (FCoV or FIPV). In the genus *Torovirus* (Snijder and Horzinek 1993), the *Equine torovirus* (EToV) (or Berne virus) is the sole representative, thus far, that can be amplified and studied in cultured cells. Bovine, porcine, and possibly human toroviruses have been identified but have not yet been propagated in cell culture. The name coronavirus is derived from the solar corona-like (Latin *corona*: crown) appearance of virus particles in negatively stained electron micrographs, whereas the name torovirus is derived from the curved tubular (Latin torus: lowest convex molding in the base of a column) morphology of the torovirus nucleocapsid structure (Figure 1a).

The outcome of arterivirus infection can range from an asymptomatic, persistent carrier-state to lethal hemorrhagic fever. The arterivirus family (Plagemann 1996; Snijder and Meulenberg 1998; Snijder and Meulenberg 2001) currently consists of a single *Arterivirus* genus with four virus species, of which the prototype *Equine arteritis virus* (EAV) is the best-studied representative in terms of molecular biology. Arteritis (inflammation of an artery), one of the disease symptoms caused by EAV, gave the arterivirus family its name. EAV and the *Porcine reproductive and respiratory syndrome virus* (PRRSV), of which different genotypes emerged in Europe and North America during the late 1980s, are the two arteriviruses that cause significant disease in livestock. Arteriviruses spread primarily via the respiratory route, but sexual transmission by persistently infected animals is an important secondary cause of infection in the case of EAV and PRRSV.

In the family *Roniviridae* (for *ro*d-shaped *ni*dovirus), the prototype of the genus *Okavirus* is the Gill-associated virus (GAV) (Spann et al. 1995; Cowley et al. 2000) of which the black tiger prawn (*Penaeus monodon*) appears to be the natural host. GAV can cause devastating infections in cultured prawns, where it targets the lymphoid organ (or 'oka') in particular.

KEY COMMON FEATURES OF NIDOVIRUSES

Nidoviruses are enveloped, positive-strand RNA viruses that share a similar genome organization and similar replication strategy (Figure 2) that is driven by the gene encoding the large nonstructural or 'replicase' proteins. Phylogenetic analysis of key domains in the nidovirus replicase gene revealed that these elements are much more closely related to each other than to the corresponding sequences in the genomes of other positive-strand RNA viruses (see below and Figure 3 for details). Thus, the nidovirus replicase represents a separate lineage in RNA virus evolution (den Boon et al. 1991; Gorbalenya and Koonin 1993; Gorbalenya et al. 1989).

Nidoviruses have diverged profoundly since they originated from their common root. They differ significantly in terms of the size of their genome, virion morphology, host range, and various biological properties. However, a number of features, although not diagnostic of this virus order alone, are thought to be common to nidoviruses. Still, it should be noted that, in particular for toroviruses and roniviruses, many of these properties have not been studied in great detail and the list below may be subject to future updates.

- Nidoviruses have positive-strand RNA genomes consisting of one single-stranded, linear RNA molecule that contains a 5′ cap structure and a 3′ poly(A) tail.
- The general nidovirus genome organization (with some exceptions) is: 5′ untranslated region – replicase – envelope proteins – nucleocapsid protein – 3′ untranslated region (however, note that the ronivirus N protein gene is located upstream of the envelope protein gene (Cowley et al. 2004)).

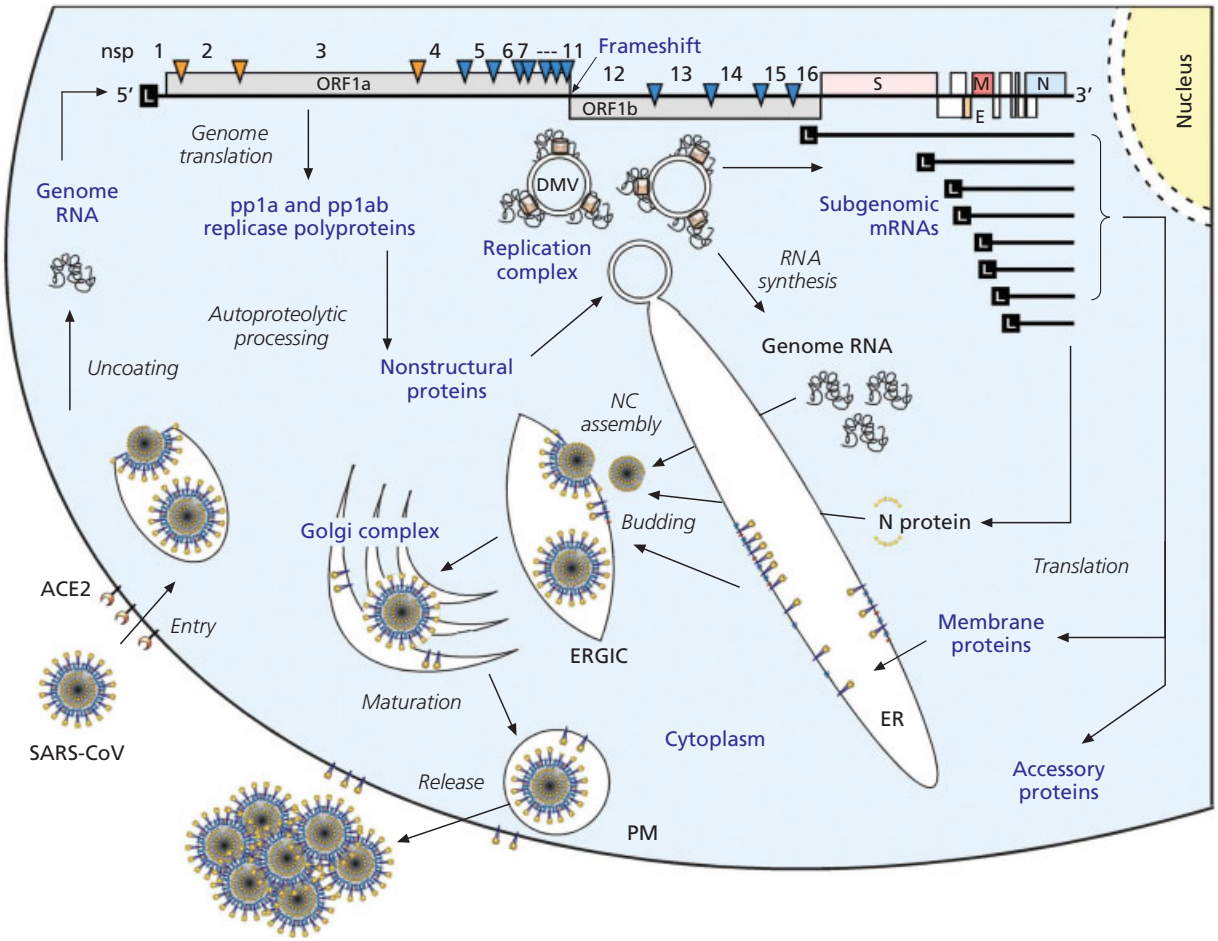

Figure 2 *Overview of the nidovirus life cycle, using SARS-CoV as an example. Depicted is the cytoplasmic replication cycle of a typical coronavirus (see text and for more details). Infection starts with binding of the virus to a specific receptor on the host cell's plasma membrane (PM) (in this case angiotensin-converting enzyme 2 (ACE2)), entry by fusion of viral envelope and cellular membrane, and release of the viral plus-strand RNA genome into the cytoplasm. Subsequently, the genome is translated by host ribosomes to produce the polyprotein precursors (pp1a and pp1ab) to the nonstructural proteins (nsp) that drive viral RNA synthesis. Following limited autoproteolysis of these 'replicase' polyproteins, a complex for viral RNA synthesis is assembled on cytoplasmic double-membrane structures (double-membrane vesicles (DMVs)). This complex produces minus-strand RNAs (not shown) that serves as template for genome replication and the synthesis of a nested set of subgenomic mRNAs. The latter, which contain a common leader sequence (L) identical to the 5′ end of the genome, are translated to express the genes that are encoded in the 3′-proximal third of the genome. Translation of the smallest subgenomic mRNA yields the viral nucleocapsid protein (N) that assembles into new nucleocapsid (NC) structures together with newly generated genome RNA. Other subgenomic mRNAs encodes viral envelope proteins that are (largely cotranslationally) inserted into membranes of the host cell's exocytic pathway and migrate towards the site of virus assembly (the ER–Golgi intermediate compartment (ERGIC) in the case of coronaviruses). Following budding of the NC through membranes that contain viral envelope proteins, the new virion ends up in the lumen and, while undergoing additional maturation steps in, for example, the Golgi complex, leaves the cell via the exocytic pathway.*

- The nidovirus replicase gene comprises two large and overlapping open reading frames (ORF) that encode the replicase polyproteins pp1a and pp1ab. Expression of the downstream ORF1b is mediated by programmed −1 ribosomal frameshifting (FS).
- An RNA structure (slippery sequence plus RNA pseudoknot) mediates programmed ribosomal frameshifting in the ORF1a/1b overlap region (Brierley et al. 1989).
- In the infected cell, nidovirus gene expression is mediated by a set of at least three 3′ co-terminal subgenomic mRNAs that are thought to be produced from a set of complementary templates of the same sizes. Generally, only the ORFs contained within the 5′ unique regions of each mRNA, i.e. the regions not found in the next smallest mRNA are expressed.
- Viral RNA synthesis occurs on intracellular, virus-induced (double) membrane structures.
- Nidovirus particles contain a lipid envelope.
- The envelope of nidovirus particles contains an integral, triple-spanning membrane (m) protein.
- Nidovirus particles are assembled intracellularly, by budding into the lumen of membrane-bound organelles.

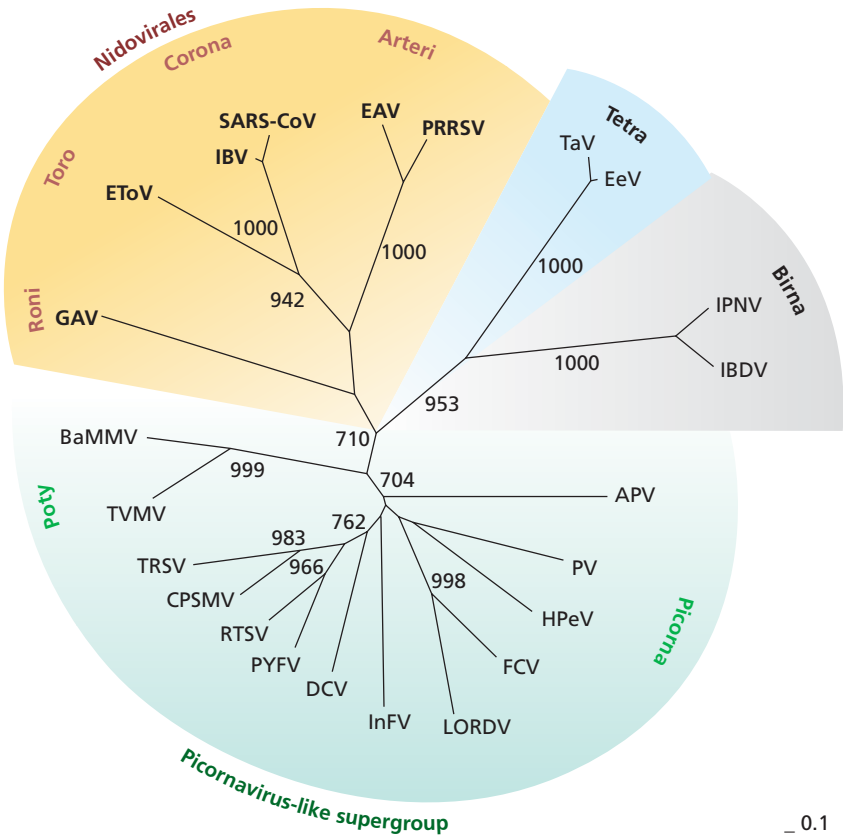

Figure 3 *Unrooted phylogenetic tree of RNA-dependent RNA polymerase (RdRp) domains of* Nidovirales *and other (super)groups of positive-strand and double-stranded RNA viruses. The most conserved part of RdRps from representative viruses in the* Picornaviridae, Dicistroviridae, Sequiviridae, Comoviridae, Caliciviridae, Potyviridae, Coronaviridae, Roniviridae, Arteriviridae, Birnaviridae, Tetraviridae, *and unclassified insect viruses was aligned. An unrooted neighbor-joining tree was inferred by the ClustalX1.81 program. For details of the analysis, see Gorbalenya et al. (2002). All bifurcations with support in >700 out of 1 000 bootstraps are indicated. Different groups of viruses are highlighted with different colors. The tree was modified from Gorbalenya et al. (2002) and Spaan et al. (2005). Virus families/groups and abbreviations of viruses included in the analysis:* Coronaviridae*: avian infectious bronchitis virus (IBV), severe acute respiratory syndrome virus (SARS-CoV) and equine torovirus (EToV);* Arteriviridae*, equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus strain VR-2332 (PRRSV);* Roniviridae*: gill-associated virus (GAV);* Picornaviridae*, human poliovirus type 3 Leon strain (PV) and parechovirus 1 (HPeV); iflavirus, infectious flacherie virus (InFV); unclassified insect viruses, Acyrthosiphon pisum virus (APV);* Dicistroviridae*, Drosophila C virus (DCV);* Sequiviridae*, rice tungro spherical virus (RTSV), and Parsnip yellow fleck virus (PYFV);* Comoviridae*, cowpea severe mosaic virus (CPSMV) and tobacco ringspot virus (TRSV);* Caliciviridae*, feline calicivirus F9 (FCV), and Lordsdale virus (LORDV);* Potyviridae*, tobacco vein mottling virus (TVMV) and barley mild mosaic virus (BaMMV);* Tetraviridae*, Thosea asigna virus (TaV) and Euprosterna elaeasa virus (EeV);* Birnaviridae*, infectious pancreatic necrosis virus (IPNV) and infectious bursal disease virus (IBDV).*

Nidoviruses also share a number of unique features that could be considered as genetic markers of this order. All of them are associated with the replicase gene, which contains a conserved series of key functional domains (a constellation abbreviated as M–3CLpro-M–FS–RdRp–ZF–HEL–NendoU; see below) including:

- A cluster of domains, encoded by ORF1a, consisting of a main proteinase (3C-like proteinase; 3CLpro), which has a chymotrypsin-like fold and substrate specificity resembling that of picornavirus 3C proteinases, and flanking transmembrane (M) domains (M-3CLpro-M);
- An ORF1b-encoded protein that combines an N-terminal (putative) multinuclear zinc-finger (ZF) domain with a C-terminal NTP-binding/5′-to-3′-helicase domain (HEL);
- An ORF1b-encoded uridylate-specific endoribonuclease (NendoU).

The exact role of these unique genetic markers in the nidovirus life cycle remains largely unknown. The presence of a common ‘leader’ sequence at the 5′ end of all viral mRNA species (Spaan et al. 1983; Baric et al. 1983; Lai et al. 1984; de Vries et al. 1990) was initially considered a major hallmark of nidoviruses, but recently proved to be a property that is not universally conserved

amongst members of this virus order (Cowley et al. 2002; van Vliet et al. 2002).

MAIN DIFFERENCES BETWEEN MAJOR NIDOVIRUS BRANCHES

Despite the common features of their genome and molecular biology (see also below), evolutionary distances within the order *Nidovirales* are vast and there are major differences between the virus families and genera that are united in this taxon. A striking difference that separates the arteriviruses from other nidoviruses is the smaller size of their genome (12.7 to 15.7 kb versus more than 27 kb for the other nidoviruses). Accordingly, the arterivirus replicase polyproteins are less than half the size of their coronavirus, torovirus, and ronivirus counterparts. Despite this difference, coronaviruses and arteriviruses share a common property that is not conserved in toroviruses and roniviruses, as they have a common leader sequence at the 5′ end of all viral mRNAs, genomic and subgenomic.

Most of all, however, the features that distinguish the various nidovirus branches from each other are found at the level of virion structure and the properties of structural proteins (see Figure 1 and for details). Arterivirus are spherical with a diameter of about 60 nm and lack prominent surface projections. Coronavirus particles have a diameter of 120–140 nm (including the extended peplomers). Toroviruses have a virion size similar to coronaviruses, while the bacilliform ronivirus particles are 150–200 nm long, have a diameter of 20–30 nm, and are also studded with prominent peplomers projecting approximately 11 nm from the surface. The nucleocapsid structure is helical–spherical for coronaviruses, helical–tubular for toroviruses and roniviruses, and isometric for arteriviruses. The nucleocapsid proteins of the four virus groups do not appear to share any common ancestry and their sizes range from 50 to 60 kDa for coronaviruses to only 12–14 kDa for arteriviruses. Also, the number of viral envelope proteins is quite variable, ranging from a single, post-translationally cleaved spike glycoprotein in roniviruses to a combination of six integral envelope proteins in arteriviruses. Whereas coronaviruses, toroviruses, and roniviruses carry a prominent spike structure (an oligomer of the S protein) on their surface, such structures have not been observed for arteriviruses. However, a common feature of all nidoviruses is the presence of an integral, triple-spanning membrane (M) protein in the viral envelope, which plays a crucial role during virus assembly.

A final difference that should be highlighted is the presence of genes encoding so-called 'accessory proteins' in coronavirus genomes. In the case of arteriviruses, toroviruses, and probably also roniviruses, all genes downstream of the replicase gene encode structural proteins. However, in coronaviruses several nonstructural proteins are encoded in this part of the genome. These genes differ (in terms of position and protein encoded) between the different subgroups that are recognized among coronaviruses (for example, see the small undesignated genes dispersed among the *S*, *E*, *M*, and *N* protein genes in the 3′ proximal region of the SARS-CoV genome in Figure 2). A common feature, however, is that these genes appear to be dispensable for replication in cell culture. This suggests that the products of these genes exert their function at the level of the interaction with the host and may be important for the virus in, for example, countering antiviral responses triggered by the infection.

GENERAL SCHEME OF THE NIDOVIRUS LIFE CYCLE

Our knowledge of the nidovirus life cycle is largely based on cell culture-based studies with a limited number of viruses from the arterivirus and coronavirus families. Consequently, the description that follows (and the example of the SARS-CoV life cycle presented in Figure 2) should not be considered as definitive for all nidoviruses.

Nidoviruses attach to their host cell by binding to a receptor on the cell surface. These host molecules are mostly proteins, but in some cases carbohydrates may be involved. A number of cellular receptors have been identified and studied in detail for coronaviruses (Yeager et al. 1992; Williams et al. 1991; Li et al. 2003) and for the arterivirus PRRSV (Vanderheijden et al. 2003). Fusion of the nidoviral and cellular membrane is mediated by one of the major surface glycoproteins. Since the spike (S) protein of several coronaviruses displays fusion activity at neutral pH, fusion of the viral envelope and the plasma membrane is considered to be one route of entry for this virus group. However, endocytosis of coronaviruses has been described, which is the same infection route used by arteriviruses. Fusion of the viral envelope and a cellular membrane (either plasma membrane or endosome) results in the release of the viral nucleocapsid into the cytoplasm of the host cell (see for details). Uncoating of the viral genome, which must precede genome translation, has not been studied in any detail.

The nidovirus genome is translated by host ribosomes that bind to the cap structure at the 5′ end of the genomic RNA and subsequently scan the template to find the translation initiation codon of replicase ORF1a. Following ORF1a translation, 60–80 percent of the ribosomes terminate at the ORF1a stop codon. However, the remaining ribosomes shift into the −1 reading frame just upstream of the ORF1a stop codon, at a specific heptanucleotide sequence (the slippery sequence), and continue translation in replicase ORF1b (Brierley et al. 1989; Snijder et al. 1990a; den Boon et al. 1991; Cowley et al. 2000). Consequently, genome translation leads to the synthesis of two replicase polyproteins, pp1a and pp1ab,

with pp1ab being a carboxyl-extended version of pp1a. The −1 frameshift is stimulated by an RNA pseudoknot structure (i.e. two stem–loop structures that interact by base-pairing between sequences in the loops of the two hairpins) that probably induces a ribosomal pause over the slippery sequence. Due to the ribosomal frameshift mechanism, the ratio between translation of ORF1a and ORF1b is constant, which is thought to be an important part of the regulation of nidovirus replicase expression.

Nascent replicase polyproteins have never been detected in nidovirus-infected cells due to the speed of autoproteolytic processing, which seems to be initiated before the completion of ORF1a translation. The processing of the N-terminal 20–25 percent of the pp1ab polyprotein, is controlled by one or more so-called accessory proteinases that initiate autoproteolysis, while the main proteinase (3CLpro) is responsible for the processing of the downstream and most conserved part of the polyproteins (reviewed by Ziebuhr et al. (2000)). The accessory proteinase domains are located in the ORF1a-encoded region of the replicase and belong to the papainlike superfamily of cysteine proteinases (Gorbalenya et al. 1991). They are either autoproteinases that liberate the replicase subunit of which they are part (arteriviruses), or they can cleave up to three sites in the N-terminal half of pp1a (coronaviruses). The C-terminal half of pp1a and the ORF1b-encoded part of pp1ab are cleaved at eight to 11 sites by the viral main proteinase, which belongs to the chymotrypsin-like superfamily of proteinases (Gorbalenya et al. 1989; Snijder et al. 1996; Ziebuhr et al. 2003; Anand et al. 2002; Barrette-Ng et al. 2002). Replicase proteolysis is a crucial and probably highly regulated process, which should produce the right cleavage products in the right amounts at the right moment. The number of mature replicase products (nonstructural proteins or nsps) ranges from 12 or 13 in arteriviruses to 16 in most coronaviruses. By analogy with other RNA viruses, it may be expected that certain processing intermediates also have specific functions in the nidovirus life cycle.

Either during or shortly after translation, and possibly regulated by proteolytic cleavages, most of the nidovirus replicase proteins associate with intracellular membranes to form a membrane-bound complex for RNA synthesis ('replication complex'), a common feature of animal positive-strand RNA viruses. A number of ORF1a-encoded subunits contain hydrophobic domains and become transmembrane proteins, although they cannot be classified as 'conventional' membrane proteins (e.g. a signal sequence is lacking) and their mode of membrane insertion is unclear. For arteriviruses, it has been shown that the expression of two of these proteins (nsp2 and nsp3) suffices to induce the formation of specific double membrane vesicles, which seem to be derived from the endoplasmic reticulum and carry the viral replication complex (Pedersen et al. 1999; Snijder et al. 2001). Similar structures have been identified in cells infected with coronaviruses (Gosert et al. 2002), although other studies implicated late endosomes (van der Meer et al. 1999) or autophagosomes (Prentice et al. 2004) in the formation of the coronavirus replication complex. Other replicase subunits lack hydrophobic sequences, but immunofluorescence and electron microscopy has revealed that most of them co-localize with the ORF1a-encoded transmembrane subunits. Thus, the latter are likely to form a membrane-bound scaffold for the replication complex.

The first step in viral RNA synthesis is the recognition of RNA signals that promote the association of the 3′ end of the viral genome with a complex of viral enzymes that includes the RNA-dependent RNA polymerase encoded in the 5′-proximal part of replicase ORF1b. This 'RdRp complex' initiates the synthesis of full-length minus-strand RNA (or antigenome), which in turn will be the template for the synthesis of novel genome RNA. For the latter process, recognition signals present in the 3′ end of the antigenome must be used (see
for details). The amplification of the viral RNA is asymmetric and results in the production of much more plus- than minus-strand RNA. In coronavirus-infected cells, the structures engaged in viral RNA synthesis have been characterized. Approximately 70 percent of the RNA structures that accumulate in infected cells are multi-stranded intermediates (replicative intermediates) and 30 percent are double-stranded structures (replicative forms). These estimates describe, essentially, the structures involved in plus-strand synthesis. The structures engaged in minus-strand synthesis have not yet been characterized, although it is known that minus-strand templates are unstable and turnover during viral replication (Sawicki et al. 2001).

The newly synthesized genome RNA may be utilized in translation, replication, subgenomic mRNA synthesis, and/or encapsidation. First, genome RNA is used for translation of replicase ORFs 1a and 1b, thus yielding the polyprotein precursors of the main components of the viral replicase/transcriptase complex. Second, genome RNA serves as template for antigenome synthesis in the asymmetric cycle of genome replication described above. Third, genome RNA is assumed to be the template to produce subgenome-length minus strands that are used as templates for subgenomic mRNA synthesis. A nested set of subgenomic mRNAs is produced to express the viral structural proteins (and sometimes also accessory proteins) from genes located in the 3′-proximal third of the genome (the region downstream of ORF1b), which remains inaccessible for ribosomes translating the genome RNA. The synthesis of nidovirus subgenomic mRNAs was studied extensively and this will be discussed in a separate paragraph below. Finally, the de novo synthesized genomes can be used for encapsidation into progeny virions at the end of the infection cycle.

Following the accumulation of the structural proteins, the components for new virus particles have to assemble

in the infected cell. New genomes first associate with the cytoplasmic nucleocapsid protein to form nucleocapsid structures. The viral envelope proteins are inserted into intracellular membranes, mostly by conventional cotranslational translocation. Subsequently, the envelope proteins are targeted to the site of virus assembly (usually membranes between the endoplasmic reticulum and Golgi complex; see for details). Here, they meet up with the nucleocapsid structure and trigger a budding process that results in the envelopment of the nucleocapsid by host membrane impregnated with viral envelope proteins. As a result of budding, the newly formed virus particle is located in the lumen of the membrane compartment, after which it can leave the cell by following the exocytic pathway towards the plasma membrane. During this process, additional maturation of viral proteins and/or carbohydrates attached to these proteins occurs, in particular during transit through the Golgi complex.

THE NIDOVIRUS REPLICASE/ TRANSCRIPTASE

The synthesis of nidovirus-specific RNAs is mediated by a molecular machinery known as replicase/transcriptase (for simplicity often referred to as 'replicase'), which has not been characterized in detail for any nidovirus. Much of what is currently known about this macromolecular complex derives from comparative sequence analysis (reviewed in (Gorbalenya 2001); see below) that implicated ORF1a and 1b in encoding the key elements of the nidovirus replicase/transcriptase. This prediction has only recently been verified experimentally using genetic methods for the arterivirus EAV and the coronaviruses HCoV-229E and MHV (van Dinten et al., 1997, 1999; Tijms et al. 2001; Thiel et al. 2001; Seybert et al. 2005; Ivanov et al. 2004; Siddell et al. 2001).

The nidovirus replicase gene forms the backbone of the unique genetic plan that unites the different nidovirus subgroups (den Boon et al. 1991; Gorbalenya and Koonin 1993; Gorbalenya et al. 1989). From the end of the 1980s and through the 1990s, sequence analysis of different coronavirus, arterivirus, and torovirus genomes revealed a genetic plan consisting of nontranslated regions at the 5′ and 3′ ends of the genome that flank six to 11 ORFs, some of which are partly overlapping. The 5′-proximal two-thirds of the nidovirus genome is dominated by the replicase ORFs that encode the large pp1a and pp1ab polyproteins. These polyproteins are organized around a conserved framework of a dozen replicative domains and are autocatalytically processed to numerous products at conserved interdomain junctions (Ziebuhr et al. 2000). The other ORFs, whose number varies in different viruses, are located further downstream and a nested set of subgenomic mRNAs is generated to allow their expression.

The first nidovirus genome to be fully sequenced was that of the coronavirus IBV (Boursnell et al. 1987). Pairwise and profile comparisons of the IBV pp1a/pp1ab with replicative proteins of positive-strand RNA viruses and selected cellular proteins (Gorbalenya et al. 1988, 1989) tentatively identified a total of eight domains and 13 sites of proteolytic autoprocessing in pp1a/pp1ab. This analysis proved to be quite accurate and was largely confirmed by subsequent experimental studies. In the ORF1a-encoded sequence, a 3CLpro was recognized that is flanked by two highly hydrophobic, presumably transmembrane domains. Downstream of the ORF1a/1b ribosomal frameshift site, sequences encoding a putative RdRp and a putative superfamily 1 helicase (HEL1), the latter linked to an upstream-positioned Cys/His-rich domain predicted to form a multi-nuclear Zn-finger (ZF), were correctly identified.

Three domains identified in IBV, namely 3CLpro, RdRp, and HEL1, were (distant) variants of domains that are ubiquitous in the replicative polyproteins of positive-strand RNA viruses. Like their counterparts in other viruses, the IBV RdRp and HEL1 domains were postulated to play a central role in RNA synthesis. These domains, along with 3CLpro, were found to possess unique structural properties (Gorbalenya et al. 1988, 1989). RdRp has a replacement of Gly by Ser in the GDD signature that is otherwise absolutely conserved in positive-strand RNA viruses. The substitution of this Gly residue was later found to be a common feature of all nidoviruses. HEL1 is uniquely associated with ZF in the same protein and evidence for an important role of the ZF domain in HEL1 activity and other functions has emerged (van Dinten et al. 2000; Seybert et al. 2005). Nidovirus 3CLpro has several unique features (Barrette-Ng et al. 2002; Anand et al. 2002) and the two flanking hydrophobic domains have not been observed elsewhere. Furthermore, these and other (nidovirus-specific) conserved domains are positioned in a very specific and unique order within the replicase. Taken together, these observations confirmed that coronaviruses were the prototype of a separate supergroup (an informal rank above the family level) of positive-strand RNA viruses.

Since most unique features of the proposed IBV replicase organization were derived from the analysis of weak sequence similarities, independent validation of these findings was important and became possible upon publication of the sequence of MHV (Bredenbeek et al. 1990; Gorbalenya et al. 1991; Lee et al. 1991; Bonilla et al. 1994). These analyses confirmed and extended the predictions made for IBV and also revealed that the most N-terminal ~2 000 amino acids of the replicase are poorly conserved between coronaviruses. Among the few conserved features identified in this region were the so-called X domain and two papainlike proteinase (PLpro) domains (Gorbalenya et al. 1991) that are part of the same protein (nsp3) (see Figure 2). The X

domain was shown to be a homologue of a functionally uncharacterized domain previously identified in the vicinity of the HEL1 domain in replicative polyproteins of the *Togaviridae* (Dominguez et al. 1990). It was subsequently shown to be a unique property of PLpros to contain a homologue of the Zn-ribbon domain that is commonly associated with a cellular transcription elongation factor (Herold et al. 1999). The presence of this Zn-ribbon, which is essential for the proteolytic activity of PLpro, implies a role in RNA synthesis for the nsp3 protein that contains this domain.

Further insight into the organization and evolution of coronavirus replicative polyproteins was gained by a study of the equine torovirus EToV (Berne virus) (Snijder et al. 1990a), which was found to have a coronaviruslike genome organization, including the characteristic RdRp–ZF–HEL1 domains encoded by ORF1b and a frameshifting signal at the ORF1a/1b junction. These results unequivocally established the relationship between toroviruses and coronaviruses, even though these viruses have different virion morphologies. The comparison also led to new assignments. In the C-terminal part of the ORF1b-encoded protein, a unique conserved domain of unknown function was delineated (Snijder et al. 1990a). Secondly, a surprising similarity was found between the C-terminal region of EToV pp1a and an accessory protein encoded by ORF2 in group 2 coronaviruses such as MHV (Snijder et al. 1991). This observation indicated that some replicative proteins are optional (since they are not conserved in all corona- and toroviruses) and that they can be subject to reshuffling, possibly through RNA recombination.

In 1991, the publication of the first complete arterivirus sequence, that of EAV, revealed several remarkable features of this virus group (den Boon et al. 1991). At that time, this virus, together with alphaviruses and rubiviruses, was considered to be part of the *Togaviridae* family. Although EAV has distinct virion morphology and a genome size approximately half that of an average coronavirus genome, it proved to share numerous features with coronaviruses. In particular, the ORF1b-encoded polyprotein, expressed through ribosomal frameshifting, contained distant homologues of the four previously identified conserved domains in corona- and toroviruses. Likewise, the pp1a polyprotein contained a characteristic domain set consisting of PLpro domains, as well as a 3CLpro domain embedded between two hydrophobic domains (den Boon et al. 1991).

However, at the same time, arteriviruses were also found to possess specific features. All conserved arterivirus domains were found to be smaller than their coronavirus counterparts, and the sequence similarities between ORF1a-encoded sequences of corona- and arteriviruses were so weak that a relationship could not be rigorously established (Ziebuhr et al. 2000). Unlike their coronavirus counterparts and the related picornavirus enzymes, the arterivirus 3CLpro was shown to employ Ser as the catalytic nucleophile (Snijder et al. 1996; Barrette-Ng et al. 2002). Importantly, though, the replicase domains of arteriviruses, like those of the corona- and toroviruses, were found to have no strong sequence similarities to other virus groups. All these observations were most compatible with a pronounced divergent evolution of arteriviruses and coronaviruses from a common ancestor (den Boon et al. 1991). Accordingly, these families were united in a coronavirus-like supergroup (Gorbalenya and Koonin 1993; Snijder and Horzinek 1993), which was subsequently recognized as the order *Nidovirales* (Cavanagh 1997).

The most recent, and most unexpected, extension of the nidovirus order was triggered by the sequence analysis of RNA viruses infecting prawns in Australia and Southeast Asia, GAV and Yellow head virus (YFV). The phylogenetic relationship between GAV and nidoviruses became evident from comparative sequence analyses of the replicase polyproteins, which again revealed striking similarities in organization and mode of expression (Cowley et al. 2000). Comparative sequence analysis identified ORF1b-encoded putative helicase and RdRp domains, ordered as in other nidoviruses and carrying specific sequence signatures, such as the GDD to SDD replacement in the RdRp. Collectively, the data argued that the replicases of GAV (infecting invertebrates) and other nidoviruses (infecting vertebrates) have a common ancestor. On the other hand, the presence of a number of regions with low sequence similarity in the ORF1b-encoded sequence and, in particular, the extremely poor pp1a conservation also suggested that GAV has diverged significantly from the vertebrate nidoviruses. Indeed, the only region in pp1a with significant sequence similarity proved to be a 3CLpro domain, again flanked by hydrophobic (probably membrane-spanning) domains (Ziebuhr et al. 2003).

Finally, the impulse given to the analysis of the nidovirus replicase by the SARS outbreak of 2003 should be mentioned. The relatively distant position of the SARS-coronavirus (SARS-CoV) among previously identified coronaviruses, and the impact of the disease that it causes, resulted in renewed in-depth analyses of all genes and proteins of coronaviruses (Rota et al. 2003; Marra et al. 2003; Thiel et al. 2003; Snijder et al. 2003; Stadler et al. 2003; Yan et al. 2003; Ziebuhr 2004b). At the level of the replicase, distant relationships between cellular enzymes involved in RNA metabolism and five nidovirus domains were tentatively identified (Snijder et al. 2003; von Grotthuss et al., 2003; Yan et al. 2003) and partially verified by subsequent experimental analysis (Ivanov et al. 2004; Bhardwaj et al. 2004). Thus, it was proposed that adenosine diphosphate-ribose1″-phosphatase (ADRP) activity is associated with the coronavirus X-domain (Gorbalenya et al. 1991) and cyclic phosphodiesterase (CPD) activity with the domain found at the C-terminal region of pp1a in EToV and in the ORF2 protein of group 2 coronaviruses (Snijder et al.

1991). New functional assignments were also made for an array of domains conserved in the carboxyl-terminal region of pp1ab (now named nsp14, 15, and 16 in coronaviruses) that is comprised of a 3′-to-5′ exonuclease (ExoN), a uridylate-specific endoribonuclease (NendoU), and an *S*-adenosylmethionine-dependent ribose 2′-*O*-methyltransferase (2′-*O*-MT). These three domains are conserved in coronaviruses, toroviruses, and roniviruses, but only the NendoU domain is present in arteriviruses. It was proposed that these five nidovirus enzymes might cooperate in a manner described for their cellular homologues. In this cooperation NendoU, ExoN, and 2′-*O*-MT might provide specificity and the CPD and ADRP may modulate the pace of the reaction(s) in a single or several pathways. The expected functional hierarchy of the five nidovirus enzymes may be reflected in their level of evolutionary conservation, with the most conserved NendoU playing a key role in the initiation of a cascade of reactions (Snijder et al. 2003).

In summary, the comparison of nidovirus replicases leads to the conclusion that the basis of nidovirus common ancestry lies in the enzymatic functions that drive virus replication. Comparative analysis of pp1a and pp1ab (particularly between coronaviruses and arteriviruses) reveals a remarkable colinearity in organization, which contrasts with the overall low sequence similarity and size difference. As our understanding of the genetic blueprints for the different nidovirus branches continues to be refined, novel details of the nidovirus-specific replication machinery are coming to light. Despite many overall similarities, the enormous evolutionary distance between the different groups united in the order *Nidovirales* guarantees that many minor and possibly also major differences will emerge during the coming years. Advanced bioinformatics, structural studies, biochemistry, virus genetics, and molecular virology each play a specific and valuable role during the functional dissection of this unique positive-strand RNA virus replicase.

SUBGENOMIC RNA SYNTHESIS

The synthesis of one or multiple subgenomic mRNAs is a mechanism that many positive-strand RNA viruses have evolved to selectively express structural and accessory proteins from ORFs other than those encoding the major replicase subunits (reviewed in Miller and Koev (2000)). In nidoviruses, these ORFs are located downstream of the replicase ORFs 1a and 1b. Accordingly, nidovirus RNA synthesis also entails, in addition to the replication of the full-length genome, the production of a set of subgenomic mRNAs. The nidovirus genome and subgenomic mRNAs form a 3′ co-terminal nested set of three to 10 mRNAs, depending on the particular virus species. The 5′-proximal part of each subgenomic mRNA encompasses a specific ORF (or sometimes several ORFs) from the 3′-proximal genome region, which thus becomes accessible for translation by host ribosomes.

For a long time, it was thought that the hallmark of nidoviruses (mostly coronaviruses at that time) was the fact that the viral subgenomic mRNAs are composed of regions that are noncontiguous in the viral genome (Spaan et al. 1983; Baric et al. 1983; Lai et al. 1984). All subgenomic mRNAs of coronaviruses, and later also arteriviruses (de Vries et al. 1990), were found to carry a common 5′ leader sequence, which is a copy of the 5′-terminal sequences of the genomic RNA. Thus, these subgenomic mRNAs (and the genome) share both 5′- and 3′-terminal sequences. In some respects, their mosaic nature resembles that of eukaryotic mRNAs generated by splicing.

More recently, however, it has become clear that the generation of subgenomic mRNAs containing noncontiguous sequences is not universal amongst the nidoviruses. First, evidence was obtained that makes it unlikely that ronivirus subgenomic mRNAs contain a common leader sequence (Cowley et al. 2002). Shortly afterwards, it was found that only one of the subgenomic mRNAs (RNA 2) of the torovirus EToV shares a short 5′-proximal leader sequence with the genome, whereas the smaller subgenomic mRNAs (like those of roniviruses) do not contain such a common leader sequence (van Vliet et al. 2002). Thus, there are differences in the mechanisms used to generate nidovirus subgenomic mRNAs and more studies are needed to identify the unique aspects of roniviruses and toroviruses RNA synthesis. However, the differences described above, rather than being viewed as fundamental, may transpire to be 'variations on a theme.' Thus, we will describe the discontinuous mode of subgenomic RNA synthesis used by coronaviruses and arteriviruses, which has been studied in much more detail, as a basis for identifying mechanistic principles that, eventually, may be related back to the unique nidovirus replicase functions that have been identified recently.

Despite the similarities between the structure of spliced eukaryotic mRNAs and the subgenomic mRNAs of coronaviruses and arteriviruses, the underlying mechanisms that generate these RNAs probably differ dramatically. The coronavirus/arterivirus subgenomic RNAs are generated in the cytoplasm of the infected cell via a unique mechanism that does not involve the post-transcriptional processing of a larger precursor to convert it into a smaller mature product (Jacobs et al. 1981; den Boon et al. 1995b; Yokomori et al. 1992), as is the case with host mRNAs generated by splicing (a nuclear process). Rather, nidovirus subgenomic RNAs derive from a process of discontinuous RNA synthesis that appears to be a variant of the similarity-assisted RNA recombination that can operate during genome replication (for a review, see Nagy and Simon (1997)).

Over the years, different models have been proposed to explain the discontinuous step in arterivirus and

coronavirus subgenomic RNA synthesis (reviewed in van der Most and Spaan (1995), Brian and Spaan (1997), Lai and Cavanagh (1997), Snijder and Meulenberg (1998, 2001), Lai and Holmes (2001)). In the two most prominent models (commonly referred to as 'leader-primed transcription' and 'discontinuous minus strand synthesis', see Figure 4), it is proposed that short conserved transcription-regulating sequences (TRS) play a key role in connecting the common leader sequence to each of the subgenomic RNA 'bodies' (the variable part of the mRNA that is identical to a part of the 3′-proximal region of the genome). These TRS elements are present at the 3′ end of the leader (leader TRS) and at the 5′ end of each of the regions in the genome that corresponds to a subgenomic RNA body (body TRSs). In the subgenomic RNA, a copy of the TRS connects the leader and body regions that are noncontiguous in the genome. Base-pairing between the leader TRS (+TRS) (in the genome) and the complement of the body TRSs (−TRS) (in the nascent minus strand) was shown to be essential for subgenomic mRNA synthesis (van Marle et al. 1999; Pasternak et al. 2001, 2003; Zuniga et al. 2004; Curtis et al. 2004).

According to the 'leader-primed transcription' model, subgenomic RNA synthesis would be primed by free leader molecules whose +TRS would base-pair with one of the body TRS complements in the anti-genome. Subsequently, using the anti-genome template, the leader primer would be extended with the body part to complete the subgenomic mRNA (Spaan et al. 1983;

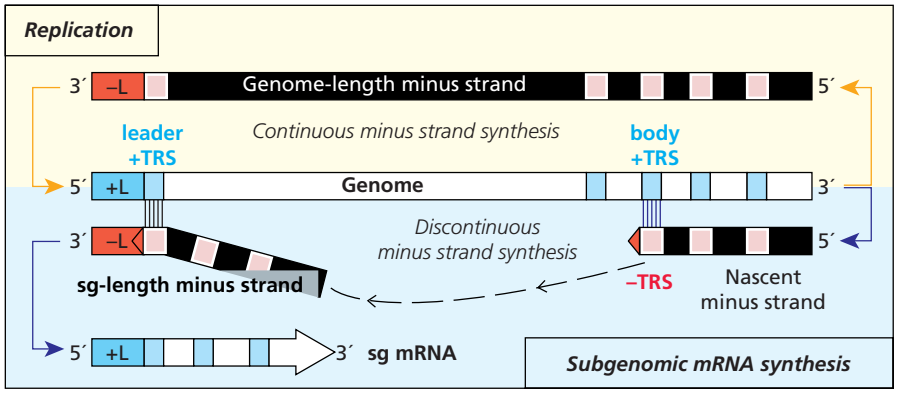

(a)

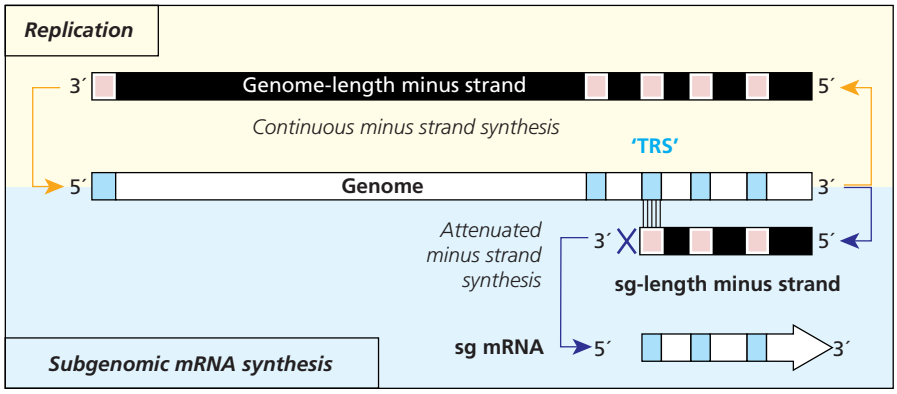

(b)

Figure 4 *Models for subgenomic mRNA synthesis from minus-strand subgenome-length templates in different nidoviruses.* **(a)** *Model for coronaviruses and arteriviruses, which have a common 5′ leader sequence on all viral mRNAs. Discontinuous extension of minus-strand RNA synthesis (Sawicki and Sawicki 1995) has been proposed as the mechanism to produce subgenome-length minus-strand templates for subgenomic mRNA synthesis. Obviously, minus-strand RNA synthesis can also be continuous (top), yielding the genome-length minus-strand required for replication. During discontinuous minus-strand synthesis (bottom), the replicase/transcriptase is proposed to be attenuated at one of the body TRSs in the 3′-proximal part of the genome. Subsequently, the nascent minus strand, with the complement of the body TRS at its 3′ end, is redirected to the 5′-proximal part of the template, where its 3′ end can base-pair with the complementary leader TRS. Next, the subgenome-length minus-strand is completed by extension with the antileader sequence and used as template for mRNA synthesis.* **(b)** *Ronivirus subgenomic mRNAs and all but the largest subgenomic mRNAs of toroviruses do not contain a common 5′ leader sequence (Cowley et al. 2002; van Vliet et al. 2002), although conserved ('body TRS-like') sequences are found upstream of each of the genes in the 3′ proximal part of their genome. It is thought that these viruses may employ a variant of the mechanism explained in* **(a)**, *in which attenuation of minus-strand RNA synthesis does occur, but the discontinuous step required for antileader addition does not take place. Rather, the attenuated minus-strand RNA can serve directly as template for subgenomic mRNA production.*

Baric et al. 1983; Lai et al. 1984). This model was partly based on the fact that complements of subgenomic RNAs (subgenome-length minus strands) were initially not detected in cells infected with MHV (Lai et al. 1982). However, after the subsequent discovery of such molecules in coronavirus- and arterivirus-infected cells (Sethna et al. 1989; Sawicki and Sawicki 1990; den Boon et al. 1996), an alternative model for subgenomic mRNA synthesis was proposed (Figure 4a) (Sawicki and Sawicki 1995). According to this model, the discontinuous step in subgenomic RNA synthesis does not occur during plus, but during minus-strand RNA synthesis. The body TRSs in the plus-strand template would attenuate minus-strand synthesis, after which the nascent minus strand, having a TRS complement at its 3′ end, would be transferred to base-pair with the leader TRS, which may be presented as part of a stem–loop structure (Chang et al. 1996; van Marle et al. 1999; van den Born et al. 2004). The subgenome-length minus strands would then be completed by extension with the complement of the leader sequence and subsequently be used as templates for the synthesis of subgenomic mRNAs. In recent years, this model, which was originally proposed by Sawicki and Sawicki, has gained considerable experimental support from both biochemical and genetic studies (Baric and Yount 2000; Sawicki et al. 2001; Pasternak et al. 2001).

The mechanism of discontinuous subgenomic RNA synthesis, as proposed for coronaviruses and arteriviruses, is a unique strategy for the generation of viral subgenomic transcripts. It contrasts with a mechanism used by many other RNA viruses that have been found to produce subgenomic mRNAs by internal initiation of transcription from 'promoters' in the antigenome (Miller et al. 1985). However, the mechanism used by coronaviruses and arteriviruses may share similarities with that of other positive-strand RNA viruses that employ premature termination (attenuation) of minus-strand RNA synthesis (reviewed in White (2002)). Also in the latter case, subgenome-length minus strands are produced that serve as the templates for subgenomic mRNA synthesis, but their synthesis does not involve the discontinuous extension of the nascent minus-strand RNA.

One of the most interesting questions that remains to be resolved is whether attenuation of minus-strand RNA synthesis is used by roniviruses and toroviruses (van Vliet et al. 2002). This property might explain the conservation in torovirus (Snijder et al. 1990b; van Vliet et al. 2002) and ronivirus (Cowley et al. 2002) genomes of sequences that appear to be the equivalent of the body TRSs of coronaviruses and arteriviruses. Whereas in coronaviruses and arteriviruses the attenuation step would be followed, according to the model of Sawicki and Sawicki, by extension of the nascent minus strand with the antileader sequence, the attenuated minus-strand products of roniviruses and toroviruses may be directly used as templates for subgenomic mRNA synthesis (Figure 4b). Thus, attenuation of minus-strand RNA synthesis may be the common feature that unites the mechanisms used to produce subgenomic mRNAs in different nidoviruses, while the process of discontinuous RNA synthesis may be restricted to coronaviruses and arteriviruses. This conclusion provokes, of course, the question of the functions of the coronavirus and arterivirus common leader sequence at the level of, for example, regulation of RNA synthesis and mRNA translation and, obviously, of alternative mechanisms used by nidoviruses that generate mRNAs that lack a common leader sequence.

NIDOVIRUS EVOLUTION

Comparative genomics and the phylogenetic analysis of nidovirus replicative domains clearly established nidoviruses as a distinct evolutionary lineage among positive-strand RNA viruses. The main nidovirus branches have diverged profoundly from each other in the course of evolution. This is evident from the enormous diversity among nidovirus enzymes, which exceeds that of their cellular homologues. For instance, nidovirus main proteinases have evolved several variants of the most conserved part of the enzyme (Gorbalenya et al. 1989; den Boon et al. 1991; Anand et al. 2002; Barrette-Ng et al. 2002; Ziebuhr et al. 2003). In nidovirus proteinases, the otherwise uniform catalytic site may include either a serine or a cysteine nucleophile and other structural variations not encountered in the cellular homologues that were identified in the three main kingdoms of life. Low replicase fidelity and, in particular, a high frequency of RNA recombination have been implicated in the generation of the nidovirus diversity. To achieve this diversity in the restricted and relatively slowly evolving environment of the infected host cell, these mechanisms must have been at work over a considerable period of time, possibly many millions of years (Koonin and Gorbalenya 1989). Given the considerable diversity of the conserved replicase among nidoviruses, it may not be surprising to see that three families of nidoviruses employ seemingly unrelated sets of structural protein genes that evolve faster than the replicases of the same origin. It has been hypothesized that a major split in nidovirus evolution, giving rise to viruses with remarkably different genome sizes, may have been coupled with the acquisition of different nucleocapsid protein genes by corona- and arteriviruses (Godeny et al. 1993).

How did the nidovirus genetic plan come to life? How have nidoviruses evolved replicative polyproteins of such outstanding complexity? There are two basic ways to increase complexity – either by acquiring domains from an outside source or by duplication of a pre-existing domain of the parental genome. These processes depend on intergenomic and intragenomic recombination, respectively. For example, most nidoviruses encode two

or more papainlike proteinase (PLpro) domains that form family-specific branches that are well separated from their relatives of other origin (Ziebuhr et al. 2000). This implies that an amplification of PLpro domains has contributed to the enlargement of the nidovirus genome (den Boon et al. 1995a; Ziebuhr et al. 2001). However, it remains an open question how many of such events occurred and when they took place – in the ancestral nidovirus lineage or later, in individual nidovirus branches.

The nidovirus-replicative domains show remote sequence similarity with replicative proteins of other (super)groups of positive-strand RNA viruses. In particular, the coronavirus ADRP and nidovirus HEL1 domains cluster with homologues encoded by viruses from the *Alphavirus*-like supergroup (Gorbalenya et al. 1988; Gorbalenya et al. 1991). In contrast, the coronavirus RdRp and main proteinase (3Clpro) domains have specific affinity with homologous enzymes in plant potyviruses (Gorbalenya et al. 1989, 2002) and the coronavirus PLpros may be grouped with the leader proteinase (Lpro) of animal aphthoviruses (Gorbalenya et al. 1991). Both poty- and aphthoviruses belong to the picornavirus-like supergroup. Sequence affinity with the potyvirus 3CLpro was also documented for the ronivirus main proteinase (Cowley et al. 2000; Ziebuhr et al. 2003). Furthermore, and in apparent contradiction to the relationships cited above, the main proteinase of arteriviruses clusters with homologues encoded by plant sobemo- and luteoviruses (Snijder et al. 1996; Ziebuhr et al. 2000). Moreover, all these proteins also have complex, albeit very distant, relationships to a variety of cellular homologues (prototypes).

These mosaic relationships between different groups of positive-strand RNA viruses suggests that their replicative domains are derived from several common gene pools and that their genomes have been built by reshuffling these domains. This hypothesis implies that the nidovirus replicative polyproteins may, in fact, be of chimeric origin. On the other hand, no evidence has been obtained so far that any of the nidovirus-replicative domains is evolutionarily interleaved with related domains of other origin. Thus, it remains possible that all or some of these similarities to members of other virus supergroups have been preserved in the course of a continuous process of profound evolution of positive-strand RNA viruses, including nidoviruses, from a common root (Gorbalenya and Koonin 1993). More analyses and new sequences are needed for a more detailed reconstruction of the evolution of nidovirus genomes in general and the nidovirus replicase gene in particular.

From a practical perspective, the vast genetic diversity of nidoviruses presents the basis for designing molecular probes of different selectivity and constitutes a formidable challenge in the development and application of therapeutics. The first genome-based and immunochemical probes for the detection of multiple and individual nidoviruses have been developed by targeting their most and least conserved components, respectively. The list of functions that are unique and well conserved in the order *Nidovirales* or its separate branches is constantly growing and provides targets for specific drugs. To be effective, these drugs must be able to cope with viruses that have shown an amazing structural plasticity during their evolution.

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Parvoviruses

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CHARACTERISTICS OF THE VIRUSES

INTRODUCTION

As their name implies, parvoviruses, from the Latin *parvus* meaning small, are amongst the smallest of the known animal viruses, having nonenveloped icosahedral protein virions approximately 250 Å in diameter. These particles, which comprise only DNA and protein, are quite dense and extremely rugged. They can survive many inactivation procedures, including most used to protect the blood supply, and remain fully viable at ambient temperatures for months or years. Some members of the family are major pathogens of insects and domestic animals, but only one, human parvovirus B19, has so far been identified as a human pathogen, and is the causative agent of fifth disease (erythema infectiosum). Many parvoviruses are essentially apathogenic, and over the past few years this characteristic has helped them gain recognition as effective vectors for the safe delivery of vaccines or therapeutic genes.

In the known biosphere, only parvoviruses have DNA genomes that are both single-stranded and linear. Parvoviruses package a single genomic molecule between 4 and 6 kb in length, which terminates in short palindromic sequences that can fold back on themselves to create duplex hairpin telomeres. These terminal hairpins are essential for the virus's unusual replication strategy, and hence serve as an invariant hallmark of the family. DNA replication proceeds through a series of duplex intermediates in the host cell, via a characteristic unidirectional strand-displacement mechanism called 'rolling-hairpin' synthesis, organized by the major viral nonstructural protein. Transmitted as single-stranded progeny, parvoviruses do not arrive in their host cell with a complementary copy of their genome sequence against which environmental damage can be corrected. This may well contribute to the observation that their genetic mutation rates are higher than those seen in double-stranded DNA viruses, and approach those more commonly associated with some RNA viruses. However, their tendency to drift genetically is held severely in check by the compact nature of the genome, and the structural constraints on its few gene products, which ensure that only a limited spectrum of mutations are compatible with progeny viability.

Parvovirus particles exhibit T = 1 icosahedral symmetry, but are generally constructed from 2–4 capsid protein size classes, usually called VP1 to VP4. Of these, VP1 is the longest, at 80–96 kDa, while the remainder comprise a nested set of polypeptides derived from its carboxy terminus (VP2 64–85 kDa, VP3 60–75 kDa, and VP4 49–52 kDa), with VP2 or VP3 being the principal capsid component. Particles are built up from 60 equivalent copies of this large carboxy-terminal core sequence, and the amino-terminal

extensions carried by some of the polypeptides are alternately concealed or exposed at the particle surface during different stages of the infectious cycle, displaying stage-specific trafficking signals or effector domains. Notably, VP1-specific sequences from most genera contain the enzymatic core of a phospholipase A2 (PLA2) molecule, which is essential for infectious entry and appears to be a unique feature of this family (Zadori et al. 2001). Virions lack essential lipids, and none of the viral proteins are known to be glycosylated, but they are modulated post-translationally by important phosphorylation events. Parvovirus virions are exceptionally stable, and quite simple antigenically. In nature only a single serotype of any particular species is generally found circulating in its host population, and this has led to the use of individual serotype, defined by neutralization of infectivity in cell culture, hemagglutination-inhibition, or specific enzyme-linked immunosorbent assay (ELISA), as the major criterion for taxonomic species demarcation (Tattersall et al. 2005). Just two antigenic sites, defined by mutations that confer resistance to neutralization by monoclonal antibodies, have been identified for canine parvovirus (CPV). Despite their differences in capsid serology, the nonstructural proteins of most species in a genus do show antigenic cross-reactivity, so that genera-specific infections can often be diagnosed serologically.

While adeno-associated viruses (AAV), from the genus *Dependovirus*, generally require coinfection with a helper adenovirus or herpesvirus in order to complete their replication cycle, the great majority of family members are capable of helper-independent, so-called 'autonomous,' replication. Viral genomes typically contain just two gene cassettes, of which one encodes the nonstructural proteins (called Rep in the AAVs and NS in other species), which are essential for viral gene expression and DNA replication, while the other encodes the overlapping set of capsid polypeptides. The limited coding potential of these genomes therefore renders them highly dependent on the synthetic machinery provided by the host cell for their own, preferential, replication. In contrast to most other DNA viruses, parvoviruses do not encode proteins that can coerce resting cells to enter cell division. Nevertheless, they do require host cells to enter S phase in order to provide the extensive deoxynucleotide triphosphate pools and active cellular DNA synthetic apparatus required for their replication, and that are not present in interphase cells. As a consequence, autonomously replicating parvoviruses cannot grow in resting cell populations, and amplify best in populations of rapidly dividing cells, both in vitro and in vivo. This lack of any encoded positive growth regulators means that parvoviruses are universally nontumorigenic, and indeed, for reasons that are not yet clear, they are often specifically oncosuppressive, exhibiting an enhanced ability to replicate in transformed cells.

AAVs overcome their limited gene repertoire by resorting to a pattern of latent infection in which they generally integrate into the host-cell genome, only switching to a productive life cycle when their host cell becomes infected with a 'helper' virus. In this situation, the helper inadvertently activates resident AAV genes, while also reprogramming the host's cell cycle for its own use, sending the cell into S phase. Resulting helper virus replication is paralleled, or even overtaken, by that of the resident AAV, culminating in cell death and the release of infectious progeny of both viruses. In recent years, this capacity of AAV to integrate, or otherwise persist in host cells, has been exploited for the safe and efficient delivery of a wide range of therapeutic genes, so that AAVs are now among the most promising vector systems available for long-term gene substitution or correction strategies. In most of these vectors all of the viral genes are deleted to accommodate the therapeutic transgene, so that these constructs cannot give rise to infectious progeny.

Characteristics of the viral life cycle inevitably dictate the types of disease parvoviruses are able to induce. On one hand, AAVs have been selected for their ability to remain essentially invisible over prolonged periods of time, and to specifically exploit coinfection with potentially tumorigenic helper viruses, while the helper-independent viruses require cells of specific differentiated phenotypes which must also be undergoing cell division, and hence DNA replication, of their own volition. These viruses are therefore parasites of specific, rapidly dividing cell populations, and generally only give rise to overt disease in situations where large numbers of their target cells continue to replicate in the adult, or where the timing of organ development in the fetus means that transiently replicating cell populations, once damaged, can no longer be effectively regenerated, resulting in fetal or neonatal death, or teratogenesis. In healthy adults, the immune response mounted against otherwise innocuous viruses can also sometimes exacerbate the pathogenesis of infection, as manifest, for instance, by the rashes and polyarthropathy which often follow infection of otherwise healthy people by human parvovirus B19.

HISTORY AND TAXONOMIC RELATIONSHIPS WITHIN THE FAMILY *PARVOVIRIDAE*

In 1970 the taxonomic family, *Parvoviridae*, was established to encompass all small nonenveloped viruses with approximately 5 kb linear, self-priming, single-stranded DNA genomes. Although genome sequences were not then available to confirm their phylogenetic relationships, the DNA sequence data amassed since that time confirm that all viruses in the family share a common evolutionary history. As delineated in Table 1, the family *Parvoviridae* is currently divided into two subfamilies on the basis of host range: the *Parvovirinae*,

Table 1 *Taxonomic structure of the family* Parvoviridae

Subfamily *Parvovirinae*	Subfamily *Densovirinae*
Genus	Genus
Parvovirus	*Densovirus*
Erythrovirus	*Iteravirus*
Dependovirus	*Brevidensovirus*
Amdovirus	*Pefudensovirus*
Bocavirus	

which infect vertebrate hosts, and the *Densovirinae*, infecting insects and other arthropods (Tattersall et al. 2005). This chapter will focus exclusively on members of the *Parvovirinae* subfamily. These are currently divided into five genera, as detailed in the phylogenetic tree in Figure 1, but we will mostly consider the properties of species belonging to the three major genera, namely the dependoviruses, predominantly the helper-dependent AAVs, and the autonomously replicating parvoviruses and erythroviruses. The latter include the human pathogen, parvovirus B19, and are named for the striking tropism of this agent for cells of the erythroid lineage.

The genus *Parvovirus*

In 1959, a small stable virus, subsequently named *Kilham rat virus* (KRV), was isolated from lysates of an experimental rat tumor (Kilham and Olivier 1959). In the following decade a number of other physically similar agents were identified as contaminants of laboratory tumors that were being serially transplanted in normal or immunosuppressed rodents, and as contaminants of cell cultures or other virus stocks in routine laboratory use (Tattersall and Cotmore 1986). Although lacking known host species, some of these isolates were injected into rodents where they caused a broad spectrum of pathology, which varied with the virus serotype and age of the host, from apparently asymptomatic viremia to teratogenesis and fetal or neonatal death (Toolan 1961). Concurrently, an infectious agent responsible for epidemics of enteritis, panleukopenia, and congenital cerebellar ataxia in domestic cats was identified, called *Feline panleukopenia virus* (FPV), and it soon became apparent that this shared the same characteristic size and genome structure as the so-called 'rodent' parvoviruses isolated in the laboratory (Kilham et al. 1967; Gorham et al. 1966). Transplacental transmission has been described for several viruses in the genus, and under experimental conditions their host range can often be extended to include a large number of vertebrate species. Since the capsids of most species hemagglutinate red blood cells of one or other mammalian species, this provides a facile assay for virion detection and quantitation. These viruses, listed in Table 2, are now all classified in the genus *Parvovirus*, with the murine virus, *Minute virus of mice* (MVM) (occasionally referred to as *Mice minute virus* (MMV)), as the type species.

Much of our knowledge of the whole parvovirus family, and the nature of parvoviral infections, has been derived from the study of these autonomously replicating viruses, which generally grow efficiently in cell culture and are readily susceptible to experimental manipulation. Mature virions of most species in the genus contain DNA strands that are predominantly negative-sense with respect to transcription, while one virus, LuIII, packages approximately equimolar positive and negative-sense strands. This unusual variability is less significant than at first suspected, since it is caused by differential rates of initiation from the two viral replication origins, rather than by any strand-specific packaging signal or mechanism. The linear single-stranded DNA genome, 5.0–5.3 kb in length, has hairpin structures at both the 5′ and 3′ ends. However, the 3′-terminal, or left-end, hairpin is 121 nucleotides (nt) in length, while the 5′, or right-end, structure is 248 nts long, and the left- and right-end hairpins are quite different from one another, both in sequence and predicted secondary structure. The intervening single-stranded region of the genome contains two mRNA transcription units, with promoters at map units 4 and 38 (where map units represent 1 percent segments of the genome, starting from the left-hand end) and a single functional polyadenylation site, located close to the right-hand end.

The genus *Dependovirus*

The mid-1960s also saw the discovery of a group of physically similar, but defective viruses, which were dubbed 'adeno-associated viruses' because they were common contaminants of laboratory adenovirus stocks. Populations of mature dependovirus virions contain equivalent numbers of positive and negative-sense single DNA strands, each approximately 4.7 kb in length, packaged into separate virions. The genome has three mRNA promoters, at map units 5, 19, 40, and inverted terminal repeats (ITR) of 145 nts, of which the first 125 nts form an imperfect palindrome that folds to create the hairpin-like telomeres. To date, no AAVs have been associated with disease, although they appear to be ubiquitously infectious in humans and primates, and are commonly isolated from adenovirus-infected tissues. At the time of writing, nine distinct full-length AAV genomes have been sequenced (Table 1), but this number is likely to expand rapidly as more potential host species are examined. The best characterized *Dependovirus* is the type species, AAV2, but AAV1, AAV2, AAV3, and AAV4 are extensively homologous at the DNA level. While AAV serotypes 2 and 3 have frequently been isolated from humans, serotypes 1 and 4 are simian isolates, and AAV6 appears to be a recombinant between AAV1 and

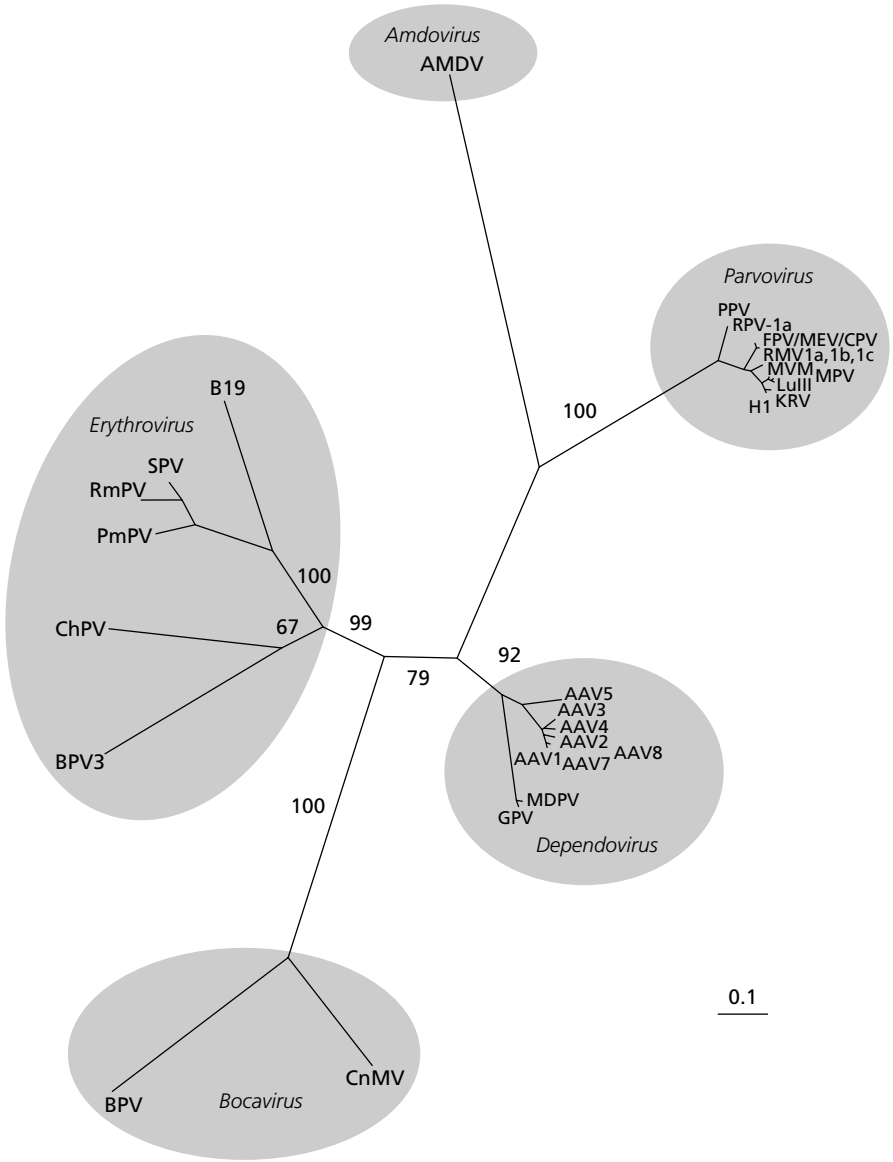

Figure 1 *Phylogenetic relationship between the nonstructural genes of members of the subfamily* Parvovirinae. *The tree was constructed with programs included in the Phylip package (courtesy of Z. Zadori and P. Tijssen).*

AAV2, and is now regarded as a strain of AAV1, since it is specifically neutralized by AAV1-specific antiserum. AAV5 is somewhat less related and has only been isolated once, from humans, where it appears to circulate in conjunction with a herpesvirus helper. Two further closely-related but distinct serotypes, AAV7 and AAV8, were recently isolated from monkeys, and more disparate avian (AAAV) and snake (SAAV) isolates have also recently been cloned and sequenced. For these viruses, efficient replication is dependent upon helper adenoviruses or herpes viruses, both in vitro, and by serological correlation, in vivo. However, in vitro, under certain conditions such as exposure to genotoxic agents or the synchronization of cell replication with hydroxyurea, AAV growth can be detected in the absence of helper viruses.

Originally, as the name *Dependovirus* implies, the genus comprised only helper-dependent viruses, but phylogenetic analysis, as illustrated in Figure 1, now places the autonomously replicating *Duck parvovirus* and *Goose parvovirus* (GPV) firmly within this genus. The *Duck parvovirus* species currently comprises two closely related strains, Muscovy duck parvovirus (MDPV) and Barbarie duck parvovirus (BDPV). These avian parvovirus species also have much larger ITRs, of between 444 and 457 nts, than those of the AAVs. They also differ from the AAVs in exhibiting marked pathogenicity in their natural hosts. The epidemics they cause in commercial flocks of domestic fowl led to their initial identification, in Hungary, where the diseases they elicit have significant agricultural impact.

Table 2 *Members of the subfamily* Parvovirinae

Species	Acronym
Genus *Parvovirus*	
Minute virus of mice	(MVM)
Feline panleukopenia virus strains:	
Feline parvovirus	(FPV)
Canine parvovirus	(CPV)
Mink enteritis virus	(MEV)
Raccoon parvovirus	(RPV)
Hamster parvovirus	(HaPV)
H-1 parvovirus	(H-1PV)
Kilham rat virus strains:	
Kilham Rat virus	(KRV)
H-3 virus	(H-3V)
LuIII virus	(LuIIIV)
Mouse parvovirus 1	(MPV-1)
Porcine parvovirus	(PPV)
Rat minute virus 1	(RMV-1)
Rat parvovirus 1	(RPV-1)
Genus *Dependovirus*	
Adeno-associated virus 1 strains:	
Adeno-associated virus 1	(AAV-1)
Adeno-associated virus 6	(AAV-6)
Adeno-associated virus 2	(AAV-2)
Adeno-associated virus 3	(AAV-3)
Adeno-associated virus 4	(AAV-4)
Adeno-associated virus 5	(AAV-5)
Adeno-associated virus 7	(AAV-7)
Adeno-associated virus 8	(AAV-8)
Avian adeno-associated virus	(AAAV)
Serpentine adeno-associated virus	(SAAV)
Bovine parvovirus 2	(BPV-2)
Duck parvovirus strains:	
Barbarie duck parvovirus	(BDPV)
Muscovy duck parvovirus	(MDPV)
Goose parvovirus	(GPV)
Genus *Erythrovirus*	
Human parvovirus B19 strains:	(B19V)
B19-Au, B19-Wi, B19-A6, B19-LaLi, and B19-V9	
Simian parvovirus	(SPV)
Pig-tailed macaque parvovirus	(PmPV)
Rhesus macaque parvovirus	(RmPV)
Bovine parvovirus type 3	(BPV-3)
Chipmunk parvovirus	(ChpPV)
Genus *Amdovirus*	
Aleutian mink disease virus strains:	(AMDV)
ADV-G (Gorham)	(ADV-G)
ADV-Utah	(ADV-U)
ADV-Pullman	(ADV-P)
Genus *Bocavirus*	
Bovine parvovirus 1	(BPV)
Canine minute virus	(CnMV)

The genus *Erythrovirus*

Human parvovirus B19, the only known human pathogenic member of the *Parvoviridae*, was discovered serendipitously in 1975 as an antigen present in a serum sample bearing the laboratory code 'B19,' from an apparently healthy donor whose blood was being screened for hepatitis reactivity (Cossart et al. 1975). Electron microscopy revealed that the antigen was associated with a virus particle of approximately 23 nm diameter, and the isolate was initially described as 'parvovirus-like' merely because of its size and appearance. Although around 60 percent of normal adults were subsequently found to have antibody to B19 virus (Cohen et al. 1983), suggesting common childhood exposure, the infection was initially thought to be asymptomatic. However, 5 years later the same antigen was identified in the blood of two soldiers suffering from mild fever, and in the next year a survey of sera archived at King's College Hospital, London, suggested an etiologic role for the virus in a type of transient aplastic crisis experienced by children with sickle cell anemia (Pattison et al. 1981). B19 virus has since been shown to be responsible for transient aplastic crisis in patients with a variety of hemolytic anemias. In 1983, the virus was linked sero-epidemiologically to erythema infectiosum (EI), a widespread childhood rashlike disease also referred to as fifth disease (Anderson et al. 1983), and in the next year it was shown that intrauterine transmission of B19 from an infected mother could sometimes lead to hydrops fetalis, particularly in the second trimester of pregnancy. By 1984, the B19 genome had been characterized, cloned, and sequenced (Summers et al. 1983; Cotmore and Tattersall 1984; Shade et al. 1986), allowing it to be placed firmly in the subfamily *Parvovirinae*. However, these analyses revealed that B19 was phylogenetically distant from viruses in the two existing genera (Dependovirus and Parvovirus), so that soon afterward the new genus Erythrovirus was created, with B19 virus as its type species. Populations of mature B19 virions contain equal numbers of positive and negative sense DNA strands of around 5.5 kb, with inverted terminal repeats of 383 nts in which the first 365 nts are palindromic and fold into complex hairpin telomeres. To date this virus cannot be efficiently propagated in any established cell line, so that virus stocks have predominantly been isolated from viremic human sera. Productive infection does occur in some primary erythroid progenitor cells present in primary human bone marrow explants stimulated with erythropoietin (EPO), which has allowed some aspects of the molecular biology of the virus to be studied, and recently an infectious clone of the viral genome was created in a bacterial plasmid (Zhi et al. 2004). This gives rise to limited progeny virus after transfection into the EPO-stimulated erythroid cells, allowing hope for

future rapid advances in the genetic dissection of viral gene products and synthetic processes.

Additional erythroviruses have also been identified in other primate species (listed in Table 2), and these viruses appear to share the exquisite tissue specificity of B19 for erythroid progenitor cells and accordingly give rise to similar disease spectra (O'Sullivan et al. 1994; Green et al. 2000). Subsequently, a more distantly related virus was isolated from Manchurian chipmunks (Yoo et al. 1999), and while this could potentially provide a useful, nonprimate, model system for studying erythrovirus infection, currently little is known about this virus. Recent advances in viral diagnostics using polymerase chain reaction (PCR) technology have also allowed the identification of related viral sequences in sera from healthy bovines, suggesting that these animals likely harbor another apathogenic or weakly pathogenic erythrovirus species (Allander et al. 2001).

The *Amdovirus* and *Bocavirus* genera

Recently the taxonomy of the *Parvoviridae* has been rationalized to more closely reflect their phylogeny by clustering viruses according to the similarity of the gene sequences of their encoded proteins. As shown in Figure 1, this has led to the reapportioning of some disparate autonomously replicating viruses from the genus *Parvovirus* into two new genera: the *Amdoviruses*, containing a single outlying species, *Aleutian mink disease virus* (AMDV); and the *Bocaviruses*, which currently has two members, *Bovine parvovirus* (BPV) and *Canine minute virus* (CnMV). Despite their phylogenetic isolation, most features of these viruses are shared with members of the genus *Parvovirus*. Mature AMDV virions contain a negative-strand DNA of 4 748 nts, with different palindromic sequences at each terminus. Permissive replication is observed in cell culture for only one isolate, ADV-G, which replicates in Crandell feline kidney cells, although restricted replication is also observed in cells bearing Fc receptors, such as macrophages, where the virus's ability to infect may be antibody-dependent (Dworak et al. 1997). There are several highly pathogenic strains of AMDV, and evidence of infection with this virus has been detected in most mustelids, skunks, and raccoons, being of particular economic impact for the mink industry. Virion structure differs slightly from members of the genus *Parvovirus*. Apart from its divergent DNA sequence, the major distinguishing features of AMDV are its VP1 N-terminus, which is much shorter than those of other *Parvovirinae*, and does not contain the phospholipase 2A enzymatic core, and the fact that in vivo its capsid proteins are highly susceptible to proteolysis, so that intact particles contain few full-length polypeptides (Aasted et al. 1984).

The *NS1* and *VP1* genes of the *Bocaviruses*, BPV and CnMV are 34 to 41 percent similar to one another, but their genomes are very distinct from all other viruses in the *Parvovirinae* subfamily. Somewhat larger than other parvoviral genomes, at 5.5 kb, the genomes of *Bocaviruses*, like those of members of the *Parvovirus* and *Amdovirus* genera, have unique termini. BPV is also notable because it packages 90 percent negative-sense and 10 percent positive-sense DNA and because it encodes a 22.5-kDa nuclear phosphoprotein, NP-1, that is distinct from any other parvovirus-encoded polypeptide.

STRUCTURE AND ORGANIZATION OF PARVOVIRUS COMPONENTS

Structure and assembly of the capsid

Parvoviruses elaborate empty capsids and full virions that are nonenveloped, 18–26 nm in diameter, and exhibit T = 1 icosahedral symmetry. The largest capsid polypeptide is routinely designated VP1, the next VP2, and so on, and they constitute a nested set of polypeptides that share a common carboxy-terminal sequence. Sixty copies of this core sequence are assembled to form the particle, with the majority being derived from either VP2 or VP3 polypeptides, but with the VP1 molecules contributing between nine and 12 copies, which are essential for infectivity. The virion has an M_r of about $5.5–6.2 \times 10^6$, with an S_{20w} of 110–122S, and a buoyant density of 1.39–1.43 g/cm^3 in CsCl. The infectious particle comprises between 70 and 80 percent protein, with the remainder being DNA, and mature virions are stable in the presence of lipid solvents, upon exposure to pH 3–9 and, for most species, incubation at 56°C for at least 60 min. The three-dimensional structures of several parvoviruses, namely CPV, FPV, MVM, porcine parvovirus (PPV), AAV2 and galleria mellonella densovirus (*Gm*DNV), have been determined to near atomic resolution by X-ray crystallography. The common core structural feature is an eight-stranded antiparallel β-barrel, a structure shared with many viral structural polypeptides, in which the β-strands are connected by elaborate and highly variable loops that make up most of the viral surface (Chapman and Rossmann 1993). The amino-terminal peptide domains of the larger proteins are submolar, so that their disposition cannot be deduced from X-ray data. Viral genomes are thought to be packaged vectorially in a 3′ to 5′ direction into pre-assembled 'empty' particles, whose X-ray structure appears almost identical to that of the infectious virion. Viral preparations frequently contain large numbers of these empty capsids, which have a density of 1.32 g/cm^3 in CsCl and a sedimentation coefficient of 70S, as well as the 'full,' DNA-containing virions, subgenomic defective DNAs (p = 1.33–1.39 g/cm^3), and particle-assembly intermediates of various densities.

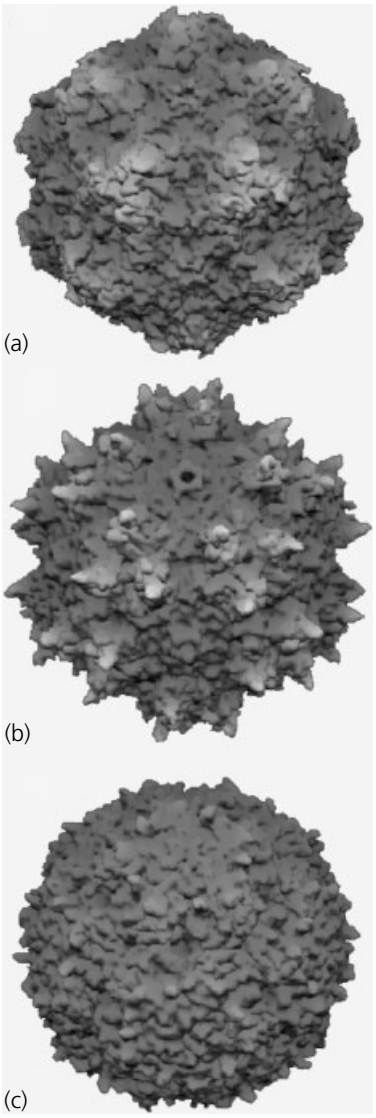

Figure 2 *Space-filling models of the capsid structures of* **(a)** *canine parvovirus (CPV),* **(b)** *adeno-associated virus 2 (AAV-2), and* **(c)** Galleria mellonella *densovirus (*Gm*DNV). Each model is drawn to the same scale and is shaded according to distance from the viral center. In each case, the view is down a two-fold axis at the center of the virus, with three-fold axes left and right of center, and five-fold axes above and below (courtesy of M. Chapman).*

The parvoviral capsid shell displays icosahedral symmetry, but its surface topology can vary substantially between genera, as illustrated in Figure 2 for members of the *Parvovirus*, *Dependovirus*, and *Densovirus* genera. In general, there is an elevated 'spike' surrounding each of the twenty three-fold symmetry axes in the particle, deep depressions at the two-fold axes. A hollow cylindrical structure surrounds each of the 12 icosahedral five-fold axes, which contains a central pore that connects the inside of the virion with the particle exterior. In B19 virus and AMDV, for which there is cryo-electron microscopic imaging and/or low resolution X-ray data, these pores appear occluded, and in full virions of MVM or CPV, from the genus *Parvovirus*, each pore contains a single copy of the glycine-rich, amino-terminus-proximal sequence from a VP2 molecule, positioned so that the amino-terminal 25 amino acids are externalized (Tsao et al. 1991; Agbandje-McKenna et al. 1998). On the outer virion surface these cylindrical structures are themselves encircled by deep canyonlike depressions of unknown function. Neutralizing antibody-binding sites generally map to the three-fold spike, or to its shoulders, and sequences that determine viral host range and oligosaccharide recognition and, in some viruses, receptor binding, lie in the two-fold depression and up the adjacent edge of the three-fold spike. In virions some of the single-stranded DNA also displays icosahedral symmetry, so that about a third of the genome can be visualized, by crystallography, within the particle shell. This DNA is oriented with its bases pointing outwards, forming a number of hydrogen bonds with amino acids of the inner surface of the capsid shell (Xie and Chapman 1996; Agbandje-McKenna et al. 1998).

A single molecule of the viral replication initiator protein (called Rep 68/78 in AAV, but NS1 in most other members of the family) is located on the outside of newly released virions, covalently attached to the extreme 5′ terminus of the genome by a short single-stranded DNA 'tether' sequence which projects through the capsid shell at an unknown location (Cotmore and Tattersall 1989).

Structure of the parvoviral genome

Infectious particles contain a single copy of the linear, nonpermuted, monopartite DNA genome, in which a relatively long single-stranded coding region (approximately 5 kb) is bracketed by short (121–418 nts) palindromic terminal sequences, capable of folding into hairpin duplexes. These duplex viral telomeres, which can be structurally quite complex, are critical for both DNA replication and encapsidation. While they are the essential hallmarks of the parvoviral genome, they vary markedly in size and structure between genera and even between species within the same genus.

With the exception of members of the *Densovirus* and *Pefudensovirus* genera of insect parvoviruses, the viral genome is monosense, that is, it codes for protein only on one strand. On this strand, designated the positive strand, the genes are organized into two separate expression cassettes. By convention, transcription is represented as proceeding left to right on the DNA, placing the 5′ end of the positive strand and the 3′ end of the negative at the left side of the genetic map, as illustrated in Figures 3–5. The open reading frames (ORF) within the left cassette give rise to a small number of nonstructural proteins which are involved in gene

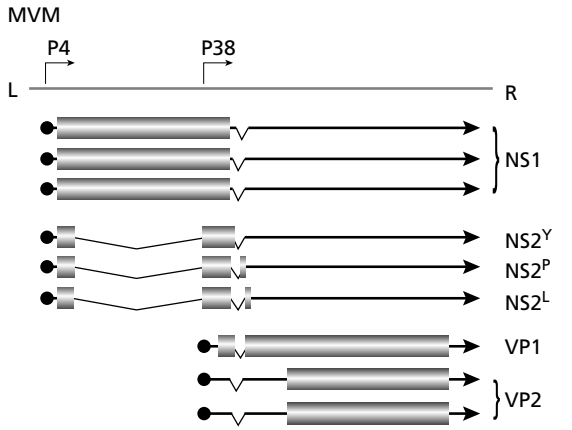

Figure 3 *Gene organization and transcription scheme for members of the* Parvovirus *genus, as shown for* Minute virus of mice *(MVM). Encoded polypeptides are shown as horizontal cylinders. The left ends of the mRNAs (thick lines) are the sites of the mRNA caps (filled circles), the right ends are the polyadenylation sites (arrowheads); introns are indicated by thin carets. Rightward arrows marked with a P indicate the start sites for transcripts driven by promoters whose location is shown in map units from the left-hand end.*

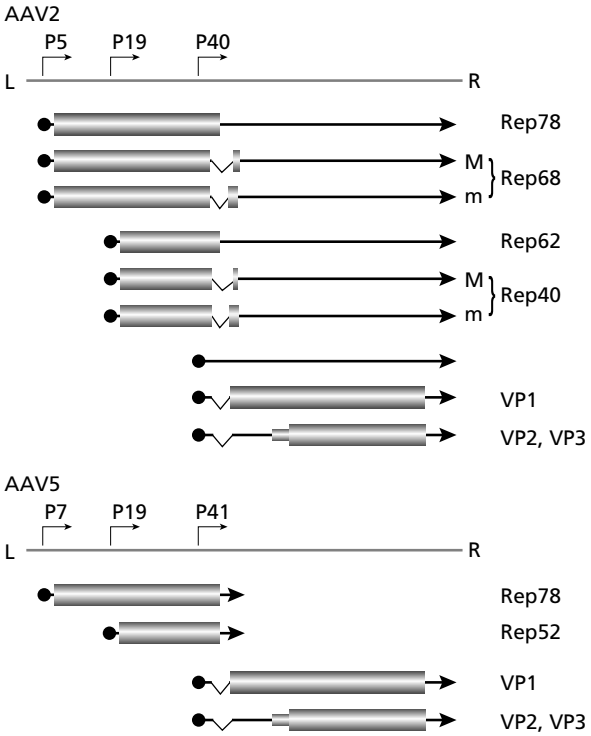

Figure 4 *Gene organization and transcription scheme for members of the* Dependovirus *genus, as shown for adeno-associated virus 2 (AAV-2) (top) and adeno-associated virus 5 (AAV5) (bottom), annotated as described for Figure 3. The narrower cylinder represents the N-terminal region of VP2, translation of which is initiated at a weak, in-frame, ACG start codon.*

regulation and DNA replication, while those encompassed by the right cassette program synthesis of the overlapping set of capsid proteins.

PARVOVIRUS GENOMES

For MVM, the type species of the *Parvovirus* genus, alternate splicing events orchestrate viral gene expression, as shown in Figure 3 (Morgan and Ward 1986; Pintel et al. 1983). Transcripts encoding the NS proteins are all made from the promoter at 4 map units. Singly-spliced transcripts encode only the multifunctional replication initiator protein, NS1, while further P4-derived transcripts are spliced into an alternate reading frame by removal of the major intron, and encode small nonstructural polypeptides, in order of abundance, NS2P, NS2Y, and NS2L, the extreme carboxy termini of which are different due to the use of alternative 5′ and 3′ splice sites bordering the small intron (Cotmore and Tattersall 1990; Morgan and Ward 1986). One function of NS1 is to up-regulate the P4 promoter itself, and this positive feedback loop appears to be a part of the 'hard-wiring' of infection that insures rapid viral takeover of the cell. A second promoter at 38 map units is *trans*-activated by NS1, as infection progresses (Clemens and Pintel 1988), and drives synthesis of the structural gene transcripts. All P38 transcripts contain a 280 nt 5′ untranslated region located upstream of the minor splice region, and the use of the same alternative 5′ splice sites that are present in the P4 transcripts, regulates synthesis of the capsid proteins. A transcript that employs the downstream 5′ and 3′ splice sites encodes the minor VP1 polypeptide, translation of which initiates at a methionine codon between the two alternate 5′ splice sites. In the more abundant transcripts, which employ the upstream 5′ splice site, this initiation codon is spliced out, and translation of the major coat protein VP2 initiates from a start codon nearly 400 nts further downstream of the splice.

Although parvoviruses may encode several smaller accessory nonstructural proteins, such as the NS2 species

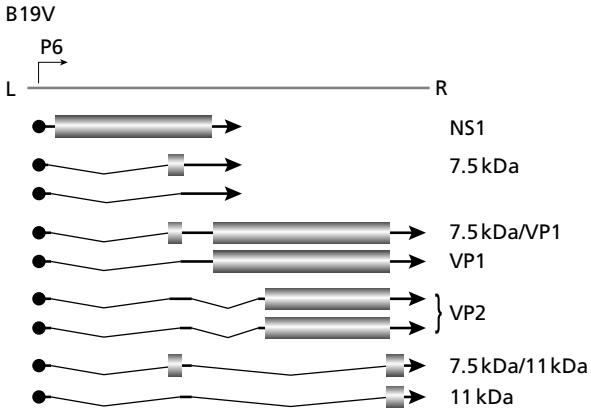

Figure 5 *Gene organization and transcription scheme for members of the* Erythrovirus *genus, as shown for human parvovirus B19 (B19V), annotated as described for Figures 3 and 21.4.*

depicted in the MVM map, only the replication initiator protein, NS1, is absolutely required for replication in all cell types (Bodendorf et al. 1999; Cater and Pintel 1992; Naeger et al. 1990). NS1, or Rep68/78 as it is known in AAV, is an ATP-dependent, site-specific DNA-binding protein with helicase activity, which initiates replication at specific viral origin sequences by introducing a site-specific single-strand nick, thus providing a base-paired 3′ nucleotide to serve as a primer for successive rounds of strand displacement DNA synthesis (Cotmore and Tattersall 1996). The *trans*-esterification reaction that creates this nick leaves NS1/Rep covalently attached to the 5′ nucleotide at the nick site, where it may serve as the 3′ to 5′ replicative helicase. All replicons that use this type of replication mechanism encode their own initiator proteins, and two protein motifs in NS1, representing a putative metal ion coordination site and the active site tyrosine of the nickase, can be traced from the *Parvoviridae* to their prokaryotic 'cousins,' the *Microviridae* (Koonin and Ilyina 1993). MVM NS1 is an 83-kDa nuclear phosphoprotein which self-associates in the presence of ATP to form an oligomeric complex that is capable of binding to a consensus DNA motif found both in the viral origins and at many other locations throughout the genome (Cotmore et al. 1995; Christensen et al. 1995). However, it is only able to nick the DNA in the context of the viral origins, where it encounters the appropriate consensus nick sequence and specific host-derived accessory proteins (Christensen et al. 1997; Cotmore et al. 2000).

Parvoviral replication initiators have evolved into highly pleiotropic proteins, playing multiple roles in the viral life cycle. In addition to their site-specific nicking function, they act as potent transactivators of viral gene transcription, binding to their recognition sequences in viral promoters and activating transcription through acidic carboxy-terminal domains (Legendre and Rommelaere 1994). In MVM, NS1-binding sites are reiterated so frequently that any sequence of 100 base pairs or more contains a site, and some carry multiple tandem and inverted reiterations (Cotmore et al. 1995). This suggests that NS1 likely plays a significant role in viral chromatin structure and progeny strand packaging.

DEPENDOVIRUS GENOMES

Dependoviruses generally have three transcriptional promoters. These are located at 5, 19, and 40 map units along the genome of the type species, AAV2, and transcribe viral mRNAs, as shown in Figure 4, in a temporally regulated fashion throughout infection (Mouw and Pintel 2000; Marcus et al. 1981). P5 transcripts are the first to be expressed, followed by those from P19 and P40, whose protein products are required later in the viral life cycle. P5 transcripts encode the replication initiator proteins Rep78 and Rep68, which appear to serve almost identical functions although they differ slightly in sequence due to splicing of the centrally positioned intron. Spliced messages link the body of *Rep* to a small carboxy terminal segment downstream of the major 3′ splice site, to make the smaller Rep68 protein. In unspliced messages, the sequence encoding the Rep protein body continues on through most of the intron, producing the larger Rep78 protein. Likewise, P19 transcripts encode two Rep forms, Rep52 and Rep48, which differ similarly at their carboxy termini. P40 transcripts are also spliced to alternative 3′ splice sites, and since the downstream acceptor is more efficient than the upstream one, major and minor spliced species are again generated. As an interesting counterpoint to the situation described above for the parvoviruses, in AAV2 the minor VP1 polypeptide initiates from a start codon located between the two 3′ splice sites, rather than between the two 5′ sites, as in MVM. The other AAV2 capsid proteins are then translated from the major, differentially spliced, transcript that lacks the initiation codon used for VP1. The major, and smallest, capsid protein, VP3, initiates from the first AUG codon in this transcript, while the less abundant VP2 species initiates from an upstream in-frame ACG codon. As a result, capsid proteins, VP1, 2, and 3, accumulate in an approximately 1:1:10 ratio, each having identical carboxy-terminal sequences but with VP1 being 137 residues longer than VP2, which is in turn 65 residues longer than VP3. While all AAV2 transcripts coterminate at the right end of the genome, AAV5 has recently been shown to exhibit an additional level of transcriptional control, as shown in the lower panel of Figure 4. Here most of the transcripts encoding nonstructural genes, driven in this case by promoters at 7 and 19 map units, terminate at a central polyadenylation site, located just downstream of the P41 promoter. Cleavage and polyadenylation at this internal site precludes alternative splicing in the Rep transcripts, so that only a single form of the P5 and p19 products are generated (Qiu et al. 2004).

ERYTHROVIRUS GENOMES

The human erythrovirus B19 employs a genetic strategy distinct from those of the other genera, as shown in Figure 5, in that it contains only one promoter, at 6 map units (Ozawa et al. 1987). An unspliced transcript terminating at a central polyadenylation site encodes NS1, while a set of full length transcripts that are spliced in the NS region, encode VP1 and VP2. Two small ORFs can also be accessed by alternatively spliced mRNAs, giving rise to 7.5- and 11-kDa nonstructural proteins. The 11-kDa protein contains three proline-rich regions that conform to consensus SH3 (Src homology 3) ligand sequences (Fan et al. 2001); however, functions for these two protein species remain to be defined. B19 uses two alternative polyadenylation sites, situated similarly to those present in AAV5, as discussed above.

Alternative use of poly(A) sites appears to be controlled by the host cell in a way that suggests that it may act as a major factor in viral tropism (Liu et al. 1992). In a productive infection of erythroid precursors, transcripts terminating at both sites are observed, and give rise to the full array of viral gene products. However, in transfected, nonpermissive cells the central polyadenylation site predominates, and viral gene expression may be largely confined to the synthesis of NS1. This situation is predicted to lead to cytopathogenesis due to the toxic nature of NS1 (Moffatt et al. 1998; Ozawa et al. 1988; Poole et al. 2004), without the production of progeny virus, and may explain in vivo observations of tissue destruction in the absence of B19 growth.

MECHANISMS OF INFECTION

Early events

Steps involved in delivering the viral genome to the host nucleus are illustrated in Figure 6, specifically for members of the genus *Parvovirus* such as MVM. In general, viruses need to achieve a sequence of interactions with the host cell to bring about infection, starting with adsorption to one or more cell surface receptors, internalization (generally in some sort of vesicle), transfer across the cell's delimiting lipid bilayer into the cytoplasm, uncoating leading to exposure of the viral genome, and delivery to the appropriate cellular replication compartment. This pathway is so complex and multifaceted that, while entry is not the only determinant of tropism, it often plays a major role in cell specificity.

Rather than using a single molecule as a cell surface receptor, many viruses employ two more-or-less separate classes of molecules: an 'attachment' receptor, or co-receptor, which effectively accumulates virus in the vicinity of the cell surface; and an infectious-entry receptor that mediates entry into the cytoplasm. While some parvoviruses are known to employ this strategy, the extent to which they rely on multiple surface interactions appears to vary from species to species, and within a species from host cell to host cell, so that few general rules are apparent. MVM binds to sialoglycoproteins present at about 5×10^5 copies per cell on murine fibroblasts, and both binding and infection are neuraminidase-sensitive, indicating a critical role for specific oligosaccharide side chains in these steps. However, at present it is not clear if these comprise one specific cell surface molecule that mediates MVM entry, in addition to others that merely effect attachment, or if all 5×10^5 receptors are equipotent. In contrast, FPV and CPV virions can bind to neuraminidase-sensitive *N*-glycolyl neuramininic acid side chains on some host-cell types, which presumably only function as attachment receptors, since infectious entry is insensitive to neuraminidase and is now known to be mediated by binding to host species-specific protein domains on cell surface transferrin receptor (TfR) molecules (Parker and Parrish 2000). AAVs also use a variety of surface receptors, many of which are widely distributed, but nevertheless fine

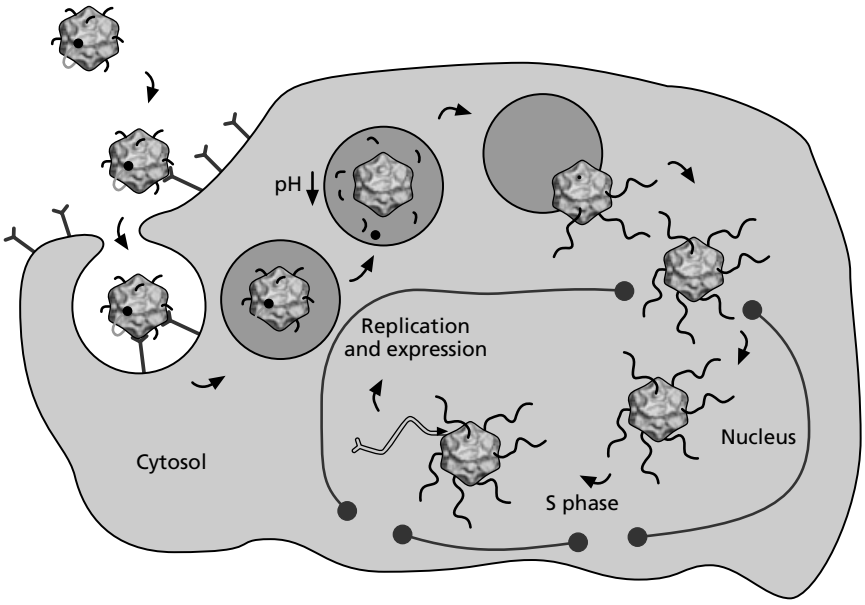

Figure 6 *Entry pathway for a generic parvovirus, as described in the text predominantly for MVM. Short black lines extruding from the virion represent VP2 N-termini, and the single line with a terminal ball represents the tether sequence and its covalently attached NS1 molecule. Longer, gray lines represent the VP1-specific N-terminal extruded upon entry into the cytosol, and the thin double line attached to nuclear virions represents the emerging genome in the process of being converted into double-stranded DNA. See text for additional details.*

details of these interactions have a major impact on effective cell tropism. AAV2 and AAV3 bind the ubiquitous glycosaminoglycan, heparan sulfate, albeit with slightly different specificities (Summerford and Samulski 1998). This may not be all that is required to mediate entry, since AAV2 has also been shown to interact specifically with either α_v, β_5 integrin or basic fibroblast growth factor receptor 1 in ways that ultimately enhance viral gene transduction and hence may function by promoting entry (Summerford et al. 1999; Qing et al. 1999). AAV4 and AAV5 do not bind heparan sulfate, but instead use cell surface carbohydrates carrying the α 2–3 form of neuraminic acid for cell attachment, AAV4 preferentially binding *O*-linked carbohydrates while AAV5 prefers *N*-linked forms (Kaludov et al. 2001). AAV5 then appears to recruit platelet derived growth factor (PDGF) receptors as its primary receptor (Di Pasquale et al. 2003). This is an unexpected finding, since AAVs generally maintain a low profile in their host, and might be expected to enter cells by stealth, whereas PDGF receptors normally respond to ligand-induced cross-linking by autophosphorylating their cytoplasmic tyrosine kinase domains, leading directly to activation of many growth factor response pathways in the host cell. It will be interesting to see whether AAV5 binding does indeed induce receptor-mediated activation, and if so, whether such changes are important in infectious entry by this virus. Finally, the human parvovirus B19 binds to cells of the erythroid lineage via the erythrocyte glycolipid globoside P antigen (Brown et al. 1993). However, a number of cell types that express globoside are not susceptible to infection, suggesting that there could be additional infectious entry receptor(s) for B19 virus.

Following receptor binding, all parvoviruses are rapidly internalized from the cell surface by receptor-mediated endocytosis, predominantly via clathrin-coated pits, and enter an endosome compartment that is sensitive to lysosomotropic agents such as bafilomycin, indicating that low endosomal pH is somehow essential for infection. While members of the genus *Parvovirus* remain sensitive to bafilomycin for up to 6 h after internalization, AAV2 appears to transit into a pH-independent compartment much faster, suggesting that aspects of their penetration mechanisms may be different. Exposure to low pH might be required during entry because it induces essential conformational changes in the virion, because it insures normal endosomal trafficking, or because the viruses specifically need to transit a hydrolase-rich late endosomal/lysosomal compartment to ensure successful entry. Which of these possibilities pertains is currently unclear and is likely to vary between viral species. Indeed, for MVM all three of these processes may be important. Thus, as illustrated in Figure 6, phosphoserinerich amino-terminal peptides from MVM VP2 molecules are displayed at the surface of mature virions, and in some cell types these peptides are known to mediate nuclear export (Maroto et al. 2004), so that removal of all such signals during entry may be essential for the success of subsequent nuclear translocation. This suggests that infectious entry may occur via a late endosomal or lysosomal route, since these are rich in proteases and nucleases, and such exposure would also explain why genomes lose their covalently-linked 5′ NS1 molecules, and the nucleotide 'tether' sequence, prior to arrival in the nucleus (Cotmore and Tattersall 1989).

MVM particles also appear to be stabilized by an acid environment. Amino-terminal sequences of VP1 are known to be essential for entry, but this region is highly protease-sensitive and is sequestered within the virion prior to its entry into the cell. However, the VP1-specific region can become accessible at the particle surface following conformational transitions, inducible in vitro by heating, which are potentiated by the removal of VP2 amino termini, but stabilized by exposure to pH 6.5 or below. Such transitions externalize the VP1 N-terminus and the viral DNA, while leaving the DNA still firmly attached to the otherwise intact particle via its left-end hairpin (Cotmore et al. 1999). Enhanced virion stability during exposure to low pH could thus serve to protect essential hydrolase-sensitive viral structures within an obligate late endosomal/lysosomal entry compartment.

Vesicle trafficking is a complex process that leads to particle delivery to many different cell locations, and trafficking studies typically compound this problem by the use of high multiplicities of input virus needed for signal detection. For example, AAV5 has been reported to enter early endosomelike structures and the cisternae of the trans-Golgi network, but also to accumulate in lysosomes, particularly when applied at high multiplicities (Bantel-Schaal et al. 2002). The high particle-to-infectivity ratios of many viruses also add to this problem, since most of the internalized viruses may enter dead-end circuits that never provide access to the nucleus. This conundrum is well illustrated by recent studies with CPV, which is internalized via an interaction with surface TfRs and presumably remains physically associated with its receptor for at least 4 h, since infectious entry can be blocked throughout that period by intracytoplasmic injection of antibodies directed against the cytoplasmic tails of the receptor (Parker and Parrish 2000). The normal cellular uptake and complex recycling patterns of TfRs have been well studied, and are known to depend upon the presence of a YTRF (Tyr–Thr–Arg–Phe) motif in its cytoplasmic tail. However, when these sequences were deleted or mutated, or polar residues introduced into the TfR transmembrane domain, which vastly increased receptor degradation, virus infection remained efficient (Hueffer et al. 2004). This suggests that infectious entry may involve a minority of TfRs that take, or are directed by the virus to take, an atypical pathway.

Exactly where, or how, these viruses penetrate the endosomal bilayer remains open to conjecture, but CPV infectivity can be blocked by the intracytoplasmic

injection of various antibodies directed against structural determinants in the capsid, indicating that there must be an essential, capsid-associated, cytoplasmic phase (Vihinen-Ranta et al. 2000). VP1 amino-termini carry a PLA2 active site, which is highly conserved among the *Parvoviridae* and appears to be essential for infectivity at some stage following endosomal accumulation, although its role and site-of-action remain to be completely elucidated (Zadori et al. 2001). However, anti-VP1 specific antibodies injected into the cytoplasm have been shown to block CPV infectivity, suggesting that structural transitions leading to exposure of this peptide must accompany entry into the cytoplasmic compartment in vivo, perhaps during transit through the bilayer, as suggested in Figure 6.

The VP1-specific N-terminal region also contains karyopherin-alpha binding sequences, which probably serve to traffic particles to the nuclear pore (Lombardo et al. 2002). Parvoviral particles are very stable and are small enough to be transported through the nuclear pore, so that during infectious entry, viral DNA may remain encapsidated until it is inside the nucleus. Docking of intact particles at the nuclear pore, and/or their translocation into the nucleus has yet to be demonstrated for members of the *Parvovirinae* during natural infection. However, this is likely since recombinant AAV particles have been repeatedly observed in the nucleus, and CPV virions microinjected into the cytoplasm accumulated in the nucleus over a period of 2 h or more where they could be detected with antibodies specific for intact particles (Vihinen-Ranta et al. 2000). Surprisingly, the protracted accumulation of intact recombinant AAV2 virions in the nuclei of liver cells has recently been associated with poor expression of virally-coded transgenes, whereas exactly similar genomes pseudotyped into AAV6 or AAV8 coats were uncoated more rapidly and transcribed more efficiently. This has led to the hypothesis that, for optimal expression, AAV virions must be trafficked to a specific site in the nucleus, where uncoating is efficiently induced by interaction with unidentified nuclear factors, and that AAV2 particles fail to achieve this intranuclear translocation (Thomas et al. 2004).

Parvoviruses are unable to induce resting cells to enter S phase, and it is not until infected cells enter S phase of their own volition that viral transcription normally initiates. Since the viral promoters are packaged as single-stranded DNA, acquisition of a complementary strand necessarily precedes viral gene expression, and its synthesis likely represents the first S phase dependent step in the viral life cycle. Initial transcription of MVM also depends upon the availability of the host transcription factor E2F to activate its P4 promoter, so that viral transcription is optimized for expression during S phase (Deleu et al. 1999). As early MVM gene products accumulate, host-cell DNA synthesis is terminated and progression through the cell cycle is suspended, but the molecular mechanisms underlying these interactions have yet to be elucidated. In contrast to the helper independent parvoviruses, productive AAV infection relies upon the ability of its helper virus to drive resting cells into S phase, and even in the absence of cellular replication some viral transcription may occur at high input multiplicities, from duplex episomes generated by the annealing of opposite sense input strands (Nakai et al. 2000), as will be discussed later.

DNA replication

Parvoviral DNA is replicated through a series of duplex, concatemeric intermediates by a strand displacement mechanism called rolling hairpin replication (RHR), depicted in Figure 7 (Cotmore and Tattersall 1996; Tattersall and Ward 1976). The replication fork is aphidicolin-sensitive, requires PCNA, is unidirectional and results in the synthesis of a single, continuous DNA strand, indicating that synthesis is probably mediated predominantly by DNA polymerase δ and its accessory proteins (Ni et al. 1998; Christensen and Tattersall 2002). Short imperfect palindromic sequences at each end of the genome repeatedly fold and unfold during this process, first creating duplex hairpin telomeres in which the 3′ nt of each strand is paired to an internal base to create a DNA primer, and then unfolding to allow the hairpin to be copied. Together with a few flanking nucleotides, these palindromes contain all of the *cis*-acting information needed for both viral replication and packaging. During RHR they effectively serve as 'toggle-switches,' reversing the direction of synthesis at each end of the linear genome, thus adapting the more ancient rolling-circle replication strategy (Ilyina and Koonin 1992) for the amplification of a linear chromosome.

Since MVM packages negative-sense strands predominantly, and hence the 3′ end of the DNA is always at left end of the incoming genome, it is easier to describe the rolling hairpin process for this virus, but exactly parallel steps ensue for incoming positive strands of all genera, except that replication would initiate at the right-end of such genomes. Thus, for MVM in step (i) of Figure 7, the base-paired 3′ nucleotide of the left-end hairpin is used by a host polymerase to prime conversion of virion DNA to the first duplex intermediate. This generates a monomer length duplex molecule in which the two strands are covalently continuous at the viral left-end telomere. Synthesis of this intermediate precedes viral gene expression. The cellular replication fork assembled on such viral templates appears to be unable to displace and copy the right-end hairpin sequence, so the 3′ end of the new DNA strand is ligated to the 5′ end of the hairpin by a host ligase, creating a covalently continuous duplex molecule (Cotmore and Tattersall 1996) (step ii). Replication

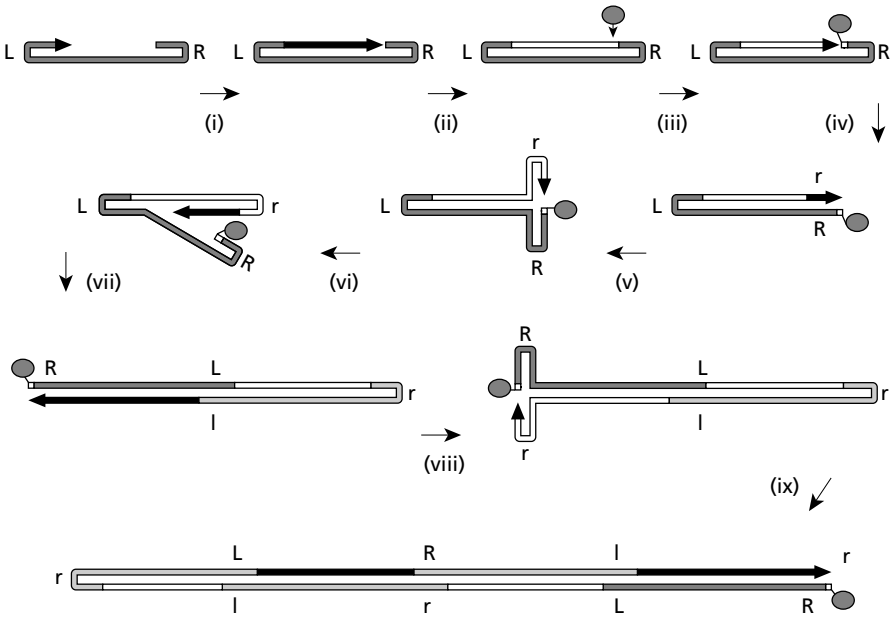

Figure 7 *Parvoviral 'rolling hairpin' DNA replication scheme. The viral genome is represented by continuous line, shaded dark gray for the original genome, light gray for progeny genomes, and black for newly synthesized DNA, the 3′ end of which is indicated by the arrowhead. The gray sphere represents an NS1 molecule. The letters L and R represent left-end and right-end palindromic sequences, respectively. Upper and lower case represent 'flip' and 'flop' versions of these sequences, respectively, which are inverted complements of one another. See text for further explanation.*

beyond this point requires expression of the viral initiator protein, NS1 in MVM or Rep68/78 in AAV, which nicks the ligated strand, as illustrated in Figure 7, step (iii), in a reaction that is assisted by a host DNA-bending protein from the HMG1 family. This requirement for HMG1 appears to vary somewhat between genera, being essential for the MVM right-end telomere, but merely enhancing initiation at the AAV2 hairpins (Costello et al. 1997; Cotmore and Tattersall 1998). Nicking is achieved by a transesterification reaction, which liberates a base-paired 3′ nt that supports the assembly of a new replication fork, while leaving NS1 covalently attached to the 5′ end of the DNA at the nick, where it is believed to function as the 3′ to 5′ replicative helicase of the subsequent replication fork (Christensen and Tattersall 2002; Smith and Kotin 1998; Wu et al. 1999; Zhou et al. 1999). NS1 also recruits the cellular RPA (replication protein A) complex, which binds and stabilizes single-stranded DNA, while AAV appears to favor adenovirus single-stranded binding protein, although in either case the availability of such factors appears rate-limiting for the progress of this strand displacement mechanism (Ward et al. 1998; Christensen and Tattersall 2002).

A complex sequence of melting and reannealing reactions then ensues, step (iv–v) Figure 7, in which the replication fork, assisted by the initiator protein, first unfolds and copies the hairpin, in a process called 'hairpin transfer,' thus replacing the original sequence of the terminus with its inverted complement (step iv). Since the terminal sequences are imperfect palindromes, and this inversion occurs with every round of replication, progeny genomes comprise equal numbers of each terminal orientation, dubbed 'flip' and 'flop.' In MVM the entire sequence of reactions from the introduction of the NS1-mediated nick to the generation of a duplex extended-form telomere is referred to as 'hairpin resolution,' while in AAV the equivalent sequence of events is called 'terminal resolution' (Snyder et al. 1990; Cotmore and Tattersall 1996). A general rule seems to be that viruses with identical termini, like the AAVs and B19, replicate both termini by hairpin resolution, and thus have flip and flop forms of each telomere, while viruses with disparate termini, like MVM, replicate the right-end by hairpin resolution and the left by an asymmetric mechanism, called junction resolution, which conserves a single sequence orientation and uses different structural arrangements and cofactors to activate the NS1 nickase, as discussed below.

The extended-form duplex termini generated in step (iv), Figure 7, are then melted out and the strands reformed into hairpinned 'rabbit ear' structures, in a process facilitated by the direct binding of NS1 to symmetrical copies of its duplex recognition sequence surrounding the axis of the extended palindrome (Willwand et al. 2002; Ward and Berns 1995) (step v). This allows the 3′ nucleotide of the newly synthesized DNA strand to pair with an internal base, thus priming synthesis of additional linear sequences (step vi). The result of rolling hairpin synthesis is the accumulation of

palindromic duplex dimeric (step vii) and tetrameric (step ix) concatemers, in which alternating unit length genomes are fused in left-end–left-end and right-end–right-end orientations. Although such concatemers are generated during AAV replication, they tend to be heterogeneous because both ends of the genome can be excised by equivalent reactions, whereas in MMV, left-end–left-end dimer duplexes accumulate, awaiting resolution by the more complex, and apparently slower, junction resolution process.

During junction resolution, left-end sequences are replicated and separated asymmetrically, so that new termini are created in a single sequence orientation, designated 'flip.' This effectively allows the two arms of the hairpin to be dedicated to different roles in the viral life cycle, the outboard arm supporting resolution, while the inboard arm potentially provides upstream elements for the nearby transcriptional P4 promoter. The 121 base left-end telomere of MVM has an important unpaired 'bubble' element in the hairpin stem, where a trinucleotide on the inboard arm opposes a mismatched dinucleotide. During rolling hairpin synthesis this hairpin is unfolded and copied to form the left-end–left-end junction, which links monomer genomes in the dimer intermediate (Figure 7, step vii), and within which these asymmetric bubble nucleotides segregate to opposite sides of the junction. It is this duplex junction structure, rather than a single hairpinned terminus, which contains the replication origin required for copying and separating MVM left-end sequences. The junction contains one candidate nick site for NS1 on each arm of the palindrome, located on opposite strands, but only the origin on the arm carrying the 'bubble' dinucleotide has the precise spatial organization required for nicking to occur. This is because, at the left-end origin, activation requires a cellular protein, called parvoviral initiation factor (PIF), to bind the viral DNA in exact juxtaposition to NS1 (Christensen et al. 1999). This interaction occurs only across the dinucleotide, and not the trinucleotide, bubble spacer element. PIF effectively stabilizes the binding of NS1 to its recognition element in the active origin, allowing it to use its 3′ to 5′ helicase activity to melt the duplex DNA at the adjacent nick site (Christensen et al. 2001). This is an essential prerequisite for nicking because, like other rolling-circle initiator proteins, NS1 is only able to nick its cognate site if this is presented as single-stranded DNA. The formation of individual nicking complexes and the biochemistry of these reactions have been studied extensively, but are largely outside the scope of this article, and the interested reader is referred to the more detailed discussions in the references cited above.

Following introduction of a single nick in the active arm of the junction, the 3′ to 5′ helicase activity of NS1 destabilizes the palindrome, frequently promoting its rearrangement into a cruciform intermediate that is essential for junction resolution. Replication forks that assemble after the junction reconfigures into this cruciform synthesize DNA in the flip orientation, creating a heterocruciform structure, which then undergoes a form of hairpin transfer. Ultimate resolution of the resulting, partially single-stranded, product requires the introduction of an NS1-mediated nick in the 'inactive' arm of the junction introduced as this is transiently presented in single-stranded form to the nickase (Cotmore and Tattersall 2003). Operationally, whether or not this final single-strand nick occurs is thought to depend upon the efficiency of hairpin resolution at the right end of the genome, since forks returning from the right end could recreate the partially resolved duplex junction before the single strand nick occurs. Thus the speed of initiation at the right-end of the genome influences the products generated at the left-end of the genome, a theme which becomes important when considering which strands are ultimately packaged, as discussed below. Although junction resolution appears complex, it uses a series of reactions that are characteristic of parvoviral replicons and their replicator proteins, and may represent a way of resolving junctions so that each arm could be dedicated to a different activity. That this might indeed be the case is suggested by studies with MVM genomes that have been mutated to present an active origin on both arms of the left telomere. Such mutations are invariably lethal, but allow the selection of viable variants in which the origin associated with the inboard arm has been inactivated by a variety of secondary mutations, suggesting that restricting cleavage at the inboard site is essential (Burnett and Tattersall 2003).

Capsid assembly and packaging

Late in infection, viral capsid gene expression predominates because NS1/Rep is able to strongly *trans*-activate the P38 promoter (Rhode 1985; Labow et al. 1986). As illustrated for MVM in Figure 8, VP1 and VP2 are synthesized in approximately the ratio required for capsid assembly. Partially assembled capsid subunits, probably in the form of trimers, exhibit a structure-dependent nuclear localization signal on a VP-common β-strand that will eventually form part of the internal surface of the capsid. This directs translocation of the intermediates into the nucleus, where they are assembled into empty capsids, which are believed to be the precursors of full virions. For AAV2 assembly appears to occur within the nucleoli, which also become highly disorganized in many other parvovirus infections (Singer and Rhode 1978; Wistuba et al. 1997). In MVM infection of murine cells some aspect of the continued synthesis and assembly of the capsid precursors requires the small nonstructural polypeptide NS2 (Cotmore et al.

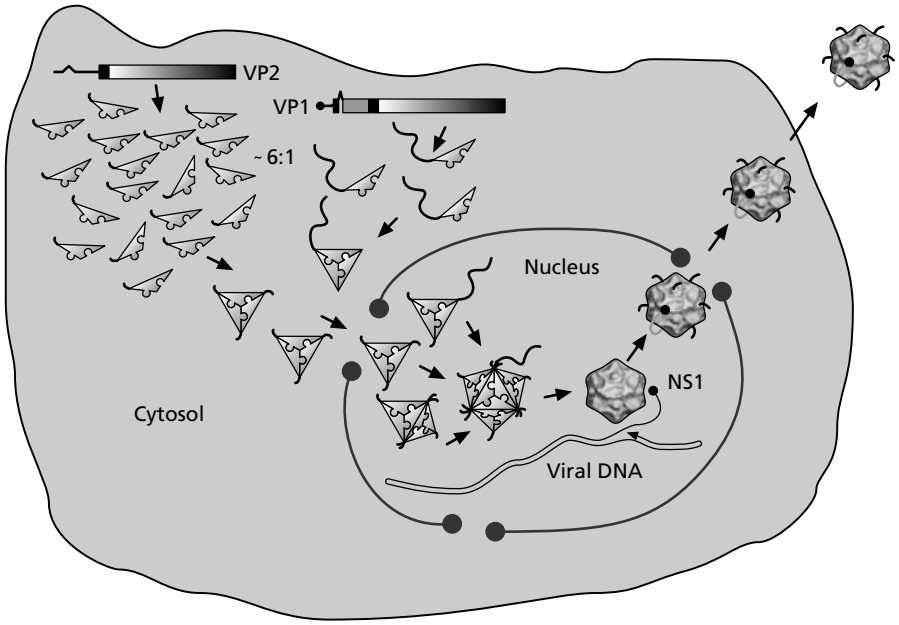

Figure 8 *Assembly and packaging pathway for a generic parvovirus, as described in the text predominantly for MVM. Lines and blocks at top left indicate the P38-driven transcripts and their encoded protein sequences. The core VP sequence that is the building block for the particle is shown as a gray gradient-filled box in the coding diagram, and a triangular icon as its translated form. Shading of VP components and identity of other symbols are described in the legend to Figure 6. See text for additional details.*

1997). Significantly, when empty capsids are assembled, the VP1 and VP2 N-termini are internalized, so that both the VP1-specific and VP-common nuclear localization signals are hidden within the particle, while the VP2 N-termini, which contain a nuclear export signal discussed later, are also effectively concealed from the export machinery. Thus empty capsids appear to be entirely neutral with regard to intercompartmental trafficking.

As assembled capsids accumulate, progeny single-stranded DNA synthesis begins to predominate over duplex DNA amplification. How this switch is controlled is unknown, but progeny synthesis is entirely dependent upon the availability of preformed capsids, and most of the single-stranded monomeric DNA in the cell appears to be encapsidated. Exactly how DNA strands are selected for packaging remains uncertain, but the minimal requirement appears to be the presence of an active nick site (Nony et al. 2003; Musatov et al. 2002; Huser et al. 2003). Since the viral initiator proteins, NS1 and Rep68/78, become covalently attached to the DNA at such sites, and because these proteins bind efficiently to intact capsids (Dubielzig et al. 1999), they may well mediate the initial interactions. Selection of positive or negative sense DNA is not directed by strand-specific packaging signals, but rather reflects the relative efficiencies of the viral replication origins associated with the two genomic termini (Chen et al. 1989; Cotmore and Tattersall 2005). However, since packaging requires ongoing viral DNA synthesis, it is not simply a process of melting out and sequestering preformed DNA strands (Myers and Carter 1980; Zhou and Muzyczka 1998). Strands are packaged vectorially, in a 3′ to 5′ direction, driven in AAV2 by the helicase activity of the small Rep proteins, Rep40 and Rep52 (King et al. 2001). As DNA is translocated into the MVM particle, approximately 25 amino acids of a VP2 N-terminus becomes externalized through the pore in each of the 12 cylindrical turrets protruding from the capsid surface at the fivefold symmetry axes (Agbandje-McKenna et al. 1998; Xie et al. 2002; Cotmore et al. 1999). This region of the VP2 polypeptide carries a series of phosphorylated serine residues and can be shown to function as a nuclear export signal, causing full virions to be translocated into the cytoplasm, and hence out of the cell via an as yet unknown route, prior to lysis (Maroto et al. 2000; Maroto et al. 2004). Thus, the act of packaging converts a trafficking-neutral capsid to a nuclear export competent virion.

NS2 is involved in this early exit pathway, even in cells where it is not required for capsid assembly, and part of this function appears to be mediated via a binding motif for the nuclear export protein, Crm1, located in its central exon (Ohshima et al. 1999; Miller and Pintel 2002; Bodendorf et al. 1999). During normal infection, NS2 is predominantly cytoplasmic, but mutations in the Crm1 binding site reveal it to be a constitutively nuclear protein, which is normally transported out of the nucleus via a Crm1-dependent pathway. Early virion release is impaired in such mutants, but it is not yet clear whether Crm1-complexed NS2 simply acts by inducing essential changes in the cellular environment,

or whether it acts through a direct interaction with the particle. Ultimately infections are generally lytic, and cells remain actively synthesizing viral DNA until necrotic or apoptotic lysis occurs at about 16–20 h after entry into S phase (Ran et al. 1999; Rayet et al. 1998), which results in significant release of residual progeny virions.

HOST RANGE

Tissue and host species specificity

Lytic growth is modulated by developmentally regulated factors operating in the host at the cellular level. Significant differences in target tissue specificity clearly exist between different parvovirus species. These can often be explained by the use of a different cell surface receptor repertoire by each virus serotype, as discussed elsewhere in this chapter, although postentry blocks to viral growth have also been identified in some virus–host combinations. However, differences in pathogenic potential exist not only between virus serotypes, but between virus strains of the same serotype, as seen within the MVM, PPV, FPV, and AMDV species. The prototype strain of MVM, MVMp, productively infects murine fibroblasts, but cannot establish infection efficiently in T lymphocytes. The isolation of a second strain of minute virus of mice, MVMi, as an immunosuppressive agent from a murine lymphoma (Bonnard et al. 1976), indicated that a mutable genetic component of the virus played a role in determining the type of differentiated cell the virus can lytically infect. For MVM, this discrimination is mediated by a region of the viral capsid called the allotropic determinant, which acts in *cis* with the genome it encapsidates (Gardiner and Tattersall 1988a,b). The determinant involves a small group of amino acids that map near the surface of the virion, grouped along the edge of the spike at the threefold symmetry axis and down into the depression at the two-fold axis (Agbandje-McKenna et al. 1998). Here a coordinated change in just two amino acid residues in the capsid of the lymphotropic murine virus MVMi allows it to extend, or even switch, its host range, gaining the ability to replicate productively in murine fibroblasts (Ball-Goodrich and Tattersall 1992).

Genetically and pathogenically distinct strains of PPV also exhibit allotropic specificity determined in a similar way, which controls their ability to replicate in particular porcine cell lines in vitro (Bergeron et al. 1996), and AMDV replication has been similarly linked to a cluster of capsid residues (Fox et al. 1999). Studies on MVM, PPV, and AMDV strains have shown that infection of the 'wrong' host cell is profoundly restricted at an, as yet, unidentified step, which occurs after virus internalization from the cell surface, but before the onset of transcription and DNA amplification. Significantly, while each MVM strain is reciprocally restricted in each other's host cell, both strains replicate efficiently in somatic cell hybrids between the two host-cell types, implying that restricted infections fail to establish because of the absence of a differentiated host factor in the restrictive host that is usually exploited by the restricted strain in its permissive cell type. However, the molecular identities of the host factors that mediate these strain-specific tropisms remain elusive, and may well vary between virus–host combinations.

While control of species host range among viruses from the FPV/CPV group is determined by a similar cluster of surface amino acid changes in the capsid (Chang et al. 1992; Parrish 1991; Truyen and Parrish 1992), these changes are now known to act via species-specific interactions with the viral cell surface receptor, recently shown to be the TfR (transferrin receptor). Thus, while all of the viruses can bind to feline and human TfR, and use these receptors to enter and infect cells, canine cell infection is a specific property of CPV and depends on the ability of this virus to bind the canine TfR (Govindasamy et al. 2003; Parker et al. 2001; Hueffer et al. 2003). The specific contact established between CPV or FPV capsids and the TfR also appears to be important, since modifications introduced into either the virus or receptor can allow cell surface binding to occur without leading to infection (Hueffer et al. 2004). For these viruses, the critical step likely involves receptor-mediated internalization of the virus in a way which selectively leads to its successful translocation across the endosomal bilayer, but how or where this operates remains uncertain.

The situation for MVM appears to be substantially different. Both fibrotropic and lymphotropic strains of MVM bind to sialoglycoproteins present at about 5×10^5 copies per cell on murine fibroblasts, and compete for binding to these receptors (Spalholz and Tattersall 1983). While all of these receptors could be equipotent, it is still possible that different minor classes of specific cell surface molecules mediate cytoplasmic delivery of MVMi and MVMp, while the more abundant species merely effect surface attachment. Thus a cytoplasmic delivery mechanism, like that conjectured for CPV, remains a possibility for MVM whereby both virus strains are internalized into endosomes on a variety of receptors, but can only penetrate the bilayer successfully from a particular receptor species. Precedent for this type of restriction has been documented for the 'small plaque' mutants of polyomavirus (Bauer et al. 1999), which vary from wildtype in binding avidly to entry-incompetent receptors. However, restriction to the extent seen for MVM, which would require most of the virus to be bound on incompetent receptors, has yet to be described. Alternatively, capsid-mediated MVM host range restriction could operate at a later stage in the life cycle, and involve a selective interaction between the virion and cell type-specific host molecules that mediate

nuclear entry, viral uncoating, or the assembly of the initial viral transcription templates. In this respect, it is interesting to note that recent studies suggest that the uncoating of recombinant AAV particles shows virus species-specificity in rat liver cells, with AAV6 and AAV8 virions being uncoated much more efficiently than their AAV2 counterparts, possibly because the latter are not delivered to nuclear loci containing host factors that mediate this transition (Thomas et al. 2004).

Tissue specificity can also be mediated in MVM after the onset of infection, by sequences that map to a region in the nonstructural gene containing the splice control regions for the major intron (Rubio et al. 2001; Gardiner and Tattersall 1988b; Colomar et al. 1998). Splicing efficiency at this intron, which can be modulated by single nucleotide mutations in the putative splice branch point region, determines the relative levels of NS1 and NS2 transcripts generated during infection, with growth in fibroblasts apparently requiring the more efficient NS2 splice configuration, while the opposite is true for growth in lymphocytes. However, the level at which this tropism operates remains to be determined. The absolute ability to express NS2 has also been identified as a host range determinant, since MVMp NS2-null mutants, which cannot express any of this protein, are completely defective for growth in murine fibroblasts, although they are able to replicate in a number of cell types, notably transformed human fibroblasts (Naeger et al. 1990). At present it is not clear whether this phenomenon represents a true host species restriction or if transformation of a normally nonpermissive host cell can sometimes complement for the lack of NS2, thus rendering the cell permissive for NS2-null virus replication.

Oncotropism

The affinity of parvoviruses for dividing cells is also reflected in their ability to interfere with, and suppress, another type of rapid cellular proliferation in their hosts, namely, neoplastic disease. Infection with autonomous parvoviruses has been shown to suppress tumor formation by a large number of viruses and carcinogens. For example, KRV suppresses leukemia induction in rats by Moloney murine leukemia virus (MoMLV) (Bergs 1969), and H1 infection of hamsters suppresses tumor formation by either adenovirus or dimethylbenzanthracene (Toolan and Ledinko 1968; Toolan et al. 1982). Similarly, AAV2 has been shown to inhibit the oncogenicity of tumor viruses and transformed cells (de la Maza and Carter 1981; Katz and Carter 1986), although in this case the effects may predominantly reflect the complex interactions between AAVs and their helper viruses.

Despite their apparent lack of natural infectivity for humans, rodent parvoviruses can grow in many human cell lines in vitro, and this is especially true if the cells are transformed by agents such as gamma-rays, nitroquinolines, or SV40 (Cornelis et al. 1988). Extensive studies over the past two decades, focusing on MVM and H1, have defined in vitro analogues of this oncotropism, and have established that it is a cell-intrinsic, rather than an immune-mediated phenomenon. Sensitization to parvovirus-mediated cell killing has been demonstrated following transformation of rodent and human fibroblasts, human keratinocytes and human mammary epithelium. Transforming agents used have varied from activated oncogenes such as h-*ras* and v-*src*, to c-*myc* and papovaviral T antigens (Rommelaere and Cornelis 1991). The biochemical basis for such sensitization is complex, and can take several forms. In some cells it manifests as a dramatic, transformation-associated increase in viral DNA amplification, while in others it can present as transcriptional up-regulation of the P4 promoter, combined with a significant lowering of the threshold concentration of viral NS proteins above which cell survival is drastically impaired (Caillet-Fauquet et al. 1990). In *ras*-transformed cells, a significant proportion of this P4 promoter up-regulation is known to be controlled by two separate transcription factor-binding sites upstream of its TATA box, one specific for members of the CREB/ATF family, while the other binds factors belonging to the Ets family (Perros et al. 1995; Perros et al. 1999; Fuks et al. 1996). Transformation-induced modulation of normal cell cycle control thus appears to promote viral replication, and pathogenicity, by impinging upon several different processes in the viral life cycle. Since it clearly does not relate entirely to their requirement for rapidly dividing cells, it is not clear why the parvoviruses should exhibit these oncotropic properties, particularly those controlled by the P4 promoter. It may be that this property has been fortuitously selected for by its natural growth in developing, particularly neonatal, hosts, where aspects of the intracellular milieu of its target cell populations may be mimicked by some of the events that contribute to transformation in the adult cell types. In support of this possibility, is the recent observation that *LacZ* expression is controlled in a cell-type-specific and differentiation-dependent pattern during embryogenesis in mice transgenic for an MVM P4-driven β-galactosidase reporter gene, indicating that P4 is markedly responsive to developmental signals (Davis et al. 2003).

Persistence: latency and cryptic infection

The first direct evidence for parvoviral latency came from the observation that adenovirus infection of some primary human embryonic kidney cultures would yield infectious AAV particles that were not detectable in the original adenovirus stock. Subsequently, it was shown that propagation of human Detroit 6 cells,

infected with AAV in the absence of adenovirus, led to the gradual disappearance of demonstrable AAV, which could then be recovered by subsequent adenovirus infection. This led to the hypothesis that AAV could persist in cells in a latent, inheritable form (Berns et al. 1975). The advent of Southern blotting allowed the identification of AAV genomes in such cells, integrated into chromosomal DNA as tandem repeats (Cheung et al. 1980). Subsequent studies have shown that the integration of AAV genes is dependent upon the presence of the *Rep* genes. While the *Rep* gene products up-regulate their own synthesis during coinfection of AAV2 with a helper adenovirus, when AAV2 infects on its own this feed-back effect of Rep is reversed and Rep production is shut down. However, between 1 000 and 4 000 Rep molecules are produced per cell, and this is enough to catalyze the integration of the viral genome into a specific site at chromosome 19q13.3-qter in the human genome (Kotin et al. 1990). Specific integration at this chromosomal locus requires the interaction of Rep with a target DNA sequence that has key features in common with the origin of AAV DNA replication embedded in each viral terminal repeat (Kotin et al. 1992). When the chromosome 19 sequence, known as AAVS1, is placed in a plasmid, it can serve as a substrate for Rep-dependent DNA replication in an in vitro system (Urcelay et al. 1995), and when present as a 'knock-in' transgene in the mouse X-chromosome, it will direct site-specific integration of AAV2 in murine cells (Young et al. 2000). Cloned into an Epstein–Barr virus (EBV) episome and transfected into human cells, the AAVS1 sequence supports site-specific AAV2 integration in vivo, which provides a system where integration can be genetically manipulated (Giraud et al. 1994). Studies using this system have confirmed that AAVS1 must be able to function as an AAV replication origin in order to serve as an integration target site (Linden et al. 1996). In addition, it appears to be important that chromatin associated with the AAVS1 locus is in an open configuration, as shown by the presence of a nearby active gene, encoding slow skeletal troponin T (Dutheil et al. 2000), a DNase I hypersensitive site (Lamartina et al. 2000), and an upstream insulator element that blocks the spread of heterochromatin over the locus (Ogata et al. 2003). In the absence of the Rep 68/78 protein, AAV2 still integrates into the host genome, but at multiple, and apparently random, sites, although there still seems to be a bias towards actively transcribed genes (Nakai et al. 2003). It seems likely that these integration events involve pre-existing double-strand breaks in the host chromosome (Song et al. 2001).

Early studies showed that the autonomously replicating parvoviruses do not integrate into host DNA during a normal infection cycle (Richards and Armentrout 1979). While no stable cell lines carrying integrated genomes resulting from helper-independent parvoviral infections have been reported, MVM has been shown to integrate site-specifically into an episomal EBV-based shuttle vector in a permissive cell, provided the left-end viral replication origin, containing a nick site for NS1, is present (Corsini et al. 1997). Interestingly, no integration was detected into episomes harboring a functional right-end origin. Isolation of such integrants was achieved by recovering the targeted shuttle vector by transfection of DNA derived from the dying cell culture into a bacterial host. This suggests that the reason stable integrants cannot normally be obtained from parvovirus-infected cell cultures is that such interactions, if or when they occur, do not confer protection against subsequent lytic infection, and thus the integrants cannot survive as clonal lines. Significantly, it has also been shown recently that MVM genomes that lack active *NS1* genes also integrate efficiently in a nonsite-specific fashion, at an efficiency that is comparable to that of Rep-negative AAV (Hendrie et al. 2003). Thus, random integration is possibly a feature common to all parvoviral genomes in situations where they are unable to express their NS genes and kill their host cell.

Although the helper-independent parvoviruses are inherently lytic, they are also exquisitely dependent on the host cell-cycle program, because while they are unable to force cells to proliferate, they require their host to enter S phase to support replication and transcription. This leads to another potential form of persistence, in noncycling cells, which has been dubbed cryptic infection, to distinguish it from the latent infections described previously for AAV. In cryptic infection, the viral life cycle does not proceed further than nuclear sequestration of the incoming virion, and is thus completely silent with respect to viral gene expression. Thus, in resting peripheral blood lymphocyte cultures, infectious virus has been observed to remain sequestered and initiate replication only after the addition of phytomitogens (Paul et al. 1979). This type of interaction is inherently unstable, and cannot persist by vertical transmission, in vitro or in vivo, because when a cryptically infected host cell enters S phase, in response to an exogenous signal, its resident virus will initiate replication, express its genes, and the cell would subsequently die (Tattersall and Gardiner 1990).

The application of highly sensitive PCR techniques to patient-derived material has recently led to the realization that B19 genomes are retained at low levels in a variety of tissues in infected individuals over the course of many years (Soderlund-Venermo et al. 2002). Possibly such viruses emerge at intervals, and boost host immunity, explaining why anti-B19 antibody titers are routinely maintained for life. At present, it has not been established whether the B19 genomes found in each of these situations persist as cryptic or latent state, as intact virions, integrated proviruses or episomal complexes.

PATHOGENESIS OF PARVOVIRAL INFECTION

INFECTIONS WITH MEMBERS OF THE *PARVOVIRUS*, *AMDOVIRUS*, AND *BOCAVIRUS* GENERA

Epidemiology

Parvoviruses are generally widely disseminated in their natural hosts. In part this is due to their high level viremias and efficient transmission via bodily secretions and excretions, and in part to the fact that infectious particles are exceptionally rugged, so that they survive outside their host for extended periods. Thus when CPV, a new virus in the feline panleukopenia serotype, emerged as a pathogen of dogs in 1978, it spread through most of the world within a few months, probably because it remained fully viable in fecal material transported on the shoes of airline passengers. Exactly how long shed virions retain viability in nature is undocumented, but they are resistant to most normal ambient temperature ranges, for instance, the half-life for MVM infectivity is in excess of 9 months at refrigerator temperatures. Statistics for virus distribution in wild animal populations are not readily available, but many, such as MPV and H1, are known to be widespread. Infection is generally acute and self-limiting, with transient viremias that last for 7–10 days while the host seroconverts, but some viruses, such as KRV, persist despite high neutralizing antibody titers, so that infected individuals may routinely shed virus for many months (Robey et al. 1968). With the advent of sensitive PCR technology it has become apparent that even following acute, apparently self-limiting infections in immune-competent hosts, low levels of viral DNA are routinely retained in some tissues for many years, although whether this DNA is episomal, cryptic, or integrated is uncertain.

Wild populations of the natural host generally have a high incidence of neutralizing antibody, so that most mothers transmit immunity to their offspring. Thus, infection of neonatal or nursing animals as maternal antibody wanes leads to an active, long-lasting immune response, rather than overt disease. As a consequence, clinically manifest infection is a rare event under endemic conditions, and is generally observed only in immunocompromised individuals. However, in veterinary medicine, vaccination of the entire population is a routine procedure because in domestic animal populations, epizootic and even panzootic, crises may develop if a virus is introduced into an immunologically naive host population.

Pathogenesis

Since their discovery at the end of the 1950s, viruses from the genus *Parvovirus* have been recognized as potential pathogens, although many of them have coevolved with their host to such an extent that infection generally remains subclinical. The spectrum of parvovirus disease thus ranges from acute lethal infections, through slowly progressing, sometimes chronic, illness, to transient subclinical malaise. Somewhat surprisingly, there are no known members of the genus *Parvovirus* that productively infect humans, although several can enter and initiate infection in normal human tissues in culture, or replicate productively in transformed human cell lines.

The types of pathology associated with viral infection can be readily understood by considering a few basic biological principles that control the viral life cycle. Firstly, parvoviruses all exhibit tissue specificity, which limits their lytic potential to cells of particular differentiated types, although this specificity may vary widely between virus species. As discussed previously, tissue specificity is frequently mediated by the presence or absence of appropriate cell surface receptors and coreceptors. However, it also commonly occurs at later stages in the infectious cycle, since the limited genetic complexity of these viruses means that they are peculiarly dependent upon exploiting the synthetic pathways of their chosen host. Disease potential is also controlled by their lack of growth control genes, so that, unlike most DNA viruses, they have no mechanism for forcing noncycling host cells to enter S phase, even though they depend absolutely upon cell functions expressed transiently during this phase of the cell cycle. Thus productive viral replication, and even viral gene expression, is restricted to proliferating cell populations. As lytic parasites of dividing cell populations, their ability to cross the placenta means that they can be potent teratogenic agents, causing fetal and neonatal abnormalities by destroying specific cell populations that are rapidly proliferating during the course of normal development. Indeed, the first autonomous parvoviruses were discovered because of their teratogenic potential in laboratory rodents. These same tissues are often quiescent, and therefore resistant, in the adult.

The age of the infected host thus profoundly influences susceptibility, since it determines the availability of mitotically active cells of the correct differentiated type. Age also influences the viruses' pathologies by determining the degree to which tissue regeneration can take place, so that viruses that replicate in tissues or organs capable of efficient regeneration, such as the liver in a young animal, lead to acute but self-limiting disease with no permanent damage, while the destruction of tissues with limited regenerative potential, such as the developing cerebellum, leaves permanent defects (reviewed in more detail in Siegl 1984, 1988).

INFECTION OF FETAL AND NEONATAL HOSTS

Fetuses and neonates are particularly susceptible to disease. If infection occurs early in pregnancy, the effects on the fetus tend to be generalized, and often

fatal, thus viruses infecting pigs, cows, rodents, and mink are commonly associated with fetal wastage. By the late pregnancy, and in neonatal animals, infection tends to be less devastating, coinciding with the increasing ability of the developing host to mount an effective immune response. As the patterns of cell division and differentiation change during development, infection also becomes more organ-specific and leads to distinct syndromes. Thus, infection with FPV or some of the rodent parvoviruses late in gestation specifically induces cerebellar ataxia by lytically targeting, or inducing apoptosis in, cells in the outer germinal layer of the differentiating cerebellum. Newborn mink infected with AMDV develop a severe, potentially fatal, respiratory distress syndrome, resulting from destruction of the type 2 alveolar pneumocytes that are proliferating during this period, while puppies infected with CPV in a time window between 8 days before and 5 days after birth show evidence of viral replication in the developing myofibers, leading to a fatal interstitial myocarditis (reviewed in Bloom and Young 2001). Most dramatically, lytic replication of some rodent parvoviruses in osteogenic tissues that are active during the perinatal period can lead to severe craniofacial and dental deformities, the so-called osteolytic syndrome, which results in offspring with small flat faces, microcephalic heads, protruding eyes and tongue, missing or abnormal teeth, and fragile bones (Siegl 1984, 1988).

INFECTIONS OF MATURE HOSTS

In older animals, more tissues are mitotically quiescent, so that acute disease is generally limited. However, some adult tissues, such as gut epithelium and the lymphopoietic system do retain large numbers of cycling cells. The small subset of autonomous parvoviruses that are capable of effectively targeting these cells, most notably viruses from the FPV/MEV/CPV complex, typically cause fatal disease in adult animals involving extensive destruction of gut epithelium and reticuloendothelial cells, while less extreme forms of leukopenia are also associated with PPV, BPV, and some rodent virus infections. Hepatitis is another common outcome of parvoviral disease in the post neonate, associated with protracted mitotic activity in the maturing liver, or damage-induced cell proliferation in adult tissue. While overt liver disease is a major symptom of GPV infections of goslings, hepatic damage associated with most viruses is rarely lasting because hepatocytes regenerate efficiently following immune clearance of the infecting virus. Patterns of acute disease in the adult thus directly reflect the replicating target tissues available at that time, and are often different from the patterns established by the same virus in the fetus or neonate (Siegl 1984, 1988).

A second, chronic, type of disease may also result from viral infection in the mature animal, associated with the immune response of the host. Thus, AMDV gives rise to a persistent infection in adult mink involving the antigen-presenting cells of the immune system, which ultimately results in immune-complex mediated glomerular nephritis. As a result, while vaccination is generally protective against most acute parvoviral disease, it can be contraindicated for viruses such as AMDV where it actually serves to intensify the pathology (Bloom and Young 2001).

INFECTIONS OF IMMUNOCOMPROMISED HOSTS

Infection of normal and mutant mice with MVM has shown that otherwise lethal pathogenic interactions between the virus and its host are effectively controlled by the host's immune system. Thus, MVMi is essentially apathogenic in adult animals (Segovia et al. 1995), but susceptible newborn mice, with immature immune systems, develop lethal myeloid depression. In vitro, the virus readily kills committed granulocyte–macrophage and erythroid progenitors, as well as pluripotent hematopoietic progenitors, derived from the bone marrow of normal mice (Segovia et al. 1991). Moreover, infection of adult SCID mice, homozygous for the severe combined immunodeficiency mutation, results in a profound leukopenia by the end of the first month and death within 4 months (Segovia et al. 1999). In these animals, the virus causes a novel dysregulation of hemopoiesis, in which primitive hemopoietic progenitors are targeted. This results in severe leukopenia, although circulating platelet and erythrocyte numbers remain stable. In the bone marrow of infected mice, granulocyte–macrophage lineage cells are seen to be markedly depleted, but erythroid progenitors undergo a twofold absolute increase (Segovia et al. 1999), indicating that compensatory mechanisms are mounted specifically by the erythroid lineage in order to maintain effective erythropoiesis. Thus, in immunocompromised adult hosts, complex pathological responses to MVMi infection can emerge that are not apparent in normal mice, a situation that parallels human erythrovirus infections of similarly immunocompromised individuals, as discussed later.

Use as vectors for gene therapy and vaccine delivery

Autonomous parvovirus vectors intended for eventual therapeutic use in humans have been modeled to date on three rodent parvoviruses, MVM, H1, and LuIII, and fall into two main classes. In the first type, the entire coding region of the virus is replaced with transgene encoding sequence, a strategy also employed in the majority of AAV vectors currently in use, discussed below. In the autonomous viruses, these complete coding replacement vectors, mostly based on an infectious clone of LuIII, have been constructed using either the endogenous viral P4 promoter, or a heterologous

mammalian promoter, to drive transgene expression (Maxwell et al. 2002). In the latter case, the only viral components remaining in the vector sequence are the terminal regions, which are used to replicate and package the construct driven by nonstructural and capsid proteins provided in *trans* from a helper plasmid. These vectors have been tested for their efficiency at transducing reporter genes, such as luciferase, into a variety of target cell types. Since helper plasmids can be organized to express VP1 and VP2 species from different parental viruses to construct a single packaged vector, these vectors have been used to map viral tropic determinants to individual structural protein species (Maxwell and Maxwell 2004; Maxwell et al. 1995).

The second type of vector retains the nonstructural gene of the virus and has the transgene substituted for sequences normally encoding the capsid proteins. These capsid replacement vectors, although crippled for the production of progeny, retain the replicative and cytotoxic functions of NS1, and therefore are designed to target cells for transgene expression concomitant with cell destruction. This arrangement also allows for high level transgene expression, both by virtue of being driven off the strongly transactivated P38 promoter and because vector genomes can replicate upon uncoating in the target cell, thus extensively amplifying the P38 transcription templates. In addition to transducing reporter transgenes, vectors of this type have been constructed to express suicide genes, as well as various cytokines, such as IL2, IL4, IP-10, or MCP-3 and other immunomodulatory molecules (Cornelis et al. 2004; Russell et al. 1992; Wetzel et al. 2001; Giese et al. 2002). The rationale behind such vectors is to exploit the natural oncotropism of the parental virus to specifically target transgene expression to tumor cells, with the anticipated result of selectively killing tumor cells or augmenting host antitumor immune responses (Haag et al. 2000; Giese et al. 2002; Cornelis et al. 2004).

The unique life cycle of the autonomous parvoviruses has also been exploited to construct vaccine vectors capable of eliciting lifelong immunity in mice following a single inoculation. By transducing antigen genes into resting lymphocytes, these vectors appear to establish cryptic infection, during which sporadic vector activation primes and continually boosts both humoral and cell-mediated Th1-like immune responses (Palmer et al. 2004).

A consistent problem faced in the successful production of parvoviral vectors has been the generation of contaminating replication competent virus following cotransfection of vector and helper plasmids. This presumably occurs as a result of recombination between NS and capsid gene sequences present in the separate vector and helper plasmids, and considerable effort has been expended to abrogate this phenomenon. Success in this has been achieved by reducing the overlap between the two viral components, and where this is impractical, by altering the DNA sequence underlying the coding sequence through the overlap, by introducing deleterious mutation into one copy of the homologous region, or combinations of these approaches (Dupont et al. 2001; Brandenburger and Velu 2004; Palmer and Tattersall 2000; Brown et al. 2002), such that vector stocks can now be generated with extremely low or undetectable contamination with replication competent virus.

INFECTIONS WITH THE *DEPENDOVIRUS* GENUS

Epidemiology

AAVs were first identified in the mid-1960s as 20-nm particles often found contaminating human and monkey adenovirus preparations. Currently, there are seven recognized human/simian serotypes, of which AAV2, AAV3, and AAV5 were isolated directly from human tissue. More specifically, serotypes 2 and 3 are frequently isolated from multiple tissues in conjunction with adenoviruses, whereas AAV5, the most distantly related member of the group by DNA and protein sequence criteria, has been isolated once, from a condylomatous genital wart, where its natural helper was likely a herpes virus (Georg-Fries et al. 1984). Antibodies against all three are found in about 50 percent of adults, with peak titers occurring between the ages of 10 and 20 (Blacklow et al. 1968a; Georg-Fries et al. 1984). In contrast, AAV4 is probably derived from a simian reservoir, since antibodies to it are very common in nonhuman primates, but are detected at much lower frequencies in humans, with a peak incidence of about 10 percent between the ages of 2 and 5 (Blacklow et al. 1968b). Antibodies reactive to AAV1 are found in a high proportion of both human and simian populations, so that its reservoir is questionable. AAV6 appears to be a hybrid recombinant between AAV1 and AAV2, which can be neutralized with anti-AAV1 sera (Rutledge et al. 1998). The remaining serotypes, AAV7 and AAV8, were isolated from Rhesus monkey tissues. Neutralizing antibodies directed against them are rare in the human population and, when present, are of low titer (Gao et al. 2002).

A surprisingly diverse series of unique AAV proviruses have also been rescued from the tissues of various nonhuman primates with, for example, a single lymph node from one rhesus monkey yielding several quite distinct viruses. This heterogeneity appears to result from AAV sequence evolution within an individual animal due, in part, to extensive homologous recombination between a limited set of coinfecting parental viruses, leading to the formation of swarms of quasispecies (Gao et al. 2003). Interstrain and interserotype genome recombination of this type has also been observed for members of other parvoviral genera

(Lopez-Bueno et al. 2003; Gottschalck et al. 1991), and is likely a common source of diversity in situations where multiple related viruses are able to infect the same host tissue.

AAV-like sequences have also been found throughout the *Mammalia*, commonly being detected in conjunction with species-specific adenoviruses. Recently, avian and snake viruses, AAAV and SAAV, have been cloned and sequenced (Bossis and Chiorini 2003; Farkas et al. 2004). Since they are not associated with pathology, such contaminants are rarely noticed, but are likely ubiquitous throughout the higher vertebrates.

Pathogenesis

AAV infection appears to occur predominantly via respiratory, fecal–oral, and conjunctival routes (Schmidt et al. 1975; Blacklow et al. 1967), although sexual transmission has also been suggested (Han et al. 1996; Georg-Fries et al. 1984). Despite the demonstrable presence of low levels of AAV DNA in many tissues, and their high seroprevalence, no study has convincingly linked AAV to any human disease. Studies designed to investigate the teratogenic potential and tissue distribution of AAVs following transplacental infection in mice and rhesus monkeys similarly failed to suggest pathologic sequelae, and recent interest in these viruses as vectors for gene therapy, discussed below, means that there have now been a large number of detailed animal studies of vector AAVs (predominantly AAV2) in mice, rats, rabbits, and dogs, without identifying reasons for clinical concern. In addition, AAV infection has been shown to inhibit transformation of cells by adenoviruses both in vitro and in animal models in vivo, leading to the suggestion that these ubiquitous viruses might even carry out a form of cancer surveillance in the human population. Results of some serological studies appear compatible with this possibility (Sprecher-Goldberger et al. 1970, 1971; Mayor et al. 1976; Georg-Fries et al. 1984; Han et al. 1996), but the idea still remains controversial.

Use as recombinant vectors for gene therapy

AAV vectors are currently being evaluated in clinical trials for the gene therapy of cystic fibrosis, hemophilia B, and muscular dystrophy (Wagner et al. 2002; Kay et al. 2000; Greelish et al. 1999; Aitken et al. 2001), while many, more preliminary, studies are currently probing their potential use to alleviate a broad range of genetic disorders. The simplicity of the AAVs, their apparent lack of pathogenicity or immune stimulation, and their broad tissue specificities, coupled with their ability to integrate into the host genome and thus achieve stable vertical transmission in dividing cells, render them exceptionally attractive for this purpose. Moreover, they can theoretically be made to integrate preferentially into a single locus in the human genome, on chromosome 19 (19q13-qter), thus limiting the danger of insertional mutagenesis associated with other viral vectors (such as retroviruses) that integrate randomly into host DNA. However, integration into this specific locus requires low level expression of the *Rep* gene, and most AAV vectors explored to date have this sequence deleted in order to avoid its potential cytotoxicity and to increase the genetic capacity of the vector. Thus they resemble the complete coding replacement vectors discussed above for the autonomous parvoviruses. In the absence of *Rep*, vector genomes still integrate with high efficiency into host DNA, but at apparently random sites, that may be the result of insertion into pre-existing double-stranded breaks (Zentilin et al. 2001; Miller et al. 2003).

Since integration generally appears to require ongoing DNA synthesis, it may well be restricted to target cells that are able to enter S phase. This is an important consideration for many gene therapy applications where gene delivery to nondividing cells is required. Surprisingly, however, studies with AAV vectors have shown that relatively stable transgene expression can also be achieved without cell division or integration. Instead, incoming positive- and negative-sense viral genomes appear to become uncoated and rearranged in nondividing cells, where they can be shown to reside as transcriptionally active, monomeric, or concatemeric duplex episomes. It is likely that incoming parental plus and minus strands, derived from separate virions, can anneal during or after uncoating to create these duplexes (Nakai et al. 2000), although various repair strategies can also be invoked, rendering cell division unnecessary for vector persistence and expression. Linear forms of these duplex episomes are commonly observed, particularly in cells with defective repair pathways (Song et al. 2001), but the hyper-recombinogenic nature of the AAV ITR generally results in concatemerization and ultimately circularization of the duplexes, in the absence of either *Rep* or cellular S-phase-dependent DNA replication (Yang et al. 1999).

Recognizing the potential cell cycle independence and transcriptional potency of duplex genomes has also led to the development of 'self-complementary' (scAAV) vectors (McCarty et al. 2001). These have one mutated ITR, which cannot be nicked, flanked by an inverted pair of genomes that are approximately half as long as the wild-type virus and end in a competent ITR, so that progeny are excised and packaged as self-complementary sequences separated by a single, uncleavable, hairpin. As predicted, when used to transduce various nondividing mouse tissues, such as liver, muscle, or brain, these vectors routinely gave more rapid gene expression and higher transduction efficiencies than their single-stranded counterparts. This may, in part, also reflect enhanced genome

survival, since naked single-stranded DNA is likely susceptible to rapid degradation in the host nucleus, making immediate conversion into duplex forms a priority for effective transgene delivery. However, viral capsids exhibit a strict DNA packaging limit, which means that sequences of more than about 5 kb cannot be encapsidated. Since scAAV vectors must carry two copies of the transgene, they have even more limited payloads, of around 2 150 basepairs of nonviral sequence, and hence can only transduce small proteins, ribozymes or anti-sense RNAs.

The transgene size limitation, which might be thought to severely restrict the usefulness of all AAV vectors can, in fact, be overcome by packaging single or multiple exons of large genes, bracketed by intronic sequences, into separate recombinant AAVs. Upon coinfection of the same nucleus, the recombinant genomes can circularize and recombine, creating concatemeric episomes in which the various gene sequences are transcribed in order, and the resulting mRNAs spliced, to yield the desired transgene product (Sun et al. 2000; Yan et al. 2000).

One further potential limitation to the use of AAV vectors is tissue specificity or, more accurately, the levels of therapeutic transgene expression that can be achieved in specific target tissues, since low level expression in most cells is possible with the original AAV2-based constructs. As discussed previously, AAV2 exhibits broad tissue specificity, using a variety of cell surface molecules including heparin sulfate proteoglycan, human fibroblast growth factor receptor and α_v,β_5 integrin as receptors or coreceptors, but it has subsequently become apparent that capsids from other AAV serotypes might prove more efficient for gene delivery to certain tissues. For example, AAV5 particles, which enter by binding to sialic acid residues and polypeptides from the PDGF family, appear to be more efficient at binding and delivering transgenes to cells of the airway epithelia, offering prospects for improved transgene delivery to the lungs (Walters et al. 2001; Rooney et al. 2002), while AAV6 and AAV8 are more efficient than AAV2 in transducing the liver (Grimm and Kay 2003; Gao et al. 2002). In the latter case, the improvement is apparently due to intranuclear uncoating efficiencies, which mean that AAV2 genomes are released slowly and are likely degraded prior to duplex episome formation (Thomas et al. 2004). Efforts to fine-tune the AAV particle to maximize gene delivery to particular cell types has thus led to an explosion of interest in the alternate capsid hypervariable regions available in nature, discussed above, and in the mapping and experimental manipulation of receptor-binding sites on the capsid surface, generating capsids that are targeted to alternate cell surface molecules. Use of such custom-designed AAVs may also ultimately overcome the problem of widespread immunity to the traditional serotypes in the human community, opening the possibility of delivering these rugged, resistant particles by in vivo, systemic routes, rather than the more technically demanding localized or ex vivo applications in current use.

Research on the use of AAV vectors to redress specific clinical conditions is expanding rapidly, although a description of these applications is beyond the scope of this chapter and has been extensively reviewed elsewhere (Carter et al. 2004). Suffice it to mention here that recombinant AAV vectors have been demonstrated to be efficacious in the treatment of many animal disease models, including those for cystic fibrosis, hemophilia, muscular dystrophy, rheumatoid arthritis, and lysosomal storage diseases, as well as several ophthalmic diseases and diseases of the central nervous system. Thus the AAVs appear to offer promise as remarkably versatile and unusually safe gene vectors, while the intense research interest in their biology that this has generated continues to reveal fascinating and unexpected details of their life cycle. With such interest and analysis, it seems likely that a whole family of vectors, fine-tuned to particular tissues and diseases, could rapidly become available.

INFECTIONS WITH THE *ERYTHROVIRUS* GENUS

Epidemiology of B19 virus

The human erythrovirus B19 is distributed globally, and infection is common, so that in Europe, the United States, and Asia around 60 percent of adults have anti-B19 IgG antibodies. It is mostly endemic, potentially emerging at any time during the year, although, in temperate climates, extended outbreaks commonly occur in late winter to early summer. These are particularly apparent in children of elementary school age, but infection continues at a lower rate throughout life. Once infected, immunity generally persists for life, and is a reliable marker of past exposure. In a typical study in England and Wales, anti-B19 IgG was found in 5–15 percent of children between the ages of 1 and 5 years, 50–60 percent of people 16–40 years old, and in excess of 85 percent of people over 70 (Cohen and Buckley 1988).

B19 virus is transmitted primarily via respiratory droplets, and virus can be detected in throat swabs during the week following infection, although exactly where the virus is replicating locally during this time is not known. Transient high titer viremias ensue during which virus can be present at concentrations of up to 10^{12} particles/ml of blood, depending upon the genetic make-up of the patient, as discussed later. However, the incidence of virus in donated blood is generally very low, with only around one in 20 000 to one in 40 000 units containing significant titers of virus, even in an

epidemic season (Cohen et al. 1990), so that infection via transfusion of individual blood units is rare. In contrast, blood products prepared from pools of donated blood can present a problem because B19 is small enough to escape removal by filtration, and is resistant to the heat and solvent treatments traditionally used to inactivate potential microbiological contaminants in such products. As a result, virus from a few viremic blood units appear to have been widely disseminated in blood products such as Factor VIII (Mortimer et al. 1983b), efficiently transmitting B19 virus infection. Thus, the incidence of B19 virus infection among hemophiliacs has historically been high (up to 90 percent), and the probability of infection correlates with the amount of clotting factor received (Azzi et al. 1999; Rollag et al. 1991). Since 2002, producers of plasma derivatives have quantitated and controlled B19 DNA in their source materials, to minimize the risk of transmission.

Diagnosis of B19 infection

Since the virus cannot be propagated effectively in tissue culture, diagnosis of acute infection relies on serologic tests and on the direct demonstration of viral DNA by hybridization or PCR (Zerbini et al. 2002). Antiviral antibodies can be measured with commercial enzyme-linked IgG or IgM capture assays, which usually employ recombinant particles as a target. The presence of IgM antibodies is indicative of recent infection, since these reliably appear within a few days of the onset of infection and persist for 2 or 3 months. Retrospective information on B19 virus infection is obtained by serological survey, with IgG and IgA antibodies typically persisting for life, possibly in response to the reactivation of latent virus. Diagnosis of persistent infection relies on DNA tests, because antibody production may be low or absent in these cases. DNA hybridization using an essentially full-length probe is sensitive enough to detect serum B19 virus levels during acute transient aplastic crisis or in chronically immunosuppressed patients, but the levels of viremia that occur during infection of normal individuals may be missed, as the technique has a baseline detection limit of around 10^6 genomes per ml. The advent of PCR-mediated gene-amplification greatly increased the sensitivity of B19 DNA detection, although this technique readily generates false-positives due to contamination. PCR results can sometimes be difficult to interpret, because low levels of virus routinely persist for months after infection in serum, bone marrow, skin, or synovial tissue from otherwise normal individuals, so that low level detection does not necessarily indicate acute infection. Moreover, the oligonucleotide primers selected may not detect extreme sequence variants of B19, such as the V9/A6 variants discussed later, whereas DNA hybridization does.

Clinical manifestations of B19 virus infection

Many cases of B19 virus infection are asymptomatic. Its most common clinical presentation in hematologically normal children is erythema infectiosum, a mild febrile illness that often presents as a 'slapped cheeks' rash or as a 'lacy' reticulate rash covering the trunk and arms. This common childhood infection was originally called fifth disease, being the fifth in a classic list of childhood rubelliform illnesses, and was well known to pediatricians for the 80 years prior to the recognition of parvovirus B19 as its sole etiologic agent (Anderson et al. 1983, 1984). The rash may be evanescent, and recurrences can be provoked by exposure to sunlight, emotion, heat, or exercise, but it frequently escapes detection in people with dark skin. About 10 percent of EI cases in children have been associated with arthropathy, although retrospective sero-epidemiological studies suggest that an equal number of arthropathies may occur in infected children not presenting with classic EI rash symptoms. Joint pains can persist for months, to a year or more, and are sometimes diagnosed as juvenile rheumatoid arthritis. In adults the classic rash symptoms are somewhat rare, although more prevalent in women than men, and arthralgia and inflammatory arthritis are much more frequent consequences of B19 infection, occurring in about 50 percent of patients. Specifically, nearly two-thirds of female and one-third of male adults experiencing a primary B19 virus infection also experience moderately severe symmetrical polyarthritis involving peripheral finger, wrist, ankle, or knee joints. This effectively means that around 15 percent of new cases of arthritis are the result of B19 virus infections. While arthropathies can sometimes be prolonged and severe enough to fulfill the diagnostic criteria for rheumatoid arthritis, B19 virus infection does not lead to articular erosion, and the arthropathy typically resolves within a few months even in more severe cases.

A characteristic biphasic clinical course of viremia followed by immune complex disease, has been described in both natural and experimental B19 virus infections of normal, otherwise healthy, individuals. In a study of human adult volunteers, viremia was detected 1 week after experimental intranasal infection of seronegative individuals, and was accompanied by a mild, flu-like illness with fever, malaise, myalgia, and itching (Anderson et al. 1985). During the viremic phase, virus is excreted from the respiratory tract, which likely represents the natural mode of transmission. Viremia is coincident with a transient, but absolute, reticulocytopenia and accompanying lymphopenia, neutropenia, and thrombocytopenia. Since the erythrocyte half-life in normal, healthy individuals is several times longer than this period of profound virus-induced bone marrow failure, frank anemia does not develop, and while the

hematological consequences of B19 virus infection are diagnostic, they are not clinically significant. Toward the middle of the second week following infection, virus-specific IgM levels rise, coincident with a fall in detectable circulating virus and the restoration of normal hematological parameters. A brief overshoot of reticulocyte numbers often prefaces this return to normality. IgM, which normally persists for 2–3 months, is followed by IgG, appearing at around 2 weeks after infection and persisting thereafter. The appearance of IgG ushers in the second phase of symptoms beginning 17–18 days after infection, which appear to be the result of immune complex formation. These include both the lacy rashes typical of EI and arthralgia, which normally last for 3–4 days, but can be protracted. Although the major protective immune response to B19 virus is humoral, some studies of cellular immune responses have been reported. These demonstrate that infected individuals mount a robust Th1-like $CD4^+$ T-cell response to viral components, and generate $CD8^+$ cytotoxic T cells that can kill cells pulsed with peptides derived from the B19 NS1 protein sequence (Klenerman et al. 2002).

Ongoing or recent B19 virus infection has also been linked to some forms of idiopathic thrombocytopenic purpura, myocarditis, acute fulminant liver failure accompanied by aplastic crisis, neuropathy, complex regional pain syndrome, neuralgic amyotrophy, and virus-associated hemophagocytic syndrome, although a causal etiology for B19 in these syndromes has not been firmly established. Viral entry and early gene expression can occur in some tissues, such as hepatocytes, that are not susceptible to productive infection. In such cases NS1-mediated apoptosis can sometimes be demonstrated in vitro, and provides a credible biological basis for the clinical observations (Poole et al. 2004). These possible disease associations are discussed in more detail elsewhere (Young and Brown 2004), although the full spectrum of virus-induced disease has still to be unraveled.

The major risk for serious pathologic outcome in normal individuals arises from exposure to B19 virus during pregnancy. A clear connection has been established between B19 virus infection, especially during the second trimester, and the development of non-immune hydrops fetalis, a potentially lethal condition for the fetus that occurs in approximately one of 3 000 pregnancies, and of which 10–20 percent are now believed to be due to B19 virus infection. About 50 percent of pregnant women lack circulating anti-B19 IgG, and thus are potentially susceptible to primary infection, and prospective studies suggest that the risk of transplacental transmission for infected women is about 30 percent, with a 5–9 percent risk of fetal loss. Thus most B19 virus infections during pregnancy do not lead to fetal loss. However, the virus replicates productively in dividing cells of the erythroid lineage, so that transplacental infection of the fetus during the second trimester, in particular, leads to destruction of the rapidly proliferating erythroblasts that are present in erythroid sinuses in the liver at this stage of fetal development. In hydrops cases, fetal infection results in severe anemia, high-output cardiac failure, fluid accumulation and death, although intrauterine transfusion of packed red cells can substantially lower the mortality rate. Fetal B19 virus infection may also cause fetal or congenital anemia, abortion, or stillbirth, although the risk of these occurring as a consequence of a primary B19 virus infection is apparently quite low. Surprisingly, this clear association between fetal pathology and B19 virus has not led, to date, to demand for a protective vaccine. Non-infectious empty recombinant B19 particles have been found to be immunogenic in normal volunteers, and might prevent fetal hydrops if 'at risk,' seronegative women were immunized early in pregnancy. Vaccine-mediated expression of the highly-immunogenic VP1-specific polypeptide, which carries phospholipase A2 activity on the outside of recombinant particles, might possibly be of concern, although such sequences could presumably be mutated or eliminated from a vaccine construct with relative ease.

Since B19 pathogenesis results predominantly from the destruction of rapidly proliferating eythroid precursors, in individuals with genetic defects that result in a shortened erythrocyte half-life and an expanded erythroblast population, anemia develops rapidly, reaches exceptionally high levels, and can require intervention. Thus transient aplastic crisis has been observed as an inevitable consequence of B19 virus infection in patients with single gene defects such as sickle cell anemia, α- or β-thalassemia, hereditary spherocytosis, stomatocytosis, or elliptocytosis, deficiencies in glucose-6-phosphate dehydrogenase, pyruvate kinase, or pyrimidine-5-nucleotidase, as well as in other hematopoietically compromised conditions, such as cold and heat antibody-mediated or chronic autoimmune hemolytic anemia, paroxysmal nocturnal hemoglobinuria, or malarial infection (Heegaard and Brown 2002). The aplastic crisis can be resolved by medical intervention, such as red cell transfusion. Otherwise it will run its course until the humoral immune response clears the virus and erythropoiesis is restored. This type of crisis only occurs once in the life of a patient and, although self-limiting, can cause severe, life-threatening anemia that can precipitate congestive heart failure, cerebrovascular events, and acute splenic sequestration (Smith-Whitley et al. 2004).

Finally, failure to produce neutralizing antibodies to B19 virus can occur in a variety of immunodeficiency states with congenital, iatrogenic, or infectious causes. Typical EI rashes do not develop in such patients, but there are high titers of circulating virus, and its persistence can lead to pure red cell aplasia, with depletion of serum reticulocytes and erythroid precursors from the bone marrow. Pure red cell aplasia of this type has been seen in Nezelof syndrome, an inherited combined immunodeficiency disorder, in patients being treated with cytotoxic chemotherapy or immunosuppressive

drugs, often after organ transplantation, and in patients infected with human immunodeficiency virus (Heegaard and Brown 2002). Immunosuppression can sometimes reactivate latent virus infections, although which tissues serve as the virus reservoir is not currently known. Persistent infection usually responds well to parenteral administration of commercial immune globulin, which invariably contains anti-B19 antibodies because of their prevalence in the donor population, but such intervention commonly leads to the rashes and arthropathy seen in acute infection. More detailed accounts of the clinical outcomes of B19 virus infection are to be found in recent reviews (Heegaard and Brown 2002; Young and Brown 2004).

Genomic polymorphism in B19

The DNA sequence of B19 was first determined from a bacterially cloned copy of a viral genome, B19-Au, isolated in Georgia, USA, from the serum of a child with homozygous sickle cell disease undergoing transient aplastic crisis (Shade et al. 1986), and this has become the reference sequence. Since that time a large number of isolates have been sequenced, or partially sequenced throughout the world, and, until 1999, all genomes appeared highly conserved. In general, they showed less than 2 percent nucleotide divergence, spread throughout the entire virus although certain regions, such as the *NS* gene, were particularly conserved. Minor antigenic and genetic differences between isolates were sometimes observed, manifest as differences in monoclonal antibody specificity, restriction fragment polymorphism, or single-stranded conformational polymorphism, but these did not appear to correlate with differences in the tissue specificity of the virus or its disease spectra. However, B19 diagnosis commonly relies on the identification of viral DNA by PCR, and this test inevitably favors the detection of conserved genomes.

Accordingly, in 1999, the blood of a French child suffering from transient aplastic anemia was found to contain a new type of virus, called V9, which eluded detection by the then-current PCR technique (Nguyen et al. 1999). At the nucleotide level, the isolate was more than 11 percent divergent from published B19 sequences (Heegaard et al. 2001), although certain regions were relatively conserved and allowed the design of new PCR assays using primers that would detect both V9 and B19 DNA. An extensive prospective study conducted in France between 1999 and 2001 subsequently revealed that V9-related viruses circulate beside B19 viruses at a significant frequency (11.4 percent) in the French population. Such genomes were surprisingly absent from large groups of North American and Danish samples analyzed at that time, suggesting that such variants might be epidemiologically linked to a particular geographic area, in this case France. An extraordinary aspect of V9 is that, despite its DNA sequence divergence, it is 96–97 percent homologous to B19-Au at the protein level. Thus, the great majority of its nucleotide changes are silent mutations that do not affect the amino acid sequence of the viral proteins, which therefore react with most anti-B19 antibodies (Heegaard et al. 2001). Another new variant, designated A6, has since been isolated in America, which is 88 percent similar to B19 virus and 92 percent similar to V9 at the nucleotide level (Nguyen et al. 2002), and an essentially similar mutant was detected in Finland, where it was called K71 or Lali (Hokynar et al. 2002). At present there is no established explanation for the emergence of these hypermutated variants, however, relationships between specific genotypic variants and specific disease states have been looked for, but not found.

In vitro cellular correlates of B19 pathology

Parvovirus B19 binds to the blood group P antigen, a neutral glycolipid called globoside that is expressed predominantly on human erythrocytes and erythroid progenitors. This functions as a productive cell surface receptor for B19, consistent with its observed tropism (Brown et al. 1993). In the human population, about one in 200 000 individuals genetically lack P antigen, and show no serologic evidence of past B19 virus infection. Bone marrow cultures derived from these individuals fail to support the growth of B19 virus in vitro, even at a high multiplicity of infection (Brown et al. 1994), indicating that the presence of P antigen is necessary for infection. However, it may not be sufficient. Thus, while P antigen is present on a number of cells such as fetal myocytes, megakaryocytes, endothelial cells, as well as the hepatocytes discussed previously, these cell types do not appear to be permissive for B19 replication, suggesting that additional factors may be required for complete replication. Some studies indicate that entry coreceptors, such as β-integrins, may be required, but intracellular permissivity factors are also indicated, since in culture many nonpermissive cells appear to support early, but not late, transcription (Liu et al. 1992). This abrogates capsid protein synthesis, but still allows expression of the potentially cytotoxic NS1 molecule of the virus (Moffatt et al. 1998; Ozawa et al. 1988), which in vitro can be shown to induce caspase-mediated cell death (Poole et al. 2004).

Development of a cell culture system that supports productive B19 replication has been extremely difficult, and at present, there is no animal model for the virus. In primary human bone marrow cultures, B19 virus inhibits colony formation by late erythroid progenitors and the more primitive burst-forming erythroid progenitors, while leaving differentiation of myeloid precursors unperturbed (Mortimer et al. 1983a). It appears from both in vivo and in vitro studies, that the pluripotent erythrogenic stem cell is resistant to virus infection,

while in the presence of erythropoietin, B19 virus will grow in later progenitors derived from human bone marrow, fetal liver, and peripheral or umbilical blood. Infection of such erythroid precursors in vitro with B19 leads to a cytopathic effect characterized by the presence of giant pronormoblasts, which appear to be equivalent to the 'lantern cells' that can be observed in vivo in the bone marrow of patients undergoing B19-associated transient aplastic crisis. These cells exhibit large eosinophilic nuclear inclusion bodies and cytoplasmic vacuolization, and electron microscopy reveals nuclei with marginated chromatin that contains virus particles. While such explanted cells cannot be maintained in long-term culture, B19 virus has also been shown to propagate in a small number of permanent cell lines of megakaryoblastoid and erythroleukemic origin, although net synthesis of virus has not been achieved in these systems (Heegaard and Brown 2002).

An infectious plasmid clone of B19 has recently been constructed, and shown to give rise to detectable levels of infectious progeny when transfected into the erythropoietin-responsive cell line UT7/Epo-S1 in vitro (Zhi et al. 2004). It seems likely that mutational analysis of this clone should allow rapid progress in understanding the molecular biology of this ubiquitous human fellow traveler, which will hopefully also inform our understanding of B19 pathology. At the core of this pathology is the destruction of rapidly proliferating erythroid precursors, which derives from the narrow tissue tropism of the virus and its absolute dependence on the presence of rapidly dividing cell populations. Thus, despite the organizational differences between individual genera in the *Parvovirinae*, the pathogenic potential of B19 follows the same underlying biological principles that determine disease throughout the autonomously replicating viruses from this family.

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Adenoviruses

WILLIE RUSSELL

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The term adenoviruses was coined in 1956 (Enders et al. 1956) to describe infectious agents that had been isolated from human adenoids (Rowe et al. 1953) and were similar to those associated with outbreaks of respiratory disease in military recruits (Hilleman and Werner 1954). The virus family was initially characterized in terms of crossreactivity in serological tests and it soon became apparent that adenoviruses sharing this common antigen were to be found in a wide variety of species. Fundamental studies on adenoviruses and their interactions with their host cells were greatly stimulated by the discovery by Trentin and his colleagues (Trentin et al. 1962) that some human adenovirus serotypes could induce tumors in hamsters. However, later studies could not demonstrate any association with human tumors. This finding, coupled with the observations that adenoviruses did not appear to be associated with any serious morbidity and that they could readily be grown in a wide variety of cell lines, led to their being used extensively as model laboratory systems to study the molecular basis of virus infection (for a review, see Russell 2000).

PROPERTIES OF THE VIRUSES

Classification

Adenoviruses have been characterized primarily on the basis of a common genus-specific antigen and a very characteristic morphology. Serotypes are classified by their ability to induce specific neutralizing antibodies. A serotype can therefore be defined as one that either exhibits no cross-reaction in neutralization with other animal typing antisera or shows a homologous:heterologous neutralization ratio of >16 (in both directions). For ratios of 8 or 16, a serotype assignment is made on the basis of other serological, biophysical, or biochemical differences.

Four families are now recognized using phylogenetic calculations based on distance matrix or parsimony analysis coupled with protein or DNA alignment on any suitable gene. These are the *Mastadenoviridae* isolated from mammals (bovine, canine, caprine, equine, human, murine, ovine, porcine, simian); the *Aviadenoviridae* isolated from birds (duck, fowl, goose, pheasant, turkey); the *Atadenoviridae* isolated from some bovines, birds, and snakes, and the *Siadenoviridae* isolated from frogs, fish, and turkeys, giving a total of at least 170 different viruses. However, the most intensively studied adenoviruses are the 51 serotypes isolated from humans (Russell and Benko 1998; Benko et al. 2000).

Morphology and structure

The virions are non-enveloped, 80–110 nm in diameter and their capsids have icosahedral symmetry (Figures 1 and 2). They have 240 nonvertex capsomeres (hexons) 8–10 nm in diameter and 12 vertex capsomeres (pentons). The pentons consist of penton bases and attached knobbed fibers, each in close association with a ring of five hexons; these are termed the peripentonal hexons. Each of the penton bases has one (or occasionally two) fibers that protrude 9–30 mm from the capsid. Several other structural polypeptides (IIIa, VI, VIII, IX) are associated with the capsid and serve to cement the hexons and to link the capsid to the core (Stewart et al. 1993). The latter consists of a double-stranded DNA genome with a covalently linked terminal

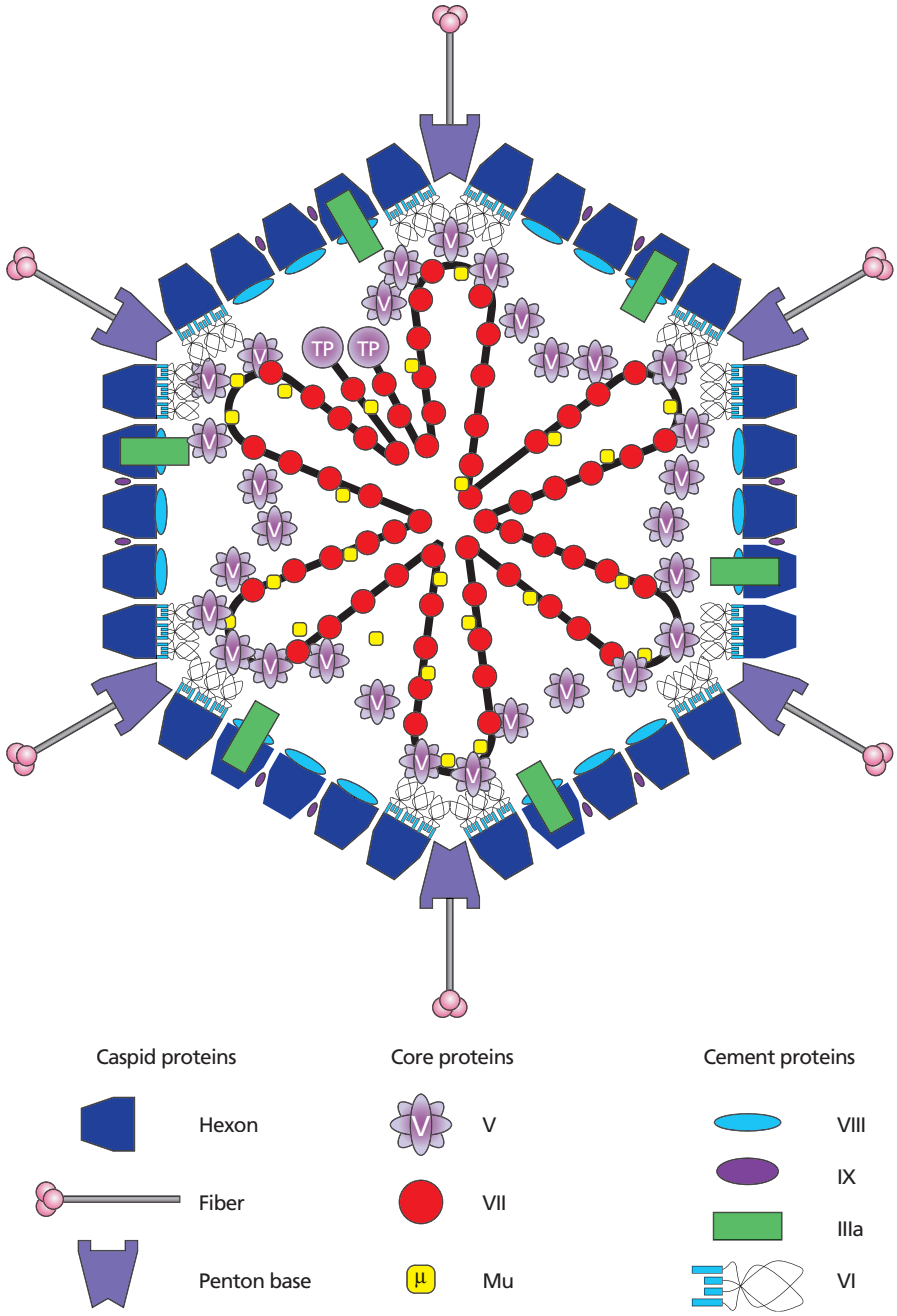

Figure 1 *Structure of adenovirus. The locations of the capsid and cement components are reasonably well defined. By contrast, the dispositions of the core components are largely conjectural. (Redrawn from Figure 1 of Russell (2000), with permission of the Society for General Microbiology)*

protein (TP) in tight association with three other polypeptides, V, VII, and mu (Figure 1). The structure of this nucleoprotein core has not been established and the core polypeptides are shown in hypothetical locations in Figure 1. The major capsid protein, the hexon, has been crystallized and its three-dimensional structure shown to be based on a composite of three identical and interlocking polypeptide chains that form a triangular tower superimposed on a pseudohexagonal base (Stewart and Burnett 1995). The fiber knob has also been crystallized and a structure obtained (van Raaij 1999). The virus is stable between pH 6.0 and 9.5, is resistant to chloroform, ether, and fluorocarbons, and on heating to 56°C disintegrates, releasing core structures (Russell et al. 1967).

On examination by electron microscopy, some preparations of adenoviruses are seen to be contaminated by small round adeno-associated viruses (Figure 2). These ssDNA parvoviruses belong to the family *Dependoviridae* (see Parvoviruses) and require adenoviruses (or herpesviruses) for their replication.

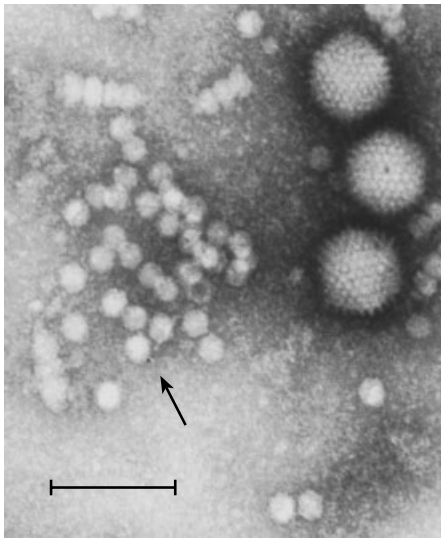

Figure 2 *Three adenovirus virions with dependoviruses (arrowed), negatively stained with phosphotungstic acid. Scale bar, 100 nm.*

Genome structure and function

The adenovirus virion contains a single linear dsDNA genome of approximately 20–25 kb. A virus-coded terminal protein is covalently linked to the 5′ end of each DNA strand. The genome of the prototype human adenovirus type 2 has 35 937 bp and contains inverted terminal repeats (ITR) of 103 bp. A large number of other adenoviruses have been completely sequenced and all contain ITRs, but of variable length. Viruses replicate in the nuclei of a wide variety of cells of the species that they normally infect, giving a characteristic pattern of nuclear staining. Some adenoviruses can infect cells of other species; such infections are often abortive and, under suitable conditions, may give rise to morphological transformation and thereby provide model systems for oncogenesis (see below).

Entry of virus is by attachment of the fiber via its knob (Figure 1) to cell receptors, the major one being termed coxsackie adenovirus receptor (CAR) (Bergelson et al. 1997). Some human serotypes can also utilize sialoglycoproteins as receptors (Arnberg et al. 2002). It is interesting that human adenovirus 41 has two fibers and only one recognizes the CAR. Since this virus readily infects cells of the gastrointestinal tract and produces diarrhea, it seems likely that one of the fibers may adsorb to enterocytes. Internalization of the virus particles with the penton bases interacting with cellular integrins via clathrin-coated vesicles is followed by transfer into endosomes. Thereafter, the virus capsid is partially disrupted by a virus-coded protease (Greber et al. 1996) and then transported to the nuclear pores where the virus DNA, probably in close association with the core components, is delivered into the nucleus and becomes attached to the nuclear matrix via the viral terminal protein (Fredman and Engler 1993). A series of nuclear and nucleolar perturbations then follow in which transcription and virus DNA replication are prominent (see Russell and Matthews 2003)

The adenovirus genome becomes available for transcription using cellular transcription factors that are abundant in the nuclear matrix. This process is well regulated and involves the synthesis of four major classes of ‘early’ transcripts (E1–E4), minor ‘intermediate’ transcripts, and late transcripts (L1–L5). The last are initiated at the ‘major late promoter’ and, allied to a tripartite leader formed by a series of splicing events, drive the synthesis of most of the structural proteins; the ‘early’ transcripts are also subject to complex splicing mechanisms. The overall nature of transcriptional regulation has not been completely elucidated but it has been suggested that as well as splicing control, the topology of the virus genome can be affected by the core proteins limiting template availability for transcription. After synthesis of the early transcripts and synthesis of ‘early’ proteins [e.g. virus DNA-binding protein (DBP)], further alterations in template topology and availability could permit virus DNA replication, thereby creating new progeny templates for late transcription (see Figure 3). However, all these transcriptional events are subject to other regulatory controls of a quite complex nature (see Russell 2000).

Virus DNA replication

Adenovirus DNA synthesis is initiated at either end of the linear double-stranded DNA and three ‘early’ viral proteins are required together with at least three cellular proteins. The initial event involves the interaction of one of the early proteins, the precursor of the terminal protein (pTP), with specific terminal DNA sequences followed by association with another early protein (polymerase) and then the formation of an ester bond between the phosphate of dCMP (C being the first nucleotide in the DNA sequence) and a serine residue in pTP. This can then function as a primer to extend a progeny DNA strand using one strand of the virus DNA as template; this process utilizes the viral polymerase and the cooperation of three other proteins, two of them cellular. The latter (NF1 and NF3) are also transcription factors and bind to specific sequences in the viral DNA, presumably facilitating the formation of the initiation complex. The other early virus protein, the DBP, also plays a role in virus DNA initiation as well as facilitating strand separation during elongation and binding to the progeny DNA strands. A third cellular protein (NF2) seems to be required for complete elongation of the progeny strand and appears to be equivalent to cellular topoisomerase I. Synthesis of the second strands takes place after that of the first strands and the formation of

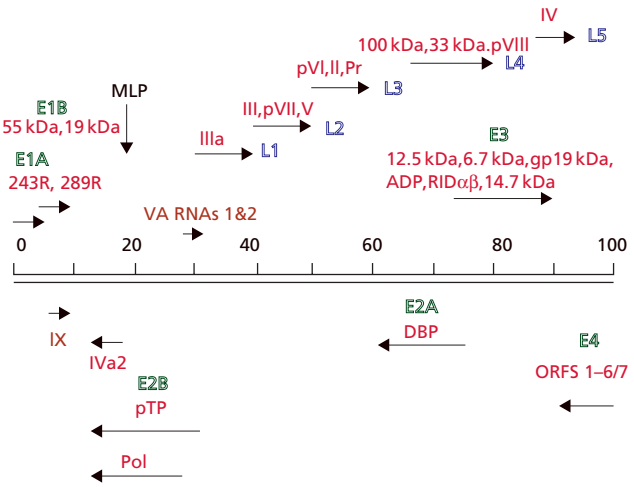

Figure 3 *Transcription of the adenovirus genome. The early (E) transcripts are outlined in green. The components of each cassette are named in a conventional manner, e.g. some are merely numbered as open reading frames (orfs) and others defined by their molecular weights or by the number of amino acids. The late (L) transcripts are outlined in blue. MLP refers to the major late promoter. The transcripts IX, IVa2 and the VA RNAs are transcribed late but independently of the MLP. The VA RNAs are not translated and play a significant role in combating cellular defense mechanisms. Arrows indicate the direction of transcription. (From Figure 2 of Russell (2000), with permission from the Society for General Microbiology.)*

a 'panhandle' structure formed by the interacting ITRs, thus allowing replication by a similar mechanism – for a recent review see Liu et al. (2003).

Polypeptides and maturation of the virion

Translation of the adenovirus transcripts begins quite early in infection and continues uninterrupted in concert with virus assembly for up to about 40 hours when both cellular and virus protein synthesis are shut off. In tissue culture systems this process is very inefficient and normally a vast excess of virus capsid proteins are made.

A number of adenovirus genomes have been completely sequenced and the dispositions of the polypeptide genes have been mapped. While there are some differences in the number and position of the polypeptides on the genome across the adenovirus family, on the whole there is good conservation of genome structure. It is also apparent that, by virtue of its splicing mechanisms, the genome makes efficient use of template information and synthesizes about 40 different polypeptides, 13 of these being incorporated into the mature virus particle (see Figure 1). Three of the nonstructural proteins synthesized early in infection are involved in virus DNA replication and are derived from the E2 transcription region. The remainder of these 'early' nonstructural proteins seem to function as modulators of the host cell, so that virus transcription and replication are favored, and to counteract the host immune response. It should be noted that not all the 'late' genes are structural: a major product of ca. 100 kDa seems to function as a chaperone for other structural proteins but is not itself encapsidated. Two other gene products, polypeptides IX and IVa2, are regulated independently of the others and, whereas IX is clearly a structural component, the evidence that IVa2 is structural is not very strong.

The maturation of adenovirus is intimately linked to the functioning of the adenovirus gene-coded 23 kDa protease (Webster et al. 1989). This enzyme is synthesized late in infection and performs cleavages, leading to maturation, on six virus structural proteins: IIIa, pVI, pVII, pVIII, pTP, and protein p-mu. The enzyme is a cysteine protease of an unusual subset and recognizes consensus sequences (M,L,I)XGX↓G or (M,L,I)XGG↓X (Webster et al. 1989). It also has the remarkable characteristic of requiring for activation a disulfide-bonded dimer of an 11-residue peptide derived from the C terminus of polypeptide pVI (Webster et al. 1993). Investigations have also shown that, in an in vitro assay, pVI will bind to the principal capsid component, the hexon, but binding is very significantly enhanced whenever pVI is cleaved by the protease (Matthews and Russell 1995). This suggests that a primary maturation event is the interaction of adenovirus protease with pVI in a 'young' virion, i.e. that formed by the introduction of virus DNA and associated proteins into the precapsid. The protease is then activated, allowing cleavage of pVI to VI accompanied by tight binding to hexons, probably also with conformational changes leading to the proteolytic cascade that marks virion maturation. The encapsidation process is also governed by the presence in the virus DNA of a packaging sequence at the conventional left end and seems to involve the binding of the virus-coded IVa2 protein (Zhang et al. 2001).

These events take place in the cell nucleus and many thousands of virions can be assembled, often in

paracrystalline arrays. In some cells the assembly process seems to be inefficient, so that many structural proteins (mostly capsomers) are made in excess and these can also form paracrystalline structures. Release of the virus results from permeabilization of nuclear membranes largely mediated by one of the nonstructural virus proteins (Tollefson et al. 1996). Abortive infections by adenovirus can occur whereby some of the early genes are expressed and, given the appropriate circumstances, can lead to morphological transformation and tumor formation in some animal model systems. There are also indications that adenoviruses can persist in vivo, engendering life-long immunity – see Lukashok and Horwitz (1998).

Antigens

As noted above, adenoviruses can be characterized on the basis of their crossreacting (or 'group') antigens and further subdivided into serotypes based on neutralization. Most adenoviruses can hemagglutinate rat or monkey cells and this characteristic has been used to subgroup the human adenoviruses and to confirm serospecificity (Table 1). These subgroups also correlate to some extent with clinical patterns (see Table 2) and with the ability to induce tumors in hamsters. The utility of these serological assays has undoubtedly been enhanced by the relatively large amount of antigens (mostly associated with the capsomers) released during infection.

The serotype-specific antigens are located on the surface of the virions and are responsible for the formation of the neutralizing antibodies. Both hexons and fibers can induce neutralizing antibodies, whereas pentons and fibers appear to function in hemagglutination. The hexon serotype-specific antigens can be assigned to the tower regions of the structure whereas the genus-specific antigens seem to reside on the basal surfaces of the hexons at the region where they interact with each other and with polypeptide VI.

A range of hexon and fiber monoclonal antibodies has been prepared and has been instrumental in defining type and group specificities as well as antigenic relationships both within and between subgroups (Watson et al. 1988).

Classification of adenoviruses by serological procedures was later supplemented by restriction enzyme analysis of virus DNA extracted from infected cells. By using a defined and limited range of restriction enzymes, patterns characteristic of adenovirus serotypes can be obtained; furthermore, this approach allows further analysis of serotypes into defined 'genotypes' – see Kajon et al. (1996). However, with the increasing availability of sequence data it has become possible to apply phylogenetic analyses to adenovirus classification and this has now become the method of choice and can even be applied in the absence of isolation of the infectious virus (e.g. Benko et al. 2002).

CLINICAL AND PATHOLOGICAL ASPECTS

Adenoviruses are widespread in many species and can be isolated from both sick and healthy individuals. Antibodies can be detected in almost all humans, reflecting infection early in childhood and possibly lifelong persistence in adenoid and lung tissues. Mortality and morbidity associated with infection are relatively low and undoubtedly ensure that the virus retains its biological niche. Nevertheless, pneumonia and gastrointestinal disease can be serious especially when immunological status is impaired – as is the case for patients with the acquired immunodeficiency syndrome (AIDS) and those undergoing transplantation procedures.

Clinical manifestations

Adenovirus infections are mostly asymptomatic but may be associated with diseases of the respiratory, ocular,

Table 1 *Subgrouping of human adenoviruses: hemagglutination and oncogenicity*

Subgroup[a]	Serotypes	Species giving hemagglutination titers	Oncogenicity in hamsters
A	12, 18, 31	Rat (incomplete)	High
B	3, 7, 11, 14, 16, 21, 34, 35	Monkey	Weak
C	1, 2, 5, 6	Rat (incomplete)	No
D	8, 9, 37	Rat, mouse, human, guinea pig, dog	No
	10, 19, 26, 27, 36, 38, 39	Rat, mouse, human	
	13, 43	Rat, mouse, human, monkey	
	15, 22, 23, 30, 44–47	Rat, mouse, monkey	
	17, 24, 32, 33, 42	Rat, mouse	
	20, 25, 28, 29	Rat, monkey	
E	4	Rat (incomplete)	No
F	40, 41	Rat (atypical)	No

a) The subgrouping is based on a variety of criteria: differential hemagglutination, fiber length, oncogenic potential in rodents, percentage DNA homology, G+C content of viral DNAs, and the ability to recombine with other members of the subgroup (see Horwitz 1990 for a more detailed explanation). Table derived from information presented by Hierholzer (1992) and Horwitz (1990).

Table 2 *Subgrouping of human adenoviruses: association with disease*

Syndrome	A	B	C	D	E	F
Upper respiratory illness	. . .	All	. . .	. . .	. . .	. . .
Lower respiratory illness	. . .	3, 7, 21	. . .	. . .	4	. . .
Pertussis syndrome	. . .	. . .	5	. . .	. . .	. . .
Acute respiratory disease	. . .	7, 21	. . .	. . .	4	. . .
Pharyngoconjunctival fever	. . .	3, 7	. . .	. . .	4	. . .
Epidemic keratoconjunctivitis	. . .	. . .	. . .	8, 19, 37	. . .	. . .
Acute hemorrhagic conjunctivitis	. . .	11	. . .	. . .	. . .	. . .
Acute hemorrhagic cystitis	. . .	7, 11, 21, 35	. . .	. . .	. . .	. . .
Immunocompromised host disease	31	All	All	29, 30, 37, 43, 45	. . .	. . .
Infant gastroenteritis[a]	31	. . .	2	. . .	. . .	40, 41
Central nervous system disease	. . .	3, 7	. . .	. . .	. . .	. . .
Sexually transmitted disease[b]	. . .	. . .	2	19, 37	. . .	. . .

a) Gastroenteritis is due predominantly to types 40 and 41 and to a lesser extent to types 2 and 31.
b) Penile and labial ulcers and urethritis. Table derived mainly from information presented by Hierholzer (1992) and Horwitz (1990).

and gastrointestinal systems (Table 2). Volunteers have been infected by rubbing virus into their conjunctivae or by administration of aerosols but, in general, it has been difficult to demonstrate unequivocally that the viruses cause disease, because they can be found in apparently normal tonsils and adenoids. Infections with human adenovirus serotypes 1, 2, 3, 5, and 6 usually take place in early childhood, giving mild respiratory infections. It has been suggested that these infections may well induce sufficient immunological defenses to modulate the progress of subsequent infections by related serotypes. The importance of adenoviruses in pediatric respiratory tract infection is not as great as that of respiratory syncytial and parainfluenza viruses, and it has been estimated that only about 5–10 percent of pediatric respiratory infection can be attributed to them. Nevertheless, acute respiratory infections occur in children and in immunocompromised children disseminated disease (with some mortality) has been reported, particularly in association with types 3 and 7 and even with type 5 (Munoz et al. 1998). Adenoviruses have also been reported to account for 10 percent of pneumonia in children and are a particular threat in developing countries where there is malnutrition and exposure to measles (for a review see Wadell 2000). Respiratory infections may also be important in military recruits, of whom 30–80 percent may become infected, usually with types 4, 7, 14, or 21; up to 20 percent may require hospitalization. This morbidity had such an effect on training schedules that the US government invested heavily in the development of appropriate vaccines. Explosive outbreaks have also been reported in boarding schools and other institutions where young people live in close association.

Pharyngoconjunctival fever is another well-defined clinical syndrome that is characterized by conjunctivitis, fever, pharyngitis, and enlargement of the adenoids. This is usually associated with types 3, 4, and 7 and can be disseminated via poorly chlorinated swimming pools. Epidemic keratoconjunctivitis ('shipyard eye') was first associated with adenovirus type 8, but other serotypes (e.g. 19 and 37) have also been incriminated in this syndrome. Adenovirus type 11 has been associated with hemorrhagic cystitis in school-age boys and cases have been reported after renal and bone marrow transplantation. Adenoviruses are often a serious problem in patients undergoing immunosuppressive treatment and in AIDS patients. Indeed, a range of new serotypes was first isolated from patients with AIDS (e.g. the latest serotypes 50 and 51 – see Schnurr and Dondero 1993; De Jong et al. 1999), suggesting that persistent long-term infection by adenoviruses kept under control by immune surveillance may be the usual situation.

The role of adenoviruses in gastrointestinal disease is, however, much more important; infection of the colon and the gut can cause severe diarrhea and acute gastroenteritis, especially in developing countries. Serotypes 40 and 41 seem to be mainly responsible for these outbreaks and the viruses can be detected in large numbers in the feces of infected children. Up to 15 percent of children hospitalized with acute gastroenteritis can be attributed to these enteric adenoviruses, second only to rotaviruses as the cause of infantile viral diarrhea. It is interesting that fecal shedding of adenoviruses appears to be a characteristic of all serotypes, but types 40 and 41 are particularly associated with enteric disease. The tropisms of these serotypes differ from those of others in that they fail to infect standard tissue cell lines such as HeLa and HEp-2 and require primary human kidney cells or the adenovirus-transformed human cell line 293 (Graham et al. 1977).

Adenovirus infections are common in many animal species. As in humans, they appear to be mostly asymptomatic and there is therefore a paucity of information on the mode and mechanics of virus transmission. There is some evidence that sometimes a virus that is asymptomatic in its natural host can produce disease in other

species. Thus epidemics of reduced laying and production of soft shell or shell-less eggs in some chicken flocks have been attributed to a duck adenovirus (EDS'76). As in humans, most of the animal adenoviruses can be isolated from the tissues of healthy animals and there is very little evidence that they cause disease in wild populations. Nevertheless, two serotypes of canine adenovirus (CAdV) do seem to cause different patterns of disease: CAdV-1 is responsible for infectious canine hepatitis and, along with CAdV-2, is among the viruses causing the 'kennel cough' syndrome. Epizootic infections with CAdV-1 in foxes, bears, wolves, coyotes, and skunks have also been reported. CAdV-2, on the other hand, seems to be confined to infections of the canine respiratory tract (reviewed by Russell and Benko 1998).

Epidemiology

Adenovirus infections are widespread and appear to be transmitted primarily by the fecal–oral rather than the respiratory route. The latter may, however, be more important for the lower numbered human serotypes. Seasonal variation of adenovirus epidemics is well recognized. Most outbreaks of pharyngoconjunctival fever in school-age children occur in summer; by contrast, epidemics in military recruits appear almost exclusively in the winter.

Infections with adenoviruses occur early in life: by 5 years of age almost all children have been infected by at least one, generally a low-numbered serotype. The epidemiology of individual serotypes has been elucidated in more detail by using restriction enzyme analysis to distinguish between isolates of type-specific viruses. In this way, the provenance of particular genotypes within these serotypes (e.g. Ad8, Ad19, Ad37) can be traced in the population. Such studies suggest that some genotypes may be associated with increased morbidities. They also indicate that multiple persistent infections probably provide opportunities for recombinational events (for a review see Wadell 2000).

Pathogenesis and pathology

In susceptible cells, adenoviruses cause early rounding and aggregation, followed by the appearance of characteristic basophilic nuclear inclusions. In organ cultures of human embryonic nasal mucosa and trachea, they infect and destroy the ciliated epithelium and it is likely that the same process occurs in vivo. Although organs outside the respiratory tract may be affected (eg liver and kidney) during severe adenovirus infection, replication rarely occurs unless the patient is immunologically compromised.

Considerable progress has been made in our understanding of adenovirus pathogenesis by studies in cotton rats (*Sigmodon hispidus*). These animals are susceptible to intranasal infection with human adenovirus type 5 (and other members of subgroup C), developing pulmonary histopathology very similar to that in human cases. The virus replicates in the bronchiolar epithelial cells, but in situ hybridization also shows early gene expression in macrophages/monocytes and in both alveoli and hilar lymph nodes. It is interesting that only early gene expression is needed to produce the pathology, of which there is an 'early' and a 'late' phase (for a review see Ginsberg 1999).

It is now well established that at the molecular level host pathology is largely governed by the success or otherwise of the ability of the invading virus to evade the host protective responses. In general, host cells have a range of strategies to combat infection: these can be considered as innate and adaptive. In the first category can be included the natural antimicrobial peptides (defensins) released by epithelial cells. Some tissues on receiving the appropriate signal (e.g. when a virus particle interacts with the plasma membrane) are able to release multiple chemokines which in turn recruit neutrophils and invoke an inflammatory response. One key factor in regulating the innate response is the activation of the cellular transcription factor NFκB (Dixit and Mak 2002).

Interferons are an important component of the innate response but adenoviruses are generally refractive to interferons mainly because their early gene products can down-regulate components of the cellular pathways that are activated by interferons. Another mechanism that can be utilized by cells under attack is to commit suicide by switching on their apoptosis circuits. A key regulator of these events is the tumor suppressor p53 which controls both cell cycle arrest and apoptosis (Ljungman 2000). Once again, early virus gene products can interfere with these pathways in a variety of ways (for a review see Russell 2000).

Immune response

The response to adenovirus infection by the immune system is efficient and well orchestrated and undoubtedly accounts for the generally low levels of morbidity. Cell-mediated responses are mounted but the virus can counteract most of these by means of products expressed from the early genes. For example, one of these is an abundant transmembrane protein which binds class I major histocompatibility complex (MHC) antigens and retains the complex in the endoplasmic reticulum. Since surface expression of class I antigens complexed with viral peptides is required for recognition and lysis by virus-specific cytotoxic T cells (CTL), this strategy effectively thwarts the host CTL response. Although CTLs are one means by which the host eliminates virus-infected cells, there are others, involving secretion of cytokines such as tumor necrosis factor (TNF) and other early virus gene products are able to interfere with these pathways.

The proliferative T-cell responses in humans are mediated by $CD4^+$ T cells, and structural proteins appear to be important targets. In addition, proliferative responses to the uncommon adenovirus type 35 (Ad35) have been found in individuals without serological evidence of previous Ad35 infection. These findings suggest that $CD4^+$ cells recognize conserved antigens and that this may play a role in modulating infections with a range of serotypes. The mechanisms of the cellular and humoral immune responses have been of intense interest following the development of recombinant adenovirus constructs as vehicles for gene therapy. However, expression of the correct gene is quite often only transient and this seems to be associated with the development of an effective host immune response. For a review of the characteristics of early gene products and adenovirus vectors see Russell 2000.

Diagnosis

Human adenovirus infections are ideally diagnosed by isolation of virus from appropriate clinical samples in a variety of sensitive indicator cell cultures such as HeLa, HEp-2, KB, or A549. The use of the 293 line of adenovirus-transformed cells (Graham et al. 1977) also facilitates the isolation of the enteric adenoviruses 40 and 41. Rounding and aggregation of cells appear after incubation for 1–4 weeks. The culture fluids containing virus and soluble antigens can be used to characterize the virus further by neutralization and by hemagglutination inhibition assay (see Table 1).

More sensitive and rapid tests have been introduced over the last few years and have found a place in many diagnostic laboratories. They include, for example, enzyme immunoassays and immunoelectron microscopy. Characterization of serotypes by restriction enzyme mapping has also been of significant value (see above). However, the most sensitive technique for virus recognition is the polymerase chain reaction (PCR) using primers based on those base sequences that are common to the genus. A number of different primers have been utilized. This technique can be exquisitely sensitive and be used where cellular techniques have not been successful (e.g. in blood and urine samples) (Echavarria et al. 1998). PCR has also been utilized successfully for environmental detection using air filters (Echavarria et al. 2000).

Current developments are using 'real-time' PCR to obtain more semiquantitative data (Houng et al. 2002). The advent of automatic sequencing has added yet another dimension to diagnosis and should assist in more precise characterization of isolates.

Control

Vaccination has been used with some success to control epidemics of adenovirus infection in military recruits and in bovines. Both inactivated and live adenovirus vaccines have been used in military recruits but live vaccines proved more effective in reducing the incidence of disease. Initial development concentrated on type 4 serotype; unattenuated virus was fed to volunteers in enteric-coated capsules to establish an asymptomatic infection in the lower alimentary tract. The virus induced moderate amounts of neutralizing antibody and, more importantly, did not spread to susceptible contacts. The type 4 vaccines grown in human diploid cells have been given to several hundred thousand military recruits with no untoward side-effects and with a significant reduction in morbidity. A triple vaccine incorporating types 4, 7, and 21 was also successful (Takafuji et al. 1979). Vaccine production ceased in 1995 and by 1999 the supplies had been exhausted and, not surprisingly, adenovirus respiratory diseases are again becoming a problem in military recruits. Vaccines have not, in general, been considered for use in the general population since the cost of development, production, and control would be quite substantial and would appear not to be commensurate with the risk of clinically significant infection. However, this position may change with the increasing use of transplantation therapies.

There has been one report of the use of normal human immunoglobulin for combating disseminated adenovirus infection (Munoz et al. 1998) and also one report of the successful use of ribavirin (Arav-Boger et al. 2000) but, as yet, there have been no other signs of a substantiated effective antiviral chemotherapy.

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Papillomaviruses

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BACKGROUND

Papillomaviruses are small double-stranded DNA viruses that induce warts on cutaneous and mucosal surfaces in many vertebrates, including humans. The infectious nature of warts was shown unequivocally in 1907 by the Italian physician Ciuffo, who demonstrated person-to-person transmission of lesions by cell-free wart filtrates. Seminal aspects of the biology and, in particular, the oncogenic potential of this group of viruses were demonstrated by Richard Shope in the 1930s, when he showed that the cottontail rabbit papillomavirus (CRPV) was the etiologic agent in cutaneous papillomatosis in the rabbit. The use of the electron microscope as a research tool in biology in the 1950s revealed papillomaviruses to be the infectious agent for human warts, but although the viruses were known in 1964 to have a genome consisting of circular double-stranded DNA, biochemical characterization of their proteins was slow because no tissue culture system for virus growth was available. Virus particles had to be obtained from clinical lesions, and the amount of virus in warts was highly variable; thus virions were abundant in plantar warts but sparse in laryngeal and genital warts. These handicaps severely limited immunologic and virologic studies, and until the mid-1970s the general view was that there was only one human papillomavirus (HPV), and that tissue location rather than virus type dictated the morphology and behavior of the clinical lesions at a specific epithelial surface.

The advent of molecular cloning and recombinant DNA technologies in the 1970s resulted in a complete reversal of this view, revealing an astonishing plurality of both animal and human papillomaviruses. Papillomaviruses (PV), it emerged, were absolutely species specific, such that human PVs could only infect humans, rabbit PV's only rabbits, and so on. They were also exquisitely tissue trophic, and the different clinical lesions were distinct diseases caused by infection with specific groups of HPV types. It became clear that within a species the individual viruses showed a predilection for either skin or internal squamous mucosae, and that within the groups of skin or mucosal viruses they could be separated into high- or low-risk types, depending upon their oncogenic potential. This was shown dramatically in the genital tract. Genital warts were shown to be associated predominantly with HPV-6 and -11 (low-risk human papillomavirus (LR-HPV)) – viruses that were almost never detected in carcinomas, whereas HPV-16, -18, and their close relatives (high-risk human papillomavirus (HR-HPV)) were associated with intraepithelial lesions, particularly in the cervix, and were detected in more than 99 percent of cervical cancers. This association with premalignant and malignant disease in humans dramatically broadened the spectrum of HPV pathogenicity, revealing these viruses

to be not just the cause of cosmetic nuisances but also important human pathogens causing life-threatening diseases.

CLASSIFICATION

The papillomaviruses are widespread in nature, infecting a wide range of species (Stanley et al. 1997). Those viruses infecting ungulates are a distinctive group in that they induce fibropapillomas with a distinctive fibroproliferative component in addition to the epithelial wart. The best studied of this group is bovine papillomavirus type 1 (BPV-1), a virus that can infect rodent cells in vitro and induce morphologic transformation, providing a biological assay with which to analyze those viral functions necessary for the induction of cell proliferation. Papillomaviruses are classified according to the species of origin and the degree of relatedness of the viral genomes according to their sequence homology. The virus types are therefore genotypes, not serotypes. PVs have been divided into five clades or supergroups, designated A–E (Chan et al. 1995) (Figure 1). The association of specific HPV types and human cancers has focused attention very much on the human viruses. Initially HPV types were distinguished from each other on the basis of molecular hybridization in liquid medium: 50 percent or less homology with a known HPV type defined a new virus type. At the 1995 meeting of the International Papillomavirus Workshop it was agreed that classification be based on the *L1* gene, and that to be classified as a new type there should be 10 percent or more sequence dissimilarity in *L1* compared to all known types. To date, more than 80 HPV types have been fully sequenced, and in addition many more partially characterized types, based on

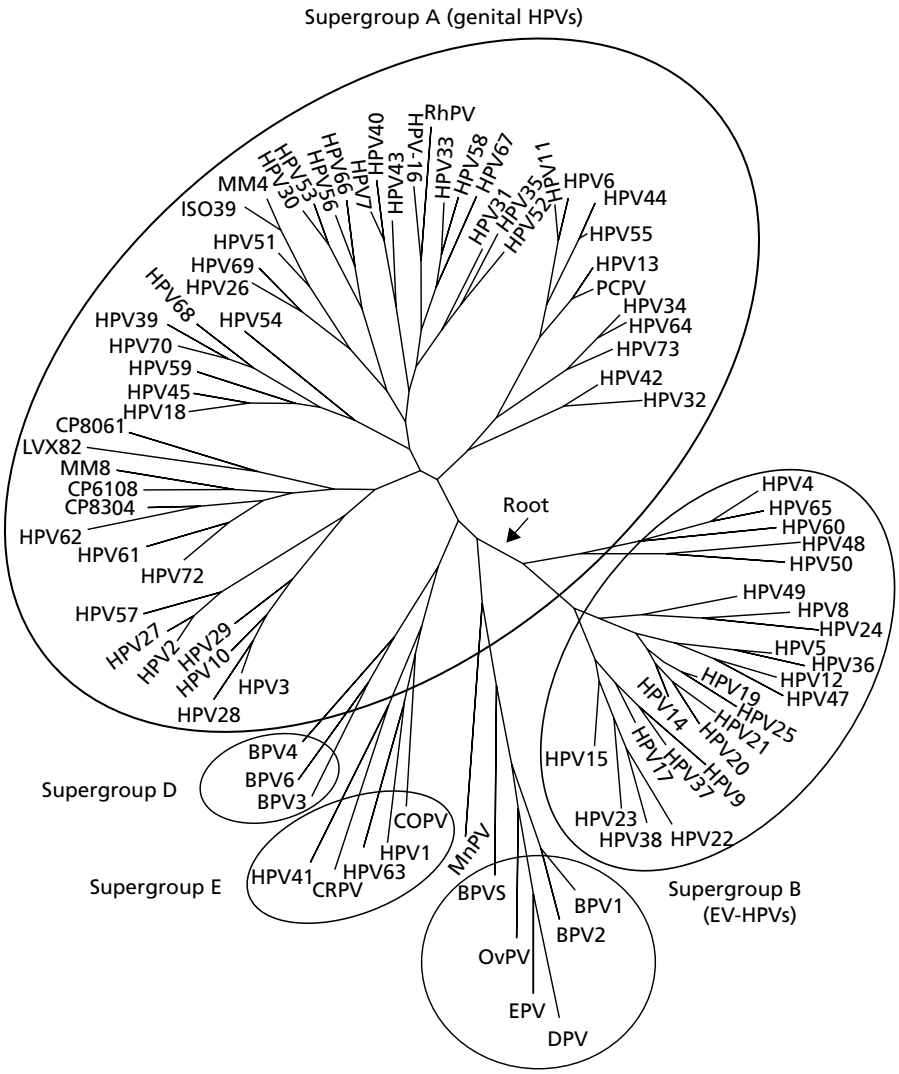

Figure 1 *Phylogenetic tree of animal and human papillomaviruses. The classification is based on nucleotide sequence comparisons using a 291-bp segment of L1. Redrawn from Chan et al. 1995, with permission.*

the *L1* open reading frame (ORF) only, have been identified (see the 'HPV sequence database' at www.stdgen.lanl.gov/stdgen/virus/hpv). Phylogenetic relationships between HPV types based on genome sequence variation indicate how these viruses have evolved and their relationship to each other. Interestingly, the clinical distinctions between skin and mucosal warts and benign and malignant lesions correlate with the virus phylogenetic tree.

BIOLOGY OF PAPILLOMAVIRUSES

Viral structure

PV particles are approximately 55 nm in diameter, harboring a single molecule of circular dsDNA of approximately 7.9 kb contained within a spherical icosahedral capsid composed of 72 pentameric capsomeres arranged on a T = 7 surface lattice. The capsomeres exist in two states, one capable of making contact with five neighbors in the 12 pentavalent capsomers, and the other capable of making contact with six neighbors in the 60 hexavalent capsomers (Baker et al. 1991) (Figure 2). The capsid is composed of the two structural proteins, the major capsid protein L1 and the minor L2. L1 is about 55 kDa in size and represents about 80 percent of the total viral protein. Expression of L1, either alone or with L2 via mammalian expression systems, results in the formation of virus-like particles (VLP) (Figure 2) that are morphologically identical to intact virus particles (Zhou et al. 1991; Kirnbauer et al. 1992; Rose et al. 1993). L1 VLPs are highly immunogenic, generating serum neutralizing antibodies (Christensen et al. 1994), and it is thought that the major neutralizing epitope is within L1. L2 is about 70 kDa, and although not essential for assembly its presence increases the efficiency of VLP production and stability of the VLPs (Hagensee et al. 1993; Kirnbauer et al. 1993; Zhou et al. 1993).

Genome organization of the papillomaviruses

The papillomaviruses exhibit a high degree of conservation of genomic organization (Figure 3). The genome can be divided into three domains: a noncoding upstream regulatory region (URR) (also known as the 'long control region'), an 'early' region comprising the ORF E6, E7, E1, E2, E4, and E5 that encode proteins required for regulation of viral DNA replication and viral gene expression, and a 'late' region encoding the L1 and L2 viral capsid proteins. The prefix 'E' or 'L' relates to the position of the ORFs within the genome, and also the timing of expression relative to vegetative viral replication. Only one strand of the dsDNA serves as the template for viral gene expression, coding for a number of polycistronic mRNA transcripts. Transcription is regulated by enhancer sequences located in the URR, which are bound by a number of cellular factors as well as the viral E2 product. The transcription start sites of viral promoters differ depending on the virus type, but in all types promoter usage is differentiation dependent. Figure 4 gives a detailed outline of the genomic organization and transcription map of HPV-16.

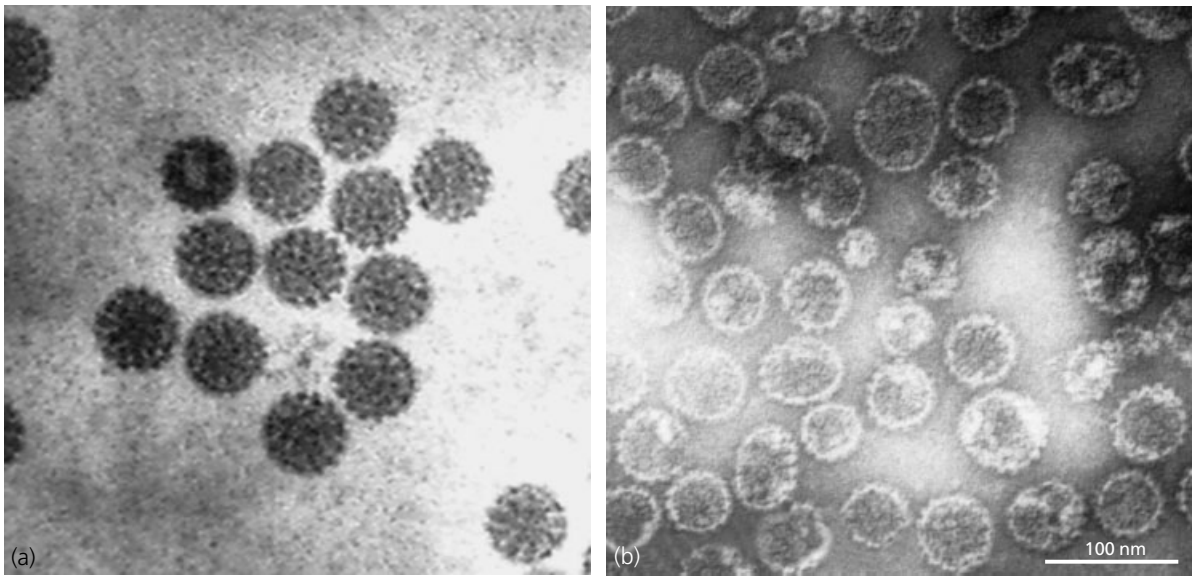

Figure 2 *Virus morphology.* **(a)** *Electron micrograph of negatively stained canine oral papillomavirus particles. The virions are about 55 nm in diameter.* **(b)** *HPV 16 L1 virus-like particles (VLPs) produced by expressing L1, the major capsid protein, in a baculovirus expression system. Scale bar = 100 nm.*

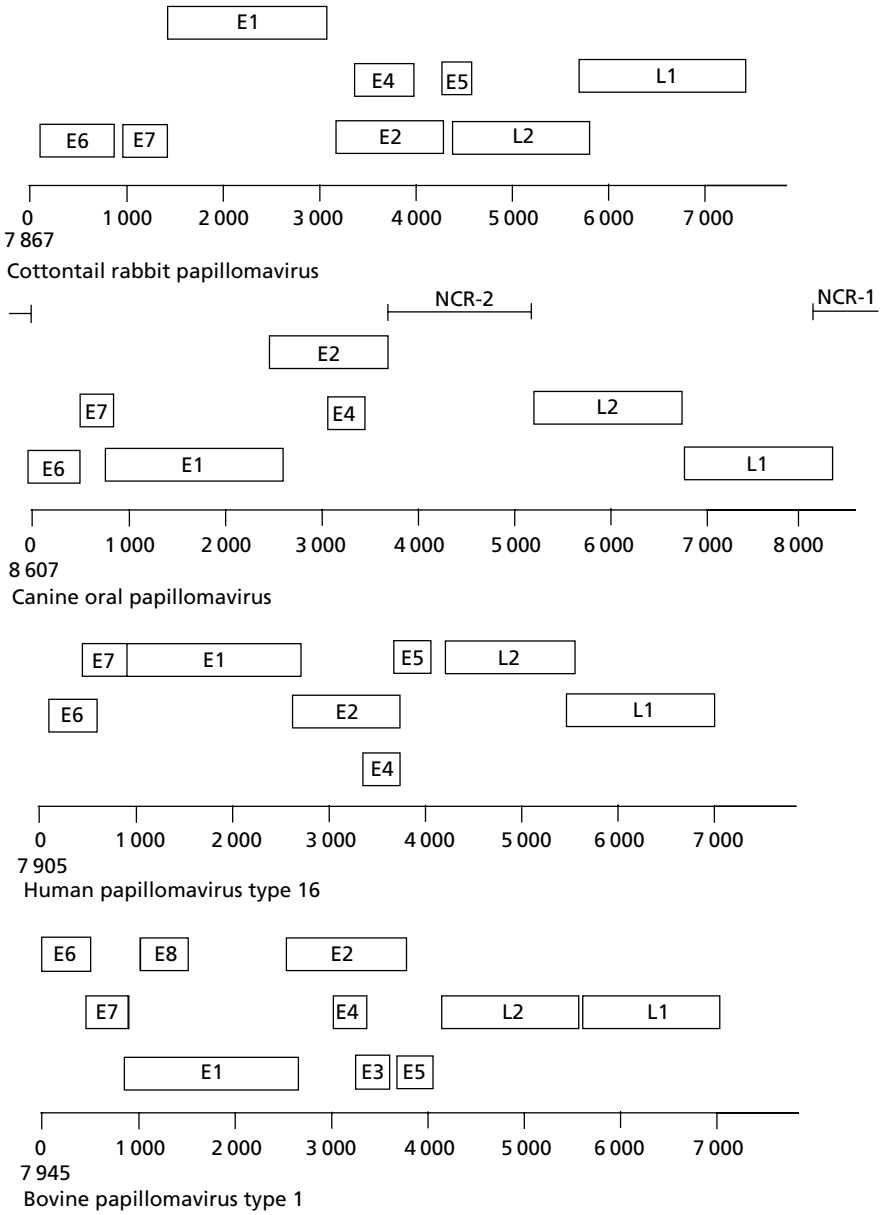

Figure 3 *The genomic structure of four papillomaviruses. A high degree of conservation of genomic organization is evident.*

THE INFECTIOUS CYCLE

The virus lifecycle is the key to understanding the pathogenesis of papillomavirus-associated disease. These viruses have an absolutely restricted host range and tissue specificity; in the natural infection papillomaviruses, with the exception of the supergroup C viruses, only infect human keratinocytes or cells with the potential for squamous maturation, such as the reserve cells of the uterine cervix. There is no tissue culture system that supports a complete infectious cycle, i.e. infection of cells in culture with virus and the subsequent generation and release of new infectious virus particles. It is unlikely that the cellular receptor for the virus defines the epithelial tropism, as virions and VLPs will bind to and enter mouse fibroblasts. The nature of the receptor(s) is unclear. The evidence that the α6β4 integrin is the primary receptor (Evander et al. 1997) has not been supported in all studies (Sibbet et al. 2000), and although there is good evidence that papillomaviruses can bind heparin and cell surface glycoproteins (Joyce et al. 1999) on keratinocytes, such binding is characteristic of many viruses.

The complete infectious cycle is absolutely dependent upon the differentiation program of the keratinocyte. Virus infects keratinocytes in the basal layer of the epithelium, but only in terminally differentiated keratinocytes are viral capsid proteins and virus particles

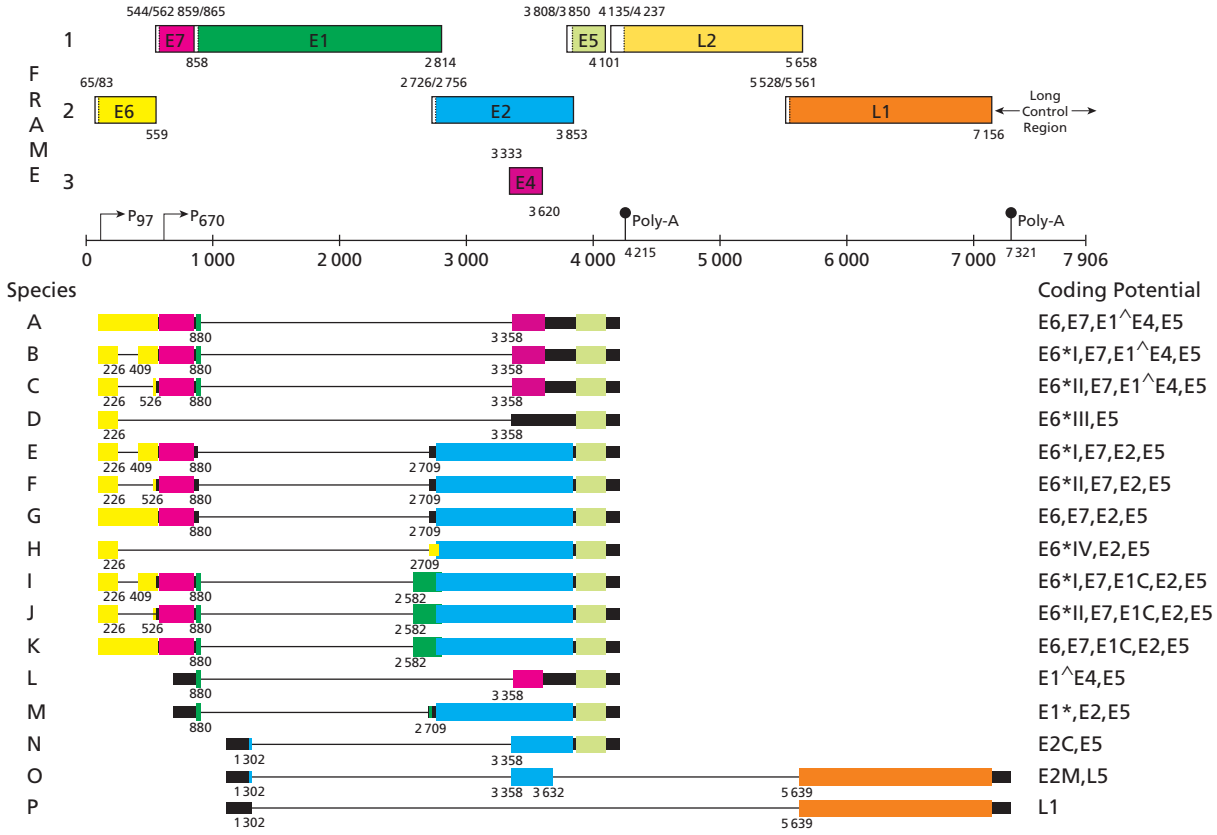

Figure 4 *Genomic organization and transcription map of HPV-16. Organization of the linearized HPV-16 genome is presented, illustrating ORFs of functional significance, well-characterized promoters (P97 and P670) and poly(A) signals. The lower panel illustrates previously characterized polycistronic mRNA species coded by the virus. These have been isolated from transfected cells and a variety of tumor cell lines and lesions. Thin black lines and colored bars represent introns and exons, respectively, and the coding potential of each species is indicated to the right. The P97 promoter is active in monolayer culture as well as systems permitting keratinocyte differentiation, and hence is the best characterized.*

assembled (Figure 5). Squamous epithelia are usually composed of 20–30 layers of cells exhibiting a coordinated pattern of gene expression concurrent with differentiation. It is thought that HPV infections occur via microwounds, which expose basal cells to viral entry. Despite the lack of unequivocal evidence, the current hypothesis is that the specific target cell for infection is the keratinocyte stem cell that resides within the hair follicle and the basal layer of squamous epithelium (Stanley 2001a). Following infection, viral genomes are established as stable episomes within the host nucleus by amplifying to approximately 20–100 copies per cell: these subsequently replicate coordinately with cellular DNA replication in a phase of plasmid maintenance.

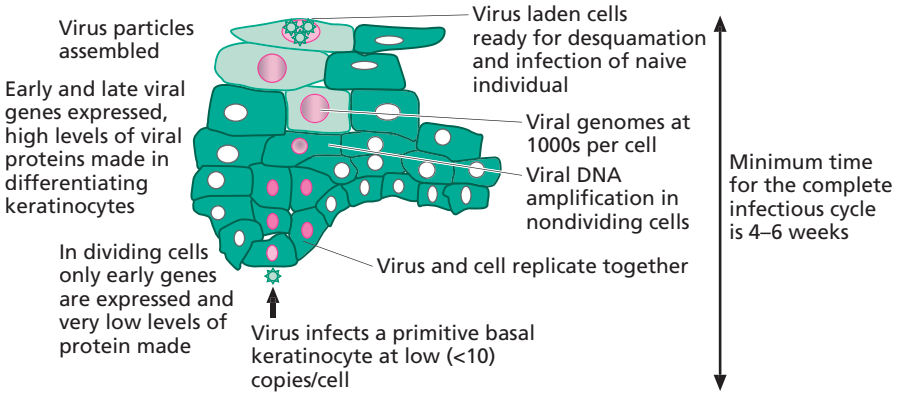

Figure 5 *The* Papillomavirus *infectious cycle*

As infected cells migrate up from the basal region and undergo differentiation, cellular replication ceases and, coordinate with the expression of differentiation-specific markers such as filaggrin, activation of differentiation-dependent late gene expression and viral capsid assembly occurs, along with amplification of viral DNA to thousands of copies per cell (Stanley 1994; Stanley et al. 1989). Koilocytes, the pathognomonic cells of papillomavirus infections, are the location of viral DNA amplification, late gene expression, and viral assembly. Productive infection only slightly alters the normal differentiation programme, with the most significant change being the retention of nuclei throughout all layers.

Our knowledge of the temporal and spatial patterns of viral gene expression comes mainly from animal models such as the dog, cow, and rabbit, and the rodent xenograft system first described by Kreider and colleagues (Kreider et al. 1987), which is permissive for vegetative growth of some HPVs. These studies show that in the first 3–4 weeks after infection with large amounts of virus, viral DNA cannot be detected in the epithelium by in situ hybridization (Stoler et al. 1990; Nicholls et al. 1999). At 4–5 weeks viral DNA can be detected in the lower layers of the epithelium and transcripts for the early genes are present. At 5–8 weeks strong signals for viral DNA are detected in the middle to upper layers of the epithelium, and in some cells viral copy number can be of the order of 1 000 or more per cell. E6 and E7 mRNAs are present at low levels in the lower dividing epithelial layers but are highly abundant in the upper differentiating layers. At about 8 weeks post-infection L1 and L2 capsid protein expression can be detected in the most superficial cells of the epithelium. The sequence of events for one of these experimental models, the canine oral papillomavirus (COPV), a low-risk mucosal virus infecting the oropharynx of dogs, is shown in Figure 6. There are variations in this spatial and temporal expression pattern and these have recently been elegantly outlined (Peh et al. 2002). Importantly, the high-risk genital HPVs (16, 18, etc.) show a similar spatial and temporal pattern of gene expression, but the regulation of the early genes *E6* and *E7* is under very tight control in the proliferating compartment of the epithelium, with very low levels of mRNA expression in these cells. E6 and E7 mRNAs are expressed abundantly only in the upper stratum spinosum and granulosum (Durst et al. 1992; Higgins et al. 1992).

TARGET CELL FOR INFECTION AND VIRAL LATENCY

The dependence upon the keratinocyte lineage for viral gene expression extends to the target cell for infection. In experimental CRPV infections, viral gene transcription is detected first in the hair follicle, in cells that have the characteristics of stem cells (Schmitt et al. 1996) This is also true in COPV, where viral DNA amplification and early gene transcription are first detected at the extreme tips of the rete ridges, thought to be the site of interfollicular keratinocyte stem cells (Figure 6). If the target cell for infection is the stem cell then this does offer an explanation for some interesting aspects of papillomavirus biology. There is, for example, the phenomenon of the lag phase between infection and the first detection of viral DNA and early gene expression. The length of this lag in experimental models such as the rabbit, the dog, and rodent xenografts of HPV 11, is consistently between 3 and 5 weeks. Anecdotal evidence in humans suggests a similar time lag for genital warts (Oriel 1971). One attractive explanation for this is that the virus infects a stem cell in the G_0 phase of the cell cycle. The 3–5-week delay before viral gene expression is detectable would then reflect the time for the stem cell to enter G_1, divide, and for committed daughter cells to enter the transit amplifying population of basal and parabasal cells permissive for viral gene expression.

Dependence upon the stem cell milieu for infection and immediate early viral gene expression would also partly explain the phenomenon of latency. HPV is frequently present as a latent infection in the female genital tract (Toon et al. 1986), and the frequent lesion recurrence observed in respiratory papillomatosis is generally believed to reflect reactivation of latent infection, rather than reinfection (Abramson et al. 1987). This is supported by the observation that histologically normal, but HPV positive, laryngeal tissues express low-abundance transcripts coding for E1 and E2 (Maran et al. 1995). Similarly, in the rabbit, low doses of CRPV lead to viral persistence and expression of truncated E1 and E2 transcripts, but no lesion unless – and until – reactivation is induced by skin irritation (Amella et al. 1994). Furthermore, CRPV can persist at the site of regressed papillomas in 60 percent of cases (Selvakumar et al. 1997) with occasional lesion recurrence, and similar phenomena are seen in BPV (Campo et al. 1994) and COPV infections (Moore et al. 2002). Latency is a poorly understood aspect of papillomavirus biology but clearly of importance for therapeutic and prophylactic intervention strategies.

REGULATION OF GENE EXPRESSION

Papillomavirus transcription is complex owing to the presence of multiple promoters and the differential production of mRNA species in different cells; viral gene expression is regulated both temporally and spatially. Transcription has been most extensively analysed for BPV-1 (Baker 1993), but information is available for the high-risk genital HPVs (Doorbar et al.

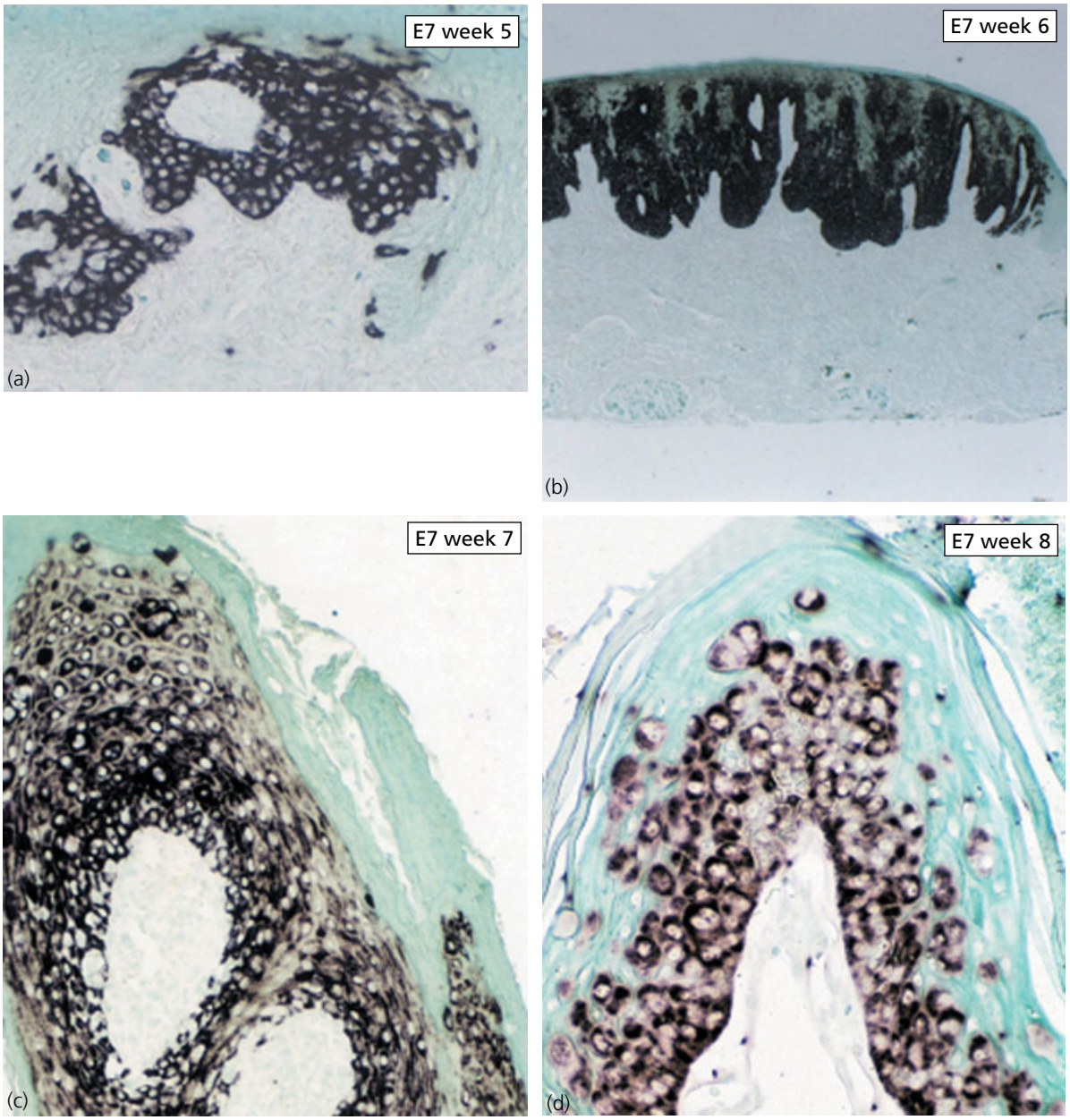

Figure 6 *Temporal and spatial expression of* Papillomavirus *genes in canine oral papillomavirus infection.* **(a–d)** *In situ RNA hybridization of experimental COPV lesions with digoxigenin-labeled E7 probe. Abundant transcripts in detected in early lesions. At week 6 there are abundant basal layer and some suprabasal transcripts persisting through to week 8. (Continued over)*

1990; Stubenrauch and Laimins 1999) and the following discussion concentrates on these.

Early gene expression

Early gene expression of genital HPVs is positively and negatively modulated by a complex interplay between a variety of cellular transcription factors and the viral E2 protein. Early polycistronic transcripts of the HR-HPVs are initiated at the early promoter upstream of the E6 ORF and terminate at a polyadenylation site at the end of the early region (Figure 3). The promoter, known as P97 in HPV-16 and HPV-31, and P105 in HPV-18 by virtue of the nucleotide from which transcription is initiated, comprises binding sites (Desaintes and Demeret 1996) for a number of cellular factors that are essential for viral transcription (see Figure 4). Binding sites for the viral E2 protein are also located within the early promoter. These allow E2 to exert its function as a transcriptional regulator.

Early gene transcription is further regulated by a *cis*-acting transcriptional enhancer located in the central

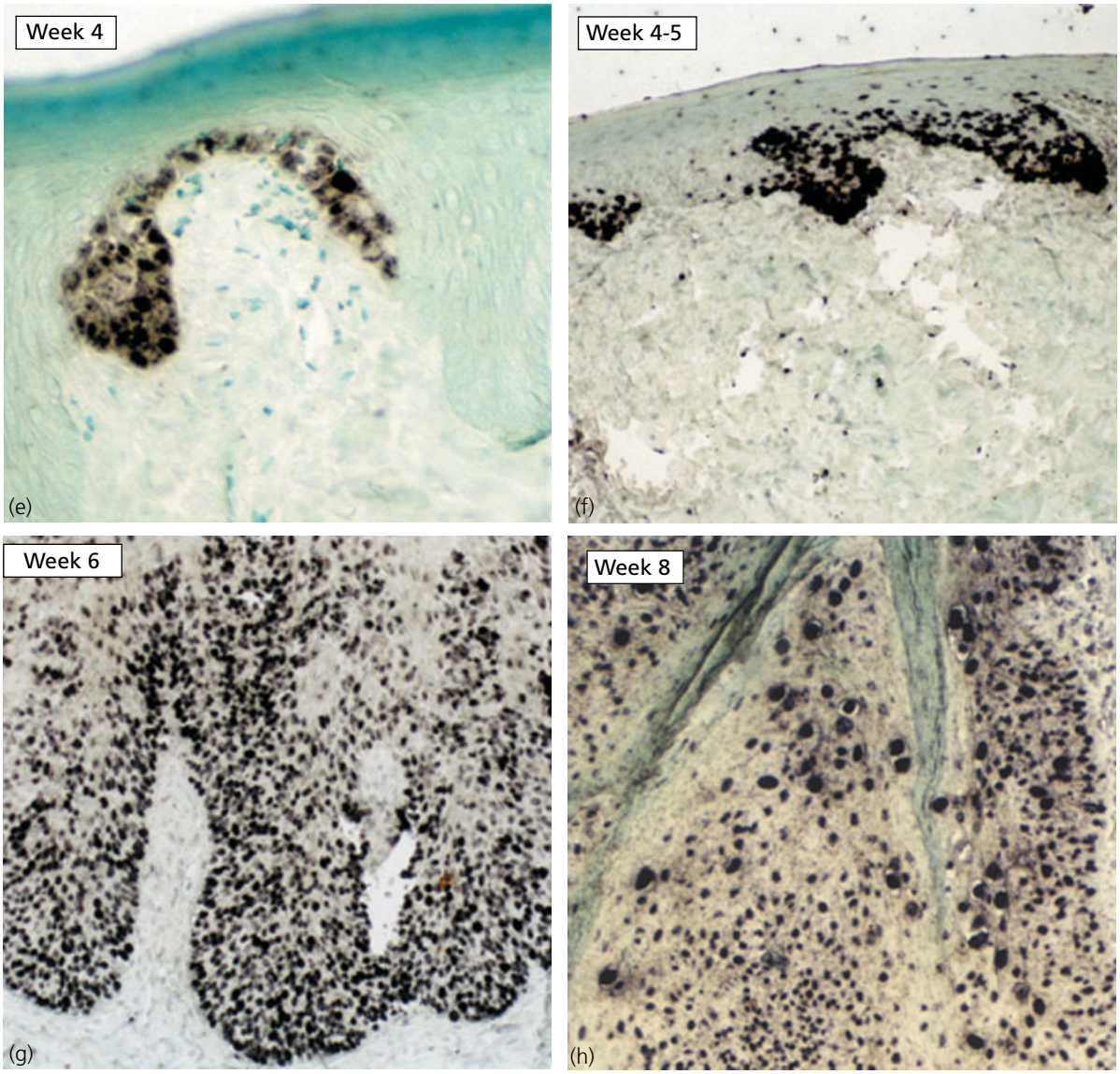

Figure 6 *Temporal and spatial expression of* Papillomavirus *genes in canine oral papillomavirus infection. (Continued)* **(e–h)** *In situ DNA hybridization of experimental COPV lesions with a digoxigenin-labeled genomic COPV probe. Viral DNA was undetectable from weeks 1 to 3 post infection. By week 4, viral DNA was found in multiple clusters each arising from a rete ridge. By 6 weeks almost all basal cells and a majority of suprabasal cells were positive for viral DNA. At week 8 sporadic cells in the more superficial epithelium had the strongest signal, together with vacuolated cytoplasm typical of koilocytes. (Continued over)*

region of the URR. This is a major determinant of HPV epithelial tropism, as it is only active in epithelial cells. The epithelial specificity of action appears to be conferred not through the action of keratinocyte-specific activators, but rather through the combinatorial action of multiple ubiquitous cellular transcription factors (Desaintes and Demeret 1996). Although the binding elements for these factors, as well as their spatial organization, vary according to viral type, organization of the enhancer appears quite conserved among the genital HPVs (O'Connor et al. 1995). In the case of the HPV-16 enhancer (see Figure 7), identified binding sites that have also been shown to regulate a number of other genital HPVs include those for NF1 (Apt et al. 1993), AP-1 (Chan et al. 1990), Oct-1 (O'Connor and Bernard 1995; Sibbet and Campo 1990), TEF-1 (Ishiji et al. 1992), and YY1 (O'Connor et al. 1996). Activity of the early promoter is differentiation dependent, with extremely low levels of early transcripts being detected in basal cells of infected epithelium, and expression increasing in the intermediate layers (Durst et al. 1992; Higgins et al. 1992; Stoler et al. 1992; Cheng et al. 1995).

There is an important difference in the expression of the E6 and E7 mRNAs of the HR-HPVs and the LR-HPVs. The HR-HPVs are expressed from a single

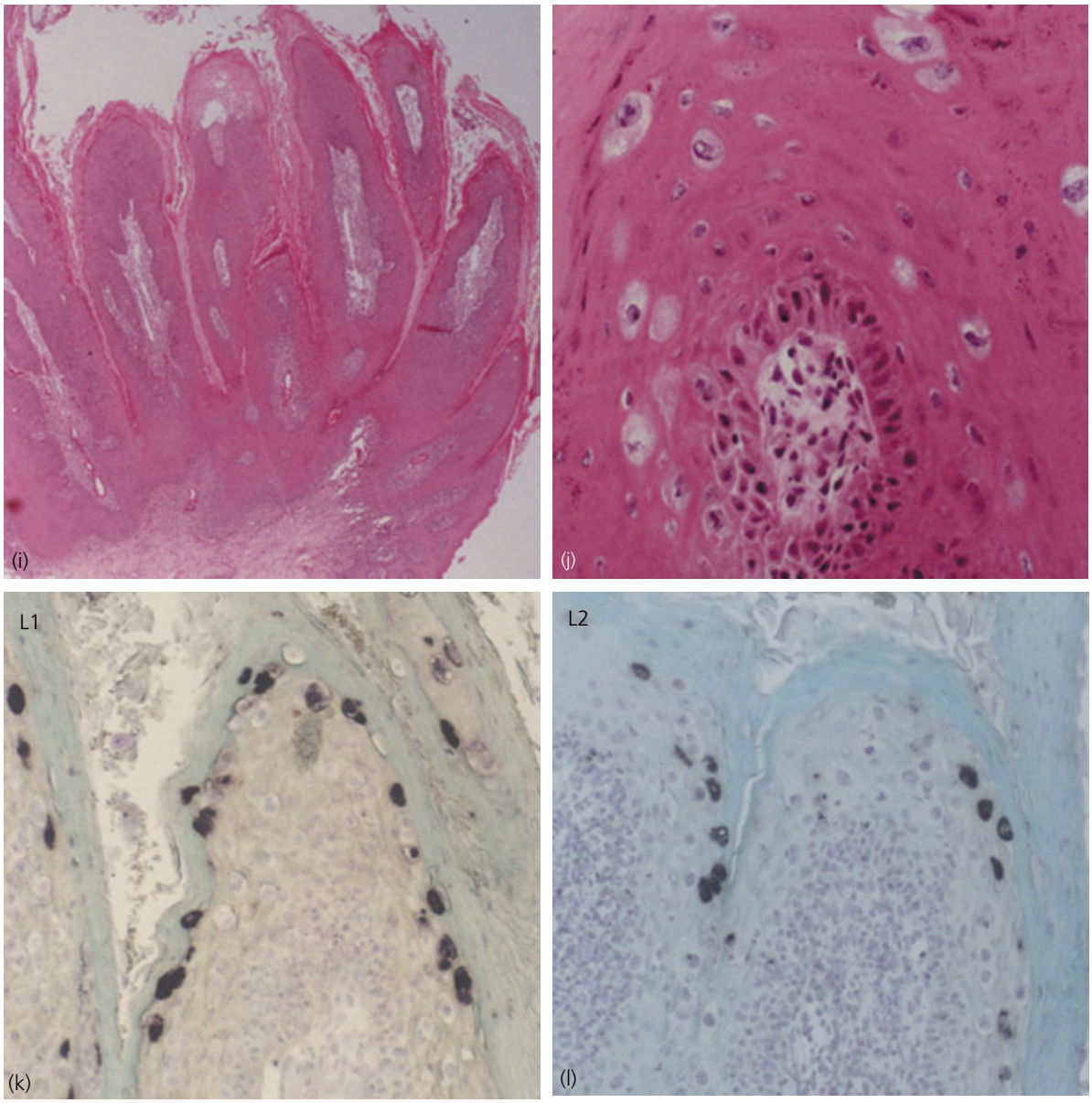

Figure 6 *Temporal and spatial expression of* Papillomavirus *genes in canine oral papillomavirus infection. (Continued)* **(i–l)** *COPV wart morphology at week 8 post infection showing the elongated filiform papillae typical of mature warts* **(i–j)**. *Koilocytes were present in the papillae by week 7* **(k–l)**. *Probes for transcripts from L1 and L2 both detected occasional positive superficial cells at 7 weeks, and abundant positive superficial cells by 8 weeks.*

promoter that directs the expression of mRNAs for E6 and E7 with splices in the *E6* gene. The mRNAs with spliced E6 splice to the 5′ end of the E6 ORF (E6*) to a transition frame with a stop codon allowing sufficient spacing for translation reinitiation of the E7 ORF. In contrast, the *E6* and *E7* of the LR-HPVs are expressed from two independent promoters (Chow and Broker 1997).

Late gene expression

The application of in vitro systems, such as organotypic raft cultures (Asselineau et al. 1986), that induce stratification and terminal differentiation of keratinocytes has permitted analysis of late gene expression (Wilson et al. 1992). The raft culture system can fully support the late stages of the virus lifecycle (Bedell et al. 1991; Stanley 1994). In genital HPVs a differentiation-dependent promoter has been detected that has a transcription start site within the E7 ORF (see Figure 4) (Grassmann et al. 1996; Hummel et al. 1992). Most polycistronic late transcripts initiated from this promoter have coding potential for the E1–E4 fusion protein (Hummel et al. 1995). More recently, it has been shown that transcripts encoding E1 and E2 are also generated from the late

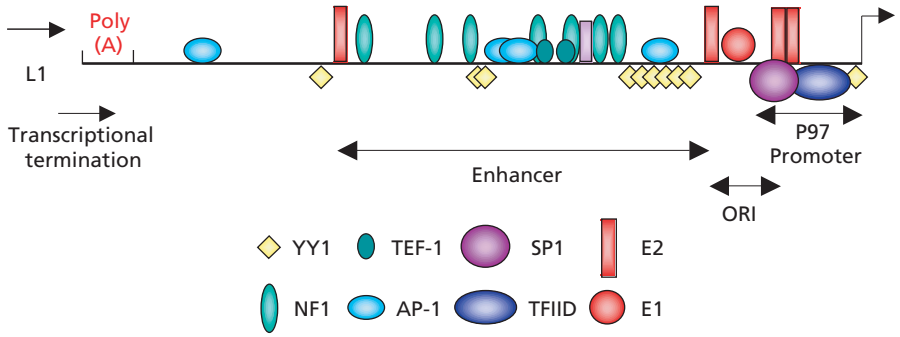

Figure 7 *Schematic representation of the HPV-16 URR (O'Connor et al., 1996). The E2 binding sites divide the URR into three functional distinct regions referred to as the 5′, central, and 3′ segments. The 5′ segment of the URR contains a transcription termination signal (poly(A)), the central segment contains the epithelial cell-specific enhancer comprising the majority of the transcription factor binding sites, and the 3′ segment contains the origin of replication (ORI), the E1 binding site, and the P97 promoter. Shown are the binding sites for a number of transcription factors with a role in transcriptional control of all genital HPVs.*

promoter (Klumpp and Laimins 1999). Also generated are transcripts coding for the L1 and L2 capsid proteins, which are translated from mRNAs that utilize the late polyadenylation signal located within the URR (Terhune et al. 1999). Importantly, the organotypic culture systems show clearly that the induction of late gene expression requires that the viral genomes be maintained as extrachromosomal elements, and that the induction of terminal differentiation alone is insufficient (Frattini et al. 1996).

REGULATION OF HPV REPLICATION

Viral replication is dependent on the action of viral proteins and components of the cellular replication machinery, including DNA polymerase α-primase, DNA polymerase δ, replication protein A (RPA), replication protein C (RPC), proliferating cell nuclear antigen (PCNA), and topoisomerases I and II (Desaintes and Demeret 1996). These are not present normally in differentiating cells and are induced as a consequence of the viral E7 protein. In transient replication assays (Chiang et al. 1992; Del Vecchio et al. 1992; Ustav et al. 1991) and in cell-free systems (Kuo et al. 1994) the viral proteins E1 and E2 are required for efficient replication of viral origin of replication (ORI)-containing plasmids. However, in certain transient replication studies low-level replication of ORI-containing plasmids has been observed if E1 alone is expressed (Gopalakrishnan and Khan 1994; Sakai et al. 1996), indicating that E2 has an auxiliary function in viral replication and that E1 is the principal replication protein. E2 is considered a specificity factor, directing the replication initiator E1 preferentially to viral DNA to initiate highly efficient virus-specific replication. However, E2 mediates interaction of PV genomes with host chromosomes, suggesting that during plasmid maintenance E2 ensures that viral genomes are segregated to daughter cells in approximately equal numbers (Skiadopoulos and McBride 1998).

PROPERTIES AND FUNCTIONS OF VIRAL ORFS

Many biochemical properties and functions of PV ORFs were originally elucidated using BPV-1 as a model system. Many of these functions have proved applicable to HPVs.

E1 ORF

The product of the E1 ORF is a 600–650 amino acid nuclear phosphoprotein with structural and sequence similarities to regions of the simian virus 40 (SV40) large T antigen required for viral replication initiation. Consistent with a replication initiator role for the E1 protein, it binds the viral origin ORI and exhibits ATPase and ATP-dependent helicase activities (Desaintes and Demeret 1996). Hence, together with the product of the E2 ORF, expression of *E1* is critical for efficient viral DNA replication and thus plays an essential role in maintenance of episomal viral genomes during the HPV lifecycle. *E1* is the most conserved of all the PV genes, and hence *E1* gene products of different PVs are expected to be comparable in terms of both structure and function.

E1 forms complexes with E2 in solution (Blitz and Laimins 1991; Frattini and Laimins 1994; Mohr et al. 1990). Although E1 alone can bind with low affinity to the A/T-rich sequence in the viral ORI (Frattini and Laimins 1994), E1:E2 complexes bind the ORI with higher affinity and specificity (Kuo et al. 1994), which is attributable to the presence of E2-binding sites flanking the single E1-binding site in the viral ORI (see Figure 2). E2 acts as a specificity factor to promote highly efficient virus-specific replication. In the current model, the E1:E2:ORI complex constitutes the inactive preinitiation complex. This serves as a precursor for the recruitment of additional E1 molecules and the formation of a multimeric E1 complex bound to the ORI

(Lusky et al. 1994). In vitro, this is accompanied by displacement of E2 from the origin (Lusky et al. 1994). E1 assembles into hexameric structures, with hexamer formation concomitant with ATPase activity and DNA unwinding (Sedman and Stenlund 1998). Thus, the hexameric form of E1 is the enzymatically active ATP-dependent helicase required during the elongation phase of replication to unwind DNA ahead of the replication fork (Hughes and Romanos 1993).

E1 also interacts with host replication factors that are required for viral replication. For example, it interacts with DNA polymerase α-primase and brings it to the viral ORI in order to initiate replication (Masterson et al. 1998). Thus the combination of the multimeric E1:ORI complex and recruited cellular factors constitutes the active replication 'initiation' complex.

E2 ORF

The product of the E2 ORF is a regulator of both viral transcription and replication. E2 is a nuclear phosphoprotein comprising an N-terminal transactivation domain and a C-terminal DNA-binding domain, both highly conserved among all PVs, separated by a nonconserved 'hinge' region of variable length. E2 binds to DNA as a dimer, with α-helices from each monomer forming specific contacts with DNA. The consensus E2-binding site is the palindromic sequence ACCGNNNCGGT, of which there are four in the viral URR of genital HPVs (see Figure 7). The role of E2, together with E1, in viral replication has been discussed above.

Most experiments have demonstrated that, unlike BPV-1 E2, the E2 proteins of mucosal HPVs act as transcriptional repressors of the early viral promoter (Bernard et al. 1989). Repression depends on the interaction between E2 and its promoter proximal binding sites at the transcriptional control region, and this is proposed to hinder assembly of the transcription initiation complex (Dong et al. 1994). However, it has also been demonstrated that E2 can *trans*-activate gene expression from the early promoter when specific E2-binding sites are mutated (Thierry and Howley 1991) One model suggests that at low concentration E2 binds the high-affinity promoter distal E2-binding site and *trans*-activates the early promoter, whereas at higher concentrations the promoter proximal sites are bound and transcription is repressed (Steger and Corbach 1997). The E2 of both high- and low-risk genital HPVs can also influence transcription of host genes, and can downregulate the promoter of the reverse transcriptase component of telomerase (Lee et al. 2002).

E4 ORF

The most abundant mRNA species detected in productive HPV infection initiates from the late promoter, splices a short 5′ sequence of the E1 ORF to the E4 ORF, and codes for the major product of the E4 ORF, the E1–E4 fusion protein (referred to hereafter as E4) (Desaintes and Demeret 1996). E4 proteins of cutaneous and mucosal HPVs vary greatly in terms of sequence homology and biological properties. The E4 of the mucosal PVs is a cytoplasmic phosphoprotein of around 90 amino acids that can self-associate to form multimeric species (Bryan et al. 1998). It comprises relatively conserved N-terminal and C-terminal regions, separated by a highly variable region. Although E4 is located in the early gene region, transcripts with coding potential for E4 are expressed at a high level late in the productive lifecycle through activation of the differentiation-dependent late promoter located in the E7 ORF. The function of E4 is poorly understood, but current hypotheses suggest that it may function at the level of control of viral replication and/or release of infectious virions.

In cultured human keratinocytes E4 has been shown to associate with the keratin intermediate filament (IF) network and, in the case of HPV-16, to cause its collapse (Doorbar et al. 1991; Roberts et al. 1993). A leucine-rich N-terminal motif was a crucial determinant in mediating cytoplasmic localization of E4, whereas C-terminal residues mediated self-association, association with the keratin IF network, and efficient disruption of the keratin IF network (Bryan et al. 1998; Roberts et al. 1997). Because of the observed cytopathic effect, it has been hypothesized that E4 may enhance the release of infectious virions in superficial layers by promoting cell lysis (Doorbar et al. 1991). However, although colocalization of E4 with the keratin IF network was demonstrated within HPV-infected epithelium, collapse of the network was not (Sterling et al. 1993; Pray and Laimins 1995). More recently, it has been shown that E4 binds and disrupts specific components of the cornified cell envelope (CCE), contributing to its altered physical characteristics of HPV-11-infected epithelium (Brown and Bryan 2000; Bryan and Brown 2000). Given that one of these is increased cell fragility, the hypothesis that E4 may enhance virion release may hold true, via disruption of the CCE and/or collapse of the keratin IF network.

Expression of HPV-16 *E4* in SiHa cells results in arrest of the cell cycle at G2 and colocalization of the Cdc2/cyclin B mitosis-promoting complex with E4 at the keratin IF network (Davy et al. 2002). Because nuclear localization of this complex is required for entry into mitosis, cytoplasmic sequestration would account for the observed G2 arrest. Thus, it has been suggested that E4-induced G2 arrest, combined with the viral oncogene cell cycle progression out of G_1 phase, creates a 'pseudo S-phase' environment ideal for replication of the viral genome.

E5 ORF

The contribution of the E5 protein to the HPV lifecycle is poorly understood. However, given that it is among

the most abundant transcripts in low-grade intraepithelial lesions, its cellular effects are likely to be important in productive HPV infections. HPV-16 E5 is a small hydrophobic protein of approximately 90 amino acids in length. Apart from its overall hydrophobic sequence, it does not share significant sequence homology with BPV-1 E5 (DiMaio and Mattoon 2001). Like BPV-1 E5, HPV-16 E5 localizes to the Golgi apparatus, endoplasmic reticulum, and nuclear membranes (Conrad et al. 1993), but unlike its bovine counterpart has only weak transforming activity (DiMaio and Mattoon 2001). The transforming capacity of HPV16 E5 correlates with its ability to inhibit downregulation of the EGF receptor in human keratinocytes (Straight et al. 1993). This is consistent with the enhanced transforming capacity of E5 in the presence of EGF (Bouvard et al. 1994). It has been shown that E5 interacts with the 16 kDa component of the vacuolar proton ATPase (Conrad et al. 1993). This impairs its function in acidification of endosomes, which is essential for their proteolytic functions (Conrad et al. 1993; Straight et al. 1995). Hence, the current model is that HR-HPV E5 transforms cells by increasing their sensitivity to extracellular EGF levels by increasing receptor recycling to the membrane (DiMaio and Mattoon 2001).

E6 ORF

The viral oncoproteins E6 and E7 are the major transforming and immortalizing proteins of the oncogenic or HR-HPVs. The transforming and immortalizing capacity of the oncoproteins of specific HPV types correlates with viral oncogenicity in vivo. Thus, the association of HR-HPVs, but not LR-HPVs, with the development of cervical carcinoma is attributable to differences in the activity of the E6 and E7 proteins. However, the properties of these proteins must be viewed primarily in terms of their function in the virus lifecycle, as it is in this context that they have evolved.

STRUCTURE AND LOCALIZATION OF E6

The HPV E6 protein is a polypeptide of approximately 150 amino acids. Although there is sequence variation of E6 in different HPV types, all retain strict conservation of four C–X–X–C motifs that allow the formation of two zinc fingers (Barbosa et al. 1989; Grossman and Laimins 1989). Owing to low levels of endogenous E6, detection of its intracellular location has been difficult. However, induction of high-level E6 expression with a baculovirus vector reveals localization to the nucleus, and also to nonnuclear membranes (Grossman et al. 1989), consistent with the notion that E6 protein is multifunctional.

INTERACTION BETWEEN E6 AND P53

A large number of cellular targets of HR-HPV E6 have been defined, although the best studied is the interaction with the tumor suppressor gene (TSG) *TP53*. The E6 protein of HR-HPVs has been shown to bind in vitro translated p53 (Werness et al. 1990), resulting in degradation of the p53 via the ATP-dependent ubiquitin pathway (Scheffner et al. 1990). This is consistent with the observation that low levels of wildtype p53 are detected in HR-HPV-positive cervical cancer cell lines and E6-immortalized human mammary epithelial cells (Band et al. 1991; Hubbert et al. 1992; Scheffner et al. 1991). There are conflicting reports as to whether E6 of LR-HPV can bind p53 (Crook et al. 1991; Lechner and Laimins 1994; Werness et al. 1990). However, this is a low-affinity interaction compared to that of HR-HPV E6, and does not induce degradation of p53. Thus, the oncogenicity of HPVs correlates with an ability to bind and induce the degradation of p53. Indeed, the demonstration that HR-HPV E6-expressing cells do not generally undergo cell cycle arrest in response to DNA damage shows that the HR-HPV E6/TP53 interaction can abrogate the normal function of this TSG (Kessis et al. 1993).

The induction of degradation of p53 by E6 is mediated by a cellular protein E6-associated protein (E6-AP) which can bind E6 of HR-HPVs, but not the E6 of LR-HPVs, in the absence of p53 (Huibregtse et al. 1991; Scheffner et al. 1993). E6 recruits E6-AP to prevent the accumulation of p53.

ADDITIONAL CELLULAR TARGETS OF E6

E6 has binding partners apart from p53 (zur Hausen 2000) (Table 1). The consequences of these various protein/protein-binding reactions with regard to E6 oncogenicity are not currently understood, but they do involve key aspects of cellular function and behavior in the maintenance of tissue architecture, the disruption of which accompanies carcinogenic progression. However, given the low levels of E6 expressed in HPV-positive lesions, it is uncertain which interactions take place in an infected cell during permissive viral growth.

FUNCTION OF E6 IN THE VIRUS LIFE CYCLE

Viral DNA amplification occurs in terminally differentiated postmitotic cells and, because the virus requires the host DNA replication machinery to achieve this, a central role of the E7 protein is to induce S-phase entry. However, one response of a normal cell to the induction of inappropriate DNA replication – for example during differentiation, at confluence, or upon growth factor withdrawal – is growth arrest or apoptosis via induction of p53. Indeed, E7-expressing primary keratinocytes show an increase in p53 levels (Demers et al. 1994). Through its ability to bind and degrade p53, the E6 protein mediates inhibition of apoptosis, a function elegantly demonstrated in transgenic mice expressing the HPV-16 oncoproteins in the ocular lens. The expression

Table 1 *Additional cellular targets and properties of the E6 oncoprotein*

Cellular binding protein or property	Notes	References
ERC-55	Calcium-binding protein of unknown function. The mouse homologue is the vitamin D receptor-associated factor that transduces the growth-suppressive effects of vitamin D. Thus, the association with E6 may inhibit these growth-suppressing signals	Imai et al. 1991
Paxillin	Focal adhesion protein that transduces signals from the plasma membrane to focal adhesions and the actin cytoskeleton. The association of E6 possibly disrupts growth-inhibitory signals from cell–cell contact	Turner 2000
hDLG	Membrane-associated PDZ motif containing protein that is the human homologue of *Drosophila* discs large TSG. Binds APC and negatively regulates cell-cycle progression. E6 induces proteasome-mediated degradation and could inhibit the inhibitory hDLG/APC interaction and also cell–cell contact	Gardiol et al. 1999
MCM7	Required for formation of the DNA replication licensing system, which allows initiation of cellular DNA replication and restricts it to a single round per cell cycle. The E6 association may be a normal requirement for the viral lifecycle	Kukimoto et al. 1998
Interferon regulatory factor (IRF-3)	Activated in response to virus infection to induce expression of the antiviral type I interferon (IFN) IFN-β. The E6 interaction does not cause degradation of IRF-3, but reduces expression of IFN-β. This is likely to be of significance in the normal viral lifecycle	Ronco et al. 1998
Enhanced degradation of MYC and BAC	BAK is a proapoptotic member of the BCL-2 family, expressed in differentiated epithelial cells. MYC can also induce apoptosis if proliferation is unscheduled. The E6-induced degradation of MYC and BAK may partly explain the TP53-independent inhibition of apoptosis by E6. This may also be of significance in the normal viral lifecycle	Thomas and Banks 1999
Induction of hTERT expression	Retrovirally delivered E6 can induce high levels of telomerase activity in preimmortal keratinocytes. This is mediated by activation of the hTERT promoter. The mechanism of activation is poorly understood, but as upregulation of the hTERT promoter correlates with the ability of E6 to bind E6-AP, it is possible that E6 targets a negative regulator of expression for degradation	Klingelhutz et al. 1996

of E7 alone induced cellular proliferation in spatially inappropriate regions and apoptosis of these cells before differentiation could occur. The expression of E6 alone inhibited the apoptosis that was observed in the lenses of nontransgenic embryos, whereas co-expression of E6 and E7 resulted in abrogation of the E7-induced apoptosis, and led to tumor formation in 40 percent of adult mice (Griep et al. 1993; Pan and Griep 1994). The ability of E6 to bind and degrade BAK and MYC (see Table 2) is consistent with inhibition of p53-independent apoptosis.

THE E6* PROTEIN

A number of early transcripts of high-risk, but not low-risk, HPVs utilize a splice donor site within the E6 ORF to yield coding potential for a number of nontransforming truncated E6 proteins known as E6* (Schwarz et al. 1985). It has been shown that E6* is capable of interacting with both the full-length E6 protein and E6-AP in vitro, blocking the association of E6 with TP53 (Pim et al. 1997). As a consequence, E6* proteins can inhibit the E6-induced degradation of TP53 (Pim et al. 1997; Shally et al. 1996). The role of E6* in the viral lifecycle remains unclear, however.

E7 ORF

STRUCTURE AND LOCALIZATION OF E7

The HPV E7 protein is a phosphoprotein of approximately 100 amino acids. Consistent with its multifunctional nature, E7 has been detected at a variety of locations within the cell, including the cytoplasm, nucleus, and nucleoli (Greenfield et al. 1991; Smotkin and Wettstein 1987). The N-terminal region of E7 comprises two regions known as conserved domains 1 and 2 (CD1 and CD2). CD2 contains a 'pocket protein'-binding L–X–C–X–E motif, and also a consensus phosphorylation site for casein kinase II (CK II). A third conserved region, CD3, resides at the C terminus and comprises two C–X–X–C motifs with the ability to bind zinc and form a zinc finger (Barbosa et al. 1989; McIntyre et al. 1993). This domain confers the ability of

Table 2 *Additional cellular targets of the E7 oncoprotein*

Cellular binding protein	Notes	References
$p21^{WAF1/CIP1}$ and $p27^{KIP1}$	Inhibitors of cyclin A/E-dependent kinases, with a role in cell-cycle arrest, differentiation, and apoptosis. Association with E7 abrogates these activities. Effects on terminal differentiation-associated growth arrest may possibly play a role in the viral life cycle	Funk et al. 1997
TATA box-binding protein	Component of the TFIID transcription factor complex. Interaction of E7 may control expression of genes related to E7-associated transformation	Massimi et al. 1997
JUN	Member of the AP-1 family of transcription factors. The E7 interaction can induce transcription from JUN-responsive promoters. However, JUN also binds RB and induces activation of AP-1-regulated genes in differentiated keratinocytes. The interaction of E7 with JUN inhibits this activation. Thus, E7 can act as both a positive and negative regulator of JUN-responsive promoters	Antinore et al. 1996
M2 pyruvate kinase (M2-PK)	This is the first example of a cytoplasmic, noncell cycle-associated target of E7. In its tetrameric form, M2-PK promotes flux of metabolites through the glycolytic pathway. Binding of E7 converts M2-PK to a dimeric form, which decreases this flux. This increases the pool of metabolites available for biosynthesis of nucleic acids. Thus, this interaction may play an important role in the viral lifecycle by increasing the pool of nucleic acids available for viral replication	Zwerschke et al. 1999

E7 to form homodimers, and mutational analysis has shown that it also contributes to the transforming potential of the HR-HPV E7 proteins (Edmonds and Vousden 1989; McIntyre et al. 1993).

INTERACTION OF E7 WITH RB AND OTHER POCKET PROTEINS

The best-characterized interaction of HPV E7 is with the TSG RB. A significant breakthrough regarding the molecular basis of transformation by HPV E7 was the demonstration that HPV-16 E7 can bind pRB both in vitro and in vivo (Munger et al. 1989). Furthermore, the affinity of this association is greater for HR-HPV than for LR-HPV E7 (Munger et al. 1989), suggesting that this property is likely to contribute to the oncogenic potential of HR-HPVs. Indeed, mutational analysis has demonstrated that E7-induced transformation is impaired if the pRB/E7 association is disrupted (Barbosa et al. 1990; Edmonds and Vousden 1989; Watanabe et al. 1990). E7 preferentially binds the hypophosphorylated form of pRB (Imai et al. 1991), disrupting the pRB/E2F association and thus inducing the expression of E2F-dependent genes required for G_1–S progression (Chellappan et al. 1992). It has also been shown that HR-HPV E7 induces degradation of pRB via the ubiquitin-mediated proteasomal pathway (Boyer et al. 1996). Thus, efficient abrogation of pRB function by E7 is mediated by protein degradation as well as binding and, consistent with this, the transforming capacity of E7 mutants correlates more closely with their ability to degrade pRB rather than just bind it (Jones and Munger 1996).

E7 also binds the pRB-related pocket proteins p107 and p130 (Dyson et al. 1992), which are also involved in cell-cycle regulation via association with E2F proteins. However, given that similar sequences of E7 are required to mediate interactions with all the pocket proteins, it is difficult to determine which of these are the most functionally significant targets of E7 using mutational analysis.

ADDITIONAL CELLULAR TARGETS OF E7

Mutational studies have shown that the E7/pRB interaction is necessary, but not sufficient, for cellular transformation (Edmonds and Vousden 1989; Phelps et al. 1992). Consistent with this, a number of additional cellular targets of E7 are now recognized (Table 2). These are likely to contribute to the ability of E7 to modulate the cell cycle as well as other cellular activities. As with E6, a number of these targets are more efficiently abrogated by E7 from HR-HPVs, and thus may further contribute to the oncogenic potential of HR-HPVs. However, the low level of E7 protein detected in clinical lesions raises the issue of which of these interactions are relevant in permissive infection.

FUNCTION OF E7 IN THE VIRUS LIFECYCLE

Because they do not encode a unique DNA polymerase, papillomaviruses are completely dependent upon the host DNA replication machinery for generation of progeny virions. Given that viral vegetative DNA replication occurs in postmitotic differentiated cells, this presents a problem for the virus. In a usual differentiation program, as postmitotic differentiated cells migrate

up the epithelium their nuclei are gradually degraded and no longer active. Thus, an important role of the virus is to maintain suprabasal cells in a replication-competent state and reactivate the host DNA replication machinery while preserving the differentiation process to allow late gene expression.

It has been shown that the E7 of HR-HPVs and, to a lesser extent, LR-HPVs under the control of the homologous URR, is sufficient to induce expression of proliferating cell nuclear antigen (PCNA) and induce cellular DNA synthesis within suprabasal cells (Cheng et al. 1995). Thus, induction of entry into S-phase and the activation of host DNA replication machinery is a crucial role of the E7 protein. Unscheduled exit from G_1 can lead to apoptosis, and this is overcome by the viral oncoprotein E6. The cellular targets of E7 are consistent with this function. Through its interaction with members of the RB family of pocket proteins, E7 can induce the activity of the E2F family of transcription factors that control the expression of genes required for entry into S-phase.

Abrogation of the effects of cyclin-dependent kinase inhibitors (CDKI) such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ is another important role of E7 in the viral lifecycle. CDKIs have roles both in cell-cycle regulation and in keratinocyte differentiation (Missero et al. 1995; Di Cunto et al. 1998) In vitro studies have shown that E7 inhibits the cell-cycle arrest function of both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ when E7-expressing keratinocytes are induced to differentiate (Jones et al. 1997; Ruesch and Laimins 1998); PCNA is induced within these cells, and markers of terminal of differentiation are expressed. The abrogation by E7 of keratinocyte differentiation-associated CDKIs (Missero et al. 1995, 1996; Di Cunto et al. 1998) may be associated with its ability to maintain a replication-competent state while permitting the development of a differentiated environment compatible with viral late functions.

L1 and L2 ORFs

The products of the L1 and L2 ORFs are expressed in only the most terminally differentiated keratinocytes and form the viral capsid. The capsid comprises 360 molecules of L1, arranged as 72 capsomers, in an icosahedral surface lattice. Recombinant HPV11 L1 with cysteine-to-glycine mutations forms capsomeres but cannot assemble into capsid-like structures, suggesting that disulphide bonds mediate capsomere binding in the papillomavirus capsid (Li et al. 1998). Cryo-electron microscopy indicates that the L2 protein is situated at the center of pentameric capsomeres in mature virions (Trus et al. 1997).

A number of studies have demonstrated that L2 is required for encapsidation of viral genomes into infectious virions (Roden et al. 1996; Stauffer et al. 1998; Zhou et al. 1993). In the model for L2-mediated assembly of papillomavirus virions, L2 localization to subnuclear domains causes the subsequent recruitment of E2 with bound viral genome and L1 (Okun et al. 2001). This L2–L1–E2-genome association confers an appropriate environment and/or concentration of components for virion assembly. The E2-binding capacity of L2 is important to confer selective encapsidation of PV DNA, and hence this is another possible function of E2-binding sites within the viral genome. Given that virions containing E2 protein have not been detected, it is proposed that E2 acts catalytically in the process of DNA encapsidation during virion assembly.

CLINICAL ASPECTS OF HPV INFECTION

Nongenital skin warts

These are common benign proliferations that occur most frequently in children and young adults. The classification of cutaneous warts includes verruca vulgaris (common warts; Figure 8) verruca plana (plane warts), verruca plantaris (deep plantar warts), mosaic plantar warts, butchers' warts, and erythrodysplasia verruciformis. Although not absolute, each classification is associated with specific HPV types, clinical and histologic appearance, and prognostic features (Moore and

Figure 8 *Common wart showing the characteristic appearance of a rough keratotic papule. Lesions may be multiple or single on cutaneous surfaces, with the most common sites being the dorsal aspects of hands and fingers. Photograph courtesy of Dr Libby Edwards.*

Tyring 2001). Butchers and meat handlers have an unusually high prevalence of hand warts, most of which are caused by HPV-7 and attributed to the combination of maceration and trauma to which these workers are subjected. Studies of warts in children show that spontaneous regression will occur within 2 years in about 60 percent of cases; the remaining third show a spectrum of responses, but eventually most lesions resolve (Massing and Epstein 1963). Immunosuppression as a consequence of transplantation, malignant, or infectious disease (HIV) increases the incidence of skin warts. These lesions are larger, more persistent, and refractory to treatment than in immunocompetent individuals, and HPV types usually identified in Epidermodysplasia verruciformis (EV) (Table 3) are frequently detected in addition to the common cutaneous HPVs.

Epidermodysplasia verruciformis

EV is a genodermatosis and is an HPV-associated, lifelong, genetic disease (for review see (Majewski and Jablonska 2001). The warts appear in early childhood, become widespread, tend not to regress, and invasive cancers can develop from them (Figure 9). EV is an autosomal recessive condition and is associated with specific HPV types (Orth et al. 2001) (Table 3) that persist for life, generating clinical lesions that are

Table 3 *Clinical lesions associated with HPV*

	HPV Types	
Lesion	**Frequent association**	**Infrequent association**
Skin warts		
Plantar warts	1	2, 4, 63
Common warts	2, 27	1, 4, 7, 26, 28, 29, 57, 60, 65
Flat warts	3, 10	2, 26, 27, 28, 29, 41, 49
Erythrodysplasia verruciformis-specific skin lesions	5, 8, 17, 20	9, 12, 14, 15, 19, 21–25, 36, 38, 46, 46, 47, 50
Malignant and premalignant skin lesions		
Bowens disease of the skin	2, 16, 34	
Skin cancers in patients with Erythrodysplasia verruciformis	5, 8	14, 17, 20, 47
Skin cancers in renal transplant patients	1–6, 8, 10, 11, 14–16, 18–20, 23-25, 27, 29, 36, 38, 41, 47, 48	
SCC of the finger	16	
Benign head and neck lesions		
Oral papillomas and leukoplakias	2, 6, 11, 16	7
Focal epithelial hyperplasia	13, 32	
Laryngeal papillomas (RRP patients)	6, 11	
Conjunctival papillomas	6, 11	
Nasal papillomas		6, 11, 57
Malignant head and neck lesions		
Laryngeal cancer		6, 11, 16, 18, 35
Oral cancer		3, 6, 11, 16, 18, 57
Tonsillar/pharyngeal cancer		16, 18, 33
Esophageal cancer		6, 11, 16, 18
Nasal cancer		16, 57
Benign anogenital lesions		
Condyloma acuminata	6, 11	30, 34, 33, 40, 41, 42, 44, 42, 44, 45, 54, 55, 61
Malignant and premalignant anogenital lesions		
CIN, VAIN, VIN, PAIN, PIN	6, 11, 16, 18, 31	30, 34, 35, 39, 40, 42–45, 51, 52, 56–59, 61, 62, 64, 66, 67, 69
Cervical cancer	16, 18, 31, 45	6, 10, 11, 26, 33, 35, 39, 51, 52, 5, 56, 58, 59, 66, 68, plus unclassified types
Buschke–Löwenstein tumors	6, 11	

Abbreviations CIN, cervical intraepithelial neoplasia; PAIN, perianal intraepithelial neoplasia; PIN, penile intraepithelial neoplasia; RRP, recurrent respiratory papillomatosis; SCC, squamous cell carcinoma; VAIN, vaginal intraepithelial neoplasia; VIN, vulval intraepithelial neoplasia.

Figure 9 *Erythropdysplasia verruciformis characterized by flat wart-like lesions or reddish macules, mainly on the face, trunk, and extensor surfaces of arms and legs (Gross et al. 1997 with permission).*

refractory to treatment. In the immunocompetent host, EV HPV types are present in a latent form in the skin but do not manifest themselves clinically unless the individual is immunosuppressed, in which case EV types can be identified in cutaneous neoplasms. Overall, the evidence indicates that the defect in EV is an inadequate cell-mediated immune response to the EV HPV subset. Consistent with this notion is the fact that cutaneous responses to contact sensitizers such as dinitrochlorobenzene (DNCB) are absent in EV patients. An important feature of the carcinomas in EV is that they develop on sun-exposed surfaces, suggesting that UV light is a cofactor in EV HPV viral oncogenesis.

Nonmelanoma skin cancer

Nonmelanoma skin cancer (NMSC) (basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)) are the most prevalent malignancies in fair-skinned individuals worldwide. Epidemiologic studies have implicated HPVs in these cancers, particularly in those arising in immunosuppressed individuals (for review see Harwood and Proby 2002). Depending on the sensitivity of the assays HPV DNA can be detected in up to 80–90 percent of SCCs arising in transplant patients. EV HPV types predominate, but no specific HPV is associated with malignancy (Harwood and Proby 2002). The prevalence of HPV DNA in premalignant lesions such as keratoacanthomas, actinic keratoses, and carcinoma in situ in transplant patients can be as high as in SCCs. The same HPV types predominate in these lesions and in BCCs, although HPV prevalence is lower in the latter (Berkhout et al. 2000).

NMSCs in immunocompetent individuals also harbor HPV DNA but the prevalence is lower (0–50 percent for SCC, 31–44 percent for BCC). However, HPV DNA can be detected in normal skin biopsies in 30–35 percent of individuals, and EV HPV types predominate (Berkhout et al. 2000). Importantly, EV HPVs have been detected in benign hyperproliferative skin disorders such as psoriasis, where 90 percent of lesional skin biopsies contain HPV, particularly HPV-5 and HPV-36 (Favre et al. 2000). This raises the possibility that these associations are not causal but represent reactivation of latent HPV genomes in proliferating keratinocytes, and at present the evidence that HPV is the initiating factor in NMSC is inconclusive.

Aerodigestive tract

Papillomatosis can occur anywhere in the aerodigestive tract – the oral cavity, nasal sinuses, hypopharynx, larynx, bronchial tree, and esophagus. In the oral cavity HPV infection is associated with a range of benign proliferative lesions (Table 4), of which squamous papilloma caused by HPV-6 or -11 is the most common (Figure 10). HR-HPV types are detected in dysplasias and squamous cell carcinomas, but these types are also present in normal oral mucosa and the etiologic role of HPV in oral cancer is questionable. However, the high prevalence of HPV-16 and HPV-33 in tonsillar carcinomas does imply a causal role for HPV in these cancers, and there are some laboratory data to support this (Snijders et al. 1994).

Papilloma is one of the most common benign laryngeal tumors, but its prevalence is difficult to estimate. The incidence of recurrent respiratory papillomatosis (RRP) in children is estimated at 4.3/100 000, and among adults 1.8/100 000 (Derkay 1995). HPVs are the causal agent, with HPV-11 infections predominating followed by HPV-6, -16, and -18 (Dickens et al. 1991). Mixed infections are common. Histologically the lesions are benign, but morbidity is significant as recurrence and/or dissemination affect the patency of the airways. Clinical behavior is variable, and lesions may regress, persist, or progress to malignancy. The latter is cofactor dependent and smoking is a significant risk factor here. Treatment is usually endoscopic excision under magnification. The frequent recurrence and often confluent spread of these lesions make treatment difficult, and patients usually require multiple surgical interventions. Noninvasive effective therapies are desperately required for these individuals.

GENITAL TRACT INFECTIONS

HPV infection of the anogenital skin and mucosae results in lesions with two morphologies: anogenital

Table 4 *HPV types in benign lesions of the oral mucosa*

Lesion	HPV types found	
	Frequently	Infrequently
Normal mucosa	6, 11, 16, 18	7, 31, 33, 59, 61
Verruca vulgaris	2, 4, 57	1, 6, 11, 16
Squamous papilloma	6, 11	2, 13, 16, 32, 31, 33, 35
Focal epithelial hyperplasia	13, 32	6, 11, 16, 24
Koilocytic dysplasia	16, 18, 31, 33, 35	6, 11
Oral epithelial dysplasia	16, 18	2, 6, 11, 31, 33, 35
Leukoplakia	6, 11	2, 16, 18
Oral warts in HIV+ patients	7, 32	2, 6, 11, 13, 16, 18, 55, 59, 69, 72, 73

warts (condyloma acuminata) and squamous intraepithelial lesions (SIL). Condylomata are associated predominantly, but not exclusively, with infection by LR-HPV types and have a low to negligible risk of malignant progression (Table 3). SIL are associated with both low- and high-risk genital HPV types, and high-risk HR-HPV-infected lesions can progress to malignancy. The majority of epidemiologic studies have been focused on HPV infection in the lower genital tract of women, and relatively little is known about nongenital HPV infection and genital HPV infection in men. In women, genital HPV transmission is primarily by sexual contact after the sexual debut, and there is a strong positive trend between prevalence of infection and increasing number of sexual partners (IARC 1996).

Condyloma acuminata

External anogenital warts are the commonest viral sexually transmitted disease (PHLS Communicable Disease Centre 1989). They have been recorded as occurring at an incidence rate of 2.4 cases/1000, with a peak attack rate of 1.2 percent in men and women aged 20–24 years (Persson et al. 1996). The warts are usually exophytic, often mutiple lesions that can occur anywhere on the external genitalia, but are found mainly the perineum and anus in women (Figure 11a) and on the penis and anus in men (Figure 11b). Most lesions regress spontaneously over time if left alone, but in a fraction of infected individuals they can persist for years. They are more frequent in the immunosuppressed, and are refractory to treatment in these patients. Most lesions are caused by HPV-6, about 15 percent are attributable to HPV-11, and a tiny fraction to other HPV types, including HPV-16. Current treatments suffer from high recurrence rates, although trials with the immunomodulator Imiquimod report significantly reduced recurrences post-therapy (Edwards et al. 1998). A rare anogenital form of condyloma acuminata is the Buschke–Löwenstein tumor, which is locally invasive but nonmetastasizing. These lesions usually contain HPV-6 or -11, and rarely HPV-16 (Boshart and zur Hausen 1986).

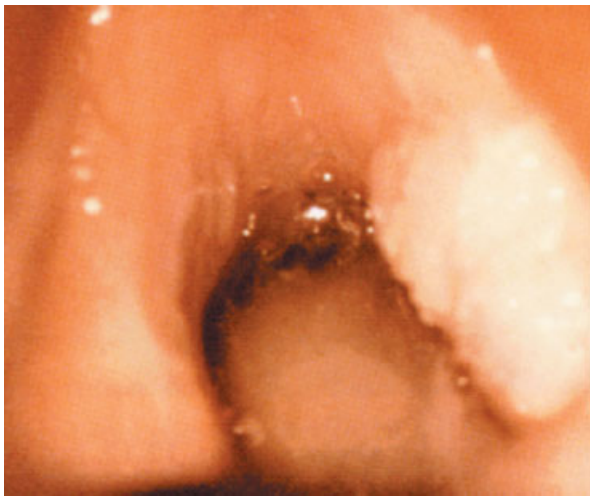

Figure 10 *Solitary adult-onset laryngeal papilloma, associated predominantly with HPV-11 (Gross et al. 1997, with permission).*

Squamous intraepithelial lesions and anogenital cancers

Squamous intraepithelial lesions (SIL) are classified histologically and form a distinct spectrum of histological atypia. Three grades of cervical intraepithelial neoplasia (CIN) are recognized in the European classification: CIN 1 – mild, CIN 2 – moderate, and CIN 3 – severe. The Bethesda classification for cytology used in the USA recognizes two classes: low-grade squamous intraepithelial lesions (LGSIL) (CIN 1) and high-grade squamous intraepithelial lesions (HGSIL) (CIN 2/3). In the vagina, vulva, penis, and anus a similar but not identical spectrum of changes can be identified – VAIN, VIN, PAIN, and PIN – but the likelihood of progression of these lesions to frank malignancies is still unclear. It is probable, but not unequivocally proven, that the majority of these intraepithelial lesions are a result of HPV infection. LGSIL at any site can be associated with both high- and low-risk HPV types, although high-risk types predominate. HGSIL are associated

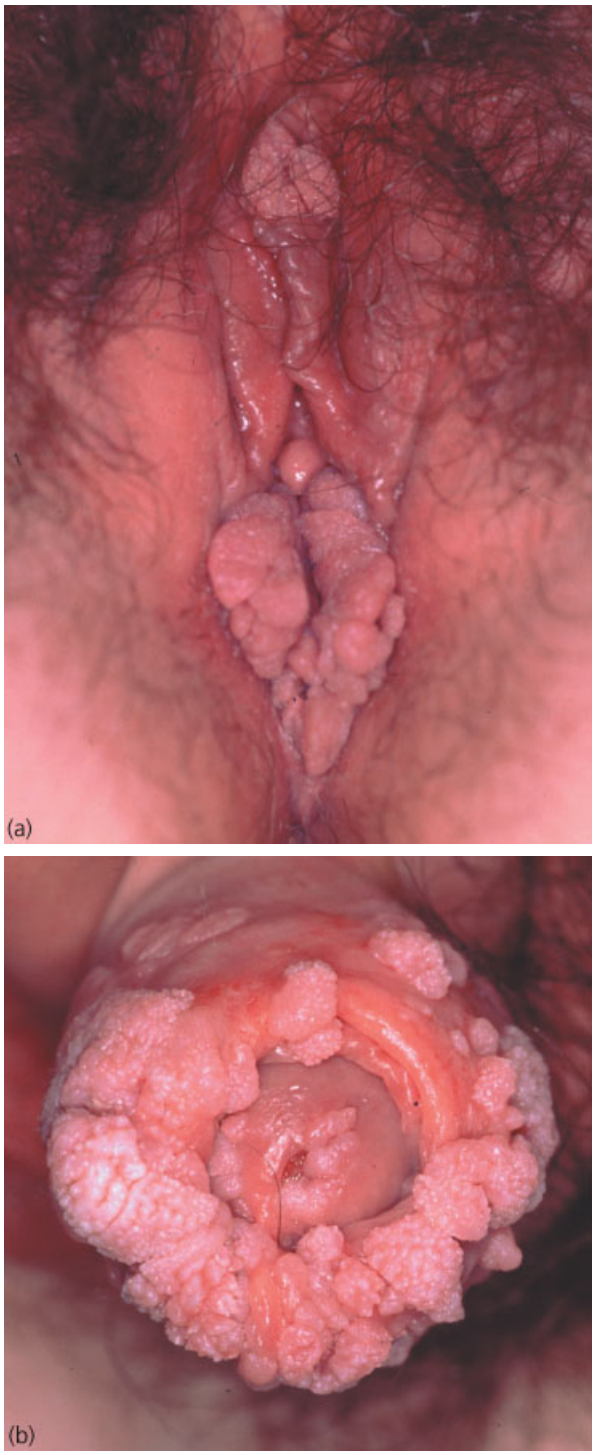

Figure 11 *Condyloma acuminata associated principally with HPV 6.* **(a)** *Severe vulval warts in a 35-year-old woman.* **(b)** *Penile and prepuce warts in a 34-year-old HIV-positive man. Immunosuppression is frequently associated with florid condylomata that are refractory to treatment. Photographs courtesy of Dr Colm O'Mahony.*

almost exclusively with high-risk types. The gross appearance of low- and high-grade cervical SIL is shown in Figure 12.

The epidemiological evidence relating HPV infection to cervical cancer is very strong and the association is considered to be causal (IARC 1996). In all methodologically sound case–control series using reliable HPV DNA detection methods the association between HR-HPVs and cervical carcinoma or the high-grade precursor lesions is very strong with ORs over 15 (reviewed in (Muñoz and Bosch 1996). The strength of this association rules out the possibility of chance, bias, or confounding. Furthermore, the association is consistent, as equally strong associations are found in countries with a high or low incidence of cervical cancer (Muñoz et al. 1992). Reliable large-scale prospective data are available, and it is clear that infection with

Figure 12 *Intraepithelial lesions.* **(a)** *Low-grade cervical intraepithelial neoplasia seen as a raised white area on the posterior lip of the cervix; both low- and high-risk HPVs can be detected in these lesions.* **(b)** *High-grade intraepithelial neoplasia and invasive carcinoma of the cervix, associated with high-risk HPVs, particularly HPV-16 (Gross et al. 1997 with permission).*

HR-HPV types precedes the development of HGSIL (Schiffman and Brinton 1995). The epidemiological evidence alone indicates that more than 95 percent of cervical cancers are attributable to HPV infection (Walboomers et al. 1999). A summary of the natural history of cervical HPV infection is as follows:

- Transient genital HPV infections are quite common (prevalence about 11–20%) in young sexually active women;
- Most infections are subclinical and resolve. Most lesions are self limiting proliferations (LGSIL) and resolve;
- A minority of women develop persistent infections with focally high levels of DNA;
- Some of these persistent infections with high risk HPVs progress to HGSIL;
- Some HGSIL progress to carcinoma.

Mechanisms of genital HPV oncogenesis

As discussed above, experimental studies show that the *E6* and *E7* genes of the high-risk genital viruses encode oncoproteins that deregulate key controls in cell proliferation. E7 binds the retinoblastoma protein pRb, effectively blocking the transcriptional repressor function of this protein and overriding the G_1/S checkpoint of the cell cycle. The E6 of the HR-HPVs binds to p53 to form a stable complex which, in combination with E6-AP, undergoes ubiquitin-mediated proteolysis. The ability to form the E6–E6-AP–p53 complex seems to be restricted to the HR-HPVs. This interaction with p53 is crucial, compromising the ability of the cell to effect growth arrest in the face of DNA damage. As a consequence of these interactions cell-cycle checkpoints are bypassed, mutations may be accumulated, and the HPV-infected cell may acquire the immortal phenotype. However, malignant transformation is a rare consequence of infection with HR-HPVs, only a fraction of infected individuals progress to CIN 3, and only 30–50 percent of CIN-3 progress to invasive cancer. In the normal viral infectious cycle viral gene expression is tightly controlled and E6/E7 expression is confined to nondividing differentiated cells, where the oncogenic potential of these genes is not allowed to flourish. HPV oncogenesis requires the deregulation of viral and cellular gene expression so that early genes are inappropriately expressed in dividing cells. In cervical carcinomas and many HGSIL this deregulation is achieved by integration of the HPV genome into host chromosomes (Daniel et al. 1995; Schwarz et al. 1985).

TRANSMISSION

Transmission occurs primarily through person-to-person contact, with shedding of viral particles from the infected individual to microwounds on the skin or mucosa of a naïve recipient. Genital HPVs are transmitted primarily, but not exclusively, via sexual contact. There is evidence for transmission from the fingers in individuals who have genital warts (Strauss et al. 1999) and for vertical transmission of HPV-16 resulting in oral infection (Puranen et al. 1997). A history of genital warts is a risk factor for juvenile recurrent respiratory papillomatosis (Shah et al. 1998), suggesting intrapartum infection.

LABORATORY DIAGNOSIS

HPVs cannot be grown efficiently in tissue culture or in animal models. Diagnosis of HPV infection relies therefore on the direct detection of HPV genomes and/or gene products by molecular hybridization procedures, usually preceded by nucleic acid amplification, in fresh or fixed tissue biopsies, tissue scrapings, and exfoliated cells. HPV detection and typing are tests not routinely available in diagnostic laboratories.

Serology

Individuals infected with HPV do make a type-specific serum antibody response to the major viral capsid protein L1 (Nonnenmacher et al. 1995; Steele and Gallimore 1990). Virions are not available for ELISA, but L1 VLPs have been used widely as the target antigen in ELISA in seroepidemiologic studies (Stanley 1997). Unfortunately, owing to the highly variable interval between infection, lesion development and seroconversion, plus the relatively low sensitivity of the assay, the detection of anticapsid serum antibody is not useful in diagnosis in the individual patient.

IMMUNITY

HPV infections are common, but the immunopathogenesis of these infections is still poorly understood. The majority of studies have focused on genital infections, and it is clear that these are common in young sexually active individuals, the majority of whom apparently clear the infection without overt clinical disease. Those who develop warts or low-grade intraepithelial lesions also in most cases mount an effective cell-mediated immune response and the lesions regress. Nonregressing anogenital warts and LGSIL are not associated with inflammation or histological evidence of immune activity, but regression of anogenital warts is accompanied by a dense mononuclear cell infiltrate dominated by T cells and macrophages and a cytokine expression characteristic of a Th1 cell-mediated immune response (Coleman et al. 1994). Animal models support this and provide evidence that the response is regulated by $CD4^+$ T cell-dependent mechanisms (Nicholls et al. 2001). The

central importance of the $CD4^+$ T-cell population in the control of HPV infection is shown by the increased prevalence of HPV infections in individuals immunosuppressed as a consequence of either organ transplantation or HIV infection (Benton and Arends 1996). Although it seems clear that the $CD4^+$ T-cell subset is critical for the induction and regulation of the host response to HPV, the antigenic targets and the nature of the effector response are still not known unequivocally. Evidence from both animal models and human infections indicates that one of the dominant responses is to E2 antigens, but responses to other early proteins E6 and E7 have also been identified (Stanley, 2001b). There is increasing evidence that both NK cells and antigen-specific CTLs are important effectors, but these responses are poorly understood.

Neutralizing antibody to the L1 capsid protein accompanies the induction of successful cell-mediated immunity, and these responses are certainly protective against subsequent viral challenge in natural infections in animals, suggesting that vaccines inducing serum neutralizing antibodies to L1 will be protective (Stanley 1997). Prophylactic immunization with VLP subunit vaccines composed of the major capsid protein L1 of some of the genital HPVs are now in phase III clinical trials. The vaccines are immunogenic, inducing type-specific responses; they are safe and early results indicate efficacy (Koutsky et al. 2002).

If the cell-mediated response fails to induce lesion regression and viral clearance then a persistent viral infection results that is, in part, due to operational immune tolerance. This seems to be reflected by the detection in vitro, in persistently infected individuals, of enhanced cellular and humoral responses to early viral proteins but the failure to clear virus in vivo (de Gruijl et al. 1996a; de Gruijl et al. 1996b). The importance of the MHC in susceptibility to or protection from *Papillomavirus* infection and associated neoplastic disease is supported by data from animal models and clinical studies (Beskow et al. 2001), but this area remains a crucial one for further investigation. The increasing understanding of the mechanisms by which the host responds effectively to HPV infection, and the reasons for the failure of these defense mechanisms in a minority of circumstances, has led to the development of therapeutic immunologically based strategies targeted to both high- and low-risk viruses.

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Polyomaviruses

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INTRODUCTION

During the last 30 years polyomaviruses, in particular SV40, have been extensively utilized by molecular biologists to decipher complicated biological events including gene transcription, RNA processing, control of RNA translation, and DNA replication in eukaryotic cells. In recent years, these viruses have received special attention in the field of biomedical science due to their involvement and association with diseases in humans, ranging from demyelinating disorders and nephropathy to various types of cancers including brain tumors, lung malignancy, and lymphomas. These new developments have encouraged many biomedical and basic scientists to employ a variety of approaches and techniques to unravel the interaction of polyomaviruses with their specific host cells, and decipher the pathogenesis of the diseases associated with polyomaviral infection. Here, we have provided an overview highlighting the general features of polyomaviruses that include their structural organization, gene regulation, and replication prior to focusing our attention on three members of this group of viruses, JCV, BKV, and SV40, due to their increasing importance in the clinical setting. We further emphasize JCV due to its established association with the fatal demyelinating disease of the brain and its association with neural and nonneural malignancies.

GENERAL DESCRIPTION

Polyomaviruses have been isolated from a number of species including monkeys, rodents, and birds, as well as humans. The common feature of the members of the family *Polyomaviridae* rests on their unique morphology which includes a 45-nm icosahedral particle comprised of three capsid proteins, and their genome which consists of double-stranded circular DNA of approximately 5 000 nucleotides in size.

While productive infection with polyomaviruses is generally species-specific, expression of the viral genome appears to be less restricted and occurs in a broad range of species. Nevertheless, transcription activation of the viral promoter is tissue-specific for some members of the polyomavirus family. Humans are the established host for BKV and JCV, while primates, including rhesus monkeys, African green monkeys, baboons, and stump-tailed macaques are the hosts for simian virus 40 (SV40), lymphotropic papovavirus (LPV), simian agent 12 (SA12), and stump-tailed macaque virus (STMV), respectively. Detection of SV40 in several human tumors in recent years has raised the possibility of humans as co-hosts for this virus. Mice are the host for polyomavirus (PYV), while rabbits and hamsters are considered the hosts for rabbit kidney vacuolating virus (RKV), and hamster polyomavirus (HaPyV), respectively. Bovine polyomavirus (BPyV) and budgerigar fledgling polyomavirus (BFPyV) target cattle and birds, respectively. In general, polyomaviruses possess an oncogenic potential in several experimental animals and it is believed that the viral early protein plays a role in the oncogenicity of the virus.

In permissive hosts, lytic infection of the cells with polyomaviruses begins by binding of the virion to the cell surface proteins. For example, the association of SV40 with class I major histocompatibility complex is an early event for infection of monkey kidney cells with the

virus. Human polyomaviruses, such as JCV, however, appear to employ different cell surface receptors, most probably *N*-linked glycoprotein containing terminal α (2–6) linked sialic acid for its attachment to permissive cells (Atwood 2001). In either case, it is believed that the major capsid protein, VP1, plays a critical role in binding of the virus to its cell surface receptor. Once attached to the cell membrane, the virus internalizes through the process of endocytosis, and after uncoating, viral DNA appears in the nucleus. The viral DNA is present in a chromatin-like structure associated with cellular histone protein H2A, H2B, H3, and H4. The genome of the polyomavirus is divided into early and late regions on the basis of whether they are expressed before or after DNA replication. Viral proteins are encoded on both DNA strands and are transcribed divergently from a central regulatory region. The early genes encode the large and small tumor antigens, multifunctional proteins that regulate progression of the viral lytic cycle, and deregulate several important pathways in host cells. The small t- and large T-antigens have a common amino terminus and arise from alternatively spliced transcripts. In more recent studies, an altered isoform of T-antigen, named T′, has been detected in JCV infected cells (Frisque 2001). In SV40, a third protein named SELP, whose open reading frame is located in the leader of some early DNA species, has been reported yet its function remains unknown (Khalili et al. 1987b). Other polyomaviruses, including JCV and BKV, also possess an early leader open reading frame which exhibits greater than 50 percent homology with SELP. The late genes encode structural components of the viral capsid proteins, VP1, VP2, and VP3. VP1 is encoded by the 3′ end of the late region and is translated from a distinct mRNA in a different reading frame than the other two capsid proteins, VP2 and VP3. Both VP2 and VP3 are encoded in the 5′ end of the late region and are translated from a common mRNA. The VP1 mRNA leader sequence codes for a small protein named 'Agnoprotein' which is found only in SV40, JCV, and BKV polyoma species.

The full lytic infection cycle of polyomaviruses can be divided into two phases: the early phase occurs prior to viral DNA replication and the late phase, which includes all events such as viral DNA replication, expression of the viral late genes, assembly of progeny viral particles, and viral release. During the early phase, the virus attaches to the cell surface, enters the cell through endocytosis, transports to the cell nucleus and after uncoating, the viral early gene begins to transcribe and synthesize viral early RNA, and after translocation, the early proteins, T-antigen, and its various isoforms. Once T-antigen is accumulated in the nucleus of the cells, it cooperates with cellular DNA replication factors to stimulate viral DNA replication, and at the same time enhances the level of viral late gene expression. The products of the late genes including the capsid proteins, VP1, VP2, and VP3, enter the nucleus and participate in the assembly of viral progeny through the association of the viral capsid proteins with the newly synthesized viral DNA. One of the most intense areas of research on polyomaviruses has been on the regulation of viral gene transcription, cell type-specific activation of the viral genome, and the control of viral DNA replication. The DNA regulatory sequence which is responsible for transcription and replication of the viral genome spans the 400–500 nucleotides which are positioned between the viral early and late genomes.

This region is composed of many *cis*-acting regulatory motifs that provide targets for binding of cellular transcription factors. Of note, the transcription of polyomaviruses is dependent on the host transcriptional machinery. The association of the proper transcriptional factors with the viral DNA sequence potentiates RNA polymerase II and its subunits to initiate viral early gene transcription. Lengthy studies on the identification of cellular factors that stimulate the SV40 promoter led to the identification of several important proteins, such as Sp1 and the AP1 family, which exhibit a broad spectrum of regulatory functions upon eukaryotic gene transcription. It was also noted that the interaction of cellular proteins with the viral DNA sequence may not be sufficient for stimulation of viral promoters, suggesting that cross-communication and indirect interaction may play a critical role in viral gene expression.

HUMAN POLYOMAVIRUSES

JCV

The infection process of JCV, as of any nonenveloped small DNA virus, begins with the attachment of the viral particles to the cell surface receptors. Recent reports indicate that JCV enters the susceptible cells through clathrin-dependent endocytosis and α(2–3)-linked sialic acid appears to be critical in this process (Atwood 2001; Liu et al. 1998a,b; Pho et al. 2000). Subsequently, the viral genome is targeted into the nucleus by an unknown mechanism. Infectious virions are formed after DNA replication and expression of capsid proteins and finally, viral particles are released upon cell death.

The nucleotide sequence analysis of JCV genome has revealed that it is composed of three functional regions including the viral early coding region, the viral late coding region, and the viral noncoding regulatory region (Frisque et al. 1984) (Figure 1). Comparison of JCV sequences with two other closely related polyomaviruses, BK virus and SV40, has revealed that it exhibits extensive sequence homology in coding regions to its counterparts. However, the sequences within the regulatory region of JCV show the greatest degree of divergence from those of BK virus and SV40. Studies over the years have shown that the regulatory region of

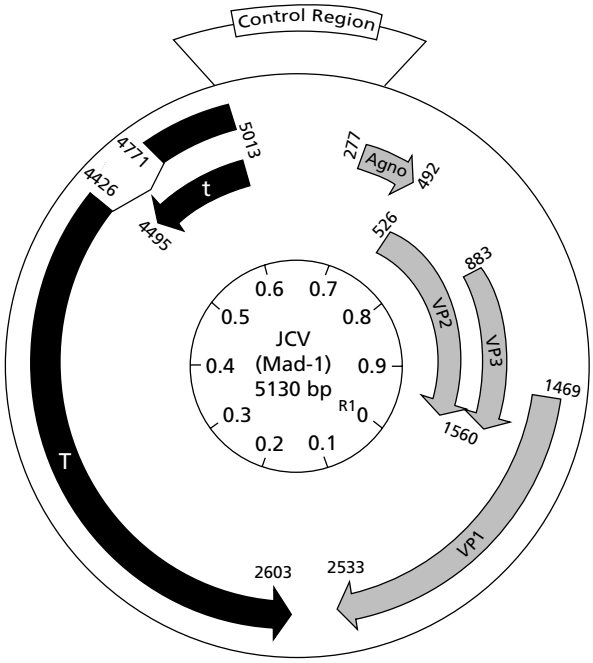

Figure 1 *Circular map of the JCV genome. Schematic representation of the JCV genome. Directions of the early and late coding regions are depicted with arrows. Early genes encode small t- and large T-antigens. Late genes encode the viral capsid proteins, VP1, VP2, and VP3, and Agnoprotein. The regulatory region is located between the two coding regions.*

JCV confers, at least in part, the tissue specificity of JCV gene expression (Ahmed et al. 1990a; Amemiya et al. 1989, 1992; Chowdhury et al. 1990, 1992, 1993; Khalili et al. 1988; Tada and Khalili 1992; Tada et al. 1989, 1991).

The regulatory region of JCV is composed of the origin of DNA replication, promoter elements for both early and late genes and *cis*-acting enhancer elements (Figure 2). The JCV origin of DNA replication is a 98-bp element located between the NF-κB motif and the first tandem repeat of JCV Mad-1 strain. It shows extensive homology to the SV40 origin (Frisque and White 1992; Kim et al. 2001). The enhancer elements are composed of two complete 98-bp tandem repeats (Mad-1 strain), each of which contains its own TATA box located in the early site of individual repeat. The first TATA box with respect to the origin of DNA replication is involved in the positioning of the transcription start sites for viral early genes (Frisque and White 1992; Kim et al. 2001; Lynch and Frisque 1990, 1991). The second TATA box does not appear to have a similar function for the viral late genes. The remaining regions within the 98-bp repeats have been shown to confer crucial *cis*-acting elements which were shown to serve as binding sites for several transcription factors (Frisque and White 1992; Kim et al. 2001; Major et al. 1992; Raj and Khalili 1995).

The regulatory region of JCV appears to be hypervariable in nature (Vaz et al. 2000; Yogo et al. 2001; Zoltick et al. 1995). Comparison of the regulatory sequences among a number of JCV isolates revealed that the hypervariability is mostly confined to 98 base pair tandem repeat region (Figure 2). Based on the variations including deletions and duplications, JCV isolates are classified into two classes or groups (Frisque and White 1992; Vaz et al. 2000; Yogo et al. 2001; Zoltick et al. 1995). The class I viruses are characterized by the presence of the 98-bp tandem repeat within its regulatory region. The Mad-1 strain of JCV belongs to class I. The class II viruses contain strains that exhibit variations from the regulatory region of the class I with deletions and insertions. Ninety-eight base pair repeats appear to vary in size and the distal repeat with respect to the origin of DNA replication lacks the TATA box sequences. There tends to be a full length or partial duplication of the 23-bp sequence element usually occurring at nucleotide 36 of the first tandem repeat (Frisque and White 1992). Although the mechanism by which these deletions and duplications occur and give rise to new strains of JCV remains unknown, it is generally

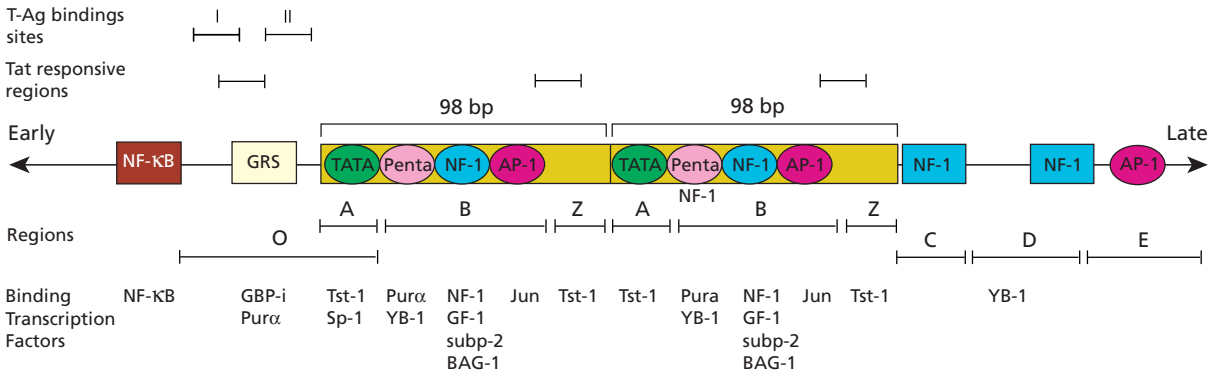

Figure 2 *Multiple transcription factor binding sites in the enhancer/promoter region of Mad-1. Transcription factors that have been shown to bind to the promoters are illustrated at the bottom of the schematic.* Cis*-acting elements are depicted in the boxes or circles in the schematic of the promoter. T-antigen binding sites and Tat responsive elements are indicated. Arbitrary subregions including A, B, Z, C, D, E, and O are indicated.*

accepted that both classes are derived from a common ancestral form of the JCV archetype strain which is mainly detected in the kidney (Gardner et al. 1971). It is postulated that alterations within the regulatory region of the JCV archetype strain can result in novel regulatory rearrangements of the virus with altered features. The new virus is then capable of replicating in new cells and tissues (Frisque and White 1992).

The Mad-1 strain of JCV has been used for most of the replication and transcriptional studies of JCV. As a result, the descriptions of the viral promoter-enhancer elements and the participation of these elements in viral gene transcription and DNA replication will be discussed with respect to Mad-1. In addition to containing the NF-κB motif and origin of DNA replication, the regulatory region of Mad-1 also confers two exact 98-bp tandem repeats (Figure 2) (Frisque and White 1992), which dictate the transcription of early and late genes in opposite directions. The *cis*-acting elements present within and around the 98-bp repeats were shown to be targets for tissue-specific and ubiquitous transcription factors (Figure 2). In addition to these cellular factors, the viral regulatory proteins, including large T-antigen and Agnoprotein, were also shown to regulate expression of the viral promoters (Raj et al. 1996; Safak et al. 2001, 2002). As stated earlier large T-antigen is a multifunctional phosphoprotein involved in both viral early and late gene expression, as well as in T-antigen-dependent viral DNA replication. Together with the cellular factors which are required for DNA replication, T-antigen binds to the viral origin of DNA replication, unwinds it and initiates replication bidirectionally (Lynch and Frisque 1990; Tavis and Frisque 1991). T-antigen regulates its own transcription via an autoregulatory loop, and plays a major role in the transcriptional switch from viral early to late gene expression (Khalili et al. 1987a; Lashgari et al. 1989).

The regulatory region of JCV is subdivided into arbitrary regions for the sake of simple transcriptional analysis (Figure 2). The cellular factors that interact with B region for example are extensively studied and include a 45 kDa protein (Khalili et al. 1988; Raj et al. 1998), GF-1 (Kerr and Khalili 1991), YB-1 (Raj et al. 1996), Purα (Chen et al. 1995), and AP-1 (Amemiya et al. 1992; Sadowska et al. 2003), Subp-2 (Fukita et al. 1993; Kerr and Khalili 1991), and BAG-1 (Devireddy et al. 2000). Computer-aided experimental analysis of the regulatory region have revealed the presence of multiple NF-1 binding sites included in the B, C, and D regions (Raj and Khalili 1995). The NF-1 transcription factor was shown to be involved in both viral transcription and replication (Kumar et al. 1993; Sock et al. 1993; Tamura et al. 1988, 1990). GF-1 is a partial recombinant protein cloned by Kerr and Khalili (1991) from a human fetal brain expression library and represents a human homologue of the cloned murine Sμbp-2 protein (Fukita et al. 1993). Its binding specificity is similar to that of NF-1. GF-1 was found to *trans*-activate viral early and late promoters (Kerr and Khalili 1991). YB-1 and Purα were shown to interact with this region. Recently, a novel Bcl-1-interacting protein, BAG-1, was cloned from p19 embryonic carcinoma cells using the JCV NF-1-binding site as a probe. It is expressed ubiquitously and regulates JCV early and late promoters through the NF-1 binding site (Devireddy et al. 2000). Another cellular factor that interacts with the B region is c-Jun, a member of the AP-1 family of transcription factors. The members of this family are known as the immediate–early inducible proto-oncogenes. Its binding activity to the B region appears to be modulated by NF-1 transcription factor which binds to adjacent NF-1-like sequences (Amemiya et al. 1992). It has been demonstrated recently that it interacts directly with its target sequences and activates transcription from the JCV promoters (Amemiya et al. 1992; Sadowska et al. 2003). Like AP-1, NF-κB, and GBP-i represent inducible cellular factors that modulate gene expression from viral early and late promoters. NF-κB represents a large family of transcription factors which are inducible in response to a wide variety of extracellular stimuli including the presence of phorbol esters and cytokines. The NF-κB family of proteins was shown to play a role in determining the basal and induced levels of transcription from viral early and late promoters (Ranganathan and Khalili 1993). While constitutively expressed subunits p50 and p52 activate transcription from the D domain (Raj et al. 1996), subunit p65 activates transcription from the NF-κB motif (Ranganathan and Khalili 1993). p50/p65 were also shown to indirectly influence JCV gene transcription through a 23-bp element present within the regulatory regions of many JCV variants (Safak et al. 1999). GBP-i interacts with GRS sequences and suppresses viral late promoter activity (Raj and Khalili 1994). Another regulator of viral early and late gene expression is the transcription factor Tst-1, a member of the well-characterized tissue-specific and developmentally regulated POU family of transcription factors. It was found to have distinct binding sites at A and Z regions. Tst-1 regulates viral early and late gene promoters by interacting with the A region (Renner et al. 1994; Wegner et al. 1993). In addition, the physical interaction of Tst-1 with JCV T-antigen was shown to lead to synergistic activation from both promoters (Renner et al. 1994). Even though a potential binding site for a ubiquitously expressed transcription factor, Sp-1, exists within the regulatory region of JCV, its function in the viral transcription is largely unknown. However, a polymerase chain reaction (PCR)-amplified variant of JCV was recently shown to confer a functional Sp-1 binding site. Transcriptional studies using Sp-1 as a *trans*-activator revealed that Sp1 stimulates transcription from the viral early promoter (Henson et al. 1992; Henson 1994). In addition to its own regulatory proteins, the JCV genome was also shown to be cross-

regulated by the regulatory proteins encoded by other viruses outside the polyomavirus family, including the HIV-1 Tat protein and the cytomegalovirus (CMV) immediate–early *trans*-activator protein 2 (IE2) (Tada et al. 1990; Winklhofer et al. 2000).

Compared to its counterpart, SV40, JCV exhibits a relatively long and inefficient lytic cycle which does not appear to conform with the progression of the viral illness, progressive multifocal leukoencephalopathy (PML). However, there is now experimental evidence to account for this discrepancy. In addition to its transcriptional regulation by a number of cellular factors and its own large T antigen, the JCV promoter has also been shown to be regulated by additional factors including cytokines, growth factors associated with immunosuppression, helper viruses such as human immunodeficiency virus type 1 (HIV-1) (Atwood et al. 1995; Tada et al. 1990), and cytomegalovirus (Heilbronn et al. 1993; Winklhofer et al. 2000). Growth factors and cytokines appear to mediate direct or indirect effects on viral promoters by induction of transcription factors, including GBP-i, NF-κB, and c-Jun (Raj and Khalili 1994; Ranganathan and Khalili 1993; Sadowska et al. 2003). An HIV-1-encoded transregulatory protein, Tat, was shown to transactivate the JCV promoter by interacting with two distinct regions within the viral regulatory region (Chowdhury et al. 1990, 1992, 1993; Tada et al. 1990).

NEUROTROPIC FEATURES OF THE JCV GENOME

Observations from both promoter-swapping experiments and somatic cell hybridization studies have indicated that the *cis*-acting promoter/enhancer elements of JCV and tissue specific cellular factors are the two main determinants that contribute to the narrow host range and neurotropism of JCV. The promoter/enhancer swapping experiments between SV40 and JCV in transgenic mice (Feigenbaum et al. 1992) and transfection studies in tissue culture indicated that narrow host range and cell specificity of JCV may be largely attributed to the JCV promoter/enhancer elements, as well as to the unique characteristics of JCV T antigen (Feigenbaum et al. 1987; Frisque and White 1992; Lashgari et al. 1989; Lynch et al. 1994; Tada et al. 1989). Reporter gene experiments using the regulatory region of JCV in in vivo and in vitro transcription assays (Ahmed et al. 1990a,b) indicated that expression of the viral early promoter is substantially higher in glial cells than nonglial cells, pointing to the importance of tissue-specific factors in JCV neurotropism (Ahmed et al. 1990b). However, JCV DNA replication and late gene expression can occur when large T-antigen is provided in nonglial cells (Lashgari et al. 1989). Somatic cell hybridization studies between transformed hamster glial cells expressing viral T antigen and mouse fibroblast cells supported the finding from transfection studies and suggested the presence of positive regulatory factors in glial cells and negative regulatory factors in nonglial cells (Beggs et al. 1988).

DISEASES ASSOCIATED WITH JCV INFECTIONS

Progressive multifocal leukoencephalopathy

PML is a white matter disease of the central nervous system resulting from the lytic infection of oligodendrocytes by JCV (Berger and Concha 1995; Major et al. 1992; Walker et al. 1973). The JCV archetype strain initially causes a latent infection in the kidneys. Immunosuppression of the host system due to an underlying disease, such as Hodgkin's lymphoma, lymphoproliferative disease, or acquired immune deficiency syndrome (AIDS) leads to the reactivation of the JCV archetype strain. During this reactivation period, the virus undergoes rearrangement in its regulatory region resulting in the Mad strains. The Mad strains then travel to the brain by an unknown mechanism, perhaps through B lymphocytes, and infect the myelin-producing cells of the central nervous system, the oligodendrocytes. Infection of oligodendrocytes results in the destruction of myelin, creating sporadic plaques of demyelination in the brain. Hence, demyelination occurs as a multifocal process, rarely unifocal, and develops in the white matter of the cortex, though plaques may also occur in any region of the central nervous system (CNS) including the brainstem and cerebellum, but excluding the spinal cord (Brooks and Walker 1984; Thomson and Jones 1968). Clinically, visual deficits are a common sign and symptom of PML at the time of presentation, accounting for 35 to 45 percent of cases, while motor weakness accounts for 25 to 33 percent of cases, and mental deficits (emotional liability, difficulty in memory, and dementia), account for 33 percent of cases at presentation (Berger and Concha 1995). PML usually progresses to death within 4–6 months. In occasional cases, clinical signs and symptoms appear to remain stable for a long period of time (Berger and Concha 1995; Major et al. 1992; Zoltick et al. 1995).

Historically, PML-like pathological cases were described by German pathologist Hallervorden as early as 1930 (Hallervorden, 1930) in a monograph entitled *Unique and non-classifiable processes*. However, the term PML was not crystallized as a distinct entity until the late 1950s. Astrom et al. described the illness in 1958 on the basis of its unique pathological features: demyelination, abnormal oligodendroglial nuclei, and giant astrocytes (Astrom et al. 1958). Descriptions made by these investigators were consistent with the development of a multifocal distribution of small or confluent white matter lesions in the cerebellum, basal ganglia, thalamus, and brain stem. Although the cause of these lesions was not known at that time, involvement of a virus in the pathogenesis of PML was suspected when inclusion bodies were seen in the nuclei of damaged

oligodendrocytes (Cavanaugh et al. 1959). Several years later, the use of electron microscopy for the identification of the suspected particles revealed that these particles resembled a polyomavirus in the enlarged nuclei of oligodendrocytes (Zu Rhein 1969; Zu Rhein and Chou 1965). Several attempts were made to isolate the suspect virus without success because of its unrecognized and unique biological properties, such as narrow host range and tissue specificity. In 1971, when Padgett and colleagues used brain tissue from a patient with PML as a source of innoculum in cell cultures derived from human fetal brain, they were successful in isolating a human polyomavirus from long-term cultures mainly consisting of glial cells (Padgett et al. 1971). This was the first direct evidence suggesting that a neurotropic viral agent was associated with the occurrence of PML. It was a coincidence that a virus similar in structure found in the urine of a patient who underwent a renal transplantation was also isolated at the same time (Gardner et al. 1971). Both viruses were associated with immunosuppressive states and named with the initials of donors, JC virus (JCV) for the patient with PML and BK virus (BKV) for the patient undergoing a renal transplantation.

Lytic infection of oligodendrocytes by JCV results in the destruction of the myelin-producing cells. The primary function of these glial cells is to myelinate the axons that project from the neuronal cell bodies of the overlying cortex. Destruction of these cells initially leads to microscopic lesions (Berger 2000; Major et al. 1992) (Figure 3b), but as the disease progresses, demyelinated areas become enlarged and eventually may coalesce, making them visible upon gross examination of cut sections (Astrom et al. 1958) (Figure 3a). In most cases astrocytes, the other component of glial cells, are also affected by infection exhibiting enlarged, lobulated, and bizarre looking nuclear structures (Major et al. 1992; Walker et al. 1973) (Figure 3b). Lipid-laden macrophages are frequently found in the areas of demyelination. Perhaps they are recruited into the CNS as a result of immune reactivation to phagocytize the myelin breakdown products. In most cases, due to the association of PML with AIDS, large numbers of HIV-infected macrophages are also found in extremely extensive necrotic lesions (Berger et al. 1987). It is not clear, however, how these immune cells infiltrated the areas of demyelination. One possible explanation is that JCV infection may recruit HIV-infected macrophages into the demyelinated areas or alternatively, uninfected macrophages may become infected by HIV after they are recruited into the nervous system.

Association of JCV with cancer

JCV causes tumors in experimental animals It is well established that polyomaviruses including JCV, BKV, and SV40 induce a variety of tumors in experimental animals (Krynska et al. 1999a; London et al. 1978, 1983; Varakis et al. 1978; Walker et al. 1973). They have the ability to induce neoplastic cell transformation in tissue culture as well. Shortly after its isolation from a brain tissue sample of a PML patient, JCV, like SV40, was confirmed to have tumorigenic potential in tissues of neuronal origin (Varakis et al. 1978; Walker et al. 1973). In this line of experiments, inoculation of JCV into several experimental animals resulted in a variety of tumors depending on the animal type, age, and site of inoculation. For example, more than 80 percent of newborn Syrian hamsters when inoculated intracerebrally and subcutaneously with the Mad-1 strain of JCV developed medulloblastomas, glioblastomas, and neuroblastomas (Varakis et al. 1978; Walker et al. 1973). In addition, the presence of an entire biologically active JCV genome was demonstrated when cells from these tumors were cocultivated with permissive glial cells (Walker et al. 1973). In another experiment, JCV was inoculated intraocularly into newborn hamsters. Abdominal neuroblastomas were developed in some cases. Metastases occurred to the bone marrow, lymph nodes, and liver (Walker et al. 1973).

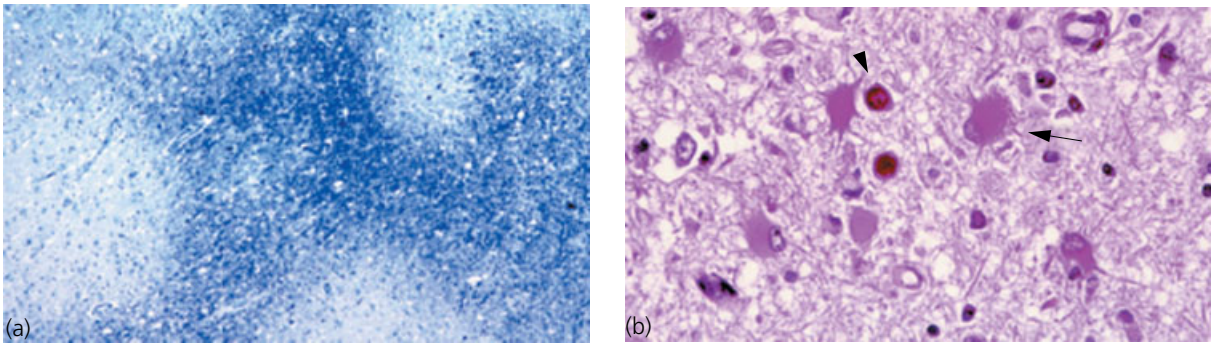

Figure 3 *Histopathological examination of a progressive multifocal leukoencephalopathy case.* **(a)** *A special staining for myelin demonstrates several areas of myelin loss in the white matter (Luxol Fast Blue, original magnification ×40).* **(b)** *Enlarged transformed astrocytes (arrow), and oligodendrocytes harboring intranuclear inclusion bodies (arrowhead) are present within the demyelinated plaques (hematoxylin-eosin, original magnification ×400).*

Interestingly, unlike the other members of the polyomavirus family (BK virus and SV40), JCV was shown to have the potential to induce tumors in nonhuman primates, i.e. monkeys. In order to mimic a case resembling PML in humans, owl and squirrel monkeys were inoculated with JCV subcutaneously, intraperitoneally, or intracerebrally (London et al. 1978, 1983). After inoculation, animals developed tumors at varying time intervals. For instance, one owl monkey developed a malignant cerebral tumor similar to an astrocytoma seen in humans 16 months after inoculation. Another developed a malignant neuroblastoma 25 months after inoculation.

Transgenic mice were used to mimic the acute demyelination seen in PML-affected brain tissue. In this line of experiments, a transgenic mouse line was created with the regulatory and coding sequences for JCV T-antigen (Small et al. 1986b). Some of the offspring from this transgenic mouse exhibited a mild to severe tremor phenotype. Hypomyelination and dysmyelination were observed in the CNS, but not in the peripheral nervous system. In addition, dysmyelination was further characterized in transgenic mice by Trapp et al. (1988) by examining the expression of JCV T-antigen and myelin-specific genes. Initial examination of brains from transgenic mice revealed that the amount of myelin sheath wrapped around the axons was relatively low. The expression levels of proteolipid protein (PLP), myelin basic protein (MBP), and myelin-associated glycoprotein (MAG) genes appeared to be normal. However, respective protein levels for each gene were shown to be reduced. Although the mechanism by which T-antigen plays a critical role in the reduction of respective protein levels in the brain of transgenic mice remains unknown, several suggestions were made in this regard. T-antigen may exert its function by altering the levels of both proteolipid and myelin basic protein synthesis or by inhibiting the maturation of oligodendrocytes. Additionally, JCV T-antigen may mediate its tumorigenic potential through its interaction with tumor suppressor genes, such as p53 and retinoblastoma gene products, Rb. Consequently their functions appear to be disturbed by the presence of T-antigen in the cell cycle. Coimmunoprecipitation assays using cellular extracts from JCV-transformed glial cells have revealed that JCV T-antigen forms complexes with Rb, p53, and p107 (Monier 1986). Supporting these findings, a report by Rencic et al. (1996) suggests a role for T-antigen in the induction of an oligoastrocytoma in an immunocompetent patient. This group also described the formation of different tumors in tissues derived from neuronal origin in transgenic mouse models (Franks et al. 1996; Gordon et al. 2000; Krynska et al. 1999a). The JCV early gene, large T-antigen, under the control of its own promoter was utilized to create these transgenic animal models. Histological and histochemical analysis of the tumor masses revealed the expression of the JCV oncogenic protein large T-antigen in tumors but not in control tissues. Oddly, transgenic animals created with the early region of JCV archetype strain (Krynska et al. 1999a) did not show any sign of hypomyelination in the central nervous system that is a feature that was seen with a transgenic animal created by Small et al. (1986b). On the contrary, cerebellar tumors which resemble human medulloblastomas were induced in those transgenic animals (Krynska et al. 1999a). In another transgenic line, half of the animals developed large, solid masses within the base of the skull by 1 year of age. Histological evaluation of the tumors by location and immunohistochemical studies demonstrated that these tumors arose from the pituitary gland (Gordon et al. 2000).

Detection of JCV in human tumors In addition to the induction of tumors in experimental animals, the JCV genome has also been detected in a variety of human tumors, which increases the likelihood of the involvement of JCV large T-antigen in tumor formation. In fact, Richardson, who first described PML (Richardson 1961), reported an incidental case of oligodendroglioma in a patient with concomitant chronic lymphatic leukemia and PML. Following this report, several more cases describing the concomitant occurrence of PML with different human tumors were also reported. Sima et al. reported the association of PML with multiple astrocytomas in 1983 (Sima et al. 1983). Similarly, Castaigne et al. (1974) described a case where a patient with a long history of immunodeficiency syndrome, in addition to PML, showed numerous foci of anaplastic astrocytes. Examination of the demyelinating lesions by electron microscopy demonstrated the presence of viral particles in both oligodendrocytes and astrocytes within PML foci, but not in the neoplastic astrocytes (Castaigne et al. 1974). A recent report by Shintaku and colleagues revealed dysplastic ganglion-like cells in a patient with PML (Shintaku et al. 2000). A large number of dysplastic or dysmorphic cells which showed properties of neurons were detected in the cerebral cortex. The expression of JCV large T-antigen, but not the viral capsid proteins, was detected in the infected neurons.

In addition to the cases of concomitant PML and cerebral neoplasm, JCV has been shown to be associated with human brain tumors in the absence of PML lesions. Boldorini et al. reported the detection of JCV DNA in the brain tumors of an immunocompetent patient with a pleomorphic xantoastrocytoma (Boldorini et al. 1998). Detection of JCV viral DNA and expression of large T-antigen were reported by Rencic et al. in tumor tissue from an immunocompetent HIV-negative patient with an oligoastrocytoma (Rencic et al. 1996). These two cases demonstrated the association of JCV in brain tumors of immunocompetent non-PML patients. Such findings further prompted attempts to establish the association of JCV with different types of brain tumors

in humans. Analysis of multiple brain tumors for the detection of JCV genome revealed that 57.1 percent of oligodendrogliomas, 83.3 percent of ependymomas, 80 percent of pilocytic astrocytomas, 76.9 percent of astrocytomas, 62.5 percent of oligoastrocytomas, and 66 percent of anaplastic oligodendrogliomas contained JCV early gene sequences (Del Valle et al. 2001, 2002a). Further, the JCV genome has been repeatedly detected in pediatric cerebellum tumors, medulloblastomas, and the viral early protein and the late auxiliary protein, Agnoprotein have been detected in tumor cells (Figure 4) (Del Valle et al. 2002b; Krynska et al. 1999b).

JCV genomic DNA has also been detected in tumor tissues that arise from other than neural origin. Recent reports indicate that the JCV genome is detected in the gastrointestinal tract and solid non-neural tumors such as colorectal cancers (Enam et al. 2002; Laghi et al. 1999; Ricciardiello et al. 2000, 2001).

BKV

Similar to JCV, BK virus (BKV) is widespread in the human population. Greater than 80 percent of the world's population is seropositive for viral proteins. The BKV genome is composed of 5 153 nucleotides and has a typical polyomavirus genomic organization. BKV shares greater than 75 percent sequence identity with JCV, with great divergence within the 300–500-bp noncoding regulatory region. The control region of BKV exhibits sequence variation among the various isolates as a result of nucleotide insertions, deletions, and rearrangements. The control region of the BKV archetype spanning a 375-bp sequence is comprised of several regulatory motifs serving as the potential binding site for regulatory proteins such as Sp1, NF-1, NF-κB, c-Jun, AP1, and many others. These elements are scattered within five arbitrarily divided regions named O (142 bp), P (68 bp), Q (39 bp), R (63 bp), and S (163 bp). In a typical BKV serotype 1 Dunlop strain, the control region is comprised of O (142 bp), three imperfect P regions: P (68 bp), P (50 bp), P (64 bp), and S (63 bp). It is postulated that rearrangement in the control region alters the architectural organization of the viral enhancer/promoter permitting efficient expression of the viral genome in the host. Lytic infection of BKV begins with the expression of the early genome whose product, T-antigen, orchestrates viral DNA replication and late gene activation. Similar to JCV, primary infection with BKV is usually asymptomatic and occurs during childhood with the highest incidence of transmission between the ages of 1 and 6 years. While the route of infection has not been well established, the association of mild upper respiratory illness and tonsillitis during exposure to the virus and subsequent seroconversions have led to speculation that the upper respiratory tract is the route of primary infection with BKV. After primary infection, BKV is likely distributed to all organs via peripheral blood cells and establishes a harmless latency, most notably in the kidney. Reactivation of BKV has been observed in the urinary tract under certain physiological conditions such as pregnancy; kidney, bone marrow, and heart transplantation; immunodeficiency disorders; systemic lupus erythematosus; and immunosuppressive therapy. After reactivation of BKV, an inflammatory syndrome affecting several organs has been detected. It is hypothesized that changes in the immune system and other physiological conditions cause dysregulation of cytokines and immunomodulators that lead to potentiation of transcriptional factors and/or suppression of transcriptional inhibitors that are involved in the control of BKV enhancer/promoter activities. Supporting this notion, it was demonstrated that interaction of nuclear factor (NF-1) with its target sequence within the BKV

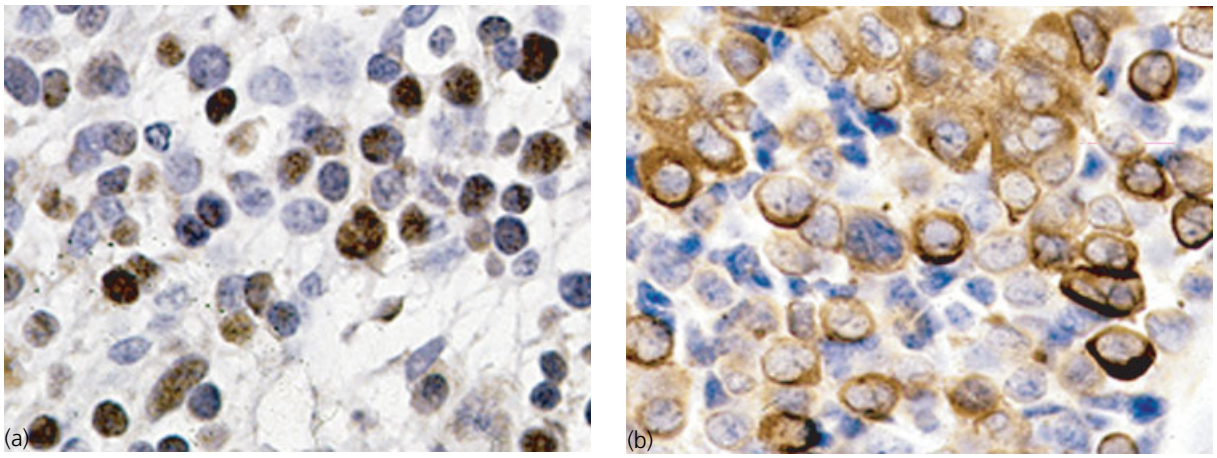

Figure 4 *Immunohistochemical detection of JC viral proteins, T-antigen and Agnoprotein in human medulloblastomas: High magnification view of a human medulloblastoma, demonstrates the presence of T-antigen in the nuclei of approximately 60 percent of the neoplastic cells (***(a)** *T-antigen 1:100 dilution, original magnification ×100). When tested with an anti-Agno antibody the cells show a robust cytoplasmic immunoreactivity (***(b)** *Agno 1:2 000 dilution, original magnification ×100).*

promoter results in suppression of viral late transcription. The presence of NF-κB, on the other hand, suggests a role for NF-κB inducible pathways via the p50/p65 transcription factor upon stimulation of cells with various tumor necrosis factors, cytokines, and immunomodulators, such as TNF-α and IL-2. Also, activation of the viral promoter can occur upon hormonal imbalances; the presence of estrogen responsive and glucocorticoid responsive elements suggest activation of BKV gene transcription by estrogen-induced transcription factors. During pregnancy, maximum reactivation of the virus is observed during the third trimester when the levels of estrogen and progesterone are at their highest, pointing to a role for glucocorticoid responsive element (GRE) and estrogen responsive element (ERE) in the induction of BKV gene transcription.

BKV AND HUMAN DISEASES

Hemorrhagic cystitis and nephritis

Reactivation of BKV and its appearance in urine is frequently observed in pregnant women without any sign of illness. Considering the kidney as a primary site for BKV reactivation, much attention has been focused on the role of BKV in renal allograft dysfunction and failure. BKV-associated nephropathy (BKVN) ranges from asymptomatic hematuria to hemorrhagic cystitis in renal graft transplants, as well as in patients with bone marrow transplantation (Andrews et al. 1988; Bedi et al. 1995; Bogdanovic et al. 1998; Chan et al. 1994; Coleman et al. 1973; Vögeli et al. 1999). In addition, BKV-associated ureteric stenosis and tubulointerstitial nephritis has been reported in individuals with renal allograft transplantation (Coleman et al. 1978; Gardner et al. 1971, 1984; Mathur et al. 1997; Nebuloni et al. 1999; Pappo et al. 1996; Purighalla et al. 1995; Smith et al. 1998). Hemorrhagic cystitis associated with BKV has also been seen in AIDS patients (Gluck et al. 1994). It has been speculated that treatment of recipients of renal allograft transplants with immunosuppressive drugs leads to reactivation of latently infected BKV in the donated organs. The reactivation of BKV that is histologically observed by areas of by tubular necrosis is in concurrence with the loss of organs. While there is no direct evidence for the induction of nephropathy by BKV in humans, earlier observations from experimental animals is consistent with the assumption that BKV is able to induce nephropathy in humans. According to that observation, a member of the polyomavirus family named cynomolgus polyomavirus induces interstitial nephritis, ureteritis, and enteritis, the symptoms which are seen in BKVN in immunosuppressed cynomolgus monkey (Van Gorder et al. 1999).

Reactivation of BKV and renal tubular injury has been repeatedly observed among AIDS patients. The clinical symptoms caused by BKV activation in AIDS patients includes meningoencephalitis, retinitis, and nephritis (Bratt et al. 1999; Nebuloni et al. 1999).

While there is no established treatment for patients with BKV-associated renal disease, it is believed that a decrease in the doses of immunosuppressive drugs in patients who undergo kidney allograft transplantation may result in viral clearance.

Association of BKV with cancer

BKV causes tumors in experimental animals Similar to its close family member JCV, BKV is tumorigenic in several experimental animals. For example, intracerebral or intravenous inoculation of BKV in hamster results in the genesis of a broad range of tumors, such as ependymoma, neuroblastoma, pineal gland tumors, fibrosarcoma, osteosarcoma, and tumors of pancreatic islets (Corallini et al. 1977, 1978, 1982; Costa et al. 1976; Dougherty, 1976; Greenlee et al. 1977; Nase et al. 1975; Noss and Stauch 1984; Noss et al. 1981; Shah et al. 1975; Uchida et al. 1976; van der Noordaa 1976). Inoculation of BKV in rats and mice caused formation of fibrosarcoma, liposarcoma, osteosarcoma, glioma, choroid plexus papilloma, and nephroblastoma (Corallini et al. 2001; Noss and Stauch 1984; Noss et al. 1981). It is believed that the oncogenicity of BKV is due to the expression of T-antigen, which has a transforming activity in cell culture. The evidence for the involvement of BKV T-antigen in tumorigenicity of the virus stems from a study in transgenic mice in which the affected animals expressing BKV T-antigen developed renal tumors, lymphoproliferative disease, and hepatocellular carcinoma (Dalrymple and Beemon 1990; Small et al. 1986a). The T-antigen of BKV has the potential to associate with tumor suppressors such as p53 and the pRb family to functionally inactivate these important cell cycle regulatory proteins (Dyson et al. 1989, 1990; Harris et al. 1996; Kang and Folk 1992; Shivakumar and Das 1996). In addition to its role in the deregulation of cell cycle pathways, BKV T-antigen may be involved in genomic instability and random chromosomal abnormalities. While the mechanism whereby BKV T-antigen induces chromosome aberrations remains unknown, several characteristics of the protein, such as the ability of T-antigen to associate with topoisomerase I, p53, and helicase activity may contribute to this event. Indeed, other viral proteins, including small t-antigen and the late regulatory protein, Agnoprotein, may also play a role in the tumorigenicity of BKV, as well as its ability to cause chromosomal damage.

Detection of BKV in human cancer The oncogenic potential of BKV in several experimental animals, the transforming activity of BKV T-antigen in vitro, and its tumorigenicity in transgenic animals prompted many investigators to search for the presence of BKV in various human tumors. Examination of 74 human brains

by conventional hybridization techniques revealed the presence of episomal free DNA of BKV in 19 samples (Corallini et al. 1987a). BKV DNA was also detected in significant numbers (44 percent) of tumors in pancreatic islets (Corallini et al. 1987b). In recent years, the search for the presence of BKV DNA in several laboratories using more sensitive assays, i.e. PCR, revealed the presence of BKV DNA in 50 out of 58, 74 out of 83, and 18 out of 18 brain tumors (De Mattei et al. 1995; Flaegstad et al. 1999; Martini et al. 1996). Using the same techniques, other laboratories failed to detect BKV DNA in ten brain tumor samples (Völter et al. 1997). The brain tumors which were studied included ependymomas, meningiomas, glioblastomas, gliomas, neuroblastomas, oligodendrogliomas, spongioblastomas, choroid plexus papillomas (Corallini et al., 1977, 1987b; De Mattei et al. 1994, 1995; Dörries et al. 1987; Negrini et al. 1990). While there is no established role for BKV in human neoplasia, it is evident that studies to establish a correlation between BKV and tumors of the central nervous system and urinary tract are essential.

SV40

SV40 represents the most studied member of the family *Polyomaviridae*. Originally isolated from monkeys, in recent years it has been repeatedly detected in a selected group of human tumors. The control region of SV40, responsible for transcription of the viral early and late genome, is comprised of three 21 base pair GC-rich repeats and two 72-bp tandem repeats with multiple binding sites for DNA binding transcription factors. A TATA box positioned in front of the GC-rich motif provides a site for the formation of a transcription initiation complex. By utilizing the SV40 regulatory sequence as a model for studying regulation of eukaryotic promoters, investigators were able to discover several key general transcription factors such as the families of Sp1, AP1, etc., to decipher complicated events involved in the initiation and elongation of the transcription process. The origin of viral DNA replication is located near the TATA box and contains the core origin and the auxiliary sequences which enhance the efficiency of viral DNA replication. The interaction of the viral early protein, T-antigen, with the sequences surrounding the origin of DNA replication is pivotal for the start of viral DNA replication indicating that expression of the early genome is the first step in the lytic cycle of SV40.

The early genome of SV40 has two transcription units that are transcribed from a region near the origin of DNA replication (Khalili et al. 1986). The early–early (E_E) transcription unit whose RNA is responsible for production of large and small T-antigen predominates at the early times after infection. The late–early (L_E) transcription unit whose RNA start sites are located upstream of the E_E TATA box, have coding sequences for both small t- and large T-antigen, and a small 2–7 kDa protein, the SV40 early leader protein (SELP) (Khalili et al. 1987b). The L_E transcription unit functions at late times after infection, and while the function of SELP remains to be investigated, it is evident that transition of the sites of early RNAs from E_E to L_E position results in the inclusion of two AUGs in the leader of the RNA. These additional AUG decrease the utilization of the AGG which is responsible for T-antigen production, thus bringing additional steps to regulate early gene transcription at the translational level. The large T-antigen of SV40 has a modular structure with several biological activities including ATPase, DNA helicase, DNA-binding, and protein-binding activities. Large T-antigen can undergo post-translational modifications, events that could potentiate its function upon the viral life cycle and/or host-cell behavior.

Replication of viral DNA is a complicated process that requires a functional origin of replication, large T-antigen with its DNA binding and helicase activities, and the host replication machinery. DNA replication usually occurs between 4 and 8 h after infection and is followed by the enhanced transcription of the late region of this virus. Results from a large volume of molecular studies have revealed that direct and indirect association of T-antigen with several cellular transcription factors is involved in the replication of viral late gene expression. Once the viral late proteins are produced, consistent with the general scheme of polyomaviruses, the assembly of the virion occurs in the nuclei of the cells.

SV40 AND HUMAN DISEASE

It is believed that the introduction of SV40 into the human population is related to the development and distribution of poliovaccines. Evidently, the initial preparations of poliovaccines that were inadvertently contaminated with SV40 caused exposure of hundreds of millions of persons to the virus. It is estimated that more than 98 million people in the United States who received poliovaccine between 1955 and 1963 were exposed to SV40 (Shah and Nathanson 1976). Other poliovaccine preparations using adenovirus types 3 and 7, which were utilized between 1961 and 1965, were also contaminated with SV40 (Lewis et al. 1973). In light of cell culture studies showing the transforming ability of SV40 T-antigen and results from animal experiments verifying the oncogenicity of T-antigen, the observed distribution of SV40 in a large population of humans via the poliovaccine has become an important subject in cancer development in humans.

SV40 caused tumors in experimental animals

In 1962, several laboratories demonstrated that SV40 can induce tumors in Syrian hamsters (Eddy et al. 1962; Girardi et al. 1962). The type of tumors induced by

SV40 was dependent on the route of viral inoculation. Systemic inoculation of SV40 resulted in the development of mesotheliomas, osteosarcomas, and lymphomas, while intracranial inoculation of hamsters with SV40 led to the development of brain tumors, such as ependymoma, and subcutaneous inoculation caused soft tissue sarcoma. Transgenic mice containing the SV40 early genome developed choroid plexus papilloma in which T-antigen expression was detected.

SV40 and human tumors

Results from many studies in recent years point to the possible role of SV40 in cancer development in humans (Gazdar et al. 2002). The first evidence of the association of SV40 with human cancer was presented in 1974 (Soriano et al. 1974) in a patient with metastatic malignant melanoma in which viral proteins were present in the lung, liver, and muscle metastases, but not normal tissues. Subsequently, SV40 has been detected by standard Southern blot and gene amplification in adult, as well as pediatric tumors of the brain including choroid plexus, ependymomas, and medulloblastomas (Bergsagel et al. 1992; Huang et al. 1999; Lednicky et al. 1995).

In one study the presence of SV40 DNA sequences has been reported in 32 percent of osteosarcomas and 41 percent of several other tumors (Carbone et al. 1996). Perhaps the most studied cases related to SV40 and cancer are malignant mesotheliomas in which nearly half of the cases contain the viral genome (Bocchetta et al. 2000; Carbone et al. 1994, 2002; Cicala et al. 1993; Rizzo et al. 1999, 2001; Shivapurkar et al. 1999, 2000; Testa et al. 1998; Toyooka et al. 2001). In the most recent studies, the SV40 sequence has been found in non-Hodgkin's lymphomas (Shivapurkar et al. 2002; Vilchez et al. 2002). Continuous detection of SV40 DNA in certain human tumors suggests that this DNA tumor virus has the ability to play a role in human disease. The inactivation of several tumor suppressors, deregulation of the cell cycle, and several other signaling pathways can serve as a potential path for the genesis of cancer by SV40 in experimental animals and perhaps in humans as well.

CONCLUDING REMARKS

The polyomaviruses have a long history of being utilized as a tool to decipher the interaction of viruses with their host cells, and to investigate pathways involved in the regulation of eukaryotic gene expression, mRNA translation, and DNA replication. Further, the polyomavirus early protein, such as SV40 T-antigen in the early years of the 'fight against cancer' has provided critical insight into the mechanism involved in the development of neoplasia. More recent studies from several basic science laboratories along with clinical observations have brought polyomaviruses to center stage in medical virology due to their involvement in various human diseases as outlined in this chapter. It is, therefore, essential to once again pay special attention to these viruses, and by employing more novel approaches and technology, study the various cellular events that can occur upon infection. The outcome will provide a better understanding of the pathogenesis of diseases associated with polyomaviruses. This information will facilitate development of effective antiviral drugs and vaccines for the prevention and treatment of diseases associated with polyomaviruses.

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Herpesviruses: general properties

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INTRODUCTION

The involvement of herpesviruses in a range of prominent medical or veterinary diseases makes them one of the most important virus families. Their ubiquitous occurrence, genetic complexity, evolutionary diversity, and widely differing biological properties have motivated great research effort worldwide, so that our understanding of these agents is now considerable. This is particularly apparent in the area of molecular genetics, and we can expect the knowledge gained to be applied increasingly to key questions about herpesvirus biology and to the development of effective therapies.

In this overview, we have focused on the best characterized member of the family, herpes simplex virus type 1 (HSV-1). Although a number of properties are shared by herpesviruses, many are not. For this reason, we have included pertinent information on the other human herpesviruses and, in a few cases, herpesviruses with nonhuman hosts. In this review, it is impossible to develop a comprehensive bibliography for such a vast area of research. Consequently, we have chosen to describe the accepted picture without extensive citation, instead referring to reviews and a limited selection of seminal or recent research papers.

CLASSIFICATION

The family *Herpesviridae*

The family *Herpesviridae* comprises over 120 viruses that infect a wide range of vertebrates, including fish, amphibians, reptiles, birds, marsupials, and other mammals including humans, and at least one invertebrate, the oyster (Minson et al. 2000). These viruses are ubiquitous and highly successful parasites, but each is usually restricted in natural infection to a single species and spreads from host to host by direct contact or by the respiratory route. Herpesviruses have large enveloped virions containing double-stranded linear DNA genomes that exhibit considerable diversity of size and structure. They vary greatly in their pathology and biology, but one common feature is their ability, following primary infection, to establish lifelong latent infections that can recrudesce to cause a second round, or even recurrent rounds, of disease. Herpesviruses are well adapted to their hosts, and it is not uncommon for primary or recurring latent infections to be inapparent or trivial in the natural setting. Under certain conditions, however, particularly those resulting in a degree of immune suppression, herpesviruses can be life-threatening. Several herpesviruses have also been implicated in various types of cancer.

On the criteria of host range, duration of reproductive cycle, cytopathology, and characteristics of latent infection, mammalian and avian herpesviruses have been divided into three subfamilies: the *Alpha-*, *Beta-* and *Gammaherpesvirinae*. Each subfamily is subdivided: for example, subfamily *Gammaherpesvirinae* comprises the *Lymphocryptovirus* and *Rhadinovirus* genera. In this review, however, we have also employed a simplified terminology used widely in the literature; for example, using α-herpesvirus (subfamily) and α_1-herpesvirus (genus) instead of *Alphaherpesvirinae* and *Simplexvirus*.

The herpesvirus nomenclature recommended by the International Committee on Taxonomy of Viruses (ICTV) uses the taxonomic unit to which the natural host belongs followed by a number. This rational scheme is used for many herpesviruses, but most human herpesviruses are generally known by their common names.

Alphaherpesvirinae exhibit a variable host range in cell culture, have a short growth cycle, spread rapidly with efficient destruction of infected cells and are neurotropic in that they have the capacity to establish latent infections primarily in sensory ganglia. *Betaherpesvirinae* are typified by a narrow host range and a long reproductive cycle in cell culture with slow virus spread. Infected cells frequently become enlarged and carrier cultures may be established. Viruses may become latent in secretory glands and lymphoreticular cells. *Gammaherpesvirinae* are associated with lymphoproliferative diseases in their natural hosts, and infect lymphoblastoid cells in vitro. Some cause lytic infections in certain epithelial and fibroblastoid cell lines. Viruses are specific for B or T lymphocytes, in which infection is frequently arrested without production of infectious progeny, and latent virus may be found in lymphoid tissue. The term 'lymphotropic' has been used to describe these viruses, but this is arguably a misleading term as lymphocytes are at best semipermissive.

This classification scheme, which is based predominantly on biological features, has been largely vindicated by studies of genetic relationships deduced from analyses of DNA sequence data. Genetic data have now superseded biological properties for classifying herpesviruses.

Human herpesviruses

To date, eight different herpesviruses have been identified whose natural host is humans (Table 1), all but two of which (HSV-2 and HHV-8) are ubiquitous in human populations. Three of these viruses have been discovered within the last 20 years. HHV-6 was first isolated from patients with a variety of lymphoproliferative and immunosuppressive disorders (Salahuddin et al. 1986), and exists in two closely related forms (Ablashi et al. 1993), one of which (HHV-6B) has been implicated as a causative agent of exanthem subitum (or roseola infantum), a common disease of infancy (Yamanishi et al. 1988). HHV-7 was first isolated from activated $CD4^+$ cells from a healthy person (Frenkel et al. 1990), and has also been implicated in exanthem subitum (Tanaka et al. 1994). HHV-8 was discovered by the detection of DNA sequences in Kaposi's sarcoma (KS) tissue from acquired immunodeficiency syndrome (AIDS) patients, but not in normal tissue (Chang et al. 1994). These sequences were subsequently detected in KS tissue from patients displaying the classic or endemic African forms of the disease and, often in association with Epstein–Barr virus (EBV), in AIDS-related primary effusion lymphomas. HHV-8 is now accepted as a causal agent of KS and, as a human tumor virus, is the subject of extensive research effort (Schulz 1998; Moore and Chang 2001a). Studies of HHV-8 counterparts in nonhuman primates have indicated that a ninth human herpesvirus species related to HHV-8 might exist (Schultz et al. 2000). To date, however, evidence has not been forthcoming.

Genome sequences for all known human herpesviruses are now available. Consequently, we know a great deal about how the genes are organized and what their functions are. Relating this type of information to general and particular aspects of herpesvirus pathogenesis presents an exciting challenge and has as an objective the control or prevention of infection.

Table 1 *Human herpesviruses*

Virus	Abbreviation	Genus	ICTV species name	G+C content (mol%)	Genome size (bp)
Herpes simplex virus type 1	HSV-1	α_1	Human herpesvirus 1	67	152 261
Herpes simplex virus type 2	HSV-2	α_1	Human herpesvirus 2	69	154 746
Varicella-zoster virus	VZV	α_2	Human herpesvirus 3	46	124 884
Epstein–Barr virus	EBV	γ_1	Human herpesvirus 4	60	171 823
Human cytomegalovirus	HCMV	β_1	Human herpesvirus 5	57	230 283[a]
Human herpesvirus 6[b]	HHV-6	β_2	Human herpesvirus 6	43	159 321 161 573 162 114
Human herpesvirus 7[b]	HHV-7	β_2	Human herpesvirus 7	40	144 861 153 080
Kaposi's sarcoma-associated herpesvirus[b]	HHV-8 (KSHV)	γ_2	Human herpesvirus 8	54	~140 500/801[c]

a) Laboratory strain (AD169; 229 354 bp) plus a 929 bp region deleted from a clone sequenced originally. Clinical isolates are approximately 5 kbp larger.
b) More than one strain has been sequenced.
c) Expressed as size of unique region/size of terminal repeat. Sequence at right end of unique region is unknown for the two sequenced isolates.

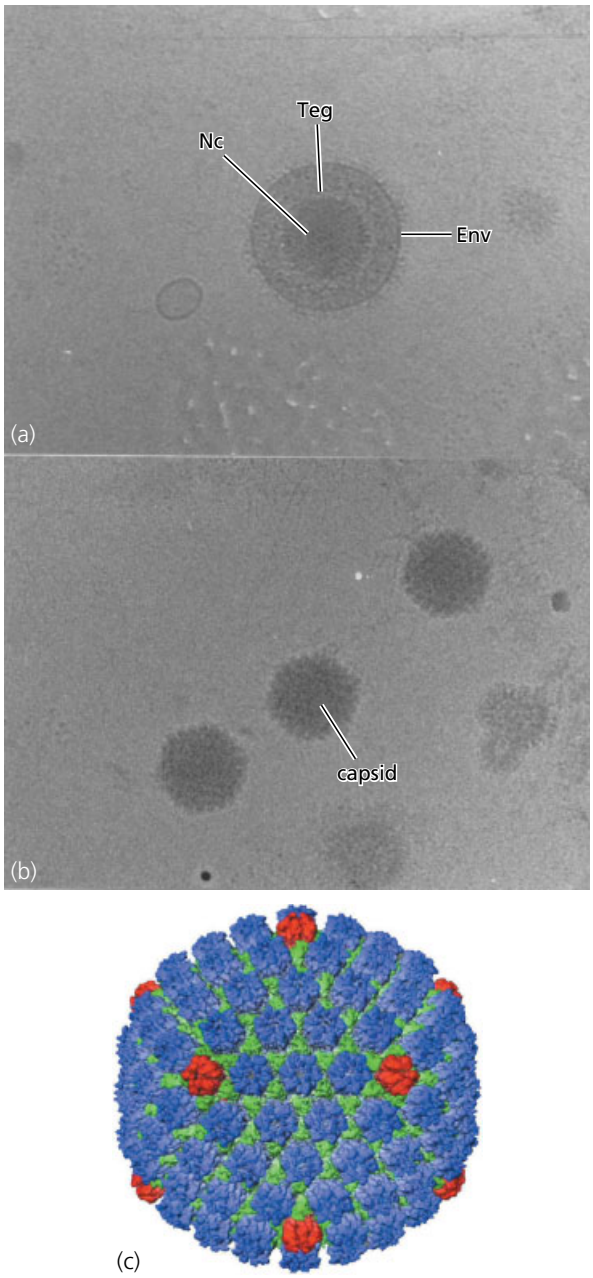

Figure 1 (a) *Electron cryomicrographs of* **(a)** *virions and* **(b)** *capsids of HSV-1. Visible in* **(a)** *are the capsid (Nc), tegument (Teg) and envelope (Env) with associated glycoprotein spikes (courtesy of Dr. F.J. Rixon);* **(c)** *three-dimensional reconstruction of the HSV-1 capsid surface structure. The view is along the two-fold axis. The hexons, pentons, and triplexes are shown in blue, red, and green, respectively (courtesy of Drs. H. Zhou and W. Chiu).*

MORPHOLOGY AND STRUCTURE

Morphology is the main factor in assigning viruses to the family *Herpesviridae*. Virions are comprised of four distinct structural elements: envelope, tegument, capsid, and core (Dargan 1986; Figure 1). The virion and capsid have diameters of 200–250 nm and 125 nm, respectively.

The lipid envelope is visible in negatively stained preparations as a pleomorphic structure with many short glycoprotein spikes. Contained within the envelope is the characteristic icosadeltahedral capsid, which in negatively stained preparations appears as an orderly array of stain-penetrated capsomers. Forming the icosahedron are 12 pentavalent capsomers at the vertices, 60 hexavalent capsomers at the 20 faces, and 90 hexavalent capsomers along the 30 edges – a total of 162 capsomers (Wildy et al. 1960). Electron-dense material is clearly present between the envelope and capsid, and the term 'tegument' has been used to describe this poorly understood region, which contains more than 20 viral proteins. Electron cryomicroscopy has been used to study frozen, hydrated herpesviruses, particularly capsids (Zhou et al. 2000). This approach allows direct examination of the three-dimensional structure without the use of stain or fixative. When combined with computer-assisted image enhancement, considerable surface detail is visible (Figure 1). Electron cryomicroscopic studies have also shown that the densely staining spherical core inside the capsid consists of the viral DNA packed at high density apparently without internal protein associations. Descriptions of toroidal structures detected in the core in earlier studies (e.g. Furlong et al. 1972) persist into the modern literature but are now accepted as artifacts of negative staining techniques.

REPLICATION

The replication cycle has been studied most fully with HSV-1, and this is the basis of the brief account given here. Fuller accounts of specific aspects of this subject are given in subsequent sections. An outline of the events that occur during HSV-1 replication is shown in Figure 2.

General aspects of the HSV-1 replication cycle are identical for HSV-2, but for other human herpesviruses less information is available, largely because of difficulties in propagating the viruses. No truly permissive cell culture system exists for EBV or HHV-8, and studies have been largely restricted to examination of latently infected cell lines. The diverse nature of human herpesviruses implies that details of their patterns of replication will vary. Nevertheless, replication cycles are all consistent with a sequential order of three major regulated phases of transcription and protein synthesis (Roizman and Knipe 2001).

The external glycoproteins of the virion have important roles in adsorption of virus to cells and subsequent penetration (Spear et al. 2000). Fusion of the virus envelope with the plasma membrane occurs at the cell surface rather than in internalized vesicles. Capsids are transferred to the nucleus via microtubules (Dohner et al. 2002), and viral DNA and at least some tegument proteins enter the nucleus, where transcription,

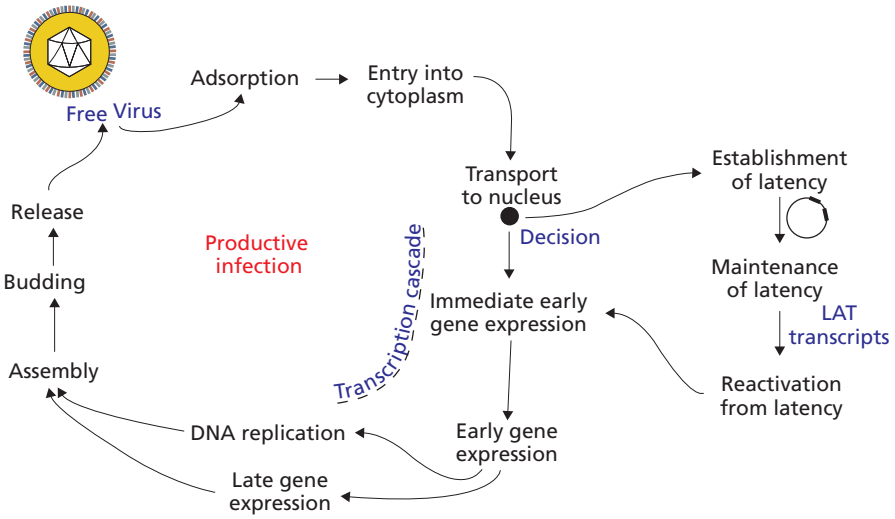

Figure 2 *The replication cycle of HSV-1*

replication of viral DNA and capsid assembly take place.

Viral DNA is transcribed into mRNA throughout infection by host RNA polymerase II, with various viral factors participating at all stages (Wagner 1985). Initially, a series of immediate–early (IE), or α genes is expressed whose transcription is independent of viral protein synthesis and is greatly enhanced by a tegument protein (O'Hare 1993). Certain IE proteins are *trans*-acting regulators of virus genes, and initiate a cascade of expression of early (or β) and late (or γ) genes (Honess and Roizman 1974). The products of early genes, defined as the subset expressed in the presence of functional IE products and before the onset of DNA replication, include several enzymes involved in DNA replication and nucleotide metabolism and a subset of glycoproteins. Late genes are dependent on functional IE proteins for expression, and in addition full levels of late protein synthesis require DNA replication. They encode mainly virion proteins. Small noncoding RNAs characteristic of γ-herpesvirus genomes are probably transcribed by RNA polymerase III.

A subset of viral genes is involved in DNA replication and packaging of DNA into capsids. DNA synthesis is initiated from one or more origins of DNA replication, generating head-to-tail concatemeric genomes apparently by a rolling circle mechanism (Boehmer and Lehman 1997). Concatemers are then cleaved and packaged into preformed capsids.

The process by which virion maturation occurs has long been controversial, but it is known that several genes are involved in directing envelopment and egress. The following scheme is probably correct (Elliott and O'Hare 1999; Skepper et al. 2001; Mettenleiter 2002). Capsids gain certain tegument proteins in the nucleus and then a membrane upon budding into the space between the two nuclear membranes. The membrane is lost on transit to the cytoplasm, where additional tegument proteins are added. Tegumented capsids then gain their envelope by budding into a component of the Golgi apparatus, or vesicles derived therefrom, and are released from the infected cell by reverse endocytosis.

The effects of these processes on host cells vary with the different viruses, and for an individual virus, depend on the cell type. The time taken for the cytopathic effect to become evident may range from hours to days, and cells may lyze, round up, or fuse. Also, the proportions of particles that are released from infected cells and the fraction that are enveloped are very variable.

Infection with HSV-1 leads to a rapid inhibition of host macromolecular synthesis. The initial phase of host shutoff is mediated by a virion component. Reduction of the functional half-lives of both infected cell and viral mRNAs by the virion shutoff component could promote rapid changes in viral gene expression in response to alterations at the level of transcription.

During infection, viral proteins interact with each other and with cellular proteins, and an increasing number of methods are available to detect such interactions. However, demonstrating physiological significance is often not straightforward and demands particular rigor. The great majority of studies of the replication cycle are carried out in vitro on infected tissue culture cells. Investigations of the replication cycle in vivo are revealing fresh insights and new gene functions. For example, analysis of the neural circuit-specific spread of α-herpesviruses (Enquist 2002). In addition to a lytic cycle of infection, all herpesviruses examined to date are able to undergo a latent cycle, discovering the molecular mechanisms of which presents further challenges to researchers (see Alphaherpesviruses: herpes simplex and varicella-zoster).

GENOME STRUCTURE

Types of structure

Herpesvirus genomes range in size from 125 to 245 kbp and nucleotide compositions from 32 to 74 percent G + C (Honess 1984). The γ-herpesviruses exhibit a global deficit in the 5′-CG dinucleotide, presumably as an evolutionary result of latency in dividing cells (Honess et al. 1989). The genome structures of many herpesviruses are known, but the majority of viruses in this family have not been examined at this level. Figure 3 illustrates the six types of genome arrangement that have been characterized thus far. A common characteristic is the presence of repeated regions at the ends of, or internally within, the genome. Similar genome structures have arisen independently during herpesvirus evolution, implying that this feature is not a reliable taxonomic tool in the absence of other data.

The simplest genome arrangement is that of group 1, which consists of a unique sequence flanked by a direct repeat. It was first described for channel catfish virus (CCV), and has since been found for equine herpesvirus 2 (EHV-2; γ_2-herpesvirus), and HHV-6. The genome of murine cytomegalovirus (MCMV; β_1-herpesvirus), also has this arrangement, but the direct repeats are much smaller.

Group 2 genomes contain a short sequence repeated a variable number of times at the termini instead of a single direct repeat, and are found among the γ_2-herpesviruses, such as HHV-8. The addition of a variable number of the terminal repeats in inverse orientation internally results in the group 3 genome structure, which has been described for cottontail rabbit herpesvirus, also a γ_2-herpesvirus. This genome exists in four equimolar isomers in virion DNA, since inversion of the two unique regions can occur by recombination between inverted repeats. The group 4 structure results from the presence of an internal set of direct repeats unrelated to the terminal set and is typified by EBV. The two unique regions do not invert in such a structure because of lack of homology between internal and terminal repeats.

In the group 5 structure, two unique regions (U_L and U_S) are each flanked by unrelated inverted repeats (TR_L/IR_L and TR_S/IR_S). The two orientations of U_S are present in equimolar amounts in virion DNA, but U_L is found predominantly or completely in a single orientation. Group 5 genomes are characteristic of α_2-herpesviruses, such as varicella zoster virus (VZV), but a separately evolved example is present in a fish herpesvirus, salmonid herpesvirus 1.

The group 6 structure is the most complex, although it was the first type to be described, for HSV-1 (Sheldrick and Berthelot 1974). It is similar to that of group 5, except that TR_L/IR_L is much larger, and the two orientations of U_L, as well as those of U_S, are present in equimolar amounts in virion DNA. HSV-2 and bovine herpesvirus 2, also an α_1-herpesvirus, and HCMV share this structure. Unlike group 5 genomes, group 6 genomes are terminally redundant, possessing a short sequence (termed the *a* sequence, some 400 bp in HSV-1) that is repeated directly at the genome termini and inversely at the IR_L–IR_S junction (Wagner and Summers 1978).

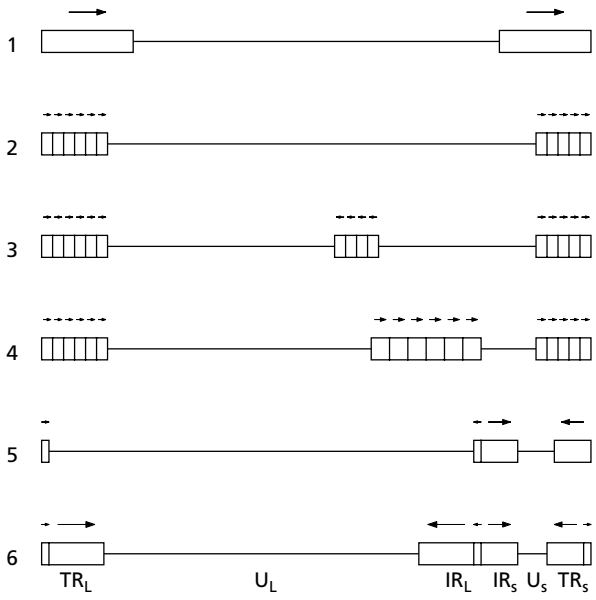

Figure 3 *Types of herpesvirus genome structure. The genomes are not drawn to scale. Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientations of repeats are shown by arrows. The nomenclature used to designate regions of the HSV-1 genome are shown at the foot of the figure (from Davison and McGeoch 1995, courtesy of Cambridge University Press).*

Human herpesviruses

Figure 4 shows a scale representation of human herpesvirus genome structures. The HSV-1 and HSV-2 genomes exhibit a group 6 structure and are essentially colinear. Preparations of virion DNA from both viruses contain equivalent amounts of four isomers that differ in the relative orientations of U_L and U_S (Hayward et al. 1975), a phenomenon termed genome isomerization. These isomers appear to be functionally equivalent, and one has been chosen arbitrarily to serve as a prototype for depicting restriction endonuclease sites and the gene arrangement. As the *a* sequence contains signals for cleavage and packaging concatemeric DNA generated during viral DNA replication, the presence of internal copies as alternative sites, in combination with homologous recombination events between TR_L and IR_L or TR_S and IR_S, provides a means for generating the genome isomers.

The VZV genome, as an example of a group 5 structure, is not terminally redundant although sequences

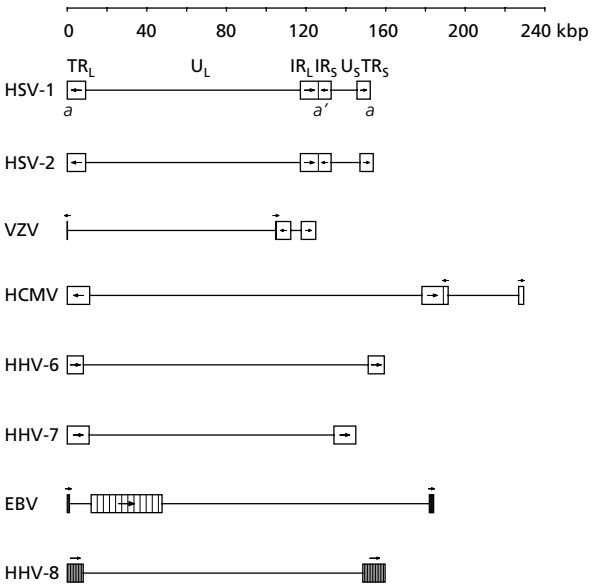

Figure 4 *Sizes and structures of human herpesvirus genome shown to scale. Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientations of repeats are shown by arrows. The nomenclature used to designate regions of the HSV-1 genome is shown at the top of the figure. The sequence that is directly repeated at the genome termini and also present in inverse orientation is shown as* a *and* a′*, respectively.*

involved in cleavage and packaging of replicated DNA are present at the genome termini. U_L is flanked by a small inverted repeat of 88 bp that results in 5 percent of virions containing genomes with U_L inverted, and U_S is found equally in both orientations.

The general arrangement of the HCMV genome resembles that of HSV-1, with a similar arrangement of unique and repeated elements and the presence of four equimolar genome isomers, although it is 60 percent larger. TR_L/IR_L is much smaller in clinical isolates than in the sequenced laboratory strain (Prichard et al. 2001). Phylogenetic studies indicate that the genome structures of HSV-1 and HCMV developed independently. HHV-6 and HHV-7 are the only known β_2-herpesviruses. Although genetically related to HCMV, they share the distinct group 1 genome structure.

EBV DNA displays a linear, noninverting structure. The terminal regions consist of up to 12 copies of a tandem repeat (523 or 538 bp), and the internal repeat region comprises between six and 12 tandemly reiterated copies of a 3072 bp sequence. The HHV-8 genome is typical of that of γ_2-herpesviruses, consisting of a unique region flanked by a variable number of copies of an 801 bp terminal repeat.

In addition to large scale repeats, most human herpesvirus genomes contain several regions consisting of tandem reiterations of short sequence elements. The presence of the repeated elements in variable copy numbers results in genome size heterogeneity. These sequences may be coding or noncoding; in the former case, they exist as multiples of 3 bp and encode repeated amino acid sequences. A further type of genome heterogeneity is represented by the loss or gain of restriction endonuclease sites and is presumably due to differences in individual nucleotides. The DNA cleavage patterns of epidemiologically unrelated HSV-1 or HSV-2 isolates are not identical, and are stable when viruses are passed in cell culture. This characteristic has an important diagnostic use and has been employed to examine modes of transmission and epidemiology of HSV-1 and HSV-2 in human populations (Umene and Sakaoka 1999). Sequence data are being employed increasingly in studies of this type.

GENETIC CONTENT

Arrangement and expression

The complete (or almost complete) DNA sequences of 29 herpesviruses have been published to date, several for more than one strain. These are listed in Table 2. In addition, a substantial amount of data is available for parts of other herpesvirus genomes. Analyses of these sequences have given a detailed view of genetic content and usually form the starting point for experimental studies on particular genes.

The number of protein-coding genes estimated from sequence analyses ranges from about 70 in smaller genomes (e.g. HSV-1 and VZV) to over 160 in the larger (e.g. HCMV). In general, the great majority of the DNA is protein-coding, and genes are arranged about equally between the two strands. Regions of overlap between genes in different reading frames on the same strand or on opposing strands are infrequent and usually not extensive. Most genes are expressed as single exons from their own promoters, but it is common for families of genes arranged tandemly on the same strand to share a polyadenylation site downstream from the most 3′ member (Wagner 1985). Few α-herpesvirus genes specify spliced mRNAs, but splicing is more common in the β- and γ-herpesviruses. Most splicing in EBV specifically involves genes expressed during latency (Kieff and Rickinson 2001), particularly a family of genes distributed over 100 kbp that is expressed by differential splicing of transcripts from a common promoter. Complete transcript maps are not available for any herpesvirus, but transcriptome analyses have been derived from oligonucleotide microarray data for several, such as HSV-1 (Stingley et al. 2000). Microarray technology has also been used to investigate changes in cellular gene expression during infection with herpesviruses, such as HCMV (Zhu et al. 1998).

HSV-1

HSV-1 is the best characterized herpesvirus at the molecular level. The genome is considered to contain 74

Table 2 *Herpesvirus genome sequences*

Virus	Genome (bp)	GenBank accession	Primary reference
Alphaherpesvirinae			
Simplexvirus (α_1)			
Herpes simplex virus type 1	152261	X14112	McGeoch et al. (1988)
Herpes simplex virus type 2	154746	Z86099	Dolan et al. (1998)
Varicellovirus (α_2)			
Varicella-zoster virus	124884	X04370	Davison and Scott (1986)
Simian varicella virus	124138	AF275348	Gray et al. (2001)
Equine herpesvirus 1	150224	M86664	Telford et al. (1992)
Equine herpesvirus 4	145597	AF030027	Telford et al. (1998)
Bovine herpesvirus 1	135301	AJ004801	Schwyzer and Ackermann (1996)
Mardivirus (α_3)			
Marek's disease virus type 1	177874	AF243438	Tulman et al. (2000)
Marek's disease virus type 2	164270	AB049735	Izumiya et al. (2001)
Turkey herpesvirus	159160	AF291866	Afonso et al. (2001)
Betaherpesvirinae			
Cytomegalovirus (β_1)			
Human cytomegalovirus[a]	230285	X17403	Chee et al. (1990)
Chimpanzee cytomegalovirus	241087	AF480884	Davison et al. (2003)
Muromegalovirus			
Murine cytomegalovirus	230278	U68299	Rawlinson et al. (1996)
Rat cytomegalovirus	230138	AF232689	Vink et al. (2000)
Roseolovirus (β_2)			
Human herpesvirus 6	159321	X83413	Gompels et al. (1995)
	161573	AB021506	Isegawa et al. (1999)
	162114	AF157706	Dominguez et al. (1999)
Human herpesvirus 7	144861	U43400	Nicholas (1996)
	153080	AF037218	Megaw et al. (1998)
Unassigned			
Tupaiid herpesvirus	195859	AF281817	Bahr and Darai (2001)
Gammaherpesvirinae			
Lymphocryptovirus (γ_1)			
Epstein–Barr virus	184113	M35547	
		V01555	Baer et al. (1984)
		M80517	Parker et al. (1990)
	171823	AH507799[b]	Unpublished
Marmoset lymphocryptovirus	149696	AF319782	Rivailler et al. (2002a)
Rhesus lymphocryptovirus	171096	AY037858	Rivailler et al. (2002b)
Rhadinovirus (γ_2)[c]			
Herpesvirus saimiri	112930/1444	X64346	Albrecht et al. (1992)
Herpesvirus ateles	108409/1582	AF083424	Albrecht (2000)
Alcelaphine herpesvirus 1	130608/1113	AF005370	Ensser et al. (1997)
Human herpesvirus 8	~140500/801	U75698	Russo et al. (1996)
		U93872	Neipel et al. (1997)
Rhesus rhadinovirus	131364/~2100	AF083501	Searles et al. (1999)
	130733/?	AF210726	Alexander et al. (2000)
Murine herpesvirus 68	118237/1213	U97553	Virgin et al. (1997)
	118311/1239	AF105037	Nash et al. (2001)
Bovine herpesvirus 4	108873/2267	AF318573	Zimmermann et al. (2001)
Equine herpesvirus 2	184427	U20824	Telford et al. (1995)
Undefined subfamily			
Ictalurivirus			
Channel catfish virus	134226	M75136	Davison (1992b)

?, not known
a) See footnote (a) in Table 1.
b) Updated version of previous accession numbers.
c) Except for EHV-2, expressed as size of unique region/size of terminal repeat. Sequence at right end of unique region is unknown in the two HHV-8 isolates.

different genes, three of them present twice in the inverted repeats. The gene arrangement is shown in Figure 5. Data continue to accumulate on the properties and functions of the proteins encoded by these genes, and the current state of knowledge is summarized in Table 3. Several other potential protein-coding regions have been identified that overlap extensively with recognized genes, but their functional significance remains dubious (McGeoch and Davison 1999).

Overall, in excess of 85 percent of the DNA is protein-coding, and genes are tightly packed with about equal numbers oriented rightwards and leftwards. Several examples of two or more genes specifying mRNAs that share a 3′ terminus are visible in Figure 5. Larger mRNAs within these nested families contain more than one reading frame, and conventional wisdom suggests that translation initiates at the AUG codon proximal to the mRNA 5′ end. Owing to this nested arrangement, promoter sequences for certain internal mRNAs lie within the protein-coding sequences of overlapping mRNAs. HSV-1 has four genes that are expressed as spliced mRNAs, two (UL15 and RL2) with introns in protein-coding regions, and two (US1 and US12) with a common spliced 5′ noncoding leader in TR_S/IR_S. In addition, the HSV-1 latency-associated transcripts (LAT), which probably do not encode proteins, are also spliced. The LATs are the only transcripts expressed during latency (see Alphaherpesviruses: herpes simplex and varicella-zoster).

Presumably, all the genes present in a herpesvirus genome are required for natural growth and transmission of the virus. However, over half of the genes present in HSV-1 are 'nonessential', in that each of them may be removed individually without eliminating viral growth in cell culture (Roizman and Knipe 2001; see Table 3). Many of these mutants are attenuated when assayed in animal models.

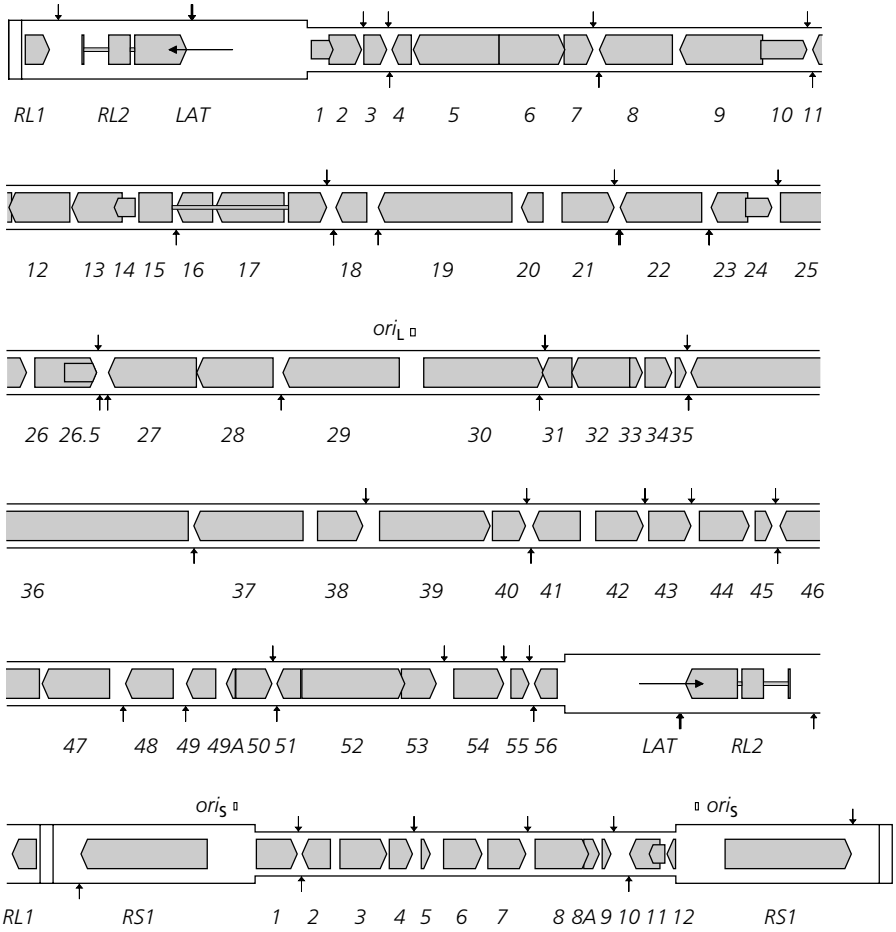

Figure 5 *Arrangement of genes in the HSV-1 genome. The genome is shown in six panels, each 25 kbp long. Inverted repeats are denoted by the thicker parts of the genome outline, and protein-coding regions are shown as shaded horizontal arrows. For the sake of clarity, the prefixes 'UL' (58 genes) and 'US' (13 genes) have been omitted from the gene nomenclature given below the genome; see Figure 4 for the locations of these regions. The major LAT RNA, presumed to be derived from a stable intron generated from a larger transcript, is also indicated by a horizontal arrow. Introns in genes* RL2 *and* UL15 *are shown as narrow bars; genes* US1 *and* US12 *are also spliced, but the intron is not shown because the 5′ exon is noncoding. Possible polyadenylation sites for mRNAs are indicated by vertical arrows above and below the genome for right and left oriented genes, respectively. The locations of* ori_s *and* ori_L *are shown by white rectangles above the genome (adapted from Davison (1992a), by courtesy of R&W Publications).*

Table 3 *Properties of HSV-1 proteins*

Gene	Predicted mass	Status	Function or properties
RL1	26194	NE	Neurovirulence factor (ICP34.5); inhibits host-controlled shutoff of protein synthesis
RL2	78452	NE	IE protein (ICP0; Vmw110); promiscuous transcriptional activator; role in ubiquitin-proteosome pathway
UL1	24932	E	Envelope glycoprotein L; complexes with glycoprotein H (UL22)
UL2	36326	NE	Uracil-DNA glycosylase
UL3	25607	NE	Nucleolar phosphoprotein
UL4	21516	NE	Nuclear protein
UL5	98710	E	Component of DNA helicase–primase complex; possesses helicase motifs
UL6	74087	E	Minor capsid protein; role in DNA packaging; putative portal protein
UL7	33057	NE	Tegument protein
UL8	79921	E	Component of DNA helicase-primase complex
UL9	94246	E	Binds to origins of DNA synthesis; possesses helicase motifs
UL10	51389	NE	Envelope glycoprotein M; disulfide-linked to UL49A protein
UL11	10486	NE	Myristylated tegument protein; role in virion envelopment
UL12	67503	(E)	Deoxyribonuclease; role in maturation/packaging of DNA
UL13	57193	NE	Tegument protein; probable serine-threonine protein kinase
UL14	23454	NE	Tegument protein; role in egress
UL15	80918	E	Role in DNA packaging; putative ATPase subunit of terminase; complexes with UL28 protein
UL16	40440	NE	Tegument protein
UL17	74577	E	Tegument protein; role in DNA packaging
UL18	34268	E	Capsid protein (VP23); component of intercapsomeric triplex
UL19	149075	E	Major capsid protein (VP5); forms hexons and pentons
UL20	24229	E/NE	Integral membrane protein; role in egress of nascent virions; host range phenotype
UL21	57638	NE	Tegument protein
UL22	90361	E	Envelope glycoprotein H; complexes with glycoprotein L (UL1); role in cell entry
UL23	40918	NE	Deoxypyrimidine kinase (dPyK or TK)
UL24	29474	NE	Nuclear protein
UL25	62664	E	Capsid-associated tegument protein
UL26	62466	E	Autocatalytically cleaved by N-terminal protease domain to give capsid protein (VP24) from N terminus and minor scaffold protein (VP21) of immature capsids
UL26.5	33758	(E)	Cleaved by UL26 protein to give major scaffold protein (VP22a) of immature capsids
UL27	100287	E	Envelope glycoprotein B; role in cell entry
UL28	85573	E	Role in DNA packaging; complexes with UL15 protein
UL29	128342	E	Single-stranded DNA-binding protein
UL30	136413	E	Catalytic subunit of replicative DNA polymerase; complexes with UL42 protein
UL31	33951	(E)	Nuclear matrix phosphoprotein involved in nuclear egress; complexes with UL34 protein
UL32	63946	E	Role in DNA packaging
UL33	14436	E	Role in DNA packaging
UL34	29788	(E)	Type II nuclear membrane protein involved in nuclear egress; complexes with UL31 protein
UL35	12095	NE	Capsid protein (VP26); located on tips of hexons
UL36	335841	E	Very large tegument protein
UL37	120549	E	Tegument protein
UL38	50260	E	Capsid protein (VP19C); component of intercapsomeric triplex
UL39	124043	E/NE	Ribonucleotide reductase large subunit (RR1)
UL40	38017	E/NE	Ribonucleotide reductase small subunit (RR2)
UL41	54914	NE	Tegument protein; virion host shutoff factor; RNase component
UL42	51156	E	Processivity subunit of replicative DNA polymerase; complexes with UL30 protein
UL43	44905	NE	Probable integral membrane protein
UL44	54995	NE	Envelope glycoprotein C; role in cell entry

(*Continued over*)

Table 3 *Properties of HSV-1 proteins (Continued)*

Gene	Predicted mass	Status	Function or properties
UL45	18178	NE	Type II membrane protein associated with envelope/tegument
UL46	78239	NE	Tegument protein; modulates IE gene transactivation VP16
UL47	73812	NE	Tegument protein; modulates IE gene transactivation by VP16
UL48	54342	E	Tegument protein (VP16; α-TIF; Vmw65); transactivates IE genes
UL49	32252	NE?	Tegument protein (VP22); capable of intercellular transport to uninfected cell nuclei; bundles microtubules
UL49A	9201	NE	Envelope protein disulfide-linked to glycoprotein M; glycosylated in some herpesviruses
UL50	39125	NE	Deoxyuridine triphosphatase (dUTPase)
UL51	25468	NE	Tegument protein
UL52	114416	E	Component of DNA helicase-primase complex
UL53	37570	(E)	Glycoprotein K; role in egress
UL54	55249	E	IE protein (ICP27; Vmw63); post-transcriptional regulator of gene expression
UL55	20491	NE	Nuclear protein
UL56	25319	NE	Membrane protein
RS1	132835	E	IE protein (ICP4; Vmw175); transcriptional regulator
US1	46521	E/NE	IE protein (ICP22; Vmw68); host range determinant
US2	32468	NE	Virion protein; interacts with cytokeratin 18
US3	52831	NE	Probably serine-threonine protein kinase; anti-apoptotic role; involved in nuclear egress
US4	25236	NE	Envelope glycoprotein G
US5	9555	NE	Envelope glycoprotein J; anti-apoptotic role
US6	43344	E	Envelope glycoprotein D; role in cell entry
US7	41366	NE	Envelope glycoprotein I; complexed with glycoprotein E in Fc receptor
US8	59090	NE	Envelope glycoprotein E; complexed with glycoprotein I in Fc receptor
US8A	16801	NE	Nucleolar phosphoprotein
US9	10026	NE	Type II membrane protein associated with envelope/tegument; role in axonal localization of viral membrane proteins
US10	34053	NE	Tegument protein
US11	17756	NE	Virion protein; double-stranded RNA-binding protein; prevents cellular PKR activation
US12	9792	NE	IE protein (ICP47; Vmw12); interferes with maturation of MHC class I molecules

Developed from Davison and Scott (1986), McGeoch et al. (1988), (1993), McGeoch (1989), McGeoch and Schaffer (1993), Davison (1993), Ward and Roizman (1994), and Davison (2000).
Genes in bold type are thought to have been inherited from the ancestor of the α-, β-, and γ-herpesviruses, and all but *UL9*, *UL23*, and *UL40* are conserved in each subfamily. The status of each gene in cell culture is indicated: E, essential; NE, non-essential; NE?, probably essential; (E), a mutant is viable, but very disabled; E/NE, non-essential under certain conditions.

Other herpesviruses

Comparisons of amino acid sequences predicted from DNA sequence data have shown that, despite substantial differences in nucleotide composition or genome structure, members of the same subfamily share the great majority of genes in a very similar layout. For example, all but six VZV genes have counterparts at equivalent locations in the HSV-1 genome. The utility of comparing a less well understood genome (e.g. VZV) with a better characterized one (HSV-1) depends on this genetic correspondence. When viruses in the three subfamilies are compared, however, divergence is much greater, with a set of 'core' genes conserved in blocks that have been rearranged during evolution. These genes number 43 and were inherited from the common ancestor of the α-, β-, and γ-herpesviruses. They are indicated in bold type in Table 3, and presumably function in processes that are central to growth and survival. Three core genes are found in only two of the three subfamilies, and this probably reflects genetic loss from one lineage. Genes encoding thymidine kinase and the small subunit of ribonucleotide reductase, for example, are not present in the β-herpesviruses. The 'noncore' genes are characteristic of each subfamily, and some are unique to one or a few very closely related viruses. Many are involved in aspects of pathogenesis that contribute to survival of the virus in its particular ecological niche. Most genes involved in control processes and latency also fall into this category.

Herpesvirus evolution

Analyses of the genetic relationships between α-, β-, and γ-herpesviruses implies that they have evolved from a common ancestor by processes including substitution, deletion, and insertion of nucleotides to modify genes or produce genes de novo and recombination processes resulting in gene duplication and divergence, capture of host genes, and gene rearrangement (McGeoch and Davison 1999). The results of phylogenetic studies (McGeoch et al. 1995, 2000) are in general accord with the notion that herpesviruses have generally (but not universally) coevolved with their hosts, and this has allowed a timescale to be proposed for the evolution of mammalian herpesviruses (Figure 6). These studies provide a firm basis for herpesvirus classification based on genetic relationships rather than on phenotypic properties.

Herpesviruses of birds and reptiles are similar to those of mammals, grouping with the α-herpesviruses. The family also contains two other major clades: fish and amphibian herpesviruses, and invertebrate herpesviruses (McGeoch and Davison 1999). The fish herpesviruses, exemplified by CCV, are only marginally related to herpesviruses that infect mammals or birds (Davison 1992b). However, evidence relating to capsid structure and assembly indicates that these herpesviruses share a common ancestor presumably dating from the time when the lineages leading to fish and other vertebrates separated (approximately 400 million years ago). The two viral lineages seem to have diverged so far that their shared inheritance is no longer discernible by amino acid sequence comparisons. The single known herpesvirus of an invertebrate, which infects several bivalve species including oysters, is very tenuously related to both fish and mammalian herpesviruses. The common ancestor of modern invertebrate and vertebrate herpesviruses is proposed as having existed about a billion years ago.

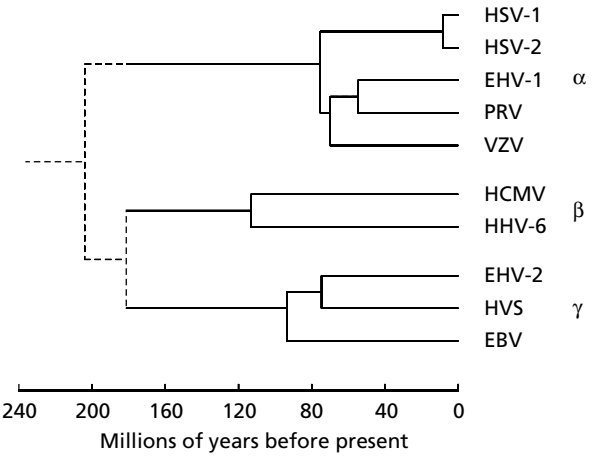

Figure 6 *A phylogenetic tree for the herpesviruses derived from sequence comparisons, with a time-scale based on the hypothesis that viruses cospeciate with their hosts. Broken lines indicate regions of lower confidence. PRV, pseudorabies virus (from McGeoch et al. 1995, with permission from Elsevier).*

GENE FUNCTION

Determination of function

Computer-aided comparisons of predicted amino acid sequences have facilitated the identification of some herpesvirus gene functions, but the majority of evidence has come from direct genetic and biochemical experimentation based on sequence information. Most information has been gained for HSV-1, and derived functions of core genes have been imputed to homologous genes in other herpesviruses. Considerable progress has also been made with noncore genes, including those of HCMV, EBV, and HHV-8. The major categories of gene functions are described below.

Control

Some proteins are involved in control processes, the most intensively studied acting directly or indirectly at the level of transcription to regulate gene expression during the infectious cycle. One such, the HSV-1 virion transactivator (Campbell et al. 1984), an essential structural component of the tegument termed VP16 (Vmw65 or α-TIF; gene *UL48*), acts to stimulate selective transcription of IE genes. Although the transactivation function of VP16 is not essential for virus growth, mutants in the appropriate domain initiate replication inefficiently at low multiplicities of infection and are avirulent in some animal model systems. Induction of HSV-1 IE genes may determine aspects of cell tropism and could play a role in reactivation from latency. Transactivation by a related protein may also occur in other α-herpesviruses, but it is not clear that the mechanism is the same. An unrelated virion protein transactivates HCMV promoters (Liu and Stinski 1992).

Coordinate induction of HSV-1 IE genes requires a consensus DNA signal (TAATGARAT) that is well conserved upstream of all five IE genes. This positive regulation of IE transcription acts through the cellular Oct-1 DNA-binding protein, and studies of VP16 function have shed light on the assembly of multicomponent transcriptional complexes (O'Hare 1993). Four IE proteins are involved in regulation of early and late virus genes (Everett 1987). Two, ICP4 (Vmw175; gene *RS1*) and ICP27 (Vmw63; gene *UL54*) are essential for replication, but precisely how these two proteins function remains obscure. ICP4 is required throughout productive infection (Watson and Clements 1980), and acts at the transcriptional level exhibiting nonspecific DNA-binding. ICP27 is a multifunctional protein that regulates transcriptional and post-transcriptional processes (Phelan

and Clements 1998). Among its post-transcriptional effects are inhibition of splicing of viral and cellular transcripts and promotion of nuclear export of viral RNAs (Koffa et al. 2001). ICP0 (Vmw110; gene *RL2*) is not absolutely required for HSV-1 replication, but is required for reactivation from latency. It has a role in overcoming suppression of viral genomes by degrading a number of cellular proteins via the ubiquitin-proteosome pathway (Everett 2000), and acts as a ubiquitin ligase in vitro (Boutell et al. 2002). Mutants of ICP22 (Vmw68; gene *US1*) are viable, but affected in host range in cell culture. The functions of this protein are not yet understood.

Considerable information is available on regulation of β-herpesvirus gene expression, largely from HCMV studies, particularly on gene functions that promote the regulated cascade of gene expression and on the viral *cis*-acting DNA sequences involved (Mocarski and Tan Courcelle 2001). Similarly, EBV and HHV-8 have been well studied both in terms of gene expression during latent infection of B-lymphocytes, and of lytic gene expression occurring following treatment of latently infected cell lines with various inducers (Kieff and Rickinson 2001; Moore and Chang 2001b).

These studies have shown that all herpesviruses examined encode strong transcriptional regulators that are expressed in the IE replication phase. However, the relevant genes appear to have developed separately in the herpesvirus lineages. This diversity is also shown by the DNA sequences that regulate synthesis of these proteins. Perhaps a similarity in tertiary structure enables these proteins to act in similar ways; alternatively, they may exert their effects by interaction with different host cell factors.

Other virus proteins modulate replicative processes in the infected cell. Amongst the α-herpesviruses, two protein kinases (encoded by genes *UL13* and *US3*, the former conserved in β- and γ-herpesviruses) are likely to act in this way. Numerous viral and cellular targets have been identified, but their physiological relevance, and hence the true roles of the enzymes, remain to be identified. The virion host shutoff function encoded by gene *UL41* is a component of an RNase that reduces the cytoplasmic stability of both cellular and viral mRNAs and serves to switch off synthesis of host cell proteins (Lu et al. 2001). This activity may also contribute to the downregulation of early viral gene expression. Viruses in each subfamily also have sophisticated genetic mechanisms for suppressing the host's immune response.

Nucleotide metabolism

Herpesviruses specify several enzymes involved in nucleotide metabolism or DNA repair (Morrison 1991). It is likely that the genes encoding these enzymes were captured at various times from the cell. Virus-specified enzyme activities generally have biochemical properties that differ from their cellular counterparts, and this is an important feature for specific targeting of certain antiviral compounds (Coen 1992).

Two HSV-1 enzymes catalyze reactions in the biosynthesis of DNA precursors: thymidine kinase (TK) and ribonucleotide reductase (RR). HSV-1 TK (gene *UL23*) appears to be evolutionarily derived from cellular deoxyribonucleoside kinase, which is known in humans as mitochondrial thymidine kinase 2 (Johansson et al. 1999). In being able to phosphorylate thymidine and deoxycytidine, the viral enzyme is more accurately described as a deoxypyrimidine kinase. HSV-1 TK activity is important for the inhibitory action of nucleoside analogs, such as acyclovir, which are phosphorylated to their monophosphate forms by the viral enzyme and subsequently to the di- and tri-phosphate forms by cellular enzymes. HSV-1 TK is not essential for viral replication in actively growing cells, but is required for growth in resting cells. The pathogenicity of TK-deficient viruses is reduced in animal models, presumably because TK activity is required to raise dTTP levels in targeting nondividing cells. As might be expected, the enzymatic properties of TK enzymes specified by different herpesviruses differ, for example in their relative ability to phosphorylate thymidine and deoxycytidine. The TK gene is not ubiquitous: the α- and γ-herpesviruses (and even CCV) contain TK genes, but the β-herpesviruses such as HCMV and HHV-6, do not, presumably because of gene loss in that lineage.

RR catalyzes the reduction of nucleoside diphosphates to deoxynucleoside diphosphates, and comprises a large (RR1) and a small (RR2) subunit encoded by genes *UL39* and *UL40*, respectively (Connor et al. 1994). It occupies a central role in metabolic routes for the supply of DNA precursors, and is required for efficient growth of virus in cell culture. RR-deficient viruses grow slowly and are nonpathogenic. Kinetic experiments have shown that RR2 is one of the earliest HSV-1 gene functions to be expressed. The genes encoding both subunits of RR are present in α- and γ-herpesvirus genomes. However, only RR1 is encoded by β-herpesviruses, and the absence of active site residues indicates that it is probably inactive. CCV lacks both genes. Interest has focused on the intersubunit interaction as a target for antiviral compounds, and this in turn has turned attention on essential interactions between other herpesvirus proteins, such as those involved in DNA replication, capsid assembly and DNA packaging (Marsden 1992).

HSV-1 encodes a deoxyuridine triphosphatase (dUTPase; gene *UL50*), which is not essential for growth in cell culture. This enzyme catalyses conversion of dUTP to dUMP, which can then be methylated to dTMP by cellular thymidylate synthase. The important function of viral dUTPase in infected cells, however, may be to reduce misincorporation of uracil during

DNA synthesis. A dUTPase gene is present in all herpesviruses studied to date, and appears to have developed in the α-, β-, and γ-herpesviruses by capture of the gene, followed by gene duplication and fusion of the two protein-coding regions. An analysis of conserved motifs casts strong doubt, however, on whether the β-herpesvirus gene is a functional dUTPase.

HSV-1 gene *UL2* encodes a uracil-DNA glycosylase, which is likely to function in removing spontaneously deaminated cytosine and misincorporated uracil residues from viral DNA and thus reduce mutations. This gene is highly conserved in the α-, β-, and γ-herpesviruses (not CCV), but is not essential for growth of HSV-1 in cell culture.

Other enzymes with roles or potential roles in nucleotide supply have been characterized in certain herpesviruses. Thymidylate synthase (TS), which catalyzes methylation of dUMP to dTMP, is encoded by VZV and certain γ_2-herpesviruses, such as HHV-8. Dihydrofolate reductase, which catalyses reduction of dihydrofolate to tetrahydrofolate, an essential step in purine synthesis and in the action of TS, is also specified by certain γ_2-herpesviruses. γ-Herpesviruses contain one or more copies of genes derived from cellular phosphoribosylformylglycinamidine synthase, which is involved in purine biosythesis. CCV possesses duplicated deoxynucleoside monophosphate kinase genes apparently captured from a bacteriophage.

DNA replication

Seven HSV-1 proteins constitute essential components of the DNA synthesis machinery, including a replicative DNA polymerase comprising two subunits, a single-stranded DNA-binding protein, three constituents of a helicase–primase complex and a protein that recognizes the origins of viral DNA replication (Challberg 1991). There has been significant progress in understanding the roles of these proteins, and several interactions in the replication complex have been characterized (Stow 2000). The possible involvement of cellular proteins, however, has not been investigated in any detail, and no origin-dependent in vitro DNA replication system has been developed. The replicative machinery of α-, β-, and γ-herpesviruses is in broad outline the same, since counterparts of six of the seven proteins are present in all mammalian herpesviruses. However, each subfamily utilizes a different means of directing the replication complex to viral DNA, and this is reflected in the lack of conservation of the α-herpesvirus origin-binding protein in the other subfamilies (except the β_2-herpesviruses) and the observation that each subfamily uses a different type of origin of replication. These differences have presumably arisen as a result of evolutionary pressures exerted on each virus during the lytic and latent cycles of infection.

HSV-1 has an origin of replication (ori_S) which, being located in TR_S/IR_S, is present in two copies in the genome (Figure 5). In addition, a related origin (ori_L) is located near the center of U_L. Other α-herpesviruses also possess ori_S at an equivalent location. Most have ori_L, in some cases at a different location in U_L, but VZV lacks this origin of replication. Both copies of ori_S or the single copy of ori_L may be deleted from the HSV-1 genome without affecting viability. Ori_S and ori_L are related in sequence and consist of an AT-rich palindrome flanked by short sequences that are recognized by the origin-binding protein, and both probably function during lytic replication. EBV also has two unrelated origins of DNA replication but, in contrast to HSV-1, these function at different stages of the life cycle (Hammerschmidt and Mankertz 1991). Ori_P is required for maintaining genome copy number in dividing cells, and is recognized by an EBV-encoded protein, EBNA-1. Ori_{Lyt}, which is required for lytic amplification of EBV genomes, consists of a much more extended region than HSV-1 ori_S or ori_L, and contains several important sequence elements. Ori_{Lyt} has also been identified in the HCMV and HHV-8 genomes (Hamzeh et al. 1990; AuCoin et al. 2002), and ori_P in the HHV-8 genome (Garber et al. 2001).

DNA polymerase has been well characterized at the genetic and biochemical levels. The catalytic subunit (gene *UL30*) is the target of antiviral agents, such as phosphonoformic and phosphonoacetic acids, which mimic dNTPs, and is the site of genetic resistance to such compounds. The associated, smaller subunit of DNA polymerase (gene *UL42*) increases the processivity of the holoenzyme. Two of the components of the helicase-primase complex (genes *UL5* and *UL52*) are essential for both enzyme activities; the UL5 protein contains a helicase domain. The third component (gene *UL8*) has an auxiliary function, and also interacts with the origin-binding protein. The origin-binding protein (UL9) consists of a homodimer, with each subunit comprising a domain that binds to the origin of replication and a helicase domain that may function in unwinding the origin to allow access to the replication complex. The single-stranded DNA-binding protein (gene *UL29*) also interacts with the origin-binding protein.

DNA packaging

The events that occur during packaging of viral DNA into immature capsids are not well understood. As with capsid assembly, this area attracts interest because of the possibility of antiviral intervention in essential herpesvirus-specific processes. Indeed, it is encouraging that one class of compound (benzimidazole ribonucleosides) has been shown to inhibit the machinery involved in DNA packaging (Underwood et al. 1998).

Eight HSV-1 genes have thus far been identified from their phenotypic effects on mutant viruses as having roles in DNA packaging (*UL6*, *UL12*, *UL15*, *UL17*, *UL25*, *UL28*, *UL32*, and *UL33*). It is unlikely that the products of these genes are all central to the packaging mechanism. The UL6, UL25, and UL17 proteins are virion components. The UL6 protein is exclusively part of the capsid and is thought to form the portal for entry of DNA (Newcomb et al. 2001). At least a proportion of the UL25 protein is capsid-associated, and the UL17 protein is in the tegument. The UL15 protein is of interest because it bears sequence similarity to ATPases and to the large subunit of the bacteriophage T4 terminase, which is responsible for energy-driven insertion of DNA into the capsid. This observation suggests that some aspects of HSV-1 DNA packaging might find parallels in bacteriophage T4, and supports the speculative view that herpesviruses and T4 may share a common, but very ancient, evolutionary origin. The *UL15* gene is unusual in that has highly conserved counterparts in all herpesviruses and is expressed as a spliced mRNA in the vertebrate viruses. The UL15 protein interacts with the UL28 protein. UL12 encodes an alkaline exonuclease, which may be responsible for resolving DNA structures produced during replication.

Virion structure

DEGREE OF COMPLEXITY

About half of the total number of HSV-1 genes specify components of the viral particle. These proteins include eight capsid constituents, at least 20 tegument components, and more than ten proteins, most glycosylated, in the envelope. The proteins making up the capsid are better characterized than those in the envelope or tegument. Capsid proteins can be envisaged as providing a robust, self-assembling container for the viral genome, tegument proteins as ancillary factors responsible for enhancing viral infection, and envelope proteins as molecules involved in allowing infecting virions to enter cells efficiently and facilitating mature virions to exit the infected cell.

CAPSID

The proteins that make up HSV-1 capsids are conserved in other herpesviruses, and the capsid structures of widely diverged members (such as HSV-1 and CCV) are similar. Thus, it is likely that the same basic mechanism for capsid maturation is fundamental to all herpesviruses. The proteins that make up the HSV-1 capsid have undergone extensive characterization (Homa and Brown 1997), and the structure of the capsid has been analyzed in some detail by electron cryomicroscopy. A single protein, VP5 (gene *UL19*), makes up the hexameric and pentameric capsomers of the HSV-1 capsid, and the intercapsomeric regions are composed of a complex (known as a triplex) of two copies of VP23 (gene *UL18*) plus a single copy of VP19C (gene *UL38*). The external tips of hexameric capsomers are decorated with a small protein, VP26 (gene *UL35*). The capsid also contains a protease, VP24 (gene *UL26*). The protein encoded by gene *UL6* is present in a small number of copies per capsid.

The steps that occur during capsid formation are reminiscent of those that take place during maturation of certain DNA-containing bacteriophages, such as T4, in that replicated DNA enters preformed immature capsids containing a core of scaffolding proteins, which are expelled in a process involving specific proteolytic events. The HSV-1 scaffold proteins are encoded by the overlapping genes *UL26* and *UL26.5* (Liu and Roizman 1991; Figure 5). The UL26.5 protein is identical to the C-terminal portion of the UL26 protein. Proteolytic action of the N-terminal domain of the UL26 protein on the UL26 protein itself at two locations, one central and one near the C terminus, and on the UL26.5 protein at the site near the C terminus generates VP24 (the protease) and VP21 (the minor scaffold protein) from the UL26 protein, and VP22a (the major scaffold protein) from the UL26.5 protein. During DNA packaging, VP21 and VP22a are expelled from the capsid interior, but VP24 is retained.

The molecular events that occur during capsid maturation have been unraveled with the aid of a system in which the HSV-1 proteins are expressed from baculovirus recombinants (Thomsen et al. 1994; Tatman et al. 1994), and, more recently, with purified proteins (Newcomb et al. 1999). VP5, VP23, VP19C are essential for capsid assembly, plus either the gene UL26.5 protein which gives rise to VP22a or, at a reduced efficiency, the gene UL26 protein which gives rise to VP21 and VP24. The proteolytic function of the UL26 protein is thus not essential in this system, but its requirement for virus production during HSV-1 infection indicates that it is involved in removal of the scaffold proteins during DNA packaging. It appears that the immediate product of capsid assembly is a spherical 'procapsid' which angularizes to a capsid during DNA packaging (Trus et al. 1996).

TEGUMENT

The tegument has a complex composition, and demonstrates icosahedral structure only in the region immediately adjacent to the capsid surface (Chen et al. 1999). It is a feature of all members of the family but, in contrast to capsid proteins, most components of the tegument are specific to a particular subfamily or genus and are therefore likely to have important roles in modulating the virus–host interaction, rather than as passive structural components of the virion. Examples of such roles for HSV-1 tegument proteins are the virion host shutoff

factor (gene *UL41*), VP16 (gene *UL48*) and the UL13 protein kinase. It is likely that additional functions for tegument proteins will emerge as this area of research develops.

ENVELOPE

At least 11 HSV-1 genes encode membrane-associated glycoproteins, and most of these are present in the virion envelope. The glycoproteins are located in membranes by virtue of one or several hydrophobic domains, and thus are exposed on the exterior of infected cells or virions. Also, some are functionally associated with initial virus–host interactions (Shukla and Spear 2001) and are therefore likely to be of considerable importance for pathogenicity. For this reason, and because of their utility in vaccine development, herpesvirus glycoproteins have been the subject of intense scrutiny.

Many glycoproteins are specific to genera or subfamilies. For example, three HSV-1 glycoproteins lack counterparts in VZV (gD, gG, gJ). Only gB, gH, gL, gM, and gN (which is not glycosylated in some herpesviruses, including HSV-1) have counterparts across the α-, β-, and γ-herpesviruses. HCMV contains extensive families of related glycoprotein genes that appear to have been generated by gene duplication and divergence (Chee et al. 1990), and thus represent a striking evolutionary experiment in generating genetic diversity.

The initial interaction of HSV-1 with cells involves the binding of gC or gB to heparan sulfate proteoglycans present on the cell surface (Spear et al. 2000). gD then binds to one of several receptors in the TNF-receptor family or immunoglobulin superfamily or to specific forms of heparan sulfate. The viral and cellular membranes fuse in a process that requires gB, gD, gH, gL, and the gD receptor, and the capsid and tegument are released into the cytoplasm. Like HSV, HCMV uses heparan sulfate as a cell surface receptor for the initial binding of virus. EBV, however, binds to cells in a different manner. The EBV virion glycoprotein gp350/220, which lacks sequence counterparts in the α- and β-herpesviruses, binds the cellular receptor CD21 that is found on B-lymphocytes (Kieff and Rickinson 2001). The nature of the receptor for EBV on epithelial cells remains unresolved. HHV-8 appears to enter cells via heparan sulfate and an integrin receptor (Akula et al. 2002).

Of the HSV-1 glycoproteins that are not required for growth of virus in cell culture, three interact with key elements of the immune system: gC binds to the C3b component of the complement system and gE and gI form a complex that can bind the Fc constant region of immunoglobulin G. Of the essential glycoproteins, gH and gL associate during maturation in endoplasmic reticulum and are present as a heterodimer in virions. Also, gM is disulfide-linked to gN. HSV-1 is unusual in that gB in other herpesviruses is processed by a trypsin-like cleavage into a disulfide-linked dimer.

Pathogenesis

Some viral proteins have specific roles in disease processes, including latency. These proteins help to fit a virus to an ecological niche and are usually specific to a subfamily or genus. They may have little effect on the growth of virus in cell culture, but may profoundly influence virus virulence in vivo. Functional analyses of such genes are fundamental to our understanding of herpesviruses as infectious agents. This information is crucial for the design of safe and effective herpesvirus vectors for gene replacement and tumor therapy and for the development of live vaccines. The mechanisms used by herpesviruses to control the processes of natural infection are steadily being elucidated, and the knowledge gained is likely to lead to novel therapies. Herpesviruses are seen increasingly as sophisticated manipulators of various cellular mechanisms and defenses in sophisticated ways, including the cell cycle, apoptosis and various aspects of the immune response (Roizman and Knipe 2001; Hengel et al. 1998).

Herpesvirus genes that modulate the immune response appear to be more numerous in the β- and γ-herpesviruses (Johnson and Hill 1998; Reddehase 2000; Neipel et al. 1997). Among the α-herpesviruses, in addition to gC, gE, and gI which can interact with the immune system, a gene specific to HSV-1 and HSV-2 (gene *US12*) encodes an IE protein (ICP47 or Vmw12) which interferes with maturation of MHC class I proteins in infected cells by interacting with the TAP peptide transporter complex (York et al. 1994). The product of gene *RL1* (ICP34.5) is specific to HSV-1 and HSV-2 and related to proteins implicated in cell differentiation, and has a profound effect on neuropathogenicity (Chou et al. 1990). It appears to function by promoting dephosphorylation of eIF-2α and thereby inhibits host-controlled shutoff of protein synthesis (He et al. 1997). RL1 null mutant viruses are not neurovirulent in vivo, and exhibit limited replication at peripheral sites, but fail to replicate in neurons of the peripheral nervous system. These viruses can, however, establish latent infections from which they can be reactivated. RL1 mutants are currently being evaluated for their possible utility in human tumor therapy (Markert et al. 2001).

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Alphaherpesviruses: herpes simplex and varicella-zoster

ANTHONY C. MINSON

Herpes simplex virus 1
Varicella-zoster virus 7
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Herpes simplex virus (HSV) types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) are alpha herpesviruses that are related in their genetic and biological properties. Their genomes are composed of homologous sets of genes organized in a co-linear fashion; they cause productive cytolytic infection of epithelial cells, and establish life-long latent infections in sensory nerve ganglia. There are, however, important biological differences:

- VZV infection has a viremic phase whereas HSV infectivity is usually limited to the epithelium and sensory nerves at the infection site
- HSV is transmitted only by contact whereas VZV is airborne and contagious
- VZV is highly species-specific whereas HSV can infect, experimentally, a wide range of species.

Indeed, our knowledge of HSV pathogenesis and immunity derives largely from studies of the virus in rodents. HSV-1 and HSV-2 are closely related: they crossreact antigenically, exhibit extensive nucleotide sequence homology and their genes are functionally homologous because stable intertypic recombinants can be generated readily in tissue culture. However, despite the fact that HSV-1 and HSV-2 can infect similar sites in humans, recombination between these viruses does not appear to occur in vivo, and genetic comparison suggests that the two subtypes have evolved independently for more than 5 million years (McGeoch and Cook 1994).

HERPES SIMPLEX VIRUS

Clinical manifestations

HSV infects mucosal epithelium or damaged cutaneous epithelium, and infection can therefore occur at a variety of sites, but oral and genital infections are most common. HSV-2 is predominantly responsible for genital infection but HSV-1 contributes significantly, representing 30–50 percent of genital isolates in some studies (Buckmaster et al. 1984; Ross et al. 1993). By contrast, HSV-2 is rarely isolated from oral lesions. The basis of this preference for different mucosal surfaces by the two virus types is unknown but the predominance of HSV-2 'below the waist' and HSV-1 'above the waist' extends to other sites. Thus anal lesions, cutaneous lesions of the thighs and buttocks, and infections of the neonate during birth are more frequently caused by HSV-2 whilst eye infections and cutaneous lesions of the head and neck are usually caused by HSV-1. Regardless of the site of infection or the virus type, HSV enters sensory nerve endings during primary infection and is transported to the neuronal cell body where latent infection is established. Since oral and genital infections are by far the most common, the trigeminal and sacral ganglia are the usual sites of latent infection by HSV-1 and HSV-2, respectively. The majority of the adult population is latently infected with HSV, but it is clear that infection rates greatly exceed clinical disease rates and many, perhaps most, individuals who are latently

infected with HSV have no recollection of primary infection and do not suffer recurrent lesions.

Primary oral infection presents as an acute gingivostomatitis comprising painful ulcers in the mouth, which resolve within a few weeks, often accompanied by fever and enlargement of local lymph nodes. A significant proportion of sore throats is also thought to be caused by primary oral HSV, and this may be the most common manifestation of primary infection. During the course of primary infection the virus establishes a lifelong latent infection in neurons of the trigeminal ganglia, and 'reactivation' results in the reseeding of virus into the oral epithelium and shedding in saliva. A minority of seropositive people suffer periodic clinical recurrences, the classic 'cold sores,' crops of vesicles on the mucocutaneous junctions of the mouth and nose which ulcerate, crust, and heal within 4–7 days (Figure 1). Some of these individuals recognize a prodromal tingling or burning sensation which heralds the development of the recurrent lesion. Recurrences are provoked by a variety of stimuli, including sunlight, physical trauma, stress, respiratory infections, and hormonal change, but it is not known whether these stimuli act by a common pathway. The development of a cold sore requires reactivation of the latent virus in the ganglia followed by the establishment of a focus of epithelial infection; a provoking stimulus might operate either by triggering a reactivation in the ganglia or by modifying the susceptibility of the relevant epithelium such that a ganglionic reactivation, which would otherwise have been silent, results in a clinical recurrence.

Genital herpes consists of painful vesicles on the genitalia or anal region. Aseptic meningitis has been reported as a complication of primary infection, and proctitis has been reported in homosexual men. HSV-2 appears to be almost exclusively responsible for these complications. As with oral infection, many primary genital infections are inapparent and the first indication of infection may be a recurrence, an episode that has been termed 'initial infection' to distinguish it from the primary infection (Corey et al. 1983). Genital herpes infections recur at a higher frequency than oral infections, and genital HSV-2 infections recur more frequently than genital HSV-1 infections (Lafferty et al. 1987). The frequency and severity of recurrent episodes usually decrease with time but recurrent genital herpes is a painful and distressing disease, and many patients suffer frequent recurrences for many years. Reactivation of latent virus and shedding in genital secretions occur at a much higher rate than clinical disease.

Figure 1 *Vesicular cold sores due to recurrent herpes simplex. (Photograph courtesy of Dr. Jane Sterling, Addenbrooke's Hospital, Cambridge, UK: Copyright Addenbrooke's NHS Trust.)*

Infection of the skin, manifest as a crop of classic herpetic vesicles, can occur on any part of the body, most commonly the head or neck, and probably always results from the contamination of damaged skin by virus in saliva or genital secretions. Infection may result from intimacy or activities likely to cause abrasion, such as wrestling (herpes gladiatorum) or rugby (scrum pox). Lesions on the fingers (herpetic whitlows) are an occupational hazard for healthcare workers. A particularly severe disease results from infection of eczematous skin (eczema herpeticum), in which the virus spreads widely, causing large areas of ulceration.

Herpes infections of the eye are second only to oral and genital infections in frequency. Primary infections are associated with keratoconjunctivitis and are often bilateral whereas recurrences are usually unilateral and result in dendritic ulcers or stromal involvement. Repeated recurrences may lead to scarring and opacification of the cornea, and consequent sight impairment.

Invasion of the central nervous system (CNS) is manifest as herpes encephalitis, a cytolytic infection, primarily of neurons, with a propensity to focus in the temporal lobes (see Infections of the central nervous system). Untreated, the disease has a mortality of >70 percent and most survivors are neurologically impaired. All age groups are susceptible but the disease is rare; about one case per million population is reported each year in the UK and the USA. Symptoms generally begin with fever and headache, rapidly followed by disorientation and progressive deterioration in consciousness, but the symptoms are not diagnostic and can be confused with other syndromes. Predisposing factors are unknown. Herpes encephalitis can apparently result from primary or recurrent infection (Nahmias et al. 1982; Whitley et al. 1982), but the route to the CNS is uncertain. A focal encephalitis, similar to that observed in humans, results from infection of mice by the olfactory route (Anderson and Field 1983). Aciclovir therapy has greatly reduced the morbidity and mortality of herpes encephalitis but effective treatment depends on prompt diagnosis.

The neonate is highly susceptible to HSV infection, and neonatal infection is frequently fatal. The disease presents classically as a generalized infection affecting multiple organs, including the lung, liver, adrenals, CNS, and skin, but in some cases the infection is more limited, involving the skin or the CNS or both. The prognosis is very poor for those with disseminated or CNS infection.

In the USA neonatal herpes is estimated to occur with an incidence of 1 per 2 000–5 000 deliveries and is perceived as a major public health problem, but in the UK the disease is rare (Sullivan-Bolyai et al. 1983; PHLS Report 1987), an unexplained discrepancy that cannot be accounted for by differences in the overall frequency of genital herpes in the two populations. The infection is most commonly acquired during birth from a genital infection of the mother but infections in utero and in the immediate postnatal period have also been documented and probably represent a significant proportion of the total. Primary infection in the mother close to term presents a much higher risk to the neonate than does recurrent infection (Prober et al. 1987), probably owing to the higher virus titers present during primary infection and perhaps to the protective effect of maternal antibody present in the neonate exposed to recurrent infection. Delivery by cesarean section is usually recommended when genital lesions are present at term. Superficial damage to the skin by fetal scalp monitors is thought to provide the virus with a route of entry, and the use of scalp monitors is contraindicated if the mother has a history of recurrent genital infection.

Not surprisingly, the immunocompromised patient has an increased susceptibility to HSV infection. The most common manifestation is a reduced capacity to control recurrent oral lesions which occur more frequently and spread more widely on the head and neck and to the respiratory tract (e.g. Smyth et al. 1990). These infections resolve slowly, if at all, in the absence of therapy. Although these infections can be very severe, they are nevertheless limited to the skin and mucosa, even in profoundly immunosuppressed patients. Disseminated, generalized infection, as seen in the neonate, has been reported in adults very rarely.

Epidemiology

A wide range of mammalian species can be infected experimentally with HSV but there is no evidence that this occurs naturally. Humans are the only natural host, and latent virus in trigeminal and sacral ganglia is the reservoir. The source of transmitted virus is oral or genital secretions, and transmission requires contact. There is no seasonal variation in disease incidence. Because HSV-2 is primarily a sexually transmitted disease and occurs only rarely as an oral infection, evidence of HSV-2 infection is found almost exclusively in adolescents and adults, whereas HSV-1 is a common infection of childhood. Despite this straightforward picture, which emerges from a large number of seroepidemiological and virological studies, precise data on the incidence of HSV-1 and HSV-2 infections in different study populations have been difficult to obtain because of the high proportion of asymptomatic infections and problems in distinguishing antibodies to the two subtypes.

In many populations, virtually all children are infected with HSV-1 during the first 5 years of life, but seroconversion rates vary greatly with geographical area and socioeconomic group. In higher socioeconomic groups in industrialized countries, seroconversion may be as low as 20 percent during the first 5 years, rising to 40–60 percent during adolescence and early adulthood. Young children secrete virus asymptomatically more frequently and for much more prolonged periods than seropositive adults; this, together with higher levels of contact activity, probably accounts for the high attack rates in this group. Seroepidemiological surveys emphasize the view that HSV-2 is transmitted primarily by the sexual route: antibodies specific for HSV-2 are virtually nonexistent in nuns but are found in the majority of prostitutes, and the appearance of HSV-2 antibodies correlates with the onset of sexual activity. The reported overall incidence of HSV-2 antibodies in the adult population varies in different studies, and many historical surveys suffer from technical problems associated with the serological crossreactivity of HSV-1 and HSV-2. More recent studies estimate that 15–25 percent of the adult US population is infected with HSV-2, and broadly similar data have been obtained in the UK (Ades et al. 1989), though prevalence rates vary significantly among different ethnic groups. As noted above, HSV-1 can also be transmitted by the sexual route, and in some studies nearly 50 percent of genital isolates were of this type (Ross et al. 1993). It is supposed, though not proven, that the large number of HSV-1 genital infections and the occasional HSV-2 oral infection result from orogenital sex.

Pathology and pathogenesis

The portal of entry in primary infection is the damaged skin or mucosa and the classic lesion is a vesicle beneath the keratinized squamous epithelial cells (Figure 2).

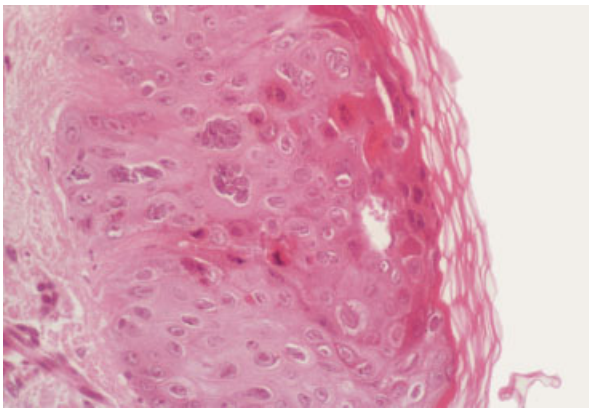

Figure 2 *Herpes simplex vesicle: infection is limited to epithelial cells, which become disorganized and exhibit characteristic nuclear inclusions. Multinuclear cells result from cell fusion. The lesion shown is at a relatively early state and contains very few inflammatory cells. (Photograph courtesy of Dr. Jane Sterling, Addenbrooke's Hospital, Cambridge, UK.)*

The infection of epithelial cells is cytolytic; the cells lose adhesion, occasionally become multinucleate as a result of virus-induced cell fusion and contain Cowdry type A nuclear inclusions. The vesicle drains and the lesion crusts before healing occurs, sometimes with residual scarring, and draining lymph nodes are commonly enlarged during this process. Recurrent lesions are morphologically and histologically similar but are usually less extensive, and lymph node swelling is inapparent. In the immune competent patient the lesion resolves within 7–10 days of its appearance.

LATENCY AND REACTIVATION

During primary infection of the skin or mucosa the virus enters sensory nerve endings and is transported by fast retrograde axonal flow to the neuronal bodies of the dorsal root ganglia innervating the site of infection. In animal models this is followed by a period of limited virus replication in the ganglia, during which virus antigen can be detected in a small number of neurons. Within about 10 days, no infectious virus can be detected in the ganglia or at the site of inoculation, but the presence of latent virus can be detected for the lifetime of the infected animal by dissection and in vitro culture of the relevant sensory ganglia (Stevens and Cook 1971; Hill et al. 1975). Similarly, 'in vitro reactivation' of latent virus can be achieved by culture of trigeminal ganglia from cadavers of HSV-1 seropositive humans.

Many lines of evidence show that HSV establishes a true latent infection rather than a low level chronic infection. Mutant viruses that are incapable of replicating at the internal temperature of the mouse establish and maintain latency (McLennan and Darby 1980). Long-term treatment of infected mice or humans with aciclovir does not cure latency (Fife et al. 1994); the state of the viral genome in latent infection is unique. HSV DNA within virus particles is a linear molecule whereas viral DNA in latently infected ganglia is endless and is thought to be present as a circular episome (Mellerick and Fraser 1987). The latently infected ganglion contains a unique set of transcripts, the 'latency-associated transcripts' (LAT) synthesized from a single promoter (the LAT promoter) situated in the repeat sequences flanking the unique long region of the viral genome (Stevens et al. 1987). These transcripts are readily detected by in situ hybridization in latently infected human trigeminal ganglia or in ganglia from experimental animals, and they are found exclusively in neurons (Figure 3) – consistent with other evidence that this cell type is the site of latent infection. The function of the latency-associated transcripts is uncertain. Mutations that modify the transcripts, or abolish their synthesis, do not prevent the virus from establishing or maintaining latent infection in animal models. Indeed, despite extensive study, no HSV gene has been identified that is essential for latent infection, and one view is that latency requires no gene function but occurs by default if the productive cycle fails. On this supposition the transcriptional machinery of the neuron is the key factor in latency and reactivation: if the neuron fails to transcribe the viral immediate–early genes (see Herpesviruses: general properties), the productive cycle cannot be initiated and the viral genome is latent by default, but at some later time perturbation of the sensory nerve may change the transcriptional program of the neuron. The viral immediate–early genes may then be expressed, the productive cycle is initiated, and the latent virus reactivates. The evidence for this mechanism is largely negative but is consistent with the characteristics of an in vitro latency model in which the level of expression of one of the immediate–early genes appears to be a key factor in determining whether the virus enters the lytic cycle or remains latent (Preston 2000). Evidence is accumulating that the LAT has anti-apoptotic functions and this protects the neuron during establishment of latency and during reactivation. This would account for the fact that HSV mutants unable to synthesize LATs are capable of establishing latency but do so with reduced frequency and reactivate less efficiently (Jones 2003).

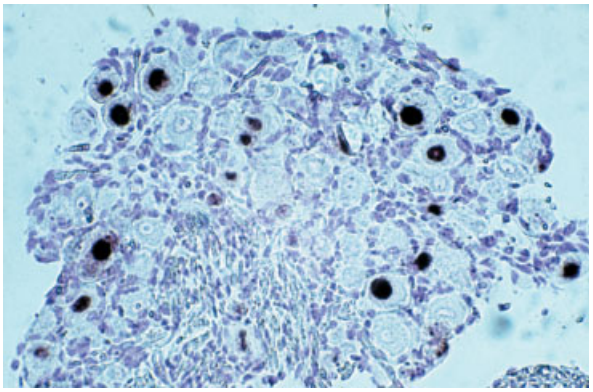

Figure 3 *Virus-specific transcripts in a sensory ganglion from a mouse latently infected with* HSV-1. *LATs are detected by hybridization in situ within the nuclei of a minority of sensory neurons. No viral antigens, productive cycle transcripts or virus particles are detectable. Latent infection of the tissue can be demonstrated by explant culture. (Photograph courtesy of Dr. R Lachmann, Department of Pathology, University of Cambridge.)*

Perturbation of the sensory nerve can induce HSV reactivation, an observation first made by Cushing (1905), who noted the frequent appearance of cold sores after sectioning the posterior sensory root of the trigeminal ganglion, a result that has been confirmed in animal models. Furthermore, virus in latently infected rat sympathetic ganglia cultured in vitro reactivates after withdrawal of nerve growth factor (Wilcox et al. 1990). Nevertheless, the specific neuronal changes that lead to reactivation are unknown. A wide variety of stimuli provoke clinical recurrence but, as noted earlier, many of these stimuli may act to enhance virus replication at

the periphery rather than to induce reactivation in the ganglion. What is certain is that reactivation and virus shedding are much more common than clinical recurrence. The fate of the neuron in which reactivation occurs is also unknown. It is axiomatic of HSV infection that the productive cycle is cytolytic, yet reactivation and seeding of epithelium with infectious particles must require productive infection in the neuron. There is no evidence of sensory loss, even in patients who suffer frequent recurrences over a very long period, and it is uncertain whether reactivation results in the loss of the neuron or if, unlike other cell types, the neuron can survive virus replication.

Immune response

HSV infection in humans induces a vigorous humoral and cell-mediated response, but studies of the details are confounded by the very large number of antigens specified by the virus (>70) and by the technical problem of examining the response to primary infection. Most studies in humans have been of the response to latent infection or recurrent disease, whereas much information on the primary response derives from experiments in inbred mice.

Infection is followed within a few days by the appearance of IgM antibodies, closely followed by IgG and IgA. IgG persists indefinitely and antibodies against at least 30 different viral polypeptides have been identified. The surface glycoproteins of the virus (of which there are at least 10) are among the most immunogenic and these are also the targets for complement-dependent and complement-independent antibody-mediated neutralization. Glycoprotein D, delivered as a subunit protein or using live vectors, is probably the most potent inducer of neutralizing antibody and, in humans, anti-gD titers correlate with serum neutralizing titers. The role of antibody in infection is, however, uncertain. High levels of passive antibody protect mice from infection and modulate infection (Simmons and Nash 1985) but B-cell-deficient mice recover more or less normally from infection and agammaglobulinemic patients do not suffer particularly severe HSV infections (Corey and Spear 1986; Simmons and Nash 1987). While antibody can protect against infection, at least in mice, the cell-mediated responses are of central importance in controlling an established infection since, in humans and in mice, T-cell deficiencies result in severe disease and inefficient clearance of virus. The importance of different T-cell subsets and of innate cellular defense mechanisms varies in mice, depending on the strain and infection route. Nevertheless, $CD4^+$ T cells predominate in clearance of infection from the skin and mucosa (Nash and Gell 1983; Schmid 1988), whereas classic major histocompatibility complex (MHC) class I-restricted cytotoxic T cells are readily detected in mice but difficult to detect in humans. There is evidence that $CD8^+$ T cells control HSV in the nervous system but by a cytokine-mediated mechanism rather than by direct cytotoxicity (Simmons and Tscharke 1992). Innate defenses are also likely to be of central importance. HSV has a very broad cell tropism yet systemic generalized disease is found almost exclusively in the neonate and not in profoundly immunosuppressed children or adults. In mice, interferon responses, NK cell activity, and macrophage function have all been shown to control HSV infection, and deficiencies in these defenses may contribute to the susceptibility of the neonate (Cunningham and Merigan 1983; Lopez 1985; Wu and Morahan 1992). Severe HSV infection in a child with an NK-cell deficiency has been reported (Biron et al. 1989) but there are conflicting data on the importance of NK cells in controlling HSV (Bukowski and Welsh 1986).

It is worth reiterating that studies of the response to HSV in the mouse are almost exclusively concerned with primary infections. Recurrent infections do not occur in inbred mice, and in guinea pigs and rabbits, which exhibit recurrent disease, the immune response is not amenable to detailed analysis. In humans, recurrent episodes are not usually accompanied by significant changes in antibody levels but changes in T-cell proliferative responses have been reported. Comparisons of immune responses in seropositive patients with and without recurrent disease have given no indication of a consistent quantitative or qualitative difference in response in these two groups, and it may be that other factors, such as latent virus load, rather than the immune response, determine recurrence frequency. Nevertheless, the fact that recurrences usually decrease in frequency with time and that immunocompromised patients suffer more recurrent episodes suggests that recurrences can be suppressed by an acquired immune response. This is an issue of some consequence because the identification of the key elements of the response might allow the development of immunotherapeutic approaches to the prevention of recurrent disease. Immune responses to HSV infection have been reviewed by Daheshia et al. (1998) and Nash (2000).

HSV has evolved a number of mechanisms to evade the immune response. Glycoprotein C binds to the C3b complement component and inhibits the complement cascade (Fries et al. 1986). The glycoprotein E/glycoprotein I complex binds to Fc regions of IgG and inhibits Fc-mediated effector mechanisms (Frank and Friedman 1989; Bell et al. 1990). The US12 gene product binds the transporter associated with antigen processing (TAP) and prevents antigen presentation on MHC class I molecules (York et al. 1994; Hill et al. 1995). None of these evasion mechanisms operates efficiently in mice, and there is good reason to doubt, therefore, whether immune control of HSV in mice precisely mimics events in humans.

Diagnosis

HSV infection is usually diagnosed by virus culture or by polymerase chain reaction (PCR). The virus is labile and sensitive to desiccation, so swabs should be kept in transport medium and cultured as soon as possible. HSV grows rapidly in a variety of fibroblasts and epithelial cell types, causing a characteristic cell rounding or 'ballooning' which develops within a few days and spreads rapidly. Unlike laboratory-passaged strains, which are often syncytial, fresh isolates cause little, if any, cell fusion. In combination with clinical presentation, the development of characteristic cytopathic effect (CPE) in culture is usually sufficient for confident diagnosis, but if necessary this can be confirmed by a variety of immunocytochemical or antigen capture tests. The sensitivity of these tests allows diagnosis within 24 hours of culture, before extensive CPE develops, and also allows distinction between HSV-1 and HSV-2 isolates with type-specific reagents. Primary infections can also be diagnosed serologically by the detection of virus-specific IgM or by the rising IgG titer. A variety of tests are available but enzyme-linked immunosorbent assay (ELISA) is the most widely used. The diagnosis of herpes encephalitis presents a special problem and rapid diagnosis is of importance because of the urgency of appropriate treatment. Diagnosis used to require the identification of virus or virus antigen in brain biopsies, but is now achieved by detection of viral DNA in the cerebrospinal fluid using PCR (Lakeman et al. 1995). The sample must, however, be taken before aciclovir therapy is begun.

The distinction between serum antibodies to HSV-1 and HSV-2 is rarely of clinical relevance but is crucial in seroepidemiological surveys of infection incidence. Serum against either virus reacts with both types in all assays, and historical methods of measuring type-specific antibody such as cross-adsorption and kinetic neutralization were time-consuming and unreliable. More recent methods are based on the observation that glycoprotein G of HSV-1 and HSV-2 is highly immunogenic and shows very little antigenic cross-reaction. Glycoprotein G, purified from infected cells or prepared as a recombinant protein, can therefore be used as a target for type-specific antibody detection using standard enzyme-linked assays (Lee et al. 1986; Sanchez-Martinez et al. 1991; Ho et al. 1992). Although these methods are clearly an improvement on previous approaches, discrepant results have been reported when compared with western blot assays, which are generally regarded as well validated but relatively tedious (Ashley et al. 1988; Safrin et al. 1992).

Control

Like other nonepidemic diseases transmitted by contact, HSV infection cannot be controlled by public health measures. Reduction in sexual transmission can be attempted by public education on the risks of unprotected sex. Despite education campaigns aimed at reducing the spread of the human immunodeficiency virus (HIV), the reported incidence of genital herpes has risen in the UK (Barton 1995), though it is questionable whether this represents a real increase in transmission frequency.

VACCINATION

No licensed vaccine is currently available for prophylaxis against HSV. Indeed, the fact that reinfection is possible and that restimulation of the response by a recurrence does not prevent further recurrences has been used to argue that the development of a successful vaccine is impossible. Two lines of evidence suggest that this is not so. First, infection by HSV-1 decreases the incidence and severity of subsequent HSV-2 genital infections (Mertz et al. 1992). Second, a variety of immunogens, including killed virus, glycoprotein subunits, viral antigens expressed by live vectors, and attenuated or disabled HSV, will all protect mice and guinea pigs from challenge infection. As noted previously, the virus has evolved immune evasion mechanisms that do not operate efficiently in rodents, and data obtained from animal models must be treated with caution. Nevertheless, these results suggest that a protective response can be mounted, and the successful development of attenuated vaccines against other human and animal alphaherpesviruses gives some confidence in the principle of vaccination against HSV. Many candidate vaccines have been developed based on the production of immunogenic subunits or the construction of rationally attenuated or disabled live HSV. A number of candidates have been studied in clinical trials, though most trials tested therapeutic benefit against established recurrent infection rather than prophylactic efficacy. The difficulty of conducting a fully controlled prophylactic trial, and in particular of monitoring subclinical infection in participants, is illustrated by the study conducted by Mertz et al. (1990). Progress and prospects in the development of HSV vaccines has been reviewed by Whitley and Roizman (2002) and Koelle and Corey (2003).

CHEMOTHERAPY

HSV, together with other human herpesviruses, is among the most studied targets for antiviral chemotherapy, a subject covered in detail elsewhere (see Immunoprophylaxis of viral diseases). Nucleoside analogues such as idoxuridine, trifluorodeoxyuridine, and adenine arabinoside were used for many years to inhibit viral DNA replication, but these analogues lacked selectivity and their cytotoxicity limited their use to topical treatment in all but the most life-threatening circumstances. The development of aciclovir transformed the treatment of HSV infections. Aciclovir

is a guanosine analogue in which the sugar is replaced by an acyclic ring lacking the 2′ and 3′ carbons. The analogue is converted to the 5′-monophosphate by the HSV-specific thymidine kinase (TK) and then to the triphosphate by cellular kinases. The viral DNA polymerase incorporates the triphosphate into the growing viral DNA where it acts as a chain terminator. The inability of host-cell kinases to convert aciclovir into its monophosphate accounts primarily for its selective action against HSV-infected cells and its nontoxicity, although, in addition, the viral DNA polymerase has a higher affinity than its cellular counterpart for the triphosphate. Aciclovir is available in a number of formulations and can be administered topically, orally, or intravenously. Its oral bioavailability is 15–30 percent and plasma half-life about 2–3 hours, so relatively frequent doses are required to maintain therapeutic concentration. The route of administration depends on the site and severity of infection: topical application is used for eye infections and oral lesions, oral administration for severe genital infections and intravenous administration for encephalitis and life-threatening infection of immunocompromised patients. A cream formulation is available without a prescription ('over the counter') in the UK for cold sore treatment, but not in the USA.

Mutants of HSV resistant to aciclovir arise readily in culture, almost invariably due to loss of a functional virus TK gene. This gene function is irrelevant for virus growth in vitro but its loss results in severe attenuation in vivo. Alternative routes to resistance are point mutations in the TK gene or DNA polymerase gene that reduce enzyme affinity for aciclovir and aciclovir triphosphate, respectively (Furman et al. 1981; Larder et al. 1983; Larder and Darby 1984). Resistance to therapy has been observed most frequently in immunocompromised patients receiving long-term therapy. The mutants isolated from these patients include those with altered TK or DNA polymerase enzymes, but the most frequent finding is of a heterogeneous population of viruses with a range of TK activities from entirely deficient to almost normal. The basis of this form of resistance is uncertain, but it is known that TK-negative viruses can be complemented, in vivo, by a wild-type virus (Efstathiou et al. 1989) and it appears that aciclovir selective pressure, particularly in the immunocompromised, can maintain a dynamic mixture of TK-kinase-positive and -negative viruses.

During the past 10 years, a second generation of anti-herpes drugs based on aciclovir, with the same mechanism of action, has been developed (famciclovir, valaciclovir). A second class of analogues with anti-HSV activity are pyrophosphate analogues which act as inhibitors of HSV DNA polymerase. Foscarnet is the sole example in clinical use. Topical application is well tolerated but intravenous administration can cause nephrotoxicity and severe nausea. Foscarnet has no advantage over aciclovir but is of value in treating aciclovir-resistant infections.

Finally, anti-herpes chemotherapy suppresses virus growth but has no apparent effect on latent infection. Patients who have been treated with aciclovir continuously for as long as 6 years experience recurrences after drug withdrawal (Fife et al. 1994).

VARICELLA-ZOSTER VIRUS

It has been recognized for many years that varicella (chickenpox) and zoster (shingles) are caused by the same virus and that zoster results from the reactivation of an endogenous virus acquired during primary varicella. Our understanding of the pathogenesis of VZV derives almost entirely from clinical studies. The virus is highly species-specific in vivo, though it has been adapted to grow in guinea-pig cells and isolates thus adapted will cause a varicella-like disease in guinea pigs (Pavan-Langston and Dunkel 1989). A closely related virus, simian varicella virus, has been isolated from Old World monkeys (Soike et al. 1984; Padovan and Cantrell 1986).

Clinical manifestations

VARICELLA

Chickenpox is one of the common childhood exanthems, affecting most children during their early school years. The incubation period is about 2 weeks, after which the characteristic rash appears, composed of macules which rapidly develop into fluid-filled vesicles. These vesicles crust within a few days and heal, usually without scarring, within a few weeks. The lesions appear in crops, so any one area will contain macules, vesicles, and crusted lesions (Figure 4). The rash tends to be centripetal and is variable in severity; a high density of vesicles is present on all parts of the skin and on mucosal membranes of the mouth and genitalia in some cases,

Figure 4 *Chickenpox macules and vesicles. (Photograph courtesy of Dr. Jane Sterling, Addenbrooke's Hospital, Cambridge, UK: Copyright Addenbrooke's NHS Trust.)*

while at the other extreme the number of vesicles may be very small or the infection inapparent. The accompanying fever is usually low grade, but reflects the severity of the rash and persists as long as new lesions continue to appear. The incidence of complications in normal children is low, the most common problem being secondary infection of lesions and consequent scarring, a problem that can usually be solved by antibiotic treatment. Central nervous system involvement is rare and may take the form of an invasive encephalitis or a transient ataxia, or may be a prelude to Reye's syndrome. Although primary varicella is usually a benign disease of the normal child, infection of malnourished children is much more severe (Salomon et al. 1966). The main groups in which complications occur are, however, the adult, the neonate, and the immunocompromised.

Infection of adults (Figure 5) is generally more severe than in children; the vesicles heal more slowly, secondary bacterial infection and scarring are more common, and the accompanying fever is higher and more prolonged. The most serious complication is varicella pneumonia which appears a few days after onset of the rash and usually resolves in the immunocompetent patient, though there may be residual nodular calcification. Pregnant women are thought to be particularly prone to varicella pneumonia, symptomatic pneumonia being reported in as many as 10 percent of primary varicella cases, with occasional mortality, though some studies report much lower frequencies of morbidity and mortality (Brunell 1992).

Primary varicella during pregnancy places the fetus at risk from two routes: infection in utero and neonatal infection. Infection in utero can result in congenital malformations which include limb hypoplasia, cicatrizing skin lesions, and sensory and motor deficiencies. These conditions are associated with maternal infection during the first 20 weeks of pregnancy. Primary varicella in the mother close to term is associated with severe disease in the neonate: symptoms appear in the infant about 7 days after birth and include pneumonia and visceral involvement, particularly of the liver. A full account of maternal, fetal and neonatal infections with VZV is given in Infections of the fetus and neonate, other than rubella.

Varicella is severe and often life-threatening in immunocompromised patients. Leukemic children form a major risk group but patients undergoing chemotherapy or steroid treatment or who have any acquired or inborn immune deficiency are at risk. The most common complications are varicella pneumonia, visceral involvement, and CNS infection, but also include hemorrhagic varicella ranging from bleeding into lesions to purpura fulminans (Feldman et al. 1975).

HERPES ZOSTER

Like HSV, VZV establishes a life-long latent infection of sensory dorsal root ganglia, but, because VZV is disseminated, latency is established in multiple ganglia. Reactivation and recurrences take the form of a unilateral rash, herpes zoster, limited to a single dermatome, the area of skin innervated by a single sensory ganglion. The rash appears most commonly in the thoracic dermatomes (Figure 6), reflecting the high density of vesicles in this area during primary varicella, but also occurs (in about 10 percent of cases) as cranial zoster, often with eye involvement and may, in rare instances, appear on almost any part of the skin or mucosa (Figure 7). In healthy people zoster is relatively uncommon; many people never experience it and few have more than one or two episodes, although it is common to suffer an episode in old age.

Figure 5 *Adult with chickenpox: slow healing and resolution of vesicles. (Photograph courtesy of Dr. Jane Sterling, Addenbrooke's Hospital, Cambridge, UK: Copyright Addenbrooke's NHS Trust.)*

Figure 6 *Thoracic zoster. (Photograph courtesy of Dr. Jane Sterling, Addenbrooke's Hospital, Cambridge, UK: Copyright Addenbrooke's NHS Trust.)*

Figure 7 *Mandibular zoster. (Photograph courtesy of Dr. Jane Sterling, Addenbrooke's Hospital, Cambridge, UK: Copyright Addenbrooke's NHS Trust.)*

The rash of herpes zoster is usually preceded by pain in the involved dermatome, which begins a few days before the rash appears and remains during its development. The rash is composed of varicella-like vesicles but often so densely packed as to appear as a continuous area of eruption (Figure 6). The rash develops over the course of about a week and usually crusts and heals in 2–3 weeks, but the course is variable and, particularly in the elderly, the symptoms are often more severe and prolonged. Unlike oral HSV recurrences, no stimuli have been identified that provoke zoster. The susceptibility of the elderly to zoster has been interpreted to reflect a waning immunity, a view consistent with the increased frequency of zoster in the immunocompromised patient, but it is probable that the immune system controls reactivated virus rather than prevents reactivation. The detection of VZV DNA fragments in circulating mononuclear cells of healthy patients (Gilden et al. 1983) implies that reactivation of latent virus may be much more common than clinical zoster. *Zoster sine herpete* is the term applied to a syndrome in which a typical zoster prodrome is unaccompanied by the subsequent development of a zoster rash (Easton 1970; Gilden et al. 1992a).

Complications associated with zoster are uncommon in children and young adults but occur frequently in the elderly and the immunocompromised. Post-herpetic neuralgia is the most common complication and is defined as pain lasting longer than 1 month after lesions heal. It is rare in patients under 40 years of age but occurs in 50 percent of patients over 60 and, although the pain usually resolves in a few months, it can last for a year or more and is a major cause of morbidity in the elderly. It is most frequently associated with zoster of the ophthalmic branch of the trigeminal (cranial zoster), and cases of sufficient severity and protraction may require surgical ablation of the ganglion. In some instances, anesthesia of the involved dermatome occurs and may result in residual palsy. The pathogenesis of post-herpetic neuralgia is unknown.

Other complications of zoster usually reflect underlying immunological defects. Zoster occurs with high frequency in immunocompromised patients and is unusually severe. Failure to control the recurrences is often illustrated by the spread of vesicles beyond the dermatome in which the initial rash occurs. The disease may become widely disseminated and correspond to severe varicella with involvement of the lungs or CNS. In these circumstances zoster may be fatal, most commonly as a result of pneumonia.

Epidemiology

VZV infects only humans. The reservoir is latent virus in sensory ganglia. Primary infection causes varicella, a common disease of childhood. Only a small minority of young adults remain uninfected but by late middle age infection and immunity are virtually universal in industrial societies. Infection is usually accompanied by the characteristic syndrome; asymptomatic infection does occur, but the proportion of subclinical infections is difficult to estimate and is based largely on patient recall. The disease occurs in epidemics which, in temperate zones, usually appear in late winter every 2 or 3 years in young schoolchildren. Airborne spread has been demonstrated (Gustafson et al. 1982) and the attack rate is high: more than 60 percent within households, and 20 percent in society as a whole (Yorke and London 1973), a rate lower than observed for measles. However, data collected before the advent of measles vaccination revealed that more young adults had been infected with VZV than with measles, a reflection of the fact that measles infection occurs almost exclusively during epidemics whereas varicella infection often occurs in unrecorded 'mini-epidemics' or as isolated cases resulting from contact with zoster. The risk of varicella infection through contact with zoster within a household has been estimated to be about threefold lower than through contact with varicella (Hope-Simpson 1965).

Zoster exhibits no seasonal incidence, and is not correlated with outbreaks of varicella. Despite reports of 'outbreaks' of zoster, all evidence favors the view that zoster is the reactivation of latent virus rather than a transmissible syndrome and reports of 'outbreaks' almost certainly refer to statistical anomalies. In immunocompetent people the incidence of zoster correlates only with age, the disease incidence being approximately proportional to the age of the study group.

Pathogenesis and pathology

Like other relatively benign diseases of humans, varicella pathogenesis cannot be studied in detail in the normal host. Our understanding of VZV pathogenesis derives, at least in part, from studies of severe or fatal disease and by analogy with other, better studied, exanthems. The route of entry is probably the upper respiratory tract or oropharynx where replication in epithelial cells occurs. During the incubation period of about 2 weeks, cell-associated virus can be detected in the bloodstream, primarily in mononuclear cells, and skin lesions are then initiated by infection of capillary endothelial cells. Whether the systemic amplification of virus is limited to circulating cells and capillary endothelium in the normal individual is unknown. In fatal varicella of the newborn or immunosuppressed, productive lesions can be found on all mucosal surfaces and in the parenchyma of almost every organ, and it is probable that limited replication at these sites contributes to the viremia in normal benign infection. The cutaneous vesicles of varicella are histologically similar to those of herpes simplex. Vesicles on mucosal surfaces rapidly develop into shallow ulcers. The vesicle contains large numbers of free virus particles, suggesting that replication in epithelial cells results in secretion of free virus, whereas in most cell types VZV is strongly cell associated. The production of free virus in the oral cavity and upper respiratory tract is the probable major source of transmission. The patient is infectious for a few days before lesions appear until a few days after new lesions cease to appear.

The virus establishes latent infection in sensory ganglia. VZV DNA can be detected in the thoracic ganglia of seropositive cadavers, and similar results have been obtained with simian varicella virus (Gilden et al. 1983; Mahalingham et al. 1991). By analogy with HSV, access to the ganglia is assumed to be from the skin via sensory nerve endings, but a hematogenous route cannot be excluded. VZV latency is poorly understood by comparison with HSV latency. The virus has not been reactivated from human ganglia by culture in vitro, the physical state of the viral genome has not been ascertained, and the pattern of transcription during latency, in contrast to HSV, appears to involve the immediate–early productive cycle genes (reviewed by Gilden et al. 1992b). Indeed, there is some disagreement as to the cell type in which latent infection is established, and true latency, as opposed to chronic infection, is not proven (Gilden et al. 1987; Croen et al. 1988). Recurrent infection with herpes zoster, is, by analogy with HSV, assumed to result from reactivation of latent virus in a sensory ganglion and seeding of infectious virions into epithelium via sensory nerve endings, a view entirely consistent with the appearance of zoster within a single dermatome. The details of these events are uncertain. As noted earlier (see section on Herpes zoster), clinical zoster is an infrequent event and most triggering conditions, such as cytotoxic drug treatment, lymphoma, or heavy metal poisoning, are immunosuppressive, suggesting a failure to control reactivated virus rather than stimulating reactivation. The frequency of reactivation rather than recurrence is unknown. The pain associated with zoster and the frequency and prolonged nature of postherpetic neuralgia distinguish VZV recurrences from those of HSV, and it is proposed that these features reflect more widespread growth and consequent inflammation in sensory ganglia during VZV recurrence. This plausible explanation implies a quantitative difference between VZV and HSV recurrence but fundamental differences in the nature of latency, reactivation, and recurrence of the two viruses cannot be excluded.

Immune response

Primary infection results in life-long immunity to exogenous reinfection. Humoral response, T-cell proliferative responses and cytotoxic T-cell responses are all detectable soon after symptoms appear, rise during convalescence, and remain detectable throughout life. Recurrence is accompanied by a pronounced anamnestic response, an observation in contrast to HSV recurrence. Antibodies to at least 35 different VZV antigens have been identified in convalescent sera, including neutralizing antibodies directed against viral glycoproteins, but, despite the use of passive immunoglobulin in therapy, there is little evidence that humoral responses are important in controlling infection. No correlation has been found between titer or spectrum of VZV-specific antibodies and severity of varicella or the recurrence or severity of zoster (Brunell et al. 1987). Agammaglobulinemic patients do not suffer from unusually severe varicella or from frequent or severe zoster. By contrast, many studies have demonstrated a correlation with deficiencies in cell-mediated immunity and the severity of varicella infection and the frequency and severity of zoster. T-cell proliferative responses, cytotoxic T-cell responses and interferon responses are all detected in human infection and in animal models (Arvin 1992; Rotbart et al. 1993). Responses to individual virus antigens, both structural and immediate early nonstructural proteins, have been found, but the importance of particular responses to individual antigens has yet to be determined. The decline in cell-mediated responses to VZV in the elderly is thought to be responsible for the increased frequency of zoster in this group (Miller 1980; Berger et al. 1981), and is consistent with the occurrence of zoster in immunocompromised patients. The maintenance of adequate levels of cell-mediated immunity to varicella in the population is therefore important but it is uncertain whether restimulation of the response occurs primarily through subclinical reactivation or

results from re-exposure to varicella. Evidence exists for both mechanisms (Meyers et al. 1980; Arvin et al. 1983), but their relative contribution is of importance in predicting the possible consequences of mass vaccination and the need to vaccinate seropositive people carrying wild VZV.

Diagnosis

Virus particles are present at high concentrations in vesicular fluid and can be cultured in a variety of human and primate cell lines. The cytopathic effect is focal and develops slowly with typical cell 'ballooning' and fusion. Immunofluorescence of infected cultures with specific antibody confirms the presence of VZV and allows more rapid diagnosis. The virus in culture is very strongly cell-associated and efficient storage of isolates requires freezing of viable infected cells. Electron microscopic examination of vesicular fluid reveals the presence of large numbers of herpesvirus particles, a method used historically to allow a rapid distinction between chickenpox and smallpox but which cannot distinguish VZV and HSV. Detection of VZV DNA by PCR is now widely used, particularly for diagnosis of severe disease where rapid differential diagnosis is required.

A wide range of serological tests for VZV-specific IgG have been described, including complement fixation, latex agglutination, radioimmunoassay, and ELISA. ELISAs for VZV-specific IgG and IgM are commercially available. A suspect rash can be confirmed as varicella by the presence of IgM or by a rising IgG titer. Zoster can be similarly confirmed because, in contrast to HSV, recurrence is usually associated with a significant rise in antibody titer. A skin test for cell-mediated immunity has been described (Kamiya et al. 1977; Takahashi et al. 1992) and is thought to provide a relevant measure of immunity to zoster.

Control

EPIDEMIOLOGICAL CONTROL

No attempt is made to limit the spread of varicella in the general population because, as a rule, acquisition of the virus early in life is preferable to infection as an adult. Within hospitals, however, VZV infection has serious consequences for high-risk groups such as premature babies, leukemic children and transplant recipients, and patient isolation may be necessary to protect these groups. Healthcare staff can be monitored for immunity to VZV, and seronegative staff who suspect they have contracted varicella or staff suffering a zoster recurrence should avoid contact with high-risk patients.

VACCINE

A live attenuated vaccine was developed in Japan by passage of the Oka strain in guinea pig cells and its efficacy was first reported by Takahashi et al. (1974). Since then the vaccine has been used extensively in normal and immunocompromised children and adults. The vaccine is licensed as a childhood vaccine in the USA and has recently been licensed in the UK for particular risk groups. Japanese and US experience with the vaccine is broadly similar (reviewed by Gershon et al. 1992 and Arvin 2001). A single vaccine dose gives >90 percent seroconversion and an 85 percent protection against subsequent varicella, which, when they occur, are mild. Both humoral and cell-mediated immunity are long-lasting but it is uncertain whether this is due to subclinical challenge with circulating virus. Vaccination of leukemic children provides similar levels of protection but vaccination of adults results in somewhat lower protection rates. There are few side effects of the vaccine in healthy children, some 5 percent suffering a mild rash occasionally accompanied by fever, but in leukemic children side effects are more noticeable, about half the vaccinees developing a rash, in some cases resembling typical varicella. Nevertheless, the vaccine-related reactions in these patients are benign compared with the risk of varicella infection. Vaccination of seropositive adults results in an enhancement of cell-mediated immunity and may therefore be of value in protecting the elderly against zoster (Berger et al. 1984; Takahashi et al. 1992).

It is not certain whether the vaccine establishes latent infection in all recipients. A few leukemic vaccinees have experienced zoster due to recurrence of the vaccine strain but these individuals suffered a varicella rash following vaccination, consistent with the expectation that latency and recurrence are related to virus load in the skin during primary infection. Because most healthy vaccinees have no rash, it is reasonable to suppose that they will not suffer recurrence, and to date this is the case. In view of its safety and efficacy, the Oka strain has been proposed for universal vaccination of children. It has been used successfully in combination with measles, mumps, and rubella vaccinations.

PASSIVE ANTIBODY

Passive immunization with specific zoster immunoglobulin (ZIG) (derived from donors with high anti-VZV titers) has been used to prevent or modify infection in high-risk patients who are thought to have come into contact with varicella or zoster (Gershon et al. 1974; Zaia et al. 1983; Berger et al. 1984; Takahashi et al. 1992). Examples include non-immune pregnant women, immunocompromised patients, premature babies in special care units, and neonates born to mothers with varicella at term.

CHEMOTHERAPY

VZV, like HSV and other alphaherpesviruses, specifies a TK and is sensitive to aciclovir. VZV is considerably less

sensitive than HSV, however, and much higher doses are required to achieve therapeutic benefit. Nevertheless, aciclovir is now widely used in the treatment of varicella and zoster (Whitley 1992, reviewed by Griffiths 1995). Treatment of uncomplicated varicella in children with oral aciclovir within 24 hours of the onset of the rash resulted in fewer skin lesions and reduced fever but the benefit is relatively small. Similar treatment of adults, who suffer more severe varicella, achieved essentially similar results but the benefit was more pronounced. Oral aciclovir is used both prophylactically and therapeutically in immunocompromised patients at risk from or suffering from varicella. Because of the relatively poor oral bioavailability of the drug, hospitalized patients with severe varicella are treated intravenously.

Oral aciclovir reduces pain and duration of the rash in zoster patients but has little or no effect on post-herpetic neuralgia. Two pro-drug derivatives of aciclovir, famciclovir, and valacilovir, with similar modes of action but different pharmacokinetic properties are now licensed for use against zoster. Their greater bioavailability offers advantages in some clinical settings (Carrington 1994; Murray 1995) (see also Antiviral chemotherapy).

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Betaherpesviruses: cytomegalovirus, human herpesviruses 6 and 7

WILLIAM BRITT

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Betaherpesviruses that infect humans include human cytomegalovirus (CMV), human herpesvirus 5, human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7). These viruses share many common biological characteristics, including restricted in vivo and in vitro cell tropism and an extended replicative cycle. Furthermore, productive virus replication, including the production of progeny virus is restricted to cells of host origin, suggesting that these viruses co-evolved with their animal hosts. This is perhaps best illustrated by cytomegaloviruses, a group of viruses with common replication strategies and conserved blocks of genes, but viruses from each mammalian species also encode additional viral genes unique to their species of origin. This observation suggests that these viruses have developed strategies and captured host genes that favor their replication and persistence in their specific host. Similarly, the in vitro and in vivo replication of betaherpesviruses is highly restricted to specific cell types and cells at particular stages of differentiation. They are also characteristically highly cell associated and replicate slowly as compared with alphaherpesviruses. The genome of betaherpesviruses range in size from the largest of all human herpesviruses, human CMV with a genome of nearly 260 kb pair to HHV-6 with a genome of 180 kb pairs. Of the well-studied betaherpesviruses, only human CMV and the closely related chimpanzee CMV contain repeated sequences at the termini of a unique long and short component of its genome, thereby permitting these components to be arranged in four isomeric forms, similar to the genomic organization of herpes simplex virus (Mocarski and Tan Courcelle 2001; Davison et al. 2003). The remaining members of this group of viruses have only a single genomic structure with repeats at the junction of the long and short regions of the genome. There are antigenic cross-reactivities between conserved structural and nonstructural proteins encoded by these viruses (Adler et al. 1993; Loh et al. 1994). Finally, infections caused by betaherpesviruses are usually subclinical and acquired at an early age in most populations. Life- or organ-threatening disease following infection is usually limited to immunocompromised hosts. More recently, chronic persistent infection with these viruses has been suggested to contribute to chronic inflammatory and proliferative diseases such as atherosclerosis and multiple sclerosis, and perhaps human cancers. The spectrum of disease associated with persistent infections caused by these viruses remains far from being fully defined.

CYTOMEGALOVIRUS

Infection with CMV is common in all human populations and infrequently associated with symptomatic illness in normal hosts. In contrast, it is a major cause of multi-organ disease in immunocompromised patients, the severity of disease being related to the degree of immunosuppression. Permissive infection in tissue culture is restricted to a very limited number of cell types, and then only in cells of human origin. CMV infections in susceptible hosts are widespread with involvement of all organ systems. Although most investigators believe that CMV can establish a persistent infection and early studies utilizing murine CMV

demonstrated a latent infection, only relatively recently have investigators demonstrated a true latent infection with human CMV (Jordan 1983; Soderberg-Naucler et al. 1997). The role of latent infection in the transmission of CMVs by blood or during allograft transplantation is well accepted; however, the importance of latent versus a persistent productive infection in the maintenance of the virus in the individual and a population remains unclear. Finally, it is of some interest that human CMV can be found in tissue from a variety of inflammatory and neoplastic diseases, raising the possibility that this virus may directly or indirectly contribute to chronic inflammatory disease and cancer.

Clinical manifestations

Although CMV rarely causes symptomatic infection in normal hosts, it has been estimated to cause between 20 and 50 percent of cases of heterophile-negative infectious mononucleosis (Klemola et al. 1970). A characteristic syndrome of fever, hepatitis, and atypical lymphocytosis following administration of blood products for cardiac surgery (postperfusion syndrome) has been attributed to CMV and represents virus transmission from blood products (Perillie and Glenn 1962; Reyman 1966). Other disease states including rheumatological diseases have been linked to CMV, although the biological and epidemiological relevance of these associations remains unclear. Recent studies have renewed interest in a possible role of CMV in the development of atherosclerotic coronary artery disease and animal models of disease have been developed which support a potentially important role of this virus in atherosclerotic heart disease (Adam et al. 1987; Petrie et al. 1988; Speir et al. 1994; Zhou et al. 1996; Zhu et al. 1999; Streblow et al. 2001; Libby 2002). Similarly, CMV gene products and nucleic acids have been detected in autopsy tissue from human brain and colon tumors and have raised the possibility that this large DNA virus could contribute to the pathogenesis of cancer (Cobbs et al. 2002; Harkins et al. 2002). Although these findings are provocative, it is unclear whether these findings represent an association between CMV and cancer or merely represent the detection of a CMV as a passenger in these tissues.

Three groups of patients are at risk for invasive acute CMV disease: (1) newborn infants infected in utero, (2) immunocompromised allograft recipients and (3) patients with AIDS. Infants infected in utero may be born with subclinical infections or may have a constellation of clinical abnormalities characteristic of what has been termed cytomegalic inclusion disease (CID) of the newborn (Jesionek and Kiolemenoglou 1904). Clinical findings include hepatosplenomegaly, jaundice, thrombocytopenia, purpura, microcephaly, chorioretinitis and, rarely, pneumonia in approximately 10 percent of congenitally infected infants (Table 1) (McCracken et al. 1969; Boppana et al. 1992). Mortality rates range from 11 to 20 percent in symptomatic infection, and up to 50 percent of long-term survivors of symptomatic infections will have deficits in perceptual and cognitive functions (McCracken et al. 1969; Alford 1984; Boppana et al. 1992; Boppana et al. 1999). A significant number of infants with asymptomatic infections are also at risk of developmental abnormalities, hearing loss being the most common (Yow et al. 1988; Fowler et al. 1992, 1997). Persistent infection with prolonged viral shedding is nearly universal in infants with either symptomatic or asymptomatic congenital infection (Stagno et al. 1975; Alford 1984).

CMV infections in the post-transplant period are a major cause of morbidity and mortality in allograft recipients. Infections can develop following reactivation of persistent or latent virus in the previously infected recipient, as the result of reinfection by virus present in the transplanted organ, or less frequently following transfusion of blood from a CMV-infected donor. The severity of infection depends on several characteristics but the two most consistently observed risk factors for severe infection are: (1) transplantation of an organ from a previously infected donor into a CMV non-immune host and (2) significant immunosuppression, including that caused by specific therapies directed at T-lymphocyte function (Singh et al. 1988; Weir et al. 1988; Fishman and Rubin 1998; Rubin 2002). Several studies using quantita-

Table 1 *Clinical manifestations of CMV infection in different patient populations*

	Clinical manifestations	
Population	**Acute infection**	**Long term**
Immunocompetent	Subclinical infection, mononucleosis syndrome	Prolonged virus shedding; inflammatory vascular disease
Immunocompromised:		
Fetuses	Hepatitis, encephalitis, retinitis, thrombocytopenia microcephaly	Hearing loss, cognitive dysfunction, developmental delay, prolonged virus shedding
Allograft recipients	Fever, hepatitis, pneumonitis, hematological abnormalities	Graft dysfunction/loss, excessive long-term mortality; transplant vasculopathy
AIDS	Retinitis, encephalitis, colitis, oesophagitis	Vision loss, wasting syndrome, decreased survival time, encephalopathy

tive measures of CMV DNA in blood have demonstrated that the viral burden of an individual patient, regardless of assignment of risk based on donor and recipient serological status, has significant predictive value of invasive CMV disease (Cope et al. 1997; Emery et al. 2000). More recent studies in hematopoietic transplant recipients have been consistent with earlier observations suggesting that the use of steroids in the post-transplant period were associated with an increased risk invasive CMV disease, presumably as a result of lymphotoxic activity of these reagents, including their activity against natural killer cells. CMV infection in allograft recipients can range from asymptomatic excretion to fulminant multi-organ disease. The most common clinical syndrome associated with CMV infection in the post-transplant period is fever, leucopenia, and mild to inapparent hepatic dysfunction (Table 1). Pneumonia attributed to CMV in the post-transplant population continues to carry a significant mortality rate. Infection of the central nervous system (CNS), including the retina, is rare in transplant patients. Long-term effects of chronic infection in allograft recipients, especially cardiac allograft recipients, include a characteristic vascuolopathy described as transplant vascular sclerosis or transplant vasculopathy (Grattan et al. 1989; Koskinen et al. 1999; Streblow et al. 2001). This disease has been faithfully recapitulated in rodent models and disease appears directly related to CMV infection and virus-gene expression in the endothelium of the allograft (Lemstrom et al. 1997; De La Melena et al. 2001; Streblow et al. 2003). The vascular disease in the allograft has been proposed to represent an accelerated variant of atherosclerotic coronary heart disease in the normal host and may offer clues to the mechanism of virus-induced vascular disease.

In certain HIV-infected populations, the incidence of CMV infection is extraordinarily high with rates exceeding 95 percent (Drew et al. 1981; Collier et al. 1987). Prior to the widespread introduction of effective antiretroviral therapy (ART) for the control of HIV replication, CMV was the most common cause of clinically important opportunistic infection in long-lived AIDS patients in the USA (Gallant et al. 1992; Pertel et al. 1992). Although numerous reports documented CMV infection in every organ system in this patient population, the gastrointestinal tract, retina and brain represent the more commonly described sites of CMV disease (Jacobson and Mills 1988; Francis et al. 1989; Schmidbauer et al. 1989; Vinters et al. 1989; Pertel et al. 1992). Colitis, persistent diarrhea with wasting, vision-threatening retinitis, and encephalopathy are common manifestations of CMV infection in patients with end-stage HIV infections (Table 1). With the advent of multidrug therapy for the control of HIV replication, the incidence of invasive CMV disease has fallen dramatically, such that new cases of retinitis in many study populations are very infrequent and develop only in patients who either present to medical care with invasive CMV disease or fail ART.

Epidemiology

The epidemiology of CMV infections can be most readily understood by examining the sources of virus, identifying populations at risk for exposure to infectious virus, and defining patients with deficits in immunity who are at risk of severe CMV infections. CMV can be cultured from most body fluids for extended periods following primary infection with significant amounts of infectious virus being found in urine, genital secretions (including semen), saliva, and breast milk. Virus is also readily transmitted by cellular elements in blood products, especially leucocytes, and by solid organs during transplantation (Prince et al. 1971; Ho et al. 1975; Gnann et al. 1988; Bowden 1991). Periodic reactivation or persistent low-level shedding from these sites is also a characteristic of CMV-infected hosts. In fact, it could be argued that, for the human host, persistent infection in the form of continuous low level replication is likely to be more frequent than periodic reactivation from latency. Sources of infection in normal hosts include exposure to virus-infected body fluids, such as occurs in crowded living conditions in developing countries and in child-care centers in developed societies. In both situations there is frequent infection of children and of susceptible child carers (Murph et al. 1986; Pass et al. 1987; Adler 1989; Murph et al. 1998). An additional source of virus exposure in infancy is breast feeding with transmission occurring in 30–70 percent of infants breast fed by mothers with serological evidence of previous infection (Dworsky et al. 1983). In adolescents and adults, CMV infection is acquired commonly through sexual exposure (Chandler et al. 1985; Handsfield et al. 1985; Chandler et al. 1987; Collier et al. 1987; Fowler and Pass 1991). Overall, it has been estimated that the rate of infection after childhood is ca. 1 percent per year (Stagno et al. 1984). Reinfections with genetically different strains of virus has been well documented in normal hosts, including children in day care and women attending sexually transmitted disease clinics (Chandler et al. 1985; Handsfield et al. 1985; Murph et al. 1986; Collier et al. 1989; Bale et al. 1996; Boppana et al. 2001). Finally, it is also likely that normal individuals can be infected with several genetically unique strains of CMV, raising questions about the definition of protective immune reactivity in patients following an acute infection.

Clinically significant infections develop in patients with deficits in immunity resulting from pharmacological immunosuppression, acquired deficits in immunity such as HIV infection or developmental immaturity of the immune system. The natural history of CMV infection in transplant recipients suggests that infection is nearly universal in those exposed, but that clinical disease is dependent on specific risk factors. In all but bone marrow

allograft recipients, a major and perhaps the most important risk factor, is the transplantation of an organ from a donor with previous infection into a CMV non-immune recipient (Ho 1991; Rubin 2002). Disease rates as high as 70 percent in renal allograft recipients following CMV mismatched donor–recipient transplantation have been reported (Rubin 2002). In the past, mortality rates in excess of 50 percent in cardiac/lung transplants limited CMV mismatched donor–recipient transplantation in some centers (Smyth et al. 1991). As the intensity of immunosuppression increases, so does the severity of CMV infections, as illustrated by the often fatal CMV infections that occur in bone marrow allograft recipients (Meyers et al. 1982; Winston et al. 1990; Schmidt et al. 1991; Zaia 2002; Boeckh et al. 2003). More recent findings have suggested that mismatched donor: recipients loci encoding natural killer (NK) cell recognition: activation antigens may also lead to significant CMV infections in the post-transplant period. If confirmed, this finding would argue for a critical role of innate immunity in resistance to CMV disease in these patients.

The natural history of CMV infections in AIDS patients has been most closely related to the depletion of $CD4^+$ lymphocytes. Several prospective studies documented high rates of CMV shedding in almost all AIDS patients, but invasive disease was more commonly observed in patients with <50 $CD4^+$ lymphocytes/mm^3 (Gallant et al. 1992). Reinfection is common and isolation of multiple strains of virus from individual patients has been reported (Drew et al. 1984; Spector et al. 1984; Chern et al. 1998). Characteristics of CMV infection that lead to severe disease and specific organ involvement are not understood, but evidence has been presented that residual virus-specific immunity may modulate disease progression (Boppana et al. 1995; Autran et al. 1997; Komanduri et al. 1998; Alberola et al. 2000; Alberola et al. 2001).

Congenital CMV infection is the most common viral infection of the human fetus (Alford 1984). In the USA the incidence of congenital CMV infection is ca. 1 percent in live births (Fowler et al. 1993; Britt and Alford 1996). In the developing world, rates of congenital infections as high as 2–3 percent have been described, suggesting that immunity does not prevent intrauterine infection (Yamamoto et al. 2001). An estimated 10 percent of infected infants will have significant infections that result in long lasting CNS sequelae (Alford; Fowler et al. 1993; Britt and Alford 1996; Boppana et al. 1999). The characteristics of the maternal infection have been claimed to be of paramount importance to the outcome of fetal infection; pre-existing maternal immunity can limit damage to the fetus, a situation analogous to that in allograft patients (Fowler et al. 1993). Primary gestational infection with CMV results in a 35–50 percent fetal transmission rate, whereas gestational reactivation or reinfection in women with preconceptional immunity is associated with a fetal infection rate of between 1–3 percent (Alford 1984; Britt and Alford 1996). Furthermore, it is estimated that fetal infection following primary maternal infection during pregnancy results in damage three times more often than infection that follows reactivation or reinfection in women with preconceptional immunity (Fowler et al. 1993). More recent findings have challenged these claims. These data include the finding that maternal reinfections with a genetically different strain of CMV do occur at a significant rate (10–12 percent) and that maternal reinfections can lead to damaging congenital infection (Ahlfors et al. 1999; Boppana et al. 2001). Furthermore, at least two different groups of investigators have reported that congenital infections following non-primary maternal infections can result in a similar incidence of long term sequelae as observed in infants infected as a result of primary maternal infections during pregnancy (Ahlfors et al. 1999, 2001; Boppana et al. 1999). Naturally acquired intrapartum or postnatal CMV infection in normal newborn infants has not been associated with severe infections and the permanent sequelae characteristic of infants infected in utero (see Table 2) (Alford 1984; Britt and Alford 1996).

Pathogenesis

In the context of currently available information, the pathogenesis of CMV infection must be viewed as a lytic viral infection resulting from a failure of host immunity to control effectively viral replication and spread. This model of CMV pathogenesis is accurately reflected by the virus burden as measured by polymerase chain reaction (PCR) or virus isolation during acute infection (Lockridge et al. 1999; Emery et al. 2000; Humar et al. 2002). However, it is almost a certainty that CMV exerts its pathogenetic potential through nonlytic mechanisms as well. These may include modulation of normal host cell functions by limited expression of its genome, and perhaps by affecting neighboring cellular functions through the induction of chemokine and cytokine production. Other pathways of host-cell damage may include the generation of host-derived immunopathological responses that result in significant organ damage, a mechanism that would explain the association of CMV with diseases such as atherosclerosis (Grattan et al. 1989; Epstein et al. 1999; Streblow et al. 2001; Libby 2002; Streblow et al. 2003). Such a mechanism has also been offered as an explanation of the severe pneumonia associated with CMV infection in bone allograft recipients and would be consistent with very early descriptions of this disease that indicated that only a few areas of the lung were infected with CMV (Myerson et al. 1984; Grundy et al. 1987). Perhaps the most convincing evidence of the importance of nonlytic mechanisms leading to disease have been provided by animal models of CMV infection leading to allograft disease, specifically transplant vascular sclerosis (Lemstrom et al. 1997;

Table 2 *Acquisitions of CMV infection in different populations*

Community acquired		Hospital acquired	
Age	**Mode of acquisition**	**Sources of CMV infections**	**Risks associated with virus transmission**
Perinatal	Intrauterine fetal infection (congenital)	Blood products	Blood products from seropositive donors
	Intrapartum		Multiple transfusions
	Breast milk acquired		White blood cell containing blood products
Infancy and childhood	Exposure to saliva and other body fluids	Allografts	Allograft from seropositive donors
			Graft rejection
Adolescence and adulthood	Sexually transmitted		
	Exposure to young children		

Koskinen et al. 1999; Streblow et al. 2003). Experimental systems including animal models have illustrated the role for virus-induced amplification of host inflammatory functions such as expression of adhesions molecules, induction of chemokine expression, and activation of transcriptional factors such as NF-κB that are central to the host inflammatory responses to virus infected cells. Such mechanisms could explain the association between CMV infection, virus gene expression in inflammatory diseases as exemplified by allograft dysfunction, and graft loss.

Pathological findings in CMV infection include evidence of virus infection in almost all organ systems (Becroft 1981; Britt and Alford 1996). Histological findings include large refractile cells (cytomegalic) with so-called owl's eye intranuclear inclusions. In some instances, organ dysfunction exceeds what would be expected from the number of infected cells detected by routine histopathological techniques. This discrepancy was initially confirmed using more sensitive techniques for demonstration of CMV (Myerson et al. 1984). Thus, the pathogenic mechanisms which account for end organ disease associated with CMV replication are unclear, as has been stated above and will likely not be explained by qualitative measures of virus within involved tissue.

Immune responses

Protective immune responses against CMV have not been definitely identified, and may not be the same in different populations. Both cellular and antibody responses to CMV participate in the immune responses that are thought to resolve infection in normal hosts (Rasmussen 1990; Walter et al. 1995; Britt and Alford 1996; Alberola et al. 2000, 2001). Most investigators who have suggested a dominant role for one arm of the immune system in protective immune responses against CMV have based these claims on findings from transplant recipients and patients with AIDS (Reusser et al. 1991; Walter et al. 1995; Autran et al. 1997; Komanduri et al. 1998; Aubert et al. 2001; Boeckh et al. 2003). In these patients, disease was associated with a decrease in either antibody or cellular responses to CMV as a result of immunosuppressive drug therapy or HIV infection. However, it must be noted that components of protective immunity in normal individuals have yet to be fully defined and that the clinical outcome in the post-transplant period that was previously attributed cellular immune effecter functions can also be related to the presence of virus neutralizing antibodies (Snydman 1993; Schoppel et al. 1997; Alberola et al. 2000). In fact, recent studies in a simian model of CMV infection in a rhesus macaques infected with a simian immunodeficiency virus have argued for a role of virus neutralizing antibodies and disease control (Kaur et al. 2003). Lastly, the importance of NK cells responses in control of murine CMV is well documented and further demonstrated by the presence of viral genes that specifically blunt the NK response (Krmpotic et al. 2002). Very recent findings would argue for a similar role of NK cells in the control of CMV infections in transplant recipients. Thus, a precise role for specific effector functions of the immune system in control of virus replication in infected host remains undefined.

In both solid organ and bone marrow transplant recipients, decreased cellular responses, especially CMV-specific $CD8^+$, MHC-restricted T lymphocyte responses, have been correlated with more severe CMV infections in the post-transplant period (Reusser et al. 1991). Reconstitution of these responses with in vitro expanded CMV-specific cytotoxic T lymphocytes has been shown to reduce post-transplant mortality secondary to CMV (Gavin et al. 1993; Walter et al. 1995). However, several studies from this same institution have shown a similar correlation of survival with the presence of virus-specific $CD4^+$-specific lymphocyte responses, especially in the late (>100 days) post-transplant period (Li et al. 1994; Walter et al. 1995; Boeckh et al. 2003). The advent of effective antiviral therapy has altered the natural history

of CMV in the transplant population such that CMV disease more commonly occurs late (180 days) in the post-transplant period (Boeckh et al. 2003). Thus, the value of a transient $CD8^+$ antiviral response provided by transferred antiviral T cells could require reassessment, particularly in light of recent experimental findings that have demonstrated the critical importance of antigen-specific $CD4^+$ responses in the maintenance of $CD8^+$ responses to infectious agents (Sun and Bevan 2003). As noted above, studies in patients with AIDS have clearly demonstrated that protective immune effector functions correlate with the restoration of CMV-specific $CD4^+$ responses (Autran et al. 1997; Komanduri et al. 1998). Findings from pregnant women have also suggested a correlation of cellular responses to CMV and intrauterine transmission, but these studies remain controversial (Gehrz et al. 1981). In AIDS patients with invasive CMV disease, cellular responses are absent because of lymphocyte depletion, yet recent improvements in the analysis of these responses have permitted the detection of CMV-specific responses in AIDS patients with severe depletion of peripheral $CD4^+$ lymphocyte numbers, indicating that only limited residual cellular immunity is required for control of CMV replication (Autran et al. 1997; Waldrop et al. 1997; Komanduri et al. 1998; O'Sullivan et al. 1999). Cellular immune responses appear to be directed primarily at the protein components of virion tegument, the products of the UL83 open reading frame (ORF), phosphoprotein 65 (pp65), and the UL32 ORF, phosphoprotein 150 (pp150). Although other CMV-encoded proteins such as the products of the *IE-1* gene elicit cellular responses, responder cell frequency analysis has indicated that these two proteins represent dominant targets of cellular immunity (Kern et al. 1999; Kern et al. 2002; Gallez-Hawkins et al. 2003). The role of cytokines in cellular responses against CMV is unknown; however, in animal models of human CMV disease, interferon (IFN)-γ has been shown to have significant antiviral activity. Finally, it has been reported that CMV infections are associated both with decreased cellular immune responses in normal individuals and with decreased CMV-specific responses in congenitally infected children (Gehrz et al. 1977; Pass et al. 1983). More recent studies using sensitive techniques such as intracellular cytokine staining following antigen exposure have demonstrated that congenitally infected infants do indeed have cellular immune responses to CMV. Depression in cellular immune responses may also contribute to superinfection with fungal pathogens in transplant recipients infected with CMV. This clinical association remains unproven but is widely held by transplant physicians.

ANTIBODY RESPONSES

Serological responses to CMV are complex because of the large number of virus proteins. Several studies have documented responses to a number of the more immunogenic proteins of CMV in both normal and immunocompromised hosts (Zaia et al. 1986; Hayes et al. 1987; Landini et al. 1988, 1993; Britt et al. 1995; Schoppel et al. 1997). These studies have provided important clues for identification of viral-encoded proteins that induce protective antibody responses as well as antibody responses which could be used in the diagnosis of acute and past CMV infections. The only well-characterized in vitro assay of antibody activity that correlates with patient outcome is virus neutralizing antibody (Adler et al. 1995; Boppana and Britt 1995; Schoppel et al. 1997). Virus-neutralizing serological responses are limited to antibodies against virus-encoded glycoproteins, as these represent the only class of viral proteins that are surface exposed in a native form. The majority of virus-neutralizing antibodies are directed against the products of the UL55 (gB), UL75 (gH), and UL73/UL100 (gM/gN) ORFs (Britt et al. 1990; Rasmussen et al. 1991). Studies have suggested that immunization with these proteins can induce neutralizing antibodies as well as antibodies reactive with the surface of virus-infected cells (Rasmussen 1990; Britt et al. 1995; Pass et al. 1999). The identification of intense and durable antibody responses to components of the viral tegument, both pp65 and pp150, has provided a rationale for including these proteins in a recombinant protein based diagnostic assay for serological reactivity to CMV (Landini 1993). Conversely, the presence of significant IgM responses to a nonstructural protein, pp50 (UL44), has led to its use as a serological target for detection of IgM and, therefore, recent infection (Landini 1993; Lazzarotto et al. 1998).

Diagnosis

The diagnosis of CMV relies either on demonstration of the agent (virus, viral proteins, nucleic acids) in body fluids, and/or tissue or on serological responses in a patient with clinical findings consistent with CMV infection. Because of the ubiquitous nature of CMV, it is important that a distinction be made between the detection of CMV and demonstration of CMV in the context of an infectious syndrome compatible with CMV. Multiple methods of recovering virus have been employed, including culture on permissive cells such as primary human fibroblasts and centrifugation enhanced culture followed by early antigen detection with monoclonal antibodies. This latter technique is widely used and can provide positive results within 24 h. In contrast, conventional tube culture may require up to 4 weeks to reveal cytopathology (Pass et al. 1995; Revello and Gerna 2002).

Techniques of viral antigen detection have been relatively limited with the exception of the antigenemia assay (van der Bij et al. 1988; Gerna et al. 1991; Miller et al. 1991; Boeckh and Bowden 1995), which uses the

immunological detection of the virus structural protein pp65 within polymorphonuclear leucocytes. Because this assay can be quantified relatively easily, it has been used to predict the likelihood of invasive disease in transplant patients and patients with AIDS (van der Bij et al. 1988; van den Berg et al. 1989). It is a widely used assay in many transplant centers and seems to be of practical value for the management of transplant patients. It is less sensitive than PCR but this may offer some advantage, including a greater positive predictive value for presence of disease because the increased sensitivity of PCR has limited its predictive value in many circumstances.

Detection of viral nucleic acids has been used extensively to document CMV infections. Dot-blot hybridization, in situ hybridization, and PCR have all been applied to clinical specimens. PCR is the most widely used assay and in certain clinical situations, such as screening newborn urine for CMV, is a highly sensitive method of diagnosis. PCR of plasma and peripheral blood buffy coat cells has been used to detect significant infection in transplant recipients and AIDS patients (Spector et al. 1992; Boeckh et al. 1997; Cope et al. 1997; Razonable et al. 2002). More recent modifications of the PCR, including real-time PCR, that permit quantitation of viral burden has provided significant new information relevant to our understanding of CMV infections in the immunocompromised patient. Spector and colleagues were one of the first groups to demonstrate that the viral burden was predictive of the likelihood of an individual patient developing invasive disease (Spector et al. 1996). Several additional reports have also demonstrated the value of this approach (Cope et al. 1997; Emery et al. 2000; Zaia 2002). Real-time PCR with extended linear ranges of nucleic acid detection have permitted rapid and accurate measurement of virus burden in a variety of patient specimens.

Serological assays of antibody reactivity to CMV include nearly every imaginable technology. Detection of IgG is straightforward and seropositivity is evidence of infection only, and relative titers are usually of little clinical value. Seropositivity does not indicate active virus shedding, but should be taken as evidence of the potential infectivity of blood or organs from the individual. The measurement of CMV-specific IgM responses offers the possibility of distinguishing between acute infection and past infection (Stagno et al. 1985; Kropff et al. 1993). Unfortunately, this assay is often associated with low sensitivity and, occasionally, low specificity (Stagno et al. 1985; Landini 1993; Revello and Gerna 2002). Furthermore, the presence of rheumatoid factors may lead to false positive IgM results (Griffiths et al. 1982). Lastly, IgM antibodies are not present in the absence of IgG reactivity, so competition for antigenic sites between IgG and IgM antibodies is possible. IgM antibodies can persist for significant periods of time post acute infection and in some patients can persist for extended periods of time. The use of IgM reactivity to establish the chronicity of an infection is controversial and up to 40 percent of patients with past infection will have IgM anti-CMV antibodies (Revello and Gerna 2002) More recently, several laboratories have demonstrated that the measurement of anti-CMV IgG avidity can help distinguish between recent and past infection with CMV (Lazzarotto et al. 1998, 1999; Revello and Gerna 2002) This approach has been incorporated into an algorithm for the diagnosis and management of CMV infections during pregnancy (Bodeus et al. 2002; Revello and Gerna 2002).

Control

ANTIVIRAL THERAPY

Two agents with activity against CMV, ganciclovir and foscarnet are in current clinical use (Coen 1992). Both have significant toxicity, and resistant isolates have developed during treatment. Their use in AIDS patients is problematic since neither is virucidal and, when treatment is interrupted, virus replication resumes. Two additional compounds have recently been licensed for treatment of CMV infections in AIDS patients, specifically retinitis – Cidofovir and Fomivirsen.

Ganciclovir is a nucleoside analogue that inhibits chain elongation both by its activity as a chain terminator and through direct inhibition of the viral DNA polymerase (Sullivan et al. 1992, 1993). It is inactive until phosphorylated by the phosphotransferase activity of the UL97 ORF (Sullivan et al. 1992). It has been used to treat invasive infections in transplant recipients, retinitis and colitis in AIDS patients, and as part of a protocol to examine its efficacy in limiting sequelae in infants with congenital CMV infections (Meyers 1991; Spector et al. 1993) (Kimberlin et al. 2003). It also has some value as an antiviral prophylaxis in bone marrow transplant recipients (Goodrich et al. 1993; Seu et al. 1997; Ljungman 2001). Ganciclovir treatment causes primarily bone marrow toxicity with neutropenia as a major adverse effect. Ganciclovir is very poorly absorbed when given orally but dosing with sufficient drug can achieve similar serum levels as an intravenously given drug and similar therapeutic efficacy (Paya et al. 2002). However, its valine ester, valganciclovir, is readily absorbed and equivalent serum levels can be achieved with standard oral dosing regimens (Gane et al. 1997; Miller et al. 1997; Pescovitz et al. 2000; Curran and Noble 2001). This formulation of ganciclovir has been licensed for treatment of CMV infections in patients with AIDS following the demonstration of similar efficacy and safety profile of the oral valganciclovir when compared to intravenous ganciclovir for the treatment of CMV retinitis (Lalezari et al. 2002; Martin et al. 2002). Debate continues over the use of ganciclovir as

prophylaxis or as treatment when virus replication is detected (preemptive therapy). Both approaches have been shown to modify CMV disease in the post-transplant period (Emery 2001; Ljungman 2001; Singh 2001).

Foscarnet is an inhibitor of CMV DNA polymerases (Snoeck et al. 1993). It is also active against a wide variety of viral polymerases. This agent has been used extensively in AIDS patients and has demonstrated efficacy in CMV retinitis (Palestine et al. 1991). It causes significant renal toxicity, thus limiting its use under certain situations arising transplant recipients. This agent has been used extensively in patients who fail ganciclovir therapy.

Cidofovir and Fomivirsen are two compounds that were licensed for the treatment of CMV retinitis in AIDS patients. Cidofovir is a nucleoside analogue and inhibits CMV DNA replication in much the same manner as ganciclovir, with the exception that it does not require phosphorylation (De Clercq 1998). This latter property makes it useful for the treatment of patients who fail ganciclovir therapy secondary to mutations in the UL97 ORF (De Clercq 1998). Unfortunately, this agent has significant nephrotoxicity that has limited its use in almost all patient populations. Fomivirsen (ISIS 2922) is the first antisense RNA compound licensed for treatment of a human disease and is active against CMV in vivo and in vitro. The clinical experience with this compound is limited because of the changing natural history of opportunistic infections in AIDS patients which has followed HAART, but injection of this compound into the eye appears to be effective therapy for HCMV retinitis.

VACCINES

Past and recent epidemiological studies have argued that CMV is a vaccine-modifiable disease (Fowler et al. 1993; Fowler et al. 2003). Previous and current approaches for vaccine development have focused on replicating live viral vaccines. These have been referred to as attenuated viruses; however, as CMV does not exhibit identifiable virulence markers, this claim remains contentious. Vaccination of renal allograft recipients with one of these candidate viruses, the Towne strain of CMV, provided some evidence that it could induce protective responses (Plotkin et al. 1991). In a recent study, this vaccine failed to protect normal women from reinfection following exposure to children in a group care setting (Adler et al. 1995). Thus, it is unclear whether this vaccine in its current formulation is sufficiently immunogenic to induce protective immunity. Clinical trials are currently underway using a recombinant glycoprotein B combined with an adjuvant as an alternative approach. Although this vaccine appears to induce antibodies, including virus neutralizing antibodies, the durability of this response appears to be limited. It should be noted that recent studies have indicated that reinfection of previously immune women with genetically different strains of virus are relatively common in some populations, raising questions about the nature of protective immunity and therefore current vaccine strategies that attempt to recapitulate natural immunity (Boppana et al. 2001).

HUMAN HERPESVIRUSES 6 AND 7

HHV-6 and HHV-7 are two relatively recently described human viruses whose clinical importance remains to be fully defined. Isolation of HHV-6 from AIDS patients initially prompted speculation that it was an important cofactor in this disease (Salahuddin et al. 1986). This initial excitement was subsequently tempered by findings which demonstrated that infection with HHV-6 was common in all populations and that persistent shedding was characteristic of the biology of this virus. A second lymphotrophic betaherpesvirus, HHV-7, was isolated from $CD4^+$ T lymphocytes of normal individuals (Frenkel et al. 1990). Both viruses are acquired at an early age and seroprevalence is in excess of 60–80 percent by adulthood in most populations. Genetically, these viruses are closely related to CMV and exhibit genetic homology with CMV over an estimated two-thirds of their genomes (Lawrence et al. 1990; Inoue et al. 1993; Gompels et al. 1995; Megaw et al. 1998). A single serotype of HHV-7 has been described, whereas HHV-6 can be divided into two groups, A and B, based on biology, serological reactivity, and genetic composition (Ablashi et al. 1991; Schirmer et al. 1991). HHV-7 utilizes $CD4^+$ as its receptor whereas the receptor for HHV-6 is CD46, the same receptor utilized by measles virus (Lusso et al. 1994; Santoro et al. 1999). Subsequent studies have indicated that the CXC chemokine receptor 4 is not a coreceptor for HHV-7 as was initially proposed (Zhang et al. 2000).

Clinical manifestations

Recognizable clinical syndromes associated with HHV-6 and HHV-7 infection and confirmed by virological studies have been limited to primary infections in children. HHV-6 is the etiological agent of exanthem subitum (roseola), a common febrile illness of early childhood (Yamanishi et al. 1988). The clinical findings of this illness include high fevers (>39°C) of 3–5 days duration, nonspecific findings such as pharyngeal injection and diarrhea, and, perhaps most characteristically, rapid defervescence followed nearly coincidentally by the appearance of a generalized maculopapular rash (Okada et al. 1993; Asano et al. 1994; Hall et al. 1994; Suga et al. 1997; Caserta et al. 1998, 2001; Clark 2000). In contrast to this classical presentation, other studies have suggested that only about 10 percent of infants with primary HHV-6 infection exhibit the full spectrum

of exanthem subitum (Pruksananonda et al. 1992; Caserta et al. 2001). Interestingly, only the HHV-6B variant has been associated with exanthema subitum (Dewhurst et al. 1993). Symptoms including irritability, bulging anterior fontanelle, and seizures in a significant percentage of infants with roseola have been suggested as evidence of CNS infection (Kondo et al. 1993; Asano et al. 1994; Hall et al. 1994; Yoshikawa and Asano 2000). It has been suggested that primary infection with HHV-6 (strain B) and invasion of the CNS may predispose to the development and recurrence of febrile seizures (Kondo et al. 1993; Caserta et al. 1994; Yoshikawa and Asano 2000). Consistent with a direct role of HHV-6 in the development of seizures, other studies have documented the presence of HHV-6 nucleic acids in the cerebrospinal fluid (CSF) from patients with acute HHV-6 infection and also in the brain tissue from both immunocompetent and immunocompromised patients (Yoshikawa et al. 1992; Suga et al. 1993; Luppi et al. 1994; Ohashi et al. 2002). From these and other studies it appears that HHV-6 is neurotropic. HHV-6, and possibly HHV-7, are important causes of acute febrile illnesses in childhood, accounting for nearly 20 percent of hospital emergency room visits in the USA (Hall et al. 1994). Primary infection with HHV-7 is less well described but appears to cause a clinical syndrome very similar to that reported for HHV-6 with a possible difference being that primary HHV-7 infection occurs at an older age (Tanaka et al. 1994; Asano et al. 1995; Torigoe et al. 1995; Caserta et al. 1998). At least one report suggested that HHV-7 may be associated with the common childhood exanthem, pityriasis rosea, while other reports have failed to confirm this association (Drago et al. 1997; Kosuge et al. 2000). Clinically apparent infections in normal adults with either virus have been infrequently reported but, when diagnosed, resemble infectious mononucleosis (Steeper et al. 1990; Akashi et al. 1993). Reactivation of HHV-6, and possibly of HHV-7, often follows febrile illnesses, and reactivation of HHV-6 follows HHV-7 infection in vitro and in vivo (Kusuhara et al. 1991; Frenkel and Wyatt 1992; Asano et al. 1995). HHV-6 has also been associated with several rare disorders, including sinus histocytosis syndrome or Rosai–Dorfman disease (Foucar et al. 1990; Levine et al. 1992). It has been argued that HHV-6, and possibly HHV-6 infection, are associated with chronic fatigue syndrome; however, several studies have failed to demonstrate an increased incidence of infection with either of these viruses and this syndrome (Braun et al. 1995; Reeves et al. 2000). Clinical reports have linked HHV-6 with multiple sclerosis (MS) based on increased immunological responses to HHV-6, including HHV-6 specific IgG and IgM antibodies in patients with MS (Soldan et al. 2000). The experimental findings linking HHV-6 with the chronic demyelinating disease include: (1) detection of HHV-6 DNA by PCR in CSF from patients with MS at a higher frequency than controls; (2) detection of HHV-6 sequences in oligodendrocytes adjacent to plaques in the brain tissue from patients with MS but not control patients; (3) HHV-6-specific immunohistochemical staining of oligodendrocytes from plaques obtained from patients with MS but not control patients (Wilborn et al. 1994b; Challoner et al. 1995; Friedman et al. 1999; Akhyani et al. 2000; Knox et al. 2000; Tejada-Simon et al. 2002). In contrast, other studies have not reproduced these early findings (Hebart et al. 1997; Coates and Bell 1998; Mirandola et al. 1999). To date, definitive evidence of causality has not been presented but the association between HHV-6 and MS is intriguing and mandate additional studies to define this relationship.

Infection with HHV-6 can result in severe organ involvement, especially in immunocompromised hosts. It has been associated with a variety of specific clinical entities, including encephalitis, pneumonitis, hepatitis, and bone marrow dysfunction (Asano et al. 1990; Tajiri et al. 1990; Carrigan et al. 1991; Yoshikawa et al. 1992; Cone et al. 1993; Knox and Carrigan 1994; Singh 2000). This latter disorder may result from HHV-6 infection of hematopoietic progenitor cells, contributing to graft dysfunction in bone marrow transplant recipients (Knox and Carrigan 1992; Carrigan and Knox 1994). The proposed mechanisms responsible for bone marrow suppression in the bone marrow recipient have included both HHV-6 mediated cytolysis of progenitor cells and/or suppression of colony forming units through stimulation of cytokine production (Knox and Carrigan 1992; Drobyski et al. 1993). However, it should also be noted that 30–45 percent of hematopoietic cell allograft recipients develop HHV-6 viremia or HHV-6 DNAemia (Imbert-Marcille et al. 2000; Caserta et al. 2001; Yoshikawa et al. 2002; Boutolleau et al. 2003). Thus, it has been difficult to ascribe specific disease syndromes to HHV-6 infection/reactivation in these patients because the majority of allograft recipients with HHV-6 do not have symptoms attributable to this virus. The importance of HHV-6 in the solid organ allograft recipients is also unclear even though up to 50–60 percent of transplant recipients will reactivate the virus in the post-transplant period (Schmidt et al. 1996; Griffiths et al. 1999; Griffiths et al. 2000). Perhaps more interesting is the association between the reactivation of HHV-6 and CMV in the post-transplant period and the association between severe CMV disease and concomitant HHV-6 infection (Dockrell et al. 1997; Lowance et al. 1999; Humar et al. 2000; Kidd et al. 2000; DesJardin et al. 2001). As noted above, several studies have suggested that HHV-6 (or HHV-7) may play a role in graft dysfunction in the post-transplant period, including the graft rejection; however, these findings must be interpreted in the context of the use of potent immunosuppressive agents to maintain graft function and the reactivation of a variety of herpesviruses including HCMV (Okuno et al. 1990; Griffiths et al. 1999).

Although numerous case reports have linked HHV-6 infection to specific organ damage in the post-transplant period, it remains to be determined whether HHV-6 is a major cause of morbidity in this patient population because many of the patients in these studies were also infected with other agents such as HCMV. Alternatively, it has been argued that the success of antivirals in limiting post-transplant complications associated with CMV replication may also be explained by the activity of these compounds against HHV-6. Similarly, in patients with AIDS, HHV-6 and HHV-7 have been associated with a variety of clinical findings but their role, if any, in AIDS pathogenesis is unclear. In vitro studies have shown that HHV-6 can activate $CD4^+$ expression in $CD4^-$ cells raising the possibility that HHV-6 could increase the number of target cells for HIV infection in the HIV infected host (Lusso et al. 1991; Flamand et al. 1998). In contrast, other studies have suggested that HHV-6 infection can suppress HIV replication in dendritic cells and that HHV-6 can down-regulate the HIV coreceptor, CXC chemokine receptor 4 (CXCR4), in $CD4^+$ lymphocytes (Asada et al. 1999; Hasegawa et al. 2001). In clinical studies, there appears to be no quantitative relationship between the level of HHV-6 in peripheral blood mononuclear cell (PBMC) and $CD4^+$ lymphocyte counts in patients with AIDS, although HHV-6 dissemination appears to be more widespread in AIDS patients than controls (Fairfax et al. 1994; Clark et al. 1996; Fabio et al. 1997). In contrast, primary HHV-6 infection has been associated with more rapid progression of HIV disease in children (Kositanont et al. 1999). Whilst some investigators have suggested that HHV-6 and HHV-7 may function as cofactors in the development of the immune deficiency associated with HIV infection, there is currently little definitive information to support this hypothesis.

Epidemiology

The seroprevalence of HHV-6 increases rapidly through early childhood such that over 90 percent of infants in developed countries will be seropositive by the age of 2 years (Saxinger et al. 1988; Balachandra et al., 1989; Okuno et al. 1989). Serological reactivity in adults is also nearly universal. The vast majority of newborn infants are antibody positive, presumably because of transplacentally acquired maternal antibody, but become seronegative by 6 months of age (Balachandra et al. 1989; Yanagi et al. 1990). Approximately 10 percent of infants less than 1 month old can have detectable HHV-6 DNA in peripheral blood mononuclear cells, suggesting that in a minority of cases HHV-6 is acquired in the perinatal period (Hall et al. 1994). Because the rate of infection as measured by seroconversion increases rapidly during the interval between 6 and 12 months with a peak age of virus acquisition between 6–9 months of age, it has been hypothesized that maternal antibody may provide protective immunity early in infancy. In North America, HHV-6B is almost always associated with primary infection in infancy in contrast to the epidemiology in Zambia in which the majority of infections in infancy are associated with HHV-6A (Dewhurst et al. 1993; Kasolo et al. 1997). Co-infection with HHV-6A and 6B is common (Cone et al. 1996). In contrast, the seroprevalence of HHV-7 increases steadily throughout childhood and by 10 years of age almost all individuals are seropositive (Wyatt et al. 1991). Furthermore, the prevalence of HHV-7 antibody reactivity in different geographical locations is more variable than that reported for HHV-6 (Wyatt et al. 1991; Yoshikawa et al. 1993; Ablashi et al. 1994). Serological reactivity for HHV-6 does not prevent infection with HHV-7 as evidenced by the later age of acquisition of HHV-7 as compared to HHV-6 and the frequent occurrence of HHV-6 and HHV-7 infection in older patients. Reactivation of HHV-6 occurs in about 40 percent of seropositive women during pregnancy and HHV-6 DNA was reported in about 1 percent of cord blood (Dahl et al. 1999). Other reports have suggested that intrauterine transmission of either virus is exceedingly rare (Balachandra et al. 1989; Yanagi et al. 1990; Aubin et al. 1992). Furthermore, HHV-6 can be found in the cervix but perinatal transmission appears infrequent (Okuno et al. 1989; Leach et al. 1993).

HHV-6 and HHV-7 can be readily isolated in the early post-transplant period from both solid organ and bone marrow allograft recipients. Increases in serological responses and viremia, as measured by culture and/or PCR, are commonly detected in up to 80 percent of transplant recipients in the first 3 months after transplantation (Okuno et al. 1990; Wilborn et al. 1994b; Chan et al. 1997; Imbert-Marcille et al. 2000; Yoshikawa et al. 2002; Boutolleau et al. 2003). More recent studies have indicated that HHV-7 was isolated in the post-transplant period as often as HHV-6 in HCMV-infected patients and when HHV-7 DNA was assayed by PCR, HHV-7 was detected more frequently than in HHV-6 in the post-transplant period (Kidd et al. 2000; Mendez et al. 2001; Lautenschlager et al. 2002; Boutolleau et al. 2003). In addition, HHV-6 antigens can be detected in up to 60 percent of renal allograft biopsies, a finding consistent with the proposed role of HHV-6 in graft rejection (Hoshino et al. 1995; Griffiths et al. 1999). However, as noted previously, the contribution of HHV-6 (and presumably HHV-7) to post-transplant morbidity and mortality remains uncertain and recent studies have demonstrated a significantly lower incidence of HHV-6 reactivation than HCMV in heart-lung transplant recipients (Michaelides et al. 2002). Furthermore, HHV-6 DNA could be detected in lung tissue in patients, both bone marrow transplant recipients and controls, who were HHV-6 seropositive (Cone et al. 1993). Similarly, the rate of HHV-7 detection in a group of stem cell

transplant recipients was nearly equivalent in peripheral blood specimens analyzed pre- and post-transplant (Boutolleau et al. 2003). Thus, it remains difficult to assign causality to HHV-6 and/or HHV-7 in post-transplant syndromes such as pneumonitis because of the near universal recovery of HHV-6 and the frequent presence of other agents such as HCMV or adenovirus in the same biopsy tissue from transplant patients.

Sources of virus include exposure to saliva, genital secretions, and PBMC (Levy et al. 1990a; Wyatt and Frenkel 1992; Black et al. 1993; Cone et al. 1994; Leach et al. 1994). Although breast milk would seem to be a likely source of HHV-6 and HHV-7, current understanding of the natural history of these infections suggests that breast milk is not a common mode of transmission. There appear to be no significant differences in the rate or age of acquisition of HHV-6 between breast-fed and nonbreast-fed infants (Takahashi et al. 1988). This epidemiologic observation is consistent with at least one study indicating that HHV-6 could not be detected in breast milk (Dunne and Jevon 1993). Limited studies in families have suggested that exposure to respiratory secretions is the most likely mode of intrafamilial spread of HHV-6 (Mukai et al. 1994). HHV-6 and HHV-7 are persistently excreted in the saliva following primary infection and in individuals with past infections, although it appears that HHV-7 is more frequently detected in saliva than HHV-6 (Levy et al. 1990b; Wyatt and Frenkel 1992; Black et al. 1993; Mukai et al. 1994; Kidd et al. 1996; Lucht et al. 1998). HHV-7 has been isolated from saliva in 60–80 percent of seropositive adults (Black et al. 1993; Hidaka et al. 1993; Black and Pellett 1999). Both viruses can be detected in PBMC from seropositive individuals using PCR, suggesting that blood may be an important source of virus transmission in hospitalized patients (Cone et al. 1994; Kidd et al. 1996).

The biological behavior of the virus in tissue culture, including such characteristics as the cellular tropism of the different HHV-6 variants and the virus yield from cord blood lymphocytes, initially suggested the possibility of variants of HHV-6 (Wyatt et al. 1990; Ablashi et al. 1993; Gompels et al. 1993). Additional studies have documented the presence of two genetically and serologically distinct variants of HHV-6, type A and type B (Ablashi et al. 1991; Schirmer et al. 1991). Comparison of the genomes from prototypic HHV-6A and HHV-6B viruses have revealed identical nucleotide sequence over 75–97 percent of the genome, depending on the region of the genome analyzed (Gompels et al. 1995). Overall, there is approximately 95 percent identity for most genes found in conserved gene blocks, but only 75 percent conservation in genes found in HHV-6 and not in other betaherpesviruses. Interestingly, recombination between variants occurs uncommonly, if at all. These variants are also associated with specific diseases as illustrated by the finding that over 95 percent of cases of roseola in the USA are caused by group B viruses (Dewhurst et al. 1993). Furthermore, HHV-6B, but not HHV-6A, has been shown to replicate in salivary glands as evidenced by its isolation from saliva (Harnett et al. 1990; Levy et al. 1990a; French et al. 1999). Studies from several different transplant centers have also documented that the B variant is more frequently associated with infections in the post-transplant period (Frenkel et al. 1994; Wilborn et al. 1994a; Boutolleau et al. 2003). The basis for the association between the B variant of HHV-6 and specific clinical syndromes remains unexplained. Together, these findings have been used by some investigators to suggest that HHV-6a and HHV-6b are distinct betaherpesviruses and not merely variants of one another.

Pathogenesis

Little is known about the pathogenesis of HHV-6 and HHV-7 infections; however, it must be assumed that, as the majority of infections are subclinical, normal host responses can effectively limit virus-induced cellular damage. In addition, there are no consistent and specific histopathological changes in target organs that suggest direct virus mediated cytopathology (Kurata et al. 1990). Because the severity of roseola can be related to the level of HHV-6 viremia, viral load may be an important determinant of this disease, and presumably of organ damage (Asano et al. 1991). Recent findings in transplant patients infected with HHV-6 also argue for a correlation between virus load and clinical disease (Ljungman 2002; Zerr et al. 2002; Boutolleau et al. 2003) The pathogenetic mechanism(s) leading to the clinical syndrome of roseola are unknown, but the lymphocyte tropism of the virus suggests that cytokines could contribute to the symptoms associated with this infection. HHV-6 infection of lymphocytes induces production of a variety of lymphokines, including TNF, IL-1β, IL-10, IL-12, and interferons (Flamand et al. 1991; Flamand et al. 1996; Campadelli-Fiume et al. 1999). Furthermore, HHV-6 encodes at least two chemokine receptor (UL12, UL51), of which at least one, UL12, is a functional receptor (Isegawa et al. 1999). Together with its lymphotropic behavior, it is almost certain that many of the clinical findings associated with primary infection with HHV-6 can be related to host derived inflammatory responses and not virus-induced cytopathology. Using techniques that detect viral nucleic acids or viral antigens in the absence of histopathological changes, HHV-6 has been demonstrated in alveolar macrophages from transplant patients with pneumonitis, hepatocytes from patients with hepatitis, and brain tissue from patients with encephalitis (Asano et al. 1990; Carrigan et al. 1991; Carrigan and Knox 1994; Cone et al. 1994). It has also been suggested that HHV-6 infection in bone marrow transplant recipients may contribute to more severe graft-versus-host disease (Wilborn et al. 1994a).

As noted previously, the possible role of HHV-6 in the pathogenesis of end organ diseases in transplant recipients is controversial because of the presence of other opportunistic pathogens and the near universal presence of HHV-6 and HHV-7 in the adult population (Cone et al. 1994, 1999; Black and Pellett 1999).

If the relationship between HHV-6 infection and disease can be considered to be incompletely defined, the relationship between specific syndromes and clinical disease and HHV-7 infection is unknown. HHV-7 infection is associated with reactivation of HHV-6 and, therefore, the assignment of the relative contribution of each virus to a clinical illness is impossible. However, the later age of HHV-7 acquisition and the lack of definable syndromes associated with HHV-7 infection suggest that its potential to cause disease in the normal host is limited. In the immunocompromised host however, HHV-7 has been proposed to be an important cofactor in the severity of disease associated with human cytomegalovirus infection in the post-transplant period (Kidd et al. 2000; Tong et al. 2000; Mendez et al. 2001). An interesting finding in these studies was that disease association between these viruses was only demonstrated when the quantity of virus in blood was measured and not just the qualitative presence or absence of either virus. Thus, it would appear that clinically significant interactions between these viruses require increased viral loads, a feature unique to patients with more significant immune dysfunction. In vitro, HHV-7 has been shown to induce $CD4^+$ lymphocyte death by both direct cytolysis and apoptosis, raising the possibility that active HHV-7 replication could contribute to the immune suppression in the post-transplant period (Secchiero et al. 1997). Perhaps this or a related mechanism could explain the role of HHV-7 in severe HCMV infection in the post-transplant period.

The association of HHV-6 infection with central nervous dysfunction, most commonly manifest as seizure activity, is particularly intriguing. Roseola is frequently associated with irritability and a bulging anterior fontanelle, two nonspecific findings of CNS involvement in this childhood infection. Case reports have provided evidence of CNS infection by HHV-6, including detection of HHV-6 nucleic acids in the CSF and in brain tissue from fatal cases (Asano et al. 1990; Ishiguro et al. 1990; Yoshikawa et al. 1992; Yoshikawa and Asano 2000). Virus could also be demonstrated in endothelial cells in the brain of fatal cases of HHV-6 encephalitis, suggesting the possibility of vasculitis as a cause for CNS disease (Yoshikawa and Asano 2000). HHV-6 has also been detected in a variety of cell types in brain tissue from HHV-6 infected patients with AIDS, including astrocytes, oligodendrocytes, microglia, and neurons (Ohashi et al. 2002). Other studies have also demonstrated, by PCR, viral nucleic acid in the CSF (Kondo et al. 1993; Suga et al. 1993; Achim et al. 1994; Caserta et al. 1994; Wilborn et al. 1994a). Interestingly, in several of these studies, including the largest in terms of number of patients, virus could not be isolated from the CSF (Hall et al. 1994). Because of the propensity of HHV-6 to reactivate after a febrile illness, including HHV-7 infection, the demonstration of viral nucleic acid in the CSF without additional evidence of tissue invasion or local replication of virus, suggests only an association between HHV-6 and CNS dysfunction and not causality. Although HHV-6 nucleic acids have been detected in normal hosts with encephalitis and compelling arguments can be made for this virus as a cause of sporadic encephalitis, definitive evidence for a direct role in childhood seizures associated with primary infection is lacking (Yoshikawa et al. 1991). Similarly, primary HHV-7 infection has been associated with the development of seizure activity (Torigoe et al. 1995; Caserta et al. 1998). It has been suggested that primary HHV-7 infection is more frequently associated with seizures in young infants, but little definitive evidence is available to support a direct role for this virus in CNS dysfunction in the normal host.

Since their discovery, HHV-6 and HHV-7 have been suspected to have a role in the pathogenesis of HIV infection. Several biological characteristics support this suspicion. These include: (1) growth in T lymphocytes in vivo and in vitro, (2) the use of CD4 as a cellular receptor by HHV-7, (3) induction of CD4 expression after infection of lymphocytes, and (4) transactivation of the HIV long terminal repeat (Ensoli et al. 1989; Takahashi et al. 1989; Ablashi et al. 1991; Lusso et al. 1991, 1994; Martin et al. 1991; Kashanchi et al. 1994; Wang et al. 1994; Zhou et al. 1994; Lusso and Gallo 1995). However, epidemiological studies have provided little definitive evidence suggesting that either HHV-6 or HHV-7 plays an important role in the progression to AIDS in HIV-infected individuals (Fox et al. 1988; Spira et al. 1990; Fairfax et al. 1994; Lusso et al. 1994). Furthermore, HHV-6 has been shown to inhibit the replication of HIV in vitro (Levy et al. 1990b). In contrast to this early finding, other studies have suggested that co-infection of HIV-infected cells with lower inocula of HHV-6 may actually lead to enhanced HIV replication (Lusso et al. 1994). Thus, it remains to be determined whether co-infection with HHV-6 or HHV-7 contributes to the progression of HIV infections.

Immunity

Immunological responses in normal hosts are sufficient to limit the replication and spread of HHV-6 and HHV-7 as evidenced by the infrequent development of symptomatic infection and the self-limited nature of the infection. The natural history of HHV-6 infection suggests that passively acquired immunity in early infancy is sufficient to prevent infection. It is unclear what role maternal immunity plays in the natural history of HHV-7. Host immune responses that limit reactivation in

normal hosts and curtail invasive infection in immunocompromised hosts are unknown, but, if analogous to other herpesvirus infections, are probably virus-specific cellular responses. The frequent shedding of both viruses in patients undergoing immunosuppressive therapy or individuals with HIV is consistent with a critical role for cellular immunological effector functions in limiting virus replication.

CELLULAR IMMUNE RESPONSES

HHV-6 infection can induce the production of antiviral cytokines, including interferons. NK cell activity and interferon-α levels have also been reported to be elevated in acute HHV-6 infection and in vitro studies have suggested that these activities may play a role in controlling HHV-6 in replication in vivo (Kikuta et al. 1990; Takahashi et al. 1992). Analysis of $CD4^+$ lymphocyte clones derived from infected individuals has revealed that, although most were specific for HHV-6, about 10 percent responded to both HHV-6 and HHV-7 and an even smaller number were cross-reactive with HHV-6, HHV-7, and CMV (Yasukawa et al. 1993). Group A and B variants of HHV-6 could be distinguished by reactivity of 7 percent of the clones. MHC-restricted cytotoxic T cell clones specific for HHV-6 infected cells have also been isolated (Yakushijin et al. 1992). The antigen specificity of the cellular responses against HHV-6 and HHV-7 remains to be defined.

ANTIBODY RESPONSES

The serological responses to HHV-6 and HHV-7 are extraordinarily complex. In the case of HHV-6, IgM antibodies specific for HHV-6 appear 5–7 days after the onset of clinical symptoms and peak around 2–3 weeks (Suga et al. 1993). The IgG antibody response to HHV-6 can first be detected 7–10 days after resolution of clinical symptoms and remain detectable for the life of the host (Ueda et al. 1989). The serological reactivity to HHV-7 appears to have similar kinetics (Yoshida et al. 2002). A large number of virus-encoded proteins are recognized by convalescent sera, but to date the antibody components of protective immunity have not been defined. Immune precipitation of radiolabelled virus-infected cell proteins with convalescent serum revealed over 20 different proteins could be precipitated (Balachandran et al. 1991). At least five glycoproteins were precipitated from infected cells (Balachandran et al. 1991). More recent studies have identified specific viral proteins that are targets of antibody responses including the gB and gH HHV-6 homologues (Ellinger et al. 1993; Liu et al. 1993; Qian et al. 1993). In addition, the major glycoprotein complex of the A variant of HHV-6, gp82-105, has been shown to be a target of neutralizing antibodies (Pfeiffer et al. 1993). A major antigenic protein of HHV-6, designated p100, is the homologue of CMV pp150 (UL32) and probably represents a dominant target of virus-specific antibodies (Neipel et al. 1992). Cross-reactivity exists between conserved structural proteins such as the major capsid protein and the nonstructural protein CD41 (homologue of CMV pp50 (UL44)) (Loh et al. 1994). Interestingly, cross-reactivity has also been shown between the major envelope glycoprotein of CMV (gB) and HHV-6 (Adler et al. 1993). The serological response to HHV-7 is equally complex, with over 15 radiolabelled infected cell proteins demonstrable by immune precipitation (Foa-Tomasi et al. 1994). Several HHV-7 encoded proteins have been shown to be recognized by immune human serum, including an 80 kDa glycoprotein which probably represents the HHV-7 homologue of gH, the gB homologue, an immunogenic tegument protein of estimated mass of 85 kDa (U14), and p86 (U11) (Foa-Tomasi et al. 1996; Nakagawa et al. 1997; Stefan et al. 1997; Stefan et al. 1999; Franti et al. 2002). Antigenic cross-reactivity between HHV-6 and HHV-7 encoded proteins has also been demonstrated and likely is an explanation for the confusion that surrounds the interpretation of seroprevalence studies (Foa-Tomasi et al. 1994, 1996; Black and Pellett 1999). Currently, routine serological assays cannot distinguish between the two variants of HHV-6.

Diagnosis

The diagnosis of HHV-6 and/or HHV-7 infection requires the detection of virus, viral antigens or viral nucleic acids in an appropriate specimen from a patient with a clinical syndrome compatible with infection by either of these viruses. HHV-6 and HHV-7 can be recovered with a reasonable degree of success from children in the early phase of exanthem subitum by cocultivation of peripheral blood lymphocytes with umbilical cord lymphocytes. Both HHV-6 and HHV-7 can also be recovered from saliva, although less consistently than from lymphocytes. Other cells suitable for virus isolation include phytohemagglutinin-stimulated PBMC. Cell lines such as HSB2, Sup T1, and Molt-3 have been used to propagate HHV-6, although tropism for specific cell lines may vary (Osman et al. 1993). Isolation of HHV-6 remains a reliable method for the diagnosis of active HHV-6 infection (Carrigan and Knox 2000). Cell lines permissive for HHV-7 have not been described. Viral antigens within infected cells can be readily detected by immunological assays that incorporate HHV-6 or HHV-7 specific monoclonal antibodies (Yamamoto et al. 1990; Ablashi et al. 1993; Parker and Weber 1993). Monoclonal antibodies can be used to distinguish between HHV-6A and HHV-6B (Foa-Tomasi et al. 1994). Perhaps the most useful assays for HHV-6 and HHV-7 are PCR amplification of specific regions of the viruses. These can be carried out on a number different clinical specimens, including PBMC, saliva, and plasma. The judicious selection of primers allows HHV-7 and the two variants of HHV-6 to be distinguished from one another

(Aubin et al. 1994; Yamamoto et al. 1994; Secchiero et al. 1995). Quantitative PCR assays aid in distinguishing between asymptomatic shedding of HHV-6 and/or HHV-7 and invasive disease (Clark et al. 1996; Kidd et al. 1996; Kidd et al. 2000). Finally, an antigenemia assay similar to the widely employed HCMV antigenemia assay has been used to identify active HHV-6 infection in transplant recipients (Lautenschlager et al. 2002).

Serological assays have yet to be rigorously standardized and, because of significant cross-reactivity between HHV-6 variants and HHV-7, confirmatory testing including absorption with heterologous viral antigens should be considered (Braun et al. 1995; Ward et al. 2001). Antibody avidity assays have been reported that can distinguish between primary HHV-6 and HHV-7 infections (Ward et al. 2001). With the identification of specific viral proteins and their corresponding ORFs, recombinant antigen-based serological assays could overcome issues of cross-reactivity and the high seroprevalence rates in the population.

Control

Although ganciclovir and foscarnet are virustatic for both HHV-6 and HHV-7, current in vitro testing of antiviral susceptibility of these viruses is poorly standardized (Williams 1992). As an example, reports have indicated that some strains of HHV-6 may be relatively resistant to ganciclovir whereas others are susceptible (Akesson-Johansson et al. 1990). Therefore, it is unclear whether adequate in vivo concentrations of drug can be achieved; however, a study in hemapoietic stem cell transplants suggested that ganciclovir treatment resulted in a significant fall in HHV-6 viral burden (Zerr et al. 2002). The HCMV UL97 homologue in HHV-6 (UL69) has been assigned to orf UL69 based on its expression in a recombinant insect virus and its capacity to phosphorylate ganciclovir (Ansari and Emery 1999). Consistent with this finding, some isolates have exhibited drug sensitivities comparable to those reported for CMV. Clinical indications for antiviral therapy will most likely be limited to immunocompromised patients with significant end organ disease; end organ disease such as pneumonitis, in which biopsy tissue could be used to confirm a clinical diagnosis, could be one group that may benefit from antiviral therapy.

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Human herpesvirus 8

BERNHARD FLECKENSTEIN AND FRANK NEIPEL

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PROPERTIES OF THE VIRUS

DISCOVERY OF THE VIRUS

Kaposi's sarcoma (KS), first described in 1872 by the Viennese dermatologist Moriz Kaposi (Kaposi 1872) in the classic form, has since been known as a peculiar semi-malignant tumor of the skin. It afflicted mainly elderly men of southern or east European descent. The frequent appearance of an unusually aggressive form of KS in young homosexual men was one of the observations marking the advent of the acquired immune deficiency syndrome (AIDS) epidemic in developed countries (Friedman Kien et al. 1982). The uneven distribution of KS amongst different transmission groups for the human immunodeficiency virus (HIV) resulted in the hypothesis that an environmental factor or a transmissible agent other than HIV is involved in KS pathogenesis (Beral et al. 1990). Most notably, whereas more than 20 percent of homosexual and bisexual AIDS patients developed KS, only 1 percent of age- and sex-matched men with hemophilia suffered from this uncommon tumor, suggesting – amongst others – transmission of a KS-related virus by sexual practice. Application of representational difference analysis (Lisitsyn et al. 1993) a polymerase chain reaction (PCR)-based method, resulted in the identification of two short DNA fragments from a new herpesvirus (Chang et al. 1994). The virus is now termed human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV). The latter name reflects a finding that was previously claimed in the paper describing the identification of HHV-8 (Chang et al. 1994). HHV-8 DNA is regularly found in KS tissues, but not in uninvolved skin or the vast majority of other malignancies. These include most solid tumors as well as various lymphomas. The few notable exceptions identified to date are two types of B-cell lymphoma or lymphoproliferative lesion, namely primary effusion lymphoma (PEL), and the plasma-cell-rich variant of multifocal Castleman disease (MCD). Similar to KS, HHV-8 DNA is almost always present in PEL and in the majority of MCD cases, suggesting a causative role of the virus in oncogenesis.

CLASSIFICATION

HHV-8 is the first and only known human member of the genus *Rhadinovirus* or γ_2-herpesvirus. This classification is based on DNA homology between the HHV-8 genome and that of other γ_2-herpesviruses (McGeoch 2001).

Related primate rhadinoviruses

Rhadinoviruses have been known for many years as animal viruses infecting primates (Fleckenstein and Desrosiers 1982; Fleckenstein et al. 1978), rodents and ungulates (Table 1). These viruses are usually widespread and generally apathogenic in their natural host. However, upon infection of closely related but distinct species, several animal rhadinoviruses are known to induce diseases, such as malignant catarrhal fever in

Table 1 *The genus* Rhadinovirus

Virus type	Natural host	Pathogenicity in natural host	Foreign hosts	Pathogenic properties in foreign hosts
Human herpesvirus type 8	Human	Kaposi's sarcoma, MCD, PEL		
Herpesvirus saimiri	Squirrel monkey		Marmosets, etc.	Polyclonal malignant T-cell lymphomas
Herpesvirus ateles	Spider monkey		Marmosets, etc.	Polyclonal malignant T-cell lymphomas
Rhesus monkey rhadinovirus	Rhesus monkey	Lymphadenopathy		
Retroperitoneal fibromatosis-associated herpesvirus of *Macaca mulatta*/*Macaca nemestrina*	*Macaca mulatta*/*Macaca nemestrina*	Retroperitoneal fibromatosis?		?
Pan troglotydes rhadinovirus-1	Chimpanzee	?	?	?
Gorilla rhadinovirus-1	Gorilla	?	?	?
Alcelaphine herpesvirus type 1	Wildebeest		Cattle, buffalo	Malignant catarrhal fever
Ovine herpesvirus type 2	Sheep		Cattle	Malignant catarrhal fever
Bovine herpesvirus type 4	Cattle			
Equine herpesvirus type 2	Horse	Transient immunosuppression in foals		
Herpesvirus sylvilagus	Cottontail rabbit	(Benign) lymphoproliferative syndrome		
Murine herpesvirus-68	Wood mouse		Mouse	Benign splenomegaly

Various primate- and nonprimate rhadinoviruses, their natural host and, if applicable, foreign hosts as well as pathogenic properties in a foreign host are given. Please note that in the case of Pan troglotydes rhadinovirus-1, Gorilla rhadinovirus-1, and the retroperitoneal fibromatosis-associated rhadinoviruses, the complete virus has not thus far been isolated. Only fragments from the DNA polymerase gene could be amplified and sequenced using a degenerate PCR approach.

cattle or T-cell lymphoma in certain neotropical primates (Table 1).

HERPESVIRUS SAIMIRI

Herpesvirus saimiri is a well-studied example of this group (Fickenscher and Fleckenstein 2001). Herpesvirus saimiri is apathogenic in its natural host, the squirrel monkey *Saimiri sciureus*. However, closely related new world primates like the marmoset succumb with highly malignant T-cell proliferation upon experimental infection with herpesvirus saimiri. Certain strains of herpesvirus saimiri effectively transform cultured human T-lymphocytes (Biesinger et al. 1992). The genes responsible for growth transformation are essentially known, and nontransforming deletion mutants have been generated. In contrast to HHV-8, the closely related herpesvirus saimiri can be effectively propagated in cell culture. It has thus been used as a model system for HHV-8 (Lee et al. 1998; Jung et al. 1999).

RHESUS RHADINOVIRUS

The discovery of HHV-8/KSHV as the first human rhadinovirus in 1994 stimulated the search for rhadinoviruses in other Old World primates. Serological studies using HHV-8-derived antigens indicated that a herpesvirus closely related to HHV-8 may exist in rhesus monkeys. Co-cultivation of lymphocytes from seropositive rhesus monkeys with fibroblasts resulted in the isolation of a novel rhesus rhadinovirus (RRV) (Desrosiers et al. 1997). The complete genomic sequences from two independent RRV isolates are available (Alexander et al. 2000; Searles et al. 1999). This revealed that RRV is indeed more closely related to HHV-8 than the prototypic rhadinovirus herpesvirus saimiri (HVS). The genomes of RRV and HHV-8 are essentially colinear. RRV contains (at least) 84 genes, 80 of which are homologous to genes found in HHV-8. Thus, at this point in time RRV is the closest known relative of HHV-8 which has been fully sequenced. RRV seems to be very widespread amongst monkeys kept in captivity. No clear disease association has been identified for RRV in otherwise healthy macaques as yet. However, inoculation with RRV resulted in the development of a multifocal lymphoproliferative disease when rhesus macaques were immunosuppressed as a consequence of infection with simian immunodeficiency virus (Alexander et al. 2000; Wong et al. 1999). In contrast to HHV-8, RRV can be readily propagated in cell culture.

RETROPERITONEAL FIBROSIS RHADINOVIRUSES

In a second approach targeted at the identification of Old World primate rhadinoviruses, Rose and co-workers studied tissue specimens from simian retroperitoneal fibromatosis (RF). RF has been identified as an infrequent disease syndrome occurring in immunosuppressed macaques (Giddens et al. 1985). RF lesions consist of an aggressively proliferating fibrous tissue with a high degree of vascularization and thus remotely resemble KS. Transmission studies indicated that an infectious agent may be involved in RF pathogenesis (Giddens et al. 1985). By using a degenerated PCR technique, fragments of a herpesvirus DNA polymerase gene were identified in RF tissues from two macaque species, *Macaca nemestrina* and *Macaca mulatta* (Rose et al. 1997). Sequence comparisons indicated that at least the DNA polymerase genes of these two novel rhadinoviruses, tentatively termed RFHVMm and RFHVMn, are more closely related to KSHV than the DNA polymerase genes of RRV. However, attempts to isolate these viruses on cultured cells or to obtain additional sequence information have not been successful so far.

HHV-8 VIRION MORPHOLOGY

The morphology of the enveloped HHV-8 virion is comparable to other herpesviruses. The three-dimensional structure of the human herpesvirus 8 capsid has been determined by cryo-electronmicroscopy using viral particles produced from cultured primary effusion lymphoma (PEL) cells at a resolution of 22–24 Å (Trus et al. 2001; Wu et al. 2000). The HHV-8 capsid is similar to those of other herpesviruses, especially herpes simplex virus-1. The icosahedral capsid shell is composed of 162 capsomers (12 pentons, 150 hexons).

MOLECULAR BIOLOGY

Genome structure

The HHV-8 genome is a linear, double-stranded DNA of approximately 160 kbp and has the overall structure typical for rhadinoviruses (Figure 1). A complete rhadinovirus genome is usually termed M genome, as it is of inter*m*ediate density (M-DNA). The γ_2-herpesviruses were termed rhadinoviruses utilizing the ancient Greek word for fragile, because this M-DNA tends to split into two fractions of DNA molecules with highly different density, the L-DNA containing genes (low density, low G+C content) and the terminal repetitive H-DNA (high density, high G+C content). The latter is, as far as currently known, without coding capacity. The almost complete nucleotide sequence of HHV-8 was determined from both a PEL cell line (Russo et al. 1996) and from a KS biopsy specimen (Neipel et al. 1997b). The central low-GC (53.3 percent G+C, L-DNA) coding fragment is flanked by numerous tandem repeats of high GC content (84.5 percent G+C, H-DNA). The L-DNA contains at least 89 open reading frames, 67 of which have homologues in the closely related γ_2-herpesvirus prototype herpesvirus saimiri (Figure 1). The overall amino acid sequence identity of these 67 HHV-8 reading frames to homologues identified in herpesvirus saimiri ranges from

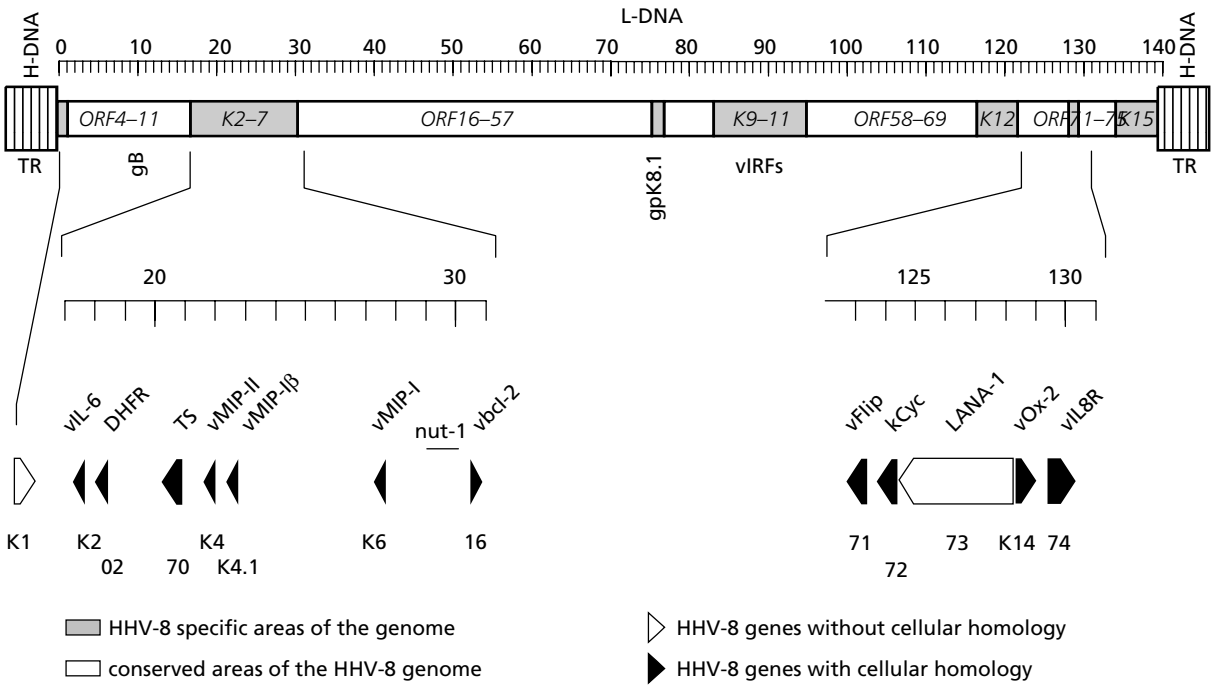

Figure 1 *Specific traits of the HHV-8 genome in a linear representation. A central low-GC (53.3 percent G+C,* L-DNA*) coding fragment is flanked by numerous tandem repeats (TR) of high G+C content (84.5 percent G+C,* H-DNA*). The L-DNA contains at least 89 open reading frames, most of which have homologues in other herpesviruses. These blocks of conserved herpesvirus genes are symbolized by open boxes in the upper half of the figure. They are interrupted by nonconserved areas shown in light gray. Most of the HHV-8 genes thought to be directly involved in pathogenesis – either by direct growth transformation or through paraendocrine mechanisms – are located within these HHV-8-specific areas. They are shown enlarged in the lower part of the figure. Black arrows indicate HHV-8 genes with homology to cellular genes. HHV-8 genes without known homology to cellular genes are shown as open arrows. HHV-8 reading frames that share homology with herpesvirus saimiri were assigned the corresponding reading frame numbers following the nomenclature by Albrecht et al. (1992).* HHV-8 *genes without recognizable homologues in herpesvirus saimiri were numbered separately and given the prefix K (K1–K15) (Russo et al. 1996). DHFR, dihydrofolate reductase; gB, glycoprotein B; gpK8.1, glycoprotein K8.1; kCyc, HHV-8 cyclin-D homologue; nut-1, nuclear tRNA-like transcript; TS, thymidylate syntase; vbcl-2, viral bcl-2; vFlip, viral FLICE inhibitory protein; vIL-6, viral interleukin-6; vIL8R, viral interleukin-8 receptor; vIRFs, viral interferon response factor homologue; vMIP-I/II, viral macrophage inflammatory protein-I/II; vMIP-1β, open reading frame with homology to macrophage inflammatory protein-1β or macrophage chemoattractant protein; vOx-2, viral Ox-2 homologous gene.*

22.4 to 66 percent (average, 42 percent). Conserved genes are usually found in a comparable genomic position and orientation. Thus, HHV-8 genes that share homology with herpesvirus saimiri are numbered from left to right according to their position on the herpesvirus saimiri genome. Open reading frames which do not share recognizable amino acid homology with genes in herpesvirus saimiri are numbered with the prefix K (Figure 1). To date, 19 genes have been identified which are not clearly homologous to genes identified in herpesvirus saimiri (K1–K15, K4.1, K4.2, K8.1, K10.5). Frequently, these 'K'-genes are strikingly homologous to known cellular genes. They code for proteins interfering with the immune system, for enzymes of the nucleotide metabolism, and for putative regulators of cell growth (Figure 1).

Virus-coded proteins

Human herpesvirus-8 DNA is present in the vast majority of KS spindle cells and primary effusion lymphoma B cells. The virus establishes a latent infection in these cells: virus progeny is not produced and only a limited set of viral genes is expressed. Whereas the HHV-8 genome is quickly lost from cultured KS spindle cells, cultured PEL cells maintain the viral genome. The full lytic program is spontaneously activated in only a few cells of a PEL culture (<1 percent). However, lytic replication can be induced in up to 5–40 percent of PEL cells by the addition of phorbol esters and/or sodium butyrate (Renne et al. 1996). Genes expressed during the HHV-8 lytic replication cycle have been essentially studied on cultured PEL cells treated with such agents. Most of these genes have pronounced homology to those of other herpesviruses. Although de novo infection of cultured cells has not been studied extensively, HHV-8 lytic genes have been divided into immediate–early, early, and late depending on their temporal expression following reactivation from latently infected, cultured cells: (1) transcription of immediate–early genes is inducible by TPA/butyrate even in the presence of agents blocking protein biosynthesis; (2)

expression of early genes requires the presence of immediate–early protein, but takes place in the absence of DNA replication; (3) transcription of late viral genes does not take place when DNA replication is inhibited. This temporal gene expression pattern where one set of genes is activated by genes expressed earlier during the lytic cycle was initially defined using herpes simplex virus and de novo infection of cultured cells. The situation in HHV-8 is different so far, as most data on temporal gene regulation were derived from latently infected PEL cells where lytic replication of the virus is activated by phorbol esters or sodium butyrate. In an alternative classification, HHV-8 transcripts were divided into three groups according to their inducibility by tetradecanoylphorbol acetate (TPA) (Sarid et al. 1998). Class I genes were transcribed constitutively, i.e. under standard growth conditions without the addition of stimulating agents like TPA; their expression did not increase when the lytic cycle was induced by TPA. Class I genes were mapped to a region close to the right end of the L-DNA encoding ORF73 (latent nuclear antigen 1, LANA), ORF72 (viral cyclin-D, vCyclin) and ORF71 (viral flice inhibitory protein, vFLIP). They constitute bona fide latently expressed genes. Class II transcripts were detectable without TPA treatment, but clearly induced to higher transcription by the addition of TPA. Essentially, immediate–early and early genes belong to this intermediate class. Until today, three immediate–early transcripts have been identified in HHV-8: ORF4.2, ORF45, and ORF50 (Zhu et al. 1999). Class III transcripts were not detectable without stimulation of cultured PEL cells by TPA or butyrate. Essentially, they comprise the late, structural proteins of HHV-8.

Lytic regulatory proteins

The lytic regulatory proteins of HHV-8 studied so far include open reading frame 50, also termed replication and transcription activator (RTA), Lyta (lytic transactivator) or ART (activator or replication and transcription), K8 (also termed K-bZip), and ORF45. As in vitro transmission of the virus is still laborious and inefficient, most data were obtained from PEL cells or transfection studies using permanent cell lines.

ORF50 codes for the key immediate–early regulator protein required for the switch from latent to lytic infection. ORF45 seems to be involved in counteracting the interferon response very early in infection. K8 protein is expressed with early kinetics and plays a role in HHV-8 DNA replication.

ORF50/K-RTA

ORF50 is translated from a 3.6-kb spliced mRNA that also contains the reading frames coding for K8. Due to an additional 5′ exon, the whole ORF50 protein comprises 691 amino acids with a calculated molecular weight of 73.7 kDa. It shares limited homology with the R-*trans*-activator of EBV (16 percent amino acid identity, 30 percent similarity), and was hence initially termed K-RTA. It is well established that RTA or ART of HHV-8 is the key immediate–early regulator protein of HHV-8. In cultured PEL cells, RTA is both sufficient and required for the switch from latent infection to lytic replication (Lukac et al. 1998, 1999). It *trans*-activates the expression of several early and delayed-early genes of HHV-8, including K8, ORF57 (Byun et al. 2002), and ORF59. In addition, RTA of HHV-8 interacts with CREB binding protein (CBP) and thus represses transcriptional activity of p53 and p53-induced apoptosis (Hwang et al. 2001). By direct interaction with cellular STAT3, ORF50 mediates STAT3 nuclear translocation, dimerization, and activation of STAT3-dependent transcription without prior phosphorylation of STAT3 (Byun et al. 2002).

ORF45-PROTEIN

A second immediate–early transcript of 1.7 kb was mapped to the ORF45 locus. ORF45 has the capacity to encode a protein of 407 amino acids and 43.3 kDa. Genes homologous to ORF45 of HHV-8 are conserved amongst the γ-herpesviruses. HHV-8 ORF45 seems to interact with cellular interferon-regulatory factor 7 (IRF-7) (Zhu et al. 2002). It inhibits IRF-7 phosphorylation and accumulation in the nucleus. Phosphorylation and nuclear translocation of IRF-7 normally results in activation of type I interferon genes. Thus, ORF45 could inhibit the activation of interferon very early in viral infection by blocking phosphorylation of IRF-7.

K8/K-BZIP

K-bZip is a 237 amino acid early nuclear protein. It includes a basic leucine zipper motif (K-bZIP), and is distantly related to the EBV lytic-cycle Z *trans*-activator (ZTA) protein. However, in HHV-8 the lytic cycle is induced by ORF50, and the role of HHV-8 K8 seems to be distinct. A 1.3-kb transcript that is not detectable until delayed early in the HHV-8 infectious cycle seems to contribute largely to K8/K-bZip protein synthesis (Saveliev et al. 2002). More compatible with the delayed early expression of K8 is its role as a negative regulator of ORF50 expression (Izumiya et al. 2003) and its function in DNA replication. Both in infected cells and in transfection assays, K-bZip is recruited into viral DNA replication compartments (Wu et al. 2001). Recent data indicate that K8, which also locates to the POD/ND10 domains, mediates arrest of the cell cycle in G1 phase (Wu et al. 2002). There is increasing evidence that herpesviral DNA replication takes place preferentially in G1 arrested cells to avoid competition with host-cell DNA replication (Flemington 2001). Thus, in contrast to its distant relative ZTA in EBV, the K-bZip of HHV-8

plays an important role in DNA replication and is hence also termed RAP (replication associated protein).

Virion proteins

VIRAL CAPSID PROTEINS

Detailed study of HHV-8 proteins forming the viral nucleocapsid has been hindered by the lack of an efficient lytic cell culture system. The major capsid protein is encoded by ORF25. Triplex proteins are encoded by ORF62 and ORF26, also termed minor capsid protein. The immunogenic ORF65 protein is also part of the nucleocapsid (Trus et al. 2001).

GLYCOPROTEINS

As in other herpesviruses, HHV-8 envelope glycoproteins play a crucial role in target cell recognition and entry. HHV-8 encodes at least five envelope glycoproteins, four of which (gB, gH, gL, gM) are conserved throughout the *Herpesviridae*. The function of two glycoproteins, gB and the HHV-8-specific K8.1, has been studied in more detail. Both are involved in binding of HHV-8 to its cellular attachment or entry receptors, heparan sulfate, and integrin $\alpha_3\beta_1$, respectively.

Glycoprotein K8.1

The gene encoding glycoprotein K8.1 (gpK8.1) is located approximately in the middle of the HHV-8 genome (Figure 1) within a block of conserved genes. However, as the designation as gpK8.1 indicates, it does not share recognizable homology with genes present in any other herpesvirus. gpK8.1 is translated from alternatively spliced messages giving rise to two variants, gpK8.1A and gpK8.1B, the larger A form being predominant. gpK8.1 is a major target for the humoral immunoresponse, and the glycoprotein is thus frequently a component of serological assays for HHV-8 (Chandran et al. 1998; Raab et al. 1998). gpK8.1 is part of the virion envelope and involved in attachment to target cells by binding to cell surface heparan sulfate (Birkmann et al. 2001).

Glycoprotein B

The conserved herpesvirus glycoprotein B (gB) is encoded close to the left end of the HHV-8 L-DNA (Figure 1). Like gpK8.1, gB has been shown to be part of the virion envelope. It binds to cell surface glycosaminoglycans (Akula et al. 2001). In addition, gB binds to integrin $\alpha_3\beta_1$, and this interaction seems to be involved in the entry of HHV-8 into target cells (Akula et al. 2002). The use of integrins as an entry receptor suggest that HHV-8 enters the cell by endocytotic pathways. This, however, is rather unusual for a herpesvirus. Although endocytosis of virions has been shown to occur with other herpesviruses, especially herpes simplex virus (Nicola et al. 2003), the biologically relevant way of infection by a herpesvirus is usually fusion of the virion envelope with the cytoplasma membrane at neutral pH.

Latent proteins

It is a common feature of oncogenic viruses that lytic replication is turned off in the vast majority of transformed cells. Essentially, this holds true for HHV-8. At least in Kaposi's sarcoma lesions and primary effusion lymphoma cells, expression of HHV-8 structural proteins is observed in only few cells (Figure 2a, b), whereas the viral genome is present in almost all tumor cells in a latent state with only few genes being expressed (Figure 2c, d). Although this observation does clearly not exclude that the lytic cycle genes play a role in HHV-8 pathogenesis, the few latent genes expressed by the vast majority of cells are particularly important for understanding HHV-8 biology and pathogenesis. Unfortunately, known latently expressed genes of EBV and, with few exceptions, HVS are not conserved in KSHV. Until recently, latent transcripts have been identified from only three loci of the HHV-8 genome. The major latency locus was found close to the right end of the genome (Figure 1). It comprises genes encoding the latency associated nuclear antigen 1 (LANA-1), a viral cyclin D homologue (k-cyclin, Figure 2d), and the viral anti-apoptotic flice inhibitory protein (v-Flip). Several transcripts of K12 (Kaposin) are also detectable both in KS and PEL. The third locus maps to the middle of the genome where various genes with homology to the interferon-regulatory protein family of transcription factors can be found. At least in PEL cells, two of these transcripts can be detected in latent infection.

LATENT NUCLEAR ANTIGEN 1 (LANA-1, LNA-1)

An area close to the right end of the HHV-8 L-DNA was consistently found to be transcriptionally active in latency. It comprises *orf71/vFLIP*, *orf72/k-cyclin*, and *orf73/LANA-1*. LANA-1 (LNA-1) was initially identified as the major antigen detected by KS patient sera in latently infected PEL cells (Gao et al. 1996a; Rainbow et al. 1997). It is now clear that LANA-1 is important for maintenance of the episomal HHV-8 genome in latently infected cells (Ballestas et al. 1999; Ballestas and Kaye 2001). It can therefore be seen as a functional analog of the Epstein–Barr virus nuclear antigen 1, which mediates maintenance of the latent EBV-episome by binding to the plasmid origin of replication, oriP. However, the *cis*-sequence required for HHV-8 LANA-1 to fulfill its function seems to be located within the high-density terminal repeats (Hu et al. 2002). Due to their high GC-content, the H-DNA differs clearly

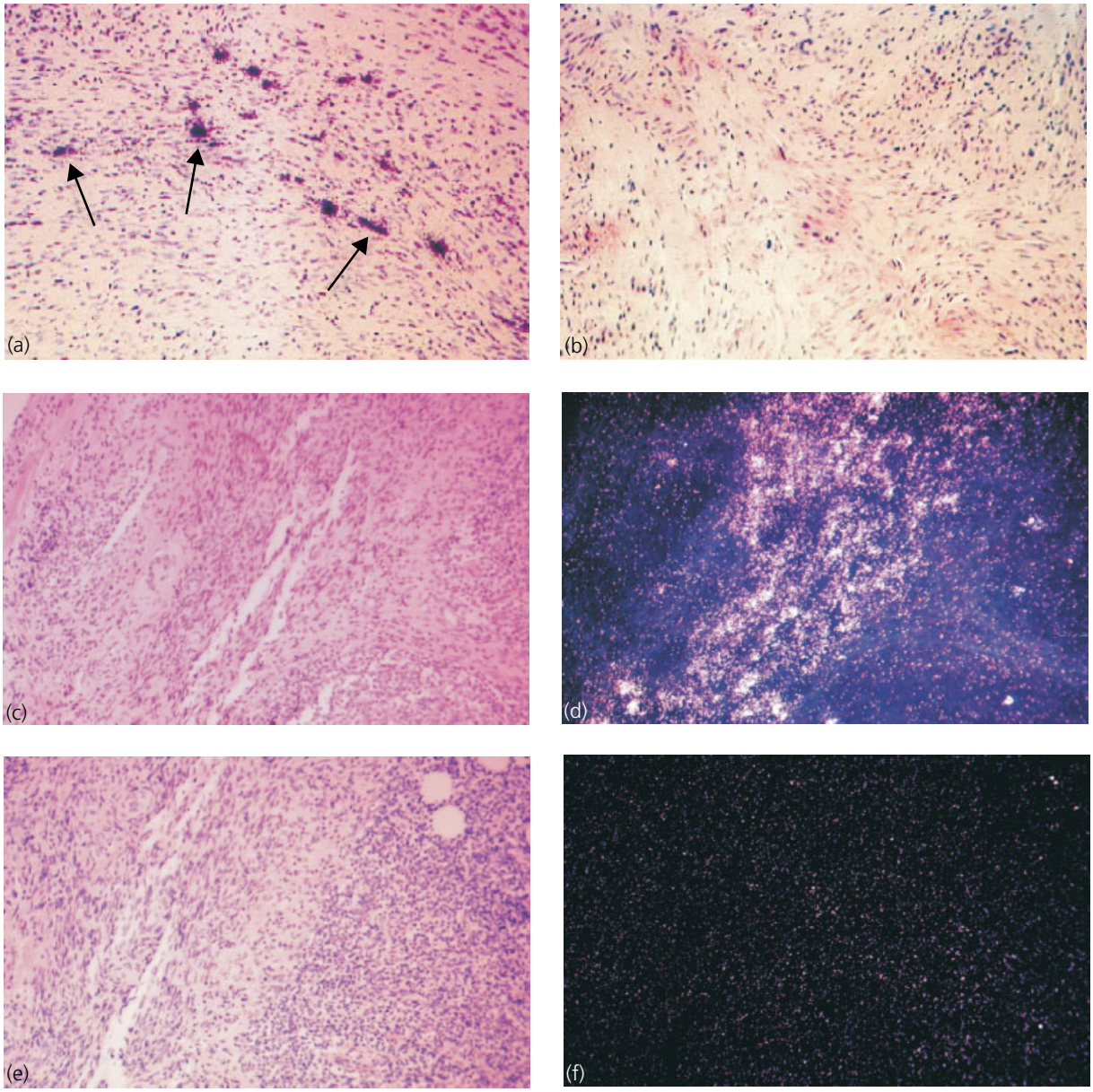

Figure 2 *Expression of HHV-8 genes in Kaposi's sarcoma. Lytic cycle genes like the minor capsid protein are detectable in only a few cells in a KS lesion* **(a)**. *The majority of spindle cells carry the HHV-8 genome in a latent state, and transcripts mapping to the k-cyclin locus are detectable* **(d)**. **(a)** *Productively HHV-8-infected cells in KS tumors were identified by in situ hybridization with strand-specific radiolabeled RNA probes directed against the mRNA coding for the HHV-8 minor capsid protein (VP23) Signals can be seen overlying single cells* **(a)**, *which are arranged in clusters between the KS spindle cells. HHV-8-positive cells are marked by arrows. Their distribution suggests that at least part of the positive cells are monocytes. Magnification, ×3 500.* **(b)** *Control hybridization. No staining was observed when a surplus of the unlabeled sense-strand RNA probe was applied to the tissue sections simultaneously with the radiolabeled antisense RNA probe. Magnification ×3 500. (c,d) Prominent signals in the majority of KS spindle cells were obtained when a probe against k-cyclin was used (c, bright field; d, dark field). A consecutive section of the same KS tissue specimen was hybridized with a HHV-8 minor capsid protein probe (VP23; e, bright field; d, dark field). The VP23 gene was not expressed in this section, indicating a more predominantly latent infection than in the sample shown in* **(a)** *(modified from Stürzl et al. (1999) and Blasig et al. (1997) with permission).*

from oriP or other eukaryotic origins of DNA replication. In addition to its well established function in maintenance of the viral genome, LANA-1 has been described to inhibit p53 and to provide protection from cell death (Friborg et al. 1999; Radkov et al. 2000). Recent data indicate that LANA stimulates S-phase entry by stabilizing β-catenin (Fujimuro et al. 2003). In summary, the latency-associated nuclear antigen 1 of HHV-8 is a multifunctional protein involved in maintenance episomal DNA, transcription regulation, and cell cycle control possibly contributing to cell transformation.

VFLIP

The viral FLIP (FLICE/caspase-8 inhibiting protein) is homologous to a cellular apoptosis inhibitor. Bi-cistronic transcripts comprising both the viral cyclin-D homologue and vFlip are detectable in latently infected cells (Figure 2b). It may play an important role in HHV-8 pathogenesis by blocking apoptosis induced by antiviral cytotoxic T cells (Thome et al. 1997) or growth factor withdrawal (Sun et al. 2003).

K-CYCLIN

Transcription of the HHV-8 encoded cyclin D homologue (k-cyclin) is driven from the same latent promoter as transcription of ORF73/LANA. It is the first gene on a bicistronic message also encoding vFLIP. k-Cyclin has been shown to associate with the catalytic subunit cdk6 to form an active holoenzyme (Li et al. 1997; Godden-Kent et al. 1997). This complex is able to phosphorylate retinoblastoma protein and to stimulate cell cycle progression from G1 to S in quiescent fibroblasts. It is especially important that the activity of the k-cyclin/cdk6 holoenzyme was resistant to inhibition by CDK inhibitory proteins $p21^{cip1}$ and $p27^{Kip1}$ (Swanton et al. 1997). These distinctive features of k-cyclin biochemistry were expanded by two papers published later (Ellis et al. 1999; Mann et al. 1999). Not only is the k-cyclin/cdk6 complex resistant to inhibition by $p27^{kip1}$, it also has a broader substrate range including $p27^{kip1}$ and substrates of cdk2. Phosphorylation of $p27^{kip1}$ results in the proteolytic degradation of this CDK-inhibitor and progression of cells arrested in G1 to S (Ellis et al. 1999; Mann et al. 1999). Deregulation of the G1/S restriction point is a feature shared by many tumor cells and DNA tumor viruses (reviewed by Pines (1995)). It is therefore intriguing to speculate that k-cyclin is involved in HHV-8 pathogenesis. However, it must be noted that at present there is no direct, experimental evidence for a transforming ability of the cyclin D homologue encoded by HHV-8, and the viral cyclin homologue has been shown to be dispensable for oncogenic transformation by the closely related herpesvirus saimiri (Ensser et al. 2001).

K12

A HHV-8 transcript of 0.7 kb (T0.7) was originally described as being abundantly expressed in nonstimulated PEL cells. T0.7 mRNA, which contains the putative open reading frame K12, is also detectable in the majority of KS spindle cells. The oncogenic potential of K12 in rodent fibroblast transformation assays was not very pronounced (Muralidhar et al. 1998) and the data remain controversial. It is therefore not yet possible to assess the oncogenic potential of K12 or T0.7 with certainty.

VIRAL INTERFERON REGULATORY FACTORS

The HHV-8 genome contains four genes with homology to cellular interferon regulatory factors (vIRFs), termed K9, K10, K10.5, and K11. At least one of these transcription factors, K10.5, is expressed in latently infected PEL cells. Whereas the vIRF-1 encoded by K9 has been shown to antagonize the action of class I interferons, the function of the latently expressed K10.5 is less clear as yet. It is interesting, however, to note that its closest cellular homologues are IRF-4 and the interferon consensus sequence binding protein, ICSBP. Expression of both IRFs is restricted to lymphoid cells. IRF-4 is clearly important for differentiation and proliferation of B-lymphocytes (Pernis 2002).

Inflammatory cytokines and related genes encoded by HHV-8

HHV-8 codes for several proteins which are not generally conserved amongst the different herpesvirus families, but which share striking homology with genes of the host cell. It is very likely that these genes have been captured from the host cell during evolution (Table 2). Interestingly, whereas the cellular genes usually consist of several exons, their viral homologous are usually translated from nonspliced messenger RNA. It is thus likely that these genes have been acquired from cellular mRNAs after reverse transcription. With the few exceptions discussed above (k-cyclin, vFlip, K10.5), these virus-cell homologous genes are not expressed in latently infected cells, but seem to be active early in the lytic replication cycle. Typically, they are coding for proteins interfering with the immune system, for enzymes of the nucleotide metabolism, and for putative regulators of cell growth. Thus, their main function seems to be to provide an environment suitable for replication of the virus by counteracting early immune responses (IRFs, anti-apoptotic proteins like vbcl-2, complement regulatory protein homologue CCPH), providing sufficient pools of nucleotides for viral replication or to increase the number of target cells for HHV-8 infection (macrophage inflammatory protein homologues, viral interleukin-8 receptor, interleukin-6). It seems to be a 'rule of thumb,' that such viral genes encode proteins which are usual functional agonists of their cellular counterparts. However, they frequently escape from the usual regulatory mechanisms. Examples are receptors with constitutive activity (e.g. the viral interleukin-8 receptor), a viral cyclinD homologue that escapes from control by cyclin inhibitory proteins, or cytokines like the viral interleukin-6 that are able to bind to a cellular receptor without additional molecules. It is possible that HHV-8 encoded cytokines or cytokine receptors are involved in KS or PEL pathogenesis by paraendocrine mechanisms. Although the HHV-8 genome is present in a latent state in the vast majority of tumor cells, a few cells in a lesion express HHV-8 lytic cycle genes.

VIRAL INTERLEUKIN-6

The open reading frame K2 of HHV-8 encodes viral IL-6 (vIL-6), a protein with moderate yet significant

Table 2 *HHV-8 genes putatively involved in pathogenesis*

ORF	Gene product	Suggested function in pathogenesis	Experimental evidence
K1	Nonconserved transmembrane glycoprotein	Positional and (possibly) functional analogue to other rhadinoviral oncoproteins	Transformation of Rat1 cells; recombinant herpesvirus saimiri: lymphocyte immortalization, lymphoma induction (Lee et al. 1998)
K2	Viral IL-6	Paracrine growth stimulation independent of gp80	Proliferation of B cells; IL-6Rα chain not required (Chatterjee et al. 2002; Burger et al. 1998; Molden et al. 1997)
vMIPs (K4, K4.1, K5)	Viral macrophage inflammatory proteins	Angiogenesis; attraction of Th2 lymphocytes	Binding to both CC and CXC receptors; attraction of eosinophils; induction of angiogenesis (Boshoff et al. 1997)
K9, K10, K10.5, K11	Interferon response factor homologues	Interfering with the antiproliferative action of IRF-1	K9: antagonistic to IRF-1, transformation of 3T3 cells (Li et al. 1998)
K12	Kaposin A	Transformation	Transformation of Rat-3 cells; increased cellular adhesion
ORF16	vbcl-2	Stabilizing productively infected cells	Anti-apoptotic activity
ORF71	vFLIP	Stabilizing latently infected cells	In situ: increased expression correlates with reduced apoptosis
ORF72	k-Cyclin	Dysregulated cell cycle progression, inhibits cell cycle arrest by p53	Activates cdk6, evades inhibition by CDK-inhibitors, destabilizes p27 (Swanton et al. 1997)
ORF73	LANA-1	Maintains latent viral infection	Mediates HHV-8 DNA persistence (Ballestas et al. 1999)
ORF74	vIL-8R	Angiogenesis, transformation of endothelial cells	Constitutively active; induces VEGF secretion, transforms rodent fibroblasts; KS-like lesions in transgenic mice (Bais et al. 1998; Yang et al. 2000)
K15	Integral membrane protein reminiscent of EBV LMP-2	Differentiation and proliferation of B lymphocytes	

EBV, Epstein–Barr virus; IL-6, interleukin-6; IL-6Rα, interleukin-6 receptor α-chain (gp80); IRF, interferon regulatory factor; k-cylin, KSHV-encoded cyclin D homologue; LANA-1, latent nuclear antigen-1; LMP-2, latent membrane protein-2 of Epstein–Barr virus; ORF, open reading frame; VEGF, vascular endothelial cell growth factor; vFLIP, viral FLICE inhibitory protein; vIL-8R, viral interleukin-8 receptor homologue.

homology to the multifunctional human cytokine IL-6 (hIL-6) (Neipel et al. 1997a). The vIL-6 cytokine is functional and supports the proliferation of both murine and human IL-6-dependent cells (Burger et al. 1998). Both human and viral IL-6 are produced in KS lesions and the proliferation of cultured spindle cells can be stimulated with oncostatin M and hIL-6 (Masood et al. 1994; Miles et al. 1992). Nevertheless, the relevance of IL-6 for KS pathogenesis has been questioned as some groups were not able to detect expression of the IL-6 receptor α-chain (IL-6Rα) by KS spindle cells (Murakami Mori et al. 1995). The IL-6R consists of two chains. The β-chain, also termed gp130, is almost ubiquitously expressed. It is responsible for ligand-dependent signal transduction and is shared by several receptors of four-helical cytokines such as IL-6, IL-11, ciliary neurotrophic factor, cardiotrophin-1, oncostatin M, and leukemia inhibitory factor. The β-chain alone is not sufficient for hIL-6 binding. hIL-6 first needs to bind to the second chain (IL-6Rα), and this complex then associates with gp130. There is compelling evidence that IL-6Rα is not required for binding of vIL-6 to the signal transducer gp130 (Chow et al. 2001; Li et al. 2001; Molden et al. 1997). Thus, vIL-6 can induce gp130 signal transduction on almost any cell. This may be important for autocrine growth stimulation of HHV-8 infected cells, as expression of the gp80 chain is reduced by interferon-α. By producing vIL-6 which is not dependent on gp80, HHV-8 infected cells escape from one of the mechanisms of growth control by interferon-α (Chatterjee et al. 2002).

CHEMOKINES ENCODED BY HHV-8

Three genes with homology to the family of CC chemokines are located in the same nonconserved region of the HHV-8 genome as vIL-6 (Figure 1, Table 2).
The proteins encoded by the open reading frames K4 and K6 are homologous to the macrophage inflammatory protein 1α (MIP-1α). They have been termed vMIP-II and vMIP-I, respectively (Nicholas et al. 1997; Russo et al. 1996). The amino acid sequence identity of these viral proteins to their cellular counterpart is only about 30 percent, but 48 percent of the viral sequences are identical to each other. This could indicate a gene duplication event that occurred in the evolution of the genes rather than independent acquisition from the host cell genome. Another HHV-8 reading frame, K4.1, is also homologous to the CC chemokine family, with similarity to both MIP-1β and macrophage chemoattractant protein. Functional data are available for vMIP-I (K6) and vMIP-II (K4). vMIP-II binds efficiently to the CC-chemokine receptor CCR3 (Boshoff et al. 1997). In contrast to cellular MIP-1α, it does also bind to CXC-chemokine receptors such as CXCR4, albeit with lower affinity. The CC-chemokine receptor CCR3 is known to be relevant for the chemoattraction of both eosinophil granulocytes and Th2 lymphocytes. Correspondingly, a potent chemoattractive effect of vMIP-II on eosinophils was described (Boshoff et al. 1997). In contrast to cellular MIP-1α and to the CC-chemokine RANTES, both vMIP-I and vMIP-II were highly angiogenic in the chorioallantoic assay (Boshoff et al. 1997). The chemokine homologues encoded by HHV-8 could thus contribute to two features of KS histology: the presence of inflammatory infiltrates containing Th2 lymphocytes, and the prominent angiohyperplasy.

VGPCR/VIL-8R

Similarly to most other rhadinoviruses, HHV-8 encodes a protein with homology to the family of G-protein coupled receptors (GPCR) with seven transmembrane regions (Table 2). The HHV-8 gene is most homologous to the human IL-8R and hence termed vIL-8R. This viral receptor is capable of activating the protein kinase C pathway, regardless of whether a suitable chemokine is present or not. This constitutive activity was sufficient to enhance the proliferation of transfected primary rat fibroblasts (Arvanitakis et al. 1997). Moreover, the transfection of vIL-8R induced focus formation in fibroblasts with an efficiency comparable to the m1 muscarinic receptor, but independently of an extracellular ligand. vIL-8R transformed cells efficiently formed tumors in nude mice (Bais et al. 1998). Transgenic mice were generated that expressed vIL-8R under control of the CD2 promoter on lymphoid cells. Interestingly, this resulted in angioproliferative lesions in multiple organs. Histologically, these lesions strikingly resembled KS (Yang et al. 2000). Thus, the constitutively signaling viral IL-8R homologue is another potential HHV-8-encoded oncogene.

CLINICAL AND PATHOLOGICAL ASPECTS

INTRODUCTION

Extensive studies on HHV-8 molecular epidemiology have shown that, although not discovered until 1994, HHV-8 has most likely been present in the human population for at least 35 000–60 000 years (Hayward 1999). Thus, HHV-8 is a typical herpesvirus which has evolved to stay precisely balanced with its host organism. As a consequence, HHV-8 infection is usually benign and often asymptomatic. Nevertheless, persistent HHV-8 infection is clearly associated with three diseases: Kaposi's sarcoma, multifocal Castleman's disease, and primary effusion lymphoma, which represent a spectrum from a hyperproliferative process (Kaposi's sarcoma) to true monoclonal lymphoma (primary effusion lymphoma). All three diseases occur more frequently in immunosuppressed patients, especially in HIV-infected patients.

EPIDEMIOLOGY

HHV-8 DNA is almost invariably detectable in all epidemiological forms of Kaposi's sarcoma, in primary effusion lymphomas, and most cases of multifocal Castleman's disease. In contrast, attempts to amplify HHV-8 DNA from various other malignant and non-malignant diseases, as well as healthy individuals were either unsuccessful or difficult to reproduce. Thus, it appears that at least amongst populations of the northern hemisphere, the detection of HHV-8 DNA is restricted to KS, PEL, and MCD, where it is regularly found. Most human tumor viruses are relatively widespread when compared to the frequency of the malignancy they cause. This is true for Epstein–Barr virus, the human herpesvirus most closely related to HHV-8, and for human papillomaviruses. The epidemiology of AIDS-associated KS in the metropolitan areas of the northern hemisphere suggested that a relatively infrequent, sexually transmitted agent is involved in KS pathogenesis (Figure 3). This poses three important epidemiological questions: firstly, is the HHV-8 prevalence significantly increased in KS risk groups, as compared to the general population? Second, does the infection precede KS development? Third, are the modes of transmission compatible with the claimed transmissible agent? Nucleic acid detection is not sufficient to answer these questions, as the high level of viral replication found in immunosuppressed patients makes detection of viral genomes much more likely in this group. Serological studies are more suitable. The first serological studies were solely based on immunofluorescence techniques using HHV-8-positive cell lines derived from primary effusion lymphomas (Cesarman et al. 1995b). In the meantime, enzyme linked immunosorbent assays utilizing purified virus, recombinant antigen, or synthetic peptides are also available. The validity of these assays, especially when it comes to the crucial question of seroprevalence in persons not at risk for KS, is still under debate (Pellett et al. 2003). This is essentially due to the high interassay variability observed with low-titered sera from non-KS patients. However, as justified as it may be, this debate should not obscure three important findings: firstly, the prevalence of (high) anti-KS antibody titers correlates very well with KS. This is true both when various AIDS-transmission groups are compared within one geographic area, and when areas of low KS prevalence (e.g. Northern Europe or the United States) are compared with regions where KS is endemic, e.g. Mediterranean Europe or parts of Africa (Gao et al. 1996b; Kedes et al. 1996; Miller et al. 1996; Simpson et al. 1996). HHV-8 seroprevalence is in the range from 0 to 5 percent in the general population of northern Europe and the United States. The increased incidence of Kaposi sarcoma in Italy is reflected by a higher HHV-8 seroprevalence, especially in southern parts of Italy, including Sicily and Sardinia. Depending on the geographic area, HHV-8 seroprevalence in Italy can be as high as 45 percent (Santarelli et al. 2001), with an average of about 12 percent in Mediterranean countries. In sub-Saharan Africa, however, where KS has been described as the most frequent malignancy even before the beginning of the AIDS epidemic, HHV-8 seroprevalence is usually above 60 percent. Second, HHV-8 seroconversion or at least a marked increase in anti-HHV-8 antibody titer precedes KS development in homosexual AIDS patients (Gao et al. 1996a; Martin et al. 1998). Third, high anti-HHV-8 antibody titers were also associated with the number of homosexual partners and a history of other sexually transmitted diseases (Martin et al. 1998). In summary, serological data largely agree that the titer of anti-HHV-8 antibodies reflects KS or the risk of KS development. Although some uncertainty still remains with the current serological assays and reactivation of HHV-8 cannot be excluded as the reason for the markedly increased anti-HHV-8 titers in KS patients, the high levels of antibodies in groups at risk of KS and seroconversion preceding the onset of the disease clearly hint at a pathogenetic role of HHV-8 infection in KS.

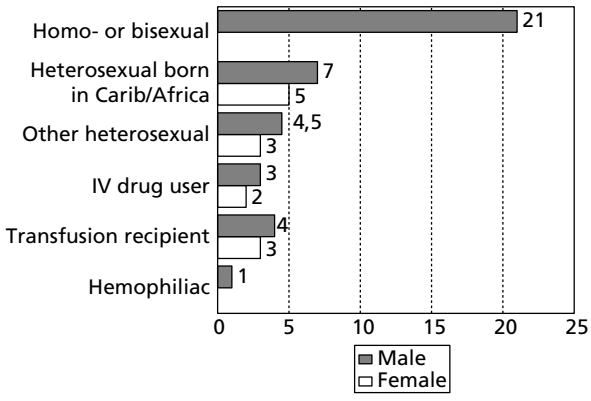

Figure 3 *Percentage of AIDS patients with Kaposi's sarcoma by HIV transmission group. The data are taken from Beral et al. (1990). A total of 90 990 persons with AIDS were included in the study. Whereas 21 percent of the HIV-infected patients in the homo- or bisexual transmission group developed KS, only 1 to 4 percent of patients infected via blood or blood products suffered from this tumor.*

CLINICAL MANIFESTATIONS

Primary infection

Like other herpesviruses, HHV-8 is usually entirely benign under normal conditions. Nonsexual transmission is most likely predominant in areas of high HHV-8 prevalence, e.g. southern European and African countries, and the virus has been detected in the saliva of children seropositive for HHV-8, making it likely that transmission via saliva is a main route in this age group.

However, modes and routes of transmission in areas of low HHV-8 prevalence are less clear. The epidemiology of AIDS-associated KS in these areas suggests transmission in the context of sexual activity. Among men, HHV-8 seroprevalence correlates with the number of homosexual partners and is a risk factor for KS development (Martin et al. 1998). Primary infection is usually asymptomatic but may be associated with a febrile illness and maculopapular rash in children (Andreoni et al. 2002). After primary infection, life-long latency is likely established in memory B cells which becomes only manifest as a disease if the virus–host balance is severely disturbed, e.g. by immunosuppression. In the latter case, three malignant diseases are clearly associated with HHV-8 infection: Kaposi's sarcoma, primary effusion lymphoma, and multifocal Castleman disease.

Kaposi's sarcoma

EPIDEMIOLOGY

Kaposi's sarcoma occurs in four epidemiological variants. The classical form afflicts mainly elderly men of southeastern European descent. It was described in 1872 by Moriz Kaposi as 'sarcoma idiopathicum multiplex hemorrhagicum.' The incidence of classic KS in northern Europe is less then 1/100 000 total population, and about ten-fold higher in endemic regions of southern Europe. The male/female ratio of classical KS is about 10/1. African endemic KS had already been described before the advent of the human immunodeficiency virus. The prevalence of African endemic KS is as high as 12 percent in certain areas of sub-Saharan Africa (Wabinga et al. 2000). Iatrogenic KS occurs in therapeutically immunosuppressed patients. The incidence of post-transplant KS correlates with the general HHV-8 seroprevalence. It is thus more frequent in southern Europe and the Arabian peninsula, where as much as 5 percent of kidney transplant recipients may develop KS (Lesnoni La Parola et al. 1997; Qunibi et al. 1988). The unusual distribution of AIDS-associated KS, the fourth epidemiological form, triggered the search for an infectious agent other than HIV in KS (Beral et al. 1990). At the beginning of the AIDS epidemic, up to 30 percent of male homosexual HIV patients developed this unusually aggressive form of KS, in contrast to only 1 percent of age- and sex-matched HIV-infected patients with hemophilia.

SYMPTOMS AND SIGNS

Initially, KS lesions present as pink or red macular eruptions that progress to plaques and nodules, which subsequently frequently ulcerate. The lesions are often accompanied by lymphedema, which, especially at the nodular and ulcerating stage, cause discomfort and pain. From the very beginning, the clinical presentation of all forms of KS is characterized by a multifocal, often symmetric distribution of the lesions. The most important predilection site is the skin, especially the skin of the lower limbs. However, lymph nodes and viscera are involved in at least 50 percent of cases, even in the more benign classic KS. The disease may afflict any organ with the apparent exception of the central nervous system. The progression from patch to plaque and nodule may take several years in classic KS, and single lesions may even disappear spontaneously. Classic KS is a chronic disease that progresses slowly over many years. Mean survival time of classic KS is 10–15 years and many patients die of an unrelated cause. Other forms of KS, however, are much more aggressive and may progress quickly, often within a few month. This holds particularly true for AIDS-associated KS (Figure 4). A standardized staging system for KS has been developed by the AIDS Clinical Trial Group (Krown et al. 1997). African endemic KS occurs in at least three different clinical pictures: a more slowly progressing form in young adults that resembles European classic KS, an aggressive cutaneous form with frequent visceral involvement that is fatal within 5–7 years, and a very aggressive lymphadenopathic form that occurs in young children (mean age, 3 years; male/female ratio, 3/1).

PATHOLOGY

Although Kaposi's sarcoma occurs in four distinct epidemiological forms which also differ in clinical course and prognosis, all forms share the same histopathology (Figure 2) and can be considered one disease entity.

At least in its early stages, KS is not a typical sarcoma. It develops in a polyclonal fashion. Multiple lesions appear simultaneously, often with an almost symmetrical distribution which is not compatible with metastatic tumor growth. In contrast to other sarcomas, classic KS may wax and wane over decades, and single lesions may disappear completely. This was also observed for the more aggressive AIDS-associated form of KS, where complete remissions were seen during antiretroviral combination therapy (Lebbe et al. 1998). Although molecular data on the clonality of several KS lesions in a single patient are conflicting, it appears that, at least in its early stages, KS is not a monoclonal tumor (Delabesse et al. 1997; Gill et al. 1997). However, late nodular lesions may eventually progress to true sarcoma as indicated by clonality (Gill et al. 1998; Rabkin et al. 1997). The histological picture of (early) KS lesions is not typical for other sarcomas. The lesions contain many different cell types. The so-called KS spindle cells are considered the tumor-defining, malignant cell type of the disease. KS spindle cells are thought to be derived from lymphatic endothelial cells (Weninger et al. 1999). However, they are not the dominant cell type until late stage disease. KS lesions begin as subtle neovascularization (Ruszczak et al. 1987a), and the pathognomonic spindle cells are not yet detectable. In addition, KS

Figure 4 *AIDS-associated Kaposi's sarcoma. One of the first AIDS patients seen in New York in 1981. Initially, the 36-year-old man presented with only a few macular KS lesions on his face and oral mucosa, and there were no lesions seen on his back* **(a)**. *A follow-up photograph of the same patient was taken 6 months later* **(b)**. *It illustrates the rapid development of disseminated Kaposi's sarcoma plaques in immunosuppressed patients (from Friedman-Kien,* Color atlas of AIDS, *with permission).*

lesions contain infiltrating inflammatory cells and multicentrically arising vascular endothelia forming capillaries and other vessels (Ruszczak et al. 1987b). The proliferation of cultured spindle cells is highly dependent on inflammatory cytokines and, with a few exceptions, KS spindle cells are not tumorigenic in nude mice (Nakamura et al. 1988; Salahuddin et al. 1988).

THERAPY

Treatment of Kaposi's sarcoma depends not only on the extent of the tumor and organ involvement, but especially on the type of KS (Hengge et al. 2002). A staging system has been provided by the AIDS Clinical Trials Group (Krown et al. 1997). Classic KS is initially treated with local therapy (excision of single lesions, radiation, intralesional and topical chemotherapy using vinblastine or vincristine). However, the lesions usually reappear. Systemic chemotherapy is used when classic KS is more advanced, disseminated, and symptomatic. The more aggressive forms of African KS (except lymphadenopathic KS in children) also respond well to systemic chemotherapy. Cytotoxic agents used in KS chemotherapy include liposomal doxorubicin, bleomycin, vinca alcaloids, and paclitaxel. Since it is known that HHV-8 is most likely required albeit not sufficient to cause KS, specific antiviral treatment has also been used in KS therapy. HHV-8 replication can be inhibited with foscarnet and ganciclovir in cell culture systems. In one study, a 75 percent reduction in the rate of new KS was seen after high-dose ganciclovir treatment for cytomegalovirus retinitis (Martin et al. 1999). However, data on the treatment of pre-existing KS lesions with ganciclovir or foscarnet are less consistent. The use of interferon-α seems to be more promising. A reduction in up to 30 percent of individuals has been reported following the therapy with high doses of interferon-α (Krown 2001). The therapeutic efficacy of interferon-α is not only due to its antiviral activity. Class I interferons have also been shown to reduce cell proliferation and angiogenesis. Iatrogenic KS usually regresses after reduction of immunosuppressive therapy. However, when immunsuppressive therapy is reinitiated, KS lesions often reappear. Thus, chemotherapy is often used in more disseminated disease. Local or systemic therapy does not increase the survival time of patients with AIDS-KS. The most effective treatment for AIDS-KS is highly active antiretroviral therapy (HAART) (Cattelan et al. 2002). Reconstitution of the immune system by HAART frequently results in complete remission of AIDS-KS.

PRIMARY EFFUSION LYMPHOMA

Primary effusion lymphoma is a rare non-Hodgkin B-cell lymphoma which is primarily seen in immunosuppressed patients. In contrast to KS, which has features of both a hyperproliferative inflammatory lesion and malignant neoplasia, PEL represents a more typical, monoclonal neoplastic process. The name primary effusion lymphoma or body-cavity-based lymphoma (BCBL) indicates that the disease manifests by pleural, pericardial, or peritoneal effusion, often without a solid tumor. Primary effusion lymphomas regularly contain HHV-8, and 50 percent of the cases are also positive for Epstein–Barr virus. Whereas HHV-8 is invariably

present in PEL, it has not been found in any other lymphoma (Cesarman et al. 1995a). PEL cells do not express B- or T-cell antigens. It is indicated by immunoglobulin rearrangement, lack of Bcl-6 expression, and expression of syndecan-1 that PEL cells are derived from post-germinal B cells. AIDS-associated PEL is usually highly aggressive with a mean survival from diagnosis of 5 to 7 months. Several permanent cell lines have been established from PEL cases. The HHV-8 genome is maintained in cultured PEL cells in a latent state with only a few genes being expressed. However, lytic replication can be induced in cultured PEL cells by chemicals such as tetra-phorbol acetate or sodium butyrate. Thus, cell lines derived from PEL are at present an invaluable tool in KS research.

MULTIFOCAL CASTLEMAN'S DISEASE

Multifocal or multicentric Castleman's disease is an atypical lymphoproliferative disorder. The plasma cell type, but not the hyaline vascular type, of MCD is associated with HHV-8 infection and epidemiologically related with both AIDS-associated and classic KS. However, in contrast to both KS and PEL, even the plasma cell type of MCD is not invariably positive for HHV-8. This may indicate that MCD represents a diverse group of lesions. Clinically, MCD is characterized by generalized lymphadenopathy, hypergammaglobulinemia, and fever. Histological examination of affected lymph nodes reveals large follicles, expanded germinal centers separated by vascular lymphoid tissue. HHV-8 harboring cells in MCD resemble immunoblasts which reside in the mantle zone of B-cell follicles. These isolated immunoblasts may eventually form monoclonal, microscopic lymphomas (Dupin et al. 2000), and monoclonal plasmablastic lymphoma may develop in MCD patients. The plasmablastic, HHV-8 associated variant of MCD is frequently resistant to systemic chemotherapy and lethal. Systemic symptoms of MCD have ben shown to correlate with increased serum levels of interleukin-6. Treatment with both anti-IL-6 antibodies, as well as an antibody directed against the interleukin-6 receptor efficiently reduces fever, fatigue, and lymphadenopathy. Whereas HAART has clearly reduced the incidence of KS and PEL, an increase in fatal MCD has been observed following highly active antiretroviral therapy (Zietz et al. 1999).

MODELS OF PATHOGENESIS

Consistent detection of viral DNA in tumor tissue is not sufficient to prove that a virus is involved in oncogenesis. In theory, different roles are possible for HHV-8 in KS pathogenesis (Neipel et al. 1997c), ranging from a mere 'passenger model,' where HHV-8 would be an innocent bystander, to the role of a directly transforming virus. Several lines of evidence strongly suggest that HHV-8 plays an essential role in KS pathogenesis: the virus is invariably present in KS, HHV-8 infection precedes KS development, the virus is present in the tumor cells themselves, and it is rather infrequently found outside the population at risk of KS. Moreover, HHV-8 belongs to a group of herpesviruses known to be oncogenic. It is difficult, however, to assess the pathogenic potential of HHV-8 more reliably without identification of the genes and mechanism involved in pathogenesis. Research on HHV-8 pathogenesis is hampered by the lack of a cell culture system for transformation as it exists for EBV or herpesvirus saimiri. In addition, several attempts have been made to establish animal models for HHV-8 proliferative diseases. Unfortunately, infections of various primates did not result in overt disease. Studies using rodents transgenic for single HHV-8 genes were more successful. However, results from such experiments must be interpreted with great caution, no matter how tempting the resemblance of lesions in mice transgenic for vIL8R to KS may be (Yang et al. 2000). In summary, several genes with oncogenic potential have been identified in cell culture transformation assays or transgenic animals, and various models were developed. However, statements on their relevance in KS, PEL, and MCD remain speculative to date.

At least two concurring models exist for the role of HHV-8 in KS pathogenesis. The 'cytokine model' emphasizes the role of inflammatory cytokines induced or produced by HHV-8. A closer look at the clinical course and histopathology of KS raises doubts about the relevance of 'classical,' transforming genes for the pathogenesis of this peculiar tumor. The peculiar pathology of KS hints at a more complex, indirect mechanism of pathogenesis. Based on clinical observations and data derived from cell culture systems, models of KS pathogenesis were developed before HHV-8 was discovered. Several groups agreed upon the notion that KS develops as an interplay of inflammatory cytokines and angiogenic factors (Ensoli and Stürzl 1998; Samaniego et al. 1998), although the cytokines focused on various different reports. Amongst them are basic fibroblast growth factor, vascular endothelial growth factor (VEGF), IL-6, tumor necrosis factor (TNF)α and β, and oncostatin M. Interestingly, HHV-8 encodes or induces several cytokines with intriguing similarity to the cellular factors shown to be required for in vitro models of KS. An example is VEGF, which is secreted by cells expressing the constitutively active vIL-8R. This leads to a model of KS pathogenesis, where increased secretion of both viral and cellular cytokines, the latter in part induced by HHV-8, promote inflammatory infiltrates (vMIP, vIL-6), angiogenesis (vMIP, vIL-8R), and enhance spindle cell proliferation (vIL-6, vIL-8R via VEGF). The reliable presence of HHV-8 in the spindle cells points to additional factors beyond those induced through the 1–3 percent productively infected cells, voting for a role more compatible with a typical 'onco-

genic transformation' model by latently expressed viral genes.

Starting from the close relationship to EBV and herpesvirus saimiri, it is intriguing to assume that transmembrane proteins mimicking constitutively active receptors mechanisms – similar to those identified in EBV and herpesvirus saimiri – might be relevant for HHV-8 pathogenesis. At first sight, HHV-8 genes K1 and K15 might fall into this category. However, attempts to detect expression of these genes in latently infected tumor cells remained unsuccessful, voting against a role of K1 and K15 in transformation. Only few viral genes were found expressed in latently infected cells. These include the viral interferon regulatory factor K10.5, LANA-1, the viral cyclin D homologue, and possibly vFLIP. Although clearly not considered a common rhadinoviral oncogene (Ensser et al. 2001), the viral k-cyclin may be such a factor in KS. It is transcribed in the majority of latently infected tumor cells, and deregulation of the G1/S checkpoint is a feature common to various malignancies and human tumor viruses. k-Cyclin has been shown to overcome arrest in G1 and to force progression of the cell cycle. One physiological function of the restriction point in G1/S is to block cell division in the case of DNA damage. This is usually achieved through the activation of p53 which in turn results in an increased synthesis of cdk inhibitors such as p21. However, the k-cyclin/cdk6 complex is resistant to inhibition by several cdk inhibitors (Swanton et al. 1997). Thus, k-cyclin may not only be responsible for increased proliferation of HHV-8-infected endothelial and spindle cells, but sustained expression of k-cyclin may also result in the unrestricted acquisition of somatic mutations, eventually resulting in the malignant phenotype and monoclonality associated with advanced stage disease. As described above, LANA-1 seems to be a multifunctional protein. It is not only required for maintenance of the viral episome, but also in transcription regulation and cell cycle control possibly contributing to cell transformation. The role of K10.5 remains speculative. Its similarity to the cellular genes IRF-4 and interferon consensus sequence binding protein suggests that it may play a role in lymphocyte development.

Thus, KS may result from a complex interplay of both viral and cellular cytokines and angiogenic factors, induced by a sustained inflammatory reaction initiated by up to 3 percent productively infected cells. The viral cyclin and perhaps other latency-associated proteins, such as LANA-1, might further enhance the proliferation of KS cells and favor the development of truly malignant cells by indirect means, e.g. the reduced control of accidental DNA damage. As KS is an unusual malignancy, resembling hyperplastic, angioproliferative inflammatory changes rather than true sarcoma, such a multistep/multifactorial model might be more compatible with KS pathogenesis than classical transformation models by viral oncogenes (Stürzl et al. 2001), as described for EBV, herpesvirus saimiri, and possibly for HHV-8-associated B-cell malignancies (PEL).

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Gammaherpesviruses: Epstein–Barr virus

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PROPERTIES OF THE VIRUS

INTRODUCTION

The Epstein–Barr virus (EBV) was discovered in 1964 (Epstein et al. 1964) in the course of a sustained search (Epstein 1999) for a possible causative virus in tumor samples from cases of endemic (African) Burkitt's lymphoma (BL). EBV is a very ancient parasite of humans. The fact that EBV-like herpesviruses have been known for many years in Old World apes and monkeys (Deinhardt and Deinhardt 1979) and have now recently been found in New World monkeys (Cho et al. 2001, Wang et al. 2001) indicates that such viruses were already infecting early primates some time before the Old World/New World evolutionary split took place, about 35 Myr ago based on palaeontology (McGeogh and Cook 1994) or 45 Myr ago based on more recent DNA sequence data (Kumar and Hedges 1998). EBV infects almost all of the world's adult population and, like all human herpesviruses, establishes lifelong persistence. However, despite the trivial nature of the infection in most individuals, the virus is the most potent immortalizing agent for mammalian B cells in vitro, is associated with a spectrum of human malignant proliferations in vivo, and causes rapidly fatal malignant tumors on experimental injection in cottontop tamarins.

EBV has a restricted host range, with humans as its natural host, but certain nonhuman primates can be infected experimentally. The virus is B lymphotropic in vivo and readily infects B lymphocytes in vitro (Pattengale et al. 1973), resulting in B-cell immortalization and the generation of continuously proliferating lymphoblastoid cell lines (LCL) (Pope et al. 1968). EBV also infects squamous epithelial cells in certain pathological conditions in vivo (see later under Nasopharyngeal carcinoma and Oral hairy leukoplakia), and such cells can support full virus replication. However, in vitro infection of primary epithelial cells is problematic, and thus no fully permissive system is available for the study of individual replicative gene functions, in contrast to latent infection, which has been extensively investigated because of the ease with which immortalization of B cells can be brought about.

CLASSIFICATION

EBV is a member of the *Herpesviridae* and is in the *Gammaherpesvirinae* subfamily, genus *Lymphocryptovirus*. This classification is based on DNA homology between the EBV genome and that of other gammaherpesviruses, the G+C content of the genome and the lymphotropism of the virus.

MORPHOLOGY AND PARTICLE STRUCTURE

Like other herpesviruses, EBV has a protein core containing DNA, a nucleocapsid composed of 162 capsomers that forms an icosahedral protein shell, and

an outer envelope derived from cellular membranes and containing virus-coded glycoprotein spikes.

MOLECULAR BIOLOGY

Genome structure and function

The EBV genome is a linear molecule of double-stranded DNA that contains 184 kb (www.med.ic.ac.uk/ludwig/EBV.htm) and has coding potential for about 70 proteins. EBV DNA contains several internal repeats interspersed with unique sequences and flanked by two terminal repeat sequences (Figure 1). EBV was the first herpesvirus to be cloned (Dambaugh et al. 1980) and sequenced (Baer et al. 1984). The nomenclature for the promoters and open reading frames within the viral genome has been assigned using the abbreviated name of the *Bam*HI restriction fragment: for example, BARF1 is the first, rightward open reading frame in the *Bam*HI A fragment of the EBV genome.

Two types of EBV exist, 1 and 2 (also called A and B) (Dambaugh et al. 1986), with an overall DNA sequence homology of 70–85 percent (Sample et al. 1990). They differ in the regions of the viral genome coding for the Epstein–Barr virus nuclear antigens (EBNA) 2, 3A, B, C (Rowe et al. 1989) and the Epstein–Barr virus-encoded small RNAs (EBER) (Arrand et al. 1989). These differences are reflected in biological diversity, type 1 viruses being more efficient than type 2 at immortalization of B lymphocytes in vitro (Rickinson et al. 1987). Type 1 EBV predominates worldwide. Type 2 is rare in western societies but more common in African communities. Dual infection may occur in individuals with immunosuppression (Zimber et al. 1986; Sculley et al. 1990). However, no type-specific disease associations have been noted. In addition to EBV type variation, individual viral isolates differ in the number of tandem repeats in their internal DNA repeat sequences. This heterogeneity can be used to define individual EBV isolates (Falk et al. 1995).

Latent infection of a B lymphocyte is associated with circularization of the EBV genome to form an episome (Lindahl et al. 1976), which later amplifies to give multiple copies. Genome circularization is achieved by covalent linkage of the terminal repeat elements, and the number of repeat sequences varies with different circularization events. Thus, when the viral episome is passed from one latently infected cell to a daughter cell (without linearizing) the number remains the same, indicating viral clonality. Estimation of the terminal repeats can therefore be used to determine whether virus infection of cells occurred before or after cell proliferation in tumor samples (Raab-Traub and Flynn 1986).

Virus-coded proteins

On infection of B lymphocytes in vitro, EBV establishes a latent type of infection with the expression of a restricted number of viral genes (latent genes) and continued cell survival and division without the production of viral progeny. The characteristics of these latent genes and their products are described below and listed with their alternative names in Table 1; the locations of their open reading frames are shown in Figure 1. This type of infection can be switched to a lytic infection with new virus production and cell death by certain stimuli that induce B-cell activation/differentiation and thereby cause the cell to become permissive for viral replication (Crawford and Ando 1986).

LATENT PROTEINS

The latent gene products include six EBNAs (1, 2, 3A,B,C leader protein (LP)) and three membrane proteins (latent membrane proteins (LMP) 1, 2A, B). In addition, two small RNAs (EBERs 1 and 2) are transcribed by RNA polymerase III but are not translated. The EBNAs initiate from promoters in either the W or the C fragments of the genome as long polycistronic messages which are subsequently spliced to give indivi-

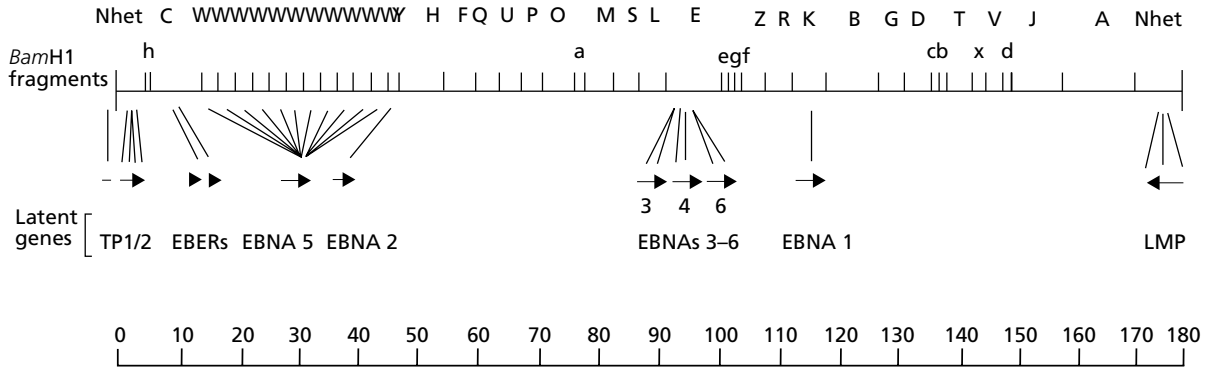

Figure 1 *Linear representation of the Epstein–Barr virus genome.* Bam*HI fragments are shown above and the location of the latent gene open reading frames is shown below. The arrows indicate the direction of transcription. The scale is in kilobases. EBERS, EB-coded small RNAs; EBNA, EB viral nuclear antigen; LMP, latent membrane protein.*

Table 1 *EBV latent genes*

Open reading frame[a]	Gene product	Alternative nomenclature	Mol. wt (kDa)	Cellular location	Required for immortalization	Suggested functions/ properties
BKRF1	EBNA1	–	65–85	Nucleus	+	Plasmid maintenance
BYRF1	EBNA2	–	86	Nucleus	+	Viral oncogene, transactivator of viral and cellular genes
BLRF3–BERF1	EBNA3A	EBNA 3	140–157	Nucleus	+	Viral oncogene, transcriptional regulator
BERF2a–BERF2b	EBNA3B	EBNA 4	148–180	Nucleus	-	NK
BERF3-BERF4	EBNA3C	EBNA 6	160	Nucleus	+	Viral oncogene, transcriptional regulator
BRWF1	EBNA (LP)	EBNA 5	20–130	Nucleus	+/-	Co-activates EBNA2 responsive genes. Increases efficiency of immortalization
BNLF1	LMP1	LMP	58–63	Membrane	+	Viral oncogene, transactivates cellular genes, mimics ligation of CD40
BARF1/ BNRF1	LMP2A	Terminal protein (TP)1	53	Membrane	-	Mimics BCR to enhance B-cell survival, inhibit B-cell activation and entry into lytic cycle
BNRF1	LMP2B	TP2	40	Membrane	-	NK
BCRF1	EBER1, 2	–	–	Cytoplasm/ nucleus	-	Regulation of PKR activity, inhibits apoptosis
BARF0	*Bam*HI A transcripts		–	Cytoplasm	-	NK

BCR, B-cell receptor; EBNA, EB virus nuclear antigen; LMP, latent membrane protein; LP. leader protein; NK, not known; PKR, double-stranded RNA protein kinase.
a) Abbreviated using the *Bam*HI(B) fragment (A–Z) LF or RF for left- or rightward open reading frame (ORF) followed by the number of the gene in a particular ORF.

dual mRNAs. In contrast, the LMPs each initiate from their own promoters. EBNAs 2, 3A,C and LMP1 have been shown by analysis of recombinant mutant viruses to be essential for immortalization, whereas EBNA3B and LP, LMP2A, B and EBERs 1 and 2 are not (for a review see Bornkamm and Hammerschmidt 2001). In addition to these proteins, complex transcripts arising from the *Bam*HI A open reading frame have been demonstrated in latently infected B cells, some of which may be translated into proteins, but their role in the life-cycle of EBV remains unclear (Smith 2001).

EBNA1

EBNA1 is a highly stable, DNA-binding nuclear phosphoprotein that is coded for by the BKRF1 open reading frame. It has a molecular weight ranging from 65 to 85 kDa, the variation being due to the different lengths of the repeat sequences in the molecule. EBNA1 protein consists of about 641 amino acids, is proline-rich, and contains a glycine–alanine repeat sequence; it binds to a specific DNA sequence by the carboxy terminus domain, which is rich in acidic and basic residues. Multiple EBNA1-binding sites occur in the latent viral origin of replication (*oriP*) domain, where the binding of EBNA1 is the only viral factor required for maintenance of the viral episome (Yates et al. 1985). This binding also enhances transcription of EBNA1 and other latent viral genes because it activates the *oriP* enhancer, which regulates the EBNA promoter, Cp, and the LMP promoter (Gahn and Sugden 1995).

EBNA1 binds nonspecifically to metaphase chromosomes, thereby ensuring the equal partitioning of the viral episomes into the two daughter cells at mitosis. Thus EBNA1 expression is a requirement not only for the maintenance of the viral episome in latently infected cells, but also for the synchronization of virus genome replication with cell division.

Mice expressing EBNA1 as a transgene in B cells develop B-cell lymphoma, suggesting a direct role for EBNA1 in B-cell oncogenesis; however, the mechanism involved in this process is unknown (Wilson et al. 1996).

The glycine–alanine domain of EBNA1 inhibits proteosome-mediated degradation of EBNA1 and thereby prevents EBNA1 peptides being displayed on the cell surface bound to MHC class 1 molecules. Thus EBNA1-expressing cells are not recognized by $CD8^+$ cytotoxic T cells (Levitskaya et al. 1995).

EBNA2

EBNA2 is an acidic, nuclear phosphoprotein with a molecular weight of 86 kDa and is coded for by the BYRF1 open reading frame. The protein is a transactivator of viral and cellular genes and is essential for the immortalization of B lymphocytes in vitro (Cohen et al. 1989). EBNA2 does not bind directly to DNA, but interacts with a variety of cellular factors to induce B-cell activation. Three domains of EBNA2 have been identified as essential for immortalization. The acidic activator domain near the carboxy terminus (amino acids 420–464) recruits basal and activating transcription factors (TF) IIB, CBP/p300, TBP, TAF40, and TFIIH, causing transcriptional activation (Johannsen et al. 1995). The second essential domain between amino acids 180 and 337 interacts with the DNA-binding proteins RBP-Jk (also called CBF1), which is ubiquitous, and PU1, which is expressed in a restricted number of hemopoietic cell types, including B cells (Ling et al. 1994; Johannsen et al. 1995). As a result of these interactions EBNA2 transactivates the latent viral genes LMP1, 2A, 2B and the Cp promoter, which gives rise to EBNAs 1, LP, 2, 3A, B, C transcripts. A number of cellular genes, including the oncogenes c-*fgr* and c-*myc* and the B-cell activation molecules CD21 and CD23, are also upregulated by EBNA2. RBP-Jk naturally interacts with the evolutionarily conserved Notch protein signaling pathways, and EBNA2 appears to exploit these in the immortalization process (Hsieh et al. 1997). The third essential domain in EBNA2 is within the most conserved regions of the gene at the amino and carboxy termini, only one of which is required for immortalization. These regions aid the formation of the homotypic and heterotypic associations necessary for EBNA2 to recruit multiple transcription factors (Tsui and Schubach 1994).

The *EBNA2* gene shows considerable DNA sequence diversity between type 1 and 2 EBV, with an overall homology of about 55 percent.

EBNA3A, B, C

These three hydrophilic nuclear proteins are coded for by the BERF open reading frames and clearly arise from a common origin. Their molecular weights range from 140 to 180 kDa, owing to the variable repeat sequences present in each protein. Expression of EBNA3A and C is required for immortalization, whereas that of EBNA3B is not (Tomkinson et al. 1993). All three proteins are transcriptional regulators and bind to RBP-Jk, effectively competing with EBNA2 binding and thereby regulating its function (Zhao et al. 1996). EBNA3A and 3C can repress transcription when targeted to DNA, and have oncogenic activity in cooperation with mutant *ras* (Touitou et al. 2001; Hickabottom et al. 2002).

EBNA-LP

EBNA-LP is a nuclear phosphoprotein that is encoded by the BWRF1 open reading frame, which forms either the leader sequence of the EBNA mRNAs or, by alternative splicing, EBNA-LP mRNA. Because of the DNA repeats in this part of the genome, EBNA-LP protein consists of multiple repeat sequences of 22 and 44 amino acids flanked by unique amino and carboxy termini. Because the DNA repeat sequences vary in number in different virus isolates, the size of EBNA-LP protein varies accordingly. Immunoblotting of EBNA-LP in electrophoretically separated proteins from EBV-infected B cells reveals a ladder of bands differing by 6–8 kDa and corresponding to products of 20–130 kDa, an effect that is probably due to differential splicing of the mRNA.

The function of EBNA-LP is unknown and it is not strictly essential for immortalization, although it increases growth efficiency of infected B cells (Hammerschmidt and Sugden 1989). The protein contains a domain which coactivates gene transcription with EBNA2, and another that inhibits this effect. However, overall EBNA-LP has an activating role which is essential for efficient immortalization.

LMP1

LMP1 is a cytoplasmic and membrane protein that is coded for by the BNLF1 open reading frame. It has a molecular weight of 58–63 kDa and is phosphorylated on serine and threonine residues. The protein associates with the cytoskeleton in the plasma membrane, appearing as discrete patches on staining. LMP1 mRNA is the most abundant viral transcript found in latently infected B cells. The primary amino acid sequence of LMP1 suggests an integral membrane protein with a short 23 amino acid cytoplasmic amino terminus, a large 200 amino acid cytoplasmic carboxy terminus, and six hydrophobic transmembrane segments forming three external loops. A smaller LMP1 mRNA also exists, coding for a protein that lacks the amino terminus of the full-length protein, is expressed in lytically infected cells, and is present in the virus particle.

Full-length LMP1 is a classic viral oncoprotein that is essential for B-cell immortalization (Kaye et al. 1993). Transfection studies using the *LMP1* gene under a heterologous promoter have shown that it induces loss of contact inhibition and anchorage dependence, altered cell morphology, and tumorigenic potential in rodent fibroblasts (Wang et al. 1985). In human EBV-negative B-cell lines and primary B cells, LMP1 induces the changes associated with immortalization. These include an increase in cell size and steady-state calcium levels, upregulation of cell activation and cell adhesion molecule (CAM) expression, as well as upregulation of the cytoskeletal protein vimentin, CD54, and interleukin-6

(IL-6) (Wang et al. 1988). LMP1 expression protects B cells from cell death, probably through induction of the anti-apoptotic protein A20. LMP1 induces cellular genes by activation of the transcription factor NFκB (Hammarskjold and Simurdo 1992) via an interaction with tumor necrosis factor (TNF)-associated cell cytoplasmic factors (TRAFs). Cell activation by this pathway is similar to that of the B-cell surface molecule CD40, which mediates activation on B-cell-receptor engagement by antigen, and LMP1 can partially mimic this function in CD40 knockout mice (Uchida et al. 1999). Studies with LMP1 deletion mutants show that these effects are mediated by the amino terminus of LMP1. Sequencing studies on *LMP*1 genes from EBV isolates from different geographical locations have identified a common 30-bp deletion in the C terminus of LMP1 as well as several point mutations compared to the prototype B95.8 sequence (Hu et al. 1991). This deletion confers increased tumorigenicity in rodent fibroblasts and epithelial cells (Hu et al. 1993), and has been suggested to be more commonly associated with lympholiferative disorders and nasopharyngeal carcinoma than the undeleted form. However, more recent studies show that the incidence of the deletion in tumors reflects the incidence in the general population in each geographical location (Khanim et al. 1996).

LMP2A, 2B

The sequences that make up the LMP2 open reading frame are located at the two ends of the linear EBV genome in BARF1/BNRF1 and are completed only when the genome has circularized to form an episome (Laux et al. 1988). LMP2A and B are produced by alternative splicing of the mRNA, giving rise to proteins of 53 and 40 kDa, respectively. Neither is essential for immortalization of B cells in vitro. Both are hydrophobic membrane proteins with 12 transmembrane segments and 27 amino acid carboxy termini, but whereas LMP2A has 119 amino acids in the amino terminus domain LMP2B has only three. Both LMP2A and B localize to patches in the plasma membrane, and LMP2A colocalizes with LMP1. The amino terminus of LMP2A contains an immunoreceptor tyrosine-based activation motif (ITAM) similar to that found in the B-cell receptor (BCR) molecule, where the phosphorylated motif plays a central role in activation and differentiation processes through its interaction with src and Syk protein tyrosine kinases (PTK). LMP2A associates with these PTKs through its ITAM (Longnecker et al. 1991), and this interaction inhibits calcium mobilization following BCR stimulation of B lymphocytes latently infected with EBV. In this way LMP2A may prevent fortuitous activation of the viral lytic cycle (Miller et al. 1994). LMP2A transgenic mice show abnormal B-cell development, with survival of immunoglobulin negative B cells in peripheral lymphoid tissue (Caldwell et al. 1998). Thus it seems possible that LMP2A can promote B-cell proliferation and survival in the absence of BCR signals, and thereby promote long-term EBV latency in B cells.

The function of LMP2B is not known, but it may play a role in regulating LMP2A function.

EBERs 1 and 2

These two small nonpolyadenylated RNA molecules are coded for by the BCRF1 open reading frame and are the most abundant RNAs in latently infected B cells (10^4–10^5 copies per cell). They are localized mainly in the nucleus, where they bind to the La protein and the large ribosomal subunit protein L22 (Clemens 1994). Although the significance of this complex formation is not known, EBERs show sequence homology to the VA1 and 2 RNAs of adenovirus, which also bind La and are thought to be involved in RNA splicing. EBERs are not required for the in vitro immortalization process or for virus replication (Swaminathan et al. 1991) but, like VA1, bind to and regulate the activity of the protein kinase PKR. This interaction may inhibit the antiviral and antiproliferative effects of interferon, because PKR is induced by, and mediates, interferon activity (Clemens 1994).

Recently EBER expression has been shown to increase the tumorigenicity of Burkitt's lymphoma cell lines by upregulating the expression of Bcl-2 (Komano et al. 1999), but the significance of this finding to BL pathogenesis has not so far been elucidated.

LYTIC PROTEINS

Genes expressed during the EBV lytic cycle have been characterized in LCL that have been induced by various agents (zur Hausen et al. 1978; Luka et al. 1979; Tovey et al. 1978); such genes have extensive homology with those of other herpesviruses. Like other herpesviruses they follow an orderly sequence of expression, each set being activated by the previous set. Lytic genes can be divided into immediate–early genes which are transcribed prior to viral protein synthesis; early genes which are transcribed in the absence of viral DNA synthesis; and late genes which represent the structural proteins and are expressed only after viral DNA synthesis. However, EBV is unique among herpesviruses in possessing a set of latent genes that are already transcribed in infected B cells and may therefore influence subsequent lytic gene expression, although a viral transactivator of the immediate early genes has not been identified among them.

Immediate–early gene products

EBV possesses two genes that can be classified as immediate–early genes: *BZLF1* (Z) and *BRLF1* (R). These two genes code for nuclear proteins which are

DNA sequence specific acidic transactivators and are derived from three mRNA species, one of which contains both R and Z sequences. R and Z coordinately upregulate expression of the early genes by binding to specific sites in the promoter regions. The early promoter elements in the duplication left (DL) and right (DR) regions of the EBV genome, which also include the origin of lytic viral DNA replication (orilyt), contain multiple Z binding sites, and code many early transcripts, including *BHRF1* and *BHLF1* mRNAs.

Z is a DNA-binding protein that shows homology to the DNA-binding domain of the c-*jun* oncogene and binds to AP-1 sites (Farrell et al. 1989). The Z and R promoters (Zp, Rp) contain AP-1 sites and so Z transactivates its own activity as well as that of R. Z interacts with NF-κB and p53 in vivo (Gutsch et al. 1994), and these interactions are thought to inhibit p53-mediated apoptosis during lytic replication and prevent toxic levels of Z accumulating late in the lytic cycle.

Two of the early EBV nuclear proteins (BSMLF1 and BMRF1) may also cooperate in the transactivation of the early genes.

Early gene products

First identified by the staining pattern of sera containing antibodies to EBV when applied to the Raji cell line, which lacks expression of the late genes, the early gene products (early antigens (EA)) were characterized as diffuse (D) (nuclear and cytoplasmic staining) and restricted (R) (nuclear staining) (Henle et al. 1971). It is now known that these complexes consist of approximately 30 proteins, most of which have enzymic functions required for viral DNA replication. Many of these were identified by their homology with early genes from other herpesviruses (Table 2).

Late gene products

Viral capsid proteins This antigen complex (VCA) consists of the nonglycosylated viral structural proteins. They have not yet been analyzed in detail because of the lack of a fully lytic system for EBV in vitro. The major capsid protein is coded for by the BCLF1 open reading frame, other components possibly arising from BNRF1 and BXRF1.

BCRF1 is a gene expressed only late in the lytic cycle that shows ca. 90 percent amino acid sequence homology with the human *IL-10* gene. The protein has been shown to have similar properties to human IL-10, and probably acts to reduce T- and NK-cell responses on initial infection and reactivation in vivo.

EBV glycoproteins The EBV-coded glycoproteins are involved in viral infectivity and spread and show extensive homology with herpes simplex glycoproteins (Table 3). Ten have been identified, most of which are inserted into the membranes of the infected cell, and several become components of the viral envelope (membrane antigens (MA)).

The major viral envelope glycoprotein, gp340/220, is coded for by the BLLF1 open reading frame. The two forms of the protein (molecular weights 340 and 220 kDa) are created by alternative splicing of the mRNA. The protein is found in the Golgi apparatus as well as the plasma membrane of infected cells. The

Table 2 *Early proteins involved in EBV replication*

Open reading frame	Mol. wt (kDa)	Cellular localization	Herpes simplex virus homology/ putative function
BMLF5	–	–	DNA polymerase
BALF5	–	–	DNA polymerase
BXLF1	–	–	Thymidine kinase
BGLF5	–	–	Alkaline exonuclease
BORF1	140	–	Ribonucleotide reductase
BARF1	38	–	Ribonucleotide reductase
BMRF1	47	Nucleus	DNA polymerase-associated protein
BALF2	135	–	Single-strand DNA-binding protein
BBLF4	–	–	Helicase/primase
BSLF1	–	–	Helicase/primase
BCRF2/3	–	–	Helicase/primase
BHRF1	18	Nuclear and cytoplasmic membrane	*bcl-2* homologue
BKRF3	–	–	Uracil DNA glycosylase
BMLF1	–	Nucleus	Transactivator
BBLF2/3	–	–	Spliced primase–helicase complex component

Table 3 *EBV glycoproteins*

Open reading frame	Mol. wt (kDa)	Putative function	Present in viral envelope
BLLF1	340/220	Receptor (CR2) binding. HSV gC functional homologue	Yes
BALF4	110.0	Virulence factor. HSV gB homologue	No
BXLF2	85	Fusion of viral and cellular membranes. Complexes with gp42 and gp25. HSV gH homologue	Yes
BZLF2	42	Binds MHC class II – viral penetration. Complexes with gp85 and gp25. Functional HSV gD homologue	Yes
BKRF2	25	Complexes with gp85 and gp42. HSV gL homologue	Yes
BILF2	78/55	–	Yes
BDLF3	150	–	Yes

HSV, herpes simplex virus.

mature protein is heavily *N*- and *O*-glycosylated, about 50 percent of the molecule being carbohydrate. Gp340/220 mediates virus attachment to the B-cell surface by binding to the EBV receptor CR2 (also called CD21) (Nemerow et al. 1987). Thus both naturally occurring and experimentally induced antibodies to gp340/220 prevent infection by blocking attachment.

Gp85 is coded for by the BXLF2 open reading frame and has homology to the herpes simplex virus glycoprotein gH. Gp25 is required for the correct folding, transport to the cell surface and function of gp85. These proteins are localized to the plasma membrane and viral envelope, where they form a trimolecular complex with gp42. Gp42 is coded for by the BZLF2 open reading frame and is involved in penetration of the virus through the cell membrane during B-cell infection by its binding to HLA class II molecules on the B-cell surface. This glycoprotein is not required for viral entry into epithelial cells that are HLA class II negative (Borza and Hutt-Fletcher 2002).

Gp110 is coded for by the BALF4 open reading frame and has homology with the herpes simplex virus glycoprotein gB. It is *O*- and *N*-glycosylated and is localized to the nuclear and cytoplasmic membranes of infected cells. Gp110 has an important role as a virulence factor; high levels of expression can determine infection of cells other than B lymphocytes (Neuhierl et al. 2002).

Immortalization

Binding of EBV to B lymphocytes occurs through a ligand–receptor interaction between the major viral envelope glycoprotein gp340/220 and the cell surface complement receptor molecule CR2 (CD21) (Nemerow et al. 1987). CR2 is a member of the immunoglobulin superfamily and is expressed on all mature circulating B lymphocytes. It forms part of a signal transduction complex including CD19, TAPA1, and Leu-13 (Bradbury et al. 1992); it is postulated that the binding of EBV to CR2 induces initial B-cell-activating signals, but these have not yet been clearly defined. Once bound, the trimolecular complex of gp85, 42, and 25 on the viral envelope initiate fusion with the cell membrane, releasing the capsid into the cytoplasm. The viral genome rapidly enters the cell nucleus where it circularizes to form an episome, which later amplifies to give multiple copies per cell.

EBNAs 2 and LP, originating from the W promoter (Wp), are the first virus-coded proteins to be detected, at around 12–15 h post infection. EBNA2 protein then generally initiates a promoter switch from Wp to Cp (Woisetschlaeger et al. 1991) by complexing with RBP-Jk at the EBNA2 response element upstream of Cp, thereby allowing expression of all the EBNA proteins by 24 h. EBNA and LP also upregulate the LMP promoters, so that full latent viral gene expression is achieved by 48 h post infection.

Cell enlargement and activation with the expression of the B-cell activation marker CD23 occur at around 48 h post infection and can be attributed mainly to the action of LMP1 (Wang et al. 1990). By 72 h some cells have progressed through the G_1, S, and G_2 phases of the cell cycle and are entering mitosis. The cells then continue to cycle indefinitely, giving rise to an immortalized LCL.The phenotype of cells in an LCL is similar to thatof antigen-stimulated lymphoblasts, with the expression of the B-cell-activation markers typical of this stageof B-cell differentiation (e.g. CD23, CD39, CD30, CD70, ki24) as well as the CAMs, CD11a, CD54, and CD58.

A few cells in an LCL (0–5 percent) enter the lytic cycle, a switch that seems to be triggered by physiological stimuli such as differentiation of the cell (Crawford and Ando 1986), and which can be induced by B-cell-activating agents such as phorbol esters (zur Hausen

et al. 1978), sodium butyrate (Luka et al. 1979), and anti-immunoglobulin (Tovey et al. 1978). Cells of LCLs are immortal but cytogenetically normal. They are not, by definition, transformed cells because they do not form colonies in soft agar or give rise to tumors in nude mice (Nilsson et al. 1971). These characteristics differ from those of EBV-carrying cell lines derived from BL biopsy material (see EBV gene expression).

EBV replication

The induction of the lytic cycle in B lymphocytes or squamous epithelial cells infected in vitro is associated with differentiation of the cell (Crawford and Ando 1986; Kirimi et al. 1995), suggesting that the availability of cellular transcription factors determines the outcome of infection. In addition, the lytic cycle can be activated by transfection of the *BZLF1* gene or superinfection with the P3HR1 strain of virus, which contains defective virus in which *BZLF1* is adjacent to Wp (Miller et al. 1984).

Replication of EBV DNA begins at the origin of lytic replication, which is 1.4 kb in length and contains many inverted repeat sequences (Hammerschmidt and Sugden 1988). *ori*lyt acts as an amplicon, giving rise to many linear concatemers of the genome by the rolling circle method, and EBV-coded DNA polymerase is required for this process. The terminal repeat elements are required for cleaving and packaging the newly formed virions.

EBV gene expression

Infection of B lymphocytes in vitro leads to initiation of transcription of the polycistronic EBNA mRNA from Wp with an EBNA2-induced switch to Cp after 48–72 h (Woisetschlaeger et al. 1991). This results in expression of all the EBNA proteins as well as expression of the three latent membrane proteins from their own promoters. In addition, EBERs and *Bam*H1 A transcripts are detectable, and this gene expression pattern is termed full latent gene expression, or latency type III (Rowe et al. 1992). However, in vivo, other patterns of viral gene expression have been identified. In BL cells, which are small, nonactivated cells expressing the cellular markers of a germinal centre B cell (CD10, CD77), only EBNA1 protein, EBERs and *Bam*H1 A transcripts are detected (latency type I) (Rowe et al. 1986). The EBNA1 mRNA initiates from a promoter in the Q region of the genome (Qp). Wp and Cp are silent and have been shown to be methylated (Masucci et al. 1989). However, when BL cells are cultured, demethylation often occurs, associated with a drift to full latent viral gene expression and a lymphoblastoid phenotype (Rowe et al. 1987).

An alternative pattern of transcription (latency type II) is seen in Reed–Sternberg cells in EBV-positive Hodgkin's disease (Deacon et al. 1993) and the malignant epithelial cells of nasopharyngeal carcinoma (Brooks et al. 1992). Here EBNA1 is expressed from Qp, and LMP1 and 2 are also expressed in the absence of the EBNAs. EBERs and the *Bam*H1 A transcripts are again detectable (Table 4).

Viral gene expression in circulating latently infected memory B cells in vivo is a controversial issue, as RT-PCR is the only technique with sufficient sensitivity to detect these rare cells. This technique does not give an indication of the genes expressed in individual cells or determine whether translation to protein occurs; however, the most regularly detected transcripts are EBERs and LMP2.

CLINICAL AND PATHOLOGICAL ASPECTS

INTRODUCTION

As a very ancient parasite of humans, EBV has established a delicately balanced relationship with its host that is almost entirely benign under normal conditions. Natural primary infection is usually inapparent, and is always followed by a lifelong, silent carrier state that becomes manifest as disease only if the virus–host balance is disturbed (Table 5).

EPIDEMIOLOGY

EBV is found in all human populations and, under natural conditions, primary infection occurs in early

Table 4 *Latent viral gene expression in EBV-associated tumors*

	EBNA						LMP				
Tumor	1	2	3A	3B	3C	LP	1	2A	2B	EBERs	EBNA promoter usage
Burkitt's lymphoma	+	–	–	–	–	–	–	–	–	+	Qp
B lymphoproliferative disease	+	+	+	+	+	+	+	+	+	+	Cp/Wp
Nasopharyngeal carcinoma	+	–	–	–	–	–	+/–	+	+/–	+	Qp
Hodgkin's lymphoma	+	–	–	–	–	–	++	+	+	+	Qp

EBNA, EB viral nuclear antigen; EBERs, EB virus-coded small RNAs; LMP, latent membrane protein; LP, leader protein.

Table 5 *Conditions associated with EBV infection in humans*

Condition	Occurrence
Clinically silent childhood primary infection	Universal in developing countries; common in developed countries
Delayed primary infection (teenagers/young adults)	Seen in developed countries: ca. 50% are clinically silent, as in childhood, ca. 50% have acute infectious mononucleosis – usually resolves; may become chronic
Primary infection with fatal outcome	Very rare familial X-linked lymphoproliferative disease (Duncan syndrome)
Lymphomas in immunodepression	In organ graft recipients
	In AIDS patients
Oral hairy leucoplakia	In AIDS patients and other T-cell immunodeficiencies
Endemic Burkitt's lymphoma	Common in children where falciparum malaria is hyperendemic
Nasopharyngeal carcinoma	Common in south Chinese and Inuit adults
Hodgkin's disease	Developed countries: *ca*. 50% are EBV associated. Developing countries: <90% EBV associated
Tumors with tenuous or suspected links to EBV infection	Some T-cell lymphomas, leiomyosarcomas, salivary gland cancers, undifferentiated carcinoma of the stomach

childhood; thus, in developing countries, 99.9 percent of all children are infected by 2–4 years of age, depending on geographical region. However, in industrialized countries with high standards of hygiene a considerable number of people do not become infected as young children, the percentage of each age group remaining free of infection as teenagers or young adults depending in western societies on socioeconomic status: the higher the standard of living, the greater the percentage (Henle and Henle 1979b). Among the very affluent as many as 50 percent of young adults may never have been infected.

Primary infection in early childhood is symptom-free and leads only to the generation of antibodies to the viral and virus-determined antigens (Henle and Henle 1979b) and to the development of specific cytotoxic T lymphocytes (Murray et al. 1992). These humoral and cellular immunological responses are maintained continuously thereafter and are responsible for keeping the lifelong EBV infection in check; the virus persists as a latent infection in a few circulating B lymphocytes (Lewin et al. 1987) and as a productive infection somewhere in the mouth and pharynx, the urogenital tract and, perhaps, the salivary glands. EBV is shed into the buccal fluid in readily detectable amounts in about 20 percent of those who have been infected, and in small amounts from time to time in many of the remainder (Yao et al. 1985); the virus has also been detected in genital secretions (Sixbey et al. 1986; Israele et al. 1991). The nature of the permissive cells producing infectious virions in the oro- and nasopharynx was for long a continuing controversy (Allday and Crawford 1988; Niedobitek and Young 1994): was the virus coming from squamous epithelial cells or from intraepithelial B lymphocytes? Evidence suggesting that it might only be the latter has recently emerged because in the absence of lymphocytes, as occurs in patients with X-linked agammaglobulinemia, EBV infection does not seem to take place (Faulkner et al. 1999). However, even if this observation appears to indicate that EBV does not normally infect and replicate in squamous epithelial cells of the mouth and pharynx, doubts and problems remain; squamous epithelial cells become infected in several conditions and an explanation of how and why the virus infects them is still required. Furthermore, such cells are capable of virus production; the latent EBV infection always carried by the malignant epithelial cells of nasopharyngeal carcinoma can be activated in vitro into a replicative cycle (Trumper et al. 1976), with release of infectious virus (Trumper et al. 1977), and virions are regularly produced in the squamous epithelial cells of oral 'hairy' leukoplakia (Greenspan et al. 1985).

Nevertheless, it has been clear for a great many years that virus in the buccal fluid is the main source of transmission of the infection in the population, by droplets and casually contaminated objects in the case of young children, and by direct salivary transfer during kissing among the sexually active (Hoagland 1955).

Those who miss the clinically silent natural primary infection in childhood are likely, sooner or later, to undergo delayed primary infection. Although 50 percent of such delayed infections are symptom-free, as in childhood, the other 50 percent are accompanied by disease: classic infectious mononucleosis (IM) (Sawyer et al. 1971; Hallee et al. 1974).

CLINICAL MANIFESTATIONS

Infectious mononucleosis

The fact that it is the teenagers and young adults in the upper classes of western countries who tend to escape silent infection as children makes IM predominantly a disease of the most affluent groups, and causes it to be exceptionally rare in developing countries. Although most cases of IM occur in adolescents and young adults,

older children and the middle-aged may occasionally develop the disease as a consequence of primary EBV infection, and rarely also the elderly. IM has long been known to be associated with kissing among young people (Hoagland 1955), and the pattern of acquisition results from a healthy carrier who is shedding virus in his or her saliva passing this during close buccal contact directly into the oropharynx of a partner who has not been primarily infected in the usual way as a child. This method of spread explains why case-to-case infection and epidemics are not seen with IM, and why the incubation period – perhaps 30–50 days – is difficult to calculate. Primary EBV infection giving IM-like symptoms may also be transmitted by blood transfusion (Gerber et al. 1969) or organ grafting (Haque et al. 1996) from an infected donor to a previously uninfected recipient.

SYMPTOMS AND SIGNS

There may be vague prodromal indisposition or an abrupt onset with sore throat, fever and sweating, anorexia, headache, and marked malaise. Brief dysphagia and orbital edema may be noticed. Severe edema of the tonsils and pharynx can sometimes cause pharyngeal obstruction. Erythematous rashes occur in a small number of untreated patients, but affect most of those who have been taking ampicillin. The pharynx, fauces, soft palate, and uvula are red and swollen, and may develop a greyish exudate. Generalized lymphadenopathy is almost invariable, most marked in the cervical region, with symmetrical, discrete, slightly tender glands; splenomegaly is seen in 60 percent of cases, an enlarged liver in 10 percent, and actual jaundice in about 8 percent. Crops of palatal petechiae are found in about one-third of patients. If mild, IM may resolve in days, but usually continues for 1–2 weeks, and is often followed by a period of marked lethargy before complete recovery. The length of convalescence may be influenced by psychological factors, but about one case in 2 000 continues in a truly chronic or recurrent form for several months or even years; exhaustive investigation of such patients has sometimes revealed minor immunological defects (Virelizier et al. 1978).

In contrast, an extremely rare genetically determined, X-linked lymphoproliferative condition (XLP disease or Duncan syndrome) first described by Bar et al. (1974) is due to a specific inability to respond normally to EBV during primary infection, and the affected young males of certain kindred die from IM owing to progressive destruction of vital organs and multisystem failure (Gaspar et al. 2002). The gene for XLP syndrome has now been cloned (Coffey et al. 1998; Sayos et al. 1998); it lies on the long arm of the X chromosome and codes for a protein responsible for controlling normal T-cell activation. Because the gene is mutated in XLP syndrome cases, failure of this control probably permits the cytotoxic T cells generated by primary EBV infection to extend their action from the true targets provided by EBV-infected B cells to include the destruction of normal cells of the patient's vital organs (Howie et al. 2000).

COMPLICATIONS

Serious but rare complications include secondary bacterial throat infections, traumatic rupture of the enlarged spleen, asphyxia from pharyngeal edema, massive hepatic necrosis, Guillain–Barré syndrome, and autoimmune manifestations such as thrombocytopenia and hemolytic anemia (Finch 1969).

PATHOGENESIS

The very long evolutionary association of EBV with its human host (more than 35–45 Myr) has ensured that it can establish an inapparent infection in early childhood which thereafter persists for life (Henle and Henle 1979b). When delayed primary infection occurs as a result of the high living standards of modern western countries, young adults encounter the virus for the first time at an age when the mode of infection and size of infecting dose are different from those in children: children come into contact with saliva from a shedder as airborne droplets or contamination on some sucked object, and thus receive a much smaller amount of virus than a young person taking in large quantities of virus-containing saliva from such a carrier during mouth-to-mouth kissing (Hoagland 1955). An exaggerated immunological response to a very large infecting dose of EBV fits well with the changes of IM: large numbers of activated $CD8^+$ T cells stimulated by a mass of infected B cells (expressing all the known latent viral genes) in the circulation, in lymph nodes, tonsils, lymphoid tissue of the mouth and pharynx, spleen, liver, and elsewhere. These T cells kill EBV-infected target cells (Callan et al. 1998), secrete multiple lymphokines (Foss et al. 1994), and are thus the cause, by immunopathological mechanisms, of the sore throat, fever, malaise, lymphadenopathy, and hepatosplenomegaly.

IMMUNE RESPONSES AND DIAGNOSIS

During acute IM, serum IgM and IgG antibodies to VCA and IgG anti-EA antibodies appear early, whereas anti-EBNA1 antibodies are not usually detectable until convalescence (Henle and Henle 1979b). On recovery, IgM anti-VCA and IgG anti-EA disappear but IgG antibodies to VCA and EBNA1 are maintained for life (Figure 2) (Henle and Henle 1979a).

Because it is not usually possible to document seroconversion, the presence of IgM antibodies to viral capsid antigen provides the most reliable diagnostic test. This may be corroborated by finding anti-EA antibodies (usually of D type) or the absence of antibodies to EBNA1, or both (Henle and Henle 1979a). These tests

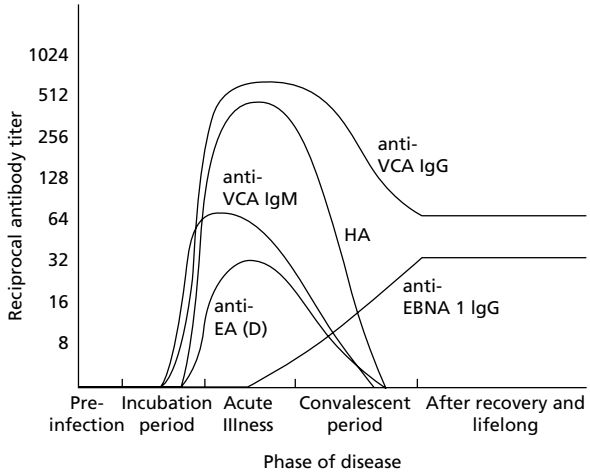

Figure 2 *Epstein–Barr virus-specific antibody response in infectious mononucleosis. Typical serum antibody titers as assessed by indirect immunofluorescence are indicated. EA (D), early antigen (diffuse); EBNA, EB viral nuclear antigen; HA, heterophile antibody; VCA, viral capsid antigen.*

depend on immunofluorescence staining of B-cell lines expressing viral antigens, are time-consuming, and are usually carried out only in larger laboratories. A number of commercial ELISAs are available but they lack the sensitivity of immunostaining. The rapid screening Monospot test is more widely used; this has been adapted from the earlier Paul–Bunnell test for IgM heterophil antibodies which agglutinate red cells from nonhuman sources. Although the heterophil antibodies are not directed against viral-coded antigens they are present in up to 85 percent of IM sera, but it must be remembered that cases of Monospot-negative IM are frequent outside the classic 15–25-year age range, and false positives may occur in pregnancy and autoimmune disease (Okano et al. 1988). An additional diagnostic feature of IM is the presence of a lymphocytosis of up to 15×10^9/l, most cells having an 'atypical' morphology. The clinical and hematological findings and a positive Monospot test give a reliable diagnosis, but if the latter is negative, tests for IgM anti-VCA antibodies must be done. Retrospective diagnosis may be possible if suitable samples are available to allow seroconversion to, or a rising titer of, EBNA1 IgG antibody to be detected.

Endemic ('African') Burkitt's lymphoma

Classic BL (Burkitt 1963) is a B-cell tumor found in parts of Africa and Papua New Guinea, where the temperature does not fall below 16.5°C or the annual rainfall below 55 cm (Burkitt 1970a). BL is overwhelmingly a disease of children, and is extremely rare over the age of 14 years. In endemic areas it is more common than all other childhood cancers added together. There is no evidence in the tumor belt for any tribal or racial susceptibility (Burkitt 1963). Endemic BL is distinct from the 'Burkitt-like' tumors (sometimes called 'American' or 'sporadic' BL) seen sporadically everywhere in the world, which have a different age incidence, anatomical distribution and response to therapy. The term 'Burkitt type' is sometimes also applied to certain leukemias because of morphological resemblances of the malignant cells.

Some 97 percent of endemic BL carries the EBV genome, whereas only 12–25 percent of the sporadic tumors are EBV-positive (Ziegler et al. 1976); it would seem that the 3 percent of EBV-negative BLs occurring in endemic areas are in fact sporadic BLs that occur at low incidence worldwide.

The association between EBV and endemic BL is so close that it is now generally accepted that the virus is a necessary (albeit not solely sufficient) element in causation of the disease. It is an essential link, together with cofactors, in a complicated chain of events that leads to the malignant change (Epstein and Achong 1979). BL cells contain the EBV genome, but only EBNA 1 is expressed. Hyperendemic falciparum malaria is an important cofactor, and its spread by anopheline mosquitoes that require warmth and moisture explains the climate dependence of the tumor (Burkitt 1969).

SYMPTOMS AND SIGNS

The tumor is usually multifocal, occurs in a variety of sites, and the symptoms depend on the anatomical location of the lesions (Burkitt 1963). Tumors in the maxilla or mandible are present in 70 percent of patients and are the presenting feature of most of these (Figure 3). They may be multiple in two, three or even all four

Figure 3 *A 9-year-old girl with a typical Burkitt's lymphoma of the right upper maxilla presenting through the orbit. The Epstein–Barr virus was first identified in cultured malignant lymphoblasts from this tumor.*

quadrants of the jaws, and are almost always accompanied by tumors elsewhere. A rapidly growing mass, loosening of teeth, and exophthalmos due to orbital spread are the main consequences. Abdominal tumors are also common, involving retroperitoneal nodes, liver, ovaries, intestine, and kidneys. BL may present in the thyroid, the adolescent female breast, the testicles, the salivary glands, and the skeleton. Extradural spinal tumors cause paraplegia of rapid onset. An unusual and characteristic feature of this lymphoma is its failure to involve the spleen and the peripheral lymph nodes.

Clinically, the tumors are firm, very rapidly growing, and painless; even when large, they cause minimal constitutional disturbance. The clinical signs are determined by the tumor sites, but wherever these may be BL grows relentlessly and, in the absence of treatment, death ensues within a few months (Burkitt 1963).

PATHOGENESIS

Molecular biological investigations are successfully unraveling the sequence of EBV gene expression that enables the virus to immortalize normal human B lymphocytes in vitro into continuously growing cell lines. Explanations are also beginning to emerge as to how the viral genes, in combination with cofactors, can bring about the malignant change leading to BL. There are currently two possibilities:

- EBV infection in the presence of the impaired immunological control brought about by hyperendemic malaria leads to a high level of EBV-driven B-cell proliferation, perhaps by similar mechanisms to in vitro immortalization; this proliferation in turn increases the chances of one or another of three BL-specific chromosomal translocations (t8:14; t8:22; t2:8) occurring, each of which can deregulate the c-*myc* oncogene on chromosome 8 by bringing it under the influence of an immunoglobulin gene promoter on chromosomes 14, 22, or 2. This deregulation causes malignant change in the affected cell and the rapid outgrowth of a clone of tumor cells (Klein 1987).
- The chronic immunological stimulation of hyperendemic malaria generates a high turnover of pre-B cells in the bone marrow; one or more of these early lymphocytes acquires one of the three chromosomal translocations involving c-*myc* deregulation by an error occurring during immunoglobulin gene rearrangement, and this renders the affected cell responsive to growth signals; infection of such a cell by EBV finally provides viral functions conferring malignancy and a tumor clone grows out (Lenoir and Bornkamm 1987).

However, with either possibility the virus–host balance is disturbed by hyperendemic malaria, and additional molecular explanations continue to emerge (Farrell et al. 1991, Kelly et al. 2002).

IMMUNE RESPONSES AND DIAGNOSIS

In the appropriate geographical regions endemic BL can often be diagnosed on clinical grounds, but this should be confirmed by histological examination of a biopsy specimen. In addition, antibodies to EBV antigens have a unique pattern in BL: titres rise as the disease progresses and fall as tumors regress in response to treatment. These changes may be used to assess disease outcome. IgG antibodies to VCA are found at 8–10 times higher geometric mean titers than in healthy controls matched for age, gender, and residence. IgG antibodies to EA-R and MA are also detectable (Henle et al. 1969), and likewise vary with disease progression; successful treatment causes a rise in anti-MA and a fall in anti-EA-R, whereas the reversal of this pattern usually heralds a recurrence of the tumor.

THERAPY

Surgery and radiotherapy are not effective, but excellent results are obtainable with moderate courses of chemotherapy (Burkitt 1970b). Cyclophosphamide is the drug of choice, and dramatic and sustained resolution is not uncommon when tumors are small.

Lymphomas in immunosuppressed individuals

The lifelong EBV infection present in all normal seropositive individuals is controlled by cell-mediated immune mechanisms. In primary and secondary suppression of cellular immunity this control of persisting EBV infection is lost, leading to increased virus replication in the oral cavity, increased numbers of virus-carrying B lymphocytes in the circulation, and increased levels of serum antibodies to lytic-cycle EBV antigens (VCA, EA). When this occurs it is sometimes described as a 'reactivated infection', although the condition is clinically silent. However, on occasions the loss of control results in EBV-associated lymphoproliferative disease. This type of disease also occurs when immunosuppressed individuals undergo primary infection and fail to mount the normal immunological response to the virus (Thomas et al. 1991).

ORGAN GRAFT RECIPIENTS

It has long been known that organ graft recipients who receive lifelong immunosuppressive drugs to prevent graft rejection have an increased risk of developing lymphoproliferative disease and lymphoma (28–100 times that of normal controls) (Penn 1983). Most of these conditions, known as post-transplant lymphoproliferative disease (PTLD), are of B-cell origin, contain the EBV genome, and express viral antigens in their cells. Lymphoproliferative disease has two forms of clinical presentation. In about 50 percent of cases it occurs within the first year after transplantation, in a young age

group, has IM-like symptoms, and is associated with primary EBV infection in patients who were seronegative at the time of grafting. The second type of presentation occurs in older patients late after transplantation, and takes the form of a localized mass, commonly in the gut, central nervous system, or transplanted organ (Hanto et al. 1981, 1985). Biopsy of the lesion reveals large-cell lymphoma, which is usually monoclonal in origin, although progression from a polyclonal B-cell proliferation probably occurs early in the disease (Frizzera et al. 1981, Frizzera 1987). The fact that all the EBV latent viral genes are expressed in these lymphoproliferative diseases, just as with lymphoblastoid cell lines immortalized by the virus in vitro, suggests that EBV is of prime importance in the etiology of the lesions (Thomas et al. 1990), although other events – perhaps cytogenetic abnormalities – may be necessary to cause the monoclonal growth.

Reduction of immunosuppressive drugs, with or without acyclovir therapy, has caused complete and often sustained regression of the tumors in many cases (Starzl et al. 1984). This has therefore become the first line of treatment, cytotoxic drugs and/or radiotherapy being used only when there is no response or after recurrence. EBV-specific cytotoxic T cells grown in vitro have been given after bone marrow transplantation to protect against such EBV-driven lymphoproliferation prior to regeneration of the immune system (Rooney et al. 1995), and to treat PTLD in bone marrow and solid organ transplant recipients (Haque et al. 2002).

AIDS PATIENTS

Two types of lymphoma are seen with increasing frequency in AIDS patients: large-cell lymphoma and Burkitt's lymphoma. Both of these may be associated with EBV (Kalter et al. 1985).

Large-cell lymphomas similar to those seen in organ graft recipients occur in severely immunocompromised AIDS patients; their distribution is extranodal and involves many unusual sites, the most common being the central nervous system. These lymphomas have a strong association with EBV, which reaches 100 percent in cerebral tumors (Herndier et al. 1994). The clinical presentation depends on the site involved, but the progression is rapid, with a mean survival time from diagnosis of 3–4 months. The treatment of choice is radiotherapy, although the results are disappointing, mainly because patients in the terminal stages of the underlying AIDS are in such poor general condition.

Burkitt's lymphoma occurs earlier in the course of human immunodeficiency virus (HIV) disease, while the immune system is still relatively intact, and is therefore more amenable to treatment. About 50 percent of these lymphomas contain EBV DNA.

Hodgkin's disease

There has long been a suspicion that EBV may play some part in the induction of Hodgkin's disease (HD) because of the similar socioeconomic epidemiology of HD and IM, and because of the well established fact that within 5 years of IM there is a four- to sixfold increase in the likelihood of developing HD (Mueller et al. 1989). In recent years, evidence has been obtained that about 60 percent of HD is EBV associated, and this mainly involves the mixed cellularity type. However, surprisingly, the majority of EBV-associated mixed cellularity HD cases do not occur in the post-IM age group (Jarrett 2002).

In EBV-associated HD EBV DNA is carried and viral proteins expressed (latency II) in Reed–Sternberg and the tumor reticulum cells (Weiss et al. 1989; Herbst and Niedobitek 1994), and both these cell types are now known to be of germinal center B-cell origin (Marafioti et al. 2000). These intriguing findings appear to implicate EBV in certain types of HD.

T-cell lymphomas

EBV DNA has been detected with varying frequency in different types of T-cell lymphoma. Detection rates of up to 97 percent have been recorded in angioimmunoblastic lymphadenopathy (Ott et al. 1992), 84 percent in nasal lymphoma (previously called midline granuloma) (Harabuchi et al. 1990), and in 46 percent of peripheral T-cell lymphoma (Jones et al. 1988). In addition, sporadic cases of EBV-positive angiocentric T-cell lymphoma and of T-cell lymphoma related to immunosuppression and chronic EBV infection also occur. In these lesions the cell phenotype is usually that of clonal, mature T cells, which more often express the CD4 than the CD8 marker. The EBV gene expression is usually of the latency II type, but the latency III pattern has also been recorded.

Although it is now clear that EBV can infect T cells under certain conditions, the involvement of the virus in the pathogenesis of T-cell lymphoma remains to be elucidated.

Various other malignancies

Recent reports have linked EBV to various cancers, including some thymic epithelial tumors (Leyvraz et al. 1985; Wu and Kuo 1993), some undifferentiated salivary gland carcinomas (Hamilton-Dutoit et al. 1991) and, more regularly, to several types of gastric carcinoma (Takada 2000); surprisingly, smooth muscle tumors carrying EBV have also been reported in immunosuppressed children (Lee et al. 1995; McClain et al. 1995). Little is known about the EBV gene expression in any of these tumors, and further work is required before a definite association can be established.

Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) arises from the squamous epithelial cells of the postnasal space; primary tumors are not found outside this well-defined anatomical site. About 70 percent of NPC are undifferentiated in type; the remainder show squamous differentiation. The tumor occurs rarely throughout the world, but has a very high incidence in southern Chinese wherever they live, and in the Inuit and related races of North America and Greenland. Among populations with a high incidence, NPC is the most common cancer of men and the second most common of women. There is also a high incidence of NPC in Malays, Dyaks, Indonesians, Filipinos, and Vietnamese, and a moderately raised incidence in a belt across North Africa, down through the Sudan and into the Kenyan Highlands. Comprehensive reviews have been given by Shanmugaratnam (1971, 1978). The tumor is usually a disease of middle or old age, except that in medium–high incidence areas of Africa it has bimodal age peaks, the first involving children and young people under 20 years of age (Cammoun et al. 1974), and a second affecting much older people. The tumor cells of undifferentiated NPC always carry the EBV genome (Klein 1979; Niedobitek et al. 1991), and most authorities agree that the virus plays a role in causation as a necessary but not the sole element in the etiological complex.

SYMPTOMS AND SIGNS

About two-thirds of patients present with one or more symptoms caused by the local effects of the tumor: nasal obstruction, discharge or bleeding; deafness, tinnitus, or earache; ocular paresis from tumor spread to affect cranial nerves. The remaining third complain only of cervical lymph node enlargement due to metastatic spread from a primary tumor that is frequently occult. The signs result either from local tumor spread, causing soft tissue distortion around the postnasal space or cranial nerve palsies, or as a consequence of lymphatic spread to cervical and later supraclavicular lymph glands, which become hard and fixed. Bloodborne metastases may be in any organ but are frequent in bones, liver, and lungs, the signs depending on the organ involved (Shanmugaratnam 1971).

PATHOGENESIS

Little is known of EBV gene function in normal infected epithelial cells, but viral gene expression in the malignant cells of NPC is well characterized; although restricted, it is less so than in BL cells with EBNA1 detected in every tumor cell of undifferentiated NPC and LMP1 in a variable proportion (latency type II) (Young et al. 1988). In addition to the virus, racial predisposition plays a role, and, among southern Chinese, a modest genetic predisposition has also been demonstrated (Simons et al. 1974; Chan et al. 1983). Environmental cofactors associated with the Chinese way of life have emerged from studies on migrants (Buell 1974; Henderson 1974), and two likely candidates have been identified:

- Traditional herbal remedies (taken as snuff) made from dried plants of the *Euphorbiaceae* and *Thymeliaceae* families, which contain tumor-promoting phorbol esters (Hirayama and Ito 1981).
- Traditional salt fish dishes, which contain carcinogenic nitrosamines (Huang et al. 1978).

Because EBV replicates in the nasopharynx of all carriers, and because southern Chinese always undergo primary infection as very young children (Henle and Henle 1979b), the taking of snuffs containing phorbol esters is significant because these chemicals are also powerful activators of EBV replication (zur Hausen et al. 1979) as well as having tumor-promoting effects. Thus, if virus production is greatly increased in people with a genetic predisposition to NPC, there will be an unusually large pool of EBV-infected cells that might progress to malignancy, perhaps through a combination of EBV *LMP1* oncogene function with nitrosamine carcinogenicity and phorbol ester tumor-promoting effects.

IMMUNE RESPONSES AND DIAGNOSIS

The diagnosis of NPC is usually made histologically on a biopsy sample from the primary tumor or from an enlarged cervical lymph gland. In addition, serum antibody titers show a characteristic and specific pattern of reaction, regardless of geographical location, and can therefore be used for diagnosis; IgG and IgA antibodies to VCA and EA-D are raised, and IgA against these antigens can be found in the saliva (Klein 1979). In the high incidence areas of southern China, mass screening of susceptible populations for serum IgA antibodies to VCA has been successful in detecting very early cases of NPC (Zeng et al. 1982), an important achievement because such early lesions usually respond very well to treatment.

THERAPY

The treatment of choice is radiotherapy, which gives 5-year survival rates of 60 percent or more in the earliest stages of the disease. Of those who survive, 70 percent remain permanently free of relapse (Ho et al. 1983). More advanced stages have correspondingly worse prognoses.

Oral hairy leukoplakia

This unusual condition is seen fairly frequently in HIV-positive homosexual men, often before AIDS develops, but sometimes heralding or accompanying the onset of

clinical AIDS. Painless white patches occur on the tongue and/or the lateral buccal mucosa; the lesions are usually multiple, measure up to 3 cm in diameter, are slightly raised, and have a 'hairy' or corrugated surface (Greenspan et al. 1985).

The squamous epithelial cells affected by this condition contain large amounts of actively replicating EBV (Greenspan et al. 1985). Acyclovir arrests the virus replication and the lesions regress, but only for the period during which drug treatment is continued.

VACCINES AGAINST EBV

In the mid-1970s evidence linking EBV to some causative role in endemic BL and NPC was sufficiently strong to warrant the suggestion that vaccine prevention of the virus infection might be a way of decreasing the incidence of the associated cancers in high-risk populations (Epstein 1976). Although BL is of outstanding importance as a model for human viral carcinogenesis, and is exceedingly common in endemic areas, in terms of world cancer it is insignificant; however, NPC is a major oncological problem for very large populations, justifying efforts to control it through the development of a vaccine against EBV. Vaccine protection against IM would also be useful.

An extended program of work on an MA-based anti-EBV vaccine (Epstein 1986, 1994) succeeded in establishing that the purified gp340 component of MA induced virus-neutralizing antibodies, and indeed cytotoxic T-cell responses (Wilson et al. 1999), on injection into cottontop tamarins. Furthermore, the latter – the only animal known to respond with lesions to experimental EBV infection – when immunized in this way were protected against a 100 percent carcinogenic challenge dose of virus (Epstein 1986, 1994). Recently, gp340 purified from cultures of mammalian cells into which the *gp340* gene had been engineered (Jackman et al. 1999) was used in a successful phase I trial in human volunteers (Gilbert 1999). A phase II trial subsequently conducted for a major pharmaceutical company in a larger group of volunteers has given preliminary results that show some encouraging protective effects (Bollen 2002); a phase II placebo-controlled trial is currently in progress.

Other approaches are also going forward. Recombinant vaccinia viruses expressing gp340 have been made, and a small scale vaccination experiment using this in nine Chinese children has given some evidence of protection (Gu et al. 1995), but a wildtype strain of vaccinia was employed for the construct which would not be acceptable for general application. A quite different project has sought to induce specific cytotoxic T-cell immunity, not antibodies: selected EBV epitopes have been fused together in a synthetic polyepitope protein for injection, not to prevent infection, but to limit EBV replication after infection by destruction of virus-producing cells by the stimulated T cells, and thereby perhaps decrease disease manifestations. Preliminary trials to explore this technique in volunteers have begun (Khanna et al. 1999).

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Poxviruses

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INTRODUCTION

The molecular aspects of poxvirus replication are considered in Poxvirus replication. This chapter considers the origin of vaccinia virus and its use to eradicate smallpox, the classification and phylogenetic relationships of poxviruses, how these viruses are transmitted and cause disease in their hosts and how they may be controlled. Lastly, the potential use of poxviruses in bioterrorism is discussed.

WHAT IS A POXVIRUS?

Poxviruses are large viruses that have double-stranded DNA genomes $\geqslant$135 kb. The virions are large and complex, are surrounded by a lipid envelope and lack icosahedral or helical symmetry. Unlike most DNA viruses, poxviruses replicate in the cell cytoplasm and therefore encode the great majority of the proteins needed for their transcription and DNA replication. Poxviruses also encode many virulence factors that are nonessential for virus replication in cell culture, but help the virus to survive in its particular host(s). These viruses infect a wide range of vertebrate and invertebrate species, although this chapter is very largely restricted to poxviruses of chordates. Some poxviruses cause severe disease such as smallpox, camelpox, or myxomatosis, but equally poxviruses may cause only asymptomatic infections. Historically, these viruses have played important roles in human medicine and biomedical science. The most serious human poxvirus, variola virus, caused the disease smallpox. This disease was eradicated in 1977 but, remarkably, the origin of the smallpox vaccine (vaccinia virus) remains an enigma of virology. For reviews of poxvirus properties see

Poxvirus replication, as well as Fenner (1996) and Moss (2001).

HISTORICAL ROLES OF POXVIRUSES

Poxviruses have played important roles in human history, medicine, and biological science. Smallpox was a devastating disease that killed up to 40 percent of those infected and has influenced the history of mankind (Hopkins 1983; Fenner et al. 1988; Glynn and Glynn 2004). It is likely that this disease became prevalent after *Homo sapiens* adopted intensive agriculture rather than living as a hunter-gatherer. This profound change in our behavior took place between 5 000 and 10 000 BC and enabled human population densities to increase so that diseases such as smallpox and measles could remain endemic. It has been estimated that populations of at least 300 000 are needed for measles to survive in mankind. In popula-

tions smaller than this, an epidemic would leave too few nonimmune hosts for the virus to be transmitted and so it could not persist in the population. Smallpox is less easily transmitted from person to person than measles, and so the population needed for smallpox to remain endemic was smaller and might be closer to 100 000. It is uncertain exactly when smallpox appeared in man, but examination of the mummified corpse of the Egyptian pharaoh Ramses V, who died in 1160 BC, revealed characteristic dermal pocks, suggesting he may have died from the disease. Smallpox probably existed in man for many centuries previously, although there is no proof of this.

Smallpox was caused by Variola virus, a member of the genus *Orthopoxvirus* (Table 1). When this disease was introduced into hitherto naive populations the effect was devastating. For instance, the introduction of smallpox into Central America in the early sixteenth century decimated the local American Indian population and helped the Spanish invaders to conquer the Aztec nation with 500 soldiers. Smallpox had a profound effect on the population of Europe and it is estimated to have killed between 200 000 and 600 000 people annually during the sixteenth and seventeenth centuries. Even during the twentieth century, smallpox was a major cause of premature mortality. In 1967 there were 131 418 cases of smallpox reported from 44 countries, but due to under-reporting the real number of cases may have been nearer to 10 million (Henderson 1976). For reviews of smallpox in history, the reader is referred to excellent texts on this topic (Hopkins 1983; Fenner et al. 1988; Glynn and Glynn 2004).

THE ERADICATION OF SMALLPOX

The eradication of smallpox has been described in a monumental book *Smallpox and its eradication* (Fenner et al. 1988) and only an outline is given here. Before Edward Jenner introduced vaccination in 1796, the only means of preventing smallpox was variolation (also called inoculation). This involved the deliberate inoculation of infectious variola virus into the skin (usually the upper arm) of the patient brave enough to be infected. The matter inoculated came directly from a patient with smallpox, or was taken from such a patient and stored in dry form until use. Introduction of the virus into the skin gave a less serious disease than that following natural infection via the respiratory tract. Nevertheless, variolation could, and did, cause serious disease and approximately 1 percent of variolated patients developed smallpox and died. Moreover, both these patients and those who survived the infection were able to transmit the infection to others and start epidemics. So variolation was a dangerous practice and was tolerated only because of the tremendous fear of contracting smallpox naturally.

Jenner himself was a variolator and was well aware of the risks and perhaps this contributed to his search for an alternative and safer way to prevent smallpox. Jenner noticed that milkmaids who sometimes became infected on their hands with cowpox virus derived from the cows they milked were spared from smallpox when an epidemic would rage through the community. They were also resistant to variolation. Although Jenner was not the first to notice these facts, he was the first to test systematically the hypothesis that it was the prior

Table 1 Poxviridae

Subfamily	Genus	Species[a]
Chordopoxvirinae	*Orthopoxvirus*	***Vaccinia virus***[b], *Variola virus*[c], *Cowpox virus*[d], *Camelpox virus*, *Monkeypox virus*, *Ectromelia virus*, *Raccoonpox virus*, *Volepox virus*, *Taterapox (gerbil) virus*
	Parapoxvirus	***Orf virus*** (contagious pustular dermatitis), *Pseudocowpox virus*, *Bovine papular stomatitis virus*
	Capripoxvirus	***Sheeppox virus***, *Goatpox virus*, *Lumpy skin disease virus*
	Leporipoxvirus	***Myxoma virus***, rabbit (Shope) fibroma virus, *Hare fibroma virus*, *Squirrel fibroma virus*, *Rabbit fibroma virus*
	Suipoxvirus	***Swinepox virus***
	Molluscipoxvirus	*Molluscum contagiosum virus*
	Yatapoxvirus	***Yaba monkey tumor virus***, Yaba-like disease virus, *Tanapox virus*
	Avipoxvirus	***Fowlpox virus***, *Canarypox virus*, *Turkeypox virus*, *Pigeonpox virus*
Entomopoxvirinae	*A*	
	B	Amsacta moorei, Melanoplus sanguinipes
	C	

a) Type species in bold.
b) Includes buffalopox virus and rabbitpox virus as subspecies.
c) Includes variola major and variola minor (alastrim).
d) Includes isolates from felines and captive exotic species.

infection with cowpox virus that caused immunity to smallpox. For this he rightly deserved the accolade for the discovery of vaccination. On 14 May 1796, Jenner vaccinated a boy, James Phipps, with material taken from the hand of a milkmaid, Sarah Nelmes, who had been infected from her cow, Blossom. Subsequently, Jenner demonstrated that Phipps was resistant to variolation. His work was submitted to the Royal Society of London, but not accepted. After being unable to publish his discovery, Jenner performed more vaccinations to obtain additional data in support of his theory. He showed by challenging some of these vaccinees that they too were resistant to variolation (Figure 1). In 1798, he wrote his famous article *An enquiry into the causes and effects of variolae vaccinae, a disease discovered in some of the western countries of England, particularly Gloucestershire, and known by the name of Cowpox* and published this privately (Jenner 1798). The success of vaccination was evident rapidly and enabled Jenner to predict in 1801, that 'the annihilation of the smallpox, the most dreadful scourge of the human species, must be the final result of this practice' (Jenner 1801). This prophecy was correct but took another 176 years to be fulfilled. Jenner's discovery of vaccination was not patented and he did not seek financial reward from it. Perhaps partly in view of this, in 1802 the British Parliament debated his discovery and awarded him a sum of £10 000, a princely sum in the early nineteenth century. In the same year, the American president, Thomas Jefferson, said of vaccination and Jenner, 'Medicine has never before produced any single improvement of such utility. You have erased from the calendar of human afflictions one of its greatest.' Five years later the British Parliament awarded Jenner another £20 000. In view of the success of vaccination and the dangers inherent in variolation, the latter practice was banned in the UK by Act of Parliament in 1840.

Given that vaccination was so effective in preventing smallpox, why did eradication take so long? Factors that contributed to this were the shortage of vaccine, loss of vaccine potency, and the refusal of some people to be vaccinated due to fear of vaccination or prejudice. From Jenner's discovery until the third quarter of the twentieth century, the vaccine supply was inadequate. Cowpox was a relatively rare disease and even though vaccine was also derived from horses, where it caused a disease called 'the grease,' vaccine shortage was a frequent problem. To overcome this, virus was passaged in humans by arm-to-arm transfer, indeed Jenner himself did this in the second series of vaccinations described in the enquiry (Figure 1). This method was used to disseminate the vaccine throughout Europe and the Spanish adopted it to transfer the vaccine to Central America in 1803. Twenty-two orphan children were taken on a voyage across the Atlantic Ocean and groups of children were vaccinated regularly en route so that virus was still growing on one or more children when the ship reached the Americas.

The process of arm-to-arm transfer provided an increased supply of vaccine but was also dangerous because it disseminated other pathogens and both measles and syphilis were transmitted in this way. Consequently, arm-to-arm transfer was banned in the UK by the Vaccination Act of 1898, but not before several epidemics of other diseases had been started. In 1885, in a shipyard in Bremen, Germany, an outbreak of

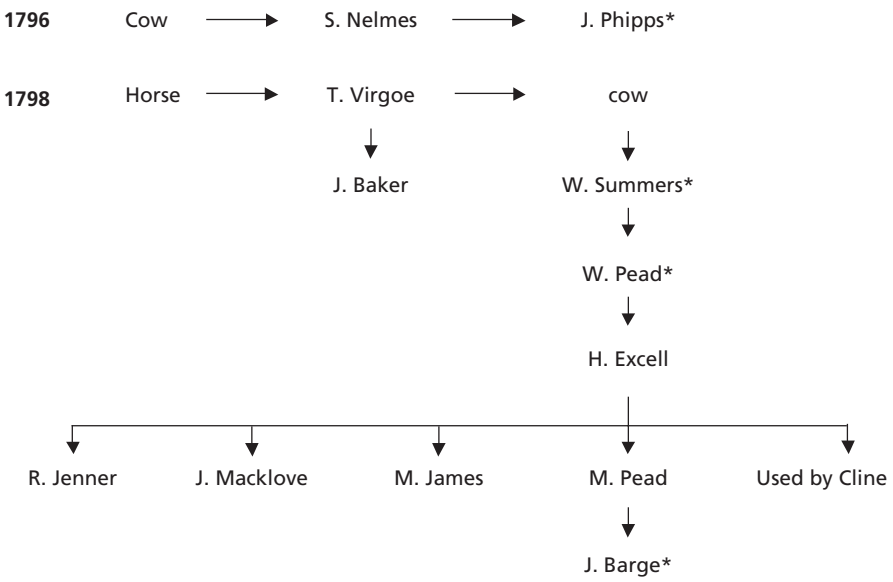

Figure 1 *The series of vaccinations performed by Jenner (1798). Nelmes and Virgoe were infected accidentally from a cow and a horse, respectively. An asterisk indicates that this person was resistant to subsequent variolation (redrawn from Baxby (1981) with permission).*

infectious hepatitis, very likely caused by hepatitis B virus, was blamed on a contaminated batch of smallpox vaccine composed of human lymph. This represented the first documented epidemic of infectious hepatitis B virus (Lurman 1885).

From the 1880s onwards, vaccine was produced on the skin of calves and became known as calf lymph vaccine. This was more plentiful, more easily standardized and free from some other human pathogens. Its proper use led to the containment of smallpox in parts of the world, but in many places the disease remained endemic. This was due partly to inadequate control of vaccine potency and this was a particular problem in tropical regions. In the early 1950s this problem was overcome by Leslie Collier and co-workers at the Lister Institute in London who developed a method to freeze-dry the vaccine such that it was stable at ambient temperature for long periods (Collier 1954, 1955). In fact the vaccine prepared in this way has remarkable stability and can resist incubation at 37°C for 3 months or even 100°C for 1 h. This was a very major advance, for now the vaccine could be used throughout the world and reconstituted under field conditions without loss of potency.

In 1959, the Soviet Union proposed to the World Health Organization that smallpox should be eradicated from the world. This proposal was endorsed enthusiastically, but despite the availability of stable vaccine there was relatively little progress during the next decade and, as mentioned above, smallpox was still endemic in at least 44 countries in 1967. After 1967, the vaccination policy was modified. Hitherto the objective was to vaccinate everyone, but obtaining blanket vaccination was virtually impossible. After 1967, ring vaccination was adopted and was more effective. This relied upon surveillance, quarantine, and vaccination. Photographs of smallpox patients were distributed widely and those identifying a case of smallpox were rewarded financially. Once identified, the patient was quarantined and all contacts were traced and vaccinated. These contacts were observed and if they developed fever, they were quarantined, and their contacts were traced and vaccinated. By this method, the spread of infection was halted and the focus of infection snuffed out. This was highly effective so that by 1975 the last case of smallpox occurred in the Indian subcontinent and the last naturally occurring case of smallpox was recorded in October 1977 near Mogadishu in Somalia.

Sadly, this was not the last case of smallpox and there was an outbreak in Birmingham, UK in 1978 following the escape of variola virus from a laboratory. In this outbreak one person died. Notably this fatality occurred despite the patient having been vaccinated twice previously, once as a child and once 12 years before contracting the disease. So immunity is not lifelong and this was recognized by the World Health Organization who recommended that those working in areas where smallpox was endemic should be vaccinated every 3 years. Of all the claims made by Jenner in 1798 about vaccination, nearly everything was correct except his belief that vaccination provided lifelong immunity.

It is largely because of the tragedy in Birmingham in 1978 that today the stocks of variola virus are held in only two locations in the world, in the Centers for Disease Control and Prevention, Atlanta, USA and in Vector, Novosobirsk, Russia. These stocks are stored in maximum security (BSL4) laboratories and all work with live virus requires prior approval by the World Health Organization which also inspects these laboratories regularly. The chance of an escape of virus from these sites is at best remote, but the concern about the potential use of variola virus in bioterrorism (see below) derives not so much from these virus repositories but from the possibility that the virus is held elsewhere illegally.

THE ORIGIN OF VACCINIA VIRUS

The virus used by Jenner in 1796 as the first human vaccine may have been cowpox virus, although this is uncertain because the original virus is not available for characterization. We know only that the virus was taken from the hand of Sarah Nelmes, who presumably was infected from her cow, and this virus was used to immunize James Phipps (Jenner 1798). However, vaccine was not only acquired from cows. In the series of vaccinations in 1798, described in Jenner's Enquiry, it is stated that Thomas Virgoe was infected from a horse and from Virgoe the virus was used to vaccinate a 5-year-old boy, John Baker (Figure 1). In addition, in 1813 and 1817, Jenner sent vaccine derived from horses to the National Vaccine Establishment, which was set up in England to provide smallpox vaccine. Vaccines were therefore derived from both cows and horses.

In 1939 in Liverpool, UK, Alan W. Downie showed that all the vaccines used to prevent smallpox in the twentieth century formed a homogeneous group that was distinct from both cowpox virus and variola virus and represented a distinct species of Orthopoxvirus that became known as vaccinia virus (Downie 1939a, b). If Jenner had used cowpox virus in 1796, sometime between then and 1939 this was replaced by vaccinia virus. Exactly when this took place is uncertain, but it is likely to have been early during the nineteenth century. Several anecdotes support his conclusion. First, the smallpox vaccine that was taken to the USA in 1856, was passed serially thereafter and became the New York City Board of Health vaccine, is vaccinia virus and not cowpox virus. Second, pathologists from the late nineteenth century who studied the cytopathic effect induced by the smallpox vaccine, noted the eosinophilic B type inclusion bodies that are made by both vaccinia virus and cowpox virus, but did not report the more obvious A type inclusion bodies that are made by cowpox virus and not vaccinia virus. Lastly, the drawings made of lesions induced by the vaccine used by Jenner are

similar to those induced by the vaccine in use today. So it is possible that Jenner used vaccinia virus, or cowpox virus, or both, but at different times. Both these viruses have a broad host range and may infect many species.

This origin of vaccinia virus was reviewed by Baxby (1981) and he suggested that it might be horsepox virus. If so, perhaps our language is wrong; for the name of the smallpox vaccine should be equinia virus (not vaccinia virus), and we use equines (not vaccines) to equinate people by the process of equination! It is remarkable that vaccinia virus is the only vaccine to have eradicated a human disease and yet its origin and natural host remain an enigma. It is also remarkable, in comparison with the regulatory paperwork needed today before any clinical trial may be undertaken, that for smallpox:

- there was no ethical or safety evaluation given to the first use of the vaccine, or the challenge of vaccinated subjects;
- the paper reporting the use of the vaccine to prevent smallpox did not survive peer-review;
- there was no properly controlled clinical trial to demonstrate the efficacy of the vaccine;
- there was (and is) no understanding of how the vaccine worked.

Yet there was anecdotal evidence that the vaccine worked: namely the global eradication of smallpox.

OTHER CONTRIBUTIONS OF VACCINIA VIRUS TO BIOMEDICAL SCIENCE

Despite its uncertain origin, vaccinia virus has played several seminal roles in biological science and medicine. In addition to its use to eradicate smallpox, vaccinia virus was the first mammalian virus to be purified, analyzed chemically, and titrated accurately. Work with vaccinia virus in the 1960s discovered the first virus-encoded nucleic acid polymerase (Munyon et al. 1967) and this discovery prompted the search for polymerases in other viruses. The study of vaccinia virus enzymes that catalyze the addition of a methylated cap structure at the 5′ end (Martin and Moss 1975) and a poly(A) tail at the 3′ end of mRNAs (Brown et al. 1973) have been informative for the study of these cellular processes. In 1982, genetically engineered vaccinia viruses were constructed that expressed genes derived from other organisms (Mackett et al. 1982; Panicali and Paoletti 1982) and such recombinant viruses were shown to have potential as vaccines against other infectious agents (Panicali et al. 1983; Smith et al. 1983a,b). This concept pioneered the development of genetically engineered viruses as live vaccines, a concept applied subsequently to other viruses and micro-organisms. More recently, studies of poxvirus virulence have revealed a wide range of proteins that inhibit specific components of the host immune response to infection (Seet et al. 2003). Lastly, studies of the interaction of vaccinia virus with the host cell have discovered how these viruses exploit cell biology to enable their entry into, movement within, and exit from the cell (Smith et al. 2003). Although smallpox has been eradicated and poxviruses now cause little human disease, there are compelling reasons to continue to study these viruses.

CLASSIFICATION

The *Poxviridae* is divided into the *Entomopoxvirinae* and *Chordopoxvirinae* subfamilies that infect insects and chordates, respectively (Table 1). The entomopoxviruses are subdivided into three genera (*A*, *B*, and *C*), although genome sequence data from two members of group B (Melanoplus sanguinipes and Amsacta moorei) have revealed considerable diversity suggesting that these viruses might be reclassified in different genera (Afonso et al. 1999; Bawden et al. 2000). The genomes of these entomopoxviruses are rich in adenine and thymine (A+T), 81.5 and 81.7 percent. These viruses are not considered further here and the reader is referred to other reviews of this topic (Moyer 1994, 1999).

The chordopoxviruses are now considered in greater detail. This subfamily is divided into eight genera: *Orthopoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Avipoxvirus*, *Yatapoxvirus*, *Suipoxvirus*, *Capripoxvirus*, and *Parapoxvirus* (see Table 1). Within each genus there are distinct species and often there are multiple strains of each species. For instance, the genus *Orthopoxvirus* contains several members, one of which is *Vaccinia virus* and there are a score or so different *Vaccinia virus* strains. Historically, the definition of a genus is that its members are immunologically cross-reactive and cross-protective, whereas viruses of different genera are neither cross-reactive nor cross-protective. This has been a reasonable method of classification and is largely supported by the analysis of virus genome sequences that are becoming available.

Sequencing of additional poxviruses from divergent species may cause an increase in the number of genera. For instance, poxviruses have been isolated from north American deer (Afonso et al. 2002), Finnish reindeer (Tikkanen et al. 2004), farmed Nile crocodiles (Horner 1988), Mongolian horses (Tulman et al. 2002a), South African sea lions (Wilson and Poglayen-Newall 1971), lizards (Stauber and Gogolewski 1990), kangaroos (Bagnall and Wilson 1974), squirrels (Thomas et al. 2003), South American rodents (Ueda et al. 1978; Esposito et al. 1980), and chameleons (Jacobson and Telford 1990). In addition, orthopoxviruses that resemble vaccinia virus strains were isolated from buffaloes in India (Dumbell and Richardson 1993), and cattle (de Souza Trindade et al. 2003) and rodents (Fonseca et al. 1998; da Fonseca et al., 2002) in South America. Possibly these viruses became endemic after transmission from man following widespread use of vaccinia virus as the smallpox vaccine.

IDENTIFICATION OF POXVIRUSES

Traditionally, the identification of poxviruses has been based upon biological observations, such as species of origin, growth properties in animal tissue or on the chorioallantoic membrane of the fertile hen's egg, reactivity with specific sera, and electron microscopy. These observations remain useful, but the rapid identification of a new virus as a poxvirus is now usually done by electron microscopy, polymerase chain reaction (PCR) (Meyer et al. 1994; Ropp et al. 1995; Heine et al. 1999), and genome sequencing. PCR shows great sensitivity, speed, and specificity. For instance, using specific sets of oligonucleotide primers it is possible to say not only that a new isolate is an orthopoxvirus, but which species it is. This was useful for the identification of the agent causing fever and generalized rash in humans in the midwest of USA during 2003 as monkeypox virus (Reed et al. 2004). PCR-based detection methods have been developed, validated, and deployed to detect variola virus that might be deliberately released by bioterrorists (see below) (Sofi Ibrahim et al. 2003).

PHYLOGENETIC RELATIONSHIPS

Genomes of representative members of all *Chordopoxvirus* genera have been sequenced (see http://www.poxvirus.org for details) and all except the parapoxviruses (that were sequenced most recently) have been used in phylogenetic comparisons (McLysaght et al. 2003; Upton et al. 2003; Gubser et al. 2004). These genomes range in size from 135 kb (Yaba monkey tumor virus) (Brunetti et al. 2003) to 288 kb (a pathogenic strain of fowlpox virus from the Animal Health Inspection Service Center for Veterinary Biologics, Ames, IA, USA) (Afonso et al. 2000) and from 36 percent A+T in genus *Parapoxvirus* to 75 percent A+T in capripoxvirus. All these genomes share a similar overall arrangement. In the central region (approximately 100 kb) the genes are conserved and many of these encode proteins that are essential for virus transcription, DNA replication, or assembly of new virions (Figure 2). In this region there are 90 genes that are conserved in all sequenced chordopoxviruses, but this number decreases to 49 when the entomopoxviruses are included, reflecting the greater diversity of the latter group. Genes in the terminal regions of the chordopoxvirus genomes are more variable and most are nonessential for virus replication in cell culture. Indeed, outside the central conserved region there is no gene that is conserved in all sequenced chordopoxviruses. These nonessential genes encode luxury functions that aid the virus in vivo and may function to inhibit aspects of the host immune response to infection, or influence host range and virulence (Smith and McFadden 2002). The types of genes that are present here reflect the different life styles that these viruses have evolved with their hosts.

A phylogenetic comparison of the chordopoxviruses (excluding parapoxviruses) using 17 of the 49 genes that are conserved in all poxviruses revealed that the sequenced *Chordopoxvirus* genera fall into four groups (Gubser et al. 2004) (Figure 3). An analysis using a different collection of genes reached a very similar conclusion (McLysaght et al. 2003). The most divergent group is the *Avipoxvirus* genus represented by *Fowlpox virus* (FPV). This avian virus has the largest genome (288 kb), the greatest number of genes that are unique to any genus (113), and the most divergent genome organization. Whereas genomes of all the other *Chordopoxvirus* genera have a consistent order and orientation of genes within the conserved, central region, FPV differs in that there are genome inversions and translocations (Figure 2).

The next most divergent group is the genus *Molluscipox virus*. This contains a single member, *Molluscum contagiosum virus*, of which there are two types, that infects only man. This virus also has many (70) unique genes that are not found in other *Chordopoxvirus* genera. It also lacks many genes found in other poxviruses such as those involved in immune regulation and instead has its own unique set of immunoregulators (Senkevich et al. 1996, 1997).

The third group includes the genera *Leporipoxvirus*, *Suipoxvirus*, *Yatapoxvirus*, and *Capripoxvirus*. These genomes are all smaller than those of the *Orthopoxvirus* genus and have several common features. For instance, the counterpart of *Vaccinia virus* strain Copenhagen gene *C7L* is present in these genera in the central region of the genome rather than being near the left end in members of the genus *Orthopoxvirus* (Figure 2).

The last group is represented by genus *Orthopoxvirus*. Orthopoxviruses have genomes of approximately 200 kb that are rich in adenine and thymine (67 percent) and form a tight cluster. They are distinguished from other chordopoxviruses by the presence of genes corresponding to *Vaccinia virus* strain Copenhagen genes *F14L*, *E7L*, and *O2L* within the central conserved region. Orthopoxviruses have been of greatest importance to man and most members of this genus have been sequenced. In addition to *Variola virus*, this genus includes *Vaccinia virus*, *Cowpox virus*, *Ectromelia virus*, the cause of mousepox, *Monkeypox virus*, and *Camelpox virus* (Table 1). Some of the names of these species are misnomers; for instance, neither *Cowpox virus* nor *Monkeypox virus* are naturally resident in cows or monkeys, instead they have rodent or squirrel reservoirs and may be transmitted to cows, monkeys, man, and other species as zoonoses (Khodakevich et al. 1987a; Crouch et al. 1995; Kulesh et al. 2004).

Although the parapoxviruses were not included in the above analysis, the genomes of orf virus and *Bovine papular stomatitis virus* were sequenced recently (Delhon

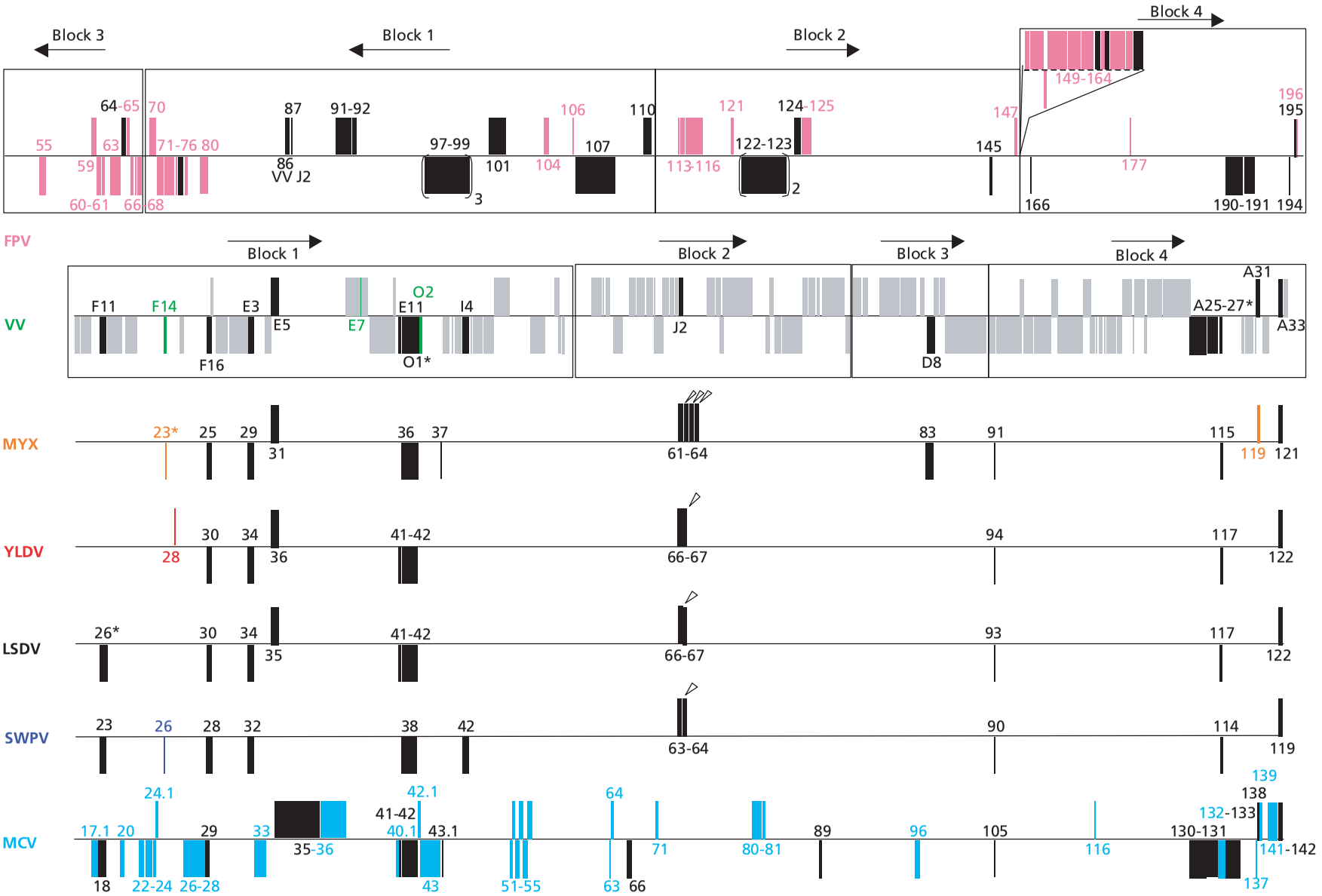

Figure 2 *Comparison of the gene content of the central region of the prototype virus from different Chordopoxvirus genera. vaccinia virus strain Copenhagen (VV) is the reference against which the other viruses (FPV, fowlpox virus; MYX, myxoma virus; YLDV, Yaba-like disease virus; LSDV, lumpy skin disease virus; SWPV, swinepox virus; MCV, Molluscum contagiosum disease virus) are compared. For each virus, genes (vertical bars) that are transcribed rightwards or leftwards are shown above or below the horizontal line, respectively. The 90 genes that are conserved in all sequenced chordopoxviruses are shown in gray for vaccinia virus. Genes conserved between two or more viruses from the different genera (but not one of the 90 conserved genes) are shown in black and genes that are unique to one genus are represented in the same color as the virus name. Genes that are present in only some members of a specific genus, but are fragmented or absent in other members of that genus, are indicated with an asterix. Block 1, counterparts of vaccinia virus strain Copenhagen F9L-G4L; block 2, counterparts of vaccinia virus strain Copenhagen G5R-D2L; block 3, counterparts of vaccinia virus strain Copenhagen D3R-A1L; block 4, counterparts of vaccinia virus strain Copenhagen A2L-A34R. The arrows illustrate the orientation of the blocks within the vaccinia virus and fowlpox virus (FPV) genomes. Arrowheads indicate the positions of orthologs of VV-COP gene C7L (Redrawn from Gubser et al. (2004) with permission).*

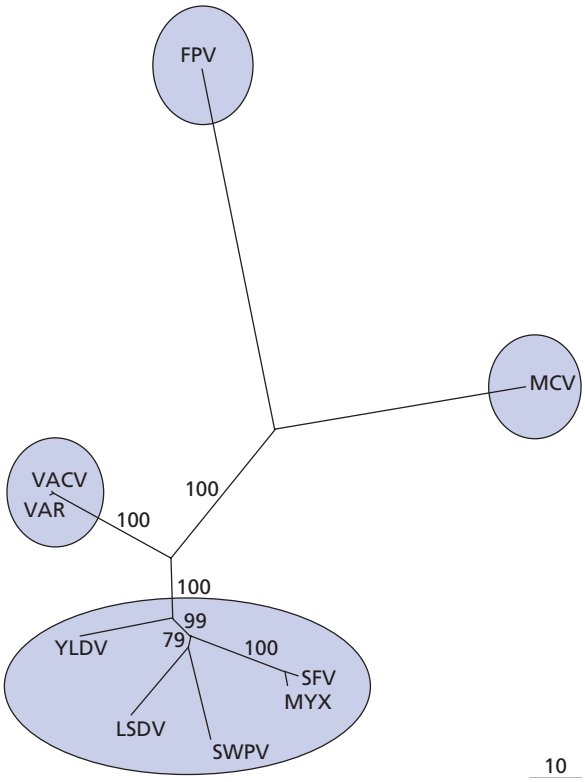

Figure 3 *Phyogenetic comparisons of chordopoxviruses. The amino acid sequence of 17 poxvirus proteins (corresponding to vaccinia virus strain Copenhagen E9L, I7L, I8R, G9R, J3R, J6R, H2R, H4L, H6R, D1R, D5R, D6R, D11L, D13L, A7L, A16L, and A24R) were aligned and an unrooted maximum-likelihood tree was obtained using the program ProML from the Phylip package version 3.0. The bootstrap values from 1 000 replica samplings and the divergence scale (substitutions/site) are indicated. Abbreviations as in Figure 2. Redrawn with modifications from Gubser et al.(2004) with permission.*

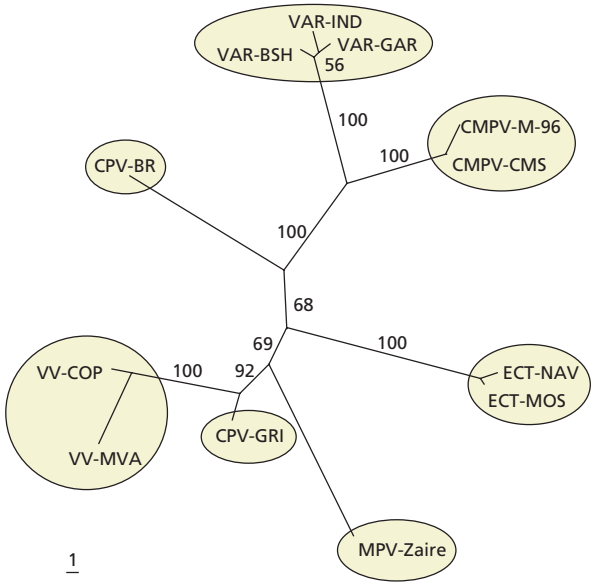

Figure 4 *Unrooted maximum likelihood phylogenetic tree showing the relationships of 12 orthopoxviruses. The amino acid sequences of proteins corresponding to vaccinia virus strain Copenhagen C6L, C7L, N1L, K2L, F2L, F4L, F6L, F8L, A56R, B1R, B5R, B15R (all encoded in the terminal regions of the genomes) were aligned and used to generate the tree using the program ProML. The bootstrap values from 1 000 replica samplings and the divergence scale (substitutions/site) are indicated. VAR-IND, variola virus strain India 1967; VAR-BSH, variola virus strain Bangladesh 1975; VAR-GAR, variola virus strain Garcia 1966; CPV-BR, cowpox virus Brighton Red; CPV-GRI, cowpox virus GRI-90; CMPV-CMS, camelpox virus strain CMS; CMPV-M-96; camelpox virus M-96; ECT-Nav, Ectromelia virus strain Naval; ECT-MOS, Ectromelia virus strain Moscow; VV-COP vaccinia virus strain Copenhagen; VV-MVA, vaccinia virus strain modified virus Ankara; MPV-Zaire, monkeypox virus strain Zaire. Redrawn with modifications from Gubser et al. (2004) with permission.*

et al. 2004), and phylogenetic trees including these viruses or sequences from other parapoxviruses (Tikkanen et al. 2004) showed that parapoxviruses are quite separate from other *Chordopoxvirus* genera, and the nearest genus is *Molluscipoxvirus*. The parapoxviruses share with molluscipoxviruses a high GC content and lack several genes encoding enzymes for nucleotide metabolism that are found in other chordopoxviruses.

A phylogenetic comparison of the orthopoxviruses using 12 genes that are conserved in the terminal regions of these genomes (Figure 4) showed that *Variola virus* and *Camelpox virus* are closely related (Gubser and Smith 2002), but *Monkeypox virus* is one of the most divergent viruses of this genus despite causing a disease in man similar to smallpox (Gubser et al. 2004). Another study using a different collection of genes conserved in these viruses came to a similar conclusion (McLysaght et al. 2003). These observations imply that one or more of these viruses have jumped species during their evolution; for *Variola virus*, a human virus, is closer to a virus that infects only camels than to *Monkeypox virus* that infects man, primates, and other species. Different strains of most *Orthopoxvirus* species form a tight cluster, consistent with their classification, however, with *Cowpox virus* the two sequenced members, Brighton Red (BR) and GRI-90 (Shchelkunov et al. 1998), are sufficiently divergent to justify their reclassification as separate species (Gubser et al. 2004). It will be interesting to compare the sequences of *Vaccinia virus* and *Cowpox virus* strains with the geographical site of isolation. Another interesting conclusion from the phylogenetic study of McLysaght et al. (2003) was that the rate of gene acquisition by the orthopoxviruses has been greater than that for *Leporipoxvirus*, *Suipoxvirus*, *Capripoxvirus*, and *Yatapoxvirus*.

There has also been significant gene loss during the evolution of the orthopoxviruses from the immediate ancestor of this group. This was shown by several genes being disrupted by mutation in one of more virus, but intact in at least one other (Aguado et al. 1992; Gubser and Smith 2002). An example is a gene encoding a soluble binding protein for interleukin (IL)-1β in many strains of *Vaccinia virus* and *Cowpox virus* (Alcami and

Smith 1992, 1996; Spriggs et al. 1992), but the disruption of this gene in all sequenced strains of variola virus. Similarly, a gene in *Vaccinia virus* that encodes an enzyme that synthesizes steroid hormones (Moore and Smith 1992) is disrupted in *Variola virus* strains into small fragments (Aguado et al. 1992; Shchelkunov et al. 1995).

There has also been recombination between *Orthopoxvirus* genomes during the evolution of these viruses. Evidence for this came from comparing the sequences of genes from the terminal regions of *Cowpox virus* strain BR and *Ectromelia virus* strain Naval with the corresponding genes from (1) the *Variola virus*/*Camelpox virus* subgroup; and (2) the *Vaccinia virus* subgroup (Gubser et al. 2004). This revealed that genes from the left end of *Cowpox virus* BR were closer to the *Vaccinia virus* subgroup than to the *Variola virus*/*Camelpox virus* subgroup. However, genes from the right end of *Cowpox virus* BR had the opposite relationship. Conversely, genes taken from the right end of *Ectromelia virus* Naval were closer to the *Vaccinia virus* subgroup, whereas genes from the left end were roughly equidistant from either group (Gubser et al. 2004).

VIRION STRUCTURE

This topic has been the subject of much debate and disagreement and is considered in detail in
Poxvirus replication. It is mentioned briefly here because the conclusion of this author differs from that of Moyer and Condit. Using vaccinia virus as the model poxvirus, there is general agreement that there are two forms of infectious virus produced from each infected cell, the intracellular mature virus (IMV) and extracellular enveloped virus (EEV), for review see Smith et al. (2002). There is also agreement that EEV contains one more membrane than IMV, but the number of membranes around IMV, how this (these) are formed and how each form of virus enters cells have been disputed. Originally, IMV was proposed to be surrounded by a single membrane that was synthesized de novo (Dales and Siminovitch 1961). Later, this model was challenged by workers who claimed there were two closed apposed membranes derived from the intermediate compartment of the cell, although it was acknowledged by these authors that 'the membranes of this cisterna are so tightly apposed that it is often difficult to distinguish the two bilayers' (Sodeik et al. 1993). Subsequently, measurement of the thickness of the IMV membrane structure found only a single lipid bilayer of 5 nm (equivalent to the thickness of cell membranes) and no continuity was detected with cell membranes (Hollinshead et al. 1999). Moreover, clear images were presented showing continuity between a single IMV membrane and the plasma membrane during IMV entry and no other membranes were visible (Armstrong et al. 1973; Chang and Metz 1976). Taken together, these data are interpreted by this author as unequivocal evidence that IMV has only a single membrane and therefore EEV has two. The challenge for the future is to elucidate how a single membrane is formed in the cytoplasm.

DISEASES CAUSED BY POXVIRUSES

Smallpox

Smallpox was a disease of man only and was a life-threatening systemic infection that spread through the body in several phases. Its impact on human health and its influence on our history can hardly be overstated. Even today, 25 years after the eradication of this disease, it has an impact as a potential bioterror weapon and has forced responses from governments around the world (see below).

Infection by variola virus was initiated by inhalation of virions released from the respiratory tract of infected patients or from scab material that had been ground into dust. A detailed study of the spread of virus from the respiratory system to other organs has been impossible in man for obvious reasons, but the mechanism of spread is believed to be similar to that of Ectromelia virus in mice, which causes the systemic disease mousepox (Buller and Palumbo 1991; Fenner 1992). With Ectromelia virus, infection spreads from the epithelium to the local lymph nodes where the virus replicates and is released into the blood stream (primary viremia) as free virions or within infected white cells. From here the virus causes an extensive infection of the reticuloendothelial system and higher levels of virus are released into the bloodstream (secondary viremia). Finally, the virus reaches the dermis where it induces formation of the characteristic skin lesions. With smallpox, there was a long incubation period before the patient became ill (8–14 days, average 11). Illness started with malaise, fever, aches, and prostration. The skin lesions progressed from papules to macules to blisters with a clear fluid containing virus. Eventually the lesions dried forming scabs that detached leaving a local scar. Once all scabs had detached from the body, the patient was no longer infectious. Smallpox lesions had a characteristic centrifugal distribution that was helpful in its diagnosis and distinction from chickenpox caused by a herpes virus, varicella-zoster virus. Smallpox lesions were abundant on the face, hands, and feet and less so on the trunk. In contrast, lesions caused by chickenpox are more abundant on the trunk than the periphery.

The mortality resulting from infection by variola virus depended upon whether infection was caused by variola major or variola minor and whether there was existing immunity. Infection by variola major in unvaccinated patients or immunologically naive populations caused up to 40 percent mortality, whereas with variola minor (also called alastrim) mortality was approximately 1 percent

(Fenner et al. 1988). Factors that predisposed to greater disease severity were immunological deficiency and pregnancy. The degree of protection provided by existing immunity depended on the time since vaccination, for epidemiological evidence showed that immunological protection does not endure for life. Those immunized long ago do have residual humoral or cell-mediated immunity or both (Hammarlund et al. 2003; Terajima et al. 2003), but it is uncertain if this will prevent infection or disease. Existing immunity may protect against mortality in most cases, but those infected might still transmit virus to others. Despite the sequencing of the genomes of minor and major strains of variola virus, it has not been possible to elucidate the basis for this large difference in virulence (Shchelkunov et al. 2000).

Monkeypox

This disease is caused by monkeypox virus, so called because it was isolated first from captive monkeys in Copenhagen in 1958, but its natural host is probably rodents and or squirrels in central and west Africa (Khodakevich et al. 1987a, b; Jezek and Fenner 1988; Kulesh et al. 2004). Monkeypox virus causes a disease in man that is clinically similar to smallpox and, until 2003, outbreaks were associated with significant mortality (1–10 percent). One important difference between monkeypox and smallpox is that the former is transmitted less efficiently from human to human and so outbreaks of monkeypox in man have tended to be isolated index cases or with very short chains of human transmission. The recognition of monkeypox as a human disease during the latter stages of the smallpox eradication campaign was of concern to the World Health Organization because it was feared that this virus might mutate into a variola-like virus and cause increased human-to-human transmission. Worse, if this virus retained its broad host range and became endemic in nonhuman hosts, it might be introduced repeatedly into man from an animal reservoir. However, these fears were largely dispelled by the sequencing of the monkeypox virus genome that showed that, within the *Orthopoxvirus* genus, monkeypox virus is one of the more divergent viruses from variola virus (Shchelkunov et al. 2001) and so evolution into a variola-like virus seems unlikely by passage and mutation.

In 2003, there was an outbreak of monkeypox in the USA that was caused by the importation of Gambian rodents (Di Giulio and Eckburg 2004; Reed et al. 2004). These rodents carried monkeypox virus without overt signs of illness, but transmitted the virus to American rodents, including prairie dogs, when cohoused in pet shops. The infected prairie dogs became ill and, as household pets, transmitted infection to man by close bodily contact, skin abrasions, or bites. Of the 81 humans infected, many became seriously ill, but there were no fatalities. Human infection was attributed to contact with infected animals rather than human-to-human transmission.

The epidemic outbreak of a febrile illness associated with a pustular rash caused concerns in USA that smallpox had re-emerged, and this fear was increased when the cause of the disease was diagnosed by electron microscopy as a poxvirus. However, these fears were allayed promptly when monkeypox virus was identified by PCR. Genome sequencing showed that the isolates from man and prairie dogs were very closely linked and were distinct from the strain of monkeypox virus that was sequenced from central Africa (Zaire) (Shchelkunov et al. 2001). This difference may explain the lack of mortality during the USA epidemic, compared with outbreaks in central Africa where mortality was up to 10 percent. Alternatively, the infected populations might have different inherent susceptibility to the disease or the disease outcome was affected by different quality of health care.

The episode of monkeypox in USA in 2003 has caused legislation banning the importation of exotic animals to be enacted and importation must now be subject to careful screening and quarantine.

Cowpox virus

Cowpox virus, like monkeypox virus, is a misnomer as the virus is not naturally resident in cows and its reservoir is probably rodents (Baxby et al. 1994; Crouch et al. 1995; Chantrey et al. 1999). However, cowpox virus has a broad host range and has infected many animals including rodents, large felines, domestic cats, cows, man, and elephants. In man, cowpox virus infections are usually single lesions (sometimes with satellites) on the hands that were acquired by handling infected animals. These can be painful and take weeks to resolve fully, but usually heal without lasting adverse effects, except for a local scar. The sequences of two cowpox virus strains (Brighton Red and GRI-90) showed that these viruses are quite diverse (see above) (Gubser et al. 2004); however, among the orthopoxviruses, the cowpox virus genomes have the greatest number of intact genes and the fewest number of broken genes fragments. This led to the suggestion that cowpox virus was closest to the ancestral poxvirus from which the other orthopoxviruses evolved (Shchelkunov et al. 1998).

Molluscum contagiosum virus

Molluscum contagiosum virus (MCV) is a strictly human virus that causes benign skin proliferations (often in children) that may persist for a year or more but then resolve. In patients with immunodeficiency caused, for instance, by infection with human immunodeficiency virus (HIV), MCV infections are more severe and a

greater problem. MCV cannot be cultivated in cell culture and its genome has diverged significantly from other chordopoxviruses (Senkevich et al. 1996). Seventy out of the 182 MCV genes are not found in other poxviruses, and some of these encode immunomodulatory proteins unique to MCV (Senkevich et al. 1997). A notable feature of MCV lesions is the walling off of the lesion from the underlying tissue and the restricted infiltration of inflammatory cells into the lesion itself. This is likely to be due to one or more of the immunomodulatory proteins encoded by MCV. The center of the MCV lesion is often packed with new virions that are shed from the lesion surface into the environment. Transmission is by close contact with virus shed form the skin surface.

Parapoxvirus

The genus *Parapoxvirus* includes viruses that infect sheep, goats, and deer (Table 1) and two members of this genus have been sequenced (Delhon et al. 2004). The disease caused by Orf virus has been called contagious pustular dermatitis or scabby mouth and causes significant economic loss in sheep due to the painful lesions on lips and teats that discourage feeding. Virus is shed into the environment and can be transmitted to new stock from infected pasture, or by direct animal to animal contact. A notable feature of orf infections is that animals can be reinfected indicating that prior infection does not induce solid immunity. For a review of *Parapoxvirus* biology, see Robinson and Lyttle (1992). Orf virus lesions have a peculiar pathology and lesions are highly vascularized and this may be due in part to expression of a virus-encoded vascular endothelial growth factor (Lyttle et al. 1994). The gene encoding this protein is nonessential for growth in cell culture but influences the outcome of infection in vivo (Savory et al. 2000). Orf infection can be transmitted to man where it causes dermal skin lesions that can be painful.

Other parapoxviruses include pseudocowpox virus, viruses that infect seals, and a virus of reindeer (Tikkanen et al. 2004). Historically, confusion between cowpox virus and pseudocowpox virus caused Jenner's claim about the efficacy of vaccination against smallpox to be challenged. The use of pseudocowpox virus, which was also obtained from the teats of cows, provided no protection against smallpox because the virus belongs to a different genus, and this caused some to state that Jenner was incorrect.

In cases where the genomes of members of this genus have been analyzed, the genomes are rich in guanosine and cytosine (G+C) (64 percent for Orf virus), but despite this, these viruses have a gene content and genome organization that is conserved with other chordopoxviruses. Overall, they are closest to the genus *Molluscipoxvirus*, and share some features with MCV, such as a G+C-rich genome and few genes for nucleotide metabolism (Delhon et al. 2004).

Leporipoxvirus

The leporipoxviruses infect rabbits. myxoma virus was isolated from its natural host in South America (*Sylvilagus* spp.) in which it does not cause severe disease, but in the European rabbit (*Oryctolagus cuniculus*) it causes the disease myxomatosis (McFadden 1988). Myxoma virus was used in the 1950s to control the huge population of European rabbits that had built up in Australia and its release and adaptation to its host provides an excellent example of how virus and host co-evolve (Fenner and Ratcliffe 1965). Initially, after the release of virulent virus most of the rabbits died and only virulent virus was isolated from the field. Subsequently, the virus strains present in the rabbit population were of more modest virulence, and the surviving rabbits became more resistant to the original virus. This demonstrates that evolution favors the emergence of pathogens of modest virulence. The basis for the changes in virulence of the myxoma viruses isolated from Australia is not understood.

The genome sequences of myxoma virus (Cameron et al. 1999) and Shope fibroma virus (Willer et al. 1999) showed more diversity exists between these leporipoxviruses than between members of genus *Orthopoxvirus*. But like the orthopoxviruses, the terminal regions of the leporipoxvirus genomes encode a diverse range of immunomodulatory proteins that inhibit aspects of the immune response to infection and that have been subject to genetic manipulation and functional characterization (Nash et al. 1999). The virulence of myxoma virus has been assessed largely in the European rabbit where it causes a fatal disease rather than in its natural host where the asymptomatic nature of the infection makes study of pathogenesis more difficult.

Capripoxvirus

The capripoxviruses infect sheep, goats, and cattle in parts of Africa and Asia and are a cause of significant mortality, morbidity, and economic loss. Transmission of infection can be via direct contact and entry through skin abrasions, close airborne transmission, or via arthropod vectors. Comparisons of the genomes (Gershon and Black 1988) and antigenicity (Kitching et al. 1986) of these viruses indicated that they are closely related; nonetheless, these viruses vary considerably in virulence and attenuated strains have been used as live vaccines. The genomes of lumpy skin disease virus and several sheeppox and goatpox viruses have been sequenced and compared (Tulman et al. 2001, 2002b). These studies identified some differences between virulent and attenuated strains near the genome termini, but which changes are responsible for attenuation is unknown. Like other chordopoxviruses, these viruses contain many genes encoding immunomodulators near the genome termini and several of these are unique to the genus *Capripoxvirus*.

Avipoxvirus

The genus *Avipoxvirus* includes a wide range of viruses that are distributed worldwide. Some of these viruses may cause severe disease and are a cause of economic loss in the poultry industry. Respiratory infection is usually more severe than dermal infection. The sequence of the prototype avipoxvirus, fowlpox virus, shows that genus *Avipoxvirus* is the most divergent genus of the *Chordopoxvirus* genera and has the largest genome and the greatest number of genes unique to this genus (see above). A notable feature of some strains of fowlpox virus was the presence of the provirus genome of an avian retrovirus, reticuloendotheliosis virus, integrated into the avipoxvirus genome (Hertig et al. 1997). Although there was no evidence of reverse transcriptase activity in fowlpox virus-infected cells, the integrated retrovirus is infectious because transfection of isolated virus DNA into uninfected cells yielded infectious retrovirus. Comparison of the genome sequences of a virulent (Afonso et al. 2000) and attenuated fowlpox virus genomes (Laidlaw and Skinner 2004) have identified many changes and the basis for attenuation is likely to be multifactorial.

Yatapoxvirus

The genus *Yatapoxirus* (Table 1) contains three members, tanapox virus, yaba monkey tumor virus (YMTV), and yaba-like disease virus (YLDV) and the genomes of the latter two viruses have been sequenced (Lee et al. 2001; Brunetti et al. 2003). *Tanapox virus* was first isolated in East Africa where it caused a febrile illness associated with one or more poxviruslike lesions, but the virus has also been isolated in Zaire and may be present across tropical Africa. The natural host is uncertain but the ability to cause disease only in monkeys would be consistent with a simian origin. Serological evidence of infection in African monkeys (Tsuchiya and Tagaya 1971; Downie 1974) established that these animals may be infected, but it remains unclear if monkeys are the natural host, or whether infections in monkeys are zoonoses after transmission from another reservoir. Human infections were more common close to rivers or following periods of flooding suggesting transmission by a biting arthropod. YMTV causes histiocytomas in rhesus monkeys and man (Niven et al. 1961).

POXVIRUSES AND BIOTERRORISM

The terrorist attacks in Washington DC and New York in 2001 and the subsequent release of anthrax spores in the USA prompted an urgent evaluation of whether there was adequate preparation against microbial pathogens that might be used in bioterrorism. If terrorists were prepared to commit suicide and mass murder by flying aeroplanes into civilian populations, it seemed probable that they might also be willing to obtain, grow, and release pathogenic micro-organisms. Top of the list of potential bioterror agents was variola virus. At the end of 2001, the preparedness for a smallpox epidemic was far from adequate. Smallpox vaccination had been discontinued for approximately 30 years and so roughly 40 percent of the population was immunologically naive for orthopoxviruses. Furthermore, it was uncertain if those vaccinated several decades previously had retained sufficient immunity to provide protection against variola virus. The stocks of vaccine were both old and limited. Indeed, during the 1990s, the World Health Organization (WHO) had ordered the destruction of millions of doses of vaccine that were deemed no longer required. The release of variola virus in a densely populated public place, perhaps a center of public transportation, might cause infection of many citizens. Given that smallpox takes an average of 11 days to develop following infection, those infected might have traveled far and wide and disseminated the infection to others before the outbreak was realized. Faced with this situation, health authorities would have an immensely challenging task of identifying all those exposed initially and all the contacts of those exposed. It was likely that the supply of vaccine would rapidly become exhausted.

Faced with this scenario, several governments initiated programs to replenish their stocks of smallpox vaccine, to increase surveillance for smallpox and to vaccinate some of their citizens. The US Government purchased sufficient vaccine to immunize each of its citizens and vaccination programs were initiated to protect approximately 500 000 first-line responders during the first quarter of 2003, approximately 11 million by the end of 2003 and, possibly, the whole population thereafter. In other western countries, the response was less radical. The UK Government vaccinated only 350 healthcare workers (www.doh.gov.uk) and bought sufficient vaccine to immunize the population if there were an outbreak. Elsewhere in Europe, some governments simply purchased vaccine, but did not vaccinate their citizens.

The decision not to vaccinate large fractions of the population was driven mostly by the fears of complications with the existing smallpox vaccines. During mass smallpox immunizations, it was recognized that immunization could cause complications such as progressive vaccinia, eczema vaccinatum, and neurological conditions with a death rate of approximately 1–5 per million vaccinees (Lane et al. 1969; Feery 1977). Those with immunological deficiency or eczema were more susceptible and pregnancy was also a condition that increased the risk of complications or their severity (Fenner et al. 1988). Today, the frequency of complications would probably be greater because a significant fraction of the population is immunosuppressed due to HIV infection and more people have eczema. In view of this, and to minimize the risk of complications, potential vaccinees

(and their close contacts) were screened carefully for contraindications to vaccination and vaccinees were monitored after vaccination. Despite this, complications occurred in the USA and the vaccination program has largely halted after approximately 500 000 vaccinations. An unexpectedly frequent complication was myocarditis (Cassimatis et al. 2004) that had been reported more rarely during previous smallpox vaccination programs (Feery 1977).

The strain of vaccine to be used in the new vaccination programs and to be stockpiled for national reserves was a source of contention. The smallpox vaccines strains that were widely used to achieve global eradication were Lister and New York City Board of Health (Wyeth) and these were effective at preventing smallpox by all strains of variola virus. Although, these vaccines were known to be effective, they were also known to have an imperfect safety record (Lane et al. 1969; Feery 1977). These vaccines had similar rates of complication, although precise comparisons are difficult because of differing populations in which these vaccines were used and different methods of assessing complication rates. Alternative vaccines based upon more attenuated strains of vaccinia virus such as LC18m8 in Japan (Hashizume et al. 1985) and modified virus Ankara (MVA) in Germany (Stickl et al. 1974) had been developed and shown to be safer in man, but their effectiveness at preventing smallpox was unproven because those immunized were never exposed naturally to variola virus. Faced with the choice of using an established vaccine that worked but caused complications, or a safer vaccine that was not proven to be effective, most governments bought the old vaccine. However, these new stocks of vaccine stocks were manufactured under modern good manufacturing practice conditions (in some cases after plaque purification) in sterile tissue culture and with rigorous screening for exogenous pathogens. In contrast, the old smallpox vaccines had been grown on the side of sheep or cattle and contained significant bacteriological contamination.

In countries where a cohort of first-line responders has been vaccinated, who would be able to treat smallpox patients without contracting and spreading the disease, there will be a need to maintain vaccination for as long as the perceived threat of smallpox remains. WHO recommended revaccination every 3 years for those in areas where smallpox was endemic.

ANTI-POXVIRUS DRUGS

The availability of an effective vaccine against smallpox was a disincentive for the development of effective drugs for combating smallpox or other poxvirus infections. However, the worries of bioterrorism and the potential to enhance the virulence of pathogenic poxviruses by genetic engineering (Jackson et al. 2001) have increased the need for effective drugs. Recent drug screening programs have identified several potentially useful drugs. The first of these is cidofovir, a licensed antiherpes virus drug used to combat human cytomegalovirus infections. Cidofovir prevents replication of many orthopoxviruses (including variola virus) in cell culture and can protect against orthopoxvirus infection in animal models (Bray et al. 2000). There are some disadvantages to cidofovir, however, such as renal toxicity and the need for the drug to be administered by intravenous injection. These difficulties are being overcome by the development of chemically modified versions of cidofovir. These can be taken orally, have enhanced activity against poxviruses, and, importantly, are no longer toxic for kidneys (Ciesla et al. 2003) but accumulate in the respiratory system, the site of primary infection by variola virus. Preliminary animal experiments show that the drug can protect against orthopoxviruses in vivo (Buller et al. 2004).

Cidofovir's mode of action is by inhibition of the virus DNA polymerase, but other virus proteins that are essential for virus replication are also being considered as targets for antipoxvirus drugs. These include dUTPase, topoisomerase, and proteins critical for virus morphogenesis. With all of these new drugs, although it will be possible to determine if they can protect against orthopoxviruses in animal models, it will be impossible to demonstrate efficacy against smallpox in man. This poses a regulatory problem for licensing.

IMMUNE RESPONSE TO SMALLPOX VACCINATION

During the smallpox eradication campaign, the immune response to vaccination had been evaluated with techniques that by today's standards were primitive. Moreover, the importance of immunity to the antigens specific to the EEV had not been fully appreciated and the immune response was evaluated predominantly against IMV. Tests such as hemagglutination inhibition (HAI), complement fixation, and plaque neutralization were employed, although the latter test was almost invariably performed against IMV. However, it was known that although vaccines composed of inactivated IMV induced very good levels of neutralizing antibody to IMV in man or animals, they failed to confer protection because they did not induce immune response to EEV antigens, for reviews see (Boulter and Appleyard 1973; Smith et al. 2002). Overall, there was only a rudimentary understanding of the immunological responses to smallpox vaccination and there was no clear benchmark that correlated with protection from smallpox. Instead a recent vaccination scar (within 3 years) was considered the best evidence.

Against this background, the recent smallpox vaccination programs in the USA and UK have provided an

opportunity to evaluate the response to smallpox vaccination using modern immunological techniques. These have included measurement of both cell-mediated and humoral immunity and have analyzed the antibody responses to IMV, EEV, and specific antigens from either virion. This is providing a benchmark against which the immunity remaining in individuals immunized decades previously or the immune response induced by new smallpox vaccines can be compared. An encouraging finding is that the immunological responses to vaccination are retained for several decades (Hammarlund et al. 2003; Terajima et al. 2003), although there must be caution in interpreting this as evidence of protection against smallpox.

The continuing worry about smallpox and bioterrorism, and the inadequate safety profile of traditional smallpox vaccines is prompting more research to develop safer vaccines. Strains LC16m8 and MVA are both being evaluated. MVA, in particular, shows promise. It was established that primary infection with MVA is safe in man and that this primary vaccination would cause the response to a subsequent vaccination with conventional vaccine to be modified, indicating existing immunity. MVA can also induce protection against challenge with other orthopoxviruses, such as vaccinia or monkeypox in rodent and animal models (Drexler et al. 2003; Earl et al. 2004). However, its efficacy in man remains unproven and a recent report showed that two immunizations with MVA induced inferior protection against monkeypox virus challenge than a single immunization with a conventional vaccine (DRYVAX) (Earl et al. 2004).

LC16m8 also has a good safety record in man and primary vaccination induced less severe reactions than those induced by the Lister strain from which LC16m8 was derived (Hashizume et al. 1985). However, a potential difficulty with LC16m8 is a mutation in the B5R gene that prevents expression of the B5R protein (Takahashi-Nishimaki et al. 1991). As well as being required to make a normal plaque size, B5R has been identified as the only EEV-specific protein that is the target for antibodies that neutralize EEV infectivity (Galmiche et al. 1999; Law and Smith 2001; Hooper et al. 2003). Given that responses to EEV are important for protection against orthopoxviruses, the omission of *B5R* is a potential problem for this vaccine. It would seem logical in the design of new smallpox vaccines to include this antigen.

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Poxvirus replication

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POXVIRUS CLASSIFICATION AND VIRION STRUCTURE

Poxviruses are one of the most complex DNA-containing virus families. The strategy for growth and reproduction of this family of viruses is sufficiently robust and adaptable to have become established in a variety of eukaryotic hosts from insects, birds, and reptiles to higher vertebrates including man. Taxonomically, the *Poxviridae* family comprises two subfamilies: the *Chordopoxvirinae* (viruses of vertebrates) and the *Entomopoxvirinae* (viruses of insects) (Moyer et al. 2000) (Table 1). There are eight *Chordopoxvirinae* genera: *orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus*, of which four (*orthopoxvirus*, *Parapoxvirus*, *Yatapoxvirus*, and *Molluscipoxvirus*) can cause human infections. Of these, the most clinically significant diseases are those caused by the orthopoxvirus *Variola virus* (VARV) (smallpox) and *Monkeypox virus* (MPXV), and the molluscipoxvirus *Molluscum contagiosum virus* (MOCV) (Esposito and Fenner 2001). There are three genera of entomopoxviruses, designated A, B, and C, based mainly on host range. Many entomopoxviruses await further classification.

Poxvirus family members share a number of unique features including:

- a large complex enveloped virion that appears as an enveloped, brick-shaped structure and is of sufficient size to be visualized under a light microscope
- the packaging in virions of a variety of enzymes including all those required to carry out transcription of poxvirus early genes from the DNA contained within the virus
- a cytoplasmic site of replication
- a double-stranded genomic DNA which can range in size from approximately 144 kb (Yaba-like disease virus, Lee et al. 2001) to nearly 300 kb (*Fowlpox virus*, Afonso et al. 2000) and up to nearly 400 kb for some entomopoxviruses (Moyer et al. 2000)
- a hairpin loop of DNA comprised of imperfectly paired heteroduplex DNA present at both termini of the genome
- a complex mechanism for assembly of infectious virions.

The overall virus growth cycle is depicted in Figure 1 p. 3 and will be expanded upon throughout this chapter.

Vertebrate poxviruses also share a co-linear core of conserved genes located within the central-most region of the genome (Figure 2 p. 3). These core genes comprise about 60 percent of the coding capacity of the virus. They are generally required for virus growth and are thus associated with essential virus functions such as transcription and DNA replication. On the other hand, the 'variable regions,' comprising the remainder of the genome extending to both termini, contain genes many of which are not essential for growth, including those devoted to control of host range as well as the responses of the cell or animal host to infection. While entomopoxviruses also contain most genes of the common

Table 1 *Family* Poxviridae *taxonomy*

Subfamilies	Genera	Member viruses	Features
Chordopoxvirinae	*Orthopoxvirus*	Camelpox,[c] cowpox,[c] ectromelia,[c] MPXV,[c] raccoonpox, skunkpox, Uasin Gishu,[a] vaccinia,[b,c] variola,[c] volepox	Brick-shaped virion, DNA ca. 200 kbp, G+C ca. 36%, wide to narrow host range, variola (smallpox), vaccinia (smallpox vaccine)
	Parapoxvirus	Auzduk disease,[a] chamois contagious ecthyma,[a] orf,[b] pseudocowpox, parapox of deer, sealpox[a]	Ovoid virion, DNA ca. 140 kbp, G+C ca. 64%
	Avipoxvirus	Canarypox, fowlpox,[b,c] juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox,[a] penguinpox,[a] sparrowpox, starlingpox, turkeypox	Brick-shaped virion, DNA ca. 260 kbp, G+C ca. 35%, birds, arthropod transmission
	Capripoxvirus	Goatpox, lumpy skin disease, sheeppox[b]	Brick-shaped virion, DNA ca. 150 kbp, ungulates, arthropod transmission
	Leporipoxvirus	Hare fibroma, myxoma,[b,c] rabbit fibroma,[c] squirrel fibroma	Brick-shaped virion, DNA ca. 160 kbp, G+C ca. 40%, leoporids and squirrels
	Suipoxvirus	Swinepox[c]	Brick-shaped virion, DNA ca. 170 kbp, narrow host range
	Molluscipoxvirus	MOCV	Brick-shaped virion, DNA ca. 180 kbp, G+C ca. 60%, human host, localized tumors, contact spread
	Yatapoxvirus	Tanapox, Yaba monkey tumor[b]	Brick-shaped virion, DNA ca. 145 kbp, G+C ca. 33%, primates and ? rodents
Entomopoxvirinae	*Entomopoxvirus A*	Melontha melontha[b]	Ovoid virion, DNA ca. 260–370 kbp, Coleoptera
	Entomopoxvirus B	Amsacta moorei[b,c]	Ovoid, DNA ca. 236 kbp, G+C ca. 18%, Lepidoptera and Orthoptera
	Entomopoxvirus C	Chrionimus luridus[b]	Brick-shaped virion, DNA ca. 250–380 kbp, Diptera
	Unclassified	Melanoplus sanguinipes[c]	

a) Probable member of genus.
b) Prototypal member.
c) Completely sequenced.

vertebrate poxvirus core, the genes are not conserved in the typical co-linear vertebrate virus core fashion, but rather are dispersed throughout the genome. The significance, if any, of the altered organization pattern in entomopoxviruses is not clear.

In the case of vertebrate viruses, while the diseases caused by individual viruses can vary greatly, the molecular events which govern basic functions such as transcription, DNA synthesis, and morphogenesis are quite similar. What distinguishes individual viruses physiologically, and is likely responsible at least in part for the distinct clinical syndromes, are the genes associated with host range and the different sets of immunomodulatory genes, found mainly in the variable regions of the genome. Much of what we know about *orthopoxvirus* genes of this nature has been derived from studies with Vaccinia virus. We will discuss some of these host range and immunomodulatory genes later in this chapter.

Some comment on the poxvirus gene nomenclature used for this chapter is in order. The first poxvirus genome to be sequenced in its entirety was vaccinia Copenhagen (Goebel et al. 1990). The open reading frame (ORF) designations adopted for the Copenhagen sequence were based on the *Hin*dIII restriction map of the genome and the direction of transcription. For example, the viral DNA topoisomerase was designated as H6R, the sixth ORF from the left end of the *Hin*dIII H DNA fragment, transcribed in a rightward direction. Because it was the first complete vaccinia sequence available, the Copenhagen nomenclature became the standard in the field. However, the vaccinia strain WR has been the de facto strain used for experimentation for more than 30 years, even though the complete WR genome sequence only recently became available. Recent nomenclature rules adopted by the International Committee on Taxonomy of Viruses (ICTV) and applied to the more recently sequenced poxvirus genomes uses a linear designation beginning at the left-hand end of the genome and continuing to the right end of the genome. Using the newer nomenclature, the WR ortholog of the vaccinia Copenhagen *H6R* gene is

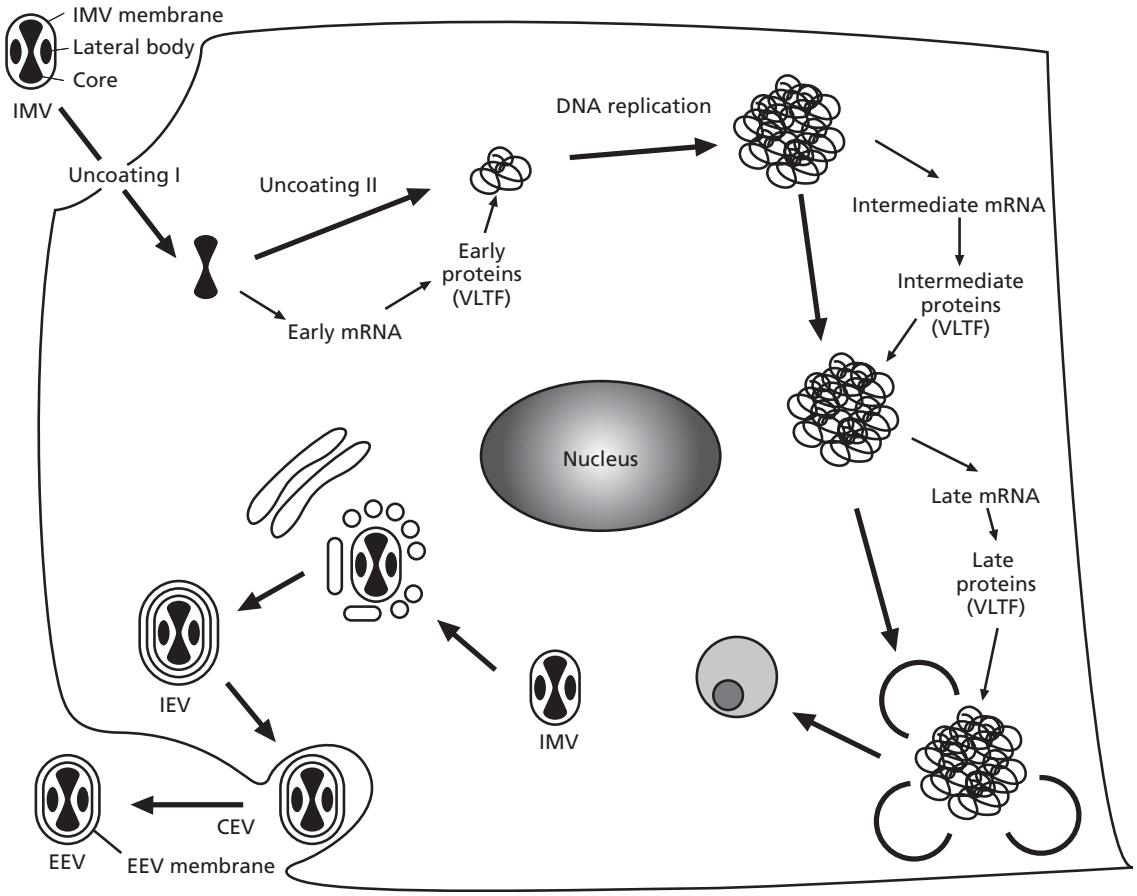

Figure 1 *The poxvirus growth cycle. Features of growth shared by all poxviruses include a temporal pattern of gene expression, a cytoplasmic site of development. Orthopoxviruses, such as variola virus and vaccinia virus, produce two distinct forms of virions. Initially, IMV is the first infectious virus formed. A small portion of that virus is wrapped within two additional layers of Golgi-derived membranes to form IEV. IEV is externalized by fusion of the outermost membrane of IEV with the plasma membrane of the cell to form CEV. CEV released from the cell surface produces free EEV. CEV, cell-associated enveloped virus; EEV, extracellular enveloped virus; IEV, intracellular enveloped virus; IMV, intracellular mature virus; VETF, viral early transcription factors; VITF, viral intermediate transcription factors; VLTF, viral late transcription factors.*

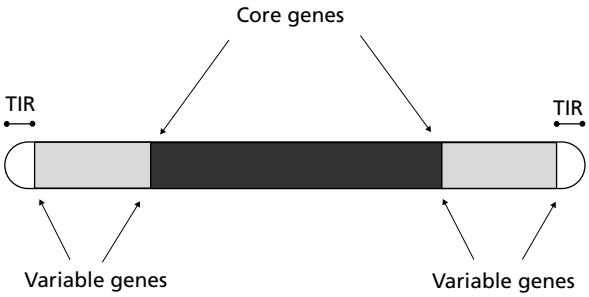

Figure 2 *The genetic organization of vertebrate poxviruses. The poxvirus is comprised of double-stranded DNA but is variable, ranging from 130 to 330 kb in individual viruses. The central-most region of the viral chromosome contains a generally conserved linear core of genes, most of which are shared by all vertebrate poxviruses. Genes within the regions to the left and right of the core gene region are the most variable and serve to distinguish the individual members of the family. Genes within this region tend to be virus-specific. The termini of the genome are highlighted by imperfectly paired TIRs. The two DNA strands are linked, resistant to exonuclease digestion, and thus form a loop or hairpin.*

designated as *VACV-WR_104*. So while acknowledging the history of the field and the use of Copenhagen nomenclature in much of the literature to date, yet mindful that vaccinia WR is the prototypic vaccinia strain and that nomenclature procedures have been changed, we have designated ORFs by both the original Copenhagen and the newer vaccinia WR designations where possible.

Virions and virion structure

The poxvirus virion structure is both unique and unconventional compared to other viruses. In general, poxvirus virions bear none of the classical features of a helical or icosahedral nucleocapsid, and while they are enveloped, the structure and biogenesis of the envelope is unusual.

Mature poxvirus virions exist in four forms which differ in the number of membranes and hence surface antigens surrounding the particle (reviewed by Smith et al. 2002). The four forms are designated intracellular

mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV) (Figures 1 and 11). IMV, the first assembled and simplest infectious form of virus, is a membraned particle that, as the name implies, remains inside cells following virus maturation (Boulter and Appleyard 1973). IEV is an intermediate in the formation of CEV and EEV, these latter two being infectious extracellular forms of virus. IEV is essentially IMV that has acquired two additional membranes via wrapping in Golgi-derived cisternae (Ichihashi et al. 1971). CEV is derived from IEV by fusion of the outermost IEV membrane with the plasma membrane (Blasco and Moss 1992). CEV is thus essentially an IMV particle that is wrapped in one additional lipid bilayer and that remains attached to the outer surface of the cell. EEV is CEV that has been released from the surface of the cell (Boulter and Appleyard 1973). IEV contains an additional seven membrane proteins not found in IMV, while CEV and EEV each contain five of these seven proteins (Smith et al. 2002). Although it is a membraned particle, IMV is extraordinarily stable, and therefore presumably provides the virus with a mechanism for maintaining infectivity in the environment between hosts over long periods of time. EEV and CEV are thought to be critical for cell-to-cell spread during infection. Thus, collectively, the multiple forms of poxviruses encompass all of the advantages of both enveloped and non-enveloped viruses.

EEV is experimentally distinguished from IMV based on antigenic differences due to the presence of different surface components, and by the fact that the extra lipid envelope confers a lighter buoyant density to the EEV particle compared to IMV (reviewed by Smith et al. 2002). The distinction between the various forms of virus is also clinically significant. It has been proposed that prevention of disease caused by orthopoxviruses is primarily related to EEV particles rather than IMV. For example, during the smallpox eradication program, some vaccines were prepared from inactivated virus obtained following disruption of infected cells (IMV). These vaccines were largely ineffective in preventing orthopoxvirus infections in humans (Kaplan 1962; Kaplan et al. 1962, 1965). Indeed, the relative efficacy of anti-IMV and EEV antisera to prevent spread of the IMV-induced infections in animals has clearly shown that anti-EEV antibodies are of much greater benefit even when there is a subsequent virus challenge (Boulter et al. 1971; Boulter and Appleyard 1973). Although EEV production is typically low compared to IMV, there are two experimental methods by which to assay and verify the presence of EEV. The first is the test for 'comets' (Law et al. 2002) where strains that produce high levels of EEV, such as vaccinia strain IHD (International Health Division) or rabbitpox virus (RPV), are allowed to grow on cell monolayers in the absence of the typical agar overlay. Such strains will generate 'comet-shaped' rather than spherical plaques, resulting from release of EEV from the primary plaque and spread of the infection to distant sites on the monolayer. Comets are inhibited by antisera to EEV but not IMV. The second method represents a modified neutralization assay in which EEV is pretreated with antibody against inactivated IMV. EEV escapes neutralization by this antibody and will produce a plaque when titered.

The structure of intact IMV, the simplest and predominant form of the virus, has been extensively studied by electron microscopy (EM). Analysis of whole virions by cryo-electron microscopy, which is thought to preserve best the natural surface features of the virion, reveals a rectangular structure with dimensions of 350×270 nm, including a 30 nm thick 'surface domain' which is less electron dense relative to the inner domain of the particle (Figure 3) (Dubochet et al. 1994; Griffiths et al. 2001b). By contrast, conventional EM of negatively stained, dehydrated samples of purified IMV reveal numerous rodlike protrusions, called surface tubule elements (STE), on or near the surface of the virion (Figure 3) (Wilton et al. 1995). Cross-sections of IMV reveal four distinct virion substructures: the envelope, the lateral bodies, the core wall, and the core (Figure 3) (Dales and Pogo 1981; Ichihashi et al. 1984; Griffiths et al. 2001a, 2001b). In many sections, the core and its wall possess a biconcave dumbbell or peanut shape; however, in many sections the core appears round, rectangular, or even somewhat irregularly shaped, ultimately suggesting a three-dimensional structure similar to an elongated biconcave disk. The core wall is a bilaminar structure, with an inner smooth layer approximately 5 nm thick and an outer layer, sometimes referred to as the 'palisade layer,' made up of regularly arranged projections approximately 10 nm in length and 5 nm in diameter (Easterbrook 1966). The lateral bodies are uniform in electron density and fill the space between the concavities in the core and the envelope. The envelope, which contains the lipid outer membrane, is also a bilaminar structure, with an inner layer approximately 5 nm thick and an outer layer approximately 9 nm thick (Hollinshead et al. 1999).

IMV substructure

The precise biochemical make-up of the poxvirus virion has been investigated by controlled degradation of purified virions coupled with deductions made from studies of virus assembly. Virions contain as many as 100 proteins, most, if not all, of which are virus coded (Essani and Dales 1979; Jensen et al. 1996). The precise substructure of the virion envelope is controversial: while some studies conclude that the envelope contains a single lipid bilayer, others conclude that it contains two tightly apposed bilayers (Hollinshead et al. 1999; Sodeik and Krijnse-Locker 2002) (see section on Poxvirus morphogenesis: IV and IMV membrane formation, below).

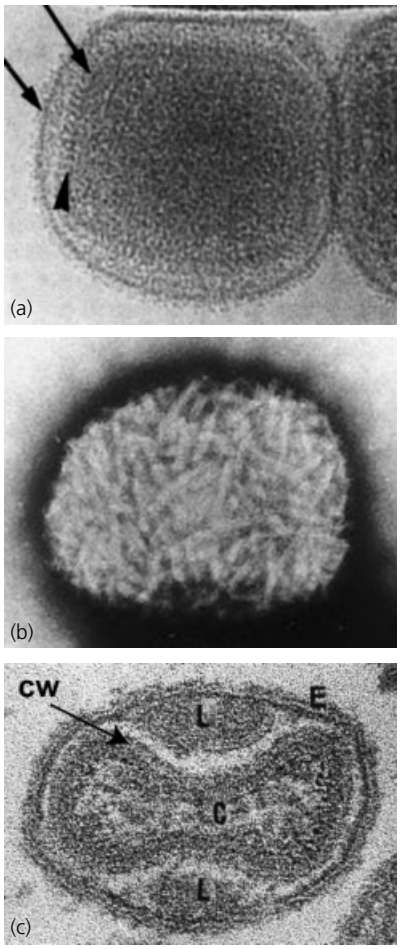

Figure 3 *Vaccinia virion structure.* **(a)** *Cryo-election microscopy of positively stained isolated particles. Two layers are evident (arrows) that are separated by a spike or spicule layer (arrowhead).* **(b)** *Negatively stained isolated particles showing surface tubule elements.* **(c)** *Thin section of a vaccinia virion showing envelope (E), lateral bodies (L), and the core (C) surrounded by the core wall (CW).* **(a)** *is reproduced from Griffiths et al. 2001b;* **(b)** *from Wilton et al. 1995;* **(c)** *from Pogo and Dales 1969.*

Treatment of virus with neutral detergent and a reducing agent solubilizes the envelope and as many as a dozen envelope-associated proteins (Easterbrook 1966; Ichihashi et al. 1984; Jensen et al. 1996). Under appropriate conditions, some of these solubilized proteins are contained in tubular structures which may be equivalent to the STEs seen in dehydrated, negatively stained intact IMV (Wilton et al. 1995). Several of the IMV envelope proteins exist in a disulfide-bonded complex (Rodriguez et al. 1997). Upon solubilization of the outer envelope, the virion core, which now assumes a more rectangular or brick-shaped structure, can be isolated with the lateral bodies still attached (Easterbrook 1966). The lateral bodies can be removed with controlled trypsin treatment and therefore are proteinaceous in nature. The exact chemical composition of the core wall is unclear. While some investigators contend that the core wall contains a true lipid bilayer (or two tightly apposed bilayers), others argue that it contains no lipid (Wilton et al. 1995; Griffiths et al. 2001b). At least one of the major virion structural proteins, 4b, localizes to the outer surface of the core wall (Ichihashi et al. 1984; Wilton et al. 1995). Within the core resides the viral DNA complexed with several virus-coded DNA-binding proteins (Ichihashi et al. 1984).

Because poxviruses initiate infection and thereafter express information from a DNA molecule in the cell cytoplasm, the virions contain the full complement of enzymes required for synthesis of early viral mRNA. Virion cores are thus a rich source of the viral enzymes necessary for viral transcription and modification (Kates and McAuslan 1967; Kates and Beeson 1970a; Shuman and Moss 1990) (Table 2). The subviral core is capable of synthesizing authentic early viral mRNAs in vitro which are capped, methylated, and polyadenylated (Kates and Beeson 1970b; Wei and Moss 1975; Bossart et al. 1978; Pelham et al. 1978). The enzymology of viral mRNA metabolism is discussed in detail below (see section on Poxvirus transcription and regulation of viral gene expression). The precise localization and organization of viral enzymes within the core is not known. Presumably, as the virus particle transverses the cellular membrane during the entry process, partial uncoating of the intact IMV must occur, thereby creating the transcriptionally active core (Pedersen et al. 2000).

In addition to the enzymes and factors listed in Table 2, which have an obvious direct role in RNA metabolism, the virion contains other enzymes with activities suggesting roles in nucleic acid or protein modification (Table 3). These include a topoisomerase (H6R/VACV-WR_104), an RNA helicase (I8R/VACV-WR_077), two protein kinases (F10L/VACV-WR_049 and B1R/VACV-WR_183), a protein phosphatase (H1L/VACV-WR_099), a glutaredoxin (O2L/VACV-WR_069), and three thiol oxidoreductases (G4L/VACV-COP_099, E10R/VACV-COP_082, and A2.5L/VACV-WR_121). Mutants in the RNA helicase are defective in the virus core-directed in vitro transcription reaction in a fashion which suggests that the RNA helicase may play a role in transporting newly synthesized early viral mRNA out of the core particle (Gross and Shuman 1996). For reasons unknown, mutants in the H1L phosphatase are also defective in early viral transcription (Liu et al. 1995). The F10L protein kinase seems to play a role in regulation of virion morphogenesis via phosphorylation, discussed in more detail below (see section on Poxvirus morphogenesis: IV and IMV membrane formation) (Traktman et al. 1995). The B1R kinase plays an essential role in viral DNA replication, discussed in more detail below (see section on The structure and replication of poxvirus DNA: The enzymology of DNA replication) (Rempel and Traktman 1992). While the O2L glutaredoxin is nonessential, the G4L, E10R, and A2.5L thiol reductases form a complex which comprises a complete disulfide-bonding pathway which targets at least two other vaccinia

Table 2 *Virus-coded mRNA metabolism enzymes and factors*

Function/Enzyme or factor[a]/Encoding gene(s) (common/Copenhagen/WR)[b]	Mol mass (kDa)	Virion[c]	Homologies; alternate functions	Reference[d]
RNA polymerization				
RNA polymerase				
RPO 147/J6R/VACV-WR_098	147	Yes	Homology to eucaryotic RNA polymerase	Broyles and Moss 1986
RPO 132/A24R/VACV-WR_144	133	Yes	Homology to eukaryotic RNA polymerase	Amegadzie et al. 1991b
RAP 94/H4L/VACV-WR_102	94	Yes	Early promoter specificity; early termination	Ahn et al. 1994
RPO 35/A29L/VACV-WR_152	35	Yes		Amegadzie et al. 1991a
RPO 30/E4L/VACV-WR_060	30	Yes	TFIIS homology; VITF-1	Ahn et al. 1990a
RPO 22/J4R/VACV-WR_096	21	Yes		Broyles and Moss 1986
RPO 19/A5R/VACV-WR_124	19	Yes		Ahn et al. 1992
RPO 18/D7R/VACV-WR_112	18	Yes		Ahn et al. 1990b
RPO 7/G5.5R/VACV-WR_083	7	Yes	Homology to eukaryotic RNA polymerase	Amegadzie et al. 1992
mRNA capping				
Capping enzyme				
DIR/VACV-WR_106	97	Yes	Early termination, intermediate transcription	Morgan et al. 1984
D12L/VACV-WR_117	33	Yes	Early termination, intermediate transcription	Niles et al. 1989
(Nucleoside-2′-*O*-)-methyltransferase				
VP39/J3R/VACV-WR_095	39	Yes	Poly(A) polymerase subunit; intermediate/late elongation	Schnierle et al. 1992
mRNA polyadenylation				
Poly(A) polymerase				
VP55/VACV-WR_057	55	Yes		Gershon et al. 1991
VP39/J3R/VACV-WR_095	39	Yes	mRNA cap methylation; intermediate/late elongation	Gershon et al. 1991
Early transcription factors				
VETF				
A7L/VACV-WR_126	82	Yes		Gershon and Moss 1990
D6R/VACV-WR_111	73	Yes		Gershon and Moss 1990
Early termination factors				
VTF				
D1R/VACV-WR_106	97	Yes	mRNA capping, intermediate transcription	Shuman et al. 1987
D12L/VACV-WR_117	33	Yes	mRNA capping, intermediate transcription	Shuman et al. 1987
Nucleoside phosphohydrolase I				
D11L/VACV-WR_116	72	Yes		Deng and Shuman 1998

(*Continued over*)

Table 2 *Virus-coded mRNA metabolism enzymes and factors (Continued)*

Function/Enzyme or factor[a]/Encoding gene(s) (common/Copenhagen/WR)[b]	Mol mass (kDa)	Virion[c]	Homologies; alternate functions	Reference[d]
H4L				
RAP 94/H4L/VACV-WR_102	94	Yes	RNA polymerase subunit; early promoter specificity	Mohamed and Niles 2001
Intermediate transcription factors				
Capping enzyme				
D1R/ VACV-WR_106	97	Yes	Early termination, mRNA capping	Vos et al. 1991
D12L/ VACV-WR_117	33	Yes	Early termination, mRNA capping	Vos et al. 1991
VITF-1				
RPO 30/E4L/VACV-WR_060	30	Yes	RNA polymerase subunit; TFIIS homology	Rosales et al. 1994a
VITF-3				
A23R/VACV-WR_143	44	No		Sanz and Moss 1999
A8R/VACV-WR_127	33	No		Sanz and Moss 1999
Late transcription factors				
VLTF-1				
G8R/VACV-WR_086	30	No		Keck et al. 1990
VLTF-2				
A1L/VACV-WR_119	17	No		Keck et al. 1990
VLTF-3				
A2L/VACV-WR_120	26	No		Keck et al. 1990
VLTF-4				
H5R/VACV-WR_103	22	No		
Intermediate/late elongation factors				
Negative elongation/RNA release factor				
A18R/VACV-WR_138	57	Yes		Lackner and Condit 2000
Positive elongation factor				
G2R/VACV-WR_080	26	No		Black and Condit 1996
VP39/J3R/VACV-WR_095	39	Yes	mRNA cap methylation; poly(A) polymerase	Latner et al. 2000

a) Multiple genes indented under a single enzyme/factor designates a multisubunit enzyme/factor.
b) See text for description of nomenclature. Many genes do not have a 'common' name, thus only the Copenhagen and WR designations are used.
c) Designates whether or not the gene product is packaged in virions.
d) A single reference is given that identifies the gene or assigns an alternative function. See text for additional references.

structural proteins and is essential for successful virion morphogenesis (Senkevich et al. 2002). While the biochemistry of the topoisomerase is understood in exquisite detail, the role of this enzyme in poxvirus replication is not known because no virus mutants in the *H6R* gene have been isolated (Tian et al. 2003).

THE STRUCTURE AND REPLICATION OF POXVIRUS DNA

The structure of poxvirus DNA

There are several observations and structural features relevant to poxvirus DNA replication which must be considered within the context of replication. First is the rather unusual structure of the viral DNA, which is comprised of linear double-stranded DNA, terminating in inverted repeats linked through an imperfectly paired DNA 'loop' or 'hairpin' (Figures 2 and 31.4a). Second, during the process of replication, concatemeric intermediates are formed, necessitating the need to excise functional monomeric molecules as well as to recreate the terminal hairpins or loops at the end of the genome. Third, consistent with the cytoplasmic nature of these viruses, DNA replication does not occur within the nucleus, which demands that the virus encode a variety of replicative enzymes whose cellular equivalents are confined to the nucleus.

Table 3 *Vaccinia virion enzymes*[a]

Function/Enzyme or factor[b]/Encoding gene(s) (common/Copenhagen/WR)[c]	Mol. mass (kDa)	Comment	Reference[d]
Protein modification			
F10L ser/thr kinase *F10L/VACV-WR_049*	52	Required for virion morphogenesis	Traktman et al. 1995
B1R ser/thr kinase *B1R/VACV-WR_183*	34	Required for DNA replication	Rempel and Traktman 1992
Tyr/Ser protein phosphatase *VH1/H1L/VACV-WR_099*	20	Required for early transcription	Guan et al. 1991
Thiol oxidoreductase *E10R/VACV-COP_082*	11	Required for virion morphogenesis	Senkevich et al. 2002
G4L/VACV-COP_099	14	Required for virion morphogenesis	Senkevich et al. 2002
A2.5L/VACV-WR_121	9	Required for virion morphogenesis	Senkevich et al. 2002
Glutaredoxin *O2L/VACV-WR_069*	12	Non-essential	Rajagopal et al. 1995
Nucleic acid topology			
Topoisomerase *H6R/VACV-WR_104*	37	Function unknown	Cheng et al. 1998
RNA helicase *I8R/VACV-WR_077*	78	Required for early transcription	Gross and Shuman 1996

a) Enzymes in this table are those that have not been shown to play a direct role in transcription. See Table 2 for virion transcription enzymes.
b) Multiple genes indented under a single enzyme/factor designates a multisubunit enzyme/factor.
c) See text for description of nomenclature. Many genes do not have a 'common' name; thus only the Copenhagen and WR designations are used.
d) A single reference is given which identifies the gene or assigns a function. See text for additional references.

The terminal most region of DNA comprising the hairpin or loop abuts a series of tandem repeats of various sizes (Figure 4b). The DNA within the hairpin or loop per se that 'caps' the larger terminal inverted sequence region (TIR) serves to link the two complementary strands and creates a telomere comprised of an imperfectly paired A+T-rich region of DNA (Figure 4b, c). The hairpins exist in two forms, sometimes call 'flip' and 'flop,' which are perfect inverse complements of each other, such that when base paired with each other they form a perfect duplex (Figure 4c, d). The duplexed flip and flop hairpins form the junction between concatemers (Figure 4d), and have important implications in the mechanism of DNA replication described below. Following and next to each terminal hairpin, the TIRs present at both the left and right extremes of the molecule continue inward. The total length of TIRs can vary from as little as 58 bp in variola virus (Shchelkunov et al. 1995) to more than 12 kb in Shope fibroma virus, a *Leporipoxvirus* (Willer et al. 1999). Indeed, there is at least one example where the TIRs have been shown to exhibit marked variation in size and sequence (up to 30 kb) from different plaque isolates of one virus (Figure 5) (Moyer and Graves 1981). A possible mechanism for the generation of variable TIRs is discussed later in the chapter. Because the TIR sequences are by definition repeated at both termini of the genome, ORFs contained within the TIRs are diploid. The sequences at the very left or right extremes of the TIRs nearest the hairpin which contain the series of small tandem, conserved repeats also contain a sequence which serves as a 'nicking site' to initiate the DNA replication process. This nicking site may also be involved in the maturation of mature length molecules from replicative DNA concatemeric intermediates (Moyer and Graves 1981). Viral DNA concatemers are not only a key feature and intermediate in poxvirus genome replication but they also allow for rapid evolution, adaptation, and diversity at the terminal extremes of the molecule which in turn constitute the primary genetic distinctions among individual poxviruses.

Several additional features of the general organization of individual poxvirus genes are noteworthy. All genes are uninterrupted, linear, and without introns. Generally, there is little overlap between individual genes. While transcription of genes can occur in either direction, genes at the termini of the viral chromosome tend to be transcribed predominantly towards the end of the chromosome.

The mechanism of DNA replication

DNA replication not only serves to provide DNA for progeny virus but also provides a functional template for the temporal expression of intermediate and late

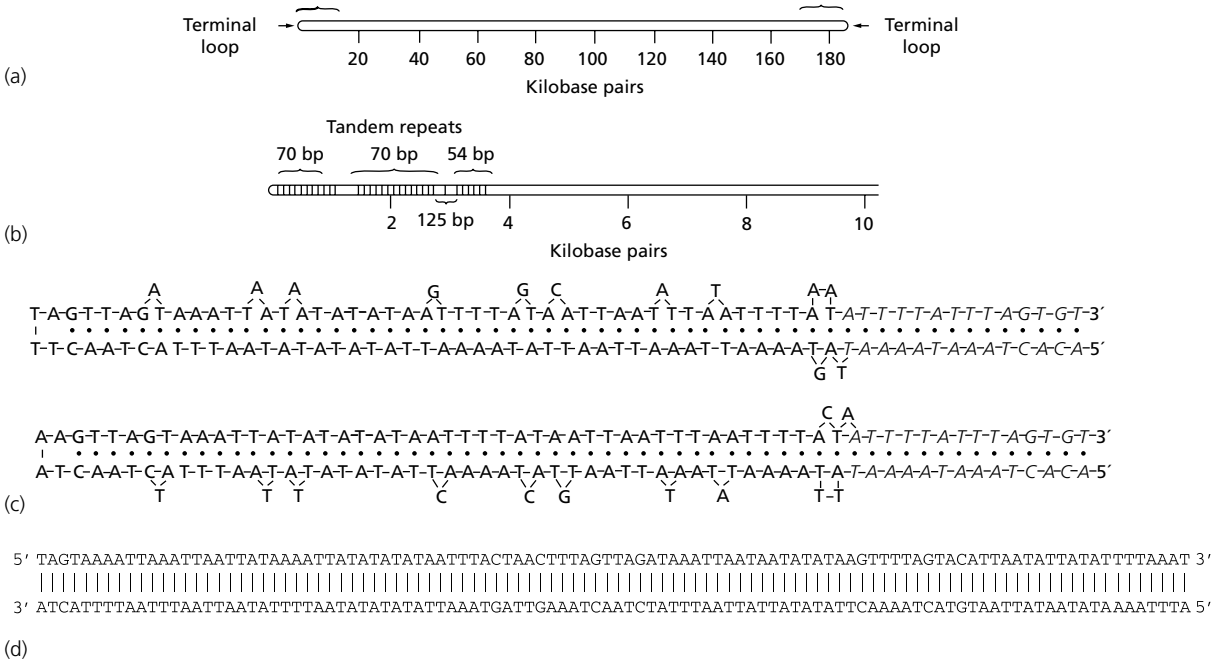

Figure 4 *Structural features of the vaccinia virus genome. From the top down is shown:* **(a)** *a unit length genome emphasizing size, inverted terminal repetition, and hairpin (terminal) loops;* **(b)** *an enlargement of the inverted terminal repeat, emphasizing the tandem repeats within the terminal repeat;* **(c)** *the two hairpin loops (non-italic font) as they are joined to the terminal repeats (italic font);* **(d)** *the duplex structure formed at a concatemer junction, consisting of the unfolded hairpins base paired to each other. (Modified from Moss 2001)*

viral genes. Synthesis of DNA occurs within the cytoplasm and is readily visualized by electron microscopy as electron-dense bodies or 'factories' which serve as the site of initiation for the assembly of mature virions. The mechanism of DNA synthesis takes into consideration the following features of viral DNA already discussed: the TIRs, the terminal DNA hairpin loops, and the generation of replicative concatemeric DNA.

The onset of replication is heralded by introduction of a nick within one or both inverted terminal repeats (ITR), most likely within 200 bp of the end of the molecule (Du and Traktman 1996). The nicking can occur at either or both termini, but for simplicity is only shown at one end (Figure 6). Strand extension at the 3′ terminus of the nick with concomitant strand displacement allows the terminal nucleotides to be copied. Hybridization of the newly synthesized terminal-most nucleotides to the parental strand leads to formation of flip and flop hairpins (Figure 4c; Baroudy et al. 1982). Thereafter polymerization continues, eventually giving rise to concatemers comprising complete genomic units of fused head-to-head or tail-to-tail genome length monomers (Moyer and Graves 1981). Additional complexity can be conferred to replicating DNA through either strand invasion by other molecules, by initiating a second replication cycle on molecules actively replicating or through recombination. There are a number of biochemical studies consistent with the proposed mechanism.

Concatemeric DNA must be resolved and unit length genomes excised in order to assemble mature virus. A graphic illustration of a possible mechanism for this process is shown in Figure 7, p. 12, in which resolution is initiated by a sequence-specific nick. The detailed requirements for concatemer resolution include a small recognition cleavage sequence (Delange et al. 1986; Merchlinsky and Moss 1986, 1989) present as an inverted repeat on both sides of the fused RR or LL hairpin loop contained within the L–RR–LL–R head-to-head or tail-to-tail concatemers that results from the replication process. It has been proposed that resolution may also occur via site-specific recombination and oriented branch migration (Merchlinsky 1990), or by nicking and sealing of extruded cruciform, holiday-like structures (McFadden et al. 2003).

It is possible for concatemers to exist as large cruciform structures and deletions can readily occur within such structures. During genome maturation, such deletion events allow for expansion and contraction of the terminal regions of the chromosome and creation of TIRs of varying length as described (Figure 8, p. 13). The terminal variability is theoretically limited only by the need to retain the essential genes in or at the conserved core and the *cis*-sequences required for replication (Moyer et al. 1980; Moyer and Graves 1981) and provides a mechanism for rapid evolution of genes within this region. The documented ability of terminal regions of the virus to expand and contract implies that

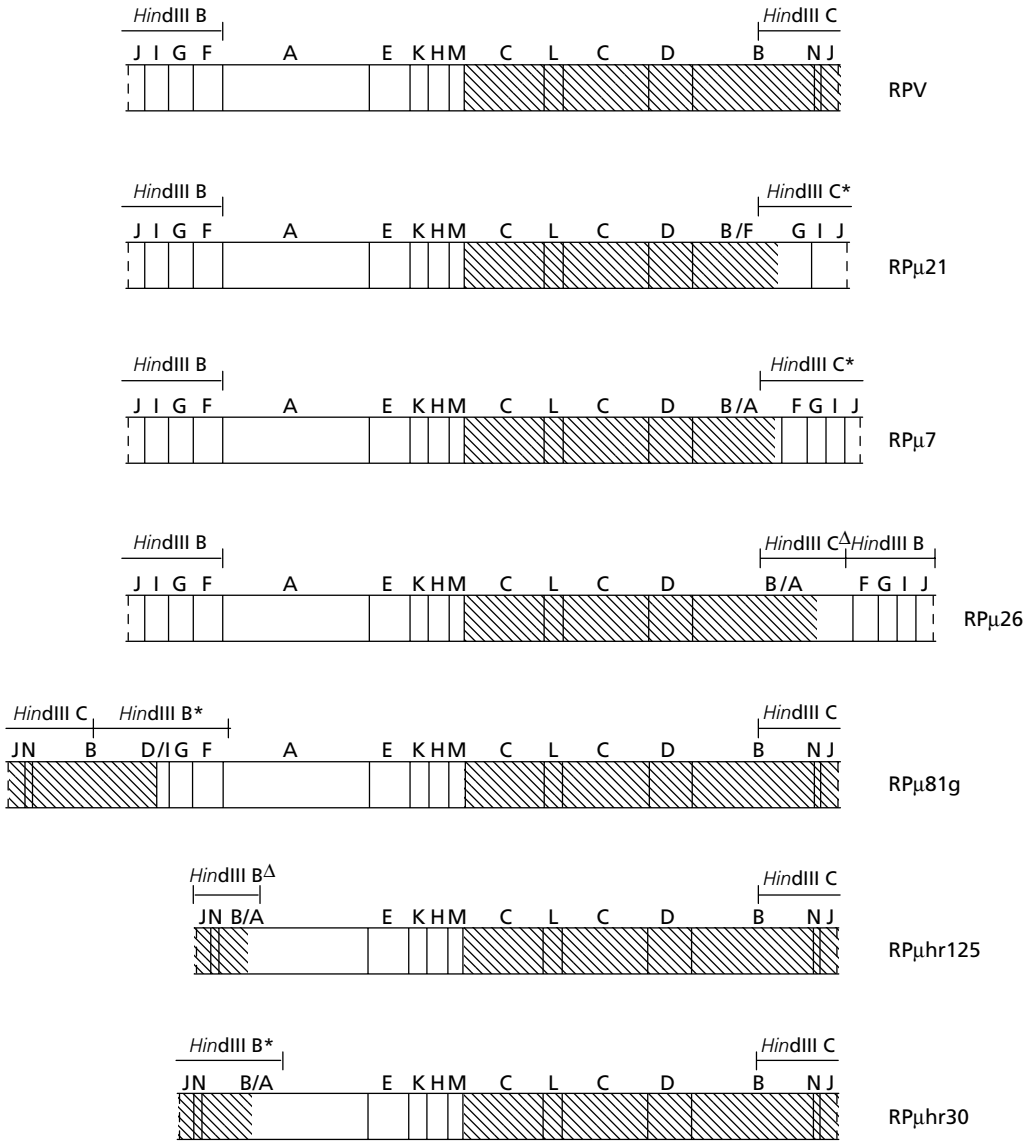

Figure 5 *Variation in size of poxvirus terminal repeats. The* XhoI *maps of RPV and six TIR variants are shown. The genome of RPV and all variants are sized to scale. Genome sizes are drawn to scale. The right and left halves of the parental RPV genome are distinguished by shading. The mutants RPμ21, RPμ7, and RPμ26 have expanded terminal repeats derived from the left region of wild-type RPV. The TIRs of RPμ26 are sufficiently large to contain many functional genes. The mutants RPμ81g, RPμhr125, and RPμhr30 likewise have expanded terminal repeats compared to wild-type RPV (from Moyer and Graves 1981). The generation of expanded TIRs through duplication and transposition of either left or right regions of the genome is likely a consequence of replication (see text).*

the amount of DNA packaged within a virion is not fixed and is determined by the location of the concatemer resolution sequences rather than the amount of DNA within a given chromosome. Indeed, it has been shown the amount of DNA packaged can be far larger than the minimum genome normally associated with a given virus and the entire bacteriophage lambda chromosome can be stably maintained within vaccinia virus (Smith and Moss 1983). This genomic plasticity has been exploited in the creation of a wide variety of recombinant poxviruses with large inserts of foreign DNA for a variety of applications.

The enzymology of DNA replication

Although poxviruses encode many enzymes associated with aspects of DNA synthesis, those that are conserved among many members are likely to constitute the 'basic' or core replication machinery (Table 4, p. 14). A subset of these conserved proteins, have been shown through the study of mutants, to exhibit defects in DNA synthesis (Table 4, top). The role of other conserved genes is in post-synthetic steps of DNA maturation or is unknown. The subset of proteins known to be involved in DNA synthesis include a DNA polymerase (ORF

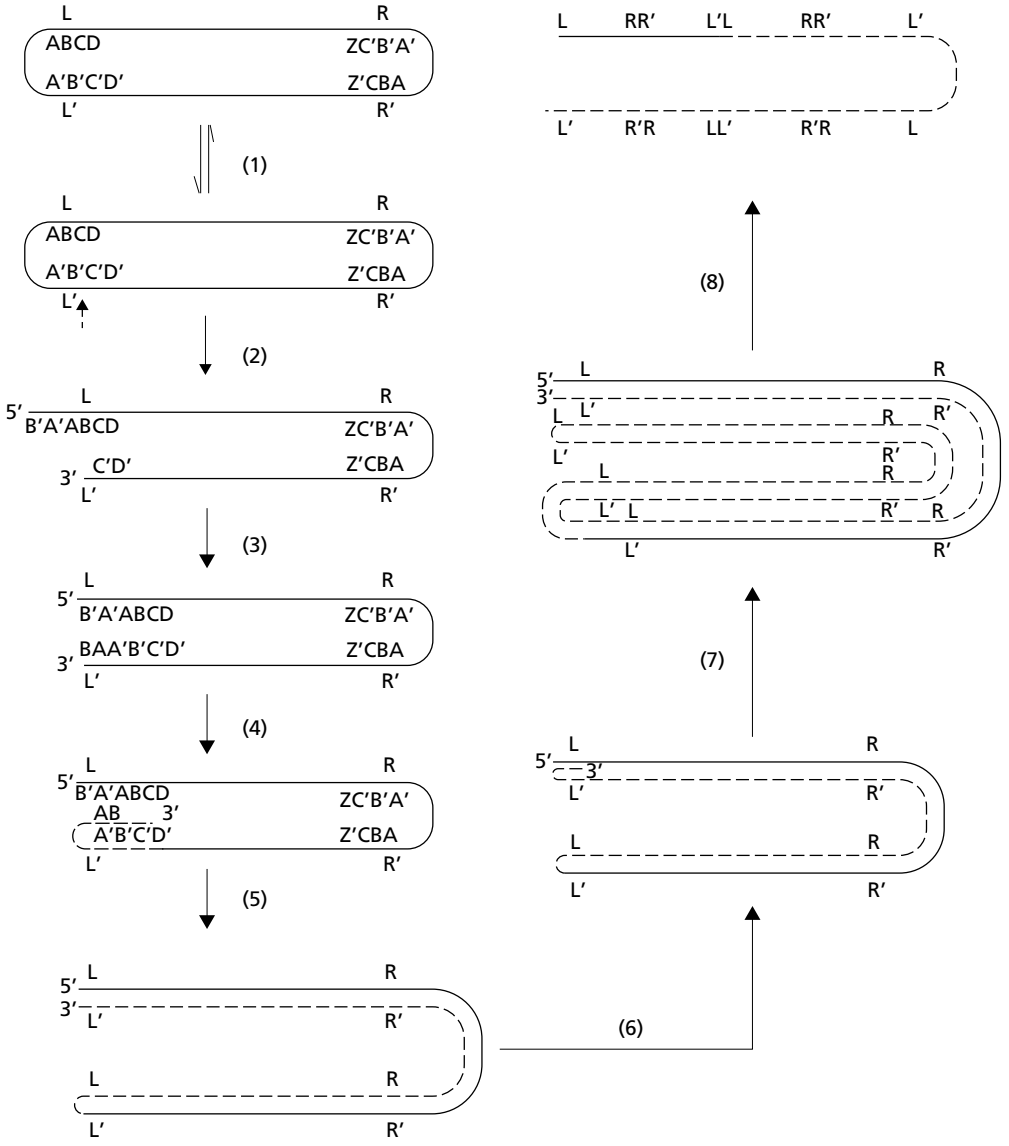

Figure 6 *The replication of* Orthopoxvirus *DNA. The viral genome is indicated at the top left with 'L' and 'R' orientation of the molecule as indicated. Replication begins with introduction of a nick near one or both termini of the genome (indicated schematically by arrow between B′ and C′) followed by strand extension. Parental DNA is indicated by solid lines, newly synthesized DNA by dashed lines. Following strand extension, self-primed replication creates a new hairpin, replication of the genome, and creation of a concatemeric genomic dimer. See text for details. (From Moyer and Graves 1981)*

E9L,VACWR 065) (McDonald and Traktman 1994), a uracil DNA glycosylase (ORF D4R, VACWR 109) (Stuart et al. 1993; Upton et al. 1993; Millns et al. 1994), a serine/threonine protein kinase (ORF B1R, VACWR 183) (Banham and Smith 1992), an NTPase (ORF D5R, VACWR 110), (Evans and Traktman 1992; Evans et al. 1995), and ORF A20, VACWR 141, a processivity factor (Ishii and Moss 2001a); (Punjabi et al. 2001) which acts in concert with the E9L DNA polymerase. The single known exception to the universal conservation of these proteins is B1R, which is absent from MCV. Inactivation of any of these proteins leads to a DNA negative phenotype and is lethal to the virus.

The DNA polymerase is a 110 kDa protein with a 3′-exonuclease proofreading activity and is a target of several antiviral drugs such as aphidicolin (DeFilippes 1984), cytosine arabinoside, and phosphonoacetic acid (Traktman et al. 1989; Taddie and Traktman 1993). Recently, A20R has been shown to function as a processivity factor for the DNA polymerase and to interact with the D4R and D5R proteins as well (McCraith et al. 2000; Ishii and Moss 2001a; Klemperer et al. 2001; Punjabi et al. 2001; Ishii and Moss 2002). Temperature-sensitive mutants in either protein lead to rapid inhibition of DNA synthesis (Sridhar and Condit 1983; Punjabi et al. 2001).

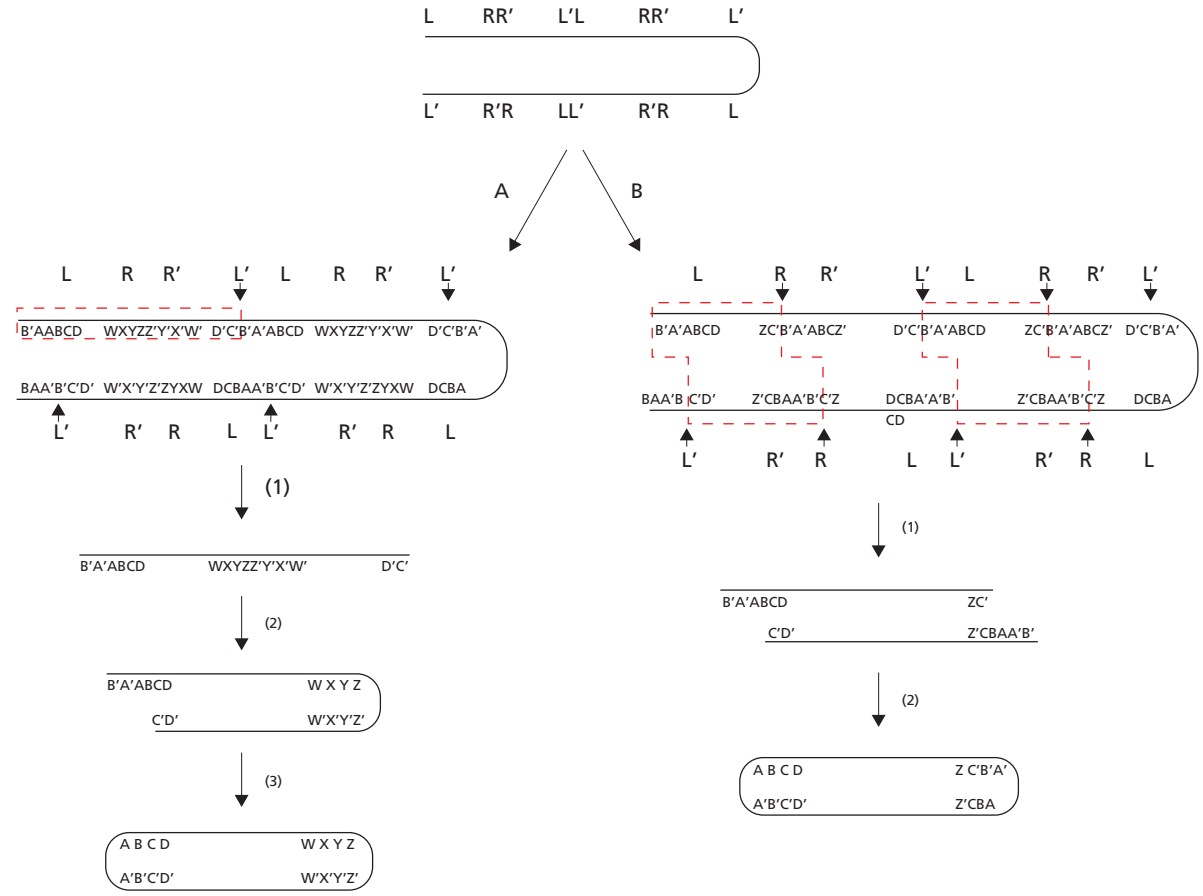

Figure 7 *Generation of mature poxvirus virion DNA from replicative concatamers. Generation of monomeric genomic DNA is initiated by single-stranded nicking (B′C′) as indicated by the arrow. Genomic DNA can be derived either in a* cis *(left) or* trans *(right) fashion from the concatemer as indicated by the red boxes. The designations L and R serve to orient the left and right termini of the molecules and where L′ and R′ are sequences complementary to those of L and R, respectively. (From Moyer and Graves 1981)*

The uracil DNA glycosylase is a small 25 kDa protein and in most systems typically functions as a repair enzyme, excising from DNA uracil residues which arise from misincorporation of dUMP by the DNA polymerase or by deamination of cytosine. The ultimate result of this activity is to leave a gap that is filled by the DNA polymerase. Therefore, the discovery that this protein is absolutely required for the replication of poxvirus DNA (Stuart et al. 1993; Millns et al. 1994; Ellison et al. 1996) was somewhat surprising. Recently, the conserved reactive site of the glycosylase was mutated, eliminating the enzymatic activity which excises uracil from DNA, yet the protein could still function in the process of DNA replication (De Silva and Moss, 2003). Therefore, while the D4R protein is required for DNA replication, the uracil DNA glycosylase activity per se is not. Interestingly, virus containing the enzymatically inactive glycosylase gene, while normal for DNA replication, is attenuated when the virus was used to infect mice.

The NTPase is a 785 amino acid protein which exhibits DNA-independent hydrolysis of both ribo- and deoxy-NTPs. Temperature-sensitive mutants in the protein exhibit a 'fast stop' inhibition of DNA synthesis at nonpermissive temperatures (Evans et al. 1995) as well as being impaired in homologous recombination (Evans and Traktman 1992). As might be expected at nonpermissive temperatures, intermediate and late gene expression is severely affected (Diaz-Guerra and Esteban 1993).

The BIR serine/threonine protein kinase is a 34 kDa protein. When a virus containing thermosensitive mutations in this gene are incubated at nonpermissive temperatures, DNA synthesis ceases. This protein is expressed early in infection but is packaged into mature virions (Banham and Smith 1992; Lin et al. 1992; Rempel and Traktman 1992). One substrate of the B1R kinase is the *H5R* gene (Beaud et al. 1995), which also forms a complex with B1R (McCraith et al. 2000). However, the role of the H5R phosphoprotein is complex and appears to be involved both in the transcription of late viral genes (Kovacs and Moss 1996) as well as morphogenesis (Demasi and Traktman 2000).

The *A50R/VACV-WR_176* gene, encodes a 63 kDa DNA ligase (Kerr and Smith 1991; Parks et al. 1994,

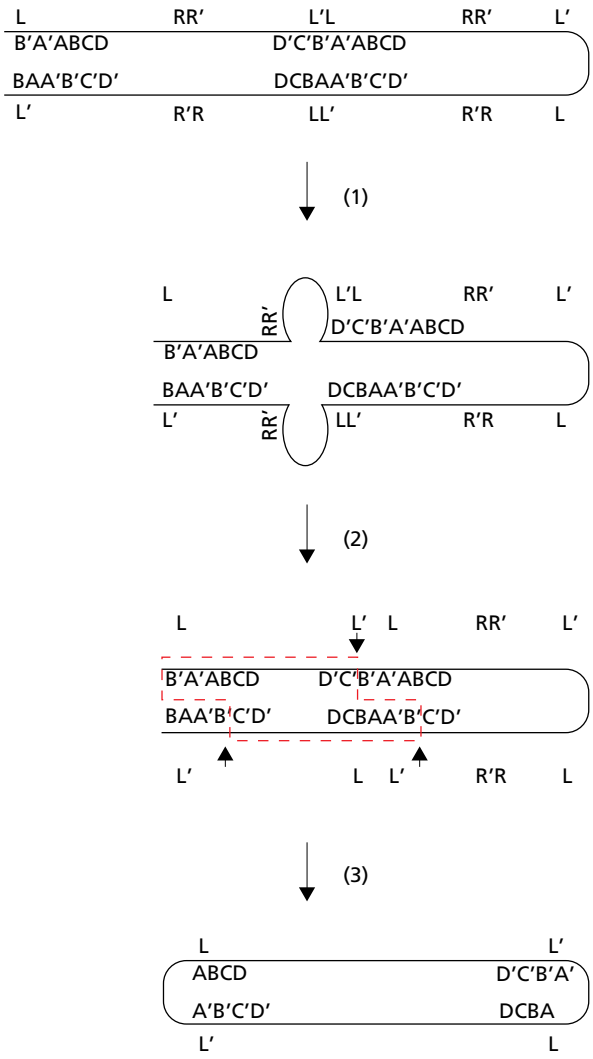

Figure 8 *Mechanism for expansion and contraction of TIR regions. A replicative, concatemeric DNA intermediate is shown. Deletion from within the array (step 1) followed by molecular excision is governed by the hypothetical concatemeric resolution signal (B′C′) and indicated by the red box. Limits of the deletion are theoretical determined by viability of the deleted genomic molecule. In the example shown, the TIR is expanded by addition of sequences from the left end of wild-type molecule. (From Moyer and Graves 1981)*

1998). Disruptions of the DNA ligase lead to a cell-line-dependent phenotype. In some cell lines, there is poor growth of virus accompanied by a two- to tenfold decrease in DNA synthesis. In other cell lines, there is little if any effect (Parks et al. 1998). This difference may be due to the levels of available host DNA ligase present in the cytoplasm.

A number of the other conserved proteins are assumed to be required for later steps in the replicative process but direct genetic evidence is lacking for most of them. Mutations in these genes would be predicted to not lead to defects in DNA synthesis per se, but rather to defects in maturation of the newly synthesized DNA which in turn leads to inhibition of progeny virus formation. The *A22R/VACV-WR_142* gene encodes a resolvase and has been shown by the use of conditional, inducible mutants to be required for resolution of concatemeric DNA into monomers (Garcia et al. 2000; Garcia and Moss 2001).

The function of two of these conserved genes is not yet clear. The *H6R/VACV-WR_104* gene encodes a 314 amino acid type 1 topoisomerase (Shuman and Moss 1987; Shuman et al. 1989), which is the smallest known topoisomerase. Attempts to isolate mutant virus deleted for this gene were unsuccessful, suggesting the gene is essential for growth (Shuman et al. 1989). Furthermore, no conditional lethal mutant has yet been isolated so the precise function of the topoisomerase in virus growth remains to be elucidated. The 34 kDa *I3L/VACV-WR_072* gene encodes a single-stranded DNA-binding protein (Rochester and Traktman 1998; Tseng et al. 1999). The I3L protein has been found localized to virosomes or factories during DNA replication (Rochester and Traktman 1998). When DNA synthesis is blocked, the protein is found in distinct foci within the cytoplasm (Domi and Beaud 2000). It has been suggested that these cytoplasmic spots are specialized sites where DNA release from incoming virus cores accumulates (Welsch et al. 2003). Elucidation of the precise role of both the *H6R* and *I3L* genes in the replicative process awaits isolation of conditional lethal mutants.

Recombination is an essential feature of DNA replication. Indeed, recombination between genetically marked viruses or transfected plasmids and viral DNA is rampant, which in practice facilitates generation of genetically engineered viruses. The processes of recombination and replication are linked and mutations which impair recombination are also impaired in DNA replication (Merchlinsky 1989; Colinas et al. 1990; Evans and Traktman 1992).

Finally, there are a number of poxvirus genes devoted to the purpose of maintaining intracellular nucleotide pools which serve as substrates for DNA synthesis. These enzymes, many of which are not absolutely essential for growth include:

- the 177 amino acid *J2R/VACV-WR_094*) which encodes a thymidine kinase (Hruby and Ball 1982) active as a tetramer (Black and Hruby 1990)
- the *A48R/VACV-WR_178* protein, a 204 amino acid thymidylate kinase (Hughes et al. 1991)
- a 147 amino acid dUTPase, the product of gene *F2L/VACV-WR_041* (Broyles 1993)
- the heterodimeric ribonucleotide reductase comprising the 771 amino acid *I4L/VACV-WR_073* and the 319 amino acid *F4L/VACV-WR_043* subunits (Slabaugh and Mathews 1986; Slabaugh et al. 1988; Tengelsen et al. 1988; Rajagopal et al. 1995).

Table 4 *Poxvirus genes involved in DNA replication*

VACCOP ORF	VACWR ORF	Size (aa)	Description
Orthopoxvirus proteins required for DNA synthesis			
A20R	141	49.1 kDa (426)	DNA polymerase processivity factor
B1R	183	34 kDa (300)	Serine/Threonine phosphokinase
D4R	109	25 kDa (218)	Uracil DNA glycosylase
D5R	110	90 kDa (785)	Nucleoside triphosphatase
E9L	065	116 kDa (1 006)	DNA polymerase
Orthopoxvirus proteins required for other aspects of DNA replication			
A22R	142	22 kDa (187)	Holliday junction resolvase
H6R	104	36 kDa (314)	DNA topisomerase
I3L	072	24 kDa (269)	ssDNA-binding protein
A50R	176	63 kDa (552)	DNA ligase
D5R	110	90 kDa (785)	Nucleotide phosphatase

Poxvirus genes involved in DNA replication are divided into two groups: viral genes directly involved in the synthesis of DNA for which genetic evidence (*ts* mutants exist) (top) or those genes involved in replication and/or post-replicative events for which genetic evidence needed to conclude these genes are essential for replication is generally lacking.

POXVIRUS TRANSCRIPTION AND REGULATION OF VIRAL GENE EXPRESSION

Overview

Gene expression during poxvirus infection occurs in a tightly regulated temporal cascade featuring sequential synthesis of early, intermediate, and late gene (the latter two are termed postreplicative) products. The regulation of poxviral gene expression is most dramatically and elegantly revealed in a simple protein metabolic labeling experiment in which infected cells are pulse labeled with a radioactive amino acid at varying times after infection and the proteins synthesized during each pulse are visualized by gel electrophoresis and autoradiography (Figure 9). Typically this experiment reveals a rapid shutoff of host protein synthesis within the first 4 hours of infection, a burst of early protein synthesis which peaks at 2 hours post-infection and disappears by 6 hours post-infection, synthesis of intermediate proteins which peaks by 4 hours post-infection and decays thereafter, followed by synthesis of late proteins which peaks by 6 hours post-infection and persists through at least 16 hours of infection. Importantly, viral DNA replication initiates with the peak of early protein synthesis, and is a prerequisite for intermediate and late protein synthesis (Pennington 1974).

Mechanistically, gene expression is regulated at the level of transcription (reviewed by Condit and Niles 2002; Broyles 2003). The early, intermediate, and late gene classes are distinguished by class-specific transcriptional promoters and cognate *trans*-acting factors. In general, each gene class encodes factors required for transcription of the succeeding gene class, resulting in the observed temporal cascade of gene expression (Kovacs et al. 1994). Thus, early mRNAs, transcribed by enzymes packaged within infecting virions, encode intermediate gene transcription factors; intermediate genes encode late gene transcription factors; late genes encode early gene transcription factors which are packaged into virions together with RNA polymerase and other RNA metabolism enzymes for the next round of infection. As noted above, both intermediate and late transcription are coupled to DNA replication, that is, inhibitors of DNA replication prevent synthesis of intermediate and late RNAs (Oda and Joklik 1967; Vos and Stunnenberg 1988; Baldick and Moss 1993). Transcription of intermediate and late genes also requires the synthesis of new RNA polymerase, which is synthesized early during infection (Hooda-Dhingra et al. 1989).

All poxvirus genes apparently contain their own promoters; polycistronic mRNAs with internal ribosome entry sites have not been described, and poxviral mRNAs are not spliced. Regardless of gene class, poxvirus promoters are relatively simple and are contained entirely within approximately 30 nt upstream from the transcription initiation site (Davison and Moss 1989a, 1989b; Baldick et al. 1992). Importantly, several genes have been described which contain compound promoters and are therefore expressed at multiple stages of infection (Wittek et al. 1980; Broyles and Pennington 1990).

As stated previously, because poxviruses replicate in the cytoplasm of infected cells, the virus encodes virtually all of the enzymes required for regulated synthesis and modification of mRNAs suitable for translation in a eukaryotic environment. Thus at least 23 virus genes

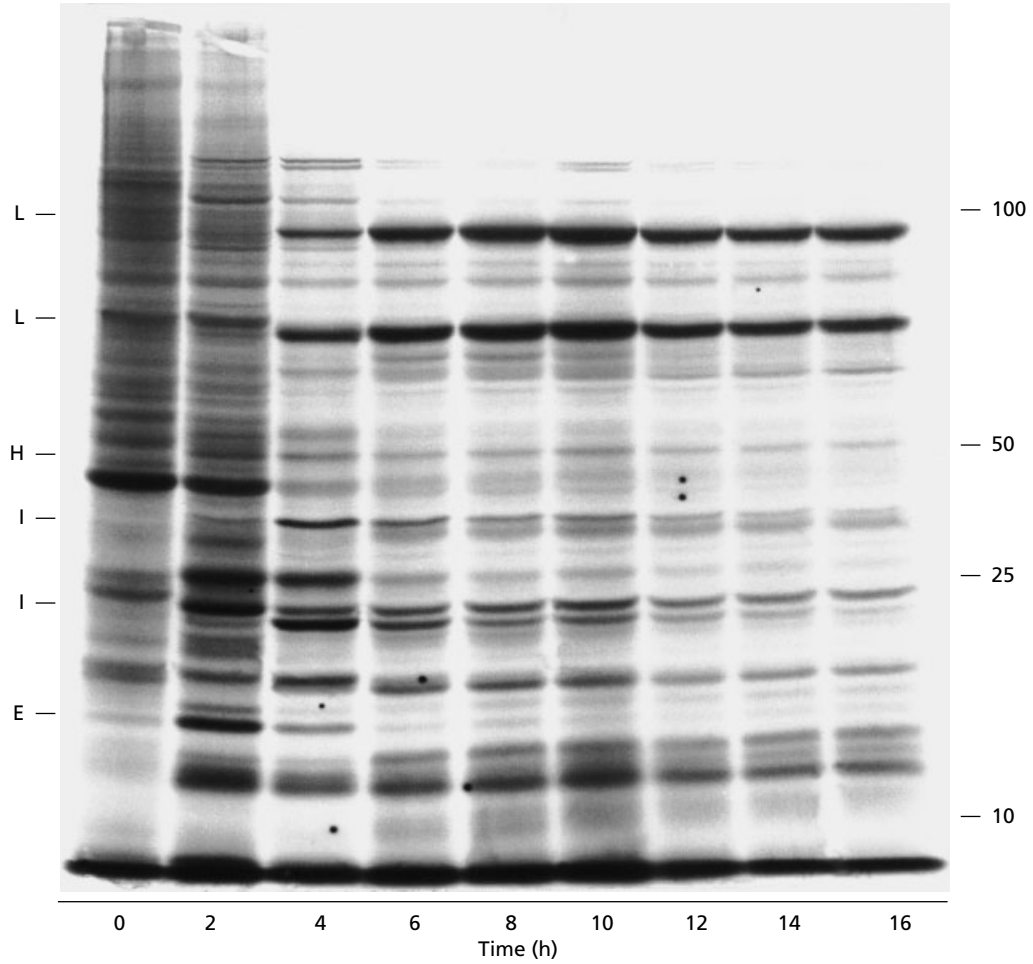

Figure 9 *Kinetics of gene expression in vaccinia virus-infected cells. Cells were infected with vaccinia virus at a multiplicity of infection of 10 plaque-forming units/cell, and metabolically pulse labeled with [^{35}S]methionine for 15 minutes at 2-hour intervals following infection. Proteins were separated by SDS-PAGE. An autoradiograph of the dried gel is shown. Each lane on the autoradiogram reveals the proteins that were being synthesized at the time after infection that the radiolabel was present, indicated under each lane. Molecular mass markers are shown at the right of the autoradiogram. At the left is indicated the migration of selected late (L), intermediate (I), and early (E) viral proteins, and a host (H) protein.*

have been described which play a direct role in viral mRNA synthesis and modification (Table 2). Interestingly, viral transcription may not be completely independent of the host; a limited number of host proteins have also been identified which may play a role in virus transcription (Rosales et al. 1994b; Broyles et al. 1999; Wright et al. 2001).

In the paragraphs below, we first describe the enzymes and reactions common to synthesis and modification of all classes of poxviral mRNA, and then focus in detail on differences in the expression of each gene class.

RNA polymerase

The virus-encoded RNA polymerase is a multisubunit enzyme which exists in two forms, one specific for early genes and one specific for intermediate and late genes (Table 2). The two forms of the enzyme contain eight subunits in common; the only difference between the two forms of the enzyme is that the early gene-specific enzyme contains one additional subunit, RAP94 (H4L/VACV-WR_102) (Nevins and Joklik 1977; Baroudy and Moss 1980; Ahn et al. 1994). Among the eight common subunits, the two largest, RPO147 (J6R/VACV-WR_098) and RPO132 (A24R/VACV-WR_144), share extensive homology with the largest subunits of both eukaryotic and prokaryotic multisubunit RNA polymerases (Broyles and Moss 1986; Patel and Pickup 1989; Amegadzie et al. 1991b). Another subunit, RPO30 (E4L/VACV-WR_060), also shows homology with the eukaryotic transcription elongation factor TFIIS (Ahn et al. 1990a). Interestingly, this subunit also serves as an intermediate gene transcription factor (Rosales et al. 1994a). The smallest vaccinia RNA polymerase subunit, RPO7 (G5.5R/VACV-WR_083), shows homology to the smallest subunit of eukaryotic RNA polymerase II

(Amegadzie et al. 1992). Neither of the remaining four common subunits nor RAP94 show significant homology to other eukaryotic or prokaryotic proteins.

Both forms of the viral RNA polymerase, RAP94+ and RAP94−, are found in virions as well as in the cytoplasm of infected cells. The activities of these enzymes are mutually exclusive, that is, the RAP94+ enzyme transcribes only early viral genes and not postreplicative genes, whereas the RAP94− enzyme transcribes only postreplicative genes and not early genes (Wright and Coroneos 1995). Importantly, this means that the RNA polymerase which transcribes either early or postreplicative genes retains a memory of the class of promoter at which transcription was initiated. This impacts significantly on termination of transcription, which occurs differently on early and postreplicative genes, as described in more detail below.

mRNA capping

All classes of poxviral mRNAs contain a 5′ cap-1 structure, in which the first transcribed nucleotide is modified by addition of a 7-methylguanine in a 5′– 5′ triphosphate linkage, and is also methylated on the 2′-hydroxyl group of the ribose moiety (Wei and Moss 1975). The entire process comprises four reactions carried out by two virus-coded enzymes (Table 4). The first three reactions are carried out by the mRNA capping enzyme, a heterodimer composed of the 97 kDa *D1R* gene product (*D1R/VACV-WR_106*) and the 33 kDa *D12L* gene product (*D12L/VACV-WR_117*) (Martin et al. 1975; Morgan et al. 1984; Niles et al. 1989). The capping enzyme first removes the gamma phosphate from a nascent, triphosphorylated mRNA, then adds a GMP in a 5′–5′ linkage, and finally methylates the added guanine in the 7 position to yield a cap-0 structure. The triphosphatase and guanylyltransferase active sites are located on the large D1R subunit of the enzyme, whereas the guanine methyltransferase active site is located on the small, D12L subunit of the enzyme (Shuman and Morham 1990; Higman et al. 1994; Mao and Shuman 1994). Methylation of the ribose on the first transcribed nucleotide requires the cap-0 RNA (guanylylated and methylated on the cap guanine base, but not ribose methylated) as a substrate, and is catalyzed by a monomeric (nucleoside-2′-*O*-)-methyltransferase, the 39 kDa product of the *J3R* gene (*J3R/VACV-WR_095*), to yield a cap-1 structure (Barbosa and Moss 1978a; Barbosa and Moss 1978b; Schnierle et al. 1992). Interestingly, both the capping enzyme and the (nucleoside-2′-*O*-)-methyltransferase are multifunctional enzymes. The D1R/D12L capping enzyme also participates in early gene transcription termination, intermediate gene transcription initiation, and may also play a role in telomere resolution during processing of replicated viral DNA concatemers to monomeric genomes (Shuman et al. 1987; Carpenter and DeLange 1991; Vos et al. 1991; Hassett et al. 1997). In addition to its role in mRNA capping, the J3R (nucleoside-2′-*O*-)-methyltransferase serves as a stimulatory subunit for the viral poly(A) polymerase, and also as a transcription elongation factor for postreplicative genes (Gershon et al. 1991; Latner et al. 2002). All three genes involved in the capping reactions are essential virus genes; however, the phenotypes of mutants in these genes do not obviously relate to the capping reaction per se. Specifically, temperature-sensitive mutants in either of the mRNA capping enzyme subunit genes (*D1R* or *D12L*) are unaffected in viral gene expression but defective in telomere resolution and morphogenesis (Carpenter and DeLange 1991; Hassett et al. 1997). Formation of the cap-0 structure is presumed to be essential for mRNA stability and translation during virus infection, but this has never been demonstrated in poxviruses directly. The J3R protein is essential only for its transcription elongation function; selective inactivation of the (nucleoside-2′-*O*-)-methyltransferase activity of the J3R protein to produce a virus which synthesizes only cap-0 mRNAs has no apparent effect on virus growth (Latner et al. 2002).

Polyadenylation

All classes of poxviral mRNAs are polyadenylated by a virus-coded heterodimeric enzyme consisting of the 55 kDa *E1L* gene product (*E1L/VACV-WR_057*) and the 39 kDa *J3R* gene product (*J3R/VACV-WR_095*) (reviewed by Gershon 1998) (Table 2). The catalytic site for polyadenylation resides in the E1L protein; however, the E1L protein on its own can processively add only approximately 35 adenylate residues to an RNA 3′ end (Gershon and Moss 1992). In the presence of the J3R subunit, full length poly(A) tails averaging 100 nt in length are synthesized; thus the J3R protein is described as the stimulatory or processivity subunit of the poly(A) polymerase (Gershon and Moss 1993a). The poly(A) polymerase is relatively promiscuous and will polyadenylate virtually any available RNA 3′ end providing it contains uridylate residues positioned at specific sites near the RNA 3′ end (Gershon and Moss 1993b). The poly(A) stimulatory and ribose methyltransferase activities of the J3R protein are independent of one another, and occupy opposite faces of an oblate sphere (Hodel et al. 1996; Gershon et al. 1998).

Early gene transcription

Early vaccinia genes are transcribed in the cell cytoplasm from partially uncoated virion cores. Early transcripts are detectable within 20 minutes following infection, they reach a peak of synthesis by 100 minutes following infection, and decline thereafter with a half-life of approximately 30 minutes (Baldick and

Moss 1993). Early gene products number at least 50, representing at least 25 percent of the coding capacity of the vaccinia genome, and include enzymes required for DNA replication, new viral RNA polymerase and capping enzyme, host defense functions, plus intermediate gene-specific transcription factors (viral intermediate transcription factors (VITF)) required for transcription from intermediate transcriptional promoters.

Mutational analysis demonstrates that poxvirus early transcriptional promoters contain an A/T rich critical core region located between −13 and −27 relative to the RNA start site (Davison and Moss 1989a). Transcription usually starts with a purine residue, but otherwise the residues between −1 and −12, described as a 'spacer' region, are insensitive to substitution mutation. Early transcription start sites are usually located approximately 5–30 nt upstream of the ATG translation initiation codon for a given gene; however, early mRNA 5′ untranslated regions of up to 600 nt in length have been reported (Lee-Chen et al. 1988; Pacha et al. 1990; Meis and Condit 1991).

The viral early transcription factor (VETF) is a heterodimeric promoter-binding ATPase composed of the 82 kDa *A7L* gene product (*A7L/VACV-WR_126*) and the 74 kDa *D6R* gene product (*D6R/VACV-WR_111*) (Broyles and Fesler 1990; Gershon and Moss 1990) (Table 2). VETF is the only member of the poxvirus transcription factors that has been shown to bind directly and specifically to its cognate promoter. VETF makes sequence-specific contacts with the promoter core region, and also makes DNA contacts in a nonsequence-specific fashion directly downstream of the initiator (Broyles et al. 1991; Cassetti and Moss 1996). Upon binding to the promoter, VETF recruits RNA polymerase to the promoter and then dissociates, hydrolyzing ATP in the process (Broyles 1991). As noted above, the RAP94 subunit of the RNA polymerase is also required for initiation at early promoters.

Termination of early gene transcription is an energy-dependent, sequence-specific event requiring virus-coded *trans*-acting factors (reviewed by Condit and Niles 2002). The sequence which triggers early gene transcription termination is UUUUUNU, occurring in the nascent RNA (Yuen and Moss 1987; Shuman and Moss 1988). Termination occurs 30–50 nt downstream from this sequence. Three viral proteins are required for early termination, the capping enzyme (called the viral termination factor (VTF) in the context of termination), the RAP94 early RNA polymerase subunit, and a 72 kDa DNA-dependent ATPase, called NPH I, the product of gene *D11L* (*D11L/VACV-WR_116*) (Shuman et al. 1987; Christen et al. 1998; Deng and Shuman 1998; Mohamed and Niles 2001) (Table 2). NPH I provides the energy required for termination, and directly contacts RAP94 (Mohamed and Niles 2000). The activity of the capping enzyme in termination is independent of its mRNA capping activity (Luo et al. 1995; Yu and Shuman 1996). Otherwise, the precise roles of these three proteins in early gene termination and the mechanism of sequence recognition remain to be determined.

Intermediate gene transcription

Intermediate transcription is triggered by the synthesis of intermediate gene transcription factors coupled with the initiation of viral DNA replication (Vos and Stunnenberg 1988). Intermediate mRNAs are detectable by 100 minutes post-infection and peak by 120 minutes post-infection, declining thereafter with a 30 minute half-life (Baldick and Moss 1993).

The identification of intermediate genes from among postreplicative genes is not straightforward and requires one or more specific in vivo or in vitro tests. For example, individual candidate genes may be tested for expression in vivo after infection with a temperature-sensitive mutant virus which is specifically defective in a late transcription factor (Carpenter and DeLange 1992). Only seven intermediate genes have so far been positively identified, and these encode a variety of functions including some host defense functions, DNA and RNA metabolism functions, and, importantly, viral late transcription factors (VLTF) which are required for recognition of late viral transcriptional promoters (Keck et al. 1990; Zhang et al. 1992; Xiang et al. 1998). Many of the postreplicative genes which are now classified as late will probably ultimately prove to be intermediate when subjected to appropriate tests for expression.

Intermediate gene promoters consist of two critical elements, an initiator region, TAAA, located at −1 to +3 relative to the RNA start site, and an A/T rich core element located at −13 to −26 (Baldick et al. 1992). The spacer region from −2 to −12 is insensitive to substitution mutation. The TAAA initiator motif may be located directly upstream from or even incorporate the translation initiating ATG. When initiating at intermediate promoters, RNA polymerase apparently retranscribes the AAA sequence of the initiator multiple times, slipping upstream between transcription events without releasing the nascent RNA, resulting in synthesis of a 30 nt nontemplated 5′ 'poly(A) head' (Bertholet et al. 1987; Schwer et al. 1987; Baldick and Moss 1993). (Because of this slippage reaction, the +1 nucleotide cannot be unambiguously assigned; it is arbitrarily and logically designated as the upstream A in the TAAA initiator motif.) No function for the poly(A) head has been demonstrated; however, it would provide a 5′ untranslated region for mRNAs transcribed from genes in which the promoter is directly abutted to the translation initiation ATG.

Four factors have been identified which are required for maximal expression of intermediate genes in vitro (Table 2). These include the heterodimeric D1R/D12L mRNA capping enzyme, and three intermediate

transcription factors, VITF 1, VITF 2, and VITF 3 (Vos et al. 1991; Rosales et al. 1994a, 1994b; Sanz and Moss 1999). (Earlier, independent experiments identified the mRNA capping enzyme and one additional intermediate gene transcription factor, VITF-B; the relationship between VITF-B and VITF 1–3 has not been determined; Vos et al. 1991.) VITF 1 and 3 are virus-coded, and VITF 2 is a cellular protein. VITF 1 is identical to the 30 kDa RNA polymerase subunit RPO30, the product of the *E4L* gene (*E4L/VACV-WR_060*). VITF 3 is a heterodimer comprising the 34 kDa *A8R* gene product (*A8R/VACV-WR_127*) and the 45 kDa *A23R* gene product (*A23R/VACV-WR_143*). The cellular protein remains to be identified. The precise mechanism of action of these proteins in intermediate gene transcription remains to be determined. In addition to these four factors, evidence suggests that the cellular transcription factor YY1 may play a role in transcription of some intermediate genes (Broyles et al. 1999; Broyles 2003). Specifically, YY1 is mobilized from the cell nucleus into the cytoplasm following poxvirus infection, and binds in vitro to intermediate promoters containing the sequence TAAATGG.

Termination of intermediate and late gene transcription occurs by a similar mechanism and is fundamentally different from termination of early gene transcription (reviewed by Condit and Niles 2002). During transcription of intermediate genes, the early transcription termination signal, UUUUUNU, is ignored, presumably because the RNA polymerase which transcribes intermediate genes lacks RAP94 (Condit et al. 1996a). Instead, RNA polymerase transcribing intermediate genes apparently terminates inefficiently at multiple sites, such that each intermediate gene is transcribed to yield an extremely 3′-end heterogeneous family of transcripts ranging in size from 1 to 4 kb, regardless of the size of the gene being transcribed (Mahr and Roberts 1984). Genetic and biochemical experiments have identified three virus-coded factors which influence the length of intermediate transcripts, and therefore play a role in intermediate gene transcription elongation or termination (Table 2). Specifically, genetic inactivation of the *A18R* gene (*A18R/VACV-WR_138*) results in the synthesis of longer than normal intermediate mRNAs, while conversely, genetic inactivation of either the *G2R* (*G2R/VACV-WR_080*) or *J3R* (*J3R/VACV-WR_095*) genes results in the synthesis of 3′-truncated mRNAs (Black and Condit 1996; Xiang et al. 1998; Latner et al. 2000). The complementary phenotypes of these mutants is reinforced by the fact that mutations in either *G2R* or *J3R* will suppress mutations in *A18R* (Condit et al. 1996b; Latner et al. 2000). The 56 kDa *A18R* gene product is a DNA-dependent ATPase and a 3′–5′ DNA helicase, and will catalyze release of RNA from a transcription elongation complex in an energy-dependent fashion in vitro (Bayliss and Condit 1995; Simpson and Condit 1995; Lackner and Condit 2000). The A18R protein is therefore likely to be a transcription termination factor, consistent with the phenotype of A18R-deficient mutants in vivo. As described above, the J3R protein also serves as the mRNA cap (nucleoside-2′-*O*-)-methyltransferase, and the poly(A) polymerase stimulatory factor. The transcription elongation activity of J3R is genetically separable from and therefore functionally independent from its other two activities (Latner et al. 2002). The 26 kDa G2R protein has no significant homology to other characterized proteins. In vitro assays for the transcription elongation functions of J3R and G2R have yet to be developed.

Late gene transcription

Late viral mRNAs are detectable by 140 minutes post-infection, and continue to be synthesized throughout infection (Baldick and Moss 1993). Genes currently classified as late genes may comprise as much as 75 percent of the vaccinia genome; however, some of these are likely to be intermediate genes as discussed above. Late mRNAs encode the majority of virus structural and assembly functions and virion enzymes, including the VETF which is packaged into newly assembled virions along with RNA polymerase and RNA modification enzymes for use in the next round of infection.

Late transcriptional promoters are very similar to intermediate transcriptional promoters. Late promoters consist of an AT-rich core located at −11 to −16 relative to the RNA start site, and a TAAAT initiator motif located between −1 and +4 (Davison and Moss 1989b; Broyles 2003). The similarity between late and intermediate promoters is illustrated by the observation that mutation of an ACA trinucleotide to CCC at positions −15 to −17 in the gene *I3L* intermediate promoter changes the promoter to a late promoter (Hirschmann et al. 1990). Like intermediate transcripts, late gene mRNAs contain a 5′ poly(A) head, synthesized via RNA polymerase slippage at the TAAAT initiator motif (Patel and Pickup 1987; Ahn and Moss 1989).

Genetic and biochemical experiments have identified at least five VLTFs which are required for maximal transcription from late promoters (Table 2). VLTF 1–4 are virus-coded factors. VLTF 1–3, identified through genetic experiments, are the products of the intermediate genes *G8R* (*G8R/VACV-WR_086*, 30 kDa), *A1L* (*A1L/VACV-WR_119*, 17 kDa), and *A2L* (*A2L/VACV-WR_120*, 26 kDa), respectively. VLTF 4 is the product of gene *H5R* (*H5R/VACV-WR_103*), a 22 kD early phosphoprotein which stimulates late transcription in vitro. A fifth cellular factor, VLTF-X, which stimulates transcription from late vaccinia promoters in vitro, has recently been identified as consisting of the heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3 (Wright et al. 2001). The specific roles of each of the late transcription factors has yet to be determined.

Elongation and termination of late mRNAs appears to occur by mechanisms identical to intermediate mRNAs. Specifically, late mRNAs are 3′-end heterogeneous, and their length is regulated by the products of genes *A18R*, *G2R*, and *J3R* (Condit and Niles 2002).

Interestingly, a few late poxvirus mRNAs possess homogeneous 3′ ends, and in the case of the cowpox virus *ATI* gene, it has been determined that the homogeneous 3′ end is generated by a sequence-specific, post-transcriptional endoribonuclease cleavage reaction catalyzed by a virus-induced enzyme (Howard et al. 1999). Whether this cleavage reaction is limited to just a few mRNAs or plays a broader role in infection remains to be determined.

Additional regulation: host shutoff and RNA turnover

Sequential synthesis of transcription initiation factors accounts only for the onset of synthesis of successive classes of poxvirus gene products; additional mechanisms must regulate the shutoff of host gene expression and the decay in expression of early and intermediate genes as infection progresses. One factor which clearly plays a role in the decay of cellular and viral gene expression is a global reduction in stability of mRNA induced by poxvirus infection. For example, β-actin mRNA, which normally has a half-life of 10 hours in uninfected cells, is rapidly degraded upon vaccinia infection (Rice and Roberts 1983). In general, late viral mRNAs have a half-life of less than 60 minutes (Oda and Joklik 1967). In addition, host-gene expression may be altered by virus infection both by inhibition of cellular RNA polymerase and by inhibition of translation of cellular mRNA (Puckett and Moss 1983; Cacoullos and Bablanian 1993) .

POXVIRUS MORPHOGENESIS

Overview

Electron microscopic examination of poxvirus-infected cells reveals discrete stages of virus morphogenesis with characteristic intermediate structures associated with each stage (Dales and Pogo 1981; Griffiths et al. 2001a, 2001b; Risco et al. 2002; Sodeik and Krijnse-Locker 2002). The earliest evidence of poxvirus infection is the appearance in the cytoplasm of discrete granular foci which are largely devoid of normal cellular organelles. These foci, called ‘viroplasm’ or ‘viral factories,’ contain replicating viral DNA and are often surrounded by cellular membranes derived from the endoplasmic reticulum (Tolonen et al. 2001). As the infection progresses, viral factories increase in size. The first evidence of virion morphogenesis is the appearance within the viroplasm of rigid, crescent-shaped membrane structures (cupules in three dimensions) which are precursors to the IMV membrane (Figure 10). The origin of these viral membranes is discussed in more detail below. Crescents evolve into circles (spheres in three dimensions), called immature virions (IV), which enclose relatively dense viroplasm (Figure 10). Some IV contain internal ‘nucleoids,’ which are unbounded, extremely electron-dense, DNA-containing structures presumed to be precursors to the virion core (Figure 10). Formation of these nucleoid-containing IV (IVN) is associated with viral DNA packaging, described in more detail below. IVN then mature to IMV containing the classical biconcave core, core envelope, lateral bodies, and outer membrane (Figure 1). Maturation of IV to IMV is essentially a metamorphosis which is presumed to take place via rearrangement of the contents of IVN; while structures which are thought to be intermediates in the IVN to IMV transition have been observed, they are

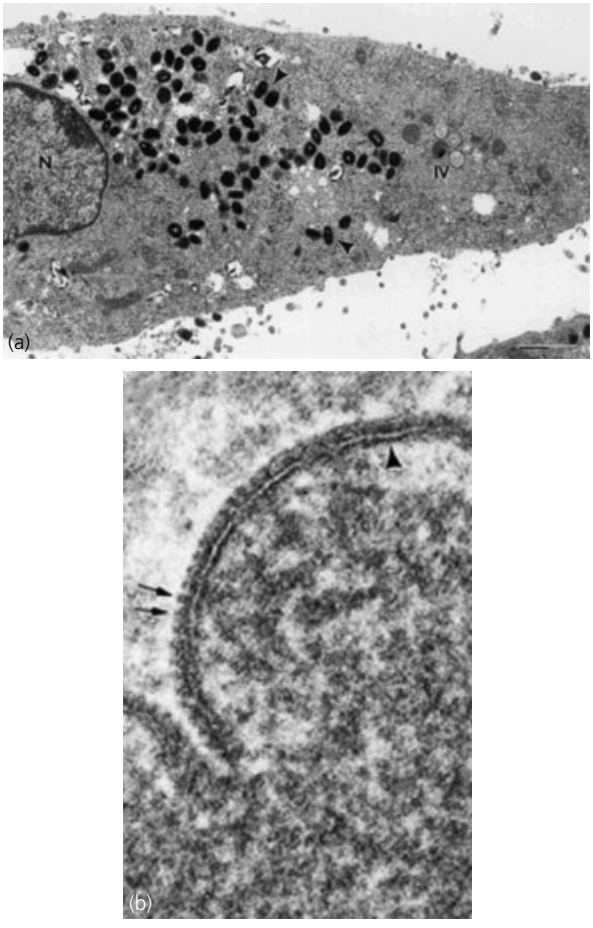

Figure 10 *Electron microscopy of vaccinia virus-infected cells.* **(a)** *Low-magnification field of infected HeLa cells 24 hours post-infection, showing the characteristic accumulation of spherical IV and IVN (marked IV), and dense brick-shaped mature viruses (arrowheads).* **(b)** *High-magnification field showing viral crescents. Arrow head indicates the crescent internal membrane. Arrows indicate spikes typical of crescents. (From Risco et al. 2002)*

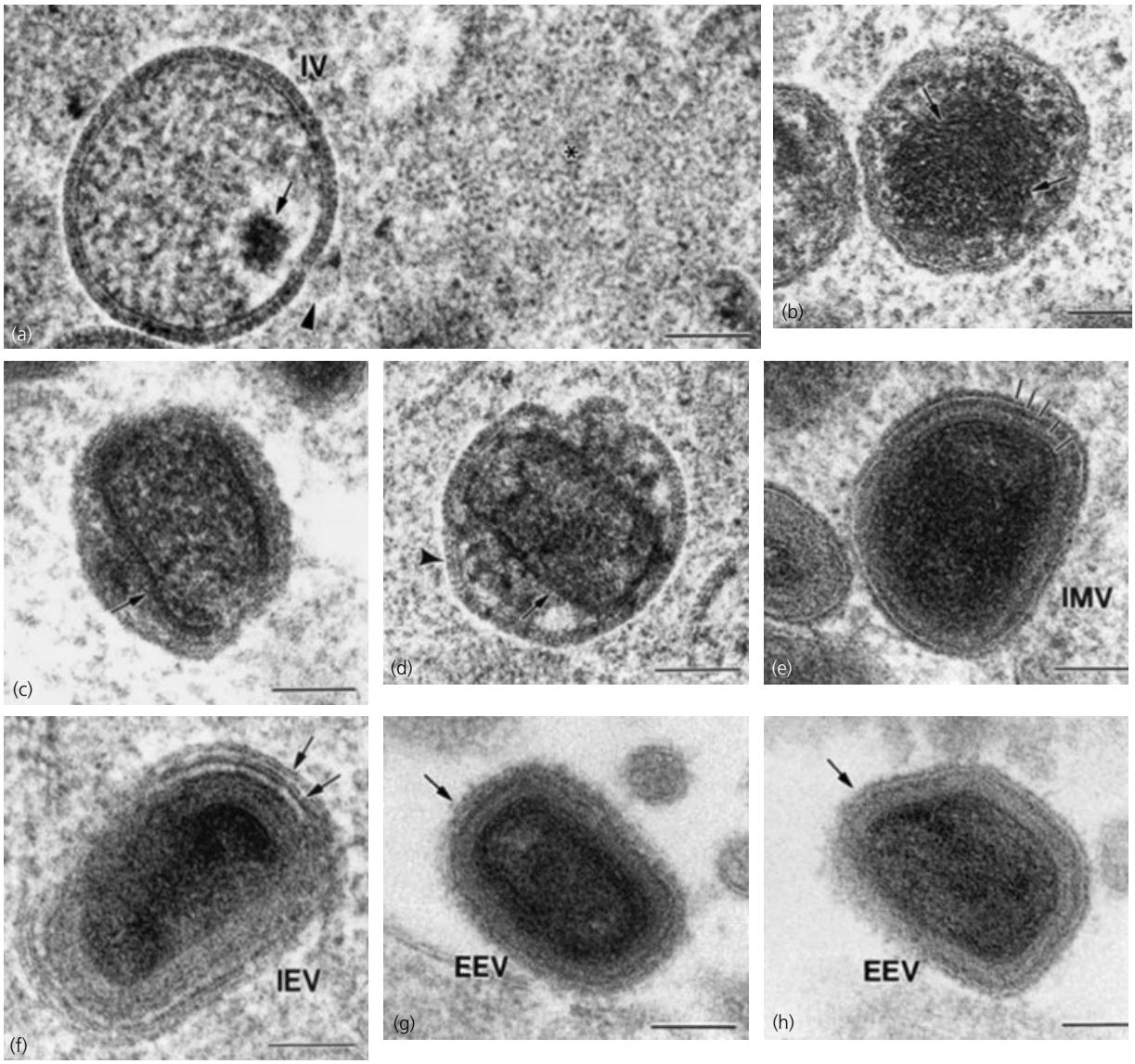

Figure 11 *Intermediates in virus assembly.* **(a)** *IV particle packing DNA (arrow); pore in the IV (arrowhead).* **(b)** *Spherical dense particles with fibrous, DNA-like, internal material (arrows).* **(c)** *and* **(d)**. *Potential intermediate maturation stages in the construction of the internal viral core (arrows) and the IMV.* **(e)** *IMV.* **(f)** *IEV with the additional double membrane (arrows).* **(g)** *and* **(h)** *Two different section planes of EEV, which have an external fuzzy coat (arrows). Bars, 100 nm. (From Risco et al. 2002)*

relatively rare and therefore the process of IMV maturation is probably rapid (Figure 11). Maturation from IVN to IMV is accompanied by proteolysis of several major virion structural proteins; mutation of one of the viral proteases involved in virion protein cleavage interrupts morphogenesis at the IVN to IMV transition (Ericsson et al. 1995; Byrd et al. 2002). Maturation from IVN to IMV is also accompanied by microtubule-mediated migration of the particles from viroplasm toward the periphery of the cell (Sanderson et al. 2000). More than 90 percent of virus does not mature past the IMV stage; a small percentage matures further to eventually be exported from the cell as EEV (Boulter and Appleyard 1973). As described earlier, EEV formation involves first the formation of IEV. IEV consists of IMV surrounded by two additional membranes, acquired via wrapping of IMV in trans-Golgi-network-derived cisternae which have been modified by the incorporation of specific virus proteins (Figure 11) (Schmelz et al. 1994). IEV are mobilized to the undersurface of the plasma membrane on microtubules, where the outermost IEV membrane fuses with the plasma membrane to form CEV (Blasco and Moss 1992; Hollinshead et al. 2001). CEV thus consists of IMV wrapped in one additional membrane, and remains attached to the external surface of the plasma membrane. Actin filament bundles then form beneath CEV pushing outward, resulting eventually in the formation of long, specialized actin-containing microvilli, each of which contains a single

CEV perched at the tip (Stokes 1976; Cudmore et al. 1996). CEV is eventually released from the surface of the cell to become EEV, which, like CEV, consists of IMV surrounded by one additional membrane. Both CEV and EEV mediate short-range and long-range cell-to-cell spread of virus, respectively (Blasco and Moss 1992; Law et al. 2002).

Some poxviruses, including certain strains of cowpox, ectromelia, raccoonpox, fowlpox, and canarypox, also produce an occluded form of virus. These viruses encode an A-type inclusion protein which is produced in large amounts late in infection and which condenses into large, electron dense aggregates in the cell cytoplasm (McKelvey et al. 2002). A-type inclusions are embedded with numerous IMV (Figure 12). While no function for A-type inclusions has been proven, it seems reasonable to assume that they would provide increased environmental stability for the embedded IMV.

Over the past decade, information regarding the contribution of individual virus genes to virus morphogenesis has been gathered by studying virus assembly following infection with mutants in at least 32 different virion proteins. The mutants which have been characterized can be roughly staged into six categories corresponding to the major intermediates in virus assembly (one recent representative reference is provided for each class):

- mutants defective in viral membrane synthesis which accumulate viroplasm devoid of crescents (Demasi and Traktman 2000)
- mutants defective in IV synthesis which accumulate viroplasm containing crescent membranes but no complete IV (Chiu and Chang 2002)

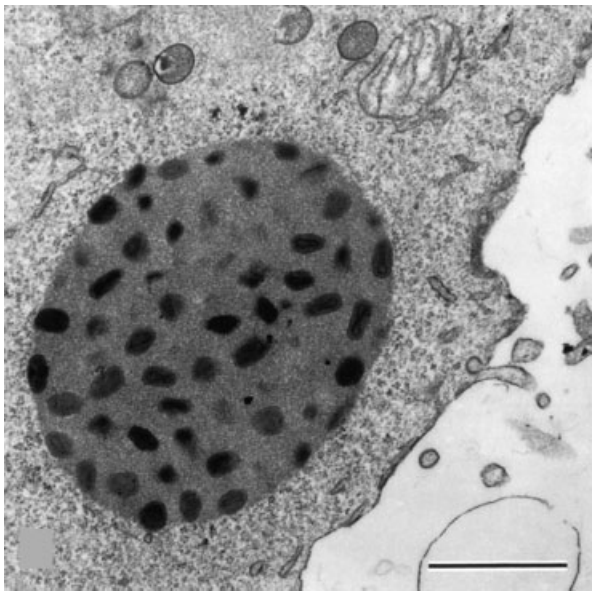

Figure 12 *A-type inclusions in cells infected with cowpox virus. The inclusion contains multiple IMV. Note the IV and IVN immature particles above the inclusion. Bar, 1 000 nm. (From McKelvey et al. 2002)*

- mutants defective in IVN synthesis which accumulate viroplasm containing IV (Yeh et al. 2000)
- mutants defective in IMV synthesis which accumulate viroplasm containing IV and IVN but no IMV (Garcia and Moss 2001)
- mutants which accumulate normal looking IMV which are not infectious (Liu et al. 1995)
- mutants defective in EEV synthesis which accumulate IMV (Sanderson et al. 2000).

Mutants in several of these categories accumulate structures which are not normally seen during wild-type virus infections; such structures may be either normal intermediates in virus assembly or dead-end defective products. Logically, mutants in several IMV membrane proteins are defective in crescent or IV formation (Rodriguez et al. 1995, 1998; Wolffe et al. 1996; Traktman et al. 2000), mutants in several core proteins are defective in maturation of IV to IMV (Klemperer et al. 1997; Williams et al. 1999), and mutants in several EEV membrane proteins are defective in maturation of IMV to EEV (Blasco and Moss 1991; Engelstad and Smith 1993; Wolffe et al. 1993). However, it is also true that mutants in several core proteins and enzymes affect viral membrane formation (Wang and Shuman 1995; Demasi and Traktman 2000), and conversely that mutants in some membrane proteins affect the transition from IV to IMV (Ravanello and Hruby 1994). These genetic studies have also revealed that poxviruses encode a complete pathway for disulfide bond formation which is required for formation of at least some of the disulfide bonds critical for virus structure and assembly (Senkevich et al. 2002). Mutation of any of the genes in the pathway interrupts morphogenesis at the IV to IVN stage.

IV and IMV membrane formation

Two distinct models have been proposed to describe the origin and structure of viral crescents and the IMV membrane. One model proposes that the crescent membranes comprise a single lipid bilayer which arises de novo, that is without continuity with any other intracellular membrane structure (Dales and Mosbach 1968; Hollinshead et al. 1999). This de novo model is based on the observation that, using conventional EM techniques, the crescents and the IMV membrane have dimensions consistent with a single lipid bilayer, and that in most standard EM preparations of virus-infected cells, no convincing connection can be observed between the crescents and any other intracellular membrane system. The notion of de novo biogenesis of a lipid bilayer membrane with free ends and two cytoplasmic surfaces is unprecedented in cell biology. An alternative model which is more consistent with established cell biology principles is that the crescents are formed from membrane cisternae derived from the endoplasmic reticulum–Golgi intermediate

compartment (ERGIC), which, through incorporation of viral proteins, collapse into rigid crescents, such that the crescents and IMV membranes actually comprise two tightly apposed lipid bilayers (Sodeik and Krijnse-Locker 2002). This 'double membrane' model is supported by EM images of IV prepared by freeze substitution, and by EM examination of thin cryosections of infected cells, which under some circumstances reveal apparent localized separation of two membranes within a crescent. In addition, cryosections stained with antibodies to both cellular and viral proteins show labeling of ERGIC membranes with viral proteins, and continuity between crescents and cell-derived ERGIC membrane cisternae (Sodeik et al. 1993; Salmons et al. 1997; Rodriguez et al. 1997; Risco et al. 2002). The double-membrane model is further supported by the clear demonstration of a remarkably similar mechanism of virion membrane biogenesis during maturation of another cytoplasmic DNA virus, African swine fever virus (Andres et al. 1998). Interestingly, infection with mutants in either of the poxvirus IMV membrane proteins A17L or A14L results in aberrant or deficient synthesis of crescents, and accumulation of numerous small vesicles containing viral membrane proteins (Rodriguez et al. 1995, 1998; Wolffe et al. 1996; Traktman et al. 2000). It has been proposed that these vesicles may normally be intermediates in crescent formation (Traktman et al. 2000). The vesicles could be shed from the ERGIC, and would normally be short lived. Crescents formed by fusion and collapse of these vesicles would comprise tightly apposed double membranes, but consistent continuity with the intermediate compartment would be rare, thus explaining the apparent de novo evolution of viral membrane crescents. A similar model of viral membrane formation proposes that the IV membrane is formed by the fusion of ERGIC-derived, viral protein-modified tubules (Risco et al. 2002). Phosphorylation may play a critical role in regulating viral membrane formation, since both the A17L and A14L membrane proteins are phosphorylated by the virus-coded F10L kinase, and mutational inactivation of the F10L kinase abrogates crescent formation (Traktman et al. 1995; Betakova et al. 1999; Derrien et al. 1999).

Viral DNA packaging

The mechanism by which viral DNA is packaged into virions is incompletely understood. Viral DNA concatemers are resolved to unit length genomes during infection with virus mutants that are incapable of forming even the earliest crescent membranes; therefore the final stages of viral DNA processing do not seem to be coupled to morphogenesis (Demasi and Traktman 2000). In theory, unit length viral DNA genomes could be incorporated into IV during membrane synthesis and subsequently condense into nucleoids. However, several investigators have observed IV which are incompletely sealed and which possess a chromatin- or nucleoidlike material positioned across a small opening or pore in the IV, sometimes apparently continuous with the internal nucleoid (Figure 11) (Morgan 1976; Ericsson et al. 1995; Risco et al. 2002). These images are interpreted to mean that DNA is specifically packaged late during IV assembly to form IVN. Interestingly, mutation of the viral gene *A32L*, which contains a predicted ATP/GTP-binding site 'P loop' motif, interrupts virus assembly at the IV to IVN transition, resulting in the accumulation of reduced amounts of IVN and large amounts of a novel, electron-dense spherical particle which does not contain DNA (Cassetti et al. 1998). These observations are consistent with a late DNA packaging model and suggest that the *A32L* gene product may mediate DNA packaging.

One group has proposed recently that viral DNA is packaged through an association of DNA with ERGIC and a second infolding of the DNA-membrane complex into the IV membrane during IVN morphogenesis (Griffiths et al. 2001a, 2001b). This model predicts that the core wall is in fact a double membrane similar to the proposed double-membrane structure for the IV and IMV membrane. Further research is required to substantiate this model.

POXVIRUS GENETICS

Overview

Genetic manipulation of poxviruses has had a broad impact on both poxvirology in particular and biomedical science in general. The influence of poxvirus genetics can be broken down into three specific areas:

- construction of virus mutants for use in determining the function of individual poxvirus genes
- use of recombinant poxviruses for protein over-expression
- use of recombinant poxviruses as vaccine vectors.

Genetic manipulation of poxviruses has taken liberal advantage of both classical 'forward' genetic techniques and modern 'reverse' genetic techniques. Classical forward genetics consists of chemical mutagenesis and random isolation of temperature-sensitive, host-range, drug-resistant, and drug-dependent mutants, followed by employment of genetic mapping procedures to determine which genes have been affected. Modern reverse genetics consists of site-specific engineering of the viral genome, from alteration of existing poxvirus genes in situ to introduction of foreign genes and heterologous transcriptional operons into the poxvirus genome.

Regardless of the application, reverse genetic engineering of poxvirus genomes follows the same fundamental 'in vivo recombination' protocol (Nakano et al. 1982). Specifically, if cells are first infected with a poxvirus and then transfected with a DNA molecule

which has homology with the infecting poxvirus genome, the transfecting DNA molecule will undergo homologous recombination with the infecting genome. A simple example of this principle is marker rescue mapping of temperature-sensitive mutants of vaccinia virus (Thompson and Condit 1986). In this example, cells are infected with mutant virus and transfected with candidate DNA molecules derived from wild-type virus. Homologous recombination between the mutant genome and a transfected DNA molecule which contains the wild-type allele of the gene will generate wild-type virus, thus mapping the temperature-sensitive lesion to a specific rescuing DNA fragment. Several variations on the basic in vivo recombination scheme have been derived which improve the efficiency of the procedure and/or enhance selection for recombinant genomes (Chakrabarti et al. 1985; Franke et al. 1985; Fathi et al. 1986; Falkner and Moss 1990; Merchlinsky and Moss 1992; Scheiflinger et al. 1992). In general, poxvirus recombination is efficient and the genome contains numerous nonessential insertion sites and can accommodate large amounts of foreign DNA, so that almost any recombinant that can be imagined can be constructed. Recently, a novel engineering scheme has been developed in which the entire vaccinia genome is contained in a bacterial artificial chromosome, where it can be engineered and subsequently reactivated by infection of cells infected with a nonreplicating helper fowlpox virus (Domi and Moss 2002).

In the sections that follow, we consider briefly the principles and practice of each of the specific areas of poxvirus genetics described above.

Construction of virus mutants for use in determining the function of individual poxvirus genes

Several laboratories have used classical forward genetic methods to assemble collections of conditional lethal mutants to be used for the study of the function of individual poxvirus genes (reviewed by Condit and Niles 1990). Recently, a complete complementation analysis combining two independent mutant collections has been conducted, resulting in a combined collection of 138 temperature-sensitive mutants comprising 53 complementation groups (Lackner et al. 2003). These temperature-sensitive mutants, along with numerous drug-resistant, drug-dependent, and host-range mutants, have been and will continue to be a powerful resource for determining poxvirus gene function. However, given that the poxvirus genome contains approximately 200 genes, and assuming that approximately 150 of these genes are essential for virus growth in culture, even this combined collection provides only about one-third coverage of the genome. With this in mind, two alternative methods for reverse genetic engineering of mutants in specific poxviral genes have been developed.

One reverse genetic method for creating conditional lethal poxvirus mutants involves regulating transcription of the poxvirus gene of interest using elements from a bacterial operon (Fuerst et al. 1989; Rodriguez and Smith 1990; Traktman et al. 2000). In its simplest form this involves construction of a recombinant poxvirus which constitutively expresses a bacterial repressor protein, for example the *lac* repressor, and which also contains a cognate operator (that is a repressor-binding site) between the transcriptional promoter and the coding sequence of the target gene to be regulated. In such a recombinant the expression of the gene can be controlled by use of an inducer molecule during virus infection, for example IPTG in the case of *lac* repressor control. Appropriate regulation of the target gene sometimes requires more baroque constructions. For example, recombinants can be constructed which contain bacteriophage T7 RNA polymerase under *lac* repressor control, and in which the target gene is regulated by both a T7 RNA polymerase promoter and a *lac* operator (Ward et al. 1995). Numerous inducible recombinant poxviruses have been constructed which have proven invaluable for the study of individual poxvirus genes. One drawback of this approach is that some poxviral genes, most notably early genes, cannot be cleanly regulated.

An alternative approach to creating conditional lethal poxvirus mutants is the construction of temperature-sensitive mutants using clustered charge to alanine scanning mutagenesis (Hassett and Condit 1994). Clusters of charged residues are identified within the linear amino acid sequence of a given target gene, and all of the charged residues in a given cluster are changed to alanine in a cloned copy of the gene. Individual clustered charge to alanine mutants are then recombined into the viral genome and recombinant viruses are tested for temperature sensitivity. Empirically, approximately 30 percent of clustered charge to alanine mutants prove to be temperature-sensitive, theoretically because the charge clusters normally reside on the surface of a protein, and the genetic neutralization of the charge weakens protein–protein interactions. Useful clustered charge to alanine temperature-sensitive mutants have been created in several vaccinia genes which were inaccessible via bacterial operon regulation (Hassett et al. 1997; Demasi and Traktman 2000; Ishii and Moss 2001b; Punjabi et al. 2001).

Use of recombinant poxviruses for protein overexpression

The technical foundation for use of poxviruses to overexpress proteins is a recombinant poxvirus which constitutively expresses the bacteriophage T7 RNA polymerase (called here vvT7) and a target gene which is expressed from a bacteriophage T7 transcriptional

promoter (Fuerst et al. 1986). Depending on the goal of the expression, the target gene may be presented in one of three different forms. The target gene may be contained on a plasmid, in which case expression is achieved by infection with the vvT7 and transfection with the target plasmid. The target gene may be recombined into another poxvirus, in which case expression is achieved by coinfection with vvT7 and the target gene-containing virus. Lastly, the target gene may be recombined into vvT7 itself as described above, providing that the system is under inducible control of a bacterial operon system.

Overexpression of proteins from poxvirus vectors has three principal uses (Moss 1996). First, the system can provide for eukaryotic expression of large quantities of protein for purification and use in biochemical studies (Bayliss and Condit 1995). Second, the system can be used for transient expression of proteins in eukaryotic cells in order to study protein function, for example function of paramyxovirus replication proteins (Smallwood et al. 2002). Third, the system has been used to launch synthesis of viral genomes and viral gene products for construction of recombinant viruses, for example rhabdoviruses (Roberts and Rose 1998).

Use of recombinant poxviruses as vaccine vectors

Infection of animals or humans with recombinant poxviruses which express foreign antigens under the control of poxvirus transcriptional promoters can result in a robust humoral and cellular immune response to the foreign antigen (Paoletti 1996). This strategy has potential for providing immunization against organisms for which conventional vaccines are lacking. Recombinant poxvirus vaccines have been used with clinical success in animals; for example, a vaccinia recombinant that expresses the rabies glycoprotein has been used in the wild to control the spread of rabies virus (Pastoret and Brochier 1996). While recombinant poxvirus vaccines would undoubtedly be effective in humans, none is currently licensed for human use, primarily because of safety concerns associated with the poxvirus vector. Vaccinia vectors with increased safety features have been created by deleting numerous genes nonessential for growth in culture, and alternative poxvirus vectors have been developed, most notably attenuated vaccinia virus strains (Modified Vaccinia Ankara) and avipoxvirus vectors such as canarypox (Paoletti et al. 1995).

POXVIRUS GENES RESPONSIBLE FOR IMMUNE EVASION, HOST RANGE, AND CONTROL OF APOPTOSIS

Poxviruses adopt a wide variety of strategies to ensure survival. These strategies include the shutoff of host transcription, synthesis of different forms of infectious virus particles with differing surface components that facilitate cell spread and viremia, and assembly of virus particles into environmentally resistant inclusion bodies. In addition, as the virus family evolved and became established in different vertebrate hosts or sites within a host, poxviruses developed the means to capture essential host genes which then evolved to target critical aspects of host defenses such as host range, inflammation, and other immunomodulatory mechanisms which serve to enhance virus survival. As described previously, many of these modulatory genes are located within the variable or noncore region of the poxvirus genome and are non-essential for virus growth in cell culture. There has been much speculation as to how poxviruses which develop in the cytoplasm might capture cellular genes. One clue is suggested by the finding that strains of fowlpox virus have incorporated a reticuloendotheliosis virus (a retrovirus) within the fowlpox virus genome (Hertig et al. 1997). It is possible that the reverse transcriptase encoded by a retrovirus might reverse transcribe cellular transcripts which might subsequently be incorporated into the poxvirus genome.

Many of the poxvirus-encoded immune modulatory genes are shared by a number of poxviruses, particularly those that cause similar diseases even though the viruses belong to different genera. However, these genes can also be quite different in the case where two viruses cause very dissimilar disease, as is the case if one compares one of the orthopoxviruses such as vaccinia virus or variola virus to MCV. Both these viruses employ the same basic molecular strategies of replication, transcription, contain the same central core of conserved genes and have a similar genome size. However, variola virus is associated with a severe, generalized disseminated infection with high morbidity and mortality whereas the disease caused by MCV in normal individuals is typically rather benign, albeit persistent, and consists of single or multiple 2–5 mm non-inflammatory, painless, papular, dermal lesions, which can arise on various parts of the body. These lesions may persist for months or longer and frequently resolve without treatment. In immunocompromised individuals, the disease is frequently more severe and causes severe discomfort and disfiguration (Esposito and Fenner 2001) (see Virus infections in immunocompromised patients).

It is likely that the genes contained within the variable regions of these two viruses play a major role in the disease process. In the case of MCV, these genes would be consistent with virus growth restricted to the dermis and be particularly effective against the host immune defenses operative at that site. On the other hand, vaccinia virus or variola virus might be expected to encode an entirely different coterie of genes best suited to overcome immune and host responses directed against an overt generalized infection. While

cautioning against oversimplification, this is a useful model to consider. These genes are summarized in Table 5.

In the discussion that follows, we focus our attention on vaccinia virus, much of which extrapolates to variola virus and to a lesser extent MCV because these are the most significant from the point of view of human disease. Much of what we know about virus-encoded immune modulatory genes encoded by poxviruses causing general, disseminated infections is derived from studies on the prototype orthopoxvirus vaccinia virus and from other poxviruses, most notably leporipoxvirus myxoma virus, and thus must be extrapolated to variola virus. We take note of the fact that there is some data to suggest that variola orthologs of vaccinia virus genes may be particularly attuned to human hosts (Rosengard et al. 2002) in much the same way as myxoma virus immunomodulatory genes tend to be particularly effective within rabbits. In the case of MCV, the virus can not be grown in cell culture so that study of MCV genes must be done either on the expressed and purified protein or following cloning of the MCV gene into a surrogate virus, typically vaccinia virus. There are at least five excellent, comprehensive reviews of poxvirus immunomodulatory proteins (Moss et al. 2000; Moss and Shisler 2001; Shisler and Moss 2001a; Alcami 2003; Seet et al. 2003). The following discussion draws from all these reviews.

General considerations of poxvirus-mediated control of the host response to infection

Generally, the initial exposure of a given host to viruses, including poxviruses, involves macrophage and lymphocytes and leads to inflammation in response to tissue damage and to the production of interferon. Interferon in turn stimulates natural killer (NK) cells which can kill virus-infected cells as a first line of defense in a non-specific fashion independent of the more sophisticated, adaptive immune response. Similarly, the activation of the complement cascade also functions as a first line of defense. Synthesis of antigenic virus proteins are then processed by antigen-producing cells such as dendritic cells and macrophages. This processing generates peptides which then complex with the major histocompatibility complex (MHC) and are presented by these antigen-presenting cells to lymphocytes. There are

Table 5 *Poxvirus immunomodulatory, anti-apoptotic and host range genes*

Virus	Gene		Protein	Description
Molluscum contagiosum virus	**MCV ORF**		**Size (aa)**	**Cellular homolog**
	080R		395	MHC class I α-chain homolog
	033L		579	Thymocyte antigen? CDIc T-cell surface antigen?
	066L		220	Glutathione peroxidase
	159R		241	Caspase-8 and FADD apoptosis-like inhibitor
	148R		104	Chemokine homolog
	054		235	IL-18 binding protein
Vaccinia virus	**VVCOP ORF**	**VACWR ORF**	**Size (aa)**	**Description**
	C3L	*025*	263	Complement control protein
	K3L	*034*	88	eIf2-α homolog
	E3L	*059*	190	dsRNA binding protein
	B8R	*190*	272	IFN-α receptor decoy
	B19R	*200*	353	IFN-α/β binding protein
	Absent	*013*	126	IL-18 binding protein
	B13/14R	*195*	345	Serpin (SPI-/crmA)
	A46R	*172*	240	TIR-like
	A52R	*178*	190	TIR-like
	A53R	*179*	103	TNF-like receptor*
	C22L/B28R	*004, 215*	122	Soluble TNFR
	A41L	*166*	219	Chemokine-binding protein, unknown ligand, anti-inflammatory
	A38L	*162*	277	CD47-like protein
	K2L	*033*	369	Serpin (SPI-3)
	A39R	*163*	295	Semaphorin
	A44L	*170*	346	Hydroxysteroid dehydrogenase
	C23L (*B29R*)	*218*	244	Type II chemokine-binding protein
	B16R	*197*	326	IL-1β binding protein
	C12L/B22R	*205*	353	Serpin (SPI-1)
	K1L	*032*	284	Host range, ankyrin repeat protein
	C7L	*021*	150	Host range, ankyrin repeat protein

a number of lymphocytes including $CD8^+$ T lymphocytes (CTL) that expand to clear infected cells and that comprises the basis of cell-mediated immunity (CMI). There are two types of T-helper cells that contribute to CTL activity. Type I $CD4^+$ helper T lymphocytes are involved in inducing longlasting CTL activity and the type 2 $CD4^+$ helper T lymphocytes interact with B cells to produce neutralizing antibodies, which prevent newly released virus from initiating subsequent infection (humoral immunity). CTLs recognize antigen in conjunction with class I MHC, whereas $CD4^+$ helper cells recognize antigen presented in conjunction with MHC class II (Esposito and Fenner 2001).

Orthopoxviruses have genes designed to neutralize both the interferon and complement responses. There is no evidence to suggest that MCV controls the complement response nor is involved in the control of interferons to anywhere the same degree as the orthopoxviruses. Following the innate response, in a naive host, the CTL response occurs and is primarily responsible for clearing an infection (Buller and Palumbo 1991; Palumbo et al. 1994; Ada and Blanden 1994; Barry and Bleackley 2002). However, antibodies formed soon thereafter may be instrumental in preventing newly synthesized particles from accessing sites not readily accessible to adaptive CTLs such as the skin. Therefore, it is reasonable to assume that virus-coded genes that deflect host responses would be designed not only to interfere with antigen presentation in the context of class I and II MHC but also to interfere with the consequences of that antigen presentation, namely virus clearance and ultimately immunological memory. There is some evidence to suggest that MCV controls the MHC but little evidence to suggest that orthopoxviruses do so directly.

However, a significant and general effort is mounted by all poxviruses against cytokines and chemokines, which are involved in both innate and adaptive immunity. The virus-mediated deflection of these responses serves to mask the infection by various means and 'buy time' during which the infection becomes established. orthopoxvirus-encoded proteins include those that interfere with complement, various cytokines (tumor necrosis factor (TNF), interferons (IFN)-α, β, and γ, interleukins (IL)-1β and IL-18), and Toll-like receptors (TLR) (Table 5). There are also poxvirus proteins designed to bind to and interfere with cellular chemokines as well as poxvirus-encoded chemokine-like molecules. Some of these and additional proteins interfere with aspects of inflammation, apoptosis, and host range. The repertoire of MCV appears to be more limited. As mentioned, it is likely that MCV controls the MHC system. MCV proteins that inhibit apoptosis, an IL-18 binding protein and a chemokine homologue have been identified. Each of these activities is described in more detail below. The orthopoxvirus ORF designation uses the dual nomenclature of vaccinia virus Copenhagen for convenience but cross-referencing to the ortholog in vaccinia WR is shown in Table 5.

Control of the MHC by MCV (MC080 and MC033)

A striking feature of MCV infections is the absence of inflammation at the sites of replication even in immunocompetent individuals (Heng et al. 1989). Also, lesion areas are devoid of NK, Langerhans, and T cells and no longer display β_2-macrogobulin reactivity (Viac and Chardonnet 1990). There are two MCV proteins which could potentially interact with or replace components of the MHC class I system. The *MC080R* gene of MCV encodes an MHC class I (α-) heavy chain homologue, which theoretically could compromise normal MHC class I function. However, the function of the MC080R protein is unclear because complexes of MC080R and β_2-macroglobulin form only at high concentrations of MC080R and are not detected on the surface of cells (Senkevich and Moss 1998). It is also noteworthy that even when complexes were detected under conditions when MC080R was overexpressed, HLA-A2 appears to successfully compete with the MC080R protein for binding to β_2-microglobulin (Senkevich and Moss 1998). Hence the mechanism by which MC080R acts is unclear. The *MC033L* ORF has some homology to thymocyte antigen and to the CD1c T-cell surface glycoprotein but the mechanism of action is unknown.

Myxoma virus, like vaccinia virus, causes a generalized disseminated infection and down-regulates class I MHC epitopes (Boshkov et al. 1992) through the M153R protein. The M153R protein contains a leukemia-associated protein (LAP) domain and localizes to the endoplasmic reticulum and is believed to target β_2-macroglobulin-associated MHC I molecules both at the cell surface and in the post-Golgi compartment for retention and subsequent lysosomal degradation (Guerin et al. 2002). There is little evidence to suggest direct control of the MHC by vaccinia virus. In fact, were MHC presentation significantly affected by vaccinia virus, vaccinia would not be nearly as effective as a vaccine vector as it appears to be (Nash et al. 1999; Moss 2001).

Complement (ORF C3L)

The complement control protein (VCP) was one of the first poxvirus anti-host defense genes discovered (Kotwal and Moss 1988; Kotwal et al. 1990). The protein is characterized by short consensus repeat sequences (SCR) characteristic of complement regulatory proteins (Kotwal and Moss 1988). VCP inhibits both the classical and alternative activation of complement by inhibiting C3b and C4b proteins, which are key in the formation of active complement (Kotwal et al. 1990). VCP also

inhibits the antibody-dependent, complement-mediated neutralization of IMV. Deletion of the *VCP* gene from vaccinia virus leads to production of smaller lesions in infected animals (Isaacs et al. 1992). It is interesting to note that the corresponding variola virus gene is much more efficient than the vaccinia VCP in inhibiting human complement (Rosengard et al. 2002). Another orthopoxvirus protein that contains SCRs, is the *B5R/VACV-WR_187* gene product (Takahashi-Nishimaki et al. 1991; Engelstad et al. 1992). B5R is a component of EEV membranes but is not found in IMV (Herrera et al. 1998). Despite the presence of SCRs, B5R does not appear to exert any effects on complement.

Interferons (IFN) (ORFs B8R, E3L, and K3L)

IFN-γ

The role of IFNs in controlling virus infections is well known. IFNs regulate a number of intracellular signaling pathways through kinase activation and these pathways are targeted in some fashion by both the orthopoxviruses and MCV, albeit differently. IFN-γ acts to up-regulate the double-stranded RNA-dependent protein kinase, phosphokinase R (PKR), a key component in cellular signaling pathways. In the case of orthopoxviruses, IFN-γ action is regulated both intracellularly by the *K3L* and *E3L* genes and extracellularly by a soluble, secreted type II IFN receptor (B8R).

The orthopoxvirus-secreted IFN-γ receptor (B8R) serves as an IFN-γ decoy binding protein, which acts to sequester IFN-γ and to prevent productive binding of the cytokine to cellular receptors. This decoy protein, typical of many 'viroceptors,' contains sequences similar to the cytokine-binding domain but lacks the transmembrane and cytoplasmic signaling sequences. While the vaccinia IFN-γ receptor decoy binds IFN-γ from many species, a similar protein isolated from myxoma virus binds only rabbit IFN-γ, indicative of adaptation to the rabbit host (Mossman et al. 1995) and the comparable gene of ectromelia virus is specific for mouse IFN-γ (Smith and Alcami 2002). Deletion of the IFN-γ receptor from vaccinia virus leads to attenuation of the virus in mouse models (Verardi et al. 2001).

The proteins E3L and K3L are involved in anti-host defense strategies that regulate the IFN responses through accessory cellular proteins including PKR, a protein induced by IFN, and the enzyme 2′,5′-oligoadenylate synthetase (OAS) (Sen 2001). Both E3L and K3L can effectively prevent PKR-mediated inhibition of protein synthesis at the level of RNA or translation, respectively. The intracellular *K3L* gene, which resembles the protein translation factor eIF2α, acts as a nonfunctional substate for PKR, which is induced by IFN-γ (Davies et al. 1993). The *E3L* gene encodes a double-stranded RNA-binding protein of either 20 or 25 kDa. Hence, by binding double-stranded RNA, the E3L protein prevents double-stranded RNA activation of PRK (Chang and Jacobs 1993; Yuwen et al. 1993). There is also some evidence to suggest that E3L can interact directly with PKR to form an inactive heterodimer (Romano et al. 1998). The binding of E3L to dsRNA also serves to abrogate activation of OAS. Initially synthesized as an inactive enzyme, OAS requires dsRNA for activation, a step also prevented by the binding of E3L to dsRNA. Once activated, OAS synthesizes 2′–5′-linked oligoadenylate which serves as a cofactor for ribonuclease L. Ribonuclease L cleaves both ribosomal and mRNA, leading to inhibition of protein synthesis at the RNA level. Deletion of the *E3L* gene has a major effect on virulence and deletion of either gene leads to a reduced host range of the virus which is discussed further under poxvirus genes that affect host range.

As is discussed below, like IFN-γ, IL-β is also controlled in an intra- and extracellular fashion. The dual means by which the virus acts to prevent the action of these cytokines suggests that the pathways controlled by these cytokines are very important in counteracting the virus infection.

IFN-α/β

Orthopoxviruses, including variola virus, encode a type I interferon binding protein (ORF B19R) which binds both IFN-α and β (Symons et al. 1995). Unlike the IFN-γ soluble receptor just described, this protein has little or no similarity to the cellular counterpart. A folding of the immunoglobulin domains contained in the viral IFN-α/β binding protein and the fibronectin type III domains of the cellular receptor have been proposed to account for their interaction with the same ligands (Alcami 2003). The protein binds to and inhibits the type I interferons IFN-α,β as well as IFN-δ and ω (Symons et al. 1995; Liptakova et al. 1997; Vancova et al. 1998; Smith and Alcami 2002). Deletion of the gene leads to attenuation of the virus (Verardi et al. 2001).

Interleukin (IL)-18-binding proteins (VACV-WR_013, MC054)

The IFN pathway is targeted indirectly by proteins which bind to and functionally eliminate IL-18, a potent inducer of IFN-γ. An IL-18-binding protein is encoded by some but not all strains of vaccinia virus and is absent in strain Copenhagen (Symons et al. 2002). This protein also has little or no sequence similarity to the cellular IL-18-binding protein. An equivalent protein is encoded by MCV. Originally, IL-18 was identified as a powerful inducer of IFN-γ, but also induces the synthesis of a number of the cytokines and chemokines, which in turn can influence the Th1 or Th2 cellular response and influence activation of NK and cytotoxic T cells

(Nakanishi et al. 2001). Deletion of the IL-18-binding protein encoded by vaccinia virus WR results in attenuated infections (Symons et al. 2002). Deletion of the IL-18-binding protein gene encoded by ectromelia virus, a pathogen of mice, also resulted in virus attenuation (Born et al. 2000).

The MCV genomic sequence indicates that none of the plethora of soluble cytokine-receptor or cytokine-binding proteins or decoy receptors found in the orthopoxviruses are encoded by MCV. However, as stated above, the virus does encode an IL-18-binding protein (MC054) (Xiang and Moss 1999). Strong binding of IL-18 has been observed with MC054 (Xiang and Moss 1999). Two additional MCV proteins encoded by ORFs, MC051 and MC053, are related to MC054, but these two proteins lack conserved residues associated with high-affinity binding to IL-18. Therefore, it is likely these two proteins bind other ligands (Xiang and Moss 2001). Unlike the orthopoxvirus IL-18-binding proteins, the MC054 protein contains a long C-terminal extension. The C-terminal tail binds with high-affinity to glycosaminoglycans and hence to cell surfaces (Xiang and Moss 2003). MC054 contains a furin cleavage site which separates the glycosaminoglycan-binding C-terminal tail of the molecule from the IL-18-binding portion of the protein (Xiang and Moss 2003). This allows for a secreted as well as cell-bound form of the IL-18-binding protein. It has been proposed that the C-terminal tail may function to alter the half-life of the IL-18-binding protein and serve to concentrate the protein in the vicinity of MCV-infected cells where it is most needed. The cleaved form may allow IL-18 binding and sequestration activity to leave the site of infection for activity at distal sites.

Interleukin (IL)-1β (ORFs B16R, B13R/B14R, A46R, and A52R)

Vaccinia virus encodes a secreted IL-1β-decoy receptor (ORF B16R), (Alcami and Smith 1992; Spriggs et al. 1992) but interestingly, the gene is present but inactive in all variola virus strains examined to date. This decoy receptor binds IL-1β but not IL-1α or the host IL-1 receptor antagonist (Alcami and Smith 1992; Spriggs et al. 1992; Smith and Alcami 2000). Deletion of this gene from vaccinia virus suggested that IL-1β serves to control fever during poxvirus infections (Alcami and Smith 1996). Virus lacking a functional B16R is attenuated when animals are infected via the intracranial route (Spriggs et al. 1992) but exhibits enhanced virulence during intranasal infections (Alcami and Smith 1992).

IL-1β, like IFN-γ, is also regulated both intracellularly as well as extracellularly. In the case of IL-β, intracellular regulation is mediated by one of the three virus-encoded serpins, crmA/SPI-2 (ORF B13R/B14R). The protein encoded by crmA/SPI-2 is a potent inhibitor of caspases-1 and 8 and perhaps 10 as well as granzyme B. Hence this protein has the unusual property of inhibiting both serine (granzyme B) and thiol proteases (caspases). Caspase-1 is responsible for synthesis of active, proinflammatory IL-1β from the inactive precursor proIL-1β (Ray et al. 1992) as well as activating IL-18 from proIL-18 (Gu et al. 1997). Therefore, crmA/SPI-2 can influence the levels of both IL-β and IL-18. The ability to inhibit caspase-8 can serve to block apoptosis via several pathways including not only extrinsically induced apoptosis induced by Fas/Fas ligand and granzyme B, but also apoptosis intrinsically mediated by caspase-8. However, deletion of this gene from vaccinia virus or cowpox virus leads to only modest attenuation (Tscharke et al. 2002).

Finally, IL-β can potentially be regulated intracellularly in one additional way. After ligand engagement, IL-1β receptor-mediated signaling can be disrupted by poxvirus proteins that contain signaling components of Toll-like receptors (TLR). TLRs are important in innate immunity and serve as pattern recognition receptors on cells of the innate immune system (O'Neill 2000). Variola virus encodes one such gene, vaccinia virus two (*A46R* and *A52R*). Each of these proteins contains a Toll/IL-1 receptor (TIR) domain motif within the cytoplasmic portion of the molecule which allows for interaction with adaptor molecules to prevent intracellular signaling downstream of the IL-1 receptor (Bowie et al. 2000). Of the two vaccinia proteins, A52R appears the more effective in inhibiting activation of NF-κB, a transcription factor involved in cytokine expression (Bowie et al. 2000). As might be anticipated, proteins such as A52R appear to endow the virus with the ability to inhibit a number of signaling pathways which are initiated from TIR domain-containing cell surface receptors such as TLRs and IL-1R (Harte et al. 2003). Deletion of A52R from vaccinia virus has no effect on growth or plaque size but the virus is attenuated in intranasally infected mice as evidenced by milder signs of illness and less weight loss (Harte et al. 2003).

Tumor necrosis factor (TNF) (ORFs C22L, B28R)

TNFs are potent proinflammatory cytokines secreted by macrophages and activated T cells comprise a family of powerful proinflammatory cytokines (Smith et al. 1994). There are at least three classes of TNFs: TNF, lymphotoxin (LT)-α and LT-β. All are active as trimers that recognize two members of the superfamily of TNF receptors (TNFR), TNFRI (p55) and TNFRII (p75). The main effect of host-encoded TNF is to induce an antiviral state that controls lysis of infected cells (Locksley et al. 2001). Poxviruses encode a number of homologues of the TNFR

and some viruses, for example cowpox virus, encode four (Hu et al. 1994; Smith et al. 1996; Loparev et al. 1998; Saraiva and Alcami 2001) designated as CrmB, CrmC, CrmD and CrmE (Cunnion 1999; Saraiva and Alcami 2001). Variola virus also encodes a TNFR homologue as does vaccinia virus strain Lister; however, many vaccinia strains lack any TNF receptor homologue (Alcami et al. 1999). Vaccinia virus strain Copenhagen encodes an inactive gene, which, because it is located in the TIR of the genome, is present in two copies (*C22L*, *B28R*). These virus-coded TNFRs have sequence similarity to extracellular domains of both p55 and p75 of the host cell but lack the anchoring C-terminal transmembrane domain that anchors the cellular protein on the cell surface (Smith et al. 1991; Upton et al. 1991; Schreiber et al. 1996; Loparev et al. 1998). These molecules function as decoy TNF-binding proteins, binding TNF to prevent TNF from interacting with the appropriate cellular receptor (Cunnion 1999; Xu et al. 2000). Another member of this family of proteins, found in cowpox virus, is a protein called VD30, which resembles cellular CD30 and binds to the cognate cellular target CD153 (Panus et al. 2002). In the case of myxoma virus, deletion of the viral TNFR homologue gene leads to a strong attenuation of the virus (Upton et al. 1991).

Regulation of chemokines (ORFs C23R, B29R, and MC148R)

Chemokines are small molecules generally involved in attracting leukocytes to the site of damage produced in response to various cellular insults including infection. However, within the context of virus infection, there are numerous possibilities for the possible function of virus-encoded chemokines and chemokine receptors (Alcami 2003). Chemokine agonists might attract leukocytes capable of supporting virus infection or induce migration of Th2 cells and thereby alter the immune response to infection. Chemokine agonists might also induce angiogenesis and/or contribute to infection pathology. Virus-encoded antagonists might prevent migration of immune cells (Th1 cells) that lead to an antiviral response. The regulation of chemokines may also alter critical signaling pathways in such a way as to promote virus infection. Viral-encoded chemokine receptors could lower the concentration of critical cytokines at localized sites of infection and could down-regulate the production of the chemokine itself, which would allow for immune evasion. Cellular-bound chemokine receptors could, in response to the appropriate chemokine, lead to migration of the particular cell leading to spread of the viral infection. Finally, the receptors could induce expression of chemokines and thereby modulate the immune response or contribute to immunopathology. The poxviruses have the potential to regulate these responses by encoding chemokine-binding proteins, chemokine receptors, or chemokine ligand mimics (Kotwal 2000; Lalani et al. 2000; Mahalingam and Karupiah 2000; Mahalingam et al. 2000; Murphy 2001). Binding proteins have been grouped into low-affinity (type I) and high-affinity chemokine-binding proteins (type II). Orthopoxviruses encode a high-affinity (type II) binding protein found in two copies within the ITRs in vaccinia virus (C23R, B29R) but only one copy in variola virus, which has far smaller ITRs. This protein, also called vCCI or CBR II, binds CC chemokines but not CXC or C chemokines. The binding to CC chemokines serves to prevent binding of the CC chemokines to the appropriate G-protein-coupled receptor (Graham et al. 1997; Smith et al. 1997; Alcami et al. 1998; Smith and Alcami 2000). The orthopoxvirus protein binds to a large variety but not all CC chemokines (Burns et al. 2002). Despite the ability to bind to a wide spectrum of CC chemokines, disruption of the gene in vaccinia virus leads to only modest attenuation but an increased leukocyte infiltration (Martinez-Pomares et al. 1995; Graham et al. 1997; Lalani et al. 1999).

Rather than encode chemokine-soluble receptors or binding proteins, MCV encodes a chemokine homologue (MC148R) (Senkevich et al. 1996). The protein is a secreted 104 amino acid protein related to the CC-chemokine family (Bugert et al. 1998) but contains an N-terminal deletion which precedes a dicysteine motif that is critical for activation of receptors. Therefore, the protein can engage or titrate receptors in a nonproductive fashion and function as an antagonist. Indeed, the protein inhibits binding, signaling, and chemotaxis of leukocytes to various CC and CXC chemokines (Krathwohl et al. 1997; Damon et al. 1998). While the protein has only been studied in the context of vaccinia virus recombinants, or as a purified protein, it can be argued that MC148R exhibits anti-inflammatory effects because it can inhibit allograft rejection in transgenic mice (DeBruyne et al. 2000).

3β-Hydroxysteroid dehydrogenase (ORF A44L)

The A44L protein catalyzes the synthesis of immunosuppressive steroids and is capable of converting pregnenolone to the steroid hormone progesterone (Moore and Smith 1992). Deletion of this gene from vaccinia virus leads to somewhat smaller dermal lesions and attenuation (Moore and Smith 1992; Sroller et al. 1998; Tscharke et al. 2002).

Semaphorin (A39R)

Semaphorins are a highly conserved family of regulatory proteins defined by the presence of a SEMA domain and an extracellular component that determines receptor-binding specificity. Semaphorins can be anchored to membranes, occur as transmembrane

components, or be secreted (Spriggs and Sher 1999; Tamagnone and Comoglio 2000). Semaphorins originally were not associated with the immune system; they serve as axonal guidance clues in the developing nervous sytem. However, they also serve as chemoattractants or chemorepellants in the immune system. Recent evidence suggests additional roles for semaphorins in regulating the immune response. The semaphorin SEMA4D (CD100) SEMA4D (CD100), expressed constitutively by T cells, enhances the activation of B cells and dendritic cells (DC) through its cell-surface receptor, CD72. SEMA4A, when expressed by DCs, is involved in the activation of T cells through interactions with TIM2. Emerging evidence indicates that additional semaphorins and related molecules are involved in T-cell antigen-presenting cell (APC) interactions (Kikutani and Kumanogoh 2003). The vaccinia virus protein (A39R) encodes a 55 kDa protein with 25 percent homology to cellular semaphorins (Kolodkin et al. 1993; Comeau et al. 1998). It is believed that the A39R protein interacts with an appropriate semaphorin protein receptor, VESPR (also referred to as CD232 or plexin C1) to induce production of the cytokines IL-6 and IL-8 to control inflammation (Comeau et al. 1998). Deletion of the gene from vaccinia virus led to increased size and prolonged persistence of skin lesions but in a virus-strain-dependent manner (Gardner et al. 2001). It has been suggested that the protein has direct or indirect proinflammatory effects (Gardner et al. 2001).

Serpins

Orthopoxviruses encode three serpins (serine proteinase inhibitors) designated as SPI-1 (C12L), SPI-2/crmA (B13R/B14R), and SPI-3 (K2L). The leporipoxviruses also encode three serpins, designated SERP1, SERP2, and SERP3. The number of serpins encoded by an individual virus can vary. The avipoxviruses can encode five or more. The serpin most universally distributed appears by sequence to be an ortholog of SPI-2/crmA. SPI-2/crmA is functionally very similar to SERP2 and SPI-3 is very similar to SERP1. MCV encodes no serpins.

SPI-3

SPI-3 (K2L, VACWR033) and SERP1 of myxoma virus are biochemically very similar. The proteinases inhibited by SERP1 include plasmin, tissue plasminogen activator, urokinase, and thrombin (Lomas et al. 1993; Nash et al. 1998). These proteases are the same as those inhibited by the SPI-3 protein even though the two proteins are only 29 percent identical (Turner et al. 2000). Nevertheless, SPI-3 can not replace SERP1 to maintain virulence in myxoma virus (Wang et al. 2000) and deletion of SPI-3 from vaccinia virus or cowpox virus leads to little if any attenuation of the virus in animals. SPI-3 inhibits orthopoxvirus-infected cell fusion, a property absent in SERP1 (Wang et al. 2000). The myxoma virus SERP1 is an extremely powerful anti-inflammatory protein (Upton et al. 1990; Macen et al. 1993) which when deleted leads to increased inflammation and a marked attenuation and more rapid clearance of myxoma virus in rabbits (Upton et al. 1990; Macen et al. 1993). Purified SERP1 has been shown to prevent inflammation following balloon angioplasty (Lucas et al. 1996; Liu et al. 2000) and allograft and xenograft transplant rejection in animal models (Miller et al. 2000).

SPI-2/CRMA

The *SPI-2* gene (*B13R/B14R* in vaccinia virus), was originally discovered in cowpox virus where the gene was designated as *crmA*. The role of this protein in regulating IL-1β and IL-18 has been discussed earlier in this chapter. In addition to being an effective inhibitor of caspase-1, it is also an effective inhibitor of caspase-8 and perhaps caspase-10. Inhibition of caspases-8 and/or 10 targets key apical caspases in the pathways of apoptosis, initiated by caspase-8, TNF- or Fas-ligands (Enari et al. 1995; Tewari and Dixit 1995; Dobbelstein and Shenk 1996; Kettle et al. 1997). This serpin can also inhibit the serine protease granzyme B, a product of NK and cytotoxic T cells, delivered to target cells to initiate perforin-dependent apoptotic killing (Quan et al. 1995; Tewari et al. 1995). Therefore SPI-2/crmA is referred to as a cross-class inhibitor because of activity against both thiol and serine proteases. This cross-class specificity is shared by the myxoma virus SERP2 protein (Turner et al. 1999). Deletion of SERP2 from myxoma virus leads to attenuation, increased inflammation, and apoptosis of lymphoid cells (Petit et al. 1996; Messud-Petit et al. 1998). Despite the overt functional similarities between SERP2 and the orthopoxvirus SPI-2/crmA, deletion of the gene from vaccinia virus (Kettle et al. 1995) or cowpox virus (Thompson et al. 1993) leads to little if any attenuation of intranasal virus infection of animals. However, when virus is introduced into the ear pinnae of the mouse, deletion of the *SPI-2/crmA* gene leads to enhanced virulence (Tscharke et al. 2002). In addition to serpin-mediated inhibition of apoptosis, a second activity, as yet unidentified, also directly modulates the mitochondrial apoptotic pathway by influencing the permeability transition core (Wasilenko et al. 2001).

SPI-1

Although grouped for simplicity with the other serpins, the *SPI-1* gene is best known as a host-range gene and is discussed below.

Host-range genes

SPI-1 (ORF *C12L/B22R*)

The orthopoxvirus serpin SPI-1 inhibits cathepsin G, a major constituent of neutrophils (Moon et al. 1999) and

deletion of the gene leads to loss of the ability of the virus to grow on human and swine cells (Ali et al. 1994). The phenotype conferred on infected cells appears perhaps to be virus dependent. In the case of RPV, infection of nonpermissive human cells leads to viral DNA replication and full expression of viral proteins and an apoptoticlike phenotype (Brooks et al. 1995) but with vaccinia virus, the nonpermissive infection was characterized by very low levels of intermediate and late viral mRNAs (Shisler et al. 1999). Deletion of the gene from the virus has only modest effects on virulence (Blake et al. 1995).

K3L AND *E3L*

The orthopoxvirus genes *K3L* and *E3L* have been previously discussed in the context of their involvement in controlling the interferon response. They are also required for broad virus host range. The *E3L* gene product, which binds double-stranded RNA, is required for replication in HeLa cells, but not BHK cells. The *K3L* gene functions as a competitive inhibitor for eIF2α phosphorylation. BHK cells are believed to contain relatively low levels of PKR. It is believed that during infection of BHK cells, there are sufficient levels of *K3L*, even in the absence of *E3L*, to prevent activation of PKR, thus offering an explanation for why *K3L* is required for replication in BHK cells. The reverse situation exists for HeLa cells where *E3L* is required. In this situation, the levels of *E3L* are sufficient to sequester all virus-induced dsRNA, alleviating the need for *K3L* (Langland and Jacobs 2002). Infection of nonpermissive cells with virus deleted for *E3L* also leads to apoptosis and the virus is profoundly attenuated when used to infect mice either intracranally or intranasally (Brandt and Jacobs 2001). Infection of nonpermissive cells with virus deleted for *K3L* also leads to apoptosis but the virus has not yet been tested in mice (B. Jacobs, personal communication).

CHOHR (*CP77*), *K1L*, AND *C7L*

Three orthopoxvirus host-range genes have been identified. All three gene products contain ankyrin repeats. The genes are dissimilar in size and sequence but are functionally related. The *CP77* gene of cowpox virus encodes a 77 kDa protein that confers on the virus the ability to grow on Chinese hamster ovary (CHO) cells (Spehner et al. 1988). The corresponding gene is present intact in variola virus (*D6L*, Garcia strain) but is truncated and nonfunctional in vaccinia virus strain WR (*B24L*) and deleted in vaccinia virus Copenhagen. Vaccinia can not grow on CHO cells. The *K1L* and *C7L* genes which encode 30 and 17 kDa proteins, respectively, and the *CP77* gene are under certain conditions functionally interchangeable and somewhat hierarchical in terms of control of host range. Each will allow growth of virus in porcine or human cells. Similarly, the *K1L* and *CP77* genes are interchangeable in that both allow virus growth in RK13 cells. Defects in *K1L* (Ink et al. 1995; Chung et al. 1997) have been linked to induction of apoptosis during infection of the respective nonpermissive cells, but apoptosis is likely not the primary cause of the abortive infection. When virus defective in *K1L* is used to infect nonpermissive RK13 cells, early transcripts are synthesized but early protein synthesis is arrested (Ramsey-Ewing and Moss 1996; Chung et al. 1997). Vaccinia virus infection of CHO cells is characterized by a block in the translation of intermediate mRNAs which prevents synthesis of late mRNAs (Ramsey-Ewing and Moss 1995). There appears to be no effect of either the the *K1L* or *CP77* gene in animal models (Chen et al. 1992, 1993). Less is known about the function of the *C7L* gene (Oguiura et al. 1993).

Inhibition of apoptosis

A number of the genes previously discussed control apoptosis. These include the orthopoxvirus caspase and granzyme B inhibitor *SPI-2/crmA*. Viral proteins which target the IFN response pathway, such as the decoy receptors and the *E3L* and *K3L* genes, can also be viewed as inhibitors of apoptosis because induction of both PKR and OAS which are intimately controlled by these viral proteins also induce apoptosis by activating caspase-8 (Gil and Esteban 2000). MCV encodes some unique regulators of apoptosis.

MC66

The MC66 ORF encodes an inhibitor of ultraviolet- or peroxide-mediated apoptosis (Shisler et al. 1998). The protein has homology to human glutathione peroxidase and is a member of the class of selenocysteine-containing proteins (Moss et al. 2000) that can be considered to function as an antioxidants. This MCV selenoprotein protects human keratinocytes against the cytotoxic effects of ultraviolet irradiation and hydrogen peroxide, providing a mechanism for a virus to defend itself against environmental stress. Enzymes such as these convert hydrogen peroxide into water and oxygen, which together with catalase and superoxide dismutase reduce levels of oxygen-reactive metabolites, which if left unchecked, would damage the cellular environment or induce apoptosis.

MC159R

Both MC159R and the related protein M160R each contain two death effector domains (DED) (Senkevich et al. 1997), which are critical motifs involved in regulating apoptosis. MC159 binds to Fas-associated death domain (FADD) as well as procaspase-8 and acts to protect transfected cells against death effector filament formation and apoptosis induced by either Fas ligand or TNF-α (Bertin et al. 1997; Hu et al. 1997; Thome

et al. 1997; Tsukumo and Yonehara 1999). Proteins such as this are referred to as viral FLICE inhibitory proteins (vFLIP) because of their ability to complex with procaspase-8 or FADD to prevent activation of procaspase-8 to active caspase-8. The function of MC160R, despite having two DED domains, is less clear. While the MC160R protein does have some anti-apoptotic activity, it is weak (Hu et al. 1997). Furthermore, the protein undergoes degradation by caspases in the absence of MC159R, indicating that one role of MC159R is to stabilize MC160R (Shisler and Moss 2001b).

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Orthomyxoviruses: influenza

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INTRODUCTION

Influenza viruses are unique in their ability to cause recurrent seasonal epidemics of varying severity, as well as global pandemics during which acute febrile respiratory disease occurs explosively in all age groups. Excess hospitalization and death often accompany less serious but widespread morbidity during both seasonal epidemics and pandemics of influenza.

Two features of influenza virus replication and evolution account for much of the epidemiological success of these viruses. First, there is the ability of novel influenza A viruses that circulate naturally in avian reservoirs to emerge unpredictably, either through genetic reassortment or through direct transmission, and spread in a naïve human population to cause an influenza pandemic. Second, there is the relatively rapid and unpredictable antigenic change that accompanies the evolution of influenza viruses once they have become established in humans. Although influenza viruses have been studied intensively for more than 70 years and have often been used as a research model for other pathogens, much remains to be learned about influenza virus replication, pathogenicity, epidemiology, immunology, and the factors that lead to the development of influenza pandemics.

Historical record

Influenza has probably existed from antiquity, although the lack of specific pathognomonic signs makes this assertion less definite than in the case of smallpox or cholera (Kilbourne 1987). Even so, historical records of rapidly spreading 'catarrhal fevers' in Great Britain (Table 1) suggest that major influenza epidemics affected human populations as early as the sixteenth century. Pandemic outbreaks are probably a more recent development, paralleling the increases in the world population and the growth of mass transportation systems. Animals may have played a leading role in past influenza epidemics. In the eighteenth and nineteenth centuries, for example, outbreaks of respiratory disease among horses were recorded concurrently with outbreaks in humans (Table 1). Although direct evidence for horse-to-human transmission of influenza viruses is lacking, the sizeable concentrations of these animals near previous centers of human population makes them attractive candidates as disease intermediaries (Hirsch 1883). In modern times, pigs have been accorded a prominent role in the generation of major influenza outbreaks (Goldfield et al. 1977; Scholtissek et al. 1985) (see 'Ecology of influenza'). Until recently, direct avian-to-human transmission associated with fatal outcome in infected individuals was believed not to occur. This changed in 1997 when 18 individuals – six of whom died – were infected with H5N1 avian viruses in Hong Kong (Claas et al. 1998a, b; Subbarao et al. 1998). Since then, direct avian-to-human transmission has been reported in several other incidents, such as human infection with H7N7 avian virus in the Netherlands in 2003 (Fouchier et al. 2004; Koopmans et al.

Table 1 *Influenza: a summary of major epidemics affecting Great Britain and other regions of the world, 1510–1890*

Epidemic year(s)	Season	Prevalence[a]	Temporally associated animal disease	Anomalous symptoms
1510	...	Pandemic	Murrain in cattle	'Gastrodynia'
1557	Autumn	Pandemic	...	Double tertian fever
1580	Autumn	European	Murrain in beasts (Kent)	Parotid swelling
1658	Spring	Britain, Europe	...	'Cephalic affection'
1675	Autumn	Britain, Europe	...	...
1688	...	Dublin	'Nasal defluxin' in horses	...
1693	Autumn	Britain, Europe	Antecedent nasal discharge in horses	...
1710	Spring	Britain, Europe	...	'Quick pulse'
1729	Winter	England, Europe	...	...
1732/3	Spring to winter	Pandemic	Cough in horses	Parotid swelling
1737/8	Autumn	England, Europe, North America	'Disease among horses'	...
1743	Spring	Britain, Europe	'Cough among horses'	...
1758	Autumn	North America, Europe, Britain	...	...
1762	Autumn	Britain, Europe	Antecedent 'horse colds' (1760)	Variable mortality
1767	Summer	Britain, Europe, North America	Dogs and horses, horse cold	...
1775	Autumn	Pandemic	Disease in dogs and horses	Prurigo, erysipelas, pustules
1782	Spring	Pandemic	...	...
1802/3	Spring	Britain, Europe	Cattle and domestic animals	'Interchanging with scarlatina'
1833	Spring	Pandemic	Concurrent disease in horses	...
1836/7	Winter	Pandemic	...	...
1847/8	Autumn	Pandemic	...	...
1889/90	Winter	Pandemic	...	Increased mortality age 20–60 (Jan. 1890)

From Kilbourne (1987).
a) Pandemic implies historical evidence of involvement of more than two continents.

2004) or H5N1 avian viruses in Hong Kong in 2003 (Peiris et al. 2004) and in Vietnam and Thailand where 42 cases and 30 deaths were reported through September of 2004 (Tran et al. 2004) (www.who.int/en/). Although only limited human-to-human transmission occurred, these episodes clearly emphasize the crucial role of avian species in influenza virus epidemiology.

Seroarcheology

Testing of sera from older adults for influenza virus antibodies (so-called seroarcheology) had suggested that the influenza in humans between 1889 and 1898 was caused by influenza A(H2N2) viruses (see Classification section), whereas epidemics or pandemics occurring in 1899–1917 and in 1918–1957 were the result of H3N8 and H1N1 viruses, respectively (Masurel and Marine 1973; Masurel and Heijtink 1983; Mulder et al. 1958; Noble 1982; Rekart et al. 1982). A more recent careful analysis of data from these earlier publications, using the clearly documented emergence of H1 viruses in 1918 as a model, indicates that the H3 subtype is linked to the pandemic of 1889–91, not that of 1900 as often quoted, and that the H2 subtype was unlikely to have caused a pandemic in the late 1800s (Dowdle 1999). Recent pandemic strains share the hemagglutinin (HA) and neuraminidase (NA) subtypes of these earlier strains; however, they are probably not direct descendants, as they show a close relationship to avian viruses (Scholtissek et al. 1978c; Webster and Laver 1972). Thus, the similarity of the HAs and NAs of recently circulating human viruses to those of the past may reflect certain properties of the surface molecules that favor viral replication in human hosts. Indeed, of the many HA and NA subtypes maintained in nature, only three had become established in humans: H1N1, H2N2, and H3N2 subtypes. However, a reassortant H1N2 virus emerged in the human population in 2001 (Gregory et al. 2002), and has since become established (Figure 1).

Virus isolation

The historical record of influenza viruses remained sparse until technological advances permitted their isolation. In 1901, a 'filterable agent' was isolated from chickens suffering from fowl plague (it was later classified by

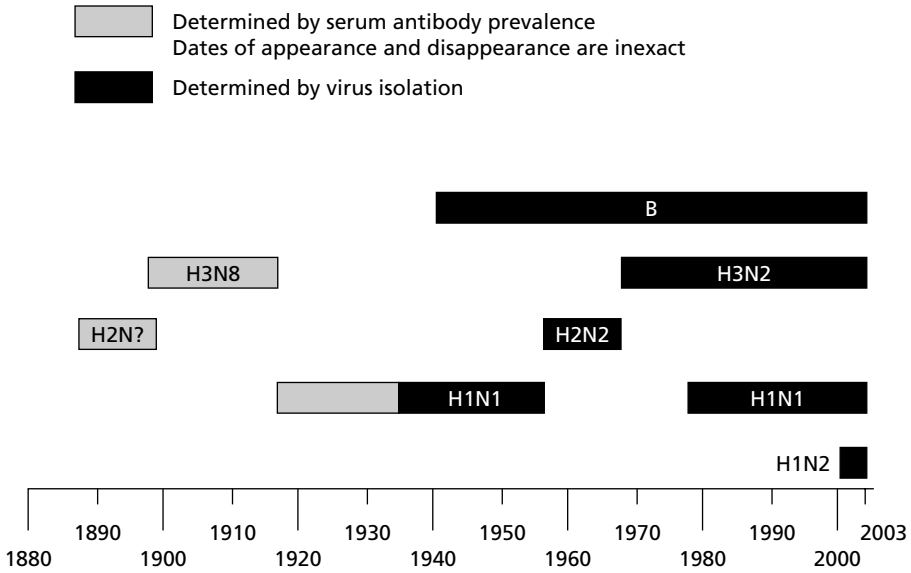

Figure 1 *Eras of prevalence of influenza A and B viruses. The periods designated in black were defined by virus isolation while the periods designated in gray were approximated by determining serum antibody prevalence in retrospective serological studies.*

Schäfer (1955) as an influenza A virus). Smith et al. (1933) inoculated ferrets intranasally with human nasopharyngeal washes, which produced a form of influenza that spread to the animals' cage mates. The human virus [A/Wilson-Smith(WS)/33(H1N1)] was transmitted from an infected ferret to a junior investigator on the project (later Sir Charles Stuart-Harris), from whom it was subsequently re-isolated. An antigenically distinct virus, isolated by Francis (1940), was classified as a type B strain (B/Lee/40) to distinguish it from the 1933 isolate. The third major type of influenza virus, influenza C, was first isolated in 1947 by Taylor (1949).

Major research advances

Influenza research gained enormous momentum when it was shown that the virus could be isolated by inoculating samples into the chorioallantoic membrane of fertilized hens' eggs (Burnet 1940; Francis and Magill 1937). Hirst (1941) and McClelland and Hare (1941) discovered that influenza virus particles agglutinate the erythrocytes of fowl as well as of other animal species. This advance established the concept of hemagglutinin inhibition in the detection of specific serum antibodies, making it possible to distinguish between viruses of the same type. Hirst (1950) further demonstrated the presence of a receptor-destroying enzyme, now known as neuraminidase. Later biochemical work (Gottschalk 1957) revealed that influenza viruses contain the HA and NA as major structural and antigenic components of the virus particle.

Subsequent advances included identification of the segmented nature of the RNA genome and assignment of corresponding protein products (McGeoch et al. 1976; Palese 1977); discovery of the antiviral compound amantadine (Davies et al. 1964); introduction of 'split' and subunit vaccines (Webster and Laver 1966); experimental work on cold-adapted vaccines; and elucidation of influenza gene replication (Inglis et al. 1979; Krug et al. 1979; Lamb and Choppin 1979). More recently, the viral genome has been completely sequenced; the three-dimensional structures of the HA (Wilson et al. 1981) and the NA (Varghese et al. 1983) have been resolved by X-ray crystallography; or the ion channel activity of the M2 protein discovered (Pinto et al. 1992; Sugrue et al. 1990b).

The establishment of reverse genetics techniques that allow the artificial generation of virus from cloned cDNA provided perhaps the most significant stimulus to influenza research in the past two decades. In 1989, a virus was generated that contained a viral RNA segment derived from cloned cDNA (Luytjes et al. 1989), followed by the generation of influenza A virus entirely from plasmids in 1999 (Fodor et al. 1999; Neumann et al. 1999). These techniques facilitated studies of the role of influenza virus proteins in the viral life cycle, identification of the determinants of viral pathogenicity, development of influenza virus vaccines, and exploration of influenza virus as a vaccine vector.

Despite this progress, much remains to be learned about influenza viruses, particularly the molecular mechanism of influenza pathogenesis, interaction between viral and host gene products, and the mechanisms giving rise to new pandemic strains.

CLASSIFICATION

The *Orthomyxoviridae* family consists of five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*,

Thogotovirus, which includes the *Thogoto virus* and *Dhori virus*, and *Isavirus*, which includes infectious salmon anemia virus (ISAV) (Klenk et al. 1995; van Regenmortel 2000). Orthomyxoviruses (Greek: orthos, straight or correct; myxa, mucus) contain segmented, linear, and negative-sense (complementary to mRNA) single-stranded RNA. The number of RNA segments differs among the genera: eight for influenza A, B and ISAV, seven for influenza C, six for *Thogoto virus*, and probably seven for *Dhori virus*. Accordingly, influenza A and B viruses contain HA and NA activities in different glycoproteins, whereas influenza C viruses lack NA, containing instead a hemagglutinin–esterase fusion (HEF) protein. *Thogoto virus* and *Dhori virus* possess a single glycoprotein (GP) that is not related to any known influenza virus protein but is related to the gp64 glycoprotein of baculoviruses (Morse et al. 1992). The virion has a molecular weight of 250 000 kDa and buoyant density in aqueous sucrose of 1.19 g/cm^3. Virions are sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde, irradiation, and oxidizing agents.

Influenza A viruses are further classified into subtypes based on the antigenicity of their HA and NA molecules. Currently, there are 16 recognized HA subtypes (Fouchier et al. 2005) (H1, H2, etc.) and nine NA subtypes (N1, N2, etc.). The full nomenclature (WHO Memorandum 1980) for each new isolate includes the type of virus, the host of origin (except for human), geographical site of isolation, strain number, and year of isolation. The antigenic description of the HA and NA is given in parentheses. For example, a type A virus isolated in Memphis, Tennessee, from a mallard duck in 1995 with a strain number of 123 and an H3N8 subtype, would be designated A/mallard/Memphis/123/95 (H3N8). So far, no antigenic subtypes have been identified among the influenza B and C viruses. *Thogoto virus* and *Dhori virus* do not cross-react antigenically.

MORPHOLOGY AND STRUCTURE

All influenza viruses have a segmented negative-sense RNA core surrounded by a lipid envelope (Figure 2). The A and B types are distinguished by two integral membrane glycoproteins, HA and NA, that protrude from the virion surface. Influenza C virus, by contrast, contains only one type of membrane glycoprotein, HEF. A third transmembrane protein, encoded by the *M2* gene of influenza A viruses and the *BM2* gene of influenza B viruses, serves as an ion channel. Within the lipid envelope exists the matrix (M1) protein. The RNA segments are associated with nucleoprotein (NP) and

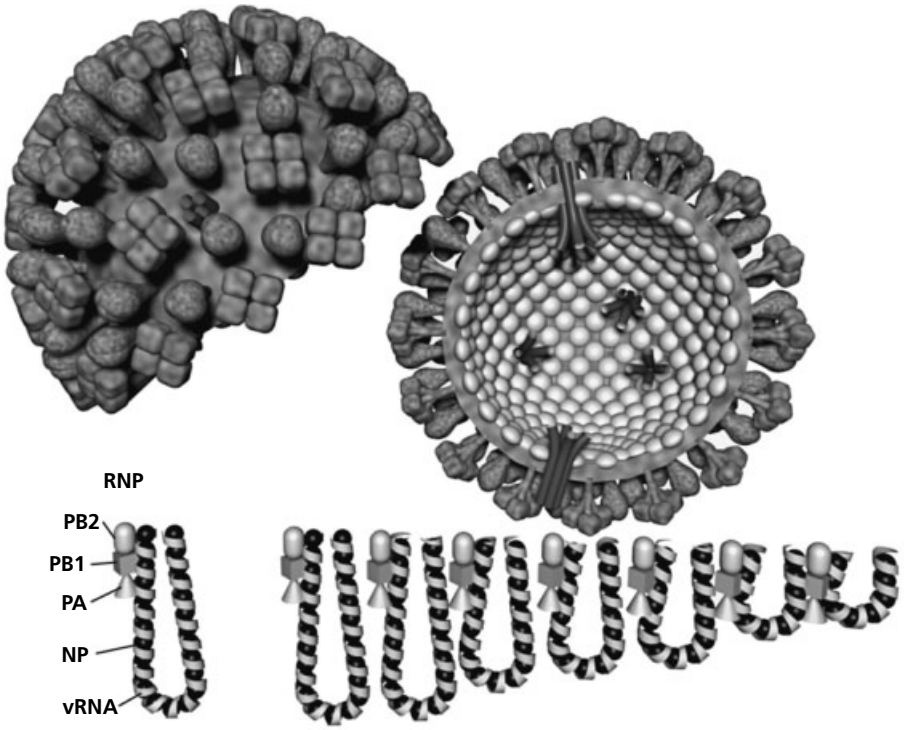

Figure 2 *Schematic diagram of an influenza A virion. The virion contains hemagglutinin (HA) and neuraminidase (NA) spikes in addition to a third membrane protein, M2. Within the viral envelope one finds ribonucleoprotein (RNP) consisting of RNA segments associated with nucleoprotein (NP), and the PA, PB1, and PB2 polymerase proteins. Three polymerase proteins are associated with the ends of vRNAs (insert). The precise location of M1 in virions is unknown, although it is associated with virion envelope, RNP, and NS2. (Courtesy of Yuko Kawaoka).*

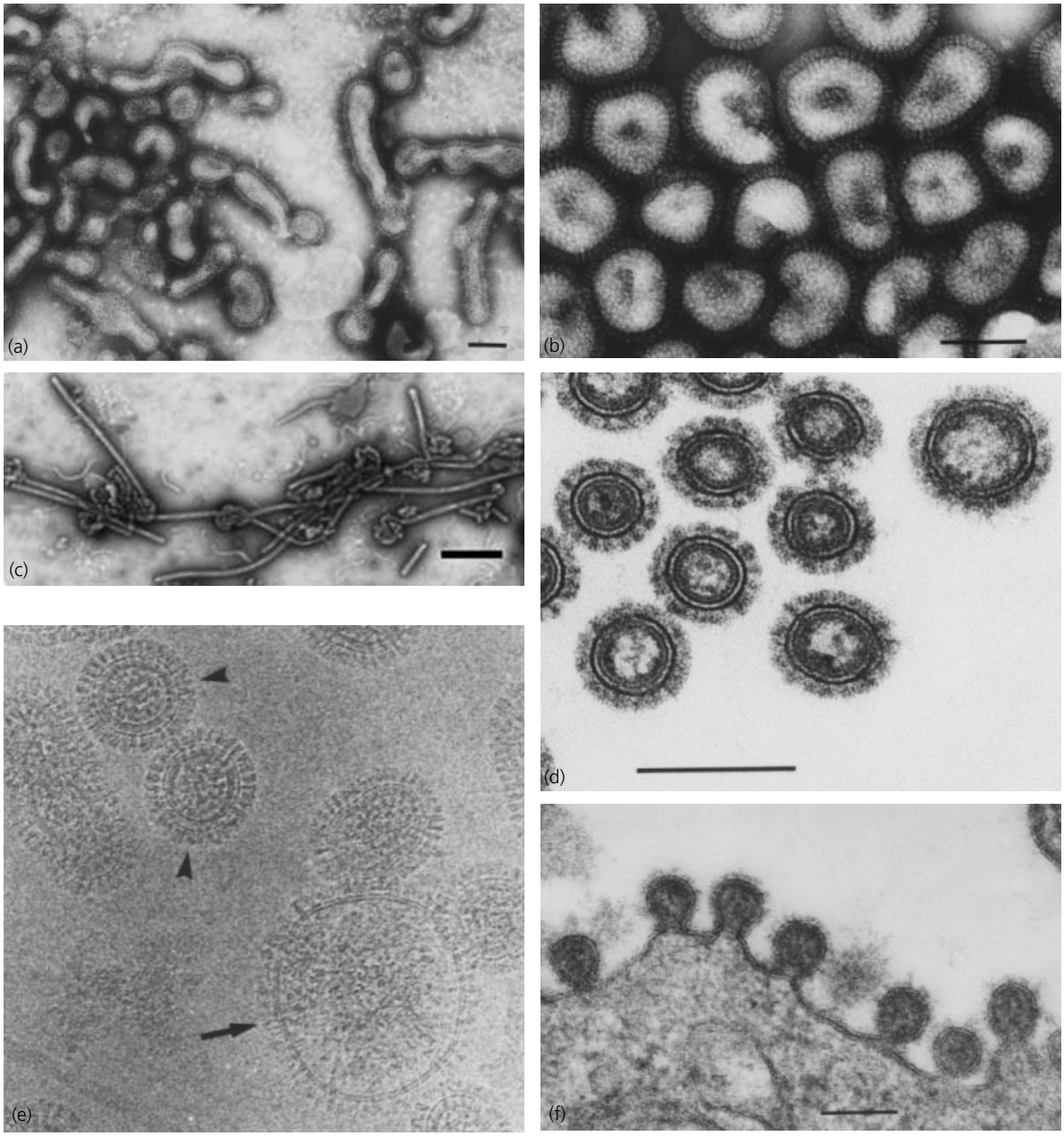

Figure 3 *Influenza A virus visualized by transmission electron microscopy. Two types of negatively stained virions are apparent:* **(a)** *filamentous and pleomorphic and* **(b)** *largely spherical. Bar = 100 nm. (Courtesy of Dr. K.G. Murti) (Murti and Webster 1986).* **(c)** *Filamentous A/Udorn/72 virus. (Courtesy of Dr. T. Noda.)* **(d)** *Sectioned virions with internal structures exposed. Bar = 100 nm.* **(e)** *High-resolution electron micrograph of influenza virus. Note the two distinct types of virions: those with thick membranes (arrowhead) and those with typical thin lipid bilayers (arrow). The former group possesses more M1 protein than does the latter. (Courtesy of Dr. Y. Fujiyoshi) (Fujiyoshi et al. 1994).* **(f)** *Virus budding from plasma membrane. Bar = 100 nm.*

three large polymerase proteins, designated PA, PB1, and PB2 on the basis of their overall acidic or basic amino acid composition, that are responsible for RNA replication and transcription (Krug et al. 1989).

Influenza virus particles are pleomorphic (Hoyle 1968) with spherical or filamentous morphology, or a mixture of both (Figure 3). Among clinical isolates that have undergone a limited number of passages in eggs or tissue culture, there are more filamentous than spherical particles, whereas extensively passaged laboratory strains consist almost exclusively of spherical virions (80–120 nm in diameter). Despite their distinctive shape, the filamentous virions possess many of the serological, hemagglutinating, and enzymatic characteristics of the spherical particles. The morphology of influenza virions seems to be primarily determined by the *M* gene (Bourmakina and Garcia-Sastre 2003; Roberts and Compans 1998), although both the *HA* and *NP* genes likely contribute (Jin et al. 1997; Mitnaul et al. 1996; Smirnov et al. 1991).

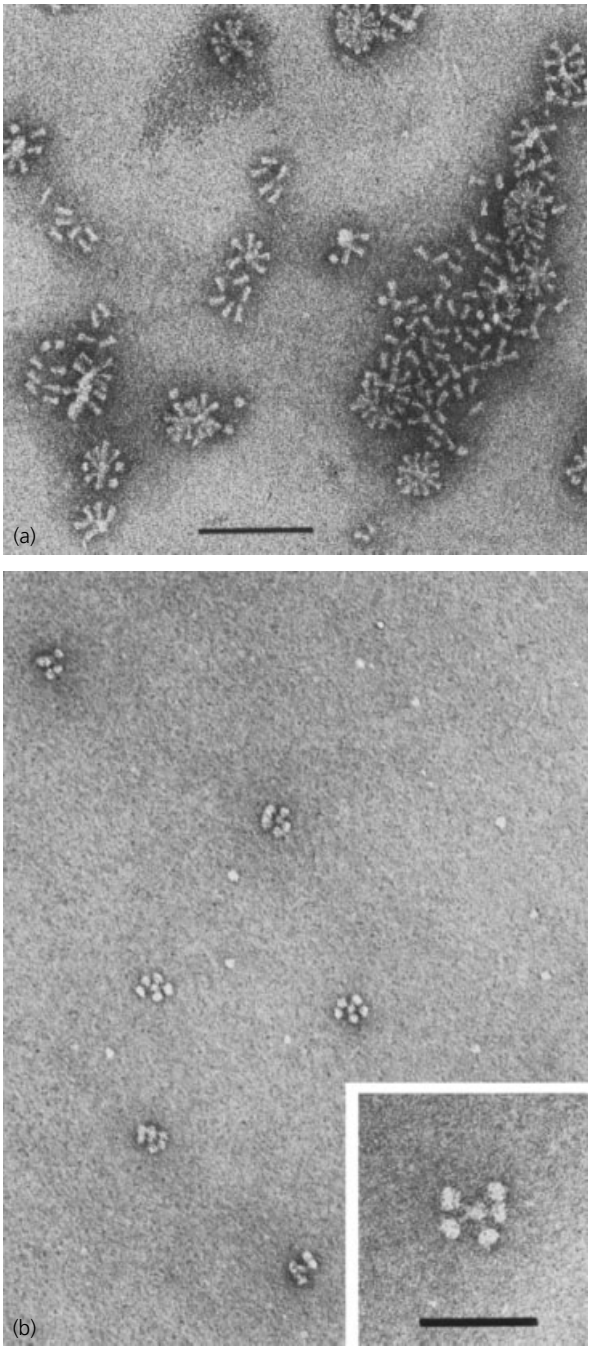

Figure 4 (a) *Hemagglutinin (HA) (bar = 100 nm) and* **(b)** *neuraminidase (NA) (bar = 50 nm) proteins isolated from detergent-disrupted virus. The molecules associate by hydrophobic C- (HA) and N- (NA) terminal sequences after the detergent has been removed. (Courtesy of Dr. K.G. Murti.) (Murti and Webster 1986).*

The HA and NA molecules that stud the surface of influenza A and B viruses range from 10 to 12 nm in length. The mean ratio of HA to NA spikes is ca. 5:1. The HA spikes are rod-shaped, whereas the NA spikes resemble mushrooms with slender stalks (Figure 4). They are not distinguishable by electron microscopy unless either the HA or NA is removed from the virion surface with a protease or rosettes of the HA and NA are formed after virions are treated with detergent. The NA distribution on virions remains uncertain. If the HA is removed with trypsin, the NA seems to be evenly distributed (Erickson and Kilbourne 1980); however, by immunoelectron microscopy with monoclonal antibodies, but not polyclonal antibodies, it seems to be clustered (Amano et al. 1992; Murti and Webster 1986). The HEF glycoproteins on influenza C viruses (8–10 nm long) are organized as hexagonal arrays of latticelike structures on the virion surface (Flewett and Apostolov 1967; Hewat et al. 1984) (Figure 5).

Within the lipid envelope, the viral RNA is associated with NP and three polymerase proteins, PA, PB1, and PB2. The organization of this ribonucleoprotein (RNP) complex within virions remains unclear. Electron microscopy has revealed helical structures in spontaneously lysed virions and in those partially disrupted with detergent (Figure 6). Similar structures can be made only with use of purified M1 protein (Ruigrok et al. 1989), indicating a significant role for this component in the generation of the RNP complex, with which M1 is known to associate (Hara et al. 2003; Kawakami and Ishihama 1983). Isolated RNP forms rod-shaped, right-handed helices that vary in length from 50 to 150 nm (Compans et al. 1972). Three-dimensional reconstitution of artificially generated viral RNPs reveal a circular shape composed of nine NP monomers, two of which form contacts with the polymerase complex (Martin-Benito et al. 2001). Each NP monomer interacts with approximately 24 nucleotides (Ortega et al. 2000) without apparent sequence specificity, and exposes the bases to the solvent (Baudin et al. 1994; Klumpp et al. 1997). The 5′ and 3′ terminal ends of viral RNA, which are complementary to each other, form a panhandle-like structure (Hsu et al. 1987).

The M1 protein is the primary determinant of virus budding (Gomez-Puertas et al. 2000; Latham and Galarza 2001) and its role in this process may be similar to that of Ebola matrix or retroviral gag proteins (Edbauer and Naso 1984; Harris et al. 1999; Jasenosky et al. 2001). Immunogold labeling with monoclonal antibodies to M1 failed to decorate the protein in virions unless they were first treated with a protease or a detergent (Murti et al. 1992). Recent cryo-electron microscopic studies suggest that M1 can modify the lipid bilayer, causing thickening of the viral envelope (Fujiyoshi et al. 1994) (see Figure 3e).

GENOME STRUCTURE AND FUNCTION

Influenza A and B viruses possess eight single-stranded RNA segments, each encoding at least one protein, whereas influenza C viruses contain only seven segments. Genome lengths differ widely among the three types of influenza viruses, some variations also being found among strains within the same type. Type B

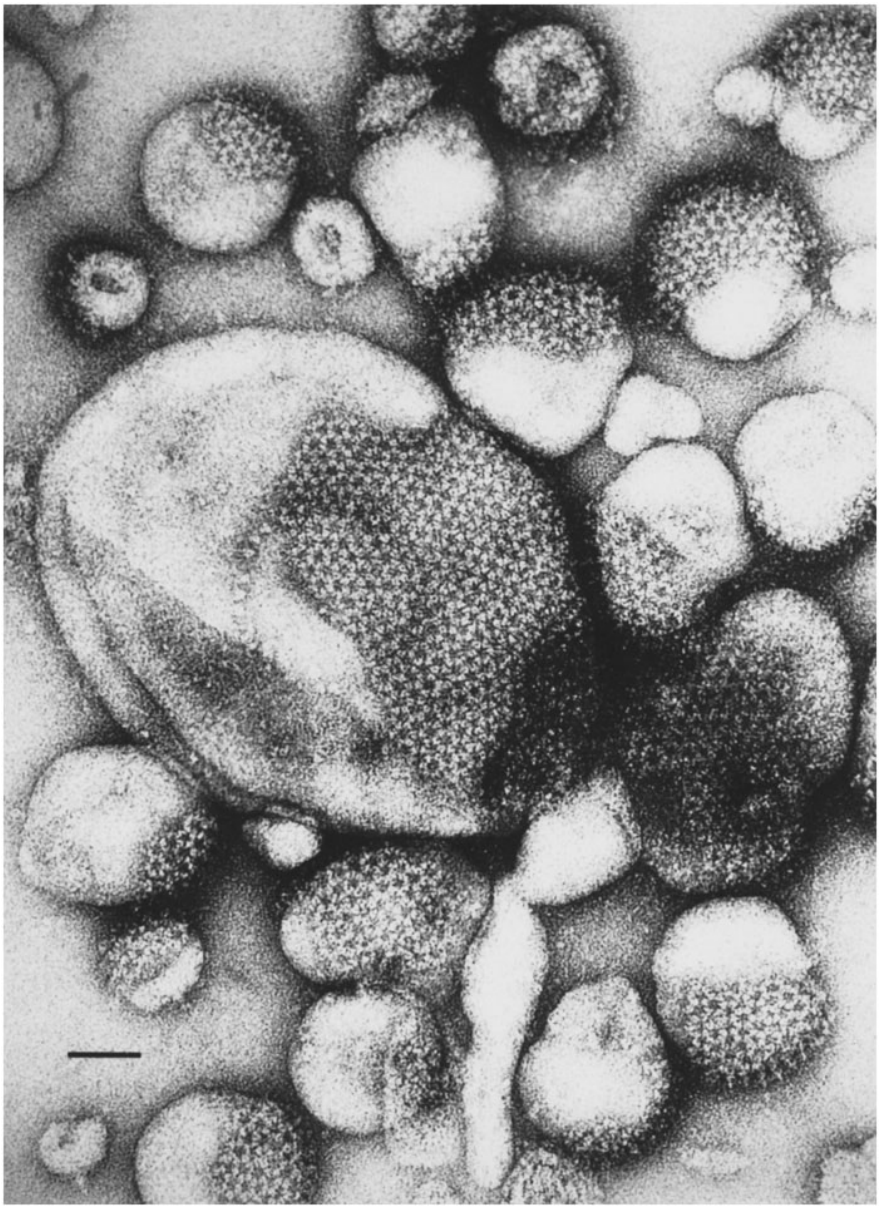

Figure 5 *Negatively stained influenza C virions with an array of extended glycoproteins. Bar = 50 nm. (Courtesy of Dr. R.W.H. Ruigrok.) (Hewat et al. 1984).*

viruses have the longest genome (ca. 14 600 nt) followed by A (ca. 13 600 nt) and then C (ca. 12 900 nt). Within each type, the lengths of genes other than the HA and NA (for A and B viruses) and the HEF (for C viruses) are highly conserved. The viral genome constitutes 2 percent of the mass of the virion.

In each of the eight RNA segments of all influenza A viruses, the first 12 nucleotides at the 3′ end and the last 13 at the 5′ end are highly conserved and contain promoter activity (Fodor et al. 1994; Fodor et al. 1995; Li and Palese 1992; Neumann and Hobom 1995; Parvin et al. 1989; Piccone et al. 1993; Seong and Brownlee 1992a; Seong and Brownlee 1992b; Yamanaka et al. 1991b) (Figure 7). Similar structures have been identified in the RNA segments of influenza B (conserved sequence: 5′ AGUAG(A/U)AACAA and 3′ UCGUCUUCGC) and C (conserved sequence: 5′ AGCAGUAGCAA and 3′ UCGU(U/C)UUCGUCC) viruses as well. The promoter regions of influenza A and B virus RNAs are interchangeable (Crescenzo-Chaigne et al. 1999; Muster et al. 1991), although the chimeric virus resulting from such modification is attenuated (Muster et al. 1991). The partially complementary 5′ and 3′ ends of the vRNA or cRNA, respectively, were thought to form a panhandle structure (Hsu et al. 1987). However, extensive characterization of promoter mutants indicated that the promoter region adopts a so-called 'corkscrew' structure characterized by intrastrand – rather than interstrand – base-pairing between the 5′ and 3′ terminal nucleotides (Flick et al. 1996; Flick and Hobom

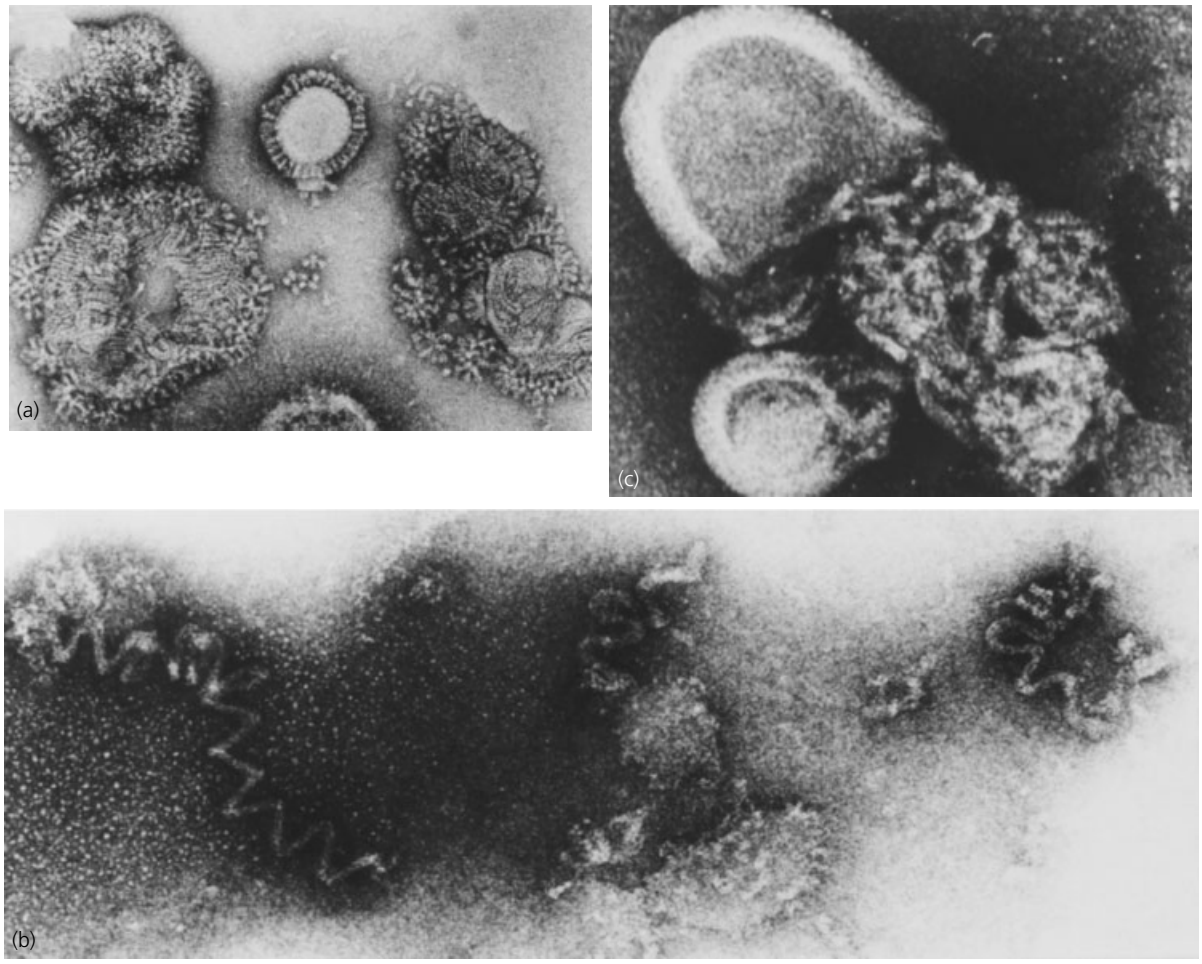

Figure 6 *Electron micrograph of the internal structure of an influenza virus.* **(a)** *Naturally disrupted virions.* **(b)** *Extended coil structures resulting from detergent treatment of virus.* **(c)** *M1 protein reconstituted into liposomes. (Courtesy of Dr. R.W.H. Ruigrok, Ruigrok et al. 1989.)*

1999). Similar promoter structures have been identified for influenza C virus (Crescenzo-Chaigne and van der Werf, 2001) and Thogotovirus (Weber et al. 1997).

Most viral genes encode a single protein (Table 2); the exceptions are the *M* and nonstructural (*NS*) genes of all influenza viruses and the *NA* gene of type B virus, that encode two proteins each (Lamb and Horvath 1991). The *NS* gene of type A virus encodes both a 26-kDa (NS1) protein translated from unspliced mRNA and a 14-kDa (NS2) protein translated from spliced mRNA (Inglis et al. 1979; Lamb and Choppin 1979) (Figure 8a), which share the same AUG initiation codon and nine subsequent amino acids. A similar protein-coding strategy is employed by the type B and C influenza viruses. Some influenza A virus *PB1* genes encode a second polypeptide, termed PB1-F2, which is encoded in the +1 reading frame (W. Chen et al. 2001) (Figure 8c).

By contrast, each type of influenza virus possesses a different mechanism for expression of *M* gene products. The *M* gene of type A viruses generates an unspliced transcript encoding the M1 protein, as well as two other alternatively spliced RNAs, designated M2 and mRNA3, which differ in their use of 5′ splice sites (Inglis and Brown 1981; Lamb et al. 1981) (Figure 8b). However, a translation product of mRNA3 has not been found in vivo. The leader sequence of the M2 mRNA contains the AUG initiation codon and codons for eight subsequent residues that are shared with the M1 protein; this region is followed by a sequence encoding 88 residues in the +1 reading frame. The nine amino acid peptide encoded by mRNA3 has not been detected in infected cells. By binding to the 5′ end of the viral M1 mRNA in a sequence-specific, cap-dependent manner, the viral polymerase complex promotes splicing at the M2 mRNA 5′ site over the mRNA3 5′ site (Shih et al. 1995). The *M* gene of influenza B viruses also encodes two proteins, M1 and BM2, which are translated via a termination-reinitiation scheme of tandem cistrons; a pentanucleotide sequence, UAAUG, contains the termination codon for the M1 and the initiation codon for the BM2 (Horvath et al. 1990) (Figure 8d). Finally, in contrast to the scheme employed by influenza A viruses, type C viruses rely on spliced transcripts to produce M1 protein

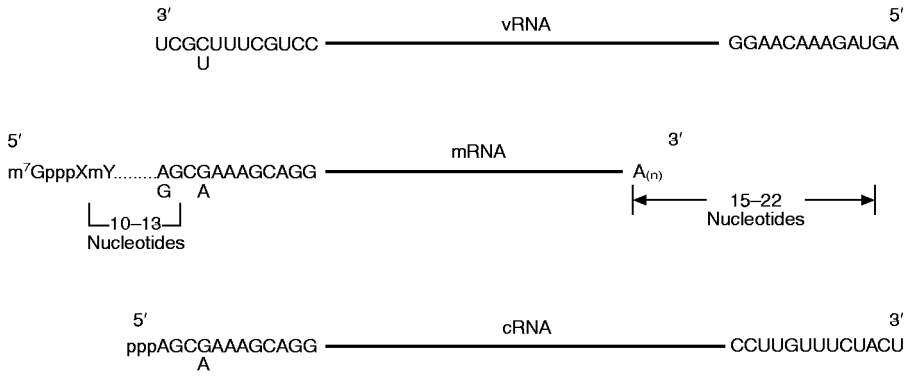

Figure 7 *Schematic diagram illustrating the differences between influenza virus virion RNA (vRNA) segments, mRNAs, and full-length cRNA (template for vRNA). The conserved 12 nucleotides at the 3′ end and 13 nucleotides at the 5′ end of each of the influenza A virus vRNA segments are indicated. Similar conserved sequences are found at the 3′ and 5′ ends of the influenza B and C virus RNA segments.*

(Yamashita et al. 1988) (Figure 8f). Splicing of the primary transcript introduces a translational termination codon, resulting in the synthesis of the M1 protein of 242 amino acids. The unspliced mRNA has the coding capacity for a 374 amino acid protein (P42) which is proteolytically cleaved to yield CM2 and M1′ (Hongo et al. 1999; Pekosz and Lamb 1998). CM2 is composed of 115 amino acids corresponding to the carboxy-terminal region of the primary transcript (Hongo et al. 1994) and has biochemical properties similar to those of the influenza A virus M2 protein (Hongo et al. 1997).

Still another mechanism of viral protein expression is represented by the influenza B virus *NA* gene, which gives rise to a bicistronic mRNA containing two initiating AUG codons that are separated by four nucleotides (Figure 8e). The 100 amino acid NB protein is translated from the 5′ AUG codon, while the 466 amino acid NA protein is translated from the second AUG codon. Even though the NA initiation codon is positioned downstream of the NB initiation site, greater amounts of NA protein (1:0.6) accumulate in cells (Williams and Lamb 1989).

Replication

The replication cycle of influenza viruses has been studied most extensively with type A strains; therefore, unless otherwise noted, the processes described below refer to viruses of that type (Figure 9).

ATTACHMENT TO UNCOATING

An influenza virus infects cells through binding of its HA or HEF protein to the cell's sialyloligosaccharide receptor. After binding, the attached virion undergoes endocytosis (White et al. 1983). The low pH of the late endocytotic vesicle triggers a conformational change in the cleavage-activated HA (Skehel et al. 1982), initiating fusion of the viral and vesicular membranes. Fusion releases the contents of the virion into the cytoplasm of the cell (uncoating). Before fusion, M2 proteins (BM2 protein for type B viruses, and possibly NB for type B viruses), by ion channeling, introduce protons into the inside of the virion, exposing the core to low pH (Hay 1992; Helenius 1992; Pinto et al. 1992; Sugrue et al. 1990b) (Figure 10). Such an event is thought to promote dissociation of the M1 from the RNP by disrupting the low pH-sensitive interaction between these molecules (Zhirnov 1990), allowing the RNP to migrate to the nucleus through the nuclear pore in an ATP-dependent manner (Kemler et al. 1994; Martin and Helenius 1991). Exposure of the viral core to low pH, however, is not required for dissociation of M1 from RNP in type C viruses (Zhirnov and Grigoriev 1994).

TRANSCRIPTION AND TRANSLATION

Once the RNP migrates into the host-cell nucleus, the associated polymerase complexes (PA, PB1, and PB2) begin primary transcription of mRNA (Krug et al. 1979), which requires cooperation with ongoing transcription by cellular RNA polymerase II. The reason for this requirement is that initiation of influenza virus mRNA synthesis depends on m^7GpppXm-containing capped primers (10–13 nt long, containing 5′-GCA-3′ at their 3′ proximal ends) that are generated from host cell RNAs by an influenza virus-encoded, cap-dependent endonuclease (Krug et al. 1989; Plotch et al. 1981). The endonuclease activity resides in the PB1 subunit (M.L. Li et al. 2001) and requires the presence of influenza viral RNA (Hagen et al. 1994); however, the requirement of both the 5′ and 3′ ends of vRNA remains controversial and may depend on the experimental parameters (Cianci et al. 1995; Li et al. 1998; Rao et al. 2003).

mRNA synthesis begins with incorporation of a G residue complementary to the penultimate C residue on the vRNAs (Beaton and Krug 1981; Kawakami and Ishihama 1983). Chain elongation is carried out by the PB1 subunit, which contains four consensus motifs for nucleic

Table 2 *Influenza A virus genome RNA and protein coding assignments*

Segment	Length (nucleotides)[a]	Encoded polypeptide	Nascent polypeptide length (amino acids)	Experimentally determined mol. wt (kDa) of polypeptides	Carbohydrates	Approx. copy no. per virion	Remarks
1	2 341	PB2	759	87	–	30–60	Component of RNA transcriptase complex
							Host-cell capped mRNA recognition and binding
2	2 341	PB1	757	96	–	30–60	Component of RNA transcriptase complex
							RNA-dependent RNA polymerase activity; capped mRNA endonuclease activity
		PB1-F2[b]	87	10.5	–	2 650	Induction of apoptosis
3	2 233	PA	716	85	–	30–60	Component of RNA transcriptase complex
							Required for replication; possible role in transcription
4	1 778	HA	566	63	+	500	Surface trimer glycoprotein
							Cleaved into HA1 and HA2
							Major antigenic determinant
							Functions in virus binding to cell surface receptors and fusion
5	1 565	NP	498	56	–	1 000	Associated with RNA segments to form ribonucleoprotein
6	1 413	NA	454	60	+	100	Surface tetramer glycoprotein; neuraminidase activity
							Functions in virus release; target of neuraminidase inhibitors
7	1 027	M1	252	27	–	3 000	Major virion component; involved in RNP transport out of nucleus
		M2	97	14	–	20–60	Coded from spliced mRNA; ion channel activity; target of adamantanes
8	890	NS1	230	26	–	NA	Nonstructural protein; inhibits mRNA transport from nucleus; interferon antagonist
		NS2(= NEP)	121	14	–	130–200	Coded from spliced mRNA; viral nuclear export protein

a) The lengths of the *HA* and *NA* genes differ among the strains.
b) Not encoded by all influenza A viruses; NA, not applicable.

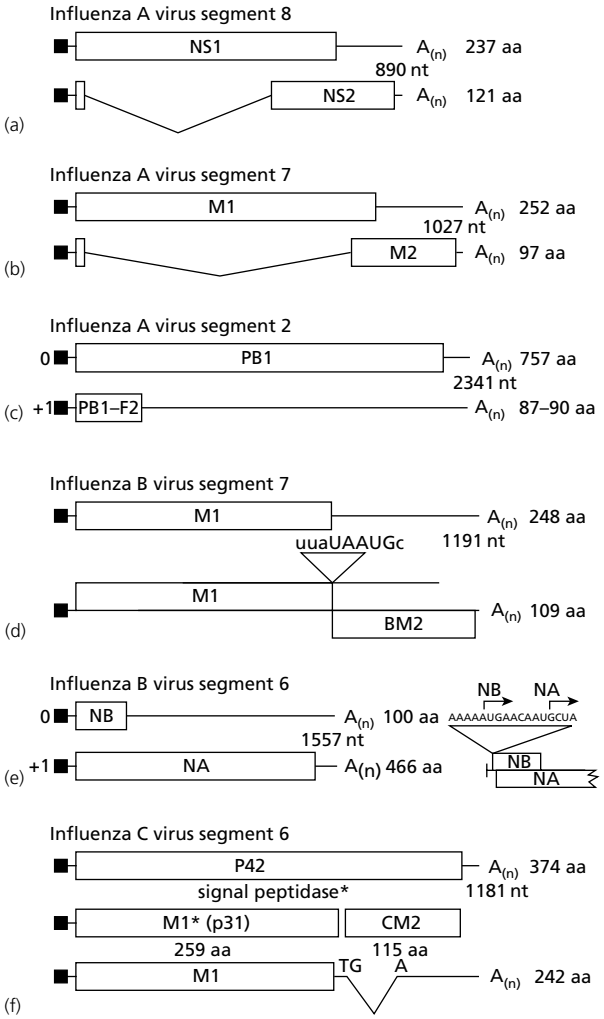

Figure 8 *Coding strategies of influenza virus genes.* **(a)** *Influenza A virus NS1 and NS2 mRNAs and their coding regions. NS1 and NS2 share ten amino-terminal residues, including the initiating methionine. The reading frame of NS2 mRNA (nucleotide positions 529–861) differs from that of NS1.* **(b)** *Influenza A virus M1 and M2 mRNAs and their coding regions. M1 and M2 share nine amino-terminal residues, including the initiating methionine; however, the reading frame of M2 mRNA (nucleotide positions 740–1 004) differs from that of M1.* **(c)** *Influenza A virus PB1 segment coding regions. Based on Kozak's rule, PB1 translation initiation may be inefficient, thereby allowing translation initiation of the PB1-F2 open reading frame by ribosomal scanning.* **(d)** *Influenza B virus RNA segment 7 open reading frames and the organization of the open reading frames used to translate the M1 and BM2 proteins. The stop–start pentanucleotide is also illustrated.* **(e)** *Open reading frames in influenza B virus RNA segment 6, illustrating the overlapping reading frames of NB and neuraminidase (NA). Nucleotide sequence surrounding the two AUG initiation codons, in mRNA sense, is shown to the right.* **(f)** *Influenza C virus mRNAs derived from RNA segment 6. The unspliced and spliced mRNAs encode P42 and M1, respectively. Cleavage of P42 by a signal peptidase yields M1′ (p31) and CM2. Thin lines at the 5′ and 3′ termini of the mRNAs represent untranslated regions. Introns in the mRNAs are shown by V-shaped lines; filled rectangles at the 5′ ends of mRNAs represent heterogeneous nucleotides derived from cellular RNAs that are covalently linked to viral sequences (Virus Taxonomy 2005).*

acid polymerases (Argos 1988; Biswas and Nayak 1994; Ishihama and Barbier 1994; Poch et al. 1989) as well as nucleotide binding sites (Asano et al. 1995; Asano and Ishihama 1997; Romanos and Hay 1984). Transcription continues until it reaches the poly(A) addition site, located 15–22 nt from the 5′ end of the vRNA. Three elements are critical for polyadenylation: a stretch of uridines near the 5′ end of the virion RNA (optimal with 5–7 residues and 16 nt from the 5′ end), which serves as the poly(A) signal (Li and Palese 1994; Luo et al. 1991; Poon et al. 1999; Zheng et al. 1999); the RNA duplex of the promoter structure; and specific nucleotides near the 5′ end of the vRNA (Pritlove et al. 1999). The current model predicts threading of the vRNA through the polymerase complex which is stably bound to the 5′ end of the vRNA throughout transcription (Fodor et al. 1994; Hagen et al. 1994). In this model, the polymerase complex bound to the promoter region would serve as a physical barrier that causes reiterative copying at the neighboring uridine stretch. The primary transcripts are then used in the production of viral proteins by the cell's cytoplasmic translation machinery. Three polymerase proteins (PA, PB1, and PB2), as well as NP, NS1, and NS2 proteins, are transported to the nucleus. The negative-strand genomic segment and the positive-strand antigenomic segments, but not viral mRNA, are coated with NP. NS1 inhibits the transport of cellular mRNA to the cytoplasm by binding to the poly(A) region (Qiu and Krug 1994) and maximizing the availability of capped primers for viral mRNA synthesis. The products of viral mRNA synthesis change as infection proceeds, indicating a temporal form of regulation (Hatada et al. 1989; Hay et al. 1977). Early in infection, the synthesis of mRNAs encoding NP and NS1 dominates; later the production of mRNAs for HA, NA, and M1 increases, while transcripts for the polymerase proteins are relatively low throughout the infection cycle, except at the earliest time. The relative amounts of mRNAs correlate with the amounts of their corresponding proteins, indicating that viral gene expression is regulated at the transcriptional level, in addition to the temporal control mechanisms governing the translational efficiency of viral mRNA (Yamanaka et al. 1988, 1991a). The production of full-length viral complementary RNA (cRNA) was believed to be delayed until viral protein had been synthesized. In this model, newly synthesized NP promoted 'read-through' of the poly(A) site, allowing the synthesis of cRNA transcripts (Beaton and Krug 1986; Shapiro and Krug 1988) which in turn serve as templates for the production of vRNA. Recent findings, however, suggest that cRNA synthesis occurs early in infection as well; at this time point, cRNA may be degraded rapidly by cellular nucleases, while increasing amounts of polymerase proteins may protect the cRNA later in infection (Vreede et al. 2004). Equimolar quantities of cRNA are synthesized throughout

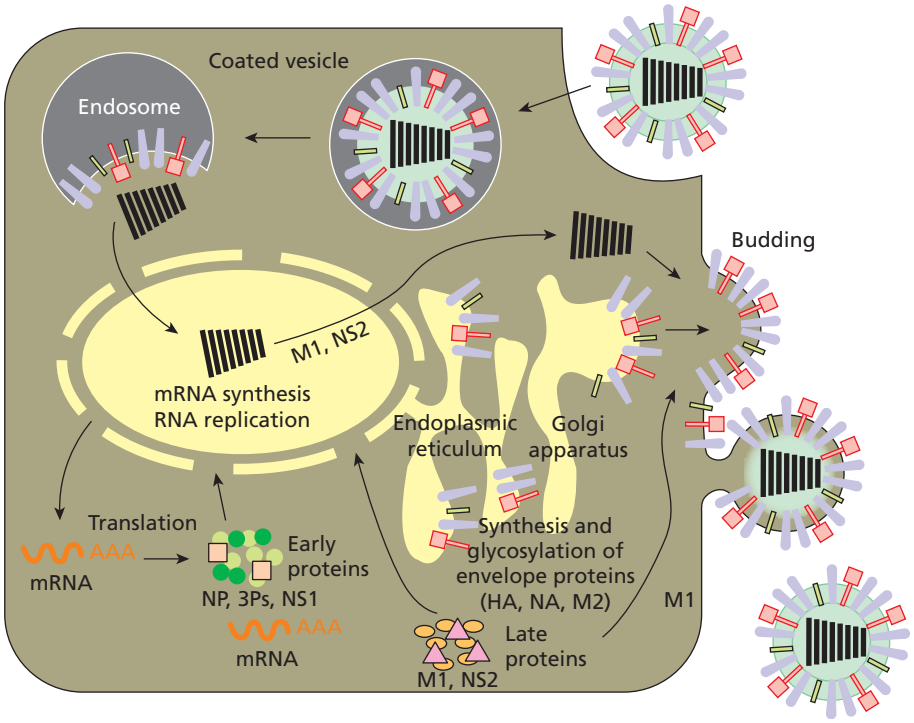

Figure 9 *Replication cycle of influenza A virus, from binding of the virus to the host cell surface to its exit from the plasma membrane. Ps, polymerase proteins. (Lamb et al. 1994.)*

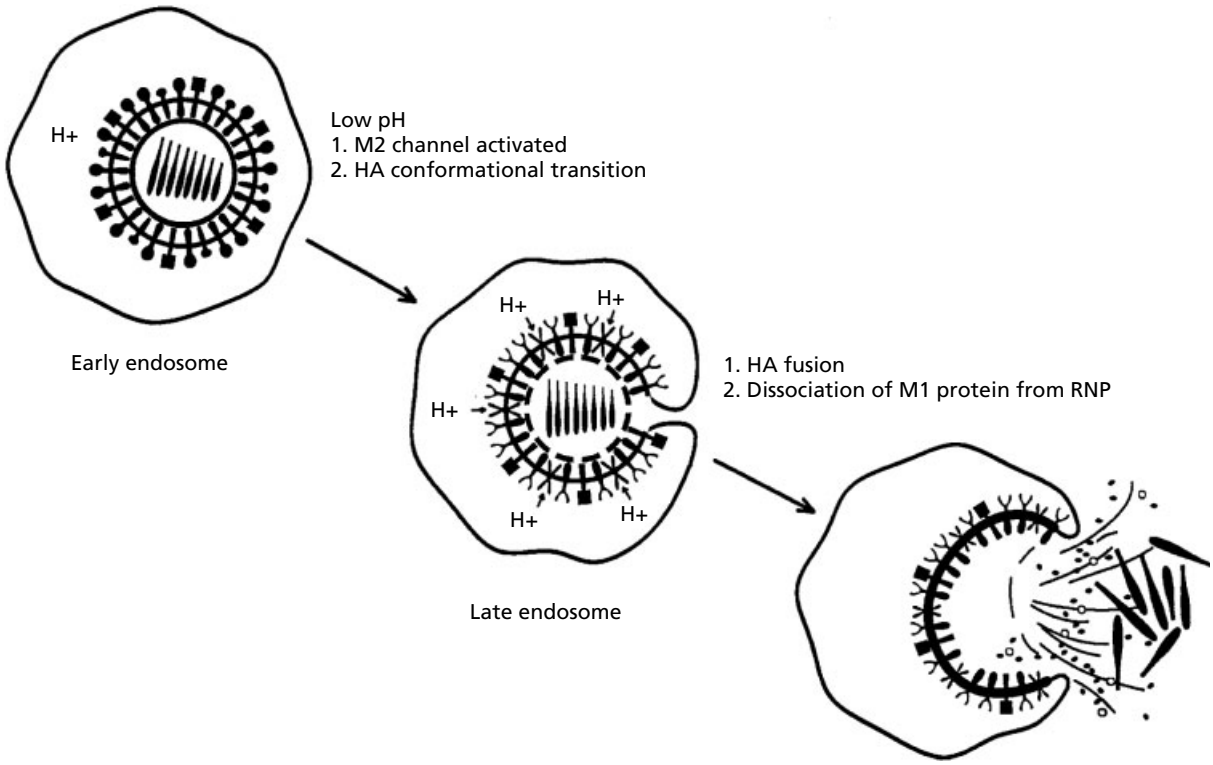

Figure 10 *Ion channel activity of the M2 protein upon virus entry. The flow of ions from the lumen of the endosome through the M2 channel into the virion interior allows the dissociation of protein–protein interactions between M1 and RNP, facilitating RNP transport to the nucleus. (Lamb et al. 1994; © Mary K. Bryson.)*

infection (Hay et al. 1977), indicating a lack of regulation of this process.

After their translation in the endoplasmic reticulum (ER), viral membrane proteins – HA, NA, and M2 for the type A viruses, NB for type B and HEF for type C – are translocated into the lumen of the ER, where they undergo oligomerization prior to their transport to the Golgi apparatus (Doms et al. 1993) and subsequent transport to the plasma membrane. The proteins are glycosylated in the ER (high mannose type), and then processed further in the Golgi to contain oligosaccharide of the complex type.

VIRION MORPHOGENESIS AND BUDDING

Nuclear export of vRNP complexes is mediated by the NS2 protein (Neumann et al. 2000; O'Neill et al. 1998) now also referred to as nuclear export protein (NEP), which interacts with the cellular export factor CRM1 (Neumann et al. 2000). In addition, the M1 protein is also required for nuclear export of vRNP complexes (Bui et al. 2000; Huang et al. 2001; Martin and Helenius 1991; Whittaker et al. 1995) and may execute its function by bridging viral ribonucleoprotein complexes and NS2 (NEP) (Sakaguchi et al. 2003). Electron microscopy of influenza virus-infected cells has not identified viral core structures, nor has an association of RNP with the plasma membrane been found; rather, RNP seems to associate with viral membrane proteins only when the virion is beginning to assume its shape (Patterson et al. 1988). Segment-specific virion incorporation signals have been identified on viral RNAs (Fujii et al. 2003; Watanabe et al. 2003) that promote their efficient incorporation into virions. These signals are located in the nontranslated regions at both ends of the vRNAs and extend into the translated regions, thereby conferring segment specificity. Although influenza virus can accommodate more than eight viral segments (Enami et al. 1991; Scholtissek et al. 1978b) – a finding that suggested random incorporation of vRNAs – efficient packaging relies on cis-acting signals that govern the incorporation of eight viral RNA segments into virions. The mechanism for selective vRNA virion incorporation remains to be determined. Few details of the final assembly process are known. Presumably, RNP buds outward through the cell membrane. Substantial amounts of M1 are found in the cytoplasm throughout the infection period. M1 interacts with cellular membranes (Bucher et al. 1980; Gregoriades and Frangione 1981; Hay 1974; Kretzschmar et al. 1996), and induces the formation of viruslike particles (Gomez-Puertas et al. 2000; Latham and Galarza 2001). Because at least a fraction of M1 is associated with RNP in the virion (Kawakami and Ishihama 1983; Rees and Dimmock 1981), this protein possibly serves as a molecular 'glue', interacting with RNP on the one hand and with HA, NA, or M2 on the other (Ali et al. 2000; Barman et al. 2001; Hara et al. 2003). Such interactions may function as a budding signal.

Polypeptides: nonstructural and structural

Influenza A virus comprises nine structural and one nonstructural protein (see Table 2), compared with nine structural and two nonstructural proteins in influenza B virus, and six structural and three nonstructural proteins in influenza C virus. For some influenza A viruses, an additional polypeptide is expressed from the *PB1* gene (see Table 2 and below).

POLYMERASE PROTEINS

The exact nature of the polymerase complex remains under investigation. Trimeric complexes composed of PB2, PB1, and PA are formed in virus-infected cells (Detjen et al. 1987; Honda et al. 1990; Kato et al. 1985; Kawakami and Ishihama 1983) and in transfected cells expressing all components of vRNP complexes (Digard et al. 1989; Honda et al. 2002; Hwang et al. 2000). Functional studies suggest that dimeric complexes consisting of PB1–PB2 or PB1–PA have transcriptase or replicase activity, respectively (Honda and Ishihama 1997; Honda et al. 2002; Lee et al. 2002); however, all three polymerase subunits may be required for efficient replication and transcription (Fodor et al. 2002; Perales and Ortin 1997). The polymerase complex recognizes the influenza virus promoter structure in a sequence-specific manner (Honda et al. 1987; Tiley et al. 1994) and also interacts with NP (Biswas et al. 1998). PB1 forms the structural backbone of the polymerase complex by interacting with both PB2 and PA (Honda and Ishihama 1997; Ishihama 1996; Ohtsu et al. 2002; Perez and Donis 2001; Toyoda et al. 1996). Artificially assembled vRNP complexes allowed the determination of a three-dimensional structure that revealed a compact polymerase complex with points of interaction with NP (Area et al. 2004) (Figur 11). The three polymerase proteins are highly conserved among type A viruses (Gorman et al. 1990b; Kawaoka and Webster 1989; Okazaki et al. 1989).

PB1

This protein is required for the initiation and elongation of newly synthesized viral RNA (Braam et al. 1983). Conserved PB1 residues Ser444–Asp445–Asp446 of type A viruses (443–445 for type B and 445–447 for type C), which resemble the signature sequences of other viral RNA polymerases, may form the core of the transcriptase/replicase activity (Biswas and Nayak 1994). Binding of PB1 to the viral promoter structure (Gonzalez and Ortin 1999a, b) activates the protein's capped mRNA endonuclease activity (Cianci et al. 1995; Gonzalez and Ortin 1999a, b; Li et al. 1998; M.L. Li et al. 2001; Rao et al. 2003). PB1 contains two discontinuous regions, both essential for nuclear localization (Nath and Nayak 1990).

Some influenza A virus *PB1* genes contain a second open reading frame yielding the so-called PB1-F2 polypep-

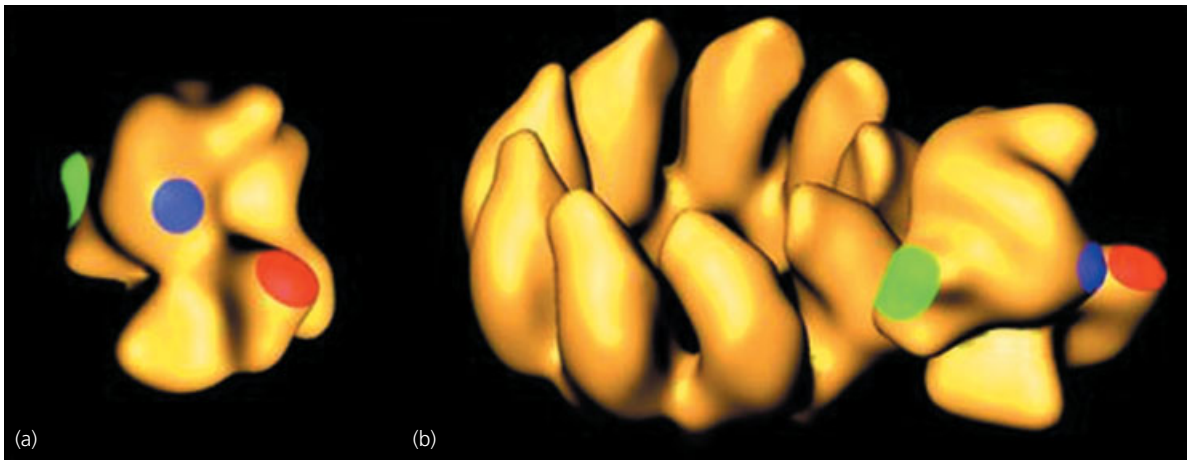

Figure 11 *Three-dimensional model for the influenza virus polymerase complex based on electron microscopic images. The colored areas depict the location of subunit domains: N-terminal region of PB2 (red), C-terminal region of PA (blue), C-terminal region of PB1 (green).* **(a)** *Polymerase model;* **(b)** *position of the polymerase subunits relative to the ringlike RNP structure. (Courtesy of Dr. J. Ortin; Area et al. 2004.)*

tide (W. Chen et al. 2001). The 87 amino acid peptide localizes to mitochondria where it induces apoptosis (D. Chen et al. 2001; Gibbs et al. 2003). The absence of this open reading frame from some animal (particularly swine) virus isolates leaves in question the overall contribution of this peptide to influenza virus pathogenesis.

PB2

PB2 is crucial for recognizing and binding to type I cap structures of cellular mRNAs (Blaas et al. 1982; Plotch et al. 1981; Ulmanen et al. 1983). Several amino acids/amino acid stretches at position 242–282 (Honda et al. 1999), 533–564 (M.L. Li et al. 2001), and/or 363 and 404 (Fechter et al. 2003) have been identified as critical for cap-binding. PB2 is essential for viral mRNA synthesis, although its requirement for replication remains controversial (Gastaminza et al. 2003; Nakagawa et al. 1995). Two signals mediate nuclear localization of influenza A PB2 protein, one of which is involved in PB2 perinuclear binding (Mukaigawa and Nayak 1991).

PB2 plays a role in host range restriction (Almond 1977; Subbarao et al. 1993; Yao et al. 2001). In 1997, H5N1 avian influenza viruses infected humans in Hong Kong, resulting in fatal outcomes in several individuals. Reverse genetics experiments demonstrated a critical role of amino acid 627 of PB2 for efficient replication in mice (Hatta et al. 2001); a mutation from glutamic acid (found in avian isolates at this position) to lysine (found in all human influenza viruses) enhances viral replication in mice (and likely in humans), leading to increased virulence. This finding was substantiated by the identification of the same Glu-to-Lys replacement in an H7N7 influenza virus isolated from a Dutch veterinarian who succumbed to influenza virus infection in 2003 (Fouchier et al. 2004), and in H5N1 viruses isolated from Vietnamese influenza virus victims in 2004 (K.S. Li et al. 2004). This replacement affects the level of viral RNA transcription/replication (Crescenzo-Chaigne et al. 2002; Massin et al. 2001; Naffakh et al. 2000; Shinya et al. 2004). However, in the ferret model, increased virulence has not been associated with this change (Zitzow et al. 2002) and viruses without this mutation have been isolated from severe and fatal human cases of influenza (Shaw et al. 2002; Ruben Donis unpublished).

PA

The PA protein is an essential component of the viral polymerase complex; however, its exact role in replication or transcription is less well defined than those of PB2 or PB1. PA is required for viral RNA replication (Honda and Ishihama 1997; Honda et al. 2002; Krug et al. 1989; Lee et al. 2002), possibly by acting as an elongation factor (Fodor et al. 2003) or by facilitating the binding of PB1 to viral RNA (Lee et al. 2002). It has also been implicated in transcription (Fodor et al. 2002). PA reduces the half-lives of co-expressed proteins (Sanz-Ezquerro et al. 1995, 1996) but the significance of this proteolytic activity remains obscure (Naffakh et al. 2001; Perales et al. 2000). In addition, a serine protease activity has been described for this protein (Hara et al. 2001) which is not critical for viral replication in cell culture (Fodor et al. 2002), but does support efficient viral growth (Toyoda et al. 2003). Expression of PA does not result in complete nuclear localization, which can only be achieved after coexpression of PB1 or NS1 (Nieto et al. 1992).

HEMAGGLUTININ

Encoded by the fourth largest RNA segment, the HA accounts for about 25 percent of viral protein and is distributed evenly on the surface of virions. It is responsible for the attachment and subsequent penetration of viruses into cells. The HA spikes, approximately 14 × 4 nm, protrude from the virion surface (Laver and

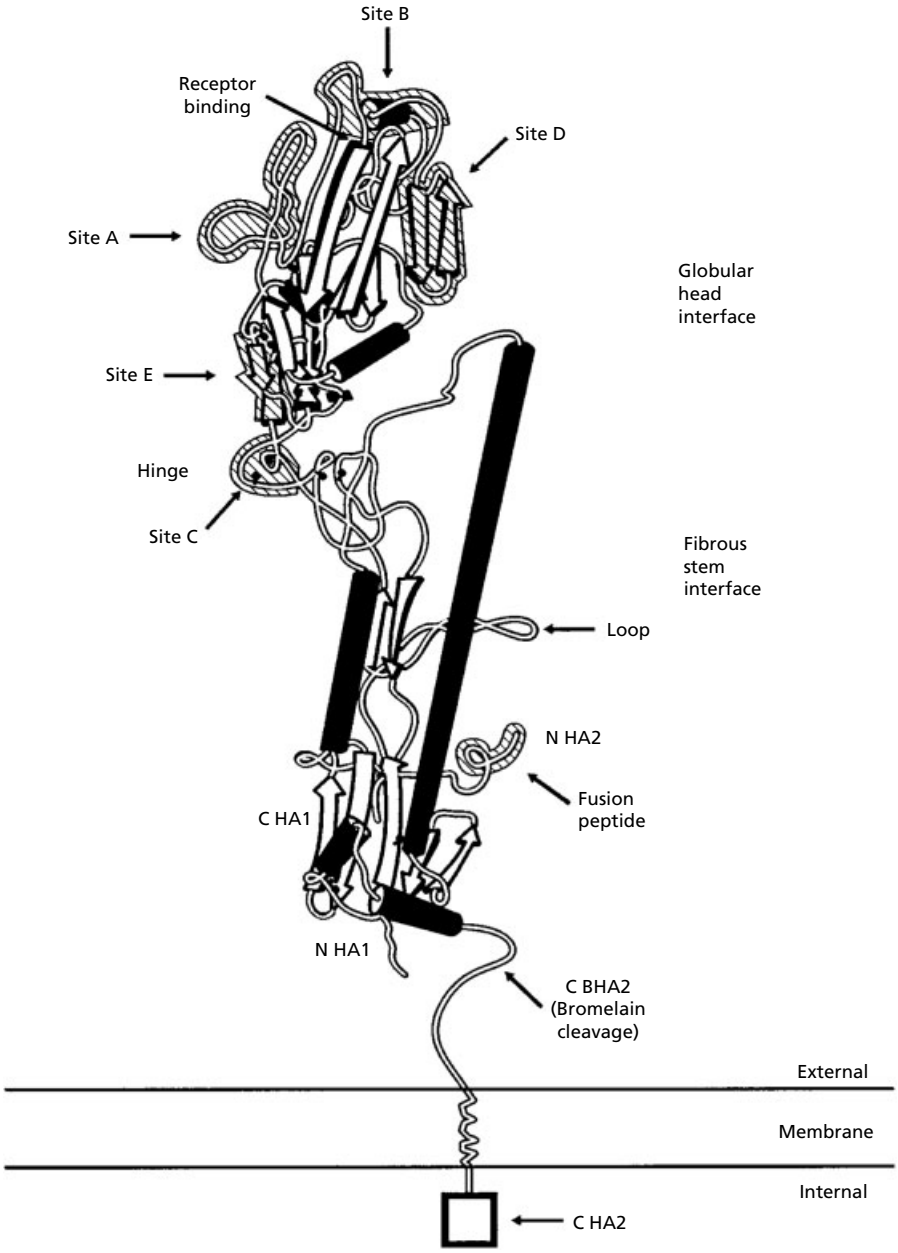

Figure 12 *Schematic diagram of the Hong Kong hemagglutinin (HA) monomer, showing folding of the HA1 and HA2 polypeptides. The shaded areas show the putative locations of the five independent antigenic areas (sites A–E). Note that the fusion peptide is also buried in the intact molecule and is exposed only after a conformational change at low pH (Bullough et al. 1994; Wilson et al. 1981).*

Valentine 1969) (Figure 12, and see Figure 3). The HA homotrimer (Wiley et al. 1977) is synthesized as a single polypeptide chain (HA0) that undergoes post-translational cleavage by cellular proteases (Klenk et al. 1975; Lazarowitz and Choppin 1975). The resulting HA1 and HA2 subunits (36 and 27 kDa) are covalently attached by a disulfide bond, while the 'two-chain' monomers are associated noncovalently to form trimers. An N-terminal signal sequence is removed. HA cleavage is required for infectivity (Klenk et al. 1975; Lazarowitz and Choppin 1975) to expose the hydrophobic amino terminus of HA2, which mediates fusion between the viral envelope and the endosomal membrane (White 1992).

Three-dimensional structure

For the ectodomain of human H3N2 virus, X-ray crystallographic structures have been determined for three different conformations: Bromelain-cleaved soluble HA (BHA) of A/Aichi/2/68(H3N2) (Wiley et al. 1981; Wilson et al. 1981; Wilson and Cox 1990) which represents the conformation of cleaved HA, the uncleaved HA0 precursor (Chen et al. 1998), and fragments of low pH-treated BHA (Bizebard et al. 1995; Bullough et al. 1994). Furthermore, the HA structures of the 1918 pandemic virus (Stevens et al. 2004), of H3 and H5 avian viruses, and an H9 swine virus have been deter-

mined (Ha et al. 2001; Ha et al. 2003). The BHA is 13.5 nm long and 1.4–4 nm in triangular cross-section, and contains all of the HA1 and the first 175 of the 221 amino acids of the HA2 subunit; it lacks only the hydrophobic membrane-anchoring peptide (Brand and Skehel 1972). The HA is folded into two structurally distinct domains, a globular head and a fibrous stalk. The globular head is entirely composed of HA1 residues and contains an eight-stranded antiparallel β-sheet. This framework supports the receptor-binding site, which is surrounded by highly variable antigenic loop structures. The fibrous stalk region, more proximal to the viral membrane, consists of residues from both HA1 and HA2. The cleavage site between HA1 and HA2 is located in the middle of the stalk. The trimeric structure is principally stabilized by the fibrous stem regions with a rather loose association of the globular heads. HA0 and cleaved HA1+HA2 are superimposable except for the region spanning the cleavage site. In uncleaved HA, the cleavage site forms a prominent surface loop in the middle of the stalk. A cavity is located next to the cleavage site that is partially filled by the carboxy terminus of HA1. Upon cleavage, the carboxy terminus of HA1 becomes exposed on the trimer surface, ca. 2.2 nm from the terminus of HA2, indicating significant rearrangement and conformational change after cleavage of the HA0. The hydrophobic amino terminus of the HA2 (fusion peptide) becomes buried in the trimeric structure, filling the cavity. The cleaved HA is presumed to be metastable.

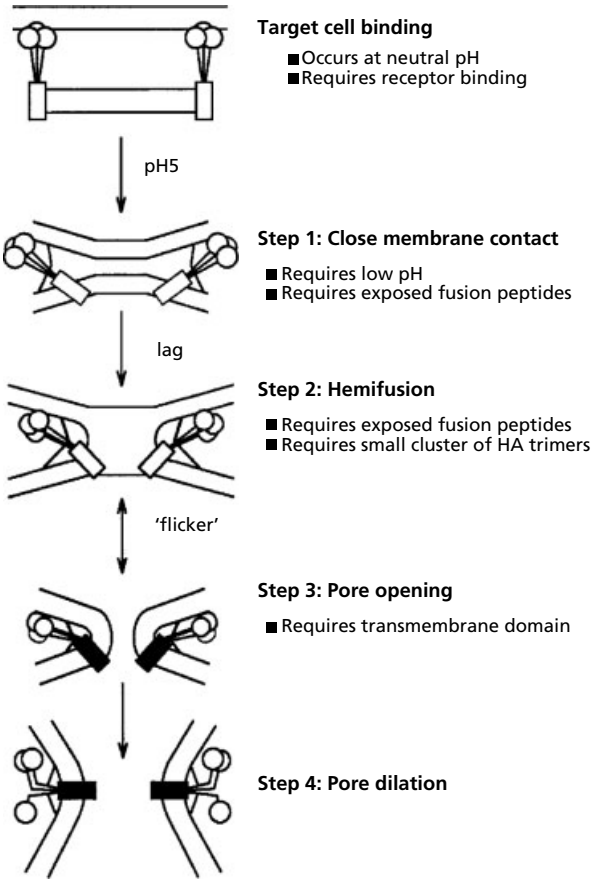

Figure 13 *Steps in HA-mediated membrane fusion. Processes described in the diagram occur after virus attachment to a cell membrane (White 1994).*

Fusion

The fusion of influenza viruses to the endosomal membrane is mediated by the HA (White 1992) (Figures 13 and 14). Under conditions of neutral pH, the fusion peptide, which forms a small part of the amino terminus of the HA2, is located in the fibrous stem of the molecule (ca. 3.5 nm away from the viral membrane and, hence, 10 nm from the target endosomal membrane), and is well integrated into the subunit interface by a network of hydrogen bonds. The importance of the peptide in HA-mediated fusion is evident from the ability of mutations in this region that alter (Daniels et al. 1987) or abolish (Gething et al. 1986b) fusion activity. When the pH is ca. 5 (late endosomal pH), the tertiary structure of the HA is altered (Skehel et al. 1982; White and Wilson 1987) (Figure 14). This change is critical for fusion of the viral and endosomal membranes (Skehel et al. 1982; Stegmann et al. 1990; White and Wilson 1987). The three-dimensional structure of the HA1 globular head remains largely unaltered (Bizebard et al. 1995), although partial dissociation of the globular heads may occur (Godley et al. 1992). By contrast, HA2 undergoes a refolding event in which the fusion peptide is relocated more than 100 Å toward the target membrane. At neutral pH, HA2 amino acid residues 38 to 55 form a short α-helix, followed by an extended loop (residues 56–75) and a long α-helix (residues 76–126) (Wilson et al. 1981). By contrast, the X-ray crystallographic structure of proteolytic fragments of the low pH-induced form of HA2 (Bullough et al. 1994; Carr and Kim 1993; Chen et al. 1999b) reveals a triple stranded α-helical coiled-coil formed by residues 40–105. Residues 106 to 112 form a loop that connects to a shorter α-helix (residues 113 to 129) that runs antiparallel to the long α-helix. The amino- and carboxy-terminal amino acids of HA2 are connected through numerous interactions that hold these residues in place at the 'tip' of the structure. The conformational states of the HA after exposure to low pH may differ among different subtypes of the molecules (Melikyan et al. 1999; Puri et al. 1990).

The exact mechanism of membrane fusion is the subject of intensive investigation and is currently believed to involve the following steps (discussed in detail in Bentz and Mittal (2003), Skehel and Wiley (2000), and Stegmann (2000), (Figure 13). Fusion is initiated by low pH that triggers a conformational change to expose the fusion peptide, which becomes inserted into the target membrane. Next, the outer leaf-

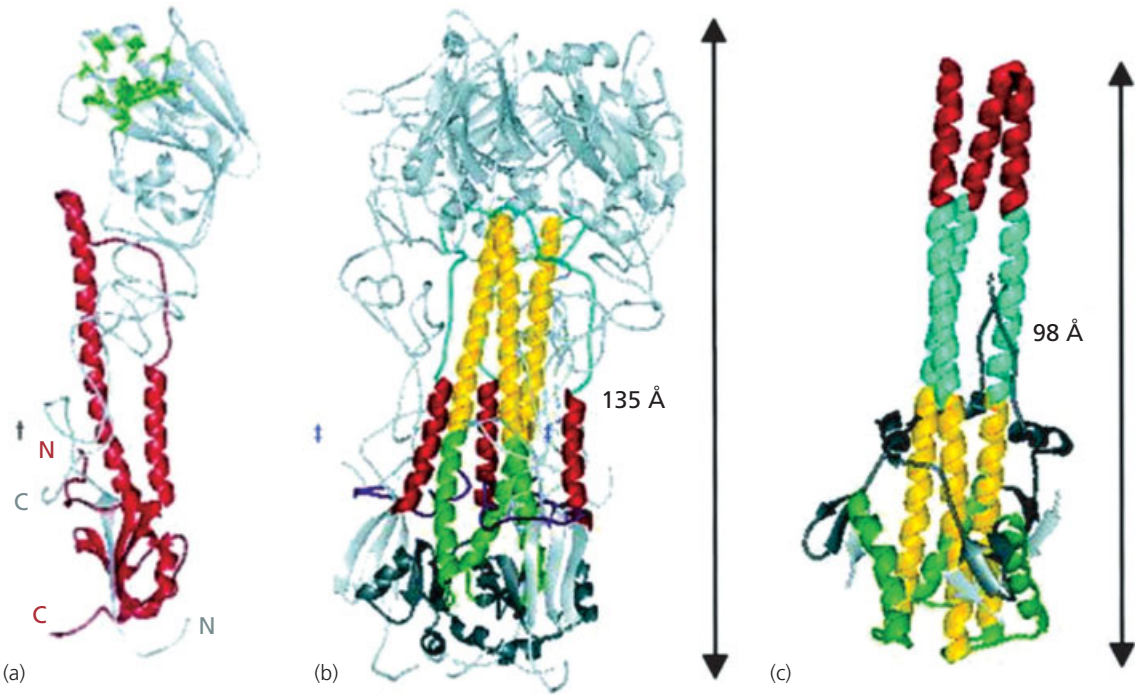

Figure 14 *Ribbon diagram of HA in the bromelain-digested neutral pH form shown for a single monomer* **(a)** *or the HA trimer* **(b)***; ribbon diagram of the low pH, trimeric fragment composed of amino acids 38–175 of HA2 which are linked through disulphide bonds to residues 1–27 of the HA1 subunit* **(c)***. In* **(a)***, HA1 and HA2 are depicted in gray and red, respectively, while the receptor-binding residues are shown in green. In* **(b)** *and* **(c)***, the helices/coils shown in red (amino acids 38–55), blue (amino acids 56–75), green (residues 106–129), and dark gray (amino acids 130–175) undergo a conformational change upon exposure to low pH, while amino acids 76–105 (yellow) maintain their conformation. In* **(b)***, the fusion peptides of the individual monomers are depicted in magenta (courtesy of Dr. I. Bahar) (Isin et al. 2002).*

lets of the membrane bilayer fuse (hemifusion), followed by fusion of the inner monolayer. Further observations suggest that oligosaccharides in the stem stabilize HA in a conformation prone to undergo structural changes that are necessary for fusion (Ohuchi et al. 1997). The length of the HA cytoplasmic tail affects fusion activity (Ohuchi et al. 1998) and the transmembrane region of HA has an important, though still undefined, role in the fusion process, as glycosylphosphatidylinositol-anchored HA is impaired in its ability to form pores (Armstrong et al. 2000; Kemble et al. 1994; Markosyan et al. 2000; Nussler et al. 1997).

Folding, assembly, and intracellular transport

During its synthesis in the ER, the HA interacts transiently with the BiP/GRP78 protein (Gething et al. 1986a; Hurtley et al. 1989) and calnexin (Chen et al. 1995; Hammond et al. 1994) before acquiring high mannose-type oligosaccharide and forming trimers, a prerequisite for its transport out of the ER (Doms et al. 1993). Through interaction with *N*-linked glycans in the HA, lectin chaperones such as calnexin and calreticulin regulate and facilitate HA folding (Daniels et al. 2003). Cysteine residues in the ectodomain are essential both for efficient folding and for stabilization of the folded molecule (Segal et al. 1992). Disulfide bond formation occurs co-translationally. In the Golgi apparatus, the oligosaccharides of the HA are further processed to the complex type. The HAs of virulent avian H5 and H7 viruses are cleaved in the trans-Golgi or trans-Golgi network by ubiquitous proteases (see below). In polarized cells, the final step of HA maturation is transport to the apical cell surface (Roth et al. 1983); its transport from the trans-Golgi network is energy-dependent (Gravotta et al. 1990). The proteolipid MAL, a non-glycosylated integral membrane protein that is a component of the apical transport pathway, is required for HA apical transport (Puertollano et al. 1999). Signals responsible for apical transport reside in the transmembrane and/or cytoplasmic domains (Brewer and Roth 1991; Lin et al. 1998). The generation of basolateral-sorting signals in the HA cytoplasmic tail directed 50–60 percent of the mutant HAs to the basolateral membrane; however, the mutant virus budded almost exclusively from the apical surface, indicating that factors other than the HA-sorting signal contribute to virus budding (Barman et al. 2003; Mora et al. 2002). The transmembrane and cytoplasmic domains contain signals for specific incorporation of the HA into virions in studies with a complementation assay (Naim and Roth 1993), although an HA tail-less mutant replicated almost as well as its parent (Jin et al. 1994).

Lipid rafts are sphingomyelin- and cholesterol-enriched microdomains in the cellular membrane. They are believed to provide platforms for the assembly and budding of viruses, likely by increasing the local concentration of viral structural proteins. HA associates with rafts through its transmembrane domain (Scheiffele et al. 1997; Shvartsman et al. 2003; Takeda et al. 2003); however, deletion of the HA cytoplasmic tail affects raft association as well (Zhang et al. 2000). Wild-type HA forms clusters at the surface of infected cells; by contrast, a mutant that lost the ability to associate with rafts was distributed randomly (Takeda et al. 2003). A mutant virus containing the 'nonraft' HA was characterized by reduced budding and fusion activity (Takeda et al. 2003).

HA cleavage

A link between HA cleavability and virulence is well established in avian influenza A viruses (Horimoto and Kawaoka 2001; Klenk and Rott 1988; Steinhauer et al. 1991; Webster and Rott 1987). In virulent H5 and H7 avian viruses, the HAs contain multiple basic amino acids at the cleavage site, which are cleaved intracellularly by endogenous proteases. By contrast, in avirulent avian viruses as well as non-avian influenza A viruses, with the exception of H7N7 equine viruses (Kawaoka 1991), the HAs lack a series of basic residues and are not subject to cleavage by such proteases (Bosch et al. 1979; Bosch et al. 1981). Thus, the tissue tropism of viruses may be determined by the availability of proteases responsible for the cleavage of different HAs, leading to differences in virulence.

Two groups of proteases seem to be responsible for HA cleavage. One includes enzymes recognizing a single arginine and able to cleave 'avirulent'-type HAs, such as plasmin (Lazarowitz et al. 1973), blood-clotting factor X-like protease (Gotoh et al. 1990), tryptase Clara (Kido et al. 1992), and bacterial proteases (Tashiro et al. 1987). The second group, which remains to be identified in vivo, comprises ubiquitous intracellular subtilisin-related proteases, furin, and PC6, which cleave virulent-type HAs with multiple basic residues at the cleavage site (Horimoto and Kawaoka 1994; Stieneke-Grober et al. 1992). Studies with HA cleavage mutants have demonstrated that the number of basic amino acids at the cleavage site and the presence or absence of a nearby carbohydrate affect HA cleavability by intracellular proteases in an interrelated manner (Kawaoka et al. 1984; Kawaoka and Webster 1988, 1989; Ohuchi et al. 1989; Vey et al. 1992). The proposed sequence requirement for HA cleavage by intracellular proteases, in the absence of a nearby carbohydrate (at residue 11 in H5 numbering) is Q–R/K–X–R/K–R (X = non-basic residue). If the carbohydrate moiety is present, virulence is maintained only if two basic amino acids are inserted (Q–X–X–R/K–X–R/K–R), or if the conserved glutamine at position –5 or the proline at position –6 is altered (i.e. B(X)–X(B)–R/K–X–R/K–R (B = basic residue)). In addition, the amino acid immediately downstream of the cleavage site (the amino terminal residue of the HA2) affects HA cleavage by intracellular proteases (Horimoto and Kawaoka 1995). The HA cleavage enzyme seems to be located in either the trans-Golgi or the trans-Golgi network, to be calcium-dependent, and to have an acidic pH optimum (Klenk et al. 1974, 1984; Walker et al. 1992). HA cleavage can be augmented by the ability of the A/WSN/33 (H1N1) NA protein to sequester plasminogen (Goto and Kawaoka 1998), hence increasing the local concentration of this ubiquitous protease precursor. This NA function relies on the presence of a carboxy-terminal lysine residue and the absence of an oligosaccharide side chain at position 146 (N2 numbering).

Oligosaccharide side-chains

The location and number of glycosylation sites are not conserved among HAs of different strains and subtypes (Goto and Kawaoka 1998; Nobusawa et al. 1991). Rather, these sites are scattered throughout the HA, although they tend to cluster around the antigenic sites on the globular heads. The HAs of individual virus strains contain from 5 to 11 sites, with the glycosylation sequence around residues 20–22 conserved in type A strains, except in some virulent avian strains in which the HA is cleaved by intracellular proteases (corresponding to residue 11 in the H5 numbering system; see section on HA cleavage). Some 17–20 percent of the total protein surface of the H3 HA could be covered by carbohydrate. The presence or absence of oligosaccharide side-chains in the HA (reviewed in Schulze (1997), affects antibody (Skehel et al. 1984) and $CD4^+$ T cell (Jackson et al. 1994) recognition of the molecule, calnexin/calreticulin binding (Hebert et al. 1996), receptor binding affinity and/or specificity (Gambaryan et al. 1998; Gunther et al. 1993; Matrosovich et al. 1999; Ohuchi et al. 1997; Tsuchiya et al. 2002), virulence (Hartley et al. 1997; Naeve et al. 1984), replication (Wagner et al. 2002), host range (Romanova et al. 2003), and fusion activity (Ohuchi et al. 1997). Reverse genetics studies demonstrated that growth restriction due to the lack of HA glycosylation site(s) can be partially overcome by the introduction of an NA of a different subtype (Baigent and McCauley 2001; Wagner et al. 2000), indicating that a functional balance between HA and NA is critical for efficient virus replication. Moreover, in H3 (Gallagher et al. 1992) and H7 (Roberts et al. 1993) HAs, no particular oligosaccharide side-chain is required for folding, intracellular transport or function of the molecule; however, at least two or three oligosaccharide chains (depending on the source of the HA) must be present to ensure transport of the molecule to the cell surface.

Acylation

The three cysteine residues in the carboxyl-terminal region of HA2 are acylated with palmitic (Schmidt 1982) by a thioester linkage (Veit et al. 1990). The lack of HA acylation affects HAs differently depending on the subtype. For instance, loss of palmitic acid does not impede the fusion activity of H3 (Steinhauer et al. 1991) or H7 HA (Philipp et al. 1995; Veit et al. 1991), but does affect syncytial formation of the latter (Fischer et al. 1998). An H3 virus with deletion of the HA cytoplasmic tail, which contains the acylation site, was generated by reverse genetics and replicates almost as well as its parent (Jin et al. 1994). On the other hand, a virus with a substitution at the carboxy-terminal cysteine of HA (Cys563 in WSN HA numbering) was not generated by reverse genetics, possibly because of structural constraints rather than the altered palmitylation (Zurcher et al. 1994). For H1 HA, replacement of the conserved cysteine residue did not affect hemifusion but restricted fusion pore formation, suggesting that palmitic acids facilitate the transition from hemifusion to fusion pore formation (Sakai et al. 2002). Results for the H2 HAs are more controversial. One report stresses the importance of each of the cysteine residues for fusion activity (Naeve and Williams 1990), while another indicates the absence of such an effect (Naim et al. 1992).

Receptor binding

Influenza A and B virus HAs bind to oligosaccharide-containing terminal sialic acids, including (α2,6)sialyllactose, *N*-acetylneuraminic acid-α2,6-galactose-(NeuAcα2,6Galβ1),4Glc and (α2,3)sialyllactose, NeuAcα2,3Galβ1,4Glc. Topologically, the binding site is a depression. The amino acid residues that contact the terminal sialic acids (Weis et al. 1988, 1990) are highly conserved among the different HA subtypes (Nobusawa et al. 1991). The hydroxy groups at C7 and C8 of the glycerol side-chain and the *N*-acetyl group, but not the hydroxy group of C9, are important for recognition by the HA (Kelm et al. 1992; Matrosovich et al. 1992). A second ligand-binding site, for which the affinity of sialyllactose is four times weaker than that for the primary site, has been identified in the HA (Sauter et al. 1992). The biological significance of the secondary ligand binding site is unknown.

The receptor specificity of the HA differs among influenza A viruses: most avian and equine influenza viruses bind preferentially the NeuAcα2,3Gal linkage, whereas human and classic H1N1 swine influenza viruses preferentially bind the NeuAcα2,6Gal linkage on the cell surface sialyloligosaccharides (Rogers and Paulson 1983; Rogers et al. 1983b; Rogers and D'Souza 1989). Early studies suggested that epithelial cells in human trachea contain sialic acid (SA) α2,6Gal but not SAα2,3Gal sialyloligosaccharides on the cell surface (Baum and Paulson 1990), and that virus with NeuAcα2,6Gal specificity binds epithelial cells lining human trachea, whereas those with NeuAcα2,3Gal specificity do not (Couceiro et al. 1993). Recent findings using cultures of differentiated human airway epithelial cells indicate that SAα2,6Gal sialyloligosaccharides are expressed on nonciliated epithelial cells, whereas ciliated cells (which represent a minor cell population of the epithelium) express SAα2,3Gal sialyloligosaccharides (Matrosovich et al. 2004). In accordance with this finding, human viruses infect preferentially nonciliated cells, while avian viruses infect ciliated cells. These results support recent documentation of human infection by avian viruses (Claas et al., 1998a, b; Fouchier et al. 2004; K.S. Li et al. 2004). However, it remains unknown whether the prevalence of different cell types in this in vitro system correlates with that in a natural setting (i.e. human respiratory tract). Thus, it has yet to be established whether the relative inefficiency of human infection by avian viruses (Beare and Webster 1991) is due to a limited number of cells susceptible to avian viruses in human airway or other unknown restrictions.

Epithelial cells in duck intestine (the replication site of avian influenza viruses) and those in horse trachea contain SAcα2,3Gal but not SAα2,6Gal sialyloligosaccharides (Ito et al. 1997; Ito and Kawaoka 2000). It is interesting that epithelial cells in pig trachea contain both types of sialyloligosaccharides (Ito et al. 1998) which explains why both human and avian viruses replicate efficiently in pigs (Kida et al. 1994). Thus, the receptor specificities of the viruses correspond to the presence of the receptor at the replication site of the virus (reviewed in Ito and Kawaoka (2000)). The HAs of influenza B viruses also preferentially recognize NeuAcα2,6Gal linkages (Xu et al. 1994). However, an avian H5N1 virus isolated from an individual in Hong Kong in 1997 bound to NeuAcα2,3Gal-containing receptors but not to those containing an α2,6-linkage (Matrosovich et al. 1999), indicating that receptor specificity (while being an important determinant of host range restriction) does not prohibit avian-to-human transmission.

Human H1 viruses from the 1918 and 1957 pandemic period seem to recognize both SAα2,6Gal- and SAα2,3Gal-containing receptors, while those isolated after 1977 have human-like receptor binding specificity (Rogers and D'Souza 1989). X-ray crystallographic studies of the pandemic 1918 HA (H1 subtype) and related human and swine HAs suggest that a combination of (minor) structural modifications allowed the 1918 HA to bind to human receptors, despite its avian-like amino acid sequence (Gamblin et al. 2004; Stevens et al. 2004).

In addition to the importance of linkage specificity between sialic acid and galactose (i.e. α2,3 vs α2,6 linkage), the sialic acid species (i.e. *N*-acetyl vs *N*-glycolyl sialic acid) is also involved in host range restric-

tion of influenza viruses. Influenza viruses differ in their recognition of *N*-acetyl vs *N*-glycolyl sialic acid (Higa et al. 1985); correspondingly, the prevalence of sialic acid species differs among animal species. *N*-glycolyl sialic acid linked to galactose by α2,3 linkages (NeuGcα2,3Gal) is prevalent in epithelial cells of horse trachea, allowing replication of viruses recognizing this glycoconjugate moiety but not of those recognizing *N*-acetyl sialic acid (Suzuki et al. 2000). Likewise, NeuGcα2,3Gal is a determinant of host range restriction in ducks (Ito et al. 2000).

Receptor specificity is largely determined by the nature of the amino acids at position 226 and 228. For human H2 and H3 viruses, substitution of Gln-226 with Leu and Gly-228 with Ser shifts receptor specificity from avian to human receptor binding (Connor et al. 1994; Naeve et al. 1984; Rogers et al. 1983a). Human H3 virus replication in ducks depends on the presence of avian-like amino acids at these positions (Hinshaw et al. 1983b; Naeve et al. 1983, 1984; Vines et al. 1998). In addition to these positions, substitution of residues at position 136, 190, 195, or 225, affect receptor binding affinity or specificity to various extents (Martin et al. 1998; Matrosovich et al. 2000; Nobusawa et al. 2000).

Host-cell-mediated selection of antigenic variants

The HA antigenicity of human influenza A and B viruses grown in embryonated hens' eggs differs from that of viruses isolated and passaged in a variety of cell cultures, including chicken embryo fibroblasts (Katz et al. 1987; Katz and Webster 1992; Robertson et al. 1987; Schild et al. 1983); even so, minor egg isolates contain the same antigenic phenotype as the tissue culture-grown viruses (Oxford et al. 1991). A similar phenomenon was discovered by Burnet and Clarke (1942) as 'O (as original)–D (as derived)' variation during passages of human viruses in eggs; O form virus agglutinated human or guinea-pig erythrocytes in preference to fowl erythrocytes, while D form viruses agglutinated them equally. Because tissue culture-grown viruses contain the same amino acid sequences of the HA as those replicating in humans, mutants are selected during replication in eggs (Katz et al. 1990; Rajakumar et al. 1990; Robertson et al. 1990). This finding is supported by the greater efficiency of primary isolation of human virus in cell culture than in eggs (Dumitrescu et al. 1981; Monto et al. 1981). Comparison of the HA sequence among viruses recently isolated in eggs or cell culture revealed mutations around the receptor binding pocket. Growth of human isolates in the allantois of eggs leads to the selection of receptor-binding variants (Ito et al. 1997; Mochalova et al. 2003; Smirnov et al. 2000) and results in enhanced receptor-binding affinity (Gambaryan et al. 1999). A/Hong Kong/156/97 virus grown in cell culture was significantly more pathogenic in mice than its egg-grown counterpart (Hiromoto et al. 2000); sequence analysis revealed amino acid changes in HA, PB1, PA, NP, and NS1. Inactivated vaccines prepared from tissue culture-grown viruses induce better protective immunity than do those from egg-grown viruses in animal models (Katz and Webster 1992; Newman et al. 1993; Wood et al. 1989) as well as in humans (Newman et al. 1993), although differences were not found when animals were immunized with vaccinia viruses that expressed the HAs of viruses grown in eggs or tissue culture (Rota et al. 1987, 1989).

NP

The NP (reviewed in Portela and Digard (2002)), the most abundant component of RNP, is a type-specific antigen associated with viral RNA. It covers 20–24 nucleotides (Compans et al. 1972; Duesberg 1969; Jennings et al. 1983; Ortega et al. 2000), exposing the bases to the outside and inducing melting of the secondary structure (Baudin et al. 1994; Klumpp et al. 1997). Thus, vRNA wraps around the NP scaffold. The RNA-binding region of the NP of influenza A virus has been mapped between residues 91 and 188 (Albo et al. 1995; Kobayashi et al. 1994). By electron microscopy, purified NP has a rodlike shape with dimensions of 6.2×3.5 nm and mostly exists in polymeric forms, ranging from trimers to large structures that resemble intact viral RNP (Ruigrok et al. 1989) (Figure 15). However, the formation of helical structures similar in conformation and density to intact viral RNP requires the presence of vRNA and M1 (Huang et al. 2001).

NP may undergo several stages of maturation, in which early disulfide-linked NPs are converted into disulfide-free NP which forms noncovalently stabilized oligomers (Elton et al. 1999; Prokudina et al. 2004; Zhao et al. 1998). It possesses several signals that regulate its nuclear import early in infection, but cytoplasmic accumulation late in infection (Bullido et al. 2000; Davey et al. 1985; Neumann et al. 1997; O'Neil and Palese 1995; Wang et al. 1997; Weber et al. 1998). Migration back to the cytoplasm presumably occurs as a component of RNP (Martin and Helenius 1991; Rey and Nayak 1992) and cytoplasmic accumulation requires interaction of NP with actin filaments (Digard et al. 1999). These transport processes are regulated by phosphorylation (Bui et al. 2002; Bullido et al. 2000; Neumann et al. 1997). NP also interacts with CRM1 (Elton et al. 2001), a cellular nuclear export factor; however, the significance of this finding for virus replication is not yet known. Free NP is needed for vRNA synthesis (Beaton and Krug 1986) and NP mutants have been identified that lead to defects in RNA replication, presumably by affecting the protein's ability to interact with the polymerase complex (Biswas et al. 1998; Mena et al. 1999). NP is also thought to play a role in host range restriction, as indicated by successful, host-depen-

dent rescue of the ts phenotype (Scholtissek et al. 1985) and restriction of avian–human influenza A virus reassortants for replication in primates (Clements et al. 1992; Murphy and Clements 1989; Snyder et al. 1987). This evidence notwithstanding, direct support for this role of NP is lacking.

NA

The tetrameric NA protein of influenza A viruses (Figure 16), a type II glycoprotein with its amino terminus inside the cell and its carboxyl terminus outside (Air and Laver 1989), is one of two major glycoproteins on the virus surface. It has an uncleaved amino-terminal signal/anchor domain and a six amino-acid tail (Blok and Air 1982b; Fields et al. 1981). Although the precise location of the amino-terminal six polar residues is unknown, they are presumed to be exposed to the cytoplasm. The NA has a box-shaped head ($10 \times 10 \times 6$ nm) comprising four co-planar and roughly spherical subunits and contains the enzyme-active center and major antigenic sites (Colman et al. 1983; Varghese et al. 1983) (Figure 17). The stalk is centrally attached to the head with a hydrophobic region by which the stalk is embedded in the viral membrane (Blok and Air 1982b; Fields et al. 1981). The stalk region is flexible in length and sequence. In contrast to the remainder of the NA, the six amino-acid cytoplasmic tail is highly conserved among all NA subtypes of influenza A virus (Blok and Air 1982a, b). This region is important for incorporation of the NA into virions but is not essential for virus replication (Bilsel et al. 1993; Garcia-Sastre and Palese 1995; Jin et al. 1994). Although deletion of five of these residues (leaving the initiating methionine) does not affect the viability of viruses, it causes attenuation because of the reduction in virion NA molecules. Mutant viruses lacking the NA or NA and HA cytoplasmic tails contain more filamentous than spherical particles (Jin et al. 1997; Mitnaul et al. 1996). An HA/NA-tail-less mutant had reduced vRNA-to-protein content (Zhang et al. 2000), suggesting an interaction of the NA and/or HA cytoplasmic tails with other viral or cellular components during vRNA virion incorporation and virion formation.

Reverse genetics experiments demonstrated that the NA stalk can be deleted almost completely or be extended by 58 amino acids to accommodate a variety of foreign sequences (Castrucci and Kawaoka 1993; Castrucci et al. 1994; Luo et al. 1993). In addition to laboratory isolates, avian viruses, especially those circulating in land-based birds (e.g. chickens) contain deletions in the NA stalk region (Banks et al. 2001; Bender et al. 1999; Blok and Air 1982b; Blok et al. 1982; Matrosovich et al. 1999; Spackman et al. 2003). 'Short-NA-stalk' viruses are characterized by mutations in HA1 which could affect receptor binding (Baigent and McCauley 2001; Banks et al. 2001; Bender et al. 1999; Matrosovich et al. 1999; Mitnaul et al. 2000; Spackman et al. 2003); these mutations likely compensate for a decreased ability to release viruses with shortened stalks. The occurrence of interrelated mutations indicates that the balance between HA and NA functions is critical for efficient influenza virus replication (reviewed in Wagner et al. (2002)).

A recombinant virus expressing reduced levels of NA was attenuated in mice (Solorzano et al. 2000). NA-deficient viruses, generated by repeated passage in the presence of bacterial NA and antibodies to viral NA, replicate in cell culture when a bacterial NA is added exogenously, as well as in animals (Hughes et al. 2000; Liu and Air 1993; Liu et al. 1995). These viruses possess

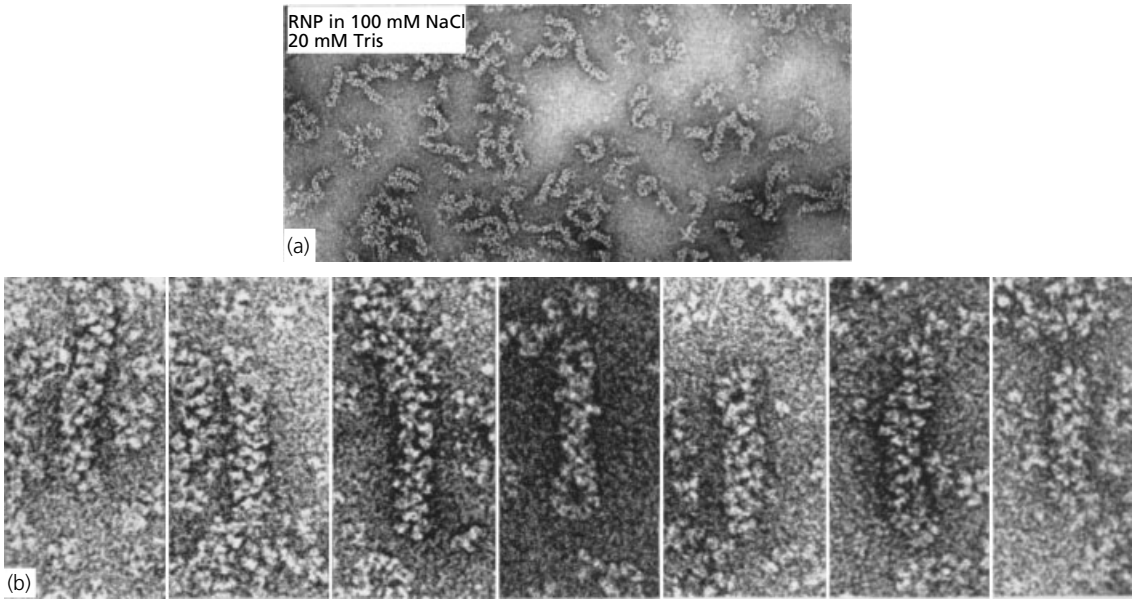

Figure 15 *Morphological features of RNP* **(a)** *and NP* **(b)**. *(Courtesy of Dr. Rob W.H. Ruigrok) (Ruigrok and Baudin 1995)*

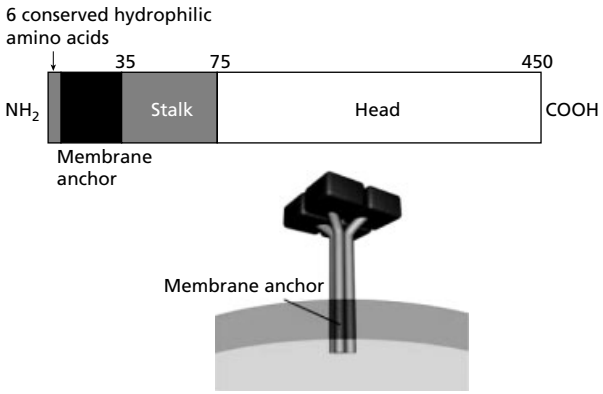

Figure 16 *Schematic diagram of the neuraminidase molecule. (Courtesy of Yuko Kawaoka) (Webster et al. 1984).*

internal deletions of 800–900 nt, leaving regions encoding the cytoplasmic and transmembrane domains and part of the stalk. Whether the truncated NA molecule exists in infected cells remains unknown. With the identification of segment-specific virion incorporation signals at the 5′ and 3′ ends of viral RNAs (Fujii et al. 2003), it seems more likely that the terminal NA sequences are maintained to ensure efficient virion incorporation. Analysis of NA-deficient viruses revealed mutations around the HA receptor-binding pocket (Hughes et al. 2000), further emphasizing the importance of a balance between HA and NA functions.

The NA plays a role in host range restriction (Hinshaw et al. 1983b). While the sialidase activity of most human and pig isolates becomes undetectable below pH 4.5, duck isolates retain their enzymatic activity under these conditions (Takahashi et al. 2001), consistent with virus propagation in the acid environment of the digestive tract. NA is also important for plaque formation by A/WSN/33(H1N1) in Madin–Darby bovine kidney (MDBK) cells and for the neurovirulence of this virus (Nakajima and Sugiura 1980; Schulman and Palese 1977; Sugiura and Ueda 1980). These phenotypes were characterized by loss of the oligosaccharide sidechain from the WSN NA, which is conserved among the NAs of all other influenza A viruses (Li et al. 1993). This observation is now explained by HA cleavage promoted by plasminogen-binding activity of WSN NA (see HA cleavage section) (Goto and Kawaoka 1998). In addition, NA seems to induce apoptosis (Mohsin et al. 2002; Morris et al. 2002), likely through the activation of transforming growth factor beta (TGF-β) (Schultz-Cherry and Hinshaw 1996), although the significance of this finding for influenza pathogenesis is not known.

Three-dimensional structure

The NA three-dimensional structure has been solved in both type A and B viruses. Despite the 28 percent homology between these two types of viruses, the overall folding patterns of their NAs are almost identical

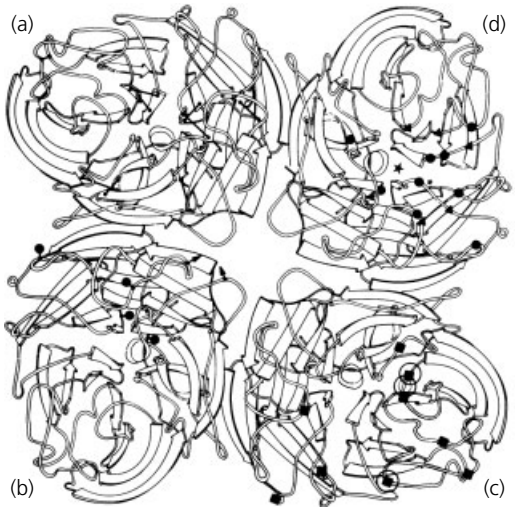

Figure 17 *Schematic illustration of the neuraminidase tetramer viewed from above, down the symmetry axis. The four subunits highlight different features of the structure:* **(a)** *disulfide bonds;* **(b)** *carbohydrate attachment sites at positions 86, 146, 200, and 234 (circle), and metal ligands Asp-113 and Asp-114 (arrows);* **(c)** *residues that change in N2 (squares) and N9 (black circles) variants selected with monoclonal antibodies;* **(d)** *conserved acidic (circles) and basic (triangles) residues in influenza A and B neuraminidase and the sialic acid binding site (star). (Courtesy of Dr. G.M. Air) (Air and Laver 1989).*

(Burmeister et al. 1992; Colman et al. 1983; Janakiraman et al. 1994; Varghese et al. 1983), and type B NA can functionally replace the type A enzyme (Ghate and Air 1999). The tetrameric NA protein has circular four-fold symmetry stabilized by calcium. The polypeptide chain folds into six topologically identical four-stranded antiparallel β-sheets arranged like propeller blades (Figure 17). The product of catalysis, sialic acid, is bound in a large pocket on the distal surface, flanked and surrounded by nine acidic residues, six basic residues and three hydrophobic residues – each of which is strictly conserved in all known influenza viral NA sequences. Alteration of more than half of these residues results in molecules deficient in enzyme activity (Lentz et al. 1987). The three-dimensional structure of the NA led to the synthesis of a potent sialidase inhibitor, 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid (von Itzstein et al. 1993), which inhibits the NA activity of both type A and B viruses (inhibition constants, 10^{-9}–10^{-10} m). The structures of NA complexed with the inhibitor 2,3-didehydro-2-deoxy-n:-acetylneuraminic acid (DANA) (Smith et al. 2001) and of a mutant NA (E119G) that is resistant to the inhibitor 4-guanidino-Neu5Ac2en (4-GuDANA) (Colacino et al. 1997) have been solved. The latter structure suggests that the E119G replacement destabilizes the NA tetramer.

Sialidase activity

The NA catalyzes the cleavage of the α-ketosidic linkage between a terminal sialic acid and an adjacent sugar

residue (Gottschalk 1957). The pH optimum of the enzyme ranges from 5.8 to 6.6, with an apparent K_m of 0.4 mm with use of N-acetylneuraminyllactose (Drzeniek and Kaluza 1972; Mountford et al. 1982). Removal of sialic acid residues by the NA promotes both entry and release of virus from infected cells (Palese et al. 1974). Avian NAs cleave NeuAcα2,3Gal in preference to NeuAcα2,6Gal. For human N2 NAs, a shift from NeuAcα2,3Gal to NeuAcα2,6Gal linkages has been observed since their introduction into the human population (Baum and Paulson 1991), presumably corresponding to the preferential recognition of the latter linkages by the HA molecule. The NA substrate specificity is determined by the nature of the amino acid at position 275 (Kobasa et al. 1999). In addition to substrate specificity, the specific activity of NA is also critical for the adaptation of avian viruses to growth in humans (Kobasa et al. 2001).

Folding, assembly, and intracellular transport

Synthesized as a monomer in the ER and translocated into the lumen of that structure, the NA rapidly forms a disulfide-linked dimer, which then non-covalently associates to form a tetramer ($t_{1/2}$ ca. 1.5–2 min). After transiently associating with the BiP/GRP78 ($t_{1/2}$ ca. 5 min), the NA is transported through the Golgi apparatus, where it acquires complex carbohydrates, rendering it resistant to endo H ($t_{1/2}$ ca. 25 min), and finally to the cell surface (Hogue and Nayak 1992; Saito et al. 1994). The mature NA contains signals for apical transport in its cytoplasmic and transmembrane domains (Barman and Nayak 2000; Kundu and Nayak 1994; Zhang et al. 2000). The transmembrane domain also contains a signal for raft association which does not overlap the apical sorting signal (Barman and Nayak 2000).

HA activity

All avian virus NAs possess HA activity (Hausmann et al. 1995; Kobasa et al. 1997; Laver et al. 1984). The HA activity can be transferred to NAs lacking this activity by altering amino acid residues that are located apart from the sialidase-active site (Nuss and Air 1991), indicating discrete HA- and NA-active sites in the NA molecule. A mutant virus with an altered HA-binding site displayed a significantly reduced hemadsorption activity and a reduction in NA enzymatic activity, but was not impaired in its ability to replicate in an avian host (Kobasa et al. 1997); thus, the biological significance of the HA activity in NA remains unknown.

NB

The dimeric, type II integral membrane protein NB (Williams and Lamb 1986) is unique to type B viruses. It is abundantly expressed in infected cells and incorporated into virions (Brassard et al. 1996). NB is composed of an 18 amino acid ectodomain, a 22 amino acid transmembrane domain, and a 60 amino-acid cytoplasmic tail (Brassard et al. 1996; Williams and Lamb 1988). Structural similarity with the M2 protein and ion channel activity (Sunstrom et al. 1996) led to the speculation that NB is the equivalent of the type A M2 protein. However, reverse genetics experiments demonstrated that NB is not critical for virus replication in vitro (Hatta and Kawaoka 2003). The role of this protein in viral replication remains obscure.

M1

The most abundant virion protein and a type-specific antigen of influenza viruses, the M1 has long been thought to be located underneath and adds to the rigidity of the lipid bilayer, although direct evidence for such a role is lacking. Immunogold labeling with anti-M1 monoclonal antibodies failed to decorate the M1 in virions unless they were first treated with a protease or a detergent (Murti et al. 1992). Cryo-electron microscopic studies suggested that the M1 can modify the lipid bilayer, causing the viral envelope to thicken (Fujiyoshi et al. 1994) (see Figure 3e). M1 is the primary determinant of virus budding and assembles into viruslike particles that are released into the medium (Gomez-Puertas et al. 2000). Furthermore, it determines the morphology of influenza virions (Bourmakina and Garcia-Sastre 2003; Roberts and Compans 1998), although other gene products may contribute (Jin et al. 1997; Mitnaul et al. 1996; Smirnov et al. 1991). Crystal structures of the N-terminal domain (amino acids 1–162) at neutral or acidic pH show a long ribbon of stacked dimers at neutral pH, while dimers were rotated in relation to each other at acidic pH (Harris et al. 2001; Sha and Luo 1997).

Located in both the nucleus and the cytoplasm (Patterson et al. 1988; Smith et al. 1987), this protein contains a karyophilic signal at residues 101–105 (RKLKR) (Ye et al. 1995). Depending on the nature of the replacement, mutations in this region have different outcomes on virus replication (Hui et al. 2003; Liu and Ye 2002). Replacement of residues 100–104 with the 'late domain' motifs PTAP or YPDL yielded virus with wild-type phenotypes (Hui et al. 2003), suggesting that this region of M1 may have a function similar to the late domains of Ebola virus or retrovirus matrix proteins. Structural similarities between M1 and human immunodeficiency virus (HIV) matrix and capsid proteins substantiate this assumption (Harris et al. 1999). In accordance with its proposed role, M1 has multiple lipid-binding regions (Bucher et al. 1980; Gregoriades and Frangione 1981; Hay 1974; Kretzschmar et al. 1996). In the cytoplasm, it is associated with cytoskeletal elements (Avalos et al. 1997).

M1 is required for nuclear export of vRNP complexes (Huang et al. 2001; Martin and Helenius 1991;

Whittaker et al. 1995), a function that relies on the association of M1 with vRNP complexes (Sakaguchi et al. 2003). M1 may therefore bridge the viral nuclear export factor NS2(NEP) and components of vRNP complexes such as PA (Hara et al. 2003).

The M1 proteins of influenza A and B viruses have a 'zinc finger' motif (Wakefield and Brownlee 1989), and purified virions contain zinc (Elster et al. 1994); however, the amount of zinc is not correlated with the RNA-binding activity of the protein and replacement of the conserved cysteine and histidine residues in the zinc finger motif did not significantly impair virus replication in cell culture (Hui et al. 2003; Liu and Ye 2002). Although serine residues between amino acids 108 and 126 of A/WSN/33(H1N1) M1 are phosphorylated (Gregoriades et al. 1990), the role of this property in viral replication is unknown; it may be important for viral replication involving intracellular movement of the protein (Whittaker et al. 1995). The influenza B M1 protein is also phosphorylated. M1 also inhibits RNP transcription activity (Perez and Donis 1998; Ye et al. 1989; Zvonarjev and Ghendon 1980), and thus is considered to serve as a molecular switch that initiates the final step of virus assembly. Furthermore, M1 contains RNA-binding domains (Eister et al. 1997; Ye et al. 1989, 1999), which have been mapped between residues 90–109 and 129–164.

One of the *M* gene products (most likely M1) contributes to the dominance phenotype shown by cold-adapted A/Ann Arbor/6/60(H2N2) in coinfection studies with other strains, both in vitro and in vivo (Whitaker-Dowling et al. 1990, 1991; Youngner et al. 1994). M1 may also be linked to rapid virus growth (Baez et al. 1980; Yasuda et al. 1994), presumably defined by the strength of its association with vRNP complexes (Liu and Ye 2002).

M2

The integral homotetrameric M2 membrane protein (Holsinger and Lamb 1991; Lamb et al. 1985; Panayotov and Schlesinger 1992; Sugrue and Hay 1991), abundantly expressed at the surface of virus-infected cells, is, nevertheless, a relatively minor component of virions (Zebedee and Lamb 1988). Sharing eight amino-terminal residues with M1, the M2 protein comprises 97 amino acids: 24 as the ecto-, 19 as the transmembrane, and 54 as the cytoplasmic domain. M2 functions as a pH-activated ion channel that permits protons to enter the virion during uncoating (Pinto et al. 1992; Sugrue et al. 1990b) and that modulates the pH of intracellular compartments, an essential function for the prevention of acid-induced conformational changes of intracellularly cleaved HAs (H5 and H7 HA subtypes of virulent avian influenza A virus) in the trans-Golgi network (Ohuchi et al. 1994; Sugrue et al. 1990b; Takeuchi and Lamb 1994). Both the imidazole ring of His-37 and the indole moiety of Trp-41 are critical for the activation and regulation of the ion channel (Okada et al. 2001; Smondyrev and Voth 2002; Tang et al. 2002; Zhong et al. 2000). The activity of the M2 ion channel is blocked by the anti-influenza drug amantadine hydrochloride (see Chemotherapy section). The M2-associated ion channel activity resides in the transmembrane region (Duff and Ashley 1992), the primary site of mutations (residues 27, 30, 31, and 34) in amantadine-resistant mutants (Hay et al. 1985). Amantadine presumably blocks the ion channel activity of M2 by steric hindrance, resulting from insertion of the active drug between Val-27 and Ser-31 of the M2 molecule (Duff et al. 1994). Reverse genetics allowed for testing of viruses with mutations or deletions in the transmembrane region of M2 (Takeda et al. 2002; Watabe et al. 2001). While such viruses are viable, they are impaired in their growth. These findings indicate that influenza A virus can undergo limited replication in cell culture without M2 ion channel activity; this activity, however, is required for efficient viral replication.

The functional role of the M2 cytoplasmic region, the longest among the influenza viral membrane proteins, is unknown but is important for virus replication (Castrucci and Kawaoka 1995). M2 co-precipitates with the RNP core prepared from purified virus (Bron et al. 1993), indicating its high affinity for this protein, a property that presumably involves the M2 cytoplasmic tail. The M2 protein forms a homotetramer by non-covalent association of M2 dimers disulfide-linked at Cys-17 or -19, or both (Holsinger and Lamb 1991; Panayotov and Schlesinger 1992; Sugrue and Hay 1991). None of the cysteine residues in the M2 is essential for viral replication (Castrucci et al. 1997), although disulfide bond formation stabilizes the M2 tetramer (Holsinger and Lamb 1991). M2 proteins are palmitylated at Cys-50 with the exception of those in H3N8 equine viruses, but are not essential for virus replication (Sugrue et al. 1990b; Veit et al. 1991). The protein is also phosphorylated, mainly at Ser-64 (85 percent), but also at Ser-82, -89, and -93 (Holsinger et al. 1995); however, replacement of Ser-64 did not affect virus replication in vitro or in vivo (Thomas et al. 1998). The M2 ectodomain may play a role in virion incorporation (Park et al. 1998).

OTHER *M* GENE PRODUCTS OF TYPE B AND C VIRUSES

BM2, encoded by the *M* gene of type B virus (Horvath et al. 1990), is expressed in large amounts late in infection and has been detected in virions (Odagiri et al. 1999). It has no obvious structural similarities with type A M2 protein (Briedis et al. 1982); however, it is an oligomeric – most likely tetrameric – integral membrane protein, and its transmembrane domain contains histidine and tryptophane residues at the same relative location and spacing as those critical for the ion channel activity of type A M2 protein (Paterson et al. 2003). In fact, BM2 possesses ion channel activity and prevents

co-expressed HA from adopting the low pH-induced conformation during transport to the cell surface (Horvath et al. 1990). These findings, together with reverse genetics studies demonstrating that BM2 is critical for the viral life cycle (Hatta et al. 2004; Jackson et al. 2004), indicate that BM2 is the equivalent of the type A M2 protein.

CM2, which resides in infected cells (Hongo et al. 1994), is generated by proteolytical cleavage of a precursor protein translated from unspliced transcripts of the *M* gene (Hongo et al. 1999; Pekosz and Lamb 1998). It is composed of 115 amino acids corresponding to the carboxy-terminal region of the precursor protein and has biochemical properties similar to the influenza A virus M2 protein (Paterson et al. 2003; Pekosz and Lamb 1997). The protein is palmitylated and phosphorylated, and the latter modification seems to accelerate tetramer formation (Z.N. Li et al. 2001; Tada et al. 1998). While these findings suggest a role of CM2 as the type C ion channel protein, direct experimental evidence is missing.

NS1

The only nonstructural protein of influenza A virus, NS1 is made in abundance during early infection. It is encoded by a co-linear mRNA, consists of 202–238 amino acids, is phosphorylated (Pekosz and Lamb 1998), and contains two karyophilic signals (Greenspan et al. 1988) as well as a latent nuclear export signal (Li et al. 1998).

The major function of NS1 is the inhibition of the cellular, anti-viral interferon (IFN) response (Garcia-Sastre et al. 1998) (reviewed in Garcia-Sastre (2001) and Katze et al. (2002)) to ensure efficient viral replication in IFN-competent hosts. In virus-infected cells, double-stranded RNA (dsRNA) is believed to trigger the activation of transcription factors such as ATF-2/c-Jun, NF-κB, or IFN-regulatory factors (IRF) that regulate IFN-β production. Similarly, IFN-α is also induced in response to virus infection. IFN induces the phosphorylation of STAT1 and STAT2, which, after dimerization and recruitment of IRF-9, activate the transcription of IFN-stimulated genes (ISG). ISGs comprise a large number of genes that establish an antiviral state in cells, including those encoding dsRNA-activated protein kinase (PKR) (PKR), Mx proteins, or proteins responsible for mRNA degradation such as 2′–5′-oligoadenylate synthetase (OAS) and RNase L. NS1 seems to interfere with these pathways by inhibiting the activation of transcription factors (Ludwig et al. 2002; Smith et al. 2001; Talon et al. 2000; Wang et al. 2000) and PKR (Bergmann et al. 2000; Hatada et al. 1999; Lu et al. 1995). It may execute its function by binding to dsRNA; however, direct protein–protein interactions between NS1 and components of the IFN system cannot be ruled out. The H5N1 avian viruses isolated in Hong Kong in 1997 induce high levels of proinflammatory cytokines, particularly tumor necrosis factor (TNF)-α and IFN-β (Cheung et al. 2002), while being resistant to the antiviral effects of these cytokines (Seo et al. 2002). These properties have been linked to the *NS* gene or NS1 protein, respectively, further emphasizing the protein's contribution to virulence (Cheung et al. 2002; Seo et al. 2002).

Besides its role in the regulation of the cellular IFN response, NS1 acts as a post-transcriptional regulator by inhibiting mRNA splicing and the nuclear export of cellular mRNA (Alonso-Caplen et al. 1992; Chen and Krug 2000; Krug et al. 2003; Lu et al. 1994; Qian et al. 1994), thereby maximizing the availability of substrate for capped primers and promoting viral mRNA synthesis. Interaction of NS1 and the cellular poly(A)-binding protein II (PABII) with the 30-kd subunit of cleavage and polyadenylation specificity factor (CPSF) blocks the binding of this protein to the AAUAAA sequence of some cellular pre-mRNAs, hence inhibiting their polyadenylation (Nemeroff et al. 1998). For some cellular mRNAs, short poly(A)-stretches have been detected whose elongation is blocked by the interaction of NS1 with PABII (Chen et al. 1999a). Both species – non-polyadenylated mRNAs and mRNAs with short poly(A) tails – remain in the nucleus where they become substrates for the endonucleolytic activity of the polymerase complex. In vitro, inhibition of splicing of mRNAs is mediated by binding of NS1 to a stem-bulge in U6snRNA (Wang and Krug 1998), which plays a critical role in the assembly/activity of spliceosomes. By contrast, in vivo, inhibition of splicing is brought about by binding of NS1 to the 30-kd subunit of CPSF (Y. Li et al. 2001). The interaction of NS1 with the 30-kd subunit of CPSF also induces the transcription of antiviral genes in an IFN-independent manner (Kim et al. 2002; Noah et al. 2003).

Two functional domains have been identified for NS1. The amino-terminal 73 amino acids constitute the RNA-binding domain and are sufficient for dimerization, as shown by structural analysis and experimental findings (Chien et al. 1997; Liu et al. 1997; Wang et al. 1999). Although mutational analysis suggested that amino acids 134–161 form the 'effector' domain that is required for the inhibition of nuclear export of poly(A)-containing cellular mRNAs (Qian et al. 1994), the entire carboxy-terminal half of the protein (amino acids 74–237) might be necessary.

NS1 induces apoptosis in infected cells (Schultz-Cherry et al. 2001), a phenotype that relies on the RNA-binding/multimerization, but not the effector, domain. However, mutations in the RNA-binding/multimerization domain are not sufficient to inhibit the induction of apoptosis, indicating that other viral proteins such as NA (Morris et al. 1999; Schultz-Cherry and Hinshaw 1996) also contribute to the induction of apoptosis.

NS2(NEP)

Encoded by a spliced mRNA (Inglis et al. 1979; Lamb and Choppin 1979), the NS2(NEP) protein comprises 121 amino acids (Lamb and Lai 1980), is phosphorylated, localizes to both the nucleus and cytoplasm of infected cells, and is incorporated into virions (Richardson and Akkina 1991). NS2(NEP) contains a classical nuclear export signal (NES) in its amino-terminal region (O'Neill et al. 1998) and interacts with the cellular nuclear export factor CRM1 (Neumann et al. 2000), which mediates export of proteins containing classical NESs. In cells infected with viruslike particles lacking NS2(NEP), viral RNP complexes are retained in the nucleus. Likely, NS2(NEP) functions by connecting the cellular export machinery with vRNPs through M1 (Akarsu et al. 2003; Yasuda et al. 1993). Based on its role as the viral nuclear export protein, NS2 is now also referred to as NEP.

The NS2 proteins of type A viruses are more conserved than the NS1 protein (76.9 percent vs 64.3 percent); however, neither product is conserved to the extent of other internal proteins (Buonagurio et al. 1986; Kawaoka et al. 1998; Ludwig et al. 1991; Nakajima et al. 1990a, b; Suarez and Perdue 1998).

HEF

The HEF protein of influenza C virus, which is synthesized as a single polypeptide with subsequent trimer formation and then cleaved into two disulfide-linked subunits, HEF1 and HEF2 (Formanowski and Meier-Ewert 1988; Herrler et al. 1979; Herrler and Klenk 1991; Hewat et al. 1984), exists on the virion surface as projections and has a reticular structure consisting mainly of hexagons (Flewett and Apostolov 1967) (see Figure 5). Its structure has been determined by X-ray crystallography (Rosenthal et al. 1998; Zhang et al. 1999). The HEF is composed of an extended, membrane proximal stalklike domain (composed of both HEF1 and HEF2 amino acids) and a membrane distal globular head that consists of HEF1 residues. The head region contains the receptor binding site which is situated on top of the protein. Two regions of significant sequence difference in HEF1, when compared to HA1 (residues 41–73 and 311–366), form the esterase domain which is located under the receptor-binding site. This confirms earlier findings that suggested separate esterase and receptor-binding sites, as di-isopropyl fluorophosphate inhibits only the esterase activity (Muchmore and Varki 1987). Calcium ion is essential for maintenance of the HEF structure. The HEF facilitates binding of influenza C virus to its cell surface receptor, an oligosaccharide containing a terminal 9-*O*-acetyl-*N*-acetylneuraminic acid (Herrler et al. 1985; Rogers et al. 1986).

The receptor-destroying enzyme (esterase) contains the 'catalytic triad' of a serine (position 57), a histidine (position 355), and an aspartic acid residue (position 352) (Rosenthal et al. 1998) that are characteristic for the active sites of esterases. The 9-*O*-acetyl group is critical for the binding of HEF, which does not recognize *N*-acetyl or *N*-glycolyl sialic acid (Rogers et al. 1986; Suzuki et al. 1992). Unlike the NA of type A and B viruses, HEF does not catalyze the cleavage of the α-ketosidic linkage between terminal sialic acid and an adjacent sugar residue, but instead catalyzes the cleavage of the 9-*O*-acetyl group of 9-*O*-acetyl-*N*-acetylneuraminic acid (Herrler et al. 1985; Vlasak et al. 1987). The protein also possesses fusion activity, which requires a low pH optimum (between 5.0 and 5.7) (Herrler et al. 1988; Ohuchi et al. 1982). The acetylesterase activity may be required for entry of the C virus into target cells (Strobl and Vlasak 1993), but not for hemolysis (Herrler et al. 1992). The host cell-dependent variant selection also occurs in influenza C viruses with mutations in the HEF protein (Umetsu et al. 1992). HEF is also acylated, but with stearic acid instead of palmitic acid, as in type A and B HAs (Veit et al. 1990).

Reverse genetics

A major research advance occurred when Palese and colleagues established a system which allows one to generate influenza viruses containing genes derived from cloned cDNA (Enami et al. 1990; Luytjes et al. 1989). While this system allowed, for the first time, modification of the influenza viral genome, it was limited by its low efficiency and the requirement for selection systems (reviewed in Neumann and Kawaoka (1999, 2001, 2002) and Neumann et al. (2002)). In 1999, a system was established for the de novo generation of influenza A virus from plasmids (Neumann et al. 1999) (Figure 18). This system relies on the cellular enzyme RNA polymerase I for intracellular synthesis of influenza viral RNA. Artificial virus generation therefore requires fusion of cDNAs encoding all eight influenza viral genes to RNA polymerase I promoter and terminator sequences, and transfection of these transcription units into eukaryotic cells. Viral RNA synthesized by cellular RNA polymerase I is then replicated and transcribed by the viral polymerase and NP proteins that are provided from RNA polymerase II-driven protein expression plasmids. Although this approach requires cotransfection of cells with 12 different plasmids, it routinely yields 10^8 infectious viruses per ml of supernatant derived from transfected cells. Modifications include the use of ribozymes for the generation of vRNA 3′ ends (Fodor et al. 1999), or – through the combination of RNA polymerase I and II promoters on one plasmid – the synthesis of vRNA and mRNA from one template, thereby reducing the number of plasmids required for rescue to eight (Hoffmann et al. 2000). These approaches also allow the artificial generation of influenza B virus (Hoffmann et al. 2002; Jackson et al. 2002) and Thogotovirus

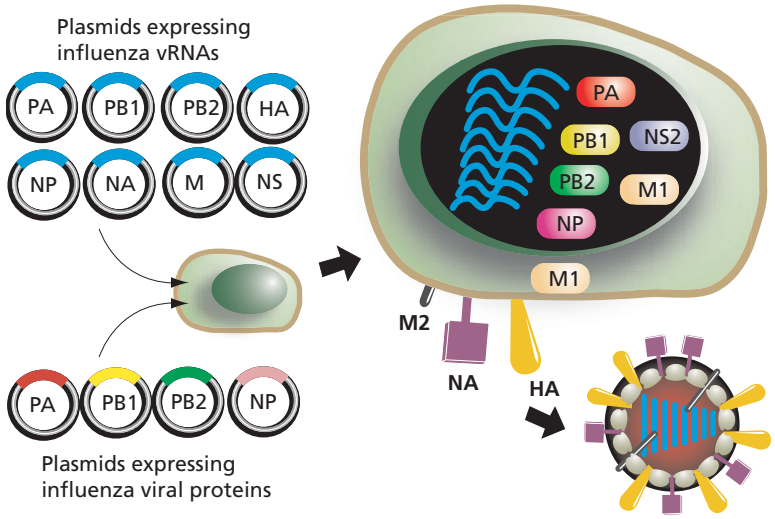

Figure 18 *Reverse genetics procedure. Influenza viral cDNAs are cloned in negative-sense orientation between RNA polymerase I promoter and terminator sequences. In plasmid-transfected cells, all eight influenza vRNAs are transcribed by the cellular enzyme RNA polymerase I. The three polymerase proteins and NP are provided from RNA polymerase II-driven protein expression plasmids. (Courtesy of Yuko Kawaoka.)*

(another member of the *Orthomyxoviridae* family) (Wagner et al. 2001).

The ability to modify the viral genome in any way desired has promoted research in the field tremendously. Current advances attributable to reverse genetics include the identification of factors determining viral pathogenicity (Hatta et al. 2001; Seo et al. 2002), the ongoing reconstruction of the 1918 pandemic virus (Basler et al. 2001; Reid et al. 1999, 2000, 2002; Tumpey et al. 2004), the re-assignment of functions encoded by the influenza B virus BM2 and NB proteins (Hatta and Kawaoka 2003; Hatta et al. 2004; Jackson et al. 2004), and the characterization of influenza viral proteins (Barman et al. 2003, 2004; Bourmakina and Garcia-Sastre 2003; Cross et al. 2001; Elleman and Barclay 2004; Hatta et al. 2001, 2002; Hui et al. 2003; Liu and Ye 2002; Neumann et al. 2000; Wagner et al. 2000; Yao et al. 2001). Moreover, reverse genetics is expected to have an enormous impact on public health by allowing the design and/or characterization of conventional (inactivated) or live attenuated vaccine viruses (de Wit et al. 2004; Hoffmann et al. 2002; Jin et al. 2003, 2004; M. Liu et al. 2003; Ozaki et al. 2004; Schickli et al. 2001; Subbarao et al. 2003; Watanabe et al. 2002; Webby et al. 2004), replication-incompetent virus-like particles (Neumann et al. 2000; Watanabe et al. 2002), or by exploring the potential of influenza virus as a vaccine vector (Shinya et al. 2004; Watanabe et al. 2003).

CLINICAL MANIFESTATIONS AND PATHOGENESIS

The spectrum of symptoms occurring during influenza virus infections is highly variable, ranging from mild respiratory disease with rhinitis or pharyngitis to primary viral pneumonia with a fatal outcome. Rates of asymptomatic infection may be nearly as high as those for symptomatic infection during some epidemics (Foy et al. 1976; Hayslett et al. 1962; Noble 1982). Often a physician's diagnosis is based on the patient's symptoms and the knowledge that influenza activity is occurring in the community. The presence of fever and cough along with sudden onset help distinguish influenza from other respiratory infections caused by other co-circulating respiratory pathogens; however, the physical signs and symptoms of influenza are not distinct enough from those of other respiratory infections to make a definitive clinical diagnosis without laboratory confirmation. In addition, symptoms in certain populations, notably babies, young children, and elderly people, may be atypical. However, during times when influenza is known to be circulating in a community, physicians participating in various studies have been able to diagnose influenza among adults with fever and cough with reported sensitivities and specificities of between 63 and 78 percent and 55 and 71 percent, respectively, compared with viral culture (Boivin et al. 2000; Monto et al. 2000).

Adults

Early symptoms in adults typically include fever, chills, headache, sore throat, dry cough, myalgias, anorexia, and malaise. A fever of 38–40°C that peaks within 24 h of onset is common, but peaks as high as 41°C can also occur. Pyrexia typically lasts 3 days but may last from 1 to 5 days or longer. Other symptoms that occur less frequently include substernal soreness, photophobia and other ocular symptoms, nausea, abdominal pain, and diarrhea (Douglas 1975; Nicholson 1992). Although most

symptoms typically resolve within a week, cough and malaise may persist for 1 or more weeks after fever has subsided. Type A influenza viruses of the H1N1, H2N2, and H3N2 subtypes as well as type B influenza viruses cause a similar spectrum of illness (Frank et al. 1985; Nicholson 1992; Spelman and McHardy 1985). However, in general, the frequency of severe infections requiring hospital admission or causing death is lower for influenza B than for influenza A(H3N2) viruses (Thompson et al. 2003, 2004). Elderly, debilitated people may have signs and symptoms that are not immediately recognized as influenza. Symptoms in uncomplicated disease in this group include lassitude, anorexia, cough, rhinitis, unexplained fever, general malaise, and confusion.

Infants and children

Influenza A and B viruses are significant causes of both upper and lower respiratory tract illness in children (Glezen 1980; Glezen et al. 1980a; Kim et al. 1979). In children, symptoms are similar to those in adults, but gastrointestinal symptoms such as vomiting, abdominal pain, and diarrhea are seen more frequently. Maximum temperatures also tend to be higher in children than in adults, and febrile convulsions can occur. In addition, myositis, croup, and otitis media occur more frequently in children. Influenza infection of neonates can be life-threatening and may be manifest only as an unexplained febrile illness (Meibalane et al. 1977). Hospitalization rates for infants and young children may increase during influenza outbreaks (Glezen et al. 1980b; Kim et al. 1979).

Complications

Complications of the upper respiratory tract after influenza infection include bacterial sinusitis and otitis media. Lower respiratory tract complications include exacerbation of chronic obstructive pulmonary disease and chronic congestive heart failure, croup, bronchitis, bronchiolitis, wheezing attacks in asthmatics, and pneumonia (primary viral pneumonia, secondary bacterial pneumonia or combined bacterial and viral pneumonia) (Betts 1995; Nicholson 1992).

Primary influenza pneumonia, which often develops abruptly and progresses rapidly, was described in detail during the 1957/8 Asian influenza pandemic (Hers et al. 1957; Louria et al. 1959). This type of pneumonia is uncommon, and occurs mainly among those at increased risk for complications of influenza. Rapid respiration rate, tachycardia, cyanosis, high fever, and hypotension are frequent symptoms. Diffuse pulmonary infiltrates and acute respiratory failure with a high mortality rate are also features of this disease. Combined viral and bacterial pneumonia is more common than primary viral pneumonia and may be clinically indistinguishable from it.

Secondary bacterial infections typically occur 5–10 days after initial onset of influenza symptoms and are responsible for most pneumonias during influenza epidemics. Productive cough, pleuritic chest pain, and chills are common symptoms of this type of pneumonia. *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Hemophilus influenzae* are the organisms most commonly involved (Schwarzmann et al. 1971). These illnesses respond to appropriate antimicrobial agents and have a lower case fatality rate than primary viral pneumonia.

Other reported but less frequent complications of influenza include myositis, myocarditis and pericarditis, acute renal failure, encephalopathy, encephalitis, transverse myelitis, Guillain–Barré syndrome and a range of other neurological complications, Reye's syndrome, and toxic shock syndrome (Betts 1995; Noble 1982).

Higher rates of spontaneous abortion, stillbirths, and premature births were reported among pregnant women during the major pandemics of 1918/19 and 1957/8 (Bland 1919; Hardy et al. 1961; Woolston and Conley 1918); increased maternal risk for death following influenza infection was also documented (Eickhoff et al. 1961; Greenberg et al. 1958). In addition, case reports (Irving et al. 2000; Schoenbaum and Weinstein 1979; Shahab and Glezen 1994) and a retrospective study during 17 interpandemic influenza seasons demonstrated an increased risk for influenza-related hospitalization for women in their second or third trimesters of pregnancy (CDC 2004).

Serious complications of influenza most often occur in people 65 years of age and older, in the very young, and in those of any age with underlying chronic cardiac, pulmonary, or metabolic disease (Barker and Mullooly 1980). Complications in elderly people, particularly among those with pulmonary, cardiovascular, or other chronic diseases, account for most of the mortality in influenza epidemics (Eickhoff et al. 1961). The highest rates of hospitalization and death following influenza infection occur in those at opposite ends of the age spectrum, i.e. less than two or 65 years and older. Recent studies have shown that otherwise healthy children aged 6–23 months of age are at a substantially increased risk for influenza-related hospitalization, compared to older healthy children and healthy adults (Izurieta et al. 2000; Neuzil et al. 2000). In general, hospital admissions are 10–20 times higher than the mortality rate in the elderly (Barker and Mullooly 1980; Glezen et al. 1987), but are an order of magnitude higher for children under 5 years of age (Thompson et al. 2004).

Pathogenesis

Much remains to be learned about the pathogenesis of influenza virus replication and its relationship to the clinical manifestations and complications of the infection.

Many studies to investigate the pathogenesis of influenza infections were conducted during the 1957/8 pandemic of Asian influenza (Hers and Mulder 1961; Walsh et al. 1961). These studies demonstrated that the principal site of replication is the columnar epithelial cells, but histological studies indicate that viral replication can occur throughout the respiratory tract. Infected ciliated columnar cells become vacuolated and lose their cilia, and infected mucosal and ciliated epithelial cells become necrotic and desquamate. In studies in which bronchoscopy was conducted on, or nasal and bronchial biopsy specimens were taken from, individuals with uncomplicated acute influenza infections, inflammation of the larynx, trachea, and bronchi, and desquamation of ciliated columnar epithelium into the lumen of the bronchus were observed. Regeneration of the respiratory epithelial cells takes ca. 3–4 weeks, during which time pulmonary function abnormalities may persist (Hall et al. 1976). In these typical cases of influenza in which infection is confined to the respiratory tract, prostration, fever, and myalgia often seem to be disproportionate to objective clinical signs or observed pathological changes.

Lungs from fatal cases of primary viral pneumonia most notably show hyaline membrane coverage of alveolar walls together with extensive intra-alveolar edema and hemorrhage. Tracheitis and bronchitis are also observed (Hers and Mulder 1961; Martin et al. 1959). Patients with secondary bacterial pneumonia have changes characteristic of bacterial pneumonia in addition to the tracheobronchial findings of influenza.

The occurrence of systemic illness and fever suggests dissemination of virus via the blood stream, but systematic studies (Kilbourne 1959; Minuse et al. 1962) and limited case reports (Lehmann and Gust 1971; Naficy 1963) suggest that viremia is detected only rarely.

Apoptosis

Influenza A and B viruses induce apoptosis in both permissive (MDCK) and non-permissive cells (Takizawa et al. 1993; Hinshaw et al. 1994), which can be blocked by the *bcl*-2 product (Hinshaw et al. 1994). Apoptosis induced by influenza virus infection seems to involve activation of the Fas antigen-encoding gene (Takizawa et al. 1995) as well as the PKR, and IFN type I secretion. The latter sensitizes cells to apoptosis signaling through FADD/caspase-8 activation (Balachandran et al. 2000). The role of apoptosis in the clinical signs and symptoms of influenza is controversial. Indirect evidence indicates that programmed cell death could help reduce viral load and pathology (inflammation and tissue damage), yet others have found that activation of caspase 3, a critical enzyme involved in apoptosis, enhances influenza replication (Wurzer et al. 2003). The PB1-F2 protein induces apoptosis in various cell types, including human monocytic cells, leading to the hypothesis that this protein functions to kill host immune cells responding to influenza virus infection (Chen et al. 2001). Influenza virus-infected cells undergoing apoptosis are readily engulfed by macrophages (Shiratsuchi et al. 2000). Infection of macrophages but not dendritic cells can trigger apoptosis (Bender 1998). It has also been reported that specific types of differentiated airway cells may undergo necrotic cell death, promoting inflammatory damage in the airway (Brydon et al. 2003).

MX PROTEIN

The pathogenesis of influenza virus infection is modulated by parameters specified by both the virus and the host. Although the available evidence suggests that numerous host genes have antiviral functions, most of them have not been identified and characterized. One exception is the Mx family of proteins, which mediate inhibition of orthomyxovirus replication (Staeheli 1990). This family of proteins is induced directly by viral infection as well as by type I interferons and may act either in the cytoplasm, as in the case of the human MxA protein, or in the nucleus, as in the case of the mouse Mx1 protein (Pavlovic et al. 1992; Noah et al. 2003). The Mx proteins belong to the dynamin superfamily of large GTPases (reviewed in Haller and Kochs 2002). The Mx GTPases seem to constitute an intracellular surveillance system that monitors the cell for viral infection by sensing viral nucleocapsids or viral ribonucleoprotein complexes. These viral components are thought to be derailed in their intracellular traffic and rendered inactive by sequestration. Specifically, MxA inhibits viral protein synthesis and genome amplification, suggesting that MxA protein interfered with either transcription, intracytoplasmic transport of viral mRNAs, viral protein synthesis, or translocation of newly synthesized viral proteins to the cell nucleus (Horisberger 1992; Turan et al. 2004; reviewed in Haller and Kochs 2002).

Immune response

INNATE IMMUNITY

During primary infection with influenza, the early host protective response involves the immediate action of cells such as phagocytes, natural killer (NK) cells, and B cells, as well as soluble factors including complement, IFN, cytokines, and chemokines. Cells and soluble factors converge to reduce the permissiveness of cells and tissues for the replication and spread of the invading virus (Kase et al. 1999; Kaufmann et al. 2001; Medzhitov and Janeway 2000; Siren et al. 2004). This early host response is triggered by receptors which recognize non-specific alarm signals of viral infection (Coccia et al. 2004; Ehrhardt et al. 2004; Lund et al. 2004; Yoneyama et al. 2004). These receptor molecules are expressed in the germline of the host. In contrast, the receptors and effectors of the adaptive immune system (antibodies and

T cell receptors) result from gene rearrangements and are pathogen-specific, leading to the establishment of immunological memory. Germline immune receptors such as the Toll-like receptors (TLR) are expressed in a variety of cells such as macrophages, dendritic cells, and intestinal epithelium. TLR-3 and TLR-7 are involved in the recognition of viral dsRNA and ssRNA respectively, promoting dendritic cell maturation and activation of IFN production (Lund et al. 2004). Viral dsRNA is also involved in the activation of proteins with antiviral activity such as the kinase PKR, which inhibits protein translation and may trigger apoptosis (Katze et al. 1988). The cellular response to infection has been studied using DNA microarray techniques that allow the analysis of the gene expression changes in cultured infected cells (Geiss et al. 2001, 2002). Using this approach, it was noted that influenza infection causes profound changes in the expression of a large number of cellular genes involved in interferon production and signaling, apoptosis, and oxidative stress. Influenza-infected patients trigger the activation of antiviral genes and production of cytokines, such as interleukin (IL)-6 and IFN-alpha which reach their highest concentration in nasal fluids within 48 h of infection, correlating with the onset of fever (Hayden et al. 1998). Other cytokines, such as TNF-alpha and IL-8 reach maximum levels 4–5 days after infection. IL-8 is temporally correlated with lower respiratory tract involvement. Influenza viruses have evolved mechanisms to counter the innate antiviral responses of the host. The viral NS1 protein antagonizes interferon production and the activation of PKR in influenza-infected cells (Garcia-Sastre et al. 1998; Lu et al. 1995; Wang et al. 2000).

Besides its importance in the early arrest of viral replication, the innate immune responses to influenza interface with and condition the acquired cellular immune response. IFN and certain cytokines induced by viral infection, such as IL-12, have a powerful influence on the nature of the adaptive immune response (Janeway and Medzhitov 2002). Evidence from a murine model of influenza shows that type I IFN plays a critical role coordinating the immune response to viral infection, by reducing inflammation (Durbin et al. 2000). The enhanced pathology that was observed in mice that lacked IFN production was not attributable to the absence of the antiviral activity of IFN but to the excess of pro-inflammatory mediators.

A new type of innate antiviral mechanism, termed RNA interference (RNAi), may also play a role in controlling influenza replication during the early stages of infection. This system involves the formation of a ribonucleoprotein complex that targets viral RNA for degradation in a sequence specific manner. Experimental studies in tissue culture and in laboratory animals showed that RNAi can cause a marked reduction of influenza virus replication. The influenza virus NS1 protein was found to antagonize RNAi activity, suggesting that this innate immune mechanism may be functional in vivo. It is not known if this antiviral mechanism is functional in human influenza infection (Ge et al. 2003, 2004; W.X. Li et al. 2004; Tompkins et al. 2004).

ADAPTIVE IMMUNITY

Antigen-nonspecific innate immune mechanisms generally inhibit, but do not eliminate, viral infection. They restrict viral replication until the more effective adaptive response is fully engaged. The adaptive immune response to infection with influenza viruses leads to induction of both virus-specific B- and T-cell immune responses to clear infection and generate long-lasting specific immunologic memory. Antibodies are made against the viral external glycoproteins HA and NA as well as the internal type-specific proteins NP and M1 (see section on Antigens). Neutralizing antibody directed against the HA is the primary immune mediator of protection from infection and clinical illness due to influenza viruses. Both mucosal and systemic immunity contribute to resistance to infection and disease (Clements et al. 1983, 1986). Influenza-specific lymphocytes have been detected in blood and lower respiratory tract secretions of infected subjects (Jurgensen et al. 1973; Yewdell and Hackett 1989). The primary cytotoxic T-cell response is detectable in blood after 1–2 weeks but is relatively short lived (Ennis et al. 1981). Evidence from animal models indicates that cytotoxic T-cell responses at mucosal surfaces play an important role in host protection (B. Liu et al. 2003a).

HUMORAL IMMUNE RESPONSE

Antibody to HA mediates neutralization of virus, complement fixation, prevention of virus attachment, and antibody-dependent cellular cytotoxicity. Resistance to infection is correlated with serum anti-HA antibody levels (Dowdle et al. 1973; Hobson et al. 1972) and can be demonstrated by protection against challenge after passive transfer of immune serum in a mouse model (Virelizier 1975). More recent studies have shown that protection from live virus challenge is associated with local neutralizing antibody and secretory IgA as well as serum anti-HA antibody (Clements et al. 1986; Johnson et al. 1986). Although antibodies to NA are inefficient in neutralizing influenza viruses, they restrict virus release from infected cells, reduce the intensity of infection and enhance recovery (see section on Antigens).

Serum antibody to HA can persist for decades and retrospective serosurveys suggest that a limited number of influenza subtypes have recycled (Masurel and Marine 1973). Decade-long persistence of immunity was demonstrated dramatically when influenza A (H1N1) strains, similar to viruses that had circulated in 1950, spread throughout the world during 1977/8. Little disease occurred in individuals born before 1950, indi-

cating that substantial immunity remained after almost 30 years. Disease occurred in people <20 years old, however, irrespective of whether they were infected by influenza viruses of the H3N2 subtype. This and numerous observations during the pandemics of 1957 and 1968 suggest that intersubtypic immunity is weak in humans. In contrast, intrasubtypic immunity to influenza A (H3N2) viruses in adults can last for 4–7 years and include two or more variants of the same subtype, depending on the extent of antigenic drift (Couch and Kasel 1983).

The fact that a repeat infection with an antigenically related strain boosts the antibody response to the first virus encountered was originally described in 1953 by Davenport and colleagues (1953). This phenomenon, known as 'original antigenic sin,' is believed to be a selective anamnestic response orientated toward both the HA and the NA antigens experienced during the original infection. Thus, cross-reactive epitopes will stimulate a predominant secondary antibody response whereas new epitopes in the reinfecting variant induce a primary response. The precise importance of this phenomenon to immunity induced by infection or vaccination is unknown, but observations on original antigenic sin suggest that induced immunity during sequential infections may be biased toward older, less relevant strains rather than the current infecting strain.

Sequence analysis of the HA genes of monoclonal antibody escape variants, along with field isolates and location of amino acid changes on the three-dimensional structure of the HA, have defined five antibody-combining sites. The relationship of these sites to antibodies produced via natural infection has yet to be determined precisely, but the specificity of the antibody response to HA seems to be limited in humans and may vary from individual to individual (Natali et al. 1981; Wang et al. 1986). A limited range of specificities of anti-HA antibodies in individual mice and rabbits have also been demonstrated (Lambkin et al. 1994; Lambkin and Dimmock 1995). These findings may have implications for antigenic drift and the epidemiological success of influenza viruses in humans.

Among children with no previous exposure to influenza viruses, vaccination with live-attenuated influenza viruses results in the appearance of serum anti-HA IgM, IgG, and IgA antibodies within 2 weeks. IgM and IgA antibodies decline after this time, but the IgG response peaks after ca. 6 weeks, declines over the next 6 months or so, and then remains relatively stable for 2–3 years (Murphy and Webster 1996; Murphy et al. 1982). Antiviral IgA, IgG, and IgM responses can be detected in nasal secretions in most individuals and persist for several months (Murphy and Clements 1989; Wright et al. 1983). A secondary response to infection in primed young adults results in serum IgG and IgA and mucosal IgA in most cases. Serum hemagglutination inhibition (HAI) responses decline initially after infection, but may then remain quite stable for years (Ada and Jones 1986). Serum antibodies to NA are rarely induced in primary infection and generally occur after re-exposure to NA of the same subtype. Antibodies to type-specific NP and M1 proteins are boosted via reinfection and may help diagnose a recent exposure to virus.

T-CELL RESPONSES

Antibodies and T cells play complementary roles in clearing the infection and promoting recovery. $CD4^+$ (Th1 and Th2) and $CD8^+$ T-cell responses to influenza are type-specific and are largely cross-reactive among influenza A viruses of different subtypes. T-cell recognition of antigen is restricted by the major histocompatibility complex (MHC) molecules, and the ability to respond to a given viral epitope depends on the HLA phenotype of an individual, a fact that complicates various T-cell vaccine approaches to vaccination against influenza. In naturally infected humans the $CD4^+$ cell response recognizes epitopes on the internal proteins NP and M1 as well as the surface proteins NA and HA.

$CD4^+$ T cells provide help for the activation of $CD8^+$ T cells by virtue of their ability to activate antigen presenting cells (dendritic cells, macrophages, etc.) primarily via CD40–CD40L interactions. Activated antigen presenting cells are essential for CD28 signaling to $CD8^+$ T cells leading to vigorous protective cytotoxic cell response to influenza infection (Prilliman et al. 2002; Schoenberger et al. 1998). The $CD4^+$ T cells are indispensable for an effective B cell antibody response to influenza. $CD4^+$ T cells can also enhance the proliferation of virus-specific $CD8^+$ T cells and maintaining $CD8^+$ T-cell memory capacity (Doherty et al. 1997).

The $CD8^+$ cytotoxic T lymphocyte response is directed to multiple surface and internal influenza proteins, in which many epitopes are potentially recognized, as determined by their binding to class I MHC molecules (Gianfrani et al. 2000; Gotch et al. 1987; Jameson et al. 1998; Robbins et al. 1997; Yewdell and Hackett 1989). However, a limited subset of immunodominant epitopes is recognized by lymphocytes primed by influenza infection (Belz et al. 2000; Yewdell and Bennink 1999).

Cytolytic $CD8^+$ effector cells fall into two subpopulations based on cytokine secretion. Both subpopulations lyse targets by production of perforin or Fas. Type 1 $CD8^+$ T cells (Tc1) secrete IFN-γ, whereas type 2 $CD8^+$ T cells (Tc2) secrete IL-4 and IL-5. Influenza virus infections elicit both Tc1 and Tc2 cells specific for class I-restricted epitopes. Tc1 cells are thought to contribute to the protective antiviral response, whereas the Tc2 component may act to limit the lung-associated pathology associated with influenza virus infection. Recent animal model studies have shown that both $CD4^+$ and $CD8^+$ T cells can contribute to immunity to influenza viruses. Animals that are deficient in both

$CD4^+$ and $CD8^+$ T cells do not survive influenza infection, but animals deficient in only $CD4^+$ or $CD8^+$ T cells are able to clear virus (Eichelberger et al. 1991a, b; Flynn et al. 1998; Lightman et al. 1987). The increased severity of influenza infections in geriatric patients has been correlated with a combination of functional impairments in their T-cell responses to influenza (Deng et al. 2004; Katz et al. 2004; Po et al. 2002).

The emergence of viral variants with altered surface antigens is a hallmark of influenza biology. Selection of antigenic variants is not limited to mutations in the HA and NA which allow escape from antibody neutralization. Evidence from influenza infections in humans and experimental animals shows that T-cell responses can select escape viruses with amino acid changes in the T-cell epitopes of the viral proteins (Boon et al. 2002; Price et al. 2000).

ANTIGENS

Polymerase proteins

There is no detectable antibody response to the polymerase proteins in convalescent sera. Their extremely low abundance in infected cells may contribute to this observation. These proteins have not been examined extensively for antigenic variants, although monoclonal antibodies to PA and PB2 are now available (Barcena et al. 1994). Cytotoxic T lymphocytes can recognize each of the three polymerase proteins (Bastin et al. 1987b; Bennink et al. 1984, 1987; Reay et al. 1989). Some viral strains produce an additional polypeptide, termed PB1-F2, encoded by the *PB1* gene segment (W. Chen et al. 2001). The PB1-F2 protein is translated from a downstream AUG initiation codon in an alternative reading frame. PB1-F2 is a mitochondria-targeted pro-apoptotic protein that has been associated with some emerging pandemic strains. This polypeptide induces CD8 T-cell responses in mice.

HA

The HA antigen is the major inducer of protective immunity to influenza viruses. Influenza A virus HAs show subtype specificity and are thought to possess five antigenic sites in the three-dimensional structure (Wiley et al. 1981; Wiley and Skehel 1987), although some subdivision and overlap of these areas have been noted (Caton et al. 1982; Daniels et al. 1983). For H3 virus strains (Wiley et al. 1981; Wiley and Skehel 1987), the sites are A, B, C, D, and E, and for the H1 strains they are Ca1, Ca2, Cb, Sa, and Sb (Caton et al. 1982). Each of these sites was derived from amino acid sequence changes in antigenic variants selected with monoclonal antibodies, as well as in natural variants. Monoclonal antibodies to each of the five sites neutralize the infectivity of the virus (Wiley and Skehel 1987). Mutations that result in gain or loss of N-linked glycosylation sites in the HA can result in major changes in the antigenic structure of the virus (Skehel et al. 1984).

Helper T lymphocytes recognize several distinct regions of the HA1 and HA2 subunits located either on the surface or the interior of the intact molecule, as well as conformational determinants on the HA (Yewdell and Hackett 1989). Helper T lymphocyte recognition includes specificity for determinants on the variable regions of the molecule to which antibodies are also directed (Hackett et al. 1983; Mills et al. 1988). The immunodominant peptide most readily recognized by both subtype-specific and cross-reactive helper T lymphocyte clones is HA1 306–328 (H3 numbering system). Most memory T-cell clones recognize sites B, C, and E (HA1 56–76; H3 numbering) (Barnett et al. 1989; Graham et al. 1989). Residues important in antibody recognition affect T-cell recognition (Barnett et al. 1989; Graham et al. 1989), indicating that both B and T cells may recognize similar sites on the HA and hence that both systems may provide selective immune pressure. Cytotoxic T cells recognize both the HA1 and HA2 subunits (Wabuke-Bunoti and Fan 1983; Wabuke-Bunoti et al. 1984).

NP

Although a type-specific protein, the NP of influenza A viruses shows antigenic variation (Herlocher et al. 1992). It contains at least three non-overlapping antibody-binding areas (Van Wyke et al. 1980), one of which is highly conserved. Monoclonal antibody binding to this site inhibits transcription of viral RNA in vitro (Van Wyke et al. 1981). The NP epitopes recognized by dominant helper T lymphocytes in humans are located in residues 206–229, whereas those of mice are found in residues 260–283, with the distinct specificity of these regions depending on haplotypes. As a major antigen recognized by cytotoxic T lymphocytes, the NP possesses several T cell-specific epitopes that are conserved among human influenza A viruses (Bastin et al. 1987a; Bodmer et al. 1988; Yewdell and Hackett 1989). Transfer of cytotoxic T lymphocytes specific to NP protects mice from lethal influenza challenge (Taylor and Askonas 1986). The protective role of HLA-restricted cytotoxic T lymphocytes that recognize NP epitopes is evidenced by the identification of virus variants with altered NP epitopes which emerged in sequential chronological order with the cognate CTL; indicative of escape from CTL immunity (Boon et al. 2002).

NA

The subtype-specific influenza A virus NA has four antigenic sites, each consisting of multiple epitopes

(Webster et al. 1984). These antigenic sites differ in that antibodies to some, but not other, sites inhibit enzyme activity. Amino acids that change during antigenic drift cluster mainly in the distal surface loops connecting the various strands of β-sheets (Air et al. 1985).

Antigenic structures of the N9 NA have been extensively studied. Analysis of the crystal structure of a complex of N9 NA from A/tern/Australia/G75 with the Fab fragment of monoclonal antibody NC41 (Colman et al. 1987; Tulip et al. 1992) shows that the antibody contacts the NA over a surface area of 900 $Å^2$, comprising 19 amino acid residues localized on five polypeptide loops surrounding the enzyme active site. Site-specific mutagenesis indicates that, of the 19 amino acids in this epitope, only a few provide the critical contacts required for antibody recognition (Nuss et al. 1993). These findings support the observation of limited sites of sequence changes and local changes to the structure in escape mutants (Tulip et al. 1991; Varghese et al. 1988; Webster et al. 1987), implying (1) that antibody escape mutants are selected only when they contain changes at critical sites, or changes that introduce bulky side-chains capable of sterically preventing antibody attachment, and (2) that the other contact residues are providing 'passive surface complementarity' (Nuss et al. 1993). Antibodies to NA are inefficient in neutralizing the virus, but reduce plaque sizes in cell culture (Jahiel and Kilbourne 1966; Kilbourne et al. 1968; Rott et al. 1974). In agreement with these findings, immunity to NA in natural infection has only a small role in influenza protection as exemplified by the 1968 pandemic, whose causative agent possessed the NA from a previously circulating human virus; the immunity to the NA that existed in the human population did not protect humans from pandemic influenza (Cockburn et al. 1969). However, in experimental infections in animal models, passive transfer of monoclonal antibodies to NA or immunization with the vaccinia virus expressing the NA protected animals against lethal challenge (Webster et al. 1988) or reduced virus titers (Rott et al. 1974; Schulman et al. 1968). Helper and cytotoxic T lymphocyte responses to NA were found in influenza virus-infected mice, but were limited compared with responses to the HA, NP, and M1 (Caton and Gerhard 1992; Hurwitz et al. 1985; Reay et al. 1989; Wysocka and Hackett 1990).

M1

Even though this protein is considered a highly conserved type-specific antigen, some variations have been found among influenza A strains studied with a panel of monoclonal antibodies (Herlocher et al. 1992; Van Wyke et al. 1984). Helper T lymphocytes specific for the M1 protein are cross-reactive between viruses of different subtypes (Hurwitz et al. 1985). M1 is also recognized by cytotoxic T lymphocytes (Gotch et al. 1987). A CTL epitope in the M1 protein that is recognized by human CD8 T cells was shown to mediate protection in transgenic mice expressing the cognate human HLA molecule (Plotnicky et al. 2003).

M2

Antibodies to the M2 protein have been found in humans infected with influenza A virus (Black et al. 1993). Passively transferred monoclonal antibodies reduced virus replication in mice (Treanor et al. 1990), and it has recently been shown that M2 protein plays a role in recovery from infection in mice (Slepushkin et al. 1995). Some antigenic variation can be found among the M2s as a result of amino acid changes in the ectodomain (Zebedee and Lamb 1988). However, antibodies elicited by M2 have been shown to mediate protection against different virus subtypes in animal models (Neirynck et al. 1999). Cytotoxic T lymphocytes specific for M2 have been isolated from convalescent mice and humans (Gianfrani et al. 2000; Jameson et al. 1998).

NS1

The influenza A virus NS1 proteins from different animal species cross-react with polyclonal antibodies. Although a panel of monoclonal antibodies distinguishes avian virus NS1 from those of human, swine, and equine strains, there is no evidence of antigenic variations among the latter group of proteins (Brown et al. 1983). Cytotoxic T lymphocytes recognizing NS1 are highly cross-reactive (Bennink and Yewdell 1988).

NS2

The antigenic properties of NS2 are poorly understood; however, mouse cytotoxic T lymphocytes specific for this protein have been found (Yewdell and Hackett 1989).

Host range restriction

Multiple genetic factors affect host range restriction for influenza viruses. Changes in host range can parallel changes in pathogenicity. At present, more is known about how particular amino acid changes at positions in or near the receptor-binding pocket of the hemagglutinin confer different receptor specificities and thus different host ranges. These receptor-binding specificities correspond to the composition of the receptor at the replication site in the host (see section on Receptor binding).

There is also indirect evidence that other genes, particularly the *NP* and *NA* genes, play a role in host range restriction. For example, replacement of the *NP* gene of an avian virus with the *NP* gene of a human virus alters

host range (Scholtissek et al. 1978a), and phylogenetic analyses of *NP* gene sequences have shown that there are five distinct host-specific lineages (Gammelin et al. 1990; Gorman et al. 1990a). Other internal genes of influenza A viruses also apparently play a role in host range and virulence (Klenk and Rott 1988). The PB2 subunit of the polymerase complex plays a role in the restriction of avian influenza viruses for certain mammalian species (Subbarao et al. 1993). Recent studies revealed that amino acid 627 of PB2 modulates the replication efficiency of avian influenza viruses in mice (Hatta et al. 2001).

EPIDEMIOLOGY

Although human type A and B influenza viruses were not isolated until 1933 and 1940, respectively, descriptions of epidemics and pandemics of respiratory disease with characteristics suggestive of influenza have been recorded for over four centuries (see section on Historical record). Certain well documented epidemiological features of modern epidemics of influenza also emerge from the early accounts. Epidemics of varying severity occurred at irregular intervals, caused the highest mortality in elderly people, and were thought to have spread from Asia. These records detail epidemics of cough, fever, chills, and muscle aches that affected many people of different ages within a short time.

An epidemic of influenza is an outbreak of disease in a circumscribed location, which may affect a town, a city, or an entire country. Localized epidemics within a community often have a characteristic pattern in which the epidemic begins abruptly, peaks within 2–3 weeks and has a total duration of 5–8 weeks (Glezen and Couch 1978). The spread of influenza virus through a community typically causes large increases in medical visits for febrile respiratory disease (Glezen 1982). Absence from school due to influenza often occurs early in the epidemic, and school children are believed to be important in disseminating the virus in the community. Although reports of increased numbers of children with febrile respiratory illness are often the first indication that influenza is circulating in a community, it is not uncommon for a laboratory-confirmed outbreak in a nursing home to be the first report that influenza is present. Absence from work, hospitalization for pneumonia, and other complications of influenza, and death due to pneumonia and influenza and its complications all tend to peak later during an epidemic.

The size of outbreaks and epidemics that occur during the interpandemic intervals is quite variable, but are almost always smaller than those that occur following the introduction of a new virus subtype. The size of epidemics and their impact reflect the interplay between the extent of antigenic variation of the virus, the extent of immunity in the population and the population groups that are most affected in a given year.

A pandemic of influenza is an epidemic of disease that involves most if not all age groups on several continents. Influenza experts agree that at least three true pandemics of influenza have occurred during the past century: Spanish influenza in 1918, Asian influenza A(H2N2) in 1957 and Hong Kong influenza A(H3N2) in 1968. Human infections with highly pathogenic avian influenza A(H5N1) viruses have raised the specter of the emergence of another influenza pandemic due to unprecedented human exposure to these viruses causing epizootics in several Asian countries in 2004.

Current epidemiology

The simultaneous circulation of two subtypes of influenza A together with influenza B viruses since 1977 has made the current epidemiology of influenza unusually complex. Influenza B viruses have circulated continuously in humans since their first isolation in 1940, whereas influenza A(H3N2) viruses have been present since their emergence in pandemic form in 1968. Unlike the situation in 1957 and again in 1968 when new pandemic strains totally replaced the previously circulating influenza A strains, the influenza A(H1N1) viruses that emerged in 1977 were unable to supplant viruses of the A(H3N2) subtype. Instead, two distinct subtypes of influenza A viruses have co-circulated worldwide since then (see Figure 1).

Prevalence of these three groups of viruses may vary temporally and geographically within a country (Chapman et al. 1993) and between countries and continents during a given influenza season (World Health Organization 1996). In the USA the circulation of influenza A(H3N2) viruses has, in recent years, been associated most often with excess pneumonia and influenza (P and I) mortality. Major peaks of excess P and I mortality were documented by the US 121 Cities Mortality Reporting System during the 1989/90 and 1993/4 influenza seasons, during which influenza A(H3N2) viruses accounted for over 98 percent of the viruses reported.

Transmission and seasonality

It is generally accepted that influenza viruses are spread primarily by aerosols of virus-laden respiratory secretions that are expelled into the air during coughing, sneezing, or talking by an infected person (Betts 1995; Moser et al. 1979). Spread by direct contact is, however, also possible. The incubation period for influenza is relatively short (1–4 days), and the explosive nature of influenza epidemics and pandemics and simultaneous onset in many people suggest that a single infected person can transmit the virus to a relatively large number of susceptible individuals.

It is also generally accepted that influenza viruses are maintained in humans only by direct person-to-person spread, as there is no firm evidence for reintroduction from latently or persistently infected individuals. This is supported by evidence from local and global surveillance of influenza viruses, which has shown that antigenic variation and the consequent epidemiological behavior of influenza viruses follow a relatively uniform pattern, each successive antigenic variant replacing the previously circulating one in such a way that the co-circulation of distinct antigenic variants of a given subtype occurs for relatively short periods (Stuart-Harris et al. 1985; Cox and Bender 1995; Smith et al. 2004). These data have shown that consecutive epidemics in a community are caused by the reintroduction of antigenically drifted, genetically distinct influenza viruses. Intensive community-wide surveillance in large population centers has shown that influenza activity can often be detected during the summer months (Fox et al. 1982; Monto and Kioumehr 1975). Global influenza surveillance also indicates that influenza viruses are generally isolated every month from humans somewhere in the world (Noble 1982; World Health Organization 1996).

The seasonality of influenza activity has been well documented in many countries with temperate climates in Europe, North America, and Asia. In these countries, influenza epidemics generally occur from December to March. Evidence that survival of influenza viruses in an aerosol is favored by low relative humidity and low temperature (Hemmes et al. 1960; Schaffer et al. 1976) is often cited as an explanation for winter seasonality of influenza in temperate climates. Seasonality in tropical or subtropical climates is less well studied; however, influenza viruses may be isolated throughout the year, with peaks of activity often occurring in the summer months (Reichelderfer et al. 1989). Other reports have suggested that peak influenza activity in tropical and subtropical areas may follow changes in weather such as the onset of the rainy season (Rao et al. 1982). The environmental and epidemiological factors responsible for these observations remain to be elucidated.

Antigenic drift and its impact

The epidemiological success of influenza viruses is largely due to the two types of antigenic variation that occur in the HA and the NA. Antigenic variation renders an individual susceptible to new strains – despite previous experience with other influenza viruses. The first kind of variation, called antigenic drift, occurs with both influenza A and B viruses, and is the gradual alteration of the structure of the protein by single amino acid substitutions (mutation) in the HA and NA within type B influenza viruses or within a given subtype of influenza A viruses. This kind of variation is the result of positive selection of spontaneous mutants by neutralizing antibodies (Bush et al. 1999; Plotkin and Dushoff 2003). The amino acid changes result in the inability of antibody to previous strains to neutralize the mutant virus. Variations in the amino acid sequences of the HA and NA occur at a rate of <1 percent per year during antigenic drift.

Antigenic drift variants are responsible for periodic epidemics that occur between pandemics. Thus, influenza among humans is caused by distinguishable antigenic variant strains of influenza A and B viruses that emerge and become predominant over a period of 2–5 years, only to be replaced by the subsequently successful antigenic variant.

Phylogenetic analyses have revealed that *HA* genes of human influenza A and B viruses have a regular relationship between the isolation date and their position on the evolutionary tree. When evolutionary rates are estimated by regression of the year of isolation against the number of nucleotide or amino acid substitutions from a common ancestor (A/Puerto Rico/8/34, A/USSR/90/77, A/Aichi/2/68, and B/Lee/40 for the old A(H1N1), contemporary A(H1N1), A(H3N2), and B viruses, respectively), it is apparent that the *HA* genes of human influenza A viruses of the H1N1 and H3N2 subtypes evolve at about the same rates, whereas *HA* genes of influenza B viruses evolve more slowly (Table 3). Recent phylogenetic analyses have also shown that, although co-circulating lineages of *HA* genes of influenza B viruses can co-exist longer than for influenza A *HA* genes, the overall patterns and the rates of evolution of the *HA* genes of these two types of influenza viruses are more similar than was previously believed (Cox and Bender 1995).

When amino acid changes in the HAs of field strains of influenza A(H3N2) viruses are plotted on the three-dimensional structure of the HA, it is apparent that much of the surface of the molecule has been altered by amino acid substitutions during the circulation of these viruses since 1968 (Figure 19). At least four changes that can be assigned to at least two antigenic sites have been observed between major epidemic strains of human influenza A viruses (Wilson and Cox 1990). Thus, the HA molecule seems to have a vast potential for antigenic change within a subtype as well as between subtypes without jeopardizing its function. This feature along with the apparent extensive nature of the antigenic regions in the HA molecule (Caton et al. 1982; Wilson and Cox 1990) make it extremely difficult to predict future antigenic variants of influenza before they arise.

MORBIDITY

Quantifying morbidity due to influenza is difficult. The spectrum of symptoms occurring during influenza virus infections is quite variable. In addition, many other respiratory pathogens cause influenza-like illness, so

Table 3 *Evolutionary rates for the HA1 portion of the* HA *gene and protein*

Virus	Period	Number	Nucleotide changes per nucleotide site per year	Amino acid changes per amino acid site per year
H1N1	1934–1957	24	3.9×10^{-3}	5.3×10^{-3}
H1N1	1977–1996	146	2.7×10^{-3}	4.2×10^{-3}
H3N2	1968–1996	340	3.7×10^{-3}	4.1×10^{-3}
B	1940–1996	168	1.2×10^{-3}	1.1×10^{-3}

parallel laboratory studies are essential to define influenza-related morbidity. Many methods have been used for estimating the impact of influenza illnesses in a community or country, including prospective surveillance of illnesses and influenza infections in all members of a defined population. Regular interviews of a randomly selected population to detect all symptomatic respiratory illnesses, coupled with laboratory studies to detect symptomatic and asymptomatic influenza infections, provide the most precise measurement of age-specific influenza morbidity and rates of asymptomatic infection. A number of these community- and family-based studies have been carried out during pandemic and interpandemic influenza outbreaks (Foy et al. 1976; Hall et al. 1971, 1973; Jennings and Miles 1978; Jordan et al. 1958; Monto and Ullman 1974; Philip and Bell 1961). These studies have shown that influenza epidemics disrupt school attendance and work productivity, place heavy demands on health care systems, and result in morbidity and mortality among high-risk populations almost every year.

During average epidemics overall infection rates are estimated to be 10–20 percent, but in selected populations or age groups, attack rates of 40–50 percent are not unusual. It is apparent from studies conducted during both pandemic years and interpandemic periods that age-specific attack rates are highest in school children (Glezen 1996). This correlates well with the observation that school absenteeism is followed by employee absenteeism during influenza epidemics. Elderly people are often admitted to hospital during the latter part of the epidemic. Both type A and B influenza viruses are important causes of respiratory infection within nursing homes and other long-term care facilities.

MORTALITY

One of the hallmarks of influenza is the increase in mortality during pandemics and many epidemics. William Farr (1885) is credited with developing the concept of 'excess mortality', i.e. the number of deaths observed during an epidemic of influenza-like illness in excess of the number expected. Estimating influenza-associated excess mortality provides one of the most objective measures of the severity of influenza epidemics and pandemics. Methods for determining the baseline or expected number of deaths for the winter season in the absence of an influenza epidemic have changed over the years. Various statistical approaches, ranging from a moving average to cyclical regression models and autoregressive integrated moving average (ARIMA) time series analyses have been used to calculate the baseline and threshold values (Choi and Thacker 1982; Collins and Lehmann 1951; Lui and Kendal 1987; Simonsen et al. 1997; Thompson et al. 2003).

Increased influenza mortality results not only from pneumonia and influenza (P and I) but also from cardiopulmonary and other chronic diseases that can be exacerbated by influenza (Collins and Lehmann 1951; Eickhoff et al. 1961). Quantification of deaths caused by influenza is complicated by the fact that information recorded on death certificates may fail to indicate that influenza was a cause of death because a laboratory diagnosis was not made or because influenza was not listed as a primary underlying or contributory cause of death. Therefore, calculations of influenza-associated excess mortality are based both on excess deaths from P and I as well as from all causes. Although excess mortality occurs primarily in elderly people, it can occur in all age groups, particularly among individuals who are at increased risk for complications of influenza. Excess mortality has been documented during circulation of both type A and B influenza viruses, but has been associated most often with circulation of influenza A subtypes H2N2 and H3N2. Since 1986, excess mortality in the USA has been associated most frequently with the circulation of influenza A(H3N2) viruses; ca. 90 percent of these influenza-associated excess deaths have occurred in individuals 65 or more years of age (CDC 2004).

It is estimated that >20 000 influenza-associated excess deaths occurred in the USA during each of nine different epidemics between 1972 and 1991; >40 000 influenza-associated deaths occurred during each of three of these epidemics (Simonsen et al. 1997) (Table 4).

ANTIGENIC SHIFT AND ITS IMPACT

Only influenza A viruses exhibit the second, more dramatic, kind of antigenic variation, called antigenic shift. Antigenic shift is the appearance in the human population of a new subtype of influenza A viruses containing a novel HA or a novel HA and NA immunologically distinct from isolates circulating previously. When antigenic shift occurs, the HA of the new strain

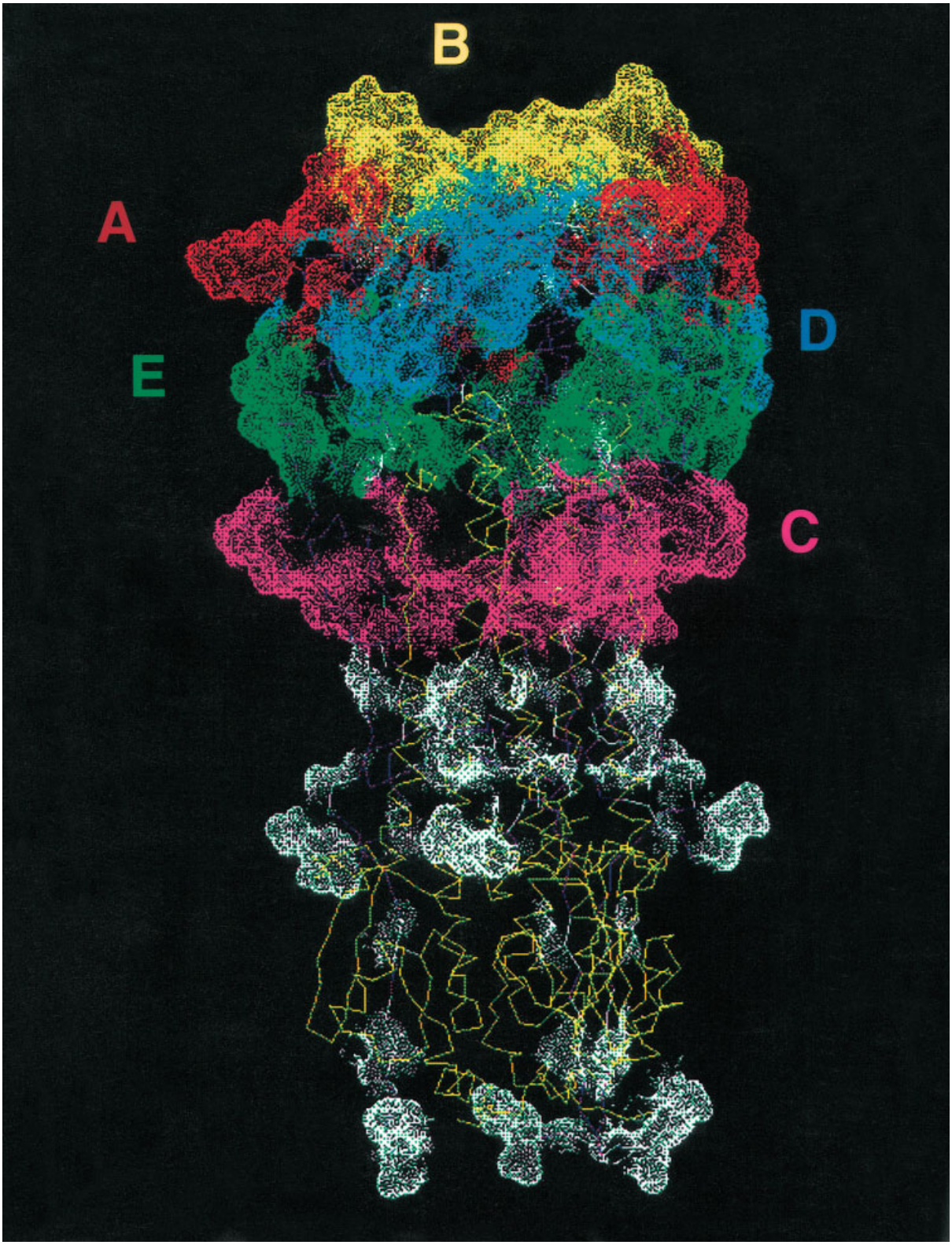

Figure 19 *Natural antigenic variation in the hemagglutinin (HA) of the H3 subtype of influenza A viruses circulating between 1968 and 1996. The HA trimer is depicted with the HA polypeptide backbone represented by an α-carbon trace in violet for the HA1 domain and in yellow for the HA2 domain. The solvent-accessible surface of residues that have changed during this period are represented by a dot surface. Antigenic regions are color coded and designated A (red), B (yellow), C (magenta), D (blue), and E (green). Amino acid residues shown in white are surface accessible but not assigned to antibody-combining sites. This graphic representation demonstrates that most of the surface-accessible amino acids in the globular head region of the HA have changed during this 28-year period of circulation in humans of influenza A(H3N2) viruses.*

Table 4 *Influenza excess mortality*

Period	Excess deaths
'Spanish' influenza	
September 1918 to April 1919	500 000
'Asian' influenza	
September 1957 to March 1969	69 800
'Hong Kong' influenza	
September 1968 to March 1969	33 800
Total	603 600
Interpandemic years	
1957–1990	600 800

would be expected to vary at the amino acid level 20–50 percent from the corresponding protein of previously circulating strains. Antigenic shift is responsible for worldwide pandemics, which occur at irregular and unpredictable intervals.

Although 16 subtypes of influenza HA have been identified in avian species, epidemics and pandemics of influenza among humans during this century seem to have been caused by viruses with HAs of only three different subtypes: H1, H2, and H3 (see Figure 1). The most severe recorded pandemic of influenza was that of Spanish influenza in 1918–1919.

Retrospective serological and genetic evidence suggests that this pandemic was caused by viruses closely related to classic swine influenza A(H1N1) virus, but its origin is unknown (Reid et al. 2004). Outbreaks of disease occurred almost simultaneously in North America, Europe, and Africa during the spring and early summer of 1918 (Crosby 1989). During the autumn of 1918 a second, more serious, wave of disease occurred. This wave peaked by the end of October but was followed by yet another wave of disease in mid-winter. Illness rates of nearly 40 percent occurred among school children during the autumn wave in the USA (Frost 1920). The differentiating features of this pandemic are the large numbers of cases with pneumonia and the unusually high case fatality rates, particularly in young healthy adults between the ages of 20 and 40 (Frost 1919). The social and medical consequences of the Spanish influenza pandemic are difficult to imagine today. About half of the 2 billion people living at that time were infected and at least 20 million of them died (Noble 1982). Hospitals were overflowing and there was a general shortage of medical services and vast disruption of community life.

Records indicate that the 1957/8 pandemic of Asian influenza A(H2N2) began in February in the southern Chinese province of Guizhou, spreading during March to Yunan province and during April to Singapore and Hong Kong (Stuart-Harris et al. 1985). The first virus isolates were obtained in Japan during May. This pandemic strain possessed completely different HA and NA antigens from the formerly circulating H1N1 viruses and spread rapidly worldwide by November 1957. Although H2N2 viruses were first isolated in the UK and the USA in June or July of 1957, peak incidence of the disease did not occur until October. This first wave of disease in both countries was followed by a second in January 1958; both waves were accompanied by excess mortality, primarily in the elderly. The timetable of key events for the pandemic of Asian influenza shown in Table 5 illustrates the short time frame for response to this pandemic and the missed opportunities for immunization after the first wave of disease. The highest attack rates during this pandemic were >50 percent in children aged 5–19 (Glezen 1996). Total influenza-associated excess mortality in the USA during this pandemic was estimated to be 69 800 (Noble 1982).

Viruses causing the Hong Kong H3N2 pandemic were first isolated in Hong Kong in July 1968. These viruses had a different HA antigen but shared the N2 NA antigen with the previously circulating Asian viruses. Widespread disease with increased excess mortality was observed in the USA during the winter of 1968/9, but in many other countries, including the UK, this did not happen until the winter of 1969/70 (Stuart-Harris et al. 1985). Total influenza-associated excess mortality in the USA for this pandemic was estimated to be 33 800 (Noble 1982).

In May 1977 the first outbreaks of disease caused by re-emerging influenza A(H1N1) viruses were observed in Tientsin, China (Kung et al. 1978). The H1N1 virus spread to other parts of Asia and reached Russia by November. Spread to Europe, North America, and the southern hemisphere followed. Illness occurred almost exclusively among people less than 20 years of age and the highest attack rates of >50 percent were among school children. These viruses were antigenically and genetically similar to H1N1 viruses that had circulated widely during the early 1950s (Kendal et al. 1978; Nakajima et al. 1978). The absence of morbidity in people over 20 years of age has been explained by the fact that they were infected with similar viruses during the 1950s. Some experts have suggested that these H1N1 viruses may have been 'genetically frozen' in nature, while others have speculated that these viruses may have accidentally escaped from a laboratory; the source remains unknown.

Although antigenic shift seems to have been a requirement for the appearance of true pandemic influenza, the emergence of influenza A viruses of a new subtype in humans does not necessarily result in a pandemic (Dowdle and Millar 1978).

Ecology of influenza

The term influenza was used to describe a variety of acute respiratory and other diseases of a number of animal species, especially horses (see section on Histor-

Table 5 *Timetable of events for the Asian influenza pandemic of 1957/58*

Date	Event
February 1957	Outbreaks of influenzalike illness in Guizhou Province, China
Early March 1957	Outbreaks of influenzalike illness in Yunan Province, China
Mid March 1957	Outbreaks widespread in China
April 1957	Outbreaks in Hong Kong, Singapore, and Taiwan
Mid May 1957	Virus isolated in Japan
June/July 1957	Virus first isolated in the USA. Outbreaks reported
September 1957	Widespread occurrence of influenza begun in the USA
October 1957	Peak incidence of disease occurred in the USA. Attack rates were highest among school children and young adults
November 1957	Incidence of disease declined in the USA. First peak of pneumonia and influenza-related deaths observed
Early December 1957	Cumulative total of 60 million doses of vaccine released in the USA; much of the vaccine was unused
January/February 1958	Second peak of pneumonia and influenza-related mortality observed with a higher proportion than usual of deaths in the elderly. It was recognized retrospectively that a second wave of disease occurred in older adults and the elderly

ical record), for many years. However, influenza A viruses have been isolated only from humans, swine, horses, cats, dogs, a variety of sea mammals, and a wide variety of both domestic and wild birds, including ducks, geese, terns, shearwaters, gulls, turkeys, chickens, quail, and pheasants (Easterday 1975; Hinshaw et al. 1980; Webster et al. 1992; Dubovi et al. 2004; Keawcharoen et al. 2004).

Studies in Asia, Australia, Europe, and North America have revealed that viruses with all 16 subtypes of HA and nine subtypes of NA have been isolated from ducks and other feral water birds (Easterday 1975; Rohm et al. 1996; Webster et al. 1992) (Table 6), an observation suggesting that avian species serve as the primary reservoir for the emergence of new pandemic strains (Webster et al. 1992). These studies have also suggested that aquatic birds are likely to be the only source for influenza viruses in other species. Infections in avian species by most influenza viruses are asymptomatic, virus replicating preferentially in cells lining the intestinal tract (Webster et al. 1978). High titers of these viruses are excreted into their water habitat by asymptomatic birds. Avirulent influenza infections in birds may be the result of influenza viruses adapting to the host over many centuries. However, a few members of the H5 and H7 subtypes can cause a lethal systemic infection owing to the susceptibility of the HAs of these viruses to cleavage in many host tissues. The large reservoir for the 16 subtypes of influenza A viruses ensures their perpetuation in nature. The avian origin of strains of influenza in humans, cats, seals, whales, pigs, horses, and domestic poultry has been documented (Webster et al. 1992; Webster and Kawaoka 1994; Li et al. 2004).

Phylogenetic studies have revealed clues that have changed our thinking about the ecology and origins of influenza A viruses of different subtypes. There are species-specific lineages of viral genes (Bean et al. 1992; Gorman et al., 1990a, b; Ito et al. 1991; Kawaoka et al. 1989), and it appears that all mammalian influenza viruses originate from the avian reservoir (Shortridge 1992; Webster and Kawaoka 1994). Besides the phylogenetic evidence supporting an avian ancestry of mammalian viruses, there is evidence from antigenic and structural studies in support of this hypothesis. Antigenic analyses of the earliest human isolate available (A/PR/8/34) suggested a direct descent from the avian-like H1N1 pandemic strain of 1918 (Brownlee and Fodor 2001). The atomic structure of the HA from the 1918 pandemic virus, A/South Carolina/1/18, has clearly revealed that the sialic acid binding pocket is very similar to that of avian influenza HA, suggesting an avian origin of this gene (Stevens et al. 2004).

Poultry, especially chickens, quail, and geese, are playing an increasingly significant role in the ecology of influenza. They are susceptible to infection with a variety of influenza subtypes from migratory waterfowl. Viral circulation in flocks, initially without causing substantial pathology, leads to the adaptation to the new species. The process of adaptation of influenza strains of the H5 and H7 subtypes to terrestrial poultry often results in acquisition of mutations at the cleavage site of the HA. These changes render the HA highly cleavable by ubiquitous proteases throughout the body which allows the virus to spread systemically. Consequently, these viruses are lethal for many bird species. The closeness of domestic poultry to human habitats and retail sales of infected live poultry have resulted in the transmission of these highly pathogenic avian viruses to humans, leading to several fatal infections in healthy children and adults (K.S. Li et al. 2004; Peiris et al. 2004; Subbarao et al. 1998).

Avian influenza H9N2 caused illness in three people in Hong Kong in 1991 and 2003, but retrospective serosurveys detected subclinical infections in the general

Table 6 *Influenza A viruses isolated from avian species, examples of HA and NA subtypes*

HA subtype designation	NA subtype designation	Avian influenza A viruses
H1	N1	A/duck/Alberta/35/76(H1N1)[a]
	N8	A/duck/Alberta/97/77(H1N8)
H2	N9	A/duck/Germany/1/72(H2N9)[a]
	N9	A/duck/Germany/1/72(H2N9)
H3	N8	A/duck/Ukraine/1/63(H3N8)[a]
	N8	A/duck/England/62(H3N8)
	N2	A/turkey/England/69(H3N2)
H4	N6	A/duck/Czechoslovakia/56(H4N6)[a]
	N3	A/duck/Alberta/300/77(H4N3)
H5	N3	A/tern/South Africa/61(H5N3)[a]
	N9	A/turkey/Ontario/7732/66(H5N9)
	N1	A/chick/Scotland/59(H5N1)
H6	N2	A/turkey/Massachusetts/3740/65(H6N2)[a]
	N8	A/turkey/Canada/63(H6N8)
	N5	A/shearwater/Australia/72(H6N5)
	N1	A/duck/Germany/1868/68(H6N1)
H7	N7	A/fowl plague virus/Dutch/27(H7N7)[a]
	N1	A/chick/Brescia/1902(H7N1)
	N3	A/turkey/England/63(H7N3)
	N1	A/fowl plague virus/Rostock/34(H7N1)
H8	N4	A/turkey/Ontario/6118/68(H8N4)[a]
H9	N2	A/turkey/Wisconsin/1/66(H9N2)[a]
	N6	A/duck/Hong Kong/147/77(H9N6)
H10	N7	A/chick/Germany/N/49(H10N7)[a]
	N8	A/quail/Italy/1117/65(H10N8)
H11	N6	A/duck/England/56(H11N6)[a]
	N9	A/duck/Memphis/546/74(H11N9)
H12	N5	A/duck/Alberta/60/76(H12N5)[a]
H13	N6	A/gull/Maryland/704/77(H13N6)[a]
H14	N4	A/duck/Gurjev/263/83(H14N4)[a]
H15	N8	A/duck/Australia/341/83(H15N8)[a]
	N9	A/shearwater/Australia/2576/83(H15N9)
H16	N3	A/black-headed gull/Sweden/5/99

a) Reference strains

population (Peiris et al. 1999). The H9N2 viruses have also circulated extensively in swine herds in Asia and elsewhere. Because H9N2 viruses can readily bind sialic acid receptors present in airway epithelium of humans and other mammals, they constitute potential pandemic candidates to be monitored in surveillance programs (Matrosovich et al. 2001).

SWINE INFLUENZA

Koen (1919) reported the occurrence of an epizootic of respiratory disease among swine in Iowa, which seemed to be a new clinical entity and which co-incided with the start of the 1918 pandemic of influenza in the Midwest of the USA. Because the clinical symptoms of the disease were similar to those occurring in humans, the disease was called swine influenza. Shope (1931) demonstrated the viral cause of swine influenza in 1930, 3 years before it was shown that influenza is a viral infection in people. Retrospective serological studies in humans (Davenport et al. 1953) and subsequent investigations (reviewed by Stuart-Harris et al. (1985)) provided strong circumstantial evidence that the Shope strain was antigenically close to the virus responsible for the 1918 pandemic of Spanish influenza.

The North American Swine influenza A subtype H1N1 viruses spread from the US to Europe in 1976 (Nardelli et al. 1978). This lineage was replaced in 1979 by another H1N1 virus that had been introduced from birds into pigs (Pensaert et al. 1981). Swine influenza strains are enzootic in pig populations in many parts of the world and have also infected turkeys in the USA (Hinshaw et al. 1983a).

A number of cases or outbreaks attributable to swine H1N1 virus transmission from ill swine to people have been documented, and each is connected with concern

stemming from the association of swine disease and antibodies to swine influenza viruses with the 1918 Spanish influenza pandemic. This concern is best exemplified by the swine influenza immunization campaign that was begun in the USA after a swine influenza virus (A/New Jersey/8/76) was isolated from a young military recruit who died of pneumonia (Goldfield et al. 1977).

Serological evidence suggested that this virus was antigenically similar to the viruses believed to have caused the 1918 pandemic and that the virus had spread to other recruits (Dowdle and Millar 1978). Since that time, additional fatal cases of swine H1N1 influenza in humans have been documented, but these viruses have so far shown a limited capacity to spread in humans.

Swine influenza virus is one of the most common causes of respiratory disease in swine, and serological surveys in the USA have indicated that nearly half of the herds have antibody against this virus. Clinical evidence indicates that influenza A strains vary in the severity of disease that they cause in infected swine. The relative prevalence of different influenza A viruses in swine varies throughout the world, but it is clear that a large reservoir of influenza A viruses exists in pigs worldwide.

Critical to the hypothesis that pigs may play a crucial role in the emergence of new pandemic strains of influenza is the observation that swine are susceptible to infection by swine, avian, and human influenza viruses (see section on Receptor binding). This susceptibility to a variety of subtypes of influenza viruses provides the opportunity for genetic reassortment in swine. These findings underscore the possible role of swine as a 'mixing vessel' for the emergence of novel influenza strains (Scholtissek et al. 1985). Furthermore, classic A(H1N1) swine viruses, avian A(H1N1) viruses, and human A(H1N1) (H3N2), and type C influenza viruses have all been isolated from naturally infected swine. Avian–swine influenza reassortants were first detected in Europe in 1979 (Scholtissek et al. 1983) and continue to circulate in pigs in that part of the world. Human–avian H3N2 viruses emerged between 1983 and 1985 in Italy and have been isolated repeatedly from pigs in Europe (Webster et al. 1992).

Human H3N2 influenza entered the North American swine herds around 1995 and gave rise to double-reassortants with classical swine influenza genes in which the surface and PB1 protein genes are of human origin. These reassortants later acquired two avian internal protein genes (PA and PB2) in addition to the human and swine genes seen in the double reassortant (Zhou et al. 1999). These triple reassortants became widespread in 1998 and have circulated widely in the pig population since then (Webby et al. 2000). Experimental infection of pigs with avian viruses representing 13 of the 16 HA subtypes has also been reported (Kida et al. 1994).

DIAGNOSIS

A number of techniques have been developed for the diagnosis of influenza virus infections. Virus isolation in cultured cells or in fertilized hens' eggs or demonstration of a four-fold or greater rise in specific antibodies between acute and convalescent sera are techniques that have been used for many years. More recently, detection of viral antigens directly in clinical specimens by immunological methods and detection of viral nucleic acids by hybridization or using polymerase chain reaction (PCR) has greatly increased the speed of laboratory diagnosis.

Virus isolation and propagation

Embryonated hens' eggs, a number of primary tissue culture systems and continuous cell lines such as Madin–Darby canine kidney (MDCK; American Type Culture Collection, Rockville MD) cells can be used to isolate and grow influenza viruses for identification or research purposes. Recognition that proteolytic cleavage of the HA is necessary for viral infectivity and that inclusion of trypsin in the tissue culture fluid cleaves the HA expanded the culture systems available for influenza isolation and propagation. Replication of influenza viruses in all of its laboratory hosts is often detected by agglutination of erythrocytes added to culture fluid or by hemadsorption of erythrocytes to infected cells. On initial passage in eggs or tissue culture, some influenza viruses preferentially agglutinate guinea-pig over chicken erythrocytes, but on continued passage the viruses may preferentially agglutinate erythrocytes from chickens (Burnet and Bull 1943).

ISOLATION IN EMBRYONATED EGGS

Soon after the first identification of human influenza A viruses, Burnet (1936) reported that embryonated hens' eggs could serve as a host system for their propagation. This host system is still used for vaccine production and for generating large quantities of influenza virus that are occasionally necessary for research. To isolate both type A and type B influenza viruses, clinical samples are inoculated into the amniotic and allantoic cavities of 10–11-day-old embryonated hens' eggs. The eggs are usually incubated at 33–34°C for 2–3 days before the virus is harvested. Most type A and B influenza viruses that are originally isolated in eggs will grow well in the allantoic cavity after one or two passages. Type C influenza viruses, on the other hand, grow only in the amniotic cavity and are best propagated in 7–8-day-old embryonated hens' eggs after 5 days' incubation.

ISOLATION IN TISSUE CULTURE

Primary cynomolgus or rhesus monkey kidney cells (PMKC) are susceptible to a variety of respiratory viruses, including influenza viruses. Disadvantages with

these cells include their cost and the presence of spumaviruses. Influenza A, B, and C viruses can also be isolated in the MDCK cell line in the presence of trypsin. Primary monkey kidney and MDCK cells are most often used for the primary isolation of influenza viruses from humans.

Influenza virus isolation is still used in many laboratories worldwide. When performed correctly with properly collected specimens along with good quality laboratory cells and reagents, this method is highly sensitive. The shell vial tissue culture isolation method combines rapid detection of virus in the inoculated cells after 48–72 h, increased sensitivity being obtained by centrifugation of specimens onto the cells. Monoclonal antibodies are often used for immunofluorescent detection of viral antigens in the inoculated cells (Kalin and Grandien 1993). Obtaining isolates for further antigenic and genetic characterization is an essential part of worldwide surveillance for the emergence of significant antigenic variants of influenza. Detection of these new variants may signal the need to revise the formulation of the influenza vaccine or to recommend other measures for public health control.

Serology

Serology may establish that infection by influenza viruses has occurred when the virus is not detected by any other method. Serological tests include complement fixation (CF), hemagglutination inhibition (HAI), neutralization, and enzyme immunoassays (EIA). These techniques are the fundamental tools in epidemiological and immunological studies as well as in the evaluation of the immunogenic properties of the vaccine.

The CF test measures antibodies against the NP and thus allows type-specific detection of antibodies to type A and B influenza viruses rather than a subtype- or strain-specific diagnosis. This test is relatively insensitive in detecting rises in pairs of serum titers from individuals with a recent infection, but can be used when more specific reagents are not available. HAI and neutralization are more sensitive and measure antibodies against subtype- and strain-specific antigens. EIA tests can be configured according to the nature of the antigens and the isotype of immunoglobulin chosen for the assay (Ziegler et al. 1995).

The HAI test is the serological test most often applied to influenza viruses (Figure 20). Because of the instability of HA, the dilution of antigen used must be precisely determined by HA titration each time the HAI test is performed. The test is also complicated by the presence in sera of several species of nonspecific inhibitors of hemagglutination. These inhibitors interact with the HA molecule and prevent the agglutination of erythrocytes, even in the absence of specific antibodies. The non-specific inhibitors fall into three classes: α inhibitors, which are present in human serum and are heat-stable sialylated glycoproteins that inhibit hemagglutination but do not neutralize viral infectivity; β inhibitors, which are also present in human serum and but are heat-labile and have neutralizing activity; and γ inhibitors, which are present in horse serum and are heat-stable sialylated sialoproteins with neutralizing activity. The α and γ inhibitors function as receptor analogues by competing with red cell receptors for binding to HA. Removal of nonspecific inhibitors from sera by using receptor-destroying enzyme or periodate is an essential step in the HAI test (Kendal et al. 1982). In addition, interference by antineuraminidase antibody and variability of viruses in avidity for red cells and antibody can influence the test results.

Rapid virus diagnosis

POINT-OF-CARE TESTS

The availability of influenza antiviral drugs has increased both physician and patient demand for rapid diagnosis. Rapid EIAs for influenza A and influenza B viruses which produce a result in 15 to 20 min are commercially available (Johnston and Bloy 1993; Ryan-Poirier et al. 1992; Todd et al. 1995; Waner et al. 1991). A positive result can be obtained with as few as 20 influenza virus-infected cells or 2 000 infectious virus particles. There currently are many commercially available point-of-care tests that rely on antigen detection in clinical specimens. Most of the tests use an immunoassay method to detect viral NP antigen, while one uses an enzyme-based chromogenic indicator to detect the presence of viral NA. These tests have a sensitivity of 50 to 100 percent and a specificity of 50 to 100 percent according to the manufacturers. Variation in sensitivity and specificity may depend on the study design, the type of sample collected, the timing of sample collection, the age of the patient, and other factors. Such tests show promise in certain clinical settings; however, the sensitivity and specificity of these tests under field conditions may be lower than reported in the published controlled studies. Furthermore, the positive predictive value of these tests may be quite low early in the influenza season, when influenza infections are relatively rare. All of these tests are less sensitive and specific than more traditional laboratory diagnostic methods such as virus isolation and serological diagnosis by the HI test, but they allow a test result to be obtained in time to treat the patient. These tests are useful in clinical laboratories of hospitals and nursing homes to detect institutional influenza outbreaks. However, because both false-positive and false-negative results are reported frequently for these point-of-care rapid tests, it is important to test multiple samples from the same outbreak before making decisions about treatment or prophylaxis for institutionalized patients.

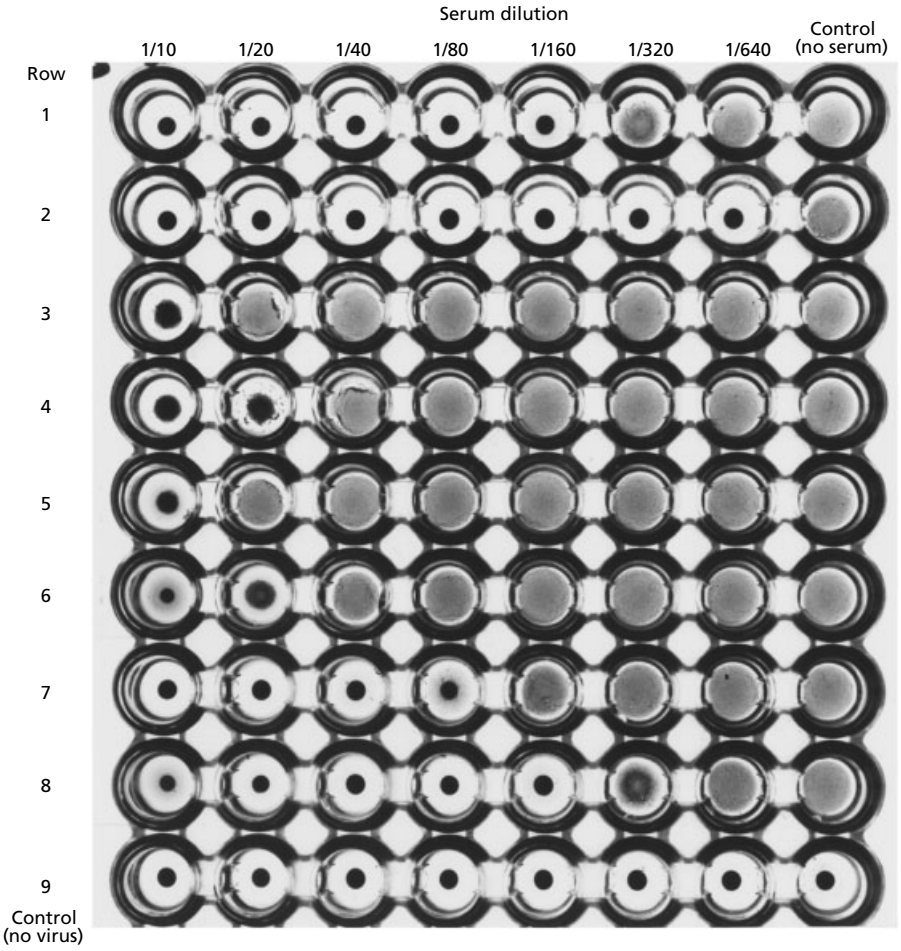

Figure 20 *Hemagglutination inhibition (HAI) test for influenza antibodies. The HAI is a competitive test in which antibodies present in an immune serum compete with red blood cells to bind the viral hemagglutinin. Row 1 shows results when antibodies are present and virus-induced hemagglutination of erythrocytes is inhibited with a consequent formation of erythrocyte 'buttons.' Row 3 shows results obtained when antibody is not present to inhibit viral hemagglutination.*

MOLECULAR METHODS

Molecular methods are being applied more widely in the diagnosis of influenza virus infections as well as in the characterization of influenza virus isolates. Molecular techniques are likely to supplant virus culture as the 'gold standard' for virus detection because they are more sensitive and offer advantages of speed and more precise characterization over other, more traditional laboratory methods. Abundant sequence data derived from isolates collected over several decades and from many geographic regions are available for selecting conserved target sequences to allow the identification of all isolates within a subtype, to discriminate between subtypes, and to distinguish between strains within a subtype. The genetic analysis of a large number of influenza viruses isolated throughout the world each year provides information for a timely update of vaccine strains and of molecular reagents for diagnosis. With currently available technology, updating molecular probes is more rapid and practical than updating MAbs on a regular basis.

For reverse transcriptase polymerase chain reaction (RT-PCR), viral nucleic acids are extracted from clinical specimens, allantoic fluid of embryonated hen eggs, or cell culture material by using the guanidium thiocyanate method (Boom et al. 1990) or commercial kits. cDNA is synthesized by in vitro reverse transcription of viral RNA primed either by specific synthetic oligonucleotides matching known nucleotide sequences on the viral genes or by random hexamers. This cDNA is amplified with specific primers and DNA polymerase. Finally, the amplified product is detected by any of numerous methods developed for this purpose.

Use of RT-PCR amplification of influenza virus RNA to diagnose influenza has been reported by Zhang and Evans (1991), Claas et al. (1992, 1993), Ellis et al. (1997a), Stockton et al. (1998), and Wright et al. (1995). Wright and colleagues developed a multiplex RT-PCR for type- and subtype-specific identification of currently circulating human influenza viruses. Three sets of oligonucleotide primers were used to identify the *NP* gene of influenza A viruses, the *NS* gene of influenza B viruses,

the *HA* genes of influenza A subtypes H1 and H3, and the *NA* gene of influenza A subtypes N1 and N2. A type- and subtype-specific multiplex nested RT-PCR format was evaluated on 619 specimens collected during the 1995 to 1996 influenza season in England (Ellis et al. 1997a).

The superiority of RT-PCR over virus culture recently has been reported in several studies (Boivin et al. 2001; Cherian et al. 1994; Ellis et al. 1997a; Herrmann et al. 2001; Kehl et al. 2001; Plakokefalos et al. 2000; Zambon et al. 2001). Nested RT-PCR and similar primers were used for most of these studies. Conserved matrix gene primers were used for typing samples as influenza A or B virus, and primers designed for detecting the HA and NA genes were used for subtyping influenza A virus specimens. In the largest of these studies, data were available for 1 033 patients; of these, 730 patients were positive by RT-PCR, 629 were positive by serological testing, and 579 were positive by virus culture (Zambon et al. 2001). RT-PCR is a useful method for rapidly processing large numbers of clinical isolates for influenza diagnosis in centralized laboratories that have sufficient resources and expertise.

A new approach to influenza diagnosis is to use DNA microarrays containing nucleic acid probes for typing and subtyping influenza viruses and to identify common respiratory viral pathogens such as influenza A, B, and C viruses, respiratory syncytial virus, parainfluenza viruses, and adenoviruses. One study demonstrated that a model microarray containing multiple probes derived from cloned PCR-amplified fragments of influenza A and B virus matrix, HA, and NA genes correctly identified complementary influenza virus-specific DNA (J. Li et al. 2001). Although more extensive studies are needed, microarray technology appears to hold future promise for rapid and accurate diagnosis and large-scale surveillance.

PCR combined with endonuclease restriction analysis has been applied to the genetic characterization of influenza viruses. For this technique, oligonucleotide primers are designed to amplify a product that contains unique restriction enzyme cleavage sites. This method can discriminate between two lineages of influenza A (H3N2) viruses which circulated simultaneously during an influenza season (Ellis et al. 1997b; Zou 1997); can determine the genetic stability of cold-adapted, reassortant live vaccine strains (Klimov and Cox 1995); and allows the rapid identification of drug-resistant influenza virus isolates (Klimov et al. 1995).

CONTROL

The morbidity and mortality caused by influenza virus infections and the disruptive effects of epidemics and pandemics on the community justify strenuous efforts to prevent this disease. Two measures are currently available to reduce the impact of influenza: immunoprophylaxis with influenza vaccines and chemoprophylaxis, or therapy with the antiviral drugs. Research to develop additional influenza vaccines and antiviral agents may result in additional products for the prevention and control of influenza.

Immunization

Vaccination is currently the most effective measure for reducing the impact of influenza. Immunization is focused mainly on individuals at increased risk of complications of influenza infection and on individuals who might transmit influenza to such people (CDC 2004; Fedson et al. 1995; Nicholson et al. 1995). For example, influenza vaccine is currently strongly recommended in the USA for anyone $\geqslant$6 months of age who, because of age or an underlying medical condition, is at increased risk for complications of influenza. They include people $\geqslant$65 years of age; people in residential or nursing homes; those with chronic disorders of the pulmonary or cardiovascular systems, including children with asthma; children aged 6–23 months; women who will be pregnant during the influenza season; anyone who has required regular medical follow-up or hospitalization during the preceding year because of chronic metabolic diseases (including diabetes mellitus), renal dysfunction, hemoglobinopathies, or immunosuppression (including immunosuppression caused by medications); and children and teenagers (6 months to 18 years of age) who are taking aspirin in the long term and may be at risk of developing Reye syndrome after influenza. People who should receive vaccination to reduce the likelihood of transmission to high-risk groups include: physicians, nurses, and other personnel in both hospital and outpatient care settings; staff, visitors, and volunteers in residential and nursing homes; providers of home care to individuals at high risk and household members (including children) of such people.

Vaccination is generally considered safe for anyone who can eat foods containing egg. Hypersensitivity to hens' eggs, the substrate for vaccine production, is the only contraindication. Desensitization to eggs followed by vaccination can be considered for individuals in high-risk groups.

VACCINE FORMULATION

Each February the World Health Organization makes recommendations concerning the antigenic properties of the strains of influenza that are to be used in vaccines during the following influenza season. These recommendations are based on data gathered by the four World Health Organization (WHO) Collaborating Centers (Atlanta, USA; London, UK; Melbourne, Australia; and Tokyo, Japan) together with data gathered by many national WHO collaborating laboratories. The objective

Table 7 *Influenza vaccine strains recommended by WHO, 1987/8 to 2004/5 for the northern hemisphere*

Year	H1N1	H3N2	B
1987/8	A/Singapore/06/86-like[a]	A/Leningrad/360/86-like	B/Ann Arbor/1/86-like
1988/9	A/Singapore/06/86-like[a]	A/Sichuan/02/87-like	B/Beijing/1/87-like
1989/90	A/Singapore/06/86-like[a]	A/Shanghai/11/87-like	B/Yamagata/16/88-like
1990/1	A/Singapore/06/86-like[a]	A/Guizhou/54/89-like[c]	B/Yamagata/16/88-like
1991/2	A/Singapore/06/86-like[a]	A/Beijing/353/89-like	Either B/Yamagata/16/88-like or B/Panama/45/90-like
1992/3	A/Singapore/06/86-like[a]	A/Beijing/353/89-like	Either B /Yamagata/16/88-like or B/Panama/45/90-like
1993/4	A/Singapore/06/86-like[a]	A/Beijing/32/92-like	B/Panama/45/90-like
1994/5	A/Singapore/06/86-like[b]	A/Shangdong/09/93-like	B/Panama/45/90-like
1995/6	A/Singapore/06/86-like[b]	A/Johannesburg/33/94-like	B/Beijing/184/93-like[e]
1996/7	A/Singapore/06/86-like[b]	A/Wuhan/359/95-like[d]	B/Beijing/184/93-like[e]
1997/8	A/Johannesburg/82/96-like	A/Wuhan/359/95-like[d]	B/Beijing/184/93-like[e]
1998/9	A/Beijing/262/95-like	A/Sydney/5/97-like	B/Beijing/184/93-like[e]
1999/00	A/New Caledonia/20/99-like	A/Sydney/5/97-like	B/Beijing/184/93-like virus or B/Shangdong/7/97-like[f]
2000/01	A/New Caledonia/20/99-like	A/Moscow/10/99-like[g]	B/Sichuan/379/99-like
2001/02	A/New Caledonia/20/99-like	A/Moscow/10/99-like[g]	B/Sichuan/379/99-like
2002/03	A/New Caledonia/20/99-like	A/Moscow/10/99-like[g]	B/Hong Kong/330/2001-like
2003/04	A/New Caledonia/20/99-like	A/Moscow/110/99-like[g]	B/Hong Kong/330/2001-like
2004/2005	A/New Caledonia/20/99-like	A/Fujian/411/2002-like	B/Shanghai/361/2002-like[h]

a) Most countries used the antigenically equivalent A/Taiwan/1/86 virus.
b) Most countries used the antigenically equivalent A/Texas/36/91 virus.
c) Most countries used the antigenically equivalent A/Shanghai/16/89 virus.
d) Most countries used the antigenically equivalent A/Nanchang/33/95 virus.
e) Most countries used the antigenically equivalent B/Harbin/7/94 virus.
f) Most countries used the antigenically equivalent B/Yamanashi/166/98 virus.
g) Most countries used the antigenically equivalent A/Panama/2007/99 virus.
h) Most countries used the antigenically equivalent B/Jiangsu/10/2003 or B/Jilin/20/2003 virus.
i) Most countries used the antigenically equivalent A/Wyoming.

is to match the antigenic properties of the HA and NA of the recommended vaccine strains as closely as possible with those of strains that are emerging and are likely to circulate the following winter.

Three types of data are used to make the WHO influenza vaccine strain recommendations each year. First, data are obtained from reference antigenic and genetic analyses of influenza viruses isolated through WHO's global influenza surveillance network. These data are used to detect new antigenic variants that might have emerged since the previous recommendations were issued and to determine how these variants are genetically and antigenically related to other circulating strains. Second, epidemiological and virological data are combined to determine whether newly identified variants are detected in association with outbreaks of disease in multiple locations or are associated only with a single case or a few sporadic cases. Third, the ability of the existing vaccine strains to induce an antibody response in humans to the newly detected variants is examined. These three types of data are combined to determine what new antigenic variants are circulating, whether these variants are associated with significant disease, and whether immunization with the existing vaccine could protect against disease caused by the new variants.

Influenza vaccines in recent years have been trivalent, containing strains of the two influenza A subtypes that are circulating along with a representative type B strain (Table 7). Viruses used by vaccine manufacturers for the type B component of the vaccine are naturally occurring virus strains that replicate to relatively high titers in the allantoic cavity of embryonated hens' eggs. The strains most often used for the type A components are high-growth reassortant viruses that contain internal genes that specify the property of high growth in eggs derived from the A/PR/8/34(H1N1) laboratory adapted strain together with the surface glycoproteins, HA and NA, from the recommended field strain. Vaccine strains are grown individually in the allantoic cavity of embryonated hens' eggs and then purified and concentrated by zonal centrifugation or column chromatography and finally inactivated with formalin or other chemicals. Vaccines are routinely standardized by means of single radial diffusion tests (Wood et al. 1981) to contain 15 μg HA per virus strain per dose; the quantity of NA is not standardized and may vary between vaccines.

Influenza vaccines currently in use are live-attenuated vaccines or inactivated subvirion (SV), or surface antigen vaccines. Live-attenuated vaccine contains avirulent replicating virus; SV vaccine contains detergent-

disrupted inactivated virus; surface antigen vaccines contain isolated HA and NA proteins (CDC 2004). Although current viruses are still produced by growing viruses in the allantoic sac of embryonated hens' eggs, numerous refinements have improved the immunogenicity and reactogenicity of current inactivated vaccines; they include the use of zonal centrifugation, the use of ether or other lipid solvents to disrupt the virus, the introduction of high-yield reassortants to improve yields, and the development of better methods to quantify the amount of viral antigens present in the vaccines. Thus modern influenza vaccines are associated with minimal side effects. Up to one-third of inactivated vaccine recipients may feel some discomfort at the vaccination site for up to 2 days after vaccination; only about 1–2 percent have fever, malaise, myalgia, or other systemic reaction, which may begin 6–12 h after vaccination and persists for 1–2 days (Betts 1995; CDC 2004).

EFFECTIVENESS OF INACTIVATED INFLUENZA VACCINES

Inactivated parenterally administered influenza vaccines have been in use since the 1940s. A large number of trials in both military and civilian populations have demonstrated the efficacy of inactivated vaccines in the prevention of naturally occurring outbreaks of influenza A(H1H1), (H2N2), and (H3N2) viruses (Ada et al. 1987; Couch et al. 1986; Dowdle 1981; Monto and Terpenning 1996). The large variations in vaccine efficacy and clinical effectiveness reported in the literature are due to differences in the ages and immunocompetence of the vaccine recipients, the degree of antigenic similarity between the vaccine and circulating virus strains, the vaccine potency, the extent and intensity of transmission of influenza, and other respiratory viruses circulating during the study period, as well as the surveillance and laboratory methods used for the studies. Current inactivated vaccines protect 70–90 percent of normal healthy adult recipients against naturally occurring disease when the antigens of the vaccine and those of the circulating influenza viruses are closely related. In people >65 years of age (a group that is a primary target of vaccination programmes in a number of countries), the antibody response to HA is reduced and clinical effectiveness of vaccine is lower. This is probably a reflection of reduced immunocompetence (Ershler et al. 1984). For older people living in the community, the effectiveness of influenza vaccine in preventing hospitalization for P and I ranges from 30 percent to 70 percent. Among elderly people living in nursing homes, inactivated vaccines confer less protection against illness than in younger healthy individuals; they are more effective in preventing severe illness or secondary complications requiring hospitalization (50–60 percent) and death (80 percent) than in protecting against disease (30–40 percent) (CDC 2004).

Chemotherapy

The chemically related drugs amantadine and rimantadine hydrochloride interfere with the replication cycle of all subtypes of type A but not type B influenza viruses (Hayden 1996; Van Voris and Newell 1992). Amantadine and rimantadine, its α-methyl derivative, share mechanisms of action, antiviral spectrums, oral bioavailability, prolonged plasma half-life, and clinical efficacy. Both drugs inhibit the replication of influenza A viruses at low, clinically achievable concentrations of <1 μg/ml (Tominack and Hayden 1987), but concerns about side-effects involving the central nervous system have limited the use of amantadine.

Amantadine and rimantadine are 70–90 percent effective in preventing illness caused by naturally occurring strains of type A influenza viruses when administered prophylactically to healthy adults or children when influenza viruses are circulating. These drugs can also reduce the severity and duration of illness caused by influenza A viruses when administered within 48 h of onset of symptoms.

The antiviral activity of both of these compounds is exerted through interference with two different ion channel functions of the viral M2 protein (see section entitled M2). The first involves inhibition of the acid-mediated dissociation of the matrix protein from the RNP complex within endosomes early in replication; this dissociation is essential for initiating viral replication (Pinto et al. 1992; Sugrue et al. 1990a). A second effect on virus maturation relating to a low pH-mediated alteration of the HA protein during its transport to the cell surface occurs during the replication of certain avian influenza viruses (Ohuchi et al. 1994; Sugrue et al. 1990a; Takeuchi and Lamb 1994).

Influenza A viruses are cross-susceptible and cross-resistant to amantadine and rimantadine. Viral strains responsible for pandemics and epidemics in recent years have all been drug-sensitive (Hayden 1996), but resistance to these compounds is readily selected by growth in the presence of these drugs either in vivo or in vitro. Resistance is associated with single nucleotide changes in RNA segment 7 and corresponding amino acid substitutions at one of four sites (amino acids 26, 27, 30, and 31 for human influenza A viruses) in the transmembrane region of the M2 protein (Hay 1992). Resistant viruses can emerge when either of these drugs is administered for treatment in adults or children. Although the frequency of the emergence of resistance is not firmly established, resistant variants have been recovered from ca. 30 percent of treated children or adults (Hall et al. 1987; Hayden et al. 1989). Nevertheless, treated individuals who shed resistant viruses recover rapidly and the duration of shedding is relatively short, so the clinical significance of the emergence of resistance in an individual is unclear. Apparent transmission of resistant virus

associated with failure of drug prophylaxis has been documented in nursing home residents receiving amantadine and in household contacts of rimantadine-treated individuals (Degelau et al. 1992; Hayden et al. 1989; Houck et al. 1995; Mast et al. 1991). Resistant viruses seem to be pathogenic and can cause typical influenza, but resistant viruses are no more transmissible or virulent than strains sensitive to amantadine and rimantadine (Hayden 1996).

Neuraminidase inhibitors

Multi-cycle replication of influenza virus in the host requires an active neuraminidase on the virus particles (see Table 2 and NA section). Zanamivir and oseltamivir are chemically related drugs known as neuraminidase inhibitors that have antiviral activity against both influenza A and B viruses (Hayden 1999, 2000). Both zanamivir and oseltamivir are approved for treating uncomplicated influenza infections. Zanamivir is approved for persons aged $\geqslant$7 years, and oseltamivir is approved for patients aged $\geqslant$1 year. Oseltamivir is administered orally, whereas zanamivir is for inhalation. When administered within 2 days of illness onset to otherwise healthy adults, zanamivir and oseltamivir can reduce the duration of uncomplicated influenza A and B illness by approximately 1 day, compared with placebo (Hayden et al. 1997; Makela et al. 2000; Demichelli et al. 2000). The course of oseltamivir therapy for persons with influenza A illness is 5 days. Oseltamivir is the only drug that has been approved for prophylactic use, being restricted to persons aged $\geqslant$13 years (Monto et al. 2002). Up to 82 percent of febrile, laboratory-confirmed influenza illnesses were prevented by oseltaminvir prophylaxis (Hayden et al. 1999; Monto et al. 1999). Besides their traditional use for the currently circulating strains of influenza A and B, neuraminidase inhibitors could prove critical to protect the health of the population at risk of infection by an emerging pandemic strain of influenza virus, until a suitable vaccine becomes available.

Emergence zanamivir and oseltamivir-resistant variants can be induced in influenza A and B viruses in vitro, but induction of resistance requires multiple passages in cell culture (Gubareva et al. 1997). Development of viral resistance to zanamivir and oseltamivir during treatment has been identified but does not appear to be frequent, with children providing a higher yield of resistant variants (Kiso et al. 2004; McKimm-Breschkin 2003). Available diagnostic tests are not optimal for detecting clinical resistance to the neuraminidase inhibitor antiviral drugs, but new diagnostic tests may revert this situation (McSharry et al. 2004). Coordinated surveillance activities that have been conducted since 1999 to detect the emergence of neuraminidase resistant variants provide the necessary information regarding the continuity of drug efficacy (Zambon and Hayden 2001).

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General properties of the paramyxoviruses

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This chapter provides a general overview of the structure and replication of viruses in the family *Paramyxoviridae*. Subsequent chapters describe each group of viruses in greater detail including the diseases associated with these viruses.

CLASSIFICATION

The family *Paramyxoviridae* includes several important human pathogens, as well as viruses affecting various species of mammals, birds, and reptiles. Together with the families *Rhabdoviridae* and *Filoviridae* they form the order *Mononegavirales*. Members of this order have a nonsegmented, single-stranded, negative-sense RNA genome, a conserved gene order, and similar replication strategy. Paramxyoviruses are pleomorphic, enveloped viruses that contain helical nucleocapsids and replicate in the cytoplasm of infected cells. The family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, which are distinguished by the diameter and pitch of their nucleocapsids, the number of genes and the presence or absence of a modified transcript of their phosphoprotein (*P*) genes. Several new genera have been added to the family over the past few years. At present, the subfamily *Paramyxovirinae* contains five genera, while the *Pneumovirinae* contains two (Mayo 2002). Viruses representing each of the subfamilies and genera are listed in Table 1. Members of the same genus share antigenic cross-reactivity and a higher degree of nucleotide sequence similarity. Distinguishing features of the genera in the subfamily *Paramyxovirinae* include the presence or absence of hemagglutination and neuraminidase activity, the length and conservation of intergenic regions, and the organization and coding capacity of the *P* gene. The two genera of the subfamily *Pneumovirinae* are characterized by the presence or absence of the *NS1* and *NS2* genes and their gene order (Table 2, Figure 1).

A number of new paramyxoviruses have been isolated recently, but have not yet been assigned to a genus. Sixteen paramyxovirus isolates from reptilian species (Ahne et al. 1999) have been described; the archetype is fer-de-lance virus (Kurath et al. 2004), which has been completely sequenced. This virus has a novel gene located between the *N* and *P* gene, which has no counterpart in any of the other viruses of the family. A number of paramyxoviruses has been isolated from rodents, including Nariva virus and Mossman virus (Roos and Wollman 1979; Miller et al. 2003). Tupaia virus was discovered in a cell line derived from tree shrews (Tidona et al. 1999) and Salem virus was isolated from horses (Renshaw et al. 2000). The natural distribution and pathogenic potential of these viruses are mostly unknown.

MORPHOLOGY AND GENERAL PROPERTIES

Paramyxoviridae are pleomorphic viruses with lipid envelopes derived from the plasma membrane of the

Table 1 *Genera and representative species of the family* Paramyxoviridae

Genera and representative species
Subfamily *Paramyxovirinae*
Genus *Respirovirus*
Sendai virus[a]
Human parainfluenza viruses, types 1 and 3
Bovine parainfluenza virus type 3
Genus *Rubulavirus*
Mumps virus[a]
Human parainfluenza viruses, types 2, 4a, and 4b
Simian virus 5
Genus *Morbillivirus*
Measles virus[a]
Canine distemper virus
Rinderpest virus
Genus *Henipavirus*
Hendra virus[a]
Nipah virus
Genus *Avulavirus*
Newcastle disease virus[a]
Avian paramyxoviruses 1–9
Subfamily *Pneumovirinae*
Genus *Pneumovirus*
Human respiratory syncytial virus[a]
Bovine respiratory syncytial virus
Genus *Metapneumovirus*
Turkey rhinotracheitis virus[a]
Human metapneumovirus

a) Type species

infected cell (Klenk and Choppin 1969, 1970). Virus particles range from 150 to 400 nm in diameter and are generally spherical, but can also be filamentous. The virions contain two viral transmembrane glycoproteins that appear as spikes of varying length on electron micrographs. The envelope surrounds the nucleocapsid, which consists of the RNA genome encapsidated by the viral nucleoprotein. Nucleocapsids of *Paramyxovirinae* measure 18 nm in diameter, with a pitch of about 5.0 nm. The nucleocapsids of the *Pneumovirinae* are more slender, with a diameter of 13–14 nm and a pitch of 6.5–7.0 nm (Compans and Choppin 1967a,b; Finch and Gibbs 1970).

Paramyxoviruses are transmitted via close contact or the airborne (droplet or aerosol) route. The two transmembrane glycoproteins serve as attachment and fusion proteins. After attachment to a receptor on the surface of the host cell, fusion of the virion envelope and the host-cell membrane takes place at neutral pH. Fusion can also occur between infected and neighboring uninfected cells, resulting in the characteristic cytopathic effect of syncytium formation in cell culture or the appearance of multinucleated giant cells in host tissues. Cell lysis is another common outcome of infection with paramyxoviruses. In addition to the receptor-binding function, the attachment proteins of the genera *Morbillivirus*, *Respirovirus*, *Rubulavirus*, and *Avulavirus* also agglutinate red blood cells, and the attachment proteins of the latter three possess neuraminidase activities (Lamb and Kolakofsky 2001).

The density of the virus particles is 1.18–1.20 g/cm^3. Protein comprises most of the virion composition by weight (69–74 percent), lipid makes up 20–25 percent, and carbohydrate comprises 5–6 percent of the virion weight. The genomes of all paramyxoviruses are composed of single-stranded RNA with a density of approximately 1.3 g/cm^3. Reagents that disrupt lipids, such as organic solvents and detergents, as well as formaldehyde and oxidizing agents, destroy the infectious nature of the viruses. The viruses are also inactivated by ultraviolet light, heating, and drying.

GENOME STRUCTURE AND ORGANIZATION

The complete genomic sequences of representative viruses from every genus of the family are available on Genbank. Genomes of the *Paramyxoviridae* range from 15 000 to more than 18 000 nucleotides in length. Within the subfamily *Paramyxovirinae*, the gene order of *N–P–M–F–H/HN/G–L* is conserved; however, two members of the genus *Rubulavirus* and at least one member of the genus *Avulavirus* possess a gene encoding a small, hydrophobic protein (*SH*) that is located between the *F* and *HN* genes (Figure 1).

All members of the subfamily *Pneumoviridae* contain an *SH* and *M2* gene. The positions of the *SH*, *F*, *G*, and *M2* genes differ between the pneumoviruses and the metapneumoviruses (Figure 1). Furthermore, the 'normal' gene order of *F* followed by *G* is reversed in the pneumoviruses. The pneumoviruses are also unusual in that they have two additional genes, *NS1* and *NS2*, preceding the *N* gene.

The total length of the genomes (in nucleotides) of all members of the subfamily *Paramyxovirinae* is evenly divisible by six. This 'rule-of-six' proposes that one nucleocapsid protein binds to six nucleotides, and that the position of the nucleotides of certain regulatory elements within a group of six has consequences for their function (Calain and Roux 1993). The rule of six does not appear to apply to the *Pneumovirinae* (Samal and Collins 1996).

The promoters for both transcription and replication lie in the noncoding regions at the genomic termini. The leader sequence at the 3′ terminus of the genome is approximately 50 nucleotides in length for all *Paramyxoviridae* and contains promoter signals for both transcription and replication. The length of the trailer at the 5′ terminus varies between 20 and 114 nucleotides and only contains the complement of a promoter for replication. The terminal 12–13 nucleotides are very well

Table 2 *General properties of the genera of the subfamily* Pneumovirinae

Characteristic	Genus	
	Pneumovirus	***Metapneumovirus***
Genome size	15 kb	13 kb
Virion characteristics:		
Hemagglutination	–	–
Neuraminidase	–	–
Spike length	10–12 nm	13–14 nm
Structural proteins (sizes in kDa)[a]:		
Polymerase protein	200	200
Attachment protein	84–90	83
Fusion protein:		
(F0)	68–70	68
(F1)	47	53
(F2)	20	15
Nucleocapsid protein	42	43
Phosphoprotein	34	40
Matrix protein	26	35
Small hydrophobic protein	8–30	19[b]
Nonstructural proteins	NS1, NS2, M2-1, M2-2	M2-1, M2-2

a) Sizes for type species of each genus.
b) Calculated molecular mass.

conserved within a genus and have a high degree of complementarity with each other.

Each gene contains *cis*-acting, noncoding regions preceding and following the open reading frame (ORF). These regions contain conserved transcriptional start and stop signals. The mRNA stop signal includes several U residues, which serve as templates for polyadenylation of the mRNAs. Within most of the genera, the intergenic regions are conserved trinucleotides. However, the rubulaviruses and the viruses in the subfamily *Pneumoviridae* have intergenic regions with lengths varying from one to 90 nucleotides, with no conservation of sequence (Hardy et al. 1999). Finally, in the morbilliviruses, there is a long untranslated region of approximately 1 000 nucleotides between the M ORF and the F ORF. A similar phenomenon is observed in the henipaviruses, where the 5′ and 3′ untranslated regions for most of the genes are longer than in those of the other paramyxoviruses, especially in the *P* gene (Harcourt et al. 2000). The functional significance of these long, noncoding regions is unknown.

REPLICATION

Attachment and penetration

Attachment of virus particles to the host-cell membranes occurs through the viral glycoprotein, which is variably called G, H, or HN, depending on the presence or absence of hemagglutination (H) or neuraminidase activity (N) (Table 2; Table 3). Respiroviruses and rubulaviruses bind to sialic acid-containing proteins or lipids, while pneumoviruses attach to glycosaminoglycans containing heparin sulfate and chondroitin sulfate (Feldman et al. 1999). In contrast, morbilliviruses bind specific receptor proteins on the cell surface. For measles virus, two receptors have been identified, CD46, a protein involved in the regulation of complement activation (Dorig et al. 1993; Naniche et al. 1993), and signaling lymphocyte activation molecule (SLAM) (Tatsuo et al. 2000). Canine distemper virus

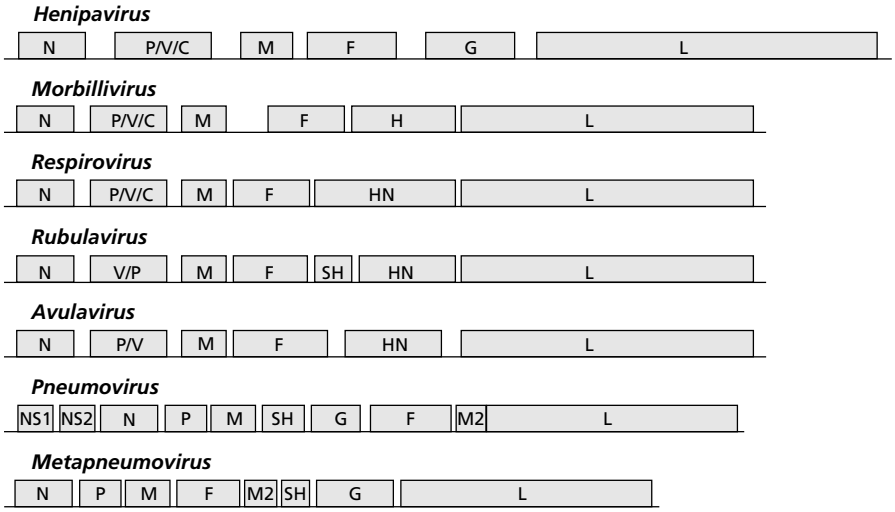

Figure 1 *Schematic representations of the genomes of viruses in the family* Paramyxoviridae. *Figure shows representative genome from each genus. Shaded boxes represent individual genes. Thin lines represent noncoding and intergenic regions. C, C protein; F, fusion protein; G, glycoprotein; H, hemagglutinin protein; HN, hemagglutinin-neuraminidase; L, polymerase protein; M, matrix protein; N, nucleoprotein; NS, non-structural protein; P, phosphoprotein; SH, small hydrophobic protein; V, V protein.*

Table 3 *General properties of the genera of the subfamily* Paramyxovirinae

Characteristic	Genus				
	Respirovirus	***Rubulavirus***	***Morbillivirus***	***Henipavirus***	***Avulavirus***
Genome size	15–16 kb	15–16 kb	15–16 kb	18–19 kb	15–16 kb
Virion characteristics:					
Hemagglutination	+	+	+	–	+
Neuraminidase	+	+	–	–	+
Spike length	8 nm	8 nm	8 nm	8–17 nm	8–12 nm
Structural proteins (sizes in kDa)[a]:					
Polymerase protein	230	200	200	200	220
Attachment protein	71	79	78	74	74
Fusion protein:					
(F0)	65	74	60	61	66
(F1)	51	61	41	49	53
(F2)	13	16	20	19	10
Nucleocapsid protein	58	72	60	58	56
Phosphoprotein	79	45	72	98	53–56
Matrix protein	38	40	38	42	41
Small hydrophobic protein		6[b]			16[b,c]
V protein		25			
Nonstructural proteins	C, C′, Y1, Y2, V, W, X[b]	V, W, I[b]	C, V	C, V, SB[b], W	V, W

a) Sizes for type species of each genus.
b) Some members of the genus only.
c) Calculated molecular mass.

and rinderpest virus bind the canine and bovine homologues of SLAM (Tatsuo et al. 2001). Specific receptors have not yet been identified for other members of the family.

Following attachment, the fusion (F) glycoprotein mediates fusion of the viral envelope with the plasma membrane of the host cell. Fusion occurs at neutral pH and requires the co-operation of the F and G/H/HN proteins. It is postulated that engagement of the receptor triggers conformational changes in F that initiate fusion. As a result of the fusion process, the nucleocapsid is released into the cytoplasm (Lamb 1993).

Transcription and replication

Primary transcription begins immediately after release of the nucleocapsid into the cytoplasm. The viral RNA-dependent RNA polymerase complex is contained in the virion. The nucleocapsid serves as the template for RNA synthesis and does not appear to disassemble. The polymerase complex enters the negative sense genomic template at the promoter region in the 3′ leader to initiate transcription. Each gene is transcribed into a monocistronic mRNA, regulated by the transcriptional start and stop sites at the beginning and end of the gene. The transcripts are capped and methylated at the 5′ terminus and polyadenylated at the 3′ terminus by the viral polymerase complex. The template for the poly(A) tail is the short poly(U) sequence that is part of the transcription stop signal following each gene. It has been suggested that the polymerase stutters at the stop signals to synthesize poly(A) tails of the mRNAs. While in transcription mode, the polymerase pauses at the gene stop signal, traverses the intergenic region, and reinitiates transcription at the next gene start signal. During this process, some polymerase molecules disengage from the template. Since there is only a single promoter at the 3′ terminus of the genome, the disengaged polymerase molecules can reinitiate transcription only at the 3′ terminus. This process leads to a gradient of transcription, in which the genes located near the 3′ terminus of the genome are more frequently transcribed than the genes at the 5′ terminus. Thus, regulation of viral gene expression is achieved by the establishment of a message gradient (Cattaneo et al. 1987; Homann et al. 1990).

The polymerases of all members of the subfamily *Paramyxovirinae* utilize a process termed RNA editing to insert nontemplated G nucleotides at a specific site to modify some of the mRNAs transcribed from the *P* gene (Thomas et al. 1988). This process results in a shift of the reading frame, beginning at the editing site, in the edited transcripts. The resulting proteins share the same amino terminal sequences as the proteins translated from the unedited transcripts, but have unique carboxyl termini. The protein translated from the unedited

transcript is the phosphoprotein (P), while the V protein is encoded by the edited transcript. The exception is found in the rubulaviruses where the unedited transcript codes for the V protein, and the P protein ORF is only accessible through editing. The editing site is highly conserved among the *Paramyxovirinae*. By contrast, all members of the subfamily *Pneumovirinae* produce only the P protein from the *P* gene. An interesting characteristic of the human and bovine respiratory syncytial viruses is that the transcriptional start site for the *L* gene lies within the *M2* gene (Collins et al. 1987).

The translation of primary transcripts leads to the accumulation of viral proteins, and a switch from transcription to replication. In replication mode, the polymerase complex enters the same (or an overlapping) promoter in the 3′ leader sequence, but ignores all transcriptional regulatory signals to transcribe a full-length, positive-sense antigenome. This requires concomitant encapsidation of the nascent strand. It is assumed that the nascent 5′ termini contain specific nucleation sites for encapsidation, although nonspecific encapsidation of nonviral RNA has been reported for several paramyxoviruses. A promoter in the 3′ untranslated region of the new antigenome (the complement to the trailer sequence) serves as a starting point for the synthesis of new, negative-sense genomes. The production of antigenomes and genomes depends on the continuous availability of nucleoprotein. It has long been assumed that the availability of nucleoprotein regulates the switch from transcription to replication; however, there is some evidence that this is not the case (Fearns et al. 1997). New genomes can serve as templates for secondary transcription.

Virus assembly

The assembly of virus particles requires cessation of genome replication, preparation of completed nucleocapsids for packaging, and preparation of regions of the plasma membrane to accept nucleocapsids for budding. The matrix (M) proteins of Sendai virus and measles virus have been shown to inhibit viral transcription in vitro, this function may be involved in the cessation of replication (Suryanarayana et al. 1994; Ogino et al. 2003). Polymerase complexes remain associated with the packaged nucleocapsid and serve to initiate the next cycle of infection. While the nucleocapsid is assembled in the cytoplasm, the glycoproteins are synthesized in the endoplasmic reticulum and undergo maturation during their transport through the Golgi network to the cell membrane. The M protein clearly plays a role in the interaction between the nucleocapsid and the membrane, but it does not appear to be the driving force behind vesicle formation (Schmitt et al. 2002). Budding of virus particles occurs at specialized membrane locations called lipid rafts, which are characterized by a high content of cholesterol and sphingolipids. While viral glycoproteins are concentrated in lipid rafts (Manie et al. 2000), host proteins are excluded from the viral membrane. The cytoplasmic tails of the F and G/H/HN proteins play a role in interaction with the M protein (Ali and Nayak 2000), transport to cell surfaces (Parks and Lamb 1990), and association with rafts (Dolganiuc et al. 2003). The orientation of particle release from the apical membrane may depend on the interaction of M and the cytoplasmic tails of the glycoproteins (Naim et al. 2000). In Sendai virus, interaction of the M protein with the microtubule network is required for apical release of the virus (Tashiro et al. 1993, 1996).

POLYPEPTIDES

Nucleoprotein

As a result of the location of the N gene at or near the 3′ terminus of the genome, the nucleoprotein (N) is the most abundant structural protein in infected cells and virions. N proteins vary in size from 391 to 553 amino acids and play a pivotal role in virus replication. Both genomic and antigenomic RNA must be encapsidated by N to serve as template for transcription or replication. The nucleocapsid binds the polymerase complex, through interaction with the P subunit of that complex. N–N binding sites and separate sites for the interaction of soluble and assembled N with P have been mapped for several members of the family. Structurally, N proteins can be divided into a core, consisting of the amino terminal 80 percent of the protein, and a carboxyl terminal tail. While the core is relatively well conserved among members of a genus, the tail is highly variable, negatively charged and phosphorylated (Hsu and Kingsbury 1982; Curran et al. 1993; Ryan et al. 1993). Presumably the assembled N protein also interacts with the M protein during virion assembly (Coronel et al. 2001). Despite the close association of N with RNA, classic RNA-binding motifs are absent from the predicted amino acid sequences examined (Dreyfuss et al. 1993).

Phosphoprotein

Members of the subfamily *Paramyxovirinae* have evolved unique mechanisms to maximize the available coding capacity of their genes without expanding their overall genome size. These mechanisms include leaky ribosomal scanning, internal ribosomal initiation, and use of non-AUG start codons (Curran and Kolakofsky 1988). Secondly, transcriptional diversity is generated by RNA editing. In contrast, members of the subfamily *Pneumovirinae* encode only a P protein from the cognate gene, thus defining a major difference between the subfamilies.

All *Paramyxovirinae* express a P protein, although the strategy for expression varies among the genera. All genera except for the rubulaviruses express the P from unedited mRNA, while insertion of a single G residue provides access to the V ORF. In rubulaviruses, the unedited transcript codes for the V protein, and the P protein is expressed from an edited message containing an insertion of two G residues. The P proteins are the most variable of the paramyxovirus structural proteins with only 22–40 percent amino acid homology being observed between each genus. The P proteins vary in size from 241 to 709 amino acids and are essential components of the viral polymerase complex.

P proteins are believed to serve as scaffolding proteins by binding both the nucleocapsid and the polymerase (L) proteins. In addition, P proteins act as chaperones for the N protein during nucleocapsid assembly. The active form of P is a tetramer and binding sites for P–P, P–L, P–N, and P–nucleocapsid interactions have been mapped for members of the respiroviruses and morbilliviruses. With the exception of the P–N-binding site, all are located in the unique carboxyl terminal domain. None of the proteins translated from edited mRNAs or alternative reading frames possess these sites. However, the binding site for the soluble N lies within the amino terminus of P and is shared with the V and W proteins (Curran et al. 1995). While information about the P proteins of the *Pneumovirinae* is not as detailed, the available data indicate that the functional domains are similar to those of the *Paramyxovirinae* (Hengst and Kiefer 2000).

The amino terminal domain of the P proteins (and the V proteins) of respiroviruses, morbilliviruses, and henipaviruses is 231–403 amino acids in length, acidic in nature and contains most of the serine and threonine phosphorylation sites. The amino terminal domain of the P proteins of the rubulaviruses and avulaviruses are smaller (134–164 amino acids) and basic in nature. Based on some limited sequence similarity, it has been speculated that the amino terminus of the P and V proteins of rubulaviruses share characteristics with the C proteins found in the other genera of the subfamily *Paramyxovirinae*. In this context, it is interesting that the rubulaviruses are the only genus in the subfamily that does not express a C protein.

V protein and other editing products

Most members of the subfamily *Paramxyovirinae* edit their *P* gene mRNA cotranscriptionally to access additional reading frames. The only exception is human parainfluenzavirus type 1, which has nine stop codons in the V reading frame (Rochat et al. 1992). V proteins are fusion proteins containing the terminal domain of P and a relatively short, conserved cysteine-rich C terminus. As for the P proteins, the amino terminus of the V protein of the rubulaviruses is basic, while the other genera express V proteins with acidic amino termini. The V proteins of the rubulaviruses also differ from those of the other genera by their localization in the nucleus of infected cells, the presence of RNA-binding domains, and the incorporation into virions (Thomas et al. 1988; Takeuchi et al. 1990). V proteins of the other genera are nonstructural proteins and localized in the cytoplasm of infected cells. The seven conserved cysteines in the unique carboxyl terminus of V proteins form zinc-binding domains.

The V proteins of rubulaviruses and morbilliviruses have been shown to interfere with the innate immune system by inhibiting interferon signaling (He et al. 2002; Rodriguez et al. 2002; Palosaari et al. 2003). The inhibition involves sequestering and/or degrading either STAT1 or STAT2 (Parisien et al. 2001). V proteins have been shown to bind a host-cell protein, damage-specific DNA-binding protein (DDB1) (Lin et al. 1998). V proteins inhibit genome replication in a dose-dependent fashion (Curran et al. 1991). Recombinant viruses defective in V expression have been generated for several species, demonstrating that V is dispensable for replication in cell culture. However, all of these viruses are attenuated in animal models, indicating that V proteins are virulence factors, probably due to their role as interferon antagonists (Kato et al. 1997; Schneider et al. 1997; Durbin et al. 1999).

The editing process is not limited to the addition of one G (or two in the rubulaviruses). The number of inserted Gs and relative abundance of edited transcripts vary between viruses. In several viruses, additional proteins have been detected, although their functions have not been determined. The insertion of two nucleotides opens the third reading frame in all genera, except rubulaviruses. These proteins include the W protein of Sendai virus and the D proteins of human parainfluenzavirus 3 and bovine parainfluenzavirus 3. In the rubulaviruses, addition of two Gs accesses the P ORF, but addition of one G leads to the production of the I protein.

C proteins

An overlapping ORF that encodes one or more short (156–215 amino acids), basic proteins can be found near the 5′ end of the P mRNA of the respiroviruses, morbilliviruses, and henipaviruses. The respiroviruses express up to four carboxyl coterminal proteins from different start sites in this ORF (C′, C, Y1, and Y2) (Curran and Kolakofsky 1989), while the other two genera express only a single C protein. Since the entire ORF is located upstream of the editing site, the C proteins are expressed from both edited and unedited transcripts of the *P* gene. Translation of some of the C related proteins of the respiroviruses start at non-AUG codons (Curran and Kolakofsky 1989). Most of these proteins are nonstructural, although small amounts of the C

protein of Sendai virus are found in virions (Yamada et al. 1990).

Several C proteins have been shown to interfere with the interferon response in a manner very similar to that described for V proteins (Gotoh et al. 1999; Shaffer et al. 2003). This function appears to be redundant in at least some viruses that express both C and V proteins. The C proteins of Sendai virus and measles virus have been shown to inhibit replication and transcription (Reutter et al. 2001; Curran et al. 1992), and the C of Sendai may have an additional role in virus assembly (Hasan et al. 2000). Recombinant viruses defective in C expression have been generated for several species, demonstrating that C is dispensable in cell culture (Baron and Barrett 2000; Radecke and Billeter 1996). However, many of these C minus viruses demonstrated altered growth characteristics and attenuation in vitro, and all of them were attenuated in animal models, indicating that C proteins are virulence factors (Valsamakis et al. 1998).

Matrix protein

As noted earlier, the matrix (M) proteins are the most likely candidates for interaction with lipids, the cytoplasmic tails of the glycoproteins (Sanderson et al. 1993) and the newly formed nucleocapsids (Stricker et al. 1994; Yoshida et al. 1986) in preparation for budding (Peeples 1991). The M proteins vary between 245 and 375 amino acid residues in length and are nonglycosylated proteins of an amphipathic nature. They contain both hydrophobic amino acids and basic amino acids. The latter lead to a net positive charge of +14 to +17 at neutral pH (Bellini et al. 1986; Sheshberadaran and Lamb 1990). However, despite the hydrophobic amino acids, M proteins do not contain transmembrane domains. Thus, it is believed that these proteins interact with the inner surfaces of the virion lipid envelope and plasma membrane. The M protein may be involved in the cessation of replication, orientation of particle release from the apical membrane, and interaction with the microtubule network (Tashiro et al. 1993, 1996).

Fusion protein

The F protein promotes fusion between the viral envelope and the plasma membrane of the host cell, as well as fusion between an infected cell and surrounding uninfected cells. These fusion events take place at neutral pH. F proteins are more highly conserved than the attachment proteins and form homotrimers. They are type I integral membrane glycoproteins, which have carboxyl termini extensions into the cytoplasm, while the amino terminus extends into the extracellular milieu. In all paramyxoviruses, F is synthesized as an inactive precursor, F_0. This precursor is proteolytically cleaved into two fragments, F_1 and F_2, which are linked by disulfide bonds and constitute the active form of the molecule. They contain a signal sequence at the amino terminus that is cleaved when the molecule is delivered to the endoplasmic reticular membrane for completion of synthesis. F proteins further contain a hydrophobic domain (known as a stop transfer signal) at the C terminus of F_1 that anchors the protein in the membrane. A short cytoplasmic tail of between 20 and 40 amino acid residues in length extends into the cytoplasm (Figure 2). Although regions of extensive amino acid homology are not obvious among the genera, there is similarity in the number and location of cysteine residues suggesting similar folded structures (Morrison and Portner 1991).

Cleavage of F_0 by intracellular or secreted host endoproteases creates two disulfide-linked subunits, F_1 and F_2. The efficiency of cleavage affects the host range and tissue tropism of the virus, such that greater cleavage efficiency is generally associated with disseminated infections and increased virulence, especially in Newcastle disease virus (de Leeuw et al. 2003). The F protein cleavage sites contain either a single or multiple basic amino acids. Viruses that contain a multibasic sequence are cleaved in the trans-Golgi network by a protease with the sequence specificity of R–X–K/R–R. An endoprotease, furin, has been identified as one protease having such properties (Barr 1991; Hosaka et al. 1991; Ortmann et al. 1994). Cleavage sites containing a single basic residue are cleaved by proteases expressed at the cell surface. Several candidate proteases have been identified (Gotoh et al. 1992). The cleavage site of the henipaviruses is unique in having the only F_1 protein that begins with a leucine instead of a phenylalanine (Wang et al. 2001). Most members of the genus *Pneumovirus* have two cleavage sites, both of which are required for activation (van den Hoogen 2004).

The larger subunit, F_1, contains a hydrophobic fusion peptide at its amino terminus (Figure 2). The fusion peptide, which is highly conserved among the *Paramyxovirinae*, mediates the fusion of the two membranes. There is less conservation in the fusion peptide within the members of the subfamily *Pneumovirinae*, and very little conservation exists between the fusion peptides of members of the two paramyxovirus subfamilies.

F_1 proteins of the *Paramyxovirinae* contain two heptad repeats (HR) (McGinnes et al. 2001). These are three to four repeats of seven amino acids, with hydrophobic amino acids in positions a and d. It is thought that engagement of the viral attachment protein triggers conformational changes in F that promote fusion (Dutch et al. 2001). The six HR in the F homotrimer form a six-helix bundle that represents the most stable form of the molecule and is required for fusion. HR A occurs adjacent to the fusion peptide and may be involved in the conformational alteration of this peptide that enhances fusion (Chambers et al. 1990). HR B is located adjacent to the transmembrane region. Peptides corresponding to HRs can inhibit fusion (Yao and

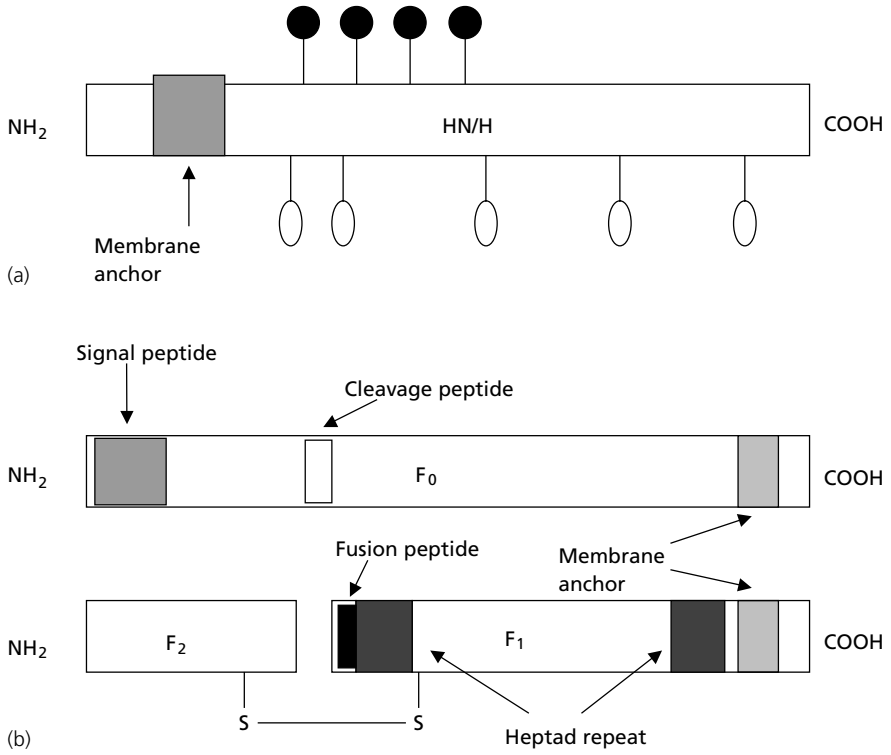

Figure 2 *Schematic diagrams depicting the most salient features of the attachment protein and fusion protein of the family* Paramyxoviridae. **(a)** *Figure shows an idealized HN or H attachment type II glycoprotein. The stop transfer or membrane anchor sequences are shown near the amino terminus of the molecule. The lollipop structures in black along the top of the diagram display the clustering of* N*-linked glycosylation sites observed in the* H *genes of many morbilliviruses, and the open structures display the more general distribution observed for the HN proteins. The G proteins (not shown) share the same orientation in membrane, contain both* N*-linked and* O*-linked glycosylation sites, and are about one-half the size of either HN or H.* **(b)** *The fusion (F) protein is a type I glycoprotein. The uncleaved form of F, F_0, is shown with a signal peptide at the NH2 terminus, which permits entry into the Golgi endoplasmic reticular lumen for membrane-associated protein synthesis. The cleavage peptide, made up of one or more basic residues, is indicated by the open box. Finally, the membrane anchor sequence at the COOH terminus is depicted. Also shown is the cleaved form of F_0 composed of F_1 and F_2 peptides. Note creation of a new NH_2 terminus on F_1 containing the fusion peptide. Regions of F1 containing heptad repeats for the* Paramyxovirinae *subfamily are also clearly labeled. The F protein contains* N*-linked sugar residues primarily on the F_2 molecule, but often on the F_1 as well. The F_1 and F_2 subunits are covalently linked via a single disulfide bond. (Figure courtesy of K. Sleeman.)*

Compans 1996; Young et al. 1997). The F proteins of paramyxoviruses share important structural features with the fusion proteins of viruses from other families including human immunodeficiency virus, Ebola virus, and influenza A virus. The common features include the structure of the core trimer, proteolytic cleavage requirement, and the position and composition of the HRs (Baker et al. 1999; Lamb et al. 1999).

F proteins of most genera are glycosylated on the F_1 and F_2 subunits except for the morbilliviruses and henipaviruses, in which only the F_2 subunit is glycosylated. Although the attachment proteins are the major targets of neutralizing antibodies, antibodies raised against F proteins are also able to neutralize infections.

Attachment protein

The attachment proteins of the *Paramyxovirinae* are type II integral membrane proteins. Such proteins have their amino termini extending into the cytoplasm from the inner surface of the plasma membrane and the carboxyl termini extending from the outer surface of the plasma membrane into the extracellular milieu (Figure 2). The attachment proteins of the morbilliviruses are referred to as H because they possess hemagglutination activity. The attachment proteins of respiroviruses, rubulaviruses, and avulaviruses have hemagglutination and neuraminidase activities and are abbreviated as HN. Since the attachment proteins of the henipaviruses and the *Pneumovirinae* do not possess hemagglutination nor neuraminidase activities, these proteins are referred to as G. The attachment proteins of the two subfamilies share no sequence homologies and therefore are treated separately in the following paragraphs.

There is considerable sequence homology between the H proteins of morbilliviruses and the HN proteins of the respiroviruses, rubulaviruses, and avulaviruses, while the G proteins of the henipaviruses are quite

different. The predicted amino acid sequences of HN proteins range in size from 565 to 582 amino acids, while the H proteins of the morbilliviruses and the G proteins of henipaviruses are larger having 602–617 amino acids. The HN protein of NDV is expressed as an inactive precursor, HN0, which is activated by removal of a carboxyl terminal fragment (Nagai et al. 1989). The HN/H/G proteins are glycosylated with *N*-linked oligosaccharides only, there are between three and eight potential sites per molecule. The general structure of the attachment proteins consists of a cytoplasmic domain, a membrane-spanning region, a stalk region and a globular head. The crystal structure of the NDV HN globular head has been determined (Crennell et al. 2000). Based on these data and extensive computer modeling, the globular head of the attachment proteins of the *Paramyxovirinae* is a propeller-shaped superbarrel of six cyclically arranged beta-sheets, and these proteins are believed to be disulfide-linked homodimers, two of which form noncovalently linked tetramers (Langedijk et al. 1997; Malvoisin and Wild 1993).

The attachment protein promotes fusion, as the ability of the F protein to fuse membranes depends on the presence of the homologous HN/H/G. The exception to this is the *Rubulavirus*, simian virus 5, which does not require the HN protein for fusion. It is believed that HN/H/G positions the membranes for access by the fusion peptide and/or causes a conformational change in F that allows fusion to occur. The fusion-helper function of HN/H/G only occurs with homologous F proteins or those of closely related viruses within the same genus (Bossart et al. 2002).

The HN glycoproteins of the paramyxoviruses and rubulaviruses employ sialic acid-containing cell surface molecules as receptors. The membrane distal globular head region contains the sialic acid-binding site. The HN proteins of respiroviruses proteins contain a conserved amino acid sequence (NRKSKS), similar to the sialic acid-binding site of influenza virus neuraminidase (Morrison and Portner 1991). The neuraminidase activity cleaves sialic acid residues, presumably to prevent budding virions from self-aggregation. Two cellular receptors have been identified for measles virus, CD46, a complement regulatory protein, and human SLAM (Dorig et al. 1993; Naniche et al. 1993; Tatsuo et al. 2000). The canine and bovine homologues of human SLAM serve as receptors for the morbilliviruses, canine distemper virus and rinderpest virus (Tatsuo et al. 2001).

The HN/H is also responsible for the ability of the viruses to bind erythrocytes. Whether there are two separate sites on HN proteins for hemagglutination and neuraminidase activity, or only one, remains unclear. The attachment proteins of the *Paramyxovirinae* are usually highly antigenic and are a major target for neutralizing antibodies.

The G proteins of the subfamily *Pneumovirinae* have little in common with the attachment proteins of the *Paramyxovirinae*, apart from their type II glycoprotein configuration and attachment property. G proteins are about half the size of HN and H proteins, the putative ORF of the G protein of human metapneumovirus is only 263 amino acids long (van den Hoogen 2004). They lack neuraminidase activity, and, with the exception of pneumonia virus of mice, also lack hemagglutination activity. There is little sequence homology even within the subfamily. G proteins probably exist as noncovalently linked trimers or tetramers (Wertz et al. 1989; Collins and Mottet 1992). The major distinguishing feature of the G proteins of the pneumoviruses is the abundance of *O*-linked sugar residues. *O*-linked glycosylation occurs at serine or threonine residues, which comprise 30 percent of the amino acid residues on the G protein of respiratory syncytial virus (Wertz et al. 1989; Collins and Mottet 1992). In addition, there are three to seven potential sites for *N*-linked glycosylation. *N*-linked glycosylation seems to occur co-translationally and is a requirement for the proper folding of the G proteins, as with the attachment proteins of the *Paramyxovirinae*. *O*-linked oligosaccharides are added after the folding is completed. The high carbohydrate content explains the difference between the predicted and apparent molecular weight of the G protein of respiratory syncytial virus (32.6 vs 90 kDa) (Wertz et al. 1985). While it is known that human respiratory syncytial virus attaches to glycosaminoglycans containing heparin sulfate, no specific receptors for this virus or other members of the subfamily have been identified.

Large (polymerase) protein

The gene closest to the 5′ terminus of the genome and the least abundant protein is the viral RNA-dependent RNA polymerase or large (L) protein. The protein accounts for 40–50 percent of the coding capacity of the viral genome. The large size of the protein (2 000–2 300 amino acid) reflects the many enzymatic and binding functions ascribed to this protein. The L proteins of the metapneumoviruses are the smallest, with only 2 004 amino acids for avian paramyxovirus (van den Hoogen 2004). The L protein is assumed to carry out all the enzymatic activities of the polymerase complex, namely initiation, elongation, and termination of both mRNA transcription and genome replication. mRNA transcription includes capping and methylation of the 5′ end of messages, as well as polyadenylation. The templates for poly(A) tails are the short poly(U) sequences that are part of the transcription termination signals of the genes. It is assumed that the polymerase moves back and forth over these sequences to synthesize poly(A) tails of more than 100 nucleotides. This process has been called ‘stuttering.’ A similar slippage effect is the basis for the

'editing' of the P mRNAs of the *Paramyxovirinae*. Whether L is involved in phosphorylation of other proteins remains uncertain.

It has recently been shown that the L proteins of respiroviruses form homo oligomers (Smallwood and Moyer 2004). Since P proteins form homo tetramers (Tarbouriech et al. 2000) and the ratio of L to P in the polymerase complex has been shown to be 1:4, the minimal polymerase complex would be L2P8; however, the exact composition is unknown. Since L proteins also interact with C proteins (Horikami et al. 1997; Sweetman et al. 2001; Smallwood and Moyer 2004), they must contain L–L-, L–P-, and L–C-binding sites, several of which have been mapped for morbilliviruses, respiroviruses, and rubulaviruses.

A comparison of the amino acid sequences of the L proteins of several members of the order *Mononegavirales* lead to the identification of six domains, which are characterized by a higher level of similarity (Poch et al. 1990). Within these domains lie shorter regions of even higher conservation that are assumed to be important for polymerase function. Several putative sites for template binding, ATP binding, and nucleotide binding have been identified. A highly conserved GDN motif in domain III may be the catalytic site for phosphodiester bond formation and be analogous to the GDD catalytic site in poliovirus. Interestingly, the amino acids flanking this motif are glutamine (QGDNQ) in all members of the family except in L proteins of the henipaviruses, which have a QGDNE motif (Harcourt et al. 2001).

L proteins are believed to function only in combination with homologous N and P proteins and on templates with homologous termini, although a few exceptions among closely related members of the same genus have been noted (Curran and Kolakofsky 1991). Therefore, binding sites and promoters have co-evolved. Data derived from experimental respiratory syncytial virus and bovine parainfluenzavirus 3 vaccine candidates demonstrate that mutations in L can play a role in viral attenuation (Crowe et al. 1996; Juhasz et al. 1997).

Small hydrophobic proteins

Simian virus 5 and mumps virus (genus *Rubulavirus*), avian paramyxovirus 6 (genus *Avulavirus*), and all members of the subfamily *Pneumovirinae* have genes encoding small hydrophobic proteins (SH). These proteins have little in common beyond the fact that they are integral membrane proteins and they share no sequence homologies. They vary in length from 44 to 183 amino acids. Some are type II membrane proteins (Bukreyev et al. 1997), while others are type I proteins (Takeuchi et al. 1996). The SH proteins of the metapneumoviruses have two potential transmembrane regions (van den Hoogen 2004). The SH proteins of simian virus 5 and human respiratory syncytial virus are structural proteins; however, this has not been proven for some of the other viruses. Spontaneous deletions of the gene or expression of defective SH proteins have been described for human respiratory syncytial virus and mumps virus strains, and recombinant viruses with deletions of the *SH* gene grow well in cell culture (Takeuchi et al. 1996). However, a recombinant human respiratory syncytial virus with a deletion of the *SH* gene was attenuated in chimpanzees (Bukreyev et al. 1997). The SH protein of simian virus 5 appears to be involved in the suppression of apoptosis (He et al. 2001).

NS1 and NS2 proteins

The members of the genus *Pneumovirus* encode two nonstructural proteins, NS1 and NS2. Their genes are located at the 3′ terminus of the genome, which leads to high levels of transcription. NS1 is 136–139 amino acids long, and NS2 is 124 amino acids in length. NS1 inhibits both transcription and replication in minigenome replication assays and NS2 had a similar, but much less pronounced effect (Atreya et al. 1998). Recombinant viruses with deletions of one or both genes demonstrate that these proteins are dispensable for growth in cell culture; however, the mutants are attenuated both in cell culture and in chimpanzees (Jin et al. 2000). NS1 and NS2 of bovine respiratory syncytial virus and human respiratory syncytial virus have been shown to protect the virus from the effects of interferon-α/β (Schlender et al. 2000; Bossert and Conzelmann 2002). Interestingly, the inhibition of interferon required expression of both proteins, indicating a cooperative action. It appears that the function of the NS proteins may be comparable to that of the C and V proteins of the *Paramyxovirinae*.

M2-1 and M2-2 proteins

All members of the subfamily *Pneumoviridae* have an *M2* gene, which expresses two proteins, M2-1 and M2-2. The M2-1 of human respiratory syncytial virus, also called the 22K protein, is 194 amino acids in length, and is a hydrophilic, phosphorylated, structural protein found associated with the nucleocapsids (Garcia et al. 1993). It acts as a processivity and antitermination factor in transcription (Collins et al. 1996; Hardy and Wertz 1998). The protein is required to transcribe longer genes completely and leads to an increase in read-through transcripts at intergenic junctions. M2-1 has no effect on replication. A C_3H_1 motif near the amino terminus, which may bind zinc, is conserved in all M2-1 proteins and is required for the function of the protein (Hardy and Wertz 2000). M2-1 binds RNA and the N protein (Cuesta et al. 2000). Deletion of the M2-1 ORF does not produce viable virus.

The M2-2 protein is translated from a reading frame that overlaps the end of the M2-1 ORF. The M2-2 protein of human respiratory syncytial virus is 90 amino acids in length and nonstructural. Deletion of the ORF produced a virus viable in cell culture, showing that the gene is dispensable; however, the mutant was attenuated in cell culture and in chimpanzees (Teng et al. 2000). The M2-2 minus mutant was further characterized by a shift from replication to transcription, with increased and continued mRNA production and decreased replication, suggesting that this protein is involved in regulating the balance of transcription and replication (Bermingham and Collins 1999).

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Morbilliviruses: measles virus

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INTRODUCTION

Acute measles is a well-defined clinical entity normally contracted by children and young adults. The causative agent, Measles virus (MeV), is an efficient pathogen, persisting in nature in populations large enough to support it, although it causes an acute infection only once in a lifetime of the individual. The virus is monotypic and antigenically stable, however, several genotypes can be distinguished some of which are inactive, while others are still active and are drifting on a genetic level worldwide. MeV is a highly successful virus, which has efficiently exploited its potential for spread. Although nonhuman primates are susceptible to infection, transmission from animals is not an important means of introducing the disease into a community. The virus may persist on rare occasions in single individuals for years, but these persistent infections are not associated with periodic shedding of infectious virus. A single attack of measles is sufficient to confer long-lasting, probably life-long immunity to re-infection. Consequently, in order to remain endemic in a given community, the virus must rely on the infection of unprotected susceptible individuals, mainly small infants with waning levels of maternal antibodies. The efficiency of this process is documented by the first known report of measles (in Egyptian hieroglyphics), which failed to recognize the infectious nature of the illness, and described it as a normal part of child growth and development.

In the prevaccine era, the maximum incidence of measles was seen in children aged 5–9 years in developed countries. Outbreaks and epidemics centered around elementary schools, while younger children acquired measles as secondary cases from their school-age siblings. By the age of 20, approximately 99 percent of subjects tested had been exposed to the virus. The introduction of the measles vaccine had a significant impact on the age incidence and percentage of measles cases in different age groups. In countries with an optimal vaccine implementation, measles infection has shifted to the teenage group, whereas in areas with inadequate vaccine programs children up to 4 years of age reveal a high primary measles attack rate (Bellini et al. 1994). In contrast, in developing countries, where measles has its greatest incidence in children under 2 years of age, the disease is a serious problem with a high mortality (up to 10 percent), with malnutrition being a precipitating factor for the severity of the disease. Therefore, the pattern of epidemiology observed differs markedly in different parts of the world, and a thorough understanding of this is essential to the development of successful vaccination programs.

CLASSIFICATION

Measles virus (MeV) is a member of the order *Mononegavirales* which includes the families *Rhabdo-*, *Filo-* and *Paramyxoviridae*. As a *Paramyxovirus*, MeV reveals structural and biochemical features associated

with this group (see General properties of the Paramyxoviruses). As it lacks a virion-associated neuraminidase activity and causes formation of intranuclear inclusion bodies, it has been grouped into a separate genus, *Morbillivirus* of which it is the type species. Other members of this genus include *Peste des petits ruminants virus* (PPRV), which infects sheep and goats, *Rinderpest virus* (RPV), which infects cattle, *Canine distemper virus* (CDV), which infects dogs, *Phocine distemper virus* (PDV), which infects seals and sea lions, dolphin morbillivirus (DMV), and porpoise morbillivirus (PMV) (see Paramyxoviruses of animals). All these viruses exhibit antigenic similarities, and produce similar diseases in their host species. Since MeV most closely resembles RPV, it has been suggested that the virus evolved in an environment in which cattle and humans lived in close proximity. Thus, measles is a relatively new disease of humans and a disease of civilization.

MORPHOLOGY, STRUCTURE, PHYSICOCHEMICAL, AND BIOLOGICAL PROPERTIES

MeV particles consist of a lipid envelope surrounding the viral RNP complex which is composed of genomic RNA associated with proteins (Figure 1a). Two transmembrane proteins (fusion (F) and hemagglutinin (H) proteins) project from the envelope surface. Both proteins extend through the virion envelope and appear on its inside surface. The H protein is anchored near its amino terminus (type II glycoprotein), and the F protein near its carboxy terminus (type I glycoprotein). One or both of the cytoplasmic domains functionally and probably physically interact with the matrix (M) protein (Cathomen et al. 1997a; Moll et al. 2001, 2002) which also links the envelope to the RNP core structure. The viral genomic RNA is fully encapsidated by nucleocapsid (N) protein and associated with the polymerase complex, consisting of the phosphoprotein (P) and the large (L) proteins, to form the RNP core structure that resists RNase degradation (Griffin and Bellini 1996). The virions are highly pleomorphic with an average size of 120 to >300 nm and both filamentous and irregular forms are known. The virion shown in an electron micrograph is bounded by a lipid envelope which bears a fringe of spike-like projections (peplomers) 5–8 nm long (Figure 2). The membrane below the spikes is 10–20 nm in thickness and the enclosed helical viral RNP core has a diameter of 17 nm and a regular pitch of 5 nm. The cargo space of the particles may vary from

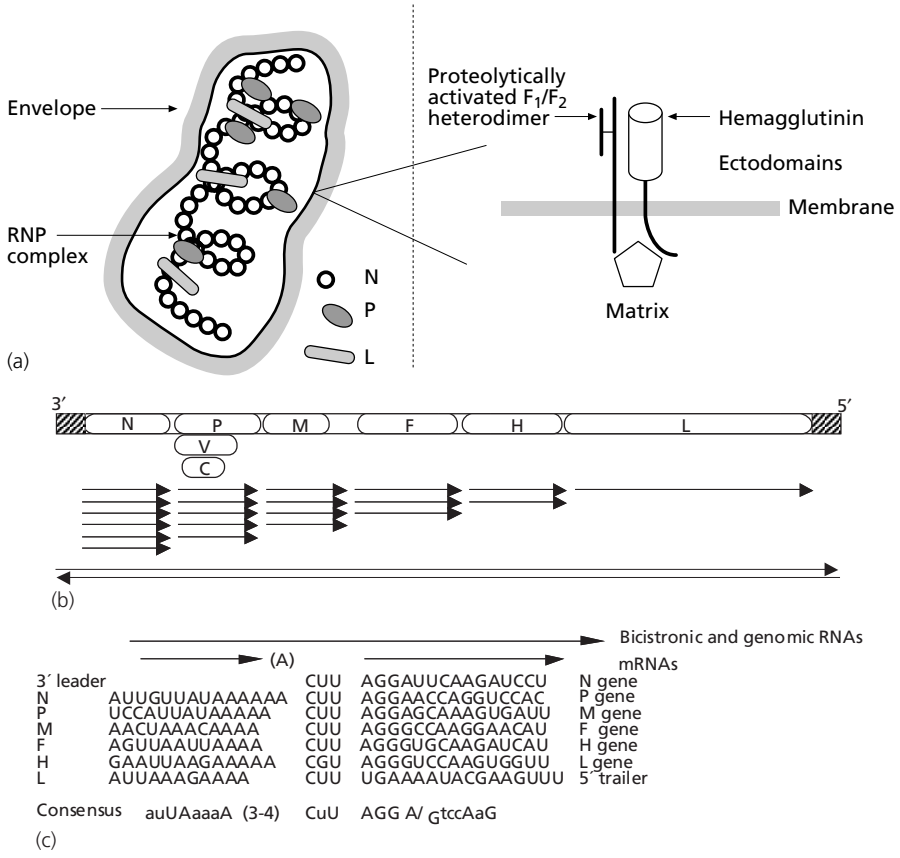

Figure 1 *MeV: Structure, genome, and replication.* **(a)** *Schematic representation of the pleomorphic MeV particle (left) and (right) the organization of its membrane proteins.* **(b)** *Genomic organization, open reading frames and representation of monocistronic and genomic transcripts.* **(c)** *Intergenic boundaries separating MeV transcription units and their consensus sequence.*

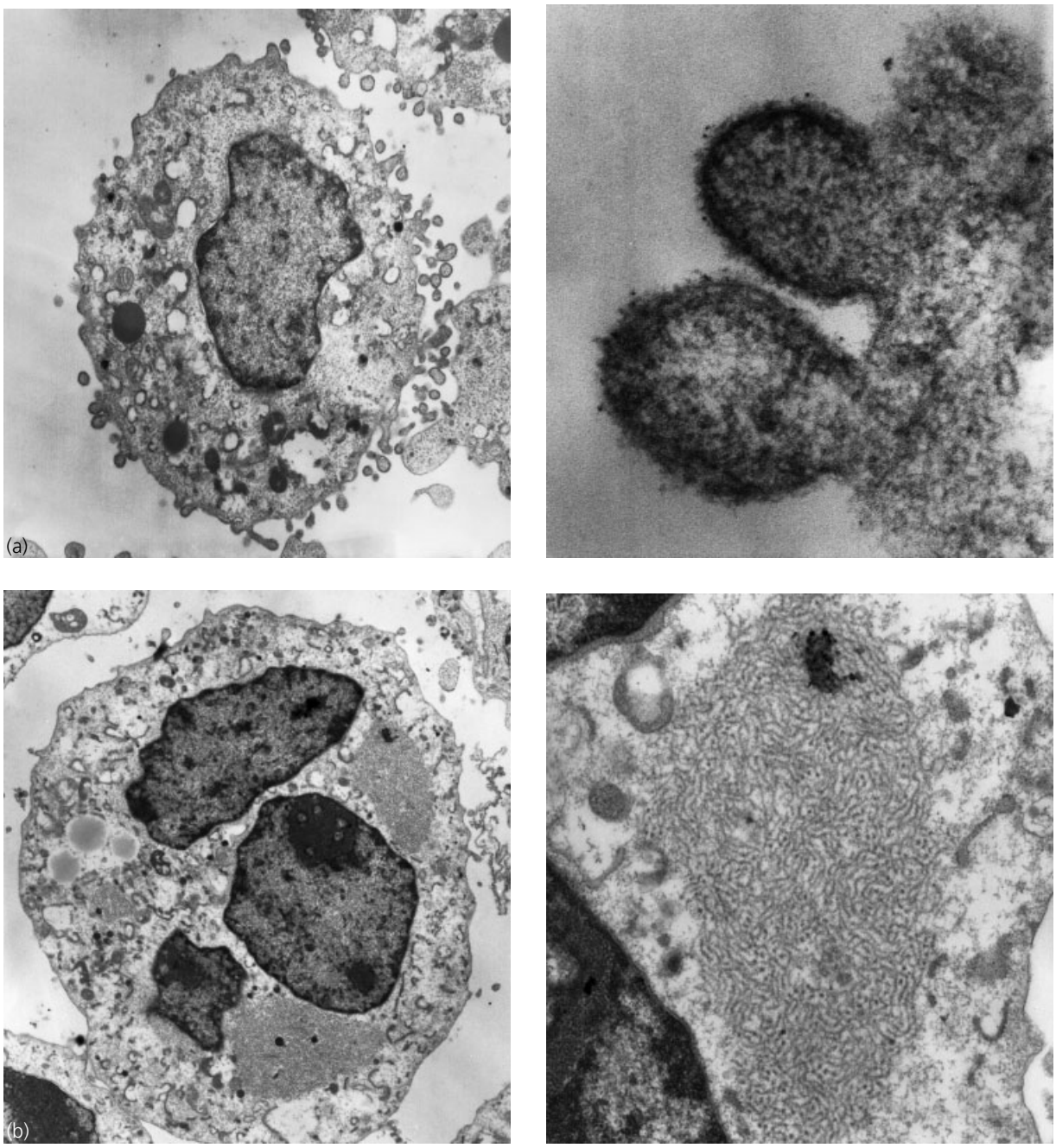

Figure 2 *MeV virions budding from the surface of infected cells* **(a)**, *left panel, and magnified, (right panel) and viral nucleocapsids accumulating in large cytoplasmic aggregates* **(b)**, *left panel, and magnified, (right panel) (kindly provided by Jürgen Schneider-Schäulies).*

3×10^5 to $>10^7$ nm^3 and could accommodate >30 genomes. In fact, packaging of multiple genomes within a single particle in vitro has been confirmed (Rager et al. 2002). Immediately below the membrane M proteins appear as a shell of electron dense material. The structure of the virion explains much of the early data concerning the stability of the virus. The integrity of the particle envelope is essential for infectivity which is inactivated by any compound which disrupts this structure, such as detergents or other lipid solvents including acetone or ether. Particles are acid labile and inactivated below pH 4.5, although they remain infectious between pH 5–9. MeV is thermolabile and has a half-life of 2 h at 37°C. Viability of the virus is maintained for 2 weeks at 4°C, but it is inactivated after 30 min at 56°C. Thermolability is probably due to an effect on the internal structure of the particle, since the hemagglutinin is relatively temperature resistant. The virus can be stored for

prolonged periods at −70°C and is amenable to freeze-drying. These properties have important consequences for the transport and storage of the vaccine.

Unlike other members of the morbilliviruses, MeV displays hemagglutination activity. This is easily demonstrated using monkey erythrocytes from rhesus, patas, and African green monkeys and baboons, but not from humans. MeV lacks detectable neuraminidase activity and does not attach to receptors containing sialic acid. Consequently, once attached to a red cell, MeV does not re-elute rapidly. H protein inserted into membranous structures is active in the hemagglutination test (HA). Virus particles separated by isopycnic centrifugation have a buoyant density of 1.23 g/cm^3, where HA activity is also detected. A large amount of hemagglutinating material is also found in the upper regions of the gradient (light hemagglutinin), which most likely represents H protein inserted into empty membranous cell fragments, or defective virus particles. Some lymphotropic MeV wild-type strains have very low hemagglutination activity which may be due to an additional *N*-linked glycosylation site within their H proteins or their low affinity for CD46, the MeV receptor expressed on monkey erythrocytes (see below). H is a major viral immunogen, and H-specific antibodies have hemagglutination-inhibiting (HI) and virus-neutralizing (NT) activities as they block the viral attachment to target cells. These antibodies do not prevent progressive viral cell-to-cell spread mediated by the F protein. The ability to lyze red blood cells once the virus has bound is mediated by the F protein. As with hemagglutination, this property is artificial, but hemolysis (HL) provides a convenient measure of F protein activity which is more sensitive to both pH and temperature than HA, and is optimal at 37°C and pH 7.4. The ability of the MeV to fuse at neutral pH accounts for the characteristic cytopathic effect (CPE) induced by these viruses, the formation of giant cells.

Genome structure, function, and replication

The 15 894 bases long viral genome is a nonsegmented RNA molecule of negative polarity that is entirely complexed with N proteins and the rigidity of this interaction is believed to account for the absence of genome recombination in the *Mononegavirales*. Each N protein covers six nucleotides, and this is thought to be the reason why only viral genomes whose number of nucleotides is a multimer of six are efficiently replicated by the viral polymerase. This constraint seems to be essential since in recombinant MeV genomes, hexameric genomic length ensuring accurate RNA encapsidation has been recently found to be actively restored (Rager et al. 2002). As long as the rule of six (Calain and Roux 1993) is maintained, MeV tolerates substantial variations in total genome length since recombinant genomes carrying foreign genes as diverse as reporter genes, heterologous envelope genes, or growth factors specificity domains displayed on the H protein can be rescued and stably propagated (Schneider et al. 2000). The standard genome is organized into six contiguous, nonoverlapping transcription units that are separated by (except for the M/F gene boundary) short noncoding regions containing signals for transcription termination, polyadenylation, and transcriptional re-initiation (Figure 1b, c) (Bellini et al. 1994). The genome is flanked by noncoding 3′ leader and 5′ trailer sequences containing specific encapsidation signals and the viral promoters used for viral transcription and/or replication of genomic and antigenomic RNAs (Horikami and Moyer 1995; Parks et al. 2001) (Figure 1c).

During transcription, the polymerase complex enters the genomic RNP at its 3′ end. After synthesis and termination of a short leader RNA, transcription is resumed at the gene start sequence 3′ of the N coding region and pauses at the gene stop sequence where, by a slippage mechanism, a poly(A) stretch is added. After liberation of the N mRNA, transcription of the downstream P mRNA is initiated. By the same stop–restart mechanisms, mRNAs for the other downstream genes are produced. As the polymerase tends to fall off the template at the intergenic boundaries, the probability of transcriptional re-initiation is lower than one, reflected by a decreasing transcriptional efficiency of downstream mRNAs (transcriptional gradient). The differential abundancies of the mRNAs directly translate into the relative amounts of proteins produced. Occasionally, the polymerase complex also ignores the intergenic stop/start signals giving rise to bi- or tricistronic, polyadenylated transcripts. Replication of the genome, or antigenome, respectively, tightly couples elongation and encapsidation. The switch from the transcription to the replication mode of the polymerase complex is thought to be dependent on the availability of N proteins for encapsidation.

From the *P* gene, two nonstructural proteins, C (20 kDa) and V (46 kDa) are expressed. While C protein is encoded within a separate reading frame (Bellini et al. 1985), V protein is translated from co-transcriptionally edited P mRNA. While its amino-terminal domain is identical to that of P, V protein has a unique cysteine-rich domain at the carboxy terminus (Wardrop and Briedis 1991). Editing (nontemplated addition of guanosine (G) ribonucleosides) is an intrinsic activiy of the MeV polymerase and occurs in about 50 percent of the P mRNAs. Additionally, an extremely low abundant protein, termed R, was reported to be expressed from the P mRNA and this required frame-shifting (Liston and Briedis 1995). As a pecularity of the morbilliviruses, an approximately 1 kb GC-rich region separates the M and the F ORFs. While several ORFs have been predicted for this region, these could only be accessed by translation from a rare bicistronic transcript

via ribosomal reinitiation. None of the putatively encoded proteins has yet been detected in infected cells.

MeV structural and nonstructural proteins

Since its first isolation, dissection of the MeV structure and replication strategy has been greatly aided by the development of tissue culture systems, the availability of monoclonal antibodies and, in particular, the availability of molecular biological approaches and techniques. The definition of MeV protein functions in more detail has long been limited by the lack of a system that allows a reverse genetic approach. As for other members of the *Mononegavirales*, plasmid-based systems to rescue infectious MeV in tissue culture have been developed and allow the introduction of stable alterations into the viral genome (Combredet et al. 2003; Radecke et al. 1995; Schneider et al. 1997; Takeda et al. 2000).

The viral RNP and nonstructural proteins

The N (nucleocapsid) protein (60 kDa; 525 amino acids (aa)), the most abundant of the MeV proteins, fully encapsidates the genomic RNA and this is the unique target for the viral polymerase to initiate transcription and replication. The protein is phosphorylated on serine and threonine residues (Gombart et al. 1995), and possibly tyrosine residues in persistent infections (Segev et al. 1995). It acts to condense the viral leader containing RNAs into a smaller, more stable, and more readily packaged form. This gives the nucleocapsid its helical form and 'herringbone' appearance in the electron micrograph (Figure 2). When expressed in the absence of viral RNA, N proteins, having undergone conformational maturation, aggregate into nuclear and cytoplasmic nucleocapsid-like structures. The N-terminal 398 amino acids are required for self-aggregation (Bankamp et al. 1996; Spehner et al. 1997) (Figure 3), while the C-terminal 125 residues are located outside the nucleocapsid. Since it contains a nuclear localization signal, N can be transported into the nucleus. The formation of high affinity protein complexes with the phosphorylated P (for which two noncontiguous domains (aa 4–188 and 304–373) are essential) prevents both nuclear translocation and self-aggregation of N proteins and encapsidation of cellular RNAs during replication of the viral genome (Bankamp et al. 1996; Huber et al. 1991). In addition, there is evidence for the formation of N/V complexes (Tober et al. 1998) (Figure 3). N protein also interacts with cellular proteins. A regulatory domain interacting with Hsp72 has been mapped within the C-terminal 24 amino acids of N, and this may be important for the enhancing effect of heat shock on MeV replication (Vasconcelos et al. 1998; Zhang et al. 2002). Moreover, direct interaction of N protein with IRF-3 and an IRF-3-associated virus-activated kinase may be involved in regulating interferon production during infection (Ten Oever et al. 2002) (Figure 3).

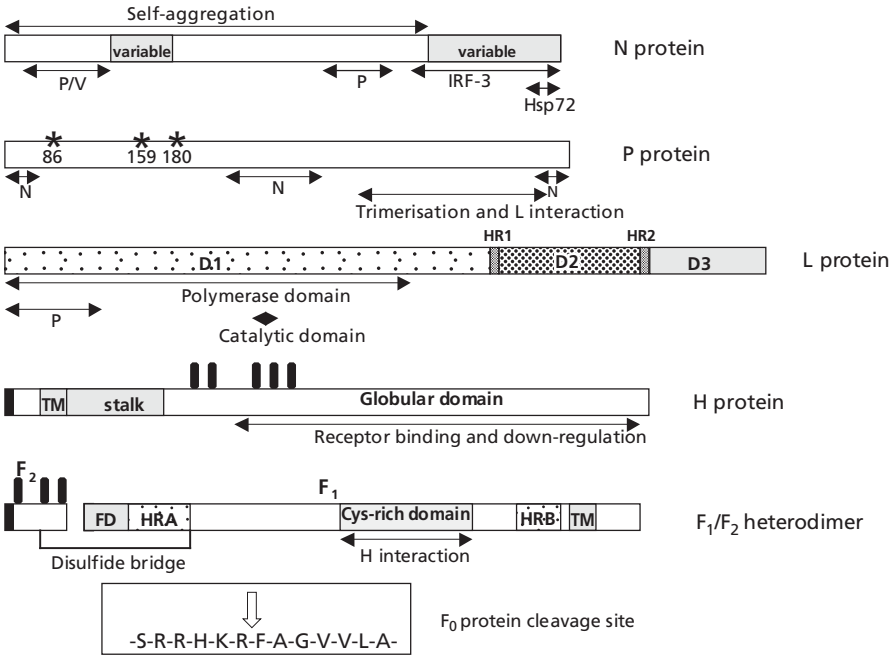

Figure 3 *Schematic representation of the major MeV structural proteins. Functional domains or those identified as essential for complex formation with MeV or cellular proteins are indicated by arrows. Phosphorylation sites within the P protein are indicated as asterisks, gylcosylation sites within the F and H proteins are marked. The sequence of the cleavage site within the F_0 protein precursor is indicated at the bottom. D1–D3, domains 1–3 within the L protein; FD, fusion domain; HRA and HRB, heptad repeat domain A and B; HR1, HR2, hinge region 1 and 2 separating domains within the L protein; Hsp72, heat shock protein 72; IRF-3, interferon response factor 3; TM, transmembrane domain.*

The P protein (72 kDa; 507 aa) is abundant in the infected cell, and only small amounts are packaged into virions. The protein is phosphorylated on serine residues 86, 151, and 180 most likely by the cellular casein kinase II (Das et al. 1995). As for the N protein, phosphorylation on tyrosine residues may occur in persistent infection (Ofir et al. 1996). Phosphorylation of P protein is essential for its function as polymerase cofactor. The protein oligomerizes into homotrimers via a predicted coiled-coil region in its carboxy-terminal domain (Curran et al. 1995; Harty and Palese 1995). Domains important for the interaction with the N protein reside in both the amino and carboxy terminus (Harty and Palese 1995), while a small central region (aa 204–321) was also found essential for the ability of P protein to induce N protein redistribution (Huber et al. 1991). Equally important for both transcription and replication, stable complexes between P and L have to be formed, and apparently L protein is stabilized by this interaction. Moreover, P is thought to act as *trans*-activator regulating L protein functions (Horikami and Moyer 1995) (Figure 3).

The diffusely distributed cytoplasmic V protein (46 kDa) is expressed from P mRNAs edited by insertion of a nontemplated G residue at position 751 after three genomically encoded Gs (Cattaneo et al. 1989a; Wardrop and Briedis 1991). As V protein shares the aminoterminal 231 amino acids with the P protein, it is also phosphorylated at the very same serine residues and is capable to form complexes via this domain with the N protein, but not to oligomerize or form complexes with the L protein (Liston et al. 1995; Tober et al. 1998). Its 68 amino-acid unique C-terminal domain is cysteine-rich and has zinc-binding properties (Cattaneo et al. 1989a; Liston and Briedis 1994).

The highly basic C protein (28 kDa) is also translated from the P mRNA by usage of a initiator codon 19 nucleotides downstream from that for P (Bellini et al. 1985). There is no evidence for its interaction with other MeV proteins, however both V and C proteins may interact with as yet undefined cellular proteins (Liston et al. 1995). The role of C and V proteins in MeV replication is uncertain. Recombinant MeV in which these reading frames were ablated can be rescued and efficiently amplified in standard cell cultures (Radecke and Billeter 1996; Schneider et al. 1997). These proteins may, however, regulate transcription and/or replication in specialized cell types and in MeV pathogenicity in vivo (Escoffier et al. 1999; Patterson et al. 2000; Tober et al. 1998; Valsamakis et al. 1998). As seen with C and/or V proteins of closely related viruses (Goodbourne et al. 2000), recent studies document that the nonstructural MeV proteins efficiently interfere with signaling via the IFN-α/β, and possibly also the IFN-γ, receptor (Palosaari et al. 2003; Shaffer et al. 2003; Takeuchi et al. 2003; Yokota et al. 2003).

The L protein (220 kDa; 2 183 aa) is expressed at low levels in infected cells and packaged into virions in only catalytic amounts. It is a multifunctional RNP-specific RNA polymerase producing mRNAs, replicative intermediates and progeny viral genomic RNAs (Bankamp et al. 2002). It also has multiple enzymatic activities (see also General properties of the Paramyxoviruses). Common to RNA polymerases, it contains a conserved duplicated GDDD motif flanked with hydrophobic residues (Bankamp et al. 1999; Blumberg et al. 1988). The polymerases of the *Mononegavirales* may function as multidomain proteins, and three well-conserved domains (D1, D2, and D3) which are linked by two variable hinges (H1 and H2) were defined by sequence alignments (Figure 3). Interestingly. D3 apparently enjoys a limited conformational independence from the other domains, since insertion of foreign reading frames into H1, but not H2, abolished replication of recombinant MeV (Duprex et al. 2002). Physical interaction with its co-factor, the P protein, requires the presence of the N-terminal 408 amino acids of the L protein.

The envelope proteins

The RNP core structure is surrounded by a lipid envelope with two glycoproteins on its external surface, the hemagglutinin (H) and the fusion (F) protein that are organized as functional complexes and appear as spikes in the EM. Both proteins are also expressed on the surface of infected cells. The H protein mediates viral attachment to receptors on the surface of target cells and also assists the F protein in causing fusion. Both proteins are protease-sensitive, as virions appear smooth under the EM following protease treatment. They can be isolated by gentle detergent lysis of the virion, although F tends to remain strongly associated with actin filaments. The spikes aggregate, presumably via the hydrophobic tails of both proteins which normally anchor them in the lipid bilayer.

The H protein, a type II glycoprotein, can be isolated as a tetrameric complex from the cell membrane. Analogous with the structure of the NDV HN protein, the ectodomain of the MeV H protein is thought to be organized into a membrane proximal stalk and a membrane distal globular head region composed of a six wing propeller structure (Crennell et al. 2000; Langendijk et al. 1997). It is folded and dimerized in the ER (Plemper et al. 2001a). Amongst the 13 strongly conserved cysteine residues within its ectodomain residues 287, 300, 381, 394, 494, 579, and 583 (all located within the globular domain) were found essential for oligomerization and folding (Hu and Norrby 1995). Moreover, cysteine 154 in the stalk region is essential for the formation of disulfide-linked dimers (Plemper et al. 2000; Santiago et al. 2002). Five potential glycosylation sites, four of which are used, are bunched within a region of 70 amino acids (aa 168–238), and glycosylation was found to be essential for proper folding, export from

the Golgi, antigenicity, and hemadsorption, probably by stabilizing the highly complex tertiary structure of the protein (Figure 3). Amino acids important for the differential interaction of MeV strains with their cellular MeV receptors, CD46 and CD150, respectively, which include aa 451, 481, 546, and 473–477, also map within the H protein ectodomain (Bartz et al. 1996, 1998; Hsu et al. 1998; Lecouturier et al. 1996; Patterson et al. 1999). In addition to its receptor binding function, H exerts a helper function in F-mediated membrane fusion probably by directing the fusion domain into the optimal distance to the target cell membrane and stabilizing the interaction (Nussbaum et al. 1995). The cytoplasmic domain of the H protein was found to be important for efficient cell surface transport of the molecule and harbors a basolateral sorting signal (Moll et al. 2001, 2002).

The F protein, a type I glycoprotein, is synthesized as inactive precursor (F_0; 60 kDa) from a mRNA which contains an up to 585-nt long 5′ noncoding GC-rich region that is thought to regulate its translational efficiency (Wild and Buckland 1995). The protein is glycosylated, oligomerizes, and is transported to the surface. The precursor is biologically activated by proteolytic cleavage at a conserved multibasic site (aa 108–112) by a subtilisin-like protease resident in the trans-Golgi to yield a disulfide-linked, biologically active F_1 (40kD)/F_2 (18 kDa) heterodimer (Bolt and Pedersen 1999; Sato et al. 1988). Glycosylation of the F_0 precursor is an essential prerequisite for cleavage and transport, and all potential *N*-glycosylation sites (aa 29, 61, and 67) reside within the F_2 subunit (Wild and Buckland 1995) (Figure 3). Because they most likely alter the conformation of this subunit, mutations of any of these sites affect cell surface transport, proteolytic cleavage, stability, and fusogenic activity of the F protein (Hu et al. 1995). The F_1 subunit reveals a topography typical for type I fusion proteins with an amino-terminal stretch of hydrophobic residues (the fusion domain), and two amphipatic α-helical domains, one of which is adjacent to the fusion domain, the other (containing a leucine-zipper motif) N-terminal of the transmembrane region (Bellini et al. 1994; Lambert et al. 1996). A central, cysteine-rich region (aa 337–381) is thought to mediate interaction with the H protein (Fournier et al. 1997; Wild and Buckland 1995) (Figure 3). Homo- and hetero-oligomerization of both glycoproteins takes place in the ER (Plemper et al. 2001b), and the strength of the F/H interaction and the fusogenic activity of the complex are also influenced by their cytoplasmic tails, both independent and dependent of their interaction with the M protein (Moll et al. 2001, 2002; Plemper et al. 2001a). Interestingly, the 33-aa long cytoplasmic tail of the F_1 subunit is frequently mutated in persistent infections (Billeter and Cattaneo 1994).

The M protein The M protein (37 kDa), translated from a mRNA with a 400 nt 3′ noncoding region (Bellini et al. 1986), is found associated with nucleocapsids and the inner layer of the plasma membrane in infected cells (Bellini et al. 1994; Griffin and Bellini 1996). Interaction of M protein with the cytoplasmic domains of one or both MeV glycoproteins has been documented in physical and functional terms (Naim et al. 2000; Plemper et al. 2001b). Thus, M protein was found to modulate the fusogenic activity of the F/H complex (Cathomen et al. 1997a,b) and interactions of M with the glycoprotein cytoplasmic tails allow M-glycoprotein co-segregation from to the apical surface in polarized cells (Naim et al. 2000). Recombinant MeVs carrying deletions of major parts of the *M* gene bud highly inefficiently thus supporting the initial suggestions that this protein might be essential in this process (Cathomen et al. 1997a). M protein may also negatively regulate MeV transcription when bound to RNPs (Suryanarayana et al. 1994). Mutations within the *M* reading frame are often found in persistent infections (Billeter and Cattaneo 1994).

THE REPLICATION CYCLE

Receptor interactions and entry

The expression of specific receptors available for the interaction with the MeV H protein on the surface of susceptible target cells is one of the most important determinants of viral tropism. During natural infection, MeV reveals a pronounced tropism for hematopoetic cells, but also enters into and replicates in a variety of cell types as it does in tissue culture. Thus, the receptor should be expressed on most human cells both in vivo and in vitro. This is the case for the first MeV receptor identified CD46 (membrane co-factor protein (MCP)), a member of the 'regulators of complement' (RCA) gene family (Doerig et al. 1993; Naniche et al. 1993) (Figure 4 and Table 1). CD46 is expressed on all nucleated cells, and is absent from human erythrocytes, which explains why these cannot be agglutinated by MeV H protein. By means of alternative splicing, several isoforms of CD46 are generated from a precursor mRNA in a tissue-specific manner and all of them support MeV uptake (Buchholz et al. 1996a; Johnstone et al. 1993; Manchester et al. 1994). The ectodomain of CD46 consists of four common membrane distal short consensus repeats (SCR 1–4), while the composition of the membrane proximal serine/threonine/proline (STP) segments and the intracellular carboxytermini (Cyt1 and Cyt 2) varies between the CD46 isoforms (Johnstone et al. 1993). CD46 normally binds complement components C3b and C4b via the SCR3 and 4 domains thereby preventing their deposition and formation of an attack complex on the cell surface (Liszewski et al. 1991). In contrast, an extended binding site for MeV H protein maps to SCR 1 and 2 (Casasnovas et al. 1999; Manchester et al. 1995). Glycosylation of these domains is apparently required (Maisner et al. 1994, 1996). The cytoplasmic tail of CD46 is dispensable for its MeV

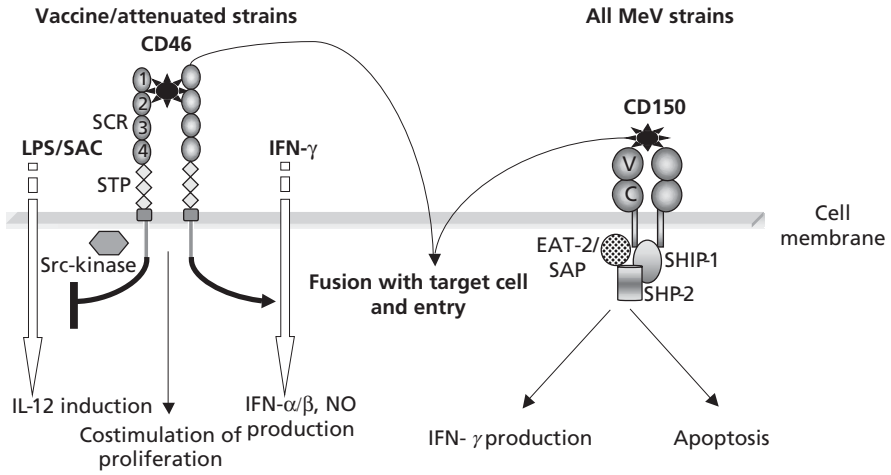

Figure 4 *Interaction of measles virus (MeV) with cell-surface receptors and consequences. MeV entry into target cells is supported by CD46 (mainly for attenuated strains) and CD150 (for all MeV strains). Both molecules have signaling properties. Tyrosine phosphorylation of the cytoplasmic domain of CD46 occurs in a Src family kinase-dependent manner, and CD46 ligation by MeV has been linked to enhancement of interferon γ (IFN-γ)-primed release of IFN-α/β and of NO production in CD46-transgenic murine monocytes. CD46-ligation can also suppress lipopolysaccharide (LPS) or* Staphylococcus aureus *Cowan strain I (SAC)-stimulated release of interleukin 12 (IL-12) from monocytes. Ligation of CD150 by specific antibodies triggers its activation, and the subsequent signaling is dependent of the presence of adaptor molecules such as SAP (for signaling lymphocyte activation molecule (SLAM)-associated protein) in T and natural killer (NK) cells and EWS/FLI1- associated transcript 2(EAT-2) in antigen-presenting cells. Triggering of CD150 is involved in recruitment and/or activity of phosphatases with protein (SHP-2; or Src-homology domain 2 (SH2)-domain-containing inositol phosphatase) or dual (SHIP-1; both protein and phospholipid) specificity, and in the induction of IFN-γ or cellular apoptosis. Established consequences of MeV-dependent regulation of signaling by CD150- or CD46-mediated signaling are indicated in plain text and those which have not yet been shown to be exerted by MeV in italics.*

receptor function (Varior-Krishnan et al. 1994), it has, however, signaling properties (Figure 4). MeV infection of cells of expression of H protein alone leads to CD46 down-regulation (Bartz et al. 1996; Krantic et al. 1995; Lecouturier et al. 1996; Schneider-Schaulies et al. 1996). Although CD46 was identified as MeV receptor by all criteria in human cells, transgenic expression of this molecule confered susceptibility to MeV infection in only some rodent cell lines and to intracerebral, but not peripheral infection in rodents indicating that as yet

Table 1 *Cellular surface molecules interacting with the MeV H protein*

	CD46	CD150	TLR2
Superfamily	Regulators of complement activity	Immunoglobulins	Pattern recognition receptor
Ectodomain	Four short consensus repeats and a variable number of serine–threonine–proline-rich repeats	One variable and one constant Ig-like domain	Repetitive leucine-rich repeats
Tissue distribution	All nucleated human cells	Activated T and B cells, thymocytes, differentiated monocyte/macrophages and dendritic cells	High on professional antigen-presenting cells
Cellular function	Protection from complement mediated lysis	Regulation of lymphocyte function	Activation of antigen-presenting cells
Endogenous ligand	C3b/C4b complement components	Self-ligand	None
MeV interaction			
Interacting MeV	Mostly vaccine and adapted strains	All MeV strains	MeV wild-type strains
Inhibition with specific antibodies	Yes	Yes	Yes
Regulation of host-cell functions	Yes	Yes	Yes
MeV uptake in transfected CHO cells	Yes	Yes	No

poorly defined determinants, including intracellular factors and possibly interferon, restrict MeV replication (Evlashev et al. 2001; Horvat et al. 1996; Mrkic et al. 1998; Naniche et al. 1993; Niewiesk et al. 1997b; Patterson et al. 2002; Vincent et al. 2002). It became, however, clear that the ability to use CD46 as entry receptor is confined to certain MeV strains, mainly vaccine strains and wild-type strains adapted to growth on Vero cells, while most lymphotropic wild-type strains and fresh isolates fail to interact with and to down-regulate CD46 (Bartz et al. 1998; Hsu et al. 1998). These and all other MeV strains tested as yet attach via their H proteins to CD150, a CD2-like molecule of the Ig superfamily (Erlenhoefer et al. 2001, 2002; Hsu et al. 2001; Minagawa et al. 2001; Ono et al., 2001a,b; Tatsuo et al. 2001) (Figure 4 and Table 1) with H proteins able to interact with both receptors revealing a higher kinetic binding rate for CD46 (Santiago et al. 2002). Amino acids essentially involved in the interaction of CD150 and the H protein have been mapped (Ohno et al. 2003; Vongpunsawad et al. 2004). CD150 is expressed by activated and memory T and B cells, and immature thymocytes, but not on freshly isolated monocytes and immature dendritic cells, but can, however, be induced on these cells upon stimulation (Aversa et al. 1997; Minagawa et al. 2001). For monocytes, this induction is mediated by interaction of the H protein of wild-type MeV strains with Toll-like receptor 2 (TLR2), which per se does not serve as entry receptor (Bieback et al. 2002) (Table 1). As for CD46, the most membrane distal portion of CD150, the V domain, is important for MeV binding (Ono et al. 2001b) (Figure 4), and the molecule is also down-regulated by MeV infection or H protein interaction (Erlenhoefer et al. 2001). Amino acids determining differential receptor usage of MeV H mainly include aa 481 and aa 546 and 451 (Bartz et al. 1998; Hsu et al. 1998; Lecouturier et al. 1996; Rima et al. 1997; Schneider et al. 2002). Similar to CD46, the cytoplasmic domain of CD150 has signaling properties (Latour et al. 2001; Mikhalap et al. 1999; Morra et al. 2001) (Figure 4). The ability of CD150 to confer susceptibility to MeV wild-type infection of lymphocytes, activated monocytes, and dendritic cells is clearly established (Hsu et al. 2001; Minagawa et al. 2001; Ohgimoto et al. 2001; Schneider et al. 2002; Tatsuo et al. 2001). Although CD150 can be induced on monocytes by TLR2 signaling, the question of how lymphotropic strains can enter into these natural target cells in vivo, the mode of MeV entry in CD150-negative cell types such as endothelial, epithelial (during acute measles), and brain cells (as prerequisite for central nervous system (CNS) persistence) is unknown (McQuaid and Cosby 2002). There is evidence for both low affinity interaction of these strains with CD46 and the existence of additional MeV receptors (Manchester et al. 2002; Takeuchi et al. 2002). For viral entry, interaction of the viral glycoproteins with cell surface molecules other than the known binding receptors may also be required. A role for both substance P receptors and moesin in this context has been suggested but not clarified (Dunster et al. 1994; Harrowe et al. 1992; Schneider-Schäulies et al. 1995b).

The presence of entry receptors is, however, not the sole determinant of tissue tropism and permissivity, as intracellular factors, particularly those associated with cellular maturation and activation, are also essential. Thus, the rodent adapted CAM strains of MeV (by as yet unknown receptors) and the Edmonston vaccine strain (and recombinant derivatives of this strain, by transgenic CD46) enter into and replicate well in neuronal cells of newborn and suckling, but not adult rodents (Duprex et al. 1999a, 2000; Finke and Liebert 1994; Lawrence et al. 1999, 2000; Liebert et al. 1990; Rall et al. 1997) and this is reflected by differential permissivity of maturing neuronal cultures in vitro (Duprex et al. 2000). MeV permissiveness in these systems is confined to the CNS, while intracellular factors restrict viral replication in most rodent cells in vivo and in vitro (see above). In human hematopoetic cells, viral replication is apparently dependent on cellular activation (Fugier-Vivier et al. 1997; Servet-Delprat et al. 2000b).

Intracellular replication, assembly, and budding

As all members of the *Mononegavirales*, MeV replicates exclusively in the cytoplasm of a permissive host cell. MeV transcription is initiated after specific attachment of the polymerase complex to the alleged promoter located within the 3′ end of the genome (Parks et al. 2001) (Figure 1b) and progresses to the 5′ end by transcribing monocistronic mRNAs. The tendency of the polymerase complex to disassociate from and reassociate with the template at the gene boundaries is apparently also controlled by host-cell factors, which is reflected by cell type-specific, differential slopes of the transcription gradient (Cattaneo et al. 1987; Griffin and Bellini 1996; Horikami and Moyer 1995; Schneider-Schaulies et al. 1989, 1995c). In the replication mode, the polymerase complex reads through the intergenic boundaries to yield a copy of the entire viral genome, the replicative intermediate. These full-length plus strands are about 100-fold less abundant than those of negative polarity. As replication requires a continuous supply of N protein, it is dependent on protein synthesis. In spite of the ability of the P protein to retain the N protein in the cytoplasm (Huber et al. 1991), intranuclear inclusion bodies consisting of N protein aggregates are typically seen late in infection. Cytoskeletal components may play a role during measles transcription/replication (Moyer and Horikami 1991). It is, however, likely that they are involved in transport of mature intracellular RNPs initially formed in the perinuclear area to the cell membrane (Duprex et al. 2000). They, or associated proteins, could

also be involved in the lateral movement of the MeV glycoproteins in the membrane required for patching and subsequent budding (Lydy et al. 1990). This may particularly refer to recruitment of the H protein into lipid rafts which may serve as platforms for MeV assembly. Upon co-expression with the F protein which has the intrinsic ability lo localize in rafts, the H glycoprotein is dragged into the rafts, and assembly of internal MeV proteins into these membrane domains requires the presence of the MeV genome. Synthesis, oligomerization, and maturation of the MeV glycoproteins has been referred to under MeV structural and nonstructural proteins. A sorting signal within their cytoplasmic domains mediates targeting of both MeV F and H proteins to the basolateral membrane in polarized cells, and interactions of M with the glycoprotein cytoplasmic tails allow M-glycoprotein co-segregation to the apical surface, suggesting a vectorial function of M to retarget the glycoproteins for apical virion release (Moll et al. 2001; Naim et al. 2000). The interaction of the cytoplasmic domains of the glycoprotein with M protein also negatively regulates the fusogenic activity of the complex (Cathomen et al. 1997a; Moll et al. 2001, 2002; Naim et al. 2000), and this may be important in viral spread by cell–cell fusion from the basolateral membrane of polarized epithelial cells and in brain tissue (Cathomen et al. 1997a; Ehrengruber et al. 2002; Moll et al. 2002; Naim et al. 2000). Functional inactivation of the M protein reading frame as often seen in persistent brain infections has thus been proposed to favor transneuronal spread in the absence of infectious virus production, which may occur even independent of the H protein (Duprex et al. 1999b, 2000; Ehrengruber et al. 2002; Lawrence et al. 2000; Patterson et al. 2001). Typically for morbilliviruses, the fusogenic activity of the F/H complex requires two conformational changes within the F protein, one of which is induced upon its proteolytic activation, the second after attachment of the H protein to its respective receptor(s) (Baker et al. 1999; Lamb 1993). The mechanism of budding is unclear, but the ability of M protein to aggregate in a crystalline array could confer upon it the capacity to distort the membrane into an outward-facing bulge, and ultimately to bud off the nucleocapsid inside a small vesicular structure. M is thought to act as a trigger in this process, and the absence of functional M protein has been suggested as a crucial element in persistence of MeV. The time taken for MeV replication in a permissive host cell is variable and becomes shorter as the virus adapts to growth in vitro. The growth of the Edmonston strain in Vero cells, is complete within 6–8 h, while for other strains, particularly fresh isolates, replication times of 7–15 days are not uncommon. Similarly, the alterations of host-cell macromolecule synthesis may vary between individual strains and cell types. Cell loss by fusion usually exceeds that caused by infection, and in some cell types, MeV infection induces apoptosis directly (Auwaerter et al. 1996; Grosjean et al. 1997; Okada et al. 2000).

Persistent infections

Persistent MeV infection can be easily established in tissue culture by several methods including passage of virus at high multiplicity with the generation of defective interfering particles, passage of infected cells in the presence of antibody, cultivating cells surviving lytic infection or infection with replication incompetent strains (Griffin and Bellini 1996). A variety of cell types can be persistently infected by one of these methods and factors favoring establishment of persistency include intrinsic or activation/differentiation-dependent regulation of viral RNA or protein synthesis (Schneider-Schaulies et al. 1992, 1993). Heat shock, IFN-inducible or RNA-binding proteins were found to be involved (Leopardi et al. 1993; Ogura et al. 1988) as were increased levels of cyclic AMP which may correlate with restrictions of MeV replication during neuronal differentiation in vitro and in vivo (Duprex et al. 2000; Schneider-Schaulies et al. 1989, 1993; Yoshikawa and Yamanouchi 1984). Moreover, signaling cascades triggered after interaction of MeV H protein with antibodies were found to further down-regulate MeV RNA synthesis in persistently infected neuronal cells (Schneider-Schaulies et al. 1992). There is, however, also evidence that the ability to establish persistent infection may depend on the MeV strain, which, in most cases, is produced in very limited amounts from persistently infected cells (Cattaneo et al. 1988b). In these viruses, mutations within the envelope protein genes lead to production of unstable or functionally impaired proteins (Billeter and Cattaneo 1994; Rima et al. 1995; Schneider-Schaulies et al. 1995c). Cellular protein synthesis is relatively unaffected by persistent MeV infection, although signaling cascades may be altered and specific cellular surface proteins down-regulated (Barrett et al. 1985; Meissner and Koschel 1995; Ofir et al. 1996; Schneider-Schaulies et al. 1992; Segev et al. 1994, 1995). Multinucleated giant cells due to cellular fusion are absent from these cultures, and this is consistent with stable down-regulation of the MeV H receptors from the cell surface (Bartz et al. 1996; Krantic et al. 1995; Schneider-Schaulies et al. 1996).

Measles genotypes

MeV is a serologically monotypic virus. Neutralizing antibody elicited to the Edmonston vaccine strain of measles virus is capable of neutralizing wild-type virus both in vitro and in vivo. With the advent of nucleic acid sequencing methods, regions of genetic variability along the measles genome have been defined (Rima et al. 1995) and exploited for use in what is currently known as ‘molecular epidemiology.’ The concept is very similar to that used to monitor polio control and eradication efforts. Maximum variability between strains of measles virus was observed within the last 450 nucleotides of the *N* gene, with as much as 12 percent nucleotide varia-

bility having been noted. The sequences of this region of the *N* gene and, when finer resolution is necessary, those encoding the entirety of the *H* gene, form the basis of differentiating lineages of wild-type and vaccine strains of MeV (Rota et al. 1996). In 1998, the World Health Organization (WHO) convened a meeting to establish a standard nomenclature for strain assignation. For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit, while related genotypes are grouped by clades (WHO 1998). WHO currently recognizes eight clades designated A, B, C, D, E, F, G, and H. Within these clades, there are 22 recognized genotypes. Genotypes are designated A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, E, F, G1, G2, G3, H1, and H2. Some clades contain only a single genotype and, in such cases, the genotype designation is the same as the clade name. Other clades, such as clade D, contain multiple genotypes, and are designated by using the clade letter (in upper case) and genotype number (for example, D1, D2). Several of the genotypes, E, F, G1, D1, appear to be extinct or inactive, and representatives of these have not been isolated for at least 15 years. However, the sequences of the inactive genotypes are maintained in the set of WHO reference sequences for completeness (WHO 2001).

Genetic characterization of measles viruses has proven to be a powerful adjunct to the standard epidemiologic techniques that are used to study the transmission of measles. Genotyping data help suggest or confirm putative sources of virus or suggest a source when one is not obvious from epidemiological data. Genotypic data can also help to establish or to refute putative links between spread cases and outbreaks. Molecular surveillance is most beneficial when it is possible to observe the change in viral genotypes over time in a particular region because this information, when analyzed in conjunction with standard epidemiologic data, has helped to document the interruption of transmission of endemic measles. Thus, molecular characterization of MeVs has provided a valuable tool for measuring the effectiveness of measles control programs (Rota and Bellini 2003; Rota et al. 2004).

Vaccine strains differ from the wild-type isolates, and SSPE-derived sequences are much more similar to those seen in wild-type viruses. Based on these sequence similarities, it has been possible to identify wild-type MeVs having circulated in a given population as likely infectious agents found later in SSPE brain material. These findings resulted in two important conclusions: firstly, SSPE develops after infection with a wild-type MeV and not following vaccination, and secondly, circulating wildtype viruses, and not particular neurotropic strains, initially infect the CNS. So far, evidence indicates that measles is an antigenically stable virus and that the development of complications is not determined solely by the virus. Susceptibility of the host, age, and immune status at the time of infection, and possibly other factors, are almost certainly more significant than the invading virus. There is, however, no evidence to suggest that the currently used vaccines are not able to control MeV infection with viruses of differing genotypes.

CLINICAL AND PATHOLOGICAL ASPECTS

Acute measles

Measles, an inevitable disease of childhood prior to the vaccine era, has been studied in detail and clinical features are well documented. The course of acute measles is illustrated diagrammatically in Figure 5.
The virus is acquired via the upper respiratory tract, with tracheal and bronchial epithelial cells being important targets (Sakaguchi et al. 1986). Subsequently the virus reaches the draining lymph nodes from where, after a first round of amplification, it spreads to the rest of the reticuloendothelial system and respiratory tract through the blood (primary viremia). Giant cells containing inclusion bodies (Warthin–Finkeldy cells) are formed in lymphoid tissue and also on the epithelial surfaces of the trachea and bronchi. About 5 days after initial infection, the virus overflows from the compartments in which is has previously been replicating. The viremic spread of MeV to a variety of organs occurs almost completely cell-associated (by infected lymphocytes and monocytes (Esolen et al. 1993; Schneider-Schaulies et al. 1991) with little evidence of plasma viremia (Forthal et al. 1992). Consequently, MeV can be reisolated from the cellular, but usually not the cell-free fraction of blood samples before and at the time of the rash, and this is greatly assisted upon mitogen activation of the cells. MeV spreads to numerous organs (including viscera, bladder, kidney, and skin) where it replicates primarily in endothelial cells, epithelial cells and monocyte/macrophages. Giant cells are formed in all infected tissues, where lymphoid hyperplasia and inflammatory mononuclear cell infiltrates are characteristically seen. Infection of endothelial cells in small vessels is accompanied with vascular dilatation, increased permeability, and inflammation. After about a 10–14 days clinically latent incubation period, the patient enters the prodromal phase which lasts about 2–4 days. Infection-mediated destruction of cells of the thin epithelia of the respiratory tract and conjunctiva lead to the symptoms then observed. These consist of fever, malaise, sneezing, rhinitis, congestion, conjunctivitis, and cough, and a transitory rash can sometimes develop which has an urticarial or macular appearance, which disappears prior to the onset of the typical exanthema. At this time giant cells are present in the sputum, nasopharyngeal secretions, and urinary sediment cells (Katz 1995). Virus is present in blood and secretions, and the patient is

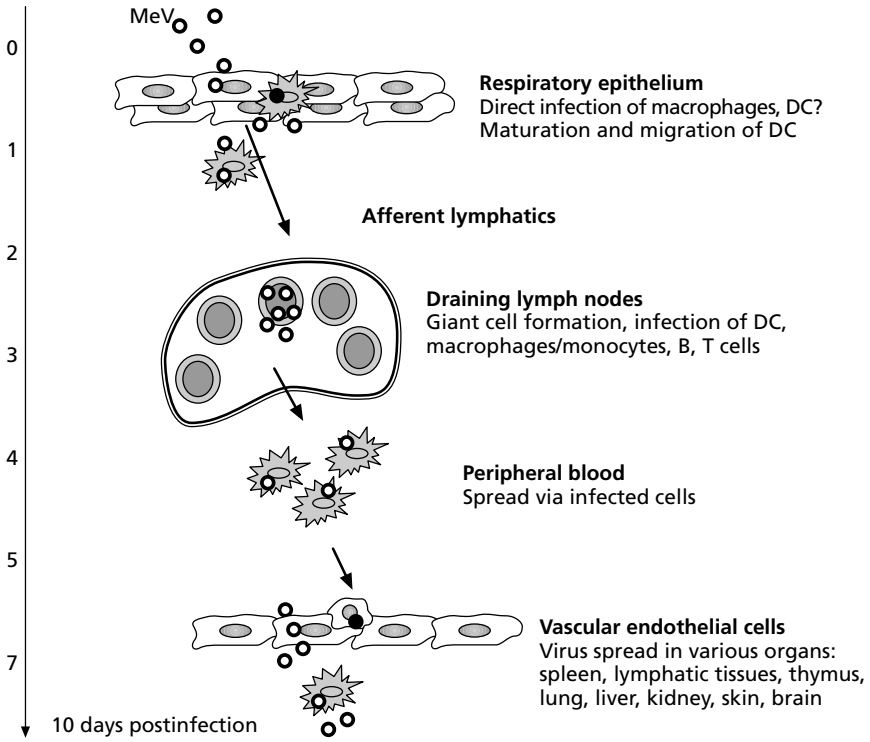

Figure 5 *Schematic representation of MeV (indicated by dots) uptake and dissemination.*

highly infectious. During this period Koplik's spots (raised spots with white centers, 1–3 mm, on an erythematous base), the pathognomonic enanthema of measles, appear on the buccal and lower labial mucosa opposite the lower molars. These begin to fade some 2–4 days after the onset of the prodromal phase as the rash develops.

The distinctive maculopapular rash appears about 14 days after exposure and starts behind the ears and on the forehead, then spreads within 3 days to involve the face, neck, trunk, and upper and lower extremities. The rash can become confluent, especially on the upper body and, once the entire body is covered, fades on the 3rd or 4th day. Histologically, the rash is characterized by vascular congestion, edema, epithelial necrosis, and round cell infiltrates. MeV antigen is absent from the spot and rash lesions, but is concentrated near blood vessels and in the endothelial cells of the dermal capillaries. The containment of infection in the skin is thought to be due to the development of cytotoxic T cells and to interferon production. As the rash results from accumulated damage to the vascular walls caused by this DTH reaction, it is often not observed in the immunosuppressed. Respiratory manifestations develop on the basis of diffuse mucosal inflammation in response to widespread MeV infection of epthelial cells and consequent inflammation of the lower respiratory tract. Gastrointestinal symptoms, mainly diarrhea, are common, while electrocardiographic abnormalities seen in about 30 percent of children with uncomplicated measles usually remain clinically insignificant. Although cerebrospinal fluid (CSF) pleocytosis and EEG changes are frequently seen in uncomplicated measles, neurologic disease in the course of, or after, measles is considered a complication rather than a pathognomonic finding. Once the exanthem has reached its height, the fever usually falls and the conjunctivitis as well as the respiratory symptomatology begins to subside. Antibody titers rise and virus shedding decreases from this point. Normally, the patient shows a rapid improvement. Continuation of clinical symptoms of the respiratory tract or fever suggests complications. On rare occasions, measles follows a hemorrhagic course, and this is characterized by high fever, seizures, delirium, respiratory distress, and hemorrhage into the skin and mucous membranes. In partially immune children, which include those with residual maternal antibodies or individuals who have received immune globulin as post exposure prophylaxis, measles can follow a special course, referred to as modified measles. Occasionally, this infection has also been seen in the course of live vaccine failure. It is usually characterized by a prolonged incubation period, mild prodrome, and sparse, discrete rash of short duration, and mild illness.

Atypical measles

This form of a measles was observed after incomplete measles vaccination prior to the exposure to natural MeV. The majority of reported cases received either

several doses of inactivated vaccine or a combination of inactivated vaccine followed by live-attenuated vaccine. The formalin-inactivated vaccine was administered in the United States from 1963 to 1967, and was withdrawn from the market soon after notification of the first cases of atypical measles (Clements and Cutts 1995). Therefore, this disease which was commonly associated with edema of the extremities and frequently with lobular or segmental pneumonia (accompanied by pleural effusion, which results in respiratory distress with dyspnea) is no longer seen. Its pathogenesis is still not fully understood. Hypotheses put forward included abnormally intense cellular immune responses, the inability of the inactivated vaccine to induce local respiratory tract immunity, and lack of production of F-specific antibodies allowing viral spread from cell to cell in the presence of neutralizing antibodies (Merz et al. 1980). As revealed more recently in macaques, the inactivated vaccine fails to induce affinity maturation of complement-fixing antibodies and the disease most likely results from a boosted anamnestic response and is mediated by immune-complex formation and eosinophilia in the presence of fusion-inhibiting antibodies (Polack et al. 1999, 2003).

Complications of measles infection

Complications of acute measles are relatively rare, and the majority results from opportunistic secondary infection of necrotic surfaces, such as those in the respiratory or gastrointestinal tract. Bacteria and other viruses can invade to cause pneumonia or other complications such as otitis media, bronchitis, or severe forms of diarrhea. The most severe complications caused directly by MeV are giant cell pneumonia and measles inclusion body encephalitis (MIBE), both of which occur in the immunocompromised patient, as well as postinfectious encephalitis (PIE) and subacute sclerosing panencephalitis (SSPE) in which no underlying susceptibility factor has been identified. Other unusual manifestations which may complicate acute measles are myocarditis, pericarditis, hepatitis, appendicitis, mesenteric lymphadenitis, and ileocolitis. If measles infection occurs during pregnancy spontaneous abortions or stillbirth may occur as well as an increased rate of low birth-weight infants. Congenital malformations have also been reported.

RESPIRATORY COMPLICATIONS

Symptomatic giant cell pneumonia is seen primarily in the immunocompromised due to unlimited and massive spread of virus to the lower respiratory tract (Breitfeld et al. 1973; Pullan et al. 1976). It is, however, mainly due to secondary bacterial and viral infections that most cases of severe pneumonia complicating measles and leading to chronic pulmonary diseases develop on the basis of the immunosuppression associated with measles (Gremillion and Crawford 1981; Katz 1995).

NEUROLOGICAL COMPLICATIONS

Postinfectious encephalitis (PIE) and measles inclusion body encephalitis

Transient EEG abnormalities as observed in some patients with acute measles suggests CNS involvement is not uncommon, although it is still a matter of controversy of whether and how MeV may reach this compartment. Acute postinfectious encephalitis (PIE) during the course of measles is a severe complication and is reported in approximately 0.1 percent of cases. In general, about 15 percent of cases are fatal, and 20–40 percent of those who recover are left with lasting neurological sequelae. Encephalitis usually develops when exanthem is still present within a period of 8 days after the onset of measles, but occasionally during the prodromal stage. PIE is characterized by resurgence of fever, headache, vomiting, seizures, cerebellar ataxia, and coma. In common with PIE induced by other viruses, this condition reveals perivascular cuffing, gliosis, and the appearance of fat-laden macrophages near the blood vessel walls and demyelination consistent with immunopathology (Gendelman et al. 1984). CSF findings in measles encephalitis consist usually of mild pleocytosis and absence of measles antibodies. Long-term sequelae include selective brain damage with retardation, recurrent convulsive seizures, hemi-, and paraplegia. Since there is little evidence for MeV RNA or antigen in the brain (Gendelman et al. 1984; Moench et al. 1988), PIE is most likely due to virus-induced autoimmunity to brain antigens (Johnson et al. 1984). In CSF specimens of PIE patients, myelin basic protein (MBP) was detected as a consequence of myelin breakdown and PIE patients and experimentally infected animals may exhibit a proliferative T-lymphocyte response to MBP (Johnson et al. 1984; Liebert et al. 1988). Since PIE is characterized by demyelinating lesions in association with blood vessels as in experimental allergic encephalitis, an MBP-specific lymphoproliferative response in measles infection is considered to be of pathogenetic importance. Mechanisms involved in triggering a T cell-mediated autoimmune response are unknown, and may include molecular mimicry or a deregulation of autoreactive cells occurring secondary to viral infections of lymphocytes.

As measles inclusion body encephalitis (MIBE) only develops in immunosuppressed patients, mainly in children with leukemia undergoing axial radiation therapy (Bitnun et al. 1999; Roos et al. 1981), it can be regarded as an opportunistic MeV CNS infection. In contrast to PIE, viral RNA and antigens are abundant in post mortem brain tissue (Baczko et al. 1988; Bitnun et al. 1999; Roos et al. 1981). The incubation period ranges from a few weeks to 6 months, and the patients often

present without a rash. The condition commences with convulsions, mainly myoclonic jerks, and is frequently confused with subacute sclerosing panencephalitis (SSPE). The seizures are often focal and localized to one site. Other findings include hemiplegia, coma, or stupor depending on the localization of the infectious disease process within the CNS. The disease progresses much faster than SSPE and death occurs within weeks or a few months. No or only low titers of MV-specific antibodies are detectable in the CSF.

Subacute sclerosing panencephalitis (SSPE)

SSPE, a fatal, slowly progressing degenerative CNS disease is seen in children and young adults usually 6–8 years after measles, although it can also occur up to 20–30 years after primary infection. Boys are more likely to develop SSPE, but the overall incidence is low (about 2–7 cases per 100 000 of acute measles). Half of the SSPE patients have contracted measles before the age of 2 years, and no unusual features of acute measles have ever been demonstrated. SSPE starts with generalized intellectual deterioration or psychological disturbance which may last for weeks to months and only be recognized as illness until more definite signs appear. These are neurological or motor dysfunctions, such as dyspraxia, generalized convulsions, aphasia, visual disturbances, or mild, repetitive simultaneous myoclonic jerks. The invasion of the retina by MeV leads, in 75 percent of cases, to a chorioretinitis of the macular area followed by blindness. The disease proceeds to progressive cerebral degeneration leading to coma and death within 1–3 years; more rapid forms are, however also known (Katz 1995).

Neuropathologically, a diffuse encephalitis affecting both the white and the gray matter is observed, characterized by perivascular cuffing, diffuse lymphocytic infiltration, and gliosis. Fibrous astrocytes, neurons, and oligodendroglial cells contain massive amounts of intranuclear inclusion bodies (Cowdry bodies), which contain MeV nucleocapsid structures, while giant cell formation or membrane changes consistent with virus maturation or release are not seen (Budka et al. 1982). The lesions lead to characteristic, pathognomonic EEG changes consisting of periodic high-amplitude slow wave complexes, which are synchronous with myoclonic jerks recurring at 3.5–20-s intervals and are remarkably stereotyped. They are bilateral, usually synchronous, symmetrical, consist of two or more delta waves with bi- or polyphasic appearance and may disappear as the disease progresses. Another pathognomonic finding is the state of humoral hyperimmunity against MeV in serum and CSF. The isotypes of MeV-specific antibodies found in CSF include IgG, IgA, and IgD, and in this compartment the immune response is of restricted heterogeneity (Doerries et al. 1988). For diagnostic purposes, determination of measles antibody in the CNS may be sufficient with, if necessary, demonstration of MeV-specific heterogeneity by isoelectric focusing in combination with an immunoblot technique.

OTHER CHRONIC DISEASES

Unlike its clear role in the etiology of the diseases outlined above, MeV has also been implicated in the pathogenesis of Paget's disease (Basle et al. 1986), chronic active hepatitis (Robertson et al. 1987), multiple sclerosis (Allen and Brankin 1993; Haase et al. 1981), and Morbus Crohn (Uhlmann et al. 2002). For each of these diseases an etiological link to measles or measles vaccination is controversial and for none has a definite role for MeV been identified (Birch et al. 1994; Duclos and Ward 1998; Moench et al. 1988).

PATHOGENESIS OF MEASLES AND PERSISTENT MeV CNS INFECTIONS

MeV can replicate in a variety of tissues, including cells of the immune system, and its interaction with the immune system is responsible for some of the key features of the disease. A DTH reaction is implicated in the production of the rash and the ability of the virus to cause a transient, generalized immunosuppression accounts to a major extent for complications associated with acute measles.

The study of MeV pathogenesis in animal models is almost exclusively confined to nonhuman primates which are permissive for intranasal MeV infection and develop disease which shares important features with that seen in humans (van Binnendijk et al. 1995). Attempts to induce measles-like disease in small animals by this route have largely failed (Liebert and Finke 1995). In rodents, MeV replication is impaired due to intracellular restriction as both rats and mice genetically engineered to express CD46, one of the MeV receptors, fail to replicate the virus after peripheral infection (Niewiesk et al. 1997b). Intracerebral infection of small rodents, both transgenic and nontransgenic, has, however, significantly contributed to the understanding of MeV CNS pathogenesis (Liebert and Finke 1995). For unknown reasons, cotton rats reveal a certain permissivity to intranasal MeV infection (Wyde et al. 1992), and, as with acute measles, experimental infection of these animals is accompanied by features compatible with immunosuppression.

Acute measles

MeV receptor interactions in pathogenesis

The usage of cellular receptors, which are discussed under The replication cycle, is an important aspect of MeV pathogenesis. Both CD46 and CD150 are down-regulated from the cell surface after contact with their respective interacting MeV H proteins and after infection (Erlenhoefer et al. 2001; Krantic et al. 1995; Schneider-

Schaulies et al. 1995a). For CD46, at least one consequence of MeV-induced down-regulation is known. Corroborating the 'natural' function of CD46 as a complement regulator, MeV-induced CD46 down-regulation enhances sensitivity to lysis by activated complement of lymphocytes in vitro (Schneider-Schaulies et al. 1996; Schnorr et al. 1995). CD46 can act as a signaling molecule, and ligation of this molecule phosphorylation of tyrosine residues within its cytoplasmic domain 2 occurs in a src family-dependent manner (Wang et al. 2000) (Figure 4). Coligation of CD3/CD46 by antibodies enhances T-cell proliferation and activation of signaling molecules, such as by $p120^{CBL}$, LAT, CrkL, Vav, Rac, and ERK indicating that CD46 can act as a co-stimulatory molecule (Astier et al. 2000; Zaffran et al. 2001). In CD46 transgenic murine macrophages, MeV-infection induced NO and type I IFN production in the presence of IFN-γ (Katayama et al. 2000) and CD46 ligation by MeV, CD46-specific antibodies or C3b/C4b complement components, interfered with stimulated IL-12 synthesis in monocyte cultures (Figure 4) (Karp et al. 1996). Moreover, the ability of CD46-specific antibodies to induce the generation of regulatory T cells in vitro upon CD3 co-ligation has been described (Kemper et al. 2003). Whether this also applies to CD46 ligation by MeV needs to be determined. Consequences of CD150 down-regulation or ligation by MeV remain speculative. Signaling events triggered after ligation of this molecule are complex and can lead to activation but also cellular apoptosis (Mikhalap et al. 1999). Coupling of CD150 to intracellular signaling pathways requires adaptor proteins, such as EAT-2 in antigen-presenting cells (Morra et al. 2001) or SAP in T and NK cells (Latour et al. 2001). In lymphocytes, CD150 ligation by antibodies recruits SHIP-1, a dual specific phosphatase, and thereby regulates recruitment of intracellular signaling molecules (Latour et al. 2001) (Figure 4). IFN-γ production from $CD4^+$ T cells can be enhanced upon CD150 ligation (Aversa et al. 1997), and thus it is tempting to speculate that downregulation of this molecule may play a role in impaired Th1 responses as seen in measles (Griffin 1995). Recent evidence, however, suggests that CD150 is more involved in regulating IL-4, rather than IFN-γ, release and thus, consequences of MeV interaction with this molecule remain speculative (Wang et al. 2004).

IMMUNE RESPONSES

MeV induces a marked immune activation as evidenced by spontaneous proliferation of PBMC, polyclonal activation of B cells, expression of activation markers on T cells, and increased plasma levels of cytokines and soluble surface proteins, such as CD4 and IL-2R (Griffin 1995). This activation is co-incident with a generalized immune suppression, and both may continue for many weeks after apparent recovery from measles.

Immune activation

Activation of $CD8^+$ T cells during measles is amply evidenced by proliferation of these cells and the appearance of MeV-specific HLA-restricted $CD8^+$ cytotoxic T lymphocytes in the blood at the time of the rash, some of which develop into CD8 memory cells (van Binnendijk et al. 1990). MeV antigens inducing $CD8^+$ cytotoxic T cells have not been completely identified, however, the F protein is one target for MHC class I-restricted T cells (van Binnendijk et al. 1989). A recent study suggests that the CD8 response plays an important role in the control and clearance of MeV infection (Permar et al. 2003a). $CD4^+$ T cells are also activated in response to MeV infection. Virion protein uptake to an endosomal compartment for subsequent MHC class II-dependent presentation can be triggered by the interaction of H protein with CD46 (Gerlier et al. 1994). $CD4^+$ T cells proliferate during the rash, and classical $CD4^+$ T cell responses such as MeV-antigen-stimulated cytokine production and MHC class II-restricted antigen-specific proliferation are stimulated (Griffin 1995). During the prodrome, plasma levels of IFN-γ, neopterin, and soluble IL-2R rise, followed by elevation of IL-2, soluble CD4, and CD8 at the time of the rash (Griffin et al. 1989, 1992). Subsequently, IL-4 becomes elevated, and this may persist for prolonged periods. This pattern of cytokine production is consistent with an early activation of $CD8^+$ and Th1 $CD4^+$ T cells followed by a predominant Th2 $CD4^+$ T-cell response during recovery (Figure 6). Whether or not Th1 responses are less efficiently generated after vaccination has not been entirely resolved (Gans et al. 1999; Griffin and Ward 1993; Schnorr et al. 2001).

Antibodies, which appear at the time of the rash, are initially of the IgM and then switch to IgG1 and IgG4

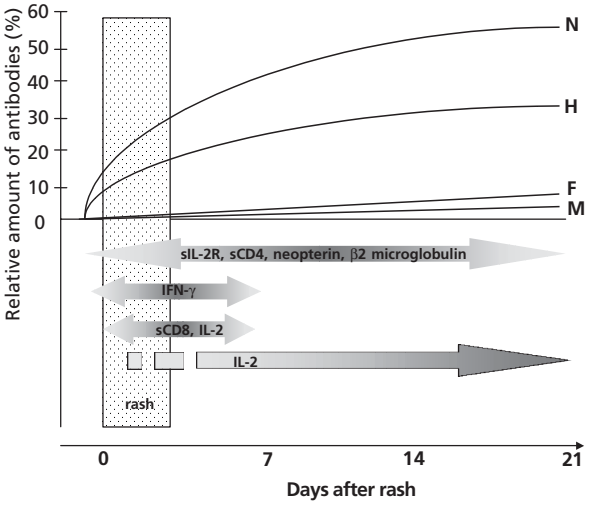

Figure 6 *Activation of immune responses during measles. Relative amounts of antibody produced to individual MeV proteins (upper part) and parameters (plasma levels of cytokines and soluble CD4, CD8, IL-2R, and β2 microglobulin, bottom part) in the course of acute measles.*

isotype (Griffin 1995). IgA, IgM, and IgG antibodies are found in secretions. Though antibodies are generated to most viral proteins, the most abundant and rapidly produced antibodies are N-specific (Figure 6). Antibodies to the H protein are neutralizing and inhibit agglutination of monkey erythrocytes by MeV by blocking the interaction of H protein with monkey CD46. Antigenicity of the H protein is variable, and up to six major, mainly conformational, epitopes were mapped by competitive binding of monoclonal antibodies (Carter et al. 1983). H-specific antibodies may also inhibit hemolysis, which is, however, a major property of F-specific antibodies. Up to seven linear epitopes within the F protein are recognized by polyclonal sera, and monoclonal F-specific antibodies can cross-react with cellular heat shock proteins (Sheshberadaran and Norrby 1984).

Early nonspecific and innate immunity

Unlike adaptive immunity, the role of early nonspecific and innate immunity in measles is poorly understood. NK-cell activity may be slightly reduced in measles (Griffin and Ward 1990). While production of type I IFN has been documented after vaccination, the production of this cytokine has not been unequivocally observed in patients with measles infections at the time of the rash (Shiozawa et al. 1988). As suggested by recent evidence, MeV vaccine and wild-type strains may also differ in their ability to induce type I IFN and to evade from IFN-mediated control in vitro in PBMC cultures (Naniche et al. 2000). The quality, magnitude, and duration of MeV-specific adaptive immunity is, however, likely to be governed to a major extent by its interaction with professional antigen-presenting cells such as tissue resident macrophages and DCs which reside at or close to its entry sites and fulfill a sentinel, but most likely also a Trojan horse function for MeV transport to the draining lymph nodes. In vitro, both immature and LPS- or CD40-matured DC are infectable with MeV, albeit virus production from pure DC and transmission of virus in DC/T cell co-cultures is very limited (Fugier-Vivier et al. 1997; Grosjean et al. 1997; Klagge et al., 2000; Schnorr et al. 1997b; Servet-Delprat et al. 2000b). There is evidence that wild-type MeV reveal a particular tropism for these cells, which is essentially determined by the interaction of the H protein with CD150 (Ohgimoto et al. 2001; Schnorr et al. 1997b). MeV infection triggers rapid maturation of immature DC as indicated by up-regulation of MHC class II and co-stimulatory molecules, and this again seems to occur more efficiently with certain wild-type strains (Dubois et al. 2001; Klagge et al. 2000; Servet-Delprat et al. 2000b). Cytokines induced in DC as well as in monocytes by MeV infection include IL-1-α/β, IL-1R antagonist, IL-6, TNF-α, type I IFN, and low amounts of IL-12p35 and IL-12p40 (Bieback et al. 2002; Klagge et al. 2000; Schnorr et al. 1997b; Servet-Delprat et al. 2000b), some of which trigger DC maturation or are directly involved in polarizing ensuing T-cell responses (Figure 7a). As outlined under The replication cycle, TLR2 signaling by wildtype MeV is involved in maturation of monocytes (Bieback et al. 2002). Signaling pathways triggered by its activation include the MAP kinases, the Akt kinase, and the NF-κB pathway, and ultimately result in production of proinflammatory cytokines, chemokines, as well as co-stimulatory molecules and CD150 (Aderem and Ulevitch 2000; Bieback et al. 2002). Activation of monocytes and DC is also induced by non-TLR2 agonistic attenuated MeV strains, and this may relate to the ability of intracellular MeV to activate NF-κB directly (Dhib-Jalbut et al. 1999; Hummel et al. 1998). Alternatively, induction of type I IFN does occur particularly with attenuated MeV strains, which, however, again, could be dependent on activation of a TLR, namely TLR3, which recognizes double-stranded RNA (Doyle et al. 2002). Interestingly, TLR3 was found to be up-regulated by laboratory, but not wild-type MeV strains as a consequence of IFN-induction (Tanabe et al. 2003).

MeV-induced immunosuppression

At the same time, when MeV-specific immunity is efficiently generated, a general suppression of responsiveness to other pathogens is established. This was recognized long before the virus was isolated and is the major reason for the constantly high morbidity and mortality rate associated worldwide with acute measles. Typically, patients reveal a marked susceptibility to opportunistic infections and lymphopenia affecting both B and T cells (Okada et al. 2000). The latter is not associated with a decrease in thymic output (Permar et al. 2003b) and may therefore result from lymphocyte depletion due to viral infection, apoptosis or aberrant homing of T cells (Addae et al. 1995; Auwaerter et al. 1996; Nanan et al. 1999; Okada et al., 2000). Immunosuppression, however, is observed up to weeks after the onset of the rash when the lymphocyte counts have returned to normal and MeV-infected cells are, if at all, present with only low frequency (Okada et al. 2001). Key features of MeV-induced immunosuppression are inhibition of DTH responses and a restricted ability of lymphocytes to proliferate in response to recall antigens, as well as polyclonal stimulation (Borrow and Oldstone 1995; Schneider-Schaulies et al. 2001) (Table 2). As only a few infected cells are usually detected, several hypotheses have been put forward to explain this finding which include the production of inhibitory factors by infected cells that have not yet been identified (Fujinami et al. 1998). Characteristics of MeV-induced immunosuppression are compatible with deregulation of APC viability and functions, which would be expected to be of significant impact on the induction, efficiency and quality of T-cell responses (Table 2). MeV infection causes maturation of immature DC, yet this may be incomplete, and

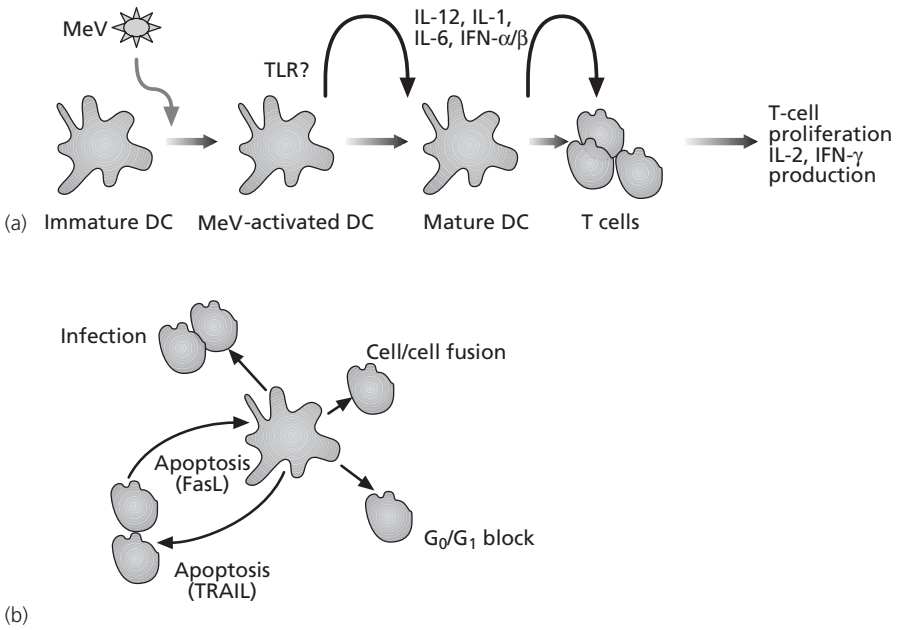

Figure 7 *Impact of measles virus (MeV) on dendritic cell (DC) maturation and T-cell activation.* **(a)** *Immature DCs, are activated and mature upon infection or surface interaction with MeV as indicated by up-regulation of MHC and costimulatory molecules and the induction of cytokines, which act as autocrine and paracrine factors further triggering maturation. Both cell–cell contacts and cytokines activate and shape T-cell responses, and this may effectively occur after uptake, processing, and presentation of viral components early after DC infection.* **(b)** *With on-going viral replication, MeV-infected DCs promote DC/T cell fusion and T-cell infection, inhibition of T-cell proliferation by expressing high levels of the MeV glycoprotein complex and T-cell apoptosis by TRAIL expression. DC depletion may result from fusion or FasL-induced apoptosis by T cells.*

Table 2 *Characteristic findings related to MeV-induced immunosuppression*

	Findings
In vivo	Susceptibility to opportunistic infections
	Loss of delayed type hypersensitivity responses
Ex vivo	Leukopenia
	MeV-infected peripheral blood mononuclear cells
	Cytokine imbalance
	Long-term suppression of IL-12 production
	Loss of proliferative responses of lymphocytes
In vitro	
Antigen-presenting cells	Apoptosis and cytotoxicity
	Infection and fusion
	Aberrant terminal maturation
	Suppression of stimulated IL-12 release
B cells	Growth arrest
	Production of inhibitory soluble mediators
T cells	Depletion by aberrant homing and apoptosis
	Production of soluble inhibitory mediators
	Loss of stimulated proliferative responses due to infection or cell-surface signaling by the MeV glycoprotein complex

impaired production of IL-12 from these cells may occur (Atabani et al. 2001; Fugier-Vivier et al. 1997; Servet-Delprat et al. 2000b). The role of IL-12 depletion in MeV-induced immunosuppression is, however, unclear as yet (Hoffman et al. 2003). In MeV-infected DC/T cell cocultures, fusion and apoptosis of both DC and T cells was seen. While the latter has been linked to MeV-mediated upregulation of TRAIL on DC and FasL on T cells (Servet-Delprat et al. 2000a; Vidalain et al. 2000), the first is exerted by the MeV glycoprotein complex expressed on DC at high levels late in infection (Ohgimoto et al. 2001). It is, however, also evident that MeV-infected DC fail to stimulate allogenic T-cell proliferation and actively inhibit mitogen-stimulated expansion without grossly interfering with the viability of these cells (Fugier-Vivier et al. 1997; Grosjean et al. 1997; Klagge et al. 2000; Schnorr et al. 1997b; Steineur et al. 1998). The inhibitory phenotype of MeV-infected DC has been linked to direct negative signaling to T cells via the MeV glycoproteins rather than to soluble inhibitory mediators (Dubois et al. 2001; Klagge et al. 2000) (Figure 7b). Expression of the MeV glycoprotein complex on infected or transfected cells or virions was found necessary and sufficient to induce a state of proliferative unresponsiveness to polyclonal and CD3/CD28 stimulation in lymphocytes (Niewiesk et al. 1997a; Schlender et al. 1996; Schneider-Schaulies et al. 2001).

For this to occur, surface contact of uninfected cells and UV-irradiated virions or cells expressing a complex of H protein and proteolytically activated F protein is required, while soluble inhibitory mediators are not involved (Weidmann et al. 2000a,b) (Figure 8a). Contact-mediated inhibition is not associated with the induction of apoptosis, but rather T-cell arrest in the late G_1 phase of the cell cycle as determined by characteristic deregulations in the expression levels and activity of S-phase entry control proteins (Naniche et al. 1999; Niewiesk et al. 1999, 2000; Schnorr et al. 1997a). Not surprisingly, intracellular signaling pathways in T cells are directly targeted by cell surface interaction with MeV. Thus, IL-2- or anti-CD3/CD28-dependent activation of the phosphoinositol-3-kinase/Akt kinase pathway, which is involved in regulating cell cycle control proteins, is disrupted after MeV contact (Avota et al. 2001) (Figure 8b). There is evidence that the N protein may also contribute to suppression of lymphocyte functions. It was found to inhibit antibody production in vitro by binding to FcγIIR on B cells (Ravanel et al. 1997), and to induce sustained calcium influx, as well as a G_0/G_1 arrest of thymic epithelial cells and other cell types (also including lymphocytes) by binding to an unknown, ubiquitously expressed receptor (Laine et al. 2003).

Persistent measles: CNS infections

MEASLES INCLUSION BODY ENCEPHALITIS (MIBE)

As this condition is confined to patients with underlying immunodeficiencies, it is not usually accompanied by intrathecal antibody synthesis. The unprotected cells develop both nuclear and cytoplasmic massive inclusion bodies consisting of virus nucleocapsids (Roos et al. 1981). Infectious virus has only been occasionally isolated by conventional methods from brain tissue, suggesting defects in replication (Baczko et al. 1988, 1993; Ohuchi et al. 1987; Roos et al. 1981). This assumption has been supported by immunohistological and molecular biological studies in MIBE (Baczko et al. 1988). In brain tissue, only N and P, but not the envelope proteins were consistently detected. In

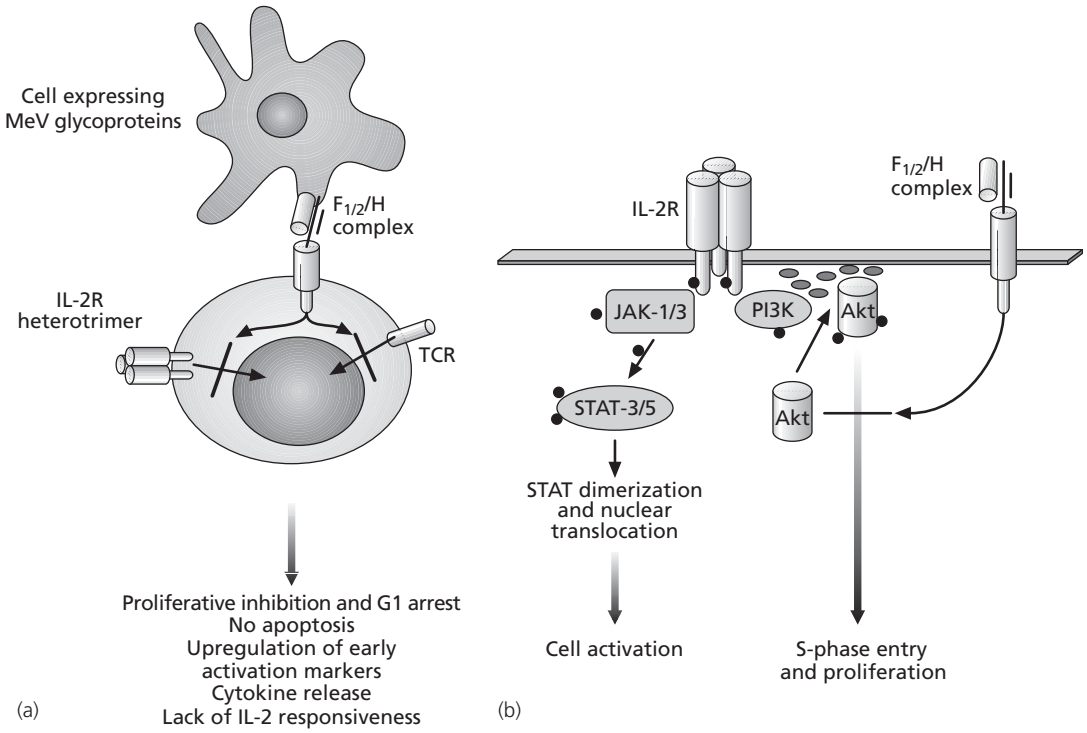

Figure 8 *Mechanisms of MeV contact mediated T-cell inhibition and consequences.* **(a)** *The MeV $F_{1/2}$/H complex induces by interaction with an, as yet unknown, receptor proliferative arrest in lymphocytes, which causes their arrest in the G1 phase of the cell cycle. In primary T cells, mitogen-induced expression of activation markers and cytokines is not affected. The IL-2R heterotrimer is expressed to normal levels, yet exogenous IL-2 does restore T-cell proliferation.* **(b)** *Signaling via the interleukin 2 receptor (IL-2R) on T cells involves phosphorylation of the cytoplasmic domains of its β- and γ-subunits. This provides docking sites for the Janus kinases JAK-1/3 which, when autophosphorylated, provide docking sites for and phosphorylate STAT-3 and STAT-5, which then dimerize and translocate to the nucleus. IL-2R signaling also activates phosphatidylinositol 3-kinase (PI3K), which catalyzes phosphorylation of inositol-phosphates. As second messengers, these recruit the Akt kinase to the cell membrane where this kinase is phosphorylated and subsequently regulates the activity of downstream targets. In T cells, this pathway was found to be particularly important for S-phase entry and proliferation. Contact with the MeV $F_{1/2}$/H complex abolishes the IL-2-dependent activation of the PI3K/Akt pathway, but not that of the JAK/STAT pathway.*

contrast, the mRNAs specific for the viral proteins were detectable in brain-derived total RNA samples with the mRNAs for the envelope proteins being under-represented. The latter failed to direct the synthesis of the corresponding proteins in vitro and this restriction was partially explained by a high rate of mutations distributed over the entire MeV genome (Cattaneo et al. 1988a,b; 1989b). Defects in MeV mRNA transcription and envelope protein synthesis apparently did not largely affect the activity of the RNP complex which spread to different areas of the patient's brain. As infectious virus particles may never be formed due to the restriction of the envelope proteins and giant cell formation has never been observed, the spread is thought to occur by microfusion events.

SUBACUTE SCLEROSING PANENCEPHALITIS (SSPE)

MeV was first implicated in the etiology of this disease by immune fluorescence in 1967 and this has since been confirmed by electron microscopy, immunoelectron microscopic (IEM) methods, and finally by the successful rescue of virus by co-cultivation techniques. Despite this, mechanisms underlying the transport of virus into the CNS, establishment and initial maintenance of persistence, and production of disease are still largely unknown. The virus is thought to gain entry to the CNS during viremia in acute measles by infected lymphocytes or infection of cerebral endothelial cells (Kirk et al. 1991; McQuaid and Cosby 2002), but once there, replication most likely proceeds only slowly. It is also not known to what extent virus replication per se is responsible for the development of lesions, or what part is played by the immune system.

Virological aspects

In SSPE, free infectious virus has never been isolated either from the brain or from CSF, and histopathological examinations have consistently failed to reveal the morphological changes associated with virus maturation. As with MIBE, giant cells and thickening of the plasma membrane at points of budding were never seen suggesting the absence of viral glycoproteins. Viral nucleocapsids present in the cytoplasm are randomly scattered and show no sign of regular alignment beneath the plasma membrane. As budding viral particles and infectious virus are not detected, the infection may spread slowly in a strictly cell-associated manner. The ability of MeV to spread transneuronally in the absence of virus production has recently been confirmed in tissue culture and this can occur in the absence of CD46 (Duprex et al. 1999b, 2000; Ehrengruber et al. 2002; Lawrence et al. 2000). Nevertheless, CD46, which is expressed on certain brain cells such as oligodendrocytes and a subset of neurons, is apparently down-regulated from MeV-positive cells in lesions (McQuaid and Cosby 2002; Ogata et al. 1997).

In SSPE brain sections, only the expression of the MeV N and P proteins and not that of the envelope proteins was consistently detected in infected cells (Liebert et al. 1986). Molecular biological studies on SSPE brain tissue revealed extensive transcriptional and translational alterations affecting mainly MeV envelope-protein specific genes (Baczko et al., 1986, 1988; Cattaneo et al. 1987, 1988b). As in MIBE, mRNAs specific for these genes were detected at only low copy numbers and were highly impaired in directing in vitro protein synthesis. A high rate of mutations was found all over the MeV genome. While the *M* gene was most affected, the *F*, *H*, *P*, and *N* genes were mutated to about the same extent, and the L gene was most conserved (Cattaneo et al. 1988a,b; 1989b). Mutations introduced were either point mutations, or appeared as clustered transitions which are thought to result from the activity of a cellular enzyme complex that actively modifies viral genetic information (Baczko et al. 1993; Cattaneo et al. 1988b). As revealed by detailed analyses, mutated MeV genomes may clonally expand in SSPE (Baczko et al. 1993). As a result of either of these events, translation of viral mRNAs was completely abolished or led to the synthesis of truncated or unstable MeV proteins. With respect to the overall high degree of conservation of the *F* gene within all MeV strains known (Rima et al. 1995), it is remarkable that truncations, mutations or deletions in the cytoplasmic domain of the F protein are almost universal (Billeter and Cattaneo 1994). These findings explain the absence of infectious MeV particles and cell fusion in infected SSPE brain tissue, which both require biologically active MeV envelope proteins. Virus can, however, occasionally be rescued from brain tissue obtained post mortem by cocultivation (Griffin and Bellini 1996). Some of these SSPE isolates have been proven invaluable tools to study virological parameters and specific genetic defects in SSPE, others, however, most likely represent contaminations and are in fact ordinary laboratory MeV strains (Rima et al. 1995). It is still unclear whether true SSPE isolates represent the dominant MeV population in the infected brain or rather small subpopulation of replication/maturation competent viruses or revertants that have been selected by the isolation procedure.

Factors involved in the establishment of persistence by a nondefective MeV in brain tissue are largely unknown. Differential interaction of the M protein with the viral RNP has been suggested for negative regulation of MeV transcription (Suryanarayana et al. 1994). There is, however, evidence that factors intrinsic to the host cell in the brain intimately control and downregulate the efficiency of MeV gene expression. In tissue culture experiments with cells of neural origin and in brain material of experimentally infected animals, evidence has been provided that intracellular factors control the efficiency of

MeV replication (Schneider-Schäulies et al. 1989). These may include unknown factors associated with brain cell differentiation (Schneider-Schäulies et al. 1993; Yoshikawa and Yamanouchi 1984), as well as the type I IFN-inducible MxA protein, which negatively regulates MeV transcription in brain cells, but not in monocytes and fibroblasts (Schneider-Schäulies et al. 1994; Schnorr et al. 1993). In addition, the cellular enzyme activity actively modifying the primary sequence of viral RNAs has been demonstrated in these cells. Thus, intracellular factors present in brain cells act to slow down viral replication after primary infection thereby preventing a rapid host-cell destruction. This may even be enhanced by virus neutralizing antibodies which were found to down-regulate MeV transcription by activating as yet unknown signaling cascades (Schneider-Schaulies et al. 1992; Segev et al. 1994). There is also evidence that MeV may regulate intracellular signaling cascades in a cell type-specific manner since it activates the transcription factor NF-κB in glial, but not neuronal cells (Dhib-Jalbut et al. 1999). Since activation of NF-κB is an essential prerequisite for the induction of most genes associated with cellular defense (such as proinflammatory cytokines), this may interfere with initial control of infection. Differential regulation of cytokines produced in primary and persistently infected glial cells has also been documented (Schneider-Schäulies et al. 1993). Whether these control mechanisms are efficient in establishing and maintaining persistent infection or other factors, such as the introduction of mutations into the viral genome are required, has not been resolved.

Critical components for disease progression and clinical appearance also remain speculative. Infection-induced loss of brain cells is likely to contribute, and this may occur to a certain extent during the asymptomatic phase. Since this is not brought about by giant cell formation, apoptosis as seen in neuronal and macroglial cells both in SSPE brain tissue and experimental CNS infection in mice is likely to be important (Anlar et al. 1999; Manchester et al. 1999). There is, however also evidence from in vitro experimentation that MeV infection does not grossly interfere with brain cell viability, but may specifically target expression of proteins required for luxury functions (Meissner and Koschel 1995).

Immunological aspects

Studies performed in SSPE patients did not reveal any evidence for a global defect in immune responses, which could account for the establishment of a persistent MeV CNS infection. A virus-specific humoral hyperimmune response is pathognomonic for SSPE. There is a significant production of MeV-specific antibodies by plasma cells residing in the CNS leading to marked elevations in the level of CSF immunoglobulin (Connolly et al. 1971). As opposed to the polyclonal response observed in serum, MeV-specific antibodies produced in the CNS are of restricted heterogeneity leading to the appearance of oligoclonal immunoglobulin bands (Doerries et al. 1988). This suggests that antibody in the CSF is made locally by lymphocytes that have invaded this compartment from the periphery in response to antigens present in the CNS. In serum specimens, antibodies of the IgG and IgD type with specificity to all MeV proteins are present, although recognition of the M protein is low or sometimes absent, whereas antibodies in CSF samples, which are of the IgG, IgA, and IgD types, generally fail completely to detect M protein (Hall et al. 1979). Studies performed on the cellular immunity in SSPE revealed that MeV-specific lymphoproliferation is similar to that of normal measles-seropositive individuals. Similarly, other measures of cellular immunity such as skin test responses to recall antigens and proliferative responses to mitogens are normal (Dhib-Jalbut et al. 1989). The mononuclear inflammatory response in the brain includes $CD4^+$ and $CD8^+$ T cells, monocytes, and Ig-secreting B cells (Nagano et al. 1991). MHC class I and class II expression is increased in the brain, and β2 microglobulin, soluble IL-2R, and soluble CD8 are elevated in CSF (Mehta et al. 1992).

EPIDEMIOLOGY

Measles infections are transmitted via aerosol or droplet exposure generated from a cough or sneeze or via contaminated fomites. The disease is so highly contagious that susceptible individuals who come into close contact with measles patients have a 99 percent probability of acquiring the disease. Measles disease is believed to have emerged as human populations began to organize into larger communities. These communities had sufficient birth cohorts that provided a renewable source of susceptible individuals, thus permitting sustained endemic transmission of the virus within the population. The epidemiology of measles virus differs in unvaccinated versus vaccinated populations. In the prevaccine era, greater than 90 percent of individuals would acquire measles infections before 10 years of age. In unvaccinated populations, measles causes periodic epidemics with interepidemic periods of 2–5 years. These periods decrease as population size and density increase, which is directly related to the availability of susceptible individuals for disease transmission.

In vaccinated populations, the interval between measles outbreaks increases and sufficiently high levels of vaccination can interrupt endemic transmission. With vaccination, the age distribution of cases is determined by which groups are likely to lack vaccine or measles induced immunity. In the United States, an extensive effort to achieve high levels of first dose measles vaccination at 12 to 15 months of age, and addition of a recommended second dose of vaccine in school-aged children has resulted in a decrease in reported measles cases from 400 000 to 500 000 per year in the 1960s to a

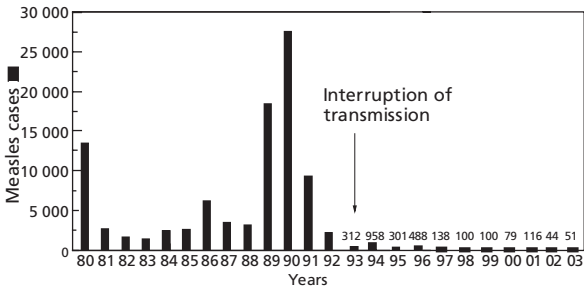

Figure 9 *The number of reported cases of measles per year ranging from 1980 to 2003. The arrow indicates the year of interruption of transmission based on no reported cases of measles for a 12-week period in 1993.*

record low of approximately 100 cases per year from 1998 to 2003 (Figure 9) (CDC 2004). It is likely that endemic transmission of measles in the United States was interrupted in 1993 and subsequent cases have occurred through periodic introduction of measles from other countries followed by limited spread (Bellini and Rota 1998; Rota et al. 2004). Given widespread international travel, measles will continue to occur even in highly vaccinated populations until global eradication is achieved.

Measles remains a formidable disease of children in many areas of the world. The World Health Organization estimates that over 30 million cases occur annually. Approximately 775 000 measles-associated deaths occur annually worldwide, and about 500 000 deaths occur in Africa alone (WHO 1999). In marked contrast, effective use of available live-attenuated measles vaccines, and a variety of multidose vaccination strategies have combined to eliminate measles in many large geographic regions including the Americas, many Scandinavian countries, and the United Kingdom. The WHO and United Nations Children's Fund have announced a strategic plan aimed at enhancing measles control and reducing global mortality by 50 percent based on the 1999 data by 2005. Most regions of the WHO have established strategic plans and tentative time-lines for measles elimination, but a decision to move to global eradication has not yet been made.

DIAGNOSIS

Before the widespread use of measles-containing vaccines, acute measles was diagnosed on the basis of the distinctive symptoms and laboratory confirmation was seldom necessary. As vaccination programs expand, the diagnostic acumen of many physicians to recognize acute measles has decreased (Helfand et al. 2004). Moreover vaccination itself has led to the emergence of unusual presentations and milder forms of measles. For these reasons and the fact that more patients are either being placed on immunosuppressive regimens, or have immunosuppressive illnesses such as acquired immune deficiency syndrome (AIDS), the need for laboratory diagnosis/confirmation of MeV has increased.

Microscopy

On direct examination, production of multinucleate giant cells with inclusion bodies is pathognomonic for measles during the prodromal phase. Such cells are detectable in the nasopharyngeal secretions (NPS). Clear identification of giant cells and Cowdry inclusion bodies is facilitated if the smear is first fixed in formalin and then stained with hematoxylin or eosin. Both direct and indirect immunofluorescence (IF) have been widely used to stain cells shed in nasal secretions, although it may be necessary to remove antibodies that already coat virus antigens with a low pH buffer. Stained cells include macrophages and ciliated cells, as well as giant cells. Urinary sediment cells have also been examined with a high degree of success. IF-positive cells may be shed in the urine from 2 days before to up to 5 days after the appearance of the rash. This method may therefore be more applicable in later stages than examination of NPS specimens. Such cells may also be present in the urine 4–16 days after vaccination with the live vaccine. IF is useful for the diagnosis of measles in the pre-eruptive phase. Immunoperoxidase histochemical stains have also been used, and the use of monoclonal antibodies has improved sensitivity and reliability of virus detection (Zaki and Bellini 1997).

Serology

In common with other infections, diagnosis of measles may be made if antibody titers rise by more than four-fold between the acute and the convalescent phases or if measles-specific IgM is found. Traditional antibody tests such as hemagglutination inhibition (HI), plaque-reduction neutralization (PRN), and enzyme-linked immunoassay (EIA) have been used extensively in the serologic diagnosis of measles. Because of the availability of sensitive and specific commercial kits, EIA has become the most widely used test format. These tests also have the ability to measure measles-specific IgM, as well as IgG responses and therefore have particular importance in diagnosis as well as measles control programs. Some of the available kits were found to have sensitivities and specificities that compared favorably with plaque reduction neutralization (de Sousa et al. 1991; Ratnam et al. 1995). Most laboratories have trained personnel and are already equipped to run EIA tests.

The recommended laboratory method for the confirmation of clinically diagnosed measles is a serum-based IgM EIA. Several serum-based capture and indirect IgM EIAs are now commercially available for use by public health, clinical, and commercial laboratories and, for the

most part, are used worldwide. The network of laboratories within the Pan American Health Organization supported the measles eradication efforts throughout the Americas using EIA as the primary confirmatory assay. The WHO Global Measles Laboratory Network has also recommended the use of the IgM EIA for the laboratory diagnosis of measles. The EIA tests can be done using a single serum specimen, are relatively rapid (2–6 h) and simple to perform, and can be used to diagnose acute measles infection from the time of rash onset until at least 4 weeks after rash onset. Thus, the IgM EIAs fulfill all the basic criteria for the accurate, effective, and efficient diagnosis of measles. Both indirect and IgM capture formats have been used (Erdman et al. 1991; Hummel et al. 1992). Though IgM capture is often regarded as the more sensitive format, studies that have compared the capture and indirect formats found that some of the commercial indirect EIA kits had sensitivities and specificities that equaled those of the capture format (Arista et al. 1995; Ratnam et al. 2000).

Isolation and detection of viral RNA

Until recently, the lack of sensitive cell lines for efficient growth of measles virus made cell culture isolation difficult, even when optimal specimens for virus isolation were available. Previous tissue culture and cell lines, such as primary monkey kidney cells and Vero cells, were considered to be permissive for measles infection. However, it is now recognized that these cells do not possess the appropriate receptor(s) for most wild-type viruses, which explains the low frequency of measles isolation when using these cell types. An Epstein–Barr virus transformed marmoset B lymphocyte cell line (B95a) was demonstrated to be approximately 100 to 1 000 times more sensitive than Vero cells and is currently the cell line of choice for measles virus isolation (Kobune et al. 1990). Attempts at measles isolation indicated that the best success is attained when specimens are collected within 0–5 days of rash onset. Although virus isolation has never been used for routine diagnosis of measles infection, use of the method is encouraged particularly in outbreak settings where there is a requirement for molecular epidemiologic data.

For virus isolation, washings and swabs are collected and mixed with buffered salt solution (pH 7.2) containing antibiotics. Urinary sediment cells are collected by low-speed sedimentation and treated similarly. Measles can be isolated directly from blood, but efficiency is increased if lymphocytes are first separated on a Ficoll gradient and stimulated with mitogen before use. MeV isolated in B95a cells differed in some biological properties from those adapted to Vero cells, suggesting MeV is subject to host cell-mediated selection. It is therefore recommended to include attempts in these cell lines in MeV isolation. Cytopathic effects (CPE) develop usually between 48 h and 15 days, and consist either of a broad syncytium or of a stellate form. Both types reveal inclusion bodies which may be present in both nucleus and cytoplasm. It is not known what governs the different forms of CPE, but the availability of cellular nutrients as well as the type of virus has an effect. Uninoculated identical cultures should also be maintained as controls. If CPE is slow or not clear, it is possible to test whether the cells contain MeV antigens or RNA by appropriate detection methods. RT-PCR-based detection of viral RNA is best applied in specific instances, such as suspected infection of the immunosuppressed in the absence of rash when only limited antiviral immunoglobulins might be expected, developing pneumonia without a rash, or unexplained encephalitis. Finally, both techniques can be attempted as a means of retrospective diagnosis using tissue obtained post mortem. RT-PCR analyses using MeV *N* and/or *F* gene-specific primers are the most commonly performed tests and employ a wide variety of specimens from which RNA is extracted and used as template, including serum, nasopharyngeal aspirates, urine sediment cells. Tissue samples including lung, kidney, skin, and brain are also amenable for testing by RT-PCR following appropriate chaotropic salt treatment and nucleic acid extraction.

Two new approaches show promise in terms of alternatives to specimen collection for the diagnosis of measles: the use of oral fluid and the use of blood spots on filter paper. Oral fluid samples have been used both for detection of measles-specific IgM and IgG, as well as rubella and mumps (Helfand et al. 1996; Perry et al. 1993). This technique is already being used for routine measles surveillance in the UK (Jin et al. 2001). The use of oral fluid samples has appeal because the technique is noninvasive, it can be used for rubella testing, it does not require processing of the sample in the field, and it may be possible to detect not only measles-specific IgM, but measles virus genome for molecular characterization (Riddell et al. 2001). However, oral fluid samples have the disadvantage that the specimens often have heightened background reactivity in commercial assays, or the commercial assays are not sufficiently sensitive when oral fluid specimens are used. Both factors make the use of oral fluid specimens less useful in settings with low measles incidence.

Like oral fluid samples, blood spots collected on filter paper do not require processing in the field, a cold chain does not appear necessary, they can be used to test for rubella as well as for measles (Condorelli et al. 1994, 1998; Helfand et al. 2001), and they can be used for molecular characterization of the measles virus genome (de Swart et al. 2001), although virus isolation would not be possible. In addition, the eluted serum from blood spots can likely be tested in commercially available measles EIA assays without the loss of sensitivity and specificity (de Swart et al. 2001; Helfand et al. 2001), making this technology very attractive for widespread use. Although

still considered an invasive technique as a method for sample collection, fingerstick is often more acceptable to parents than phlebotomy (Wassilak et al. 1984).

TREATMENT

Measles is an acute self-limiting disease, which, in the absence of complications, will run its course without the need for specific intervention. Treatment is supportive and there are no proven effective antiviral compounds available. Infection of the undernourished, the immunocompromised or children suffering from chronic debilitating diseases is more serious. Such patients as well as children of less than 1 year of age and pregnant women after household contacts can be protected by the administration of human anti-measles gammaglobulin (0.25–0.5 ml/kg). If this is given within the first 3 days after exposure, it is usually effective (CDC 1989). The effectiveness is diminished if the globulin is given 4–6 days after exposure, and abolished if more than 6 days have elapsed. Ribavirin has been used to treat pneumonitis and SSPE (Mustafa et al. 1993). Respiratory tract infections cause considerable damage to the ciliated epithelium and this may lead to superimposed bacterial infections. In the case of a pneumonia it is sometimes difficult to distinguish between primary viral infections and the superimposed bacterial infection. In any case, treatment with antibiotics is required.

Treatment of acute measles postinfectious encephalitis is only symptomatic and supportive, and does not differ from any other postinfectious encephalitis. Careful attention to fluid and electrolyte balance is essential. Seizure control requires anticonvulsive drugs. Application of steroids has not been shown to be beneficial. No specific therapy is known for SSPE. A variety of approaches have been attempted, but as yet no convincing results effects have been demonstrated and almost all cases have proved fatal. Evaluation of the effectiveness of treatment is extremely difficult. First, the course of SSPE is highly variable and spontaneous remissions are common. Secondly, SSPE is a rare disease, and clinical trials are therefore inevitably based on a very small number of patients and interpretation is difficult. Isoprinosine (inosiplex) and interferon (also applied in combination) have been widely used for treatment of SSPE without, however, preventing the lethal outcome of the disease (Gascon 2003). In the case of isoprinosine, it seems likely that treatment may lengthen survival time if given early in the disease.

PREVENTION

Vaccination with measles-containing vaccine is the only effective way to prevent measles infections. The PAHO region of the WHO has effectively eliminated measles using live-attenuated measles vaccine. Although the vaccine strategies in the PAHO member countries markedly varied, all strategies provided vaccinees with the opportunity to receive two doses of vaccine (de Quadros et al. 2004). Following a resurgence in measles disease between 1989 and 1991, the United States adopted a two dose vaccination schedule (American Academy of Pediatrics 1998). The first dose is administered at 12–15 months of age, and currently the second dose can be given any time between 4 weeks later and school entry. At present, the vaccine coverage in the US is estimated to be 93 percent (Hutchins et al. 2004). As a result of the improved immunization strategy, the number of reported measles cases has dramatically dropped (Figure 9) and most of the reported cases can be attributed to importations. In Germany and the UK, where vaccination is not mandatory, philosophical opposition to and distrust of vaccination has led to a lower acceptance rate and less dramatic results have been achieved.

In developing countries, the consequences of MeV infection are most severe. Factors, such as the presence of maternal antibody, underlying infectious diseases, and malnutrition, profoundly interfere and confound vaccine efficacy in age groups most vulnerable to measles. Moreover, large influxes of migrating refugees from areas beset by conflict or natural disaster have resulted in the re-introduction of measles into populations that have undergone mass vaccination efforts, thus complicating the assessment of vaccine efficacy. Although the live-attenuated measles-containing vaccines have been successfully used to control and eliminate measles in the Americas and elsewhere (see below), the experience in Africa has led some to consider development of alternative vaccines, such as high-titered vaccines, DNA-based vaccines with or without adjuvants, thermostable dried powder vaccines, and more. With the exception of the high-titered vaccines, which are no longer recommended by the WHO following their association with adverse outcomes (Aaby et al. 1993; Holt et al. 1993), none of the other alternatives have been clinically tested. Nonetheless, any vaccine used in mass vaccination campaigns must be safe, as measles is not normally a life-threatening illness, efficacious, and affordable for mass administration. Its effectiveness should also be long-lasting, if not life-long.

Live vaccine

The first attenuated vaccine strain was the Edmonston B strain produced by serial passage of the virus in human kidney cells, human amnion cells, chick chorioallantoic membrane and, finally, chicken embryo fibroblasts (Rota et al. 1994). This vaccine was administered intramuscularly or subcutaneously 12–18 months after the disappearance of maternal antibodies. Vaccination resulted in 95 percent seroconversion of recipients, but side-effects of mild measles (fever and rash) were common (5–10 percent). Consequently, the vaccine was administered along with immunoglobulin (Ig) to modify the side-

effects (Markowitz and Katz 1994). The side-effects and necessity to provide Ig led to the development of the further attenuated and less reactogenic Schwarz and Moraten strains. These were derived from the Edmonston B vaccine by further passage in the chick embryo at lowered temperature. Further Medical Research Council (UK) trials demonstrated that these were indeed less reactogenic and the incidence of postvaccination febrile convulsions was reduced from 7.7 to 1.9 per 1 000 recipients. Both vaccines produced a 95 percent seroconversion rate, although antibody titers induced by the Schwarz strain declined more rapidly than those produced in response to the Moraten strain but remained at protective levels.

In recent years, the Edmonston B vaccine strain was further passaged in human diploid cells. This vaccine, referred to as Edmonston Zagreb strain, has been shown to produce higher seroconversion rates than the Schwarz vaccine when administered at the same age. Similar observations have been made with the AIK-C vaccine, produced in Japan, but whose lineage stemmed from the Edmonston A virus. All WHO measles vaccines that are currently in use are derived from Edmonston Enders A or B seed stocks. The attenuated strains now in use are so reduced in virulence that encephalitis has only been noted in about one in 1 million vaccine recipients as compared to one per 1 000–5 000 children with natural measles. The measles vaccine is administered subcutaneously usually between the ages of 9 and 20 months for primary vaccination. Humoral immune responses, as defined by HI, NT, and enzyme-linked immunosorbent assay (ELISA), are good and protective as long as the vaccine is given after waning of the maternal antibodies. As with acute measles, the major isotype of MeV-specific antibodies is IgG1. Levels of antibodies induced are generally lower than after natural infection and may decay more rapidly, however, are still measurable in most individuals 30 years after immunization in the absence of boosting infections (Dine et al. 2004). Activation of cell-mediated immunity is generally thought to be similar to that of acute measles in that both MeV-specific $CD4^+$ and $CD8^+$ T cells are stimulated, although CTL-responses after restimulation in vitro are considerably lower than after natural infection. Also similar as with acute measles, administration of live measles vaccine is associated with transient lymphopenia, loss of DTH skin test responses to recall antigen, and decreased in vitro proliferative responses to mitogens. Both leukopenia and atypical lymphocytosis have also been found after revaccination.

Effectiveness of vaccination in the control of measles

In the prevaccine era, an estimated 4–5 million cases occurred annually in the USA and by the age of 15 years 95 percent of the population had seroconverted. Following the rigorous implementation of the MeV vaccination program in the United States, the case reports fell dramatically from 500 000 annually to 26 000 in 1978 and 1 500 in 1983. From 1984 to 1988 only 3 700 cases were reported (Figure 9). As a result of measles vaccination, the mortality and PIE have also declined and the available experience indicates that SSPE can also be prevented by measles vaccination (Halsey 1990). However, in 1989 and 1990 a dramatic increase in acute measles cases was observed in the USA which rose in 1989 to 18 193 and in 1990 to 27 786 cases (Figure 9) (Atkinson et al. 1992). The two major causes for this resurgence of measles were the unappreciated low vaccine coverage among preschool-aged children, particularly in urban settings, and vaccine failure (waning immunity) in a low percentage of school-aged children who received only a single dose of vaccine. As a result of this measles epidemic and to address the issue of possible waning immunity, the American Academy of Pediatrics (AAP) and the Immunization Practices Advisory Committee of the USA (ACIP) recommended a change from a one-dose to a two-dose schedule for measles vaccination, as discussed earlier. In 1998, both groups issued a uniform recommendation for a second dose before school entry, and further recommended that all school-aged children receive a second dose of vaccine before 2001 (American Academy of Pediatrics 1998; Watson et al. 1998). The elimination of measles in the USA has taken a strong political and social commitment, uniting the efforts of many professional groups such as the ACIP, AAP, American Academy of Family Physicians (AAFP), state and local governments, and a number of community-based organizations. In 1999, single dose coverage at 2 years and at school entry was 92 and 98 percent, respectively. Seventy percent of school-aged children were estimated to have received two doses of vaccine in 1998 (CDC 2000; Kolasa et al. 2004). The two-dose schedule is only one strategy used to ensure high vaccine coverage in the quest for measles elimination. PAHO has used live-attenuated MeV in a strategy known as keep-up, catch-up, follow-up. This strategy combines routine vaccination (keep-up) with mass vaccination campaigns (catch-up), and 4-year follow-up campaigns (follow-up) (de Quadros et al. 1996). Both this schedule and the two-dose schedule employ the live-attenuated vaccine and are predicated on effective and efficient implementation of routine immunization programs. Furthermore, both strategies provide at least a second immunization opportunity for the vaccinee. Many countries in various regions of the WHO have adopted different variations of the two opportunities for immunization strategy for the interruption of measles transmission with remarkable results (Strebel et al. 2004). When used in conjunction with the two opportunities for measles vaccination, the current live-attenuated measles vaccine has proven to be efficacious, effective, and safe. Initial vaccination can be

administered at 6 months of age to children living in high-risk areas (areas with a large inner-city urban population where more than five cases among preschool-aged children occurred during each of the last 5 years or with recent outbreaks among unvaccinated preschool-aged children). Vaccination at ages younger than 12 months is associated with reduced efficacy, thus a second dose of vaccine is critical to ensure protection for those children. In these cases, the second dose should be administered before the determined average age of onset of natural measles for the region/country.

Side-effects of live vaccination, adverse reactions, precautions, and contraindications

The vaccine has an excellent record of safety. Although on rare instances a transient rash or low-grade fever are observed after 5–12 days in some vaccinees, these remain otherwise asymptomatic as for the vast majority of recipients. As with the administration of any agent that can induce fever, some children may have a febrile seizure. Although children with a history of seizures are at increased risk for developing idiopathic epilepsy, febrile seizures following vaccinations do not increase the probability of subsequent epilepsy or neurologic disorders. Most convulsions following measles vaccination are simple febrile seizures, and they affect children without known risk factors. Nevertheless, parents of children who have a personal or family history of seizures should be advised of the small increased risk following measles vaccination which is, however, by far outweighed by the benefits of the protective effects. CNS conditions, such as encephalitis and encephalopathy, have been reported with a frequency of less than one per million doses administered, an incidence which is lower than that of encephalitis of unknown etiology. This finding suggests that the reported severe neurologic disorders temporarily associated with measles vaccination were not caused by the vaccine. Live measles vaccine should not be administered to women who are, or are considering becoming, pregnant within the next 3 months because of the theoretical risk of fetal infection. The decision to administer or delay vaccination because of a current febrile illness depends on the cause of the illness and the severity of symptoms. Hypersensitivity reactions following the administration of live measles vaccine are rare and usually occur at the injection site. Vaccine is contraindicated in persons with a history of anaphylactic reactions to eggs or gelatin following ingestion. Individuals who have experienced anaphylactic reactions to neomycin should not be given the vaccine (Duclos and Ward 1998).

Replication of vaccine viruses can be enhanced in persons with immune-deficiency diseases and in those with immunosuppression, as occurs with leukemia, lymphoma, generalized malignancy, or therapy with alkylating agents, antimetabolites, radiation, or large doses of corticosteroids. Thus, patients with such conditions or therapies (except for HIV infection) should not be given live measles vaccine. Short-term corticosteroid therapy does not contraindicate live measles vaccination. The increasing number of infants and preschoolers with HIV infection in certain countries has directed special attention to the appropriate immunization of these children. Asymptomatic children in need of measles live vaccination should receive it without prior evaluation of HIV infection state. Moreover, vaccination should be considered for all symptomatic HIV-infected children, including those with AIDS, since measles in these children can be severe. Limited data on measles vaccination among both asymptomatic and symptomatic HIV-infected children indicate that the vaccine has not been associated with severe or unusual adverse events, although antibody responses have been unpredictable (Moss et al. 1999, 2003). Exposed symptomatic HIV-infected (as well as other immunocompromised) persons should receive high doses of measles Ig regardless of their previous vaccination status.

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Rubulavirus: mumps virus

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IDENTIFICATION OF THE VIRUS AND CLASSIFICATION

As a disease, mumps has been known for centuries (Gordon and Kilham 1949). A population of roughly 200 000 people is needed to sustain mumps infection and this density was first achieved about 4 000 to 5 000 years ago (Carbone and Wolinsky 2001). The first description of a mild epidemic illness with swelling in front of the ear, sometimes accompanied by painful swelling of the testes, is accredited to Hippocrates in the fifth century BC. The name mumps may be derived from the Old English verb meaning 'to sulk' or from the Scottish verb meaning 'to speak indistinctly' (Gershon 1995).

As early as 1908 Granata suggested, from experiments in rabbits, that mumps disease was caused by a filterable agent (Granata, cited in Wollstein 1916). However, it was Johnson and Goodpasture (1934) who first induced parotitis in rhesus monkeys by inoculating fresh saliva from acute mumps cases directly into Stensen's duct, and then passed the infection to further monkeys by inoculation of a sterile parotid gland extract by the same route. A major step forward was the growth of monkey-passaged mumps virus in the yolk sac, amniotic sac, and allantoic sac of developing chick embryos (Habel 1945; Levens and Enders 1945). This was followed by primary isolation from human saliva (Beveridge et al. 1946) and cerebrospinal fluid (CSF) (Henle and McDougall 1947), and eventually the isolation and passage of mumps virus in cultures of Hela and monkey kidney cells (Henle and Deinhardt 1955).

Mumps virus is classified in the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus,* based on virus morphology, genome organization, genetic sequences, and the biological activity of viral proteins. Also classified in this genus are the human parainfluenza viruses 2, 4a, and 4b, *Newcastle disease virus*, *Avian paramyxoviruses 2 to 9*, *Simian virus 5*, *Simian virus 41*, *Mapuera virus*, and *Porcine rubulavirus* (van Regenmortel et al. 2000).

PHYSICAL PROPERTIES

Mumps virus morphology

Mumps virions grown in cell culture are heterogeneous in size and shape, and consist of pleomorphic particles measuring between 100 and 600 nm in diameter. There is an outer lipid membrane on the surface of which are glycoprotein projections measuring 8–17 nm. This membrane envelops the nucleocapsid that consists of a coiled helical structure with a mean diameter of 17 nm, a central core of 4–5 nm, a regular periodicity of 5 nm, and a unit length of 1 μm (Figure 1). In addition to the roughly spherical virions, filamentous and other bizarre forms commonly occur (Horne et al. 1960; Finch and Gibbs 1970; Kelen and McLeod 1977).

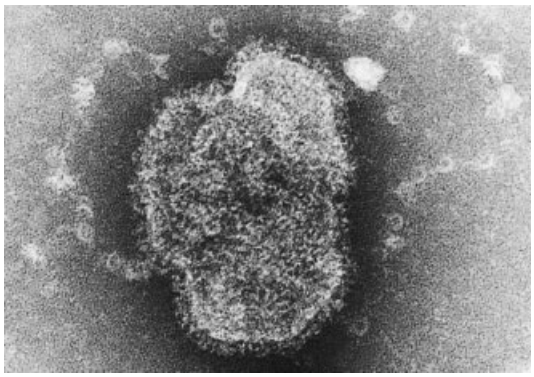

Figure 1 *Electron micrograph of a mumps virus negatively stained with phosphotungstic acid. The helical nucleocapsid can be seen extruding through the outer membrane at the bottom right of the virion (×200 000) (courtesy of Dr. H. Appleton).*

Virus stability

Mumps virus infectivity is lost within 3–4 days at room temperature, but may remain for several weeks at 4°C, and for years at −50°C or below. The presence of protein, for example 1 percent bovine albumin, 0.5 percent gelatin, or 2 percent serum, stabilizes the virus which otherwise loses 90–99 percent infectivity within 2 h at 4°C in a protein-free medium. Mumps virus infectivity is destroyed by heat (55°C for 20 min) and by reagents which disrupt lipids such as organic solvents, e.g. ether, acetone, or chloroform, detergents, oxidizing agents, β-propiolactone, 0.2 percent formalin at 4°C for 2 h, low pH, drying, and intense ultraviolet radiation. Mumps virus antigens are not destroyed by these treatments, however (Kelen and McLeod 1977; Hopps and Parkman 1979).

GENOMIC STRUCTURE

Organization of the genome

The mumps virus genome consists of a nonsegmented single-stranded RNA macromolecule of negative polarity that contains 15 384 nucleotides (nt), based on several mumps strains that have been completely sequenced (Elango et al. 1988, 1989a, b; Elliott et al. 1990; Okazaki et al. 1992; Clarke et al. 2000; Jin et al. 2000). There are seven genes coding for six major structural proteins that were identified by many investigators, and a seventh small hydrophobic protein (SH) of uncertain function (Figure 2, Table 1).

The NP, P, and L proteins

The NP, P, and L proteins are components of the viral nucleocapsid (NC). The nucleocapsid-associated protein (NP) is a major NC associated protein and protects the genomic RNA from cellular proteases. NP contains 549 amino acids (aa) and has an estimated molecular weight of about 61.4 kDa (Tanabayashi et al. 1990a). The NP is susceptible to proteolytic degradation following phosphorylation (McCarthy and Lazzarini 1982). Three antigenic sites have been identified residing within the 74 aa C-terminal region (aa 475–549), between aa 412 and aa 475, and within the N-terminal half of NP (Tanabayashi et al. 1990a). Amino acid substitutions identified in mumps strains are located mainly in the C-terminal half of NP (Jin et al. 2000). The primary structure of NP is well conserved among phenotypically divergent mumps virus strains (Carbone and Wolinsky 2001).

The 1 320 nt phosphoprotein (*P*) gene (including 3′ and 5′ noncoding regions) encodes a protein of 391 aa. The P protein is phosphorylated and has a predicted mass of 41.6 kDa. P is also NC-associated and forms part of the transcriptase complex. The generation of the P protein requires the insertion of two nontemplated guanosine residues into the *P* gene transcript at a G-rich region of the *P* gene. An alternative transcription pathway leading to a 224 aa virion (V) protein is produced in the absence of RNA editing by G-nucleotide insertion (Elliott et al. 1990; Paterson and Lamb 1990; Carbone and Wolinsky 2001). A relative abundance of cysteine residues is present at the C terminus of the V protein, and this feature indicates that the cysteine-rich V protein may have a regulatory function in RNA transcription (Paterson and Lamb 1990).

The large (*L*) gene is 6 925 nt in length excluding the polyA tract, and encodes a putative protein of 2 261 aa with a predicted molecular weight of 256.6 kDa (Okazaki et al. 1992). The L protein is associated with the viral nucleocapsid structure as part of the transcriptase complex. There are important motifs including the active polymerase catalytic site (aa 778–780), an RNA template contact region (aa 555–643) and an ATP binding site (aa 1814–1818) (Okazaki et al. 1992; Tanabayashi et al. 1992).

The HN, F, and M proteins

Two surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F), together with nonglycoprotein M (matrix), form the virus envelope. The putative HN protein consists of 582 aa and has a molecular weight of about 64 kDa. The HN protein mediates adsorption of the virus to the host cell. A hydrophobic domain of 19 residues (aa 35–53) at the N-terminus of HN, which appears to anchor the protein in the viral envelope, is conserved (Waxham et al. 1988). An A for G substitution at nt position 1081 resulting in a change from Glu^{335} to Lys^{335}, which has been suggested as a marker for virulence, was found in some strains (Brown et al. 1996; Brown and Wright 1998), but was not observed in others (Jin et al. 2000). There are nine potential glycosylation

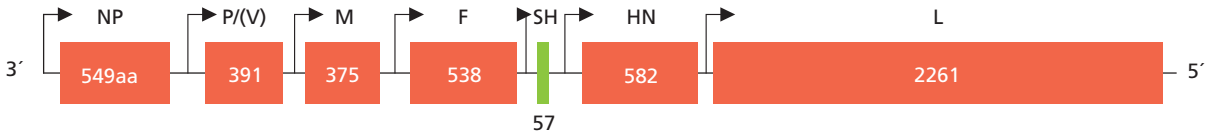

Figure 2 *The structure of the mumps virus genome showing the seven coding regions. The numbers in the boxes denote the number of amino acids in each gene product.*

sites and 17 cysteine residues within the putative HN protein, which are important antigen recognition sites.

The *F* gene encodes a 538 aa protein with a calculated molecular weight of about 58.8 kDa (Waxham et al. 1987). The functionally important domains within the *F* gene include the hydrophobic amino terminus domain (aa 20–23) which is presumed to be the region for initiating the fusion process, and the sequence Arg–Arg–His–Lys–Arg (aa 98–102), recognized by a host-cell protease which activates the F protein (Waxham et al. 1987). The mature form of the F protein is a disulfide-linked complex of F_1 and F_2, a sole cysteine in F_2 providing the only disulfide link with the membrane-bound F_1 (Carbone and Wolinsky 2001). Depending on the strain there are six or seven potential glycosylation sites and 14 cysteine residues within the F protein. Serine at position 195 was reported to be responsible for the in vitro cell fusion observed with certain mumps virus strains (Tanabayashi et al. 1993).

The M protein is composed of 375 aa and has an estimated mass of about 41.6 kDa. It plays a critical role in the assembly of mumps virions by mediating the alignment of the nucleocapsid beneath virus-modified areas of the host cell membrane before budding of the virion (Matsumoto 1982). The M sequence of various mumps strains is highly conserved, the aa differences amongst strains ranging between 0.3 and 1.9 percent (Tanabayashi et al. 1990b; Jin et al. 2000).

The SH protein

The small hydrophobic (SH) protein is a putative membrane protein (Takeuchi et al. 1996) of unknown function. SH mRNA is found in mumps virus-infected cells and encodes a 57 aa molecule (Elango et al. 1989b). The *SH* gene is the most variable part of the mumps virus genome and phylogenetic relationships among mumps strains have been determined using SH gene sequences (Afzal et al. 1997a; Wu et al. 1998; Jin et al. 1999). In contrast to other genes, where nt and aa divergence range from 3.2 to 4.0 percent and 0.5 to 3.0 percent, respectively (Jin et al. 2000), variations between strains of up to 20 and 41 percent, respectively, are seen in the *SH* gene (Jin et al. 1999). This confirms that the *SH* gene is the most suitable target for genotyping (Afzal et al. 1997a; Orvell et al. 1997; Wu et al. 1998), and sequence analysis of the *SH* gene has proved to be a useful tool for studying the molecular epidemiology of mumps (Cohen et al. 1999; Jin et al. 1999, 2004).

The 3′ and 5′ noncoding regions

The 3′ and 5′ noncoding regions (NTR) are situated at the start and end of each gene, as well as between each gene (intergenic). These regions are involved in transcript termination and in initiating the addition of the poly(A) tails that are present at the ends of all viral transcripts. A high degree of variation was noted at the intergenic junctions amongst mumps strains (Jin et al. 2000), as well as in other rubulaviruses (Bellini et al. 1998). The terminal RNA regions of the genomic and antigenomic RNAs of the paramyxoviruses are known to contain sequences essential for RNA replication and transcription (Elango et al. 1988; Harty and Palese 1995). The 5′ NTR of the *F* gene is critical for pathogenicity in other paramyxoviruses (Evans et al. 1990;

Table 1 *Position and size of mumps virus gene products*

Gene	3′ NTR (number of nts)	ORF position (nt numbers)	5′ NTR (number of nts)	Intergenic sequence (number of nts)
3′ Leader	55	–	–	–
NP	90	146–1 795	111	2
V/P	70	1 979–2 653/3 152	74	1
M	36	3 264–4 391	90	1
F	63	4 546–6 162	48	7
SH	50	6 268–6 441	92	2
HN	78	6 614–8 362	66	1
L	8	8 438–15 223	137	–
5′ Trailer	24	–	–	–

nt, nucleotide; NTR, nontranslated region; ORF, open reading frame.

Valsamakis et al. 1998). However, the significance of variations in the NTR of mumps strains, which may affect biological function, remains unclear.

Strain variation

It is generally believed that RNA viruses are prone to rapid mutation (Domingo et al. 1997), and genetic mutations could accumulate during virus transmission, cell adaptation or in vitro replication. Although mumps virus is serologically monotypic, distinct lineages of wildtype viruses exist and have been observed to cocirculate globally since genetic characterization of viruses became widely available in the 1980s. Genotypes A–J have so far been reported for mumps virus. The strain diversity was identified based on the nucleotide sequence of the most variable gene, the *SH* gene (Afzal et al. 1997a; Orvell et al. 1997; Jin et al. 1999, 2004; Kim et al. 2000; Tecle et al. 2001; Uchida et al. 2001). Studies demonstrating the overall stability of the mumps genome support the use of the *SH* gene, which shows the greatest diversity, for genotyping of mumps virus strains (Jin et al. 2000). Genetic characterization of wild-type viruses has become invaluable for mumps control by identifying the transmission pathways of the viruses (WHO 2001) and oral fluid samples may be used for this purpose (Jin et al. 2004). However, there is insufficient evidence to show that mumps genotypes are associated with particular clinical symptoms or that the current vaccine would not protect against all the genotypes (Yates et al. 1996). Further studies of mumps virus diversity are required to understand its evolution, to define the relationship between strains and to understand the effects of these mutations on biological functions.

BIOLOGICAL PROPERTIES

In vitro replication

Paramyxovirus infection of a susceptible cell includes the steps of adsorption, penetration, internalization of the virion, and uncoating (Galinski and Wechsler 1991). The F and HN proteins mediate fusion of the viral envelope with the plasma membrane, although the cell receptors for mumps virus have not yet been identified. Subsequently, virus nucleocapsids are released into the cytoplasm (Lamb 1993). Viral proteins are synthesized in the cytoplasm of infected cells, and the assembly of virions occurs on the plasma membrane by a budding process. Regions that contain the fully processed and glycosylated HN and activated F proteins are prepared on the plasma membrane to accept the nucleocapsids for budding. The M protein is thought to interact with both the cytoplasmic portions of the glycoproteins and with the nucleocapsids at the budding site. The final assembly process of mumps virus packaging and bud formation remains unclear (Bellini et al. 1998).

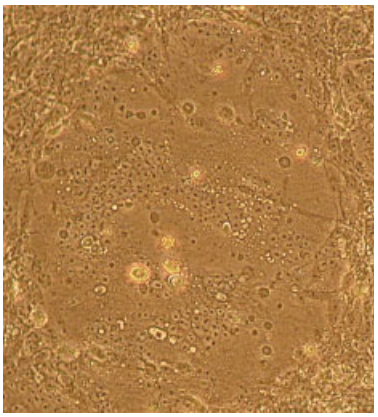

Figure 3 *Syncytial cytopathic effect produced by the replication of a* mumps virus *isolate in Vero cell cultures.*

Mumps virus will grow in vitro in the allantoic cavity, amniotic cavity, and yolk sac of fertile hens' eggs, and in a number of primary cell cultures, such as chicken embryo fibroblasts, monkey kidney cells, monkey testicular cells, human embryonic kidney, human amnion, and bovine kidney cells. Many semicontinuous mammalian cell strains and continuous cell lines are also susceptible to mumps virus including Vero, BS-C-1, LLC-MK2, MDBK, MDCK, Hela, Hep-2, KB, B95a, BHK, and human fibroblasts (Shelokov et al. 1958; Hopps and Parkman 1979; Carbone and Wolinsky 2001; Knowles and Cohen 2001). Mumps virus replication has been reported in human fetal pancreatic beta cells (Notkins 1977; Vuorinen et al. 1992) and human leukocyte cultures (Duc-Nguyen and Henle 1966), and persistent mumps virus infection occurred in vitro in synovial cells, although viral antigens were not expressed on the surface of infected cells (Huppertz 1994). Typically a syncytial cytopathic effect (CPE) is seen within 3–7 days after inoculation (Figure 3), but vacuoles, spindlelike cells, or cell pyknosis may also occur or CPE may be minimal (Gresser and Enders 1961; McCarthy et al. 1980). Syncytium formation is dependent on cell fusion for which expression of the glycoproteins HN and F on the cell surface are essential, together with the presence of the amino acid serine at position 195 in the fusion protein (Tanabayashi et al. 1993).

Hemagglutination and hemadsorption

Mumps virus hemagglutinates guinea pig erythrocytes at 4–37°C, and also those of chickens, rodents, simians, and humans. However, elution occurs rapidly above 4°C (Beveridge and Lind 1946; Kelen and McLeod 1977). Furthermore, due to the expression of HN on the cell surface, mumps virus-infected cells, with or without CPE, hemadsorb guinea-pig, chicken, rhesus, sheep, and

human O red blood cells at 4 and 20°C (Shelokov et al. 1958; Duc-Nguyen 1968; Person et al. 1971).

PATHOGENESIS

Mumps virus is usually spread by droplet infection (Gordon and Kilham 1949; Lerner 1970; Balraj and Miller 1995). There is an initial incubation period of 16–18 days, range 14–24 days, during which virus first replicates in the mucosa of the upper respiratory tract or eye. Infection then spreads to draining lymph nodes where further virus multiplication takes place, followed by a transient viremia, at which time virus dissemination occurs to other organs of the body (Kilham 1948; Lerner 1970; Carbone and Wolinsky 2001). Mumps virus can probably infect most organs of the body, but has a predilection for glandular tissue: most frequently affected are the salivary glands, testes, pancreas, and brain, and less frequently the heart, kidneys, ovaries, breasts, epididymis, prostate, joints, labyrinth, spinal cord, lung, liver, spleen, and thyroid. Mononuclear cell inflammation and edema occur in tissues during the incubation period and contribute to the tissue damage. Interferon is probably also important in pathogenesis, and can be detected in serum and saliva during the first 3 days of illness (Lerner 1970). The peripheral white blood cell count early in disease ranges between 2 400 and 30 000/mm^3 usually with a predominance of lymphocytes (Lerner 1970).

Infectious mumps virus is present in the blood for only a day or two at the onset of disease, but can be detected in the throat or saliva from 6 days before the onset of symptoms and may continue for up to 10 days after onset (Henle et al. 1948b; Kilham 1948; Brunell et al. 1968; Lerner 1970), cessation of excretion correlating with the local production of mumps-specific secretory IgA (Chiba et al. 1973). Mumps virus is also present in the urine for up to 14 days after the onset of illness (Utz et al. 1964; Lerner 1970). Kilham (1951) reported isolation of mumps virus from the breast milk of a woman who had parotitis 2 days before delivery, and Taparelli et al. (1988) isolated mumps virus from vaginal secretions. Mumps virus was recently isolated from semen during orchitis (Jin et al. 2004).

Mumps virus infects the ductal epithelium of the parotid gland producing intracytoplasmic inclusions, and the infected cells desquamate into the lumen of the duct. The duct is blocked by these cells and by leukocytes and other debris, causing dilatation above the blockage. There may also be an acute inflammatory infiltrate of periductal tissue involving lymphocytes and macrophages (Weller and Craig 1949; de Godoy et al. 1969). The lymphatics surrounding the gland become obstructed, with the production of edema that may spread down over the chest wall. Serum amylase levels usually rise coincident with the parotitis (Azimi et al. 1969; Lerner 1970).

Mumps virus infects the testes and much more rarely the ovaries. The seminiferous tubules are probably the site of virus multiplication leading to blockage of the tubules with epithelial cell debris and polymorphonuclear leukocytes, accompanied by local infiltration of lymphocytes and macrophages and interstitial edema. Partial atrophy of the testis may follow (Gall 1947; Carbone and Wolinsky 2001; Gnann 2002). The epididymis, spermatic cord, and prostate may also be affected by inflammation. Mumps virus has been isolated from testicular biopsies taken during orchitis (Hook et al. 1949; Bjorvatn 1973), and at postmortem from an 8-year-old boy with parotitis and meningoencephalitis, but no orchitis (Bistrian et al. 1972).

Involvement of the brain is common as shown by CSF pleocytosis in over half of mumps cases, although many do not have central nervous system (CNS) symptoms (Finkelstein 1938; Bang and Bang 1943; Lerner 1970). Usually the meninges are infected rather than the parenchyma. However, in mumps meningoencephalitis the basal leptomeninges, ependyma, and choroid plexus are infiltrated by lymphocytes and plasma cells. There is widespread edema, gliosis, perivascular cuffing, vascular dilatation, and engorgement, and degeneration of neurones (Taylor and Toreson 1963). Lesions may also be found in the spinal cord and mumps virus may be isolated from the brain postmortem (de Godoy et al. 1969). In secondary mumps postinfectious encephalomyelitis, the CNS lesions are caused by immunological destruction of the myelin sheaths. There is perivascular infiltration of mononuclear cells, edema, scattered foci of neuronophagia, microglial proliferation, swelling of astrocytes, and perivascular demyelination throughout the parenchyma (Donohue et al. 1955; de Godoy et al. 1969; Bistrian et al. 1972).

An acute interstitial inflammatory response may be present in the pancreas, with a mononuclear cell infiltrate, serous edema and swelling, and varying degrees of degeneration of exocrine epithelial cells. The islets of Langerhans may also be affected. The histology is similar to that in the parotid gland (Weller and Craig 1949; Craighead 1975).

The effect of mumps virus on the heart was described at postmortem in an 8-month-old girl (Brown and Richmond 1980). The heart was enlarged due to gross dilatation of all the chambers, there was pericardial effusion with mild pericarditis, and diffuse infiltration of the myocardium by lymphocytes, and occasional polymorphonuclear leukocytes. Mumps virus was isolated from the myocardium.

Transient subclinical renal dysfunction occurs in 100 percent of patients with mumps, with proteinuria in 20 percent and microscopic hematuria in 40 percent (Utz et al. 1964). Mumps virus replicates in renal tubular epithelial cells and the ureter (Weller and Craig 1949). A renal biopsy in a girl with persistent hematuria showed mild mesangial proliferative glomerulonephritis

with deposition of IgA and IgM, C3, and mumps antigen in the glomerulus (Lin et al. 1990). A case of immunoglobulin A nephropathy in association with mumps infection was also reported by Fujieda et al. (2000). Kabacus et al. (1999) described an interstitial mononuclear cell infiltrate, edema, and focal tubular epithelial damage in the kidney at postmortem of a 14-year-old girl with nephritis.

Placental mumps infection may or may not lead to infection of the fetus. In one report there was a diffuse proliferative necrotizing villitis with areas of necrosis and mineralization in the fetal viscera. Intracytoplasmic inclusions were present in chorionic and fetal tissues (Garcia et al. 1980). Kurtz et al. (1982) reported mumps virus isolation from fetal tissues of a spontaneous first trimester abortion occurring on the 4th day of illness, and Takahashi et al. (1998) detected mumps virus RNA by reverse transcription polymerase chain reaction (RT-PCR) in cord blood cells of an infant with severe pulmonary symptoms whose mother was infected with mumps late in pregnancy.

CLINICAL FEATURES

Mumps is an acute infection mostly of children and young adults. Infection is subclinical overall in one quarter to one third of cases (Maris et al. 1946; Philip et al. 1959; Reed et al. 1967; Levitt et al. 1970; CDC 1989), but this depends on age. Between 10 and 14 years of age, symptoms are present in up to 90 percent of cases, whereas in children 2–3 years of age subclinical infection is frequent. Immunity following infection is usually lifelong, but re-infection can occur, sometimes symptomatically (Gut et al. 1995). The case fatality ratio is 1.6 to 3.8 per 10 000 (Carbone and Wolinsky 2001). The symptoms of mumps infection can be variable. There may be a prodromal phase of 1–7 days during which the temperature rises to 37.2–38.3°C, although it may reach above 40°C, and there may be headache, myalgia, anorexia, and malaise. The most common clinical presentation of mumps infection is swelling of the parotid gland which occurs in over 90 percent of symptomatic cases and is very often bilateral. In 75 percent of cases the contralateral gland swells 1–5 days after the first as symptoms in the first gland are subsiding. Initially there is pain or tenderness near the angle of the jaw and perhaps also earache. Maximum pain and swelling usually occurs within 48 h of onset, lasting for up to a week or more (Lerner 1970; Carbone and Wolinsky 2001), and is accompanied by dryness of the mouth. The skin over the gland is hot and flushed. Mumps infection in children under 5 years of age frequently presents as upper respiratory tract symptoms in the absence of parotitis (Cooney et al. 1975). The submaxillary and sublingual glands may also occasionally be involved.

Many other organs may be affected. The frequency of complications follows the frequency of infection, except for orchitis and oophoritis that occur much more often after puberty, and meningoencephalitis which is two to three times more common in males than in females. Mumps infection has not been reported to be more severe in immunocompromised children than in normal children, but the duration of symptoms and viral shedding may be prolonged (de Boer and de Vaan 1989; Tolpin 1991).

Orchitis occurs in about 20–38 percent of postpubertal male patients and much less frequently in prepubertal boys and infants (Werner 1950; Philip et al. 1959; Reed et al. 1967; Lerner 1970; Levy and Notkins 1971; CDC 1989). There may or may not have been preceeding parotitis 3 to 7 days earlier (Lerner 1970; Beard et al. 1977). Following experimental mumps infection, virus was isolated from saliva 10 days before the onset of orchitis (15 days postinfection) in a child who developed orchitis without parotitis (Henle et al. 1948b). Orchitis is usually unilateral, although bilateral involvement is seen in 17–40 percent of cases, and the second testicle may swell as the swelling in the first is subsiding (Werner 1950; Philip et al. 1959; Beard et al. 1977). There may be severe local pain, warmth, and tenderness, together with chills, sweating, swinging temperature, headaches, backache, nausea, and vomiting. The testicles may swell three- to four-fold within 5 days. There may also be associated epididymitis in 85 percent of cases (Lerner 1970). Testicular atrophy may result in one third to half the patients with orchitis. This is bilateral in 10 percent of those with atrophy, but mostly only one testicle is severely damaged (Werner 1950; Lerner 1970; Levy and Notkins 1971; Beard et al. 1977; CDC 1989). The sperm count may be normal, but transient or prolonged azoospermia may occur and antisperm antibodies may be produced (Jalal et al. 2004). Sterility is infrequent (CDC 1989).

Oophoritis, manifested by ovarian pain and pelvic tenderness, may occur in 0.5–7 percent of postpubertal females infected with mumps (Reed et al. 1967; Person et al. 1971; ASID 1974), but the symptoms are usually not as severe as orchitis in males. No association with infertility has been reported. Mastitis has been reported in 31 percent of postpubertal females in association with mumps infection, but the symptoms are mild and fleeting (Philip et al. 1959).

Symptoms reflecting central nervous system involvement may occur in up to 15 percent of mumps cases (Brown et al. 1948; CDC 1989), and are two to three times more frequent in males than in females (Azimi et al. 1969; Johnstone et al., 1972; ASID 1974; McDonald et al. 1989). A spectrum of disease is seen from mild CSF pleocytosis to severe encephalitis (Modlin et al. 1975). Most patients will have meningitis with fever, severe headache, vomiting, neck stiffness, and photophobia, but up to one quarter of these may also have symptoms of cerebral involvement, such as drowsiness,

convulsions, slurred speech, twitching or rigidity of limbs, or altered sensory perception, denoting meningoencephalitis (Philip et al. 1959; Reed et al. 1967; Azimi et al. 1969; Lerner 1970; Johnstone et al. 1972; McDonald et al. 1989; Sosin et al. 1989). However, little change is usually seen in the EEG, even when clinical seizures are present (Gibbs et al. 1964).

Before mumps vaccination was introduced mumps was the most frequent cause of aseptic meningitis in the UK (Johnstone et al. 1972). Mumps meningitis may precede parotitis by a week or follow up to 3 weeks later, and in up to half of patients may occur in the absence of parotitis (Kilham 1949; Azimi et al. 1969; Wilfert 1969; Lerner 1970; Johnstone et al. 1972; Wolontis and Bjorvatn 1973). Mumps meningitis is usually mild and self-limiting, subsiding after 2–10 days without serious sequelae, but following mumps meningoencephalitis sequelae may persist for months to years. There may be difficulty in memory and learning, focal motor and sensory signs (Julkunen et al. 1985), ataxia, and behavioral disturbances along with electroencephalographic changes (Koskiniemi et al. 1983), as well as chronic encephalitis, seizures, and intellectual deterioration, polio-like paralysis or coma, and very rarely quadriplegia, single nerve palsy or weakness of the facial nerve, hydrocephalus, and psychomotor retardation (Bang and Bang 1943; Philip et al. 1959; Lerner 1970; Johnstone et al. 1972; Gonzalez-Gil et al. 2000; Carbone and Wolinsky 2001; Gnann 2002). The fatality rate following mumps encephalitis is between 0.5 and 2.3 percent (Modlin et al. 1975; CDC 1989).

Secondary postinfectious encephalitis involving demyelination may rarely occur 1–4 weeks after mumps infection with the sudden onset of fever, severe chills, paresis, convulsions, or coma, which may be accompanied by involuntary movements, cranial nerve lesions, ataxia, and upper motor neurone signs (Donohue et al. 1955; Taylor and Toreson 1963). Other very rare neurological complications of mumps include transverse myelitis, nystagmus, Ménière's disease, and bilateral neuroretinitis (Venketasubramanian 1997; Bansal et al. 1998; Khubchandani et al. 2002).

Deafness may occur secondary to endolymphatic labyrinthitis manifested by tinnitus, vertigo, and vomiting, in 0.02–0.3 percent of mumps patients. The deafness is usually unilateral and occurs with the same frequency in males and females (Bang and Bang 1943; Lerner 1970; Davis and Johnsson 1983). Mumps virus was grown at operation from inner ear perilymph (Balraj and Miller 1995). Deafness is usually transient (Vuori et al. 1962), but permanent sensorineural hearing loss may occur in up to one in 20 000 cases (ASID 1974; CDC 1989). Total bilateral deafness has been reported following asymptomatic mumps infection (Unal et al. 1998).

Pancreatitis characterized by upper epigastric pain, nausea, and vomiting, may occur following mumps infection, with preceding parotitis being present in only two thirds of cases (Philip et al. 1959; Reed et al. 1967; Lerner 1970; Craighead 1975). Severe hemorrhagic pancreatitis associated with mumps has also been described (Feldstein et al. 1974). Despite many reports of a temporal association between mumps infection and the onset of diabetes mellitus, a causal relationship has not as yet been demonstrated (Craighead 1975; Notkins 1977; Carbone and Wolinsky 2001).

Electrocardiographic abnormalities may be seen in association with mumps infection, beginning 1–4 weeks after onset and lasting from 1 week to several months (Bengtsson and Örndahl 1954; Lansdown 1978). Cardiac symptoms are, however, rarely present although mild pericarditis and interstitial lymphocytic myocarditis have been reported (Lansdown 1978; Brown and Richmond 1980), and fatalities may occur following cardiac involvement (Lansdown 1978; Brown and Richmond 1980; Kabacus et al. 1999). A reported association between mumps infection and endocardial fibroelastosis is controversial (Hopps and Parkman 1979; Ni et al. 1997).

The joints may occasionally be affected by mumps virus infection, leading to mild or moderate migratory mono- or polyarthritis. This complication occurs mainly in young adults, with a male to female ratio of 5:1. Arthritis may appear up to 3 weeks before parotitis or follow up to 2–3 weeks later, usually lasting for 2 weeks or occasionally up to 6 months, but no residual joint damage results (Caranasos and Felker 1967; Gold et al. 1968; Lerner 1970; Gordon and Lauter 1984).

Transient renal dysfunction is frequent during mumps infection but rarely severe. However, a fatal case of interstitial nephritis and myocarditis was reported in a 14-year-old girl (Kabacus et al. 1999). Mumps infection has been temporally associated with subacute thyroiditis (Levy and Notkins 1971; Person et al. 1971; Bell et al. 1997), and mumps virus was grown from thyroid tissue in the acute stage of infection (Eylan et al. 1957; Lerner 1970). Transient abnormalities in liver function tests are occasionally seen in mumps infection, but clinical hepatitis is very rare (Lerner 1970; ASID 1974).

Fetal wastage may occur, usually within 2 weeks of clinical disease in the mother, in up to 27 percent of cases following mumps infection in the first trimester of pregnancy, rarely during the second trimester, and not in the third trimester or postnatally (Philip et al. 1959; Siegel et al. 1966; CDC 1989). Intrauterine mumps infection late in pregnancy may lead to severe pulmonary symptoms in the infant (Jones et al. 1980; Groenendaal et al. 1990; Takahashi et al. 1998), and perinatal infection in young infants may result in upper respiratory symptoms, thrombocytopenia, splenomegaly, and fever (Lacour et al. 1993). There is evidence that premature infants may be more susceptible than full-term infants to postnatal mumps infection (Glick et al. 1998). Clinical mumps is rare in infants during the first 2 years of life, but orchitis has been reported.

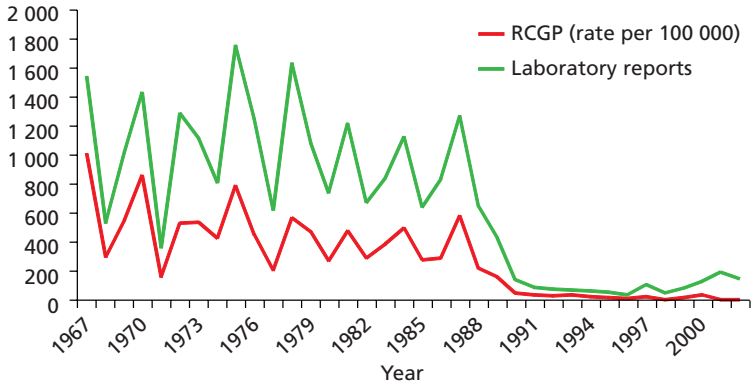

Figure 4 *Consultation rates and laboratory reports for mumps in England and Wales: 1967–2002. Source of data: Birmingham Research Unit, Royal College of General Practitioners (RCGP) and Health Protection Agency Communicable Disease Surveillance Centre*

THE IMMUNE RESPONSE FOLLOWING MUMPS VIRUS INFECTION OR VACCINATION

Both natural mumps infection and vaccination with live-attenuated vaccine induce cell-mediated immunity (CMI), humoral immunity (IgG, IgM, IgA) (Brown et al. 1970; Sarnesto et al. 1985), and long-term protection. Mumps-specific cytotoxic T cells and lymphoproliferative responses are present in the blood, peaking 2 weeks after natural infection or a live vaccine booster dose and lasting for years (Ilonen 1979; McFarland et al. 1980; Chiba et al. 1982; Gans et al. 2001). Mumps-specific cytotoxic T cells and sensitized lymphocytes can also be detected in the CSF of patients with mumps meningitis (Fryden et al. 1978; Kreth et al. 1982; Fleischer and Kreth 1983).

During mumps infection a rise in serum antibody occurs, mumps-specific IgG being maximal during the third week of clinical disease (Ukkonen et al. 1981). Most IgG is of subclass 1, but IgG3 is also present (Sarnesto et al. 1985; Gut et al. 1995). A switch from low to high avidity IgG occurs about 180 days after infection (Narita et al. 1998). Mumps-neutralizing antibody is detectable 2 days after onset of parotitis in some patients, and peaks at 2–4 weeks (Brunell et al. 1968). Low, protective levels of antibody persist thereafter (Meyer et al. 1966; Lerner 1970), but may be boosted by natural re-infection. The level of mumps-neutralizing antibody detected after vaccinaton with live vaccine was found to be lower than after natural infection (Ennis et al. 1969). Immunity to clinical mumps is reported to correlate with a neutralizing antibody titer of >1 in 8, but the importance of protective cell mediated immunity is not known.

Mumps-specific IgM antibody is usually detectable in serum and saliva by 2–4 days after onset, and begins to wane in the first 2–6 months depending on the test used for detection (Brown et al. 1970; Chiba et al. 1973; Bjorvatn 1974; Ukkonen et al. 1981; Gut et al. 1985; Sakata et al. 1985). Anti-mumps IgM has also been reported in cases of secondary vaccine failure (Narita et al. 1998). Anti-mumps IgA antibody appears in serum and oral secretions during the first week of disease, and following vaccination, and persists for weeks to months (Chiba et al. 1973; Bjorvatn 1974; Friedman et al. 1983; Tanaka et al. 1992; Gut et al. 1995).

EPIDEMIOLOGY

Mumps infection occurs worldwide, man being the only natural host for the virus. Before the introduction of vaccination, mumps was a common endemic disease of children and young adults, most clinically apparent infections occurring between the ages of 4 and 14 years, with the peak at 5–9 years. Mumps is rare under 9 months of age (RCGP 1974; Modlin et al. 1975; Galbraith et al. 1984; CDC 1989), but in a study in England 63 percent of children had anti-mumps antibody by 5 years of age and 87 percent were seropositive at 10 to 12 years (Morgan-Capner et al. 1988). This indicates that much infection occurs earlier than is apparent from the clinical data (Galbraith et al. 1984). Cooney et al. (1975) emphasized the role of young children in the spread of mumps infection via saliva due to the mild respiratory illness that occurs at this age. Urban communities have a higher prevalence of mumps infection than rural communities. In England, the peak incidence of mumps infection occurred every 2–3 years in the prevaccination era (Figure 4) (RCGP 1974; Galbraith et al. 1984; Anderson et al. 1987; Balraj and Miller 1995), but in less densely populated or remote areas outbreaks occur every 2–7 years. Mumps infection is seasonal in temperate climates in the northern hemisphere, with most cases occurring in late winter and spring (Lerner, 1970; RCGP 1974; Cooney et al. 1975; Modlin et al. 1975). However, seasonal variation has not been reported in tropical countries. Outbreaks of mumps have been reported in isolated communities (Philip et al. 1959; Reed et al. 1967), and are a particular problem in

crowded environments, such as military establishments (Gordon and Kilham 1949), schools and colleges (London and Yorke, 1973; CDC 1989) and large social events involving young adults with close contact (Bell et al. 1997). There is only one serotype of mumps virus and immunity after natural infection is usually lifelong (Lerner 1970). However, reinfection has been reported in 1–2 percent of cases.

Since vaccination was introduced an at-risk cohort of older age groups has emerged (CDC 1989; Sosin et al. 1989; Bell et al. 1997; Visser et al. 1998) as predicted by Galbraith et al. (1984). The age group with the highest incidence of mumps infection changed from those under 15 years of age to those over 15 years of age 7 years after the introduction of MMR vaccination at 15 months of age in both England and Wales (Gay et al. 1997) and in Croatia (Bakasun 1997). An outbreak of mumps in 1996 in a secondary school in England was reported amongst 13–14 year olds who were too old to have been vaccinated with MMR, but too young to have natural immunity from previously circulating virus (Wehner et al. 2000).

TREATMENT

The treatment for mumps infection is symptomatic, i.e. rest and analgesics. Morphine may be needed for the relief of orchitis. Corticosteroids may be given for severe parotitis or orchitis. Gray (1973) reported that prednisolone reduced the pain of orchitis, but had no effect on the spread of infection from one testicle to the other, or on testicular atrophy. In another study of adult men with parotitis, intramuscular injection of 20 ml of gammaglobulin from human mumps convalescent serum significantly reduced the development of orchitis. There was no protective effect, however, from normal gammaglobulin (Gellis et al. 1945). Several recent studies reported a favorable outcome following experimental treatment of mumps orchitis with IFN-α-2B (Erpenbach 1991; Ruther et al. 1995; Ku et al. 1999), although in one study testicular atrophy was not prevented (Yeniyol et al. 2000).

PREVENTION

Isolation of patients is not effective in controlling the spread of mumps infection due to virus shedding by patients before symptoms are apparent and also by those who are infected subclinically (Henle et al. 1948b; Brunell et al. 1968; Lerner 1970). It was reported that the spread of mumps in a community outbreak was reduced by the early mass use of hyperimmune gammaglobulin (Copelovici et al. 1979). Normal or hyperimmune gammaglobulin given after exposure does not, however, protect from clinical mumps (Enders 1946; Reed et al. 1967; CDC 1989). Similarly vaccination with inactivated or live vaccine late in the incubation period does not prevent clinical mumps (Meyer et al. 1966; Ennis et al. 1969), although the use of killed vaccine or MMR in susceptible groups of children or adults may contribute to the control of a mumps outbreak (Habel 1951; Ramsay et al. 1991).

Mumps infection has now been successfully controlled by vaccination in several countries. In the mid to late 1940s inactivated and live attenuated vaccines derived from monkey tissue or egg-passaged mumps virus, were tried in experimental animals and humans (Enders 1946; Enders et al. 1946; Henle et al. 1951a). A killed vaccine was introduced in 1950, but was discontinued in the USA in 1978 (Habel 1951; CDC 1989). Seroconversion was demonstrated after vaccination with the killed vaccine in 92 percent of recipients as determined by single radial hemolysis (SRH) or enzyme-linked immunosorbent assay (ELISA), but in only 58 percent of recipients by hemagglutination inhibition (HI) (Julkunen et al. 1984). In most cases, the antibody reacted with denatured HN or F protein. Killed vaccines give protection in about 80 percent of recipients, but it is only short-term protection and antibody subsequently wanes (Henle et al. 1951a; Lerner 1970). Anti-mumps IgM antibody is not detectable by radioimmunoassay (RIA) and cell mediated responses are delayed (Ilonen et al. 1981).

Live-attenuated mumps vaccines grown in cell culture were developed in the 1960s. Attenuation was achieved by serial passage of the virus in embryonated hens' eggs followed by chick embryo cell culture. The first safe and effective live-attenuated mumps vaccine was licensed in the USA in 1967 (Modlin et al. 1975; CDC 1989). Seroconversion occurs in up to 97 percent of vaccinees within 4 weeks of vaccination with the live vaccine as detected by virus neutralization, in 75–94 percent by SRH or ELISA and in 38–67 percent by HI (Hilleman et al. 1968; Brunell et al. 1969; Ennis et al. 1969; Modlin et al. 1975; Julkunen et al. 1984; CDC 1989). Whilst 6-month-old infants were reported to have limited humoral immune responses to live mumps vaccine, probably due to the presence of maternal antibody, the cell-mediated response was equivalent to that of older infants (Gans et al. 2001). Protection lasts for at least 20 years (CDC 1989). This long-lasting protection post-vaccination may be due to boosting with naturally circulating wildtype virus in populations with only partial vaccination coverage (Modlin et al. 1975; Weibel et al. 1978). Even if mumps infection in a population is not prevented, fewer complications occur (Visser et al. 1998). Single mumps vaccine was later combined with measles and rubella in the live MMR vaccine. The serological response to mumps and long-term protection were reported to be the same following the combined vaccine as with the single live mumps vaccine (Hilleman et al. 1968; Modlin et al. 1975; Weibel et al. 1978). Vaccination with MMR produces mumps-specific

gamma interferon in over 90 percent of vaccinees (Nakayama et al. 1990). In England and Wales, MMR was introduced in 1988, and was given at 12–15 months of age together with a catch-up program for preschool children (Gay et al. 1997). By 1998 childhood vaccination against mumps was being carried out with varying schedules in 82 countries (Galazka et al. 1999).

Since the introduction of mumps vaccination, the number of mumps cases have fallen dramatically in several countries (Galazka et al. 1999). In England and Wales the incidence of mumps was reduced by about 90 percent (Figure 4) (Balraj and Miller 1995; Gay et al. 1997), and by 98 percent over an 18-year period in the USA (CDC 1989). Mumps outbreaks have, however, recently been reported in vaccinated populations (Hersh et al. 1991; Briss et al. 1994; Cheek et al. 1995; Dias et al. 1996; Germann et al. 1996). These may be due to primary or secondary vaccine failure (Briss et al. 1994; Narita et al. 1998; Afzal and Minor 1999), and occurred mostly after only one dose of vaccine (Hersh et al. 1991). Gay et al. (1997) reported that 11 of 14 children aged 1–9 years confirmed with mumps after 1994 had previously been vaccinated. In the UK, 15–19 percent of those vaccinated at 12–18 months of age were found to be seronegative to mumps 4 years later by plaque reduction neutralization (PRN) (Miller et al. 1995) or ELISA (Pebody et al. 2002). Johnson et al. (1996) found that 11–13 year olds who had been vaccinated at 15 months of age had a lower mean titer of anti-mumps antibody than 4–6 year olds, although the prevalence of antibody was the same in each group. Therefore, although the incidence of mumps is reduced following one dose of vaccine, infection is not eliminated, and the risk of infection in older age groups leading to more complications is increased (Gay et al. 1997). A second dose of MMR at 4–6 years of age was recommended in the USA in 1989 (Galazka et al. 1999) and in the UK in 1996 (Pebody et al. 2002). In Sweden, it is given at 12 years of age (Broliden et al. 1998). A second MMR dose given at 11–13 years of age was reported to be as effective at boosting antibody levels as that given at 4–5 years of age (Johnson et al. 1996). In Finland, a two-dose MMR immunization program was introduced in 1982, and with very high childhood vaccination rates indigenous mumps had been eradicated by 1996. Between 1997 and 1999, only four cases of mumps occurred in Finland, all originating from outside the country, and no secondary cases occurred. Furthermore, infection did not spread from neighboring Russia where large mumps outbreaks were occurring in the unvaccinated population despite frequent travel between the two countries (Peltola et al. 2000).

Live mumps vaccine is contraindicated in immunosuppressed patients and pregnant women, in whom killed vaccine may be used. MMR vaccine may be given to asymptomatic HIV-infected children (CDC 1989; Dunn et al. 1998; Carbone and Wolinsky 2001; Gnann 2002).

The strain of mumps virus used in the original live vaccine was the Jeryl Lynn strain (Modlin et al. 1975). Subsequently the Urabe and Rubini strains have also been widely used in the vaccine following various attenuation protocols, as well as other strains including Leningrad-3, Hoshino, L-Zagreb, S-12, Torii, Miyahara, NK-M46, Sofia 6 (Brown and Wright 1998; Ito and Tsurudome 2002). Vaccination with the Jeryl Lynn strain has been associated with parotitis in vaccinees (Balraj and Miller 1995). Fever, rash, pruritis, and purpura may also be temporally related to vaccination (Modlin et al. 1975; CDC 1989). Mumps virus was not isolated from blood, urine, or saliva postvaccination with the Jeryl Lynn strain (Ennis et al. 1969), or from the throat after vaccination with the Miyahara or Hoshino strains, although mumps RNA was detected in the throat up to 26 days postvaccination with the latter two strains (Nagai and Nakayama 2001). However, in two of three seronegative pregnant women who were vaccinated 7–10 days prior to planned abortion, mumps virus was isolated from placental, but not fetal, tissues (Yamauchi et al. 1974). Mumps infection occurring in four army recruits who received MMR vaccine 16 days earlier, was shown to be caused by wild-type and not vaccine virus (Cohen et al. 1999). Several vaccines are now known to be mixtures of related strains (Boriskin et al. 1992; Afzal et al. 1993; Brown et al. 1996; Brown and Wright 1998). Recently a new vaccine, RIT/4385, derived from one of the two strains in Jeryl Lynn, has been evaluated in Europe (Usonis et al. 1999).

The live Urabe Am 9 strain mumps vaccine was developed in Japan (Yamanishi et al. 1973) and licensed in 1979. It was introduced in other countries in the 1980s to improve efficacy. High rates of seroconversion with the Urabe vaccine were subsequently reported in children from several countries including Brazil, South Africa, India, and China (Galazka et al. 1999), and significantly more children had mumps neutralizing antibody 4 years after receiving MMR vaccine containing the Urabe strain than vaccine containing the Jeryl Lynn strain (Miller et al. 1995). Mumps vaccine containing the Urabe strain of virus was found to be associated with parotitis in 0.7 percent of vaccinees (Balraj and Miller 1995), vaccinelike virus being isolated from the throat during parotitis (Forsey et al. 1990). Orchitis and transmission of virus to siblings was also reported (Sugiura and Yamada 1991; Sawada et al. 1993). More significantly, however, the Urabe strain was found to be associated with an unacceptable level of meningitis in vaccinees (Brown et al. 1991). Meningitis, found to occur within 2–4 weeks of vaccination, was first reported in Canada (McDonald et al. 1989) and subsequently in the UK, Belgium, France, Germany, and Japan (Balraj and Miller 1995). Vaccinelike virus was recovered from the CSF in between one in 1 000 and one in 11 000 vaccinees (Forsey et al. 1990; Brown et al. 1991; Sugiura and Yamada 1991; Miller et al. 1993). Meningitis was also a

problem with two other mumps vaccines produced in Japan, from the Torii and Hoshino strains of virus (Ueda et al. 1995), with the Sofia-6 strain grown in guinea-pig cells and used in Bulgaria (Odisseev and Gacheva 1994), and with the Leningrad-3 and L-Zagreb strains in Slovenia and Croatia (Galazka et al. 1999). There was a predominance of meningitis in males, but the illness was mild with no sequelae (Sugiura and Yamada 1991; Balraj and Miller 1995). Urabe containing vaccines were subsequently withdrawn from many markets (Brown et al. 1991; Galazka et al. 1999). In the UK, Urabe containing MMR was replaced with vaccine containing the Jeryl Lynn strain in 1992, and no further cases of meningitis occurred postvaccination (Gay et al. 1997). Urabe Am9 vaccine is a mixture of strains, one of which has an A at nucleotide position 1081 of the HN gene leading to the amino acid Lys at position 335, which has been suggested by some authors (Brown et al. 1996) to be important in the causation of meningitis. It was suggested that this strain pre-existed in the vaccine virus rather than arising as a revertant (Brown and Wright 1998).

The Rubini strain of mumps virus was attenuated by passage in human diploid cells for use as a vaccine, and licensed in Switzerland in 1985 (Glück et al. 1986). It had a high apparent immunogenicity and a low incidence of side effects. However, recent mumps outbreaks in Switzerland and Singapore (Germann et al. 1996; Goh 1999), Portugal (Dias et al. 1996; Afzal et al. 1997b), Spain (Pons et al. 2000; Montes et al. 2002), and Italy amongst populations vaccinated with this strain suggest a low protective value (Germann et al. 1996; Chamot et al. 1998). Primary vaccine failure is probably occurring (Goh 1999), but secondary vaccine failure has also been suggested (Afzal and Minor 1999).

LABORATORY INVESTIGATIONS

Differential diagnosis

Parotid swelling may be due to a number of causes other than mumps virus infection, and laboratory confirmation of mumps is necessary in isolated cases. These causes include: other virus infections such as parainfluenzaviruses 1 and 3, influenzavirus A, coxsackie virus, lymphocytic choriomeningitis virus (LCMV), echovirus, human immunodeficiency virus, Epstein–Barr virus, herpes simplex virus (HSV) and varicella-zoster virus; acute and chronic conditions such as tumors, Sjögren's syndrome, sarcoidosis, calculus, cirrhosis, diabetes mellitus, malnutrition, chronic renal failure, tuberculosis, amyloidosis, intraparotid hemorrhage, suppurative infections; and medications such as iodides, phenothiazines, phenylbutazone, didanosine, thiouracil (Tolpin 1991; Carbone and Wolinsky 2001; Gnann 2002). Similarly, orchitis or testicular enlargement may be due to enteroviruses, mycoplasmas, gonococcal infection, tuberculosis, and non-infectious causes such as tumors, hernia, and torsion of a maldescended testicle (Gray 1973). Another complication of mumps infection, aseptic meningitis, may also be due to infection with other viruses including enteroviruses, LCMV, arboviruses, and HSV (Johnson 1982). In the absence of parotitis, laboratory confirmation of mumps infection is needed even during an epidemic.

Determination of immunity or susceptibility to mumps infection

The mumps skin test that was once widely used for determining immunity to mumps infection has been found to be unreliable, and results do not correlate with the presence of neutralizing antibody (Brunell et al. 1968; St Geme et al. 1975). Early serological tests used to detect antibody to mumps included CFT, HI, and virus neutralization (Henle et al. 1951b; Bashe et al. 1953; Buynak et al. 1967), although the HI test was not recommended for determining immune status because residual inhibitors in the serum can mask low titers of antibody (Kelen and McLeod 1977). There was variation in sensitivity between the tests with antibody remaining detectable by neutralization, but not by CFT or HI for months to years after infection (Ennis et al. 1968; Kelen and McLeod 1977). This, together with the specificity of the neutralization test, led to virus neutralization becoming the standard test for assessing past mumps infection (Meyer et al. 1966; Brunell et al. 1968; Kenny et al. 1970). Subsequently further tests were developed for the detection and quantification of anti-mumps antibody: indirect fluorescent antibody test (IFAT) (Brown et al. 1970), RIA (Daugharty et al. 1973; Forghani et al. 1976), hemolysis-in-gel (Grillner and Blomberg 1976; Väänänen et al. 1976), single radial immunodiffusion, hemolysis inhibition, and mixed hemadsorption (Norrby et al. 1977), enhanced virus neutralization (Sato et al. 1978), and ELISA (Leinikki et al. 1979; Popow-Kraupp 1981; Meurman et al. 1982). ELISA later became the assay of choice because of its sensitivity, specificity, speed, and low cost, and the large numbers of samples that can be tested simultaneously (Leinikki et al. 1979; Glikmann and Mordhorst 1986; Linde et al. 1987; Juto et al. 1989). Harmsen et al. (1992) reported a high level of correlation between mumps antibody as measured by indirect ELISA and that measured by a virus neutralization enzyme immunoassay, supporting an earlier suggestion that antibody detected by ELISA probably reflects immune status (Leinikki et al. 1979). This correlation was not, however, confirmed in a later study (Pipkin et al. 1999). Recently a quantitative immuno-PCR assay was developed in an attempt to increase the sensitivity and specificity of mumps antibody detection (McKie et al. 2002).

Diagnosis of recent mumps infection

SEROLOGICAL DIAGNOSIS

A serological diagnosis of mumps infection can be made by detection of seroconversion or a four-fold rise in mumps-specific IgG antibody in paired acute and convalescent sera, or the presence of mumps-specific IgM antibody in a single acute sample (Ukkonen et al. 1981; Meurman et al. 1982; Glikmann et al. 1986) or from a neonate (Takahashi et al. 1998). Two antigens were originally identified in the CFT, S antigen (soluble, nucleoprotein) and V antigen (surface glycoprotein, HN). Antibody to S antigen rises in the first 2 weeks of infection and then declines rapidly, becoming undetectable within a few months, whereas antibody to V antigen rises to high titer after the first week and persists for years (Henle et al. 1948a). Thus, a comparison of anti-S and anti-V titers could be used to identify recent mumps infection. An antibody rise may also be detected by neutralization, but it was reported that for maximal detection of antibody early after natural infection, and more especially after vaccination, a complement-mediated neutralization test was needed (Hishiyama et al. 1988).

Although mumps-specific IgM antibody can be detected in serum by several different assays, IFAT (Brown et al. 1970; Bjorvatn 1974), the hemadsorption immunosorbent test (Denoyel et al. 1982; van der Logt et al. 1982) and RIA (Daugharty et al. 1973), ELISA is now the most generally used assay (Meurman et al. 1982; Gut et al. 1985). An antibody capture format (MACRIA or MACELISA) gives rise to fewer false-positive and false-negative results than does the indirect test (Sakata et al. 1985). Recently a mumps MACELISA has been developed using recombinant nucleoprotein antigen (rNP) and an anti-rNP monoclonal antibody, which has equivalent sensitivity to the MACRIA (Samuel et al. 2002).

Saliva (i.e. oral fluid) samples are easier to collect than serum and the process is minimally invasive (Parry et al. 1987). Solid phase antibody capture immunoassays are particularly suited to the detection of low level antibody present in saliva, and thus saliva has been used as an alternative to serum for anti-mumps IgM detection in conjunction with both MACRIA (Perry et al. 1993; Jin et al. 2004) and MACELISA (Frankova and Sixtova 1987; Wehner et al. 2000). Mumps-specific IgA antibody is transiently present in saliva and may also detected by RIA (Friedman et al. 1983) or ELISA (Frankova and Sixtova 1987).

A problem encountered in the interpretation of mumps antibody tests is the presence of cross-reacting antibody to the parainfluenza viruses (Kelen and McLeod 1977; Hopps and Parkman 1979). This can be a particular problem with ELISA (Fedova et al. 1992; Harmsen et al. 1992), although less of a problem is found with IgM tests than with IgG (Brown et al. 1970; Meurman et al. 1982; Gut et al. 1985; Linde et al. 1987). Specificity in the mumps ELISA can be enhanced by the use of both an antibody capture format and a nucleoprotein antigen (Glikmann and Mordhorst 1986; Glikmann et al. 1986; Frankova et al. 1988). The effect of different antigens in the ELISA was also reported by Linde et al. (1987), who found that IgG antibody to mumps nucleoprotein rose earlier after infection than antibody to the glycoprotein, as had been reported respectively for the S and V antigens in the CFT (Henle et al. 1948a). Linde et al. (1987) and Gut et al. (1995) found that a high titer of anti-mumps IgG3 antibody to nucleoprotein antigen was diagnostic for recent mumps infection and could be used to distinguish mumps infection from infection with the parainfluenza viruses. Lehtonen and Meurman (1986) reported the use of an IgG avidity ELISA to distinguish early infection with mumps from parainfluenza virus infection.

DETECTION OF MUMPS VIRUS, ANTIGEN, AND RNA

In the acute stage of disease, mumps virus can be isolated from blood (Kilham 1948), saliva, throat washings, a swab of Stensen's duct orifice, or urine (Person et al. 1971). Mumps isolation from saliva is most successful during the first 4–5 days of symptoms (Chiba et al. 1973). Infectious mumps virus is present in urine for up to 2 weeks after onset, being isolated from 71 percent of samples taken during the first 5 days and from 58 percent taken between days 6 to 10 (Utz et al. 1964). The cell cultures susceptible to mumps virus are given above, but primary monkey kidney cells or human embryo kidney are probably the optimum cultures for primary isolation of virus (Utz et al. 1957; Person et al. 1971; Lennette et al. 1975; Kelen and McLeod 1977; Hopps and Parkman 1979). Characteristic CPE is not always present, but virus replication can be detected by hemadsorption using guinea-pig erythrocytes (see above). The isolate may be identified as mumps by IFAT, HI, hemadsorption inhibition, virus neutralization, ELISA, or more recently, by RT-PCR (Wolontis and Bjorvatn 1974; Lennette et al. 1975; Hopps and Parkman 1979; van Tiel et al. 1988; Boriskin et al. 1993). Rapid detection of virus may be achieved by an initial shell vial cell culture step followed by IFA, Vero, and LLC-MK2 being found to be the most sensitive cell lines followed by MDCK, MRC5, and HEp-2 (Germann et al. 1998; Reina et al. 2001). Alternatively when Vero cells were first inoculated and incubated overnight to amplify the virus followed by RT-PCR a sensitivity of detection of 1–20 units of infectious virus was reported (Boriskin et al. 1993). Mumps antigens can be detected by IFAT in nasopharyngeal cells or by EIA directly in saliva, and mumps RNA can be detected in saliva, throat swabs, urine, or umbilical cord blood by RT-PCR (Cusi et al. 1996; Afzal et al. 1997b; Takahashi et al. 1998; Jin et al.

2004). The timing of the samples is important, however, 69 percent of oral fluids being positive up to 7 days after onset. This falls to 20 percent between 8 and 14 days and 1.6 percent if taken later (Jin et al. 2002).

MUMPS MENINGITIS

CNS abnormalities can be detected in the majority of patients with symptoms of mumps infection (Bang and Bang 1943). There is frequently a CSF pleocytosis with, usually, <500 lymphocytes/mm^3 (Finkelstein 1938; Azimi et al. 1969; Johnstone et al. 1972), but >10 000 lymphocytes/mm^3 have been reported (Lerner 1970). The cell count peaks during the first week of illness, and wanes gradually over several weeks or months (Kilham 1949; Wilfert 1969; Wolontis and Bjorvatn 1973; Fryden et al. 1978; Vandvik et al. 1978; Link et al. 1981). The CSF glucose concentration is usually normal, but levels down to 17–41 percent of the serum level in 6–29 percent of all cases have been reported (Azimi et al. 1969; Wilfert 1969; Lerner 1970; Johnstone et al. 1972). Protein content is elevated in 39–70 percent of all cases, up to 100 mg/dl and occasionally exceeding 700 mg/dl (Kilham 1949; Azimi et al. 1969; Wilfert 1969; Fryden et al. 1978). Albumin indices are also elevated and may remain so for months (Link et al. 1981), reflecting damage to the blood–brain barrier.

Anti-mumps IgG, IgM, and IgA antibody can be detected in the CSF during mumps meningitis (Morishima et al. 1980; Vartdal and Vandvik 1983), and the nonspecific and specific antibody index for all three classes of antibody is also raised (Forsberg 1986). The IgG and IgM antibody are oligoclonal, whereas the IgA is polyclonal (Vartdal and Vandvik 1983). Between 30 and 43 percent of all patients with mumps meningitis have intrathecal synthesis of IgG and demonstrable oligoclonal immunoglobulins during the first 2 weeks of CNS symptoms, rising to 75 percent between 4 and 7 weeks, which may persist for more than 2 years even with apparently complete clinical recovery (Fryden et al. 1978; Vandvik et al. 1978; Link et al. 1981). In patients with clinical sequelae following mumps encephalitis intrathecal production of anti-mumps antibody may continue for up to 14 years (Julkunen et al. 1985). Using RIA, however, it was subsequently found that up to 91 percent of patients with acute meningitis showed mumps-specific IgG in the CSF (Reunanen et al. 1982), and using ELISA 66 percent had IgM (Forsberg 1986). Mumps-specific IgM antibody detected in the CSF by MACELISA showed no cross-reaction with the parainfluenza viruses (Glikmann et al. 1986).

In cases of mumps meningitis virus particles can be seen in the CSF by electron microscopy (Kelen and McLeod 1977). Mumps virus antigen can be detected in cells from the CSF by IF (Lerner 1970; Boyd and Vince-Ribarić 1973; Lindeman et al. 1974), or by a rapid capture ELISA (Chomel et al. 1997). It was reported that mumps virus can be isolated from the CSF in at most half of those with meningitis (Utz et al. 1957; Azimi et al. 1969) within 8–9 days of the onset of neurological symptoms, 2–3 days being the optimum period for detection (Kilham 1949; Wolontis and Bjorvatn 1973). However, in a recent study, mumps was detected using nested RT-PCR in CSF from 96 percent of patients with clinical CNS disease and a diagnosis of mumps compared to only 39 percent detected by virus isolation (Poggio et al. 2000).

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Respiroviruses: parainfluenza virus

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INTRODUCTION

Respiratory tract infections caused by respiroviruses, and specifically the human parainfluenza viruses, cause many of the most significant childhood diseases in both developed and underdeveloped areas of the world. This chapter is divided into two parts: the first part discusses properties of respiroviruses including human parainfluenza viruses types 1 and 3, and the second part discusses clinical and pathological aspects of infection with these pathogens.

PROPERTIES OF THE VIRUSES

Classification and structure

The respiroviruses comprise a genus within the *Paramyxoviridae* family whose members include the human pathogens parainfluenza virus types 1 and 3, bovine parainfluenza virus, *Sendai virus* (a mouse pathogen), and a number of other less-studied members, including murine parainfluenza virus type 1 and simian respirovirus 10. These viruses have features that distinguish them from other members of the *Paramyxovirinae*. They all possess both hemagglutinating and neuraminidase activities on their receptor-binding protein, and all have the ability to encode a nonstructural C protein. Like all other members of the *Paramyxovirinae*, these viruses, although pleimorphic, are roughly spherical in shape, are approximately 150-400 nm in diameter and possess a lipid envelope composed of host cell lipids and viral glycoproteins that is derived from the plasma membrane of the host cell during budding. Figure 1 shows a schematic diagram of a representative respirovirus particle. All contain a negative-strand nonsegmented single-stranded RNA genome that is encapsidated by the viral nucleocapsid protein and forms a helical nucleocapsid (Choppin and Compans 1975). The viral RNA of each is approximately 15 500 nucleotides in length. Like all negative-strand RNA viruses, the respiroviruses encode and package an RNA-dependent RNA polymerase in the virion particles. The polymerase activity is only manifest on the RNA/NP helical nucleocapsid template and is attributed entirely to two viral proteins, L and P. There are approximately 50 P proteins and 300 L proteins per virion nucleocapsid (Lamb et al. 1976).

The negative-strand RNA genome of all respiroviruses contains six genes arranged in the order 3′-NP–P/V/C–M–F–HN–L-5′. The *NP* gene encodes the nucleocapsid protein that binds to the viral genome and antigenome during RNA synthesis, forming the characteristic helical nucleocapsids. The *P* gene of all respiroviruses encodes the phosphoprotein P (a subunit of the viral polymerase), another protein termed C, and for several members (Sendai and bovine parainfluenza type 3) also encodes a V protein via nontemplated nucleotide addition to the mRNA, a process referred to as RNA editing (see below). The *M* gene encodes a nonglycosylated membrane-associated protein, termed membrane or matrix protein. The M protein binds both to viral nucleocapsids and to viral glycoproteins that have been inserted into the host cell membrane, playing

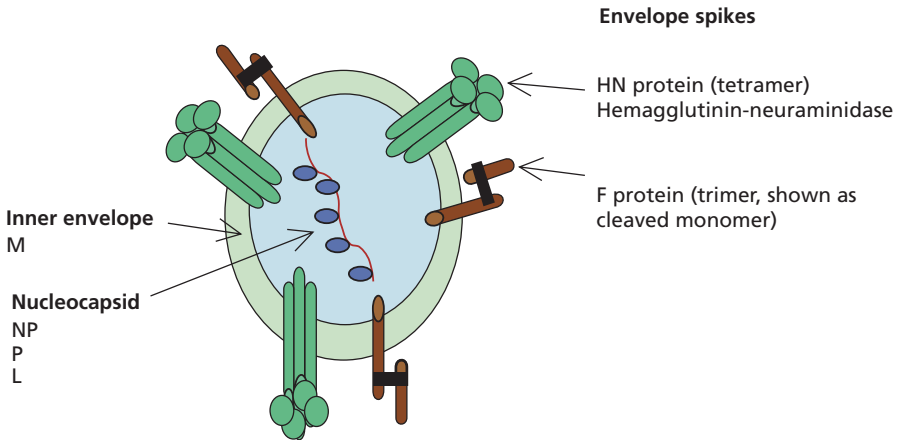

Figure 1 *Schematic diagram of a parainfluenza virus*

a role in virus assembly. The M protein also lends structure to the viral envelope, forming a protein layer between the lipid envelope and the helical nucleocapsid within. The F and HN genes encode the two integral membrane glycoproteins, the fusion (F) protein and the hemagglutinin-neuraminidase (HN). The F glycoprotein mediates fusion between the viral and host-cell membranes during infection, while the HN glycoprotein, possessing both receptor-binding and receptor-destroying activities (i.e. hemagglutinating and neuraminidase functions), is responsible for attachment of the virus particle to receptors on the cell, interaction with the F protein to mediate fusion between the viral and cell membranes, and release of progeny virus particles from infected cells. The *L* gene, comprising approximately 50 percent of the coding capacity of the virus, encodes the L protein, the catalytic subunit of the viral RNA polymerase.

Viral entry and membrane fusion: the roles of the surface glycoproteins

A characteristic feature of paramyxovirus infection in cell culture is the induction of cell fusion at neutral pH, resulting in the formation of syncytia. While several elements of the fusion process have been extensively studied, there remains much to learn about the precise role of each of the surface glycoproteins and the critical determinants of fusion. Several basic features of the glycoproteins are shown in Figure 2. Infection of cells by respiroviruses is initiated by attachment of the virus to the host cell through interaction of the HN glycoprotein with a neuraminic acid-containing molecule on the host cell surface. The fusion glycoprotein F is synthesized as a single polypeptide chain (F0) that forms a trimer before being cleaved by host-cell proteases to yield a membrane-distal (F2) and membrane-anchored subunit (F1). The new N-terminal region of the membrane-anchored subunit F1, termed the fusion peptide, contains hydrophobic residues that insert into target membranes during fusion at neutral pH (reviewed in Hernandez et al. (1996)). Penetration and uncoating of the virus occur by fusion of the viral envelope with the plasma membrane of the cell, resulting in the release of the viral nucleocapsid into the cytoplasm. Infection also results in fusion between cells, which involves the interaction of F and HN proteins expressed on the surface of an infected cell with the membrane of an adjacent uninfected cell. The role of the receptor binding surface glycoprotein, HN, in this process has been the focus of much investigation in recent years, and is discussed in detail below.

THE HEMAGGLUTININ-NEURAMINIDASE

The HN proteins are type II transmembrane glycoproteins, oriented such that their NH_2 termini extend into the cytoplasm while the C termini are extracellular. The neuraminidase activity of HN is important for removing sialic acid residues from the viral and cellular surfaces to prevent self-aggregation and to allow for the release of newly formed virus. HN is present on the cell surface as a homotetramer composed of disulfide-linked homodimers (Markwell and Fox 1980; Thompson et al. 1988; Ng et al. 1989; Russell et al. 1994). The molecule contains a cytoplasmic domain, a membrane-spanning region, a stalk region, and a globular head. The membrane distal globular head region contains the sialic acid-binding site, which mediates attachment to sialic acid-containing receptor molecules on the cell surface to initiate infection. The recent publication of a suite of crystal structures of Newcastle disease virus (NDV) HN (Zaitsev et al. 2004) and parainfluenza virus type 3 HN (Lawrence et al. 2004) (shown in Figure 3 complexed with sialic acid) demonstrate the locations of the active site residue (T193 for parainfluenza virus type 3 HN) on the globular head of the molecule. These structures, when taken together with experimental data about the HN, promise to reveal much about the functions of HN and its interaction with F (see below).

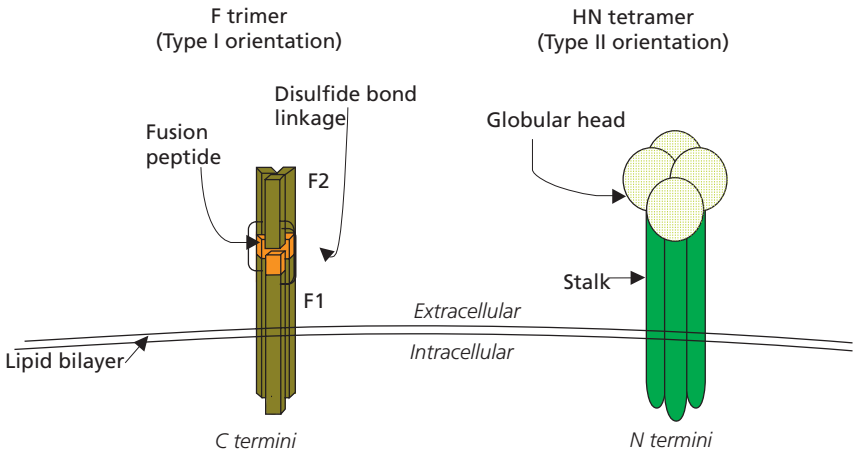

Figure 2 *F (fusion) and HN (receptor-binding) proteins*

RECEPTOR MOLECULES FOR PARAINFLUENZA VIRUSES

The receptors for parainfluenza viruses are known to be sialoglycoconjugates based upon their destruction by neuraminidases. Beyond this general classification, little is known. For human parainfluenza virus type 3 (HPIV-3) and other respiroviruses, the virus appears to be specific in its use of receptors (Moscona and Peluso 1996; Suzuki et al. 2001) in that it cannot make use of all potential sialic acid-containing surface molecules. For Sendai virus, specific gangliosides have been shown to be capable of functioning as receptors. Sendai virus binds to and fuses with liposomes containing gangliosides (Haywood 1974; Haywood and Boyer 1982), and three gangliosides belonging to the same family were shown to function as receptors for this virus in cultured cells (Markwell et al. 1981). It was shown that the minimal structure recognized by Sendai virus is NeuAcα2,3Gal. While glycolipids have been shown to function in initiating infection by Sendai virus, cell surface glycoproteins also contain the NeuAcα2,3Gal sequence as one possible type of carbohydrate modification. A number of studies have used sialidases to destroy receptors, followed by sialyltransferases to resialylate the cell surface in order to determine the important sialic acid component of the receptor (Paulson et al. 1979; Markwell and Paulson 1980), but these experiments do not determine whether the biologically important glycoconjugate is a lipid or a protein since these enzymes can use either glycolipids or glycoproteins as substrates. In addition, although various studies with Sendai virus have assessed binding to erythrocytes or liposomes (Haywood 1974; Suzuki et al. 1985), it remains unclear which molecules are actually used on target cells. For example, Sendai virus was shown to bind to heparin, and this binding inhibited Sendai fusion with erythrocyte ghosts or liposomes (Ohki et al. 1992; Zschornig et al. 1993). Recently, it was suggested that HPIV-3 can utilize heparan sulfate (HS) during the cell entry process (Bose and Banerjee 2002). HPIV-3 interacted with HS-agarose in vitro, and cellular entry was reduced if HPIV-3 was preincubated with soluble HS or if target cells were treated with heparinase to remove cell surface HS. However, inhibition was incomplete, suggesting at least that additional cell surface molecules

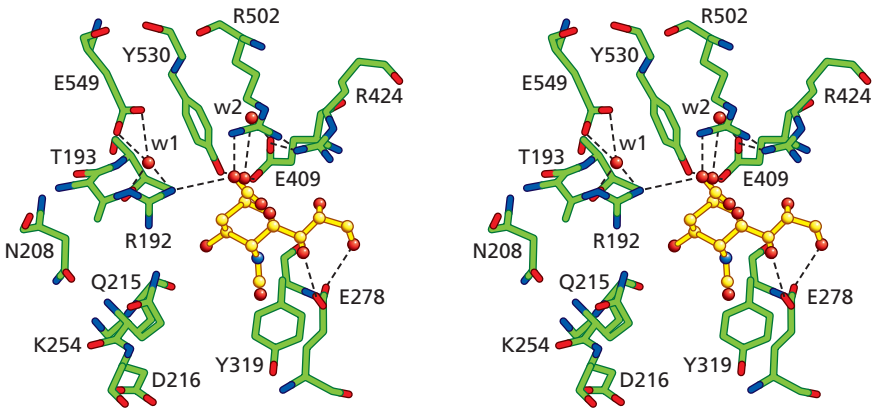

Figure 3 *HN active site (parainfluenza type 3) complexed with sialic acid (from Lawrence et al. 2004).*

are required. It is not known whether HS interacts with the HPIV-3 HN or F proteins. It is clear that HS is not used as the primary entry receptor, but it is possible that HS plays a secondary role and augments the efficiency of entry for HPIV-3, as well as Sendai viruses. While paramyxoviruses have many biological properties in common, several lines of investigation have now highlighted important differences between viruses of this family in regard to the viral entry and fusion processes, and it remains important to identify target receptors used by respiroviruses.

THE DUAL FUNCTION OF THE HN MOLECULES: A BALANCE OF ACTIVITIES

Unlike influenza viruses, in which separate surface glycoproteins are responsible for the neuraminidase and receptor-binding activities, the respirovirus HN is a dual function molecule. The existence of separate sites for the receptor binding and neuraminidase functions of the HN molecule has been debated extensively. Studies performed in various paramyxovirus family members with mutations in different HN residues have addressed this question by attempting to ascribe functional roles to these residues. The varied results failed to elucidate definitively whether the dual HN functions reside in the same or distinct sites. In studies of HPIV-3, both the neuraminidase and receptor binding activity of HN was inhibited by sialic acid analogues such as DANA (Levin Perlman et al. 1999) and 4-GU-DANA (Greengard et al. 2000). Passaging of HPIV-3 in the presence of 4-GU-DANA yielded an HN variant, ZM1, in which an alteration of residue 193 increased the receptor binding, as well as the neuraminidase activity, and conferred 4-GU-DANA resistance for both activities (Murrell et al. 2003). Analysis of this (4-GU-DANA-resistant) variant ZM1 supported the possibility of a single site capable of affecting both binding and enzymatic properties (Murrell et al. 2003) and the presence of a single, multifunctional site on HPIV-3 HN therefore appeared likely. A single site was also implicated in a study of bovine parainfluenza virus 3 (BPIV3) in which residue 193, the HPIV-3 193 correlate, affected both hemagglutinating and neuraminidase activities and thus suggested a single site (Shioda et al. 1988). Several studies employing monoclonal antibodies to select for Sendai virus escape variants postulated an HN with dual sites (Portner 1981; Portner et al. 1987; Thompson and Portner 1987; Gorman et al. 1990; Lyn et al. 1991). Initial crystallographic studies suggested that a single site on NDV HN was responsible for both activities (Crennell et al. 2000). However, a more recent study has identified an additional potential NDV HN receptor-binding site (Zaitsev et al. 2004). Recently, a third function of HN has become clear. Upon binding to receptor, parainfluenza HN plays a critical role in 'triggering' F to undergo its final conformational alteration that permits fusion (Porotto et al. 2003). This is discussed in detail below.

For influenza virus, the balance of NA and HA activities is important for the retention of in vivo viability. If virus binds receptor avidly, then entry will be efficient, while release will be hindered resulting in loss of viability. If receptor binding is reduced drastically, then entry of the virus is compromised resulting in a similar loss of viability. It is the balance of these two functions that determines the functional characteristics of influenza virus, including its ability to escape inhibition by a molecule like 4-GU-DANA. Balance of the dual functions of HN is also important for the functional characteristics of HPIV-3, even though the distinct biological activities of receptor binding and receptor destruction reside on a single molecule. For example, the fusogenic HPIV-3 variant C-0 incurred a single amino acid change that altered only its receptor binding affinity (Moscona and Peluso 1993). Another fusogenic HPIV-3 variant, C-28, possessed a single alteration that reduced the neuraminidase-specific activity while sparing its receptor binding affinity (Huberman et al. 1995). However, both variants exhibit similar highly fusogenic phenotypes distinct from wildtype HPIV-3. Thus, it is possible to affect HN functions separately, and, similar to influenza virus, the balance of binding and release influences the outcome of HPIV-3 infection. It is likely that the quantitative relationship between HN's receptor-binding and receptor-destroying functions determines the outcome of infection by parainfluenza viruses. It has been shown that virion release depends on this balance (Porotto et al. 2001). The ability of HN to trigger F to fuse (see below) is intimately related to HN's receptor bound state. Therefore, the balance between HN's two functions – binding and release from receptor – ultimately determines the third key HN function, the promotion of fusion, and thus viral entry (Porotto et al. 2001, 2003). Figure 4 presents a model of the several points of HN action in the cell during infection.

VIRAL INTERFERENCE

Viral interference is characterized by resistance of infected cells to infection by a challenge virus. This type of interference has been documented for several paramyxoviruses, including Sendai virus (Kimura et al. 1976) and NDV (Bratt and Rubin 1967a, b; Bratt and Rubin 1968) and was presumed to be due to the destruction of viral receptors by the viral neuraminidase. For HPIV-3, it was shown that interference is mediated by HN, and that the HN-mediated interference effect can be attributed to the presence of an active neuraminidase enzyme activity (Horga et al. 2000), indicating that the mechanism for attachment interference by a paramyxovirus is attributable to the viral neuraminidase.

THE FUSION PROTEIN

The fusion protein F, shown schematically in Figure 5, is important in the fusion process of all

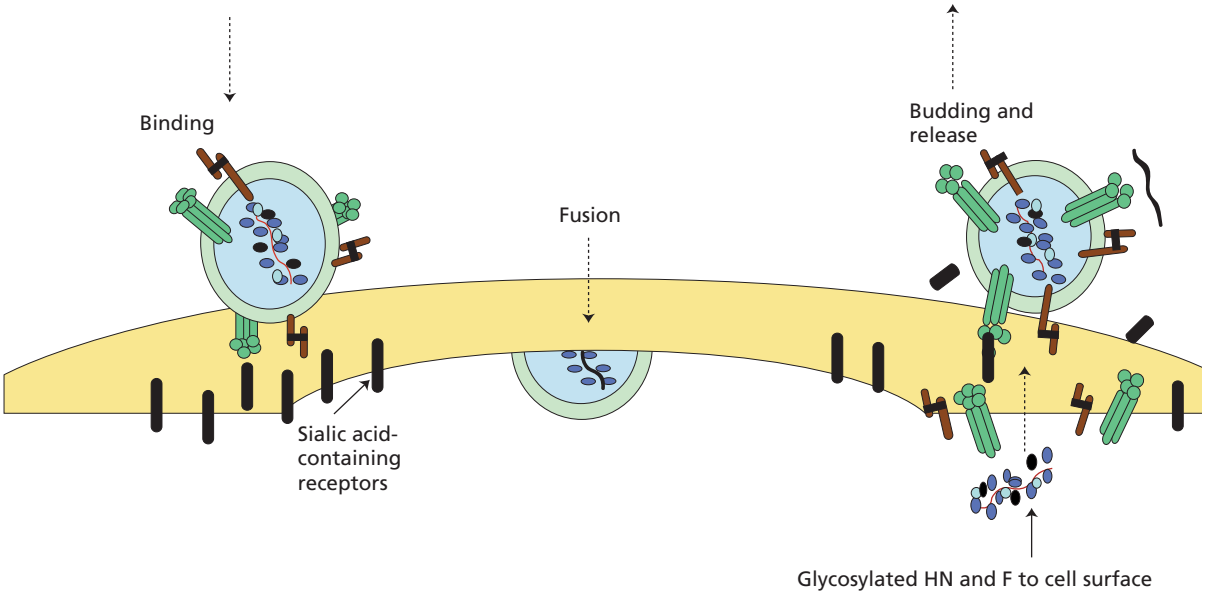

Figure 4 *Points of HN action*

paramyxoviruses (Lamb 1993; Plemper et al. 2003). The F proteins are type I integral membrane glycoproteins, synthesized as the inactive precursor F0, which trimerizes in the endoplasmic reticulum (Dutch et al. 2000). F0 is cleaved by a host cell protease during its transit to the cell surface to produce the active protein, which consists of two disulfide-linked subunits, F1 and F2 (Homma and Ouchi 1973; Scheid and Choppin 1974). After this cleavage, a new hydrophobic N-terminus is exposed. This is the fusion peptide, the region directly involved in mediating fusion of the viral envelope to the plasma membrane of the host cell. The F protein cleavage sites contain multiple basic amino acids, and are cleaved in the trans-Golgi network by subtilisin-like endoproteases, furin and Kex2, with the sequence specificity of R–X–K/R–R. For several paramyxoviruses (e.g. Sendai, NDV) the efficiency of cleavage has been shown to directly correlate with host range and tissue tropism of the virus, with greater 'cleavability' associated with virulence (Nagai et al. 1976, 1979; Nagai and Klenk 1977; Garten et al. 1980, 1992; Pritzer et al. 1990; Tashiro et al. 1990, 1992; Klenk and Garten 1994; Ortmann et al. 1994). It is also is likely that, by playing a role in determining the

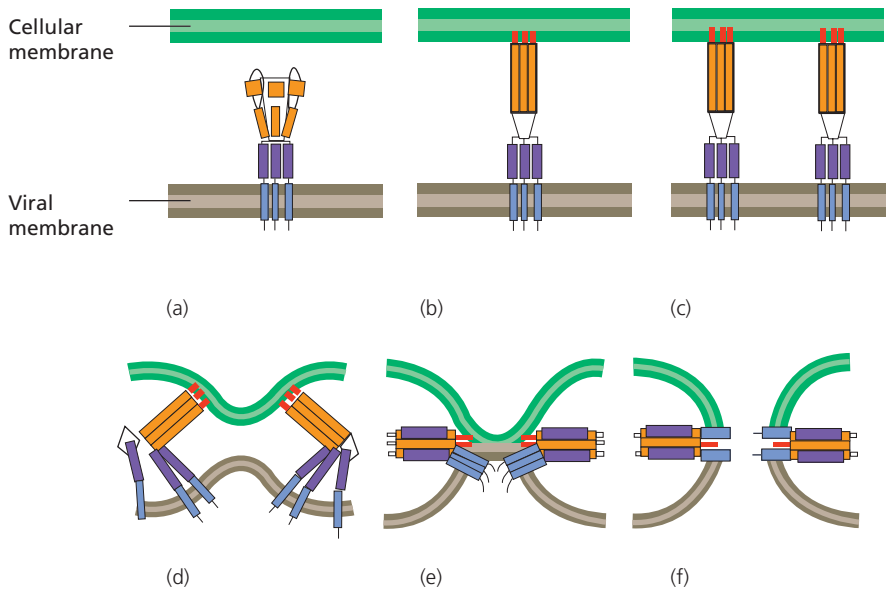

Figure 5 *Schematic general model of fusion protein-mediated membrane fusion (from Jardetzky and Lamb 2004)*

budding efficiency from different cell types, the F protein affects cell and host tropism for the parainfluenza viruses (Okada et al. 1998). F proteins contain a hydrophobic domain (stop transfer signal) at the C terminus that anchors the protein in the membrane. It has been shown that the fusion activity of F is regulated in part by the cytoplasmic domain of the protein (Dutch and Lamb 2001; Tong et al. 2002; Seth et al. 2003).

FUSION MECHANISMS AND THE ACTIVATION OF F PROTEIN

The available data on viral fusion proteins of paramyxoviruses indicate that before initiating the fusion process, fusion proteins remain in a metastable state, which requires a trigger of some sort to induce the conformational change that leads to exposure of the fusion peptide (for a review, see Jardetzky and Lamb (2004)). A second 'activation' step is required for fusion readiness. For the parainfluenza viruses, this step requires interaction of the HN protein with its receptor. The triggering of the conformational change from a metastable fusion protein to a fusion-active state was initially elucidated for influenza HA (Carr and Kim 1993; Chen et al. 1998). The mechanism of this fusion activation in paramyxovirus F molecules is thought to have analogies to the influenza HA. The possibility of a common process is supported by the fact that the post-fusion conformation of HA, as well as several paramyxovirus F proteins, consist of a six-helix coiled-coil formed by three heterodimers of two heptad repeat (HR) regions (HRA and HRB). In this 'final' structure, the fusion peptide and the transmembrane domain are brought into proximity, allowing for membrane fusion (Skehel and Wiley 1998). For paramyxoviral F proteins, as well, the final F structure in which the fusion peptide is exposed requires formation of this stable six-helix structure leading to membrane merger (Baker et al. 1999). A schematic diagram of the process, from a recent review by Jardetsky and Lamb, is shown in Figure 5. Step (a) shows the metastable fusion protein; in (b) and (c) the fusion peptides are released and inserted into the target membrane. The proteins refold into a trimeric hairpin shape (d) and in (e) a helical coiled-coil rod forms next to the fusion peptide and refolding relocates the fusion peptides and transmembrane regions to the same end of this coiled coil, with the free energy that results from this step bringing the membranes closer together. With the completion of refolding, the most stable form of the protein is formed (f) and fusion is complete (Jardetzky and Lamb 2004).

The paramyxovirus F1 ectodomain contains two conserved heptad repeat (HR) regions, designated HRA and HRB, located near the fusion peptide and the transmembrane domains. Peptides derived from the HRA and HRB regions have been used to inhibit fusion intermediates of F (Lamb and Kolakofsky 2001) and thereby to elucidate the mechanism whereby F mediates fusion. The HRA and HRB peptides are thought to inhibit fusion by preventing the F protein from forming the helical bundles required for fusion.

While for influenza virus the 'trigger' leading to conformational change of the fusion protein is the low pH of the endosome, for paramyxoviruses, which fuse at neutral pH at the cell surface, this trigger is likely to be HN-receptor binding. While the F protein is the molecule directly responsible for fusion of the lipid bilayers, the receptor-binding protein HN has also been shown to be essential for cell fusion mediated by HPIV. Interaction of HN with its receptor is required in order for F to promote membrane fusion during viral infection (Moscona and Peluso 1991; Horvath et al. 1992; Lamb 1993; Bagai and Lamb 1995). It has been proposed that upon binding sialic acid receptor, HN undergoes a receptor-induced conformational change, which in turn triggers a conformational change in F that allows fusion to occur (Lamb 1993; Porotto et al. 2003). Lamb recently showed that coexpression of HN with F of SV5 results in F protein susceptibility to HRA peptide inhibition, supporting the notion that HN binding to receptor induces a conformational change in F (Russell et al. 2001).

The triggering mechanism of paramyxovirus F at neutral pH is an area of active investigation. Lamb has shown that the free energy generated by the formation of the coiled-coil structure in F is the driving force for membrane fusion (Russell et al. 2001). There is evidence that the HN and F proteins interact (Tanabayashi and Compans 1996; Yao et al. 1997; Tong and Compans 1999). Recent work has supported the notion that HN interacts specifically with F protein to enable F to mediate fusion, and has generated models for this process. While, as above, co-expression of SV5 HN with F resulted in F protein susceptibility to HRA-region peptide inhibition, suggesting that HN binding to receptor induces a conformational change in F, the F protein fusion intermediate that was inhibited by the HRB peptide bound RBC independently of HN (or after HN had already exerted its action on F), and is likely to be the prehairpin stage of F immediately preceding membrane merger (Russell et al. 2001). The HRB peptide (corresponding to the region near the TM domain) binds F after the activation has already occurred, and F has been triggered to insert in the target membrane (and fusion at that point is independent of HN). The HN–receptor interaction is thus likely to be required for F protein function quite early in the triggering process. Many of the steps in the fusion and entry process remain to be understood. In particular, why is there a requirement for HN receptor binding, in order to initiate fusion promotion? What is the relationship between the 'docking' function of HN and its role in fusion promotion? A current model for paramyxovirus

glycoprotein function proposes that HN attachment to receptor leads to interaction, which then triggers F (Russell et al. 2001). Alternatively an HN–F complex may form during trafficking to the surface, and HN attachment may lead to a conformational change in HN with a resulting release of F's fusion peptide (Russell et al. 2001). Models of possible mechanisms whereby binding of HN to receptor may contribute to activation of F are illustrated in Figure 6. The role of cell membrane constituents, including lipids and lipid rafts, in the organization of the glycoproteins for the fusion and entry process, and the potential role of cytoskeletal molecules in this process (Pastey et al. 1999; Pastey et al. 2000), remain to be clarified.

Primary transcription and replication

ROLES OF NONCODING SEQUENCES

As mentioned in Classification and structure, all respiroviruses contain six genes arranged in the order 3′-NP–P/V/C–M–F–HN–L-5′. In addition to the six mRNA-encoding portions of the genomic RNA, there are extragenic nucleotide sequences that control polymerase function. At the ends of the genome are short transcribed noncoding sequences that serve as polymerase-binding sites, as well as encapsidation signals. These sequences are called leader and antileader (or trailer) for the 3′ and 5′ ends, respectively. The leader RNA contains the signal to initiate binding of the NP protein to a nascent RNA product, and the promoter, or viral RNA polymerase binding site, on the negative-strand RNA template. The antileader (trailer) serves a similar dual function during the second stage of genome RNA replication, when full-length encapsidated plus-stranded RNA-containing nucleocapsids (antigenomes) are copied into progeny negative-strand RNA-containing nucleocapsids that are then packaged into progeny virions.

At the start of each gene, there are conserved sequences of ten nucleotides in length (3′-UCCRXXUUXC). Between each gene there is a trinucleotide sequence (3′-GYY) and at the end of each gene 11 conserved bases (3′-UXXUXXUUUUU), where R is a purine, Y is a pyrimidine, and X indicates no consensus (Kolakofsky et al. 1998). These sequences are believed to signal initiation of transcription and polyadenylation and termination of the mRNA. Except for the small number of intergenic nucleotides, all of the RNA of the viral genome is transcribed into either mRNA or leader RNAs, and most of the transcribed RNA is translated into viral proteins.

THE REPLICATION CYCLE

Replication of the virus occurs exclusively in the cytoplasm. Infecting virions fuse with the plasma membrane of the cell, releasing the viral nucleocapsid into the cytoplasm. The nucleocapsid contains the genome RNA in tight association with the viral NP protein, in the form an RNAse-resistant helical structure that is stable in

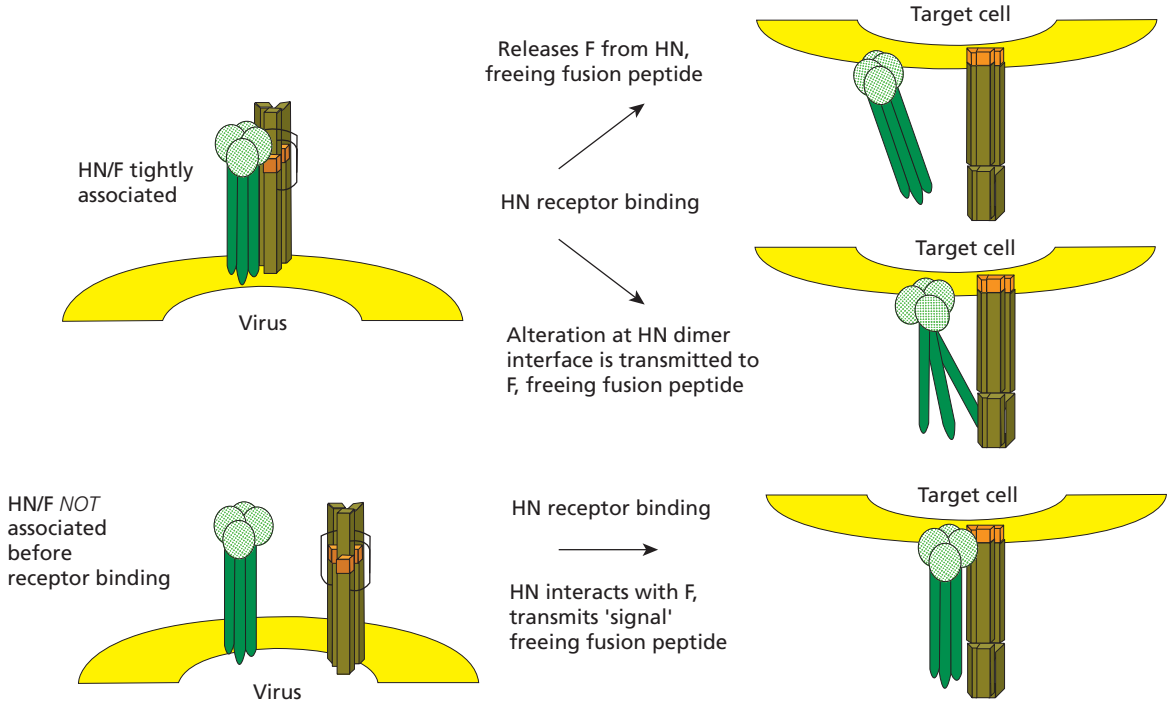

Figure 6 *Models for the role of HN–receptor interaction in F activation*

cesium chloride and readily visualized in the electron microscope. This RNA/protein complex is the template for both transcription and genome RNA replication, and there is no evidence that the structure uncoats further. Figure 7 represents this process, showing schematically that: (1) transcription and genome RNA replication both occur using the viral RNP nucleocapsid as a template; (2) during transcription multiple subgenomic mRNA molecules are made; (3) each subgenomic mRNA molecule is capped, methylated, and polyadenylated; and (4) during genome replication a full-length plus-stranded copy is made and encapsidated. This full-length positive-sense strand is the template for new progeny minus-sense nucleocapsids.

The virally encoded RNA polymerase is bound to the nucleocapsid as a complex of P and L proteins. Neither of these proteins bind to isolated viral RNA, indicating that protein–protein interactions are necessary in order for binding to occur. Thus, the infecting virion delivers an RNA–protein complex into the cell that is capable of transcribing and replicating the viral RNA genome. Purified virions can transcribe the viral RNA genome into multiple subgenomic capped and polyadenylated mRNAs if disrupted with a mild detergent and incubated in a simple salt mixture containing RNA precursors. However, genome RNA replication does not occur unless these in vitro systems are supplemented with cell extracts that are capable of supporting ongoing protein synthesis. These observations illustrate the fact that there are fundamental differences between the two processes of RNA synthesis that must occur during the growth of respiroviruses (transcription and genome RNA replication). One process, genome RNA replication, requires concurrent protein synthesis and perhaps multiple host-cell factors, while the other, transcription, occurs independently from host factors other than energy sources and nucleotide supply. Both processes are described in more detail below.

The first step in the viral growth process, termed primary transcription to denote transcription directly from the infecting nucleocapsid template, initiates at the exact 3′ end of the genome in a primer-independent manner, first transcribing the short (approximately 50 nt) uncapped leader RNA. The synthesis of the leader RNA is followed sequentially by each of the other viral mRNAs in the order NP-P/V/C–M–F–HN–L. There is a polarity to the transcription process, so that genes located at the 3′ end of the viral genome are transcribed more frequently than those at the 5′ end. Each of the mRNAs is transcribed and present in infected cells as a function of its relative location on the negative sense viral genome. This polarity of transcription is a property of the viral coded RNA-dependent RNA polymerase complex.

The NP transcript is most abundant in infected cells and the L protein transcript is least abundant. The polymerase appears to pause at each intergenic junction and either reinitiates transcription at the next gene downstream or, alternatively, detaches from the nucleocapsid template and reinitiates upstream at the 3′ leader region that contains the polymerase binding site or promoter. This 'decision' made by the polymerase at each gene junction, together with the fact that there is a single polymerase entry site at the 3′ end of the genome, explains the sequential and polar nature of transcription. Transcription of each gene depends upon successful transcription and termination of the preceding upstream gene. Each mRNA is capped and methylated at its 5′ end and polyadenylated at its 3′ end, all carried out by the viral polymerase complex. Polyadenylation of the transcripts occurs through a polymerase chattering mechanism at a conserved stretch of U residues (U_5) at the end of each gene. The transcription of the *P* gene is more complex, and is discussed in more detail below.

THE 'RULE OF SIX'

The genomes of respiroviruses contain a total number of nucleotides that are evenly divisible by six. Taking advantage of the ability to generate recombinant infectious viruses from DNA clones ('reverse genetics'), it was observed that recombinant Sendai virus genomes that have a nucleotide number not divisible by six were

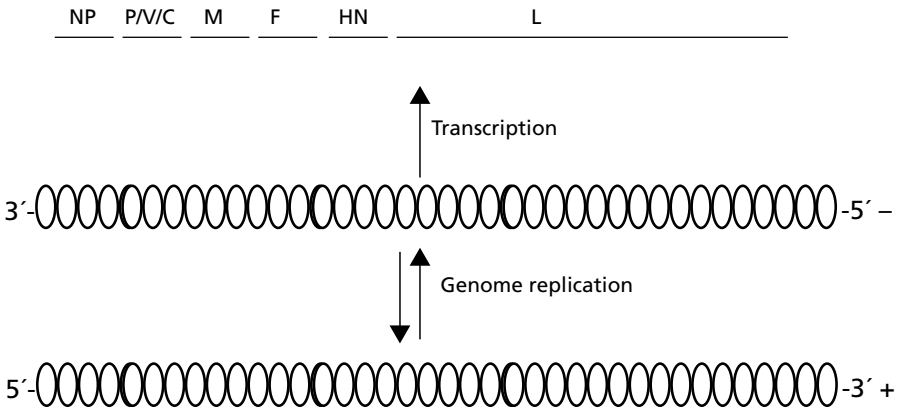

Figure 7 *Transcription and genome RNA replication of respiroviruses*

still assembled efficiently into nucleocapsids, but these genomes were very inefficiently transcribed or replicated (Pelet et al. 1996). This requirement for an optimal nucleotide number, or length evenly divisible by six, is referred to as the 'rule of six.' According to this model, the phasing of the nucleocapsid protein subunit on the viral genome is such that each protein subunit interacts with six nucleotides, and this phasing is important to maintain proper alignment of the 3′ *cis*-nucleotide sequences that are recognized by the viral RNA polymerase complex during transcription and replication. Experimental evidence for this model has been obtained (Pelet et al. 1996; Kolakofsky et al. 1998; Vulliemoz and Roux 2001), and suggests that there is an optimal spacing for promoters on the viral genome relative to the NP proteins.

POLYCISTRONIC mRNAS AND ALTERNATE READING FRAMES

For five of the six genes, transcription generally leads to generation of a single mRNA species that encodes a single protein molecule. However, the *P* gene of respiroviruses encodes a number of proteins and, in several members of the family, more than one mRNA is synthesized. Each of the *P* genes of all of the members of this virus genus is transcribed directly into an mRNA that encodes the P protein. This mRNA also encodes a number of smaller protein products in an alternate reading frame from the P protein, and hence the P mRNA is bi-cistronic. The largest open reading frame encodes the P protein, of 568–603 amino acids in length. The second ORF, near the 5′ end of the mRNA, is translated into a number of proteins called C, C′, Y1, and Y2. These 'C' proteins, as they are commonly called, are a nested set of carboxy-coterminal proteins, with each protein encoded in the same reading frame, but differing by its start site on the mRNA. Not each member of the family encodes each protein. Sendai virus encodes all four, HPIV-1 encodes C′ and C, while HPIV-3 and BPIV-3 encode just the C protein (Giorgi et al. 1983; Curran and Kolakofsky 1988). In Sendai virus the C, Y1, and Y2 proteins are synthesized by ribosomal choice at separate AUG codons, while C′ is initiated using an ACG codon. All of these proteins are small (175–215 amino acids) and basic, with isoelectric points of over 10. The functions of the C proteins are incompletely understood. The C protein of HPIV-3 and the C proteins of Sendai virus are apparently required for virus growth in cultured cells since recombinant viruses that do not express these proteins replicate poorly (Kurotani et al. 1998; Latorre et al. 1998). In addition, as discussed below, these proteins may function in pathogenesis in the infected host.

NONTEMPLATED NUCLEOTIDE ADDITION

The *P* gene of most respiroviruses, in addition to encoding the P and 'C' proteins, also encodes at least one other protein using a second mRNA transcribed from the gene (HPIV-1 is the exception). The process by which this second mRNA is produced has been referred to as RNA editing, or nontemplated nucleotide addition. A sequence of nucleotides in the *P* gene is recognized by the viral polymerase as a signal for 'stuttering,' and the polymerase either ignores this stuttering signal and transcribes the P mRNA, or stutters and incorporates one or two nontemplated G residues at the precise editing site on the mRNA (Thomas et al. 1988; Vidal et al. 1990a, b; Hausmann et al. 1999a, b). The consequence of this nontemplated nucleotide addition is that the resultant mRNA allows access to a conserved short open reading frame called V. The V protein contains the N-terminus of the P protein fused to the V ORF. This V ORF is highly conserved among the viruses, is cysteine-rich and has been shown to bind zinc (Paterson et al. 1995). It is dispensable for viral growth in cell culture, and appears to function as a pathogenesis factor (see Interaction with the host cell). In several respiroviruses, addition of two nontemplated G residues allows access to a second ORF termed D (Galinski et al. 1992). Thus the *P* gene of most respiroviruses encodes a number of protein products through alternate codon usage and RNA editing.

THE SWITCH FROM TRANSCRIPTION TO REPLICATION

Replication of the viral genomic RNA is a process both linked to and distinct from transcription of the genomic RNA. Unlike transcription, replication of the viral RNA genome depends on ongoing protein synthesis, and therefore is linked to prior transcription of the viral genome. In addition, during the process of genome RNA replication, the nascent RNA molecule is assembled into the characteristic helical nucleocapsid structure. During replication all signals to the viral polymerase are ignored, including the transcriptional termination signals at the ends of each gene and the capping, methylation, polyadenylation, and RNA editing signals that are present on the template RNA. The viral genome and antigenome contain 3′U-OH and 5′ pppA, respectively, rather than a cap and a poly(A) tail as in the mRNAs encoded by the genome.

Both transcription and genome RNA replication require the nucleocapsid template and the L and P proteins. The requirement for ongoing protein synthesis in order for RNA replication to occur is in part a consequence of the fact that the newly synthesized replication molecules are coassembled into nucleocapsids, and therefore NP protein is required in stoichiometric levels corresponding to the viral genome and antigenome RNAs (approximately 2 500 NP molecules per genome RNA). The NP protein that serves as the substrate for this assembly (NPo) is complexed with the P protein (Horikami et al. 1992). Binding of the P protein to free NPo is thought to prevent the self-assembly of free NP

protein into higher order structures, and thus P protein acts as a molecular chaperone, delivering the NP to the assembling nucleocapsid while not, itself, becoming a part of the structure. The second stage of genome RNA replication occurs when the newly replicated and encapsidated full-length plus-stranded RNA (antigenome)-containing nucleocapsids are copied into negative-strand RNA-containing progeny nucleocapsids identical to the nucleocapsid that is packaged into progeny viral particles. These progeny viral nucleocapsids are templates for transcription and can greatly amplify the amount of viral mRNA and protein produced in an infected cell.

THE *NP* GENE

The nucleocapsid protein NP of respiroviruses is encoded by the 3′-proximal gene and is the most abundant viral protein in the infected cell. The protein in Sendai virus is 524 amino acids in length and is phosphorylated at its C-terminus (Hsu and Kingsbury 1982; Ryan et al. 1993). The protein's main function is to coassemble with genomic and antigenomic RNAs to form the template for transcription and replication, the genome- and antigenome-containing nucleocapsid. When NP is expressed in the absence of other viral proteins it self-assembles into structures resembling viral nucleocapsids, suggesting that it can form higher order assemblies through protein–protein interactions. In infected cells, NPo is complexed with the P protein. Interaction with P protein prevents aggregation of free NPo, and it is the NPo–P protein complex that serves as the substrate for assembling NP/RNA nucleocapsid complexes. In addition to NP–NP and NPo–P interactions in nucleocapsid assembly, the NP protein on the nucleocapsid binds to the viral RNA and to the P subunit of the RNA polymerase of the virus. It has been demonstrated that the N-terminal 400 amino acids of the NP protein of Sendai virus are required for assembly into nucleocapsid templates (Buchholz et al. 1993; Curran et al. 1993). Further, these same 400 N-terminal amino acids of NP also retained the NPo–P interacting activity (Homann et al. 1991; Buchholz et al. 1994; Myers and Moyer 1997; Myers et al. 1997; Myers et al. 1999). The C-terminal portion of the protein appears to be necessary for binding of the P protein to the nucleocapsid (Ryan et al. 1993; Buchholz et al. 1994).

THE *L* GENE

The L proteins of all respiroviruses are greater than 2 000 amino acids in length and are encoded by approximately half of the viral genome. L is the least abundant protein in virus particles and in infected cells. It is likely that all of the enzymatic activities required for mRNA synthesis and genome RNA replication (polymerization, capping, methylation, polyadenylation) are encoded within this large multifunctional protein. However, a detailed understanding of the structure/function of the L protein has yet to be obtained.

Sequence alignments have identified six conserved domains in the protein, (I–VI) (Poch et al. 1990; Sidhu et al. 1993). Several of the domains contain conserved sequence motifs that suggest possible functions. Domain II has a conserved charged RNA binding motif found in several other viruses (Smallwood et al. 1999). Domain III has a motif found in several other virus L proteins that has phosphodiester bond-forming activity (Smallwood et al. 2002). Domain VI contains a potential nucleotide binding motif. Domain III contains a motif found in the active site of several positive-stranded RNA virus polymerases and is believed to contain the polymerase active site (Jin and Elliott 1992; Sleat and Banerjee 1993; Schnell and Conzelmann 1995). Domains IV and V have no homology with any known polymerase-associated motifs.

The L protein, in order to be functional, must complex with the P protein and via this interaction bind to the nucleocapsid template. The P protein binding site has been localized to the N-terminal 350 amino acids of the L protein of Sendai virus (Holmes and Moyer 2002). A similar picture is emerging for the L protein of HPIV-3, where a short stretch of amino acids at the very N-terminus of the L protein interacts with the P protein of the virus (Malur et al. 2002). The L protein itself is an oligomer, and the oligomerization domain has been mapped to the N-terminal 174 amino acids (Cevik et al. 2003). However, L protein expressed alone is rapidly degraded, and its stability is increased by coexpression of the P protein (Horikami et al. 1997). It has been proposed that the L protein first oligomerizes, and then these oligomeric complexes interact with a complex of the P protein forming a stable and active RNA transcriptase/replicase complex (Cevik et al. 2003). The stoichiometry of the proteins in this polymerase complex have been suggested to be either L2–P4 or L2–P8. What is certain (see below) is that the P protein is a tetramer, while the state of the L protein is less certain at this time.

THE *P* GENE

The *P* gene of respiroviruses encodes a number of protein products, as described above. The P protein is the only one of these proteins that is essential for RNA synthesis. P is a phosphoprotein that acts as a molecular chaperone for the NPo protein in nucleocapsid assembly. However, the role that phosphorylation plays in modulating the function of the protein, if any, is uncertain (Hu et al. 1992, 1999). P is also an essential component of the viral RNA polymerase, forming a complex with the L protein both in solution and on the template, as well as serving to mediate binding of the polymerase complex to the nucleocapsid via P–NP interactions. Several facts have emerged concerning the

structure/function of the P protein. Firstly, it oligomerizes into a tetramer. A partially resolved molecular structure suggests the P protein complexes with itself via coiled-coil motifs (Tarboubiech et al. 2000a, b). Second, it complexes with the L protein to form the polymerase complex (Curran et al. 1994; Smallwood et al. 1994; De et al. 2000; Bousse et al. 2001). Third, it mediates binding of the polymerase to the nucleocapsid template; L does not bind to the nucleocapsid template alone (Horikami and Moyer 1995). Last, P acts as a molecular chaperone for NP during nucleocapsid assembly and prevents NP from self-assembling into higher order structures (Curran et al. 1995b; Horikami et al. 1996).

Several of the domains on the P protein that participate in these varied activities have been mapped. The C terminus of the protein is capable of replacing the intact protein in supporting transcriptional activities (Curran et al. 1994). This fragment contains the domains/motifs required for oligomerization of the protein, for its interaction with and stabilization of the L protein complex, and for its ability to bind to the nucleocapsid template (Ryan and Portner 1990; Curran et al. 1994, 1995a; Smallwood et al. 1994; Bowman et al. 1999; De et al. 2000; Bousse et al. 2001). The only domain or motif so far mapped to the N-terminus is that required for its chaperone function(Curran et al. 1995b).

Assembly and budding

Respiroviruses are formed by budding of newly replicated and assembled nucleocapsids containing the viral RNA genome along with the P and L proteins through modified areas of the plasma membrane that contain the F and HN transmembrane glycoproteins and the M protein. In polarized epithelial cells, the viruses bud from the apical surface of the cell. A detailed understanding of the final assembly and budding process is lacking. However, it is likely that the nonglycosylated M protein binds to the nucleocapsid, condensing it, and preventing it from being transcribed or replicated. The M protein also likely interacts with the cytoplasmic tails of the HN and F proteins, and thereby mediates the alignment of the nucleocapsid and the areas of the plasma membrane containing viral glycoproteins in preparation for budding (Mottet et al. 1996, 1999; Coronel et al. 1999, 2001; Ali and Nayak 2000; Takimoto et al. 2001).

The M proteins of respiroviruses are small basic proteins and are a major component of virions. Their role in budding has been clearly shown by demonstrating the formation of virus-like particles in cells expressing just the M protein of HPIV-1 (Coronel et al. 1999, 2001). Interestingly, when NP was coexpressed, the budded particles contained structures closely resembling helical viral nucleocapsids. In similar systems, expression of the F protein alone led to the formation of virus-like particles (Takimoto et al. 2001). These data indicate that there are inherent properties of more than one respirovirus protein that can lead to the formation of virus particles during the budding process, and there is specificity in protein–protein recognition during this budding process.

Interaction with the host cell

EVASION OF HOST DEFENSE MECHANISMS

Host cells have developed a variety of countermeasures in the face of viral infection. In some of these, an intracellular environment is created that is less hospitable for virus replication (the interferon (IFN)-induced antiviral state). Some cell types undergo apoptosis, which limits viral replication. Many viruses have been found to posess strategies for evading these host immune responses, particularly the innate immune response that plays a key role in controlling virus infection. Many viruses encode gene products that are capable of inhibiting the host interferon response (Katze et al. 2002). For the paramyxoviruses, evasion of this response has been found to be mediated by the C and V proteins.

The Sendai virus *C* gene was one of the earliest examples of an overlapping gene (Giorgi et al. 1983). The *C* gene open reading frame is found overlapping the amino-terminal end of the open reading frame for the P and V proteins (which are also expressed from the *P* gene mRNA via the mechanism of mRNA editing (Giorgi et al. 1983; Bellini et al. 1985), discussed in detail in Primary transcription and replication). C proteins carry a net positive charge and are expressed from the second ORF (+1 with respect to P). For Sendai virus, evasion of the interferon system is accomplished in several ways by the C protein (Garcin et al. 2003). Sendai virus C protein binds STAT1, preventing its activation in response to IFN, and the carboxyl part of the C protein mediates this activity. A phenylalanine at position 170 of C is also critical for blocking STAT1 activation (Gotoh et al. 2001; Takeuchi et al. 2001; Garcin et al. 2002). In addition, Sendai C protein targets STAT1 for degradation, and the amino-terminal residues of the C proteins mediate this activity (Garcin et al. 2003).

The V proteins require the pseudotemplated addition of a single G residue to switch from the N terminal P to C terminal V ORF. V proteins of the parainfluenza viruses are fusion proteins containing a negatively charged and phosphorylated N terminus and a short, cysteine-rich zinc-binding domain at their C terminus (Paterson et al. 1995; Lamb and Kolakofsky 2001). The V proteins are involved in a wide range of mechanisms for evading the immune response. These have been found thus far to include prevention of apoptosis (He et al. 2002; Wansley and Parks 2002), cell-cycle alterations (Lin and Lamb 2000), inhibition of double-

stranded RNA signaling (He et al. 2002; Poole et al. 2002), and prevention of IFN biosynthesis (He et al. 2002; Poole et al. 2002; Wansley and Parks 2002). V protein expression has been shown for several paramyxoviruses to inhibit IFN-responsive STAT proteins. The strategies whereby different V proteins inhibit STAT proteins have been found to be highly diverse. Most information about the function of these V proteins have been derived from study of closely related paramyxoviruses. For example, SV5 V proteins target STAT1 for degradation (Didcock et al. 1999; He et al. 2002), while HPIV type 2 V protein targets STAT2 (Parisien et al. 2001; Andrejeva et al. 2002). Mumps virus V protein can target both STAT1 (Nishio et al. 2002) and STAT3 (Ulane et al. 2003) and can also interact with cellular RACK1, potentially modifying IFN receptor activity (Kubota et al. 2002). Nipah virus V protein sequesters STAT1 and STAT2 in high-molecular-weight cytoplasmic complexes, thus preventing STAT protein nuclear accumulation (Rodriguez et al. 2002).

USE OF CELLULAR MACHINERY FOR VIRAL ENTRY AND/OR ASSEMBLY

After entering the target cell via fusion with the cell membrane, and release of the viral molecules into the cytoplasm, respiroviruses carry out their replicative cycle within the cytoplasm (as discussed in Primary transcription and replication) and then assemble and bud at the cell surface (discussed in Assembly and budding). The potential role of cytoskeletal molecules in the process of viral entry (Pastey et al. 1999, 2000), as well as the fascinating question of how the various viral constituents are directed during the parasitic phase of the life cycle to the correct cellular locations, remain to be clarified. It is possible that entry, fusion, and/or budding involve the participation of the cellular actin cytoskeleton, as well as microtubule function (Tashiro et al. 1993; Gupta et al. 1998; De and Banerjee 1999; Bose et al. 2001). In addition, host cellular proteins that are involved in cell mobility could be involved. For the related virus respiratory syncytial virus (RSV), it was shown that F binds RhoA, a small GTPase of the Ras superfamily, in yeast two-hybrid interaction studies (Pastey et al. 1999). RhoA controls cellular functions including actin reorganization, cell morphology, and motility. The interaction with RSV F was shown in vivo by mammalian two-hybrid assay and in RSV-infected HEp-2 cells by coimmunoprecipitation. Cells that overexpressed RhoA were used to show that RhoA correlated with fusogenicity by RSV, suggesting RhoA could play a role in RSV-induced syncytium formation. Subsequently, RhoA peptides were used to show that cell–cell fusion by HPIV-3 could be inhibited by a RhoA peptide corresponding to the region that binds RSV F (Pastey et al. 2000), suggesting a common component of the entry/fusion mechanisms involving interaction between the F proteins and RhoA.

CLINICAL AND PATHOLOGICAL ASPECTS OF INFECTION WITH HUMAN PARAINFLUENZA VIRUSES

Croup (laryngotracheobronchitis)

ETIOLOGY

The parainfluenza viruses types 1, 2, and 3, taken together, are the most important cause of croup. HPIV-3 alone is responsible for approximately 11 percent of pediatric respiratory hospitalizations (Murphy 1988; Chanock 1990) and is a predominant cause of croup in young infants. HPIV 1 and 2 tend to infect older children and adolescents. Infection with HPIV in immunocompromised children (for example, transplant recipients) has been associated with a wide range of disease, from mild upper respiratory symptoms to severe disease requiring mechanical ventilation and leading to death, especially in young children, those whose infections occur soon after transplant, and those with the most intensive immunosuppression (Apalsch et al. 1995).

Other viral causes of croup include adenovirus, influenza A and B, rhinoviruses, RSV, measles and enteroviruses. Bacterial causes (such as *H. influenzae*, Group A hemolytic strep, *C. diphtheriae*, *S. pneumoniae*, *S. aureus* and *Mycoplasma pneumoniae*) are much less frequent. While RSV is the leading cause of acute lower respiratory tract infection in young children and causes a significant percentage of croup, it is more commonly seen as a cause of bronchiolitis. Influenza viruses (A and B) are also important causes of serious lower respiratory tract disease in children. Influenza A is an important cause of croup, as well as pneumonia and pharyngitis-bronchitis. Diphtheria caused by *Corynebacterium diphtheriae* is now a rare cause of croup. However, this organism may infect the larynx, trachea, and bronchi by extension from a pharyngeal infection or by primary infection, and result in a croupy cough with stridor.

EPIDEMIOLOGY

HPIV1 causes large seasonal outbreaks of illness, with rises in the number of croup cases every other year (odd-numbered years). HPIV-2 outbreaks generally follow those caused by HPIV-1. HPIV-3, in contrast, causes outbreaks each year, generally in the spring or summer (Hall 2001).

PATHOGENESIS IN THE HUMAN

Parainfluenza viruses replicate in the epithelium of the upper respiratory tract, and spread to the lower respiratory tract within 3 days. The pathogenesis in the lung

has not yet been well studied as it has for RSV. Epithelial cells of the small airways become infected with resultant necrosis and imflammatory infiltrates. The interplay between virus-induced cell damage, beneficial immune responses and inflammatory responses that contribute to disease for HPIV-3 is still unknown, and this is an area of active investigation for parainfluenza virus research.

CLINICAL MANIFESTATIONS

Croup is the most common clinical manifestation of HPIV infection. Inflammatory obstruction of the airway produces the clinical picture in all forms of infectious croup, which include acute laryngitis, laryngotracheitis, and laryngotracheobronchitis. A mild upper respiratory syndrome usually precedes the clinical manifestations of croup. The clinical findings are produced by inflammatory obstruction of the airway in all forms of the illness. The severity and extent of the infectious process (and, likely, of the inflammatory response) determine the sites of obstruction in the laryngotracheobronchial tree. Milder cases are characterized by hoarseness and a barking ('croupy') cough. Low-grade fever, loss of appetite, and malaise may be the only constitutional signs, respiratory distress is absent or minimal, and the condition responds promptly to appropriate treatment and subsides in a few days. In patients with severe acute laryngitis the infection may descend rapidly to the trachea and bronchi, causing increased respiratory distress evidenced by nasal flaring and inspiratory retractions. In these cases, the site of obstruction is usually the subglottic area. Hoarseness is more marked, and breathing may become rapid and labored, with inspiratory stridor and retraction of the suprasternal notch, the supraclavicular spaces, the substernal region, and the intercostal spaces. Expiratory wheezes and various types of bronchitic rales are heard on auscultation of the chest. The illness can rapidly progress to respiratory failure with hypoxia, weakness, decreased air exchange, and death. Agitation, crying, and manipulation of the airway can aggravate the respiratory distress. The white blood cell count is frequently normal or mildly elevated, and radiographs classically show a 'steeple sign' indicative of subglottic edema.

In patients with infection limited to the laryngeal area, chest auscultation reveals inspiratory stridor and impaired aeration. The main site of obstruction lies below the vocal cords, where subglottic tissues bulge to meet in the midline. An exudate may add to the airway obstruction. In laryngotracheobronchitis, the most extensive type of croup, expiratory wheezes rales are heard on auscultation of the chest. Atelectasis resulting from plugging of a bronchus with exudate may result in localized areas of absent breath sounds, bronchial breathing, and dullness to percussion. If a main bronchus becomes plugged, cyanosis worsens, and the heart and mediastinum may be shifted to the affected side.

DIAGNOSIS

Diagnosis of HPIV infection in the clinical setting can be made by viral isolation, or by detection of antigen or nucleic acid. All three types of methods are currently available, and the use of reverse transcription polymerase chain reaction (RT-PCR) allows for the diagnosis of several respiratory viruses simultaneously. A multiplex RT-PCR assay for detecting HPIV 1, 2, and 3 was developed in 1995 (Fan and Henrickson 1996) and there is currently a highly sensitive and specific multiplex assay for detecting these viruses along with RSV and influenza viruses (Fan and Henrickson 1996; Fan et al. 1998; Kehl et al. 2001). These assay kits, as well as kits for antigen detection, allow for screening of children and are likely to be used more commonly in the future as more therapies for pediatric respiratory viruses become available.

TREATMENT

Novel antivirals for HPIV are under investigation, including molecules designed to inhibit viral fusion and entry, but no specific therapy is available as of November 2003. Most children with croup do not require hospitalization, and treatment is supportive, aimed at maintaining the airway and hydration. Avoiding agitation of the child is important, in order not to compromise the airway. If necessary, humidified supplemental oxygen may be administered by hood, mask, or nasal cannula. Steroids are beneficial in the treatment of croup (Kairys et al. 1989; Orlicek 1998; Wright et al. 2002) and nebulized steroids can be effective (Husby et al. 1993; Klassen et al. 1994, 1998; Johnson et al. 1998). Racemic epinephrine or L-epineprhine has been successfully used for the treatment of more severe cases of croup (Kuusela and Vesikari 1988; Waisman et al. 1992).

PREVENTION

The development of a vaccine for the parainfluenza viruses has been hampered by the need to induce an immune response in young infants whose immature immune systems and whose maternal antibody both interfere with the development of an adequate immune response. Experimental vaccines are under evaluation, with reasonable expectation that a vaccine for HPIV-3, and perhaps also HPIV1, will soon be feasible. Several strategies have been recently evaluated for HPIV-3 vaccines. Bovine parainfluenza type 3, attenuated in humans by nature of host range, has been shown to be infectious in seronegative recipients, with most vaccinees shedding virus or developing an immune response (Clements et al. 1996; Karron et al. 1996). Cold-adapted mutants of parainfluenza have been evaluated in both chimpanzees and in healthy infants. A cold-passaged mutant (cp45) was evaluated in seronegative chimpanzees and found to be attenuated in the upper and lower

respiratory tracts. Immunized animals were resistant to wildtype PIV-3 challenge. The vaccine was also well tolerated when given intranasally to HPIV-3-seropositive and -seronegative children. One dose of vaccine induced a serum hemagglutination-inhibiting antibody response in 81 percent of vaccinees (Hall et al. 1993; Karron et al. 1995a, 1995b, 1995c). As for RSV, it is likely that with the recent increase in our understanding of the molecular contributions to attenuation, engineered live HPIV-3 vaccines can be designed to be ideally attenuated and immunogenic (Skiadopoulos et al. 1999a, b, c; Tao et al. 1999), and that this technology will enhance our ability to rapidly develop effective parainfluenza virus candidate vaccines suitable for children in the future.

Acute bronchiolitis

ETIOLOGY

While RSV is the leading cause of bronchiolitis in young children, accounting for 50–90 percent of all bronchiolitis hospitalizations, after RSV the human parainfluenza viruses type 3, and to a lesser extent type 1 and 2, are the most common causative agents of bronchiolitis (Collins et al. 1996).

PATHOGENESIS

Bronchiolitis is the result of a balance between cellular damage mediated by the viral pathogen and injury caused by the immune response of the host (Welliver 2000). Virus first replicates in the nasopharyngeal epithelium and spreads to the lower respiratory tract. Viral replication in the small airways causes inflammation, sloughing and necrosis of epithelium. Edema and increased mucous secretion cause plugging of the small airways, atelectasis, airway narrowing, and obstruction. Primary infection tends to be the most severe, and it is unclear whether this is due to immunopathologic mechanisms, immunologic immaturity or simply to the small size of the airways of the infected infants.

The immunology and immunopathogenesis of HPIV infection are complex and not fully understood, although it is clear that both humoral and cellular components of the immune system contribute to both pathogenesis and protection. Understanding of the interplay between virus-induced cell damage, beneficial immune responses and inflammatory responses will be key for the development of safe vaccine strategies and effective therapeutic interventions. This has been important, for example, in understanding mechanisms that may have contributed to enhanced disease observed after the administration of a formalin-inactivated RSV vaccine to infants (Scott et al. 1978; Connors et al. 1992; Waris et al. 1996; Prince et al. 1999, 2001; Openshaw et al. 2001). The pathologic changes that are present in children that have died with parainfluenza infection suggest exaggerated inflammation (Aherne et al. 1970; Downham et al. 1975).

HPIV primary infection does not confer permanent immunity, and in fact repeated reinfection within a year of the previous infection is common in young children. Although reinfection with HPIV-3 can occur, immunity is usually sufficient to restrict virus replication from the lower respiratory tract and prevent severe disease. Mucosal IgA levels correlate with protection from replication of parainfluenza viruses in humans (Smith et al. 1966; Tremonti et al. 1968). Cell-mediated immunity is also an important contributor to prevention of disease: HPIV-3 infection of T cell-deficient infants can result in fatal giant-cell pneumonia (Smith et al. 1966; Tremonti et al. 1968) and HPF-induced pneumonia has a 30 percent mortality in bone marrow transplant recipients (Wendt et al. 1992). The desirablilty of a vaccine that would protect against HPIV-1, -2, and -3 is clear. An inactivated HPIV-1, -2, -3 vaccine used in infants in the late 1960s was immunogenic, but did not offer protection from infection (Chin et al. 1969; Fulginiti et al. 1969), and live attenuated viruses are now being considered as vaccine candidates (Clements et al. 1991; Karron et al. 1995a, 1995b). This underscores the importance of better defining the virus–host interactions that result in pathology versus immunity. The cotton rat (*Sigmodon hispidus*) is an excellent model for HPIV lower respiratory infection (Porter et al. 1991) and for testing potential therapies (Prince and Porter 1996), since experimental infection leads to bronchiolitis and interstitial pneumonia, mimicking human disease. When cotton rats were infected with wild-type HPIV-3 and with variant viruses containing single amino acid changes in the receptor-binding protein HN the pathology observed in lungs of infected animals was distinct: infection with viruses with variant HN resulted in far more severe alveolitis and interstitial pneumonitis even though there was little difference in magnitude and duration of virus replication (Prince et al. 2001). These findings are reminiscent of the cases of unexplained severe HPIV-3 disease in presumably normal children, and present a model to explore the role of HN–receptor interaction in immunopathogenesis that may translate into better understanding of HPIV-3 disease.

CLINICAL MANIFESTATIONS

Bronchiolitis usually begins as an ordinary upper respiratory tract infection with nasal discharge, cough, slight fever, fretfulness, and loss of appetite. The infant may rapidly worsen and develop rapid labored breathing, with retraction of the intercostal spaces, use of accessory respiratory muscles and cyanosis. Increasing obstruction leads to progressive hypoxemia, followed by exhaustion and death. Respirations are rapid, shallow, difficult, and often wheezy, with a rate of 60 to over 80 per minute. Inspiratory retraction is seen in the

suprasternal notch and the intercostal and subcostal spaces. Cyanosis appears or is intensified during coughing or crying and can be continuous if the obstruction is severe.

Physical findings include overinflated lungs with hyperresonance to percussion. The diaphragm is depressed, and expiration is prolonged, and wheezing is prominent. Initial hypoxemia may be followed by respiratory acidosis and hypercapnia. Hyperinflation and diffuse interstitial pneumonitis are seen on chest X-ray in severe disease.

Risk factors predisposing to serious disease include underlying medical conditions such as prematurity, cardiopulmonary disease, immunodeficiency, metabolic disease, and some neuromuscular disorders. Assessment of the risk for progression to severe disease includes evaluation of underlying medical conditions, and the overall clinical status of the child. An oxygen saturation less than 95 percent by pulse oxymetry is a reliable objective predictor of progression to severe disease.

TREATMENT

The treatment of acute bronchiolitis should aim to relieve bronchiolar obstruction, correct hypoxemia and acidosis, control potential cardiac complications, provide supportive measures, and prevent or treat secondary bacterial infection.

Management of bronchiolitis is mainly supportive and symptomatic, aimed at providing adequate oxygenation and hydration. Close monitoring of the respiratory status is essential and cardiovascular support may be required in the presence of manifestations of developing heart failure. If the child has very severe bronchiolitis that progresses in spite of these measures, assisted ventilation may be needed.

The use of alpha- and beta-adrenergic agonists remains controversial. The individual response to these therapies is variable and their use must be assessed in each individual case. A trial of inhaled bronchodilators and evaluation of the response is recommended in most cases, particularly in patients with past history of reactive airway disease or asthma. If there is no significant improvement, the therapy may be discontinued.

Cellular damage caused by the immune system of the host is an important component in viral bronchiolitis. Because of this element of inflammation, corticosteroids have been considered and studied for the treatment of bronchiolitis, but to date these studies have failed to demonstrate significant efficacy. Other antiinflammatory agents, such as cromolyn sodium, budesonide, antileukotrienes, and antikinins, are currently being evaluated.

Pneumonia

ETIOLOGY

A variety of pathogens are etiologic agents of pneumonia in children in addition to the human parainfluenza viruses, including RSV and influenza virus and numerous bacterial agents. The incidence of viral pneumonia far outstrips that of bacterial and fungal pneumonia. The clinical manifestation of these various agents is dependent on the pathogen, the child's age and the immune status.

CLINICAL MANIFESTATIONS AND DIAGNOSIS

The clinical diagnosis of pneumonia is supported by the findings of fever, tachypnea, and crackles on lung examination. For many patients, this evaluation is sufficient and the need for pursuing further diagnostic evaluation, including complete blood count, chest radiograph, and blood culture, is based on the degree of toxicity and illness of the child. The most reliable indicator of a positive chest radiograph is the clinical finding of tachypnea. Approximately two out of three of patients with pneumonia present with this finding.

Clinical evidence may not be sufficient to differentiate between viral and bacterial etiologies. Conjunctivitis and otitis media are more often associated with bacterial than viral pneumonia (Ramsey et al. 1986), and wheezing is more commonly associated with viral etiologies (Turner et al. 1987; Forgie et al. 1991). The chest radiograph does not differentiate bacterial from viral pneumonia when radiographs are compared in a blinded fashion (McCarthy et al. 1981; Courtoy et al. 1989). Elevated CRP and absolute neutrophil are more reliable indicators of bacterial pneumonia than clinical and radiologic evidence (Korppi et al. 1997; McIntosh 2002).

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Pneumovirus and Metapneumovirus: respiratory syncytial virus and human metapneumovirus

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With the isolation of respiratory syncytial virus (RSV) in 1956, and its association with serious lower respiratory tract infection in young children, the significance of RSV as an important human pathogen was established. Recent discovery of human metapneumovirus (hMPV), a respiratory virus biologically and clinically related to RSV, has highlighted the importance of understanding the virus–host relationship between RSV and hMPV. The biology, replication, and virus and host factors that influence virus replication, immunity, and disease pathology are discussed.

HUMAN RESPIRATORY SYNCYTIAL VIRUS – THE TYPE SPECIES *PNEUMOVIRUS*

The *Paramyxoviridae* family includes numerous important human and avian respiratory tract pathogens. Human respiratory syncytial virus (HRSV) is a member of the *Pneumovirinae* subfamily and type species member of the *Pneumovirus* genus. RSV was first isolated in 1956 as the chimpanzee coryza agent (CCA) during an outbreak of respiratory illness in a chimpanzee colony (Morris et al. 1956). Shortly thereafter, RSV was isolated from an infant with bronchopneumonia, and from another infant with bronchitis (Chanock and Finberg 1957). It was named respiratory syncytial virus because of its characteristic ability to induce syncytia in cell lines. RSV is now recognized as a ubiquitous virus, and the most important cause of serious lower respiratory tract illness in infants and young children worldwide, as well as an important pathogen in the elderly, and those with compromised cardiac, pulmonary, or immune systems (Fixler 1996; Couch et al. 1997; Falsey 1998; Hall 1999; Simoes 1999; Falsey and Walsh 2000; Billings et al. 2001; CDC 2002; Staat 2002). RSV, followed by the parainfluenza viruses, is the primary cause of hospitalization for respiratory tract illness in young children. The infection rate in young children approaches 70 percent in the first year of life (Glezen and Denny 1973), and in the USA, lower respiratory tract disease develops in 20–30 percent of

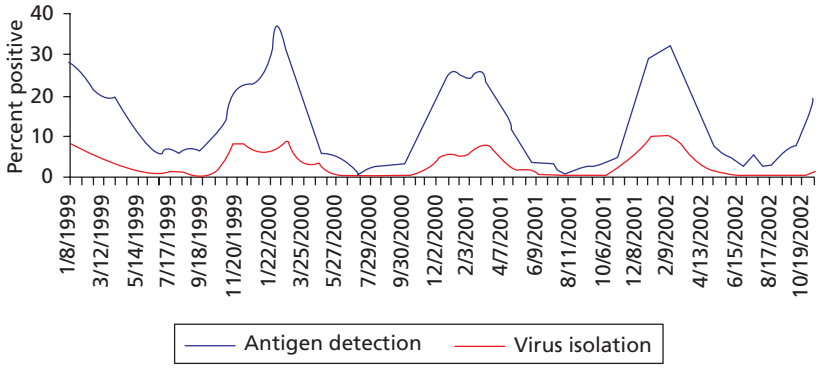

Figure 1 *The percent positive RSV tests in the USA from 1999 to 2002 by week of report to the National Respiratory and Enteric Virus Surveillance System. Weekly laboratory test result data are collected on a voluntary basis from reference laboratories in the USA. In the USA, RSV community outbreaks typically occur during late fall, winter, and early spring. There may be variation in the timing of outbreaks between regions and between communities in the same region.*

infected children requiring hospitalization (Shay et al. 1999). In immune compromised patients, or bone marrow or lung transplant recipients, RSV may cause respiratory failure leading to mortality rates of 70–100 percent (Hertz et al. 1989). RSV is a seasonal virus with peak rates of infection occurring annually during cold seasons in temperate climates, and in the rainy season in tropical climates. In the USA, most RSV infections occur during a period from October through March with peak activity usually in January or February (Figure 1). There are two major groups of RSV strains, A and B, and both strains circulate concurrently. The clinical severity of RSV infection has not been conclusively linked with infection by A or B strain. The significant public health burden mediated by RSV infection is exemplified by the dramatic infection rate in younger children, the percent of children hospitalized because of RSV-associated lower respiratory tract disease, and by the substantial mortality in the young and immune compromised. Despite four decades of efforts to unravel the complexities of RSV–host interactions, there is no effective vaccine to control RSV infection, and only limited prophylactic or therapeutic treatments. Although this work has led to a greater understanding of RSV immunobiology, significant efforts are still needed to understand the mechanisms of immunity and disease pathogenesis associated with infection, and their relationship to the newly discovered hMPV, in order to develop effective vaccines and treatments.

AVIAN PNEUMOVIRUS (APV) – THE TYPE SPECIES *METAPNEUMOVIRUS*

APV is a member of the *Pneumovirinae* subfamily, and type species member of the *Metapneumovirus* genus. In the USA, APV was first isolated in 1997 from turkeys with clinical signs of respiratory disease (Senne et al. 1997; Njenga et al. 2002). It is a ubiquitous virus that has been isolated from fowl in Africa, Europe, and North America and South America (Njenga et al. 2002). The clinical signs of infection in turkeys include conjunctivitis, rhinitis, sinusitis, and tracheitis, which led to it initially being named *Turkey rhinotracheitis virus* (Collins and Gough 1988; Jirjis et al. 2002a). APV causes upper respiratory infection in both turkeys and chickens, and may rapidly spread through flocks via respiratory aspirate (Alkhalaf et al. 2002). Infection by APV infection is generally not associated with mortality, but is associated with high morbidity and exacerbated secondary infections, although it is possible to have infection without clinical signs (Seal 2000; Alkhalaf et al. 2002; Chary et al. 2002). Recovery usually takes place within 10–12 days. Morbidity may be 100 percent, and mortality, usually due to secondary bacterial infections, ranges from 0 to 90 percent (Alkhalaf et al. 2002; Jirjis et al. 2002a; Njenga et al. 2002). All ages of fowl are susceptible; however, APV disease is more severe in young birds. There are two strains of APV recognized, A and B, based on differences in the *G* glycoprotein of the virus (Njenga et al. 2002). Nucleotide sequence analysis of the *G* glycoprotein gene has indicated that isolates from the UK and France are similar and belong to strain A, and isolates from Spain, Italy, and Hungary are identical, and belong to strain B (Njenga et al. 2002).

CLASSIFICATION

RSV is a nonsegmented negative-strand RNA virus belonging to the family *Paramyxoviridae* (order *Mononegavirales*) which consists of two subfamilies and five genera. *Morbillivirus*, represented by *Measles virus*, *Rubulavirus*, represented by *Mumps virus* that includes parainfluenza virus 2 (PIV2), parainfluenza virus 4, *Newcastle disease virus* (NDV), and *Simian virus 5* (SV-5), *Respirovirus*, represented by *Sendai virus* that includes parainfluenza virus 1 and parainfluenza virus 3 form the subfamily *Paramyxovirinae* (Figure 2). The

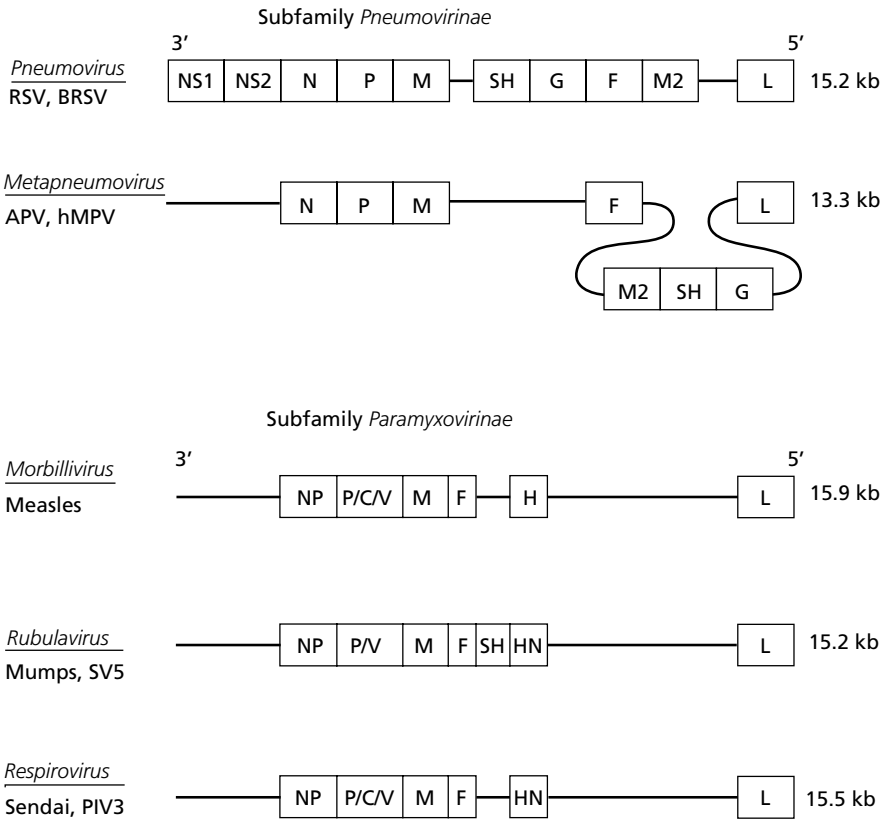

Figure 2 *Comparison of gene maps of members belonging to the* Pneumovirinae *and* Paramyxovirinae *subfamilies of nonsegmented negative-strand RNA viruses. The gene alignment is illustrated as genomic RNAs from 3′ to 5′. Each box represents a separate encoded mRNA, and the acronyms for the encoded proteins. The box sizes and spacing are not to scale. Protein names: NS, nonstructural protein; N/NP, nucleocapsid protein; P, phosphoprotein; M, matrix protein; M2, second matrix protein; SH, small hydrophobic protein, G/HN/H, attachment protein; F, fusion protein; L, large polymerase protein; C, nonstructural protein; V, cysteine-rich protein. The* Rubulavirus SH *gene is indicated for mumps virus and SV-5 members; however, NDV, PIV2, and simian virus 41 do not have an* SH *gene.*

Pneumovirus genus, represented by RSV, includes bovine, ovine, and caprine RSV, and the *Metapneumovirus* genus is represented by APV (or *Turkey rhinotracheitis virus*) and tentatively includes hMPV, forming the subfamily *Pneumovirinae* (Figure 2). The *Morbillivirus*, *Rubulavirus*, and *Respirovirus* genera are highly interrelated, sharing significant amino acid homology for many proteins; however, the *Pneumovirus* genus is substantially different. The pneumoviruses have fusogenic activity (Zimmer et al. 2001), but do not hemagglutinate or have hemolytic activity (Richman et al. 1971), and neuraminidase activity is absent in both *Morbillivirus* and *Pneumovirus*. Pneumoviruses also differ from other paramyxoviruses in the diameter of the helical nucleocapsid that is 12–15 nm, rather than 18 nm (Bhella et al. 2002). In addition, pneumoviruses encode a larger number of mRNAs, but the lengths of genomic RNAs are similar to paramyxoviruses (Figure 2).

Pneumovirus genome contains two nonstructural genes (*NS2*, *NS1*) followed by eight other genes namely, nucleocapsid (*N*), phosphoprotein (*P*), matrix (*M*), small hydrophobic (*SH*), surface attachment glycoprotein (*G*), surface fusion glycoprotein (*F*), second matrix (*M2*), and RNA-dependent RNA polymerase (*L*) in the order 3′-*NS1–NS2–N–P–M–SH–G–F–M2–L*-5′. By contrast, the *Metapneumovirus* genome lack *NS* genes, and has a different gene order given by 3′-*N–P–M–F–M2–SH–G–L*-5′.

PNEUMOVIRUS PROTEINS

Electron microscopy (EM) and negative staining have shown that pneumovirus virions are pleomorphic, consisting of a nucleocapsid surrounded by a lipid envelope with a diameter ranging from 150 to 300 mm (Joncas et al. 1969; Norrby et al. 1970; Bachi and Howe 1973). A thin section EM of RSV particles budding from the plasma membrane of HEp-2 cells is shown in Figure 3. The lipid bilayer that surrounds the nucleocapsid is derived from the host-cell plasma membrane during the budding process, and contains three virally encoded surface transmembrane glycoproteins: G, F, and SH. The virion matrix proteins, M1 and M2, are non-glycosylated, and the N, P, and L proteins associate with genomic RNA to form the nucleocapsid. The NS1

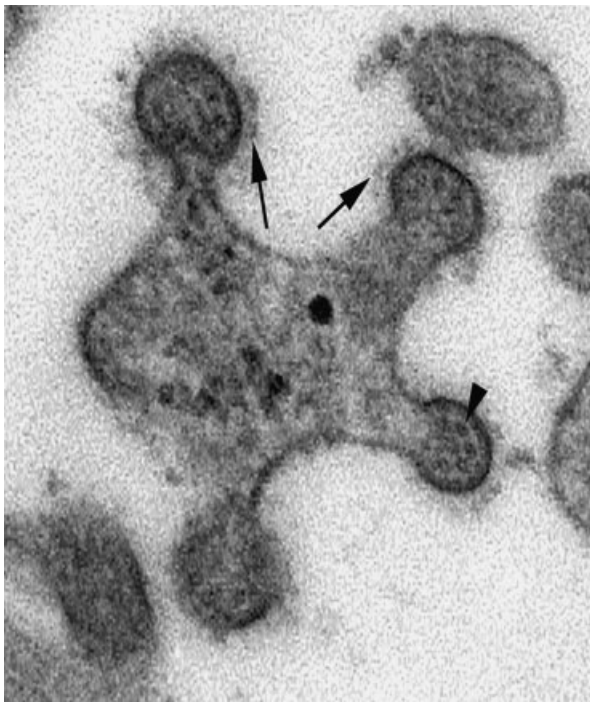

Figure 3 *RSV budding from the plasma membrane of HEp-2 cells. Cross-sections of the viral nucleocapsid (arrowhead) are enclosed within the viral envelope which has prominent surface projections (arrows). (Courtesy of C.S. Goldsmith, J. Hierholzer, and E.L. Palmer, Centers for Disease Control and Prevention, Atlanta, GA.)*

and NS2 proteins are not readily detectable in virions, but are found in RSV-infected cells.

The RSV genome consists of 15 222 nt that are transcribed into 11 major subgenomic mRNAs, each of which codes for a unique protein (Huang and Wertz 1982; Collins and Wertz 1983; Collins et al. 1984a, 1986; Dickens et al. 1984). The surface fusion glycoprotein (F), in combination with the surface attachment glycoprotein (G), is responsible for attachment, and the F glycoprotein is responsible for virus penetration and fusion of the viral envelope with host cell membranes and formation of syncytia. The F glycoprotein is structurally and functionally related to other paramyxoviruses (Collins et al. 1984b; Spriggs et al. 1986). It is a type I membrane glycoprotein of approximately 70 kDa that contains a cleaved N-terminal signal sequence anchored in the membrane by a C-terminal membrane anchor sequence allowing for >90 percent of the N-terminal molecule to be extracellular (Arumugham et al. 1989; Paradiso et al. 1989). The F glycoprotein is synthesized as precursor protein (F_0) in the endoplasmic reticulum consisting of a F_2 domain at amino acid positions 1–130, a cleavage site at amino acid positions 131–136, and an F_1 domain at amino acid positions 137–574. The F_0 protein precursor is cleaved by a trypsinlike protease into disulfide-linked F_1 and F_2 subunits in the trans-Golgi, constituting the biologically active form of the molecule. The F_2 domain is modified by the addition of N-linked sugars, and the F glycoprotein is expressed as a trimer or tetramer (Collins et al. 1984b; Johnson and Collins 1988).

The G glycoprotein has a number of genetic, structural, and functional differences that are unique among the *Paramyxoviridae* viruses, and most virus attachment proteins in general (Satake et al. 1985; Wertz et al. 1985, 1989). The G glycoprotein forms from a polypeptide precursor (32 kDa) that ranges in amino acid length (292–299 amino acids) depending on the virus strain (Martinez et al. 1999; Sullender et al. 1991). The precursor is co-translationally modified by the addition of high mannose N-linked sugars to form an intermediate of 45 kDa, that is followed by addition of *O*-linked sugars in the trans-Golgi to achieve a mature form of approximately 90 kDa (Wertz et al. 1989; Collins and Mottet 1992). Most of the carbohydrate is of the O-linked variety, thus the G glycoprotein has high serine, threonine, and proline content (Collins 1990; Collins and Mottet 1992). The high proline content may reduce highly folded secondary structure contributing to an extended structure. The G glycoprotein is also palmitylated (Collins and Mottet 1992). The G glycoprotein is a type II glycoprotein with a single N-terminal hydrophobic region (amino acids 38–66) that serves as a signal peptide and membrane anchor (Wertz et al. 1985; Vijaya et al. 1988; Roberts et al. 1994; Lichtenstein et al. 1996). Proximal to the membrane anchor region is an extracellular ectodomain. In the middle of the ectodomain is a short protein segment and four cysteine residues (173, 176, 182, and 186) that are highly conserved in all RSV isolates (Sullender et al. 1991; Teng and Collins 2002). This region contains a CX3C chemokine motif (amino acids 182–186) that may facilitate virus attachment to cells expressing the CX3C chemokine receptor, CX3CR1 (Tripp et al. 2001). Flanking this region are two glycosylation sites that are highly divergent among all RSV isolates (Cane et al. 1991; Sullender et al. 1991). The G glycoprotein is expressed both membrane-bound and secreted by initiation of translation at an alternate in-frame AUG codon located in the middle of the hydrophobic transmembrane region (Roberts et al. 1994). Approximately 15 percent of the G glycoprotein synthesized in infected cells is secreted in a soluble form lacking the cytoplasmic domain and part of the signal-anchor sequence, but retaining the same characteristics as the membrane bound form, e.g. glycosylation and antibody reactivity (Hendricks et al. 1987, 1988).

The SH protein is a short (64 amino acids) minor surface glycoprotein with unknown function. Unpublished results from our laboratory suggest that SH expression may regulate apoptosis by inducing expression of the anti-apoptosis gene, *IEX-1L* (Domachowske et al. 2000) in RSV-infected human epithelial cells. The SH protein is membrane-anchored at a centrally located anchor sequence such that only a third of the molecule

is extracellular. SH accumulates in the infected cell in different forms (Sho, SHg, SHp, SHt), but the SHo unglycosylated species is the most abundant form (Olmsted and Collins 1989; Collins et al. 1990a). All forms of SH, except for SHt, are transported to the cell surface, and only Sho and SHp are incorporated into the virion (Collins and Mottet 1993). The significance of the different forms is unknown, but conservation among human and bovine strains of RSV suggests that they may be involved in virus attachment, penetration, or viral uncoating (Collins et al. 1990a; Anderson et al. 1992), or may be important in regulating apoptosis.

RSV has three nucleocapsid-associated proteins, N, P, and L. The RNA binding protein, N, is a multifunctional protein of 391 amino acids that has a central role in transcription and replication of viral genomic RNA (Collins et al. 1984a, 1985). The phosphoprotein (P) is a key component of the viral RNA-dependent RNA polymerase complex, having two general functions as a transcription and replication factor, and as a chaperone for soluble L protein (Horikami et al. 1992). The P protein is smaller (241 amino acids) than most paramyxovirus P proteins (391–602 amino acids) and is dispensable for virus replication in vitro (Villanueva et al. 2000). However, phosphorylation of P protein is required for efficient virus replication in vitro and in vivo (Villanueva et al. 2000; Khattar et al. 2001). The large viral polymerase, L, is the least abundant of the structural proteins. The L protein is similar in length to other paramyxovirus counterparts (approximately 2 200 amino acids), but there is limited sequence homology within the paramyxoviruses (Stec et al. 1991). The N, P, and L proteins are the minimal *trans*-acting proteins required for RNA replication (Yu et al. 1995); however, RSV transcription and replication appears to lack the requirement that template length be an even multiple of an integer such as six, which is obligatory for nucleocapsid function of other paramyxoviruses, such as measles virus and Sendai virus (Samal and Collins 1996).

Unlike other negative-strand RNA viruses, pneumoviruses such as RSV have two matrix proteins, M and M2 (Collins et al. 1984a; Huang et al. 1985). The M protein is smaller (256 amino acids) than similar paramyxovirus counterparts (335–375 amino acids), and has limited sequence homology (Satake and Venkatesan 1984). The M2 protein (194 amino acids) is unique to pneumoviruses. M and M2 are nonglycosylated proteins that appear to be virion-associated. The M protein appears to functionally inactivate nucleocapsid transcription prior to packaging, and to mediate nucleocapsid association with the nascent envelope. The M2 protein appears to function as a transcription factor to increase polymerase processivity, and enhance readthrough of intergenic junctions during virus transcription (Hardy et al. 1999; Hardy and Wertz 2000). It has a Cys_3H_1 zinc-binding motif that is essential for its function, and has been shown to interact with the N protein (Hardy and Wertz 2000).

Of the pneumovirus proteins, the function of NS1 and NS2 proteins are the least defined. NS1 and NS2 are considered nonstructural proteins as only trace amounts are detected in the virion (Huang et al. 1985; Collins et al. 1990a; Evans et al. 1996). Unique to pneumoviruses, the nonstructural proteins are encoded by separate mRNAs. NS1 and NS2 may act cooperatively to antagonize interferon (IFN)-α/β-induced antiviral responses, and NS2 protein appears to be rapidly processed and secreted (Schlender et al. 2000; Gotoh et al. 2001). Studies with recombinant RSVs with deletions in the *NS1* and *NS2* have shown that these genes are dispensable for virus replication in vitro; however, they provide auxiliary functions for efficient RSV replication in vitro and in vivo (Jin et al. 2000).

METAPNEUMOVIRUS PROTEINS

The *Metapneumovirus* genus has two members that cause respiratory disease, APV and the recently discovered hMPV (van den Hoogen et al. 2001; Stockton et al., 2002). Similar to the pneumoviruses, electron microscopy and negative staining have shown that metapneumovirus virions are pleomorphic, consisting of a nucleocapsid surrounded by a lipid envelope with a diameter ranging from 150 to 300 mm (Figure 4). APV has similar morphology and structural polypeptides compared with those of bovine, human, and murine pneumoviruses, but lacks the *NS* genes, making the genome shorter (13.3 kb). APV possesses a helical nucleocapsid 14 nm in diameter, and eight viral polypeptides with a gene order given by 3′-*N–P–M–F–M2–SH–G–L*-5′. hMPV has the same gene order.

The APV F glycoprotein consists of 538 amino acids, and the F1 and F2 subunits contain 102 residues (including the F1–F2 connecting peptide RRRR) and 436 residues, respectively. The F glycoprotein protein has approximately 40 percent amino acid identity with the F glycoprotein of RSV (*Pneumovirus* genus), but half that with members of the other two genera (*Respirovirus* and *Morbillivirus*) in the *Paramyxoviridae* family (Njenga et al. 2002). The putative hMPV F glycoprotein open reading frame encodes a 539 amino acid protein. Amino acid sequence analysis indicates 81 percent identity to group C APV, and only 10–20 percent homology with other paramyxovirus counterparts. The F1 and F2 subunits are similar to APV, and contain a conserved cleavage site (RQSR). Interestingly, a second cleavage site (RARR) occurring in RSV does not occur in metapneumoviruses. Similar to pneumoviruses, two heptad repeats (HRA and HRB) are located adjacent to the fusion peptide and transmembrane region, and are required for viral fusion.

The APV *G* gene is 1 193 nt in length and encodes a protein of 391 amino acids, which is larger than the RSV

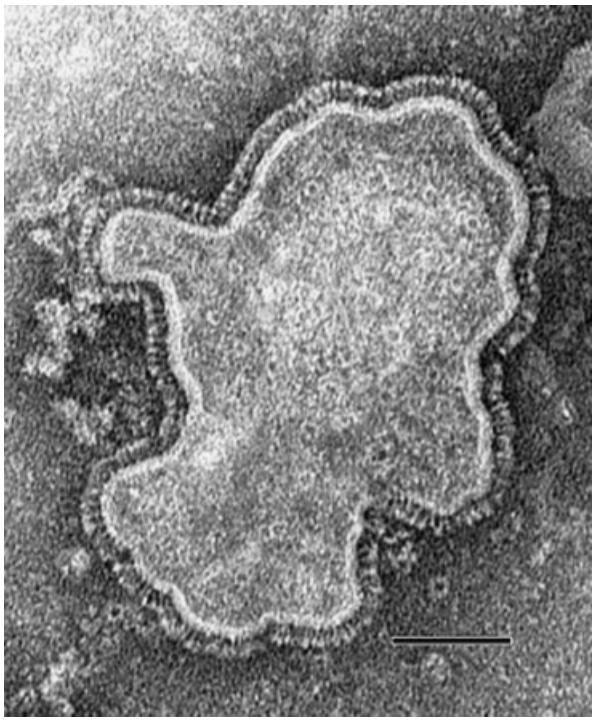

Figure 4 *A negative-stained electron micrograph of hMPV. This pleomorphic form of the virus is stain-penetrated, to permit visualization of portions of the virus envelope and nucleocapsid. A border composed of hMPV surface proteins occurs around the virus periphery. Bar marker represents 100 nm. (Courtesy of Charles Humphrey, Centers for Disease Control and Prevention, Atlanta, GA.)*

G glycoprotein (292–299 amino acids) (Ling et al. 1992). The APV *SH* gene is 589 nt in length, encoding a protein of 174 amino acids that is considerably larger than the RSV SH protein (64 amino acids) (Ling et al. 1992). Hydrophobicity profiles of APV SH and G glycoproteins show considerable similarity to those of the SH and G glycoproteins of RSV. The nucleotide sequences of the putative attachment protein (G glycoprotein) of APV isolates suggests that there are at least two distinct subgroups (A and B), similar to the grouping described for RSV (Juhasz and Easton 1994). Within the subgroups, there is nearly 100 percent amino acid identity; however, between the subgroups, there is considerable divergence. The hMPV *SH* gene encodes a 183 amino acid protein, which is the largest of all pneumovirus or metapneumovirus SH glycoproteins. The amino acid composition and hydrophobicity profile is similar to APV and pneumoviruses; however, hMPV SH glycoprotein contains 10 cysteine residues, compared to 16 for APV. The putative hMPV G glycoprotein open reading frame encodes a 236 amino acid protein. Several smaller open reading frames that lack start codons occur immediately following, or are in frame with, the G glycoprotein open reading frame, suggesting that additional coding sequences are expressed as separate proteins, or are expressed through RNA-editing events. The hMPV G glycoprotein open reading frame is predicted to contain five N-linked glycosylation sites, similar to APV (Juhasz and Easton 1994; Njenga et al. 2002), but lower than RSV which has seven sites (Wertz et al. 1985). The predicted hydrophobicity profile of hMPV G glycoprotein is similar to the pneumoviruses, and the overall organization is consistent with an anchored type II transmembrane protein. However, the hMPV G glycoprotein lacks the cysteine secondary structure and CX3C chemokine motif in the G glycoprotein of pneumoviruses.

The APV nucleocapsid protein gene (*N*) comprises 1 197 nt, forming a single major open reading frame that potentially encodes a protein of 391 amino acid residues (Li et al. 1996). The predicted N protein is identical in length to human, bovine, and ovine RSV, and overall is approximately 41 percent similar, but has some regions of >90 percent identity (Njenga et al. 2002). The hMPV N protein is a 394 amino acid protein similar to APV. Amino acid sequence analysis indicates 88 percent overall identity to group C APV, but only 10 percent overall identity with other paramyxoviruses.

The APV phosphoprotein gene (*P*) comprises 855 nt containing one open reading frame encoding a protein of 278 amino acids (Ling et al. 1995). Comparison of the overall APV P protein sequence with that of the other pneumoviruses reveals amino acid identities ranging from 31 to 35 percent; however, there is much higher identity (approximately 70 percent) in a region of 57 residues that include a heptad repeat sequence. The putative hMPV P protein open reading frame encodes a 294 amino acid protein that shares 68 percent amino acid homology to group C APV. Similar to RSV and group C APV, the hMPV P protein open reading frame lacks cysteine residues.

The APV large viral polymerase gene (*L*) comprises 6 099 nt, and encodes a single major open reading frame of 2 004 amino acids (Randhawa et al. 1996). The APV L protein is the smallest described for any nonsegmented, negative-strand RNA virus. The protein contains six linear noncontiguous domains, a consensus ATP-binding site, and four polymerase motifs previously described for the L proteins of negative-strand RNA viruses. The *L* gene of hMPV encodes a 2 005 amino acid protein, and contains a consensus ATP-binding motif. Domain III in the hMPV L protein (amino acids 625–847) has 83 percent amino acid sequence identity with APV, and approximately 70 percent identity with RSV.

The APV M protein contains a hydrophobic domain common to pneumoviruses (Seal 1998). The M protein nucleotide sequence of group A strains is approximately 75 percent similar to group B strains (Seal 2000). The APV *M2* gene contains two overlapping open reading frames that encode the M2-1 protein of 184–186 and 71–73 amino acids that do not show any similarity to the equivalent RSV protein. The sequences of the intergenic regions of APV reveal the gene order to be 3′-*M–F–M2*-5′,

compared to the 3′-*M–SH–G–F–M2*-5′ gene order of pneumoviruses. Determination of the nucleotide sequences in APV has established that *NS1* and *NS2* genes are absent in APV. The hMPV *M* gene, like APV, has two overlapping open reading frames, the first of which encodes a 187 amino acid M2-1 protein having 84 percent sequence identity to group C APV, the second of 71 amino acids. The hMPV M2-1 protein has three cysteine residues located within the first 30 amino acid residues conserved among all pneumoviruses, suggesting the possibility of a zinc-binding protein. Amino acid sequence comparison of the M2-2 open reading frame indicates approximately 60 percent identity to group C APV.

POLYMORPHISMS AMONG PNEUMOVIRUS AND METAPNEUMOVIRUS

The genomic organization of pneumoviruses and metapneumovirus are comparable, but there are distinct variations. Pneumovirus genomes (15.2 kb) contain two nonstructural genes (*NS1* and *NS2*), followed by the nucleocapsid (*N*), phosphoprotein (*P*), matrix (*M*), small hydrophobic (*SH*), surface glycoprotein (*G*), fusion (*F*), second matrix (*M2*), and large RNA-dependent, RNA polymerase (*L*) genes. By contrast, metapneumovirus genomes are smaller (13.4 kb), lack *NS1* and *NS2* genes, and the gene order is 3′-*N–P–MF–M2–SH–G–L*-5′. Genetic and antigenic polymorphisms are well documented for pneumoviruses such as RSV, where the *SH* and *G* genes have been shown to have the greatest diversity among strains (Sullender et al. 1990, 1991, 1998; Mlinaric-Galinovic et al. 1994; Zheng et al. 1999). For RSV A and B strains, there is approximately 25 percent antigenic relatedness overall, with F glycoproteins approximately 50 percent related and G glycoproteins approximately 1–10 percent related (Johnson et al. 1987a; Hendry et al. 1988). Divergence in the G glycoprotein is greatest in the ectodomain and related to poorly conserved *N*- and *O*-linked sugar acceptor sites (Johnson et al. 1987b; Sullender et al. 1991). A high percentage of nucleotide sequence differences occur in the G glycoprotein for A and B strains, for which nearly half encode amino acid substitutions (Cane et al. 1991; Sullender et al. 1991). It is possible that G glycoprotein diversity among RSV strains provides an advantage to the virus to evade previously induced immunity in the population. Sequence information indicates that multiple genotypes circulate simultaneously in the same community, and similar genotypes circulate in widely separated communities during different years (Peret et al. 1998, 2000). Immune pressure may contribute to diversity in the G glycoprotein, since it is one of the two major neutralizing antigens (the other the F glycoprotein), the ectodomain is extracellular, and sequence diversity is correlated with antigenic diversity.

Metapneumoviruses such as APV appear to have similar genetic and antigenic polymorphisms as the pneumoviruses. Comparison of the nucleotide and amino acid sequences of APV isolates indicate that the G glycoprotein is extremely variable between strains (38 percent nucleotide identity and 56 percent amino acid predicted sequence identity), but there is high nucleotide sequence identity (>99 percent) among strains (Bayon-Auboyer et al. 2000; Seal 2000; Njenga et al. 2002). APV strains also vary in patterns of neutralization by monoclonal antibodies reactive to the G glycoprotein (Njenga et al. 2002). Similar to RSV, the F glycoprotein and M protein of APV have extensive nucleotide and amino acid sequence identity within strains, but lower identity between strains (Naylor et al. 1998; Seal 2000; Seal et al. 2000). Sequence comparison between hMPV and APV shows high levels of nucleotide identity, and absence of *NS1* and *NS2* genes. Partial N, M, F, and L sequence comparison of hMPV isolates indicates that hMPV has two strains or clusters with 90–100 percent nucleotide identity within strains, and 80–90 percent identity between strains. Comparison of the four strains of APV to hMPV indicates 56–88 percent amino acid sequence identity within the nine open reading frames, and comparison of N, P, M, F, and M2 proteins indicated 80 percent overall amino acid identity. The APV *G* gene sequence is currently unavailable, but partial sequence information (personal communication) suggests that it has low sequence identity to hMPV.

VIRUS REPLICATION

Virions primarily attach to cells through the G glycoprotein. Attachment appears to be principally mediated by interaction of heparin-binding domains on the G glycoprotein with cell surface glycosaminoglycans (Krusat and Streckert 1997; Bourgeois et al. 1998; Feldman et al. 1999). However, recent evidence indicates that a CX3C chemokine motif located in the conserved region of the G glycoprotein (amino acids 182–186) may facilitate attachment through interaction with the CX3C chemokine receptor, CX3CR1 (Tripp et al. 2001), although this region is not required for attachment (Teng and Collins 2002). The G glycoprotein itself is not required for virion attachment to cells. Mutant RSV lacking *SH* and/or *G* genes have been shown to infect cells, likely through interaction with the F glycoprotein (Karron et al. 1997a; Teng et al. 2001; Techaarpornkul et al. 2002). Pneumoviruses penetrate cells by fusion with the plasma membrane, a process associated with the F glycoprotein (Bachi 1988). Penetration involves incorporation of the viral envelope into the cell membrane, and nucleocapsid release into the cytoplasm (Levine and Hamilton 1969; Hierholzer and Tannock 1986; Routledge et al. 1987; Arslanagic et al. 1996). The L protein initiates viral transcription and replication proceeds in the cytoplasm without nuclear involvement (Fearns and Collins 1999).

Transcription of pneumovirus mRNAs occurs in a 3′ to 5′ order from a single promoter near the 3′ end. Polymerase-mediated transcription results in a series of subgenomic mRNAs that are co-linear copies of the genes with no evidence of mRNA editing or splicing (Dickens et al. 1984; Huang et al. 1985; Kuo et al. 1996; Harmon et al. 2001; Krempl et al. 2002). Sequence and transcriptional mapping analysis of RSV mRNAs indicates that each transcript begins at the first nucleotide of the gene-start signal, is capped at the 5′ end, and polyadenylated at the 3′ end. Both mRNAs and proteins can be detected by 4 hours post-infection with peak mRNA synthesis and protein expression occurring 16–20 hours post-infection. The level of protein expressed is related to mRNA abundance (Dickens et al. 1984). In general, there is decreasing amounts of mRNAs with increasing gene distance from promoter sequence. RNA replication is dependent on active protein synthesis.

VIRUS ASSEMBLY

Virions assemble at the plasma membrane. Inclusion bodies containing viral ribonucleoprotein cores appear immediately below the plasma membrane. Nucleocapsids localize with cell membrane containing membrane viral glycoproteins. Virions mature in clusters at the apical surface in a filamentous form associated with caveolin-1, and extend from the plasma membrane (Brown et al. 2002). Experiments using human primary airway epithelial cell cultures have shown that ciliated epithelial cells are targeted by RSV, and that infection and shedding occurs via the apical membrane, and virus may be spread to neighboring ciliated cells by the motion of the cilia (Zhang et al. 2002).

INFECTION AND EXPERIMENTAL ANIMAL MODELS

Pneumoviruses and metapneumoviruses generally infect the superficial layers of the respiratory epithelium, but can grow in a wide variety of mammalian cells. There appear to be few differences in susceptibility to infection for most epithelial cell types, but significant differences in virus replication. For example, human nasal epithelial cell cultures, human bronchial epithelial cell cultures, and a human bronchoepithelial cell line (BEAS-2B) are equally susceptible to RSV infection; however, bronchial cells release less infectious virions than nasal cells (Becker et al. 1992), and RSV replicates better in ciliated epithelial cells compared to non-ciliated epithelial cells (Henderson et al. 1978; Zhang et al. 2002). In addition, a human epithelial cell line, HEp-2, and an African green monkey cell line, Vero, are commonly used to propagate pneumoviruses; however, only Vero cells effectively support metapneumovirus propagation (van den Hoogen et al. 2001). APV has been successfully isolated from infected turkeys using day-old chicken or turkey embryos, and chicken embryo fibroblasts and Vero cells support virus replication (Yachida et al. 1978; Goyal et al. 2000; Jirjis et al. 2002b; Patnayak et al. 2002).

Viral infection of susceptible cell lines results in a majority of cell-associated virions. A large fraction of the cell-associated virions are aggregated, and many virions released from the infected cell are defective and non-infectious (Levine and Hamilton 1969; Bachi 1988). Both pneumoviruses and metapneumoviruses are rather unstable, and rapidly lose infectivity during storage at −70°C, during handling, and during purification procedures. Freezing virus stocks in media containing 50 mM HEPES and 100 mM magnesium sulfate tends to stabilize the virions. Plaque assays can be used to readily quantify pneumoviruses; however, metapneumoviruses do not mediate quantifiable cytopathology or plaque formation (van den Hoogen et al. 2001). This limitation can be overcome by acetone fixation of infected cells and immunostaining to quantitate infected cells.

For pneumoviruses, a variety of experimental animals may be useful for experimental evaluation of infection or disease pathogenesis; however, there is great variation in permissiveness to infection. For RSV, the only experimental animal that approaches human permissiveness to infection and disease is the chimpanzee (Belshe et al. 1977; Collins et al. 1990b). Since this animal model is limited, the majority of RSV studies have focused on mice and cotton rats, which are semipermissive for RSV replication, but fail to fully emulate RSV disease associated in humans (Byrd and Prince 1997). BALB/c mice are the most commonly used experimental animal model for RSV infection, and much has been learned from its use; however, they appear less permissive to RSV infection than cotton rats (Prince et al. 1978, 1979, 1986). Histopathological studies of RSV disease in cotton rats has shown to better emulate the human condition than similar studies in mice. The choices for experimental animal models of metapneumovirus infection are less diverse. hMPV appears to be primarily a human pathogen; however, recent studies from our laboratory have shown that hMPV can infect and replicate in BALB/c mice, and there is strong preliminary evidence that cotton rats are also susceptible to infection (personal communication). APV is a pathogen of a variety of birds including turkeys, ducks, pheasants, and ostriches causing rhinotracheitis, and possibly a pathogen of chickens associated with swollen head syndrome (Cook 2000; Catelli et al. 2001; Shin et al. 2001).

DISEASE PATHOGENESIS

RSV is the leading cause of severe lower respiratory tract infection in infants and young children worldwide (Simoes 1999; Hall 2001; Krilov 2001; Law et al. 2002; McNamara and Smyth 2002; Weisman 2002), and may

lead to a wide spectrum of respiratory illnesses. RSV infection is most common during infancy and early childhood (CDC 2000), but may also occur in adults, the immune compromised, and the elderly (Englund et al. 1991; Couch et al. 1997; Hall 1999; Han et al. 1999; Simoes 1999; Falsey and Walsh 2000). In infants, 25–40 percent of infections result in lower respiratory tract involvement, including pneumonia and bronchiolitis (Shay et al. 1999; Hall 2001). Viral replication initiates in the upper respiratory tract; however, it can spread to the lower airways by aspiration of secretions or via the respiratory epithelium involving the bronchi, bronchioles, and alveoli, and may continue to macrophages and monocytes (Domurat et al. 1985; Midulla et al. 1989; Becker et al. 1992). Infection is associated with necrosis of the bronchiolar epithelium, destruction of the ciliated epithelial cells, and a peribronchiolar infiltrate of lymphocytes and mononuclear cells (Price 1990; Lugo and Nahata 1993). Interalveolar thickening and filling of alveolar spaces with fluid released from edematous submucosal and advential tissues may lead to airways obstruction (Price 1990; Panitch et al. 1993). The characteristics of the immune response to RSV are not fully elucidated, and it is possible that damage of the epithelium and the bronchiolar ciliary cells may be associated with the host immune response, particularly RSV-specific $CD8^+$ cytotoxic T cells (CTL) (Garofalo et al. 1996).

RSV illness frequently begins with rhinorrhea and low-grade fever that is often accompanied by cough and wheezing (Price 1990; Lugo and Nahata 1993). At the beginning of illness, RSV replicates in the nasopharynx, reaching titers between 10^4 and 10^6 $TCID_{50}$/ml of nasal secretion in infants (Hall et al. 1975, 1976; Hall 1977). The titer deceases over time, and most patients recover within 1–2 weeks post-infection. Some infants continue to shed virus for 3 weeks after hospitalization, and disease severity may correlate with the duration of virus shedding (Hall et al. 1976). Approximately 60 percent of primary RSV infections are confined to the upper airways; however, during a period of 2–5 days, infection may progress to lower respiratory tract involvement. The mechanism for viral spread to the lower airways is not known, but may occur by epithelial ciliary action, or through aspirated secretions; however, RSV can spread cell to cell without emerging into the extracellular space. Severe disease generally involves lower respiratory tract infection, and physical examination may reveal otitis media, rales, and diffuse wheezing (Anderson 2000; Greenberg 2001; Panitch 2001; West 2002). The severity of the disease is directly related to the age of the patient. Infants under 6 months of age are the most severely affected, likely due to smaller, more easily obstructed airways and decreased ability to clear secretions. Illness may be particularly severe in premature infants, and in those with congenital cardiac or pulmonary disease (Krilov 2001; Shay et al. 2001; Aujard and Fauroux 2002; Law et al. 2002). Apnea occurs in approximately 20 percent of infants hospitalized with RSV bronchiolitis (Bruhn et al. 1977; Church et al. 1984). The frequency of apnea increases significantly in premature infants, occurring early in the course of RSV disease. Apnea rarely lasts more than a few days, but about 10 percent of these patients require intubation and mechanical ventilation. The mechanisms that contribute to apnea are not well understood, but recent studies in mice suggest a link between RSV G glycoprotein binding to the CX3C chemokine receptor, CX3CR1, induction of the tachykinin, substance P, and subsequent depression of respiratory rates (Tripp et al. 2003).

In adults, the most common symptoms of RSV infection are those of the common cold which include rhinorrhea, sore throat, and cough. In the elderly, RSV may cause significant lower respiratory tract disease including pneumonia and some mortality (Han et al. 1999; Falsey and Walsh 2000). RSV is also a significant cause of morbidity and mortality in patients undergoing bone marrow or organ transplantation (Wendt 1997; Billings et al. 2002; Ison and Hayden 2002; Small et al. 2002). RSV reinfections occur frequently and are often associated with illness (Henderson et al. 1979); however, the cumulative effect of multiple re-infections appears to temper subsequent disease, an effect that is likely related to developing immunity. The severe disease observed in immune compromised or suppressed patients, and in experimental animal models, indicates that cell-mediated immunity is an important mechanism of host defense against RSV (Fishaut et al. 1980; Graham et al. 1991).

DIAGNOSIS

Parainfluenza viruses and bacteria that can cause respiratory tract illness complicate the diagnosis of RSV infection in infants less than 6 months of age. Presumptive diagnosis of RSV infection in young children can be made on the epidemiologic setting that includes seasonality and community outbreaks of diagnosed RSV infection. The specific diagnosis is established by isolation of RSV from respiratory secretions, including sputum, throat swabs, or nasopharyngeal washes. Infections in older children and adults cannot be differentiated with certainty from those caused by other respiratory viruses. Examining increases in titer of neutralizing serum antibodies may aid diagnosis of RSV infection in older children and adults; however, serological tests depend on fourfold or greater rises in complement-fixing or neutralizing antibody titers for useful diagnosis, and are less sensitive in children under 4 months of age (Richardson et al. 1978; Brandenburg et al. 1997). In addition, serological diagnosis requires comparison of acute and convalescent phase serum specimens, and is therefore not useful during acute illness. Virus obtained through sputum, throat swabs, or nasopharyngeal washes

can be detected by tissue culture and immunostaining, by immunofluorescence, ELISA, or other techniques including detection of nucleic acid by reverse-transcriptase polymerase chain reaction (RT-PCR) (Paton et al. 1992; Falsey et al. 2002; Coiras et al. 2003).

MANAGEMENT AND TREATMENT

Infection with RSV is usually self-limiting, but may require supportive care including administration of oxygen, intravenous hydration, and intubation. Bronchodilators may be used if wheezing is part of the clinical presentation. Inhaled salbutamol may have some beneficial effects as a bronchodilator in moderate bronchiolitis (Kellner et al. 1996); however, racemic epinephrine appears to substantially improve airway resistance and clinical scores (Sanchez et al. 1993). Steroids, which are effective for alleviating wheezing in asthma, appear to have limited beneficial effect in RSV bronchiolitis (Roosevelt et al. 1996; De Boeck et al. 1997). The only specific antiviral drug licensed for RSV treatment is ribavirin. Ribavirin, a guanosine analogue, has antiviral activity in tissue culture and cotton rats (Hruska et al. 1982), but its effectiveness in decreasing the severity of clinical symptoms in young patients is questionable. When delivered as a continuous aerosol, ribavirin appears to have modest effects on the duration of virus shedding (Hall et al. 1983, 1985; Taber et al. 1983), but its use is now recommended only for immune compromised patients and those who are severely ill.

To prevent or reduce severe lower respiratory tract infection by RSV, prophylactic or therapeutic treatment with purified human intravenous immunoglobulin (Ig)G, RSV-IVIG (RespiGAM™), or humanized anti-F glycoprotein monoclonal antibody, palivizumab (Synagis™) has been investigated. RespiGAM™, given parentally (2 g/kg/day) to infants and young children with RSV bronchiolitis or pneumonia was shown to reduce the amount of RSV shed; however, the mean duration of hospitalization was not affected by treatment (Hemming et al. 1987). However, RespiGAM™ given monthly by intravenous infusion has been shown to reduce RSV-associated hospitalization by approximately 40 percent, and significantly reduced the severity of symptoms in high-risk patients who acquired RSV (Groothuis et al. 1993; Simoes et al. 1998). Synagis™ exhibits neutralizing activity and, like RespiGAM™, if administered monthly (15 mg/kg) has been shown to reduce RSV-associated hospitalization by approximately 55 percent (CDC 1998). Since monthly treatment with RespiGAM™ or Synagis™ appears necessary to reduce RSV-associated hospitalization, other agents that may be used in conjunction with these treatments to ameliorate RSV-associated disease are being investigated. Recent studies examining prophylactic or therapeutic treatment of RSV-infected BALB/c mice with neutralizing anti-F glycoprotein and anti-substance P monoclonal antibodies have shown that prophylactic or therapeutic treatment with anti-substance P antibodies promptly reduces pulmonary inflammation, while anti-F antibody treatment reduces the virus load (Haynes et al. 2002), suggesting that combined antiviral and anti-inflammatory (substance P) antibody treatment may be effective in treating RSV disease in humans.

Small molecule inhibitors of RSV infection or replication have received some attention, particularly drugs that target the fusion process. Some peptide fusion inhibitors have been designed to interfere with heptad repeat domains in the F glycoprotein and disrupt their interaction, required during conformational rearrangement of the F glycoprotein that occurs in the later stages of the fusion process (Shigeta 2000; De Clercq 2002). A small-molecule inhibitor of RSV fusion is a peptide comprising amino acids 77–95 of RhoA (Pastey et al. 1999, 2000). RhoA is a small GTP-binding protein that has been shown to mediate virus-induced syncytium formation (Pastey et al. 1999, 2000; Gower et al. 2001). In studies of mice, in vivo administration of the RhoA peptide was shown to be effective in decreasing RSV titers if given at the time of infection; however, treatment at 4 days post-RSV infection was less effective at reducing viral titers, and did not alter the degree of pathogenesis when compared to mice treated with phosphate-buffered saline (PBS) alone. A similar picture has emerged for a variety of other small-molecule inhibitors that appear to be effective at inhibiting RSV infection in vitro, but their solubility, bioavailability, and effectiveness in vivo remains to be elucidated.

VACCINE DEVELOPMENT

RSV infection may cause severe lower respiratory tract infections early and late in life, and repeat infections with the same, or different strains of RSV are common. These indications suggest a lack of durable immunity. For an RSV vaccine to be effective, it must confer protection better than that associated with natural RSV infection, and for infants, must do so in the first weeks of life. These obstacles may require different vaccines and vaccination strategies. Early attempts to develop a safe and effective RSV vaccine failed. A formalin-inactivated, alum-precipitated RSV (FI-RSV) vaccine preparation that was administered intramuscularly to young children in an RSV vaccine trial in 1969 was not protective and when vaccinated children were subsequently exposed to natural RSV infection, they developed enhanced pulmonary disease with several fatalities (Kapikian et al. 1969; Kim et al. 1969). The enhanced pulmonary disease has been attributed to altered immunity to the FI-RSV vaccine; however, the exact mechanism is not completely understood. Recent studies from our laboratory examining FI-RSV-enhanced disease have shown that the absence of the G glycoprotein or G glycoprotein CX3C motif during FI-RSV

vaccination or RSV challenge of FI-RSV-vaccinated mice, or treatment with anti-substance P or anti-CX3CR1 antibodies, reduces or eliminates enhanced pulmonary disease, modifies T-cell receptor (TCR) Vβ usage, and alters CC and CXC chemokine expression by pulmonary leukocytes (Haynes et al. 2003). These results suggest that the G glycoprotein, and in particular the G glycoprotein CX3C motif, may be key in the enhanced inflammatory response to FI-RSV vaccination, possibly through the induction of substance P.

The disastrous results associated with FI-RSV vaccination were unexpected since natural RSV infection induces a protective although incomplete immune response to infection. Thus, RSV vaccine efforts have been focused on development of attenuated vaccines. Development of temperature-sensitive or cold-passaged vaccine candidates and subsequent examination in adults indicated some effectiveness, but the attenuated vaccine candidates proved too unstable or virulent in children, and often reverted back to wild-type virus (Pringle et al. 1993; Karron et al. 1997b; Crowe et al. 1999; Gonzalez et al. 2000; Wright et al. 2000; Crowe 2001b; Krilov 2001). Clinical trials with candidate subunit vaccines have been explored. A series of trial studies examined the effectiveness of a full-length F glycoprotein vaccine (PFP) purified from RSV-infected cells (Paradiso et al. 1994; Tristram et al. 1994; Falsey and Walsh 1996; Dudas and Karron 1998; Groothuis et al. 1998; Gonzalez et al. 2000). An indication from the studies suggested that PFP was safe, but was only moderately immunogenic. A different series of clinical trials examined the effectiveness of a recombinant protein (BBG2Na) consisting of amino acids 13–230 of the RSV G glycoprotein fused to the C-terminal domain of streptococcal G protein (Libon et al. 1999; Kneyber et al. 2000; Goetsch et al. 2001; Power et al. 2001). These studies showed that BBG2Na was moderately immunogenic in adults, but did not offer significant protection. Investigation of other candidate subunit vaccines is proceeding, including development of DNA vaccines. Effective subunit vaccines may be useful in RSV seropositive groups at high risk, or immunizing pregnant women to enhance protection of their newborns (Crowe 2001b; Kneyber and Kimpen 2002).

Reverse genetics employing full-length RSV complementary DNA to produce transcripts of infectious RNA offers many advantages in RSV vaccine design. The entire RSV genome can be precisely manipulated to offer optimal immunogenicity and attenuation. Reverse genetics has been used to delete nonessential genes in the RSV genome with the hope of restricting replication in vivo, but not in vitro, thus permitting efficient vaccine production (Collins and Murphy 2002). Studies with recombinant RSV have shown that five RSV genes, *NS1*, *NS2*, *SH*, *G*, and *M1*/*M2* can be deleted or silenced individually, or in certain combinations, without dramatically altering viral replication in vitro (Collins and Murphy 2002). Attenuation studies in chimpanzees have shown that recombinant RSV lacking the *SH* gene is least attenuated, whereas recombinant RSV lacking the *M2* gene is most attenuated (Teng et al. 2000). Attenuation of RSV vaccine candidates may also be achieved by taking advantage of the natural host restriction of RSV (Buchholz et al. 2000). Bovine RSV is over-attenuated and poorly immunogenic in seronegative chimpanzees (Buchholz et al. 2000); however, bovine RSV replication and immunogenicity can be improved by replacing the *F* and *G* genes with human RSV *F* and *G* genes (Schmidt et al. 2002). Strategies that focus on systemic replacement or deletion of RSV or bovine RSV genes in chimeric recombinant RSV are anticipated to yield useful vaccine candidates. Another advantage of recombinant RSV is the ability to alter or silence regions that may be associated with altered immunity or pathogenesis. For example, the RSV G glycoprotein has been shown to contain a CX3C chemokine motif (amino acids 182–186) that can bind to the CX3C chemokine receptor, CX3CR1, and mediate leukocyte chemotaxis in a fashion similar to fractalkine (Tripp et al. 2001). Recent studies from this laboratory suggest that the G glycoprotein, and in particular the G glycoprotein CX3C motif, is key in the enhanced inflammatory response to FI-RSV vaccination, possibly through the induction of substance P (Haynes et al. 2003). Therefore, ablation of this region may enhance the efficacy of recombinant RSV vaccine candidates.

IMMUNOLOGY AND HOST FACTORS

Innate immunity is the first line of defense providing early resistance to virus infection through a variety of host components. For respiratory viruses, innate immunity may be first mediated by mucus secreted by the membranes lining respiratory tract. Mucus acts as a protective barrier to block the adherence of respiratory viruses to epithelial cells, and ciliary movement, coughing, and sneezing removes virus particles trapped within mucus. In addition, a family of proteins called collectins has the ability to recognize foreign carbohydrate patterns, interact with phagocytic cells, and induce opsinization of the particles containing foreign carbohydrates (Lu et al. 2002; Shepherd 2002). The collectins, especially mannose-binding protein, and alveolar surfactant molecules SP-A and SP-D, are important agents in innate immunity (McCormack and Whitsett 2002), and SP-A is important in innate immunity to RSV infection (LeVine et al. 1999). SP-A binds to the RSV F glycoprotein, but not to the G glycoprotein, and neutralizes RSV infection in a calcium-dependent fashion (Ghildyal et al. 1999). If viruses penetrate to the respiratory epithelium, destructive soluble factors including enzymes and cytokines are released by the infected cells, or by resident phagocytic cells. Infected epithelial cells and alveolar macrophages release chemokines and pro-inflammatory

cytokines that include tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and CC and CXC chemokines (Becker et al. 1991; Arnold et al. 1995; Saito et al. 1997; Zhang et al. 2001). Expression of these soluble mediators of immunity contributes to airway inflammation, immune cell trafficking, bronchial hyperresponsiveness, and exacerbates mucus production. The CC chemokines, such as the macrophage inflammatory proteins (MIP), regulation on activation, normal T-cell expressed and secreted (RANTES), and eotaxin recruit a variety of granular cells to the site of infection or inflammation (Saito et al. 1997; Olszewska-Pazdrak et al. 1998). RANTES, eotaxin, and MIP-1α are potent chemokines for eosinophils and increased levels, or mRNA, in airway secretions or peripheral blood leukocytes have been found in infants with RSV bronchiolitis (Hornsleth et al. 2001; Noah et al. 2002; Smyth et al. 2002; Tripp et al. 2002). The CXC chemokine IL-8, a potent chemoattractant of neutrophils, has been found in secretions of RSV-infected children (Sheeran et al. 1999; Noah et al. 2002; Smyth et al. 2002), and experimentally infected adults (Noah and Becker 2000). Eosinophils are part of the nonspecific innate immune response and participate in inflammatory reactions. They contain cationic molecules that are useful for destroying infectious agents, especially helminths. Neutrophils are nondividing, short-lived cells containing primary azurophilic granules and secondary granules. The primary azurophilic granules contain myeloperoxidase together with other antimicrobial agents including defensins and cathepsin G. The secondary granules contain lactoferrin and other enzymes. Alveolar macrophages and dendritic cells are long-lived, tissue-resident cells that have an important role in phagocytosis and antigen presentation to the T-cell compartment. In this role they recognize and remove virally infected epithelial cells and debris, and present viral antigen to T cells, thereby initiating the adaptive immune response. Contact with virally infected cells, and the process of phagocytosis, activates macrophages and dendritic cells to secrete soluble mediators including interferons, lysozyme, and other factors that inhibit viral replication and upregulate the inflammatory response (Krilov et al. 1987; Lewis et al. 1989; Tsutsumi et al. 1996). Recognition of pathogens by phagocytic cells is achieved by pattern recognition receptors encoded in the germline (Hallman et al. 2001; Armant and Fenton 2002). The pattern recognition receptors (PRR) have broad specificity and recognize pathogen-associated molecular patterns (PAMP) that differ from pathogen to pathogen, but are not found in the host (Underhill and Ozinsky 2002). Thus, PAMPs are perceived as molecular signatures of infection by PRRs, resulting in activation of innate and adaptive immune responses (Barton and Medzhitov 2002; Sabroe et al. 2002). There are two major groups of PRRs – those that are secreted in the blood and lymph, and those on the surface of cells. Secreted PRRs include components of the complement fixation pathway, e.g. C2, C3, and C4, and cell-surface PRRs include the Toll-like receptors (TLR) (Akira et al. 2001). There are 10 known TLRs, and three other candidate molecules, that specialize in recognition of different PAMPs. For example, TLR2 generally recognizes peptidoglycan of gram-positive bacteria, while TLR4 generally recognizes lipopolysaccharide in the outer membrane of gram-negative bacteria (Akira et al. 2001). Interestingly, the RSV F glycoprotein has been shown to stimulate innate immunity through activation of the shared components of CD14 and TLR4 (Kurt-Jones et al. 2000), and TLR4 appears to be important in the innate immune response to RSV infection (Haynes et al. 2001). TLR4-deficient mice challenged with RSV exhibit impaired natural killer (NK) cell and $CD14^{+}$-cell pulmonary trafficking, deficient NK-cell function, impaired IL-12 expression and impaired virus clearance. Activation of TLRs on phagocytic cells can trigger adaptive immune responses by up-regulation of T-cell co-stimulatory molecules and cytokines required for T-cell activation. In this role, phagocytic cells, particularly dendritic cells, have a key role in coupling innate and adaptive immune responses.

The adaptive immune response includes humoral and cell-mediated immunity. Antibodies located in the serum, lymphatics, and in secretory pathways mediate humoral immunity. There are five different classes of antibodies or immunoglobulins (Ig) known as IgD, IgA, IgM, IgE, and IgG, four subclasses of IgG, and two subclasses of IgA. Soluble antibodies and those on the surface of B cells recognize antigens in the native form. Resistance to respiratory virus infection in the upper airways is mediated by local, secretory IgA, and is transitory. More durable humoral resistance to infection seems to be associated with IgM, and especially IgG antibodies. In the newborn, passively acquired maternal IgG antibodies provide some protection from infection, a factor that may contribute to the reason why children under 8 weeks of age are rarely infected with RSV (Crowe 2001a). Maternal antibody levels diminish during the first 6 months of life (Brandenburg et al. 1997), leaving infants unprotected against RSV infection; however, serum and secretory antibodies appear within days of a primary RSV infection (McIntosh et al. 1978; Welliver et al. 1980). The serum and secretory antibody titers produced in infants are significantly lower than those of older subjects (Murphy et al. 1986a; de Sierra et al. 1993), and wane several months after primary infection of infants (Welliver et al. 1980). The diminished serum and neutralizing antibody response has been attributed to suppressive effects of maternally transferred antibodies (Crowe 2001a; Wright et al. 2002); however, immunological immaturity may also be important. Post-infection, serum antibody levels generally wane; however, higher levels are maintained over a longer period after repeated RSV infection (Murphy et al. 1986b; Wagner et al. 1989). As the infant matures,

the presence of neutralizing IgA in respiratory secretions produced by local mucosal B cells may become more important. Studies of experimentally induced RSV disease in healthy adult volunteers indicate that the presence of nasal IgA neutralizing antibody correlates more closely with protection than does the presence of serum antibody (Mills et al. 1971). Complement is an important component of innate immunity and the humoral response to RSV infection. RSV-infected epithelial cells can activate complement by both the classical and alternative complement pathways (Edwards et al. 1986; Smith et al. 1981), and complement is important for enhanced antibody-mediated virus neutralization.

Cell-mediated immunity to respiratory virus infection involves $CD8^+$ CTLs, $CD4^+$ helper T cells, and innate immune cells, e.g. NK cells. The role of cell-mediated immunity in virus clearance is difficult to assess in humans; however, studies in patients with deficiencies in cellular immunity have shown that cellular immunity has a pivotal role in protection from severe RSV disease and limiting virus shedding (Fishaut et al. 1980; Chandwani et al. 1990). In vitro studies of human peripheral blood mononuclear cells (PBMC) have shown that NK cells and $CD4^+$ and $CD8^+$ T cells have potent antiviral responses. Exposure of adult PBMC to RSV immediately up-regulates NK activity, a feature dependent upon IL-15 induction (Fawaz et al. 1999). Infants hospitalized because of RSV infection have increased numbers of $CD16^+/CD56^+$ NK cells, and express T-helper-1 (Th1) and T-helper-2 (Th2) cytokines, and enhanced CC chemokine messenger RNA (Tripp et al. 2002). Antibody-dependent cellular cytotoxicity (ADCC), a feature associated with NK cells has been detected in human PBMC from RSV-infected individuals using serum antibodies and antibodies from nasopharyngeal secretions (Scott et al. 1977; Meguro et al. 1979; Cranage et al. 1981; Kaul et al. 1982). RSV-specific CTL responses in PBMC from infants with acute RSV infection can be detected rapidly after infection (Isaacs et al. 1987; Chiba et al. 1989). The response is age-dependent, as >65 percent of infants 6–24 months of age, and approximately 35 percent of infants under 5 months of age exhibit RSV-specific cellular cytotoxicity. Examination of the CTL repertoire of adults to RSV proteins showed that N, SH, F, M, M2 and NS2 proteins are targeted by CTLs with no predilection toward a particular major histocompatibility complex (MHC) phenotype (Bangham et al. 1986; Cherrie et al. 1992). Most data concerning RSV-specific $CD4^+$ or $CD8^+$ T-cell responses are derived from experimental animal studies, as it is difficult to assess responses in the lungs of infected patients, particularly infants and young children. Examining alterations of $CD4^+$ or $CD8^+$ T-cell responses in PBMC may be useful as a correlate of immune status; however, studies that examine bronchial lavage cells from infants infected with RSV may provide better information. For example, a study that examined bronchial lavage cells from infants infected with RSV identified large numbers of neutrophils in the upper airway (93 percent) and lower airway (76 percent), but few $CD4^+$ and $CD8^+$ T cells with median $CD4^+/CD8^+$ ratios being 22:1 in the upper airway, and 15:1 for the lower and upper airways (Everard et al. 1994). These results suggest that neutrophils probably have a major role in immunity and possibly RSV disease, and counter the hypothesis that excessive lymphocyte cytotoxicity is associated with RSV bronchiolitis.

The role of $CD4^+$ and $CD8^+$ T lymphocytes in terminating RSV infection, causing disease, and protecting from re-infection have been investigated using experimental animal models, particularly BALB/c mice. In BALB/c mice, the M2, F, and N proteins are the major targets for CTLs (Nicholas et al. 1990; Openshaw et al. 1990; Openshaw 1995b). Both $CD4^+$ and $CD8^+$ T-cell subsets have been shown to be involved in terminating RSV replication, as antibody depletion of both T-lymphocyte subsets markedly prolongs RSV replication (Graham et al. 1991). In addition, both $CD4^+$ and $CD8^+$ T cells have been shown to contribute to immunity and disease pathogenesis, although $CD8^+$ T cells appeared to have a dominant role in controlling RSV disease (Varga and Braciale 2002). Investigation of the RSV proteins associated with host resistance has shown that sensitization with F or G glycoproteins induces almost complete resistance to RSV challenge despite depletion of both $CD4^+$ and $CD8^+$ T cells prior to challenge, indicating that host resistance is mediated by the humoral response to F or G glycoproteins (Connors et al. 1992a). However, host resistance may also be mediated by sensitization with the M2 protein, and depletion of $CD8^+$ T cells abrogates M2-associated protection, while depletion of $CD4^+$ T cells has an intermediate effect (Connors et al. 1992a). Although both $CD4^+$ and $CD8^+$ T cells contribute to disease pathogenesis, passive transfer of RSV-specific $CD4^+$ T cells into RSV-immune mice mediates more severe immunopathology and pulmonary eosinophilia compared to transfer of $CD8^+$ T cells (Alwan et al. 1992). Distinct types of pulmonary disease are mediated by functionally different T-cell subsets reactive to different RSV proteins. Adoptive transfer of T-cell lines recognizing G glycoprotein into RSV-infected mice induces severe illness, characterized by pulmonary eosinophilia and hemorrhaging, compared to transfer of M2-specific, or F-glycoprotein-specific T-cell lines (Alwan et al. 1994). It is likely that Th2-type cytokine expression by the G-glycoprotein-specific T-cell lines contributed to the disease severity, since these cells were $CD4^+$ and expressed IL-4 and IL-5, whereas M2-specific or F-glycoprotein-specific T-cell lines were $CD4^+$ or $CD8^+$ and expressed Th1-type cytokines. The relationship of Th1- to Th2-type cytokine expression appears important in the outcome of RSV immunity or disease pathogenesis (Lemanske 1998; Welliver 2000;

Graham et al. 2002). Primary RSV infection induces a mixed Th1- and Th2-type cytokine response with limited disease pathogenesis (Openshaw et al. 2002), and from animal studies, it appears that early IFN-γ expression is key in controlling the Th1/Th2 cytokine balance (Spender et al. 1998; Boelen et al. 2002; Openshaw et al. 2002). Absence of IFN-γ early after primary infection or during subsequent RSV infections has been shown to result in a predominant Th2-type cytokine response and increased disease severity (Hussell et al. 1997; Boelen et al. 2002; Durbin et al. 2002). The relationship between phenotypes associated with cell activation and cytokine expression was investigated in RSV-infected BALB/c mice (Tripp et al. 2000c). In this study, $CD54^+$ and $CD102^+$ lymphocytes expressed higher levels of IL-2, IL-4, IL-5, and IFN-γ compared to $CD44^+$, $CD49d^+$ or $CD62L^{lo}$ lymphocytes, and DNA analysis of lymphocytes expressing IL-2 or IFN-γ revealed higher G2/M levels compared to lymphocytes expressing IL-4 or IL-5, suggesting greater activation of Th1-type lymphocytes in the lungs in response to RSV infection.

IMMUNOPATHOLOGY AND THE RESPONSE TO RSV PROTEINS

Immune-mediated pathology was first indicated in clinical trials of FI-RSV vaccine parentally administered to infants who subsequently developed enhanced pulmonary disease upon natural RSV infection (Chin et al. 1969; Fulginiti et al. 1969; Kapikian et al. 1969; Kim et al. 1969). The vaccine failure is generally attributed to poor protection, and an unbalanced immune response to vaccination (Murphy et al. 1986c). The poor protection may be linked with a weak neutralizing secretory IgA response associated with parental vaccination, or an unbalanced humoral response to vaccination. FI-RSV vaccinees developed high titers of serum antibodies to the F glycoprotein, but these antibodies did not exhibit effective neutralizing activity (Murphy et al. 1986c). In addition, vaccinees had low levels of serum antibodies to the G glycoprotein, suggesting that G glycoprotein immunogenicity was altered by formalin-inactivation. Lung histopathology of FI-RSV vaccinees who died revealed extensive infiltration of neutrophils and eosinophils that is not associated with primary RSV infection in mice (Chin et al. 1969; Graham et al. 1993; Waris et al. 1996). Unfortunately, investigation of the $CD4^+$ and $CD8^+$ T-cell response was not available during this vaccine trial, thus cell-mediated responses to FI-RSV vaccination have been inferred from experimental animal studies.

FI-RSV vaccine studies in experimental animal models, particularly mice and cotton rats, suggest that enhanced pulmonary disease is primarily mediated by $CD4^+$ T cells and Th2-type cytokines, as depletion of $CD4^+$ T cells or IL-4 or IL-10 abrogates enhanced disease (Connors et al. 1992b, 1994). The RSV G glycoprotein appears to sensitize for enhanced disease, skewed Th2-type cytokine and chemokine responses, and to enhance pulmonary expression of substance P, a neurogenic mediator of inflammation (Johnson et al. 1998; Johnson and Graham 1999; Graham et al. 2000; Tripp et al. 1999, 2000a, b, d). G glycoprotein sensitization for enhanced pulmonary disease has been shown to be associated with T-cell epitopes on the G glycoprotein comprising amino acids 185–193 (Srikiatkhachorn et al. 1999), 184–198 (Tebbey et al. 1998), or 193–202 (Sparer et al. 1998); however, the response mediated by the G glycoprotein may not be epitope-specific, since the form and site of administration of G glycoprotein available for antigen processing and presentation has been shown to be an important factor sensitizing for enhanced pulmonary disease (Bembridge et al. 1998). G-glycoprotein-associated Th2-type responses including high IL-4, IL-5, and IL-13 cytokine expression by infiltrating leukocytes, pulmonary eosinophilia, and extensive inflammation upon live RSV challenge (Openshaw 1995a; Graham et al. 2000; Openshaw et al. 2001). The T-cell repertoire is also altered by G-glycoprotein sensitization, characterized by constrained TCR $V\beta14^+$ usage by $CD4^+$ T cells responding to RSV challenge, and in vivo elimination of TCR $V\beta14^+$ cells has been shown to abolish Th2-type pulmonary disease (Varga et al. 2001). A recent study from our laboratory indicates that FI-RSV-enhanced disease is associated with G-glycoprotein expression, and in particular the G-glycoprotein CX3C motif, and that pulmonary substance P expression contributes to the enhanced inflammatory response associated with FI-RSV vaccination (Haynes et al. 2003). Interestingly, G-glycoprotein-specific cytotoxic T-cell responses are not readily detected in humans or mice; however, mice sensitized to G glycoprotein generate Th1 and Th2 $CD4^+$ T-cell responses (Hancock et al. 1996). By contrast, mice sensitized to RSV F glycoprotein induce potent cytotoxic T-cell responses, and Th1 $CD4^+$ T-cell responses (Alwan and Openshaw 1993).

In addition to sensitizing for Th2-type $CD4^+$ T-cell responses and altering the T-cell repertoire, the G glycoprotein has been associated with other immune modulatory features. BALB/c mice infected with wild-type RSV have markedly reduced pulmonary trafficking of neutrophils and NK cells, increased pulmonary substance P expression, and reduced CC and CXC chemokine mRNA expression by bronchoalveolar lavage (BAL) cells compared to mice infected with an RSV mutant lacking *G* and *SH* genes (Tripp et al. 1999, 2000a, 2000d). It is possible that skewed Th2-type $CD4^+$ T-cell responses associated with G glycoprotein expression are affected by reduced CC and CXC chemokine expression. MIPs and inflammatory protein (IP)-10 are chemotactic for cells bearing the chemokine receptors CCR1, CCR5, and CXCR3 (Kabashima et al. 2001; Luther and Cyster 2001; Sauty et al. 2001). These chemokine receptors are preferentially expressed on

Th1 cells (Loetscher et al. 1998; Odum et al. 1999; Syrbe et al. 1999), thus decreased expression of MIP and IP-10 may impair recruitment of Th1-type cells favoring Th2-type cell recruitment. The G glycoprotein can also mimic the activities of the only known CX3C chemokine, fractalkine (Tripp et al. 2001). G glycoprotein contains a CX3C chemokine motif that interacts with the CX3C chemokine receptor, CX3CR1. The structural similarity between the G glycoprotein CX3C motif and the fractalkine CX3C motif is shown in Figure 5. G glycoprotein CX3C with CX3CR1 can compete with fractalkine for binding to CX3CR1, induce leukocyte chemotaxis in a fashion similar to fractalkine, and facilitate virus infection. The results suggest that G glycoprotein interaction with CX3CR1 may be important to the biology of RSV infection and possibly disease pathogenesis.

The form of the G glycoprotein appears to be important in immunity and disease pathogenesis. The secreted form of the G glycoprotein is more potent in inducing pulmonary eosinophilia than the membrane-anchored form (Johnson et al. 1998), and sensitization with the secreted form is associated with increased expression of IL-5, IL-13, and eotaxin (Johnson and Graham 1999). Pulmonary eosinophilia mediated by soluble G-glycoprotein sensitization does not require IL-4 expression, but does require IL-5, and if both IL-4 and IL-13 are inhibited at the time of immunization, the development of pulmonary eosinophilia upon RSV challenge is reduced (Johnson and Graham 1999). By contrast, presentation of RSV F in a soluble form does not sensitize for pulmonary eosinophilia, but does induce IL-4 and IL-5 (Bembridge et al. 1999). RSV-specific $CD8^+$ T cells and IL-12-activated NK cells appear to regulate development of G-glycoprotein-mediated enhanced disease. If RSV-specific $CD8^+$ T cells are present at the time of G-glycoprotein sensitization, the development of pulmonary eosinophilia and enhanced disease is reduced (Alwan et al. 1994; Hussell et al. 1997). Similarly, IL-12-activated NK cells can reduce pulmonary eosinophilia mediated by G-glycoprotein sensitization, but do not enhance the severity of illness in the absence of $CD8^+$ T cells (Hussell and Openshaw 2000).

EPIDEMIOLOGY

Viral respiratory tract infections can be severe during infancy, and viral bronchiolitis is the most common cause of infant and young children hospitalization in the developed world (Leader and Kohlhase 2002; Staat 2002). RSV is the primary cause of lower respiratory tract infections among infants and young children, accounting for approximately 70 percent of all cases of viral bronchiolitis (Glezen et al. 1986). During a 17-year study period in the USA, an estimated 1.65 million hospitalizations for bronchiolitis occurred among children younger than 5 years, and 57 percent of these hospitalizations occurred among children <6 months of age (Shay et al. 1999). RSV activity in the USA monitored by the National Respiratory and Enteric Virus Surveillance System at the Centers for Disease Control and Prevention (CDC) has shown that RSV outbreaks occur between October and April (Figure 1). Examining the number of bronchiolitis hospitalizations in 1994–1996 from November through April in the USA, it is estimated that 51 240–81 985 annual bronchiolitis hospitalizations among children younger than 1 year were related to RSV infection (Shay et al. 1999). In the USA, the risk of primary RSV infection in infants <12 months of age ranges from 50 to 70 percent, and approximately 30–40 percent of children in this age group develop lower respiratory tract illness (Parrott et al. 1973; Glezen et al. 1986). By age 2, most children have experienced at least one RSV infection (CDC 2000), and the risk for lower respiratory tract illness remains high with re-infection, and through the first 5

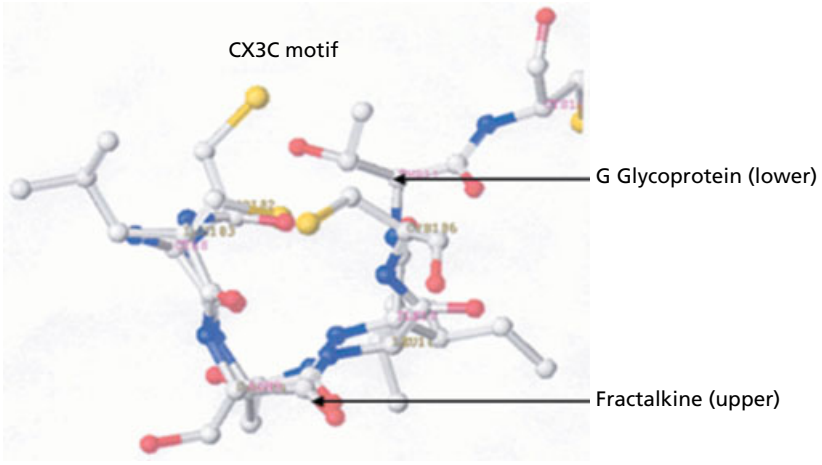

Figure 5 *Superimposed images of the CX3C motif of fractalkine and RSV G glycoprotein modeled using the molecular modeling program SYBYL. Forcefields were generated using SYBYL and charges calculated using the Gasteiger–Huckel and Kollman methods.*

years of life (Glezen et al. 1986; Wright et al. 1989). The risk of serious RSV infection is high in patients with underlying disorders of the cardiac, pulmonary, or immune system (Wendt 1997; Englund et al. 1999; Falsey and Walsh 2000; Whimbey and Ghosh 2000; Hall 2001; Aujard and Fauroux 2002; Greenberg 2002; Ison and Hayden 2002). Diagnostic studies using RT-PCR indicate that RSV may cause approximately 50 percent of all respiratory tract illness in adult patients. RSV is recognized as a major cause of serious disease in older adults and the elderly, and is associated with high morbidity and some mortality in older persons residing in nursing homes and in the community (Falsey 1998; Han et al. 1999). The relationship of serum antibody to RSV and risk of RSV infection suggests that older adults with low titers of serum neutralizing antibody may be at greater risk of developing symptomatic RSV infection than those who have high antibody titers (Falsey and Walsh 1998). Applying rates of RSV detection from prospective studies of lower respiratory tract illness in adults to national hospital discharge diagnosis and death certificate data, it is estimated that RSV causes 14 000–62 000 hospitalizations and 1 500–6 700 deaths each year in the USA in older adults and the elderly (Han et al. 1999). The impact of RSV strain variation on the epidemiology of RSV disease is not completely understood; however, RSV outbreaks appear to be community-based. The circulation patterns of RSV A and B strains appear genotypically distinct among communities with limited diversity between strains of the same genotype, but greater diversity in genotype in strains from different communities (Peret et al. 1998, 2000). Isolates of RSV strain A appear to be more common than strain B, and individual outbreaks of RSV infection may differ substantially in strain predominance; however, no substantial linkage has been made between strain variation, hospitalization, and disease severity. RSV is a major cause of nosocomial infections (Hall 1983), particularly for premature infants and the immune compromised (CDC 2002; Hall 2000; Jones et al. 2000; Heerens et al. 2002).

Nosocomial pneumonia is a common hospital-acquired infection in children, and is often fatal. Viruses, predominantly RSV, are the most common cause of pediatric nosocomial respiratory tract infections (Hall 1983; Mlinaric-Galinovic and Varda-Brkic 2000). Risk factors for nosocomial pneumonia include hospitalization during the RSV season, age, underlying chronic disease, admission to an intensive care unit, intubation, burns, surgery, immune suppression, and long hospitalization. The morbidity and cost associated with nosocomial RSV infection of infants is striking (Macartney et al. 2000). About 40 percent of nosocomial RSV infections are associated with lower respiratory tract disease in this young age group (Hall 1983). RSV appears to be nosocomially transmitted by fomites on the hands of hospital personnel (Hall et al. 1980). Medical staff infected with community-acquired or nosocomially acquired RSV are frequently unaware of RSV infection because infection may present as mild flulike illnesses. However, RSV infection may debilitate up to 40 percent of those infected, resulting in absence from work (Hall et al. 1978). The key to control of nosocomial transmission of RSV is compliance with guidelines for prevention and surveillance described by the CDC. Education of hospital personnel about the nature of RSV, active surveillance among patients, careful hand washing, and use of gloves and barriers are important in controlling infection (Snydman et al. 1988; Madge et al. 1992; Goldmann 2001); however, prevention through the use of masks and gowns has not been successful (Hall and Douglas 1981; Murphy et al. 1981).

The disease burden of hMPV is not well known. Recent identification of hMPV in the Netherlands was made from respiratory samples submitted for viral culture during the winter season associated with RSV infection (van den Hoogen et al. 2001). Approximately half the initial 28 samples were cultured from infants <1 year of age, and 96 percent were isolated from children <6 years of age. Seroprevalance studies of children aged 6–12 months indicated that approximately 25 percent had detectable antibodies to hMPV, and by age 5 all patients showed evidence of past exposure or infection. In Australia, three of 200 randomly chosen respiratory samples negative for known respiratory pathogens, were positive for hMPV on culture, RT-PCR, or both (Howe 2002). From Canada, 11 isolates from 10 patients aged 2 months to 87 years of age with acute respiratory tract illness during the winter season were identified as hMPV at the CDC (Peret et al. 2002). Two major groups or strains of hMPV were identified in Canadian isolates based upon sequence diversity. Similar to RSV, some of the hMPV isolates did not originate from a single outbreak, but were from unrelated cases of respiratory illness. The serological studies of hMPV infection in the Netherlands, and epidemiological features associated with studies at the CDC and in Australia, suggest that hMPV likely infects most children by age 5, co-circulates with RSV, and may cause repeated infections.

PERSPECTIVE

RSV disease remains a major public health problem, and limited prophylactic or therapeutic treatments are available. Significant progress is being made toward understanding the virus–host relationship related to immunity and disease pathogenesis, and these studies have led to several promising RSV vaccine candidates that are under clinical evaluation. The development of a safe and efficacious RSV vaccine remains a challenging task, as does preventing infection or controlling RSV infection in infants, the elderly, or immune-compromised patients. Success in these areas will likely require

different vaccine and treatment strategies. Our limited understandings of durable immunity to RSV, immune-modulating activities of viral proteins, enhanced disease mechanisms associated with formalin-inactivated RSV vaccines and mechanisms that contribute to immunity or disease complicate vaccine development. Development of successful prophylactic or therapeutic treatments to reduce virus load and inhibit inflammation remains a priority. Progress towards these goals is occurring through experimental animal studies; however, more rapid translation of the findings to humans is required. The discovery of hMPV and its clinical relationship to RSV emphasizes the need for a better understanding of the disease burden associated with hMPV infection, the relationship between RSV and hMPV immunity, and the associated disease pathogenesis.

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Paramyxoviruses of animals

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This chapter describes the clinical and pathological aspects of infections caused by viruses of the family *Paramyxoviridae*, subfamily *Paramyxovirinae* that occur in birds and mammals (Figure 1). The emphasis is on viruses that are important veterinary pathogens, as well as those that also cause severe disease when transmitted to humans.

MORBILLIVIRUSES

Within the family *Paramyxoviridae*, members of an antigenically closely related group of viruses, the genus *Morbillivirus* (Figure 2) are responsible for some of the most devastating viral diseases in domestic and wild animal populations (Barrett 2001). Generally each virus species causes severe clinical signs in only one order of mammals, but the full host range in terms of infection is unknown. They share many clinical and pathological characteristics; in particular they are all highly immunosuppressive (Heaney et al. 2002). Despite this, animals which recover from an infection develop strong cellular and humoral immune responses, which give subsequent life-long protection, and can also give cross-protection against infection with other morbilliviruses.

Rinderpest

Rinderpest, also known as cattle plague, can infect a variety of cloven hoofed animals, for example cattle, buffalo, yak, and antelope, where it generally causes an acute febrile illness 3–15 days after infection. The term 'cattle plague' is now usually used to denote a severe outbreak of this disease in nonendemic areas, whereas 'rinderpest' is the term used most often in endemic regions. The last outbreak of classical cattle plague with very high mortality and economic losses occurred in Pakistan in 1994–1995 (Rossiter et al. 1998). In its most severe form it has been characterized as the disease of the three Ds: discharge, diarrhea, and death. Historically, rinderpest has been a major problem in Europe and Asia, but it was eliminated from Europe by the beginning of the twentieth century using zoosanitary control measures. Sporadic introductions, which were quickly contained, occurred in Europe, the Americas, and Australia in the early twentieth century. This led to the establishment of the Office International des Epizooties (OIE), an international veterinary organization which acts as a World Health Organization for animal diseases. As the disease was being eliminated from Europe it was introduced into Africa in about 1887 and caused a major pandemic on that continent killing over 90 percent of the cattle and buffalo, resulting in great social and economic hardship (Barrett and Rossiter 1999; Mack 1970).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Virus enters by the respiratory route and replicates in the draining lymph nodes of the head and throat. White blood cells, lymphoblasts, and macrophages become infected and the virus spreads via the blood and lymph to other lymphatic tissues, and then to the lungs, mucous membranes, and gastrointestinal tract. Virus excretion begins 24–48 h before the onset of clinical signs and lasts for 7–10 days. Typically, the animal develops nasal and ocular congestion, which progresses to give profuse lacrimal and nasal discharges which can become mucopurulent. Necrotic lesions appear on all mucous

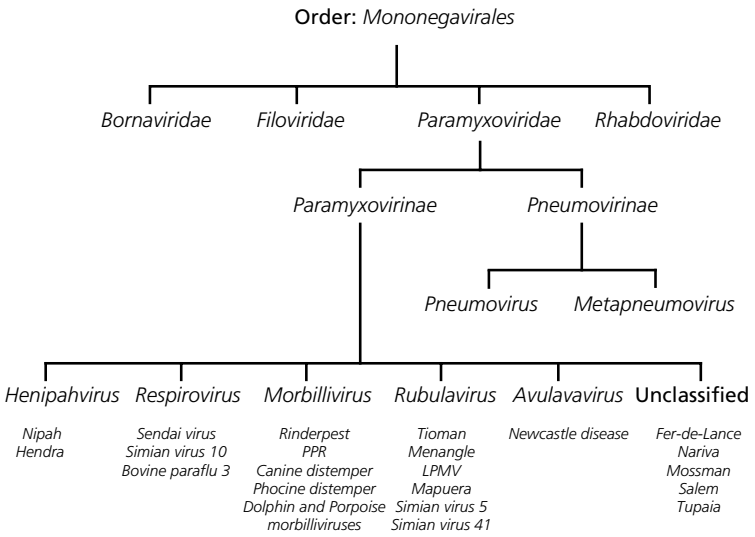

Figure 1 *Classification of the viruses within the order* Mononegavirales. *Family* Paramyxoviridae *is divided into two subfamilies, and there are five genera within the subfamily* Paramyxovirinae.

membranes, especially in the mouth where the lips, gums, buccal papillae, and tongue can be affected. Lesions on the mucous membranes and in the gastrointestinal tract are responsible for the severe manifestations of the disease. The animals become depressed, anorexic, and emaciated because the mouth lesions make eating painful. The intestinal pain causes them to arch their backs and the hair becomes rough and soiled by the mucopurulent discharge and watery, often bloody, diarrhea. Severe diarrhea results in dehydration and the animals have sunken eyes and reduced skin turgor. Pregnant animals usually abort and recovery is prolonged in survivors, but results in life-long protection from disease. Pathological findings include severe lymphodepletion, necrosis of the Peyer's patches and lesions in many parts of the digestive tract. The blood becomes concentrated and slow to circulate and the blood vessels in the longitudinal folds of the large intestine become distended and appear prominent, giving a marked striped appearance, the so-called zebra stripes. The lungs are generally minimally affected in rinderpest infections. Mortality can be as high as 70–90 percent in such severe cases, but there is considerable variation in the severity of symptoms, and even asymptomatic infections can occur, depending on the virulence of the virus strain involved and the host's innate resistance. Cattle of *Bos indicus* species are more resistant than *Bos taurus* species (Scott 1990). In endemic areas, mortalities of

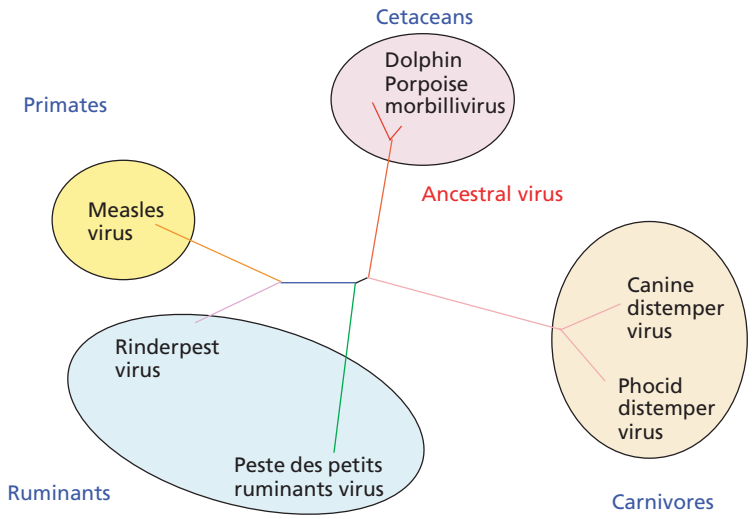

Figure 2 *Phylogenetic tree showing the relationships between the different morbilliviruses based on partial sequence of the* P *gene. A universal primer set was used to amplify a 429-base pair DNA fragment as described by Barrett et al. (1993). The tree was derived using the PHYLIP DNADIST and FITCH programmes (Felsenstein 1997). The branch lengths are proportional to the mutational differences between the viruses and the hypothetical common ancestor that existed at the nodes in the tree.*

30 percent, or less, are more normal. The pathogenesis of rinderpest infection has been extensively described (Brown and Torres 1994; Wamwayi et al. 1995; Wohlsein et al. 1993, 1995). The disease follows a similar course in wildlife species, except that skin lesions and corneal opacity are often seen, especially in buffalo and kudu, and this greatly increases the risk of predation (Kock et al. 1999).

EPIZOOTIOLOGY

The virus is present in all secretions and excretions from the infected animal but, since the virus is labile, it requires close contact for infection to occur, most often by infected air droplets. Contaminated fomites can also be a source of infection but nearly all outbreaks can be traced to the introduction of unvaccinated stock infected with the virus (Wamwayi et al. 1992; Rossiter 2001a; Rossiter et al. 1998). In endemic areas, rinderpest disease is most often seen in animals less than 2 years of age, as older animals are generally immune due to previous exposure. Maternal immunity protects the very young for up to 6 months. Until the mid-1990s, rinderpest was endemic in many parts of southern Asia, the Middle East, and Africa (Rweyemamu and Cheneau 1995); however, the intense vaccination campaigns carried out over the past decade have reduced the endemic region to a small area of eastern Africa on the borders of Somalia and Kenya. There are three distinct lineages of the virus, two African and a third found exclusively in Asia (Barrett et al., 1998) (Figure 3). The virus remaining in this last endemic focus is of African lineage 2 and appears to be maintained in cattle as a mild infection, but it becomes clinically apparent when it spreads, by means of trade and cattle movement, to areas where there are susceptible wildlife species such as buffalo, eland, and kudu (Kock et al. 1999). It has been suggested that rinderpest is the archetypal morbillivirus and it is likely that other morbilliviruses evolved form a ruminant virus of this type (Norrby et al. 1985).

CONTROL

All morbilliviruses are very sensitive to inactivation by heat, ultraviolet (UV) light, and chemicals, especially ones that disrupt the lipid envelope, so zoosanitary measures are very effective in controlling these infections. There is no effective treatment for rinderpest, but infection can easily be prevented by vaccination using a tissue culture attenuated vaccine developed by Plowright and Ferris (1962). There is only one serotype of rinderpest virus, as is the case for other morbillivirus species, and the attenuated vaccine is safe and gives life-long protection with a single shot. The main disadvantage of the vaccine is the inability to distinguish the vaccinated from naturally infected and recovered animals in serological assays. This would be a very useful characteristic to have at this final stage in the global eradication campaign and research is in progress to develop marker vaccines (Walsh et al. 2000).

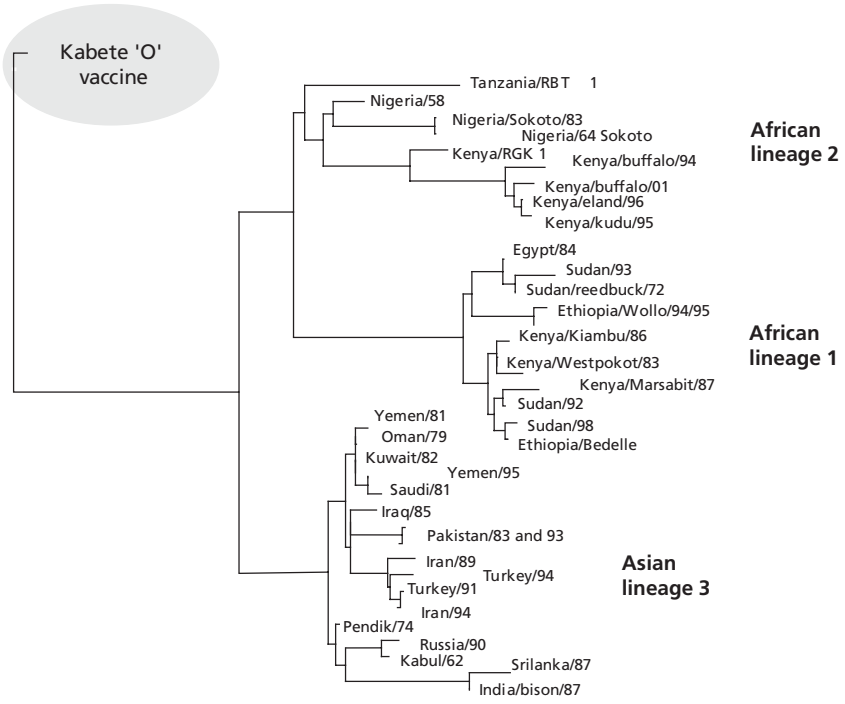

Figure 3 *The relationships between the different rinderpest viruses based on partial sequence data derived from the various fusion protein genes. A specific primer set was used to amplify a 372-base pair DNA fragment as described by Forsyth and Barrett (1995). The tree was derived as described in Figure 2.*

In nonendemic areas, a quarantine and slaughter policy has proved the most effective means of control. However, in endemic regions, or where cultural considerations prohibit slaughter, mass vaccination accompanied by restriction of animal movement and market closures are the control measures used. Because of the severe economic losses incurred as a result of rinderpest in Africa and Asia in the 1980s, a concerted international control and vaccination program was set up. This is now known as the Global Rinderpest Eradication Programme (GREP) with a target of eradication by 2010.

LABORATORY DIAGNOSIS

Rapid and accurate diagnosis of rinderpest has important implications for disease control programs as the clinical signs can be confused with those of other less serious diseases, such as bovine viral diarrhea and malignant catarrhal fever viruses. Classic cattle plague can be diagnosed clinically in nonendemic areas relatively easily but mild rinderpest, or disease in vaccinated populations, can only be diagnosed with certainty by laboratory testing. The virus can be detected by isolation in tissue culture cells (either primary bovine kidney cells or B95a cells), by antigen detection using various assays, for example agarose gel immunodiffusion, counter-immunoelectrophoresis, or preferably immunocapture enzyme-linked immunosorbent assay (ELISA) (Anderson et al. 1996; Libeau et al. 1994). A pen-side test, based on the use of virus-specific monoclonal antibodies, has been developed to detect virus antigen in samples taken in the field and this has greatly helped veterinarians investigating a potential outbreak to diagnose the disease and differentiate it from others with similar clinical manifestations (Wambura et al. 2000). Recently, reverse transcription polymerase chain reaction (RT-PCR) has been the method of choice for laboratory diagnosis as the resulting PCR product can be sequenced to give valuable information on the strain of virus involved (Forsyth and Barrett 1995; Barrett et al. 1998). Antibody responses can be detected in surviving animals, or following vaccination, by virus neutralization assays; however, for the large numbers of sera that are required to be tested for control programs a competitive ELISA has been developed which has proved highly robust and is routinely used in laboratories in countries where the virus remains a threat (Anderson and McKay 1994; Anderson et al. 1996). This assay is also being used for serosurveillance purposes to ensure that countries are free, and remain free, of disease.

Peste des petits ruminants virus

Peste des petits ruminants (PPR) is a disease mainly of sheep and goats, which is clinically very similar to rinderpest in large ruminants. Where it occurs it is considered to be one of the most economically important diseases in these species. Generally goats are more sensitive to PPR than sheep, but that can be strain-dependent. The disease was first seen in West Africa in the 1940s (Gargadennec and Lalanne 1942), but is now known to be widely distributed in sub-Saharan Africa. In the 1990s the Arabian peninsula, the Middle East, and most parts of the Indian subcontinent were swept by an epizootic of PPR where it has remained (Dhar et al. 2002; Ozkul et al. 2002).

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Like rinderpest, peste des petits ruminants virus (PPRV) infection is characterized by high fever, ocular and nasal discharges, necrosis in the mouth, and inflammation of the gastrointestinal tract leading to diarrhea and dehydration (Rossiter 2001b). However, unlike rinderpest in cattle, interstitial and suppurative pneumonia is commonly found in PPRV-infected animals and secondary bacterial infections (usually *Pasteurella* spp.) are often a complicating factor and so it can be easily confused with contagious caprine pleuropneumonia (CCPP). The pathological findings are very similar to those described for rinderpest infections, apart from the more marked lung involvement and severe bronchopneumonia. Giant cells are seen in the bronchioles and terminal alveoli, usually with intracytoplasmic and intranuclear inclusion bodies (Rossiter 2001b). Mortality rates in PPR epizootics can range from 10 to 90 percent and may vary with the host's innate resistance, body condition, age, complications resulting from secondary bacterial and parasitic infections, and perhaps, the virulence of the virus involved (Kitching 1988). However, little research has been carried out to establish these as facts.

EPIZOOTIOLOGY

The virus circulates in endemic areas in sheep and goats, but the full host range of this virus is unknown. There is no indirect transmission of the virus and no known wildlife reservoir; however, captive wildlife are known to be highly susceptible. Several species of wild antelope died as a result of infection in a zoo in the United Arab Emirates (Furley et al. 1987) and recently an epizootic killed many gazelle in a game park in Saudi Arabia (Abu Elzein et al. 2004). In West Africa, PPR is more prevalent in the more humid areas and shows a seasonal pattern. In the Middle East, the intensive trading of small ruminants for religious festivals spreads the virus. Due to the clinical and morphologic similarities of rinderpest and PPR, there was considerable confusion regarding which virus was circulating in large and small ruminants, especially in Asia, as these strains of rinderpest were known to infect sheep and goats and result in clinical disease. PPRV infection has been reported in buffalo in India and in cattle in West Africa (Anderson

and McKay 1994; Govindarajan et al. 1997). This can interfere with the take of the rinderpest vaccine and cause confusion in serological surveillance; however, if the GREP is successful, this will no longer be an issue. PPRV isolates have been grouped into four distinct lineages on the basis of fusion (F) protein gene sequence similarities and they reflect the geographical distribution of virus strains (Shaila et al. 1996; Dhar et al. 2002) (Figure 4).

CONTROL

Zoosanitary measures, as applied to rinderpest control, are effective but often difficult to implement because of the large numbers of animals involved and the nomadic nature of many small ruminant pastoralists. These are often amongst the poorest members of society and depend very heavily on small ruminants for economic survival. Vaccination of herds is the most effective means of controlling PPR and at first, because of the close antigenic relatedness, the rinderpest vaccine was used to protect animals against PPR, but a homologous vaccine has now been developed and is widely used to control the disease (Couacy-Hymann et al. 1995). In endemic areas, captive wild animals should be kept well separated from local sheep and goat flocks, as the safety of the PPR vaccine in wild species has not yet been established.

LABORATORY DIAGNOSIS

The virus can be isolated most easily in primary sheep or goat kidney or skin cell cultures and it can also be grown after several blind passages in Vero cells. Laboratory techniques for detecting the virus have developed in parallel with those for rinderpest because of the need for differential diagnosis of the two infections and specific antibody, antigen and RNA detection tests are now available to distinguish the two viruses (Forsyth and Barrett 1995; Anderson et al. 1996; Libeau et al. 1994).

Canine distemper virus

Canine distemper virus infects a variety of carnivores, most notably the dog, mink, and ferret among domestic species. The epizootiology in wild carnivores is poorly understood but infections in these species have been more extensively studied in recent years.

CLINICAL AND PATHOLOGICAL MANIFESTATIONS

Disease is most often seen in young animals, but can also occur in older animals. Infection begins in the upper respiratory tract and, after an incubation period which can range from 1 to 6 weeks, spreads in a similar fashion to other morbilliviruses, that is from the local lymph nodes via the lymph and blood to other lymphatic

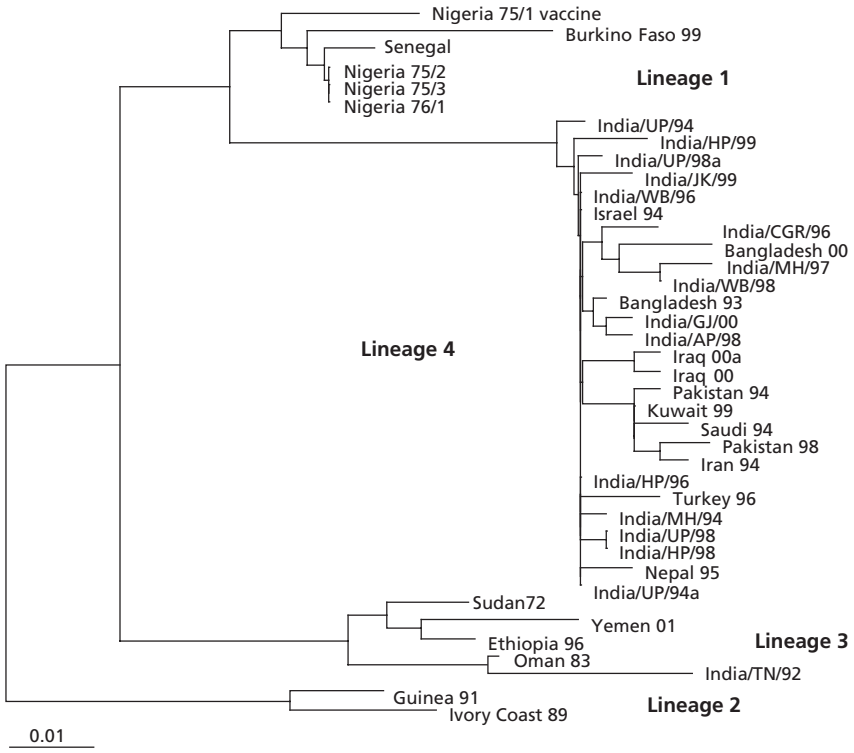

Figure 4 *The relationships between the different peste des petits ruminants viruses based on partial sequence data derived from the fusion protein gene. A specific primer set was used to amplify a 372-base pair DNA fragment as described by Forsyth and Barrett (1995). The tree was derived as described in Figure 2.*

tissues and then to the upper and lower respiratory tracts, gastrointestinal mucosa, and brain. Following an initial febrile response, infected animals suffer depression and develop mucopurulent nasal and ocular discharges, vomiting, diarrhea, and pneumonia, signs commonly seen in other morbillivirus infections. A dry cough may develop which progresses to a moist cough. However, the most striking difference between canine distemper and the ruminant morbilliviruses is the virus' ability to infect neurons and other cells of the nervous system, but the neurotropism of isolates can also vary considerably (Stettler et al. 1997). Brain infection results in a nonsuppurative encephalitis, meningoencephalitis, neuronal necrosis, and demyelination. Neurological signs such as seizures, tremors, disorientation, incoordination, coma, or behavioral changes are often present and can develop acutely weeks or months later. Another aspect of the disease that differs from that caused by rinderpest and PPR infections is a hyperkeratosis that commonly develops on the foot pads (so-called 'hard pad'). There can be considerable variation in the severity of disease and this is partly related to differences in the infecting strain, often referred to as a biotype, and the individual and species involved (Appel 1987). A strong humoral antibody response to the virus may result in mild or inapparent disease and the virus is cleared quickly but if a weak response is mounted, and the animal recovers from the inevitable disease, it can shed virus for 2 to 3 months. While early development of circulating antibody has been shown to be crucial for protection and recovery from canine distemper infections that is not the case for the ruminant morbilliviruses where a cell-mediated response appears to be more important for protection.

EPIZOOTIOLOGY

Canine distemper is found worldwide, with the exception of hot arid regions, and results in periodic disease outbreaks, mostly seen in domestic dogs, but a variety of wildlife species are highly susceptible. For example, disease has been observed in several species of large cat in American zoos, lions and hyenas in the Serengeti plains in Africa, javelinas in Arizona, ferrets, mink, raccoons, coyotes, badgers, foxes, bears, pandas, and seals (Appel 1987; Appel and Summers 1995; Haas et al. 1996; Harder et al. 1996; Roelke-Parker et al. 1996). A retrospective study of pathological specimens from large cats that died of unknown causes in a Swiss zoo indicated that canine distemper was not a new infection in these species (Myers et al. 1997). Ferrets are particularly susceptible to distemper infections and the virus has threatened the survival of the black-footed ferret, a highly endangered species in the USA (Carpenter et al. 1976). Virus is usually maintained in dog populations by transmission to susceptible young via respiratory, ocular, and nasal secretions, which contain the virus. Like all morbilliviruses, it is highly contagious but requires close contact for transmission. In enzootic areas, the density of the susceptible population is an important factor in maintenance and spread and disease is most often seen in young animals after the maternal antibody has waned. In isolated populations, as for other morbilliviruses, epizootics occur which can be severe and affect all age groups (Leighton et al. 1988).

CONTROL

Modified live virus vaccines, the Onderstepoort and Rockborn strains developed in the 1950s, have been very successful in controlling disease in domestic dogs and mink. Maternal antibody decreases the effectiveness of the vaccine, so vaccine should be given after maternal antibody begins to wane at 6–9 weeks of age (Chapuis 1995). Most vaccination protocols recommend giving a second dose at 12–15 weeks of age to ensure an effective vaccination. Live virus vaccines have also been used in nondomestic species, but this is risky and they should only be used if proven safe in that species. Vaccine-induced immunosuppression can be a problem in animals such as the panda (Bush et al. 1976; Deem et al. 2000). Threatened and endangered wild carnivores are best protected by ensuring good vaccination cover in domestic dogs living near wildlife reservations and minimizing contact. Domestic dogs were considered the most likely source of the virus that infected the lions, hyenas, and wild dogs in Africa (Roelke-Parker et al. 1996).

LABORATORY DIAGNOSIS

The virus can be isolated in cell culture or virus-specific antigens and RNA can be detected in infected secretions, blood, and affected tissues. Vero and dog kidney cells have been used for virus isolation, although not all strains grow well in these culture systems. Pulmonary alveolar macrophages and mitogen-stimulated canine or ferret blood lymphocytes are the most sensitive cell culture system for canine distemper virus (Appel et al. 1992). Viral antigens can be detected by immunofluorescence in conjunctival smears or by immunohistochemistry carried out on fixed tissues. Viral RNA can be detected by RT-PCR assays using specific primer sets (Forsyth and Barrett 1995; Kennedy et al. 2000). The most important differential diagnosis is for rabies virus because of the implications for human health.

Morbillivirus infections of aquatic mammals

Morbillivirus infections in marine mammals were unknown before 1987, but since then several newly recognized members of the genus *Morbillivirus* have been identified as the etiological agent of disease and mortality in the orders *Pinnipedia* (seals) and *Cetacea* (whales, porpoises, and dolphins). The first to be recog-

nized was phocine (seal) distemper virus, which had severe ecological consequences for the European harbor seal (*Phoca vitulina*). Thousands of this species died along the coasts of northern Europe in 1988 (Osterhaus and Vedder 1988). The virus was also identified in European gray seals (*Halichoerus grypus*), but they have proved to be more resistant to the virus. Subsequently two other morbilliviruses were isolated from marine mammals, namely the dolphin and porpoise morbilliviruses (Barrett and Rima 2002; Kennedy et al. 1988; Domingo et al. 1990). The genetic similarity of the dolphin and porpoise viruses would indicate that they are variations of the same virus and they are collectively referred to as the cetacean morbillivirus (Barrett et al. 1993). Canine distemper virus has also been shown to infect and kill several species of seal (Kennedy et al. 2000; Grachev et al. 1989; Mamaev et al. 1995).

CLINICAL MANIFESTATIONS

Seals infected with either phocine distemper virus or canine distemper virus show a loss of body condition (reduced blubber thickness) along with the normal signs of a morbillivirus infection, fever, serous or mucopurulent oculonasal discharge, conjunctivitis, dyspnea, diarrhea, lethargy nervous signs, and abortion in pregnant females. Pneumonia was the main gross pathological finding in seals with the airways containing mucopurulent, blood-stained, or frothy exudates. Central nervous system (CNS) degeneration was also observed. Clinical data for infected cetaceans are less comprehensive as sick animals have not been observed under confined conditions and no experimental infections have been carried out. During the epizootic in the Mediterranean in 1990–1991, many striped dolphins (*Stenella coeruleoalba*) were found to be in poor body condition and loss of fat stores led to decreased buoyancy. Skin lesions and necrosis of the buccal mucosa, lymphodepletion, and hemoconcentration were commonly observed. Other clinical signs in striped dolphins included tachycardia, abnormal respiratory rates, lethargy, and reduced sound emissions. Bronchopneumonia was the most marked pathological feature in dolphins, along with CNS degeneration. The majority of the sick seals and dolphins had nonsuppurative encephalitis, some with signs of demyelination, which explained their behavioral changes (Domingo et al. 1992; Duignan et al. 1992).

EPIZOOTIOLOGY

Phocine distemper virus

The small size and scattered nature of European seal populations means it is unlikely that they could maintain this virus in circulation and indeed the virus disappeared from these populations following the epizootic in 1988. In 2002, another outbreak, following an almost identical course, occurred. Infection most probably results when interspecies transmission introduces or reintroduces the virus to a susceptible population. The similarities in the course of the two European outbreaks may be explained by the migratory and breeding habits of the seals and there is a growing consensus among some seal biologists that gray seals may be key vectors in the transmission of phocine distemper between harbor seal colonies in European waters. This is especially so where large geographical jumps are made. Harbor seals usually return to the same haulout, whereas gray seals do not and move much greater distances between these sites. Another factor may be the relative resistance of this species to the virus enabling it to transmit without necessarily showing severe clinical disease. Since it is likely that most virus transmission occurs on land, their mixing at shared haulout sites most probably explains the timing in early summer to autumn. More than 20 000 seals died during each of the two European phocine distemper outbreaks. The original source of virus in the 1988 and 2002 outbreaks was probably Arctic harp seals (*Phoca groenlandica*), which came into contact with northern European gray seals (Dietz et al. 1989). Serological evidence suggests that the disease is endemic in many species of Arctic seals and has affected Atlantic walruses (*Odobenus rosmarus*), but the full host range and clinical disease induced in most species has not been fully determined (Duignan et al. 1997; Nielsen et al. 2000). Phocine distemper virus can also threaten terrestrial mammals and during the 1988 epizootic in Europe the virus clinically infected farmed mink in Denmark (Blixenkrone-Møller et al. 1990) and there is evidence for phocine distemper and dolphin distemper virus antibodies in Canadian terrestrial carnivores (Philippa et al. 2004).

As mentioned above, phocine distemper is not the only morbillivirus known to cause disease in seals and canine distemper virus infection can also result in significant mortality in these species. Immediately prior to the European seal epizootic, during the winter of 1987–1988, Lake Baikal seals (*Phoca sibirica*) suffered an unusual and severe mortality and there was no possible epidemiological connection between the two events (Grachev et al. 1989). Subsequently canine distemper virus, rather than phocine distemper virus, was shown to have been the etiological agent in the outbreak (Mamaev et al. 1995). Similarly, mass die-offs occurred in Caspian Sea seals (*Phoca caspica*) in 1997 and 2000, which were also attributed to canine distemper virus (Forsyth et al. 1998; Kennedy et al. 2000). Although not proven, the source of the virus is likely to have terrestrial carnivores, such as wolves, which prey on seal pups. Canine distemper virus has also been implicated in the deaths of thousands of crabeater seals (*Lobodon carcinophagus*) in the Antarctic in the 1950s. Since there are no terrestrial carnivores in the Antarctic, it is thought likely that sledge dogs used at that time were the source of the virus, or possibly the animals were infected by contact with carnivores in New Zealand or South America

during migrations. A subsequent serological survey of Antarctic seals showed them to have a high prevalence of canine distemper-specific antibodies (Bengtson et al. 1991). All morbillivirus infections in seals are clinically similar and so differential diagnosis has to be made, which is most easily done by RT-PCR using universal morbillivirus primer sets and primer sets specific for each virus species (Mamaev et al. 1995; Kennedy et al. 2000).

Cetacean morbillivirus

Cetacean species in all the major oceans of the world have been reported to be seropositive for this virus (Duignan et al. 1996; Van Bressem et al. 2001). The pilot whale (*Globicephala* spp.) appears to be the reservoir and vector for transmission of the cetacean morbillivirus to other species. They move in large groups, known as pods, over great ocean distances and have a worldwide distribution. Over 90 percent of pilot whales involved in mass strandings between 1982 and 1993 were morbillivirus seropositive (Duignan et al. 1995a, b). Although the first report of a cetacean morbillivirus was in 1988 in a porpoise caught off the coast of Northern Ireland (Kennedy et al. 1988), subsequently it was shown that a mass die-off of Atlantic bottlenose dolphins (*Tursiops truncatus*) that occurred along the Atlantic coast of the USA in 1987–1988 was probably the result of morbillivirus infection since more than 50 percent of the animals were found to be morbillivirus positive (Lipscomb et al. 1994). Originally the mortality was thought to have been due to brevitoxins produced by red tides of dinoflagellates. Another epizootic occurred in the Gulf of Mexico in 1993–1994 (Lipscomb et al. 1994). There is also serological evidence of exposure of Florida manatees (order *Sirenia*) to morbillivirus infection but no reports of disease or unusual mortality in this species have been reported (Duignan et al. 1995c).

Some evidence also links the cetacean morbillivirus with seal mortality, although not on the same massive scale seen with phocine or canine distemper viruses. A minor outbreak of disease in seals occurred 1998 along the Belgian and northern French coasts and morbillivirus antigen and nucleic acid were detected in tissues from sick animals which were genetically closely related to either the cetacean morbillivirus or canine distemper virus (Jauniaux et al. 1998). The rare monk seal population of the eastern Mediterranean was not severely affected during an epizootic of this virus which killed thousands of striped dolphins in these waters in 1990–1991; however, the cetacean morbillivirus was found in carcasses of monk seals that died in large numbers off the coast of Mauritania in 1997 (Van de Bildt et al. 2000). At the time, an algal bloom was also considered to be an exacerbating or primary factor, which may have contributed to the very high mortality among adult seals at that time (Harwood 1998).

The long-term effects on marine mammal populations infected with morbilliviruses is not clear and will depend on whether the species concerned is already at dangerously low numbers, for example the Mediterranean monk seals and the Caspian Sea seals (Kennedy et al. 2000; Osterhaus et al. 1992). The catastrophic die-off in one of the few remaining colonies of monk seals off the Mauritanean coast in 1997 wiped out an estimated 70 percent of the population within the space of 1 month, a figure representing about one-third of the total world population, leaving only a few hundred remaining (Harwood 1998). However, in populations with healthier numbers, the situation is not so serious and following the 1988 epizootic the European seal populations recovered their normal levels within a few years.

CONTROL

Control of wildlife diseases is extremely difficult and vaccination poses great logistical problems. One exception has been the success in controlling rabies in wildlife using the vaccinia virus recombinant vaccine, but this required very long and careful development (Pastoret and Brochier 1996). Similar attempts at vaccinating seals with a vaccinia recombinant expressing the canine distemper virus fusion (F) protein failed as the virus did not replicate and elicit an immune response (Van Bressem et al. 1991). Vaccination of wildlife also raises ethical questions concerning the effects of the disturbance such an intervention would have on the population and whether there would be uncontrolled spread of the vaccine in the environment. Protection of vulnerable animals in marine wildlife parks and seal sanctuaries would be acceptable as the animals could be confined until the excretion of any vaccine virus had ceased. In any event, there is no currently licensed vaccine for use in these species. Because of the close antigenic relationship between morbilliviruses, strong cross-protection to infection is given by vaccines for other morbilliviruses. An experimental CDV-ISCOM vaccine has been shown to protect seals from PDV, but the duration of the immunity induced remains uncertain (Visser et al. 1992). Canine distemper vaccines have not been fully tested for safety in seals and these vaccines have been shown to cause death in other wildlife species (Bush et al. 1976; Carpenter et al. 1976; Sutherland-Smith et al. 1997); however, a small trial of live attenuated canine distemper vaccine in seals was successfully carried out in England during the 2002 phocine distemper outbreak. The consensus at present is that wildlife is best protected by vaccination to control diseases in domestic species, for example vaccination of cattle has proved an effective means of eliminating rinderpest from wildlife in Africa, but this is not an option for protecting marine mammals from morbillivirus infection since the marine morbilliviruses are rarely found in terrestrial mammals. Because of the threat posed by canine distemper to

seals, it is recommended that unvaccinated dogs should not be allowed to mix with seals and sledge dogs are no longer used in the Antarctic.

AVULAVIRUSES (NEWCASTLE DISEASE VIRUS)

Newcastle disease virus (NDV) is responsible for outbreaks of severe disease among poultry throughout the world. Since outbreaks reported to the OIE result in severe trade limitations, this disease has serious economic consequences. NDV, also called fowl pest or avian paramyxovirus type 1 (APMV-1), is a member of the subfamily *Paramyxovirinae*. Recently, avian paramyxoviruses have been designated as a new genus, *Avulavirus* (Chang et al. 2001). The genomes of several rubulaviruses, except NDV, contain a small hydrophobic (SH) protein gene that is also not present among the respiroviruses (Lamb and Kolakofsky 2001). Interestingly, the avian paramyxovirus-6 (APMV6) genome was reported to contain a putative SH protein gene (Chang et al. 2001).

Clinical manifestations of infection

NDV causes epidemic disease in a wide range of domesticated and wild birds, most notably among poultry (Alexander 1995). It has been isolated from all orders of birds. The incubation period averages 5–6 days, with a range of 2–15 days. The type and severity of disease varies with the infecting virus strain, as well as the age and species of the infected bird. For example, younger chickens are subject to more severe disease than older chickens. Although there is a continuum of clinical presentations, isolates have been separated arbitrarily into four groups based on the presence of enteric or neural disease and the speed with which they kill embryonating hens' eggs. All strains reduce egg production in chickens.

NDV strains are divided into three pathotypes based on disease severity (Alexander 1997). Lentogenic strains are associated with no, or mild, respiratory tract symptoms and some have been developed into live virus vaccines. Young birds are most likely to develop illness when infected with these strains. The mesogenic form is associated with an acute respiratory illness of moderate severity that is sometimes associated with life-threatening nervous system complications. Neurotropic velogenic strains are associated with respiratory and neurological disease, such as paralysis of the legs. In outbreaks, death can occur in 5–10 percent or more of infected birds. Viscerotropic-velogenic strains cause an acute, often lethal, infection with hemorrhagic gastrointestinal lesions. In outbreaks, mortality rates can be as high as 90 percent.

Virulence of NDV is dependent on the amino acid sequence of the cleavage site of the F protein (Glickman et al. 1988), which determines the ability of the F protein to be cleaved by cellular proteases (Gotoh et al. 1992). Mesogenic or velogenic strains have dibasic amino acids in the F protein cleavage sequence, while the F proteins of lentogenic NDV strains do not have this motif (Glickman et al. 1988).

Both wild-type and vaccine strains of NDV can infect humans (Chang 1981). After an incubation period of 1–4 days, the patient sometimes develops conjunctivitis, which is usually unilateral and, occasionally, systemic symptoms such as fever, myalgia, and malaise. Symptoms usually resolve within a week.

Epizootiology

NDV can spread by inhalation or ingestion of infectious virus. Large amounts of infectious virus can be excreted in the feces, which is likely to be the source of infectious virus in most instances. NDV can be introduced into new populations through natural movement of infected birds (e.g. migration, shipping infected birds from one location to another) or by fomites such as contaminated food, equipment, or water. The virus can be transmitted quickly and efficiently in crowded populations, such as poultry houses. Humans presumably become infected by contamination of the hands and autoinoculation of the conjunctiva or by inhalation of aerosolized virus. Most human infections have occurred after exposure to wild-type virus in slaughterhouses or laboratories or while administering the vaccine.

Pathology, pathogenesis, and immune response

The virus infects via the respiratory or gastrointestinal tract or the conjunctivae. With virulent strains, the virus first causes a local lytic infection and then the bird becomes viremic and the virus spreads to infect multiple organs, including the brain, heart, kidneys, and reticuloendothelial system. The speed and efficiency with which it infects different tissues seem to be key to its virulence and this, in turn, depends on the host species and strain of NDV. Efficient cleavage of the glycoproteins in a wide range of cell types is characteristic of highly virulent strains (Rott and Klenk 1988). The titer of the inoculum can also alter the course of the infection. A low-titer inoculum can ameliorate disease caused by a virulent strain.

Vaccination or naturally acquired infection confers resistance to further disease. Passively acquired antibody can also provide protection from disease. It has not yet been determined which components of the immune response are responsible for controlling and clearing established infection in the bird.

Laboratory diagnosis

Diagnosis of NDV is often important in the context of control measures that are to be undertaken, and requires isolation and the characterization of the virus as virulent, avirulent, or possibly vaccinelike. Respiratory, fecal, or cloacal specimens from live birds or tissue specimens from affected organs from dead birds can be used to isolate the virus. Cell culture systems or embryonic chicken (or other fowl) eggs from flocks known to be pathogen-free or NDV-free can be used to isolate the virus. The virus is then characterized by determining its virulence in embryonating hens' eggs, day-old chicks or 6-week-old chickens. Molecular methods to characterize strains and track transmission are being developed and may provide alternative ways to determine virulence (Seal et al. 1995; Alexander 1999).

Antibodies against NDV can be detected by a variety of tests, including neutralization, hemagglutination inhibition and enzyme immunoassays (EIA). Antibody tests have been used primarily to demonstrate past infection or a response to vaccination.

Control

Vaccination and transmission control programs have been used to prevent NDV infection. Live virus vaccines developed from B1 or Lasota lentogenic strains are most widely used. For poultry, the vaccines can be administered in a variety of ways: orally in water, by intranasal or intraocular inoculation, or by aerosols. Quarantine of imported birds and quarantine and slaughter of infected flocks have been an integral part of NDV control in a number of countries.

Other avian paramyxoviruses

Eight other serotypes of avian paramyxoviruses have been isolated from a variety of birds. Although NDV is the most important pathogen from an economic standpoint, other viruses in the group have sometimes been associated with serious disease.

HENIPAVIRUSES

Viruses belonging to the recently described genus *Henipavirus* within the family *Paramyxoviridae* emerged during the past decade. These viruses were initially identified as the etiologic agents responsible for outbreaks of febrile respiratory illnesses of veterinary livestock. When transmitted to humans from infected animals, these viruses were responsible for severe encephalitic or respiratory diseases that often had fatal outcomes (Murray et al. 1995a; Chua et al. 2000). The two viruses in this genus, Hendra virus and Nipah virus are different from most other paramyxoviruses because they have a broad host range both in vivo and in vitro. Fruit-eating bats appear to be a natural reservoir for the henipaviruses, although isolation of these viruses from this reservoir has been infrequent. Humans are infected via intermediate hosts such as horses or pigs, by exposure to infected fruit bats, or by direct human-to-human transmission.

Hendra virus is the type species of the genus *Henipavirus*, and both Hendra virus and Nipah virus have genetic characteristics and replication strategies that are similar to those of other members of the subfamily *Paramyxovirinae*. The henipaviruses also have several unique genetic features that distinguish them. The genomes of Hendra virus and Nipah virus are 18 234 and 18 246 nucleotides, respectively, making them the largest paramyxovirus genomes reported (Harcourt et al. 2001; Wang et al. 2000). The increased size is due to a longer open reading frame (ORF) encoding the phosphoprotein (P) and the unusually long nontranslated regions that flank each gene (Harcourt et al. 2000, 2001; Wang et al. 1998, 2000; Yu et al. 1998a,b). The *P* gene of these viruses encodes three nonstructural proteins (C, V, and W) in addition to P. The C protein is encoded by a second ORF that initiates 23 nucleotides downstream of the P ORF translational initiation site (Harcourt et al. 2000; Wang et al. 1998). In addition to the C ORF, HV has another ORF in the *P* gene that encodes a putative, arginine rich, small basic protein (SB) of 65 amino acids (Wang et al. 1998), which has not yet been detected in infected cells (Wang et al. 2001).

The *P* genes of both Hendra virus and Nipah virus contain an RNA editing site that it is identical to the editing site found in measles virus (MeV) (Harcourt et al. 2000; Wang et al. 1998). The addition of a single G allows expression of the V protein, while addition of two Gs allows expression of a protein analogous to the W protein of Sendai virus (Delenda et al. 1998; Harcourt et al. 2000; Vidal et al., 1990). The V and W proteins appear to be virulence factors since cells expressing both V and W of Nipah virus block activation of an IFN-inducible promoter in primate cells (Park et al. 2003). Expression of IFN-α and IFN-γ-driven reporter genes was inhibited by the expression of the V of Nipah virus or HV in the presence of IFN. The V of Nipah virus formed high molecular weight complexes of 300–500 kDa with STAT1 and STAT2, which prevented both the activation and nuclear transportation of STAT1 (Rodriguez et al. 2002, 2003).

The L proteins of the henipaviruses have the six linear domain structures found within the polymerase proteins of all viruses in the order *Mononegavirales*. A highly conserved GDNQ sequence within domain III is conserved in most of the *Mononegavirales* viruses but is changed to GDNE in the henipaviruses and tupaia virus (Poch et al. 1990; Tidona et al. 1999; Harcourt et al. 2000; Wang et al. 2001).

The cleavage site of the fusion (F) protein of the henipaviruses contains a single basic amino acid and thus does not contain the R–X–R/K–R consensus sequence for furin proteases that is seen in the morbilliviruses, rubulaviruses, and pneumoviruses (Lamb and Kolakofsky 2001). The cellular protease(s) that cleaves the F of henipaviruses is unidentified, though basic amino acids are not required for cleavage (Moll et al. 2004). The henipaviruses are unique in that they have a leucine residue at the amino terminus of F_1 instead of the phenylalanine found in most other members of the family *Paramyxoviridae* (Harcourt et al. 2000; Wang et al. 2001). The G proteins of the henipaviruses have no known hemagglutinin or neuraminidase activities (Harcourt et al. 2000; Yu et al. 1998a).

Epidemiology and transmission

Hendra virus has been associated with two human and 17 horse deaths in three separate incidents in Australia during 1994–1999 (Murray et al. 1995a, 1995b; Selvey et al. 1995). The last known incident of Hendra virus infection occurred during January 1999 in Australia and involved a fatality in a single, adult mare, but no human infections (Field et al. 2000; Hooper et al. 2000). Antibodies to Hendra virus were detected in several fruit bat species in Queensland and the virus isolated from a fruit bat was indistinguishable from that isolated from horses and humans (Young et al. 1996; Halpin et al. 1999, 2000) suggesting that these bats may be a natural reservoir for Hendra virus. Transmission to horses may have occurred through the ingestion of material recently contaminated by the urine or infected fetal tissue of fruit bats (Halpin et al. 1999; Field et al. 2000).

The first known human infections with Nipah virus occurred during an outbreak of severe febrile encephalitis in peninsular Malaysia and Singapore in 1998–1999. Direct contact with pigs was the primary source of human infection (CDC 1999a, b; Chua et al. 2000). This outbreak began in October 1998 near the city of Ipoh and then spread southward in conjunction with the movement of pigs, resulting in three other clusters of human disease near Kuala Lumpur. In Singapore, abattoir workers who slaughtered pigs imported from outbreak-affected areas in Malaysia were exclusively affected (Paton et al. 1999; Chew et al. 2000). A total of 265 patients (104 fatal) with viral encephalitis and 11 patients (one fatal) with laboratory-confirmed Nipah virus disease were reported in Malaysia and Singapore, respectively. Adult males who were primarily involved in pig farming or pork production activities accounted for the majority of cases. Although Nipah virus is excreted in respiratory secretions and urine of patients (Chua et al. 2001), a survey of healthcare workers in Malaysia demonstrated no evidence of human-to-human transmission (Mounts et al. 2001).

Because of the virologic similarities between Nipah virus and Hendra virus, surveillance for the natural reservoir for Nipah virus was focused on bats. Neutralizing antibodies to Nipah virus were found in four fruit-bat species and one insectivorous-bat species in peninsular Malaysia (Yob et al. 2001), and antibodies have been detected in Cambodian bats (Olson et al. 2002). Nipah virus has recently been isolated from the urine of Malaysian fruit bats roosting on Tioman Island (Chua et al. 2002).

Nipah virus has been established as the cause of fatal, febrile encephalitis in human patients in Bangladesh during the winters of 2001, 2003, and 2004. A Nipah virus-like virus was identified as the cause of the outbreaks in 2001 and 2003 based on results from serologic testing (Hsu et al. 2004); however, Nipah virus was isolated from patients with acute disease in 2004. Though antibodies to Nipah virus were detected in fruit bats from the affected areas in 2004, an intermediate animal host was not identified. It is likely that the virus was transmitted directly from bats to humans. Human-to-human transmission of Nipah virus was also documented during the outbreak in Bangladesh (ICDDRB 2004a,b; WHO 2004).

Clinical and pathological manifestations

Little is known concerning the clinical disease and associated pathology in the natural hosts of the henipaviruses, and studies are in progress to further address these questions. Natural or experimental Hendra virus infections of bats are asymptomatic though the bats seroconvert and viral antigen and some live virus can be detected (Williamson et al. 1998; Halpin et al. 2000; Field et al. 2001). Horses and cats naturally infected with Hendra virus show clinical signs that include depression, anorexia, profuse nasal discharge, and labored breathing with pulmonary edema and congestion. Pneumonic and hemorrhagic lesions are seen in the lungs and death occurs within 2 days of infection. Virus can be isolated from the urine of infected animals (Williamson et al. 1998; Field et al. 2001). Experimental infection of horses was less severe than natural disease. Nipah virus infection of golden hamsters results in fatal neurological disease, thus providing a small animal model for studying the pathogenesis of Nipah virus (Wong et al. 2003).

In the first reported cases of Hendra virus infection in humans, patients had abrupt onset of an influenza-like illness, characterized by myalgia, headaches, lethargy, and vertigo (Selvey et al. 1995). Based on the exposure history of the two people involved, the incubation period for the acute disease caused by Hendra virus is approximately 5–7 days. One patient remained lethargic for 6 weeks but recovered fully, while the other succumbed to infection. In the second reported Hendra

virus incident, the single patient initially presented with features of meningitis, including headache, drowsiness, vomiting, and neck stiffness. Following complete recovery, this patient presented 13 months later with neurological disease and died shortly thereafter.

The onset of Nipah virus disease is abrupt, usually with the development of fever and severe encephalitis. Fever (97 percent), headache (65 percent), dizziness (36 percent), vomiting (27 percent), and reduced level of consciousness (21 percent) were the most common clinical features noted during the outbreaks in Malaysia and Singapore (Goh et al. 2000). In addition to the neurological symptoms described above, acute respiratory distress was also noted in many patients during the Nipah virus outbreak in Bangladesh in 2004 (ICDDRB 2004a, b). The exact incubation period of Nipah virus disease in humans is not known but ranged from several days to 2 months, and was 2 weeks or less in the majority of patients.

The pathogenesis of Hendra virus in humans showed a severe interstitial pneumonia (Selvey et al. 1995; Murray et al. 1995a) and leptomeningitis (O'Sullivan et al. 1997). In contrast, a multiorgan vasculitis associated with infection of endothelial cells was the main pathologic feature of Nipah virus infection (Parashar et al. 2000) with infection being most pronounced in the central nervous system with necrotic foci of neuronal degeneration. Evidence of endothelial infection and vasculitis was also seen in other organs, including the lung, heart, spleen, and kidney. Nipah virus has been isolated from cerebrospinal fluid, tracheal secretions, throat swab, nasal swab, and urine specimens of patients (Goh et al. 2000; Chua et al. 2001).

During the outbreak in Malaysia, direct contact with pigs was the primary source of human Nipah virus infection, with no evidence of human-to-human transmission (Parashar et al. 2000). Although pigs showed extensive infection of the upper and lower airways, with evidence of tracheitis, and bronchial and interstitial pneumonia, the mortality rate was less than 5 percent. A harsh, nonproductive cough (called the mile-long cough) was a prominent clinical feature, but other signs such as lethargy or aggressive behavior indicated some neurological involvement (Chua et al. 2000; Hooper and Williamson 2000; Hooper et al. 2000). Serologic studies demonstrated evidence of infection among other species of animals, including dogs and cats on and near farms with Nipah virus-infected pigs (Chua et al. 2000; Hooper et al. 2000), though it is unclear whether humans were at risk from exposure to infected animals other than pigs (Goh et al. 2000; Parashar et al. 2000).

Control

Since interruption of transmission of Hendra and Nipah viruses from their reservoirs to intermediate hosts or directly to humans is difficult, early identification of infected animals and humans and use of appropriate personal protective equipment in healthcare and agricultural settings are the keys to prevention. Since person-to-person transmission of Nipah virus has been documented, those in contact with patients, including healthcare workers, should use standard droplet precautions during contact with secretions, excretions, and body fluids of patients.

No vaccines or antiviral drugs are currently available for Hendra virus or Nipah virus. In vitro, ribavirin has a demonstrated antiviral effect against Hendra virus. During the Nipah virus outbreak in Malaysia, patients receiving ribavirin either orally or intravenously showed a lower mortality rate (Chong et al. 2001).

Laboratory diagnosis

Analysis of serum samples from outbreaks indicated that Nipah virus and Hendra virus infections generate a humoral immune response in humans. IgM was present in serum shortly after the onset of Nipah virus infection and nearly all patients were antibody-positive by the third day (Ramasundrum et al. 2000). IgG was detected 10–29 percent of Nipah virus-infected patients within the first 10 days of illness, and in 100 percent of patients after 17–18 days.

Because of their ability to cause severe disease in animals and humans, work with live Nipah virus and Hendra virus must be conducted at BSL-4. ELISA assays to detect IgG and IgM antibodies are routinely used to detect Hendra virus and Nipah virus infections. These assays often use inactivated or expressed antigens and, therefore, have the advantage that they do not need to be conducted at BSL-4. Also, IgG antibodies can be detected by using standard serum neutralization tests. Immunohistochemistry has proven extremely useful for detecting Nipah virus antigens in fixed tissue samples. Both Hendra virus and Nipah virus can be isolated on Vero-E6 cells from cerebrospinal fluid, nasal samples, and urine samples from infected individuals (Goh et al. 2000; Chua et al. 2001). Various RT-PCR assays have been used to detect RNA from Hendra virus and Nipah virus in a variety of clinical samples and tissues (Halpin et al. 2000; Daniels et al. 2001; Guillaume et al. 2004).

RUBULAVIRUSES

Several viruses in the genus *Rubulavirus* have been isolated from animals. During the search for the reservoir of Nipah virus in Malaysia, a previously undescribed rubulavirus, Tioman virus, was isolated from a small number of fruit bats. Tioman virus has not been associated with disease in bats or any other animal (Chua et al. 2002). Menangle virus is antigenically related to Tioman virus, and was originally isolated from stillborn piglets in a piggery in New South Wales Australia in 1997. Infections resulted in degeneration of

the brain and spinal cord and an increase in the number of abortions and stillbirths associated with deformities. Fruit bats living near the affected piggeries were seropositive for antibodies to Menangle virus. Two of over 250 humans who worked in the piggery also had antibodies to Menangle virus and reported an influenza-like illness following exposure to the infected pigs (Chant et al. 1998; Philbey et al. 1998). No further outbreaks of Menangle virus have been reported. Another porcine paramyxovirus, La-Piedad Michoacan-Mexico virus (LPMV), was identified as the agent responsible for fatal disease in piglets and has been endemic in Mexico since 1980. Bats do not play a role in the tranmission of LPMV (Linne et al. 1992; Salas-Rojas et al. 2004). Another respirovirus, Mapuera virus, has also been detected in bats (Henderson et al. 1995).

UNASSIGNED PARAMYXOVIRUSES

A number of new paramyxoviruses have been isolated recently from various animal species, but have not yet been assigned to a genus. Sixteen paramyxovirus isolates from reptilian species (Ahne et al. 1999) have been described; the archetype is fer-de-lance virus, which causes highly pathogenic respiratory infections in snakes. This virus has been completely sequenced and has a novel gene located between the *N* and *P* genes, which has no counterpart in any of the other viruses of the family. Fer-de-lance virus has been proposed as the type species for a new genus, *Ferlavirus*, within the subfamily *Paramyxovirinae* (Kurath et al. 2004). A number of paramyxoviruses have been isolated from rodents, including Nariva virus and Mossman virus (Roos and Wollmann 1979; Miller et al. 2003). Tupaia virus was discovered in a cell line derived from tree shrews (Tidona et al. 1999) and Salem virus was isolated from the mononuclear cells of a horse during an outbreak of disease in the USA in 1992 (Renshaw et al. 2000). Mossman, Tupaia, and Salem viruses are unclassified members of the subfamily *Paramyxovirinae* that are most closely related to the morbilliviruses and henipaviruses (Miller et al. 2003). The natural distribution and pathogenic potential of these novel paramyxoviruses are mostly unexplored.

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Coronaviruses, toroviruses, and arteriviruses

STUART G. SIDDELL, JOHN ZIEBUHR, AND ERIC J. SNIJDER

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PROPERTIES OF THE VIRUSES

Introduction

Coronaviruses, toroviruses, and arteriviruses are enveloped, positive-strand RNA viruses that infect vertebrates and can cause disease in their natural hosts. The genome organization and replication strategy are similar in all three groups of viruses but the differences in morphology and in genome size of the arteriviruses clearly set them apart. Coronaviruses and toroviruses are usually associated with respiratory or enteric disorders, although other organs (for example, the central nervous system (CNS)) can also be involved. The outcome of arterivirus infection can range from an asymptomatic, persistent carrier-state to lethal hemorrhagic fever.

The combination of features that distinguishes coronaviruses, toroviruses, and arteriviruses from other positive-strand RNA viruses are primarily in the structure and function of the replicase gene:

- The replicase gene comprises two overlapping open reading frames (ORF), and expression of the downstream ORF is mediated by –1 ribosomal frameshifting.
- Sequence analysis has identified common coding regions, e.g. motifs characteristic of proteinase, polymerase, helicase, and endoribonuclease, that are located at specific positions within the replicase gene. The signature organization of these domains can be expressed as (M-3CL-M)–RdRp–(Zn-HEL)–NendoU, where M-3CL-M represents a chymotrypsin-like proteinase (with a substrate specificity resembling that of picornavirus 3C proteinases) flanked by two hydrophobic transmembrane domains, RdRp is RNA-dependent RNA polymerase, (Zn-HEL) is a putative multinuclear Zn-finger-like domain associated with a superfamily 1 helicase domain and NendoU is a uridylate-specific endoribonuclease.
- In the infected cell, viral gene expression is mediated by a set of four or more 3′ co-terminal subgenomic mRNAs. Only the ORFs contained within the 5′ unique regions of each mRNA, i.e. the regions not found in the next smallest mRNA, are expressed as protein.

The features that distinguish coronaviruses, toroviruses, and arteriviruses from each other are two-fold:

1. There are significant differences in structure and function of the nucleocapsid protein gene. The molecular weights of the nucleocapsid protein are 50–60 kDa for coronaviruses, ca. 19 kDa for toroviruses and 12–14 kDa for arteriviruses. The nucleocapsid structure also has different morphologies: helical-spherical for coronaviruses, helical-tubular for toroviruses, and isometric for arteriviruses.
2. In addition to the common motifs listed above, coronaviruses and toroviruses have motifs indicative of additional replicase gene functions related to RNA processing. These include a putative exonuclease (ExoN), 2′-*O*-ribose methyl transferase (2-*O*-MT) and cyclic phophodiesterase (CPD).

A number of other features help to define and distinguish these three groups of viruses. They include the organization of the virus genome, genome size, evidence of discontinuous RNA synthesis during subgenomic mRNA production, the structure and function of the virus envelope (glyco)proteins and the intracellular budding of virus particles (de Vries et al. 1997; Gonzalez et al. 2003; Holmes 2001a; Lai and Holmes 2001; Snijder et al. 2003a; Snijder and Meulenberg 1998; van Vliet et al. 2002; Ziebuhr et al. 2000).

Classification

At the present time the coronaviruses and the toroviruses represent two genera of the family *Coronaviridae*. The arteriviruses are designated as a separate, monogeneric family, the *Arteriviridae* (they were previously assigned to the family *Togaviridae*). The families *Coronaviridae* and *Arteriviridae* are united in the order *Nidovirales* (Cavanagh 1997). The viruses that currently comprise the families *Coronaviridae* and *Arteriviridae* are listed, with their abbreviations and natural hosts, in Table 1.

Twenty four viruses are now classified as species in the *Coronaviridae* (Spaan et al. 2005). The prototype of the genus *Coronavirus* is avian *Infectious bronchitis virus* (IBV). The name coronavirus is derived from the solar coronalike (Latin *corona*: crown) appearance of virus particles in negatively stained electron micrographs. The prototype of the genus *Torovirus* is *Equine torovirus* (previously Berne virus), the name torovirus being derived from the curved tubular (Latin *torus*: lowest convex molding in the base of a column) morphology of the nucleocapsid structure. In addition to the viruses listed in Table 1, a further coronavirus, rabbit coronavirus (RbCoV) is considered as a tentative species of the genus and there have been recent reports of a novel human coronavirus, HCoV-HKUI (Woo et al. 2005) and a bat coronavirus, BaCoV (Poon et al. 2005). Also, although *Feline infectious peritonitis virus* (FIPV), *Sialodacryoadenitis virus* (SDAV), *Human coronavirus NL63* (HCoV-NL63), and *Porcine respiratory coronavirus* (PRCoV) are now recognized as coronavirus species,

Table 1 *Coronaviruses, toroviruses, and arteriviruses*

Natural host	Virus	Abbreviation
Coronaviruses		
Chicken	*Infectious bronchitis virus*	IBV
Cattle	*Bovine coronavirus*	BCoV
Dog	*Canine enteric coronavirus*	CCoV
Cat	*Feline coronavirus*	FCoV
Cat	*Feline infectious peritonitis virus*	FIPV
Humans	*Human coronavirus 229E*	HCoV-229E
Humans	*Human coronavirus NL63*	HCoV-NL63
Humans	*Human coronavirus OC43*	HCoV-OC43
Humans	*SARS-coronavirus*	SARS-CoV
Humans	*Human enteric coronavirus*	HECoV
Mouse	*Murine hepatitis virus*	MHV
Rat	*Rat coronavirus*	RtCoV
Rat	*Sialodacryoadenitis virus*	SDAV
Pig	*Porcine epidemic diarrhoea virus*	PEDV
Pig	*Transmissible gastroenteritis virus*	TGEV
Pig	*Porcine hemagglutinating encephalomyelitis virus*	HEV
Pig	*Porcine respiratory coronavirus*	PRCoV
Turkey	*Turkey coronavirus*	TCoV
Pheasant	*Pheasant coronavirus*	PhCoV
Shearwater	*Puffinosis coronavirus*	PCoV
Toroviruses		
Horse	*Equine torovirus*	EToV
Cattle	*Bovine torovirus*	BToV
Humans	*Human torovirus*	HToV
Pig	*Porcine torovirus*	PToV
Arteriviruses		
Horse	*Equine arteritis virus*	EAV
Mouse	*Lactate dehydrogenase-elevating virus*	LDV
Monkey	*Simian haemorrhagic fever virus*	SHFV
Pig	*Porcine reproductive and respiratory syndrome virus*	PRRSV

limited sequence data suggests that they could also be considered as variants of *Feline coronavirus* (FCoV), RtCoV, *Human coronavirus 229E* (HCoV-229E), and *Transmissible gastroenteritis virus* (TGEV), respectively. The sequence relationships of coronaviruses and toroviruses have been analyzed in detail (Gonzalez et al. 2003) and a revision of the current taxonomy is expected.

Four viruses are classified as species in the *Arteriviridae* (Snijder et al. 2005). The prototype of the family is *Equine arteritis virus* (EAV). The name arterivirus is derived from arteritis (inflammation of an artery), a disease caused by EAV. Previously, *Porcine respiratory and reproductive syndrome virus* (PRRSV) was also referred to as swine infertility and respiratory syndrome virus (SIRSV) or Lelystad virus. The four arterivirus species are antigenically distinct and no detailed serological cross-reactions have been reported. Phylogenetic analysis of arterivirus replicase proteins indicates that the four arterivirus species each constitute a branch within the genus, with each species represented by a cluster of strains. *Lactate dehydrogenase-elevating virus* (LDV) and PRRSV are most closely related to each other.

Finally, it should be mentioned that sequence analysis of *Gill-associated virus* (GAV) and *Yellow head virus* (YHV), which infect prawns, indicates that they are related to coronaviruses, toroviruses, and arteriviruses (Cowley et al. 2000) and justifies placing them in a genus *Okavirus* in the family *Roniviridae* in the order *Nidovirales* (Gonzalez et al. 2003). However, these viruses will not be discussed further in this chapter.

Morphology and structure

Coronaviruses are pleomorphic but roughly spherical enveloped particles, 120–160 nm in diameter with a characteristic 'fringe' of surface projections 20 nm long. An inner 'fringe' of short surface projections is sometimes seen on murine hepatitis virus (MHV), bovine coronavirus (BCoV), turkey coronavirus (TCoV), and human coronavirus OC43 (HCoV-OC43) particles. Toroviruses are also pleomorphic, enveloped particles, 120–140 nm in diameter; they are disk-, kidney- or rod-shaped and, like the coronaviruses, are decorated with 15–20 nm surface projections. The nucleocapsid of both coronaviruses and toroviruses is helical but differs in morphology. Coronavirus nucleocapsids are extended when the structure is relaxed but they may be organized into spherical, possibly icosahedral, superstructures in the virus particle (Escors et al. 2001; Risco et al. 1996; Salanueva et al. 1999). A gap separating the internal core from the envelope has been observed in coronaviruses using cryo-electron microscopy. The nucleocapsids of toroviruses are tubular. Arteriviruses are 45–60 nm in diameter and consist of an isometric nucleocapsid of 25–35 nm that is surrounded by a lipid envelope. No prominent surface projections are seen on the virion but a surface pattern of relatively small and indistinct projections has been observed. The morphological features and the assignment of structural proteins to the coronavirus, torovirus, and arterivirus virions are illustrated in Figure 1.

Coronaviruses have an estimated molecular mass of 400×10^6 kDa and a buoyant density in sucrose of 1.15–1.20 g/cm^3. The buoyant density in CsCl is 1.23–1.24 g/cm^3 and the sedimentation coefficient $S_{20,w}$ is 300–500. Coronaviruses are sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde, oxidizing agents, and UV-irradiation. Some coronaviruses are resistant to acid pH and/or dessication. Toroviruses have a buoyant density of 1.14–1.18 g/cm^3 in sucrose and an estimated sedimentation coefficient ($S_{20,w}$) of 400–500. Virus infectivity is stable between pH 2.5 and 9.7 but rapidly inactivated by heat, organic solvents, and irradiation. Arteriviruses have a buoyant density in sucrose of 1.13–1.17 g/cm^3 and 1.17–1.20 g/cm^3 in CsCl. The sedimentation coefficient is 214S–230S. Temperature, pH, and lipid solvents affect virion stability and virions are highly unstable in solutions containing low concentrations of detergents.

Genome structure and function

The genome of coronaviruses and toroviruses is a positive-strand RNA molecule of ca. 27 000–31 500 nucleotides (nt). This estimate of size is based on the nucleotide sequence of nine complete coronavirus genomes and the nucleotide sequence of the equine torovirus (EToV) genome (see www.ncbi.nlm.nih.gov/entrez/) (van Vliet et al. 2002). The coronavirus and torovirus genomic RNAs have a 5′ cap structure, and are polyadenylated at their 3′ end. It is generally accepted that the genomic RNA of coronaviruses and toroviruses is infectious, a conclusion that is based on the initiation of infection in susceptible cells with RNA extracted from purified virions and the initiation of infection with transcripts from cDNA clones corresponding to full-length coronavirus genomic RNA (Almazan et al. 2000; Casais et al. 2001; Thiel et al. 2001a; Yount et al. 2000; Yount et al. 2003; Yount et al. 2002).

The positive-strand RNA genome of arteriviruses is significantly smaller than that of coronaviruses and toroviruses. Sequence analysis of the EAV, LDV, and PRRSV genomes indicates a size of ca. 12 700–15 700 nt (see www.ncbi.nlm.nih.gov/entrez/). The genomic RNA of arteriviruses is polyadenylated at the 3′ end. It has been reported that the genomic RNA of simian haemorrhagic fever virus (SHFV) has a 5′ cap structure (Sagripanti et al. 1986). Cap analogue was found to be an essential component of the in vitro transcription reactions used to generate infectious RNA from PRRSV and EAV full-length cDNA templates (Meulenberg et al. 1998; van Dinten et al. 1997).

REGULATORY ELEMENTS

The genomes of coronaviruses and toroviruses contain a 5′ non-translated region (NTR) of several hundred

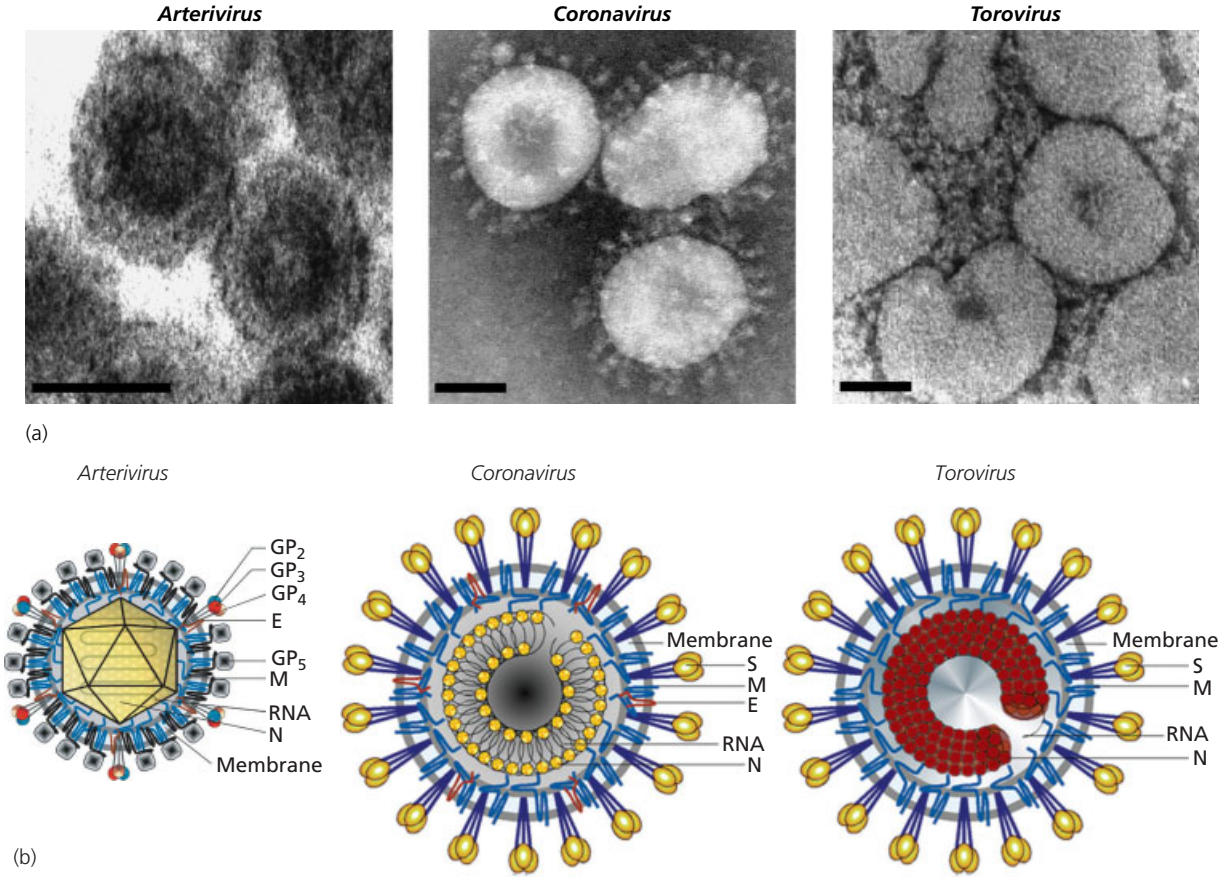

Figure 1 *Electron micrographs and schematic representations of coronavirus, torovirus, and arterivirus virion structure showing the locations of the structural proteins. The stoichiometry of the virion components is shown arbitrarily. E, envelope protein; GP_5, major glycoprotein; GP_2, GP_3, GP_4, minor glycoproteins; M, membrane protein; N, nucleoprotein; S, spike glycoprotein. The scale bar represents 50 nm.*

nucleotides. This region is almost devoid of AUG codons but a small ORF of 3–11 codons is conserved in the 5′ NTR of viruses examined to date. It has been speculated that this 'mini-ORF' may play a role in regulating translation from the genomic RNA (Senanayake and Brian 1999). The 5′ NTR of arteriviruses is relatively short, about 150–220 nt. Although small ORFs have been identified in this region for EAV, PRRSV, and SHFV (Snijder and Meulenberg 1998). The EAV intra-leader ORF was shown to be completely dispensable for EAV replication in cell culture (Molenkamp et al. 2000).

By analogy with other positive-strand RNA viruses, it can be expected that secondary (or tertiary) structures in the 5′ NTR of coronaviruses, toroviruses, and arteriviruses will have a role in the synthesis and packaging of virus RNA. Van den Born and colleagues (2004) have predicted and analyzed the secondary structure of the 5′ proximal region of the EAV genome. Their analysis suggests that secondary structures in this region play a role in the promotion of nidovirus plus-strand RNA synthesis from genomic and subgenomic templates. It remains to be shown exactly which of the predicted structural elements are involved and if, for example, additional RNA–protein interactions are important. Similar secondary structures have also been predicted in the 5′ NTR of other arteriviruses, coronaviruses, and toroviruses (Chang et al. 1996; Shieh et al. 1987; Van den Born et al. 2004).

As is described in more detail in the section 'Transcription,' coronavirus and arterivirus gene expression is mediated by the production of a set of subgenomic mRNAs in the infected cell. An element that plays an important role in this process is the so-called transcription-regulating sequence (TRS). In coronaviruses and toroviruses, this motif is a species-specific AU-rich sequence of ca. 10 nt, located within the 5′ NTR and upstream of the ORFs that are destined to become 5′ proximal in the subgenomic mRNAs. In the arteriviruses, the motif is also an AU-rich, species-specific sequence of 6 nt. Secondary structure predictions of the 5′ NTR of many coronaviruses and arteriviruses (Van den Born et al. 2004) places the 5′ TRS (which is termed the leader TRS, see below) in a hairpin loop structure referred to as the 'leader TRS hairpin' or LTH. This structure is thought to be critical for the process of discontinuous RNA synthesis that takes place during the

production of coronavirus and arterivirus virus minus-strand RNA.

As for many other viruses, the packaging of coronavirus and arterivirus genomes is a process that involves the interaction of virus structural proteins and elements in the genomic RNA. It has been shown for the coronaviruses MHV and BCoV that the packaging of genomic RNA is, at least partially, mediated by an RNA element located at the 3′ end of the replicase gene (Cologna and Hogue 2000; Fosmire et al. 1992). In contrast, the packaging signal of TGEV has been located at the 5′ end of the genome, specifically, within the first 640 nt (Escors et al. 2003). Also, it has been shown that DI-RNA sequences from the 5′ and 3′ NTR of IBV are required for minigenome incorporation into viral particles (Dalton et al. 2001). It is possible that for some coronaviruses, the packaging signal may be comprised of multiple elements but more data are needed to resolve this apparent discrepancy. The packaging signals of arteriviruses have not been mapped.

The 3′ NTR of coronaviruses and arteriviruses comprises 60–150 nt (arteriviruses) or 200–500 nt (coronaviruses and toroviruses) following the last functional ORF and preceding the polyadenylate tract. It can be expected that secondary (or tertiary) structures in the 3′ NTR will have important roles in virus replication. Indeed, at the very 3′ end of the MHV 3′ NTR an extensive complex structure containing multiple stem loops and bulges has been identified and shown to have a role in DI-RNA replication (Liu et al. 2001). This structure incorporates a common motif, $^{G}/_{U}$GGAAGAGC$^{U}/_{C}$, that is found 70–80 nt from the 3′ end of all coronavirus 3′ NTRs examined to date. Also, the 3′ NTR of the MHV and BCoV genomes has been shown to include phylogenetically conserved structures, the 3′ NTR bulged stem loop and the 3′ NTR pseudoknot, that are postulated to represent a molecular switch that controls an, as yet unidentified, aspect of viral RNA synthesis (Goebel et al. 2004). There has been no detailed analysis of the arterivirus 3′ NTR and the only significant nucleotide sequence conservation that has been found in the 3′ NTR is located immediately upstream of the polyadenylate tract. However, it has become clear that RNA signals involved in arterivirus replication extend into the coding sequences in the 3′-proximal region of the genome (Molenkamp et al. 2000). In the case of PRRSV, a so-called 'kissing interaction' between the loop sequences of RNA hairpin structures in the 3′ NTR and the nucleocapsid protein gene was recently found to be crucial for minus-strand RNA synthesis (Verheije et al. 2002b).

OPEN READING FRAMES

The available data suggest that coronavirus and arterivirus genomes contain between six and 14 functional ORFs. The easiest to define are those located toward the 3′ end of the genome, encoding the structural proteins (Figure 2). For coronaviruses and toroviruses, these are the spike (S), membrane (M), and nucleocapsid protein (N) ORFs and for the arteriviruses these are ORFs encoding the major surface glycoprotein (GP_5), three minor glycoproteins (see the section on structural proteins) and the envelope (E), membrane (M), and nucleocapsid (N) proteins. The gene order, 5′-surface glycoprotein(s) ORF(s)/membrane protein ORF/nucleocapsid protein ORF-3′, is conserved in the genome of all coronaviruses and arteriviruses studied to date.

The genomes of coronaviruses contain a fourth structural protein ORF, encoding the E (envelope) protein, located 5′ proximal to the M protein ORF. The genomes of toroviruses and a subset of coronaviruses (e.g. HCoV-OC43, MHV, BCoV) also contain an additional structural protein gene, the hemagglutinin-esterase (HE) ORF. The location of this ORF in coronaviruses is 5′ proximal to the *S* protein gene, and in toroviruses it lies between the *M* and *N* protein genes (Cornelissen et al. 1997; Snijder et al. 1991). It seems that the *HE* gene is not essential and may be converted to a pseudogene; for example, during prolonged passage of virus in tissue culture. Recently, it has been suggested that the SARS-coronavirus (SARS-CoV) genome encodes an additional structural protein (currently known as the 3a protein) that is associated with the spike glycoprotein (Zeng et al. 2004).

In the arteriviruses, the glycoprotein encoded by the PRRSV ORF2a, its homologues encoded by ORF2b in EAV and LDV, as well as the EAV and PRRSV ORF3 and ORF4 glycoproteins, were experimentally identified as minor virion components (de Vries et al. 1992; Faaberg and Plagemann 1995; Meulenberg and Petersen-den Besten 1996; Snijder et al. 1999; van Nieuwstadt et al. 1996; Wieringa et al. 2002). It should be noted that the PRRSV ORF3 glycoprotein was suggested to be a minor structural glycoprotein in a European PRRSV isolate (van Nieuwstadt et al. 1996) but a nonstructural protein in a Canadian isolate (Mardassi et al. 1998). Finally, a small, nonglycosylated envelope protein, encoded by ORF2a in EAV and ORF2b in PRRSV, was detected in virus particles and designated E (for envelope) protein (Snijder et al. 1999; Wu et al. 2001).

In the SHFV genome, the region corresponding to that between ORF 1b and ORF 5 in the other three arteriviruses contains three additional ORFs, covering about 1.6 kb (Smith et al. 1997). On the basis of limited sequence similarities, it was postulated that these SHFV ORFs (named ORFs 2a, 2b, and 3) have arisen from the duplication of the SHFV homologues of EAV/LDV/PRRSV ORFs 2–4 by an RNA recombination event (Godeny et al. 1998). Except for this putative triple gene duplication in SHFV, the genetic composition and gene order of all arteriviruses are identical.

With respect to ORFs that encode nonstructural proteins, the corona- and arterivirus genome is dominated by the replicase gene (also referred to as the RNA polymerase gene or RNA polymerase locus). The

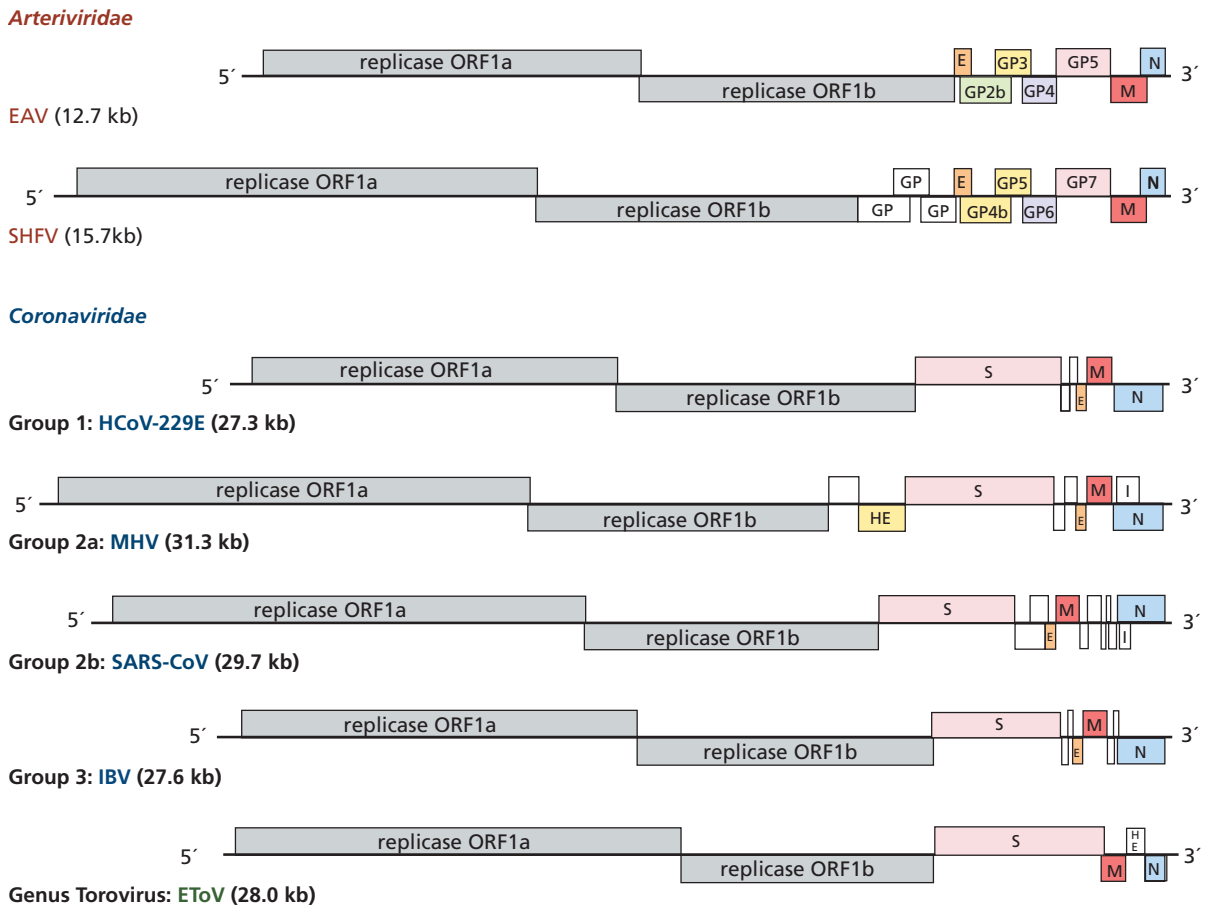

Figure 2 *Genomic structure of coronaviruses, toroviruses, and arteriviruses. The genomic ORFs of representative arteri- and coronaviruses are indicated and the names of the replicase protein and structural protein genes are given. References to the nomenclature of the accessory protein genes can be found in the text. Note that the* HE *protein gene of EToV is a pseudogene but that a functional HE protein is expressed from the BToV genome (Cornelissen et al. 1997). Note also that the arterivirus and coronavirus genomes are drawn to different scales.*

replicase gene consists of two large ORFs encompassing ca. 10 000 nt (arteriviruses) or 20 000 nt (coronaviruses and toroviruses) toward the 5′ end of the genome. These ORFs, ORF1a and ORF1b, overlap in the –1 reading frame. The extent of the overlap varies but invariably encompasses a specific sequence motif, UUUAAAC (or GUUAAAC in the case of EAV), which is referred to as the 'slippery' sequence. Immediately downstream of the slippery sequence is a characteristic RNA structure, the pseudoknot, which can be formed by base pairing. Together, these two elements mediate –1 ribosomal frameshifting during expression of the replicase gene (Brierley 1995). The translation products of the 1a and 1b ORFs are extensively processed by virus-encoded proteinases and, as described below in the section entitled Replicase, produce a large number of proteins that assemble to form a functional replication-transcription complex.

In contrast to the replicase gene, the pattern and arrangement of the remaining nonstructural protein ORFs in the genomes of nidoviruses depend on the virus genus. In the toroviruses and arteriviruses, there is no evidence for nonstructural genes other than the replicase gene. In the coronaviruses there is a diverse and complex pattern of nonstructural genes, even for viruses that are considered to be closely related. The reasons for this complexity are probably two-fold. First, many coronavirus genes seem to encode proteins with accessory functions. Thus, under certain conditions (for example, adaptation and propagation in cultured cells), mutations accumulate which lead to the inactivation and even deletion of these genes. Second, not only divergence from a common ancestor but also RNA recombination seems to have been a major driving force in the evolution of nidoviruses (see also The order *Nidovirales*). It is particularly interesting to note that the SARS-CoV genome encodes a large number of nonstructural proteins that are probably not involved in RNA synthesis and that many of the ORFs encoding these proteins appear to have no counterparts in other coronaviruses.

Replication

A hallmark of the order *Nidovirales* is the production of a set of multiple, subgenomic, 3′ co-terminal mRNAs in

the infected cell. The subgenomic mRNAs are synthesized in nonequimolar amounts but in relatively constant proportions throughout the replication cycle. The structural relationship of these subgenomic mRNAs to the genomic RNA is illustrated for MHV, EAV, and EToV in Figure 3. The replication of the coronavirus MHV and the arterivirus EAV have been studied most intensively and it is assumed that these viruses set a paradigm for other nidoviruses. However, it is also clear that the replication of coronaviruses, toroviruses, and arteriviruses differs, at least in some details. One particularly notable difference is the fact that, in contrast to the subgenomic mRNAs of coronaviruses and arteriviruses, all but the largest of the subgenomic transcripts of toroviruses lack a 5′ leader sequence derived from the 5′-proximal region of the genome (see below).

TRANSCRIPTION

The process by which nidoviruses generate multiple subgenomic mRNAs in the infected cell is complex and not fully understood. A detailed description of the extensive and sometimes conflicting literature in this area is beyond the scope of this chapter and only a summary of the most important aspects is presented here. For more information, the reader is referred to the

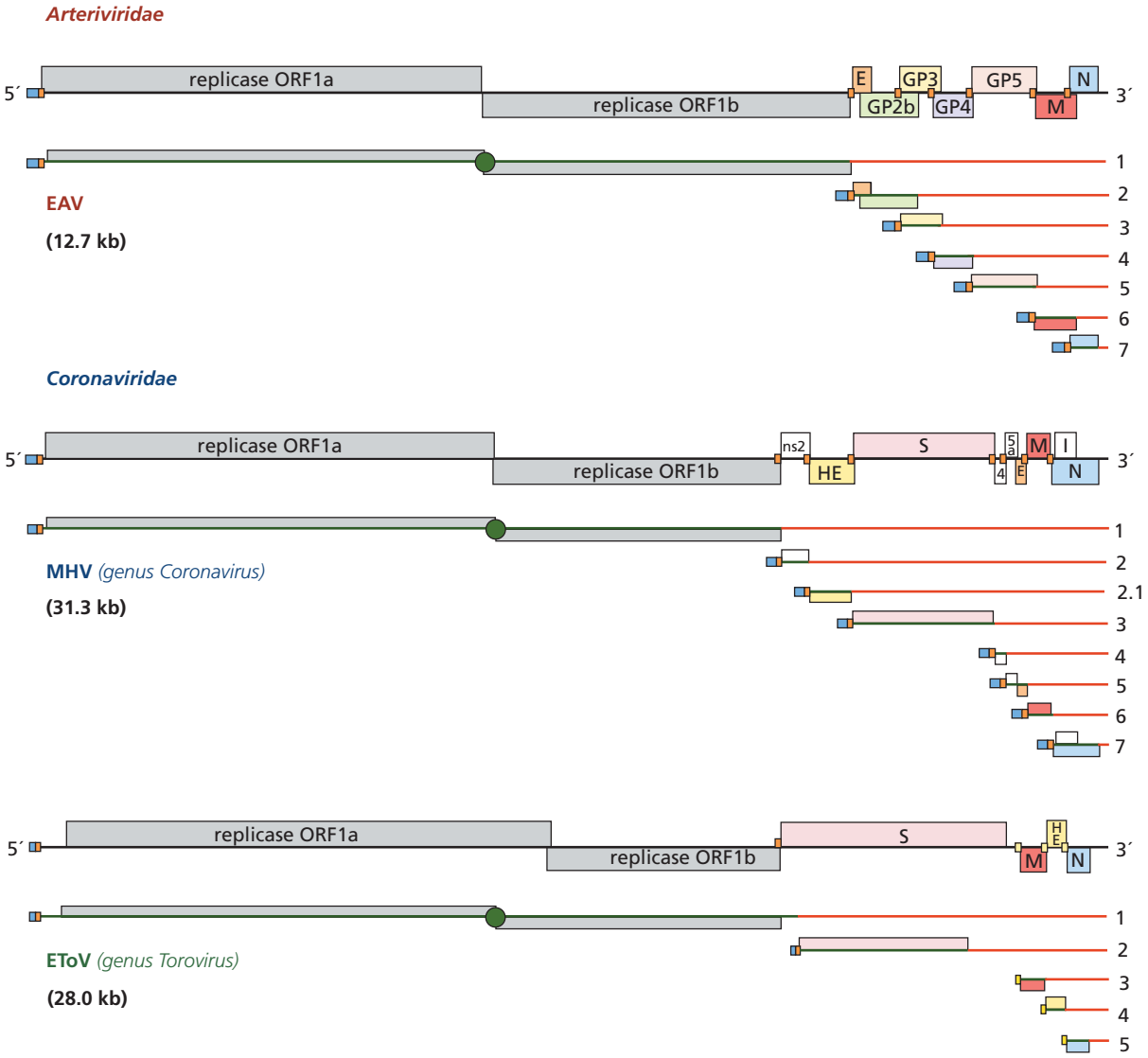

Figure 3 *The genome expression strategies of EAV, MHV, and EToV. The figure shows the genomic organization of the viruses and the structural relationships of the genome-length and subgenome-length mRNAs. The leader sequence and TRS sequences found at the 5′ end of EAV and MHV mRNAs, as well as the genome-length and subgenome-length mRNA2 of EToV are indicated as blue and orange boxes, respectively. The TRS-like element found at the 5′ end of EToV subgenome-length mRNAs 3, 4, and 5 is indicated as a yellow box. The ribosomal frameshifting element found in genome-length mRNA1 is indicated as a green circle and the translated region of each mRNA is indicated in green (the translationally silent regions are indicated in red). Only the translated ORFs are indicated for each mRNA.*

published literature and references therein (Alonso et al. 2002; Baric and Yount 2000; Curtis et al. 2004; Lai and Holmes 2001; Pasternak et al. 2001, 2004; Sawicki et al. 2001; Snijder and Meulenberg 2001; van der Most and Spaan 1995; van Vliet et al. 2002; Zuniga et al. 2004).

- The polycistronic genomic RNA of nidoviruses is infectious.
- Coronavirus and arterivirus subgenomic mRNAs are comprised of non-contiguous sequences. At their 5′ end they have a leader sequence of 65–100 nt (coronaviruses) or 150–210 nts (arteriviruses) that is derived from the 5′-proximal region of the genome. In toroviruses, only the largest subgenomic mRNA contains a short leader sequence derived from the 5′ end of the genome. All smaller torovirus subgenomic mRNAs do not contain this leader sequence.
- The site at which the leader and body of the mRNA are joined is defined by the position of a specific TRS, a conserved regulatory sequence preceding the mRNA body (body TRS). This sequence is also present at the 3′ end of the leader (leader TRS). The TRS is often referred to as the intergenic region or leader-body junction site in the older literature on coronaviruses and arteriviruses, respectively. In the case of all but the largest of the torovirus subgenomic mRNAs, the 5′ end of the mRNA is defined by a similar conserved regulatory sequence, apparently without the subsequent acquisition of the genomic 5′ leader sequence via a discontinuous RNA synthesis event as is found in coronaviruses and arteriviruses.
- The fusion of leader and body sequences of subgenomic mRNAs, as it occurs in coronaviruses and arteriviruses and the largest subgenomic mRNA of toroviruses, relies on a mechanism in which specific base-pairing interactions between RNA sequences and higher order RNA structures play an important role. This process appears to resemble sequence similarity-assisted RNA recombination, as it occurs in many positive strand RNA viruses (Nagy and Simon 1997).

In the coronaviruses:

- The bulk of the mRNA in the cell is produced from transcription intermediates that contain subgenome-length, negative-strand RNA. There is one transcription intermediate per mRNA species.
- The bulk of the subgenome-length negative-strand RNAs has an antileader sequence at their 3′ end.
- The subgenome-length RNAs (positive or negative strand) are not able to initiate a transcription complex.

It has also been shown that EAV-infected cells contain a set of subgenome-length transcription intermediates and that each intermediate contains a subgenome-length minus-strand RNA with an antileader sequence (den Boon et al. 1996).

These observations have been interpreted using two different models that essentially attempt to explain three basic questions. (1) How do subgenome-length templates arise? (2) How are the leader and body sequences of sg mRNAs joined? (3) What is the role of the conserved transcription-regulating sequences during subgenomic RNA synthesis?

The first model proposes that the genomic RNA enters the cell and is translated to produce RNA replicase that is used to synthesize a full-length negative-strand copy of the genome, the so-called antigenome. Transcription is initiated from the 3′ end of the antigenome and a leader transcript, identical to the 5′-proximal part of the genome, is synthesized. This leader RNA is then used to prime transcription of the different mRNA bodies, a process for which base pairing of the leader TRS (the TRS at the 3′ end of the leader RNA) with each of the body TRS complements in the antigenome would be a necessary interaction to synthesize a complete set of subgenomic mRNAs. The essential features of this model are that the discontinuous step takes place during positive-strand RNA synthesis and that the complement of each body TRS acts essentially as a promoter for subgenomic mRNA synthesis.

The second model has a different 'scenario.' Again, the genomic RNA enters the cell and is translated to produce RNA replicase. The RNA replicase is then used, however, to produce not only antigenome but also a set of subgenome-length negative-strand RNAs, the 3′ ends of which are defined by the body TRSs. These negative-strand RNAs then acquire an antileader sequence by a discontinuous 3′ terminal extension process that involves a crucial base pairing interaction between the body TRS complement at the 3′ end of the nascent minus strand and the leader TRS in the 5′ end of the genomic template (Pasternak et al. 2001; Zuniga et al. 2004). Subsequently, the subgenome-length negative-strand RNAs are completed by addition of the antileader sequence (the complement of the genomic leader sequence) and the subgenome-length minus-strand templates carry the RNA replicase into a transcription complex and act as templates for viral mRNA synthesis in the cell. In this model, discontinuous RNA synthesis takes place during the production of negative-strand RNA synthesis and the body TRSs act essentially as attenuators of RNA synthesis (Sawicki and Sawicki 1998).

Using the newly developed reverse genetics systems for arteriviruses (Meulenberg et al. 1998; van Dinten et al. 1997) and coronaviruses (Almazan et al. 2000; Thiel et al. 2001a; Yount et al. 2000), two important aspects of nidovirus discontinuous RNA synthesis were recently addressed (Pasternak et al. 2001; van Marle et al. 1999a; Zuniga et al. 2004). The first, which applies to both transcription models, is the base-pairing interaction between the leader TRS and the complement of the body TRS in the viral negative strand. By site-directed

mutagenesis, it was shown that disruption of this base-pairing interaction can dramatically affect subgenomic mRNA synthesis, whereas the introduction of compensatory mutations could restore subgenomic mRNA synthesis. The second aspect is the origin of the TRS that forms the leader-body junction in the mRNA molecule. Whereas the first model predicts this sequence to be a copy of the leader TRS, the discontinuous minus strand extension model predicts exactly the opposite, i.e. the sequence in the mRNA should be a copy of the body intergenic region or TRS. Using EAV TRS mutants with partial transcriptional activity, it was demonstrated that, indeed, the body TRS determines the sequence at the mRNA leader-body junction. Together with biochemical data obtained for coronaviruses (Baric and Yount 2000; Sawicki et al. 2001), these results provide strong evidence for the discontinuous minus strand extension model.

The remarkable recent data on the structure of torovirus subgenomic mRNAs (van Vliet et al. 2002), and the reported absence of a common 5′ leader sequence in roniviruses (see The order *Nidovirales*), have made it clear that the production of subgenomic RNAs by discontinuous RNA synthesis is not a universal feature of nidoviruses. The largest subgenomic mRNA of toroviruses is most likely generated in a manner similar to that proposed above for coronaviruses and arteriviruses. Namely, a short piece of identical sequence, present both at the 3′ end of the leader and the 5′ end of the body segment, functions as the cross-over site for an RNA recombination-like process during negative-strand RNA synthesis. This process may be enhanced by a predicted RNA hairpin located just upstream of the proposed attenuation site. However, for the remaining subgenomic RNAs of toroviruses, and for those of roniviruses, it was proposed that minus-strand synthesis is attenuated at the conserved ('TRS-like') sequence upstream of the body segment and that these attenuated minus-strand RNAs can function directly as templates for subgenomic mRNA synthesis. It is important that the minus-strands generated in cells infected with roniviruses and toroviruses are investigated in detail, to confirm that subgenome-length minus-strands indeed exist and result from attenuation of minus-strand RNA synthesis. However, if this is the case, it may be that the attenuation of minus strand RNA synthesis is, in fact, the common feature that unites the mechanisms used to produce subgenomic mRNAs in different nidoviruses. This conclusion would then provoke the question of the functions associated with the nidovirus leader sequence and, ultimately, the nature of the cis-acting signals needed for the promotion of nidovirus plus strand RNA synthesis. Finally, it should be noted that, in addition to the interactions described above, many other, as yet undiscovered, RNA–RNA, RNA–protein and protein–protein interactions are expected to play a role in nidovirus subgenomic mRNA synthesis (Pasternak et al. 2000; Shi et al. 2000; Tijms et al. 2001; van Dinten et al. 2000; Yu and Leibowitz 1995; Zhang and Lai 1995).

TRANSLATION

The production of a 3′ co-terminal set of subgenomic mRNAs in the infected cell allows for the expression of genes that are located internally on the nonsegmented genome. By and large, the viral mRNAs are thought to be functionally monocistronic although usually, with the exception of the smallest mRNA, they are structurally polycistronic. This conclusion is based mainly on the in vitro translation of coronavirus mRNAs and conforms to the conventional 'ribosome scanning' model of translational initiation. Thus, taking MHV as an example (Figure 3), the smallest mRNA, mRNA 7, encodes the nucleocapsid protein, mRNA 6 encodes the membrane protein, mRNA 3 encodes the spike protein, and so on. If the genomic sequence is known and the position of the functional body TRSs or intergenic regions has been determined by sequence analysis of mRNAs, a 'translational model' can be deduced for each of the viruses belonging to the order *Nidovirales*. It should be noted, however, that for arteriviruses, toroviruses, and even the majority of coronaviruses, this 'model' still needs to be confirmed by experiment.

The model outlined above describes the basic translational strategy of nidoviruses. However, Figure 3 indicates that there have to be exceptions. For example, MHV has no subgenomic mRNAs that could account for the expression of ORFs 1b, E, or I in this manner. It is now clear that nidoviruses also use a variety of alternative translation strategies to express some of their gene products.

In several coronaviruses (Brierley 1995; Brierley and Pennell 2001; Thiel et al. 2003), EToV (Snijder et al. 1990) and EAV (den Boon et al. 1991) the region of RNA that encompasses the overlap of ORFs 1a and 1b is able to mediate a high frequency of –1 ribosomal frameshifting. Thus, ORF1b is expressed as a fusion protein with the ORF1a gene product. As described above, ribosomal frameshifting is mediated by a 'shifty' sequence, the actual position at which frameshifting takes place, and an RNA pseudoknot which is believed to slow or halt the ribosome as it translates this sequence. The most likely mechanism of frameshifting is simultaneous slippage of two ribosome-bound tRNAs present in the aminoacyl and peptidyl sites of the ribosome during decoding of the 'shifty' sequence. The mechanistic aspects of nidovirus –1 ribosomal frameshifting have been analyzed in some detail (Brierley et al. 1997; Liphardt et al. 1999; Marczinke et al. 2000; Napthine et al. 1999).

The subgenomic mRNAs encoding the E proteins of MHV (mRNA 5) and IBV (mRNA 3) contain two and three ORFs, respectively, in their 5′ unique regions. The

E ORF is 3′-proximal in both cases and the mRNAs are functionally bi- and tricistronic, respectively (Budzilowicz and Weiss 1987; Liu et al. 1991). Therefore, the expression of the E ORF has to involve the internal initiation of protein synthesis. The unique region of both mRNAs contains an element that is able to mediate the internal entry of ribosomes and initiate protein synthesis in a cap-independent manner (Jendrach et al. 1999; Liu and Inglis 1992). This element has not yet been characterized in detail. A similar element has also been recognized in the mRNA 3 of certain TGEV strains (O'Connor and Brian 2000). In the arterivirus group, mRNA 2 of EAV was proposed to be a functionally bicistronic mRNA from which both the GP_{2b} glycoprotein and the newly discovered E protein are expressed (Snijder et al. 1999). Likewise, mRNA 2 of LDV and PRRSV and mRNA 4 of SHFV are probably used to express the corresponding genes of these other arteriviruses. Another mRNA of SHFV, mRNA 2, was proposed to be functionally bicistronic but, in this case, the expression of two ORFs (2a and 2b) from this mRNA remains to be demonstrated (Godeny et al. 1998).

Sequence analysis of the nucleocapsid protein gene of SARS-CoV, MHV, and related coronaviruses (Parker and Masters 1990; Snijder 2003a) has revealed an ORF, the internal or I ORF, which, in BCoV, is expressed in infected cells and encodes a 23-kDa, membrane-associated protein (Senanayake et al. 1992). The internal protein is translated from the same mRNA as the nucleocapsid protein, and it has been suggested, mainly on theoretical grounds, that the initiation of internal protein synthesis conforms to the 'leaky ribosomal scanning' model proposed by Kozak (Kozak 1999). Similar considerations apply to some of the small ORFs in the 3′-proximal region of the SARS-CoV genome (Snijder et al. 2003a).

Nonstructural polypeptides

REPLICASE

The replicase gene of viruses belonging to the order *Nidovirales* encodes two polyproteins of extraordinary size and complexity. The translation of ORF1a theoretically produces a polyprotein, pp1a, of 450–500 kDa (coronaviruses) or 190–260 kDa (arteriviruses). Alternatively, as described above, –1 ribosomal frameshifting is used to express an ORF1ab-encoded fusion protein, pp1ab, with a potential molecular weight of 750–800 kDa (coronaviruses) or 350–420 kDa (arteriviruses). Moreover, the polyproteins themselves are not the end products of replicase gene expression. Instead, they are co- and post-translationally cleaved by viral proteinases that are part of these polyproteins to produce a complex array of functional gene products.

Sequence analysis of the predicted nidovirus replicase polyproteins has revealed a number of putative functional domains that appear to have been conserved during evolution (Brown and Brierley 1995; de Vries et al. 1997; Gorbalenya 2001; Gorbalenya et al. 1989a; Snijder et al. 2003a; Snijder and Spaan 1995; Ziebuhr 2004a,b). These domains and their approximate positions are illustrated in Figur 4.

The ORF1a-encoded part of nidovirus pp1a and pp1ab polyproteins contains several proteinase domains, which are of two types. First, papainlike cysteine proteinase (PLpro) domains are located in the N-proximal half of the polyprotein. These may be single domains (SARS-CoV) or multiple domains (e.g. MHV and PRRSV). Nidovirus PLpro domains are generally linked with zinc-finger domains (Figure 4) (Snijder et al. 1995; Herold et al. 1999; Tijms et al. 2001) and have similar (sometimes overlapping) substrate specificities (Ziebuhr et al. 2001). At most PLpro cleavage sites, small aliphatic residues are conserved at the P1 and P1′ positions (Ziebuhr et al. 2000). Second, towards the C-terminal end of pp1a, a 3C-like proteinase domain (3CLpro) is found. In coronaviruses (and roniviruses), the active site nucleophile of this proteinase is a cysteine residue, whereas its arterivirus counterpart contains an active site serine (Hegyi et al. 2002; Liu and Brown 1995; Lu et al. 1995; Snijder et al. 1996; Ziebuhr et al. 1997, 2003). The 3CLpros of coronaviruses and arteriviruses are members of the large superfamily of chymotrypsin-like proteolytic enzymes that share a two-β-barrel fold (Gorbalenya et al. 1989b). There are significant structural differences between the coronavirus and arterivirus 3CLpros, for example, with respect to their sizes, subdomain organizations, and principal catalytic residues (Anand et al. 2002, 2003; Barrette-Ng et al. 2002). Despite these differences, the 3CLpros of coronaviruses and arteriviruses have similar substrate specificities (Hegyi and Ziebuhr 2002; Snijder et al. 1996; Thiel et al. 2003; Wassenaar et al. 1997; Ziebuhr et al. 1995, 2000). Thus, both enzymes cleave at sites that have a glutamine (coronaviruses) or glutamic acid (arteriviruses) at the P1 position and a small residue (usually glycine, alanine, or serine) at the P1′ position.

An important secondary function of pp1a appears to be the anchoring of the nidovirus replication complex to intracellular membranes (see below). Hydrophobic domains that flank the 3CLpro at either side are conserved in the pp1a proteins of all nidoviruses (Gorbalenya 2001; Snijder and Meulenberg 1998; Ziebuhr 2004a) (Figure 4). In the ORF1b-encoded product, which is expressed only as part of pp1ab, four domains have been conserved in all nidoviruses. The first is a RNA-dependent RNA polymerase module (RdRp) that is also found in a wide variety of putative RNA-dependent RNA polymerases. The alteration of the polymerase 'core' motif from glycine–aspartic acid–aspartic acid (GDD) to serine–aspartic acid–aspartic acid (SDD) is a feature that seems to be characteristic for the nidoviruses (Gorbalenya et al. 1989a). The second is a

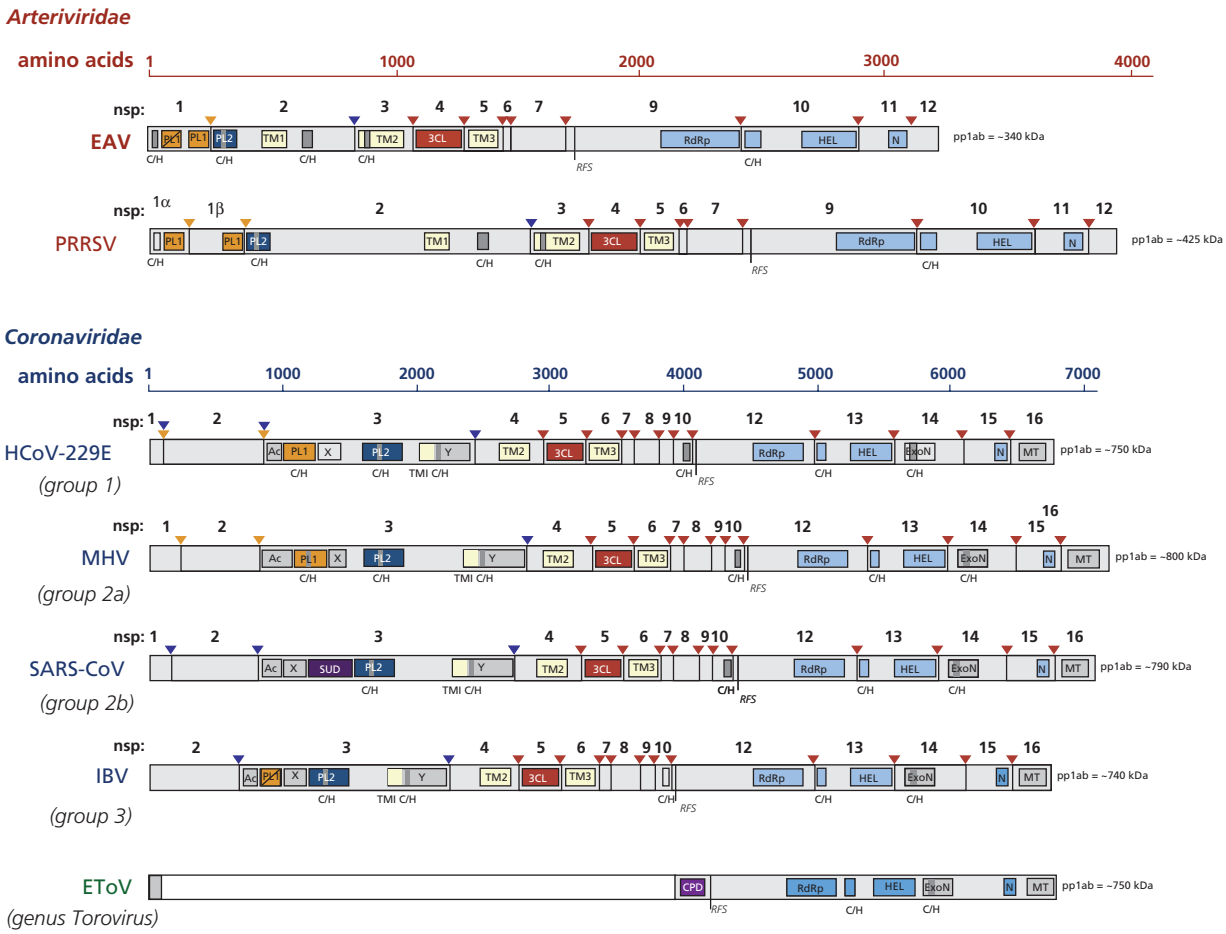

Figure 4 *The replicase gene organization of representative arteriviruses and coronaviruses. The replicase gene organization is depicted in the form of the polyprotein pp1ab. The delineation of amino acids encoded in ORF1a and ORF1b is indicated as RFS (ribosomal frameshift) and arrows represent sites in pp1ab that are cleaved by papainlike proteinases (orange and blue) or the main (3CL) proteinase (red). The proteolytic cleavage products are numbered and within the cleavage products, the location of domains that have been identified as structurally or functionally related are highlighted. These include domains with conserved Cys and His residues (C/H), putative transmembrane domains (TM), domains with conserved features (Ac, X, and Y), and domains that have been associated with proteolysis (PL1, PL2, and 3CL), RNA-dependent RNA synthesis (RdRp), helicase (HEL), exonuclease (ExoN), uridylate-specific endoribonuclease (N), and methyl transferase (MT) activities. Note that the arterivirus and coronavirus polyproteins are drawn to different scales.*

cysteine–histidine-rich motif that has been proposed to function as multinuclear zinc-finger (ZF) domain and was implicated in viral RNA synthesis (van Dinten et al. 1997, 2000). The third, and part of the same protein as the ZF domain, is a helicase domain. The location of the helicase domain downstream of the polymerase module in the polyprotein is unusual and characteristic of nidoviruses. And fourth is a conserved domain near the C-terminal end of pp1ab that was recently identified as a uridylate-specific endoribonuclease (NendoU) (Ivanov et al. 2004b; Snijder et al. 2003a). There are a number of (putative) additional RNA-processing activities (Snijder et al. 2003a) that appear to be differentially conserved among nidoviruses, indicating that the enzymology of viral RNA synthesis and/or some virus–host interactions may differ among the various nidovirus families and genera. Thus, for example, all nidoviruses (except for the arteriviruses) have been predicted to encode 3′-to-5′ exoribonuclease and 2′-*O*-ribose methyltransferase functions in ORF1b (Figure 4). Coronaviruses (but not arteriviruses and roniviruses) also encode a putative ADP-ribose 1″-phosphatase activity that is conserved in only very few plus-strand RNA viruses (Gorbalenya et al. 1991; Snijder et al. 2003a). Finally, toroviruses and group 2 coronaviruses (excluding SARS-CoV) encode a putative cyclic phosphodiesterase that is located at the C-terminus of the torovirus ORF1a encoded polyprotein, whereas the coronavirus homologue is expressed from a sg RNA (rather than being part of the replicase polyprotein itself) (Snijder et al. 2003a). The activities of most of the nidovirus RNA-processing enzymes remain to be characterized.

Compared with other positive-strand RNA viruses, the biochemical analysis of functions encoded in the

nidovirus replicase is still at an early stage (for a comprehensive review, see Ziebuhr (2004a)). There have been occasional reports of RNA-dependent RNA polymerase activity in subcellular fractions of coronavirus-infected cells or coronavirus-infected cells permeabilized with lysolecithin. However, there is no evidence that these systems can initiate RNA synthesis and their application has been very limited. Coronavirus RdRps and their nidovirus relatives have been classified as an outgroup of superfamiliy 1 RdRps (Koonin 1991). A molecular model for the coronavirus RdRp domain, which resides in the C-terminal two-thirds of nsp12 (Gorbalenya et al. 1989a) (Figure 4), has recently been reported (Xu et al. 2003). Furthermore, it has been proposed that the MHV nsp12 interacts through a protein segment located upstream of the C-terminal RdRp core domain with ORF1a-encoded proteins, such as nsp4 ($3CL^{pro}$), nsp8, and nsp9 (Brockway et al. 2003). Crystal structure analysis of SARS-CoV nsp9 and biochemical data have shown that nsp9 is a single-stranded RNA-binding protein (Egloff et al. 2004; Sutton et al. 2004) and genetic data obtained for MHV have implicated nsp10 in minus-strand synthesis (Siddell et al. 2001). Taken together, the data suggest that the small proteins encoded by the 3′-proximal region of ORF1a may have co-factor functions for the RdRp and be directly or indirectly involved in viral RNA synthesis.

In the replicase polyprotein, the RdRp domain is C-terminally flanked by a multidomain protein, called nsp10 in arteriviruses and nsp13 in coronaviruses, which contains N-terminal ZF and C-terminal helicase domains (Figure 4). Nidovirus helicases have been shown to have NTPase, RNA 5′-triphosphatase as well as RNA and DNA duplex-unwinding activities (Bautista et al. 2002; Heusipp et al. 1997; Ivanov et al. 2004a; Ivanov and Ziebuhr 2004; Seybert et al. 2000a, 2005; Tanner et al. 2003). Coronavirus and arterivirus helicases appear to act processively and unwind their substrates with 5′-to-3′ polarity (Ivanov et al. 2004a; Ivanov and Ziebuhr 2004; Seybert et al. 2000a, 2005). The N-terminal ZF was recently shown to be required for the enzymatic activities of nidovirus helicases (Seybert et al. 2005). Both the in vitro activities of nsp10/nsp13 and RNA replication of EAV in tissue culture were found to be extremely sensitive to substitutions of conserved Cys and His residues in the ZF domain (Seybert et al. 2005; van Dinten et al. 2000). Furthermore, point mutations that selectively inactivate EAV sg RNA synthesis (van Dinten et al. 1997, 2000; van Marle et al. 1999b) were identified and mapped to the region connecting the ZF and helicase domains of the protein. The flexibility of this spacer was postulated to be essential for the function of the protein in sg RNA synthesis. Furthermore, the most N-terminal cleavage product of the EAV replicase, nsp1, was implicated in the regulation of sg RNA synthesis. The protein was shown to be dispensable for genome replication, but absolutely required for sg RNA synthesis (Tijms et al. 2001).

Recently, manganese-dependent, uridylate-specific endoribonuclease activities (NendoU) were established for nsp15 of SARS-CoV and HCoV-229E (Ivanov et al. 2004b; Snijder et al. 2003a; Bhardwaj et al. 2004) (Figure 4). The nsp15 activity was shown to be critically involved in coronavirus RNA synthesis. Although nsp15 is capable of cleaving both single-stranded and double-stranded RNA in vitro, the biologically relevant substrate was proposed to be double-stranded RNA, which is cleaved more effectively and specifically at GU and GUU sequences (Ivanov et al. 2004b). 2′-*O*-ribose-methylated RNA substrates proved to be resistant to cleavage by nsp15, indicating that the putative 2′-*O*-ribose methyltransferase domain residing adjacent to NendoU in the coronavirus replicase polyprotein may control or modulate the ribonucleolytic activity of nsp15.

Also, there are an ever-increasing number of reports on the characterization of arterivirus and coronavirus proteinases and the proteolytic processing of the replicase gene products. A detailed review of these data is beyond the scope of this chapter (for recent reviews, see Ziebuhr 2004a; Ziebuhr et al. 2000). Briefly, the papain-like proteinases seem to be largely responsible for the processing in *cis* of N-terminal and N-proximal products from the ORF1a-encoded region of the replicase polyproteins, whilst the 3C-like proteinases are likely to function mainly in *trans* and are responsible for self-excision and processing of the pp1a and pp1ab polyproteins downstream of the proteinase domain itself (Figure 4). Because of their key role in polyprotein processing, nidovirus 3C-like proteinases are considered to be attractive targets for antiviral (in particular, anti-SARS) drug development (Anand et al. 2003; Davidson and Siddell 2003). Over the past years, significant progress has been made in the functional and structural characterization of these enzymes. Thus, for example, crystal structures of several coronavirus and arterivirus 3C-like proteinases have been reported and the binding modes of proteinase substrates have been elucidated by X-ray structure analysis of inhibitors bound to the active sites of TGEV and SARS-CoV 3CLpros, respectively (Anand et al. 2002, 2003; Barrette-Ng et al. 2002; Shi et al. 2004; Yang et al. 2003). The structural information provides an excellent basis for rational drug design and, because of the conserved substrate specificity of coronavirus 3CLpros, even the development of broad-spectrum proteinase inhibitors suitable for the therapy of human and animal coronavirus infections may be a tractable goal (Anand et al. 2003; Hegyi and Ziebuhr 2002; Thiel et al. 2003). Also, other nidovirus replicative proteins, such as the RNA-processing enzymes, RNA-binding proteins, RdRp, and the helicase, might be targets for selective antiviral therapies (Davidson and Siddell 2003), although their characterization is still at an early stage.

INTRACELLULAR LOCALIZATION OF THE REPLICASE

Over the past decade, the association of their replication complex with intracellular membranes (of different origin) has emerged as a common feature of positive-strand RNA viruses that replicate in eukaryotic cells (see, for example, Ahlquist et al. 2003; Mackenzie et al. 1999; Rust et al. 2001; Salonen et al. 2003) and references therein). The association of viral replicases with (modified) cellular membranes is thought to be an important advantage in creating a suitable (micro)-environment for viral RNA synthesis. Furthermore, the formation of membrane-bounded complexes may aid in shielding double-stranded RNA replication intermediates from double-stranded RNA activated host defense mechanisms such as RNA interference and interferon-induced pathways.

The subcellular localization of the replicase has only been studied in detail for the arterivirus EAV and for the coronavirus MHV. In the case of EAV, most replicase subunits (including RdRp and helicase) localize to virus-induced double-membrane structures that are likely derived from the endoplasmic reticulum (ER) (Pedersen et al. 1999; van der Meer et al. 1998). These paired membranes and double membrane vesicles (DMV) were shown previously to be induced by arterivirus infection. DMVs that strikingly resemble those seen in virus-infected cells could also be induced by expressing a part of the EAV pp1a protein (nsp2 and nsp3) (Pedersen et al. 1999; Snijder et al. 2001), indicating that their generation does not depend on viral replication or the presence of ORF1b-encoded protein functions. All cleavage products of the EAV pp1a and pp1ab polyproteins (with the exception of a fraction of nsp1; Tijms et al. 2002) and de novo synthesized RNA (as visualized by BrUTP labeling) colocalize to membranes in the perinuclear region of infected cells (Pedersen et al. 1999; van der Meer et al. 1998). Also, a substantial proportion of the EAV nucleocapsid protein was found in this region of the cell, but its involvement in genome replication or subgenomic (sg) RNA synthesis is highly unlikely, since nucleocapsid gene expression can be inactivated without a significant effect on either process (Molenkamp et al. 2000; Tijms et al. 2002).

For the only coronavirus studied in some detail thus far, MHV, the situation is less clear-cut, which may be partially due to the use of (i) a variety of cell lines, (ii) different cellular marker proteins, and (iii) antisera recognizing different MHV replicase subunits (compare, for example, Bost et al. 2001; Denison et al. 1999; Gosert et al. 2002; Shi et al. 1999, van der Meer et al. 1998). The majority of the MHV replicase subunits were found to be associated with intracellular membranes. During the peak of MHV RNA synthesis, key nonstructural proteins, like RdRp and helicase, de novo synthesized RNA, and also the viral nucleocapsid protein, were found to colocalize in punctate cytoplasmic foci (Denison et al. 1999; Shi et al. 1999; van der Meer et al. 1998). Ultrastructural studies of the replicase complex in MHV-infected cells have remained equivocal thus far. While the results of one study pointed mainly to membranes of (late) endosomal origin as the site of viral replication (van der Meer et al. 1998), a more recent study (Gosert et al. 2002) identified virus-induced double-membrane structures, resembling those implicated in the RNA synthesis of arteriviruses. Although all reports support the membrane association of MHV (and more recently also SARS-CoV) RNA synthesis, a variety of different cellular compartments (including ER, Golgi complex, endosomes, and autophagosomes) have been implicated in viral RNA synthesis (Ivanov et al. 2004a; Prentice et al. 2004a,b). Furthermore, the localization of, in particular, the MHV helicase protein, was reported to change later in infection (Bost et al. 2001).

Although further studies are necessary before definite conclusions can be reached, it does appear that major differences between arterivirus and coronavirus replication complexes may exist. In general, it is unclear how the nidovirus replicase associates with membranes. The most relevant questions are: (i) the mechanism of membrane insertion of the replicase subunits that anchor the complex, (ii) the coordination of proteolytic processing and membrane association, (iii) the role of protein–protein interaction (between viral proteins and possibly between viral and host proteins), and (iv) the interaction with host membranes, including the formation of double membranes and DMVs.

ACCESSORY PROTEINS

In the case of the arterivirus EAV, it has been shown that none of the ORFs downstream of the replicase gene are required for genome replication or subgenomic (sg) mRNA synthesis (Molenkamp et al. 2000). Each of these genes seems to encode a structural protein that is essential for the production of infectious progeny virus (Wieringa et al. 2003a). In the case of coronaviruses, there are a variety of proteins of unknown function encoded downstream of the replicase gene. Little more is known about them than their predicted molecular weights and putative structural features deduced from computer-assisted analyses (Brown and Brierley 1995) (see Figure 4). They do not appear to be essential for coronavirus sg RNA synthesis (Thiel et al. 2001b). The expression of some of the accessory proteins has been confirmed in coronavirus-infected tissue culture cells (reviewed in Brown and Brierley 1995) and evidence of post-translational modification (e.g. phosphorylation of the BCoV ORF2 protein) (Cox et al. 1991) has been obtained. Recently, the crystal structure of the SARSCoV ORF7a protein revealed an Ig-like fold for this intracellular membrane protein (Nelson et al. 2005). The picture, however, remains very incomplete. Despite

this lack of information, two interesting observations have been made. First, for some coronaviruses, variants have been obtained that are defective for the expression of one or more of these proteins but are able, nevertheless, to replicate normally in tissue culture. Thus, for example, MHV variants defective for the expression of the ORF2, ORF4 or ORF5a gene products have normal properties in vitro (Schwarz et al. 1990; Yokomori and Lai 1991). Consistent with this idea, other studies show that deletion of the accessory genes from the MHV or FIPV genomes, result in replication-competent viruses that are, nevertheless, attenuated in mice and cats respectively, suggesting that these proteins provide a selective advantage in the natural host (de Haan et al. 2002b; Haijema et al. 2004). Second, it seems that the functions carried by some proteins of the *Coronaviridae* may be looked upon as 'genetic cassettes.' Thus, for example, the MHV CPDase domain is encoded by ORF2 whereas its EToV homologue is encoded at the 3′ end of the ORF1a (Snijder et al. 1991). Another example of this concept is the proteins encoded by ORFs 4a and 4b of HCoV-229E, which seem to be equivalent to the single protein encoded by ORF3 of porcine epidemic diarrhoea virus (PEDV) (Duarte et al. 1994). In a similar vein, SARS-CoV has been predicted to encode as many as eight accessory proteins. Also in this case, it seems that (at least some of) these proteins are not essential in tissue culture. Thus, for example, a deletion in ORF7b was observed upon passage of the Frankfurt 1 strain of SARS-CoV in tissue culture (Thiel et al. 2003). Furthermore, SARS-CoV isolates obtained from SARS patients differed from SARS-CoV-like viruses isolated from Himalayan palm civets (and one human isolate, GZ01) by a 29-nucleotide deletion in ORF8 (Guan et al. 2003), generating two smaller ORFs, 8a and 8b. The biological significance of the deletion (e.g. in terms of pathogenesis and/or adaptation to the human host) remains to be investigated. Thus far, only one of the SARS-CoV accessory proteins, the ORF3a protein, has been characterized in some detail (Yu et al. 2004; Zeng et al. 2004). Sequence comparison of diverse SARS-CoV isolates suggests a correlation of specific substitutions in the ORF3a and spike protein genes, suggesting that the biological function of the ORF3a protein is closely associated with spike protein functions (Zeng et al. 2004). The study suggests that the ORF3a protein associates with the SARS-CoV spike protein by intermolecular disulfide bridges and it is proposed that the ORF3a protein may be an additional structural protein.

Structural polypeptides

Whereas the RNA replicase provides clear evidence of an evolutionary link between viruses of the order *Nidovirales*, the structural proteins indicate a complex phylogeny, probably involving both recombination and evolution from a common ancestor. For example, the nucleoproteins of corona-, toro-, and arteriviruses show no sign of any relationship (apart from the features common to almost all nucleoproteins) whilst the envelope proteins of coronaviruses and toroviruses are clearly related both structurally and functionally. The structural proteins of coronaviruses, toroviruses, and arteriviruses are listed in Table 2.

NUCLEOCAPSID AND MEMBRANE POLYPEPTIDES

The nucleoprotein of corona-, toro-, and arteriviruses is a basic phosphoprotein that associates with the genomic RNA to form the nucleocapsid structure. However, the

Table 2 *Structural proteins of corona-, toro-, and arteriviruses*

Protein		Molecular weight (kDa)			Glycosylation	Abundance	Comments
		Corona	Toro-	Arteri-			
Nucleoprotein	N	50–69	19	12	No	Major	Basic, phosphorylated
Membrane	M	23–35	27	16	Only coronavirus	Major	Multi-membrane-spanning protein
Spike	S	180–220	200	...[a]	Yes	Major	Peplomer structure with coiled-coil elements
Envelope	E	9–12	...	7–9	No	Minor	Integral membrane protein
Hemagglutinin esterase	HE	65	65	...	Yes	Major	Subset of coronaviruses, disulfide-linked homodimer
Glycoprotein	GP_2	...	...	23–28	Yes	Minor	Part of $GP_2/Gp_3/GP_4$ heterotrimer
Glycoprotein	GP_3	...	...	18–29	Yes	Minor	Part of $GP_2/Gp_3/GP_4$ heterotrimer
Glycoprotein	GP_4	...	...	16–20	Yes	Minor	Part of $GP_2/Gp_3/GP_4$ heterotrimer
Glycoprotein	GP_5	...	...	30–45	Yes	Major	Disulfide-linked heterodimers with M

a) No such protein described.

nature of this association must be very different for the three groups of viruses. First, the sizes of the nucleocapsid proteins differ markedly and, secondly, the three groups of virus structures have very different morphologies. In a relaxed configuration, the coronaviruses and toroviruses have a helical nucleocapsid, which is unique among positive-strand RNA enveloped viruses, whilst the arteriviruses have an isometric (cubical) nucleocapsid. Recent data suggests that the coronavirus nucleocapsid protein binds to RNA with little sequence specificity (Narayanan et al. 2003a). Sequence comparisons and some partial structural information on coronavirus and arterivirus nucleocapsid proteins suggest a multidomain structure (Huang et al. 2004; Laude and Masters 1995), but the specific functions of the various domains remain to be elucidated. The partial crystal structure of the PRRSV N protein suggests that it may represent a new class of capsid-forming protein, with domains distinctly different from those of other known enveloped viruses (Doan and Dokland 2003). Both coronavirus and arterivirus nucleocapsid proteins assemble into higher order structures in virus-infected cells and when expressed as recombinant proteins (He et al. 2004; Narayanan et al. 2003b; Surjit et al. 2004; Wootton and Yoo 2003). Also, it has been noted that both the coronavirus and arterivirus nucleocapsid proteins have nucleo-nucleolar transport signals and a fraction of the protein synthesized in infected cells localizes to the nucleolus (Hiscox et al. 2001; Rowland et al. 2003; Tijms et al. 2002). However, it is not clear if this phenotype is linked, for example, to the viral modulation of nucleolar function, or simply a case of fortuitous molecular mimicry.

Coronaviruses and arteriviruses both have a multi-membrane-spanning protein, M. For most M proteins, it is thought that one of the hydrophobic transmembrane domains functions as an internal signal sequence, although for the M proteins of TGEV, FCoV, and CCoV an amino terminal signal sequence is present. Transient expression studies have revealed that the M protein also carries a signal for retention in the Golgi apparatus (coronavirus) or endoplasmic reticulum (torovirus and arterivirus) of the cell. Only when it is associated with other components of the virion does the M protein proceed beyond these compartments. Despite the striking similarities in structure of coronavirus and arterivirus M proteins, there are also significant differences. For example, only the M proteins of coronaviruses are glycosylated. In BCoV, MHV, and HCoV-OC43, *O*-glycosylation has been demonstrated; for the other coronaviruses, *N*-glycosylation is found (de Haan et al. 2002a; Rottier 1995).

Recent studies have shed some light on the role of the M protein in coronavirus budding. It appears that the coronavirus M protein may exist in two conformations. In TGEV, approximately two-thirds of the molecules are triple-membrane spanning and have a N_{exo}-C_{endo} configuration: the remaining molecules are quadruple-membrane spanning and have a N_{exo}-C_{exo} configuration. The molecules with an intravirion carboxyl-terminus are thought to interact with the nucleocapsid structure (Escors et al. 2001). Consistent with this conclusion, studies on MHV have suggested that the M protein interacts with the nucleocapsid structure, either by an M protein–N protein interaction (Kuo and Masters 2002) or by specifically binding to the packaging signal in the genomic RNA (Narayanan et al. 2003a). In any case, the M protein would link the incorporation of nucleocapsid structures to the virus budding process. Finally, de Haan and colleagues have shown that there are strong homotypic interactions between MHV M proteins and they suggest that these lateral interactions within the viral membrane generate the major driving force for envelope formation (de Haan et al. 1998, 2000). As discussed below, the coronavirus M protein is also believed to interact with the spike glycoprotein. Very little is known about the functional role(s) of the arterivirus M protein. It has been shown that the EAV and LDV M and GP_5 proteins are associated in virus particles as disulfide-linked heterodimers and that covalent linkage is essential for virus infectivity (Snijder et al. 2003b).

The coronavirus envelope protein, E, is predicted to have a hydrophobic region, located in the amino terminal half of the polypeptide, which is flanked by charged residues. Adjacent to this hydrophobic domain is a cysteine-rich region, followed by an abundance of charged residues in the carboxy half of the polypeptide (Siddell 1995). These features are indicative of an integral membrane protein, and a membrane location for the E proteins of IBV, TGEV, and MHV virions has been demonstrated. Recently, a cation-selective ion channel function has been demonstrated for the SARSCoV E protein (Wilson et al. 2004). The E protein, together with the M protein, has been shown to facilitate coronavirus particle formation (Bos et al. 1996; Fischer et al. 1998; Vennema et al. 1996) and recent data has shown that the E protein accumulates in and induces curvature into the pre-Golgi membranes where coronaviruses assemble by budding (Arbely et al. 2004; Raamsman et al. 2000). Although there is clear evidence for the importance of the E protein in the assembly of coronaviruses, it is not absolutely essential (Kuo and Masters 2003). The E protein is also reported to induce apoptosis (An et al. 1999). The identification of the E protein of arteriviruses has further extended the collection of structural proteins of this virus group (Snijder et al. 1999; Wu et al. 2001). The hydropathy profile of this small hydrophobic non-glycosylated protein suggests that it is an integral membrane protein with an uncleaved signal-anchor sequence in the central part of the molecule.

MAJOR GLYCOPROTEINS

The coronavirus and torovirus spike glycoproteins have a number of features in common: a large number (18–35) of potential *N*-glycosylation sites, an N-terminal

signal sequence, a C-terminal transmembrane anchor domain, putative heptad repeat domains and, with the exception of some coronaviruses (e.g. TGEV, FCoV, SARSCoV, and HCoV-229E), a centrally located, arginine-rich cleavage site for a 'trypsinlike' proteinase. When post-translational cleavage takes place (which varies according to the virus and the cell) the quaternary structure of the protein is an oligomer (dimer or trimer), each monomer being comprised of two subunits, S1 (amino terminal) and S2 (carboxyl terminal) that are noncovalently linked. The S1 subunit is thought to be a globular protein that comprises the bulbous head of the peplomer. The heptad repeat domains, which are located in the S2 subunit, are thought to generate intrachain coiled-coil secondary structure (more precisely, antiparallel six helix bundles) and provide the stalk of the peplomer with its rigid, elongated structure (Cavanagh 1995). More detailed structural analysis of the coronavirus spike protein fusion core suggests that it shares significant similarity with both the low pH-induced conformation of influenza hemagglutinin and the fusion core of human immunodeficiency virus (HIV) gp41 (Bosch et al. 2003; Ingallinella et al. 2004; Tripet et al. 2004; Liu et al. 2004; Xu et al. 2004a, b). This suggests that the mechanisms for the viral fusion of coronaviruses might be quite similar to those of other class I virus fusion proteins. The MHV S protein is acylated, and it has been speculated that fatty acid chains are associated with a cluster of cysteine residues located in the C-terminal, hydrophilic tail of the protein. The same region has been implicated in spike protein-mediated membrane fusion (Chang et al. 2000; Ye et al. 2004). Finally, the assembly of the coronavirus spike protein into virions involves an interaction between the charge-rich carboxyl-terminal region of the endodomain, most probably with the membrane protein (de Haan et al. 1999).

The spike protein of coronaviruses has at least two major functions in virus replication. First, it binds to cellular receptors to initiate the infection process. The S protein–receptor interaction is complex and may involve the recognition of carbohydrates as well as protein. However, the major class of protein receptor for MHV has been identified as isoforms of the Bgp glycoprotein, a group of murine, carcinoembryonic antigen (CEA)-related glycoproteins in the biliary glycoprotein subgroup (Dveksler et al. 1993; Hemmila et al. 2004). Similarly, the metalloproteinase, aminopeptidase N (also called CD13 in humans) has been identified as the protein receptor for TGEV and HCoV-229E (Delmas et al. 1992; Yeager et al. 1992). Finally, angiotensin-converting enzyme 2 (ACE2) has been identified as a functional receptor for the SARS-CoV (Li et al. 2003; To and Lo 2004). It seems clear that the coronavirus spike protein is a major determinant of cell and species tropism (Casais et al. 2003; Haijema et al. 2003). The second major function of the S protein is to mediate membrane fusion. This activity is necessary for the fusion of viral and cellular membranes at the cell surface during the initial stages of infection, and it may also occur later in infection by extensive formation of syncytia. In the coronaviruses, virus entry is thought to take place at the cell surface or within endosomes prior to their acidification. However, mutants of MHV that depend on low pH for fusion activity have been isolated (Gallagher et al. 1991) and SARS-CoV S-pseudotyped lentiviral vectors also display pH-dependent fusion (Yang et al. 2004a). Cleavage of the S protein seems to enhance its cell-to-cell fusion activity but is not required for virus infectivity (de Haan et al. 2004; Gombold et al. 1993). Bosch and colleagues have recently identified a putative fusion peptide in the S protein of a number of coronaviruses (Bosch et al. 2004).

The arterivirus major glycoprotein, GP_5 is sometimes considered as 'equivalent' to the coronavirus S protein but its structure is completely different and it appears to lack some of the important biological functions ascribed to the coronavirus spike glycoprotein. Recent analyses suggest that it is a polytopic class I protein; after removal of the signal peptide, the processed protein consists of an N terminal ectodomain (about 30 amino acids for LDV and PRRSV, 95 amino acids for EAV) which is *N*-glycosylated, an internal hydrophobic region that crosses the membrane three times and a C-terminal endodomain of 50–75 amino acids (Snijder et al. 2003b). The ectodomain of the EAV GP_5 protein carries one or two polylactosaminoglycans, a very unusual modification for a viral glycoprotein. The corresponding LDV protein was shown to carry 1–3 polylactoaminoglycans that strongly influence immunogenicity, sensitivity to antibody neutralization and neuropathogenicity (Li et al. 1998). At the present time, there is only limited information on the functions that are likely to be associated with this protein. The PRRSV GP_5–M protein hetrodimer has been implicated in attachment to a heparin-like receptor on the surface of porcine alveolar macrophages (Delputte et al. 2002) but studies with EAV chimeras make it clear that a factor other than the GP_5 ectodomain may determine the host specificity of arteriviruses (Dobbe et al. 2001). Likewise, the tropism of PRRSV M protein mutants carrying the ectodomain of other arteriviruses was unchanged, indicating that both ectodomains in the GP_5–M heterodimer can be replaced without a detectable effect on cell tropism (Verheije et al. 2002a). Recently, it has been shown that porcine sialoadhesin mediates the internalization of PRRSV into alveolar macrophages and that the presence of sialic acid on the virus is essential for attachment (Delputte and Nauwynck 2004; Vanderheijden et al. 2003).

The hemagglutinin-esterase glycoprotein is a structural protein that is restricted to a subset of the *Coronaviridae*. A strong hemagglutinating property and a second, shorter, fringe of peplomers on the virion surface characterize this subset, which includes HEV, BCoV, MHV, HCoV-OC43, and TCoV (Brian et al.

1995). Amongst the toroviruses, BToV has been shown to express a hemagglutinin-esterase protein (Cornelissen et al. 1997). It should be noted that some other coronaviruses (for example, TGEV and IBV) can also display strong hemagglutinating activity but this is a property of the surface glycoprotein S and is usually cryptic: treatment of the virus with, for example, neuraminidase is required to 'activate' hemagglutination. Finally, even for hemagglutinating coronaviruses, variants have been found that, for one reason or another, have lost the ability to express a functional HE protein. Thus, for example, the MHV strain A59 is defective in this respect. In the same way, it seems likely that the single isolate of EToV studied to date, which clearly carries the remnants of an HE protein gene but does not express an HE protein, is also a variant virus.

In the case of MHV, the HE protein is a disulfide-linked homodimer. The monomer polypeptide (65–75 kDa) has an N_{exo}–C_{endo} configuration and is *N*-glycosylated in the ectodomain. Transient expression of the MHV and BCoV HE proteins has shown that the protein has both hemagglutinin and esterase activities (Pfleiderer et al. 1991; Yoo et al. 1992). The acetylesterase activity of BCoV is specific for *N*-acetyl-9-*O*-neuraminic acid (Vlasak et al. 1988). Comparison of the coronavirus HE protein sequence and the sequence of the influenza C virus HEF protein reveals a remarkable degree of conservation, including, for example, many cysteine residues and the active site motif 'phenyalanine–glycine–aspartic acid–serine–arginine' (FGDSR) (Vlasak et al. 1988). It has been speculated that the *HE* gene of coronaviruses has been acquired by an RNA recombination event between ancestral nidoviruses and influenza C viruses (Luytjes et al. 1988; Snijder et al. 1991).

The importance of the coronavirus HE protein is not yet clear. On the one hand, there is evidence for a functional role: for example, monoclonal antibodies specific for the BCoV HE protein are able to neutralize virus infectivity. On the other hand, MHV A59, which does not have an HE protein, is able to replicate quite normally in tissue culture. Moreover, it has been shown that, even for coronaviruses that express high levels of HE, the HE–ligand interaction alone is not sufficient to mediate infection (Gagneten et al. 1995; Popova and Zhang 2002). One possibility is that the interaction of HE with *N*-acetyl-9-*O*-neuraminic acid serves to concentrate virus on the cell membrane and thereby facilitates the interaction of the coronavirus S protein with its specific protein receptor molecule. As the levels of cell surface *N*-acetyl-9-*O*-neuraminic acid can differ dramatically in vivo, this interaction may still be an important determinant of pathogenicity.

MINOR GLYCOPROTEINS

The minor arterivirus glycoprotein encoded by ORF2b (GP_{2b}; EAV/LDV) or ORF2a (GP_{2a}; PRRSV) is a conventional class I integral membrane protein with an N-terminal signal peptide, a C-terminal transmembrane segment, and 1–4 potential *N*-glycosylation sites. The protein is significantly under-represented in EAV particles (at least in relation to its intracellular abundance) (de Vries et al. 1992) and was shown to occur in EAV-infected cells in a variety of monomeric conformations and complexed with other minor glycoproteins (de Vries et al. 1995; Wieringa et al. 2003a). Complex formation is a prerequisite for GP_{2b} incorporation into virions, although the equivalent PRRSV protein was reported to be present in virions as a monomer (Meulenberg and Petersen-den Besten 1996). Recent mutagenesis studies using an EAV infectious cDNA clone revealed that cysteine residues in the GP_{2b} ectodomain form both intramolecular and intermolecular cysteine bridges, the latter involving a cysteine of the GP_4 ectodomain (Wieringa et al. 2003b).

The PRRSV and EAV GP_4 proteins were also identified as minor glycoprotein constituents of the virus particle (van Nieuwstadt et al. 1996; Wieringa et al. 2002). They contain an N-terminal signal sequence, a C-terminal membrane anchor and a small endodomain, which are typical properties of class I integral membrane proteins.

The EAV GP_3 protein is a heavily glycosylated integral membrane protein with an uncleaved N-terminal signal sequence and a hydrophobic C-terminal domain. The protein is inserted into the lipid bilayer by either or both of its hydrophobic termini and has no detectable endodomain (Hedges et al. 1999; Wieringa et al. 2002). Like GP_{2b} and GP_4, EAV GP_3 localizes to the endoplasmic reticulum, both in infected cells and in expression systems (Wieringa et al. 2002). There is no doubt about the presence of GP_3 in EAV particles (Wieringa et al. 2002), but conflicting data on the presence of PRRSV GP_3 in virions have been reported. Whereas GP_3 was detected in purified European PRRSV by using specific monoclonal antibodies (van Nieuwstadt et al. 1996), the protein could not be immunoprecipitated from a purified virus preparation of a North American isolate (Mardassi et al. 1998). However, small amounts of the latter protein were detected in a soluble form in the medium. Topological studies on the corresponding LDV protein also suggested it to be a secretory protein but, thus far, this protein could not be detected in cell lysates or the medium of infected cells (Faaberg and Plagemann 1997).

Recent studies on the GP_{2b}, GP_3, and GP_4 proteins of EAV have revealed various important properties of the arterivirus minor glycoproteins. By separately knocking out the expression of each of the EAV structural proteins in the context of a full-length EAV cDNA clone, it was shown that both major and minor structural proteins are required for the production of infectious progeny virus (Molenkamp et al. 2000). Subsequently, it was found that knock-out mutants for minor structural protein expression produce noninfectious subviral particles consisting of GP_5, M, N, and the viral RNA genome (Wieringa et al. 2004; Zevenhoven-Dobbe et al. 2004). These subviral particles did not differ from wild-type

virus in terms of buoyant density, major structural protein composition, and appearance in electron microscopy. When one of the proteins GP_{2b}, GP_3, or GP_4 was missing, the incorporation of the other minor GPs was blocked and that of the E protein was greatly reduced (Wieringa et al. 2004). The absence of the E protein completely blocked the incorporation into virions of the three minor glycoproteins, which are present in equimolar amounts and form a heterotrimeric complex (Wieringa et al. 2003a). In infected cells, covalently linked GP_{2b}/GP_4 heterodimers associate with GP_3 to produce a $GP_{2b}/GP_3/GP_4$ complex that is required for incorporation of the three glycoproteins into virions. Following virus release, GP_3 becomes disulfide-linked to the GP_{2b}/GP_4 heterodimers, a postassembly maturation event that yields a complex of three covalently bound glycoproteins. Consequently, EAV particles contain both GP_{2b}/GP_4 heterodimers and $GP_{2b}/GP_3/GP_4$ heterotrimers (Wieringa et al. 2003a).

The above data indicate that EAV N, M, and GP_5 are the only structural proteins required for virus assembly, but that the disulfide-linked $GP_{2b}/GP_3/GP_4$ heterotrimer is required for virus infectivity, most likely because this complex is essential for attachment to the host cell surface. The higher order structure of the arterivirus E protein is unknown, but also this protein is essential for infectivity (Molenkamp et al. 2000) and may, in fact, be associated with the $GP_{2b}/GP_3/GP_4$ trimer (Wieringa et al. 2003b).

Immune responses

HUMORAL IMMUNITY

The surface glycoprotein, S (coronaviruses and toroviruses), and the major glycoprotein, GP_5 (arteriviruses), are the major inducers of neutralizing antibody during natural infection (Balasuriya et al. 2004a; Chirnside et al. 1995; Ostrowski et al. 2002; Spaan et al. 1988; Wissink et al. 2003). In the specific case of SARS-CoV, it also appears that the S protein is the only significant protective antigen among the structural proteins (Buchholz et al. 2004). The antigenic structure of the coronavirus S protein has been studied and the picture that emerges is complex (Cavanagh 1995). Essentially, the majority of virus neutralization (VN) epitopes seem to be located on the S1 subunit (or the amino terminal half of the S protein if it is not cleaved) and they are 'conformational' in the sense that they are comprised of amino acids that are brought into close proximity by the folding of the protein (Zhou et al. 2004). The VN epitopes are clustered into domains, one of which is usually immunodominant, although 2–3 domains may carry VN epitopes. Glycosylation seems to be an important component of VN epitope structure. Some studies have indicated that VN epitopes may be located on the S2 subunit (or carboxyl half of the protein) and that amino acids in the S2 subunit can modulate the recognition of VN epitopes located in the S1 subunit (Zhang et al. 2004a). It should be kept in mind that, although many VN epitopes have been defined, the mechanisms of virus neutralization remain essentially unknown.

Although the S and GP_5 proteins are the prime inducers of humoral immunity, antibody responses to other structural proteins may also have a role in protection. Some monoclonal antibodies specific for the MHV or TGEV M protein can neutralize virus infectivity in vitro (Fleming et al. 1989; Risco et al. 1995) and, as already mentioned, HE-specific antibodies are able to neutralize BCoV infectivity. It has been shown that E-specific antibodies are able to neutralize MHV infectivity in the presence of complement (Yu et al. 1994). The relevance of these observations to the situation in vivo is, however, unclear. The arterivirus, PRRSV, can be neutralized by monoclonal antibodies recognizing the ectodomain of the GP_4 protein (Meulenberg et al. 1997; van Nieuwstadt et al. 1996) and recombinant EAV N protein is able to induce neutralizing antibodies in mice (Tobiasch et al. 2001). Finally, immunization studies using alphavirus-based expression vectors have shown that the co-expression of EAV GP_5 and M is required for the induction of neutralizing antibodies in mice and horses (Balasuriya et al. 2000, 2002), although other reports indicate that the GP_5 ectodomain alone may suffice (Castillo-Olivares et al. 2001). A recombinant EAV lacking the immunodominant region of the GP_5 ectodomain was found to be viable and able to induce protective immunity in horses, indicating that immune effector mechanisms other than neutralizing antibodies binding to this region of GP_5 play an important role in protection against infection (Castillo-Olivares et al. 2003b).

CELL-MEDIATED IMMUNITY

As for most other viruses, the cell-mediated immune response to coronavirus and arterivirus infection is less well characterized than the humoral immune response. There is good evidence that the cell-mediated response is important in a variety of natural infections (Castillo-Olivares et al. 2003a; Loa et al. 2001; Paltrinieri et al. 1998) but, for technical reasons, most of the information we have relates to murine infections (MHV or LDV), often in an experimental setting. The coronavirus S, M, and N proteins, as well as the arterivirus N protein, are targets for cellular immune recognition (Plagemann 1996; Yang et al. 2004b). In a few cases, specific T-cell epitopes have been defined and helper or cytotoxic functions have been attributed to T-cell subpopulations. There is some preliminary evidence that nonstructural proteins may also represent T-cell immunogens (Stohlman et al. 1993).

In addition to MHC-restricted immune responses, coronavirus infections elicit lymphokine responses. Recent studies indicate that SARS-CoV infection elicits a response that is characterized by elevated levels of IL-6 cytokine, MCP-1, and IP-10 chemokines (Jones et al. 2004; Zhang et al. 2004b). Also, it is interesting to

note that the SARS-CoV nucleocapsid protein has been shown to regulate cellular signaling (Subbarao et al. 2004). MHV infection of 129/Sv mice triggers a transient increase in the level of IL-12 (p40) mRNA present in pooled spleen cells (Coutelier et al. 1995). Also, the M protein of TGEV can induce, directly or indirectly, the production of interferon-α in lymphocytes (Baudoux et al. 1998, de Haan et al. 2003). The significance of these types of response for innate immunity has not yet been defined but there seems little doubt that they play an important role in particular situations.

CLINICAL AND PATHOLOGICAL ASPECTS

Introduction

The spectrum of disease caused by coronaviruses and arteriviruses is summarized in Table 3. As is the case with many viruses, the majority of infections are asymptomatic, perhaps reflecting the pathogenic equilibrium reached during the co-evolution of host and pathogen. Many of the clinical diseases listed in Table 3 are, in fact, manifested only in immunodeficient animals (e.g. neonates or during pregnancy). In contrast, when a nonnatural host is infected, or when the natural host is infected by an unusual route or virus variant, fulminant disease can also ensue. In the following review, only natural infections of major importance are discussed. For a discussion of less common and experimental infections with coronaviruses and arteriviruses the reader is referred to other review articles (Dales and Anderson 1995; Foley and Leutenegger 2001; Garwes 1995; Haring and Perlman 2001; Holmes 2001b; Koopmans and Horzinek 1995; Paul et al. 2003; Plagemann et al. 1995).

Coronaviruses

HUMAN CORONAVIRUSES (INCLUDING SARS-COV)

Human coronaviruses (HCoV) are accepted as a major cause of respiratory tract illness in humans. HCoVs are

Table 3 *Coronavirus and arterivirus infections in natural hosts*

		Infection						
Virus	**Host**	**Respiratory**	**Enteric**	**Reproductive**	**Neurologic**	**MPS**	**Other**	**Clinical disease**
Coronaviruses								
IBV	Chicken	++	...	+	...	...	+[a]	Infectious bronchitis
BCoV	Cattle	...	++	...	...	...	...	Neonatal calf diarrhoea, winter dysentery
CCoV	Dog	...	++	...	...	...	...	Enteritis
FCoV	Cat	+	++	...	+	...	+[b]	Enteritis, infectious peritonitis
HCoV-229E	Human	++	...	...	...	...	...	Common cold
HCoV-OC43	Human	++	?	...	...	...	...	Common cold
SARS-CoV	Human	++	+					Severe acute respiratory syndrome
MHV	Mouse	+	++	...	+	...	+[c]	Enteritis, rhinitis, hepatitis
RtCoV	Rat	++	...	...	...	...	+[d]	Pneumonitis
PEDV	Pig	...	++	...	...	...	...	Epidemic diarrhoea
TGEV	Pig	+	++	...	...	...	...	Transmissible gastroenteritis
HEV	Pig	+	...	...	++	...	...	Vomiting and wasting disease
TCoV	Turkey	...	++	...	...	...	...	Transmissible enteritis
Toroviruses								
EToV	Horse	...	...	...	...	...	...	?
BToV	Cattle	+	++	...	...	...	...	Gastroenteritis
HToV		...	++	...	...	...	...	Gastroenteritis
PToV		...	++	...	...	...	...	Gastroenteritis
Arteriviruses								
EAV	Horse	+	...	+	...	++	+[e]	Rhinitis, abortion
LDV	Mouse	...	...	...	+	++	...	Generally asymptomatic
SHFV	Monkey	...	...	...	...	++	...	Generally asymptomatic
PRRSV	Pig	+	...	+	+	++	...	Pneumonia, abortion

MPS, mononuclear phagocyte system. ++, main target for infection; +, secondary target for infection. ?, circumstantial evidence of infection or disease. Other targets of infection are: [a]kidney; [b]serous membranes; [c]liver; [d]salivary, lacrimal glands; [e]arteries.

grouped into three major groups that are prototyped by HCoV-229E, HCoV-OC43, and SARS-CoV. HCoV-229E and HCoV-OC43 are mainly associated with common colds (Myint 1995). It has been estimated that up to 30 percent of clinical cases are due to infections with these two viruses. Typically, after an incubation period of about 3 days, patients develop general malaise, headache, nasal discharge, sneezing, and a mild sore throat (Tyrrell et al. 1993). About one-tenth of patients also have a fever and one-fifth will have a cough. Although most of these infections remain restricted to the upper respiratory tract there is evidence that HCoV-229E and HCoV-OC43 may also cause severe lower respiratory tract illness (El-Sahly et al. 2000; Falsey et al. 2002; Pene et al. 2003; Vabret et al. 2003; van Elden et al. 2004). Also, HCoV-NL63, a recently isolated HCoV that is closely related to HCoV-229E, was reported to be associated with lower respiratory tract infections, such as pneumonia and bronchiolitis, in young children (Fouchier et al. 2004, van der Hoek et al. 2004; Esper et al. 2005). Very recently, yet another human coronavirus, HCoV-HKU1, has been isolated from patients with pneumonia (Woo et al. 2005). HCoV-229E and HCoV-OC43 have also been associated with diseases outside the respiratory tract, for example with gastroenteritis (Zhang et al. 1994) and, possibly, neurological diseases (Arbour et al. 2000; Stewart et al. 1992). HCoV infections are thought to be transmitted by aerosols. The peak incidence of infection is in the winter and spring (Lina et al. 1996). Somewhere between 10 percent and 20 percent of individuals have serological responses indicative of HCoV-229E or HCoV-OC43 infection in any one year, although this figure may increase to 35 percent in some years (Monto and Lim 1974). A high percentage of infections occur despite pre-existing antibody, and the frequency of infection gradually diminishes with age (Callow et al. 1990). Little is known about the pathogenesis and immune response to HCoV-229E and HCoV-OC43 infection, mainly because there is no animal model available. Replication of the virus is optimal at 33–34°C and takes place in ciliated cells of the nasal epithelium (Afzelius 1994). Serum antibodies rise about 1 week after infection, peak at 2 weeks, and decline to low levels after about 12–18 months. The serum antibody response is mainly directed toward the surface protein although membrane- and nucleoprotein-specific immunoglobulins are also synthesized.

With very few exceptions, HCoVs are difficult to isolate and, even after adaptation to tissue culture, propagation in vitro is inefficient. Thus, the isolation of HCoVs in organ or tissue culture and the identification of HCoVs by electron microscopy or immunoelectron microscopy is possible only in specialized laboratories. Until a few years ago, the diagnosis of HCoV-229E and HCoV-OC43 infection relied mainly on serological methods, including, for example, enzyme-linked immunosorbent assay (ELISA), neutralization, immunofluorescence, western-blotting, and complement fixation (Myint 1995). More recently, the detection of viral antigens and, in particular, RT-PCR methods have become the standard approach to the rapid diagnosis of 229E and OC43 infections (Hays and Myint 1998; Sizun et al. 1998; Stephensen et al. 1999; Vabret et al. 2001; Vallet et al. 2004; van Elden et al. 2004). Despite these advances, the detection of HCoV-229E and HCoV-OC43 infections is not generally done in routine diagnostic laboratories.

Another disease caused by a human coronavirus is the severe acute respiratory syndrome (SARS), a life-threatening and rapidly progressing form of pneumonia that first emerged in Guangdong province, China, in November 2002. Between February and July 2003 the virus infected more than 8 400 people worldwide and at the end of the epidemic more than 800 deaths had been recorded. SARS was shown to be caused by a previously unknown coronavirus, which was named SARS-CoV (Drosten et al. 2003; Ksiazek et al. 2003; Kuiken et al. 2003; Peiris et al. 2003a). The virus is generally believed to have evolved from an animal coronavirus that only recently crossed the species barrier to humans, although the natural reservoir of SARS-CoV remains to be identified (Guan et al. 2003). There is limited evidence that a very low number of people already had antibodies to SARS-CoV before the SARS outbreak, suggesting previous exposure to the same or a related coronavirus (Zheng et al. 2004). SARS-CoV is only distantly related to other animal and human coronaviruses (Marra et al. 2003; Rota et al. 2003) and has been classified (as the prototype of a new subgroup) in the coronavirus group 2, which also includes HCoV-OC43 (Gorbalenya et al. 2004; Snijder et al. 2003a). SARS-CoV is spread mainly by the respiratory and, possibly, fecal–oral routes (Ho et al. 2003b; Poutanen and McGeer 2004; Seto et al. 2003). Transmission requires close contact and, therefore, often occurs in hospital and health care settings from patients who are severely ill. It is generally believed that the virus is not transmitted from asymptomatic cases to contacts (Lee et al. 2003b). The clinical course of SARS is characterized by early nonspecific influenza-like symptoms, including persistent fever, chills, rigors, myalgia, and general malaise. After several days, respiratory symptoms (dry cough, rhinorrhea, shortness of breath) and gastrointestinal symptoms (nausea, vomiting, diarrhea) develop in most cases (Lee et al. 2003c; Loutfy et al. 2003; Ooi and Daqing 2003; Peiris et al. 2003a,b; Rainer 2004). About 8 days after the onset of fever, there is a rapid, often bilateral progression of ground glass opacies on chest radiographs. In a significant proportion of patients, multifocal and diffuse consolidation develops. The lung pathology may be associated with further complications, such as pneumomediastinum and pneumothorax, resulting in dramatically decreasing arterial oxygen saturation (Ooi and Daqing 2003; Peiris et al. 2003b). About 20 percent

of the patients deteriorate with evidence of an acute respiratory distress syndrome (ARDS), requiring admission to an intensive care unit and mechanical ventilation. The overall case-fatality rate determined at the end of the outbreak in July 2003 was calculated to be about 11 percent. The fatality rate was extremely low in children (close to 0 percent), whereas in patients older than 65 years the mortality approached 50 percent. The laboratory diagnosis of SARS is based on RT-PCR and serology. RT-PCR is done from nasopharyngeal aspirate and swab samples and, if available, specimens from the lower respiratory tract (Emery et al. 2004). Viral RNA is also detectable in feces, serum, plasma, and urine. Viral load in respiratory samples and fecal samples peak at around 10–12 days and 15–17 days, respectively, after illness onset (Chan et al. 2004). To detect SARS-CoV infection as early as possible (i.e. in the first week of illness), multiple clinical samples should be collected and analyzed simultaneously. Positive RT-PCR results must be confirmed using primers specific for different parts of the SARS-CoV genome and tests should be done with multiple clinical samples. Seroconversion is a relatively delayed phenomenon and, consequently, not suitable as a rapid diagnostic test. Generally, SARS-CoV-specific IgG becomes detectable after 10–15 days of illness or even later. Serological assays, therefore, remain the gold standard for retrospective studies but are less suitable for rapid diagnosis. Anti-SARS-CoV IgG in the serum can be detected by immunofluorescence, enzyme-linked immunosorbent assays, and neutralization tests, which correlate very well with each other. The first serological assays to be developed and used during the SARS epidemic in 2003 were based on virus antigen produced from SARS-CoV-infected cells. This rapid development was possible because SARS-CoV, in contrast to most other HCoVs, grows to high titers in cell culture (for example, in Vero cells). Meanwhile, numerous diagnostic assays based on recombinant nucleocapsid (N), spike (S), and membrane (M) protein antigens are available, providing convenient tools that do not require biosafety level 3 laboratories. SARS-CoV antibodies show only very little cross-reactivity with antibodies specific for other human coronaviruses (Ksiazek et al. 2003). Negative SARS-CoV serology at 28 days after onset of symptoms is generally accepted to exclude the diagnosis of SARS-CoV infection.

To date, there have been no high-quality studies of any antiviral or immunosuppressive therapy. The few available studies indicate that therapies based on antiviral agents, such as ribavirin and oseltamivir, and immunosuppressive steroid therapy, may have severe side effects but there is only anecdotal evidence that these drugs significantly reduce the severity of the disease (Cyranoski 2003; Ho et al. 2003a; Loutfy et al. 2003; So et al. 2003; Tsang et al. 2003). However, SARS-CoV and other human coronaviruses proved to be sensitive to treatment with type I interferons, both in cell culture and animal models (Cinatl et al. 2003; Haagmans et al. 2004; Hensley et al. 2004; Hertzig et al. 2004), suggesting that type I interferons are promising drugs for SARS treatment protocols, which is consistent with pilot (mainly uncontrolled) clinical studies (Cinatl et al. 2004).

Thus far, the pathogenesis of SARS has been only poorly understood. The fact that, despite significantly reduced virus load, some patients deteriorate in the second week of illness suggests that the lung damage may be due to immunopathological mechanisms rather than virus replication. Animal models suitable to address this possibility and to investigate SARS-CoV pathogenesis in general are becoming increasingly available (Fouchier et al. 2003; Martina et al. 2003; Subbarao et al. 2004, ter Meulen et al. 2004; Wentworth et al. 2004; Yang et al. 2004b). The SARS outbreak has inspired many studies into virtually every aspect of SARS-CoV biology. The studies revealed promising therapeutic approaches, some of which might be used to develop drugs that are suitable to treat SARS-CoV infections. Candidate antivirals include fusion inhibitors (Bosch et al. 2004; Liu et al. 2004), inhibitors of the SARS-CoV main proteinase (Anand et al. 2003; Bacha et al. 2004; Hsu et al. 2004), recombinant antibodies (ter Meulen et al. 2004), and small compounds identified by screening of drug libraries (Wu et al. 2004). Finally, promising candidate vaccines have been developed that proved to be protective in SARS animal models (Bisht et al. 2004; Buchholz et al. 2004; Bukreyev et al. 2004; Yang et al. 2004b). The rapid progress in the improvement of diagnostic tests and the development of vaccines and antivirals should help to control future outbreaks of SARS more effectively.

AVIAN INFECTIOUS BRONCHITIS VIRUS

IBV is a pathogen of economic importance to the poultry industry worldwide. It causes a highly contagious disease affecting the respiratory, reproductive, neurological, and renal systems of chickens. IBV infection results in a drop in egg production in adult birds and damages the developing reproductive system in young birds. Infection of the respiratory tract, most probably by aerosols, is the most important route of transmission. Young chickens are most susceptible to infection and the clinical signs include nasal discharge, sneezing, coughing, and tracheal rales. IBV infection predisposes chickens to infection with secondary pathogens. Even young chickens will usually recover from an uncomplicated IBV infection, but the incidence of IBV-related nephritis, which can result in high mortality rates, seems to be increasing (Cavanagh and Naqi 2003; Cook and Mockett 1995; Ignjatovic and Sapats 2000; Lee et al. 2004).

Chickens respond to IBV infection by producing specific humoral antibodies of the IgM, IgG, and IgA

subclasses. The major viral antigens are the S and M proteins, and the S protein is a major immunogen. High concentrations of VN antibodies are thought to prevent the spread of virus from its primary replication site to the reproductive and renal organs. Local immune responses, in particular IgA, play a significant role in controlling the respiratory infection. Finally, cell-mediated immune responses also develop after IBV infection. Cell-mediated immune responses to the S, N, and M proteins have been demonstrated (Ignjatovic and Galli 1993). At the moment, however, the relationship between cell-mediated immune responses and the degree of protective or long-term immunity has not been defined.

The diagnosis of IBV infection, in common with most other viruses, depends on three approaches: (1) isolation of the virus; (2) demonstration of a specific antibody response, usually by serological methods; and (3) demonstration of viral nucleic acid in clinical material.

IBV can be isolated in tracheal organ cultures and embryonated eggs and propagated in tissue cultures. Infected allantoic fluid and tracheal organ culture supernatant often contain enough virus particles to be visualized directly by electron microscopy. Alternatively, IBV isolates can be identified by immunofluorescence using polyclonal antisera or by antigen assays based on monoclonal antibodies. The existence of strain-specific monoclonal antibodies has also led to the development of serotype-specific ELISAs (Karaca and Naqi 1993).

A range of serological tests are available to detect IBV antibodies. They include immunofluorescence, hemagglutinin inhibition, ELISA, and virus neutralization. Probably the most sensitive tests are ELISAs using purified virus as antigen. In the future, it can be expected that these types of test will be significantly improved by the use of recombinant antigens (Chen et al. 2003; Guy et al. 2002; Wang et al. 2002). It is possible to detect IBV nucleic acids directly in clinical material. PCR is a rapid and sensitive method to amplify and identify IBV RNA (Jackwood et al. 2003; Liu et al. 2003). Combined with nucleotide sequencing (Lee et al. 2003a), PCR will probably also be the preferred method for distinguishing between IBV serotypes.

At the present time, the control of IBV relies almost exclusively on vaccination and management. Both live attenuated and inactivated vaccines are available. It is generally believed that both vaccines can be used, usually in combination, to induce high levels of humoral antibody and that immunity represents protection against reproductive and renal disease rather than protection against respiratory infection (Cook and Mockett 1995). There are also two major problems associated with the use of vaccine. First, there are many serotypes of IBV and, although it is not necessary to develop a different vaccine for each serotype, occasionally serotypes emerge against which existing vaccines are not fully effective. Secondly, although some serotypes probably emerge by antigenic drift, there is at least circumstantial evidence to suggest that they may also emerge by recombination, possibly involving the live vaccine virus itself (Cavanagh 2003; Lee and Jackwood 2000).

PORCINE TRANSMISSIBLE GASTROENTERITIS VIRUS

The severity of disease caused by porcine transmissible gastroenteritis virus TGEV is related to the age of the animal. Infection of the piglet during the first 2 weeks of life causes vomiting followed by profuse, watery diarrhea that eventually results in dehydration and frequently death within 2–5 days. In older pigs, similar clinical signs are manifested but the mortality rate drops as the body weight of the animal at the time of infection increases. Transmission of TGEV is primarily from the ingestion of contaminated material, usually feces or milk, and possibly via aerosols (Enjuanes and Van der Zeijst 1995; Garwes 1995). TGEV has been reported in a large number of countries, but with different incidence and prevalence of disease. In countries where pig breeding has not been intensified, transmissible gastroenteritis is essentially epizootic with an incidence of disease every 2–3 years. In this situation, new outbreaks of disease are probably due to the cyclic reintroduction of virus. In countries with intensive pig breeding, transmissible gastroenteritis infection has become essentially enzootic. In this situation, the virus probably establishes a persistent infection in adult animals, in the gut or respiratory tract.

The pathogenesis and immune response to TGEV infection, especially in newborn animals, are quite well understood. The virus primarily infects and replicates in the apical tubovascular system of villous absorptive cells in the jejunum and ileum. Clinical signs seem to result from an increase in osmotic pressure in the lumen of the small intestine, caused by a failure to absorb milk lactose combined with an abnormal level of sodium secretion into the lumen (Garwes 1995). Because immunity requires the secretion of antibodies into the lumen of the gut, the rapid progression of the disease in the newborn animal probably provides insufficient time for immunity to develop, irrespective of whether the animals have reached a state of immune competence. The main source of immune protection for neonates, therefore, has to be antibodies provided by the mother in colostrum and milk (de Diego et al. 1992). Surprisingly, it has been shown that colostrum from nonimmune sows can also provide some degree of protection (Enjuanes & Van der Zeijst 1995).

Several techniques are available for the rapid diagnosis of TGEV infection, including immunofluorescence, ELISA, nucleic acid hybridization, and RT-PCR (Enjuanes and Van der Zeijst 1995; Kim et al. 2000, 2001, Liu et al. 2001a; Paton and Lowings 1997). Most importantly, a panel of type-, group-, and interspecies-

specific monoclonal antibodies have been developed to distinguish between viruses of the TGEV cluster, including PRCV (Sanchez et al. 1990). As described above, immune protection of piglets can be best achieved by natural lactogenic immunity provided by sows, by artificial lactogenic immunity using serum or protective monoclonal antibodies or, perhaps, by transgenic lactogenic immunity (Castilla et al. 1998). Virulent TGEV strains can be used to produce a solid immunity for both pregnant sows and their offspring (de Diego et al. 1992) but there is an obvious danger associated with this approach. Attenuated strains of TGEV and the PRCV variant have also been tested as vaccines but they have only limited efficacy (Saif and Wesley 1992). Efforts are being made to develop more effective vaccines. These may include noninfectious antigens that are targeted to the gut, and the use of live vectors with enteric tropism (Chen and Schifferli 2003, Lamphear et al. 2004; Liu et al. 2001b; Saif 1999; Sola et al. 2003).

FELINE CORONAVIRUS

Feline coronaviruses are a common cause of enteric disease in cats and, occasionally, they cause an immune-mediated disease known as feline infectious peritonitis (FIP). The enteric disease results in mild, transient diarrhoea that is self-limiting. FIP, however, is invariably lethal. Two distinct forms of FIP exist: the wet form, which is characterised by abdominal, and occasionally, pleural effusions, and the dry form, which is characterized by granuloma formation on the surface of tissues. In the dry form, the CNS is commonly involved but any organ may be affected. Feline coronavirus infection is enzootic in virtually all environments where large numbers of cats are kept and transmission is predominantly by the feco-oral route (de Groot and Horzinek 1995).

The patho-etiology of FIP is not well understood. It is known that purebred cats are predisposed to the development of FIP and the majority of cats develop FIP before 2 years of age (Foley et al. 1997; Poland et al. 1996). A widely held view is that FIP occurs when a cat is exposed to feline coronavirus variants that have mutated in the host and gained the ability to replicate in macrophages (Vennema et al. 1998). There is, however, no conclusive evidence to support this hypothesis. Also, it has been noted that, at least in experimental infections, seropositive cats develop an accelerated fulminating course of disease and this has been interpreted as evidence of 'antibody-dependent enhancement.' However, similar effects are seldom seen in natural infections (Hohdatsu et al. 1998). Finally, it has been postulated that a deregulated cytokine/chemokine response and the lack of an effective cell-mediated immune response plays a central role in the development of FIP (Paltrinieri et al. 2003). However, the available data is still preliminary.

With the exception of some laboratory-adapted strains, feline coronaviruses cannot be easily propagated in cell culture. Therefore infections are diagnosed by serological methods or, if the animals are shedding virus in the faeces, by using RT-PCR (Addie and Jarrett 2001; Addie et al. 2004; Addie et al. 2003). However, there is no specific diagnostic test for animals that are at increased risk of developing FIP or are in the earliest stages of the disease. Indeed, definitive diagnosis of FIP can only be made by histopathological examination of biopsy material or post-mortem material. As there is no effective treatment for FIP, environmental controls are currently the most powerful and widely used tools for the control of feline coronavirus infection. These include good hygiene, isolation, and early weaning. In the longer term, there is a clear need for an effective vaccine and/or the development of antiviral drugs that could be used to protect animals at greatest risk.

Toroviruses

BOVINE TOROVIRUS (PREVIOUSLY BREDA VIRUS)

There is clear evidence that torovirus infections are quite common in vertebrate species. Despite their potential veterinary and clinical relevance, the virus group has not been studied in great detail, in particular since propagation of toroviruses in cell culture has proven to be very difficult. Serological evidence of torovirus infection has been obtained in all ungulates that were tested using a bovine torovirus (BToV) neutralization assay (horses, cattle, sheep, goats, and pigs) and in rats, rabbits, and some species of feral mice (Weiss et al. 1984). Torovirus-like particles have also been observed and, in some cases, isolated from stool specimens from horses, cattle, pigs, humans, cats, and dogs (Koopmans and Horzinek 1995; Kroneman et al. 1998). Four torovirus genotypes were recently distinguished, exemplified by BToV (also known as bovine enteric torovirus), porcine torovirus (PToV), EToV, and human torovirus (HToV). In addition, sequence analysis revealed ample evidence for recurring intertypic recombination and predicts the existence of at least two additional, hitherto unknown, torovirus genotypes (Smits et al. 2003). It has been speculated that toroviruses may be associated with enteric disease in many species but, as yet, the only disease that can be definitely ascribed to a torovirus is BToV-related gastroenteritis in cattle.

Seroepidemiological studies in a number of countries show that the infection of cattle with BToV seems to be common throughout the world. In dairy cattle herds in the Netherlands, 85–95 percent of animals have antibodies to BToV by 1 year of age (Koopmans et al. 1989). Transmission probably takes place via the oral or the respiratory routes. BToV infections are usually limited to the gut, although there is some evidence for a

possible respiratory involvement. The clinical signs of infection are watery diarrhea with dehydration, weakness, and depression. Predominantly affected are young animals 2–3 weeks of age; by the time they are 3–4 months old, they have only very mild or no diarrhea, in association with virus shedding. There is some preliminary evidence that persistent BToV infection can be established in a herd (Koopmans et al. 1990).

The pathogenesis of BToV infection centers on the destruction of crypt and villus epithelial cells, particularly in the small intestine. Although the epithelial cells are rapidly replaced, the new cells lack a mature brush border and thus present insufficient absorptive surface and digestive enzymes. This decrease in absorptive and digestive capacity causes an accumulation of lactose in the gut lumen, which in turn results in water and electrolytic retention leading to diarrhea. At least in young animals, the immune response following infection is probably of secondary importance, because protection depends mainly on maternal antibodies derived from colostrum and milk.

Attempts to isolate and propagate toroviruses have proved unsuccessful. In cell or tissue culture, BToV as well as HToV and PToV, have not been replicated to date. The isolation of EToV was a unique event that could be repeated only with the field sample of a single horse. The diagnosis of BToV infection has thus relied on immunological methods for the detection of BToV antigen or BToV-specific antibodies. However, there is evidence that BToV-infected animals shed ELISA-detectable amounts of virus for only 2–3 days (Woode et al. 1982), and the purification of BToV antigens from the feces of experimentally infected gnotobiotic calves is a laborious and expensive procedure. Nucleic acid hybridization, RT-PCR, and ELISA methods have been developed for the detection of BToV in clinical material (Hoet et al. 2003; Koopmans et al. 1991, 1993) and are used for diagnostic purposes and epidemiological studies. The control of torovirus-related disease currently depends on good management practices.

Arteriviruses

EQUINE ARTERITIS VIRUS

EAV was first isolated in 1953 from an aborted equine fetus. Serological evidence suggests that EAV is widespread in the horse population (Timoney and McCollum 1988) but infections are generally subclinical and usually lead only to a mild, often unrecognized, infection of the respiratory tract. If the virus does cause overt disease, the clinical symptoms are acute anorexia and fever, usually accompanied by palpebral edema, conjunctivitis, nasal catarrh, and edema of the legs, genitals, and abdomen (Plagemann and Moennig 1992). The clinical symptoms of EAV infection vary widely, probably reflecting, at least in part, differences in virulence among EAV variants. Almost all naturally infected horses recover from EAV infection. Donkeys are the only other known host for the virus (Paweska 1994).

The primary mode of EAV transmission is assumed to be via the respiratory route by contact with aerosol secretions from acutely infected animals. The name 'equine arteritis virus' was derived from the characteristic necrosis of small muscular arteries following infection. Of greatest concern to the horse breeding industry, however, is the fact that the virus frequently causes abortion in pregnant mares (Golnik et al. 1986). Furthermore, a carrier state exists in seropositive stallions in which EAV is produced in the semen (Balasuriya et al. 2004b). These 'shedding stallions' may thus infect brood mares by the venereal route.

Studies of experimentally infected horses have indicated that, as with other arteriviruses, the initial replication of EAV takes place in lung macrophages. The virus infects the bronchial lymph nodes and then spreads throughout the body via the circulatory system. The cause of abortion in pregnant mares has not yet been elucidated. EAV-induced, macrophage-derived cytokines may contribute to the pathogenesis of EAV and the magnitude of the cytokine response may be linked to the virulence of the infecting virus strain (Moore et al. 2003). Neutralizing antibodies, mainly of the IgG class, are induced within a week of EAV infection, and this clears the virus from the circulation. Clearly, however, this does not always prevent the virus from establishing a persistent infection, particularly in stallions. Neutralizing antibodies generally persist for years, and protective immunity is presumed to be lifelong. Only one EAV serotype has been recognized, although European and North-American genotypes can be discriminated (Stadejek et al. 1999). As described above, the EAV GP_5 glycoprotein is the main inducer of neutralizing antibodies (Balasuriya et al. 2004a; Balasuriya et al. 2002; Castillo-Olivares et al. 2001; Chirnside et al. 1995).

Traditionally, the diagnosis of EAV infection has been based on the isolation and propagation of virus in tissue culture. The clinical materials most often used are nasopharyngeal, vaginal and rectal swabs or a buffy coat fraction from blood or semen. Rabbit kidney (RK-13), baby hamster kidney (BHK-21), and Vero cells are susceptible to infection and develop characteristic cytopathic effects.

Attempts to isolate virus from field cases have sometimes proved difficult. Several immunological assays for measuring EAV-specific antibodies in horse sera are available but the detection of neutralizing antibodies ($TCID_{50}$ and plaque reduction assays) are used most commonly. The determination of the nucleotide sequence of the EAV genome has also allowed the development of assays based on RT-PCR (Chirnside and Spaan 1990; Gilbert et al. 1997; St-Laurent et al. 1994). The control of EAV infection relies on a combination of vaccination and containment (Chirnside 1992;

Plagemann and Moennig 1992). At present, a live-attenuated and a killed virus vaccines are available. The live vaccine induces long-lasting protection against clinical disease but does not prevent reinfection with wild-type virus or temporary virus shedding (McCollum 1986). To avoid this problem, a formalin-inactivated vaccine (Fukunaga et al. 1984) has been developed. Increased knowledge of the antigenic properties of the EAV GP_5 protein may provide the basis for the development of an EAV subunit vaccine (Balasuriya et al. 2002; Castillo-Olivares et al. 2001; Chirnside et al. 1995; Giese et al. 2002). Furthermore, the development of reverse genetics systems for EAV (van Dinten et al. 1997) has opened the possibility of the development of vaccines based on the genetic engineering of the virus genome (Castillo-Olivares et al. 2003b; de Vries et al. 2001; Zevenhoven-Dobbe et al. 2004).

Porcine reproductive and respiratory syndrome virus

PRRSV is responsible for respiratory disease in pigs of all ages and for abortions and stillbirths in pregnant sows. The clinical signs of infection are transient fever, anorexia, and respiratory distress. Mortality is generally seen only in young piglets and is often associated with secondary infections (Meredith 1993; Plagemann 1996). Within the herd, PRRSV is thought to be transmitted mainly via aerosols but, as in the case of EAV, sexual transmission also occurs (Sur et al. 1997). The virus is present in nasal secretions and swine can be readily infected by intranasal inoculation. Fecal–oral transmission may, however, also take place and infection between herds may involve the movement of infected animals or fomites. In pregnant sows, PRRSV can be transmitted transplacentally, leading to reproductive failure in late gestation. The disease associated with PRRSV first appeared in the USA in the late 1980s. The first incidence of the disease in Europe was in northern Germany in 1991 and the virus is now common in the Netherlands and the UK. As with TGEV, the disease associated with PRRSV infection was originally epizootic, but more recently the incidence of acute disease has decreased and mild, even unapparent, infections are becoming more commonplace.

Lung macrophages are the primary site of PRRSV infection and replication. Their destruction is probably the cause of the increased susceptibility of infected pigs to secondary bacterial infections. The virus may also spread to other organs. In addition to interstitial pneumonia, lymphocytic encephalitis is a common lesion found in PRRSV-infected animals. The course of development of anti-PRRSV immunity is unconventional and many issues remain to be studied in detail (Murtaugh et al. 2002). The virus does not elicit the strong innate immune response seen in many other viral pathogens. Humoral and cell-mediated immunity is induced and clears the virus from the circulation but not from lymphoid tissues, where the infection becomes persistent. As with other arteriviruses, the initial phase of PRRSV infection is characterized by transient suppression of the cellular immune response coupled with an enhanced response to T-cell-dependent and T-cell-independent antigens (Murtaugh et al. 1993). The weak cell-mediated immune response suggests that PRRSV suppresses T-cell recognition of infected macrophages (Xiao et al. 2004). PRRSV-specific humoral antibodies can be detected 1–2 weeks after infection and peak after 5–6 weeks. The neutralizing antibody component reaches a maximum after 4–6 weeks and, thereafter, virus is generally cleared from the circulation. There is evidence that virus may persist in swine herds for periods of 2 years or longer but it is not clear whether this represents persistence in the individual animal, reinfection of animals, or the infection of animals that have escaped earlier encounters with the virus.

The diagnosis and control of PRRSV infections still represents a significant problem. In tissue culture, PRRSV is most successfully isolated and propagated in primary pig lung macrophages. However, the costs involved preclude this as a routine diagnostic procedure. A number of continuous cell lines can be used for virus propagation but they do not seem to be equally susceptible to all isolates. Serologically, PRRSV infections can be detected by virus neutralization, immunofluorescence, and immunoperoxidase staining. However, it is clear that many isolates of PRRSV differ antigenically and it will be some time before the extent of this variation has been fully characterized (Drew et al. 1995). Finally, the detection of PRRSV RNA using RT-PCR is under development (Legeay et al. 1997; Suarez et al. 1994; Van-Woensel et al. 1994) and promises to be the method of choice for the detection of low levels of viral RNA in the tissues of infected pigs. Control measures essentially consist of the eradication of infected herds and restrictions on the movement of pigs from enzootic areas. Live-attentuated and killed vaccines are available to protect pigs. However, adverse effects of vaccination of Danish pig herds with a modified live PRRS vaccine have been described (Botner 1997), which were probably caused by reversion of the vaccine virus to virulence. Acute PRRS-like symptoms, including an increasing number of abortions and stillborn piglets, were experienced in vaccinated herds. Furthermore, vaccine virus was transmitted from vaccinated to nonvaccinated boars in several cases, resulting in viremia and shedding of vaccine virus into semen (Madsen et al. 1998). In this respect, the killed vaccines are safer but are less efficacious in the induction of protection. Promising results were obtained in DNA vaccination experiments with plasmids expressing the GP_5 protein of PRRSV (Pirzadeh and Dea 1998). Neutralizing antibodies and lymphocyte proliferation

responses were detected in DNA-vaccinated pigs and the spread and clinical signs of challenge virus was reduced. As in the case of EAV, the immunogenic properties of the GP_5 ectodomain (Plagemann 2004; Wissink et al. 2003) suggest that the PRRSV major glycoprotein might be a good candidate for a DNA-based subunit vaccine. In addition, following the development of reverse genetics systems for PRRSV (Meulenberg et al. 1998; Nielsen et al. 2003; Truong et al. 2004), the first results from studies using vaccine viruses that have been attenuated by genetic engineering have recently been reported (Verheije et al. 2003).

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Picornaviruses

PHILIP MINOR

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The picornaviruses cause a number of diseases of animals and humans of varying degrees of severity. The earliest record of a human disease believed to be caused by a virus is an Egyptian funerary stela of about 1 300 BC depicting a priest with the typical withered limb and dropped foot deformity of poli omyelitis, caused by *Poliovirus*, a picornavirus of the *Enterovirus* genus. In 1909, the virus was transmitted from the spinal cord of a fatal case to monkeys by Landsteiner and Popper. Enders et al. (1949) showed that poliovirus could be grown in primate cells of non-neural origin, and the isolation of other enteroviruses followed. Coxsackie virus was isolated from two children with paralytic disease from the town of Coxsackie in New York State in 1948 by the inoculation of newborn mice with fecal extracts (Dalldorf and Sickles 1948). Robbins and co-workers isolated echoviruses (enterocytopathic human orphan viruses) from healthy children in 1951. The echo-, polio- and coxsackie viruses, with more recently discovered viruses identified by number, are classified as enteroviruses, as shown in Table 1. The classification of the picornaviruses has been extensively reviewed and modified over the last few years and now relies mainly on molecular approaches.

The *Rhinovirus* genus of the picornavirus family is one of the causative agents of the common cold. Kruse (1914) first demonstrated that a cold could be transmitted by bacteria-free filtrates. The Common Cold Research Unit at Salisbury (UK) was established in 1946 and investigation of transmission of the common cold to human volunteers eventually led to the cultivation of the virus in vitro. In the USA, two cytopathogenic agents, JH and 2060, were described (Price et al. 1959) and were originally classified as echovirus 28, later being reclassified as *human rhinovirus A*, the type species of the genus (Kapikian et al. 1967). In 1960, Tyrrell and Parsons found that other viruses that caused common colds could be propagated in cultures of human embryonic kidney cells rolled at 33°C in medium with low pH (Tyrrell and Parsons 1960). This observation and the introduction of semicontinuous strains of diploid human embryonic lung fibroblasts (Hayflick and Moorhead 1961) led to a rapid increase in the number of

Table 1 Enterovirus *serotypes*

Group[a]	Major disease
Polioviruses 1–3	Paralytic poliomyelitis Aseptic meningitis
Coxsackie viruses A1–22, 24	Aseptic meningitis Herpangina Conjunctivitis (A24)
Coxsackie viruses B1–6	Aseptic meningitis Fatal neonatal disease Pleurodynia Myo- or pericarditis
Echoviruses 1–9, 11–21, 24–27, 29–34	Aseptic meningitis Rashes Febrile illness
Enteroviruses 68–71	Conjunctivitis (enterovirus 70) Polio-like illness (enterovirus 71)

Echovirus 9 = coxsackie virus A23; echovirus 10 = reovirus; echovirus 28 = rhinovirus; echovirus 34 = coxsackie virus A24.

a) In the current report of the International Committee on Taxonomy of Viruses these viruses are prefixed 'human' to distinguish them from animal viruses where relevant.

viruses isolated. Rhinoviruses were also isolated from horses (Plummer 1963) and cattle (Bögel and Böhm 1962).

The Rhinovirus Collaborative Programme of the World Health Organization authenticated 100 serotypes of rhinovirus over the next 20 years (Hamparian et al. 1987).

Foot-and-mouth disease virus (FMDV) was the first non-plant virus to be discovered (Loeffler and Frosch 1898).

PROPERTIES OF THE VIRUSES

The picornaviruses are small RNA-containing viruses. The complete sequences of the genomic RNA of many strains of picornaviruses have been determined, the first being that of the type 1 poliovirus strain Mahoney (Kitamura et al. 1981), and the atomic structures of representative viruses of each of the various genera have been resolved by X-ray crystallography (Hogle et al. 1985; Rossmann et al. 1985; Ming et al. 1987; Acharya et al. 1989). The discussion of the detailed molecular virology that follows refers to poliovirus except where stated otherwise.

Classification

The six genera that make up the *Picornaviridae* are:

- the enteroviruses (Table 1)
- the rhinoviruses
- the aphthoviruses, which cause foot-and-mouth disease of cattle
- the cardioviruses of mice, which include mengo virus and *Encephalomyocarditis virus* (EMCV) as well as Theiler's virus
- the hepatoviruses, which include hepatitis A viruses (described in Hepatitis A and E)
- the parechoviruses, which encompass the viruses previously designated echovirus 22 and echovirus 23.

Comparisons of the sequences of the virus genomes play an essential role in the classification scheme. In particular, Theiler's virus and *Hepatitis A virus* were moved from the enteroviruses mainly because of the nature of their genomic organization and sequence, although they also have particular pathogenic features, whereas echovirus 22 and echovirus 23 appear to be typical enteroviruses in all except their sequences, which are very different.

Molecular classification of the picornaviruses

Theiler's virus of mice is an acid-stable picornavirus which produces symptoms analogous to those of poliomyelitis in humans, and for many years it was classified as an *Enterovirus*. Determination of the sequence and later the structure of the virus showed that it was much more closely related to the cardioviruses of mice such as EMCV, and it was reassigned on this basis. Likewise, *Hepatitis A virus* was categorized as an *Enterovirus*, largely on the basis of its acid stability and its occurrence in the stool of infected individuals, but its pathogenesis is strikingly different from that of other enteroviruses and its sequence is uniquely placed among those of the other picornaviruses. It was also therefore reclassified.

Sequence comparisons made it clear that the rhinoviruses were very similar to the enteroviruses and a reevaluation of the whole basis of the classification took place, which has revealed the relationships between picornaviruses and enabled subclassifications to be made. The increasing use of molecular methods in clinical virology makes this a valuable and logical development.

Complete sequences of many picornaviruses are known and viruses can be classified into the appropriate genus on the basis of comparison of the amino acid sequences of any part of the coding region of the genome, including those encoding the structural proteins, P1, or the polymerase 3D (King et al. 2000). Dendrograms showing the relationships between the different picornavirus genera based on the P1 region are shown in Figure 1. Variation between genera is typically greater than 70 percent in terms of sequence, and the rhinoviruses and the enteroviruses are surprisingly closely related. If the nucleic acid sequence of the 5′ noncoding region is considered, however, only two clusters are evident.

Subclusters can be identified within a genus giving rise to the concept of the species. Members of a species are defined as sharing greater than 70 percent amino acid identity within P1, greater than 70 percent amino acid identity within the nonstructural proteins 2C and 3D combined, sharing a limited range of host cell receptors and natural host range, and having a degree of compatibility in certain aspects of virus multiplication. Species within the *Enterovirus* genus are shown in Figure 2. *Poliovirus* is currently classified in a different subspecies to the other group C species. There is reason to believe that the members of a species are able to exchange segments of their genomes by recombination more or less freely. Thus within the B species the P1 region is sufficient to identify a virus as a coxsackie virus B1 distinct from a coxsackie virus B3, for example, while comparisons of the nonstructural protein 3D are believed to be only able to identify a virus as a B species. Similarly within the C species it is not currently possible to distinguish a coxsackie virus A21 strain from a poliovirus by examination of the nonstructural proteins. This finding, if confirmed, implies that viruses within a species infect the same cell types and show a great deal of compatibility between their genomes, while

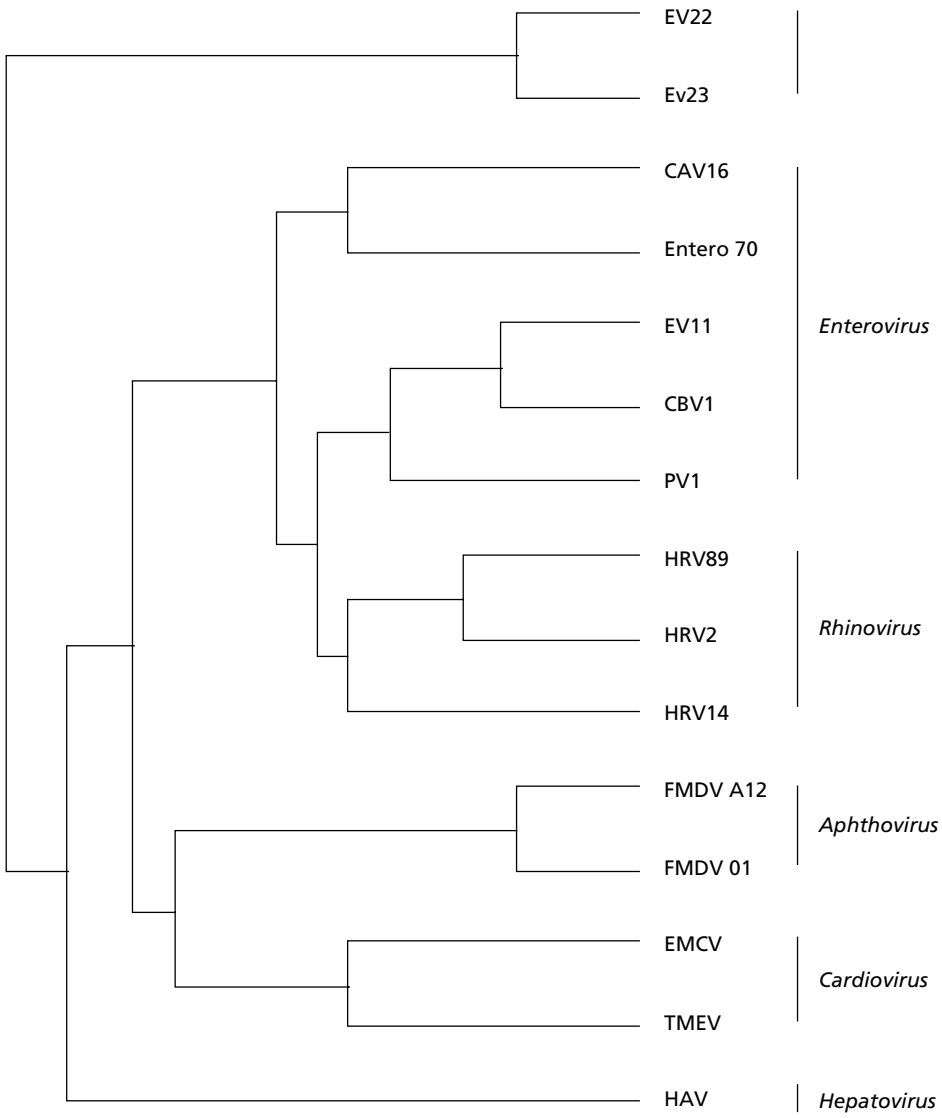

Figure 1 *Clustering of the picornavirus genera on the basis of comparison of the complete sequences of the genome. The viruses designated EV22 and EV23 (enterovirus 22 and enterovirus 23) are now classified as parechovirus 1 and parechovirus 2, respectively, on the basis of their highly distinct genomic sequences.*

those of different species are either unable to infect the same cell type naturally or have genomes which are incompatible with each other when segments are exchanged. Polioviruses which are recombinants between the vaccine strains and unknown species C enteroviruses have been isolated on several occasions, as discussed below (Kew et al. 2002).

Physical properties of picornaviruses of different genera

The viruses of the picornavirus genera share a number of characteristics (Palmenberg 1987), including size, icosahedral morphology, lack of a lipid envelope, a positive-sense RNA genome and the general arrangement of their structural proteins. The sedimentation coefficient is ca. 155–160S. The enteroviruses, cardioviruses, hepatoviruses, and parechsoviruses have a buoyant density in cesium chloride of 1.34 g/cm^3, the rhinoviruses of 1.40 g/cm^3, and the aphthoviruses of 1.43 g/cm^3. The enteroviruses and parechoviruses, unlike rhinoviruses or aphthoviruses, are totally stable at pH 4 and resistant at pH 2, and this property formed the basis of early classification schemes for the picornaviruses.

The genomes of both the aphtho- and the cardioviruses include a tract of cytidine residues not found in the other genera, and share a similar genomic structure distinct from that of the enteroviruses and rhinoviruses, as described below. The properties of the picornavirus genera are summarized in Table 2, p. 5.

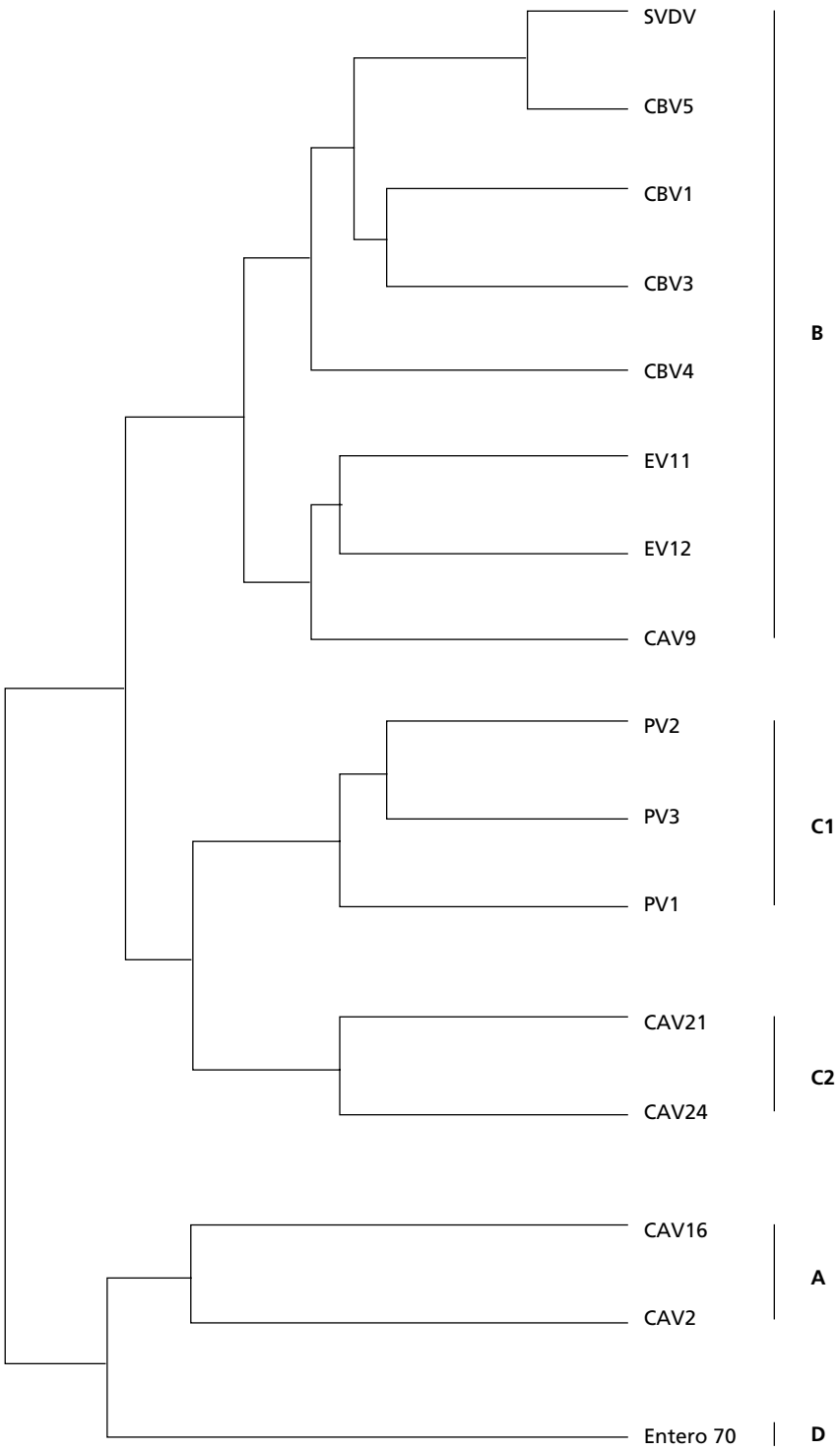

Figure 2 *Clustering of the human enteroviruses based on comparisons of the sequences of the structural proteins P1, showing the occurrence of species (clusters) A, B, C, and D.*

Biological and antigenic properties of subgroups

The enteroviruses are divided into various biological subgroups, which in general reflect the sequence classifications imperfectly. However, some are apparently related both serologically and by the sequence of the P1 region. For example, poliovirus 1 and poliovirus 2 share common antigens. Cross-relationships also exist between coxsackie viruses A3 and A8, A11 and A15, A13 and A18, echoviruses 1 and 8, 6 and 30, and 12 and 29.

Table 2 *Properties of the picornavirus genera*

Property	*Enterovirus*	*Hepatovirus*	*Rhinovirus*	*Cardiovirus*	*Aphthovirus*	*Parechovirus*[a]
Particle size	27 nm	27 nm	27 nm	27 nm	27 nm	27 nm
Sense or virion RNA	Messenger	Messenger	Messenger	Messenger	Messenger	Messenger
Sedimentation coefficient (S) of virion	156	156	149	156	142–146	156
RNA in virion (%)	31	31	31	34	38	31
Size of virion proteins (kDa)						
VP1	35	35	35	34	29	30.5
VP2	30	30	30	30	30	38
VP3	24	24	23	23	22	30
VP4	7	?[b]	7	7	8	. . .
Poly(A) tract	+	+	+	+	+	+
Size of genome						
kb	7 445	7 450	7 155	7 832	8 400	7 340
kDa	2 200	2 200	2 200	2 300	2 500	2 200
Buoyant density in CsCl (g/cm^3)	1.34	1.34	1.41	1.34	1.43	1.36
Acid stability	+	+	–	+	–	+
Poly(C) tract	–	–	–	+	+	–
Base composition						
A (%)	30	29	32	26	26	32
U (%)	24	33	28	25	21	29
C (%)	23	16	20	26	28	19
G (%)	23	22	20	23	25	20

a) Echoviruses 22 and 23.
b) It is not clear whether hepatoviruses contain VP4.

In general, the echoviruses may be biologically distinguished from the coxsackie viruses by their failure to cause pathological changes in newborn mice. However, most strains of echovirus 9 can produce flaccid paralysis in newborn mice, a finding that led to its early classification as coxsackie virus A23. Some strains of enterovirus 71 grow better in newborn mice than in cultures of primate cells. Conversely, coxsackie virus A9 is not pathogenic for mice unless passaged in cell culture, in which it grows readily, thus resembling the echoviruses.

The neutralization of rhinovirus infectivity by antisera is largely type specific, and is the basis of the numbering system by which rhinoviruses are classified into 100 serotypes (Hamparian et al. 1987). Minor serological variations may exist within a serotype. Within type 1 there are two subtypes, A and B: the low degree of cross-reaction between these subtypes may be reciprocal or one-way (Monto and Johnson 1966). 'Prime' strains of rhinovirus 22 that are antigenically broader than the prototype strain have been isolated (Schieble et al. 1970; Hamparian et al. 1987).

Resemblances to other viruses

A number of other viruses seem to be superficially similar to the picornaviruses. Astroviruses, which contain RNA and lack a lipid bilayer, are of similar size to picornaviruses, although with distinct morphology in electron micrographs. The caliciviruses are similar, but significantly larger, with a particle diameter of 30 nm, a characteristic morphology, and a capsid consisting of a single protein species. Other small round viruses are found in the intestinal tract and may be confused with the true enteroviruses; their relationship, if any, with the enteric picornaviruses is not clear (see Human enteric RNA viruses: Astroviruses).

However, the picornaviruses are similar in many ways, including particle structure and genomic organization to the spherical plant viruses such as cowpea mosaic virus and to insect viruses such as cricket paralysis virus. Similarly, the picornaviruses and togaviruses share some features of genomic strategy and organization. These relationships, which have been revealed by detailed molecular biological characterization of the viruses, suggest intriguing possibilities for the evolutionary origin of picornaviruses (see, for example, Rossmann 1987).

Morphology and structure

Picornaviruses usually appear in electron micrographs as roughly spherical particles not penetrated by negative

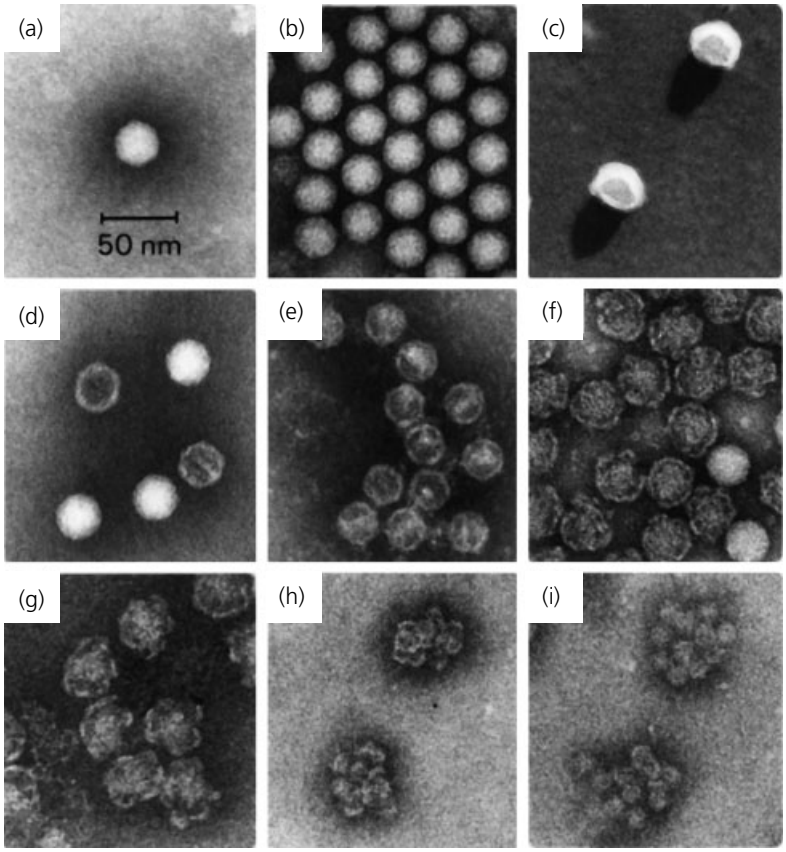

Figure 3 *Electron micrographs of poliovirus; all specimens (except c) stained with 4 percent sodium silicotungstate and all magnified ×200 000.* **(a)** *Whole virus particle (D antigen) ca. 30 nm in diameter and displaying a hexagonal profile.* **(b)** *High concentration of virus in hexagonal array.* **(c)** *Freeze-dried virus with low angle platinum shadowing (print reversed to give black shadows); virus particles produce either pointed or blunt-ended shadows, which are consistent with an icosahedral particle.* **(d)** *Penetration of stain into empty particles reveals outer capsid.* **(e)** *Empty particles (C antigen) showing small central unstained portions representing incomplete contents.* **(f)** *Flattened particles showing complete contents and outer capsid.* **(g)** *Flattened particles showing breakdown of capsid into angular or crescentlike portions.* **(h and i)** *Particles heated at 56°C for 2 min produce aggregates of ca. 12 crescentlike or circular structures.*

staining methods. Figure 3 demonstrates some of the features of poliovirus, but similar results would be obtained with all picornaviruses. The viruses are 25–30 nm in diameter (Figure 3a), and can occasionally present a hexagonal appearance (Figure 3a) or form hexagonal arrays (Figure 3b). Similarly, shadowed preparations can produce blunt-ended or pointed shadows, consistent with an icosahedral structure (Figure 3c). In preparations positively stained after drying of the virus from ethanol where the RNA is extruded, the capsid may remain negatively stained and so the thickness of the virion wall can be seen to be ca. 2.5 nm. A similar thickness has been determined for the empty capsids, which are in general penetrated by negative stain (Figure 3d), although frequently there are regions from which the stain is excluded (Figure 3e). When the particle is distorted during drying, it may expand and break into circular or crescent-like portions (Figure 3g). Similarly, heating (Figure 3h, i) can give rise to 12 crescentic or circular structures, which are believed to correspond to the pentamers of the icosahedron.

Understanding of picornaviruses was extended immensely by the determination of the atomic structures of rhinovirus 14 (Rossmann et al. 1985), the type 1 poliovirus strain Mahoney (Hogle et al. 1985), the cardiovirus mengo virus (Ming et al. 1987) and FMDV (Acharya et al. 1989). The structure of the type 1 poliovirus strain Mahoney is shown in Figure 4. Although all picornavirus structures solved to date are strikingly similar to each other, and to the spherical plant viruses such as southern bean mosaic virus and tomato bushy stunt virus, there are also clear differences between them.

The capsid of poliovirus is composed of 60 protomers, each being made up of one molecule of each of the four virion proteins VP1, VP2, VP3, and VP4. The protomers are arranged with icosahedral symmetry. Each of the 20 faces of the icosahedron is made up of three protomers, orientated so that the 12 apices of the icosahedron are occupied by five copies of VP1 while the center of each face is occupied by three copies each of VP2 and VP3, alternating around the threefold axis of symmetry. The overall thickness of the shell is about 3 nm, extending

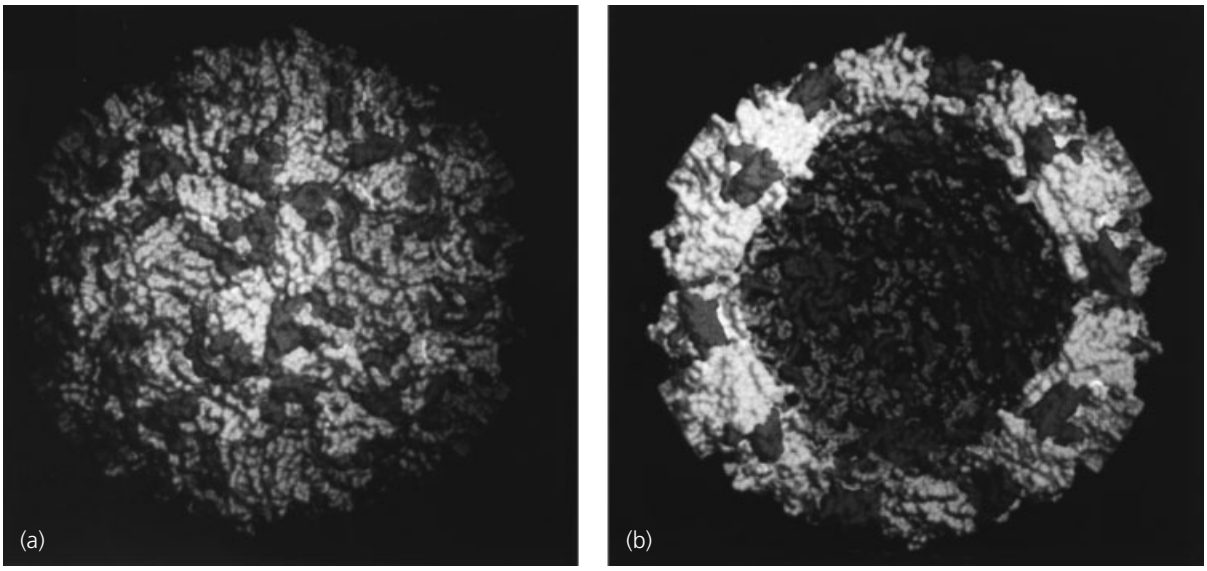

Figure 4 *Structure of the Mahoney strain of type 1 poliovirus showing* **(a)** *the complete capsid, and* **(b)** *the capsid with one pentamer removed to reveal the inside of the virion.*

11–14 nm from the particle center. However, the apices of the icosahedron and the face at the threefold axis of symmetry form two elevated features separated by a cleft or 'canyon' that circles the apex, which, for the enteroviruses and rhinoviruses, is believed to be the site by which the virus attaches to the specific receptor site on the cell.

The structural organization of the individual proteins that make up the virion is similar, (Figure 5). Each protein has a wedge-shaped core structure composed of eight strands of protein, shown as broad arrows in Figure 5, arranged in an antiparallel β-sheet array, the eight strands collectively forming a barrel (termed the β barrel). The core structure of polioviruses, rhinoviruses, and other spherical viruses, including plant viruses, are very similar in this respect. The features that are unique to the individual virus, including antigenic sites, arise from the loops that join the different β strands and the sequences at the N and C termini.

The narrow end of the β barrel of VP1 is located near the icosahedral apex of the virus while the corresponding regions of VP2 and VP3 alternate around the threefold axis of symmetry. VP4 forms a lattice around the inside of the pentameric apex. Interactions between adjacent pentamers include regions of secondary structure in the form of β sheets between the β barrels of VP2 and VP1.

In addition to the protein components of the virus shell, the N terminus of each VP4 protein is covalently bound to a myristic acid residue (Chow et al. 1987). The myristate sequences penetrate the pentameric apex, possibly forming a framework for assembly.

The structures of poliovirus and rhinovirus are very similar but differ from that of the cardioviruses, typified by mengo virus (Ming et al. 1987), where the pentameric apex is surrounded by five distinct pores rather than a canyon. The 15 C-terminal residues of VP1 are disordered but exposed on the virus surface, whereas all C-terminal residues of VP1 are ordered in the poliovirus and rhinovirus.

The particle shell of FMDV is relatively smooth because of the truncation of certain loops, and there is no canyon or series of pits around the pentameric apex (Acharya et al. 1989). One loop in VP1 forms a disordered surface structure known to be a major antigenic site. The C terminus of VP1 is also a disordered surface feature lying in the region of the disordered loop. A residue in VP2 is covalently linked by a disulfide bridge to a residue in the disordered VP1 loop, and is therefore itself faint in the structure. It is believed that the highly exposed, disordered loop forms the site on the virus by which it attaches to the host cell, in contrast to the other picornaviruses which attach to the cell via the canyon or pit regions.

Genome structure and function

Sequences of the genomes of many picornaviruses representing strains from each genus have been determined. The genomic RNA, constituting about 33 percent of the mass of the virion, varies in size from about 7 200 nt for rhinovirus to 8 500 nt for FMDV (mol. mass 2 000–3 000 kDa). Virion RNA is of messenger sense and therefore infectious for cells, although less so than intact virus. The layout of the genome of poliovirus is presented in Figure 6. The 5′ terminus of the RNA is covalently linked to a small protein (VPg) of 22 amino acids via the phenolic hydroxyl group of the tyrosine at residue 3 from the N terminus, a residue conserved in all picornaviral sequences of VPg to date. The following 740 nucleotides of the RNA constitute the 5′ noncoding region preceding the coding region of the genome,

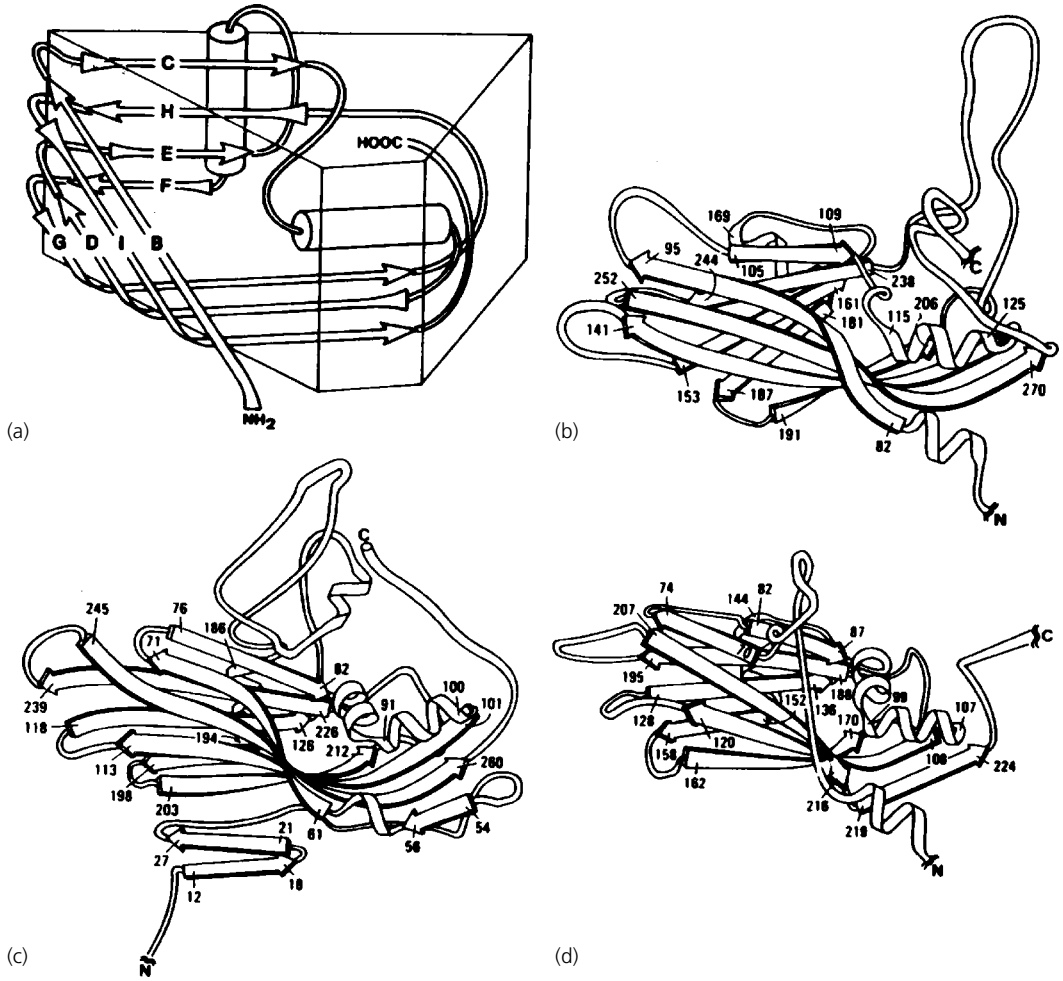

Figure 5 *Structure of the proteins making up the poliovirus capsid:* **(a)** *schematic diagram of β-barrel structure (arrow) and connecting α helices (cylinders);* **(b)** *VP1;* **(c)** *VP2;* **(d)** *VP3.*

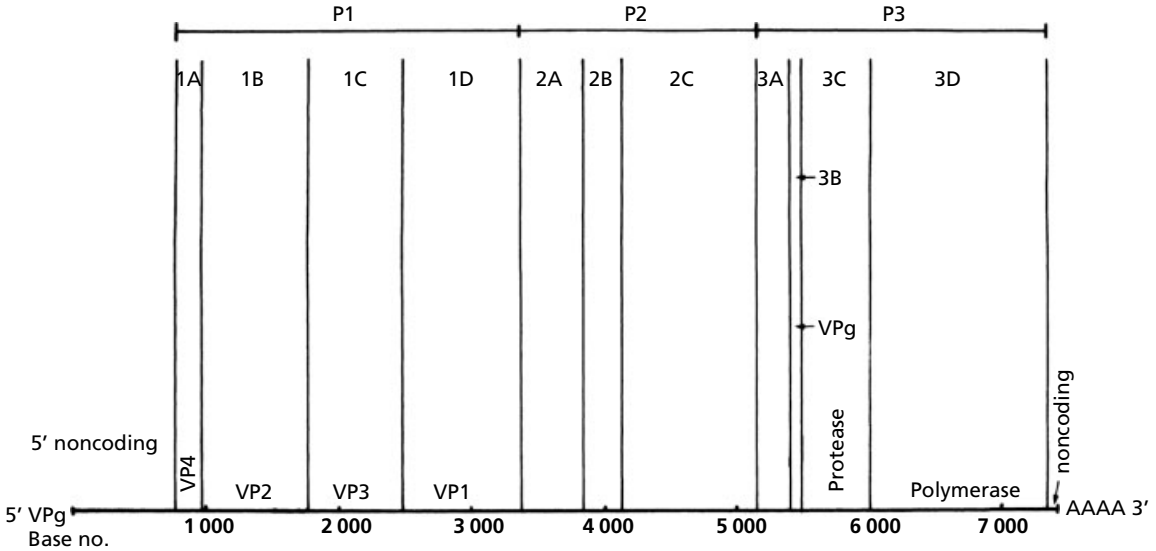

Figure 6 *Organization of the genome of enteroviruses showing the 5′ and 3′ noncoding regions and the location of the regions coding for the various proteins. Nomenclature is according to Rueckert and Wimmer (1984) and the common name where appropriate.*

which is translated as a single large protein cleaved by virus-encoded proteases as it is translated and post-translation. The genome terminates in a short untranslated 3′ noncoding region of about 70 nt, to which a sequence of 40–100 adenosine residues is attached, comparable to that found on many eukaryotic mRNAs. Sequence analysis of viral proteins has defined the genomic location and proteolytic cleavage sites used in processing. The structural proteins are encoded in the order VP4, VP2, VP3, and VP1, or 1A, 1B, 1C, 1D in the formal nomenclature (Rueckert and Wimmer 1984), from the 5′ end, making up the P1 region of the polyprotein which is excised as a single unit during replication. The nonstructural proteins P2, consisting of proteins 2A, 2B, and 2C, and P3, consisting of proteins 3A, 3B, 3C, and 3D, are also considered to be single units. The rhinoviruses closely resemble the enteroviruses except that the 5′ noncoding region lacks the poorly conserved 100 nt region immediately preceding the open reading frame.

The genomic organization of the cardioviruses and aphthoviruses differs from this general structure in that they encode a leader protein (L) before the P1 region and contain a polycytidylate tract in the 5′ noncoding region. In addition, protein 2A of FMDV is very small, and the genome includes three distinct copies of the protein VPg, any of which may be covalently linked to virus-derived RNA.

Complete cDNA copies of the picornavirus genome can be generated and used either to recover infectious virus directly from the DNA (Racaniello and Baltimore 1981) or, after linking to transcription systems such as SP6 or T7, to generate infectious RNA. It is thus possible to study the effect of defined genetic manipulations on the phenotype of the virus. For technical reasons, complete infectious copies of aphthovirus or cardiovirus genomes are more difficult to produce than those of the other genera.

Antigens

Most studies of the antigenic properties of enteroviruses, and of poliovirus in particular, have been concerned with humoral immunity to structural proteins, which is believed to be the major factor in protection from disease, although some work has concerned sites involved in recognition by T cells (Mahon et al. 1995). In infection by FMDV, however, a major immune response is directed against the infection-associated antigen (IAA) which has been shown to correspond to the nonstructural RNA polymerase protein 3D.

Antigenic differences may occur between different isolates; for example, isolates of type 3 polioviruses in Finland during 1984/85 differed from classic type 3 strains (Huovilainen et al. 1987) and UK strains of coxsackie virus B5 from 1973 were very different from the coxsackie virus Faulkner strain isolated in 1952.

The infectious virus particles of polioviruses and other picornaviruses differ antigenically from the empty capsids. Mayer et al. (1957) resolved poliovirus preparations into distinct fractions by centrifugation on a sucrose step gradient; most of the infectious material appeared in the fourth layer (D) and reacted preferentially with convalescent sera, but viral antigens could also be detected in the lighter third layer (C) which reacted preferentially with acute phase sera. D antigenic material corresponds to infectious particles, and C to empty capsids. Others showed that the natural virus expressed one antigenic determinant (termed N antigen), whereas material heated at 56°C for 30 minutes expressed another (H antigen) (Hummeler and Tumilowicz 1960); in general, N and D antigens are equated, as are C and H antigens. Relatively mild treatments have a pronounced effect on antigenicity (Le Bouvier 1955). Rhinoviruses also exhibit D and C antigen characteristics (Butterworth et al. 1976).

Monoclonal antibodies derived from mice immunized with poliovirus can bind to the D or to the C antigenic forms of the virus (Rombaut et al. 1983), or to both (Ferguson et al. 1984). In addition, some D-specific antibodies bind to subunits that are precursors to virions in the assembly pathway, whereas others bind only to mature virus. The particular sites recognized by antibodies were identified by generating antigenic mutants resistant to neutralization by monoclonal antibodies (Minor et al. 1986a; Page et al. 1988).

The specific amino acid substitutions found in antigenic mutants of poliovirus selected with murine antibodies are largely located in the loops decorating the β barrels and clustered into several non-overlapping sites on the surface of the virion. The regions in which mutations have been identified are shown in Table 3, and each site is complex and conformational in nature. For poliovirus of both type 2 and type 3, site 1 seems to be the major target of the murine immune response, whereas antibodies against the corresponding region of type 1 have not been induced by immunization with native virus.

Antibodies directed against site 3 of poliovirus serotypes 1 and 3 tend to be highly strain specific, even to the extent that antigenic drift can be demonstrated during the period of excretion by vaccinees.

Neutralizing antibody is difficult to induce with isolated viral proteins or with short peptides based on sequences found within them except where, as in FMDV, the immunogenic sequence is disordered in the structure (Bittle et al. 1982). The difference is attributable to the highly integrated and conformational nature of the antigenic sites. Even where viruses have been constructed in which entire sites have been exchanged between different serotypes (Burke et al. 1988; Murray et al. 1988) and can have antigenic and immunogenic properties of both parents, the inserted site is imperfectly expressed (Minor et al. 1990, 1991). The viruses usually grow poorly.

Table 3 *Antigenic sites for the neutralization of poliovirus and Rhinovirus by monoclonal antibodies*

Poliovirus			Rhinovirus 14		
Site	**Amino acids involved**		**Site**	**Amino acids involved**	
1	VP1	89–100	Nim 1a	VP1	91, 95
	VP1	142	Nim 1b	VP1	83, 85, 138, 139
	VP1	166	. . .	. . .	. . .
	VP1	253	. . .	. . .	. . .
2	VP1	220–222	Nim II	VP1	210
	VP2	164–170	. . .	VP2	158, 159, 161, 162
	VP2	273	. . .	. . .	. . .
3	VP1	286–290	Nim III	VP1	287
	VP3	58–60	. . .	VP3	72, 73, 75, 78, 203
	VP3	70–72	. . .	. . .	. . .
4	VP3	77–80	. . .	. . .	. . .
	VP2	72	. . .	. . .	. . .

Similar antigenic studies have been performed for rhinovirus 14. Sherry and colleagues (1986) identified four antigenic sites recognized by monoclonal antibodies (Table 3). All are surface features and in general the antigenic structures identified in all picornaviruses correspond to all of the prominent surface features in the structure, which are usually loops between strands of β barrels (Minor 1990). In contrast to poliovirus, no sites are strongly immunodominant or recessive.

When virus infection is initiated, the capsid undergoes a major rearrangement with the extrusion of the N terminus of VP1, which is normally internal. Antibodies to this region have been described in post-infection human sera and are believed to have neutralizing capacity.

Replication

ENTRY INTO HOST CELLS

Viral RNA from poliovirus is able to induce a single round of replication in rabbit kidney cells, which are not normally susceptible to infection (Holland et al. 1959), indicating that the blockage is at the level of virus entry. Saturable receptors have been demonstrated (Lonberg-Holm et al. 1976) and competition experiments showed that the three serotypes of poliovirus attach to the same site, distinct from that used by other enteroviruses. Monoclonal antibodies directed against cellular components have been raised that specifically block the binding of poliovirus (Minor et al. 1984), coxsackie viruses (Campbell and Cords 1983), and rhinovirus 14.

The gene coding for the poliovirus receptor was identified by Mendelsohn et al. (1986, 1989). It is a three-domain protein of the immunoglobulin superfamily with an unglycosylated molecular mass of 42 kDa. The cellular receptor for most rhinoviruses is the cell adhesion protein ICAM-1, a five-domain protein of the same superfamily (Greve et al. 1989; Staunton et al. 1989).

Other picornavirus receptors identified include decay accelerating factor (DAF), implicated in the complement pathway as the receptor for echovirus 7, among others (Ward et al. 1994) and integrin VLA2 for echovirus 1 (Bergelson et al. 1992). The possible need for additional molecules in attachment or penetration is suggested by the failure of mouse cells transfected with the genes for either ICAM-1 or DAF to support the growth of rhinovirus 14 or echovirus 7. Moreover, whereas mouse cells transfected with the poliovirus receptor are highly susceptible to poliovirus (Pipkin et al. 1993), it has been suggested that CD44 may also play a role (Shepley and Racaniello 1994). The poliovirus receptor and susceptibility to coxsackie virus B3 and echovirus 11 have been shown to be encoded on human chromosome 19 (Medrano and Green 1973; Miller et al. 1974). Transgenic mice carrying the gene for the human poliovirus receptor are susceptible to poliovirus infection by a variety of routes, developing paralysis similar to that seen in primates (Ren and Racaniello 1992) and such animals have been extensively used in pathogenesis studies in place of primates. A summary of the receptor sites used by some picornaviruses is given in Table 4, but the situation is complex except for polio in that the receptor sites listed alone do not in general confer susceptibility when transfected into nonsusceptible cells alone (Rieder and Wimmer 2002).

UNCOATING

When virus is allowed to bind to cells at room temperature and the cultures are then incubated at 37°C, up to 90 percent of the virus may be released into the medium. The released virus (A particles) has lost VP4, and has converted from D (N) antigenicity to C (H) antigenicity, although it retains the virion RNA (Joklik and Darnell 1961). Similar particles can be identified in the cells, and their production can be catalyzed by soluble receptor in the case of poliovirus.

PROTEIN SYNTHESIS

The genomic RNA is infectious and translated into virion proteins directly. The 5′ terminus of many

Table 4 *Receptors and accessory factors used by picornavirus genera and species*

Genus	Species	Receptor	Accessory factors
Enterovirus	**B**		β_2–m
	Coxsackie virus A9	$\alpha_v\beta_3$; alternative receptor likely	MAP-70
	Coxsackie virus B1–6	HCAR	. . .
	Coxsackie viruses B1, 3, 5	DAF (= CD55)	$\alpha_v\beta_6$
	Echoviruses 1, 8	VLA-2 (= $\alpha_2\beta_1$)	. . .
	Echoviruses 3, 6, 7, 11, 12, 13, 19, 21, 24, 25, 29, 30, 33	DAF	β_2–m, CD59
	Echovirus 6	Heparin sulfate?	. . .
	Echovirus 69	ND	. . .
	C		
	Polioviruses 1–3	CD155 (Pvr)	. . .
	Coxsackie viruses A13, 17, 18, 20, 21, 24	ICAM-1	. . .
	Coxsackie virus A21	DAF	62
	Coxsackie virus 24v	ICAM-1, second receptor?	. . .
	D		
	Enterovirus 70 (68)	DAF	. . .
	E		
	Bovine enterovirus types 1 and 2	ND	. . .
Parechovirus	Human echovirus types 22 and 23	$\alpha_v\beta_3$	. . .
Rhinovirus	Major receptor group rhinovirus (>90 serotypes)	ICAM-1	. . .
	Minor receptor group rhinovirus (>10 serotypes)	LDL receptor related	. . .
Hepatovirus	Hepatitis A virus	HAVcr-1	. . .
Aphthovirus	FMDV	$\alpha_v\beta_3$, $\alpha_v\beta_6$	. . .
	A12 strains of foot-and-mouth disease virus 0_1	Heparan sulfate	. . .
Cardiovirus	Encephalomyocarditis virus, mengo virus	VCAM-1, sialylated glycophorin	. . .
	Theiler's murine encephalomyocarditis virus	ND	. . .
	Vilyuisk virus	ND	. . .

eukaryotic mRNA species is blocked by a 'cap' or 7-methylguanosine residue linked in the reverse orientation to the 5′ terminal residue by phosphate groups. Ribosomes bind close to the free capped 5′ end of the RNA and then 'scan' the sequence for the first suitable initiation codon (defined in part by the nucleotides that flank it) at which protein synthesis commences. Picornavirus RNAs are translated by another mechanism in which the 5′ noncoding region acts as an internal initiation site for translation not requiring a free terminus (Pelletier and Sonenberg 1988). Following the initiation of viral protein synthesis, host protein synthesis is rapidly shut off by mechanisms that are not fully understood but include the proteolytic cleavage of initiation factors required for the cap-dependent protein synthesis of the host cell by a mechanism involving the virus-coded protease 2A.

POST-TRANSLATION PROCESSING

The proteases of polioviruses and other enteroviruses are extremely specific and have been proposed as possible targets for chemotherapeutic agents. In the case of poliovirus, the P1 protein comprising all the structural proteins is excised as a single unit by protease 2A, which cleaves between a tyrosine–glycine pair. This has the effect of removing the structural from the nonstructural proteins before they are processed any further. The corresponding protease in coxsackie virus B3 is believed to cleave between a threonine–isoleucine pair. Later processing is carried out by protease 3C or the precursor molecule 3CD. It may be difficult to identify the active moiety, which may be an intermediate or a final product of the cleavage pathway. The processing protease in poliovirus cleaves between glutamine–glycine pairs, including those found in the P1 region between VP2 and VP3, and VP3 and VP1. The exposure and possibly the amino acid sequence surrounding the dipeptide govern whether cleavage occurs, as many such pairs are not cleaved. A similar sequence specificity seems to function in coxsackie virus B viruses, but the situation is less clear in viruses such as hepatitis A virus, or other picornaviruses, such as EMC, in which cleavage does not seem to involve a specific dipeptide (Palmenberg 1987).

Two further proteolytic cleavages take place in the viral growth cycle. One occurs in protein 3D, at a tyrosine–glycine pair, and is thus probably performed by protein 2A. This may have the effect of reducing the amount of active polymerase in the cell, but the advantage of this is not understood, especially as at least the Sabin type 3 vaccine strain of poliovirus and viruses related to it do not carry out this step, although they grow to high titer in vivo and in vitro. The final cleavage involves the formation of VP2 and VP4 from the precursor VP0, which occurs as the last stage of maturation.

REPLICATION OF RNA

The synthesis of RNA requires an RNA template that is read from the 3′ to 5′ direction, whereas protein synthesis requires an RNA template read in the opposite direction. The two processes are therefore mutually exclusive, and the factors influencing the fate of a particular RNA molecule are not known.

The replication of poliovirus RNA requires the synthesis of both negative and positive strands of RNA (Kuhn and Wimmer 1987; Paul 2002). Messenger-sense (positive-strand) RNA is synthesized in a great excess over the complementary-sense RNA. Infected cells contain complete double-stranded copies of one positive and one negative strand of viral RNA, termed replicative forms (RF) and partial double-stranded structures termed replicative intermediates (RI). All nascent strands of polio RNA of either sense are covalently linked to VPg at the 5′ terminus. This protein is absent from RNA associated with ribosomes. The association of VPg with nascent RNA presumably accounts for the fact that inhibitors of protein synthesis, such as puromycin, rapidly inhibit RNA synthesis.

All of the nonstructural proteins are involved in RNA replication in some way. Viral protein 3D is present in replication complexes isolated from infected cells (Lundquist et al. 1974) and is able to synthesize poly(U) from a poly(A) template if primed with oligo(U) (Flanegan and Baltimore 1977). Protein 3B (VPg) is associated with nascent RNA strands. Guanidine inhibits poliovirus RNA synthesis, and mutants that are either resistant to it or dependent on its presence for growth possess base changes in protein 2C, which is therefore also implicated in synthesis in some way. This protein may influence host range in polioviruses and in rhinoviruses. It is therefore likely that some host factor is involved, although this could, for example, involve structural factors contributed by the host, such as cell membranes.

The detailed mechanism of viral RNA synthesis is controversial, although it is accepted that it occurs in particulate or membranous regions of the infected cell. Replication of virus in vitro has been reported in protein translation systems primed with polioviral RNA. Protein 3D is the main component of replicative complexes which may be prepared from infected cells by mild detergents. Such complexes synthesize both virion (+) and complementary (−) sense RNAs in vitro. Three models for RNA synthesis have been put forward.

1. Dasgupta et al. (1980) reported the presence of a 67 kDa protein able to initiate viral RNA synthesis in the absence of oligo(U). The protein has kinase activity, and the implication of host proteins in viral nucleic acid synthesis in other systems is well documented. However, this factor [host factor (HF)] leaves no clear role for VPg.
2. Flanegan and co-workers (Young et al. 1985) have reported a host enzyme-terminal uridylate transferase (TUT) – which is able to add uridine residues to the 3′ termini of viral nucleic acid. Uridine bases added to the 3′ end of the virion RNA that carries a poly(A) tail would form a tail that could loop back and anneal to the poly(A) sequence as a primer for 3D. The 3′ terminus of the complementary RNA is UUUUAA, so there is a short sequence for such a hairpin structure to form and prime RNA synthesis on the negative-sense RNA. An analogous mechanism has been suggested in the replication of parvoviruses. The hairpin structure would be cleaved by VPg or a precursor, in the course of which VPg would be transferred to the 5′ end of the nascent strand.
3. There is a proposal that uridine residues are added to VPg to make VPg pUpU, which then functions as a primer. Such an intermediate has been reported.

Combinations of these models have been proposed and the reader is referred to Paul (2002) for further details and discussions of the experimental evidence available.

ASSEMBLY OF CAPSIDS

The structural proteins are synthesized and processed as a unit and assemble to form pentamers of VP0, VP1, and VP3 (Putnak and Phillips 1981). Isolated pentamers (or 14S particles) are able to assemble into procapsids, which are complete but empty viral shells, sedimenting at 75S. It is possible to prepare soluble fractions from infected cells containing 14S subunits able to assemble to give 75S empty shells. If the pH is allowed to fall, however, the 75S units become altered, adopting the C antigenic form, and are no longer dissociable.

The last step in the maturation of the infectious virion is the cleavage of VP0 to give VP2 and VP4. It has been proposed that this occurs autocatalytically by insertion of the nucleic acid as a proton donor in the presence of a serine residue at position 10 of VP2 close to the cleavage point (Rossmann et al. 1985). However, specific mutagenesis of residue 10 does not prevent cleavage of VP0. A possible model for virion assembly is that the empty 75S capsids act as a dissociable reservoir of structural proteins. As the units reassociate around the genomic RNA, the cleavage of VP0 to VP2 and VP4 occurs, making assembly essentially irreversible under

the conditions pertaining in the infected cell. This scheme remains speculative but plausible.

Effects on host cells

Replication of enteroviruses is normally accompanied by apoptosis of the host cell. The viruses almost certainly encode proteins that affect this process, but this remains a subject of study. Non-lytic persistent virus infections have been reported for poliovirus (Sarnow et al. 1986), hepatitis A virus, echovirus 6, and the coxsackie virus B viruses (Matteucci et al. 1985) in appropriate cells. There is evidence for the persistent enterovirus infection in vivo of humans and rats (Bowles et al. 1986; Schnurr and Schmidt 1988; Yousef et al. 1988).

Resistance to physical and chemical agents

Enteroviruses can remain viable for years at −20°C and −70°C, and for weeks at 4°C, but lose infectivity slowly at room temperature and with increasing rapidity as the temperature is raised. Their inactivation at all environmental temperatures is inhibited by molar magnesium chloride; this property has led to the widespread use of magnesium chloride as a stabilizer of oral poliovaccine.

In the absence of organic matter, enteroviruses are rapidly inactivated by ultraviolet light and usually by drying.

Enteroviruses are insensitive to alcoholic and phenolic disinfectants, ether, chloroform, and deoxycholate. Treatment with formaldehyde (0.3 percent), 0.1 M HCl, or free residual chlorine (0.3–0.5 p.p.m.) causes rapid inactivation. The activity of chlorine is diminished by a high pH, low temperature, and the presence of organic matter, and it may therefore be less effective when used under field conditions.

CLINICAL AND PATHOLOGICAL ASPECTS

Enteroviruses

The alimentary tract is the predominant site of replication of enteroviruses. Although infection is usually asymptomatic, enteroviruses are recognized causes of paralytic poliomylitis, encephalitis, aseptic meningitis, myocarditis, conjunctivitis, and numerous other syndromes associated with target organs outside the alimentary tract. The serotypes comprising each group and the main disease caused by them are listed in Table 1.

HOST RANGE

The only natural host of polioviruses are humans, although most strains will infect Old World monkeys and chimpanzees. However, cotton rats, mice and hamsters, and fertile hens' eggs may be infected by strains adapted in the laboratory. Other enteroviruses, notably the coxsackie viruses, will infect mice under laboratory conditions. Swine vesicular disease virus (SVDV) is closely related to coxsackie virus B5.

PATHOGENESIS

Enteroviruses infect via the mouth or oropharynx. The incubation period is usually 7–14 days, with extremes of 2–35 days. Virus infects the mucosal tissues of the pharynx, gut or both, finally entering the bloodstream and gaining access to cells of the reticuloendothelial system and specific target organs such as the meninges, myocardium, and skin. Enteroviruses can generally be recovered from the pharynx during the first week of illness and from the feces for 1–4 weeks after onset of illness; they have been isolated from cerebrospinal fluid (CSF), spinal cord, brain, heart, conjunctivae, and skin lesions.

Two or more enteroviruses may propagate simultaneously in the alimentary tract but usually multiplication of one virus interferes with the growth of the heterologous type.

Poliomyelitis

Poliomyelitis is the most serious disease caused by any of the enteroviruses. According to one view (Bodian 1955), the virus first multiplies in the tonsils and Peyer's patches of the small intestine before spreading to the more distant lymph nodes and thence by a viremia to other sites, including the spinal cord. According to another view (Sabin 1955), the virus multiplies at mucosal surfaces and accumulates in the draining lymph nodes where some, but not all, strains may replicate. From there virus seeds peripheral sites, including peripheral nerves, and the virus generated at the distant sites is the source of the detectable viremia. Antibodies appear early and are protective even when given as passive immunoglobulin (Hammon et al. 1953).

Virus may pass to the spinal cord along nerves under certain conditions; for example, in children with inapparent infection either at the time of tonsillectomy or at the time of injection with material causing local inflammation.

The anterior horn cells of the spinal cord are most readily infected by poliovirus, but in severe cases the intermediate gray ganglia and even the posterior horn and dorsal root ganglia may also be involved. In the brain, the reticular formation, the vestibular nuclei, the cerebellar vermis, and the deep cerebellar nuclei are most often affected.

Other enteroviruses

The pathogenesis of nonpolio enteroviruses is similar to that of poliovirus in the initial stages but the target

organs vary. For example, echoviruses 6 and 9 may infect the CNS, causing meningitis. Coxsackie virus A7 and enterovirus 71 can cause paralysis, and others, such as coxsackie virus B viruses, may infect the heart or muscle.

Coxsackie virus A24 and enterovirus 70 resemble rhinoviruses, being spread by fomites and direct inoculation of the conjunctiva by contaminated fingers. The incubation period is very short (12–48 hours); recovery is usually complete within 1–2 weeks.

HOST FACTORS

Age is an important determinant of the severity of enterovirus disease, the incidence of some syndromes being greatest in neonates (e.g. myo- or pericarditis).

Malnutrition may worsen disease. When marasmus was induced in normally resistant postweaning mice, challenge with coxsackie virus B3 gave more severe disease.

Physical exertion while incubating poliovirus infection is associated with a higher incidence or greater extent of paralysis. The severity of paralysis is increased in monkeys infected experimentally with poliovirus and made to swim until exhausted. Similarly, exercising mice infected with coxsackie virus A9 or coxsackie virus B3 results in increased virus titers in their myocardium, in higher mortality rates, or both.

Pregnancy and parturition may be associated with a greater risk of paralysis with poliovirus infection or a greater risk of myocarditis with coxsackie virus B infection. Hypo- or agammaglobulinemic individuals with impaired or absent B-cell function but normal T-cell function clear enterovirus infections more slowly than those with a normal immune system. This provides further evidence for the importance of humoral immunity in the control of enteroviral infection. Maternal antibody may not protect from infection but will prevent disease.

CLINICAL SYNDROMES

The description of clinical syndromes below is not exhaustive. Further details concerning enteroviral diseases may be found in reviews by Moore and Morens (1984); Melnick (1990), and Bendinelli and Friedman (1988).

Most enterovirus infections produce clinically inapparent or minor illness. The consequences of infection, when they do occur, range from specific clinical syndromes, such as fatal paralytic or cardiac illness, to minor undifferentiated febrile illnesses. Asymptomatic infection rates are reported as 90–95 percent for the polioviruses, 76 percent for the coxsackie viruses and 43 percent for the echoviruses.

Poliomyelitis

Exposure to poliovirus may have the following consequences:

- **inapparent infection** (90–95 percent)
- **'abortive'** or **'minor' illness** (4–8 percent) with symptoms of upper respiratory tract infection, gastrointestinal upset, or influenza-like illness. The patient recovers within a few days
- **non-paralytic poliomyelitis** (1–2 percent), an illness of about 2–10 days similar to 'aseptic' meningitis, often accompanied by back pain and muscle spasm. Recovery usually is rapid and complete
- **paralytic poliomyelitis** (0.1–2 percent). The major illness, paralysis, usually follows a prodromal illness similar to the minor illness described above, especially in children. Thereafter the predominant feature is flaccid paralysis resulting from lower motor neuron damage. Paralytic poliomyelitis is termed **spinal** if the lower spine is involved or **bulbar** (from 'bulb' or medulla oblongata) if the upper spine and brainstem are involved. Bulbar poliomyelitis is the more serious because it affects the respiratory system.

A paralysed patient may recover completely or have varying degrees of residual deficit. A review of 203 cases between 1969 and 1981 in the USA (Moore et al. 1982) showed that residual paralysis was minor in 11 percent and significant or severe in 79 percent; 10 percent of the cases were fatal.

Post-polio syndrome has been described in which individuals who have survived an initial attack suffer a degeneration of function many years later. This may be anatomic in origin as advancing age results in the loss of nerve cells from an already damaged pathway. Sequences of enterovirus-related nucleic acid have been reported in the CSF of such cases, however, and some workers believe that the virus may persist.

'Aseptic' meningitis

Nonbacterial 'aseptic' meningitis (AM) is a common manifestation of enterovirus infection. Pleocytosis of the CSF is characteristically lymphocytic but may show transient predominance of polymorphonuclear leukocytes when cell counts are high in the early stage of illness, temporarily mimicking bacterial meningitis. Recovery is usually complete within a few weeks, although irritability and fatigue may persist for some weeks and there is a small risk of serious neurological damage in the first year of life (Wilfert et al. 1981). Second attacks of AM may occur from infection with other serotypes.

Paralytic disease

Polioviruses have been the dominant viral cause of paralysis in most of the world but are approaching eradication as described later. Sporadic cases of paralysis associated with coxsackie virus A7 continue to be reported (Gear 1984).

Paralysis has also been reported in association with coxsackie viruses B2–6 and echovirus types 3 (transverse myelitis), 4, 6, 9, 11, and 19.

Enterovirus 71 is capable of causing severe CNS disease with persisting flaccid paralysis, although it can also cause meningitis, encephalitis, and hand-foot-and-mouth disease (HFMD), depending on the outbreak (Melnick 1984).

Enterovirus 70, an agent of acute hemorrhagic conjunctivitis (AHC), has caused poliomyelitis-like illness (Kono et al. 1977).

Encephalitis

The dominant enterovirus associated with outbreaks of encephalitis is enterovirus 71, although poliovirus, coxsackie viruses A9, B3, B5, and B6 and echoviruses 6, 9, 17, and 19 have all been isolated from CSF or brain tissues.

Myalgic encephalomyelitis (postviral fatigue syndrome)

Multiple symptoms are associated with this illness (Behan 1980), but always include extreme muscle fatigue following slight physical effort, muscle pain, and psychological upset. The lack of objective physical signs, pronounced emotional lability, and neuroticism and the unknown nature of the disorder have made it easy for some physicians to dismiss the illness as hysterical. Myalgic encephalomyelitis (ME) is not a fatal disease but recovery may take months or years; relapses during periods of physical or mental stress are common. Chronic infection with enteroviruses has been reported (Yousef et al. 1988).

Pleurodynia (Bornholm disease)

The group B coxsackie viruses are the main identified causes of pleurodynia, also known as Bornholm disease, epidemic myalgia, or the devil's grippe. The chest pain is spasmodic, intensified by movement and may be severe. Abdominal pain resulting from involvement of the diaphragm occurs in approximately half the cases; in children this may be the chief complaint. The illness lasts for 2–14 days and is self-limited; recovery is complete although short relapses are common.

Sporadic cases of pleurodynia have also been associated with coxsackie viruses A4, A6, and A10; coxsackie virus A9 has been implicated in this syndrome and also in chronic diseases of the muscle and joints.

Congenital and neonatal infection

Prematurity, low birth weight, onset of illness within the first few days of life, occurrence during the 'enterovirus season' and recent antepartum or postpartum febrile illness in the mother are risk factors for the most severe, generalized and fatal neonatal enteroviral disease, most commonly associated with the coxsackie virus B, particularly coxsackie virus B2, and coxsackie virus B4. Neonatal illness has seldom been attributed to coxsackie virus A viruses but echoviruses (e.g. echoviruses 4, 9, 11, 17–20, 22, and 31) have been incriminated in both nursery outbreaks and sporadic infections. Fatal cases of echovirus infection generally present with progressive severe hepatitis and CNS involvement, whereas fulminant coxsackie virus B infections can be distinguished by the presence of severe myocarditis.

Cardiac disease

Severe myocarditis is usually a prominent feature of disseminated enterovirus infections of neonates; less severe myocarditis or pericarditis may follow infection of older children and adults. It has been estimated that ca. 5 percent of all symptomatic coxsackie virus infections induce heart disease.

Nucleic acid hybridization has been used to detect enterovirus RNA in myocardial biopsies. Replicating enterovirus RNA was found in 19 of 81 patients with suspected myocarditis (Kandolf 1988) but in none of the controls. Endomyocardial biopsies from >100 patients with heart muscle disease were investigated by Archard et al. (1988). Enterovirus genomic RNA was detected in 60 percent with active myocarditis and 47 percent with healing myocarditis; tissue from cases of other specific heart muscle diseases was consistently negative. All patients who made a coxsackie virus B-specific IgM response also had detectable enterovirus RNA in biopsy tissue. Enterovirus-infected myocardial cells have also been detected by hybridization in situ in dilated myocardiomyopathy (Kandolf 1988). Persistence of enteroviral genomes may therefore be implicated in chronic heart disease.

Mucocutaneous syndromes

Herpangina Herpangina is characterized by fever and a vesicular exanthem that typically involves the fauces and soft palate. It is chiefly associated with coxsackie virus A types 1–10, 16, and 22, and less commonly with coxsackie viruses B1–5 and some echoviruses. The disease occurs during the summer season and generally involves clusters of patients. Herpangina mainly affects young children but occasionally is seen in young adults. The illness is self-limited, with recovery in a few days.

Hand-foot-and-mouth disease (HFMD) HFMD is predominantly a childhood illness, usually associated with infection by coxsackie virus A16 or enterovirus 71. Occasionally, cases due to coxsackie viruses A5, A7, A9, A10, B2, and B5 have been reported. Typical cases of HFMD have vesicles and ulcers mainly in the front of the mouth, most frequently on the tongue, and a vesicular exanthem, mostly localized on the palms and soles;

virus can be isolated readily from these sites. During outbreaks, ca. 60–70 percent of cases show the complete clinical picture of HFMD. Recovery from HFMD is usually complete within 1–2 weeks.

Swine vesicular disease (SVD) in humans Swine vesicular disease virus (SVDV) is a porcine enterovirus that is closely related to coxsackie virus B5 (Zhang et al. 1993). The disease in pigs was first recognized in Italy in 1966. Since then SVD has been seen throughout Europe as well as in Hong Kong and Japan. The lesions in pigs resemble those caused by foot-and-mouth disease and other vesicular diseases of pigs. No vesicles have been seen in workers with pigs affected with SVD although, in laboratories, inapparent human infection has occurred and antibodies have been detected. There are on record four human cases with presumptive evidence of infection with SVDV. One patient had a severe illness with fever, myalgia, weakness, and abdominal pain, in two the disease was mild and the fourth had meningitis. In three of these patients infection occurred after exposure to pigs; the other was exposed to the virus in the laboratory.

Exanthems Usually transient and erythematous (rubelliform, maculopapular, rarely morbilliform, or vesicular) exanthems have been reported in infections by enteroviruses, notably during outbreaks of infection with coxsackie viruses A9, A16, and B5 or echoviruses 4, 9, and 16. Rash is seldom the sole clinical feature in symptomatic enterovirus infections. Fever, malaise, cervical lymphadenopathy, and aseptic meningitis may also occur in patients with rash. Rashes associated with coxsackie virus A9 and echovirus 9 can mimic rubella virus infection so closely as to merit diagnostic differentiation, particularly in pregnant women. Congenital abnormalities following maternal enterovirus infection have only rarely been reported.

Acute epidemic hemorrhagic conjunctivitis In 1970 there was a large epidemic of acute epidemic hemorrhagic conjunctivitis (AHC) in Singapore with more than 60 000 reported cases. The causal agent was identified as a variant of coxsackie virus A24. After an incubation period of 18–48 hours, mild to severe conjunctivitis developed, with subconjunctival hemorrhage in a minority of cases; recovery was usually complete within 1–2 weeks. Epidemics of AHC recurred in southeast Asia in 1975 and again in 1985 and in the Americas.

Enterovirus 70 first emerged in an epidemic of AHC in Ghana in 1969, spread through the coastal areas of Africa, eventually reaching India, southeast Asia, and Japan by 1970, ultimately involving hundreds of millions of people. Serological surveys in Ghana, Indonesia, and Japan confirmed that the virus was not prevalent before the pandemic. The illness had a short incubation period (24 hours), acute subconjunctival hemorrhage being the most characteristic sign; corneal involvement was usually transient and the prognosis good. The first outbreak in the western hemisphere was in Brazil in 1981 from where it then spread north, reaching Florida.

Rare cases of neurological complications accompanying or following enterovirus 70 conjunctivitis have been reported.

Respiratory disease

Respiratory illness caused by enteroviruses cannot be distinguished clinically from that caused by viruses such as rhinoviruses, parainfluenza viruses, respiratory syncytial virus, and adenoviruses. However, these viruses predominate in the winter months, whereas respiratory infection by enteroviruses typically occurs during the summer and autumn.

Diabetes

A possible association of insulin-dependent diabetes mellitus (IDDM) with coxsackie virus B infection was first suggested by Gamble in 1969. Reviews by Gamble (1980); Barrett-Connor (1985) and Toniolo et al. (1988) provide comprehensive interpretations of the available data.

A long euglycogenic period, characterized by the presence of islet cell antibodies (ICA) and T-lymphocyte responses, precedes the appearance of hyperglycemia following the near total disappearance of the β cells of the islets of Langerhans. There is some correlation of HLA haplotype (DR3 mainly in males, DR4 mainly in females) with this illness, suggesting that an autoimmune process is responsible for the chronic and irreversible loss of β cells. What triggers pancreatic autoimmunity in individuals is unknown.

Strains of coxsackie virus B4 and coxsackie virus B5 shown to be diabetogenic in mice have been isolated from people developing diabetes (Toniolo et al. 1988). It has now been shown that most of the group B coxsackie viruses are potentially able to infect and damage pancreatic endocrine cells, causing hyperglycemia. Serological studies have demonstrated that about one-third of the cases of IDDM in children are probably preceded by coxsackie virus B infection, although this may be a precipitating rather than causative factor.

Gastroenteritis

Although enteroviruses readily replicate within the gut, they are seldom the cause of gastroenteritis when this is the predominant feature.

Hepatitis

Hepatitis can accompany generalized coxsackie virus B and echovirus infections in neonates or occasionally in older children as well as following infection with hepatitis A virus (Hepatitis A and E).

Rhinoviruses

Rhinoviruses are the major cause of common colds. In the US National Health Interview Survey for 1985, colds were associated with 161 million days of restricted activity and accounted for 26 million days of school absenteeism, 23 million days lost from work and 27 million visits to physicians. More than 800 oral cold remedies are available and the annual expenditure on such treatments exceeds $2 billion in the USA.

CLINICAL MANIFESTATIONS

About 30 percent of human volunteers inoculated intranasally with nasal secretions or tissue culture fluid containing rhinoviruses develop colds, whereas less than 5 percent of volunteers given uninfected material have symptoms (Tyrrell 1965). When virus is given to adults without antibody, up to 100 percent develop colds (D'Allessio et al. 1976). At least 15 different serotypes have been given to volunteers, with essentially the same results.

In studies of university students (Gwaltney and Jordan 1966; Hamre et al. 1966; Phillips et al. 1968), industrial workers (Hamparian et al. 1964; Gwaltney et al. 1966), military personnel (Bloom et al. 1963), and civilian families (Elveback et al. 1966; Higgins et al. 1966; Hendley et al. 1969; Monto and Cavallaro 1972; Fox et al. 1975, 1985), rhinoviruses were isolated from 7–40 percent of people (including children) with upper respiratory illness but from less than 2 percent of healthy people. These findings probably underestimate the importance of rhinoviruses, because virus can be isolated from only half of known infections. The same epidemiologic studies also indicate that between 10 percent and 40 percent of rhinovirus infections are subclinical, with substantial differences in pathogenicity between serotypes.

Rhinovirus infections are more common and severe in young children than in adults (Fox et al. 1975). Rhinoviruses are also associated with lower respiratory disease in children or those with a history of chronic respiratory disease. However, in controlled studies, rhinoviruses were found in 3–8 percent of children in hospital either with lower respiratory illness or without respiratory symptoms (Chanock and Parrott 1965; Holzel et al. 1965; Stott and Walker 1967; Mufson et al. 1970). Hence, the precise role of rhinoviruses in acute respiratory disease of children is unclear (Cherry 1973). In children with a history of wheezy bronchitis or asthma, rhinoviruses are associated with up to 33 percent of acute attacks, and virus was found more often in sputum than in the nose or throat, suggesting that virus multiplies in the lower respiratory tract (Horn et al. 1979).

Rhinoviruses can cause lower respiratory tract illness in adult volunteers infected with virus in a fine particle aerosol (Cate et al. 1965). Transient abnormal pulmonary function has also been found in a small proportion of healthy volunteers after intranasal instillation of rhinovirus and in a minority of natural infections. Although rhinoviruses have been isolated postmortem from the lungs of adults who died of pneumonia, they are not implicated as a significant cause of adult pneumonia. However, in people with chronic bronchitis, rhinovirus infections have been associated with 12–43 percent of exacerbations and virus frequently seems to invade the lower respiratory tract (Stott et al. 1968). Although Buscho and colleagues (1978) found that rhinoviruses could infect patients without exacerbating their chronic bronchitis, such patients are more susceptible to rhinovirus infection than are otherwise healthy people. Furthermore, Horn and Gregg (1973) found lower respiratory tract signs in 85–90 percent of rhinovirus infections in adults with a previous history of asthma or bronchitis but in only 5 percent of such infections in otherwise healthy adults.

Bovine rhinovirus types 1 and 3 have been isolated from cattle with respiratory disease but there is little evidence to show that they are clinically important (Stott et al. 1980; Yamashita et al. 1985). There is one reported isolation of bovine type 2 virus (Reed et al. 1971). Similarly, although infection with equine rhinoviruses is widespread in horses, their precise contribution to the problem of respiratory disease is not yet clear (Powell et al. 1978).

PATHOGENESIS

Hand contact rather than aerosol is the prime mode of transmission of rhinoviruses (Gwaltney et al. 1978). Virus has not been recovered from air in rooms occupied by infected people and it is not possible to transmit rhinovirus colds between volunteers separated by a double wire mesh allowing free flow of air but no direct contact. Under the same conditions, coxsackie virus A21 was readily transmitted. Aerosols produced by talking, coughing, and sneezing derive primarily from the saliva, which has a low titer of virus, and not from the nasal secretions in which titers are highest.

By contrast, rhinovirus can readily be isolated from the hands of people during the acute stage of a cold. Furthermore, the hand contacts the eye or nose, on average, once every 3 hours. Thus, in a comparison of routes of rhinovirus transmission, 11 or 15 hand-contact exposures initiated infection but only 1 of 22 aerosol exposures was successful. Epidemiologic findings indicate that colds spread poorly in workplaces where many people share the same air space but direct contact is minimal. By contrast, transmission occurs readily within families where direct contact is frequent.

Rhinoviruses replicate in and destroy the ciliated epithelium of both nasal and tracheal mucosae, but grow to highest titer in nasal epithelium (Hoorn and Tyrrell

1965). Ciliated cells are extruded from the epithelium, leaving a generally smooth surface (Reed and Boyde 1972). Mycoplasma replication is enhanced in such virus-damaged tissue (Reed 1972), which is also probably more susceptible to secondary invasion by bacteria and mycoplasmas. Although the target cell in the lower respiratory tract is not known, type II pneumocytes may be infected by rhinoviruses in vitro (Tyrrell et al. 1979). Rhinoviruses are rarely found outside the respiratory tract, partly because their growth is restricted by temperatures above 37°C.

Host responses to infection may cause the vascular engorgement, increased vascular permeability with transudation of serum proteins and increased mucus production that are characteristic of colds. Host responses include release of inflammatory mediators, such as kinins; influxes of inflammatory cells, including polymorphonuclear leukocytes; interferon; and probably neuroreflexes and associated cholinergic stimulation and neuropeptide release (Naclerio et al. 1988). Further evidence for the role of kinins is provided by the finding that intranasal instillation of bradykinin causes some of the symptoms and alterations in nasal mucus seen in rhinovirus colds.

IMMUNE RESPONSES

Rhinovirus infection stimulates an antibody response in 47–77 percent of cases (Fox et al. 1975). The response is greater in adults than in children. Severe lower respiratory infection induces higher antibody titers than mild upper respiratory infection. Furthermore, certain serotypes are more effective antigens than others. Specific antibody in the circulation is related to protection against reinfection of volunteers. In families in which exposure to known serotypes occurred, pre-existing serum antibody was 52–69 percent effective in preventing infection. Rhinovirus infections also stimulate local IgA production. Whether secretory IgA in the mucus of the upper respiratory tract or circulating antibody in the serum is the more important in protection remains contentious (Perkins et al. 1969; Douglas and Couch 1972).

Foot-and-mouth disease

Foot-and-mouth disease is a highly infectious disease of ungulates. There are a few reports of infection in humans and experimental infections of laboratory animals are readily initiated. Seven serotypes of FMDV are recognized – O, A, C, SAT1, SAT2, SAT3, and Asia 1 – and existing vaccines are based on inactivated preparations of the appropriate serotype.

The first signs of disease in cattle are dullness, anorexia, and rising temperature, followed by nasal discharge and the appearance of vesicles in the mouth and on the feet. Initially, the vesicles in the mouth are present on the tongue, hard palate and dental pad, lips and muzzle, and on the feet in the interdigital space or along the coronary band, giving rise to lameness. The incubation period varies from 1 to 14 days.

The main routes of infection are inhalation, ingestion, or penetration of the epithelium. Virus may persist in the pharyngeal area for prolonged periods.

EPIDEMIOLOGY

Epidemiology is discussed in two sections: enteroviruses and rhinoviruses.

Enteroviruses

MODE OF TRANSMISSION

Humans are the only known reservoir for members of the human enterovirus group. Virus is generally shed for longer periods in feces (1 month or more) than from the oropharynx, and fecal contamination is the usual source of infection although enterovirus 70, the agent of acute hemorrhagic conjunctivitis, has been found almost exclusively in conjunctival and throat specimens.

INFLUENCE OF CLIMATE

In temperate climates enteroviruses are more prevalent in the summer and autumn (i.e. when warmth and humidity are at their peak). In tropical climates they tend to circulate all the year round or are associated with the rainy season.

SPREAD WITHIN FAMILIES

Young children are the usual reservoir of enterovirus infection. The secondary attack rates in susceptible family members are reported as 92 percent for polioviruses, 76 percent for coxsackie viruses and 43 percent for echoviruses. The greater spread of polioviruses and coxsackie viruses may be related to their longer periods of excretion. Secondary coxsackie virus infections were more common in mothers (78 percent) than in fathers (47 percent). Coxsackie viruses spread to 75 percent of exposed susceptibles but to only 25 percent of exposed people who already had antibody to the infecting type; echoviruses infected 43 percent of susceptibles and only one person with homotypic antibody.

EPIDEMIOLOGICAL SURVEILLANCE

International data

In 1963 the World Health Organization established a system for the collection and dissemination of information on viral infections. Although the level of reporting may be low and variable, the data are of interest in identifying viruses implicated in clinical syndromes. Examples from 1975 to 1983 are shown in Tables 5 and 6.

The commonest coxsackie virus reported was coxsackie virus A9, the serotype most readily detectable in

Table 5 *Reports of coxsackie virus infections by main clinical features, 1975–83*

	Coxsackie virus A infections		Coxsackie virus B infections	
Systems affected	**Number**	**Predominant serotypes**	**Number**	**Predominant aerotypes**
Central nervous system				
Total	1 627	A9	4 364	B5, 4
Paralytic	68	A9, 1	112	B4, 5
Cardiac	57	A9	596	B4, 2
Muscle/joint	35	A9	302	B4
Skin/mucosa	1 262	A16	360	B5, 4
Respiratory	741	A9	2 880	B4, 2
Gastrointestinal	996	A9	2 921	B4
Ophthalmic	30	A24	33	B4
Others	644	...	2 169	...
No illness/data	395	...	1 309	...
Totals	5 787	...	14 934	...

Data from the Global Surveillance Programme, World Health Organization, Geneva.

cell cultures. Coxsackie virus A16 is the main cause of HFMD. Over all, coxsackie virus B4 was the serotype associated with the widest range of syndromes; coxsackie virus B5 was the predominant serotype linked with nonparalytic CNS infections and rashes.

Almost half the reported echovirus infections were associated with CNS disease, especially aseptic meningitis (Table 6). Echoviruses 6, 9, 11, 19, and 30 are regularly encountered during outbreaks of aseptic meningitis, sometimes accompanied by a rash in echovirus 6 and 9 infections. Parechovirus 1, echovirus 22 is usually associated with a 'failure to thrive' syndrome in infants less than 1 year old and is seldom associated with CNS infection. The apparent role of coxsackie viruses A and B and, to a lesser extent, the echoviruses in paralytic disease should be interpreted with caution because most reports are based on virus isolation from feces alone.

Table 6 *Reports of echovirus infection by main clinical features, 1975–83*

	Echovirus infections	
Systems affected	**Number**	**Predominant serotypes**
Central nervous system		
Total	16 668	E30, 11, 19, 9, 6
Paralytic	178	E11, 9
Cardiac	266	E11, 6, 22
Muscle/joint	223	E11, 6
Skin/mucosa	976	E9, 11
Respiratory	4 620	E11, 22
Gastrointestinal	7 763	E11, 22
Ophthalmic	72	E11
Others	4 106	...
No illness/data	3 497	...
Total	38 191	...

Data from the Global Surveillance Programme, World Health Organization, Geneva.

Infections due to enterovirus serotypes 68–71 reported to WHO during 1975–83 and the associated illnesses are listed in Table 7.

Illness due to enteroviruses 68 and 69 is uncommon. Although the figures clearly show the association of enterovirus 70 with eye infections, they fail to reveal the pandemic nature of this virus in acute hemorrhagic conjunctivitis; enterovirus 70 is difficult to isolate in cell culture and this alone might explain the discrepancy. Enterovirus 71 is more readily isolated from clinical specimens, and its associations with CNS infections and HFMD (or both concurrently) are apparent from Table 7.

Peak virus activity was seen in July in the northern hemisphere, and during December to January in the southern hemisphere.

As shown in Table 8, enterovirus infections were more common in children, especially in those aged 1–4 years.

Rhinoviruses

Rhinovirus infections are seen throughout the year but are most prevalent in the spring and autumn when sudden changes in temperature are common. It has been suggested that rapid temperature changes are closely associated with the incidence of colds (Hope-Simpson 1958). Rhinoviruses are distributed throughout the world, and antibodies to them have been detected even in the remote communities of Alaskan Eskimos, Pacific Micronesians, and Kalahari Hottentots.

The multiplicity of rhinovirus serotypes is a major factor in their epidemiology. Several serotypes circulate simultaneously within a community, and an individual may be infected with two serotypes at the same time (Cooney and Kenny 1977). Some serotypes persist continuously in a population for several years whereas others may appear transiently. The distribution of sero-

Table 7 *Reports of enterovirus 68–71 infections in 217 cases by main clinical features, 1975–83*

	Number of infections			
Systems affected	**Ev 68**	**Ev 69**	**Ev 70**	**Ev 71**
Central nervous system				
Total	1	0	1	93
Paralytic	0	0	1	3
Cardiac	0	0	0	0
Muscle/joint	0	0	0	0
Skin/mucosa	0	0	0	41
Respiratory	1	1	0	9
Gastrointestinal	0	4	0	10
Ophthalmic	0	0	33	0
Others	0	0	0	10
No illness/data	1	2	0	10
Totals	3	7	34	173

Data from the Global Surveillance Programme, World Health Organization, Geneva.

types has changed over the years. Before 1967 most isolates belonged to the first 55 serotypes; in 1968 and 1969 fewer than half the isolates were serotypes 1–55 and an increasing number were types 56–89. Between 1970 and 1975, 50 percent of rhinoviruses isolated did not belong to the first 89 types (Fox 1976). However, more recent publications indicate that over 90 percent of rhinoviruses isolated can now be identified as known serotypes (Fox et al. 1985; Hamparian et al. 1987). Some serotypes, such as 1B, 12, 15 and 38, are isolated more often than others, possibly because they spread more effectively. Certainly, the more frequently isolated serotypes cause a higher secondary attack rate in family surveys (Fox et al. 1975). However, it must be remembered that frequency of isolation may just reflect the sensitivity of the cell cultures used rather than the distribution of viruses in a population.

LABORATORY DIAGNOSIS

Enteroviruses

Detailed descriptions of the principles and procedures for the diagnosis of enterovirus infections are published elsewhere (Grist et al. 1979).

COLLECTION OF SPECIMENS

The usual specimens for virus isolation are feces (or rectal swabs) and throat swabs. **Fecal** excretion of virus commences within a few days of infection and may continue for weeks in children with poliovirus and coxsackie virus infections, although it rarely exceeds 1 month with the echoviruses; in adults it is usually of shorter duration (1–2 weeks). Excretion in the feces is often intermittent, so, ideally, more than one sample should be collected at least 1–2 days apart. Isolation of virus from pharyngeal secretions is possible up to 1 week after onset of symptoms. Culture of virus from the CSF is an essential part of the routine laboratory diagnosis of patients with aseptic meningitis.

VIRUS ISOLATION IN CELL CULTURES

Polioviruses, the coxsackie B group viruses, and the echoviruses have been readily isolated in kidney cell cultures prepared from rhesus or cynomolgus monkeys or baboons which may not be readily available nowadays. Other susceptible cultures can be obtained from human amnion, diploid cells of human embryo lung, and the RD cell line derived from a human rhabdomyosarcoma. Polioviruses can be isolated in HEp-2c cells or the L20B line (Pipkin et al. 1993).

IDENTIFICATION OF SEROTYPES

Serotypes may be identified by reference antisera. These LBM pools (devised by Lim, Benyesh, and Melnick) are issued in freeze-dried form to reference centers after formal requests to WHO, Geneva. There are eight pools of antisera (A–H) against 42 enteroviruses that grow readily in cell culture and a further seven pools (J–P) of antisera against 19 coxsackie virus A serotypes that grow readily only in newborn mice (Table 9) (Melnick and Wimberly 1985).

ANTIGENIC VARIATION

Antigenic variation in the enteroviruses can give rise to prime strains that may not be neutralized by sera to the prototype strain, but can induce antibodies that neutralize the homologous and prototype strains equally well. Intratypic variants have also been described for coxsackie viruses A24, B1–4, B6, echoviruses 4, 9, and

Table 8 *Global surveillance of enteroviral infections by age, 1975–83*

	Percentage in each age group					
Viruses (no. of infections)	**<1 year**	**1–4 years**	**5–14 years**	**15–24 years**	**25+ years**	**Unknown**
Coxsackie virus A (5 787)	18	41	20	5	11	5
Coxsackie virus B (14 934)	25	34	18	5	14	4
Echovirus (38 191)	28	26	24	7	11	4
Enteroviruses 68–71[a] (217)	25	32	22	3	14	3

Data from the Global Surveillance Programme, World Health Organization, Geneva.
a) Details available from only 217 of total of 864 infections reported to WHO.

Table 9 *Type-specific antisera included in each of eight 'intersecting' LBM pools A–H*

Pool	Enteroviruses represented in each pool			
A	A7	B1, 4	E1, 4, 5, 7, 15, 29, 33	None
B	A7, 9	B2	E2, 3, 9, 19, 21, 26	P2
C	None	B1, 3, 5	E2, 6, 12, 24, 29, 30	P1
D	None	B2	E6, 13, 14, 16, 25, 26, 32, 33	P3
E	None	B4, 5	E5, 11, 13, 17, 18, 22, 30, 32	P2
F	None	B6	E7, 9, 14, 18–20, 26, 27, 29	P1
G	A9	B3	E4, 5, 16, 17, 20, 23, 30, 31	None
H	A16	B6	E1, 3, 9, 12, 22, 23, 32	P3

Antisera against 19 additional coxsackie A viruses are contained in pools J–P. Data from Melnick and Wimberly (1985).

33 and enterovirus 70. Unlike prime strains, intratypic variants have a narrower antigenic spectrum than their corresponding prototype strains.

Aggregation of virions may also cause problems in the neutralization test. The Pesascek strain of echovirus 4 is poorly neutralized by homologous antisera, whereas the Du Toit strain is readily neutralizable. Virus in non-neutralizable aggregates was found to constitute up to 30 percent of untreated Pesascek stock virus preparations but only 0.1 percent of Du Toit stocks. Methods used to disaggregate enteroviruses include filtration or treatment with chloroform.

VIRUS ISOLATION IN NEWBORN MICE

Coxsackie virus A is not readily detected in cell cultures (Table 10). Isolation requires that specimens should be injected into litters of newborn mice (24–48 hours old) by intracerebral, intraperitoneal, and subcutaneous routes. The group A viruses induce general myositis of striated muscle, causing flaccid paralysis and death. The B group cause spastic paralysis and degenerative changes in the brain and necrosis of the brown fat.

TEST FOR ANTIBODIES

A few diseases – notably pleurodynia, HFMD, and acute hemorrhagic conjunctivitis – are so regularly associated with particular serotypes (viz. coxsackie viruses B1–5, coxsackie virus A16, and enterovirus 70, respectively) that their serological diagnosis is often feasible; in other situations virus isolation is the best approach. Antibody titers are compared in paired sera, the first sample being collected in the acute phase and the second 1–14 days later. Enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay may be used as well as neutralization or, in some cases, hemagglutination inhibitor. Such methods can be used to assay for IgM specifically, and thus identify acute infections.

MOLECULAR BIOLOGICAL METHODS

Polymerase chain reaction (PCR) is increasingly applied to clinical specimens and isolates, particularly using primers able to amplify a wide range of enterovirus sequences, which can then be studied further. The use of PCR obviates the need for specific sera if regions of the genome encoding structural proteins are amplified (see above under Classification). Isolation may also be unnecessary, and this may change the spectrum of enteroviruses detected, specifically where they are difficult to grow in culture as for the coxsackie A viruses. Viral sequences have been commonly reported from samples of CSF and serum which is usually a poor source for virus isolation. The advantage of isolation is that it provides permanent specimens for later study. It is likely to become less common except in specialized centers in the future.

Rhinoviruses

Rhinovirus infections are normally diagnosed by isolation of virus from respiratory secretions. As for the

Table 10 *Comparative sensitivity of enterovirus isolation systems*

Virus group	Primary monkey[a]/ baboon	Primary human amnion	Human embryo lung (semi-continuous)	RD[b]	Newborn mice
Poliovirus	++	+	+	++	−
Coxsackie virus A	±[c]	±	±[d]	+	++
Coxsackie virus B	++	±	±	−	+
Echovirus	++	+	+	++	±
Enteroviruses	+	Variable	+	Variable	±

a) Rhesus or cynomolgus.
b) RD, human rhabdomyosarcoma continuous cell line.
c) Coxsackie virus A7, A9, A10, A16.
d) Coxsackie virus A21: −, nil; ±, poor; +, good; ++, very good.

enteroviruses, serological techniques have only limited value because of the large number of serotypes and the absence of a specific rhinovirus group antigen.

Rhinoviruses are most often isolated from nasal washings but throat or nasal swabs and sputa are suitable alternative samples. Virus is rarely isolated from blood.

Specimens should be transported to the laboratory in a buffered medium containing about 1 mg of protein (e.g. bovine plasma albumin) per milliliter and inoculated into cell cultures as soon as possible. Most rhinoviruses are strictly species-specific. Therefore, apart from the few strains that grow in monkey kidney cells, human rhinoviruses will grow only in cells of human origin. Semicontinuous cell strains from human embryonic lung are the most widely used for virus isolation, although certain virus strains are isolated more readily in diploid cells from human embryonic kidney or tonsils. Semicontinuous cell strains from different human embryos vary considerably in their sensitivity to rhinoviruses and these differences are not always revealed by titration of laboratory passaged viruses (Stott and Walker 1967; Fox et al. 1975). A continuous line of HeLa cells, sensitive to rhinoviruses, may also be used for primary isolation (Cooney and Kenny 1977). Bovine rhinoviruses are usually isolated in secondary calf kidney cells. All cell cultures inoculated with rhinoviruses should be rolled at 33°C in medium with pH below 7.6. Virus-infected cultures develop small areas of refractile cells, usually within 7 days. Such cytopathic agents are tentatively identified as rhinoviruses by demonstrating that they resist chloroform or ether, pass through a 50 nm filter, grow in the presence of bromodeoxyuridine (a DNA inhibitor) and are destroyed at pH 5. Final identification of a rhinovirus serotype requires neutralization by specific antiserum but this procedure can be simplified by combining antisera into a scheme of pools (Kenny et al. 1970).

Organ cultures of human embryonic trachea or nasal epithelium support the growth of some rhinoviruses that cannot be isolated in cell cultures. Although most of these viruses can subsequently be passaged in cell culture, some apparently replicate only in organ cultures.

When the infecting serotype is known, as in volunteer trials, virus antigen can be detected in nasal washings by ELISA, using specific polyclonal or monoclonal antibodies.

Molecular methods can also be applied. Deoxyoligonucleotides complementary to conserved sequence of the 5′ noncoding region hybridize to the RNAs of all 57 rhinovirus serotypes so far tested and also detect virus in nasal washings specifically and efficiently (Bruce et al. 1988). This approach has the further advantage that cDNA to this region of viral RNA may be specifically amplified by PCR, increasing the sensitivity of the technique.

Serological diagnosis of rhinovirus infection by demonstrating rising antibody titers is practicable only when the serotype of the infecting virus is known. The microneutralization test has been widely used for the detection of rhinovirus antibodies. The hemagglutination inhibition test is particularly useful for the rapid screening of volunteers for antibodies before inoculation (Reed and Hall 1973) but can, of course, be used only for serotypes that hemagglutinate. This restriction does not apply to ELISA or complement fixation tests.

CONTROL OF ENTEROVIRAL INFECTIONS

There are no established antiviral agents for the prevention or treatment of enteroviral diseases although a number of experimental drugs have been used in clinical trials. Pleconaril, an agent which inserts into the viral capsid and prevents the virus binding to the cell and subsequently uncoating, has been used in trials to attempt to interrupt chronic enterovirus infections of hypogammaglobulinemic patients. Quarantine of patients, or contacts, is of little use because most infections are inapparent and spread among close contacts is rapid. A degree of control can be achieved in special baby-care units (where enterovirus infections can often prove fatal), by 'barrier nursing,' an increase in general hygiene of the staff, and exclusion of staff and parents with even 'minor' illnesses. Similar precautions in eye clinics may help when acute hemorrhagic conjunctivitis is present in the community.

Use of immunoglobulin

Commercially prepared γ-globulin may be useful in preventing enteroviral disease, especially in nurseries threatened by serious outbreaks of group B coxsackie viruses. Its use halted an outbreak of echovirus 11 in a premature baby unit in England in 1983.

Prophylaxis: rhinoviruses

The use of formalin-inactivated rhinovirus 1A vaccine to induce antibody was first described in 1963 (Doggett et al. 1963; Mufson et al. 1963). Subsequently, similar inactivated vaccines made from rhinoviruses 2 and 13 have been administered by subcutaneous, intramuscular, or intranasal routes and have protected volunteers against live virus challenge. The problem of numerous serotypes with little or no cross-protection remains.

SPECIFIC THERAPY

In a review of chemotherapy for rhinovirus infections, Sperber and Hayden (1988) have pointed out that treatment may be aimed at either the virus or the symptoms it induces.

Many compounds specifically inhibit rhinoviruses in vivo but few have beneficial effects against infection in vivo, although some are used in symptom relief. Interferon, zinc salts, synthetic antiviral agents, and monoclonal antibodies have been tested against rhinovirus colds in placebo-controlled trials.

Natural and recombinant human interferons have been tested clinically. Recombinant interferon-α2, administered intranasally, protects against natural and experimental rhinovirus infections. However, no therapeutic benefit is observed even if treatment is started within 30 hours of virus challenge.

A number of compounds are now available that specifically bind to rhinovirus capsids and block uncoating. The Sterling–Winthrop compound disoxaril and its derivatives have powerful antiviral effects both in vitro and in animals inhibiting viral uncoating. It has been established by X-ray crystallography that this drug acts within the VP1 β barrel of the capsid. Two other compounds, dichloroflavan and R61837, also inhibit rhinovirus uncoating, and probably bind to a similar region of the capsid. In clinical trials, dichloroflavan was ineffective but R61837 had significant benefit when given intranasally before an experimental rhinovirus challenge. Enviroxime, a benzimidazole derivative, seems to inhibit the formation of the viral RNA polymerase replication complex. When given orally and intranasally before the challenge, enviroxime significantly reduced production of nasal mucus. However, no therapeutic benefit has yet been demonstrated.

Murine monoclonal antibodies directed against the major group cell receptor compete with rhinoviruses in vitro and displace previously bound virions from cells, thus inhibiting infection. One of these monoclonal antibodies, administered intransally and prophylactically to humans, modified experimental rhinovirus infection both clinically and virologically.

A major pharmacokinetic obstacle to effective therapy is the maintenance of an adequate concentration of drug in respiratory mucosae where rhinoviruses replicate. Furthermore, successful therapies must work rapidly and be free of any side effects.

Prophylaxis: the eradication of poliomyelitis

Polioviruses are believed to have been established throughout most of the population of the world from ancient times, surviving in an endemic form by infection of susceptible infants. Because of the universal presence of antibody to all three serotypes in women of childbearing age, and the protective effect of maternal antibody on disease but not gut infection, most infants would be infected while still protected. Improvements in standards of hygiene in the late nineteenth century led to a delay in the exposure of infants to an age when maternal antibody had declined to non-protective levels. Patterns of disease then changed from the relatively uncommon and endemic kind to the occurrence of large epidemics, and poliomyelitis also became known as infantile paralysis. Safe and effective polio vaccines were developed and used in the 1950s and had an immediate and impressive effect in developed countries. The incidence of poliomyelitis in the USA between 1951 and 1979 is shown in Figure 40.7.

INACTIVATED POLIO VACCINE

The first of the currently used vaccines to be developed was the formalin-inactivated preparation of Salk (1960). Virus was treated with low concentrations of formalin under carefully controlled conditions to ensure that infectivity but not immunogenicity was lost. The resulting inactivated polio vaccine (IPV) is administered

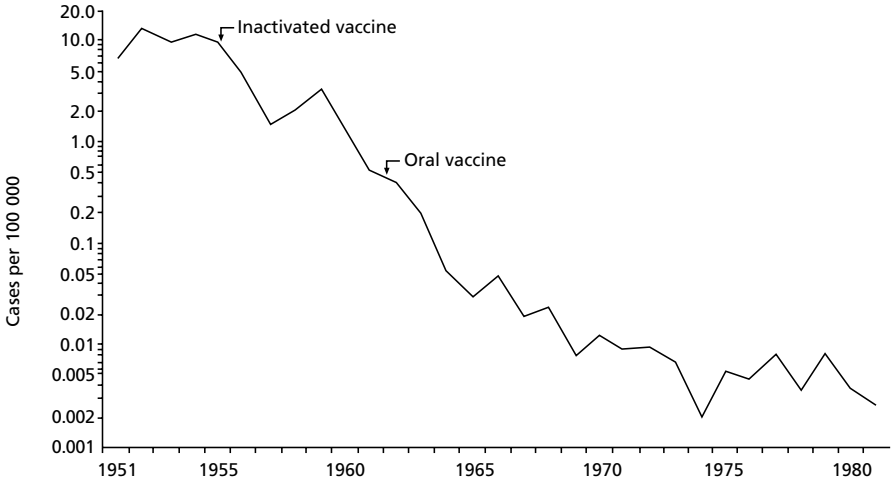

Figure 40.7 *Incidence of paralytic poliomyelitis in USA from 1951 to 1979, showing the effect of vaccination*

by injection; in the early days there were significant problems of production, particularly of the type 1 component which is the least immunogenic of the serotypes in IPV. Immediately after the licensure of the vaccine, batches were used that contained imperfectly inactivated virus, resulting in paralysis in recipients. This was the Cutter incident (Nathanson and Langmuir 1963), and is attributed to the presence of aggregates in which the virus at the center of a clump was protected from inactivation. Production was revised at once to introduce filter steps before and after treatment to remove aggregates, and no case of poliomyelitis attributable to IPV has been reported since. However, filtration removed viral antigen, making the supply problems more intense, and it was not until the 1980s with the development of improved high-density cell culture systems that high-potency IPV [enhanced potency IPV (EIPV)] became available. Its use in developed countries is increasing at the expense of the live attenuated vaccine.

LIVE POLIO VACCINE

The relative merits of inactivated polio vaccine and live attenuated vaccines were and remain a matter of heated debate. Both are highly effective and safe, but it is argued that the live, oral polio vaccine OPV developed by Sabin (Sabin and Boulger 1973), which imitates natural gut infection, also stimulates gut immunity effectively, preventing reinfection and thus interrupting transmission. Whilst it can be shown that even recent OPV vaccinees can be reinfected by wild-type strains, OPV is effective in breaking transmission in epidemics. This may be due to gut immunity or to simple competition. OPV is known to spread from person to person to some extent, which can be an advantage if not all susceptible individuals are immunized directly. Where countries have used IPV alone, unimmunized subpopulations can suffer outbreaks. For example, a religious group in Holland experienced outbreaks of type 1 in 1976 and type 3 in 1993.

The Sabin vaccine strains were developed by passage in vivo or in vitro and selected for reduced neurotropism by tests in primates. The molecular basis of this attenuation for primates was studied intensively in the 1980s and 1990s (Minor 1992).

The type 3 strain was studied in particular because it failed the primate safety test more often than types 1 and 2. Complete sequences of the genomes of the virulent precursor strain (Leon), the vaccine strain (Leon 12_{a1b}), and a virulent revertant isolated from a fatal case of vaccine-associated poliomyelitis were determined and compared to identify features common to the virulent strains that distinguished them from the vaccine strain. Recombinant viruses were made in which portions of the attenuated vaccine strain genome were exchanged for the equivalent region of the Leon genome to identify changes that would attenuate. Finally, a deattenuated version of the Sabin type 3 vaccine strain was made in which all bases thought to have an attenuating effect were reverted to the virulent form. The conclusion from these studies was that only two mutations were responsible for the great majority of the attenuation of the Sabin type 3 vaccine strain. They were a base change at residue 472 in the 5′ noncoding region, and a coding change leading to a substitution of a phenylalanine residue for a serine residue at position 91 of the capsid protein VP3. The 5′ noncoding change leads to a loss of efficiency in the initiation of protein synthesis in in vitro translation systems (Svitkin et al. 1985), and the change in VP3 affects capsid assembly and is responsible in large part for the fact that growth of the Sabin vaccine strain is greatly inhibited at high temperatures. Other changes may make some contribution to the attenuated phenotype, but these two cause the only effects readily detectable in the standard virulence tests.

Comparable recombinants were made between the type 1 Sabin and the virulent type 1 Mahoney strains. The relatively large number of differences between the strains made detailed analysis more difficult, but it was concluded that a major attenuating mutation was to be found at base 480 in the 5′ noncoding region, comparable to that found in type 3, and that other mutations were scattered through the genome, most if not all in the structural proteins. For type 2, there is evidence for a mutation in a similar location in the 5′ noncoding region at residue 481, and in structural protein VP1 at residue 143. There is evidence from examination of the rare cases of vaccine-associated poliomyelitis that occur that the mutations identified in the animal model systems also attenuate the vaccine for humans.

THE ERADICATION OF WILD POLIOMYELITIS

In 1988 the World Health Organization declared the goal of eradicating all wild-type poliovirus and thus the cases of poliomyelitis that they caused by the year 2000. While this target date has passed, there is every possibility that the goal will be achieved in the near future. The reader is referred to the website for current information (www.polioeradication.org). Both live and killed vaccines have been shown to be highly effective but the live attenuated vaccine has been the most widely used because of ease of administration and cost; it is also proven to be able to interrupt epidemics of disease, although initially it was widely considered that it was not effective in developing countries. Various reasons were given to explain this, including possible interference with vaccine take by intercurrent intestinal infections, but the main reasons are probably twofold. First, the infrastructure in tropical countries is usually less well developed than in the temperate climates where the vaccine was shown to work, so that the quality of the vaccine could be very poor by the time it was used. Second,

infection with poliovirus in temperate climates is markedly seasonal, with transmissions occurring only in the warmer summer months. Thus, when following the usual practice of immunizing children at a set age, the pool of susceptible individuals could be reduced greatly during the winter, when no virus transmission occurred, so that transmission was more difficult in the summer when the virus returned. By contrast, in tropical climates infection is year round, although there may be an element of seasonality. It is therefore a matter of chance whether an individual encounters the vaccine or the wild-type virus first, and there is no period of respite when immunization can make sufficient inroads on the susceptible population to affect person-to-person spread.

The solution to the problems was first to develop systems (cold chains) by which the quality of vaccines could be maintained by keeping them cold from the point of manufacture to the point of use. Second, the vaccine was used in a way that mimics an epidemic, namely by immunizing very large numbers of susceptibles within a very short period. This has taken the form of National Immunization Days (NID), which may involve the vaccination of 120 million children in a single country in a single day. As all susceptible intestines are occupied by vaccine, or are immune because of the vaccine, transmission of the wild-type virus is interrupted and in practice the wild-type virus can be eradicated in a particular limited region by a few well-conducted NIDs. Problems of access to regions of conflict or areas of particularly poor infrastructure arise, but the effect of the strategy has been to reduce the number of countries in which the virus is endemic to ten or less as of late 2002, with most concern arising from India and Nigeria. The Americas were certified free of polio in 1991, the Western Pacific Region of WHO including China in 1994, and the European Region in 2002. Even areas such as the Democratic Republic of Congo where savage wars are in progress and numbers of vaccine workers have been killed in the course of immunization programs are credibly free of poliomyelitis caused by the wild-type virus. Whilst wild-type poliovirus is still present in a few parts of the world, and in 2002 there were setbacks in both India and Africa in terms of numbers of cases identified, it is likely that the last wild-type 2 virus in the world was isolated in 1999, and that sooner rather than later the other two serotypes will also be eliminated.

One of the important factors in monitoring the progress of this exercise was the demonstration that virus transmission in a particular region had been interrupted. This depended on the specific identification of virus strains, accomplished by sequencing a relatively small part of the genome encoding the region around the *VP1-2A* gene (Rico-Hesse et al. 1987) or the region encoding the whole of VP1. Such sequences can be used to construct dendrograms of relatedness, and it has been possible to identify 'genotypes' of the different serotypes, defined as strains differing by 15 percent or less in sequence. The genotypes were found to be geographically clustered; thus an isolate from the Middle East would closely resemble other isolates from the Middle East, including those made some time ago. It was therefore possible to identify a particular virus as indigenous to the country of origin or imported, and if so, from where. For example, in 1984 a small outbreak of type 3 poliomyelitis occurred in Finland in which there were nine paralytic cases and one case of aseptic meningitis. Circulation of the strain was widespread despite the use of IPV in Finland, and there was some evidence that the strain was antigenically unusual. Genomic analysis revealed that the strain originated from the Middle East, possibly imported by soldiers serving with the UN.

CESSATION OF VACCINATION

One of the anticipated benefits of eradication is the cessation of vaccination, with consequent savings. It is also likely to be difficult and possibly unethical to maintain vaccination programs after the disease is eradicated. Stopping vaccinating is easier said than safely done, however. The molecular basis of the attenuation of the live vaccine strains of poliovirus developed by Sabin established in the 1980s and 1990s (Minor 1992) shows that for each of the strains there were relatively few mutations involved, two for each of the type 2 and 3 strains and a few more for the type 1 strain. In each case a mutation in the 5′ noncoding region had an effect with one or more mutations in the structural proteins and so far as can be seen there are no attenuating mutations in the nonstructural proteins.

The vaccines are extremely safe and effective but they all derive from original circulating natural isolates; the type 3 strain came from a fatal case. Moreover, it has been conclusively demonstrated that in rare instances, estimated at about one per 750 000 first-time vaccinees or one in 2 million overall, the vaccine can itself cause poliomyelitis in recipients or their contacts. In view of the small number of attenuating mutations, this is not surprising.

However, even when normal healthy vaccinees were examined it was found that the virus was capable of extraordinarily rapid and accurate adaptation and mutation (Minor et al. 1986b). For type 3, the mutation in the 5′ noncoding region, which is one of two that attenuate, the virus was lost at the latest within 6 days and usually within 3. The other mutation in the structural proteins is usually suppressed by changes at other positions rather than back mutation at about 11 days after vaccination; the suppressor mutations may affect other steps in the assembly of the virus than that affected by the attenuating mutation, and therefore compensate for its effects indirectly.

In addition, it was found that recombination between the viruses in the vaccine was extremely common. In

fact it has been found to be the rule that a child excreting type 3 poliovirus more than 11 days after vaccination will be producing a recombinant virus in which the structural proteins derive from type 3 and the nonstructural proteins from types 1 or 2. Complex recombinants of all three serotypes are common.

The fact that the vaccine strains were derived from transmissible paralytic circulating strains of virus, that they can, albeit rarely, cause paralysis in recipients and in their contacts, coupled with the rapid evolution of the virus in vaccine recipients, suggests that it should be possible to select an epidemic, transmissible paralytic strain of poliovirus from the vaccine given the right conditions. Current observations confirm this possibility and give cause for concern.

PERSISTENCE AND CIRCULATION OF VACCINE-DERIVED VIRUS

One strategy for the cessation of vaccination would be simply to stop when it was certain that wild-type virus had been eradicated. The assumption on which this is based is that the vaccine virus does not circulate readily, and that it will therefore die out before sufficient susceptible individuals accumulate to maintain it in circulation. It is possible that this strategy would be successful. In certain countries vaccine is given at one set time. In Cuba, vaccine is only given twice at the beginning of the year and virus cannot be isolated from sewage specimens 6 months later. It has been shown that on average a vaccinee excretes poliovirus for about 5 or 6 weeks, with only one percent continuing for 10 weeks. It is believed that immunocompetent individuals never excrete the virus for longer than 6 months. Moreover, early studies showed that in the population studied which was in Louisiana, USA, the virus died out after three person-to-person transmissions. Thus virus excretion in the main is of limited duration and transmission is not very effective.

However, there have been several separate incidents in which small outbreaks of poliomyelitis have occurred after the eradication of the wild-type and where the causative virus is clearly related to the vaccine strain (Figure 40.8). They include type 2 strains isolated in Egypt between 1988 and 1993, strains responsible for an outbreak on the island of Hispaniola in Haiti and the Dominican Republic, which caused more than 20 cases in 2000–01 (Kew et al. 2002), a very small outbreak in the Philippines in 2001, and in Madagascar in 2002. The strains had some features in common. It is possible to date the origin of the strain by examining the sequence of the viral genome. During epidemics, the sequence drifts at a remarkably constant rate of about 3 percent for the silent mutations that do not cause amino acid changes, or about 1 percent if all mutations are considered (Martin et al. 2000a, 2000b). The rate seems to be independent of the type or the specific outbreak, and based on this molecular clock the strains in the incidents listed above had been circulating undetected for 1–2 years. When they were examined in more detail, it was found that all were recombinant viruses between the vaccine strains and an unknown virus, which was not any of the vaccine strains. As poliovirus was either rare or eradicated in each of the countries concerned, it is assumed that the partner virus was a nonpolio group C enterovirus. As described above, the sequence data available suggest that recombination between members of this group occurs readily. In Hispaniola and in Madagascar there were several distinct recombinant viruses, indicating that multiple recombination events had taken place. The reason why all so far have been recombinants is not known, nor is there any understanding of what makes the viruses readily transmissible when the parent vaccine strain is not. The level of virus excreted is very similar in the outbreak cases and in normal vaccinees; it is tempting to speculate that the recombinant nature of the viruses plays a part, but it may be fortuitous. As stated above, all the vaccine strains originated from circulating strains of some sort and in principle it should be possible for them to regain transmissibility, particularly in the light of the rapid adaptation of the viruses in vaccine recipients. The origin of the strains is also speculative, but plausible. When poliomyelitis is eradicated from a region, the motivation for continuing vaccination dwindles and other health issues get attention. In Haiti, vaccine uptake was about 50 percent; in the other regions it was higher, typically about 80 percent overall. Thus while some of the susceptible children were given live vaccine, which they excreted, others were not, a situation that persisted for several years. This scenario is ideal for the selection of transmissible viruses and it seems almost inevitable that they would arise wherever vaccine programs gradually fade away. The solution could be to immunize the population to high coverage and then stop vaccinating altogether. This would at least reduce the time available for selecting transmissible strains.

CHRONIC EXCRETION OF POLIOVIRUS

A second hazard to the cessation of vaccination arises from the existence of hypogammaglobulinemic patients who may excrete virus for very long periods. So far as is known, such patients are relatively rare; at the time of writing the total number is fewer than 20. Moreover, there is no documented case of any of them acting as a source of infection for others. Some excrete virus for a prolonged period of 1 or 2 years and then stop spontaneously for known or unknown reasons; in one case excretion of virus stopped 3 years after a *Shigella* infection but usually there is no identifiable cause. However, there are instances where virus excretion had persisted for 10 years or more before it was detected, based on the sequence of the viruses, where the molecular clock seems to operate at the same rate as in an epidemic. Most patients have been identified because they develop poliomyelitis; exceptions include

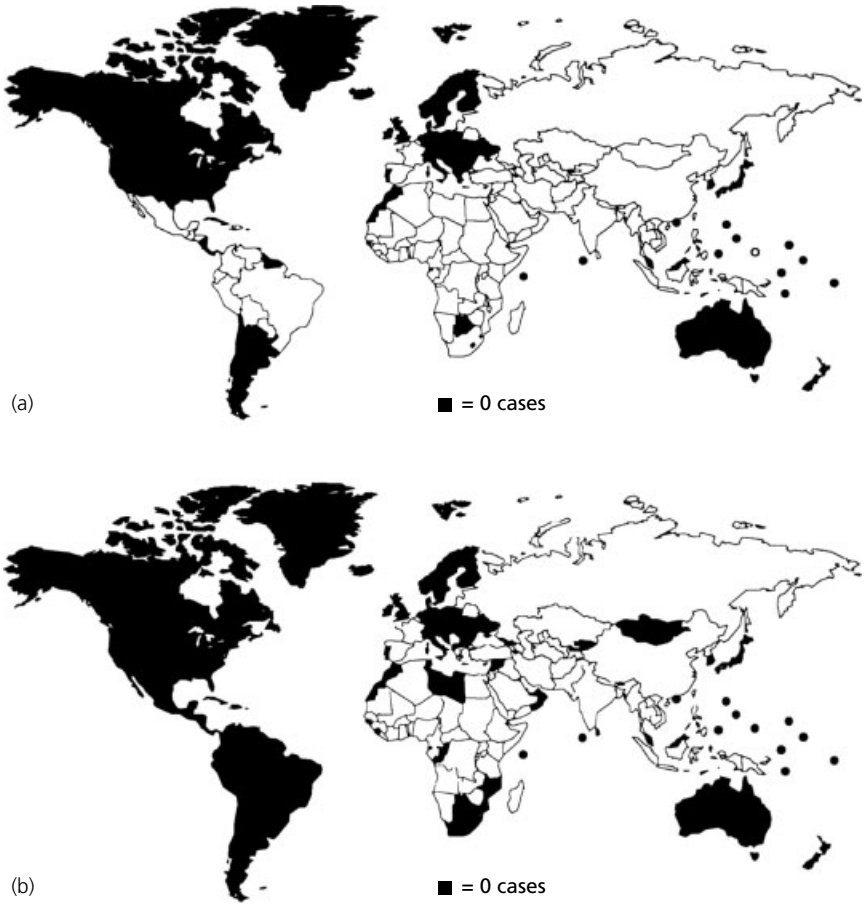

Figure 40.8 *Global distribution of poliomyelitis in* **(a)** *1988 and* **(b)** *1994. Shaded areas indicate countries with no reports of poliomyelitis due to wild-type virus.*

two patients who were deliberately given vaccine in the 1960s to investigate residual responses and protect them from wild-type poliovirus which was epidemic in the UK at the time. Some patients have been discovered by accident in the course of other investigations. Probably the best documented case is an individual who is currently known to have been excreting poliovirus in 1995 and was still doing so in 2002. Based on the sequence data, the patient is likely to have been excreting virus for 20 years. The virus is excreted at high levels in the stool and is highly virulent in animal models; it has also lost all molecular markers of attenuation. It seems likely that such individuals could act as origins of the virus in a post-eradication setting. They are rare, but nonetheless they occur. As hypogammaglobulinemia is mainly a disease of Caucasians, it may be that the hazard they pose is less by virtue of the high living standards and level of public hygiene than if they occurred in poorer parts of the world. The possible effect of AIDS has been raised; so far as is known, it does not increase the risk of long-term excretion of poliovirus, which is associated with a defect in humoral immunity. Extensive studies have not been carried out, however.

POSSIBLE SOLUTIONS

At the time of writing, the solution to the persistence of poliovirus from whatever source is not clear. Many developed countries have switched to the use of inactivated polio vaccine which at worst will maintain immunity while the significance of the issues raised can be evaluated. It has been proposed that vaccination should continue indefinitely, or that it should cease abruptly, which is likely to make the occurrence of the circulating strains found in Hispaniola and the other instances cited above less likely. New vaccines based on stabilized strains or nonreplicating vaccines have also been suggested. The possibility of bioterrorism or the escape of wild-type poliovirus from laboratory or other sources, or even the re-evolution of the virus from one of the other group C enteroviruses means that some reserve vaccine stock will be required. The form it will take and the strategies for its use remain to be established.

FUTURE PROSPECTS

Poliomyelitis due to wild-type virus is very likely to be eliminated in the near future. At that point the only way

of contracting poliomyelitis will be by vaccination, which will therefore presumably stop. The strategy for doing so has not yet been elaborated, but the risk is that the vaccine strains will occupy the niche occupied by wild poliovirus, and, in view of their ability to adapt, that they will become virulent. It will therefore be necessary to protect individuals for some time until it can be shown that the vaccine-derived strains are not circulating freely but have died out. It is likely that this will be done by changing to the use of IPV rather than OPV, accompanied by suitable environmental monitoring. In Finland after the 1984/85 outbreak, OPV was used extensively but was only detectable in sewage for a period of a few months. It is therefore possible that circulation will not be prolonged, and that poliomyelitis will become the second disease to be eliminated, after smallpox.

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Human enteric RNA viruses: astroviruses

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Astroviruses are a relatively newly classified family of nonenveloped small RNA viruses. They appear to be widespread in nature and the list of family members and their known host species is increasing steadily. Most members are enteric and are an important cause of diarrhea and vomiting in the young of both humans and animals. In common with most agents of this type, the viruses seem to be highly resilient and survive well in the environment.

Human astroviruses (HAstV) were the first to be identified and were observed during electron microscopic examination of diarrheal stools from infants (Appleton and Higgins 1975; Madeley and Cosgrove 1975a). Their distinctive and unique appearance under negative stain (Figure 1) immediately suggested a novel type of virus. The particles were rounded in shape, 28 nm in diameter with a smooth margin, and, most distinctively, approximately 10 percent of them displayed a five- or six-pointed star motif on their surface. The term 'astrovirus' (Greek: astron – star) was coined to refer to this feature (Madeley and Cosgrove 1975b). Viruses showing this characteristic appearance have since been found in the stools of a variety of mammals including cattle (Woode and Bridger 1978), sheep (Snodgrass and Gray 1977), pigs (Bridger 1980), cats (Hoshino et al. 1981; Harbour et al. 1987), dogs (Williams 1980), mink (Englund et al. 2002), and mice (Kjeldsberg and Hem 1985). All were associated with mild diarrhea, generally in the young of the species. Astroviruses have also been found to infect birds including turkeys (McNulty et al. 1980), chickens (Yamaguchi et al. 1979), and ducks (Gough et al. 1984), but in avian species the symptoms may vary. The duck

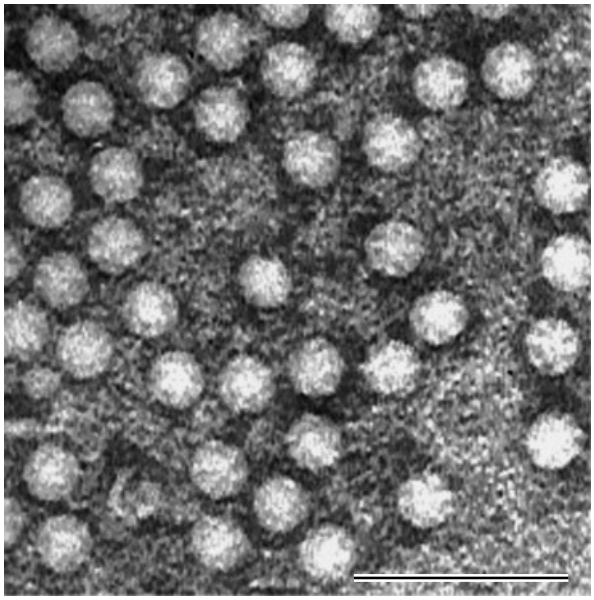

Figure 1 *Human fecal astroviruses negatively stained with phosphotungstic acid and viewed under the EM. Star-like motifs are visible on the surface of some particles, those showing both five- and six-pointed stars are visible. Bar 100 nm*

astrovirus DHV II causes a necrotic hepatitis, turkey astroviruses (TAstV) are associated with immunosuppressive disease or diarrhea, and the chicken virus causes nephritis. Astroviruses are a significant cause of mortality in all these species.

As is the case with other viruses infecting the gut, astroviruses are also observed in stools from symptom-free individuals and are also found in association with other enteric viruses. These features complicate attempts to assess the significance of astroviruses in human disease. Electron microscopy (EM)-based studies showed relatively few diarrheas were astrovirus-associated even though infection was known to be widespread. This in turn suggested that infections must usually be relatively mild or inapparent. Although this remains probable, recent evidence suggests that the 'typical' astrovirus structure described above may not have been a reliable feature for identification and studies using alternative technologies indicate a more significant virus association with symptomatic illness.

Initial attempts to culture these viruses failed and so the first investigations were carried out using ovine astrovirus (OAstV) since this could be obtained relatively easily from experimentally infected lambs (Herring et al. 1981). A major landmark in astrovirus research occurred with the adaptation of HAstV to cell culture; Lee and Kurtz (1981), working in Oxford, UK, repeatedly passaged virus from an adult volunteer through primary human embryo kidney cells in the presence of trypsin. Although the virus did not cause cytopathic effect in these cells, its presence could be demonstrated by immunofluorescence. After six passages it was able to infect a continuous monkey kidney cell line (LLC-MK2 cells), again requiring the addition of trypsin and without the production of cytopathic effect. This cultivation method enabled the production of antigens and subsequently both polyclonal (Lee and Kurtz 1982) and monoclonal (Herrmann et al. 1988) antibodies to HAstV were produced. These led to the recognition of antigenic variation between viruses and five serotypes were identified (Kurtz and Lee 1984). This list has now reached eight serotypes of HAstV. There are thought to be at least two serotypes of bovine astrovirus (BAstV) but apart from this, serotypic variation amongst animal strains is unknown. Enzyme-linked immunoassays were also developed (Herrmann et al. 1990), and these are now available commercially superseding to a large extent diagnosis by EM.

The Oxford adapted astrovirus strains continue to form the basis for much astrovirus research and although these represent each of the five serotypes initially identified, the procedure required to produce more isolates is relatively cumbersome and the number of characterized clinical isolates remained small. This bottleneck was overcome with the finding that a continuous human gut cell line (CaCo-2) was able to support the growth of HAstV directly from stool specimens without the need for prior adaptation; most clinical samples could now be isolated routinely by conventional laboratory procedures (Willcocks et al. 1990).

Subsequently, molecular cloning techniques were applied to both the Oxford strains and isolates from elsewhere, leading to the complete sequence determination of several virus genomes. The information derived from this has in turn led to the development of more sensitive detection systems using the reverse transcription-polymerase chain reaction (RT-PCR). All these advances have in turn fed an increase in our understanding of astrovirus genomic structure and replication strategy that ultimately led to the recognition of the unique identity of these agents and to the definition of a new family of viruses – the *Astroviridae* (Monroe et al. 1995).

CLASSIFICATION

In the 1970s, the application of EM to human stools revealed a number of small round viruses. These were originally classified according to their morphology and in 1982, Caul and Appleton proposed an interim classification scheme dividing them into 'featureless' and 'structured' particles. The featureless viruses included the enteroviruses, parvoviruses, and parvovirus-like particles, whilst the 'small round structured viruses' included the astroviruses, human caliciviruses (now termed sapoviruses) and the Norwalk-like viruses (now termed noroviruses). The 'structured' group was further divided into smooth-edged particles (the astroviruses) and those whose particles had a ragged outline (the sapo- and noroviruses). Since this time, increased knowledge of the protein composition and replication strategies of these viruses has permitted their more detailed and definitive classification. Astroviruses and human caliciviruses were soon recognized as distinct entities and the term small round structured virus (SRSV) was then used simply to refer to noroviruses.

Historically, astroviruses were identified as such simply from their morphology. Virus particles were nonenveloped and were thought to contain a single-stranded, presumptive positive-sense RNA genome but little more was known about them. These features are shared by two other families of animal viruses (the *Picornaviridae* and the *Caliciviridae*). Astroviruses were better defined when the growing database of their properties enabled their ready differentiation from other virus families. Features of polypeptide composition and genome structure were used to distinguish astroviruses from those other families above and thus permit their formal classification as a distinct virus family, the *Astroviridae* (Monroe et al. 1995). In common with modern trends in virus taxonomy, astroviruses are increasingly defined and grouped according to their genomic sequences; indeed, avian nephritis virus (ANV) was first isolated in 1976 but it was not until 2000 that molecular

Table 1 *Human and animal astroviruses*

Genus	Species	Serotypes	Host	Disease	Abbreviation
Mamastrovirus	*Human astrovirus*	8	Humans	Gastroenteritis	HAstV
	Bovine astrovirus	2	Cattle	None	BAstV
	Ovine astrovirus	1	Sheep	Gastroenteritis	OAstV
	Porcine astrovirus	1	Pigs	Gastroenteritis	PAstV
	Feline astrovirus	1	Cats	Gastroenteritis	FAstV
	Canine astrovirus[a]	1	Dogs	Mild enteritis	CAstV
	Murine astrovirus[a]	1	Mice and other rodents	Diarrhea	MAstV
Avastrovirus	*Duck astrovirus*	1	Ducks	Hepatitis	DAstV
	Turkey astrovirus type 1	1	Turkeys and chickens	Gastroenteritis	TAstV-1
	Turkey astrovirus type 2	. . .	Turkeys	Poultry enteritis mortality syndrome	TAstV-2
	Avian nephritis virus	1	Chickens	Acute nephritis	ANV

a) Tentative member of the *Astroviridae* awaiting molecular characterization.

cloning and sequence determination of the virus genome indicated that it should be classified within the *Astroviridae* (Imada et al. 2000). At first only one genus (*Astrovirus*) was recognized within the family. As sequence data accumulated, it became clear that the astroviruses obtained from mammals resembled each other more closely than those newly characterized viruses isolated from birds. The avian viruses likewise appear to be more closely related to each other than to astroviruses of mammals. Thus the *Astroviridae* have now been divided into two genera, *Mamastrovirus* (mammalian viruses) and *Avastrovirus* (avian astroviruses). The members currently assigned to each genus are listed in Table 1. Sequence comparisons such as these have the potential to illuminate much of the history of this virus family and this matter is discussed again under sequence phylogeny (see below).

BIOPHYSICAL PROPERTIES

Astrovirus particles are nonenveloped and have a buoyant density of between 1.34 and 1.40 g/ml in cesium chloride. HAstV particles have been reported with densities of 1.35–1.37 g/ml (Caul and Appleton 1982; Kurtz and Lee 1987; Willcocks et al. 1990; Matsui et al. 1993). Porcine astrovirus (PAstV) has been reported to have a buoyant density of 1.34–1.35 g/ml (Shimizu et al. 1990) and ovine of 1.38–1.40 g/ml. Aggregates of OAstV produced two peaks on cesium chloride density sedimentation: 1.365 and 1.39 g/ml (Herring et al. 1981). Similarly two distinct bands of 1.32 and 1.34 g/ml (Willcocks et al. 1990) and 1.33 and 1.37 g/ml (Matsui et al. 1993) were obtained using HAstV grown in vitro. Since the polypeptide composition of each band appeared identical in both cases, it had been speculated that the material of lower density consisted of empty virions. This was confirmed when Matsui and colleagues analyzed both peaks by hybridization analysis and showed that the lower density material contained no viral RNA (Matsui et al. 1993).

In common with other nonenveloped viruses, astroviruses show the expected resistance to lipid solvents such as chloroform and are able to resist a variety of detergents (non-ionic, anionic, and zwitterionic). They are relatively resistant to inactivation by chlorine, titer is not reduced at levels below 30 p.p.m. of free chlorine. The virus can withstand heating to 60°C for 5 but not for 10 minutes (Kurtz and Lee 1987). As enteric viruses, they also display resistance to inactivation at acid pH, although stability is actually quite broad and the viruses withstand a range of pHs from 2 to 11 (Kurtz and Lee 1987). Amongst the enteric viruses their general stability appears second only to hepatitis A virus (Carter and Adams 2000) and they persist well in the environment. These properties have an important influence on the potential routes for transmission of the virus and this is discussed below.

MORPHOLOGY

Electron microscopy

HAstV particles of 'typical' morphology were originally reported as approximately 28 nm in diameter with a clearly defined margin and a characteristic star-like motif in their center. However, the characteristic star-like motif is visible only on some 10 percent of the virions (Figure 1) – or fewer in some preparations (Madeley 1979). The stellate structures are five- or six-pointed, but neither of their centers is penetrated by stain. Consequently both stars have a characteristic solid white center in electron microscopy (EM). This contrasts with the six-pointed 'star of David' feature visible on the surface of caliciviruses when viewed along their threefold axis of symmetry. The surface features of

caliciviruses are ring-like hollows in which stain can accumulate. Calicivirus stars thus have a dark center surrounded by darker patches (Carter and Madeley 1987). It is not clear how the astrovirus star motifs are produced but insight may come from recent analyses of frozen particles using the technique of cryoelectron microscopy.

Astrovirus particles are usually attributed a smooth margin; however, in many micrographs there are indications of spike-like protrusions. Furthermore, particles are often present in very large numbers in stool samples and can sometimes be seen in paracrystalline arrays. Even when packed closely together, the edges of the particles seem not to make contact and it has been suggested that this even spacing between the virions could imply the existence of surface projections beyond the resolution of the electron microscope (Risco et al. 1995).

Particle size differs depending on the virus; OAstV has been reported to have a diameter of 29 nm (Gray et al. 1980), BAstV had an average diameter of 30 nm (but varied between 27 and 35 nm) (Woode et al. 1984). Interestingly, cell culture grown virus particles appear to be slightly larger, bovine astrovirus serotype 2 propagated in primary bovine kidney cells was 34 nm in diameter (varied between 30 and 37 nm) (Aroonprasert et al. 1989). This difference in diameter found between viruses propagated in cell culture and those derived from the natural host is also seen in HAstV and in general, HAstV particles grown in vitro and released from cell cultures appear less distinct and more ragged-edged than those observed in stool preparations. In fact, their average diameter has been reported to be as large as 34 nm for particles derived during the first five passages in CaCo-2 cells and such particles exhibited a remarkable similarity to caliciviruses in appearance (Willcocks et al. 1990; Risco et al. 1995). Viruses of more classical appearance were observed in later micrographs. Risco and colleagues (1995) similarly described virus released from LLC-MK2 cells and visualized in the electron microscope by negative staining as having a yet larger particle diameter (41 nm) and exhibiting a clear layer of surface spikes as suggested above.

Studies by Risco and colleagues (1995) also shed some light on this variability in appearance. These workers found that the larger particles shed from cell culture could be induced to display star-like motifs after a brief exposure to alkali (pH 10 for 10 minutes). Exposure like this is not likely under conditions of natural infection or sample collection, and most EM stains are acidic, but nonetheless local alkaline concentrations might conceivably occur during sample preparation and grid drying. The atypical larger particles were also infectious and it was proposed that these larger particles with the surface projections may represent the true native form of the virus, and the so-called 'typical' particles bearing surface stars might result from artifacts during preparation. However, it should be borne in mind that the gut is a complex environment; although astroviruses can be grown in CaCo-2 and LLC-MK2 cells, it is likely that neither mimics exactly the true host cell, the enterocyte. Similarly, production of infectious virus requires supplementation with a protease (trypsin) and capsid protein processing appears to proceed both inside and outside the cell and may involve several stages (see below). Perhaps other extracellular factors are needed to allow released virions to 'mature' fully, although present in the intestinal lumen these would be absent in cell cultures. Consequently, it is not clear at present whether the star-bearing astroviruses represent an artifactually rearranged ragged-edged particle, or whether the ragged particles might represent partially matured forms of those showing surface stars. However, it is clear that both types are infectious and that this variation may well have affected some of the EM-based studies of virus prevalence.

Cryo-electron microscopy

Cryo-electron microscopy uses computer analysis of digitized images of hydrated virus particles to generate an electron density map and eventually a three-dimensional reconstruction of the particle. The technique is powerful and recent data have been obtained using Oxford adapted virus grown in LLC-MK2 cells. The final reconstructions (Figure 2) show that astrovirus particles consist of a smooth but rippled icosahedral capsid some 33 nm in diameter. The ripples do give a faint indication of star-like patterning on the capsid surface but this is not a prominent feature in these images. Thirty dimeric spikes arise from this capsid and are situated at the twofold axes of symmetry. These extend some 5 nm from the particle surface to give an overall diameter around 41–43 nm. Little detail was obtained concerning the structure of the RNA within the particle but it appeared to adopt a partially icosahedral confirmation.

THE VIRION-ASSOCIATED PROTEINS

The polypeptide composition has been determined for several serotypes of HAstV as well as the ovine and porcine viruses. The results have shown heterogeneity both in the number and the size of the structural proteins. Initial studies on OAstV found two polypeptides of about 33 kDa (Herring et al. 1981), Shimizu and colleagues (1990) purified PAstV grown in cell culture in the presence of trypsin and identified five polypeptides (of 39, 36, 31, 30, and 13 kDa).

The protein composition of various serotypes of HAstV has been investigated. All were analyzed following propagation in the presence of trypsin. Kurtz and Lee (1987) identified proteins of 36.5, 34, 33, and 32 kDa in an HAstV-4 isolate and later Kurtz (1989) found proteins of 34, 33, 26.5, and 5.2 kDa in HAstV-1.

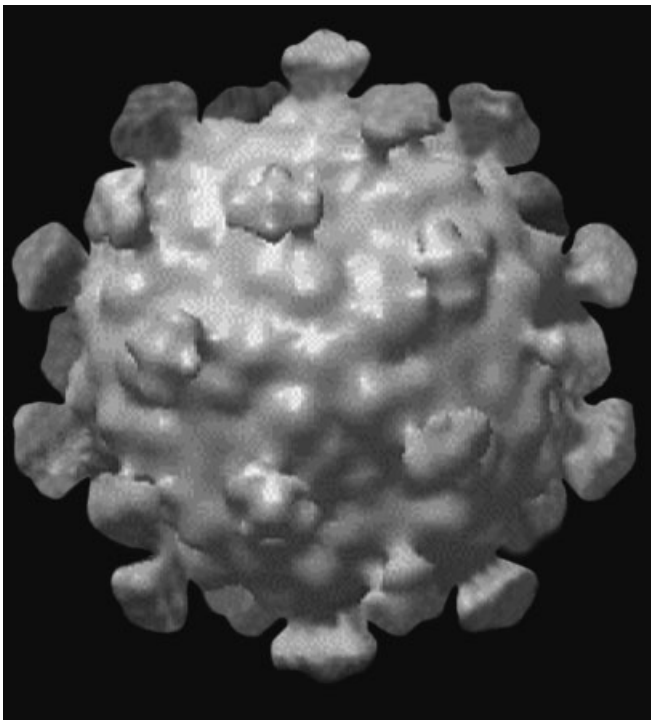

Figure 2 *Cryo-electron microscopic reconstruction of cell-culture-grown human astrovirus (resolution 2.4 nm). Structural predictions are derived by mathematical amalgamation of digitized images of frozen hydrated particles observed under the EM. The reconstruction shows surface projections rising from a smoothly rippled capsid surface. A five-pointed star-like feature is visible as a raised area in the center of the capsid surface. Image reproduced (with permission) from Matsui et al. 2001. We thank Dr S. Matsui and Dr M. Yeager for provision of this illustration.*

The small protein has never been confirmed but this may be due to variation between isolates or to differences in the polyacrylamide gel analysis system used. Willcocks and colleagues (1990) examined a serotype 1 astrovirus propagated in CaCo-2 cells and found proteins of 33.5, 31.5, and 24 kDa. Monroe and colleagues examined an HAstV-2 isolate adapted to growth in LLC-MK2 cells and found a similar pattern with proteins of 31, 29, and 20 kDa when the virus was propagated in the presence of trypsin (10 μg/ml). When trypsin was absent, virions contained a single protein of 90 kDa. Treatment of these virions with trypsin restored the normal pattern of polypeptides, confirming that the 90 kDa protein was an immature precursor to the normal forms (Monroe et al. 1991b). These reports have been combined to suggest a consensus protein composition for HAstV, although the actual sizes seem to vary with strain (Willcocks et al. 1992; Monroe et al. 1995). HAstV were thought to contain at least three structural proteins. Together the sum of their molecular masses suggested that they would account for almost all of the structural protein precursor. Two of these proteins formed a doublet on polyacrylamide gel analysis around 33 kDa, whilst the third was smaller and variable in size between isolates. This protein was not always detected, and was shown to be stripped from the virion by detergent treatment, which suggested that it may be loosely associated on the surface of the particle and thus be easily lost (Willcocks et al. 1990). Recently, Belliot and colleagues compared structural protein sizes in HAstV 1–5 and reconfirmed the basic pattern above; the largest two proteins varied very little between serotype and were always seen as a doublet of about 33 kDa. The third protein was consistently observed and varied between 25 and 28 kDa. This protein was largest in HAstV-2 and HAstV-4 and most variable in size amongst HAstV-5 isolates (Belliot et al. 1997a). The origin of these proteins is discussed below.

GENOME ORGANIZATION

The astrovirus genome consists of a single strand of positive-sense RNA of approximately 6 800 nucleotides, the 3′ terminus is polyadenylated (Jiang et al. 1993; Lewis et al. 1994; Willcocks et al. 1994b). The structure of the 5′ terminus is not known but it is anticipated that it is covalently linked to a small virus-encoded protein (VPg) as has been found in the calici- and picornaviruses.

The complete nucleotide sequences were first determined for HAstV serotypes 1 [Oxford reference strain adapted to growth in LLC-MK2 cells (Lewis et al. 1994) and Newcastle strain isolated in CaCo-2 cells (Willcocks et al., 1994b)] and 2 (Oxford reference strain; Jiang et al. 1993). All astrovirus genomes sequenced to date (including those of animal strains) show the same basic

organization. The RNA contains three sequential open reading frames (ORF) termed 1a, 1b, and 2 (Figure 3) and has a short nontranslated region at each end of the genome (approx. 85 bases at the 5′ end and 83 at the 3′ end). The sizes of these ORFs for representative types of astrovirus are given in Table 2. The nonstructural proteins are encoded in the 5′ section of the genome in ORFs 1a and 1b; the structural proteins are specified by ORF 2. However, whilst both ORF 1a and ORF 2 possess 5′ initiation codons in a good context for activity (Kozak 1989), and whilst ORF 1a closes at a termination codon, there is no obvious start codon for ORF 1b and no separate mRNA for this region has ever been observed. ORF 1b is encoded in the −1 frame relative to ORF 1a, and examination of the 71 nt region of overlap (Figure 4) revealed features resembling the translational frameshifting elements seen in other virus families (e.g. the retroviruses (Jacks et al. 1988), coronaviruses, and arteriviruses (Bredenbeek et al. 1990)). These features comprised a 7 nt AU-rich sequence typical of 'slippery' sequences at which translating ribosomes could move back one base into ORF 1b. Slippery sequences alone have little effect and all are combined with features of strong secondary structure. In the astroviruses this is a stem–loop rather than a pseudoknot (Jiang et al. 1993; Lewis et al. 1994; Willcocks et al. 1994b). These features are conserved in all sequences determined to date. Mapping studies have supported the existence of the predicted secondary structures (Marczinke et al. 1994). Testing of this region of the RNA by translation in vitro has confirmed that it does direct −1 ribosome translation frameshifting at the ORF 1a/1b junction with an efficiency of approximately 5 percent in vitro (Marczinke et al. 1994). This efficiency is about the same as that seen in retroviruses, but experiments in which the same signals were introduced into living cells indicated that frameshifting may be rather more efficient (25–28 percent) when translation takes place inside the cell (Lewis and Matsui 1996). Frameshifting ribosomes avoid the termination codon that closes ORF 1a and progress instead into ORF 1b. The product is an ORF 1a/1b fusion protein. Since not all ribosomes slip, this mechanism results in the manufacture of rather more ORF 1a products than those of ORF 1b. This latter frame encodes the virus RNA-dependent RNA polymerase which may be required in smaller amount. Thus the frameshifting events may be viewed as a refinement in the replication process, minimizing production of unnecessary RNA polymerase without requirement for a separate mRNA.

Replication of the virus

Replication of astrovirus is relatively poorly understood although the derivation of infectious clones of HAstV-1 (Geigenmuller et al. 1997) and ANV (Imada et al. 2000) should enable further analysis of the astrovirus life cycle and determination of structural features essential for replication and encapsidation.

The cell-surface receptor for astroviruses has not been identified; experiments with polarized gut epithelial cells (CaCo-2) have suggested that the receptor may be located on the basolateral rather than the apical membranes in this case (Willcocks et al. 1992). This appears unusual at first sight, but other enterically infecting viruses such as murine reovirus also show such

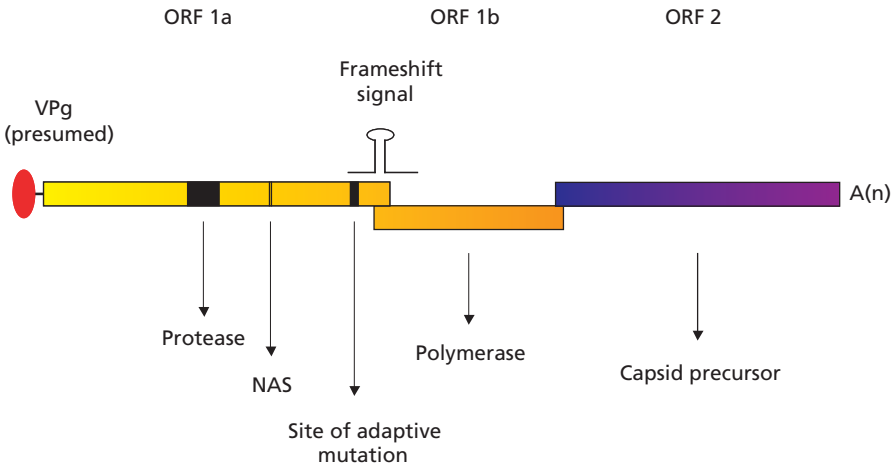

Figure 3 *Genome organization of human astrovirus. The basic three-open reading frame structure of* HAstV-1 *is illustrated (not to scale). A presumptive VPg is indicated at the 5′ terminus but its presence has not been formally demonstrated and the poly(A) tail is indicated at the 3′ terminus. The slippery sequence/stem–loop structure driving the frameshifting expression of ORF 1b is indicated at the overlap of ORFs 1a and 1b. These ORFs encode the virus nonstructural proteins and are both expressed from the genome. ORF 2 specifies precursors to virion structural proteins and is expressed from a subgenomic mRNA. The relative positions of functional motifs and other features of interest are indicated. Protease, site of virus serine protease; NAS, location of the bipartite nuclear addressing sequence; Site of adaptive mutation, location of repetitive sequences apparently deleted between strains and that may assist growth in some cell types.*

Table 2 *Genome and open reading frame sizes for characterized* Astroviridae

Astrovirus	Genome size (if complete)	ORF 1a amino acids	ORF 1b amino acids	ORF 2 amino acids	Accession number
HAstV-1 (Newcastle)	6 813	924	512	786	NC_001943
HAstV-1 (Oxford)	6 771	920	519	787	L23513
HAstV-2	6 797	920	519	796	L06802
HAstV-3	6 815	927	515	794	AF141381
HAstV-4	. . .	. . .	. . .	771	AB025801–AB025812
	. . .	. . .	. . .	771	Z33883
HAstV-5	. . .	. . .	. . .	783	AB037274–AB037273
HAstV-6	. . .	. . .	. . .	778	Z66541
	. . .	. . .	. . .	. . .	AB031030–AB031031
	. . .	. . .	. . .	. . .	AB013618
HAstV-7	. . .	. . .	. . .	791	AF248738
	. . .	. . .	. . .	791	Y08632
HAstV-8	6 759	921	518	842[a]	AF260508
	. . .	. . .	. . .	782	Z66541
TAstV-1	7 003	1 099	512	671	NC_002470
	. . .	. . .	. . .	. . .	Y15936
TAstV-2	7 355	1 125	527	724	AF206663
ANV	6 927	1 029	483	683	NC_003790
FAstV	. . .	. . .	. . .	816	AF056197
PAstV	. . .	. . .	. . .	783	Y15938
	. . .	. . .	. . .	776	AB037272
OAstV	6 440	844	523	762	NC_002496
	. . .	. . .	. . .	. . .	Y15937

a) Sequence reveals 60 additional residues at the N terminus preceding the usual start sequence MASK. . . . It is not known if these are present in the astrovirus mRNA and consequently whether they are translated to protein. Similar sequences present in some strains of HAstV-1 are not transcribed into subgenomic mRNA.

a distribution of receptors. In that case it is believed that virus is taken up whole through the M cells overlying the Peyer's patches, and released on the other side, so gaining access to the basolateral cell surfaces. In this regard it may be significant that BAstV has an affinity for M cells (see below). Cell penetration proceeds via absorptive receptor-mediated endocytosis. Virus entry to the cytoplasm is presumably triggered by pH decrease in the endosome since procedures that elevate intra-endosomal pH have been shown to reduce virus infection (Donelli et al. 1992). The astrovirus genome is infectious if transfected into susceptible cells so it is assumed that translation of the incoming genome is sufficient to initiate the replication process. Full-length transcripts of a cDNA clone of the virus are also infectious on transfection but a synthetic cap structure must be added. This result is reminiscent of that obtained for the caliciviruses (Sosnovtsev and Green 1995) and suggests that some structure at the 5′ terminus (presumably a VPg) is required to initiate translation and that the role of this structure can be substituted by a cap. Proteins are generated from the genome by direct translation to express ORF 1a and via a −1 ribosomal frameshifting event to express ORF 1b. Processing of the polyproteins thus produced is described below.

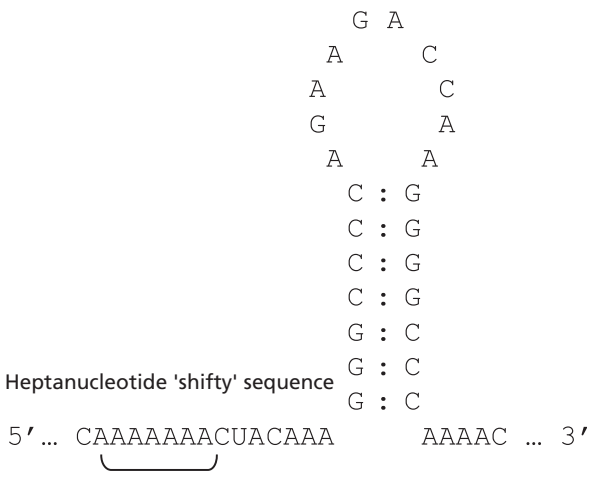

Figure 4 *Frameshift signal derived from* HAstV-1. *The sequence illustrated shows the A-rich heptanucelotide slippery or shifty sequence preceding the stable stem–loop structure. The stem is highly stable and GC-rich; sequence of the stem and the loop appear less important than the overall stability of the structure (Marczinke et al. 1994).*

There have been reports of a nuclear involvement in both BAstV and HAstV replication. Aroonprasert and colleagues (1989) studied BAstV infection in vitro using immunofluorescent antibodies and found fluorescence initially in the cytoplasm of infected cells, followed shortly by brilliantly fluorescing intranuclear granules, suggesting nucleolar involvement. Similarly, experiments

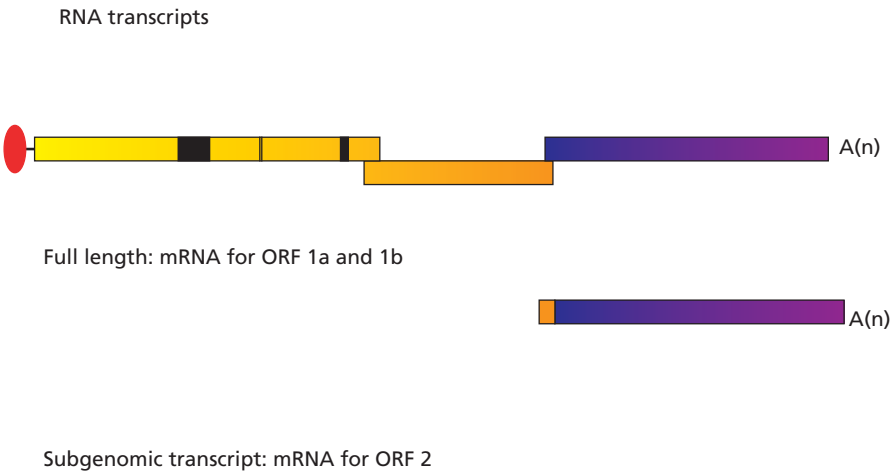

Figure 5 *Astrovirus-induced intracellular RNA. Full-length genome is present and acts as mRNA for ORFs 1a and 1b. A subgenomic mRNA is also synthesized to express the virus ORF 2 proteins. This is co-terminal with the virus genomic 3′ end and extends partially into ORF1b sequences. However, these precede the initiation codon and are not translated. In some viruses, ORF 2 in the genome is potentially longer than the subgenomic mRNA. In these cases any potential initiation codon (s) opening ORF 2 in the genomic RNA are usually in a poor context for activity and are not transcribed into the subgenomic mRNA. The subgenomic mRNA thus presents the ORF 2 initiation codon in the best context for activity as the first such codon in the message. Translation commences with the sequence 'M A S K ---' in almost all cases.*

studying HAstV replication in LLC-MK2 cells showed fluorescence in the nucleus at relatively late times in infection (W.D. Cubitt, personal communication). This fluorescence could suggest movement of structural protein to the nucleus since it has been detected with sera raised to the capsid protein. However, early cloning experiments used such sera to select clones expressing virus antigen (Matsui et al. 1993) and this approach led to the identification of regions specifying the so-called immunoreactive epitopes. Although one of these was derived from the capsid protein as expected, another was surprisingly located in the nonstructural genes close to the nuclear locating signal. When sera were raised to this part of the nonstructural protein, they also reacted with infected cell nuclei (Willcocks et al. 1999). The role of the nucleolus or nucleus in astrovirus infection is not known but replication is not affected by actinomycin D and thus DNA-directed information flow is presumably not required.

A second viral mRNA is also found during astrovirus replication (Monroe et al. 1991b); this subgenomic RNA is approximately 2 800 bases long and is also polyadenylated. It has been found by hybridization and sequence analysis to be co-terminal with the 3′ end of the genome (Matsui et al. 1993; Willcocks and Carter 1993) and is also believed to bear a VPg at its 5′ end (Figure 5). The subgenomic RNA has been found only in infected cells and does not become packaged into virus particles. During infection it is synthesized in five- to tenfold molar excess over the genomic RNA, presumably to allow synthesis of structural proteins in larger quantities than the nonstructural (Monroe et al. 1993). Since this RNA is not present in the virus, it must be generated from the incoming full-length genomic RNA; however, little is known of RNA synthesis by this virus. Promoters have not been characterized and it is not known whether the subgenomic mRNA is subsequently replicated independently via its own separate negative-strand template, or produced only from the full-length antigenome. The 5′ terminus of the subgenomic RNA has been mapped (Monroe et al. 1993) and primer extension analysis reveals the bulk of the product to commence at position 4 359 in the sequence (coordinates refer to HAstV-1). Although the bulk of the molecule does indeed extend in a 3′ direction from this position, a small fraction of the product is slightly longer by 12 or 16 bases (Willcocks and Carter 1993).

Early in infection, capsid antigen is detected in the cytoplasm as a diffuse fluorescence in the perinuclear area. As infection proceeds this becomes more granular, and EM shows dense deposits of virus capsids in these areas (Aroonprasert et al. 1989). Cytopathic effect is not well observed because the trypsin required to allow the particles to mature causes detachment of the cells such that cytopathic effect is not visible. The exception to this has been in some CaCo-2 cell cultures where a shriveling and rounding of the cells can be seen. In this case cytopathic effect is usually limited to the margins of the cell sheet (Willcocks et al. 1990). This is presumably because receptors are located on the basolateral membranes of the cells and the virus has ready access to these only at the edges of the monolayer. Most others are presumably shielded below the tight junctions that bind the cells together. Virus is presumed to be released by cell lysis.

PROTEIN CODING AND EXPRESSION

ORFs 1a and 1b specify the virus nonstructural proteins; the arrangement of the sequences for which a potential role has been identified is given in Figure 3. ORF 1a has the potential to specify a 101 kDa protein that, in common with other positive-strand RNA viruses, is subsequently cleaved to release functional processed molecules. Both the polyprotein and a number of cleavage products have been identified inside cells, although function has not been allocated to all of them. Sequence analysis has revealed the presence of a chymotrypsin (3C)-like serine protease (Jiang et al. 1993); this is in contrast to the picorna- and caliciviruses which have a cysteine residue at the active site of their 3C protease. However, no sequence motifs indicative of RNA helicase, methyltransferases, or papain-like proteases have been detected in astroviruses. It is possible that small RNA viruses of this type do not require a helicase for efficient replication (Koonin 1991b; Jiang et al. 1993). It has been suggested that astrovirus may encode a VPg, based on the absence of a methyltransferase and the similarity of the polymerase to those of other VPg-containing viruses. Sequence analysis has indicated that it might be encoded upstream of the protease but VPg has not been observed experimentally (Matsui and Greenberg 1996). Towards its C-terminus, ORF 1a contains a bipartite nuclear localization signal (Willcocks et al. 1992; Jiang et al. 1993). Antisera raised to this region of the polyprotein have shown that nonstructural proteins do enter the cell nucleus during infection (Willcocks et al. 1999), but the purpose of this movement is not clear. This region of ORF 1a is also the site of a mutation that seems to accompany adaptation of HAstV to growth in continuous cell lines of nongut origin (e.g. LLC-MK2 cells). The Oxford adapted viruses were obtained by serial passage in primary human embryo kidney. During this process the host cell range of the virus became altered. Comparison with samples of the same virus that had been passaged in volunteers rather than human embryo kidney, cells showed that the cell-adapted virus contained a deletion of 45 nt. This removes a partially repetitive 15 amino acid sequence. Isolates made directly in gut cell lines (e.g. CaCo-2 cells) did not contain this deletion (Willcocks et al. 1994a). It has been suggested that this deletion might improve 'fit' between a virus protein and a cellular protein with which it must interact, thus extending host cell range. However, other features of this region also seem variable between strains and so the extent to which this modification represents adaptation to a particular host cell type rather than a coincidental or strain-related variation is not fully understood.

ORF 1b encodes a 59 kDa polypeptide whose amino acid sequence is that of an RNA-dependent RNA polymerase; it contains all the motifs typical of a polymerase belonging to supergroup I (Jiang et al. 1993). This group includes polymerases of the picorna- and caliciviruses, as well as some plant viruses (such as the bymoviruses and potyviruses), although the similarities between astroviruses and the plant viruses in this region do not necessarily indicate a common origin for the viruses (Koonin 1991a).

ORF 2 encodes the virion structural proteins and is expressed from a subgenomic mRNA that is 3′ co-linear with the genome (Monroe et al. 1993; Willcocks and Carter 1993). The structural proteins are produced as a precursor (90 kDa), which is then proteolytically cleaved. This protein has a well-conserved N terminus which is highly basic, and contains a serine/arginine repeat motif (Jonassen et al. 2001). This is similar to the basic region found in some coronaviruses (those related to transmissible gastroenteritis virus), and is also reminiscent of some plant viruses where such a sequence is involved in RNA binding (Carter 1994). By contrast, the 3′ end of ORF 2 specifies a highly acidic region. The function of these two areas is not known, but it is likely that these regions of strong charge localization do play a role in protein–protein interactions or protein–RNA interactions.

A highly conserved 35 nt stem–loop structure is located near the ORF 2 stop codon in all the astrovirus sequences examined to date (except that of TAstV-2). This feature straddles the termination codon and is formed from both translated and nontranslated sequences. The function of this structure is not known, but it is likely to be vital to the virus. Any change in one nucleotide of the stem structure is always accompanied by a compensatory mutation in the other strand of the stem such that base pairing and thus secondary structure is maintained (Jonassen et al. 1998).

Processing of nonstructural proteins

The nonstructural proteins are generated from two polyproteins corresponding to ORF 1a (101 kDa) or the ORF 1a/1b fusion resulting from ribosomal frameshifting during translation (160 kDa). The processing of these proteins remains to be fully elucidated but several workers have now examined processing either in infected CaCo-2 cells, transfected baby hamster kidney (BHK) cells or in vitro. A synthesis of the resultant data is presented in Figure 6.

Processing has been examined during productive infections of CaCo-2 cells using sera raised to specific sections of the nonstructural protein ORFs. Sera recognizing the C-terminal third of ORF 1a (residues 643–920 in HAstV-1) detected multiple cleavage products; the complete ORF 1a product was not observed, the largest protein being only 75 kDa. This indicated that some 26 kDa had been rapidly cleaved from the N terminus of ORF 1a and could not be detected using this serum. The 75 kDa protein appeared to be further cleaved,

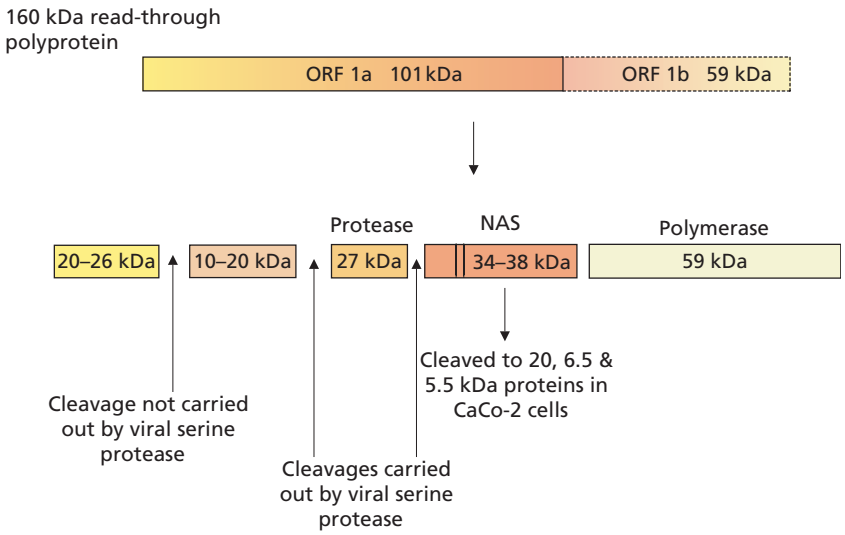

Figure 6 *Processing of astrovirus nonstructural proteins. Here the processing of the ORF1a/1b fusion generated by read-through translation of both ORFs is illustrated; 20–26 kDa is cleaved from the N terminus by a nonviral protease soon after translation (whilst other cleavages are performed by the virus-encoded enzyme). This releases the 27 kDa protease molecule and a presumptive second protein that has not been directly observed. The third section released from ORF 1a (34–38 kDa) includes the bipartite nuclear addressing signal (NAS) and is further cleaved to at least three products 20, 6.5, and 5.5 kDa in size. The ORF 1b product (polymerase) is believed to be cleaved at or near the frameshift position and is not markedly cleaved subsequently.*

producing a 34 kDa C-terminal product that could be detected by the serum used. Any other product(s) that derived from the N terminus of the molecule were not detectable by this method. The 34 kDa molecule appeared to be unstable and did not accumulate to high levels in the cell; instead it appeared to be processed to cleavage products of 20, 6.5, and 5.5 kDa that were observed to accumulate. The proteins that would be recognized by this serum would include any that contain the nuclear localization signal, and these were identified in the cell nucleus (Willcocks et al. 1999).

ORF 1a sequences have also been expressed in BHK cells transfected with expression constructs containing ORF 1a (Geigenmuller et al. 2002a). These workers also observed a cleavage near the N terminus, removing in this instance a 19 kDa section. This cleavage also took place if the virus protease was inactivated, thus confirming that it is not mediated by this enzyme and is probably a host-cell-specified event. Further processing was dependent on a functional viral protease and led to a product of 27 kDa that spanned the serine protease motifs. This corresponds to the 27 kDa protein identified in astrovirus-infected CaCo-2 cells and which can now be identified as the virus protease itself.

Gibson and colleagues (1998) reported the in vitro expression of both the full-length ORF 1a polyprotein and that of the combined ORF 1a/1b but presented no information on processing. A second study examined processing in vitro using antisera raised to synthetic peptides; a single autocatalytic event was observed in the ORF 1a polyprotein. This yielded an N-terminal 64 kDa and a C-terminal 38 kDa product. This cleavage was accomplished by the virus-specified serine protease since mutation of the serine at the active site obviated this event (Kiang and Matsui 2002). The 38 kDa protein is probably analogous to the 34 kDa protein identified in CaCo-2 cells and their nuclei. The removal of 26 kDa from the N terminal was not observed, indicating again that this may be a host-cell-dependent cleavage.

Willcocks and colleagues (1999) also detected synthesis of the polymerase (ORF 1b product) in CaCo-2 cells using sera raised to the expressed ORF 1b protein. A single product of 59 kDa was detected. This is approximately the size expected for the whole of ORF 1b (786 aa). This suggests that little if any internal cleavage takes place in the polymerase and similarly that most or all of ORF 1a is effectively removed when the fusion protein is processed.

Processing of structural proteins

The astrovirus structural protein is translated from ORF 2 as a 90 kDa precursor protein and it has long been recognized that trypsin plays a vital role in cleavage of this precursor (Monroe et al. 1991b). Willcocks and colleagues (1990) showed that particles released in the absence of trypsin were non-infectious. Bass and Qui (2000) confirmed this finding and showed that such particles contained only minimally processed capsid precursor protein; 71 amino acids were cleaved from the N terminus intracellularly to generate a 79 kDa capsid precursor from which particles were formed. Treatment of these particles with trypsin after release led to the formation of three structural proteins and increased infectivity by 50 000–100 000-fold. Other workers have

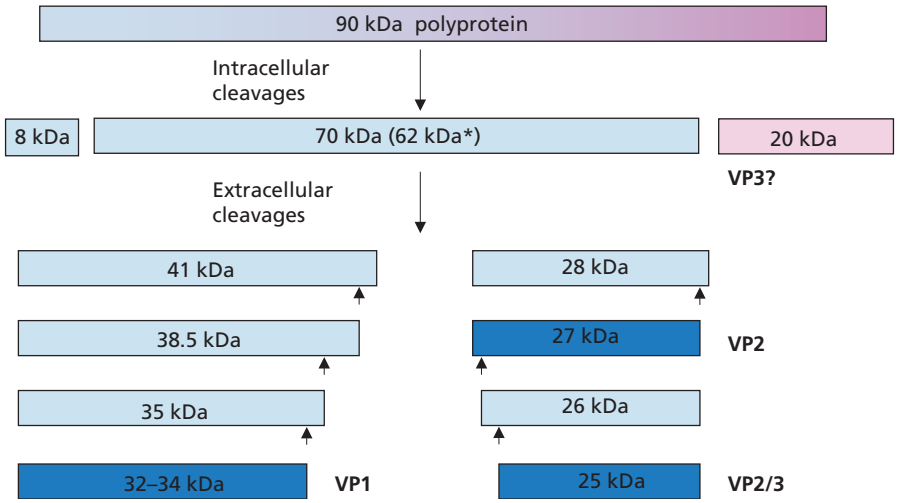

Figure 7 *Processing of astrovirus structural proteins. The illustration attempts to bring together data from a variety of sources, not all of which are compatible. Consequently the actual processing pathways used by any one virus may vary depending on either serotype of virus and/or host cell. In the model presented, primary translation generates a molecule of 90 kDa (VP90) that is subsequently processed. One report states that 8 kDa may be cleaved from the N terminus rapidly after translation (indicated by '*') but this does not appear to be general and does not occur in other systems where it has been sought. VP20 is cleaved from the C terminus and at least one report finds that this occurs intracellularly. In this case VP20 was not subsequently present in the virion (Mendez et al. 2002) and particles were formed exclusively from VP70. Other reports indicate that particles form from VP90 (and thus must include residues derived from VP20) (Geigenmuller et al. 2002b). Thus the backbone of the virion may indeed be formed from sequences contained within VP70 but the C-terminal residues contained within VP20 may be superficially located. Once cleaved (intracellularly or extracellularly), they may readily dissociate from the particles. Maturation of VP70 (or VP90 where particles are constructed from this protein) occurs extracellularly and is mediated by trypsin. Several sequential cuts are made and the efficiency of each may vary with both the strain of virus (and the accessibility of the scissile bond) and the activity of trypsin supplied in the growth medium. As a result, particle compositions may appear complex but VP32–34, and the overlapping proteins VP25 and VP27 may predominate (indicated in deeper color). Differences in the extent of maturational cleavage could also explain the lack of stoichiometric relationship observed in some preparations where levels of VP27 appear far lower than those of VP25. Past attempts to use a conventional nomenclature in which virus proteins are numbered VP1–3 are complicated. This nomenclature is best avoided since the proteins could be potentially assigned different names depending on the degree of cleavage and/or VP20 dissociation in any one preparation (indicated above).*

not observed this trimming of the N terminus and report structural proteins in the virion that extend completely to the N terminus of the protein. (Geigenmuller et al. 2002b; Mendez et al. 2002).

Determination of the cleavage sites and the manner of processing of virion proteins has been complicated by uncertainty as to the protein composition of the virions (see above). In a study of the Oxford HAstV-2 strain, Sanchez-Fauquier and colleagues (1994) found that the capsid precursor was cleaved to three polypeptides (34, 29, and 26 kDa) in the presence of trypsin. These sizes would account for almost all the structural protein precursor. However, N-terminal sequencing revealed surprising features. VP29 and VP26 were not distinct molecules. Instead, these moieties overlapped substantially; they shared a common C terminus and were both derived from the center of the precursor protein. The residues extending from the N terminus of the precursor to the N terminus of VP29 could form the 34 kDa protein (although this was not confirmed), but this analysis left the fate of some 27 kDa from the C terminal of the precursor protein unexplained. A further study of structural protein processing in HAstV-8 offered a possible explanation for these findings (Figure 7). Mendez and colleagues (2002) found that 20 kDa was cleaved from the C terminus of the capsid precursor intracellularly. This molecule was not detected in virions and its fate has not been determined. The remaining 70 kDa molecule was processed extracellularly and only to completion when high levels of trypsin (>100 μg/ml) were present. The 70 kDa polyprotein was cleaved firstly to 41 and 28 kDa products and subsequently the size of each was further reduced through a series of trimming cuts. Three products were prominent in preparations of virions; VP34 and two forms derived from VP28: VP25 and VP27. The relationship between these two molecules agrees with the findings of Sanchez-Fauquier et al. (1994). The variability in the detection of the third virion protein could then result simply from differing efficiencies of trypsin cleavage and this might also explain some of the earlier reports of multiple protein components in the virion.

However, this explanation also fails to provide a role for the C-terminal 20 kDa removed intracellularly from the precursor in these experiments. This section of the protein is highly variable in sequence between serotypes. This suggests exposure to host antibody selection pressure and thus implies a structural (or at least extracellular)

role. Furthermore, its presence on the same mRNA as the structural proteins would suggest that it is manufactured in similar amount. Geigenmuller and colleagues (2002b) infected CaCo-2 cells with mutant virus bearing a tag sequence at either the N or C terminus of the capsid protein. They also observed the removal of up to 20 kDa from the C terminus of the capsid precursor protein, but in this case the process was inefficient; the majority of the protein remained uncleaved regardless of the position of the tag. However, particles were observed in these cells, suggesting that C-terminal cleavage is not essential for particle formation and consequently C-terminal sequences can be incorporated at least into immature forms of the capsid inside the cell. Although these facts are suggestive of a structural role, this region has not been demonstrated in mature, extracellular virions by N-terminal sequence determination, indicating that it may be lost during particle maturation or sample preparation in the laboratory.

SEROLOGY

In the 1980s, Kurtz and Lee identified five serotypes of HAstV (HAstV-1–5) by immunofluorescence and immunosorbent electron microscopy (Lee and Kurtz 1982; Kurtz and Lee 1984). Each of these reference strains had been adapted to growth in LLC-MK2 cells (Lee and Kurtz 1981). Since then, three more serotypes have been recognized (HAstV-6–8); all were originally isolated in the UK (Lee and Kurtz 1994; Kurtz, unpublished data). However, they have remained uncommon in the UK. The HAstV are antigenically distinct from the animal viruses (Kurtz and Lee 1984); however, feline astrovirus (FAstV) is detected efficiently by enzyme-linked immunosorbent assay (ELISA) kits engineered using sera and monoclonal antibodies raised to HAstV.

In many viruses, genogroup analysis based on sequence phylogeny is superseding conventional classification by serotyping. Although genogroup analysis can be performed on any part (or the whole) virus sequence, serotyping by definition depends only on the structural proteins. For the most part, genogroupings are based on nonstructural sequences since these are well conserved and thus relatively easily accessed by PCR for sequence determination. In general, this type of analysis does give similar, if not identical, patterns regardless of the section of the genome used for analysis, but even so the potential for recombination events that might combine structural genes from one serotype with nonstructural genes from another should not be ignored. These problems can be largely overcome in the case of the astroviruses provided that the structural protein is used for genotyping. Noel and colleagues (1995) examined a short PCR product derived from ORF 2 of each serotype and showed that genotyping in this instance led to the same result as serotyping. Since then, additional capsid precursor sequences have become available, but the coincidence of the two types of grouping has been maintained. Wang and colleagues (2001) compared capsid precursor sequences of 46 strains of HAstV and found that the genogroups obtained corresponded exactly to the serotypes. Comparisons have shown that within any given serotype, where more than one ORF 2 complete sequence is available, the strains are more than 94 percent identical. By contrast, the highest identity level seen between serotypes was 75 percent between types 5 and 6 (Mendez-Toss et al. 2000).

Jonassen and colleagues (2001) examined variation within the capsid sequence and also found that the N-terminal half was well conserved, while the C-terminal portion was hypervariable with large inserts or deletions. Neutralizing monoclonal antibodies prepared to the astrovirus capsid have all been found to be specific to the VP29 protein encoded in the midsection of the capsid polyprotein (Bass and Upadhyayula 1997).

The largest protein, on the other hand, is more conserved, and a common epitope has been predicted from sequence analysis (Mendez et al. 2002). All the serotypes of HAstV are recognized by a single non-neutralizing monoclonal antibody produced by Herrmann and colleagues (1988). The monoclonal antibody is directed at the capsid protein (Lewis et al. 1994), but the site it recognizes has not been fully defined. Current data suggest that the antibody binds to two discontinuous regions of the capsid protein, both located in the largest protein VP34 between residues 82–115 and 215–260 (residue numbers given for HAstV-1 strain A2/88 Newcastle) (M.M. Willcocks et al., unpublished data). This monoclonal antibody has been used to develop a commercial ELISA for astrovirus and also reacts with FAstV.

SEQUENCE PHYLOGENY

Genome nucleotide sequences

The complete nucleotide sequences have now been determined for four serotypes of HAstV:

1. HAstV-1 both Oxford (Lewis et al. 1994) and Newcastle (Willcocks et al. 1994b) strains
2. the Oxford strain of HAstV-2 (Jiang et al. 1993)
3. HAstV-3 from Germany (Oh and Schreier 2001)
4. HAstV-8 from Mexico (Mendez-Toss et al. 2000).

Three of the avian astroviruses have been sequenced, two from the turkey [TAstV-1; (Jonassen et al. 1998) and TAstV-2 (Koci et al. 2000a)] and one from the chicken (ANV; Imada et al. 2000). One other mammalian virus, that of the sheep, has also been sequenced – OAstV (Jonassen et al. 2001). The sequence of the structural gene *ORF2* has been obtained for the remaining serotypes of HAstV: HAstV-4 (Willcocks et al. 1995), HAstV-5 (Wang et al. 2001; S.S. Monroe et al., unpublished data), HAstV-6 (M.M. Willcocks et al.,

I. Oishi et al., both unpublished data), and for HAstV-7 (Jonassen et al. 2001; Walter et al. 2001b). Structural gene sequences are also available for the feline (M.M. Willcocks et al., unpublished data) and porcine astroviruses (Jonassen et al. 2001; Wang et al. 2001).

An analysis of the nine complete sequences available showed that they could be divided into two groups (Figure 8), with one containing the mammalian astroviruses (OAstV being an outlier) and the second containing the avian astrovirus sequences. The same two groups were also derived using either ORF 1a or 1b alone for the comparison (Lukashov and Gouldsmit 2002). Comparison, by these authors, of all the available ORF 2 sequences groups all the HAstV serotypes together with the feline virus being most closely related to them, followed by the porcine, and with the ovine capsid sequence showing considerable variation from the other mammalian capsid nucleotide sequences. Again the avian astroviruses formed a separate cluster. The authors speculate that the topology of the phylogenetic trees suggests that cross-species transmission may have occurred during astrovirus evolution with a PAstV-like virus being passed to cats and then to humans. This sort of cross-species transfer occurring during virus speciation and over evolutionary time must be clearly distinguished from zoonotic infections whereby animal viruses may infect humans, although this may also occur (see below). Grouping of the structural proteins on the basis of their more conserved sections should indicate underlying relatedness between viruses and strip out the effects of antigenic variation driven independently by immune responses in different host species. When performed this way, FAstV was even more closely grouped with the human strains, again indicating an evolutionary relationship (Wang et al. 2001). A comparison based on ORF 2 amino acid sequences reached similar conclusions (Jonassen et al. 2001).

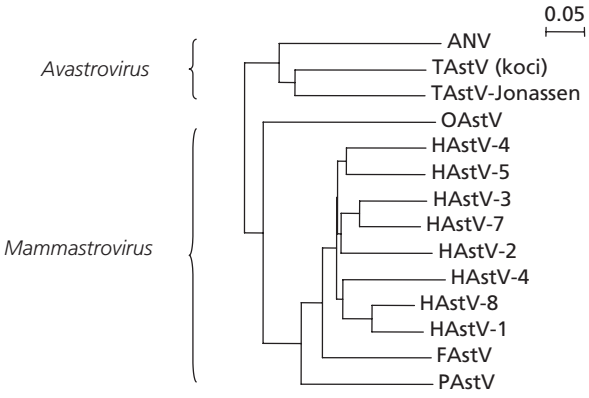

Figure 8 *Phylogenetic analysis of representative astrovirus capsid sequences. Sequences fall into two clear genogroups that have now been recognized as separate genera:* Mamastrovirus *containing sequences from mammalian viruses and* Avastrovirus *containing sequences from avian isolates. FAstV groups closest to human strains whilst OAstV is an outlier from the* Mamastrovirus *group.*

Analysis of virus sequences can always be complicated by the potential for recombination between genomes. The two phylogenetic groups identified above are not affected by these concerns, but recombination between human astroviruses can almost certainly take place. Four isolates of HAstV-5 from Houston, TX and Mexico City were analyzed at the junction of ORF 1b to ORF 2. These new isolates were closely related to serotype 3 in ORF 1b but closest to serotype 5 in ORF 2. These data, combined with the strong sequence conservation in this region seen in all HAstV serotypes, indicated a possible recombination site at the ORF 1b/ORF 2 junction (Walter et al. 2001a). Recombination events like this could exchange capsid protein genes between viruses in the case identified between serotypes of human virus, but presumably this could also exchange genes between an animal and a human virus under appropriate circumstances.

TRANSMISSION

Person to person

The virus is transmitted by the fecal–oral route and therefore spreads readily among babies and small children where infection is most common. There have been many reports of outbreaks in childcare facilities and schools (Lew et al. 1991; Mitchell et al. 1993, 1999b). Prevention or control of astrovirus infection in such settings is generally best achieved by careful attention to hygiene (hand washing, cleaning, or disposal of potentially contaminated items). It has been suggested (Abad et al. 2001) that astroviruses are able to survive on inert surfaces long enough for fomites to play a role in transmission of astrovirus diarrhea, and disinfection of any potentially contaminated surfaces, door handles, etc., is also required. The virus has been shown to be relatively resistant to disinfection by ethanol or isopropanol (Kurtz et al. 1980). It is also relatively resistant to inactivation by heat, acidification, detergent treatment, and treatment with phenolic, quaternary ammonium chloride, or benzalconium chloride-based products (Kurtz and Lee 1987). However, 70 percent methanol was found to decrease infectivity by 3 $\log_{10}$ and 90 percent decreased it to less than 10 infective units/ml and proprietary virucidal surface cleaners are effective. Since the virus is relatively temperature stable, attention should be paid to the operating temperature of bedpan washers. Infected individuals should be separated from those not infected and particular care should be taken to ensure that those who handle food do not continue to work while infected.

Foodborne

Foodborne transmission is not generally assumed to be significant in the case of astrovirus infection. This arises

since the bulk of astrovirus infections occur in the young, an age group where direct fecal–oral person-to-person transmission is more likely. However, foodborne transmission between adults is likely to be more significant, and although most individuals are likely to have had prior experience of astrovirus types 1 and 2 (with resulting protective or ameliorating antibody memory), many would not have encountered the rarer serotypes which could therefore induce symptomatic illness. Astrovirus outbreaks have been associated with the consumption of shellfish contaminated with sewage (Kurtz and Lee 1987). A recent study in France found that 17 percent of oysters sampled were contaminated with the virus; and that, when sampling was carried out in an area routinely impacted by human sewage, 50 percent of mussels examined contained detectable levels of viral RNA (Le Guyader et al. 2000). Mussels and cockles are generally cooked before consumption and regulations brought in to control hepatitis A virus contamination will also control astroviruses. Astroviruses have been associated with single-exposure outbreaks similar to those more usually associated with Norwalk viruses, but these seem relatively uncommon. These have been well studied in Japan where Oishi and colleagues (1994) reported data from a multi-school outbreak in Osaka; and Utagawa and colleagues (1994) reported three outbreaks associated with restaurants and schools. In each case, astroviruses were the organisms most frequently identified although other agents were also present in some samples from the outbreak. Consequently, the contribution of individual viruses to the overall pattern of these events is not completely clear. Nevertheless, the potential for widespread and significant astrovirus-induced foodborne illness must be acknowledged.

Astroviruses survive well in water (see below) and transmission from contaminated streams has been reported (Cubitt 1991). They are also relatively difficult to inactivate with chlorine (the normal method of rendering water potable) being considerably more resistant than poliovirus (Abad et al. 1997). If the virus survives chlorination, then further inactivation could be quite slow, astroviruses can persist for a long time in dechlorinated tapwater: after 60 days the infectivity was reduced by 3.6 logs when the water was maintained at 20°C and only 2 logs at 4°C (Bosch et al. 1997). Astrovirus has been detected in water samples from an area where a concurrent astrovirus-induced gastroenteritis outbreak had occurred (Pinto et al. 1996).

Environmental transmission

Until recently relatively little was known of the circulation of astroviruses through the environment and particularly through surface waters and water treatment systems. A recent survey of seawater bathers in the UK implied that astrovirus survived well enough to pose a risk of infection in coastal waters and the presence of virus in shellfish (above) is also indicative. Higher levels of gastroenteritis were reported by surfers compared to age-matched controls who did not sea-bathe. Of the surfers surveyed, 93 percent showed serological evidence of exposure to HAstV-4 compared to 39 percent of controls (Myint et al. 1994). Molecular methods have detected astroviruses in 21 percent of river water samples and 3 percent of Dam water samples in South Africa (D. Steele, personal communication). Virus was found in samples where all usual microbiological indicators of fecal pollution were absent or within acceptable limits implying that astrovirus may present a risk in samples that could be assessed as 'safe' by usual methods (Taylor et al. 2001). The stability of the virus means that particles survive well and are detectable in waters flowing both into and out of water treatment works, indicating that viruses survive at least some forms of water processing. A quantitative assessment of astrovirus in such waters found that titers of 4×10^5– 1.4×10^7 particles per 100 ml in the water flowing into treatment plants and levels of 6×10^2–9×10^4 per 100 ml in the treated water flowing out (P. LeCann, personal communication). Astrovirus (and enteroviruses) has also been detected in sludge (Chapron et al. 2000). Consequently, it is hard to avoid the conclusion that astroviruses represent a hitherto underestimated environmental contaminant. Given recent data from Koopmans and colleagues (1998) that exposure to one serotype carries little protective effect against infection by other serotypes, then adults will continue to be at risk from the higher serotypes to which they are only rarely exposed.

PREVALENCE

Even from the first small-scale studies it was apparent that HAstV infections are very common, exposure occurs early in life (at least to the more common serotypes), and seropositivity rises steeply in early childhood. A survey in the UK found that 64 percent of children had antibodies to HAstV-1 by 4 years of age and this figure rose to 87 percent by age 10 (Kurtz and Lee 1978). A more recent age-stratified assessment of seroconversion to types 1 and 6 in London showed that children rapidly seroconverted to type 1 as maternal immunity waned. Infections commenced around 5–6 months of age and by age 5 nearly 90 percent of samples were positive for type 1 antibody. Seroconversion to type 6 was seen to commence slightly later (7–11 months) and to rise more slowly, reaching only some 20 percent even by adulthood (Kriston et al. 1996). A similar study addressing seroconversion in the USA found again that seroconversion to type 1 occurred rapidly after loss of maternal antibody, reaching 94 percent by age 6–9 years. In this case infections by HAstV-3 were also studied and a similar pattern observed. Infections occurred during the same time frames as those by HAstV-1 but were less

frequent, with 55 percent of 6–9-year-olds remaining seronegative (Mitchell et al. 1999a). Midthun and colleagues (1993) found 45 percent of adults were seropositive for type 5 virus, 55 percent having presumably not contracted the virus. A seroprevalence survey in the Netherlands reached similar conclusions (Koopmans et al. 1998), finding antibodies to HAstV-1 in 91 percent; HAstV-2 in 31 percent; to HAstV-3 in 69 percent; to HAstV-4 in 50 percent and to HAstV-5 in 36 percent of persons sampled.

Several surveys have examined the incidence of astrovirus in diarrheal stools. In general, these fall into two classes: those using predominantly EM as the means of diagnosis, and those using alternative techniques (RT-PCR or ELISA). The former class generally report astroviruses at a frequency of 1–5 percent in diarrheal stools, whilst the latter find an increased prevalence and report values in the region of 5–15 percent. The second type of survey generally suggests that astrovirus is second only to rotavirus as a cause of enteric illness in children. A potential explanation of this discrepancy is addressed under diagnostic methods. Despite their differences, these surveys do reveal some underlying patterns, as follows.

First, where seasonality was detected, astrovirus incidence mirrored that of rotavirus. A peak of infection was observed in the winter months in Europe and the developed world (Guix et al. 2002; Lew et al. 1990; Maldonado 1996; Monroe et al. 1991a; Mustafa et al. 2000; Rodriguez-Baez et al. 2002; Sakamoto et al. 2000). Infection peaks in the rainy seasons elsewhere, e.g. Guatemala (Cruz et al. 1992) and rural Mexico (Maldonado et al. 1998). Infections in Japan showed a peak between March and April (Utagawa et al. 1994). The explanation of seasonality is not clear for any enteric virus, although it possibly involves contributions from a variety of factors. These may include: an increased efficiency of person-to-person transmission in colder weather (due to increased virus survival in the environment and more crowding in public places); the possibility of watercourse contamination (due to increased rainfall and blockage of drains, etc., by leaves); and even an increase in the levels of virus in filter-feeding shellfish consumed in the colder months.

Second, whilst co-circulation of different serotypes, and also of distinct genetic variants within a serotype, is often seen (McIver et al. 2000; Traore et al. 2000), serotype 1 is usually the most common virus detected, a finding expected from the seroprevalence studies above. Kurtz and Lee (1984) determined the serotypes of astroviruses acquired in the community in Oxford (UK) and found serotype 1 accounted for 77 percent of virus identifications in clinical samples. Serotypes 2–5 each accounted for 5–7 percent of the isolates. This was, however, a relatively small survey, but a more recent repeat (covering the period 1976–92) yielded similar data (Lee and Kurtz 1994). Serotype 1 was again the most common (65 percent), with all the other serotypes having much lower incidence (HAstV-2 and HAstV-4 both 11 percent; HAstV-3 9 percent; HAstV-5 2 percent; and HAstV-6 and HAstV-7 both less than 1 percent). These findings are repeated across the world, serotype 1 being the most common serotype found in surveys carried out in a variety of geographical regions (Australia – Palombo and Bishop 1996; Chile – Gaggero et al. 1998; Mexico – Mendez-Toss et al. 2000; Korea – Kang et al. 2002; and Spain – Guix et al. 2002).

Third, this relative prevalence of serotype 1 is not universally true. Some fluctuation in relative prevalence of the individual serotypes is observed. In the UK survey described above, serotype 1 was the most common throughout the period surveyed except in 1991 when serotype 2 was the most prevalent (Lee and Kurtz 1994). Analysis of the age-stratified seroprevalence studies in London also indicates that infection by serotype 1 viruses may have increased in the years since 1990–91 and a sharp decrease in the seroprevalence rates (from 90 to 50 percent) is observed in children above the age of 6 years. This suggests that infection was less common when these individuals were aged <12 months, the peak age period for acquisition of the virus. Evidence from seroconversion of seawater bathers (Myint et al. 1994) and from serotyping studies in Oxford (Willcocks et al. 1995) indicate that serotype 4 increased in prevalence in the UK in 1993. Similarly, HAstV-2 was found as the most common serotype in Mexico City in two reports (Guerrero et al. 1998; Walter et al. 2001b), and in Malawi, HAstV-1 and HAstV-2 were found in approximately equal incidence (Cunliffe et al. 2002).

In common with rotavirus and the noroviruses, astrovirus is frequently acquired nosocomially. Two surveys in California found astrovirus and rotavirus at a similar frequency in the hospital setting. In both surveys, about half the astrovirus infections were thought to have been nosocomially acquired (Shastri et al. 1998; Rodriguez-Baez et al. 2002). The later survey (conducted 1998–2000) reported that 21 percent of hospitalized children (<6 years) had symptoms of gastroenteritis during their stay. Astrovirus was detected in 5.2 percent of the samples and 75 percent of these infections were symptomatic. Rotavirus accounted for only marginally more positive samples at 6.8 percent, suggesting that astrovirus has a similar incidence to rotavirus in this context. Rotavirus infections were, however, more severe and more likely to lead to dehydration (Rodriguez-Baez et al. 2002). Dennehy and colleagues (2001) obtained similar results in Australia; rotavirus was found to be the major cause of nosocomially acquired infection with astrovirus accounting for 16 percent. Community-acquired astrovirus was observed at a rate of 6.8 percent of samples. Once more rotavirus infection was clinically more severe. A survey in France examined the incidence of norovirus and astrovirus in hospitalized patients.

Infections were assigned as community or nosocomially acquired, depending on the delay between admission and development of illness. Where this period exceeded typical incubation times, infection had presumably been acquired in hospital. Approximately half the astrovirus infections identified and a third of the norovirus infections were found to be nosocomial (Traore et al. 2000). Foley and colleagues (2000) also found an approximately equal incidence of astrovirus and norovirus in presumed nosocomial infections in Ireland. Thus the majority of reports find that infection by all three agents can occur at about the same frequency in the hospital setting.

A further question to be considered is to what extent if any, animal strains of astrovirus might regularly cross-infect between species to reach humans. Sequence relatedness between feline and human astroviruses does suggest an evolutionary relationship, but this neither implies nor excludes more frequent traffic of viruses between these species. Certainly a recent large-scale survey of infectious intestinal disease in England and Wales found that recent contact with pets (cats and dogs) with diarrhea (but not with healthy animals) did carry an increased risk of developing intestinal disease. However, these cases tended to be associated with failure to detect a causative organism which was surprising (Food Standards Agency 2000). This in turn suggests that any zoonotically infecting agent might not be detected by the usual diagnostic methods used. Recent experimental data have shown that FAstV can infect human cells (CaCo-2) in culture, inducing cytopathic effect and leading to the release of progeny particles. These could not, however, be passaged further even when trypsin treated. This suggests that humans might be infectable with FAstV. Recombinant FAstV antigen was then used to modify an enzyme immunoassay (EIA) already developed for the type-specific detection of antibody to human astroviruses (Mitchell et al. 1999a). This test was then used to examine a panel of human sera previously characterized for antibody to HAstV types 1 and 6 (Kriston et al. 1996). The FAstV antigen was found to react with a panreactive non-neutralizing monoclonal antibody (Herrmann et al. 1988) but had no reactivity with rabbit sera raised to individual serotypes 1–7 of human astrovirus. This survey found that 7/262 (2.6 percent) of sera showed a specific reaction towards the feline virus antigen that did not correlate with cross-reacting seroconversion to any of the HAstV strains tested. Furthermore, samples from two different infants were negative for all seven human astroviruses tested, yet were positive for antibody recognizing the feline virus (W. D. Cubitt, unpublished observations). These data suggest that feline virus can infect humans, inducing seroconversion. However, it is not clear whether this is associated with any illness or if the infection is 'dead-end' and cannot be spread further between humans.

CLINICAL MANIFESTATIONS

Human and mammalian astroviruses

Human astroviruses are generally associated with fairly mild, self-limiting gastroenteritis in infants and young children. To some extent this view is likely to be an oversimplification (reviewed by Glass et al. 1996) Recent studies report that astrovirus is the second most common viral agent (after rotavirus) found in young children with diarrhea (Herrmann et al. 1991). However, astroviruses are also frequently identified in symptom-free individuals and it is clear that infection does not always result in clinical signs. The highest reported incidence was observed in rural Mayan infants. In this study 61 percent of a 3-year birth cohort showed astrovirus infection. Astrovirus was associated with 26 percent of all diarrheas observed and 17 percent of astrovirus positives were associated with diarrhea and a further 8 percent with other symptoms. In this study, astrovirus was found to be the major pathogen in that community, outstripping even rotavirus in incidence (Maldonado et al. 1998). However, findings from other surveys have revealed infection rates and symptom associations that were somewhat lower, e.g. 4.9 percent in Barcelona (Guix et al. 2002) and varied between 1.4 and 4.4 percent from 1995 to 1998 in Melbourne, Australia (Mustafa et al. 2000). However, it does not necessarily follow that illness is mild and astroviruses do result in hospitalization for severe diarrhea. In a study of such admissions in South Africa, rotavirus was found to be the most common viral cause at just under 30 percent but astroviruses were a highly significant second at 7.4 percent (D. Steele, personal communication). Even in the USA it has been estimated that astroviruses account for some 5 percent of all hospital admissions due to diarrhea (CDC 1990). There is currently no vaccine for astrovirus infection but an active program has targeted rotavirus for many years. Should such a vaccine enter general use, the relative significance of astrovirus-induced disease would rise.

The virus has an incubation period of 3–4 days that is followed by the appearance of diarrhea (typically two to six motions/day). This is the predominant clinical symptom of infection by these viruses and is accompanied by shedding of virus, which generally lasts for 2–3 days but may persist for up to 14 days (Kurtz and Lee 1987). Other reported symptoms include nausea and vomiting, headache, fever, and abdominal discomfort. A survey in London, UK found that 82 percent of children infected with astrovirus experienced diarrhea, 64 percent vomiting, 25 percent abdominal discomfort, and 18 percent mild dehydration (Nazer et al. 1982). A larger scale survey of infectious intestinal disease in the UK has analyzed symptoms reported by those infected with astroviruses: all those affected reported diarrhea as the

major symptom with 60 percent% of infected children described as incapacitated by the infection (Food Standards Agency 2000).

Further astrovirus-induced illness is not always mild in adults. An outbreak of severe gastroenteritis amongst French military recruits was associated with HAstV-3 (Belliot et al. 1997c) whilst the Food Standards Agency (2000) study in England and Wales found diarrhea as the major symptom in astrovirus-infected adults attending their GP surgeries; 65 percent described it as 'severe' and nearly 90 percent were 'incapacitated' by the illness. Abdominal pain and loss of appetite were also common in both children and adults but febrile effects (temperature, muscle ache, etc.) seemed more frequent in adults than in children and vomiting was more frequent in children (75 percent) than in adults (29 percent). It may well be that many adults remain at risk of symptomatic infection by higher serotypes of astrovirus to which cross-protective immunity resulting from infection with serotype 1 is poor (Koopmans et al. 1998).

Symptom severity has been investigated by means of volunteer studies: bacteria-free filtrates of astrovirus-containing stools have been introduced orally to adult volunteers; in general these have shown that the virus has low pathogenicity in healthy adults. Kurtz and colleagues (1979) inoculated eight volunteers with HAstV-1 and found that only one developed diarrheal illness (and shed large numbers of astrovirus particles) and a second volunteer reported mild symptoms (accompanied by a lower level of virus shedding). However, most of this panel would have been exposed previously to this virus serotype. A second study in the USA exposed 19 volunteers to serotype 5 virus; 47 percent of these persons had undetectable titers of astrovirus-specific serum antibodies when inoculated. One volunteer developed mild illness and nine had a serological response to the virus but did not report symptoms (Midthun et al. 1993). The conclusion from these studies is that astrovirus infection is usually relatively mild in otherwise healthy adults whilst the clinical pattern tells us that this cannot be universally true.

Astrovirus infections may be especially significant in vulnerable groups; outbreaks have also been reported in residential communities of elderly people (Gray et al. 1987; Lewis et al. 1989). In Marin County, California (Oshiro et al. 1981), 51 percent of the residents of a convalescent hospital (and 12 percent of the staff) developed symptoms. Most experienced minor illness lasting about 3 days and consisting only of diarrhea, although 13 percent of the patients also experienced fever. In the immunocompromised, virus shedding has been reported for several months following infection (Noel and Cubitt 1994) and the gastroenteritis caused may be severe (Coppo et al. 2000). Infections of hospitalized children undergoing immunosuppressive regimens may be prolonged and can prove difficult to eradicate from the unit (Cubitt et al. 1999). Prolonged infections may also be occurring in Bangladesh where a study found astrovirus was more frequently present in children <5 years who suffered from persistent diarrhea (15 percent) than in those with acute and short-lived illness (4 percent) (Unicomb et al. 1998). This suggests that in Bangladesh astrovirus may be an important contributor to the prolonged illness (defined as >14 days' duration) observed in 7 percent of diarrheal patients. In this setting, prolonged diarrhea is a serious condition and frequently results in death. However, it was not determined whether astrovirus was actually the cause of this persistent condition or simply secondary to it, nor was astrovirus linked to clinical severity. There have been few reports of any long-term consequences of astrovirus infection; however, short-term lactose intolerance and occasionally cows' milk intolerance lasting several months have been reported (Nazer et al. 1982; Kurtz and Lee 1987).

In other mammals, it is also generally the young of the species that show symptomatic infection and again diarrhea is the predominant symptom. These include lambs (Snodgrass and Gray 1977), calves (Woode and Bridger 1978; Woode et al. 1984), pigs (Shirai et al. 1985; Shimizu et al. 1990), mink (Englund et al. 2002), and cats (Hoshino et al. 1981; Harbour et al. 1987). The virus is clearly responsible for illness in the sheep and in cats it was severe enough to require hospitalization. However, in pigs and calves it appears relatively mild and the bulk of any symptoms observed may be due to coinfection by other agents.

Avian astroviruses

In birds, the range of symptoms produced by astrovirus is more diverse and generally more severe than in mammals. Astrovirus infection (DHV II) causes a rapidly fatal hepatitis in ducklings (Gough et al. 1984). Mortality rates can exceed 25 percent and have been reported up to 70 percent. Symptoms usually appear in 3–6-week-old birds. Examination revealed that the ducklings died of acute hepatitis with hepatocellular necrosis and generally, hyperplasia of the bile duct. Astrovirus-like particles were found by EM in the liver and feces and experimental transmission of the virus resulted in hepatitis and death within 2–4 days in five of 20 ducklings inoculated. Thus far the UK is the only reported country in which DHV II has struck and the origin of the virus remains unknown but is believed to result from contact with wild birds. The virus caused no symptoms in mature ducks, suggesting that adult birds might harbor the infection.

An avian astrovirus was first reported as a cause of gastroenteritis and mortality in turkey poults (6–11 days of age) in 1980 (McNulty et al. 1980). Since then there have been other reports of astrovirus outbreaks in turkeys resulting in enteritis and stunting of growth (Reynolds and Saif 1986). These features have been

linked to a temporary reduction in maltase activity in the small intestine, resulting in disaccharide maldigestion (Thouvenelle et al. 1995). More recently TAstV has been implicated as the causative agent of poult enteritis mortality syndrome (PEMS) in turkeys. This emerging disease is characterized by enteritis, growth depression, lymphoid atrophy, and, interestingly, immunosuppression (Koci et al. 2000a). The virus can be detected in the intestines, thymus, and bursa of infected birds. The virus can reduce macrophage viability and phagocytosis in vitro, thus leading to increased susceptibility of infected turkeys to secondary bacterial infections (Qureshi et al. 2001).

ANV infection of young chickens results in growth retardation of the birds by causing acute interstitial nephritis (Imada et al. 1979). The virus has been shown to be widely distributed in chickens, and turkeys may also be susceptible (Imada et al. 1980). Field strains of the virus show vastly different degrees of pathogenicity ranging from subclinical infections to death (Frazier et al. 1990; Shirai et al. 1991).

PATHOGENESIS

HAstV pathogenesis has not been extensively studied; duodenal biopsy taken during infection showed that the virus had infected the epithelial cells of the lower part of the intestinal villi (Phillips et al. 1982). In lambs experimentally infected with OAstV, lesions were seen throughout the small intestine, especially in the midgut and ileum (Snodgrass et al. 1979). The virus was found to replicate in the mature columnar epithelial cells situated in the apical two-thirds of the villi (virus was detected in the cytoplasm of these cells by EM). The damaged cells were replaced by immature crypt cells – resulting in villus atrophy and malabsorption typical of virus-induced enteric illness (Gray et al. 1980). By contrast, BAstV infection was confined to the specialized M cells that overlay Peyer's patches in the jejunum and ileum of the calves; these infected cells were then shed and replaced by immature crypt cells (Woode et al. 1984). It has been suggested that the limited number of susceptible cells in the bovine gut compared to that in the sheep could explain the lack of symptoms in experimentally infected calves (Kurtz and Lee 1987).

Little is known of immunity to astroviruses. Certainly antibody is developed early in life but Koopmans and colleagues (1998) suggest that little heterotypic protection may result and cross-neutralization activity was not detected in those serotypes where it was sought. HLA-restricted $CD4^+$ T cells specific for astrovirus have been identified in the duodenal mucosa of eight of eight normal (asymptomatic) individuals and these were thought to be of the Th1 phenotype. The fact that astrovirus-specific T cells can be isolated from normal small intestinal biopsies strongly suggests that the precursor frequency of virus-specific T cells in the mucosa is likely to be higher than in peripheral blood. This could suggest that the gut immune system frequently encounters these viruses, regularly reactivating any T cells to proliferate. The presence of these cells might then promote immunity to reinfection (Molberg et al. 1998). All the T-cell clones tested recognized a recombinant HAstV-1 capsid protein, demonstrating that the T-cell epitopes concerned must reside in that protein. The extent to which this cellular immunity is heterotypic has not been determined (O. Molberg, personal communication).

DETECTION AND DIAGNOSIS

Electron microscopy

Astroviruses were originally detected, and for many years could only be diagnosed by EM. This has almost certainly resulted in a reduced efficiency of diagnosis since the technique relies on specimens that contain high levels of virus particles exhibiting good morphological preservation. Astroviruses may be shed in large numbers during infection (up to 10^{10} particles/g feces; Kurtz and Lee 1987), but this is by no means always the case; particles may be hard to find and their preservation may not be good. It has been found that the virions are unstable if pelleted from cesium chloride gradients and are better purified using potassium tartrate (Ashley and Caul 1982). Astrovirus morphology may itself be inherently variable (see section on Morphology, above). Although a typical astrovirus can be safely identified as such, specimens lacking these features cannot readily be discounted. If stars are not visible, a featureless particle would be observed and these can easily be mistaken for parvovirus-like agents. Reanalysis by culture and blotting of a panel of EM-identified parvoviruses revealed the majority to be astrovirus (Willcocks et al. 1991).

Oliver and Phillips (1988) carried out retrospective analyses of samples that had originally been classified on the basis of their appearance in the electron microscope and showed that several samples (14 of 53 re-examined) originally identified as 'small round' or 'parvovirus-like' viruses were in fact astroviruses. This particular confusion is significant since some laboratories have reported the small round virus (parvovirus-like) agents as the second most frequently identified virus (Lew et al. 1990). Alternatively, particles may be larger than expected, (possibly coated in antibody) and the margins of the particle might not be smooth. Indeed, Marin County virus was initially identified as a calicivirus due to these affects (Oshiro et al. 1981). These variations in structural parameters have undoubtedly clouded attempts to assess the significance of astroviruses in disease. The recent large-scale survey of infectious intestinal disease in the UK has identified astroviruses by EM, using ELISA methods only to confirm those found as positive by this

means. Presumptive 'parvoviruses' have not been further examined. This is likely to underestimate the occurrence of astrovirus and this survey has yielded incidence figures similar to other EM-based surveys rather than the higher values usually associated with ELISA methods. One major advantage of EM, however, is that it is a 'catch-all' diagnostic tool, allowing any other potential pathogens in the specimen to also be detected. A survey in London, UK found that of 28 infants infected with astrovirus 16 were also infected with another enteric pathogen (Nazer et al. 1982). In patients shedding fewer particles, immune electron microscopy has proved a valuable tool (Berthiaume et al. 1981).

Cell culture

It was initially difficult to establish cell culture methods for astroviruses, and to date only the porcine and human strains can be cultured in vitro. Both were originally propagated in renal cells of the host species (Lee and Kurtz 1981; Shimizu et al. 1990). The viruses were only able to grow with the addition of trypsin (at 5–10 μg/ml) to the culture medium. In the absence of trypsin, infection could occur but did not give rise to infectious progeny (Lee and Kurtz 1981). This trypsin level caused disruption of the cell monolayer and no cytopathic effect was apparent. However, Hudson and colleagues (1989) did succeed in developing both a plaque assay for three of the serotypes (again with the addition of trypsin to the overlay), and a virus neutralization assay. PAstV required the addition of 0.5 μg trypsin to grow and limited cytopathic effect was visible in the porcine embryonic kidney cells (Shimizu et al. 1990).

Since these experiments, three human cell lines have been identified that are able to support the replication of HAstV directly from clinical samples. Two of these (CaCo-2 and T84) are gut-cell lines and one (PLC/PRF/5) is derived from a hepatoma.

Willcocks and colleagues (1990) found that a human colonic carcinoma cell line (CaCo-2 cells) could be infected with HAstV. Trypsin was required (at 5 μg/ml) for productive growth and at this concentration these cell monolayers remained relatively intact. Cytopathic effect became apparent from 2 days post-infection. These cells also supported the partial replication of FAstV in that infections could be established using viruses from feline diarrheal feces; mRNA and protein synthesis occurred and progeny particles were released. However these were found to be non-infectious and infectivity could not be rescued by addition of trypsin.

Taylor and colleagues (1997) found that a human hepatoma cell line (PLC/PRF/5) was also able to support the replication of human astroviruses directly from clinical samples. Trypsin was required (10 μg/ml) and the disruption caused by this to the cell monolayers did not permit detection of any cytopathic effect. Virus growth was monitored by EM or indirect immunofluorescence. In addition to these cells, the Oxford adapted astrovirus strains have a wider host cell range (Brinker et al. 2000).

Although cell culture is essential for isolating viruses from clinical samples, it is not generally the method of choice for diagnostic purposes because it is labor intensive, slow, expensive, and less sensitive than immunologically based or PCR-based assays. It has, however, been used successfully to enhance the detection level of RT-PCR for samples where virus particles should be highly dilute, e.g. drinking water. Concentrated samples were propagated in CaCo-2 cells before analysis by RT-PCR (Abad et al. 1997). A similar approach has been used in environmental water samples (where propagation in PLC/PRF/5 cells was employed; Marx et al. 1995) or in sludge biosolids (where integrated cell culture/RT-PCR was coupled with nested PCR; Chapron et al. 2000).

Enzyme immunoassays

Herrmann and co-workers (1988) developed an enzyme immunoassay (EIA) for astrovirus, using a monoclonal antibody to the group antigen to capture virus; this detected all astroviruses grown in cell culture. These workers later modified the test to detect astroviruses in stool samples (Herrmann et al. 1990). Compared with immune electron microscopy, this assay has as high a sensitivity (91 percent) and specificity (98 percent). Moe and colleagues (1991) developed a modified version of the assay using a biotinylated detector antibody. These assays have been particularly useful in epidemiologic studies, where large numbers of samples need to be assayed (Herrmann et al. 1991; Lew et al. 1991; Moe et al. 1991; Cruz et al. 1992; Walter et al. 2001b). A latex agglutination test specific for HAstV-1 has also been reported for this application (Kohno et al. 2000).

Modifications to these tests using recombinant antigen have also allowed the development of ELISA or immune fluorescence-based methods to detect serum antibody and thus determine the prevalence of antibodies to astroviruses in various communities. When used in this way, baculovirus-expressed antigen reacts in a serotype-specific manner, allowing antibody to each serotype to be assessed independently (Kriston et al. 1996; Mitchell et al. 1999a).

Molecular methods

A dot-blot hybridization procedure for detecting astroviruses in fecal samples has been described (Willcocks et al. 1991) but has only been used in a small-scale study (Silcock et al. 1993). A liquid hybridization assay was successfully tested on 26 wild strains of the virus (Belliot et al. 2001). As sequence data has accumulated a number of RT-PCR tests have been developed (Monroe et al. 1993; Jonassen et al. 1995; Noel et al. 1995; Yue

and Ushijima 1996; Sakamoto et al. 2000; Traore et al. 2000). These have facilitated the rapid diagnosis of human astrovirus outbreaks (Cubitt et al. 1999). Oligonucleotide primers designed to match conserved sequences can detect all serotypes. Primer pairs have been reported targeting the 3′ end of the genome (Monroe et al. 1993; Jonassen et al. 1995), the RNA-dependent RNA polymerase gene (Belliot et al. 1997b) and from the 5′ end of the capsid gene (Sakon et al. 2000). RT-PCR has also been used for genotyping of human astroviruses by sequencing of a 348-bp amplimer from the capsid gene (Noel et al. 1995). However, this method has epidemiologic rather than diagnostic applicability. An RT-PCR has also been reported for the detection of TAstV in PEMS-affected commercial turkey flocks (Koci et al. 2000b).

The technique has also been useful in confirming results with specimens that gave ambiguous results in other tests (Grohmann et al. 1993) and has been able to provide more detail on outbreaks. Mitchell and colleagues (1995) used EIA and RT-PCR to screen fecal specimens in an outbreak of gastroenteritis in a child-care center. EIA found that stools from 75 percent of the infants, 50 percent of the toddlers, and 20 percent of the children were positive, whilst using RT-PCR increased the numbers to 100, 79, and 90 percent, respectively. This changed the interpretation of the outbreak; the virus had not selectively infected some of the children but had in fact infected almost all of them. The number of days of virus shedding was also found to be longer using this more sensitive method and increased from 1 to 9 days (as detected by EIA) to up to 35 days (as detected by PCR).

CONCLUSIONS

Astroviruses are becoming increasingly appreciated as agents of human disease to which we are regularly exposed from each other and the environment, and possibly from animals. Infections within institutional settings, often affecting those at particular risk, pose a particular problem. The significance of these agents as the second most frequent cause of gastroenteritis in children worldwide, and their possible association with prolonged illness in the third world, demands attention. Rotavirus remains the single most significant cause of childhood diarrhea and rightly receives the attention required to develop vaccines. However, control of rotavirus infection does not control gastroenteritis; vaccinees would remain susceptible to astrovirus infection and this could lead to a perceived lack of vaccine efficacy that could restrict vaccine uptake.

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Human enteric RNA viruses: Noroviruses and Sapoviruses

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HISTORICAL PERSPECTIVES

Diarrheal disease in humans, clinically distinct from bacterial gastroenteritis, was first reported in the southern USA. It was originally described as 'hyperemesis hiemis' or winter vomiting disease (Zahorsky 1929), a term that reflects its seasonality. The illness occurred in widespread epidemics with a high secondary attack rate, and was documented as a short, self-limiting episode of vomiting with some diarrhea. Later, this syndrome was also recognized by clinicians in the UK (Bradley 1943; Gray 1939; Miller and Raven 1936). Its distinction from bacterial gastroenteritis was confirmed by investigations of institutional outbreaks in which stool cultures were consistently negative for pathogenic bacteria.

Between the 1940s and the early 1970s, a variety of synonymous terms were used for this syndrome, including nonbacterial gastroenteritis, epidemic gastroenteritis, acute infectious nonbacterial gastroenteritis, and winter vomiting disease. The transmissibility of an agent by oral inoculation of human volunteers with a fecal filtrate was repeatedly demonstrated (Adler and Zickl 1969; Clarke et al. 1972; Dolin et al. 1971; Gordon et al. 1947; Jordan et al. 1953; Reimann et al. 1945). This approach established the possibility of a viral etiology, and although the natural history of this transmissible agent was comprehensively described, all attempts to isolate a bacterial pathogen or a viral agent failed.

An outbreak of epidemic gastroenteritis in a primary school in Norwalk, Ohio, USA led to confirmation of the viral etiology of winter vomiting disease (Kapikian et al. 1972). Fecal filtrates from affected children were fed to volunteers, whose stools were examined by immunoelectron microscopy. A previously undescribed virus-like particle, 27 nm in diameter, was detected and subsequently named Norwalk virus. Further studies in volunteers established Norwalk virus as the agent responsible for the original outbreak. Seroconversions or rising IgG titers, as well as virus excretion, were demonstrated in infected volunteers, and the histopathology of jejunal biopsies from those with acute disease gave new insights into the cell tropism of Norwalk virus in the small intestine (Agus et al. 1973; Schreiber et al. 1973). Further volunteer studies by American workers identified viruses morphologically similar to the Norwalk virus in two outbreaks of nonbacterial gastroenteritis. These viruses were termed Montgomery County and Hawaii, respectively, after the locations of the outbreaks. This mode of nomenclature became common (Caul 1988) and laboratories continue to name strains after the location of isolation.

Soon after the discovery of the Norwalk virus, morphologically indistinguishable viruses from clinically similar outbreaks were reported. The agents responsible for these virologically characterized outbreaks were termed Hawaii (Thornhill et al. 1977; Wyatt et al. 1974), Snow Mountain (Dolin et al. 1982; Morens et al. 1979) and Taunton (Caul et al. 1979; Pether and Caul 1983).

Volunteer studies were central to the understanding of the transmissibility, pathogenesis, and immunobiology

of the Norwalk and Hawaii viruses (Parrino et al. 1977; Schreiber et al. 1973; 1974; Wyatt et al. 1974) and established a previously undescribed and poorly characterized group of related viruses as an important cause of epidemic gastroenteritis. The Norwalk-like viruses cause acute, explosive diarrhea, vomiting, or both, and are highly infective, with rapid secondary spread. Large outbreaks have been described in semiclosed communities throughout the world as a result of person-to-person spread (Caul 1988; Christensen 1989; Kaplan et al. 1982; Pether and Caul 1983). Point-source outbreaks resulting from the ingestion of sewage-contaminated water, contaminated shellfish or food (Morens et al. 1979; Murphy et al. 1982) emphasize the great public health importance of these viruses. There are major problems in controlling such outbreaks, and the economic implications, including those applicable to the food and shellfish industries, are being increasingly recognized.

The widespread application of electron microscopy (EM) to the examination of diarrheal stool samples resulted in major advances in our understanding of the etiology of nonbacterial gastroenteritis. Rotaviruses were rapidly identified as major pathogens in endemic infantile gastroenteritis, and other viruses, including 'fastidious' adenoviruses, were added to the list of enteric 'diarrhea' viruses. In addition to these distinctive viruses there were many reports of an apparently wide range of small-round viruses (SRV) approximately 30 nm in diameter. In many studies SRVs were identified by immunoelectron microscopy (IEM), which masks surface morphology, and often resulted in a confusing picture of the etiology of nonbacterial gastroenteritis. Other SRVs were identified from their classic surface structure, and detailed descriptions of the morphology of the newly described astroviruses and 'classic' caliciviruses were published (Madeley 1979).

In 1982 an 'interim classification scheme' was presented (Caul and Appleton 1982) as an to aid national surveillance within the UK and to establish which SRVs were causally related to viral gastroenteritis. The authors concluded that the causative agents of diarrheal disease were the SRVs that possessed surface morphology to which descriptive terms could be applied. These viruses included the previously described astroviruses, 'classic' caliciviruses, and a third group of viruses that shared the features of an amorphous surface structure and 'ragged'-edged virions. This third group was represented by the prototype Norwalk virus, and within the interim scheme were termed small-round structured viruses (SRSV). A prerequisite for classification under this scheme was that all viruses were examined in the absence of antibody to ensure that any surface structure would not be obscured. SRSVs emerged as the most important cause of epidemic nonbacterial gastroenteritis worldwide, with viruses of this group causing similar illnesses.

This morphological classification scheme recognized fundamental differences between astroviruses, the classic caliciviruses, and SRSVs, and was supported by epidemiological and immunological distinctions (Blacklow and Greenberg 1991; Caul 1988). The molecular characterization of these three different viruses showed that they contained positive-sense single-stranded RNA genomes. The genome structure of astroviruses, however, is very different from that of the SRSVs and 'classic' caliciviruses, and so the astroviruses have been assigned to a separate virus family and are discussed in Human enteric RNA viruses: Astroviruses. SRSVs and the 'classic' caliciviruses both belong to two separate genera within the *Caliciviridae*, which from 1998 to 2002 were temporarily called Norwalk-like viruses (NLV) and Sapporo-like viruses (SLV), respectively. The ICTV has now settled on a new nomenclature for these genera, namely Noroviruses and Sapoviruses, reflecting the place of discovery of each of the prototypes. The clinical, epidemiological and pathological aspects of Noroviruses and Sapoviruses are summarized (Table 1).

On the basis of sequence analysis and genome organization, human enteric caliciviruses have been assigned to two separate genera within the *Caliciviridae* (Jiang et al. 1993; Lambden et al. 1993; Dingle et al. 1995). The SRSVs or Norwalk-like viruses belong to the *Norovirus* genus and the Sapporo-like or 'classic' caliciviruses are members of the *Sapovirus* genus. Two other genera are included in the family and contain viruses pathogenic for animals. The *Vesivirus* genus includes *Feline calicivirus* and *Vesicular exanthema of swine virus*. The fourth genus, *Lagovirus*, is represented by the *Rabbit hemorrhagic disease virus* (RHDV) and the closely related *European brown hare syndrome virus* (EBHSV).

NOROVIRUSES

Classification and morphology

The prototypic Norwalk virus was originally described as non-enveloped, round, 27-nm particles with a 'ragged' outer edge but lacking a definite surface structure (Kapikian et al. 1972). The low titer of virions found in stool samples, together with their small size and amorphous structure, has made them difficult to detect by direct EM. Many other Noroviruses have been described and have in common a buoyant density of 1.33–1.41 g/cm^3, an inability to propagate in vitro, and an RNA genome of positive polarity (Greenberg and Matsui 1992).

Characteristically, Noroviruses possess a single capsid protein (Greenberg et al. 1981), but lack of a cell-culture system has restricted the production of capsid antigen to recombinant heterologous expression systems. Capsid proteins expressed in insect cells using

Table 1 *The clinical, epidemiological and pathological aspects of Noroviruses and Sapoviruses*

Appearance	Genome structure	Clinical features	Examples
Noroviruses			
Feathery, ragged outline No distinctive surface structure Some preparations resemble classic calicivirus No cell-culture system	ssRNA, +ve polarity, polyadenylated, ca. 7.7 kb group I and 7.5 kb group II ORF1 nonstructural polyprotein ORF2 capsid ORF3 small basic protein Possible subgenomic RNA	Winter vomiting disease Epidemic nausea vomiting and diarrhea Acquisition of antibodies is gradual, vomiters are more likely to seroconvert Peak illness in adults No immunity Clinical features identical for group I and group II viruses	**Group I:** *Norwalk virus*: (school outbreak, USA 1969) Southampton virus (family outbreak, UK 1991) **Group II:** Hawaii virus: (family outbreak, USA 1972) Lordsdale virus (hospital outbreak, UK 1993)
Sapoviruses Distinctive cup-shaped surface depressions, giving classic 'Star of David' morphology No cell-culture system	ssRNA, +ve polarity, polyadenylated, ca. 7.3 kb ORF1 nonstructural polyprotein contiguous with and fused to capsid gene ORF2 small basic protein Subgenomic RNA and VPg present in animal viru	No seasonal variance Predominantly pediatric illness Antibodies acquired at young age Diarrheal illness usually mild	**Group I:** *Sapporo virus*: outbreak in an orphanage Sapporo and Japan, 1982 Manchester virus: sporadic case of acute vomiting and diarrhea in a 6-month-old child (1993) **Group II:** Bristol virus: sporadic case 15-month -old child (1998) London virus: 8-month-old infant hospitalized for diarrhea and vomiting (1992)

baculovirus vectors spontaneously assemble to form virus-like particles and are released into the cell culture supernatant (Dingle et al. 1995; Jiang et al. 1992; Jiang et al. 1995b). These virus-like particles are larger in diameter and have the appearance of empty virions (Figure 1).

Cryo-electron microscopy and computer image processing techniques have determined the empty virion structure to a resolution of 2.2 nm (Rothnagel et al. 1994) (Figure 2). The empty capsids are composed of 90 dimers of the capsid structural protein and are 38 nm in diameter, with T = 3 icosahedral symmetry. Archlike capsomers formed from dimers of the capsid structural protein and deep depressions at the five- and three-fold axes are distinctive features. Each virus-like particle has 32 surface hollows surrounded by the protruding arches. Twelve hollows are at the icosahedral fivefold axis and have a small hump at the center. The other 20 hollows at the three-fold positions seem to be flat. This distinctive molecular architecture has also been described for a primate calicivirus (Prasad et al. 1994).

The structure of recombinant Norwalk virus capsids at near-atomic resolution has been determined by X-ray crystallography (Prasad et al. 1999, 2000). The structure of the capsid protein is organized into two domains joined by a flexible hinge (Figure 3). The inner shell (S) domain is composed of the N-terminal 225 residues and is involved in the formation of the icosahedral capsid shell. The shell domain also shows the classic eight-stranded β barrel structure typical of many viral capsid proteins. The protruding (P) domain forms prominent structures extending from the surface of the shell, and is formed from the C-terminal half of the protein. The P domain is further organized into two subdomains (P1 and P2), and it has been suggested that these structures may be involved in binding to cellular receptors and may also be the determinants of strain specificity (Prasad et al. 1999). Mutational analysis of the critical residues involved in VLP formation (Bertolotti-Ciarlet et al. 2002) has shown that complete removal of the P domain resulted in the formation of smooth particles with a diameter slightly less than that of the complete rNV VLPs. These studies demonstrated that the shell domain alone of the capsid protein was able to initiate spontaneous assembly into particles, but the P domain was required for particle stability.

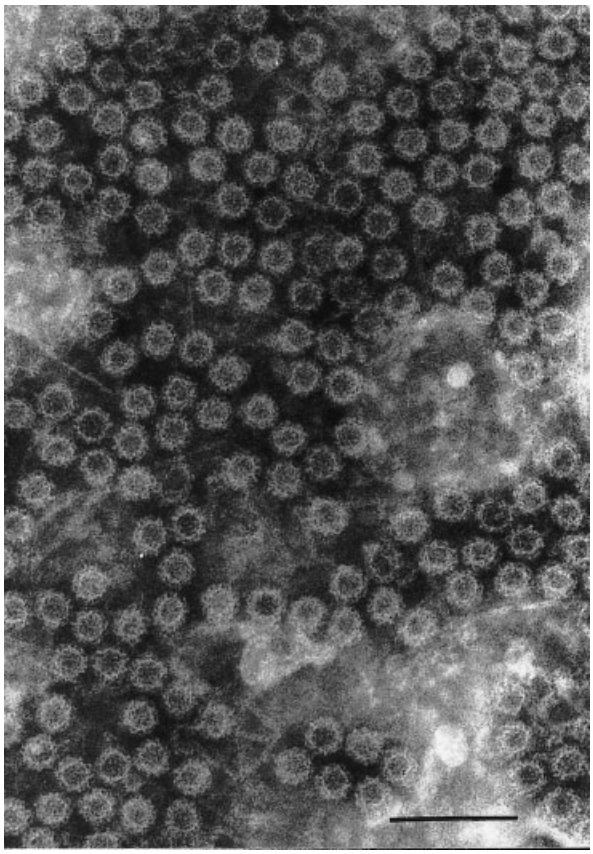

Figure 1 *Electron micrograph showing recombinant virus-like particles produced by expression of Southampton virus capsid protein from insect cells. Bar, 100 nm.*

Genome organization

Since the first reports of the molecular cloning of the Norwalk virus genome in 1990, rapid progress has been made in the genome analysis of Noroviruses. Virus purified from feces of volunteers infected with Norwalk virus was used to generate a library of cDNA clones representing most of the viral genome. Authentic clones were identified by hybridization to post- but not preinfection stool nucleic acid. Sequence information indicated that the Norwalk virus genome is single-stranded positive-sense RNA of about 7.5 kb and polyadenylated at the 3′ terminus (Jiang et al. 1990). In one of the viral clones a motif characteristic of viral RNA-dependent RNA polymerases was identified. Matsui and colleagues (Matsui et al. 1991) then cloned a region of the Norwalk virus genome that was immunoreactive when expressed in recombinant λ phage. Following these early studies a number of complete genome sequences of human Noroviruses (Dingle et al. 1995; Green et al. 2002; Hardy and Estes 1996; Jiang et al. 1993; Katayama et al. 2002; Lambden et al. 1993; Lambden et al. 1995; Pletneva et al. 2001; Schreier et al. 2000; Seah et al. 1999; Someya et al. 2000) have been determined, together with numerous partial sequences covering the RNA polymerase and capsid regions in the 3′ half of the genome.

Phylogenetic analyses of the RNA polymerase and capsid regions of the genome indicate that human Noroviruses are divided into two genetic groups (Ando et al. 1994; Cubitt et al. 1994; Green et al. 1994; Wang et al. 1994). The genome of group I viruses is slightly larger (7.7 kb) than the group II genome (7.5 kb), although computer analyses reveal that the reading frame usage of these two genetic groups is very similar. The genomes of both group I and group II Noroviruses (Figure 4) are characterized by a large 5′ open reading frame (ORF) encoding a nonstructural polyprotein of approximately 1 700 amino acids, followed by a smaller ORF encoding the capsid structural protein.

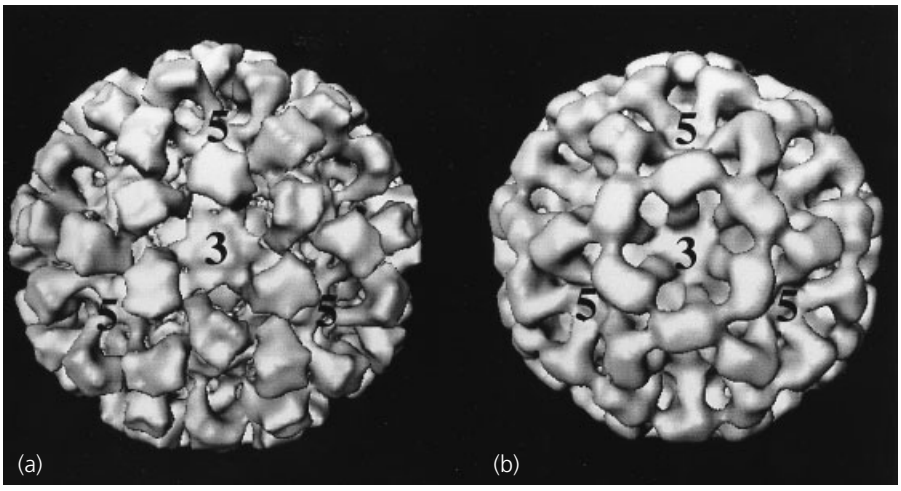

Figure 2 *Computer-generated images from cryo-electron microscopic preparations of* **(a)** *recombinant Norwalk virus capsids and* **(b)** *primate calicivirus. The images are viewed along the threefold axes. (Photograph courtesy of Dr B.V.V. Prasad, Baylor College of Medicine, Houston TX, USA.)*

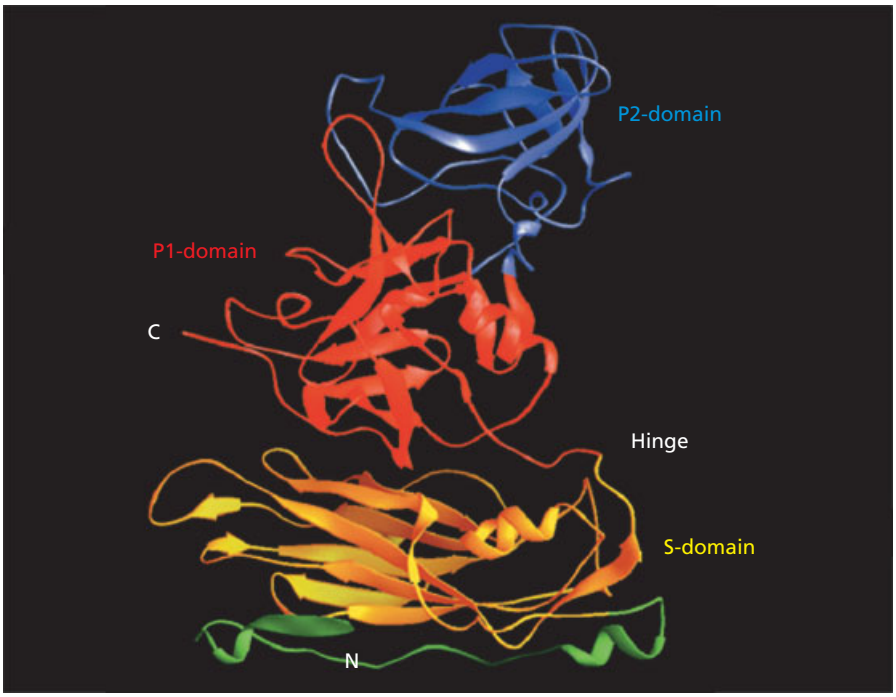

Figure 3 *Ribbon representation showing the structure of recombinant Norwalk virus capsid protein. The N-terminal arm, S domain, and P1/P2 subdomains are colored green, yellow, red, and blue, respectively. The N and C termini, together with the hinge region between the shell and protruding domains, are indicated. (Image courtesy of Dr B.V.V. Prasad, Baylor College of Medicine, Houston TX, USA.)*

At the 3′ end of the genome is a small ORF, predicted by computer analysis to encode a basic protein. This genome organization clearly distinguishes Noroviruses from other positive-strand RNA viruses, such as the picornaviruses.

The smaller size of the group II *Norovirus* genome is attributable to its smaller ORF1. The nucleotide (nt) sequence in the 5′ region of ORF1 shows significant diversity between the two genetic groups, although distal to this variable region both groups show the characteristic motifs of the NTPase, cysteine protease, and RNA polymerase in the same relative genomic positions. Sequence diversity at the 5′ end of the genome may reflect fundamental differences between the two groups of viruses in terms of secondary structures or regulatory signals. The first 110 nucleotides of the Norwalk virus genome have been shown to form stable complexes with several cellular proteins from HeLa and CaCo cell extracts (Gutierrez-Escolano et al. 2000). The proteins La, PCBP-2, PTB, and hnRNPL, which are required for translation in poliovirus and hepatitis C virus, may also be essential for translation of caliciviral RNA.

The capsid coding region in the *Norovirus* genome is similar to the arrangement in feline calicivirus (FCV), in which ORF2 is frame-shifted relative to ORF1 so that the N terminus of the capsid protein overlaps the C terminus of the putative RNA-dependent RNA polymerase. However, the extent of reading frame overlap differs between the two genetic groups of Noroviruses, being 17 nt in group I and 20 nt in group II. The small 3′ ORF is also in a different reading frame from the capsid-encoding ORF2. The first residue of the initiator codon of ORF3 overlaps the last base of the terminator codon of the capsid gene. A characteristic feature of the animal caliciviruses is the production of a 3′ co-terminal polyadenylated subgenomic RNA coding for the capsid and small 3′ ORF. This subgenomic RNA is thought to provide an additional message for the production of capsid protein, and in the case of RHDV this additional RNA is packaged into mature virions (Meyers et al. 1991). It is not yet clear whether Noroviruses produce a subgenomic message, although detection of RNA of over 2 kb by Northern blot analysis of total stool RNA from a volunteer infected with Norwalk virus suggested that this virus also produces a subgenomic RNA (Jiang et al. 1993). A characteristic feature of the animal calicivirus genomic and subgenomic RNAs is that the 5′ termini are highly conserved (Lambden and Clarke 1995). The 5′ terminal sequence of ORF1 is repeated around the 5′ terminal region of ORF2, suggesting that human enteric caliciviruses, like their animal counterparts, also produce a subgenomic RNA. The 5′ genomic and subgenomic termini of both groups I and II Noroviruses begin with the sequence GU, and by analogy with the animal caliciviruses this terminal sequence is linked to the VPg

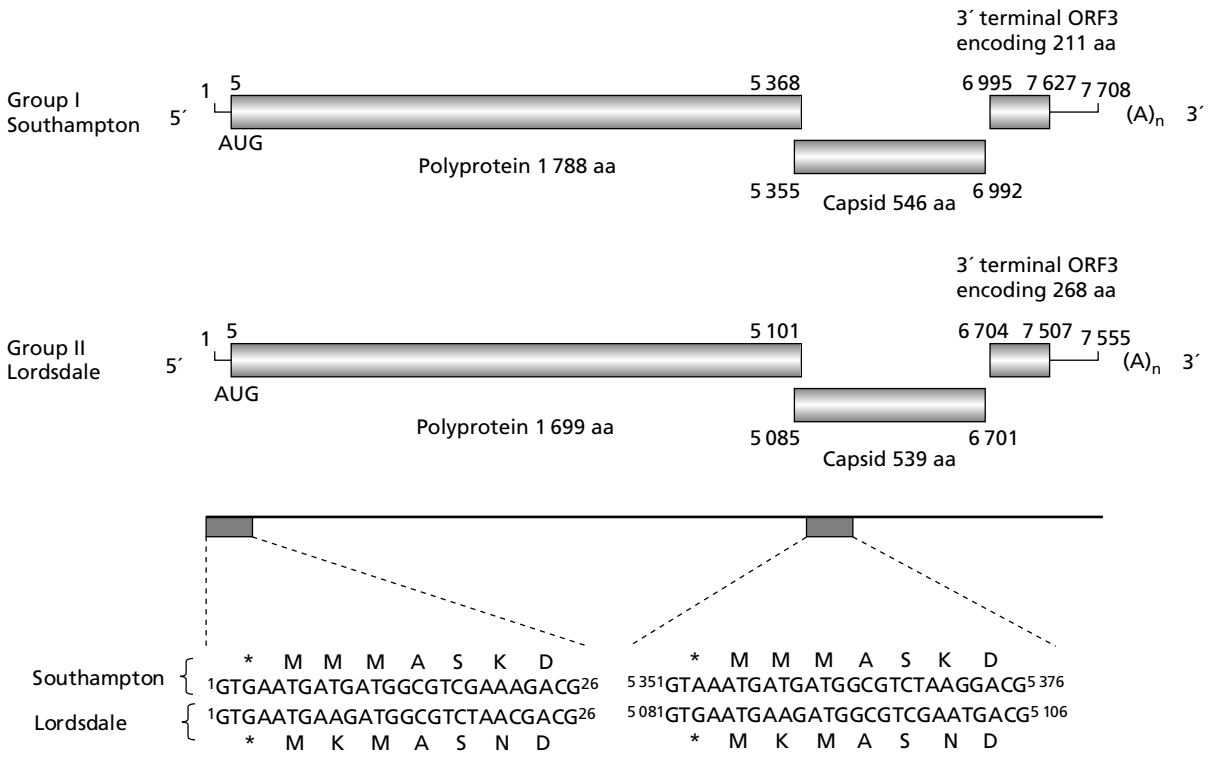

Figure 4 *Comparison of the genome structures of groups I and II Noroviruses. Shaded boxes represent computer-predicted open reading frames. Translation products and their sizes are indicated on the open reading frames. The conserved sequences at the 5′ genomic termini and the predicted 5′ termini of the subgenomic RNAs of groups I and II Noroviruses are aligned beneath the genome maps. Southampton virus (EMBL/GenBank Accession No. L07418); Lordsdale virus (EMBL/GenBank Accession No. X86557).*

protein. The conserved GU residues at the 5′ genomic termini appears to be a common feature of the *Caliciviridae*. The amino acid involved in the linkage between VPg and the RHDV genome has been identified as Tyr-21 (Machin et al. 2001).

Viral proteins

Highly purified Norwalk virus recovered from CsCl equilibrium density gradients contains a single protein of 59 kDa (Greenberg et al. 1981). The single capsid protein was identified by radioimmunoprecipitation of purified virions with acute and convalescent sera from infected volunteers. Similar studies with the antigenically distinct Snow Mountain agent revealed a major structural polypeptide of 62 kDa (Madore et al. 1986). In addition to the 59 kDa capsid protein of Norwalk virus, a soluble protein with a molecular weight of 30 kDa was also detected in the supernatant of fecal suspensions. It has been estimated that up to 50 percent of the excreted antigen is present as soluble protein (Jiang et al. 1992). Until recently the source of the soluble protein remained uncertain, and was suggested to be an immunogenic nonstructural protein or a cleavage product derived from the capsid protein. However, analysis of virus-like particles expressed from insect cells infected with recombinant baculovirus demonstrated that the 30 kDa protein is a specific cleavage product of the viral capsid (Hardy et al. 1995). Recombinant Norwalk virus treated with trypsin released a 32 kDa protein, and analysis of the N-terminal sequence of this product revealed that a trypsin-specific cleavage occurred at amino acid residue 227. The 30 kDa soluble protein reacted with rabbit polyclonal antisera raised against recombinant Norwalk virus capsids, and analysis of the N-terminal sequence of the approximately 30 kDa soluble protein showed it to be identical to the 32 kDa trypsin cleavage product. In addition, it was shown that the 32 kDa proteolytic cleavage product is derived from soluble capsid protein and not intact assembled virions. These data suggest that the Norwalk virus capsid undergoes proteolytic cleavage during the course of infection. Japanese isolates antigenically related to the Hawaii virus also demonstrated immunoreactive structural proteins of 63 and 30 kDa by Western blot analysis (Hayashi et al. 1989; Oishi et al. 1992).

Several groups have described capsid gene sequences from a number of different *Norovirus* isolates that encode proteins with predicted molecular weights of approximately 60 kDa. Computer alignments of the capsid sequence data support the division of Noroviruses

into two major genetic groups. The capsid proteins have approximately 68 percent amino acid sequence identity within a genetic group and 40 percent between groups. However, the *Norovirus* capsids show only limited homology with the corresponding proteins in members of the other genera. A dendrogram showing the phylogenetic relatedness of caliciviruses is shown in Figure 5. The prototype Norwalk virus and the antigenically distinct Southampton virus are both members of genetic group I, together with the Desert Shield virus. A UK isolate, Lordsdale virus, has been assigned to genetic group II, along with Snow Mountain agent, Hawaii virus, Toronto virus, and Mexico virus.

The first major ORF encodes a polyprotein that is presumably processed by a proteolytic cascade analogous to that of picornaviruses. Computer analysis of the ORF1 sequence data has identified conserved amino acid motifs typical of the picornaviral 2C NTPase, 3C protease, and RNA-dependent RNA polymerase. NTPase activity has been demonstrated in recombinant p41of Southampton virus expressed as an *Escherichia coli* fusion protein (Pfister and Wimmer 2001). Sequence comparisons of group I and II Noroviruses reveal, apart from the short conserved sequence motifs, extreme sequence diversity at the 5′ proximal regions of the genomes. In RHDV the genomic and subgenomic RNAs are linked at their 5′ termini to a small protein analogous to picornaviral VPg, although the caliciviral protein (approximately 15 kDa) is considerably larger than the picornaviral equivalent (22 amino acids). The presence of a VPg-like protein, encoded by ORF1 in Noroviruses, is strongly inferred by analogy with the animal caliciviruses.

Although no specific biological function has been assigned to the translation product of ORF3 it has been reported that this protein is present in mature virions (Glass et al. 2000). A role for ORF3 protein as a minor capsid protein has also been reported in vesiviruses (FCV) (Sosnovtsev and Green 2000) and lagoviruses (RHDV) (Wirblich et al. 1996). The ORF3 translation product shows considerable sequence variation both within and between genera, which is surprising if the predicted protein product has the same functional role in all caliciviruses. It has been proposed that this basic protein may play a role in RNA packaging and assembly of the mature virion.

Polyprotein processing

In common with picornaviruses, caliciviral proteins are synthesized as a large polyprotein precursor which is subsequently processed in a proteolytic cascade by the viral 3C-like protease. In vitro transcription and translation of genomic clones does not appear to generate the intact polyprotein, suggesting that proteolytic processing probably occurs cotranslationally. Using in vitro mutagenesis and expression of the active protease in *E. coli*, five cleavage sites have been identified in Southampton *Norovirus* (Figure 6) (Liu et al. 1996; Liu et al. 1999b). Two of the cleavages in Southampton virus are at QG dipeptides and are responsible for the release of the p48 N-terminal protein and p41 NTPase from the polyprotein. Three further cleavages at EA and EG dipeptides generate the p22 (3A), p16 (VPg), p19 (3C protease), and p57 (RNA-dependent RNA polymerase). The preference for a glutamate residue at the P1 position of the cleavage dipeptide is also evident in FCV (Sosnovtsev et al. 1998, 2002) and RHDV (Wirblich et al. 1995; Meyers et al. 2000). The amino acid at the P1′ position of the cleavage dipeptide is more variable, but there appears to be a marked preference for glycine residues. The P4 position N terminus of the scissile bond also appears to be important in efficient processing (Hardy et al. 2002).

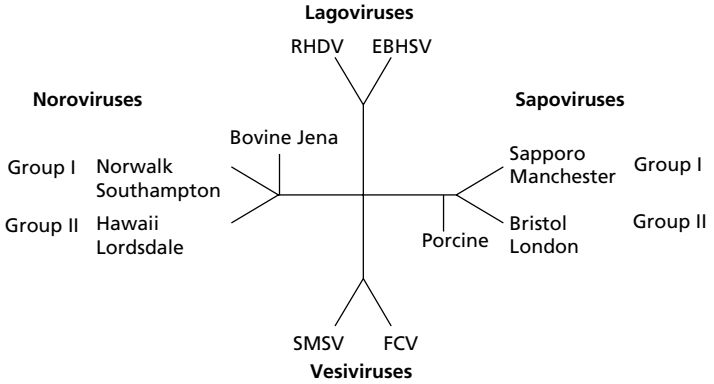

Figure 5 *Diagrammatic representation of the phylogenetic relationship between members of the* Caliciviridae*: adapted from (Robinson et al. 2002). Assignments are based on computer analyses of the complete amino acid sequence of the capsid proteins.* Norovirus *(Southampton, L07418; Norwalk, M87661; Lordsdale, X86557; Hawaii, U07611; Bovine, AJ011099);* Sapovirus *(Sapporo, U65427; Manchester, X86560; Bristol, AJ249939; London, U95645; Porcine, AF182760); FCV (Feline calicivirus, M86379); SMSV (*San Miguel sealion virus*, M87481); RHDV (*Rabbit hemorrhagic disease virus*, M67473); EBHSV (*European brown hare syndrome virus*, Z69620). The EMBL/GenBank accession numbers are shown in parentheses.*

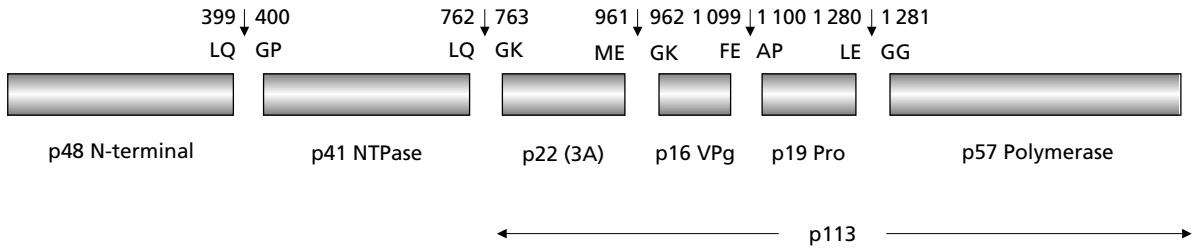

Figure 6 *Proteolytic cleavage map of the Southampton virus polyprotein encoded by ORF1. The shaded boxes represent the mature cleavage products of the nonstructural polyprotein. Cleavage sites are indicated between the boxes, and the amino acid coordinates at the P1/P1′ cleavage dipeptide are given each side of the arrows. The calculated molecular masses and functions of the cleavage products are indicated below the boxes. The large primary cleavage product (p113) observed in in vitro translation reactions extends from amino acid 763 to the carboxy terminus of the 3D polymerase and is indicated by the arrow below the cleavage map.*

Antigenic variation

Several approaches have been used in an attempt to evaluate antigenic relationships between the Noroviruses. Using human volunteers, cross-challenge studies have demonstrated that Norwalk and Hawaii viruses are antigenically distinct (Kapikian 1994), and further volunteer studies with the Montgomery County agent identified a one-way cross-protection with Hawaii virus. There was short-term homologous immunity after challenge of humans with all three viruses (Dolin et al. 1972; Wyatt et al. 1974).

The antigenic relatedness of Norwalk, Hawaii, and Snow Mountain viruses was also studied by testing serological responses in volunteers. Using an enzyme immunoassay (EIA) variable degrees of antigenic relatedness were observed, with a two-way cross-reaction between Snow Mountain and Hawaii virus and a one-way cross-reaction between Norwalk and Snow Mountain viruses (Madore et al. 1990). These results may reflect heterologous responses and contrast with the greater specificity of IEM or solid-phase immunoelectron microscopy (SPIEM), which is based on the use of intact virions (from fecal specimens) and convalescent sera.

Four antigenic types of *Norovirus* were originally described in the UK, identified by IEM or SPIEM (Lambden et al. 1993; Lewis 1991; Lewis 1990), six in the USA (Lewis et al. 1995) and nine in Japan (Okada et al. 1990). However, all the above methods suffered from the same technical drawbacks of poorly defined and limited reagents, which meant it was not possible to compare the differently defined antigenic 'types'. Although these were useful methods in the early days of research they have largely been replaced by modern molecular methods.

A definitive approach for serotyping Noroviruses based on neutralization is not possible because there is no cell-culture system. The ability to express the capsid protein in insect cells where it assembles to form virus-like particles, first demonstrated for Norwalk virus (Jiang et al. 1992), was a significant advance that has provided a renewable source of capsid antigen for many different strains. Apart from their use in structural studies, purified VLPs have been used to raise polyclonal and monoclonal antisera for the development of new diagnostic reagents. Several antigen capture EIAs have been developed using polyclonal antisera to VLPs from different animal species. Evaluation of these assays has shown that they are highly *Norovirus* strain specific; thus EIAs for Norwalk or Mexico virus will detect only homologous antigens (Graham et al. 1994; Jiang et al. 1995a). Phylogenetic analysis of capsid sequences suggests that the clustering of strains that show high levels of sequence conservation is also reflected by immunological relationships defined by EIA. However, some caution must be exercised in interpreting the meaning of these observations, as immunological relationships defined by EIA may still mean that viruses are not serotypically related as defined by virus neutralization.

Clinical manifestations

Detailed information on the clinical, immunological, and pathological aspects of Noroviruses have been established from investigations on volunteers (Kapikian 1994), in whom infections by Norwalk virus, Hawaii agent, Montgomery County agent, and the Snow Mountain agent are clinically indistinguishable (Morens et al. 1979; Wyatt et al. 1974). Following transmission by the fecal–oral route, volunteer and epidemiological studies have established an incubation period of approximately 24 h (Kapikian 1994), with a dose-dependent range of 18–48 h (Blacklow and Herrmann 1988). Symptoms are sudden in onset and are accompanied by nausea and vomiting, which can be projectile and severe. Low-grade fever and diarrhea usually occur, the latter being relatively mild. In contrast to bacterial gastroenteritis, diarrheal stools do not contain blood, mucus, or white cells. Other symptoms include mild abdominal pain or cramps, malaise, and headaches. Vomiting may arise from a decrease in gastric motility (Meeroff et al. 1980), giving rise to a reflux action into the stomach. Respiratory

involvement has not been observed, and nasopharyngeal washings from an acutely ill volunteer did not induce disease on passage to other volunteers (Dolin et al. 1972).

In general, *Norovirus* infections are self-limiting and affected patients rarely need to be hospitalized. There have, however, been occasional reports of severe dehydration that required the administration of intravenous fluids. Deaths associated with *Norovirus* infections are exceptionally rare and have not been directly attributed to this group of viruses (Kaplan et al. 1982).

Pathogenesis

Volunteer studies show that as few as 10–100 infectious particles may be needed to initiate infection. Replication is considered to occur in the mucosal epithelium of the small intestine, although direct evidence is lacking. Light microscopy of jejunal biopsies from volunteers infected with Norwalk virus showed partial flattening and broadening of the villi, with disorganization of the mucosal epithelium (Agus et al. 1973; Schreiber et al. 1973). The lamina propria was infiltrated with mononuclear cells and vacuolization of mucosal epithelium was noted. Crypt cell hyperplasia was common, and, at the ultrastructural level, mucosal epithelial cells showed dilatation of the rough and smooth endoplasmic reticulum, with an increase in multivesiculate bodies. Microvilli were significantly shortened, and amorphous electron-dense material was present in the expanded intercellular spaces. The absence of Noroviruses in damaged mucosal cells was noteworthy. The results of light and electron microscopy in volunteers infected with the Hawaii agent were similar, and, again, virus particles were not identified in biopsies (Dolin et al. 1975; Schreiber et al. 1974). In general, *Norovirus* infections seem to cause mild atrophy of the villi of the small intestine, assumed to arise from limited virus replication that damages the mucosal cells. The appearance of mucosal lesions was paralleled by a decrease in brush border enzymes, which returned to normal values during convalescence (Agus et al. 1973).

Epidemiology

Noroviruses are now established as the most important cause of epidemic nonbacterial outbreaks of gastroenteritis worldwide. National surveillance and diagnosis by EM of outbreaks of nonbacterial gastroenteritis in the UK have shown that Noroviruses are a more common cause of infective gastroenteritis than *Salmonella* or *Campylobacter* (Food Standards Agency 2000). The infectious intestinal disease study conducted in England and Wales between August 1993 and January 1996 suggested that at least 600 000 cases of gastroenteritis annually could be attributed to *Norovirus* infections (Food Standards Agency 2000). This figure is likely to be a considerable underestimate, as the relatively insensitive method of EM was chosen for *Norovirus* detection. There has also been an increasing prevalence of Noroviruses in recent years, with a strong suggestion of a winter seasonality culminating with a major epidemic in the UK in January 2002. Similarly, since October 2002 several states in the USA have reported an increase in outbreaks of *Norovirus* infection (www.cdc.gov/mmwr/PDF/wk/mm5203.pdf).

The diversity of settings associated with these economically important viruses is demonstrated by work conducted in the USA soon after the discovery of the Norwalk virus. This virus originally caused a community-wide outbreak in Norwalk, Ohio; Hawaii and Montgomery County agents were responsible for small family outbreaks (Thornhill et al. 1977), whereas the Snow Mountain agent caused an outbreak at a holiday camp in Colorado (Morens et al. 1979). The Taunton agent caused considerable disruption in a hospital in southern England (Caul et al. 1979; Pether and Caul 1983). Subsequent surveillance and investigations of outbreaks in many parts of the world identified the potential of Noroviruses for causing epidemic gastroenteritis in semiclosed or community-wide populations, for example families, healthcare institutions, holiday locations including cruise ships, educational establishments, and the catering industry. Outbreaks occur among children and adults, but rarely among neonates or very young children. Although considerable data are available on the causal role of Noroviruses in outbreaks, less is known about their role in endemic disease, perhaps because of the relative mildness of the illness. Hospitalization of individual cases is thus unusual, and opportunities to investigate sporadic cases are scarce.

Despite the lack of secondary spread by the respiratory route, it is clear that many of the explosive outbreaks described cannot be due solely to fecal–oral transmission. Noroviruses can be detected in vomitus (Greenberg et al. 1979b): the projectile vomiting associated with acute infections and the consequent formation of aerosols offers an alternative mode of spread (Caul 1994a; Chadwick and McCann 1994; Sawyer et al. 1988; Ho et al. 1989). This, in conjunction with the low infectious dose, would explain the rapid, explosive outbreaks commonly observed in semiclosed communities. The epidemiology of *Norovirus* infections should thus include not only person-to-person spread but also mechanical transmission from hand to mouth. The likelihood of spread from inhalation of aerosol by contacts in close proximity to the index case has been suggested but not proven (Caul 1994a).

In addition to these modes of transmission, Noroviruses are often associated with point-source outbreaks (Caul et al. 1993; Kapikian 1994; Okada et al. 1990). In many cases a likely vehicle of transmission has been identified, for example in the USA, contaminated water

supplies – e.g. municipal water, drinking water on cruise ships, recreational swimming water (lake or pool), and commercially produced ice-cubes – have been incriminated (Blacklow and Greenberg 1991; Cukor and Blacklow 1984; Greenberg et al. 1979a). Foodborne outbreaks are well recognized, and food-specific attack rates have incriminated salads, bakery products, fresh fruit, cold foods, sandwiches, and cooked meat The source of the contamination has often been identified as a symptomatic food handler (Pether and Caul 1983; Reid et al. 1988). The secondary attack rate of point-source outbreaks is often high. In the UK, 20–25 percent of *Norovirus* outbreaks were associated with contaminated food (Caul 1994b), and 26 of 38 outbreaks in Japan were considered to be foodborne (Okada et al. 1990). The application of molecular epidemiology (RT-PCR) has increased our knowledge of such outbreaks and the role of the food handler in transmission. The secondary attack rates in outbreaks in the USA reported by the Centers for Disease Control and Prevention (CDC), Atlanta, ranged between 4 and 32 percent, but attack rates of more than 50 percent have often been reported in semiclosed communities. Outbreaks have been prolonged by the introduction of susceptible individuals into an infected environment (e.g. aboard cruise ships and in hospitals).

Contaminated shellfish are a major source of epidemic gastroenteritis (Appleton and Pereira 1977; Christopher et al. 1978; Kaplan et al. 1982; Caul 1994b; Morse et al. 1986). The most commonly reported are the bivalve molluscs (oysters, cockles, and mussels), which are filter-feeders and become infected by Noroviruses from raw sewage. Noroviruses are the major cause of viral gastroenteritis arising from eating shellfish in the UK, and both small and large outbreaks have frequently been reported around the world (Kohn et al. 1995; Le Guyader et al. 2000; Murphy et al. 1979).

Seroepidemiology

The inability to propagate Noroviruses in cell culture and the lack of a suitable animal model severely limited seroprevalence studies in the past. American workers developed a number of crude serological assays, using fecal extracts containing Norwalk virus, to assess the acquisition of Norwalk antibody in different populations (Greenberg et al. 1979a; Kapikian et al. 1978). Antibodies were acquired gradually during childhood, about 20 percent of children under 5 years old being positive. The prevalence of antibody rose rapidly to 45 percent in 18–35-year-olds, and to 55–60 percent in those aged 45–65. These findings are in accordance with the prevalence of *Norovirus* infections in older children and adults, and contrast with those of rotaviruses and *Sapovirus* in developing countries, where antibody is acquired very early in life. In Bangladesh 100 percent of children had acquired antibody by the age of 5, whereas in the former Yugoslavia the seroprevalence rate was intermediate between that of the USA and those of less developed countries (Kapikian 1994); similar results have been reported from Taiwan and the Philippines. The prevalence of Norwalk virus antibody in blood donors in the USA, Belgium, Yugoslavia, and Switzerland ranges from 54 to 77 percent, and emphasizes the ubiquitous nature of this agent. Compelling evidence that Norwalk virus was responsible for many outbreaks throughout the world in the 1970s has been demonstrated by RIA tests (Kapikian 1994). All these studies were performed when Norwalk virus was circulating in the community.

The expression of recombinant Norwalk virus capsid protein (Jiang et al. 1992) in insect cells, and subsequently the expression of the capsid protein from a number of other Noroviruses, has allowed extensive seroepidemiological studies to be performed using defined reagents in an ELISA format. Importantly, it was shown that recombinant Norwalk virus antigen performs identically to natural Norwalk virus antigen purified from feces of infected volunteers (Green et al. 1993). Seroepidemiological studies using recombinant *Norovirus* capsid antigens have yielded generally similar results: newborn to 6-month-old children had higher serum antibody levels than children aged 6–11 months, representing the decay of maternal antibodies in the early months of life (Cubitt et al. 1998; Dimitrov et al. 1997; Gray et al. 1993; Parker et al. 1994; Pelosi et al. 1999). *Norovirus* antibody prevalence increases in the first 2 years of life, reaching 90 percent in individuals over 60 years old. The prevalence of antibodies to genogroup II Noroviruses appears to be greater, and there are some differences in the rate of antibody acquisition between studies. However, it is likely that these assays detect a range of heterologous responses to Noroviruses, rather than antibodies specific for a single virus type.

Some 20 percent of individuals appear to be resistant to *Norovirus* infections (Graham et al. 1994). Norwalk virus VLPs were used in an interesting study to investigate their binding properties to epithelial cells (Marionneau et al. 2002). This study showed that Norwalk virus VLPs attach to the surface of epithelial cells, but only for secretor phenotype individuals, who represent 80 percent of American/European populations. Inactivation of the *FUT2* gene (chromosome 19) is the predominant mutation that defines the nonsecretor phenotype. Competitive binding studies using H1 and/or H3/4 trisaccharides confirmed that Norwalk virus VLPs uses these as ligands for binding to epithelial cells of secretors, and this may in part account for the susceptibilty of the general population. A separate volunteer challenge study (Hutson et al. 2002) showed that patients with the B blood group antigen have a decreased risk of *Norovirus* infection; however, this work did not evaluate the secretor status of the individuals tested. Recently it was shown, using a human challenge model, that 29 percent

of a study population was homozygous recessive for the *FUT2* gene, and that these individuals were resistant to infection following challenge. However, some individuals in the susceptible population were resistant to infection, suggesting an immune component to resistance (Lindesmith et al. 2003).

Laboratory diagnosis

VIRUS ISOLATION

There are no reports of the isolation of Noroviruses in either cell or human intestinal organ cultures. Tests with a wide range of animals have also failed to identify a suitable model (Kapikian 1994), although recently it was reported that macaques could be experimentally infected with human Noroviruses (Subekti et al. 2002). Some success has been achieved with the transmission of Norwalk virus in chimpanzees, in which serological responses and excretion of Norwalk virus antigen in stools were described (Wyatt et al. 1978). It is, however, unlikely that isolation in cell cultures will ever be used for diagnosis, as in vitro methods are too slow and labor-intensive.

ELECTRON MICROSCOPY

EM is the most commonly used laboratory diagnostic method for direct detection of Noroviruses in stools. All Noroviruses are indistinguishable on EM when examined by routine negative staining methods (1.5 percent phosphotungstic acid at pH 6.5). Virions possess an amorphous surface structure, lacking a defined symmetry, with a ragged outline (Figure 7a) that probably explains the wide range of particle diameters (32–38 nm) reported. It is essential that preparations of Noroviruses are examined in the absence of antibody, which can mask the surface structure and lead to incorrect identification (Caul and Appleton 1982).

Noroviruses are commonly excreted in feces as small aggregates and in very low numbers at 10^6 viruses/g of stool (relative to other enteric viral infections, e.g rotaviruses and adenoviruses). These concentrations are close to the limit of sensitivity of the EM, so careful examination of preparations is necessary. Contamination with other proteins in clinical samples often obscures Noroviruses, and can be overcome by applying SPIEM with human convalescent sera. Samples prepared in this way are superior to conventional methods of virus concentration used in EM and significantly increase the detection rate of Noroviruses. SPIEM is as sensitive as molecular methods for detecting Noroviruses (Green et al. 1995) and has the advantage of speed that is so important to the management of outbreaks. EM has the added value of being a 'catch-all' system, whereby all viruses associated with gastroenteritis can be detected. EM will probably retain an important role in the investigation of future *Norovirus* outbreaks, for which rapid diagnosis is a prerequisite.

SEROLOGICAL METHODS

The preparation of hyperimmune antisera to recombinant *Norovirus* capsid antigens has facilitated the development of an EIA for identifying naturally acquired or experimentally induced Norwalk virus antigen and antibody (Jiang et al. 1992; Treanor et al. 1993). Application of these new assays to volunteer studies has provided new insights into Norwalk virus infection (Graham et al. 1994), showing that seroconversions were highest among volunteers who experienced vomiting. The assays for Norwalk virus are highly specific, revealing more subclinical infections, longer periods of virus excretion (up to 7 days), and higher infection rates than were previously recognized (Graham et al. 1994). Subsequent to the development of the Norwalk virus EIA a range of antigen capture EIAs were developed based on polyclonal antisera from different animal species for the capture and detection of *Norovirus* antigens in human fecal specimens (Jiang et al. 1995a, b; Leite et al. 1996; Green et al. 1997; Hale et al. 1999; Vipond et al. 2000).

All these assays show a high degree of type or strain specificity, and as yet it has not been possible to develop a 'catch-all' antigen trap EIA. The EIA format for detection of Noroviruses in clinical specimens offers considerable advantages over RT-PCR. EIA is cheap, rapid, and simple, and thus easy to deploy in the routine diagnostic setting. Recently the first commercially available EIA has been brought to the diagnostic marketplace. This contains a mixture of polyclonal sera for antigen capture and monoclonal antibodies for detection, and is designed to detect a range of both genogroup I and genogroup II human Noroviruses.

RT-PCR

The characterization of the *Norovirus* genome has allowed the application of molecular techniques to the diagnosis of *Norovirus* infection. The scientific literature now abounds with RT-PCR-based studies. Most diagnostic RT-PCR approaches are based on the RNA polymerase and capsid regions of Noroviruses. Both these regions show considerable heterogeneity, allowing the division of human strains into a minimum of two genetic groups (Green et al. 1994) and many different clusters or clades. Molecular epidemiology is a powerful tool for the investigation of point-source outbreaks, particularly for examining shellfish (Atmar et al. 1993) and in monitoring the spread of Noroviruses (Green et al. 1995). These techniques are not used for routine diagnosis because of cost considerations and the need for specialized laboratory equipment (e.g. thermal cyclers), and are thus restricted to the research or reference laboratory.

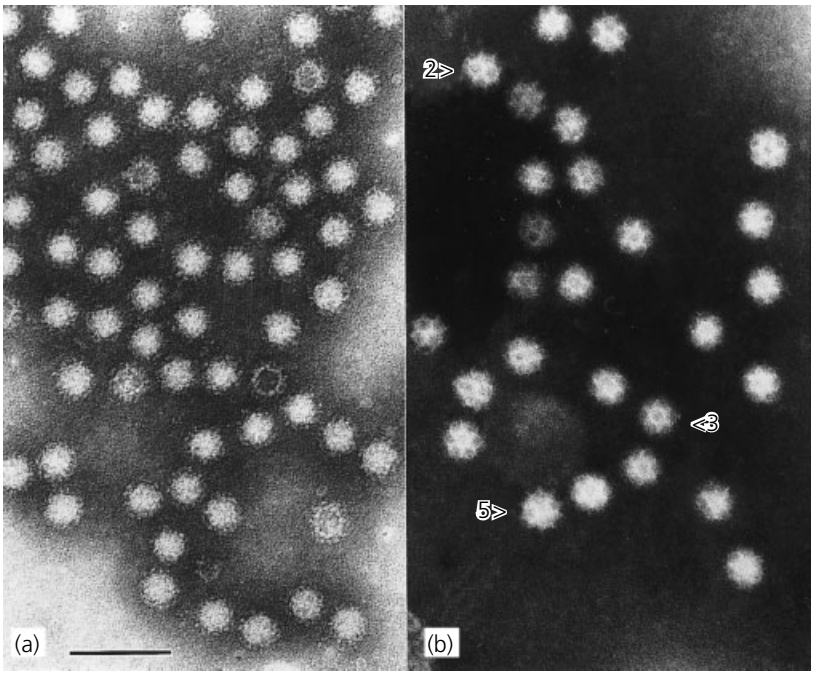

Figure 7 *Electron microscopic appearance of human enteric caliciviruses.* **(a)** *Noroviruses lack the distinctive surface morphology characteristic of Sapoviruses, although a ragged edge is clearly visible surrounding some of the particles.* **(b)** *The distinctive surface structures on a* Sapovirus *viewed along the two-, three- and fivefold (indicated) axes of symmetry. Bar, 100 nm for each panel.*

An electronic system (CaliciNet) has been developed at CDC Atlanta to fingerprint strains of Noroviruses that are associated with foodborne outbreaks (www.cdc.gov/foodsafety/fsactivities.htm).

Prevention and control

In volunteers, Noroviruses induce both short- and long-term immunity but, until the mechanisms are fully understood, it is unlikely that a vaccine can be developed (Green et al. 1995).

The high infectivity of Noroviruses and the explosive outbreaks of gastroenteritis that often occur in semi-closed communities present a major challenge to control of infection and require aggressive intervention (Caul 1994a; Chadwick and McCann 1994). Measures for interrupting the various modes of transmission (see Epidemiology) must include 'enteric' precautions, but these alone are ineffective and should be supplemented by measures to deal with patients who vomit (Chadwick et al. 2000). Symptomatic healthcare workers should be excluded from contact with patients for at least 2 days after resolution of symptoms. Effective handwashing should be routine. People caring for patients should wear disposable gloves, gowns, and masks when cleaning vomit, fecal material, or contaminated clothing. Soiled linen and clothes should be handled carefully, to minimize the generation of aerosols, and transported in plastic bags to the laundry. All potentially contaminated surfaces in toilets, bathrooms, and rooms occupied by patients should be disinfected with a chlorine-based product and cleaned with a hot detergent solution. Contact with other patients should be avoided.

Common-source outbreaks are well documented. Symptomatic food handlers have often been incriminated and must be excluded from the workplace for at least 2 days after resolution of symptoms. Decontamination of all potentially infected surfaces in the kitchens and associated rest and toilet facilities is essential (Caul et al. 1993). Suspect water supplies may require shock chlorine treatments. Shellfish are a particular problem in the transmission of Noroviruses, and the most effective preventive measure is to cultivate them in clean water, as depuration processes are not effective in eliminating Noroviruses from shellfish.

SAPOVIRUSES

Distinctive virus particles 30-40 nm in diameter and differing from both astroviruses and the Noroviruses were first recognized in fecal samples by negative staining techniques in 1976 (Madeley and Cosgrove 1976). These viruses, displaying a distinctive typical calicivirus morphology, have since been assigned to a separate genus (*Sapovirus*). Viral particles viewed along their threefold axes of symmetry possess a central stain-filled cup (calyx) surrounded by six peripheral cups, giving rise to the 'star of David' appearance. Viewed along their two-fold axes of symmetry they have four cups (Figure 7b). Particles viewed along their fivefold axes present as 10-pointed spheres and may not be identified

so readily as the classic particles described above. Unlike astroviruses, which possess a smooth, entire edge, caliciviruses have a ragged outline which makes accurate measurement difficult, and explains the particle diameters of 31–38 nm quoted by most workers. Sapoviruses have a buoyant density of 1.37–1.38 g/cm^3 (Terashima et al. 1983). The history of these viruses has been reviewed (Chiba et al. 2000). The significance of molecular data to differentiate between classic caliciviruses and Noroviruses was first recognized in our laboratory (Lambden et al. 1994). Further extensive sequencing studies have shown that the classic caliciviruses have a different genome structure from the Noroviruses, and phylogenetic analysis clearly places them in a different genus. The prototype strain *Sapporo virus* was isolated from an orphanage in Sapporo, Japan (Chiba et al. 1979).

Detailed ultrastructural studies on caliciviruses showing the typical morphology have been performed only on animal caliciviruses (Prasad et al. 1994; Zheng et al. 2001). The three-dimensional structure of the viruses was determined by cryo-electron microscopy and computer image-processing techniques. The primate calicivirus virions are 40 nm in diameter and have a T = 3 icosahedral symmetry, with 32 surface depressions at the five- and three-fold axes of symmetry (Figure 2). The surface depressions are surrounded by 90 archlike capsomers, each of which is a capsid protein, in an arrangement similar to that seen in the Noroviruses.

Genome organization

Sequence analyses of isolates of Sapoviruses from the UK, USA, and Japan have been used to assign this group of viruses to a separate genus (Berke et al. 1997; Jiang et al. 1997; Lambden et al. 1994; Liu et al. 1995; Matson et al. 1995; Noel et al. 1997; Vinjé et al. 2000).

Only two complete genome sequences of human *Sapovirus* (UK/Manchester/93; UK/Bristol/98) have so far been described (Liu et al. 1995, 1997; Robinson et al. 2002), and the sequence and genome organization of these viruses support their allocation to a genus separate from the Noroviruses. The complete sequence of the Manchester isolate consists of 7 431 nt, with the first predicted in-frame AUG codon at position 13. The 3′ end of the genome contains 84 untranslated nucleotides, followed by a polyadenylate tail. The genetically distinct Bristol isolate has a genome of 7 490 nt, with the predicted translation initiator codon at position 14. The 3′ untranslated region of Bristol virus consists of 140 nt preceding the poly (A) tail. The small ORF overlapping the start of the capsid encoding region of the genome of Manchester virus was not present in the Bristol isolate, suggesting that this region of the genome may not encode a biologically active product. This overlapping sequence was also missing from the closely related London/92 isolate (Jiang et al. 1997). In common with all caliciviruses examined to date, Sapoviruses also possess the small 3′ terminal ORF. Sequence comparisons and phylogenetic analyses suggest that the Sapoviruses form distinct genetic clusters in a manner analogous to the Noroviruses (Berke et al. 1997; Vinjé et al. 2000). A recent analysis has suggested that there may be two discrete genetic clades of human Sapoviruses, together with genetically distinct animal counterparts (Robinson et al. 2002), mirroring the situation in the *Norovirus* genus (Figure 5).

A fundamental difference between Noroviruses and Sapoviruses is that the region of the genome predicted to encode the structural capsid protein of Sapoviruses is in the same frame as ORF1 and contiguous with the RNA polymerase coding region, resulting in one large fused polyprotein (Figure 8). This genome organization is also found in the rabbit hepatotropic calicivirus, RHDV, and contrasts with those of all Noroviruses sequenced to date. The consequence of such a genome organization is that an additional proteolytic cleavage is required to release the RNA polymerase from the capsid protein.

A major feature that distinguishes the caliciviruses from the picornaviruses is the production of one or more species of subgenomic RNAs during replication. In the case of RHDV, a subgenomic RNA is also encapsidated into mature virus particles (Meyers et al. 1991; Zheng et al. 2001). The existence of a subgenomic RNA has not been established for Sapoviruses, and only indirect evidence exists for Norwalk virus. The 5′ terminal sequences of the putative subgenomic RNAs share sequence homology with the 5′ genomic RNA termini, although the level of sequence identity was less than the analogous conserved motifs observed both for Noroviruses and animal caliciviruses. The conservation of sequence motifs suggests an important regulatory role for these regions of the genome, perhaps serving as promoters for the synthesis of genomic and subgenomic RNAs from negative-strand intermediates.

Viral proteins

The lack of a productive cell culture system for Sapoviruses and the resistance of adults to experimental infection (Cubitt 1994) has greatly impeded analysis of the viral polypeptides. During acute infection the viruses are, however, shed in relatively high concentrations. A single fecal specimen obtained during an outbreak of acute gastroenteritis in an orphanage in Sapporo, Japan, was used for the purification of virions (Terashima et al. 1983). This study showed that classic *Sapovirus* particles contain a single polypeptide of 62 kDa that could be precipitated with convalescent (4 weeks) but not acute-phase (2 days) serum from the same patient.

The large ORF1 has a coding potential of 2 280 amino acids and includes conserved motifs typical of the 2C NTPase, 3C protease, and 3D RNA polymerase in

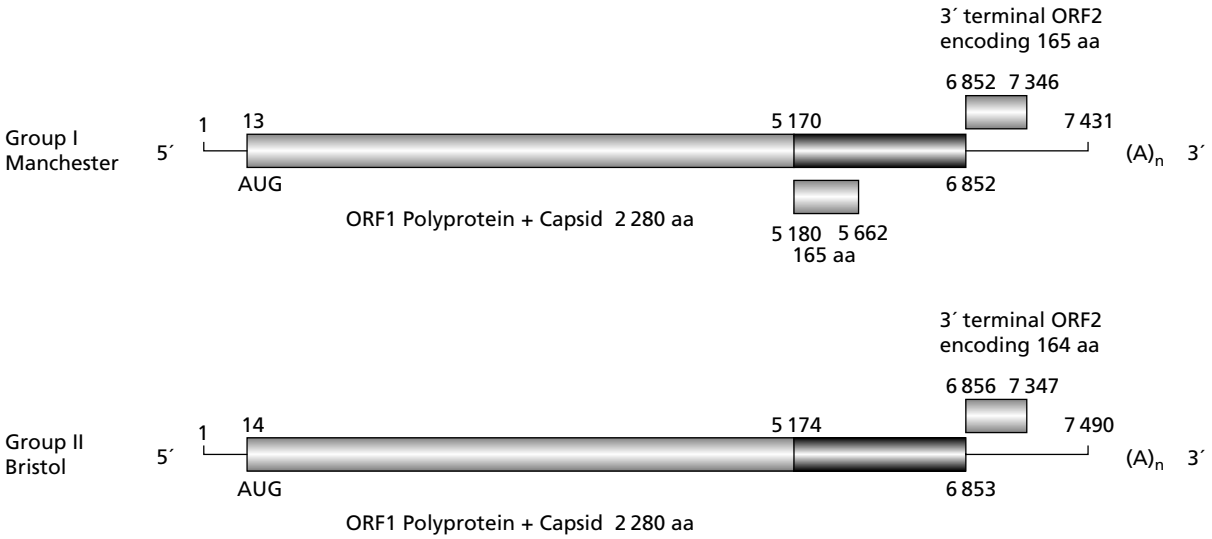

Figure 8 *Comparison of the genome structures of groups I and II Sapoviruses (Manchester, EMBL/GenBank Accession No. X86560; Bristol, EMBL/GenBank Accession No. AJ249939). Shaded boxes represent computer-predicted open reading frames. Translation products and their sizes are indicated on the open reading frames. The darker shaded area at the 3′ terminus of ORF1 represents the capsid coding region which is fused to the ORF1 nonstructural polyprotein. In group I Sapoviruses an additional small protein (161 aa) encoded in an alternative reading frame is predicted within the capsid coding region.*

relative genomic positions similar to those of other caliciviruses. Sequence comparisons of Sapoviruses with other caliciviruses, including the Noroviruses, show only 20–40 percent amino acid identity in the functional domains of the nonstructural polyprotein. Relatively little is known about polyprotein processing in Sapoviruses, except that in vitro protein synthesis in a coupled transcription translation reaction yields a very different cleavage pattern from the Noroviruses (Clarke and Lambden 2000). The exact cleavage dipeptide has not been experimentally determined for Sapoviruses, but it is likely that these viruses will show a similar site preference for EG residues.

Clinical manifestations

Symptomatic *Sapovirus* infection occurs most often in infants and young children, and appears indistinguishable from the milder forms of rotavirus infection (Cubitt 1987; Cubitt and McSwiggan 1981; Sakai et al. 2001; Suzuki et al. 1979). Transmission is by person-to-person spread through the fecal–oral route, and this is probably important in maintaining an endemic state in some semi-closed communities. Contaminated shellfish, cold foods, and drinking water have also been implicated as vehicles of infection (Cubitt 1988).

The incubation period is 1–3 days and symptoms persist for an average of 4 days. Diarrhea and vomiting are common, whereas fever with upper respiratory symptoms is less so. There is no evidence that Sapoviruses replicate in respiratory mucosal cells or that respiratory spread occurs. As respiratory symptoms have been described mainly in young children, they may arise from one of the concomitant respiratory viral infections common in this age group.

Pathogenesis

In contrast to Noroviruses there have been no reports of ultrastructural studies of human *Sapovirus* infections in the gut. Analogy with all the other 'diarrhea viruses' would suggest that human Sapoviruses replicate in mucosal cells lining the villi of the small intestine. In limited adult volunteer studies the virus was administered by the nasal/oral route (Cubitt 1994). Symptoms were inapparent, or ranged from mild to moderately severe, where the latter included nausea, vomiting, diarrhea, pyrexia, and abdominal pains. These clinical features resemble those in naturally acquired infections.

Epidemiology

Most of our data on the epidemiology of Sapoviruses derives from EM observations and seroprevalence studies using purified viruses or hyperimmune sera in blocking ELISAs, although latterly some RT-PCR surveys have been conducted. When the Sapoviruses were first discovered it was not possible to assign a causal role because 20 percent of children excreting them were asymptomatic. Subsequently, an outbreak of gastroenteritis in an infant and junior school in north London was investigated and a *Sapovirus* was shown to be the causal agent (Cubitt et al. 1979) This outbreak was mistakenly described as 'winter vomiting disease,'

although spread to others in the school and to home contacts was negligible, being perhaps limited by their immunity. These epidemiological and immunological features are not consistent with 'winter vomiting disease' caused by Noroviruses. Sapoviruses have since been established as enteric pathogens (Christensen 1989; Cubitt 1994). In children hospitalized with sporadic diarrhea, excretion rates of 0.9–6.6 percent have been reported (Cubitt and McSwiggan 1981; Suzuki et al. 1979). A similar excretion rate (2.9 percent) was reported in a daycare center in the USA (Matson et al. 1989). All these studies suggest that Sapoviruses are a minor cause of clinically significant disease. In contrast with infection in older children, neonatal infection is often subclinical (Matson et al. 1990). These observations are supported by data from the IID study in England and Wales which showed that Sapoviruses were found most frequently in children between the ages of 1 and 4 years, and constituted 7 percent of the viruses identified in the general practice component of this study (Food Standards Agency 2000). This is in agreement with an RT-PCR survey conducted in Japan, where 6.6 percent of patients with gastroenteritis were positive for Sapoviruses (Okada et al. 2002), and a survey of children aged 2–24 months in Finland, where 9.2 percent of stool specimens were positive (Pang et al. 2001).

Sapovirus infections are endemic in the UK, usually appearing sporadically in young children, although community and nosocomial outbreaks have also been recognized. Outbreaks have been reported in Japan (Chiba et al. 1979; Oishi et al. 1980), Australia (Grohmann et al. 1991), England (Cubitt et al. 1979, 1980; Cubitt and McSwiggan 1981; Gray et al. 1987; Humphrey et al. 1984), Canada (Spratt et al. 1978), and Scandinavia (Kjeldsberg 1977), not only among children but also among elderly patients in nursing homes, where the attack rates ranged from 50 to 70 percent. Outbreaks among adults and elderly patients are unusual and may represent infections that have arisen because of waning immunity. A distinct seasonality has not been described for *Sapovirus* infections, primarily because too few cases were reported from which to analyze seasonality.

Seroepidemiology

Progress in understanding the seroepidemiology of Sapoviruses has been dependent on using viruses from fecal specimens. Attempts at expression of the human *Sapovirus* major capsid protein in insect cells using recombinant baculoviruses have met with mixed fortunes. Whereas some laboratories have reported success in obtaining recombinant VLPs with a structure and morphology similar to those of the native virions, in most cases these VLPs are unstable. Fortunately, it has been possible to purify sufficient *Sapovirus* particles from stool samples to produce diagnostic antigen trap ELISAs. Such an ELISA was also used in a blocking assay presenting captured antigen from stool specimens to measure antibodies to Sapoviruses in 128 serum samples from an age-stratified population from the USA. This important study showed a high incidence of antibody in young children, reaching 100 percent in the 4–6-year age group (Nakata et al. 1988). Thus immunity to Sapoviruses appears to be long-lived, in sharp contrast to *Norovirus* infections, where the short-term immunity allows symptomatic reinfections (Greenberg and Matsui 1992).

In Japan, antibody to *Sapovirus* is rapidly acquired between 6 months and 2 years of age, with a prevalence of 30 percent, rising to 65 percent in 2–5-year-olds and approximately 90 percent in older children and adults (Nakata et al. 1985; Sakuma 1981). Tests on pooled γ-globulin and serum samples in all continents confirm these findings (Christensen 1989). The high seroprevalence in early childhood agrees with the reported excretion rates of Sapoviruses in young children. The paucity of reports of *Sapovirus* infection in hospitalized children with diarrhea suggests that asymptomatic – or at least clinically insignificant – disease in childhood is common.

Laboratory diagnosis

VIRUS ISOLATION

Human Sapoviruses cannot be grown in cell culture, although there is a single report of serial propagation of human *Sapovirus* in dolphin kidney and HEK cells in the presence of trypsin (Cubitt and Barrett 1984). In this work, cytoplasmic replication was demonstrated by immunofluorescence and by radiolabeling of polypeptides. This report has not been confirmed, and many other workers have failed to propagate human Sapoviruses in vitro.

ELECTRON MICROSCOPY

The unique morphology of Sapoviruses allows definitive identification by EM (Madeley 1979), and micrographs should always be checked to confirm direct observations with the EM. The characteristic morphology has been the main criterion for identifying Sapoviruses. If the morphology suggests a *Sapovirus* but is not convincing, RT-PCR analysis may confirm the diagnosis (Honma et al. 2000; Robinson et al. 2002). EM is currently the only routine method available for laboratory diagnosis, and requires skilled operators to achieve consistent results.

IMMUNOASSAYS

The need for alternative methods of identifying caliciviruses in stool samples has been generally recognized.

Nakata and co-workers (Nakata et al. 1983) found that a solid-phase RIA using a hyperimmune guinea-pig serum was more sensitive than EM. This assay was later modified to an EIA of similar sensitivity (Nakata et al. 1988). No commercially produced ELISA tests are available to the routine laboratory.

SEROLOGY

Specific IgM, seroconversions or rising titers of specific IgG have been observed following infection (Chiba et al. 1979; Cubitt et al. 1979; Cubitt et al. 1980; Cubitt et al. 1981; Nakata et al. 1985; Sakuma 1981). These techniques, although useful in epidemiological studies, are not ethically justified for diagnosing enteric 'diarrhoea virus' infections in young children because they involve taking blood samples.

PREVENTION AND CONTROL

The measures needed to prevent the spread of calicivirus infections are the same as those recommended in the previous section on Prevention and control.

ANIMAL ENTERIC CALICIVIRUSES

Following the discovery of human enteric caliciviruses similar viruses were found in cattle and swine. These viruses were either observed directly by EM or, in some cases, their presence was inferred by a positive polymerase chain reaction (PCR). With improved detection technology it is likely that other mammalian enteric caliciviruses are yet to be discovered, and although there is no evidence for cross-species infection, an animal reservoir for human infection remains a possibility.

Noroviruses

Early studies revealed the presence of viruses resembling Noroviruses in fecal specimens from cattle (Almeida et al. 1978; Granzow and Schirrmeier 1985; Günther et al. 1984; Woode and Bridger 1978). The first bovine Noroviruses were described in England and, following the tradition established for the human Noroviruses, are called Newbury agents 1 and 2 after the geographical location where they were discovered (Woode and Bridger 1978). The Newbury agent 1 (NA1), like the human Noroviruses, has a single major capsid protein (Dastjerdi et al. 2000). Following the discovery of the Newbury agents similar viruses were also found by two independent groups in the former East Germany (Granzow and Schirrmeier 1985; Günther et al. 1984). Since these early studies the application of RT-PCR to fecal specimens from farm animals has revealed the presence of *Norovirus*-like sequences in both swine (Sugieda and Nakajima 2002; Sugieda et al. 1998) and cattle (van der Poel et al. 2000).

The complete genome sequence of a bovine virus (Jena virus (JV)) has been determined (Liu et al. 1999a) and shows that the JV genome organization is very similar to that of the human Noroviruses. Sequence analysis of the capsid region of Newbury agent 2 (NA2) has confirmed the relationship of the bovine Noroviruses to the human Noroviruses, and phylogenetic analysis suggests that the bovine Noroviruses belong to a distinct genetic group (Belliot et al. 2001). Bovine Noroviruses do not grow in cell culture; however, calves have been used in an experimental setting to study infection. Newborn calves and calves up to 60 days of age are susceptible to infection with bovine Noroviruses (Bridger 1990). Virus replication occurs in the enterocytes of the small intestine, showing a similar tissue and cell tropism to that observed for human Noroviruses (Hall et al. 1984).

EM studies from the 1980s suggested that bovine Noroviruses are 'commonly found in the British calf population' (Bridger et al. 1984). Recently the capsid protein of JV has been expressed in insect cells using a recombinant baculovirus vector. The protein assembled to form VLPs, and this allowed the development of a diagnostic ELISA and an ELISA to study the seroprevalence of JV. The antigen capture ELISA was used to survey fecal specimens collected from calves in dairy herds in Germany, and showed that 8.9 percent of the samples were positive for JV infection. Seroepidemiological data from the same study indicated that 99.1 percent of cattle have antibodies to JV. The very high seroprevalence for JV suggests that bovine *Norovirus* infections are endemic, and the high antibody levels in older cattle suggests that they are constantly re-exposed to infection. These observations, coupled to the findings of RT-PCR surveillance in other countries which has identified *Norovirus* sequences in bovine and swine, confirms the widespread nature of *Norovirus* infections, and it would seem likely that Noroviruses remain to be found infecting other mammalian species. It has been suggested that animal Noroviruses could be a source of infection for humans. Although there are no data on the susceptibility of humans to animal *Norovirus* infections (with the exception of a single report), no human Noroviruses have been shown to infect and cause disease in other mammalian species. Thus the bovine system may provide an important animal model for studying the human viruses.

A bovine calicivirus morphologically indistinguishable from the Noroviruses was recently sequenced in the USA (Smiley et al. 2002). This virus (BEC NB) was originally observed in 1980, along with a coronavirus, in a fecal specimen from a calf in Nebraska, Ohio. However, the genome organization of BEC NB resembled that of the Sapoviruses, with the ORF1 polyprotein fused to and contiguous with the capsid encoding region of the genome. In experimental infections of calves, BEC NB showed similar intestinal lesions and tropism to the bovine Noroviruses. Phylogenetic analysis

suggests that BEC NB may be a representative of new genus of enteric caliciviruses.

Sapoviruses

Membership of the *Sapovirus* genus is defined on the basis of phylogeny and the organization of the viral genome. An enteric calicivirus infecting swine was first described in the USA in 1980 (Saif et al. 1980). Experimental infections with this virus (Cowden PECV) using gnotobiotic piglets established a causal role in enteric disease. Infection results in villous atrophy and profuse diarrhea. The adaptation of Cowden PECV to growth in cell culture marked an important and significant breakthrough in calicivirus research, as this is the only enteric calicivirus that can be cultured. Following serial passage through gnotobiotic piglets the virus was adapted to grow in primary porcine embryonic kidney cells (Flynn and Saif 1988), and then in a continuous pig kidney cell line (Parwani et al. 1991). The key factor in this success was the incorporation of the intestinal contents of uninfected gnotobiotic piglets into the cell culture medium. The growth-promoting factors of the intestinal contents of piglets have been investigated in depth (Chang et al. 2002). Pretreatment of cells or PECV prior to infection (30 min) with intestinal contents, followed by removal, failed to support viral growth, suggesting that cells needed constant signaling/stimulation from intestinal contents to allow viral growth. Transfection with synthetic genomes of PECV (bypassing receptor binding by the virus) only produced infectious progeny when intestinal contents were continually present. However, the addition of suramin, together with other adenylate cyclase inhibitors, restricted the growth-promoting effect of intestinal contents, suggesting that replication of PECV is dependent on a cAMP signaling pathway that is induced by intestinal contents. Interestingly, the cell culture-adapted virus did not produce clinical disease in experimentally infected piglets (Guo et al. 2001b), suggesting that the cell culture adaptation process had attenuated the virus. The complete genome sequences were determined for wildtype (wt) and cell culture-adapted strains (tc) of Cowden PECV. The genome organization of Cowden PECV conforms closely with that of human Sapoviruses (see Genome organization). PECV tc showed two silent mutations and two amino acid changes in ORF1, and five nucleotide changes in the capsid that result in four amino acid changes compared to PECV wt. It was speculated that the changes in the capsid sequence are responsible for the attenuation of the cell culture-adapted virus (Guo et al. 1999) The capsid protein of Cowden PECV has been expressed in insect cells using a recombinant baculovirus, and undergoes spontaneous assembly to form virus-like particles that are morphologically and antigenically similar to the native virus (Guo et al. 2001c). Insect cells infected by the recombinant baculovirus were used to investigate seroresponses of experimentally infected piglets, and an antigen trap ELISA was developed using hyperimmune sera raised to the VLPs. A preliminary survey using this ELISA showed that variants of PECV were circulating in local swine herds.

It seems likely that there are as yet undiscovered Sapoviruses infecting other animal species. Confirmation that a candidate calicivirus belongs to the *Sapovirus* genus requires phylogenetic and genome organization analyses. An enteric calicivirus was described in mink, but this virus could not be grown in cell culture. RT-PCR of a fragment of the *RNA polymerase* gene showed that the mink virus was closely related to PECV (Guo et al. 2001a). However, the mink from the farm where the virus was discovered had been fed raw offal from pigs, casting doubt on the precise origin of the virus.

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Reoviruses, orbiviruses, and coltiviruses

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INTRODUCTION AND GENERAL CLASSIFICATION

The *Reoviridae* family contains ten genera:

- *Orthoreovirus*, *Rotavirus*, *Orbivirus*, all infecting animals and humans
- *Coltivirus* infecting insects, rodents, and humans
- *Aquareovirus* infecting fish and molluscs
- *Cypovirus* infecting insects
- *Fijivirus*, *Phytoreovirus*, and *Oryzavirus* infecting plants and insects (Mertens et al. 2000; Nibert and Schiff 2001).
- *Seadornavirus* infecting insects and humans (Attoui et al. 2000)

In this chapter *Orthoreovirus* is called **reovirus**, the term being an acronym derived from **r**espiratory, **e**nteric, **o**rphan viruses. Only the mammalian genera are discussed; the rotaviruses are reviewed in Rotaviruses.

Members of the *Reoviridae* have the following features in common:

- They contain a genome of 10–12 segments of double-stranded RNA (dsRNA).
- They form icosahedral, nonenveloped particles of approximately 70–80 nm in diameter with a double- or triple-shelled capsid.
- They replicate in the cytoplasm of cells without complete uncoating, produce 5′ end-capped, non-polyadenylated mRNAs, and form intracytoplasmic inclusion bodies.

In comparison to many other viruses, the *Reoviridae* are relatively resistant to inactivation by heat, lipid solvents, and a wide range of pH (with the exception of the orbiviruses, which lose infectivity at low pH).

Full-length sequences of the genomes of several strains of reo-, rota-, and orbiviruses are known, and the protein-coding assignments have been established. The overall genome size in bp (genomic molecular mass in kDa) is 23 540 bp (7 770 kDa) for reoviruses (type 3 reovirus), 18 555 bp (6 120 kDa) for rotaviruses (SA 11 rotavirus), and 19 218 bp (6 340 kDa) for orbiviruses [bluetongue virus type 10 (BTV 10)] (Mertens et al. 2000). The 5′ and 3′ untranslated regions (UTR) of all reovirus RNAs are short (reovirus: 5′-UTR 13–32 nt, 3′-UTR 35–83 nt; rotavirus: 5′-UTR 9–49 nt, 3′-UTR 17–182 nt; orbivirus: 5′-UTR 11–34 nt, 3′-UTR 31–116 nt).

Reoviruses have been isolated from humans but are rarely associated with disease. They cause systemic disease in rodents (mice), and the mouse model of reovirus infection has been used extensively to study mechanisms of viral pathogenesis (Virgin et al. 1997). Rotaviruses are the major cause of infantile gastroenteritis worldwide and the causative agent of acute diarrhea in the young of many mammalian species (e.g. calves, piglets, and lambs). Orbiviruses mainly infect animals, producing severe disease (e.g. African horse sickness, epizootic hemorrhagic disease in cattle and deer, and bluetongue disease in sheep). *Coltivirus* causes Colorado tick fever in humans. Mammalian reoviruses have a broad host range and frequently contaminate water supplies. Rotaviruses are most commonly spread by the fecal–oral route, and orbiviruses are transmitted by a variety of ticks and mosquitoes.

REOVIRUSES

Genome, gene protein assignment, and structure

Orthoreoviruses (reoviruses) are found worldwide in a wide range of vertebrates. They cause hepatic and neurological disease in mice, diarrhea in cattle and sheep, and respiratory tract infections in dogs. In birds, reovirus infections are associated with hepatitis, arthritis, and diarrhea. There are three serotypes of mammalian reovirus, each represented by a prototype strain isolated from a human host: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D).

The reovirus genome consists of ten segments of dsRNA that fall into three size classes, which for strain T3D are: large (L), 3 854–3 916 bp; medium (M), 2 203–2 304 bp, and small (S) 1 189–1 416 bp (Nibert and Schiff 2001). The viral proteins are designated according to size: **λ** for proteins encoded by large gene segments, **μ** by medium segments, and **σ** by small segments. Each gene segment is monocistronic with the exception of the *S1* gene, which encodes the viral attachment protein, σ1, and a small nonstructural protein, σ1s. Several protein products are modified post-translationally. The genome segments, their size in bp, and the size, number of molecules, and location in the virion, post-translational modifications, and some of the recognized functions of the protein products are shown in Table 1.

Reovirus virions consist of two concentric protein shells, called outer capsid and core. Three different types of reovirus particles are formed during the process of viral disassembly (Figure 1, p. 4):

1. Virions contain the full complement of eight structural proteins. These represent the infectious form of the virus and are capable of spread from host to host.
2. Infectious subvirion particles (ISVP) lack **σ**3, contain particle-associated cleavage fragments of **μ**1 called **δ** and **ϕ**, and have an extended form of **σ**1. ISVPs are formed in the intestinal lumen or in the endocytic pathway.
3. Cores are devoid of outer-capsid proteins. These particles are enzymatically active and catalyze synthesis of viral RNA.

There are 11 reovirus proteins, eight structural proteins (λ1, λ2, λ3, μ1, μ2, σ1, σ2, and σ3) and three nonstructural proteins (μNS, σNS, and σ1s). Image reconstructions of cryo-electron micrographs of reovirus particles (Metcalf et al. 1991; Dryden et al. 1993, 1998) (Figure 1) combined with X-ray crystallographic analysis of cores (Reinisch et al. 2000) and outer-capsid proteins (Olland et al. 2001; Chappell et al. 2002; Liemann et al. 2002) have revealed the particle structure in great detail. The core lattice is formed by 120 copies of λ1. Channels of λ1 are found at the fivefold symmetry axes of the icosahedral core particle. The λ3 (12 copies) and μ2 (20 copies) proteins are located at the internal aspect of these channels. The λ3 protein is the viral RNA-dependent RNA polymerase (RdRp) and is closely associated with the ten segments of genomic dsRNA. The crystal structure of λ3 reveals a cage-like molecule, with independent channels for template and product (Tao et al. 2002). On top of the λ1 shell are 150 copies of σ2 that act as a clamp to stabilize the λ1 lattice. The λ2 protein (60 copies) forms pentamers at the fivefold symmetry axes. 'Turrets' of λ2 reach to the outer capsid. The λ2 protein plays an important role in viral RNA synthesis by serving as the capping enzyme complex. The outer shell is formed by 600 copies of μ1 and 600 copies of σ3 that interact extensively to form heterohexamers (Liemann et al. 2002). The μ1 protein is myristoylated at its N terminus and is involved in viral penetration of host-cell membranes. The σ3 protein serves as a protective cap for μ1 and renders the virus stable in the environment. The σ1 protein (36 copies) is the viral attachment protein and forms trimers at the icosahedral vertices. The N terminus of σ1 is embedded in the turrets of λ2.

Replication

The reovirus replication cycle is entirely cytoplasmic (Figure 2, p. 4). Many of the features of reovirus replication are similar, though not identical, to those of rotaviruses (for further details, see Nibert and Schiff 2001 and Rotaviruses).

Reovirus infection is initiated by the binding of the σ1 protein to cell-surface receptors. The σ1 protein is a trimer consisting of an elongated fibrous domain – the tail – that inserts into the virion and a virion-distal globular domain – the head (Fraser et al. 1990). Type 3 σ1 contains two receptor-binding domains, one in the head that binds to junctional adhesion molecule 1 (JAM1) (Barton et al. 2001b) and another in the tail that binds α-linked sialic acid (Chappell et al. 2000). In T3D σ1, these domains are dissociable by treatment of σ1 with intestinal proteases, such as trypsin or chymotrypsin (Nibert et al. 1995). Type 1 σ1 binds cell-surface carbohydrate as well, but this molecule has not been identified. For sialic-acid-binding reovirus strains, the initial interaction between the viral particle and the host cell is likely mediated by sialic acid due to the high surface concentration of this carbohydrate (Barton et al. 2001a). By virtue of its rapid association rate, virus binding to sialic acid likely adheres the virion to the cell surface, thereby enabling it to diffuse laterally until it encounters JAM1. Such an adhesion-strengthening mechanism of viral attachment has been shown for several other viruses including herpesviruses and lentiviruses.

Table 1 *Gene protein assignments and functions of mammalian reovirus proteins (T3D)*

RNA		Proteins					
Segment	Size (bp)	Designation	Size (kDa)	Number of molecules/virion	Location	Modification	Functions
L1	3 854	λ3	142	ca. 12	Core	. . .	RdRp
L2	3 916	λ2	145	60	Core spike (outer capsid)	. . .	Guanylyltransferase; methyltransferases
L3	3 901	λ1	143	120	Core	Zn metalloprotein	Binds RNA; NTPase? RNA helicase? RNA triphosphatase?[a]
M1	2 304	μ2	83	ca. 20	Inner capsid	. . .	Binds RNA; NTPase?
M2	2 203	μ1	76	600	Outer capsid	N-myristoylated	Role in penetration and transcriptase activation
		μ1C	72	. . .	. . .	Cleavage product of μ1	. . .
M3	2 241	μNS	80	. . .	Nonstructural	Phosphoprotein	ssRNA-binding; core binding; role in RNA assortment or replication?
		μNSC	75	. . .	Nonstructural	. . .	Unknown
S1	1 416	σ1	49	36	Outer capsid	. . .	Viral attachment protein; hemagglutinin; type-specifying antigen
		σ1s	14	. . .	Nonstructural	. . .	Unknown (dispensable in cell culture)
S2	1 331	σ2	47	150	Inner capsid	. . .	Binds ds RNA
S3	1 198	σNS	41	. . .	Nonstructural	. . .	ssRNA-binding; role in assortment or replication?
S4	1 196	σ3	41	600	Outer capsid	Zn metalloprotein, sensitive to protease degradation	dsRNA-binding; effects on translation

Modified from Nibert and Schiff 2001.

a) Possibly encoded by the M1 gene segment μ2 protein (Tao et al. 2002).

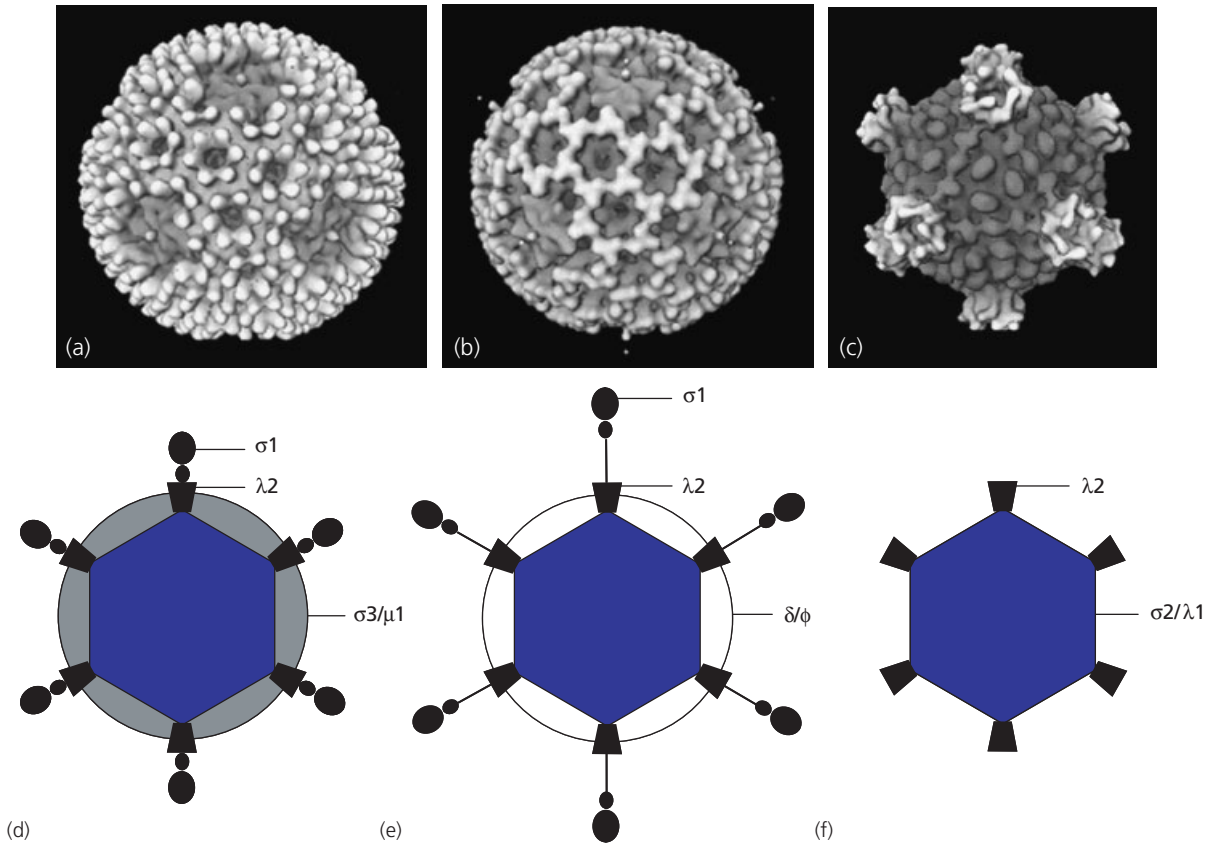

Figure 1 *Reovirus virions, ISVPs, and cores.* **(a–c)** *Surface-shaded views of the reovirus strain T1L* **(a)** *virion,* **(b)** *ISVP, and* **(c)** *core. The images were obtained using cryo-electron microscopy and three-dimensional image reconstruction. Each particle is viewed along a threefold axis. Figure modified from Centonze et al. 1995 with permission.* **(d–f)** *Schematic representations of reovirus* **(d)** *virion,* **(e)** *ISVP, and* **(f)** *core. Major viral structural proteins are indicated.*

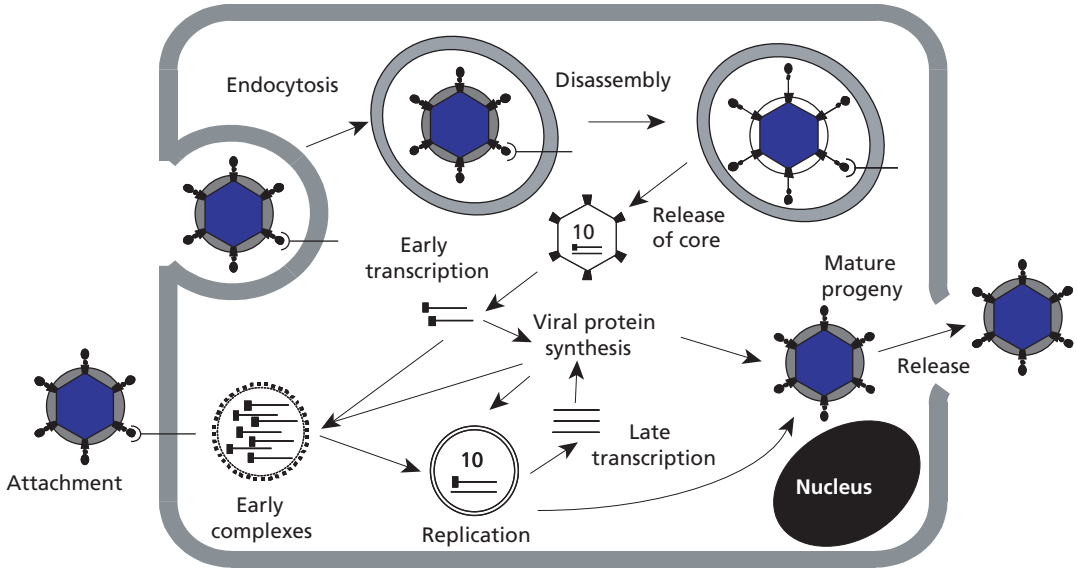

Figure 2 *The reovirus replication cycle. Reovirus virions attach to cellular receptors and enter cells by receptor-mediated endocytosis. Within an endocytic compartment, the viral outer capsid is removed to generate ISVPs. ISVPs penetrate membranes of endocytic vesicles and release the viral core into the cytoplasm. Transcription of the viral genome occurs within the core, and nascent mRNAs are released through turret-like openings at the viral vertices. Viral mRNAs are used as template for viral protein synthesis and replication of viral genomic dsRNA. Particles containing dsRNA segments are termed replication particles. These particles are transcriptionally active and yield additional viral mRNAs, resulting in an amplification of viral protein synthesis. Following addition of viral outer-capsid proteins to the replication particles, progeny virions are assembled and exit the cell.*

Following attachment to cell-surface receptors, reovirus enters cells by receptor-mediated endocytosis. In the endocytic compartment, virions undergo proteolysis by endocytic proteases, resulting in generation of ISVPs. The endocytic cysteine-containing proteases cathepsin B and cathepsin L convert virions to ISVPs in mouse fibroblasts (Ebert et al. 2002). ISVPs also can be formed extracellularly in the murine intestine or by treatment with intestinal proteases in vitro. During virion-to-ISVP conversion, σ3 is degraded and lost from virions, σ1 undergoes a conformational change, and μ1 is cleaved to form particle-associated fragments δ and ϕ (Figure 1). Removal of σ3 is thought to expose hydrophobic domains in μ1 that facilitate interactions of ISVPs with endosomal membranes, leading to delivery of core particles into the cytoplasm (Nibert et al. 1991; Liemann et al. 2002).

Transcription of the reovirus genome is fully conservative, i.e. both strands of the genome remain within the cores. The nature of the functional reovirus transcriptase is not fully understood. The λ1, λ2, λ3, and μ2 proteins all likely play important roles in viral RNA synthesis, with λ3 serving as the viral RdRp. Only positive-sense transcripts are produced during primary transcription, and these transcripts are capped, as the core contains virus-encoded capping enzymes. The λ2 protein has guanylyltransferase and methyltransferase activities (Luongo et al. 2000; Reinisch et al. 2000). Nascent transcripts leave the core via channels formed by pentamers of λ2 at the vertices of the fivefold symmetry axes. Transcripts are produced in a ratio according to their size; S-class RNAs are more abundant than M-class RNAs, which are in turn more abundant than L-class RNAs. Capped, full-length RNAs act as both mRNAs and templates for negative-strand RNA synthesis within progeny cores.

Replication of reovirus genomic dsRNA segments occurs within newly formed cores, although the mechanisms underlying this process are poorly understood. Plus-strand RNA is thought to be packaged during assembly of cores, where it serves as template for minus-strand synthesis. The assembly of the correct number of RNA segments into cores is highly regulated, but the process ensuring that each core receives a single copy of each RNA is unknown. Following synthesis of dsRNA, the nascent cores become transcriptionally active and enter a second round of mRNA synthesis. In contrast to the products of primary transcription, these secondary transcripts are not capped.

Both capped and uncapped RNAs serve as translational templates. There are different translation efficiencies of reovirus mRNAs, suggesting post-transcriptional control. The variability in translation is in part due to the sequence context of the initiator AUG (especially at the −3 and +4 positions) (Kozak 1981). The 3′-UTRs of some reovirus mRNAs also contain translational control sequences (Mochow-Grundy and Dermody 2001). During reovirus infection, most protein synthesis in the cell is viral in origin. Outer-capsid protein σ3 may play a central role in this process through its effects on the activity of the interferon-induced enzyme, protein kinase RNA (PKR). This effect is thought to relate to the affinity of σ3 for dsRNA and its capacity to compete with PKR for dsRNA binding (Imani and Jacobs 1988; Lloyd and Shatkin 1992; Schmechel et al. 1997). Sequestration of dsRNA by σ3 presumably attenuates PKR activation, thereby impeding the capacity of PKR to phosphorylate eIF-2a.

The morphogenesis of reovirus particles is complex. The earliest detectable newly formed particles contain single-stranded RNA (ssRNA). These particles, termed 'replicase particles,' are actively engaged in genomic dsRNA synthesis. Once dsRNA is synthesized, the replicase particles initiate secondary transcription and are termed 'transcriptase particles.' The additional uncapped transcripts lead to increased protein synthesis. Outer-capsid proteins condense onto the transcriptase particles, silencing transcription, to form mature, double-shelled reovirus virions. Viral nonstructural proteins are involved in collecting ssRNA prior to formation of replicase particles (Antczak and Joklik 1992) and may play additional roles in particle morphogenesis (Broering et al. 2000; Becker et al. 2001). A peculiar feature of reoviruses is the occurrence of oligonucleotides (di- to nonanucleotides) in viral particles. These RNA species appear to be synthesized following particle assembly and are likely to represent aborted products of transcription (5′ ends of mRNAs).

The mechanism by which progeny virions are released from infected cells has not been determined, but it is thought that reovirus egress occurs by cell lysis. However, most virus in infected cell lysates remains bound to cell fragments, which probably reflects the association of viral replication centers with cytoskeletal components. Reovirus induces apoptosis of infected cells. Thus, it is possible that virus may be released in association with membrane-bound apoptotic bodies.

Numerous attempts have been made to establish a reverse genetics system for reoviruses. Several reports describing such a system (e.g. Roner et al. 1990; Roner and Joklik 2001) await wider application.

Apoptosis induced by reovirus infection

Reovirus induces the morphological and biochemical hallmarks of apoptosis in cultured cells and in vivo. Strain-specific differences in the capacity of reovirus to induce apoptosis are determined by the σ1-encoding *S1* gene (Tyler et al. 1995). Binding of σ1 to both sialic acid (Connolly et al. 2001) and JAM1 (Barton et al. 2001b) is required to achieve maximal levels of apoptosis.

However, viral attachment is not sufficient to elicit an apoptotic response. Inhibitors of acid-dependent viral disassembly block apoptosis by reovirus (Connolly and Dermody 2002), indicating a requirement for post-attachment entry steps in apoptosis induction. Viral transcription, however, is dispensable for apoptosis by reovirus, as inhibitors of viral RNA synthesis do not diminish the capacity of reovirus to induce apoptosis (Tyler et al. 1995; Connolly and Dermody 2002). These findings suggest that apoptosis induced by reovirus is triggered by a signaling pathway initiated by early steps in the viral replication cycle.

A critical component of the signaling cascade that leads to apoptosis of reovirus-infected cells is the transcription factor NF-κB. Within hours following viral adsorption, reovirus activates NF-κB, and this activation is required to induce an apoptotic response (Connolly et al. 2000). In some cell types, reovirus infection leads to expression of death receptors DR4 and DR5 and their pro-apoptotic ligand TRAIL (Clarke et al. 2000), suggesting apoptosis is induced by an extrinsic cell-death pathway. However, mitochondrial injury during reovirus infection has been documented (Kominsky et al. 2002), providing evidence that intrinsic signals are also involved in apoptosis induced by reovirus infection.

Pathogenesis: the mouse model

The elucidation of viral genetic determinants of the pathogenesis of reovirus disease has led to the establishment of general principles of viral disease mechanisms (for reviews, see Virgin et al. 1997; Tyler 2001).

This analysis is based on two main observations:

- Different reovirus serotypes cause different disease manifestations in the CNS of newborn mice (reovirus type 1: hydrocephalus; reovirus type 3: encephalitis).
- Different reovirus serotypes have different RNA and protein migration profiles by polyacrylamide gel electrophoresis (PAGE) (Figure 3).

Because reovirus has a segmented genome, 'reassortant genetics' can be used to identify specific viral genes involved in various aspects of virus–host interaction. Parental virus strains that differ with respect to a given phenotype (e.g. tissue tropism) are used to generate progeny reassortant viruses containing different combinations of gene segments from each of the parents (Figure 4). Careful observation of mice infected with various reassortant viruses has revealed the relative contributions of individual genes or sets of genes and their products to the pathogenic process.

Viruses produce disease by interacting with the host in a discrete series of steps:

- entry into host
- primary replication
- spread through the host
- specific cell and tissue tropism
- host immune response.

Following oral inoculation, reovirus is taken up by Peyer's patches via the M cells (Wolf et al. 1981), specialized gut epithelia overlying the lymphoid tissue of the Peyer's patches. Virus then disseminates to various sites within the host including the CNS. However, not all reovirus strains are capable of growth in the intestine and systemic spread following oral inoculation. Strain T3D is neurovirulent after intracranial (Weiner et al. 1980b; Morrison et al. 1993) or intramuscular (Tyler et al. 1986) inoculation but does not infect the murine intestine after oral inoculation. By contrast, T1L produces high titers in intestinal tissue and spreads systemically (Rubin and Fields 1980; Keroack and Fields 1986; Bodkin and Fields 1989) (Table 2, p. 8).
Using T1L × T3D reassortant viruses, it was shown that the σ1-encoding *S1* gene and the λ2-encoding *L2* gene are the primary determinants of strain-specific differences in reovirus growth in the intestine (Bodkin and Fields 1989).

Following oral or intramuscular inoculation, strains of both serotypes invade the CNS, yet by different routes and with distinct pathological consequences (Table 2).
T1 reovirus spreads to the CNS hematogenously and infects ependymal cells (Weiner et al. 1980b; Tyler et al. 1986), resulting in hydrocephalus (Weiner et al. 1977). By contrast, T3 reovirus spreads to the CNS neurally and infects neurons (Weiner et al. 1980b; Tyler et al. 1986; Morrison et al. 1991), causing lethal encephalitis (Weiner et al. 1977; Tardieu et al. 1983). T1L × T3D reassortant viruses were used to show that the pathways of viral spread (Tyler et al. 1986) and tropism for neural tissues (Weiner et al. 1980b; Dichter and Weiner 1984) segregate with the σ1-encoding *S1* gene (Weiner et al. 1980a; Lee et al. 1981). Thus, σ1 determines the CNS cell types that serve as targets for reovirus infection.

Reovirus pathogenesis is not restricted to the CNS. Reovirus infection causes pathology and physiological dysfunction in a wide range of organs and tissues including the hepatobiliary system, myocardium, lungs, and endocrine tissues. Reovirus hepatobiliary disease (Wilson et al. 1994) and myocarditis (Sherry et al. 1989) in mice have become particularly well-established experimental models of viral injury at these sites.

Myocarditis induced by reovirus is unusual in comparison to other viral causes of myocarditis in that the pathogenesis is not immune-mediated. Instead, reovirus cytopathicity is a direct cause of myocyte injury, which results from a complex interplay of the interferon (Sherry et al. 1998; Noah et al. 1999; Azzam-Smoak et al. 2002) and apoptotic pathways (DeBiasi et al. 2001). Efficiency of viral RNA synthesis is a key factor in determining the extent of myocardial injury (Sherry et al. 1996). Accordingly, viral gene segments encoding proteins involved in

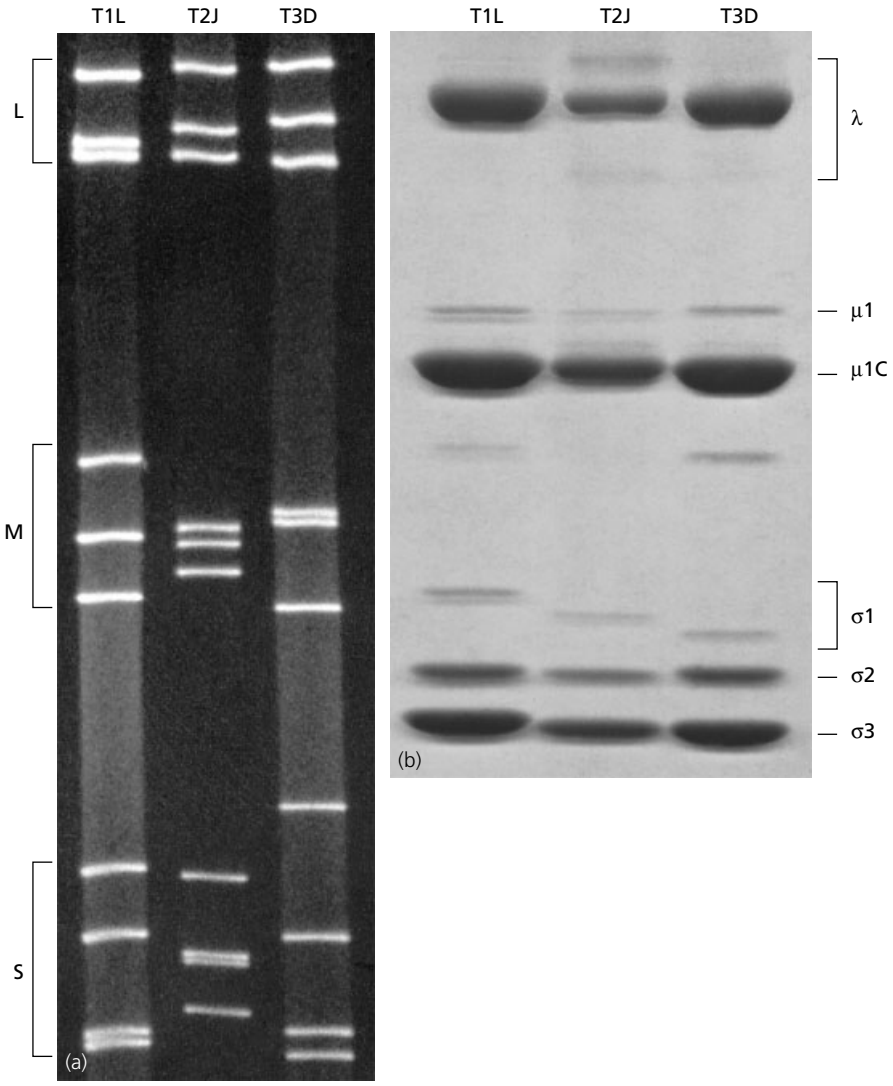

Figure 3 *Electrophoretic profiles of* **(a)** *genomic dsRNA and* **(b)** *structural proteins of prototype reovirus strains T1L, T2J, and T3D. Equal numbers of reovirus particles were resolved by acrylamide gel electrophoresis.* **(a)** *Viral dsRNA gene segments were visualized following staining of the gel with ethidium bromide. Gene segment size classes, large (L), medium (M), and small (S), are indicated.* **(b)** *Viral structural proteins were visualized following staining of the gel with Coomassie blue. Viral proteins are indicated. This figure was prepared with the assistance of J. Denise Wetzel, Vanderbilt University.*

viral transcription and genome replication play important roles in determining strain-specific differences in the capacity of reovirus to induce myocarditis (Sherry and Fields 1989; Sherry and Blum 1994; Sherry et al. 1998).

Illness in humans

Reoviruses are recognized and well-studied pathogens of mice and other rodents, but it has been difficult to link reovirus infections to disease in humans. Although infections of humans are common, most infections are asymptomatic. Reovirus infections are occasionally associated with enteritis, rhinitis, and CNS disease in infants and children (Tyler 2001).

Reovirus has also been linked to neonatal biliary atresia in humans (Tyler et al. 1998). While a definitive causal relationship between reovirus infection and biliary atresia has not been established, it is quite provocative that infection of newborn mice (ND4 Swiss Webster) with a reovirus type 3 strain that utilizes sialic acid as a coreceptor was associated with viral replication in bile duct epithelium and abnormalities in hepatobiliary function very similar to those accompanying bile duct obstruction in humans, including hyperbilirubinemia, elevated levels of serum alkaline phosphatase, and steatorrhea (for details see Barton et al. 2003; for review Forrest and Dermody, 2003).

Epidemiology

By the end of childhood, most humans have antibodies against each of the three reovirus serotypes (Jackson

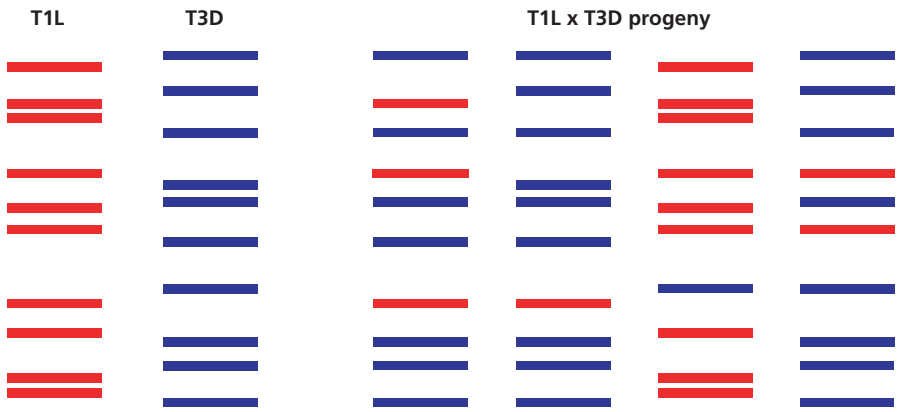

Figure 4 *Generation of reassortant viruses. Biological polymorphisms exhibited by different reovirus strains can be mapped to specific gene segments through the use of reassortant viruses. Reovirus strains are distinguishable by signature electrophoretic profiles of their dsRNA gene segments (i.e. electropherotypes). Co-infection of cells with different strains produces a collection of progeny reassortant viruses containing various combinations of gene segments from each parent. A phenotypic difference between two parental strains can be genetically mapped by screening the reassortant viruses in appropriate assays and correlating expression of the phenotype with a specific parental gene segment. The electropherotypes of prototype strains T1L and T3D are illustrated in this figure.*

Table 2 *Properties of reovirus pathogenesis attributable to σ1 protein*

	Functional property				
Viral serotype	**Intestinal growth**	**Pathway of spread**	**CNS tropism**	**Tail receptor**	**Head receptor**
Type 1	Yes	Hematogenous	Ependymal cells	Unknown	JAM1
Type 3	Variable[a]	Neural	Neurons	Sialic acid	JAM1

a) Some type 3 reovirus strains can infect the intestine and disseminate systemically. Others fail to infect intestinal tissue and do not spread to distant sites.

et al. 1961; Lerner et al. 1962; Leers and Rozee 1966). In a study conducted at a pediatric hospital in the USA, the incidence of anti-reovirus antibodies increased from less than 25 percent in those less than 1 year of age to greater than 70 percent in those over 3 years of age (Lerner et al. 1962). Reovirus-specific antibodies are also found in most mammals. Reovirus infections do not appear to have a seasonal distribution, although the original isolates were from patients with diarrheal illnesses occurring during the summer months. Reoviruses are commonly found in environmental water sources (Adams et al. 1982; Ridinger et al. 1982; Dahling et al. 1989), and it has been suggested that human fecal contamination is a major source of virus in water supplies (Matsuura et al. 1993).

Reovirus oncolysis

Transformed cells are substantially more permissive for reovirus infection than untransformed cells (Strong and Lee 1996). This property correlates with an activated *Ras* signaling pathway (Strong et al. 1998). Reovirus kills transformed cells in animals (Coffey et al. 1998) and is being investigated for the treatment of some forms of cancer in humans.

ORBIVIRUSES

Genome, gene protein assignment, and structure

The genome of orbiviruses consists of 10 segments of dsRNA of three size classes termed *L1–L3*, *M4–M6*, and *S7–S10*. Full-length sequences of each of the genome segments of BTV 10 have been determined (Roy 1989). Protein-coding assignments have been established for each of the BTV genes (Roy 2001). The genome segments, their size in bp, and the size, number of molecules, and location in the virion, post-translational modifications, and some of the recognized functions of the protein products are shown in Table 3.

With the exception of the *S10* RNA, orbivirus genes are monocistronic, encoding seven structural proteins (VP1–VP7) and four nonstructural proteins (NS1–NS3, NS3A). The 5′ and 3′ termini of the orbivirus RNA segments are conserved and have inverted complementarity (Roy 2001), theoretically allowing formation of 'panhandle' structures, a property common to other members of the *Reoviridae*.

The inner core of the virion consists of the segmented genome with which three minor proteins, VP1, VP4, and

Table 3 *Gene protein assignments and functions of orbivirus proteins (BTV 10)*

RNA segment		Protein				
Designation	Size (bp)	Designation	Deduced mol. mass (kDa)	Number of molecules per virion	Location in virion	Function (modification)
L1	3 954	VP1	149.6	12	Inner core	RdRp
L2	2 926	VP2	111.1	180	Outer capsid (exposed)	Viral attachment protein; hemagglutinin; type-specifying neutralization antigen
L3	2 772	VP3	103.3	120	Core (subcore layer)	Scaffold protein for VP7; interaction with genomic RNA
M4	2 011	VP4	76.4	ca. 24	Inner core	Guanylyltransferase; methyltransferases; NTPase; RNA 5′-triphosphatase; pyrophosphatase
M5	1 639	VP5	59.2	360	Outer capsid (under surface)	Glycoprotein; crossreactive antigen; not neutralization-specific; virus penetration; fusogenic; positioning of VP2?
M6	1 770	NS1	64.4	. . .	Nonstructural	Tubule formation; translocation of virus particles?
S7	1 156	VP7	38.5	780	Core surface	Group-specific antigen; core binding to insect cells
S8	1 124	NS2	41.0	. . .	Nonstructural	Phosphoprotein; binds ssRNA; morphogenesis (cytoplasmic inclusions)
S9	1 046	VP6	35.8	ca. 72	Inner core	Binds ssRNA and dsRNA; helicase; ATPase
S10	822	NS3	25.6	. . .	Nonstructural	Glycoprotein; virus release
	. . .	NS3A	24.0	. . .	Nonstructural	Function unknown

Slightly modified from Roy 2001, with permission of author and publisher.

VP6 (encoded by the *L1*, *M4*, and *S9* RNAs, respectively), are closely associated. These proteins serve as the viral RdRp, guanylyltransferase, and RNA-binding proteins, respectively. The inner core is surrounded by an inner capsid layer composed of VP3 trimers (interior of the inner capsid; encoded by the *L3* RNA), which form a scaffold for a layer of VP7 trimers (exterior of the inner capsid; encoded by the *S7* RNA). The structure containing the genome and proteins VP1, VP4, VP6, VP3, and VP7 is also referred to as the viral core. The core is surrounded by the outer capsid, which consists of VP5 (encoded by the *M5* RNA), a glycoprotein, and VP2 (encoded by the *L2* RNA). VP2 is the most variable of the BTV proteins and carries major epitopes determining serotype and neutralization specificity. VP2 is the orbivirus attachment protein and hemagglutinin (Roy 2001). Antibodies directed against VP2 protect animals from infection, but antibodies directed against VP5 do not.

There are four orbivirus nonstructural proteins:

- NS1 (encoded by the *M6* RNA)
- NS2 (encoded by the *S8* RNA)
- NS3/NS3A (encoded by the *S10* RNA, initiating from in-frame AUG codons and differing in size by 1.5 kDa).

The precise functions of the orbivirus nonstructural proteins are not fully understood (Roy 2001).

Although the three main genera of the *Reoviridae* (reo-, rota-, and orbiviruses) have unique characteristics in structure, replication, pathogenesis, and epidemiology, these viruses exhibit remarkable parallels in the functions of their structural proteins (Table 4). All these viruses have a virion-associated RdRp and proteins with RNA-capping activity. They also share structural proteins that act as scaffolds in several layers of the capsid and others that interact with receptors, hemagglutinate, and determine neutralization (serotype) specificity. There is increasing evidence that the nonstructural proteins have similar functions in morphogenesis, virion assembly, and release, but the parallels in the functions of these proteins are less obvious.

Replication

Orbiviruses are unusual among the *Reoviridae* in that they replicate in both mammalian and insect cells. This property reflects the capacity of these viruses to productively infect cells at very different temperatures (37°C and 22–27°C, respectively).

Table 4 *Functional similarities of structural proteins of* reovirus*, rotavirus, and* bluetongue virus[a]

		Protein designation (and coding RNA segment)					
Location in virion	**Function**	**Reovirus**		**Rotavirus**		**Bluetongue virus**	
Core (inner layer)	RdRp	λ3	(*L1*)	VP1	(1)	VP1	(*L1*)
	Guanylyltransferase, methyltransferases	λ2	(*L2*)	VP3	(3)	VP4	(*M4*)
	Scaffolding protein;	λ1/σ2	(*L3*/*S2*)	VP2	(2)	VP3	(*L3*)
	RNA binding	μ2	(*M1*)			VP6	(*S9*)
Inner capsid (middle layer)	Structural protein	μ1/μ1C	(*M2*)	VP6	(6)	VP7	(*S7*)
Outer capsid (outer layer)	Structural protein	σ3	(*S4*)	VP7	(7[b])	VP5	(*M5*)
	Attachment protein	σ1	(*S1*)	VP4	(4)	VP2	(*L2*)

Slightly modified from Mattion et al. 1994, with permission of authors and publisher.
a) Functional analogies of the nonstructural proteins (reovirus μNS, σNS, and σ1s; rotavirus: NS1–NS5; and bluetongue: NS1–NS3, NS3A) are difficult to define at present.
b) Depending on the strain, VP7, NSP2, and NSP3 are encoded by RNA 7, 8, or 9.

Orbiviruses attach via VP2 to sialic-acid-containing receptors on the surface of mammalian cells. It is possible that receptors in addition to sialic acid are used for viral attachment in a manner analogous to that of other members of the *Reoviridae*; however, such receptors have thus far not been identified. Core particles, which lack VP2 and VP5, adsorb well to and infect insect cells, suggesting that VP7 at the exterior of the core interacts with insect cell receptors. Virus enters mammalian cells by endocytosis, after which the outer capsid (VP2 and VP5) is removed. The resulting core particle is transcriptionally active and catalyzes synthesis of viral mRNAs. The viral RdRp has a temperature optimum in vitro of 28°C but retains activity at 37°C (Van Dijk and Huismans 1982), reflecting adaptation of viral replication to different types of host cells. VP4 mediates capping enzyme activities, resulting in the production of capped mRNAs (Martinez-Costas et al. 1998; Ramadevi et al. 1998; Ramadevi and Roy 1998). Viral proteins are synthesized from 2 to 14 hours after infection and accumulate in the cytoplasm. The viral NS1 protein forms extensive tubules throughout the cytoplasm, but their function is unknown (Eaton et al. 1990). Orbivirus release is thought to be accomplished by cell lysis.

Core-like particles (CLP) and virus-like particles (VLP) largely self-assemble when combinations of viral proteins (CLPs: VP3 and VP7; VLPs: VP2, VP3, VP5, and VP7) are overexpressed simultaneously in insect cells using baculovirus recombinants (French et al. 1990) (Figure 5).

Detailed morphological studies of VLPs (Hewat et al. 1994) and biochemical analyses of individual viral proteins have led to the identification of protein

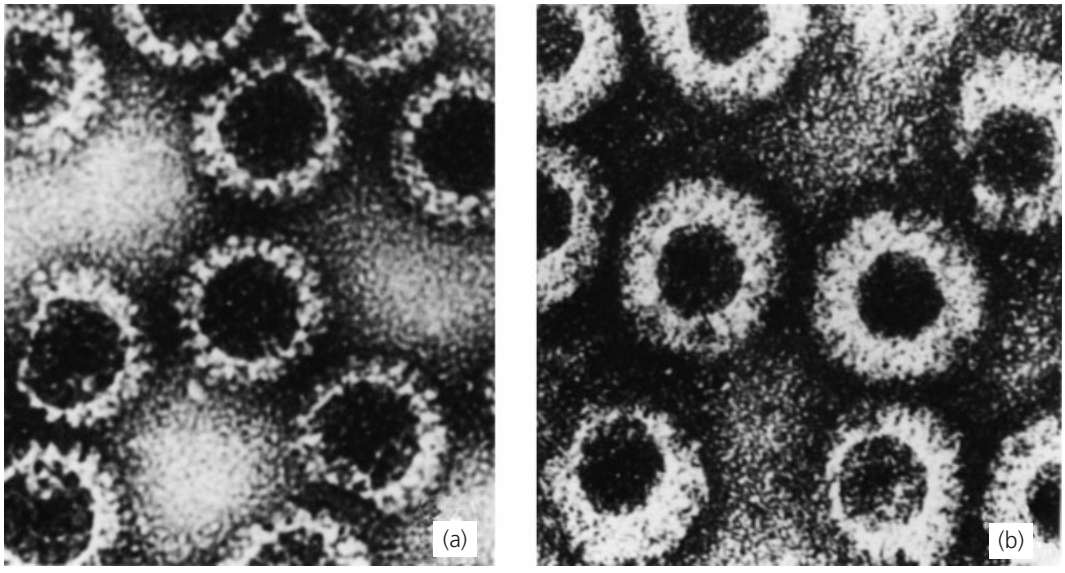

Figure 5 (a) *CLPs and* **(b)** *VLPs of BTV. The particles were obtained by coexpression of baculovirus recombinants in insect cells (CLPs: VP3 and VP7; VLPs: VP2, VP3, VP5, and VP7). Particles were purified and examined by cryo-electron microscopy and three-dimensional image reconstruction. (From French et al. 1990 with permission of the authors and publisher.)*

domains responsible for protein–protein and protein–RNA interactions (reviewed by Roy 2001). The structure of VP7 has been solved using X-ray crystallography, and its domain structure is known in atomic detail (Grimes et al. 1995). The structure of the BTV core also has been solved using X-ray crystallography, revealing significant insights into the nature of the enzymatic activities of the core and providing clues to mechanisms of RNA packaging (Grimes et al. 1998).

Pathogenesis

In mammals, BTV replicates mainly in the lymphoreticular system. The virus has a particular affinity for endothelial cells of capillaries, causing ischemic lesions, inflammatory changes, and edema. There is a prolonged viremia that lasts for up to 1 month, although neutralizing antibody appears 7–10 days following infection (Parsonson 1990). Different BTV serotypes are capable of gene segment reassortment in vitro and in their natural hosts (Samal et al. 1987), giving rise to considerable diversity among field isolates.

Illness

The incubation period for BTV-induced disease is 6–9 days. Illness starts with fever, followed by vomiting and hemorrhage. Bronchopneumonia occurs and can be fatal. Disease severity can vary, and many BTV infections are subclinical. Symptomatic disease is observed in 10–30 percent of infected animals, and mortality is approximately 5 percent (Parsonson 1990). Besides sheep, cattle are commonly infected, although these infections are usually asymptomatic. BTV can infect the fetus and is associated with abortions and fetal abnormalities in sheep and cattle (Parsonson 1990).

Epidemiology

BTV constitutes one of at least 14 serogroups of orbiviruses (Mertens et al. 2000) and occurs in 24 serotypes. These viruses infect sheep, cattle, goats, and wild ungulates in many countries of the tropical and subtropical zones (Parsonson 1990). Their distribution is supported by a variety of insect vectors (*Culicoides* midges, ticks, and mosquitoes) in which the virus replicates (Mellor 1990). Other orbivirus serogroups, such as African horse sickness virus (AHSV), occurring in horses, donkeys, and dogs, and epizootic hemorrhagic disease virus (EHDV), occurring in deer, also have diverged into serotypes. Sequence comparisons of BTV, AHSV, and EHDV indicate that there is considerable conservation of the *VP3* gene product (58–79 percent). There is less conservation of the *VP7* gene product (43–63 percent), the protein specifying the group antigen, and less still of the *VP2* gene product (32–45 percent), which specifies viral serotype (Roy 2001).

Vaccines

Antibodies specific for BTV VP2 are neutralizing and provide effective homotypic protection (Huismans et al. 1987). Generation of CLPs and VLPs by co-expression of viral genes in insect cells infected with baculovirus recombinants (e.g. French et al. 1990) (Figure 5) has enabled the development of vaccine candidates (Roy et al. 1992; Roy and Sutton 1998). These particles, formulated either as mixtures derived from different serotypes or as particle chimeras, have the potential to elicit broad protective immune responses. A vaccine of inactivated particles of AHSV serotype 4 has been shown to fully protect against homologous challenge (House 1998).

COLTIVIRUSES

Coltiviruses possess a genome of 12 segments of dsRNA and are now classified as a separate genus of the *Reoviridae* (Mertens et al. 2000). Within the *Coltivirus* genus, two groups and several subgroups have been defined based on sequence comparisons of RNA segments (Attoui et al. 1998). These viruses are isolated from ticks, mosquitoes, rodents, and humans. Colorado tick fever virus (CTFV) can cause a fatal disease in humans. Different isolates of CTFV differ mostly in RNA segments 4 and 6, suggesting that protein products of these genes are located in the outer capsid and carry neutralization-specific epitopes (Bodkin and Knudson 1987).

CTFV is endemic in the Rocky Mountain region of the USA and Canada. In the insect vector, which includes ticks of many species, CTFV infects the midgut, from which it spreads to other organs including the salivary glands, allowing transmission to mammalian hosts by bite. As rodents have prolonged CTFV viremia, they may serve as a reservoir for tick infection in nature. However, a variety of other mammals (e.g. marmots, deer, elk, and sheep) can also be infected. Humans are infected by bite but are not thought to generate sufficient viremia to maintain the transmission cycle (Monath and Guirakhoo 1996).

CTFV can be isolated from the erythrocytes of humans for as long as 120 days after infection, whereas it disappears from plasma very quickly (Bowen 1988). This cell-associated viremia is likely to originate from infection of bone marrow stem cells; from these cells, virus is continuously dispersed into the periphery in maturing erythrocytes. Interestingly, the viremia persists in the presence of a strong neutralizing antibody response (Monath and Guirakhoo 1996).

After an incubation period of 4 days (range 1–19 days), humans infected with CTFV develop fever (possibly due to elevated interferon levels), leukopenia, gastrointestinal symptoms (20 percent of cases), rash (10 percent), and meningoencephalitis (3–7 percent). A severe hemorrhagic form of the disease (purpura,

epistaxis, and gastrointestinal bleeding) is rarely seen. The case-fatality rate is less than 0.1 percent.

CTFV can be isolated from the blood of infected persons using continuous cell lines (e.g. Vero and BHK-21 cells), and viral antigen can be detected in erythrocytes by immunofluorescence testing (IFT). Serological diagnosis can be accomplished by IFT using infected Vero cells as antigen or by enzyme-linked immunosorbent assay (ELISA).

Treatment of Colorado tick fever is supportive. Ribavirin has been used in cases of severe hemorrhagic disease. However, controlled clinical trials to test its efficacy have not been reported.

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Rotaviruses

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Rotaviruses were discovered in 1973 as being associated with diarrheal disease in children (Bishop et al. 1973; Flewett et al. 1973) and have since been recognized as the major cause of gastroenteritis in infants and young children and in a wide range of animal species. In humans, they cause 500 000–600 000 deaths each year (Miller and McCann 2000), and there is an urgent need for a vaccine. For reviews of other viral causes of gastroenteritis, see Blacklow and Greenberg (1991), Kapikian (1994), Hart and Cunliffe (1997), Chiba et al. (1997), Desselberger (1998a), Chadwick and Goode (2001), Cohen et al. (2002), Desselberger and Gray (2003), and Adenovirus; Human enteric RNA viruses: astroviruses; and Human enteric RNA viruses: noroviruses and sapoviruses.

GENOME, GENE PROTEIN ASSIGNMENT, AND STRUCTURE

Rotaviruses have a genome of 11 segments of double-stranded RNA (dsRNA) which can be easily separated by polyacrylamide gel electrophoresis. The RNA segments code for six structural (VP1, VP2, VP3, VP4, VP6, VP7) and six nonstructural proteins (NSP1–NSP6) (Estes 2001). The structural proteins make up a triple-layered particle and are located in the core (inner layer; VP1–VP3), inner shell (intermediate layer; VP6) and outer shell (outer layer; VP4, VP7). The wheel-like (Latin: *rota*) appearance by electron microscopy of the triple-layered particles is diagnostic (Figure 1a). Figure 1b shows diagrammatically the gene coding assignment, the position of the structural proteins in the particles and their three-dimensional (3D) structure as obtained by image processing techniques from cryo-electron micrographs.

Further details of the 3D structure have been elucidated in a number of excellent studies by the groups of Prasad (Prasad et al. 1988, 1990, 1996; Shaw et al. 1993; Prasad and Chiu 1994; Lawton et al. 1997a, b, 1999, 2000; Prasad and Estes 1997; Crawford et al. 2001; Pesavento et al. 2001); Yeager (Yeager et al. 1990, 1994), Rey (Petitpas et al. 1998; Lepault et al. 2001; Mathieu et al. 2001) and Hewat (Thouvenin et al. 2001). According to their findings, the genome is comprised within the core shell formed by VP2. The transcription complex, consisting of VP1 and VP3, is attached at the 12 fivefold symmetry positions on the inside of the shell. The addition of VP6 (260 trimers) produces double-layered particles, and further addition of VP7 (780 molecules) and VP4 (60 dimers) leads to the formation of triple-layered particles which are the infectious virions after VP4 cleavage. The triple-layered capsid is ordered along five-, three-, and two-fold symmetry axes and is perforated by 132 aqueous channels of types I–III (located in the three symmetry positions). The type I channels play an important role in transcription (see below).

Complete gene–protein assignments have been achieved for several *Rotavirus* strains. Table 1, p. 3

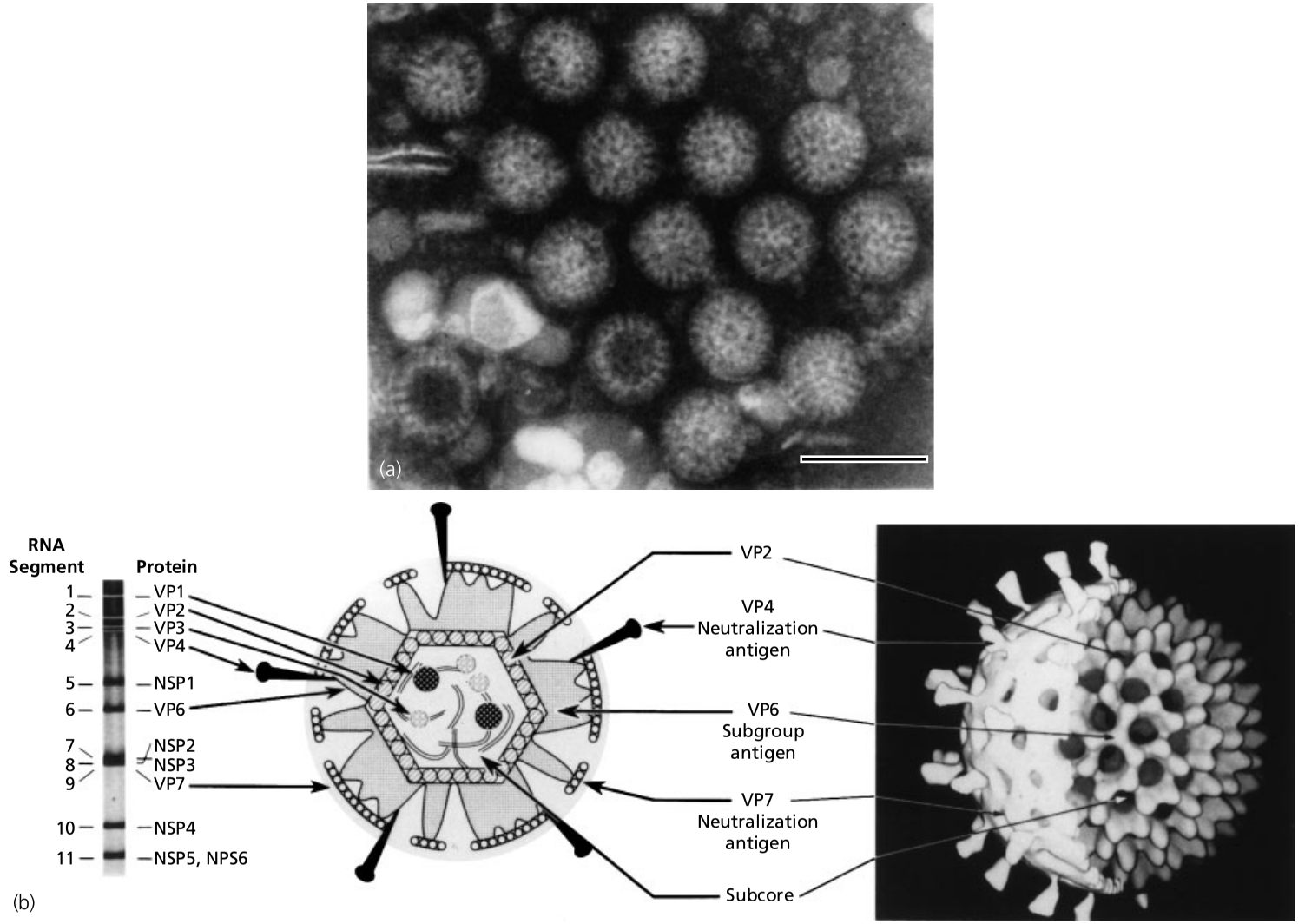

Figure 1 (a) *Rotaviruses in the fecal suspension of an infant suffering from acute gastroenteritis. Electron micrograph, PTA stain. (Courtesy of Dr. E.A.C. Follett, Regional Virus Laboratory, Ruchill Hospital, Glasgow. Bar, 100 nm.)* **(b)** *RNA profile (10 percent SDS polyacrylamide gel, silver stain), protein products (gene protein assignment) and particle structure (diagram and reconstruction from a cryo-electron micrograph) of rotaviruses. (From Mattion et al. 1994, with permission of the authors and publisher)*

Table 1 *Genes, gene protein assignments, and functions of proteins of group A* Rotavirus

RNA segment		Protein product					
No.	Size (bp)	Description	Deduced mol. mass (kDa)	Location	No. of molecules per virion	Post-translational modification	Functions
1	3 302	VP1	125.0	Inner core	12	–	RNA-dependent RNA polymerase; binds ssRNA; complex with VP3
2	2 690	VP2	94.0	Core	120	Myristylation	Binds RNA; required for replicase activity of VP1
3	2 591	VP3	88.0	Inner core	12	–	Guanylyltransferase; methyltransferase; complex with VP1
4	2 362	VP4	86.8	Outer capsid (dimer)	120	Proteolytic cleavage to VP5* and VP8*	Hemagglutinin; cell attachment; neutralization antigen (ab protective); fusogenic; protease-enhanced infectivity; virulence (mice, piglets)
5	1 611	NS53, NSP1 (VP5)	58.7	Nonstructural	NA[c]	–	Binds RNA; virulence (mice); nonessential for replication (some strains); interacts with IRF-3
6	1 356	VP6	44.8	Inner capsid (trimer)	780	Myristylation	Group- and subgroup-specific antigen; protection (intracellular neutralization)? Required for transcription
7[a]	1 059	VP9 (NSP3)	34.6	Nonstructural (dimer)	NA	–	Binds RNA (3′ end); competes with cellular PABP for interaction with eIF-4G1 (translation); inhibits host-cell translation
8[a]	1 104	NS35, NSP2 (VP8)	36.7	Nonstructural (octamer)	NA	–	Binds RNA; NTPase; helicase; involved in +strand RNA packaging; virulence (mice)
9[a]	1 062	VP7	37.4[b]	Outer capsid (trimer)	780	Cleavage of signal sequence; glycosylation	Neutralization antigen (ab protective); binds Ca^{2+}
10	751	VP12 (NSP4)	20.3	Nonstructural	NA	Glycosylation (VP10, NS28); trimming	Intracellular receptor (morphogenesis); viral enterotoxin (secreted cleavage product); protection by specific ab; virulence (mice, piglets)
11	667	VP11 (NSP5)	21.7	Nonstructural	NA	O-glycosylation, phosphorylation	Binds RNA; protein kinase; interacts with NSP2, VP2, and NSP6
		NSP6	12.0	Nonstructural	NA		Interacts with NSP5

Modified from Estes (2001).

a) This gene protein assignment is of the SA11 rotavirus strain.

b) Second in-frame initiation codon located 30 codons downstream (deduced mol. mass 33.9 kDa).

c) NA, not applicable.

lists the RNA segments and their products for the simian rotavirus SA11 strain; also noted are post-translational modifications and functions as far as known.

CLASSIFICATION INTO SUBGROUPS, SEROTYPES, AND GENOTYPES

A classification of rotaviruses has been derived from immunological characteristics of various components of the particles and from genomic composition (Estes 2001). Groups A–E can be differentiated according to lack of serological crossreactivities of the inner capsid protein VP6 with polyclonal and monoclonal antibodies. There may be two more groups, F and G (Bridger 1987). Within group A rotaviruses, subgroups (I, II, I+II, nonI, nonII) were defined according to exclusive reactivities of two VP6-specific monoclonal antibodies (Greenberg et al. 1982). Types within group A are determined by cross-neutralization studies as serotypes (Offit et al. 1986; Green and Kapikian 1992) or by sequence comparison as genotypes. (Typing of rotaviruses within other groups is rudimentary.) As the two surface proteins (VP4 and VP7) carry neutralization-specific antigens, a dual classification scheme, similar to that developed for influenza viruses, has been established, differentiating G types (for VP7 which is a **g**lycoprotein) and P types (for VP4 which is a **p**rotease-sensitive protein, being cleaved post-translationally into its subunits VP5* and VP8*). So far, 14 G types and at least 20 P types have been differentiated, indicating extensive genomic diversity within group A rotaviruses (Estes 2001). As these proteins are coded for by different RNA segments and as rotaviruses of the same group reassort readily in doubly infected cells, both in vitro (Garbarg-Chenon et al. 1984; Graham et al. 1987) and in vivo (Gombold and Ramig 1986; Ward et al. 1990), the observed diversity resulting from various combinations of VP7 and VP4 types is very large. Whilst the correlation of G serotypes and genotypes is practically complete, for many P genotypes no serotype has been established yet. Therefore P serotype and genotype are designated separately but jointly (where available), the latter in square brackets: for example, the human Wa strain is classified as G1P1A[8], the human DS-1 strain as G2P1B[4], the equine strain Eq/L338 as G13P12[18], the bovine strain Bo/993/83 as G7P[17], etc.

REPLICATION

Rotaviruses spread via the oral–fecal route and infect the small intestine after oral ingestion. Multiplication occurs in the mature epithelial cells at the tips of the villi of the small intestine. Rotaviruses grow well in secondary monkey kidney cells and in immortalized monkey kidney cell lines (MA104, BS-C1) in the presence of trypsin, and therefore their replication in vitro could be studied in detail (Estes 2001). Figure 2 is a diagrammatic presentation of these events. Triple-layered particles (i.e. the infectious virions) attach to the host cells via the outer-layer protein VP4 (Ludert et al. 1996). Virus entry is by receptor-mediated endocytosis or direct penetration (Cuadras et al. 1997; Gilbert et al. 1997). The cellular receptor or receptors have not been fully characterized yet, but some animal strains use sialic acid located on glycolipids (Ciarlet and Estes 1999; Ciarlet et al. 2002b). Other strains seem to recognize galactose (Jolly et al. 2001). In addition to glycolipids, several integrins have been proposed to mediate uptake, possibly in a post-attachment step, acting as co-receptors (Coulson et al. 1997; Guerrero et al. 2000; Hewish et al. 2000). The heat-shock cognate (Hsc) protein 70 may also be involved as a co-receptor (Guerrero et al. 2002). For porcine rotavirus, a ganglioside receptor has been described (Rolsma et al. 1998). Replication is exclusively in the cytoplasm. After removal of the outer capsid proteins, the viral RNA-dependent RNA polymerase (coded for by RNA 1) is activated in double-layered particles (Cohen et al. 1979), and by use of the VP1/VP2/VP3 transcription complex (Lawton et al. 2000) large numbers of positive-strand RNA molecules are transcribed and exit from the double-layered particles via their 12 aqueous channels located on the vertices of the fivefold symmetry axes. This process is ATP-dependent. Transcription is asymmetric, i.e. only positive-strand RNAs appear as transcription products. Multiple RNA molecules can be transcribed from a single actively transcribing particle. The new RNA molecules act as mRNAs, and their translation products start to accumulate in the cytoplasm. NSP3 is intimately involved in translation by binding to the 3′ end of mRNA and to the eukaryotic translation factor eIF-4G (Vende et al. 2000). Molecules of mRNA seem to be pulled through VP2 oligomers (the nascent core) by means of a complex formation with NSP2 which has NTPase activity and acts as a molecular motor (Taraporewala et al. 1999; Patton 2001; Schuck et al. 2001; Taraporewala and Patton 2001). NSP5 that interacts with VP2 and NSP2 (Berois et al. 2003) is also involved at this stage. Double-layered particles are formed, consisting of VP1, VP2, VP3, and VP6 and several of the nonstructural proteins, and containing one genome equivalent of packaged single-stranded RNA which is then replicated to form dsRNA. Double-layered particles accumulate and form pseudocrystalline aggregates termed viroplasm (= intracytoplasmic inclusion bodies). It is not clear at present how the very tight packaging control of rotaviruses (and of other *Reoviridae*) is achieved. When budding through the rough endoplasmic reticulum (RER), whereby NSP4 acts as an intracellular receptor for VP6, double-layered particles incorporate VP7 and VP4 to form the third, outer layer (a transient, RER-derived envelope is shed before complete maturation). Triple-layered infectious virions are released by cell lysis. VP4 may reach the plasma membrane through

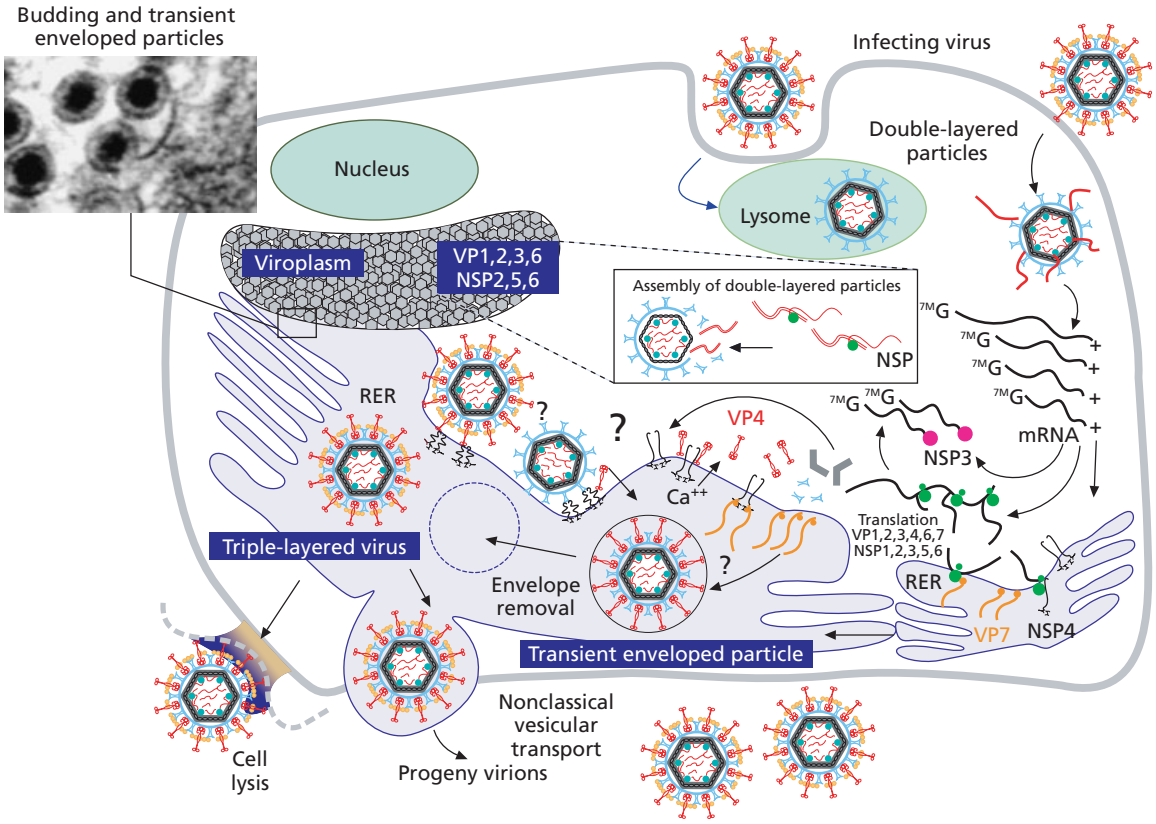

Figure 2 *Major features of the* Rotavirus *replication cycle. For details see text. Triple layered rotavirus particles (TLPs, i.e. infectious virions) attach to the surface of the host cell by its outer layer protein, VP4, interacting with sialic acid (in glycolipids) or other receptors on the cell surface. Virus entry is by receptor mediated endocytosis. The outer layer proteins VP4 and VP7 are removed (possibly in lysosomes). The resulting double layered particles (DLPs) are transcription active, i.e. they synthesize and extrude large numbers of mRNAs (specific for all RNA segments) that are capped but not polyadenylated. Only positive sense transcription products are made. The mRNAs are translated into proteins using the cellular machinery. This process is facilitated by the viral NSP3 interacting with the 3′ end of the mRNA and forming a complex with the eukaryotic translation factor eIF4G. Rotavirus cores are formed, consisting of VP2, VP1, and VP3, into which rotavirus mRNA molecules are incorporated. The rotavirus proteins NSP2 and NSP5 are intimately involved in this process which is ATP dependent. Within the cores the ssRNA segments are replicated to form the dsRNA genome. The early packaging process is tightly controlled. Rotavirus cores interact with VP6 to form DLPs which in turn aggregate into pseudocrystalline arrays termed 'viroplasm' (i.e. intracytoplasmic inclusion bodies). DLPs then bud through the rough endoplasmic reticulum (RER); this step is facilitated by interaction of NSP4 that has become part of the RER membranes with DLPs via VP6 (NSP4 acting as an intracellular receptor for DLPs). In the RER, DLPs acquire VP7 and VP4 to become TLPs. During this process particles are transiently enveloped; however, the envelope is shed before complete maturation (this step is a unique characteristic of rotavirus replication that is still poorly understood). TLPs are then released by the cell undergoing lysis or by a 'non-classical' vesicular transport involving interaction with lipid 'rafts' near the plasma membrane. (Redrawn from Estes 2001, with permission of the publisher)*

the microtubule network and interact with cholesterol liposphingolipid rafts, possibly promoting assembly near the plasma membrane (Nejmeddine et al. 2000; Sapin et al., 2002; not shown in Figure 2) (For further details of structure–function correlations and replication see Prasad and Estes 1997; Lawton et al. 2000; Estes 2001). In immunodeficient hosts and under certain experimental conditions, rotaviruses undergo genome rearrangements (for review: Desselberger 1996).

PATHOGENESIS: ANIMAL MODELS

Cell death and desquamation reduce digestion and adsorption of nutrients (primary malabsorption) and lead to villous atrophy. This is followed by a reactive crypt-cell hyperplasia accompanied by increased secretion, which is thought to contribute to the severity of diarrhea. The local pathogenesis is shown diagrammatically in Figure 3.

Several animal models for rotavirus infections are being explored (mice, rabbits, piglets, rats; Saif et al. 1994; Burns et al. 1995; Conner and Ramig 1996; Guérin-Danan et al. 1998; Ciarlet et al. 2002a). Of those, piglets and rats are infectable with human strains and produce diarrhea; they are therefore of particular interest. Many features of rotavirus infections, particularly pathogenicity and immune responses, have been studied in animal models to a greater extent than in man (see below).

The viral factors determining pathogenicity of rotaviruses have been investigated in several animal models

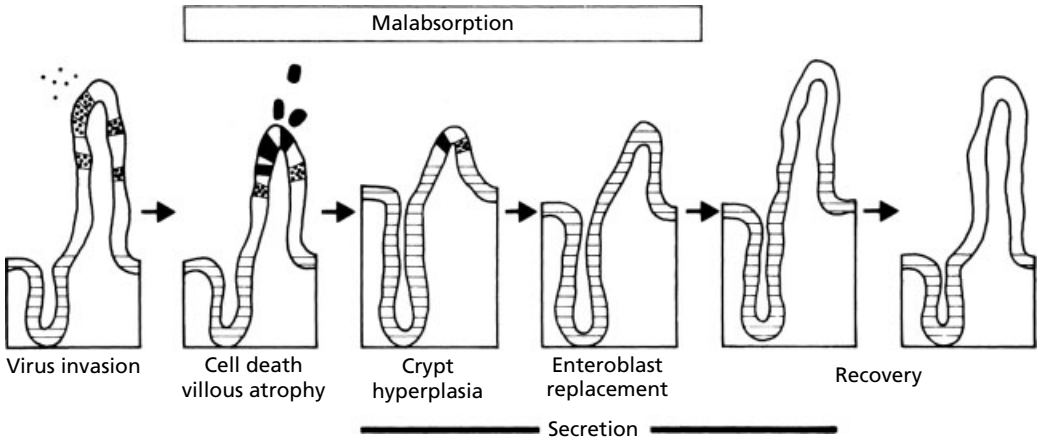

Figure 3 Rotavirus *pathogenesis (diagram). Development of damage to gut mucosa and ensuing diarrhea. (Redrawn from Phillips 1989, with permission of the author and publisher.)*

(piglets, mice, rabbits). The product of RNA segment 4, VP4, is likely to be a major determinant (Offit et al. 1986; Gorziglia et al. 1988; Bridger et al. 1992, 1998; Burke et al. 1994a, b), but products of other structural genes (RNA 3 coding for VP3 and RNAs 8 or 9 coding for VP7) and nonstructural genes (RNA 5, coding for NSP1, RNA 8 coding for NSP2, and RNA 10 coding for NSP4) have also been associated with pathogenicity (Table 2) (Broome et al. 1993; Hoshino et al. 1993, 1995; Ball et al. 1996 ; review by Burke and Desselberger 1996).

The discovery of NSP4 as an enterotoxin (Tian et al. 1994, 1995; Ball et al. 1996) has allowed the old observation that rotavirus-infected animals exhibit profuse diarrhea prior to the detection of histological lesions to be explained. NSP4 or a peptide thereof (aa 114–135) induce dose- and age-dependent diarrhea in laboratory animals (mice, rats) in the absence of histological changes (Ball et al. 1996) (Figure 4).

NSP4 produces an increase in intracellular Ca^{2+} concentration (Tian et al. 1994), disturbing the cellular homeostasis. Recently it was found that a peptide of NSP4, that is active as an enterotoxin is secreted from infected cells (Zhang and Zeng 2000) and is able to induce intracellular Ca^{2+} elevation and diarrhea in mice. It is thought that the secreted protein binds to a (still hypothetical) receptor and thus affects uninfected cells (Zhang and Zeng 2000; Tafazoli et al. 2001). A model of the NSP4 action is proposed in Figure 5, p. 8.

Antibody to NSP4 has been shown to reduce the severity of diarrheal disease in suckling mice (Estes 2003). The effect of rotavirus infection on the enteric nervous system also may play a role in pathogenesis (Lundgren et al. 2000).

ILLNESS AND TREATMENT

After a short incubation period of 24–48 hours, the onset of illness is sudden with watery diarrhea, vomiting, and rapid dehydration. Untreated rotavirus infection is a major cause of infant death in the developing world. It should be noted, though, that the clinical symptoms of rotavirus infection may vary widely: asymptomatic infections of neonates with so-called nursery strains have been described (Hoshino et al. 1985; Gorziglia et al. 1988), but also central nervous system infections (Iturriza-Gómara et al. 2002a) and chronic infections and hepatitis in children with immunodeficiencies (Gilger et al. 1992).

Table 2 Rotavirus *genes implicated in pathogenicity in different hosts*

Gene segment	Gene product	Host	Reference
3	VP3	Pig	Hoshino et al. 1993, 1995
4	VP4	Mouse	Offit et al. 1986
		Human	Gorziglia et al. 1988
		Pig	Bridger et al. 1992; Burke et al. 1994a, b; Hoshino et al. 1993, 1995
5	NS53 (NSP1)	Mouse	Broome et al. 1993
7	NS35 (NSP2)	Mouse	Broome et al. 1993
7 or 8	NSP3	Mouse	Mossel and Ramig 2002, 2003
8 or 9	VP7	Pig	Hoshino et al. 1993, 1995
10	NS28 (NSP4)	Pig	Hoshino et al. 1993, 1995
		Mouse, rat	Tian et al. 1994, 1995; Ball et al. 1996; Zhang and Zeng 2000; Tafazoli et al. 2001; Yu and Langridge 2001 ; Estes 2003

Modified from Burke and Desselberger 1996.

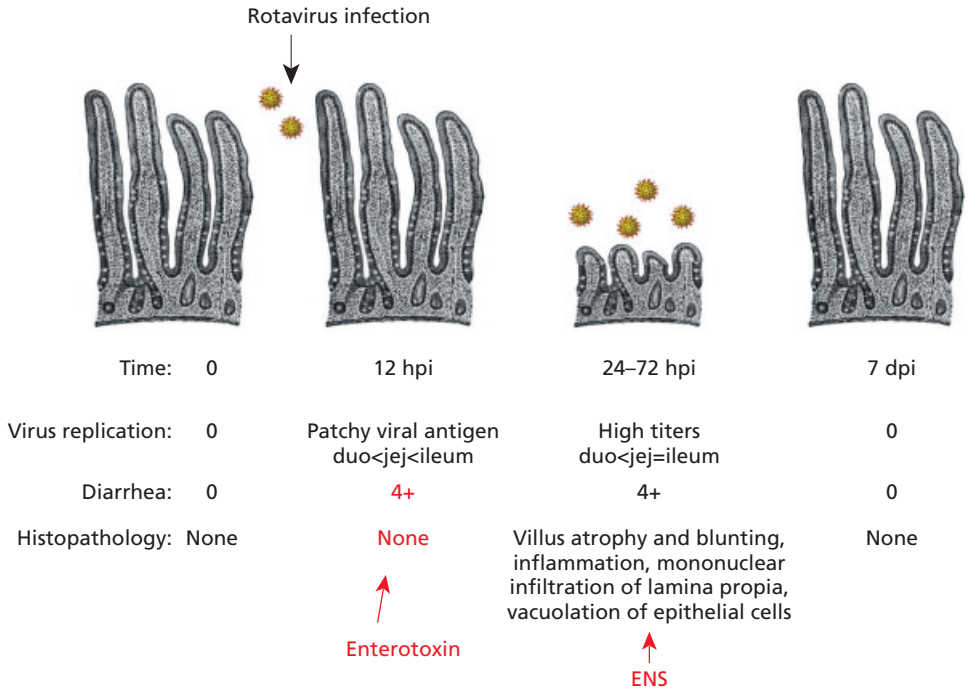

Figure 4 Rotavirus *pathogenesis. Correlation of virus replication, histopathology, and disease over time. Enterotoxin, NSP4; ENS, enteric nervous system. (From Estes 2001 with permission of the publisher)*

Treatment is by oral, subcutaneous, or intravenous rehydration (Bhan et al. 1994; International Study Group on Reduced-osmolarity ORS Solutions 1995; Desselberger 1999). Table 3 gives the WHO-approved formulae of oral rehydration solutions (ORS). There is no approved specific antiviral treatment (Desselberger 1999). Oral immunoglobulins seem to shorten the period of virus shedding and diarrhea but are not in routine therapeutic use (Guarino et al. 1994; Bass 2003).

DIAGNOSIS

The number of virus particles in the gut at the peak of the diarrhea can be as high at 10^{11}/ml of feces. Diagnosis is therefore relatively easy using electron microscopy, enzyme-linked immunosorbent assays (ELISA) or passive particle agglutination techniques.

Electron microscopy allows quick identification of the pathognomonic double- and triple-layered rotavirus particles. These particles differ morphologically from other viruses causing acute gastroenteritis: enteric adenoviruses, human caliciviruses (noro- and sapo-viruses), astroviruses, and small round viruses (SRV) (Doane 1994) (see Adenovirus; Human enteric RNA viruses: astroviruses; and Human enteric RNA viruses: noroviruses and sapoviruses).

Serological assays to detect rotavirus antigen are used widely, mostly applying direct or indirect ELISAs. The tests can be calibrated and quantified and are easily performed. If the detecting anti-rotavirus antibody is type-specific, the test can be used for G and P typing; this, however, depends on the presence of triple-layered virus particles in the clinical specimen. Another frequently used rapid technique is a passive particle agglutination test (Ruggeri et al. 1992). Details of procedures are described by Yolken and Wilde (1994).

The viral genome can be detected easily after phenol extraction of RNA from crude or semipurified rotavirus-containing specimens and separation by polyacrylamide gel electrophoresis (PAGE) followed by silver staining (Herring et al. 1982). Nowadays, the procedure is of value mainly in outbreak investigations.

The use of rotavirus-specific oligonucleotide primers in reverse transcription-polymerase chain reactions (RT-PCR) has allowed not only sensitive detection but also subgroup determination and typing for both G and P types when the subgroup- and type-specifying sequence diversities are exploited (Gouvea et al. 1990; Gentsch et al. 1992; Iturriza-Gómara et al. 2002c, 2003b). For rotavirus typing, RT-PCR has become the method of choice (Iturriza-Gómara et al. 1999, 2000).

Human rotaviruses can be grown in vitro on monkey kidney cells in the presence of trypsin (Ward et al. 1984), but the procedure is not in routine use.

IMMUNE RESPONSE AND CORRELATES OF PROTECTION

After neonatal or primary rotavirus infection a mainly serotype-specific humoral immune response is elicited, but there is also partial protection against subsequent

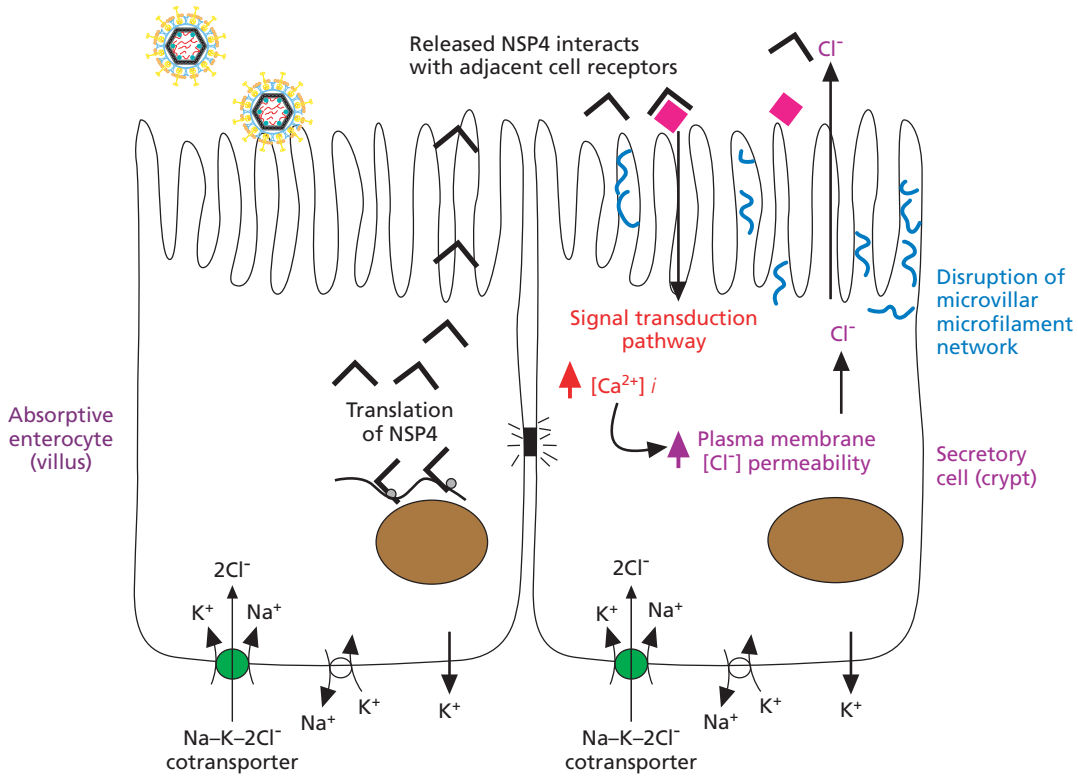

Figure 5 *Proposed model of NSP4 action.* Rotavirus*-infected enterocytes produce virus-specific proteins including NSP4. NSP4 is released from cells by lysis or secretion, and this extracellular NSP4 acts in a paracellular fashion by binding to an as yet unidentified receptor for NSP4 on adjacent cells. This triggers a rapid response involving a signal transduction pathway that mobilizes intracellular Ca^{2+} and results in increased plasma membrane Cl^- permeability. The secretory cells are likely to be crypt cells but might also be villus enterocytes. The plasma membrane permeability is age-dependent, based on studies in CFTR knock-out mice (Morris et al. 1999). Extracellular NSP4 also causes a delayed disruption of the organization of filamentous actin and transepithelial resistance (Tafazoli et al. 2001). ∧, NSP4; ◆, (putative) NSP4 receptor. (Redrawn from Estes et al. 2001)*

heterotypic infections (Bishop et al. 1983; Chiba et al. 1986; Friedman et al. 1988). The exact correlates of protection remain to be determined (Offit 1994), but the best are provided by coproantibodies of the IgA subclass (Coulson et al. 1992; Matson et al. 1993; Feng et al. 1994, 1997; Yuan et al. 1996; To et al. 1998) although not in all cases (O'Neal et al. 2000). There seems to be protection from severe disease after natural infection and vaccination (see below) even if the serotype of the challenging virus differs from that of the first infection or vaccination (Ward et al. 1992; Offit 1994; Velazquez et al. 1996). In the mouse model, homologous protection from infection depends on the level of mucosal IgA antibodies; heterologous protection can also be achieved, but depends on dose and strain and also seems to be correlated with the ability of the heterologous strain to stimulate detectable rotavirus-specific intestinal IgA antibody (Feng et al. 1994, 1997). In mice, rotavirus VP6-specific IgA monoclonal antibodies that lack direct neutralizing activity can protect from primary infection. It has been proposed that the mechanism of protection is by intracellular viral inactivation following transcytosis of IgA (Burns et al. 1996; Herrmann et al. 1996; Feng et al. 1997; Schwartz-Cornil et al. 2002) as experimentally shown for influenza viruses (Mazanec et al. 1995), but firm data on 'intracellular neutralization' have not become available so far for rotaviruses. The extent to which heterotypic protection is efficient in humans remains unclear (Offit 1994). There are rotavirus-specific cytotoxic T-cell responses but their exact role in overcoming the infection and protecting against subsequent infections is not clear (Offit et al. 1993; Offit 1994; Franco and Greenberg 1995).

Table 3 *Oral rehydration solutions (ORS) for the treatment of* Rotavirus*-related diarrhea*

	Osmolarity (mmol/l)	
Component	**WHO ORS**	**'ORS light'**
Sodium	90	60
Potassium	20	20
Chloride	80	50
Citrate	10	10
Glucose	111	84
Total, osmolarity	**311**	**224**

Modified from Desselberger (1999).

EPIDEMIOLOGY

The epidemiology of group A rotavirus infections is complex, as at any one time and in any geographical location rotaviruses of different G types co-circulate (e.g. Noel et al. 1991; Gentsch et al. 1996; Ramachandran et al. 1998). The relative incidence of G types also changes over time in the same location. Approximately 95 percent of co-circulating strains are types G1–G4 in most places, typically G1P1A[8], G2P1B[4], G3P1A[8], and G4P1A[8], but other G types may be represented at high frequencies, particularly in tropical areas (Gentsch et al. 1996; Leite et al. 1996; Ramachandran et al. 1996; Cunliffe et al. 1999; Iturriza-Gómara et al. 2000; for review: Desselberger et al. 2001). Recently, G9 rotaviruses have been isolated as one of the predominant outbreak strains in several locations in the USA (Ramachandran et al. 1998) and in the UK (Iturriza-Gómara et al. 2002b). Group B rotaviruses have caused outbreaks of diarrhea in children and adults in China (for review see Hung 1988; Fang et al. 1989) and have been isolated from sporadic cases of gastroenteritis in Calcutta, India (Krishnan et al. 1999; Kobayashi et al. 2001). Group C rotaviruses are associated with small outbreaks in humans (Caul et al. 1990; Jiang et al. 1995).

Besides the accumulation of point mutations (genomic drift; Iturriza-Gómara et al. 2003b), gene reassortment upon dual infection with co-circulating human, or human and animal strains (genomic shift; Iturriza-Gómara et al. 2001, 2003b) is likely to play a major role in generating the increasing diversity of rotaviruses. Although reassortment of two different viruses with 11 gene segments theoretically predicts 2^{11} different progeny viruses (including the parent strains), constraints to reassortment have been identified in naturally circulating rotaviruses (Graham et al. 1987). The genes encoding the structural proteins VP6, VP7, and VP4 reassort independently, although some constraints among the genetic lineages of these proteins seem to exist (Iturriza-Gómara et al. 2001, 2003b). By contrast, the genes encoding VP4 and NSP4 are inextricably linked in progeny, possibly through their intense interaction during morphogenesis (Iturriza-Gómara et al. 2003a).

As for group A rotaviruses (Estes 2001), there is a large animal reservoir for group B rotaviruses. Whilst transmission of animal rotaviruses to man has been demonstrated, it is not clear at present to what extent animal rotaviruses can be transmitted to man (Das et al. 1993; Gentsch et al. 1996).

VACCINE DEVELOPMENT

Rotavirus infections have been recognized as a major viral infection in children worldwide (Bern and Glass 1994), and therefore development of vaccine candidates has been a major goal and has been in progress since the early 1980s. Results have been mixed for some time, owing to the enormous genomic and antigenic diversity of rotaviruses (for review see Kapikian 1994; Vesikari 1994; Desselberger 1998b). Mainly animal rotaviruses (of simian or bovine origin) have been used as live attenuated vaccines. In many cases, protection from infection and/or mild disease was only modest (40–50 percent). By contrast, 70–80 percent protection from severe disease including dehydration was recently achieved (Rennels et al. 1996; Joensuu et al. 1997; Pérez-Schael et al. 1997), particularly when applying a cocktail of viruses, for example as a tetravalent vaccine containing a rhesus monkey rotavirus (RRV) of G3 type and three monoreassortants, individually carrying the *VP7* gene of human serotypes G1, G2, and G4 in the RRV genetic background. A tetravalent RRV-based reassortant vaccine (Rotashield®) received Food and Drug Administration (FDA) approval as a universal vaccine in the USA in August 1998, and recommendations for its usage have been issued (Centers for Disease Control and Prevention 1999a). More than 1.5 million doses were administered in the following 10 months. During that time cases of intussusception were observed in vaccinees, particularly within 3–7 days after the first dose with a relative risk of 27.9 (95 percent confidence interval 10.8–72.1; Murphy et al. 2001), and attributable risks of between one case of intussusception in every 4 700–9 500 children vaccinated (Murphy et al. 2001) and one case in 11 000 children vaccinated (Kramarz et al. 2001) have been calculated. These observations led the Centers for Disease Control and Prevention (CDC) and the American Academy of Pediatrics to withdraw the recommendation for use in infants (Centers for Disease Control and Prevention, 1999b, c). Recent ecological studies have failed to demonstrate an increase in the incidence of intussusception during the time of usage of the Rotashield vaccine (Chang et al. 2001; Simonsen et al. 2001), suggesting that the attributable risk may be even smaller.

However, due to the urgent need for a rotavirus vaccine, the research for its development is ongoing. There are several other live attenuated candidate vaccine strains: the bovine WC3 virus (G6P7[5]) and G1 and P1A[8] reassortants thereof (Treanor et al. 1995; Clark et al. 1996), the attenuated human strain 89-12 (G1P1A[8]: Bernstein et al. 1998, 1999), and multivalent vaccines comprising representative strains carrying G1–G4 and G9. Two live, attenuated oral rotavirus candidate vaccines, consisting of a) a pentavalent WC3-based, human reassortant vaccine (RotaTeq™), and b) a monovalent strain 89-12-based vaccine (Rotarix™), are in advanced stages of large phase III trials (Heaton 2004, De Vos 2004), and are likely to enter the licensure process in 2005. Rotarix™ has already been approved in Mexico in early 2005.

In addition, new approaches to immunization are under investigation: applying inactivated virus or synthetic peptides (Conner et al. 1993), enhancing rotavirus immunogenicity by microencapsidation (Offit et al. 1994), using virus-like particles (VLP) obtained by co-expression of viral proteins (VP2, VP4, VP6, and VP7) from baculovirus recombinants in insect cells (Crawford et al. 1994; Conner et al. 1996; O'Neal et al. 1997; Ciarlet et al. 1998) or using DNA vaccines (Herrmann et al. 1996; Chen et al. 1997, 1998). Expression of rotavirus proteins in plants (potatoes, bananas) from vectors downstream of plant-specific promoters is also under investigation ('edible vaccines,' Arntzen 1997; Yu and Langridge 2001; Mason et al. 2002). In view of the heterotypic immune responses observed after natural infection and immunization, it must be determined which of the epidemiologically important human P and G rotaviruses are mainly required for protection (Offit 1994).

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Rubella

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HISTORICAL INTRODUCTION

Rubella was first described in the mid-eighteenth century by two German physicians, de Bergen and Orlow. At that time it was frequently known by the German name 'Röteln,' and it was due to the early interest of the German physicians and the general acceptance of a German name that the disease subsequently became known as 'German measles.' For many years, German measles was frequently confused with measles and scarlet fever, other infectious diseases presenting with rash, and at one time was considered to be a cross between them. The clinical differences between these diseases were recognized in the nineteenth century and rubella was accepted as a distinct disease by an International Congress of Medicine in London in 1881 (Smith 1881). The disease received comparatively little attention, for infection was generally mild and severe complications were rare, until the 1940s when the association between maternal infection and such congenital defects as cataract, heart disease, and hearing loss was first recognized (Gregg 1941). This report is discussed in more detail later.

Rubella virus (RUBV) was not isolated in cell culture until 1962, when Parkman and colleagues (1962) detected the presence of RUBV in primary vervet monkey kidney cell cultures by means of the interference technique and Weller and Neva (1962) reported unique cytopathic effects in primary amnion cell cultures. Tests for neutralizing and hemagglutination inhibition (HAI) antibodies were reported in 1962 (Parkman et al. 1962, 1964) and 1967 (Stewart et al. 1967) respectively. These tests allowed seroepidemiological investigations to be conducted. The fine structure of RUBV was not determined until 1968 as it is difficult to obtain the high titers of virus required for electron microscopy (Best et al. 1967; Holmes et al. 1968). Much of the work on the molecular structure and replication of RUBV was carried out in the late 1980s and early 1990s, some time after similar work on other viruses. This was probably because RUBV is slow to grow in cell culture, high levels of virus are difficult to produce consistently and the high G+C content of the genome made sequencing difficult.

PROPERTIES OF THE VIRUS

Classification

Rubella virus, an enveloped single-stranded RNA virus, belongs to the family *Togaviridae* (Togaviruses) and is probably distantly related to the alphaviruses (Frey 1994). Unlike other togaviruses, RUBV has no known invertebrate host and has been placed by itself in the genus *Rubivirus*.

Morphology and structure

The virion has a mean diameter of 58 nm with a 30 nm core (Figure 1) (Best et al. 1967; Holmes et al. 1968; Murphy et al. 1968). The core is surrounded by a lipoprotein envelope with surface spikes 5–8 nm in length. In thin sections of infected cells an electron-lucent zone is seen between the core and the envelope. The virion is

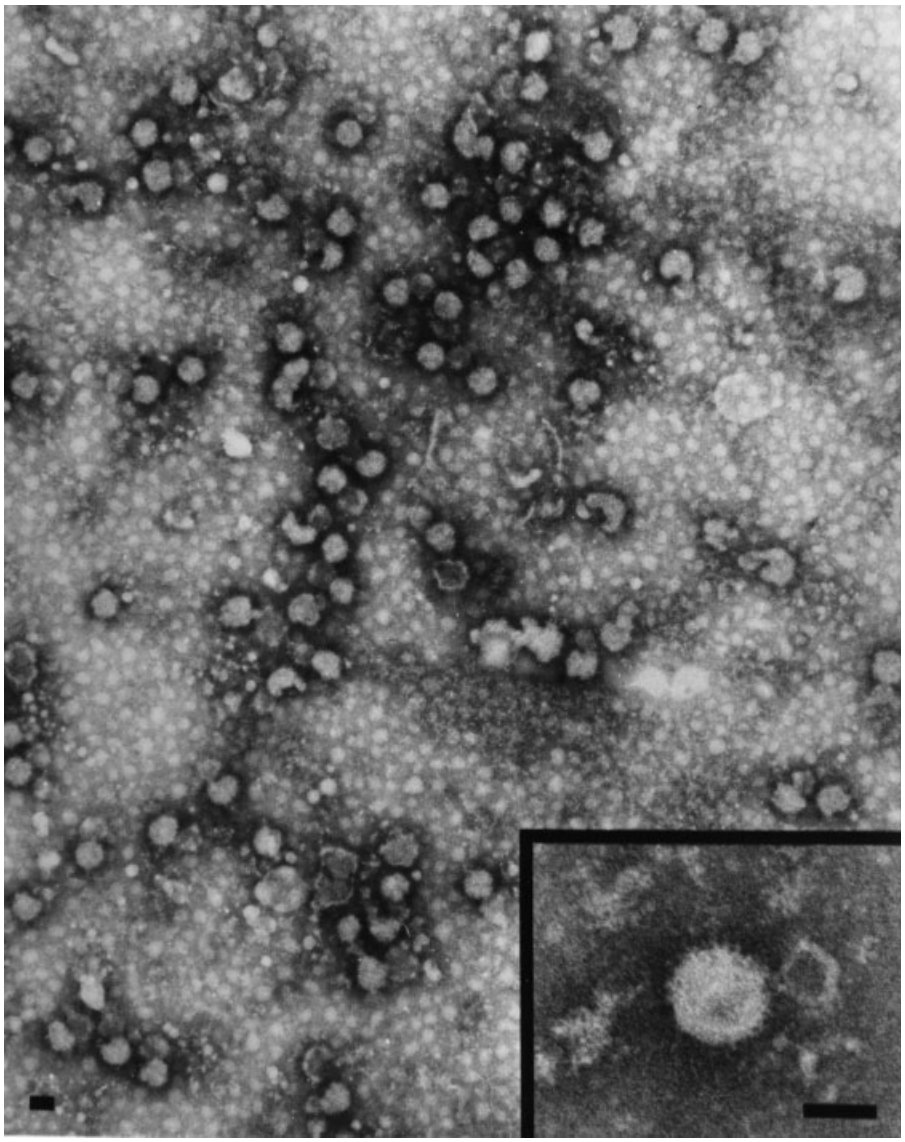

Figure 1 *Negatively stained preparation of rubella virus. Inset, an enlarged particle, showing spikes. Bar, 50 nm (kindly provided by Dr. I. Chrystie).*

pleomorphic, owing to the delicate nonrigid nature of the envelope. The symmetry of the nucleocapsid has been difficult to establish because of its instability, but rotational analysis of thin sections of rubella virions suggests that the core has a T = 3 icosahedral symmetry and 32 capsomers (Matsumoto and Higashi 1974).

Physical properties of RUBV are shown in Table 1 and were reviewed by Horzinek (1973, 1981). The stability of the virus is enhanced by the addition of proteins and $MgSO_4$ to the suspending medium. Because of the lipid content of the viral envelope, RUBV is inactivated by detergents and organic solvents. The effects of these and other chemicals have been reviewed (Parkman et al. 1964; Norrby 1969; Plotkin 1969; Horzinek 1973, 1981; Herrmann 1979).

Genome organization

The RUBV genome consists of a single strand of positive sense RNA, 9 762 nucleotides (nt) in length, which is capped at the 5′ end and polyadenylated at the 3′ end. The cap is required for efficient translation as it serves as a ribosome recognition site (Oker-Blom 1984; Frey 1994). The genome contains two nonoverlapping open reading frames (ORF) separated by a linker region of 123 nucleotides (nt) in the same translation frame. The 5′ proximal ORF extends from nt 41 to 6 388 and encodes the p200 polyprotein precursor for the nonstructural proteins (NSP) p150 and p90 (Dominguez et al. 1990; Pugachev et al. 1997a; Liang and Gillam 2000). The 3′ proximal ORF extends from nt 6 512 to 9 700, and encodes the structural proteins (SP): capsid (C), and

Table 1 *Physical and morphological properties of rubella virus*

	Properties		Reference
Virus particle			
Diameter	40–70 nm		Reviewed by Horzinek (1973)
Buoyant density	in sucrose: 1.16–1.19 g/ml		
	in $CsCl_2$: 1.20–1.23 g/ml		Reviewed by Horzinek (1973)
Sedimentation coefficient	240S		Thomssen et al. (1968)
	342S		Russell et al. (1967)
	350 ± 50S		Bardeletti et al. (1975)
Nucleocapsid			
Diameter	30–40 nm		Reviewed by Horzinek (1973)
Symmetry	Icosahedral		Reviewed by Horzinek (1973)
Buoyant density	in $CsCl_2$: 1.4 ± 0.4 g/ml		
Sedimentation coefficient	150S		Vaheri and Hovi (1972)
Molecular weight	2 600–4 000 kDa		
Nucleic acid	Single strand of RNA		Kenney et al. (1969)
Buoyant density	1.634 g/ml		Hovi and Vaheri (1970)
Sedimentation coefficient	38–40S		Sedwick and Sokol (1970)
Molecular weight	3 200–3 500 kDa		
Length of surface projections	5–6 nm		Holmes et al. (1969); Smith and Hobbins (1969)
Chemical composition	RNA 2.4%	Lipid 18.8%	Voiland and Bardeletti (1980)
	Proteins 74.8%	Carbohydrate 4.0%	
Major polypeptides	Envelope	E1 58 kDa	Oker-Blom et al. (1983)
		E2 42–47 kDa	Waxham and Wolinsky (1985)
	Nucleocapsid	C 33–35 kDa	
Thermal stability			
4°C	Stable for ⩾7 days		Fabiyi et al. (1966)
37°C	Inactivated at 0.1–0.4 $\log_{10}$ $TCID_{50}$/ml per h		Parkman (1965)
56°C	Inactivated at 1.5–3.5 $\log_{10}$ $TCID_{50}$/0.1 ml per h		Parkman (1965)
70°C	Inactivated at 5.5 $\log_{10}$ $TCID_{50}$/0.1 ml per 0.5 h		Kistler and Sapatino (1972)
−70°C	Stable		Parkman et al. (1964)
Freeze drying	Stable		Parkman et al. (1964)
pH sensitivity	Stable at pH 6.0–8.1		Schell and Wong (1966); Norrby (1969)
	Unstable at more acid and alkaline pH		
UV sensitivity	Inactivated within 40 s		Fabiyi et al. (1966)
1 350 W/cm^2	Inactivated at 7.0 $\log_{10}$ $TCID_{50}$/0.1 ml per h		Kistler and Sapatino (1972)
Photosensitivity	Labile, K = 0.07/min in PBS		Booth and Stern (1972)
Sonication	Stable for ⩾9 min		Schell and Wong (1966)
Chemical sensitivity	Formaldehyde, lipid solvents, detergents e.g. SDS, Nonidet P-40, Tween 80		Parkman et al. (1964); Chantler et al. (2001)

$TCID_{50}$, 50% tissue culture infective dose.

glycoproteins E1 and E2 (Dominguez et al. 1990; Frey 1994; Yao et al. 1998). The gene order is 5′-p150–p90–C–E2–E1-3′ (Forng and Frey 1995). The genomic RNA serves as the template for synthesis of a complementary negative-sense RNA strand, which in turn, is the template for synthesis of positive-sense 40S genomic and 24S subgenomic RNA, from which the SPs are translated (Figure 2).

The base composition of the genome is 14.9 percent A, 15.4 percent U, 30.8 percent G and 38.7 percent C, making the G+C composition 69.5 percent, the highest reported for an RNA virus. This initially made sequence determination difficult, and errors were made in the first sequences reported (Dominguez et al. 1990). Genome-length cDNA clones have been produced and used to synthesize infectious RNA transcripts (Wang et al. 1994).

Virus life cycle

ATTACHMENT AND ENTRY

RUBV can replicate in a variety of cell lines which suggests that the receptor for RUBV is a ubiquitous molecule (reviewed by Frey 1994). The receptor has not yet been identified, but as membrane lipid molecules play an essential role it may be a lipid–carbohydrate complex (Mastromarino et al. 1990). The reproductive life cycle of RUBV takes place in the cytoplasm. Like most enveloped animal viruses, RUBV probably enters cells by receptor-mediated endocytosis and uses endocytic machinery to travel within the cytoplasm (Sodeik 2000). During endocytosis, the virion is internalized in a coated vesicle, which is transported to the endosomal compartment. The low pH environment of the endosome triggers a conformational change in the envelope glycoproteins that favors the fusion of viral and endosomal membranes (Katow and Sugiura 1988; reviewed by Frey 1994). The low pH also causes the C protein to undergo a structural change which makes it more hydrophobic, and allows uncoating of nucleocapsid to occur (Mauracher et al. 1991; Lee and Bowden 2000). Both events suggest that the low pH environment mediates delivery of viral genomic RNA into the cytoplasm.

REPLICATION, TRANSCRIPTION, AND TRANSLATION

The replication cycle of RUBV in vertebrate cell cultures is slow and less efficient than that of alphaviruses. Peak virus production usually occurs at 48 h postinfection (Hemphill et al. 1988; Frey 1994). Replication of RUBV has been suggested to occur in ‘replication complexes’ that appear as membrane-bound cytoplasmic vacuoles, lined internally with vesicles and associated with the rough endoplasmic reticulum (RER). The vesicles within the complex appear to be the sites of viral replication, and may also protect the genomic RNA from cellular ribonucleases. The replication complexes also appear to serve as sites for nucleocapsid assembly (Lee et al. 1994; Lee and Bowden 2000). Some RUBV replication complexes are modified lysosomes, characterized by the presence of degenerating material within the vacuole. However, others may be endosomal in origin, as observed in alphavirus-infected cells (Togaviruses) (Magliano et al. 1998).

Following the uncoating of capsid and release of genomic RNA into the cytoplasm, the 40S genomic RNA serves as the template for nonstructural protein (NSP) synthesis. The NSPs are translated from the 5′ proximal ORF as a 2 116 amino acid polyprotein, p200 (200 kDa), which is proteolytically cleaved to produce two nonstructural proteins p150 (residues 1–1 301, 150 kDa) and p90 (residues 1 302–2 116, 90 kDa) (Marr et al. 1994; Forng and Frey 1995; Chen et al. 1996). Preceding the NSP-ORF there are three potential AUG start sites at nucleotides 3, 41, and 57. Translation of the NSP-ORF is initiated at AUG_{41}, whereas AUG_3 and AUG_{57} are in a different reading frame and terminate at nucleotides 54 and 90, respectively. Neither of these short ORFs are essential for virus replication (Pugachev et al. 1997a; Pugachev and Frey 1998a).

The cleavage of the p200 polyprotein precursor is mediated by an RUBV-encoded protease within the C-terminal region of p150, which is homologous to several other viral and cellular papain-like proteases (Gorbalenya et al. 1991; Marr et al. 1994). The catalytic dyad is formed by residues Cys-1152 and His-1273 and cleavage takes place at Gly-1301 in the sequence Gly_{1300}–Gly_{1301}–Gly_{1302} (Marr et al. 1994; Chen et al. 1996; Liang and Gillam 2000). Site-directed mutagenesis of Gly-1301 to Ala or Val, and of Gly-1300/1302 to Val abrogates cleavage and results in non-infectious virus (Chen et al. 1996; Liang and Gillam 2000).

The 40S positive-sense genomic RNA also acts as the template for the synthesis of a complementary negative-sense strand of RNA. The negative strand, which is present in double-stranded form, is in turn the template for synthesis of the positive-sense 40S genomic RNA and 24S subgenomic RNA. p200 is the principal RNA-dependent RNA polymerase (RdRp) for negative-strand RNA synthesis, and cleavage products p150/p90 are required for positive-strand (genomic and subgenomic) RNA synthesis (Liang and Gillam 2000, 2001). Thus the switch from the synthesis of negative to positive-strand RNA is regulated by NSP processing. The p150 protein has also been shown to be localized in RUBV replication complexes (Kujala et al. 1999). Negative strand RNA synthesis continues to accumulate until 10 h postinfection, and remains constant thereafter. Synthesis of positive-strand RNAs (genomic and subgenomic) begins 10 h postinfection and escalates dramatically. This replication pattern resembles that of alphaviruses where negative-strand synthesis ceases after 4–6 h and switches to positive-strand RNA synthesis (Sawicki et al. 1981; Liang and Gillam 2001).

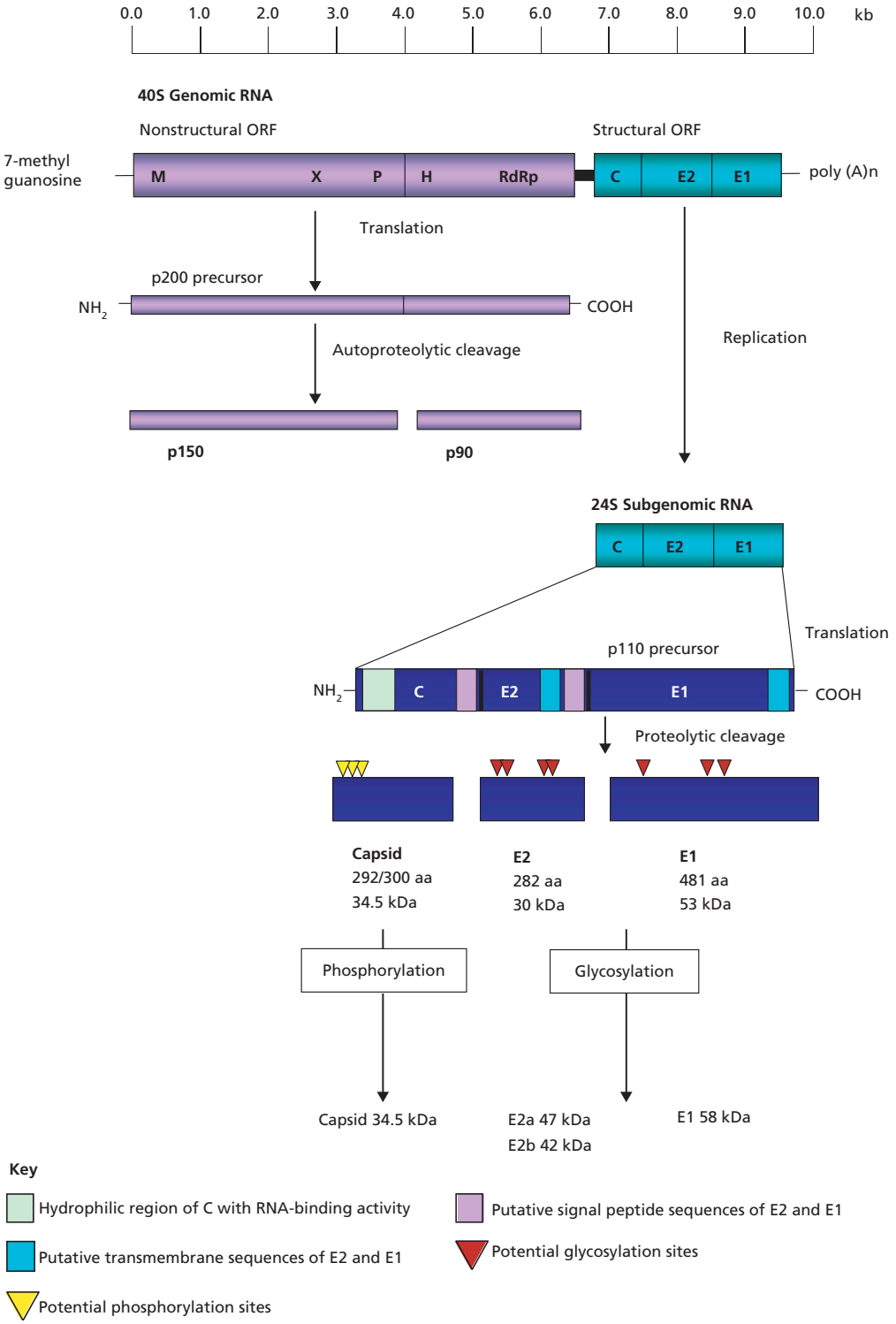

Figure 2 *Schematic representation of the steps involved in the replication translation, and processing of Rubella virus structural and nonstructural proteins. The RUBV genome consists of two long nonoverlapping ORFs, the 5′ ORF encodes the nonstructural proteins and the 3′ ORF encodes the structural proteins. The translation of RUBV RNA produces the p200 precursor, which is cleaved to produce the p150 and p90 nonstructural proteins. This initiates the synthesis of the full-length negative-strand RNA. The negative strand acts as a template for the synthesis of the full-length positive strand RNA for new viral progeny and the 24S subgenomic RNA. The 24S subgenomic RNA is translated into the p110 polyprotein precursor, which is proteolytically cleaved and post-translationally modified to produce the structural proteins C, E2, and E1. Within the 5′ nonstructural ORF are putative amino acid sequence motifs for methyltransferase (M), RNA-dependent RNA polymerase (RdRp), helicase (H), and papain-like cysteine protease (P) activity. The X motif indicates a region of unknown function, which has homology to alphaviruses, hepatitis E virus, and coronaviruses.*

The subgenomic RNA is translated to produce a 1 063 amino acid polyprotein precursor, p110 (110 kDa), which is proteolytically cleaved to produce the SPs C, E2, and E1 (Figure 2) (Kalkkinen et al. 1984; Oker-Blom 1984). The cleavage of p110 is thought to occur at signal peptide sequences that precede the amino termini of E2 and E1. The E2 and E1 signal peptides remain attached to the carboxy termini of C and E2, respectively, and direct the insertion of these proteins into the endoplasmic reticulum (ER) (Suomalainen et al. 1990; Marr et al. 1991; Baron et al. 1992). The capsid protein lacks the autoprotease activity of other alphavirus C proteins and cleavage of p110 is mediated by a host-cell signalase found within the lumen of the ER (Clarke et al. 1988; Frey and Marr 1988; Oker-Blom et al. 1990). The E2 signal peptide is required for RUBV glycoprotein-dependent localization of capsid to the juxtanuclear region and subsequent virus assembly in the golgi (Frey 1994; Qiu et al. 1994; Law et al. 2001). Retention of the E2 signal peptide on the carboxy terminus of capsid is unique to Rubella virus within the *Togaviridae* (Law et al. 2001).

Following proteolytic cleavage E1 and E2 form disulfide-linked heterodimers before they exit the ER. Heterodimerisation of E1 with E2 is required for E1 to be correctly folded and transported from the ER to the Golgi and the cell surface. Incorrectly folded proteins are retained in the ER (Frey 1994). An internal hydrophobic domain in E1 (aa 81–109) plays a major role in the formation of the E1–E2 heterodimer (Yang et al. 1998). The capsid protein forms dilsulfide linked homodimers (Waxham and Wolinsky 1983; Baron and Forsell 1991). Newly synthesized 40S genomic RNA is packaged with the RUBV capsid protein to form nucleocapsids. A 29 nt sequence (nt 347–375), within the genomic RNA has been identified as a binding domain for capsid, and a peptide region (amino acids 28–56) with specific RNA binding activity has been identified in the capsid (Liu et al. 1996; Wolinsky 1996).

DEFECTIVE INTERFERING (DI) RNAS

Serial undiluted passage of RUBV in Vero cells results in the production of DI RNAs, approximately 7 000 and 800 nt in length (Frey and Hemphill 1988). Characterization of these DI RNAs revealed that the 7 000-nt species contained a 2 500–2 700-nt deletion in the SP-ORF, and the 800 nt species was the subgenomic DI RNA synthesized from the larger species (Derdyn and Frey 1995). These are the only types of DI RNA which are continuously generated. Although DI RNAs are generated during persistent infection, their presence is not necessary to establish persistence (Frey and Hemphill 1988).

VIRUS RELEASE

Newly synthesized RUBV particles are released by budding from both the plasma membrane and internal membranes. The nucleocapsid core buds from the modified internal membranes and subsequently attains E1 and E2 glycoproteins and the host-cell lipids it requires to form the viral envelope. The mechanism by which the nucleocapsid interacts with E2–E1 heterodimers and the precise mechanism of budding is not known. However, E1 transmembrane and cytoplasmic domains have been found to be important in the late stages of virus assembly. The cytoplasmic domain of E1 may interact with the capsid protein to drive the budding process (Yao and Gillam 1999; Lee and Bowden 2000).

In vitro, virus release varies according to the type of cells infected. Vero cells release more virus into the cell culture medium than BHK-21 cells, as virus maturation only occurs at the plasma membrane and less intracellular virus is produced (Bardeletti et al. 1979). In BHK-21 virus maturation occurs both at the plasma membrane and at the Golgi and vacuoles of the cytoplasm. Wolinsky (1996) suggested that the capacity to mature within intracellular vacuoles, avoiding the host's immune system, may enable the virus to establish persistent infections in humans. Pathak et al. (1994), using immunoelectron microscopy, reported spherules (pleomorphic membrane-bound structures containing a dense thread-like structure) as well as virions budding from the surface of infected Vero cells.

EFFECT ON THE HOST CELL AND ULTRASTRUCTURAL STUDIES

RUBV infection in Vero cells does not affect total cellular RNA synthesis, and total cell protein synthesis is only modestly inhibited (Hemphill et al. 1988). However, this effect may be cell-type dependent as, in contrast, others have reported an increase in cellular metabolism (Vaheri and Cristofalo 1967; Bardeletti 1977).

In RUBV-infected cell cultures, virus-producing cells display intracellular membrane alterations and vacuolation approximately 2 days after infection. Membrane alterations included proliferation and distension, as well as the appearance of crystalline inclusions and annulate lamellae (Bardeletti et al. 1979; Pathak et al. 1994). Freeze-substitution electron microscopy of RUBV-infected cells has revealed the presence of homogeneously dense particles lacking a virus core within Golgi stacks, which coexist with mature virions, and appear to be virion precursors (Risco et al. 2003).

Large osmiophilic inclusion bodies containing myelin whorls and densely packed vesicles of varying size have been observed in the epithelial cells of human fetal nasal and tracheal organ cultures infected in vitro with RUBV (Kistler et al. 1967). The cytoplasmic ground substance as well as the extracellular space in close vicinity to the microvilli of these cells contained numerous viruslike particles with a diameter of 380–700 Å. It is possible that the inclusion bodies described by these authors correspond to the replication complexes analyzed by Lee and Bowden (2000).

RUBELLA VIRUS-INDUCED APOPTOSIS

RUBV-induced cytopathic effect has been shown to be due to caspase-dependent apoptosis in a number of susceptible cell lines (Pugachev and Frey 1998b; Megyeri et al. 1999; Hofmann et al. 1999; Duncan et al. 1999; Cooray et al. 2003; Domegan and Atkins 2002). Replicating virus is required for the induction of apoptotic cell signaling, which begins early post-infection (Megyeri et al. 1999; Cooray et al. 2003). The genetic determinants of cytopathogenicity have been mapped to the NSP-ORF (Pugachev et al. 1997b), but these have not been demonstrated to be directly involved in RUBV-induced apoptosis. Expression of the capsid protein alone was shown to induce cell death in transfected RK13 cells (Duncan et al. 2000). However, this function of the RUBV capsid is disputed by Hofmann et al. (1999), who demonstrated that expression of the entire SP-ORF reading frame did not cause apoptosis. Whether RUBV-induced apoptosis is dependent upon p53 is uncertain. Although p53 and p21 protein expression, and *p21* gene expression is detected in RUBV-infected Vero cells (Megyeri et al. 1999), p53 null neurons die in the same way as normal neurons in response to RUBV infection (Domegan and Atkins 2002). Anti-apoptotic protein Bcl-X is able to confer partial protection against RUBV infection in BHK-21 cells, but not in RK13 cells (Duncan et al. 1999).

REGULATORY ELEMENTS

The Rubella virus genome contains four regions of nucleotide sequence, which are thought to function as regulatory signals for RUBV replication, and have homology to the alphavirus genome (Dominguez et al. 1990; Frey 1994). Two of these regions are located at the 5′ and 3′ termini of the RUBV genomic RNA and are predicted to form stable stem-loop (SL) structures, and are termed the 5′ (+) SL and 3′ (+) SL, respectively. The 5′ (+) SL is located between nucleotides 15 and 65, it is preceded by a 14-nt single-stranded leader (ss-leader) sequence and consists of a terminal loop and a hinge region. A complementary equivalent of the 5′ (+) SL is present at the 3′ end of the viral minus-strand RNA (termed the 3′(−) SL), and is thought to serve as a promotor for initiation of positive-strand genomic RNA by the viral replicase (Pugachev and Frey 1998a). The 3′ (+) terminal 305 nt of the RUBV genome is predicted to form four stable SL structures. The first two SLs (SL1 and SL2) are located at the end of the the E1 coding region, and the second two (SL3 and SL4) within the 59-nt 3′ untranslated region (UTR) that precedes the poly(A) tract. An inverted repeat sequence of 12 nt preceding the 59-nt 3′ UTR and capable of forming a SL structure, is required for initiation of negative-strand synthesis (Nakhasi et al. 1990, 1994).

Pogue et al. (1993) analyzed the transcriptional activity of the 5′ and 3′ ends of the genome by constructing chimeras of chloramphenicol acetyl transferase (CAT) RNA flanked by the 3′(+) or 5′(+) SL sequences. The translational efficiencies of the 5′(+) and 3′(+) SLs were measured by the level of CAT activity from the chimeras in transfected cells. Both 3′ and 5′(+) SLs were found to be necessary for efficient translation of CAT RNA in vitro and in vivo.

The third region of homology consists of 23 nucleotides upstream of the subgenomic (SG) RNA start site (nt −46 to −23). This site has been termed the SG promoter and synthesis of SG RNA is initiated in this region on a negative-strand copy of the genomic RNA (Frey 1994; Tzeng and Frey 2002). In contrast, the SG promoter region of alphaviruses is immediately adjacent to the RNA start site. The region necessary for synthesis of RUBV SG RNA and production of viable virus has been mapped to −28 nt upstream (5′) through to at least +6 nt downstream (3′) of the SG start site (Tzeng and Frey 2002). The predicted secondary structure around the SG promoter region consists of two stem-loop structures SLI (−20 to −47 nt) and SLII (−16 to SG start site). However only SLII lies within SG promoter region and may be necessary for promoter function (Tzeng and Frey 2002).

A fourth predicted regulatory region lies 224 nt downstream of the 5′ end and consists of a stretch of 46 nt. However, unlike alphaviruses, this region does not form a predicted double stem-loop configuration, and its functional significance is unknown.

INTERACTION OF THE RUBV GENOME WITH CELLULAR PROTEINS

Cellular host factors are often involved in RNA virus replication and translation. Cellular proteins with RNA recognition elements may contribute enzymatic, structural, and regulatory activities required for RNA synthesis. A number of cellular proteins have been identified which interact with the RUBV genome.

The SL at the 3′ (+) end of the genome is able to bind to three host-encoded cytosolic proteins (61, 63, and 68 kDa). Phosphorylation of the host proteins is necessary for this interaction (Nakhasi et al. 1990). The 61 kDa protein has been purified and identified as the simian homolog of human calreticulin (Singh et al. 1994; Nakhasi et al. 1994). Phosphorylation of calreticulin is required for RNA-binding activity and calreticulin autophosphorylates itself at Ser and Thr residues. RNA binding and autophosphorylation activities were localized to the N-terminal 180 amino acids (aa). RNA binding activity was abrogated by deletion of the first ten aa residues, whereas autophosphorylation activity was localized to aa 60–180 (Singh et al. 1994; Atreya et al. 1995). The specific binding of calreticulin to the 3′(+) is important in regulating RUBV RNA negative strand synthesis.

UV cross-linking and RNA gel shift assays were used to identify host-cell proteins which interacted with the 5′

(+) SL. Two host proteins (53 and 59 kDa) present in both RUBV infected and uninfected cells were found to interact specifically with the 5′ (+) SL. Human serum with anti-Ro/SS-A antigen specificity immunoprecipitated the 53 and 59 kDa protein-RNA complexes containing the 5′ (+) SL RNA (Pogue et al. 1993). Serum from individuals with various immune disorders, which recognized La, or Ro and La autoantigens, immunoprecipitated ribonucleoprotein complexes containing both the 3′ and 5′ (+) SL RNAs. Like Ro, the La autoantigen was found to interact specifically with the 5′ (+) SL (Pogue et al. 1996). Identification of autoantigens binding to RUBV RNA suggests they may play a role in replication and pathogenesis.

Polypeptides

The SP ORF has two closely spaced AUG initiation codons, which may both be used indiscriminantly to produce capsid protein of 292 or 300 aa in length (Clarke et al. 1988). The capsid protein is phosphorylated, nonglycosylated, and has a molecular weight of 33 kDa. The protein is highly basic due to a high proportion of proline and arginine residues, which probably contribute to dimerization and binding of the 40S genomic RNA within the viral nucleocapsid. RUBV capsid protein is phosphorylated prior to virus assembly (Garbutt et al. 1999). A number of Ser/Thr residues within the capsid are potential phosphorylation sites, but site-directed mutagenesis experiments identified Ser 46, within the RNA binding region, as the most significant (Law et al. 2003). Capsid phophorylation is not essential for virus replication, but mutation of Ser 46 to Ala impairs virus growth and cytopathic effect. Hyperphosphorylated capsid mutants bind viral RNA more efficiently than wildtype capsid, and phosphatase treatment of wildtype improved RNA binding. This suggests that the capsid undergoes a dephosphorylation step prior to interaction with genomic RNA and nucleocapsid assembly (Law et al. 2003).

Host-cell mitochondrial protein p32 has been shown to bind to the N-terminal capsid domain and may regulate nucleocapsid assembly (Garbutt et al. 1999; Mohan et al. 2002). The exact physiological function of p32 is unknown, although it was originally identified as a C1q binding protein, it is capable of binding to a wide range of cellular and viral proteins (Beatch and Hobman 2000; Mohan et al. 2002). Binding of capsid to p32 at the mitochondria and overexpression of the SPs, results in clustering of the mitochondria to the perinuclear region.

E2 is 282 aa in length, and in most RUBV strains sequenced, it contains four potential *N*-linked glycosylation sites (Asn-X-Ser/Thr/Cys), where X can be any residue except proline. E2 also contains *O*-linked carbohydrates (Lundstrom et al. 1991). More than two forms of E2 occur (42–47 kDa) which differ in their degree of glycosylation (Bowden and Westaway 1985; Waxham and Wolinsky 1985).

E1 is 481 aa in length, has a molecular weight of 58 kDa, and three potential *N*-linked glycosylation sites (Figure 2) (Kalkkinen et al. 1984; Bowden and Westaway 1985; Waxham and Wolinsky 1985). E2–E1 heterodimers are membrane-bound and exposed on the surface of the virus particle. E2 is less accessible to the action of enzymes and antibodies than E1, suggesting that E2 is buried under E1 in the E2–E1 heterodimer (Waxham and Wolinsky 1985).

The RUBV structural proteins have been expressed in *Escherichia coli* and mammalian cells using Sindbis virus and vaccinia virus vectors, and in insect cells using baculovirus-expression vectors. Recombinant proteins have been purified from all of these systems (Seppanen et al. 1991; Londesborough et al. 1992; Oker-Blom et al. 1995; Chen et al. 1995; Orellana et al. 1999; Johansson et al. 1996). E1 and E2 have been expressed in soluble form (Hobman et al. 1994c; Seto et al. 1995), and virus-like particles containing the three structural proteins have been produced in transient and stably transfected CHO and BHK-21 cells (Hobman et al. 1994a, 1994b; Qiu et al. 1994).

RUBV NSPs contain a number of motifs of functional significance (Figure 2). P150 contains a region of homology to alphavirus NSP3, termed the X-motif, which is located adjacent to the protease domain, between aa 834 and 940. This motif has only been found in the genomes of the alphaviruses, Rubella virus, Hepatitis E virus, and possibly coronaviruses (Dominguez et al. 1990; Gorbalenya et al. 1991; Frey 1994). The X motif is necessary for *trans* cleavage activity of the NSP protease, but the exact function is still unknown. It is speculated that the proline-rich region within the X-motif may provide a protein–protein interaction domain that enables protease to easily gain access to its *trans* substrate (Liang and Gillam 2000). A methyltransferase (MT) motif has been identified at the N-terminus of p150 (Rozanov et al. 1992). It consists of a hydrophobic segment followed by a glycine-rich loop and is believed to have a role in the capping of viral RNA (Koonin 1993).

The second NSP, p90, contains two global amino acid motifs, which are conserved among positive-sense RNA viruses: a GDD motif (aa 1 966–1 968) indicative of RdRp activity, and a GKS/T motif indicative of helicase activity (Dominguez et al. 1990; Frey 1994; Pugachev et al. 1997a). Mutation of either Asp-1967 or Asp-1968 residues to Ala within the GDD motif are lethal. Mutation of Gly-1966 to Ala blocks virus replication and impairs infectivity, demonstrating that RUBV does have RdRp catalytic activity (Wang and Gillam 2001). This supports the role of p90 in positive-strand RNA synthesis.

ANTIGENIC COMPOSITION

Antigenic properties

Only one serotype of rubella virus has been described, and the virus is serologically distinct from other togaviruses. Early work on RUBV identified hemagglutinating (HA), complement-fixing (CF), precipitating (Le Bouvier 1969) and platelet-aggregating (PA) (Penttinen and Myllyla 1968) antigens. Kobayashi (1978) demonstrated that RUBV also has hemolytic activity.

The HA activity is associated with the surface projections on the viral envelope. It can be obtained from the supernatant fluid of infected BHK-21 cell cultures if these are maintained on serum-free medium or if nonspecific inhibitors of hemagglutination (serum lipoproteins) are first removed by kaolin treatment from the serum included in the maintenance medium (Stewart et al. 1967). Alternatively, treatment of the RUBV harvest with EDTA will separate HA from nonspecific inhibitors by removing Ca^{2+} (Furukawa et al. 1967). Rubella HA can also be prepared by alkaline extraction of infected BHK-21 or Vero cell cultures (Halonen et al. 1967). Although treatment of virus with ether destroys the HA activity, treatment with ether and Tween 80 retains this activity and increases the titer of the antigen, because of the formation of subunits. Ca^{2+} is required for the attachment of HA to erythrocytes. Optimum conditions for hemagglutination and further details of the nonspecific inhibitors of HA and methods for their removal from test sera were reviewed by Herrmann (1979).

Three CF antigens have been described: (1) a large particle antigen with a density of 1.19–1.23 g/ml in sucrose gradients and associated with the infectivity and HA activity; (2) a small particle ('soluble') antigen, which is probably a subunit of the protein coat of the virus (Schmidt and Styk 1968); and (3) a 150S particle, which appears to be associated with the ribonucleoprotein core of the virus (Vesikari 1972). High-titer preparations of RUBV can be used as CF antigens. These are usually prepared either by concentration of infected cell culture fluids or by alkaline extraction of infected cells (Herrmann 1979). BHK-21 and Vero cells are used for the production of suitable high-titer virus.

B-cell epitopes

During rubella infection, antibody responses are produced against all three structural proteins, but the E1 protein appears to be immunodominant (Table 2) (Cusi et al. 1989; Chaye et al. 1992a; Wolinsky et al. 1991). Studies using murine monoclonal antibodies have identified several epitopes on E1; three of these exhibit neutralizing or hemagglutinating activity, and have been mapped to residues 245–285 (Terry et al. 1988). A further well-conserved neutralization domain has been identified within residues 211–239 (Chaye et al. 1992b; Mitchell et al. 1992b; Wolinsky et al. 1993). Human sera react with synthetic peptides comprising residues 214–285 of the E1 protein, suggesting that this region may represent a major neutralization domain. A recombinant protein containing these epitopes is recognized by most rubella antibody-positive human sera (Starkey et al. 1995). Murine and human antibody-binding domains also occur outside this region (Ilonen et al. 1992; Mitchell et al. 1993; Newcombe et al. 1994). A neutralization epitope has also been identified on the E2 glycoprotein (1–26) (Dorsett et al. 1985); other epitopes have been located between amino acids 51 and 105. At least two epitopes are present on the C protein (residues 9–29 and 64–97) (reviewed by Frey 1994). However, the epitopes that confer protection are probably conformational (Wolinsky et al. 1991) and have not yet been identified.

T-cell epitopes

Rubella virus-specific cellular immune responses have been measured in vitro by lymphoproliferation assays and lymphocyte-mediated cytotoxicity assays. All three structural proteins contain T-cell epitopes. Immunoreactive regions have been identified within capsid protein residues 9–29, 119–152, 255–280, E2 residues 54–74 and E1 residues 213–239, 273–284, 358–377, and 389–422 (Ou et al. 1993; reviewed by Chantler et al. 2001). MHC-restricted cytotoxic T-cell responses have been described (reviewed by Chantler et al. 2001). The significance of these T-cell epitopes in relation to protection is unclear.

Biological, antigenic, and nucleic acid sequence variation

Differences in the biological activity of attenuated and wild-type strains have been reported. The attenuated Cendehill strain has lower infectivity for rabbits and other laboratory animals than wild-type strains (Zygraich et al. 1971; Gill and Furesz 1973). Ohtawara and colleagues (1985) demonstrated that the growth of Japanese vaccine strains is restricted at 39°C, the body temperature of the rabbit, suggesting that the attenuated strains are temperature-sensitive mutants. Chantler and colleagues (1993) have shown that the vaccine strains RA27/3 and Cendehill differ from wild-type strains in growth characteristics, plaque morphology, and temperature sensitivity. They have suggested that the processing in the Golgi of the viral glycoproteins from the attenuated strains was slower than for wild-type strains, particularly in synovial cells (a human chondrocyte cell line and synovial membrane cells). The growth of the HPV77-DE5 vaccine strain was similar to the M33 strain

Table 2 *Antigenic regions of RUBV structural proteins identified as B-cell epitopes*

Structural protein	Amino acid residues	Hemagglutination activity	Neutralization activity	References
E1	157–176			Chaye et al. (1993)
	202–283	+	+	Wolinsky et al. (1991)
	208–239		+	Corboba et al. (2000)
	212–286			Starkey et al. (1995)
	213–239	+	+	Mitchell et al. (1993)
	214–233		+	Chaye et al. (1992b)
	214–240	+		Chaye et al. (1992b)
	219–233		+	Chaye et al. (1992b)
	219–239			Mitchell et al. (1993)
	221–239		+	Wolinsky et al. (1993)
	243–286		+	Starkey et al. (1995)
	245–251	+	+	Terry et al. (1988)
	250–252			Lozzi et al. (1990)
	258–277			Mitchell et al. (1993)
	260–263		+	Lozzi et al. (1990)
	260–266	+	+	Terry et al. (1988)
	274–285	+	+	Terry et al. (1988)
	374–390			Chaye et al. (1993)
E2	?		+	Green and Dorsett (1986)
	10–36			Mitchell et al. (1993)
	1–115			Wolinsky et al. (1991)
	151–170			Mitchell et al. (1993)
	244–263			Mitchell et al. (1993)
C	1–30			Ou et al. (1992)
	9–29			Wolinsky et al. (1991)
	64–97			Wolinsky et al. (1991)
	96–123			Ou et al. (1992)

from which it was derived. More recently, the slower replication of the Cendehill strain has been confirmed and both wildtype and attenuated strains have been shown to induce apoptosis (Domegan and Atkins 2002; Cooray et al. 2003).

Many studies in the late 1960s and 1970s failed to reveal any antigenic variation among RUBV strains and there was no reliable test to distinguish wild and attenuated strains. However, differences have been observed by cross-neutralization tests and neutralization kinetics (reviewed by Banatvala and Best 1990). More recent studies with monoclonal antibodies used in neutralization, HAI and enzyme immunoassay (EIA) revealed no significant differences between nine RUBV strains tested, which included the vaccine strains Cendehill, RA27/3, HPV77-DE5, and TO336 (Best et al. 1992). However, Dorsett et al. (1985) demonstrated strain-specific epitopes within the E2 glycopolypeptide when disrupted virus was used.

RUBV is genetically stable when compared to other RNA viruses. Although there is only one serotype of Rubella virus (Best et al. 1992), two genotypes have been identified (Bosma et al. 1996; Frey et al. 1998). Genotype 1 was isolated from cases in Europe, North America, and Japan between 1966 and 1996. Genotype 2 was isolated from cases in India and China in the 1990s and has more recently been detected in Italy (Zheng et al. 2003). A variable region (aa 697–800) has been identified within the gene coding for NSP1 (Hofmann et al. 2003). Nucleic acid sequencing has revealed that five characteristic nucleotide changes found within a 1 300 nucleotide sequence of the E1 coding region of the RA27/3 vaccine strain distinguishes it from other isolates of RUBV (Frey and Abernathy 1993; Frey 1994). Despite this, the deduced amino acid sequence of RUBV is remarkably stable (0.0–2.9 percent) although the amino acid sequence of E1 is more variable (Bosma et al. 1996; Frey et al. 1998; Hofmann et al. 2003).

CLINICAL AND PATHOLOGICAL ASPECTS

Introduction

In 1941, N. McAlister Gregg, an Australian ophthalmologist, published his now famous retrospective study 'Congenital cataract following german measles in the mother,' in which he showed that, if acquired in early pregnancy, rubella could cause congenital malformations

(Gregg 1941). Seventy-eight babies, all with a similar type of congenital cataract, were born in New South Wales after an extensive rubella epidemic there in 1940 and many of the mothers gave a history of rubella, usually in the first or second month of pregnancy. Congenital defects of the heart were also noted in 66 percent of cases whose cardiac condition was recorded. These findings were soon confirmed in Australia, and deafness was also noted in many congenitally infected infants. Microcephaly, dental defects, and low birth weight were also reported.

Despite confirmation of Gregg's original observation, an annotation in the *Lancet* (Editorial 1944) suggested that additional studies were required, as it could not be proved that the illness with rash experienced by these mothers was in fact rubella and that it was unlikely that such an association would have previously gone unnoticed. However, Hope-Simpson (1944) reported in the *Lancet* congenital cataract and heart defects in two babies in England after epidemic rubella. Similar defects had been noted before Gregg's original observation, but their significance had not been appreciated. Additional retrospective studies reporting congenital defects induced by rubella in early pregnancy were subsequently carried out (reviewed by Hanshaw et al. 1985).

Variable results were obtained in retrospective studies carried out before the isolation of rubella virus in 1962. When the starting point for investigations was an infant with one or more rubella-induced deformities results suggested that a very high proportion of mothers who had rubella during pregnancy were delivered of infants with congenital malformations. The outcome of pregnancies in which maternal rubella was followed by the birth of unaffected infants was not recorded. In 1940, when rubella was at its peak incidence in New South Wales, Gregg and colleagues (1945) reported that 96 percent of infants whose mothers had had rubella in early pregnancy suffered from congenital defects that were confined to cases in which maternal rubella had occurred before the 16th week of gestation.

In the period 1963–1964, within a year of the first reports of RUBV isolation and detection of rubella antibodies by neutralization tests, the USA experienced one of the most extensive outbreaks of rubella ever recorded. During this epidemic it was shown that maternal rubella could result in a generalized and persistent fetal infection and that infants excreted virus not only at birth but also for many months after this, despite developing rubella-specific antibody responses. In addition, the spectrum of anomalies was much wider than had hitherto been described. The impact of this epidemic stimulated research into the development of rubella vaccines and, following this, attenuated vaccines were licensed for use, in the USA in 1969 and in Britain in 1970. Congenitally acquired rubella, therefore, became a potentially preventable disease. National programs, which achieved high uptake rates, have recently shown a marked reduction in both postnatally and congenitally acquired rubella.

Postnatally acquired infection

CLINICAL AND VIROLOGICAL FEATURES

After an incubation period of 14–21 days, the characteristic features of rubella, rash, and lymphadenopathy may appear. In young children the onset of illness is usually abrupt. Such constitutional symptoms as fever and malaise may be present for a day or two before onset of the rash, but they usually subside rapidly after its appearance. Older children and adults may experience more pronounced constitutional symptoms 3–4 days before the rash appears, and during this prodromal phase an enanthem consisting of erythematous pinpoint lesions on the soft palate may be present. The exanthem is usually discrete, in the form of pinpoint maculopapular lesions. It appears first on the face and spreads rapidly to the rest of the body; lesions on the body may coalesce. The rash usually persists for about 3 days, occasionally longer, but may be fleeting. The mechanism by which rash is induced has not been established. Although immunopathological mechanisms may be responsible, RUBV has been isolated from skin biopsy specimens taken not only from areas with rash, but also from parts of the skin without rash and from the skin of patients with subclinical infection (Heggie 1978). Furthermore, the development of rash may be prevented by the administration of pooled human immunoglobulin, although this does not prevent viremia. Patients may complain of tender lymph nodes when or just before the rash appears. Follow-up studies of susceptible people exposed to rubella have revealed that lymphadenopathy may be present 7–10 days before the onset of rash, and sometimes for an even longer period after it has disappeared. Suboccipital, postauricular, and cervical lymph nodes are most frequently affected. Rubella is rarely associated with severe complications. Encephalitis may occur in one in 5 000–10 000 cases, but in general the prognosis is good (Krugman and Ward 1968). Very occasionally, there is clinical evidence of thrombocytopenia, which may result in purpuric rash, epistaxis, hematuria, and gastrointestinal bleeding.

The most common complication of postnatally acquired rubella is joint involvement. Although this is rare among children and adult males, it may occur in up to 60 percent of postpubertal females. Symptoms generally develop as the rash subsides and vary in severity from mild stiffness of the small joints of the hands to a frank arthritis with severe pain, joint swelling, and limitation of movement. The finger joints, wrists, knees, and ankles are most frequently affected. The duration of these symptoms is usually about 3 days, but occasionally they may persist for up to a month. Rubella-induced arthralgia is not associated with any sequelae. Arthralgia

also occurs in postpubertal females after administration of rubella vaccine. The mechanism by which naturally acquired and vaccine-induced infection causes arthralgia is probably complex. Thus, joint symptoms may result from direct infection of the synovial membrane by virus, as RUBV has been isolated from the joint aspirates of vaccinees with vaccine-induced arthritis (Weibel et al. 1969). Furthermore, studies in vitro have shown that attenuated virus strains will replicate in human synovial membrane cell cultures (Grayzel and Beck 1971). However, an immune mechanism is probably also involved, because, in addition to virus, joint aspirates have been shown to contain rubella-specific IgG (Ogra and Herd 1971; Mims et al. 1985), which suggests that joint symptoms may be induced by immune complexes. It is therefore of interest that the presence of rubella antibody containing immune complexes in the serum has been associated with a high incidence of joint symptoms following rubella vaccination (Coyle et al. 1982). However, hormonal factors may also play a role, for, in addition to being common in postpubertal females, the development of joint symptoms appears to be related to the menstrual cycle. After rubella vaccination, they are most likely to occur within 7 days of the onset of the cycle (Harcourt et al. 1979).

RUBV has been suggested as a possible cause of chronic inflammatory joint disease, but studies on patients with that disease have yielded conflicting and unconfirmed results. In the 1980s it was claimed that RUBV could be detected in synovial fluid or the synovium, as well as in lymphocytes from patients with both rheumatoid arthritis and seronegative arthritis (i.e. rheumatoid factor negative), but these reports have not been confirmed. However, it is known that RUBV is able to establish persistent infection in vivo (Waxham and Wolinsky 1984), as well as in human synovial cell cultures and organ cultures (Cunningham and Fraser 1985; Miki and Chantler 1992) and that high levels of rubella antibody are found in joint fluid (Mims et al. 1985). Although there is no doubt that RUBV may be isolated from synovial fluid after naturally acquired infection or vaccination and from occasional patients with chronic inflammatory joint disease (Report 1994; Bosma et al. 1998), recent studies show that rubella vaccination is not associated with chronic inflammatory joint disease.

DIFFERENTIAL DIAGNOSIS

Rubella is often difficult to diagnose clinically because the illness may present atypically with minimal lymphadenopathy and an evanescent rash, and, conversely, typical rubelliform rashes may be induced by other viruses (Ramsay et al. 2002). Thus, such viruses as enteroviruses, human herpesviruses 6 and 7, West Nile virus and dengue virus may occasionally induce rubella-like rashes. Chikungunya virus and Ross River virus (Togaviruses) and parvovirus B 19 (Parvoviruses) may induce not only a rubella-like rash, but also arthralgia. Serological studies have shown that there is poor correlation between a past history of rubella and immune status, particularly among adults (Brown et al. 1969). This is not surprising because not only may rubella present atypically, but disease without rash or subclinical infection may occur in up to 25 percent of children (Krugman and Ward 1954; Green et al. 1965).

The relation between clinical and virological features is shown in Figure 3. The patient is potentially infectious for a prolonged period, as pharyngeal virus excretion may occur for up to a week before onset of rash and persist thereafter for 7–10 days. Virus may also be detected in the stools and urine, but excretion occurs for a shorter time. These are not suitable specimens from which to isolate virus and do not play an important role in virus transmission. Viremia is present for about a week before the onset of rash, but as this appears rubella antibodies develop and viremia terminates.

EPIDEMIOLOGY

Rubella has a worldwide distribution. In temperate climates, before the introduction of rubella vaccination, epidemics usually occurred in spring and early summer, rubella occurring less commonly among preschool children than among schoolchildren and young adults. Women of child-bearing age were often infected as a result of exposure to their own children or at work. Seroepidemiological studies showed that the proportion of seropositive people increased progressively with age, about 50 percent of 9- to 11-year-old children being immune. Among women of child-bearing age, the proportion increased to about 80–85 percent (Dowdle et al. 1970). In industrialized parts of the world (e.g. UK, USA, Sweden, and Finland), which have achieved high uptake rates for rubella vaccine, postnatally and congenitally acquired rubella are now rare. However, in the UK, there is still a pool of rubella-susceptible males who have not been vaccinated. Many young men were infected in localized outbreaks of rubella in 1993 and 1996 (Figure 4), transmitting infection to some susceptible pregnant women, most of them being young primigravidae and many of whom were immigrants (Miller et al. 1997, www.hpa.org.uk/infections). Immigrants from Asia are at particular risk, as up to 23 percent may be susceptible (Tookey et al. 2002). Despite the success of the rubella vaccination program in the USA, a number of cases of congenitally acquired rubella were reported in California among immigrant women and among the Amish population in Pennsylvania in 1989/1990, this resulting from infection of unvaccinated women (Lindegren et al. 1991).

During the last few years, several studies have demonstrated that there is a considerable problem of

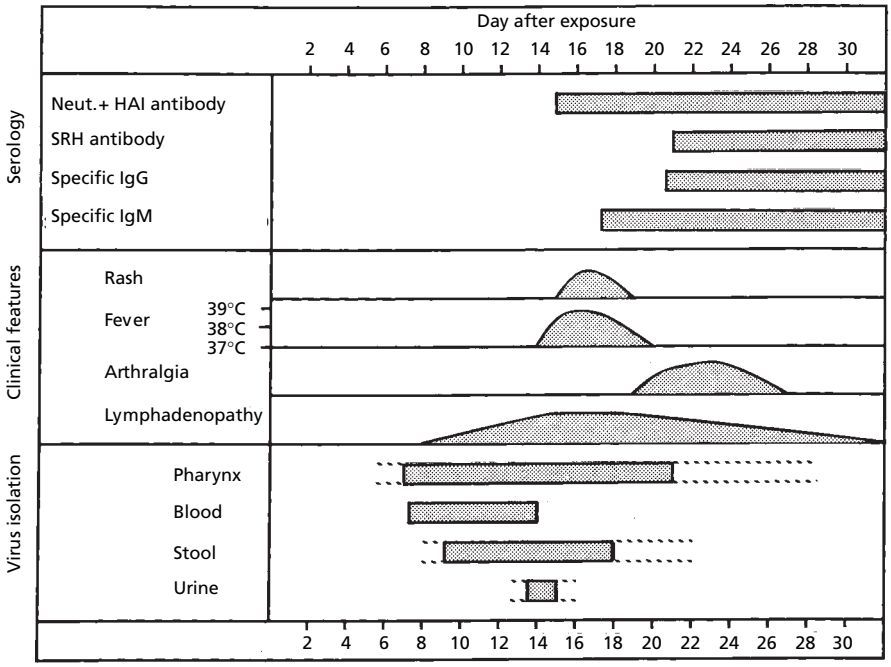

Figure 3 *Relationship between clinical and virological features of postnatally acquired rubella. HAI, hemagglutination inhibition; Neut., neutralizing; SRH, single radial hemolysis.*

congenital rubella in developing countries (Cutts et al. 1997; WHO 2000a, c). Such countries have an annual incidence of 0.6–4.1 cases per 1 000 live births, usually associated with rubella outbreaks. This is similar to the incidence in developed countries before the introduction of rubella vaccination. Epidemics of rubella occur every 4–7 years. The proportion of women susceptible to rubella has been shown to be 15–20 percent in many areas, although there may be considerable variation between different areas of a country, with susceptibility usually being higher in rural than in urban areas (Cutts et al. 1997). In some countries ⩾25 percent are susceptible. Particularly high susceptibility rates occurred in island populations, including Jamaica and Trinidad and Tobago. An increasing number of countries are addressing this problem by including rubella vaccination in their national immunization programs (see Vaccination programs).

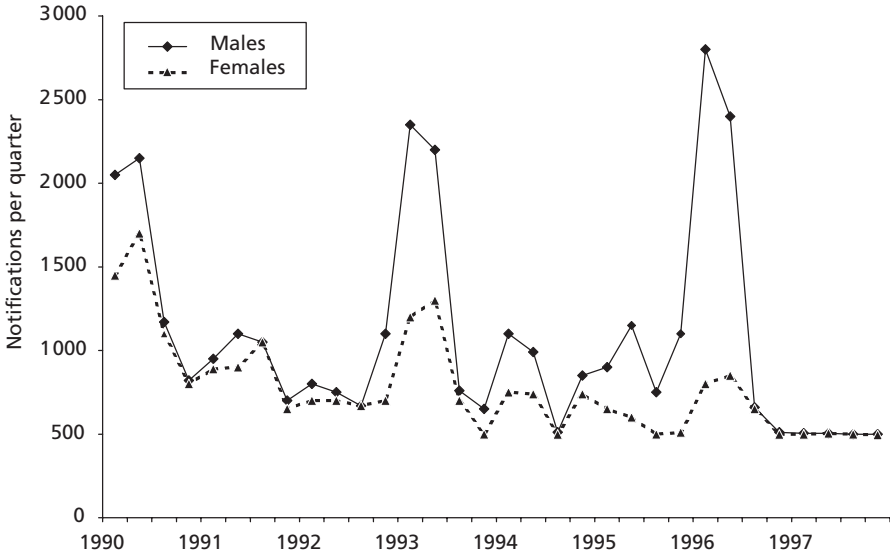

Figure 4 *Epidemic periodicity of rubella in males and females in England and Wales. Laboratory reports 1990 to 1997 (Miller et al. 1997).*

IMMUNE RESPONSES

Rubella-specific IgG, IgM, and IgA responses develop rapidly after the onset of rash. Rubella-specific IgG persists for life, but may decline to low levels in old age. The IgG response is predominantly IgG1. IgG3 is detected in most sera from cases of recently acquired rubella and IgG4 occasionally in sera from cases of remote rubella (Stokes et al. 1986; Thomas and Morgan-Capner 1988). Rubella-specific IgM usually appears within 4 days of onset of rash and persists for 4–12 weeks, but detection depends on the sensitivity of the technique employed. Specific IgM may sometimes persist for up to 1 year after both naturally acquired infection and rubella immunization (Pattison et al. 1975; O'Shea et al. 1985). Serum and nasopharyngeal IgA responses are detectable for at least 5 years after infection. The specific serum IgA response is exclusively IgA1. Specific IgD and IgE responses develop rapidly after onset of infection and persist for at least 6 months. The results of Mitchell and colleagues (1992a) suggest that males have a more rapid antibody response than females. Methods available for the detection and quantification of rubella antibodies are discussed under Laboratory diagnosis.

Immunoblotting for detection of polypeptide-specific antibodies is technically difficult. However, Zhang et al. (1992) demonstrated that antibodies to E1 and E2 were detected more effectively under nonreducing conditions than in the presence of 2-mercaptoethanol. The response to E1 is greater than to E2 and there is a strong response to the capsid protein (C) (Zhang et al. 1992; Nedeljkovic et al. 1999). Antibodies to E1 are essential for protection (Cusi et al. 1995). Avidity of IgG antibody to E1 matures during the 2 years after rubella infection, but IgG anti-E2 and anti-C show minimal avidity maturation (Mauracher et al. 1992).

A decrease in total leukocytes, neutrophils, and T cells, and a transient depression of lymphocyte responsiveness to mitogens and antigens such as purified protein derivative (PPD) is seen after rubella (Buimovici-Klein et al. 1976; Maller et al. 1978; Niwa and Kanoh 1979; Hyypiä et al. 1984). Cell-mediated immune responses, measured by lymphocyte proliferation assays, develop within a few days of onset of rash and persist for many years. Lymphokine secretion has also been detected (Honeyman et al. 1974; Buimovici-Klein and Cooper 1985). MHC class I-restricted $CD8^+$ cytotoxic T lymphocytes have been demonstrated in rubella-immune individuals (Lovett et al. 1993).

RE-INFECTION

Natural infection is followed by protection from reinfection, except in rare cases. However, evidence of reinfection may occasionally be obtained by demonstrating a significant increase in antibody concentration following natural and experimental exposure to rubella. Such reinfection is generally asymptomatic (Horstmann et al. 1970; Vesikari 1972). Reinfection in pregnancy is hazardous only if viremia occurs, and this has rarely been documented in experimental studies (O'Shea et al. 1983). Following maternal reinfection during the first 16 weeks in pregnancy, the risk of fetal infection has been estimated to be no more than 8 percent, and fetal damage is rare (Best et al. 1989; Morgan-Capner et al. 1991). Although it is possible that, in such cases, transmission of virus to the fetus may be due to a specific defect in the maternal immune response, rubella reinfection is not associated with a lack of neutralizing antibodies or persistent impairment of rubella-specific lymphoproliferative responses (O'Shea et al. 1994). Sequence changes have not been identified in the E1 ORF of isolates from cases of reinfection (Bosma et al. 1996). Further studies are required to determine whether reinfection is due to a failure to produce an immune response to the protective epitopes of the virus. Reinfection in pregnancy will be eliminated if high rates of rubella vaccination are achieved and maintained among the target groups (see Vaccination programs). Rubella reinfection has been reviewed by Best (1993) and Bullens et al. (2000).

Congenitally acquired infection

CLINICAL MANIFESTATIONS

Although the early retrospective enquiries emphasized the frequency and importance of such defects as congenital anomalies of the heart and eyes, and deafness, it was not until follow-up studies had been carried out on infants whose mothers had had rubella during the extensive 1963–1964 outbreak in the USA that it was fully appreciated that congenital rubella frequently caused widespread multisystem disease. Follow-up studies showed that congenital rubella syndrome (CRS) was not a static disease and that prolonged careful evaluation of infants at risk was necessary before some or all of the features of CRS were apparent. The broader range of anomalies described after the US 1963/1964 and subsequent outbreaks were probably not due to any change in viral virulence, but rather to more careful and prolonged observation. Careful scrutiny of the records of infants with CRS who were born before these outbreaks revealed that such anomalies as thrombocytopenic purpura and osteitis, although not reported in the literature, occurred fairly frequently.

Cooper (1975) divided clinical features associated with rubella infection into those that were transient, developmental, or permanent (Table 3). The pathogenesis of transient lesions is not understood, but they are usually present only during the first few weeks of life, do not recur and are not associated with the development of permanent sequelae. Intrauterine growth retardation resulting in low birth weight but at a normal gestational

Table 3 *Clinical features associated with congenitally acquired rubella (adapted from Cooper 1975)*

	Common	Uncommon
Transient	Low birth weight	Cloudy cornea
	Thrombocytopenic purpura	Hepatitis
	Hepatosplenomegaly	Generalized lymphadenopathy
	Bone lesions	Hemolytic anemia
	Meningoencephalitis	Pneumonitis
Developmental	Sensorineural deafness	Severe myopia
	Peripheral pulmonary stenosis	Thyroiditis
	Mental retardation	Hypothyroidism
	Central language defects	Growth hormone deficiency
	Diabetes mellitus	'Late onset disease'
Permanent	Sensorineural deafness	Severe myopia
	Peripheral pulmonary stenosis	Thyroid disorders
	Pulmonary valvular stenosis	Dermatoglyptic abnormalities
	Patent ductus arteriosus	Glaucoma
	Ventricular septal defect	Myocardial abnormalities
	Retinopathy	
	Cataract	
	Microphthalmia	
	Psychomotor retardation	
	Microcephaly	
	Cryptorchidism	
	Inguinal hernia	
	Diabetes mellitus	

age ('small for dates' babies) is among the most common of the transient features. Thus, Cooper and colleagues (1965) found that about 60 percent of infected infants fell below the tenth, and 90 percent below the 50th percentile.

A petechial or purpuric rash is also common, particularly among infants whose mothers had had maternal rubella in early pregnancy (Figure 5) (Cooper et al. 1965; Horstmann et al. 1965). However, low birth weight and a purpuric rash are seldom the sole manifestations of congenital rubella. These infants may have other anomalies, such as congenital heart and eye defects, although they may not always be apparent at birth. Infants with thrombocytopenic purpura generally have a platelet count ranging from 3 000 to 100 000/mm^3, this being associated with a decreased number of megakaryocytes, but of normal morphology, in the bone marrow. In general, the platelet count rises spontaneously during the first month of life, although (rarely) some infants die from such complications as intracranial hemorrhage.

OCULAR DEFECTS

Many of the ocular defects characteristic of CRS were described by Gregg (1941), who drew particular attention to pigmented retinopathy and cataract. Pigmented retinopathy may be present in up to 50 percent of infants with CRS (Menser and Reye 1974; Vijayalakshmi et al. 2002) and may provide a useful aid in clinical diagnosis. The macular area of the retina is generally affected, but the lesions rarely impair vision. Cataracts may be unilateral or bilateral. Although usually present at birth, they may not be visible until several weeks later (Murphy et al. 1967). Lesions may be subtotal, consisting of a dense pearly-white central opacity (Figure 6) or, total with a more uniform density throughout the lens. Microphthalmus and iris hypoplasia are often associated with congenital cataract, but glaucoma is less common. However, it is important to recognize glaucoma as it can rapidly lead to blindness. Strabismus and nystagmus are also common (Vijayalakshmi et al. 2002).

Microphthalmia and glaucoma result from disturbances in organogenesis, and retinopathy and cataract result from intrauterine tissue destruction. However, delayed manifestations of congenital infection have also been recorded including lens changes, chronic uveitis, glaucoma, choroidal neovascularization, corneal hydrops, and keratoconus. Mechanisms postulated include virus persistence in the eye, resulting in RUBV-induced reduced growth rate and lifespan of cells, autoimmune phenomena or virally induced vascular damage and reactive hypervascularization (reviewed by Arnold et al. 1994).

DEAFNESS

Of the permanent defects, the most common is sensorineural deafness. This results from rubella-induced

Figure 5 *Purpuric rash in newborn infant with congenitally acquired rubella, who was subsequently found to have congenital heart disease and cataract.*

damage to the organ of Corti. However, central auditory impairment may also occur. Hearing loss, which may be unilateral or bilateral, mild or profound, may sometimes be the only rubella-induced congenital anomaly.

Peckham (1972) followed up 218 children who were apparently normal at birth, but who had been exposed to rubella in utero. When assessed for hearing loss at the age of 1–4 years, 50 (23 percent) were deaf. When 85 were re-examined between the ages of 6 and 8 years, further hearing defects were detected in another nine children. Of the children with hearing defects, 90 percent were seropositive. Because rubella antibodies are uncommon before the age of 4 years, it is particularly important to follow up infants with persistent rubella antibody so that hearing defects can be recognized as early as possible. Hearing defects can be detected in early infancy by testing auditory brainstem responses and otoacoustic emissions (Van Straaten et al. 1996).

Figure 6 *Congenital rubella cataract in a 9-month-old infant. Cataract present in the left eye was surgically removed.*

HEART DISEASE

Congenital anomalies of the cardiovascular system are responsible for much of the high perinatal mortality associated with CRS. Numerous studies have shown that the most common lesions are persistence of a patent ductus arteriosus, proximal (valvular) or peripheral pulmonary artery stenosis, and a ventricular septal defect (Sperling and Verska 1966; Hastreiter et al. 1967; Cooper 1975).

Occasionally, neonatal myocarditis is found, often associated with other cardiac malformations (Korones et al. 1965). Rubella-induced damage to the intima of the arteries may result in obstructive lesions in the renal and pulmonary arteries (Rorke and Spiro 1967; Phelan and Campbell 1969).

DEFECTS IN THE CENTRAL NERVOUS SYSTEM

About 25 percent of infants who present at birth with clinical evidence of CRS have central nervous system (CNS) involvement, usually in the form of a meningoencephalitis. Such infants are often lethargic at birth, but may become irritable and often exhibit evidence of photophobia. They have a full anterior fontanelle, pleocytosis, and an increased amount of protein in the cerebrospinal fluid (CSF) (Desmond et al. 1967). The outcome is variable and unpredictable. Although approximately 25 percent of infants presenting with meningoencephalitis at birth may, by the age of 18 months, be severely retarded, suffering from communication problems, ataxia, or spastic paresis, others appear to progress well neurologically despite poor development in the first 6 months of life.

Rubella panencephalitis is rare. Only about 50 cases have been reported, the majority as a consequence of congenital rubella infection, although it has also been reported following postnatally acquired disease. Most patients with CRS develop clinical signs between the ages of 8 and 20 years (reviewed by Frey 1997). RUBV has been recovered from the brain both with and without cocultivation techniques. It has also been recovered from patients' lymphocytes (Cremer et al. 1975; Wolinsky et al. 1979). Elevated levels of rubella-specific IgG and occasionally IgM may be detected in the serum.

The CSF may contain elevated concentrations of protein and immunoglobulin. Oligoclonal bands and a high CSF: serum rubella antibody ratio may be present suggesting intrathecal production of antibodies (Wolinsky et al. 1982).

Histological studies show panencephalitis with a perivascular inflammatory response, as well as a vasculitis. Rubella antigens have not been detected in brain sections by immunofluorescence. It has been postulated that post-rubella panencephalitis may be mediated by immune complexes (Waxham and Wolinsky 1984) or by virus-mediated autoreactivity to brain antigens (Martin et al. 1989).

DIABETES

Diabetes mellitus was originally believed to be a rare complication of CRS. However, follow-up studies of infants infected in utero during the Australian and US epidemics of 1940 and 1963/1964, respectively, have shown that nine of 45 (20 percent) of Australian and 30 of 242 (12.4 percent) of US children eventually developed insulin-dependent diabetes mellitus (IDDM). Up to 40 percent of CRS patients have impaired glucose tolerance (See and Tilles 1998). A long latent period is characteristic, the mean age of children developing IDDM in the US study being 9 years; all the Australian patients were in their third decade (Menser et al. 1978; Ginsberg-Fellner et al. 1985). Lymphocytic infiltration of the pancreas of an infant with CRS, but without IDDM may suggest that RUBV can initiate a train of events that subsequently results in IDDM in later life (Bunnell and Monif 1972). It seems likely that autoimmune mechanisms are involved in the pathogenesis of IDDM, because the HLA types in these patients are typical of those with autoimmune disease, there being a significant increase in prevalence of HLA-DR3, some increase in HLA-DR4 and a virtual absence of HLA-DR2. In addition, islet cell antibodies have been detected in 20 percent of these patients. These antibodies have a cytotoxic effect on cultured islet cells and predict the diabetic state. Although autoimmune responses may play an important role, the mechanism by which RUBV might trigger them remains to be established. An experimental study employing human fetal islet cells showed that RUBV induced a depression of immunoreactive secreted insulin without being cytolytic (Numazaki et al. 1990). A further study suggested that autoimmune phenomena might be involved, because immunoreactive epitopes in the RUBV capsid shared antigenicity with islet β cell protein (molecular mimicry) (Karounos et al. 1993).

BONE DEFECTS

Bone lesions may be detected by X-ray. Irregular areas of translucency are present in the metaphyseal portion of the long bones, but there is no evidence of periosteal reaction in over 20 percent of infants with congenital rubella (Cooper et al. 1965). These lesions generally resolve within 1–2 months. Cooper et al. (1965) detected these characteristic radiological changes in a fetus of 18 weeks' gestational age, suggesting that the process inducing such changes begins in early gestational life.

LATE ONSET DISEASE

Between the ages of about 3 and 12 months, some infants may present with such features as a chronic rubelliform rash, persistent diarrhea and pneumonitis. Marshall (1973) referred to this syndrome as 'late onset disease.' Although mortality is high, some infants show a dramatic response to treatment with corticosteroids. This syndrome may reflect an immunopathological phenomenon. Circulating immune complexes that appear to contain rubella antigen have been demonstrated in infants with late onset disease (Tardieu et al. 1980), and Coyle and colleagues (1982) demonstrated rubella antibody containing immune complexes in children with congenital rubella who developed new clinical problems some years after birth.

Some developmental defects may take many months or years to become apparent, but then persist permanently. Failure to recognize such defects in early infancy may not always be the result of difficulty in their detection. There is evidence which suggests that such defects as perceptive deafness, CNS anomalies and some ocular defects may actually develop or become increasingly severe some considerable time after birth. Menser and Forrest (1974) showed that it might be up to 4 years before the first rubella defects were recognized. Further defects might continue to be recognized up to the age of 8 years. The progressive nature of congenitally acquired disease is emphasized by the finding that children with previously stable congenital rubella-induced defects developed a widespread subacute progressive panencephalitis with progressive motor retardation as late as the second decade in life (Townsend et al. 1975; Weil et al. 1975).

LONG-TERM FOLLOW-UP OF CRS

As a result of the extent and severity of rubella-induced congenital malformations, children who survive will need continuous specialized management, education, and rehabilitation. However, a study carried out on 50 25-year-old patients with CRS born in Australia after the 1940/1941 epidemic showed that, although many were deaf or had eye defects, they had developed far better than had been anticipated when assessed in early childhood. Many had married and produced normal children, and all but four were employed, most patients being of average intelligence (Menser et al. 1967a). Follow-up studies so far reported on children with CRS following the 1963/1964 US rubella epidemic suggest that it may have had a more catastrophic impact on the lives of affected children than did the 1940/1941

Australian epidemic (Cooper 1975). This may be a reflection on the more modern methods of treatment available to the children born after this more recent epidemic. Many might not have survived previously.

Although congenitally acquired rubella is now rare in countries that have adopted rubella vaccination programs, a heavy burden from previous epidemics persists. A 20-year follow-up on 125 patients infected during the extensive 1963–1965 USA outbreak showed that, although many patients had multiple defects, ocular disease was the most commonly noticed disorder (78 percent) followed by sensorineural deafness (66 percent), psychomotor retardation (62 percent), and cardiac anomalies (58 percent). A 60-year follow-up of the Australian cohort has shown that the prevalence of diabetes, thyroid disorders, early menopause, and osteoporosis is higher in those with CRS than in the general population (Forrest et al. 2002).

Pathogenesis

The fetus is at risk during the period of maternal viremia, because placental infection may occur at this time. The most likely source of virus is from the maternal viremia. Virus may also be excreted via the cervix for up to 6 days after the onset of rash (Seppala and Vaheri 1974), and, because virus may exist in the genital tract for even longer, placental infection by direct contact or from ascending genital infection cannot be excluded.

After infection in early pregnancy, rubella induces a generalized and persistent virus infection in the fetus, which may result in multisystem disease. Töndury and Smith (1966) conducted histopathological studies on the products of conception from mothers clinically diagnosed as infected with rubella: anomalies were present in 68 percent of 57 fetuses when maternal rubella was contracted in the first trimester. When contracted in the first month of pregnancy, 80 percent were abnormal, sporadic foci, or cellular damage being present in the heart, inner ear, lens, skeletal muscle, and teeth. It was suggested that RUBV enters the fetus via the chorion, in which it induces necrotic changes in the epithelial cells, as well as in the endothelial lining of the blood vessels. The damaged endothelial cells are desquamated into the lumen of the vessel and then transported as virus-infected 'emboli' into the fetal circulation to settle in and infect various fetal organs. Lesions in the chorion were present as early as the tenth day after the onset of maternal rash. Fetal endothelial damage was distributed widely and probably resulted from viral replication rather than from antibody-mediated damage, because the most extensive histopathological changes were present at a gestational period before the fetal immune defense mechanism was sufficiently mature to be activated. Indeed, a characteristic feature of rubella embryopathy following maternal rubella in early gestational life is the notable absence of an inflammatory cell response (Töndury and Smith 1966).

At least three mechanisms have been suggested for inducing fetal damage: a virus-induced retardation in cell division, apoptosis (Pugachev and Frey 1998b), and tissue necrosis. Studies in vitro on embryonic cell cultures and rubella-infected fetuses suggest that RUBV may induce chromosomal damage and cause cells to divide more slowly than those that are uninfected (Plotkin et al. 1965). This may be due to a specific protein that reduces the mitotic rate of infected cells (Plotkin and Vaheri 1967). If retardation of cell division occurs during the critical phase of organogenesis, it is likely to result in congenital malformations. It has also been shown that the organs of rubella-infected infants are smaller and contain fewer cells than those of uninfected infants (Naeye and Blanc 1965). The fetal endothelial damage induced by rubella infection may cause hemorrhages in small blood vessels, leading to tissue necrosis and further damage of malformed organs over a longer period. Such organs as the liver, myocardium, and organ of Corti may be affected. Studies on the products of conception obtained from virologically confirmed cases of rubella during the first trimester have shown that the fetus is almost invariably infected, regardless of the time at which infection has occurred during this period (Rawls 1968; Thompson and Tobin 1970). It has also been suggested that RUBV may interfere with organ growth by preventing the assembly of actin and interrupt the cell cycle by binding to the tumor suppressor protein pRB (reviewed by Lee and Bowden 2000).

RUBV is isolated infrequently from neonates whose mothers developed infection after the first trimester, possibly because by then fetal immune mechanisms can effectively terminate infection. More mature fetal tissues do not have a reduced susceptibility to infection, for studies in vitro have shown that RUBV will replicate as well in organs derived from fetuses of 12–13 weeks' gestational age as in those of younger fetuses (Best et al. 1968). Nevertheless, even though severe congenital anomalies are rarely encountered following rubella after the first trimester, serological evidence of fetal infection has been shown to occur in 25–33 percent of infants whose mothers acquired maternal rubella between the 16th and 28th weeks of gestation (Cradock-Watson et al. 1980; Vejtorp and Mansa 1980).

Persistence of virus

Following intrauterine infection in early pregnancy, RUBV persists throughout gestation and can be isolated from most organs obtained at autopsy from infants who die in early infancy with severe and generalized infections. Virus may also be recovered from the nasophar-

yngeal secretions, urine, stools, CSF, and tears of survivors. RUBV can be isolated from nasopharyngeal secretions of most neonates with severe congenitally acquired disease, but by the age of 3 months the proportion excreting virus has declined to 50–60 percent and by 9–12 months to 10 percent (Cooper and Krugman 1967). Particularly during the first few weeks after birth, those with severe disease may excrete high concentrations of virus and readily transmit infection to rubella-susceptible contacts. RUBV may persist in infants with CRS in secluded sites for even longer. Thus, RUBV has been recovered from a cataract removed from a 3-year-old child (Menser et al. 1967b) and from the CSF of children with CNS involvement up to the age of 18 months (Desmond et al. 1967). Rubella antigen was detected in the thyroid from a 5-year-old child with Hashimoto's disease by immunofluorescence (Ziring et al. 1977), and by cocultivation techniques RUBV was recovered from the brain of a child who developed rubella panencephalitis at the age of 12 years (Cremer et al. 1975; Weil et al. 1975). Experimental studies have shown that, within the CNS, the astrocyte is the main cell type in which RUBV replicates with high concentrations of virus being expressed. Intrauterine infection involving these cells may perhaps induce focal areas of necrosis resulting in the pattern of neurological deficit observed in CRS (Chantler et al. 1995).

How RUBV persists throughout gestation and for a limited period during the first year of life has not been clearly established. Possible mechanisms include defects in cell-mediated immunity (CMI), poor interferon synthesis, and the possibility that a limited number of infected fetal cells give rise to infected clones which persist for a limited period. It has also been suggested that selective immune tolerance to the RUBV E1 protein may play a role (Mauracher et al. 1993). Studies in vitro show that RUBV replicates in T lymphocytes and macrophages and can also persist in B lymphocytes, causing inhibition of host-cell protein synthesis (Chantler and Tingle 1980; van der Logt et al. 1980). Infection of macrophages may interfere with their interactions with T cells. Postnatally acquired rubella causes a transient reduction in lymphocyte responses to phytohemagglutinin (Buimovici-Klein et al. 1976; Maller et al. 1978; Vesikari 1980), as well as a decrease in the numbers of T cells (Niwa and Kanoh 1979). CRS might be expected to cause an even greater reduction in responsiveness. Indeed, significantly diminished lymphoproliferative responses to phytohemagglutinin and rubella antigen, as well as diminished interferon synthesis, were demonstrated in 40 congenitally infected children aged 1–12 years (Buimovici-Klein et al. 1979). Impairment of CMI responses was related to the gestational age at which maternal infection occurred, and was greatest in infants whose mothers acquired rubella in the first 8 weeks of pregnancy. Hosking and colleagues (1983) suggested that children with nerve deafness due to CRS could be distinguished from those with immunity due to postnatally acquired rubella by their failure to produce lymphoproliferative responses to rubella antigen. O'Shea and colleagues (1992) also found that 10 of 13 (80 percent) children with CRS under the age of 3 years failed to mount a lymphoproliferative response. Congenitally infected infants also have impaired natural killer cell activity (Fuccillo et al. 1974) and persistent T-cell abnormalities (Rabinowe et al. 1986). Defective CMI responses may persist into the second decade of life, well beyond the time when RUBV can be recovered from accessible sites.

Risks to the fetus

Whether maternal rubella induces fetal damage that causes intrauterine death or the birth of a malformed infant depends on the gestational age at which maternal rubella occurs, although other factors may also be involved. Maternal rubella may result in spontaneous abortion in up to 20 percent of cases (Siegel et al. 1971). This occurs most commonly when maternal infection is acquired during the first 8 weeks of pregnancy. To this must be added fetal wastage from therapeutic abortion following virologically confirmed rubella.

MATERNAL RUBELLA IN THE FIRST TRIMESTER

Many early prospective studies, the results of which are still quoted, underestimated the risks of congenital malformations, because maternal infections were included that were not rubella-induced and some rubella-infected infants were not followed up for sufficiently long. It is now known that 75–100 percent of infants born to mothers infected at this time will be congenitally infected and most of those infected will have associated defects (Enders 1982; Miller et al. 1982; Grillner et al. 1983). Many infants, although apparently normal at birth, if followed up for periods ranging from a few months to some years, may eventually be shown to have such defects as perceptive deafness or minimal CNS anomalies. Figure 7 relates the gestational age of maternal rubella to the clinical manifestations of congenitally acquired disease among 376 infants infected in utero during the 1964 epidemic in the USA. When maternal infection is acquired during the first 8 weeks of pregnancy – the critical phase of organogenesis – cardiac and eye defects are likely to occur. Retinopathy, hearing, and CNS defects are more evenly distributed throughout the first 16–20 weeks of gestational life.

MATERNAL RUBELLA AFTER THE FIRST TRIMESTER

RUBV is seldom isolated from infants whose mothers acquired rubella after the first trimester, although studies in vitro have shown that fetal tissues, regardless of gestational age, are susceptible to infection (Best

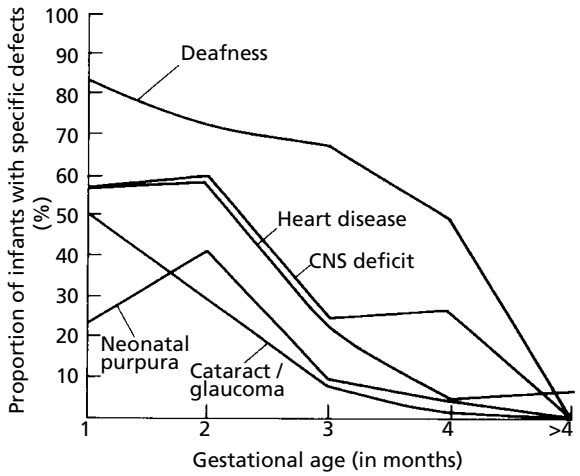

Figure 7 *Relation of clinical manifestations of congenital rubella to time of maternal infection, extrapolated from Cooper et al. (1969).*

et al. 1968). Indeed, serological studies confirm that a high proportion of infants are infected as a result of maternal rubella contracted after the first trimester, rubella-specific IgM being detected in 25–33 percent of infants whose mothers had rubella between the 16th and 20th weeks of pregnancy (Cradock-Watson et al. 1980). However, because organogenesis is complete by 12 weeks and in more mature fetuses immune responses may limit or terminate infection, such infants rarely have severe or multiple anomalies. Deafness and retinopathy, which per se does not affect vision, are likely to be the only anomalies commonly associated with rubella after the first trimester. When the results of four studies conducted in different countries are combined it is seen that the risk is about 17 percent when infection occurs at 13–16 weeks and about 5.9 percent after infection at 17–20 weeks (Peckham 1972; Miller et al. 1982; Grillner et al. 1983; Munro et al. 1987). Figure 7 shows that deafness is usually the sole clinical manifestation of fetal infection occurring between 13 and 16 weeks, and is relatively common, but that deafness or any other defects are only rarely encountered after this time.

PRECONCEPTUAL RUBELLA

Studies conducted in Germany and Britain indicate that preconceptual rubella does not result in transmission of RUBV to the fetus. Thus, Enders and colleagues (1988) found no serological or clinical evidence of intrauterine infection in 38 infants whose mothers' rashes appeared before or within 11 days after their last menstrual period (LMP). However, the fetus of a mother whose rash appeared 12 days after her LMP became infected, and all of ten mothers who developed rash 3–6 weeks after their LMP transmitted infection to their fetuses.

LABORATORY DIAGNOSIS

Rubella antibody screening

Tests for rubella antibodies are extensively used to identify susceptible women who should be offered rubella vaccination. In the UK, all antenatal patients are tested, as should women presenting to their general practitioners and 'well woman' clinics who have no history of vaccination or a previous positive antibody result (Department of Health 2003). A number of tests are available commercially. Enzyme-linked immunosorbent assay (EIA) is used widely because it is readily automated and can easily be included in automated antenatal screening. Latex agglutination (LA) has the advantage that a result is available in a few minutes. Single radial hemolysis (SRH) plates may be prepared in the laboratory with commercially available reagents. HAI is not recommended for screening since false-positive results may occur as a result of incomplete removal of β lipoprotein inhibitors of HA, and because it is more time consuming and labor intensive. These techniques have been described in detail by Best and O'Shea (1995). Vaccination should be offered to women who are seronegative or have antibody concentrations <10 IU/ml.

Rubella antibodies may be detected in oral fluid by IgG-capture radioimmunoassay (RIA) (GACRIA) (Parry et al. 1987). The use of oral fluid rather than serum has several benefits for seroepidemiological studies, especially those involving children and in developing countries, since it can be obtained by a noninvasive technique (Eckstein et al. 1996; Nokes et al. 1998).

Most rubella antibody tests use whole virus as antigen, but peptides and proteins produced by recombinant techniques have been evaluated (Mitchell et al. 1992b; Hobman et al. 1994c; Starkey et al. 1995) and some methods seem promising. One commercial test employed virus-like particles produced by expression in BHK cells (Grangeot-Keros et al. 1995).

Virus isolation and identification by RT-PCR

RUBV may be detected in clinical samples by isolation in cell culture or by reverse transcription polymerase chain reaction (RT-PCR). Preferred samples are a throat swab or oral fluid. Virus isolation techniques are rarely used for the diagnosis of postnatally acquired rubella because this may be established more reliably and rapidly by serological methods. However, virus isolation may be of value for determining the duration of excretion in congenitally infected infants, as they may transmit infection to susceptible contacts. RUBV isolates are also required to study the molecular epidemiology of the virus.

RUBV can be identified by the production of CPE in RK13, SIRC, and certain sublines of Vero cells, by interference in primary vervet monkey kidney (VMK) (Parkman et al. 1962) and other monkey kidney cultures, or by using immunofluorescence or immunoperoxidase for detection of antigen in such cells as RK13, BHK-21, and Vero. The most sensitive technique for isolation of RUBV is one or two passages in Vero cells, followed by passage in RK13 cells, in which the virus may be identified by immunofluorescence employing hyperimmune sera or monoclonal antibodies or by RT-PCR. These techniques have been described in detail by Best and O'Shea (1995). However, as virus isolation is labor intensive and a minimum of 3 weeks is required to obtain a result, this technique is available only in a few specialized laboratories.

Nested RT-PCRs for the detection of RUBV RNA employing primers from the E1 region have been described by Bosma et al. (1995b); Revello et al. (1997); Vyse and Jin (2002) and Li Jin (personal communication, 2003). Results of these assays compared well with virus isolation. RT-PCR is now used for the pre- and postnatal diagnosis of congenital rubella and is useful for detection of RUBV RNA in oral fluid when serological results are equivocal (Vyse and Jin 2002).

Laboratory diagnosis of postnatally acquired infection

A clinical diagnosis of rubella is unreliable and therefore laboratory confirmation is required, particularly for the diagnosis of rubella-like illness during pregnancy and for contacts of pregnant women. The detection of rubella-specific IgM is the method of choice. Specific IgM antibodies may be detected by indirect and M-antibody capture enzyme immunoassays. Care should be taken to ensure that the test chosen has a high degree of specificity and sensitivity (Hudson and Morgan-Capner 1996; Tipples et al. 2004). Rubella-specific IgM usually appears within 4 days of onset of rash and persists for 4–12 weeks. More than 4 weeks after onset, detection will depend on the sensitivity of the technique employed. The diagnosis should be confirmed by detecting a significant rise in antibody concentration or by testing a further serum for specific IgM. A significant rise in antibody concentration may be detected by a variety of methods, including HAI, EIA, or LA titration. Seroconversion can also be detected by SRH. Although HAI antibodies develop within a day or 2 of onset of rash, antibodies detected by EIA, LA, or SRH may be delayed until 7–8 days (Figure 3).

Rubella-specific IgG and IgM antibodies may be detected in oral fluid using antibody capture radioimmunoassays. Results correlate well with serum antibodies (Perry et al. 1993; Vyse et al. 1999). The optimum time for detecting specific IgM is 1–5 weeks after onset of illness. This method is particularly useful when testing children and has been used to demonstrate that many rubella-like rashes are caused by other viruses or bacteria (Ramsay et al., 1998, 2002).

RE-INFECTION

Reinfection is associated with a rise in antibody concentration, sometimes to very high levels. An IgM response may also be present, but is usually lower and more transient than that following primary infection. An accurate history of rubella contact, rubella vaccination, and previous rubella antibody screening is required in order to interpret results. It may be particularly difficult to distinguish between a primary infection and reinfection, if blood is not obtained shortly after contact or if sera taken prior to contact (e.g. for screening purposes) are not available. However, reinfection may be distinguished from primary infection by examining the antigen-binding avidity of specific IgG, because the avidity of specific IgG from cases of recent primary rubella is low compared with that from people with remote infection or reinfection (Thomas and Morgan-Capner 1991).

Testing for reinfection is not indicated in populations where most women are rubella seropositive and there is a low incidence of rubella (Morgan-Capner and Crowcroft 2002).

ASSESSMENT OF THE RISK TO WOMEN EXPOSED TO OR WHO DEVELOP RUBELLA-LIKE ILLNESS IN PREGNANCY

Precise details of the date of onset of illness, presence, and distribution of such clinical features as rash, lymphadenopathy, and arthralgia should be obtained from pregnant women who present with rubella-like clinical features. If there is a history of contact with a rubella-like illness the date, duration, and type of contact (e.g. casual or more prolonged household contact) should be obtained, in order to interpret the results of virological investigations. In addition, enquiry should be made about results of previous screening tests for rubella antibodies and history of rubella vaccination.

Patients who have been exposed to rubella should be carefully followed up serologically because retrospective studies have shown that some women who delivered infants with CRS gave no history of a rubella-like illness during pregnancy. Blood should be collected from pregnant women with rubella-like features as soon as possible after the onset of symptoms. Provided that a blood sample is obtained within the first 3–4 days, it is usually possible to detect a significant rise in antibody concentration and rubella-specific IgM in a second blood sample taken a few days later.

Although most patients develop rubella antibodies within a few days of onset of symptoms, antibody responses may very occasionally be delayed for as long as 10 days. This underlines the importance of collecting further blood samples from patients who remain sero-

negative. Many patients present in the postacute phase of their illness, at which time antibody concentrations are likely to have reached maximum concentrations. It must be emphasized that there is no particular concentration of antibody ascertained by any test that can be regarded as indicative of recent or current infection. Because a virological diagnosis is usually required quickly for obstetric reasons, such patients should be tested for rubella-specific IgM with minimum delay.

Women who present within the incubation period and who have antibodies may be reassured, although it is often wise to obtain a second blood sample 7–10 days later to ensure that antibody concentrations are stable. The interpretation of results may depend on the accuracy of the history given by the patient. Sometimes an earlier blood sample is available (e.g. taken at the first antenatal visit) which can be tested in parallel with a sample taken after contact. If there is no change in antibody concentration the patient can be reassured with confidence that she was already immune.

Patients presenting after an interval greater than the incubation period and who are seropositive are more difficult to assess because antibody levels may already have reached their maximum. The sera of such patients should therefore be tested for rubella-specific IgM. Detection of rubella-specific IgM in women who give no history of a rubella-like rash or contact with such a rash, should be interpreted with caution. In some patients, the specific IgM may persist for months or years after infection and vaccination (Banatvala et al. 1985; Thomas et al. 1992) and false-positive results may occur in patients with autoimmune diseases and other infections. In such cases it is necessary to use further tests, such as specific IgG avidity and western blot to determine whether recent infection has occurred (Pustowoit and Liebert 1998; Thomas et al. 1999; Best et al. 2002).

Because rubella is unlikely to be acquired as a result of casual or brief contact (e.g. while shopping or in public transport), seronegative patients who experience this type of exposure should be reassured that the risks of acquiring rubella are small. Nevertheless, it is essential to follow up such people serologically. Those exposed more closely over a longer period are at greater risk, but even they may be reassured, particularly during nonepidemic times, by being told that the clinical diagnosis of rubella is often incorrect. Anxiety may be allayed by testing the index case, in order to confirm or refute the diagnosis. Patients who have been followed up, but who remain seronegative, should be offered rubella vaccination in the immediate postpartum period.

Laboratory diagnosis of congenital rubella

POSTNATAL TESTS

The National Congenital Surveillance Programme in the UK classifies suspected cases of congenital rubella according to the criteria listed in Table 4. The congenital rubella syndome (CRS) case classification for the United States has been revised (Reef et al. 2000).

A diagnosis of CRS can be established by:

- Detection of rubella-specific IgM in cord serum or serum samples obtained in early infancy. An IgM antibody capture assay is generally preferred for this purpose (Chantler et al. 1982; Hudson and Morgan-Capner 1996). Specific IgM has been detected by M-antibody capture radioimmunoassay (MACRIA) in all symptomatic infants up to the age of 3 months, in 90 percent of infants aged 3–6 months, in fewer than 50 percent of infants aged 6–12 months and only occasionally in children over 1 year old (Chantler et al. 1982). However, commercially available enzyme immunoassays (indirect and M-antibody capture formats) have not been evaluated for the diagnosis of CRS and may be less sensitive. The absence of specific IgM by IgM antibody capture assays in the neonatal period virtually excludes congenital rubella.
- Detection of persistent rubella IgG antibody in serum or oral fluid at a time when maternal antibodies are no longer detectable (approximately 8 months). This may be a useful technique when patients present too late for the detection of specific IgM.
- Isolation of RUBV or detection of RUBV RNA by RT-PCR (Bosma et al. 1995b) in specimens (such as pharyngeal swabs) taken from infants during early infancy. Facilities for RUBV isolation are not widely available. RT-PCR can also be applied to lens

Table 4 *Congenital rubella: case classification criteria (Miller et al. 1994)*

Congenital rubella infection	No rubella defects, but congenital infection confirmed by isolation of virus, or detection of specific IgM or persistent IgG in infant
Congenital rubella syndrome	
Confirmed	Typical rubella defect(s) plus virus-specific IgM or persistent IgG in infant; or two or more rubella defects plus confirmed maternal infection in pregnancy
Compatible	Two or more rubella defects with inconclusive laboratory data, or single rubella defect plus confirmed maternal infection in pregnancy
Possible	Compatible clinical findings with inconclusive laboratory data, e.g. single defect plus probable maternal infection in pregnancy
Unclassified	Insufficient information to confirm or exclude

aspirates from cataracts in order to establish a diagnosis of CRS (Bosma et al. 1995a).

RUBV can be isolated from the stools, urine, tears, CSF, and nasopharyngeal secretions of infants with CRS. Cooper and Krugman (1967) isolated RUBV from the nasopharynx of almost all severely infected infants at birth, but by the age of 3 months the proportion declined to about 60 percent and, by 9–12 months, to approximately 10 percent. Virus excretion has not yet been tested by RT-PCR, which may detect virus excretion for longer. However, RUBV may persist at other sites for even longer (see Persistence of virus). RUBV can be isolated from most of the organs obtained at autopsy from severely infected infants who die in early infancy. Babies excreting virus may transmit infection to susceptible contacts. Therefore, until virus is no longer being excreted, women of childbearing age, some of whom may be in the early stages of pregnancy, should be dissuaded from visiting such babies until serological tests confirm that they are immune.

Rubella-specific lymphoproliferative assays (O'Shea et al. 1992) and tests for low avidity specific IgG (Thomas et al. 1993) may be of value for retrospective diagnosis of CRS in children between the ages of 1 and 3 years.

Children with CRS may lose antibodies to the E1 glycoprotein (de Mazancourt et al. 1986; Mitchell et al. 1992b; Mauracher et al. 1993; Meitsch et al. 1997) and consequently HAI antibodies may not be detected (Cooper et al. 1971; Ueda et al. 1975). Forty-one percent of 40 persons with CRS tested at 60 years of age had no rubella antibodies detectable by EIA (Forrest et al. 2002). In order to determine the immune status of such persons in later life, it may be necessary to use tests such as western blot, which detect antibodies to individual RUBV polypeptides.

PRENATAL TESTS

Prenatal diagnosis of CRS may provide a more accurate estimate of risk when the mother is reluctant to have a therapeutic abortion following rubella in the first trimester, when maternal reinfection is confirmed or suspected and when serological results are equivocal, for example when rubella-specific IgM is detected in a woman with no history of a rubella-like illness. A prenatal diagnosis may be made by testing amniotic fluid for RUBV RNA by RT-PCR, and fetal blood for RUBV RNA and rubella-specific IgM. The detection of RUBV RNA in amniotic fluid and fetal blood has sensitivities of 87.5 percent and positive predictive values of 100 percent (Enders 1998) when tested by the RT-PCR method described by Bosma et al. (1995a). Amniotic fluid can be taken after 15 weeks' gestation and $\geqslant$8 weeks after onset of maternal rubella infection. However, as false-negative results may occur, it is desirable to test further amniotic fluid, as well as fetal blood at 22–23 weeks' gestation.

As the fetus may not produce sufficient IgM before 22 weeks' gestation, fetal blood should be obtained by cordocentesis after that time. It is advisable to test fetal blood for rubella-specific IgM with more than one assay as levels may be low. Although this approach means that the patient may have to wait some time for the final test, it is possible that false-negative results may be obtained before this time (Tang et al. 2003).

Prenatal diagnosis has also been discussed by Revello et al. (1997); Enders (1998) and Katow (1998).

PREVENTION

Rubella vaccination

Rubella and congenitally acquired rubella are preventable, and the elimination of CRS in some countries with national vaccination programs results from the administration of attenuated rubella vaccines.

RUBELLA VACCINES

The first attenuated strain of rubella was developed by the National Institutes of Health in the USA. This strain was isolated from a military recruit with acute rubella and attenuated by 77 passages in VMK cell cultures. Following preliminary trials in primates in which this vaccine was shown to be protective, controlled trials were conducted among institutionalized children, the vaccine strain having been passaged a further five times in duck embryo fibroblasts. Within a short time, further vaccine strains were prepared, being attenuated in primary rabbit kidney (Cendehill) and in human diploid cell cultures (RA27/3) (Proceedings 1969). The vaccine containing the RA27/3 strain, originally isolated from the fetal kidney of a rubella-infected conceptus, is the most widely used worldwide, although a number of vaccine strains developed in Japan and China are now used in these countries (Perkins 1985). The accumulated data from trials employing rubella vaccines show that they are immunogenic, protective, and well tolerated. Vaccinees excrete virus via the nasopharynx, but infection is not transmitted to susceptible contacts. The development and properties of different rubella vaccines have been reviewed by Banatvala and Best (1989) and Best (1991). Rubella vaccine is now usually given with measles and mumps vaccines as the triple vaccine measles, mumps, and rubella (MMR) (Salisbury and Begg 1996).

VACCINATION PROGRAMS

Rubella vaccines were licensed in the USA in 1969 and in the UK in 1970. In the USA a policy of universal childhood immunization was adopted, this being aimed at interrupting transmission of virus by vaccinating preschool children, thereby reducing the risk of pregnant women being exposed to rubella. Because high uptake

rates were achieved, this policy markedly reduced the incidence of postnatally acquired and CRS (Figure 8). However, in 1989/1990 rubella occurred among unvaccinated women in some parts of the USA, resulting in cases of CRS (Lindegren et al. 1991). Rubella vaccine is now given as the MMR vaccine at 12–15 months and 4–6 years of age. Those who miss the second dose should be offered it at 11–12 years (Centers for Disease Control and Prevention 2001a).

A selective vaccination program was initially adopted in the UK, this being directed first at prepubertal schoolgirls, women at particular risk of acquiring rubella (e.g. nurses and school teachers), and seronegative adult women identified by rubella antibody screening. Because complete vaccination of the target population was an unrealistic goal, the vaccination program was augmented in 1988 by offering rubella vaccine to preschool children of both sexes (Banatvala 1987) and rubella is now given with measles and mumps (MMR) vaccine in two doses at 12–15 months and 4–5 years of age. This resulted in a fall in the number of reported cases of rubella, congenital rubella, and of terminations of pregnancy because of rubella (Figure 9). Following the administration of MR vaccine to all 5- to 16-year-old school children in late 1994 to prevent a predicted measles epidemic, it was agreed to discontinue vaccination of schoolgirls, but to continue to ensure that susceptible pregnant women were vaccinated postpartum.

Although a significant proportion of young adult males remains susceptible, high rates of vaccine uptake among preschool children together with the markedly reduced susceptibility among pregnant women have resulted in CRS being rare in the UK. Thus, in contrast to 1984 in which 52 cases were reported, in 1996 only 12 cases were notified to the National Congenital Rubella Surveillance Programme. Two of the 12 mothers had acquired the infection while abroad (Tookey and Peckham 1999). Seven babies with CRS were born in the 2-year period 2000–2001. Five of the seven mothers had acquired rubella abroad (Rahi et al. 2001; P Tookey, personal communication, 2002). It will be unfortunate if CRS returns in the future, as a consequence of the fall in the uptake of MMR due to the unjustified public concern about its safety (see Vaccine reactions).

Most countries in Europe administer MMR vaccine between the first and second birthdays; and many now offer a second dose. Although uptake of rubella vaccine is poor in some countries in Europe, Sweden and Finland have eliminated rubella by use of two-dose MMR programs (Peltola et al. 1994; Böttiger and Forsgren 1997). A review published by the World Health Organization (WHO) in 1997 highlighted the problem of congenital rubella in developing countries (Cutts et al. 1997). A meeting was held in 2000 to consider methods for surveillance of rubella and congenital rubella and the use of rubella vaccines in developing countries (World Health Organization 2000a). WHO also published guidelines for the surveillance of rubella and CRS (World Health Organization 1999) and a position paper on rubella vaccines (World Health Organization 2000b). Many developing countries have introduced rubella vaccination, since it has been shown to be cost-effective (Hinman et al. 2002), and by 2002, 123 (57 percent) of all countries/territories in the world had included rubella vaccination in their national immunization programs (Figure 10). Forty-one of the 44 countries in the Pan American Health Organization (PAHO) had incorporated MR or MMR into their childhood immunization programmes by 2002 (World Health Organization 2003a). Although it is desirable to give rubella vaccine to children with measles (as MR or

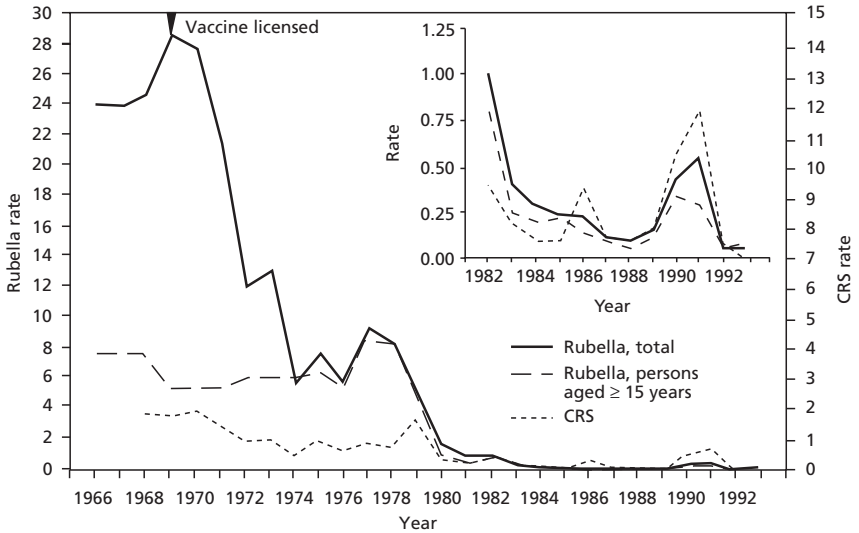

Figure 8 *Incidence rates of rubella and congenital rubella syndrome (CRS) in the USA, 1966–1993; cases of rubella reported to the National Notifiable Disease Surveillance System per 100 000 population; cases of CRS reported to the National CRS Registry per 100 000 live births (from Centers for Disease Control and Prevention, 1994).*

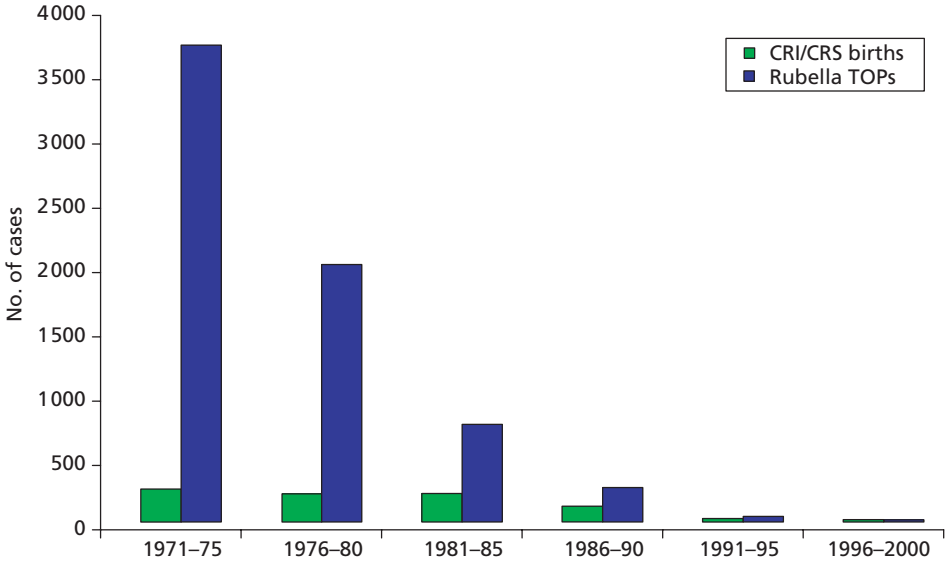

Figure 9 *Congenital rubella births and rubella-associated terminations of pregnancy (TOP) in England and Wales (data from National Congenital Rubella Surveillance Programme 2003, www.ich.ucl.ac.uk).*

MMR), it is also necessary to vaccinate susceptible women of child-bearing age, as childhood vaccination alone will increase the risk to susceptible adults (Robertson et al. 1997, World Health Organization 2000a, c). WHO recommend that surveillance for rubella, CRS, and rubella vaccine coverage should be monitored (World Health Organization, 1999, 2003a), since it is necessary to have a vaccine uptake of at least 80 percent among children to prevent circulation of rubella.

IMMUNE RESPONSES

About 95 percent of vaccinees develop an immune response. Serum antibodies develop between 10 and 28 days after vaccination and reach maximum levels about 6 months later. Occasionally, antibody responses may be delayed for up to 8 weeks. Failure to respond may result from concurrent infections, pre-existing low levels of antibody undetectable by less sensitive assays, or the presence of passively acquired antibody acquired maternally, via blood transfusion or administration of immunoglobulin. Failure to comply with the manufacturer's recommendations during storage or after reconstitution may result in virus inactivation and loss of potency of the vaccine.

Following vaccination, antibodies to the major structural proteins E1, E2, and C may be detected, and rubella-specific IgG, IgA and IgM responses are present. Specific IgM responses are detected in about 70 percent of vaccinees. Occasionally, rubella-specific IgM may persist at low levels for up to 4 years after vaccination (O'Shea et al. 1985). Virus-specific IgA concentrations decline more rapidly than specific IgG. There is a transient oligomeric (10S) IgA response, which is succeeded by a 7S response that may persist at low levels for 10–12 years after vaccination. The RA27/3 vaccine induces a secretory IgA response which persists for up to 5 years (O'Shea et al. 1985).

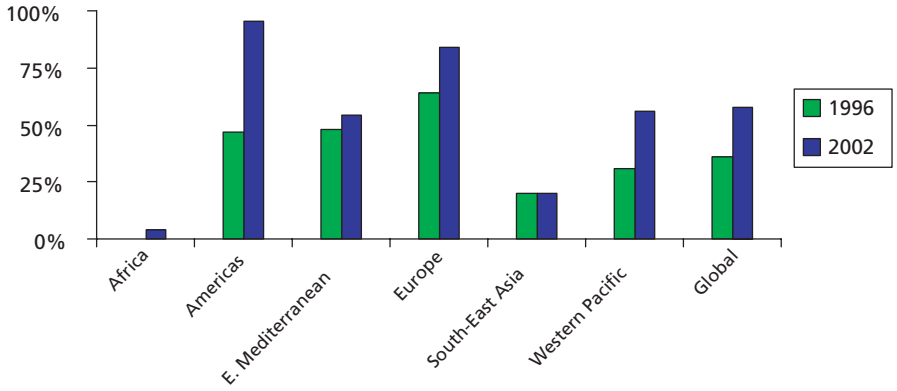

Figure 10 *Percentage of countries/territories, by WHO region and globally, with rubella vaccine in the national immunization system, 1996 and 2002 (Source: WHO Department of Vaccines and Biologicals, December 2002).*

Although serum antibody levels detected by HAI and SRH persist for at least 21 years in most vaccinees, in approximately 10 percent the levels decline to <15 IU/ml within 5–8 years. A small number may become completely seronegative (reviewed by Banatvala and Best 1989). However, when volunteers with low levels of antibody were challenged intranasally with high titer RA27/3 vaccine, most appeared to be protected although a transient low level of virus-specific IgM was present in four of 19 and a transient viremia was detected in one of 19 volunteers (O'Shea et al. 1983). A transient lymphoproliferative response has been detected following rubella vaccination (Buimovici-Klein and Cooper 1985).

VACCINE EFFICACY AND RE-INFECTION

The protective efficacy of rubella vaccines is about 95 percent (reviewed by Plotkin 1999). Experimental studies demonstrate that reinfection (see relevant sections on reinfection) is more likely to occur in those whose immunity is vaccine-induced than in those with naturally acquired immunity (reviewed by Best 1991). Re-infection is less likely to occur after vaccination with the RA27/3 vaccine strain, than with the Cendehill and HPV-77 vaccine strains, which is why the RA27/3 strain is now used in most rubella vaccines (see above).

Vaccination is recommended for adult women who have rubella antibodies <10 IU/ml, but some of these women will fail to mount a booster response, as seen in vaccine trials (Best 1991). Therefore, no more than two doses of vaccine are indicated for such women. However, it may be of value to test the sera from these women for rubella IgG using more than one technique (Best, 1991; Best and O'Shea 1995). Following vaccination, women may be considered immune if antibodies $\leqslant$10 IU/ml are detected by two different techniques. Survivors of CRS may fail to respond to rubella vaccine.

VIRUS EXCRETION

Rubella vaccine may be detected in nasopharyngeal secretions from most vaccinees 6–29 days after vaccination, as well as in the breast milk of lactating women vaccinated postpartum. Although lack of transmission to susceptible contacts may result from low concentrations in nasopharyngeal secretions, it is possible that attenuation may alter the biological properties of the virus, resulting in poor replication in the respiratory tract. Very occasionally, virus has been transmitted to breastfed infants, but they do not develop any clinical features of infection.

VACCINE REACTIONS

Rubella vaccine is well tolerated although lymphadenopathy, rash, and arthropathy may occur between 10 and 30 days after vaccination. In general, such reactions are less severe than following naturally acquired infection. Indeed, vaccinees may fail to notice their lymphadenopathy and, if rash occurs, it is usually macular, faint, and evanescent. Joint symptoms are related to age and gender, being rare in children of both sexes (2.5–10 percent), but occurring in up to 58 percent of post-pubertal females. Most commonly, the small joints of the hands are affected, but the knees, wrists, and ankles may also be involved. Symptoms rarely persist for longer than a week. However, a small proportion of vaccinees may develop arthritis with pain and limitation of joint movement, which may occasionally recur intermittently. Joint symptoms rarely result in absence from work.

Although it has been suggested that MMR is associated with inflammatory bowel disease and autism, several longitudinal studies have failed to confirm an increased incidence among MMR vaccinees. A number of prestigious groups and committees have examined the evidence and stated that there is no good scientific evidence to support this theory (Chief Medical Officer 2001; Institute of Medicine 2001; Miller, 2001; World Health Organization 2003b). The association is circumstantial as these diseases are first detected at about 15 months of age, the age at which MMR is given to children. However, due to the publicity generated in the media, public concern has resulted in a serious fall in the uptake of MMR in some parts of the UK, which has lead to outbreaks of measles (Jansen et al. 2003).

CONTRAINDICATIONS

As with other live vaccines, immunocompromised patients should not be vaccinated. Such people include those with malignant disease and those being treated with cytotoxic drugs, radiotherapy, or corticosteroids. Thrombocytopenic patients should not be vaccinated. HIV-positive people, with or without symptoms, are exceptions to these recommendations because they may be vaccinated with such live vaccines as MMR and polio. HIV-positive people do not react adversely to such live vaccines but, if unprotected, may experience serious complications, particularly if infected with measles.

Live vaccines may be administered concurrently, preferably at different sites (except in the case of MMR). If this is not possible, the two live vaccines should be separated by an interval of 3 weeks. A similar period should be allowed between the administration of rubella vaccine, or MMR, and BCG. If a vaccinee has a febrile illness, it is prudent to postpone rubella vaccination until the individual has recovered. The rubella vaccines may contain such antibiotics as neomycin, kanamycin, or polymyxin, and anyone with established hypersensitivity to these antibiotics should not be vaccinated. As passively acquired antibodies may interfere with vaccine-induced responses, rubella vaccination

should be delayed for 3 months after blood transfusion or administration of immunoglobulin. However, the administration of anti-D immunoglobulin does not suppress vaccine-induced immune responses, although it may be prudent to confirm seroconversion 2–3 months after vaccination.

VACCINATION DURING PREGNANCY

Although wild-type strains of rubella are teratogenic, the accumulated data from studies reported in different countries, in which 661 rubella-susceptible women were vaccinated during pregnancy, have shown that this has not resulted in rubella-induced permanent fetal defects. Forty-four percent of these women were vaccinated during the high-risk period between 2 weeks before and 6 weeks after conception. Vaccine strains may, however, infect the conceptus because rubella vaccine strains have been isolated from the placenta, kidney, and bone marrow for up to 94 days after vaccination. Furthermore, rubella-specific IgM responses or antibodies persisting for up to a year have been detected in about 3 percent of infants delivered of mothers inadvertently vaccinated during early pregnancy. Among the 661 infants born to women who were inadvertently vaccinated during pregnancy, only one rubella-IgM positive infant had a heart murmur which resolved by 2 months of age (P. Tookey, personal communication 2000; reviewed by Best and Banatvala 2004).

The theoretical maximum risk of rubella vaccine-induced major malformations based on a 95 percent confidence limit of the binomial distribution has been calculated to be 1.2 percent, this being less than the 3 percent of major malformations occurring in 'normal' pregnancies (Bart et al. 1985; Enders 1985). These encouraging reports have resulted in a substantial reduction in terminations of pregnancy being carried out as a result of inadvertent vaccination in pregnancy in Britain. Nevertheless, it is still recommended that pregnant women should not be vaccinated against rubella and that pregnancy should be avoided for 1 month after vaccination (Salisbury and Begg, 1996; Centers for Disease Control and Prevention 2001b).

Passive immunization

Normal human immunoglobulin does not confer any clearcut protection (Report 1970). This preparation may reduce the incidence of clinically overt maternal infection, but subclinical infection may nevertheless occur, with a prolonged incubation period. Because inapparent infection is accompanied by viremia, fetal damage is not prevented when it is used in pregnancy. However, it is possible that the administration of normal human globulin reduces the level of fetal infection or damage, or both (Peckham 1974; Hanshaw et al. 1985).

High-titer rubella immunoglobulin has been used experimentally to determine whether infection induced by rubella vaccine can be prevented (Urquhart et al. 1978). This preparation has not been properly evaluated in the field, but may be recommended for the few susceptible pregnant women who come into contact with clinical rubella and for whom therapeutic abortion is unacceptable.

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Flaviviruses

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PROPERTIES OF THE VIRUSES

This family of enveloped, positive-strand RNA viruses contains over 70 members, of which about 50 are arthropod-borne; many are pathogenic for humans and other vertebrates. The family name is derived from that of the type species, *Yellow fever virus* (YFV) (Latin: *flavus* = yellow).

Classification

The family *Flaviviridae* contains three genera: *Flavivirus*, *Pestivirus*, and *Hepacivirus*, and the unassigned GBV viruses (GBV-A, GBV-B, and GBV-C). All members of the *Flavivirus* genus are antigenically related. Serological crossreactions are most evident in hemagglutination-inhibition (HI) tests, and plaque reduction neutralization tests (PRNT) show the greatest specificity. Seven subgroups or complexes of flaviviruses have been defined on the basis of cross-neutralization tests (De Madrid and Porterfield 1974), and this subdivision has recently been substantially confirmed and extended in a more comprehensive study (Calisher et al. 1989). Flaviviruses can also be divided into three biological subsets based upon their mode of transmission, by mosquitoes, ticks, or by having no known arthropod vector. Table 1 shows the relationship between the subgroups determined by the PRNT and the mode of transmission of currently named flaviviruses.

Three pathogens of farm animals: hog cholera virus (HCV) or *Classical swine fever virus* (CSFV), bovine viral diarrhea virus (BVDV), and *Border disease virus* (BDV), constitute the *Pestivirus* genus. The mosquito cell-fusing agent is an additional member of the *Flaviviridae* but is outside the genus *Flavivirus*. Hepaciviruses as well as the unassigned GBV viruses, both agents of hepatitis, are the topic of another chapter and are not further discussed here.

Morphology and virion structure

Flaviviruses possess an isometric core, 30–35 nm in diameter, that contains a nucleocapsid or core (C) protein complexed with single-stranded positive-sense RNA. This core is surrounded by a lipid bilayer containing an envelope (E) protein and a membrane (M) protein, giving a total virion diameter of about 45 nm. The flavivirus E protein is a class II fusion protein. The M protein is first synthesized as a precursor protein, prM. The virion structure of dengue virus (DENV) virus has recently been solved using cryo-electron microscopy, and differs from the structure of recombinant subviral particles (RSP) derived from mammalian cells transfected with a plasmid containing only the genes for the prM and E proteins of tick-borne encephalitis virus (TBEV) (Ferlenghi et al. 2001; Kuhn et al. 2002). The virion envelope contains an icosahedral scaffold of 90 E protein dimers (Kuhn et al. 2002).

Pestiviruses are 40–60 nm particles that contain an electron-dense core of about 30 nm. The virion is enveloped with the E^{rns}, E1, and E2 proteins associated with envelope. Purified pestiviruses sometimes show a racquet type of morphology (Renard et al. 1985).

Genome structure and function

The genome of flaviviruses is a molecule of single-stranded, positive-sense RNA, approximately 11 kb in

length containing short untranslated regions at 3′ and 5′ ends, a 5′ cap and a non-polyadenylated 3′ terminus (Figure 1). There is no subgenomic mRNA in flavivirus-infected cells. Complete genome sequences are known for YFV (Rice et al. 1985; Chambers et al. 1989), West Nile virus (WNV) (Castle et al. 1985; Wengler et al. 1985, 1987; Nowak et al. 1989; Lanciotti et al. 1999), all four serotypes of DENV (Hahn et al. 1988; Mackow et al. 1987; Osatomi and Sumiyoshi 1990; Fu et al. 1992), Japanese encephalitis virus (JEV) (Sumiyoshi et al. 1987), Kunjin virus (KUNV) (Coia et al. 1988), Powassan virus (POWV) (Mandl et al. 1993) and TBEV (Pletnev et al. 1990). A number of partial genome sequences, primarily of the E proteins,

Table 1 *Relationship between* Flavivirus *subgroup and mode of transmission*

Tick-borne viruses		No-vector viruses		Mosquito-borne viruses	
Virus	**Subgroup**	**Virus**	**Subgroup**	**Virus**	**Subgroup**
Karshi virus (KSIV)	1	*Carey Island virus* (CIV)	1	Alfuy virus (ALFV)	(3)
KFDV	1	*Phnom Penh bat virus* (PPBV)	1	*Japanese encephalitis virus* (JEV)	3
Langat virus (LGTV)	1	*Apoi virus* (APOIV)	2A	*Kedougou virus* (KEDV)	3
Louping ill virus (LIV)	1	*Bukalasa bat virus* (BBV)	2A	*Kokobera virus* (KOKV)	3(5?)
Negishi virus (NEG)V	1	*Dakar bat virus* (DBV)	2A	*Koutango virus* (KOUV)	3
Omsk hemorrhagic fever virus (OHFV)	1	*Entebbe bat virus* (ENTV)	2A	Kunjin virus (KUNV)	3
Powassan virus (POWV)	1	*Rio Bravo virus* (RBV)	2A	*Murray Valley encephalitis virus* (MVEV)	3
Royal Farm virus (RFV)	1	*Saboya virus* (SABV)	2A	*Saint Louis encephalitis virus* (SLEV)	3
Tick-borne encephalitis virus (TBEV)	1	*Cowbone Ridge virus* (CRV)	2B	Stratford virus (STRV)	3
Meaban virus (MEAV)	1A	*Jutiapa virus* (JUTV)	2B	*Usutu virus* (USUV)	3
Saumarez Reef virus (SREV)	1A	*Modoc virus* (MODV)	2B	*West Nile virus* (WNV)	3
Tyuleniy virus (TYUV)	1A	*Sal Vieja virus* (SVV)	2B	*Yaounde virus* (YAOV)	(3)[a]
Gadget's Gully virus (GGYV)	U	*San Perlita virus* (SPV)	2B	Spondweni virus (Spondweni)	4
Kadam virus (KADV)	U	*Aroa virus* (AROAV)	U	*Zika virus* (ZIKV)	4
		Batu Cave virus (BCV)	(U)[a]	*Bagaza virus* (BAGV)	5
		Cacipacore virus (CPCV)	U	*Israel turkey meningo-encephalitis virus* (ITV)	5
		Montana myotis leukoencephalitis virus (MMLV)	U	*Ntaya virus* (NTAV)	5
		Sokuluk virus (SOKV)	U	*Tembusu virus* (TMUV)	5
		Tamana bat virus (TABV)	U	*Yokose virus* (YOKV)	5
				Banzi virus (BANV)	6
				Bouboui virus (BOUV)	6
				Edge Hill virus (EHV)	6
				Potiskum virus (POTV)	(6)[a]
				Uganda S virus (UGSV)	
				Dengue virus 1 (DENV-1)	7
				Dengue virus 2 (DENV-2)	7
				Dengue virus 3 (DENV-3)	7
				Dengue virus 4 (DENV-4)	7
				Bussuquara virus (BSQV)	U
				Ilhéus virus (ILHV)	U
				Jugra virus (JUGV)	U
				Naranjal virus (NJLV)	U
				Rocio virus (ROCV)	U
				Sepik virus (SEPV)	U
				Wesselsbron virus (WESSV)	U
				Yellow fever virus (YFV)	U
Total:	14		19		36=69

Based on data from De Madrid and Porterfield 1974 and from Calisher et al. 1989.
U, unrelated to any other virus.
a) Batu Cave virus, Potiskum virus, and *Yaounde virus* were not examined in either series, but are included for completeness.

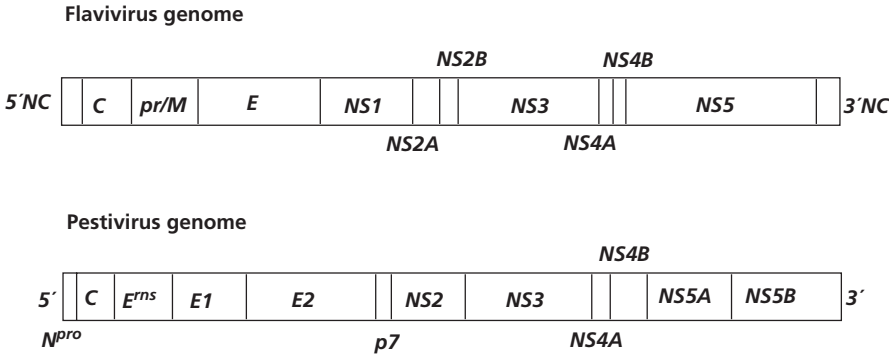

Figure 1 *Flavivirus and Pestivirus genome organization.*

are also available. A single long open reading frame (10 223 nt in the case of YFV) spans almost the entire length of the RNA. The gene order is 5′-*C–prM–(M)–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5*-3′ (Rice et al. 1985) (Figure 1).

The genome of pestiviruses has been shown to be more similar to hepaciviruses than flaviviruses and is reported to be 12.5 kb in length. The 3′ end of the genomic RNA is not polyadenylated (Renard et al. 1987), and unlike flaviviruses, the pestivirus RNA lacks a 5′-cap structure (Brock et al. 1992). The pestivirus genome encodes a single open reading frame with a theoretical coding capacity of about 4 000 amino acids. Complete genome sequences have been determined for CSFV (Meyers et al. 1989; Moormann et al. 1990) and BVDV (Collett et al. 1988; Deng and Brock 1992), and BDV (Becher et al. 1994, 1998). Similar to flaviviruses, the genes encoding the structural proteins are located at the 5′ end of the genome. The gene order is 5′-*Npro–C–Erns–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B*-3′ (Collett et al. 1989).

Polypeptides: structural and nonstructural

Cell-associated flaviviruses have a C protein with a molecular mass of 12–13 kDa surrounded by a lipid bilayer containing the two membrane glycoproteins E and prM (mol. mass 50 and 22 kDa, respectively). Extracellular virions contain the shorter M protein (mol. mass 8 kDa) instead of prM. Seven nonstructural proteins have been well described (see section below on Replication). Because of the continued medical significance of the flaviviruses, the structure and function of the E protein (approximately 500 amino acids) have been intensely investigated.

Three antigenic domains (A, B, and C) have been identified on the E protein (Mandl et al. 1989; Heinz and Roehrig 1990; Roehrig et al. 1998). The A domain (amino acids 50–130 and 185–300) is a linearly discontinuous domain that is divided by the C domain (amino acids 130–185). The A-domain structure is stabilized by five disulfide bridges and contains mostly conformationally dependent virus-neutralizing epitopes. Much of the antigenicity of the B domain (amino acids 300–400) requires a disulfide bond between Cys 11 and Cys 12. The A, B, and C domains constitute the ‘head’ of the E glycoprotein. A 100 amino acid stalk (approximately amino acids 400–500) that includes a 50 amino acid hydrophobic tail (amino acids 450–500) anchors the molecule in the virion envelope. Unlike other enveloped viruses, there is essentially no cytoplasmic tail on the E protein. The three-dimensional molecular structure of the amino terminal 380 amino acid tryptic fragment of the TBEV E protein homodimer has recently been solved (Rey et al. 1995). The protein folds into three distinct domains (I, II, and III), which correlate well to the previously defined domains C, A, and B. Because the locations of the E protein Cys residues are conserved among all flaviviruses, it is generally assumed that this overall structure is the same for all flaviviruses (Figure 2).

Three major nonstructural proteins (NS1, NS3, and NS5) can be found in flavivirus-infected cells. The NS1 protein is a membrane-bound glycoprotein whose function has not been fully determined. It is possible that this protein is involved in virion morphogenesis. The NS2A and NS3 proteins appear to function in concert as a virus-specific protease (Yusof et al. 2000; Leung et al. 2001). The NS3 protein also exhibits helicase activity (Utama et al. 2000; Matusan et al. 2001). The crystal structure of a portion of the NS3 proteins has recently been solved (Valle and Falgout 1998; Krishna Murthy et al. 1999; Murthy et al. 1999, 2000). The NS5 protein is the virus RNA-dependent RNA-polymerase.

Pestivirus proteins also appear to be synthesized as a polyprotein. Four structural proteins: C (14 kDa), and three envelope proteins – E^{rns} (44–48 kDa), E1 (33 kDa), and E2 (55 kDa) – have been identified. The envelope glycoproteins, E1 and E2, form dimers through disulfide bridging in both infected cells and virions. The nonstructural protein N^{pro} (23 kDa) that has autoproteolytic activity is located 5′ of the C protein. This protein has no analogue in the flaviviruses. Pestiviruses also do not

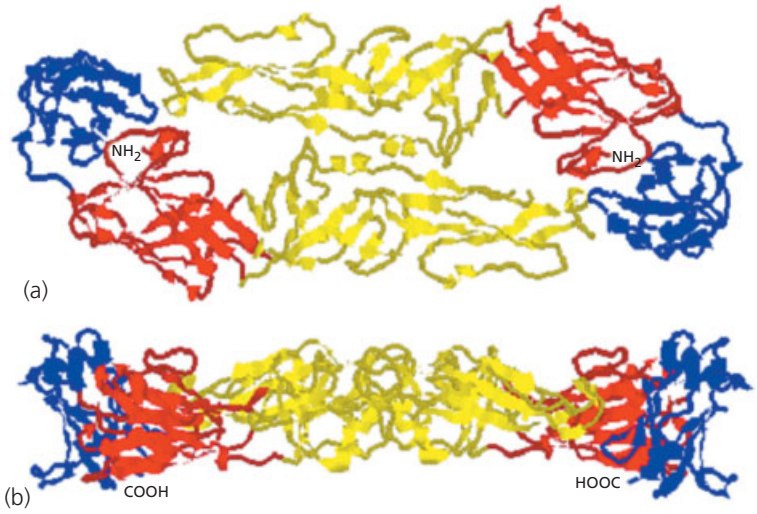

Figure 2 *E-glycoprotein structure. Homodimer of the TBE E protein (a.a. 1–395). Color domains: Domain I (C-domain), red; Domain II (A-domain), yellow; Domain III (B-domain), blue.* **(a)** *top view;* **(b)** *side view.*

appear to have a protein analogue of the flavivirus NS1 protein. Another nonstructural protein, NS2/NS3, is located 3′ of the envelope genes and apparently functions as a viral protease involved in polyprotein processing (Wiskerchen and Collett 1991). The NS2/NS3 protein is cleaved to NS2 and NS3 in some pestiviruses. Both the NS2/NS3 and the cleaved NS3 must have serine protease activity, even though the catalytic motif is located in the N-terminal region of NS3. At least three other nonstructural proteins have been identified: NS2, NS5A, and NS5B. The NS5B contains sequence motifs similar to flaviviral RNA polymerase.

Antigens

The E protein is the viral hemagglutinin, which also elicits neutralizing, enhancing, and protective antibodies. The C protein contributes to the group reactivity detected by the complement fixation (CF) test. The NS1 protein is responsible for inducing the soluble complement-fixing antigen detected in virus-infected cells. Monoclonal antibodies (mAb) specific for the NS1 protein can protect animals from virus challenge (Schlesinger et al. 1987; Henchal et al. 1988; Zhang et al. 1988; Falgout et al. 1990; McCown et al. 1990).

The pestivirus E^{rns} and E2 are primarily responsible for eliciting pestivirus neutralizing antibodies (Weiland et al. 1990, 1992). Because it is difficult to elicit virus-neutralizing antibody specific for the E1 protein, it is thought that most of this protein is buried in the virus envelope, similar to the flavivirus M protein.

Replication

Although it has not been conclusively proven, evidence is accumulating that flaviviruses infect cells by attaching to cellular receptors through the E-protein domain III (Bhardwaj et al. 2001; Crill and Roehrig 2001; Hurrelbrink and McMinn 2001). Flavivirus cellular receptor moieties have been implicated but not been identified conclusively (Chen et al. 1997; Lee and Lobigs 2000, 2002; Chu and Ng 2002; Germi et al. 2002). After endocytosis, the nucleocapsid is released into the cytoplasm by membrane fusion catalyzed by a low-pH dependent conformational change in the E protein. All flavivirus E proteins have a highly conserved sequence (amino acids 98–110) that has chemical similarities to regions shown to be responsible for cell-membrane fusion in orthomyxoviruses and paramyxoviruses.

The viral mRNA is translated as a polyprotein, which undergoes extensive co-translational and post-translational proteolytic processing and cleavage by cellular signalase(s). Cleavage generates the primary amino acid sequence of the mature structural proteins, prM and E, and the amino terminus of the following nonstructural protein, NS1. The amino-terminal portion of the polyprotein is released as a molecule, the anchored C protein, which is associated with membranes of the rough endoplasmic reticulum. The anchored C protein is converted to mature C protein by removal of the carboxyl-terminal hydrophobic segment. Cell-associated virions are constructed from the proteins C, prM, and E. The prM protein is cleaved in the Golgi vesicles, by a furin-like enzyme resulting in loss of the amino-terminal fragment of this protein and release of mature virions from infected cells. It is currently believed that the prM protein functions as an E-protein chaperone, protecting it from acid-catalyzed denaturation during virus maturation. The cleavage sites generating the nonstructural proteins NS2A, NS2B, NS4A, and NS4B have been identified.

Flavivirus infection causes virus-specific cytopathic effects in mammalian cells. Flavivirus cell killing has recently been identified as an apoptotic event (Despres et al. 1996, 1998; Liao et al. 1997, 1998; Marianneau et al. 1997, 1998a, b, c, 1999; Avirutnan et al. 1998; Couvelard et al. 1999; Kamalov et al. 1999; Balachandran et al. 2000; Jan et al. 2000; Matthews et al. 2000; Ho et al. 2001; Prikhod'ko et al. 2001, 2002; Su et al. 2001, 2002; Lin et al. 2002; Parquet et al. 2002; Sauerwald et al. 2002; Shafee and AbuBakar 2002). Cytopathic effects in flavivirus-infected arthropod cells are not as dramatic. The site of flavivirus budding is not fully defined. Nucleocapsids do not appear to accumulate at the plasma membranes, indicating that budding may be quite efficient.

The processing of the pestivirus structural proteins has been described (Rümenapf et al. 1993). The pestiviral envelope glycoproteins are produced as a precursor protein, E^{rns}/E1/E2, which is rapidly cleaved to E^{rns}/E1 and E2. The E^{rns}/E1 precursor is then processed to E^{rns} and E1. The E^{rns} protein has no hydrophobic anchor sequence and can be found in infected cell supernatant. This cleavage pattern is similar to that seen with the flavivirus prM/E protein.

Flavivirus vaccines

The live-attenuated YFV vaccine is historically one of the most successful virus vaccines ever developed. It has low reactogenicity and excellent immunogenicity. Recently, however, a low rate of both neurotropic and viscerotropic adverse reactions following vaccination with YFV vaccines have been documented (Vasconcelos et al. 2001; Cetron et al. 2002). These reactions have primarily been associated with vaccination of the elderly, and preliminary genomic sequence data from recovered YFV vaccine show no signs of reversion. Further study is necessary to determine the reasons behind these new observations.

Modern approaches based on recombinant DNA technology are now being applied to flavivirus vaccine development. These approaches utilize DNA copies of the viral genome or individual viral genes to prepare either attenuated chimeric flaviviruses or direct DNA vaccines. Chimeric viruses based on either the YFV or DENV genome have been prepared (Bray and Lai 1991; Guirakhoo et al. 1999, 2000, 2001, 2002; Monath et al. 1999, 2000, 2002; Butrapet et al. 2000; Huang et al. 2000; Arroyo et al. 2001). Some of these chimeric vaccines have passed preclinical testing and are being prepared for human clinical trials. DNA-based vaccines have been prepared from number of flaviviruses (Konishi et al. 1997, 2000a, b, 2001; Chang et al. 2000, 2001; Hunt et al. 2001; Konishi and Fujii 2002). Preliminary tests of a DNA vaccine for WN virus have suggested that it may be effective in horses (Davis et al. 2001).

CLINICAL AND PATHOLOGICAL ASPECTS

Diseases caused by flaviviruses: general features

The flaviviruses known to cause human disease are listed in Table 2, several of which may also cause disease in wild or domestic animals. For example, in South Africa, Wesselsbron virus (WESSV) causes disease in sheep, and to a lesser extent goats and cattle (Coetzer and Theodoridis 1982); Kyasanur Forest disease virus (KFDV) has killed monkeys in India (Sreenivasan et al. 1979); and YFV can kill South American monkeys in nature and Asian monkeys in the laboratory, but not African monkeys. Israel turkey meningo-encephalitis virus (ITV) has caused economic losses in turkey farms in Israel and is also present in South Africa (Barnard et al. 1980).

CLINICAL MANIFESTATIONS

Flaviviruses cause a spectrum of clinical manifestations ranging from asymptomatic infections to undifferentiated febrile illnesses, fevers with rash or arthralgia or both, to hemorrhagic fevers, hepatitis, and encephalitis, which may result in death. The same virus can cause a variety of syndromes.

ARTHROPOD VECTORS

Much is known about the epidemiology of viruses such as DENV, YFV, JEV, St. Louis encephalitis virus (SLEV), and Central European TBEV, but there are also many uncertainties about these and other flaviviruses. For example, there are substantial differences between the natural history of YFV in Africa and in the New World (Downs 1982). YFV and DENV are classic examples of mosquito-borne flaviviruses, being transmitted in urban outbreaks by *Aedes aegypti* in both Africa and the Americas (YFV) and globally in the tropics (DENV) by a variety of mosquito species in the forest cycle. DENV are the only arboviruses that have fully adapted to a human–mosquito–human cycle and no longer depend on the forest cycle for maintenance. It has also been recovered from ticks in West Africa (Germain et al. 1982), although it is not known whether these arthropods contribute to the maintenance of the virus in nature.

In general, mosquito-borne flaviviruses are more common in the tropics, whereas tickborne flaviviruses tend to be prevalent in more temperate regions. The flaviviruses without a known vector are associated with rodents and bats, and are thought to be transmitted by contact with infective secretions. Only one virus in this subset, Dakar bat virus (DBV), is known to infect humans naturally, but several other nonvector-borne flaviviruses have caused laboratory infections. Transovarial, vertical,

Table 2 *Flaviviruses known to cause human disease*

Virus	Geographical distribution	Other features
Mosquito-borne viruses		
Banzi virus (BANV)	S and E Africa	2 cases only
Bussuquara virus (BSQV)	Brazil, Colombia, Panama	1 case only
Dengue viruses 1–4 (DENV types 1–4)	Worldwide in the tropics	Tropics and subtropics where the virus and a *Stegomyia* vector exist
Edge Hill virus (EHV)	Australia	
Ilhéus virus (ILHV)	C and S America	
Japanese encephalitis virus (JEV)	E, SE, S Asia, W Pacific	
Kedougou virus (KEDV)	Africa	Laboratory infection
Kokobera virus (KOKV)	Australia	
Kunjin virus (KUNV)	Australia, Sarawak	
Murray Valley encephalitis virus (MVEV)	Australia, New Guinea	
Rocio virus (ROCV)	Brazil	Epidemics of encephalitis
St. Louis encephalitis virus (SLEV)	N America, Panama, Jamaica, Trinidad, Brazil, Argentina	
Sepik virus (SEPV)	New Guinea	1 case only
Spondweni virus (SPOV)	E, W & S Africa	
Usutu virus (USUV)	E, W, Central Africa, Central Europe (Austria)	Found in birds in Austria
Wesselsbron virus (WESSV)	E, W, S Africa, Thailand	
West Nile virus (WNV)	E, W, S Africa, S and SE Asia, India, Mediterranean area, N America	Large epidemic throughout USA
Yellow fever virus (YFV)	W and Central Africa, S and Central America	Periodical epidemics, e.g. Ethiopia, Nigeria
Zika virus (ZIKV)	E and W Africa, Malaysia, Philippines	1 case in Uganda
Tickborne viruses		
Absettarov virus (ABSV)	Europe	
Hanzalova virus (HANV)	Europe	
Hypr virus (HYPRV)	Europe	
Karshi virus (KSIV)	Asia	
Koutango virus (KOUV)	Africa	
Kumlinge virus (KUMV)	Europe	
Kyasanur Forest disease virus (KFDV)	Mysore, India	
Langat virus (LGTV)	Malaysia	Only experimental cases proven
Louping ill virus (LIV)	British Isles	
Negishi virus (NEGV)	Asia	
Omsk hemorrhagic fever virus (OHFV)	The former Soviet Union	
Powassan virus (POWV)	Canada and USA	
Tick-borne encephalitis		
Western equine encephalomyelitis virus (WEEV)	Europe from Scandinavia to Balkans and from Germany to the former Soviet Union	
Eastern equine encephalomyelitis virus (EEEV)	Eastern Europe and the former Soviet Union	
No known arthropod vector		
Apoi virus (APOIV)	Japan	Laboratory infection
Dakar bat virus (DBV)	W, E and Central Africa, Madagascar	2 cases only
Phnom Penh bat virus (PPBV)	Cambodia, Malaysia	
RBV	USA, Mexico	

and trans-stadial transmission have been documented with several mosquito-borne and tickborne flaviviruses (Rosen 1987).

PATHOGENESIS

Flavivirus infections in humans cause three main disease patterns – nonspecific febrile illness (pantomorphic), encephalitis (neuromorphic), and hemorrhagic fever (viseromorphic) – which generally reflect the tropisms of the viruses for different target organs (Monath 1986). Symptoms in most flavivirus infections probably result from the direct effects of virus replication in the various target organs, which include the brain, liver, skin, lymphoid tissue, and cells of the mononuclear phagocyte lineage. Most flaviviruses are pantomorphic, causing only a nonspecific febrile illness in a large proportion of infections. Some, however, have virulence and evolutionary host characteristics that make them more neuromorphic or viseromorphic, thus causing encephalitis or hemorrhagic fever, respectively.

Both viral and host factors influence the pathogenesis of flaviviral infections of humans. Many flaviviruses have been shown to vary naturally in virulence characteristics as a result of selection pressures associated with the arthropod vector and/or vertebrate host. In addition, host factors such as age, immune status, and genetic background may influence pathogenesis. A mouse model of natural resistance to flavivirus infection has been developed (Brinton et al. 1998; Sangster et al. 1998; Shellam et al. 1998; Brooks and Phillpotts 1999; Urosevic et al. 1999; Silvia et al. 2001; Mashimo et al. 2002; Perelygin et al. 2002). This resistence appears to be associated, at least in part, with the intrinsic antiviral interferon response.

IMMUNE RESPONSE

Antigenically related flaviviruses may co-exist within similar habitats. There is extensive crossreactivity between flaviviruses in serological tests, and while there is no cross-protective immunity, heterologous flavivirus antibody may modulate clinical illness. This is especially important with DENV, in that infection with a single serotype provides homotypic immunity, but may enhance infection with a heterologous DENV and increase the risk of dengue shock syndrome (DSS) or dengue hemorrhagic fever (DHF).

DIAGNOSIS

Isolation of virus provides definitive evidence of flavivirus infection. Development of rapid and sensitive assays to detect viral RNA in tissue have extended the abilities to identify virus when viral titers are too low for isolation, or specimens have not been handled properly (Briese et al. 2000; Lanciotti et al. 2000; Lanciotti and Kerst 2001; Hunt et al. 2002; Komar et al. 2002). In practice, however, infections are diagnosed most frequently on the basis of serological tests, which include the PRNT, CF, and HI, and ELISA for virus-specific IgM and IgG (Roehrig 2000). A variety of flavivirus derived murine mAbs are available for virus identification (Roehrig 2000).

CONTROL

Measures directed towards vector control provide the only practical means of limiting virus transmission for most flaviviruses. Vaccines are available for YFV, JEV, and TBEV.

IMPORTANT MOSQUITO-BORNE DISEASES

Yellow fever virus

Although yellow fever virus (YFV) is believed to have originated in Africa, the first recorded outbreak was in Mexico in 1648. This was followed during the seventeenth, eighteenth, and nineteenth centuries by numerous urban epidemics in the West Indies, Central and South America, and the eastern part of the USA as far north as New York. Epidemics in more temperate regions of the western hemisphere were the result of introductions through seaports and of transport of mosquito vectors and viruses along commercial shipping routes. The work of the US Army Commission in Cuba established that urban transmission of YFV from human to human was infected by *Ae. aegypti* mosquitoes (Reed 1902), and subsequent eradication measures against this mosquito, and immunization using a live attenuated virus vaccine (see below), effectively controlled urban YF in the Americas and West Indies, respectively. However, the disease persisted sporadically in forest areas of both Africa and South America as a consequence of sylvatic cycles involving monkeys and forest-dwelling mosquitoes, e.g. *Haemagogus* and *Sabethes* spp. in South America, *Ae. africanus* in East Africa, and a variety of *Aedes* spp. in West Africa (Strode 1951). In the past 20 years, *Ae. aegypti* has reinfested nearly all tropical American countries, putting this region at the highest risk of urban epidemics of YF in more than 50 years.

In humans, YF illness varies from an inapparent infection to a fulminating disease terminating in death; three stages (infection, remission, and intoxication) can be commonly recognized. After an incubation period of 3–10 days, there is sudden onset of fever, rigors, headache, and backache. The patient is usually intensely ill and restless with flushed face, swollen lips, bright red tongue, and congested conjunctivae; many patients suffer from nausea and vomiting. A bleeding tendency may be seen early on. This stage of active congestion is followed after 2–3 days by a brief remission and then a resumption of febrile illness. The facial edema and flushing are replaced by a dusky pallor, the gums

become swollen and bleed easily, and there is a pronounced hemorrhagic tendency with black vomit, melena, and ecchymoses. The pulse rate is slow, despite the high fever, and the blood pressure falls, resulting in albuminuria, oliguria, and anuria. Death, when it occurs, is usually within 6–7 days of onset, and is rare after 10 days of illness. The jaundice, which gives the disease its name, is generally apparent only in convalescing patients. In patients with intoxication, mortality may occur in 20–50 percent. In a recent outbreak in Nigeria, mortality was over 50 percent (Nasidi et al. 1989). Most patients with severe disease will have leukopenia, thrombocytopenia, elevated liver enzymes, and coagulation defects. At autopsy the organs most affected are the liver, spleen, kidneys, and heart. Typically, midzonal necrosis is apparent in the liver, affecting cells around the periphery of the lobule and sparing areas around the central vein. Hyaline necrosis is evident and Councilman inclusion bodies are usually present.

Treatment is supportive and confined to nonspecific measures, including maintenance of fluid and electrolyte balances and replacement of any substantial amounts of blood lost through hemorrhage. Laboratory diagnosis can be made by virus isolation, serology, polymerase chain reaction (PCR), and immunohistochemistry. One dose of live-attenuated 17D vaccine provides complete protection for 10 years. A French vaccine prepared from a neurotropic strain of YFV has been widely used in Africa. It confers good protection and is cheaper than the 17D vaccine, but because it has caused adverse reactions, including a number of cases of encephalitis, its use is limited.

Dengue/dengue hemorrhagic fever (DHF)

There are four DENV serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. These viruses are closely related to each other antigenically, and crossreactivity occurs in serological tests. However, there is no lasting cross-protective immunity; cross-protection between DENV serotypes lasts for only a few months. Thus, individuals can have as many as four dengue infections in their life, one with each serotype.

Major DENV epidemics have been caused by all four DENV serotypes. Generally one serotype predominates in an area until increased herd immunity decreases transmission. Then another serotype predominates. DENV changes genetically during natural transmission, and significant biological differences have been shown between strains of the same serotype (Russell and McCown 1972; Gubler and Rosen 1977; Gubler et al. 1978).

The epidemiology of the four DENV serotypes is similar (Gubler 1988, 1998). All have a worldwide distribution in the tropics and are maintained in most tropical urban centers in a mosquito–human–mosquito cycle. In many urban centers, multiple virus serotypes co-circulate (hyperendemicity). The principal mosquito vector is *Ae. aegypti*, an African species that was spread around the world during the seventeenth, eighteenth, and nineteenth centuries with the slave trade and shipping industry. This species became highly adapted to living in intimate association with humans and is a highly efficient epidemic vector in urban settings. Secondary vectors include other *Ae. stegomia* species such as *Ae. albopictus*, *Ae. polynesiensis*, and *Ae. scutellaris* spp. These secondary vector species can transmit outbreaks, but may be more important as maintenance vectors. DENV are also maintained in forest cycles in Asia and Africa. These are thought to be primitive cycles and involve monkeys and canopy-dwelling *Aedes* spp. mosquitoes that rarely come in contact with humans. Current evidence suggests that these forest cycles are not important in contributing to urban epidemics (Rico-Hesse 1990).

Infection with DENV causes a spectrum of clinical illness, ranging from inapparent infection to mild nonspecific viral syndrome to classical DENV fever to severe and fatal hemorrhagic disease. The clinical picture of classical DENV fever has been described in experimentally infected volunteers (Siler et al. 1926; Simmons et al. 1931). Classic DENV fever is seen in adults and older children. After an infective mosquito bite, there is an incubation period of 4–6 days (range 3–14 days), followed by the sudden onset of fever (which is often biphasic), severe headache, chills, and generalized pains in the muscles and joints. A maculopapular rash generally appears on the trunk between the third and fifth day of illness and spreads to the face and extremities. Lymphadenopathy, anorexia, constipation, and altered taste sensation are common. Occasionally, petechiae are seen on the dorsum of the feet, legs, hands, axillae, and palate late in the illness. In young children, the illness is usually mild, but may be severe. The illness generally lasts for 5–7 days, after which recovery is usually complete, although convalescence may be prolonged. There is leukopenia with a relative lymphocytosis, and thrombocytopenia may occur. Liver enzymes may be elevated and hemorrhagic manifestations may occur.

DHF is a severe form of DENV infection characterized by sudden onset of fever, usually of 2–7 days' duration, and nonspecific signs and symptoms. The critical stage of DHF occurs 24 hours before to 24 hours after the temperature falls to or below normal. During this time, hemorrhagic manifestations usually occur, and signs of circulatory failure may appear. The patient may become restless or lethargic, experience acute abdominal pain, and have cold extremities, skin congestion, and oliguria, usually on or after the third day of illness. Clinical laboratory tests at this time will show thrombocytopenia (platelet count $<100\ 000/mm^3$) and evidence of a vascular leak syndrome, which may cause hypovolemia, shock, and death. The most common hemorrhagic manifestations are skin hemorrhages, but epistaxis, bleeding

gums, gastrointestinal hemorrhage, and hematuria may occur.

DHF is most commonly observed in children under the age of 15 years in Asia, but in the Pacific and Americas, DHF also occurs in adults (Gubler et al. 1978; Guzman et al. 1984a, b; Schatzmayr et al. 1986; Dietz et al. 1996). The pathogenesis of DHF is still not well understood. It is hypothesized that classical DHF with a capillary leak syndrome has a unique immunopathologic basis associated with heterologous dengue antibody-dependent enhancement of viral infection of cells of the mononuclear phagocyte lineage (Halstead 1980). Infection of these cells stimulates the release of a vasoactive mediator(s) that apparently causes increased vascular permeability; if not promptly detected and corrected, this can lead to hypovolemia, shock, and death. Although the risk of DHF is higher in children experiencing a second dengue infection, DHF also occurs in patients experiencing primary infections with DENV, suggesting that heterologous DENV antibody (previous infection) is not a necessary prerequisite for DHF. Furthermore, some strains of DENV cannot be enhanced in vitro (Rosen 1977; Gubler et al. 1978). Both field and laboratory evidence has been accumulating in recent years that supports a more prominent role of viral factors in the pathogenesis of DHF and suggesting that virus strain and serotype are also important risk factors for severe disease (Rosen 1986b, 1989; Gubler 1988, 1998; Trent et al. 1989; Rico-Hesse et al. 1997). Another complicating factor is that DEN infections with severe hemorrhage, but no evidence of a capillary leak syndrome, may have another pathogenetic mechanism (Gubler 1988). Because there is not a good animal model for DHF, a full understanding of the pathogenetic mechanism of severe dengue disease will have to await careful field studies in dengue endemic areas.

DENV infection can be diagnosed by virus isolation, serology, PCR, and immunohistochemistry. Although laboratory tests are reliable for diagnosis, they are not rapid and should not be used to make management and treatment decisions in a clinical setting. Instead clinicians should rely on clinical laboratory tests that can be used to monitor whether there is a capillary leak.

Case-fatality rates can be as high as 20–40 percent in DHF/DSS, but can be reduced with early diagnosis and proper case management using fluid replacement therapy. On average, DHF case-fatality rates are almost 5 percent. There is no vaccine for DENV/DHF, although significant progress has been made in developing both live attenuated vaccine candidates and second-generation recombinant candidate vaccines using infectious clone technology in recent years (Kinney and Huang 2001). Currently, disease prevention depends exclusively on mosquito control and avoiding mosquito bites.

Japanese encephalitis

Japanese encephalitis virus (JEV) is widespread throughout Asia, from the maritime provinces of Russia to South India and Sri Lanka (Umenai et al. 1985; Rosen 1986a). JEV is the most important cause of arboviral encephalitis globally with more than 45 000 cases reported annually. In recent years, JEV, which has been limited to Asia, has expanded its geographical distribution with outbreaks in the Pacific (Paul et al. 1993; Hanna et al. 1995). Epidemics occur in late summer in temperate regions, but the virus is enzootic and occurs throughout the year in many tropical areas of Asia. JEV is maintained in a natural enzootic cycle involving culicine mosquitoes and water birds. The virus is transmitted to man by *Culex* mosquitoes, primarily *Cx. tritaeniorhynchus*, and related species, which breed in rice fields. Pigs are the primary amplifying hosts of JEV in the peridomestic environment.

The incubation period of JE is 5–14 days. Onset of symptoms is usually sudden, with fever, headache, and vomiting. The illness resolves in 5–7 days if there is no CNS involvement. In those patients with CNS involvement, lethargy is common, faces are expressionless, and there are sensory and motor disturbances affecting speech, the eyes, and limbs. There may be confusion and delirium progressing to coma, with convulsions in children as a presenting sign. Weakness and paralysis may affect any part of the body. The lesions are generally upper motor neuron in character. Neck rigidity and a positive Kernig sign are found, and reflexes are abnormal. Initial leukocytosis is followed by leukopenia. The mortality in most outbreaks of JE is less than 10 percent, but is higher in children and has exceeded 30 percent in some outbreaks in India and in Korea (Umenai et al. 1985). Neurological sequelae in patients who recover are reported in up to 30 percent of cases.

A formalin-inactivated vaccine prepared in mice infected with the Nakayama strain of JEV is used widely in Japan, Korea, Taiwan, and Thailand; in China, an inactivated vaccine prepared in primary hamster kidney cell cultures has been given to millions of children. A live-attenuated vaccine prepared in primary hamster kidney cell culture has also given very promising results in children in China (Xin et al. 1988).

St. Louis encephalitis

St. Louis encephalitis (SLE) is one of the most important mosquito-borne encephalitides in the USA, causing periodic epidemics in the midwest and southeast. SLEV is prevalent throughout the western hemisphere from Canada to Argentina, but epidemics are unknown in South and Central America (Monath and Tsai 1987). SLEV is maintained in a culicine mosquito–bird–mosquito cycle, with periodic amplification by peridomestic birds

and *Culex* mosquitoes. In Florida, the principal vector is *Cx. nigripalpus*, in the Midwest, *Cx. pipiens pipiens* and *Cx. p. quinquefasciatus* and in the western United States, *Cx. tarsalis*.

Only a small proportion of SLE infections lead to clinical illness. Onset of illness is usually insidious, with a nonspecific prodrome. The disease is generally milder in children than in adults. In those children who do have severe disease, however, there is a high rate of encephalitis. The elderly are at highest risk for severe disease and death. No vaccine is yet available, and prevention, therefore, is by mosquito-control measures.

West Nile virus

West Nile virus (WNV) is closely related to JEV, Murray Valley encephalitis virus (MVEV), and SLEV. It, like the other flaviviruses that cause encephalitis, has a natural maintenance cycle involving wild birds and mosquitoes. Historically, the geographical distribution of WNV included Africa, Central Asia, the Middle East, and Europe. In 1999, WNV was identified in the western hemisphere for the first time in New York City and has since spread throughout most of the USA, southern Canada, and the northern part of Mexico along the US boarder (Gubler et al. 2000; Marfin et al. 2001; Petersen and Roehrig 2001; Campbell et al. 2002; Petersen and Marfin 2002; Roehrig et al. 2002). Genomic sequencing studies have identified two lineages of WNV (Mathiot et al. 1990; Lanciotti et al. 1999, 2002; Scherret et al. 2001). Lineage 1 contains viruses that have caused major human outbreaks in Africa, Europe, the Middle East, and the USA. Also included as a lineage 1 WNV is the Australian flavivirus, KUNV. KUNV normally causes mostly asymptomatic infections, but occasional cases of encephalitis have been reported (Muller et al. 1986). Lineage 2 WNVs are primarily enzootic viruses that circulate in Africa and have not been associated with human epidemics. The lineage 1 North American WNV is most closely related to a genotype in Israel (Jia et al. 1999; Lanciotti et al. 1999), indicating that this was the most likely source of the North American WNV.

An interesting aspect of the North American/Israeli strain of WNV is its virulence for some of its avian reservoirs, especially members of the family *Corvidae* (e.g. crows, blue jays, etc.), humans, horses, and a number of other animal species. High virulence is not common among other strains of WNV and avian virulence in the form of dead bird surveillance has been exploited as a particularly effective method to identify WNV activity in North America (Eidson et al. 2001a, b). To date, more than 150 species of birds and 19 other mammalian species have been shown to be infected with WNV in the USA (Komar et al. 2002). Based on seroprevalance of WNV-reactive antibodies in birds and their large populations, passerine birds appear to be major avian reservoirs for WNV in North America. Thousands of horses have been infected with WNV in the USA since 1999, with a case-fatality rate of about 25 percent. Studies suggest, however, that the horse is a dead-end host for WNV (Bunning et al. 2002). In addition, WNV morbidity and/or mortality have been documented in other mammals. A killed WNV vaccine has been developed for horses. Beginning in 2001, it has been used extensively in the USA. How, or if, this vaccination program has modified current the equine epizootic in North America is not known. A DNA vaccine also shows promise for disease prevention in horses (Davis et al. 2001).

The principal mosquito vectors belong to the genus *Culex*, including *Cx. univittatus* in Africa, *Cx. pipiens* and *Cx. modertus* in the Middle East and Europe, and the *Cx. vishnui* and *Cx. tritaeniorhynchus* complexes in Asia. While *Cx. pipiens pipiens* and *Cx. p. quinquefaciatus* appear to be primary vectors among the bird reservoirs in North America, at least 40 other species of mosquitoes have been shown to be infected with WNV, and several of them are being investigated as possible bridge vectors to humans and horses (Nasci et al. 2001; Turell et al. 2001, 2002; Dohm et al. 2002). In addition, WNV has been isolated from both soft and hard ticks, which may be involved in the maintenance cycle in some areas (Gromashevsky et al. 1973; Lvov et al. 2000).

Human infection is usually mild and generally presents as a nonspecific viral syndrome. In those cases with more severe disease, meningoencephalitis is the most common complication. In the USA, WNV encephalitis can present as a poliomyelitis-like syndrome (Glass et al. 2002; Leis et al. 2002). The incubation period can range from 2 to 15 days post-infection. A single case of hepatitis caused by WNV has been reported from Africa. The case-fatality rate in severe cases is about 10 percent, but like SLE, may be much higher in elderly people above the age of 50. The summer of 2002 in the USA saw the largest human outbreak of WNV meningo-encephalitis ever documented with more than 3 800 human cases. Results of human serosurveys in the USA suggest that 1:150 WNV infections progress to encephalitis in persons less than 65 years, but may be as high as 1:50 in older individuals (Mostashari et al. 2001).

Recent studies have also demonstrated that WNV can be transmitted through transfusion or transplantation of virus-contaminated blood or other tissues, through human breast milk, or transplacentally. While these may be infrequent events, the blood contamination may necessitate screening of blood products for the presence of WNV during intense human viral outbreaks.

There is no human vaccine for WNV. Prevention relies on mosquito control and protection from mosquito bites to reduce the risk of exposure. Definitive diagnosis can only be made using laboratory tests to isolate the virus or detect specific antibodies.

Murray Valley encephalitis

Murray Valley encephalitis (MVE) is a zoonosis that occurs only in Australia and New Guinea. The virus is closely related to JEV, WNV, SLEV, and several other flaviviruses, all of which are zoonoses and cause encephalitis in humans. MVEV, first isolated in 1951, has been and is responsible for major epidemics of encephalitis in Australia. Like other flaviviruses of this group, the virus is believed to be maintained in a natural cycle involving water birds and mosquitoes. However, this is not proven and serological evidence suggests other hosts are commonly infected. The major mosquito vector appears to be *Cx. aunutirostris*, although other species such as *Ae. normanensis* and other *Aedes* spp. may play a role, especially in the maintenance cycle. The inapparent to apparent ratio infection is about 500:1. Clinical disease is characterized by sudden onset of a nonspecific viral syndrome with fever, headache, nausea/vomiting, anorexia, and myalgias, followed by drowsiness, malaise, irritability, mental confusion, and meningismus. There is a pleocytosis and leukocytosis. In severe cases, there may be hyperactive reflexes, spastic paresis, convulsions, coma, and death. The case-fatality rate is about 20 percent; neurological sequaleae occur in a significant number of those patients that survive. All age groups are affected.

Viremia in humans has not been documented in MVEV infections, and it is likely that they are dead-end hosts. The pathological changes that occur in the central nervous system are similar to those caused by other viral zoonoses that cause encephalitis. Definitive diagnosis must rely on laboratory tests that detect specific antibody to MVEV.

There is no vaccine for MVE. Prevention relies on mosquito control and individuals taking responsibility to protect themselves against mosquito bites.

Other mosquito-borne infections

ILHÉUS VIRUS

Ilhéus virus (ILHV) is active over wide areas of Central and South America, including Trinidad, and is probably maintained in a forest cycle involving birds and mosquitoes. Most human infections are asymptomatic, but occasional cases of encephalitis are recognized.

ROCIO VIRUS

Rocio virus (ROCV) is antigenically related to SLEV and is known only from Brazil. Clinically it causes CNS disease similar to other flaviviruses that cause encephalitis. It has caused major epidemics in Brazil, principally in males 15–30 years of age (de Souza Lopes et al. 1981; Iversson 1988).

SPONDWENI VIRUS

Spondweni virus (SPOV) is present in South Africa, and has produced overt human disease in a few expatriates in West Africa (Wolfe et al. 1982).

USUTU VIRUS

The historical range of Usutu virus (USUV) is in East, West and Central Africa. Recently, however, USUV has been isolated from birds in Austria (Weissenbock et al. 2002). A single case with fever and a rash has been reported.

WESSELSBRON VIRUS

Wesselsbron virus (WESSV) has wide distribution in Africa and causes epizootics in sheep, producing abortion and death in newborn lambs and ewes, and less severe disease in goats and cattle (Coetzer and Theodoridis 1982). The virus also infects man in nature and in the laboratory (Justines and Shope 1969).

TICK-BORNE INFECTIONS

Tick-borne encephalitis

Tick-borne encephalitis (TBE) is caused by two closely related viruses that are distinct biologically. The eastern subtype causes Russian spring–summer encephalitis (RSSE) and is transmitted by *Ixodes persulcatus*. The western subtype is transmitted by *Ixodes ricinus* and causes Central European encephalitis (CEE). The latter name is somewhat misleading, since the condition occurs in foci extending from Scandinavia in the north to Yugoslavia and Greece in the south.

Of the two subtypes, RSSE is the more severe infection, having a case-fatality rate of up to 25 percent in some outbreaks, whereas mortality in CEE seldom exceeds 5 percent. Both viruses are maintained in natural cycles involving a variety of mammals and ticks; the latter serve as the main reservoir host by passing the viruses from generation to generation by transovarial and trans-stadial transmission. Human exposure occurs in the spring and summer months in temperate zones and in fall and winter in the Mediterranean, when the ticks are more active. Most human exposure is associated with work or recreational activities in enzootic areas. The incubation period is 7–14 days. Infection usually presents as a mild, influenza-type illness or as benign, aseptic meningitis, but may result in fatal meningoencephalitis. Fever is often biphasic, and there may be severe headache and neck rigidity, with transient pareses of the limbs, shoulder girdle, or, less commonly, of the respiratory musculature. A few patients are left with residual flaccid paralysis (Ackermann et al. 1986). Although the great majority of TBE infections follow

exposure to ticks, infection has occurred through the ingestion of infected cows' or goats' milk.

Inactivated vaccines are available against both eastern and western subtypes of TBE. The vaccine against the western subtype is highly effective (Kunz et al. 1980). Definitive diagnosis can only be achieved by specific laboratory tests to detect antibody.

Louping ill

Louping ill (LI) is derived from an old Norse word meaning 'to leap.' Louping ill virus (LIV) is a member of the TBEV complex. It is known in the British Isles as a disease of sheep characterized by CNS manifestations, in particular, cerebellar ataxia, paralysis, and death. It has never been a serious hazard to humans: most reported cases are the result of laboratory infections. A few natural infections do occur in persons closely associated with sheep. The illness is generally biphasic with encephalitic involvement in the second stage. The vector is *I. ricinus* and the virus is maintained in rodents, deer, and ground-living birds (Reid 1988).

Powassan virus

Powassan virus (POWV) is a member of the TBEV complex. It is thought to be a rare cause of acute viral CNS disease in Canada and the USA, but is also present in the former Soviet Union, where it has been recovered from mosquitoes, ticks, and humans. It was first isolated from the brain of a 5-year-old child who died in Ontario in 1958, and has since caused a number of cases of encephalitis in Canada and the eastern USA (Artsob 1988). However, with intensified surveillance for neurological disease caused by WNV in the northeastern USA, four cases of POWV encephalitis have been documented (Centers for Disease Control and Prevention 2001). Patients who recover may have residual neurological sequelae. In addition to isolations from humans, the virus has been recovered from ticks (*I. marxi, I. cookei*, and *Dermacentor andersoni*) and from the tissues of a skunk (*Spiligale putorius*) (Johnson 1987). There is clinical and serological evidence that POWV can cause fatal encephalitis in horses, which may be confused with rabies encephalitis (Little et al. 1985).

Kyasanur Forest disease

Kyasanur Forest disease virus (KFDV) was first recognized in 1957 during a fatal epizootic affecting wild monkeys in Mysore (Karnataka) State, India. Since then, thousands of infections have occurred in forest workers with a mortality of up to 10 percent. Until recently it was found only in southwestern India. The virus has been isolated from monkeys, humans, and ticks. The principal tick vector appears to be *Haemaphysalis spinigera*; the vertebrate reservoir host is uncertain (Banerjee 1988). Human infection with KFDV causes a spectrum of illness ranging from inapparent infection to severe hemorrhagic disease. Hemorrhagic manifestations are common and may involve the skin, gums, and gastrointestinal tract. Although CNS involvement is uncommon, there may be signs of meningeal irritation and stiff neck. An inactivated vaccine is available in Europe.

An apparent subtype of KFDV has been isolated from fatal human cases of hemorrhagic disease in Jeddah, Saudi Arabia, associated with meat handlers (Zaki 1997; Besaud 2005). The virus was subsequently isolated from other patients with illness ranging from fatal hemorrhagic disease to viral syndrome. All known human infections to date have been associated with handling meat or drinking unpasteurized camels' milk. The virus appears to be associated with sheep and camels, and to be transmitted by ticks, but no data are available on either the natural vertebrate host or the arthropod vector.

Omsk hemorrhagic fever

Omsk hemorrhagic fever virus (OHFV) is also caused by a tickborne Flavivirus. It was first isolated in association with human disease in Siberia in 1945; illness was characterized by biphasic fever with lymphadenopathy and hemorrhages from mucous membranes. OHFV is antigenically distinct from the eastern and western subtypes of TBEV. Infection may be acquired from ticks, usually *Dermacentor pictus* or *D. marginatus*, or from exposure to infected water voles or muskrats (Lvov 1988).

Other tick-borne infections

MEABAN VIRUS, SAUMAREZ REEF VIRUS, AND TYULENIY VIRUS

These viruses are associated with seabird ticks in France, Australia, and subarctic regions of the USA and the former Soviet Union (Clifford et al. 1971; Lvov et al. 1971; St George et al. 1977; Chastel et al. 1985).

NEGISHI VIRUS

Negishi virus (NEGV) has caused at least two fatal cases of encephalitis in Japan (Okuno et al. 1961). Although tick transmission has not been proven, this virus is closely related to other tick-borne flaviviruses (De Madrid and Porterfield 1974; Furuta et al. 1984; Calisher et al. 1989).

PESTIVIRUS INFECTIONS

Bovine viral diarrhea

Infection with BVDV occurs worldwide in many wild and domestic bovine species. Two clinical syndromes are

recognized in cattle, namely bovine viral diarrhea and mucosal disease. The former is a mild, transient infection with high morbidity but low mortality, which is followed by immunity. Viral infection during pregnancy can result in abortion or the birth of persistently infected offspring, some of which may have teratogenic defects. Two biotypes of virus have been recognized, only one of which is cytopathic. When persistently infected, antibody negative animals carrying the noncytopathic strain virus are reinfected with a serologically related cytopathic strain, they develop mucosal disease, which carries a high mortality (Baker 1987; Brownlie et al. 1987).

Antibodies reactive with pestivirus antigens have been detected in the serum of two children with microcephaly (Potts et al. 1987). With a mAb prepared against BVDV Yolken et al. (1989) detected pestivirus antigens in the feces of infants with gastroenteritis.

Border disease

BDV of sheep, originally recognized in the Scottish border country, occurs also in New Zealand. The causative virus is closely related to that of bovine viral diarrhea (Horzinek 1981; Nettleton 1987).

Classical swine fever (hog cholera)

This important disease of pigs occurs worldwide and is associated with persistent infections, abortion, and congenital malformations (Liess 1987).

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Togaviruses

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CLASSIFICATION

Two decades ago the *Togaviridae* family included a wide variety of small, enveloped viruses with single-stranded RNA genomes of positive polarity of about 4×10^6 Da. These RNAs could serve directly as messenger RNA and were packaged into nucleocapsids demonstrating cubic symmetry. The family name was derived from the Latin *toga* (a Roman mantle or cloak) and refers to the lipid envelope. However, since the *Togaviridae* was first defined, its contents have changed significantly based on accumulated data about viral replication and nucleotide sequences of the genomes. Flaviviruses, pestiviruses, and arteriviruses, which were temporarily brought together with alphaviruses and rubella virus, were finally classified into different families (Weaver et al. 2000). Currently the Togaviridae is comprised of two genera, *Alphavirus* and *Rubivirus*. Twenty-eight known alphaviruses are grouped into eight complexes, the prototypes of which are *Western equine encephalitis virus* (WEEV), *Eastern equine encephalitis virus* (EEEV), *Venezuelan equine encephalitis virus* (VEEV), *Semliki Forest virus* (SFV), *Middelburg virus* (MIDV), *Ndumu virus* (NDUV), *Trocara virus* (TROCV), and *Salmon pancreas disease virus* (SPDV) (Table 1). Partially or completely sequenced genomes of different alphaviruses reveal at least 60 percent nucleotide sequence identity in the nonstructural and 40 percent or more identity in the structural proteins. Sequencing and phylogenetic studies have indicated that WEEV, *Highlands J virus* (HJV) and *Fort Morgan virus* (FMV) are recombinant New World alphaviruses whose glycoproteins were derived from a Sindbis-like ancestor, the remainder of the genome being derived from an EEE-like ancestor (Figure 1, p. 3). Phylogenetic studies suggest only three clearly defined virus groups: a VEE/EEE group, an SF group (including MIDV), and a Sindbis group (Powers et al. 2001).

Most alphaviruses are described as arthropod-borne, or arboviruses that are maintained in nature through biological transmission among vertebrate hosts by arthropod vectors, predominantly mosquitoes (Griffin 2001a). However, the recent finding of salmon pancreas disease and sleeping disease alphaviruses indicates that arthropod vectors may not be required for the circulation of all alphaviruses.

Alphaviruses have highly differing abilities to cause disease in humans and animals. Some of them, including VEEV, EEEV and WEEV, can cause severe epidemic encephalitis, with a high mortality rate. Infection by others usually leads to either serious, but not life-threatening, illnesses or clinically inapparent infection with mild symptoms, including headache, fever, skin rashes, and arthritis. Many alphaviruses (at least mosquito-transmitted ones) are believed to have similar lifecycles, structures of viral particles, and replication strategies (Griffin 2001a).

The genus *Rubivirus* (Rubella) consists of a single known member, *Rubella virus*. Alphaviruses and rubella virus do not have significant primary sequence

Table 1 *List of alphaviruses*

Antigenic complex	Species (abbreviation)	Subtype	Clinical syndrome	Distribution
Barmah Forest	*Barmah Forest virus* (BFV)		Febrile illness, rash, arthritis	Australia
Eastern equine encephalitis (EEE)	EEE virus (EEEV)		Febrile illness, encephalitis (none recognized in Latin America)	North, Central, South America
Middelburg	*Middelburg virus* (MIDV)		None recognized	Africa
Ndumu	*Ndumu virus* (NDUV)		None recognized	Africa
Semliki Forest	*Semliki Forest virus* (SFV)		Febrile illness	Africa
	Chikungunya virus (CHIKV)		Febrile illness, rash, arthritis	Africa
	O'nyong-nyong virus (ONNV)		Febrile illness, rash, arthritis	Africa
	Getah virus (GETV)		None recognized	Asia
	Bebaru virus (BEBV)		None recognized	Malaysia
	Ross River virus (RRV)	Sagiyama	Febrile illness, rash, arthritis	Australia, Oceania
	Mayaro virus (MAYV)		Febrile illness, rash, arthritis	South and Central America, Trinidad
	Una virus (UNAV)		None recognized	South America
Venezuelan equine	VEE virus (VEEV)	IAB	Febrile illness, encephalitis	North, Central, South America
encephalitis (VEE)		IC	Febrile illness, encephalitis	South America
		ID	Febrile illness, encephalitis	South America, Panama
		IE	Febrile illness, encephalitis	Central America, Mexico
	Everglades virus (EVEV)		Febrile illness, encephalitis	Florida (USA)
	Mucambo virus (MUCV)		Febrile illness, myalgia	South America, Trinidad
		strain 71D1252	Unknown	Peru
	Tonate virus (TONV)		Febrile illness, encephalitis	Brazil, Colorado (USA)
	Pixuna virus (PIXV)		Febrile illness, myalgia	Brazil
	Cabassou virus (CABV)		None recognized	French Guiana
	AG80-663 virus (AGV)		Febrile illness, myalgia	Argentina
	Mosso das Pedras virus (MDPV)		None recognized	Brazil
Western equine encephalitis (WEE)	*Sindbis virus* (SINV)		Febrile illness, rash, arthritis	Africa, Europe, Asia, Australia
		Babanki	Febrile illness, rash, arthritis	Africa
		Ockelbo	Febrile illness, rash, arthritis	Europe
		Kyzylagach	None recognized	Azerbaijan, China
	Whataroa virus (WHAV)		None recognized	New Zealand
	Aura virus (AURAV)		None recognized	South America
	WEEV	Several	Febrile illness, encephalitis	Western North, South America
	Highlands J virus (HJV)			Eastern North America
	Fort Morgan virus (FMV)	Buggy Creek	None recognized	Western North America
Trocara	*Trocara virus* (TROV)		None recognized	South America
Salmon pancreas disease (SPD)	SPD virus (SPDV)		Pancreatic disease (salmon)	Atlantic Ocean and tributaries
		Sleeping disease virus	Sleeping disease (trout)	Worldwide

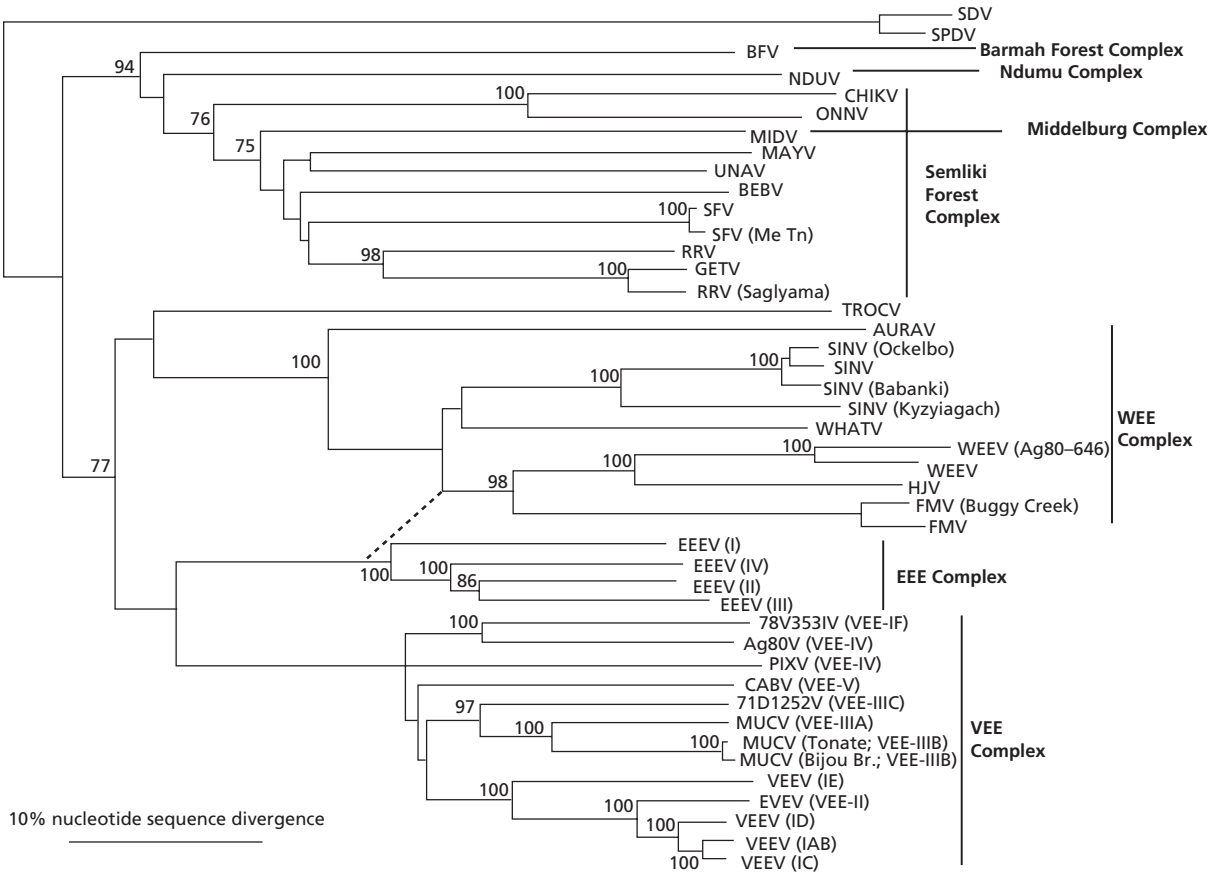

Figure 1 *Phylogenetic tree of all* Alphavirus *species, and selected subtypes and variants (in parentheses). Virus abbreviations are found in Table 1. Numbers refer to bootstrap values for clades defined by the adjacent node.*

homology in structural and nonstructural proteins. However, the coding strategies, their genomes, mechanisms of replication and structure of viral particles demonstrate a number of similarities, suggesting a common ancestry and supporting their inclusion in the same family *Togaviridae*.

VIRION STRUCTURE

Alphavirus virions are about 70 nm in diameter and are composed of an icosahedral nucleocapsid with T = 4 symmetry, surrounded by a lipid envelope containing glycoprotein spikes. The three-dimensional organization of viral particles has been determined for Ross River virus (RRV), Sindbis virus (SINV), and VEEV, which represent three different *Alphavirus* complexes. Electron cryomicroscopy and image reconstruction demonstrated that for these viruses the envelope glycoproteins are arranged on the outer surface of particles in a very similar way. The 240 heterodimers of E1 and E2 are combined into 80 trimers forming glycoprotein spikes that are distributed on the surface of viral particles in a T = 4 icosahedral lattice that mirrors the symmetry of the nucleocapsid. The regularity of spike distribution is determined by the direct interaction of glycoprotein heterodimers with capsid protein subunits. The E1 glycoprotein, which is structurally homologous to the envelope glycoprotein of flaviviruses, appears to lie parallel to the lipid envelope (Lescar et al. 2001), whereas the E2 glycoprotein projects outward from the virion to form the spikes.

GENOME STRUCTURE AND REPLICATION

The *Alphavirus* genome is a single-stranded RNA molecule of positive polarity nearly 12 000 nucleotides in length, with a sedimentation coefficient of ca. 42–49S (Schlesinger and Schlesinger 2001; Strauss and Strauss 1994). The genomic RNA mimics the structure of cellular messenger RNA: it contains a 5′ methylguanylate cap structure and a 3′ polyadenylate sequence. The nonstructural viral proteins coded by the 5′ two-thirds of the genome are translated directly from this RNA immediately following its introduction to the cytosol. Structural proteins are coded by the subgenomic RNA that corresponds to the 3′ one-third of the genome. The subgenomic 26S RNA, transcribed from the subgenomic promoter located on the minus-strand intermediate of

the viral genome, is also capped and polyadenylated. The full-length minus-strand intermediate is synthesized on the template of viral genomic RNA and serves as a template for both subgenomic and genomic RNAs. The general scheme of *Alphavirus* replication is demonstrated in Figure 2. Synthesis of all three RNAs is asymmetric and highly regulated. Minus-strand synthesis is detectable only within the first 4 h post infection, whereas later only plus-strand RNAs (genome and subgenome) are being synthesized. This regulation is achieved via the sequential processing of the nonstructural proteins. Initially they are synthesized for different alphaviruses as two polypeptides, P123 and P1234, or just single P1234. This difference in synthesis of nonstructural proteins depends on the presence of the opal codon at the end of nsP3. Some alphaviruses contain this terminating codon, and P1234 can be synthesized only upon readthrough of the UGA that occurs with 5–20 percent efficiency. Other alphaviruses contain arginine or cysteine codons in place of UGA, and only P1234 is translated. This polyprotein comprises sequences of all four nonstructural proteins, nsP1, nsP2, nsP3, and nsP4, and its processing is mediated by the protease domain of nsP2. First autoproteolytic cleavage is performed in *cis* and results in the release of P123 and nsP4. These two proteins form the viral component of the replicative complex, and this replicative complex is active for minus-strand synthesis but is inefficient in generating the plus-strand genome and subgenomic RNAs. Further cleavage of nsP1/nsP2 and nsP2/nsP3 junctions is also performed by nsP2 protease, this time in *trans*, which changes the template specificity of replicative complex by making it incapable of minus-strand RNA synthesis and significantly increasing its activity in plus-strand RNAs production. The time of genomic and subgenomic RNA synthesis concurs with the most efficient release of viral particles, which requires high-level production of viral genomes and structural proteins for packaging. In spite of a great deal of progress in understanding *Alphavirus* genome replication, the detailed mechanism of viral nonstructural protein functioning is not completely understood. nsP1 possesses both guanine-7-methyltransferase and guanyltransferase activities required for capping of both genomic and subgenomic RNA. This protein also appears to be involved in binding of the replicative complexes to the membranes. nsP2 has a number of activities. Its amino terminal domain is considered to have helicase function,

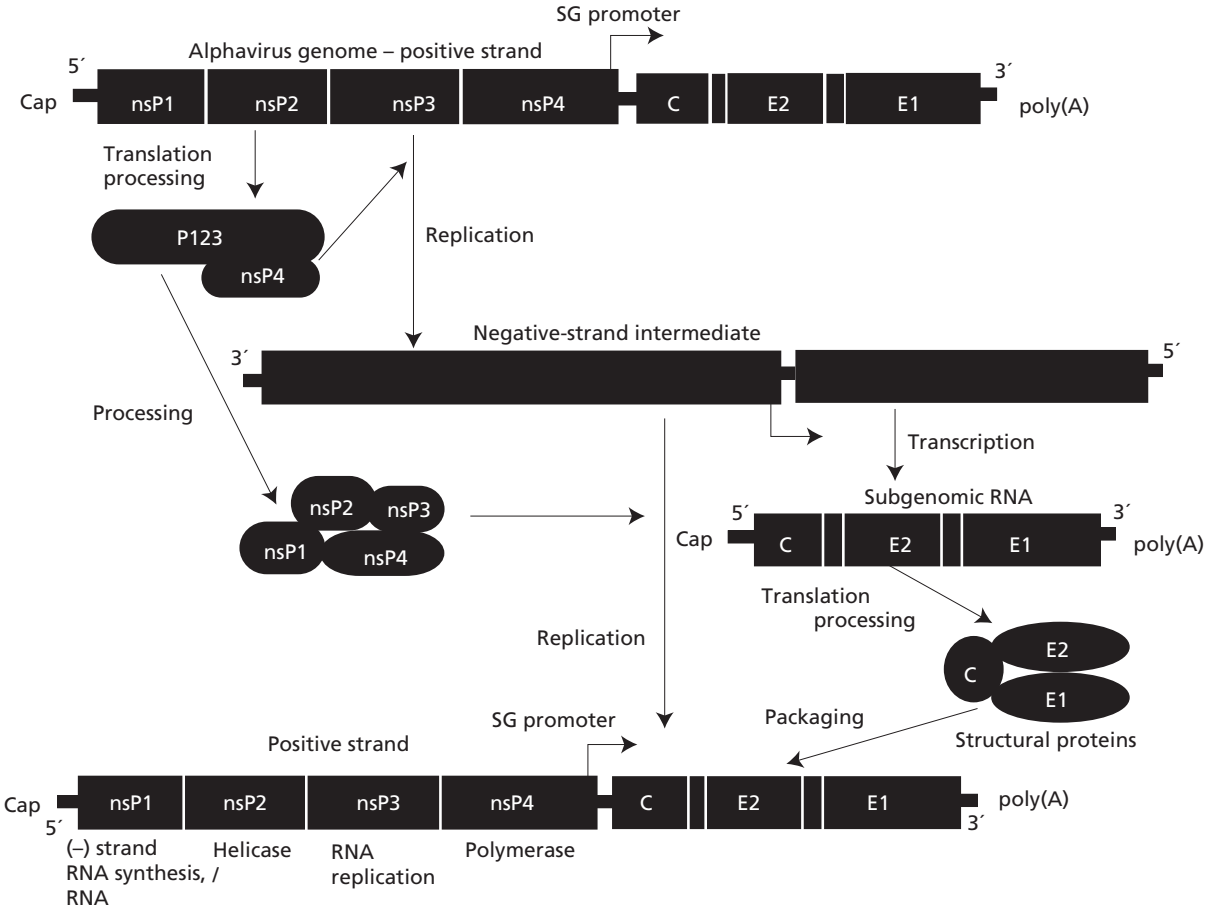

Figure 2 *Organization of the* Alphavirus *genome and schematic presentation of* Alphavirus *replication*

and the carboxy terminal part of the protein is a protease that orchestrates the processing of nonstructural proteins. Point mutations in this protein or in the nsP2/nsP3 cleavage site change the ratio of genome/subgenome RNA synthesis, suggesting that this protein is involved in the functioning of the subgenomic promoter. This protein accumulates in infected cells in a ten-fold excess to nsP4, and a significant fraction is transported to the nucleus. A number of mutations in this protein convert cytopathic alphaviruses, SINV and SFV into noncytopathic variants, which can persist in some cell lines of vertebrate origin (Frolova et al. 2002). These facts suggest that, in addition to its role in RNA replication, nsP2 may have additional functions in virus–host cell interactions. nsP3 is the least-studied protein, with unidentified functions. It can tolerate extensive deletions in C-terminal sequence that have very low sequence identity among alphaviruses, but mutations in the other fragments can make virus replication temperature sensitive. nsP4 is the RNA polymerase and contains the characteristic GDD motif found in all RNA-dependent polymerases. Mutations in this protein can make replicative complexes incapable of both minus- and plus-strand RNAs synthesis.

Alphavirus genomes contain a number of conserved sequences, so-called *cis*-acting elements that are required for replication. These short sequences include a 19-nt conserved sequence element (CSE), which immediately precedes the 3′ terminal poly(A) tract. The location of the 19-nt CSE suggests that it is an important element of the promoter for minus-strand RNA synthesis. Mutations in this sequence are either lethal or significantly alter virus replication. Another conserved sequence, 24-nt CSE, was identified as a promoter for transcription of the subgenomic RNA that functions on the minus-strand replicative intermediate. The 5′ termini of viral genomes appear to contain at least two functional elements. The 51-nt CSE (nt 155–205 of SINV) is located inside the nsP1-coding sequence and is predicted to form two short stem-loop structures. This CSE was shown to function as a replicational enhancer that operates in a host-dependent manner. Point mutations in this element alter virus replication in more mosquito cells than in cells of mammalian origin. The 5′UTR of alphaviruses was always believed to contain a promoter element that is required for initiation of the plus-strand RNA synthesis, and mutations in the 5′UTR significantly downregulate virus replication. The 5′ *cis*-acting elements not only determine plus-strand RNA synthesis when present on the 3′ end of minus-strand replicative intermediate, but control the minus-strand synthesis as well. The compelling evidence suggests that the 5′ and 3′ ends of *Alphavirus* genome RNAs interact to initiate replication, and this interaction is probably achieved by bringing them into close proximity via translation initiation factors.

STRUCTURAL PROTEINS

Alphavirus structural proteins are translated from the subgenomic RNA. Like the nonstructural proteins, they are synthesized as a single polypeptide that is cleaved both co- and post-translationally. Structural proteins are coded in the order capsid (C), E3, E2, 6K, and E1. The capsid is 260–270 amino acids in length (30–34 kDa), and the crystal structure has been solved for Sindbis virus. The capsid protein is synthesized first, possesses a serine protease activity, but in contrast to the nonstructural protease, the nsP2, it acts only in *cis* and cleaves itself from the polypeptide chain that is still being translated. This cleavage releases the signal peptide from the E3 (10 kDa) protein, leading to translocation of both E3 and E2 (PE2, sometimes called P62) into the endoplasmic reticulum (ER). PE2 remains attached to membranes via hydrophobic amino acids in the carboxy terminal part that function as a membrane anchor. The very end of the PE2 contains a signal sequence for translocation into the ER of the amino terminal part of the next short protein, 6K, whose role in virus particle formation is not well understood. In turn, the C-terminal part of 6K serves as the signal peptide required for transport of the last protein, E1, into ER. The latter protein also possesses a hydrophobic anchor on the C terminus. Proteolytic cleavages between PE2 and 6K, as well as between 6K and E1, are performed by cell signalases. The last, very critical, cleavage in the E3/E2 cleavage site is accomplished by a furin-type protease and is required for viral infectivity. During transport to the plasma membrane in secretory vesicles, the envelope proteins are postranslationally modified: E2 (420–424 amino acids) and E1 (438–442 amino acids) acquire oligosaccharides, become palmitoylated, and form heterodimers.

Formation of *Alphavirus* particles is based on specific RNA–protein and protein–protein interactions. The nucleocapsid protein is capable of selectively packaging viral genomes. It recognizes the encapsidation signal(s) located in the sequence of the nonstructural proteins in the viral genomic RNA and, after further oligomerization, 240 molecules of the capsid protein form an icosahedral nucleocapsid. During the final steps of viral replication, the capsid protein molecules in completely or partially assembled nucleocapsids interact with the cytoplasmic domain of E2 in E1–E2 heterodimers on the cell membrane. This leads to envelopment of the nucleocapsid by the plasma membrane containing glycoprotein spikes, and to the release of virus particles. This stage of virus maturation is not well understood, but it has been demonstrated that nucleocapsids undergo conformational changes and heterodimers of glycoproteins appear to assemble into trimeric spikes.

Because of their close association, E1 and E2 share a number of biological functions and antigenic properties.

The glycoprotein spike functions both in attachment to cellular receptors and in fusion with cellular membranes. The E2 glycoprotein elicits high-titer virus-specific neutralizing antibody. Two domains capable of eliciting neutralizing antibody have been identified in the E2 glycoprotein: amino acids 114–120, 180–220 (or 230–250 for RRV) using monoclonal antibody neutralization escape variants and rapid penetration variants. Antibodies to the carboxyl-proximal domain neutralize virus infectivity by blocking virion attachment to susceptible cells. Antibodies to the E2 glycoprotein can also block virus hemagglutination, presumably because of its close association with E1. The amino terminal 25 amino acids of the VEEV E2 glycoprotein protect mice from virus challenge. Mutations in the E2 glycoprotein have been associated with differences in virulence and the ability to infect and replicate in mosquitoes.

The E1 glycoprotein contains a highly conserved amino acid sequence (amino acids 80–96), which has sequence and chemical similarities to regions responsible for cell membrane fusion in orthomyxoviruses and paramyxoviruses. Mutation of this region alters the fusion characteristics of the virus. Because cell-membrane fusion seems, therefore, to be mediated by the E1 glycoprotein, it is not surprising that this glycoprotein elicits potent hemagglutination-inhibiting antibody. Virus-neutralizing anti-E1 antibodies have also been identified. Binding of these antibodies has been associated with E1 amino acid 132 using monoclonal antibody neutralization escape variants. Anti-E1 antibodies are usually not serotype specific and fix complement well.

Many of the biologically important epitopes on both the E1 and the E2 glycoproteins are conformational. This result makes mimicking these antigens in subunit or peptide vaccines difficult. The C protein elicits broadly cross-reactive antibody and is also a major complement-fixing antigen.

VIRUS–HOST CELL INTERACTIONS

Most data on *Alphavirus* replication in vitro were derived from experiments on relatively nonpathogenic members of the genus, SINV and SFV, but are probably applicable to most members of the genus. In general, alphaviruses can infect a wide variety of hosts and cell lines of vertebrate and invertebrate origin. Infection is initiated by virion attachment to cellular receptors. Their broad host range strongly indicates that alphaviruses can either attach to multiple receptors on the cell surface or use a very conserved protein as a receptor. Both of these assumptions appear to be correct. Several receptors have been identified for SINV in different cells (reviewed by Strauss and Strauss 1994). At the same time, it was unambiguously demonstrated that after adaptation of SINV or VEEV to BHK-21 cells that are traditionally used for their propagation, both viruses accumulate point mutations in structural glycoprotein E2, causing them to bind efficiently to heparan sulfate-like molecules (Griffin 2001a). The E2 is a protein that interacts directly with cell receptors. It is generally believed that amino acids 180–220 are directly involved in this interaction, because a considerable fraction of antibodies with neutralizing activity are specific to this peptide. Alphaviruses enter the cells via the endocytic pathway. Next, internalized virus-containing vesicles transform into endosomes, and an acidic pH leads to conformational changes in the glycoprotein spikes, trimerization of the E1 subunits, and fusion of viral and endosomal membranes. The E1 glycoprotein contains a very conserved peptide (amino acids 80–96) that was suggested to have fusogenic activity. It was recently demonstrated that the three-dimensional structure of E protein of tick-borne encephalitis virus (*Flavivirus* genus) and E1 of SFV have a number of similarities, particularly in the position of their fusogenic domains (Lescar et al. 2001). Fusion of the membranes leads to the release of nucleocapsids into the cytoplasm, and the next steps of virus disassembly are performed by ribosomes. *Alphavirus* capsid proteins have a very high affinity to ribosomes, and translocation of capsids to ribosomes results in uncoating of viral genomes and initiation of replication.

Replication of alphaviruses profoundly affects the metabolism of vertebrate cells with the inhibition of protein, RNA, and DNA synthesis playing the central role. Replication appears to activate two competing processes. First, viruses change the cellular environment to meet the needs of their propagation. At the same time, cells respond to virus replication by down-regulating virus growth and activating signaling pathways, followed by secretion of cytokines that prevent dissemination of the infection. The balance between these two mechanisms appears to determine the outcome of infection on the cellular level. In spite of very extensive investigation, it is still undetermined as to what mechanism or combination of different mechanisms defines the five- to ten-fold inhibition of translation that takes place 4–8 h post-infection. However, the translational shutoff is specific to cellular mRNAs. Alphavirus subgenomic RNA is translated very efficiently in this significantly changed intracellular environment owing to the unique structural element, a translational enhancer, located downstream from the initiating AUG. This enhancer and high concentration of the subgenomic RNA make this template the only RNA translated in the cells during the late stage of the infection.

Downregulation of transcription is the second major event in alphavirus-infected cells (Frolova et al. 2002). Both messenger and ribosomal RNAs are synthesized less efficiently, if at all, in permissive cell lines. Transcriptional shutoff may play an important role in the inhibition of interferon (IFN) production by infected

cells and a more efficient dissemination of the infection. Owing to dramatic changes in the intracellular environment, cells die within 24–48 h of infection, and their death is usually accompanied by apoptotic changes (Griffin 2001a).

TRANSMISSION CYCLES AND DISTRIBUTION

Most alphaviruses are **ar**thropod-**bo**rne, or **arbo**viruses, and are maintained in nature through biological transmission between susceptible vertebrate hosts by hematophagous arthropods. Arboviruses multiply and produce viremia in the vertebrate, replicate in the tissues of arthropods – obligately in the midgut and salivary glands – and are transmitted to new vertebrates when the arthropod takes a subsequent blood meal after a period of extrinsic incubation. The term arbovirus has no taxonomic significance and encompasses a heterogeneous group of viruses in seven taxonomic families, including the *Togaviridae* (alphaviruses only).

All alphaviruses are zoonotic and have at least two hosts, one a vertebrate and the other a hematophagous arthropod. The majority of alphaviruses are maintained in complex lifecycles involving one or more nonhuman vertebrate reservoir hosts and one or more arthropod vectors; an example is EEEV (Figure 3). These cycles remain undetected until humans or domesticated animals encroach on an enzootic focus, or the virus escapes the primary cycle via a secondary vector or vertebrate host as the result of some ecological or genetic change. Humans and domestic animals generally become involved only after the virus is brought into the peridomestic environment by a bridge vector, or after they enter an enzootic transmission focus. Humans and domestic animals, which frequently develop clinical illness, are usually considered 'dead-end' hosts because they do not produce viremia sufficient to infect vector arthropods and contribute to the transmission cycle, or because they are not bitten by a sufficient number of vectors when viremic. Many alphaviruses have several different vertebrate hosts and some are capable of

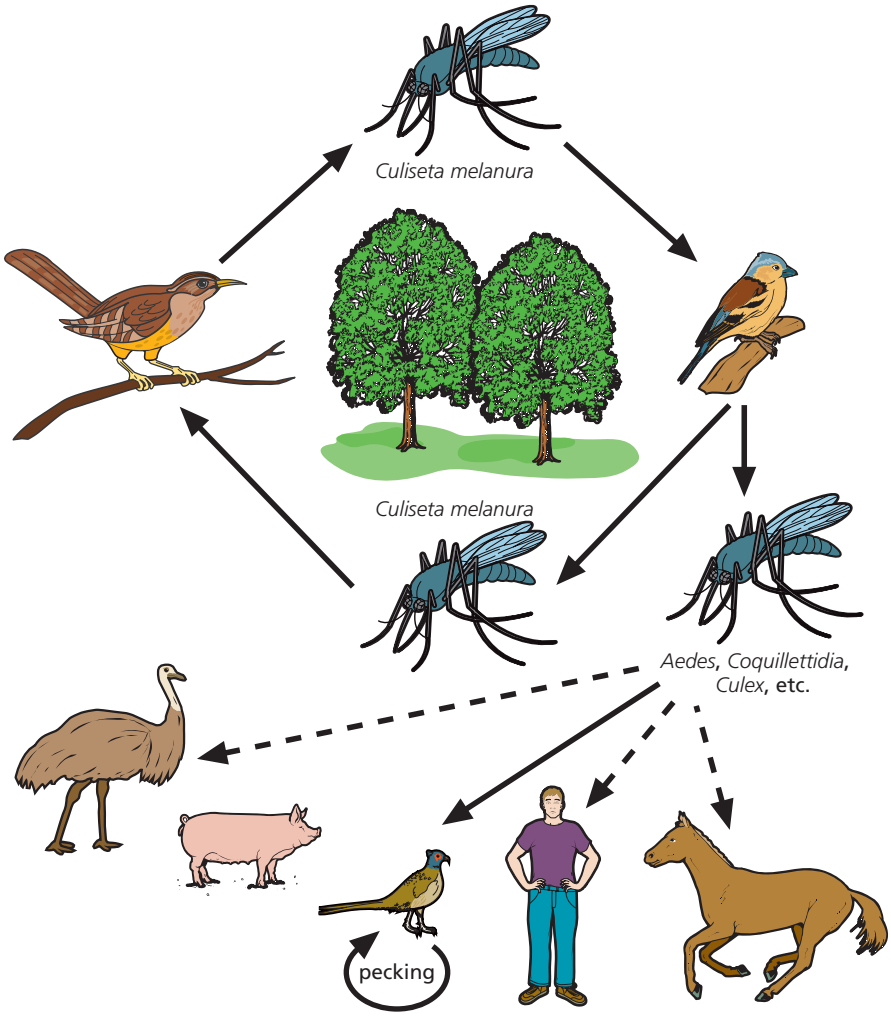

Figure 3 *Transmission cycle of Eastern equine encephalitis virus. Dashed arrows indicate dead-end transmission to hosts that generally do not infect additional vectors or vertebrates.*

transmission by more than one vector. A few alphaviruses, such as Chikungunya virus (CHIKV) and potentially VEEV, cause sufficient viremia in humans to be transmitted in a human–arthropod–human cycle. Transovarial transmission in nature has been demonstrated for SINV and RRV, and may facilitate maintenance of these alphaviruses in nature.

Every *Alphavirus* has its own distinct ecology. For some, such as SINV, EEEV (Figure 3) and WEEV (Figure 4), birds are the principal reservoir host, and others such as VEEV rely principally on rodents. The reservoir hosts of many alphaviruses are not known with certainty. Several, including CHIKV, EEEV, WEEV, VEEV, and SINV, have been isolated from bats, and these hosts deserve further study. All alphaviruses infecting humans are mosquito-borne. Several alphaviruses, including MIDV, CHIKV, and SINV, have also been isolated from ticks, but the significance of these findings is unknown. FMV and Bijou Bridge (a variant of Tonate virus in the VEE complex) are believed to use as their vectors cimicid bugs that occupy bird nests.

Alphaviruses have a nearly worldwide geographical distribution. Previously only the Antarctic area was believed to be *Alphavirus*-free, but a recent discovery of southern elephant seal *Alphavirus* (La Linn et al. 2001) indicates that our knowledge of their distribution is probably far from complete. The majority of alphaviruses are found in tropical developing countries. The greatest number have been described from Africa and South America, where the flora and fauna are diverse and extensive ecological and epidemiological studies have been conducted. The smaller number of viruses described from Asia, which has ecological diversity comparable to that of Africa and tropical America, may simply reflect the lack of surveillance in that region. The geographical distribution of each *Alphavirus* is limited by the ecological parameters governing its transmission cycle, including temperature, rainfall patterns,

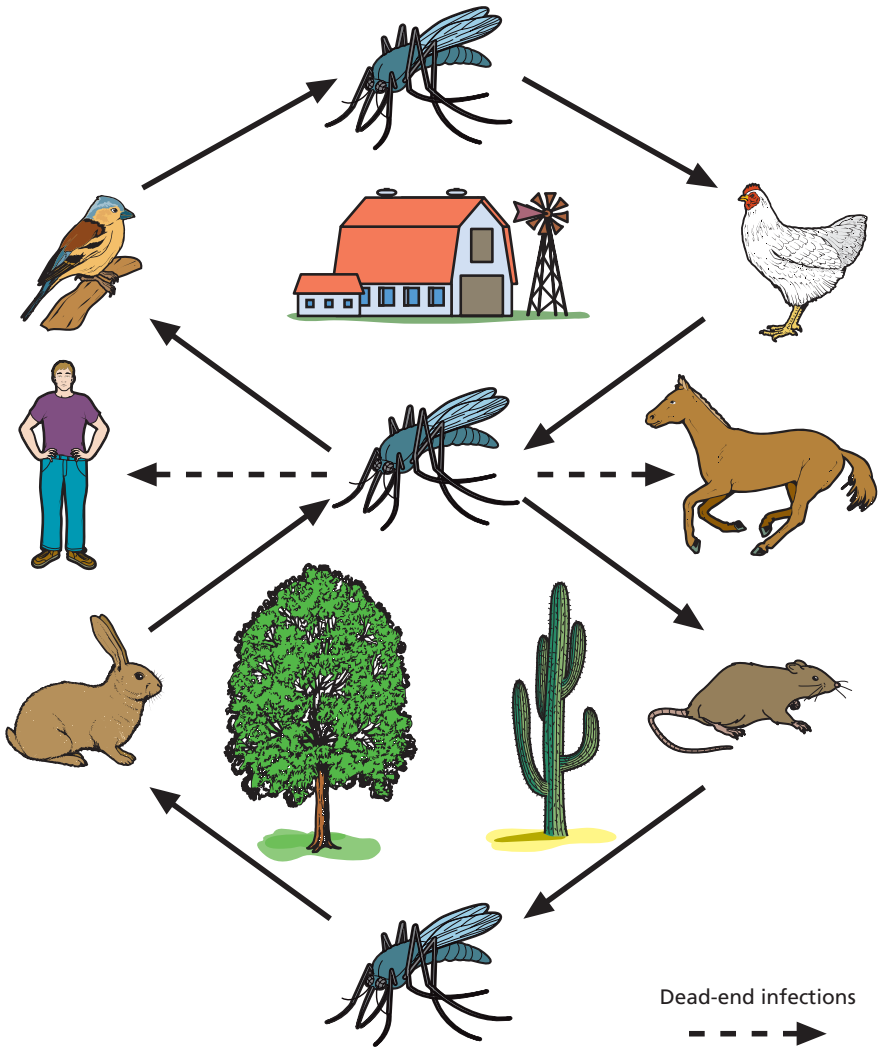

Figure 4 *Transmission cycles of Western equine encephalitis virus*

distribution of the arthropod vector(s), and vertebrate reservoir host(s).

Mosquitoes are by far the most important *Alphavirus* vectors, and bugs (*Cimicidae*) are also involved in the transmission of some alphaviruses. In addition to arthropod transmission, several alphaviruses, such as VEEV, are highly infectious by aerosol and numerous laboratory infections have occurred. Because of their aerosol infectivity, stability, and high degree of virulence, VEEV, EEEV, and WEEV are considered potential agents of biological warfare and terrorism.

DISEASES CAUSED BY ALPHAVIRUSES

Of the 28 currently described alphavirus species, at least 13 are known to cause disease in humans (Table 1). The majority of *Alphavirus* infections in humans cause a nonspecific febrile illness that is easily mistaken for infection by a respiratory virus such as influenza, or for dengue in the tropics. Onset is usually sudden, with fever, headache, myalgias, malaise, and occasionally prostration. The same virus can cause a variety of clinical syndromes. Infection with some alphaviruses, such as VEEV, EEEV, and WEEV, may lead to more severe disease, such as encephalitis, often with a fatal outcome or permanent neurological sequelae. These and some other alphaviruses are also responsible for disease in animals, notably *Equidae*. Often only a small proportion of people infected with these potentially encephalitic arboviruses progress to overt encephalitis; most develop only the prodromal, nonspecific stage of the infection, which may even be asymptomatic.

PATHOGENESIS

In vertebrates, initial sites of replication include skeletal muscle (EEEV, WEEV, SFV, RRV, SINV, and Getah virus (GETV)), and Langerhans' cells in the skin, leading to infection of the draining lymph node (VEEV, and probably others) (Griffin, 2001a; 2001b; Tsai et al. 2002). Plasma viremia is probably the main form of dissemination to other tissues and organs. Viruses such as RRV that cause a rash replicate in the skin, and RRV and CHIKV are also believed to replicate in striated muscle. Experimental studies have shown that invasion of the central nervous system (CNS), when this occurs, generally follows initial virus replication in various peripheral sites and a period of viremia exceeding a threshold for CNS entry. Entry to the CNS through the olfactory tract has been demonstrated for VEEV in a mouse model, but has not been investigated for most alphaviruses, and pathogenesis in humans is very poorly understood. CNS invasion may also occur through endothelial cells.

Alphaviruses can also cause pathology in their mosquito vectors, though vector pathogenesis has only been investigated thoroughly in a few cases. WEEV and EEEV kill midgut epithelial cells, their initial sites of replication, and this pathology may hasten dissemination of virus from the midgut to the salivary glands by facilitating passage through the basal lamina. Although mosquito pathology reduces the survival of mosquitoes infected with EEEV, the late timing of mortality may result in little effect on virus transmission.

IMMUNE RESPONSE AND DIAGNOSIS

Alphaviruses are highly immunogenic, and both cellular and humoral immune mechanisms contribute to recovery following natural or experimental infection. Humoral immunity is believed to be more important for protection (Tsai et al. 2002).

Alphavirus infections are diagnosed on the basis of either immunoglobulin M (IgM) and immunoglobulin G (IgG) serological responses, or by isolation of virus from acute-phase serum. Serological tests commonly employed include IgM or IgG ELISA, plaque reduction neutralization, hemagglutination inhibition, or complement fixation. Virus isolation is readily accomplished using a variety of vertebrate cells, such as Vero or baby hamster kidney (BHK), or using mosquito cells such as C6/36. Intracranial inoculation of newborn mice is more sensitive than cell culture for some alphaviruses. Alphaviruses are seldom recovered from the CNS, including the cerebrospinal fluid (CSF), except from fatal cases. A large array of antialphavirus murine monoclonal antibodies is available for virus identification.

CONTROL OF ALPHAVIRAL DISEASE

Because most alphaviruses are maintained in zoonotic transmission cycles, little can be done in an ecologically acceptable manner to control levels of virus circulation. The interruption of transmission to humans by mosquito control provides the only effective approach to control most alphaviral diseases. Control of some alphaviral diseases such as EEE relies on early detection of enzootic amplification in reservoir hosts (e.g. birds), followed by mosquito control to reduce vector populations in inhabited regions. Sentinel animals can also be used to detect virus circulation, or testing of potential mosquito vector pools for virus infection. For equine encephalitides such as EEE and VEE, equine cases usually precede human infection by a few weeks, and are useful indicators of the need for vector control (Weaver 2001a, b).

Mosquito control to reduce vector populations usually relies on the large-scale application of adulticides – or sometimes larvicides – in regions of enzootic or epidemic transmission. The inaccessibility of many enzootic habitats necessitates aerial insecticide applications. For many alphaviral diseases, personal protection against mosquito bites is the most effective means of human disease prevention. This is especially important

for individuals who reside, work, or recreate near habitats known or suspected of harboring enzootic transmission. Diethylmethylbenzamide (DEET) (35 percent formulations recommended; 10 percent for children) is the most effective mosquito repellent generally approved for use on the skin, and permethrin can be applied to clothing and camping gear to enhance protection. Education of persons likely to contact enzootic habitats is also important for preventing human infection.

There are no licensed human alphavirus vaccines. Equine vaccines are available for EEEV, WEEV, VEEV, and GETV. For some alphaviruses, such as VEEV, that require equines for efficient amplification to infect large numbers of people, equine vaccination can be effective in limiting human disease.

ENCEPHALITIS AND OTHER IMPORTANT ALPHAVIRUS DISEASES

Eastern equine encephalitis

HISTORY AND DISTRIBUTION

EEEV was first isolated in 1933 from the brain of an infected horse, and in Argentina in 1930 (identified retrospectively). EEEV occurs in hardwood swamp habitats near the eastern seaboard, the Gulf coast, and at some inland midwestern locations of the USA, and in Canada, some Caribbean Islands, Mexico, and Central and South America (Scott and Weaver 1989; Weaver 2001a). Small outbreaks of human disease have occurred in the USA, the Dominican Republic, Cuba, and Jamaica. More extensive equine epizootics are common during the summer in temperate areas of the USA and Argentina, and as far north as Québec, Ontario, and Alberta in Canada. Four distinct antigenic variants representing distinct genetic lineages of EEEV have been delineated; three occur in Latin America and do not appear to cause human disease. The fourth is represented by all strains from North America and the Caribbean, and is highly virulent for people.

TRANSMISSION CYCLES

EEEV circulates nearly continuously in tropical and subtropical locations, and appears to overwinter in temperate areas of the USA by an unknown mechanism (Weaver 2001a). In North America, enzootic circulation involves passerine bird reservoir hosts and *Culiseta melanura*, an ornithophilic mosquito that is found in hardwood swamp habitats (Figure 3). The distribution of EEEV closely approximates that of *Cs. melanura*, suggesting that it is the principal enzootic vector. Larvae of *Cs. melanura* develop at the base of trees or under roots in swampy, peat soils. Because humans, equines, and most other animals apart from passerine birds are believed to be dead-end hosts, and *Cs. melanura* is probably by far the most efficient vector, human and equine cases are generally found in close proximity to swamp habitats; epizootic transmission probably involves bridge vectors such as *Coquillettidia perturbans*, *Ochlerotatus canadensis*, and *Oc. sollicitans*, which feed on both birds and mammals and can transmit to humans, horses, and other hosts. Other species, such as *Aedes vexans* in temperate regions and *Oc. taeniorhynchus*, *Culex taeniopus*, and *Cx. nigripalpus* in the tropics, may be involved in transmission to humans and equines. The EEEV transmission cycle in Latin America is poorly characterized, but probably involves enzootic vectors in the subgenus *Culex (Melanoconion)*, and possibly birds and rodents as reservoir hosts.

DISEASE AND VIRAL GENETICS

Although not well documented, rates of inapparent (encephalitis) infection suggest that most human EEEV infections result in a nonspecific prodromal illness characterized by fever, headache, myalgias, photophobia, and dysesthesias (Tsai et al. 2002). The incubation period is 4–10 days, and the prodromal phase evolves over several days to longer than a week before the onset of encephalitis in some patients, especially young children and the elderly. The development of encephalitis is typically accompanied by severe frontal headache, dizziness, vomiting, lethargy, and later, neck stiffness, confusion, delirium, and drowsiness, sometimes leading to coma. Patients may have other neurological disorders, such as abnormal reflexes, spasticity, paralysis, and cranial nerve palsies, and infants may have convulsions and bulging fontanelles. The CSF is under pressure, has elevated protein, and pleocytosis is common. EEEV infection is also accompanied by visceral and pulmonary congestion. In the brain, loss of neurons, perivascular cuffing, microglial proliferation, and focal inflammatory infiltration are the most typical findings of human encephalitis. Vascular lesions are also common, with thrombi and extravasation of red cells, and necrosis and demyelinization also occur. Apoptosis occurs in glial and inflammatory cells, but is less commonly associated with neuronal cell death. Overall mortality rates during more recent outbreaks have averaged about 33 percent.

An EEEV vaccine is not available for general human use, although formalin-inactivated vaccines are effective in horses if administered frequently.

Genetically and antigenically, EEEV is highly conserved in North America, where a single, highly conserved lineage has evolved since 1933 (Weaver 2001a). The North American strains have also caused outbreaks as far south as Mexico and the Caribbean. EEEV strains isolated in Latin America are more genetically and antigenically diverse. There is no evidence that Central/South American strains of EEEV

cause human encephalitis, or even a febrile illness. Detection of human antibodies in South America suggests that the explanation for the lack of human encephalitis cases there is related to a difference in human virulence between the North and South/Central American virus lineages.

Western equine encephalitis

HISTORY AND DISTRIBUTION

WEEV was first isolated in California in 1930 from the brain of a horse, and remains an important cause of encephalitis in *Equidae* and in humans in North America, mainly in western parts of the USA and Canada (Reisen 2001; Reisen and Monath 1988). Sporadic cases also occur in Central and South America.

TRANSMISSION CYCLES

In the western USA the enzootic cycle of WEEV occurs mainly during the summer and involves passerine birds, in which the infection is inapparent. *Cx. tarsalis*, a mosquito associated with agriculture and irrigation systems, is the principal vector (Figure 4). A summer cycle involving jackrabbits and *Ae. melanimon* or *Oc. dorsalis* mosquitoes has also been documented in desert environments. The mechanism of WEEV overwintering in temperate climates is unknown, but chronic infection of birds has been observed after experimental infection. WEEV has also been isolated from snakes and from a variety of mammal species. In Argentina, *Oc. albifasciatus* is a probable mosquito vector.

DISEASE AND VIRAL GENETICS

Human WEE cases are usually first seen in June or July in the northern hemisphere. Most WEEV infections are asymptomatic or present as mild, nonspecific illness with fever, headache, and fatigue. Patients with clinically apparent illness usually have a sudden onset, with fever, headache, chills, nausea, myalgia, vomiting, anorexia, and malaise, followed by altered mental status, weakness, dizziness, vomiting, and signs of meningeal irritation (Tsai et al. 2002). Some patients progress to neck stiffness, weakness, and generalized tremulousness, and occasionally stupor or coma. Children, especially those under 1 year old, are affected more severely than adults and may be left with permanent sequelae, which are seen in 5–10 percent of patients. Overall mortality in apparent cases is 4 percent and is highest among the elderly.

Several genetic lineages and antigenic subtypes of WEEV have been delineated. Interestingly, unlike EEEV, some lineages of WEEV range in distribution from North to South America, and others appear to be limited to the tropics or subtropics. Virulence differences have been detected in mice, but it is unknown whether there are differences in virulence for humans.

Venezuelan equine encephalitis

HISTORY AND DISTRIBUTION

VEEV was first isolated in Venezuela in 1938 from the brain of a horse; like EEEV and WEEV, it causes encephalitis in Equidae and humans. VEE is an important veterinary and public health problem in Central and South America because equines remain important for agriculture and transportation in many regions (Walton and Grayson 1988; Weaver 2001b). Focal outbreaks occur periodically, with some spreading over large areas and causing up to hundreds-of-thousands of equine and human cases. A large epizootic that began in Guatemala and El Salvador in 1969 reached Texas in 1971; it was estimated that over 200 000 horses died in that outbreak, which was controlled by a massive equine vaccination programme using the live-attenuated TC-83 vaccine. Several thousand human infections were documented. A more recent VEE epidemic occurred in the fall of 1995 in Venezuela and Colombia, with an estimated 100 000 human infections.

TRANSMISSION CYCLES

Epizootic transmission involves horses, mules, and donkeys as amplification hosts, and a variety of mosquitoes in the genera *Ochlerotatus*, *Psorophora*, and others as vectors. Although infected people become viremic and capable of amplification by infection of mosquito vectors, epidemiological evidence (lack of any major epidemics in the absence of equine amplification) indicates that they are not as efficient as equines; this is probably due to the fewer mosquitoes sustained by viremic persons than by equines. Although most human VEE cases occur during equine epizootics, endemic transmission also occurs in communities in close proximity to sylvatic enzootic cycles in the absence of any equine disease.

DISEASE AND VIRAL GENETICS

VEEV is one of several alphaviruses in the VEE antigenic complex (Table 1; Figure 1). Only VEEV and Everglades virus (genetically a variant of VEEV in subtype ID) are associated with encephalitis in humans, but other VEE complex viruses, such as Mucambo, can cause febrile illness. All major human and equine VEE outbreaks have involved subtype IAB and IC strains of VEEV, which are capable of efficient equine amplification via the production of high-titered viremia (Figure 5). These equine-virulent strains apparently evolve periodically from equine-avirulent enzootic strains in subtype ID. Some outbreaks from the 1940s to

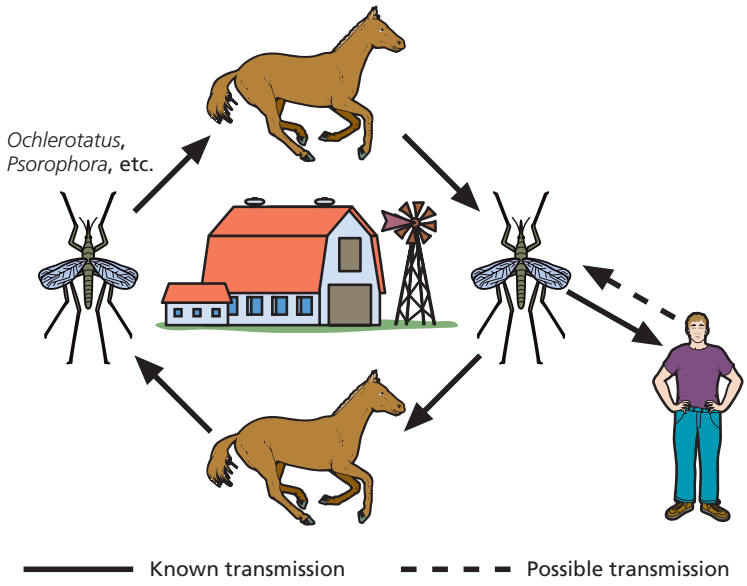

Figure 5 *Epizootic transmission cycle of Venezuelan equine encephalitis virus*

1973 may have been caused by the use of incompletely inactivated vaccines made from equine-virulent subtype IAB strains.

Enzootic strains (subtypes ID, and IE) of VEEV and all other members of the VEE complex occur in swamp or sylvatic habitats, ranging from southern Mexico to Argentina. These viruses are generally maintained in cycles involving forest-dwelling rodents and *Culex (Melanoconion)* spp. mosquito vectors. Occasional human cases or small outbreaks of human febrile illness occur when people come into contact with these enzootic cycles. The enzootic strains can also occasionally cause human encephalitis, and there may be no difference in human virulence between the epizootic (serotypes IAB and IC) and enzootic (serotypes ID and IE) VEEV strains.

In adults, VEEV infection typically leads to a nonspecific febrile illness accompanied by severe headache, fever, chills, myalgia, prostration, vomiting, diarrhea, and lymphopenia. Some patients, especially children, progress to neurological disease characterized by convulsions, disorientation, drowsiness, mental depression, seizures, and behavioral changes; permanent sequelae are common. Teratogenic effects and fetal death occur following infection in pregnant women. Infection is accompanied by diffuse congestion and edema, with hemorrhage in the brain, gastrointestinal tract, and lungs. Severe necrosis and vasculitis occur in lymph nodes, spleen, and the gastrointestinal tract, along with hepatocellular degeneration and interstitial pneumonia. During epidemics, overall mortality rates average about 0.5 percent. Although humans shed virus in the throat and are potentially infectious, epidemiological studies have failed to yield evidence of direct human–human transmission in households or healthcare settings.

Chikungunya

HISTORY AND DISTRIBUTION

Chikungunya virus (CHIKV) was first isolated from human patients and mosquitoes during an epidemic in the Newala district of Tanzania in 1952/53. The native name, which means 'that which bends up,' is derived from the main symptom of excruciating joint pains (Woodall 2001a; Jupp and McIntosh 1988). Chikungunya virus has frequently been isolated from humans and mosquitoes during epidemics in India, southeast Asia, southeast Africa and sub-Saharan Africa. The largest epidemics in recent years have been in India and Indonesia.

TRANSMISSION CYCLES

CHIKV is transmitted in Africa primarily by sylvatic *Aedes* spp., whereas *Ae. aegypti* transmits the virus in urban centres of India and southeast Asia. No vertebrate host other than humans has been discovered, although monkeys are probable reservoir hosts in Africa.

DISEASE AND VIRAL GENETICS

After an incubation period of 2–4 days there is a sudden onset of fever and crippling joint pains, which may incapacitate the patient, accompanied by chills, flushed face, headache, myalgias, backache, photophobia, and rash in ca. 80 percent of patients (Tsai et al. 2002). There may be conjunctival injection, and patients often have anorexia and constipation. The acute phase of illness lasts for 2–4 days, with recovery in 5–7 days; the fever may be biphasic. Arthralgia is the most striking sign or symptom, occurring in about 70 percent of cases. The areas affected include the metacarpophalangeal joints, wrists, elbows, shoulders, knees, ankles, and metatarsal

joints. Arthralgia may affect one or several joints. Reddening and swelling may occur, and arthralgias may persist for months in a small proportion of cases. The clinical picture resembles that of dengue fever, with which it is often confused. Differential diagnosis should include dengue, SINV, and West Nile virus.

In India and southeast Asia, Chikungunya virus has been implicated in outbreaks of hemorrhagic fever, often in association with dengue viruses. A careful review of the clinical presentation of CHIKV infection in Asia, however, suggests that, although mild hemorrhagic manifestations may occur in a small proportion of cases, Chikungunya is not a cause of severe hemorrhagic disease. Hemorrhagic complications have not been reported in association with Chikungunya infections in Africa. Phylogenetic studies and historical observations have suggested that CHIKV originated in Africa and spread to Asia from eastern Africa. Two distinct CHIKV lineages have been delineated: one contains all isolates from western Africa, and the second comprises all southern and East African strains, as well as isolates from Asia.

O'nyong-nyong

HISTORY AND DISTRIBUTION

This virus caused a major epidemic that began in Uganda in 1959 and quickly spread to Kenya, Sudan, Congo, Tanzania, Mozambique, and Malawi, affecting an estimated 2 million people and 90 percent of the population in affected areas (Woodall 2001b). A 1996 epidemic in Uganda affected more than 1 million people. The name O'nyong-nyong was given by the Acholi people and means 'joint breaker.' Igbo-ora virus, isolated in Nigeria, the Central African Republic, and the Ivory Coast during the 1960s, where it caused small outbreaks, is considered a genetic variant of O'nyong-nyong virus (ONNV). ONNV is closely related to CHIKV, and the diseases produced by the two agents are very similar. However, ONNV has a geographically limited distribution in Africa.

TRANSMISSION CYCLES

O'nyong-nyong virus is transmitted by *Anopheles gambiae* and *An. funestus* mosquitoes during outbreaks (it is the only anopheline-borne alphavirus), both of which also transmit malaria. The interepidemic maintenance cycle and reservoir hosts of ONNV have not been described, but the virus has been isolated from sentinel mice in Senegal in the absence of human disease (although human serosurveys indicated a prevalence of 55 percent there).

DISEASE AND VIRAL GENETICS

Infection with ONNV is clinically indistinguishable from that with CHIKV, and is accompanied by fever, chills, severe headache, myalgia, arthralgia, and the formation of a morbilliform rash. No human mortality has been reported.

Ross River fever

HISTORY AND DISTRIBUTION

Ross River virus (RRV) is the most common human arboviral pathogen in Australia, and over 38 000 human cases were reported between 1991 and 1998. Epidemics of benign polyarthritis have been recognized there since 1928, but the causative agent, RRV, was not isolated until 1959 from *Oc. vigilax* mosquitoes collected along the Ross River (Mackenzie 2001b; Kay and Aaskov 1988). Epidemics of polyarthritis involving several thousand cases have occurred regularly in Australia, and the virus is endemic in most coastal regions of the country, as well as inland, especially along waterways and rivers. Since the early 1980s RRV has possibly expanded its geographic distribution into the Pacific, with outbreaks in New Guinea, Fiji, American Samoa, Raratonga, Tonga, and New Caledonia.

TRANSMISSION CYCLES

The natural transmission cycle of RRV is not well understood. Serological surveys suggest that a diverse group of wild and domestic animals may be involved as reservoir hosts. Humans have significant viremia, and the virus in some epidemics has been maintained in a human–mosquito transmission cycle. In coastal regions, *Oc. vigilax* in the north and *Oc. camptorhynchus* in the south and west are important mosquito vectors. *Cx. annulirostris* is also considered an important vector in Australia. In the Pacific islands, *Ae. polynesiensis*, *Oc. vigilax* and *Ae. aegypti* are important vectors. Macropods (kangaroos) are believed to be the principal reservoir hosts in Australia, but other mammals, including flying foxes and rabbits, may also be involved.

DISEASE AND VIRAL GENETICS

Disease occurs most commonly in persons 20–50 years of age and is rare in children; women are affected more often than men. The incubation period is 5–21 (typically 7–9) days; patients with polyarthritis present with fever, arthralgia of the small joints of the hands and feet, myalgia, maculopapular or vesicular rash, paresthesiae of the palms and the soles, lethargy, and headache. Photophobia, anorexia, and lymphadenopathy may also occur. The painful arthritis frequently persists for several weeks, occasionally relapsing a year or more later, but recovery is complete and no fatalities have been reported. Subclinical infections also appear to be common. The persistence of IgM antibodies for several months after infection, and the detection of viral RNA in the synovial lining fluid by RT-PCR, suggest that replication may persist in joints, possibly associated with macrophages.

No vaccine is available. Prevention and control measures are directed at the mosquito population, and at health education on how to protect against mosquito bites. Two major RRV lineages have been identified phylogenetically, in eastern and western Australia, respectively; the eastern lineage caused the Pacific outbreaks in 1979–1980.

Other alphavirus infections

BARMAH FOREST VIRUS

First isolated in Australia from *Cx. annulirostris* mosquitoes, this virus accounts for about 10 percent of epidemic polyarthritis cases there and is second only to RRV as a human arboviral pathogen. The virus appears to be restricted to Australia, where it causes a disease characterized by fever, fatigue, arthritis, arthralgia, and rash that is clinically similar to that caused by RRV. Human infection with Barmah Forest virus (BFV) was first reported in 1986, and the first epidemic of human disease occurred in 1992 in the Northern Territory (Mackenzie 2001a). BFV cross-reacts with other alphaviruses, including RRV, by hemaglutination inhibition tests, but is distinct by complement fixation and neutralization tests.

Mammals, including marsupials, are believed to be the reservoir hosts and the virus has been isolated from a wide range of mosquito species: *Cx. annulirostris* appears to be the most widespread vector. In coastal regions, *Oc. vigilax* and *Oc. camptorynchus* are believed to be major vectors. Human infection occurs sporadically, with the majority of cases being reported from Queensland.

GETAH VIRUS

This virus was originally isolated in Malaysia from *Cx. gelidus* mosquitoes and *Cx. tritaeniorhynchus*. It is closely related to Sagiyama, Bebaru, and RR viruses, which are considered to be subtypes of Getah (Kumanomido 2001; Powers et al. 2001). Getah virus (GETV) is found throughout southeast Asia, including Japan, the Philippines, and Australia, but serological cross-reaction with RRV makes interpretation of serosurvey data difficult. GETV has not been associated with human disease, but causes severe encephalitis in horses in Japan. Antibodies have also been detected in humans, horses, and pigs, but the virus is not known to cause disease in the former two hosts. Pigs may serve as amplifying hosts during equine epizootics. GETV has been isolated from a wide variety of mosquito species, especially *Culex* spp. in Asia and Australia, and from *Aedes* spp. in Siberia.

MAYARO VIRUS

Mayaro virus (MAYV) is closely related to Chikungunya and O'nyong-nyong viruses and causes a similar illness characterized by fever, chills, headache, myalgia, and arthralgia, followed by a maculopapular rash. Arthralgia may persist for weeks or even months (Powers et al. 2001; Tesh et al. 1999). Isolated first in Trinidad from human serum in 1954, it was subsequently isolated from humans in Brazil, Colombia, French Guiana, Venezuela, Bolivia, Surinam, and Peru. Serological surveys also suggest that it occurs in Guyana, Panama, and Costa Rica. Little is known about the natural history of MAYV, but it has been isolated numerous times from *Haemagogus* spp. mosquitoes, suggesting a monkey–*Haemagogus* mosquito sylvatic transmission cycle. Evolutionary studies indicate that an ancestor of Una virus and MAYV, sister species, was introduced from the Old World, where all other members of the Semliki Forest antigenic complex occur (Powers et al. 2001).

SINDBIS VIRUS

Sindbis virus (SINV) has been designated as the prototype *Alphavirus*. It was first isolated in Egypt in 1953 but has a wide distribution in Africa, India, tropical Asia, Australia, and Europe (Griffin 2001b). Three major virus lineages have been identified by phylogenetic studies: a Paleoarctic/Ethiopian lineage, an Oriental/Australian lineage, and a distinct lineage from southwestern Australia. It rarely causes overt disease, but has been associated with febrile illnesses in Africa and India. SINV has a number of variants that have caused outbreaks of human illness in Sweden (Ockelbo disease), Finland (Pogosta disease), Russia (Karelian fever), and West and Central Africa (Babanki). Ockelbo, a SINV variant, causes fever characterized by rash. The disease is described under the names of Ockelbo disease in Scandinavia, Pogosta disease in Finland, and Karelian fever in the USSR. A single case with hemorrhagic manifestations and joint pains has been reported from Australia. SINV is maintained in enzootic cycles involving a variety of birds and mosquitoes of the genera *Culex*, *Aedes*, and *Culiseta*.

SEMLIKI FOREST VIRUS

Semliki forest virus (SFV) was isolated from *Aedes* mosquitoes collected in the course of yellow fever field studies in Uganda. Zingilamo (isolated from birds in the Central African Republic) and Me Tri (isolated from humans in Vietnam) viruses are considered strains of SFV. The virus has been used widely in laboratory studies and was regarded as nonpathogenic until it caused a single, fatal infection in a laboratory worker. Antibodies to SFV are commonly found in human serum collected from both East and West Africa, but no overt disease was recognized until 1987, when it caused a large outbreak of mild febrile illness in Bangui, Central African Republic. SFV may be maintained in a

monkey–*Aedes* mosquito cycle, and ticks may also be involved (Pfeffer 2001).

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Bunyaviridae

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PROPERTIES OF THE VIRUSES

Introduction

Viruses of the family *Bunyaviridae* are enveloped, single-stranded RNA viruses with a tripartite genome. Approximately 300 distinct viruses are included in five genera of the family: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*. Viruses of the same genus share structural, genetic, and antigenic characteristics, and often similar epidemiology. Many are arboviruses (arthropod-borne viruses), transmitted between vertebrate hosts (or plants in the case of tospoviruses) by blood-feeding mosquitoes, ticks, gnats, or thrips. A significant exception is the genus *Hantavirus*, whose known members are maintained in nature by chronically infected rodents, especially of the family *Muridae*, and are thought to be transmitted most commonly among rodents by bite and aerosol, and to humans by aerosol. Among the noteworthy hantaviruses are *Hantaan virus* and *Sin Nombre virus*, which were the first viruses known to cause hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome, respectively. Other human diseases caused by viruses within the family include California encephalitis and La Crosse encephalitis (*Orthobunyavirus*), sandfly fever and Rift Valley fever (*Phlebovirus*), Crimean–Congo hemorrhagic fever (*Nairovirus*), Oropouche fever (*Orthobunyavirus*), and others. Viruses of the genus *Tospovirus* are presently known only as plant pathogens. More than 360 plant species belonging to 50 families are known to be susceptible to infection with tospoviruses.

The history of viruses of the family *Bunyaviridae* parallels that of arbovirology. Before 1950, many viruses were investigated because of their ability to cause human disease. Thus, during the Second World War, much was discovered about sandfly fever viruses, because these viruses were responsible for significant morbidity among Allied troops stationed in the Middle East (Hertig and Sabin 1964). Sandfly (or phlebotomus) fever virus later became the prototype member of the genus *Phlebovirus*. A second complex of viruses destined to become a genus within the family also has links to military medicine. Viruses now known to be members of the genus *Hantavirus* played major roles in military campaigns in Asia, Yugoslavia, and Scandinavia during the Second World War, and again during the Korean conflict. Indeed, today these viruses continue to infect soldiers in the Balkans and elsewhere. Likewise, university researchers in California isolated the prototype strain of California encephalitis in the mid-1940s, setting the stage for the discovery of many other closely related viruses, including significant human pathogens like La Crosse virus. Investigations of the California antigenic group of bunyaviruses led to the discovery of many of the fundamental characteristics of the family: the pathogenic potential of some viruses, transovarial transmission as a method of epidemiological maintenance, and the molecular basis for our current understanding of the group (LeDuc 1987).

The type species of the family, *Bunyamwera virus*, was first isolated in Uganda from a pool of *Aedes* mosquitoes collected during studies of yellow fever (Smithburn et al. 1946). *Bunyamwera virus* was clearly unrelated either to *Yellow fever virus* or to any other arbovirus then known, but subsequent studies showed that it shares structural and biochemical properties with many other viruses that now make up the family (Smithburn

et al. 1946; Murphy et al. 1973; Bishop et al. 1980; Elliott 1990). In the 1950s, the Rockefeller Foundation founded a series of international research stations dedicated to the study of viral diseases of humans, and during this time many of the viruses now recognized as members of the family were first discovered. For example, the laboratory in Belem, Brazil, was the source of many viruses then grouped antigenically as the 'group C' arboviruses through the classic work of Clarke and Casals (group A viruses evolved to the genus *Alphavirus*, in the family *Togaviridae*, while the group B viruses have become the genus *Flavivirus* in the family *Flaviviridae*). The Bunyamwera, group C, *Guama*, *Simbu*, and other arboviruses were known for a time as the 'Bunyamwera supergroup,' then finally linked structurally and biochemically to form the genus *Bunyavirus*, family *Bunyaviridae*. Recently, the Bunyaviridae subcommittee of the International Committee of the Taxonomy of Viruses (ICTV) has proposed that the genus *Bunyavirus* be renamed *Orthobunyavirus* and this will be included in the Eighth Report of the ICTV due to be published in 2004. The Rockefeller Foundation-sponsored laboratories in Africa and India further contributed to the growing number of then known 'bunyaviruses'. Even today, newly recognized viruses isolated from arthropods or vertebrates are often found to be members of the family.

Classification

Viruses of the family *Bunyaviridae* are classified according to structural similarities, antigenic characteristics, common biochemical properties and, more recently, genetic homology. Five genera are now recognized, the largest being the genus *Orthobunyavirus*, with approximately 170 different viruses assigned to 18 groups (Table 1). The genus *Hantavirus* takes its name from *Hantaan virus*, the cause of hemorrhagic fever with renal syndrome. The hantaviruses are rapidly expanding in number, many 'new' viruses being identified by polymerase chain reaction (PCR) amplification of viral RNA obtained from field samples, but in several cases the actual viruses have yet to be isolated in a tractable form. At present, at least 26 serologically and/or genetically unique hantaviruses have been described, with many others almost certainly awaiting discovery (Table 2, p. 5). The genus *Nairovirus* is named after the virus of Nairobi sheep disease (Montgomery 1917), and contains more than 30 viruses in seven groups. The genus *Phlebovirus* has recently been expanded to include an earlier distinct genus, *Uukuvirus*, now merged with the phleboviruses on the basis of a common replication strategy and genetic similarities (Elliott et al. 1992). The most recent addition to the family is the genus *Tospovirus*, which takes its name from tomato spotted wilt virus. At present, this genus is limited to plant viruses and is transmitted by thrips (Thysanoptera). The *Tospovirus* genus encompasses at least 13 viruses, 11 of which are divided into two serogroups and two others that are serologically unrelated (Table 3, p. 6).

Within each genus, viruses were historically divided on serological characteristics into group, complex, virus, and subtype. 'Subtype' refers to viruses that can be serologically distinguished only with difficulty; named viruses can be easily distinguished; 'complex' refers to assemblies of viruses that are significantly cross-reactive; and 'group' or 'serogroup' encompasses complexes that are distantly related by serological activity (Peters and LeDuc 1991). More recent classification schemes have included the concept of a virus species, defined as a polythetic class of viruses. The interpretation of what constitutes a virus species is not consistent for each genus of the family, or among virus families in general.

Morphology and structure

Virions generally seem, by electron microscopy (EM), to be 80–120 nm spherical particles. Although pleomorphic particles are commonly observed by EM, such irregularity may be the result of desiccation, which occurs during specimen preparation, rather than inherent properties of viruses in the family (Hewlett and Chiu 1991). External features of the virions include a distinct bilaminar membrane, approximately 5 nm thick, and a fringe of surface projections approximately 5–10 nm long, comprised of the viral envelope glycoproteins, G1 and G2 (von Bonsdorff and Pettersson 1975; Hewlett and Chiu 1991) (Figure 1, p. 6). Recently, the *Bunyaviridae* subcommittee of the ICTV has proposed that G1 and G2 be renamed Gn and Gc due to difficulties in a common nomeclature across the different genera in the *Bunyaviridae*. As this nonemcalture has not been adopted yet, the glycoproteins will be termed G1 and G2 in this chapter. Because of differences in the G1 and G2 proteins of viruses in each genus, the characteristic appearance of virions varies. The surface area of the bunyavirus, La Crosse virus, was calculated to be able to accommodate from 4 700 to 12 000 tightly packed G1 and G2 proteins; however, estimates from EM studies suggest that only 270–1 400 proteins are present on a virion (Hewlett and Chiu 1991). The arrangement of the proteins, therefore, must include wide spacing between each heterodimer of G1 and G2. The most comprehensive study of surface structure and chemical composition for a virus in the family was performed with the phlebovirus Uukuniemi virus. The virion surface units of Uukuniemi virus appeared as penton–hexon clusters with a $T = 12$, $P = 3$ icosahedral surface lattice and with hexon–hexon distances of approximately 12.5–16 nm for stained viral particles and 17 nm for freeze-etched samples

Table 1 *Viruses of the* Bunyaviridae *known to cause disease in humans or domestic animals and their geographic distribution and principal vectors*

	Geographic distribution	Associated illness	Principal vector
Genus *Bunyavirus*			
Anopheles A group (12)			
Tacaiuma	S America	Human	Mosquitoes
Bunyamwera group (32)			
Bunyamwera	Africa	Human	Mosquitoes
Cache valley	N America	Human, sheep, cattle	Mosquitoes
Fort Sherman	C America	Human	Mosquitoes
Garissa	Africa	Human	Not known
Germiston	Africa	Human	Mosquitoes
Ilesha	Africa	Human	Mosquitoes
Kairi	S America	Equine	Mosquitoes
Main Drain	N America	Equine	Mosquitoes, culicoid flies
Shokwe	Africa	Human	Mosquitoes
Wyeomyia	S America	Human	Mosquitoes
Xingu	S America	Human	Mosquitoes
Bwamba group (2)			
Bwamba	Africa	Human	Mosquitoes
Pongola	Africa	Human	Mosquitoes
Group C (14)			
Apeu	S America	Human	Mosquitoes
Caraparu	S and N America	Human	Mosquitoes
Itaqui	S America	Human	Mosquitoes
Madrid	N America	Human	Mosquitoes
Marituba	S America	Human	Mosquitoes
Murutucu	S America	Human	Mosquitoes
Nepuyo	S and N America	Human	Mosquitoes
Oriboca	S America	Human	Mosquitoes
Ossa	N America	Human	Mosquitoes
Restan	S America	Human	Mosquitoes
California group (14)			
California encephalitis	N America	Human	Mosquitoes
Guaroa	S and N America	Human	Mosquitoes
Inkoo (Jamestown Canyon)	Europe, N America	Human	Mosquitoes
La Crosse	N America	Human	Mosquitoes
Snowshoe hare	N America	Human	Mosquitoes
Tahyna	Europe	Human	Mosquitoes
Guama group (12)			
Catu	S America	Human	Mosquitoes
Guama	S and N America	Human	Mosquitoes
Nyando group (2)			
Nyando	Africa	Human	Mosquitoes
Simbu group (24)			
Akabane	Africa, Asia, Australia	Cattle	Mosquitoes, culicoid flies
Ingwavuma	Africa, Asia	Pigs	Mosquitoes
Oropouche	S America	Human	Culicoid flies, mosquitoes
Genus *Hantavirus* (26)			
Andes	S America	Human	Rodent
Bayou	N America	Human	Rodent
Black Creek Canal	N America	Human	Rodent
Dobrava-Belgrade	Europe	Human	Rodent
Hantaan	Asia, Europe	Human	Rodent
Laguna negra	S America	Human	Rodent
New York	N America	Human	Rodent

(*Continued over*)

Table 1 *Viruses of the* Bunyaviridae *known to cause disease in humans or domestic animals and their geographic distribution and principal vectors (Continued)*

	Geographic distribution	Associated illness	Principal vector
Puumala	Europe, Asia	Human	Rodent
Seoul	Worldwide	Human	Rodent
Sin Nombre	N America	Human	Rodent
Genus *Nairovirus* (34)			
Crimean–Congo haemorrhagic fever (CCHF) group (3)			
CCHF	Africa, Asia, Europe	Human	Ticks
Hughes group (10)			
Hughes	N and S America	Sea birds	Ticks
Nairobi sheep disease group (2)			
Dugbe	Africa	Human, cattle	Ticks, culicoid flies, mosquitoes
Nairobi sheep disease	Africa, Asia	Human, cattle	Ticks, mosquitoes
Genus *Phlebovirus* (51)			
Sandfly fever group (23)			
Candiru complex			
Alenquer	S America	Human	?
Candiru	S America	Human	?
Punta Toro complex			
Punta Toro	N, S, and C America	Human	Phlebotomine flies
Rift Valley fever complex			
Rift Valley fever	Africa	Human, cattle	Mosquitoes
Sandfly fever Naples complex			
Sandfly fever Naples	Europe, Africa, Asia	Human	Phlebotomine flies
Toscana	Europe	Human	Phlebotomine flies
No complex assigned in sandfly fever group (16)			
Chagres	C America	Human	Phlebotomine flies
Sandfly fever Sicilian	Europe, Africa, Asia	Human	Phlebotomine flies
Uukuniemi group (12)			
St. Abbs head	Europe	Sea birds	Ticks
Grouped but unassigned viruses of *Bunyaviridae* (19)			
Bhanja group (3)			
Bhanja	Africa, Asia, Europe	Human	Ticks
Kasokero	Africa	Human	?
Yogue	Africa	Human	?
Ungrouped and unassigned viruses of *Bunyaviridae* (23)			
Bangui	Africa	Human	Mosquitoes
Issyk–Kul (Keterah)	Asia	Human	Mosquitoes, ticks
Tamdy	Asia	Human	Ticks
Tataguine	Africa	Human	Mosquitoes
Wanowrie	Africa, Asia	Human	Mosquitoes, ticks

Numbers in parenthesis indicate the total viruses within that taxon.

(von Bonsdorff and Pettersson 1975). The biochemical composition of Uukuniemi virus was estimated to be 2 percent RNA, 58 percent protein, 33 percent lipid, and 7 percent carbohydrate (Obijeski and Murphy 1977).

All viruses in the family have tripartite, single-stranded RNA genomes, with segments designated large (L), medium (M), and small (S). Each RNA is complexed with many copies of the viral nucleocapsid protein (N) to form three distinct ribonucleocapsids, which seem to be helical (von Bonsdorff et al. 1970; von Bonsdorff and Pettersson 1975). Terminal, complementary, nucleotide sequences are conserved on the L, M, and S segments for viruses within a particular genus and differ from those of viruses in other genera (Patterson et al. 1983; Collett et al. 1985; Schmaljohn et al. 1986a; Rönnholm and Pettersson 1987; Schmaljohn et al. 1987;

Table 2 *Serologically or genetically distinct viruses in the genus* Hantavirus

Virus	Abbreviation	Original source	Geographic distribution	Location of rodent host[a]	Human disease
***Murinae* subfamily-associated viruses**					
Hantaan	HTN	*Apodemus agrarius*	Korea	Asia, Europe	HFRS[b]
Seoul	SEO	*Rattus norvegicus*, *R. rattus*	Korea	Asia, Europe, the Americas	HFRS
Dobrava-Belgrade	DOB	*Apodemus flavicollis*	Slovenia	Europe, Middle East	HFRS
Thai-749	THAI	*Bandicota indica*	Thailand	Asia	unknown
***Arvicolinae* subfamily-assocated viruses**					
Puumala	PUU	*Clethrionomys glareolus*	Finland	Europe, Asia	HFRS
Prospect Hill	PH	*Microtus pennsylvanicus*	Maryland	N America	Unknown
Tula	TUL	*Microtus arvalis*	Russia	Europe	Unknown
Khabarovsk	KHA	*Microtus fortis*	Russia	Asia	Unknown
Topografov	TOP	*Lemmus sibiricus*	Siberia	Russia, Asia, N America	Unknown
Isla Vista	ISLA	*Microtus californicus*	California	N America	Unknown
***Sigmodontinae* subfamily-associated viruses**					
Sin Nombre	SN	*Peromyscus maniculatus*	New Mexico	N America	HPS[c]
New York	NY	*Peromyscus leucopus*	New York	N America	HPS
Black Creek Canal	BCC	*Sigmodon hispidus*	Florida	The Americas	HPS
Bayou	BAY	*Oryzomys palustris*	Louisiana	Southeastern	HPS
Andes	AND	*Oligoryzomys longicaudatus*	Argentina	S America	HPS
Caño Delgadito	CANO	*Sigmodon alstoni*	Venezuela	S America	Unknown
Rio Mamore	RIOM	*Olygoryzomys microtis*	Bolivia, Peru	S America	Unknown
Laguna negra	LAN	*Calomys laucha*	Paraguay	S America	HPS
Muleshoe[d]	MULE	*Sigmodon hispidus*	Texas	N America	Unknown
El Moro Canyon[d]	ELMC	*Reithrodontomys megalotis*	California	N America	Unknown
Rio Segundo[d]	RIOS	*Reithrodontomys mexicanus*	Costa Rica	Mexico, Central America	Unknown
Limestone Canyon	LSC	*Peromyscus boylii*	Arizona	N America	Unknown
Calabazo[d]		*Zygodontomys brevicauda*	Panama	S America	Unknown
Choclo[d]		*Oligoryzomys fulvesens*	Panama	S America	HPS
Araraquara[d]	ARA	*Bolomys lasiurus*	Brazil	S America	HPS
Juquitiba[d]		*Unknown*	Brazil		HPS
Castelo dos Sonhos	CAS	*Unknown*	Brazil		HPS
Maporal	MAP	*Oecomys bicolor*	Venezuela	S America	Unknown
Insectivore-associated virus					
Thottapalayam	TPM	*Suncus murinus*	India	Asia	Unknown
Other possible species and hosts (limited sequence; no other information available)					
Monongahela	MON	*Peromyscus maniculatus*	W Virginia	N America	HPS
Blue River	BR	*Peromyscus leucopus*	Oklahoma	N America	Unknown
Oran	ORN	*Oligoryzomys longicaudatus*	Argentina	S America	Unknown
Lechiguanas	LEC	*Oligoryzomys flavescens*	Argentina	S America	HPS
Bermejo	BER	*Oligoryzomys chacoensis*	Argentina	S America	Unknown
Maciel	MAC	*Bolomys obscurus*	Argentina	S America	Unknown
Pergamino	PER	*Akadon azarae*	Argentina	S America	Unknown

Data from Carey et al. (1971); Lee et al. (1978, 1982); Elwell et al. (1985); Schmaljohn et al. (1985, 1995); Avsic-Zupanc et al. (1992a, b); Elliot et al. (1994); Plyusnin et al. (1994, 1996); Song et al. (1994, 1995a, b); Rollin et al. (1995); Torrez-Martinez and Hjelle (1995); Torrez-Martinez et al. (1995); Lopez et al. (1996); Rawlings et al. (1996); Bharadwaj et al. (1997); Fulhorst et al. (1997); Johnson et al. (1997); Milazzo and Eyzaguirre (2002); Sanchez et al. (2001); Johnson and DeSouza (1999); Powers et al. (1999); Vincent et al. (2000).

a) Given as approximate distribution; many rodent species occur focally, many others have widespread distributions.

b) HFRS, haemorrhagic fever with renal syndrome.

c) HPS, hantavirus pulmonary syndrome.

d) Cell culture isolate has not been reported.

Table 3 *Serologically distinct viruses in the genus* Tospovirus

Genus *Tospovirus*	Abbreviation	Vector	Reference
Tomato spotted wilt group			
Tomato spotted wilt	TSWV	*F. occidentalis*, *F. schultzei*, *F. intonsa*, *F. fusca*, *F. bispinosa*, *T. tabaci*, *T. setosus*, *T. palmi*	de Haan et al. (1992a)
Groundnut ringspot	GRSV	*F. occidentalis*, *F. schultzei*, *F. intonsa*	de Ávila et al. (1993)
Tomato chlorotic spot	TCSV	*F. accidentalis F. schultzei*, *F. intonsa*	de Ávila et al. (1993)
Chrysanthemum stem necrosis	CSNV	*F. schultzei*, *F. occidentalis*	de Ávila et al. (1993)
Zucchini lethal chlorosis	ZLCV	*F. zucchini*	de Ávila et al. (1993)
Impatiens necrotic spot	INSV	*F. accidentalis*, *F. schultzei*, *F. intonsa*	Law et al. (1991)
Watermelon silver mottle group			
Watermelon silver mottle	WSMV	*T. palmi*	Yeh and Chang (1995)
Watermelon bud necrosis	WBNV	*T. palmi*	Jain et al. (1998)
Peanut bud necrosis	PBNV	*T. palmi*	Satyanarayana et al. (1996)
Melon yellow spot[a]	MYSV	*T. palmi*	Kato et al. (2000)
Iris yellow spot	IYSV	*T. tabaci*	Cortes et al. (1998)
Ungrouped			
Peanut yellow spot	PYSV	*S. dorsalis*	Satyanarayana et al. (1998)
Peanut chlorotic fan-spot	PCFV	*S. dorsalis*	Yeh et al. (1998)

a) Previously referred to as physalis severe mottle virus (Cortes et al. 1998, 2001).

de Haan et al. 1990, 1991; Elliott et al. 1991; Kormelink et al. 1992a) (Table 4). Base pairing of these RNAs to form panhandle structures (Figure 2, p. 8) can result in noncovalently closed circular RNAs and nucleocapsids (Pettersson and Bonsdorf 1975; Obijeski et al. 1976; Hewlett et al. 1977; Raju and Kolakofsky 1989) (see Figure 1).

At least one each of the L, M, and S ribonucleocapsids are required for viral infectivity. However, equal numbers of nucleocapsids may not always be packaged

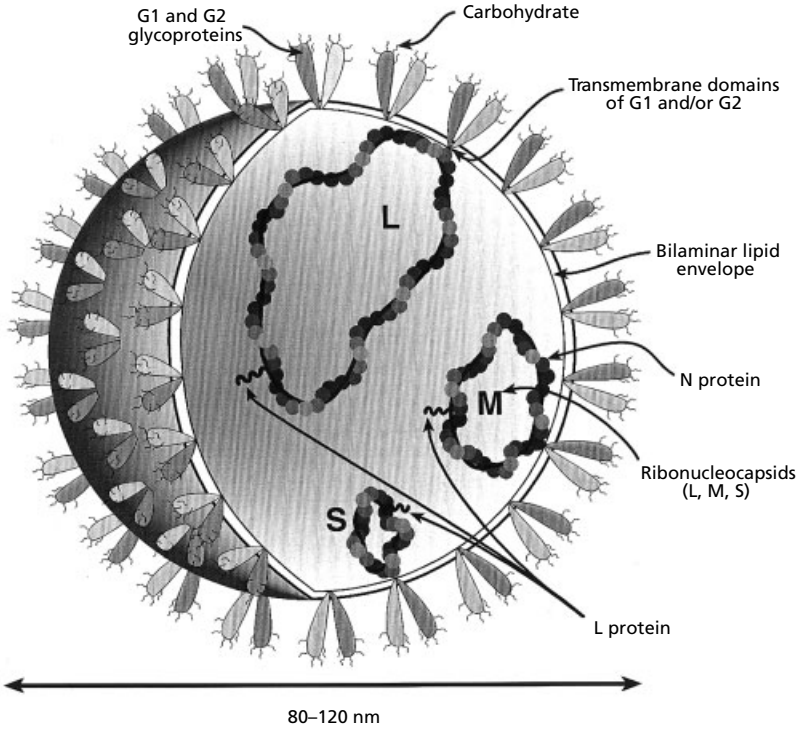

Figure 1 *Structure of a typical virus particle in the family* Bunyaviridae. *Individual components of the virus are described in the text.*

Table 4 *Consensus 3′ terminal nucleotide sequences of the L, M and S genome segments of representative members of the* Bunyaviridae

Genus	Virus	Genome segment	Gene size (nucleotides)	Nucleotide sequence[a]	GenBank No.
Orthobunyavirus	Bunyamwera	S	961	3′-UCAUCACAUGAGGUG	D00353
		M	4458	3′-UCAUCACAUGAUGGC	M11852
		L	6875	3′-UCAUCACAUGAGGAU	X14383
Hantavirus	Hantaan	S	1696	3′-AUCAUCAUCUGAGGG	M14626
		M	3616	3′-AUCAUCAUCUGAGGC	M14627
		L	6533	3′-AUCAUCAUCUGAGGG	X55901
Nairovirus	Dugbe	S	1712	3′-AGAGUUUCUGUUUGC	M25150
		M	4888	3′-AGAGUUUCUGUAUGG	M94133
		L	12255	3′-AGAGUUUCUGUAGUU	U15018
Phlebovirus	Rift Valley fever	S	1690	3′-UGUGUUUCGAGGGAUC	X53771
		M	3884	3′-UGUGUUUCUGCCACGU	M11157
		L	6404	3′-UGUGUUUCUGGCGGGU	X56464
Tospovirus	Tomato spotted wilt	S	2916	3′-UCUCGUUAGCACAGUU	D00645
		M	4821	3′-UCUCGUUAGUCACGUU	S48091
		L	8897	3′-UCUCGUUAGUCCAUUU	D10066

a) Sequences identical on all three genome segments are underlined.

in mature virions (Bishop and Shope 1979). Differing complements of ribonucleocapsids were suggested to be related to virion size differences noted by EM (Talmon et al. 1987). Varying amounts of virus complementary-sense RNAs (cRNAs) were also identified in virions, but the significance, if any, of this finding is not known (Raju and Kolakofsky 1989; Simons et al. 1990; Kormelink et al. 1992a).

In addition to N, G1, and G2, virions also contain a polymerase protein (L) that is needed to copy the negative-sense viral genome into messenger-sense RNA(s) (Ranki and Pettersson 1975; Bouloy and Hannoun 1976; Schmaljohn and Dalrymple 1983; Patterson and Kolakofsky 1984; Gerbaud et al. 1987a; Adkins et al. 1995) (see Figure 1). The exact placement of the L protein within virions is not known, but it is believed to be associated with each of the ribonucleocapsids.

Genome structure and function

The expression strategies of the L, M, and S segments of viruses in the five genera of the family *Bunyaviridae* include both similarities and marked differences (Figure 3, p. 9). All viruses encode their structural proteins (N, G1/G2, and L) in the S, M, and L cRNA, respectively. Viruses in each genus, except for those in the genus *Hantavirus*, also encode nonstructural proteins in their M and/or S segment vRNA or cRNA. The G1 and G2 proteins of all viruses are believed to be encoded as a polyprotein precursor that is cleaved cotranslationally, although direct evidence for this has been obtained only for phleboviruses (Ulmanen et al. 1981; Rönnholm and Pettersson 1987; Suzich and Collett 1988; Andersson et al. 1997a). The L segments of all viruses described to date encode only the L protein and have <200 nucleotides of noncoding information (Elliott 1989; Schmaljohn 1990; de Haan et al. 1991; Muller et al. 1991). There is no evidence for additional coding regions in either the L segment cRNA or the vRNA but see Nairoviruses below.

ORTHOBUNYAVIRUSES

Orthobunyaviruses produce both N and a nonstructural protein, NS_S, from overlapping reading frames found in the S segment cRNA (Gentsch and Bishop 1978; Cash et al. 1979; Bishop et al. 1982; Fuller and Bishop 1982; Akashi and Bishop 1983; Cabradilla et al. 1983; Fuller et al. 1983; Akashi et al. 1984; Bouloy et al. 1984; Elliott 1985; Gerbaud et al. 1987b). Only one S segment mRNA species was detected for the orthobunyaviruses; thus, a single mRNA must be used for translation of both N and NS_S (Bouloy et al. 1984; Patterson and Kolakofsky 1984; Eshita et al. 1985; Saeed et al. 2000). The orthobunyavirus M segment encodes an NS_M protein between coding information for G2 and G1 (Eshita and Bishop 1984; Lees et al. 1986; Grady et al. 1987; Fazakerly et al. 1988; Nakitare and Elliott 1993).

HANTAVIRUSES

Hantaviruses seem to have the simplest genome expression strategy of viruses in the family bunyaviridae, in that there is no clear evidence that nonstructural proteins are generated. The S segments of certain hantaviruses have an overlapping reading frame within N, which could encode a protein similar in size to that of the bunyavirus NS_S protein. However, such a protein has not yet been found (Parrington and Kang 1990;

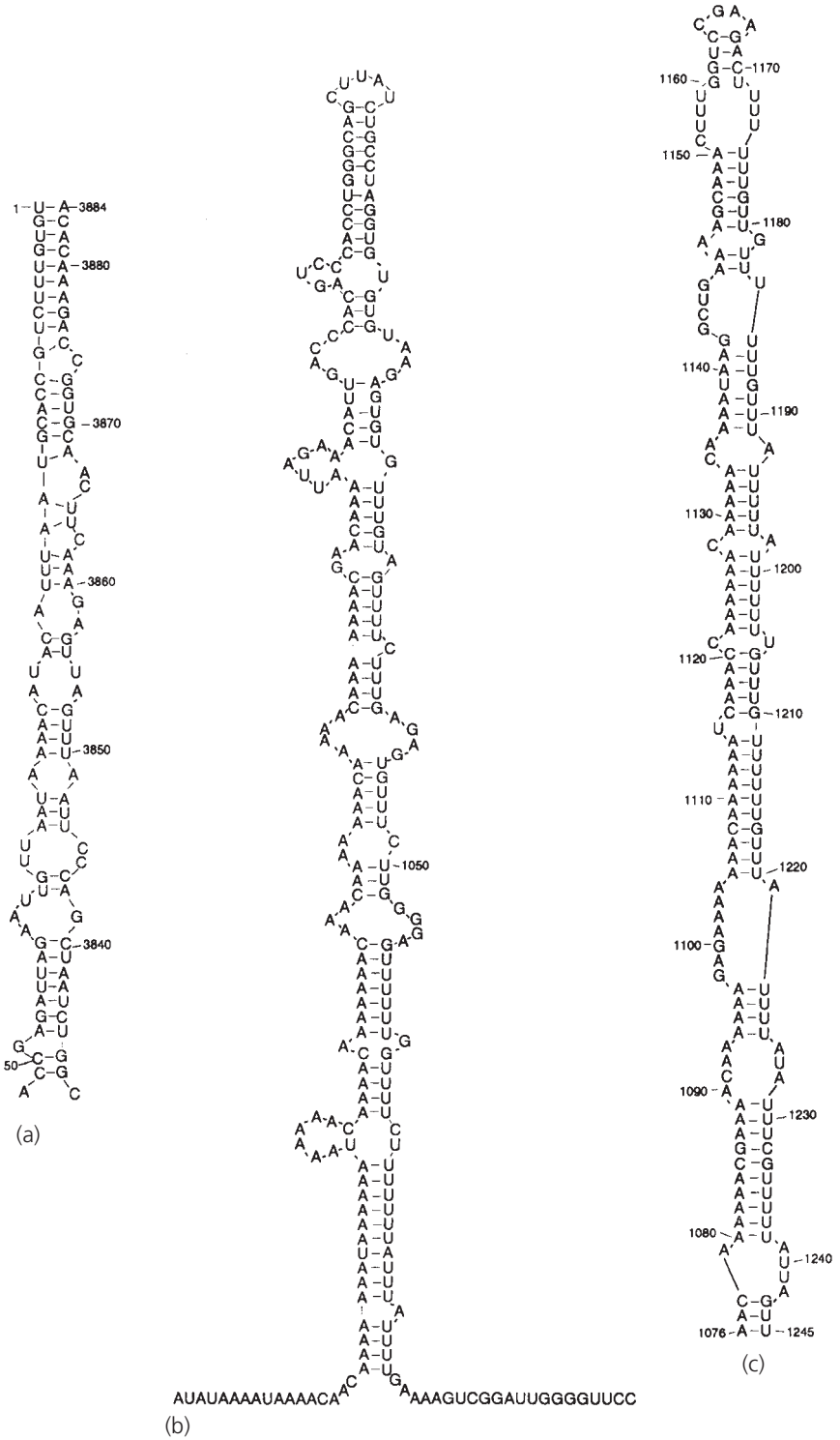

Figure 2 *Predicted panhandle structures formed by base-pairing of complementary sequences.* **(a)** *Terminal sequences of the M genome segment of the* Phlebovirus *Rift Valley fever virus (data from Collett et al. 1985).* **(b)** *Intergenic region of the ambisense S genome segment of the* Phlebovirus *Punta Toro virus (data from Emery 1987).* **(c)** *Intergenic region of the M genome segment of the* Tospovirus *Tomato spotted wilt virus (data from Kormelink et al. 1992b).*

Settergren et al. 1990; Stohwasser et al. 1990; Spiropoulou et al. 1994; Li et al. 1995). Certain of the New World hantaviruses have very large S segment 3′ noncoding regions; i.e. >700 bases for Sin Nombre virus as compared to <400 bases for prototype hantaan virus. Numerous repeated nucleotide sequences found in the

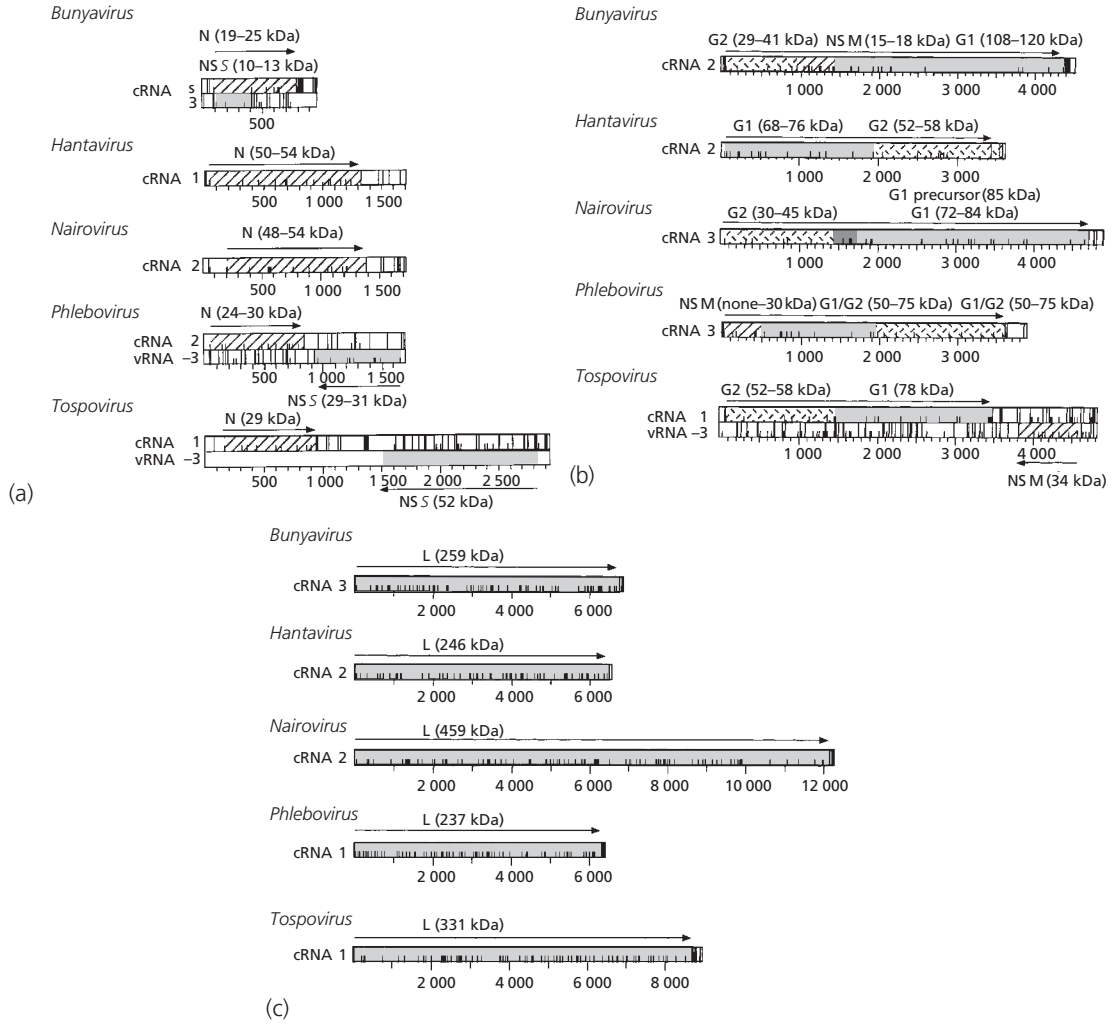

Figure 3 *Coding strategy and gene organization of the bunyaviruses.* **(a)** *S segment open reading frames encoding the N and NS$_S$ proteins are indicated by striped and stippled boxes, respectively.* **(b)** *M segment open reading frames encoding G1, G2, and NS$_M$ are indicated by stippled, cross-hatched and striped boxes, respectively.* **(c)** *L segment open reading frames encoding the L protein are indicated by stippled boxes. Translation initiation codons are represented by short hatch marks and stop codons are indicated by long hatch marks. The reading frame used, with respect to complementary sense RNA (cRNA, frames 1, 2, 3) or virus sense RNA (vRNA, frames –1, –2, –3) are listed to the left of the boxes. Nucleotide sequence numbers, with respect to the 5′ terminus of cRNA are shown below the boxes. Arrows indicate the direction of translation, 5′ to 3′, and predicted molecular weights of the gene products are shown above or below the arrows.*

Sin Nombre virus noncoding region are suggested to result from polymerase slippage, but a functional importance for their presence has not been discovered (Spiropoulou et al. 1994; Li et al. 1995; Morzunov et al. 1995; Ravkov et al. 1995). The M segments of hantaviruses have only a short potential intergenic region (e.g. $\leqslant$34 amino acids for Hantaan virus) between the sequences encoding G1 and G2, as well as little additional 5′ or 3′ noncoding information (Schmaljohn et al. 1987).

PHLEBOVIRUSES

Phleboviruses use an ambisense coding strategy to produce an NS_S polypeptide. That is, NS_S is encoded in vRNA in a reading frame that does not overlap the cRNA sequences encoding N (Ihara et al. 1984; Overton et al. 1987; Marriott et al. 1989; Simons et al. 1990; Giorgi et al. 1991). The existence of NS_S was verified in cells infected with numerous phleboviruses, including Uukuniemi virus, Rift Valley fever virus, Punta Toro virus, and Karimabad virus (Parker and Hewlett 1981; Ulmanen et al. 1981; Smith and Pifat 1982; Struthers and Swanepoel 1982; Overton et al. 1987). In vitro translation studies and northern blot analyses with viral RNAs demonstrated that N and NS_S are generated from separate, subgenomic messages (Ulmanen et al. 1981; Parker et al. 1984; Ihara et al. 1985b; Marriott et al. 1989; Simons et al. 1990). The M segment of some, but not all, phleboviruses encodes an NS_M in cRNA in the same reading frame but preceding the coding information for G1 and G2 (Collett et al. 1985). For example, the cRNA

open reading frame of Rift Valley fever virus has five in-frame translation initiation codons. Use of the first ATG resulted in production of uncleaved NS_M G2 as well as G1, whereas use of the second ATG resulted in distinct NS_M of approximately 14 kDa, G2 and G1 proteins (Collett et al. 1985; Suzich and Collett 1988; Kakach et al. 1989; Schmaljohn et al. 1989; Suzich et al. 1990). Both the NS_M G2 fusion protein and the NS_M protein are readily observed in cell cultures infected with Rift Valley fever virus. An even larger potential NS_M protein (approximately 30 kDa) with 13 inframe initiation codons is predicted from the M segment nucleotide sequence of Punta Toro virus. However, no polypeptide of this has been identified in cells infected with Punta Toro virus (Ihara et al. 1985a). There is no apparent homology between the Rift Valley fever virus and Punta Toro virus NS_M proteins, and both Rift Valley fever virus and Punta Toro virus G1 and G2 proteins can be efficiently produced in vitro in the absence of the NS_M coding information (Matsuoka et al. 1988; Suzich and Collett 1988; Suzich et al. 1990).

In contrast to those of Punta Toro virus and Rift Valley fever virus, the M segment of the phlebovirus, Uukuniemi virus, has no preglycoprotein coding region, and the amino terminus of G1 is located 17 amino acids downstream of the first (and only) initiation codon (Rönnholm and Pettersson 1987). Thus, Uukuniemi virus G1 seems to be similar to the Rift Valley fever virus NS_M G2 fusion protein. Cleavage of the G1/G2 precursor of Uukuniemi virus takes place cotranslationally in the endoplasmic reticulum, shortly after synthesis of G2 begins, and the G2 signal sequence remains attached to the carboxy terminus of G1 (Andersson et al. 1997a).

NAIROVIRUSES

The S segment of nairoviruses, like that of hantaviruses, encodes only a large N protein and has no known nonstructural protein coding information (Cash 1985; Ward et al. 1990; Marriott and Nuttall 1992). The M segment of the nairovirus, Dugbe virus, seems to encode only G2 and G1, although G1 was suggested to arise from post-translational cleavage of a slightly larger precursor (Marriott et al. 1992). The L segment of *Dugbe virus* is the largest gene of any member of the family bunyaviridae. Until recently it was not known why nairoviruses might need a polymerase protein almost twice the size of some of the other viruses in the family. Nucleotide sequencing of the L RNA of Crimean-Congo hemorrhagic fever virus revealed the segment is 12 164 nucleotides in length and encodes 3 944 amino acids. The amino terminus of the L protein encodes an ovarian tumorlike cysteine protease motif suggesting that the L polyprotein is autoproteolytically cleaved or involved in deubiquitination (Honig et al. 2004; Kinsella et al. 2004).

TOSPOVIRUSES

Both the S and the M segments of tospoviruses have ambisense coding strategies and separate subgenomic messages to generate structural and nonstructural proteins (de Haan et al. 1990; Kormelink et al. 1992a; Law et al. 1992). Unlike the NS_S of phleboviruses, which is about the same size as N, that of tospoviruses is approximately twice the size of N (Figure 3). Thus, the L segment of tospoviruses is the only one of the three gene segments that uses a strictly negative-sense coding strategy (de Haan et al. 1991).

Replication

ATTACHMENT AND ENTRY

One or both of the integral viral envelope proteins, G1 and G2, are believed to mediate attachment of virions to host-cell receptors. This was first demonstrated by proteolytic enzyme treatment of purified La Crosse virus, which resulted in 'spikeless' virus particles and a concomitant 5 $\log_{10}$ reduction in infectivity (Obijeski et al. 1976). For most viruses in the family, it is not known which of the envelope glycoproteins mediates attachment. To attempt to answer that question for bunyaviruses, La Crosse virus was treated with bromelain or Pronase, which degraded portions of G1, but left G2 uncleaved. Such treatment resulted in its complete loss of infectivity (Kingsford and Hill 1981). La Crosse virus G1 was also implicated as the attachment protein for mammalian cells because affinity-purified G1 protein could competitively inhibit attachment of radiolabeled G1 to Vero cells (Ludwig et al. 1991). In contrast, G2 did not bind to Vero cells, but did bind to cultured mosquito cells and midguts. To competitively inhibit binding of G2, both G1 and G2 were required, which led to the speculation that the G1 protein might be the viral attachment protein for vertebrates, whereas G2 is used for arthropod infection (Ludwig et al. 1991). Confirmation of this as a general feature of the bunyaviruses is needed. Although no similar information exists for viruses in other genera, there is evidence that neutralizing and hemagglutination-inhibiting (HI) sites are present on both the G1 and G2 proteins of phleboviruses (Keegan and Collett 1986; Pifat et al. 1988) and hantaviruses (Dantas et al. 1986; Arikawa et al. 1989), so it is possible that either or both of these proteins may be involved in attachment. Although cellular receptors have not been clearly identified for most viruses in the family, for two hantaviruses which cause hantavirus pulmonary syndrome, Sin Nombre virus and New York virus, integrins expressed on endothelial cells were implicated as specific receptors (Gavrilovskaya et al. 1998).

Fusion of infected cells at acidic pH has been reported for viruses in the *Bunyaviridae* (Gonzalez-Scarano et al. 1984; Arikawa et al. 1985; Gonzalez-Scarano 1985;

Gonzalez-Scarano et al. 1985). Such pH-dependent fusion is generally believed to relate to early events in internalization of viral particles. Electron microscopy of the infection process of Rift Valley fever virus revealed that virions seem to enter cells in phagocytic vacuoles (Ellis et al. 1988). This observation is consistent with a mode of entry similar to that first described for alphaviruses in which the virus is endocytosed in coated vesicles which subsequently become acidified (Marsh and Helenius 1980; Tycko and Maxfield 1982). The acidification triggers a fusion of viral membranes and endosomal membranes, and results in the release of the viral nucleocapsid into the cell cytoplasm. Whether one or both of the envelope proteins are necessary for fusion has not been determined for most members of the family. For the orthobunyavirus La Crosse virus, there is ample information to indicate that the G1 protein is involved in fusion. For example, it is known that monoclonal antibodies to G1 inhibit fusion, that G1 undergoes a conformational change at the pH of fusion and that a virus mutant with a defective G1 does not cause fusion (Gonzalez-Scarano 1985; Gonzalez-Scarano et al. 1985, 1987). Consistent with this, expression of G1 in the absence of G2 with recombinant vaccinia viruses resulted in cell fusion in vitro (Jacoby et al. 1993). These functions defined for the G1 and G2 proteins of bunyaviruses do not imply like functions for viruses in other genera.

TRANSCRIPTION

After uncoating of viral genomes, primary transcription of negative-sense vRNA to complementary mRNA occurs through interaction of the virion-associated polymerase (L protein) and the three viral RNA templates (Ranki and Pettersson 1975; Bouloy and Hannoun 1976). Transcription occurs entirely in the cytoplasm and there is no requirement for ongoing cellular transcription, as indicated by the resistance of viruses in the family to actinomycin D, an inhibitor of DNA-dependent RNA polymerases, such as host-cell DNA-dependent RNA polymerase II (Obijeski and Murphy 1977). Only the L and N proteins of Bunyamwera virus (Dunn et al. 1995) and Rift Valley fever virus (Lopez et al. 1995; Prehaud et al. 1997) were found to be necessary to reconstitute transcription in vitro. The L protein is believed to function first as an endonuclease to cleave capped oligonucleotides from host mRNAs to serve as transcription primers (Figure 4). This priming results in the presence of 5′ terminal extensions of approximately 10–18 heterogeneous nucleotides on the termini of viral mRNAs (Bishop et al. 1983; Bishop et al. 1984; Patterson and Kolakofsky 1984; Eshita et al. 1985; Ihara et al. 1985b; Collett 1986; Bouloy et al. 1990; Simons and Pettersson 1991; Kormelink et al. 1992c; Dobbs and Kang 1993; Jin and Elliott 1993a, b). For phleboviruses and tospoviruses, which have ambisense coding strategies, both the N and the NS_S subgenomic mRNA have heterogeneous, nonviral, 5′ terminal extensions (Ihara et al. 1985b; Simons and Pettersson 1991; Kormelink et al. 1992b).

Direct evidence for the presence of capped structures on the termini of the extensions was obtained by using anticap antibodies to immunoselect mRNAs (Hacker 1990; Vialat and Bouloy 1992). That the cleavage of capped oligonucleotides may not be an entirely random process is indicated by a preference for specific mono-, di- or trinucleotides at the −1 to −3 positions with respect to the 5′ terminus of mRNAs of particular viruses. For example, almost all cDNA clones of the S mRNA of the orthobunyavirus Germiston virus displayed U or G at the −1 position (Bouloy et al. 1990). Similarly, Bunyamwera virus preferred UG, but snowshoe hare virus was found to have A most commonly as the 3′ terminal nucleotide of the S mRNA extensions (Bishop et al. 1983; Eshita et al. 1985; Jin and Elliott 1993a). For phleboviruses, C was found most often at the –1 position of the M mRNA of Rift Valley fever virus and also at the –1 position of the N and NS_S mRNAs of Uukuniemi virus (Collett 1986; Simons and Pettersson 1991). For Hantaan virus, the preferred –1 nucleotide was G (Dobbs and Kang 1993; Garcin et al. 1995) and for the nairovirus Dugbe virus, C (Jin and Elliott 1993b). The reason for such preferences could imply that a restricted or specific subset of host mRNAs is used for primers, perhaps because of a need for limited base pairing with the viral genome.

Studies on the orthobunyaviruses Germiston virus and Bunyamwera virus revealed the insertion of U or GU between the primer and the 5′ terminal viral sequence. It was also noted that the 3′ most terminal nucleotides of the stolen host sequences were often similar to the 5′ terminal viral sequences, leading to the hypothesis that the insertions might arise from partial reiteration of the 5′ terminal sequences owing to a backward slippage of the viral polymerase after the first two or three nucleotides are transcribed (Vialat and Bouloy 1992; Jin and Elliott 1993a). In addition to such insertions, for orthobunyavirus and phlebovirus mRNAs, but not for the nairovirus mRNAs studied, the +1 nucleotide in the mRNA transcript is sometimes missing (Bishop et al. 1983; Bouloy et al. 1990; Simons and Pettersson 1991; Jin and Elliott 1993a, b), and for hantavirus RNAs the first three nucleotides are often absent (Dobbs and Kang 1993).These findings suggest that transcription initiation at nucleotides other than the terminal one may occur. For hantaviruses, a prime-and-realign-model for transcription was proposed which explains many of the experimental observations (Garcin et al. 1995). According to the model, priming by host oligonucleotides, with a terminal G residue would initiate transcription by aligning at the third nucleotide of the viral RNA template (C residue). After synthesis of a few oligonucleotides, the nascent RNA could realign by slipping backwards two nucleo-

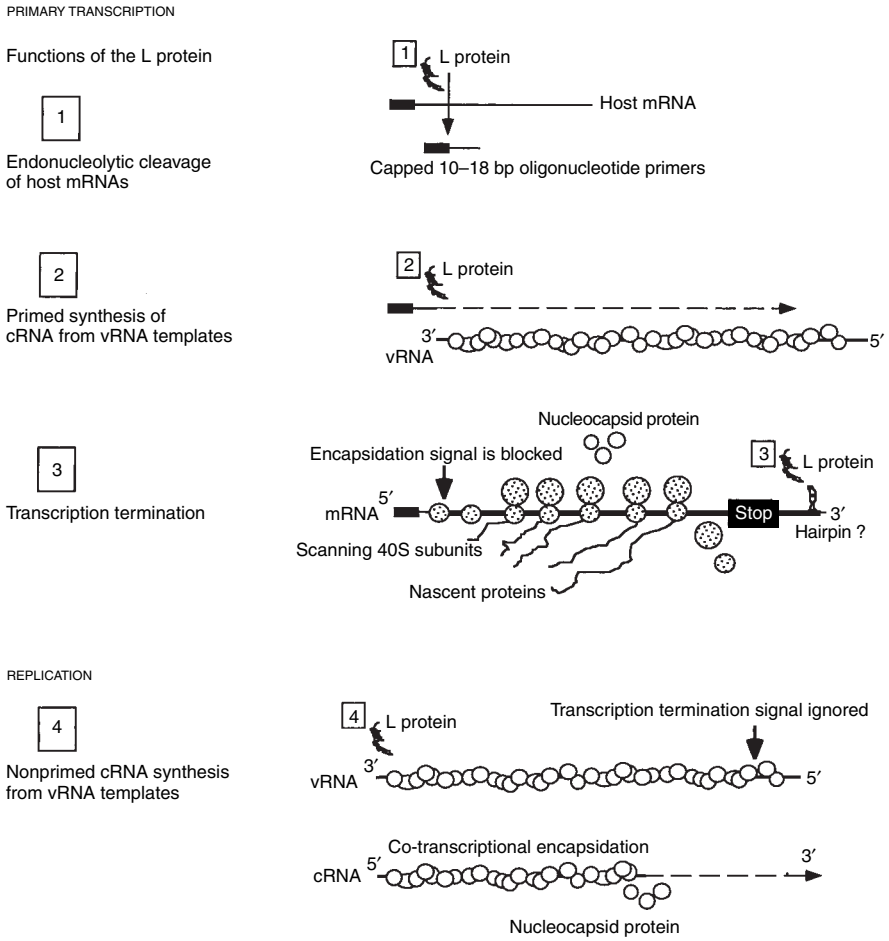

Figure 4 *Transcription and replication of the bunyaviruses. (1) The L protein cleaves capped oligonucleotides from host cell mRNAs. (2) The capped oligonucleotides are used to prime transcription of cRNA from vRNA templates. (3) Transcription is terminated at signals that may involve secondary structure or primary sequence of the vRNA template. mRNAs are not encapsidated, perhaps owing to the presence of scanning 40S ribosomes or the caps. (4) For replication to proceed, the L protein must use nonprimed synthesis and must ignore transcription termination signals to produce complete cRNA templates for further vRNA synthesis. The mechanism(s) of the switch from transcription to replication are not well defined.*

tides on the repeated terminal sequences (AUCAUCAUC), such that the G becomes the first nucleotide of the nontemplated, 5′ extensions. The frequent deletion of one or two of the triplet repeats in hantaviral mRNA supports this sort of slippage mechanism and suggests that sometimes the initial priming might start at the C residue of the third triplet in the conserved sequence rather than at the C of the second triplet. A prime-and-realign model was also suggested for transcription of hantaviral vRNA and cRNA, except that transcription is postulated to initiate with pppG alignment at the third nucleotide (C residue) of the template RNA. After synthesis of several nucleotides, the polymerase slips, allowing the nascent RNA to realign such that the initial priming G residue is overhanging. The L protein was suggested to act as an endonuclease to remove the G, leaving a monophosphorylated U residue at the nascent 5′ end. The presence of the monophosphorylated U on hantaan virus RNA was experimentally demonstrated (Garcin et al. 1995).

For the phlebovirus Rift Valley fever virus, a reconstituted transcription system was used to experimentally demonstrate that the minimal signal for transcription is the 13 3′-terminal nucleotides of the S segment vRNA or cRNA (Prehaud et al. 1997). One of the two repeated dinucleotides at the temini (UGUG) could be removed without deleterious effect on transcription, thus providing indirect support for the prime-and-realign model for phleboviruses, as well as hantaviruses. Whether this model holds for all viruses in the family remains to be determined.

At present, it is not clear what host functions are important for primary transcription of viruses in the family *Bunyaviridae*. Conflicting data concerning the need for ongoing host protein synthesis for primary transcription (as defined by the inability to achieve full length transcripts in the presence of drugs, such as cycloheximide and puromycin) were reported for viruses in the genus *Orthobunyavirus* (Abraham and Pattnaik 1983; Patterson and Kolakofsky 1984; Eshita et al. 1985;

Raju and Kolakofsky 1986, 1987a, 1989; Bellocq et al. 1987). If there is a need for ongoing protein synthesis, it may be at the level of ribosome scanning, rather than translation. This speculation was based on the finding that transcription was not inhibited by edeine, a drug that prevents 40S and 60S ribosomal subunits from complexing (Vialat and Bouloy 1992). These data are consistent with an earlier hypothesis that scanning ribosomes prevent premature termination by preventing base-pairing between the template RNA and the transcript, which would cause the polymerase to halt and terminate prematurely (Bellocq et al. 1987).

Termination of primary transcripts (i.e. mRNAs) occurs approximately 100 nucleotides from the 5′ terminus of the vRNA template, resulting in mRNAs that are truncated at their 3′ termini, as compared to cRNA (Figure 44) (Cash et al. 1979; Pattnaik and Abraham 1983; Bouloy et al. 1984; Patterson and Kolakofsky 1984; Eshita et al. 1985; Pettersson et al. 1985; Collett 1986; Raju and Kolakofsky 1987b; Bouloy et al. 1990). Potential transcription termination sites have been proposed for the S segments of La Crosse virus (Patterson and Kolakofsky 1984) and snowshoe hare virus (Eshita and Bishop 1984; Eshita et al. 1985) at or near the genomic sequence 3′-G/CUUUUU. Proposed U-rich transcription termination sites also are found on the M and S segments of Germiston virus (3′-AUGUUUUUGUU and 3′-GGGGUUUGUU, respectively) (Bouloy et al. 1990). Although such transcription termination sites are similar to those resulting in polyadenylation of mRNAs of other viruses, mRNAs of viruses in the family *Bunyaviridae* are not known to be polyadenylated (Ulmanen et al. 1981; Pattnaik and Abraham 1983; Pettersson et al. 1985). Moreover, transcription termination sites without the homopolymeric U_5 or U_6 tract have been proposed for the S and M segment mRNAs of another orthobunyavirus, snowshoe hare virus (5′-GGUGGGGGGUGGGG and 5′-GGUGGGGGGUGGGG, respectively) (Eshita and Bishop 1984) and the M segments of the phleboviruses Rift Valley fever virus and Punta Toro virus (5′-UGGGGUGGUGGGGU and 5′-GGUGAGAGUGUAGAAAG, respectively) (Collett 1986). Comparing the S segment sequences of a number of phleboviruses revealed that G-rich sequences and similar sequence motifs were found in the intergenic regions of some, but not all, phleboviruses (Giorgi et al. 1991).

Transcription termination of ambisense genes to yield structural and nonstructural protein mRNAs may occur through recognition of RNA secondary structure rather than a specific gene sequence. For example, the transcription termination sites for both the N and the NS_S mRNAs of Punta Toro virus were mapped to within 40 nucleotides of one another, and predicted hairpin structures were found in this intergenic region, as well as in the intergenic regions of the S segments of certain other phleboviruses and in both the S and the M segments of tospoviruses (de Haan et al. 1990; Kormelink et al. 1992b; Law et al. 1992) (see Figure 3). For the S segment of the phlebovirus Uukuniemi virus, although there is a noncoding region of 70 nucleotides between the N and the NS_S genes, hybridization studies demonstrated that the 3′ ends of the subgenomic messages overlap one another by about 100 nucleotides. Thus the 3′ end of the NS_S mRNA extends into the coding region of N and the 3′ end of the N mRNA terminates just before the coding sequences for N. A short palindromic sequence in the intergenic region (including the 3′ ends of each mRNA) is predicted to allow formation of an A/U-rich hairpin structure (Simons and Pettersson 1991). Such structures could affect transcription termination only if they can form while the genome is complexed with N. To date there is no direct evidence that this occurs.

Although the mechanism of transcription termination is not known, studies performed with the L protein of Bunyamwera virus expressed by vaccinia virus recombinants resulted infrequently in correct transcription termination, suggesting that an additional factor (or factors), of viral or cellular origin, may be required for consistent transcription termination (Jin and Elliott 1993a). One such factor might include interaction between the transcriptase and a newly synthesized viral protein, such as NS_S (Vialat and Bouloy 1992; Jin and Elliott 1993a). This could not be a familial characteristic, however, because hantaviruses and nairoviruses are not known to encode NS_S proteins. Also, for viruses using ambisense coding to produce NS_S, the kinetics of synthesis of N and NS_S, as well as the finding that only N mRNA can be detected in the presence of cycloheximide, suggest that replication of full-length, encapsidated cRNA must occur before synthesis of the NS_S mRNA. Thus, because NS_S appears relatively late in infection, it is unlikely to have a role in primary transcription, unless it can be generated in small amounts directly by translation of the vRNA (Simons et al. 1992).

GENOME REPLICATION

The switch from primary transcription to genome replication requires the L protein to switch from primed synthesis, by using capped host-cell oligonucleotides, to a process of independently initiating transcription at the precise 3′ end of the template and producing an encapsidated, full-length transcript. The factors dictating that vRNA and cRNA should complex with N to form nucleocapsid structures, while mRNAs should not, are not known, but it has been suggested that the added capped host cell sequences on the 5′ ends of viral messages may somehow prevent encapsidation (Raju and Kolakofsky 1989).

Unlike primary transcription, secondary transcription is clearly sensitive to inhibitors of protein synthesis, such

as cycloheximide. The need for ongoing protein synthesis for replication to proceed is likely to be due to the need for continued viral protein synthesis rather than host cell protein synthesis. Although the factors that dictate that the L protein should begin genome replication are not known for viruses in the family *Bunyaviridae*, a mechanism similar to that proposed for rhabdoviruses and paramyxoviruses seems plausible: accumulating a sufficient quantity of N seems to trigger encapsidation and to serve as an antitermination signal, thus allowing full-length genome synthesis (Blumberg et al. 1983; Wertz 1983; Patton et al. 1984a, 1984b; Arnheiter et al. 1985; Banerjee 1987; Vidal and Kolakofsky 1989). Nonstructural proteins were suggested to control the availability of the N protein of rhabdoviruses, and where available, they could possibly do the same for the *Bunyaviridae*. A model for transcription and replication based on information detailed above is presented in Figure 4.

SYNTHESIS AND PROCESSING OF VIRAL PROTEINS

S segment gene products

As described above (Orthobunyaviruses, Phleboviruses, Tospoviruses), the N and NS_S proteins encoded by the vRNA and cRNA of the S segments of bunyaviruses, phleboviruses, and tospoviruses do not originate from precursor polypeptides, and therefore processing of proteins is not required. Hantaviruses and nairoviruses encode only one product in their S segments. Post-translational modifications (e.g. phosphorylation or amidation) have not generally been defined for S segment products except for the NS_S of Rift Valley fever virus. For Rift Valley fever virus, the NS_S protein was reported to be phosphorylated (Parker et al. 1984) and to accumulate in the nuclei of infected cells where they seemed to aggregate as filamentous structures (Struthers and Swanepoel 1982). Although this is not known to be a general finding for phleboviruses, very few of them have been examined with immune sera specific for the NS_S protein. The NS_S of the tospovirus tomato spotted wilt virus was seen in association with fibrous structures in the cytoplasm of infected plants. However, it was not clear if the proteins actually formed the filaments or were just associated with them (Kormelink et al. 1991).

The NS_S protein has been found to function as an interferon antagonist and also inhibit host-cell protein synthesis (Bouloy et al. 2001; Bridgen et al. 2001). A detailed description of its function can be found in the Antigens, immune responses, and virulence section below.

M segment gene products

Historically, the designation of glycoproteins G1 and G2 comes from their relative migrations when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). G1 is the more slowly migrating polypeptide. These designations, based on apparent polypeptide size, do not always correlate with the gene order, structure or function of G1 and G2. For example, for the two phleboviruses Rift Valley fever virus and Punta Toro virus, it is known that G1 of Rift Valley fever virus is related to G2 of Punta Toro virus and vice versa (see Figure 3). Thus, the terms Gn and Gc have been proposed by the *Bunyaviridae* subcommittee of the ICTV to overcome problems with nomenclature, but have yet to be universally adopted.

Cotranslational cleavage of G1 and G2 (and in the *Bunyavirus* and *Phlebovirus* genera, NS_M) is believed to be mediated by a cellular signalase-like enzyme. Except for the *Nairovirus* Dugbe virus, which may encode G1 as a precursor, all predicted polyprotein precursors have hydrophobic regions preceding both G1 and G2 that are consistent with signal sequences that allow transport of the nascent proteins through membranes. The precursors also have variable numbers of potential transmembrane regions, and a hydrophobic sequence at the carboxy terminus, indicative of a membrane anchor region (Eshita and Bishop 1984; Collett et al. 1985; Schmaljohn et al. 1987; Fazakerley and Ross 1989; Law et al. 1992; Marriott et al. 1992). Thus, these proteins are assumed to be typical class 1 membrane proteins, i.e. with the amino terminus exposed on the surface of the virion and the C terminus anchored in the membrane.

A common property of all M segment gene products predicted from cDNA sequences studied so far is their high cysteine content (4–7 percent) and, in related viruses, the conservation of the position of the cysteine residues (Ihara et al. 1985a; Lees et al. 1986; Grady et al. 1987; Rönnholm and Pettersson 1987; Antic et al. 1992b; Kormelink et al. 1992b; Law et al. 1992; Marriott et al. 1992). These findings suggest that extensive disulfide bridge formation may occur and that the positions may be crucial for determining correct polypeptide folding. The secondary structure of the proteins can also play a role in immunogenicity, in that neutralizing or protective epitopes are often conformational and nonlinear (Battles and Dalrymple 1988; Wang et al. 1993).

All of the envelope proteins examined to date possess *N*-linked oligosaccharides. The gene sequences that define a potential *N*-linked glycosylation site are those that encode Asn–X–Ser/Thr, where X is not Pro (Hubbard and Ivatt 1981). The number of potential glycosylation sites found in the predicted M segment gene product range from four sites for certain hantaviruses to nine sites for some tospoviruses (Antic et al. 1992b; Law et al. 1992). The number of sites actually used in mature virion proteins has not been defined for most viruses. However, G2 of the phlebovirus Rift Valley fever virus was demonstrated to be glycosylated at its single available site and at least three of four possible G1 sites (Kakach et al. 1989). Analysis of the

predicted amino acid sequences of the envelope proteins of the bunyavirus snowshoe hare virus (Eshita and Bishop 1984; Fazakerly et al. 1988) and examination of glycosylated tryptic oligopeptides (Vorndam and Trent 1979) suggest that all three potential glycosylation sites on G2 are used and at least one of two sites on G1. The single glycosylation site available in the G2 protein of hantaan virus is used, and this site is conserved among numerous other hantaviruses (Schmaljohn et al. 1986b; Antic et al. 1992a). Where studied, the oligosaccharides attached to the G1 and G2 proteins were mostly high-mannose glycans, although complex and novel intermediate-type oligosaccharides were also identified on the G1 protein of Uukuniemi virus (Madoff and Lenard 1982; Pesonen et al. 1982; Schmaljohn et al. 1986b; Antic et al. 1992a; Ruusala et al. 1992).

Although not studied extensively, the NS_M proteins of the bunyavirus Bunyamwera virus and the phlebovirus Rift Valley fever virus were found not to be glycosylated (Lees et al. 1986; Collett et al. 1989; Kakach et al. 1989). For Rift Valley fever virus, however, another M segment gene product consisting of an uncleaved form of the NS_M protein and G2 is also routinely generated, and the NS_M portion of that fusion protein is glycosylated (Kakach et al. 1989). The NS_M protein of tospoviruses is not a membrane protein. It is encoded in the ambisense and is translated from a subgenomic message. Because this protein has no counterpart in any of the other genera of the family, it was suggested to be a genetic adaption of these viruses for survival in plant hosts (Kormelink et al. 1994; Storms et al. 1995).

L segment gene products

No post-translational modifications of L proteins for viruses in the *Bunyaviridae* have been described. The L protein is a multifunctional enzyme. For Bunyamwera virus and Rift Valley fever virus, the L and N proteins are essential and sufficient for transcriptase activity (Dunn et al. 1995; Lopez et al. 1995). The Bunyamwera virus L protein transcribes a synthetic template in concert with the N proteins of closely related bunyaviruses, but only inefficiently with more dissimilar orthobunyavirus N proteins (Dunn et al. 1995). The complementary terminal nucleotide sequences of viruses in the family *Bunyaviridae* that are predicted to form panhandle structures have been suggested as possible polymerase recognition structures. Although only limited information exists, in one study a point mutation resulting in disruption of the base-paired structure was found to drastically reduce Bunyamwera virus L transcriptase activity in vitro (Dunn et al. 1995).

Comparison of L protein sequences of viruses in the family *Bunyaviridae* with other RNA polymerases revealed the presence of four conserved 'polymerase motifs' (Poch et al. 1989; Jin and Elliott 1992). Mutational analysis of cDNA representing Bunyamwera virus L within these conserved regions was used to demonstrate that amino acid residues that are highly conserved among viruses in the family could not be changed, but that changes in nonconserved amino acids did not generally reduce the ability of the expressed L protein to transcribe authentic viral nucleocapsid templates in an in vitro assay (Jin and Elliott 1992; Dunn et al. 1995).

TRANSPORT

One of the earliest notable features found to distinguish bunyaviruses from all other negative-strand RNA viruses was that the viral particles are formed intracellularly by a budding process at smooth surface vesicles in the Golgi area (von Bonsdorff et al. 1970; Lyons and Heyduk 1973; Murphy et al. 1973; Bishop and Shope 1979). Recent studies with Uukuniemi virus indicated that viruses mature not only in the Golgi stacks, but also in the pre-Golgi intermediate compartment (Jäntti and Hilden 1997). Because the virus particles are too large to be transported in the 60-nm vesicles postulated to function in the intra-Golgi transport network, it was suggested that the Golgi cisternae themselves may be involved in virion transport (Jäntti and Hilden 1997). The site of virus maturation, be it either the intermediate compartment or the Golgi stacks, is at least partly dictated by the accumulation and retention of a sufficient concentration of the viral envelope proteins. The factors involved in Golgi transport and retention have been studied for viruses in the genera *Phlebovirus*, *Hantavirus*, and *Orthobunyavirus*. For most of these viruses, it appears that dimerization of the two envelope proteins in the endoplasmic reticulum must occur before they are transported to the Golgi. For Punta Toro virus, kinetic studies indicated that heterodimerization occurs between newly synthesized G1 and G2 within 3 min after protein synthesis and that the dimers are linked by disulfide bonds (Chen and Compans 1991).

For phleboviruses, orthobunyaviruses, and some hantaviruses, if dimerization does not occur, then only one protein can move to the Golgi (Matsuoka et al. 1988, 1991; Chen and Compans 1991; Rönnholm 1992; Ruusala et al. 1992). For example, in the absence of G1, G2 of Punta Toro virus was transported out of the ER and expressed on the cell surface. Removal of the carboxy-terminal anchor sequence of G1 resulted in secretion of this protein. When both proteins were expressed, however, both G1 and G2 were found in the Golgi. These studies suggest that the Golgi transport and retention signal is located on the G1 protein of Punta Toro virus (Chen et al. 1991). Similar results were obtained with the phlebovirus Uukuniemi virus, in that expression of G1 and G2 independently in COS cells resulted in translocation of only G1 to the Golgi, while G2 remained in the ER, suggesting that the signal for transport resides on G1 and that G2 is transported only by association with G1 (Rönnholm 1992).

Conclusive evidence that the region of the cytoplasmic tail adjacent to the transmembrane domain is the Golgi retention signal for phleboviruses was obtained by expression of truncated and chimeric G1 proteins of Punta Toro (Matsuoka et al. 1994, 1996) and Uukuniemi viruses (Andersson et al. 1997b). In these studies, deleting or replacing the cytoplasmic tail of G1 resulted in cell surface expression, rather than Golgi retention. Also, addition of the cytoplasmic domain to proteins normally expressed at the plasma membrane effected Golgi retention. For Punta Toro virus, the retention signal was found to be located within the first ten amino acids of the cytoplasmic domain (Matsuoka et al. 1996). Similar studies have yet to be reported for viruses in other genera of the *Bunyaviridae*.

Although Golgi retention is the norm for G1 and G2 proteins of viruses in the family, some viruses mature at cell surface membranes. For two New World hantaviruses, Sin Nombre virus and Black Creek Canal virus, envelope glycoproteins were detected on the cell surface, and virus budding from the plasma membrane was observed by electron microscopy (Goldsmith et al. 1995; Ravkov et al. 1997). Likewise, even though the phlebovirus Rift Valley fever virus matures in the Golgi in continuous cell lines, in primary rat hepatocytes budding was observed at the plasma membrane in addition to Golgi membranes (Anderson and Smith 1987). The molecular basis for these observations has not yet been determined.

For bunyaviruses and phleboviruses, which express an NS_M protein in addition to G1 and G2, all three proteins localize in the Golgi. However, NS_M is not needed for transport, as indicated by targeting of G1 and G2 to the Golgi even when they were expressed from an M segment from which the NS_M coding region had been deleted (Matsuoka et al. 1988; Wasmoen et al. 1988; Lappin et al. 1994).

The transport of tospovirus proteins within plant cells and insect cells has not been studied extensively. These viruses replicate in insects (Ullman et al. 1993; Wijkamp et al. 1993), as well as plants. Therefore, in addition to possessing the transport mechanisms described for other arthropod-borne viruses in this family, tospoviruses must also be able to traverse the plant cell wall. For tomato spotted wilt virus, the NS_M protein was found to be associated with nucleocapsid aggregates and was postulated to be involved in cell-to-cell movement of nonenveloped ribonucleocapsids (Kormelink et al. 1994). The NS_M protein was further shown to assemble into tubule-like structures that were postulated to penetrate the plasmodesmata, allowing transport of the free, nonenveloped ribonucleocapsids from cell to cell (Storms et al. 1995). The association of NS_M only with nucleocapsids, but never with mature enveloped particles (Kormelink et al. 1994) supports this hypothesis. Thus it is likely that the G1 and G2 proteins (and envelope) of tospoviruses are required for replication in their insect vectors, but not in plants (Verkleij and Peters 1983; de Resende et al. 1991).

PROTEIN INTERACTIONS IN VIRUS ASSEMBLY AND RELEASE

Unlike viruses in families such as the *Rhabdoviridae*, *Orthomyxoviridae*, and *Paramyxoviridae*, which utilize their matrix (M) protein to act as a nucleating step for assembly and to bridge the gap between the integral viral envelope proteins and their nucleocapsids, bunyaviruses that do not have an M protein must rely on direct interaction of the envelope proteins and ribonucleocapsids to trigger assembly (Smith and Pifat 1982). Because viral ribonucleocapsids and spike structures have been observed only on portions of Golgi vesicle membranes directly involved in the budding process and not on adjacent areas of the same membranes, it is likely that some sort of transmembranal recognition between the viral glycoproteins and the N protein is required for budding. Direct evidence for transmembrane regions of G1 and G2 was obtained by enzymatic digestion of exposed proteins of the phlebovirus Karimabad. In this study, it was determined that approximately 12 percent of G1 or G2, or both, was exposed on the cytoplasmic face of membranes in infected cells and was accessible to digestion. A large protease-resistant fragment was identified, which was presumably sequestered in the membrane in a manner that rendered it safe from enzymatic digestion (Smith and Pifat 1982). Candidate transmembrane regions have been predicted from hydropathic characteristics of derived amino acid sequences representing the envelope proteins of all members of the *Bunyaviridae* examined to date. For Uukuniemi virus, it has been suggested that the cytoplasmic tail of G1 (which is at least 70 residues long) is a logical candidate for interaction with the nucleocapsids (Rönnholm and Pettersson 1987; Pettersson 1991).

This cytoplasmic tail was found to be palmitylated. Although removal of palmitylation sites (cysteine residues) had no effect on the transport of G1 to the Golgi, it is conceivable that such fatty acid acylation of the cytoplasmic domain could play a role in virus assembly, perhaps by facilitating interaction with N, but this remains to be experimentally determined (Andersson et al. 1997b).

It is believed that, after the particles bud into the Golgi cisternae, they are released in individual small vesicles in a manner analogous to secretory granules of other cell types (Broadwell and Oliver 1981; Rothman 1981; Smith and Pifat 1982). The release of virus from infected cells presumably occurs when the cytoplasmic, virus-containing vesicles fuse with the cellular plasma membrane, i.e. by normal exocytosis.

Host factors involved in transport of viral proteins have not been clearly defined. However, for Black Creek Canal virus, an interaction of nucleocapsid protein with cellular actin filaments was identified and

was suggested to play a role in virus assembly or release (Ravkov et al. 1998). Because this virus is atypical of most other viruses in the family (i.e. it buds at the plasma membrane instead of in the Golgi), it is not known if this finding will prove relevant to other members of the *Bunyaviridae*.

The morphogenesis of tospoviruses in their insect vector, thrips, seems to be similar to that observed with other viruses in the family (Wijkamp et al. 1993). Differences, however, are seen in plants and plant cells. As indicated above, it is likely that the tospovirus envelope is needed only for replication in insects, and not in plants. Nonenveloped tospovirus particles are commonly seen in infected plants (Kitajima et al. 1992); defective tomato spotted wilt virus isolates, which are defective in glycoprotein synthesis and lack the lipid envelope, were found to retain the ability to spread through plant tissues at the same rate as nondefective viruses (Verkleij and Peters 1983; de Resende et al. 1991). These findings, as well as the small pore size (approximately 3 nm) of plants' plasmodesmata, suggest that infectious ribonucleocapsids, rather than complete tospovirus virions, are transported through the plant plasmodesmata (Kormelink et al. 1994).

Antigens, immune responses, and virulence

The major viral antigens are the structural proteins, N, G1, and G2. Generally, the N protein is highly antigenic, and is usually the prominent antigen detected in serological assays such as complement fixation and ELISA of infected cell-culture lysates. The G1 and G2 proteins are the basis for serological assays such as hemagglutination inhibition and plaque-reduction neutralization.

For all animal-infecting bunyaviruses, the envelope glycoproteins, G1 and G2, are presumed to be the major antigens involved in induction of immunity. Three lines of evidence support this assumption: (1) monoclonal antibodies to G1 or G2 or both, but not to N, can neutralize viral infectivity in vitro; (2) passive transfer of neutralizing, but not nonneutralizing, monoclonal antibodies or monospecific polyclonal sera to G1 or G2 or both, protects animals from challenge with homologous viruses; and (3) immunization of animals with recombinant viruses expressing G1 or G2 (or both) results in protective immunity (Dantas et al. 1986; Collett et al. 1987; Pifat et al. 1988; Arikawa et al. 1989, 1992; Dalrymple et al. 1989; Schmaljohn et al. 1990; Pekosz et al. 1995). For some viruses, such as the phlebovirus Rift Valley fever virus, although monoclonal antibodies to both G1 and G2 will neutralize virus in cell culture, only those specific for G2 were able to passively protect animals. Passively transferred polyclonal, monospecific sera to G2 were also found to be protective, but sera to G1 were not (Dalrymple et al. 1981, 1989; Keegan and Collett 1986; Smith et al. 1987). This finding was substantiated in mice immunized with recombinant vaccinia or baculoviruses expressing either G1 or G2, in that only the G2 recombinants induced a protective immune response in the animals (Dalrymple et al. 1989; Schmaljohn et al. 1989). These findings suggest that a humoral response alone to one or both of the envelope glycoproteins may be sufficient for protection from infection with viruses in the family.

Little information is available concerning the importance of cell-mediated immune responses to members of the *Bunyaviridae*. For the hantavirus, Hantaan virus-specific cytotoxic T lymphocytes (CTL) were demonstrated after restimulation of immune mouse lymphocytes with Haantan virus antigen in vitro. The CTL response was also suggested to be involved in cross-reactive protection among hantaviruses. Evidence for this was obtained by restimulating, with Hantaan virus, spleen cells from mice immunized with heterologous hantaviruses. In one instance of cross-protection (i.e. immunization with Puumala virus and protection against Hantaan virus), evidence suggested that T cells may be particularly relevant (Asada et al. 1987, 1988, 1989). A non-neutralizing, presumbably cell-mediated, protective immune response was also demonstrated in hamsters immunized with baculovirus-expressed Hantaan virus and challenged with virulent Hantaan virus (Schmaljohn et al. 1990).

Recent studies have investigated the early immune response to bunyavirus infection. The type I interferon system, that includes protein kinase R (PKR), 2′–5′ oligoadenylate synthetase/RNase L and Mx proteins, has been shown to be important for antiviral activity by blocking transcription, translation, and replication of viral RNA, including sequestion of the N protein by the Mx protein (Frese et al. 1996; Kanerva et al. 1996; Jin et al. 2001; Kochs et al. 2002; Bridgen et al. 2004) and interferon knockout mice are susceptible to La Crosse virus infection, whereas wild-type mice are not (Hefti et al. 1999). Investigation of the function of the NSs protein of phleboviruses and orthobunyaviruses has shown that this viral protein is an interferon antagonist and has a critical function of suppressing the production of interferon in virus-infected cells. Initial studies with Rift Valley fever virus showed that a strain containing a deletion in the NSs gene was attenuated and a good interferon inducer, while wild-type virus was virulent and a poor interferon inducer (Vialat et al. 2000; Bouloy et al. 2001). Thus, NSs is an important virulence factor for members of the *Bunyaviridae*. Subsequent studies using reverse genetics of Bunyamwera virus showed that mutation of NSs resulted in a virus that induced high levels of interferon, whereas wild-type virus did not (Bridgen et al. 2001; Weber et al. 2002). These studies also suggested a role for NSs in the shutoff of host-cell protein synthesis that has been substantiated by Colon-Ramos et al. (2003) who have shown the

orthobunyavirus NSs has sequence and functional activities similar to Reaper, a proapoptotic protein from *Drosophila*, including inhibition of host-cell protein synthesis. Investigation of the mechanism of action of NSs shows that NSs blocks an early step in interferon induction, namely activation of interferon transcription factors NF-κB and interferon regulatory factor 3 (IRF-3) by double-stranded RNA (dsRNA) (Weber et al. 2000; Weber et al. 2002). Kohl et al. (2003) showed that virus lacking a functional NSs induces apoptotic cell death more rapidly than wild-type virus. Although both wild-type and NSs mutant viruses activated proapoptotic transcription factor IRF-3, only wild-type virus suppressed signaling downstream of IRF-3, thus delaying cell death via IFR-3-mediated apoptosis. However, although Bunyamwera virus NSs blocks induction of interferon by dsRNA, unlike other interferon antognists, it does not appear to involve the dsRNA-activated PKR and RNase L systems (Streitenfeld et al. 2003).

In recent years animal models have been developed for hantavirus pulmonary syndrome (Hooper et al. 2001b; Milazzo and Eyzaguirre 2002) and phlebovirus hemorrhagic fever (Fisher and Tesh 2003) that will be important in elucidating the pathogenesis of bunyavirus diseases.

For tospoviruses, resistance to infection was achieved by transforming plants with viral nucleoprotein (*N*) gene sequences (de Haan et al. 1992b; Vaira et al. 1995). This protection was demonstrated to be RNA-mediated, i.e. gene silencing owing to the presence of *N* gene sequences rather than to the presence of N protein (de Haan et al. 1992b; Pang et al. 1997). In addition to immunogenicity, the viral envelope glycoproteins are also most frequently associated with virulence, although this asssociation is not absolute. By using reassortant viruses, it was demonstrated that both mouse virulence and mosquito infectivity correlated with the M segment gene products of the bunyavirus, La Crosse virus (Gonzalez-Scarano et al. 1988). However, a study of reassortants of the phlebovirus Rift Valley fever virus revealed that attenuating mutations (for mice) were present in a vaccine strain virus on all three of the genome segments (Saluzzo and Smith 1990).

CLINICAL AND PATHOLOGICAL ASPECTS

Clinical manifestations

Over 60 viruses of the family *Bunyaviridae* are known to cause disease in humans or animals. Most human infections are silent, and are detectable only by the appearance of specific antibodies. When clinical disease occurs, manifestation of infection ranges from self-limited febrile disease to potentially fatal fulminant hemorrhagic fever or encephalitis. Acute undifferentiated illness is often characterized by an abrupt onset of fever, usually accompanied by chills. Myalgia, arthralgia, headache with or without photophobia, malaise, anorexia, and nausea are common; occasionally vomiting also occurs. Gastrointestinal symptoms rarely dominate the clinical picture, however, nor are respiratory symptoms often seen, although some patients may have sore throat, cough, or even pulmonary infiltrates. A notable exception is hantavirus pulmonary syndrome. Physical examination may reveal conjunctival injection, mild adenopathy, and some abdominal tenderness. Clinical laboratory findings usually indicate a normal, decreased, or moderately elevated white blood count. The typical duration of uncomplicated illness is 2–4 days, perhaps extending to a week. A second wave of fever may occur ('saddle-back fever'), but there is no residual illness seen, although convalescence may require several days.

More severe infections may include a maculopapular rash on the trunk appearing after the onset of illness and lasting a few days (Bwamba, Oropouche, and others), bleeding (most severe in Crimean–Congo hemorrhagic fever, Garissa fever, and Rift Valley fever), encephalitis (Bhanja, California group and Rift Valley fever), hepatitis (hantaviruses and Rift Valley fever), acute kidney failure (hantaviruses), aseptic meningitis (Bwamba, Oropouche, and others), respiratory distress due to acute pulmonary edema (New World hantaviruses) and retinal vasculitis (unique to Rift Valley fever). Fatal infections have often been associated with California group viruses, especially La Crosse virus in children, Crimean–Congo hemorrhagic fever, hantaviral infections, especially hantavirus pulmonary syndrome in which case fatality rates approach 50 percent, and Rift Valley fever virus.

The most important veterinary pathogens are akabane virus, the cause of congenital malformations and abortions in cattle, sheep, and goats in Japan, Australia, South Africa, and Israel (Parsonson and McPhee 1985), Aino, Cache Valley, and Nairobi sheep disease viruses, the cause of malformations and abortions in sheep and/or cattle. Rift Valley fever virus, in addition to human infections, also causes abortion and death in domestic animals. Rift Valley fever virus is commonly found throughout subSaharan Africa, with periodic epizootic/epidemic transmission in Egypt (Meegan 1979), Yemen, and Saudi Arabia (Shoemaker et al. 2002).

IMPORTANT INFECTIONS

California encephalitis and related viruses

California encephalitis was until 2000, the most important arboviral disease seen in the USA, the vast majority of cases being detected among children infected with La Crosse virus. For example, in 1996–1997, of the 286 confirmed or probable arboviral infections, 252 (88 percent) were caused by California group viruses. In those years, the greatest number of cases occurred in

West Virginia (139 cases) with 11 other states having at least one case (Report 1998). Other pathogenic California group viruses include prototype California encephalitis virus, Guaroa, Inkoo (Jamestown Canyon), snowshoe hare virus and Tahyna virus. With the exception of Inkoo and Tahyna viruses, California group viruses are found predominantly in North America where they are transmitted by mosquitoes. Inkoo and Tahyna viruses are found in Europe, and there is serological evidence to suggest that viruses similar or identical to snowshoe hare virus are present in Asia. Tahyna-related viruses have been found in Africa. California group viruses are widely distributed, being found from the tropical zones to above the Arctic Circle (reviewed in LeDuc 1979).

The incubation period for California encephalitis patients is about 3–7 days after an infectious mosquito bite. Onset is abrupt with fever, headache, and lethargy; nausea and vomiting are common. Rash, diarrhea and arthralgia are uncommon. In milder cases, recovery occurs within a week, the only indication of neurological involvement being meningeal signs and perhaps disorientation. More severe cases develop seizures, convulsions, and loss of consciousness, with coma in some instances. Mild neurological signs, which may still be present at discharge, manifest as irritability and other conditions, but usually resolve over the course of several weeks, with little in the way of permanent illness being reported. Of 166 cases reported, two died and two had lasting hemiparesis. Patients with seizures during acute disease are at greater risk for subsequent convulsions, usually seen within a year after recovery. Intellectual and social functions do not seem to be disturbed among recovered children (Matthews et al. 1968; Sabatino and Cramblett 1968; Grabow et al. 1969; Rie et al. 1973). La Crosse encephalitis is usually a disease of children, whereas Jamestown Canyon encephalitis is found in adults.

Tahyna virus is found primarily in Europe, with a related or identical virus being found in Africa (Lumbovirus). Antibodies in humans have been found in Sri Lanka, China, and Russia. Human infection ranges from a mild febrile disease to aseptic meningitis. Pharyngitis, pulmonary involvement, or gastrointestinal disturbances, such as nausea and vomiting may be common. Although CNS involvement has been documented, serious illness or death has not been associated with Tahyna virus infections (Peters and LeDuc 1991).

Akabane virus

Akabane virus is an important veterinary pathogen causing congenital malformations, arthrogryposis, hydranencephaly, and other deformities, as well as abortions in cattle, sheep, and goats in Japan, Australia, South Africa, and Israel (Parsonson and McPhee 1985). Antibodies to akabane virus have been found in sera collected from 25 of 41 wildlife species in 11 subSaharan African countries (Al Busaidy et al. 1987). Both *Culex* mosquitoes and *Culicoides* are implicated in transmission.

Oropouche fever virus

Oropouche virus was first isolated in Trinidad from the blood of a forest worker with a mild febrile illness. Although never common in Trinidad, the virus has become a major cause of rural and urban epidemics in the Amazon region of South America, a large percentage of the resident population being infected during outbreaks. More than 500 000 cases have been recorded, primarily from the Brazilian states of Para, Amazonas, Maranhao, and Goias and more recently from Peru, Panama, Bolivia, and Costa Rica (Vasconcelos et al. 1989, 1994; Watts et al. 1997; Saeed et al. 2000). The disease is characterized by headache, fever, myalgia, arthralgia, photophobia, retrobulbar pain, nausea, and dizziness. Rash occurs in less than 10 percent of the confirmed cases. Symptoms may recur in more than half those clinically ill, usually within 1–2 weeks of the initial onset. No deaths have been reported for Oropouche fever, but meningitis or meningismus has been reported. Patients are often debilitated and prostration is common (reviewed in LeDuc and Pinheiro 1988).

Hantavirus infections

Hemorrhagic fever with renal syndrome (HFRS) has long been recognized clinically in Asia, Russia, and Scandinavia, but the etiology of the syndrome has been known only since the late 1970s. Over 200 synonyms have been used to describe HFRS, including Korean hemorrhagic fever, epidemic hemorrhagic fever, nephropathia epidemica, epidemic nephrosonephritis, and many others, often referring to geographic locations. It was not until 1978, however, that the causative agent, hantaan virus, was isolated from the lungs of the striped fieldmouse, *Apodemus agrarius* (Lee et al. 1978). Unlike other members of the family *Bunyaviridae*, the hantaviruses that infect humans are maintained in nature by chronically infected rodents. No arthropod vector is involved in their transmission cycle. Hantaan virus was named after the Hantaan River, near the demilitarized zone dividing the Korean peninsula, where the infected field mice were first captured.

Subsequent studies have demonstrated that hantaan virus represents an antigenically and genetically novel virus among the *Bunyaviridae* and was thus the prototype member for a new genus within the family (Schmaljohn and Dalrymple 1983; Schmaljohn et al. 1985). Subsequently, additional serologically related viruses have been isolated, many of which are known to cause human disease. Previously, human disease was thought to be limited to febrile illness followed by varying

degrees of kidney failure. Recently, however, a new clinical presentation has been documented: hantavirus pulmonary syndrome (Duchin et al. 1994). This syndrome is characterized by acute onset of febrile disease, followed by an often fatal respiratory distress syndrome. Recent studies have shown this new disease to be caused also by hantaviral infections, but with a unique group of antigenically and genetically related viruses found only in the Americas. Thus, it appears that two basic lineages of hantaviruses have evolved, one occurring predominantly in the Old World, specifically evolving with rodent species found there, and leading to hemorrhagic fever with renal syndrome presentations, and one in the New World causing hantavirus pulmonary syndrome, maintained in nature by New World rodent species (Figure 5; and see Table 2).

Phylogenetic studies suggest that the hantaviruses can be divided into three main groups: the Hantaan-like viruses that are carried by Murinae rodents (Old World mice and rats), Puumala-like viruses that are carried by Arvicolinae rodents (lemmings and voles), and Sin Nombre-like viruses that are carried by Sigmodontinae rodents (New World mice and rats) (Plyusnin 2002). However, a number of these 'viruses' are classified solely on genetic data for a portion of the genome in the absence of virus isolation and few serologic studies (see Table 2). No doubt more detailed studies on these viruses will become available in the future.

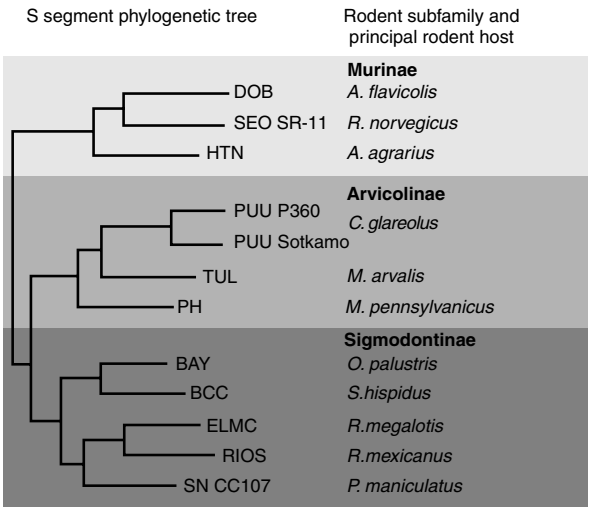

Figure 5 *Relationships of hantaviruses and their rodent hosts. Phylogenetic relationship of the complete S segments of hantaviruses is represented in a single most parsimonious tree derived by using PAUP 3.1.1 software. Horizontal lengths of branches are proportional to nucleotide sequence differences. Abbreviations for viruses and accession numbers for the S segment viral sequences are:* Dobrava virus*, DOB, L41916;* Seoul virus*, SEO, M34881;* Hantaan virus*, HTN, M14626;* Puumala virus*, PUU virus (strain P360), L11347; PUU (strain Sotkamo), X61035;* Tula virus*, TUL, Z30941;* Prospect Hill virus*, PH, X55128;* Bayou virus*, BAY, L36929;* Black Creek Canal virus*, BCC, L39949;* El Moro Canyon virus*, ELMC, U11427;* Rio Segundo virus*, RIOS, U18100;* Sin Nombre virus *(strain CC107) SN, L33683.*

The most severe form of HFRS occurs in Asia and is caused by prototype hantaan virus. It is associated with a case-fatality rate of approximately 5 percent, although this may be much higher in certain rural areas. This disease is characterized by an abrupt onset of fever, chills, malaise, myalgia, headache, dizziness, and anorexia after a variable incubation period of 2–42 days, but most often of 2–4 weeks. During this initial febrile phase, severe abdominal and back pain appear, accompanied by increasing nausea and vomiting, tenderness over the lower back, flushing of the face, neck and chest, and injection of the conjunctivae, palate, and pharynx. Petechiae may appear on the axillae, face, neck, chest, and soft palate, and hemorrhages into the conjunctivae may occur. Clinical laboratory studies reveal normal or slightly elevated white blood cell counts, decreased platelets and rising hematocrit (packed cell volume) values. Proteinuria may appear towards the end of this phase of the disease (McKee et al. 1985).

Defervescence occurs after 3–7 days and marks the start of the hypotensive phase, which may last from several hours to a few days. This phase is characterized by tachycardia, falling arterial pressure, and narrowing pulse pressure, mental changes and, in severe cases, classic shock. Bleeding tendencies continue with capillary hemorrhage and rising hematocrit values. Leukocytosis with a left shift, thrombocytopenia, and prolonged bleeding times are seen; there are high levels of proteinuria and oliguria begins. About one-third of the fatalities occur during this phase. The oliguric phase follows and lasts for 3–7 days, during which blood pressure returns to normal or is slightly elevated due to relative hypervolemia. The fall in urinary output is accompanied by elevated serum creatinine and blood urea nitrogen and other evidence of renal failure. Pulmonary edema may occur and care must be taken in fluid management. Rash and facial flushing disappear during this phase, although nausea and vomiting may continue. Severe hemorrhagic manifestations may occur, gastrointestinal or CNS bleeding being especially serious. Almost half the deaths occur during this phase, often due to pulmonary edema or infection, electrolyte imbalance, late shock, or hemorrhage into the brain. Clinical recovery begins during the diuretic phase, with the normalization of clotting and return of renal function. For the next few hours or days there is diuresis, with outputs of 3–6 liters daily. Strength and appetite improve, but fluid management must be maintained to prevent negative fluid balances which may lead to shock. The convalescent phase may require several months, and hyposthenuria may persist for months to years (McKee et al. 1985). Although complete recovery has been assumed, recent studies with Seoul virus have called this assumption into question (Glass et al. 1993). Specific therapy is not available, and careful supportive care is

essential to improving survival. Care must be consistent with the phase of the disease, careful attention being paid to fluid management. Recent studies have demonstrated that ribavirin is efficacious in treating HFRS if administered early in the course of illness (Huggins et al. 1991). Treated patients had significantly fewer deaths than those untreated, and they spent less time in each phase of the disease and were more likely to skip entire phases altogether.

Other Old World hantaviruses cause diseases similar to, though generally less severe than, classic hantaan virus infections. These include Puumala virus, the cause of nephropathia epidemica of western Europe, Scandinavia, and the western portions of Russia. Nephropathia epidemica may follow the same phases as classic HFRS, but is fatal in less than 1 percent of cases.

The severity of the clinical course of HFRS caused by Puumala virus was found to correlate statistically with specific HLA haplotypes, indicating that host, as well as virus, factors can dictate the outcome of hantaviral infection (Mustonen et al. 1996, 1998). In the Balkan region of Europe, Puumala virus overlaps in many areas with both classic hantaan virus and local strains that are capable of causing very severe disease with mortality rates at least as high as those seen in Asia. The severe HFRS of the Balkans has been associated with Dobrava virus, also reported as Belgrade virus (now known to be the same viruses) and is indistinguishable clinically from HFRS of Asia (Gligic et al. 1992; Avsic-Zupanc et al. 1992a, 1992b, 1995; Taller et al. 1993). Seoul virus, associated with *Rattus norvegicus* (brown rat), probably originated in Asia, but is now found throughout the world wherever large populations of rats exist (LeDuc et al. 1986). It causes a less severe form of HFRS that is characterized by greater liver involvement than classic Hantaan infections, and in general a lower case-fatality rate, although deaths have been recorded. Seoul virus infections are especially common in China, but have been documented in many parts of the world, including Europe and the Americas. In the USA, careful studies have associated past Seoul virus infection with an increased risk of hypertensive renal disease (Glass et al. 1993), an observation that could have significant economic implications for many developed countries (LeDuc et al. 1992).

Hantavirus pulmonary syndrome was first recognized in 1993, when an outbreak of an apparently new and highly fatal form of severe respiratory illness occurred in southwestern USA. As a result of subsequent field and laboratory investigations, the epidemiology and clinical characteristics of this new disease were described, and the name hantavirus pulmonary syndrome was proposed. It was caused by a previously unknown hantavirus, later named Sin Nombre virus (also reported as Four Corners or Muerto Canyon virus). The overall case-fatality rate was 52 percent and examination of the first 100 cases found they were distributed between 21 states. Furthermore the disease had been recognized since at least 1959 (Khan et al. 1996).

Hantavirus pulmonary syndrome has a prodrome of fever and myalgia (all of 17 patients originally described), with cough or dyspnea (76 percent), gastrointestinal symptoms (76 percent) and headache (71 percent) (Duchin et al. 1994). Physical characteristics seen among these original 17 cases include tachypnea (100 percent), tachycardia (94 percent), and hypotension (50 percent). Laboratory findings reflect leukocytosis, increased hematocrit, thrombocytopenia, prolonged prothrombin, and partial thromboplastin times, elevated serum lactate dehydrogenase, decreased serum protein concentrations, and proteinuria. Fifteen (88 percent) cases rapidly progressed to acute pulmonary edema, and 13 (76 percent) died. Predictors of death included increases in hematocrit and partial thromboplastin time, and all deaths occurred with profound hypotension (Duchin et al. 1994). Despite laboratory evidence strongly suggestive of disseminated intravascular coagulopathy, clinical signs of hemorrhage have rarely been reported (Zaki et al. 1995). Radiographic examination reveals bilateral pulmonary infiltrates in virtually all hospitalized patients, and pleural effusions in many. At postmortem examination, copious pleural effusions are often found (Zaki et al. 1995). Multiorgan involvement was found in all of 44 fatal cases examined, with generalized vascular congestion. The primary histopathological features were seen in the lung where microscopic examination revealed mild to moderate interstitial pneumonitis with variable degrees of congestion, edema, and mononuclear cell infiltrates (Zaki et al. 1995). Immunohistochemical examination of postmortem tissue from 44 fatal cases revealed viral antigen-positive cells widely distributed in various tissues, but seen predominantly within endothelial cells of capillaries and small vessels. The most intensive and extensive endothelial staining was in tissues of the lungs, where viral antigen was abundant and uniformly distributed. It involved most of the pulmonary microvasculature, although high densities of viral antigens were also observed in lymphoid follicles of the spleen and, to a lesser extent, in lymph node follicles (Zaki et al. 1995). The magnitude and extent, however, of pulmonary endothelial involvement reflect the difficulty experienced by clinicians in attempting to manage the often terminal shock syndrome.

Treatment has been prompt hospitalization, aggressive clinical management, and early pulmonary support. Ribavirin, which can be efficacious in the treatment of HFRS patients, has been administered to hantavirus pulmonary syndrome patients, but at present there are insufficient data to indicate efficacy clearly.

Crimean–Congo hemorrhagic fever virus

Crimean–Congo hemorrhagic fever (CCHF) is a severe, tickborne, acute hemorrhagic fever found throughout

much of Africa, extending northward to Albania, the former Yugoslavia, Crimea, and elsewhere in the former Soviet Union, Iraq, and much of the Middle East, and eastward into Pakistan and western China. Clinical disease has been well described from cases seen in the former Soviet Union, and nosocomial outbreaks have been common among hospitalized patients, especially those undergoing surgery. The case–fatality rate originally described for cases occurring in Crimea was 15–30 percent (Chumakov 1946); other outbreaks have had case–fatality rates as high as 40 percent (Hoogstraal 1979). Cases usually occur singly among shepherds or others with rural exposure who have been fed on by ticks, or as small clusters among individuals involved in the slaughter of an infected animal (Swanepoel et al. 1985). Nosocomial outbreaks have occurred in the former Soviet Union, the former Yugoslavia, South Africa, and the Middle East (Burney et al. 1976). They typically involve a severely ill patient with hemorrhage and a suspected acute abdomen, perforated ulcer or other diagnosis leading to surgical intervention. Inevitably the patient dies, and often the medical team, family members, or both, are infected, often with a fatal outcome.

The clinical picture of CCHF is an initial influenza-like illness followed by severe headache, joint and back pains, fever, nausea, vomiting, and photophobia. The incubation period is typically 3–6 days in nosocomial infections, and 3–12 days following tick exposure. Initial symptoms may be followed by circulatory collapse, shock, and a variety of hemorrhagic manifestations, such as epistaxis, hemoptysis, hematemesis, melena, and skin hemorrhage. Bleeding into the intestinal tract may cause the patient to present with acute abdominal pain, leading to surgical intervention and increased risk of nosocomial spread. Involvement of the central nervous system indicates a poor prognosis. Clinical laboratory data include leukopenia from the onset of illness, neutropenia, thrombocytopenia, and falling hemoglobin and erythrocyte counts in severely ill patients. The clinically apparent attack rate was estimated to be five infections per case of hemorrhagic fever in the former Soviet Union (Goldfarb et al. 1980).

Nairobi sheep disease virus

This acute, hemorrhagic gastroenteritis affecting sheep and goats in East Africa was described by Montgomery (1917), who correctly attributed transmission to the tick, *Rhipicephalus appendiculatus*. The case–fatality rate may be 70–90 percent in sheep, but goats are less seriously affected. Herdsmen tending infected animals may develop a mild, febrile illness, and laboratory infections with this virus have occurred (Swanepoel 1995). In India, Ganjam virus was isolated from ticks collected from sheep and goats in Orissa state (Karabatsos 1985). Ganjam virus is now considered to be synonymous with Nairobi sheep disease virus (Davies et al. 1978). Dugbe virus, another virus of the genus *Nairovirus*, has been isolated many times from cattle ticks in Nigeria (Kemp et al. 1971), and there have been a few Dugbe virus isolations from febrile humans.

Rift Valley fever virus

Epizootics of Rift Valley fever virus were first reported at the beginning of the twentieth century, but the virus was first isolated in the 1930s in the Rift Valley of Kenya, where sheep suffered fatal hepatic necrosis and abortion. Since then, there have been many epizootics throughout subSaharan Africa, and associated human disease has been described. In 1977, Rift Valley fever was first documented outside subSaharan Africa, when an outbreak occurred in Egypt (Hoogstraal 1979; Laughlin et al. 1979; Meegan 1979). Extensive human and animal disease occurred in Egypt, including the Nile delta region, between 1977 and 1979, but adjacent countries were spared. Transmission appears to have ceased in 1979. In 1993, however, Rift Valley fever once again appeared in Egypt, initially in Upper Egypt near Aswan, and subsequently in the Nile delta (WHO 1994). This presumed importation has likewise not spread beyond Egypt's borders, and may have been contained or eliminated by an aggressive vaccination campaign.

The disease appeared in Mauritania and Senegal in 1987 when an estimated 224 patients died in Mauritania, and many abortions occurred in sheep and goats. This epidemic was associated with flooding at the Diama Dam near the mouth of the Senegal River (Digoutte and Peters 1989). In 2000, the virus was detected in Yemen and Saudi Arabia where nearly 2 000 cases were reported with an estimated 245 deaths. Genetic studies of the virus were consistent with the theory that infection was introduced from East Africa (Shoemaker et al. 2002).

Sheep, cattle, and goats suffer 10–30 percent mortality from Rift Valley fever virus, and a large percentage of infected pregnant animals abort. Disease and death are most frequent in young animals. Native ungulates seem to be more resistant to lethal infection. Disease in humans is characterized by acute onset of fever, headache and myalgia with incapacitating prostration. The incubation period is typically 2–6 days; in most patients the illness resolves after 2–3 days' rest, although symptoms may persist for a week. A small proportion, perhaps 1 percent, of cases develop hemorrhagic fever, encephalitis, or retinal vasculitis. Hemorrhagic disease begins as typical Rift Valley fever, but patients develop gastrointestinal bleeding, become jaundiced, and may die in shock. Disseminated vascular damage and hepatic failure may contribute to the cause of death. Fatal encephalitis may also follow Rift Valley fever, which typically develops a few days to 1–2 weeks after initial recovery from classic disease. Patients develop

headache, confusion, return of fever, and various neurological signs that may lead to residual illness or death. Similarly, ocular complications appear up to 3 weeks after acute disease, presenting with rapid onset of decreased visual acuity due to retinal hemorrhage, exudates, and macular edema. Vision returns as the edema and exudates resolve, but about half the patients suffer some degree of permanent visual loss.

Sandfly fever and related viruses

Sandfly fever, also known as pappataci fever or phlebotomus fever, has been recognized as a militarily important disease since the Napoleonic Wars (Hertig and Sabin 1964). During both World Wars, foreign troops suffered significant incidence rates of sandfly fever in the Mediterranean theaters. These viruses are transmitted by the bite of infectious sandflies (genus *Phlebotomus*, thus 'phlebotomus fever'), and take their names from the sites of their original isolation. Sabin was the first to demonstrate that these two distinct viruses could cause virtually identical diseases in humans (Sabin 1951). The clinical syndrome after infections with either Naples virus or Sicilian virus is characterized by abrupt onset of fever lasting 2–4 days, headache, eye pain, photophobia, pain in the back and joints, anorexia, and malaise. Nausea and vomiting, other abdominal symptoms, sore throat, drowsiness, dizziness, and saddleback fever may also be seen, but rash or meningeal signs are uncommon (Sabin 1951; Hertig and Sabin 1964; Bartelloni and Tesh 1976). No deaths have been documented among several hundred cases studied, and no sequelae are recognized. Life-long homologous protection probably follows infection, but there is no crossprotection between Naples and Sicilian viruses.

Toscana virus was isolated in 1971 from sandflies (*Phlebotomus pernicious*) captured in the Tuscany region of central Italy (Verani et al. 1980). Subsequent investigations there have shown it to be the causative agent in many cases of summertime aseptic meningitis (Verani and Nicoletti 1995). Serological surveys have found antibodies to Toscana virus in other southern European countries as well, although its health impact in these areas is less well characterized (Balducci et al. 1985).

New World sandfly fever viruses

Alenquer, Candiru, Chagres, and Punta Toro are all New World sandfly fever viruses originally isolated from febrile patients. The clinical disease following infection with any of these viruses is virtually indistinguishable from that seen in Naples or Sicilian infections. Human cases are not common owing to the secretive nature of their New World sandfly vectors (genus *Lutzomyia*), which inhabit tropical forests and are unlikely to come into contact with large numbers of humans. Even so, antibody prevalence rates ranging from 2 to 34 percent for Punta Toro virus were detected in serological surveys of residents of rural Panama, and about half those rates for Chagres virus (LeDuc et al. 1980).

Other infections

Bangui virus causes a febrile illness with rash in the Central African Republic. It has also been recovered from a bird in the same country (Digoutte et al. 1980).

Bhanja virus was first isolated in India from *Haemaphysalis intermedia* ticks collected from goats in Ganjam district, Orissa (Shah and Work 1969). Other isolations have been made from cattle and goat ticks in West Africa, and from blood samples collected there from cattle, sheep, ground squirrels, and hedgehogs. Bhanja infection of goats is widespread in the former Czechoslovakia, Italy, and the former Yugoslavia; and many animal sera collected in Sri Lanka have antibodies against this virus. Infection of humans caused severe neurological disease and death in at least two cases in the former Yugoslavia, and laboratory infections have occurred (Vesenjak-Hirjan et al. 1980).

Bwamba virus was first isolated from acutely ill febrile patients in Bwamba County, Uganda (Smithburn et al. 1941). Later serological studies have found high prevalence rates of antibodies to this and closely related viruses throughout East, Central, West, and South Africa. Bwamba virus seems to be a common cause of febrile illness in humans in Africa, causing a self-limiting disease accompanied by rash and prostration, often with meningeal signs (Georges et al. 1980). Bwamba and a closely related virus, Pongola, have been isolated from various mosquito species, but little is known about their natural history. Kasokero virus has been isolated from bats in Uganda, where it may cause fever in humans (Kaluda et al. 1986). Laboratory infections have occurred.

Cache Valley virus has been associated with congenital malformations in sheep and cattle, and serological surveys have frequently found antibodies in domestic animals and humans throughout the Americas. Human disease is uncommon. However, a recent report of severe encephalitis and multiorgan failure attests to its ability to cause life-threatening illness (Edwards et al. 1989; Sexton et al. 1997).

Garissa virus is a reassortant bunyavirus. It was isolated from acute hemorrhagic fever cases during an epidemic of Rift Valley fever in Kenya and southern Somalia in 1997 and early 1998. Initial studies indicated that the sequences of the L and S RNA segments were nearly identical to those of Bunyamwera virus, whereas the sequence of the M segment was very different (33 percent nucleotide and 28 percent amino acid differences from Bunyamwera virus). Acute sera of 14 hemorrhagic fever cases yielded either virus isolation or PCR evidence of infection (Bowen et al. 2001). Very recent

studies have shown that Garissa and Ngari virus M segments are nearly identical in sequence while the L and S segments of Bunyamwera virus are very similar to Ngari virus. These data indicate that Garissa virus is not a reassortant but is an isolate of Ngari virus, which is a reassortant of Bunyamwera virus (Gerrard et al. 2004). Previously, Ngari virus has not been considered a cause of hemorrhagic fever and further studies are required to investigate the pathogenesis and epidemiology of Ngari virus.

Keterah virus, isolated in Malaysia from Argas ticks and from bats, is indistinguishable from Issyk–kul virus, which has been isolated from *Aedes caspius* mosquitoes and from bats (*Nyctalus noctula*, *Myotis blythii* and *Vespertilio serotinus*) and ticks (*Argasvespertilionsis*) in Kirghiz SSR, the former USSR. It has since been associated with epidemics of febrile disease in humans in that region (Lvov et al. 1984).

Nyando virus was isolated from mosquitoes in Kenya, and from humans in the Central African Republic, where it caused fever, myalgia, and vomiting (Digoutte et al. 1980).

Tamdy virus is a tick-borne virus causing a febrile illness in humans in the Asiatic regions of the former USSR (Lvov et al. 1976).

Tataguine virus is a mosquito-borne virus that causes fever, joint pains, and rash in humans in Africa (Digoutte et al. 1980).

Wanowrie virus was isolated from *Hyalomma marginatum isaaci* ticks collected from sheep in India, and from the brain of a 17-year-old girl who died in Sri Lanka after a 2-day fever with abdominal pain and vomiting (Pavri et al. 1976). The virus is also present in Egypt and Iran (Sureau and Klein 1980).

Epidemiology

GENERAL CONSIDERATIONS

Viruses of the family *Bunyaviridae* are maintained in nature in a complex life cycle, usually involving an arthropod vector and a vertebrate host. The exceptions are viruses of the genus *Hantavirus*, which are maintained by chronic infection of rodents and other small mammals. In general, each virus is specifically associated with a particular arthropod species that serves to transmit the virus, and one or more species of vertebrate hosts that serve to amplify the virus following infection through a transient viremia. Arthropod vectors, especially mosquitoes, are often seasonally abundant, which results in seasonal transmission of the viruses they transmit. During periods of adverse weather conditions, virus survival is often maintained by vertical or transovarial transmission whereby an infected female passes the viral infection on to the next generation of vectors. The virus survives in association with the dormant egg and, with hatching and subsequent development, virus transmission to vertebrate hosts takes place during blood feeding. Viruses may also be transmitted venereally from infected males to uninfected female vectors during mating. Vertebrate hosts serve to amplify the amount of virus in circulation by means of a viremia of varying intensity and duration. Uninfected vector species become infected when ingesting a viremic bloodmeal. Following an extrinsic incubation period of a few days' duration, during which the virus replicates and is disseminated throughout the vector, the newly infected vectors are then able to transmit the virus at subsequent blood feeding. Mosquitoes are the most important group of arthropod vectors, but ticks, sandflies, and biting midges (*Culicoides* spp.) are also important vectors for certain viruses.

Most of the bunyaviruses are zoonotic in nature, and humans are usually only accidental hosts that generally do not facilitate amplification of the virus in nature. Exceptions are Oropouche virus and perhaps sandfly fever viruses, in which humans seem to be important sources of virus for uninfected vectors. Selected examples of the transmission cycles of representative viruses are presented below to demonstrate their complexity.

IMPORTANT INFECTIONS

California encephalitis due to La Crosse virus

Most viruses of the genus *Bunyavirus* are transmitted by mosquitoes, and California encephalitis due to La Crosse virus may serve as an example of one of the better understood transmission cycles (see Calisher and Thompson 1983, for overview). The mosquito *Aedes triseriatus* is the principal vector of La Crosse virus. This species is common in the hardwood forests of the upper midwestern United States, where it breeds primarily in tree holes. It survives the harsh winters in the egg stage. In late spring or early summer the first adults emerge, mate, and begin to seek a blood meal. La Crosse virus is transmitted transovarially from infected adult female *Ae. triseriatus* to the next generation of eggs, so a proportion of those newly emerging adults are already infected with the virus (Watts et al. 1973, 1974). These infected females transmit the virus to susceptible vertebrate hosts at blood feeding, beginning the amplification cycle each year, whereas infected males may pass the virus to uninfected females during mating. (Male mosquitoes do not feed on blood, and thus cannot transmit the virus directly to an amplifying vertebrate host.) *Ae. triseriatus* females typically feed during the day, most frequently on squirrels, chipmunks, foxes, and rabbits, and on humans if present. Susceptible squirrels and chipmunks circulate the virus in high enough titer to infect other *Ae. triseriatus*, thus serving to amplify the prevalence of virus in the vector population. Foxes, with their larger range, may both amplify the virus among local vector populations and also disseminate it to more distant vector populations (Yuill 1983). Humans are infected

when they enter the woods for recreation or work, most clinical cases being seen among children less than 15 years old. As *Ae. triseriatus* breeds equally well in discarded tyres or other items that retain water, infected mosquito populations may flourish in close proximity to dwellings, increasing the risk of infection to residents. Prevention of California encephalitis due to La Crosse virus can be facilitated by clean-up campaigns to reduce the availability of mosquito breeding sites, but elimination of all breeding is probably impossible owing to the abundance of natural breeding sites (Francy 1983).

Oropouche virus

Although most viruses of the genus *Orthobunyavirus* are transmitted by mosquitoes, some, such as Oropouche and other members of the *Simbu* serogroup, are transmitted by biting midges (family *Ceratopogonidae*, genus *Culicoides*). Oropouche virus was first described from Trinidad, but in the past two decades has become a major public health problem in northern Brazil and adjacent Amazon Basin nations (for an overview, see LeDuc and Pinheiro 1988). This increase is almost certainly a result of the changing ecology of the Amazon Basin, deforestation leading to human settlements and large plantations of banana and cacao, with the organic waste from these and other crops serving as ideal breeding sites for the vector *Culicoides*. This has led to tremendous population densities of *C. paraensis* in many parts of the Amazon Basin. Oropouche virus is thought to be maintained in two distinct transmission cycles: silent sylvatic transmission involving nonhuman primates, sloths, and perhaps other vertebrate hosts, and an urban cycle in which humans are the primary amplifying and disseminating host and *C. paraensis* serving as the primary vector (Pinheiro et al. 1981). This species lives in close association with humans and readily feeds during daylight hours. Epidemics occur when midge populations are large, the majority of the human population is susceptible and Oropouche virus is introduced from the silent sylvatic cycle into the urban setting. Although only a small percentage of the midges are able to transmit the virus, it seems that the populations are often so abundant that transmission is sustained until the majority of those humans susceptible have been infected. Humans circulate virus in sufficiently high titers to infect feeding *C. paraensis*, and consequently may serve to introduce the virus to new areas through their travel. With the increasing deforestation of the Amazon Basin, epidemics of Oropouche and other previously uncommon viruses found there are likely to increase.

Hantaan and related hantaviruses

Unlike other viruses of the family *Bunyaviridae*, arthropod vectors do not seem to play a significant role in the transmission of the hantaviruses. It seems that the hantaviruses, perhaps like other members of the family, represent an excellent example of co-evolution of the virus with a specific vertebrate host. As information about each of the genetically unique hantaviruses accumulates, it is increasingly apparent that each virus is specifically linked to a particular species of rodent host. These associations are shown in Table 2. The virus lineages closely follow the origin of the rodent hosts. Within the rodent family *Muridae*, three subfamilies exist: *Murinae*, *Arvicolinae*, and *Sigmodontinae*; the hantaviruses maintained by rodent species of each of these subfamilies are genetically most closely related to each other, and less so to those of viruses associated with rodents of the other subfamilies (see Figure 5) (for an overview, see LeDuc 1995). Transmission of hantaviruses between rodent hosts is thought to occur by aerosol from infectious excreta, and perhaps by saliva (Glass et al. 1988; Nuzum et al. 1988). Biting may play an important role in transmission of hantaviruses among rodents as territories are set and mating occurs (Glass et al. 1988). Studies have failed to demonstrate direct transmission from infected parent rodents to their offspring. Humans are infected when they come into contact with infected rodent hosts. There is thus a strong occupational association with the risk of hantaviral infection, farmers, woodcutters, soldiers, and others with significant rural exposure being most often infected. Hantaviral infections are seasonal. They occur frequently in warmer months when recreational activities and outdoor work bring people into close contact with infected rodents. Infections caused by hantaan and Puumala virus peak in prevalence also in late fall and early winter in Asia and Europe, respectively. In cold seasons, the rodents seek shelter in homes and sheds, infecting not only male workers, but also children and women.

Crimean–Congo hemorrhagic fever virus

Crimean–Congo hemorrhagic fever (CCHF) virus serves as an excellent example of two aspects of the epidemiology of viruses in the family *Bunyaviridae*: the threat of nosocomial or other direct transmission from infected blood to susceptible humans, and transmission by ticks (for overviews, see Hoogstraal 1979; Watts et al. 1989; Swanepoel 1995). CCHF virus is widely distributed from eastern Europe to Asia and Africa. In general, its distribution follows closely that of the primary tick vectors of the genus *Hyalomma*. Immature ticks acquire infection during feeding on viremic hosts, often small mammals, or by vertical transmission from infected females to the progeny. As ticks develop, the virus may be transmitted transstadially to subsequent developmental stages, and infected ticks may pass the virus to uninfected females during mating. Adult females, including those infected venereally, may then pass the virus to subsequent generations transovarially (Gonzalez et al. 1992). Viremia occurs after infection of small mammals as well

as of domestic ruminants, which may serve to infect uninfected tick vectors. The proportion of uninfected ticks that becomes infected when feeding on a viremic host generally increases with the intensity of viremia. In addition, nonviremic transmission, as described for Dugbe virus, is also thought to occur with CCHF (Jones et al. 1987; Jones et al. 1989).

Humans become infected either through the bite of an infected tick or through direct contact with infectious blood from an infected animal. The original outbreak of CCHF, described from the Crimean peninsula in 1944–1946, involved large numbers of people exposed to ticks during post-war conditions, clearing agricultural lands that had been fallow. Today, however, most cases are seen among individuals in Bulgaria, Albania, and adjacent parts of the former Yugoslavia, sporadically in the Middle East, and as isolated cases in Africa, especially in South Africa where the virus is well known and studied. Clusters of cases occur in hospitals, especially in remote or ill-equipped clinics and surgeries, where the disease may not be recognized initially, and staff are infected by direct contact with infectious bodily fluids. Transmission of CCHF virus to workers in the laboratory setting has also occurred, leading to severe disease and fatalities.

Pathogenesis, pathology, and immune response

Human infection with a bunyavirus generally follows the bite of an infectious vector or, less frequently, inhalation of infectious virus. After an incubation period, a brief viremia often occurs, which corresponds to the period of acute illness. Signs and symptoms probably result from direct effects of virus on target organs, such as the liver or brain. Immunohistochemical analysis has found widespread hantaviral antigens in endothelial cells of the microvasculature of hantavirus pulmonary syndrome patients, which may reflect similar targets of infection with other hantaviruses (Zaki et al. 1995). Disseminated intravascular coagulopathy may occur in severe disease with hemorrhagic manifestations such as HFRS, CCHF or Rift Valley fever. A typical humoral immune response leads to cessation of viremia and clinical recovery in most cases, with immunoglobulin M (IgM) predominating initially, followed by immunoglobulin G (IgG). The cellular immune response to bunyavirus infections is not well defined, nor is the role of cytokines. A brief discussion of the pathogenesis, pathology, and immune response of selected diseases is presented in Clinical manifestations.

Diagnosis

Diagnosis is based on demonstration of the specific virus causing infection, by isolation, by detection of viral antigen or nucleic acids, or by acquisition of antibody specific for the infecting virus. Classic virus isolation techniques such as inoculation of suckling mice or cell cultures of mammalian or insect origin have been often used with considerable success. Likewise, routine serological techniques such as hemagglutination inhibition, complement fixation, immunofluorescent antibody assays, neutralization tests in mice or cell culture have been used to demonstrate rising or falling titers of antibody in paired sera from patients. These techniques are generally time consuming and expensive, and do not offer a definitive diagnosis soon enough to influence clinical management. Recently, more rapid diagnostic procedures have been developed that can offer a presumptive diagnosis within a few hours of receipt of a single acute serum sample. With enzyme immunoassays, viral antigen may be detected directly, or virus-specific IgM antibody demonstrated, generally in time to influence clinical management. For example, patients with HFRS due to hantaan virus are often positive for IgM antibodies at the time of hospital admission (LeDuc et al. 1990). Confirmation of the specific etiology helps in forecasting the course of the illness and initiating antiviral therapy such as ribavirin, which is efficacious in the treatment of HFRS (Huggins et al. 1991). Direct detection of viral nucleic acid, most often through reverse transcriptase-polymerase chain reaction amplification, and subsequent sequencing or endonuclease digestion of amplified products, has recently been applied for diagnosis, especially of hantavirus infections (Nichol et al. 1993). Acute specimens and autopsy tissues have both been used successfully to establish the diagnosis. However, the value of the technique is limited by the need for specially designed primers and very careful laboratory techniques to minimize the risk of contamination (Grankvist et al. 1992; Feldmann et al. 1993; Nichol et al. 1993). At present, these techniques are limited to research or reference laboratories and not widely available.

Prevention and control

Diseases caused by bunyaviruses have, with few exceptions, a low incidence of human infection and thus do not justify development of vaccines. An exception is HFRS, especially that due to hantaan virus in Asia, where several candidate vaccines are in development, under field trials or, in at least one case, commercially available (Lee et al. 1990; Schmaljohn et al. 1994). Most current candidates are inactivated whole virus vaccines that require multiple inoculations and boosters to ensure protection. However, recombinant candidates are also under development. In particular, DNA vaccines appear to have significant promise for Hantaan and related viruses (Hooper et al. 2001a; Custer et al. 2003). Development of live-attenuated vaccines for hantavirus

infections has been hampered by the absence of a suitable animal model that would faithfully mimic human disease (WHO 1995), although recent studies suggest some animal models may have utility (Hooper et al. 2001b; Milazzo and Eyzaguirre 2002; Fisher and Tesch 2003). Vaccines for Rift Valley fever virus have also been developed, with both inactivated and live attenuated products available for veterinary use, and an inactivated vaccine available on a limited basis for humans (Smithburn 1949; Eddy et al. 1981; Kark et al. 1982; Morrill et al. 1987, 1991). A live-attenuated candidate vaccine for humans is now in early clinical trails. Observational studies indicate that ribavirin has a beneficial effect in CCHF infections. Thus it may be that oral prophylactic treatment of exposed persons is warranted (Mardani et al. 2003).

Most often, however, prevention and control of bunyaviruses are based on avoidance of human contact with infected arthropod or small mammal vectors. Infection usually occurs when people enter into the ecological setting where viruses are being transmitted silently among infected vectors and wild vertebrate hosts. In these situations, personal protective measures, such as the liberal use of insect repellants and wearing appropriate clothing, may reduce the risk of infection. Likewise, care should be taken to ensure that houses, vacation cabins, or campsites do not attract or support breeding potential vectors. Thus, using screens on windows and doors and removing potential mosquito breeding sites, such as used tyres or other water-holding containers, will help prevent infections. In the case of rodentborne hantaviruses, special precautions are needed to rodent-proof homes and to maintain clean campsites.

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Arenaviruses

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PROPERTIES OF THE ARENAVIRUSES

INTRODUCTION

The arenaviruses are enveloped, single-stranded RNA viruses that are primarily carried by rodents and occasionally transmitted to humans. Old World and New World arenaviruses have been isolated on the African and American continents respectively (see Table 1).

The arenaviruses are important clinically as human pathogens and experimentally as models for persistent infection and cellular immune responses. The prototype virus, lymphocytic choriomeningitis virus (LCMV), was first isolated from a human diagnosed with St. Louis encephalitis; tissue homogenates passaged through monkeys and mice caused fever and aseptic meningitis (Armstrong and Lillie 1934). A year later, a filterable agent was isolated from the cerebrospinal fluids of two patients; this agent elicited similar symptoms in mice (Rivers and Scott 1935). At the same time, Traub (1935) discovered a contaminant virus in the mouse colony of the Rockefeller Laboratories. Viruses were exchanged between the three laboratories and their identity was confirmed by neutralization in vitro and cross-protection in vivo (Rivers and Scott 1936). LCMV has frequently been found as a contaminant of laboratory mice, rats, and hamsters in North America and Europe, and may have entered North America via mice from Europe. Studies of murine LCMV infection have contributed to a wealth of information on the mechanisms of viral persistence and the interactions of viruses with host immune systems (see section entitled Immune response).

In 1956, the nonpathogenic Tacaribe virus was isolated from Caribbean fruit bats (Downs et al. 1963). With the agricultural expansion in South America, two pathogenic arenaviruses emerged: Junin, isolated from humans with Argentine hemorrhagic fever (Parodi et al. 1958); and Machupo, isolated from humans with Bolivian hemorrhagic fever (Johnson et al. 1965). Collaboration between field workers, the Yale Arbovirus Laboratories and the Rockefeller Laboratories documented the morphology and antigenicity of the 'Tacaribe group' of viruses. A nonpathogenic member of this group, Pichinde virus, was isolated during a trapping programme in Colombia, and has since served in many biochemical studies (Trapido and Sanmartin 1971; Cosgriff et al. 1987; Liu et al. 1986; Qian et al. 1991). More recently, other arenaviruses have been discovered in Argentina (Oliveros virus, Bowen et al. 1996), Brazil (Cupixi, Charrel et al. 2002), Venezuela (Pirital virus, Charrel et al. 2001a), Peru (Allpahuayo virus, Moncayo et al. 2001) and the US (Whitewater Arroyo virus, Charrel et al. 2001b; Bear Canyon virus, Fulhorst et al. 2002).

Table 1 Arenaviridae*: carriers and geographical distribution (modified from Clegg 2002)*

Virus	Virus carrier	Geographical distribution	Potential for human disease
New World			
Allpahuayo	*Oecomys* spp.	Peru	None reported
Amapari	*Oryzomys goeldii*	Brazil	None reported
	Neacomys guinae	Brazil	
Bear Canyon	*Peromyscus californicus*	California, USA	None confirmed
Cupixi	*Oryzomys* spp.	Brazil	None reported
Flexal	*Neacomys* spp.	Brazil	None reported
Guanarito	*Oryzomys* spp.	Venezuela	Severe
Junin	*Calomys laucha*	Argentina	Severe
	Calomys musculinus	Argentina	
	Akodon azarae	Argentina	
Latino	*Calomys callosus*	Bolivia	None reported
Machupo	*Calomys callosus*	Bolivia	Severe
Oliveros	*Bolomys obscures*	Argentina	None reported
Parana	*Oryzomys buccinatus*	Paraguay	None reported
Pichinde	*Oryzomys albigularis*	Colombia	None
	Thomasomys fuscatus	Colombia	
Pirital	*Sigmodon alstoni*	Venezuela	None reported
Sabía	Not known	Brazil	Severe
Tacaribe	*Artibeus literatus*	Trinidad	None reported
	Artibeus jamaicensis	Trinidad	
Tamiami	*Sigmodon hispidus*	Florida, USA	None reported
Whitewater Arroyo	*Neotoma* spp.: *albigula*, *cinerea*, *mexicana*, *micropus*	New Mexico, USA	Three fatalities had a virus, 87 percent identical to WWA
Old World			
LCM	*Mus musculus*	Worldwide	Mild to severe
Lassa	*Mastomys natalensis*	West Africa	Severe, often fatal
Mopeia	*Mastomys natalensis*	Mozambique Zimbabwe	None reported
Mobala	*Praomys jacksoni*	Central African Republic	None reported
Ippy	*Mastomys natalensis*	Central African Republic	None reported

It was not until the late 1960s that the morphological similarities between LCMV and the Tacaribe group of viruses were noted: both were enveloped viruses with a granular or sandy appearance. Serological tests later confirmed the relationship and they were named arenaviruses after the Latin *arena* for 'sandy' (Johnson et al. 1965; Dalton et al. 1968; Rowe et al. 1970). When Lassa fever virus emerged in Africa, it was quickly identified as an arenavirus on morphological and serological criteria (Murphy 1975). Arenaviruses are considered 'emerging pathogens' because new isolates are coming to our attention with great frequency (Coimbra and Nassar 1994). The following publications contain more details about the arenaviruses: Oldstone (2002); Bishop (1990); McCormick (1990); Salvato (1993a); Southern (1996); Buchmeier et al. (2001), and Peters (1997).

CLASSIFICATION

The *Arenaviridae* are one of seven families of negative-strand RNA viruses, the others being *Filoviridae, Rhabdoviridae, Paramyxoviridae, Orthomyxoviridae*, *Bunyaviridae*, and *Bornaviridae*. These viruses are characterized by single-stranded, non-infectious genomic RNA that requires a virus-encoded RNA transcriptase to initiate replication. Arenaviruses have been classified by a combination of morphological characteristics, biochemical analyses, and immunological tests (Pfau 1974) and are the only family of negative-strand RNA viruses with a bisegmented genome (Pedersen 1979). Under the Old World and New World groupings are at least 22 species of arenaviruses (Table 1) with the probable addition of further viruses as surveillance continues. Genetic relationships among the arenaviruses can be depicted by a phylogenetic tree based on the sequence differences between viral nucleocapsid proteins (Figure 1). A recent comparison (Bowen et al. 2000) found the broadest range of sequences within the *Lassa* species, i.e. up to 24.1 percent nucleotide divergence and up to 12 percent amino acid divergence. However, two distinct species of New World arenaviruses, *Junin virus* and *Machupo virus*, were relatively similar to each other with 23.3 percent nucleotide divergence and 13.7 percent amino acid divergence.

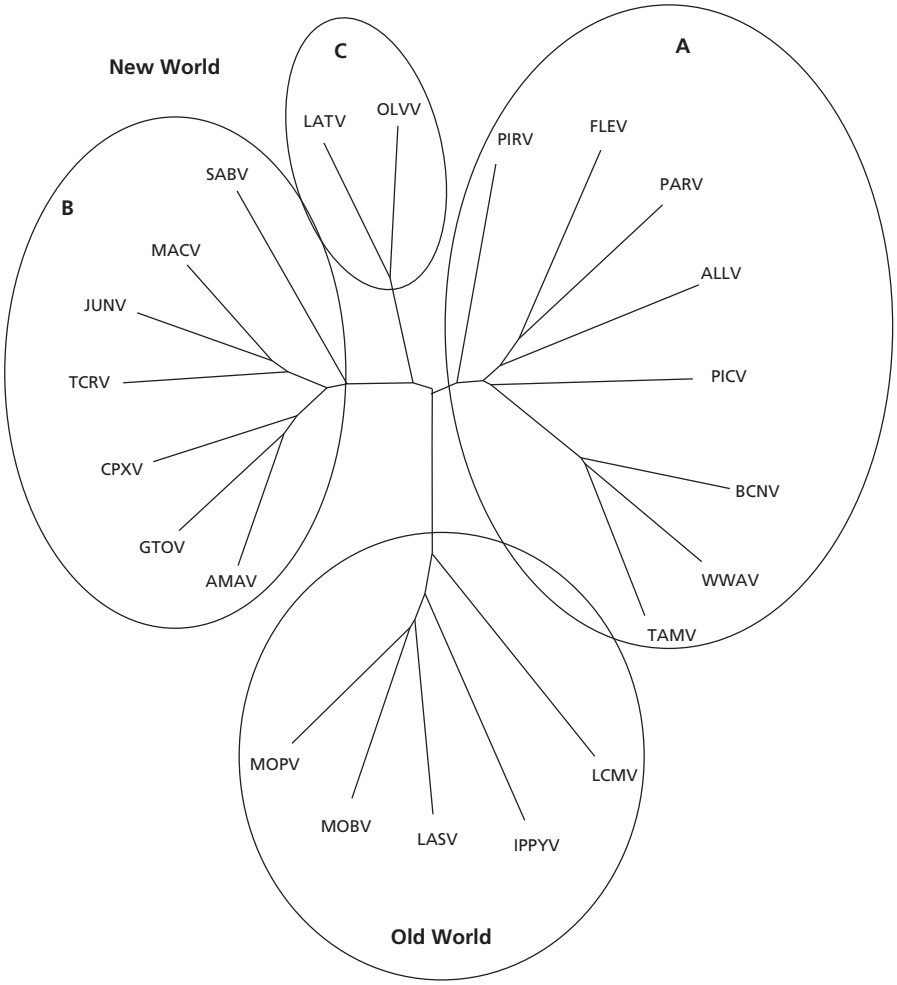

Figure 1 *Phylogenetic relationships among the* Arenaviridae. *Partial* N *gene nucleotide sequences corresponding to nt 1770–2418 of* Tacaribe virus *S RNA sequence (GenBank accession no. M20304) were aligned (Clustal X 1.81) and analyzed by Jukes–Cantor algorithm and neighbor-joining method using the MEGA v2.1 software program (modified from Bowen et al. 2000).*

Considering that picornaviruses have as much as 30 percent amino acid divergence within species (Dahourou et al. 2002), the arenaviruses are considerably subdivided. In addition to sequence criteria, classification of arenaviruses is influenced by the ability of viruses to reassort with one another, i.e. intraspecies reassortment should be possible and interspecies reassortment, as between *Lassa virus* and *Mopeia virus* (Lukashevich 1992), is rare.

DISTRIBUTION

The worldwide distribution of arenaviruses follows the distribution of their natural reservoir hosts, primarily rodents (Figure 2). Occasionally, infected humans have traveled extensively, resulting in movement of the viruses (Holmes et al. 1990).

Although there are over 30 families of rodents, the arenaviruses are associated with only two major families: the *Muridae* (e.g. house mice and rats) and the *Cricetidae* (e.g. voles, deer mice, and gerbils). The *Muridae* inhabit human dwellings and food stores, in contrast to the *Cricetidae* that live in the open grasslands. Muridae include *Mastomys*, that carries the Lassa-like viruses; *Praomys*, that carries Mobala; and *Mus*, that spread from the Old World to the rest of the globe, simultaneously dispersing LCMV. The cottonrat, *Sigmodon hispidus*, from which Tamiami virus was isolated in the USA, is related to the *Cricetidae*: *Calomys, Akadon, Oryzomys*, and *Neocomys* of South America and *Peromyscus* of North America. Tacaribe virus is an exception in that it was isolated from the fruit bat, *Artibeus*. It is notable that infections follow seasonal associations between humans and carriers; for example, the harvest season in Argentina, April to June, is also the peak time for the occurrence of Argentine hemorrhagic fever.

MORPHOLOGY AND STRUCTURE

The envelope composition of an arenavirus derives from host-cell membranes during maturation from the cell

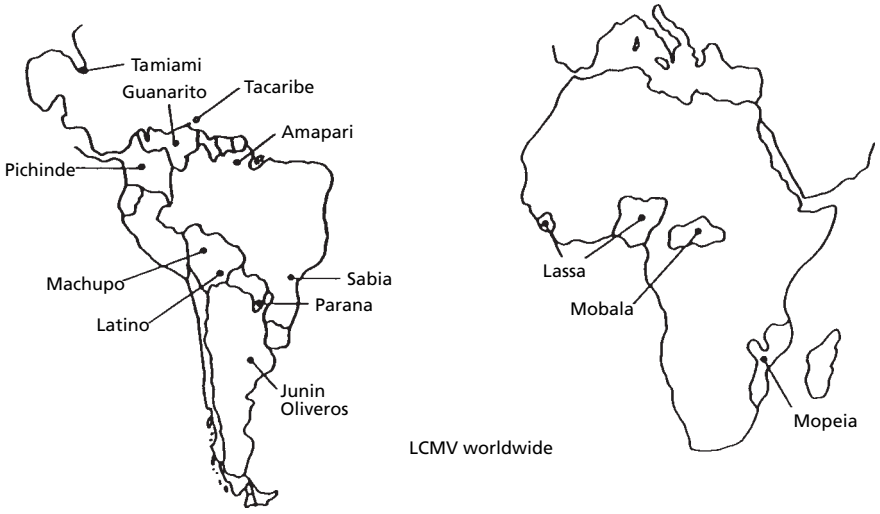

Figure 2 *Map of the distribution of Old World and New World arenaviruses. The Old World viruses originated on the European and African continents and the New World viruses originated in the Americas.*

surface (Figure 3a). The envelope is sensitive to organic solvents and detergents; treatment with these reagents can be used to separate viral cores from their envelopes. Virions are also sensitive to UV and gamma-irradiation, to heating at 56°C, and to pH outside the range of 5.5–8.5. Acid or high salt treatment of LCMV causes one of the envelope glycoprotein chains, GP-1, to dissociate from the virion surface, whereas the other chain, GP-2, remains anchored in the envelope (Di Simone et al. 1994). Acid treatment of LCMV decreases its Vero cell infectivity, which is due to virus aggregation or the loss of GP-1. Virions are generally purified from the media surrounding infected cells by a combination of precipitation with 7 percent polyethylene glycol and centrifugation: buoyant densities are 1.9–1.2 g/cm^3 in cesium chloride, 1.17–1.18 g/cm^3 in sucrose and 1.14 g/cm^3 in amidotrizoate (Buchmeier et al. 1978).

Originally, electron micrographs showed arenaviruses as highly pleiomorphic, probably because of their instability during fixation procedures. More recently, cryo-electron micrographs have revealed spherical particles with an average diameter of 90–110 nm (Burns and Buchmeier 1993) (Figure 3c, d). Glycoproteins on the surface of the virion form T-shaped spikes extending 7–10 nm from the envelope. Electron-dense granules within arenaviruses, from which the name 'arena' derives, are ribonucleoprotein particles. These include ribosomal RNA, and perhaps whole ribosomes, and more of such particles are present in late viral harvests than in early harvests (Farber and Rawls 1975; Pedersen 1979; Dutko and Oldstone 1983; Southern et al. 1987). With Pichinde virus, virion-associated ribosomes are not essential for the infectious process; viruses produced in cells containing *ts* ribosomes are infectious even at the nonpermissive temperature (Leung and Rawls 1977). Electron microscopy of disrupted virions reveals circularized strings of ribonucleoproteins (Palmer et al. 1977) that may have resulted from the self-annealing of the RNA termini that encode approximately 20 bases of complementary nucleotides.

Arenaviruses produce defective interfering (DI) particles (reviewed by Buchmeier et al. 1980a). DI particles are generated rapidly during infection of tissue cultures with LCMV, and render the cells resistant to superinfection with LCMV, less resistant to Pichinde and entirely susceptible to heterologous viruses (Lehmann-Grube et al. 1969; Welsh and Buchmeier 1979). DI particles sediment to a lower density than infectious virus and have a smaller target size for UV inactivation, and their concentration can be measured by cytopathogenicity-reduction assays. DI particles of Pichinde lack conventional S RNA (Dutko et al. 1976).

GENOME STRUCTURE AND FUNCTION

The arenaviruses contain single-stranded RNA that consists of 2 RNA segments, L ca. 7000 bases and S ca. 3400 bases. Complete sequences are now available for LCMV, Lassa, Tacaribe, and Pichinde, and partial sequences for Junin, Mopeia, and other arenaviruses (Romanowski et al. 1985; Auperin et al. 1986; Auperin and McCormick 1989; Iapalucci et al. 1989; Salvato and Shimomaye 1989; Salvato et al. 1989; Ghiringhelli et al. 1991; Harnish et al. unpublished; reviewed in Clegg 1993). Four open reading frames have been identified: a gene for the envelope glycoprotein is encoded at the 5′ end of the S RNA segment, and, in the negative sense, a gene for the nucleocapsid protein is encoded at the 3′ end of the S RNA. A gene for a small zinc-binding protein is encoded at the 5′ end of the L RNA, and, in the negative sense, a gene for the viral RNA polymerase is encoded at the 3′ end of the L RNA (Figure 4).

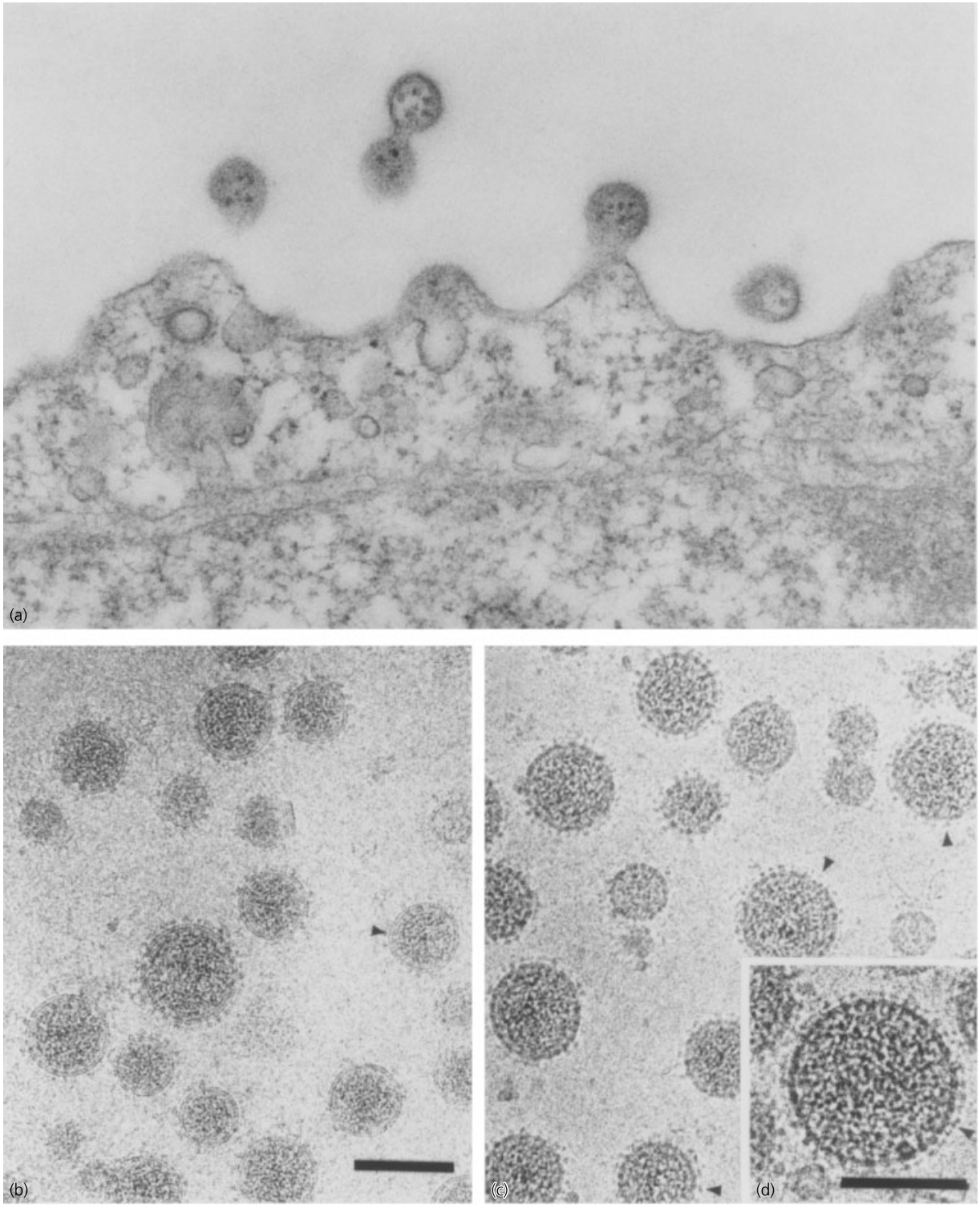

Figur 3 *Electron micrographs of LCM virions.* **(a)** *Thin section showing virions budding from infected BHK-21 cells. Typical 110 nm virions containing 20 nm electron-dense particles are evident.* **(b)** *Cryo-electron micrograph of unstained LCM virions at the 1.5 μm defocus level to emphasize lipid bilayer (see arrow) and in* **(c)** *cryo-electron micrograph of LCM at the 3 μm defocus level to emphasize surface topography. Bars = 100 nm. (Microscopy by R Milligan, Scripps Clinic and Research Foundation, San Diego. Reproduced from Burns and Buchmeier 1993.)*

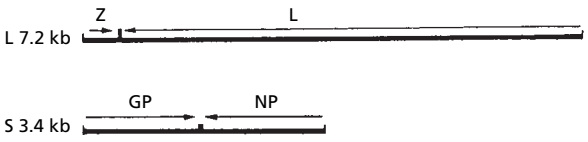

Figure 4 *The two arenavirus RNA segments, L and S, are depicted with the four encoded genes: Z (zinc-binding protein), L (polymerase), GP (envelope glycoprotein) and NP (nucleocapsid protein). (Reproduced from Salvato 1993b)*

A hallmark of the arenaviruses is their 'ambisense' coding strategy (Auperin et al. 1984). Each RNA segment encodes a gene in the positive (mRNA) sense and an additional nonoverlapping gene in the negative sense, i.e. the latter gene must first be transcribed to obtain the mRNA sense. The viruses are classified as negative strand, because the mRNA sense genes cannot function in translation directly from the genomic RNA; subgenomic mRNA copies of these genes must first be produced by transcription (Figure 5).

Strong secondary structures can be predicted for arenavirus genomic RNAs: stem–loops in the intergenic regions and base-paired 'panhandles' at the termini of the RNA segments. The terminal 20 bases of the arenavirus genomic RNA are conserved between genera and are almost identical within one arenavirus strain. It is possible to form intra- and extramolecular complexes, i.e. the 3′ and 5′ termini of the S RNA can anneal with each other or the 3′ of the S RNA can anneal with the 5′ of the L RNA, and so on. It has been suggested that this conserved terminal structure may represent a binding site for the viral RNA polymerase (Salvato 1993b) (Figure 6).

Reassortment of the two genomic RNA segments has been used to map functions to one segment or the other. Production of reassortant viruses involves infection of cultured cells with two virus isolates at once and screening for progeny virus by nucleic acid hybridization, reverse transcriptase polymerase chain reaction (RT-PCR), or by limited sequence analysis. Reassortant

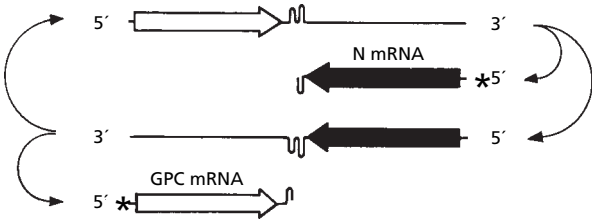

Figure 5 *The ambisense coding strategy of the arenaviruses is illustrated for the S RNA segment of* Junin virus. *First, the subgenomic nucleocapsid mRNA (N mRNA) is made and then the full-length antigenomic RNA is made (this is a 'replication intermediate'); finally, the subgenomic glycoprotein mRNA (GP-C mRNA) is made. For the L RNA a similar sequence occurs with the L and Z subgenomic mRNAs. (Reproduced from Romanowski 1993)*

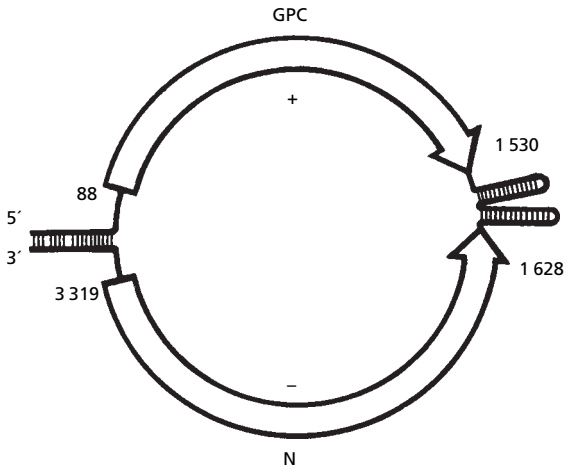

Figure 6 *The S RNA segment of* Junin virus *can be predicted to form strong secondary structures at the RNA terminus (a panhandle of the 3′ and 5′ ends) and at the intergenic region (a double stem–loop structure). The glycoprotein (GP-C) and the nucleocapsid protein (N) open reading frames are in opposite coding sense on either side of the intergenic stem–loops. (Reproduced from Romanowski 1993)*

viruses that contain the S RNA of one parental virus and the L RNA of the other can usually be isolated. This approach was used to show that the virulence of LCMV WE for guinea-pigs mapped to the L RNA (Riviere 1986), that the S RNA of LCMV Armstrong is associated both with diminished growth in C3H mice (Oldstone et al. 1985) and with the emergence of insulin-dependent diabetes in nonobese diabetic mice (Oldstone et al. 1990), and that the persistence of an LCMV Armstrong isolate depends on the L RNA (Matloubian et al. 1993). The latter case illustrates one shortcoming of this approach in that the persistent phenotype is likely to be pleiotropic, with essential genetic components on both RNAs (Salvato et al. 1991). Likewise, reassortant viruses between the Old World arenaviruses Mopeia and Lassa allowed mapping of the ability of Lassa S segment to protect against a lethal intracerebral inoculation of Lassa virus in the murine model (Lukashevich 1992).

A reverse genetics system developed by the de la Torre laboratory indicates a transcription inhibitory function for Z protein (Lee and de la Torre 2002)

REPLICATION

Tropism and virus entry

Arenaviruses replicate in a broad range of mammalian hosts and in almost every tissue of the host, reaching high titers in brain, kidney, liver, and secondary lymphoid organs (see Figure 9). Virus replication is restricted in lymphocytes, macrophages, and terminally differentiated neurons, probably because of the absence

of host cell factors (Borrow et al. 1991; de la Torre et al. 1993; Polyak et al. 1995a). Arenaviruses are often propagated in adherent cell lines such as BHK-21 cells, mouse L cells or Vero cells. The broad range of mammalian cells that can be infected suggests that the receptor for virus entry is fairly well conserved. In attempts to find the receptor for LCMV, Borrow and Oldstone (1992) used a virus overlay blot assay to identify a 160 kDa glycoprotein from rodent fibroblasts. Further studies of this protein made it clear that several arenaviruses bind to a 120–140 kDa cellular glycoprotein identified as α-dystroglycan (Cao et al. 1998). However, variations in binding affinity to α-dystroglycan were observed among the LCMV strains, with LCMV Cl –13 and WE strains being typical of high affinity binders while Arm binds at low affinity. Other arenaviruses (i.e. Lassa, Mobala, Oliveros) also bind to this glycoprotein, but Guanarito virus does not, suggesting that more than one cellular protein may serve as a receptor for arenaviruses (Buchmeier 2002).

Arenavirus entry into cultured cells is sensitive to lysosomotropic agents (Pichinde: Mifune et al. 1971; Lassa and Mopeia: Glushakova and Lukashevich 1989, Glushakova et al. 1992; LCMV: Borrow and Oldstone 1994; Di Simone et al. 1994). In the case of LCMV, this uptake was not affected by cytochalasins, so does not require clathrin-coated pits and takes place in smooth-walled vesicles by 'viropexis.' Once the virus is in the cell, the life cycle of uncoating, mRNA transcription, translation, and genome replication takes place in the cytoplasm, with some contribution from host-cell components for transcription and translation (Figure 7).

Arenavirus gene expression

The ambisense coding arrangement of the arenavirus genome provides a mechanism for temporal regulation of gene expression: NP and L mRNAs can be transcribed from the incoming genomic RNA, whereas GP and Z mRNAs are transcribed only from RNA antigenomic templates that also function as replication intermediates. Kinetic analysis of LCMV replication shows simultaneous accumulation of NP and GP mRNA (Fuller-Pace and Southern 1988), but in Tacaribe virus, newly synthesized NP mRNA accumulates before the onset of RNA replication. Furthermore, inhibition of protein synthesis during early infection prevents RNA replication but allows the continued synthesis of NP mRNA (Lopez and Franze-Fernandez 1985; Franze-Fernandez et al. 1987). For LCMV, it has been shown that the Z mRNA is included in the virion, unlike mRNAs for the other viral genes, and may be essential at an early stage of the viral life cycle (Salvato and Shimomaye 1989). Further work with inhibitors of transcription and translation will be necessary to characterize the sequence of events in arenavirus replication.

Arenavirus mRNA transcription begins with 3–7 nontemplated nucleotides: this has been demonstrated for the 5′ terminus of the NP mRNA in Tacaribe virus (Raju et al. 1990), the NP and GP mRNAs in LCMV

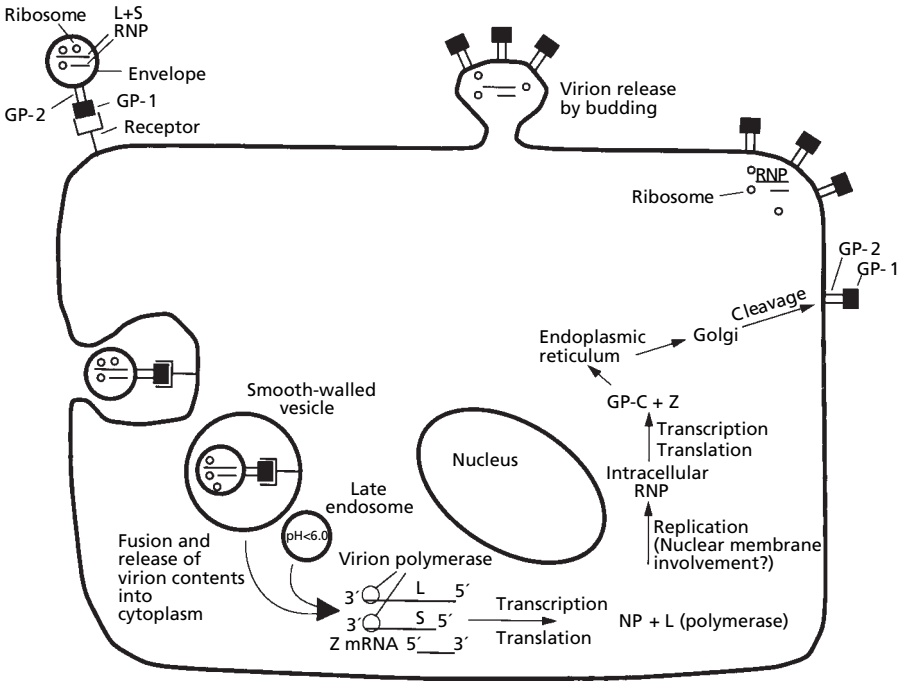

Figure 7 *Arenavirus life cycle. L, large RNA segment; S, small RNA segment; RNP, ribonucleoprotein. RNP complexes in the virion contain viral genomic RNA, Z mRNA, viral proteins, and host components such as ribosomal proteins.*

(Meyer and Southern 1993) and the NP and GP mRNAs in Pichinde virus (Polyak et al. 1995b). Genome and antigenomes generally have one nontemplated base. These may arise either by de novo synthesis or by a cap stealing mechanism as has been described for the *Orthomyxoviridae* and the *Bunyaviridae* (Garcin and Kolakofsky 1990).

The noncoding regions of arenavirus genomic RNA have the potential to form several stem–loop structures that are well placed to regulate transcription and translation. LCMV, Lassa, and Pichinde viruses each have single intergenic stem–loops on the S RNA, whereas Mopeia, Tacaribe, and Junin viruses each predict double stem–looped structures (reviewed by Clegg 1993). Translation termination codons have been described on the proximal side of the intergenic stem–loops on the S RNA and L RNA. Transcription terminations for Tacaribe virus NP, GP, Z, and L mRNAs have been described at the distal portion of each stem–loop, indicating that the secondary structure is not acting to stop transcription. Furthermore, since transcription termination has been described at multiple positions on the stem, it is likely that the polymerase is displaced from the RNA by an extragenomic factor rather than by a feature of the genome structure (Franze-Fernandez et al. 1993; Meyer and Southern 1993). Thus, since it appears that the intergenic stem–loops may help stop translation, in conjunction with the encoded stop codons, they are unlikely to serve as terminators or attenuators of transcription, and they may serve as recognition or nucleation points for virus assembly and encapsidation.

Recent studies using a LCMV RNA analogue, showed clearly that L and NP are the minimal viral factors required for efficient RNA synthesis mediated by the LCMV polymerase (Lee et al. 2000). In addition, this system allowed tests for the *cis*-elements required for transcription and replication of LCMV genome in vitro (Lee et al. 2002).

Virus–host interactions during arenavirus replication

LCMV infections in carrier mice (covered more extensively in the section entitled Clinical and pathological aspects) can persist in a broad range of tissues, without detectable cellular damage or inflammation (see Figure 9). However, some terminally differentiated cell functions or 'luxury functions' can be abolished by the presence of replicating virus. For example, a reduction of macrophage lymphokines, growth hormone, thyroid hormones, and acetylcholinesterase have been observed in persistently infected murine systems (Jacobs and Cole 1976; Oldstone et al. 1977, 1982, 1985; Rodriguez et al. 1983; Klavinskis and Oldstone 1987). The ability of LCMV to affect differentiated functions of the immune, neural, and endocrine systems depends on the genetic background of the host.

In tissue culture at low multiplicity of infection (i.e. moi <0.1) arenavirus replication is not detected for 6 h, after which cell-associated virus increases exponentially. The titer of extracellular virus reaches a maximum 36–48 h after infection. Infected cells undergo only limited cytopathic change during virus production with little or no change in the level of host protein synthesis. Long-term virus cultures are readily established.

Although arenavirus replication takes place in the host-cell cytoplasm, enucleated cells fail to support virus production. Specifically, the host-cell nucleus is required during the first 10 h of Pichinde virus infection of BHK-21 cells (Banerjee et al. 1976). Either enucleation removes a site of virus replication near the nuclear membrane or host nuclear products are required for viral replication. The latter is supported by the finding that α-amanitin, an inhibitor of host RNA polymerase II that is responsible for mRNA and hnRNA synthesis, prevents arenavirus replication. In contrast, actinomycin D, which primarily inhibits rRNA synthesis, has a lesser effect on Pichinde replication.

Host-cell ribosomes that become incorporated into nascent virions retain their capacity to catalyse protein synthesis, but are not necessary for virus replication (Leung and Rawls 1977). In Pichinde virus, hybrid molecules between viral RNA and host-cell ribosomal RNA have been observed (Shivraprakesh et al. 1988) but their functional significance is unclear. Host-cell ribosomal proteins and translation factors are definitely incorporated within virus but may simply be remnants of assembly complexes rather than the package of complete ribosome (Borden et al. 1998b).

Host-cell enzymes are essential for the maturation of arenavirus envelope glycoproteins. These are translated as a long precursor (GP-C) that undergoes glycosylation, transport, and proteolysis involving host-cell enzymes (Buchmeier and Oldstone 1979; reviewed in Burns and Buchmeier 1993). Initially, for LCMV, a 58-residue signal peptide is cleaved from the N terminus of GP-C. Further cleavage depends on glycosylation of the precursor in the Golgi or post-Golgi compartments with 5–6 *N*-linked glycosylations for the N-terminal portion of GP-C (to become GP-1) and 2 *N*-linked glycosylations for the C-terminal portion of GP-C (to become GP-2) (Wright et al. 1989). Cleavage by a furinlike protease occurs at Arg_{262}–Arg_{263}. This cleavage is followed by trimming of the N-terminal portion of GP-2 (Burns and Buchmeier 1993). A protein kinase activity capable of phosphorylating the nucleocapsid protein has been identified in preparations of LCMV (Howard and Buchmeier 1983), but it is not known whether this is a cellular or viral enzyme or if it has any essential function. Recently, it has also been shown that Lassa Gp precursor is cleaved in endoplasmic reticulum by the cellular subtilase SKI-1/S1P, an enzyme that has been

observed to be involved in cholesterol metabolism (Lenz et al. 2001). Moreover, since apparently only the cleaved glycoprotein is incorporated into virions, this cellular enzyme is necessary for the formation of infectious virus. Lassa GP-C is the first viral glycoprotein known to be processed by SKI-1/S1P and its tissue expression pattern in rats revealed highest levels of activity in liver, spleen, and adrenal glands and low levels in brain (Seidah et al. 1999). Accordingly, in human Lassa fever cases, higher viral titers are recovered from liver, spleen, lungs, kidneys, and adrenal but not from brain (Walker et al. 1982a). This finding suggests that specific inhibitors for SKI-1/S1P mediated cleavage of Lassa virus GP-C might lead to new therapeutical approaches for Lassa fever (Lenz et al. 2001).

POLYPEPTIDES: STRUCTURE AND FUNCTION

Arenavirus proteins include the 250-kDa RNA polymerase (L), the 11–14-kDa zinc-binding protein (Z) encoded on the L RNA, the 63-kDa nucleocapsid protein (N or NP) and the 75-kDa glycoprotein precursor (GP-C) encoded on the S RNA (see Figure 4). These four proteins account for the coding capacity of the virus without overlap (Salvato and Shimomaye 1989). The stoichiometry of proteins in the LCM virion has been determined by metabolic labeling (Salvato et al. 1992). Per virion, there are about 30 copies of L protein, 1500 copies of NP, 650 copies of GP-1, 650 copies of GP-2 and 450 copies of Z protein (Figure 8). According to cross-linking studies, NP and Z are associated (Salvato et al. 1992) and NP and GP-2 are associated (Burns and Buchmeier 1991). Recent immunoprecipitation and genetic studies reveal Z:L binding that explains the ability of Z to inhibit transcription (Jacamo et al. 2003).

NP is the most abundant virion protein as well as the most stable and abundant viral protein in infected cells. There are ca. 1500–2000 copies of NP per virion, or one copy per 10–20 bases of viral genomic RNA (Bishop 1990). Among the negative-strand viruses, the nucleocapsid proteins usually function as the primary structural protein of viral cores and in mediating the interaction of the RNA polymerase with the viral RNA. A phosphorylated form of NP has been detected late in acute infection and in greater abundance in persistently infected cells (Bruns et al. 1986), leading to the suggestion that phosphorylation of NP is associated with attenuation of virus production and persistence. Breakdown products of NP have also been detected both in infected cells and in virions. It is conceivable, but not yet demonstrated, that fragments of NP serve as minor structural proteins (Coto et al. 1993). A 28 kDa degradation product of NP has been identified within the nuclei of Pichinde virus-infected cells, but no significance can yet be assigned to this (Howard 1993).

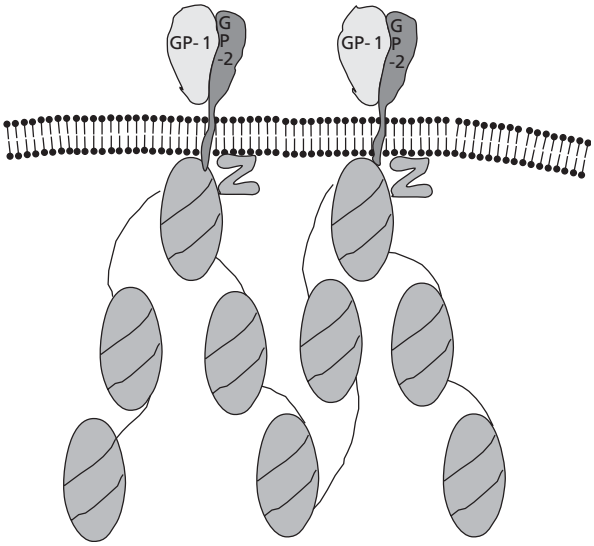

Figure 8 *Hypothetical scheme for the association of structural proteins in the LCM virion. The viral RNA is represented as a string winding around the beads that are the nucleocapsid protein molecules. The Z protein is depicted as a Z associated with the nucleocapsid proteins. GP-2 is anchored in the viral envelope and associated with NP. GP-1 is loosely associated with the surface of the virion. In each virion the proportions of Z, GP-1, and GP-2 in the virion are roughly equivalent, whereas NP is 3- to 5-fold more abundant. The polymerase or L protein accounts for only 30 molecules per virion. (Reproduced from Salvato 1993b)*

For LCMV, the envelope glycoprotein (GP) is synthesized as a 73-kDa precursor and cleaved into 43-kDa (GP-1) and 35-kDa (GP-2) polypeptides (Buchmeier and Oldstone 1979). Cleavage and maturation of the arenavirus glycoproteins, using host enzymes, has been described in the previous section. All arenaviruses that have been examined so far have potential cleavage sites in the glycoprotein precursor and two cleavage products that can be identified in protein gels. Tamiami and Tacaribe viruses are an exception in which only one cleavage product is observed in gels; however, the situation was clarified for Tacaribe virus when it was shown that the two cleavage products comigrate on gels but can be detected by analysis of the peptide termini (Burns and Buchmeier 1993).

The spikes of the virion are composed of GP-1 and GP-2: GP-2 is anchored in the membrane and associated with the nucleocapsid core through its C terminus and GP-1 is loosely attached to the virion surface through ionic interactions. Cross-linking studies indicate that GP-1 and GP-2 are present as homotetramers (Burns and Buchmeier 1991). Sequence analysis and antigenic cross-reactivity indicate that GP-2 contains a highly conserved sequence, whereas GP-1 contains the most variable sequence among the *Arenaviridae* (Weber and Buchmeier 1988; Clegg 1993). With Junin virus, rabbits and guinea-pigs inoculated with GP-2 produce neutralizing antibodies against the infectious virus (reviewed in

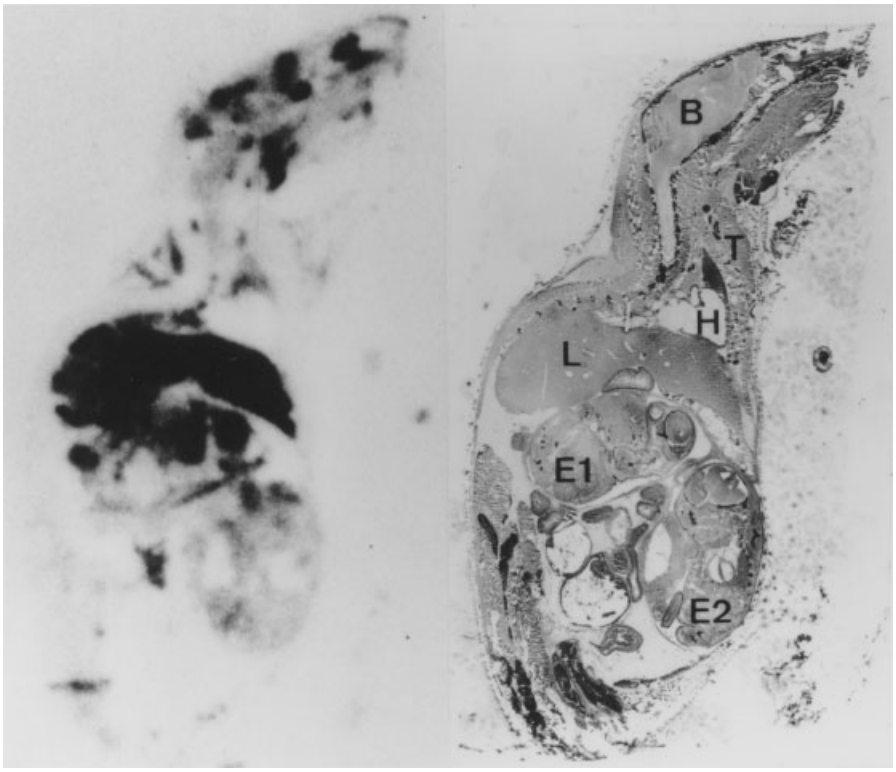

Figure 9 *Sagittal section of a pregnant mouse infected with LCMV. On the left is an autoradiograph of radioactive probes hybridizing to viral RNA within the section. On the right is the stained section: B, brain; E1, one embryo; E2, another embryo; H, heart; L, liver; T, thymus. (Reproduced from Southern et al. 1984)*

Martinez Peralta et al. 1993). In contrast, for LCMV, neutralizing antibodies predominately recognize conformational epitopes within GP-1 (Parekh and Buchmeier 1986) and this recognition depends on glycosylation and retention of disulphide bonds (Wright et al. 1989). It is likely that conformational changes of GP-1 expose domains on the spike glycoprotein that mediate virus entry into host cells (Di Simone et al. 1994).

The arenavirus L protein is the viral RNA-dependent RNA polymerase, is encoded in the 3′ ORF of the L RNA segment and has a molecular mass of 180–250 kDa. The L protein is a component of nucleocapsids since polymerase activity has been associated with these structures. The LAS L protein contains six motifs of conserved amino acids that have been found among arenavirus L proteins and core RNA-dependent RNA polymerase (RdRp) of other segmented negative-stranded (SNS) viruses (*Arena*-, *Bunya*-, and *Orthomyxoviridae*). (Lukashevich et al. 1997). Two regions found in the polymerases of arenaviruses and bunyaviruses are conserved in arenaviruses L proteins.

The zinc-binding protein of 11–14 kDa has a strongly conserved 'ring finger' motif in LCMV, Tacaribe, and Pichinde (Iapalucci et al. 1989; Salvato and Shimomaye 1989; Harnish et al. unpublished). This motif is characterized by the classic zinc-binding residues described in TFIIIA, and additional cysteine and histidine residues that help the molecule form a ring, i.e. C–X2–C–X(9–27)–C–X(1–3)–H–Y2–C–X2–C–X(4–48)–C–X2–C (Freemont 1993). Ring finger proteins generally have *trans*-regulatory functions and have been involved in protein–protein, protein–membrane and protein–nucleic acid interactions. The LCMV Z protein is a significant structural component of the virion and is able to bind isotopic zinc (Salvato and Shimomaye 1989; Salvato et al. 1992). Z protein has been detected in cytoplasm and nucleus of LCMV-infected cells and recent studies revealed that interacts with a nuclear oncoprotein, promyelocyte leukemia protein (PML), modulating the interferon response as well as ribosomal proteins (P0, P1, and P2) and transcription factors (eIF4E) performing regulatory functions in infected cells (Borden et al. 1997, 1998a, b; Campbell Dwyer et al. 2000; Djavani et al. 2001b). Garcin et al. (1993) showed that immunoprecipitation of Tacaribe Z protein blocked transcription whereas de la Torre's minigenome transcription system was inhibited by addition of excess of Z (Cornu and de la Torre, 2001, 2002). Also, the protein Z had a positive role on virus assembly using the same system (Lee and de la Torre 2002) Thus, the Z protein of the arenaviruses is most likely to be a matrix protein.

ANTIGENS

Highly accurate nucleic acid technologies (i.e. sequencing and RT-PCR) have recently superseded the use

of viral antigens in identification and classification of arenaviruses. Nevertheless, complement fixation (CF), immunodiffusion (ID), immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA), and neutralization/plaque-reduction assays have all been instrumental in the detection, diagnosis, characterization, and classification of arenaviruses (Bro-Jorgensen 1971; Brown and Kirk 1969; Chastel 1970; Geshwender et al. 1976; Simon 1970).

CF shows a strong relationship between Tacaribe, Junin, and Machupo viruses, and distant cross-reactivities between Pichinde and Tamiami viruses (Casals 1975; Casals et al. 1975; Rawls and Buchmeier 1975). By the CF test, LCMV and Lassa viruses are related to each other, but very distantly related to the New World arenaviruses. The complement-fixing antigen is probably a portion of the nucleocapsid protein (Howard 1993; Rawls and Leung 1979).

IF with antisera raised against heterologous viral antigens afforded the first evidence that Tacaribe and LCMV are related. Serological cross-reactivities between the 'type-specific' envelope proteins have been determined by IF assays in which unfixed cells, displaying viral envelope antigens, are suspended with antisera. Cross-reactivities between 'group-specific' nucleocapsid proteins are determined from acetone-fixed cells, because the primary antigen within infected cells is NP. Serological cross-reactions in patients with Bolivian and Argentine hemorrhagic fevers are detected by IF tests with fixed cultures. Acute phase and early convalescent sera have been particularly valuable. A great deal of cross-reactivity can be seen between Machupo and Junin viruses, closely followed by Tacaribe virus (Buchmeier et al. 1977).

IF has played an important role in arenavirus diagnosis. In the case of Lassa fever virus, infected cells have been fixed to glass slides that are stable for months and can be used for field work in Africa (McCormick 1987). Infected cells on slides can be treated with UV and γ-irradiation and with acetone to ensure that they are not infectious. ELISA has also been useful for diagnosis in the field, but requires more viral antigen than the fixed-cell slides.

Cross-neutralization is the most specific test of antigen relatedness between arenaviruses. Neutralizing antibodies to Lassa appear late in convalescent sera, but are usually too specific or too weak to be detected in cross-neutralization (Peters 1984). High titer animal sera can effect cross-neutralization between Junin, Tacaribe, and Machupo viruses, but cross-neutralization has not been observed between Junin and Machupo viruses with human convalescent sera, even though these are positive in CF assays. The sensitivity of the neutralization test can be increased for LCMV by adding complement or anti-γ-globulin to the test system. The incorporation of antibodies into the overlay for a plaque assay can also be used to detect neutralizing antibodies; for example, plaque size reduction was described for Pichinde virus (Chanas et al. 1980).

With the advent of monoclonal antibodies, many useful and specific reagents against arenavirus antigens became available. Initially they were used to demonstrate the relatedness or conservation of arenavirus antigens. Panels of monoclonal antibodies against LCMV and Pichinde virus showed distinct cross-reactivities with Lassa and Mopeia viruses (Buchmeier et al. 1981). Antibodies against GP-2 and NP are more broadly cross-reactive than antibodies against GP-1, which react only against a subset of the strains. For example, one LCMV GP-2 monoclonal cross-reacted with both Old World (LCMV, Lassa, Mopeia, and Mobala) and New World (Pichinde, Junin, Tacaribe, Amapari, Parana, and Machupo) viruses (Weber and Buchmeier 1988). In a similar comparison, monoclonals raised to Lassa virus were tested against Mopeia and Mobala viruses, and GP-2 cross-reactivity once again was notable (Gonzalez et al. 1984). Cross-reactivity of NP epitopes between Lassa and Mopeia has been confirmed, along with lesser reactivity to the envelope glycoprotein (Clegg and Lloyd 1984). Broad cross-reactivities observed between epitopes on Pichinde and Old World arenaviruses demonstrate that several surface antigens are conserved between the Old World and New World arenaviruses (Buchmeier et al. 1980b).

In recent studies, monoclonal and monospecific antibodies have been invaluable in determining the molecular structure and function of arenaviruses. For example, nucleic acid sequences have been confirmed to encode proteins by the use of peptide-specific antisera (Southern et al. 1987; Salvato et al. 1989), neutralizing antisera have been used to identify the viral structural proteins involved in neutralization (Burns and Buchmeier 1993, Martinez Peralta et al. 1993).

CLINICAL AND PATHOLOGICAL ASPECTS

CLINICAL MANIFESTATIONS

In animals

All arenaviruses establish persistent infection in the natural rodent host after virus infection in utero or within a few days of birth. Adult mice inoculated intracerebrally with LCMV develop tremor with characteristic extensor spasm of the legs and they finally go into convulsions and die (Oldstone 1987b). When adult mice are inoculated peripherally, the outcome is variable with a marked loss of body weight. Lassa virus causes a silent persistent infection in its natural reservoir *Mastomys natalensis,* in the same way that LCMV infects mice. In hamsters, LCMV causes symptoms similar to those in mice. Guinea-pigs infected with Junin virus develop hemorrhagic disease with extensive necrosis of lymphatic tissue and a late development of neurological

disease (Weissenbacher et al. 1975; Molinas et al. 1978). Guanarito virus causes mortality in suckling mice and adult guinea-pigs, but not in adult mice (Tesh et al. 1994). In rhesus and cynomolgus monkeys, LCMV and Lassa virus cause fever, anorexia, severe petechial rash on the face, progressive wasting and death within about 2 weeks, with a course similar to Lassa fever in humans (Jahrling et al. 1980; Peters et al. 1987). Similar manifestations have recently been described after the intravenous inoculation of rhesus macaques with a low dose (1 000 plaque forming units) of the LCMV WE strain (Lukashevich et al. 2002). Junin and Machupo virus infections of nonhuman primates simulate the disease in humans, with fever, anorexia, weight loss, and gastrointestinal symptoms. The animals die with cachexia and severe dehydration (Eddy et al. 1975; Kastello et al. 1976; Weissenbacher et al. 1979; Avila et al. 1987).

In humans

Arenavirus infections in humans range from a febrile disease with aseptic meningitis with LCMV (Armstrong and Sweet 1939) and Tacaribe (anecdotal laboratory-acquired infection in 1976; see Martinez Paralta et al. 1993) to total collapse and death with circulatory and respiratory failure with the hemorrhagic fever viruses: Lassa fever virus (Rose 1956; Buckley et al. 1970; Frame et al. 1970), Junin virus, Argentine hemorrhagic fever (Maiztegui 1975) and Machupo virus Bolivian hemorrhagic fever (Mackenzie et al. 1964; Johnson et al. 1965), Guanarito virus Venezuelan hemorrhagic fever (Peters et al. 1974) and Sabía virus infection (Coimbra and Nassar 1994). The arenavirus hemorrhagic fevers are often severe, generalized febrile diseases with multi-organ involvement and fatality rates of about 16–30 percent in untreated hospitalized patients, characterized by tissue and pulmonary edema with prominent hypovolemic shock and acute respiratory distress syndrome (Fisher-Hoch 1993). Several of the South American arenaviruses have been found by screening rodent populations but are not associated with human disease; for example, Oliveros virus is carried by 20–30 percent of *Bolomys obscuras* in the Argentine hemorrhagic fever areas (Mills et al. 1996).

LCMV infection may be asymptomatic, mild or moderately severe with central nervous system (CNS) manifestation. Lymphocytic choriomeningitis begins with fever, malaise, weakness, myalgia, and headache associated with photophobia. Anorexia, nausea, and dizziness are common (Farmer and Janeway 1942). It has also been reported that LCMV has teratogenic capacity (Baldridge et al. 1993; Barton et al. 1995)

The onset of Lassa fever is characterized by generalized symptoms, including high fever, joint pain, back pain, and severe headache, leading on to dry cough and exudative pharyngitis. In severe cases, patients have raised hematocrit (packed cell volume), vomiting and diarrhea (Fisher-Hoch et al. 1985; Johnson et al. 1987; McCormick et al. 1987a; Walker et al. 1982a). Edema and bleeding may occur together or independently. Acute neurological manifestations ranging from unilateral or bilateral deafness to moderate or severe diffuse encephalopathy with or without seizures are common in Lassa fever. A febrile, systemic illness typically progresses in 4 days to subacute or chronic neuropsychiatric syndromes, including generalized seizures, dystonia, and memory difficulties or seizures, agitation with personality and cognitive changes (McCormick et al. 1987a; Solbrig 1993). In pregnant women, Lassa fever is severe, especially during the third trimester, and fetal/neonatal loss is 87 percent (Price et al. 1988). In children, Lassa virus infection has been associated with a 'swollen baby syndrome' consisting of widespread edema, abdominal distension, and bleeding (Monson et al. 1987).

Argentine hemorrhagic fever, Bolivian hemorrhagic fever, Venezuelan hemorrhagic fever, and the newly isolated Sabía virus infection are clinically very similar (Mackenzie et al. 1964; Johnson et al. 1965; Peters et al. 1974; Maiztegui 1975; Weissenbacher et al. 1987; Coimbra and Nassar 1994). Symptoms include malaise, high fever, severe myalgia, arthralgia, anorexia, relative bradycardia, lumbar pain, epigastric pain, abdominal tenderness, conjunctivitis and retro-orbital pain, with photophobia. In severe cases there is nausea, vomiting, diarrhea, tremor, and convulsions (Vainrub and Salas 1994). Proteinuria, microscopic hematuria with subsequent oliguria, and uremia are common. Fatal cases show hemorrhagic disorders due to vascular collapse with hypotensive shock, hypothermia, and pulmonary edema. In contrast to Lassa fever, bleeding with severe thrombocytopenia is more common in Argentine and Bolivian hemorrhagic fevers (Fisher-Hoch 1993).

EPIDEMIOLOGY

Arenaviruses are zoonotic agents maintained within rodent hosts in a chronic carrier state. The majority of the rodents associated with arenaviruses are commensals or semi-commensals, living within human dwellings or in cultivated fields. The habitat preferences of each species dictate the epidemiological patterns of the diseases they cause in humans with regard to occupational, sexual, or seasonal bias (reviewed by Childs and Peters 1993).

In the case of LCMV, vertical transmission in utero is the major mechanism of viral maintenance in *Mus musculus* at the population level. Transmission through the gastrointestinal tract is the other possibility (Montali et al. 1993; Rai et al. 1996). Rodent-to-human infections probably occur through aerosols or contact with rodent blood, droplets, and fomites. Nosocomial spread of LCMV has not been reported. A longitudinal study in

the USA from 1941 to 1958 implicated LCMV infections in about 8 percent of the patients diagnosed with suspected viral meningitis, and serological studies have suggested an incidence of LCMV infection in the general population of up to 10–15 percent (Childs and Peters 1993). Most of these infections are probably mild or subclinical. Laboratory infections with LCMV are relatively common.

Human-to-human spread has been reported for Lassa fever in the community and in hospital settings (McCormick et al. 1987b), whereas only a few cases of nosocomial transmission have been reported for Bolivian hemorrhagic fever (Peters et al. 1974) and none for Argentine hemorrhagic fever virus. There have been reports suggestive of sexual transmission of Lassa and Machupo viruses from convalescing patients (Douglas et al. 1965). Aerosol spread and direct contact are the most likely routes of infection between humans. Neonates are at risk of infection through their mother's milk.

The aerosol stability of arenaviruses seems to be high. Studies with Lassa virus indicate a biological half-life of 55 min at 25°C and 30 percent relative humidity or 18 min at 25°C and 80 percent relative humidity (Stephenson et al. 1984). Arenaviruses are susceptible to heat and desiccation.

In natural primary hosts, arenaviruses can establish at least two types of chronic infections. LCMV, Junin, Machupo, and Lassa viruses can establish persistent infections resulting in long-term viremia with few signs of disease in carriers. Other arenaviruses such as Latino virus and Tamiami virus cause chronic infections without persistent viremia (Jennings et al. 1970; Webb et al. 1973). Strain and passage history of arenaviruses inoculated into experimental animals have a marked effect on lethality, tissue tropism, and development of persistent infection (Traub 1938; Dutko and Oldstone 1983). The dose of arenavirus in conjunction with the route of exposure and age of the host can have a critical effect on the type of infection that results (Webb et al. 1975).

PATHOGENESIS

In animals

Adult mice infected intracerebrally with LCMV develop an acute inflammatory leptomeningitis, and choroiditis leading to death within 6–10 days (Oldstone 1987a). When adult mice are inoculated peripherally, viremia peaks about 4–5 days after inoculation, usually causing widespread immune-mediated damage to the meninges, choroid plexus, and ependyma (Cole and Johnson 1975). LCMV is known to infect and replicate in virtually every organ examined, particularly in the spleen, liver, kidney, brain, lung, uterus, thymus, and lymph nodes (Figure 9). Immunopathology characteristic of acute murine infection with LCMV is described in the section entitled Immune response. In newborn mice, LCMV inoculation by any route leads to persistent infection. After replication in the brain, virus enters the circulation to reach tissues, serum, and urine. The pathogenesis of Lassa virus in mice is minimal and similar to infection by LCMV, whereas Junin and Machupo viruses may cause either illness and death or may induce persistence in newborn mice (Webb et al. 1975; Sabattini et al. 1977). Junin and Machupo viruses in their natural rodent hosts, Calomys spp., cause up to 50 percent fatality of infected suckling animals. Machupo virus renders the animals sterile, causes fetal mortality, and also induces hemolytic anemia with significant splenomegaly.

Histological studies of LCMV infection in hamsters and guinea-pigs show that the virus is pantropic, with high titers of virus and little evidence of histological damage. In guinea-pigs, Lassa virus causes myocarditis, pulmonary edema, and hepatocellular damage (Walker et al. 1975; Jahrling et al. 1982). Junin virus is mainly viscerotropic in guinea-pigs without evidence of an immunopathological mechanism. The animals develop hemorrhagic disease with extensive necrosis of lymphatic tissue (Weissenbacher et al. 1975; Molinas et al. 1978). Monkeys infected with Lassa virus have mild hepatic focal necrosis, pulmonary interstitial pneumonitis with interstitial edema, and focal adrenal cortical necrosis (Walker et al. 1982a). In rhesus and cynomolgus monkeys the infection causes death in 10–15 days due to vascular collapse and shock, with mild hemorrhage into mucosal surfaces (Fisher-Hoch et al. 1987). A primate model showing similar hemorrhagic fever was developed for the hepatotropic LCMV strain WE (Lukashevich et al., 2002, 2003).

In humans

The arenavirus hemorrhagic fevers in humans are often severe, generalized febrile diseases with multi-organ involvement. The hemostatic defect in these infections is characterized by platelet dysfunction and loss of integrity of the capillary bed. This presumably causes the leakage of fluids and macromolecules into the extravascular spaces and subsequent hemoconcentration, hypoalbuminemia, and hypovolemic shock. There is no evidence for major hepatorenal failure or organ destruction by direct viral replication. Renal damage in Argentine and Bolivian hemorrhagic fevers is characterized by structural damage in the distal tubular cells and collecting ducts (Cossio et al. 1975). Lungs show large areas of intra-alveolar or bronchial hemorrhage. Microscopic examinations reveal a general alteration in endothelial cells and mild edema of the vascular walls, with capillary swelling and perivascular hemorrhage (reviewed by Fisher-Hoch 1993). Necropsy findings

from a single case of Sabía virus infection show diffuse pulmonary edema and congestion with intraparenchymal hemorrhages, hepatic congestion with focal hemorrhage and necrosis, renal edema and acute tubular necrosis, splenic enlargement and congestion, and massive gastrointestinal hemorrhage (Coimbra and Nassar 1994).

The pathogenesis of CNS disease in arenavirus infections is poorly understood. Seizures, delirium, memory difficulties, and abnormal movements are the final common pathways of many metabolic changes. Failure of postmortem examinations to detect specific pathological changes in the brain of Lassa virus-infected individuals along with lower viral titers than other organs suggests that some of the neurological manifestations of these infections may not result from a direct viral effect but are due to some indirect cause (reviewed by Solbrig 1993). In 1998, Lukashevich et al. showed that Lassa virus could suppress inflammatory response in primates and in human cell cultures (Lukashevich et al. 1999). This is in contrast to guinea-pig studies (Maiztegui 1975) and in support of human studies showing that fatal infection tended to have less TNF, IP-10, and IL-8 (Mahanty et al. 2001)

IMMUNE RESPONSE

The LCMV murine model has proved to be invaluable for the study of acute and persistent infections in a natural host and has also brought to light several principles of viral immunobiology and viral immunopathology. As many of the studies on arenavirus immunology have been done in the LCMV murine model, the virus-specific immune responses in the LCMV context are summarized here.

Adult immunocompetent mice inoculated with LCMV either succumb to the disease or survive with permanent immunity. Neonatally infected mice or immunosuppressed mice inoculated with LCMV develop a lifelong persistent infection. Anti-LCMV antibodies are detectable by day 4 and LCMV-specific cytotoxic T lymphocytes (CTL) peak around 7–9 days after infection (Hotchin 1971; Lehmann-Grube 1971; Bro-Jorgensen and Volkert 1974).

Protection against an acute LCMV infection is mediated almost exclusively by $CD8^+$ CTLs (Zinkernagel and Doherty 1979; Buchmeier et al. 1980a; Lehmann-Grube et al. 1988). The same CTLs may also cause immunopathology (Hotchin 1962; Cole et al. 1972; Doherty and Zinkernagel 1974; Zinkernagel and Doherty 1979; Leist et al. 1988). The kinetics of viral spread and the T-cell response are important factors that affect the outcome of the disease (Hotchin 1962; Buchmeier et al. 1980a). After intracerebral infection, the T-cell immune response is protective only when a large number of T cells are recruited early, while relatively few choriomeningeal cells are infected. When virus spreads rapidly and too many choriomeningeal cells are infected, immune T cells cause extensive lysis and therefore lethal immunopathological disease (Oehen et al. 1991; Battegay et al. 1992).

During the acute LCMV infection, the H-2-restricted, LCMV-specific $CD8^+$ CTL response peaks at 7–9 days after infection (Marker and Volkert 1973; Zinkernagel and Doherty 1974). These responses are accompanied by and depend on high levels of *IL-2* gene transcription, IL-2 production and expression of the high affinity form of the IL-2 receptor (Kasaian and Biron 1990). A secondary stimulation of LCMV-immune mice with homologous virus results in a memory CTL response peaking earlier than in primary infections (Lehmann-Grube and Lohler 1981). The magnitude of secondary CTL response is often less than the acute response, probably because the virus is cleared so rapidly. $CD8^+$ cells have a functional autonomy during the immune response to LCMV infection and do not depend on the presence of the majority of $CD4^+$ cells to sustain either activation or proliferation in vivo (Kasaian et al. 1991).

In contrast to the fatal meningitis mediated by $CD8^+$ T cells, $CD4^+$ T cells mediate a less severe form of choriomeningitis in response to intracerebral LCMV infection. Evaluation of the immune response against LCMV in CD8-defective mice reveals a $CD4^+$ T cell-dependent immunopathology after intracerebral infection with LCMV and the clearance of LCMV by mechanisms independent of $CD8^+$ T cells (Fung-Leung et al. 1991).

Mice persistently infected with LCMV by congenital infection or neonatal inoculation do not develop a detectable CTL response and can be regarded as 'tolerant' at the level of CTLs. The characteristically subclinical persistent infections of these animals have given rise to the notion that persistence requires B-cell tolerance (Hotchin 1971). However, these mice are not completely tolerant to LCMV, because they make antibodies to all the LCMV structural proteins (Oldstone and Dixon 1967; Buchmeier et al. 1978).

LCMV infection in the mouse has been important for the study of $CD8^+$ T cells because it induces a large T-cell response that can be identified using ex vivo cytolytic assays and, more recently, Elispot and Tetramer assays, to confirm the size and specificity of the early expansion of T-cell populations. Tetrameric peptide–MHC-class-I complexes (tetramers) allow antigen-specific T cells to be tracked in time and space, as well as a detailed analysis of their surface phenotype (Altman et al. 1996).

Of all the potential peptides, one dominant epitope elicits a response up to 30 percent of the $CD8^+$ T cells in the spleen with little evidence of bystander activation (Klenerman et al. 2002). In several strains different epitopes have been identified with similarly dominant T-cell responses (Murali-Krishna et al. 1998; Gallimore et al. 1998).

The response of T cells to antigen during LCMV infection in the mouse seems to follow a bell-shaped curve with increasing antigen leading to increased T-cell activation. In conditions of extreme or prolonged antigenic stimulation, tetramer-positive T cells lose their function and then decline in number. This situation is reversible and cells recover if antigen load decreases. Lack of help, lack of costimulation and other factors might contribute to T-cell deletion as well (Ou et al. 2001).

In Lassa fever there is a substantial macrophage response, with little if any lymphocytic infiltrate. The antibodies directed against the glycoprotein and nucleocapsid protein appear early in the illness in Lassa fever, with a classic primary IgG and IgM response. The antibodies are present simultaneously with viremia in humans and primates (Fisher-Hoch et al. 1987; Johnson et al. 1987). Thus, though the antibodies are markers of acute illness, they are not involved in recovery. Neutralizing antibodies are found in only a minority of patients several months after the clearance of virus (Jahrling et al. 1980; Jahrling and Peters 1984). Thus, clearance of virus presumably depends on cell-mediated immunity. In contrast, IgG antibodies to Junin and Machupo viruses appear 12–30 days after onset and are usually associated with clinical improvement and virus clearance, and the therapeutic efficacy of immune plasma in patients with Junin infection is directly associated with the titer of neutralizing antibodies in the donor plasma (Peters et al. 1973; Enria et al. 1984). The role of cell-mediated immunity in viral clearance and subsequent protection from Junin and Machupo virus infections is not known.

IMMUNOPATHOLOGY

Efficient cell-mediated immunity is crucial for the recovery of a host from acute LCMV infection (Zinkernagel and Doherty 1979; Buchmeier et al. 1980a). In infections with a noncytopathic virus such as LCMV, the balance between the virus spread and the T-cell immune response determines whether either virus elimination (protection) or cell and tissue damage (immunopathology) predominates or whether a virus carrier state (no virus elimination, no cell and tissue damage) results. Acute LCM disease, chronic LCM-wasting disease and LCM carrier status in mice (Hotchin 1962; Zinkernagel and Doherty 1979; Buchmeier et al. 1980a) are examples of these varying equilibrium conditions between virus and immune response.

Effectors of immunopathology

Intraperitoneal or intravenous infection of adult mice with LCMV usually results in a virus-specific CTL response that clears the virus with long-lasting immunity, whereas intracerebral inoculation results in a lethal leptomeningitis associated with virus replication in the brain and an intense mononuclear cell infiltrate. Death and the associated meningitis are blocked if the mice are treated with thymectomy, immunosuppressive irradiation or immunosuppressive drugs such as cyclophosphamide; this led to the suggestion that the disease is mediated by the immune response (Haas and Stewart 1956; Rowe et al. 1963; Gilden et al. 1972). Subsequent investigations have supported the hypothesis that the pathology in acute LCMV disease is mediated by cellular immunity (Volkert and Lundstedt 1968; Cole et al. 1972; Gilden et al. 1972).

Interferon (IFN) increases the susceptibility of LCMV-infected fibroblasts to lysis by H-2-restricted CTLs (Bukowski and Welsh 1985). This enhanced lysis correlates with increased cell surface expression of MHC antigens but not of LCMV antigen. IFN-γ is an especially potent inducer of MHC expression in most cell types (Wong et al. 1984). This indicates that the limiting component in the lytic interaction is the histocompatibility antigen and that physiological influences on the expression of antigen may affect the progress of host-mediated disease. Treatment of adult mice infected intracerebrally with LCMV with antibody to IFN prevents their death, whereas untreated control mice succumb to the same dose of lethal LCMV, thus showing the role of IFN in the pathogenesis of cell damage in murine LCM (Pfau et al. 1983). The promyelocytic leukemia protein (PML) is a mediator of arenavirus sensitivity to IFN (Djavani et al. 2001a; Bonilla et al. 2002).

Murine LCMV infection is also considered the classic example of virus-induced immune complex disease. Persistent LCMV infection with circulating non-neutralizing antibodies in ratios and quantities that favor binding of complement and deposition in tissues results in immunopathology. This state is affected by host and viral genetic factors (Oldstone et al. 1983). Large quantities of deposited immune complexes along with IFN-γ seem to cause development of glomerulonephritis (Walker and Murphy 1987). Strains of mice characterized as intermediate IFN responders, such as outbred Swiss mice, have higher levels of circulating immune complexes and develop much more severe immune complex-associated glomerulonephritis than strains of low IFN responder mice, such as BALB/c mice, after LCMV infection (Woodrow et al. 1982). Antibodies to IFN diminish glomerular damage without affecting levels of circulating immune complexes (Pfau et al. 1983).

Progress of central nervous system pathology

$CD8^+$ T cells along with macrophages are thought to be the main cells that contribute to meningeal inflamma-

tion after LCMV infection (Allan et al. 1987). In addition, natural killer (NK) cells, $CD4^+$ T cells, neutrophils, and B cells are also present at the site of inflammation (Johnson et al. 1978). Large numbers of white blood cells, including CTLs, NK cells, and macrophages, are found in the cerebrospinal fluid (CSF), brain tissue, and meningeal exudate. Although the integrity of the blood–brain barrier is destroyed, the relatively non-lytic LCMV itself is unlikely to disrupt the barrier (Doherty 1973; Doherty and Zinkernagel 1974). It is more likely that the virus-induced IFN-γ initiates the infiltration of inflammatory cells across the blood–brain barrier.

The presence of CTLs in CSF has been shown by functional studies and by immunocytochemistry (Zinkernagel and Doherty 1973). CTL activity is also present in the CSF 3 days after immune-cell transfer to LCMV-infected, immunosuppressed animals. After CNS infection with LCMV, high levels of NK-cell activity are detected in CSF and cervical lymph nodes. However, meningitis presumably does not occur before development of T cell response, as NK cells probably do not play an essential role in LCMV-induced immunopathology (Allan and Doherty 1986). NK cells do not influence LCMV synthesis in vivo, but do decrease the level of Pichinde virus (Welsh 1987; Brutkiewicz and Welsh 1995).

The CTLs present in cyclophosphamide-suppressed LCMV-infected mice after passive transfer of immune cells are of donor and not of recipient origin. So $CD8^+$ T cells also seem to be essential for triggering infiltration into the CNS. Presumably, for a maximal inflammatory process to occur, MHC class I-restricted $CD8^+$ T cells proliferate first in the peripheral lymphoid tissue and then enter the brain after recognition of MHC class I-compatible infected cells at the blood–CSF barrier (reviewed by Allan et al. 1987).

Immunosuppressed mice that survive an ordinarily lethal dose of LCMV develop acute LCM disease and die when reconstituted with syngeneic LCMV-specific immune T cells (Gilden et al. 1972). Thus, it is possible to imitate the immunopathology observed in acute LCMV infection by inoculating LCMV-specific H-2-restricted CTLs into carrier mice, which in turn cause either viral clearance and recovery or CTL-mediated immunopathology and death (Allan and Doherty 1985). $CD8^+$ T cells act directly rather than by the recruitment of circulating monocytes. Lethal LCM can be prevented in the uninfected mouse by intracerebral inoculation of cloned $CD8^+$ T cells together with the virus (Baenziger et al. 1986). The co-injected $CD8^+$ T cells gradually eliminate virus-infected cells without causing the massive inflammation associated with acute LCM disease. Severe meningitis ensues in adoptive transfer of LCMV-specific CTLs only when there is matching of MHC class I antigens between donor and recipient mice. In comparison, compatibility of MHC class II antigens is less important (Doherty et al. 1976; Allan and Doherty 1985).

Immunosuppression

MECHANISM OF IMMUNOSUPPRESSION

Acute infection of adult mice with LCMV leads to a general suppression of the immunological and mitogenic responses in vivo and in vitro, which persists for 2–3 months after infection (Mims and Wainwright 1968; Bro-Jorgensen and Volkert 1972; Jacobs and Cole 1976; Brenan and Zinkernagel 1983). Although different mechanisms have been postulated to explain this phenomenon, it is still poorly defined. LCMV infects different types of lymphocytes (Gartner et al. 1986; Rosenthal et al. 1986; Ahmed et al. 1987b) and macrophages (Mims and Subrahmanyan 1966; Zinkernagel and Doherty 1979). The ability of LCMV to induce a virus carrier state conatally or neonatally and in adult mice may be due to these particular tropisms of LCMV (Ahmed et al. 1987b). Depending on the virus isolate, the virus dose, the time after infection, and the mouse strain infected, LCMV can cause a severe immune deficiency, rendering the mice more susceptible to secondary infections.

Mice persistently infected with LCMV are deficient in generating LCMV-specific CTLs and delayed type hypersensitivity responses and also make low levels of antibodies against LCMV (Buchmeier et al. 1980a). LCMV-specific immune responses are inhibited by a lack of appropriate T-helper cells, because although deletion of $CD4^+$ T cells in a transgenic mouse model does not impair the primary $CD8^+$ LCMV-specific CTL response (Rahemtulla et al. 1991) it does impair T-cell memory (Matloubian et al. 1994). The different levels of cytokines in the brain and sera of acutely and persistently infected mice indicate that the two types of infections probably activate different types of cells.

A mechanism for generalized immunosuppression has been elucidated in the case of a particular LCMV isolate. For this isolate, LCMV Armstrong clone 13 (Ahmed et al. 1984), virus replicates to high titer in the spleen, causing immune-mediated destruction of dendritic cells; hence loss of the cells needed for immune responses to other pathogens (Tishon et al. 1993; Borrow et al. 1995). Clonal deletion of LCMV-specific CTLs may occur subsequently (Moskophidis et al. 1993). The long-term effect of clone 13 infection is that reduced immune response capacity allows the virus to persist, in contrast to infection with other isolates of this strain that are cleared within weeks by a vigorous cellular immune response (Salvato et al. 1991). The persistent and rapidly cleared isolates of LCMV Armstrong are analogous to the Docile (viscerotropic) and Aggressive (neurotropic) isolates of LCMV UBC in which docility is associated with the tendency for high titer replication in the viscera that leads to suppression of the CTL response and persistent infection (Thomsen and Pfau 1993). It is important to note that viral tropism

is crucial to the pathogenesis in these cases. The different tropisms of LCMV Armstrong and its variant isolate, clone 13, are shown in Figure 10.

Suppression of cell-mediated immunity

LCMV infection causes suppression of responses to T-dependent antigens in vivo (Mims and Wainwright 1968) and to allografts (Lehmann-Grube et al. 1972; Guttler et al. 1975). This may be due to a decreased number of mature T lymphocytes in lymphoid organs as a result of an alteration of T-progenitor cells in the bone marrow (Bro-Jorgensen and Volkert 1972; Thomsen et al. 1982). Alternatively, it is suggested that the immunosuppression may be related to the lysis of infected T lymphocytes and macrophages by virus-specific CTLs (Silberman et al. 1978). In addition to unresponsiveness to antigens, the proliferative response to T- and B-cell mitogens is also depressed and a virus-induced macrophage defect is probably responsible for the abortive collaboration with T lymphocytes (Jacobs and Cole 1976).

Suppression of humoral immunity

The suppression of T-independent IgM and a strictly T help-dependent IgG immune response against a second infectious agent is observed in immunocompetent adult mice infected with LCMV but not in tolerant LCMV carrier mice or LCMV-infected mice that have been depleted of $CD8^+$ T cells with monoclonal antibodies. Thus, the immune suppression is due not to LCMV itself nor to IFN induced by it but rather to the $CD8^+$ T cell-dependent immune response against LCMV (Leist et al. 1988).

Studies of the kinetics of T cell-mediated immunosuppressive effects of LCMV infection on primary and secondary antibody responses (to Vesicular stomatitis virus (VSV)) show that LCMV-WE induced immunosuppression is absolute for primary IgG responses induced during a limited time between days 2 and 11 after LCMV infection. The kinetics of induction of the T cell-independent IgM responses closely follow that of a normal CTL response to LCMV-WE (Roost et al. 1988). LCMV infection on the same day or before (but not after) VSV infection leads to suppression of IgG responses to VSV. However, primed IgG responses are not suppressed by a subsequent LCMV-WE infection and immunosuppression is not antigen specific but general (Ruedi et al. 1990). LCMV-induced suppression is absolute to the extent that priming does not occur, since subsequent challenge with the identical VSV serotype triggers a strictly primary response.

The severity and duration of immunosuppressiveness depend on the virus dose, the virus isolate, and the mouse strain used. LCMV-WE and LCMV Docile are the most and LCMV Armstrong the least immunosuppressive. Mouse strains differ considerably with respect to extent of suppression, depending on both the MHC and non-MHC genes. H-2q and H-2k mice seem to be more susceptible to immune suppression than H-2b or H-2d mice (Roost et al. 1988).

DIAGNOSIS

Diagnosis of arenavirus infection relies mainly on isolation and subsequent identification of the virus. Serological tests can also be used to detect specific antibodies or antigens.

Virus can be isolated mainly from the blood or serum. In addition, throat swabs, CSF, urine, breast milk, or other tissues taken by biopsy or at necropsy can also be used (Monath et al. 1974; Walker et al. 1982b; Johnson et al. 1987). The most successful system for isolating arenaviruses has proved to be cell culture. In addition to cell culture, laboratory animals such as suckling mice, guinea-pigs, and hamsters can also be used, although they are more expensive and increased safety precautions are necessary. The probability and timing of virus isolation vary with each arenavirus (Table 2).

Differential diagnosis for arenavirus infections, especially for Lassa fever, includes bacterial septicemia, typhoid, paratyphoid, typhus, trypanosomiasis,

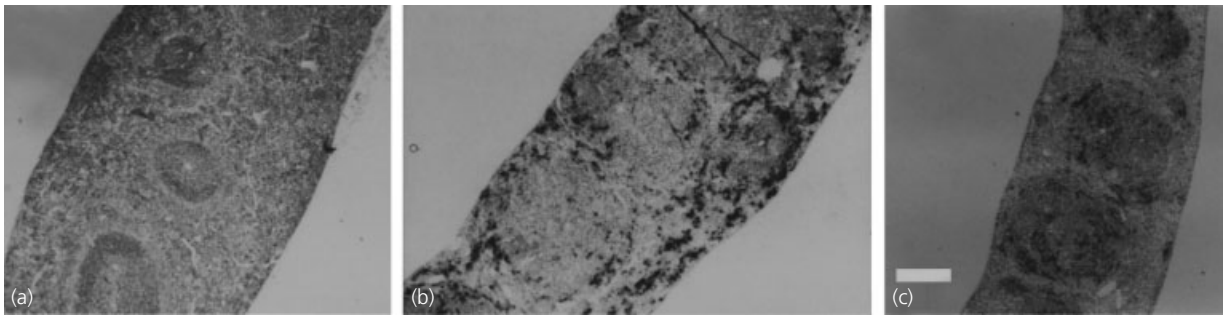

Figure 10 *Localization of LCMV nucleic acids in murine spleen by in situ hybridization with ^{35}S riboprobe specific for NP mRNA.* **(a)** *Spleen section from an uninfected control mouse (BALB/c ByJ).* **(b)** *Spleen section from a mouse infected IV with LCMV Armstrong for 3 days.* **(c)** *Spleen section from a mouse infected IV with the clone 13 variant of LCMV Armstrong for 3 days. (Reproduced from Borrow et al. 1995.)*

Table 2 *Arenavirus isolation from tissue specimens at different stages of illness*

Virus	Tissue specimen	Stage of illness
LCMV	Blood	Initial febrile stage
	CSF	During acute illness
Junin	Blood	Up to 8 days in milder cases, up to 2 weeks in severely ill patients
	Throat swab	During acute illness
	Breast milk	During acute illness
Machupo	Blood	Up to 2 weeks (intermittent viremia)
	Throat swabs	Sporadic detection
Lassa	Blood	3–5 days in milder cases, up to 4 weeks in severely ill patients
	Throat swabs	Days 5–12 of illness
	Breast milk	During acute illness
	CSF	During acute illness
	Pleural transudate	During acute illness
	Pericardial transudate	During acute illness
	Urine	Up to 1– 2 months

Data adapted from McCormick (1990).

streptococcal pharyngitis, leptospirosis, malignant malaria, and other viral hemorrhagic fevers (Howard and Simpson 1990). Nucleic acid amplification by RT-PCR along with Southern blot hybridization with virus-specific DNA probe can be used as an early, rapid, and sensitive method for detecting arenavirus nucleic acid in peripheral blood mononuclear cells (PBMC) as well as from other tissues. Studies with Junin virus-infected PBMCs and tissues have shown that this assay allows detection of Junin virus RNA in RNA extracted from 100 μl of whole blood by guanidinium thiocyanate disruption and acid phenol extraction. As little as 0.01 pfu of Junin virus can be detected in a blood sample by this assay (Bockstahler et al. 1992; Lozano et al. 1993). In situ hybridization by viral sequence-specific radioactive probes can be done directly on biopsy or autopsy specimens to detect viral RNA and has proved to be highly sensitive in experimental LCMV infections of mice (see Figure 9).

Antibodies to arenaviruses can be detected in serum by a variety of serological methods, including IF assay, ELISA, immunoperoxidase labeling, radioimmunoassay, reverse phase hemagglutination assay, and hemagglutination inhibition tests. The complement fixation test is now rarely used because it is less sensitive than other methods (serological tests and antibody levels in arenavirus infections have been reviewed by McCormick 1990). IF is often used in serological diagnoses to detect arenavirus antigens in cell monolayers that have been exposed to suspect sera. At present, ELISA is increasingly used for the diagnosis of arenavirus infections. Alternatively, known antiserum or monoclonal antibodies can be used to detect viral antigens in biopsy or necropsy materials by immunohistochemical techniques.

PROPHYLAXIS

The development of a safe and effective vaccine for arenavirus infections of humans has proved difficult. Several killed and live-attenuated vaccines have been tested for Lassa, Junin, and Machupo viruses, none of which has proved suitable for widespread human use. Many of these vaccines are still in the stage of animal trials. A new live-attenuated Junin virus vaccine, Candid:1, has been developed which has been shown to be safe and immunogenic in nonhuman primates (Barrera Oro and McKee 1991; Contigiani et al. 1991; McKee et al. 1993). Laboratory animals infected with various avirulent viruses serologically related to Lassa virus, including LCMV, Mopeia, and Mobala viruses, survived a subsequent challenge with virulent Lassa virus (Walker et al. 1982a; Jahrling and Peters 1986). Similar strategies for protection against Junin virus with heterologous live vaccines have repeatedly demonstrated protection against Junin virus in guinea-pigs and hamsters (reviewed by Barrera Oro and McKee 1991). Other vaccine trials with inactivated Lassa and Machupo viruses have given mixed results, although they are immunogenic. Immunization with inactivated Lassa virus protects *Papio hamadryas* monkeys from a subsequent challenge with Lassa virus (Krasnianski et al. 1993), but fails to protect rhesus monkeys even though there is a secondary, high titer antibody response to the major structural proteins of Lassa virus in these vaccinated monkeys (McCormick et al. 1992).

For Lassa virus, a killed antigen vaccine has proved ineffective and an attenuated virus vaccine is not available, so a live recombinant virus vaccine provides a very attractive alternative. Recombinant vaccinia virus vaccines, which express either the Lassa virus nucleoprotein or the glycoprotein gene, successfully protect guinea pigs from a lethal Lassa virus infection, but offer incomplete protection in primates (Auperin et al. 1988; Morrison et al. 1989; Auperin 1993). Fisher-Hoch et al. (2000) have tested a variety of Lassa vaccines delivered to nonhuman primates via the NYBH vaccinia vector. In this study, the authors described the outcome after the

vaccination of 44 monkeys with Mopeia or with vaccinia expressing Lassa S segment genes (*G1*, *G2*, *N*, *G1+G2* or combinations of them). A third of the monkeys were Cynomolgus macaques and the remainder were rhesus and all of them were challenged with a lethal dose (10^5 pfu) of Lassa (Josiah strain). The data indicate that vaccines delivering all genes of the Lassa S RNA (both *N* and *G*) are more protective than vaccines with only the glycoprotein genes and, later, these are more protective than vaccines with only the *N* gene. From these experiments it is apparently clear that animals with high titers of neutralizing antibodies are not protected whereas others that survived did not show high levels of neutralizing antibodies, this finding supports the need for cellular immune response to protect against the Lassa challenge (Fisher-Hoch et al. 2000). A weak but measurable cross-protection against LCMV intracranial challenge can be mediated by Lassa-specific $CD4^+$ T cells (La Posta et al. 1993; Djavani et al. 2000, 2001b). Vaccine trials so far have suggested that cell-mediated immune response must be activated to protect against challenge with arenaviruses.

Immunization with recombinant vaccinia virus that expresses the LCMV glycoprotein (VV GP) or nucleoprotein (VV NP) protects mice from LCM disease by induction of a protective CTL response in an H-2 haplotype-dependent manner (Hany and Oehen 1989; Klavinskis et al. 1990; Oehen et al. 1991). Mice can be specifically protected by subcutaneous inoculation of recombinant LCMV proteins (GP or NP) or just the T-cell epitope of the LCMV nucleoprotein as an unmodified free synthetic peptide in incomplete Freund's adjuvant (Schulz et al. 1991; Bachmann et al. 1994). Vaccination with DNA encoding the LCMV nucleoprotein or the glycoprotein also confers protection against lethal LCMV challenge and against persistent LCMV infection in an MHC-dependent manner by priming $CD8^+$ cytotoxic lymphocytes (Martins et al. 1995; Yokoyama et al. 1995).

In certain circumstances however, immunization with VV GP or VV NP aggravates disease. For example, BALB/C mice infected with a high dose of the LCMV Docile isolate usually survive, unless they are preinjected with VV NP or VV GP (Oehen et al. 1991). This suggests that low level immunization may accelerate development of disease. Vaccination may shift the balance from low (i.e. late) to high (i.e. early) responder status and may therefore prevent immunopathologically mediated disease, or it may shift the balance only slightly from a nonresponsive asymptomatic carrier to a low or intermediate responder status to cause immunopathology. Thus, as well as illustrating the potential value of CTL vaccines, these vaccine studies also highlight the limitations of subunit vaccines. To protect an outbred population in an MHC-restricted fashion, it will be necessary to make a vaccine that consists of a cocktail of relevant peptides and to ensure that none of its components aggravates the disease in a subsequent virus challenge.

TREATMENT

Immunotherapy

LCMV-induced persistent infection in mice is a classic example of viral persistence and serves as a model to study basic principles of immune clearance in persistent and disseminated infections in general. This model system makes it possible to test the potential of specific immune therapy to clear virus from a chronically infected host and to study the effector mechanisms responsible for clearing such infections.

Volkert (1963) was the first to show that the adoptive transfer of spleen cells from LCMV-challenged immune adult mice results in reduction of infectious virus in carrier mice. This has been confirmed by a number of workers (Gilden et al. 1972; Allan and Doherty 1985; Baenziger et al. 1986). Distinct patterns of viral clearance and histopathology are observed in different organs after adaptive immunotherapy of persistently infected (carrier) mice. The clearance of viral materials from the CNS is distinct in pattern and timing with clearance from other organs. Clearance from the liver, lung, spleen, lymph nodes, pancreas etc. occurs within 30 days, whereas in the brain, infectious virus is eliminated but viral antigen persists up to 90 days after immunotherapy (Ahmed et al. 1987a). The urinary system is the most resistant to immunotherapy, and the viral antigen is localized within the renal tubules in the form of antigen–antibody complexes (Oldstone and Dixon 1967).

Clearance of viral materials (infectious virus, viral nucleic acid and proteins) from several organs of persistently infected mice probably occurs by reconstitution of LCMV-specific CTLs that have malfunctioned or have been deleted during viral infection. By using mice that are recombinant in the H-2 region and by selective depletion of lymphocyte subpopulations, it has been shown that viral clearance is mediated by cooperation between virus-specific $CD8^+$ T cells and nonspecific bone marrow-derived mononuclear cells from the carrier host (Ahmed et al. 1987b). The effector mechanisms responsible for eliminating the persistent and disseminated LCMV infection of mice are dependent on the lytic ability of CTLs, because perforin-negative transgenic mice are unable to clear infection (Kagi et al. 1994).

Early success of Lassa virus immune plasma in the treatment of Lassa fever (Leifer et al. 1970) and immunotherapy of Machupo virus infections in primates (Eddy et al. 1975) showed promise for the treatment of arenavirus infections in humans. Convalescent phase plasma from Junin virus patients reduced mortality from 16 to 1 percent in those who were treated in the first 8 days of illness (Maiztegui et al. 1979), and the efficacy of

the plasma seemed to be directly related to the concentration of neutralizing antibodies of the plasma. However, a better understanding of the limitations of this approach and reduced success in subsequent cases have restricted its use. A late neurological syndrome developed 4–6 weeks after the onset of acute illness in about 10 percent of the cases treated with Junin virus immune plasma. Passive antibody therapy depends on collection of plasma from people known to have had the disease, testing the plasma or screening the donor for antibodies to bloodborne agents such as hepatitis, and proper storage of plasma until it is used. In addition, the existence of HIV and other retroviral diseases transmissible by blood products has made the further screening of plasma mandatory before use. A study of combined antibody and ribavirin therapy has been performed in the mouse model (Seiler et al. 2000).

Antiviral agents

The antiviral drug ribavirin has proved effective in the treatment of Lassa fever in laboratory animals (Jahrling et al. 1980; Jahrling et al. 1984) and in humans (McCormick et al. 1986), especially when administered during the first 6 days after the onset of illness. Later, the pathogenesis of the infection is less reversible. Patients presenting late in disease require more effective clinical management of physiological dysfunction and need other drugs which may be used to stabilize the state of shock sufficiently long to facilitate recovery and survival. Ribavirin is perhaps more effective if given intravenously than orally (McCormick et al. 1986; McCormick 1990). It is the drug of choice for treatment and for prophylaxis in cases of possible exposure to Lassa virus, in laboratory or hospitals. Studies with Junin virus infections indicate that ribavirin may also have beneficial effect in Argentine hemorrhagic fever (Enria and Maiztegui 1994). A single case of laboratory-acquired Sabía virus infection was successfully treated with intravenous ribavirin (Barry et al. 1995) at a dosage recommended by the Centers for Disease Control and Prevention (CDC) for other arenavirus infections (a loading dose of 30 mg/kg body weight, followed by a dose of 15 mg/kg every 6 h for 4 days, and then by a dose of 7.5 mg/kg 3 times daily for 6 days).

In addition, fluid, electrolyte, and osmotic imbalances must be corrected in anticipation of the development of clinical shock and broad spectrum antibiotics administered to prevent secondary bacterial infections. However, even vigorous support of this kind may be insufficient to prevent fatal progression of disease.

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Filoviruses

HEINZ FELDMANN AND HANS-DIETER KLENK

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PROPERTIES OF FILOVIRUSES

Introduction

In 1967, an outbreak of viral hemorrhagic fever occurred among laboratory workers in Europe exposed to tissues and blood from African green monkeys (*Cercopithecus aethiops*) imported from Uganda (Siegert et al. 1967; Martini and Siegert 1971). The virus, which had been isolated from a number of patients, has been called Marburg virus (MARV), after the city with the first reported cases. This episode marked the first identified outbreak of disease caused by a member of a group of viruses that have been classified in the family *Filoviridae*.

It was nearly a decade later, in 1976, when two other major filovirus outbreaks occurred in southern Sudan and northern Zaire (now Democratic Republic of the Congo). These outbreaks occurred simultaneously, but involved two biologically distinct species, Sudan ebolavirus (SEBOV) and Zaire ebolavirus (ZEBOV), of a new filovirus, Ebola virus (EBOV) (World Health Organization 1978a, 1978b; McCormick et al. 1983). In 1989, a third species of Reston ebolavirus (REBOV) emerged as the causative agent of an epizootic among a group of cynomolgus monkeys (*Macaca fascicularis*) imported from the Philippines into the United States (Jahrling et al. 1990). A similar epizootic occurred in 1992 when cynomolgus monkeys were imported into Italy from the same supplier who shipped the 1989 monkeys to the USA (World Health Organization 1992). In contrast to other human filoviruses, REBOV seemed to be less pathogenic, and no disease occurred in four humans who were infected with this species of EBOV (Peters et al. 1993). This is also reflected in a lower pathogenicity for nonhuman primates (Fisher-Hoch and Brammer 1992). EBOV was also detected in western Africa (Ivory Coast) in 1994, when a single human case was identified and a novel species Côte d'Ivoire ebolavirus (CIEBOV) was isolated (LeGuenno et al. 1995). This person was presumed to have been infected with EBOV while she was performing a necropsy on a wild chimpanzee, whose troop had undergone increased mortality, presumably due to EBOV disease.

In the past years EBOV and MARV have caused several major outbreaks of hemorrhagic fever in Central Africa. In 1995 EBOV re-emerged in the Democratic Republic of the Congo, causing a severe outbreak of hemorrhagic fever in the city of Kikwit and surrounding villages in Bandundu Province. A total of 315 cases of EBOV hemorrhagic fever were reported, of whom 244 died (77 percent) (World Health Organization 1995a). Molecular analyses identified the causative agent as ZEBOV, the glycoprotein gene differing by only 1.6 percent from the virus that caused the 1976 outbreak in northern Zaire (Democratic Republic of the Congo) (Sanchez et al. 1996). From 1994 to 1997 Gabon has suffered from three EBOV epidemics that occurred in the northeastern part of the country (Makokou). Virus isolation and sequence analysis demonstrated that all epidemics were caused by ZEBOV (Georges et al. 1999). In 1998, MARV emerged in the northern part of the Democratic Republic of the Congo near the border to Uganda and Sudan. More than 100 cases were reported with a mortality of about 70 percent (Muyembe-Tamfum 2000; Nichol 2000; Bausch et al. 2003). In October 2000, EBOV emerged in northeastern Uganda. The virus has been identified as species

SEBOV. Four hundred and twenty-five cases were confirmed with 224 deaths (World Health Organization 2001). There is currently (spring 2003) an EBOV outbreak ongoing in Congo-Brezaville that has so far claimed more than 100 human lives and has killed large parts of the local gorilla and chimpanzee population.

In addition to the major outbreaks described above, sporadic cases of filovirus hemorrhagic fever in humans have occurred in various parts of Africa. Incidences of filovirus disease in humans that have been confirmed by virus isolation are summarized in Table 1.

Classification

Filoviruses are classified in the order *Mononegavirales*, a large group of viruses that have nonsegmented negative-strand (NNS) RNA as their genomes. The family *Filoviridae* was created on the basis of unique morphologic, morphogenetic, physicochemical, and biological features of its members (Kiley et al. 1982). The family harbors two genera, *Marburgvirus* and *Ebolavirus*. The genus *Marburgvirus* is more homogeneous and consists of only a single species, but at least two different genetic lineages co-exist (Kiley et al. 1988; van Regenmortel et al. 2000; Sanchez et al. 1998a). The genus *Ebolavirus*, however, can be subdivided into four species: *Côte d'Ivoire*, *Reston*, *Sudan*, and *Zaire* (CIEBOV, REBOV, SEBOV, ZEBOV) (Feldmann et al. 1997; Sanchez et al. 1996; Feldmann et al. 2004). Nucleotide sequence comparison between MARV and EBOV shows only scattered similarities, which is in contrast to strong similarities seen between amino acid sequences of the structural proteins. Despite this amino acid similarity, there is no indication that there is any significant serological (antigenic) cross-reactivity between EBOV and MARV, but the species of EBOV share common epitopes (Richman et al. 1983; Feldmann et al. 1994). It may be that the nucleotide sequences of these agents diverged at some point in the distant past but the structural proteins have maintained similar structures and functions. Significant differences within the genus *Ebolavirus* were first noted from peptide and oligonucleotide mapping (Buchmeier et al. 1983; Cox et al. 1983), and have been confirmed by sequence analysis of the glycoprotein genes (Sanchez et al. 1996). The study by Sanchez et al. showed that all four species differ from each other to a comparable extent (37–41 percent nucleotide differences). This suggests that filoviruses have evolved into specific niches and may reflect a similar divergence in the natural hosts, assuming they have co-evolved.

Molecular analyses of the genomes of filoviruses have clearly demonstrated a close genetic relationship to the other families of the order, especially the *Rhabdoviridae* and *Paramyxoviridae*. All NNS RNA viruses share a similar genome organization: the more conserved genes that encode core and L proteins are located at the 3′ and 5′ ends, respectively, of the genomic RNA. In between these conserved areas is a more variable region that generally contains genes encoding envelope and certain membrane-associated proteins. The only exception occurs in the genomes of filoviruses, which contain a minor nucleocapsid-associated protein (VP30) in this region (Figure 1). Filovirus genomes are more complex than those of rhabdoviruses, and their organization is more like those of members of the genera *Respovirus* and *Morbillivirus* of *Paramyxoviridae*. This relationship was confirmed by comparisons of the deduced amino acid sequences of the nucleoprotein and L protein (Feldmann et al. 1993; Sanchez et al. 1993).

In terms of biohazard classification, filoviruses are classified as 'Biosafety Level 4' agents on the basis of their high mortality rate, person-to-person transmission, potential aerosol infectivity and absence of vaccines or chemotherapeutic agents. Maximum containment is required for all laboratory work with infectious material (Centers for Disease Control and Prevention 1993).

Morphology and structure

Filovirus virions are bacilliform in shape, but particles can also appear as branched, circular, U or 6-shaped and long filamentous forms. This morphology is unusual for viruses and has been important in the classification and nomenclature (Latin *filum*: thread) (Figure 1a,b). Virions vary greatly in length but show a uniform diameter of approximately 80 nm. Family members differ in length of virion particles but seem to be very similar in morphology. Peak infectivity has been associated with particles of 665 nm for MARV and 805 nm for EBOV. Virions are composed of a central core formed by a nucleocapsid or ribonucleoprotein (RNP) complex, which is surrounded by a lipid envelope derived from the host cell plasma membrane. Electron micrographs reveal an axial channel (10–15 nm in diameter) that is surrounded by a central dark layer (20 nm in diameter) and an outer helical layer (50 nm in diameter) with cross-striations of 5 nm intervals. Spikes, approximately 7 nm in diameter and spaced at intervals of approximately 10 nm are seen as globular structures on the surface of virions (Siegert et al. 1967; Peters et al. 1971; Murphy et al. 1978; Kiley et al. 1982).

The RNP complex is composed of a genomic RNA molecule and four of the seven virion structural proteins: the nucleoprotein (NP), virion structural protein (VP) 30, VP35, and the large (L) protein (Becker et al. 1998) (see Figure 1b). The genomic RNA has a molecular weight of 4 200 kDa and constitutes 1.1 percent of the virion mass (Regnery et al. 1980). The three remaining structural proteins are membrane-associated; the glycoprotein (GP) shows a type I transmembrane protein profile (Will et al. 1993; Sanchez et al. 1998b), while the VP24 and VP40 are probably located at the inner side of the

Table 1 *Outbreaks of filoviral hemorrhagic fever*

Location	Year	Virus/species	Cases (mortality)	Reference	Epidemiology
Germany/Yugoslavia	1967	MARV	32 (23%)[a]	Martini and Siegert (1971)	Imported monkeys from Uganda source of most human infections
Zimbabwe	1975	MARV	3 (33%)	Gear et al. (1975)	Unknown origin; index case infected in Zimbabwe; secondary cases were infected in South Africa
Southern Sudan	1976	SEBOV	284 (53%)	World Health Organization (1978a)	Unknown origin; spread mainly by close contact; nosocomial transmission and infection of medical staff
Northern Democratic Republic of the Congo	1976	ZEBOV	318 (88%)	World Health Organization (1978b)	Unknown origin; spread by close contact and by use of contaminated needles and syringes in hospitals
Tandala, Democratic Republic of the Congo	1977	ZEBOV	1 (100%)	Heymann et al. (1980)	Unknown origin; single case in missionary hospital; other cases may have occurred nearby
Southern Sudan	1979	SEBOV	34 (65%)	Baron et al. (1983)	Unknown origin; recurrent outbreak at the same site as the 1976 outbreak
Kenya	1980	MARV	2 (50%)	Smith et al. (1982)	Unknown origin; index case infected in western Kenya died, but physician secondarily infected survived
Kenya	1987	MARV	1 (100%)	Johnson et al. (1996)	Unknown origin; expatriate traveling in western Kenya
USA	1989/90	REBOV	4 (0%)	Jahrling et al. (1990)	Introduction of virus with imported monkeys from the Philippines; four humans asymptomatically infected
Italy	1992	REBOV	0 (0%)	World Health Organization (1992)	Introduction of virus with imported monkeys from the Philippines; no human infections associated
Ivory Coast	1994	CIEBOV	1 (0%)	LeGuenno et al. (1995)	Contact with chimpanzees; single case
Gabon	1994	ZEBOV	Unknown	Georges et al. (1999)	Several laboratory confirmed cases; outbreak associated with the death of nonhuman primates
Kikwit, Democratic Republic of the Congo	1995	ZEBOV	315 (77%)	World Health Organization (1995a)	Unknown origin; course of outbreak as in 1976
Gabon	1996	ZEBOV	50 (50%)	Georges et al. (1999)	Outbreak was linked to the butchering, transport and preparation for consumption of a dead chimpanzee
USA	1996	REBOV	0 (0%)	Rollin et al. (1999)	Introduction of virus with imported monkeys from the Philippines; no human infections associated
Gabon	1996/97	ZEBOV	60 (75%)	Georges et al. (1999)	Outbreak associated with the death of nonhuman primates
South Africa	1996	ZEBOV	2 (50%)	Richards et al. (2000)	Disease was introduced from Gabon
Northern Democratic Republic of the Congo	1998–2000	MARV	>100 (70%)	Bausch et al. (2003)	Outbreak with several introductions; cases are associated with illegal gold mining
Uganda	2000/01	SEBOV	425 (53%)	World Health Organization (2001)	Unknown origin; ongoing outbreak around Gulu close to the border to the Democratic Republic of the Congo
Congo-Brazaville	2002	Ebola	>100 (90%)		Originating from large outbreak among gorillas and chimpanzees

a) Numbers include a primary case that has been diagnosed some years after the epidemic (Slenczka et al. unpublished data).

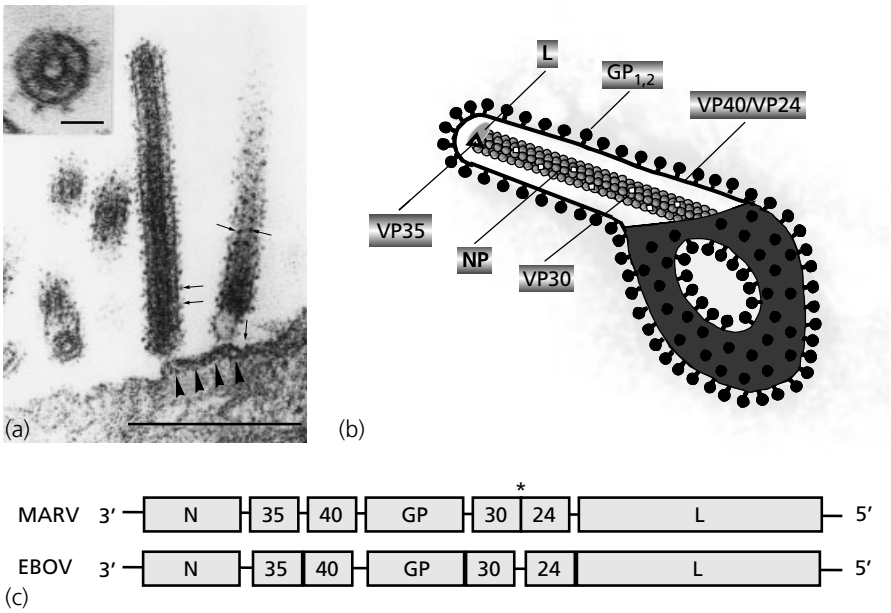

Figure 1 *Structure of filovirus particles.* **(a)** *Ultrathin sections obtained from primary cultures of human endothelial cells 3 days after infection with MARV analyzed by transmission electron microscopy. Particles consist of a nucleocapsid surrounded by a membrane in which spikes are inserted (arrows). The nucleocapsid contains a central channel (inset). The plasma membrane of infected cells is often thickened at locations where budding occurs (arrowheads) (bar, 0.5 μm; bar inset, 50 nm).* **(b)** *Schematic illustration of a filoviral particle. Four proteins are involved in nucleocapsid formation: polymerase or large (L) protein, nucleoprotein (NP), virion structural protein (VP) 30 and VP35. The glycoprotein ($GP_{1,2}$) is a type 1 transmembrane protein and anchored with the carboxy-terminal part in the virion membrane. Homotrimers of GP form the spikes on the virion surface (arrows in panel a). VP40 and VP24 are membrane-associated.* **(c)** *Schematic illustration of the filoviral genome. The genome consists of a single, negative-stranded, linear RNA molecule. G, glycoprotein gene; L, polymerase (L) gene; N, nucleoprotein gene; 24/30/35/40, virion structural protein (VP) genes; asterisk, gene overlap (altered from Feldmann and Kiley, 1999).*

membrane. Virus particles have a molecular weight of 3–6 × 10^8 and a density in potassium tartrate of 1.14 g/cm^3 (Kiley et al. 1988).

Genome structure and function

Genomes of filoviruses consist of a single negative-stranded linear RNA molecule (Kiley et al. 1982; Regnery et al. 1980). The RNA is non-infectious, does not contain a poly(A) tail, and upon entry into the cytoplasm of host cells is transcribed to generate polyadenylated subgenomic mRNA species (Kiley et al. 1982; Feldmann et al. 1992; Sanchez et al. 1993). The complete nucleic acid sequences of two different isolates of MARV (Feldmann et al. 1992; Bukreyev et al. 1995), the Mayinga isolate of ZEBOV and two isolates of REBOV (Sanchez et al. 1993; Volchkov et al. 1999, 2000a) have been elucidated. Filovirus genomes are approximately 19 kb long (MARV, 19.1 kb; EBOV, 18.9 kb) and are significantly larger than those of other *Mononegavirales*. Genes have been delineated by transcriptional signals at their 3′ and 5′ ends that have been identified by their conservation and by analysis of mRNA sequences. The following order is characteristic for filoviruses: 3′-(leader)-N-VP35–VP40–GP–VP30–VP24-L-(trailer)-5′ (Figure 1c).

Genes of filoviruses are usually separated from one another by intergenic regions that vary in length and nucleotide composition. However, some genes overlap, especially those of EBOV, and the positions and numbers of overlaps vary among filoviruses. ZEBOV possesses three overlaps, between the VP35 and VP40, GP and VP30, and VP24 and L genes, whereas MARV isolates have only one overlap, involving the VP30 and VP24 genes (Figure 1c). The length of the overlaps is centered on five highly conserved nucleotides within the transcriptional signals (3′-UAAUU-5′) that are found at the internal ends of the conserved sequences. Transcriptional start signals are conserved among filoviruses, and the sequence 3′-CUNCNUNUAAUU-5′ represents the consensus motif. Transcriptional stop signals are identical for all genes (3′-UAAUUCUUUUU-5′) except the *VP40* gene of MARV (Feldmann et al. 1992) and the *L* gene of ZEBOV (Volchkov et al. 1999) (see Figure 3). Most genes tend to possess long noncoding sequences at their 3′ and/or 5′ ends which contribute to the increased length of the genome. Extragenic sequences are found at the 3′ (leader) and 5′ (trailer) ends of the genome. Those leader and trailer sequences are complementary to one another at the extreme ends (Kiley et al. 1986; Feldmann et al. 1992; Feldman and Kiley 1999; Volchkov et al. 1999), a feature that is shared by all NNS RNA viruses.

Polypeptides

NUCLEOPROTEIN

The nucleoprotein (NP) is encoded by the 3′-most end of the linear RNA genome. The NP from various filoviruses differ slightly in their electrophoretic mobility patterns, ranging from 95 kDa (MARV) to 105 kDa (ZEBOV). The molecular weight calculated from the deduced amino acid sequences of the corresponding genes of MARV (695 amino acids) and EBOV (739 amino acids) are 78 kDa and 83 kDa, respectively, and the differences in their lengths is related to the variable C termini of the protein (Sanchez and Kiley 1989; Sanchez et al. 1992). The NP proteins possess an unusually high molecular weight compared to other NNS virus nucleocapsid proteins which range from 42 to 62 kDa. The NP protein is the major structural phosphoprotein, and only the phosphorylated form of the protein is incorporated into virions. This implies that phosphorylation is needed to form stable virion RNP complexes (Elliott et al. 1985; Elliott et al. 1993b; Becker et al. 1994). NP is the major component of the RNP complex and is tightly bound to it (see Figure 1b).

VP35: POLYMERASE COFACTOR (PHOSPHOPROTEIN EQUIVALENT)

The *VP35* gene encodes a protein that ranges from 329 amino acids for MARV to 340 for ZEBOV (Feldmann et al. 1992; Bukreyev et al. 1993; Sanchez et al. 1993). The association of this protein in the RNP complex is much weaker than that of the NP and VP30 (Elliott et al. 1985; Kiley et al. 1988). Within the complex VP35 interacts with NP and L (see Figure 1b) and the three proteins seem to be the key component of the transcription and replication machinery (Becker et al. 1998). Hydropathy plots of MARV and EBOV VP35 showed similar profiles and a prominent common hydrophilic domain in close proximity to the N-termini. This region may be involved in template binding. Data obtained with an artificial replication system revealed that VP35 is functionally equivalent to a phosphoprotein of *Mononegavirales* (Mühlberger et al. 1998, 1999). In contrast to other phosphoproteins of paramyxoviruses and rhabdoviruses, however, this protein is only weakly phophorylated (Becker and Mühlberger 1999). Finally, VP35 has been shown to function as a type I interferon antagonist (Basler et al. 2000) by interfering with IRF3 phosphorylation (Basler et al. 2003).

VP40: MATRIX PROTEIN

The VP40 of filoviruses is 303 and 326 amino acids long for MARV and ZEBOV, respectively (Feldmann et al. 1992; Sanchez et al. 1993; Bukreyev et al. 1993). This protein, which is abundant in virion particles, is not associated with the RNP complex and behaves like a membrane-associated protein when analyzed after nonionic detergent treatment of virion particles (Elliott et al. 1985; Kiley et al. 1988). VP40 is nonglycosylated, shows a predominantly hydrophobic profile, and functions as the matrix protein (see Figure 1b). VP40 is essential for assembly, and its crystal structure shows that it is composed of two structurally similar β-sandwich domains (Dessen et al. 2000; Ruigrok et al. 2000). The monomeric form of VP40 can be induced to change its conformation into oligomeric ring-like structures, which may play an important role in virus assembly and budding (Scianimanico et al. 2000). VP40 octamers have specific RNA-binding properties suggesting that this protein has additional functions in the life cycle besides promoting virion formation (Gomis Rüth et al. 2003).

GLYCOPROTEIN

The glycoprotein (GP) is encoded in the fourth gene, and is the only glycosylated structural protein of virions (Figure 1b). GP of MARV and ZEBOV are 681 and 676 amino acids in length, respectively (Will et al. 1993; Sanchez et al. 1993; Volchkov et al. 1995). They are type I transmembrane proteins and can be subdivided into a large ectodomain, a lipid membrane spanning domain of approximately 30 amino acids, and a short cytoplasmic tail of four (EBOV) and eight amino acids (MARV). GP undergoes a complex sequence of processing events in the endoplasmic reticulum (ER). This includes the removal of the signal peptide (Will et al. 1993; Sanchez et al. 1998b), *N*-glycosylation (Feldmann et al. 1991, 1994; Becker et al. 1996; Sanchez et al. 1998b), and oligomerization (Feldmann et al. 1991; Sanchez et al. 1998b). ER processing is followed by acylation in a pre-Golgi compartment (Funke et al. 1995; Ito et al. 2001), and by *O*-glycosylation and maturation of *N*-glycans in the Golgi apparatus (Feldmann et al. 1991, 1994; Geyer et al. 1992; Will et al. 1993; Becker et al. 1996). Depending on the host cell, there are wide variations in the amount of neuraminic acid present on MARV GP (Feldmann et al. 1994). Finally, GP is proteolytically cleaved into a larger amino (GP_1) and smaller carboxy-terminal (GP_2) subunit in the trans-Golgi network by a subtilisin-like proprotein convertase, most likely furin (Volchkov et al. 1998a, 2000b) (see Figure 2).

The mature envelope glycoprotein ($GP_{1,2}$) is able to form spikes on membrane surfaces without the need of other viral proteins in this maturation process (Volchkov et al. 1998b). It is anchored in the membrane via a carboxy-terminal hydrophobic domain of GP_2. The middle region of $GP_{1,2}$ is variable, extremely hydrophilic, and carries the bulk of *N*- and *O*-glycans that account for more than one-third of the molecular weight of the mature protein (Geyer et al. 1992; Will et al. 1993; Feldmann et al. 1991; Becker et al. 1996). Oligosaccharide side chains differ in their terminal sialylation patterns, which seem to be isolate-, as well as cell line-dependent (Feldmann et al. 1994). Detailed structural analyses of carbohydrates are available for MARV only

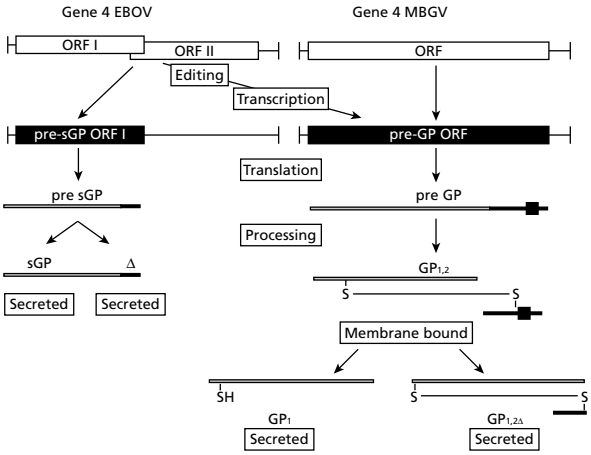

Figure 2 *Transcription and expression strategies of the glycoprotein genes of filoviruses. In contrast to MARV, the EBOV surface glycoprotein GP is encoded in two overlapping reading frames (ORF I and II), and expression of GP occurs through transcriptional editing. ORF I encodes for the precursor of the secreted small glycoprotein (pre-sGP) that is expressed from unedited transcripts and post-translationally cleaved into the soluble products sGP and Δ-peptide. Mature GP ($GP_{1,2}$) consists of the disulfide-linked (S–S) subunits GP_1 and GP_2. Significant amounts of GP_1, sGP_1, Δ-peptide, and $GP_{1,2\Delta}$ are released from cells, in soluble form (altered from Klenk et al. 1998).*

(Geyer et al. 1992). Comparison of amino acid sequences shows conservation at the amino- and carboxy-terminal ends in which the two hydrophobic domains (signal peptide, membrane anchor) and most of the highly conserved cysteine residues are located. The two most carboxy-terminal cysteine residues seem to be acylated (Funke et al. 1995; Ito et al. 2001). GP_2 contains a sequence of several uncharged and hydrophobic amino acids at a distance of 22 (EBOV) or 91 (MARV) amino acids from the cleavage site which bears some structural similarity to the fusion peptides of retroviruses (Gallaher 1996; Volchkov et al. 2000b). The special arrangement of the cysteine residues in the molecule allows an intramolecular disulfide bridge formation between the two cleavage products which results in a stem region consisting of GP_1 and GP_2 and a crownlike domain on the top formed by GP_1 carrying the mass of the carbohydrate side chains (Jeffers et al. 2002). Mature $GP_{1,2}$ is a trimer consisting of disulfide-bonded $GP_{1,2}$ molecules (Feldmann et al. 1991; Sanchez et al. 1998b). X-ray crystallography demonstrated that the central structural feature of the GP_2 ectodomain is a long triple-stranded coiled coil followed by a disulfide-bonded loop which reverses the chain direction and connects to an α helix packed antiparallel to the core helices (Weissenhorn et al. 1998a, 1998b; Malashkevich et al. 1999). During maturation GP_1 is partly shed in monomeric form after release of its disulfide linkage to the transmembrane subunit GP_2 (Volchkov et al. 1998b). Cleavage by the metalloprotease TACE results in shedding of the entire ectodomain ($GP_{1,2\Delta}$) (Dolnik et al. 2004).

In general, filovirus GPs lack significant homologies with envelope proteins of other viruses. However, a region of 26 amino acids (and less conserved surrounding sequences) in the external domain in close proximity to the transmembrane region shows significant homology to an immunosuppressive domain in envelope proteins of several retroviruses (Volchkov et al. 1992; Will et al. 1993; Bukreyev et al. 1995). This region is also the most conserved sequence in the GPs of MARV and EBOV. It is not known whether this sequence in filovirus GPs has immunosuppressive properties. As the only surface protein of virions GP functions in receptor binding and fusion (see Virus entry). Furthermore, GP is discussed (see Immune responses) as an important target for the host immune response.

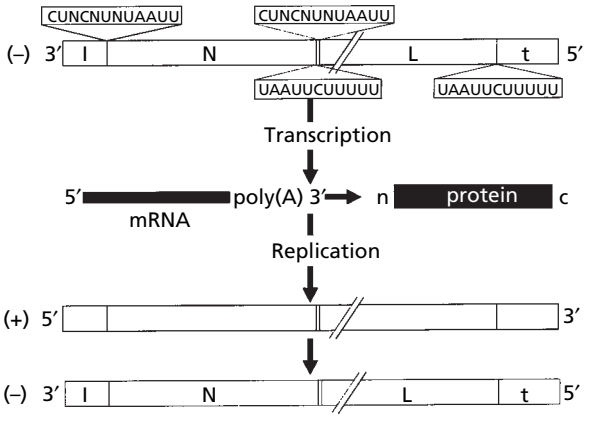

Figure 3 *The mode of transcription and replication of filoviruses, based on the data available to date. Each gene on the linear arranged nonsegmented (–)-sense genome is flanked by conserved transcriptional start (3′-CUNCNUNUAAUU-5′; indicated above) and termination signals (3′-UAAUUCUUUUU-5′; indicated underneath). Transcription starts at the 3′ end of the (–)-sense genome and leads to polyadenylated mRNA species. For replication a full-length (+)-sense antigenome is synthesized which serves as the template for the synthesis of progeny (–)-sense RNA. c, carboxy-terminal end of proteins; l, 3′ untranslated region (leader); L, viral RNA-dependent RNA polymerase; n, amino-terminal end of proteins; N, nucleoprotein; poly(A), polyadenylation of mRNA species; t, 5′ untranslated region (trailer).*

VP30: MINOR NUCLEOPROTEIN

The fifth gene encodes VP30, a protein intimately associated with the RNP complex (Elliott et al. 1985; Kiley et al. 1988) (Figure 1b). The protein has a length of 260 and 281 amino acids with ZEBOV and MARV, respectively (Feldmann et al. 1992; Sanchez et al. 1993). VP30 is the second strongest phosphorylated protein of virions (Elliott et al. 1985, 1993b; Becker and Mühlberger 1999). Using an artificial reverse genetic system (Mühlberger et al. 1999), it was shown that VP30 is essential for EBOV-specific transcription, but not replication. VP30 seems to be involved in transcription

initiation, most likely by inhibiting pausing of the transcription complex at the RNA structure of the first transcription start site. For transcription of the following genes VP30 seems to be nonessential (Weik et al. 2002).

VP24: MEMBRANE-ASSOCIATED PROTEIN OF UNKNOWN FUNCTION

VP24 is expressed from the sixth gene, and is 253 and 251 amino acids long in MARV and ZEBOV, respectively (Feldmann et al. 1992; Sanchez et al. 1993). Unlike VP40, VP24 is not completely removed from the RNP complex under isotonic conditions (Elliott et al. 1985; Kiley et al. 1988). VP24 presumably serves as a second matrix protein (see Figure 1b).

LARGE PROTEIN (RNA-DEPENDENT RNA POLYMERASE)

The large (L) protein is encoded at the 5′ end of the linear genome and has a predicted molecular weight of 267 kDa (2 331 amino acids) for MARV (Mühlberger et al. 1992) and 253 kDa (2 212) for ZEBOV (Volchkov et al. 1999). Computer-assisted comparison revealed significant homologies to L proteins of other NNS RNA viruses. Homologies are mainly located in the amino-terminal half of the protein and concentrated within three common motifs, named boxes A, B, and C. Other common features are a high content of leucine and isoleucine residues, a large positive net charge, clusters of basic amino acids interspersed with nonbasic ones, putative ATP-binding sites, two neighboring cysteine residues located in the C-terminal half of the protein, and the genome localization of the encoding gene. A highly conserved peptide motif, -GDNQ-, is located at the carboxy-terminal end of domain B and is flanked by hydrophobic amino acid residues. Similar motifs with alterations in the first amino acid have been described and discussed as catalytic sites for some RNA-dependent RNA polymerases of plant, animal, and bacterial viruses. Studies using an artificial reverse genetic system (Mühlberger et al. 1998, 1999) identified the L protein as the viral RNA-dependent RNA polymerase (see Figure 1b).

NONSTRUCTURAL GLYCOPROTEINS

The secreted glycoprotein precursor (pre-sGP) is the primary expression product of gene 4 of EBOV (Volchkov et al. 1995; Sanchez et al. 1996). For ZEBOV it has a length of 364 amino acids and shares the amino-terminal 294 amino acids with the transmembrane GP. The different carboxy terminus (69 amino acids) contains many charged residues, as well as conserved cysteine residues. sGP undergoes several co- and post-translational processing events, such as signal peptide cleavage, glycosylation, oligomerization, and proteolytic cleavage. The limiting step during maturation and transport seems to be oligomerization in the ER (Volchkova et al. 1998, 1999). After oligomerization sGP is transported into the Golgi compartments where glycosylation is completed and posttranslational cleavage by furin into the mature form sGP and a small peptide, called Δ-peptide, occurs (Volchkova et al. 1999). Due to the lack of a transmembrane anchor mature sGP is efficiently secreted from infected cells, a process which is independent of proteolytic cleavage. sGP appears as a disulfide-linked homodimer which shows an antiparallel orientation (Sanchez et al. 1998b; Volchkova et al. 1998). Dimerization is due to an intermolecular disulfide linkage between the amino and carboxy-terminal cysteine residues at positions 53 and 306, respectively. The remaining four highly conserved cysteine residues at the amino-terminus seem to be involved in the intramolecular folding of monomers (Volchkova et al. 1998).

Δ-peptide, the small cleavage product of pre-sGP, varies in length between 40 and 48 amino acids for the different EBOV. Its molecular mass of approximately 10 to 14 kDa is significantly larger than the one predicted from the amino acid sequence (approximately 4.7 kDa). The difference is due to the attachment of several *O*-glycans that carry terminal sialic acids. In this respect it differs from sGP which seems to mainly carry *N*-linked carbohydrates. As with mature sGP the peptide is secreted from infected cells (Volchkova et al. 1999).

The small secreted glycoprotein (ssGP) of EBOV resembles a natural carboxy-terminal truncated variant of sGP and is generated from gene 4 by transcriptional editing. Due to the lack of a transmembrane anchor and carboxy-terminal cysteine residues ssGP is secreted in monomeric form (Volchkov et al. 1995; Volchkova et al. 1998).

FILOVIRUS REPLICATION

Virus growth in cell cultures

The Vero cell line, especially the E6 clone, is most widely used for virus isolation and propagation. Primary virus isolation has also been successful in MA-104 and SW13 cells (McCormick et al. 1983; Jahrling et al. 1990). A variety of other cells have been tested as substrates for filovirus replication (van der Groen et al. 1978; McCormick et al. 1983; Peters et al. 1992). These include human microvascular endothelial cells (HMEC-1), primary cultures of human umbilical cord vein endothelial cells (HUVEC), and human peripheral blood monocytes/macrophages (Schnittler et al. 1993; Feldmann et al. 1996a).

MARV and ZEBOV cause lytic infections in cell culture. SEBOV and REBOV replicate more slowly upon primary isolation, and the cytopathic effect (CPE) is not as prominent as with ZEBOV. The course of infection in tissue culture can be monitored by an indirect immunofluorescence assay (IFA) or by plaque assay. In cases of little or no CPE, reverse transcriptase-polymerase chain reaction (RT-PCR) on viral RNA isolated from infected cells and tissue culture

supernatants can be helpful for quantification (Schnittler et al. 1993; Sanchez and Feldmann 1996).

Viral subgenomic RNA synthesis in tissue culture is detectable as early as 3 h after infection, reaches a maximum by 18 h, and declines thereafter. CPE is not seen before 48 h after infection. The first subgenomic RNA to be detected is NP-specific which reaches levels sufficient to produce protein by 7 h after infection. All proteins are detectable by in vitro translation of polyadenylated RNA isolated 18 h after infection, thereafter the yield of translation products decreases. Polymerase chain reaction (PCR) assays of genomic RNA of MARV particles in supernatants of infected cells indicated that the replication cycle is approximately 12 h (Sanchez and Kiley 1987; Schnittler et al. 1993; Mühlberger et al. 1996).

Virus entry

The filovirus GP mediates receptor binding and subsequent fusion with susceptible cells (Takada et al. 1997; Wool-Levis and Bates 1998; Yang et al. 1998; Chan et al. 2000b). There is evidence that MARV uses the asialo glycoprotein receptor to infect hepatocytes (Becker et al. 1995). For EBOV, it was suggested that integrins, especially the beta 1 group, might interact with the glycoprotein and perhaps be involved in virus entry (Takada et al. 2000). Other studies indicate that the folate receptor alpha serves as cofactor for entry of EBOV and MARV (Chan et al. 2001). A similar role has been postulated for C-type lectins (Alvarez et al. 2002). Fusion activity has never been experimentally demonstrated using standard methodology. Early post-infection filovirus particles are associated with coated pits along the plasma membrane indicating endocytosis as a possible mechanism for entry (Geisbert and Jahrling 1995). This is supported by studies which employed lysosomotropic agents (Mariyankova et al. 1993; Chan et al. 2000b). A fusion peptide was postulated for ZEBOV in a distance of 22 amino acids from the cleavage site (amino acids 524–539) (Gallaher 1996). Recently it was demonstrated that the same peptide induces fusion with liposomes (Ruiz-Argüello et al. 1998). This together with mutational analysis of the putative fusion domain (Ito et al. 1999) offers compelling support for a fusion peptide role for this conserved hydrophobic region in the EBOV GP. For MARV a similar putative fusion domain can be found at a distance of 91 amino acids from the cleavage site (Volchkov et al. 2000b). Receptor binding and fusion are followed by uncoating, a mechanism which is unknown.

Transcription, translation, and genome replication

Filovirus transcription and replication take place in the cytoplasm of infected cells, and the mechanisms resemble those of other NNS RNA viruses. As with other members of the order *Mononegavirales*, transcription is believed to start at the extreme 3′ end, leading to the synthesis of a short (+)-leader sequence that is terminated when the first transcription start site is encountered (Figure 3). The seven structural genes are subsequently transcribed to produce seven monocistronic polyadenylated subgenomic RNA species. There is no evidence for larger amounts of bi- or multicistronic subgenomic RNA species (Sanchez and Kiley 1987; Feldmann et al. 1992; Sanchez et al. 1993). Analyses of MARV mRNA species have shown that the 5′ ends of the transcripts are two bases shorter than previously published (Mühlberger et al. 1996). All start signals of filovirus genes contain the consensus sequence 3′-CUNCNUNUAAUU-5′. The 3′ ends of the transcripts carry a poly(A) tail generated by a stuttering mechanism of the viral polymerase at a run of five to six uridine residues located at the 5′ end of all transcription stop signals. Therefore, the sequence 3′-UAAUU-CUUUUU(U)-5′ serves as a transcription stop and polyadenylation signal. Both signals carry the pentamer 3′-UAAUU-5′, a unique feature among NNS RNA viruses. The function of the pentamer is unknown, but it could serve as the recognition site for positioning the polymerase complex. The surrounding semiconserved sequences may then mediate the exact initiation of transcription and termination/polyadenylation events.

Filovirus transcripts start precisely at the first nucleotide of the highly conserved transcriptional start signal. They contain unusually long untranslated regions especially at the 3′ ends. The 5′ end untranslated regions can build a stable secondary structure with the conserved nucleotides located in the stem region of a hairpin. Nucleotide substitutions in the conserved 5′ regions are accompanied by compensatory mutations of the complementary nucleotide thus leading to conservation of the secondary structure. Hairpin formation might play a role in transcript stability and ribosome binding (Sanchez et al. 1993; Mühlberger et al. 1996). The role of gene overlaps in regulation of transcription is unknown. Sanchez et al. (1993) proposed that, following mRNA synthesis, transcription is reinitiated by reposition of the polymerase at the downstream start site. This ‘back up’ mechanism is supported by the finding that attenuation of filovirus genes with start sites in overlaps does not occur to any higher degree, as has been noted for a much larger overlap found in the respiratory syncytial virus genome. Alternatively, the polymerase may occasionally terminate transcription without polyadenylation at the overlap and initiate transcription of the downstream gene, but there is no evidence for detectable levels of transcripts lacking poly(A) tails (see Figure 3).

The switch mechanism between transcription and replication is unknown, but as with other NNS viruses, synthesis of the NP protein could be a key factor.

Encapsidation and polymerase complex entry sites are probably located on the leader sequence, and the fact that the ends of the genome are complementary suggests a single identical encapsidation site on genome and antigenome. This would function for both transcription as well as replication (Feldmann et al. 1992). Replication involves a full-length (+)-strand antigenome which serves as the template for synthesis of (−)-strand genome molecules. Encapsidated genomic RNA forms nucleocapsids that go into the formation of new infectious virions at the cell surface (Figure 3).

In the past years artificial replication systems have been developed for MARV and EBOV which allowed more detailed study of the requirements for transcription and replication (Mühlberger et al. 1998, 1999). It could be demonstrated that NP, VP35, and L are essential and sufficient for transcription, as well as replication and encapsidation of MARV monocistronic monigenomes, whereas EBOV-specific transcription was also dependent on the presence of VP30 systems (see Polypeptides). Recently, the successful generation of infectious clones for ZEBOV was reported which will finally allow the determination of the critical steps in virus replication (Volchkov et al. 2001; Neumann et al. 2002).

The organization and transcription of the GP genes of EBOV is unusual, and involves transcriptional editing. EBOV GP genes possess two overlapping reading frames (Figure 2). Full-length GP is expressed by the addition of a single nontemplated adenosine residue at a run of seven uridine residues on the vRNA template (Volchkov et al. 1995; Sanchez et al. 1996). The primary gene product is a small nonstructural glycoprotein (sGP) that is secreted from infected cells (see Nonstructural glycoproteins). In addition, virus variants have been found after passaging in tissue culture and animals that express full-length GP from a single open reading frame. Those variants acquired a mutation that added a single uridine nucleotide at the editing site connecting the GP open reading frame (Sanchez et al. 1993; Volchkov et al. 1995). The MARV GP is expressed in a single frame and the gene does not contain sequences favoring mechanisms such as editing or frameshifting (Will et al. 1993; Bukreyev et al. 1995).

Virus assembly and exit

Virions usually bud at the plasma membrane, and the budding process is probably mediated at membrane locations where GP is incorporated. The cytoplasmic tail of GP is thought to interact with VP40 or VP24, or both. VP40 or VP24 may mediate the linkage between the RNP complexes and the transmembrane protein GP (see also Polypeptides). Particles mature preferentially in a vertical mode, but budding via the longitudinal axis has also been observed. In macrophages, budding has also been observed at intracytoplasmic membranes surrounding vacuoles which form during infection (Feldmann et al. 1996a). VP40 appears to be targeted by 'late domains' to multivesicular bodies that may play an important role in virus assembly (Kolesnikova et al. 2002; Jasenosky and Kawaoka 2004).

CLINICAL AND PATHOLOGICAL ASPECTS

Epidemiology

It is generally accepted that human filovirus outbreaks are of a zoonotic nature. Guinea pigs, primates, bats, and hard ticks have been suspected to be natural hosts, but the search for filoviruses in these and many other wild species was unsuccessful. Studies on experimentally infected wild animals have shown that fruit and insectivorous bats support replication and circulation of high titers of EBOV without necessarily becoming ill, indicating that bats could serve as the reservoir host (Swanepoel et al. 1996).

MARV and the SEBOV and ZEBOV appear to be indigenous to the African continent, and both EBOV species have been isolated from human patients only in Africa. MARV has been isolated from human patients in Africa and Europe, though the European cases were caused by a virus originating from Africa (Martini and Siegert 1971). The REBOV outbreak provided the first evidence for the presence of a filovirus outside Africa (Hayes et al. 1992). Serological studies (IFA) among captive macaques in the Philippines indicated that the source of REBOV might be wild nonhuman primates. However, IFA-detected antibodies seem to be spurious, and latent infections in nonhuman primates have never been observed (Fisher-Hoch et al. 1992).

Serological studies suggest that filoviruses are endemic in many central African regions (summarized in Feldmann et al. 1996b, 2003). Serosurveys in other countries, such as Germany, the United States, and the Philippines, have identified antibodies to filoviruses in humans as well. Serological data based on IFA are of only limited reliability, as nonspecific reactivity has been observed when filovirus antigens are used. Nevertheless, it is possible that subclinical infections caused by known or unknown filoviruses or a closely related virus (paramyxovirus?) may be responsible for a certain amount of the seropositivity detected in human populations.

Clinical manifestation

MODE OF TRANSMISSION

Person-to-person transmission by intimate contact is the main route of infection in human outbreaks. The EBOV

outbreaks in 1976 and 1995 and the MARV outbreak in 1967 all involved nosocomial transmission via contaminated syringes and needles. Therefore, extreme care should be taken with blood, secretions, and excretions from infected patients. On the basis of experiences from these episodes, isolation of patients, use of strict barrier nursing procedures (e.g. protective clothing, respirator), and measures to handle and disinfect contaminated material promptly are sufficient to prevent transmission to healthcare workers. Transmission by droplets and small-particle aerosols have been observed in outbreaks among experimentally infected and quarantined imported monkeys. This is confirmed by identification of filovirus particles in alveoli of naturally and experimentally infected monkeys and humans (Peters et al. 1991; Pokhodyaeu et al. 1991; Geisbert et al. 1992; Zaki and Peters 1997; Zaki and Goldsmith 1999). Epidemiological studies of human outbreaks, however, indicate that aerosols and droplets do not seem to be an important route of transmission.

CLINICAL SYNDROME

The onset of the disease is sudden with fever, chills, headache, myalgia, and anorexia. This may be followed by symptoms such as abdominal pain, sore throat, nausea, vomiting, cough, arthralgia, diarrhea, and pharyngeal and conjunctival injection (Figure 4). Patients are dehydrated, apathetic, disoriented, and may develop a characteristic, nonpuritic, maculopapular centripetal rash associated with varying degrees of erythema and desquamate by day 5–7 of the illness. Hemorrhagic manifestations develop during the peak of the illness; they are of prognostic value for the disease. Bleeding into the gastrointestinal tract is most prominent beside petechia and hemorrhages from puncture wounds and mucous membranes. Laboratory parameters are less characteristic, but the following findings are associated with the disease: leukopenia (as low as 1 000/μl), left shift with atypical lymphocytes, thrombocytopenia (50 000–100 000/μl), markedly elevated serum transaminase levels (AST typically exceeding ALT), hyperproteinemia, and proteinuria. Prothrombin and partial thromboplastin times are prolonged and fibrin split products are detectable. In a later stage, secondary bacterial infection may lead to elevated white blood counts.

Nonfatal cases have fever for about 5–9 days; fatal cases develop clinical signs early during infection and death commonly occurs between day 6 and 16, due to hemorrhage and hypovolemic shock. Mortality is high and varies between 22 and 88 percent depending on the virus. The highest rate has been reported for ZEBOV; REBOV seems to possess a very low pathogenicity for humans or may even be apathogenic.

Convalescence is prolonged and sometimes associated with myelitis, recurrent hepatitis, psychosis, or uveitis. There is an increased risk of abortion for pregnant women, and clinical observations indicate a high death rate for children of infected mothers (Martini and Siegert 1971; Pattyn 1978; Baron et al. 1983; Peters et al. 1996; Peters and LeDuc 1999; Sanchez et al. 2001).

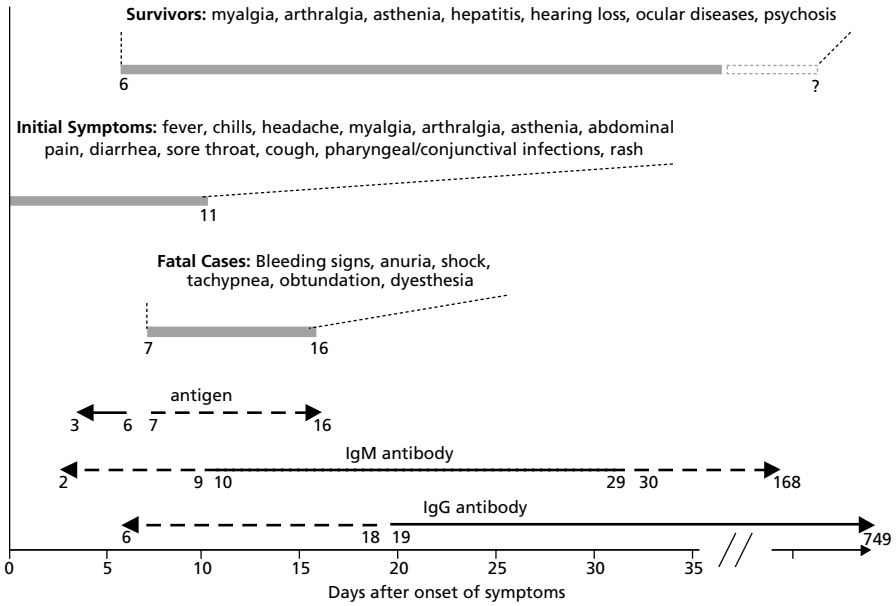

Figure 4 *Clinical course of filoviral hemorrhagic fever. The scheme presents an average clinical course of Ebola hemorrhagic fever. Key: gray bars, time interval of symptoms; black lines, period in which clinical samples are most likely be tested positive (antigen, IgM, or IgG antibodies); dashed lines, period in which a mix of positive and negative results can be obtained (antigen, IgM or IgG antibodies); numbers, days post onset of symptoms (adapted from Bwaka et al. (1999) and Rowe et al. (1999)).*

Pathology

PATHOLOGY IN EXPERIMENTAL ANIMALS

Monkeys, guinea pigs, suckling mice, and hamsters have been experimentally infected with filoviruses. MARV and ZEBOV are highly virulent for most of these species. SEBOV and REBOV are less virulent, often causing a self-limited infection in guinea pigs and monkeys.

The incubation period for rhesus and African green monkeys inoculated with MARV and ZEBOV is 4–16 days. High titers of virus can be detected in liver, spleen, lymph nodes, and lungs by onset of clinical symptoms. All these organs, especially liver, show severe necrosis due to virus replication in parenchymal cells. Little inflammatory response at those sites is typical. Interstitial hemorrhage occurs and is most prominent in the gastrointestinal tract. In infected nonhuman primates, thrombocytopenia has been found accompanied by aggregation disorders of the remaining platelets in response to agonists such as ADP and collagen (Murphy et al. 1971; Fisher-Hoch et al. 1985). Histopathological damage of the target organs is at odds with serum transferase levels showing increases in AST and ALT (AST:ALT = 7:1). This argues against hepatocellular dysfunction and raises the question for extrahepatic targets (Peters et al. 1996). Morphologic studies on REBOV-infected monkeys revealed extensive virus replication in tissue macrophages, interstitial fibroblasts of many organs, circulating monocytes/macrophages and less frequently in endothelial cells, hepatocytes, adrenal corticoid cells, and renal tubular epithelium (Geisbert et al. 1992). Similar results have been reported from monkeys experimentally infected with MARV and ZEBOV (Ryabchikova et al. 1999).

PATHOLOGY IN HUMANS

In fatal cases, generalized hemorrhage is found macroscopically in most organ systems. Microscopic changes include focal necrosis in liver, lymphatic organs, kidneys, testes, and ovaries. The liver, although always involved with large eosinophilic intracytoplasmic inclusion bodies in hepatocytes and Councilman-like bodies within necrotic foci, is not the site of massive, potentially fatal necrosis. Generalized lymphoid necrosis is characteristic for the disease, and renal tubular necrosis is commonly found in agonal stages. A diffuse encephalitis, as described for many viral infections, has been observed. In addition, focal hemorrhages have been observed. The clotting system is activated and intravascular fibrin thrombi have been observed. Viral antigen can be detected in many organs, predominantly in liver, kidneys, spleen, and adrenal glands. Viral persistence has been demonstrated for filoviruses by antigen/RNA detection or isolation from liver biopsy material, anterior chamber of the eye (4–5 weeks), and semen (12 weeks), despite an apparently normal immune response (e.g. Martini and Siegert 1971; Pattyn 1978; Peters et al. 1996; Rowe et al. 1999; Sanchez et al. 2001).

Pathophysiology

Pathophysiological changes that make filovirus infections so devastating are only partially understood. Clinical and biochemical findings support the anatomical observations of extensive liver involvement, renal damage, changes in vascular permeability, and activation of the clotting cascade. The visceral organ necrosis is a consequence of virus replication in parenchymal cells. However, no organ, not even the liver, shows sufficient damage to account for death. The role of disseminated intravascular coagulation (DIC) in pathogenesis is still controversial, because laboratory confirmation of DIC in human infections has never been demonstrated. In nonhuman primates the intrinsic clotting pathway is most affected, whereas the extrinsic pathway is spared.

Laboratory parameters in the crucial early stage of filovirus hemorrhagic fever, such as high AST/ALT ratio, normal bilirubin levels, and marked lymphopenia followed by a dramatic neutrophilia with left shift, suggest extrahepatic targets in the infection (Peters et al. 1996). As with some hemorrhagic fever with renal syndrome, dengue hemorrhagic fever, and Lassa fever, fluid distribution problems and platelet abnormalities are dominant clinical manifestations indicating dysfunction or damage of endothelial cells and platelets. Post mortem, there is little monocyte/macrophage infiltration in sites of parenchymal necrosis suggesting that also a dysfunction of white blood cells, such as macrophages, occurs. Morphological studies on REBOV-infected monkeys from the 1989 epizootic (Geisbert et al. 1992) and monkeys experimentally infected with ZEBOV (Ryabchikova et al. 1999) showed that monocytes/macrophages and fibroblasts are the preferred sites of virus replication in early stages, whereas other cell types may become involved as the disease progresses. Human macrophages in culture are also sensitive to infection resulting in massive production of infectious virus and cell lysis (Feldmann et al. 1996a). Although the studies on infected nonhuman primates did not identify endothelial cells as sites of massive virus replication, in vitro studies and investigations on human cases with EBOV hemorrhagic fever clearly demonstrated that endothelial cells are suitable targets for virus replication (Schnittler et al. 1993). Here, infection leads to complete cell lysis indicating that damage of endothelial cells may occur during infection. Recently cell destruction upon expression of EBOV glycoprotein was reported (Chan et al. 2000a; Yang et al. 2000).

Supernatants of MARV-infected monocyte/macrophage cultures can increase paraendothelial permeability in an in vitro model (Feldmann et al. 1996a). Examina-

tion for mediators in those supernatants revealed increased levels of secreted cytokines and chemokines, especially TNF-α. These data support a mechanism of a mediator-induced vascular instability that leads to increased permeability and a shock syndrome that is seen in severe cases. Hemorrhagic manifestations, however, are also likely to be caused in part by the damage inflicted upon the reticuloendothelial system as a direct result of massive virus replication. Hemorrhages occur later in infection and could be due to an extended damage which cannot be repaired by wound-healing mechanisms. The bleeding tendency is reinforced by a decrease in blood pressure as a common consequence of shock. The combination of viral replication in endothelial cells and virus-induced cytokine release from macrophages may also promote a distinct proinflammatory endothelial phenotype that then triggers the coagulation cascade (Schnittler and Feldmann 1999).

Immune responses

The mechanisms of recovery from filovirus infections in humans and wild as well as laboratory animals are not well understood. In contrast to survivors and asymptomatic cases that show IgM and IgG antibody responses to filovirus antigens, fatal filovirus infections usually end with high viremia and little evidence of a humoral immune response (Baize et al. 1999; Ksiazek et al. 1999; Leroy et al. 2000). Neutralizing anti-GP antibodies were shown to have protective and therapeutic properties in animal models (Maruyama et al. 1999; Wilson et al. 2000). Protection by convalescence sera has been reported anecdotically, but has not been evaluated in controlled clinical trials to date (Mupapa et al. 1999).

Immunosuppression, in general, seems to be an important factor in the pathogenesis of filoviral hemorrhagic fever. The mechanisms, however, leading to the immunosuppressed status of the hosts are unknown and currently being investigated. In humans and monkeys, there is extensive disruption of the parafollicular regions in the spleen and lymph nodes, and proliferation of filoviruses in macrophages (cytolytic in vitro) has been demonstrated (Murphy et al. 1971; Baskerville et al. 1985; Geisbert et al. 1992; Feldmann et al. 1996a; Ryabchikova et al. 1999). For EBOV, it has been reported that sGP interacts with the host immune response by binding to neutrophils through CD16b, the neutrophil-specific form of the Fcγ receptor III. Subsequently, sGP binding appears to inhibit early activation of these cells (Yang et al. 1998; Kindzelskii et al. 2000). This concept, however, has been challenged by a report from Maruyama and colleagues (1998). Relatively high amounts of GP_1 are released into the medium of filovirus-infected cells, and it has been discussed that this soluble glycoprotein as well as sGP may effectively bind antibodies that might otherwise be protective (Sanchez et al. 1996; Volchkov et al. 1998b; Ito et al. 2001). In addition, filovirus transmembrane glycoprotein molecules possess a sequence close to the carboxy terminus resembling a presumptive immunosuppressive domain found in retrovirus glycoproteins (Volchkov et al. 1992; Will et al. 1993; Bukreyev et al. 1995). Peptides synthesized according to this 26 amino acid long region inhibited the blastogenesis of lymphocytes in response to mitogens, inhibited production of cytokines, and decreased proliferation of mononuclear cells in vitro (Ignatyev 1999). It is not yet known, however, if the immunosuppressive domain on the GP is functional on mature molecules.

There is also evidence that filoviruses interfere with innate immunity which is generally believed to play an important role in viral replication and which, therefore, allows the cellular and the humoral arm of the immune system to clear the virus in later stages of the infection. Recent studies with EBOV have shown that VP35 blocks dsRNA- and virus-mediated induction of an IFN-responsive promoter and the IFN-β promoter. VP35 is therefore likely to function as an inhibitor of the type I IFN response in EBOV-infected cells and may be an important determinant in EBOV pathogenicity (Basler et al. 2000).

Diagnosis

In tropical settings identification of a filovirus hemorrhagic fever may be difficult, because the most common causes of severe, acute, febrile disease are malaria and typhoid fever. A wide range of infectious disease must be considered next, such as shigellosis, meningococcal septicemia, plague, leptospirosis, anthrax, relapsing fever, typhus, murine typhus, yellow fever, Chikungunya fever, Rift Valley fever, hemorrhagic fever with renal syndrome, Crimean–Congo hemorrhagic fever, Lassa fever, and fulminant viral hepatitis. Travel, treatment in local hospitals, contact with sick persons or wild and domestic monkeys are useful historical features in returning travelers, especially from Africa. Diagnosis of single cases is extremely difficult, but the occurrence of clusters of cases with prodromal fever followed by cases of hemorrhagic diatheses and person-to-person transmission are suggestive of viral hemorrhagic fever, and containment procedures have to be initiated. In filoviral hemorrhagic fever prostration, lethargy, wasting, and diarrhea seem to be more severe than observed in patients with other viral hemorrhagic fevers. The rash is characteristic and useful in differential diagnosis.

Laboratory diagnosis can be achieved in two ways: measurement of the host-specific immune response to the infection; and detection of viral antigen and genomic RNA in the infected host (Table 2, Figure 4).
Antigen detection ELISA (Ksiazek et al. 1992, 1999) and reverse transcriptase-polymerase chain reaction (Sanchez and Feldmann 1996; Sanchez et al. 1999) are the primary tests to diagnose an acute infection. For antibody detection the most commonly used assays are

Table 2 *Laboratory diagnosis*

Test	Target	Source	Remarks
Indirect immunofluorescence assay (IFA)	Virus-specific antibodies	Serum	Simple to perform, but prone to false positives and subjective interpretation
Enzyme-linked immunosorbent assay (ELISA)	Virus-specific antibodies	Serum	Specific and sensitive, but initial response slower than IFA
Immunoblot	Virus-specific antibodies	Serum	Protein-specific, but interpretation sometimes difficult
Antigen ELISA	Viral antigen	Blood, serum, tissues	Rapid and sensitive, but requires special equipment
Immunohistochemistry	Viral antigen	Tissues (e.g. skin, liver)	Inactivated material, but requires time
Fluorescence assay (FA)	Viral antigen	Tissues (e.g. liver)	Rapid and easy, but interpretation is subjective
Polymerase chain reaction (PCR)	Viral nucleic acid	Blood, serum, tissues	Rapid and sensitive, but requires special equipment
Electron microscopy	Viral particle	Blood, tissues	Unique morphology (immunostaining possible), but insensitive and requires expensive equipment
Virus isolation	Viral particle	Blood, tissues	Virus available for studies, but requires time

A surveillance assay based on formalin-fixed skin specimens has been established for the field (Lloyd et al. 1999).

direct IgG and IgM enzyme-linked immunosorbent assays (ELISA) (Ksiazek 1991; Becker et al. 1992), and IgM capture assay (Ksiazek et al. 1999). Confirmatory tests include Western blot (Becker et al. 1992; Elliott et al. 1993a) and the indirect immunofluorescence assay on acetone-fixed infected cells inactivated by γ-radiation. Electron microscopy has been particulary useful in diagnosis of filovirus infections (Siegert et al. 1967; Murphy et al. 1978; Jahrling et al. 1990). Viral structures can be visualized in culture fluid from initial passage cell cultures by negative staining, and in thin sections of any infected material. Immunohistochemistry on formalin-fixed material and paraffin-embedded tissues can be used for detection of filoviruses (Jahrling et al. 1990), as well as immunofluorescence on impression smears of tissues (Rollin et al. 1990).

Viral antigen can be detected in blood from day 3 up to 7–16 days after the onset of symptoms. With the development of more sensitive tests, such as the direct IgG and and the IgM capture ELISA (Ksiazek et al. 1999) antibody detection became more valuble in early diagnosis. Based on data from Kikwit, IgM antibodies appear between 2 and 9 days after the onset of symptoms and disappear between 30 and 168 days after onset. IgG-specific antibodies develop between day 6 and 18 after onset and persist (Rowe et al. 1999) (Figure 4).

Attempts to isolate virus from serum or other clinical material should be performed using Vero or MA-104 cells (monkey kidney cells). However, most filoviruses do not cause extensive cytopathic effect on primary isolation. Guinea pigs can be used for primary isolation of those filoviruses that initially do not grow well in tissue culture. Several passages are usually required to produce a uniformly fatal disease.

Patient management and control

Vaccination and chemotherapy for human application do not exist yet. Supportive therapy should be directed towards maintenance of effective blood volume and electrolyte balance. Shock, cerebral edema, renal failure, coagulation disorders, and secondary bacterial infection have to be managed and may be life-saving. Heparin treatment should only be considered in cases with clear evidence for DIC. Human convalescence plasma has been used to successfully treat patients (Mupapa et al. 1999). However, its general usefulness remains unconfirmed. Recently neutralizing anti-GP antibodies could be generated from different species, including human, which were immunized or infected with EBOV. The neutralizing antibodies showed protective and therapeutic properties in animal models (Maruyama et al. 1999; Wilson et al. 2000). Filoviruses are resistant to the antiviral effects of interferon, and interferon administration to monkeys has failed to increase survival rate or achieve a reduction in virus titer. Ribavirin has no effect on filoviruses in vitro and is probably of no therapeutic value.

Isolation of patients is recommended, and protection of medical and nursing staff is required. This can be achieved by strict barrier nursing techniques and the use of high-efficiency particulate air (HEPA) filter respirators for protection against aerosols when feasible. Detailed information has been published regarding management of patients with suspected filoviral hemorrhagic fever and ways to minimize spread of virus in outbreaks, especially in Africa (Centers for Disease Control and Prevention 1988; World Health Organization 1995b).

A protective vaccine would be extremely valuable for at-risk medical personnel in Africa and researchers

working with infectious filoviruses. Crossprotection among different EBOV subtypes in experimental animal systems has been reported, suggesting a general value of vaccines (Bowen et al. 1980; Fisher-Hoch and Brammer 1992). Inactivated vaccines have been developed with formalin or heat treatment of cell culture-propagated MARV and EBOV (species Sudan and Zaire) (Lupton et al. 1980; Agafonov et al. 1992). Because of the biohazard of filoviruses and the general lack of knowledge as to the pathogenic processes involved in filovirus diseases, it would be difficult to insure the safety of attenuated strains of MARV or EBOV. The transmembrane glycoprotein GP is assumed to be the major antigenic molecule of virion particles. The successful use of this protein in different immunization approaches has clearly demonstrated its immunogenic and protective properties in small animal models and nonhuman primates (Hevey et al. 1998; Vanderzanden et al. 1998; Xu et al. 1998; Pushko et al. 2000; Sullivan et al. 2000; Sullivan et al. 2003; Garbutt et al. 2004).

The importation of wild-caught monkeys is an important factor in the introduction of filoviruses into foreign human populations (Martini and Siegert 1971; Jahrling et al. 1990; World Health Organization 1992). Quarantine of imported nonhuman primates and professional handling and testing of these animals will help to minimize the risk of filovirus outbreaks in humans. Guidelines have been published for quarantine and proper handling of monkeys in medical research (Centers for Disease Control and Prevention 1990).

Filovirus infectivity is quite stable at room temperature (20°C), but is destroyed in 30 min at 60°C. Infectivity is also destroyed by ultraviolet and γ-irradiation, formalin (1 percent), lipid solvents (deoxycholate, ether), β-propiolactone, and hypochloric and phenolic disinfectants (Elliott et al. 1982; Centers for Disease Control and Prevention 1988, 1993).

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Rhabdoviruses: rabies

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INTRODUCTION

The family *Rhabdoviridae* (from Greek *rhabdos*: rod) contains hundreds of viruses whose hosts vary widely among vertebrates, invertebrates, and plants (Murphy et al. 1995; Tordo et al. 2004) in which they give rise to various diseases (Figure 1). This variability contrasts strongly with their striking similarity in morphology, structure, and mechanisms of replication. Another common feature is that all members present a danger to humans, not only from direct disease but also from livestock and crop losses.

Arthropods are their most frequent vectors and probable original reservoirs, from which they adapted to plants and vertebrates. Traces of this evolution still exist: the sigma virus exclusively infects the fruit fly *Drosophila*; the plant rhabdoviruses have mixed arthropod–plant cycles; vesiculoviruses, ephemeroviruses, and possibly lyssaviruses undergo mixed insect–mammal cycles; and some evidence even suggests that an aquatic arthropod could be involved in the transmission of fish rhabdovirus. Several insect species replicate both vertebrate and plant rhabdoviruses, but a triple insect–plant–vertebrate cycle has not so far been observed. Finally, rabies and rabies-related viruses became more narrowly adapted and now infect only mammals. The vast majority of rhabdoviruses are transmitted mechanically: in plants by injury or through arthropod or worm vectors and in vertebrates by aerosols, contact, bite, or sexual transmission. A few viruses (e.g. the sigma virus of *Drosophila*) are vertically transmitted.

For historical reasons, studies on rabies virus contributed greatly to the development of vaccines, pathology, and analysis of the immune response. Studies on vesicular stomatitis virus, which is comparatively safe to handle and replicates readily in most cell types, have provided much useful biochemical, biophysical, and molecular data. The fish, plant, and cattle rhabdoviruses, long neglected, have recently attracted increased attention because of their economic importance.

PROPERTIES OF THE VIRUSES

The *Rhabdoviridae*

The family *Rhabdoviridae* belongs to the order *Mononegavirales*, which also embraces the families *Paramyxoviridae*, *Filoviridae* and *Bornaviridae*. These viruses share a linear nonsegmented RNA genome of negative polarity embedded within a helical ribonucleoprotein complex (RNP). The criteria for *Rhabdovirus* classification have been progressively sharpened, taking into account physical, chemical, and structural properties, antigenicity and, finally, genetics. The viruses were first grouped into genera or other groups by serological cross-reactions between internal antigens and their antibodies. Subdivision into serotypes was performed by cross-reaction between envelope antigens (Schneider et al. 1973). The use of monoclonal antibodies (mAbs) further refined the classification (Rupprecht et al. 1991). Molecular biology techniques permitted phylogenetic

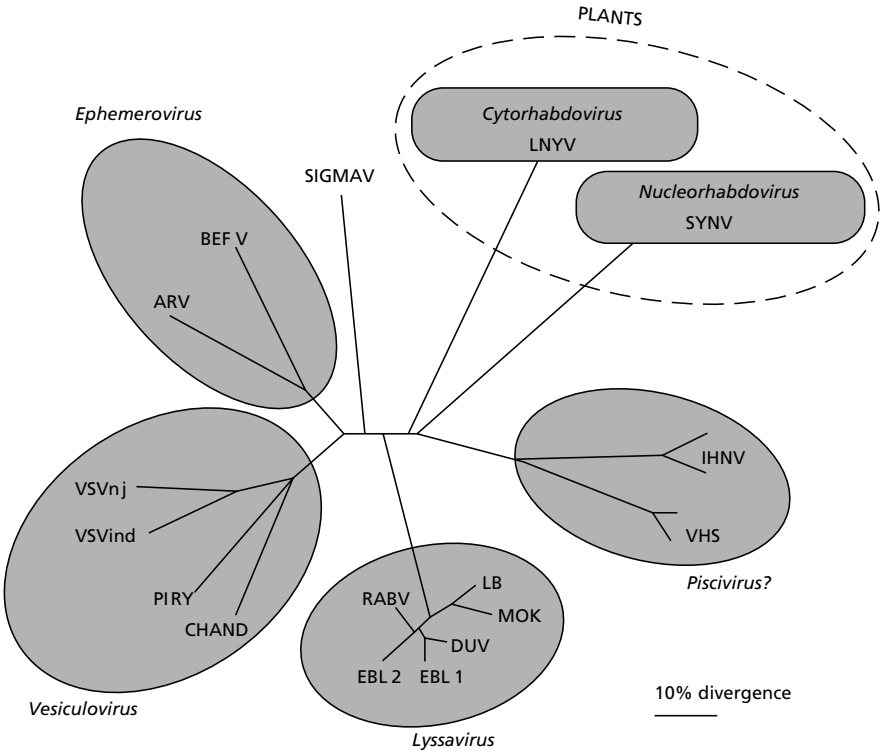

Figure 1 *Radial phylogenetic tree of the* Rhabdoviridae *family established from N protein sequence alignment using the ClustalW program (neighbor joining method). Gray circles outline the genera. ARV, Adelaide River virus; BEFV, bovine ephemeral fever virus; CHPV, Chandipura virus; DUVV, Duvenhage virus; EBLV-1 and EBLV-2, European bat Lyssavirus, types 1 and 2; IHNV, infectious hematopoietic necrosis virus; LBV, Lagos bat virus; LNYV, lettuce necrotic yellow virus; MOKV, Mokola virus; PIRYV, piry virus; RABV, rabies virus; SIGMAV, sigma virus; SYNV, sonchus yellow net virus; VHSV, viral hemorrhagic septicemia; VSIV, vesicular stomatitis virus, Indiana; VSNJ, vesicular stomatitis virus, New Jersey.*

studies and the definition of genotypes (Badrane et al. 2001; Bourhy et al. 1993a; Morzunov et al. 1995; Tordo et al. 1995; Wang et al. 1995; Tordo et al. 2004 and references herein). However, a considerable number of rhabdoviruses remain, classified only roughly on the basis of morphology, host or geographical distribution, or pathological features. Further analysis is needed to justify their inclusion in existing groups or the creation of new ones.

GENUS *VESICULOVIRUS*

The main member of the genus *Vesiculovirus* (Latin *vesicula*: blister) is vesicular stomatitis virus (VSV), which is frequently designated as the prototype of the entire family *Rhabdoviridae*. Eight species have been characterized, of which Indiana and New Jersey are the most recent (Murphy et al. 1995; Tordo et al. 2004). VSV causes a febrile illness in cattle, horses, and pigs, characterized by the appearance of blisters in the mouth, which rapidly ulcerate (see Clinical and pathological aspects: vesiculovirus infection). Other 'vesicular viruses,' which include members of other families, cause similar lesions: for example, *Picornaviridae* (foot-and-mouth disease and swine vesicular disease) and *Caliciviridae* (vesicular exanthema virus). These viruses, of great economic importance for farming, are readily distinguished by morphological or serological criteria.

GENUS *LYSSAVIRUS*

Until 1956 and the first isolations of rabies-related viruses in Africa (King et al. 1994) and Europe (Schneider and Cox 1994), rabies virus (RABV) was believed to be antigenically unique. This warranted the creation of the genus *Lyssavirus* (Greek *lyssa*: rabies) for viruses responsible for rabies-like encephalitis. The genus was at first divided into four serotypes by antigenic crossreactivity with sera and mAbs, but European bat lyssavirus (EBLV) isolates remained unclassified (Dietzschold et al. 1988; Rupprecht et al. 1991; WHO 1992). Phylogenetic analysis of the nucleoprotein and glycoprotein genes further delineated six genotypes, the first four of which matched the serotypes (1) rabies, (2) Lagos bat, (3) Mokola, and (4) Duvenhage. The other two genotypes are EBL types 1 (5) and 2 (6) (Bourhy et al. 1993a).

Genotype 1 corresponds to classic RABV. It encompasses laboratory strains used for vaccine seed or control (challenge virus standard (CVS)) and many viruses isolated from various rabid domestic or wild animals worldwide. Members of genotypes 2–6 are rabies-related

viruses, which so far have been isolated only in the Old World. They have a narrower geographical distribution than rabies and, although they occasionally infect humans and domestic animals, they seem to infect preferentially certain specific host species. Lagos bat virus, Molokai virus, and Duvenhage virus were isolated in sub-Saharan Africa, principally from frugivorous bats, small mammals (shrews and rodents), and insectivorous bats, respectively (King et al. 1994). EBLV-1 and EBLV-2 are spreading widely in Europe, from the former USSR to Spain, and mainly infect insectivorous bats of the *Eptesicus* and *Myotis* species, respectively (Bourhy et al. 1992, 1993a; Schneider and Cox 1994; Amengual et al. 1997; Serra-Cobo et al. 2002).

In May 1996, a new lyssavirus was isolated from fruit-eating bats (flying foxes, *Pteropus alecto*) on the eastern coast of Australia, a country considered to be rabies-free since 1867. The same virus was also isolated from two other species of *Pteropus*, and from an insectivorous bat. Within the two following years, two fatal cases of human encephalitis caused by this new Australian bat lyssavirus (ABLV) were confirmed. Genetic and antigenic analysis of the virus, as well as cross-seroneutralization suggested that ABLV is closer to genotype 1, but sufficiently distinct to justify a new genotype (7) (Guyatt et al. 2003). The current seven genotypes are distributed in two phylogroups with distinct pathological and immunological properties: phylogroup I includes genotypes 1 and 4 to 7; phylogroup II comprises genotypes 2 and 3 (Badrane et al. 2001; Nadin-Davis et al. 2002). This classification is however bound to evolve, particularly as surveillance for bat lyssaviruses is re-enforced (Arguin et al. 2002; Reynes et al. 2004). Already, four additional divergent lyssaviruses have been isolated in bats of Central Asia, East Siberia, and the Black Sea region and are proposed as new genotypes: Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), and West-Caucasian bat virus (WCBV) (Botvinkin et al. 2003; Kuzmin et al. 2003).

From the epizootiological point of view, rabies is maintained and transmitted by mammalian species serving as reservoirs and vectors (Baer 1991) (see section on Epizootiology and epidemiology below). They are distinguished from other mammals, which, although susceptible to infection, constitute epidemiological cul-de-sacs. Rabies exists in two major epidemiological forms: (1) canine rabies, of which stray dogs are the vectors, is responsible for >95 percent of the human cases in developing countries (Plotkin 1993; Meslin et al. 1994); and (2) sylvatic rabies, which involves wildlife worldwide. The wild vectors vary geographically and include terrestrial mammals, mainly carnivores, and bats. The last are vectors for six out of seven *Lyssavirus* genotypes characterized so far, and the exclusive vector in five of them. Phylogenetic analysis supports the evolution of lyssaviruses in bat vectors with occasional but regular spill over and host switching to carnivore vectors to extend the virus host range (Badrane and Tordo 2001). Polymerase chain reaction (PCR) technology has revolutionized the analysis of lyssavirus field isolates (Benmansour et al. 1992; Sacramento et al. 1992; Smith et al. 1992; Tordo et al. 1992a, 1993a, 1995; Bourhy et al. 1993a, b; Nadin-Davis et al. 1993, 1994; Kissi et al. 1995; Nel et al. 1993; Smith et al. 1993 and many others). In particular, phylogenetic analysis revealed that each genotype comprises variants with distinct genetic patterns typical of the geographical area and/or the species of isolation. In addition, in genotype 1 (classical rabies), a cosmopolitan lineage illustrates human influence in the worldwide spread of variant, probably transmitted by dogs present in Europe in earlier centuries (Kissi et al. 1995; Smith et al. 1992; Badrane and Tordo 2001). This variant has undergone successful adaptation to other wildlife species. Most of the current rabies vaccine strains derive from a wild strain of genotype 1 isolated by Pasteur (Tordo 1996).

GENUS *EPHEMEROVIRUS*

The genus *Ephemerovirus* was created to accommodate viruses in tropical and subtropical areas transmitted by hematophagous insects to cattle and water buffalo (Walker et al. 1992, 1994; Fu et al. 1994a). The name came from the prototype bovine ephemeral fever virus (BEFV), which was initially reported to cause a brief, transient fever. However, ephemeroviruses have also been isolated from healthy insects and cattle, as well as from cattle with acute febrile illnesses of major economic importance. BEFV has been found in Australia, Japan, China, the Middle East, and South Africa. Adelaide river virus (ARV), Kimberley virus, and *Berrimah virus* have been isolated in Australia, *Makalal virus* in Kenya, and *Puchong virus* in Malaysia (Calisher et al. 1989; Tordo et al. 2004).

Ephemeroviruses are genetically closer to vesiculoviruses (Wang et al. 1995) but are antigenically related to: (1) the unclassified rhabdoviruses Obodhiang virus, Kotonkan virus, and Kolongo virus that were isolated from, respectively, mosquitoes in the Sudan, midges in Nigeria, and birds in the Central African Republic; and (2) the rabies-related Lagos bat virus (Calisher et al. 1989). Obodhiang virus and Kotonkan virus are in turn serologically linked to the rabies-related Molokai virus (Calisher et al. 1989), which is the only lyssavirus replicating in *Aedes albopictus* cell cultures (Buckley 1975). Taken together, these data suggest that Obodhiang virus and Kotonkan virus may occupy an intermediate position between the genus *Lyssavirus*, adapted to mammals, and the genus *Ephemerovirus*, which has maintained a mixed arthropod–mammal cycle.

PLANT RHABDOVIRUSES

Although more than 100 plant viruses are listed as possible rhabdoviruses, their poor immunogenicity has

hampered serological grouping and classification was established at the genetic level (Jackson et al. 1999; Tordo et al. 2004). They are named according to the lesions they cause (e.g. necrosis, yellowing, and mosaic). Most are still classified according to the type of arthropod vector (aphid, leafhopper, etc.) (Jackson et al. 1987). Their transmission is mechanical, via arthropod or worm vectors, and most of those examined replicate efficiently in these vectors (Jackson et al. 1987). Two genera have so far been distinguished on replicative criteria. Members of the genus *Cytorhabdovirus* (prototype: lettuce necrotic yellows virus (LNYV)) replicate in the cytoplasm in association with viroplasms, and their morphogenesis takes place in vesicles of the endoplasmic reticulum. Members of the genus *Nucleorhabdovirus* (prototype: potato yellow dwarf virus (PYDV) replicate in the nucleus, morphogenesis takes place at the inner nuclear envelope and virions accumulate in the perinuclear spaces (Murphy et al. 1995; Jackson et al. 1999).

FISH RHABDOVIRUSES

The rhabdoviruses of fish cause hematopoietic necrosis, hemorrhagic septicemia, dropsy, swim bladder inflammation and hydrocephalus, diseases of major economic importance to the fish farming industry. Several are similar to vesiculoviruses based on structural similarities in protein composition and size. Phylogenetic analyses suggest placing these viruses in a separate genus, *Novirhabdovirus* (Benmansour et al. 1994; Morzunov et al. 1995; Schütze et al. 1995; Tordo et al. 2004). Fish rhabdoviruses grow in cell cultures at the ambient temperature of fish. They may be transmitted by an aquatic arthropod.

SIGMA VIRUS

Sigma virus is unique among the rhabdoviruses. It is a hereditary factor endemic in 20 percent of natural populations of the fruit fly *Drosophila*, in which it is propagated via the gametes. Sigma virus is not cytopathic and only slightly pathogenic in the infected flies, except that it confers sensitivity to CO_2. Replication is partly under the control of an antiviral host gene, *ref(2)P*, which is also required for male fertility (Dezélée et al. 1989). It has been suggested that sigma was a rhabdovirus trapped by a nonbiting fly and behaved as a hereditary factor to ensure its own survival (Brun 1984).

BIRD RHABDOVIRUSES

There have been rare isolations of rhabdoviruses from birds in America, Central Africa, and India. Very little is known from them, except that they replicate quite efficiently in insect cells (Zeller and Mitchell 1989; Travassos da Rosa et al. 2002).

Morphology and chemical composition

MORPHOLOGY

Under the electron microscope (Figure 2), animal rhabdoviruses are usually bullet-shaped with one round end and the other flat; plant rhabdoviruses are more frequently bacilliform (Figures 2 and 3); a few examples of conical forms have been observed in Obhodiang virus, Kotonkan virus, and bovine ephemeral fever virus and sometimes in RABV. The virion diameter is almost constant (50–100 nm), but lengths vary (100–430 nm), depending on the species or on the presence of defective interfering (DI) particles, which appear when the multiplicity of infection is high. The DI particles possess a truncated genome, are therefore defective in various viral functions, and must depend on infectious virions to complement their deficiency (Lazzarini et al. 1981). Their smaller genomes compete efficiently with normal genomes for replication and packaging into virions. Although their role during natural infection remains unclear, these subgenomic deletion mutants have been mimicked extensively to generate non-infectious recombinant viruses for reverse genetic studies (reviewed by Conzelmann 1996).

The virion is composed of two structural units (Figure 4). One is a central cylinder 50 nm in diameter with characteristic cross-striations composed of a tightly coiled RNP with helical symmetry. This is contained within the second unit, a lipoprotein membrane that is provided by the cell membrane during budding, and through which protrudes an array of knobbed glycoprotein spikes of about 10 nm in length.

CHEMICAL COMPOSITION

The chemical composition is approximately 74 percent proteins, 20 percent lipids (composition dependent on the host cell), 3 percent carbohydrates, and 3 percent RNA. The RNA is a single molecule of negative polarity (i.e. non-infectious). There are five major viral proteins with post-translational modifications that have been assigned either to the envelope or to the RNP structures by biochemical dissection of the virion with proteases and detergents (Delagneau et al. 1981; Tordo and Poch 1988b). From outside to inside:

- The membrane-anchored glycoprotein forms a trimer (Doms et al. 1987; Gaudin et al. 1992) that is glycosylated, and acylated with palmitic acid (Schmidt and Schlesinger 1979; Gaudin et al. 1991b). It constitutes the protruding spikes that are detected at the virion surface in electron microscopy (Delagneau et al. 1981; Gaudin et al. 1992).
- The central RNP is formed by an intimate association between the nucleoprotein (phosphorylated in RABV) and the RNA genome, which is thereby rendered insensitive to nucleases. The heavily phosphorylated

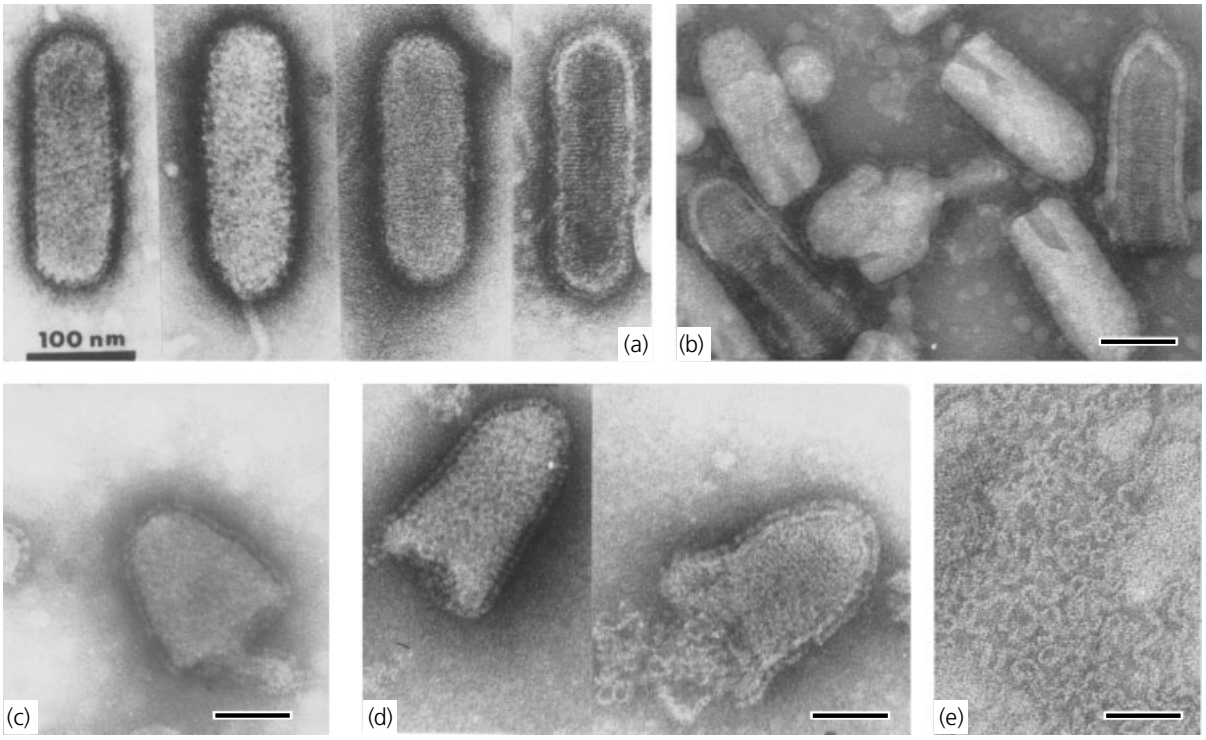

Figure 2 *Electron micrographs of rhabdoviruses.* **(a)** *Plant rhabdoviruses (courtesy of A.O. Jackson).* **(b)** *Vesiculovirus.* **(c)** *Lyssavirus (rabies virus, PV strain).* **(d)** *Lyssavirus (Mokola); one damaged virion with ribonucleocapsid protruding from the base).* **(e)** *Ribonucleocapsid RNP. All scale bars, 100 nm.*

phosphoprotein and the large protein (polymerase) are also bound to the RNP, although less intimately.

- The exact position of the matrix protein remains controversial. It is unclear if it is embedded in the inner layer of the membrane, in the central axial channel of the RNP, or both (Barge et al. 1993; Gaudin et al. 1995a). RABV matrix protein is palmitoylated (Gaudin et al. 1991a), whereas VSV matrix protein is phosphorylated (Kaptur et al. 1995). A subpopulation of M protein is ubiquitinylated in order to recruit the cellular machinery necessary for budding (Harty et al. 2001).

For historical reasons, the nomenclature of the five basic proteins differs between viruses; reports of their relative number per virion also vary owing to the different assay techniques used (reviewed by Coll 1995). Despite these discrepancies, the proteins have similar functions and similar molecular weights. We shall use the conventional abbreviations N for nucleoprotein (50–55 kDa), P for phosphoprotein (35–40 kDa), M for matrix protein (25 kDa), G for glycoprotein (60–70 kDa), and L for the large polymerase (approximately 200 kDa).

Besides these main viral proteins, several rhabdoviruses have developed additional polypeptides for specific functions (see section on Implications for the structure of the genome below, and Figure 7). These additional proteins are rarely found in the viral particle, suggesting their involvement in replicative functions or interaction with the host cell. Conversely, several cellular proteins are reported as being packaged in the RABV and VSV virions, for example cellular kinases playing a major role in activation of the phosphoprotein for replicative functions (Gao and Lenard 1995b; Gupta et al. 1995) and heat shock proteins (Sagara and Kawai 1992). Cytoskeleton-associated proteins such as actin-binding proteins (from the ezrin–radizin–moesin family) are closely associated with the membrane glycoprotein and thereby promote incorporation of actin into the virion (Sagara et al. 1995). Their involvement in virion formation during the budding process is likely but the exact role of the cytoskeleton in the replication/assembly process remains unclear.

Infection cycle in the cell

Except for the sigma virus of *Drosophila*, which is passed vertically, the vast majority of rhabdoviruses are transmitted mechanically by injury, contacts, bite, or aerosols. Tissue tropisms and pathogenicity vary with individual viruses. The main steps of cell infection, shared by all the rhabdoviruses, will now be described (Figure 5). It should be noted that the data come mainly from studies on VSV and to some extent on RABV; current researches on other rhabdoviruses may reveal variations.

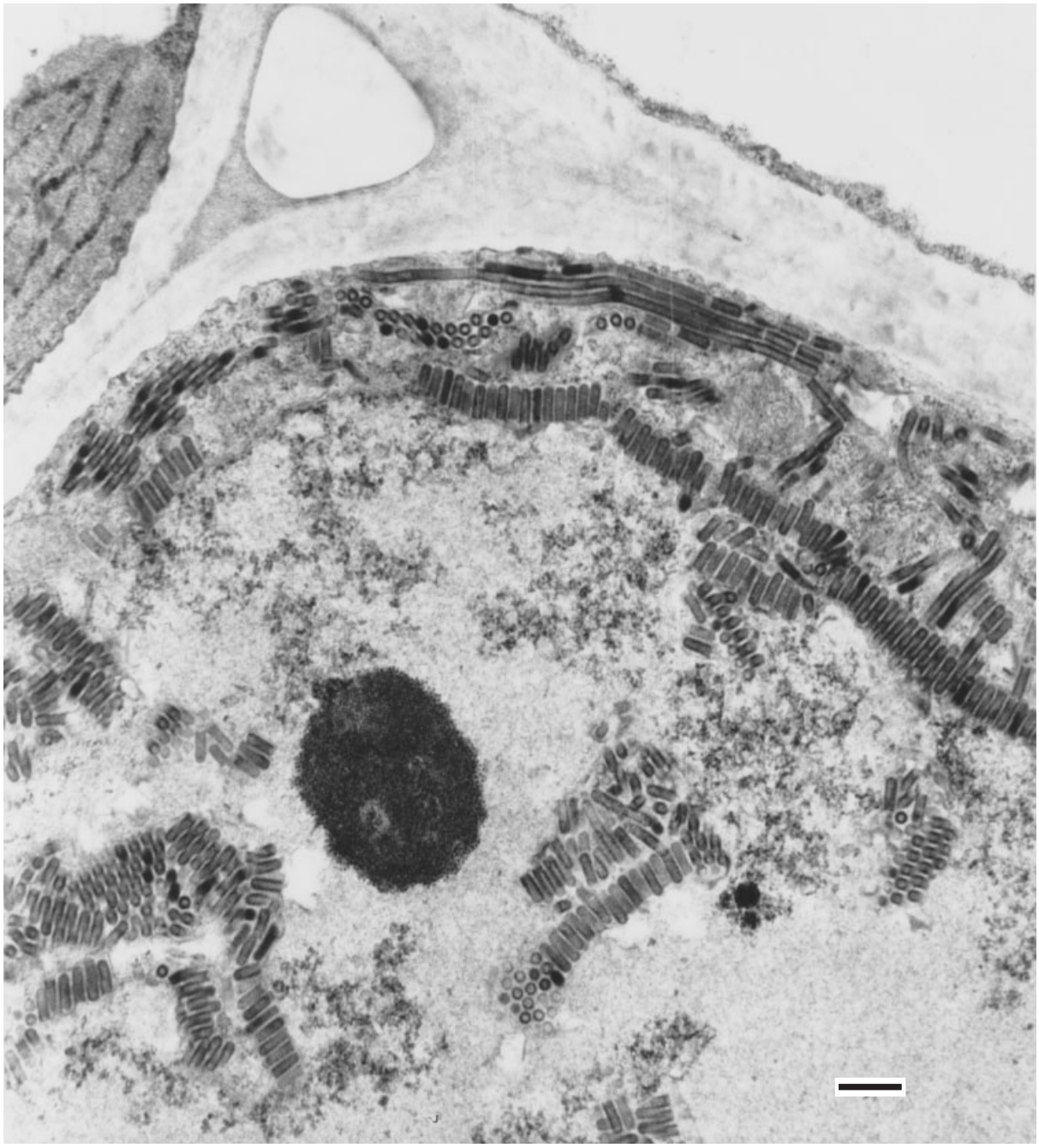

Figure 3 *Eggplant mottle dwarf virus within a cell nucleus. Note the long bacillary forms near the nuclear membrane. Bar, 4.5 μm (courtesy of R. Hull).*

PENETRATION INTO THE CELL

The attachment of the virus to the susceptible cell membrane is mediated by the glycoprotein, which recognizes specific receptors (Coll 1995). The nature of these receptors remains a matter of debate and the accumulated data reveal the complexity of the problem. In addition, most of the studies have been performed on strains that have been adapted for multiplication in established cell lines. For example, RABV isolates multiply and propagate exclusively inside neurons. Although adaptation does not abolish neurotropism, it renders the virus able to grow in nonneuronal cells. This adaptation may be due to the capability of fixed RABV strains to use ubiquitous receptors present on every cell type investigated to date. Ubiquitous receptors could be molecules such as phospholipids (Superti et al. 1984), gangliosides (Conti et al. 1988; Superti et al. 1986) or proteins (Wunner et al. 1984; Broughan and Wunner 1995; Gastka et al. 1996).

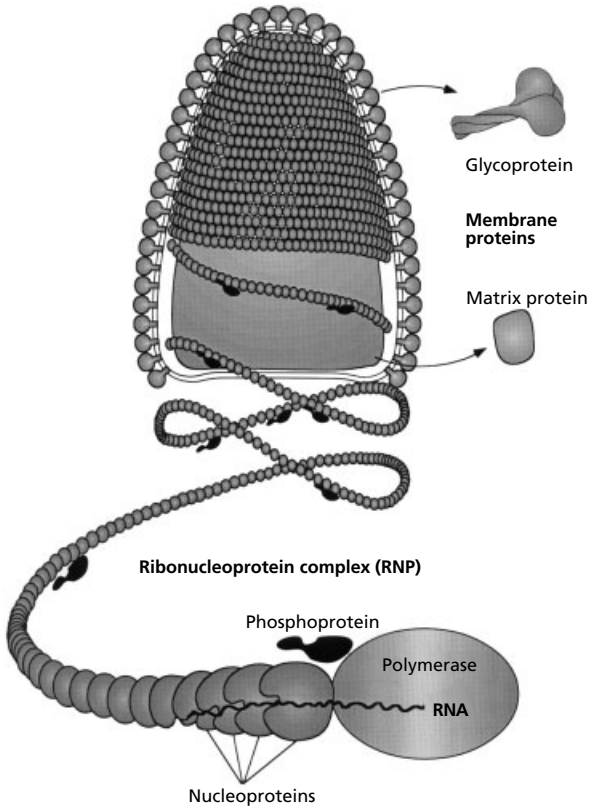

Figure 4 *Structure of the virion.*

Several studies indicated that the nicotinic acetylcholine receptor (nAChR) is a receptor for RABV (for a review, see Baer and Lentz 1991). Nevertheless, as the nAChR is located mainly on muscle cells and as RABV infects neurons that do not express nAChR (McGehee and Lorca 1995), it has been proposed that, in animals, this receptor enbles street RABV to multiply locally in myotubes only at the site of inoculation (Burrage et al. 1985). This would facilitate subsequent penetration into neurons. More recently, the neuronal cell adhesion molecule (NCAM) has been shown to facilitate viral entry in established laboratory cell lines (Thoulouze et al. 1998) and, thus, has been proposed to be another receptor for RABV. Finally, an expression cloning approach using soluble RABV glycoprotein led to the identification of the murine p75 neurotropin receptor as another putative RABV receptor (Tuffereau et al. 1998). Subsequent examination of other lyssaviruses' genotypes revealed that the glycoprotein from European bat lyssavirus 2 (but not that of the other genotypes) could also bind p75 (Tuffereau et al. 2001).

Phosphatidylserine has been proposed to be a receptor for VSV (Schlegel et al. 1983). Nevertheless, the absence of this lipid in the outer leaflet of cells (except when they are apoptotic) suggests that this putative receptor has to be considered with caution.

Very few things are known concerning the receptor of other members of the family. Recently, it has been suggested that fibronectin is a receptor for viral hemorrhagic septicemia virus (VHSV), a salmonid rhabdovirus (Bearzotti et al. 1999). This putative receptor was identified using mAbs generated against rainbow trout gonad cells. These mAbs have been selected for their ability to protect cells from VHSV infection. The blocking activity of these mAbs was also effective against other nonantigenically related fish rhabdoviruses. Interestingly, Broughan and Wunner (1995) had also suggested an involvement of fibronectin for RABV entry in BHK21 cells. Thus, fibronectin might play a general role in rhabdovirus entry.

Once bound, the virus is internalized into a cellular endosome. Then, as the pH decreases within the endosome, the viral membrane fuses with the vesicle one and the RNP is freed into the cytoplasm. Fusion of rabies virus with liposomes has been studied in detail (Perrin et al. 1982; Gaudin et al. 1991b, 1993). Fusion is only triggered at low pH and does not require any specific composition of the target membrane. It is optimal around pH 6, and is not detected above pH 6.4. These pH values are very similar to those determined for VSV (White et al. 1981; Carneiro et al. 2003) and for a fish rhabdovirus (Gaudin et al. 1999). Preincubation of rhabdoviruses at low pH in the absence of a target membrane leads to inhibition of viral fusion properties. However, loss of fusion properties can be reversed by readjusting the pH to above 7. This is the main difference between rhabdoviruses and other viruses fusing at low pH for which low pH-induced fusion inactivation is irreversible (reviewed in Gaudin 2000a).

Low pH-induced conformational changes of RABV glycoprotein and their relationships with fusion activity have been studied. At the virion surface, G can assume at least three different states that have been characterized by electron microscopy, sensitivity to proteases, and monoclonal antibody assays (Gaudin et al. 1991b, 1993, 1995b; Roche and Gaudin 2002). The native (N) state is detected at the viral surface at pH above 7. The activated (A) hydrophobic state is detected immediately after acidification. It interacts with the target membrane as a first step of the fusion process (Durrer et al. 1995). After prolonged incubation at low pH, G is in a fusion inactive conformation (I) that is antigenically distinct from the N state. There is a pH-dependent equilibrium between these states which is shifted toward the I state at low pH (Roche and Gaudin 2002). Similar results have been obtained for VSV G (Clague et al. 1990; Doms et al. 1987; Pak et al. 1997; Carneiro et al. 2003). Finally, the results obtained so far suggest that, for rhabdoviruses, the sequence of events leading to membrane fusion are very similar to that of other enveloped viruses (Gaudin 2000b) and that the fusion complex is made of several trimers (Roche and Gaudin 2002).

Once delivered into the cytoplasm the RNP is ready to serve as a template for gene expression. In the

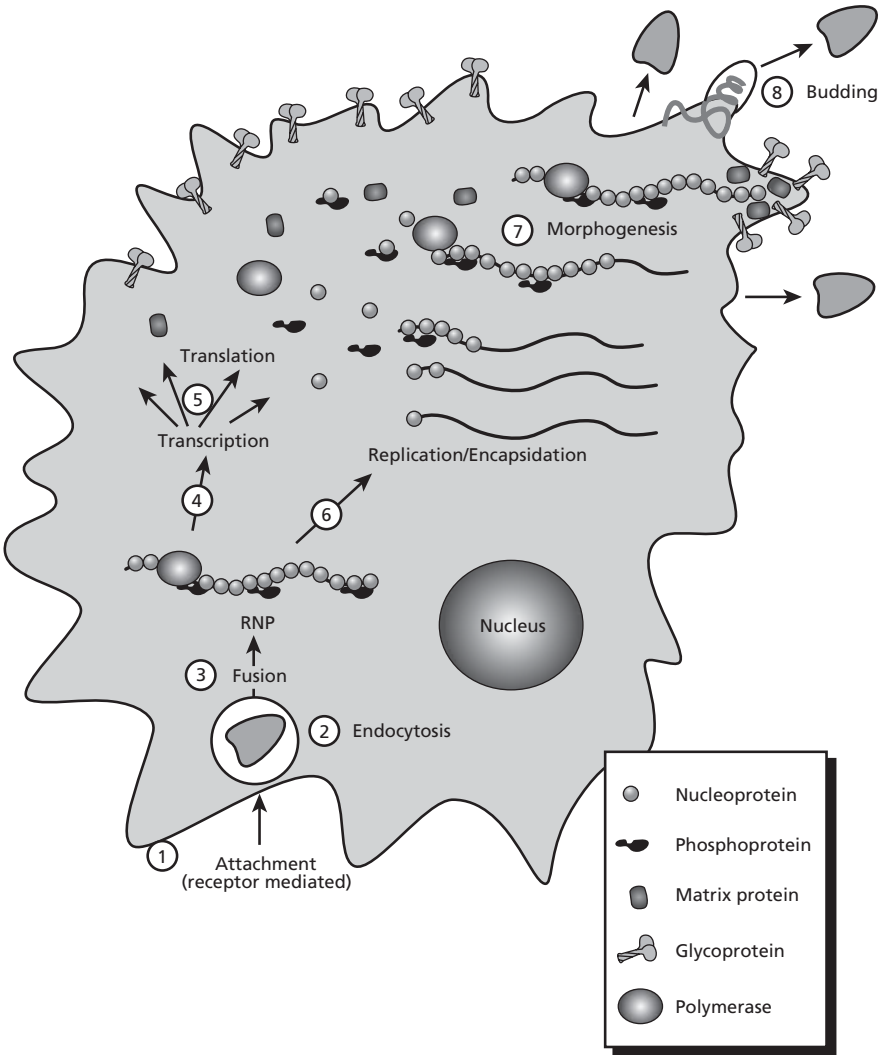

Figure 5 *Example of the rhabdoviral cell infection cycle (rabies virus).*

particular case of neuron infection by RABV, the RNP is first transported by retrograde axonal flow and replicates only in the perikaryon. Recently, it has been demonstrated that the phosphoprotein of lyssaviruses binds the LC8 light chain of the dynein motor suggesting that dynein might play a role in the transport of the RNP (Raux et al. 2000; Jacob et al. 2000). Nevertheless, as an RABV mutant, having a mutated phosphoprotein unable to bind LC8, behaves as wild-type virus even in animals, the exact role of this interaction is not clear (Mebatsion 2001).

Gene expression takes place exclusively in the cytoplasm and only a limited number of viral transcripts or proteins reach the nucleus where their exact role is not clear. In the case of vesiculoviruses, M protein, by interacting with nuclear pores, enhances the availability of the cellular translation machinery by inhibiting the export of cellular mRNAs from the nucleus (Her et al. 1997; Petersen et al. 2000; von Kobbe et al. 2000). In the case of RABV, the *P* gene products are able to interact with the nuclear PML bodies. As these nuclear structures are involved in the resistance of many cells to viral infection, this interaction is supposed to modulate the cellular innate immune response (Blondel et al. 2002).

Mechanism of genome expression

Mechanisms of rhabdovirus genome expression have been more extensively explored on the VSV model (reviews: Banerjee 1987; Tordo and Poch 1988b; Vidal and Kolakofsky 1989; Banerjee and Barik 1992; Tordo and Kouznetzoff 1993; Barr et al. 2002; Kolakofsky et al. 2004; Whelan et al. 2004). Because the rhabdovirus genome has negative polarity, it must be transcribed to produce the complementary positive-strand mRNAs. The RNP serves as template for two successive RNA synthetic functions (Figure 6): transcription then

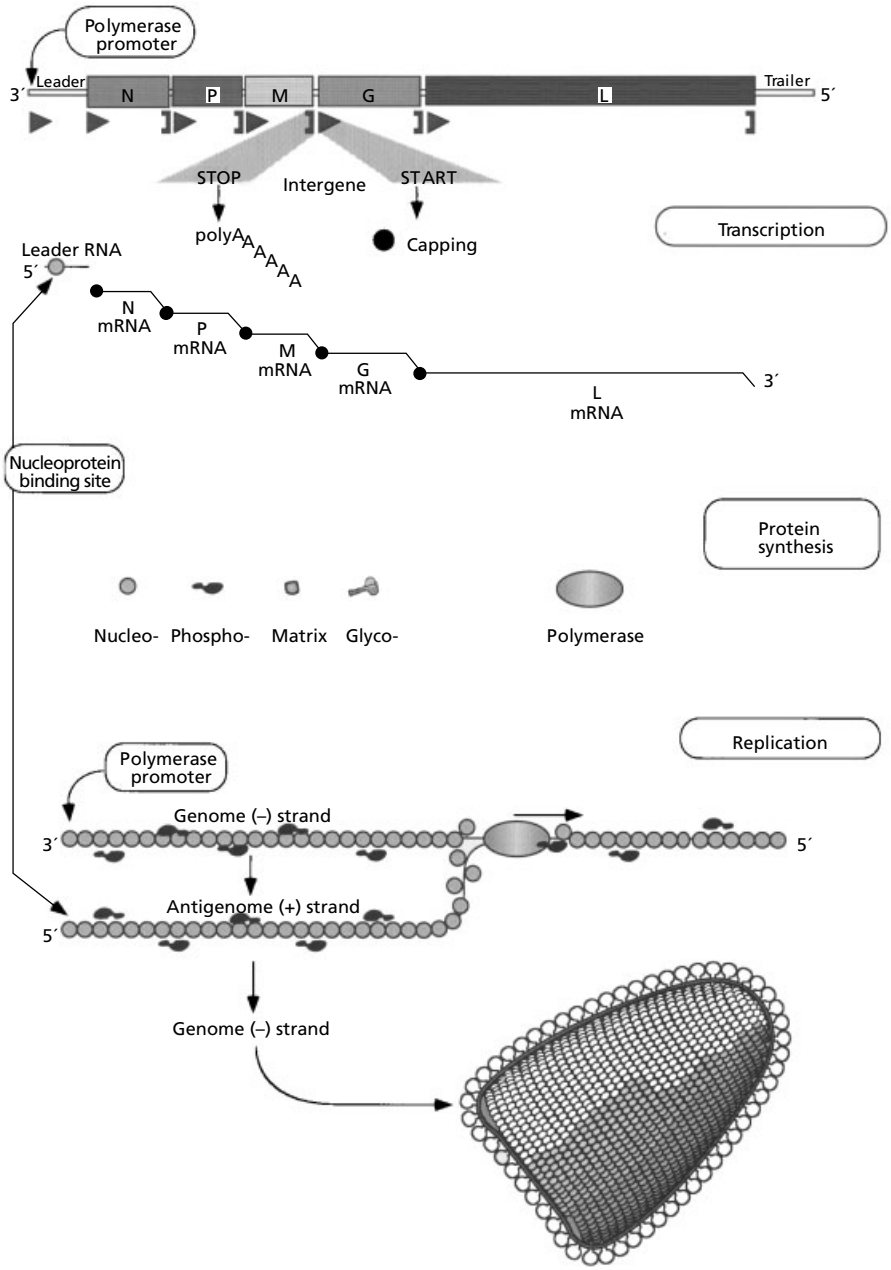

Figure 6 *Transcription, translation, and replication mechanisms of rhabdoviruses.*

replication. For both, the viral polymerase recognizes a promoter at the 3′ end of the encapsidated genome, and proceeds towards the 5′ end. There is still a debate whether there is a single 3′or two different promoters for transcription and replication. The difference between transcription and replication is determined by the processivity of the polymerase, that influences the way it progresses either sequentially or continuously. Transcription gives rise to mostly monocistronic transcripts: first a short noncapped, nonpolyadenylated leader RNA, then five (or more) mRNAs coding for the viral proteins (Figure 6). This sequential progression depends on the recognition of short conserved *cis*-acting signals, bordering the cistrons, which initiate start (and capping) and stop (and polyadenylation) of the mRNAs. When the transcriptase stops transcribing at one polyadenylation signal, it scans in both directions for the nearby (re)start mRNA signal. This strategy results in sequential transcription from the 3′ to the 5′ cistrons, associated with a progressive attenuation due to the cumulative probability of the polymerase to fall off the template during scanning. Thus, the length of the scanned intergenic region influences the efficiency of re-initiation. Bidirectional scanning permits overlapping cistrons when the polymerase backtracks and restarts within the cistron upstream.

In contrast, the replicase acts continuously and generates a full-length positive-strand antigenome that is in turn replicated into negative-strand genomes for the progeny virions. To become functional templates, the genomes and antigenomes must be encapsidated into RNP, which protects them from the action of nuclease. This is why viral protein synthesis is a prerequisite for replication. There is an entry site for the nucleoprotein at its 5′ end to promote the concomitant encapsidation of the growing RNA. During the transcription step, leader RNA carrying this entry site is released. During replication, the 5′ end remains linked to the growing RNA genome, which is progressively encapsidated.

The main event influencing continuous synthesis by the polymerase and its switch from transcription to replication mode is the concomitant (anti)genome encapsidation. Here, the amount of nucleoprotein available is decisive (Vidal and Kolakofsky 1989; Horikami et al. 1992). At low levels, the polymerase stops at the end of the leader RNA and immediately re-initiates at the start signal of the first mRNA. This initiation prevents encapsidation of the mRNAs and causes a shift to the sequential transcription mode, in which elongation is independent of encapsidation. This results in an increase in viral protein. At high levels of nucleoprotein, the concomitant encapsidation prevents the polymerase stopping at the end of the leader RNA, causing a switch into the processive mode and the replicase becomes unable to recognize the following start and stop signals.

The translation step separating transcription from replication is ensured by cellular mechanisms. The classic scanning model (Kozak 1989), predicting that ribosomes bind to the capped 5′ end of the mRNA and scan downstream, is generally accepted. Thus, most of the viral proteins start at the first AUG codon along the mRNA. Exceptions to this rule have, however, been noted during translation of the P mRNA. In addition to the normal P protein, VSV encodes two other polypeptides starting at distal AUGs, one in the same open reading frame (ORF) (Herman 1986) and one in another ORF (Spiropoulou and Nichol 1993). By contrast, RABV uses at least four AUGs in the same ORF. Translation of these proteins is initiated by a leaky scanning mechanism (Chenik et al. 1995). The resulting polypeptides are either cytoplasmic or nuclear, depending on the accessibility of corresponding export and import signals, and thereby interact with various cellular partners (Blondel et al. 2002). All the viral proteins are expressed in the cytosol by free polyribosomes, except the glycoprotein which is synthesized as a transmembrane protein by polyribosomes associated with membranes of the rough endoplasmic reticulum (RER). It is then transported to the cytoplasmic membrane via the Golgi apparatus. Because of the acidity of the RER and Golgi compartments, the glycoprotein is synthesized in a conformation inactive for fusion, thus impairing its interaction with viral cellular membranes during transport. Once at the cell surface, the pH increases and the conformation reverts to the native type (Gaudin et al. 1995b, c).

Several viruses have developed variations on this general model of expression. The ephemeroviruses use a polycistronic strategy to express both structural and regulatory genes (Wang and Walker 1993; Wang et al. 1994). In the case of vaccine strains of RABV (PV, ERA, SAD), there are alternative terminations for the matrix and glycoprotein cistrons (Tordo and Poch 1988a, Morimoto et al. 1989; Sacramento et al. 1992, Tordo et al. 1992b; Tordo and Kouznetzoff 1993). Two consecutive stop (polyadenylation) signals are alternatively recognized by the transcriptase to produce either a small or a large mRNA. It has been proposed that alternative termination, by modifying the length of the scanned intergenic region, could influence the transcription level of the distal cistron, resulting in a regulation tool for genome expression (Tordo and Poch 1988a; Tordo et al. 1992b; Tordo and Kouznetzoff 1993). This regulation could be partly under the control of specific cellular factors, reinforcing the importance of the intracellular environment for cell susceptibility noted above, and suggesting that it might be worthwhile to study rabies neurotropism at the transcriptional level.

Implications for the structure of the genome

The genome of rhabdoviruses varies in length between 11 kb (*Vesiculovirus*) and 15 kb (*Ephemerovirus*) (see Figure 7). Its structure subserves a typical expression mechanism. For both the minus-strand genome and the plus-strand antigenome the promoter for the polymerase is located at the 3′ end (Smallwood and Moyer 1993); the entry site of the nucleoprotein for encapsidation is at the 5′ end (Moyer et al. 1991) (Figure 6). As the minus-strand genome is template for either transcription or replication, while the plus-strand antigenome is only template for replication, the promoter for replication of the plus-strand antigenome is logically much stronger than that of the minus-strand genome (Barr et al. 2002; Le Mercier et al. 2003; Whelan et al. 2004). The consequence of the preservation of these important signals is that the extremities of both positive and negative strands are complementary, although a hairpin structure was never evidenced in vivo. Indeed, a minigenome consisting of only 50 residues from each end is sufficient to ensure transcription, replication, encapsidation, and envelopment (Pattnaik et al. 1995). In addition, when the complementarity between extremities is increased by mutagenesis, replication rather than transcription is favored (Wertz et al. 1994; Whelan et al. 2004). It is interesting that most of the DI particles effectively mimic such minigenomes; those with extensive complementary ends are the most efficient for interference.

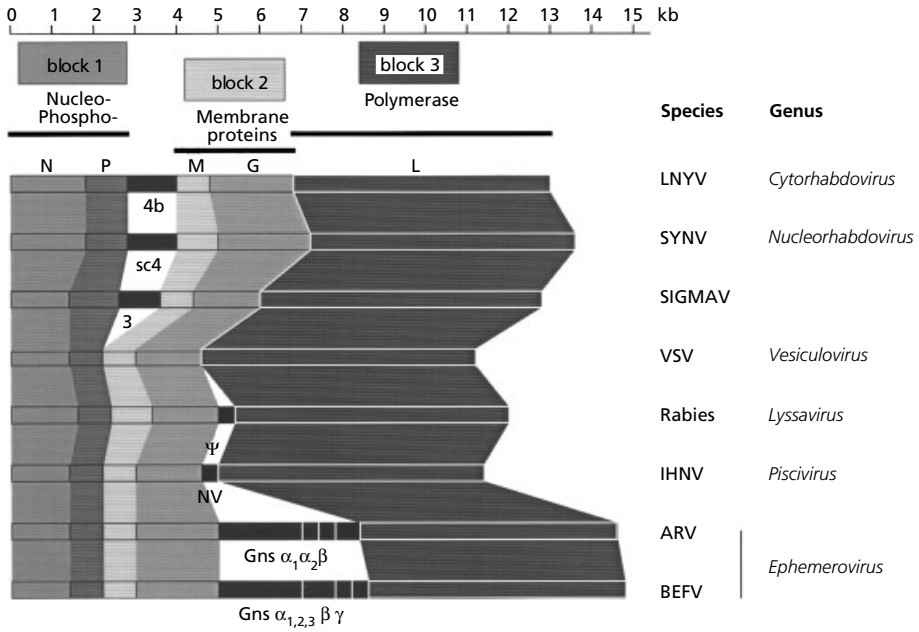

Figure 7 *Structural comparison of the rhabdovirus genomes. ARV, Adelaide River virus; BEFV, bovine ephemeral fever virus; IHNV, infectious hematopoietic necrosis virus; LNYV, lettuce necrotic yellows virus; SYNV, sonchus yellow net virus; VSV, vesicular stomatitis virus.*

Besides the conserved extremities, the genome possesses a modular structure with the juxtaposition of cistrons independently controlled by signal sequences. Indeed, the progressive decrease in the rate of transcription from the 3′ to the 5′ end implies that the position of a cistron directly influences its rate of expression. This explains why rhabdoviruses have conserved very similar genome organizations into three blocks (Figure 7) (Tordo et al. 1992a). Block 1 at the 3′ end encodes proteins required in large quantities (N, P), particularly the nucleoprotein that balances the switch between transcription and replication. Block 2 encodes the membrane proteins (M, G). Block 3 at the 5′ end encodes the viral polymerase (L), required in limited amounts. This modular structure accepts some flexibility, and in several viruses typical genes are inserted that are adapted to their particular biology. Between blocks 1 and 2, the plant rhabdoviruses encode a protease-like protein (sc4, 4b) that corresponds to the movement protein needed for passage of the virus through plasmodesmata, thus allowing cell-to-cell transmission despite the presence of the cell wall (Scholthof et al. 1994). At the same position the sigma virus encodes a protein related to the reverse transcriptase of retro-elements (ORF 3) (Landès-Devauchelle et al. 1995). Between blocks 2 and 3, the ephemeroviruses encode a second heavily glycosylated glycoprotein (Gns) which probably arose from a sequence duplication of the *G* gene (Wang and Walker 1993). Gns is nonstructural and of unknown function, as are the additional small proteins (α, β, γ) encoded consecutively (Wang et al. 1994) and the NV protein of the fish rhabdovirus IHNV (Morzunov et al. 1995; Kurath et al. 1997). It is interesting that, at the same position between blocks 2 and 3, lyssaviruses conserve a large noncoding area initially termed 'pseudogene' ψ because it could represent a vestige of the multiple changes occurring in this region during rhabdovirus evolution (Tordo et al. 1986, 1992b; Ravkov et al. 1995).

Exit from the cell

Before viral budding, transcription and replication are inhibited and, simultaneously, the RNP becomes intensively condensed and coiled. A considerable body of evidence suggests that this morphological role during virion assembly is played by the matrix protein (see Morphology and chemical composition above). In parallel, glycoproteins are concentrated in particular regions of the plasma membrane (Brown and Lyles 2003). After the cytoplasmic maturation step, the virion leaves the cell by budding, the lipid envelope being provided by the host cell membrane. Most rhabdoviruses bud from the plasma membrane and some from internal cell membranes. The plant rhabdoviruses bud either from the endoplasmic reticulum (cytorhabdoviruses) or from the inner nuclear envelope (nucleorhabdoviruses) and accumulate in perinuclear spaces (Jackson et al. 1987).

Functional role of the viral polypeptides

The advances in genetic manipulation of negative-strand RNA viruses have greatly facilitated the functional

dissection of viral genes and proteins by reverse genetics (see Conzelmann 1996 for a review, and also Lawson et al. 1995; Whelan et al. 1995; Kretzschmar et al. 1996; Schnell et al. 1996; Le Mercier et al. 2002). The use of these viruses has greatly improved the understanding of viral pathogenesis and has provided stable vectors to express foreign genes and develop new vaccines (Schnell et al. 1996; Dietzschold et al. 2003; McKenna et al. 2003; Faber et al. 2004). In addition, studies of the interactions between the proteins and the backbone RNA genome are being pursued by various physical (electron microscopy and crystallography), biochemical, and immunological methods.

THE RIBONUCLEOCAPSID COMPLEX (RNP)

The RNP is the minimum structure needed to mediate RNA synthesis. The N protein RNA is the template whereas the polymerase function is catalyzed by the L and P proteins (Conzelmann and Schnell 1994). Minus-strand as well as plus-strand genomic templates are so tightly complexed with the N protein that they are resistant to both RNases and post-translational gene silencing by short interfering RNA (Bitko and Barik 2001). The RNP complex possesses a typical helical form (Schoehn et al. 2001). Chemical probing experiments on VSV RNP showed that the N protein binds to the sugar–phosphate backbone, exposes the Watson–Crick positions of the bases to the solvent and modifies their reactivity (Iseni et al. 2000). Indeed, the nucleoprotein melts secondary RNA structure (helical activity) and favors transcription/replication without the need for dissociation. L is the RNA-dependent RNA polymerase that possesses most of the required enzymatic activities: RNA synthesis (De and Banerjee 1984); mRNA capping and methylation (Horikami and Moyer 1982); mRNA polyadenylation (Hunt et al. 1984; Hunt and Hutchinson 1993); and possibly kinase activity on the P protein (Barik and Banerjee 1992). P is a regulatory cofactor for which various functions have been proposed: mediating interaction between L and the N-RNA template (Isaac and Keene 1982; De and Banerjee 1984); transiently displacing the N protein to gain access to the RNA (Hudson et al. 1986); complexing the soluble N to maintain it in a replication-competent form for encapsidation (Masters and Banerjee 1988; Howard and Wertz 1989; La Ferla and Peluso 1989; Mavrakis et al. 2003).

The nucleoprotein N appears as a two-modular protein, with a large, compact (resistant to proteases) and well-conserved NH_2-terminal core binding the RNA, and a smaller, flexible and variable COOH tail which is exposed and ensures contact with the P protein (Banerjee et al. 1989; Iseni et al., 1998; Schoehn et al. 2001). RNA-binding sites with noncanonical motifs have been tentatively identified for RABV and viral hemorrhagic septicemia virus (Kouznetzoff et al. 1998; Said et al. 1998). Site-directed and deletion mutagenesis and two-hybrid method suggested domains important for N–N and N–P interactions (Chenik et al. 1994; Jacob et al. 2001; Schoehn et al. 2001). The rabies virus N protein N is phosphorylated (unlike VSV). The role for this phosphorylation due to a cellular kinase is unclear; phosphate hydrolysis could produce free energy for transcription and replication (Wu et al. 2002, 2003).

The interaction network of the P protein within the RNP has been extensively studied, (Chenik et al. 1994, 1998; Fu et al. 1994b; Gao and Lenard 1995a; Jacob et al. 2001; Takacs and Banerjee 1995). The rabies virus P protein seems to be composed of two independent domains that nicely outline its intermediate position between the L and N–RNA template. The NH_2 half encompasses the L binding site close to the N terminus (amino acids (aa) 1–52 particularly 1–19) as well as a weak N-binding site (aa 1–185 particularly 69–138) which probably interacts and maintains the soluble N in an encapsidation-competent form (Mavrakis et al, 2003). The COOH half comprises a strong N-binding site (aa 176–297), which likely binds the N–RNA template. The atomic structure of the COOH half (186–297) has been determined and the residues important for N–RNA binding mapped (Mavrakis et al. 2004). The COOH half is a monomer and the oligomerization of P rather involves the NH_2 half (particularly aa 52–189). The phosphorylation of P is essential for regulating its activity in transcription (Barik and Banerjee 1992; Beckes and Perrault 1992; Gao and Lenard 1995a) and replication (Chang et al. 1994). P subspecies varying in degree of phosphorylation and in physical state (soluble, multimeric, or complexed with N or L) have different activities in relation to both functions (Tuffereau et al. 1985; Gao and Lenard 1995a; Richardson and Peluso 1996). The P phosphorylation step is ensured at precise sites by different cellular kinases packaged in the virion: the casein kinase-II and an L-associated kinase (LAK) for VSV (Barik and Banerjee 1992; Beckes and Perrault 1992; Chen et al. 1997; Gao and Lenard 1995a, 1995b; Gupta et al. 1995); the protein kinase C (isomers α, β, and γ and another cellular rabies specific phosphokinase (RVPK) for rabies virus. It is interesting that protein kinase Cγ is mostly found in nerve tissue, and could provide another tissue-specific element in the regulation of rabies transcription. Phosphorylation is thought to promote conformational changes. Phosphorylation is required for oligomerization of the VSV but not of the rabies P protein (Gao and Lenard 1995b, Gigant et al. 2000). The small C and C′ basic proteins encoded in a second ORF on the *P* gene (Spiropoulou and Nichol 1993; Kretzschmar et al. 1996) are unnecessary for VSV growth in tissue culture (Kretzschmar et al. 1996). It has been suggested that they play a role in viral pathogenesis or transmission by insect vectors (Kretzschmar et al. 1996).

Due to very limited amounts in the virion as well as in infected cell, less is known on the structure–function of

the L polymerase. The region binding the P protein is located at the COOH side: in aa 1638–1873 for VSV (Canter and Perrault 1996); in the C-terminal 566 aa for rabies virus (Chenik et al. 1998). The sequence alignment of L proteins from *Mononegavirales* has provided a structure that is consistent with its multifunctional nature: six conserved domains (probably carrying the catalytic activities) are concatenated by variable hinge regions (Poch et al. 1990). The domains seem to be autonomous as suggested by complementation between L mutants (Flamand 1980). Putative functions have been tentatively assigned to several domains (Poch et al. 1990). In particular, domain III possesses four or five hyperconserved motifs similar in structure and position to those found in all RNA-dependent polymerases (Poch et al. 1989; Müller et al. 1994) and also in DNA-dependent polymerases, although more distantly (Delarue et al. 1990). Distant from this 'polymerase module,' domain VI (500 aa toward the COOH side) is typical from 2′-*O*-ribose-methyltransferases (Bujnicki and Rychlewski 2002; Ferron et al. 2002) and could be involved in the methylation during mRNA capping (Abraham et al. 1975; Gupta et al. 2002). This predictive analysis has guided the first trials of site-directed mutagenesis, which basically confirmed the functional importance of the predicted motifs (Sleat and Banerjee 1993; Schnell and Conzelmann 1995). A more systematic analysis by extensive sequence comparison between wildtype and mutant strains of VSV suggests that residues in the interdomain V–VI are essential for polyadenylation and thermosensitivity (Hunt and Hutchinson 1993). In addition, it was suggested that host factor(s) might be required for the activity of the L protein (Mathur et al. 1996).

MEMBRANE PROTEINS

M is a small protein (slightly more than 200 amino acid residues) comprising a positively charged amino terminal part (Poch et al. 1988). For VSV, this amino terminal part can be removed by proteolysis (Ogden et al. 1986). Cleavage of VSV M by thermolysin provided a resistant core (Mth) composed of two noncovalently associated fragments (residues 48–121 and 122–(or 123 or 124 depending on the thermolysin cleavage site) 229) (Gaudier et al. 2001) that could be crystallized allowing the determination of its structure (Gaudier et al. 2002).

The M protein is involved in the late steps of the infectious cycle. It condenses the RNP into a tightly coiled helical structure (Newcomb et al. 1982). This tightly coiled structure seems to be formed near cellular membranes where M is bound (Odenwald et al. 1986; Flood and Lyles 1999). Strong evidence that M plays a major role in VSV budding comes from studies of VSV temperature-sensitive mutants affected in budding that were shown to be mutated in the *M* gene and were complemented by wild-type plasmid-derived M (Flamand 1970; Lyles et al. 1996). More recently, an M-deficient RABV mutant has been obtained using reverse genetic (Mebatsion et al. 1999). In the absence of M, infectious particles were mainly cell associated and the yield of cellfree infectious virus was largely reduced demonstrating the role of RABV M in virus budding. Supernatants from cells infected with the M-deficient RABV did not contain the typical bullet-shaped rhabdovirus particles, but instead contained long, rod-shaped virions, showing severe impairment of the virus formation.

Two biochemical properties of VSV M have been studied, auto-association and membrane-binding VSV. M self-associates into large multimers at physiological NaCl concentration (McCreedy et al. 1990), a process initiated by trypsin-sensitive nucleation sites that is reversed by increasing the salt concentration (Gaudin et al. 1995a). M self-association could be important for nucleocapsid compaction and may constitute a driving force for budding. VSV M cell membrane association is also necessary for budding. This interaction was proved to be extremely stable in vivo as it is not destabilized by harsh treatments such as 2M KCl or pH 11 (Bergmann and Fusco 1988; Chong and Rose 1993, 1994). The regions of the protein involved in membrane association are not completely defined: hydrophobic photolabeling has identified the amino-terminal region of the protein (Lenard and Vanderoef 1990), but M mutants deleted from the amino-terminal domain still interact with cellular membranes, suggesting that another domain of the protein is involved (Chong and Rose 1994; Ye et al. 1994). Indeed, the structure of Mth revealed a hydrophobic patch at the surface of the protein. This patch is located close to a hydrophobic loop containing the cleavage site of thermolysin and surrounded by basic residues (two arginines and three lysines). The combined presence of hydrophobic and basic residues suggests that it is a likely candidate for being a region interacting with membranes (Gaudier et al. 2002).

The amino-terminal part of the matrix protein contains two short motifs (PPxY and PSAP) that are supposed to recruit the cellular partners involved in the ultimate step of the budding process (i.e. the fission step). The PPxY motif has been shown to be recognized by an E3 ubiquitin ligase (Harty et al. 2001) and to influence budding efficiency (Jayakar et al. 2000). The exact role of the PSAP motif remains to be determined.

The functions of M are not limited to viral assembly and budding. Both VSV and RABV M inhibits the transcription of the viral genome (Clinton et al. 1978; De et al. 1982; Finke et al. 2003). RABV M acts also as a replication stimulatory factor (Finke et al. 2003). For RABV M, a mutation has been identified that affect the function of M only in regulation of RNA synthesis, but not in assembly and budding, providing evidence that these functions are genetically separable (Finke and Conzelmann 2003).

Finally, VSV M expression causes the rounding of cells, a phenotype characteristic of VSV infection (Blondel et al. 1990; Melki et al. 1994) that is probably a consequence of M-induced apoptosis (Kopecky et al. 2001; Kopecky and Lyles 2003). As mentioned above, it also inhibits the export of RNAs from the nucleus (Her et al. 1997; Petersen et al. 2000; von Kobbe et al. 2000). This export inhibition may be the cause of M-induced inhibition of host-cell transcription (Black et al. 1993; Black and Lyles 1992). It has also been proposed that host transcription shut-off is induced by an effect of M on the transcription factor TFIID, which was found to be ineffective in the cells where M is expressed (Yuan et al. 1998). All these cytopathic effects of M protein explain why VSV is deleterious to all types of cell.

The glycoprotein (reviewed by Coll 1995) has the typical structure of group I transmembrane proteins. An NH_2 terminal signal peptide of about 20 amino acids (aa) in length serves the translocation of nascent protein through the RER membrane and is cleaved in the mature protein. A hydrophobic transmembrane segment (approximately 20 aa) remains embedded in the viral envelope. It separates the 'cytoplasmic' COOH domain (about 50 aa), interacting with internal viral proteins, from the glycosylated NH_2 ectodomain (approximately 450 aa). The glycosylation and palmitoylation sites have been mapped (Rose et al. 1984; Gaudin et al. 1991a; Shakin-Eshleman et al. 1992). The role of glycosylation in correct folding of G ectodomain has been studied in detail (Machamer and Rose 1988; Hammond and Helenius 1994; Mathieu et al. 1996; Gaudin 1997). A map of the disulfide bridges of the ectodomain of VHSV G has also been established (Einer-Jensen et al. 1998) that has allowed a general model for the rhabdoviral G (Walker and Kongsuwan 1999). It has to be noted that VSV G and some of its thermosensitive mutants have been used as model glycoproteins to elucidate how membrane proteins are transported all along the secretory pathway. Particularly, VSV G has been used to study the quality control mechanisms that operate in the endoplasmic reticulum (ER) and the downstream compartments to ensure that only proteins that are correctly folded are transported (e.g. Hammond and Helenius 1994; Cannon et al. 1996).

The important functions of the ectodomain have also been mapped. Based on sequence similarity with snake venom curare-mimetic neurotoxins (Lentz et al. 1984; Rustici et al. 1993; Tordo et al. 1993b) and further studies using anti-idiotypic antibodies (Hanham et al. 1993), the 190–203 aa sequence of rabies glycoprotein ectodomain has been implicated in the recognition of the nicotinic AChR. By selection of RABV mutants that were not neutralized by a soluble form of p75, the domain of interaction with this receptor has been shown to implicate aa 318 and 352 (Langevin and Tuffereau 2002). The region involved in fusion has also been searched by insertion and directed mutagenesis. Different sites modified the ectodomain structure in such a way that fusion activity was blocked or modified (Li et al. 1993; Zhang and Ghosh 1994; Fredericksen and Whitt 1995, 1996). However, the exact locations of the fusion peptide were mapped by photolabeling to the following sequences: aa 102–179 for RABV, 58–221 for VSV (Durrer et al. 1995). Taken together, these data suggest that a sequence corresponding to aa 118-136 of VSV enriched in prolines and aromatic residues might constitute G fusion peptide. However, the data obtained during these studies have also indicated that a second region of the protein, located in the carboxy-terminal part of the ectodomain (around aa 400), controls the low pH-induced conformational changes of G (Gaudin et al. 1996; Shokralla et al. 1998). Finally, several residues of the ectodomain seem to play a crucial role in pathogenicity and virulence (Tordo et al. 1993b). For RABV, neurovirulence is directly linked to the maintenance of a basic residue in position 333 (Tuffereau et al. 1989) that may be involved in p75 recognition (Tuffereau et al. 1998). Mutation at this residue reduced the categories of neurons sensitive to infection (Coulon et al. 1994).

Antigens

In the case of RABV, several hundred mAbs have been used to characterize the antigenic structure of G making this protein one of the best known viral antigens (Raux et al. 1995; Benmansour et al. 1991; Préhaud et al. 1988; Seif et al. 1985; Lafon et al. 1983; Wiktor and Koprowski 1980). RABV G has two major antigenic sites that are antigenic sites II and III. Antigenic site II is located between positions 34 and 42 and positions 198 and 200 (Préhaud et al. 1988). These peptides are joined by a disulfide bridge and maintained together in the tertiary structure of G (Dietzschold et al. 1982). Antigenic site III extends from amino acids 330 to 338 (Seif et al. 1985). Beside these major antigenic sites, one minor antigenic site and a few isolated epitopes have been described (Lafay et al. 1996; Raux et al. 1995; Benmansour et al. 1991; Dietzschold et al. 1990).

The immune response against lyssaviruses is complex; the response to RABV has been extensively studied. Although all the viral proteins are antigenic, their roles in protection vary (Lafon 1994; Xiang et al. 1995; Drings et al. 1999). Their respective importance has been defined by recombinant DNA techniques (Tordo 1991; Perrin et al. 2001). Two of them, G and N, are of primary importance, the phosphoprotein P being of less significance. Purified G protein protects against intracerebral challenge with RABV, and is the only antigen that consistently induces virus-neutralizing antibody (Wiktor et al. 1973). This property mainly depends on the preservation of its three-dimensional structure,

although a linear neutralizing epitope has been identified (Bunschoten et al. 1989; Benmansour et al. 1991; Ni et al. 1995). On the other hand, the glycoprotein shares with the N and P proteins of RNP the capacity to induce a cellular immune response, involving, respectively, T helper cells (Th) and cytotoxic T cells (Tc) (Lafon 1994; Xiang et al. 1995). Purified RNP protects against challenge by peripheral routes, probably through a cross-help mechanism between N-specific Th lymphocytes and G-specific B lymphocytes producing neutralizing antibodies (Dietzschold et al. 1987; Lodmell et al. 1991; Lodmell et al. 1993). An effective role of the Tc response in protection remains to be clearly demonstrated (Xiang et al. 1995). It is interesting that the G and N antigens conserve their immunogenic properties when administered orally (Hooper et al. 1994). This property is used extensively in large vaccination campaigns with vaccinia or adenoviruses expressing recombinant G protein (see Control).

Most of the current rabies vaccine strains derive from a wild strain isolated by Pasteur (Tordo 1991). This was initially passaged intracerebrally in rabbits until the incubation period shortened and became 'fixed' at 5–8 days. From this, multiple passages in animal brain and cell culture were performed. Vaccine strains belong to genotype 1 (causing classic rabies) and protect against homologous isolates (Lodmell et al. 1995), but do not protect efficiently against rabies-related viruses in genotypes 2–6 that also pose possible threats to humans (Bahloul et al. 1998; Badrane et al. 2001). Work is in progress to improve the protection offered by the vaccine by increasing the number of genotypes against which it is effective. The conserved N protein, a strong inducer of Th cells, may be important in this respect. It behaves as a superantigen in humans and BALB/c mice, activating Vβ8 and Vβ6 T cells, respectively (Lafon et al. 1992; Lafon 1994). The antigenic sites for B and T cell responses have been extensively mapped along the G, N, and P viral antigens by sequencing mutants resistant to neutralizing antibodies or by measuring the reactivity of chemically cleaved or synthetic peptides (Bunschoten et al. 1989; Benmansour et al. 1991; Ertl et al. 1991; Minamoto et al. 1994; Goto et al. 1995). The vast majority of the G protein antigenic sites seem to be conformational, sometimes involving regions that are distant from each other in the primary structure, but which are brought in proximity by protein folding and disulfide bridges. Taking into account the recent modifications in rabies epidemiology and especially the growing importance of bats in human transmission, an important effort has been done to enlarge the protection spectrum of vaccines, from anti-rabies to anti-lyssavirus status. Novel strategies have been elaborated using the versatility permitted by DNA-based immunization (Bahloul et al. 1998; Jallet et al. 1999). Plasmids expressing chimeric G proteins were constructed by fusing the COOH half of one genotype with the NH_2-half of another genotype. Upon intra-muscular injection in mice, protection was obtained against the two parental genotypes but also against other genotypes, demonstrating the possibility of increasing the spectrum from antirabies to anti-lyssavirus vaccines. Protection was also obtained by intramuscular vaccination of dogs (Perrin et al. 2000). Finally, the capability of the lyssavirus G protein to carry foreign epitopes/antigens was also demonstrated in the perspective of a future DNA multivalent vaccine against various zoonoses for carnivores (Desmezières et al. 1999).

CLINICAL AND PATHOLOGICAL ASPECTS: VESICULOVIRUS INFECTION

Vesicular stomatitis

The interest in vesicular stomatitis viruses as models for basic studies in virology and molecular biology has tended to overshadow their natural history and their role as agents of disease. The disease was first reported in livestock in the USA in 1821 (Hanson 1981). It is largely confined to the western hemisphere and reports of its occurrence elsewhere are considered to be either of importations from the West or of doubtful authenticity (Brown and Crick 1979). The virus was first isolated by Cotton (1927) and its morphology established by Chow and co-workers (Chow et al. 1954).

Disease in animals

The clinical manifestations in horses and cattle are fever, development of vesicles on the tongue, gums, and lips, and excessive salivation. Teats of milking cows may be infected and, less commonly, lesions may be seen on the feet. Animals have difficulty in eating and walking, and lose weight; milking cows stop lactating. The disease is rarely lethal except in pigs. Epizootics of vesicular stomatitis are sudden in onset (within approximately 2 weeks) and quickly involve many animals in a herd but often do not spread to adjacent farms. In the USA it occurs in the summer months in approximately 10-year cycles. In Central America, outbreaks are more frequent and possibly associated with the change from wet to dry seasons.

Disease in humans

The virus can also infect humans, usually causing a mild influenzalike illness, although more severe forms are reported. The incubation period is 1–9 days after exposure and symptoms may last 3–4 days; occasionally there may be a relapse (Sellers 1984). Infections of humans have mostly been reported in laboratory workers after aerosol inhalation or accidental contamination of eyes or abrasions. However, in some areas of North and Central

America 25–90 percent of farmers have antibody to VSV (Hanson 1981).

Transmission

The virus is found in vesicle fluid and epithelium and does not persist after recovery. It is not excreted in urine, feces, or milk. Spread by direct contact with vesicle fluid is possible, and transfer from teat lesions by milkers and milking equipment is occasionally reported. However, experimental studies have failed to demonstrate direct animal-to-animal transmission, and Sellers (1984) noted many outbreaks in which the disease did not spread to neighboring animals on the same farm. The bites of infected blood-sucking arthropods seem to be the most likely mode of transmission, demonstrated experimentally with several strains of the virus. In addition, many vesiculoviruses have been isolated in nature from mosquitoes, sandflies, midges, mites, and ticks. There are no overt pathological signs in arthropods, and how they become infected is obscure. The disease in animals being brief, with minimal and transient viremia, they are unlikely natural reservoirs of the virus. It has been suggested that some strains may be plant viruses and that animals are infected either directly, by eating infected plants, or by the bites of insects that have fed on such plants.

Vaccines

Vaccines ranging from live unmodified virus to vaccinia recombinants specific for the G protein have been used with poor success. Their development has not been pursued vigorously, both because of the self-limiting nature of the disease and because the durability of the immune response is uncertain. However, the potential of reverse genetic methods could give new perspectives in this domain (Barr et al. 2002).

CLINICAL AND PATHOLOGICAL ASPECTS: RABIES INFECTION

Although the clinical signs of rabies have been recognized for many centuries, it is mainly since the late nineteenth century that significant progress has been made in laboratory diagnosis, human postexposure treatment, control of animal rabies, and studies of the epidemiology, pathogenesis, and immune response. Currently, in most of the industrialized world, diagnostic systems are well organized, rapid, and efficient; human postexposure treatment is very effective; host species are principally wildlife; and technologically, there are excellent prospects for control or eradication of the disease in large geographical areas. In spite of these remarkable advances, rabies is still a serious problem with significant economic losses and human deaths in large parts of Africa, Asia, and South America.

Clinical aspects

FACTORS DETERMINING CLINICAL MANIFESTATIONS

The fact that not every rabid animal bite could result in clinical rabies and death has pointed out the existence of factors that could determine the clinical manifestations and lethal outcome of the disease (Hemachudha 1989). First, different species have different levels of susceptibility, latency, and infectivity (Hemachudha and Mitrabhakdi 2000). Thus, it has been possible to place species into different groups of low (e.g. opossums), moderate (e.g. nonhuman primates), high (e.g. cattle), and extremely high susceptibilities (e.g. fox) (WHO 1973). Other determinant factors are the severity of the wounds, the site of exposure, and the virus content of the saliva. For example, the incubation period is generally shorter following bite wounds to the head region than after those at the extremities, with grossly an inverse correlation between the length of the incubation period and the dose of virus. It is thought that a specific pattern of clinical manifestations could not be associated with a particular rabies virus variant, as reported by Hemachudha et al. (2003). Taken together, a combination of these factors results in the fact that the incubation period in animals may vary widely, with an usual range from 2 to 12 weeks with periods much longer than 3 months (Charlton et al. 1987), and also in the fact that in human rabies mortality after rabid animal bites varies from 35 to 57 percent (Hemachudha and Phuapradit 1997), with a few recorded cases of incubation periods of several years (Fishbein 1991; Smith et al. 1991).

CLINICAL MANIFESTATIONS IN HUMANS

Rabies is mainly transmitted through bite exposure; however, contamination can occur through aerosol, as reported in caves inhabited by rabid bats or in laboratory accidents with infected aerosolized rabid tissues. Rarely, transmission of rabies may also be associated with handling of infected carcasses, or exposure of the mucous membranes and skin abrasion to virus-laden saliva, and corneal transplantation. The transplacental route has been rarely reported in humans (Hemachudha and Mithrabhakdi 2000).

The incubation period in humans may vary from 20 to 90 days (Jackson 2003), with incubation periods as long as years that had been reported (Smith et al. 1991). The early symptoms are frequently nonspecific and may include general malaise, fever, chills, nausea, vomiting, diarrhea, headache, anxiety, apprehension, and irritability, often accompanied with itching and pruritus at the site of animal bite. After a period ranging from 2 to 10 days, neurological signs appear and the disease

may then develop according to either a furious ('encephalitic') or paralytic ('dumb') form, which may not be distinguished from magnetic resonance imaging (Laothamatas et al. 2003). Fever and proximal weakness with loss of deep tendon reflexes and urinary incontinence are universal findings. Sensory function remains intact in all modalities (Hemachudha and Phuapradit 1997).

The general features accompanying the disease are fluctuating body temperature, probably due to the effect of rabies virus infection on the hypothalamic center, myocarditis, and cerebral hypoxia due to respiratory compromise that can cause intracranial pressure, seizures, and coma (Hemachudha 1994; Warell 1976). In the furious form, signs of irritation of the central nervous system predominate, with hyperactivity (hallucinations, agitation), which can be aggravated by internal or external stimuli such as noise or light. Hydrophobia may occur in up to 50 percent of the patients (Mrak and Young 1994). Periods of hyperactivity may be interspersed with periods of calm and lucidity. Unless the patient dies early, the 'encephalitic symptoms' progress to paralysis and coma. If the disease presents as dumb (paralytic) rabies, the prodrome may be similar but the main feature is paralysis. Unless intensive care is given, patients with encephalitic rabies usually die within 7 days; those with paralytic rabies may survive as long as 2–3 weeks. A very few cases of recovery have been reported in the literature: only four patients with rabies have been reported to survive; none of them had cardinal features of rabies and were regarded as atypical rabies cases (Hemachudha and Mitrabhakdi 2000).

CLINICAL MANIFESTATIONS IN ANIMALS

It is noteworthy that most of the clinical signs of rabies are expressions of neurological dysfunctions rather than neural cell lysis (Charlton 1994). Although the clinical signs in animals are variable and may be nonspecific in the early stages of the disease, the prodromal period is generally accompanied in animals by behavioral changes. For example, during the initial prodromic phase, the dog appears as anxious and nervous (Chomel 1999) and the cat shows gait abnormality and a strange look in the eyes (Fogelman et al. 1993). Most animals become disorientated and wander aimlessly without regard for the social organization of the species.

Although there is variability in many clinical signs, the range in length of illness in most species is 1–10 days. Paralysis is fairly consistent, and is seen in nearly all cases unless death occurs early. In the dog, a furious form can be observed for 1–7 days, characterized by irritability, aggression, hypersensitivity, disorientation, and seizures (Barnes et al. 2003). In the paralytic or dumb form, which lasts 1–10 days, paralysis will affect one or more limbs, and then progresses to affect the entire nervous system, especially the cranial nerves and larynx (Chomel 1999). In horses, a predominant feature is weakness or paralysis of the hindquarters, which may be accompanied by lameness and colic (Green et al. 1992). In cattle, foamy hypersalivation and loss of appetite, voice modification, paralysis, and paresis occurred 2 days after initial signs; presence of the three major symptoms: hypersalivation, loss of appetite, and frequent mooing, should always suggest rabies infection (Chomel 1999). In wildlife, aggressive behavior is observed in up to 50 percent of the rabid raccoons, and the clinical signs are quite similar to those observed in infected skunks. In these latter, it has been shown that experimentally infected animals, after restlessness in the early stages, develop paralysis in a few days and die usually within a week (Charlton 1988, 1994; Charlton et al. 1991). Aggressive behavior has also been well documented in foxes and mongooses, which are often seen attacking domestic animals (Everard and Everard 1992). In bats, rabid animals are generally found lying on the ground, unable to fly; an experimental model of rabies virus infection has allowed to demonstrate that stable variants of the fixed rabies virus strain CVS-24 induced clinical signs such as paresis, ataxia, and inability to fly for one, whereas the second did not induce neurological signs (Reid and Jackson 2001).

Epizootiology and epidemiology

FACTORS AFFECTING THE EPIZOOTIOLOGY OF RABIES

Different species of the orders Carnivora and Chiroptera (bats) support independent epizootic cycles of different *Lyssavirus* variants. A particular species may serve as a principal host only in a limited part of its geographical distribution. The disease is transmitted regularly to a number of other mammalian species in addition to the species recognized as the principal host. The occurrence of rabies in these other species may have little or no influence on the course of an epizootic; however, their role is often not readily defined. Each principal host species has its specific pattern population biology and specific modes of social interaction. These host qualities determine which virus variants are capable of survival in this species. It is essential that an infected animal transmit the virus during a period of virus excretion to an adequate number of other susceptible individuals. For this to occur, *Lyssavirus* strains must be adapted to the physiological traits and population biology of their hosts (Bacon 1985; Wandeler 1991a; Wandeler et al. 1994). They must have a host-specific pathogenicity and pathogenesis (length of incubation period, duration and magnitude of virus excretion, duration and extent of clinical illness). We assume that each principal host has its own virus variants adapted for persistence in its populations. Thus, within the area of a principal host,

there is little virus variation among the isolates, even if they are performed in a species that is the principal host in another area. With the development of molecular and monoclonal antibody technologies it became possible to demonstrate that indeed antigenically distinct variants exhibiting phylogenetic relationships, circulate in different host populations (Rupprecht et al. 1991; Benmansour et al. 1992; Sacramento et al. 1992; Smith et al. 1992; Bourhy et al. 1993a, b; King et al. 1994; Kissi et al. 1995; Nadin-Davis et al. 1993, 1994; Nel et al. 1993; Smith et al. 1993; Tordo et al. 1993a).

TRANSMISSION OF INFECTION

Transmission of the disease usually requires bite-inflicted deposition of virus-laden saliva into tissues of the recipient animal. Virus may be excreted several days before the onset of clinical signs. The maximum recorded times are: fox, 5 days; skunk, 6 days; dog, 7 days with a Mexican isolate and 13 days with an Ethiopian isolate; cat, 3 days; bat (*Tadarida brasiliensis mexicana*), 12 days (for a review, see Charlton 1988). Excretion of virus before signs of illness is the justification for quarantine (usually 10–14 days) of clinically normal dogs and cats that have bitten humans. Occasionally, excretion of virus may be intermittent. Virus may not be isolated from glands of animals that had previously shed virus in saliva. Because of these features and the patchy nature of antigen-containing cells in salivary glands, examinations of saliva or salivary glands at the time of death may not be reliable indicators of virus transmitted in an earlier exposure to a rabid animal. There are very few data on the dose of virus received in natural infections. The carrier is defined as a clinically normal animal that secretes virus in saliva (not the immediately prodromal excretion of virus just described). There is no convincing evidence of the carrier state in field cases in North America. Most of the reports of rabies transmission by apparently healthy animals (usually dogs) and prolonged secretion in saliva are from Africa and Asia (for a review, see Fedaku 1991).

RABIES IN DOGS

One can readily distinguish between areas of the world in which dogs are the predominant hosts and those where rabies is maintained in wild animals and in which only 0.1–5.0 percent of the rabies cases reported annually are in dogs. In large parts of Asia, Africa, and Latin America, rabies in dogs is much more common, making up 95 percent or more of all diagnosed cases. Even though dog rabies is often termed 'urban rabies,' it is clearly a rural problem in many developing countries (Fedaku 1991; Bingham et al. 1999a; Wandeler and Bingham 2000). Dogs are kept and tolerated at very high numbers in most human societies, sometimes reaching densities of several thousand/km^2; this is considerably more than any wild carnivore population ever achieves (for a review, see Wandeler et al. 1993). Such high densities could facilitate enzootic canine rabies, although it is sometimes suspected that rabies in dogs is linked with wildlife rabies. There is no doubt that rabid dogs are the major source of human infection (Beran 1991). In industrialized countries of North America and Europe, where the epizootic is maintained and spread by wild carnivores, three factors may account for the low incidence of rabies in dogs: most dogs are restricted in their movements; they are kept indoors or in enclosures and leashed when outside; dog vaccination is strongly recommended or even compulsory.

RABIES IN WILD CARNIVORA

The principal rabies hosts of the order Carnivora are small- to medium-size (0.4–20 kg) omnivores, scavenging and foraging on small vertebrates, invertebrates, fruit, and refuse produced by humans. They reach high population densities (often several individuals/km^2) in and near human settlements. High intrinsic population growth rates allow rapid recovery of populations reduced by persecution or disease. They are all able to support initial epidemics of high case density and thereafter an oscillating prevalence over many years (Wandeler 1991a).

Areas for which associations of RABV variants with populations of wild Carnivora are well documented are limited to North America, Europe, parts of southern Africa, and some Caribbean islands. The red fox (*Vulpes vulpes*) is the principal rabies host in subarctic and northeastern North America, in all of central and eastern Europe, and in subarctic and temperate zones of Asia (Blancou et al. 1991). In arctic regions of North America and Asia, the arctic fox (*Alopex lagopus*) is the predominant rabies host (Crandall 1991). The viruses isolated from red and arctic foxes from fox rabies areas of North America are all closely related, but distinct from European fox isolates. Jackals (*Canis* spp.) are the principal hosts in southern Africa (Foggin 1985; Bingham et al. 1999b) and are probably involved in much larger areas in Africa and Asia. Other wildlife species that maintain independent cycles of distinct RABV variants in southern Africa are various viverrids, especially the yellow mongoose (*Cynictis penicillata*), and possibly the bat-eared fox (*Otocyon megalotis*) (King et al. 1994). The striped skunk (*Mephitis mephitis*) is the species most often diagnosed rabid in large areas of Canada and the USA (Charlton et al. 1991). Raccoon (*Procyon lotor*) rabies is at present confined to the USA, but is spreading in the southeastern and in mid-Atlantic states (Winkler and Jenkins 1991). Mongooses (*Herpestes auropunctatus*) introduced to some Caribbean islands are now the principal reservoir there (Everard and Everard 1985). Gray foxes (*Urocyon cinereoargenteus*) in some North American areas (Carey et al.

1978) and raccoon dogs (*Nyctereutes procyonides*) introduced to eastern and subarctic Europe (Cherkasskiy 1988) are sometimes suspected of supporting independent epizootics.

RABIES IN BATS

The African bat *Lyssavirus* isolates are of genotypes (serotypes) 2 and 4, while those from bats in Europe were identified as genotypes 5 and 6. Isolates from Australian Megachiroptera and from insectivorous bats are called Australian bat lyssaviruses. They form a distinct clade (genotype 7) in phylogenetic analysis (for a review, see McColl et al. 2000). American bat rabies viruses have been categorized as genotype 1, but a more detailed analysis of the large diversity of distinct isolates offers some surprises. Chiroptera have life history traits that are quite different from those of carnivorous rabies hosts: they are small, long-lived, have low intrinsic population growth rates, and are ecological specialists. The properties of lyssaviruses adapted to bats must therefore be different from those of Carnivora rabies. This statement remains a hypothesis because the population biology and epidemiology of bat rabies is insufficiently explored. A notable exception is rabies in vampire bats as described by Lord et al. (1975). An interesting feature of bat rabies is the vast antigenic diversity of isolates. In Canada alone, 12 distinct variants are recognized with mAbs. Several variants occur in a single species, and the geographical distribution of variants is overlapping (Nadin-Davis et al. 2001). This is in sharp contrast to the pattern of rabies in Carnivora, where very little epitope variation is recognized over very large areas (Nadin-Davis et al. 1999).

INCIDENCE OF RABIES IN HUMANS

In industrialized countries, rabies in humans is rare owing to low rates of exposure, high standards of health education and relatively easy access to postexposure treatment with potent vaccines. However, an estimated 35 000–60 000 people die from rabies every year worldwide. The number of people receiving postexposure treatment – mostly after dog bites – is about 3.5 million per year (Bögel and Motschwiller 1986; Bögel and Meslin 1990). Almost all deaths of humans from rabies and the vast majority of treated bite exposures occur in developing countries (Acha and Arambulo 1985). This may in part be due to a high rate of exposure to biting rabid dogs, but even if this assumption is correct, it does not fully explain the high number of rabies casualties. In view of the efficacy of modern postexposure treatment, nearly all cases of rabies in humans must be considered as failures of the medical system. For various reasons, including lack of availability or ignorance, the correct treatment was not applied or not applied in time (Wandeler et al. 1993).

PATHOGENESIS AND PATHOLOGY

Rabies virus spread

INOCULATION SITE

After contamination, it has been shown that rabies virus might infect the cells located within the site of inoculation, generally the myocytes (Figure 8) (Murphy et al. 1973; Charlton and Casey 1979). The data are confirmed by in vitro models of infection of cultured myotubes, where infection and replication of street rabies virus can be demonstrated (Tsiang et al. 1986). These data can provide an explanation for the long incubation that can be observed during natural rabies virus infection (Charlton et al. 1997). However, classical experiments have shown that in some cases rabies virus was able to leave the site within a few hours following inoculation, in agreement with a mouse model of infection where direct infection of the nerve endings without prior replication in the muscle has been demonstrated (Shankar et al. 1991).

VIRUS SPREAD TO THE CENTRAL NERVOUS SYSTEM

Whichever are the first host cells to be infected, rabies virus enters both sensory and motor fibers (Coulon et al. 1989; Lafay et al. 1991) probably through a receptor-mediated endocytosis (for the putative receptors see section on Epizootiology and epidemiology above). Studies in cultured neuronal cells indicate that after entry in axon terminals (or somatodendritic domain), rabies virus can be detected in endosomes shortly after uptake (Lewis and Lentz 1998; Lewis et al. 1998).

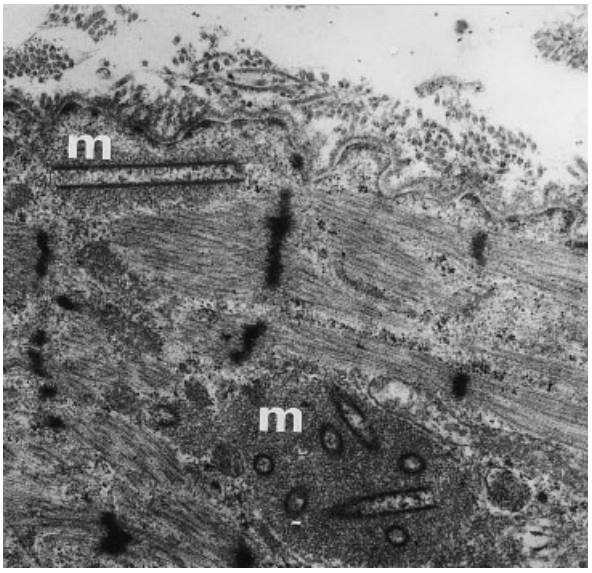

Figure 8 *Electron micrograph of two bodies of matrix (m) and anomalous tubular structures in a muscle cell of a skunk at the site of inoculation of street RABV (×2 000).*

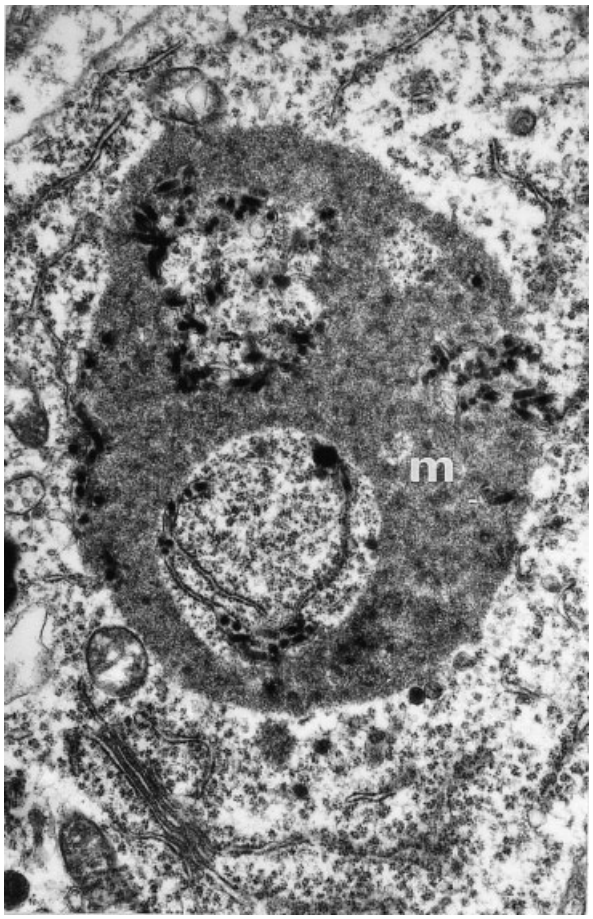

Figure 9 *Electron microscopic structure of Negri body in a neuron in skunk brain. Note the body of matrix (m) with entrapped rough endoplasmic reticulum, free ribosomes and virions (×15 000).*

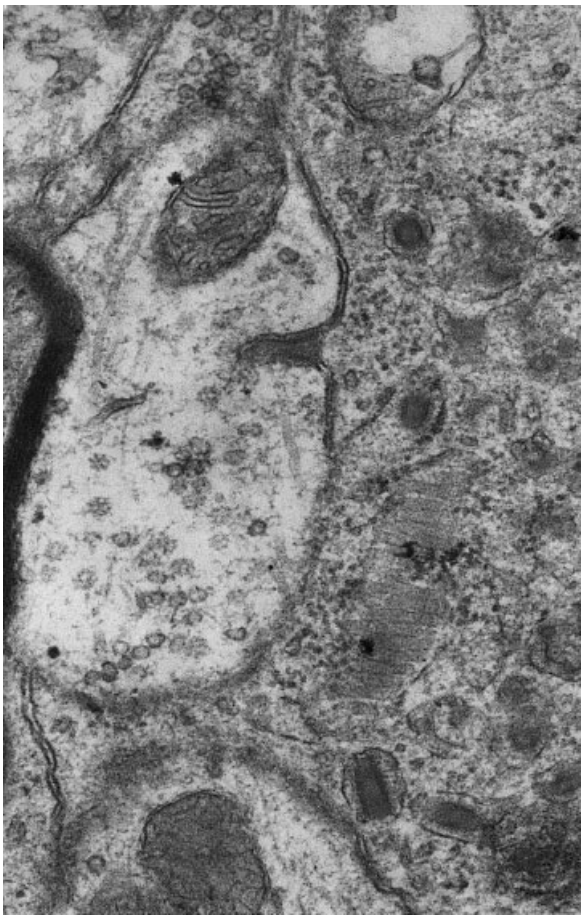

Figure 10 *Electron micrograph of skunk brain. Virion budding on neuronal plasma membrane and simultaneous endocytosis by an adjacent axon terminal (×25 000).*

Rabies virus is then transported through axonal transport in the peripheral nervous system toward the central nervous system. This transport is dependent on the microtubule network, as shown by inhibition produced by microtubule-disrupting colchicine and vinblastine (Bijlenga and Heany, 1978; Tsiang 1979). By using cultured sensory neurons in two-compartments devices, it has been demonstrated that rabies virus used the retrograde axonal transport to disseminate within the neuron, at a rate of 25–50 mm/day at least, which is compatible with a fast microtubule-based retrograde transport (Lycke and Tsiang 1987; Tsiang et al. 1991). These properties of rabies virus have been used for retrograde transneuronal tracing studies within the peripheral nervous system (Tang et al. 1999) (Figures 9 and 10).

SPREAD WITHIN THE CENTRAL NERVOUS SYSTEM

Once rabies virus has reached the spinal cord or brainstem neurons, it disseminates rapidly throughout the central nervous system. Analysis of postmortem brains of naturally infected humans indicate that almost all the brain areas may be infected: thalamus, spinal cord, pons, medulla oblongata, cerebellum, hippocampus, and striatum (Bingham and van der Merwe 2002; Vural et al. 2001). Rabies virus replication occurs primarily in the neurons (Tsiang et al. 1983; Gillet et al. 1986; Kelly and Strick 2000), although some rabies virus strains in some species have been reported to be able to infect significantly other neural cells, such as Bergmann glia (Jackson et al. 2000). The spread of rabies within the central nervous system occurs from neuron to neuron by combination of axonal transport and transsynaptic passage.

The transsynaptic passage has been studied by stereotactic inoculation experiments in the rat (Gillet et al. 1986; Ceccaldi et al. 1989), which have shown that rabies virus uptake occurs mainly at the axon terminal, following the neuroanatomical connections. This is compatible with electron microscopy studies in the skunk, showing viral budding at the postsynaptic membrane (Charlton and Casey 1979). The viral glycoprotein G is required for this transsynaptic transfer (Etessami et al. 2000) and is responsible for the diverse

distribution patterns of the different rabies virus strain in the brain (Yan et al. 2002).

The retrograde axonal transport of rabies virus in the brain has also been demonstrated by stereotactic inoculation experiments (Gillet et al. 1986; Ceccaldi et al. 1989; Etessami et al. 2000) and by neuroanatomical studies (Kucera et al. 1985). More recently, the retrograde axonal transport, transsynaptic transfer and its low cytopathic effect of rabies virus has been used for neuroanatomical tracing studies within the monkey brain (Kelly and Strick 2000). Rabies virus transport within the central nervous system is dependent on the integrity of the microtubule network (Ceccaldi et al. 1989; Ceccaldi et al. 1990). In the past few years, the molecular basis for axonal transport of rabies virus has been extensively studied since two independent groups demonstrated a strong interaction between the rabies virus phosphoprotein P and the light chain (so-called LC8 of the cytoplasmic dynein) (Jacob et al. 2000; Raux et al. 2000). These results are confirmed by reverse genetic studies showing that abolishing the P–LC8 interaction by deletion of the conserved LC8-interacting motif (K/RXTQT) in the rabies virus phosphoprotein P is able to reduce the efficiency of peripheral spread of an attenuated rabies virus strain SAD D-19 (with replacement at the Arg at position 333) (Mebatsion 2001). However, this deletion has no effect in intracranial inoculation, suggesting that other mechanisms could act together with P–LC8 interaction for rabies virus spread, although recombinant *P*-gene-deficient rabies viruses are apathogenic in adult and suckling mice, even when inoculated intracranially (Shoji et al. 2004).

Events within the central nervous system

HISTOPATHOLOGY

Relatively mild neuropathological changes are generally observed in the central nervous system of infected hosts (Iwasaki and Tobita 2002), and neuronal cell death does not seem to be prominent during the course of rabies (Ceccaldi 1999; Jackson 2003; Kelly and Strick 2000). However, according to the viral strains that are concerned, mild to severe histopathological can be observed, although they are not constant. They can vary from moderate perivascular cuffing with mononuclear cells (Foley and Zachary 1995), focal and regional gliosis, slight neuronophagia, and presence of inflammatory cells in the meninges (Charlton 1988; Perl and Good 1991) to extensive neuronal degeneration and neuronophagia, associated with widespread inflammation (Fekadu et al. 1982).

Spongiform lesions in rabies were first described by Charlton (1984) in experimentally infected skunks and red foxes, and were further found in natural infections and in other species (Foley and Zachary 1995). The

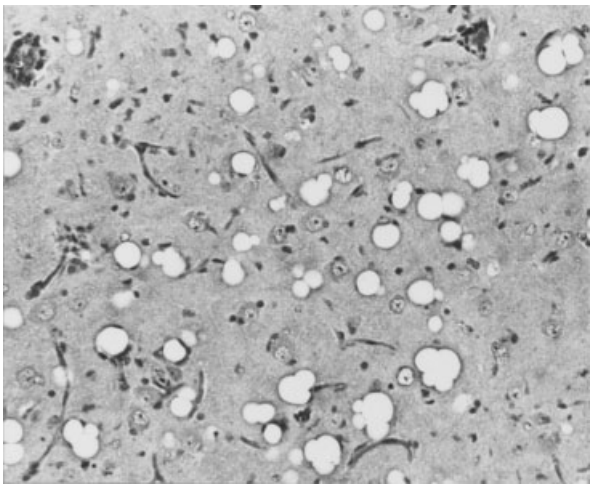

Figure 11 *Spongiform lesions in neurophil of thalamus of a skunk experimentally infected with street RABV (H & E, ×160) (reproduced, with permission of the publisher, from Charlton 1984).*

thalamus (all nuclei) and cerebral cortex are the most frequently and severely affected regions (Figure 11). Vacuolation seems to develop rapidly, probably in <2–3 days, occurs after infection by several street virus variants, and is independent of the immune response, route of inoculation, or viral preparation (Charlton et al. 1987) (Figure 12). In affected regions of the brain, the degree of vacuolation was not correlated with the amount of antigen accumulation, suggesting that spongiform change in rabies is probably an indirect effect, such as neurotransmitter imbalance.

NEURAL DYSFUNCTIONS

The fact that brain damage may be relatively limited and that neuronal death is not pronounced during rabies

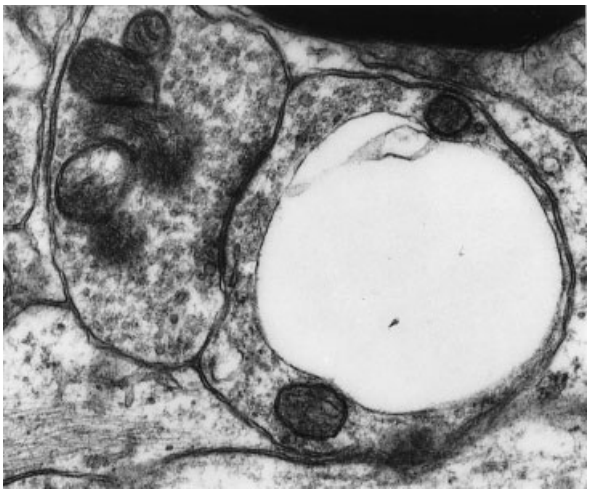

Figure 12 *Electron micrograph of a membrane-bound vacuole in the dendrite of a neuron in skunk brain (×30 000) (reproduced, with permission of the publisher, from Charlton 1984).*

has rapidly led to the hypothesis that rabies disease would be mostly due to neural dysfunction rather than neuronal cell lysis (Tsiang et al. 1983; Jackson 2002). Clinically, some data have been provided recently by magnetic resonance imaging of the brain where some patients show multifocal lesions in the brainstem and hypothalamus, with high signals on T2-weighted images in the medulla, pontine tegmentum, and hypothalamus (Pleasure and Fischbein 2000). Experimentally, it has been shown that rabies virus was able to alter the neuronal physiology at different levels, in cultured neural cells as well as in murine models. These alterations concern the release and uptake of neurotransmitters, as shown for GABA whose uptake is reduced (45 percent) in rabies virus-infected cells (Ladogana et al. 1994), or serotonin release (Bouzamondo et al. 1993). Changes in the number of receptors to neurotransmitters have also been described (Ceccaldi et al. 1993). Abnormalities are also detected in the modulation of neural receptors, such as α2-adrenoreceptors whose modulation is prevented by rabies virus, but not muscarinic receptors, of Ca^{2+} channels in neuroblastoma cells (Iwata et al. 2000). Nitric oxide neurotoxicity has been hypothesized to mediate neuronal dysfunction in rabies, since induction of inducible nitric oxide synthase mRNA and increased quantities of nitric oxide in brains of experimentally infected rodents have been reported (Koprowski et al. 1993; Hooper et al. 1995). Interestingly, inducible nitric oxide synthase inhibition is able to delay death of rabies virus-infected mice (Ubol et al. 2001).

More recently, regulation of neuronal gene expression has been described in experimentally infected murine models of rabies virus. These works report activation of immediate–early genes mRNA expression (correlated with an increase in rabies RNA (Fu et al. 1993), up-regulation of nine genes during rabies virus infection (Egr1, STAT1) (Saha and Rangarajan 2003) and dysregulation of expression of 39 genes during infection; these included genes involved in regulation of cell metabolism, protein synthesis, synaptic activity, and cell growth and differentiation (Prosniak et al. 2001).

APOPTOSIS

Apoptosis can be observed during rabies infection both in vivo in mice (Thoulouze et al. 1997), and in virus-infected mouse neuroblastoma cells which undergo chromatin condensation and DNA fragmentation within 48 h postinfection (Ubol et al. 1998). Interestingly, a bat strain and a primary canine rabies virus isolate were both able to induce apoptosis in infected mice and reactivate a developmentally down-regulated gene, *Nedd-2*, which may be required for apoptotic elimination of cells damaged by infection (Ubol and Kasisith 2000). Apoptosis could play a role during nonfatal rabies infection, where the levels of viral replication and primary degeneration of infected neurons by apoptosis could be responsible for the infiltration of T lymphocytes capable of inducing secondary degeneration of neural cells (Galelli et al. 2000). The pro-apoptotic properties of the different virus strains being dependent on the viral glycoprotein G (Préhaud et al. 2003) as well as matrix protein M (Kassis et al. 2004).

However, the fact that a silver-haired bat rabies virus variant does not induce apoptosis in the brain of experimentally infected mice suggests that apoptosis would not be an essential pathogenic mechanism for the outcome of a rabies virus infection. Furthermore other pathological processes may contribute to the profound neuronal dysfunction characteristic of street rabies (Yan et al. 2001), although numerous apoptotic neurons could be identified in the brain stem and hippocampus of the brain of a rabies- (and HIV-)infected patient (Adle-Biassette et al. 1996).

PERIPHERAL SPREAD OF VIRUS AND INFECTION OF NONNERVOUS TISSUE

While rabies virus disseminates and replicates within the central nervous system, a subsequent viral transport to peripheral tissues occurs. This transport allows infection of nonnervous tissues such as muscle, myocardium, kidney, lung, pancreas, and lacrymal glands. In a study performed in 11 human rabies confirmed cases, it has been shown that rabies virus antigens could always be found in the adrenal glands, heart, pancreas, and gastrointestinal tract (Jogai et al. 2002). In a murine experimental model of infection, Tsiang demonstrated that the mechanism for such a centrifugal spread was microtubule dependent and involved the axonal transport (Tsiang 1979). The infection of salivary glands is of particular relevance to further transmit the disease for many species (Figures 13 and 14).

IMMUNE RESPONSES

An important concern is the extent to which the expressions of the naturally occurring disease are the result of the immune response. Although conclusive evidence from natural infections is rather sparse, several reports of experimental studies suggest that the immune response can influence the susceptibility, incubation, and morbidity periods, the type of clinical signs, excretion of virus, and recovery from infection (before or, rarely, after clinical signs). In addition, these effects frequently depend on interactions of the immune response with other factors, such as animal species and age, viral strain, route of inoculation, and dose of virus.

NEUTRALIZING ANTIBODIES

Virus-neutralizing antibodies (VNA) are a critical component of immune resistance. They are produced in response to RABV glycoprotein; production is T-cell dependent, requiring $CD4^+$ T cells, as well as B cells. They are considered to be most effective in the early

Figure 13 *Electron micrograph of rabies virions in acinar lumen of submandibular salivary gland (skunk) (×30 000).*

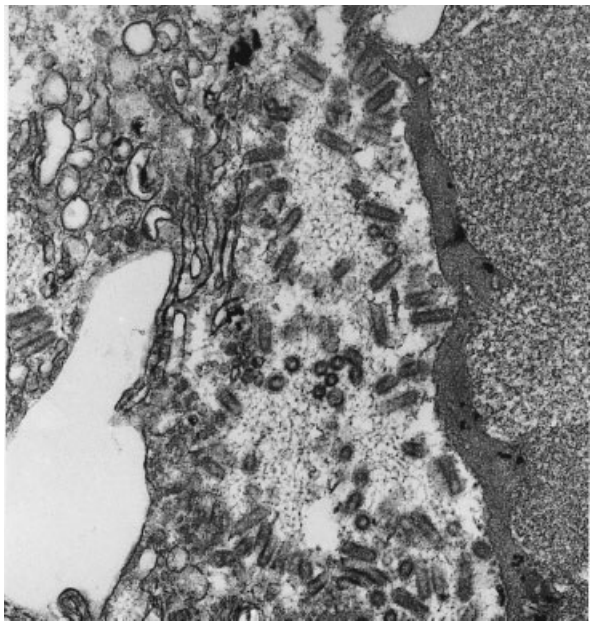

Figure 14 *Electron micrograph of mucous cell of submandibular salivary gland of skunk infected with street RABV. Viral budding on the inner surface of secretory granules (×37 500).*

stages of infection, i.e. before viral entrance into peripheral nerves. However, some weakly pathogenic strains can be cleared from the central nervous system (CNS) by VNA. Apparently, protection resides primarily with IgG rather than IgM (for reviews, see Turner 1990; Lafon 1994). Measurement of serum VNA has been and continues to be the predominant method for estimating immune resistance to rabies infection in humans, and in conjunction with challenge is frequently used in efficacy tests of vaccines in other animals.

Generally, VNA is not detected in infected humans until after the onset of clinical signs (for reviews, see Fishbein 1991; Hemachudha 1994). In animal experiments, the relation of neutralizing antibody to outcome of infection is variable. There are several reports of VNA in a small proportion of apparently healthy foxes, skunks, and raccoons in rabies enzootic areas, presumably because of previous exposure to the virus (for reviews, see McLean 1975; Winkler 1975; Blancou et al. 1991; Winkler and Jenkins 1991; Wandeler et al. 1994). The prevalence of VNA in mongooses in enzootic areas in Grenada may be high (up to 40 percent), and fluctuates with changes in the incidence of rabies. High titers of VNA may be indicative either of advanced CNS infection or of recovery. Evidence that the VNA response can influence susceptibility to rabies comes mainly from work with inbred strains of mice inoculated intraperitoneally with street RABV (for review, see Lodmell 1988): resistant and susceptible strains were, respectively, good or poor responders in terms of VNA production.

N PROTEIN

Recent advances include the demonstration, originally by Dietzschold and co-workers (1987), that N protein can induce an immune response sufficient to protect animals against challenge in the absence of detectable VNA. Priming with N protein can augment the production of VNA on subsequent inoculation of live vaccines (for review, see Fu et al. 1994). A recent study indicates that the nucleoprotein of RABV is a superantigen (Lafon et al. 1992). The significance of this in immune resistance has not yet been determined (Lafon 1994).

IMMUNOSUPPRESSION AND CELL-MEDIATED IMMUNITY

Various features of rabies have been studied by immunosuppression or other manipulations of immune mechanisms in experimental animals. The findings include an increase or a decrease in the incubation period due to immunosuppression (Tignor et al. 1974; Smith et al. 1982; Charlton et al. 1984); early death in mice due to antibody and not to immune T cells (Prabhakar and Nathanson 1981); optimum clearance of RABV from the nervous system requires both B and T lymphocytes (Kaplan et al. 1975; Miller et al. 1978; Prabhakar et al. 1981; Smith 1981); immunosuppression-facilitated centrifugal neural

migration of virus (Smith et al. 1982); and dissemination to the salivary glands (Charlton et al. 1987).

Cytotoxic T cells can be induced by rabies glycoprotein, nucleocapsid, N protein, and NS protein (for review, see Lafon 1994). The precise role of cytotoxic T cells in immunoprotection has not been resolved. Under certain circumstances, they may be critical for host defense (Nathanson and Gonzalez-Scarano 1991). Lafon (1994) has indicated that, in the absence of neutralizing antibodies, cytotoxic T cells are not sufficient to protect the host; however, their participation in combination with other immune effector mechanisms cannot be excluded. Alternatively, they may contribute to immune injury in rabies.

INFLUENCE OF IMMUNE RESPONSE ON TYPE OF RABIES

The role of the immune response in the type of clinical signs, especially encephalitic versus paralytic rabies, is now being studied. As described above, in skunks inoculated with street virus, early spread in the CNS included ventral motor neurons in the lumbar cord, the motor cortex, and other brain nuclei relevant to motor control of skeletal muscle. Assuming its deleterious effect on infected neurons, an early immune response might be more likely to induce paralytic rather than encephalitic signs. In fact, several studies do support a role for the immune response in paralytic rabies (Tignor et al. 1974; Iwasaki et al. 1977; Smith et al. 1982; Sugamata et al. 1992; Weiland et al. 1992; Lafon et al. 1994). However, Hemachudha et al. (1988) suggested that in humans, lymphocyte proliferative responses are associated with encephalitic rabies, and that paralysis in mice infected with the CVS strain of RABV did not depend on the T-cell system (Hovanessian et al. 1988). Still other studies suggest that the immune response has little effect on the type of clinical signs (Gourmelon et al. 1991; Charlton 1994).

Diagnosis

Rabies diagnosis serves epidemiological surveillance, the monitoring of disease control efforts, and to educate postexposure prophylaxis (PEP) in exposed people. Because almost all human infections result from bites by rabid animals, the decision to initiate or continue PEP often depends on the results of laboratory diagnostic tests on the animal of concern. Diagnostic programs require efficient and well-organized field inspection, specimen collection, and submission systems, adequately equipped laboratories and well-trained laboratory personnel, and effective reporting systems. Details of the laboratory procedures are contained in several excellent reviews (Webster and Casey 1988; Sureau et al. 1991; Trimarchi and Debbie 1991; Bourhy et al. 1992; Meslin et al. 1995).

NEGRI BODIES

The inclusion bodies discovered by Negri (1903) in the cytoplasm of rabies-infected neurons served as exclusive pathognomonic lesions for half a century until immunofluorescence methods were introduced in the late 1950s and early 1960s (Goldwasser and Kissling 1958). The Negri body methods are rapid, but there is a fairly high rate of false negatives (10–20 percent) when compared to antigen detection by immunofluorescence or virus isolation.

IMMUNOFLUORESCENCE

During the 1960s, the fluorescent antibody test (FAT) rapidly became the method of choice. The method can be performed on smears of brain tissue, produces results in a few hours, and, most importantly, is highly specific. With improvements in conjugates and microscopes, the rate of false-negatives has gradually decreased to about 0.1 percent. Most laboratories use immunofluorescence as a primary test. It is recommended that negative FAT results on brains from animals with a history of human exposure be subjected to a confirmatory test, such as a cell culture or mouse inoculation test.

MOLECULAR BIOLOGICAL TECHNIQUES

The 1992 WHO Expert Committee on Rabies did not recommend PCR for routine rabies diagnosis because of the obvious difficulties in preventing cross-contaminations in diagnostic laboratory settings. However, molecular techniques are becoming rapidly more accessible and sophisticated and have potential for diagnostic applications (Sacramento et al. 1991; Tordo et al. 1992b, 1995; Kamolvarin et al. 1993), particularly for antemortem confirmation of suspect human cases (Tordo et al. 1995) and for material that is not suitable for FAT.

OTHER METHODS

The rapid rabies enzyme immunodiagnosis (RREID) test has a high degree of accuracy, and has been advocated for laboratories (e.g. in developing countries) having difficulties in using the FA test, and for screening large numbers of specimens in epidemiological studies. It can be performed on heat-inactivated specimens but not on formalin-fixed specimens. Immunohistochemical (immunoperoxidase) methods and in situ hybridization (Nadin-Davis et al. 2003) are suitable when only formalin-fixed specimens are available.

Antemortem diagnosis is occasionally requested for humans with suspected rabies. Corneal impressions and skin biopsies (back of the neck above the hairline) are recommended for immunofluorescence and oral fluids for virus isolation and PCR detection of viral RNA.

IDENTIFICATION OF VIRUS VARIANTS

Molecular analysis has largely replaced serological methods for the classification of viruses. The character-

ization of selected parts of viral genomes permits reconstructing evolutionary relationships. These can be displayed as phylogenetic trees. Likelihood values can be calculated for different conceivable branching topologies. Molecular epidemiology is the reconstruction of the history of an epidemic using these powerful tools (Nadin-Davis et al. 1999). Monoclonal antibodies recognize phenotypic aspects of viral proteins and can therefore not provide phylogenetic information with the same accuracy. However, they bind to molecular surface structures recognized by a host's immune system, structures that are likely to have functional properties on which natural selection may act. While molecular studies remain fairly laborious and costly processes, the antigenic characterization with mAbs is an inexpensive 'workhorse' approach to identify large numbers of virus isolates in a diagnostic laboratory setting. Though the binding of a larger number of mAbs can be studied on infected cell cultures, it is important that the tests can be conducted on the original brains in order to remain low in cost and efforts. A panel of mAbs must discriminate between the different species and variants occurring in an area. Variants should be defined as members of a clade (branch) in a phylogenetic tree.

Control

The ultimate purpose of rabies control is the protection of humans from both infection and economic loss. In humans, rabies can be controlled by prophylactic vaccination and postexposure treatment, by reducing the risk of human exposure, or, conclusively, by eradication.

CONTROL OF RABIES IN DOMESTIC ANIMALS

Control measures may include immunization, movement restrictions, reproduction control, habitat control, and removal of stray dogs (Wandeler and Bingham 2000; Meslin et al. 2000).

Immunization

An effective way of reducing the incidence of human infection is by prophylactic immunization of those domestic animals that are the most common sources of human exposure. Rabies has a high incidence in dogs in enzootic areas where dog populations reach high densities. Well-planned and -executed vaccination campaigns drastically reduce rabies incidence in dogs and may even eliminate the disease in areas where it is not maintained by wildlife. Elimination of the disease should be the goal rather than a temporary reduction in incidence. The most economical way to achieve it is by mass vaccination performed in accordance with a comprehensive national plan. Vaccinations should be done by parenteral inoculation of a product approved by the national authorities, usually an inactivated vaccine conferring 2 years of immunity after one injection. When the appropriate technology and resources are available, the immunization of individual domestic animals should be verifiable by certification, preferably in the form of an implanted microchip.

Whenever possible, modern inactivated cell culture vaccines should be used for the immunization of dogs and other domestic animals. They combine safety with high immunogenicity. Cell lines and primary cell cultures are used as substrates for a number of different virus strains. Several manufacturers include a variety of different antigens (distemper, adenovirus, leptospirosis, parainfluenza, parvovirus) in combined vaccines. The use of live-attenuated vaccines is no longer recommended for dog immunization, except for special situations (e.g. national campaigns under economic constraints). Live recombinant vaccines and other products of genetic engineering are now becoming available.

Quarantine

Many countries have legislation for destroying or quarantining for several weeks domestic animals after possible contact with a suspect animal. A ten-day quarantine is recommended for dogs and cats that have bitten a person. The quarantined animal is killed and submitted for laboratory diagnosis if it develops suspicious symptoms during this observation period.

A number of countries that are free from rabies in wild carnivores and dogs have prevented the importation of terrestrial mammal rabies by rigorous enforcement of quarantine measures; for example, a 6-month quarantine period for imported cats and dogs in the UK. This approach is now considered unnecessary, given the availability of effective vaccines for pet animal immunization and tests for verifying an adequate antibody response.

IMMUNIZATION OF WILD ANIMALS

Control of wildlife rabies by reducing population densities of host species has been attempted repeatedly. However, the resilience of these opportunistic Carnivora to persecution, their reproductive potential, and high carrying capacities of both rural and urban habitats often nullify such efforts. A more promising approach is mass vaccination of the main hosts. Immunization of free-living wild animals is not an easy task. The wild mammal has to be lured into vaccinating itself. The methods have to be simple and efficient, so that it becomes technically and economically possible to establish the herd immunity required to eliminate rabies. Oral rabies immunization became attainable in the early 1970s when it was found that ingested attenuated rabies vaccine, ERA, immunized foxes (Black and Lawson 1970; Baer et al. 1971). This discovery suggested the possibility of administering an oral rabies vaccine to wild carnivores by bait.

Wandeler (1991b) detailed the requirements for vaccines to be used for oral immunization of free-living wild animals. Currently only live-attenuated or live recombinant vaccines are available for oral immunization. They have to infect host cells for eliciting a protective immune response. A high residual pathogenicity is a severe weakness of the initially applied ERA/SAD vaccines. The most important qualities of baits for proper vaccine delivery are that they should, as far as possible, be attractive for the target species and not for others. The delivery system must ensure mass immunization of the target species. Technical resources, administrative structures and labor needs, as well as constraints imposed by safety requirements, terrain, climate, etc., have to be taken into account.

The development of oral immunization of wildlife as an instrument for rabies control has been described by Winkler (1992). Aubert et al. (1994) have reported the extent of wildlife rabies vaccination in Europe, and Campbell (1994) has given an account of the North American situation. The first field trial with foxes was carried out in Switzerland in 1978 (Steck et al. 1982). Most areas in Switzerland and later, large areas of other European countries and Canada became free from rabies after oral immunization campaigns with ERA/SAD vaccines. Newer genetically engineered recombinant vaccines have great potential for wildlife rabies control (Blancou et al. 1986; Rupprecht et al. 1986; Yarosh et al. 1996). A vaccinia–rabies glycoprotein recombinant (V–RG) has been used successfully in Belgium (Brochier et al. 1991), Luxembourg, and in parts of France (Aubert et al. 1994). V–RG is applied in North America in attempts to control rabies in raccoons (Robbins et al. 1998), coyotes, and gray foxes.

The most important conclusion from the field applications of oral vaccination is that it appears to be possible to immunize sufficient host individuals to stop the spread of the disease into rabiesfree areas and to eliminate it from enzootic areas in terrestrial mammals. In Europe, rabies did not reappear spontaneously from an undetected reservoir after fox vaccination campaigns were discontinued, but was occasionally able to reinvade a fox population from infected contiguous areas. However, caution has to be applied when interpreting prevalence data from field studies that have no adequate controls and that cannot be repeated (Wandeler 2000).

RABIES PROPHYLAXIS IN HUMANS

The history of rabies vaccines and of postexposure treatment in humans from the time of Louis Pasteur to the modern era has been well described (Vodopija and Clark 1991; Thraenhart et al. 1994). Highly efficacious vaccines that usually provoke only minimal or no side effects are now available and can be used for pre- and postexposure immunization. All of them contain RABV antigens in a concentrated and partially purified form. Preparations widely used are the human diploid cell strain (HADES) or the Vero cell strain, which are inactivated with β-propiolactone. Unfortunately, because of economic considerations, the cheaper brain tissue-derived vaccines are still predominant in large parts of the world. These vaccines are of inferior potency and may induce severe allergic reactions that can occasionally be difficult to distinguish from clinical rabies (Hemachudha 1989).

Pre-exposure prophylaxis

Pre-exposure immunization is generally encouraged only for people at potentially high risk of exposure; for example, laboratory personnel working with rabies, veterinarians, and animal control and wildlife officers. Pre-exposure immunization consists of a series of inoculations (usually three) within 21–180 days with an approved and potent inactivated vaccine. Serum antibody titers should be monitored. For people at continuing high risk of exposure, it is recommended that booster injections are given when the serum antibody titer falls below 0.5 IU/ml (WHO 1992).

Postexposure prophylaxis

People bitten or licked by a mammal acting abnormally in a geographic area where rabies might occur must be considered as exposed and postexposure treatment should begin as soon as possible. Properly administered, it is highly effective. It consists of passive immunization with rabies immunoglobulin, and vaccination (WHO 1992). Instant thorough washing of bite and scratch wounds with water and soap or detergent is most important; the use of disinfectants is also recommended. Purified rabies immunoglobulin, of equine or, preferably, human origin (HRIG), and the first dose of a potent vaccine must be administered with as little delay as possible. Additional doses of vaccine at the officially specified intervals are necessary. Tetanus prophylaxis should also be given to people without definite evidence of recent immunization. Recommendations on what has to be considered an exposure, what vaccines to use, routes of vaccine administration, treatment schedules, and the application of immunoglobulins vary from country to country; the guidelines of the national health authority must be consulted.

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Borna disease virus

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GENERAL FEATURES

Borna disease virus (BDV) is the prototype of the family *Bornaviridae*, genus *Bornavirus*, within the nonsegmented, negative-strand (NNS) RNA viruses (order *Mononegavirales*). The name Borna refers to the city of Borna, Germany, which was the site of an equine epidemic in 1895–1896 that crippled the Saxon cavalry. This neurotropic virus appears to be distributed worldwide and has the potential to infect most, if not all, warmblooded hosts. At the time of writing it is the only known example of the family *Bornaviridae*. BDV is similar in genomic organization to other NNS RNA viruses; however, its genome (about 8.9 kb) is substantially smaller than those of *Rhabdoviridae* (about 11–15 kb), *Paramyxoviridae* (about 15–16 kb) or *Filoviridae* (about 19 kb), and BDV is distinctive in its nuclear localization of replication and transcription. Although this feature is shared with the plant nucleorhabdoviruses (Martins et al. 1998), it is unique among NNS RNA animal viruses. Genome organization and gene expression of BDV are remarkable for the overlap of open reading frames (ORF), transcription units, and transcription signals; readthrough of transcription termination signals; and differential use of translation initiation codons. There is precedent for the use of each of these strategies by the *Mononegavirales*. The concurrent use by BDV of such a diversity of strategies for the regulation of its gene expression is unprecedented in NNS RNA viruses. Furthermore, BDV uses the cellular splicing machinery to generate some of its mRNAs. Although splicing is found in *Orthomyxoviridae* (segmented, negative-strand RNA viruses), it is novel in an NNS RNA virus.

Virion molecular weight and the $S_{20,\omega}$ are not known. Partially purified BDV infectious particles have a buoyant density in CsCl of 1.16–1.22 g/cm^3, in sucrose of 1.22 g/cm^3, and in renografin of 1.13 g/cm^3. Virions are stable at 37°C and lose only minimal infectivity after 24 h of incubation in the presence of serum. Virus infectivity is rapidly lost by heat treatment at 56°C, exposure to pH 5.0, organic solvents, detergents, chlorine, formaldehyde, or UV radiation (Ludwig et al. 1988). Several complete genomic BDV sequences are now reported, including strain V (Briese et al. 1994), He/80 (Cubitt et al. 1994a), CRNP5 (a rat-passaged strain with altered pathogenicity) (Nishino et al. 2002), and No/98 (Nowotny et al. 2000; Pleschka et al. 2001). No/98, an Austrian isolate, is unusual in that it differs from other isolates by more than 15 percent at the nucleotide level; other known BDV isolates, including those where only partial sequence data are available, differ generally by less than 6 percent.

MOLECULAR BIOLOGY OF BDV

BDV genome organization

Independent cloning experiments in two laboratories, using either ribonucleoprotein (RNP) preparations or viral particles obtained from infected cultured cells with high salt release techniques, revealed a negative-polarity single-stranded RNA genome comprising approximately 8.9 kb (Briese et al. 1994; Cubitt et al. 1994a). The BDV genome is remarkably compact, encoding six major ORFs in three transcription units

framed by complementary termini similar to those of other NNS RNA viruses (Figure 1) (Briese et al. 1994; Wehner et al. 1997). The first transcription unit reveals a simple pattern with only one coded protein (nucleoprotein, N, p38/40). The second transcription unit encodes proteins X (p10) and P (phosphoprotein, p23) in overlapping ORFs. The third unit contains coding sequence for the matrix protein (M, p16), type I membrane glycoprotein (G, p57, gp94), and polymerase (L, p190). Elaboration of these proteins is dependent upon a variety of mechanisms for transcriptional, posttranscriptional, and translational control of expression, including alternative transcriptional initiation, readthrough of termination signals, splicing, and leaky ribosomal scanning (reviewed in Schneemann et al. 1995).

Replication and transcription

Replication and transcription of the BDV genome occur in the cell nucleus. Although nuclear replication and transcription are found in segmented negative-strand RNA viruses and nucleorhabdoviruses (NNS RNA viruses of plants), BDV is the only NNS RNA virus of animals with this property (Briese et al. 1992).

The 5′ and 3′ terminal nontranscribed sequences of NNS RNA viruses typically encode promoters that serve for both replication and transcription of the genome. BDV contains a 42 nucleotide untranslated region (UTR) at the 3′ end of the genome, which presumably functions as a promoter for expression of the viral (+) sense antigenome and viral transcripts, and a 54 nucleotide UTR at the 5′ end, presumably initiating

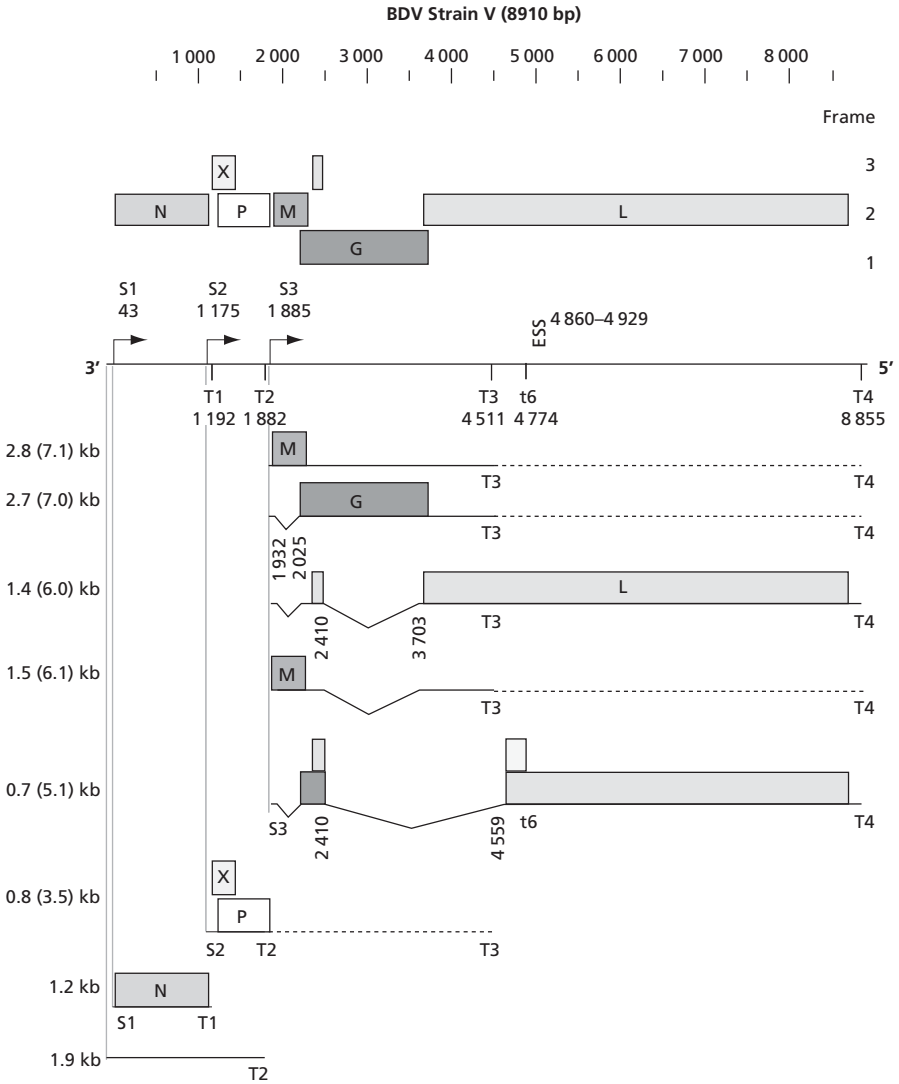

Figure 1 *BDV genomic map and transcripts. Abbreviations: S1 through S3, initiation sites of transcription; T1 through T4, and t6, termination sites of transcription. Readthrough at termination signals T2 and T3 is indicated by dashed lines; ESS, exon splicing suppressor.*

transcription of the (–) sense genome during replication (Schneemann et al. 1994). In other NNS RNA viruses the ratio or abundance of viral proteins during the replication cycle in the cell regulates the switch from transcription to production of full-length viral antigenome and genome from the same promoter sequences, rather than from different promoters. However, in vitro replication/transcription and reverse genetic systems are not yet established for BDV; thus, there are no direct data concerning how BDV regulates transcription and replication.

Transcription of the BDV genome results in the synthesis of at least six primary, polyadenylated, subgenomic RNAs with chain lengths of 0.8, 1.2, 1.9, 2.8, 3.5, and 7.1 kb (Briese et al. 1994; Cubitt et al. 1994a). The 7.1 kb RNA and 2.8 kb RNA are modified by splicing of two introns of approximately 0.1 and 1.3 kb to yield RNAs of 7.0/6.1/6.0 and 2.7/1.5/1.4 kb, respectively (Figure 1) (Cubitt et al. 1994b; Schneider et al. 1994b). The 7.1 or 7.0 kb RNAs can be further modified by alternative choice of the splice acceptor site of intron 2, generating a third intron of approximately 2.1 kb and yielding RNAs of 6.5 or 0.7 kb (or 6.6/0.8 kb if intron 1 is not spliced) (Tomonaga et al. 2000; Cubitt et al. 2001). The 1.9 kb RNA is fundamentally different from the other transcripts. It starts at the 3′ terminus of the genome rather than at the first transcription initiation signal, is not capped, and is only partly polyadenylated (Schneemann et al. 1994). Whether it represents an analog of leader-containing subgenomic RNAs found in other NNS RNA viruses or an abortive replication intermediate with a stop at the second termination signal is unknown.

The molar abundance of the individual BDV mRNAs in infected cultured cells and tissues resembles that of rhabdoviruses and paramyxoviruses, where transcription by the endogenous nucleocapsid-associated viral RNA polymerase complex occurs sequentially from the 3′ end of the genome following the order of the genes (Briese et al. 1994; Cubitt et al. 1994a). NNS RNA viruses typically contain signal sequences in the noncoding intergenic regions of the genome that specify transcriptional termination/polyadenylation and initiation by the viral polymerase complexes (Banerjee 1987). RNA circularization, followed by RT-PCR over the ligated ends and sequencing, revealed that BDV contains three semiconserved, U-rich initiation motifs that are unique to BDV, and four stop signals that, similar to other NNS RNA viruses, contain an A followed by six or seven U residues (Schneemann et al. 1994). However, the gene junctions are atypical for NNS RNA viruses as they cannot be clearly divided into discrete regions corresponding to a termination signal for one transcription unit, an intergenic region, and an initiation signal for the next transcription unit. The first and the second transcription units overlap, such that the second initiation signal, S2, lies upstream of the first termination signal, T1 (Figure 1). A similar organization is postulated to serve as an attenuation signal for control of polymerase expression in respiratory syncytial virus (Collins et al. 1987); however, in BDV, attenuation appears not to take place, as the two transcription units are expressed at similar levels (Briese et al. 1994). The second and third transcription units are separated by only two nucleotides, with the second termination signal, T2, fully contained within the third initiation signal, S3. The initiation signal S3 gives rise to two different primary transcripts: the first terminates at the third termination signal T3, whereas the second is expressed by readthrough of T3 and termination at T4 (Figure 1). Indeed, readthrough of T3 in BDV is essential for the expression of p190, the polymerase protein. Transcriptional readthrough may provide a means for regulating the expression of the BDV polymerase, a protein only needed in catalytic amounts. Consistent with this notion is the observation that levels of the 7.1 kb transcript are lower than those of the 2.8 kb transcript (Briese et al. 1994).

Splicing of BDV mRNAs

Splicing is an intriguing feature of BDV that allows for efficient use of its genome, controls the expression of three ORFs comprising the third transcription unit, and may have implications for neurotropism and pathogenesis. Introns are located at nucleotides (nt) 1932–2025 (intron 1) and 2410–3703 (intron 2) (Cubitt et al. 1994b; Schneider et al. 1994b) (Figure 1). Differential splicing and the potential for either termination or readthrough at the termination signal T3 allows for the expression of six additional RNAs (Schneider et al. 1994b). Differential splicing of the two introns regulates expression of the M, G, and L proteins. Splicing of intron 1 places the 13th amino acid (aa) residue of the M ORF in frame with a stop codon. Whereas this abrogates M expression, the resulting 13 aa minicistron facilitates G expression by ribosomal reinitiation (Schneider et al. 1997b). Preliminary data suggest that the 13 aa minicistron which facilitates G expression by ribosomal reinitiation also facilitates L expression. Splicing of intron 2 fuses 17 nt of an upstream, AUG-containing sequence with a continuous ORF comprising the remainder of the L coding sequence (nt 3703–8819) (Briese et al. 1994; Schneider et al. 1994b; Walker et al. 2000). Recently, an alternative splice acceptor site has been identified at nt 4559 (Figure 1) that is controlled by an exon splicing repressor downstream (Tomonaga et al. 2000). Splicing of intron 3 (nt 2410–4559) and transcriptional readthrough to t6 (Briese et al. 1994) may result in the expression of two additional BDV specified proteins (Tomonaga et al. 2000; Cubitt et al. 2001). In one potential product, the upstream L exon would be fused to a short ORF

terminating at t6 to yield an 8.4 kDa polypeptide. In the other, the N-terminal portion of G would be fused to the truncated L ORF to yield a 165 kDa polypeptide. Splice junctions associated with the three introns are homologous to mammalian splice junction consensus sequences and can be processed by the host-cell splicing machinery. However, the splice acceptor side at nt 4559 is not strictly conserved throughout sequenced BDV isolates; the lack of conservation in No/98 (Pleschka et al. 2001) indicates that potential protein products are not likely to be essential to the basic lifecycle of the virus.

Viral proteins

NUCLEOPROTEIN

The first ORF of the BDV antigenome codes for a protein of 40 kDa. Although participation in RNP complex formation and binding to RNA has yet to be demonstrated, its position in the viral genome, its size, and its relative abundance in infected cells suggests that it corresponds to the BDV nucleoprotein (N).

This protein, together with BDV P, comprises the soluble antigen (s antigen), a complex found in the noninfectious supernatant obtained after high-speed centrifugation of sonicated, infected brain, or cultured cells. The s antigen was a cornerstone for diagnosis of infection prior to the advent of molecular probes and recombinant proteins, and provided the first evidence for interaction of BDV proteins (von Sprockhoff 1956; Wagner et al. 1968; Ludwig et al. 1973; Haas et al. 1986; Bause-Niedrig et al. 1992).

The nucleoprotein exists as either a 40 kDa or a 38 kDa isoform. Whereas p40 uses the entire ORF, p38 initiates at the second in-frame AUG and lacks 13 aa at the amino terminus (Figure 2). It is unknown whether p38 and p40 are translated from a single transcript, although an RNA has been found that initiates downstream of S1 and encodes p38 (Pyper and Gartner 1997). Immunohistochemical and cell fractionation experiments with cells transfected for expression of p40 or p38 revealed that, whereas p40 is primarily nuclear, p38 is primarily cytoplasmic. This difference in the subcellular distribution of p40 and p38 reflects the presence in p40 of an amino terminal nuclear localization signal (NLS) ($_3$PKRRLVDDA$_{11}$) (Kobayashi et al. 1998; Pyper and Gartner 1997). The in vivo significance of the two isoforms of N is unknown. Both p38 and p40 bind the BDV phosphoprotein (P). P contains a potent NLS; thus, it is conceivable that the 38 kDa isoform may enter the nucleus through interaction with P. Interaction with P is facilitated by two motifs located in the N-terminal half of N (K_{51}–Y_{100}, and L_{131}–I_{158}; Figure 2) (Berg et al. 1998; Kobayashi et al. 2001). p38 and p40 also contain an internal leucine-rich nuclear export signal (NES) (L_{128}TELEISSIFSHCC$_{141}$) (Kobayashi et al. 2001) that overlaps with the P binding motif. It is hypothesized that the NLS-lacking p38 isoform of N, possibly in complex with mature RNPs, may redistribute to the cytosol after dissociation of bound P (Kobayashi et al. 2001).

X-PROTEIN

Unlike the first transcription unit, which is monocistronic and encodes only two isoforms of N, the second transcription unit is bicistronic and encodes two polypeptides expressed from different ORFs, X and P. X is expressed from an ORF that initiates upstream of P and overlaps with the P ORF in a +1 frame shift (Wehner et al. 1997). X and P interact in mammalian two-hybrid and coimmunoprecipitation experiments (Schwemmle et al. 1998). The site of binding on X has been localized to the N-terminal motif S_3DLRLTLLELVRRL$_{16}$ (Malik et al. 2000; Wolff et al. 2000). X is both nuclear and cytoplasmic. It has been suggested that nuclear localization of X is a result of its binding to P. Similarity of the leucine-rich N-terminal motif to NESs of several cellular and viral export proteins, such as HIV-1 Rev or PKI (see Figure 2, leucines underlined), led to speculation that X may mediate nucleocytoplasmic shuttling through its interaction with the viral RNP via P. However, recent data suggest that this motif functions as an NLS rather than an NES (Figure 2, R_6LTLLELVRRNGN$_{19}$ (Wolff et al. 2002)). X transport through the nuclear pore complex is mediated by direct binding to importin α. The role of X in the BDV lifecycle is unknown.

PHOSPHOPROTEIN

The phosphoprotein is expressed from the second ORF of the second transcription unit of the BDV genome. There is no evidence of splicing to eliminate the first AUG initiating translation of X (Briese et al. 1994; Cubitt et al. 1994a); thus, it is likely that P is expressed through a leaky scanning mechanism. The phosphoproteins of NNS RNA viruses are essential cofactors for virus transcription and replication. Their phosphorylation by cellular kinases influences the ability of phosphoproteins to form homomultimers, bind other viral proteins, and serve as transcriptional activators. Although there are no direct data concerning the function of P, it is postulated by analogy to be similar to other viral phosphoproteins. P contains two strong NLSs at its N and C termini (Figure 2) (Shoya et al. 1998; Schwemmle et al. 1999b). Mammalian two-hybrid and coimmunoprecipitation experiments indicate that P interacts with itself, X, and N. Analysis of P truncation mutants led to the identification of three nonoverlapping regions important for homo-oligomerization (aa 135–172), binding to X (aa 33–115), and to N (aa 197–201) (Figure 2) (Schwemmle et al. 1998).

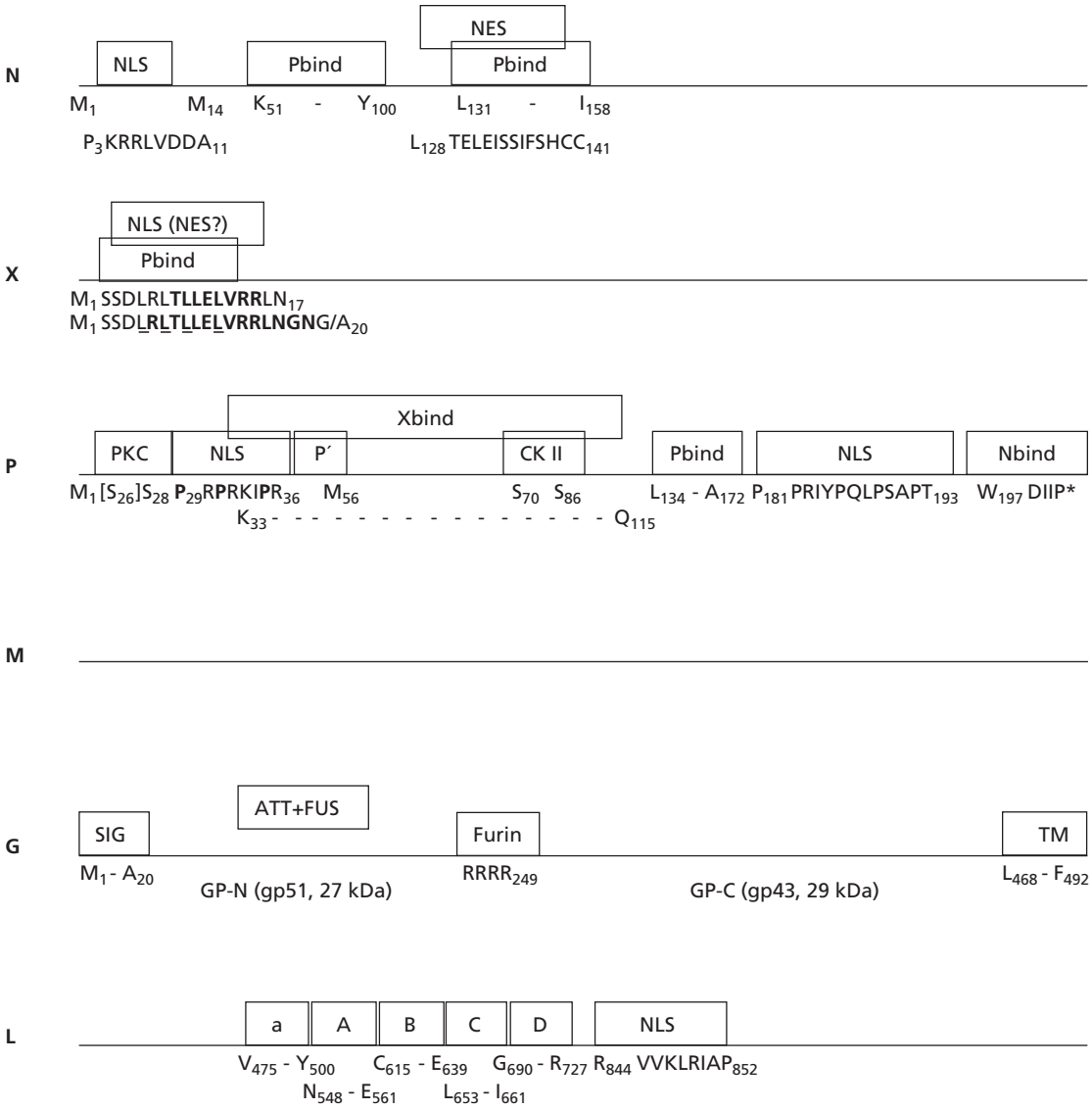

Figure 2 *Functional motifs identified in BDV proteins. Abbreviations: a, A, B, C, D, conserved L-polymerase motifs; ATT + FUS, attachment and fusion domain; CK II, casein kinase II phosphorylation; Furin, Furin cleavage site; M1 and M14 in N, and M1 and M56 in P indicate start sites of p40 and p38, or P and P', respectively; Nbind, site of interaction with N; NES, nuclear export signal; NLS, nuclear localization signal; Pbind, site of interaction with P; PKCϵ, protein kinase Cϵ phosphorylation; SIG, signal peptide; TM, transmembrane domain; Xbind, site of interaction with X.*

Investigation of the phosphorylation sites of P revealed a complex phosphorylation pattern that is unusual for NNS RNA viruses. P is phosphorylated predominantly by protein kinase Cϵ (PKCϵ) and, to a lesser extent, by casein kinase II (CK II). Peptide mapping studies identified Ser28 (and Ser26, which is not present in all strains) as site(s) for PKCϵ, and Ser70 and Ser86 as sites for CK II phosphorylation (Schwemmle et al. 1997). In other viral systems, sequential phosphorylation by different kinases may be associated with conformational changes that influence protein–protein interactions (Barik and Banerjee 1992). Whereas the PKCϵ phosphorylation site(s) of P overlap with the NLS, the CK II phosphorylation sites overlap with the region of interaction with X (Figure 2). Thus, it is conceivable that the status of P phosphorylation may influence nuclear trafficking of P (and possibly X through its interaction with P). PKCϵ is highly concentrated in limbic circuitry (Saito et al. 1993). This observation is intriguing, given the limbic distribution of BDV, and suggests that phosphorylation events may be important in BDV neurotropism.

MATRIX PROTEIN

The first ORF of the third transcription unit predicts a protein with a molecular weight of 16 kDa (p16, M). This protein was characterized as a viral product prior to

the advent of genetic analysis of BDV when a 14.5 kDa protein was isolated from the brains of neonatally infected rats (Schädler et al. 1985). Later, protein microsequencing identified this protein as the product of the 16 kDa ORF (Kliche et al. 1994). Early studies indicated oligosaccharide modification of extracted protein, and a neutralizing activity of p16-specific antibodies led to the speculation that p16 might be an unusual, glycosylated matrix protein (Kliche et al. 1994; Hatalski et al. 1995; Stoyloff et al. 1997). However, more recent work indicates that p16 is a nonglycosylated matrix protein (Kraus et al. 2001). In light of these data, the neutralizing activity of p16-specific antibodies may represent reactivity with RNPs, which are thought to be involved in the spread of BDV during infection (Cubitt and de la Torre 1994; Gosztonyi et al. 1993).

GLYCOPROTEIN

The second ORF of the third transcription unit predicts a protein with a molecular weight of 57 kDa. Posttranslational modification by N-glycosylation with high-mannose oligosaccharides yields a full-length type 1 membrane protein of 94 kDa (G, gp94) (Schneider et al. 1997a; Richt et al. 1998; Kiermayer et al. 2002) that is proteolytically processed by the cellular protease furin (Richt et al. 1998). Carboxyterminal cleavage of arginine$_{249}$ yields two fragments, GP-N (27 kDa nonglycosylated) and GP-C (29 kDa nonglycosylated) (Kiermayer et al. 2002; Richt et al. 1998) (Figure 2). Whereas precursor gp94 accumulates in the endoplasmic reticulum, the GP-C product is transferred to the cellular membrane, anchored by its C-terminal transmembrane domain. Both gp94 and glycosylated GP-C (43 kDa, gp43) are incorporated into infectious particles (Gonzalez-Dunia et al. 1997). The extensively glycosylated GP-N product (51 kDa, gp51) was recently demonstrated in infected cells and infectious particles (Kiermayer et al. 2002). Analysis of the N-glycans in gp43 and gp51 revealed maturation of the oligosaccharide chains. Whereas precursor gp94 carries only high-mannose glycans, gp43 and gp51 both contain a mixture of high-mannose and complex type glycans (Kiermayer et al. 2002).

In other viral systems glycoproteins typically mediate attachment and entry, and it has been suggested that BDV enters the cell through endocytosis followed by membrane fusion in the acidic environment of the endosome (Gonzalez-Dunia et al. 1998). Several lines of evidence indicate a role for G in BDV attachment and entry. Neutralization activity of sera from infected animals is reduced following immunoadsorption with gp94 protein; preincubation of susceptible cells with G protein interferes with infectivity (Schneider et al. 1997a); and sera raised against a bacterially expressed, truncated protein (starting at M_{150}) neutralizes BDV (Gonzalez-Dunia et al. 1997). Protease inhibitor studies indicate that cleavage of the precursor gp94 is essential for infectivity (Richt et al. 1998). Pseudotyping experiments implicate the N-terminal portion of the BDV G protein in attachment and entry (Perez et al. 2001). In view of recent data indicating the presence of GP-N in virions (Kiermayer et al. 2002), it seems likely that critical residues are positioned C-terminal of M_{150}.

Although G may be expressed from unspliced transcripts by leaky ribosomal scanning, intron 1 spliced transcripts are more likely to serve as messages for G in vivo (Schneider et al. 1997b).

POLYMERASE

The most 5′ gene of BDV occupies more than half of the BDV genome and is thought to represent the viral RNA-dependent RNA polymerase, analogous to other NNS RNA virus L polymerases. A continuous ORF with a coding capacity of 190 kDa is formed by RNA splicing of intron 2, which fuses a small upstream ORF to the large downstream ORF (Briese et al. 1994; Schneider et al. 1994b). The product of this splicing event is considered to represent the active BDV polymerase, though no functional data are currently available (Walker et al. 2000). The protein contains the known conserved L-polymerase motifs (Briese et al. 1994; Cubitt et al. 1994a) (Figure 2) and, similar to other L polymerases, it interacts with P and is phosphorylated by cellular kinases (Walker et al. 2000). The protein localizes to the nuclear compartment of infected cells (Walker et al. 2000), mediated by a strong NLS mapped to a R_{844}VVKLRIAP$_{852}$ motif in the middle of the ORF shortly after the conserved L polymerase motif D (Walker and Lipkin 2002).

PATHOGENESIS AND EPIDEMIOLOGY OF BDV

Hosts and epidemiology

A syndrome of progressive meningoencephalitis of horses and sheep consistent with BDV infection (von Sind 1767) was recognized 100 years before the disease received its name from an equine outbreak near the town Borna in Germany. Although this clinical pattern is still considered to represent classic Borna disease (BD), infection may also result in asymptomatic carrier status, subtle disturbances in learning and memory, profound disorders of behavior and movement (Narayan et al. 1983a; Carbone et al. 1989, 1991a; Rott and Becht 1995), or death. Emerging epidemiologic data, including reports of asymptomatic, naturally infected animals (Matthias 1954; Ihlenburg and Brehmer 1964; Lange et al. 1987; Kao et al. 1993; Nakamura et al. 1995, 1996; Bahmani et al. 1996; Hagiwara et al. 1997a, 2001; Reeves et al. 1998; Berg et al. 1999, 2001; Nishino et al.

1999; Yamaguchi et al. 1999; Dauphin et al. 2001; Helps et al. 2001; Horii et al. 2001; Vahlenkamp et al. 2002; Inoue et al. 2002; Yilmaz et al. 2002), suggest that the host range and geographic distribution of BDV are larger than previously appreciated (Table 1). Natural infection has been reported in a wide variety of hosts, including horses, donkeys, sheep, cattle, dogs, cats, rabbits, lynxes, foxes, and birds. Experimental infection has been achieved in many of these species, and also in rodents and primates. Whether BDV naturally infects humans remains controversial; however, there is consensus that all warmblooded animals are likely to be susceptible to infection. Although Central Europe has the highest reported prevalence of Borna disease, natural infection has been described throughout Europe, in Asia, and in North America. It is unclear whether the apparent increase in host and geographic range of BDV is due to spread of the virus, or enhanced awareness of the agent and improved diagnostic reagents.

Neither the reservoir nor the mode of transmission of natural infection is known. Natural infection of horses and sheep is typically sporadic and peaks in the spring months; epidemics of disease are infrequent (reviewed in Dürrwald and Ludwig 1997). An olfactory route of transmission has been proposed because intranasal infection is efficient and the olfactory bulbs of naturally infected horses show inflammation and edema early in the course of disease (Joest and Degen 1911; Ludwig et al. 1988). One outbreak in a zoo has been attributed to inadvertent inoculation of BDV during vaccine administration (H. Müller, personal communication). Experimental infection of neonatal rats results in virus persistence and is associated with the presence of viral gene products in saliva, urine, and feces (Sierra-Honigmann et al. 1993). Such secreta/excreta are known to be important in the transmission of other pathogenic viruses (for example, lymphocytic choriomeningitis virus, hantaviruses). Normal adult rats housed in cages separate from but adjacent to those of neonatally infected rats can become infected, suggesting the possibility of aerosol transmission (M. Hornig, M. V. Solbrig, and T. Briese, personal communication). Reports of BDV nucleic acid and proteins in peripheral blood mononuclear cells (PBMC) also indicate a potential for hematogenous transmission (Sierra-Honigmann et al. 1993; Rubin et al. 1995). The observations that rodents can be persistently infected with BDV and excrete virus suggest that they have the potential to serve as both natural reservoirs and vectors for virus dissemination. However, naturally infected rodents have not been found (Tsujimura et al. 1999; Hagiwara et al. 2001); thus, the significance of rodents for the transmission of BDV to other domesticated animals and humans remains speculative. An alternative avian reservoir is suggested by studies indicating the presence of BDV nucleic acids in the feces of wild mallards and jackdaws (Berg et al. 2001).

Natural infections

Although infection may be asymptomatic, symptomatic disease typically follows a predictable course. Clinical signs at the onset of disease in horses and sheep are nonspecific: excited or depressed behavior, hyperthermia, anorexia, jaundice, constipation, and colic (Rott and Becht 1995). Classic disease becomes apparent within 1–2 weeks. Animals maintain an upright, wide-based stance, with the head extended. Repetitive behaviors are common and may include vacuous chewing, circular ambulation, and running into obstacles. In the terminal phases of disease, horses become paretic. A distinctive decubitus posture associated with paddling movements of the legs has been described. Frequently, in late disease, the virus migrates centrifugally along the optic nerve to cause retinopathy and visual impairment. Acute mortality may be as high as 80–100 percent in horses and 50 percent in sheep (Rott and Becht 1995). Sheep that survive may have permanent neurologic deficits. Recurrence of acute disease has been described in sheep (Heinig 1969). Natural symptomatic infection with a fatal outcome has also been reported in cattle (Bode et al. 1994; Caplazi et al. 1994), cats (Lundgren and Ludwig 1993; Lundgren et al. 1995a, b; Nakamura et al. 1999b), rabbits (Metzler et al. 1978), and dogs (Weissenbock et al. 1998; Okamoto et al. 2002). Epidemics of paresis in ostriches were attributed to BDV infection; however, these data have not been confirmed (Malkinson et al. 1995).

Viral persistence without apparent disease has been described in naturally infected horses (Ihlenburg and Brehmer 1964; Lange et al. 1987; Nakamura et al. 1995; Berg et al. 1999; Hagiwara et al. 2002), sheep (Matthias 1954; Hagiwara et al. 1997a; Vahlenkamp et al. 2002), and cats (Nakamura et al. 1996; Reeves et al. 1998; Nishino et al. 1999; Helps et al. 2001; Horii et al. 2001) in Europe and Asia. There is one report indicating subclinical infection of horses in North America (Kao et al. 1993). Vertical transmission of BDV has been described in horses (Hagiwara et al. 2000).

Experimental models of bornavirus infection

A wide range of animal species has been experimentally infected with BDV. Rats (Nitzschke 1963; Hirano et al. 1983; Narayan et al. 1983a), mice (Kao et al. 1984; Rubin et al. 1993; Hallensleben et al. 1998), rabbits (Zwick and Seifried 1925; Krey et al. 1979a, b), guinea pigs (Zwick and Seifried 1925), gerbils (Nakamura et al. 1999a; Watanabe et al. 2001), tree shrews (Sprankel et al. 1978), rhesus monkeys (Zwick et al. 1927; Pette and Környey 1935; Cervos-Navarro et al. 1981; Stitz et al. 1981; Krey et al. 1982), and chickens (Zwick et al. 1926; Ludwig et al. 1988) are all susceptible to classic disease; however, the

Table 1 *Animal species reported to be infected with BDV*

Host and sample type	Country	Seropositive	Viral protein/ RNA	Seropositive and viral protein/RNA	References
Horse					
Normal	USA	3–6% (8–18/295)			Kao et al. 1993
CNS disease (B)	Germany	38% (3/8)	RNA 100% (8/8)	38% (3/8)	Zimmermann et al. 1994
CNS disease (B)	Germany	100% (4/4)	RNA 100% (4/4)	100% (4/4)	Binz et al. 1994
Normal (PBMC)	Japan	26% (15/57)	RNA 30% (17/57)	18% (10/57)	Nakamura et al. 1995
Normal (PBMC)	Iran	16.7% (12/72)	23.6% (17/72)	12.5% (9/72)	Bahmani et al. 1996
CNS disease (B)	Japan	66.7% (4/6)	66.7% (4/6)	66.7% (4/6)	Hagiwara et al. 1997b
Normal	Japan	17.8% (16/90)			Yamaguchi et al. 1999
Normal (PBMC)	Sweden	24.5% (13/53)	RNA 1.9% (1/53)	0% (0/53)	Berg et al. 1999
CNS disease (PBMC)	Sweden	57.7% (15/26)	RNA 26.9% (7/26)	19.2% (5/26)	Berg et al. 1999
Various (B)	France		RNA 4% (3/75)		Dauphin et al. 2001
Various (PBMC)	France		RNA 63.6% (14/22)		Dauphin et al. 2001
Normal	China	75% (15/20)			Hagiwara et al. 2001
Various	Japan	32% (8/25)			Yamaguchi et al. 2001
Normal	Turkey	25% (82/323)			Yilmaz et al. 2002
Various	Japan	46.4% (58/125)			Hagiwara et al. 2002
Normal	Japan	26.9% (35/130)			Inoue et al. 2002
Donkey					
CNS disease (B)	Germany	50% (1/2)	RNA 50% (1/2)	50% (1/2)	Zimmermann et al. 1994
CNS disease (B)	Germany	100% (1/1)	RNA 100% (1/1)	100% (1/1)	Binz et al. 1994
Cattle					
Normal (PBMC)	Japan	20% (15/74)	RNA 11% (8/74)		Hagiwara et al. 1996
Various (B)	France		RNA 9.7% (3/31)		Dauphin et al. 2001
Normal	China	0% (0/20)			Hagiwara et al. 2001
Sheep					
CNS disease (B)	Germany	50% (1/2)	RNA 100% (2/2)	50% (1/2)	Zimmermann et al. 1994
CNS disease (B)	Germany	100% (1/1)	RNA 100% (1/1)	100% (1/1)	Binz et al. 1994
Normal (PBMC)	Japan	36.7% (11/30)			Hagiwara et al. 1996
Normal	China	50% (10/20)			Hagiwara et al. 2001
Normal (PBMC)	Germany	11.5–19.4% (3/26–13/67)	RNA 0–14.9% (0/26–10/67)		Vahlenkamp et al. 2002

(*Continued over*)

Table 1 *Animal species reported to be infected with BDV (Continued)*

Host and sample type	Country	Seropositive	Viral protein/ RNA	Seropositive and viral protein/RNA	References
Cat					
CNS disease	Germany	7% (12/173)			Lundgren et al. 1993
CNS disease	Sweden	46% (11/24)			Lundgren et al. 1993
Various (PBMC)	Japan	8% (7/83)	RNA 13% (11/83)	0% (0/83)	Nakamura et al. 1996
CNS disease	Japan	66.7% (10/15)	RNA 53.3% (8/15)		Nakamura et al. 1999b
Normal (PBMC)	Japan	18.75% (6/32)	RNA 6.25% (2/32)	3.1% (1/32)	Nishino et al. 1999
CNS disease (PBMC)	UK		RNA 80% (4/5)		Reeves et al. 1998
Various (B)	UK		RNA 33% (6/18)		Reeves et al. 1998
FIV+	Germany	33.8% (22/68)			Huebner et al. 2001
Normal, FIV–	Germany	17.2% (32/186)			Huebner et al. 2001
CNS disease	Japan	46.2% (24/52)			Ouchi et al. 2001
Normal	Japan	23.7% (36/152)			Ouchi et al. 2001
Various	Turkey	42.5% (34/80)			Helps et al. 2001
Various	Japan	3.1% (12/383)			Horii et al. 2001
Various	Philippines	3.8% (2/53)			Horii et al. 2001
Various	Indonesia	2.0% (1/51)			Horii et al. 2001
CNS disease (B)	Sweden	40% (4/10)	RNA 100% (10/10)	40% (4/10)	Johansson et al. 2002
Lynx					
CNS disease (B)	Sweden		Protein/RNA 100% (1/1)		Degiorgis et al. 2000
Fox					
Unknown (B)	France		RNA 6.8% (4/59)		Dauphin et al. 2001
Dog					
CNS disease (B)	Austria		Protein/RNA 100% (1/1)		Weissenböck et al. 1998
CNS disease (B)	Japan	100% (1/1)	100% (1/1)	100% (1/1)	Okamoto et al. 2002
Ostrich					
CNS disease (B)	Israel		Protein 53.8% (7/13)		Malkinson et al. 1993
Birds					
Normal (F)	Sweden		RNA 33% (2/6)		Berg et al. 2001
Rodents					
Normal	China	0% (0/165)			Hagiwara et al. 2001
Normal	Japan	0% (0/106)			Tsujimura et al. 1999

Tissue examined for protein/RNA: (PBMC) peripheral blood mononuclear cells; (B) brain tissue; (F) fecal matter.

incubation period, mortality, and severity of disease vary considerably between species, between strains within a species, and depend on the immune status of the host. Whereas, in immunocompetent adult hosts, infection results in dramatic immune-mediated meningoencephalitis consistent with the classic syndrome observed in naturally infected horses and sheep, animals tolerant of infection owing to immature or compromised immune systems have a more subtle course.

ADULT RAT MODEL

Susceptibility to disease varies with the host rat strain. Wistar rats and black-hooded (BH) rats can be productively infected but have less severe disease than Lewis rats, a strain with a lesion in the hypothalamopituitary–adrenal axis associated with enhanced susceptibility to immune-mediated disorders, such as experimental allergic encephalomyelitis and adjuvant-induced arthritis (Cizza and Sternberg 1994; Sternberg et al. 1990). One report found that resistance to BD was inherited as a dominant trait in Lewis and BH hybrids that was independent of major histocompatibility complex (MHC) genes (Herzog et al. 1991). The virulence of viral strains for rats may be enhanced by serial passage of the virus in rat brain (Nitzschke 1963).

Infection is most rapidly achieved with intracranial and intranasal inoculations. Nonetheless, any route of

inoculation that allows virus access to nerve terminals (for example, intramuscular, intraperitoneal, or footpad injection) ultimately results in central nervous system (CNS) infection and classic disease. Viremia is unlikely to play a significant role in BDV dissemination and pathogenesis. Although viral gene products have been found in PBMCs of infected animals (Steinbach 1994), intravascular inoculation only infrequently results in productive infection. Several observations indicate that BDV disseminates primarily via neural networks: (1) viral proteins and nucleic acids can be traced centripetally and transsynaptically after olfactory, ophthalmic, or intraperitoneal inoculation (Carbone et al. 1987; Morales et al. 1988); (2) the onset of disease is delayed with distance of the inoculation site to the CNS (Carbone et al. 1987); and (3) migration of virus to the CNS after footpad infection can be prevented by sciatic nerve transection (Carbone et al. 1987). Irrespective of the route of inoculation, clinical disease begins coincidentally with the appearance of viral proteins in hippocampal neurons and the onset of meningitis (Carbone et al. 1987; Richt et al. 1989). It has been proposed that BDV, like rabies virus, is likely to spread as an RNP complex within neural networks (Gosztonyi et al. 1993). Although structures consistent with RNPs have been described in neurons of experimentally infected animals (Cervos-Navarro et al. 1981; Sasaki and Ludwig 1993), the form of disseminating virus is unknown.

Infection of adult Lewis rats produces a prominent neurobehavioral disorder and is characterized by pronounced immunopathology. In the acute phase (4–8 weeks post infection), cellular infiltrates ($CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, and macrophages) and Th1-type cytokines are prominent in perivascular and parenchymal regions of CNS; in the chronic phase (15 weeks post-infection and beyond), a decline in infiltrates is accompanied by an increase in Th2-type cytokines and a shift to a humoral immune response (Hatalski et al. 1998a). Although antibodies to N and P generated during the acute phase of disease are nonneutralizing (Furrer et al. 2001), antibodies with neutralizing capacity increase dramatically after the acute phase (Hatalski et al. 1995) and probably participate in restriction of the virus to neural tissues (Stitz et al. 1998a). Mechanisms contributing to viral persistence are as yet uncertain. Altered viral gene expression is an unlikely explanation as there is little substantive change in CNS over the course of disease in viral titers (Narayan et al. 1983b; Carbone et al. 1987), transcripts coding for BDV proteins, or levels of BDV N and P proteins (Hatalski 1996). Modulation of immune responses as BD progresses to the chronic phase may exert some influence on BDV persistence.

The distinct clinical and behavioral features of the immune-mediated adult rat model closely parallel the CNS pathology of the acute and chronic phases. In the acute phase, coinciding with monocyte infiltration in CNS regions of early viral burden, such as the hippocampus, amygdala, and other limbic structures (Carbone et al. 1987), animals demonstrate exaggerated startle responses and hyperactivity. As animals enter the chronic phase of infection, high-grade stereotyped motor behaviors (continuous repetition of behavioral elements, including sniffing, chewing, scratching, grooming, and self-biting), dyskinesias, dystonias, and flexed seated postures appear (Solbrig et al. 1994), in parallel with the spread of virus throughout limbic and prefrontal circuits. Up to 10 percent of animals become obese, achieving body weights up to 300 percent of normal (Ludwig et al. 1988).

Disorders of movement and behavior in adult-infected rats are associated with dysfunction in dopamine (DA) circuits (Solbrig et al. 1994, 1995, 1996a, b, 1998), as seen in many neuropsychiatric disorders (Cooper et al. 1991; Kane and Marder 1993; Anderson 1994; Hamner and Diamond 1996; Kelsoe et al. 1996; Partonen 1996; Ernst et al. 1997), and may be further linked to serotonin (5HT) abnormalities (Solbrig et al. 1995). Enhanced sensitivity of central DA systems of adult-infected BD animals to DA agonists, antagonists, and DA reuptake inhibitors is observed. Administration of the mixed-acting DA agonist dextroamphetamine (Solbrig et al. 1994), or of cocaine, a DA reuptake inhibitor, to adult-infected rats elicits increased locomotor and stereotypic behavior, indicating dose-dependent potentiation of DA neurotransmission (Solbrig et al. 1998). Low presynaptic autoreceptor doses of the direct DA agonist apomorphine reduce hyperactivity, whereas higher doses increase locomotion. Both pre- and postsynaptic sites of the DA transmitter system appear to be damaged in striatum: DA reuptake sites, as measured by mazindol binding, are reduced in caudate-putamen (Solbrig et al. 1998) and nucleus accumbens (Solbrig et al. 1996b); and postsynaptic D2, but not D1, receptor binding is markedly reduced in caudate-putamen, whereas D2 and D3 receptor binding is reduced in nucleus accumbens (Solbrig et al. 1994, 1996a, b). In contrast, postsynaptic DA receptors (D1, D2, D3) remain intact in prefrontal cortex (Solbrig et al. 1996a). Further support for D2-selective losses and resultant D1 hypersensitivity as mediators of neurobehavioral disturbances in adult BD is found in the ability to reverse locomotor hyperactivity through the administration of D1 receptor blocking agents, such as the D1 antagonist SCH23390, or clozapine, an atypical antipsychotic with mixed D1, D2, D3, and D4 antagonist activity, but not through the administration of D2-selective antagonists (e.g. raclopride) (Solbrig et al. 1994).

Neurochemical studies further support a lesion in DA transmission consistent with partial DA deafferentation and compensatory metabolic hyperactivity in nigrostriatal and mesolimbic DA systems. Decreases in DA levels exceed those in dihydroxyphenylacetic acid (DOPAC, the major metabolite of DA) levels in high

performance liquid chromatography (HPLC) analysis of tissues from striatum, nucleus accumbens, and olfactory tubercle (Solbrig et al. 1994), whereas in prefrontal cortex marked increases are noted in DOPAC (Solbrig et al. 1996a). Depletion of tyrosine hydroxylase (TH)-immunoreactive cells in substantia nigra, ventral tegmental area (Solbrig et al. 1994), and striatum (Solbrig et al. 2000), and a decrease TH protein content in striatum but not in substantia nigra pars compacta, are nonetheless accompanied by an increase in TH functional activity (Solbrig et al. 2000). Increased gene expression of neurotrophic factors that support the growth of DA-producing cells in vitro, including BDNF, NT-3, NT-4, and CNTF, may also contribute to the sensitivity to DA agonist action in adult BD (Solbrig et al. 2000).

Additional neuromodulator abnormalities are also noted in the adult model. The expression of genes for neuromodulatory substances and their associated synthesizing enzymes, including somatostatin, cholecystokinin, and glutamic acid decarboxylase, is greatly reduced during the acute phase and recovers toward normal in the chronic phase of adult BD (Lipkin et al. 1988). The cholinergic system, a major participant in sensorimotor processing, learning, and memory, is similarly affected, with a decrease in choline acetyltransferase-positive fibers as early as day 6 post-infection, progressing to nearly complete loss of these fibers in hippocampus and neocortex by day 15 (Gies et al. 1998). Preliminary work on the dysregulation of 5HT and norepinephrine (NE) systems suggests metabolic hyperactivity of 5HT, with a modest increase in the metabolite 5-hydroxyindoleacetic acid in striatum, and of NE, as evidenced by a small increase in the NE metabolite, 3-methoxy-4-hydroxyphenethyleneglycol, in prefrontal and anterior cingulate cortex regions (Solbrig et al. 1995). These changes may reflect compensatory upregulation or heterotypic sprouting following partial loss of DA afferents to these brain regions. Selective effects of BDV on 5HT and NE pre- or post-synaptic receptors have not yet been investigated. Pharmacologic and neurotransmitter-specific molecular probes have also been used to characterize endogenous opioid systems in the adult rat model. Infected animals respond abnormally to the opiate antagonist naloxone, with hyperkinesis and seizures, and also demonstrate increases in striatal preproenkephalin mRNA at 14 and 21 days (Fu et al. 1993b), and 6 weeks after BDV infection (Solbrig et al. 2002a). BDV and met-enkephalin immunoreactivity also coincide in a high percentage of cells (Solbrig et al. 2002a). Induction of the enkephalin system in adult BD may relate to increased levels of phosphorylated cyclic AMP response element binding (phosphoCREB) protein through activation by BDV of the mitogen-activated protein (MAP) kinase pathway, thus stimulating transcription factors that regulate enkephalin expression in striatum (Konradi et al. 1993). However, the mechanisms by which these changes in endogenous opioid systems occur are unclear. The marked CNS inflammation in adult-infected rats confounds the role of direct effects of the virus, virus effects on resident cells of the CNS, and cellular immune responses to viral gene products in the production of monoamine, cholinergic, and opiatergic dysfunction in BD.

NEONATAL RAT MODEL

The neonatal rat model does not show overt immunopathology; instead, despite a high virus load in the brain and lifelong persistence, animals infected within the first 12 h of life develop a milder behavioral syndrome and restricted neuropathology that may provide a more intriguing model for neuropsychiatric disorders. The cerebellar and hippocampal dysgenesis observed in neonatally infected animals is consistent with the more subtle neurodevelopmental abnormalities reported by some investigators in autism (Kemper and Bauman 1993), schizophrenia (Altshuler et al. 1987; Fish et al. 1992), and affective disorders (Soares and Mann 1997). Neonatally infected animals display a wide range of physiologic and neurobehavioral disturbances. They are smaller than uninfected littermates (Carbone et al. 1991b; Bautista et al. 1994), with no demonstrable alteration of glucose, growth hormone, or insulin-like growth factor-1 (Bautista et al. 1994) or amount of food ingested (Bautista et al. 1995); display an enhanced preference for salt solutions; and exhibit altered circadian rhythms (Bautista et al. 1994). Behavioral and cognitive changes in rats infected in the neonatal period include abnormal early locomotor development (Hornig et al. 1999), spatial and aversive learning deficits (Dittrich et al. 1989; Rubin et al. 1999), increased motor activity (Bautista et al. 1994; Hornig et al. 1999), abnormal anxiety responses (Dittrich et al. 1989; Hornig et al. 1999; Pletnikov et al. 1999a), stereotypic behaviors (Hornig et al. 1999), and reduced initiation of, and response to, nondominance-related play interactions (Pletnikov et al. 1999b). Thus, the neuropathologic, physiologic, and neurobehavioral features of BDV infection of neonates indicate that it not only provides a useful model for exploring the mechanisms by which viral and immune factors may damage developing neurocircuitry, but also has significant links to the range of biologic, neurostructural, locomotor, cognitive, and social deficits observed in a wide range of human neuropsychiatric illnesses.

CNS dysfunction in neonatally infected animals has been proposed to be linked to direct viral effects on morphogenesis of the hippocampus and cerebellum, two structures that in rodents continue to mature postnatally. Although overall architecture is maintained, granule cells of the dentate gyrus (Hornig et al. 1999; Rubin et al. 1999) and Purkinje cells of the cerebellum are lost (Eisenman et al. 1999; Hornig et al. 1999) through apoptosis (Hornig et al. 1999). The extent of neuronal loss in

the dentate gyrus is correlated with the severity of spatial learning and memory deficiencies in neonatally infected Lewis rats (Rubin et al. 1999). Testing of cerebellar function demonstrates deficits in motor coordination and postural stability (Hornig et al. 1999; Pletnikov et al. 2001), consistent with Purkinje cell losses. Further studies are needed to evaluate the mechanisms by which early postnatal exposure to BDV induces apoptotic losses and functional damage in cerebellar and limbic circuitry.

Although the cellular inflammatory response to BDV following neonatal infection is restricted, a phenomenon ascribed to the immaturity of rat postnatal immune function, a brief surge in mononuclear cell infiltrates occurs (Hornig et al. 1999), along with elevations in the expression of proinflammatory cytokine (Hornig et al. 1999; Sauder and de la Torre 1999), chemokine (Sauder et al. 2000), and chemokine receptor (Rauer et al. 2002) transcripts. However, this transient immune response does not colocalize with sites of neuropathologic damage (Weissenböck et al. 2000). Neuropathology instead parallels regions and the time course of microglial proliferation and expression of MHC class I and class II, ICAM, CD4, and CD8 molecules (Weissenböck et al. 2000). Humoral immune response to BDV in neonatally infected animals is also curtailed, with anti-BDV antibody titers remaining below 1:10 through 133 days post infection (Carbone et al. 1991b).

Reduced levels of neurotrophic factor mRNAs occur in the neonatal model (Hornig et al. 1999; Zocher et al. 2000). However, these changes are restricted to the hippocampus; thus, a different mechanism must be invoked to account for losses of Purkinje cells in cerebellum. It is conceivable that abnormal regulation of apoptosis – either failure of normal apoptotic sequences to be curtailed with age, or excess activation of apoptotic cell programs – may contribute to abnormal CNS architecture in neonatal infections with BDV or other neurotropic viruses. Excitotoxic stimulation, including activation of glutamatergic circuitry, is one factor that might trigger neuronal apoptosis. Complex alterations in mRNAs for apoptosis mediators, including increased levels of mRNAs for FAS and ICE (caspase-1), two promotors of apoptosis, and decreased mRNA for bcl-x, a factor that inhibits apoptosis, have been identified in hippocampus, amygdala, prefrontal cortex, nucleus accumbens, and cerebellum, and are consistent with the promotion of apoptosis throughout the brains of rats neonatally infected with BDV (Hornig et al. 1999).

Recent studies in tissue culture systems are providing insights into mechanisms for viral damage that may illuminate pathogenesis in neonatal infection. Inhibition of cell-to-cell spread of BDV by a MAPK/ERK kinase (MEK) inhibitor in cell culture (Planz et al. 2001b) and analyses of neuronal differentiation of PC12 cells (Hans et al. 2001) indicate an interaction of BDV with cellular MAP kinase signaling pathways. Infected PC12 cells demonstrate constitutive phosphorylation of MEK, ERK, and the transcriptional activator Elk-1; however, nuclear translocation of ERK is impaired, and cells fail to differentiate with NGF treatment (Hans et al. 2001). Inhibition of neurite outgrowth is also reported in other infected cell lines, and has been ascribed to interference by BDV P with the normal interaction between the neurite outgrowth factor, amphoterin, and its receptor, RAGE (*R*eceptor for *A*dvanced *G*lycation *E*nd-products). Complex formation between amphoterin and P leads to altered intracellular distribution of amphoterin in infected cells, with reduced levels of amphoterin and of RAGE activation at growth cones of extending cells (Kamitani et al. 2001).

MOUSE MODEL

Mice are readily infected and have high titers of virus in the brain (Kao et al. 1984). Until recently mice were considered to be relatively resistant to disease; however, two reports indicate that disease can be induced by adaptation of virus through multiple passages in mice (Rubin et al. 1993) or infection of specific host strains during the neonatal period (Hallensleben et al. 1998). As in rats, severe clinical disease is mediated by MHC class I-restricted cytotoxic T cells (Hallensleben et al. 1998; Hausmann et al. 1999). The immunopathological process mediated by $CD8^+$ T cells in a $CD4^+$ T cell-dependent fashion is most obvious in mouse strains carrying the $H\text{-}2^k$ allele of the MHC. In $H\text{-}2^k$ mice the BDV N peptide T_{129}ELEISSI$_{136}$ has been identified as the dominant cytotoxic T-cell epitope that sensitizes cells for lysis (Schamel et al. 2001).

TREE SHREWS

Little is known about BDV pathogenesis in phylogenetically higher species such as nonhuman primates and the prosimian tree shrew (*Tupaia glis*). Intracerebral inoculation of tree shrews leads to persistent infection and a disorder characterized primarily by hyperactivity and alterations in sociosexual behavior, rather than motor dysfunction (Sprankel et al. 1978). Disturbances in breeding and social behavior were most profound in animals caged in mating pairs. Females, rather than males, initiated mating, and infected animals failed to reproduce despite increased sexual activity. Although detailed neuroanatomic studies were not performed, the syndrome was interpreted to be due to neuropathological changes in the limbic system.

NONHUMAN PRIMATES

The only reported studies of experimentally infected primates employed adult Rhesus macaques (*Macaca mulatta*). These animals were initially hyperactive and subsequently became apathetic and hypokinetic. Pathology was remarkable for meningoencephalitis and retinopathy (Stitz et al. 1981).

Immune response and BDV persistence in the CNS

Neurotropism affords BDV an opportunity to persist in an environment characterized by a restricted immune response. However, this does not reflect a failure to induce an immune response. Indeed, humoral immunity appears to play a role of confinement of infection to the CNS (Stitz et al. 1998a), and cellular immunity is essential to the expression of classic disease. Early studies of BD reported a correlation between the severity of meningoencephalitis and the clinical manifestations of disease (Zwick 1939). The first direct evidence for the role of the immune response in the pathogenesis of BD emerged from studies in which splenectomized Rhesus monkeys became persistently infected but had prolonged incubation periods and less severe disease than immune competent animals (Cervos-Navarro et al. 1981; Stitz et al. 1981). Experiments with infected athymic rats or adult rats immunosuppressed by treatment with cyclophosphamide and cyclosporin A (Stitz 1991; Narayan et al. 1983a; Herzog et al. 1985; Stitz et al. 1989) provided further evidence that BD is immune mediated.

In acute BD, CNS infiltrates are comprised of macrophages, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, NK cells and, to a lesser extent, plasma cells (Deschl et al. 1990; Bilzer et al. 1995). Antibodies to CD4 and CD8 markers were initially used to deplete these cells and investigate their individual contributions to immunopathogenesis. Although depletion of either $CD4^+$ or $CD8^+$ T lymphocytes resulted in amelioration of the inflammatory response in brain and the severity of acute clinical disease (Stitz et al. 1992; Planz et al. 1995), only depletion of $CD8^+$ cells was found to reduce brain atrophy (Bilzer and Stitz 1994). Although these data may suggest that $CD4^+$ T lymphocytes are not important in BD pathogenesis, others demonstrated that the recruitment of $CD8^+$ cells is dependent on $CD4^+$ T lymphocytes (Nöske et al. 1998). It has been argued that neuronal cells express little or no MHC class I surface markers and should not be susceptible to $CD8^+$ CTL lysis (Joly et al. 1991); however, both neurons and astrocytes in BDV-infected rats have been shown to express MHC class I protein (Bilzer and Stitz 1994; Planz et al. 1993). Furthermore, lymphocytes isolated from BD rat brain were found to lyse infected target cells in an MHC class I-restricted manner (Planz et al. 1993). Adoptive transfer experiments have been pursued with N protein-specific $CD4^+$ T lymphocytes. Immunosuppressed, persistently infected rats so treated had a neurological disease that was similar but not identical to BD (Richt et al. 1989). The cellular immune response to BDV in the rat seems to be restricted to N, and the N peptide A_{230}SYAQMTTY$_{238}$ has been identified as the relevant T-cell epitope naturally recognized in the context of MHC class I in Lewis rats (Planz et al. 2001a; Planz and Stitz 1999).

In animals that progress to chronic BD there is a pronounced decrease in CNS infiltration. The numbers of macrophages, $CD4^+$ T lymphocytes, and $CD8^+$ T lymphocytes in brain are markedly reduced; however, numbers of plasma cells are increased. In contrast to the immunopathogenesis of acute BD, which has been extensively studied by several investigators, little is known about the basis for the decline in CNS infiltration in chronic infection. Potential mechanisms include altered viral gene expression or modulation of the immune response. The first possibility is unlikely because viral titers do not change substantially over the course of disease (Narayan et al. 1983a). In addition, brain levels of N and P, the only BDV proteins that can be readily quantified, and of RNAs coding for M, G, and L (Hatalski 1996), are similar in early and late disease. There is, however, evidence for modulation of the immune response over the course of BD, manifest as induction of BDV-specific type 1 T lymphocyte (Th1) tolerance. Whereas lymphocytes isolated from the brains of acutely infected rats have potent cytolytic activity, lymphocytes from brains of chronically infected rats do not lyse BDV-infected target cells (Sobbe et al. 1997). Induction of BDV-specific tolerance in chronic infection may reflect the time course for presentation of viral antigens in the thymus (Rubin et al. 1995). Alternatively, Th1 cells may become anergic or undergo apoptosis owing to the presentation of BDV antigens in brain without essential co-stimulatory signals (Schwartz 1992; Karpus et al. 1994; Khoury et al. 1995). Support for this hypothesis is found in the observation that apoptosis of perivascular inflammatory cells is most apparent at 5–6 weeks post infection, coincident with the onset of decline in encephalitis (Hatalski et al. 1998a).

Variability in cytokine expression in brain over the course of BD may also be important for modulation of the immune response. Cytokine mRNA levels have been measured in rat brain at different times post infection by RT-PCR (Shankar et al. 1992), RNase protection assay, or Northern hybridization (Hatalski et al. 1998a). Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, and IL-6 mRNAs are transiently expressed in the acute phase of disease (Shankar et al. 1992; Hatalski et al. 1998a). Message for another proinflammatory cytokine, IL-2, was detected by RT-PCR experiments throughout infection (Shankar et al. 1992); however, RNase protection assays revealed that IL-2 mRNA peaks in the acute phase of infection and declines thereafter (Hatalski et al. 1998a). IL-2 and IFN-γ are produced by Th1 cells for the recruitment and activation of CTL and the stimulation of antigen-presenting cells (Mosmann and Coffman 1989). The peak in CNS levels of these proinflammatory cytokine mRNAs in acute BD is temporally correlated with the peak of

immune cell infiltration. Cytokines produced by type 2 T lymphocytes (Th2), such as IL-4, have the potential to down-regulate the cellular immune response. IL-4 mRNA levels are elevated throughout the course of BD, but increased in the chronic phase of infection (Hatalski et al. 1998a). IL-10 mRNA peaks in the acute phase of BD and declines thereafter (Hatalski 1996). As in experimental allergic encephalomyelitis (Kennedy et al. 1992), the peak in IL-10 mRNA expression is correlated with a shift away from CNS recruitment of immune cells. The high levels of IL-4, IL-6, and IL-10 mRNAs observed in acute BD are likely to contribute to B-cell activation. Indeed, in rats, progression toward the chronic phase of disease is accompanied by an increase in brain levels of BDV-specific antibodies (Hatalski et al. 1998b).

The cytokine profile in chronic BD is consistent with modulation of the CNS immune response to reduce Th1 T-cell activation and increase the Th2 humoral response. TGF-β mRNA expression is elevated in BD rat brain from the onset of acute disease forward (Hatalski et al. 1998a), and its presence in chronic BD is compatible with a Th2 response. TGF-β has the potential to mediate inhibition of the proinflammatory effects of TNF-α, suppression of T- and B-cell growth, reduction of free radical formation, and inhibition of antibody production and CTL activity (Barnard et al. 1990; Fontana et al. 1992). Experimental administration of TGF-β to BD rats reduced the severity of encephalitis but did not prevent the onset of acute disease; thus, the role of TGF-β in BD pathogenesis remains unclear (Stitz et al. 1991).

The basis for the Th1 to Th2 switch in BD is a matter only for speculation. There are no data to indicate whether presentation of antigen on MHC class I is insufficient or activation of the Th1 cells is inefficient owing to inadequate costimulation. However, the result of this switch is consistent with the induction of Th1 down-regulation, an event that would allow survival of the host and foster viral persistence.

BDV and human disease

Recognition of BDV's broad experimental host range and the observation that disturbances in behavior in experimentally infected animals are reminiscent of some aspects of human neuropsychiatric diseases, including major depressive disorder, bipolar disorder, schizophrenia, and autism, led to the proposal that BDV might be implicated in their pathogenesis. Although there is consensus that humans are likely to be susceptible to BDV infection, the epidemiology and clinical consequences of human infection remain controversial. There have been no large controlled prevalence studies. Furthermore, methods for the diagnosis of human infection are not standardized; thus, it is difficult to pursue meta-analysis. Most reports suggesting an association between BDV and human disease have focused on neuropsychiatric disorders, including unipolar depression, bipolar disorder, or schizophrenia; however, BDV has also been linked to chronic fatigue syndrome, AIDS encephalopathy, multiple sclerosis, motor neuron disease, and brain tumors (glioblastoma multiforme) (Tables 2 and 3). The improbably broad spectrum of candidate disorders has led some investigators to propose that infection is ubiquitous, and that in some disorders elevation of serum antibody titers or the presence of viral transcripts in peripheral blood mononuclear cells or neural tissues reflects generalized (AIDS) or localized (glioblastoma multiforme) immunosuppression.

There are only infrequent reports where infectious virus has been isolated from humans (Bode et al. 1996; Nakamura 1998; Planz et al. 1999; Nakamura et al. 2000), and only two reports in which BDV gene products were found in human brain by in situ hybridization and immunohistochemistry (de la Torre et al. 1996; Nakamura et al. 2000). Methods most commonly used for serologic diagnosis of infection include indirect immunofluorescence with infected cells and Western immunoblot or enzyme-linked immunosorbent assays (ELISA) with extracts of infected cells or recombinant proteins. One study found that the majority of human serum antibodies binding BDV proteins in these assays did so with low avidity; the authors suggested, therefore, that cross-reactivity accounts for much of the seroprevalence data in BDV epidemiology (Allmang et al. 2001). However, more detailed analyses of the same sera using peptide arrays representing BDV N and P sequence, indicated that some low-avidity sera also contain low levels of high-avidity antibodies (Billich et al. 2002). Another explanation for the erratic performance of serologic assays in the Borna system may be that antibodies are bound in circulating immune complexes (CIC). In the one study based on the use of this serologic method, CICs were detected in more than 90 percent of subjects with major depressive disorder or bipolar disorder, versus 30 percent of healthy controls (Bode et al. 2001).

Infection has also been diagnosed through the demonstration of BDV transcripts and proteins in tissues or peripheral blood mononuclear cells (reviewed in Hatalski et al. 1997). Most frequently, detection of viral RNA has been achieved through nested reverse transcription-polymerase chain reaction (nRT-PCR). Although sensitive, this method is prone to artifacts because of the inadvertent introduction of template from laboratory isolates or cross-contamination of samples (Schwemmle et al. 1999a). Amplification products representing bona fide isolates are difficult to distinguish from artifacts by sequence analysis. As noted earlier, BDV is characterized by extraordinary sequence conservation. Studies of N and P sequence from widely

Table 2 *Serum immunoreactivity to BDV in subjects with various diseases.*

	Prevalence			
Disease	**Disease**	**Control**	**Assay**	**References**
Psychiatric (various)	0.6% (4/694)	0% (0/200)	IFA	Rott et al. 1985
	2% (13/642)	2% (11/540)	IFA	Bode et al. 1988
	4–7% (200–350/5000)	1% (10/1000)	WB/IFA	Rott et al. 1991
	12% (6/49)		IFA	Bode et al. 1993
	30% (18/60)		WB	Kishi et al. 1995b
	14% (18/132)	1.5% (3/203)	WB	Sauder et al. 1996
	24% (13/55)	11% (4/36)	IFA	Igata-Yi et al. 1996
	0% (0/44)	0% (0/70)	IFA/WB	Kubo et al. 1997
	2.8% (35/1260)	1.1% (10/917)	ECLIA	Yamaguchi et al. 1999
	9.8% (4/41)		IFA	Bachmann et al. 1999
	14.8% (4/27)	0% (0/13)	IFA	Vahlenkamp et al. 2000
	0% (0/89)	0% (0/210)	IFA/WB	Tsuji et al. 2000
	1.1–5.5% (1 or 5/90)	0% (0/45)	WB (N or P)	Fukuda et al. 2001
	2.1% (17/816)		ELCIA	Rybakowski et al. 2001a
	2.4% (23/946)	1.0% (4/412)	ECLIA	Rybakowski et al. 2001b, 2002
	12.6% (11/87)	15.5% (45/290)	IFA	Lebain et al. 2002
Affective disorders	4.5% (12/265)	0% (0/105)	IFA	Amsterdam et al. 1985
	4% (12/285)	0% (0/200)	IFA	Rott et al. 1985
	38 or 12% (53 or 17/138)	16 or 4% (19 or 5/117)	WB (N or P)	Fu et al. 1993a
	37% (10/27)		IFA	Bode et al. 1993
	12% (6/52)	1.5% (3/203)	WB	Sauder et al. 1996
	0–0.8% (0–1/122)	0% (0/70)	IFA/WB	Kubo et al. 1997
	2% (1/45)	0% (0/45)	WB	Fukuda et al. 2001
	92.6% (26/28)	32.3% (21/65)	CIC	Bode et al. 2001
Schizophrenia	25% (1/4)		IFA	Bode et al. 1993
	9–28% (8 or 25/90)	0–20% (0 or 4/20)	WB (N or P)	Waltrip et al. 1995
	17% (15/90)	15% (3/20)	IFA	Waltrip et al. 1995
	14% (16/114)	1.5% (3/203)	WB	Sauder et al. 1996
	20% (2/10)		WB	Richt et al. 1997
	0–1% (0-2/167)	0% (0/70)	IFA/WB	Kubo et al. 1997
	14% (9/64)	0% (0/20)	WB	Waltrip et al. 1997
	17.9 or 35.8% (12 or 24/67)	0% (0/26)	WB (N or P)	Iwahashi et al. 1997
	12.1% (38/276)		WB	Chen et al. 1999b
	10.3% (3/29)	23.1% (6/26)	IFA	Selten et al. 2000
	9% (4/45)	0% (0/45)	WB	Fukuda et al. 2001
	12.6% (11/87)	15.5% (45/290)	IFA	Lebain et al. 2002
CFS	24% (6/25)		WB	Nakaya et al. 1996
	34% (30/89)		WB	Kitani et al. 1996, Nakaya et al. 1997
	0% (0/69)	0% (0/62)	WB	Evengard et al. 1999
	100% (7/7)	33% (1/3)	WB	Nakaya et al. 1999
MS	13% (15/114)	2.3% (11/483)	IP/IFA	Bode et al. 1992
	0% (0/50)		IFA	Kitze et al. 1996
Mental health care workers	9.8% (8/82)	2.9% (8/277)	WB	Chen et al. 1999b
Family of schizophrenic patients	12.1% (16/132)	2.9% (8/277)	WB	Chen et al. 1999b
Live near horse farms	2.6–14.8% (2/78–16/108)	1% (1/100)	ELISA	Takahashi et al. 1997
Ostrich exposure	46% (19/41)	10% (4/41)	ELISA	Weisman et al. 1994

Abbreviations: CFS, chronic fatigue syndrome; CIC, circulating immune complexes; ELCIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; IP, immunoprecipitation; MS, multiple sclerosis; N, nucleoprotein; P, phosphoprotein; WB, western immunoblot.

Table 3 *BDV RNA, virus or protein in subjects with various diseases*

		Prevalence			
Disease	**Tissue**	**Disease**	**Controls**	**Divergence[a]**	**References**
Psychiatric (various)	PBMC	67% (4/6)	0% (0/10)	0–3.6%	Bode et al. 1995
	PBMC	37% (22/60)			Kishi et al. 1995b
	PBMC	42% (5/12)	0% (0/23)	0–4.0%	Sauder et al. 1996
	PBMC-coculture	9% (3/33)	0% (0/5)	0.07–0.83%	Bode et al. 1996
	PBMC	2% (2/106)	0% (0/12)		Kubo et al. 1997
	PBMC	0% (0/24)	0% (0/4)		Richt et al. 1997
	PB	0% (0/159)			Lieb et al. 1997a
	Blood	(1/1)			Planz et al. 1998
	PBMC	4% (5/126)	2.4% (2/84)		Iwata et al. 1998
	PBMC	20% (3/15)	0% (0/3)		Planz et al. 1999
	PBMC	0% (0/81)			Kim et al. 1999
	PBMC	0% (0/27)			Bachmann et al. 1999
	CSF	0% (0/27)			Bachmann et al. 1999
	PBMC	1.8% (1/56)	0.6% (1/173)		Tsuji et al. 2000
	PBMC	37% (10/27)	15.4% (2/13)		Vahlenkamp et al. 2000
	PBMC	1.1% (1/90)	0% (0/45)		Fukuda et al. 2001
Affective disorders	PBMC	33% (1/3)	0% (0/23)		Sauder et al. 1996
	PBMC	17% (1/6)	0% (0/36)		Igata-Yi et al. 1996
	Brain	40% (2/5)	0% (0/10)		Salvatore et al. 1997
	PBMC	4% (2/49)	2% (2/84)	0–5.1%	Iwata et al. 1998
	CSF	5% (3/65)	0% (0/69)	[Protein][a]	Deschle et al. 1998
	PBMC	2% (1/45)	0% (0/45)		Fukuda et al. 2001
Schizophrenia	Brain	0% (0/3)	0% (0/3)		Sierra-Honigmann et al. 1995
	CSF	0% (0/8)	0% (0/8)		Sierra-Honigmann et al. 1995
	PBMC	0% (0/7)	0% (0/7)		Sierra-Honigmann et al. 1995
	PBMC	64% (7/11)	0% (0/23)		Sauder et al. 1996
	PBMC	10% (5/49)	0% (0/36)		Igata-Yi et al. 1996
	PBMC	100% (3/3)		4.2–9.3%	Kishi et al. 1996
	PBMC	0% (0/10)	0% (0/10)		Richt et al. 1997
	Brain	53% (9/17)	0% (0/10)		Salvatore et al. 1997
	PBMC	9.8% (6/61)	0% (0/26)		Iwahashi et al. 1997
	PBMC	4% (3/77)	2% (2/84)	0–5.1%	Iwata et al. 1998
	PBMC	14% (10/74)	1.4% (1/69)		Chen et al. 1999a
	Brain	25% (1/4)		[RNA, virus, protein][a]	Nakamura et al. 2000
	PBMC	13.8% (4/29)	34.6% (9/26)		Selten et al. 2000
	PBMC	0% (0/45)	0% (0/45)		Fukuda et al. 2001
CFS	PBMC	12% (3/25)		6.0–14%	Nakaya et al. 1996
	PBMC	12% (7/57)	4.9% (8/172)		Kitani et al. 1996, Nakaya et al. 1997
	PBMC	0% (0/18)			Evengard et al. 1999
FMS	CSF	0% (0/18)	0% (0/6)		Wittrup et al. 2000
Hippocampal sclerosis	Brain	80% (4/5)			de la Torre et al. 1996
	Brain	15% (3/20)	0% (0/85)		Czygan et al. 1999
MS	CSF	11% (2/19)	0% (0/69)	[Protein][a]	Deuschle et al. 1998
	PBMC	0% (0/34)	0% (0/40)		Haase et al. 2001
Mental health care workers	PBMC	15% (7/45)	1.4% (1/69)		Chen et al. 1999a
Normal controls	PBMC		5% (8/172)		Kishi et al. 1995a
	Brain		6.7% (2/30)		Haga et al. 1997

Abbreviations: CFS, chronic fatigue syndrome; CSF, cerebrospinal fluid; FMS, fibromyalgia syndrome; MS, multiple sclerosis; PB, peripheral blood; PBMC, peripheral blood mononuclear cells.

a) Divergence of P-gene nucleotide sequence from common BDV isolates (strain V and He/80); [Protein], [virus, protein] indicates antigen analysis, or virus and antigen analysis.

disparate BDV isolates revealed variability of up to 4.1 percent at the nucleotide level and 1.5–3 percent at the predicted amino acid level (Schneider et al. 1994a); only a single sequence has been identified that shows greater divergence (Nowotny et al. 2000). Thus, similarities in sequence between putative new isolates and confirmed isolates cannot be used to exclude the former as artifacts.

VACCINES AND ANTIVIRALS

Lapinized BDV 'Dessau' was used as a vaccine for horses in regions of endemic infection in Germany for decades. Concerns about the efficacy and safety of this vaccine led to its abandonment in the western states of Germany in 1980, and in the eastern states of Germany in 1992 (Dürrwald and Liebermann 1991; Richt and Rott 2001). There are two published reports of efforts to develop vaccines in rodent models using strain He/80. In one study rats were inoculated with either high or low doses of virus attenuated by multiple passages in MDCK cells. Low-dose recipients had classic T-cell-mediated disease and succumbed to encephalitis (although with a longer course); high-dose recipients had high antibody titers to BDV and only mild disease (Oldach et al. 1995). In another study, rats were infected with a recombinant vaccinia virus expressing BDV nucleoprotein and subsequently challenged with BDV. Although vaccination resulted in enhanced viral clearance, disease was aggravated (Lewis et al. 1999). No studies are described for vaccines based on BDV G; however, results of passive immunotherapy experiments wherein a monoclonal antibody directed against G was protective suggest that this approach might be effective (Furrer et al. 2001).

Three compounds are described as having antiviral efficacy against BDV: amantadine, ribavirin, and 1-β-D-arabinofuranosylcytosine (ARA-C). Findings with amantadine are controversial. The drug was found to inhibit replication of some human BDV isolates in cell culture and improve the clinical course in some subjects with affective disorders (Bode et al. 1997; Dietrich et al. 2000). The antidepressant response to amantadine has been variously attributed to drug effects on viral replication (Bode et al. 1997; Bode and Ludwig 2001) or on brain levels of neurotransmitters implicated in depression (Lieb et al. 1997b). Interestingly, tests of animal isolates indicated no antiviral effect in vitro or in vivo (Cubitt and de la Torre 1997; Hallensleben et al. 1997; Stitz et al. 1998b). The sensitivities of the other human isolates, RW98 (Planz et al. 1999) and HuP2br (Nakamura et al. 2000), are unknown. Ribavirin inhibits transcription and replication of both He/80 and strain V BDV in a variety of cell lines (Mizutani et al. 1998; Jordan et al. 1999). In addition to its antiviral effects through the depletion of cellular GTP pools, interference with mRNA capping, and direct interaction with viral polymerases, ribavirin can promote a Th1-type immune response (Hultgren et al. 1998; Tam et al. 1999). This led to concerns that enhancement of cellular immunity might aggravate the immunopathology of BD (Sobbe et al. 1997; Hatalski et al. 1998a; Lewis et al. 1999). The efficacy of ribavirin was assessed in the rat model, wherein the drug was administered directly to the brain by intraventricular injection. Although ribavirin had no effect on viral load, treated animals had less inflammation and milder disease, presumably owing to antimitotic effects on microglia (Solbrig et al. 2002b). Ara-C, a nucleoside analog that inhibits DNA polymerase enzymes, also inhibits BDV replication. The mechanism of action remains unclear, but is postulated to be direct inhibition of the viral polymerase rather than an indirect effect mediated by host cell factors (Bajramovic et al. 2002).

The establishment of two functional BDV minireplicon assays (Perez et al. 2003; Schneider et al. 2003) was recently reported. The importance of N:P protein stoichiometry was demonstrated in both.

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Hepatitis A and E

BETTY ROBERTSON

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INTRODUCTION

Enterically transmitted viral hepatitis

Although descriptions of epidemic jaundice go back to earliest recorded human history, the infectious nature of hepatitis was not widely appreciated until this century. Investigations during the Second World War led to the first clear recognition that there were distinct forms of transmissible hepatitis: one that was acquired through ingestion of contaminated food or water ('infectious jaundice') and another that was associated with the administration of blood or blood products ('homologous serum jaundice') (Havens 1944; Barker et al. 1945). MacCallum and Bradley (1944) classified these as type A and type B hepatitis, setting in place a classification scheme that still exists. The distinguishing epidemiological features of these infectious diseases and the lack of cross-protection engendered by their respective agents were confirmed by Krugman and his associates in a classic series of studies carried out among institutionalized children (Krugman and Ward 1958; Ward et al. 1958; Krugman et al. 1960). However, the modern era of hepatitis A virology began with the identification of virus particles in human fecal samples (Feinstone et al. 1973).

The subsequent development of an immune electron microscopy assay for virus-aggregating antibodies resulted in a variety of increasingly sophisticated diagnostic tests for hepatitis A virus (HAV) infection. Widespread application of these new serologies, coupled with recently developed assays for hepatitis B virus (HBV) and its related antibody responses, led to the recognition that there were at least two additional human viruses responsible for reported cases of non-A, non-B (NANB) hepatitis. One type of NANB hepatitis, now recognized to be due to infection with Hepatitis C virus (HCV), resembled hepatitis B in that it was frequently associated with chronic liver disease and often transmitted by transfusion or other parenteral blood exposures (Choo et al. 1989). However, a distinctive agent responsible for waterborne or enterically transmitted NANB hepatitis was recognized to be the cause of large outbreaks of acute hepatitis in the Indian subcontinent and subsequently elsewhere (Khuroo 1980). These outbreaks included an epidemic in Delhi in 1955 that involved over 30 000 people and was associated with a breach in the city's sanitary water supply system. Although for many years this epidemic was considered to be due to type A 'infectious' hepatitis, serological tests carried out in the late 1970s indicated otherwise (Wong et al. 1980). The responsible agent, Hepatitis E virus (HEV), was identified by Balayan et al. (1983) in human fecal samples.

Similar and contrasting properties of HAV and HEV

HAV and HEV share a number of physical and biological characteristics (Table 1). Both are non-enveloped, RNA viruses with single-stranded genomes of positive polarity. Both display strong tropism for the liver (hepatocytes) and typically cause self-limited infections associated with acute hepatic inflammation. Above all, however, it is the fecal–oral transmission of HAV and HEV that distinguishes these viruses from other human hepatitis viruses, including HBV, hepatitis delta virus (HDV) and HCV. Both HAV and HEV are shed

Table 1 *Similar and contrasting properties of HAV and HEV*

Common properties		
Particle shape	Icosahedral	
Particle size	27–32 nm	
Chloroform sensitivity	Resistant	
Genome	ss (+)[a] 7.5 kb RNA	
Predominant transmission	Fecal–oral	
Liver disease	Acute, self-limited	
Unique properties	HAV	HEV
$S_{20,w}$	157S	183S
Bouyant density (g/cc^3)	1.33	1.29
Polypeptides	3–4 structural, 7 nonstructural	1–2 structural, 4–5 nonstructural
Stability	60°C, 1 h	Unknown
ORF	Single ORF	3 overlapping ORFs
5′ End structure	VPg	5′ Methylated cap

a) ss (+), single-stranded, positive-sense.

at high titers in the feces of infected individuals. In each case, the virus present in feces is replicated primarily in the liver and reaches the intestinal tract following secretion from the hepatocytes into biliary canaliculi and passage through the bile ducts. The absence of a lipid envelope is an important factor in this process, as it renders both HAV and HEV stable when suspended in bile. In contrast, the other human hepatitis viruses possess an outer lipid envelope and are likely to be rapidly inactivated in bile. Therefore, newly replicated HAV and HEV particles have a direct route to the outside environment that is denied the other hepatitis viruses. This allows HAV and HEV to cause explosive outbreaks of disease that are not seen with other types of hepatitis. However, neither HAV nor HEV causes persistent infections in humans and thus, unlike HBV and HCV, they have no association with chronic viral hepatitis or hepatocellular carcinoma.

Despite these similarities, HAV and HEV are very different viruses with a number of distinguishing features that result in their classification within different virus families (Table 1). These differences include fundamental aspects of virion structure and genome organization, as well as molecular mechanisms of translation and RNA replication.

HEPATITIS A VIRUS

Classification

On the basis of several features that distinguish HAV from other picornaviruses (Table 2), it is classified as the only member of the *Hepatovirus* genus of the *Picornaviridae* family (Minor 1991). Unique attributes of HAV include its tropism for the liver, its unusually slow and usually noncytolytic replication cycle, and its lack of close genetic relatedness to any other picornavirus.

Morphology of the HAV particle

The HAV virion appears as a relatively smooth, 27–30 nm rounded particle when viewed by electron microscopy in negatively stained preparations (Figure 1). Icosahedral symmetry is evident in many particles, but information concerning finer aspects of the capsid structure is limited. Infectious virions have a sedimentation constant of ca. 157S and band in CsCl at ca. 1.325 g/cm^3 (Lemon et al. 1985). Most virus preparations contain a large proportion of empty 70S capsids that are antigenically identical to virions.

Genome organization

The genomic RNA is single-stranded, ca. 7.5 kb in length and of positive polarity. The extreme 5′ end of the virion RNA is covalently coupled to a small viral protein, 3B (VPg), which is presumed to be removed from the RNA after its release from the capsid. The organization of the genome is similar to that of all picornaviruses (Figure 2, p. 4) (Najarian et al. 1985; Cohen et al. 1987c), but there are several unique features. There are at least three functionally separate domains: the 5′ and 3′ nontranslated RNA (NTR) and a single, intervening, long open reading frame (ORF) which encodes the viral polyprotein.

5′ NONTRANSLATED RNA

The 5′ NTR is approximately 735 nt long and contains an extensive secondary and tertiary RNA structure (Figure 3, p. 4). The segment from nucleotides 1–94 contains a 5′ terminal hairpin followed by 2 RNA pseudoknots (Brown et al. 1991; Shaffer et al. 1994) that, on the basis of studies of the corresponding cloverleaf-like RNA structure in poliovirus, probably play a controlling role in the initiation of positive-strand RNA

Table 2 *Properties of hepatoviruses and other picornaviral genera*

	Hepatoviruses	Aphthoviruses	Cardioviruses	Enteroviruses	Rhinoviruses
Primary host	Humans and higher primates	Cloven-hoofed mammals	Mammals	Higher primates	Mammals
Host-species restriction	Strict	Relatively strict	Relatively broad	Strict	Strict
Primary target organ	Liver (gut)	Systemic	CNS, liver	CNS, muscle (gut)	Respiratory mucosa
Dominant mode of transmission	Fecal–oral	Respiratory	??	Fecal–oral and respiratory	Respiratory
Serotypes	1	7	2	>70	>100
Particle size	27–30 nm	23–25 nm	24–30 nm	22–28 nm	24–30 nm
Buoyant density	1.33 g/cm^3	1.44 g/cm^3	1.34 g/cm^3	1.34 g/cm^3	1.41 g/cm^3
Stability <pH 7.0	pH 1.0	Nil	pH 3.0	pH 3.0	Nil
Stability >60°C	+	–	–	–	–
Genome RNA	7.5 kb	8.4 kb	7.8 kb	7.4 kb	7.2 kb
Sequence relatedness[a]	None	Cardioviruses	Aphthoviruses	Rhinoviruses	Enteroviruses
% G+C content	38	43	50	47	40
5′ poly(Y) tract	Poly(UUCC)	Poly(C)	Poly(C)	No	No
IRES structure	Type II	Type II	Type II	Type I	Type I
Leader (L) protein	Probably not	Yes	Yes	No	No
2A protease	No	Yes	Yes	Yes	Yes
Replication cycle	Very slow	Very fast	Very fast	Fast	Fast

a) to other *Picornaviridae*; Palmenberg (1989).

synthesis (Andino et al. 1993). At nucleotides 95–154 there is a unique pyrimidine-rich tract (pY1) that contains a series of repetitive (U)UUCC(C) sequence motifs assuming a poorly defined higher ordered structure, followed by a short, single-stranded domain (Shaffer et al. 1994). Both in location and in base composition, the pY1 domain resembles the much longer, nearly pure poly(C) tracts of the cardioviruses and aphthoviruses, other genera within the *Picornaviridae*. It seems that the pY1 tract is dispensable for viral

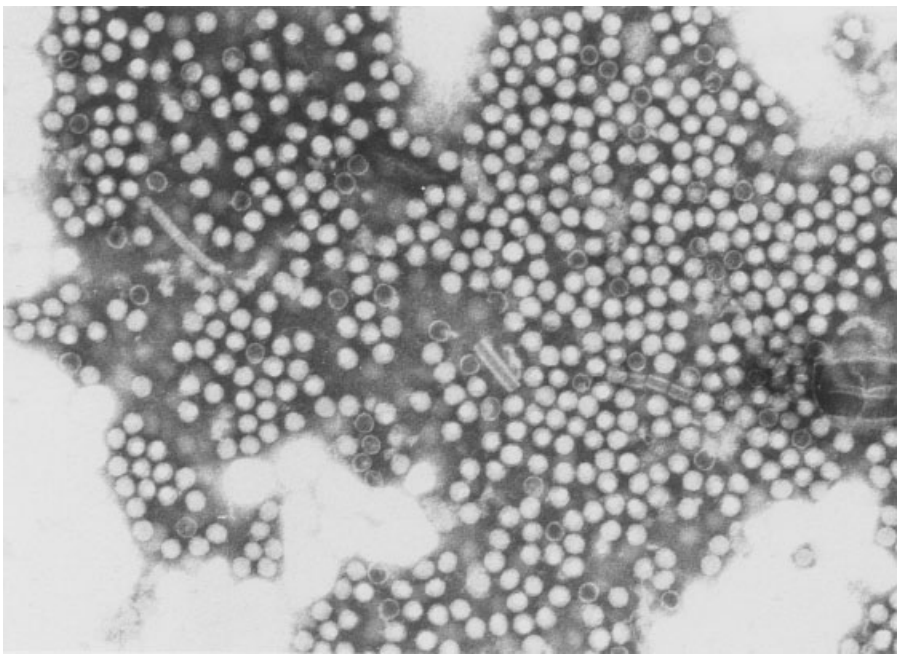

Figure 1 *Hepatitis A particle morphology demonstrated by electron microscopy. Negative-stained preparation.*

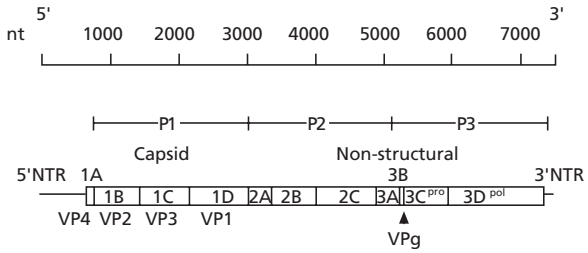

Figure 2 *Organization of the single-stranded positive-sense RNA genome of HAV.*

replication, both in primates in vivo and in cultured cells (Shaffer et al. 1994, 1995). The single-stranded domain located at nucleotides 135–155 seems to play a functional role in viral RNA replication, as deletion mutations in this region confer a temperature-sensitive viral replication phenotype due to a defect in RNA synthesis (Shaffer and Lemon 1995). Further downstream, the segment of 155–735 nt contains several complex stem–loops that comprise an internal ribosomal entry site (IRES) and direct the cap-independent translation of the long ORF (pp 696 and 699) (Brown et al. 1991, 1994; Glass and Summers 1992).

LONG ORF

The long ORF encodes a large polyprotein of c. 2200 amino acid residues that is proteolytically cleaved cotranslationally and post-translationally to generate mature viral proteins. As illustrated in Figure 2, the polyprotein can be functionally divided into three segments, P1, P2, and P3, as defined for other picornaviruses (Rueckert and Wimmer 1984). The P1 segment contains the structural proteins that form the viral capsid: 1A (also known as VP4), which is very small and not yet demonstrated to be a component of the HAV virion; 1B (VP2); 1B (VP3); and 1D (VP1). The P2 segment contains three nonstructural polypeptides, 2A, 2B, and 2C. The 2A protein appears to play a role in viral assembly and is present in immature virions as uncleaved VP1/2A (Borovec and Anderson 1993), while 2B and 2C have functions related to RNA replication (see section on Replication of HAV). The P3 segment contains four additional nonstructural proteins, including 3A (which probably helps anchor replication complexes to cellular membranes), 3B (VPg, the genome-linked protein), $3C^{pro}$ (a cysteine protease), and $3D^{pol}$ (an RNA-dependent RNA polymerase). As in poliovirus infections, some intermediate precursor proteins (e.g. 3AB, 3ABC, or 3CD) appear to have functions distinct from their processed end products (see section on Replication of HAV).

3′ NONTRANSLATED RNA

The 3′ NTR is ca. 60 nt in length and is polyadenylated at its 3′ terminus. Little is known about the functions of

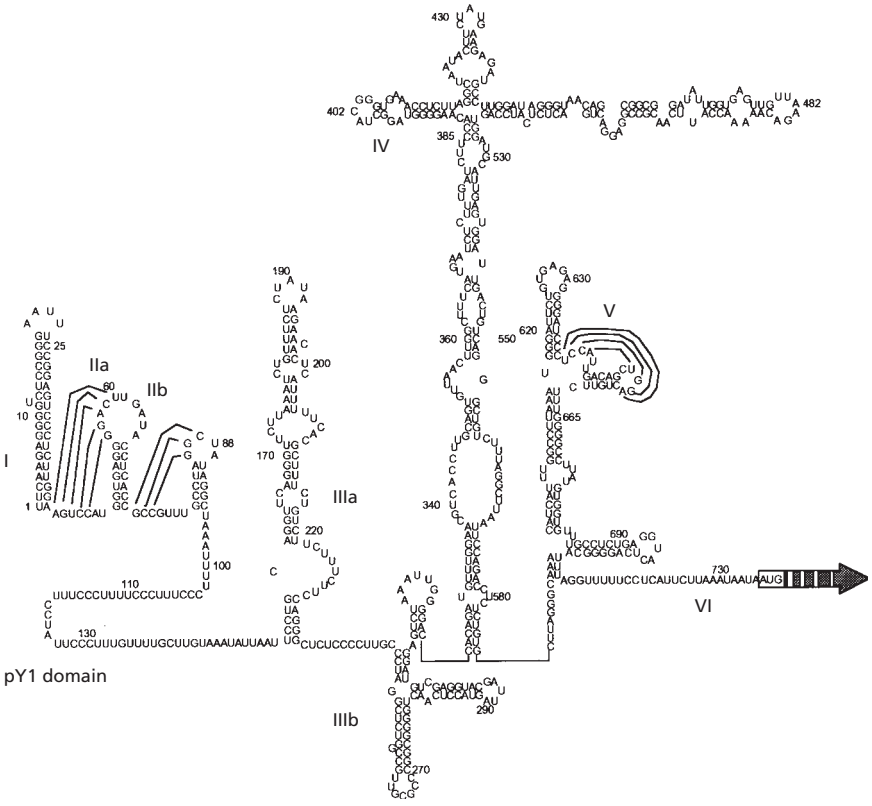

Figure 3 *Secondary and tertiary structure within the 5′ nontranslated RNA of HAV (Brown et al. 1991).*

the 3′ NTR sequence in virus replication. As with the 5′ NTR, the 3′ NTR is suspected to have substantial secondary structure and to interact with specific viral or host-cell proteins (Nuesch et al. 1993).

GENETIC VARIATION AMONG HAV STRAINS

Although the nucleotide sequences of different HAV strains have a relatively high degree of conservation, there is sufficient sequence heterogeneity to distinguish distinct phylogenetic lineages (Jansen et al. 1990; Robertson et al. 1991, 1992). A comparison of the sequences of a 168-nt segment near the VP1/2A junction of 152 different strains of HAV revealed seven distinct 'genotypes' that differed from each other at >15 percent of base positions (Figure 4) (Robertson et al. 1992). Representatives of three of these genotypes (I, II, VII) have been recovered only from human sources, whereas another genotype (III) contains viruses isolated both from humans and from captive Panamanian owl monkeys. The remaining three genotypes (IV, V, VI) are represented by single strains of simian HAV recovered from nonhuman primates (African green and cynomologus monkeys). Genotypes I and III include the vast majority of identified human strains and each contains two subgroups that differ at >7.5 percent of base positions. These have been designated subgenotypes IA and IB, IIIA and IIIB. Recently, the full genome sequences for representatives of human genotypes II (CF53/Berne, Lu et al. 2004), and VII (SLF88, Ching et al. 2002) have been determined, and the data indicate these two strains represent two subtypes of the same genotype and have been proposed to be subgenotypes IIA and IIB within genotype II (BH Robertson, unpublished observations). Similar conclusions were derived by Costa-Mattioli and coworkers (2002) based upon their evaluation of the complete VP1 region of 81 strains from France, Kosovo, Chile, Argentina, and Uruguay.

The molecular epidemiology of HAV using sequence comparison has characterized patterns of virus spread within different regions of the world and transmission patterns in outbreaks (Jansen et al. 1990; Robertson et al. 1991, 1992, 2000; Hutin et al. 1999; Diaz et al. 2001; Bruisten et al. 2001; Taylor 1997; de Paula et al. 2002; Costa-Mattioli et al. 2001a, b; Arauz-Ruiz et al. 2001; Mbayed et al. 2002). For example, multiple genotype IA virus strains recovered in China, North and Central America, and Cuba comprise separate, phylogenetically and geographically-related clusters within genotype IA while in South America no consistent pattern of virus derivation is apparent. In contrast, most HAV strains recovered in Europe show extensive genetic divergence, suggesting the absence of a dominant circulating virus and consistent with a large proportion of European cases being caused by virus strains imported from other regions (Jansen et al. 1990; Robertson et al. 1992; Costa-Mattioli et al. 2001b; Bruisten et al. 2001). Sequence analysis has identified identical sequences within common source and community wide outbreaks (Niu et al. 1992; Hutin et al. 1999; Robertson et al. 2000) and provided the ability to

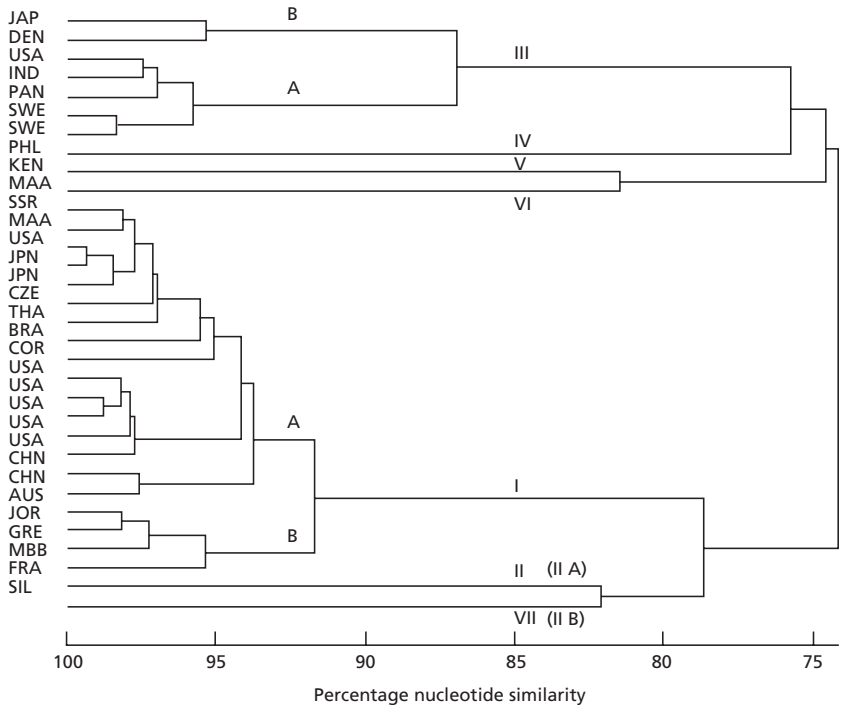

Figure 4 *Phylogenetic analysis of RNA sequences near the VP1/2A junctions of hepatovirus strains recovered from human sources in different geographic regions (genotypes I, II, III, and VII) or simian sources (genotypes IV, V, and VI) (Robertson et al. 1992).*

discriminate between outbreaks in the same city with different sources of transmission (Dentinger et al. 2001).

Structure of HAV

STRUCTURAL POLYPEPTIDES

The structural (capsid) proteins of HAV are encoded by the most 5′ (P1) region of the long ORF. The primary proteolytic cleavage of the nascent viral polyprotein seems to occur between the 2A and 2B polypeptides (Almela et al. 1991; Cho and Ehrenfeld 1991; Winokur et al. 1991; Borovec and Anderson 1993). The site of this cleavage, which is mediated by the viral protease $3C^{pro}$, was recently shown to occur at residue 867 of the polyprotein, making 2B considerably larger than in other picornaviruses (Martin et al. 1995). Subsequent cleavage of the P1-2A precursor at the VP2/VP3 and VP3/VP1 junctions by $3C^{pro}$ results in 1A1B (also known as VP0), VP3 and VP1-2A (otherwise known as PX). VP1-2A seems to be an intermediate in virus assembly, as it can be detected within infected cells in pentameric subunits and possibly in some early virions (Borovec and Anderson 1993).

The VP4 sequence, located at the extreme amino terminus of the polyprotein, includes two potential methionine initiation codons (codons 1 and 3) which precede a consensus myristylation signal (GxxxT/S) (Chow et al. 1987). This has led to speculation that 'VP4' might be cleaved during replication to place the myristylation signal at the N terminus of the polyprotein, as such a signal is present at the amino terminus of the VP4 proteins of enteroviruses and rhinoviruses. Moreover, in the genera *Aphthovirus* and *Cardiovirus* of the *Picornaviridae*, a 'leader' (L) protein, located at the amino terminus of the polyprotein, is proteolytically removed to yield the free amino end of VP4, which is then myristylated (Chow et al. 1987). Thus, the VP4 proteins of all other picornaviruses are N terminally myristylated. However, despite numerous attempts, no one has yet been able to demonstrate the presence of VP4 within the HAV particle, or to determine by labeling experiments whether any of the viral capsid proteins are myristylated. In addition, site-directed mutagenesis of the two potential initiator methionines resulted in a change in the size of VP0, proving that there is no early cleavage within the sequence of VP4 and strongly suggesting that HAV VP4 is not myristylated (Tesar et al. 1992, 1993). Thus, although myristylation of VP4 seems to be important for the assembly of other picornaviruses, it does not seem to be necessary for HAV morphogenesis.

As in other picornaviruses, cleavage of VP0 to VP4 and VP2 occurs after the encapsidation of viral RNA (Bishop and Anderson 1993), resulting in a difference in the protein profile of empty capsids and complete virions. However, this cleavage occurs slowly with HAV, a feature that might be important to pathogenesis as it may prevent the infection of intestinal epithelial cells by virus secreted in the bile into the upper intestinal tract. The precise arrangement of the polypeptides within the assembled capsid is not known, as the hepatoviruses represent the only genus of the *Picornaviridae* for which the capsid structure has not been solved by X-ray crystallography. A hypothetical structure has been proposed, modeled after the structures of mengo virus and rhinovirus (Luo et al. 1988). However, this model does not correlate particularly well with experimental data obtained from biochemical and enzymatic studies, or with analysis of neutralization-resistant escape mutants (see below). Extended incubation of virus (up to 24 h) in the presence of large amounts of protease (1–10 mg/ml) results in the cleavage of VP2 by chymotrypsin and of VP1 and VP2 by trypsin. However, protease digestion has no effect on infectivity or antigenicity of the virus (Lemon et al. 1991a). Chemical iodination of exposed tyrosine residues occurs at two surface sites, one involving VP1 and the other, VP2. The VP1 residue is located between amino acids 233 and 273, whereas the VP2 site was at Tyr^{100} (Robertson et al. 1989).

ANTIGENIC STRUCTURE

Available evidence indicates that HAV strains comprise a single serotype, and that infection or immunization with any one strain confers protection against all others (LeDuc et al. 1983; Lemon and Binn 1983a; Lemon et al. 1992a). Although antigenic differences between human and simian viruses can be detected with a selected monoclonal antibody (mAb) (Nainan et al. 1991; Tsarev et al. 1991), the simian viruses react with other mAbs as well as polyclonal antibodies raised to human strains. The dominant antigenic determinants of HAV are highly conformational, as mAbs to the virus fail to recognize any capsid polypeptide in immunoblot assays. In addition, the antigenicity of the virus is curiously monotonic. Many mAbs are able to inhibit competitively by >50 percent the binding of polyclonal antibodies to the virus, and mAbs can be classified into several different groups on the basis of their ability to block the binding of two reference monoclonals (Stapleton and Lemon 1987; Ping and Lemon 1992; Schofield et al. 2002). Some mAbs recognize antigenic determinants common to 70S empty particles and 14S pentamer subunits (which are formed by five copies of the VP2–VP3–VP1 protomer subunit), whereas others recognize an antigenic determinant that is present only on 70S particles and is thus likely to span pentamer–pentamer interfaces (Stapleton et al. 1993). With few exceptions, mAbs to the virus are able to neutralize infectivity (Ping and Lemon 1992). No monoclonal antibodies have been identified that are directed toward the binding site for the putative HAV receptor, HAVcr-1 (see section on Replication of HAV).

Neutralization-resistant HAV mutants have been produced by successive rounds of neutralization and passage of virus in the presence of mAb (Stapleton and Lemon 1987). These mutants have only a limited number of amino substitutions, all in VP3 or VP1 (Ping et al. 1988; Ping and Lemon 1992; Nainan et al. 1992). Most, if not all, of these amino acid substitutions are likely to be at residues that interact directly with the complementarity-determining regions of the cognate mAb. They thus serve to map the neutralizing epitopes of the virus. The most frequently identified mutations are at Asp^{70} of VP3, but escape mutations have also been found at Pro^{65}, Ser^{71}, and Glu^{74} of this protein. Mutation at Asp^{70} profoundly alters the antigenicity of the virus, significantly reducing the reactivity of the virus with most polyclonal antibodies (Lemon et al. 1991b; Ping and Lemon 1992). Several different amino acid substitutions have been identified at this residue. Other neutralization escape mutants have substitutions within the VP1 sequence, at Ser^{102}, Asp^{104}, Lys^{105}, Val^{171}, Ala^{176}, or Lys^{221}. Except for several mutants with different amino acid substitutions at Lys^{221} of VP1 (all of which determine resistance to a single mAb), most escape mutants demonstrate extensive cross-resistance to multiple mAbs (Ping and Lemon 1992). The simian strains of HAV all contain an Asp^{70} mutation that results in lack of recognition of these viruses by monoclonal antibodies against human HAV (Nainan et al. 1992). These data suggest that residues 65–74 of VP3 (centering on Asp^{70}) and 102–105 and 171–176 of VP1 contribute to a single immunodominant antigenic site that contains a limited number of closely related and overlapping epitopes. This interpretation is consistent with the monotonicity of the polyclonal antibody response to the virus noted above.

There is also limited evidence for a neutralization epitope located near the amino terminus of VP1. Antibodies raised against a synthetic peptide representing residues 13–24 of VP1 generated neutralizing antibodies (Emini et al. 1985), and low levels of neutralizing antibodies were found in some animals immunized with a chimeric poliovirus containing this segment of the HAV VP1 molecule placed within an antigenic surface loop of poliovirus VP1 (Lemon et al. 1992b). Because the amino terminal domain of VP1 is located internally within the crystallographically determined structures of other picornaviruses, it is likely that this epitope becomes exposed only after interaction of the virus with its putative cellular receptor.

Consistent with the conformational nature of HAV epitopes, individual recombinant HAV capsid proteins generally fail to elicit antibody responses that are reactive with the viral capsid. However, such proteins may prime for B cell responses in small animals (Johnston et al. 1988). Epitope(s) responsible for such priming reside within the carboxy terminal 156 residues of VP1 (Harmon et al. 1993). Also consistent with the conformational nature of HAV epitopes, a large number of synthetic peptides representing different segments of the HAV capsid proteins have been shown not to express any relevant HAV antigenic activity (Lemon et al. 1991c). There is data suggesting that a synthetic peptide 'mimotope' selected using a combinatorial approach may be able to stimulate production of antibodies that are reactive with the virus capsid (Mattioli et al. 1995).

Five antigenic domains over the entire HAV polyprotein using 237 overlapping 20mer peptides were identified using convalescent antisera (Khudyakov et al. 1999). These domains, probably composed of linear epitopes, include amino acids 57–90 (within VP2); amino acids 767–842 at the VP1–2A junction; amino acids 1403–1456 encompassing the P2C–P3A junction; amino acids 1500–1519 comprising the entire P3B protein; and amino acids 1719–1764 which encodes the P3C–P3D junction. In general, the nonstructural proteins have not proved to be adequate targets for antibody detection. Studies using expressed 3C protease for antibody detection demonstrated the presence of these antibodies after the acute phase of the illness, but they declined more rapidly than antibodies directed toward the capsid (Robertson et al. 1993; Stewart et al. 1997).

Replication of HAV

PROPAGATION OF VIRUS IN CULTURED CELLS

Replication of wild-type HAV is very slow and inefficient in cell culture, typically taking days to weeks to reach maximum virus yields (Provost and Hilleman 1979; Daemer et al. 1981; Binn et al. 1984). However, with patience and continued passage, virus yields are increased and the otherwise lengthy replication period is shortened. HAV variants that have been adapted to cell culture will replicate in many primate cell lines, but certain cell lines derived from African green monkey (BS-C-1 or CV-1) or fetal rhesus monkey kidney (FRhK-4) seem to be most permissive for virus growth (Provost and Hilleman 1979; Daemer et al. 1981; Binn et al. 1984). Other mammalian cell types are also permissive for limited replication of the virus (Dotzauer et al. 1994). Thus there does not seem to be a strict host species requirement in cell culture. Viruses that are well adapted to growth in monkey kidney cell cultures are often highly attenuated when inoculated into susceptible primates or humans (Provost et al. 1982; Karron et al. 1986) (see below).

The specific cell culture infectivity (cell culture infectious units per genome copy) of one virus variant (HM175/P16) that had been adapted to growth in cultured cells during 16 sequential passages in African green monkey kidney cells was >3 000 times that of the related wild-type virus (Jansen et al. 1988). However, even virus isolates that are well adapted to growth in

cultured cells replicate much more slowly than picornaviruses such as encephalomyocarditis virus (EMCV) or poliovirus (Lemon et al. 1991b). Most HAV strains fail to induce a cytopathic effect in cultured cells, although cytopathic variants have been selected from strains that are highly adapted to growth in cultured cells (Anderson 1987; Cromeans et al. 1987; Robertson et al. 1988; Lemon et al. 1991b). Such cytopathic variants may reach maximum yields in <24 h under one-step growth conditions (Lemon et al. 1991b). However, even these strains of HAV do not induce a specific shutdown in host cell macromolecular synthesis. To a considerable extent, the poor growth of HAV in cultured cells has limited investigations of the replication cycle.

In most cases, the replication of HAV in cell cultures can be detected only by immunological techniques or demonstration of the viral RNA by nucleic acid hybridization. The quantal, radioimmunofocus assay allows accurate titration of viral infectivity by immunostaining of viral replication foci developing beneath an agarose overlay over a period of 6–14 days (Lemon et al. 1983).

HAV CELL RECEPTOR

Recently, a putative receptor for HAV has been identified as a mucinlike class I integral membrane glycoprotein. This molecule (HAVcr-1) was identified by two independent groups using AGMK cells (Kaplan et al. 1996) and a marmoset–Vero cell hybrid (Ashida and Hamada 1997). It consists of an N-terminal Cys-rich region with homology to the immunoglobulin superfamily followed by a threonine, serine, proline-rich region which is similar to mucinlike proteins. The human homologue (huHAVcr-1) was cloned from human kidney and liver cDNA and determined to be 79 percent identical to the AGMK derived receptor, excluding three insertions/deletions within the mucinlike domain (Feigelstock et al. 1998). The huHAVcr-1 molecule does not appear to be liver specific, as it has been identified in all organs evaluated, with highest levels within kidney and testis. Evaluation of deletion mutants of the HAVcr-1 revealed that the Cys-rich region and the N-terminal N-glycosylation site is required for binding of HAV (Thompson et al. 1998); evaluation using immunoadhesins also revealed that it neutralizes bound HAV (Silberstein et al. 2001). Interestingly, a related protein from mice (Tim-1) and rats (Kim-1), expressed on T cells, is associated with airway hyperreactivity within inbred mice (McIntire et al. 2001).

In contrast to these findings, Dotzauer et al. (2000), have presented evidence that an alternative mechanism can be used by the virus to target it to liver cells. They propose that in addition to other host-derived materials found associated with HAV virions, IgA molecules bound to virus can serve to target virions to the liver using the asialoglycoprotein receptor. Initial infection would be from ingested IgA–virus complexes or the result of local IgA stimulated within the small intestine.

CAP-INDEPENDENT TRANSLATION OF THE VIRAL POLYPROTEIN

The first step in replication following the uncoating of the viral RNA is the cap-independent translation of the viral polyprotein. The initiation of translation is carried out under direction of the IRES located between nucleotides 155 and 735 of the 5′ NTR (Brown et al. 1991, 1994). Although the IRES shares several structural features with that of EMCV, it is ca. 50–100-fold less active in directing translation than the EMCV IRES, even in HAV permissive cells (Brown et al. 1994; Whetter et al. 1994; Schultz et al. 1996b). It is likely that weak IRES activity contributes to the slow and noncytopathic growth of HAV, because studies indicate that translation is rate limiting in infection of cultured African green monkey kidney cells with wild-type virus (Schultz et al. 1996b).

The IRES of HAV binds specifically to several cellular proteins, including polypyrimidine tract-binding protein (PTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (p39) (Chang et al. 1993; Schultz et al. 1996a). Experimental data indicate that the binding of GAPDH destabilizes RNA secondary structure and is thus likely to be detrimental to IRES-directed translation (Schultz et al. 1996a). PTB and GAPDH compete with each other for binding to certain stem–loop structures within the 5′ NTR, and the relative levels of these cellular proteins may be important in determining the translational activity of the HAV IRES (Yi et al. 2000). Specific hepatocyte factors may enhance translation directed by the HAV IRES (Glass and Summers 1993).

REPLICATION FUNCTIONS ASSOCIATED WITH NONSTRUCTURAL PROTEINS

The nonstructural proteins of HAV are responsible for proteolytic processing of the polyprotein and replication of the viral RNA. There are at least seven distinct nonstructural proteins: 2A, 2B, 2C, 3A, 3B (the genome-linked protein, VPg, which is covalently attached to the 5′ end of virion RNA), $3C^{pro}$ (the viral protease) and $3D^{pol}$ (the RNA-dependent RNA polymerase). Many of the specific functions of these proteins are not well understood.

Although the 2A proteins of other picornaviruses have protease activities and are responsible for the primary *cis*-active cleavage of the polyprotein (at either VP1/2A or 2A/2B), this is not the case for HAV. The HAV 2A protein shares no sequence identity with these other picornaviral 2A proteins and does not have proteolytic activity. Unlike other picornaviruses, primary cleavage of the HAV polyprotein precursor occurs between 2A and 2B, resulting in a P1–2A intermediate. While the 2A/2B cleavage site has been identified as

Gln^{836}/Ala^{837} and is mediated by $3C^{pro}$ (Martin et al. 1995), the cleavage of VP1–2A has been more difficult to determine. Early data suggested that this junction was cleaved by the $3C^{pro}$ (Schultheiss et al. 1994; Schultheiss et al. 1995); Probst et al. (1997) also concluded that $3C^{pro}$ is responsible for the maturation cleavage of VP1/2A between amino acids Val^{272}-Glu^{273} and Ser^{274}. In contrast, two studies provide evidence that $3C^{pro}$ is not involved in this cleavage – mutation of the only sites possible for $3C^{pro}$ cleavage did not abolish infectivity or virus maturation (Martin et al. 1999). This conclusion was supported by identification of the C-terminal amino acids of VP1 (Graff et al. 1999); the C terminus was found to be heterogeneous and composed of Leu^{270}-Pro^{271}-$Thr^{272}/Glu^{273}/Ser^{274}$. The predominant terminal amino acid was Ser^{274}, but with detectable amounts of Glu^{273} and Thr^{272}. This process had been proposed to be mediated by an unknown cellular protease. Cohen et al. (2002) created deletion mutants within the 2A region, and found that deletion of the amino terminal 40 percent abolished infectivity, while deletions in the carboxy terminal 60 percent resulted in small plaque phenotype. However, earlier studies of large (15 amino acid) deletions within 2A were found not to impair virus growth in cultured cells (Harmon et al. 1995). The N-terminal deletions would alter the VP1–2A cleavage that occurs after pentamer assembly, thereby affecting virus maturation. These studies have lead to the conclusion that there are two domains within 2A; an amino terminal molecular chaperon-like domain that directs the folding of the capsid precursor for proper 3C processing, while the C terminal domain is needed for non-$3C^{pro}$-mediated processing of the VP1–2A precursor to generate mature VP1.

The 2B and 2C proteins of HAV have functions related to replication of the viral RNA and, on the basis of studies with poliovirus, presumably play essential roles in directing the assembly of a membrane-based replicase complex (Bienz et al. 1992; Cho et al. 1994). Since mutations in 2B and 2C are important for adaptation of wild-type HAV to replication in cultured cells (Emerson et al. 1992a; Emerson et al. 1993), it is very likely that these proteins interact in an as yet undefined but highly specific fashion with cellular proteins or other components of the intracellular membrane. 2C contains a 'DEAD' motif, suggesting that it may have RNA helicase activity, and has been shown to be an NTPase. The HAV 2B and 2C proteins most probably function as a complex, as mutations in these proteins which play an important role in defining the replication properties of rapidly replicating, cytopathic viruses act in a highly cooperative fashion (Zhang et al. 1995). The 2C proteins of HAV and poliovirus are clearly related phylogenetically, and mutations that confer resistance of poliovirus to guanidine (an inhibitor of poliovirus RNA replication) map to the 2C protein. HAV replication has been suggested to be sensitive to guanidine (Cho and Ehrenfeld 1991), but this has not been found to be the case by other investigators (Siegl and Eggers 1982).

The function of the 3A protein in viral replication is also poorly defined, but it is likely to serve as an anchor for 3AB or 3ABCD within the membranous replication complexes. Mutations in 3A may play a role in determining the cytopathic phenotype, as multiple cytopathic strains that have been sequenced in this region contain deletions of one or more amino acids near the amino terminus of 3A (Lemon et al. 1991b; Beneduce et al. 1995). However, any role that mutations in 3A may play in determining this phenotype is of secondary importance to the role played by mutations in 2BC (Zhang et al. 1995). The 3B (VPg) protein is covalently attached to the 5′ nucleotide of the virion RNA (Weitz et al. 1986). Studies with other picornaviruses suggest that it is removed shortly after uncoating of the viral RNA, and that it is also likely to be present at the 5′ end of newly synthesized minus-strand RNAs. Its precise functions may include a role in priming for RNA synthesis, or possibly in cleavage of the RNA if negative-strand synthesis originates from a snap-back, self-priming mechanism.

$3C^{pro}$ is clearly the best studied of the nonstructural proteins of HAV because its three-dimensional structure has been determined by X-ray crystallography (Allaire et al. 1994). It is a cysteine protease with both structural and functional relatedness to chymotrypsin and the serine proteases. $3C^{pro}$ seems to be responsible for each of the proteolytic cleavages of the viral polyprotein (Harmon et al. 1992; Kusov et al. 1992; Schultheiss et al. 1994; Tesar et al. 1994), with the exception of the VP4/VP2 cleavage (Bishop and Anderson 1993) and the VP1/2A as indicated above. The substrate specificity of the $3C^{pro}$ enzyme has been studied extensively using synthetic peptide substrates (Jewell et al. 1992; Malcolm et al. 1992). The dipeptide sequences present at the various HAV cleavage sites are considerably more variable than in other picornaviruses. Studies with poliovirus suggest that $3C^{pro}$ has specific RNA-binding activity and interacts with the 5′ NTR during initiation of plus-strand RNA synthesis; structural studies indicate that the RNA-binding site is located on the opposite side of the molecule from the protease active site. Poliovirus $3C^{pro}$ has also been considered to contribute to host cell shutdown at the transcriptional level by directing cleavage of the human TATA-binding protein (Clark et al. 1993; Das and Dasgupta 1993). However, either the HAV $3C^{pro}$ protein lacks this activity or it is expressed at much lower levels during viral replication, because HAV infection has never been shown specifically to impair host cell metabolic processes. The putative RNA-dependent RNA polymerase activity of $3D^{pol}$ is suggested by the presence of a conserved 'GDD' amino acid motif. Thus far, however, attempts to express an active polymerase have resulted in an insoluble product with no detectable activity.

GENETIC CHANGES ASSOCIATED WITH ADAPTATION TO GROWTH IN CULTURED CELLS

The genetic changes associated with adaptation of the HM175 and GBM strains of HAV to growth in cell culture have been identified by comparisons of the nucleotide sequences of wild-type and cell culture-adapted variants (Cohen et al. 1987a; Jansen et al. 1988; Ross et al. 1989; Graff et al. 1994). These studies are important because cell culture-adapted viruses are often highly attenuated and have been used as candidate vaccines in humans. The HM175 strain has been studied intensively, with complete genomic sequences determined for three independently isolated, cell culture-adapted viruses (Cohen et al. 1987a; Jansen et al. 1988; Ross et al. 1989). The mutations associated with cell culture adaptation are scattered throughout the genome, but tend to concentrate in the 5′ NTR and P2 domains.

Several individual mutations are present in multiple, independent cell culture-adapted isolates, signifying their importance to efficient replication in cell culture. The construction of an infectious cDNA clone of the HM175 virus (Cohen et al. 1987b) has allowed a detailed molecular genetics analysis of mutations that are associated with the adaptation of HAV to growth in cultured cells. Emerson and colleagues (1991, 1992a, b) found that mutations within the P2 region (2B and 2C proteins) are necessary for efficient growth in cultured cells. A relatively conservative Val^{216} to Ala^{216} substitution in 2B (nucleotide 3889) seems to be the most important single mutation with respect to growth in cultured cells. These P2 mutations probably facilitate interactions between these viral proteins and a host cell-specific factor(s) required for replication, but further details are lacking.

Mutations within the 5′ NTR, strongly associated with host-cell restricted growth, have been demonstrated in virus grown in CV-1, BS-C-1, MRC-5, HEK, and HFS cells lines. Analogous host restricted mutations are not necessary for growth in FRhK4 cells (Emerson et al. 1991, 1992a; Funkhouser et al. 1994; Graff et al. 1994; Graff et al. 1997). The mutations responsible for this effect (Day et al. 1992) are located in or near the viral IRES, and enhance cap-independent translation (Schultz et al. 1996b). These results indicate that there are cell type-specific factors that influence the efficiency of translation directed by the HAV IRES in certain cell lines. Differing cytoplasmic levels of GAPDH and PTB may play an important role in determining the activity of these 5′ NTR mutations, as these two cellular proteins compete for binding to the 5′ NTR with opposing effects on viral translation (Chang et al. 1993; Schultz et al. 1996a).

In contrast, BS-C-1-adapted HAV was grown in guinea-pig embryo cells for five passages, and selected regions (5′ NTR, 2ABC3AB, and the 3′ NTR) were amplified and sequenced. Only two nucleotides within these regions were changed; 2B nucleotide 3679, and 2C nucleotide 4419 (Frings and Dotzauer 2001). Under these circumstances, host-cell growth restriction is not regulated by interaction of cellular factors with 5′ NTR sequences. Likewise, replication of a subgenomic replicon containing luciferase in the place of the capsid proteins within Huh-7 cells was enhanced by mutations within the P2 and P3 regions, especially mutations at 2B nucleotide 3889, while the 5′ NTR region had no effect within this system (Yi and Lemon 2002).

The nearly complete nucleotide sequences have been determined for three rapidly replicating, cytopathic variants (*rr/cpe+*) recovered from cells that were persistently infected with the HM175 virus (Lemon et al. 1991b). These clonally isolated variants contained identical mutations within the 5′ and 3′ NTRs as well as within the RNA encoding the P2 and P3 proteins (2B, 2C, 3A, and 3D), while the intervening genomic sequences contained unique silent and nonsilent mutations. This suggested that the mutations that were common to the three cytopathic variants were likely to be responsible for the *rr/cpe*$^+$ phenotype. To determine which mutations are responsible for the *rr/cpe*$^+$ phenotype, infectious cDNA chimeras were constructed that contained various segments of one of these cytopathic variants (HM175/18f) in the genetic background of a noncytopathic virus (Zhang et al. 1995). Analysis of the chimeric viruses demonstrated that complete expression of the *rr/cpe*$^+$ phenotype required the cooperative effects of multiple mutations in 2B, 2C, the 5′ NTR and the P3 regions of the genome. Thus, the *rr/cpe*$^+$ phenotype represents a super-adaptation of HAV to growth in cell culture, and reflects changes in multiple virus functions.

Clinical and pathological aspects of HAV infection

PATHOGENESIS OF HEPATITIS A

To a considerable extent, present understanding of the pathogenesis of hepatitis a derives from studies of HAV infections in several species of susceptible nonhuman primates: chimpanzees, certain species of tamarins, and New World owl monkeys (Dienstag et al. 1975; Dienstag et al. 1976; Bradley et al. 1977; Cohen et al. 1989). Although these primate species can be infected either by oral or by intravenous challenge with virus, HAV infection typically follows oral exposure to the virus in humans, due either to close personal contact with an infected individual or to the ingestion of fecally contaminated water or food. Viral replication occurs primarily within hepatocytes, and the secretion of virus into bile results in large quantities of virus being shed in the feces. The titer of virus excreted in stools may be at least as high as 10^8 particles or 10^6 infectious virions/g (Purcell et al. 1984; Lemon et al. 1990). A sustained viremia throughout the incubation period and the early

stages of acute liver disease parallels the fecal shedding of virus, but at titers that are 100–1000-fold lower (Cohen et al. 1989; Lemon et al. 1990) (Figure 5).
Early studies demonstrated viral antigen within the liver, in hepatocytes and Kupffer cells, as well as in splenic macrophages and along the glomerular basement membrane in the kidney (Dienstag et al. 1976; Mathiesen et al. 1978a, 1980; Shimizu et al. 1982). However, it is not clear whether the presence of viral antigen in phagocytic cells reflects nonspecific trapping of viral antigen or replication of virus.

The genetic basis of pathogenesis has been evaluated by Emerson and coworkers (2002) by generating 14 chimeric viruses from clonal wild-type and attenuated sequences of HM-175 which contained 19 individual nucleotide changes over the whole genome. The resulting chimeras were evaluated in tamarins for their ability to cause disease. Nucleotides 3025 and 3196 within the VP1–2A junction and nucleotides 4043, 4087, 4222, and 4563 within the 2C genome region were necessary for expression of virulence within tamarins and were associated with severity of acute hepatitis. The mutations within the 2C protein, combined with a mutation at nucleotide 3889 within 2B controlled efficient replication in cultured cells.

The relationship between pathogenesis and the infected host species was approached by inoculating simian HAV (AGM27 from African green monkeys) into five different primate species (Emerson et al. 1996). The results revealed that different clinical patterns are seen depending upon the host. The most severe disease pattern was seen in African green monkeys and marmosets while infection of rhesus monkeys and cynomologus monkeys resulted in less severe symptoms; infection of chimpanzees did not result in detectable biochemical changes or histopathology, and seroconversion was delayed until 5–6 weeks after inoculation. Cross-challenge with a standard challenge dose of wild-type human HAV HM-175 in animals who had been exposed to AGM-27 revealed that all the animals were protected from acute infection, consistent with protection due to the presence of antibodies generated by the AGM-27 infection.

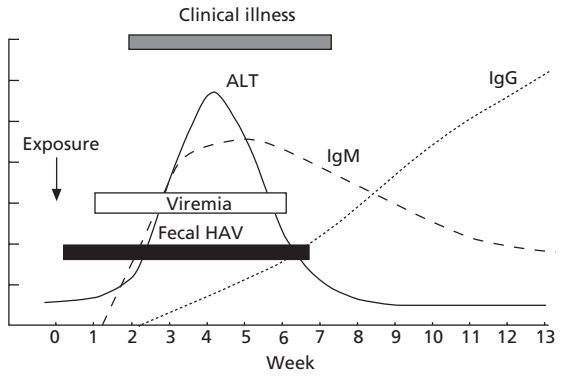

Figure 5 *Virological and pathological events during a typical infection with HAV.*

Although most if not all of the virus found in feces and blood is likely to be replicated within hepatocytes, there are limited data that indicate that HAV also infects intestinal epithelial cells in vivo. The early events in hepatitis A infection and the mechanism by which the virus reaches the liver remained poorly defined. HAV antigen has been identified within intestinal epithelial cells of experimentally infected tamarins (Karayiannis et al. 1986) as well as orally inoculated owl monkeys (Asher et al. 1995). In owl monkeys, viral antigen was observed in epithelial cells lining crypts in the small intestine within 3 days of inoculation. These findings in vivo are supported by studies demonstrating the growth of cell culture-adapted HAV in polarized cultures of colonic carcinoma cells (CaCo-2) grown on semi-permeable membranes (Blank et al. 2000). Such cells could be infected only from their apical surface, and released progeny virus in a vectorial fashion only from the apical surface. Infectious virus has also been detected at low levels within pharyngeal secretions, but later in infection when viremia was also present (Cohen et al. 1989; Asher et al. 1995). It is difficult to be certain whether this reflects virus produced locally or simply secretion of virus into saliva from the blood.

The light microscopic findings in acute hepatitis A include features common to all forms of acute viral hepatitis, including inflammatory cell infiltration, hepatocellular necrosis, and liver cell regeneration. Studies in chimpanzees demonstrated that the early histological lesions of hepatitis A infection involve periportal liver cell necrosis with relative sparing of the perivenular area of the lobule (Dienstag et al. 1976). These histological observations have been confirmed in humans undergoing biopsies at different stages of the acute disease. Portal infiltration by lymphocytes, plasma cells and periodic acid–Schiff (PAS)-positive macrophages are prominent features in early biopsies. These features are more pronounced in later biopsies (Abe et al. 1982; Teixera et al. 1982). Some biopsies taken during acute hepatitis A infection demonstrate extension of the inflammatory infiltrate from the periportal region into the hepatic parenchyma, with significant erosion of the limiting plate. Although this histological picture is more suggestive of chronic hepatitis, the histopathological features of hepatitis A are reversible and generally resolve completely given sufficient time.

CELLULAR IMMUNE RESPONSE TO HAV INFECTION

Because infection of cultured cells with wildtype HAV does not result in the cytopathic effects observed with many other well characterized picornaviruses, it is generally assumed that HAV infection is also noncytopathic in vivo and that hepatitis A is an immunopathologically mediated disease. This is probably correct, as virus repli-

cation and fecal virus shedding are maximal in the late incubation period, before the onset of symptoms or biochemical evidence of liver injury. Thus, liver injury and viral clearance are closely related. $CD8^+$, MHC class I-dependent, cytotoxic T cells that are capable of lysing autologous HAV infected cells, but not of controlling uninfected cells, are present both in the circulation and in the liver at the site of disease (Vallbracht et al. 1986; Vallbracht et al. 1989; Fleischer et al. 1990). These virus-specific T cells also produce interferon-γ (Maier et al. 1988). It is likely that interferon-γ and other cytokines secreted by virus-specific $CD8^+$ cells serve to recruit additional, less specific, inflammatory cells to the site of the infection that may be responsible for much of the liver injury. The exact mechanism by which the infection is cleared is inadequately defined, but this process probably involves a combination of antibody-mediated neutralization of infection, interferon-mediated inhibition of viral replication, and cytotoxic T cell-induction of cellular apoptosis. A prominent feature of this response may be the upregulation of the normally low levels of MHC class I markers displayed by hepatocytes in response to interferon-γ and other cytokines. Specific T-cell epitopes of HAV have not been identified.

HUMORAL IMMUNE RESPONSE TO HAV

In addition to the cellular immune responses described above, there is a vigorous antibody response to the virus during the later stages of infection. These antibodies are directed against conformational epitopes that are displayed by intact virions as well as empty viral capsids. Serum-neutralizing antibodies provide protection against reinfection with HAV, whereas cell-mediated immunity seems to be involved in viral clearance once infection has been established. Serum antibody responses are first noted at the onset of symptoms and include virus-specific IgM as well as IgG and IgA. IgM anti-HAV is detected concurrently with the onset of clinical symptoms, but IgG is also present during the acute symptomatic phase of the infection (Locarnini et al. 1979; Lemon et al. 1980). Both IgM and IgG antibodies have virus-neutralizing activities (Lemon and Binn 1983b; Lemon 1985b). The IgG response is durable and provides longstanding protection against reinfection. However, there are suggestions that it may decline to low and potentially nondetectable levels after many decades, particularly in people infected early in life. Upon re-exposure, such individuals may experience asymptomatic reinfections associated with anamnestic IgG antibody responses in the absence of detectable IgM (Villarejos et al. 1982). Although coproantibodies have been detected by immunoassay, saliva and stool do not contain virus-specific neutralizing antibodies (Locarnini et al. 1980; Stapleton et al. 1991). These data suggest that secretory antibody plays little if any role in protection against hepatitis A.

Sensitive and specific immunoassays are widely available for the detection of total and IgM-specific antibodies to HAV. However, measurements of virus-neutralizing antibody are tedious and labor intensive. The radioimmunofocus reduction assay (Lemon and Binn 1983b) or HAV antigen reduction assay (Krah et al. 1991) are the most accurate methods available for determination of virus-neutralizing antibody. In addition to antibodies directed against the virus capsid, antibodies reactive with nonstructural proteins (especially $3C^{pro}$) have also been detected. The presence of such antibodies may differentiate immunity due to natural infection from that gained by immunization with inactivated HAV vaccine (Robertson et al. 1993; Stewart et al. 1997).

CLINICAL MANIFESTATIONS

HAV causes an acute, self-limiting infection that does not progress to a chronic phase. Manifestations of the disease are generally restricted to the liver. However, although extrahepatic manifestations suggesting involvement of the central nervous system or kidneys are rare, their occurrence is well documented (Bromberg et al. 1982; Hammond et al. 1982; Malbrain et al. 1994; Ogawa et al. 1994; Zikos et al. 1995). The typical clinical course of type A hepatitis is shown in Figure 5. Following exposure, an incubation period of 15–45 days precedes the development of clinical symptoms. However, virus is present in the blood and shed in the stools within a few days of exposure (Asher et al. 1995; Ward et al. 1958; Krugman et al. 1962; Bower et al. 2000). The magnitude of the viremia and the amount of virus shed in the feces continues to increase until just before the onset of symptoms. Symptoms typically occur abruptly, with liver injury heralded by fever, myalgia, malaise, nausea, anorexia, and vomiting, accompanied occasionally by abdominal pain in the right upper quadrant. More distinctive signs of hepatitis and the disruption of normal hepatobiliary metabolism appear rapidly thereafter, including the passage of dark, 'Coca-Cola'-like urine, light, clay-colored stools, and frank icterus. These clinical findings coincide with the appearance of characteristic biochemical abnormalities, including abnormal elevations of serum levels of the liver-derived enzymes alanine aminotransferase (ALT), alkaline phosphatase (AST), and γ-glutamyl transpeptidase (GGTP), as well as increased levels of serum bilirubin. All these events signal the host immune response to the infection, including the appearance of virus-specific neutralizing antibodies (Lemon and Binn 1983b). Resolution is marked by slow recovery and often a prolonged period of convalescence. Nevertheless, serum enzyme levels are almost always normal by 6–12 months after onset of the disease, often much sooner. Rarely, however, HAV infection may trigger the onset of a chronic, autoimmune hepatitis (Vento et al. 1991).

The severity of symptoms associated with HAV infection is closely correlated with age. Acute, symptomatic,

icteric infections seem to be the rule in older adolescents and adults (Lednar et al. 1985). On the other hand, infection in children under 2 years of age is almost never recognized clinically as hepatitis (Hadler et al. 1980). Most people with antibodies to HAV have no history of acute hepatitis, probably reflecting infection at an early age many years previously. Individuals over 50 years of age at the time of infection are at substantially increased risk of developing fulminant hepatitis, and clinical evidence of hepatic failure such as ascites, bleeding diathesis or hepatic coma (Forbes and Williams 1990; Hadler 1991; Lemon and Shapiro 1994). A systematic study has not been done, but patients with pre-existing chronic liver disease due to any cause may be at increased risk of fulminant disease when infected with HAV. Fortunately, fulminant hepatitis A is relatively rare, and accounts for fewer than 100 deaths annually in the USA (Lemon and Shapiro 1994).

Cholestatic hepatitis A and relapsing hepatitis A represent two less severe complications of type A hepatitis. Cholestatic hepatitis is characterized by persistent jaundice associated with pruritus, anorexia, and weight loss. Patients eventually recover after several weeks to months, but may recover more rapidly following a brief course of corticosteroids (Gordon et al. 1984). Such therapy should be attempted with caution, and only when there is no doubt about the diagnosis. There are many reports of clinical relapses 1–3 months after apparent recovery from acute hepatitis A. Relapsing hepatitis A is characterized by secondary rises in levels of serum enzyme, persistence of IgM anti-HAV, and possibly recurrent viremia and fecal virus shedding (Sjogren et al. 1987; Glikson et al. 1992). The pathogenesis of relapsing hepatitis A is unknown, but may involve viral escape from immune surveillance.

DIAGNOSIS

The diagnosis of acute viral hepatitis is usually suggested by the presence of the typical constellation of signs and symptoms associated with elevation of serum ALT activities. However, in the absence of a specific epidemiological setting suggestive of hepatitis A, the clinical manifestations of HAV infection are not sufficiently different from those of other types of acute viral hepatitis to allow a virus-specific diagnosis to be made without serological testing. Generally, the diagnosis is confirmed by the demonstration of IgM antibodies to the virus, which are almost always present at the onset of symptoms and which persist for up to 6 months following infection. These antibodies are usually measured by solid phase, IgM-capture immunoassays (Lemon et al. 1980; Decker et al. 1981). Competitive-inhibition immunoassays allow non-isotype-specific detection of viral antibodies (Mathiesen et al. 1978b; Decker et al. 1979). When such tests are positive in the absence of IgM antibodies, they are considered indicative of the presence of IgG antibodies to the virus. This result is consistent with immunity due to prior infection or immunization against HAV.

Epidemiology of hepatitis A

TRANSMISSION OF HEPATITIS A

Almost all transmission occurs by the fecal–oral route. Within the USA, the most common risk factor for infection is close contact with a person with hepatitis A (Centers for Disease Control 1994). Person-to-person transmission accounts for most infections among household contacts as well as institutionalized individuals and children and staff in preschool daycare centers (Hadler et al. 1980). Handlers of nonhuman primates may be at risk for similar reasons. Not surprisingly, sexual behavior can influence the spread of infection, particularly among male homosexuals (Corey and Holmes 1980). Food- and waterborne transmission of HAV is also common, facilitated by the stability of the HAV virion and its relative resistance to drying (Siegl et al. 1984). Foodborne transmission of HAV may follow contamination of uncooked foods by infected food handlers, and is not infrequently implicated in common source outbreaks of disease. However, fresh produce may also be contaminated at its source, before distribution (Rosenblum et al. 1990). Finally, infection may occur following ingestion of raw or partially cooked filter-feeding shellfish collected from polluted waters, as these organisms have the ability to concentrate the virus. All these modes of transmission pose risks for travelers to HAV-endemic regions.

Although uncommon, parenteral transmission of the virus is also possible. Injecting drug users have represented a substantial proportion of cases of hepatitis A in some studies, and there is little doubt that they are at increased risk of HAV infection (Widell et al. 1983; Centers for Disease Control 1988). These infections are probably related to sharing of contaminated needles and thus to parenteral transmission of the virus, given that a relatively high level viremia is present for several weeks throughout the asymptomatic incubation period of the hepatitis A (Lemon et al. 1990). This conclusion is strongly supported by remarkable concurrent declines in the incidence of drug use-associated cases of hepatitis A, hepatitis B, and hepatitis C in the USA since 1988. This would be unexpected if cases of hepatitis A among drug users related primarily to poor sanitation. Transmission of virus by blood and blood products is also well documented, although it is much less common than with other hepatitis agents. Many such infections have been recognized in neonates who may receive split units of blood (Rosenblum et al. 1991), but transfusion-related infections have also been observed rarely in adults (Hollinger et al. 1983; Sherertz et al. 1984). Parenterally transmitted infections have been described in hemophi-

liacs receiving high-purity, solvent-detergent inactivated factor VIII (Mannucci et al. 1994; Robertson et al. 1994b), and cancer patients who received IL-2 produced in LAK cells maintained in 'normal human serum' (Weisfuse et al. 1990). With the possible exception of infections among drug users, however, parenteral transmission of HAV does not contribute significantly to the overall spread of the infection.

PREVALENCE OF HEPATITIS A

The prevalence of HAV infection varies widely by geographical region and depends on factors such as population density and the quality of public health sanitation and sewage disposal (Lemon and Shapiro 1994). It may be greatly increased during periods of social upheaval and war. The poorest of developing countries are characterized by the type I seroprevalence curve shown in Figure 6. In these countries, including many in Africa, Asia, and Central America, infections occur predominantly in very young children and are thus generally clinically inapparent. As a result, most children have antibodies against HAV by the age of 5–10 years, and disease is very infrequent in older people. With improved sanitary conditions, as found in the emerging economies of eastern Europe, the republics of the former Soviet Union and China, HAV infections tend to occur at a somewhat older age, resulting in the type II seroprevalence curve in Figure 6. In these regions, the probability of exposure and infection remains relatively high, but an increase in the mean age at infection results in a greater disease burden as adolescents and young adults are more likely to experience clinical symptoms when infected (Szmuness et al. 1977; Frosner et al. 1979; Lemon 1985a). In recent decades, improving economic conditions have resulted in changing epidemiological patterns in many developing nations (Innis et al. 1991), with the result that epidemics of hepatitis A among older children, adolescents, and young adults, previously unheard of, are now becoming apparent. Older adults and the elderly are protected from infection by virtue of the antibody they carry from infections acquired in earlier, more endemic periods.

This trend continues in relatively well developed countries such as the USA and much of western Europe, where young children are no longer commonly infected (except in the setting of daycare centers), and the burden of disease is found among adolescents and young adults (Szmuness et al. 1977; Frosner et al. 1979; Lemon and Shapiro 1994). In these countries, symptomatic HAV infections in older individuals result in substantial absence from work and associated medical expenses. The major sources of infection in these populations are community-wide outbreaks, daycare centers, and common source outbreaks. Relatively high seroprevalence in the older age groups (Figure 6) signifies not continued risk of infection over time, but rather a cohort effect from infection in previous decades when HAV infections were more prevalent (Frosner et al. 1978). Finally, in highly developed regions such as the Scandinavian countries, seroprevalence tends to follow the type IV curve shown in Figure 6. There is little endemic transmission of the virus, and disease occurs primarily in travelers to other regions and in certain high risk groups such as intravenous drug users (Frosner et al. 1979; Siebke et al. 1982; Widell et al. 1983). Despite this convenient classification, it is important to note that prevalence patterns do not follow political as much as socioeconomic borders. For example, within the USA there are communities that would fit each of the type II, III, and IV patterns.

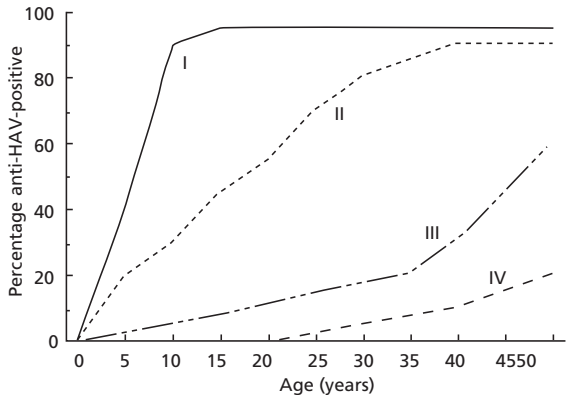

Figure 6 *Typical age-related seroprevalence curves found in populations residing in regions with high (I), intermediate (II) or low (III) endemicity for HAV infections, and in regions such as Scandinavia where endogenous transmission of HAV is now very uncommon (IV).*

Prevention of hepatitis A

To a considerable extent, hepatitis A can be prevented by good personal hygiene, adequate disposal and treatment of human waste, and provision of safe drinking water. As indicated above, HAV is rarely transmitted endemically in countries where these standards are met. However, specific measures are also available for the prevention of hepatitis A. These include passive immunoprophylaxis with pooled human immunoglobulin (IG) and active immunization with hepatitis A vaccines.

POOLED HUMAN IMMUNOGLOBULIN

Postexposure prophylaxis is generally accomplished with human IG (0.2 ml/kg), which should be given to people who are household members or sexual contacts of an infected individual (Gellis et al. 1945; Winokur and Stapleton 1992 Immunization Practices Advisory Committee 1990). Postexposure prophylaxis with IG may not prevent infection but it is remarkably effective in reducing or eliminating symptoms. It probably exerts

this effect by reducing the level of viremia, thereby reducing spread of virus within the liver (Lemon 1985a). To be optimally effective, it must be given within 2 weeks of exposure to the virus. IG is also useful for pre-exposure prophylaxis (0.2–0.5 ml/kg) and has been administered routinely to individuals traveling to HAV-endemic regions. If given before exposure, IG prevents disease for up to 4–6 months (Conrad and Lemon 1987). In this setting it is more likely to prevent infection as well as symptoms of hepatitis A, but this depends on the dose administered, the time elapsed between administration of IG and exposure to the virus, as well as the magnitude of the viral inoculum. Except for very limited travel, the use of inactivated hepatitis A vaccine is preferable and is largely replacing pre-exposure prophylaxis with IG.

HEPATITIS A VACCINES

Inactivated whole virus vaccines contain purified HAV particles (both complete virions and empty capsids) produced in cell culture and inactivated with formalin (Binn et al. 1986; Andre et al., 1990; Lewis et al. 1991). Several inactivated vaccines are licensed in their countries of origin and elsewhere, making inactivated HAV vaccine available worldwide. Most of these vaccines are adsorbed with alum (aluminium hydroxide) and all seem to be very immunogenic when administered by intramuscular injection. They are generally safe, and two have been shown in well designed clinical trials to provide a high level of protection against symptomatic hepatitis A in children (Werzberger et al. 1992; Innis et al. 1994; Lemon 1994). Antibody levels exceeding that produced by a single injection of IG are often present within 2–4 weeks of the first vaccine dose. Booster doses, given as early as one month following primary immunization, enhance the titer of neutralizing antibody and thus are likely to extend the duration of protection. Although the minimal protective level of antibody is not well defined, it is very low (<45 mIU/ml) (Stapleton et al. 1985; Werzberger et al. 1992). Although inactivated vaccines do not produce detectable secretory antibody, the prevention of intrahepatic virus replication is likely to prevent or substantially reduce the fecal shedding of virus. Thus, in contrast to inactivated poliovirus vaccines, immunization with inactivated hepatitis A vaccines should render an individual unable to transmit the infection if exposed to the virus.

The major shortcoming of inactivated HAV vaccines is that they are quite expensive and thus not available for the control of hepatitis A on a population-wide basis in countries where immunization is most needed. Although inactivated vaccines are likely to be more effective than IG in controlling community-wide outbreaks of disease (Werzberger et al. 1992; Lemon and Shapiro 1994; Prikazsky et al. 1994), the use of the vaccine in this setting is also likely to be limited by cost. Although data are limited, immunization may provide some level of protection against hepatitis A if it is administered immediately after exposure to the virus (Werzberger et al. 1992; Robertson et al. 1994a).

Several candidate attenuated HAV vaccines have been evaluated in clinical trials (Provost et al. 1986; Midthun et al. 1991; Sjogren et al. 1992). The virus strains included in these vaccines have been attenuated by passage in cell culture and require parenteral injection to initiate infection uniformly in human recipients. In general, the immune response to these candidate vaccines has been disappointing with slow, low magnitude antibody responses. This probably reflects the fact that these virus strains are highly restricted in their ability to replicate in human hosts. Although they seem quite safe in clinical trials involving limited numbers of people and are likely to be effective clinically, these candidate attenuated vaccines have generally been abandoned by commercial manufacturers in favor of the development of more profitable inactivated vaccines. However, one such vaccine has been used extensively in China (Mao et al. 1989, 1991).

A fresh approach to the selection of candidate attenuated HAV vaccine strains is likely to be required for the development of highly immunogenic attenuated vaccines. One such approach has been the creation of large deletion mutations within the 5′ NTR of a virulent, but minimally cell culture-adapted, variant of HAV (Shaffer et al. 1994). Some of these mutants have a strongly temperature-sensitive replication phenotype. However, there is no evidence thus far that these large 5′ NTR deletions result in significant attenuation of the virus in primates (Shaffer et al. 1995). A more promising alternative approach is to consider the use of simian strains of HAV, which seem to be naturally restricted in their ability to cause disease in chimpanzees (Purcell 1991), or virus chimeras containing selected sequences of human and simian HAVs. Recent data using chimeras derived from cell-adapted HM-175 (HAV/7) and the simian AGM-27 strain suggest that chimeras containing the HAV/7 background and the C terminus of the AGM-27 2C are attenuated for chimpanzees and may provide an alternative for development of an attenuated vaccine (Raychaudhuri et al. 1998).

Similarly, there has been little success in developing recombinant hepatitis A vaccines. This reflects the fact that the protective epitopes present on the surface of this picornavirus are conformationally defined by interactions between the individual capsid proteins in the assembled virus particle. However, Winokur and colleagues (1991) have demonstrated that it is possible to produce immunogenic, assembled viruslike capsids following expression of the P1 protein precursor and $3C^{pro}$ by recombinant vaccinia virus. Moreover, preliminary studies with synthetic peptide mimotopes (Mattioli et al. 1995) indicate that certain peptide motifs may successfully mimic the conformational, immuno-

dominant antigenic site of HAV, suggesting the ultimate feasibility of a synthetic peptide-based vaccine.

HEPATITIS E VIRUS

Classification

On the basis of the morphology of the hepatitis E virus (HEV) particle, the absence of a lipid envelope, the type and length of its nucleic acid and the general organization of its genome, HEV was originally classified within the *Caliciviridae* (Cubitt et al. 1995) (Table 3). However, a phylogenetic analysis of the nucleotide sequence of HEV shows that it is related more closely to the alphavirus superfamily of positive-strand RNA viruses, in particular the rubiviruses (Koonin et al. 1992) and it has subsequently been removed from the *Caliciviridae* and reassigned to an unclassified genus termed 'hepatitis E-like virus' (Berke and Matson 2000). Hepatitis E-like viruses have been identified in humans, swine, and chickens and all have similar genetic arrangements. The swine derived virus does not appear to cause any overt clinical symptoms in the pigs; in contrast, the avian HEV-like virus is associated with a hepatitis-splenomegaly syndrome in chickens within the United States. (Haqshenas et al. 2001)

Morphology and structure

As with many other features of HEV, the exact structure of the virion remains elusive owing to the absence of a reliable cell culture system that is permissive for its replication and to difficulties in obtaining large quantities of stable particles from human and nonhuman primate samples. The HEV particle is chloroform-resistant and has a morphology resembling that of human caliciviruses and Norwalk virus (Figure 7; and see Table 1). Although early reports suggested the diameter of HEV particles to be ca. 27 nm (Balayan et al. 1983), subsequent studies have shown the average diameter to be closer to 32 nm (Bradley et al. 1988). Smaller particles may result from proteolytic degradation as the virus passes through the intestine, as both particle sizes have been observed in single stool suspensions (Bradley et al. 1987). HEV-specific antibodies recognize both forms. The characteristic cup-like surface of caliciviruses is less prominent in HEV, but Markham rotational enhancement clearly indicates surface projections and valleys (Ticehurst 1991). Cryoelectron microscopy of a recombinant baculovirus expressed ORF2 product (amino acids 112–660) revealed spherical structures approximately 27 nm in diameter. These structures contained 60 copies of the recombinant ORF2, with a T = 1 lattice of icosahedral symmetry (Xing et al. 1999). However, X-ray diffraction data on the surface structure of the virus itself which is predicted to have a T = 3 lattice composed of 180 molecules is not available since sufficient amounts cannot be obtained from cell culture growth.

GENOME ORGANIZATION

The genome of HEV is a single-stranded, 7.5-kb, positive-sense RNA that is polyadenylated at its 3′ terminus (Reyes et al. 1990; Tam et al. 1991). It contains three separate ORFs flanked by short 5′ and 3′ NTRs of 27 and 68 nt, respectively (Figure 8). The presence of a methyltransferase motif within the nonstructural proteins encoded by the most 5′ ORF (ORF1) closest to the 5′ terminus is consistent with the presence of a methylated cap structure (Koonin et al. 1992) on the 5′ end of the RNA. Experimental studies have confirmed the presence of such a structure (Zhang et al. 2001b;

Table 3 *Properties of Hepatitis E virus, Calicivirus, and Rubivirus*

	Calicivirus	Hepatitis E virus	Rubivirus
Morphology	Icosahedral	Icosahedral	Spherical
Envelope	No	No	Yes
Size	35–39 nm	27–32 nm	60 nm
Bouyant density	1.33–1.39 g/cm^3	1.29 g/cm^3	1.2 g/cm^3 (?)
$S_{20,w}$	170–183S	183S	280S (?)
RNA genome:	ss (+) RNA	ss (+) RNA	ss (+) RNA
5′ structure	VPg	5′ methylated cap (?)	5′ methylated cap
3′ structure	Poly(A)	Poly(A)	Poly(A)
Length	8.2 kb	7.5 kb	9.8 kb
Subgenomic RNAs	2	2	1
Structural proteins	1 capsid protein	2 (?) (1 glycosylated)	1 capsid protein 2 glycoproteins
Nonstructural proteins	Picorna-like protease RDRP	Papain-like protease RDRP Methyltransferase	Papain-like protease RDRP Methyltransferase

RDRP, RNA-dependent RNA polymerase; ss (+), single-stranded, positive-sense.

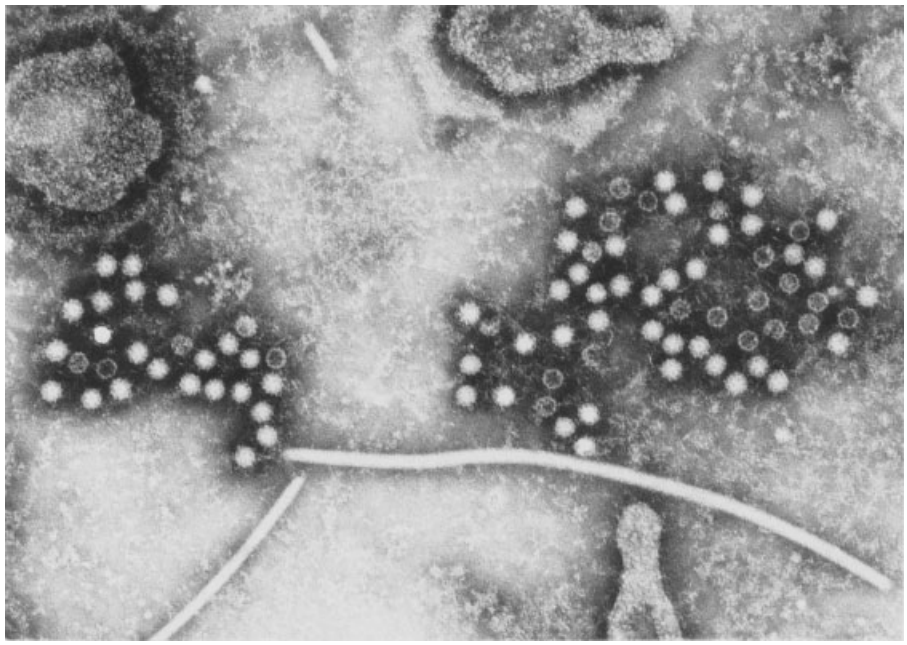

Figure 7 *Morphology of HEV particles demonstrated by electron microscopy.*

Kabrane-Lazizi et al. 1999a), identified the viral encoded capping enzyme (Magden et al. 2001) and verified that the capped structure is necessary for infectivity (Emerson et al. 2001). The ORF closest to the 3′ terminus (ORF2) is thought to encode the major structural protein. There is an additional short ORF (ORF3), which overlaps ORF1 by a single nucleotide and ORF2 by most of its length. ORF3 encodes a small protein that appears to be phosphorylated and is associated with the cytoskeleton (Zafrullah et al. 1997). The 5′ end of HEV RNA may contain a secondary structure analogous to that predicted to be present in alphaviruses. In addition, the sequence between 150 and 208 nt of HEV resembles a conserved 51 nt alphavirus sequence, whilst homology at 3602–3632 nt of HEV and junction sequences of alphaviruses suggests that this region may contain a promoter for transcription of a subgenomic RNA.

HEV PROTEINS

Most information about the proteins of HEV has been obtained from analysis of the nucleotide sequence of the virus. ORF1 encodes a relatively large polyprotein that contains consensus motifs for a methyltransferase, a papain-like cysteine protease, a helicase (NTP-binding site), and an RNA-dependent RNA polymerase similar to those found in the alphaviruses (Koonin et al. 1992).

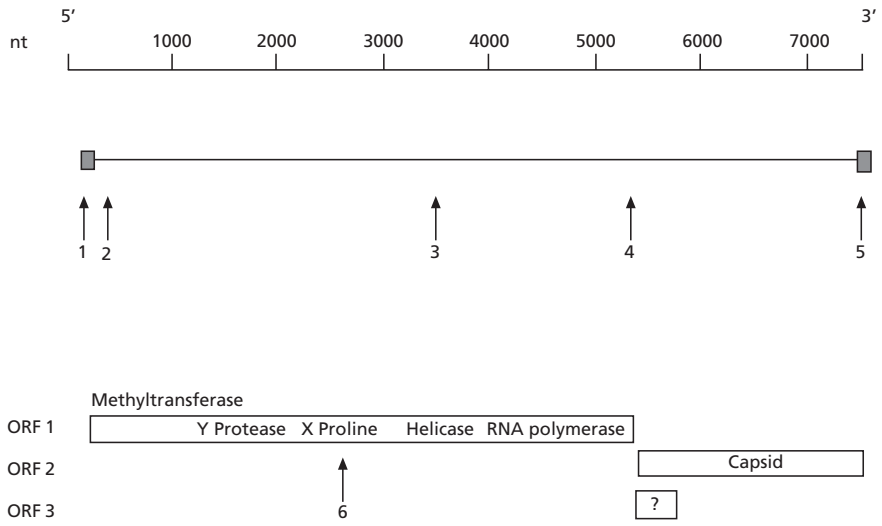

Figure 8 *Organization of the 7.5-kb single-stranded RNA genome of HEV.*

Also present in the ORF1 polyprotein is a polyproline 'hinge,' which is also found in rubiviruses, and a conserved 'X' domain, which is of unknown function but is found flanking the papain-like protease domain in other positive-strand RNA viruses. Although this polyprotein is almost certainly proteolytically cleaved into 4–5 individual polypeptides with distinct functions in replication, the details of this process are not understood and a preliminary study was unable to detect self-cleavage products using two different expression systems (Ropp et al. 2000). Several unusual features of this polyprotein distinguish HEV from members of the *Alphavirus* genus (Koonin et al. 1992). For example, a peculiar domain ('Y' domain) located downstream of the methyltransferase motif has no equivalent in the alphaviruses, although a similar domain is found in the plant virus, beet necrotic yellow vein virus. The function of this domain is unknown. Near the 'proline hinge' region, a 'hypervariable' region has also been noted whose function is not known (Fry et al. 1992, C.-C. Huang et al. 1992; Tsarev et al. 1992; Yin et al. 1993).

ORF2 encodes a 72 kDa capsid polypeptide and contains a signal peptide sequence and three potential glycosylation sites (Tam et al. 1991; Yarbough et al. 1991). The amino half of the ORF2 protein is rich in arginine residues, suggesting that it may associate with viral RNA. Cloning and expression of this product indicate that it is translocated in the endoplasmic reticulum, cleaved by signal peptidase and glycosylated (Jameel et al. 1996; Zafrullah et al. 1999). Subsequent studies have determined that ORF2 is present in both glycosylated and nonglycosylated forms but only the nonglycosylated form is stable in mammalian cells (Torresi et al. 1999). A portion of this molecule lacking the first 100 amino acids self assembles into viruslike particles (Li et al. 1997; Xing et al. 1999), and generates antibodies that are protective (Tsarev et al. 1994a; Zhang et al. 2001a). From an immunologic perspective, the ORF2 amino acids 452–617 are responsible for generating neutralizing antibodies that react with all four genotypes (Meng et al. 2001). An additional immunoreactive site is located at the amino terminus, but this site is not accessible using intact virions (Schofield et al. 2003). Although the ORF2 protein is found on the cell surface (Zafrullah et al. 1999), it is important to note that any surface lipid components of the virus would be unstable and almost certainly removed during passage of the virus through the bile.

Overlapping ORF1 and ORF2 is a small open reading frame (ORF3) that encodes a 13 kDa, cysteine-rich polypeptide that is immunoreactive with many patient sera. The genotype I ORF3 product has been shown to be a cytoskeleton-associated phosphoprotein (Zafrullah et al. 1997), with Ser^{80} serving as the phosphorylation site. Subsequent studies using a yeast two hybrid system suggests that the phosphorylated form of this protein associates with the nonglycosylated form of the ORF2 product and therefore may be involved in capsid assembly (Tyagi et al. 2002). However, the analogous product for genotype III does not contain a Ser^{80}, and large deletions have been described within this ORF in viruses recovered from two separate epidemics in India (Ray et al. 1992).

REPLICATION OF HEV

Although there are reports of HEV replication in conventional cell cultures (Huang et al. 1992; Kazachkov et al. 1992), they have not been confirmed. However, replication has been documented recently in primary cultures of hepatocytes taken from cynomolgus monkeys infected in vivo (Tam et al. 1996). The absence of a well characterized and readily available permissive cell culture system has severely limited studies of the molecular mechanisms of replication. The nonstructural proteins are presumably translated from newly uncoated RNA by a 5′ cap-dependent mechanism, providing the enzymes required for subsequent synthesis of both negative- and positive-strand RNAs. The 3′ end of the viral genome binds the viral RNA dependent RNA polymerase thereby providing a mechanism for viral RNA replication (Agrawal et al. 2001) while the ORF2 protein binds to the first 76 nt of the 5′ region of the viral RNA (Surjit et al. 2004). Two 3′ co-terminal, subgenomic messenger RNAs of 3.7 and 2.0 kb seem to be transcribed from the full-length negative-strand RNA, with the 2.0-kb message present in greater abundance than the 3.7-kb product (Tam et al. 1991). However, the role of these subgenomic RNAs in directing translation of the ORF2 and ORF3 proteins remains unclear. There is no understanding of how these processes are regulated.

GENETIC AND ANTIGENIC VARIANTS OF HEV

HEV strains seem to comprise a single serotype of antigenically related viruses, as all well-characterized strains share cross-reacting epitopes. Several studies have examined the reactivity of acute or convalescent sera with synthetic peptides or recombinant proteins expressed from ORF2 and ORF3 of different HEV strains (Yarbough et al. 1991; Kaur et al. 1992; Khudyakov et al. 1993; Li et al. 1994). Antigenic epitopes have been identified at the carboxy termini of both ORF2 and ORF3 protein products. The ORF3 epitope seems to differ between eastern and western hemisphere strains (Yarbough et al. 1991), however, the biological relevance of this finding is unclear. Multiple studies have identified that the carboxy terminal portion of ORF2 (approximately amino acids 270–660) is responsible for generating neutralizing antibodies, and that recombinant constructs containing these amino acids self assemble into viruslike particles (Li et al. 1997; Xing et al. 1999). Riddell et al. (2000), were the first to identify amino

acids 394–457 as part of the immunodominant capsid epitope using mouse monoclonal antibodies. Subsequently, Meng et al. (2001) used a PCR-based in vitro neutralization assay to evaluate the ability of synthetic peptides to inhibit binding of virus to PLC/PRF/5 cells and identified amino acids 452–617 as part of the neutralization epitope. A subsequent study characterized monoclonal antibodies derived from a chimpanzee experimentally infected with a construct including amino acids 112–607 (Schofield et al. 2003). Two antigenic reactive sites were identified in this study. One site, associated with neutralization, was localized to amino acids 452–617, while the second site was located between amino acids 112 and 150.

Despite the homogeneous antigenic reactivity, this group of viruses has a wide range of genetic diversity. Four distinct genotypes can be defined based upon complete genome sequences. Up to nine genotypes have been proposed based upon limited sequencing of 287 nucleotides within the OFR1 region (Schlauder and Mushahwar 2001) although this region has not been demonstrated to be representative of the complete genome. Genotype I contains human HEV strains recovered from endemic regions in the Far East and Central Asia – India, Myanmar, Nepal, Pakistan, China, Uzbekistan (Arankalle et al. 1999; Tam et al. 1991; Nakai et al. 2001; Gouvea et al. 1997; Shrestha et al. 2003; van Cuyck-Gandre et al. 2000; Li et al. 2002; Chatterjee et al. 1997) and African nations – Namibia, Algeria, Morocco, Chad, Egypt, and Tunisia (He et al. 2000; van Cuyck-Gandre et al. 1997; Meng et al. 1999; Tsarev et al. 1999; Chatterjee et al. 1997). These strains form a monophyletic cluster with individual branches being much more closely related genetically to each other (about 90 percent identity) than to strains representing viruses from other parts of the world. Genotype II is represented by a single sequence from Mexico (C.-C. Huang et al. 1992), although limited sequence of strains from Nigeria also appear to belong to genotype II (Buisson et al. 2000). Genotype III has been identified from human and swine sources in the United States, Japan, and the The Netherlands (Schlauder et al. 1998; Meng et al. 1997; Takahashi et al. 2001, 2003a, b; Okamoto et al. 2001; van der Poel et al. 2001; Widdowson et al. 2003); sporadic human cases in Greece, Spain, Italy, and the United Kingdom (Schlauder et al. 1999; Pina et al. 2000; Wang et al. 2001) and in swine populations in India and New Zealand (Arankalle et al. 2002, 2003; Garkavenko et al. 2001). Genotype IV strains have been characterized from human and swine sources in Taiwan, Japan, and China (Hsieh et al., 1998, 1999; Nishizawa et al. 2003; Takahashi et al. 2001, 2003a, b; Li et al. 2002; Wang et al. 2002), while human cases with this genotype have been identified in Vietnam (Hijikata et al. 2002). The tight genetic clustering observed within genotype I is not seen in the available sequences from these other strains.

Many of these sequences are derived from swine sources, and it has become obvious that there is a wide array of swine HEV sequences all over the world. However, the direct relationship between swine HEV and human disease is not obvious. Individuals with occupational exposures to swine do have a higher seroprevalence of antibodies against HEV, but this does not reflect disease (Drobeniuc et al. 2001; Meng et al. 2002). Other animal reservoirs including rats, mice, cows, sheep, goats, dogs, cats, wild boar, and deer have also been proposed based upon seroprevalence studies or virological studies (Balayan et al. 1990; Favorov et al. 1996, 2000; Kabrane-Lazizi et al., 1999b; Wang et al. 2002; Matsuda et al. 2003; Tei et al. 2003). In addition, avian HEV strains genetically related to swine and human HEV, have been characterized from chickens with hepatitis – splenomegaly syndrome in the United States (Haqshenas et al. 2001) and Australian big liver and spleen disease virus (Payne et al. 1999; Haqshenas et al. 2001).

Clinical and pathological aspects of HEV infection

PATHOGENESIS OF HEPATITIS E

Chimpanzees and several species of monkeys are susceptible to HEV infection and have served as experimental models of hepatitis E in humans (Bradley et al. 1987; Ticehurst et al. 1992; Tsarev et al. 1993a). In addition, swine, rats, and possibly other mammalian species may support replication of the virus as discussed above. The most reliable experimental animal models have been the rhesus and cynomolgus macaques, and much of our understanding of the pathogenesis of this disease is derived from experimental infections of these animals. Following intravenous inoculation of cynomolgus macaques, there is an incubation period of 2–6 weeks, which precedes the development of raised liver enzymes (Figure 9). During this period there are viremia and fecal excretion of virus, reflecting replication of virus within hepatocytes. Antibodies to HEV develop just before elevations of liver enzymes, coincident with resolution of the viremia and fecal virus shedding and reductions in the quantity of viral antigen present in the liver. A biphasic pattern of liver disease has been observed in some animals, the initial period of enzyme elevation occurring within the first 2 weeks after inoculation. Pathological changes in the liver are consistent with acute viral hepatitis (Longer et al. 1993), but not specific for any etiological agent, and include hepatocyte necrosis and inflammatory cell infiltration. In general, the overall pattern and severity of disease do not seem to be as dramatic as in humans. Although limited evidence supports the theory of replication of the virus within the gastrointestinal tract of swine, an inoculum containing $10^{6.8}$ infectious units by intravenous inocula-

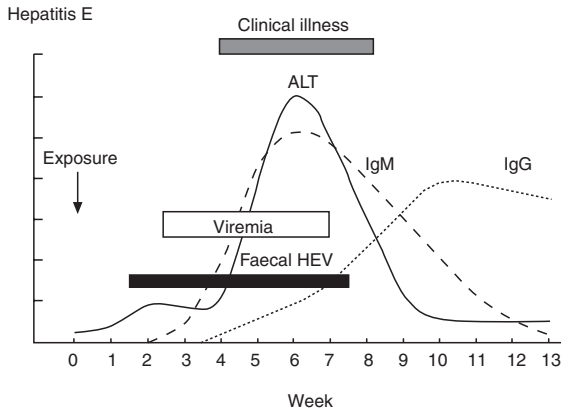

Figure 9 *Typical course of virological and pathological events during infection with HEV.*

tion failed to initiate infection when administered orally to macaques (Tsarev et al. 1994b).

There are few data about virological events in infected humans. In one of two reported human volunteer infections, raised liver enzymes and jaundice persisted for 3 months (Chauhan et al. 1993). Viremia was first detected at day 22, and terminated at day 46 when liver enzymes reached their peak value. Antibodies against HEV were first detected at day 41; their appearance was followed by declining levels of liver enzyme. Excretion of virus in stool samples was detected by RT-PCR between 30 and 50 days after ingestion. Disease was limited to a 1 month period in the other reported experimental human infection (Balayan et al. 1983). Examination of liver specimens from patients involved in outbreaks of disease attributed to HEV in Delhi, the Kashmir and (putatively) Ghana have revealed two general types of histopathological change (Gupta and Smetana 1955; Morrow et al. 1968; Khuroo 1980). A cholestatic pattern is characterized by prominent bile stasis within canaliculi and a unique glandlike transformation of parenchymal cells. There is portal and, to a lesser extent, parenchymal infiltration of lymphocytes and polymorphonuclear leucocytes. A second histopathological pattern is similar to that found in other types of acute hepatitis, and includes ballooning degeneration of hepatocytes and formation of acidophilic bodies.

IMMUNE RESPONSE TO HEV INFECTION

Antibodies to HEV develop during convalescence from the disease and are reactive with both linear and conformational epitopes of the virus. A wide variety of assays has been developed for detection of these antibodies, including various ELISAs using recombinant proteins (Goldsmith et al. 1992; He et al. 1993; Tsarev et al. 1993b) or combinations of peptides and recombinant antigens (Sallie et al. 1992; Paul et al. 1994) as antigen synthetic peptides (Coursaget et al. 1994; Favorov et al. 1994). In addition, immunoblot assays have been developed using recombinant antigens (Favorov et al. 1992; Li et al. 1994). These assays seem to vary considerably in their sensitivity and specificity, with relatively low overall concordance between results in a recent collaborative study. Synthetic peptides representing linear epitopes within the protein products of all three ORFs seem to react with serum from infected individuals (Kaur et al. 1992; Favorov et al. 1994), but peptide-based assays performed poorly over all against a reference panel of anti-HEV serum. A baculovirus-expressed, nearly complete ORF2 product forms viruslike particles and is a superior antigen because it displays conformation-dependent epitopes (Tsarev et al. 1993b). The early antibody response to this antigen includes both IgM and IgG antibodies to the virus (Tsarev et al. 1993b, 1994a), although the IgM response is variable. Antibody to the ORF2 protein was detectable in immunoblots for more than 12 months after infection, whereas antibody to ORF3 disappeared following resolution of the disease (Li et al. 1994). Nothing is known of the cellular immune responses, and there is controversy regarding the longevity of natural immunity after infection with HEV (Bryan et al. 1994; Arankalle et al. 1995). A number of early studies (Paul et al. 1994; Dawson et al. 1992; Mast et al. 1997) suggested that a measurable proportion of the population within nonendemic regions had evidence of antibodies to HEV (1.2–13 percent). Subsequent seroprevalence studies have revealed that individuals who are exposed to pigs in an occupational setting (farm workers, veterinarians) are more likely to be anti-HEV positive than a control population unexposed to pigs (Drobeniuc et al. 2001; Meng et al. 2002); likewise individuals who have increased exposure to rats have increased seropositivity against HEV (Smith et al. 2002).

CLINICAL MANIFESTATIONS OF HEPATITIS E

HEV, like HAV, causes an acute, self-limited disease that does not progress to a chronic phase. As shown in Figure 9, the incubation period is somewhat longer than that for HAV, averaging ca. 40 days. The prodromal symptoms of infection are similar to hepatitis A, including malaise, anorexia, and abdominal discomfort. A 15–20 percent case-fatality rate has been observed among women who become infected during the third trimester of pregnancy (Khuroo et al. 1981), although the clinical features of fatal disease are not well described. Fulminant disease also occurs in other settings. About 60 percent of sporadic cases of fulminant hepatitis in India are due to HEV infection (Nanda et al. 1994). The much less frequent involvement of HEV in fulminant hepatitis in developed countries (Liang et al. 1993; Sallie et al. 1994) reflects the rarity of the infection in these regions.

DIAGNOSIS

Serological diagnosis of acute infection is relatively straightforward with the assays currently available.

Detection of IgG anti-HEV in the absence of markers of other types of acute viral hepatitis is generally indicative of HEV infection. Tests for IgM antibodies have marginal sensitivity and are therefore of limited usefulness. In the past, lack of standardization and poor concordance between peptide based and recombinant ORF2 based assays for IgG anti-HEV severely limited their usefulness in population-based studies and epidemiological investigations. However, it is currently accepted that assays using the recombinant ORF2 antigen most clearly indicate evidence of infection in humans and other species.

Epidemiology of HEV

The earliest documented outbreak of hepatitis E occurred in Delhi, India, in 1955, after heavy flooding of the Yamuna River (Viswanathan 1957). Subsequent outbreaks of disease and sporadic cases of hepatitis E have been confirmed in a wide variety of tropical and developing regions, including India, China, Nepal, Pakistan, Myanmar, Indonesia, the Central Asian region of the former Soviet Union, Egypt, Ethiopia, Jordan, Algeria, the Ivory Coast, Chad, Sudan, Somalia, Ethiopia, and Mexico. In most outbreaks, contaminated drinking water has been incriminated as the source of infection. However, sporadic cases of hepatitis E also occur in these regions (Hyams et al. 1992a, b; Bile et al. 1994), suggesting that the virus is endemic. Clinically apparent hepatitis E generally occurs in individuals 25–40 years of age (Bradley 1992; Jameel et al. 1992), and the mean age at infection with HEV seems to differ markedly from the mean age of infection with HAV in regions that are endemic for both viruses. Surveys of sera collected over an interval of 10 years from an HEV-endemic region of India demonstrated that most HAV infections occurred before the age of 5 years, whereas HEV infections generally occurred after the age of 16 years (Arankalle et al. 1995). The reason for this difference is not known; serologically determined rates of infection are similar in children and adults during disease outbreaks, although adults are more likely to develop clinical evidence of hepatitis (Mast et al. 1993). Hepatitis E is an extraordinarily rare disease in the USA and Europe, although seroprevalence studies with currently available assays indicate that limited exposure to HEV or a closely related virus may indeed occur. In these regions of the world, almost all reported cases have been in travelers who have recently returned from endemic regions.

The apparent low level, endemic circulation of the virus in some regions contrasts with the fact that person-to-person transmission of HEV is unusual during epidemics (Mast et al. 1993; Aggarwal and Naik 1994). The recent data indicating markers of HEV infection in multiple animal species (pigs, deer, boar, rats, macaques) suggests that this family of viruses is ubiquitous in many parts of the world. This has led to speculation that HEV may represent a zoonotic pathogen, confined to its ecological niche until adverse circumstances, such as flooding, result in fecal contamination of the water supply (Bile et al. 1994). Another possibility is that certain individuals may shed virus for extended periods and thereby serve as reservoirs of infection (Nanda et al. 1995).

Control of hepatitis E

As with HAV, primary prevention of HEV infection rests with the provision of safe drinking water and the sanitary disposal of human waste (Bile et al. 1994), but most cases of hepatitis E occur in regions of the world where these conditions are difficult to achieve. Data concerning the protective efficacy of IG are limited and difficult to interpret (Joshi et al. 1985; Khuroo and Dar 1992). However, it is likely that anti-HEV is not present in significant titers in IG prepared from plasma collected in well developed, industrialized countries. Passive immunoprophylaxis of nonhuman primates with hyperimmune globulin has prevented biochemical and histological evidence of hepatitis although it did not prevent infection (Tsarev et al. 1994a). Both viremia and fecal virus excretion were documented in animals receiving hyperimmune globulin.

Because HEV cannot be propagated efficiently in cell culture, vaccine development has focused on recombinant immunogens. Preliminary studies suggested that immunization of cynomolgus monkeys with a recombinant, partial ORF2 protein product may have provided protection against disease, but not virus replication following challenge with a heterologous inoculum (Purdy et al. 1993). Subsequent studies have revealed that the partial recombinant ORF2 product with a molecular weight of 53 kd provides protection against disease (Tsarev et al. 1994a; Zhang et al. 2001a, 2002; Purcell et al. 2003), but not necessarily infection (depending upon the titer of the challenge inoculum) (Tsarev et al. 1997). These data are encouraging for the development of a vaccine.

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Hepatitis C virus

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INTRODUCTION

Since its discovery 15 years ago (Choo et al. 1989; Kuo et al. 1989), hepatitis C virus (HCV) has been the subject of intense research and clinical investigations as its major role in human disease emerged. Globally, HCV is estimated to infect 170 million people, 3 percent of the world's population, and creates a huge disease burden from chronic progressive liver disease. HCV has become a major cause of liver cancer and one of the most common indication for liver transplantation (reviewed in Pawlotsky (2003b), Hoofnagle (2002), and Seeff (2002)). HCV infection can be treated, but currently requires long-term medical support and follow up; current therapies are impractical for the majority of HCV carriers worldwide. The development of a protective vaccine remains a distant prospect.

Discovery of HCV

The discovery of HCV in 1989 was the dramatic end to a major research endeavor to find the causes of post-transfusion hepatitis. In the 1970s, several groups produced evidence for an infectious agent, distinct from the hepatitis A virus (HAV) and hepatitis B virus (HBV), that caused chronic hepatitis and was frequently transmitted by blood and blood products (Prince et al. 1974; Feinstone et al. 1975; Alter et al. 1975). Typically, non-A, non-B hepatitis (NANBH) was a mild disease that caused jaundice in fewer than half of cases, and was frequently asymptomatic. In its clinical features, it differed in several respects from acute HAV and HBV infection with a longer incubation period from exposure to liver function abnormalities, around 8 weeks, than is usually observed for HAV (4 weeks), but shorter than that for HBV (12 weeks). There was a high rate of chronicity following acute infection (at least 50 percent of cases, compared with around 5 percent for adult HBV infection). While chronic infection was almost invariably asymptomatic at first, a large proportion of cases eventually progressed to cirrhosis.

Progress towards identifying the cause of NANBH was hampered by the difficulty in culturing the agent in cell or organ culture, and the eventual characterization of the infectious agent of NANBH was made possible after the successful transmission of the agent into chimpanzees. Experimental data were then obtained that the agent passed through a 80-nm filter and was inactivated by lipid solvents such as chloroform; together these data suggested a small enveloped virus. Examination of liver biopsies from patients with NANBH and of experimentally infected chimpanzees helped to define the histologic features of acute and chronic disease and to differentiate it from other causes of hepatitis. Passaging and titration of the NANBH agent in chimpanzees eventually provided high titer stocks of virus that were necessary for the eventual cloning and sequence analysis of the infectious agent (Bradley et al. 1979; Bradley and Maynard 1986).

Nucleic acid (both DNA and RNA) was extracted following ultracentrifugation from a large volume of a

plasma sample with a high infectivity titer in chimpanzees (approximately 10^6 infectious units/ml). The nucleic acid was reverse-transcribed and the resulting DNA fragments cloned into the λ-gt11 bacteriophage. The DNA sequences in the resulting library were expressed as fusion proteins during replication of the bacteriophage in *Escherichia coli*. Immunoscreening of large numbers of bacteriophage plaques with antisera from patients with NANBH led to the eventual identification of an immunoreactive clone (designated 5-1-1) that appeared to be specific for the NANBH agent (Choo et al. 1989). This clone showed no similarity with any human or *E. coli* genomic sequences and produced a protein that was consistently recognized by sera from patients with NANBH, but not by sera from control individuals, or those with hepatitis of other etiologies (Kuo et al. 1989).

The 5-1-1 clone was then used as a probe to identify overlapping sequences in the λgt-11 library and this led to the assembly of a nearly complete nucleotide sequence of the NANBH agent (Choo et al. 1991). Knowledge of the length, nucleic acid composition, and genome organization of HCV has led to its classification in the *Flaviviridae* family (see Relationship of HCV to other flaviviruses) and its method of replication (see Replication of the HCV genome). From the sequence of HCV it was possible to design hybridization probes and primers for amplification of the HCV genome by polymerase chain reaction (PCR) that can be used to detect HCV RNA in sera and liver biopsies from hepatitis patients (see Diagnostic tests for HCV infection). The original 5-1-1 clone, together with an overlapping clone, c100-3, and more recently with clones from other parts of the genome, has been used to manufacture recombinant proteins for assays for antibody to HCV. This has enabled effective screening of blood donors for HCV infection to be adopted worldwide.

RELATIONSHIP OF HCV TO OTHER VIRUSES

Relationship to positive-strand RNA viruses

The sequence of the HCV genome shares several features with other positive-strand RNA viruses. Most striking is the existence of a single continuous open reading frame that occupies almost the entire genome. After translation, this is cleaved into individual proteins that are enzymes necessary for virus replication or are structural components of the virion (Figure 1). In overall genome organization and likely method of replication, HCV is most similar to members of the *Flaviviridae*, and indeed the roles of the different proteins encoded by HCV have been inferred by comparison with homologues in other flaviviruses. Although there is still no suitable method developed for the in vitro culture of HCV, expression of cloned HCV sequences in prokaryotic and eukaryotic systems, and the recently developed replicon has also allowed the replication functions of several nonstructural proteins of HCV to be identified (Figure 1) (described in detail below in the section Nonstructural proteins).

Other members of the *Flaviviridae* have many features in common with HCV. They have a similar genome size to HCV (YFV: 10 862 bases (Rice et al. 1985), compared with 9 379 of HCV (Choo et al. 1991)), and the virus envelope contains a viral encoded glycoprotein (E1). However, the homologue of HCV E2 in flaviviruses (also a membrane-bound glycoprotein: NS1) is not incorporated into the virion, being expressed only on the infected cell surface. Like HCV, the polyprotein of flaviviruses is cleaved by a combination of viral and host-cell proteases. Although there is no close sequence similarity between HCV and other flaviviruses, there are at least two regions with conserved amino acid residues that demonstrate enzymatic functions. The NS5B protein of HCV contains the GDD (glycine–aspartate–aspartate) motif that forms part of the active site in virus RNA-dependent RNA polymerases (RdRp) of positive-strand RNA viruses (Miller and Purcell 1990; Koonin 1991). Similarly, the NS3 protein contains domains associated with helicase and serine protease activity (Miller and Purcell 1990) (see Nonstructural proteins).

Shared features of HCV with pestiviruses

An important difference between members of the *Flaviviridae* is the structure of the 5′ and 3′ untranslated regions (UTR). These parts of the genome are involved in replication (see below), and in the initiation of translation of the virus genome by cellular ribosomes. Both pestiviruses and HCV have highly structured 5′ UTRs, in which internal base-pairing produces a complex set of stem–loop structures that interact with various host-cell and virus proteins during replication and translation (Smith et al. 1995; Tsukiyama Kohara et al. 1992; Wang et al. 1994; Poole et al. 1995). In both viruses, there is evidence for internal initiation of translation, in which binding to the host-cell ribosome directs translation to an internal methionine (AUG) codon (see HCV translation). This contrasts strongly with translation of flavivirus genomes which act much like cellular mRNAs, in which ribosomal binding occurs at the capped 5′ end of the RNA, followed by scanning of the sequence in the 5′ to 3′ direction with translation commencing at the first AUG codon.

Structurally, HCV is also more similar to the pestiviruses than flaviviruses, with a low buoyant density in sucrose (see Physicochemical properties), similar to that reported for pestiviruses and attributable in both cases

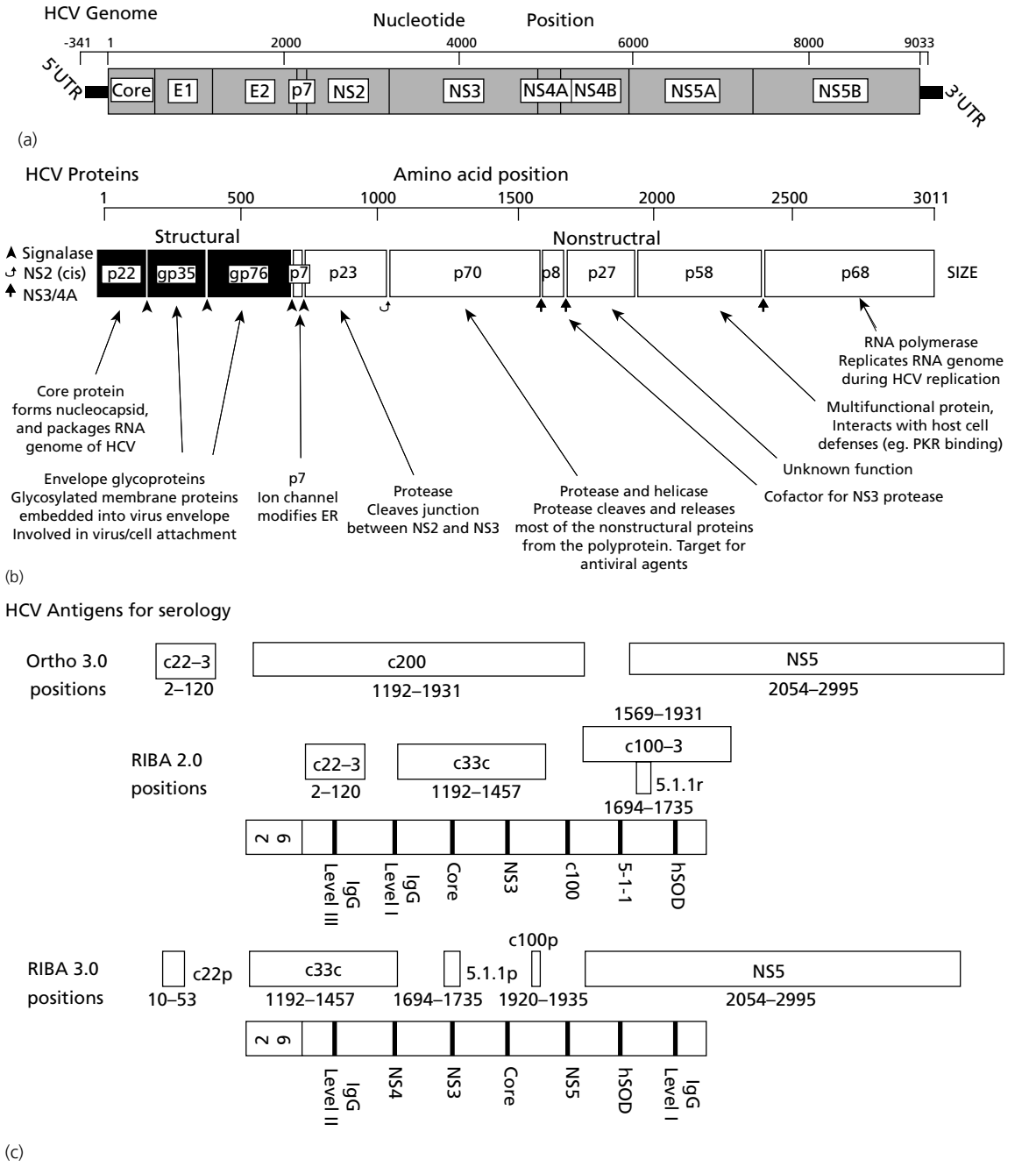

Figure 1 *Organization of HCV genome.* **(a)** *The coding sequences from structural and nonstructural proteins, and the 5′ and 3′ untranslated regions.* **(b)** *The sizes and summaries of functional properties of the mature proteins cleaved from the translated polyprotein, with the positions and enzymes responsible for the cleavage reaction indicated.* **(c)** *Regions of the genome expressed as recombinant antigens for serological assays. Nucleotide and amino acid positions numbered as in Choo et al. (1991).*

to extensively glycosylated proteins in the virus envelope. By contrast, flavivirus envelope glycoproteins contain few sites for *N*-linked glycosylation, and the virion itself is relatively dense (1.2 g/cm^3). Finally, the arrangement and number of cleavage sites of the HCV polyprotein is more similar to pestiviruses, particularly in the cleavage of NS5 into two subunits, with NS5B corresponding to the RNA polymerase.

Relationship of HCV to other flaviviruses

Shortly after the discovery of HCV, a number of new flaviviruses was found to infect humans, old and new world primate species. The most closely related virus to HCV was GB virus B (GBV-B), found to cause acute, resolving infections in captive tamarins (*Sanguinis* spp.),

which had been inoculated with a transmissible agent originally passaged from a plasma sample from a surgeon who had developed a hepatitis of unknown etiology (Simons et al. 1995). Remarkably, tamarins exposed to this in vitro passaged material were also found to be infected with a second, largely unrelated flavivirus, described as GB virus A (GBV-A). While a range of wild caught South American primate species show frequent evidence for infection with species-specific variants of GBV-A (Leary et al. 1997; Erker et al. 1998; Bukh and Apgar 1997), current surveys had not provided clear evidence for either active or past infection with GBV-B in New World primates, and its origin in the passaged material is therefore currently unclear. What has become apparent is that neither virus infected the human whose plasma originated the passaged material (both GBV-A and GBV-B were probably picked up at some point during passage through a number of different New World primate species), and they therefore do not represent human viruses. However, with knowledge of the sequences of GBV-A, it has proved possible to detect viruses distantly related to GBV-A found in humans and chimpanzees; two groups independently but simultaneously discovered in 1995 the human homolog, described as hepatitis G virus (HGV) or GB virus-C (Leary et al. 1996; Linnen et al. 1996). Shortly afterwards, a virus related to HGV/GBV-C was found to be widely distributed in chimpanzees (Adams et al. 1998; Birkenmeyer et al. 1998).

The evolutionary relatedness of HCV, GBV-B, GBV-A, HGV/GBV-C, and other flaviviruses can be shown by comparison of a region of the highly conserved RdRp (Figure 2). Flaviviruses fall into four distinct genetic groups, corresponding to (1) HCV; (2) pestiviruses; (3) an expanded group of tick and mosquito-borne viruses and nonvector-transmitted viruses infecting rodents; and finally (4) HGV/GBV-C and GBV-A. The affiliation of GBV-B remains somewhat uncertain, although most methods of phylogenetic analysis place it within the HCV group, and it resembles HCV more closely than other flaviviruses in genome structure. Using both phylogenetic information and comparisons of genome organization, the International Committee on the Taxonomy of Viruses (ICTV) has now formally classified these genetic groups into three named genera, the flaviviruses, the pestiviruses, and the hepaciviruses (containing HCV and GBV-B), and a fourth, currently unnamed and provisional genus that contains HGV/GBV-C, GBV-A, and other primate homologues (Thiel et al. 2000).

PROPERTIES OF THE VIRUS

Genome

The genome of HCV is single-stranded RNA of positive (protein coding) polarity. As with other positive-strand

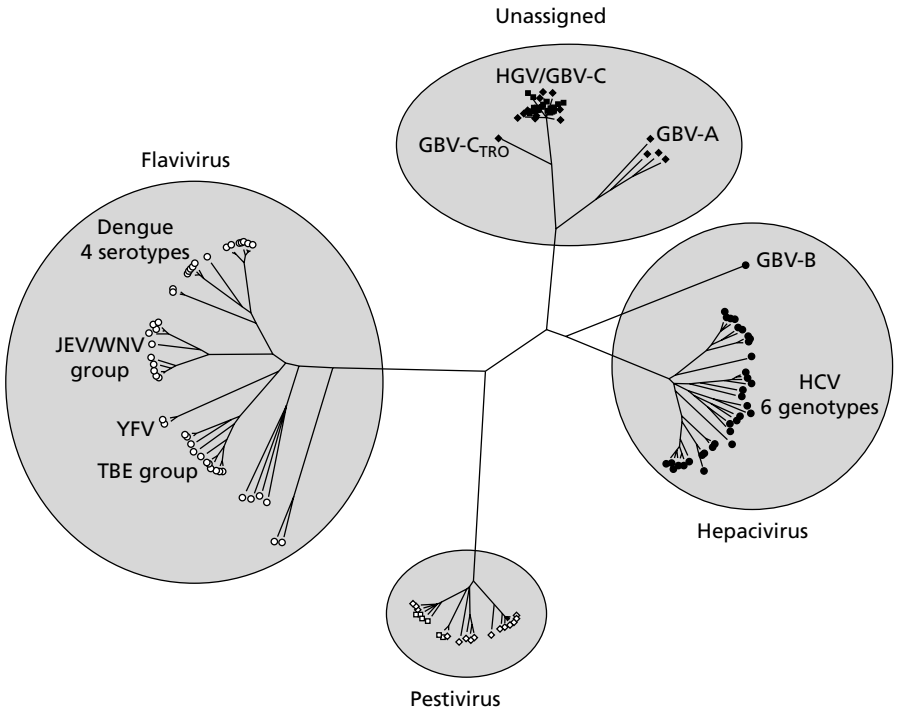

Figure 2 *Genera within the family* Flaviviridae. *Phylogenetic analysis of a conserved region of the RNA polymerase gene of flaviviruses reveals four well-defined genetic groups. These correspond to three of the current classified genera of flaviviruses, while the fourth group contains the currently unassigned HGV/GBV-C viruses and homologues in New World primates.*

RNA viruses, genomic RNA of HCV is infectious on experimental inoculation into chimpanzees (Yanagi et al. 1997). More than 98 percent of the HCV genome codes for virus proteins (9 033 of 9 397 bases of the HCV-PT genome (Choo et al. 1991)), with noncoding regions at both 5′ and 3′ ends. The 5′ UTR is 341–344 bases long, with no evidence for chemical modification of the ends, such as capping with a methyl^{7}guanosine residue as found in members of the genus *Flavivirus* and in eukaryotic mRNAs.

The 3′ untranslated region contains three domains, 25–30 poorly conserved nucleotides. a homopolymeric region comprising mainly uridine/cytosine residues, and finally, a highly conserved 'X-region' that forms a stable RNA structure comprising three juxtaposed stem–loops. There is no evidence for any covalent modifications to the genomic RNA of HCV.

Morphology

HCV remains difficult to visualize by electron microscopy. Without an efficient in vitro culture system for HCV, most attempts at visualization have used negative-staining of HCV of high titer plasma (Figure 3). In the example shown (Kaito et al. 1994), HCV forms viruslike particles of diameter 55–65 nm (Figure 3) with 6-nm spikelike projections that may correspond to the envelope glycoproteins on the virion surface. The envelope glycoproteins of HCV show several potential sites for *N*-linked glycosylation (four sites in E1, 11 sites in E2) and it is likely that the addition of relatively large carbohydrate groups contribute structurally to the outer surface of the virion, and influences the ability of antibody to neutralize infectivity.

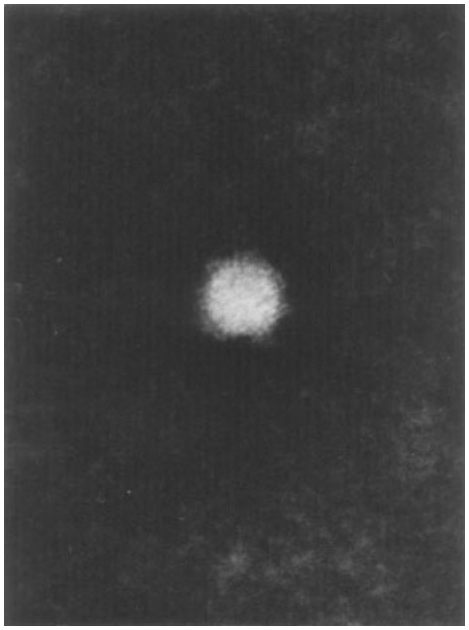

Figure 3 *Morphology of HCV virions. Visualization of an isolated virus particle of HCV by surface staining using 2 percent phosphotungstic acid, pH 6.5 (Kaito et al. 1994). Fine spikelike projections can be seen around the edge of the virus particle of approximate measured diameter of 60 nm. This figure was kindly provided by the authors.*

Physicochemical properties

The density of HCV is difficult to measure accurately because of its binding to various factors in plasma (the only available source of virion particles to study). For example, it associates to a variable extent with β-lipoprotein that reduces its density (Bradley et al. 1991; Thomssen et al. 1992). In samples from seropositive individuals, it may also be immune complexed with antibody creating a second density band of up to 1.20–1.25 g/cm^3 (Bradley et al. 1991; Miyamoto et al. 1992; Hijikata et al. 1993b; Carrick et al. 1992).

HCV is inactivated by exposure to chloroform, ether, and other organic solvents and by detergents. It is inactivated by dry heat treatment at 80°C or wet-heat treatment at 60°C, organic solvents (n-heptane) and detergents used in the manufacture of plasma-derived blood products (Mannucci 1993).

REPLICATION OF HCV

Culture of HCV in vitro

Despite considerable efforts, little progress has yet been made towards the development of a system for the culture of HCV in vitro. HCV does not produce obvious cytopathology, and the amounts of HCV released from cells infected in vitro are often low (Shimizu et al. 1992, 1993; Carloni et al. 1993; Shimizu and Yoshikura 1994; Kato et al. 1995). This may be because the cells used for culture are not representative of those infected in vivo, or because productive infection requires a combination of cytokines and growth factors found in the liver that is not reproduced in cell culture.

Transfection of full length DNA sequences of the HCV genome might be expected to initiate the full replicative cycle of HCV, as it does when similar experiments are carried out with picornavirus sequences. However, while HCV proteins are translated and can be processed into structural and nonstructural proteins, there is no evidence for replication of genomic RNA, nor the assembly of virion particles. Despite considerable efforts, the reason for this block is still not understood, and has led to alternative strategies to study the HCV replication cycle. Of these, the most successful has been the development of subgenomic or full-length genomic replicons of HCV (Lohmann et al. 1999; Ikeda et al. 2002; Blight et al. 2003; Pietschmann et al. 2002). The original clone

was constructed as a dicistronic vector containing the nonstructural genes (NS3–NS5B) and the 3′ UTR from a genotype 1b clone whose expression was driven by a powerful IRES from encephalomyocarditis virus (EMCV). Upstream was a selectable marker (*neo*) and a 5′ terminus comprising the HCV 5′ UTR (Lohmann et al. 1999). Under neomycin selection, this replicon replicates at selfsustaining levels, although this is limited to specific cell lines such as certain clones of the Huh-7 hepatoma cell line. Replication is enhanced by the appearance of a number of 'adaptive' amino acid changes in the NS3 and NS5B regions (Bartenschlager et al. 2003), even though these play no role in natural infections and actually attenuate replication in experimentally infected chimpanzees (Bukh et al. 2002).

Since this pioneering work was carried out, several groups have developed replicons based on other genotype 1b isolates (Ikeda et al. 2002) and other genotypes (e.g. 1a, 2a; (Blight et al. 2003; Kato et al. 2003)). It is also possible to create viable replicons containing the structural proteins (Pietschmann et al. 2002; Blight et al. 2003), even though these still require selection and do not generate infectious viral particles. Clearly much remains to be discovered about the cell line restrictions, the requirement for 'adaptive,' nonphysiological sequence changes, and the underlying reasons why infectious virus does not appear to be produced in any of the available systems. Nevertheless replicons are useful in studying many aspects of the replication of HCV genomic RNA; they are also increasingly used to discover more about the mode of action of interferons used for human therapy (see Antiviral treatment), and the action and development of resistance to other antiviral agents, such as protease and RNA polymerase inhibitors (Blight et al. 2000; Frese et al. 2001; Lanford et al. 2003; Wang et al. 2003; Lu et al. 2004).

Animal models

The chimpanzee is the only animal model for HCV with demonstrated susceptibility to HCV infection, and which show a course of infection resembling that in humans. In particular, exposed chimpanzees may become persistently infected, with incubation periods, seroconversion profiles, and elevation of liver enzymes (such as alanine aminotransferase) broadly similar to that of human infections. Unfortunately, the later events in HCV infection in humans, such as the development of cirrhosis and hepatocellular carcinoma typically occurring over periods of 20–30 years, make it difficult to conduct natural history studies of the main disease outcomes of HCV infection in chimpanzees (Walker 1997).

One frequently observed difference between humans and chimpanzees lies in the immune response to infection. Inoculated chimpanzees generally show a delayed, weaker, and less broadly reactive antibody response to infection (Choo et al. 1994), while the appearance of strong T-cell responses is associated with clearance of infection (Cooper et al. 1999). These differences complicate the use of the chimpanzee as a model for vaccine development (see Immunization).

With the possible exception of the gorilla (for which there are few or no data available), HCV does not infect other primates in the wild (Makuwa et al. 2003), nor can they be infected experimentally (Bukh et al. 2001; Abe et al. 1993). There is, however, one report of the unexpected ability of HCV to replicate in the tupaia (Xie et al. 1998). It is not known whether the apparent species-specificity of HCV for human and higher apes is determined at the level of entry, by intracellular blocks in the replication cycle of HCV in cells of other species, or by missing cellular factors for virus assembly and release. The current problems associated with establishing HCV in a cell culture system make this issue experimentally difficult to resolve at present.

Replication of the HCV genome

In common with other positive-strand RNA viruses, HCV replicates its RNA genome through the production of a minus-strand replication intermediate, that is an RNA copy of the complete genome that is synthesized by the activity of a virus encoded RdRp (NS5B) (Figure 1). The minus-strand copy becomes the template for the generation of positive-strand copies. Because template can be transcribed several times, many minus-strand copies can be copied from the infecting positive strand, and each of these transcripts used several times to produce positive-strand progeny sequences. In this way, a single input sequence could be amplified several thousand-fold during the course of replication within a cell.

Using a highly strand-specific PCR method, antisense HCV RNA sequences have been detected in the liver of HCV-infected individuals, directly demonstrating replication of HCV via its replication intermediate (Lanford et al. 1994). Transcription initiation is dependent on possession of intact termini to the 5′ and 3′ UTRs. It has also been recently shown that replication in the replicon system requires RNA structures in the NS5B coding region (You et al. 2004; Lee et al. 2004). While the details of its role and of adjacent RNA structures in NS5B remain to be established, it is clear from other virus systems that transcription initiation and its regulation with translation are likely to be complex, and to involve multiple long-range interactions within HCV genomic RNA and with a range of cellular proteins and RNA sequences.

HCV translation

The 5′ UTR is an essential component in the initiation and regulation of translation of the large open reading

frame (ORF) of HCV. The region is between 341 and 344 bases in length, and a combination of computer analysis, nuclease mapping experiments, and studies of covariance has allowed the RNA secondary structure to be accurately predicted (Tsukiyama Kohara et al. 1992; Wang et al. 1995; Smith et al. 1995). Similar structures have been predicted for pestiviruses (Smith et al. 1995; Wang et al. 1995) and GBV-B (Grace et al. 1999) despite their frequent lack of nucleotide sequence similarities with HCV.

Evidence that the 5′ UTR can direct the internal initiation of translation was originally obtained by in vitro translation of reporter genes downstream from the 5′ UTR sequence placed in mono- or dicistronic vectors (Wang et al. 1993, 1994; Fukushi et al. 1994). The IRES activity of the 5′ UTR directs translation from the AUG methionine codon at position 341, through binding to the 40S ribosomal subunit (Pestova et al. 1998). There is no evidence for translation from any of the variable number of AUG triplets upstream from this site. As the structure of the IRES extends into the coding region, its activity is influenced by these downstream sequences. For example, the efficiency of IRES-mediated translation is inversely related to the stability of a short stem loop (designated stem–loop IV; nucleotides −12 to +12 with respect to the initiation codon) (Honda et al. 1996). It has also been demonstrated that the first 28–42 nucleotides of the core sequence must lack RNA secondary structure to allow positioning of the ribosome at the start of the coding sequence (Rijnbrand et al. 2001).

The synthesis of HCV proteins occurs through translation and cotranslational or subsequent proteolytic cleavage of the large potential polyprotein encoded by the open reading frame (Figure 1), and is intimately connected to the formation of a replication complex of HCV that remains closely associated with the endoplasmic reticulum (ER). Initial translation of the core gene leads to a protein on the cytoplasmic side of the ER with an amino terminus embedded in the ER membrane. This hydrophobic tail is subsequently cleaved to release the core protein, and directs synthesis of the E1 glycoprotein to the luminal side of the ER. A second hydrophobic region sequence embeds E1 to the ER membrane by forming a trans-membrane anchor. A second, host-cell signal peptidase (signalase)-mediated cleavage reaction separates it from E2. Signalase subsequently mediates cleavage of E2 from p7 and p7 from NS2. The remainder of the HCV proteins are produced on the cytoplasmic side of the ER. Cleavage of NS2 from NS3 is carried out autoproteolytically by a *cis*-acting zinc-dependent protease formed by the uncleaved NS2/NS3 precursor protein (Figure 1). Subsequent translation leads to the synthesis of NS3/NS4A serine protease that mediates cleavage between NS3 and NS4A in *cis*, and *trans*-cleavage of sites between NS4A and NS4Bb, between NS4B and NS5A, and between NS5A and NS5B (Figure 1).

Virus entry, uncoating, assembly, and release

Currently there is uncertainty about the nature of the cellular receptor for HCV and the mechanism by which it enters the cell. It has been found that CD81, a tetraspanin molecule that plays a role in the conformational ordering of signaling complexes, bound to the E2 protein of HCV, and that this interaction was blocked by neutralizing antibodies to HCV (Pileri et al. 1998). More recently, using a pseudotype system in which HCV envelope proteins were co-expressed with enveloped–deleted proviral clones of human immunodeficiency virus type 1 (HIV-1) to generate infectious particles, it was shown that expression of CD81 was absolutely required for infection of hepatocytes by HCV (Zhang et al. 2004). However, CD81 is widely distributed on a variety of human cell types, and does not therefore account for apparent highly specific cellular tropism of HCV for primary hepatocytes of the HCV/HIV-1 pseudotype. It is therefore likely that CD81 represents a coreceptor for HCV and that other cell surface molecules, perhaps with a liver-specific pattern of expression, are required for essential steps in HCV-binding or -fusion reactions. Of several candidate molecules, most attention has been paid to the low density lipoprotein (LDL) receptor, as its expression is restricted to the liver, and HCV purified from plasma of HCV-infected individuals is known to associate with LDL (Agnello et al. 1999; Wunschmann et al. 2000).

Replication of HCV within the cell occurs in the cytoplasm, in membrane-bound replication complexes, containing most of the nonstructural proteins of HCV and viral RNA (Lin et al. 1997; Ishido et al. 1998; Egger et al. 2002; El Hage and Luo 2003). At present, there is little information on the process of HCV virion assembly, maturation, and release from the cell, because virus particle formation is not achievable with currently used replicons of HCV, even those that express the full complement of structural proteins. However, core, E1, and E2 proteins expressed from transfected expression vectors have been shown to localize to the endoplasmic reticulum, intermediate compartment, and the *cis*-Golgi, likely representing steps in their maturation (such as glycosylation), and ultimately, particle assembly (Martire et al. 2001; Blanchard et al. 2002). There is currently no evidence for expression of HCV proteins on the surface of infected cells.

PROTEINS OF HCV

The genome of HCV is thought to encode at least ten proteins, of which three are structural (core, E1, and E2) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Figure 1). HCV also expresses p7, a membrane-associated ion channel that may function

during virus assembly or infection. Most information about the function and enzymatic activities of the nonstructural proteins has been obtained by expression of DNA sequences corresponding to the different proteins, and by direct observations of the cellular distributions and properties of HCV proteins detected in liver or plasma in vivo.

Structural proteins

The structural proteins of HCV are encoded by sequences at the 5′ end of the genome and so are translated first (Figure 1). Expression of this part of the genome in cells (Selby et al. 1993; Ralston et al. 1993; Grakoui et al. 1993c), or in reticulocyte lysate containing microsomal membranes (Hijikata et al. 1991b; Santolini et al. 1994) leads to the synthesis of a polyprotein followed by proteolytic cleavage into the core, E1, and E2 proteins. Cleavage between the capsid protein and E1, E1 and E2, and E2 and NS2 are dependent upon the addition of microsomal membranes, implying the role of the host-cell signalase in these processing steps.

CORE

The core or capsid protein has a size of 21–22 kDa, smaller than predicted from its coding sequence of 191 amino acids, the result of cleavage of the final 17–18 amino acids post-translationally to produce the mature protein. The N terminus contains several positively charged amino acid residues, that bind to viral RNA and which are presumably involved in the virus packaging reaction. It is not known how core proteins bind together to form the HCV nucleocapsid, although nucleocapsid formation is thought to drive the budding process in the ER that leads to the formation of fully-assembled virions. It has been proposed that the core protein of HCV can be functionally divided into three domains (Hope and McLauchlan 2000): domain 1 (amino acids 1–117) forms the nucleocapsid of the virus, while domain 3 represents the final 10–12 amino acid hydrophobic leader sequence that is cleaved after translation. Domain 2 has been shown to be the region of core responsible for its targeting to lipid droplets, a process which potentially interferes with lipid metabolism within the infected hepatocyte, and which may play a role in the development of steatosis as a complication of HCV infection (Perlemuter et al. 2002). Interestingly, domain 2 is highly conserved between HCV and GBV-B, while only the capsid region (domain 1) is obviously homologous with the capsid regions of pesti- and flaviviruses (Hope et al. 2002).

ENVELOPE (E1, E2)

The envelope proteins of HCV (E1, E2) are synthesized in mammalian cells as proteins with sizes ranging from 31–35 kDa and 68–72 kDa, respectively (Hijikata et al. 1991b; Selby et al. 1993; Grakoui et al. 1993c; Ralston et al. 1993; Santolini et al. 1994). E1 and E2 are extensively glycosylated after translation at a large number of *N*-linked glycosylation sites in the extracellular domains of the proteins. Interactions between E1 and E2 have been demonstrated by immunoprecipitation experiments, in which antibody to E1 or E2 could precipitate both proteins under nondenaturing conditions (Ralston et al. 1993; Grakoui et al. 1993c; Dubuisson et al. 1994). E1 and E2 are thought to form heterodimeric structures in the virus envelope through a noncovalent interaction (Dubuisson et al. 1994; Matsuura et al. 1994; Selby et al. 1994).

The envelope proteins are likely to form the principal target of antibody-mediated neutralization of virus infectivity, although investigation of this phenomenon has been hampered by the lack of an in vitro infectivity assay, and of a suitable animal model. Recently, however, it has been possible to demonstrate neutralizing antibody responses directed at the E1 and E2 glycoproteins through the use of pseudotyped virus, comprising the envelope glycoproteins from HCV and a capsid from either HIV or a murine retrovirus (Bartosch et al. 2003; Logvinoff et al. 2004). The E2 protein contains hypervariable regions (HVR) in the N-terminal region, which evolve much more rapidly during the course of persistent infection than other regions of the genome. This variability may arise through selection of amino acid changes that allow immune escape from neutralizing antibody; persistence may therefore be accompanied by continuous virus sequence change to keep ahead of developing B-cell responses (Kumar et al. 1993; Taniguchi et al. 1993; Weiner et al. 1992; Farci et al. 2000; Kantzanou et al. 2003) (see Immune escape).

P7

The small p7 protein lies between E2 and NS2 proteins of the virus polyprotein and is thus at the junction of the structural and nonstructural gene regions (Lin et al. 1994; Mizushima et al. 1994), although it is not known whether p7 is a virion component. The 63 amino acid protein is highly hydrophobic and remains embedded in the ER membrane on translation through a transmembrane anchor of two predicted alpha helices (Carrere-Kremer et al. 2002). Recently it has been shown that p7 oligomerizes to create ion channels within membranes (Griffin et al. 2003). These pores allow transport of cations across membranes, comparable to the activity of viral viroporins, such as M2 in influenza virus. Similarly, to M2, the activity of p7 is inhibited by amantadine (Carrere-Kremer et al. 2002), an observation that suggests a new strategy to treat HCV infection. It is currently unknown at what stage in the replication cycle p7 is active or required, nor whether it is an integral component in the envelope of the mature virus particle.

Nonstructural proteins

In vitro translation of the rest of the genome leads to the production of proteins of sizes 23, 70–72, 4, 27, 56–58, and 66 kDa, corresponding to NS2, NS3, NS4A, NS4B, NS5A, and NS5B, respectively (Figure 1).

NS2

The NS2 protein is cleaved from p7 at its N terminus by ER-associated signalase. NS2 remain membrane bound, with its C terminus located through the ER membrane, and the N terminus in the cytoplasm (Santolini et al. 1995). The only function assigned to NS2 is to form one domain of a protease in combination with the first 180 amino acid residues at the N terminus of NS3 (Hijikata et al. 1993a; Grakoui et al. 1993a). This protease activity autocatalytically excises the NS2 protein from NS3 in a *cis*-cleavage reaction through a currently unclear mechanism. The dependence of the NS2/NS3 protease on Zn^{++} and its inhibition by ethylenediaminetetraacetic acid (EDTA) (that chelates divalent cations) suggests that it might be a zinc protease. However, mutation of what would be functionally cysteine and histidine amino acids in the catalytic dyad of a cysteine protease also abolished protease activity of NS2/NS3 (Hijikata et al. 1993a). The NS2 protein is widely conserved across the flavivirus family; it is possible that it plays other roles in virus replication or assembly beyond that of a self-excising protein that detaches itself from the rest of the replication complex.

NS3

The NS3 protein has a variety of enzyme activities that participate in both RNA replication and in processing of the viral polyprotein. The mature form of NS3 migrates as a protein of 67 kDa, and is generated through NS2/NS3-mediated cleavage from NS2, and NS3/NS4B-mediated cleavage from NS4A. Although not cleaved internally when expressed in mammalian cells or during virus replication, the NS3 protein can be conceptualized as comprising two independent domains with different enzymatic activities.

The first 181 amino acid residues at the N terminus of NS3 show two different protease activities. As described in the previous section, it forms part of the NS2/NS3 protease, and forms a separate, functionally distinct serine protease after cleavage (Tanji et al. 1994). The serine protease shows *cis*-acting activity in cleavage of the NS3/NS4A junction, and *trans*-activity in the cleavage of NS4A from 4B, 4B from 5A, and 5B from 5A (Eckart et al. 1993; Hijikata et al. 1993a; Grakoui et al. 1993b; Bartenschlager et al. 1993; Tomei et al. 1993). The *trans*-activity of NS3 requires NS4A as a cofactor; recent crystallographic studies have shown that NS3 and NS4A form a tightly bound protease complex (Kim et al. 1996), with extensive interactions between residues 21 and 32 of NS4A with the β-sheet of the NS3 protease core. This interaction with NS4A may chaperone or stabilize the folding of NS3 into an active conformation. The hydrophobic, predicted membrane spanning N terminus of NS4A may, in turn, anchor the protease complex to membranes and thus link it to other components of the viral replication complex. NS3 contains a structural zinc-binding site through binding with a histidine and three cysteine residues, and which supplies electrons to the catalytic site in the enzyme complex.

The substrate specificity and mechanism of action of the NS3/NS4A protease has been extensively investigated because of the possibility of developing inhibitors that could be used to treat HCV infection (see Antiviral treatment). Each of the cleavage sites in different variants of HCV conform closely to the consensus sequence D/E – X–X–X–X–C/T // S/A (where // represents the cleavage site) (Komoda et al. 1994; Kolykhalov et al. 1994; Bartenschlager et al. 1995; Leinbach et al. 1994). There is some evidence for a less stringent requirement for specific amino acids around the *cis* cleavage site (NS3/NS4A) than those cleaved in *trans* (Bartenschlager et al. 1995). Inhibitors of NS3/NS4A have now been developed that mimic the recognition sequence, and competitively inhibit the processing of the HCV polyprotein (De Francesco et al. 2003); BILN 2061 is currently in clinical trials (Lamarre et al. 2003). While the primary function of the protease domain of NS3 is processing of the HCV polyprotein, it has recently been shown that the NS3/4A protease blocks the phosphorylation and signaling function of the antiviral interferon regulatory factor 3 (Foy et al. 2003). Blocking of this cell defense pathway may contribute to the ability of HCV to persist in vivo, and to resist exogenous interferon therapy.

The much longer second enzymatic domain of NS3 shows helicase and NTPase activities. Helicases displace base-paired RNA or DNA sequences from a template strand usually in a 3′ to 5′ direction. This process requires the presence of Mg^{++} ions and energy provided by hydrolysis of ATP by a NTPase activity of the enzyme and these are combined in an energy-dependent unwinding process of template and copied RNA sequences during virus genome replication (Gwack et al. 1997; Tai et al. 1996). Several sequence motifs in NS3 (such as the DEXH box) are shared with corresponding proteins in other positive-strand RNA viruses, suggesting a degree of functional equivalence on which the HCV helicase might be modeled (Suzich et al. 1993).

NS4A

As described above, NS4A functions as part of the NS3/NS4A protease complex, with an additional role in localizing the complex to membranes. No other function of NS4A has been determined to date.

NS4B

The function of NS4B is not fully understood. After cleavage, NS4B is membrane-associated in the ER

(Hugle et al. 2001) with at least two predicted membrane-spanning domains (Hugle et al. 2001; Lundin et al. 2003), and participates in the formation of a membranous web that may act as a structural platform for HCV replication complex (Egger et al. 2002). Recently, NS4B has been shown to possess a GTPase activity that is required for replication of the HCV replicon; this finding suggests an additional enzymatic role for NS4B in the replication complex (Einav et al. 2004; Piccininni et al. 2002).

NS5A

The NS5A protein is a 56–58-kDa nonstructural protein. Although for many years after the discovery of HCV the function of NS5A was unknown, more recent research has revealed a key role for NS5A in a variety of interactions with host-cell defense mechanisms that may be associated with the ability of the virus to persist in vivo and to resist interferon therapy. On cleavage from NS4B and NS5B, NS5A is bound to the ER by the N-terminal amphipathic helix (Figure 4) and becomes associated with the viral replicase complex on the cytoplasmic side of the ER; binding to NS5B may be required for the RNA polymerase activity of the replication complex (Shimakami et al. 2004). NS5A becomes phosphorylated after translation, at one or more of several targeted serine or threonine residues at the center and C terminus of the protein. The number and position of NS5A phosphorylation sites varies between genotypes (Hirota et al. 1999; Reed et al. 1997). NS5A may additionally become hyperphosphorylated within a serine-rich region at the center of the protein (Figure 4), although this form of NS5A is not present in the replication complex, and mutations that prevent phosphorylation at positions 2 197 and 2 204 actually enhance the replication ability of the HCV replicon (see Replication of the HCV genome).

While NS5A is a necessary part of the viral replication complex, it shows a number of additional activities that are broadly aimed at combating the innate cell defenses, thus potentially contributing to the persistence of HCV and its resistance to interferon. These include the binding to and inactivation of PKR (Gale et al. 1997), blocking apoptotic pathways through sequestering p53, modulation of intracellular calcium levels, and binding to growth factor receptor-bound protein 2 (Grb-2) (Majumder et al. 2001; Gong et al. 2001; Tan et al. 1999), and induction of anti-inflammatory interleukin-8 (IL-8) secretion (Polyak et al. 2001) (recently reviewed in Macdonald and Harris 2004). Many of these interactions block the apoptotic cellular response to persistent HCV infection, while the induction of IL-8 may potentiate viral replication and inhibit intracellular IFN-responsive antiviral pathways. These different functions have been mapped to a number of different domains in the NS5A protein (Figure 4).

Interferon therapy has been shown to lead to the appearance of a number of amino acid changes in the region of NS5A that interacts with PKR (Figure 4). An association was also found between treatment response and possession of the so-called 'prototype' NS5A sequence in the region where mutations occurred (Enomoto et al. 1995). These original findings have since been confirmed in recent meta-analyses of all the available data which demonstrated a clear correlation between possession of the prototype interferon-sensitivity determining region (ISDR) sequence and treatment resistance, and a large number of diverse amino acid changes in nonresponders (Witherell and Beineke 2001; Pascu et al. 2004). It has also been shown that the same differential response exists in genotype 2a and 2b infections (Murakami et al. 1999). Prototype ISDR sequences have also been shown to be associated with higher circulating virus loads in untreated patients (Watanabe et al. 2003). It is therefore possible that PKR evasion is a key determinant in the persistence of HCV and potentially other aspects of the virus/host interaction.

NS5B

NS5B is an RNA-dependent RNA polymerase, and is responsible for the copying of the HCV genome during replication. NS5B is cleaved at its N terminus from the rest of the HCV polyprotein by the NS3/NS4A protease. NS5B remains membrane bound through a hydrophobic,

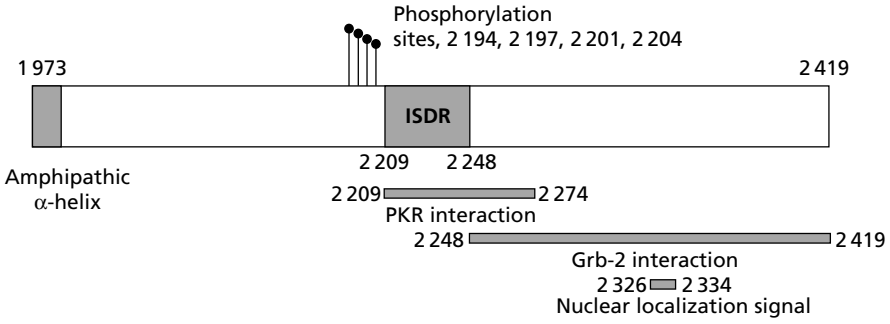

Figure 4 *Diagram of the* NS5A *gene. This shows the positions of the ER-binding amphipathic helix, the interferon-sensitivity determining region (ISDR), and regions of the protein known to interact with PKR and other cellular pathways associated with innate cell defenses against viruses. Identified sites of hyperphosphorylation found in the 58-kDa form of the protein localize to the ISDR.*

membrane-inserted anchor sequence at the carboxyl terminus of the protein. The mature form of NS5B is 68 kDa in size, is phosphorylated, and forms part of the replication complex along with other nonstructural proteins that participate in genome replication. The recently determined crystal structure (Lesburg et al. 1999) has shown NS5B to have a typical thumb/palm/finger arrangement of domains that encircle the RNA template strand, and that contains catalytic residues, such as the GDD motif characteristic of RNA polymerase of other positive-strand RNA viruses. Determining the structure of NS5B has been important for the design and optimization of activity of new classes of antiviral agents that inhibit RNA polymerase function (see Antiviral treatment). In vitro, NS5B requires a nucleic acid primer to initiate polymerization (Oh et al. 1999). The mechanism of strand initiation during genome copying is unknown, although it has recently been demonstrated that replication required one or more RNA stem–loops in the terminal NS5B region (You et al. 2004), to which NS5B directly binds (Lee et al. 2004; Cheng et al. 1999). Whether strand initiation of the opposite strand occurs through a similar mechanism, and whether there is post-transcriptional editing or modification of the newly synthesized genomic and antigenomic strands is unknown.

HCV GENETIC VARIABILITY

Nucleotide sequence variation

The first indication of the genetic heterogeneity of HCV came from comparisons of nucleotide sequences of HCV variants from Japan with that of the prototype HCV variant obtained from the USA, HCV-PT (Choo et al. 1991). For example, the complete genome sequences of HCV-J (Kato et al. 1990) and -BK (Takamizawa et al. 1991) from Japan showed 92 percent similarity to each other, but only 79 percent with HCV-PT. At that time, the former were referred to as the 'Japanese' type (or type II), while those from the USA (HCV-PT and -H) were classified as type I. However, far more divergent variants of HCV have since been found in Japan (Okamoto et al. 1992b, 1991) and elsewhere (Mori et al. 1992; Chan et al. 1992), leading to the adoption of an extended classification of HCV into types and subtypes.

Comparison of nucleotide sequences of variants recovered from infected individuals in different risk groups for infection and from different geographical regions has revealed the existence of six major genetic groups. Over the complete genome, these differ in 30–35 percent of nucleotide sites, with more variability concentrated in regions such as the E1 and E2 glycoproteins, while sequences of the core gene and some of the nonstructural protein genes, such as NS3, are more conserved. Least sequence variability between genotypes is found in the 5′ untranslated region, the 5′ end of the core gene and the 99 base pair X-tail in the 3′ UTR, where specific sequences and RNA secondary structures are required for replication and translation functions. Despite the sequence diversity of HCV, all genotypes share an identical complement of colinear genes of similar or identical size.

Each of the six major genetic groups of HCV contain a series of more closely related subtypes, typically different from each other by 20 percent in nucleotide sequences compared with the >30 percent between genotypes (Figure 5) (Simmonds et al. 1993a). Some, such as genotypes 1a, 1b, and 3a have become very widely distributed as a result of transmission through blood transfusion and needle sharing between infecting drug users (IDU) over the past 30–70 years, and now represent the vast majority of infections in western countries (Figure 5a) (see Geographical distribution of HCV genotypes). These are the genotypes most commonly encountered clinically, and for which most information has been collected on response to interferon and other antiviral treatments (see Biological differences between HCV genotypes).

A different pattern of sequence diversity is observed in parts of Africa and South East Asia. Here, there are close associations between genotypes and specific geographical regions (Figure 5b). For example, infections in western Africa are predominantly by genotype 2 (Jeannel et al. 1998; Mellor et al. 1995; Wansbrough Jones et al. 1998; Ruggieri et al. 1996; Candotti et al. 2003), while those in central Africa, such as the Democratic Republic of Congo (DRC) and Gabon are by genotype 1 and 4 (Bukh et al. 1993a; Fretz et al. 1995; Stuyver et al. 1993b; Menendez et al. 1999; Xu et al. 1994; Ndjomou et al. 2003; Mellor et al. 1995). In both regions, there is a remarkable diversity of subtypes; for example, 20 of 23 HCV-seropositive blood donors in Ghana (west Africa) were infected by genotype 2, but each corresponded to different and previously undescribed subtypes (Candotti et al. 2003). Similar diversity was found in Guinea, Benin, and Burkina Faso (west central Africa), where 18 different subtypes of genotypes 1 and 2 infected 41 HCV-infected individuals (Jeannel et al. 1998). These field observations reflect both the huge genetic diversity of genotypes 1, 2, and 4, and also its likely long-term presence in human populations in these parts of Africa. Taking this geographical mapping further, genotypes 3 and 6 show similar genetic diversity in south and eastern Asia (Tokita et al. 1994a, b, 1995; Mellor et al. 1995).

The model suggested by these genotype distributions is that HCV has been endemic in sub-Saharan Africa and South East Asia for a considerable time, and the occurrence of infection in western and other nontropical countries represents a relatively recent emergence of infection in new risk groups for infection (Simmonds

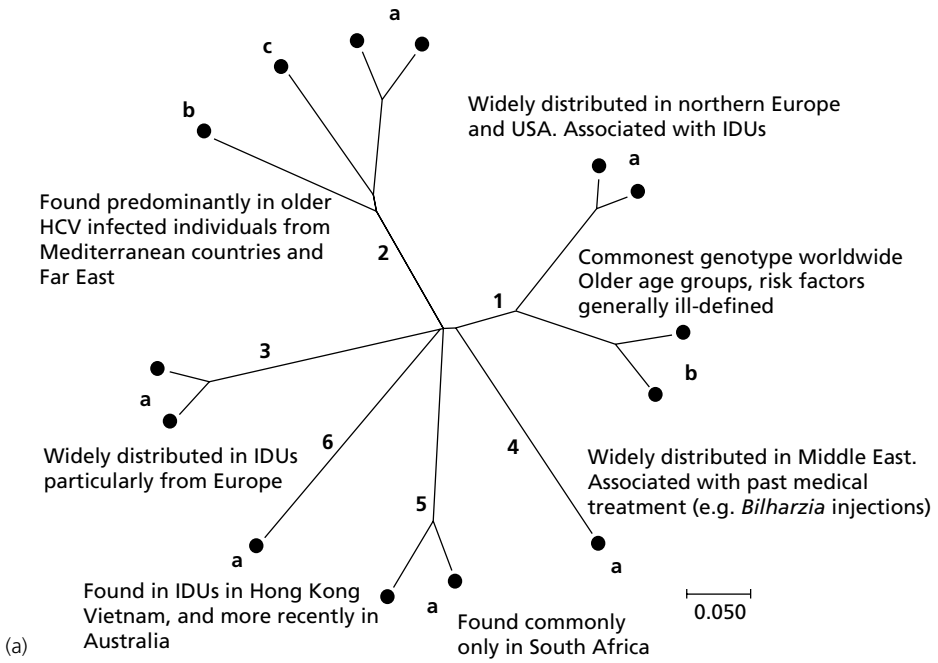

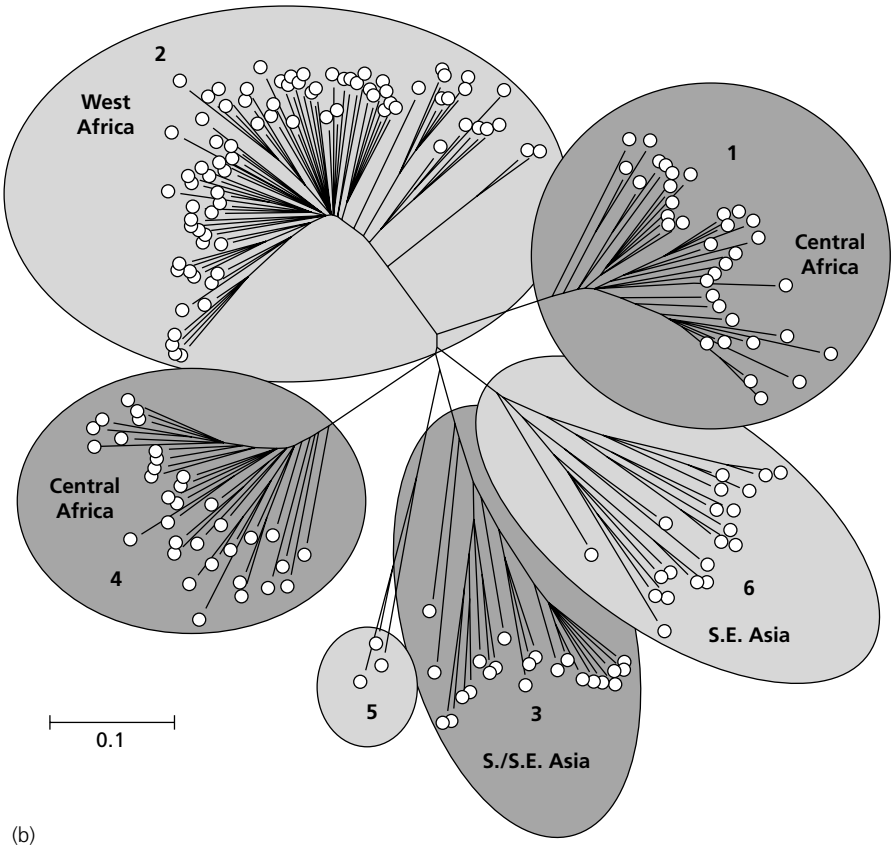

Figure 5 (a) *Evolutionary tree of the principal genotypes of HCV found in industrialized countries. Phylogenetic analysis was carried out on complete genome sequences of genotypes of HCV found in the main identified risk groups for HCV infection (IDUs, recipients of unscreened blood or blood products, other parenteral exposures). These represent the main variants believed to have become prevalent over the course of the twentieth century.* **(b)** *Evolutionary tree of all available NS5B sequences for different subtypes and genotypes HCV. This phylogenetic analysis demonstrates that HCV variants still fall into six distinct genotypes, but each contains numerous novel variants discovered in high diversity areas in sub-Saharan Africa and South East Asia. (The trees were constructed using the phylogeny program neighbor-joining implemented in the MEGA package, using Jukes-Cantor corrected distances.)*

2001; Ndjomou et al. 2003). In the twentieth century, parenteral exposure to bloodborne viruses became frequent through the widespread adoption of blood transfusion since the 1940s, the medical use of often unsterilized needles for injections and vaccinations (a practice that continues in many developing countries) and most specifically to industrialized countries, injecting drug use, and the sharing of injection equipment. These new routes for transmission plausibly account for the epidemiological and genetic evidence for recent epidemic spread of HCV over the past 50 years in Europe, Egypt, and elsewhere (Cochrane et al. 2002; Pybus et al. 2001, 2003; Ndjomou et al. 2003). However, the original routes of HCV transmission in endemic, high diversity areas are not understood, as transmission by either sexual contact or from mother to child is inefficient (Wasley and Alter 2000; Pradat and Trepo 2000; Thomas 2000), and there is little evidence historically for the widespread parenteral exposure that fueled the recent epidemic in western countries.

Nomenclature of HCV genotypes

The six main genetic groups or 'clades' of HCV have been designated as genotypes (Figure 5). Following previous proposals (Mellor et al. 1995; Mizokami et al. 1996; de Lamballerie et al., 1997), 'new' genotypes (such as 7a, 8a, 9a, and 11a) have been assigned as members of genotype 6, and genotype 10a into genotype 3. An advisory group is currently cataloging a full listing of recognized genotypes and subtypes, and resolving any inconsistencies (such as the labeling of two HCV variants with the same subtype name) for publication in 2005. The new standardized nomenclature will be adopted by public access databases that allow access to fully annotated sequences deposited on GenBank, EMBL, or DDBJ. It has also been decided not to pursue the designation of all variants within genotypes as individual subtypes. In high diversity areas such as central Africa, there may an indefinite number of 'subtypes' that would be of little value to catalog. Future assignment of subtype names will only be carried out for those where a new variant is found in three or more epidemiologically unlinked infections, thus restricting formal classification to those that are more widely distributed, and which may show specific geographical, risk group, or other epidemiological associations.

Recombination

Recombination occurs in many families of RNA viruses, its occurrence requiring both epidemiological opportunity and biological compatibility. Recombination occurs within cells infected with two or more genetically distinct variants of HCV, and thus requires both coinfection of the same individual with more than one such variant, and substantial overlaps in their geographical distributions of different genotypes to enable recombinant forms to be detected. The wide range of genotypes circulating in the main risk groups for HCV in western countries, such as 1a and 3a in IDUs, provides opportunities for recombination, as does the nature of HCV risk behavior, frequently involving multiple exposures around the time of primary infection (e.g. repeated needle-sharing with several infected individuals). Recombination may also be facilitated by the absence of protective immunity from reinfection in chronic HCV infection (Farci et al. 1992; Kao et al. 1993; Jarvis et al. 1994; Lai et al. 1994).

A convincing example of a viable, and rapidly spreading recombinant form of HCV contains structural genes from genotype 2k, a recombination point in NS2 and nonstructural genes from genotype 1b, and has been recovered from several IDUs in St Petersburg (Kalinina et al. 2002, 2004). The existence of widespread recombination would place a considerable limitation on the use of genotyping assays for HCV, as the genotype identified in the region used in the assay, such as the 5′ UTR or core gene may not correspond to the genotype of other parts of the genome which may be more relevant for determining treatment response (see Biological differences between HCV genotypes).

Biological differences between HCV genotypes

The major features of HCV structure, replication, transmission, and ability to establish persistent infection are shared between all known variants. Infection with each genotype has become widespread in human populations, and indicates that they are equally capable of maintaining infections in human populations. Despite this obvious evidence for phenotypic similarity, there is growing evidence for genotype-specific differences in persistence and interactions with innate cell defenses and the immune system that have important repercussions for current and likely future therapy.

The extensive amino acid sequence variability found in both structural and nonstructural proteins would be expected to modify profoundly immunological recognition by both antibody and T-cell receptors. For example, the envelope proteins of different genotypes differ in amino acid sequence from each other by between 34–40 and 26–29 percent for E1 and E2. By analogy, this degree of sequence variability would be sufficient to prevent neutralization of one subtype by antibody elicited by a heterologous serotype, a prediction that has been borne out in recent studies using HCV chimeras (see Replication of the HCV genome). There is also clear evidence for antigenic differences between recombinant proteins or peptides from different genotypes in enzyme-linked immunosorbent assays (McOmish et al.

1993; Simmonds et al. 1993c; Tsukiyama Kohara et al. 1993; Mondelli et al. 1994). Antigenic differences between genotypes have implications for the development of vaccines for HCV and for the optimal design of serological screening and confirmatory assays for HCV (see Diagnostic tests for HCV infection).

The most clinically apparent difference between genotypes is in their susceptibility to treatment with interferon (IFN) monotherapy or IFN/ribavirin (RBV) combination therapy. Typically, only 10–20 and 40–50 percent individuals with chronic infection with genotype 1 on monotherapy and combination therapy, respectively, exhibit complete and permanent clearance of virus infection. This long-term response rate is much lower than the 50 and 70–80 percent observed on treatment of genotype 2 or 3 infections (comprehensively reviewed in Zeuzem (2004) and Pawlotsky (2003a)). This difference has proved to be highly significant in patient management, and has led to the use of higher doses and longer durations of treatment for type 1 (and type 4) infections to achieve acceptable efficacy. In numerous multivariate analyses, genotype-specific differences in treatment response have been shown to be independent of host variables, such as stage of disease progression, age, duration of infection, sex, and HIV and other virus coinfections. It is similarly independent of virus-specific factors, such as pretreatment viral load, although this also independently correlates (inversely) with response. The underlying mechanism of these differences in response is currently unknown.

Enzymatic studies and preliminary data from clinical trials provide consistent evidence for frequent genotype-specific differences in susceptibility to recently developed protease and RNA polymerase inhibitors for HCV therapy (De Francesco et al. 2003). Antiviral agents whose modeling, screening, and optimization was based on enzymatic studies of genotype 1 protease or RNA polymerase have since been found to be poorly inhibitory against other genotypes (Holland-Staley et al. 2002). For example, the protease inhibitor, BILN 2061, showed nearly 2 logs weaker binding affinity to genotype 2 and 3 proteases than to genotype 1 (Thibeault et al. 2004). These enzymatic differences may be clinically significant; in a recent efficacy trial, nongenotype 1-infected individuals were nonresponsive or only weakly responsive to short-term treatment with the BILN 2061 protease inhibitor (Reiser 2004), in contrast to its efficacy in genotype 1 (Lamarre et al. 2003).

In contrast to the clear-cut differences between genotypes in their response to antiviral therapy, it has proven much more difficult to obtain reliable data on the differences in the natural history and pathogenicity between HCV genotypes. This is because it is difficult to obtain prospective and/or case–controlled data on the outcomes of HCV infection over the exceptionally long time over which complications of HCV infection present clinically. Biased recruitment of study cohorts towards the minority with clinically apparent disease lack the community denominator and information on durations of infection with which to estimate the time course of disease development. Longitudinal studies have shown that genotype 1 invariably appeared more likely to establish persistence, and in carriers, to be associated with more severe liver disease compared with genotypes 2 and 3 (Mazzeo et al. 2003; Yee et al. 2000; Resti et al. 2003; Franchini et al. 2001). The underlying mechanism for the greater persistence of genotype 1 infections may be related to the greater treatment resistance of this genotype (see above). Most evidence supports the hypothesis that genotype 1 is more likely to cause hepatocellular carcinoma (Di Bisceglie 1997). However, infections with genotype 3 are associated with a higher incidence of steatosis (Adinolfi et al. 2001; Rubbia-Brandt et al. 2000), thought to result from direct cytopathic damage to hepatocytes from a block in lipoprotein secretion (Serfaty et al. 2001).

CLINICAL AND PATHOLOGICAL ASPECTS

Clinical and pathological features of HCV infection

Hepatitis C infection causes an indolent and slowly progressive liver disease that is asymptomatic until the development of decompensated liver disease and, often, liver cancer. The following sections review the clinical and pathological features associated with acute and chronic hepatitis, the replication of HCV in liver and possibly at extrahepatic sites, and describes proposed mechanisms of liver damage and of persistence.

Acute hepatitis

Percutaneous exposure to HCV usually results in an asymptomatic infection (not associated with jaundice), and most people become chronic carriers of the virus (Figure 6). This contrasts strongly with the observation that few adults infected with HBV become chronically infected. The period from exposure to the development of hepatitis has been measured in a number of investigations of post-transfusional hepatitis. Most studies have reported an interval of about 8 weeks to the development of abnormal liver function tests (such as alanine aminotransferase (ALT) levels), although viremia can be detected earlier (Figure 6). Only 5 percent of individuals with acute HCV infection have symptomatic disease, although in some cases it may be severe. HCV is very rarely associated with acute fulminant hepatic failure (Wright et al. 1991; Mutimer et al. 1995a). HCV may be more likely to cause acute liver failure in the patient who is a carrier of hepatitis B and in those who acquire hepatitis B and HCV

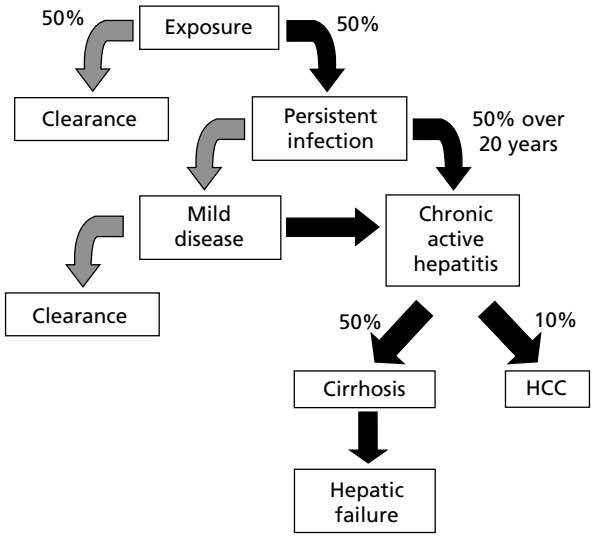

Figure 6 *Outcome of infection with HCV. Hepatitis can be detected by blood tests approximately 2 months after exposure to HCV. Most infections are asymptomatic, and a minority of patients develop jaundice. Approximately 50 percent of infected patients have persistent infection, i.e. they become HCV carriers. Of these, a proportion will progress to chronic active hepatitis, and may eventually progress to cirrhosis or hepatocellular carcinoma. The frequencies of the various outcomes are provided only as a very approximate guide, and are greatly influenced by host (particularly patient age) and viral factors (such as infecting genotype).*

simultaneously. Clinically, hepatitis caused by HCV is indistinguishable from that caused by other hepatitis viruses; jaundice may develop, but more usually symptoms are nonspecific such as fatigue, anorexia, and nausea. Most HCV-infected individuals cannot recall a history of jaundice, and HCV accounts for a small proportion of clinically apparent hepatitis cases in the community.

Viremia can be detected by PCR in the early stages of acute hepatitis, appearing at the same time or slightly earlier than abnormal levels of ALT (Figure 7). In contrast, seroconversion for antibody may be delayed for several weeks or months after the onset of hepatitis (Lelie et al. 1992), although second- and third-generation assays have closed this 'window' period to some extent. Nevertheless, reliable identification of HCV as a cause of acute hepatitis depends on the use of PCR, in contrast to acute HAV infection where IgM is generally detectable at the onset of clinical hepatitis. Histological features of acute HCV are similar to those associated with acute HAV and HBV infection; liver biopsy is rarely indicated to make a diagnosis of acute HCV infection.

Chronic hepatitis

The frequency of chronic infection following exposure to HCV is high, although there are difficulties in estimating a precise figure. For example, HCV may be identified more frequently in those with symptomatic primary infection or those with post-transfusion hepatitis (Kiyosawa et al. 1990; Gilletterver et al. 1995), both of which may be associated with an increased likelihood of chronic infection (Gordon et al. 1993; Mutimer et al. 1995b).

Individuals infected through blood transfusion and hemophiliacs, many of whom have been repeatedly exposed to HCV through the use of nonvirally inactivated clotting factor concentrates, have a high rate of persistent infection associated with active liver disease (60–80 percent). In contrast, lower rates of chronic infection and more slowly progressive disease are observed in individuals with lesser exposure to HCV. For example, only 50 percent of women exposed to a particular batch of anti-D immunoglobulin had persistent infection (Meisel et al. 1995), whereas in a similar outbreak in Ireland, also associated with the use of anti-D (Power et al. 1994), viremia was detected by PCR in only 50 percent of people remaining seropositive 17 years after exposure. Given the tendency for antibody to become undetectable following clearance of viremia, the true frequency of persistent infection in these two cohorts may be even lower.

Persistent infection with HCV is generally associated with persistent and progressive hepatitis. Chronically infected individuals generally have fluctuating or continuously abnormal levels of ALT and are viremic. However, it has been difficult to establish a correlation between the level of viremia and the severity of liver disease. For example, HCV RNA levels in asymptomatic blood donors are similar to those found in patients treated for liver disease (Smith et al. 1996; Lau et al. 1995a). Moreover, several studies have failed to document any association between the degree of viremia and level of ALT or other biochemical abnormalities associated with hepatitis.

Histological features

HCV infection causes a range of characteristic histological changes in the liver, although none allows a specific diagnosis of HCV infection to be made. The most striking feature of HCV infection of the liver is the presence of lymphoid follicles within the portal tracts (Figure 8); these are less often observed in hepatitis from other viral causes. Biopsies also typically reveal a dense periportal inflammatory process, associated with the infiltration of lymphocytes and plasma cells. Bile duct damage is often found in association with the infiltration, with vacuolization and ballooning of epithelial cells lining small bile ducts. Another common observation is the appearance of lobular hepatitis, with lymphocyte infiltration within sinusoids surrounding the hepatocytes. Steatosis is a frequent histological feature of HCV infection. It may be more commonly observed in those patients with HCV genotype 3 infection and

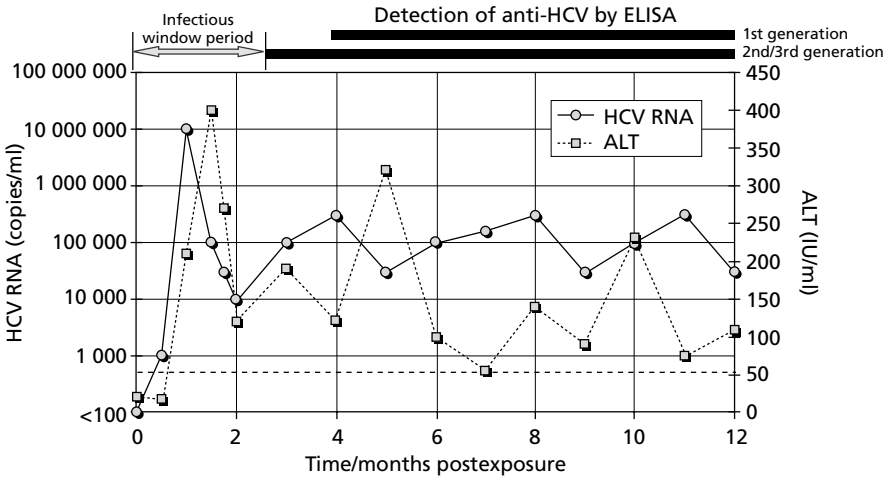

Figure 7 *Virological and biochemical markers of acute HCV infection. HCV RNA (solid line) and abnormal ALT levels (dotted line) appear approximately 50 days after exposure to HCV in a typical individual. The subsequent development of chronic hepatitis is indicated by persistent viremia and by fluctuating abnormal ALT levels. Antibody to HCV first appears after the onset of acute hepatitis, in this example leading to 'infectious window periods' of up to 60–80 days in currently used serological assays. The antibody profile elicited by infection is highly variable between individuals, although antibody to c33c is normally the first to appear.*

may resolve (along with other features of chronic hepatitis) in response to successful antiviral therapy.

The extent of histological abnormalities within biopsies can be summarized by descriptions such as lobular, chronic persistent hepatitis (CPH), and chronic active hepatitis (CAH), with or without cirrhosis, although more informative scoring systems such as the Knodell and Ishak scores have also been devised (Knodell et al. 1981; Ishak 1994). A spectrum of milder disease is generally observed in studies of asymptomatic individuals, such as those identified by blood donor screening, possibly reflecting a shorter duration of infection.

Despite sporadic publication of in situ histological demonstrations of HCV RNA and/or encoded proteins, most histopathologists struggle to demonstrate HCV in tissue sections and convincing reproducible studies using commercially available reagents are lacking.

Progression of disease

The percentage of chronically infected individuals who progress to cirrhosis and liver failure is not known. Progression to clinically significant liver damage is almost invariably very slow, although it may be faster in the context of immunosuppression. Particularly aggressive liver disease associated with HCV has been observed in immunosuppressed recipients of organ transplants and in patients with inherited immunodeficiency states (Healey et al. 1994). Studies of post-transfusion HCV highlight the following important clinical features. Many transfusion recipients are elderly (often suffering significant nonhepatic diseases) and die within two decades of infection, but not as a consequence of HCV infection. HCV-related mortality is rare within 20 years of infection; in one study, no excess mortality of HCV-infected individuals was observed over a period of 17 years (Figure 9) (Seeff et al. 1992). Cirrhosis is rarely observed within 10 years of infection, and as few as 20 percent of infected patients have cirrhosis after 20 years' follow-up. Risk factors for progression of chronic hepatitis to cirrhosis include age at time of infection (more rapid progression observed when infection occurs at older age), male gender, and concurrent alcohol consumption (Poynard et al. 1997). Recent analyses have highlighted the potential adverse impact of hepatic steatosis (and patient body mass index (BMI)) on fibrosis progression and on response to antiviral therapy. Cirrhosis may be complicated by hepatocellular carcinoma, but this rarely develops within 15 years of HCV infection (see Hepatocellular carcinoma). Some patients have asymptomatic infection, with normal liver function tests and mild hepatitis, despite 30 years of infection.

HCV may be associated with more aggressive liver disease in older than in younger people. Prognosis may also depend on viral genotype, although this is interrelated with other factors such as age at acquisition. For example, most transfusion recipients are elderly, and HCV genotype 1b is most commonly isolated from the serum of patients with transfusion-associated infection (Mahaney et al. 1994). Although it is not possible to estimate long-term prognosis at the time of infection, it is more feasible for patients with a known long duration of infection. For instance, when a 30-year-old patient has cirrhosis within 10 years of infection, there is a high likelihood that liver failure will eventually develop. However, for a 70-year-old patient with mild hepatitis despite 30 years' duration of infection, HCV is unlikely to cause death. Assessment of prognosis is an important aspect of selecting patients for treatment.

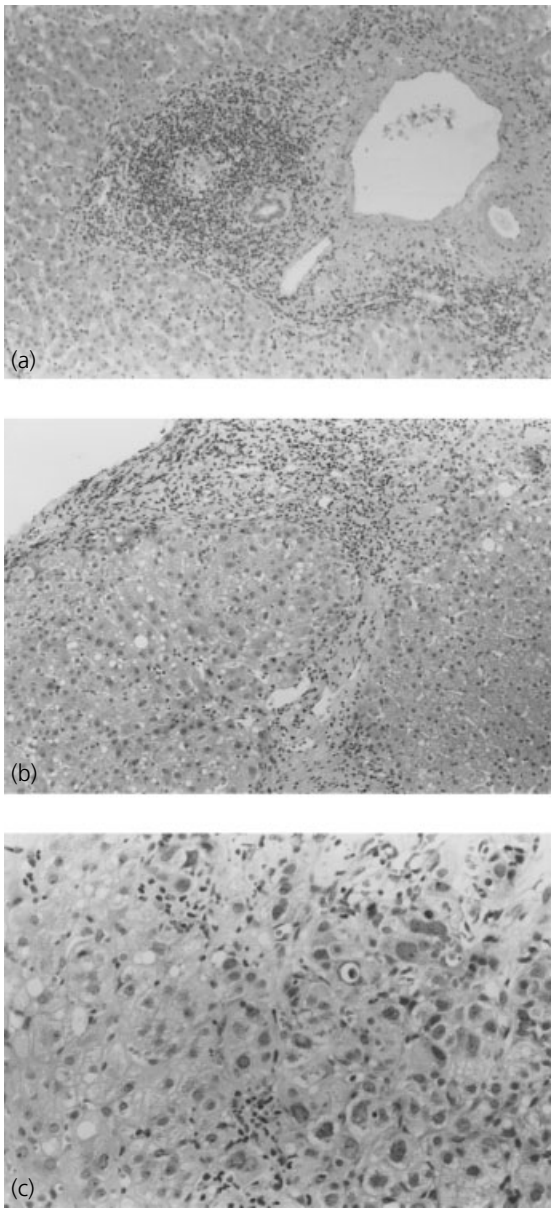

Figure 8 *Typical histopathological feature of HCV infection of the liver.* **(a)** *Typical HCV hepatitis with a large lymphoid aggregate within a portal tract showing reactive germinal center formation. Although the aggregate is close to a bile duct and adjacent to the limiting plate of the tract, there is little evidence of bile duct damage and none of interface hepatitis (piecemeal necrosis). Plasma cells and macrophages are also commonly present.* **(b)** *Cirrhosis. This is characterized by coarse fibrous septa separating nodules of regenerating hepatocytes.* **(c)** *Hepatocellular carcinoma. Tumor cells (right) are pleomorphic and hyperchromatic adjacent to regenerating hepatocytes in a cirrhotic nodule. There is a patchy chronic inflammatory infiltrate at the interface between them (sections kindly provided by Professor D.J. Harrison, Department of Pathology, University of Edinburgh).*

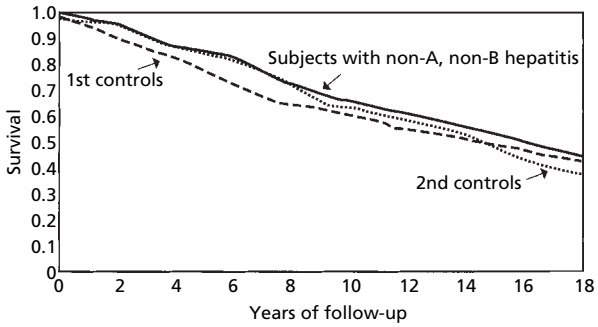

Figure 9 *Mortality associated with chronic post-transfusion hepatitis. The authors studied patients with transfusion-related non-A, non-B hepatitis (principally hepatitis C) who had been identified in five major prospective studies conducted in the USA between 1967 and 1980. Each index case was matched with two groups of noninfected transfusion recipients (first and second), and mortality rates from all causes were determined during an average follow up of 18 years. Mortality from all causes was high (about 50 percent in all groups), and the survival curves for the three groups were the same (reproduced from Seeff et al. (1992). Death from liver disease was low in all three groups, but was significantly higher in those originally given a diagnosis of non-A, non-B hepatitis (data not shown).*

After many years, liver failure may develop in patients with cirrhosis (also known as decompensated cirrhosis) (Fattovich et al. 1997). Decompensation may be manifest as hepatic encephalopathy, variceal hemorrhage, or ascites. Clinical manifestations of liver failure are not specific for HCV-induced liver disease, but are common to all forms of cirrhosis. Hepatocellular carcinoma may develop in an HCV-infected liver, but is very rarely observed in the absence of cirrhosis (see below).

In summary, most HCV infection is asymptomatic; when symptoms do develop, they are the nonspecific symptoms of liver failure.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a frequent complication of long-standing HCV infection. In many western countries such as Spain and Italy, as well as in Japan, HCV infection is found in 60–90 percent of cases of HCC. HCC develops slowly, typically appearing 30 years after infection, and being rare within 15 years (Kiyosawa et al. 1990). In most studies, HCC usually, although not invariably (Demitri et al. 1995), occurs in patients with pre-existing cirrhosis.

For these reasons, the mechanism by which HCV causes HCC is generally considered to be indirect, arising as a consequence of chronic damage and inflammation of liver cells. Unlike HBV, replication of HCV does not produce DNA sequences that could be integrated into the host genome, nor is there any evidence for transforming activity of any of the HCV gene products. Treatment of hepatitis C patients with interferon-α has been reported to greatly reduce the subsequent risk of developing HCC, even in those conventionally regarded as 'non-responders,' in whom long-term normalization of ALT was not achieved (Nishiguchi et al. 1995).

B-cell responses to HCV infection

Infection with HCV elicits humoral and T-cell-mediated immune responses. Antibody reactivity to several HCV proteins can be detected in most infected individuals, although the response is highly variable and generally weak compared with antibody responses to other viruses. There seems to be no specific pattern of antibody response that is predictive of the long-term outcome of infection.

HCV antibody has been found to rapidly decline in titer after clearance of viremia in those who spontaneously resolve infection and in those who respond to HCV therapy. For example, ten women with documented exposure to HCV infection through treatment with contaminated anti-D immunoglobulin in East Germany were found to be seropositive 10 years after exposure, but five became completely seronegative in the following 10 years, while antibody titers in the remainder fell to close to the cut-off the HCV serological assay (Takaki et al. 2000).

Until recently it has been difficult to assess the protective effect of the serological response to HCV infection. It is now possible to investigate neutralization of HCV in vitro through the production of virus pseudotypes, in which a different virus from HCV is packaged inside an HCV envelope, and whose infectivity can be more readily quantified than HCV (Flint et al. 2004; Bartosch et al. 2003; Hsu et al. 2003) (see Structural proteins). Neutralizing antibody responses can be detected in HCV-infected individuals; titers are initially low and highly strain-specific, but increase and broaden in reactivity on development of chronic infection (Logvinoff et al. 2004). Neutralizing antibody was found targeted to epitopes in the hypervariable region of the E2 envelope protein and elsewhere in the viral envelope (Bartosch et al. 2003). Neutralizing antibodies elicited by infection with genotype 1a was able to cross-neutralize pseudotypes generated from other strains of genotype 1a, as well as of genotype 1b. Despite these new observations, it remains unclear whether B-cell responses are important in controlling HCV replication in vivo, and contribute to virus clearance on initial infection or subsequently.

T-cell responses to HCV infection

A vigorous cellular immune response to HCV can be detected in infected individuals. T-helper cells obtained from peripheral blood of most HCV-infected people proliferate when exposed to recombinant HCV proteins, such as core, E1, E2, NS3, NS4, and NS5 (Botarelli et al. 1993; Ferrari et al. 1994; Schupper et al. 1993). Indeed, unlike B-cell responses, recovery from infection is associated with stronger and more sustained cytotoxic T lymphocyte (CTL) responses around the time of primary infection than in those who become persistently infected (Cooper et al. 1999; Thimme et al. 2001; Lechner et al. 2000). Proliferative responses were detected more frequently in individuals who had cleared infection, suggesting that the cellular immune response is important in the control of virus replication in vivo. In the East German women who lost HCV antibody reactivity on resolution of active infection (see previous section), all retained HCV-specific helper and cytotoxic T-cell responses more than 10 years after clearance of viremia (Takaki et al. 2000).

Proliferative responses of T-helper cells obtained from the liver showed a highly restricted reactivity to NS4, compared with the responses found in mononuclear cells of peripheral blood (Minutello et al. 1993). CTLs from the liver and peripheral blood of HCV-infected individuals react with a wide range of epitopes from both structural and nonstructural proteins (Koziel et al. 1992, 1993; Cerny et al. 1995b; Battegay et al. 1995). CTLs are likely to be important in both the control of, and recovery from, HCV infection and in the pathogenesis of liver disease.

Mechanism of persistence

Although humoral and cellular immune responses to HCV occur upon infection, these seem to be unable to clear the infection (see Chronic hepatitis) or to protect an individual from reinfection. For example, it is possible to reinfect chimpanzees repeatedly with the same strain of HCV (Prince et al. 1992; Farci et al. 1992), producing hepatitis de novo similar to that observed on primary infection. However, chimpanzees who have cleared previous infection are partly or completely resistant to reinfection with the homologous or heterologous genotypes of HCV (Lanford et al. 2004), possibly the result of strong T-cell memory responses characteristic of resolved infections. Repeated exposure of humans to HCV may also produce recurrent episodes of infection, observed for example in thalassemic patients transfused with HCV-contaminated blood (Lai et al. 1994). Infection with more than one HCV genotype is frequently observed in other multiply exposed individuals, such as hemophiliacs (Jarvis et al. 1994). Persistence of HCV and the observed susceptibility of individuals to reinfection occur despite the existence of neutralizing antibody responses to HCV, that which often shows cross-reactivity to other HCV genotypes.

Immune escape

Several observations suggest that many of the amino acid changes that occur during HCV infection are driven sequentially by selection pressures that prevent peptide binding to MHC class I or II alleles, or prevent

neutralization by antibody responses to the virus. The envelope proteins are the principal targets of humoral immune responses, and it has been hypothesized that changes in E1 and E2 alter the antigenic properties of the proteins, thereby allowing the virus to 'escape' from neutralizing antibodies (Weiner et al. 1992). In this model, persistent virus replication is achieved by continuous diversification of HCV in regions particularly sensitive to neutralizing antibody; this hypothesis is supported by the observation that much of the variability in the *E2* gene is concentrated in discrete 'hypervariable' regions (Weiner et al. 1992; Hijikata et al. 1991a; Kato et al. 1992), that may be in close proximity to parts of the protein involved in virus/cellular receptor interactions, as in influenza A virus. Supporting this hypothesis is the observation that sequence change was slower in individuals with defects in B-cell immunity (Booth et al. 1998), where there would be reduced immune selection on B-cell epitopes. Similarly, the strong and sustained CTL responses in primary HCV infection may drive sequence change in those who became chronic carriers (Chang et al. 1997; Cantaloube et al. 2003; Sheridan et al. 2004). Immune selection may also underlie the high degree of sequence variability between and within genotypes in the envelope and other regions of the genome such as NS5A.

Mechanism of liver damage

It is likely that direct cytopathic infection of cells in the liver, as well as damage secondary to the inflammatory process contributes to HCV-associated liver disease. Evidence that HCV replication itself is directly cytopathic includes the clinical observation of more severe and rapidly progressive liver disease in individuals who are immunosuppressed and who have high levels of viremia. Secondly, there is generally a direct correlation between clearance of viremia and normalization of ALT levels in people who respond to interferon treatment (see Progression of disease), implying that it is virus replication itself that is responsible for the hepatitis. These observations contrast with HBV, in which successful treatment with interferon is thought to result from augmenting CTL activity in the liver and is associated with elevated ALT levels and a transient exacerbation of hepatitis.

Immunopathology may result from cytotoxic T cell reactivity against HCV-infected cells, or the process of HCV infection might trigger an autoimmune disease in which uninfected cells become the targets. The development of an immune response to the cellular protein, GOR, in association with HCV infection could be an example of this process (Mishiro et al. 1991). Understanding the mechanism of liver disease would assist the rational development of HCV treatment (see Antiviral treatment). Standard antiviral treatment schedules including interferon-α have been shown to clear hepatitis in some individuals (Davis et al. 1989; Di Bisceglie et al. 1989), although it is not known whether it acts as a virucidal agent or if its action depends on its immunomodulatory properties such as the upregulating expression of MHC class I on hepatocytes.

Extrahepatic manifestations

In a few infected patients, HCV may be responsible for extrahepatic clinical manifestations and disease (Martin 1993). The underlying pathogenic mechanisms for these extrahepatic manifestations are varied and often poorly understood. There are at least three mechanisms by which HCV could cause such disease. First, it is possible that HCV is capable of cytopathic replication in cell types outside the liver. Secondly, HCV may trigger an autoimmune process that is directed against antigens expressed on nonhepatic cells. Thirdly, persistent HCV infection could lead to immune-complex formation with antibodies followed by deposition in small vessels.

Methods to detect extrahepatic replication of HCV include the detection of HCV protein expression by immunocytochemistry and the detection of replication intermediates by strand-specific PCR. Simple detection of HCV RNA sequences is generally insufficient evidence, as HCV may nonspecifically associate or bind to cells such as macrophages or B lymphocytes in the peripheral circulation. The detection of HCV in tissues may reflect a process of immune-complex deposition rather than replication.

Many published studies reporting the detection of replication intermediates (complementary RNA sequences) in a range of cell types used methods that were insufficiently specific. However, using a PCR-based method for detection of sequences from the core region, Lerat and co-workers (1996) reported that replication may occur in certain lymphoid cells. In contrast, no evidence for extrahepatic replication was observed in chimpanzees, using a similar assay (Lanford et al. 1995). In other studies, HCV was detected in lymphocytes infiltrating the liver by immunocytochemical and hybridization methods (Blight et al. 1994).

HCV infection is clearly associated with certain types of vasculitis and glomerulonephritis, which are caused by immune complex deposition. In southern Europe, HCV infection seems to be associated with porphyria cutanea tarda, a potentially disfiguring skin condition associated with an acquired deficiency of the liver enzyme uroporphyrinogen decarboxylase (Herrero et al. 1993). There is a putative association of HCV with other nonhepatic conditions, including Sjögren's syndrome, rheumatoid arthritis, pulmonary fibrosis, corneal ulceration, aplastic anemia, and non-Hodgkin's lymphoma. Vasculitis and glomerulonephritis are more frequent in patients with established cirrhosis.

HCV is classically associated with the small vessel vasculitis known as 'essential mixed cryoglobulinemia' (Agnello et al. 1992) (also observed in patients with chronic HBV infection). Laboratory features include the presence of rheumatoid factor activity in serum, the presence of a cryoprecipitate, complement activation, and proteinuria/hematuria. The most important clinical manifestation is purpura, which is nearly always present, and 25–50 percent of patients have renal disease. Most patients have constitutional symptoms, especially weakness and arthralgia; Raynaud's phenomenon, Sjögren's syndrome, and neuritis may also be present. Purpura may resolve with interferon-α therapy (Misiani et al. 1994).

HCV infection is also associated with membranoproliferative glomerulonephritis (MPGN) type 1 (Johnson et al. 1993), which is a consequence of immune complex deposition in the glomerular capillaries. It is associated with serum rheumatoid factor positivity, with complement activation, and with cryoglobulinemia. MPGN is classically associated with heavy proteinuria, often in the nephrotic range (more than 3.5 g per 24 h), whilst creatinine clearance is modestly impaired. Treatment with interferon-α reduces urinary protein loss, but the impact of treatment on renal outcome is uncertain (Johnson et al. 1993). It should be emphasized that HCV infection is a relatively common cause of mixed essential cryoglobulinemia and MPGN, but that HCV infection is rarely complicated by these conditions.

EPIDEMIOLOGY

Parenteral routes of transmission

In western Europe, Australia, and North America, most HCV-infected patients have a history of percutaneous exposure to the virus, and most are (or have been) injecting drug users. The seroconversion rate of IDUs has been estimated at 20 percent per year, so long-term drug users are almost invariably HCV-infected. Drug use was uncommon before the 1960s, so drug users tend to be younger than patients infected through transfusion and are also more likely to be infected with genotypes other than type 1b. Most drug users have asymptomatic infection with no history of jaundice, but have chronic hepatitis whereas some have developed cirrhosis and a few have progressed to liver failure. Since drug use is usually associated with HCV acquisition at a fairly young age (in comparison with transfusion-acquired infection) and thus with a slower rate of progression to cirrhosis, few IDUs would progress to liver failure within two (or even three) decades of infection. However, because of the lower average age of IDUs, their life expectancy from the time of infection is five or six decades; despite less aggressive infection, many will survive long enough to develop significant liver disease.

In the 1960s, clotting factor concentrates became more widely used and most patients with coagulation disorders became infected with HCV. Thousands of donors contribute to each factor concentrate, so contamination with HCV and HIV was very likely. Many recipients were young, and most developed liver disease. Some have progressed to liver failure, and died or undergone liver transplantation (Telfer et al. 1994), although for many the prognosis may have been affected by HIV coinfection. Since the introduction of effective virus inactivation for blood products in the 1980s, factor concentrates have not transmitted HCV infection.

Repeated exposure to blood and blood products (in the absence of serological screening and/or heat treatment) is clearly associated with a high risk of HCV exposure and infection. The risk associated with exposure to a single donor product (e.g. one unit of blood) was already diminishing before the availability of specific serological tests for HCV, but varied significantly from country to country. Mercenary donors are more likely to be HCV-infected, and the prevalence of donor seropositivity was significantly reduced when 'self-exclusion' policies were introduced to combat the risk of HIV transmission. For example, by the mid-1980s fewer than 1 percent of UK donors were HCV-positive (Contreras et al. 1991).

Screening of blood donors for antibodies to HCV was introduced by most blood transfusion services in 1990 and 1991. Blood donors are clearly not representative of the entire population, but scrutiny of HCV-positive donors provides some insights into the epidemiology of this infection (Alter 1995; Mansell and Locarnini 1995). The lowest seroprevalence is observed in Scandinavia and in the UK with slightly higher prevalence in North America, western Europe, and Australia. Prevalence is intermediate in eastern and southern European countries, even higher in Japan, and most prevalent in Middle Eastern (especially Egyptian) blood donors. When donor seroprevalence is low, the seropositive donor is more likely to have an identifiable risk factor for parenteral exposure (Mutimer et al. 1995b), but as donor seroprevalence increases, prior parenteral exposure becomes more difficult to identify (Esteban et al. 1991).

In countries of low and intermediate seroprevalence, seropositive donors frequently admit to prior intravenous drug use; sometimes a single exposure, 20–30 years previously. Investigation of seropositive donors confirms that the majority are viremic, and that many have chronic hepatitis and cirrhosis (Esteban et al. 1991; Irving et al. 1994; Mutimer et al. 1995b). As seropositive donors are excluded from the blood donor panels, the frequency of seropositivity falls; in the UK, for instance, true seropositivity fell from an initial rate of 0.60 to 0.15 percent. To further reduce the already small risk for transfusion-associated infection, some blood transfusion organizations have introduced nucleic acid testing of

donated products for HCV RNA (see Blood donor screening by PCR and related methods).

Transplanted organs may also transmit HCV infection, and studies in the USA and UK suggest that organ donors have a higher seropositivity rate than blood donors (Pereira et al. 1992; Wreghitt et al. 1994). The majority of high-risk blood donors are subject to self-exclusion, an option that is not available to the brain-dead organ donor. In one study, six of 554 (1.08 percent) British organ donors were HCV-seropositive in the screening assay, and four of these six infected an organ recipient.

Other routes of transmission

Most studies (and this discussion) have focused on groups at risk for HCV infection as a result of percutaneous exposure. These groups include IDUs, transfusion recipients (especially recipients of pooled plasma products such as factor VIII, anti-D) (Power et al. 1995), immunoglobulin (Healey et al. 1994), transplant recipients, hemodialysis patients, and healthcare workers (Zuckerman et al. 1994; Klein et al. 1991). Tattooing and acupuncture may also be responsible for some percutaneous exposure, and in countries of high prevalence the use of unsterilized needles for cultural rituals, medical treatment, or vaccination programs may result in HCV infection. For example, bilharzia treatment using reusable and unsterile needles in the past has frequently been suggested as a cause for the very high population prevalence of HCV infection in Egypt.

However, for many people, overt percutaneous exposure cannot be identified. For example, detailed questioning failed to identify such a cause of infection in approximately one-third of HCV-infected Scottish blood donors (Crawford et al. 1994), and this has prompted the search for other routes of transmission. There seems to be a low risk of infection associated with sexual contact with an HCV carrier. Evidence suggesting that sexual transmission may occur includes the observation that HCV seems to be more common in people who have multiple sexual partners, such as prostitutes and male homosexuals (Tedder et al. 1991; Buchbinder et al. 1994). Large-scale studies of sexual partners of HCV-positive people generally show a slightly increased likelihood of HCV infection compared with the background population (Daikos et al. 1994; Soto et al. 1994; Cerny et al. 1995a; Utsumi et al. 1995), although in two studies (both of wives of hemophiliacs) no transmissions were documented (Bresters et al. 1993a; Hallam et al. 1993). Other sexually transmitted diseases may facilitate sexual transmission of HCV, as is the case for HIV.

Barrier methods of contraception/protection are likely to prevent transmission of HCV. For casual sexual contact, such precautions are mandatory (for mutual protection from all sexually transmitted infection). When one member of a long-term sexual partnership is identified as a carrier of HCV, both members should be counseled appropriately. The other partner might request serological testing. It seems likely that most couples will not elect to change their sexual practices. HIV/HCV coinfection may increase the risk for sexual transmission of HCV, and this may be a function of the higher HCV titer which is measured in the blood of HIV coinfected patients. In this setting, barrier methods of protection are clearly appropriate.

Early studies documented a low but detectable rate (5–15 percent) of mother-to-child transmission of HCV (Novati et al. 1992; Lam et al. 1993; Wejstal et al. 1992). However, it remains unclear whether this is true vertical transmission (in utero or during birth) or if it occurs through close contact (and possibly unapparent parenteral exposure) during the postnatal period. The risk of infant infection seems to be increased when the mother is HIV/HCV coinfected, and again this may be secondary to higher virus loads associated with immunosuppression (Ohto et al. 1994). HCV transmission may also occur as a result of 'household' contact. In this setting, it is almost impossible to distinguish vertical and sexual transmission from other 'household' contact. The parents of an index case are most likely to be infected. Relative risk is lower for spouses and siblings, and lowest for children of the index case.

In western Europe, most patients with liver failure will be referred for liver transplantation. Examination of the liver transplant experience during the 1980s and 1990s suggests that HCV has been an uncommon cause of liver failure in the UK. This observation suggests the relatively recent introduction of this virus into the western European population. It is evident that HCV infection was rare in western Europe and in North America before the emergence of intravenous drug use in the 1960s. However, it is calculated that the USA experienced an annual incidence of 15 cases per 100 000 population during the 1980s (Alter 1995), predicting a national reservoir of HCV carriers of as many as 3.5 million people. That HCV currently poses less of a clinical problem in the USA and northern Europe than in southern Europe, Japan, and the Middle East is probably due to its later introduction in these populations. In the future, all countries can expect higher rates of significant liver disease from HCV, including liver cancer.

Geographical distribution of HCV genotypes

Some genotypes of HCV (types 1a, 2a, 2b) show a broad worldwide distribution, while others such as type 5a and 6a are only found in specific geographical regions. HCV infected blood donors and patients with chronic hepatitis from countries in western Europe and the USA is frequent with genotypes 1a, 1b, 2a, 2b, and 3a, although

the relative frequencies of each may vary, such as the trend for more frequent infection with type 1b in southern and eastern Europe. In many European countries, genotype distributions vary with age of patients, reflecting rapid changes in genotype distribution with time within a single geographic area.

A striking geographical change in genotype distribution is apparent between south east Europe and Turkey (both mainly type 1b) and several countries in the Middle East and parts of north and central Africa where type 4 predominates. For example, a high frequency of HCV infection is found in Egypt (20–30 percent (Saeed et al. 1991; Darwish et al. 1993; Kamel et al. 1992)), of which almost all correspond to type 4a (Simmonds et al. 1993b; McOmish et al. 1994). HCV genotype 5a is frequently found amongst NANBH patients and blood donors in South Africa, but is found only rarely in other parts of Africa or elsewhere.

In Japan and Taiwan and probably parts of China, genotypes 1b, 2a, and 2b are the most frequently found. Infection with type 1a in Japan appears to be confined to hemophiliacs who have received commercial blood products, such as factor VIII and IX clotting concentrates, provided in the USA. A genotype with a highly restricted geographical range is type 6a which was originally found in Hong Kong, where approximately one-third of anti-HCV-positive blood donors are infected with this genotype, as are an equivalent proportion in neighboring Macau and Vietnam.

Molecular epidemiology of HCV infection

Persistent infection with HCV entails continuous replication of HCV over years or decades and the large number of replication cycles, combined with the relatively error-prone RNA-dependent RNA polymerase leads to measurable sequence drift of HCV over time. For example, over an 8-year interval of persistent infection in a chimpanzee, the rate of sequence change for the genome as a whole was 0.144 percent per site per year (Okamoto et al. 1992a), similar to the rate observed in the 5′ half of the genome over 3 years in a human carrier (0.192 percent (Ogata et al. 1991)). Using this molecular clock, it is in principle possible to calculate times of divergence between HCV variants, and therefore establish their degree of epidemiological relatedness. Similarly, phylogenetic analysis of sequences from individuals exposed to HCV can be used as evidence for a common source of infection. Sequence comparisons in variable regions of the HCV genome, such as E2 and NS-5, have been used to document transmission between individuals, and to explore the possibility of nonparenteral routes of transmission such as mother to child (Weiner et al. 1993), within families (Honda et al. 1993), and by sexual contact (Rice et al. 1993; Setoguchi et al. 1994; Healey et al. 1995). For example, clustering of HCV sequences into a single phylogenetic group amongst recipients of an HCV-contaminated blood product (anti-D immunoglobulin) was still apparent 17 years after exposure (Power et al. 1995).

DIAGNOSTIC TESTS FOR HCV INFECTION

Antigens

Antibody reactivity can be detected to several linear and conformational epitopes present on both structural and nonstructural proteins of HCV. Recombinant proteins or peptides containing these antigenic regions are used for serological tests for antibody to HCV (see Figure 1), and much of our current knowledge of antibody responses to HCV has arisen from research aimed at improving antibody tests to enable effective screening of blood donors and diagnosis in patients. Cytotoxic T-cell and proliferative responses have also been detected to several peptides or recombinant proteins.

NS4

The original HCV-specific clone isolated from chimpanzee plasma was expressed in *E. coli*, the derived protein of 42 amino acids (1 694–1 735) being encoded by the NS4 region of the genome (see Figure 1). A larger clone (c100-3) assembled from several overlapping clones was expressed in yeast and formed the basis of the first commercial tests for HCV antibody. The antigen was derived from amino acids 1 569–1 931, thus spanning a small section of the NS3 region, as well as almost all of 4a and 4b. Despite its being a somewhat unnatural hybrid, use of the antigen in screening assays provided the first evidence of the importance and widespread occurrence of HCV infection. Antigen from NS4 is present in all current screening enzyme-linked immunosorbent assays (ELISA), whether singly, in combination with NS3 or in synthetic peptide form. Likewise, all commercial supplementary immunoblot assays contain NS4 components either as recombinant antigens (Chiron RIBA-2, Murex western blot, Abbott Matrix) or as synthetic peptides (Chiron RIBA-3, Organon Liatek III), which show improved specificity.

Many of the antigenic determinants in the NS4 region show considerable amino acid sequence variability between genotypes of HCV; serological reactivity to the 5-1-1 protein is absent or weak in samples from individuals infected with types 2 or 3 (Chan et al. 1991; McOmish et al. 1993). Indeed, type-specific reactivity to NS4 forms the basis for serological typing assays that can differentiate between the antibody responses elicited

by different genotypes (Simmonds et al. 1993c; Tanaka et al. 1994; Bhattacherjee et al. 1995).

CORE

Infection with HCV elicits a strong humoral antibody response to the core protein, predominantly to a series of linear epitopes at the N terminus of the protein (Nasoff et al. 1991; Sallberg et al. 1992). The incorporation of either recombinant proteins or synthetic peptides from the core region led to a great improvement in the sensitivity of HCV screening assays for HCV antibody (Chiba et al. 1991; Hosein et al. 1991; Ishida et al. 1993). Commercial screening assays have mainly used recombinant antigen (c22) comprising a major portion of the core region (Ortho 3.0, amino acids 2–120; Abbott 3.0 amino acids 1–150, Murex VK47/48), but the Sanofi Pasteur New Antigen ELISA instead uses two synthetic peptides. Similarly the most recent version of the Chiron RIBA supplementary test, RIBA-3, replaces the recombinant antigen of RIBA-2 with one synthetic peptide utilizing amino acids 10–53 resulting in much improved specificity. Liatek III incorporates two peptides from the core region, while the Murex and Matrix blots retain recombinant antigen. The amino acid sequence of the core region is highly conserved between different genotypes of HCV and consequently, most epitopes are cross-reactive, with similar reactivity observed for individuals infected with different genotypes. Those differences that do exist have been exploited in a typing assay to distinguish antibody to type 1 and type 2 (Tsukiyama Kohara et al. 1993), although the core proteins of types 1, 3, 4, 5, and 6 are probably too similar to allow this assay to be extended further.

NS3

Antibody to epitopes in the NS3 region of HCV is produced early in infection and is the first detectable in many seroconversions while in established infections, it is as common as core antibody and more frequently seen than antibody to c100 or NS5. Several manufacturers were slow to realize the benefits of incorporating antigen from this region in their screening tests and some early tests had either none or too little to be effective, thus missing viremic samples (Dow et al. 1993; Courouce et al. 1995). All current screening assays contain recombinant antigen either singly or in combination with NS4 sequences (Ortho 3.0) or c22 sequences (Abbott 3.0). In Ortho 3.0, the NS3 antigen has been modified biochemically to give increased sensitivity for NS3 antibody enabling earlier detection of seroconversions (Courouce et al. 1994) and the detection of chronically infected patients with low levels of NS3 antibody (Dow et al. 1996). All supplementary immunoblots utilize recombinant antigen from the NS3 region. Changes in the concentration of the coating antigen, c33c, in RIBA-3 have made this test particularly sensitive to NS3 antibody (Courouce et al. 1994).

NS5

Recombinant antigen from the NS5 region was incorporated in the first Wellcozyme/Murex ELISA, but appeared in the Abbott and Ortho tests only with the third generation. Both Abbott and Ortho tests use recombinant antigen from almost the whole NS5 region (amino acids 2 054–2 995), whereas in the Murex assay the size of the recombinant protein has been reduced with each succeeding test and the present Murex test contains protein from only a small segment of the NS5a region. Of the current supplementary tests, RIBA-3 and Murex blot incorporate recombinant antigen, whilst in Liatek III it is a peptide. The contribution of NS5 antigens to improved specificity and sensitivity in screening tests has been difficult to quantify. Seroconversions in which antibody to NS5 occurs first are rare, and no chronically infected patient has been found with NS5 antibody as the only marker. Specificity is not enhanced, as these tests produce significant numbers of a new population of RIBA-3, NS5 indeterminates, that are all PCR-negative; it is generally agreed that improvements in sensitivity with third generation ELISAs are associated with improved NS3 antibody detection. Inclusion in supplementary tests may have value in providing extra evidence of positivity, but the poorer specificity of NS5 antigens in these tests may compromise interpretation.

The battery of antigens used in current screening and supplementary tests will detect the vast majority of chronic, established infections. Where improvement is required is in earlier detection of seroconversion. In addition, manufacturers will need to address the problems associated with antigenic variation between different genotypes. All current screening tests are based on genotype 1 antigens, and while this genotype is widely distributed throughout the world, it is not the only, nor predominant, genotype in many countries. Diminished sensitivity to genotype 3 is seen in RIBA-2 (Damen et al. 1995) and to a lesser extent RIBA-3 (Dow et al. 1996), and this may also be reflected in the sensitivity of screening ELISAs (Neville et al. 1997). Future tests will need to incorporate antigens from various genotypes to ensure an optimal sensitivity for all (response to all).

First generation tests

The first test to specifically detect antibody to HCV was based on the c100-3 antigen (Kuo et al. 1989). Use of this simple, single antigen test in ELISA format confirmed that HCV was the major cause of post-transfusion non-A, non-B hepatitis in the USA and elsewhere (Alter et al. 1989; Esteban et al. 1990; Hopf et al. 1990; van der Poel et al. 1990), and that prevalence of

antibody to the c100-3 antigen was high in hemophiliacs (Brettler et al. 1990), drug abusers (Esteban et al. 1990), and in patients with hepatocellular carcinoma (Colombo et al. 1989; Bruix et al. 1989; Nishioka et al. 1991). The simple first generation tests for anti-HCV were valuable in establishing the importance of HCV infection worldwide, its identity with non-A, non-B hepatitis and its wider spread in the community usually via drug abuse.

Second generation tests

Once the HCV genome had been fully sequenced and the structural and nonstructural regions identified (Choo et al. 1991), several manufacturers developed second generation tests which included antigen to the core region (c22c) and one or more further nonstructural regions NS3 (c33), NS4 (c100-3), or NS5. The most widely used tests, those manufactured by Ortho Diagnostics and Abbott Laboratories, contained four antigens (5-1-1, c100-3, c22, and c33c). These tests were significantly more sensitive and specific than their first generation equivalents and their use for screening blood donations virtually eliminated post-transfusion HCV infection as a complication of transfusion (Aach et al. 1991). The improved sensitivity allowed earlier detection of seroconverting patients by some weeks (van der Poel et al. 1992) (Figure 7), and the detection of HCV antibody positivity in donors and patients previously screened as negative in the first generation assays (Yuki et al. 1992; Aoki et al. 1994).

The same series of antigens present in the second generation Ortho and Abbott tests were incorporated in an improved RIBA test by Chiron, RIBA-2 – often referred to as 4-RIBA in early reports. In this test, two bands were required for positivity, one band was termed an indeterminate result. This test was very effective in identifying ELISA false positives in first generation tests where it represented a truer confirmatory test in that it incorporated new antigens (c33c and c22) not present in the ELISA screening test (van der Poel et al. 1991; Courouce et al. 1991). There was a strong correlation between RIBA-2 positivity and viremia as detected by PCR tests (Garson et al. 1992; Larsen et al. 1992; Bresters et al. 1993b; Yun et al. 1993). The interpretation of the RIBA-2 indeterminate result, of which there were significant numbers in low risk populations such as blood donors, and the advice to give the patient/donor has proved controversial. Only PCR testing could clearly define the association with HCV infection, but few PCR-positive indeterminate individuals were found in low risk groups (Follett et al. 1991), and these were almost always associated with the c22 or c33 band (Bresters et al. 1993b). Most of the indeterminate reactivity to c22 could be shown to be due to nonspecific cross-reactivity to restricted N-terminal regions of the core antigen (Tobler et al. 1994). RIBA-2 is less likely to 'confirm' samples from individuals infected with type 1 genotypes (Damen et al. 1995; Dow et al. 1996) due to the predominantly type-specific reactivity to the NS3 and particularly NS4 proteins.

Third generation tests

Current tests for HCV antibody omit the original 5-1-1 antigen and include the NS5 antigen, although the value of this addition is questionable. Third generation ELISAs are more sensitive and detect seroconversions significantly earlier (Courouce et al. 1994; Uyttendaele et al. 1994; Vrielink et al. 1995b), but this improvement is associated with an improved reactivity of the c33 antigen component, and not with the presence of the NS5 antigen (Barrera et al. 1995; Lee et al. 1995; Courouce et al. 1995). Counterbalancing this improvement in sensitivity is some loss in specificity related to a new population of indeterminate samples associated with the NS5 antigen; NS5 indeterminate samples are invariably PCR-negative.

Significant changes were made in the updated RIBA test, RIBA-3 introduced in Europe in 1994. As well as incorporating NS5 recombinant antigen, RIBA-3 incorporates synthetic peptides for c22 and c100 replacing the recombinant antigens of RIBA-2. Improved sensitivity is again apparent and associated with the NS3 component (Courouce et al. 1994; Zaaijer et al. 1994). Specificity is greatly enhanced and use of RIBA-3 resolves many of the nonspecific problems associated with RIBA-2 (Dow et al. 1993; Zaaijer et al. 1994). Viremia and RIBA-3 positivity are strongly associated when three and four bands are present but much less so for two-band positives (Dow et al. 1996). Care is required with all PCR-negative, two-band positives, and further ELISA and supplementary tests are necessary before equating such reactivity with HCV infection, as false positives are known (Damen et al. 1995; Dow et al. 1996). Although RIBA-3 has improved sensitivity for genotypes 2 and 3 compared to RIBA-2, some lack of sensitivity to type 3 is still apparent (Dow et al. 1996).

Alternative antigen tests

Manufacturers of HCV serological assays use recombinant proteins and peptides from type 1a, although some in the USA and Europe have cloned HCV independently and produced commercial products based upon differently sourced HCV antigens. These produce tests which show different nonspecific reactivity on screening from that produced by the Chiron-based tests. Consequently, using one such test to screen ELISA reactives from an alternatively sourced antigen test could help to resolve many of the specificity problems associated with HCV ELISAs (Craske et al. 1993). However, patent

regulations have forced the removal of these tests from the marketplace and only two commercially available ELISAs with independently sourced antigen are widely available (Murex, Sanofi-Pasteur).

Alternative supplementary tests to the Chiron RIBA products are produced by several manufacturers based either on the Chiron isolate (Abbott Matrix) or independently sourced antigen (Murex Western Blot, Organon Liatek line immunoassay, Diagnostic Biotechnology blot assay). The Liatek assay most closely approaches RIBA-3 for sensitivity and specificity (Zaaijer et al. 1994). A new antigen from the E2/NS1 region of the genome incorporated into this test did not contribute to earlier detection of HCV antibody in seroconversion. Whether use of recombinant envelope antigens is helpful must await further studies. All these supplementary tests can be valuable when investigating a doubtful ELISA or RIBA result.

Genome detection

Detection of the HCV genome is indispensable for the accurate diagnosis and the complete characterization of HCV infection in a patient. Our knowledge of HCV infection has benefited immensely from use of the PCR for HCV RNA. A PCR test or equivalent is the only method of confirming HCV infection in equivocal ELISA reactives such as RIBA indeterminates, and is the only method for distinguishing between chronic and resolved infections. Retrospective studies on recipients of blood donations known to be RIBA positive or indeterminate indicate that transmission is only associated with PCR positivity (Vrielink et al. 1995a).

Detection of the virus genome is the only reliable method of following the effect of treatment with antiviral drugs on patients with chronic infection since normalization of biochemical liver function tests is not always associated with virus clearance. Similarly, chronic HCV infection with associated disease is known in patients with normal liver functions tests (Nalpas et al. 1995). Patients in early seroconversion and patients with an impaired immune response can appear falsely negative in current HCV ELISA tests and PCR is necessary for complete diagnosis in such cases (Bukh et al. 1993b). In chimpanzees, viremia can be detected by PCR within the first week after infection (Beach et al. 1992; Shimizu et al. 1990) providing a long window period when the blood is infectious but no antibody can be detected.

Blood donor screening by PCR and related methods

One of the most used applications for HCV PCR and related nucleic acid amplification tests (NAT), such as transcript-mediated amplification (TMA), has been its adoption for routine testing of blood donors in addition to serological tests. The introduction of NAT allows acutely infected donors to be detected before their seroconversion for antibody to HCV (Figure 57), and thus further reducing or eliminating the potential for transfusion-transmitted HCV infections. The introduction of NAT screening was prompted by estimates that between one in 100 000–200 000 donations would be intercepted by NAT in this 'window' period, thus preventing large numbers of transfusion-transmitted infections (Courouce and Pillonel 1996; Schreiber et al. 1996). The introduction of very large-scale NAT screening in the USA, other western countries, and Japan has led to impressive progress in establishing the infrastructure and laboratory facilities for testing, as well as driving the evolution of pooled testing in which donations are combined into small pools before testing, and positives resolved by the creation of cross-pools. In practice, donor yield has proved divergent from original estimates, with incidences of less than one per million HCV NAT-positive, seronegative donations being found in most countries. Despite the scope for HCV NAT to be combined with NAT-detection methods for other viruses currently screened for in blood donors by serology methods, the cost-effectiveness of this further testing is highly questionable (Roth and Seifried 2001; Marshall et al. 2004; Jackson et al. 2003; Simmonds et al. 2002).

HCV viral load measurement

A large number of methods have been developed in-house and commercially for the measurement of HCV viral loads in plasma, and potentially in other samples such as liver biopsy. The PCR can be made semi-quantitative by 'limiting dilution' of the test sample, in which titration of nucleic acid yields an endpoint that reflects the original concentration of HCV sequence in the original sample. These techniques require calibration against agreed HCV RNA standards (Saldanha et al. 1999), and can be time-consuming and impractical for large numbers of samples. Improvement in accuracy and practicality has been achieved through the development of competitive PCR, where the test sample is co-amplified with known amounts of a separate target sequence; the ratio of amplified HCV sequences to those of the competitive target can provide an accurate quantitation of HCV (Ravaggi et al. 1995). Because of the limited quantitative range of most tube-based quantitation methods, and the rapid plateauing of product quantity when amplification proceeds beyond the exponential phase, HCV quantitation may be more effectively carried out by real-time PCR methods (such as the LightCycler from Roche, and the TaqMan method from Perkin Elmer). These assays quantify target sequences through monitoring of product accumulation at each cycle of the amplification reaction. Product detection methods generally involve the specific detection of

amplified target sequences through hybridization to fluorimetric probes. For example, in the Lightcycler assay, two hybridization probes bind to adjacent sequences within the amplicon leaving one base in between, thus allowing fluorescence resonance energy transfer (FRET) between the donor fluor on the 3′ end of one probe and the acceptor fluor on the 5′ end of the other one. The TaqMan assay uses a single hybridization probe labeled with a reporter dye and an adjacent 'quencher' molecule that absorbs fluorescence. On binding to amplified sequences during PCR, the probe becomes degraded by exonuclease activity of *Taq* polymerase during copying, and releases the quencher and the reporter dyes into solution. Increases in the amount of amplified target therefore destroy quenching of the reporter dye, and lead to increases in fluorescent emission intensity.

For HCV, a non-amplification based method is widely used that uses highly sensitive branched DNA (bDNA) synthetic oligonucleotide probes which binds to viral RNA in the test sample that has been immobilized on a solid phase. The bDNA probes enable multiple copies of the alkaline phosphatase-labeled probe hybridize to each amplifier probe on the immobilized complex, which is detected by addition of a chemiluminescent substrate. Viral loads in the range from 1 000–10 million RNA copies/ml can be detected by this method.

The measurement of HCV viral load in plasma has a number of clinical applications and commercially available assays are widely used for prediction of treatment response, in combination with genotype determination (see Antiviral treatment). However, HCV viral loads do not predict the current severity of liver disease in infected individuals, or their likelihood of progression to clinically significant liver disease (Poynard et al. 1997). Viral load is, however, predictive of transmission risk in certain settings; for example, mother-to-infant transmission is strongly associated with higher viral loads. In the original study (Ohto et al. 1994), women who transmitted infection showed a significantly higher viral load ($10^{6.4\pm0.5}$ RNA copies/ml) than nontransmitters ($10^{4.4\pm1.5}$ RNA copies/ml).

HCV genotyping assays

Genotyping of HCV has become important in the treatment of infection (see Antiviral treatment), and for epidemiology tracing in studies of virus transmission. Genotyping assays are usually based upon the analysis of an amplified segment of the genome (such as the 5′ UTR, commonly used for virus detection and viral load monitoring; see Genome detection and HCV viral load measurement). The sequence of the 5′ UTR is highly conserved between genotypes, but genotype-specific differences comprising one or more substitutions can detected by probe hybridization (Stuyver et al. 1993a; Maertens and Stuyver 1998), by changes in restriction endonuclease sites (McOmish et al. 1993; Davidson and Simmonds 1998), or by direct sequencing of the 5′ UTR (Ross et al. 2000). As described above (see Recombination), few recombinant forms have been described to date, and for practical purposes it can be assumed that the 5′ UTR sequence predicts the genotype of the whole virus. Should more circulating recombinant forms be detected, then this assumption may have to be re-examined.

In general, the 5′ UTR is highly predictive of the genotype of the rest of the virus, reflected by the high concordance between the results of genotyping assays that use the 5′ UTR and the genotype identified by nucleotide sequencing in NS5B or other coding regions of the genome (Simmonds et al. 1993c; Tanaka et al. 1994; Lau et al. 1995a; Bhattacherjee et al. 1995; Zheng et al. 2003; Gault et al. 2003; Dansako et al. 2003). However, 5′ UTR-based assays have some limitations. For example, some genotype 6 variants found in South East Asia have 5′ UTR sequences identical to those of genotype 1a or 1b (Tokita et al. 1994a, 1995; Mellor et al. 1996), although their geographical restriction is therefore not a major problem for assays carried out for clinical purposes in the USA, other western countries, and Japan. More problematic is the inability of 5′ UTR-based assays to reliably differentiate different subtypes, such as 1a from 1b, and 2a from 2c. While such information may be relevant for epidemiological purposes, the major clinical use of genotyping assays is, however, for pretreatment prediction of response and dose scheduling of interferon therapy (see Antiviral treatment). For this application, differentiation of genotype 1 from 2 or 3 is generally all that is required, and existing assays are acceptably accurate. Serological typing methods are based upon the detection of type-specific antibody to epitopes of HCV that differ between genotypes (Bhattacherjee et al. 1995; Simmonds et al. 1993c). Such assays may show high sensitivity and reproducibility, although antigenic similarity currently precludes the separate identification of subtypes, such as 1a and 1b or 2a and 2b using the NS4 peptides alone. Serological typing assays provide the only method to determine the originally infecting genotype of individuals who have cleared viremia.

Commonly used commercially available assays for HCV genotype include the VERSANT HCV genotype assay, the TRUGENE HCV 5′ NC genotyping kit (both from Bayer) and the Murex HC03 serotyping assay. The VERSANT assay is a hybridization assay using line probes immobilized on to nitrocellulose strips that capture labeled target sequences in a number of simultaneous highly sequence-specific hybridization reactions (InnoLipa II) (Stuyver et al. 1993a; Maertens and Stuyver 1998). The TRUGENE assay automates the direct sequencing of amplicons from the 5′ UTR, although this approach can in principle be extended to

other regions of the genome that may be more informative if reliable identification of different subtypes is required. The HC03 serotyping assay identifies type-specific antibody to genotypes 1–6 by ELISA using peptide from regions in the NS4A and 4B regions.

CONTROL OF HCV INFECTION

Antiviral treatment

Since the introduction of serological screening of blood products, acute HCV infection is seldom encountered in clinical practice. Small studies show that treatment with interferon (interferon-α and -β have been used) during the acute phase may be beneficial, and substantially reduces the risk of chronic infection (Omata et al. 1991; Lampertico et al. 1994; Jaeckel et al. 2001).

Combination antiviral therapy with interferon-α and ribavirin can cure a substantial proportion of patients with chronic HCV infection (Figure 10). Most clinicians now use a pegylated form of interferon-α. 'Pegylation' involves the covalent attachment of a molecule of polyethylene glycol to an interferon-alpha molecule. This alters the pharmacological properties of the interferon-alpha to prolong the plasma half-life. As a consequence, pegylated interferons are administered only once weekly. In comparison to the combination of nonpegylated interferon and ribavirin, the use of pegylated interferon and ribavirin is associated with significantly enhanced cure rates (Manns et al. 2001; Fried et al. 2002). Favorable predictors of response to antiviral treatment include HCV genotype (good response for types 2 and 3, inferior for type 1), low pretreatment viral titer, no or minimal liver fibrosis, female gender, and low BMI. In clinical trials, cure rates of approximately 50 percent have been achieved for genotype 1 patients and 80 percent for genotypes 2 and 3. Response to treatment can be monitored by quantitative HCV serum measurements.

Interferon-α and ribavirin may also be indicated for the treatment of extrahepatic manifestations of HCV infection, such as essential cryoglobulinemia and glomerulonephritis.

The mechanism by which different genotypes might differ in responsiveness to treatment remains obscure, particularly as it is still unclear whether interferon acts as an antiviral or as an immunomodulatory agent. Infection with genotypes 1, 2, and 3 all cause progressive liver disease with no evidence that type 1 is significantly more pathogenic than other variants. Similarly, studies of both blood donors and patients have indicated that virus loads are similar among individuals infected with different genotypes (Lau et al. 1995b). It is therefore unlikely that the greater response rates achieved with types 2 and 3 are simply secondary to differences in disease severity. Sequence comparisons demonstrated a

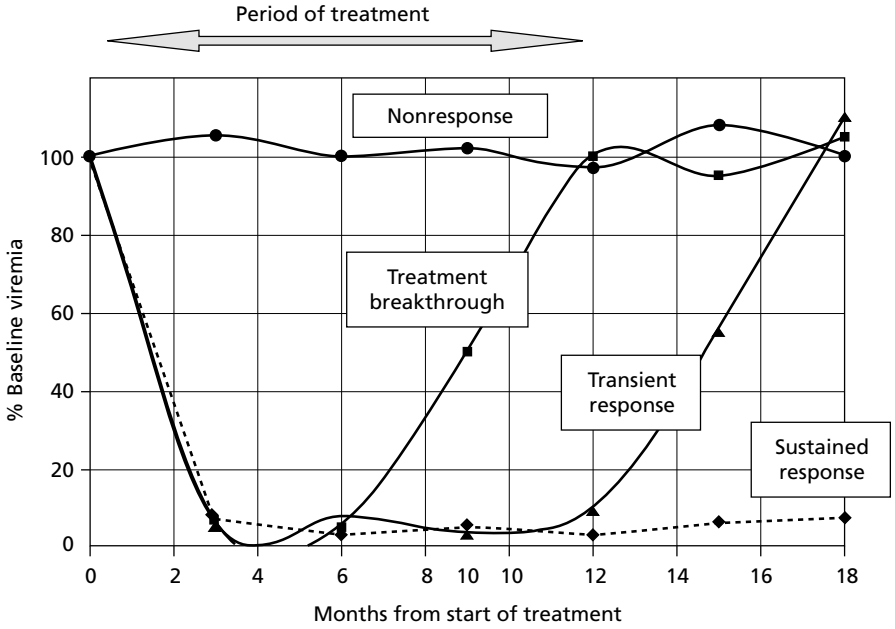

Figure 10 *Pattern of virological responses to interferon-α/ribavirin therapy. The response to HCV therapy is highly variable between individuals. A proportion of individuals, particularly those with genotype 1 infection will show no clearance of viremia after institution of therapy. Of those that clear viremia, three outcomes are commonly observed. Some individuals will relapse during treatment (treatment breakthrough), or after the end of the treatment schedule (transient response). Individuals who clear viremia and remain nonviremic in plasma over 6 months follow up (sustained response, dotted line) have cleared infection permanently. Clearance of viremia is almost invariably associated with normalization of biochemical markers of liver disease, such as ALT.*

cluster of amino acids in the NS5A region that consistently differed between responders and nonresponders to interferon treatment (Enomoto et al. 1995) (see Nonstructural proteins, NS5A).

The prospects for effective treatment of HCV is further enhanced by current progress in the development of several series of new antiviral agents that target the viral protease and RNA polymerase (De Francesco et al. 2003; Carroll et al. 2003; Sarisky 2004). The development of these small molecule inhibitors of HCV replication has been possible following the development of the HCV replicon culturing system, which allows large-scale screening of potential antiviral agents, and structure optimization by chemical modifications. Currently, the protease inhibitor, BILN2061 (Lamarre et al. 2003), and RNA polymerase inhibitors, NM283 and JT003 are in phase II clinical trial, while several more are in phase I or laboratory development. There are many parallels between the development strategy of HCV antivirals with those used for HIV-1 therapy. For example, some inhibitors of HCV RNA polymerase, such as 2-C-methyladenosine (Olsen et al. 2004), may act as chain terminating nucleoside analogue inhibitors, while nonnucleoside inhibitors, such as benzothiadiazines and benzimadoles bind noncatalytic sites in NS5B, and inhibit enzymatic activity though alterations in protein configuration (Sarisky 2004). Unfortunately, parallels with HIV therapy extend to the finding of large numbers of resistance mutations to all of the protease and RNA polymerase inhibitors developed to date (Nguyen et al. 2003; Lu et al. 2004); these spontaneously emerge after relatively short passaging of the replicon in the presence of antivirals in vitro. In the future, therapy with 2 or 3 antivirals targeting different enzymes may be effectively combined to reduce the frequency of treatment resistance.

Liver transplantation

Liver transplantation is indicated for patients with decompensated HCV cirrhosis and for some patients with hepatocellular carcinoma complicating HCV infection. In many countries, HCV-associated liver disease has become the most common indication for liver transplantation. Liver transplantation does not cure HCV infection, and reinfection of the graft is probably inevitable (Wright et al. 1992). Recurrence may be associated with hepatitis of the graft, which typically occurs within 6 months of transplantation. Short- and intermediate-term outcomes of graft infection are excellent, and reinfection has no discernible effect on patient morbidity or mortality during the first 3 years or so after transplantation. Liver disease is more aggressive in the setting of immunosuppression. There is a need to develop strategies for the treatment of HCV reinfection of the transplanted liver, which might also be applied to the treatment of other immunosuppressed patients (e.g. renal transplant patients) with hepatitis due to HCV infection.

It is clear that pegylated interferon and the combination of interferon and ribavirin can cure a proportion of patients with recurrent HCV after liver transplantation (Bizollon et al. 1997). As observed for the nontransplant patient, viral genotype appears to be the most important determinant of response to treatment. Unfortunately, antiviral treatment is poorly tolerated by post-transplant patients who are more likely to experience the adverse hematological side-effects of both interferon and ribavirin. Thus, compliance with therapy may be poor in this setting, and results (on an intention-to-treat analysis) are disappointing.

Prevention

Screening of blood donors has proved to be effective in preventing transmission of HCV infection through blood transfusion. For example, the incidence of post-transfusion hepatitis fell from 10.7 to 1.9 percent in Spain (Gonzalez et al. 1995) and from 7.7 to 2.1 percent in Japan (Japanese Red Cross Non-A, Non-B Hepatitis Research Group 1991) following the introduction of first-generation screening assays. Most of the donations that were missed using this initial test would have been identified as positive by second- or third-generation assays (Aach et al. 1991; Gonzalez et al. 1995; Yuki et al. 1992; Aoki et al. 1994). A combination of blood donor screening and virus inactivation has virtually eliminated HCV transmission by blood products, such as clotting factor concentrates and immunoglobulins.

The main continuing risks for HCV transmission are intravenous drug abuse and the use of unsterile needles for medical and dental procedures, tattooing, and other percutaneous exposures. Much of this could be prevented by education, greater availability of disposable needles and, for drug abusers, needle exchange programs. Many of the public health measures adopted to prevent transmission of HIV by parenteral routes will assist efforts at controling HCV.

Immunization

The development of a vaccine for HCV faces a series of formidable obstacles. HCV is highly heterogeneous, and effective vaccines may need to be multivalent in a way analogous to poliovirus vaccine in order to be protective against multiple serotypes. Furthermore, the high rate of chronicity and the continued susceptibility of HCV-infected individuals to reinfection (see Chronic hepatitis) had suggested that a protective immune response may be difficult to achieve by immunization. However, the protection from rechallenge observed in chimpanzees with resolved genotype 1 infections may be more analogous to the immune status of a vaccine, and

provides some optimism that antigens that provoke T-cell responses of similar intensity to natural infection may be protective, not only from homologous challenge, but also from infections with other genotypes.

Vaccine development has been relatively slow, partly because chimpanzees are the only in vivo model to investigate the efficacy of different putative HCV vaccines, making experiments and trials extremely expensive, and placing severe limitations on the number of immunization and challenge experiments that can be carried out. Despite these difficulties, encouraging results have been obtained using recombinant envelope proteins (E1 and E2) expressed in mammalian cells as immunogens. These induce a short-lived specific anti-E1 and E2 response in immunized chimpanzees, and a partially protective effect from challenge with low titers of the homologous virus (Choo et al. 1994; Houghton et al. 1998). Chimpanzees that became infected showed reduced levels of viremia on seroconversion, and greater likelihood of spontaneously clearing infection. In recognition of the likelihood that effective vaccines may have to contain immunogens that stimulate T-cell responses rather than just neutralizing antibody, many of the more recently developed candidate vaccines have used antigenic components from the core gene in the form of immune-stimulating complexes, virosomes, or liposomes (Polakos et al. 2001; Hunziker et al. 2002). Rather than immunize with viral proteins, T-cell responses may be more effectively induced with live vectors, such as rabies virus (Siler et al. 2002) or the alphavirus, Semliki Forest virus (Brinster et al. 2002). DNA-based vectors have also been used, designed to express HCV core or envelope coding sequences in situ after immunization (Brinster and Inchauspe 2001; Major et al. 1995; Forns et al. 2000; Geissler et al. 1998). These potently induce both T-cell responses and antibody reactivity to the immunizing proteins, and where tested, induce partial protection in experimentally challenged chimpanzees, with comparable modification to the course of infection noted for the original E1/E2 vaccines. While the lack of sterilizing immunity in chimpanzees induced by vaccination is somewhat less than originally hoped for, HCV vaccines do have potential prophylactic value in preventing a significant proportion of exposed individuals from developing chronic infection. If the vaccines can be manufactured on a large scale and at low economic cost, there is clearly future scope for large-scale prevention of HCV-induced liver disease on a worldwide scale.

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Hepatitis B

WOLFRAM H. GERLICH AND MICHAEL KANN

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INTRODUCTION

Epidemic jaundice was described by the Babylonians, but the infectious nature of the disease and the involvement of the liver were not recognized until the late nineteenth century. In general it was believed that bad living conditions, particularly during wars, generated the catarrhal jaundice. In 1885, Lürmann observed an outbreak of jaundice 2–8 months after people had been given smallpox vaccine. This outbreak was probably caused by hepatitis B virus, since the vaccine had been prepared from human lymph. A larger outbreak of hepatitis caused by vaccination occurred in 1937 when soldiers developed severe jaundice after receiving a yellow fever vaccine containing human serum (Findlay and MacCallum 1937). On the basis of epidemiological studies, two types of agents were suggested: type A, causing large outbreaks via the fecal–oral route, and type B, transmitted mainly by human serum. Hepatitis B was therefore also called homologous serum hepatitis. During the 1970s it became apparent that more than one virus species may transmit hepatitis predominantly by the parenteral route (hepatitis C virus and hepatitis delta virus), but the first identified virus of parenterally transmissible hepatitis was called hepatitis B virus (HBV).

Early efforts to identify HBV failed because it did not grow in tissue cultures or the usual experimental animals. Only humans and higher primates (hominoidae), for example, chimpanzees, are reliably susceptible to HBV. Eventually, the surface protein of HBV was accidentally discovered in 1965 during the search by the anthropologist B.S. Blumberg for polymorphic serum proteins as genetic markers in the blood of an Australian aborigine (Blumberg et al. 1965), and was called Australia antigen. Two years later, the association between the occurrence of the Australia antigen and serum hepatitis infection was recognized (Blumberg et al. 1967). Australia antigen was exposed at the surface of pleomorphic, spherical or filamentous particles of 17–25 nm diameters, which did not contain nucleic acid and were therefore most likely not infectious agents. Among these more numerous particles, Dane and colleagues (1970) detected some larger double-shelled viruslike particles 42 nm in diameter (Figure 1). The surface of these so-called Dane particles cross-reacted with antibodies against Australia antigen. Their significance as the potential viral agent of hepatitis B was confirmed by the detection of antibodies against the inner shell, termed core or nucleocapsid, of the Dane particle in patients with acute hepatitis B. The core antigen was called HBcAg; the Australia antigen was called hepatitis B surface antigen (HBsAg). These antigens induce the corresponding antibodies anti-HBc and anti-HBs. Anti-HBc is a serological marker for previous and current HBV infections, whereas HBsAg is a marker for a current HBV infection. Using these epidemiological markers, it became apparent that HBV is a common human pathogen that causes acute and chronic liver disease throughout the world, particularly in Southeast Asia and sub-Saharan Africa. Chronic illness develops in 5–10 percent of infected adolescents or adults, but in up to 90 percent of infected neonates. Chronic HBV infection is a major cause of liver cirrhosis and primary liver cell carcinoma (Beasley et al. 1981). About half of the world's population has had contact with HBV.

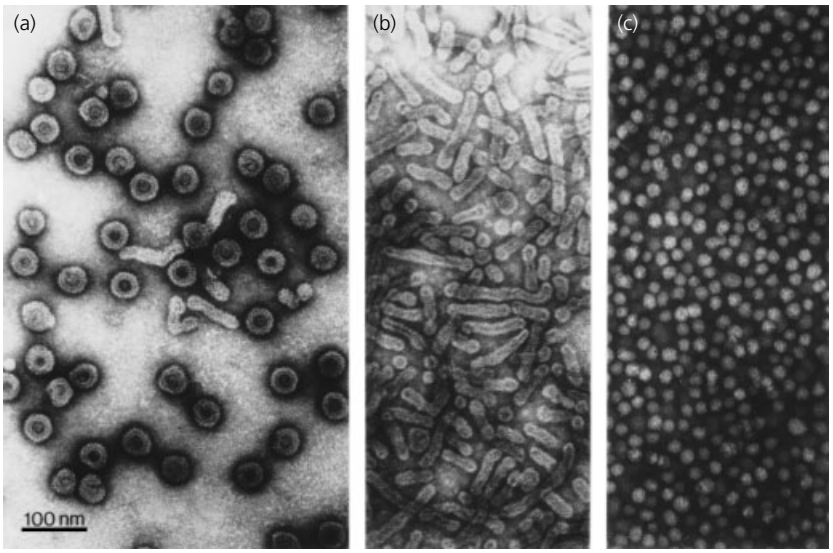

Figure 1 *Electron microscopy of* **(a)** *hepatitis B virus (Dane particles),* **(b)** *HBs filaments and* **(c)** *20-nm particles purified from HBV carrier plasma. Note the double-shelled structure of the HBV particles.*

PROPERTIES OF THE VIRUS

Classification

In 1973, an endogenous DNA polymerase activity (i.e. an enzyme that incorporates nucleotides into DNA without addition of an exogenous template) was discovered within Dane particles (Kaplan et al. 1973). The endogenous template was a circular partially double-stranded DNA of ca. 3 200 nucleotides (nt) (Landers et al. 1977). It was found that the DNA strand of negative polarity was transcribed inside the core particle from an encapsidated RNA template, suggesting a replication strategy similar to retroviruses (Summers and Mason 1982).

The observation that liver carcinoma occurred in the Eastern woodchuck (a marmot of the North American east coast) led to the discovery of the HBV-like *Woodchuck hepatitis B virus* (WHV), the closely related *Ground squirrel hepatitis virus* (GSHV), and recently in a variety of primates including chimpanzees, orangutans, gorillas, gibbons, and woolly monkeys. HBV-like virus species were also found in Peking ducks (*Duck hepatitis B virus* (DHBV)), gray herons *Heron hepatitis B virus* (HHBV), and other waterfowl. All these viruses are highly species specific; for example, the heron HBV does not infect ducks, and the woodchuck virus does not infect ground squirrels. Some of these animal models are very useful for elucidating the replication and pathogenesis of HBV. The great molecular and biological similarity of these viruses led to the definition of a common virus family, *Hepadnaviridae* (from *hepar*, liver, and DNA, for the type of the genome) (Mason et al. 2005). The mammalian viruses form the genus *Orthohepadnavirus* (HBV, WHV, GSHV). Because of significant structural differences the avian viruses form a separate genus, *Avihepadnavirus* (for example, DHBV, HHBV).

Although the genome organization, biology, and replication of hepadnaviruses are quite different from those of the *Retroviridae*, the common strategy of reverse transcription places them and the *Caulimoviridae* of plants (Toh et al. 1983) into one class of viruses. *Hepadnaviridae* and *Caulimoviridae*, with their DNA genomes, have also been called pararetroviruses, in contrast to the orthoretroviruses with an RNA genome.

Morphology and structure

All well-characterized hepadnavirus species appear after negative staining in the electron microscope as double-shelled particles of ca. 42 nm diameter (Figure 1). In mammalian hepadnaviruses the surface of the virions consists of several hundred (possibly 240) subunits comprising three different membrane-spanning polypeptides, termed L(arge), M(iddle), and S(mall) surface (HBs) proteins (Figure 2; and see Figure 6, below). These proteins are carboxy-terminally co-terminal and differ in additional amino-terminal domains. Thus, LHBs consists of an S domain and a preS domain (i.e. preS1 plus preS2 domain) (Heermann et al. 1984). The preS domain of LHBs can be localized externally or internally (Bruss et al. 1994; Lambert and Prange 2003). MHBs contains only the preS2 and the S domain whereas SHBs consists only of the S domain (Figure 2 and Figure 6). Because all HBs proteins can be glycosylated at one or two positions, six different proteins, GP42, P39, GP36, GP33, GP27, and P24, can be distinguished whereby G indicates glycosylation and the number the molecular weight (kDa). Compared with the amount of core particles, these HBs proteins are

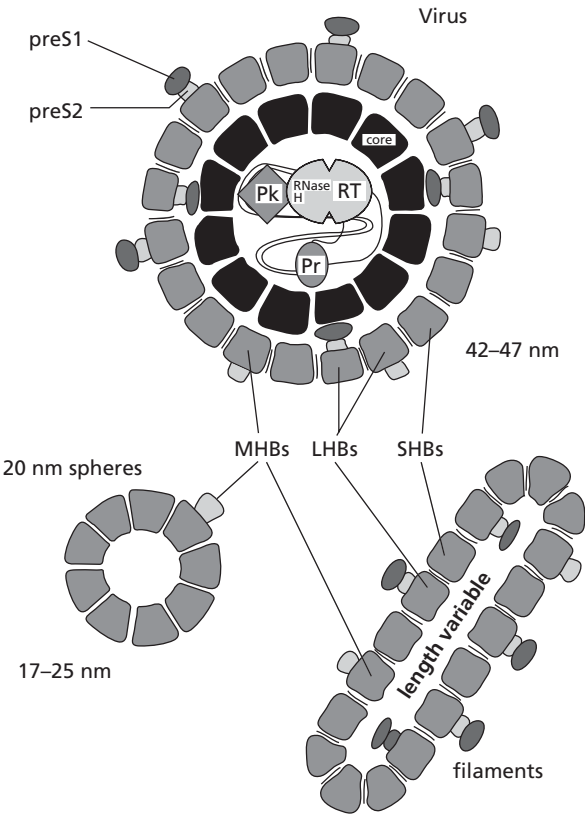

Figure 2 *Schematic presentation of hepatitis B virus and subviral HBsAg particles. DNA is drawn as a single or double line. Structural proteins: the viral polymerase is depicted in gray with a priming domain (Pr), the catalytic domain (RT), and RNase H. The nucleocapsid (core or HBc) is shown in black. Each block represents a dimer; 120 dimers comprise a particle. The surface proteins are shown in gray, with a middle gray S domain, a light gray preS2 domain, and a dark gray preS1 domain. For the topology of the surface proteins, see Figure 6. The virus particles also contain a cellular protein kinase (Pk) and cellular chaperones that are not shown here.*

overexpressed and assemble either to subviral spheres of ca. 20 nm diameter or to filamentous structures, depending on the composition of the different surface proteins (Figure 2). In serum samples of highly viremic individuals, these particles can be found in up to 1 000-fold excess.

In contrast to the mammalian hepadnaviruses, the avian hepadnaviruses are not glycosylated and have only a large (36 kDa) and a small (18 kDa) surface protein and thus do not have division of the preS domain into preS1 and preS2 subdomains. Like the mammalian hepatitis B viruses, these proteins are overexpressed, resulting in the secretion of spherical particles of ca. 40–60 nm diameter.

Both the virion and the surface antigen particles are assembled at the endoplasmic reticulum (ER) and bud to the lumen of a post-ER intermediate compartment. Thus the lipid in the outer protein shell or the HBs particles is derived from an intracellular compartment and not from the plasma membrane. In contrast to other enveloped viruses the lipid content seems to be lower, resulting in a relatively high density of 1.16 g/ml in sucrose. Furthermore, the subunits of blood plasma-derived HBs particles are cross-linked by disulfide bonds and do not disassemble after the addition of detergent.

Within virions the surface proteins enclose the core which encapsidates the viral genome. The core particles interact with the internally localized preS domain of LHBs (Bruss 1997; Ponsel and Bruss 2003) (Figures 2 and 6d). The core protein consists of ca. 185 amino acids (HBV) with a basic carboxyterminus of four arginine-rich clusters. The core molecules form dimers in the cytosol (Zhou and Standring 1992), which are linked by disulfide bridges after release from the reducing environment in the cytosol (Jeng et al. 1991).

Capsid assembly occurs spontaneously. It is initiated by trimerization of the dimer (Zlotnick et al. 1999) and results in two populations of icosahedral core particles, either with a T = 3 or a T = 4 symmetry. They are composed of 180 and 240 core proteins respectively, and show diameters of 32 and 36 nm (Crowther et al. 1994; Kenney et al. 1995). The capsids do not form a closed shell but contain 2-nm holes that allow diffusion of nucleotides into the lumen. They show spikes on their surface, which have been successfully used as carrier sites for foreign epitopes in a highly immunogenic conformation (Pumpens and Grens 2001). Although capsid formation does not require any other viral component, the presence of RNA strongly enhances the assembly. This phenomenon is caused by the stabilizing interaction of RNA and the arginine clusters. Consequently, truncated core proteins lacking the carboxyterminus show a much slower assembly kinetic and are less stable than the wild-type. The localization of the carboxyterminus is, however, flexible. While being internally localized in RNA-containing core particles (Zlotnick et al. 1999) genome maturation results in an exposure on the capsid surface (Rabe et al. 2003).

In a patient's liver or in other eukaryotic cells, but not in bacteria, the assembly of the core particles is combined with the encapsidation of a cellular protein kinase. It is known that it phosphorylates serine residues within the carboxy-terminal part of the core protein (Gerlich et al. 1982). Reports on the trapped protein kinase are divergent (Barrasa et al. 2001; Daub et al. 2002, Duclos Vallee et al. 1998; Kau and Ting 1998; Kann and Gerlich 1994; Kann et al. 1993). Possibly, different kinases can replace each other regarding their function. In infected human liver, or artificial genome-expressing cell cultures, core particles encapsidate a complex of the pregenomic RNA and the viral DNA polymerase. The polymerase transcribes the pregenomic RNA into a negative DNA strand, which is used as the template for second strand DNA synthesis. The viral polymerase consists of four domains: (1) the priming

domain; (2) a so-called spacer or tether; (3) the reverse transcriptase domain, which catalyses RNA-dependent DNA synthesis; and (4) the RNase H domain, which degrades the RNA from the resulting DNA–RNA hybrid (for review, see Nassal and Schaller 1996). Because HBV DNA in infected hepatocytes remains unintegrated as an episomal minichromosome (Bock et al. 2001), the HBV polymerase is, in contrast to retroviral polymerases, devoid of an integrase domain. Furthermore, no protease domain has been identified such as is found in retroviruses.

Genome structure and function

The HBV genome consists of a circular, partially double-stranded DNA in which the longer strand of negative polarity is ca. 3 200 nt long (Figure 3A). The 5′ end of this strand has a terminal redundancy of 8–10 nt. It is covalently bound to the primer domain of the viral polymerase. The polymerase presents the hydroxyl residue of tyrosine 96 (DHBV) or tyrosine 63 (HBV) as the acceptor for phosphodiester linkage to the first nucleotide of the negative DNA strand. The 5′ and 3′ end of this DNA strand are not covalently linked. The circular structure is caused by base pairing of the negative with the positive DNA strand at the discontinuity around the two ends of the negative DNA strand. The positive DNA strand has a defined 5′ end ca. 230 nt (HBV) upstream of the 3′ end of the negative DNA strand. The 5′ end is linked to a capped RNA oligonucleotide, 18 nt long that acts as a primer for second strand DNA synthesis and represents the undegraded 5′ end of the pregenomic RNA (see section entitled Replication, below, and Figure 3a). The length of the positive DNA strand varies between 1 100 and 2 600 nt,

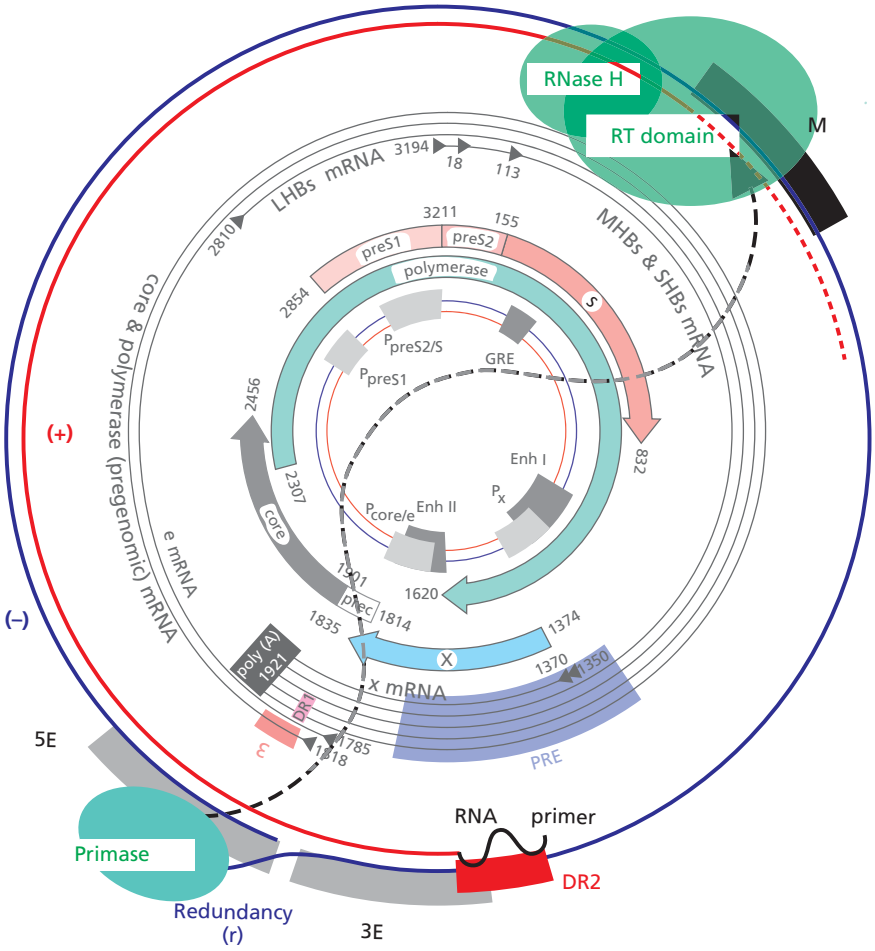

Figure 3 *Genome organization of HBV. Numbering follows the system of Galibert et al. (1979) and is shown for genotype A2 isolate 991 (GenBank association no. X51970.* **(a)** *Structure of virion DNA shown as the outer circle. The minus strand has a redundancy of 8–10 nt at the ends and is covalently linked to the primer (pr) domain of the polymerase. The active center of the reverse transcriptase domain (RT) is probably associated with the variable 3′ end of the incomplete plus strand, the RT domain is covalently linked with the primer domain via the tether domain. The plus strand starts at the 5′ end with a mRNA-derived cap and the 18 5′ terminal remaining bases of the RNA pregenome which are base-paired with the direct repeat DR2 to the minus strand. The circularization elements 5E, 3E, and M are also shown.* *(continued over)*

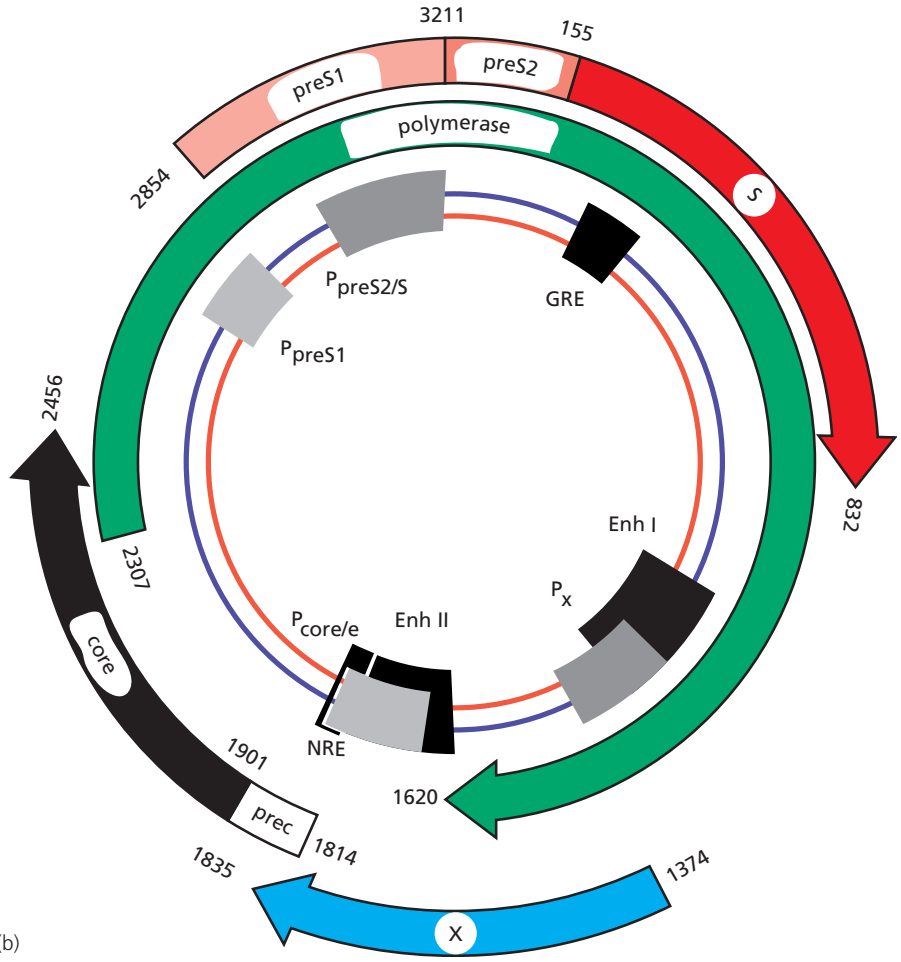

Figure 3 *Genome organization of HBV. Numbering follows the system of Galibert et al. (1979) and is shown for genotype A2 isolate 991 (GenBank association no. X51970. (Continued)* **(b)** *Open reading frames (ORFs) and some transcription regulating elements on the cccDNA. P: promoter, Enh: enhancer; GRE: glucocorticoid responsive element. Light gray promoters are liver-specific. ORFs start with the first conserved and translated start codons. Vertical lines within ORFs are internally used start codons. (Continued over)*

depending on how far the viral polymerase has proceeded. Therefore a single-stranded region of 600–2 100 nt is left and this gap can be partially filled in vitro by the viral polymerase after the addition of deoxynucleotides.

Cloning and sequencing of HBV DNA by many groups of workers confirmed the physical structure of the DNA. The genome of wild-type hepadnaviruses contains four conserved, partially overlapping, open reading frames (ORF) (Figure 3b), all on the negative DNA strand (Schlicht and Schaller 1989). Thus, the coding capacity of the 3 200 nt corresponds to 5 500 nt (had the ORFs been arranged in a linear manner). The ORFs encode:

- The core protein and an additional precore region which includes a signal peptide sequence; this additional precore peptide results in a secreted, proteolytically modified form of the core protein, called HBe (see section entitled Nonstructural proteins, below)
- The DNA polymerase
- The nested set of surface proteins
- A protein of unknown function, X protein (HBx), which was believed to be absent in the avian hepadnaviruses. Recent studies, however, describe the expression of an X-like protein from the DHBV genome via an unusual initiation site and the existence of an X-related gene in other avihepadnaviruses (Schuster et al. 2002).

The expression of the ORFs is controlled by at least four promoters, enhancers I and II (for review, see Tang and McLachlan 2001), glucocorticoid-responsive elements (GRE) increasing transcription, and a negative regulating element (NRE) that selectively inhibits the transcription from the core/precore promoter. Termination of transcription is encoded by a single polyadenylation signal so that all viral mRNAs share a common 3′ end.

On the level of the transcribed mRNAs, additional regulatory elements are found. Present on all mRNAs is the so-called post-transcriptional regulatory element (PRE), which suppresses splicing of the transcribed

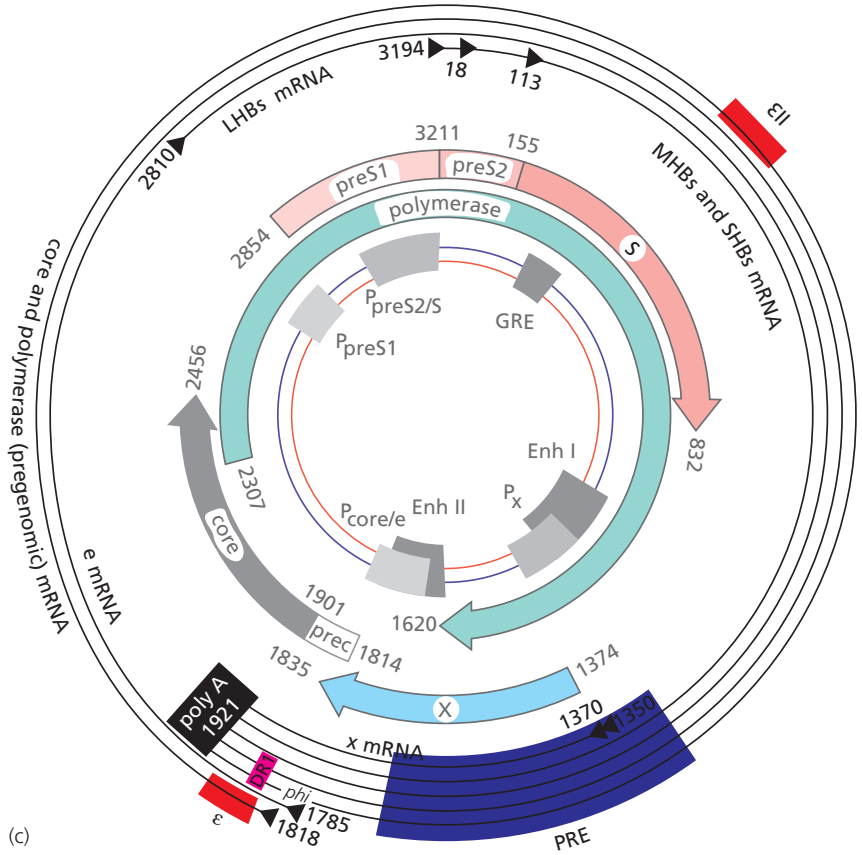

Figure 3 *Genome organization of HBV. Numbering follows the system of Galibert et al. (1979) and is shown for genotype A2 isolate 991 (GenBank association no. X51970. (Continued)* **(c)** *mRNAs of HBV. ϵ: encapsidation signal and polymerase binding site. DR1: direct repeat one PRE: post-transcriptional regulatory element preventing splicing and enabling transport to the cytoplasm.*

RNAs (Huang and Liang 1993). The pregenome contains signals to support reverse transcription and the formation of circularized DNA. This includes the ϵ signal, required for binding of the viral polymerase and subsequent initiation of reverse transcription, two direct repeats (DR) of 11 nt (orthohepadnavirus), which are termed DR1 and DR2. The minus-strand DNA, which is the product of reverse transcription of the RNA pregenome, comprises the M region, which supports the correct translocation of the polymerase to the site where subsequent plus-strand DNA synthesis is initiated. Circularization of the minus strand requires additional elements (Liu et al. 2004) as the terminal redundancy of the minus strand (r) and two flanking domains called 5E and 3E (Figure 3a) (see section entitled Replication, below).

Replication

OVERVIEW OF THE VIRAL LIFE CYCLE

Figure 4 outlines the essential steps in the viral life cycle. The mode of attachment and entry is not unequivocally identified. The uptake by clathrin-mediated endocytosis has been suggested for DHBV (Köck et al. 1996) (Figure 4). Recent data suggest actin-independence and microtubule-dependence of viral entry (Funk et al. 2004). These findings support the idea of endocytosis since endosomes are transported via microtubules towards the perinuclear region. Because hepadnaviruses multiply via RNA generated by the cellular RNA polymerase II, the DNA genome must be transported into the nucleus. Consequently, the DNA genome must escape from the endosome in a complex with viral proteins, which facilitate the transport towards the nuclear envelope. To pass the nuclear envelope, the genome must be transported through the nuclear pore complexes (NPC), which are the only sites for macromolecule exchange between the cytoplasm and the nucleus. At least inside the karyoplasm, the genome must be accessible to transcription factors and the polymerase II, which transcribes different sets of mRNAs.

For genome replication, one mRNA species of supergenomic length is used. This mRNA is translated into core protein and, in an overlapping reading frame, into the viral polymerase. This RNA can then specifically interact with the viral polymerase and cellular chaper-

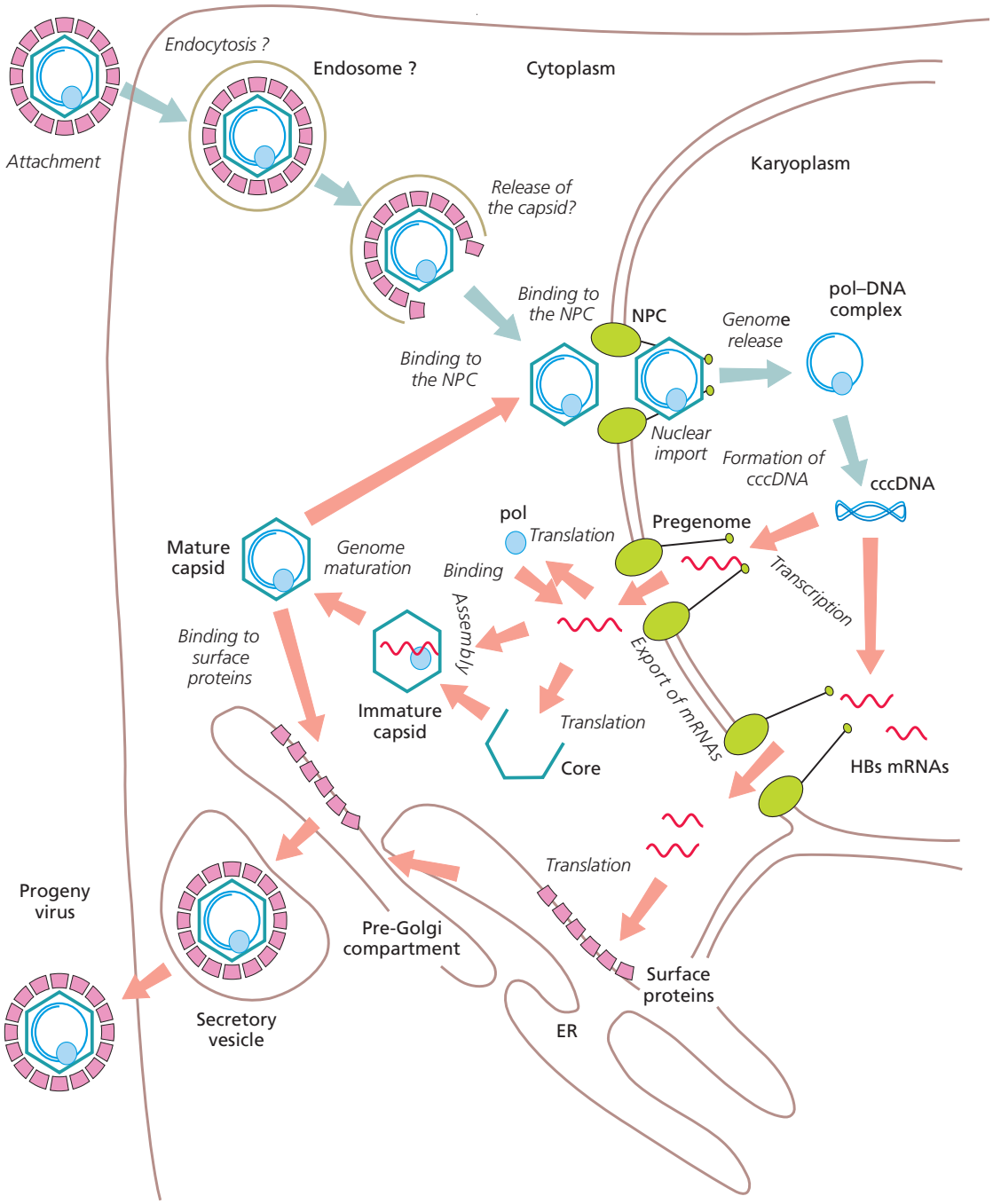

Figure 4 *Schematic presentation of the HBV replication cycle. Details of the figure are given in the text.*

ones. This complex is specifically encapsidated by the core protein into particles (Hu et al. 2002). Phosphorylation of at least some core protein subunits is required for the encapsidation (Gazina et al. 2000). Within the core particle, the encapsidated RNA serves as the template for reverse transcription and is therefore also called pregenomic RNA. The core particle containing the mature DNA genome can be either enveloped into the surface proteins and secreted as mature virus into the bloodstream or it can release the encapsidated DNA into the nucleus of the infected cell, leading to an amplification of the episomal viral DNA.

ATTACHMENT AND ENTRY

HBV infects naturally only humans and hominoidae, and is highly adapted to fully differentiated hepatocytes. Hepatocyte cultures are only susceptible for a very short time window, 1–3 days after explantation from the organ. Susceptibility can be prolonged and enhanced by addition of dimethyl sulfoxide and special hepatocyte media.

Primary duck hepatocytes are highly susceptible for DHBV and produce progeny virus after 2 days. Mammalian hepatocyte cultures are more difficult to obtain and less susceptible. Usually they require high virus input and produce little progeny virus with no spread from cell to cell. Permanent cell lines from hepatomas are usually not susceptible, but if transfected with double-stranded HBV DNA covering all genes, they express infectious HBV. Thus, the block in the susceptibility of permanent cell cultures comes at an early step in the viral life cycle before formation of covalently closed circular DNA (cccDNA). True infection requires species-, organ-, and differentiation-specific factors acting on attachment and uptake of the virus. Recently, a redifferentiated hepatoma cell line, Hepa RG, has been established which is after redifferentiation as susceptible as primary human hepatocytes (Gripon et al. 2002).

The scarcity of surgically obtained human liver and the low efficiency of the cell cultures have severely hampered studies on these early steps, but some limited data have been obtained for HBV. Additionally, studies with animal hepadnaviruses have been helpful. The preS1 protein sequence 10–36 (21–47 in HBV genotype A) is essential for binding of HBV to human hepatocyte membranes, and binding can be blocked by competing peptides or by antibodies against that sequence (Neurath et al. 1986; Pontisso et al. 1989). Such antibodies also block infection of primary human hepatocyte cultures (Maeng et al. 2000). Antibodies against SHBs in natural conformation also block infectivity, but a generally accepted role of SHBs in attachment has not been identified. Emergence of escape HBV mutants in infected anti-SHBs positive patients (Carman et al. 1997; Nainan et al. 2002) confirms the essential role of the SHBs antigen loop from amino acids 100–160 in vivo for viral spread. The woolly monkey hepadnavirus (Lanford et al. 1998) can infect human hepatocyte cultures, but much less efficiently than HBV. The determinant of the host range of this virus has been mapped to the region preS1 (1–40) that is also essential for attachment, with amino acids 5, 27, and 30 being most divergent (Chouteau et al. 2001).

In spite of their narrow host specificity, the human and the woolly monkey HBV, but not woodchuck HBV, quite efficiently infect primary hepatocyte cultures from the livers of *Tupaia belangeri* (Köck et al. 2001). These are small animals living in the tropical forests of South East Asia forming their own order (*Scandentia*) among the mammalians, unrelated to primates. They can be bred in large numbers in captivity and provide a more available source of fresh liver tissue than humans or primates. Using *Tupaia* hepatocytes for measuring infectivity of HBV, monoclonal antibodies against preS1 20–23 and against native SHBs have been found to be completely neutralizing while preS2 antibodies and a sequential SHBs antibody neutralized only partially (Glebe et al. 2003). With DHBV, both preS and S antibodies have also been found to be neutralizing (Sunyach et al. 1999). As with human HBV, the preS sequence of DHBV is essential for binding and uptake and determines the host range (Ishikawa and Ganem 1995). Infectivity of HBV and DHBV for hepatocytes requires the myristoylation of the conserved glycin 2 of the preS domains (Bruss et al. 1996). Competing preS peptides are also more potent inhibitors if myristoylated (Urban and Gripon 2002). The preS sequence of DHBV binds to duck carboxypeptidase D that is an essential, but not sufficient, receptor for infection (Kuroki et al. 1995; Urban et al. 2000). For human HBV, numerous candidate receptors have been described, binding either to preS1, preS2, or SHBs but a connection to the infection process could up to now not be demonstrated.

The preS2 domain of HBV binds a modified form of natural human or primate serum albumin (Krone et al. 1990). The nature of the modification is unknown but it can be mimicked by treatment with glutaraldehyde. Such a modified human serum albumin mediates binding of preS2-containing HBsAg particles including HBV to human hepatocyte membranes (Pontisso et al. 1989), but it is not known whether this phenomenon contributes to infection. LHBs and SHBs are essential for both morphogenesis and infectivity of HBV or hepatitis delta virus (HDV), the envelope of which is derived from HBV. MHBs is nonessential for infectivity of HBV or HDV. Natural or artificial deletion mutants without the MHBs start codon are viable in patients and in cell cultures.

Recent work suggests a slow, actin-independent entry of DHBV, the details of which are not understood (Funk et al. 2004). The receptor for DHBV, carboxypeptidase D, shuttles between plasma membranes and the Golgi apparatus and may mediate uptake of DHBV into the cells (Breiner et al. 1998). The postulated second receptor mediating entry to the cytosol is still unknown. All hepadnaviruses at the amino end of the S domain or SHBs protein contain a short hydrophobic sequence that is similar to fusion peptides of many enveloped viruses. This sequence can indeed replace the fusion peptide in influenza virus hemagglutinin in the step of membrane fusion (Berting et al. 2000) and may play a similar role during entry of hepadnaviruses. In HBV-infected *Tupaia* hepatocyte cultures, HBV binding is very efficient, but the later steps seems to occur at low efficiency (Glebe et al. 2003).

At least in DHBV, the entire capsid is transported towards the nuclear envelope (Köck et al. 2003). Unpublished data after lipofection of HBV capsids reveal that this transport is indeed facilitated by using the cellular microtubule transport machinery (Rabe and Kann 2005) as was shown for other viruses (Whittaker et al. 2000).

NUCLEAR ENTRY AND FORMATION OF EPISOMAL VIRAL DNA

Liver cells are differentiated cells of which the vast majority are nondividing. As with other viruses replicating in the nuclei of arrested cells, the hepadnaviral

genome has to travel through the cytoplasm towards the nuclear pores followed by passage through the pore. Furthermore, the conversion of the relaxed circular (rc) viral DNA to the cccDNA by cellular enzymes (Köck et al. 2003) requires its release from the capsid.

Most knowledge on the subsequent passage through the nuclear pores has been obtained by in vitro systems established for studying the nuclear import of cellular proteins. It has been shown that nucleocapsids bind to the NPC, following the 'classical' pathway of karyophilic proteins, which is mediated by the cellular proteins importin α and β (karyopherin α (and β). The exposure of a corresponding nuclear localization signal (NLS) on the surface of the nucleocapsids, which is bound by importin α, is dependent upon genome maturation and/or phosphorylation of the core subunits (Kann et al. 1999). In contrast to herpes virus capsids and adenoviruses, which also migrate to the NPC, HBV capsids are small enough to pass the nuclear pore as entire particles (Panté and Kann 2002), as demonstrated after microinjection into *Xenopus* oocytes.

After release from the capsid within the nuclear basket (Rabe et al. 2003), the partially double-stranded DNA genome with its protein primer at the 5′ end of the negative DNA strand and its RNA primer at the 5′ end of the viral positive strand, must be converted by cellular DNA modifying enzymes to a cccDNA. In fact, formation of cccDNA is the first marker of successful infection, followed by replicative intermediates after 24 h. The central role of cccDNA for transcription of viral RNAs was confirmed by infecting chimpanzees with cloned, circular HBV DNA via intrahepatic injection (Will et al. 1985). The following steps for the generation of cccDNA are implied: (1) removal of the 5′ terminal redundancies from the negative strand and removal of the covalently bound polymerase; (2) removal of the RNA primer; (3) completion of the positive DNA strand; and (4) linkage of the 5′ and 3′ ends of each strand by DNA ligase. The positive DNA strand may be filled up by the viral polymerase (Köck et al. 2003), but cellular polymerases may be also required, because treatment of primary hepatocytes with ddG, an inhibitor of cellular DNA polymerases, resulted in suppression of cccDNA formation (Köck and Schlicht 1993).

TRANSCRIPTION AND TRANSLATION

The four promoters and two enhancers of HBV control expression of at least five overlapping RNAs of different sizes (see Figure 3c) by the cellular RNA polymerase II (for a review: Moolla et al. 2002). Like most eukaryotic mRNAs, the viral transcripts include a CAP structure at their 5′ terminus and are polyadenylated at their 3′ end. The genome contains only one polyadenylation signal TATAAA, which is conserved in all mammalian hepadnaviruses. The transcripts contain many potential splice donor and acceptor sites, but they also contain a splice-suppressing sequence, homologous to the rev-responsive element (RRE) of human immunodeficiency virus (HIV). The corresponding sequence in hepatitis B viruses was termed PRE (Huang and Liang 1993). PRE acts context-dependently consistent with the observation that it has a secondary structure (Loeb et al. 2002). In contrast to the HIV-RRE however, the HBV RNA export is not sensitive to leptomycin B (Zang and Yen 1999), indicating that the nuclear export factor chromosome region maintenance (CRM)-1 is not involved whereas the corresponding WHV element is CRM-1 dependent (Popa et al. 2002). Probably, HBV-PRE acts like the constitutive transport element (CTE) found in the export of unspliced Mason–Pfizer monkey virus (MPMV) RNAs. CTE override the retention of intron-containing RNAs by a direct interaction with the export factor TAP, which is leptomycin insensitive. Polypyrimidine tract binding (PTB) protein has been reported to bind to PRE and to contribute to the nuclear export of HBV RNAs (Zang et al. 2001). HBV RNAs are post-transcriptionally down-regulated upon cytokine induction. The decrease of HBV mRNAs however is counteracted by a stabilization of the HBV transcripts mediated by the association to the autoantigen La (Heise et al. 1999). La was shown to bind to a stem–loop structure located between nts 1275 and 1291 of HBV. It was reported that the turnover of HBV RNAs depends on structural features and less on the primary nucleotide sequence (Ehlers et al. 2004).

As in most eukaryotic mRNAs, the first reading frame is most efficiently translated. Thus, the different mRNAs are named after the first encoded ORF HBe-, HBc/pol-, preS1-, preS2/S-, and X-RNA. Among these RNAs, two sets can be discriminated: RNAs of subgenomic length, encoding X and the surface proteins; and RNAs longer than the genome (supergenomic RNAs), encoding HBe, core, and polymerase. Regulation of transcription from the HBV genome has been studied in great detail.

The HBx RNA of mammalian hepadnaviruses is ca. 700 nt long, and starts at two closely situated initiation sites (Schaller and Fischer 1991). The presence of multiple binding sites for liver-specific transcription factors (hepatonuclear factors (HNF) 3 and 4; C/EBP) in its promoter, which overlaps the enhancer I, makes it likely that the transcription of this RNA is regulated in a tissue-specific manner, but it can also be expressed in a large variety of nonhepatic cell lines.

The major transcript of the HBV genome of ca. 2.1 kb does not contain a clearly defined 5′ end, and is well transcribed in a large variety of hepatic and nonhepatic cell lines (Cattaneo and Will 1983). This subspecies of mRNA contains the preS2 and S regions with their start codons, resulting in the expression of MHBs when the first AUG is used. In most translation events, the second AUG is used, leading to the expression of the small surface protein, SHBs (Gallina et al. 1992). Its promoter

is called preS2/S promoter and consists mainly of ubiquitously active SP1 binding sites (Raney et al. 1992), which explains the wide variety of cell lines and tissues in which the SHBs can be expressed.

In contrast to the above-mentioned transcripts, the LHBs-encoding mRNA of ca. 2.4 kb is transcribed in a liver-specific manner (Antonucci and Rutter 1989), requiring the presence of the liver-specific transcription factors HNF1 and 3 (Raney and McLachlan 1995). Because the expression of the encoded LHBs protein is essential for virus envelopment and secretion (Bruss and Ganem 1991), activation of the LHBs promoter seems to be an essential element in HBV–host cell restriction. In DHBV-transfected cells and DHBV-infected ducks, a common additional spliced mRNA is found with the start site of the pregenomic transcript, which seems to be essential for virus production (Obert et al. 1996). Because the resulting mRNA has the same length as the major LHBs mRNA, splicing of a 1.1 kb intron seems to be most probable. While splicing does not seem to be essential for HBV, various splice products have been identified in hepatocellular carcinoma cell lines (Rosmorduc et al. 1995) and in patients (Günther et al. 1996). A hepatitis B spliced protein (HBSP) composed of a fragment of pol and a new ORF seems to be often expressed and antibody against it may be associated with the generation of fibrosis (Soussan et al. 2003).

Two sets of supergenomic mRNAs exist, both of ca. 3.3 kb in length, being terminally redundant in ca. 150 nt. Owing to the redundancy of the RNAs, the polyadenylation signal, localized downstream of the AUG of the core ORF, must be ignored on the first passage of the transcription machinery. Apparently, this phenomenon is caused by the surrounding nucleotide sequences and by the length of the upstream region (Cherrington et al. 1992).

The transcription of these supergenomic RNAs is controlled by the core promoter and enhancer II which are liver specific owing to binding sites for nuclear hormone receptors, such as HNF4, retinoid X receptor, and peroxisome proliferator-activated receptor alpha (Tang and McLachlan 2001). The core/HBe promoter initiates the transcription of a subset of two classes of RNAs, which differ by ca. 30 nt at their 5′ end but have completely different biological functions. Only the longer mRNAs contain the AUG start codon of the preC region at their 5′ end, which is in frame with the AUG of the core reading frame. Therefore, these RNAs encode a primary translation product that differs from the core protein by 29 additional amino acids at the amino-terminal end. This additional domain, called preC, contains a hydrophobic sequence that serves as a signal sequence for secretion into the ER (see section entitled Nonstructural proteins, below). The resulting product is HBe antigen.

The genome replication is only supported by the other supergenomic transcript, which does not contain the preC AUG, but includes the initiation AUG at nt 1 900 for core protein (Ou et al. 1990). From this RNA, the overlapping *pol* ORF, beginning 407 nts downstream of the core AUG, is also translated, probably less efficiently. In contrast to retroviruses, which also have overlapping polymerase and nucleocapsid ORFs, expression of hepadnaviral polymerase occurs by de novo translational initiation and not by ribosomal frame shifting (Chang et al. 1990). The exact mechanism of pol translation remains unsolved because conflicting evidence has been reported for both an internal entry of the ribosomes at or near the pol AUG and for leaky scanning from the 5′ end of core/pol mRNA.

The ratio between pregenomic and 'preCore' HBe mRNA is variable and depends on the nucleotide sequence in the core promoter/enhancer. Particularly, mutations from A1762 to T and G1764 to A reduce HBeAg expression and enhance pregenome expression and thus replication (Buckwold et al. 1996). HNF 3β favors expression of HBe mRNA and inhibits pregenome transcription and replication (Banks et al. 2002).

In addition to cellular transcription factors, two virus encoded transcription factors regulate the gene expression of HBV. HBx protein is a promiscuous transactivator of many cellular genes and of the HBV genes (see below). The other transcription-activating element is located within the preS2 domain and acts only if located at the cytosolic side of the ER. This topology exists in LHBs protein and in carboxyterminally truncated forms of MHBs (Hildt et al. 1995). PreS2 activates the raf/MEK signal cascade that is essential for HBV replication (Stockl et al. 2003). HBx activates tyrosine kinases and calcium signaling which also activates HBV replication (Bouchard et al. 2003).

ASSEMBLY OF THE VIRAL NUCLEOCAPSID AND GENOME MATURATION

Expression of the viral nucleocapsid in *Escherichia coli* leads to core particles that, by electron microscopy, are indistinguishable from natural nucleocapsids found in infected liver (Kenney et al. 1995). (However, it should be considered that only the structure built by the first ca. 140 amino acids is depictable.) In these expression systems, devoid of any other viral protein, assembly is combined with nonspecific encapsidation of RNA. Specific encapsidation of the pregenomic RNA, as it occurs in vivo, is mediated by the epsilon (ϵ) signal and the viral polymerase (Bartenschlager et al. 1990; Hirsch et al. 1990). Mutations throughout the whole polymerase gene affect the ability for specific RNA encapsidation, suggesting a complex interaction between viral polymerase with pregenomic RNA and the core particle in which other proteins such as the cellular chaperone Hsp90 and co-chaperones may be involved (Hu and Seeger 1996; Beck and Nassal 2003).

The encapsidation signal ϵ of the pregenomic RNA was mapped within the preC region of DHBV and HBV

RNA (Nassal and Schaller 1996). Although ϵ is present in all HBV RNAs at the 3′ end and is redundant at the 5′ and 3′ ends of the supergenomic transcripts, only the 5′ terminal ϵ supports encapsidation (Junker-Niepmann et al. 1990). ϵ forms a base-paired stem–loop (see Figure 6a), conserved in its structure throughout all hepadnaviruses. Although ϵ seems to be sufficient for pregenome encapsidation by viral polymerase, additional sequence elements downstream increase the encapsidation efficiency (Hirsch et al. 1991; Calvert and Summers 1994). These elements may explain how the viral polymerase discriminates the 5′ terminal ϵ from the 3′ terminal ϵ in the super- and subgenomic RNAs. The nonreactivity of the HBe mRNA despite the 5′ terminal ϵ in encapsidation is caused by the translating ribosomes on the HBe RNA, which prevent the folding of ϵ or its interaction with the polymerase (Nassal et al. 1990). This indicates that only complete, translating 80S ribosomes are able to displace the ϵ-bound polymerase, because the pregenomic RNA must be scanned by the 40S ribosomal initiation complex for the initiation of core protein synthesis.

In addition to the polymerase and the pregenomic RNA, a protein kinase is encapsidated within core particles (Gerlich et al. 1982). This activity is associated with nucleocapsids, even when expressing the core gene alone in insect cells (Lanford and Notvall 1990). As mentioned above, the kinase is not unequivocally identified yet but it is known that it phosphorylates serine residues between the arginine clusters in the carboxy-terminal portion of the core protein (Lan et al. 1999).

Studies on the function of the protein kinase are difficult to perform since the carboxy-terminal part of the core protein is multifunctional. It comprises domains essential for RNA packaging and genome maturation (Nassal 1992). Hence, it is not surprising that point mutations of the protein kinase target sequences always resulted in a more or less complete inhibition of genome maturation. Using this approach, it has been shown that phosphorylation at serine 162 is required for specific encapsidation of the RNA pregenome (Gazina et al. 2000), mediated by the polymerase. However, phosphorylation in vitro inhibits interaction of nucleic acids and the core protein, implying that the majority of phosphorylation probably occurs after encapsidation of pregenomic RNA (Kann and Gerlich 1994).

The complex events that convert the linear RNA pregenome to the circular double-stranded DNA in the virion are outlined in Figure 5 (for review, see Ganem et al. 1994). Previous observations on the endogenous polymerase activity suggested that this enzyme is active only within nucleocapsids. However, DHBV RNA, which contains the ϵ signal, and the polymerase ORF, can be used in a cell-free translation system to express an active DHBV polymerase that can prime and elongate the first nucleotides of the negative-strand DNA (Wang and Seeger 1993). These data have been confirmed by expression of the DHBV polymerase in *Escherichia coli*, which was able to initiate reverse transcription at a synthetic template after complementation with eukaryotic chaperones Hsc70 and Hsp40 (Beck and Nassal 2003). Noteworthy, these cell-free systems could only be established for the polymerase of DHBV and allow only the addition of some nucleotides; attempts to express the HBV polymerase in a similar manner failed.

The progress on DHBV research allowed the understanding of the initial events in hepadnaviral replication. The polymerase starts at the bulge of the ϵ signal (Figure 5a) and not, as originally concluded from primer extension experiments, within the 3′ terminal DR1. For the priming of the negative-strand DNA, the hydroxyl group of tyrosine 96 of the DHBV polymerase serves as the acceptor for the synthesis of a phosphodiester bridge with the first nucleotide. After the addition of the first 4 nt (DHBV, 3 nt in HBV) encoded by the bulge, the oligonucleotide polymerase complex dissociates from the bulge, and reanneals with a sequence complementary to the 3′ terminal DR1 (Figure 5b). These findings were confirmed for the HBV polymerase by expression in yeast. However, because there are many complementary sequences to the three or four initial bases of the negative DNA strand within the hepadnavirus genomes, additional factors must be involved. These include the primary and secondary structure of the surrounding RNA sequences of the DR1 as well as the distance between initiation site and DR1 that regulate the transfer of the polymerase nucleotide complex from ϵ to a sequence element ϕ at the 3′ terminal DR1 (Tang and McLachlan 2001). At DR1, reverse transcription, that means negative-strand DNA elongation, proceeds towards the 5′ end of the pregenome. The catalytic center for the polymerization reaction contains the typical YMDD motif as has been described for retroviruses. A further similarity is that the elongation of the negative DNA strand is combined with degradation of the RNA strand in the resulting DNA–RNA hybrid. Mapping of the 3′ end of the negative DNA strand showed that it nearly coincides with the 5′ end of the pregenomic RNA. Thus, the 3′ end of the negative DNA strand is obviously specified by ‘run off’, when the polymerase reaches the end of its RNA template, resulting in the terminal redundancy of the negative DNA strand of 8–10 nt (Figure 5c). Since the RNase H domain is separated from the polymerizing catalytic center in the polymerase, an RNA oligonucleotide of 17–18 bases does not become degraded. This oligonucleotide represents the 5′ end of the pregenome including the 5′ CAP. Together or concomitantly with the polymerase, the oligonucleotide translocates to the complementary region of DR2 on the minus-strand DNA. The translocation requires a folding of the minus strand mediated by a structural element within the S ORF termed the M region (Liu et al. 2003).

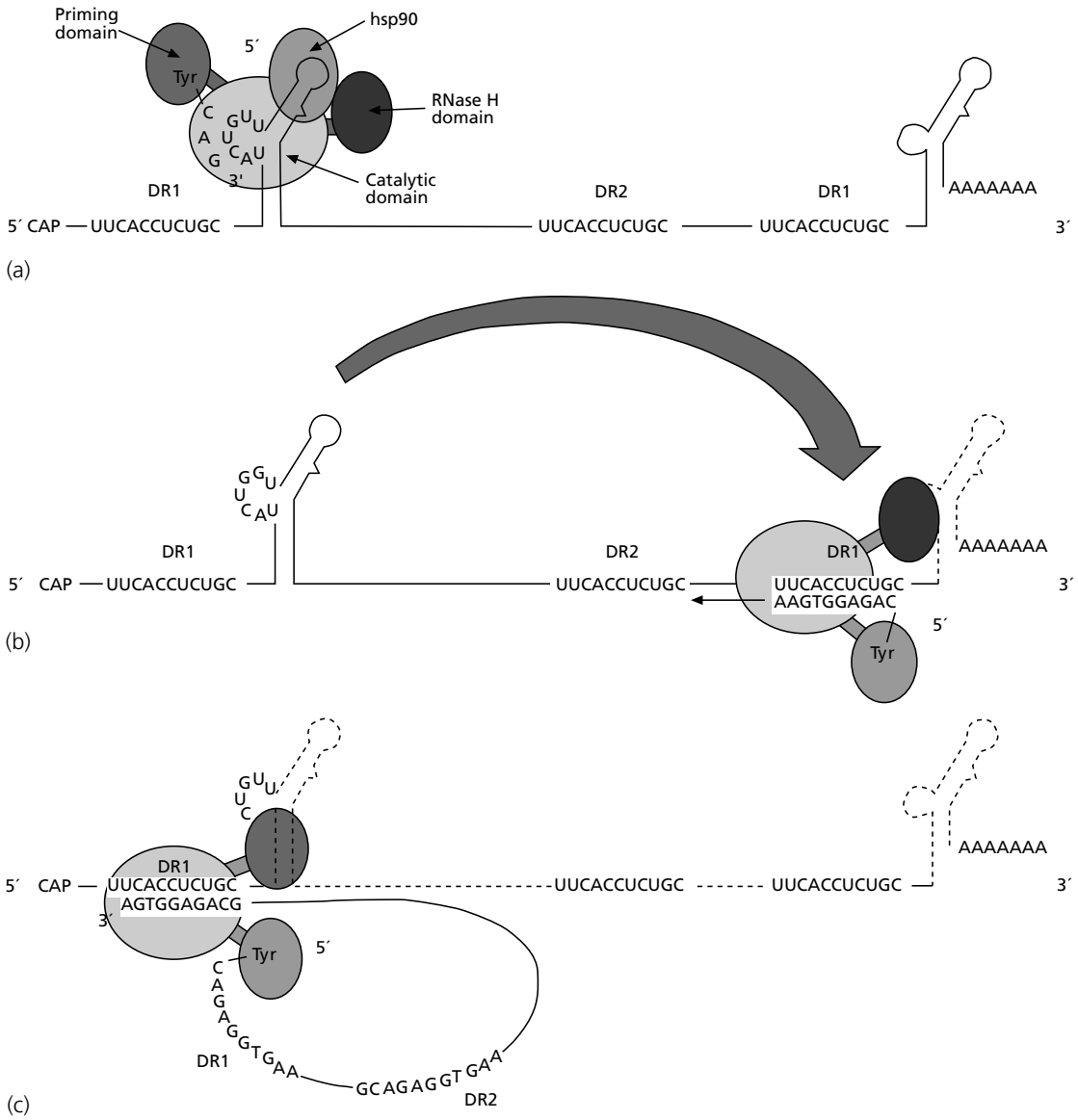

Figure 5 *Generation of the viral genome from the pregenomic RNA.* **(a)** *Binding of the viral polymerase and the cellular factor hsp90 to the encapsidation signal on the pregenomic RNA. Reverse transcription for the first 3 DNA nucleotides starts at the bulge of the stem–loop structure. The first nucleotide (C) is covalently linked to a hydroxyl group of a tyrosine within the priming domain of the polymerase.* **(b)** *Translocation (large arrow) of the polymerase–nucleotide complex to a complementary sequence on the DR1. Further negative DNA strand synthesis.* **(c)** *Completion of negative DNA strand synthesis. The degraded RNA template is depicted as a dotted line. The remaining undegraded RNA oligomer is shown in black. (continued over)*

At DR2, the polymerase uses the 3′ end of the RNA oligonucleotide as the primer for DNA synthesis, now accepting the single-stranded DNA of the minus strand as template. Since the polymerase is covalently bound to the 5′ end of the minus strand, this single strand must form a loop which narrows when the polymerase proceeds towards the 5′ end. Apparently, the distance between phosphodiester linked Tyr in the priming domain and the catalytic center of the polymerase allows that plus strand DNA synthesis reaches the 5′ end of the minus DNA strand template (Figure 5d, e). Because the negative DNA strand is linear, second-strand synthesis could run off at the 5′ end of the template, but mature HBV genome in core particles is predominantly circular. Thus, the polymerase is able to bridge the gap between the 3′ and the 5′ end of the negative DNA strand. On the basis of mutational analysis, a melting of the 3′ end of the positive DNA strand and the 5′ end of the negative DNA strand (Figure 5f) was suggested. Due to the terminal redundancy r, the 3′ end of the positive DNA strand can anneal with the complementary 3′ end of the negative DNA strand (Figure 5g). Besides

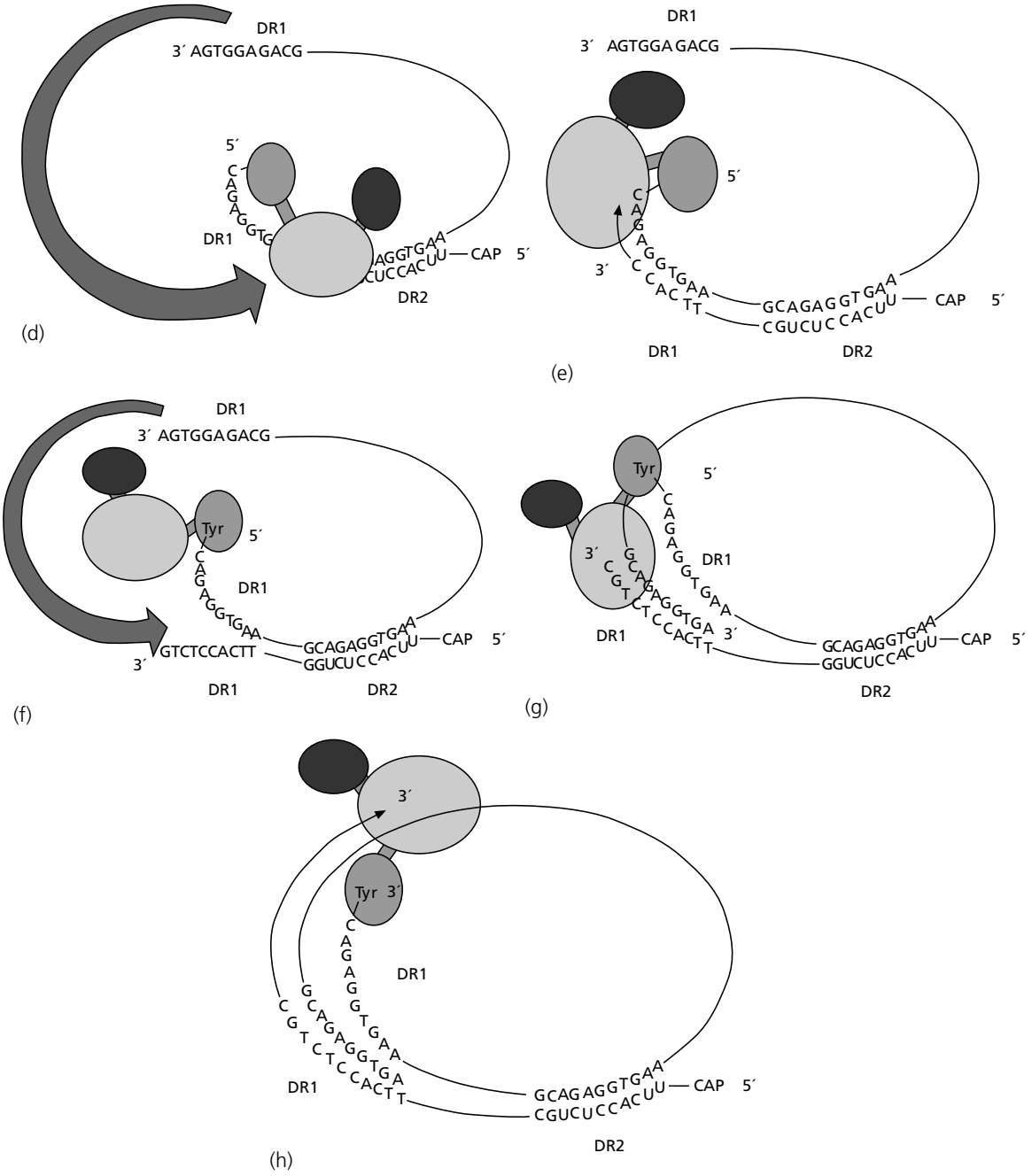

Figure 5 ***(continued) Generation of the viral genome from the pregenomic RNA.*** **(d)** *Translocation (arrow) of the RNA oligomer and the polymerase to a complementary sequence on the DR2.* **(e)** *Start of positive DNA strand synthesis, using the 3′ end of the RNA oligomer as a primer.* **(f)** *Melting of the 3′ end of the positive DNA strand and the 5′ end of the negative DNA strand. Translocation of the 3′ end of the negative DNA strand to the DR1 at the 3′ end of the positive DNA strand (arrow).* **(g)** *Annealing of the DR1 at the 3′ end of the negative DNA strand with the complementary sequence of DR1 at the 3′ end of the positive DNA strand (arrow).* **(h)** *Further positive DNA strand synthesis.*

r, however, there are additional requirements for circularization. This includes the adjacent regions termed 5E and 3E (Liu et al. 2003, 2004).

Annealing of the plus strand 3′ end and 5′ end of the minus strand reconstitute a continuous single-stranded DNA template for the polymerase. Thus, the polymerase should be able to synthesize a double-stranded circular DNA with two nicks between the 5′ and 3′ end of both minus- and plus-strand DNA. The virion, however, contains a partially double-stranded DNA with the heterogeneous 3′ ends of the plus DNA strand leaving a single-stranded gap of ca. 800 nt. The reason for this phenomenon is unknown but one may hypothesize that the limited space within the capsid does not

allow an ongoing synthesis since double-stranded DNA is less flexible than single-stranded DNA. A minority of virions contain a linear double-stranded DNA genome instead of the partially double-stranded circular DNA molecule. As shown for DHBV these virions are infectious. Within the nucleus the cccDNA is formed by circularisation of the linear DNA using nonhomologous recombination between the two ends (Yang and Summers, 1998).

TRAFFICKING OF PROGENY HEPADNAVIRAL CAPSIDS

Transport to the nucleus

Mature core particles may enter two different pathways, which are important for the viral life cycle. Because the HBV cccDNA in the nucleus is subject to slow degradation, the pool of intranuclear HBV genomes must be restored permanently to ensure viral persistence. The restoration of the pool is usually possible either by new infection of the hepatocyte with HBV, or by re-entry of viral DNA after release from the viral nucleocapsid. Amplification of cccDNA between 10 and 50 copies per cell occurs even in cultured hepatocytes that are not susceptible to DHBV and HBV infection, so it obviously does not require reinfection but nuclear entry of viral genomes from newly synthesized core particles in the cytoplasm. This 'short cut,' without secretion and reinfection, occurs predominantly at the beginning of infection. It leads to the establishment of chronic infection on the cellular level. In addition, this unusual strategy, which has only been described for pararetroviruses, avoids the appearance of the virus in the serum, and minimizes the risk of being neutralized by circulating antibodies. However, DHBV cores cannot spread from cell to cell but must be enveloped, secreted, and enter new cells (Funk et al. 2004).

The mechanism of how the viral genome is delivered to the nucleus has been studied in in vitro systems and seems to follow the same strategy as during initial infection. Apparently, the genome is transported within the core particles, which use the cellular microtubule transport system for reaching the perinuclear region, followed by an active nuclear import mediated by cellular nuclear import factors. For interaction with the import factors, the core proteins expose a 'classical' localization signal (NLS) on the capsid surface. The NLS is localized within the carboxy-terminal domain of the core proteins and within the lumen of the capsid after assembly. The exposure of the NLS on the capsid surface thus requires a structural change, which is caused by phosphorylation of the carboxy terminus (Rabe et al. 2003). The NLS is bound by the adapter protein importin α and subsequently by the cellular transport receptor importin β. Following the established pathway of nuclear import, importin β mediates the binding to the cytoplasmic fibers of the NPC and the transport through the pore into the nuclear basket (Panté and Kann 2002). Exclusively at this site, the core particles disintegrate and release the viral DNA into the karyoplasm (Rabe et al. 2003).

Envelopment and secretion

The second pathway for core particles is the envelopment into the surface proteins, followed by virus secretion. DHBV mutants defective for envelope proteins but competent for replication lead to intranuclear cccDNA accumulation up to 50 times the natural copy number. The expression of surface proteins results in envelopment of core particles and secretion of virus, and regulates amplification of cccDNA (Summers et al. 1990). After de novo infection of hepatocytes, transcription of HBs mRNA is a late event (Tuttleman et al. 1986). Thus, the regulation of DHBV cccDNA amplification via surface protein synthesis guarantees that a large pool of cccDNA has been generated in the infected cell before virus is exported.

In contrast to HDV, where only SHBs is required for assembly and secretion, assembly of HBV requires LHBs and SHBs (Bruss and Ganem 1991). Stepwise truncation of LHBs showed that only sequences proximal to amino acid 108 of preS are required for HBV assembly. A series of mutations determined that the highly conserved preS1 region between Arg103 and Ser124 at the transition to preS2 is essential (Bruss 1997). Using the same technique, envelopment-negative core mutants were identified. Although being spread throughout the primary amino acid sequence, these mutants clustered at two sites on the capsid surface. One cluster is localized in a ringlike groove around the tip of the spikes and the second cluster at the amino-terminal end of the flexible carboxy terminus of the core protein (Ponsel and Bruss 2003).

In the current model of HBV (Figure 6c,d; and see Figure 2), the preS domains in LHBs have a dual topology. The preS domain in part of the LHBs molecules is cytosolically retained by Hsc70 (Lambert and Prange 2003); the other part is exposed at the surface of HBV, allowing multiple functions of LHBs:

- Like matrix proteins of many other enveloped proteins, it mediates the budding of core particles together with their envelope proteins. For this task, a matrix protein must be fixed at the membrane, but its binding site for the viral capsid should be located on the cytosolic side.
- The preS1 domain is also the attachment site to the cell surface during infection. For this purpose the preS domain has to be accessible on the surface of the particle.

The mechanism by which the preS domain post-translationally passes through the ER or post-ER membrane is unclear. Direct passage through the lipid bilayer without the involvement of signal recognition

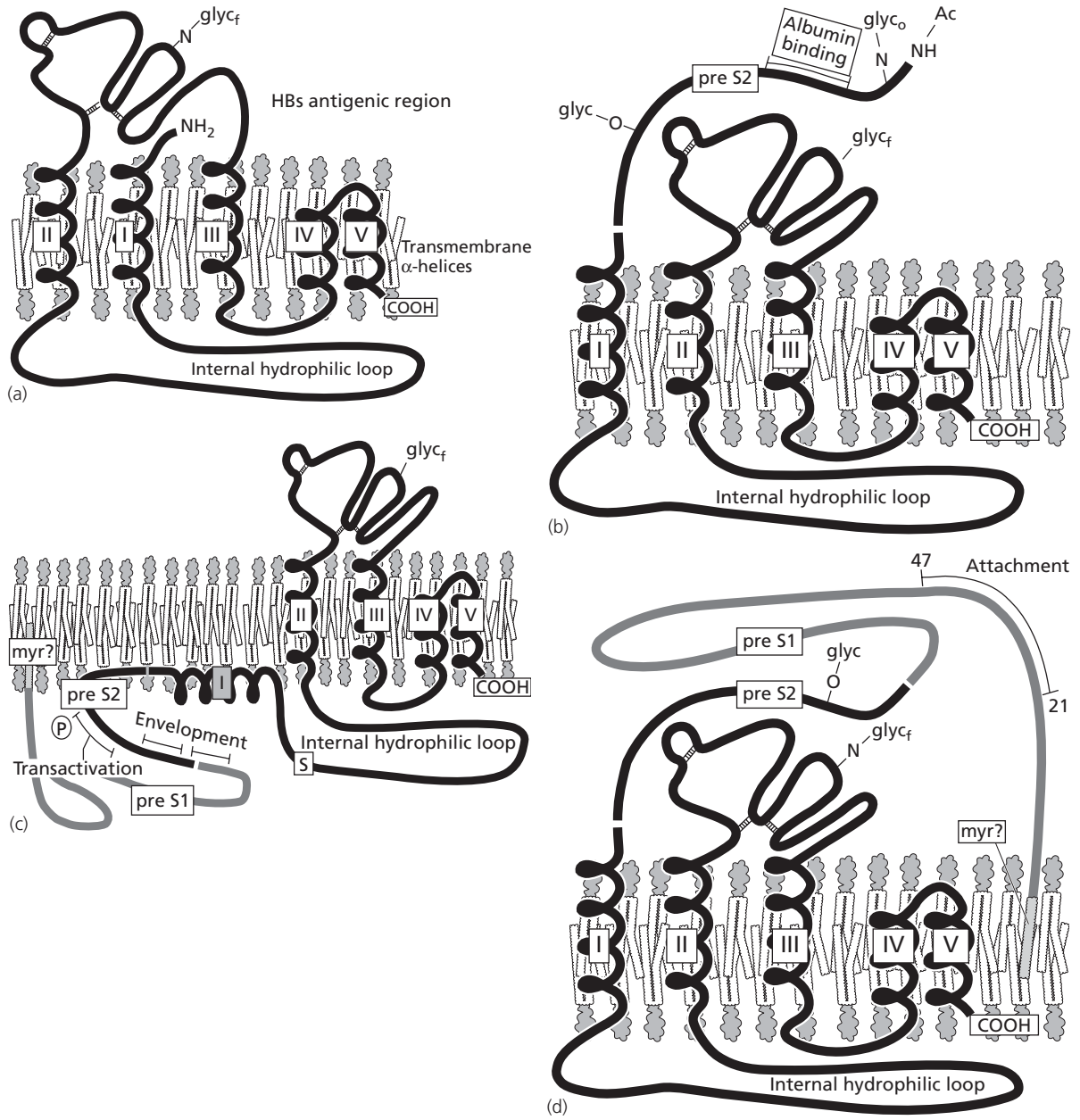

Figure 6 *Postulated topology of hepatitis B virus surface proteins.* **(a)** *SHBs;* **(b)** *MHBs;* **(c)** *LHBs with preS in the cytosol;* **(d)** *LHBs with the preS at the virus surface. In the nonassembled state, the bottom reflects the cytosolic side which represents, after encapsidation, the core particle of the inner part of the virus. The top of the figure represents nonassembled surface proteins in the lumen of the endoplasmic reticulum, after assembly and secretion the outside of the particle. The molecules of the membrane bilayer are depicted in gray and white. Predicted transmembrane helices are numbered I–V. ac: acetyl residue;* N-*glyc$_o$: obligatory complex* N-*linked glycan;* N-*glyc$_f$: facultative* N-*glycan; myr: myristylation site; O-glyc: O-linked glycan.*

particles (SRP) and secretion factors (sec) has been described for small amphipathic proteins, but the preS domain does not have this type of structure (Bruss et al. 1994). Incubation at low pH and at 37°C favors translocation of interior preS domains to the surface of virions (Bruss et al. 1994; Guo and Pugh 1997). The preS2 portion of preS carries a translocation motif that allows for energy-independent membrane passage (Oess and Hildt 2000) and may carry proteins into cells (Hafner et al. 2003). However, there are no data that this motif is involved in the translocation of the preS domain itself.

The forces that drive the budding and assembly of HBV and HBs particles are not yet well understood. The carboxy-terminal part and parts of the two hydrophilic loops in the S domain can be deleted or substituted, but most of the cysteine residues in the internal loop are essential. The high sequence conservation in the four α-helices suggests a coil-to-coil interaction,

which may contribute to the morphogenesis of virions and HBs particles (Berting et al. 1995).

The HBs particles mature in a pre-Golgi compartment. The *N*-linked glycosides of the HBs proteins at N146 of the S domain and N4 of preS2 are trimmed and modified to a complex biantennary oligosaccharide. *O*-linked glycan is linked to T37 of preS2, and an acetyl residue to the N-terminal methionine (Schmitt et al. 1999, 2004). Cultivation of HBV-expressing cells with inhibitors of glucosidase I (for example, *N*-butyldeoxynojirimycin) blocks the trimming and prevents the secretion of HBV (Block et al. 1994). MHBs is a specific target for this block of secretion (Lu et al. 1995).

HBsAg 20 nm spheres are constitutively secreted. However, HBs-filaments may be retained in the ER if they contain more LHBs than SHBs. The retention signal resides in the preS1 region (Gallina et al. 1995). MHBs does not significantly alter the secretion behavior if properly glycosylated.

Certain hepatocytes in HBsAg carriers seem to overexpress LHBs, relative to SHBs, and contain a dilated ER, which gives them a ground glass appearance in light microscopy. Such cells probably do not contribute to HBV production and are often found in so-called healthy HBsAg carriers with low viremia (Dienes et al. 1990). Only cells that express core protein, polymerase, SHBs, and LHBs protein in a well-balanced mixture are able to assemble and secrete HBV.

Nonstructural proteins

In contrast to the positive-strand RNA viruses that do not use reverse transcription, retroviruses and the hepadnaviruses do not use a major part of their coding capacity for nonstructural proteins. However, LHBs and SHBs are not only essential structural components of the virion; they occur also as secreted antigens. Furthermore, MHBs is a nonessential structural protein of mammalian hepadnaviruses.

Two proteins that are not essential for replication in cell cultures are HBe protein and HBx protein. Neither HBe protein nor HBx protein have yet been identified as a structural component of the virion. They are therefore considered here as nonstructural proteins, although their presence in low amounts in virions cannot be excluded.

HBs PROTEINS

A peculiarity of all wild type hepadnaviruses is expression of the HBs proteins in large excess. Even in HBV carriers producing many virions, empty HBs particles are present in ca. 1 000-fold greater number than virions and may reach concentrations of up to 1 g/l, as major serum proteins such as IgM. In low viremic HBsAg carriers, HBsAg may be present in particle numbers up to 10^{12}/ml even though HBV DNA is barely detectable by polymerase chain reaction (PCR). Thus, in a way, HBs proteins are nonstructural as well as structural proteins. HBsAg particles have a much longer half-life (>8 days) than virions, which disappear with a half-life of 1–3 days from the serum if replication is blocked (Chulanov et al. 2003). However, HBV-transfected hepatoma cells also produce an excess of HBs proteins. Thus, the excess of nonstructural HBs protein over virion-bound HBs protein is not only due to more rapid removal of virions from the circulation. The significance of this overexpression is not clear. It may contribute to an immune tolerance that is a precondition for highly productive persistent infection in an immunocompetent host. Furthermore the excess HBs proteins may block the HBV receptors of the host during late or persistent stages of infection and thereby allow the circulation of virions, which is a precondition for efficient transmissibility. The long delay of several weeks or even months between infection and the first appearance of virions and HBs protein is probably due to the slow replication of HBV, but may also be due in part to immediate readsorption to receptors not yet blocked. However, for DHBV intracellular down-regulation of the gp180 receptor by L-DHBs protein has been observed (Breiner et al. 2001).

The proportion of HBs proteins differs in the three morphological forms of HBs particles. Virions contain a high proportion of LHBs, HBs filaments intermediate amounts, but small amounts are present in 20 nm particles. The proportion of MHBs may be variable and is usually higher in HBs particles from high viremic carriers.

HBe PROTEIN

HBe protein was discovered by the analysis of HBsAg-positive sera from different donors by double diffusion in agar gels (Magnius and Espmark 1972). Some HBsAg carriers contained a precipitating antibody against a novel HBV antigen called e antigen (HBeAg). HBeAg was found predominantly in highly productive HBV carriers with a detectable endogenous HBV DNA polymerase, whereas anti-HBe was usually found in 'healthy' low viremic carriers. Sera from HBeAg-positive carriers typically have infectivity titers for chimpanzees of 10^8/ml or more and genome numbers of 10^9–10^{10}/ml, whereas anti-HBe- and HBsAg-positive sera have typically only ten infectious doses/ml (Shikata et al. 1977). The biochemical nature of HBe protein remained unclear until 1982, when recombinant HBc particles, devoid of any other viral component, were converted into HBeAg by treatment with protease and ionic detergents. Characterization of isolated HBe protein from the serum of HBV carriers revealed that it had an amino acid composition similar to that of HBc protein, but it had only ca. 15 kDa owing to a carboxy-terminal truncation at Val 149 of HBc.

It cannot be ruled out that a minor part of HBe protein in serum may be derived from degraded nucleocapsids or nonassembled HBc protein, but at least the major part of HBeAg, if not all, is generated by a biosynthetic pathway different from that of HBc protein. As mentioned in the section entitled Genome structure and function, above, HBV-containing cells express, besides the pregenomic RNA, a slightly longer RNA that contains not only the AUG for the HBc protein but also the AUG for the preC region 29 codons upstream. The preC sequence has the property of a signal peptide, which directs the nascent polypeptide to the membrane of the ER and causes its translocation to the ER lumen. The translocation is, however, incomplete, thus allowing the re-entry of part of the full-length HBe protein molecules with 25 kDa to the cytosol. This form of HBe protein may be transported to the nucleus because of its carboxy-terminal nuclear localization signal.

The major part of ER-associated HBe protein is cleaved at Ala19, thus generating a 22-kDa protein. During passage through the Golgi apparatus, a further protease cleaves HBe at Val149 or alternative sites downstream, generating proteins of 16–18 kDa. This cleavage is a precondition for release from the cell. HBe protein may oligomerize, but, in contrast to HBc, does not assemble to a closed particle. The tryptophan and cysteine residues in the remaining part of the preC peptide prevent assembly to corelike particles (Schlicht and Wasenauer 1991). The amount of HBeAg in the serum of HBV carriers by far exceeds the amount of HBc protein within circulating virions. Titers in enzyme immune assay (EIA) often reach 1:8 000, and detection by agar gel immune precipitation suggests amounts of several micrograms per milliliter. Thus, both major structural proteins HBs and HBc of the virion are accompanied by a large excess of slightly altered nonviral forms.

It is doubtful whether HBe protein has a direct function in the viral life cycle. The HBe mRNA does not support genome replication. HBe-negative mutated genomes of DHBV and WHV replicate in the natural hosts. Naturally occurring HBe-negative variants of HBV are present in many patients (see section Clinical and pathological aspects). It has even been found that preC-containing wild type genomes replicate less efficiently in transfected hepatoma cells than preC-negative genomes (Scaglioni et al. 1996).

The conservation of HBe protein in all known hepadnaviruses, in spite of its being nonessential, suggests that it provides an important advantage for the survival and spread of hepadnaviruses in their host population. A clue to the function of HBe protein may be the observation that newborn woodchucks infected with an HBe-negative mutant develop acute, but not chronic, infection as occurs with wild-type virus (Chen et al. 1992). It may be that HBe protein suppresses immune elimination of HBV, thus contributing to the development of a carrier state (Milich and Liang 2003). Furthermore, HBeAg is transferred in utero from a HBV carrier mother to the fetus (Wang and Zhang 2003).

HBx PROTEIN

HBV encodes a protein of 154 amino acids (141 in WHV and 138 in GSHV), the function of which is essentially unknown. The recent identification of a functional HBx protein in avian hepadnaviruses (Schuster et al. 2002) suggests that it is somehow essential during hepadnaviral replication in that it alters the host organism in a way that it is more permissive for viral replication. HBx is not essential for HBV production in transfected permanent cell lines (Yaginuma et al. 1987) or primary rat hepatocyte cultures (Blum et al. 1992). WHV genomes without a functional *HBx* gene do not replicate efficiently in woodchucks (Chen et al. 1993; Zoulim et al. 1994), but replicate sufficiently in vivo to induce immunity and may, thus, be considered as attenuated virus (Zhang et al. 2001). This observation suggests that HBx protein is not essential for low-level replication in vivo, but enhances infection (Xu et al. 2002). There may, however, also exist an effect of HBx on the immune system favoring tolerance against HBV, because HBV genomes with a truncated HBx variant have been observed preferably in immunodeficient patients (Repp et al. 1992). Evolution of such HBV genomes without a complete *HBx* gene in immunocompromised patients may indicate that HBx protein is not so necessary in patients without an efficient immune defense as in normal hosts. HBx protein may activate components of the specific and the innate immune system, for example, the nuclear factor of activated T cells NF-AT (Lara-Pezzi et al. 1998) or NF-κB (Su and Schneider 1997). HBV may enter lymphocytes and monocytes of HBV carriers and is weakly expressed. In these cells, HBx mRNA is expressed at highest levels compared to other HBV mRNAs (Stoll-Becker et al. 1997).

The genome position of the *HBx* gene and promoter next to HBV enhancer I is reminiscent of the genome position of the early 1A (*E1A*) gene of adenoviruses and of other immediate–early genes of DNA viruses. *E1A* is essential for adenovirus replication in most but not all cell types by *trans*-activating the early promoters of E2 and E4 in the adenovirus genomes. The *trans*-activating activity of HBx on HBV enhancer I and on various viral or cellular enhancers was first described in 1988 by Spandau and Lee and by Zahm et al. Numerous further studies (for example, Wu et al. 2002) confirmed the nonspecific promiscuous *trans*-activating activity of HBx (review by Murakami 2001), but usually the activation factor is low. HBx does not bind directly to DNA. If artificially expressed in the absence of other HBV genes, it is found most abundantly in the cytoplasm, but it is also present in the nucleus (Doria et al. 1995). Current evidence suggests that cytoplasmic HBx may act either

via a tyrosine kinase signaling cascade and/or mitochondrial calcium channels (Bouchard et al. 2003) but other pathways also seem to exist (Hafner et al. 2003). However, the *trans*-activating activity seems to be maintained in some systems if a nuclear localization signal is added. Many potential nuclear binding partners of HBx protein have been identified in vitro: transcription factors (TF), ATF II, and CREB, the TATA-binding protein TFIID, TFIIB, RNA polymerase subunit RPB5, a novel X-associated protein XAP-1 which may be involved in DNA repair, the ubiquitous transcription factor Oct 1, tumor suppressor protein p53, DNA repair-associated transcription factor ERCC3, and single-stranded RNA. Furthermore, binding of HBx to XAP-C7, an α-subunit of the proteasome, or to XAP-P13, a subunit of the regulatory 26S complex of the proteasome, or to a liver-specific HBx-interacting protein XIP were reported. The highly divergent results of the in vitro binding studies allow, for the moment, only one conclusion: that HBx is very sticky. The association with the proteasome appears to be significant, because proteasome inhibitors may restore HBV replication activity of HBx-deficient mutants to wild-type levels (Zhang et al. 2004). A problem with all the *trans*-activation studies is that they depend on heavy transient overexpression of both HBx proteins and the target sequence. Moreover, they were carried out in culture systems that produce HBV without the presence of HBx and may thus be irrelevant in respect to the viral life cycle.

Many viral immediate–early transcription-activating factors, such as E1A of certain adenoviruses, or P6 and P7 of certain papillomavirus types, are also tumor proteins. They bind and inactivate tumor suppressor proteins such as Rb or p53. HBx was reported to bind and inactivate essential functions of p53. An Rb-inactivating factor of HBV has not yet been reported. HBx transforms the SV40TAg-immortalized fetal mouse hepatocyte line FMH 202 to overt malignancy if expressed in large amounts (Höhne et al. 1990). Similar results were obtained in p53-negative mouse cells (Oguey et al. 1996). Of several strains of mice transgenic for HBx protein, only one strain developed liver tumors; high expression of HBx in these mice was necessary. In one transgenic mouse line, an enhanced susceptibility against chemical carcinogens was noted (reviewed by Schaefer and Gerlich 1995). In human hepatocellular carcinoma (HCC) and cirrhotic liver HBx protein may be detected with selected anti-HBx antibodies in the cytoplasm of some but not all cells (Su et al. 1998). More recently, a very clear association of anti-HBx antibodies in the plasma and HBx antigen in the liver with the presence of HCC was found in contrast to findings in chronic hepatitis B patients (Hwang et al. 2003).

While immortalized cells may become malignant under high expression of HBx, primary cells seem to go into apoptosis (Terradillos et al. 1998; Schuster et al. 2000). HBx also enhances the apoptotic effect of tumor necrosis factor (TNF) alpha (Su and Schneider 1997). It appears that overexpressed HBx interacts with a variety of apoptosis-inducing or inhibiting factors, for example, c-FLIP (Kim and Seong 2003), mitochondrial membranes (Shirakata and Koike 2003), Bcl-2 (Terradillos et al. 2002), or the Fas-ligand (Yoo and Lee 2004). The oncogenic effect of HBx seems to reside in the amino terminal portion (Gottlob et al. 1998), whereas the proapoptotic and transcription-activating effect is in the carboxy-terminal portion (Schuster et al. 2000). HBx mutants present in HCCs abrogate the antiproliferative and transactivating effects of wt HBx and may, thus, maintain oncogenicity (Sirma et al. 1999). The proapoptotic function is conserved in the avihepadnaviruses (Schuster et al. 2002).

In natural infection, HBx mRNA is usually expressed in low levels. Furthermore, the half-life of HBx seems to be very low. Even if one postulates an oncogenic potential of HBx, this may explain why acutely infected patients and chronically infected HBV patients with high replication do not develop HCC. In WHV-infected woodchucks, WHxAg is localized in the cytoplasm of infected cells, but is rarely found in HCC (Jacob et al. 1997).

It is open to question whether HBx is not oncogenic at all in natural settings, or its expression is too low, or viral factors suppress the activity of HBx. With regard to the latter possibility, it has been observed that the core gene of HBV, if co-expressed, suppresses the *trans*-activating activities of the *HBx* gene on a variety of promoters. As with oncogenes from other viruses, *HBx* may be negatively controlled by viral genes the expression of which is stimulated by *HBx*. In HCC, *HBx* may be expressed from HBV DNA integrates, which often do not encode other complete HBV ORFs.

In summary, HBx probably has a role in liver-specific expression of HBV and a weak oncogenic potential that, however, can be effective only in combination with other oncogenesis-favoring events. The significance of the proaptotic effect is not understood.

Integration of HBV DNA and liver cancer

Integration of the viral genome into the host genome is not part of the hepadnaviral life cycle, in contrast to the retroviruses. Nevertheless, integration of HBV DNA fragments into the hepatocyte genome is a frequent event during HBV infection. Even if a full-length genome would be integrated the circular HBV genome is thereby disrupted and no longer able to express a viable pregenomic RNA. (Viable HBV DNA can be integrated for experimental purposes in the form of artificial linear constructs covering the sequence of the pregenome plus homologous or heterologous upstream transcription signals.) However, subgenomic mRNAs

and viral proteins may be expressed from integrated HBV DNA fragments. Integration sites in the viral genome are very often around DR1 at one end and at variable sites at the other end. These integrates may express a carboxy-terminally truncated HBx protein fused to cellular sequences which still has *trans*-activating activity in experimental systems (Wollersheim et al. 1988). Furthermore, a cryptic polyadenylation site may be used that generates a polylysine tail at the carboxy-terminal end of a truncated HBx protein (Rakotomahanina et al. 1994).

The integrated HBV DNA is quite often composed of two, or even more, fragments that are linked together in a head-to-tail arrangement or in other complex rearrangements. Occasionally, such a rearranged DNA fragment may express truncated forms of preS/S proteins, in particular truncated MHBs and HBx. Such truncated LHBs or MHBs proteins have been found to *trans*-activate transcription factor AP1 and others, suggesting an additional growth-stimulating effect of these defective HBV proteins (Hildt et al. 1997). Such HBV DNA fragments induce malignant growth in cell cultures and nude mice (Luber et al. 1996).

With human HBV DNA, no clear preference for a defined integration site has been identified and it was noted that the integration site might be rearranged. It seems that integration of HBV DNA destabilizes the host genome. In two independent studies from Japan (Kawai et al. 2000; Okabe et al. 2000), a loss of heterozygosity was more often found in HBV-associated HCC than in nonviral or HCV-associated HCC. It has been suggested that oxidative stress – as may occur in inflammation – induces not only DNA-damage but also enhances HBV DNA integration (Dandri et al. 2002). A model has been proposed that topoisomerase I mediates the integration of HBV DNA around the DR1 site. A role of the integrated HBV DNA in the development of HCC is suggested by the fact that virtually all HCC-derived cell lines from HBV carriers contain one, or usually more, HBV DNA fragments and that the HCCs are usually of monoclonal origin. The model of insertional mutagenesis leading to deregulation of growth control genes has been verified for several human HCCs. One example is the fusion of a retinoic acid receptor β chain with 29 amino-terminal residues from preS1, which may lead to uncontrolled expression of this differentiation factor (Dejean et al. 1986). Another example is the insertion of preS2/S sequences of HBV into an intron of cyclin A, generating a preS2/2 fusion with cyclin A devoid of its amino-terminal degradation signals (Wang et al. 1990). This altered cyclin A has transforming properties in cell cultures. A third example is the activation of mevalonate kinase (Graef et al. 1994). More recently, a systematic search for HBV DNA integration sites revealed, in six of 21 isolates, insertion into cellular genes which are key regulators of cell proliferation and viability, among them the human telomerase reverse transcriptase gene (Gozuacik et al. 2001), which has also been found by others (Ferber et al. 2003). Further integration sites were reported next to the RNA-binding motif of Y chromosome gene which was found to be ectopically expressed in three of 10 HCCs from children (Tsuei et al. 2002). More typical are, however, integrations into Alu-type repeats, mini-satellite-like, satellite III or a variable number of tandem repeat sequences, suggesting a preference of HBV DNA integration into sites where other multiple insertions have already occurred during evolution (Rogler and Chisari 1992).

Development of HCC is a regular event in woodchucks chronically infected with WHV. In HCCs of this animal, insertion of WHV DNA very often occurs upstream of a functional retrotransposon encoding cellular N-*myc*2. The viral enhancers activate the cellular N-myc2, which finally leads to the development of HCC (Flajolet et al. 1998). Insertion of hepadnaviral DNA into c-*myc* sequences was observed both in woodchucks and in ground squirrels. However, in human HCC c-*myc* or N-*myc* activation was found only occasionally (Buendia et al. 1993). Hepadnaviral DNA integration has also been found in HCC from ducks, but development of HCC in ducks seems to be exclusively correlated to exposure to aflatoxin B.

HBV genotypes, HBsAg subtypes, and HBV variants

The sequencing of numerous HBV isolates in the late 1970s and early 1980s has revealed a surprisingly high degree of conservation; even rodent hepadnaviruses are ca. 60 percent homologous to human HBV. Human HBV splits into eight identified genotypes, A to H, which diverge by 8–15 percent in the DNA sequence (Norder et al. 2004). Furthermore, genosubtypes with a typical predominant ethnical distribution have been defined: A1 in Africa, Brazil, and India, A2 in Europe and USA, B1 and B2 in Japan and China, B3 in Indonesia, B4 in Vietnam, C1 and C2 in Japan, Korea, and China, C3 in Oceania, C4 in Australian aborigines), D1–D3 in Europe, the Mediterranean, Middle East, and Central Asia, D4 in East Asia, E in West Africa and Madagascar, F1 and H in Central America, F2 in South America and Polynesia. The latter observation supports the debated theory of Thor Heyerdahl that a part of the Pacific Islands was settled from South America (Norder et al. 2004). Genotype G is rare and has no clear geographical pattern. HBV has also been found in apes, every species having its own genotype(s). Due to vertical and intrafamiliar transmission, the genotype usually reflects the ethnic origin even after several generations and not the actual geographical location. Americans or Canadians of East Asian origin usually have genotype B or C, Brazilians often genotype A1 originating from Africa.

Human and ape genotypes are phylogenetically equally distant (reviewed by Bartholomeusz and Schaefer 2004), but genotypes F and H branch off and are more distant. This suggests that there may have been a common ancestor to old world primates' hepadnaviruses and most human HBV genotypes whereas genotypes F and H may have been acquired in the new world. The next relative to human and ape hepadnaviruses has been isolated from woolly monkeys (a new world monkey) (Lanford et al. 1997). Since this virus is not viable in chimpanzees, it is considered an own species as is WHV (Mason et al. 2005). Avian hepadnaviruses are only distantly related to mammalian hepadnaviruses, despite their similar genomic organization and replication strategy.

The multiple functions of the viral nucleic acid sequences for the coding of overlapping genes, regulation of replication, transcription, and assembly of the virions prevent excessive variability. A hot spot of variability between virus species is the preS1 region, probably because of its role in host specificity and because the overlapping polymerase sequence functions in this region only as spacer.

Variability in the hydrophilic loop of the SHBs protein on the virion surface or on HBs particles, respectively, determines the serological HBsAg subtype. Besides the common antigenic determinant *a*, subtype-specific alleles *d* or *y*, and *w1*, *w2*, *w3*, *w4*, or *r* were described. Combinations of these alleles define nine HBsAg subtypes according to a workshop held in 1975 in Paris. Position 122 of SHBs is lysine for determinant *d* and arginine for determinant *y*. Position 160 is lysine for the four *w* alleles and arginine for *r*. Differentiation into *w1* to *w4* is mainly based on exchanges at position 127 (Norder et al. 1994). There is no clear correlation between genotype and HBsAg reactivity. Thus, HBV genomes should preferably be characterized by their genotype. Antigenic differences, defined by reactivity with monoclonal antibodies, also exist in the preS2 and preS1 regions. The predominant sequences of an HBV isolate are usually conserved within an infection chain from person to person, suggesting low variability. Obviously the existing genotypes of HBV are optimally adapted to replication in their host population. However, HBV turns out to be a quasi species at all stages of infection if a large number of cloned genomes is analyzed.

HBV variants can be generated by point mutations, small deletions or insertions, and by splicing events. The reverse transcription of HBV is very inaccurate and whereby all possible mutations may be created in a very short time. One can estimate that a highly viremic chronic carrier generates ca. 10^{13} new viral genomes per day. At an assumed error rate of 10^{-5}, 10^{8} mutants per day are generated. While in the quasi-immunotolerant state one genome species is strongly favored, any kind of selection leads to a great variety of variants. Breakdown of immunotolerance is indicated by disappearance of HBeAg and leads to a rapid accumulation of viable and nonviable mutants (Bozkaya et al. 1996). The main targets are

- the preS1/S2 junction leading to a loss of MHBs (Blackberg and Kidd-Ljunggren 2003)
- alterations in the binding sites for transcription factors in the core promoter and enhancer region (Erhardt et al. 2000) leading to less HBeAg production and stronger replication
- inactivation of the precore region, i.e. the HBeAg leader sequence (Chu et al. 2003)
- or deletions in the core protein (Günther et al. 1996).

The type of mutations leading to a HBeAg loss of production differs with the genotype (Chan et al. 1999; Tanaka et al. 2004). X-protein deletions are often found in immunosuppressed patients (Schlager et al. 2000). All these mutations supposedly develop under the host's own immune system as the only selective pressure. Active immunization with SHBs vaccine (Lu and Lorentz 2003) or passive immunization with anti-HBs (Carman et al. 1999) may select for escape mutations in the HBsAg antigen loop. Treatment with nucleoside or nucleotide analogues selects for mutations in the reverse transcriptase domain of the HBV DNA polymerase, which also become evident in the S domain (Locarnini 2003). Often, several types of selection come together leading to complex combinations of mutations. Typically, heavily mutated variants are more often found in late stage infection or HCC.

CLINICAL AND PATHOLOGICAL ASPECTS

Normal ('wild') types of *Hepadnavirus* species are not overtly cytopathogenic. Inflammatory liver disease is caused by the host immune response against viral antigens whereby cellular cytotoxic lymphocytes exert the strongest effect (Chisari and Ferrari 1995). Consequently, symptomatic liver disease is absent in persistently infected individuals whose immune system is either immature at birth or severely impaired. Some healthy adults may also develop persistent oligosymptomatic infection without an efficient immune response; possibly immunogenetic factors predispose for persistent infection.

Another type of oligosymptomatic infection occurs when the infectious dose is very low or the immune response is rapid and efficient, or both. In such cases, silent immunization results. Acute hepatitis occurs in most immunocompetent adults who are infected by the percutaneous route with highly infectious serum or blood. A variable, but usually small, proportion of such patients develops chronic disease after acute hepatitis B. Depending on the balance between viral replication and host defense, chronic disease may cease spontaneously or proceed over years or decades to liver cirrhosis or HCC,

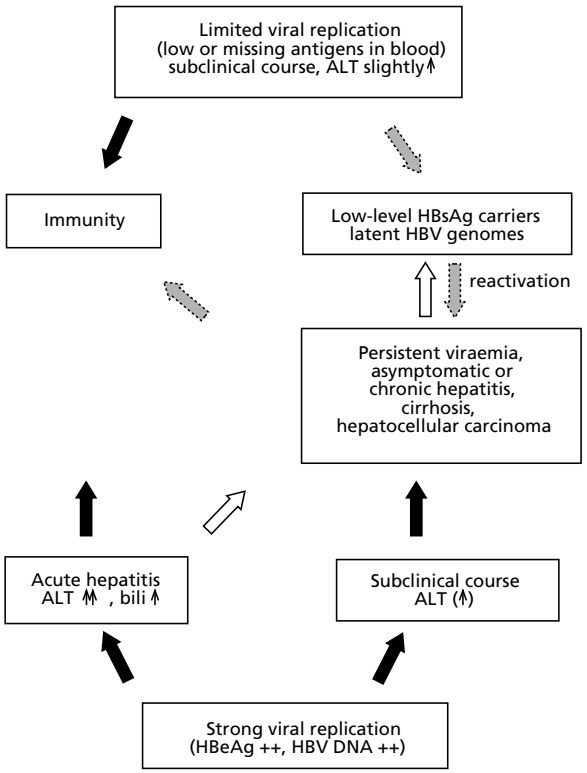

Figure 7 *Possible courses of hepatitis B virus infection. Black arrows show frequent courses, white arrows possible courses, and gray arrows rare or hypothetical events.*

or both. HCC usually develops in cirrhotic liver but may also appear independently of cirrhosis. In infections with high virus replication, extrahepatic manifestations (e.g. glomerulonephritis or periarteritis nodosa) due to circulating immune complexes may occur. Figure 7 illustrates the possible courses of HBV infection.

Clinical course of HBV infection

For more detailed descriptions and original literature, see textbooks on hepatology (e.g. Bircher et al. 1999) or on hepatitis viruses (e.g. Thomas 2000 or Lai and Locarnini 2002).

ACUTE HEPATITIS B

Diagnosis of acute hepatitis based merely on clinical symptoms is unreliable. Early nonspecific symptoms are malaise, poor appetite, nausea, and right upper quadrant abdominal pain which lasts for several days. In ca. 10 percent of patients with acute hepatitis B, a prolonged prodromal phase of up to 4 weeks occurs with serumlike sickness, including arthralgia or even frank arthritis, fever, and skin rash. In the more severe cases, after 3–6 days, fatigue and anorexia worsen and jaundice develops. Essential for the diagnosis is the determination of the alanine amino transferase (ALT) in the serum. In Germany, the upper limit of the normal range is 50 IU/l for men and 35 IU/l for women (measured at 37°C). Clinical hepatitis is associated with values higher than 500 IU/l, reaching several thousand IU/l in severe cases (Thomas 2000). The level of ALT indicates the degree of hepatocyte damage. It increases sharply at the end of the prodromal phase and usually reaches peak values shortly before the appearance of jaundice caused by levels of high bilirubin in the serum. In less severe cases, bilirubin concentrations are below 50 μmol/l; in severe cases >340 μmol/l. In contrast to cholestatic jaundice, alkaline phosphatase is only moderately increased in the serum.

The time course of a typical acute hepatitis B is shown in Figure 8a. Clinical and biochemical data do not allow distinction between infections by the various hepatitis viruses. The most reliable serological marker for acute hepatitis B is IgM antibody against HBcAg (IgM anti-HBc). With the onset of symptoms, IgM anti-HBc appears and reaches peak values within a few days.

Many clinicians rely more on the detection of HBsAg in the serum. HBsAg appears during the incubation phase (Figure 8a), depending on the infective dose and the mode of exposure, several weeks or months after the infective event. It increases over several weeks, typically to peak levels of 10 000–100 000 PEI units/ml of HBsAg (PEI = Paul Ehrlich Institute, Germany). Decrease of HBsAg concentration starts with the onset of the prodromal phase and continues until complete recovery. Most patients remain HBsAg positive for several months.

In 2–10 percent of acute cases, HBsAg may have been eliminated so rapidly that it is already undetectable in the first available serum sample. To identify HBV as the etiological agent in these cases, it is essential to obtain a quantitative IgM anti-HBc result if no other cause for the hepatitis has been identified. Some EIAs for HBsAg may detect less well variants of HBsAg with altered *a* determinant, but these variants are rare in acute hepatitis B.

Persistence

Persistence of HBsAg for more than 6 months after onset in high concentrations (>100 PEI units/ml) means that (1) the acute hepatitis B evolved to chronicity (Figure 8b); or (2) a pre-existing chronic HBV infection was superimposed by an acute hepatitis of another etiology; or (3) the acute disease may actually be an exacerbation of chronic hepatitis B. Again, a high IgM anti-HBc during the early acute phase confirms recent acute HBV infection, whereas moderate amounts or absence indicate previous HBV infection.

Full recovery

Full recovery is assumed if the ALT reaches normal or close to normal levels and if HBsAg becomes negative

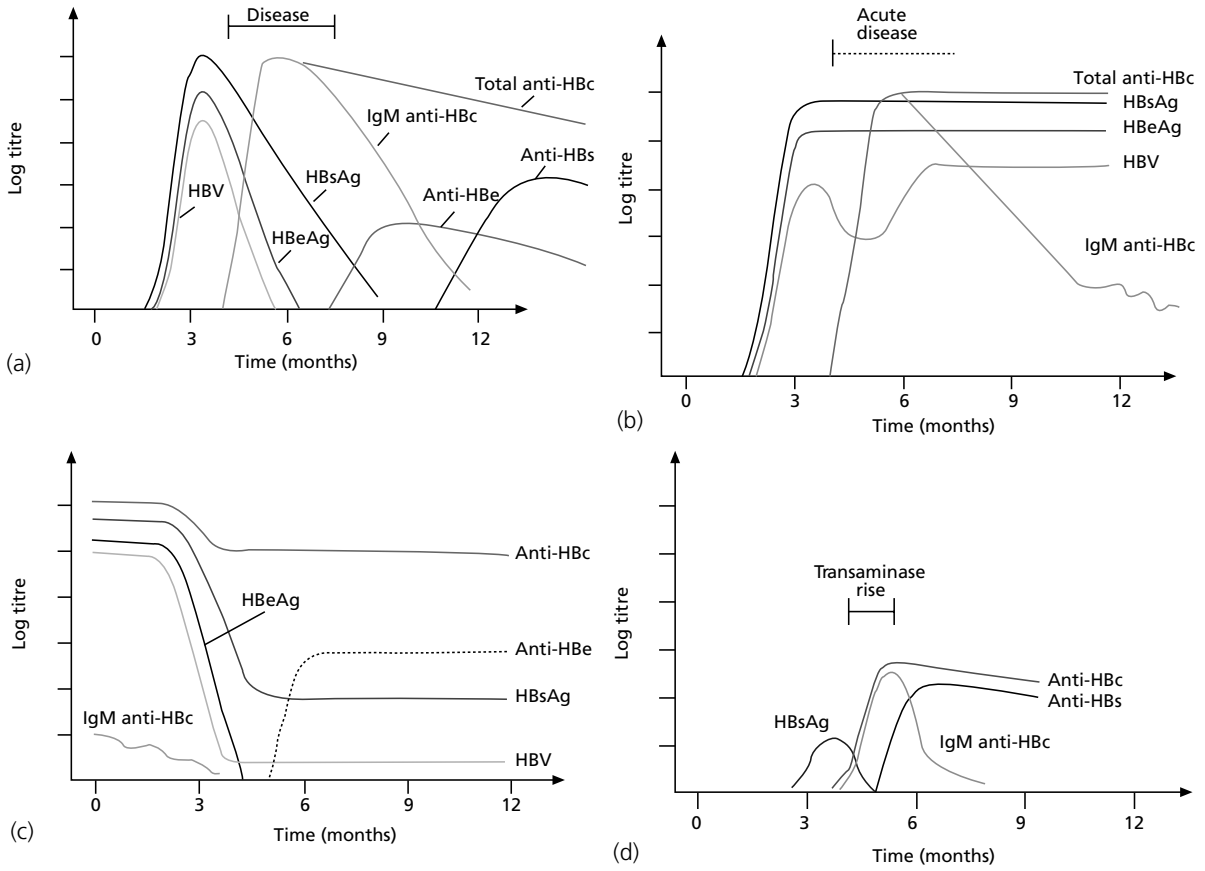

Figure 8 *Serological markers during the different courses of hepatitis B in arbitrary units:* **(a)** *acute hepatitis B, resolving;* **(b)** *acute hepatitis B, chronic evolution;* **(c)** *HBe seroconversion in chronic hepatitis B;* **(d)** *subclinical course of transient HBV infection.*

in EIA. This takes more than 6 months in some cases of protracted disease. Anti-HBs appears several weeks or even months after the disappearance of HBsAg. IgM anti-HBc falls to levels <100 PEI units, but may occasionally remain positive at low levels for several years. Anti-HBc of the IgG class may either persist lifelong or disappear after several years or decades.

HBV itself appears in the serum during the incubation period several weeks before HBsAg in amounts that allow its DNA to be detected by sensitive PCR techniques. Hybridization assays are not usually sensitive enough to detect HBV before HBsAg. Maximum concentration of 10^6–10^9 genomes/ml is reached before the onset of symptoms. About 40 percent of patients are already negative in hybridization assays (<10^6 genomes/ml) when the first serum sample is available.

Low-level persistence after recovery and reactivation

Cumulative evidence suggests that a major proportion of recovered patients retain intrahepatic HBV genomes. These continue to stimulate a cytotoxic T-cell response, which controls viral expression and replication (Rehermann et al. 1996). Occasionally, the cellular immune response is the only marker of the previous hepatitis B. Low level persistence may allow reactivation of HBV replication if the T-cell immunity breaks down, for example, after organ transplantation or during cancer therapy. Even a high anti-HBs level cannot reliably protect against reactivation because escape mutants may grow up. Cessation of immune suppression is extremely dangerous in that situation because memory T cells will proliferate and severely attack the infected liver (Westhoff et al. 2003). Thus, it is highly advisable to screen all patients before immunosuppression not only for overt (i.e. HBsAg positive), but also for occult HBV infection and to monitor for reactivation in positive patients.

Subclinical course and occult infections

The mild courses of transient HBV infection (Figure 8d) will usually go unnoticed unless sought after known exposure. For a few days, serology will show low amounts of HBV DNA, HBsAg, IgM anti-HBc, and a moderate transient rise of ALT to levels <500 IU/l for less than 4 weeks. Anti-HBc may appear at the peak of ALT, anti-HBs somewhat later. This inapparent course will often develop in healthy people

who were exposed to low doses or mucocutaneously. In completely inapparent HBV infections without ALT elevation, markers of HBV may be difficult to detect and occasionally follow unexpected patterns. Anti-HBs may occur before anti-HBc or remain the only serological marker. In some transient infections, HBV DNA may be the only marker of infection. While these 'occult' HBV infections are irrelevant for the patient and his contact persons, HBV may be transmitted if such HBV infected persons donate blood or organs, particularly liver (Allain 2004).

Fulminant course

About 0.2 percent of clinically apparent cases of acute hepatitis B are so severe that the patients develop symptoms of hepatic failure within a few days and ensuing hepatic coma. This course is often fatal. Survivors recover completely and develop immunity to HBV characterized by anti-HBs and anti-HBc. Fulminant cases are often associated with coinfection by hepatitis delta virus (see Hepatitis Delta virus) or possibly other hepatitis viruses. The significance of HBe-negative HBV variants for the development of fulminant hepatitis B is discussed controversially but they are found in ca. 50 percent of the cases.

Coinfection

Acute HBV-infection may occur together with hepatitis A virus (HAV), hepatitis C virus (HCV), HDV or HIV. None of the other hepatitis viruses aggravates the course of acute hepatitis, but HDV suppresses HBV viremia and enhances HBs antigenemia. HIV favors persistence of HBV. Pre-existent HCV may be transiently suppressed by HBV (Chulanov et al. 2003).

CHRONIC HEPATITIS B

Clinical signs of chronic hepatitis are usually mild and nonspecific. They include lack of energy, easy fatigability, and malaise. Myalgias, arthralgias, and skin rash may be present in cases with high virus replication due to circulating immune complexes. HBV-associated immune complex disease is an important cause of glomerulonephritis in tropical countries. The course of the disease is usually intermittent with acute exacerbations during which abdominal discomfort, nausea, weight loss, dark urine, jaundice, and mild fever may occur.

However, chronic HBV infection may go unnoticed until compensated or even decompensated cirrhosis of the liver is present. In this late phase of the disease the above-mentioned symptoms are enhanced and signs of hepatic encephalopathy may be present. Blood coagulation is decreased due to reduced synthesis of coagulation factors in the liver. Serum albumin is also decreased whereas IgG is increased, probably due to impaired degradation in the liver. The ALT levels are usually only moderately enhanced to, at the most, several hundred IU/l and often fluctuate. A major complication is the collateral blood circulation due to impaired blood flow through the cirrhotic liver. Breakages of the esophageal varices are life-threatening, and are a result of portal hypertension. A further consequence of portal hypertension is the development of ascites in some cases.

It is important to distinguish between disease activity reflected by markers of viral replication, antiviral immune response, and hepatocyte damage, on the one hand, and symptoms caused by the lack of functional hepatocytes and the replacement of normal liver tissue by connective tissue on the other. Whereas the viral disease activity may come to an end either spontaneously or as a result of treatment, the conversion of normal liver tissue to cirrhotic tissue is irreversible. The liver status can be assessed only by histology, liver function tests, and determination of serum proteins synthesized or metabolized by the liver. Sonography describes the shape and density of the liver and may detect an increased diameter of the portal vein due to portal hypertension.

Most patients with persistent HBV infection are not aware of a previous acute hepatitis B episode. As pointed out above, HBV persistence very often develops in individuals who are immunologically immature or somehow impaired. Perinatal HBV infection virtually always results in persistence, unless HBe-negative variants are predominant. In the early years of life, symptoms are usually absent in HBV carriers despite high virus replication. The same is true for patients who acquired infection during significant immune deficiency. However, in otherwise healthy HBV carriers, a cytotoxic immune response will eventually develop and cause chronic disease. This cytotoxic immune response is directed primarily against the HBc protein, whereas the HBs protein seems to be a minor target in chronic hepatitis B. The HBc-specific T-cell response suppresses HBV replication and may even eliminate many HBV-expressing cells. As a result, an exacerbation of chronic hepatitis B may lead to a new form of persistent HBV infection, in which HBsAg is still present in the serum but the number of virions is much lower than in the previous phase of high viremia. HBeAg disappears and anti-HBe is often present in this phase. If HBV replication is constantly controlled by the immune system at low levels, the so-called healthy HBsAg carrier status is reached. Eventually, HBsAg may also disappear from the serum. In these cases, anti-HBc is often the only marker of HBV infection. This phase of low viral activity may be reached at all degrees of irreversible liver disease. Thus, a seemingly favorable serological constellation suggesting low viral replication does not necessarily mean insignificant liver disease.

During the high viremic HBeAg-positive phase, variability of the HBV epitope regions is low. In HBeAg-negative cases, however, considerable variability is

observed. This variability may be generated by the immune elimination of the original strains.

A dangerous clinical situation occurs if a low viremic HBV carrier (with or without HBsAg or symptoms) is therapeutically immunosuppressed because of autoimmune disease, organ transplantation or anti-cancer therapy. In such cases, high HBV replication may reappear, but, as soon as the immune suppression is terminated, the existing memory cells induce a vigorous cytotoxic immune response that often results in fatal fulminant hepatitis B. In such cases, early pre-emptive therapy with an antiviral substance is indicated.

Coinfection of chronic hepatitis B patients with other hepatitis viruses and/or HIV is frequent. Intravenous drug users are often coinfected with HCV and/or HDV, male homosexuals often with HIV. Both HCV and HDV suppress replication of HBV, HCV often to the point that it becomes occult (Pontisso et al. 1993). In HIV coinfected patients, HBV may become the dominant pathogen under successful HIV therapy and requires an antiviral therapy, which is also effective against HBV (Rockstroh 2003). Superinfection of chronic hepatitis B patients with HAV does not lead to an aggravated course (Vento et al. 1998).

HEPATOCELLULAR CARCINOMA

HBV is the major cause of HCC in regions with high HBV prevalence. HCC usually develops after cirrhosis, but may also occur without cirrhosis. The involvement of HBV may not be recognized by testing for HBsAg in the serum, requiring the search for HBV DNA. In some cases in which the HCC contains integrated HBV DNA fragments no classic serological HBV marker may be present. However, in highly endemic areas of the world, people positive for HBsAg are 100 times more likely to develop HCC as compared to those who have anti-HBc and/or anti-HBs but no HBsAg (Beasley et al. 1981). An important cofactor is exposure to aflatoxin B (Ming et al. 2002). In low endemic areas the relative risk of HBsAg carriers to develop HCC is also increased. A British study found a 26-fold increased incidence of HCC in male HBsAg-positive blood donors compared to the male general population. The incidence was 33.5 per 100 000 persons year for male, but only 4.4 for female HBsAg carriers (Crook et al. 2003).

In some countries with intermediate HBV prevalence, HCV may be the major cause of HCC, but coinfection with HBV, HCV, and even HDV is quite common in HCC patients. In Italy, 45 percent of HCC patients have heavy alcohol intake, 36 percent HCV infection, and 22 percent HBV infection as risk factor for HCC (Donato et al. 1997). In Germany HBV is the leading risk factor for HCC (45 percent) followed by HCV (37 percent) and alcohol (Kubicka et al. 2000). While HBsAg persistence is associated with a highly increased risk for HCC, absence of HBsAg does not exclude development of an HBV-associated HCC (Yu et al. 1997; Tamori et al. 2003; Pollicino et al. 2004). It is believed that in addition to HBsAg, the level of HBV replication is positively correlated to the risk for HCC (Tang et al. 2004). The strong preponderance of HCC in male HBV carriers is positively correlated to the testosterone levels and to the structure of the androgen-receptor gene (Yu and Mertz 2001).

The development of HCC may be diagnosed early by quantitative measurement of α-fetoprotein (AFP) in the serum, but levels may be normal (<20 ng) before the HCC reaches a size of 3 cm. Levels above 1 000 ng/ml, or a sharp rise of AFP, indicate HCC. Abdominal imaging by ultrasound of HBsAg-positive patients, particularly those with liver cirrhosis, is advisable at intervals of 3–4 months.

Pathogenesis and immune response

The pathogenesis of HBV is based on the immune reaction. Virus replication itself is not overly pathogenic but subtle effects on hepatocyte function cannot be excluded. Transgenic mice expressing HBV or single HBV proteins are normal or almost normal. However, selective expression of LHBs in transgenic mice leads to storage of LHBs-containing filaments in the ER and pre-Golgi compartments. The LHBs-storing hepatocyte acquires a ground glass-like appearance. In mouse strains with very high expression of LHBs, hepatocytes become very sensitive to interferon-γ. Inflammatory liver disease and eventually HCC develop whereby the tumors no longer express LHBs (Rogler and Chisari 1992). LHBs containing ground glass hepatocytes are often found in healthy HBsAg, carriers but their diagnostic or pathogenetic significance in humans is not clear.

A potential pathogenic role has been suggested for HBx protein due to its apoptosis inducing properties, which may even protect against malignant transformation (Schuster et al. 2000). On the other hand, HBx has been reported to counteract p53-induced apoptosis (Wang et al. 2002) and to enhance malignancy of immortalized cells (Höhne et al. 1990; Oguey et al. 1996). HCCs usually contain variants of the *HBx* gene, which are less frequent in chronic hepatitis B patients (Yeh et al. 2000). Selective overexpression of HBx protein in a certain mouse strain has generated HCC late in life, but other mouse strains did not develop HCC. However, HBx-expressing mouse lineages were more susceptible to chemical carcinogens. Thus, HBx may be one oncogenic factor of HBV infection acting together with chronic inflammation (Nakamoto et al. 1998), insertional mutagenesis (Gozuacik et al. 2001), testosterone levels (Yu and Mertz 2001), or aflatoxin (Ming et al. 2002). Basal core promoter mutations 1762T and 1764A have been reported to be associated with HCC (Kuang et al. 2004; Kao et al. 2003), but this may be an indirect association because these mutations

appear late in infection and lead to enhanced HBV replication (Blackberg and Kidd-Ljunggren 2003).

SHBs, HBc, and HBe proteins are not pathogenic when expressed in transgenic mice. SHBs and HBe protein are secreted whereas HBc protein is stored as HBc particles in the nucleus. Cytoplasmic HBc particles are rapidly degraded whereas nuclear HBc protein has a longer half-life. All these findings are in accordance with observations on human HBV infections. HBsAg, secreted HBeAg and nuclear HBcAg are not associated with disease per se.

There may be differences in pathogenicity and virulence between the various HBV genotypes. In Africa, Europe, and the USA genotypes A, D, and E predominate which lead less frequently to perinatal transmission, probably due to a shorter period of high level viremia in females. However, their horizontal transmission is frequent in early childhood (Dusheiko et al. 1985). In Asia, with genotypes B and C, perinatal transmission is frequent and seroconversion from anti-HBeAg to anti-HBe with a concomitant decrease in viremia occurs later. With genotype C, seroconversion to HBeAg occurs still later than in genotype B resulting in an even more progressive liver disease (Kao et al. 2004) and worse response to therapy.

HBeAg-negative variants are presumed to cause a more severe acute infection but less persistence. However, after persistence has been established by the HBeAg-positive virus, HBeAg negative variants can evolve and persist further.

Virtually all infected individuals develop anti-HBc antibodies. Development of IgM anti-HBc indicates significant ongoing HBV replication and is a serological marker of inflammatory disease. The appearance of anti-HBs usually shows that HBsAg production is low or absent. However, HBsAg and anti-HBs may co-exist and, rarely, HBs escape mutants are present. Anti-preS1 and anti-preS2 appear during recovery from acute hepatitis B earlier than anti-SHBs, and disappear earlier. In some cases, however, these antibodies may also contribute to the formation of circulating HBs immune complexes in chronic HBV carriers. Anti-HBx and anti-HBpol antibodies are detected in many but not all patients with acute or chronic hepatitis B, and are neither diagnostically nor pathogenetically important. HBs and HBe immune complexes contribute to extrahepatic manifestations of HBV, but are probably not a major pathogenic factor in viral hepatitis.

Administration of anti-SHBs in high doses protects against de novo infection with HBV. The administration of anti-SHBs to individuals already infected (for example, liver transplant recipients or some HBV-infected patients) favors outgrowth of HBs escape mutants. In vitro experiments with infection of cell cultures show that anti-preS2, and in particular anti-preS1, are also neutralizing antibodies, but their protective function in vivo is not well established.

The elimination of HBV-producing cells during acute hepatitis B is mediated mainly by a strong T-cell response to HBc/e and HBs proteins (Chisari and Ferrari 1995). Passive transfer of HBs-specific T-cell clones from immunized mice to HBs-transgenic mice induces acute hepatitis. The severity of the disease correlated directly with the dose of cytotoxic $CD8^+$ cells against HBs and the amount of HBs protein in the liver. It may result in fulminant hepatitis, as shown in animals with LHBs storage. The cytotoxic $CD8^+$ cells stimulate nonspecific inflammatory cells which form necroinflammatory foci, inducing necrosis of hepatocytes. However, these nonspecific cells are not necessary for HBV elimination (Sitia et al. 2002). The histological picture of this CD8+ cell-induced disease in transgenic mice is similar to that in acute or fulminant hepatitis B of humans. HBc/e- and HBs-specific $CD8^+$ cells have been isolated readily from the blood of patients with acute hepatitis B. In contrast, chronic hepatitis B patients have only low numbers of circulating or intrahepatic $CD8^+$ cells specific for HBs or HBc. This low number may explain the persistence of HBV and the continuous inflammation at moderate levels. Control of HBV replication in liver cells does not necessarily require the elimination of all HBV-expressing cells. Activated $CD8^+$ cells secrete interferon-γ and interleukin-2 which induce secretion of TNF-α. TNF-α reduces the stability of HBV mRNAs (Heise et al. 2001) and suppresses expression of HBV or its proteins, or both, without necessarily inducing cytolysis or apoptosis. In experimentally infected chimpanzees, $CD8^+$ cells induce the elimination of cccDNA even before cytotoxicity occurs, but final elimination of cccDNA is associated with hepatic turnover (Wieland et al. 2004a). A complete elimination of all cccDNA-containing cells is, however, not always achieved. $CD4^+$ lymphocytes against HBc/e are numerous in most patients with acute resolving hepatitis, but few are found during chronic hepatitis B. $CD4^+$ cells against HBs proteins are less abundant than those against HBc/e.

The mechanisms of how HBV escapes the immune system are not understood. Even in those individuals who resolve acute hepatitis B, HBV is able to replicate for weeks or even months without inducing an immune response. Evaluation of the gene expression pattern in acutely HBV-infected chimpanzees characterized HBV as a 'stealth virus' not visible to the innate immune system and with little influence on the host cells (Wieland et al. 2004b). Only after a period of several weeks of logarithmic expansion of HBV was the immune system activated, most likely via $CD4^+$ cells and interferon-γ. It is not known which factors finally activate the immune system after a long period of inertia.

HBV persistence seems to be caused by immune evasion and nonpathogenicity of the wild-type virus. It has been suggested that intrauterine transfer of maternal HBV antigens, particularly HBeAg, generates immune

tolerance to HBV in the newborn, but most newborns of HBeAg-positive mothers respond very well to HBsAg vaccine. HBeAg in the serum may be involved in the suppression of a vigorous cytotoxic immune response against HBc protein, because newborns (both human and woodchuck) develop persistent infection only if the infecting HBV-positive serum is also HBeAg positive. However, HBeAg in cells also becomes a target for the inflammatory immune response (Milich and Liang 2003). Other potential factors supporting immune evasion are the high excess of secreted HBs proteins and the high resistance of SHBs protein to proteolytic degradation, which may impair antigen presentation.

Diagnosis

A survey of the significance of HBV parameters for different diagnostic problems is given in Table 1.

ACUTE HEPATITIS

According to the description in 'Acute hepatitis B', virtually all cases of acute hepatitis B show at the onset of symptoms easily detectable levels of anti-HBc. Thus, a test for anti-HBc clarifies whether a patient with hepatitis is infected with HBV. Lack of anti-HBc excludes HBV as the causative agent of a present acute hepatitis or of a subclinical transaminase rise (Table 1).

If anti-HBc is positive, a test for HBsAg and IgM anti-HBc should follow (Table 1). A negative or low IgM anti-HBc (<600 PEI units) excludes HBV as the cause of a clinically apparent acute hepatitis, but points to previous HBV infection, which may have resolved or be persistent. (Persistent infection is recognized by a positive HBsAg test, immunity by a positive anti-HBs.) During early or very late phases of the acute disease, IgM anti-HBc may have concentrations lower than 600 PEI units/ml. In mild HBV infections, IgM anti-HBc may also be lower (Gerlich et al. 1986).

HBsAg is at least positive in most hepatitis B-infected individuals transiently. It may be eliminated very rapidly in fulminant hepatitis with extremely strong immune elimination or in very mild infections with very low viral expression. Alternatively, HBsAg may be present, but undetectable, by the usual immune assays because of mutations in the major epitopes. Such cases can be diagnosed by the quantitative assay of IgM anti-HBc and by the test for HBV DNA using PCR. HBV infection of a patient may suggest, in addition, a risk of HDV, HCV, and HIV infection, and appropriate tests for these infections may be advisable.

Table 1 *Serological parameters of HBV infection*

Serological parameters	
Ongoing or previous infection (prevalence)	Anti-HBc
Ongoing infection	HBsAg
Time of infection	IgM anti-HBc (quantitative)
Infectivity	HBV DNA (quantitative)
Natural immunity	Anti-HBc and anti-HBs
Immunity after vaccination	Anti-HBs (quantitative)

The patient's disease status should be followed by determination of the ALT level until it is close to normal. An early favorable prognosis is possible if the HBeAg patient is negative already in the first serum sample, or HBV DNA negative in hybridization assays. These two parameters become negative in resolving cases within 4–6 weeks. Thus, a second assay of HBeAg or HBV DNA after 6 weeks, when they were initially positive, is advisable. Alternatively, the HBsAg quantity may be measured accurately by electroimmunodiffusion or a quantitative EIA. In resolving cases, HBsAg drops within 6 weeks by more than 60 percent. A definitive resolution may be assumed if HBsAg is negative and anti-HBs is positive but this may take up to one year. However, even in this condition, low-level viremia and intrahepatic persistence of HBV DNA has to be assumed in a major proportion of the convalescents.

CHRONIC HEPATITIS

Patients with symptoms of chronic hepatitis need a thorough examination of the liver, including liver function tests and, if possible, liver biopsy. The viral activity is not clearly correlated to disease activity or to the histological appearance of the liver, but HBsAg is virtually always detectable. In early phases of chronic infection, HBeAg is often present and HBV DNA detectable even by the insensitive hybridization assays. Quantities of ALT, HBV DNA, HBeAg, and IgM anti-HBc are often unstable, fluctuate or are occasionally even negative. HBeAg is sometimes absent, but HBV DNA is usually detectable however PCR may be required as a more sensitive technique for detection. The diagnostical parameters associated with persistent HBV infection are shown in Table 2.

A typical feature of late phase chronic HBV infection is the selection of variants with deletions or mutations leading to an altered expression of HBV or to altered gene products. Most frequent are mutations (stop codons, promoter inactivation, frame shifts) that abolish expression of HBeAg. Deletions also occur quite often in the B-cell epitope regions of HBcAg and preS. Deletions within the *HBx* gene are common in patients with impaired immune reactions and are often the only mutation in these people, but they are rare in HCC patients (Blackberg and Kidd-Ljunggren 2003).

INAPPARENT PERSISTENT HBV INFECTION

Infrequently, HBV infection is not recognized subjectively. Thus, HBV carriers are often detected accidentally – during blood donation, because of an occupational risk, before operations or during search for a

Table 2 *Three states of HBsAg carriership – typical parameters*

Parameters	Immune tolerance	Chronic disease	'Healthy' HBsAg carrier
Serum			
HBsAg μg/ml[a]	30–150	<30	<30
HBeAg PEI[b] – U/ml	2 000–10 000	<2 000 or absent	Negative, anti HBe^+
HBV-g.e./ml[c]	10^9–10^{10}	10^6–10^9	$<10^6$
Anti-HBc	+ to ++	+++	+ to ++
IgM-antiHBc PEI units	<50	10–500	<10
ALT IU/ml 37°C	<120	>120, variable	<50
Liver			
$HBcAg^+$ cells	90–100%	Variable, low	Negative
$HBsAg^+$ cells	90–100%	Variable, low	Variable, frequent ground glass cells, $LHBs^+$
Dynamics	Stable	Unstable	Stable
Interferon response	Transient or none	Often sustained	No treatment required

One IU HBV DNA in the WHO standard corresponds to ca. 5 g.e./ml.
a) 1 μg HBsAg = 1 000 PEI units or 2 000 WHO units. So-called ng units are often inaccurate.
b) PEI: Paul Ehrlich Institute for Sera and Vaccines, Langen, Germany.
c) g.e.: genome equivalents.

possible source of HBV infection. The most universal test to detect an ongoing or previous HBV infection is anti-HBc. However, anti-HBc may remain negative for up to 6 months post-infection, whereas HBsAg is positive much earlier. In some chronic HBV carriers who were infected perinatally, anti-HBc may remain negative lifelong although HBV replication is very strong. Anti-HBc may also be absent in reactivated HBV infections due to immune suppression or in conditions where antibody synthesis is generally suppressed. The earliest marker of HBV infection is HBV DNA in the serum if detected by a highly sensitive nucleic acid amplification technique (NAT) such as PCR.

If a person is identified as being positive for HBsAg, he or she should be tested for anti-HBc. Negative anti-HBc suggests an early phase of infection with a possible acute hepatitis B still to come, or a severe state of immune deficiency which, however, usually would have been known for other reasons. Absent IgM anti-HBc in the presence of IgG anti-HBc suggests low disease activity and a stable carrier state (Table 2). HBsAg carriers stay, by definition, HBsAg positive for more than 6 months. Positive HBeAg suggests high viremia and high infectivity, but some HBeAg-positive carriers have only moderate viremia ($<10^8$ genomes/ml) and are less infectious. Absence of HBeAg suggests low viremia ($<10^6$ HBV particles /ml) but does not exclude higher levels. A quantitative HBV DNA assay is therefore recommended. Most HBeAg-positive carriers and some HBeAg-negative carriers reach very high viremia levels of 10^9–10^{10} genome-containing HBV particles.

Chronically infected individuals may be divided into three groups, as shown in Table 2. The immunotolerant type is highly infectious but oligosymptomatic. The HBsAg carrier is also healthy but noninfectious (except for blood donation). A status between these two extremes is usually associated with liver disease that, however, may be unrecognized. In this phase, disease activity is often fluctuating. Silent HBsAg carrierlike phases may change with flareups. Viremic HBV carriers often spontaneously reach the HBsAg carrier state after one or more episodes of recurrent liver disease. Unfortunately, this disease phase may be longlasting and result in liver cirrhosis or HCC. Thus, regular monitoring of ALT levels in persistently HBsAg-positive people is advisable until it is known whether a stable state is present. HBeAg and HBV DNA should also be regularly checked if positive.

NATURAL IMMUNITY

People who have been at risk for HBV may have acquired immunity by silent HBV infection. In order to detect natural immunity the person's blood should be tested for anti-HBc, and then, if positive, for anti-HBs. Naturally immune people should be anti-HBc and anti-HBs positive (see Table 1). It has, however, been noted that anti-HBs may occasionally disappear several years after an acute hepatitis B infection. Isolated anti-HBs without previous vaccination may be nonspecific and nonprotective, but asymptomatic transient HBV infection may also lead to low levels of anti-HBs without anti-HBc. Isolated anti-HBc may have different meanings:

- The person may be in the phase after disappearance of HBsAg but before the appearance of anti-HBs (so-called window period). In this case the titer should be high.
- It may be present long after previous HBV infection in which anti-HBs has already disappeared. In this case, the titer should be low.
- It may be a response to an HBV infection, in which HBsAg is mutated, bound in immune complexes or below the detection limit. Anti-HBe may be present.

- The anti-HBc test may react nonspecifically. Usually, the signal strength is low.

If distinction between the four situations is required, an HBV DNA assay by NAT may be helpful. If it is necessary to decide whether a person is immune, vaccination against HBV with one dose of vaccine is recommended and a quantitative test for anti-HBs should be done 1–2 weeks later. Situation (2) will lead to a rapid anamnestic response with a reappearance of anti-HBs at high titers. If this does not occur, vaccination should be continued by the regular schedule with two or three further doses. In situation (3), no anti-HBs may develop. For the discussion of vaccine-induced immunity see 'Active immunization'.

DETECTION OF HBV IN TISSUE SPECIMENS

Because of its ability to generate high viremia and antigenemia, HBV infection is usually easy to diagnose with the aid of serum samples. Liver biopsies are necessary to examine the degree of inflammation, necrosis, and fibrosis, and repeated biopsies give information on the progression of the disease. Because of the risk associated with biopsy, it should be done only if the clinical, biochemical, and virological data suggest severe, progressive disease. Once the biopsy is available, it may be stained for HBsAg, preS1 antigen, HBcAg, or HBV DNA. In HBsAg-positive patients, usually no more information can be derived from these stainings than from a complete quantitative HBV serology. The situation is different in HBsAg-negative patients with liver cirrhosis or HCC. Here, a liver biopsy or tumor tissue may allow detection of extracted HBV DNA by NAT and, if highly positive, by Southern blot. Distinct bands in a Southern blot prove mono- or oligoclonal outgrowth of hepatocytes with integrated HBV DNA and, thus, HBV-associated liver disease.

DIAGNOSTIC TESTS FOR HBV

HBsAg

HBsAg is detected qualitatively by solid-phase immune assays with unlabeled anti-HBs as the carrier and labeled anti-HBs for detection in the liquid phase. Originally, radioactive iodine 125 was used as label (radioimmune assay) but today enzymes (EIA) are preferred which generate a colored or fluorescent product. Chemoluminescence labeling is also used. The specificity and sensitivity is mostly determined by the quality of the anti-HBs reagent. Most commercially available assays use either as the solid phase or as label or for both just one monoclonal antibody against SHBs. While this is sufficient for most genotypes of wild-type HBV, mutants of the *SHBs* gene may escape detection in reactivated hepatitis B (Westhoff et al. 2003), in blood donors (Jongerius et al. 1998) or in the general population (Carman et al. 1997). Use of affinity-purified polyvalent antibodies or several non-overlapping monoclonal antibodies, possibly also against preS epitopes, help to overcome this problem. Analytical sensitivity is also very important. An international standard is available for its validation which contains 33 IU/ml heat-inactivated SHBsAg of subtype adw2 and genotype A2. The detection limit should be below 0.1 IU/ml, but not all tests are so sensitive. Nonspecific reactions are usually rare (<0.1 percent) and may be verified by inhibition tests using polyvalent anti-HBs. Quantification may be achieved by using reference samples and appropriate dilutions of the sample which give a result in the proportionality range (<100 PEI units/ml) of the EIA. Results may be expressed either in PEI or in International Units /ml. One IU is 0.55 PEI-Unit. One PEI-Unit corresponds to 1 ng of fully native HBsAg. Unfortunately, many test kit producers use 'ng' units, which are calibrated with partially denatured HBsAg. Furthermore, test kits may react differently with various subtypes of HBsAg or with variants of HBsAg. A useful technique for quantifying HBsAg in the range of 1 000–100 000 PEI-units/ml is Laurell electrophoresis (Glebe and Gerlich 2004). This is the relevant range for quantitative assessment of acute and chronic hepatitis B.

Anti-HBc

Anti-HBc is usually determined by inhibition EIAs with recombinant HBcAg (expressed e.g. in *E. coli*) at the solid phase and labeled anti-HBc, the binding of which is inhibited by the anti-HBc within the samples. Sandwich assays with HBcAg at the solid phase and labeled HBcAg or labeled anti-IgG are also available. There are nonspecific inhibitors of HBcAg in human sera; nonspecific reactions may therefore be as frequent as 1 percent in the normal population. Repetition of the test using a test kit from a second producer usually yields a positive result if the reaction is specific, but a false-negative result in this second assay cannot be excluded. Divergent anti-HBc results in different tests are particularly frequent in individuals with a high risk of HBV exposure. Western blotting is not helpful and quantification of anti-HBc is not usually done. Chronic HBV carriers and acute hepatitis B patients may have end point titers up to 1:1 000 000 whereas immune people usually have titers below 1:1 000.

IgM anti-HBc

IgM anti-HBc is usually detected by the anti-μ capture EIA. This type of EIA binds via anti-μ antibodies IgM from the sample to the solid phase; thereafter labeled HBcAg is added. If the sample contains IgM anti-HBc, labeled HBcAg will be bound. Quantification is highly advisable. Results may be expressed in PEI units/ml or as an EIA index. Some commercial test kits employ very high cut-off values to give a positive result only with sera

from acute hepatitis B, but all test kits react significantly stronger with sera from patients with active chronic hepatitis than the negative control giving values up to 600 PEI units/ml. Testing for IgM anti-HBc is advisable only if the screening for total anti-HBc was positive.

Anti-HBs

Anti-HBs is usually detected by a sandwich EIA with HBsAg at the solid phase and labeled HBsAg in the liquid phase. Some producers use natural purified HBsAg from HBV carrier plasma; others use recombinant HBsAg particles from yeast or from mammalian cells devoid of preS antigen. Thus, quantitative results obtained for one serum using various test kits may differ significantly. The detection limit is usually between 1 and 10 mIU/ml and saturation of the assay is reached at 100–1 000 mIU/ml. Sera may contain more than 100 000 mIU/ml after vaccination with HBsAg. Nonspecific results are possible, but they are rare. Coincident positivity of HBsAg and anti-HBs may occur in patients with chronic hepatitis B. The anti-HBs in these patients usually reacts with HBsAg subtypes not present in the HBV carrier. Normally, anti-HBs should be assayed only in anti-HBc-positive people or after vaccination.

HBeAg

HBeAg should be determined only in people with HBsAg. It is tested by EIA and may be quantified using PEI units. Detection limits of commercial assays are between 1 and 10 PEI units/ml and high viremia is usually associated with high HBeAg titers (>2 000 PEI units/ml). Anti-HBe is a less important marker of HBV infection. However, monitoring of chronic hepatitis B patients for seroconversion (Figure 8c) from HBeAg to anti-HBe is clinically relevant. Furthermore, assay of anti-HBe may help to interpret the significance of anti-HBc without HBsAg or anti-HBs.

HBV DNA

HBV DNA has become very important in the diagnosis of HBV infections and may be helpful for answering many questions:

- HBsAg positive anti-HBc negative samples need confirmation of specificity. A highly sensitive HBV DNA assay, preferable NAT, will react positively in almost all specifically positive samples.
- In anti-HBc positive but HBsAg and anti-HBs negative samples, HBV DNA can help to identify low level HBV carriers, or carriers with a mutated HBsAg which escaped detection.
- In very early phases of infection, inapparent transient or occult persistent HBV infection, HBV DNA may be the only detectable viral marker. Persistent low level HBV infections are particularly frequent in HCV, HBV-coinfected persons.

For these applications an assay of the highest possible sensitivity is required. With todays techniques, usually few or even one DNA molecule in the tested sample volume are detectable. Thus, the sample volume is often the limiting factor. Unfortunately, some inapparent HBV infections show extremely low levels of viremia around 1 genome/ml (Kuhns et al. 2004). Nevertheless, HBV DNA assays should be employed whenever a serological pattern is inconsistent or of uncertain specificity. In-house NAT assays (if adequately validated) may be superior to commercially available assays because several target regions may be used. This can prevent false-negative results due to mutations in the primer or probe target regions.

In the 1990s, NAT was plagued by false-positive results due to cross contaminations between the amplificate and samples. The use of closed systems and/or degradation or amplificates has helped to avoid this problem during handling and extraction. However, cross contamination between samples is still a problem because sera may contain up to 10^{11} genome equivalents/ml. False-negative results due to polymerase inhibitors, false reagents, or wrong performance should be excluded by inclusion of an internal control or by spiking of a suspected false-negative sample with a known positive sample. False-negative results due to mutations in the target sequence of probes and primers remain a problem of unknown magnitude, but the existence of HBsAg true-positive, HBV DNA-negative samples give a hint to its existence. For diagnostic purposes only, NAT systems with detection of the amplificate by labeled probes should be used, because they are less prone to contamination by the amplificate. Techniques with in situ generation and measurement of the signal after each amplification step (real-time PCR) are preferable. The great advantage of these systems, besides their high contamination resistance and analytical sensitivity, is the ability to quantitate the complete range of concentrations from <100 to $>10^{10}$ g.e./ml. The TaqMan system uses a fluorescent probe, which is degraded when being annealed to the amplificate and is thereby released from a fluorescence quencher. The fluorescence resonance energy transfer (FRET) technique uses two probes with two different fluorescence dyes. FRET can only occur and generate the signal when the probes bind next to each other to the amplificate. Due to the existence of a World Health Organization (WHO) approved International standard, both assays can be standardized. The WHO has preferred not to define the HBV DNA in g.e./ml or 'copies/ml' but in International Units, because different results were obtained with different methods of detection (Saldanha et al. 2001). These differences are most likely caused by variable losses during extraction, because the HBV

DNA carries the covalently bound HBV polymerase with it. This protein alters the extraction properties of the DNA, and it is the reason why for the standardization of HBV DNA assays HB-virion-derived DNA and not cloned DNA should be used. With adequate extraction and calibration one IU corresponds to ca. 5 g.e./ml of HBV genotype A2 (Heermann et al. 1999). One picogram HBV DNA is 280 000 g.e./ml.

Real-time PCR is superior to competitive PCR because it is more sensitive and does not reach an upper limit of quantification. Hybridization assays have been used for a long time to quantitate HBV DNA, but they are too insensitive (detection limit $>10^6$ g.e./ml) for therapy monitoring or assessment of infectivity. These are the main applications of HBV DNA quantitation.

HBV DNA genotyping or resistance testing can be done via hybridization of a NAT product with commercially available sets of probes, but sequencing of a NAT product covering the SHBs/RT region is a cost-effective and more informative in-house alternative.

Epidemiology

PREVALENCE

It is estimated that 50 percent of the world's population have had contact with HBV and about 10 percent of these (ca. 350 million) have developed persistent infection. HBV carriers have a high risk of developing liver cirrhosis and a 20 to 100-fold greater risk of developing HCC. Mortality from acute hepatitis B is low (<1 percent), but much higher from chronic hepatitis B. It is estimated that 1 million people per year die from the sequelae of chronic hepatitis B. Prevalence of anti-HBc, the best marker for current or previous infection, is highly variable in different geographic regions, ethnic or behavioral groups. Areas of low prevalence of anti-HBc positivity (<5 percent) are Northern Europe, North America, and parts of South America; areas with a particularly high prevalence (>50 percent) are sub-Saharan Africa, most parts of Asia and Oceania except India, where more than 10 percent of the adult population are HBsAg carriers. In Australia, New Zealand, and Alaska the native population is much more affected than the Caucasian immigrants. There are no hints that certain races are more susceptible to persistent infection. There may also be very large differences within a country; for example, southern Italy has a much higher prevalence than most northern areas. Lower socioeconomic status is often (but not always) connected with higher prevalence. In areas of low or moderate endemicity, prevalence increases gradually with age, being very low during childhood. In areas of high prevalence most individuals have acquired the infection before school age.

The prevalence pattern is changing due to lower incidence in young people. Vaccination has an efficacy of ca. 90 percent if performed optimally. Indeed in highly endemic countries like Taiwan or Oceania, which have implemented general vaccination in childhood, the prevalence of HBsAg has decreased by a factor of five from 14–20 percent to 3–5 percent (Lin et al. 2003; Wilson et al. 2000).

INCIDENCE

In many countries, hepatitis B is a notifiable disease and, thus, numbers on the annual incidence are available. In Germany, incidence of acute hepatitis B has continuously decreased from a high level in the seventies (>10 per 100 000 person years) to 1.7 per 100 000 person years, in 2002. Similar decreases are observed in other developed countries. Thus, acute hepatitis A and C are now more frequent than hepatitis B. In Germany, the peak incidence is found in males aged between 20–29 years pointing to i.v. drug use and male homosexuality as major risk factors. It is almost certain that acute hepatitis is under-reported. The true number of cases is probably two or three times higher. The true number of new HBV infections is even more difficult to determine, because most infections are subclinical, even those that finally lead to chronic hepatitis. On the basis of 6 percent prevalence of anti-HBc in the general population of Germany, even the general population must have had an incidence of 100–500 inapparent HBV infections per 100 000 person years before the 1980s. However, today's incidence is probably much lower.

MODES OF TRANSMISSION

Because of the extremely high infectivity titer of $>10^8$/ml serum, even the slightest trace of serum is dangerous, but other bodily secretions such as saliva, ejaculate, vaginal secretions or menstrual blood may also cause transmission, and titers of 10^6/ml may be present in these materials. HBsAg and HBV DNA have been detected in urine (Knutsson and Kidd-Ljunggren 2000), but there is no epidemiological evidence that the infection is transmitted by exposure to urine. Transmission is most efficient by intravenous injection, 100 times less efficient by the intramuscular or percutaneous route and least efficient by mucosal contact. Intact skin is impermeable to HBV but open wounds allow efficient entrance. Assuming an infectivity titer of 10^8/ml serum in an unrecognized HBV carrier, 1 nl of this serum (an invisible amount) would still contain 100 infectious doses, which would almost certainly cause infection if administered by a contaminated syringe or other medical device. Even dilution of this serum by $1{:}10^5$ (e.g. a blood spill of 0.1 ml has been washed away with 10 liters of water) would leave 1 ml of this liquid still infectious when administered percutaneously. Swallowing highly infectious blood or serum, blood splashes into the eyes, and contact with open wounds almost invariably lead to infection of susceptible individuals. Heterosexual contacts are a less effective but still relatively efficient

way of transmission. Transmission has been very frequent between male homosexuals, depending on their sexual practices. The danger of transmission by intense kissing is unknown whereas bites transmit very efficiently. Experience suggests that the rate of transmission from HBV carriers to contacts is proportional to the genome titer, frequency and intensity of exposure. Genome titers $<10^6$/ml have been empirically found not to pose a risk to even the closest contact person unless contaminated syringes or similar devices are involved. HBsAg-positive mothers with a negative HBV DNA hybridization test ($<10^6$/ml) do not usually transmit HBV to their newborn babies (Ip et al. 1989). Thus, sources of infection are almost invariably (except for blood products) highly viremic HBV carriers. Even with very high titers, transmission to household contacts usually requires time and special situations; normal school or professional contact is not a risk. Transmissions occur more easily in nursery school or in injury-prone sports or jobs; for example, a young HBV carrier infected his co-pathfinders by hiking along the same thorny trail, and an HBV-positive butcher infected all his colleagues within a short time because of the many skin injuries they received during the course of their work. Nosocomial infections have been frequent in previous decades but today they occur only in rare cases of accidents or failures to follow hygiene. Transmission of HBV from patients to medical staff is a well-known risk, but the reverse is also well documented (Gunson et al. 2003). In recreational activities, devices for ear or nose piercing or tattooing have been sources of transmission, but the most significant are shared syringe and needles for intravenous drug use.

Control

Control of HBV includes counseling of HBV carriers, general hygiene, disinfection, blood screening, active and passive immunization, and treatment of persistent infection.

COUNSELING OF HBV CARRIERS

As pointed out under Modes of transmission above, even the slightest trace of highly infectious blood on a sharp item will transmit HBV if it penetrates the skin of a susceptible person. The job and the life style of a highly viremic HBV carrier must be analyzed as to whether their blood might contaminate sharp items that may percutaneously infect other people. The carrier should be informed that simple cleaning by wiping the blood away or washing with water will be insufficient to remove infectivity reliably. All highly viremic HBV carriers should be instructed to avoid injuries, to keep lost blood under control, and to disinfect items that are contaminated by their blood if they cannot be cleaned by regular washing.

Surgeons who have their hands and sharp instruments within the body of their patients cannot completely avoid small, often unnoticed, injuries. Thus, highly viremic carriers (HBeAg positive or HBV DNA hybridization positive ($>10^5$/ml)) should not operate or work as dentists. HBV transmission by HBeAg-negative and low-viremic ($<10^5$/ml) carriers has not yet been reported, except for one case (Corden et al. 2003). Operations performed by them should be safe if combined with wearing two pairs of gloves and an injury-avoiding technique. European recommendations added to this limit a safety margin and suggest that HBV carriers should have <1 000 g.e./ml when performing exposure prone procedures (Gunson et al. 2003). The main point is that all medical staff should be vaccinated and be tested at least once for HBsAg and for anti-HBs. No restrictions are necessary for noninvasive medical procedures.

Household contacts, close friends, and sexual partners should be vaccinated or (if appropriate) tested for anti-HBc and HBsAg, or both. Physicians should be informed before they do invasive procedures with the HBV carrier. To take appropriate precautions, it would be desirable that sexual partners and close social contacts should know that a person is a carrier of HBV; they may, however, fail to understand the nature of the risk and discriminate against the carrier. The HBeAg and HBV DNA status should be checked at least twice a year. The usefulness of antiviral therapy should be considered carefully, because an unsuccessful treatment might cause more harm than good.

HYGIENE

Human blood is almost ubiquitous in medical practice and laboratories, and HBV infections were once very frequent in the medical profession. Protection against the contamination with blood is now observed more strictly since the occurrence of HIV; this includes wearing gloves and facemasks and using safer equipment (for example, for blood taking) that causes fewer injuries.

Patients are now better protected by the use of disposable items, in particular syringes and needles, and by a generally higher awareness of hygiene. Breaking of hygiene rules during blood taking or injections still causes small outbreaks of HBV infection even in developed countries, and originates usually from an unrecognized highly viremic carrier. Nondisposable devices that cannot be autoclaved (e.g. endoscopes) can still pose a problem, if the available disinfection devices are not adequately used.

In dialysis centers, HBV carriers must have their own room with their own dialysis machine. Furthermore, dialysis patients should be vaccinated or – if nonresponders – monitored for HBsAg. Outbreaks of HBV have been observed in pediatric oncology wards despite good hygiene. Here patient-to-patient contact is

common but HBV infections usually go unnoticed if the patients are not screened for HBsAg. Transmission from an operated highly viremic carrier to the next operated patient occurs occasionally even in these days and points to lack of hygiene. However, general screening of all patients, or at least of all those who undergo surgery, is considered to be cost-inefficient.

Isolation of patients with acute or chronic HBV infection is not essential if they are instructed as described above, behave responsibly, and do not lose blood in an uncontrolled fashion. However, HBV-carrying children should be supervised carefully.

DISINFECTION

Modern disinfectants should be validated with test organisms. In the European Community, a standard test is required in which a working dilution of a disinfectant must reduce the infectivity titer of poliovirus by a factor of $>10^4$ within 1 hour at room temperature. Because there is no feasible infectivity test for HBV, no disinfectant is formally validated for HBV even though it is one of the most important disinfection problems in medicine. The use of duck HBV as a model that can be titrated has been generally accepted, but its structural proteins (which are the targets) and its mode of transmission (almost exclusively vertical) are very different. A better model would be woodchuck HBV but the test system is not readily available. Inactivation of HBsAg or of the viral endogenous DNA polymerase is an inadequate substitute because their connection with infectivity is not established and a reduction by a factor of $>10^4$ has not been measured. The morphological alteration and disintegration test (MADT) using purified HBV particles and electron microscopy has been of some value. Disinfectants that inactivate simian virus 40 would also be expected to inactivate HBV, whereas adenovirus may be a more sensitive virus than HBV. Recently, agents that target viral DNA can be validated by PCR. Furthermore, neutralizing epitopes may be used as targets (Jursch et al. 2002) and finally cell cultures for infectivity testing of HBV have become available (Gripon et al. 2002; Gerlich et al. 2004).

SAFETY OF BLOOD PRODUCTS AND ORGAN DONATIONS

All blood or plasma donations must be tested as a minimum requirement by licensed highly sensitive tests for HBsAg; HBsAg-positive blood or plasma must not be used. However, in developing countries with very high HBV prevalence and very limited financial resources, HBsAg testing is often not done or is suboptimal. It has been estimated that in a highly endemic country like Ghana, the risk of children <10 years for acquiring HBV is 1:11 for unscreened and 1:360 for HBsAg screened blood (Allain et al. 2003). In Germany, the residual risk of HBV in a blood donation despite HBsAg screening is 1:100 000 to 1:500 000. While this risk is very low it is still 10 times higher than for HCV or HIV transmission. A similar situation exists in most developed countries. Additional measures like screening for HBV DNA and/or anti-HBc to reduce the small residual risk of HBV transmission are in most countries optional.

Donor blood may contain HBV despite HBsAg screening, because of:

- a mix-up in the blood bank
- a very early phase of HBV infection
- a late phase of HBV infection
- an escape mutant or a variant of HBV or
- immune complexing of HBsAg.

In cases (2) to (5) above, NAT may detect HBV DNA; in cases (3) to (5), anti-HBc should be also positive. Screening of every blood donation for HBV DNA by NAT has been introduced in sample pools by blood donation services of the German Red Cross (Roth et al. 2002) and in Japan (Minegishi et al. 2003). Screening for anti-HBc by EIA in low prevalence areas has also prevented a few cases of HBV infection in the recipients, but it caused a large loss of donors. In high prevalence areas, exclusion of anti-HBc positive persons is not feasible because most donors would be positive. To avoid loss of donors, anti-HBc-positive donors should be tested for anti-HBs and only anti-HBs-negative donors excluded. Anti-HBs-positive blood is thought to transfer protective antibody. HBV DNA-positive, HBsAg-negative blood with anti-HBs did not transmit HBV (Prince et al. 2001). Besides screening for HBV DNA, vaccination of non-yet immune donors against HBV would prevent transmission of fresh HBV-infections to recipients.

Anti-HBs in large plasma pools cannot exclude infectious HBV. In 1994, a clotting factor IX preparation transmitted HBV to at least 30 people, although the donors were screened for HBsAg the pool was anti-HBs positive and the transmitted HBV was not an escape mutant. The pool was, however, HBV DNA positive when tested by a highly sensitive NAT (detection limit 10 genomes/ml) and the product was suboptimally inactivated.

Since 1995, virtually all plasma protein products have been required to be treated with one or more validated virus inactivation steps, i.e. by heat at 60–70°C for 10–30 hours in the presence of protective factors. Experience suggests that low contamination by HBV ($<10^4$ infectious doses/ml) can be completely inactivated by such a heat treatment. High contamination can be prevented by donor screening for HBsAg and NAT testing of plasma pools. Treatment of plasma with solvent/detergent is probably effective against HBV as against other enveloped viruses. Recently, methods for chemical inactivation of cellular blood products have been developed and validated with DHBV infectivity and HBV DNA reaction in PCR (Aytay et al. 2004). HBV transmission by blood or blood products should be

extremely rare exceptions today. However, if a new HBV infection occurs in a recipient, the producer of the product and the responsible public health institution should be notified and the source of infection identified.

For screening of organ and tissue donors, principally the same rules should be followed as for blood donors. However, livers from anti-HBc-positive donors will often reactivate HBV in the recipient and should be used only for HBV positive recipients or under protection with an antiviral substance and/or passive immunization.

Active immunization

All aspects of active and passive immunization have been described in detail by Ellis (1993). The first attempt to vaccinate against HBV used boiled HBsAg-containing serum as inactivated vaccine. More convincing success was obtained with HBsAg 20-nm particles purified from the plasma of HBV carriers. Residual infectivity of HBV and of other viruses potentially present in plasma was minimized by various inactivation procedures. The most frequently applied plasma-derived vaccine was treated with pepsin at pH 2, urea, and formalin. Other products were inactivated with higher doses of formalin or heating to 100°C. Plasma-derived HBsAg has been used in millions of recipients without any known transmission of viruses. Meanwhile, most countries use 'recombinant' HBsAg that has been expressed in transformed yeast cells. HBsAg expressed in mammalian cells is also used as a vaccine, but is more expensive and not generally available. Today, vaccines typically contain 10–20 μg purified 20-nm HBsAg particles as a standard dose, adsorbed to aluminium hydroxide as an adjuvant, and an antibacterial substance; some products are treated with formalin. Usually, three doses, the second 1 month after the first and the third at 6 months, are recommended for adults. For infants of HBsAg-negative mothers, combination with other vaccinations is recommended. The exact observance of the intervals is not essential, but long intervals between the second and the third dose favor development of high titers of anti-HBs. When rapid protection is required, or after exposure, a shortened schedule of 0, 1, and 2 months and a fourth dose after 6 or 12 months should be used. For infants, smaller doses (2.5–10 μg) are advisable, and for immunodeficient recipients, larger doses of 40 μg HBsAg. Vaccines with stronger adjuvants or HBs-encoding DNA are in clinical evaluation.

Indications for vaccination

The WHO has recommended the vaccination of all children worldwide during the first year of life. In areas where perinatal transmission is frequent, vaccination should start as soon as possible after birth. The same is true for children whose mothers are known to be HBsAg positive or who have not been tested for HBsAg. Post-exposure vaccination with four doses of 10 μg recombinant yeast-derived HBsAg (at 0, 1, 2, and 12 months) was reported to protect 95 percent of the babies born to HBsAg- and HBeAg-positive mothers. Most other studies on the prevention of perinatal HBV infection combined HBsAg vaccine with passive immunization (see 'Passive vaccination', below), but it is not clear whether immunoglobulins against HBV (HBIG) really improve the protection rate if highly efficacious active vaccines are used (for review, see West 1993). Studies without HBIG in South Africa (Hino et al. 2001) and in Oceania (Basuni et al. 2004) revealed a relatively low rate of vaccine failures and no selection of escape mutants whereas after passive/active immunizations escape mutants emerged (Nainan et al. 2002). Vaccination failure occurs more often in mothers with very high viremia ($>10^8$ g.e./ml). Thus, reduction of viremia to lower levels has been attempted with lamivudine therapy before delivery (Van Zonneveld et al. 2003).

In 2004, the majority of countries are following the WHO programme, but many countries with high prevalence have financial limitations and are not able to take part. Some countries with low prevalence do not consider early childhood vaccination to be necessary. In 2002, Germany had a vaccination coverage of 80 percent in early childhood and 60 percent in adolescents.

Among adults, vaccination is strongly recommended for people at risk. Everyone who is or will be in professional contact with blood from other persons, whether directly or indirectly, should be offered free vaccination against HBV by their employer or teaching institution. This includes not only medical and paramedical professions, but also people in the armed forces, the police, staff and inmates of prisons or other closed institutions, and social workers. Patients who need continuous invasive or infusion therapy should be vaccinated, as should relatives and close contacts of HBV carriers. Intravenous drug users should be offered free vaccination and also free access to syringes and needles. Travel to high-prevalence countries does not require hepatitis B vaccination unless close contact or a long stay is expected. Male homosexuals have a high risk of HBV infection and heterosexual promiscuity poses a greater risk than nonpromiscuous heterosexual activity. For many of these risk groups the combined vaccine containing HAV and HBs antigens is advisable.

Side-effects

The vaccine causes no local side effects except moderate pain at the injection site. Generalized nonspecific side effects are rare or mild. Recipients should be questioned about hypersensitivity to yeast components, formalin or the antibacterial substance, but such reactions are very rare. Induction of neurological autoimmune disorder has been suspected in some cases of

being associated with hepatitis B vaccine, but several studies did not confirm this. It should be noted that HBsAg may be detected in the serum for a few days after vaccination, and thus for one week after vaccination no blood should be donated.

Pre- and post-testing

People to be vaccinated because of already existing high risk should be tested for anti-HBc before vaccination. Ethnic origin from a high prevalence area is also a risk. If anti-HBc is positive the serum sample should be tested further and the person vaccinated as described in 'Natural immunity,' above. If anti-HBc is negative, vaccination should be started following the schedule of the vaccine producers. If there is a high probability that HBV infection is already present, the person should be tested for HBsAg, even if negative for anti-HBc. An HBsAg-positive person should not be vaccinated, although accidental vaccination is not known to be harmful.

The immune response should be controlled by a quantitative anti-HBs test 4 weeks after the third dose in anyone who is at greater than average risk. Although T-cell responses and immunological memory may be more important than the anti-HBs itself, an easily detectable anti-HBs level is a marker of protection. The detection limit is 1–5 IU/l; 10 IU/l is considered to be a marker of protection. People with less or no anti-HBs should be revaccinated immediately with a further dose. Low responders with <100 IU/l should be revaccinated after one year, all others after 10 years if the special risk is still present, but the necessity of boosters is debated.

Children who have been vaccinated during the first year of life should be revaccinated at the onset of adolescence because then they are again at increased risk. Nonresponders after four doses may receive further doses if protection is urgently requested. Response rates are >90 percent in the healthy general population, but decrease strongly with age. Further risk factors for nonresponse are male gender, obesity, and smoking. Vaccines with preS antigen or other adjuvants may induce a better response but are not yet generally available at the time of writing.

Escape mutants

Post-exposure prophylaxis by active and passive vaccination is usually successful if given soon after injury (e.g. needle-sticks) with HBV-contaminated items. Active vaccination of newborns from HBeAg-positive mothers is also successful in up to 95 percent of cases. In ca. 5 percent of the children, HBsAg may appear in spite of vaccination or never be negative from the first day. Such infants may have already been infected in utero. In some children, HBsAg appears despite seemingly protective levels of anti-HBs. These children have escape variants, detectable by sequencing of the *SHBs* gene, with mutations in the major HBs epitopes between amino acids 120 and 150. Escape variants have been described with various genotypes except for genotype A. A frequent escape mutation is glycine 145 of the S protein to arginine (Nainan et al. 2002).

Although early experiments in chimpanzees suggested that immunization with one HBsAg subtype protected against all HBV genotypes, it should be noted that most recombinant vaccines have genotype A2 or HBsAg *adw2* respectively. It is possible that genotype-specific antibodies may contribute to protection.

Experiments in chimpanzees and in cell culture suggest that anti-preS1 and partially also anti-preS2 contribute to protection. Experiments with transgenic mice suggest that helper cell epitopes of the preS region may induce a better B-cell response against SHBs. The potentially better protection of humans by preS-containing vaccines has not yet been confirmed by field studies. However, induction of anti-HBs in many non-responders to the normal recombinant SHBs vaccine has been found with a vaccine containing preS1 and preS2 (Zuckerman et al. 2001). A similar vaccine induced a stronger and more rapid anti-HBs response than the conventional vaccine (Shapira et al. 2001).

PASSIVE VACCINATION

Normal immunoglobulin does not contain enough anti-HBs to be used for passive vaccination. A special immunoglobulin is produced from donors who are naturally immune and boosted with plasma-derived vaccine or from anti-HBc-negative donors who have particularly high titers after vaccination. 15 percent HBIG for intramuscular administration must contain 200 IU/ml; 5 percent HBIG for intravenous application must contain at least 50 IU/ml. Studies in the 1970s and 1980s showed that HBV infection was prevented in newborns from HBsAg- and HBeAg-positive mothers if HBIG was given within 24 h after birth. Active vaccination seems to be more effective and does not require complementation by passive immunization, but in many countries both active and passive vaccination are still required.

Passive immunization of HBeAg-positive liver transplant recipients usually fails unless combined with antiviral therapy, but in HBeAg-negative patients continuous levels of 100 IU/l of passively administered anti-HBs are able to prevent recurrence of liver disease. Passive immunization of the recipient prevents also viral reactivation in a transplanted anti-HBc positive donor liver (Roque-Afonso et al. 2002).

Passive immunization is recommended together with active immunization after proven accidental exposure of a nonimmune person. In these situations, the blood of the 'donor' should be rapidly tested for HBsAg and, if positive, the 'recipient' should be vaccinated passively

and actively, after a blood sample has been taken for anti-HBc and anti-HBs testing.

TREATMENT

The treatment of acute hepatitis B can be directed against symptoms, but this is usually not necessary. Antiviral therapy of fulminant cases can be attempted but usually comes too late. The aim of antiviral or immunotherapy is to reduce persistent viral replication.

Immune modulators

Interferon-α and interferon-β have been used for the treatment of chronic hepatitis B. Success of interferon therapy depends very much on the status of the patient:. Patients with the so-called immunotolerant state (see Table 2) cannot usually be cured and should be monitored until they reach the state of chronic active disease, when the success rate of interferon therapy is much higher. Patients with a high probability of sustained response have typically $<10^9$ genomes/ml, low or absent HBeAg (<2 000 PEI units), low HBsAg (<30 000 PEI units/ml) and high ALT levels (>120 IU/l) (Burczynska et al. 1994; Erhardt et al. 2000). The higher the initial ALT levels are, the higher is the response rate in terms of low replication and HBeAg-negativity (Lok et al. 1998). Treatment is undertaken with quite high doses (2.5–10 MU/m^2 three times a week) if the patient tolerates the severe side effects such as flulike symptoms, depression, hair loss, leukopenia, and thrombopenia. Autoimmune antibodies should be monitored. Usually the HBV DNA concentration will fall within few weeks to levels $<10^6$ genomes/ml. Some patients do not respond at all, some may redevelop high levels of viremia even during therapy and others may become positive again soon after withdrawal of interferon. Six months of therapy are considered the minimum in responders before withdrawal is attempted. A relapse is not rare and a second course of interferon may be tried. Interferon may be harmful to patients with cirrhosis and is therefore not recommended in the terminal phases of chronic liver disease. In spite of its many disadvantages and relatively low efficacy, interferon has the advantage that in many cases it is able to induce seroconversion from HBeAg to anti-HBe and sustained cessation of viral replication, which is correlated with a very good clinical prognosis (Niederau et al. 1996).

Conjugation of interferon with polyethylene glycol (PEG) improves its pharmacological properties and has been used for therapy of chronic hepatitis B with at least as good success as standard interferon (Janssen et al. 2005). In this recent study, it was noted that genotypes A (47 percent) and B (44 percent) respond almost twice as well as genotypes C (28 percent) and D (25 percent). Combination with lamivudine did not improve the long-term response.

Immune therapy

Vaccination of chronic HBV carriers with licensed SHBs vaccines together with the application of interferon (Heintges et al. 2001) or with a MHBs-containing vaccine without interferon (Pol et al. 2001) increased the rate of HBV DNA negativation above rates in untreated controls but the effect was barely significant. Vaccination of chronic HBV patients with a preS1-, preS2-, and S-containing vaccine induced a Th2 response against these antigens but could not reduce the viremia (Jung et al. 2002). Hepadnaviral DNA vaccines or conventional protein vaccines alone or together with antivirals have been partially successful in the woodchuck (Menne et al. 2002) or duck model (Le Guerhier 2003), which warrants further studies. The successful use of a DNA vaccine in healthy humans to induce a protective Th1 and anti-HBs response is a further stimulus to follow this line (Roy et al. 2000). The optimal adjuvant and combination with antivirals has still to be found.

Antiviral substances

In the 1980s and early 1990s, only highly toxic nucleoside analogues were available. As a side-effect of HIV therapy, several nucleosidic inhibitors of reverse transcriptase were found to be active against HBV as well. Oral Lamivudine (3TC), at 100 mg per day, has been found to be very effective in reducing the viremia by several logs and to improve the HBV-associated liver disease. However, this drug and the other inhibitors of reverse transcription cannot block expression of the viral genes including the pregenome RNA and they cannot eliminate the pre-existing cccDNA of HBV. Lamivudine is able to suppress the release of mature HBV particles and infection of new target cells. Lamivudine induces an enhanced T-cell response against HBV antigen but this stimulation is transient and normally not sufficient to overcome persistent productive infection (Boni et al. 2003). Long-term therapy is necessary and well tolerated due to low toxicity. After 1 year of treatment most patients experience a clear improvement of ALT levels but more than half of them experience a relapse within 12 months after therapy. High pre-treatment levels of HBV DNA were predictive for a relapse after stop of therapy (Huang et al. 2003). Loss of HBeAg is a favorable prognostic marker for a sustained response to lamivudine. In one study (Dienstag et al. 1999), 32 percent of the patients seroconverted after 1 year on lamivudine, but usually the seroconversion rate is lower than with interferon and only twice above the control group, for example, in a study (Jonas et al. 2002) in HBeAg positive children. If HBeAg seroconversion has been reached and HBV DNA was undetectable by PCR, the favorable result is maintained in the majority (64 percent) of patients (Dienstag et al. 2003). The relapse rate can be further reduced by continuation of lamivu-

dine therapy for 6 months (Ryu et al. 2003). Further extension was useful in one study (Chien et al. 2003) but not in another (Lee et al. 2003). One predictor of HBeAg seroconversion under therapy is a high pretreatment ALT level >5-fold the upper limit of normal (Perrillo et al. 2002), furthermore a rapid decrease of HBV DNA to levels $<10^4$/ml (Gauthier and Bourne 1999).

The limiting factor for a permanent therapy with lamivudine is the development of resistance. After 1 year of lamivudine, 23 percent of patients were resistant, after 5 years 65 percent. Resistance development was associated with hepatitis flares in most cases and in 5 percent with serious liver disease-related adverse events (Lok et al. 2003). Whether lamivudine should be stopped or continued once resistance has emerged is debated. The resistance occurs with mutations in the YMDD amino acid motif of the HBV reverse transcriptase domain of M204 to I or V or S (Stuyver et al. 2001). The mutant replicates less efficiently than the wild type, but compensatory additional mutations restore viability (Bock et al. 2002). The appearance of resistance severely impairs the long-term efficacy of lamivudine in cases of chronic hepatitis B, but this does not preclude its short-term usefulness. It is useful to suppress viremia in HBV patients before liver transplantation where reinfection is usually highly detrimental. However, lamivudine alone protects only a part of the patients and needs to be complemented at least for a short period of time with HBIG (Buti et al. 2003). Lamivudine is sufficient to suppress reactivation of HBV infection by an HBsAg-negative anti-HBc-positive donor liver or in anti-HBc-positive, HBsAg-negative recipients (Nery et al. 2003). Lamivudine also supports the establishment of adoptive immunity originating from an anti-HBs-positive liver donor in a HBV-positive recipient (Lo et al. 2003). Furthermore, it is useful to prevent reactivation of hepatitis in HBsAg-positive patients receiving various forms of immunosuppression or cancer therapy (Shibolet et al. 2002; Lau et al. 2003). In highly viremic pregnant women, lamivudine may suppress HBV to levels insufficient to infect the baby (Su et al. 2004). Lamivudine is also supportive in treatment of the HBV-induced immune complex disease polyarteritis nodosa (Guillevin et al. 2004).

If lamivudine resistance has developed, Adefovir – a nucleotide analogue and licensed drug – is in most cases effective (Perillo et al. 2004). Continuation of lamivudine together with Adefovir is not superior to Adefovir alone (Peters et al. 2004). Adefovir is active in HBeAg-positive (Marcellin et al. 2003) and HBeAg-negative chronic hepatitis B (Hadziyannis et al. 2003). The response rate is similar to that of lamivudine but resistance development is rare. Since Adefovir is nephrotoxic at higher doses than 10 mg/day, and expensive, therapy can start with lamivudine under close monitoring of viremia and continued with Adefovir if resistance arises. Before licensing of Adefovir, Tenofovir, another inhibitor of reverse transcription, licensed for HIV, has been found even more effective (Dore et al. 2004) and should be considered in certain cases where both lamivudine and Adefovir have failed. Entecavir is also efficient against lamivudine-resistant HBV but further resistance can develop (Tenney and Levine 2004). Combination therapy, which is extremely valuable in HIV, has not yet been established for HBV. Combinations of Adefovir and lamivudine are not useful (Peters et al. 2004) combinations of lamivudine (Cooksley 2004; Janssen et al. 2005) or Adefovir (Marcellin and Lau 2004) with interferon were not superior to the single drugs.

Experimental drugs

Immunotherapy with various approaches has not reached advanced clinical studies. Even less developed are the various gene therapy approaches (Farrell 2000) including the most recent discovery of small interfering RNAs, which induce degradation of their target RNAs (McCaffrey et al. 2003). Inhibitory peptides which block viral attachment may also have potential (Glebe et al. 2005). In spite of modern drug design, high-throughput screening may still discover new drugs like the heteroaryl-pyrimidines, which inhibit maturation of HBV cores (Deres et al. 2003).

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Hepatitis delta virus

JOHN M. TAYLOR

Properties of the virus	1	References	5
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In 1977, the first clue to the existence of this virus came from a study of patients chronically infected with hepatitis B virus (HBV). A novel antigen, termed the delta antigen, was found in the hepatocyte nuclei of patient biopsies (Rizzetto et al. 1977). An initial interpretation was that the delta antigen might be a new variant of HBV. However, a more surprising explanation was subsequently obtained. By 1980, it was shown that this antigen was part of an infectious agent that could be transmitted to chimpanzees (Rizzetto et al. 1980). It is sometimes called the hepatitis delta agent, but more frequently hepatitis delta virus (HDV) or hepatitis D virus (HDV). Strictly speaking it is a subviral agent that is dependent upon HBV to provide the envelope proteins needed for the assembly of new virions. On one hand, this dependence of HDV replication on HBV means that many aspects of HDV and HBV biology overlap. On the other hand, the RNA genome of HDV and its strategy of replication have proven to have features unlike those of the helper HBV or any other infectious agent of animals.

PROPERTIES OF THE VIRUS

Classification

Currently, HDV is classified as the sole member of the genus *Deltavirus*. As mentioned above, it is a **satellite** of HBV, since its genome shows no obvious sequence relationship to that of HBV. The RNA genome of HDV and its mechanism of replication are unique relative to RNA viruses of animals, but, as discussed in the section on Plant viroid analogy below, they show important similarities to certain infectious agents of plants.

Virion structure

In the serum of an HDV-infected individual, there are not only HDV particles but also the non-infectious, empty, surface antigen particles that are characteristic of HBV infection. The 42 nm diameter HBV may also be found, though surface antigen particles are always in at least 100-fold excess. The hepatitis B surface antigen particles, termed HBsAg, include both 22 nm diameter roughly spherical particles and elongated filaments. Several groups have tried to purify the HDV particles away from HBV and HBsAg particles. HDV was thus estimated to be around 38–43 nm in diameter (Bonino et al. 1984; He et al. 1989; Ryu et al. 1992). The envelope proteins of these particles include the same three found in HBV. Inside the HDV particle is the RNA genome and around 70 copies of the delta antigen (Ryu et al. 1993; Gudima et al. 2002). The size and shape of this HDV ribonucleoprotein structure may be more heterogeneous than initially claimed; it is certainly not like the more defined icosahedral core structures of HBV.

The single-stranded RNA genome of HDV is in several ways fundamentally different from other RNA viruses of animals (Lai 1995). At ca. 1 700 nt it is the smallest.

- The genome has a circular conformation whereas the other viral genomes are linear.
- The circle is able to fold on itself, with Watson and Crick base pairing of ca. 70 percent of the nucleotides, forming an unbranched rod-like structure.

- On the genome is a domain of about 85 nt, which acts as a self-cleaving ribozyme. When heated in vitro, in the presence of magnesium ions, this domain catalyzes a specific transesterification reaction. The reaction can also be reversed in vitro (Sharmeen et al. 1989), although there is evidence to suggest that in cultured cells, a host factor is needed to achieve ligation (Reid and Lazinski 2000). Recent studies have determined the structure of this ribozyme (Ferre-D'Amare et al. 1998) and the folding pathway followed to achieve this structure (Chadalavada et al. 2002).

Replication

As reviewed in Hepatitis B, HBV is a hepatotropic virus but we still do not know the receptor(s) by which it enters hepatocytes. We do know, however, that antibodies against epitopes within the pre-S1 and pre-S2 domains of the HBV envelope proteins neutralize the virus. The same is true for HDV (Sureau et al. 1992). The replication of HDV in an infected animal is limited to the liver (Netter et al. 1994).

Inside infected cells there are not only HDV genomes but also complementary copies, called antigenomes. These have the same four properties mentioned for the genomes in the section on Virion structure above. In addition, the antigenomic RNA encodes, down one side of the rod-like structure, the 195 aa delta antigen. However, the antigenomic RNA, like the genomic RNA, is not translated. Translation of the delta antigen is considered dependent upon a third HDV RNA. This RNA is 5′-capped, 3′-polyadenylated, and cytoplasmic. Its size and sequence include the appropriate side of the antigenome; it is thus considered the mRNA for the delta antigen (Hsieh et al. 1990; Gudima et al. 1999). There is disagreement as to whether or not the mRNA transcription precedes that of antigenomic RNA or whether the two are separate and concurrent (Modahl and Lai 1998; Macnaughton et al. 2002). It has been estimated that an infected liver cell contains 300 000, 50 000, and 600 copies of the genome, antigenome, and mRNA, respectively (Chen et al. 1986). More recent quantitative studies indicate that during replication there is typically around 200 copies of the delta protein per molecule of genomic RNA (Gudima et al. 2002).

It is still not clear which host polymerase(s) carry out the HDV RNA synthesis. There is currently agreement that the host RNA polymerase II produces the transcripts of genomic RNA and mRNA (Modahl et al. 2000; Moraleda and Taylor 2001; Macnaughton et al. 2002). However, it has also been claimed that a different polymerase is needed to produce transcripts of antigenomic RNA (Modahl et al. 2000; Macnaughton et al. 2002).

Initially it was believed that during replication the majority of both genomic and HDV genomic RNAs were restricted to the nucleus (Taylor et al. 1987). More recent studies indicate that a significant fraction of the genomic RNA might by cytoplasmic (Gudima et al. 2002; Macnaughton and Lai 2002). There is even evidence that ribonucleoprotein complexes of HDV RNA and delta protein might undergo nuclear shuttling between the nucleus and the cytoplasm (Tavanez et al. 2002). Such complexes might also involve host proteins such as the splicing factor SC35 (Bichko and Taylor 1996), nucleolar proteins B23 and nucleolin (Lee et al. 1998; Huang et al. 2001), and the protein known as delta interacting protein A (Brazas and Ganem 1997).

During genome replication an ever-increasing fraction of the antigenomes undergo one or more post-transcriptional RNA-editing events. One such event is specific and essential for the life cycle. The adenosine located in the middle of the amber termination codon for the 195 aa delta antigen is deaminated by a host enzyme (Polson et al. 1996). Apparently only a small element of the rod-like folding structure of the antigenomic RNA is needed to define the target for this editing (Reid and Lazinski 2000). Transcription after editing leads to molecules of mRNA with the amber termination codon replaced by one for tryptophan. This altered mRNA encodes a somewhat longer protein, the 214-aa delta antigen. Both the small and large forms of the delta antigen are essential for HDV replication.

The small form of the delta antigen is required for genome replication (Kuo et al. 1989). The large delta antigen, which appears later in infection, does not support genome replication and, instead, can be a dominant-negative inhibitor of such replication (Chao et al. 1990). A more important property is that the large delta antigen is essential for the assembly of HDV ribonucleoprotein particles into new virions containing envelope proteins of the helper HBV (Chang et al. 1991). The large form becomes farnesylated at a site 4 aa from the new C terminus (Otto and Casey 1996). This modification is essential for virion formation (Glenn 1995; Glenn et al. 1998), presumably because it facilitates interactions with membranes and/or HBV envelope proteins. A monophosphorylated form of the large delta antigen has been detected, but only inside infected cells and not in serum particles (Bichko et al. 1997). Phosphorylated forms of the small delta protein have also been detected and may be needed for the support of genome replication (Mu et al. 2001).

The two forms of the delta antigen thus have quite distinct biological functions. At the same time, since they share a common 195 aa, they have some common properties (Hwang et al. 1995). These include:

- a domain involved in protein–protein dimerization and multimerization
- a bipartite signal which acts as a signal for localization to the nucleus

- a bipartite signal for protein–RNA interactions; the delta antigens have some specificity for recognizing HDV rodlike RNAs relative to other RNAs.

Both proteins are highly basic.

Experimental systems

The HBV envelope proteins are essential for virion formation, but replication of the HDV genome can be achieved in cells independent of the helper virus. Genome replication can be studied in established cell lines and even with in vitro systems.

Other than man, the only hosts that are known to allow complete replication of HDV are the chimpanzee and the woodchuck. The woodchuck hepatitis B virus (WHV) is a near relative of HBV, and it was found that HDV could replicate in woodchucks with WHV replacing HBV as helper (Ponzetto et al. 1984). Other hepadnaviruses, like duck hepatitis B virus (DHBV), are not able to support HDV replication; this may reflect the minimal ability of HDV to replicate in avian cells (Chang et al. 2000; Liu et al. 2001).

The only cultured animal cells that can be infected with HDV are primary hepatocytes. This is considered to reflect the need for the same receptors as used by the helper HBV. Thus, HDV with an HBV coat will infect human or chimpanzee hepatocytes (Sureau et al. 1991), and HDV that has been passaged in woodchucks, will infect woodchuck hepatocytes (Taylor et al. 1987). It is possible to implant human hepatocytes within the mouse and use these for HDV infection (Ohashi et al. 2000). Alternatively, injection of HDV into the mouse tail vein or peritoneal cavity can lead to infection of some mouse hepatocytes (Netter et al. 1993).

If one obviates the need for a receptor interaction by using techniques that directly fuse viral and cell membranes, then HDV can be introduced into established cell lines that are not necessarily of liver origin (Bichko et al. 1994). Under such conditions, HDV genome replication will occur. This can also be achieved by transfection of cells with HDV RNA or cDNA constructs. For RNA transfections, it is necessary that the small form of the delta antigen either is already present in the transfected cells or enters with the RNA. One interpretation is that HDV RNA will only migrate to the nucleus as a ribonucleoprotein complex with the delta antigen. In agreement with this idea, the initial requirement for a source of delta antigen can be met by providing a mRNA for this protein (Modahl and Lai 1998). Transfection can also be used to initiate HDV genome replication in mouse skeletal muscle (Polo et al. 1995a) and in mouse liver (Chang et al. 2001). Finally, using mice, made transgenic for HDV cDNA sequences, HDV genome replication can be detected in multiple tissues (Polo et al. 1995b).

If transfected cultured cells are also expressing the envelope proteins of the helper virus, then HDV virus-like particles are released. These will be infectious if not only the small but also the large envelope proteins of HBV are present; the large envelope protein contains the pre-S1, domain that is essential for HBV receptor interactions (Sureau et al. 1992).

Plant viroid analogy

Even though HDV is in many respects unique among RNA viruses of animals, it does seem to have some relatives in the plant world. Viroids, of which more than 25 have been described, are subviral agents of plants. Like HDV, they have small single-stranded RNA genomes that are circular in conformation and able to replicate via redirection of the host RNA polymerase II. In some cases they are known to contain both self-cleavage domains and the ability to self-ligate. However, unlike HDV, they are much smaller (only 240–375 nt), encode no proteins, and are not dependent in any way on a helper virus (Taylor 1999). In addition to the viroids there are also plant agents sometimes referred to as virusoids, which share similarities to HDV. However, the virusoids, unlike the viroids and HDV, replicate via an RNA polymerase provided by a helper virus (Taylor 1999).

CLINICAL AND PATHOLOGICAL ASPECTS

Transmission

Like HBV, HDV is a blood-borne pathogen. Cycles of HDV replication are dependent upon the envelope proteins produced by HBV; nevertheless, the modes of successful HDV transmission can be somewhat different from those of HBV. (Expression of envelope proteins via integrated copies of HBV DNA, might be able to support HDV replication cycles.) HDV is transmitted via contaminated needles among drug abusers and via blood products and transfusions. Sexual transmission is rare and so is perinatal transmission.

In natural situations, cycles of HDV replication fall into two classes: superinfections, where the patient is already infected with HBV, or co-infections, where the patient receives both HDV and the helper HBV at the same time. Superinfections are more likely than co-infections to lead to fulminant hepatitis. It has been hypothesized that since HDV replication may be aided by a more aggressive HBV replication, it could be the response of the host to the HBV replication that is the principal cause of pathogenesis (Casey et al. 1996). Some HDV infections pass through the acute phase and survive as a chronic infection. Such chronicity is also dependent upon a chronic HBV infection; thus, it should not be a surprise that HDV superinfections of patients already chronically infected with HBV are more likely, after the acute phase, to proceed onto chronicity.

A third mechanism of HDV infection was considered to arise in some patients with terminal HDV-associated cirrhosis that are given a liver transplantation from a virus-free donor. The interpretation was that hepatocytes of the new liver can first become infected by HDV alone; these would be nonproductive or latent infections, in which genome replication occurs but no particles are produced. Later, superinfection of such hepatocytes with HBV could lead to rescue and spread of the HDV (Rizzetto and Ponzetto 1995). Analogous latent infections were considered to be produced in experimental animals (Netter et al. 1994). More recently, the concept of latent infections, especially in humans, has been called into question because of evidence that at no time was HBV actually totally absent (Smedile et al. 1998).

Detection and clinical manifestations

There are several serum-based, commercially available, assays for HDV infection. The most widely used test is for antibodies (IgM and/or IgG) directed against the delta antigen. Tests that are more expensive are available for detection in serum of the delta antigen or the genomic RNA. Hybridization assays typically need more than 105 viral genomes per sample. By the use of reverse transcriptase to produce cDNA copies and the subsequent amplification of this cDNA with the polymerase chain reaction, as little as 10 molecules of the genome can be detected; however, sequence heterogeneity between isolates can render difficult the choice of appropriate oligonucleotide primers (Niro et al. 1997).

At one time, liver biopsy, with direct detection of delta antigen by immunohistochemistry, was considered the gold standard for detection of HDV infections (Rizzetto and Ponzetto 1995). However, in chronic infections, the number of infected hepatocytes and the level of replication per infected hepatocyte can be too low for easy detection. Not surprisingly, with more sophisticated in situ hybridization procedures, the sensitivity of detection can be increased. The possibility of adverse consequences of liver biopsy recommends against the use of this approach for routine purposes.

HDV infections may not always have a pathogenic effect. For example, on one Greek island there was an endemic chronic infection with both HBV and HDV, and yet infected individuals had no detectable signs of liver damage (Hadziyannis et al. 1993). By contrast, there have been local epidemics of HDV in the Amazon Basin of South America associated with a high incidence of fulminant hepatitis and death. It is possible that in such individuals there is an excessive HBV replication with associated immune response and liver damage and that the HDV, which after all depends upon HBV, replicates more efficiently (Casey et al. 1996). Early studies ascribed for such patients specific histological lesions, such as cytoplasmic eosinophilia, and microvesicular steatosis without an inflammatory infiltrate. Later studies argue against any direct involvement of HDV markers in such lesions. The lesions may reflect derangement in the secretory pathway of certain hepatocytes (Rizzetto and Ponzetto 1995) or the consequences of exposure to interferon presumably induced in response to the viral infection (Shimizu and Purcell 1989).

As recently reviewed (Gerin et al. 2001), the incubation period prior to an acute HDV infection is about 3–7 weeks. This is followed by a preicteric phase of about 1 week during which there can be various nonspecific symptoms, such as fatigue, lethargy, anorexia, and nausea. During this time the levels of virus in serum rise to a peak, and then fall, usually within a period of a few days. Peak titers of 10^{10}–10^{12} HDV particles per ml can be detected in the serum. Such titers are much higher than for HBV, which usually do not exceed 10^{9}–10^{10} particles per ml, but are similar to the titer of HBsAg 22 nm particles. Also detected in serum is an increase in biochemical markers of liver damage, such as alanine and aspartate aminotransferases. The appearance of jaundice defines the next phase, the icteric phase. Fatigue and nausea may persist. Serum bilirubin levels may become abnormal. Although the markers of HDV replication are decreasing, this phase may be the most severe clinical stage. At this point, a fulminant hepatitis may occur, but this is rare, though about ten times more frequent than for hepatitis induced by HBV alone, or by other hepatitis viruses. For most coinfections, the patient recovers over a period of weeks to months, and HDV infection is cleared. By contrast, with superinfections, the acute phase is usually followed by a chronic HDV infection. Such chronicity has a high chance of proceeding to cirrhosis and even hepatic failure. It has been previously thought that chronic HDV infections can progress not only to cirrhosis but also to hepatocellular carcinoma (Fattovich et al. 2000). One evaluation suggests that while chronic HDV infections frequently increase the risk of cirrhosis, they may only indirectly via HBV, increase the risk of hepatocellular carcinoma (Kew 1996).

Epidemiology and phylogenetics

HDV has been detected in many parts of the world, including Europe (Italy, Spain), Asia (China, Taiwan, Japan), the former USSR, and South America (Amazon Basin, Peru). The incidence in the USA is relatively low.

Chronic HBV infections are currently estimated at around 400 million worldwide. In 1993 it was estimated that there were around 25 million chronic cases of (both HBV and HDV) (Alter and Hadler 1993). However, worldwide the HDV levels are decreasing with the impact of HBV vaccination (which also protects against HDV), testing of blood prior to transfusions, and in some cases, altered patterns of intravenous drug usage. New cases of HDV are becoming rare and chronic HDV is being referred to as a ‘vanishing disease’ (Gaeta et al. 2000).

Primary sequence differences between isolates can be as much as 40 percent. Such sequence differences have been used to divide isolates into genotypes I–III. Genotype I contains the majority of isolates and it has been divided further into subgroups (Niro et al. 1997). Genotype II consists of several isolates from East Asia. Newer data suggest it is almost as widespread as genotype I (Ivaniushina et al. 2001). Genotype III includes only a small number of isolates from life-threatening infections in the Amazon Basin of South America. It is difficult to determine to what extent this pathogenesis is specific for the genotype of HDV relative to that due to the replication of the associated HBV, of genotype F (Casey et al. 1996; Gerin et al. 2001). Studies with transfected cells have shown that there are limitations in the ability of genomes of one genotype to be assisted in replication by the small delta proteins of other genotypes (Casey and Gerin 1998).

Pathogenicity and interactions with the helper virus

It has been reported that when the small form of the delta antigen is expressed in cultured cells there can be an associated cytopathic effect (Cole et al. 1991). In contrast to this, mice made transgenic for the expression of either the small or large forms of the delta antigen fail to show any associated pathology (Guilhot et al. 1994). Overall, it is agreed that there are no striking direct cytopathic effects of HDV (or HBV) replication (Gowans and Bonino 1993).

Nevertheless, HDV replication does have consequences for the infected cell. For example, mice experimentally infected with HDV showed that a small fraction of the hepatocytes became infected and were, within several days, somehow replaced, even in mice with a severe combined immunodeficiency (Netter et al. 1993). Also, some studies in tissue culture do support the interpretation that in cells replicating the HDV genome the rate of cell division is decreased (Bichko and Taylor 1996; Wang et al. 2001).

It has been noted that at the peak of an acute HDV infection, replication of the helper virus may be transiently suppressed (Gerin et al. 2001). However, in some cases this suppression can be as little as several-fold (Netter et al. 1994). It must be realized that there can also be evidence for 'interference' in patients infected with hepatitis C virus (HCV) superimposed on HBV and HDV (Jardi et al. 2001).

Treatment

Treatments with interferon-alpha are known to suppress particle production. However, the doses have to be very high and associated side effects are substantial. Moreover, when the patient is withdrawn from therapy, HDV production usually promptly resumes. The cure rate ascribable to such treatment, after subtraction of spontaneous cures, is probably <20 percent (Rizzetto and Ponzetto 1995; Lau et al. 1999).

A reasonable expectation was that suppression of HBV replication and assembly would indirectly suppress HDV replication. It was expected that some of the inhibitors, such as lamivudine, that act so potently on HBV replication should therefore inhibit HDV. Even the effects of interferon-alpha on HDV in large part might be indirect, via suppression of the HBV. However, from a study of four patients given 1 year of lamivudine therapy, it was observed that HDV RNA levels in the serum did not significantly decrease and liver damage continued (Lau et al. 1999).

Vaccination

In most cases, an HDV-specific vaccine is not needed since that for HBV is effective, indirectly. However, for individuals already chronically infected with HBV, an HDV vaccine might be of use. Several preliminary studies have used the delta antigen for vaccination, with variable success (Rizzetto and Ponzetto 1995).

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TT virus and other anelloviruses

MAURO BENDINELLI AND FABRIZIO MAGGI

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TT virus (TTV) is one of several human viruses discovered in recent years, following the development of sensitive techniques for showing foreign nucleic acids in tissues. Soon after the discovery, it became clear that this novel virus was just one of a vast group of related, previously unrecognized viral agents, all of which were characterized by small, circular single-stranded DNA genomes with negative polarity. Presently, all these viruses are classified within a new genus, *Anellovirus*, that includes two species clearly distinct for their different genome sizes, TTV and TT minivirus (TTMV) [previously referred to as TTV-like minivirus (TLMV)].

Knowledge on TTV and related viruses has accumulated rapidly, but many fundamental aspects remain unresolved, particularly their significance for human health. In fact, anelloviruses have been found to be amazingly widespread, with abundant viral DNA detectable in the plasma of 80 percent or more of the general population worldwide, but in spite of this and the publication of more than 500 papers, no associated clinical disease has yet been identified. This unprecedented situation has actually led to the proposal that anelloviruses should be considered completely nonpathogenic and be regarded as the first recognized viral member of the normal commensal microflora (Mushahwar 2000). This view appears, however, somewhat premature considering all the many issues that still wait to be addressed (Bendinelli et al. 2001; Simmonds 2002).

DISCOVERY

TTV was first described in 1997 by Japanese workers who detected part of its genome whilst, in an attempt to identify additional agents of human hepatitis, were comparing by representational difference analysis, the nucleic acids contained in sequential blood samples of a patient (TT) with cryptogenetic post-transfusion hepatitis (Nishizawa et al. 1997). TTV discovery was soon followed by the detection, in diseased and healthy individuals, of numerous other viruses with genome properties clearly akin to those of TTV although often very divergent from it and from each other in sequence. The latter received specific names – SANBAN, YONBAN (meaning the third and the fourth, respectively, in Japanese), PMV, SENV, KAV – or were indicated with numbers (Hijikata et al. 1999; Hallett et al. 2000; Primi et al. 2000; Takahashi et al. 2000a; Tanaka et al. 2001) but are now classified as distinct genotypes of TTV. Furthermore, polymerase chain reaction (PCR) testing of certain blood donors for TTV produced shorter amplicons than expected, and further characterization revealed the existence of additional viruses also clearly related to TTV but with smaller genomes, namely TTMV (Takahashi et al. 2000b).

VIRIONS AND GENOMES

Physicochemical and immunoelectron microscopy studies have shown that the TTV particle has a density of 1.31–1.35 g/ml and is approximately spherical, 30–32 nm in diameter, and devoid of external lipids (Okamoto et al. 1998; Mushahwar et al. 1999; Itoh et al. 2000). Although most information on virion structure has been obtained with genotype 1, there are no indications that significant genotype-related differences might exist within TTV. TTMV, however, has a smaller size, as determined by

filtration (Takahashi et al. 2000b). Like other non-enveloped viruses, both TTV and TTMV are unaffected by ether and chloroform and are believed to resist chemical and physical inactivating agents at least as effectively as parvoviruses and circoviruses (Todd et al. 2001).

Complete or near complete genome sequences, determined for a number of TTV and TTMV isolates, have revealed a high degree of divergence between the two viruses. The DNA of TTV is approximately 3 800 nt long (Erker et al. 1999; Kamahora et al. 2000; Muljono et al. 2001; Okamoto and Mayumi 2001; Hino 2002) whilst that of TTMV ranges around 2.8 kb. The general organization of the two genome types is, however, similar. Approximately one-third of their length has no coding functions (untranslated region (UTR)), but appears to control virus replication and expression through a number of regulatory sequences and secondary structures. Prominent among the latter is a stretch of approximately 100 nt with high guanine and cytosine content (GC region; Miyata et al. 1999). A number of such regulatory elements are highly conserved among TTV and TTMV isolates and are therefore exploited for designing universal primers (see below).

As deduced from the sequences, whilst no open reading frames (ORF) are present in the genomic DNA of either viruses, the antigenomes contain one large ORF (ORF1) and several partially or totally overlapping smaller ones (Kakkola et al. 2002; Yokoyama et al. 2002). ORF1 has a coding capacity of 770 and 675 amino acids in TTV and TTMV, respectively. The deduced encoded protein, which represents the putative capsid protein, contains an arginine-rich hydrophobic region at the N terminus, three hypervariable regions (HVR) (HVR-1, 2, and 3) located centrally, and variable numbers of potential glycosylation sites (Hijikata et al. 1999). The other ORFs are variously conserved amongst different genotypes. ORF2 encodes putative nonstructural proteins of approximately 120 and 100 amino acids in TTV and TTMV, respectively, which might have kinase activity in at least some genotypes. ORF3 seems to lead to two differently phosphorylated proteins, thus resembling gene *NS5A* of hepatitis C virus (HCV) (Asabe et al. 2001).

TAXONOMY AND NOMENCLATURE

At the time TTV was discovered, all known viruses of vertebrates with small circular, single-stranded DNA genomes were classified within the family *Circoviridae*, leading to the suggestion that the new virus should also be included in this family. Indeed, TTV has a high genome-relatedness to the *Chicken anemia virus* (CAV), a circovirus that causes damages to the poultry industry. However, the creation of a new virus family was also proposed (Mushahwar et al. 1999; Takahashi et al. 2000b; Okamoto and Mayumi 2001) to accommodate the considerable differences existing with other circoviruses [*Porcine circovirus* (PCV), *Beak and feather disease virus* (BFDV) of parrots], even though the latter have ambisense and not negative-strand genomes like CAV, TTV, and TTMV. More recently, following the advice of an appointed study group, the International Committee for the Taxonomy of Viruses (ICTV) has decided to classify TTV and TTMV as distinct species within a newly created, temporarily self-standing genus named *Anellovirus* (from *anellus*, Latin for ring). The ICTV has also decided to change the extended names of the two viruses in torque-teno-virus (TTV) (from the Latin words *torques* and *tenuis*, meaning necklace and thin, respectively) and torque-teno-minivirus (TTMV). This was suggested to conform to the rule that no official virus designation should be derived from people's names, without having to extensively modify the acronyms (Hino 2002).

Both TTV and TTMV show a remarkable degree of genetic heterogeneity. This diversity, which is especially high in the coding portion of the genomes (for example, amino acid divergence is as high as 68 percent in the ORF1 of isolates TA278 and SANBAN) has been exploited in order to group isolates. The criteria adopted for TTV are similar to the ones used for HCV: genotypes (identified by Arabic numbers) are defined by nucleotide divergences of 30 percent or greater, and subtypes (identified by small letters) by nucleotide divergences of between 15 percent and 29 percent. By such criteria, at least 40 genotypes of TTV, each (or most) subdivided in several subtypes, are presently known. In addition, genotypes have been clustered into five major TTV groups (designated 1–5), distinct from one another by nucleotide divergences of 50 percent or greater (Table 1). TTMV has been less investigated but existing data indicate that it may be even more diversified than TTV: comparison of full-length and ORF1 sequences has so far identified three clusters diverging by 40 percent or more at the nucleotide level (Table 1). Due to their considerable genetic distances, it is likely that in the future TTV genogroups will come to be regarded as distinct species. For both TTV and TTMV, the recognition of much greater diversity is expected with analysis of increasing numbers of sequences. It should also be noted that TTV-like viruses have been detected in several species of nonhuman primates, other mammals, and birds (Leary et al. 1999; Abe et al. 2000; Okamoto et al. 2002). From the limited amount of sequence data available, it would appear that primate viruses have undergone long-term co-evolution with their host species (Simmonds 2002).

INTERACTIONS WITH INFECTED CELLS

TTV and TTMV can been found in many tissues of the body and this, together with the demonstration of

Table 1 *Viruses recently classified in the genus* Anellovirus

Virus	Genogroup	No. of genotypes included	Representative isolates	Reference
TTV	1	6	TA278	Nishizawa et al. 1997
	2	6	PMV	Hallett et al. 2000
			KAV	Heller et al. 2001
	3	15	SANBAN	Hijikata et al. 1999
			SENV	Primi et al. 2000
	4	9	YONBAN	Takahashi et al. 2000a
			CT23	Peng et al. 2002
	5	4	JT33	Peng et al. 2002
TLMV	1	At least 4	CBD203	Takahashi et al. 2000b
			PB4TL	Biagini et al. 2001
	2	At least 2	CBD231	Takahashi et al. 2000b
	3	At least 2	NLC023	Biagini et al. 2001

replicative nucleic acid intermediates of TTV in several tissues, has suggested that both viruses might infect many different cell types (see below). However, the precise cell type(s) that support virus replication remain undetermined (Takahashi et al. 2002; Yu et al. 2002). In culture, only fresh human peripheral blood mononuclear cells have hitherto been found to permit TTV propagation (Maggi et al. 2001b; Mariscal et al. 2002).

Current understanding of TTV and TTMV replication mechanisms is scanty and mostly inferred from what is known about CAV. CAV grows in lymphoid cell lines and other cell types, is found in the nucleus, and appears to depend on cell proteins expressed during the S-phase for replication (Todd et al. 2001). TTV also appears to replicate in activated, but not resting, lymphoid cells (Maggi et al. 2001b; Mariscal et al. 2002). Viral DNA replication is believed to occur by the rolling circle mechanism. Such a mechanism, which is particularly well suited for replicating single-stranded DNA viruses, is indicated by the presence in the deduced ORF1 product of conserved Rep protein motifs typical of the many viruses that replicate in this manner (Hino 2002). It should be noted, however, that the great genetic diversity of TTV has also led to the theory that polymerases with low proofreading activity intervene (Umemura et al. 2001b). Indeed, the rates of nucleotide change reported for the HVR of TTV (up to 7.3×10^{-4} per site per year) are at least tenfold higher than in hepatitis B virus (HBV) and other DNA viruses and in a range similar to RNA viruses (Umemura et al. 2001b). Transcription profiles are poorly understood. Recently, TTV genome transcription and splicing has been found to produce three to four species of viral mRNA, transcribed from the negative strand and having different lengths but with common 5′ and 3′ terminals, in infected tissues (Okamoto et al. 2000a, 2001) and in experimental systems (Kamahora et al. 2000; Yokoyama et al. 2002).

Modes of virion assembly and release from producer cells are not known. In analogy with what is observed with other nonenveloped viruses, egress of progeny virions is likely to imply the destruction of infected cells. Genetic recombination between different genotypes appears to be a frequent event in TTV and TTMV (Worobey 2000; Biagini et al. 2001), indicating that the same cell can become infected with more than one genotype. Recombination is most often observed in the noncoding portion of the viral genome, most likely due to the high conservation of this region. As discussed below, individuals who harbor multiple genotypes are numerous, thus contributing to the explanation as to why recombination is a frequent occurrence. Available data exclude that TTV can persist as an episome or become integrated into cell DNA (Yu et al. 2002).

NATURAL HISTORY OF INFECTION

A well-documented property of both TTV and TTMV is that they readily establish chronic productive infections in many, if not all, exposed individuals. Apart from this, information about the infection course is still sketchy and almost exclusively limited to TTV. Whilst others appear likely (Okamoto and Mayumi 2001), three modes of TTV entry into the body have been clearly documented: inoculation of contaminated blood or blood-derived products (Simmonds et al. 1998; Bendinelli et al. 2001), transplacental infection (Morrica et al. 2000), and airborne infection (Maggi et al. 2003). In the latter study, 87 out of 100 young children hospitalized for acute respiratory infections were found to carry larger TTV loads in the nasal secretions than in blood and, in some, the same virus was demonstrable earlier in the nares than in the circulation, thus suggesting that the respiratory tract is a port of entry and also a site of primary virus amplification. This and other studies (Kobayashi et al. 1999; Matsumoto et al. 1999) have also shown that TTV becomes detectable in the blood within one or a few weeks after exposure, thus indicating that dissemination of infection throughout the body is rapid.

Self-limited TTV infections have been described (Lefrere et al. 1999; Matsumoto et al. 1999), and, according to a recent report (Ohto et al. 2002), their frequency depends on viral genotype; however, the possibility is not excluded that such findings reflect

post-acute reductions of viremia levels under the detection threshold rather than true virus eradications. Large fluctuations of TTV viremia levels have been observed in a number of subjects over time (Ball et al. 1999; Matsumoto et al. 1999). In any case, prolonged, possibly lifelong, TTV persistence is by far the most frequent outcome (Lefrere et al. 1999; Matsumoto et al. 1999; Takayama et al. 1999). Plasma TTV loads vary extensively in chronically infected persons, ranging between 10^2 and more than 10^8 DNA copies per ml (Pistello et al. 2001). Similarly to what has been observed with other chronic plasma viremia-inducing viruses, TTV viremia is the result of the continuous shedding into, and clearance from, the bloodstream of large numbers of virions (Table 2).

Cerebrospinal fluid is usually virus-negative in infected individuals with an intact blood–brain barrier (Maggi et al. 2001a). All other anatomic sites tested have been found to harbor variably abundant TTV. These include liver, lung, pancreas, spleen, kidney, lymph nodes, skeletal muscles, thyroid gland, bone marrow, and circulating leukocytes. For some such tissues, namely liver, bone marrow, and circulating peripheral blood mononuclear cells, there are also molecular indicators (double-stranded viral DNA and viral mRNA) that TTV replicates locally (Okamoto et al. 1998, 2000a, b, c, 2001; Kikuchi et al. 2000; Suzuki et al. 2001). However, the possibility that these findings reflect circulating virus or ubiquitous virus-infected cells, such as lymphocytes and/or macrophages, cannot be excluded at present. That proliferating hematopoietic cells might be a major source of TTV is suggested by observations that baseline viremia decreased to undetectable levels in virus carriers treated with myelosuppressive drugs (Kanda et al. 1999). The possibility has also been suggested that different TTV genotypes differ in tropism and replicate preferentially in selected body compartments (Okamoto et al. 2001).

It is probable that the extraordinary ability of anelloviruses to persist and continuously release copious progeny into the systemic circulation is due to a complex combination of virus and host factors. That primary infections generally occur early in prenatal or postnatal life (see below) may be important for persistence by inducing partial immunotolerance. Also, if productive viral replication is indeed possible only in cycling cells (Maggi et al. 2001b; Mariscal et al. 2002), then only a fraction of potentially susceptible cells might be permissive at any given time, thus allowing even supposedly cytocidal agents, such as anelloviruses, to persist. On the other hand, it might well be that immune responses elicited by anelloviruses are not particularly vigorous and/or the immune effectors produced are incapable of controlling the infection with sufficient efficacy.

Immune responses have not been properly investigated as yet, at least partly due to lack of suitable viral antigens. A few attempts to produce recombinant TTV antigens have yielded confusing or negative results. Recently, an enzyme-linked immunosorbent assay using antigen extracted from the feces of a carrier has permitted the following of the antibody responses developed by two patients with post-transfusion TTV infections. Specific IgM and IgG were detected after 10–21 and 12–17 weeks, respectively (Tsuda et al. 2001), possibly indicating that humoral responses to TTV mount slowly. Furthermore, immunoprecipitation studies have shown that a variably large proportion of total TTV found in chronically infected patients has IgG bound to its surface (Nishizawa et al. 1999; Ott et al. 2000). It would be of interest to ascertain whether such immunocomplexed virus is still infectious for cells. The fact that mixed infections by multiple TTV strains are numerous (see below) indicates that antiviral immunity does not efficiently protect against superinfection by heterologous genotypes.

Table 2 *Dynamics of chronic TTV viremia, relative to other viruses*

Virus	Mean virion half-life in plasma	New virions entering plasma per day	Reference
HBV	1.2 days	$>1.7\times10^{13}$	Whalley et al. 2001
HCV	2.7 hours	$>1.3\times10^{12}$	Neumann et al. 1998
HIV-1	< 1 hour	$>9.8\times10^{9}$	Ramratnam et al. 1999
TTV	6.5 hours	$>3.8\times10^{10}$	Maggi et al. 2001c

LABORATORY DIAGNOSIS

Reliable tissue culture and immunological methods for demonstrating TTV and TTMV or induced antibodies are lacking. Diagnosis of infection relies, therefore, solely on the detection of viral genomes in blood or other specimens. The PCR procedures most commonly used at present are listed in Table 3. Due to the great genetic diversity of both TTV and TTMV, the choice of the viral DNA segment targeted for amplification has an enormous impact on PCR assay sensitivity. For example, early tests for TTV, which targeted poorly conserved sequences in the ORF1 (Bendinelli et al. 2001), greatly underestimated the prevalence rates of infection since they almost exclusively detected genogroup 1 viruses. The UTR of both TTV and TTMV are now known to be most suitable for the design of primers capable of detecting all, or the majority of, the viral genotypes hitherto identified (Okamoto and Mayumi 2001). However, no method which has the potential to simultaneously detect all possible variants of both TTV and TTMV with a single test has yet been described. Quantitative real-time assays also exploit this region (Maggi et al. 2001c; Moen et al. 2002). On the other hand, the

Table 3 *Methods currently in use for the diagnosis of TTV and TTMV*

Virus	Purpose	Type of test	Target region
TTV	Detection	Qualitative universal PCR	UTR
	Quantitation	Real-time universal PCR	UTR
	Typing	Group-specific PCR	ORF1 (genogroups 1–3)
			UTR (genogroups 4 and 5)
		Sequencing	ORF1 (all genogroups)
TTMV	Detection	Qualitative PCR	UTR
	Quantitation	Real-time PCR	UTR
	Typing	Sequencing	ORF1

genogroup-specific PCR assays that have been proposed for typing purposes as a practical alternative to sequencing methods are UTR- or ORF1-based, depending on the specific genotype targeted. Genogroup- or genotype-specific quantitative assays may also become valuable in future studies attempting to correlate definite viral types to disease (Maggi et al. 2003).

EPIDEMIOLOGY

The viruses discussed in this chapter are certainly among the most widespread viral agents of humans. The use of sensitive and broad-range PCR techniques has indeed shown that over two-thirds of the global population carries TTV in plasma and that active TTMV infection is possibly even more common (Biagini et al. 2000, 2001). Thus, almost everyone is infected with either TTV or TTMV or both. In fact, individuals who carry multiple genetic forms of the two viruses are more the rule than the exception (Umemura et al. 2001a). Significantly, a recent report has described the presence of as many as 15 different genotypes of TTMV in the blood of an immunocompromised subject (Vasconcelos et al. 2002). Prevalence rates are already very high during the first months of life and augment only moderately thereafter (Bendinelli et al. 2001; Maggi et al. 2003), but whether the proportion of individuals who carry multiple types of the two viruses varies with age remains to be established. Gender and socioeconomic conditions appear to be of little importance for spread of the infection. Geographical variations in total and genotype-specific prevalence rates would appear to exist (Gallian et al. 2000; Muljono et al. 2001; Umemura et al. 2001a); however, systematic studies conducted using uniform protocols that might permit really meaningful comparisons are lacking.

That anelloviruses can be transmitted via blood and blood products is obvious and has been repeatedly documented. However, their amazing diffusion in the general population implies the existence of much more frequent occasions of contagion, as also corroborated by frequent virus detection in saliva, nasal and genital secretions, tears, and feces. Intrauterine transmission is a clear possibility and has been repeatedly demonstrated with the detection of TTV in cord blood (Gerner et al. 2000; Inami et al. 2000; Matsubara et al. 2000; Fornai et al. 2001). Another route that has been clearly documented is through the respiratory tract (Maggi et al. 2003). Based on indirect evidence, fecal–oral spread is also very likely (Gallian et al. 2000; Lin et al. 2000; Tawara et al. 2000). The role in human epidemiology of the TTV-like viruses detected in other species, including pet and livestock animals, is unclear (Leary et al. 1999; Okamoto et al. 2002).

There are no available assays that permit the discrimination of whether viremia-negative subjects have skipped or have resolved the infection. Investigating this issue serologically is complicated by the poor definition of viral antigens and by the fact that virus in blood is frequently heavily immunocomplexed (Nishizawa et al. 1999; Ott et al. 2000; Tsuda et al. 2001; Kakkola et al. 2002).

ASSOCIATION WITH DISEASE

To date, there are no clinical manifestations that have been unequivocally linked to anelloviruses. As discussed in detail elsewhere (Bendinelli et al. 2001), early attempts to implicate TTV in those forms of liver disease that have a putative viral cause but are unrelated to known viruses have been frustrated, albeit it is still considered probable that, in some cases, TTV infection is associated with transient and mild elevations of liver enzyme levels (Nishizawa et al. 1997; Kanda et al. 1999; Tawara et al. 2000). Other cryptogenetic diseases that have been investigated include diabetes, cryoglobulinemia, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, the Kawasaki syndrome, multiple sclerosis, and other neurological diseases. In all cases, results have excluded a TTV etiology or have been inconclusive.

As already mentioned, failure to identify associated diseases, together with the extraordinarily high prevalence rates of both TTV and TTMV active infections in the general population worldwide, has led to the hypothesis that these viruses might be completely nonpathogenic. However, at the time of writing it seems more appropriate to consider anelloviruses as 'orphans of disease,' similar to that previously done for many other viruses which in the early years after discovery could not be linked to any disease but were subsequently found

to produce significant pathologies (e.g. echoviruses, reoviruses, Epstein–Barr virus, and others). Several aspects need investigating more thoroughly. For example, the possible clinical implications of primary infections, especially in infants, have been only marginally explored (Biagini et al. 2003; Maggi et al. 2003). Similarly, only recently is it becoming appreciated that only some types of TTV and TTMV might be pathogenic but their disease potentials might be blurred by many nonpathogenic types. Certain reports suggest that TTV genotypes 1a, 12, and 16 are especially damaging to the liver (Shibata et al. 2000; Umemura et al. 2001a) although others discount these findings (Yoshida et al. 2002). The possible consequences for the host's immune system of having to deal chronically with these viruses should also be attentively investigated, especially if proliferating lymphoid cells are confirmed to be a major site of virus infection. In this regard, recent findings have shown that among a large group of infants hospitalized for acute respiratory illnesses, the ones who exhibited higher plasma and nasal levels of TTV were also the ones who had more severe disease (Maggi et al. 2003). Furthermore, circulating loads of TTV are particularly high in patients with AIDS or other immunocompromising conditions (Christensen et al. 2000; Shibayama et al. 2001), possibly contributing to immune impairment. Accounts implicating TTV or TTMV in some renal ailments (Yokoyama et al. 2002) and in certain forms of anemia, neutropenia, and thrombocytopenia (Kikuchi et al. 2000; Simmonds 2002) are also worth further scrutiny.

CONTROL

As long as their clinical implications remain undefined, there will certainly be little attention paid on how the viruses discussed in this chapter might be controlled. Their epidemiologic features leave, however, little doubt that trying to prevent infections would be extremely difficult in the absence of specific multivalent vaccines. On the other hand, TTV has proven insensitive or only transiently sensitive to interferon in patients treated for other viral infections (Maggi et al. 1999, 2001c; Dai et al. 2002), albeit combined therapy with ribavirine seems more effective and selected viral genotypes more susceptible (Umemura et al. 2002). As expected, antiretroviral drugs have also shown no anti-TTV activity (Takamatsu et al. 2001). Thus, should it one day be regarded as clinically useful, treating established infections would most likely need the development of specific antivirals.

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Retroviruses and associated diseases in humans

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INTRODUCTION AND GENERAL PROPERTIES OF THE *RETROVIRIDAE*

Retroviruses seem to have played a role in the evolution of human genetics, considering the large number of retroviral remnants within the human genome. Why, where, and how these viral residues occurred is a curious enigma of our genome. Did they arise from multiple infections, which over time devastated new evolving species of hominoids? Did such an occurrence result in survivors who either were infected with defective retroviruses or had modified their receptors to be resistant to primary infection? The answers may become apparent if, in several thousand years, remnants of human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV) are found 'fixed' in the human genome.

Within the large family of retroviruses, lentiviruses, which include the HIVs, are unusual in that they possess complexities and degeneracies that differ from the biological properties of other, more stable, primate retroviruses, such as the deltaretroviruses and the spumaviruses. Nevertheless, these agents share the common feature of utilizing a reverse transcribing enzyme to replicate their genome. Retroviruses replicate in a unique manner. An enzyme carried by the viruses reverses the usual flow of genetic information (normally from DNA to RNA (messenger RNA) and then to protein) by causing the RNA genetic information to create a DNA (proviral) intermediate. This is a characteristic feature of all retroviruses. Viral genetic information in the DNA form is contained in this 'provirus,' which can be integrated into the genome of the host cell, where it may remain latent or nonexpressing for variable periods. Only when the provirus is activated, often by first messenger signals such as cytokines or antigens, are viral proteins and new viruses made. In the mature virion the genome is diploid, carried as a 60–70S dimer complex of two identical, positive-sense, single-stranded RNA copies, whereas reverse transcriptase (RT) is a meiotic event, resulting in a double-stranded haploid DNA provirus. The proviral genome structure of retroviruses is characterized by repeated sequences at either end of the genome, the long terminal repeats (LTR). These structures play an important role in viral expression because they contain promoter and enhancer elements. The genomes of all retroviruses that are able to replicate fully always include three genes coding for the three sets of structural proteins: those of the core proteins (gag), the envelope proteins (env) and the polymerase enzyme (pol). The genome arrangement LTR–gag–pol–env–LTR is common to all known retroviruses. A simplified retroviral life cycle is depicted in Figure 1.

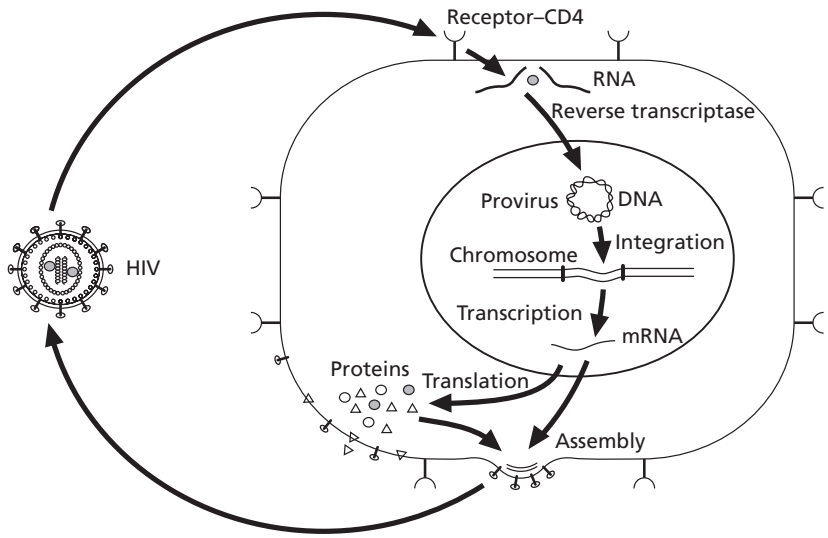

Figure 1 *Schematic representation of the life cycle of HIV. Virions attach to specific cell surface receptors. Following internalization and uncoating either via receptor-mediated endocytosis or by fusion at the plasma membrane, the reverse transcriptase in the core of the virion becomes activated and synthesizes a double-stranded DNA provirus. Circularized and linear forms of the provirus migrate to the nucleus and integrate in host chromosomal DNA. The integrated proviral genome may remain latent, but, if active, particularly in proliferating cells, it expresses positive-strand RNA. Full-length transcripts become packaged into progeny virions budding from the plasma membrane. Messenger RNA (mRNA) transcripts encode the structural and regulatory viral proteins, which can be recognized as antigens within infected cells.*

Among the viruses, retroviruses and the diseases they cause were some of the earliest to be described in detail (Weiss 1984). At the turn of the century, equine infectious anemia was one of the first animal infections recognized as having a viral etiology. Shortly afterwards, there were descriptions of filterable, transmissible agents associated with cancers among chickens that related both leukemias (avian leukosis, 1908) and solid tumours (Rous sarcoma, 1911) to infections with viruses. In 1936, breast cancer in mice was found to be transmitted by a virus in the animal's breast milk, and from the 1950s onwards leukemias in mice, cats, fish, apes, and other vertebrate hosts were found to be caused by retroviruses. Knowledge of these cancer-causing viruses has yielded important insights into the nature of neoplasia and catalyzed the search for similar agents as a cause of malignancy in humans. Despite the attraction of this idea, which implied the possibility of vaccines to prevent cancer, it was not until 1980 that the relationship between a retrovirus infection and human neoplasia was documented, leading to the eventual characterization of HTLV.

Recently, the family of *Retroviridae* has been reclassified into seven different genera (Table 1). Viruses from the genera *Lentivirus* and *Deltaretrovirus* are known to cause disease in humans while viruses from the genus *Spumavirus* have been shown to infect humans, but no disease associations yet have been found. The chapter presented here deals primarily with those non-HIV retroviruses that cause or are associated with human disease.

HUMAN T-LYMPHOTROPIC VIRUS TYPE 1 AND HUMAN T-LYMPHOTROPIC VIRUS TYPE 2

Morphology

Human T-lymphotropic virus type 1 (HTLV-1) was the first human retrovirus isolated in 1981 (Kalyanaraman et al. 1981; Poiesz et al. 1981). Shortly thereafter, Human T-lymphotropic virus type 2 (HTLV-2) was isolated from a person with hairy cell leukemia (Kalyanaraman et al. 1982). Both viruses are part of the *Orthoretrovirinae* subfamily. HTLV-1 and HTLV-2 are typical C-type retroviruses; they contain an electron-dense, centrally located nuclear core and they bud from the cell surface. Within the core are two positive-sense single-stranded RNA genomes. A unique feature about HTLV-1 and HTLV-2 is their inefficient ability to infect via cell-free virus. Rather, these viruses require cell-to-cell contact for productive infection. Despite this, electron microscopy has shown that chronically infected cells form normal viral particles that are assembled at the plasma membrane and released (Poiesz et al. 1980; Miyoshi et al. 1981; Ohtsuki et al. 1982). It is currently unknown why cell-free virus has reduced infectivity.

Genome organization

HTLV-1 and HTLV-2 are more than 9 kb in length and have similar genomic organization: *gag*, *pro*/*pol*, *env*, p*X*,

Table 1 Retroviridae *family*

Genus	Virus	Host	Growth in human cells in vitro	Mode of transmission to humans	Human disease or infection
Alpharetrovirus					
Avian leukosis virus	ALV	Birds			
Rous sarcoma virus	RSV	Birds			
Gammaretrovirus					
Moloney murine leukemia virus	MoMLV	Mice			
Feline leukemia virus	FeLV	Cats	Yes		
Porcine endogenous retrovirus	PoEV	Pigs	Yes	?[a]	?
Baboon endogenous virus	BaEV	Baboons	Yes	?[a]	?
Betaretrovirus					
Mouse mammary tumor virus	MMTV	Mice			
Mason–Pfizer monkey virus	MPMV	Monkeys	Yes	?	Infection
Deltaretrovirus					
Bovine leukemia virus	BLV	Cows			
Primate T-lymphotropic virus 1	HTLV-1	Humans	Yes	Mother to child, sexual transmission, blood	T-cell lymphoma, neurological disorders, HAM/TSP[b]
Primate T-lymphotropic virus 2	HTLV-2	Humans	Yes		
Epsilonretrovirus					
Walleye dermal sarcoma virus	WDSV	Fish			
Lentivirus					
Human immunodeficiency virus type 1	HIV-1	Humans	Yes	Mother to child, sexual transmission, blood	AIDS
Human immunodeficiency virus type 2	HIV-2	Humans	Yes		
Simian immunodeficiency virus	SIV	Monkeys	Yes	(biting, blood, other?)	Infection
Visna/maedi virus	VISNA	Sheep			
Equine infectious anemia virus	EIAV	Horses		(biting, blood, other?)	
Feline immunodeficiency virus	FIV	Cats	Yes		
Caprine arthritis encephalitis virus	CAEV	Goats			
Spumavirus					
Simian foamy virus	SFV_{CPZ}[c]	Nonhuman primate	Yes	(biting, blood, other?)	Infection

a) Risk of transmission to human from xenotransplantation.
b) TSP, tropical spastic paraparesis; HAM, HTLV-associated myelopathy.
c) No human species of *Spumavirus* (foamy) is known. Simian foamy viruses from a number of simian species have been isolated or sequences identified in humans (see section below on Zoonotic retroviral infections).

and are flanked by the LTRs (Figure 2). Further, they share approximately 65 percent of their nucleotide sequences. The *gag* gene encodes for a polypeptide precursor that contains the structural proteins, p19 (MA), p24 (CA), and p15 (NC). Sera from infected patients generally have antibodies that recognize p19 and p24. The *pro* gene encodes for the viral protease. Synthesis of the protease as part of the gag polyprotein

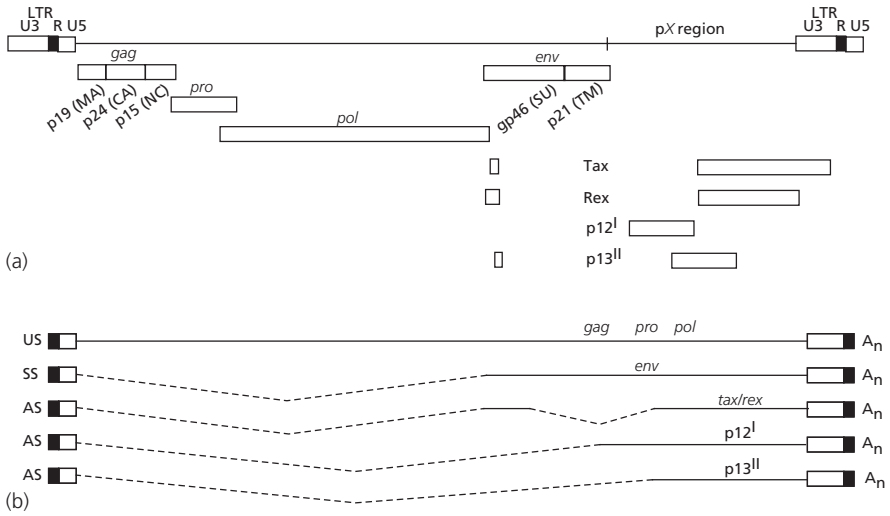

Figure 2 *Molecular genetic organization of HTLV.* **(a)** *Genomic organization. The 8.3-kb genome is flanked by long terminal repeats (LTR). Open reading frames are shown for the structural proteins, viral enzymes, and regulatory proteins.* **(b)** *RNA species. Unspliced, singly spliced, and multiply spliced RNA species are produced. Solid lines represent the exons and the dotted lines represent the intervening sequences.*

precursor is done by ribosomal frameshifting (Nam et al. 1988). The *pol* gene encodes the reverse transcriptase. The *env* gene encodes the transmembrane and external envelope glycoproteins, gp21 and gp46. The HTLV LTR is structured in a similar manner as other retroviral LTRs and contains three regions, U3, R, and U5. The U3 region contains transcriptional control sequences including the three imperfect 21 nt repeats that make up the Tax response element 1 (TRE-1), which is required for the *trans*-activation by Tax (see below) (Shimotohno et al. 1986). The U3 region also contains sequences for polyadenylation and termination of mRNAs. The R and U5 regions form the leader sequence encoded at the 5′ mRNAs. The R region is important for post-transcriptional control by Rex (see below).

As a complex retrovirus, HTLV-1 and HTLV-2 use alternative splicing and internal initiation codons to produce several regulatory and accessory proteins encoded by four open reading frames (ORF) located in the p*X* region of the viral genome between *env* and the 3′ LTR.

TAX (*TRANS*-ACTIVATING GENE)

The two originally described regulatory proteins, Tax and Rex, are generated from a doubly spliced, 2.1 kb mRNA encoded by ORF IV. The HTLV-1 *trans*-activator, Tax, is a 40 kDa protein. Tax contains a nuclear localization sequence that interacts with the cellular transcription factors, cyclic AMP-responsive element-binding protein (CREB) (Franklin and Nyborg 1995), nuclear factor-κB (NF-κB) (Sun and Ballard 1999), AP-1 (Jeang et al. 1991), and serum-response factor (SRF) (Fujii et al. 1992), and thus is a potent transcriptional activator not only from the viral promoter (Tax-responsive element), but also from enhancer elements of many cellular genes involved in host-cell proliferation. These genes include the *IL-2*, *IL-2R*, *GM-CSF*, *Lck*, and others. On its own, Tax has been shown to immortalize T lymphocytes (Rosin et al. 1998) and transform rat fibroblasts (Tanaka et al. 1990). When HTLV-1 with mutations in the CREB region or the NF-κB region of Tax was introduced into primary T lymphocytes, the NF-κB mutant lost its ability to transform the cells, indicating that the NF-κB pathway may play a critical role in the transformation process (Robek and Ratner 1999). Collectively, these data suggest that Tax plays a direct role in the development of HTLV-1-associated leukemia, adult T-cell leukemia (ATL).

REX (REGULATOR OF VIRAL EXPRESSION)

Rex is a 27 kDa phosphoprotein encoded by the ORF IV. Rex has an essential role in viral replication and the regulation of viral structural genes by functioning as a post-transcriptional regulator that increases the expression of singly spliced and unspliced viral mRNAs (Hidaka et al. 1988). Rex binds to the mRNA through a *cis*-acting stem–loop structure, Rex responsive element, which is located within the R region of the viral LTR (Ballaun et al. 1991; Bar-Shira et al. 1991; Unge et al. 1991). For the production of infectious virus, Rex-mediated regulation is required to balance the spliced and unspliced mRNAs.

ADDITIONAL HTLV-1 ACCESSORY GENES

Additional gene products have recently been identified from the two other ORFs. ORF I encodes $p12^{I}$. $p12^{I}$ is a highly hydrophobic protein that has been shown to localize to the endoplasmic reticulum with calcium-binding proteins (Ding et al. 2001). It has been shown that $p12^{I}$ is necessary for optimal viral infectivity in nondividing lymphocytes (Collins et al. 1998; Albrecht et al. 2000)

and activates NFAT-mediated transcription in lymphoid cells (Albrecht et al. 2002). Collectively these data suggest that $p12^{I}$ is important during early infection of T cells to facilitate cellular activation and viral replication. Much less is known about the ORF II product, $p13^{II}$. Recent reports have shown that $p13^{II}$ localizes to the mitochondria, altering the inner-membrane morphology and disrupting the inner-membrane potential (Ciminale et al. 1999; D'Agostino et al. 2002). These data suggest that $p13^{II}$ induces apoptosis of infected cells; however, this has not been observed, suggesting this viral protein may affect other mitochondrial functions.

Replication strategy

HTLV RECEPTOR

The identity of the HTLV-1 and HTLV-2 receptor(s) remains an enigma. It is unknown if both viruses share the same receptor, but mutual interference studies suggest that they may (Sommerfelt and Weiss 1990). Early work had proposed that the receptor may be located on human chromosome 17 (Sommerfelt et al. 1988; Gavalchin et al. 1995; Li et al. 1996); however, subsequent work failed to substantiate these results (Sutton and Littman 1996; Okuma et al. 1999; Jassal et al. 2001). Furthermore, this work has shown that the HTLV surface glycoprotein, gp46, can bind to cell lines derived from numerous species and cell types which implies broad expression of a common cellular antigen or possibly use of distinct cellular antigens for use as a receptor. It is currently unknown if one receptor or multiple receptors are required for HTLV binding and infection. Using an HTLV surface adhesion glycoprotein that binds to cell surface proteins important for HTLV-1-mediated entry, Nath et al. (Nath et al. 2003) showed binding to T cells, B cells, NK cells, and macrophage. Further, immune activation increased binding to $CD4^{+}$ and $CD8^{+}$ T cells, suggesting that the receptor(s) is up-regulated after activation of T cells (Manel et al. 2003a). Recent work has shown that treating susceptible cells with trypsin reduces infectivity, and entry was partially inhibited by ammonium chloride, suggesting that pH may play a role in entry (Trejo and Ratner 2000).

Cells infected with HTLV typically have metabolic alterations. Moreover, gammaretroviruses (Table 1) utilize receptors that belong to the multimembrane-spanning transporter family (Overbaugh et al. 2001). Together with this evidence and that presented above, Manel et al. (Manel et al. 2003b) showed that a ubiquitous glucose transporter, GLUT-1, was utilized as a receptor for HTLV-1 and HTLV-2. Interfering with the expression of GLUT-1, but not other similar proteins, reduced the binding of the HTLV-1 and HTLV-2 envelopes. In support of Igakura et al. (Igakura et al. 2003) data, the GLUT-1 expression is concentrated to the mobile membrane regions and should be involved in the 'virological synapse' (see below). Further work needs to confirm these results.

CELLULAR INFECTION

While the previous data suggest that HTLV has the potential to infect and replicate in a multitude of host species and cell types, in humans, HTLV-1 has shown a preferential tropism for $CD4^{+}$ T cells (Richardson et al. 1990). Recently, HTLV-1 was detected in $CD8^{+}$ T cells, and it was suggested that they might be a reservoir since there is low-level replication in these cells (Nagai et al. 2001). Like HTLV-1, HTLV-2 infects $CD4^{+}$ and $CD8^{+}$ T cells, but it has a broader cellular tropism in vivo as shown by detection of HTLV-2 genome in B cells, macrophage, and NK cells (Lal et al. 1995). However, the ability to replicate in non-T cells is poor as reflected in the lower proviral copy number in those non-T-cell types (Lal et al. 1995). It is currently unknown what factors in T cells, as opposed to other cell types, are required for optimal replication of HTLV-1 and HTLV-2.

HTLV rarely infects cells through cell-free virus as typified by the lack of HTLV transmission using serum/plasma as opposed to whole blood (Donegan et al. 1994). Direct cell-to-cell contact is required for efficient HTLV infection and results in syncytium formation between the infected cell and the uninfected cell. Cellular adhesion molecules that include ICAM-1, ICAM-3, and VCAM, have been shown to enhance syncytium formation (Hildreth et al. 1997; Daenke et al. 1999). It has been thought that the syncytium formation is involved in cell-to-cell infection, but until recently, how infection occurs has not been explained. Work by Igakura et al. (Igakura et al. 2003), showed that there is the formation of a 'virological synapse' that transfers HTLV between lymphocytes. These authors show that viral proteins and nucleic acids are polarized to the cell–cell junction within 40 minutes of cell contact. The polarization is accompanied by microtubule reorientation that transports Gag/nucleic acid complexes toward the cell–cell junction. The HTLV Env may have a role in the initiation of the virological synapse, but the formation of the synapse may be due to the up-regulation of certain adhesion molecules such as integrins (Dezzutti et al. 1995; Valentin et al. 1997; Yamamoto et al. 1997).

The kinetics of HTLV infection are thought to occur in a two-step process. Early after cell entry, there is a transient phase of RT followed by integration into the host's genome with subsequent persistent multiplication of infected cells. Integration is a requisite for expression of progeny virus and is responsible for persistence of the infection. In vitro studies have suggested that retroviruses may prefer to integrate into areas of the host genome that are transcriptionally active (Scherdin et al. 1990). It was also suggested that in persistently HTLV-infected cell lines, the provirus may integrate into GC-rich regions (Glukhova et al. 1999). However, in vivo work has shown

that HTLV preferentially integrates into AT-rich regions and is not associated with transcriptionally active or GC-rich regions (Leclercq et al. 2000a, b).

Genetic diversity

As mentioned earlier, HTLV-1 and HTLV-2 share 65 percent genetic identity, and have minimal genetic variability (<10 percent) among different geographical isolates. The genetic stability of HTLV-1 and HTLV-2, despite long latency periods and high proviral load, is mainly attributed to clonal expansion of HTLV-harboring cells (Cavrois et al. 1996; Wattel et al. 1996). While the misincorporation rate of the HTLV RT is much lower than that of the HIV-1 RT (Mansky and Temin 1995; Mansky 2000), this is believed to play a lesser role in the genetic stability of these viruses. The LTR region has the lowest nucleotide homology, while the regulatory genes, *tax* and *rex*, have the highest nucleotide homology. The stability of the *env* gene may be due to its inability to tolerate mutations well. Pique et al. (Pique et al. 1990) performed insertion mutational analysis and showed that the majority of the mutations resulted in a nonfunctional envelope as shown by the lack of syncytia. These data suggest that the conformational constraints for processing and fusion abilities tend to limit the variability of the HTLV-1 envelope. Further, the utilization of the 'virological synapse' may allow HTLV-1 and perhaps HTLV-2 to escape the immune pressures that result in viral evolution.

While the overall genome of HTLV is highly conserved, nucleotide divergence in the LTR has been exploited to genotypically subtype both HTLV-1 and HTLV-2 (Slattery et al. 1999). There are four genetic subtypes of HTLV-1 based on the geographical origin of the virus: cosmopolitan (subtype A), Central African (subtype B), Australo-Melanesian (subtype C), and New Central African (subtype D) (Mahieux et al. 1997; Slattery et al. 1999). Likewise, HTLV-2 has four subtypes: IIa, IIb, IIc, and IId (Switzer et al. 1995; Slattery et al. 1999). Subtype IIa is commonly found among injecting drug users (IDU) worldwide, whereas subtype IIb is found primarily among Amerindians. This genetic heterogeneity within HTLV-1 and HTLV-2 has provided valuable information on geographical clustering and viral transmission (Switzer et al. 1995; Slattery et al. 1999).

Clinical and pathological aspects

Less than 5 percent of those persons infected with HTLV-1 develop the known diseases. Approximately 1–2 percent develops an aggressive T-cell malignancy known as ATL, and about 2–3 percent develop a chronic inflammatory disease called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Additional inflammatory diseases are also associated with HTLV-1 infection and they affect the skin (infective dermatitis), joints (HTLV-associated arthropathy), or the eye (uveitis). Rarely do persons concurrently develop ATL and HAM/TSP; however, there are documented cases of HAM/TSP with the other inflammatory diseases. It is currently unknown why there are distinct disease manifestations, but it may be due to the HTLV proviral load (Nagai et al. 1998; Manns et al. 1999b; Grant et al. 2002), the host immune response to HTLV (Jacobson et al. 1990), or the genetic background of the infected persons (Manns et al. 1998; Jeffery et al. 2000; Nishimura et al. 2000). For instance, studies calculating proviral load demonstrate that the proviral load is low among asymptomatic carriers compared to persons with HAM/TSP and ATL (Nagai et al. 1998; Manns et al. 1999b), suggesting that proviral load may be an indicator for future disease development.

ADULT T-CELL LEUKEMIA

An HTLV-1-infected person has an estimated 1 percent chance of developing adult T-cell leukemia (ATL) over their lifetime. Males are approximately 1.5 times more likely to develop ATL as females. ATL is diagnosed by seropositivity for HTLV-1, the presence of morphologically distinct $CD3^+/4^+/25^+$ lymphocytes with cleaved nuclei (flower cells)(Figure 3), and elevated serum lactate dehydrogenase activity. A comprehensive study on ATL published the outcome of 818 patients (Shimoyama 1991). That study characterized four clinically distinct subtypes of ATL. Smoldering ATL has the best prognosis with a mean survival time (MST) of >2 years and a projected 4-year survival of 62.8 percent. Smoldering ATL is characterized by skin and lung infiltration of leukemic cells and a normal leukocyte count with a low number of leukemic cells. Often it is hard to diagnose from the asymptomatic carrier since in both cases abnormal lymphocytes (flower cells) are detected in blood smears. However, only in ATL can clonal integration of HTLV-1 be detected by Southern blot. Chronic ATL has a MST of 24.3 months and a projected 4-year survival of 26.9 percent. In chronic ATL, there is a high leukocyte count, and it is associated with lymphadenopathy and hepatosplenomegaly. In both smoldering and chronic ATL, there is no association with hypercalcemia and no leukemic infiltration into the central nervous system, gastrointestinal tract, or bones. Lymphoma has a MST of 10.2 months with a projected 4-year survival of 5.7 percent. Acute ATL has a MST of 6 months and projected 4-year survival of 5 percent. These are both aggressive malignancies and are associated with massive lymphadenopathy, hepatosplenomegaly, lytic bone lesions, and multiple visceral lesions with skin and lung inflitration. Lymphoma and acute ATL patients are functionally immunosuppressed and

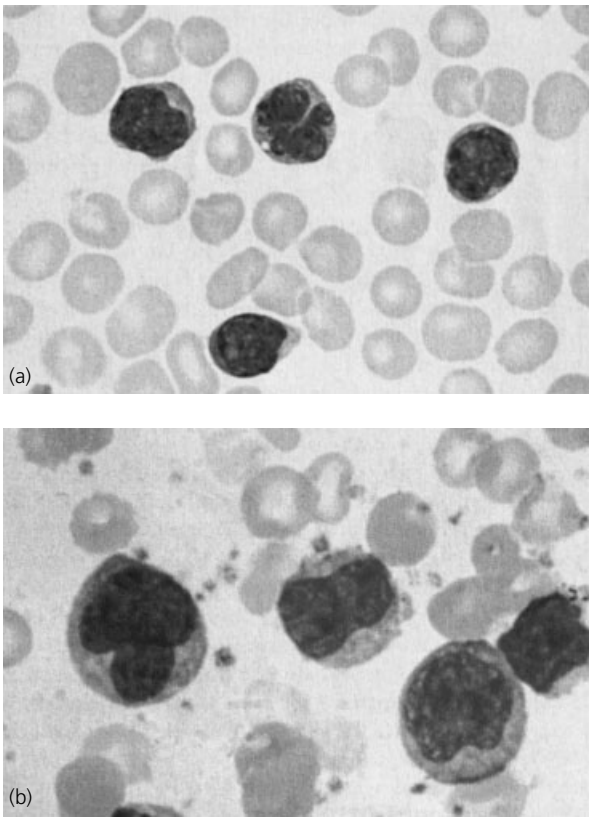

Figure 3 *Peripheral blood film of retrovirus infected patients.* **(a)** *Peripheral film of a patient with HTLV-I-associated adult T cell leukaemia/lymphoma (ATLL) showing lymphocytes with cleaved nuclei ×1000. (Courtesy of Professor D Gatovsky.)* **(b)** *Film of patient presenting with acute glandular fever illness during HIV seroconversion. Marked lymphocytosis with many abnormal immature-looking lymphocytes ×1500. (Courtesy of Dr C Ludlam.)*

may develop a variety of opportunistic infections. The difference between the lymphoma and acute types is whether or not the majority of leukemic cells is in the peripheral blood compartment. Lymphoma ATL has less than 1 percent leukemic cells present on a blood smear whereas acute ATL has a massive leukemic infiltrate in the peripheral blood.

HTLV-ASSOCIATED MYELOPATHY/TROPICAL SPASTIC PARAPARESIS

HTLV-1-infected persons have a lifetime risk of 0.25 percent for developing HTLV-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) (Kaplan et al. 1990). Interestingly, females are three times as likely to develop HAM/TSP as are males. HAM/TSP is a slowly progressing myelopathy. The clinical features include the gradual onset of muscle weakness in the legs, hyperreflexia, clonus, extensor plantar responses, sensory disturbances, bowel and bladder dysfunction, impotence, and low back pain (Nagai and Jacobson 2001). However, unlike multiple sclerosis, HAM/TSP does not show periods of spontaneous improvement without treatment. Diagnosis includes the presence of virus or HTLV-specific antibodies in the cerebrospinal fluid, brain, and spinal cord tissues. ATL flower cells are found in about half of HAM/TSP patients' peripheral blood and cerebrospinal fluid with a frequency of approximately 1 percent (Osame et al. 1987). Autopsy material from HAM/TSP patients shows loss of myelin and axons in the lateral, anterior, and posterior columns of the thoracic level of the spinal cord. This pathology is associated with an inflammatory infiltrate consisting of lymphocytes, predominately $CD8^+$ T cells, and foamy macrophages. The cytotoxic $CD8^+$ T cells are specific for HTLV-1 proteins, typically the p*X* gene products. It is thought that these HTLV-1-specific $CD8^+$ cytotoxic T lymphocytes are involved in the development of HAM/TSP by either directly lyzing infected astrocytes (Kuroda et al. 1994; Lehky et al. 1995) or indirectly by secreting proinflammatory cytokines (Tendler et al. 1991; Kuroda and Matsui 1993).

OTHER INFLAMMATORY DISEASE ASSOCIATIONS

Other inflammatory disease associations include HTLV-associated uveitis (HU), HTLV-associated arthropathy (HAA), Sjögren's syndrome, and infective dermatitis (ID). HU, literally the inflammation of the uvea, is clinically characterized by the sudden onset of moderate or severe vitreous opacities with mild iritis and retinal vasculitis in one or both eyes (Mochizuki et al. 1996). Women are affected by HU twice as often as men. Patients with HU generally have a higher proviral load than asymptomatic HTLV-1 carriers, have viral particles and HTLV-1 provirus in their eyes, and accumulate HTLV-1-infected cells in their eyes (Ono et al. 1995; Sagawa et al. 1995). HU is self-limiting or responds well to treatment with topical or systemic corticosteroids; however, up to half of affected patients relapse. Generally, the visual prognosis is good after resolution (Takahashi et al. 2000).

HAA is indistinguishable from idiopathic rheumatoid arthritis with marked thickening of the synovial lining cells and lymphocytic infiltration of the sublining layer. The characteristic histopathological finding of an accumulation of HTLV-1 atypical lymphocytes in the synovial tissue and fluid as well as antibodies against HTLV-1 antigens in synovial fluid indicates that this is HAA (Sato et al. 1991). Epidemiologically, HTLV-1-infected persons are more likely to develop a polyarthropathy resembling rheumatoid arthritis than HTLV-1 negative persons (Motokawa et al. 1996). Furthermore, the arthropathy in the HTLV-1-infected persons was milder and onset was at a more advanced age compared to HTLV-1-negative persons with rheumatoid arthritis. The presence of HTLV-1 *Tax* is a common finding in synovial cells from persons with HAA. *Tax* expression is associated with increased inflammatory cytokine expression and synovial cell proliferation (Kitajima et al. 1991;

Miyasaka et al. 1991). Subsequent work with transgenic mice expressing the HTLV-1 *Tax* gene has shown the development of a polyarthropathy resembling rheumatoid arthritis (Iwakura et al. 1991, 1998) and it was associated with inflammatory cytokine expression (Iwakura et al. 1995).

Sjögren's syndrome is an autoimmune disorder characterized by a T-lymphocytic infiltration of salivary glands leading to ductal structure destruction (Eguchi et al. 1992; Ohyama et al. 1998). Epidemiologic reports have shown high seroprevalence of HTLV-1 among patients with Sjögren's syndrome in areas endemic for HTLV-1 (Eguchi et al. 1992; Terada et al. 1994). T-lymphocytic infiltrate in the salivary glands correlates to the clinical findings of Sjögren's syndrome (Nakamura et al. 2000). Furthermore, there is an accumulation of polyclonal HTLV-1-infected T cells expressing conserved T-cell receptor motifs in the salivary glands (Sasaki et al. 2000). The *Tax* gene sequence is detected in some salivary gland specimens, and expression of gene sequences homologous to HTLV-1 *Tax* may lead to the activation of autoreactive T cells in patients with Sjögren's syndrome (Sumida et al. 1994; Mariette et al. 2000).

ID is found commonly among children in the Caribbean and Japan. The average age at disease onset is 2 years with >50 percent of patients being female. Five major criteria are used for the diagnosis of ID (La Grenade et al. 1998) and include:

1. eczema of the scalp, axillae and groin, external ear and retroauricular areas, eyelid margins, paranasal skin, and/or neck
2. chronic watery nasal discharge without other signs of rhinitis and/or crusting of the anterior nares
3. chronic relapsing dermatitis with prompt response to appropriate therapy but prompt recurrence on withdrawal of antibiotics
4. usual onset in early childhood
5. HTLV-1 antibody seropositivity.

Skin lesions associated with ID become less severe with age, presumably due to the maturation of the immune system. Epidemiologic data indicate that ID is predictive of later ATL or HAM/TSP development (Hanchard et al. 1991; Tsukasaki et al. 1994; LaGrenade et al. 1995).

HTLV-2 ASSOCIATED DISEASES

Because of the genetic relatedness between HTLV-1 and HTLV-2, it was assumed that similar diseases would be associated with both viruses. However, no diseases have been definitively associated with HTLV-2. The estimated lifetime risk of disease development for HTLV-2-infected persons is unknown, but is less than that estimated for persons with HTLV-1 infection. A few reports have described forms of leukemia/lymphoma in HTLV-2-infected persons (Rosenblatt et al. 1986, 1987; Kaplan et al. 1991), but this is rare. There also has been a report on a $CD8^+$ lymphocytosis associated with high HTLV-2 proviral copy numbers in intravenous drug users (Rosenblatt et al. 1990), but this has not been confirmed in other cohorts. Recently, HTLV-2 has been associated with a neurological disease resembling HAM/TSP (Jacobson et al. 1993; Murphy et al. 1997a; Silva et al. 2002) and ataxia (Harrington et al. 1993; Sheremata et al. 1993). These data suggest that HTLV-2-infected persons rarely develop disease; however, they should be followed up more closely for potential disease development.

IMMUNOSUPPRESSIVE NATURE OF HTLV-1 AND HTLV-2

While the majority of infected persons remain asymptomatic (>95 percent), recent studies report an increased incidence of infectious diseases (such as bronchitis and kidney and/or bladder infections) in HTLV-1- and HTLV-2-infected persons (Modahl et al. 1997; Murphy et al. 1997b, 1999) and an increased association with mortality, especially those persons with a high anti-HTLV-1 antibody titer (Arisawa et al. 1998). Higher rates of primary neoplasias have been reported in ATL patients, their siblings, and mothers as compared to HTLV-1-negative non-Hodgkin's lymphoma patients and their respective relatives in Japan (Kozuru et al. 1996). Clinical laboratory findings from asymptomatic HTLV-1 and HTLV-2-infected persons show altered blood chemistries and elevated absolute lymphocyte counts, but these findings were not significant enough to change their clinical evaluation or counseling of otherwise healthy HTLV-infected subjects (Glynn et al. 2000b). Some elderly HTLV-1-infected persons have anergic responses to purified protein derivative (PPD) (Suzuki et al. 1999). The inability to respond to PPD was found to be due in part to their inability to respond to IL-12. However, a more recent report showed that testing middle-aged HTLV-1- and HTLV-2-infected persons demonstrated no anergy to PPD, mumps, or *Candida albicans* antigens (Murphy et al. 2001). The wide spectrum of diseases indicates that further work is necessary to determine the full public health impact of HTLV infection in the general population.

Treatment

To date, therapy for ATL has had limited success. A combination of interferon-alpha (IFN-α) and zidovudine has been effective in a limited number of cases (Gill et al. 1995; Hermine et al. 1995). For persons with HAM/TSP, early disease can be alleviated by corticosteroids, and some relief is seen with zidovudine and danazol (Gout et al. 1991; Harrington et al. 1991). Lamivudine has been shown to decrease HTLV-1 proviral load, but has not been shown to clinically improve HAM/TSP patients who have established disease (Taylor et al. 1999). Despite a recent study showing that

HTLV-1 RT was equally susceptible to zidovudine, zalcitabine, didanosine, and stavudine, but resistant to lamivudine (Garcia-Lerma et al. 2001), lack of sustained clinical remission suggests that alternative treatments need to be found.

Long-term, high dose IFN-α therapy has been shown to reduce HTLV-1 proviral load and improve motor performance in six patients (Yamasaki et al. 1997). However, no randomized trials have been done to compare the effectiveness of these therapies for ATL or HAM/TSP treatment. Arsenic trioxide (As_2O_3), which causes apoptosis of HTLV-1-infected cell lines or fresh ATL cells from patients, has been proposed for the treatment of ATL (Ishitsuka et al. 1998). Its combination with IFN-α appears to have a high synergistic effect in vitro (Bazarbachi et al. 1999). The clinical application of IL-2R-directed therapy, using a humanized monoclonal antibody (or that antibody armed with radionuclides), provides a new perspective for the treatment of autoimmune disorders such as HAM/TSP and certain neoplastic diseases including ATL (Waldmann 1996). Antibodies directed toward both IL-15 and IL-2, or their receptors, inhibit almost completely the proliferation of peripheral blood mononuclear cells (PBMC) from HAM/TSP patients in vitro (Azimi et al. 1999). While these treatments show promise, they still need to be tested in a clinical setting.

Epidemiology

HTLV-1 and HTLV-2 share approximately 60 percent of their nucleotide sequences; yet they have distinct evolutionary and geographical distributions. HTLV-1 infects 15–20 million people worldwide, with endemic foci in southern Japan, the Caribbean, Melanesia, sub-Saharan Africa, and Central and South America (de The and Bomford 1993; Madeleine et al. 1993). The seroprevalence rate ranges from 3–6 percent in the Caribbean islands (Murphy et al. 1991) to approximately 27 percent in southern Japan (Mueller et al. 1996). HTLV-2 is endemic in Amerindian tribes throughout North and South America as well as pygmy tribes in Central Africa with seroprevalences ranging from 3 to 33 percent (Maloney et al. 1992; Hjelle et al. 1994). In the USA and Europe, the seroprevalence for both HTLV-1 and HTLV-2 among low-risk populations is less than 1 percent (Manns et al. 1999a). However, high-risk populations such as IDUs, who are predominately HTLV-2 infected, have a seroprevalence ranging from 0.4 to 20 percent (Kaplan and Khabbaz 1993).

HTLV-1 and HTLV-2 infections are spread through sexual, vertical, and parenteral transmission. Sexual transmission of HTLV-1 has been shown to primarily occur from male to female; however, female-to-male transmission does occur, albeit at a lower efficiency (Murphy et al. 1989; Stuver et al. 1993; Sullivan et al. 1993). Sexual transmission has been associated with older male partners, length of relationship, high anti-HTLV antibody titers, and high proviral load (Stuver et al. 1993; Kaplan et al. 1996). Less is known about the sexual acquisition of HTLV-2; however, in an HTLV-2 endemic area, there was a strong concordance of seropositivity between spouses (Vitek et al. 1995). The role of other sexually transmitted diseases in the spread of HTLV-1 or HTLV-2 is less clear. Persons engaging in high-risk sexual relationships have been shown to have a higher seroprevalence of HTLV-1 (Murphy et al. 1989; Wiktor et al. 1990; Nakashima et al. 1995). A history of having both ulcerative and nonulcerative sexually transmitted diseases has been associated with a higher prevalence of antibodies to HTLV-1, suggesting that they may affect HTLV-1 transmission (Murphy et al. 1989; Nakashima et al. 1995). Recently, cervicitis was shown to be associated with cervical shedding of HTLV-1 and thus may increase HTLV transmission (Zunt et al. 2002). More work is needed to fully address the role of sexually transmitted diseases in the sexual transmission of HTLV-1 and HTLV-2.

Vertical transmission, also known as mother-to-child transmission, primarily occurs through postnatal breastfeeding. HTLV-1 and HTLV-2-infected lymphocytes have been isolated from breast milk (Kinoshita et al. 1984; Heneine et al. 1992b). Mothers that have been shown to transmit HTLV to their children have higher viral antigen-producing capacity from isolated breast milk mononuclear cells as well as proviral load (Yoshinaga et al. 1995; Ureta-Vidal et al. 1999). Additional maternal factors that are important for transmission are high titer of HTLV antibodies (Hino et al. 1987; Hirata et al. 1992), and breastfeeding for more than 12 months (Takahashi et al. 1991; Wiktor et al. 1993; Hino et al. 1996). It was shown that reducing the length of breastfeeding significantly reduces the transmission of HTLV-1 (Takahashi et al. 1991; Wiktor et al. 1993). While the majority of vertical transmission occurs through breast milk, there is a minor population of children that becomes infected even if not breastfed. In utero transmission has been proposed as a secondary route for mother-to-child transmission. Several groups inconsistently found HTLV-1 proviral DNA in cord blood mononuclear cells from the HTLV-1-infected children, suggesting that in utero infection may not be a major route of transmission in children who were not breast-fed (Kawase et al. 1992; Katamine et al. 1994; Nyambi et al. 1996).

Transmission via saliva is considered possible but studies presenting direct evidence for this mode of transmission have not been published. HTLV-1 proviral DNA has been detected in saliva or mouthwashes from ATL (Taniguchi et al. 1993) and HAM/TSP patients (Soto-Ramirez et al. 1995; Achiron et al. 1996; Offen et al. 1998), and asymptomatic HTLV-infected carriers (Yamamoto et al. 1995; Achiron et al. 1996; Belec et al. 1996; Offen et al. 1998). Antibodies against HTLV-1 are

also present in saliva (Archibald et al. 1987; Soto-Ramirez et al. 1995; Yamamoto et al. 1995). The association of the presence of viral RNA and local IgA in saliva has been thought to provide evidence that viral replication may occur in the oral cavity of HAM/TSP patients and some asymptomatic carriers (Soto-Ramirez et al. 1995). However, the neutralizing effect of saliva IgG on syncytium formation in vitro and the saliva's ability, even in the absence of antibodies, to inhibit cell-to-cell transmission of HTLV-1 in vitro demonstrate that transmission through the saliva, if it occurs at all, would be rare (Yamamoto et al. 1995).

Injecting drug use and sex with IDUs are the most important risk factors for HTLV-2 transmission (Kaplan and Khabbaz 1993). Because of the high risk of transfusion-related transmission of HTLV-1 and HTLV-2, screening of volunteer blood donors has been implemented in Japan, several Caribbean countries, various European countries, and the USA and Canada (Whyte 1997; Courouce et al. 1999; Glynn et al. 2000a). The effectiveness of these screening programs is manifested by reduced risk of transfusion-related transmission. In the USA, the incidence rates of seroconversion associated with HTLV in blood donors is estimated to be 1.59 per 100 000 persons per year (Glynn et al. 2000a), and the residual risk of transmitting HTLV infection by transfusing screened blood is estimated to be 1 in 641 000 (Schreiber et al. 1996).

Laboratory diagnosis

The most common assays used for screening persons detect antibodies in serum or plasma. However, such assays detect only past exposure to the viruses. Direct detection of HTLV-1 and HTLV-2 is achieved through the use of nucleic acid assays such as the polymerase chain reaction (PCR).

SEROLOGICAL DETECTION

The immunodominant regions of structural proteins and regulatory gene regions have been well characterized (Lal 1996) and are the basis for the development of diagnostic assays both for detection and differentiation of HTLV-1 and HTLV-2. Typically, an enzyme immunoassay (EIA) is used as the primary screening assay, and western blot (WB) is used as a confirmatory test. Particle agglutination assays, which are based on the agglutination of HTLV-1 lysate-coated gelatin particles by antibodies in a test specimen, are also used in various countries as screening assays.

The EIA is a colorimetric test that is both sensitive and simple to perform and generally now uses purified HTLV-1 and HTLV-2-infected cell lysates and/or HTLV-1 and HTLV-2 recombinant antigens or synthetic peptides. The addition of HTLV-2 antigens to test kits significantly improved the detection of antibodies to HTLV-2, compared with results using kits that contained only HTLV-1 antigens (Andersson et al. 1999; Liu et al. 1999; Poiesz et al. 2000). Comparative analysis of various commercial screening assays containing both HTLV-1 and HTLV-2 antigens indicates that sensitivity ranges from 98.9 to 100 percent for confirmed HTLV-1-positive specimens and 91.5 to 100 percent for confirmed HTLV-2 positive specimens. Specificity ranges from 90.2 to 100 percent for both infections (Andersson et al. 1999; Poiesz et al. 2000). These assays are used for both blood donor screening and as an aid in clinical diagnosis of HTLV-1 and HTLV-2 infection and related diseases. However, the EIAs cannot differentiate between infection with HTLV-1 and HTLV-2 because of the significant homology in structural proteins between the two viruses (Courouce et al. 1999). To serologically differentiate between these viruses, WB is used.

Although WB assays using purified viral lysates are highly sensitive for detecting $p24^{gag}$ antibodies, they do not always detect antibodies to native envelope glycoproteins. Several approaches have addressed this issue in second-generation WB assays. In one, viral lysates have been spiked with recombinant p21e protein (p21e). Antibodies to p21e are detected more easily compared to those to $gp46^{env}$; however, some false-positive specimens have been identified by this test (Lal et al. 1992a; Busch et al. 1994; Kleinman et al. 1994). A second approach is a modified WB that contains type-specific $gp46^{env}$ recombinant proteins from HTLV-1 and HTLV-2, as well as a truncated form of p21e (gd21) that reduces nonspecific reactivity (Varma et al. 1995) (Figure 3). This assay not only serves as a confirmatory serological test but also permits the differentiation of infection between HTLV-1 and HTLV-2 (Varma et al. 1995). The modified WB has improved specificity and is sensitive for most subtypes of HTLV-1 and HTLV-2 (Varma et al. 1995). Specimens with reactivity to $p24^{gag}$ and p21e, but no reactivity to either $rgp46^{I}$ or $rgp46^{II}$, are referred to as HTLV-positive untypeable. PCR analysis of these untypeable specimens has identified the presence of HTLV-specific sequences (Varma et al. 1995). In rare instances, specimens with confirmed HTLV infection have reactivity to $p19^{gag}$ (in the absence of p24) and p21e (Cesaire et al. 1999; Rouet et al. 2001). Likewise, in some instances, antibody to p21e may represent an early antibody response during the 6-month seroconversion window period, and individuals with such reactivity should be retested in 6 months (Manns et al. 1992). It is the current policy of the US Public Health Service to recommend that the diagnostic criteria for WB tests require that a specimen demonstrating antibodies to $p24^{gag}$ and to native $gp46^{env}$ and/or recombinant p21e are considered seropositive for HTLV-1 or HTLV-2 (CDC and the U.S.P.H.S. Working Group 1993). Specimens reacting with any of the bands, but not satisfying the above criteria, are designated indetermi-

nate. Specimens with no immunoreactivity to any bands are considered negative for antibodies to HTLV-1 and HTLV-2 (false-positive EIA specimens).

Both of the WB assays can give indeterminate results or show immunoreactivity to a single HTLV gene product, conditions which do not meet the criteria of seropositivity (Busch et al. 1994; Varma et al. 1995). Antibody to only Gag proteins (p24, p19) is the most common indeterminate pattern that is observed in EIA-reactive specimens, and extensive PCR analysis using primers to detect multiple gene regions have failed to detect HTLV-1 or HTLV-2 proviral sequences in these seroindeterminate persons thus indicating that these individuals are not likely to be infected with HTLV-1 or HTLV-2 (Lal et al. 1992b; Medrano et al. 1997; Cesaire et al. 1999; Rouet et al. 2001). Limited studies have also established that individuals with indeterminate WB profiles generally do not have risk factors for HTLV infection (Mauclere et al. 1997; Medrano et al. 1997; Cesaire et al. 1999; Rouet et al. 2001). Such indeterminate WB results appear to represent antibodies to different viral and cellular antigens that cross-react with HTLV proteins (Mahieux et al. 2000). However, recent data suggest that a small number of patients with chronic progressive neurological disease and HTLV-indeterminate WB patterns may be due to infection with a defective HTLV or have HTLV-1 in low copy numbers (Soldan et al. 1999; Waziri et al. 2000). The possibility that such indeterminate WB results may represent a novel retrovirus with partial homology to HTLV has also been explored; however, no amplification was observed using generic PCR primers that would amplify HTLV-related viruses (Vandamme et al. 1997; Busch et al. 2000).

NUCLEIC ACID DETECTION

Amplification of proviral DNA by PCR is the preferred method for determining infection status, testing the validity of serological assays, distinguishing between HTLV-1 and HTLV-2, and studying the in vivo tissue distribution. These qualitative and quantitative PCR techniques can be used to identify HTLV infections for subtyping purposes or for determining viral load.

Two quantitative PCR procedures have been successfully used on PBMCs to confirm and differentiate between HTLV-1 and HTLV-2 infections. The first uses HTLV consensus primers that allow amplification of both viruses; typing is achieved either by hybridizing the product to an HTLV-1-specific or HTLV-2-specific probe (Heneine et al. 1992a) (Figure 4) or by specific restriction digestion patterns (Tuke et al. 1992). A second approach employs type-specific primers and probes in separate amplifications (Heneine et al. 1992a). The PCR products can be detected with labeled internal probes by Southern blot hybridization. A solid-phase, nonradioactive, EIA-based detection system has been

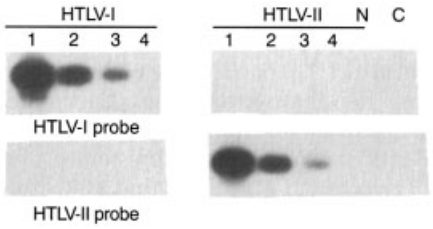

Figure 4 *Generic PCR amplification of HTLV-I and HTLV-II by SK110/SK111 and differentiation by type-specific probing. Numbers 1, 2, 3 and 4 refer to lysates of uninfected Hut-78 cells containing 1500, 150, 2 and 0 HTLV-I infected (MT-2) or HTLV-II (MO-T) cells, respectively. C, reagent cocktail control; N, Hut-78. The HTLV-I specific probe is SK112, and the HTLV-II probe is SK188.*

developed which seems highly sensitive and specific for detection and differentiation of both HTLV-1 and HTLV-2 (Dyster et al. 1994; Dezzutti et al. 1996).

Multiplex DNA PCR is a semiquantitative technique used for the simultaneous amplification, detection, and discrimination of different target sequences in one tube. In addition to primers for HTLV-1/HTLV-2 detection, primers for HIV-1/HIV-2 detection are also included. Primers for the *gag* region of HIV-1, *env* region of HIV-2, *pol* region of HTLV-1, and *Tax* of HTLV-2 are used to provide specific and sensitive coamplification of proviral sequences (Heredia et al. 1996; Vet et al. 1999). Multiplex PCR can detect fewer than ten proviral copies for each virus per sample and thus can provide an accurate measure of the number of copies of each proviral sequence that were originally present in the sample.

Additional quantitative nucleic acid assays have been developed to determine proviral DNA load. These assays include nested PCR with limiting dilution (Tosswill et al. 1998), quantitative competitive PCR (Albrecht et al. 1998), and real-time PCR (Kamihira et al. 2000; Miley et al. 2000). Primers have been designed for the *gag*, *tax*, or *pol* genes, and all are highly sensitive.

VIRUS ISOLATION, ANTIGEN DETECTION, AND REVERSE TRANSCRIPTASE ASSAYS

Viral isolation of HTLV-1 or HTLV-2 has been difficult due to the cell-associated nature of this virus. Nevertheless, cocultivation of HTLV-1- and HTLV-2-infected PBMCs with activated, allogeneic HTLV-negative PBMCs is used to obtain viral isolates. The culture supernatants are collected weekly, for up to 4 weeks, and the presence of $p19^{gag}$ antigen in the supernatant is tested using an antigen-capture EIA. Alternatively, detection of viral replication in cultures supernatants is accomplished by detecting HTLV reverse transcriptase activity using an amplified reverse transcriptase (AMP-RT) assay (Yamamoto et al. 1996). This assay was shown to detect the presence of HTLV-1 and HTLV-2 in culture supernatants that were not detected by EIA.

ZOONOTIC RETROVIRUS INFECTIONS

The identification of isolated infections with either simian immunodeficiency virus (SIV) or simian foamy virus (SFV) in occupationally exposed workers suggested that occupational contact with nonhuman primates (NHP) may be associated with risks of transmission of simian retroviruses (Khabbaz et al. 1992, 1994; Schweizer et al. 1995, 1997). The recognition that human HIV-1 and HIV-2 infections originated from simian-to-human transmission of SIV has heightened concerns of cross-species transmission of animal retroviruses. Such transmissions could occur in various settings: occupational exposures to NHPs (e.g. in biomedical research), exposures to NHPs as pets or in zoological gardens, or in occasions when NHP organs have been used in xenotransplantation. In addition to SIV and SFV, NHP are natural hosts to several exogenous retroviruses including simian T-lymphotropic viruses (STLV) and simian type D viruses (SRV) (Lowenstine and Lerche 1988).

Foamy viruses (FV), including SFV, belong to the *Spumavirus* genus in the family *Retroviridae*. FVs cause cytopathic effects with syncytium formation in vitro, but appear to be nonpathogenic in vivo. They establish persistent infections in many animal species, including nonhuman primates, cats, cows, and sea lions. SFVs infect more than 70 percent of captive nonhuman primates (Hooks and Gibbs 1975; Neumann-Haefelin et al. 1993; Schweizer et al. 1995; Meiering and Linial 2001), in which they appear to cause latent infection in almost all organs – spleen, liver, kidney, salivary gland, brain (Swack and Hsiung 1975) – and can be readily detected by PCR in PBMCs. Minimal viral replication appears to occur in the oral mucosa (Falcone et al. 1999).

Human infection with SFV

A foamy virus referred to as human foamy virus (HFV), now called prototype foamy virus (PFV), was isolated from a nasopharyngeal carcinoma specimen from a Kenyan man (Achong et al. 1971). Following its detection, seroepidemiologic studies in human populations have shown controversial results. Most studies did not find antibodies to PFV even in populations exposed to NHPs (Brown et al. 1978; Nemo et al. 1978; Schweizer et al. 1995). Some earlier serological studies reported seropositivity among certain populations from 6.9 percent among Pacific Islanders (Loh et al. 1980) to 17 percent in Kenyans (Achong and Epstein 1978) but later studies using confirmatory testing could not confirm PFV antibody presence even in sera highly reactive by enzyme-linked immunosorbent assay (ELISA) (Ali et al. 1996; Heneine et al. 2003). Most of the earlier studies did not always use adequate methods to control for false-positive results or additional confirmatory methods were not available at that time (Heneine et al. 2003).

The failure to find antibodies to PFV in sera from 250 persons, including 50 with nasopharyngeal carcinoma and Burkitt's lymphoma (Brown et al. 1978), the cross-reactivity of PFV with antibodies to SFV (Nemo et al. 1978), and sequence analyses all suggested that the PFV was most likely of chimpanzee origin (Herchenroder et al. 1994).

Recent studies, using WB for detection of antibodies and confirmatory testing for viral DNA, have documented seropositivity to SFV in 19 persons from Germany, the USA, and Canada (Schweizer et al. 1995, 1997; Heneine et al. 1998; Sandstrom et al. 2000; Brooks et al. 2002; Switzer et al. 2004). All of them have been occupationally exposed to nonhuman primates, their biological fluids, or to simian viruses in the laboratory. Sequence analysis confirmed that SFV infection has originated from baboons, chimpanzees, macaques, and African green monkeys (Heneine et al. 1998) (Figure 5). A study of Cameroonians who reported contact with nonhuman primates through hunting and butchering demonstrated SFV infection in natural settings (Wolfe et al. 2004). Ten of 1 099 (1 percent) persons had SFV WB positive results. Three WB-positive persons were also PCR-positive. Sequence analysis indicated that SFV was acquired from a mandrill, gorilla, and a DeBrazza guenon.

Characteristics of human infection with foamy viruses

Early studies raised questions about possible associations between PFV infection and a variety of human diseases (e.g. thyroiditis, amyotrophic lateral sclerosis, hemodialysis encephalopathy) (Cameron et al. 1978; Werner and Gelderblom 1979; Lagaye et al. 1992; Westphal et al. 1992; Stancek et al. 1995). Later studies, using more precise diagnostic methods, did not confirm such associations or endemic human infection (Heneine 1995; Schweizer et al. 1995, 1997; Ali et al. 1996; Rosener et al. 1996; Winkler et al. 2000).

Humans infected with SFV show persistent seropositivity by WB; testing of stored sera revealed that some persons have been seropositive for more than 20 years (Heneine et al. 1998; Switzer et al. 2004). The identification, by PCR, of two conserved SFV proviral DNA sequences in PBMCs confirms the persistent nature of the SFV infection (Heneine et al. 1998).

Although PFV can cause transient immunosuppression in experimentally infected mice and rabbits (Santillana-Hayat et al. 1993), currently there is no evidence that SFVs are pathogenic in their natural host (Weiss 1996; Linial 2000). Available data on the SFV-infected persons show that no obvious disease can be attributed to these infections at the time of their detec-

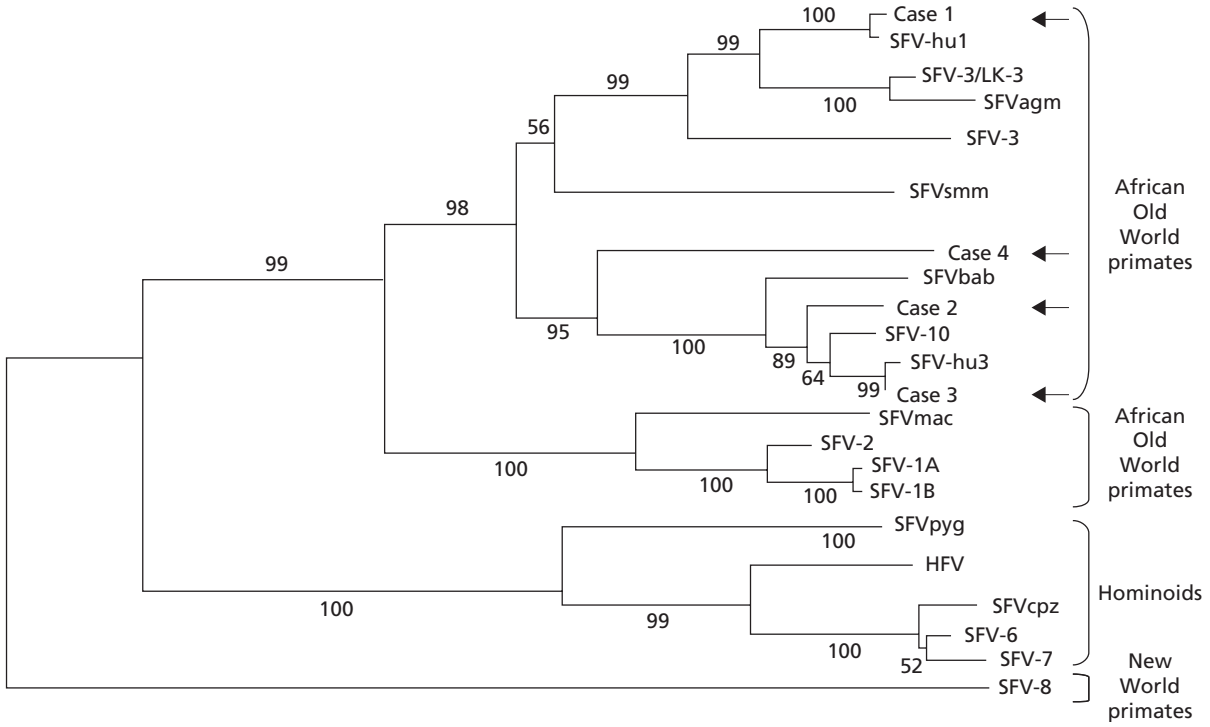

Figure 5 *NHP species origin of SFV infections in workers occupationally exposed to NHPs. Phylogenetic analysis by neighbor-joining of SFV* pol *sequences derived from peripheral blood lymphocytes of four case workers and from SFVhu1 and SFVh3, two SFV isolates obtained from case 1 and case 3, respectively. SFV sequences of different NHP species shown are SFVagm, SFVsmm, SFVbab, SFVmac, SFVpyg, obtained from African green monkey, sooty mangabey, baboon, and pygmy chimpanzee, respectively. SFVcpz and SFV-6 and SFV-7, from chimpanzees, SFV-8 from spider monkey (a New World monkey species). Numbers shown at branch nodes represent bootstrap percent values.*

tion (Schweizer et al. 1995, 1997; Heneine et al. 1998; Sandstrom et al. 2000; Brooks et al. 2002). Because FV are considered nonpathogenic and because they can infect a broad range of human cells and human cell lines in the laboratory (Meiering and Linial 2001), they are considered as potential candidates for gene transfer therapy (Bieniasz et al. 1997; Hill et al. 1999).

HTLV infections are an example that retroviruses may cause a disease in a small proportion of infected individuals and after a long latent period. To better characterize the course of SFV infection in humans, the US Centers for Disease Control and Prevention (CDC) has initiated a long-term follow-up of SFV-infected persons.

Human-to-human transmission

No evidence of male-to-female transmission in wives of six SFV-infected workers was found, suggesting that transmission of SFV among humans by sexual or less intimate contact may not occur easily (Schweizer et al. 1997; Heneine et al. 1998; Switzer et al. 2004). Four recipients of blood components from one SFV-infected donor were tested 1.9 to 7 years after transfusion and are not infected (Boneva et al. 2002). Although preliminary, these data suggest that secondary SFV transmission among humans appears unlikely and humans may be a dead-end host of the SFV infection – as is often the case with zoonoses. However, a larger number of SFV-infected persons and their contacts need to be followed for a longer period of time to confirm lack of human-to-human transmission and benign nature of the SFV infection in humans.

Other animal retroviruses

The observation that SFV appears to be readily transmissible to humans raises concerns that human contact with other domestic animals such as cats, all of which are known to harbor FV and other retroviruses (Tobaly-Tapiero et al. 2001), may be accompanied with risk of zoonosis. A study of persons occupationally exposed to cats by Butera et al. (Butera et al. 2000) found no evidence of infection with either feline foamy virus (FFV), feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV) in 203 North American feline veterinary practitioners. Persons studied reported extensive duration of work with cats and multiple high-risk exposures. Similarly, Winkler et al. (1999) found no evidence of FFV zoonosis among Australian veterinarians, although exposure to felines were not clearly defined in that study.

XENOTRANSPLANTATION AND OTHER IATROGENIC RISKS OF INFECTION WITH ANIMAL RETROVIRUSES

Particular attention has been given to risks of transfer of animal retroviruses to humans through xenotransplantation – the use of live animal tissues or cells as xenografts. Interest in xenotransplantation has increased because of its potential to provide a solution for the chronic shortage of allotransplants or to provide porcine pancreatic islet cells for treatment of diabetes, or neuron cells for treatment of Parkinson's disease. Organs from NHPs have been used with some success in the past but currently pigs are the preferred source of animal xenografts. Both NHPs and pigs harbor endogenous retroviruses, which cannot be eliminated from source animals by pretransplant screening because their DNA is part of the cell's DNA. For example, porcine endogenous retroviruses (PoEV) and baboon endogenous viruses (BaEV) can infect human cells and have been extensively studied recently as potential causes of zoonotic infections in relation to xenotransplantation. SFV and BaEV sequences were detected in multiple tissue compartments of two human recipients of baboon livers (Allan et al. 1998). The presence of baboon mitochondrial DNA was also detected in these same tissues, suggesting that xenogeneic 'passenger leukocytes' harboring latent or active viral infections had migrated from the xenografts to distant sites within the human recipients. The persistence of SFV and BaEV in human recipients in the post-transplant period reveals the potential infectious risks associated with xenotransplantation (Allan et al. 1998). More than 200 persons exposed to various porcine xenografts have been tested for PoEV without evidence for infection (Herring et al. 2001). In one of the studies, about 23 percent of persons exposed to live porcine tissue had proviral sequences detected in quantities consistent with microchimerism but not infection (Paradis et al. 1999). PoEV has also been found as a contaminant of porcine factor VIII concentrates, which are used to treat hemophiliacs (Heneine et al. 2001); however, there was no evidence for PoEV infection in 88 recipients of this product (Heneine et al. 2001).

The identification of RT activity in chick-cell-derived vaccines including measles, mumps, and yellow fever vaccines has led to studies to identify the source of the RT activity and assess the risks it poses to vaccine recipients (Boni et al. 1996; Tsang et al. 1999). These investigations indicated that the RT activity in the measles, mumps, and rubella (MMR) vaccines and in yellow fever (YF) vaccines was found to be associated with viral particles containing RNA from the endogenous avian leukosis virus subgoup E (ALV-E) and the endogenous avian virus (EAV) (Weissmahr et al. 1997; Tsang et al. 1999; Johnson and Heneine 2001; Hussain et al. 2003). Both ALV-E and EAV originate from the chick embryonic fibroblasts used to propagate the measles and mumps vaccines, or from the chick embryos used for YF vaccine production. The detection of RT activity in all vaccine lots suggested that vaccine recipients might be universally exposed to these endogenous chicken retroviral particles. However, testing of MMR and YF vaccine recipients has thus far revealed no evidence of transmission of ALV-E or EAV to vaccine recipients. No antibody reactivity to either ALV-E or EAV was found in 206 US recipients of MMR vaccine and in 43 Brazilian recipients of YF vaccine (Hussain et al. 2001; Johnson and Heneine 2001). No ALV-E and EAV sequences were also detected by PCR in serum and peripheral blood lymphocyte samples from these vaccinees (Hussain et al. 2001; Johnson and Heneine 2001). While current data continue to support the use of chick-cell-derived MMR and YF vaccines, the presence of adventitious avian retroviral particles in these vaccines has raised questions about the suitability of primary chicken cell substrates for vaccine production.

Using retroviral vectors such as the Moloney murine leukemia virus for retrovirally mediated gene transfer has proven successful in treating certain genetic conditions. However, the recently reported lymphoproliferative disease in two of ten children treated for X-linked severe combined immunodeficiency demonstrates the risks that may be associated with retroviral integration and insertional mutagenesis (Hacein-Bey-Abina et al. 2003).

RETROVIRUSES, CANCER, AND DISEASES OF UNKNOWN ETIOLOGY

Interestingly, the literature is replete with studies showing the associations of cancer with retroviruses in animals. With the exception of the HTLV, humans are somewhat void of such associations. Our genome, on the other hand, contains remnants of retroviral elements (as endogenous retroviruses), suggesting that during our evolution we encountered and symbiotically associated with these viruses, which now provides a resistance to retroviral-mediated cancers. Retroviruses and/or retroviral components have been investigated for years as the putative agent in other diseases of unknown etiology. Numerous papers attempt to assign a retrovirus, its insertion, or gene product to the cause and association with many diseases such as multiple sclerosis, osteoarthritis, systemic lupus erythematosus, Sjögren's disease, Graves' disease, just to name a few. It has been neither the intent nor the aim of this chapter to refute those claims here, but rather to present the most scientifically accepted associations of retroviruses with human disease and infection. Retroviruses are constantly emerging and their ability to zoonotically transmit, recombine, mutate,

and 'trap' other genes makes them a constant concern for the stewards of public health.

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Foamy viruses

AXEL RETHWILM

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INTRODUCTION

The family of *Retroviridae* is taxonomically divided into two subfamilies: the *Orthoretrovirinae* and the *Spumaretrovirinae,* the latter are better known as foamy viruses (FV) (Table 1). The separation of the previously homogenous group of retroviruses into the two subfamilies by the International Committee of the Taxonomy of Viruses (ICTV) reflected the molecular biology of FVs and their replication pathway, which appears to be significantly different from all other retroviruses, and have common aspects with the replication strategy of hepadnaviruses (Linial 1999; Lecellier and Saïb 2000; Rethwilm 2003b; Flint et al. 2004). In this chapter, these findings will be reviewed and the general biology of FVs will be summarized.

NATURAL HISTORY AND GENERAL PROPERTIES OF FV

FVs have been known for more than 50 years. After long term cultivation of primary monkey cells a typical foamy cytopathic effect (CPE) emerged (Figure 1) that could be transferred in a cell-free manner to uninfected cell cultures (Enders and Peebles 1954; Rustigian et al. 1955; Lepine and Paccaud 1957; Paccaud 1957; Falke 1958; Plummer 1962). Following the detection of a reverse transcriptase activity in gradient-purified virions, FV were classified among the *Retroviridae* (Parks et al. 1971). Curiously the question of the molecular basis of the FV-specific foamy degeneration of cells has not been addressed yet.

FVs have been found in the mammalian species which also naturally harbor lentiviruses, namely nonhuman primates (Hooks and Gibbs 1975; Swack 1981), felines (Lutz 1990; Pedersen 1990), bovines (Johnson et al. 1988; Amborski et al. 1989), equines (Tobaly-Tapiero et al. 2000), ovines (Flanagan 1992), and occasionally others (Hruska and Takemoto 1975; Rossignol et al. 1975; Kennedy-Stoskopf et al. 1986; Amborski et al. 1987; Meiering and Linial 2001; Saïb 2003). An overview is shown in Table 2. In particular, FVs from monkeys and great apes have been studied since the early years of FV research (Hooks and Gibbs 1975). It is a common experience that these simian foamy viruses (SFV) can influence studies on other simian (retro-) viruses, such as the immunodeficiency viruses, by reactivation from latency and destroying primary cell cultures (Voss et al. 1992; Feldmann et al. 1997).

Virus is shed in the saliva and can be easily isolated by throat swabs from infected monkeys and cats (Hooks and Gibbs 1975; Shroyer and Shalaby 1978; Alke et al. 2000). Transmission in monkeys is believed to occur by social contacts involving biting during juvenility (Meiering and Linial 2001; Falcone et al. 2003). After infection, the virus persists lifelong and can be isolated from many tissues (Hooks and Gibbs 1975; Falcone et al. 1999a; Falcone et al. 2003). Viral DNA at approximately one copy number per 10^2–10^3 cells can be found in most organs of perfused animals, although cellfree virus has not been detected in the plasma (Falcone et al. 1999a;

Table 1 *The ICTV nomenclature of the* Retroviridae

Subfamily	Genus	Example	Genome
Orthoretrovirinae	*Alpharetrovirus*	Avian leukosis virus (ALV)	Simple
	Betaretrovirus	Mouse mammary tumor virus (MMTV), Mason–Pfizer monkey virus (MPMV)	Simple
	Gammaretrovirus	Murine leukemia virus (MuLV)	Simple
	Deltaretrovirus	Bovine leukemia virus (BLV), Human T-cell leukemia virus (HTLV)	Complex
	Epsilonretrovirus	Walleye dermal sarcoma virus (WDSV)	Complex
	Lentivirus	Human immunodeficiency virus (HIV)	Complex
Spumaretrovirinae	Foamyviruses	Prototype foamy virus (PFV)	Complex

Falcone et al. 2003). The conservation of viral sequences over time is also suggestive of poor replication in vivo (Schweizer et al. 1999). Despite the prominent CPE FVs exhibit in adherent cell cultures, the in vivo infection is nonpathogenic (Linial 2000; Meiering and Linial 2001; Falcone et al. 2003). Continuous virus isolations by throat swabs and from peripheral blood lymphocytes (PBL) many years after primary infection indicate virus persistence (Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981; Meiering and Linial 2001; Falcone et al. 2003). More recently, the sequences of bovine foamy virus (BFV), feline foamy virus (FFV), and equine foamy virus (EFV) have also become available (Renshaw et al. 1991; Renshaw and Casey 1994a, 1994b; Helps and Harbour 1997; Winkler et al. 1997a; Tobaly-Tapiero et al. 2000; Hatama et al. 2001) and studies into the molecular biology, epidemiology, and persistence of FFV were performed (Winkler et al. 1998; Winkler et al. 1999; Alke et al. 2000; Hatama et al. 2001; Roy et al. 2003; Bodem et al. 2004).

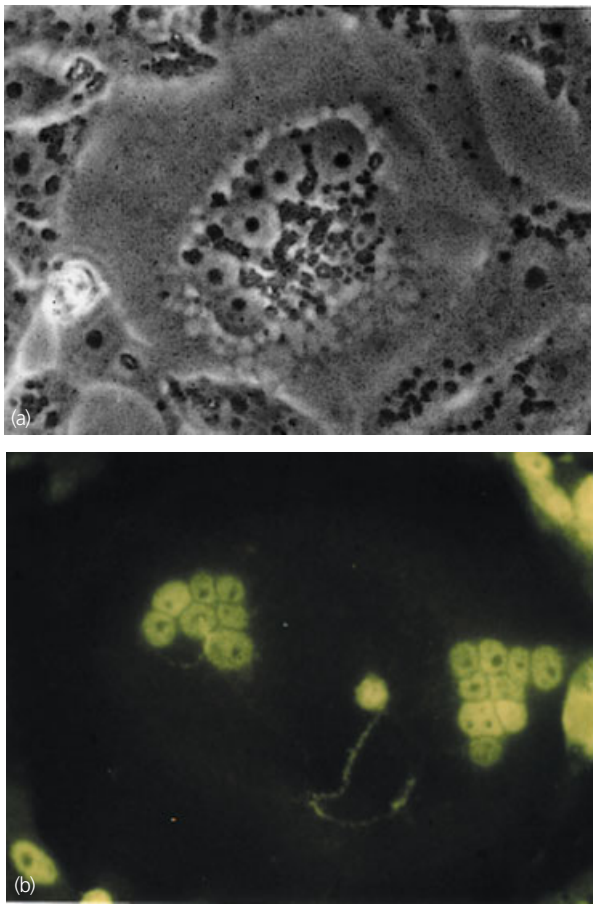

Figure 1 (a) *Micrograph of a typical FV cytopathic effect with the formation of multinucleated syncytia and vacuolation. SFVagm-infected Vero cell culture.* **(b)** *Nuclear staining in indirect immunofluorescence of SFVagm-infected cells with a SFVagm-positive monkey serum.*

In particular, the analysis of cats which were co-infected by FFV and feline immunodeficiency virus (FIV) did not reveal FFV-related diseases, which were triggered by FIV-induced immunosuppression (Zenger et al. 1993). Furthermore, the artificial immunosuppression in a limited number of SFV-infected monkeys by cyclosporin A resulted only in a higher rate of FV isolations by throat swabs, but not in disease that could be attributed to the virus (D. Neumann-Haefelin, personal communication). Similar results on virus isolation frequencies were obtained when analyzing cats co-infected with FFV and FIV (Bandecchi et al. 1992). The infection of laboratory animals, such as mice and rabbits, by FV of primate origin also leads to virus persistence in the absence of recognizable pathogenicity (Swack and Hsiung 1975; Hooks and Detrick-Hooks 1979; Brown et al. 1982; Santillana-Hayat et al. 1993; Saïb et al. 1997a; Schmidt et al. 1997b; Falcone et al. 2003).

Since human immunodeficiency virus (HIV) arose by trans-species transmissions from nonhuman primates to men (Hahn et al. 2000; Sharp et al. 2001; Peeters et al. 2002), much effort has been undertaken in identifying genuine human FV infections and a potential linkage with human diseases of a suspected infectious origin. In particular, neurological diseases were investigated after the discovery that mice transgenic for FV genes developed brain pathology (Bothe et al. 1991; Aguzzi et al. 1992; Aguzzi et al. 1993). Taking together the results from several studies, in which populations potentially at risk of contracting retroviral infections

Table 2 *Foamy virus isolates*[a]

Host species	Isolate	Key references
Macaque (*M. cyclopsis*, *M. mulatta*, *M. fascicularis*)	SFVmac	(Johnston 1961; Kupiec et al. 1991)
African green monkey (*C. aethiops*)	SFVagm	(Stiles et al. 1964)
Squirrel monkey (*S. sciureus*)	SFVsqu	(Johnston 1971)
Galago (*G. crass. panganiensis*)	SFVgal	(Johnston 1971)
Chimpanzee (*P. troglodytes* spp.)	SFVcpz	(Rogers et al. 1967; Hooks et al. 1972; Herchenröder et al. 1994)
Spider monkey (*Ateles* spp.)	SFVspm	(Hooks et al. 1973)
Capuchin (*Cebus* spp.)	SFVcap	(Hooks and Gibbs 1975)
Baboon (*P. cynocephalus*)	SFVbab	(Rhodes-Feuillette et al. 1979)
Orangutan (*P. pygmaeus*)	SFVora	(McClure et al. 1994; Verschoor et al. 2003)
Gorilla (*G. gorilla* sp.)	SFVgor	(Bieniasz et al. 1995a)
Marmoset (*C. jacchus*)	SFVmar	(Marczynska et al. 1981)
Felines	FFV	(Riggs et al. 1969; Helps and Harbour 1997; Winkler et al. 1997a)
Bovines	BFV	(Malmquist et al. 1969; Renshaw et al. 1991)
Equines	EFV	(Tobaly-Tapiero et al. 2000)

a) Modified from Meiering and Linial (2001); Falcone et al. (2003).

were screened for FV antibodies, humans do not appear to be a natural reservoir of FV (Hunsmann et al. 1990; Landay et al. 1991; Flügel et al. 1992; Folks et al. 1993; Heneine et al. 1994; Pyykkö et al. 1994; Schweizer et al. 1994; Simonsen et al. 1994; Heneine et al. 1995; Schweizer et al. 1995; Ali et al. 1996; Rösener et al. 1996).

However, additional studies identified a small population of FV-infected humans who were mostly people in contact with nonhuman primates, or material derived from them or African monkey hunters (Schweizer et al. 1997; Heneine et al. 1998; Sandstrom et al. 2000; Switzer et al. 2004; Wolfe et al. 2004). These trans-species infections were never caused by a hypothetical 'human' FV, but could always be traced back to a particular monkey virus (Heneine et al. 2003; Wolfe et al. 2004). Therefore, these isolates are called, for instance, SFVagm(hu) or SFVcpz(hu) to indicate that they were derived from humans zoonotically infected by SFV from African green monkey or chimpanzee, respectively. Virus isolations from FV-infected humans were unsuccessful from throat swabs and required PBL (Heneine et al. 1998; Heneine et al. 2003). Positive results twenty or more years after infection indicate lifelong persistence (Schweizer et al. 1997; Heneine et al. 2003; Switzer et al. 2004). As in their natural hosts, the infections of humans occur in the absence of any pathogenicity (Heneine et al. 2003). Furthermore, so far there is no indication that the virus is transmitted to other humans even by intimate contact or that FV other than those of primate origin can infect humans (Boneva et al. 2002; Heneine et al. 2003; Switzer et al. 2004). Thus humans are likely to represent a dead-end host to primate FV.

The absolute number of humans identified to be infected by SFV is small. A careful follow-up of these cases is required to ensure early recognition of a possible risk of a newly emerging pathogenic human retrovirus. However, given the very slow mutation rate of FV and their general apathogenicity, this scenario appears to be unlikely.

Curiously, the first FV that was molecularly cloned and is still the best-studied virus of this group was initially called 'human' FV, because it was reported to be derived from a human source (Achong et al. 1971; Rethwilm et al. 1987). Following sequencing of chimpanzee FVs, which revealed an almost identical nucleotide sequence (Herchenröder et al. 1994), and the large-scale sero-epidemiological studies with negative results mentioned above, the name 'human' FV was found to be inappropriate and misleading (Rethwilm 2003a). Instead, the name prototypic foamy virus (PFV) for this virus, which most likely is a chimpanzee FV, was coined (Rethwilm 2003a).

FV can infect a very large range of cell cultures (Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981). Pseudotyping experiments of vesicular stomatitis virus (VSV) with PFV envelope indicated that the unknown receptor must be ubiquitous, since even reptile cells could be infected (Hill et al. 1999). FVs make use of a pH-dependent endocytotic entrance pathway, which is vulnerable to various lysosomotropic agents (Picard-Maureau et al. 2003). Despite the ubiquitous presence of the receptor, not all cells support active virus replication. Reasons other than lack of receptor appear to be responsible for this (Meiering et al. 2001). In adherent cells, lytic infection results in cellfree virus titers of around 10^5–10^6 infectious units/ml (Yu and Linial 1993; Schmidt and Rethwilm 1995). Thus, any given cell produces only 0.1–1 cellfree progeny virus. However, the amount of cell-bound virus is approximately ten times higher (Neumann-Haefelin et al. 1983; Yu and Linial

1993). Of particular interest are cell lines of hematopoietic origin, because these cells often do not show the FV-typical giant cell CPE (Mikovits et al. 1996; Yu et al. 1996a). Yet these cells can be persistently infected and virus production is often low in the range of 10^2–10^3 infectious units/ml (Yu et al. 1996a). The virus–cell interaction in lymphoid cell lines may reflect the in vivo situation closer than fibroblastoid cells. In natural infection, lymphocytes probably disseminate the virus in the body. Active virus replication appears to be restricted to a few organs, like the oral mucosa (Falcone et al. 1999a; Falcone et al. 2003). Furthermore, gamma interferon (γ-IFN) secreted by activated lymphocytes is a potent suppressive factor of FVs that restricts virus replication in a species-specific manner (Falcone et al. 1999b). FV replication was shown to induce apoptosis (Ikeda et al. 1997; Mergia et al. 1997). However, this has not been further elucidated.

MOLECULAR BIOLOGY OF THE *SPUMARETROVIRINAE*

Morphology and composition of the FV particle

Electron microscopy studies revealed most of the details of the FV ultrastructure (Gelderblom and Frank 1987). Extracellular infectious particles are approximately 110 nm in diameter (Figure 2). They consist of an immature core of approximately 65 nm consisting of capsid (Gag) protein, which is either uncleaved or processed at the C-terminus where a 3 kD peptide is cleaved by the viral protease (see below). In primate FVs these two moieties are of 71 and 68 kD MW, which are present in around equimolar amounts in the viral particle (Hahn et al. 1994; Fischer et al. 1998). The capsid is surrounded by an envelope, in which the prominent viral glycoproteins (Env) are embedded in a trimeric arrangement (Wilk et al. 2000). In PFV the precursor of Env is of apparent 130 kD MW. Cellular proteases cleave this protein into three subunits, the 18-kDa leader peptide ($gp18^{LP}$), an around 80-kDa surface protein ($gp80^{SU}$), and the 48-kDa transmembrane subunit ($gp48^{TM}$). All three proteins are integral parts of the virion (Lindemann et al. 2001). At least in PFV another glycoprotein ($gp170^{Env\text{-}Bet}$) can be found at approximately 50 percent of the amount of $gp130^{Env}$ (Giron et al. 1998; Lindemann and Rethwilm 1998). The $gp170^{Env\text{-}Bet}$ which is only loosely, if at all, associated with the virion appears not to be required for in vitro replication (Lindemann and Rethwilm 1998). It is generated from an alternatively spliced mRNA (Giron et al. 1998; Lindemann and Rethwilm 1998) which consists of the majority of the env open reading frame (ORF) fused to the bet ORF (see below). The polymerase (pol) ORF is expressed as a 127-kDa apparent MW precursor which is cleaved by the viral protease into the 85-kDa protease-reverse transcriptase/RNase H (PR-RT/RN) and the 41-kDa integrase (IN). All three proteins are found in virions (Netzer et al. 1993; Pfrepper et al. 1998). The molar ratio of Gag to Pol proteins approximates 15:1 (M. Cartellieri and A. Rethwilm, unpublished observation).

The genomes of FVs have been studied in detail for PFV and FFV (Moebes et al. 1997; Yu et al. 1999; Roy

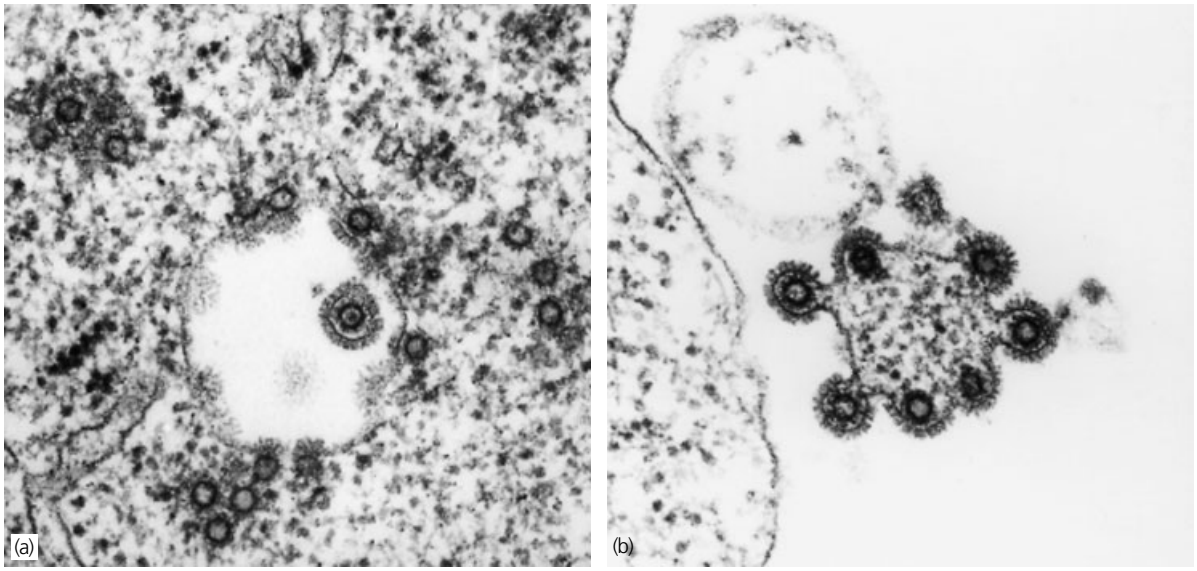

Figure 2 (a) *Electron micrograph of intracellular capsids, particles budding into intracellular compartments, and mature foamy virus.* **(b)** *Electron micrograph of budding foamy viruses revealing the prominent envelope glycoprotein spikes. The magnification in A and B is 96 000-fold. The pictures are a courtesy of Hanswalter Zentgraf (Heidelberg, Germany).* *(Continued over)*

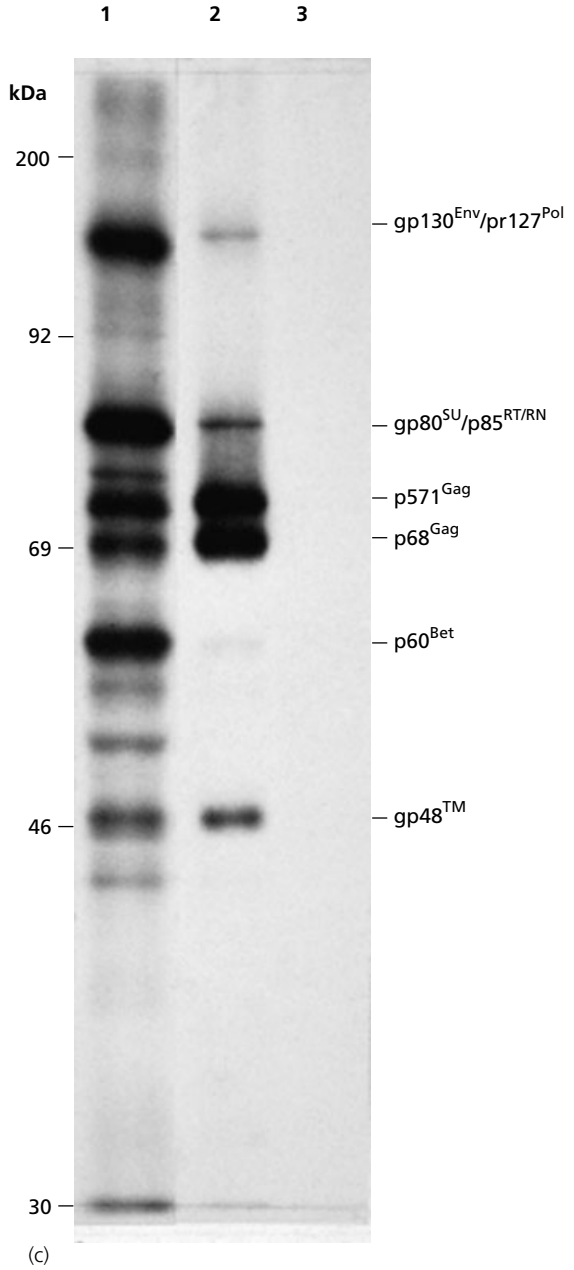

Figure 2 *(Continued)* **(c)** *Radioimmunprecipitation of metabolically labeled foamy virus proteins with positive monkey serum. Lane 1, intracellular virus proteins; lane 2, extracellular virion proteins; lane 3, lysate from uninfected cells reacted with positive serum. The molecular weight marker is indicated on the left and the main viral proteins on the right.*

et al. 2003). Interestingly, FVs contain a large amount of already reverse transcribed, mostly linear, cDNA (Yu et al. 1996a, 1999; Moebes et al. 1997; Roy et al. 2003). In orthoretroviruses, the ratio of DNA to RNA (late reverse transcripts of the *pol* or *gag* genes) is approximately one in 4×10^5 molecules. In spumaretroviruses, it was determined to be as high as one DNA in six RNA molecules (Yu et al. 1999; Roy et al. 2003). While some reports indicate that only the DNA form of the genome is relevant for replication (Moebes et al. 1997; Yu et al. 1999; Roy et al. 2003), others point to a role of RNA as well (Delelis et al. 2003).

Genome organization and expression

FV genomes are large; the PFV genome (linear viral DNA) is 13.2 kb (Figure 3) and the FFV genome 11.7 kb long. The viral genes are flanked by long terminal repeats (LTR) (of 1770 bp in PFV), subdivided into U3 (1423-bp), R (194-bp), and U5 (153-bp) regions (Maurer et al. 1988; Schmidt et al. 1997a). Adjacent to the 5′ LTR a short untranslated sequence is positioned, which is followed by the canonical *gag*, *pol*, and *env* genes. The protease is encoded in the *pol* gene. Downstream of env and overlapping the 3′ LTR, two accessory ORFs can be found in all sequenced FVs. The first harbors the *tas* gene (for *t*rans*a*ctivator of *s*pumaretroviruses) and ORF-2 gives rise to Bet, an abundant protein of ill-defined function (see below).

After provirus integration an internal promoter (IP), which is located in the *env* gene upstream of the accessory ORFs gives rise to two major transcripts (Löchelt et al. 1993b; Campbell et al. 1994; Löchelt et al. 1994; Mergia 1994; Bodem et al. 1998a) which are often spliced in the 5′ untranslated region (Figure 3). To do this the IP has some weak basal activity (Löchelt et al. 1993a). The activity of the IP is essential for the remainder of the replication cycle (Löchelt et al. 1995). One of the IP transcripts leads to the Tas protein and the other to Bet (Löchelt et al. 1994). Bet is derived from a multispliced mRNA. The most significant splice uses a splice donor (SD) in ORF-1 and an ORF-2 splice acceptor (SA). Tas activates transcription from the IP and the 5′ LTR U3 promoter. The U3 promoter has no detectable activity in the absence of Tas (Keller et al. 1991). The Gag protein is translated from a (pre-)genome length transcript. Instead of being expressed as a Gag–Pol fusion protein, FV Pol uses its own mRNA for translation as an authentic protein (Bodem et al. 1996; Enssle et al. 1996; Löchelt and Flügel 1996; Yu et al. 1996a). This transcript is spliced from the major 5′ SD in U5 to an SA which is located in the *gag* gene (Figure 3). The *pol* gene-specific splicing event appears to be critical for viral replication (Jordan et al. 1996). The transcripts for the Env protein also make use of the major 5′ SD and a major (or one of the minor) SA sites at the 3′ end of the *pol* gene (Muranyi and Flügel 1991). Despite this complex splicing pattern, a post-transcriptional regulator of FV gene expression has not been detected (Baunach et al. 1993; Yu and Linial 1993; Lee et al. 1994; Adachi et al. 1995). However, an RNA element within the *pol* gene has been described to facilitate Gag protein expression of HIV (Wodrich et al. 2001). This result awaits further substantiation.

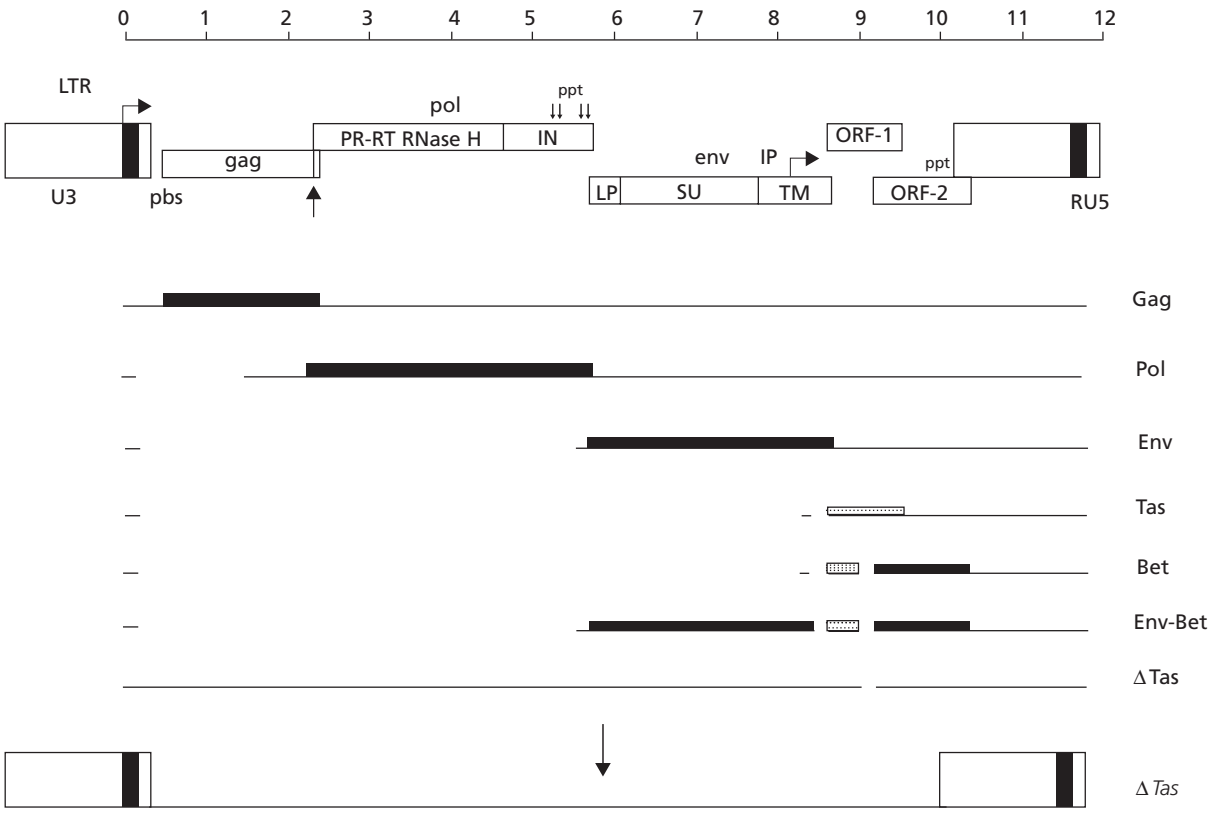

Figure 3 *Foamy virus genome organization (upper panel), main transcripts (middle panel), and ΔTas virus (bottom panel). Foamy virus genomes contain long terminal repeats (LTRs) at their ends, which are typically subdivided into U3, R, and U5 regions. First strand reverse transcription is initiated at the primer binding site (pbs). There are two polypurine tracts (ppt) for the initiation of second strand synthesis. RNA transcription initiates at the internal promoter (IP) or in the 5′ LTR. The canonical* gag, pol, *end* env *genes are indicated. Only one cleavage site for the viral protease has been identified in* gag *(arrow). The Pol precursor protein is expressed from a spliced mRNA and is also cleaved once between protease-reverse transcriptase and integrase. The Env precursor bears two cleavage sites for cellular proteases, one between the leader peptide (LP) and the surface (SU) domain and one between the latter and the transmembrane (TM) subunit. In addition, there are two further open reading frames (ORFs) downstream of* env. *ORF-1 gives rise to the Tas protein, which* trans*-activates transcription from the IP and the LTR promoter. Orf-1 transcripts are often spliced in the 5′ untranslated region (UTR) and can be generated from the IP or the 5′ LTR. This depends on the viral isolate. ORF-2 is expressed from a multispliced mRNA and gives rise to the Bet protein, which is highly expressed in infected cells, but of unknown function. Bet transcripts can also start from the IP or the main 5′ promoter. An Env–Bet fusion-protein is generated from the env mRNA that also uses the splice sites in the 5′ UTR of IP transcripts and splice sites in ORF-1 which lead to authentic Bet protein. These splicing events lead to an Env–ORF-2 fusion-protein in FFV. The function of Env–Bet (or Env–ORF-2) is ill-defined. The ΔTas transcript is of pregenomic length except for the small ORF-1 intron that has been spliced out. The ΔTas genome is transactivator-negative and helper virus-dependent (see text for details).*

Additional transcripts can be found in some FVs. Spliced tas and bet mRNAs originating in the 5′ LTR have been described for PFV, but not for FFV (Muranyi and Flügel 1991; Bodem et al. 1998a). The use of the *env* gene-located splice sites described above can give rise to a PFV Env-Bet fusion protein (Figure 3) from which the part of env coding for the membrane-spanning region of TM has been spliced out (Giron et al. 1998; Lindemann and Rethwilm 1998), or to an Env–ORF-2 fusion protein in FFV (Bodem et al. 1998a).

Aside from the (pre-)genomic transcript, a further transcript of almost (pre-)genomic length exists, from which only the small intron in the *tas* gene has been spliced out (Saïb et al. 1993). After reverse transcription this RNA leads to ΔTas virus (Saïb et al. 1993) which requires a helper virus for replication (see below).

Additional structural nucleic acid features include the primer binding side (pbs) 3′ of the 5′ LTR, which is specific for the t-RNA lysine[1,2] (Maurer et al. 1988), a dimer linkage site (Erlwein et al. 1997; Cain et al. 2001), the polypurine tract (ppt) 5′ of the 3′ LTR, and an internal stretch of purine-rich sequences which is located in the *pol* gene (Kupiec et al. 1988; Tobaly-Tapiero et al. 1991). The functional significance of this central ppt has not been completely determined yet, however, it is believed to have analogous functions to the ppt in HIV (Charneau and Clavel 1991; Charneau et al. 1992). Furthermore, RNA sequences involved in expression of structural proteins and in packaging the (pre-)genomic

transcript and the Pol protein have been identified (Erlwein et al. 1998; Heinkelein et al. 1998, 2000b; Wu et al. 1998; Park and Mergia 2000; Russell et al. 2001; Heinkelein et al. 2002b). Full functional characterization of these sequences has not yet been carried out.

Antigens and function of individual proteins

CORE ANTIGEN (GAG)

Cleavage of the Gag precursor into matrix, capsid, and nucleocapsid subunits is a feature that is conserved among *Orthoretrovirinae* and is responsible for the maturation of the viruses. The process which can be viewed as condensation of the capsid after budding (Swanstrom and Wills 1997), does not occur in FVs (Figure 2). Instead, only a small peptide is chopped of the C-terminus of FV Gag by the viral protease. This cleavage is essential for virus replication (Enssle et al. 1997; Zemba et al. 1998) and capsids consisting only of the precursor tend not to close and to show an irregular morphology (Konvalinka et al. 1995; Fischer et al. 1998).

Viral capsids serve two antagonistic roles: on the one hand they must be stable enough to protect the nucleic acid from the hostile environment and, on the other, they must easily free this nucleic acid once uptake into a susceptible cell has occurred. Orthoretroviruses have solved this problem by using protease-mediated defined cleavages of their Gag proteins, which prepare the capsids for disassembly (Wills and Craven 1991; Boulanger and Jones 1996; Kräusslich and Welker 1996; Swanstrom and Wills 1997). Therefore, the question arises how spumaretroviruses meet this challenge. In one possible scenario, disassembly of capsids occurs by FV protease-mediated cleavages of Gag early following the viral uptake (Giron et al. 1997). The identification of further Gag cleavage sites in addition to the C-terminal p3 cleavage site by in vitro PR assays and the vulnerability of the virus by mutagenesis of these sites is consistent with this hypothesis (Pfrepper et al. 1999). However, in the respective study, other possible alterations on Gag morphogenesis have not been excluded. Furthermore, recent evidence indicates that the FV capsid structure is maintained in the first phase after infection (Petit et al. 2003).

FV Gag proteins also lack other conserved sequence motifs, such as the major homology region, or the nucleic acid-binding cysteine-histidine-boxes (Wills and Craven 1991; Swanstrom and Wills 1997). Instead, PFV Gag harbors three so-called glycine–arginine-rich (GR) boxes at the C terminus (Schliephake and Rethwilm 1994). The first of these boxes binds with equal affinity to RNA and DNA (Yu et al. 1996b), the second box harbors a nuclear localization signal which is responsible for primate FV Gag proteins being transiently transported into the nucleus of infected cells (Figure 1) (Schliephake and Rethwilm 1994), while no function has been assigned to the third box. Nuclear localization of Gag, however, does not seem to be essential for FV cell culture replication (Yu et al. 1996b). In sharp contrast to the lentiviruses FV Gag proteins are much less well conserved compared to Pol and Env proteins (Figure 4). It is, therefore, not surprising that even the GR-boxes of primate FV are not conserved in the equine, bovine, and feline isolates. Furthermore, FFV Gag has been shown not to have a nuclear phase (Bodem et al. 1998b).

FV capsids assemble in the cytoplasma (Baldwin and Linial 1998; Fischer et al. 1998). A coiled-coil domain in the N-terminal half of FV Gag has been identified as being required for Gag–Gag interactions (Tobaly-Tapiero et al. 2001). While orthoretroviral Gag proteins bear all the information required for particle budding from the plasma membrane (Wills and Craven 1991; Boulanger and Jones 1996), this is not the case in FVs where Gag depends on the presence of autologous Env to export the particles, either from the plasma membrane or from the endoplasmic reticulum (ER) (Baldwin and Linial 1998; Fischer et al. 1998; Pietschmann et al. 1999). However, the addition of a heterologous plasma membrane-targeting signal to the FV Gag N-terminus, together with the change of an arginine at position 50 to alanine, leads to Env-independent budding of Gag (Eastman and Linial 2001). The mutation of an arginine in a similar position of the *gag* gene of Mason–Pfizer virus, an orthoretrovirus that, like FVs, shows cytoplasmic capsid assembly (D-type morphology), has been reported previously to redirect capsid assembly to the plasma membrane (C-type morphology) (Rhee and Hunter 1990). Thus, like orthoretrovirus capsids, the FV Gag proteins show some flexibility in the intracellular location of particle assembly.

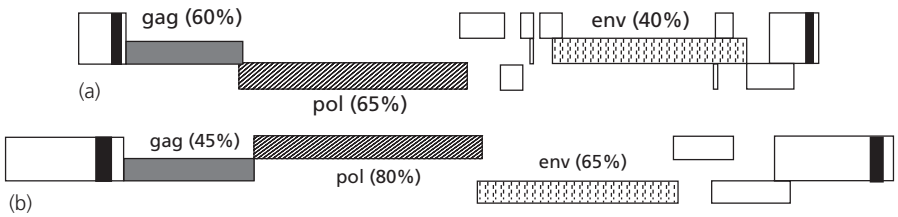

Figure 4 *Amino acids conservation among primate lentiviruses* **(a)** *and foamy viruses* **(b)**. *While in lentiviruses the Env protein is the most divergent structural viral protein, in primate FVs Gag is much more heterogenous than Env.*

RT ANTIGENS (POL)

FV Pol protein uses its own ATG start codon for expression from a spliced mRNA (Bodem et al. 1996; Jordan et al. 1996; Löchelt and Flügel 1996; Yu et al. 1996a). Sequences specifying protease, reverse transcriptase proper, RNase H, and integrase can be clearly identified on the basis of the conserved motifs of the active centers of the respective enzymes (Kögel et al. 1995a, 1995b). Unusually, the FV protease is not cleaved from the RT/RN (Pfrepper et al. 1998). The only consistent cleavage occurs between PR/RT/RN and IN (Pfrepper et al. 1998). A minor cleavage site has also been described between RT and RN of PFV (Pfrepper et al. 1998). Another peculiarity has been detected in the FFV PR. While the active center of retroviral proteases bears the strictly conserved DS/TG motif, FFV contains in this position a DSQ (Winkler et al. 1997a; Flügel and Pfrepper 2003). Nonetheless, the FFV PR containing this amino acid is enzymatically active (Flügel and Pfrepper 2003). The PFV Pol protein precursor, as well as its PR/RT/RN and IN subunits, were reported to be nuclear proteins when cells were transfected with eucaryotic expression constructs of the respective ORFs (Imrich et al. 2000). The functional significances of these findings have not been elucidated.

Since the Pol precursor protein of FVs is expressed independently from the Gag protein, FVs must have found a way of Pol incorporation into the particle in the absence of a Gag fusion partner. Interestingly, the presence of (pre-)genomic RNA is required in order to assemble Pol into viral capsids (Heinkelein et al. 2002b). In contrast to the hepadnaviruses, this RNA is incorporated in the absence of Pol into FV capsids (Baldwin and Linial 1998, 1999). Therefore, it is highly likely that in FVs (pre-)genomic RNA serves as a bridging molecule, which interacts with Gag on the one side and Pol on the other and, thereby, facilitates completion of particle assembly. The identification of separate sequences in the (pre-)genomic RNA for the interaction with Gag and Pol is consistent with this hypothesis (K. Peters, M. Heinkelein, and A. Rethwilm, unpublished results).

FV reverse transcriptase appears to be much more processive than orthoretroviral RT (Rinke et al. 2002). This is probably a prerequisite for FV reverse transcription prior to budding (see below).

ENVELOPE ANTIGENS (ENV)

The glycoproteins of FVs are another example of the highly divergent replication pathway that these viruses have developed. All orthoretroviral Env proteins are synthesized as precursors that are cleaved by cellular proteases into SU and TM subdomains which are part of the mature virion. TM is an MHC class I transmembrane protein (Einfeld 1996; Hunter 1997). It is anchored in the viral membrane and makes contacts to the SU subdomain (Swanstrom and Wills 1997). In murine leukaemia virus (MLV) and in Mason–Pfizer monkey virus, small peptides are cleaved from the cytoplasmic tail of TM to activate its fusogenic activity (Green et al. 1981; Henderson et al. 1984; Brody et al. 1992, 1994; Sommerfelt et al. 1992; Rein et al. 1994). However, this does not change the general organization of the glycoprotein complex. The orthoretroviral Env precursor bears a signal peptide sequence at the N-terminus that is responsible for ER translocation. This rather short sequence is cleaved from the remainder of the protein in the ER and degraded or further processed (Martoglio et al. 1997; Swanstrom and Wills 1997).

The signal or leader peptide of FV Env proteins is rather long and is also cleaved from the rest of the protein, however, in striking contrast to the situation in orthoretroviruses, the LP remains associated with the virion (Lindemann et al. 2001). Thus, FVs posses three glycosylated Env proteins (LP, SU, and TM), and two of these cross the viral membrane, LP at the N terminus and TM at the C terminus. Being initially synthesized as an MHC class III membrane protein (precursor), the mature Env consists of an MHC class II membrane protein (LP), the extraviral SU, and an MHC class I membrane protein (TM) (Lindemann et al. 2001). Furthermore, the LP points with its N-terminus to the viral capsid and makes contact to Gag (Lindemann et al. 2001; Wilk et al. 2001; Geiselhart et al. 2003). This is the reason for the specificity of the FV capsid–glycoprotein interaction and the molecular basis of the inability to pseudotype unmodified FV capsids with heterologous envelopes (Pietschmann et al. 1999). The mutation of only one conserved tryptophane residue in the cytoplasmic part of LP abrogates infectivity and the ability of Env to interact with Gag (Lindemann et al. 2001; Wilk et al. 2001), although the specific molecular way in which this interaction takes place is not yet known. Biophysical experiments point to contacts of LP with the Gag N terminus, which is believed to form a layer underneath the viral membrane akin to the matrix protein of orthoretrovirus Gag proteins (Wilk et al. 2001).

Not only is Env required to export FV capsids, Gag protein appears similarly to stimulate the quantitative release of Env from the plasma membrane (Pietschmann et al. 2000). The equine virus may represent an exception, because it was reported to bud exclusively from the plasma membrane (Tobaly-Tapiero et al. 2000; Lecellier et al. 2002a), while the other known FVs preferentially bud from intracellular membranes. Except in the case of EFV, an ER retrieval signal has been identified in the cytoplasmic tail of all FV TM proteins (Goepfert et al. 1995; Tobaly-Tapiero et al. 2000). This may, in the absence of Gag, hold back Env in intracellular compartments (Goepfert et al. 1997). However, additional elements in the membrane-spanning region of TM and the FV LP are at least equally important for this

retrieval (Pietschmann et al. 2000; Lindemann et al. 2001).

Another interesting feature of FV Env is the ability to self-associate and form subviral particles (SVP). Such SVPs arise by self aggregation of hepatitis B virus (HBV) surface protein in HBV infection in numbers exceeding the infectious virus and are believed to deflect the anti-HBV immune response (Gerlich 1991). While the SVPs of HBV are abundantly shed from infected cells, the PFV SVPs are, probably due to the retention of Env in cytoplasmic compartments, less abundant (Shaw et al. 2003).

NONSTRUCTURAL PROTEINS

Tas

The FV Tas proteins are approximately 209–300 amino acids in length, depending on the specific virus isolate (Rethwilm 1995; Löchelt 2003). They act as transactivators of gene expression from the two FV promoters and are required for virus replication (Baunach et al. 1993; Rethwilm 1995; Löchelt 2003). Tas augments gene expression by direct binding to DNA motifs in the IP and the U3 region of the LTR (He et al. 1996; Zou and Luciw 1996; Kang et al. 1998; Kang and Cullen 1998; Bodem et al. 2004). Tas binds to the IP with higher affinity than to the U3 promoter and to the U3 promoter with higher avidity than to the IP (Kang et al. 1998; Löchelt 2003). Between FV promoters and Tas proteins from different species there is only little conservation and almost no cross-transactivation (Löchelt 2003; Bodem et al. 2004). Tas proteins have been dissected into several subdomains: at the N terminus a region is shared with the Bet protein. The variable length of this region is responsible for the length variability of Tas proteins from different species (Löchelt 2003). The promoter-targeting domain that shows a low degree of conservation between different Tas proteins and is responsible for recognition of the DNA target sites follows the domain shared with Bet (Löchelt 2003). Between this region and the C-terminal activation domain is located the nuclear localization signal (NLS), consisting of an accumulation of basic residues (Keller et al. 1991; Chang et al. 1995; Rethwilm 1995; Bodem et al. 1998b). The approximately 30 amino acids activation domain stimulates gene expression in a wide variety of lower and higher eukaryotic cells when targeted to a heterologous promoter via an unrelated DNA-binding motif (He et al. 1993).

The DNA-binding regions and the activation domains show no homology to known motifs. However, genetic evidence indicates that FV Tas proteins belong to the acidic class of transcriptional activators (Blair et al. 1994). FV Tas proteins are believed to interact with conserved components of the transcriptional machinery. In yeast cells the ADA2 transcriptional adapter molecule has been identified and shown to be required for activation by the PFV Tas activation domain (Blair et al. 1994). FV Tas proteins can also activate gene expression by binding to recognition sequences located elsewhere in the FV or in the host cellular genome (Campbell et al. 1996; Wagner et al. 2000; Kido et al. 2002).

Since the elements in the U3 region of the LTR, which are responsive to Tas, do not completely match with the DNA-binding sites (Mergia et al. 1992; Erlwein and Rethwilm 1993; Lee et al. 1993; Renne et al. 1993), it is likely that mechanisms in addition to direct binding of Tas are involved in the transcriptional regulation of FV promoters (He et al. 1996). Possibly, Tas has DNA-binding and protein–protein interaction capabilities. The cellular promyelocytic leukaemia protein (PML) complexes with and strongly inhibits PFV Tas function (Regad et al. 2001). PML is induced by IFN and this may explain the IFN-responsiveness of FVs. However, it was shown by others that on a cellular level PML is not involved in the establishment of viral latency (Meiering and Linial 2003). Viral latency appears to be the dominant form of persistence in the infected host (Falcone et al. 2003). Nuclear factor I (NFI) was identified as another element to repress FV gene expression (Kido et al. 2003). Its contribution to the state of viral latency remains to be determined.

Bet

Because Bet is a protein derived from an mRNA spliced from ORF-1 into ORF-2 (Figure 3), only one (Tas or Bet) of the two accessory FV proteins can be expressed at a time. In lytically infected cells there appears to be a strong preference to generate the Bet protein, while Tas is relatively rare (Baunach et al. 1993). The function of PFV Bet remains elusive, since the part of Bet (ORF-2) that is nonoverlapping with Tas (ORF-1) or with other sequences, such as the downstream ppt and 3′ LTR motifs required for integration, can be deleted or functionally ablated from infectious plasmids without adversely affecting their replication competence (Baunach et al. 1993; Yu and Linial 1993; Schmidt and Rethwilm 1995). Bet is mainly cytoplasmically located, however, a small portion of PFV Bet has been reported to enter the nucleus, as well as being shed from infected cells and taken up by other cells (Lecellier et al. 2002b). In FFV, Bet is required for the release of FFV particles in a cell-type dependent manner (Alke et al. 2001).

Using a reactivation system from viral latency by the addition of the phorbol ester PMA, it was shown that Bet counteracts IP activity (Meiering and Linial 2002). As outlined above, IP activity is the start of a series of events leading to progeny virus. It is possible that in latent infection the relative expression levels of Tas and Bet, even more than in lytic infection, shift in favour of Bet. Thus, the generation of Tas versus Bet is supposed to be the molecular switch that controls the outcome of an infection: lytic (little expression of

Tas, major expression of Bet) versus latent (no expression of Tas, exclusive expression of Bet) (Meiering and Linial 2002). However, the mechanisms controlling this switch remain to be determined. In addition, it is likely that Bet serves additional functions than controlling viral latency.

Interestingly, the overexpression of PFV Bet renders cells resistant to superinfection by the autologous virus (Saïb et al. 1995; Bock et al. 1998). Once delivered as plasmid DNA into the cells, establishment of the resistant phenotype was found to be unrelated to down-regulation of viral uptake or the activity of viral promoters (Bock et al. 1998). It was, therefore, concluded that the block in replication most likely occurred between virus entry and provirus establishment (Bock et al. 1998). Any functional consequences of this for the viral replication cycle have not yet been analyzed.

Overview of the FV replication strategy

FVs appear to have acquired a unique replication strategy that has adopted aspects of the orthoretroviral and the hepadnaviral replication pathways (Nassal and Schaller 1993; Linial 1999; Lecellier and Saïb 2000; Rethwilm 2003b). An overview is presented in Figure 5. Although at first sight the FV genome organization looks like a typical complex retrovirus, the unique features cover any aspect of the replication strategy and the functions of individual proteins (Rethwilm 2003b). From provirus integration (Enssle et al. 1999; Juretzek et al. 2004), regulation of gene expression (Löchelt 2003), packaging of (pre-)genomic RNA and Pol proteins (Heinkelein et al. 2000b; Heinkelein et al. 2002b), capsid assembly (Linial and Eastman 2003), and cleavage by the viral protease (Flügel and Pfrepper 2003) to budding and Gag–Env interaction (Lindemann and Goepfert 2003), FV replication deviates from what are believed to be the standard rules of retrovirology (Vogt 1997).

Most striking is the generation of infectious full-length cDNA late in the replication cycle before budding of the particle (Yu et al. 1996a, 1999; Moebes et al. 1997; Roy et al. 2003). Aside from exiting the infected cell, this DNA can also recycle to the nucleus and integrate, resulting in the delivery of additional proviruses, at least in the absence of FV glycoprotein (Heinkelein et al. 2000a, 2003) (Figure 5). While this phenomenon is reminiscent of the life cycle of retrotransposons (Boeke and Stoye 1997), its significance for the FV replication cycle remains to be determined. The extent to which cDNA synthesis in the virus-producing cell is complete may depend on the actual cell type, which can explain why reverse transcription can also be detected early in the replication cycle (Delelis et al. 2003).

In addition, FVs generate a reverse-transcribed defective cDNA (the ΔTas genome in Figure 3) to an extent that is likely to affect biological function (Saïb et al. 1993). The ΔTas is generated from an almost pregenomic length RNA, from which ORF-1 intron, normally used to generate Bet mRNA, has been spliced out (Figure 3). Thus, ΔTas are replication-defected. Depending on the number of ΔTas proviruses, which can express some Bet protein, cells become resistant to the infection by wildtype virus (Saïb et al. 1995). However, the exact relationship between wild-type and ΔTas genome, and whether this type of interference is taking place in vivo, is not known.

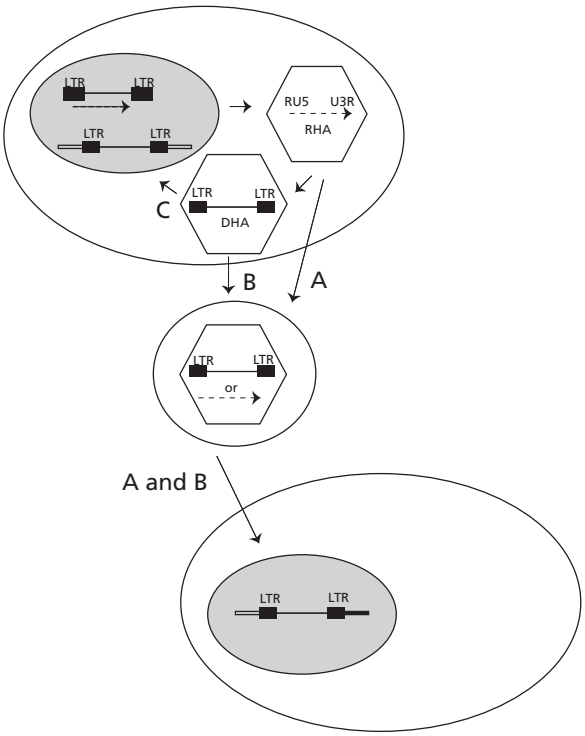

Figure 5 *Principal retroviral replication strategies. Orthoretroviruses make use of pathway A regardless of intracellular capsid assembly or assembly at the plasma membrane. Obligatory FV capsid assemble occurs in the cytoplasm. Furthermore, reverse transcription of the pregenomic RNA takes place at this stage and cDNA is exported (pathway B) or can recycle to the nucleus and integrate into the host cell genome to give rise to additional proviruses (pathway C). (Modified from Heinkelein et al. 2000a)*

While orthoretroviruses are RNA viruses replicating through a DNA intermediate, where the step of reverse transcription occurs early in the replication cycle, FVs, like hepadnaviruses, are DNA viruses, which replicate through an RNA intermediate with a reverse transcription step late in the replication cycle. Unlike the hepadnaviruses, they require integration into the host cell genome for replication, similar to orthoretroviruses (Enssle et al. 1999; Meiering et al. 2000). This raises the question as to whether FVs may represent a functional or evolutionary link between these reverse transcribing virus families (Flint et al. 2004).

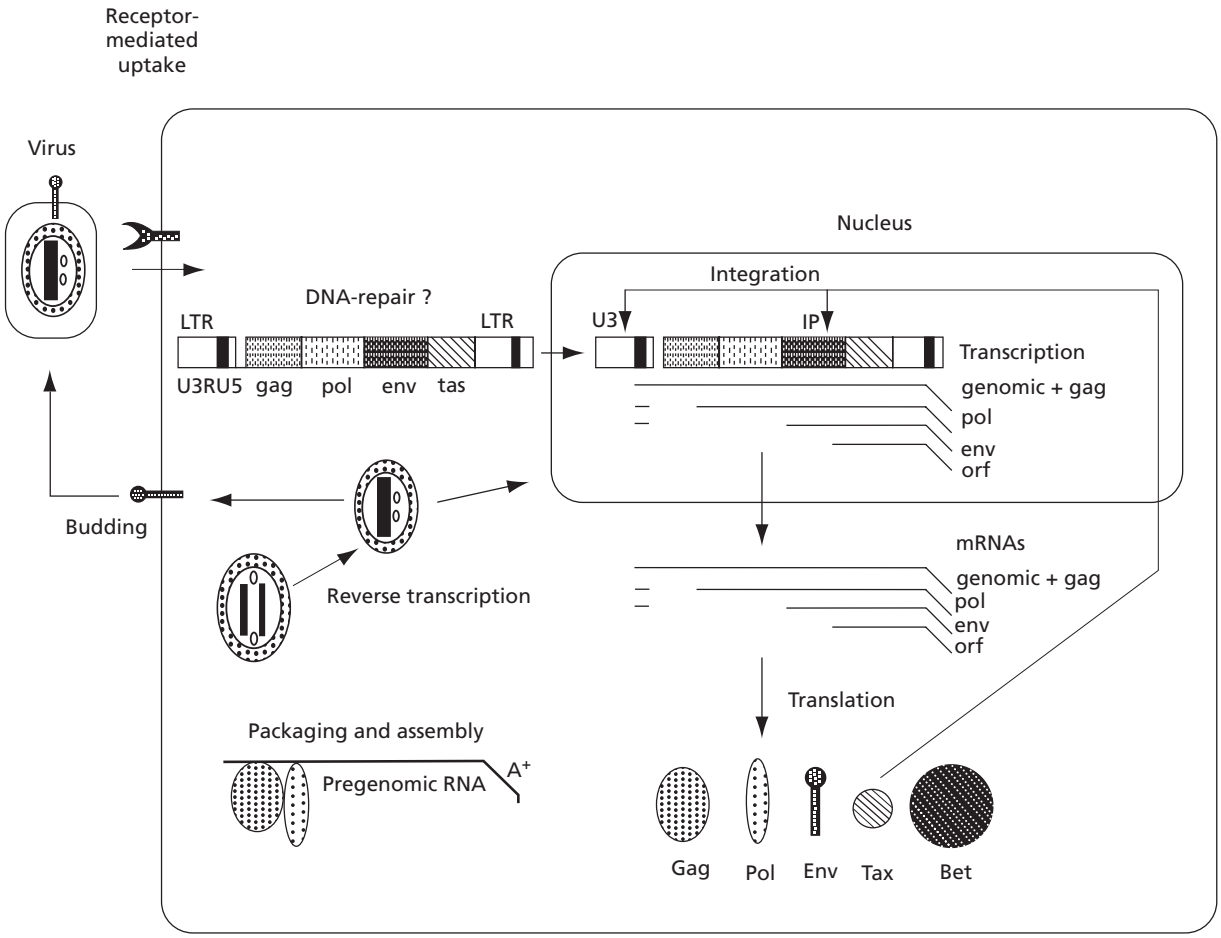

Figure 6 *Overview of the FV replication strategy. (Modified from Rethwilm 2003a)*

FV VECTORS

FV vectors based on PFV, SFV-1, and FFV have been developed over the last years (Mergia and Heinkelein 2003). Some of the vectors were replication-competent, taking advantage of the generally nonpathogenic nature of FV infection (Schmidt and Rethwilm 1995; Schwantes et al. 2002). These vectors may be used to deliver, for instance, suicide genes or genes for immunogenic proteins in vivo (Nestler et al. 1997; Schwantes et al. 2003). However, since the virus has no advantage from the additional genetic burden, these genes are usually lost quickly during replication, at least in vivo (Nestler et al. 1997; Schwantes et al. 2003).

More promising for future applications are replication-incompetent vectors based on FVs. There are several theoretical advantages of using FV vectors than MLV-derived vectors, which are the most widely and most successful retroviral vectors currently in use in clinical trials (Cavazzana-Calvo et al. 2004). First of all, the parental virus is nonpathogenic. As a consequence gene therapy with FV vectors should be safe, even in the unlikely event of generating replication-competent vector-derived retroviruses by recombination (Schenk et al. 1999; Bastone and Löchelt 2004). Furthermore, by separation of the packaging plasmids in three instead of two expression units (orthoretroviruses generate a Gag/Pol fusion protein) a further safety profile can be added to FV vectors (Heinkelein et al. 2002a; Trobridge et al. 2002a). Secondly, the genome of FVs is larger than that of MLV, possibly enabling the packaging of larger fragments of foreign DNA (Heinkelein et al. 2002a; Park et al. 2002; Trobridge et al. 2002a). Thirdly, FV can be concentrated without significant loss of infectivity, e.g. by centrifugation (Hill et al. 1999; Trobridge et al. 2002b). The receptor for FVs, although not currently known, is widespread, which allows targeting of a wide variety of cells (Mergia et al. 1996; Hill et al. 1999; Lindemann et al. 2000).

However, FVs do not infect resting cells (Bieniasz et al. 1995b; Trobridge and Russell 2004). The incoming capsid appears to be transported into the vicinity of the nucleus (Saïb et al. 1997b; Petit et al. 2003), but similar to MLV FVs appear to require the breakdown of the nuclear membrane for integration and subsequent gene expression (Hajihosseini et al. 1993). The difference from MLV is that, since FV reverse transcription has already taken place in the virus- or vector-producing

cell, the form of the nucleic acid that is delivered into the target cell consists of DNA instead of RNA. Reverse transcription can be regarded as the most vulnerable step in retrovirus replication, and due to the greater stability of double-stranded DNA compared to single-stranded RNA, FVs may be ideal to target rarely cycling cells, such as hematopoietic progenitors.

In a variety of seminal experiments it has been shown that FV vectors can target CD34 antigen-positive human hematopoietic progenitors of different sources that are able to repopulate sublethally irradiated NOD/SCID mice with an efficiency comparable to vectors derived from lentiviruses and much superior to MLV vectors, provided that the target cells were not prestimulated (Hirata et al. 1996; Josephson et al. 2002; Leurs et al. 2003; Josephson et al. 2004). The transduction of hematopoietic stem cells was demonstrated by analysis of transgene expression and vector integration site determination in lymphoid and myeloid compartments after long-term engraftment (Josephson et al. 2002; Leurs et al. 2003; Josephson et al. 2004).

However, it must be noted that packaging cells for FV vectors, which would allow to better control for the quality of the vector preparations by standard means, are not yet available (Mergia and Heinkelein 2003). Furthermore, FV capsids do not currently allow the efficient pseudotyping by heterologous glycoproteins and the FV envelope glycoprotein complex exhibits considerable cell toxicity, which makes the handling of FV vectors painstaking. It remains to be seen whether the development of adenovirus–foamy virus hybrid vectors, which create a form of in vivo packaging cell line can overcome this problem (Picard-Maureau et al. 2004; Russell et al. 2004) or whether batches of FV vectors will have to be produced by the repeated transient transfection of cells.

Furthermore, the packaging signal on the (pre-)genomic RNA and the mechanism of RNA, as well as Pol protein packaging have not yet been completely resolved (Heinkelein et al. 2000b, 2002b). It is known that at least two regions on the RNA are required for efficient packaging into particles (Erlwein et al. 1998; Heinkelein et al. 1998; Wu et al. 1998; Heinkelein et al. 2000b; Russell et al. 2001). One element resides in the 5′ leader region and extends into the *gag* gene, while the other element is located in the 3′ *pol* gene region. Thus, there is a need for a better understanding of the biochemical basis of RNA and Pol protein packaging and the mechanism of FV particle assembly to further improve current vectors and packaging constructs.

EPIDEMIOLOGY AND LABORATORY DIAGNOSIS

FV are highly prevalent in many monkey species from the Old and the New World (Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981; Swack 1981; Meiering and Linial 2001; Falcone et al. 2003). Of these, only Old World FVs are characterized in greater detail. The rate of infection in the wild can be as high as 30 percent or above (Hooks and Gibbs 1975; Swack 1981; Meiering and Linial 2001). In captivity, infection rates may be anywhere between 70 percent and close to 100 percent (Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981; Swack 1981; Rösener et al. 1996; Meiering and Linial 2001; Falcone et al. 2003). Only a weak cross-neutralization was reported for SFVs originating from different species. Trans-species transmissions in captive monkeys and even dual infections with two different SFVs has been described (Hooks et al. 1972; Hooks and Gibbs 1975; Swack 1981; Meiering and Linial 2001).

As in monkeys, the FV prevalence in cats increases with age to an incidence of over 70–80 percent in domestic or feral animals over the age of nine (Bandecchi et al. 1992). Social contact is suspected to be the most likely mode of FFV transmission (Bandecchi et al. 1992). Two FFV serotypes which do not cross-neutralize and which have substantial differences in the env *SU* genes are documented (Winkler et al. 1998; Zemba et al. 2000). Because the Gag sequences are conserved, diagnostic tests rely on the detection of antibodies to this protein (Winkler et al. 1997b).

As already mentioned above, so far there is no indication of a naturally circulating human FV. However, since the detection of an SFV infection in a monkey caretaker (Schweizer et al. 1997), more extensive investigations led by the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, USA on occupationally acquired FV infections were carried out (Heneine et al. 1998; Switzer et al. 2004). Altogether, approximately 5 percent of animal caretakers or laboratory personnel handling infected material were found to be sero- and PCR-positive (Brooks et al. 2002; Heneine et al. 2003; Switzer et al. 2004). Thus, the risk of occupational acquisition of an SFV infection appears to be much higher than that of contracting an infection with other nonhuman primate viruses (Brooks et al. 2002; Heneine et al. 2003; Switzer et al. 2004). No risk of attracting an FFV infection was found for veterinarians handling FV-infected cats (Butera et al. 2000).

Another population potentially at risk of FV infection are monkey hunters in the African bush. While an earlier investigation found no evidence of infection in a rather small sample (Goepfert et al. 1996), a more recent study identified 1 percent positives when screening over 1 000 human sera (Wolfe et al. 2004). Samples positive for FV antibodies were confirmed by nucleic acid detection and sequencing (Wolfe et al. 2004). Besides differences in the sample size, it should be noted that the methods of screening were also different in the two studies (Goepfert et al. 1996; Wolfe et al. 2004).

The method of choice to detect FV infections in humans consists of screening sera for antibody against a

cocktail of antigens derived from the SFVs potentially being transmitted. An immunoblot coated with antigen derived from two SFVs (SFVagm and SFVcpz) has beenshown to react with serum samples, which are positive for a wide variety of different SFVs (Hussain et al. 2003). Antibody response in an immunoblot against the Gag antigens usually indicates positivity (Hahn et al. 1994; Hussain et al. 2003). The detection of an antibody response against the nonstructural Bet protein (Figure 2) corroborates serological diagnosis of infection (Hahn et al. 1994). Antiglycoprotein antibodies are only detected reliably by radio-immunoprecipitation (Netzer et al. 1990). In any case positive results in antibody tests should be confirmed by PCR diagnosis using primers from regions conserved in the FV genome, such as from the integrase gene (Schweizer and Neumann-Haefelin 1995; Khan et al. 1999). When the infecting virus is known and in culture, indirect immunofluorescence also reliably detect infection (Neumann-Haefelin et al. 1983). In this case a strong nuclear fluorescence should be visible, at least in some cells when dealing with FVs of primate origin (Figure 1).

Virus isolation can be done relatively easily from throat swabs of infected monkeys or cats using a wide variety of target cells, but preferably fibroblasts, to monitor the development of CPE (Hooks and Gibbs 1975; Swack 1981). Indicator cell lines bearing the *lacZ* gene under the control of different FV LTRs have been established for easy and quick monitoring of infection (Yu and Linial 1993; Bock et al. 1998; Roy et al. 2003). However, such cell lines are not available for all FVs. Virus isolation from humans requires particular conditions, such as the isolation from PBLs and potentially the addition of anti-interferon (Heneine et al. 1998; Falcone et al. 1999b).

The methods to diagnose FV infections are not generally in use and in the case of a suspected human SFV infection, one should refer to a more specialized and experienced laboratory. SFV-infected humans should not donate organs, blood or blood products (Allan et al. 1998; Boneva et al. 2002). Whenever possible, such individuals and their close contacts should be followed for the unlikely event of the emergence of a new retrovirus adapted to replicate efficiently in the human host (Peeters et al. 2002).

Zidovudine (AZT) is the only known antiretroviral drug that efficiently blocks FV replication (Rosenblum et al. 2001). In the event of reactivating latent FVs when culturing primary monkey cells, AZT at a concentration of 5–10 μm can be tried in order to block SFV replication.

Infections with foamy viruses are not of medical importance, but the study of their molecular biology has contributed much to our understanding of the ways retroviruses replicate and the use of foamy virus vectors may open a new field in somatic gene therapy.

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Human immunodeficiency virus

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INTRODUCTION

Since the beginning of the 1980s millions of people have been infected with human immunodeficiency virus (HIV) the causative agent of the acquired immune deficiency syndrome (AIDS), a multi-systemic, deadly, and so far incurable disease. HIV infections remain one of the most devastating diseases that humanity has ever faced. Unfortunately, there is little hope that an effective vaccine will be developed in the near future, nor are there other mechanisms available to stimulate the natural immunity against HIV. Current antiretroviral treatment is based on drugs that target either the viral reverse transcriptase (RT) or the protease (PR) or the viral envelope (gp41). A drawback to these treatments is that with HIV's high rate of mutation and replication, dynamic, drug-resistant mutants are evolving, particularly when anti-retroviral treatment suppresses virus replication to only marginal levels. Cellular genes have much lower mutation rates, and a potential solution to this problem is to target host cellular factors required for HIV replication. These include enzymes and interactive proteins that are essential for replication of HIV in host cells, which can be selectively manipulated without interfering with the vital functions of the organism.

GENOMIC ORGANIZATION AND REPLICATION CYCLE OF HIV

The *Retroviridae*, a family of viruses, which include the *Human immunodeficiency virus type 1* (HIV-1) and *Human immunodeficiency virus type 2* (HIV-2), belong to the gigantic group of eukaryotic retrotransposable elements (for review see Doolittle et al. 1990). Retroviruses are distinguished by their ability to reverse transcribe their RNA genomes into DNA intermediates by using the enzyme RT. The DNA form of the provirus genome is then integrated into a host cell chromosome by action of the viral enzyme integrase (Int). All retroviruses contain at least three major genes that encode the main virion structural components which are each synthesized as three polyproteins that produce either the inner virion interior (Gag, group specific antigen), the viral enzymes (Pol, *pol*ymerase), or the glycoproteins of the virion *env*elope (Env). In addition to virus structural proteins and viral enzymes, some retroviruses code for mostly small proteins with regulatory and auxiliary

functions. While retroviruses share the same fundamental replication cycle and have the same basic genomic organization (e.g. the canonical *gag*, *pol*, and *env* genes) they do vary in the content of additional small regulatory genes and they have evolved different structures of their Gag proteins (for review see Coffin et al. 1997). The retroviral *gag* gene encodes the major structural protein and is entirely sufficient for virus particle formation (for review see Swanstrom and Wills 1997). According to the nomenclature defined by the International Committee on Taxonomy of Viruses (ICTV) convention (Hull 2001), orthoretroviruses contain RNA inside virus particles and are released from the plasma membrane. However, the assembly of orthoretroviruses follows two different strategies: Alpha-, gamma-, delta-, and lentiviruses (ICTV nomenclature) assemble on the inner leaflet of the plasma membrane and are released as immature virions. In contrast, betaretroviruses and spumaviruses first assemble in the cytoplasm to form immature particles that are then transported to the plasma membrane where virus budding occurs.

Viruses of the genus *Lentivirus* including HIV-1, HIV-2, and *Simian immunodeficiency virus* (SIV), replicate preferentially in lymphocytes and in differentiated macrophages and cause long-lasting and mostly incurable chronic diseases. In contrast to gamma-retroviruses (ICTV nomenclature for oncoretroviruses) that require the breakdown of the nuclear membrane during mitosis for viral integration into host-cell chromosomes, lentiviruses are uniquely capable of infecting nondividing cells, preferentially nondividing macrophages and resting T-cells, as is the case for HIV-1 (Lewis and Emerman 1994).

As for other retroviruses, the HIV replication cycle begins with virus attachment and penetration through the plasma membrane. HIV binds to different cell-receptors, among them the differentiation antigen, CD4, acting as primary receptor, as well as different specific chemokine receptors that act as coreceptors after binding to CD4 (for review see Weiss 2002). The first retroviral receptor ever identified was the CD4 receptor on T-cells. It was established as the primary receptor for HIV-1 and HIV-2 and SIVs in 1984 (for review see Bour et al. 1995). However, the CD4 molecule alone is not adequate to allow Env-mediated membrane fusion and virus entry, as certain primary isolates of HIV-1 preferentially replicate in T-cell lines (T-cell-line trophic, 'TCL'-tropic), while others establish productive replication only in macrophage cultures ('M'-trophic). Thus, a second class of HIV receptors was predicted and finally identified as a member of the G protein-coupled seven transmembrane domain receptor superfamily acting as so called 'coreceptors' during HIV entry (for review see Berger et al. 1999). Coreceptors, in general, function as cellular receptors for α- and β-chemokines. Two types of coreceptors arbitrate the disparity in cell-type trophism: the α-chemokine receptor CXCR4 (first described as 'fusin') that is typically present in T-cell lines, and the β-chemokine receptor CCR5 that is present on macrophages. In tissue culture, primary lymphocytes support replication of HIV, irrespective of its trophism. This phenomenon is demonstrated by the fact that cultures of activated peripheral blood mononuclear cells (PBMC) express both coreceptors, CXCR4 and CCR5. Notably, coreceptors symbolize an attractive target for antiretroviral therapy. For example, the genetic heterogeneity in coreceptor alleles determines the vulnerability to HIV infection or disease progression. A well-characterized example is the identification of a truncated form of CCR5 (termed as the 'CCR5/d32' mutation) that in its homozygous form can protect individuals from HIV infection (Deng et al. 1996; Samson et al. 1996).

Following virus entry, the viral RNA genome is transcribed into double-stranded DNA, which as part of the preintegration complex (PIC) is transported to the nucleus where the proviral genome is integrated into chromosomes. Virus entry starts with the so-called 'uncoating' of the virus which is the fusion between the virus and host cell membrane followed by the release of the viral core into the cytoplasm. The virus core comprises all viral components that remain intact after the virus membrane is removed. Following membrane fusion, the reverse transcription complex (RTC) and the PIC is released from the virus core complex. While there is evidence that the CA molecules of the incoming virus are degraded via the ubiquitin proteasome system (UPS) (Schwartz et al. 1998), the three virus proteins Int, the matrix (MA), and the viral protein R (Vpr) molecules together with the provirus genome form the PIC. Although the PIC components comprise various nuclear import signals (for review see Sherman and Greene 2002), it is assumed that additional cellular and viral factors, including a central DNA flap, which is formed during reverse transcription (Zennou et al. 2000), facilitate the import of the PIC into the nucleus. It is worth noting that virus entry involves a number of consecutive and highly organized multistep events that might explain why approximately only one out of a thousand mature HIV-1 particles is capable of establishing a productive infection.

Further postentry events such as the reverse transcription of the viral genome, the nuclear import of the PIC, the integration of proviral DNA, the activation of HIV-1 LTR promoter driven retroviral gene expression, the export, the transport, and the splicing of viral RNA will not be described in detail in this review. Rather, late steps of the HIV-1 replication cycle, such as membrane targeting of Gag and Gag–Pol polyproteins, virus assembly, as well as budding and maturation will be explained in more detail. Following the early steps of the replication cycle and upon activation of integrated provirus, viral RNAs are processed and transported into the cytosol for translation of newly synthesized

structural proteins that assemble at the plasma membrane into budding particles. The Gag polyprotein in HIV-1 and HIV-2 consists of different functional domains that mediate the recognition and binding of viral RNA, the membrane targeting of Gag and Gag–Pol polyproteins, virion assembly, and efficient particle release from the plasma membrane as the final step of virus budding (for review see Kräusslich and Welker 1996; Swanstrom and Wills 1997; Vogt 1997, 2000). The Gag polyprotein is the single viral protein sufficient and strictly required for virus particle assembly and budding, although further viral components such as the genomic RNA, the envelope, and the Gag–Pol precursor proteins are required for production of infectious progeny virions. The processing of the HIV-1 Gag polyprotein Pr55 by the viral PR generates the MA, CA, nucleocapsid (NC), and $p6^{Gag}$ proteins (Swanstrom and Wills 1997). During proteolytic processing of HIV-1 $Pr55^{Gag}$ two spacer peptides, p2 and p1, are also generated. The HIV-1 *pol*-encoded enzymes, PR, RT, and Int are proteolytically released from a large polyprotein precursor, $Pr160^{GagPol}$, the expression of which is the outcome from a rare frameshift event during $Pr55^{Gag}$ translation (for review see Freed 2001). Similar to the Gag proteins, the Env glycoproteins are also synthesized from a polyprotein precursor protein. The resulting surface (SU) gp120 and transmembrane (TM) gp41 glycoproteins are produced by a cellular protease during trafficking of gp160 to the cell membrane. While gp120 harbors the domains that mediates virus binding to CD4 and coreceptors, the gp41 anchors the TM/SU complex in the virus membrane and includes the determinants that regulate fusion between cellular and virus membranes during virus entry. The N-terminus of the extra cellular ecto domain of gp41 harbors the hydrophobic domain, the so called 'fusion peptide' that in concert with two helical motifs of gp41 regulates membrane fusion by formation of a six-helix bundle (Weissenhorn et al. 1997). The highly polymorphic SU protein gp120 is organized into five conserved domains (C1 to C5), and five highly variable domains that in most of the known SU sequences are concentrated near loop structures and are stabilized by disulfide-bond formation.

In general, Gag proteins of different retroviruses exhibit a certain structural and functional similarity: MA mediates the plasma membrane targeting of the Gag polyprotein and lines the inner shell of the mature virus particle, CA regulates assembly of Gag and forms the core shell of the infectious virus, and NC regulates packaging and condensation of the viral genome. In addition to those canonical mature retrovirus proteins, other Gag domains have been described, such as the HIV-1 $p6^{Gag}$ region that directs the incorporation of the regulatory protein Vpr into budding virions. Furthermore, $p6^{Gag}$ harbors the late (L) domain required for efficient virus budding (Göttlinger et al. 1991).

HIV particles bud from the plasma membrane as immature noninfectious viruses consisting predominantly of uncleaved polyproteins. After virus release and in concert with PR activation which is autocatalytically released from the Gag–Pol polyprotein, processing of Gag and Gag–Pol polyproteins into its mature proteins and condensation of the inner core structure occurs resulting in the formation of mature infectious virus. Besides PR and Env, at least two other viral factors are known to promote efficient virus release: the HIV-1 specific accessory protein Vpu (Strebel et al. 1988) and the $p6^{Gag}$ domain (Gottlinger et al. 1991). While Vpu supports virus release by an ion channel activity (Schubert et al. 1996b, c), $p6^{Gag}$ contains the late assembly (L) domain that is required for efficient separation of assembled virions from the cell surface by a yet undefined mechanism that is further detailed below and involves the cellular UPS (Vogt 2000).

ROLE OF CELLULAR FACTORS IN HIV REPLICATION

From entry to release and maturation into infectious progeny virions, each individual step in the HIV replication cycle exploits cellular pathways. As for most other intracellular parasites, the replication of HIV is dependent on interaction with host cell factors, and some of these are specifically incorporated into progeny virions. Among the most abundant cellular proteins, and the first ever found in HIV-1 virions, is the peptidyl-prolyl isomerase (PPIase) cyclophilin A (CypA). This protein is specifically incorporated into HIV-1 virions, but not into virions of other lentiviruses, through an interaction with a proline-rich region that is conserved in the CA of all HIV-1 Gag polyproteins. In particular, Pro-222, which is conserved in the CA region of all HIV-1 Gag polyproteins appears to be important for the interaction of HIV-1 Gag with CypA (for review see Luban 1996). Earlier studies using two-hybrid screens found that HIV-1 Gag binds to most of the known members of the family of CyPs. CyPs were originally described as binding partners of cyclosporin A (CsA), an immunosuppressive cyclic undecapeptide used clinically to prevent allograft rejection. CsA binds with nanomolar affinity to CypA and this complex inhibits calcineurin, a calcium-dependent phosphatase that regulates gene expression of various cytokine genes in activated T-cells (for review see Ivery 2000). CyPs belong to the PPIases, a group of enzymes found in organisms ranging from prokaryotes to humans that catalyze the otherwise relatively slow *cis*/*trans* isomerization of peptidyl–prolyl bonds in vitro. A large body of evidence supports a function of CypA in formation of infectious HIV-1 viruses (for review see Ott 2002; Luban 1996). These studies were based either on mutation within *gag* or the use of competitive CypA inhibitors, such as CsA that

interfere with the CypA–Gag interaction. A more conclusive proof that among the other 14 known members of mammalian CyPs, only CypA plays a functional role in supporting HIV-1 replication, was provided by selective genetic inactivation of the gene encoding CypA in human $CD4^+$ T-cells (Braaten and Luban 2001). The immunosuppressive activity of CsA is not correlated with anti-HIV activity as the non-immunosuppressive derivative NIM811 ([methyl-Ile^4]-cyclosporin) represents an even more potent inhibitor of CypA-mediated HIV-1 replication than the parental CsA. Thus, CsA and related non-immunosuppressive derivatives form an interesting class of drugs that can modulate the interaction of CypA with other proteins.

Recent investigation of the conformational heterogeneity of the proline residues in the N terminus of the HIV-1 accessory protein Vpr suggested a functional interaction between Vpr and a host peptidyl–prolyl *cis/trans* isomerase (PPIase) that might regulate the *cis*/*trans* interconversion of the imidic bond within the conserved proline residues of Vpr in vivo (Bruns et al. 2003). The physical interaction between Vpr and the major host PPIase CypA involves the N-terminal region of Vpr and includes an essential role for proline in position 35 (Zander et al. 2003). The CypA inhibitor CsA, and non-immunosuppressive PPIase inhibitors, such as NIM811 and sanglifehrin A (SFA), block expression of Vpr without affecting pre- or post-translational events, such as transcription, intracellular transport or virus incorporation of Vpr. Similarly to CypA inhibition, Vpr expression is also reduced in HIV-1-infected $CypA^{-/-}$ knock-out T-cells. These studies provide the first evidence that, in addition to the interaction between CypA and HIV-1 capsid occurring during early steps in virus replication, CypA is also important for the de novo synthesis of Vpr and that in the absence of CypA activity, the Vpr-mediated cell cycle arrest is completely lost in HIV-1 infected T-cells.

Another example of host–virus interaction that has recently received much attention is the role of the UPS in virus budding. Efficient detachment of HIV-1 from the cell membrane is strictly dependent on the function of the L domain, a tetrapeptide motif (Pro–Thr/Ser–Ala–Pro (PT/SAP)) that encompass positions seven to ten of the $p6^{Gag}$ protein (for review see Freed 2002; Greene and Peterlin 2002). This process, which involves fission of the virus particle from the cell surface following envelopment and closing of the virus and cellular membrane, is commonly described as virus budding. L domain-deficient mutants of HIV-1 are characterized by late budding arrest, where virions remaining tethered to the plasma membrane by a thin membranous stalk that does not allow complete membrane fission (Göttlinger et al. 1991; Huang et al. 1995). Similar deficiencies in budding were reported for other retroviruses with L domain mutations within Gag polyproteins (for review see Freed 2002). Although the detailed molecular mechanism(s) of how L domains regulate virus budding remain elusive, it is now generally believed that they function as a docking site for the cellular budding apparatus which normally is involved in the endocytotic recycling of cell surface-receptors (for review see Pornillos et al. 2002). Recent work provided an intriguing insight into the mechanism of how virus budding exploits a cellular machinery that is normally involved in vacuolar lysosomal protein sorting and multivesicular body (MVB) biogenesis (Martin-Serrano et al. 2003; von Schwedler et al. 2003). The recruitment of these cellular factors to the virus assembly site is facilitated by the interaction between the PTAP motif of $p6^{Gag}$ and at least one important factor which is the tumor susceptibility gene product (Tsg101) (Garrus et al. 2001; Martin-Serrano et al. 2001; Myers and Allen 2002; VerPlank et al. 2001), an E2 type ubiquitin ligase. Mechanistically, the interaction between Tsg101 and $p6^{Gag}$ is mediated by the N-terminal ubiquitin-binding domain, designated as ubiquitin E2 variant (UEV) sequence of Tsg101 that binds to the PTAP motif of $p6^{Gag}$ in a process that appears to be up-regulated when upstream lysine residues in $p6^{Gag}$ are mono-ubiquitinylated (Garrus et al. 2001; VerPlank et al. 2001; Pornillos et al. 2002; Demirov et al. 2002; Myers and Allen 2002). Recently, two additional binding partners of Tsg101 have been identified in virus budding: ALix/AIP1, a homologue of the yeast class E Vps protein Bro1 that binds to a C-terminal region of HIV-1 $p6^{Gag}$ and is also involved in L-domain function (Strack et al. 2003; von Schwedler et al. 2003), and Hrs, a regulator of MVB formation involving ESCRT recruitment to endosomes (Bache et al. 2003; Pornillos et al. 2002). The normal function of Tsg101 is to target multi-ubiquitinylated proteins into endosomal vesicles followed by MVB formation in a process that is similar to virus budding and involves the budding into the endosomal vesicles (Babst et al. 2000; Katzmann et al. 2002). Topologically, MVB generation is analogous to virus budding, since in both cases cytoplasmic material is extricated and enveloped by cellular membranes. Thus, it appears that HIV recruits the components of the MVB system to the budding machinery that follows two separate and cell-type-specific pathways: in T-cells HIV-1 buds primarily from the cell surface, while in monocytes/macrophages the virus buds into vacuoles of the MVB system (for review see Amara and Littman 2003). In general, there is mounting evidence that HIV-1 assembly and budding exploits the cellular protein sorting and trafficking pathway which itself is regulated by the UPS (for review see Schnell and Hicke 2003).

Another important example of the interaction of HIV-1 with host factors is the relationship between one HIV-1 accessory protein, the virus infectivity factor (Vif), and apolipoprotein B mRNA editing enzyme (APOBEC3G). APOBEC3G, also known as CEM15, is a member of cellular cytidine deaminase-editing enzyme

family that represents another aspect of the so-called 'innate immune system' (for review see Vartanian et al. 2003; Rose et al. 2004; Navarro and Landau 2004; Schrofelbauer et al. 2004). The antiretroviral activity of APOBEC3G is based on its unique capability to hypermutate retroviruses, including HIV-1, with terminal consequences. This innate block in virus replication is counteracted by Vif, which in the virus producer cell binds to APOBEC3G, induces its polyubiquitination, and thus proteasomal degradation (Yu et al. 2003; Mangeat et al. 2003; Harris et al. 2003; Stopak et al. 2003). In this way Vif is precluding the presence of APOBEC3G in progeny virions. As a cytidine deaminase, APOBEC3G induces G to A hypermutation in proviral DNA and, thus, acts as an inhibitor of HIV-1 replication. Mechanistically, APOBEC3G causes changes of C to T in the viral DNA minus strand (Yu et al. 2004). This, in turn, leads to insertion of G in front of C, and A in front of T during DNA plus strand synthesis. The APOBEC3G induced hypermutation can interfere with HIV-1 replication in several ways: it may cause instability of the viral DNA, interference with plus-strand synthesis, or disturb functional gene expression from the proviral genome. The antiretroviral activity of APOBEC3G depends on its encapsidation into progeny virions, which is mediated through interaction with the NC region of the Gag polyprotein and/or with viral and nonviral RNAs (Alce and Popik 2004; Svarovskaia et al. 2004). It can be assumed that virion incorporation of APOBEC3G would maneuver the enzyme into close proximity with the reverse transcription complex.

Another example for host–virus interaction unraveled recently is the interaction between cellular and viral ion channel forming proteins, namely the HIV-1 specific virus protein u (Vpu) and the widely expressed acid-sensitive two-pore K^+ background channel TASK-1 (Hsu et al. 2004, for review see Strebel 2004). Most intriguingly, this interaction may be the consequence of a so called 'molecular piracy' as the N-terminal TM domain of TASK-1 shares striking similarity to that of Vpu. Further, the physical interaction between both TM domains may cause the intercalation of TASK-1 and Vpu into hetero-oligomeric channel complexes and, thus, may explain why TASK-1 suppresses Vpu-mediated virus release while Vpu abolishes TASK-1 channel conductance. This hypothesis was supported by the observation that sole expression of the homology sharing TM domain of TASK-1 can functionally complement the virus release function of Vpu. However, independent of whether Vpu itself acts as an ion channel or instead regulates the activity of a cellular ion channel, it remains an open question how the host cell membrane conductance controls late steps in virus budding.

A further accessory HIV protein was recently shown to interact specifically with a cellular factor: the HIV-1 Nef mimics an integrin receptor signal and thus recruits a nuclear transcription repressor host protein to the cell membrane (Witte et al. 2004). As detailed below, Nef is thought to obstruct T-cell activation by recruiting certain cellular factors into a so far undefined signaling complex to the cell membrane. Specifically, Nef directs the Polycomb Group (PcG) protein Eed, which represents a nuclear repressor of transcription, to the inner leaflet of the cell membrane. The Nef-mediated re-translocation of Eed causes activation of the Tat-dependent HIV-1 LTR promoter driven transcription, most likely because of the depletion of Eed repressor from the nucleus.

Further well-characterized examples for host virus protein interaction are the virus receptors (CD4 and chemokine receptors) which enable virus entry into specific host cells (Berger et al. 1999), the role of the chromatin-remodeling system (Yung et al. 2001) and the HMG I family proteins (Farnet and Bushman 1997) for proviral DNA integration, as well as the requirement of different factors of the endosomal protein trafficking and ubiquitinglation systems for virus release (for review see Perez and Nolan 2001; Vogt 2000; Luban 2001).

MOLECULAR STRUCTURE AND FUNCTION OF HIV REGULATOR PROTEINS

In addition to the canonical retroviral Gag, Pol, and Env proteins common to replication-competent retroviruses, HIV-1 and other lentiviruses encode further small proteins with either essential regulatory (Tat and Rev) or accessory (Vpu, Vif, Nef, and Vpr) functions that serve to accelerate viral replication. While Tat is critical for transcription driven by the HIV-1 LTR promoter, Rev is essential for transport of viral RNAs from the nucleus to the cytoplasm. The function of these essential regulatory HIV genes will not be discussed in this review. The focus will be on the accessory regulator proteins, with special emphasis on Vpr and Vpu. These proteins also called 'auxiliary,' since they are not essential for HIV-1 replication in certain cell lines, yet a growing number of in vivo studies report important roles of these HIV-1 gene products in the pathology and spread of HIV-1 (for review see Cohen et al. 1996).

THE LENTIVIRUS PROTEIN R (VPR)

The highly conserved 96-amino acid, 14-kDa viral protein R (Vpr), has received considerable attention and a number of biological functions have been attributed to its presence in various cellular and extra cellular compartments. The most intensively investigated biological functions of Vpr are those affecting the translocation of the PIC of the incoming virus from the cytoplasm to the nucleus, and the arrest in the G_2 phase of the cell cycle (for a recent comprehensive resume see Sherman and Greene 2002; Sherman et al. 2002; Bukrinsky and Adzhubei 1999). The nuclear targeting

function of Vpr has been associated with HIV-1 infection of terminally differentiated macrophages. Regarding the second function of Vpr, that leads to G_2 cell cycle arrest in HIV-1-infected and/or Vpr-transfected human cells, it was suggested that this activity provides an intracellular milieu conducive to enhanced viral replication by increasing HIV-1 LTR-driven gene expression (Goh et al. 1998). This response has recently been linked with the capacity of Vpr to alter the structure of the nuclear lamina leading to transient, DNA-containing herniations of the nuclear envelope that intermittently rupture (de Noronha et al. 2001). Other studies suggest that the prolonged G_2 arrest induced by Vpr ultimately leads to apoptosis of the infected cell (Poon et al. 1998; Stewart et al. 2000). Conversely, early anti-apoptotic effects of Vpr have been described which are overlayed by its pro-apoptotic effects (Conti et al. 2000). These pro-apoptotic effects of Vpr may result from either effects on the integrity of the nuclear envelope or direct mitochondrial membrane permeabilization (Jacotot et al. 2001), perhaps involving Vpr-mediated formation of ion channels in cellular membranes (Piller et al. 1996).

Vpr is a virion-associated (Paxton et al. 1993), nucleocytoplasmic shuttling (Sherman et al. 2001) regulatory protein that is encoded by (and conserved among) primate lentiviruses, HIV-1, HIV-2, and the SIVs. Although dispensable for growth of HIV-1 in dividing cultured T cells, Vpr appears to play an important role in virus replication in vivo, since deletion of *vpr* and the related *vpx* genes in SIV severely compromises the pathogenic properties in experimentally infected rhesus macaques (Gibbs et al. 1995; Lang et al. 1993). Furthermore, HIV-2 and SIV also encode an additional Vpr-related protein, Vpx, that is believed to function synergistically with Vpr. The Vpr of HIV-1 is reported to exhibit numerous biological activities, including nuclear localization (based on the presence of at least two nuclear localization signals) (Heinzinger et al. 1994; Jenkins et al. 1998; Vodicka et al. 1998; Popov et al., 1998a, b; Sherman et al. 2000, 2001), ion channel formation (Piller et al. 1996), transcriptional activation of HIV-1 and heterologous promoters (Stark and Hay 1998; Felzien et al. 1998), co-activation of the glucocorticoid receptor (Kino et al. 1999), regulation of cell differentiation (Levy et al. 1993), induction of apoptosis (Stewart et al. 1997; Ayyavoo et al. 1997), cell cycle arrest (Levy et al. 1993; Rogel et al. 1995), and transduction through cell membranes (Henklein et al. 2000a). Although significant amounts of Vpr (approximately 0.15-fold molar ratio to viral core proteins, Müller et al. 2000) are packaged into budding HIV-1 particles in a process dependent on Vpr's interaction with the C-terminal $p6^{gag}$ domain of the Gag polyprotein precursor Pr55 (Paxton et al. 1993), the biological role(s) of virion associated Vpr remains to be fully elucidated.

As the biologically relevant activity of the multifunctional Vpr has not yet been clarified, the molecular bases for many of its effects remain elusive. To date, structural studies of the full-length molecule have been hampered by the fact that this protein does not crystallize and the use of NMR techniques is complicated by the strong tendency for Vpr to undergo self-association. In addition, Vpr's structure depends critically on the solution conditions, and an unresolved apparent heterogeneity in the oligomeric composition of the polypeptide has been observed (Wecker et al. 2002; Henklein et al. 2000b). Many structural characterizations of Vpr have shown the important influence of environmental factors and indicate that in vivo folding of Vpr will probably require the presence of structure-stabilizing interacting factors (for review see Bukrinsky and Adzhubei 1999). The secondary structures in Vpr emerging from these analyses suggest the presence of an α-helix–turn–α-helix motif between residues 17 and 48 and an amphipathic α-helix located between amino acids 53–55 and 78–83 (Schüler et al. 1999). These helices are highly conserved and most likely play a key role in self-association and the interaction of Vpr with heterologous proteins (Felzien et al. 1998). Various investigations have shown that the C-terminal domain participates in a number of specific protein–protein and protein–nucleic acid interactions, such as that with NCp7 (de Rocquigny et al. 1997), Tat (Sawaya et al. 1999), RNA (de Rocquigny et al. 2000), and adenine nucleotide translocator of the mitochondrial pore (Jacotot et al. 2000).

STRUCTURE AND FUNCTION OF THE HIV-1 SPECIFIC VPU PROTEIN

The 81 amino acid Vpu is encoded exclusively by HIV-1 (Cohen et al. 1988; Strebel et al. 1988), with one exception, the HIV-1 related isolate SIV_{cpz} that encodes a Vpu-like protein similar in length and predicted structure to the Vpu from HIV-1 isolates (Huet et al. 1990). The HIV-1 specific accessory gene *vpu* encodes an integral membrane phosphoprotein with various biological functions (for review see Bour and Strebel 2003): firstly, in the ER Vpu induces degradation of CD4 in a process involving the ubiquitin-proteasome pathway and CK-2 phosphorylation of its cytoplasmic tail (Vpu_{CYTO}) (Schubert et al. 1998; Margottin et al. 1998). In addition, there is evidence that Vpu interferes with the major histocompatibility complex (MHC) class I antigen presentation and regulates Fas-mediated apoptosis (Kerkau et al. 1997; Komoto et al. 2003; Akari et al. 2001). Secondly, Vpu augments virus release from a post-ER compartment by a cation-selective ion channel activity mediated by its transmembrane anchor (Vpu_{TM}) (Schubert et al., 1996a, and references therein). It was shown previously that Vpu can regulate cationic current when inserted into planar lipid bilayers and in *Xenopus*

oocytes (Schubert et al. 1996a; Coady et al. 1998). However, the detailed mechanism how Vpu as a membrane conductance regulator supports virus budding remains elusive. The new insights are indicating that the virus release function of Vpu_{TM} involves the mutually destructive interaction between Vpu and the K^+ channel TASK-1 (Hsu et al. 2004, for review see Strebel 2004). Thus, it is now conceivable that Vpu affects membrane conductance through tuning the activity of host-cell ion channels (for review see Lamb and Pinto 1997).

Vpu is a class I oligomeric membrane-bound phosphoprotein (Maldarelli et al. 1993) composed of an amphipathic sequence of 81 amino acids comprising a hydrophobic N-terminal membrane anchor proximal to a polar C-terminal cytoplasmic domain. The latter contains a highly conserved region, from Glu-47 to Gly-58 that is present in all analyzed Vpu sequences. It also contains the phosphorylation sites for the casein kinase two (CK-2) that regulate Vpu's function in the ER (Schubert et al. 1994; Schubert and Strebel 1994). The structure and the orientation in the membrane of the N-terminal hydrophobic Vpu_{TM}, which is primarily associated with an ion-channel activity, either by itself (Schubert et al. 1996a) or in the context of full-length Vpu (Ewart et al. 1996), was first analyzed using the synthetic peptide Vpu^{1-39} (Wray et al. 1999). The tertiary U-shaped turn structure identified in solution, however, was inconsistent with the formation of ion-conducting membrane pores in planar lipid bilayers (Schubert et al. 1996a) since it is unlikely to be able to span the bilayer. Consequently, proton-decoupled ^{15}N cross-polarization solid-state NMR spectroscopy has been employed to investigate full-length Vpu and its isolated domains oriented in phospholipid bilayers using either synthetic discretely ^{15}N-labeled amino acids (Wray et al. 1999; Henklein et al. 2000b), or uniformly ^{15}N-labeled recombinant material (Marassi et al. 1999; Ma et al. 2002). Those solid state NMR data were consistent with a transmembrane alignment of a helical polypeptide, implying that the nascent helices in the folded solution structure reassemble to form a linear α-helix involving residues 6 to 29 that lies parallel to the bilayer normal with a tilt angle of $\leqslant 30^\circ$ (Wray et al. 1999) and placing Trp^{22} near to and the Glu^{28}-Tyr-Arg-motif that is know to be important for helix termination, anchorage, and pore selectivity. Most recently, attention has focused on the ion channel activity of Vpu and its similarity to other viral ion channels (for review see Fischer and Sansom 2002). Molecular dynamic investigations of various Vpu fragments, combined with conductance measurements, provide evidence for water-filled five-helix bundles. In summary, the monomeric structure model of Vpu provides details of the topology and positions of secondary structure in the membrane bound state. However, more experimental and theoretical work is required to understand Vpu in its functional form in vivo where it exists as the phosphoprotein in multi-protein complexes involving CD4 and β-TrCp (Margottin et al. 1998), or in homo-oligomeric nonphosphorylated form required for its ion-channel activity in the cell membrane.

THE VIRAL INFECTIVITY FACTOR VIF

The 23-kDa phosphoprotein Vif represents a viral infectivity factor that is highly conserved among all lentiviruses with one exception, the equine infectious anemia virus (EIAV). Vif regulates virus infectivity in a cell-type-dependent fashion. Permissive cells like HeLa, Cos 293T, SupT1, CEM-SS, and Jurkat cells support HIV replication independently of Vif. In contrast, primary lymphocytes, macrophages, and certain cell lines that might express a specific cellular Vif interacting factor require Vif for production of fully infectious viruses (Strebel et al. 1987; Simon et al. 1998).

Until recently, the mechanism of how Vif supports formation of infectious virions was enigmatic. Contemporary findings, detailed above, support the intriguing hypothesis that at least one main function of Vif is to neutralize a natural host defense mechanism. This novel innate anti-HIV defense mechanism was recently revealed as the cellular cytidine deaminase APOBEC3G, which together with Vif is encapsidated into progeny virions (for review see Navarro and Landau 2004). Intriguing was the long known fact that Vif can act in *trans* when it is expressed in the virus producer, but not in the target cell, indicating that Vif must be situated at the virus assembly site (von Schwedler 1993). Consistent with this is the fact that relatively low levels of Vif molecules are detected in mature HIV-1 virions, yet its encapsidation is increased when unprocessed Gag or certain Gag mutants are expressed (Sova et al. 2001). Heterokaryon analysis of fusions between permissive and nonpermissive cells revealed that nonpermissive cells must contain a so far unknown, naturally occurring antiviral factor that is neutralized by Vif of the incoming virus (Simon et al. 1998a). This model of a post-entry function for Vif is further supported by the observation that Vif from diverse lentiviruses function in a species specific mode (Simon et al. 1998b). Several hypothesis have been put forward to explain the mechanism of Vif function: the activity of Vif might regulate reverse transcription and proviral DNA synthesis (Dornadula et al. 2000), Vif can support the formation of stable virus cores (Ohagen and Gabuzda 2000), or Vif might bind to viral RNA in order to support post-entry steps of virus replication (Khan et al. 2001). The discovery of the APOBEC3G–Vif interaction provides at least some explanation for these previous observations (Navarro and Landau 2004).

THE MULTIFUNCTIONAL NEF PROTEIN

Originally, the 27 kDa N-terminal myristoylated membrane-associated phosphoprotein Nef was described as a 'negative factor' that suppresses the HIV-LTR promoter activity (for review see Freed 2001; Frankel 1998; Steffens and Hope 2001; Marsh 1999). As one of the most intensively investigated HIV-1 accessory proteins, Nef has been shown to fulfill multiple functions during the viral replication cycle. However, the mechanism(s) of Nef has not yet been fully elucidated. In general, it is now widely accepted that, inconsistent with its originally 'negative' nomenclature, Nef plays an important stimulatory role in the replication and pathogenesis of HIV-1. The broad spectrum of Nef-associated activities so far described can be summarized in distinct classes: (i) modulation of cell activation and apoptosis, (ii) change in intracellular trafficking of cellular proteins, particularly of cell receptor proteins like CD4 and MHC class I, and (iii) increase of virus infectivity.

Well characterized and one of the earliest observation in the course of studying '*Nefology*' is the Nef-induced down-regulation of cell surface CD4 (Garcia and Miller 1991). This mechanism occurs on a post-translational level by augmentation of receptor internalization followed by lysosomal degradation, where Nef is binding to the adapter protein complex in clathrin-coated pits (Aiken et al. 1994). As there are at least two other mechanisms of HIV-1 receptor interference, induced by the envelope and Vpu proteins of HIV-1, it was speculated that intervention with the primary virus receptor, CD4, must be advantageous for HIV-1 replication. Indeed, excess of cell-surface CD4 was shown to interfere with encapsidation of Env and release of infectious HIV progeny viruses (Lama et al. 1999; Ross et al. 1999). Another influence on immune receptors is the Nef-mediated block in the transport of MHC-I antigen complexes to the cell surface, leading to the disturbance in the recognition of HIV-1 infected cells by cytotoxic T_{CD8} lymphocytes (CTL) (Collins et al. 1998; Schwartz et al. 1996, Le Gall et al. 2000). More recent studies described down-regulation of co-stimulatory protein CD28 induced by Nef (Swigut et al. 2001). As it appears, the mechanism(s) by which Nef blocks cell surface expression of CD4, MHC-I, and CD28 are independent and can be genetically separated (Le Gall et al. 2000; Piguet and Trono 2001; Piguet et al. 1999, 2000).

Nef is expressed relatively early in the HIV replication cycle, and there are reports that Nef is incorporated into budding virions where it can be cleaved by the viral protease. Nef is present during almost all steps of the viral lifecycle which might explain the multiple functions deployed by this accessory protein. Major attention has been focused towards the Nef-mediated modulation of the T-cell signal transduction and activation pathways (for review see Fackler and Baur 2002). Several lines of evidence supported the hypothesis that, by affecting the T cell receptor, Nef may support several aspects of the virus replication cycle, including regulation of apoptosis and immune evasion. Earlier studies in transgenic mice indicated Nef-dependent activation in T-cell signaling (Skowronski et al. 1993). Later studies indicate that Nef imitates T-cell receptor signalling motifs (Simmons et al. 2001). The Nef-induced T-cell activation requires the tyrosine kinase Zap70 and the zeta chain of the T-cell receptor (Xu et al. 1999). Further, Nef was shown to activate T-cells by binding to the Nef-associated kinase, also known as the p21-activated kinase 2 (PAK2) (Arora et al. 2000; Renkema et al. 2001). In addition to the activation of T-cells, Nef also induces cell death, although the mechanism and biological relevance of this Nef-function is less clear. It was also shown that expression of the death receptor Fas ligand (FasL) is activated by Nef and that this upregulation might increase killing of CTLs attacking HIV infected cells (Xu et al. 1997).

In terms of HIV related pathogenesis, it is becoming clear that the multifunctional Nef protein represents an attractive target for development of antiviral strategies, since this accessory protein has been explicitly characterized as a viral factor that increases the pathological potential and the replication of lentiviruses in vivo. For instance, virus isolates from so called long-term nonprogressing HIV-1 infected individuals maintain deletion in *nef* alleles (Rhodes et al. 2000). Consistent with the idea that Nef supports virus replication in vivo, Nef of SIV was shown to elevate virus replication and disease progression in SIV-infected rhesus macaques (Kestler et al. 1991).

DYNAMICS OF VIRAL REPLICATION

Before the direct quantification of plasma viral RNA was possible, estimates of viral dynamics were based on indirect parameters such as anti-HIV-1 antibody titer (Gaines et al. 1987), p24 antigenaemia (Paul et al. 1987), CD4 cell count (Goedert et al. 1987) and clinical progression. This lead to the belief that the natural course of HIV-1 infection included a 'latent stage' in which little or no viral replication occurred. It was not until 1989 that Ho et al. showed that viral replication and immune destruction was an on-going and active process. Thereafter, Pantaleo et al. (1993) and Embretson et al. (1993) demonstrated active viral replication during the 'latent' stage in tissues such as lymph nodes, tonsils and adenoids, and in cells such as T-helper cells and macrophages. The availability of drug regimes that could suppress all viral replication facilitated investigation of the dynamics of viral replication. Two groups (Ho et al. 1995; Wei et al. 1995) made use of viral load measurements to assay the rate of decline of plasma viremia in the presence of drug. They found that viral load fell exponentially within two weeks of starting therapy and that this decline was a combination of the clearance of free virus from the plasma and the removal of

infected, productive cells. Both groups independently calculated the half-life of a virion in plasma to be 2–2.1 days, a higher replication rate than previously expected, and estimated viral production to be at least 10^8 or 10^9 virions per day.

Another study (Perelson et al. 1996) exploited the new potent antiretroviral therapy to demonstrate an exponential decrease in plasma viral load and to calculate the half-life of virion clearance to be 6 hours and the daily rate of virus production to be 10.3×10^9, greater than a one-log increase on previous estimations. It was calculated that 99 percent of plasma virus was released by recently infected cells. One year later, using a triple antiretroviral regime, Perelson and co-workers (1997) demonstrated that the therapy-induced viral decline was a two-stage event. This involved loss of plasma virus and virus from recently infected cells, followed by the loss of virus from long-lived infected cells such as tissue macrophages or dendritic cells, the half-life being approximately 1–4 weeks. A third stage was also predicted in which latently-infected cells required activation prior to virus production. Ho's prediction that HIV-1 could be eradicated from latently-infected cells with 3 years of Combined Antiretroviral Therapy (CART) (Ho 1997) was undermined by data showing that virus could still be detected from these latently infected cells after more than 2 years of CART, despite an undetectable plasma viremia (Wong et al. 1997b; Finzi et al. 1997; Chun et al. 1997).

The interplay between the immune response and virion production produces the viral load 'set point', which is prognostic of clinical outcome and disease progression in an infected individual (Mellors et al. 1996). It was proposed that lymphoid tissues trap virions early in infection, but as the lymphoid structure becomes damaged more virus is released to the circulating plasma. Calculations based on CD4 cell dynamics (Ho et al. 1995; Wei et al. 1995) estimated a daily turnover of between 1.8 and 2×10^9 cells, and led David Ho to propose the 'tap and sink' analogy for immune depletion. He hypothesized that the pool of CD4 cells was maintained as fast as it was destroyed during early infection but that, with time, the tap runs dry as new cells become fewer. Eventually the sink empties. Newer techniques using glucose-labeled CD4 cells have provided an insight into real-time CD4 cell dynamics and suggest that, in fact, HIV-1 infection affects the half-life of the CD4 cells, rather than their rate of production (Hellerstein et al. 1999). Hence, although the initial effect of CART is to increase the rate of CD4 cell production, the long-term aim is to increase the lifespan of cells.

ENZYME INFIDELITY AND GENETIC VARIATION

The *Lentiviridae* undergo extensive genetic variation. Variability results in part from RT infidelity combined with a lack of 3′-5′ exonuclease activity, a proof-reading function common in mammalian polymerase enzymes which repairs incorrectly synthesized DNA. HIV-1 has one of the highest mutation rates of retrovirus RTs. In vitro studies on purified RT have estimated the error rate to be one base in every 3.4×10^5 (Preston et al. 1988; Roberts et al. 1988; Mansky and Temin 1995). When this mutation rate is considered in tandem with the estimated replication rate of ten billion new virions each day, the scope for diversity becomes apparent – every possible single point mutation is created daily in each infected individual. As a result, mutations that confer drug resistance may be present at undetectable levels prior to the commencement of anti-viral therapy. The majority of mutations are point mutations consisting of base-pair substitutions, although base insertions and deletions have been identified (Larder et al. 1999).

An alternative means by which variation may be introduced is via recombination. Each virion contains two RNA copies, both of which may contribute to a growing DNA strand during reverse transcription. Two viruses containing unique drug resistance mutations may create a single multidrug resistant virus by recombination (Moutouh et al. 1996). Following infection, the HIV-1 population develops into a 'quasi species' of genetic variants and as infection progresses, the homogeneity of the infecting population increases (McNearney et al. 1992). Viral variants may have different cell tropisms (e.g. brain and lymphoid tissue (Simmonds et al. 1991; Di Stefano et al. 1996) and co-receptor usage (Deng et al. 1996; Oberlin et al. 1996). Such genetic variation is inherent to the evolution of HIV-1 at an individual level, but also on a more global level, as illustrated by the presence of subtypes.

HIV-1 SUBTYPES

Analysis of viral sequences allows HIV-1 to be classified into three groups, representing the distinctive HIV-1 lineages, Main (M), Outlier (O) (Gurtler et al. 1994) and Non-M-Non-O (N) (Simon et al. 1998). The M group was initially subtyped following analyses of *gag* gene sequences from virus isolated in 15 countries (Louwagie et al. 1993), and has been further divided into individual subtypes (A–K) (Figure 1). Isolates containing RNA from more than one subtype are now appearing and have been termed circulating recombinant forms (CRF) (Carr et al. 1998), of which at least 14 have been identified (Robertson et al. 2000). The predominant example of a CRF is subtype AE, prevalent in Thailand. That these viruses might represent recombinants was confirmed from sequence analyses of *gag* and *env* genes (McCutchan et al. 1992; Louwagie et al. 1993), and from analyses of complete subtype AE genomes (Carr et al. 1996; Gao et al. 1996). However, the nonrecombinant subtype E 'source' isolate has yet to be identified, leading to uncertainty concerning the origins of

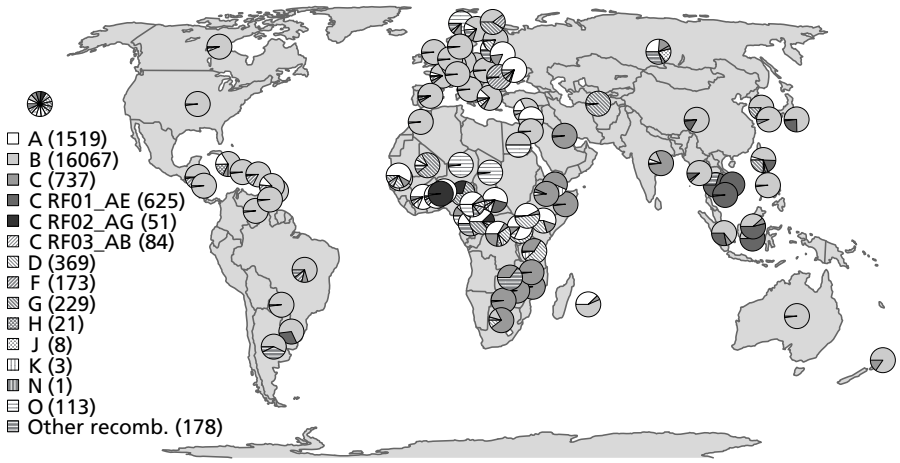

Figure 1 *HIV-1 subtype distribution worldwide. The global distribution of HIV-1 subtypes (from Los Alamos National Laboratory Database (2002) wwwl/:hiv-web.lanl.gov/geography/index.html). Subtypes are represented by color, with the green subtype B predominating in Europe and the USA and the red, blue, and pink of subtypes A, C, and D, respectively, being prevalent in sub-Saharan Africa.*

subtype AE. Sub-subtypes are lineages closely related to particular subtypes, but not genetically distant enough to justify calling a new subtype. For example, subtype F comprises sub-subtype F1 and a newly found lineage sub-subtype F2 (Triques et al. 1999). A third F sub-subtype, F3, was later reclassified as subtype K (Triques et al. 2000). The subtypes of most HIV-1 strains can be determined by sequence analysis of any region of the genome as inter-subtype nucleotide sequence divergence may exceed 20, 15, and 25 percent for *gag*, *pol*, and *env*, respectively (Robertson et al. 2000).

HIV-1 subtypes show different geographical distributions. The majority of HIV-1 infections in Europe and North America are with subtype B, while in sub-Saharan Africa, subtypes A, C, and D predominate (Figure 1). Nevertheless, in the UK 25 percent of HIV-1 infections are estimated to be caused by non-B subtypes (Parry et al. 2001). In East Africa, subtypes A and D are most common, whereas in the south and south-east, subtype C predominates, although this subtype is also spreading eastwards (Koch et al. 2001). Cameroon is a source of Group O (Mauclere et al. 1997) and group N strains (Ayouba et al. 2000). Other continents display subtype variability. For example in India and south-east Asia subtype C is common (Bollinger et al. 1995) and in Thailand subtypes B and AE predominate (Gao et al. 1996; Ou et al. 1993).

In South America subtypes B, C, and F are found (Campodonico et al. 1996; Bongertz et al. 2000), and the developing epidemic in the former Soviet Union comprises subtypes A, B, C, G, and AB (Liitsola et al. 1998; Lukashov et al. 1995).

HIV-1 and HIV-2 show 50 percent genetic similarity (Korber et al. 1995) and demonstrate differing degrees of pathogenicity, transmissibility, and drug susceptibility (Marlink et al. 1994; Pauwels et al. 1990). Subtype variability may impact on issues as diverse as vaccine design, detection, and quantification of HIV-1 infection, transmissibility, and pathogenicity.

THE CLINICAL IMPACT OF VIRAL DIVERSITY

Viral diversity impacts on the diagnosis of HIV-1 infection. The assay that is most widely applied to HIV-1 diagnosis is the enzyme-linked immunosorbent assay (ELISA). Until recently, ELISAs for the detection of antibodies to HIV-1 and HIV-2 were not equally sensitive for all subtypes. The earliest ELISAs failed to detect antibodies to group O and HIV-2, although incorporating antigens from these strains into newer assays overcame the problem (Schable et al. 1994). A recent study of the ability of six ELISAs to detect antibodies from all the major subtypes showed that the assays detected most HIV-1 group M variants (Koch et al. 2001) as well as group N (Masciotra et al. 2000).

Other clinical assays affected by viral diversity are those quantifying HIV infection. Viral load (VL) is the number of HIV-1 RNA copies per milliliter of plasma, and various techniques, based on differing technologies, are available for its quantification. The Roche Amplicor HIV-1 Monitor v1.5 assay (Roche Diagnostics, New Jersey, USA) (Mulder et al. 1994) uses reverse transcription and then PCR to amplify and quantify a region of the *gag* gene. The nucleic acid sequence-based amplification assay (NASBA) (Organon Teknika, Boxtel, The Netherlands) (Kievits et al., 1991; de Baar et al. 2001) directly amplifies RNA from *gag*. The HIV-1 Quantiplex branched DNA (bDNA) assay (Chiron Diagnostics, Emeryville, California, USA) (Pachl et al. 1995; Todd et al. 1995) uses sequential hybridization of probes targeting the *pol* gene to quantify viral RNA and the LCx quantitative assay (Abbott) uses RT-PCR amplification of *pol* (Swanson et al. 2000).

Each technique has a different sensitivity for viral load measurement, according to the subtype being tested (Alaeus et al. 1999; Holguin et al. 1999; Parekh et al. 1999; Clarke et al. 2000). This may preclude the comparison of viral loads from patients infected with different HIV-1 subtypes and, if different assays are used for consecutive readings in the same patient, any change in viral load may be difficult to interpret. For example, early versions of the Amplicor and NASBA assays detected HIV-1 subtypes A and H with lower degrees of sensitivity than other Group M subtypes (Coste et al. 1996), although comparisons of the more recent versions of these assays show that all subtypes are equally quantified (Swanson et al. 2001). As many viral load assays are PCR-based, polymorphic variability may compromise the binding of primers. Accordingly, assays can be improved by utilizing highly conserved regions of the genome, such as *pol* (Ernest et al. 2001) or by exploiting non-PCR amplification techniques, such as the bDNA assay (Parekh et al. 1999).

The influence of subtype on viral transmissibility has also been suggested by a number of studies, although data are limited. The difficulty with interpreting such studies is that there are many confounding factors. For example, coinfection with sexually transmitted pathogens such as *Neisseria gonorrhoeae* or herpes simplex virus also increase transmissibility (del Mar Pujades et al. 2002; Fawzi et al. 2001). There are data that suggest that subtype B isolates are less transmissible than non-B counterparts (Cameron et al. 1989; van Harmelen et al. 1997), and that the maternal transmission rate is lower in Tanzanian mothers infected with HIV-1 subtype D, than in those infected with A or C (Renjifo et al. 2001). Without larger controlled studies, such findings should be regarded with caution, particularly in the light of evidence to suggest that factors, such as viral load, significantly impact on virus transmission (Shaffer et al. 1999).

The degree of pathogenicity associated with HIV-1 infection is also multifactorial and, accordingly, attributing an effect to subtype is difficult. Some studies suggest that the rate of disease progression is similar, regardless of subtype (Alaeus et al. 1999; Weisman et al. 1999; Amornkul et al. 1999), whereas a study in Senegal suggested a survival advantage for those infected with subtype A HIV-1 (Kanki et al. 1999). More recently, in a study from Uganda, viruses with subtype D envelope were associated with faster disease progression (relative risk of death, 1.29; $P = 0.009$) and with a lower CD4 cell count ($P = 0.001$), compared with subtype A (Kaleebu et al. 2002).

SUBTYPE VARIABILITY AND THE PROSPECT OF A VACCINE

The lack of a vaccine for HIV-1 remains one of the major obstacles to controlling the epidemic. A number of approaches have led to clinical trials, including live vectors which express selected HIV-1 genes (Gupta et al. 2002; Corey et al. 1998; Ferrari et al. 1997), DNA vaccines (Caselli et al. 1999; Ayyavoo et al. 2000), live-attenuated *nef*-deleted virions (Khatissian et al. 2001; Wyand et al. 1999), and killed viral constructs (Moss et al. 2001). The high HIV-1 mutation rate and the development of quasi species facilitate the development of 'escape mutants' (Barouch et al. 2002) and restrict vaccine design. By using vaccines that utilize highly conserved regions of the HIV-1 genome, such as Tat and Rev, the impact of genetic variability is reduced and, accordingly, these vaccines have also been shown to be active against a range of subtypes (Goldstein et al. 2001), eliciting humoral and cellular responses (Caselli et al. 1999; Cafaro et al. 1999).

The extent of subtype effect on vaccine design remains unclear, especially as the majority of vaccines are designed to inhibit HIV-1 subtype B. Neutralizing antibodies are subtype-specific (Mascola et al. 1994), although broad cross-reactivity of neutralizing antibody activity to subtypes B and C has been reported (Bures et al. 2002; Dorrell et al. 1999). Cross-clade T-cell responses have been elicited. For example, an HIV-1 gp160-DNA construct encoding subtype B *env*, *tat*, and *rev* genes elicited specific and cross-reactive cell-mediated immune responses in mice (Arora and Seth 2001) and similar responses have been reported in human volunteers in Uganda (Cao et al. 2000; Van der Groen et al. 1998). Vaccines are being developed to express epitopes from a range of subtypes. For example, antibody responses to both subtypes AE and B have been reported using a vaccine that contains gp120 subunits from both (Berman et al. 1999). As many countries are affected by more than one subtype, it is likely that any effective vaccine will be required to induce broad responses (Agwale et al. 2002; Novitsky et al. 2001) and, accordingly, genetic libraries are being developed to document HIV-1 genetic variability with the aim of improving vaccine design (Mukai et al. 2002).

In 1991, antisera from human volunteers immunized with recombinant gp120 immunogens were examined for their capacity to neutralize field isolates of HIV-1 grown in primary peripheral blood mononuclear cells (PBMC) (McCormack et al. 2000; Beddows et al. 1999). None of the vaccine sera neutralized any primary HIV-1 isolates, even when homologous in sequence to the laboratory adapted isolates which could be neutralized by these sera. These data showed that the neutralization of primary isolates could not be achieved by sera raised to recombinant proteins, although these field isolates could be neutralized by human antisera from HIV+ subjects. Further work (Spenlehauer et al. 1998; Zhang et al. 2002) showed that the neutralization of laboratory HIV-1 strains is predominantly through a linear epitope of 33 amino acids at the tip of the third variable domain of gp120, called the V3 loop. The V3 loop is expressed

on the surface of gp120 in cell-line adapted viruses, and is also present and highly immunogenic on the surface of the recombinant gp120 molecules. However, primary isolates of HIV-1 have a more cryptic V3 epitope, and antibodies to V3 do not neutralize these primary isolates if grown in primary cells. The molecular explanation for this phenomenon was published in 1996, with the characterization of the chemokine co-receptors for HIV entry. Primary isolates use CCR5 as the principal co-receptor, which is present on activated $CD4^+$ T cells; laboratory-adapted HIV-1 isolates grown in immortalized, transformed T-cell lines use CXCR4 as the co-receptor, which is responsible for the over-expression of V3 in these isolates (see earlier section for references).

Up to the present, September 2004, it has not been possible to engineer a recombinant envelope gp120 which induces antibodies that can neutralize primary HIV-1 isolates in PBMCs. It is highly likely that such an immunogen will require extensive investigation from structural biologists who may be able to engineer a gp120 molecule in its native trimeric conformation with the capacity to induce neutralizing antibodies against primary isolates. However, no such molecule is currently on the horizon.

Notwithstanding these negative neutralization experiments, the $rgp120_{MN}$ molecule was taken to two full phase III trials in the USA, Holland, and Thailand, by VaxGen. The first of these trials in >7000 HIV-negative subjects at risk of HIV-1 infection reported in March 2003 (www.vaxgen.com/pressrom/index.html 2003; Francis et al. 2003); there was no protection against infection, with equal numbers of infections in the active and placebo groups. The negative results of this VaxGen $rgp120_{MN}$ study have been bitterly disappointing for a world desperate for a prophylactic HIV vaccine, but extremely important for science. The results confirm the predictive value of laboratory-adapted and primary isolate neutralization data, and also support the predictive value of the macaque SHIV animal challenge model (Feinberg and Moore 2002; Moore and Burton 1999).

In the absence of natural immunity to HIV-1, and without an immunogen capable of inducing neutralization of all subtypes, attention turned to other correlates of protection from HIV-1. Immunologists studied children born to HIV-infected mothers, where only 30 percent become infected; long-term survivors of HIV-1 infection who might have beneficial immune responses; the primary immune response to HIV-1 where there is some evidence of immune-mediated viral suppression and the phenomenon of highly HIV-exposed but persistently HIV-sero-negative subjects (HEPS). These HEPS have been repeatedly described throughout the AIDS epidemic in all risk-groups. Rowland-Jones (1999), studying HEPS prostitute women in Nairobi, Kenya, demonstrated that absence of HIV-1 infection, despite repeated exposure to the virus, was associated with the presence of detectable HIV-specific, HLA class-I-restricted $CD8^+$ cytotoxic T-lymphocytes (CTL). When these women ceased regular commercial sex work, exposure to HIV lessened and they became more susceptible to infection. Her hypothesis was that exposure to HIV could, in some circumstances, lead to the induction of a protective cellular immunity.

This observation has subsequently led to a considerable amount of animal and human clinical research on the induction of HIV-specific cellular immunity through immunization. McMichael's group has shown that a priming immunization with DNA followed by boosting with a modified vaccinia virus (such as modified vaccinia Ankara, MVA) bearing the same genetic insert is a potent inducer of HIV-specific CTL (Mwau et al. 2004). In the macaque model, induction of SIV-specific CTL through DNA-MVA prime–boost immunization fails to protect against challenge with a pathogenic $SHIV_{89.6}$, but does lead to significant disease attenuation in the macaques (McMichael and Hanke 2003). There are now several clinical trials in humans at phase I/II of promising CTL-inducing immunogens; these include naked DNA, adenovirus, vaccinia, alphaviruses, peptides, and viruslike particles (VLP). However, the prospect of a prophylactic HIV vaccine which does not protect, but which might slow disease progression in vaccines would be extremely difficult to test in human trials, and would not fully address the world's urgent need for the prevention of HIV infection.

Should an effective HIV vaccine be developed, the immense genetic diversity of the HIV epidemic will place great evolutionary pressure on the virus to escape from vaccine-induced immunity. As with the emergence of point mutations conferring resistance to antiretroviral drugs, so vaccine-induced immunity is likely to promote naturally occurring mutants with lower susceptibility to vaccine induced cellular immunity. Phillips and co-authors (1991) first described the phenomenon of viral escape from a CTL epitope in 1991; the potential emergence of this phenomenon will need to be investigated in parallel to phase III vaccine trials in the future.

In 2004, there is no HIV vaccine. It is generally believed that a successful HIV vaccine will need to induce both HIV-specific cellular immunity as well as potent, broadly reactive primary isolate neutralizing antibodies. The reality of the research portfolio currently is that this prospect appears to be remote.

HIV THERAPY AND THE EMERGENCE OF RESISTANCE

In the two decades since the first antiretroviral was serendipitously identified, drugs to combat HIV infection have been developed at an increasing rate. These fall into well-defined categories (Table 1), according to which aspect of the virion lifecycle is being inhibited (Figure 2). CART, consisting of at least three antiretroviral drugs, is the current standard of care for

Table 1 *Drugs designed to inhibit HIV-1*

Site of action	Drug class	Drug
Reverse transcriptase	Nucleoside analogues	Zidovudine (AZT/ ZDV)
		Didanosine (ddI)
		Zalcitabine (ddC)
		Stavudine (D4T)
		Lamivudine (3TC)
		Abacavir (ABC)
		Emtricitibine (FTC)
	Nucleotide analogues	Adefovir Dipivoxil[a]
		Tenofovir PMPA
	Nonnucleoside analogues	Nevirapine (NEV)
		Delavirdine (DLV)
		Efavirenz (EFV)
Protease	Protease inhibitors	Saquinavir (hard and soft gel) (SQV)
		Ritonavir (RTN)
		Nelfinavir (NEL)
		Indinavir (IND)
		Amprenavir (AMP)
		Lopinavir
		Tipranavir[a]
		Atazanavir[a]
Integrase	Integrase inhibitors	S-1360[a]
Co-receptors	CCR5 inhibitor	SCH-C[a]
	CXCR4 inhibitor	AMD-3100[a]
Gp41	Fusion inhibitor	Envufutide (T-20)
		T-1249[a]

Drugs that contribute to the majority of CART regimes currently prescribed. Standard CART regimes include two nucleoside analogues in conjunction with either a protease inhibitor or a nonnucleoside analogue.
a) Still in research stages, under trial or not fully licensed at time of writing.

HIV-1-infected patients (Pozniak et al. 2003), and the availability of assays in simple kit form to quantify peripheral virus load has complemented drug development to optimize patient care (Nolte 1999).

Drugs designed to inhibit HIV-1 RT

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTI)

The essential action of the NRTI class is to mimic the natural host nucleoside triphosphate molecules (dNTP), which depends on replacing the hydroxyl group at the 3′ position of the deoxyribose sugar ring in the natural dNTPs by an alternative moiety in the drug (Figure 3). For example, the azido group at the 3′ position in Zidovudine (ZDV) (3′-azido-3-deoxythymidine, an analogue of thymidine, also called AZT or Retrovir and produced by GlaxoSmithKline) promotes the drug's chain-termination activity (Figure 3). This compound was initially designed as an anti-tumor agent and was found to inhibit the HIV-1 RT (Mitsuya et al. 1985). It competes in vitro with natural host nucleosides and is incorporated into the lengthening DNA strand by the viral RT, resulting in chain termination.

The NRTIs can be divided into the thymidine analogues: ZDV and Stavudine (2′,3′-didehydro-3′-deoxythymidine) or D4T, and non-thymidine analogues: Didanosine (2′,3′-dideoxyinosine), ddI; Zalcitabine (2′,3′-dideoxycytidine), ddC; Lamivudine ((-)-2′-deoxy-3′-thiothiadine), 3TC; Emtricitibine ((-)B-l-3′-thio-2′-3′-dideoxy-5-fluorocytidine), FTC and Abacavir (IS, 4R)-4-[2-amino-6(cyclopropyl amino)-9H-purin-9-yl]-2-cyclopentene-1-methanol succinate), ABC). Since the former act predominantly in dividing cells and the latter in resting cells, combinations of the two groups are often coprescribed. An early clinical trial, designated the DELTA study (Delta Coordinating Committee 1996) which investigated whether two NRTIs in combination were more effective than AZT alone in delaying disease progression, demonstrated that combining AZT with ddI or ddC conferred a survival advantage over AZT monotherapy and heralded the concept of combination therapy.

Initial treatment with AZT monotherapy resulted in viral rebound and the discovery of a genotypic pattern of resistance, as mutations emerged initially at codons 41 and 215, then at 67, 70, 210, and 219. An alternative pathway leading to AZT resistance and broad cross-class resistance is the 'Q151M complex' (Shirasaka et al. 1995) associated with mutations at codons 62, 75, 77, and 116. These mutations seem to act via interference with the dNTP binding site (Shirasaka et al. 1995). This is seen more commonly when AZT is prescribed with ddI or ddC and confers 320-fold resistance to AZT and cross-resistance to ddI, ddC, D4T, Abacavir, and 3TC.

NUCLEOTIDE REVERSE TRANSCRIPTASE INHIBITORS (NTRTI)

The NtRTI require two intracellular phosphorylation events for activity. Two drugs originally from Gilead Sciences (Adefovir dipivoxil and Tenofovir (bis-POC PMPA)) are already monophosphorylated and, therefore, require only a single monophosphorylation for activation. Adefovir, although not licensed following problems with renal toxicity, had shown potent reductions in viral load after one year of monotherapy and appeared to have a favorable resistance profile compared to the NRTIs. It was also active against herpes simplex virus, cytomegalovirus, and hepatitis B virus (Xiong et al. 1997; Merta et al. 1990). Tenofovir which has similar activity without the toxicity, received FDA approval in the United States in 2001.

Although Adefovir has been withdrawn, it was active against viruses with the RT mutation in codon 184 and with AZT resistance (Miller et al. 1999; Gu et al. 1995).

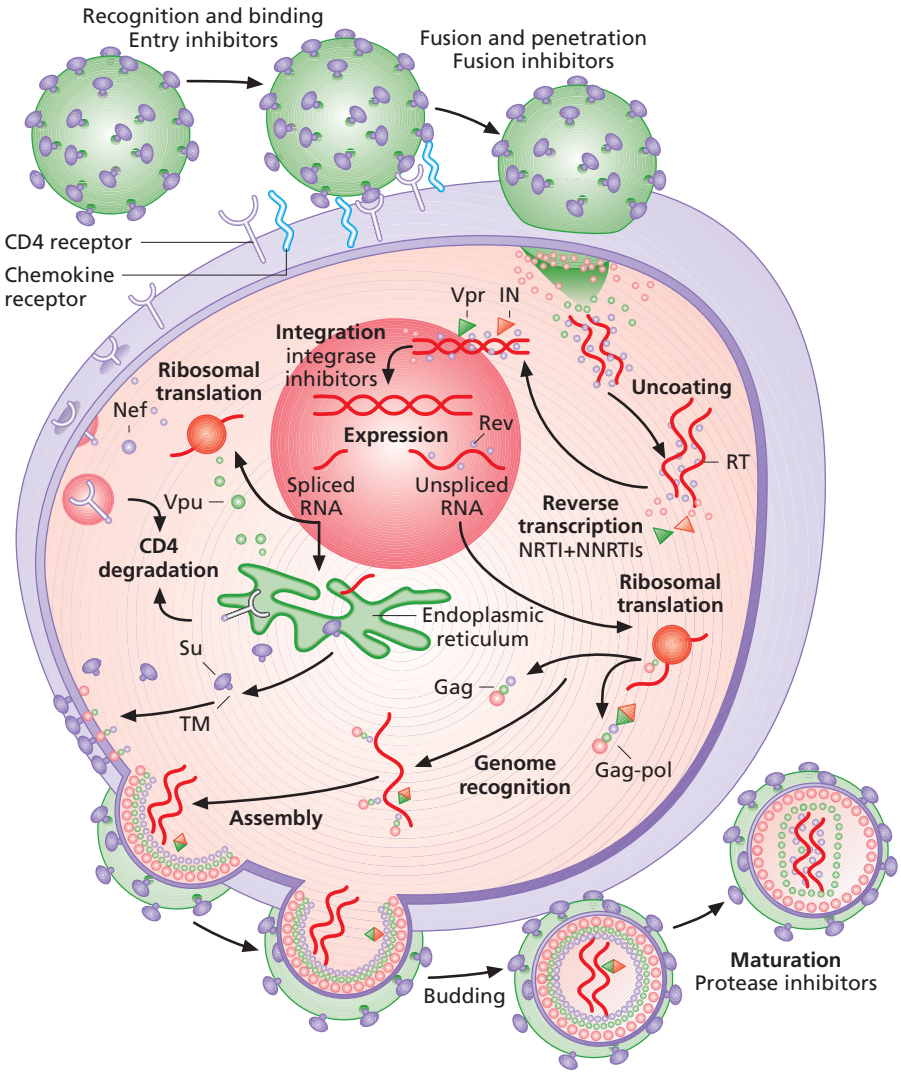

Figure 2 *The HIV-1 life cycle and site of action of antiretroviral drugs. Targets of drugs already approved for use by the FDA are shown in red. Others currently undergoing development and likely to be available for clinical trial in the near future are shown in blue. The HIV-1 core penetrates the host-cell post-entry via receptor-binding and fusion with the plasma membrane. Double-stranded proviral DNA resulting from the action of RT on the viral single-stranded RNA is translocated to the nucleus, where it is integrated into the host chromosomal DNA, courtesy of the viral integrase. Viral RNA is transcribed from the proviral DNA and viral proteins are translated from host cell ribosomes. New virions are assembled at the membrane from which they bud, maturing as they do so under the action of the viral protease. Redrawn from Pomerantz and Horn (2003) with permission.*

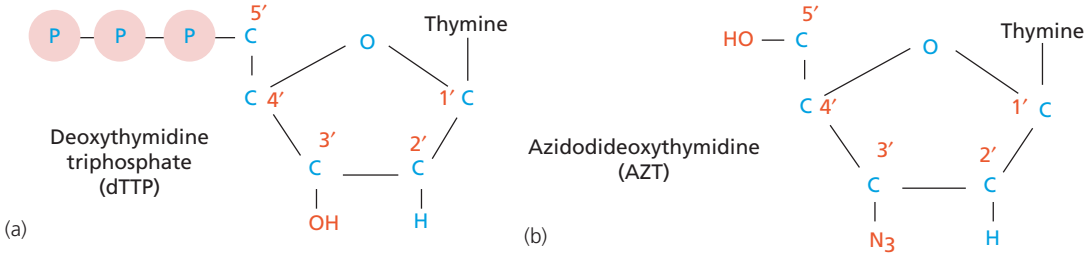

Figure 3 *Structure and mechanism of action of NRTIs. The structure of* **(a)** *the natural dNTP, deoxythymidine triphosphate (dTTP) and* **(b)** *azidodideoxythymidine (AZT, Zidovudine). The important difference is that the hydroxyl group attached to the carbon at position 3 of dTTP, which is vital to the DNA polymerase reaction, has been replaced by an azido group. Hence, when AZT is incorporated into the growing DNA strand, chain termination ensues.*

Mutations at codons 65 and 70 have been associated with resistance to Adefovir in vitro (Gu et al. 1995), and K70E has also been found in vivo (Mulato et al. 1998), although the significance of this is still undetermined. Interestingly, the activity of Adefovir against viruses containing the M184V mutation was 3–4-fold greater than against wild-type virus (Miller et al. 1999). Viruses containing the K65R and L210W mutation have reduced sensitivity to Tenofovir.

NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTI)

The NNRTIs include Nevirapine, Efavirenz, Delavirdine, and the thiobenzimidazolone (TIBO) agents. These are noncompetitive inhibitors of RT (Spence et al. 1995) and bind to a specific site associated with codons 100–108 and 181–190 that provide the hydrophobic binding pocket within the polymerase domain of the p66 subunit of RT (Spence et al. 1995). These codons are also sites for key resistance mutations.

Although potent, the NNRTIs induce early resistance (Richman et al. 1994), often associated with mutations near codons 100 to 108 and 181 to 190, the locations of the drug-binding site. The Y181C mutation emerges rapidly in vitro, to cause 100-fold decrease in susceptibility to Nevirapine (Richman et al. 1991) and highly resistant strains have been identified in vivo within eight weeks of therapy (Richman et al. 1994; de Jong et al. 1997). Drug levels which exceed the IC_{50} of the resistant strains can be achieved in vivo using the higher dose of 400 mg, although with a higher incidence of side-effects (Havlir et al. 1995).

The K103N mutation seems to produce classwide resistance, including to newer drugs such as Efavirenz (DMP-266) (De Clercq 1998), although Efavirenz maintains some activity in the presence of the Y181C mutation (Jeffrey et al. 1998). The quinolone/quinoxaline class of NNRTIs, such as Nevirapine, also develop resistance through mutation at codon 190, but at the expense of enzymatic activity (Kleim et al. 1994). The major concern with NNRTIs is that only one mutation is required to induce resistance and, hence, they should be used in combination with other antiretroviral agents.

Drugs designed to inhibit HIV-1 protease

The viral protease acts to cleave and process the precursor proteins encoded by the *gag* and *gag–pol* genes. By inhibiting this posttranslational cleavage, the resultant virions are non-infectious. The RT inhibitors only block HIV-1 replication in acutely infected cells, whereas the protease inhibitors (PI) inhibit virus production from chronically infected cells, since they act at a late stage in the lifecycle (Lambert et al. 1992). The PIs inhibit the protease enzyme by acting as peptidomimetic competitive inhibitors at the enzymatic active site. Therapy with PIs also appears to have a more suppressive effect on HIV-1 replication in lymph nodes than the RTIs (Gunthard et al. 1998a; Wong et al. 1997a).

Resistance and cross-resistance to PIs are well documented and at least 24 mutations have been identified in the 99 amino acid sequence (Figure 4). To provide prolonged efficacy, PIs need to be prescribed as part of a three-drug CART regime. Such regimes significantly reduce mortality and morbidity associated with HIV-1 infection (Palella et al. 1998) and, accordingly, have been given the acronym 'HAART,' for Highly Active Antiretroviral Therapy.

The interpretation of mutations associated with PI resistance is complex, especially with drugs such as Indinavir (Condra et al. 1996), when a large number of codons appear to be involved. Moreover, resistance is only one factor leading to drug failure. Others include the effects of bioavailability (Kupferschmidt et al. 1998; Vella and Floridia 1998; Perry and Noble 1998), cytochrome P450 metabolism (Wacher et al. 1998; Kupferschmidt et al. 1998), intolerance (Deeks et al. 1998; Sepkowitz et al. 1998; d'Arminio et al. 1998), and glycoprotein P transporter proteins (Kim et al. 1998; Wacher et al. 1998). Nevertheless, resistance remains a major cause of drug failure, and the issue of cross-resistance is becoming increasingly important, as failure on one PI may preclude the use of others (Condra et al. 1995).

A promising new class of antiretroviral agents are the 'Fusion inhibitors.' Two agents, T20 and T1249, bind to the HR1 domain of gp41 and disrupt the conformational change in this protein required for viral attachment and entry to the host cell (Kilby et al. 1998b; Lambert et al. 1999). As these drugs are peptides they cannot currently be given orally and, in addition, resistance has already been documented (Kilby et al. 1998b). Agents which bind to the co-receptors CCR5 (Simmons et al. 1997) and CXCR4 (Rusconi et al. 2000) or inhibit the -CD4 interaction (Jacobson et al. 2000) are also in development. For example, data on SCH-C, a CCR5 antagonist, have shown a 0.5 $\log_{10}$ reduction in viral load in a limited number of subjects over 10 days (Reynes et al. 2002). A CXCR4 antagonist termed AMD-3100 reduces viral load by 0.8 $\log_{10}$ RNA copies/ml (Schols et al. 2002), although antiviral potency has been questioned (Hendrix et al. 2002). The novel entry inhibitor BMS-806, is an antagonist of CD4–gp120 interactions and is still in preclinical development (Lin et al. 2002). It is active against multiple HIV-1 subtypes and against viruses displaying CXCR4 and CCR5 tropism.

MECHANISMS OF DRUG RESISTANCE

The infidelity of the retroviral RT results in a high mutation rate when viral genomic RNA is converted to DNA, as discussed. In the absence of complete viral inhibition, as might be associated with poor adherence,

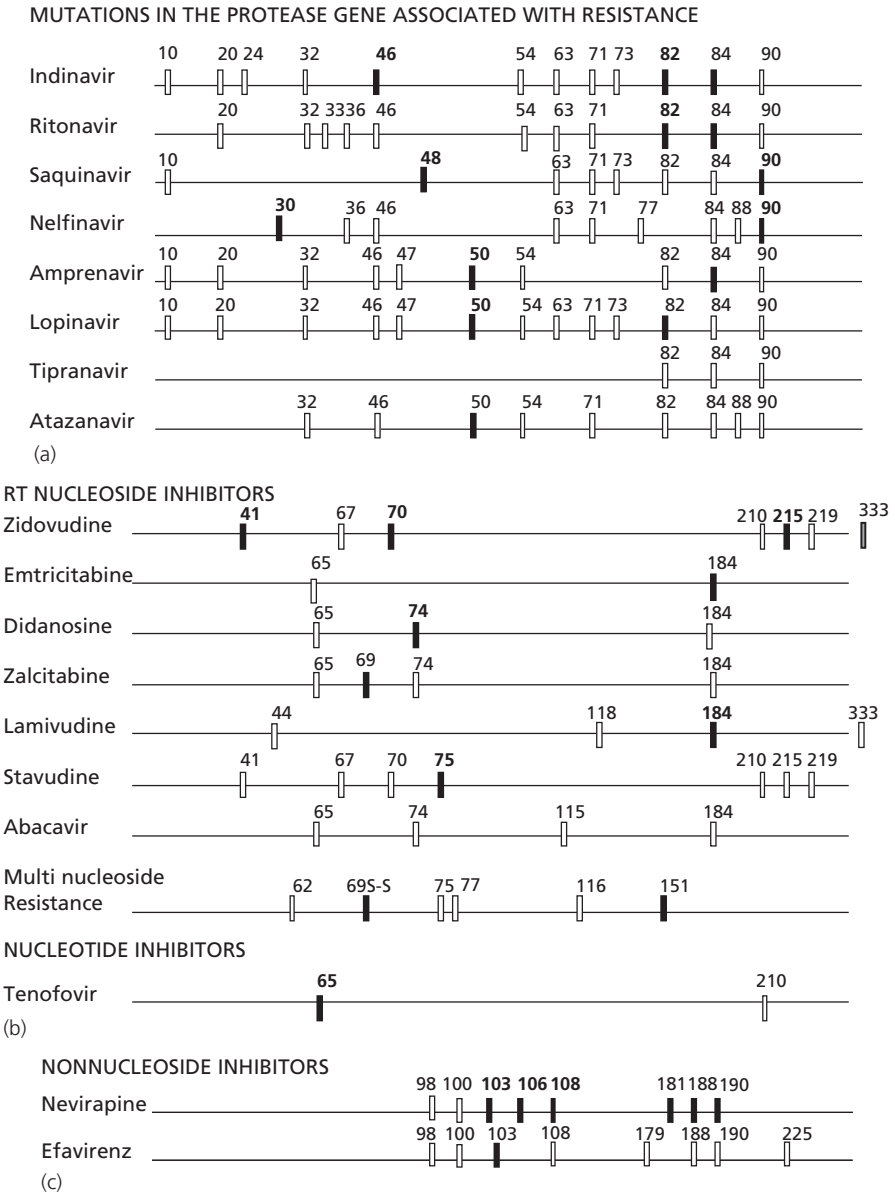

Figure 4 *Major point mutations associated with* **(a)** *PI resistance,* **(b)** *NRTI, and* **(c)** *NNRTI. Filled codons represent key mutations and white codons are secondary mutations.*

increased drug metabolism or drug sanctuary sites, there is increased opportunity for selection of drug-resistant mutants. According to the principles of natural selection, these variants soon become dominant and replicating viral RNA becomes detectable in the plasma. The recurrence of detectable plasma virus on therapy is often described as virological or therapeutic 'failure.' The point mutations in the *pol* gene that are commonly associated with drug resistance are detailed in Figure 4.

Although the genetic basis of HIV-1 drug resistance is well documented, the mechanisms underlying the loss of drug sensitivity are only now being understood. For the NRTI class of antiretroviral agents, there are two possible biochemical explanations for resistance. Either the retroviral RT develops increased ability to discriminate between natural dNTP molecules and inhibitors, or the enzyme acquires the ability to remove the chain-terminating inhibitors from the extending viral DNA in a process called 'pyrophosphorylysis' (Meyer et al. 1998; Boyer et al. 2001).

Certain mutations in HIV-1 RT decrease the activity of the nucleoside analogue by reducing its binding or altering the orientation of its binding so as to be unfavorable for incorporation. An example of such a mutation is the methionine to valine mutation at codon 184 of HIV-1 RT responsible for high-level resistance to 3TC and cross-resistance to other NRTIs (Tisdale et al. 1993). The beta-methyl group of the valine clashes

sterically with the l-oxathiolane ring of 3TC, resulting in mal-positioning of 3TC-triphosphate and a markedly reduced incorporation of 3TC and other NRTIs into the viral cDNA.

The second mechanism, pyrophosphorylysis, is the opposite reaction to reverse transcription, resulting in the removal of chain-terminating inhibitors from the extending viral DNA (Meyer et al. 1998; Boyer et al. 2001). Some AZT resistance mutations enhance the ability of HIV-1 RT to excise AZT-monophosphate from the AZT-terminated cDNA, freeing up the 3′ hydroxyl group (Figure 1) and allowing DNA polymerization to proceed. This excision reaction is catalyzed by ATP, and some AZT resistance mutations (e.g. 210W, 215Y/F) probably increase the binding affinity of ATP to the mutant enzyme. This enhanced excision capability of the mutant enzyme is not specific for AZT-monophosphate-terminated cDNA, and this helps to explain the cross-resistance observed between AZT and some other nucleoside analogues, such as D4T and Abacavir (Lennerstrand et al. 2001). Additionally, the 69SS mutation that confers multidrug resistance to the NRTI class also increases pyrophosphorylysis (Mas et al. 2000). Mutations such as M184V in RT, which confer resistance to 3TC, inhibit pyrophosphorylysis (Gotte et al. 2000). The M184V mutation alters the polymerase active site to interfere specifically with the ATP-mediated excision of AZT-MP from the end of the primer strand and, thereby, partially restores sensitivity to other NRTIs, such as AZT (Boyer et al. 2002).

The 20-year anniversary of the discovery of HIV-1 was celebrated in 2003. Within two decades research has changed the diagnosis of HIV infection in the western world from a certain death sentence to a manageable condition. However, viral latency (Wong et al. 1997b; Finzi et al. 1997), the recognition of tissue compartmentalization of replication which facilitates an evolutionary path independent of that taken by viruses in the periphery (Haggerty and Stevenson 1991; Zhu et al. 1996; Overbaugh et al. 1996; Zhang et al. 1998) and the persistence of low-level replication despite HAART (Dornadula et al. 1999; Dornadula et al. 2001), all conspire to undermine efforts to eradicate HIV-1 from infected hosts. Much remains to be understood of the way in which the virus responds in vivo to protect itself from annihilation, and further elucidation of the mechanism of replication will undoubtedly impact on the development of more novel and effective treatment regimens.

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Prions of humans and animals

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PROPERTIES OF PRIONS

Introduction

Prion diseases, also termed transmissible spongiform encephalopathies (TSE), are inevitably fatal neurodegenerative conditions that affect humans and a wide variety of animals (Aguzzi et al. 2001c). Although prion diseases may present with certain morphological and pathophysiological features that parallel other progressive encephalopathies, such as Alzheimer's and Parkinson's disease (Aguzzi and Haass 2003; Aguzzi and Raeber 1998), they are unique in that they are transmissible. Homogenization of brain tissue from affected individuals and intracerebral inoculation into another individual of the same species will typically reproduce the disease. This important fact was recognized almost six decades ago in the case of scrapie (Cuille and Chelle 1939), a prototypic prion disease that affects sheep and goats.

The agent that elicits TSEs was termed prion by Stanley B. Prusiner, and defined as 'a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids' (Prusiner 1982). However, within the present chapter the term 'prion' will be used operationally to denote the infectious agent, without implying that it possesses particular chemical or structural characteristics.

Prions certainly differ from all other known infectious pathogens in several respects. First, prions do not appear to contain an informational nucleic acid genome longer than 50 bases that would code for their progeny. Second, the only known component of the prion is a modified protein that is encoded by a cellular gene. Third, the major, and possibly only, component of the prion is the scrapie isoform of the prion protein (PrP^{Sc} or PrP-res), which is a disease-associated conformer of the cellular isoform PrP^{C} (also termed PrP-sen).

A fundamental event in prion diseases is a conformational change that occurs during the conversion of PrP^{C} into PrP^{Sc}. PrP^{C} has been identified in all mammals and birds examined to date, as well as in the frog *Xenopus laevis* (Strumbo et al. 2001), and in fish (Oidtmann et al. 2003; Rivera-Milla et al. 2003). PrP^{C} is anchored to the external surface of cells by a glycolipid moiety (Stahl et al. 1987). The function of PrP^{C} is unknown. All attempts to identify post-translational chemical modifications that distinguish PrP^{Sc} from PrP^{C} have been unsuccessful to

date (Stahl et al. 1993). PrP^{C} contains approximately 45 percent α-helix and two very short stretches of β-sheet (Riek et al. 1996). Conversion to PrP^{Sc} creates a protein that contains approximately 30 percent α-helix and 45 percent β-sheet. The mechanism by which PrP^{C} is converted into PrP^{Sc} remains unknown but PrP^{C} seems to bind to PrP^{Sc}, perhaps along with ancillary proteins, to form an intermediate complex during the formation of nascent PrP^{Sc} (Meier et al. 2003). Transgenic (Tg) mouse studies have provided genetic evidence that incoming prions in the inoculum interact preferentially with homotypic PrP^{C} during the propagation of prions (Prusiner et al., 1990; M. Scott et al. 1993).

The human prion diseases are referred to as kuru, Creutzfeldt–Jakob disease (CJD), variant CJD (vCJD), Gerstmann–Sträussler–Scheinker (GSS) disease, and fatal familial insomnia (FFI). The most common prion diseases of animals are scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) or 'mad cow' disease, and chronic wasting disease (CWD) of deer and elk (Table 1). Kuru was the first of the human prion diseases to be transmitted to experimental animals, and it has often been suggested that kuru spread among the Fore people of Papua New Guinea by ritual cannibalism (Gajdusek et al. 1966; Gajdusek 1977). The experimental and presumed human-to-human transmission of kuru led to the belief that prion diseases are infectious disorders caused by unusual viruses similar to those causing scrapie in sheep and goats. Yet a paradox was presented by the occurrence of CJD in families, first reported more than 70 years ago (Kirschbaum 1924; Meggendorfer 1930), which appeared to be a genetic disease. The significance of familial CJD remained largely unappreciated until mutations in the protein coding region of the PrP gene on the short arm of chromosome 20 were discovered (Sparkes et al. 1986; Hsiao et al. 1989). The earlier finding that brain extracts from patients who had died of familial prion diseases inoculated into experimental animals often transmit disease posed a conundrum that was resolved with the genetic linkage of these diseases to mutations of the *PRNP* gene that encodes PrP^{C} (Masters et al. 1981a; Prusiner 1989a; Tateishi et al. 1992a). To date, all cases of familial prion disease were shown to cosegregate with *PRNP* missense or, in one case, nonsense mutations.

Table 1 *Human prion diseases*

Disease	Etiology
Kuru	Infection
Creutzfeldt–Jakob disease:	
Iatrogenic	Infection
Sporadic	Unknown
Familial	*PRNP* mutation
Variant	Presumed BSE infection
Gerstmann–Sträussler–Scheinker disease	*PRNP* mutation
Fatal familial insomnia	*PRNP* mutation

The most common form of prion disease in humans is sporadic CJD (sCJD). Its cause is unknown. Many attempts to show that the sporadic prion diseases are caused by infection have been unsuccessful (Malmgren et al. 1979; Brown et al. 1987; Harries Jones et al. 1988; Cousens et al., 1990). The discovery that inherited prion diseases are caused by germline mutation of the PrP gene raised the possibility that sporadic forms of these diseases might result from a somatic mutation (Prusiner 1989a). Alternatively, since PrP^{Sc} is formed from the cellular isoform of the prion protein, PrP^{C}, by a post-translational process (Borchelt et al. 1990), sporadic prion diseases may result from the spontaneous conversion of PrP^{C} into PrP^{Sc}.

CJD was reported to have a worldwide incidence of one case per 10^{6} inhabitants annually (Masters and Richardson 1978). However, in countries that carry out active surveillance programs, reported CJD incidence is often higher (Aguzzi et al. 2000a), and in Switzerland it has reached $3.0/10^{6}$/year (Glatzel et al. 2002, 2003b), suggesting that many cases may go undetected. Less than 1 percent of CJD cases are infectious, and most of those seem to be iatrogenic. Between 10 and 15 percent of cases of prion disease are inherited, whilst the remaining cases are sporadic. Kuru was once the most common cause of death among New Guinea women in the Fore region of the Highlands (Gajdusek and Zigas 1957, 1959; Gajdusek et al. 1966) but has virtually disappeared with the cessation of ritualistic cannibalism (Alpers 1987; Mead et al. 2003). Most patients with CJD present primarily with dementia, but approximately 10 percent exhibit cerebellar dysfunction as the initial sign. People with either kuru or GSS usually present with ataxia, whereas those with FFI manifest insomnia and autonomic dysfunction (Hsiao and Prusiner 1990; Brown 1992; Medori et al. 1992b).

PrP^{CJD} has been found in the brains of most patients who died of prion disease. The term PrP^{CJD} is sometimes used when referring to the abnormal isoform of PrP in human brain. In this chapter, PrP^{Sc} is used interchangeably with PrP^{CJD}. PrP^{Sc} is always used after human CJD prions have been passaged into an experimental animal, because the nascent PrP^{Sc} molecules are produced from host PrP^{C} and the PrP^{CJD} in the inoculum serves only to initiate the process. In the brains of some patients with inherited prion diseases, as well as transgenic mice expressing mouse (Mo) PrP with the human GSS point mutation (Pro→Leu), detection of PrP^{Sc} by western blotting has been problematic despite clinical and neuropathological hallmarks of neurodegeneration (Hsiao et al. 1990, 1994), but histological techniques such as histoblotting (Taraboulos et al. 1992a) have often revealed protease-resistant PrP.

Experimental transmission of neurodegeneration from the brains of patients with inherited prion diseases to

inoculated rodents has been less frequent than with sporadic cases (Tateishi et al. 1992a). Whether this distinction between transmissible and nontransmissible inherited prion diseases will persist is unclear. Transgenic mice expressing a chimeric Hu/Mo PrP gene are highly susceptible to human prions from sporadic and iatrogenic CJD (iCJD) cases (Telling et al. 1994), and – somewhat unexpectedly – transgenic mice expressing a full-length bovine *Prnp* transgene appear to be the best model for transmission of human prions to date (Scott et al. 1999). There is hope that suitable transgenic mice may eventually make the use of apes and monkeys for the study of human prion diseases unnecessary. To date, this is not yet possible for several reasons:

- the molecular determinants that determine species barriers are not fully understood, and may encompass species-specific host factors additionally to PrP
- the peripheral pathophysiology of prion diseases may differ considerably between mice and humans.

Scrapie is the most common natural prion disease of animals. An investigation into the etiology of scrapie followed the vaccination of sheep for louping ill virus with formalin-treated extracts of ovine lymphoid tissue unknowingly contaminated with scrapie prions (Gordon 1946). Two years later, more than 1 500 sheep developed scrapie from this vaccine. In the late 1990s a similar incident led to widespread scrapie infection of Italian sheep herds. Although the transmissibility of experimental scrapie became well established, the spread of natural scrapie within and among flocks of sheep remained puzzling. Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease which could be eradicated by proper breeding protocols (Parry 1962; Parry and Livett 1973). He considered its transmission by inoculation to be of importance primarily for laboratory studies and communicable infection of little consequence in nature. Other investigators viewed natural scrapie as an infectious disease and argued that host genetics only modulates susceptibility to an endemic infectious agent (Dickinson et al. 1965).

Prion-infested offal is thought to be responsible for the epidemic of BSE (Wilesmith et al. 1992b). Prions in the offal from scrapie-infected sheep, or perhaps from rare cows affected by hypothetical 'sporadic BSE,' seem to have survived the rendering process that produced meat and bone meal (MBM). Whether the 'Ur-BSE prion' originated from sheep, or was autochtonous to cows, is a question that may not be solvable, and is of mainly academic and legal significance.

The MBM was fed to cattle as a nutritional supplement. After BSE was recognized, MBM produced from domestic animal offal was banned from further use. Since 1986, when BSE was first recognized, >180 000 cattle have died of BSE. More than 130 persons have developed a 'new variant' of CJD (Chazot et al., 1996; http://www.doh.gov.uk/cjd/stats/aug02.htm, 2002; Will et al. 1996), which is likely to represent transmission of BSE to humans (Bruce et al. 1997; Collinge et al. 1996; Hill et al. 1997a).

As we learn about the molecular and genetic characteristics of prion proteins, our understanding of prions and their place in biology will undoubtedly undergo considerable change. Indeed, the discovery of PrP, the identification of pathogenic *PRNP* gene mutations, the differences in the structures of PrP^{C} and PrP^{Sc}, as well as studies of the process by which prions move from the site of infection to the brain, have already forced us to think about these diseases from viewpoints that had not been previously considered.

Development of the prion concept

HYPOTHESES ON THE NATURE OF THE SCRAPIE AGENT

The published literature contains a fascinating record of the structural hypotheses for the scrapie agent, proposed to explain the unusual features first of the disease and later those of the infectious agent. Among the earliest hypotheses was the notion that scrapie was a disease of muscle caused by the parasite *Sarcosporidia* (M'Fadyean, 1918; M'Gowan 1914). With the successful transmission of scrapie to animals, the hypothesis that scrapie is caused by a 'filtrable' virus became popular (Cuille and Chelle 1939; Wilson et al. 1950). With the findings of Tikvah Alper and her colleagues that scrapie infectivity resists inactivation by ultraviolet (UV) and ionizing irradiation (Alper et al. 1966, 1967), a myriad of hypotheses on the chemical nature of the scrapie agent emerged, including the quite prescient conjecture that it may consist of a self-replicating protein (Griffith 1967).

BIOASSAYS OF PRION INFECTIVITY

The experimental transmission of scrapie from sheep (Gordon 1946) to mice (Chandler 1961) gave investigators a more convenient laboratory model, which yielded considerable information on the nature of the unusual infectious pathogen that causes scrapie (Alper et al. 1966, 1967, 1978; Gibbons and Hunter 1967; Pattison and Jones 1967; Millson et al. 1971). Yet progress was slow because quantification of infectivity in a single sample required holding 60 mice for at least one year before accurate scoring could be accomplished (Chandler 1961).

Attempts to develop a more economical bioassay by relating the titre to incubation times in mice were unsuccessful (Eklund et al. 1963; Hunter and Millson 1964). However, some investigators used incubation times to characterize different 'strains' of scrapie agent, whilst others determined the kinetics of prion replication in

rodents (Dickinson and Meikle, 1969; Kimberlin and Walker 1978a, 1979). Quantitative bioassays based on incubation times had been applied to the measurement of picorna and other viruses three decades earlier (Gard 1940). After scrapie incubation times were reported to be approximately 50 percent shorter in Syrian hamsters than in mice (Marsh and Kimberlin 1975), studies were undertaken to determine whether the incubation times in hamsters could be related to the titer of the inoculated sample. It was found that there was an excellent inverse correlation between the length of the incubation time and the logarithm of the dose of inoculated prions, and a more rapid and economical bioassay was developed (Prusiner et al. 1980a, 1982b). This improved bioassay for the scrapie agent in Syrian golden hamsters accelerated purification of the infectious particles by a factor of nearly 100. Still, the soaring costs of animal caretaking make it impossible for most institutions to carry out large-scale incubation-time-based prion titration studies. The recent development of a quantitative, highly sensitive cell-based bioassay by Klohn and Weissmann may foster a much-needed quantum leap in prion analytics (Klohn et al. 2003), which could prove crucial for many practical purposes, such as validation of prion decontamination procedures.

THE PRION CONCEPT

Once an effective protocol was developed for preparation of partially purified fractions of scrapie agent from hamster brain, it became possible to demonstrate that the procedures that modify or hydrolyze proteins produce a diminution in scrapie infectivity (Prusiner et al. 1981a, 1982b). At the same time, tests done in search of a scrapie-specific nucleic acid were unable to demonstrate any dependence of infectivity on a polynucleotide (Prusiner 1982; Riesner et al. 1993), in agreement with earlier studies reporting the extreme resistance of infectivity to UV irradiation at 254 nm (Alper et al. 1967).

On the basis of these findings, it seemed unlikely that the infectious pathogen capable of transmitting scrapie was a virus or a viroid. For this reason the term 'prion' was introduced to distinguish the proteinaceous infectious particles that cause scrapie, CJD, GSS, and kuru from both viroids and viruses (Prusiner 1982). Hypotheses for the structure of the infectious prion particle included:

- proteins surrounding a nucleic acid encoding them (a virus)
- proteins associated with a small polynucleotide
- proteins devoid of nucleic acid (Prusiner 1982).

Mechanisms postulated for the replication of infectious prion particles ranged from those used by viruses to the synthesis of polypeptides in the absence of nucleic acid template to post-translational modifications of cellular proteins. Subsequent discoveries have narrowed hypotheses for both prion structure and the mechanism of replication.

Considerable evidence has accumulated over the past decade supporting the prion hypothesis. But while the prion concept is now enjoying considerable acceptance, it should not go unmentioned that several core issues regarding prion replication are still entirely obscure (Chesebro 1998).

Discovery of the prion protein

Once the dependence of prion infectivity on protein was clear, the search for a scrapie-specific protein intensified. Although the insolubility of scrapie infectivity made purification problematic, this property was used by Prusiner along with its relative resistance to degradation by proteases to extend the degree of purification (Prusiner et al. 1980a, 1981b). In subcellular fractions from hamster brain enriched for scrapie infectivity, a protease-resistant polypeptide of 27–30 kDa (later designated $PrP^{27–30}$) was identified, which was absent from controls (Bolton et al. 1982; McKinley et al. 1983). Radioiodination of partially purified fractions revealed a protein unique to preparations from scrapie-infected brains (Bolton et al. 1982; Prusiner et al. 1982a).

Progress in the study of prions was greatly accelerated by the discovery of PrP and determination of its N-terminal sequence (Bolton et al. 1982; Prusiner et al. 1982a, 1984). Purification of $PrP^{27–30}$ to near-homogeneity allowed determination of its NH_2-terminal amino acid sequence. Determination of a single, unique sequence for the NH_2 terminus of $PrP^{27–30}$ permitted the synthesis of an isocoding mixture of oligonucleotides that was subsequently used to identify incomplete PrP cDNA clones from hamster (Oesch et al. 1985) and mouse (Chesebro et al. 1985). cDNA clones encoding the entire open reading frames (ORF) of Syrian hamster (SHa) and Mo PrP were subsequently recovered (Basler et al. 1986; Locht et al. 1986).

The experiments described above established that PrP is encoded by a chromosomal gene, and not by a nucleic acid within the infectious scrapie prion particle (Oesch et al. 1985; Basler et al. 1986). Levels of PrP mRNA remain unchanged throughout the course of scrapie infection – an unexpected observation which led to the identification of the normal PrP gene product, a protein of 33–35 kDa, designated PrP^{C} (Basler et al. 1986; Oesch et al. 1985). PrP^{C} is protease-sensitive and soluble in nondenaturing detergents, whereas $PrP^{27–30}$ is the protease-resistant core of a 33–35 kDa disease-specific protein, designated PrP^{Sc}, which is insoluble in detergents (Meyer et al. 1986).

THE *PRNP* GENE LOCUS

The entire ORF of all known mammalian and avian *Prnp* genes is contained within a single exon (Figure 1) (Basler et al. 1986; Westaway et al. 1987; Hsiao et al. 1989; Gabriel et al. 1992). This feature of the PrP gene eliminates the possibility that PrP^{Sc} arises from alter-

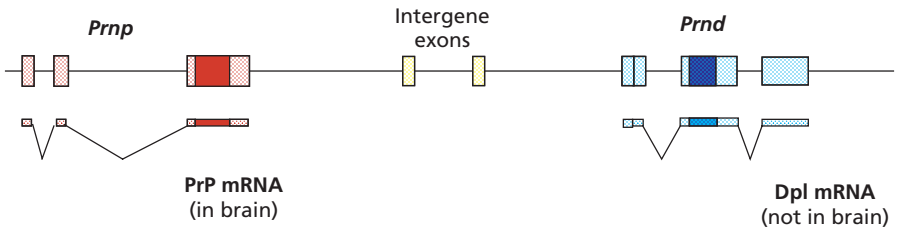

Figure 1 *Structure and organization of the chromosomal* Prnp *and* Prnd *genes. In all mammals examined the entire ORF is contained within a single exon. The 5′-untranslated region of the PrP mRNA is derived from either one or two additional exons (Basler et al. 1986; Puckett et al. 1991; Westaway et al. 1991, 1994a). Only one PrP mRNA has been detected. A prion protein homologue termed Dpl is encoded by the* Prnd *locus. PrP^{Sc} is thought to be derived from PrP^{C} by a post-translational process (Basler et al. 1986; Borchelt et al. 1990, 1992; Caughey et al. 1991b; Taraboulos et al. 1992b). The amino acid sequence of PrP^{Sc} is identical to that predicted from the translated sequence of the DNA encoding the PrP gene (Basler et al. 1986; Stahl et al. 1993), and no unique post-translational chemical modifications have been identified that might distinguish PrP^{Sc} from PrP^{C}. Thus, it seems likely that PrP^{C} undergoes a conformational change as it is converted to PrP^{Sc}. A second gene bearing remarkable structural homologies with* Prnp *was identified approximately 16 kb 3′ of the* Prnp *locus, and was termed* Prnd*. The product of this locus is termed* Doppel*.*

native RNA splicing (Basler et al. 1986; Westaway et al. 1987, 1991), although mechanisms such as RNA editing or protein splicing remain a possibility (Blum et al. 1990; Kane et al. 1990). The two exons of the SHaPrP gene are separated by a 10 kb intron: exon 1 encodes a portion of the 5′-untranslated leader sequence whilst exon 2 encodes the ORF and the 3′-untranslated region (Basler et al. 1986). The murine and ovine PrP genes are composed of three exons; exon 3 is analogous to exon 2 of the hamster (Westaway et al. 1991, 1994a). The promoters of both the SHa and Mo PrP genes contain multiple copies of GC-rich repeats and are devoid of TATA boxes. These GC nonamers represent a motif that may function as a canonical binding site for the transcription factor Sp1.

PrP genes map to the short arm of human chromosome 20 and to the homologous region of mouse chromosome 2 (Sparkes et al. 1986). Hybridization studies demonstrated <0.002 *Prnp* gene sequences per ID_{50} unit in purified prion fractions, strongly suggesting that a nucleic acid encoding PrP^{Sc} cannot possibly constitute a component of the infectious prion particle (Oesch et al. 1985). This is a major feature that distinguishes prions from viruses, including retroviruses that carry cellular oncogenes and from satellite viruses that derive their coat proteins from other viruses previously infecting cells. Although *Prnp* mRNA is constitutively expressed in the brains of adult animals (Chesebro et al. 1985; Oesch et al. 1985), it is highly regulated during development (Manson et al. 1992; Sales et al. 2002). In the septum, levels of PrP mRNA and choline acetyltransferase increased in parallel during development (Mobley et al. 1988). In other brain regions, PrP gene expression occurred at an earlier age.

PrP amyloid

The discovery of $PrP^{27–30}$ in fractions enriched for scrapie infectivity was accompanied by the identification of rod-shaped particles in the same fractions (Prusiner et al. 1982a, 1983). The fine structure of these rod-shaped particles failed to reveal any regular substructure. Indeed, the irregular ultrastructure of the prion rods differentiates them from most viruses, which have regular, distinct structures (Williams 1954), and made them indistinguishable ultrastructurally from many purified amyloids (Cohen et al. 1982). This analogy was extended when the prion rods were found to display the tinctorial properties of amyloids (Prusiner et al. 1983). These findings were followed by the demonstration that amyloid plaques in the brains of humans and other animals with prion diseases contain PrP, as determined by immunoreactivity and amino acid sequencing (Bendheim et al. 1984; DeArmond et al. 1985; Kitamoto et al. 1986; Roberts et al. 1988; Tagliavini et al. 1991).

Solubilization of $PrP^{27–30}$ into liposomes with retention of infectivity (Gabizon et al. 1987) suggests that large PrP polymers are not required for infectivity – although it is well possible that PrP^{Sc} oligomers may constitute nucleation centers pivotal to prion replication (Jarrett and Lansbury 1993; Lansbury 1994). In Tg(SHaPrP) mice inoculated with SHa prions, numerous amyloid plaques were found but none was observed if these mice were inoculated with mouse prions, indicating that amyloid formation is not an obligatory feature of prion diseases (Prusiner et al. 1990; Prusiner and DeArmond 1994). In agreement with these studies, PrP plaques are consistently found in some inherited prion diseases (Ghetti et al. 1989) but absent from others (Hsiao et al. 1991a).

Formation of PrP^{Sc}

Whether PrP^{C} is the substrate for PrP^{Sc} formation, or a restricted subset of PrP molecules are precursors for PrP^{Sc}, or whether reactive transition states exist (Weissmann 1991a), remains to be established. Several experimental results argue that PrP molecules destined to become PrP^{Sc} exit to the cell surface, as does PrP^{C} (Stahl et al. 1987) prior to its conversion into PrP^{Sc}

(Caughey and Raymond 1991; Borchelt et al. 1992; Taraboulos et al. 1992b).

Like other glycophosphatidylinositol (GPI)-anchored proteins, PrP^{C} seems to recirculate via a subcellular compartment bounded by cholesterol-rich, non-acidic, detergent-insoluble membranes denominated 'rafts' (Figure 2) (Keller et al. 1992; Anderson 1993; Shyng et al. 1994; Gorodinsky and Harris 1995; Taraboulos et al. 1995). Within the raft compartment, GPI-anchored PrP^{C} seems to be either converted into PrP^{Sc} or partially degraded (Taraboulos et al. 1995; Meier et al. 2003). After denaturation PrP^{Sc}, like PrP^{C}, can be released from the cell membranes by digestion with phosphatidylinositol-specific phospholipase C, suggesting that PrP^{Sc} is tethered only by the GPI anchor (Borchelt et al. 1993). In scrapie-infected cultured cells, PrP^{Sc} is trimmed at the N terminus to form $PrP^{27–30}$ in an acidic intracellular compartment (Caughey et al. 1991b; Taraboulos et al. 1992b). In contrast to cultured cells, the N-terminal trimming of PrP^{Sc} is minimal in brain, where little $PrP^{27–30}$ is found (McKinley et al. 1991a). Deleting the GPI addition signal resulted in greatly diminished synthesis of PrP^{Sc} (Rogers et al. 1993), and soluble dimeric PrP^{C} inhibits prion replication (Meier et al. 2003). In contrast to PrP^{C}, PrP^{Sc} accumulates primarily within cells, where it is deposited in cytoplasmic vesicles, many of which seem to be secondary lysosomes (Taraboulos et al. 1990b, 1992b; Caughey et al. 1991b; McKinley et al. 1991b; Borchelt et al. 1992). Studies of scrapie-infected brains suggest that PrP^{Sc} accumulates within either lysosomes or late endosomes (Laszlo et al. 1992; Arnold et al. 1995).

Although most of the difference in the mass of $PrP^{27–30}$ predicted from the amino acid sequence and observed after post-translational modification is due to complex-type oligosaccharides (Endo et al. 1989; Haraguchi et al. 1989), these sugar chains are not required for PrP^{Sc} synthesis in scrapie-infected cultured cells, based on experiments with the asparagine-linked glycosylation inhibitor tunicamycin, and site-directed mutagenesis studies (Taraboulos et al. 1990a).

SEARCH FOR A CHEMICAL MODIFICATION OF PRP

The discovery that the entire ORF of the PrP gene is contained within a single exon, first in Syrian hamsters and later in humans and other animals, argued that PrP^{Sc} is not generated by alternative splicing (Basler et al. 1986; Goldmann and Hunter 1988; Hsiao et al. 1989; Goldmann et al. 1991b; Westaway et al. 1994a, 1994b). This prompted a search for a post-translational chemical modification to explain the differences in the properties of these two PrP isoforms (Stahl et al. 1993). PrP^{Sc} was analyzed by mass spectrometry and gas phase sequencing in order to identify any amino acid substitutions or post-translational chemical modifications. The amino acid sequence was the same as

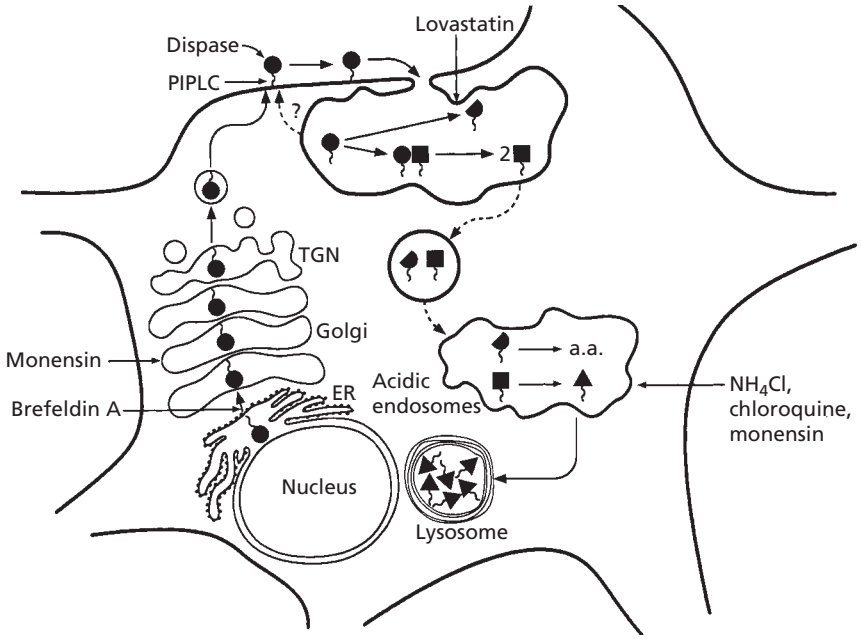

Figure 2 *Pathways of prion protein synthesis and degradation in cultured cells. PrP^{Sc} is denoted by squares; circles denote PrP^{C}. Before becoming protease-resistant, the PrP^{Sc} precursor transits through the plasma membrane and is sensitive to dispase or PIPLC added to the medium. PrP^{Sc} formation probably occurs in a compartment accessible from the plasma membrane, such as caveolae or early endosomes, both of which are non-acidic compartments. The synthesis of nascent PrP^{Sc} seems to require the interaction of PrP^{C} with existing PrP^{Sc}. In cultured cells, but not brain, the N terminus of PrP^{Sc} is trimmed to form $PrP^{27–30}$; PrP^{Sc} then accumulates primarily in secondary lysosomes. The inhibition of PrP^{Sc} synthesis by brefeldin A demonstrates that the endoplasmic reticulum (ER)–Golgi is not competent for its synthesis and that transport of PrP down the secretory pathway is required for the formation of PrP^{Sc}.*

that deduced from the translated ORF of the PrP gene, and no candidates for post-translational chemical modifications that might differentiate PrP^C from PrP^{Sc} were found (Stahl et al. 1993). These findings forced consideration of the possibility that conformation distinguishes the two PrP isoforms.

STRUCTURES OF PURIFIED PRP^C AND PRP^{SC}

Low-resolution structural studies indicated that PrP^C has a high α-helix content (ca. 40 percent) and little β-sheet (3 percent) (Pan et al. 1993). These findings were refined by Wüthrich and colleagues who determined the fine structure of PrP^C by nuclear magnetic resonance spectroscopy (Riek et al. 1996; Hornemann et al. 1997) (Figure 3a), and later also by crystallographic studies (Figure 3b) (Knaus et al. 2001). By contrast, the β-sheet content of PrP^{Sc} is ca. 40 percent and the α-helix ca. 30 percent as measured by Fourier-transformed infrared (FTIR) (Pan et al. 1993) and circular dichroism (CD) spectroscopy (Safar et al. 1993b) (Figure 4). No high-resolution structure is available for PrP^{Sc}, although interesting models have been conjectured on the basis of electron crystallography (Wille et al. 2002). That $PrP^{27–30}$ has a high β-sheet content was inferred from the green-gold birefringence of the prion rods after staining with Congo red (Prusiner et al. 1983). Indeed, $PrP^{27–30}$ polymerized into rod-shaped particles with ultrastructural appearance of amyloid (Figure 5) contains ca. 50 percent β-sheet and ca. 20 percent α-helix (Caughey et al. 1991a; Gasset et al. 1993; Safar et al. 1993a). In contrast to $PrP^{27–30}$ which did polymerize into rod-shaped amyloids, neither purified PrP^C nor PrP^{Sc} formed aggregates detectable by electron microscopy (Figure 5) (McKinley et al. 1991a; Pan et al. 1993).

It is still possible that a hitherto undetected chemical modification of a small fraction of PrP^{Sc} initiates the conversion process into PrP^{Sc}. Limitations of the current analytical techniques for proteins prevent a stringent study of this question, and it is quite possible that PrP^{Sc} consists of inhomogeneous species, only some of which are capable of propagation. Because PrP^{Sc} seems to be the only component of the 'infectious' prion particle, it is likely that this conformational transition is a fundamental event in the propagation of prions. Accordingly, denaturation of $PrP^{27–30}$ under conditions which reduced scrapie infectivity resulted in diminution of β-sheet content (Gasset et al. 1993; Safar et al. 1993b).

CONVERSION OF PRP^C INTO PRP^{SC}

Studies with Tg(SHaPrP) mice have provided genetic information about the process in which PrP^C is converted into PrP^{Sc} through the formation of a PrP^C/PrP^{Sc} complex (Prusiner et al. 1990), even if such complex was not isolated physically. The level of PrP^C expression seems to be directly proportional to the rate of PrP^{Sc} formation and, thus, inversely related to the length of the incubation time. Formation of the PrP^C/PrP^{Sc} complex was facilitated when the amino acid sequences of the two PrP isoforms were the same; SHaPrP differs from MoPrP at 16 positions out of 254. Differences in amino acid sequence delayed prion formation, resulting in a prolongation of the incubation time (Scott et al. 1989).

The protein-only hypothesis posits that the agent is devoid of nucleic acid and consists solely of an abnormal conformer of the cellular prion protein, PrP^C (Figure 6, p. 10).

In vitro conversion of recombinant ^{35}S-labeled PrP^C to a PrP^{Sc}-like protease-resistant moiety was achieved by mixing with a 50-fold excess of PrP^{Sc} (Kocisko et al. 1994). The binding of PrP^C to PrP^{Sc} depends on the same residues (Kocisko et al. 1995) that render Tg(MH2M) mice susceptible to SHa prions (M. Scott et al. 1993) and seems to be strain-dependent (Bessen et al. 1995). However, evidence is still lacking that the converted material acquires infectious properties (Aguzzi and Heikenwalder 2003).

Propagation of prions

Initial transgenetic studies had shown that the 'species barrier' between mice and Syrian hamsters for the transmission of prions can be abolished by expression of a SHaPrP transgene in mice (Scott et al. 1989). Transgenic mice expressing human (Hu) PrP were then constructed. These Tg(HuPrP) mice inoculated with human prions failed to develop CNS dysfunction more frequently than nontransgenic controls (Telling et al. 1994). Faced with this apparent dichotomy, Scott constructed mice expressing a chimeric Hu/Mo PrP transgene designated MHu2M because earlier studies had shown that chimeric Syrian hamster/mouse (SHa/Mo) PrP transgenes supported transmission of either Mo or SHa prions (Scott et al., 1992; M. Scott et al. 1993). The Tg(MHu2M) mice expressing the chimeric transgene were highly susceptible to human prions, suggesting that Tg(HuPrP) mice have difficulty converting $HuPrP^C$ into PrP^{Sc} (Telling et al. 1994). Although MoPrP and HuPrP differ at 28 residues, only nine or perhaps fewer amino acids in the region between codons 96 and 167 feature in the species barrier in the transmission of human prions into mice, as demonstrated by the susceptibility of Tg(MHu2M) mice to human prions.

When Tg(HuPrP) mice were crossed with gene-targeted mice in which the MoPrP gene had been disrupted ($Prnp^{0/0}$) (Büeler et al. 1992), they were rendered susceptible to human prions. These findings suggested that Tg(HuPrP) mice were resistant to human prions because $MoPrP^C$ inhibited the conversion of $HuPrP^C$ into PrP^{Sc}: once $MoPrP^C$ was removed by gene ablation, the inhibition was abolished (Telling et al. 1995). Whereas earlier studies argued that PrP^C forms a complex with PrP^{Sc} during the formation of nascent

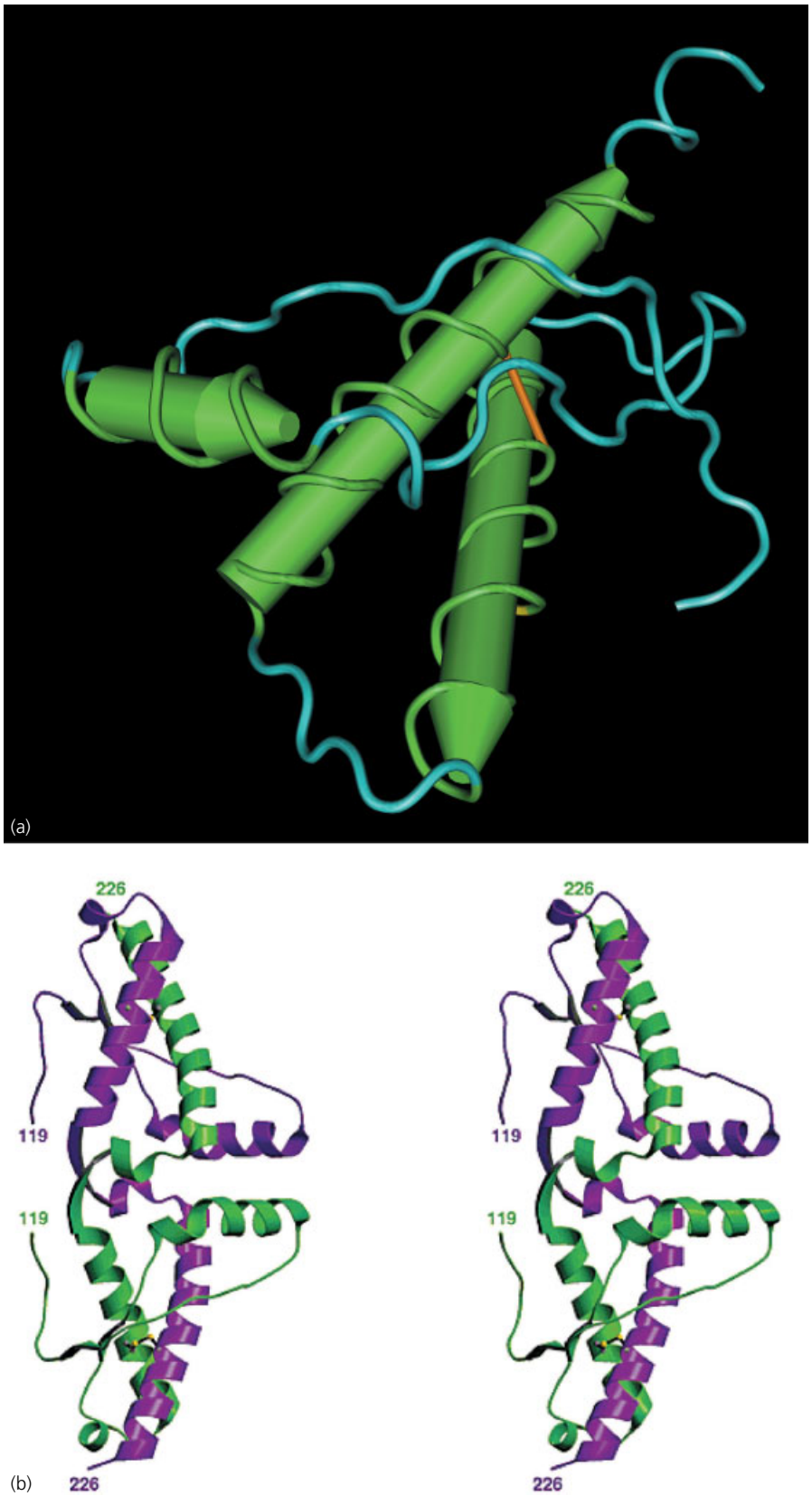

Figure 3 *The fine structure of the cellular prion protein, PrP^C.* **(a)** *Structure of the normal human prion protein, as submitted by R. Zahn, A. Liu, T. Luhrs and K. Wuthrich to the Molecular Modeling Database of the National Center for Biotechnology information (www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=19138).* **(b)** *Crystal structure of a PrP dimer (reproduced from Knaus et al. 2001).*

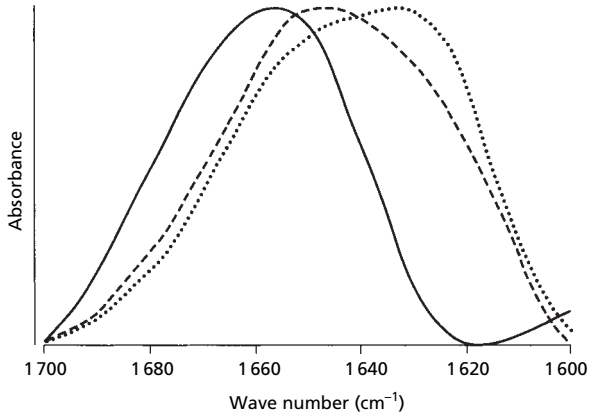

Figure 4 *Fourier transform infrared spectroscopy of prion proteins. The amide I′ band (1700–1600 cm^{-1}) of transmission FTIR spectra of PrP^{C} (solid line), PrP^{Sc} (dashed line) and PrP$^{27–30}$ (dotted line). These proteins were suspended in a buffer in D_2O containing 0.15 M sodium chloride/10 mM sodium phosphate, pD 7.5 (uncorrected)/0.12 percent ZW. The spectra are scaled independently to be full scale on the ordinate axis (absorbance). (Reproduced from Pan et al. 1993)*

PrP^{Sc} (Prusiner et al. 1990), these findings suggested that PrP^{C} also binds to one (or more than one) additional macromolecule during the conversion process. Telling and colleagues called this second macromolecule 'protein X' with the proviso that a second binding site on PrP^{Sc} might also function as protein X. However, 8 years after its initial postulation, no physical evidence for protein X has come forward, and Collinge and colleagues have observed exceptions to the genetic observations that had sparked the invention of protein X. Therefore, at present it is still possible that protein X is indeed a prion replication cofactor, but it is equally possible that it is nonexistent, that it exists but is not a protein, or that multiple host-encoded factors ('proteins X, Y, and Z') distinct from PrP may bind to PrP^{Sc} (Fischer et al. 2000; Maissen et al. 2001) and influence prion replication in vivo through a variety of direct and indirect mechanisms. On the basis of the available spectrum of data, one may speculate that the latter scenario is by far the most likely.

Transgenetics and gene targeting

Ablation of the PrP gene (*Prnp*$^{0/0}$) affected neither development nor behavior of the mice (Büeler et al. 1992; Manson et al. 1994a). In fact, *Prnp*$^{0/0}$ mice have remained healthy for more than 2 years. *Prnp*$^{0/0}$ mice are resistant to prions and do not propagate scrapie infectivity (Büeler et al. 1993; Prusiner et al. 1993; Sailer et al. 1994). *Prnp*$^{0/0}$ mice were sacrificed 5, 60, 120, and 315 days after inoculation with Rocky Mountain Laboratory prions passaged in CD-1 Swiss mice. Except for

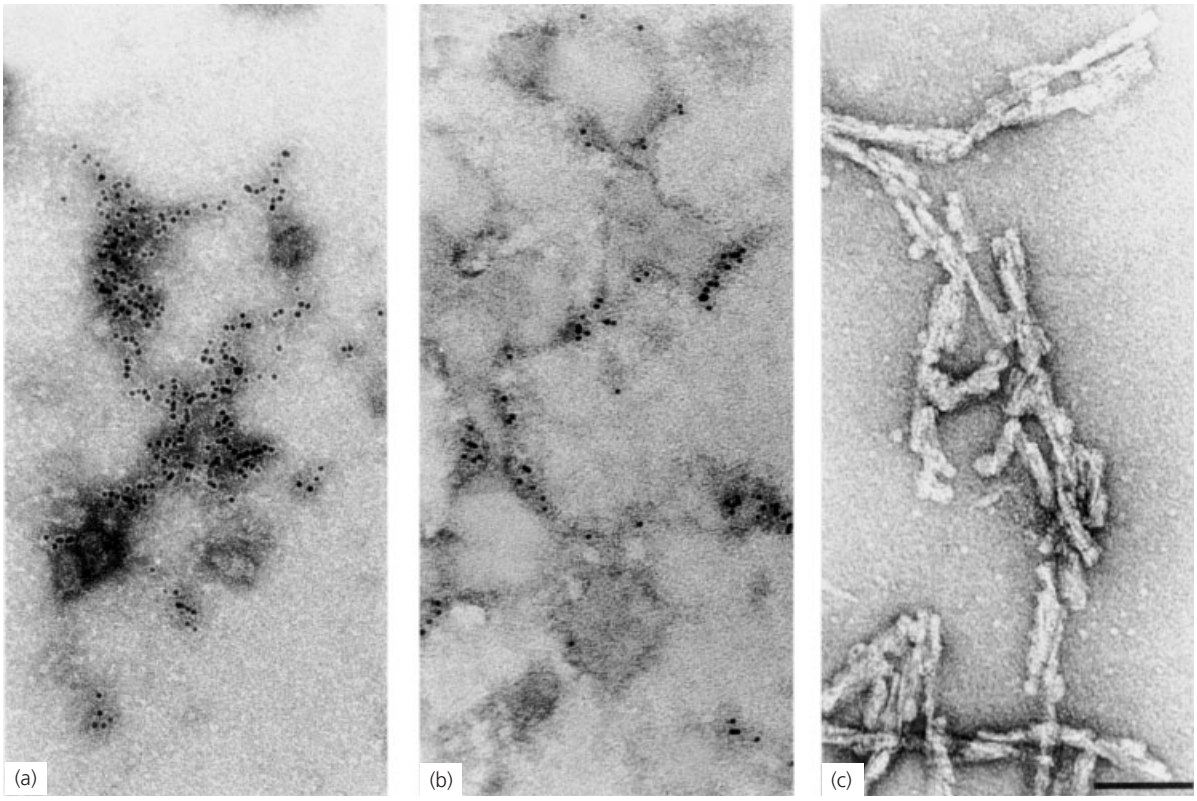

Figure 5 *Electron micrographs of negatively stained and immunogold-labeled prion proteins.* **(a)** *PrP^{C}.* **(b)** *PrP^{Sc}.* **(c)** *Prion rods composed of PrP$^{27–30}$ were negatively stained with uranyl acetate. Bar, 100 nm. (Reproduced from Pan et al. 1993)*

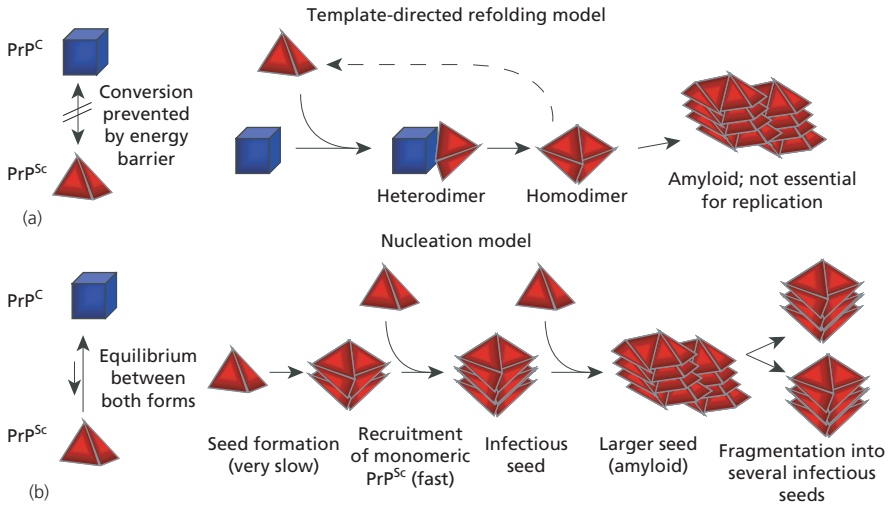

Figure 6 *Models for the conformational conversion of PrP^C into PrP^{Sc}.* **(a)** *The 'refolding' or template-assistance model postulates an interaction between exogenously introduced PrP^{Sc} and endogenous PrP^C, which is induced to transform itself into further PrP^{Sc}. A high-energy barrier may prevent spontaneous conversion of PrP^C into PrP^{Sc}.* **(b)** *The 'seeding' or nucleation-polymerization model proposes that PrP^C and PrP^{Sc} are in a reversible thermodynamic equilibrium. Only if several monomeric PrP^{Sc} molecules are mounted into a highly ordered seed, can further monomeric PrP^{Sc} be recruited and eventually aggregates to amyloid. Within such a crystallike seed, PrP^{Sc} becomes stabilized. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which can recruit further PrP^{Sc} and thus results in apparent replication of the agent.*

residual infectivity from the inoculum detected at 5 days after inoculation, no infectivity was detected in the brains of $Prnp^{0/0}$ mice. $Prnp^{0/0}$ mice crossed with Tg(SHaPrP) mice were rendered susceptible to hamster prions but remained resistant to mouse prions (Büeler et al. 1993). Since the absence of PrP^C expression does not provoke disease, it is likely that scrapie and the other prion diseases result not from an inhibition of PrP^C function due to PrP^{Sc} but rather from interference with some as yet undefined cellular process (Hegde et al. 1998, 1999).

Mice heterozygous ($Prnp^{+/0}$) for ablation of the PrP gene had prolonged incubation times when inoculated with mouse prions (Figure 7) (Büeler et al. 1994; Manson et al. 1994b). The $Prnp^{+/0}$ mice developed signs of neurological dysfunction at 400–460 days after inoculation. Therefore, the amount of available PrP^C is rate-limiting for the development of disease – a finding with significant implications for prion therapy. These findings are in agreement with studies on Tg(SHaPrP) mice in which increased SHaPrP expression was accompanied by diminished incubation times (Prusiner et al. 1990), and reinforce the idea that the concentration of PrP^C in brain is rate-limiting for its conversion into PrP^{Sc}.

Because $Prnp^{0/0}$ mice do not express PrP^C, they can be easily immunized to produce α-PrP antibodies. $Prnp^{0/0}$ mice immunized with mouse or hamster prion rods produced α-PrP antisera that bound mouse, SHa, and human PrP (Prusiner et al. 1993; Brandner et al. 1996b). These findings contrast with earlier studies in which α-MoPrP antibodies could not be produced in mice, presumably because the mice had been rendered tolerant by the presence of $MoPrP^C$ (Barry and Prusiner 1986; Kascsak et al. 1987; Rogers et al. 1991). That $Prnp^{0/0}$ mice readily produce α-PrP antibodies is consistent with the hypothesis that the lack of an immune response in prion diseases is due to the fact that PrP^C and PrP^{Sc} share many epitopes. For reasons that are not understood, $Prnp^{0/0}$ mice have not proved useful to produce α-PrP antibodies that specifically recognize CD epitopes present on PrP^{Sc} but absent from PrP^C – although such experiments have now been attempted for more than a decade.

MODELING OF GSS IN TRANSGENIC MICE

The codon 102 point mutation found in GSS patients was introduced into the MoPrP gene and Tg(MoPrP-P101L)H mice were created expressing high (H) levels of the mutant transgene product. Two lines of Tg(MoPrP-P101L)H mice spontaneously developed CNS degeneration, characterized by clinical signs indistinguishable from experimental murine scrapie and neuropathology consisting of widespread spongiform morphology and astrocytic gliosis (Hsiao et al. 1990) and PrP amyloid plaques (Hsiao et al. 1994). Excitingly, transmission of brain homogenate from Tg(MoPrP-P101L)H mice to healthy mice expressing lower levels of the same transgene (denoted Tg196/$Prnp^{0/0}$) induced spongiform encephalopathy (Telling et al. 1996).

In other studies, modifying the expression of mutant and wild-type *Prnp* genes in transgenic mice permitted experimental manipulation of the pathogenesis of both inherited and infectious prion diseases. Although overexpression of the wtPrP-A transgene ca. eightfold was not deleterious to the mice, it did shorten scrapie incubation times to as little as 45 days after inoculation with mouse scrapie prions (Telling et al. 1996).

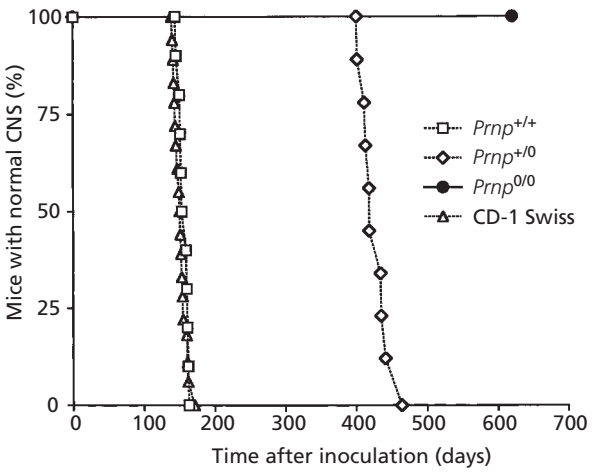

Figure 7 *Incubation times in PrP gene ablated* $Prnp^{+/0}$ and $Prnp^{0/0}$ mice as well as wild-type $Prnp^{+/+}$ and CD-1 mice inoculated with RML mouse prions. The RML prions were heated and irradiated at 254 nm prior to intracerebral inoculation into CD-1 Swiss mice (open triangles), $Prnp^{+/+}$ mice (open squares), $Prnp^{+/0}$ mice (open diamonds) or $Prnp^{0/0}$ mice (solid circle).

Prion diversity

The diversity of scrapie prions was first appreciated in goats inoculated with 'hyper' and 'drowsy' isolates (Pattison and Millson 1961b). Scrapie isolates or 'strains' from goats with a drowsy syndrome transmitted a similar syndrome to inoculated recipients, whereas those from goats with a 'hyper' or ataxic syndrome transmitted an ataxic form of scrapie to recipient goats. Subsequently, studies in mice demonstrated the existence of many scrapie 'strains' in which extracts prepared from the brains of mice inoculated with a particular preparation of prions produced a similar disease in inoculated recipients (Dickinson and Fraser 1979; Bruce and Dickinson 1987; Dickinson and Outram 1988). While the clinical signs of scrapie for different prion isolates in mice tended to be similar, the isolates could be distinguished by the incubation times, the distribution of CNS vacuolation that they produced, and whether or not amyloid plaques formed.

That such isolates could be propagated through multiple passages in mice suggested that the scrapie pathogen has a nucleic acid genome that is copied and passed on to nascent prions (Dickinson et al. 1968; Bruce and Dickinson 1987). But no evidence for scrapie-specific nucleic acid encoding information that specifies the incubation time and the distribution of neuropathological lesions has emerged from considerable efforts using a variety of experimental approaches. In striking contrast, mice expressing PrP transgenes have demonstrated that the level of PrP expression is inversely related to the incubation time (Prusiner et al. 1990). Furthermore, the distribution of CNS vacuolation and attendant gliosis are a consequence of pattern of PrP^{Sc} deposition which can be altered by both PrP genes and non-PrP genes (Prusiner et al. 1990). These observations taken together begin to build an argument for PrP^{Sc} as the informational molecule in which prion 'strain'-specific information is encrypted. Deciphering the mechanism by which PrP^{Sc} carries information for prion diversity and passes it onto the nascent prions is a challenging goal. Whether PrP^{Sc} can adopt multiple conformations, each one of which produces prions exhibiting distinct incubation times and patterns of PrP^{Sc} deposition, remains to be determined (Cohen et al. 1994). Preliminary immunochemical evidence suggests that PrP^{Sc} of different strains may expose different epitopes (Safar et al. 1998), and may display different stability to chaotropic salts (Peretz et al. 2002) – which perhaps may provide indirect evidence for conformational differences, but may also indicate association with different 'third parties' of proteic or other nature.

PRION STRAINS AND VARIATIONS IN PATTERNS OF DISEASE

Scrapie was first transmitted to sheep and goats by intraocular inoculation (Cuille and Chelle 1939) and later by intracerebral, oral, subcutaneous, intramuscular, and intravenous injections of brain extracts from sheep developing scrapie. Incubation periods of 1–3 years were common, and often many of the inoculated animals failed to develop disease (Dickinson and Stamp 1969; Hadlow et al. 1980, 1982). Different breeds of sheep exhibited markedly different susceptibilities to scrapie prions inoculated subcutaneously, suggesting that the genetic background might influence host permissiveness (Gordon 1966).

The lengths of the incubation times have been used to distinguish prion strains inoculated into sheep, goats, mice, and hamsters. Dickinson and his colleagues developed a system for 'strain typing' by which mice with genetically determined short and long incubation times were used in combination with the F1 cross (Dickinson et al. 1968, 1984; Dickinson and Meikle 1971). For example, C57BL mice exhibited short incubation times of ca. 150 days when inoculated with either the ME7 or the Chandler isolates; VM mice inoculated with these same isolates had prolonged incubation times of ca. 300 days. The mouse gene controlling incubation times was labelled *Sinc* and long incubation times were said to be a dominant trait because of prolonged incubation times in F1 mice. Prion strains were categorized into two groups based on their incubation times:

1. those causing disease more rapidly in 'short' incubation time C57BL mice
2. those causing disease more rapidly in 'long' incubation time VM mice.

Noteworthy are the 22A and 87V prion strains that can be passaged in VM mice while maintaining their distinct characteristics.

MOLECULAR BASIS OF PRION STRAINS

The mechanism by which isolate-specific information is carried by prions remains enigmatic; indeed, explaining the molecular basis of prion diversity seems to be a formidable challenge. Some investigators are still contending that scrapie is caused by a viruslike particle containing a scrapie-specific nucleic acid that encodes the information expressed by each isolate (Bruce and Dickinson 1987). To date, no such polynucleotide has been identified, despite using a wide variety of techniques, including measurements of the nucleic acids in purified preparations. An alternative hypothesis has been suggested that PrP^{Sc} alone is capable of transmitting disease but the characteristics of PrP^{Sc} might be modified by a cellular nucleic acid (Weissmann 1991b). This accessory cellular polynucleotide is postulated to induce its own synthesis upon transmission from one host to another, but there is no experimental evidence to support its existence.

Two additional hypotheses not involving a nucleic acid have been offered to explain distinct prion isolates: a non-nucleic acid second component might create prion diversity, or post-translational modification of PrP^{Sc} might be responsible for the different properties of distinct prion isolates (Prusiner 1991). Whether the PrP^{Sc} modification is chemical or only conformational remains to be established, but no candidate chemical modifications have been identified (Stahl et al. 1993). Structural studies of GPI anchors of two SHa isolates have failed to reveal any differences; it is interesting that ca. 40 percent of the anchor glycans have sialic acid residues (Stahl et al. 1992). A portion of the PrP^{C} GPI anchors also have sialic acid residues; PrP is the first protein found to have sialic acid residues attached to GPI anchors.

Multiple prion isolates might be explained by distinct PrP^{Sc} conformers that act as templates for the folding of de novo synthesized PrP^{Sc} molecules during prion 'replication.' Although it is clear that passage history can be responsible for the prolongation of incubation time when prions are passed between mice expressing different PrP allotypes (Carlson et al. 1989) or between species (Prusiner et al. 1990), many scrapie strains show distinct incubation times in the same inbred host (Bruce et al. 1991).

PrP^{Sc} species associated with two hamster-adapted scrapie strains, hyper (HY) and drowsy (DY), proved to display characteristic clinical and histopathological properties as well as distinct biochemical patterns with respect to proteinase K digestion (Bessen and Marsh 1992, 1994) – which may be explainable by the presence of different conformations of PrP^{Sc}. Analogous findings have been made with other prion strains propagated in mice. In addition, PrP^{Sc} of certain strains differ in the ratio of diglycosylated to the monoglycosylated form. Based on the fragment size and the relative abundance of individual bands, three distinct patterns (PrP^{Sc} types 1–3) were defined for sporadic and iCJD cases (Collinge et al. 1996; Parchi et al. 1996, 1999). By contrast, all cases of vCJD displayed a novel pattern, designated type 4 by Collinge, and type IIb by Parchi and Gambetti (Figure 8). Interestingly, brain extracts from BSE-infected cattle as well as BSE-inoculated macaques exhibited a type 4 pattern. Even more intriguingly, transmission of BSE or vCJD to mice produced mouse PrP^{Sc} with a type 4 pattern indistinguishable from the original inoculum (Hill et al. 1997a) – findings that impressively support the hypothesis that vCJD is the human counterpart to BSE. An additional tool, which may eventually serve to characterize prion strains, utilizes the differential affinity of certain antibodies for properly folded and denatured isoforms of PrP, thereby differentiating as many as eight different conformers (Safar et al. 1998). This provides further circumstantial evidence for the assumption that strain specificity is encrypted within the physical structure of PrP.

THE FUNCTION OF THE CELLULAR PRION PROTEIN

Despite considerable efforts, the physiological function of PrP^{C} is still unclear. A proposed role for PrP^{C} in synaptic function (Collinge et al. 1994) has been questioned by other investigators (Lledo et al. 1996) and a possible regulation of circadian rhythm by PrP^{C} has remained more-or-less anecdotal (Tobler et al. 1996). Hence, the only definite phenotype of $Prnp^{0/0}$ mice is their resistance to prion inoculation (Büeler et al. 1993). It seems, however, rather unlikely that a singular protein that is as highly conserved among species as PrP^{C}, from turtles to frogs, fish, and humans, has evolved for the sole reason of bestowing susceptibility to prion diseases.

If the function of PrP^{C} were completely unrelated to prion disease pathogenesis, PrP^{C} may be just one of many proteins whose function awaits clarification. Yet the function of PrP^{C} may very well have something to do, in a subtle way, with prion-induced damage. *Prnp* ablation does not elicit disease, even when induced postnatally (Mallucci et al. 2002): hence prion pathology is unlikely to come about by simple loss of PrP^{C} function. If PrP^{C} transduces a signal (in analogy to many other GPI-linked proteins), or possesses some enzymatic activity, one could speculate that conversion to PrP^{Sc} may alter signal transduction strength, or substrate specificity, thereby conferring a toxic dominant function. In these scenarios, understanding the function of PrP^{C} may be instrumental to deciphering prion pathology, and perhaps even to devising therapeutical approaches.

Is PrP^{C} an enzyme? Glockshuber noted that PrP^{C} has similarities to membrane-anchored signal peptidases (Glockshuber et al. 1998), but his observation has not been substantiated by functional data. PrP^{C} can bind

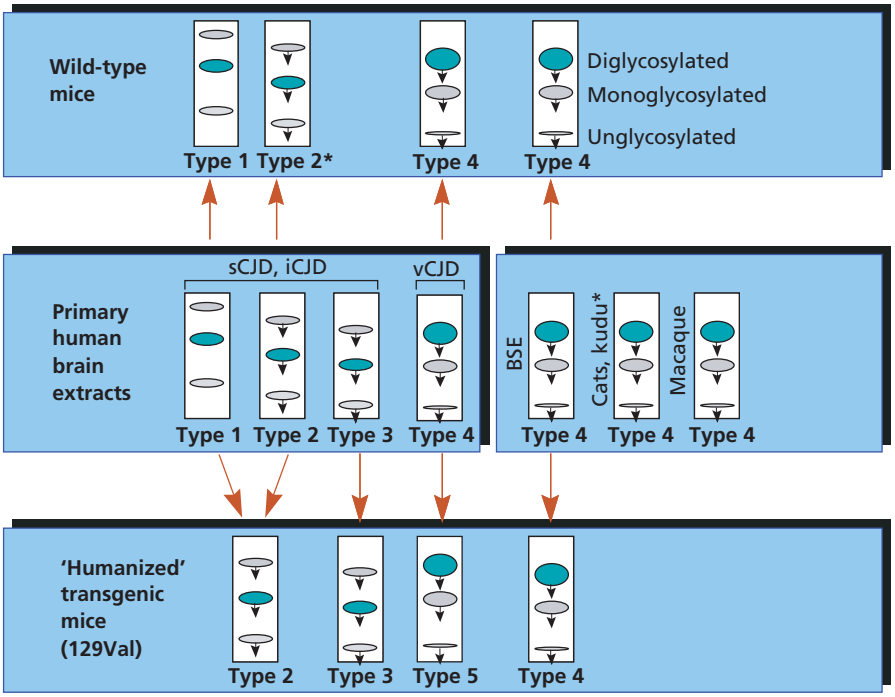

Figure 8 *Patterns of PrP glycosylation. Representation of the three glycosylated PrP^{Sc} moieties (un-, mono-, and diglycosylated PrP^{Sc}) in immunoblots of brain extracts after digest with proteinase K. Different inocula result in specific mobilities of the three PrP bands as well as different predominance of certain bands (middle panel). These characteristic patterns can be retained, or changed to other predictable patterns after passage in wild-type (upper panel) or humanized mice (PrP-deficient mice bearing a human PrP transgene, lower panel). Based on the fragment size and the relative abundance of individual bands, three distinct patterns (PrP^{Sc} types 1–3) were defined for sporadic and iatrogenic CJD cases. By contrast, all cases of vCJD displayed a novel pattern, designated as type 4 pattern.*

copper (Brown et al. 1997; Wadsworth et al. 1999), but reports of increased copper content of neurons lacking PrP^{C} have been questioned by contradictory findings (Waggoner et al. 2000). The speculation that PrP^{C} may be a superoxide dismutase (Brown et al., 1997; D.R. Brown et al. 1999) was perceived as particularly attractive in view of its multiple copper-binding sites. However, PrP^{C} does not make any measurable contribution to dismutase activity in vivo (Hutter et al. 2003; Waggoner et al. 2000). Interestingly, it was recently suggested that amino proximally truncated PrP^{C} may depress endogenous dismutase activity (Sakudo et al. 2003).

Perhaps PrP^{C} and PrP^{Sc} do not possess any intrinsic biological activity, yet they modify the function of other proteins. This supposition has prompted a search for PrP-interacting partners, and there is no dearth of PrP-binding proteins: the antiapoptotic protein Bcl-2 (Kurschner et al. 1995), caveolin (Gorodinsky and Harris 1995; Harmey et al. 1995), the laminin receptor precursor (Rieger et al. 1997), plasminogen (Fischer et al. 2000) and N-CAM (Schmitt-Ulms et al. 2001). None of these interactors, however, have yet revealed a functional pathway in which PrP^{C} would be involved in vivo. It was recently shown that PrP-deficient macrophages do not support bacterial 'swimming internalization' of the gram-negative bacterium, *Brucella abortus* (Watarai et al. 2003) and that PrP^{C} interacts with a *Brucella* heat-shock protein, Hsp60. These intriguing findings raise the question of whether PrP^{C} is a part of a general Hsp60-dependent 'danger-sensing' mechanism (Aguzzi and Hardt 2003).

THE PRION DOPPELGANGER

After the advent of large-scale sequencing efforts and genome projects, it was realized that there is an ORF directly adjacent of *Prnp* that encodes a protein sharing significant homology with PrP^{C} (Moore et al. 1999). The novel gene, *Prnd*, is located 16 kb downstream of *Prnp* in the mouse genome, and encodes a protein of 179 residues, which was termed Dpl ('downstream of the *Prnp* locus' or 'Doppel,' German for 'double') (Moore et al. 1999; Weissmann and Aguzzi 1999; Behrens and Aguzzi 2002). The *Prnd* gene is evolutionarily conserved from humans to sheep and cattle, and shows roughly 25 percent identity with the carboxy-proximal two-thirds of PrP^{C}. Structural studies indicate that Dpl contains three α-helices, like PrP^{C}, and two disulfide bridges between the second and third helices (Lu et al. 2000; Silverman et al. 2000; Mo et al. 2001). Dpl mRNA is expressed at high levels in testis, less in other peripheral organs and, notably, at very low levels in brain of adult wild-type mice. However, significant *Prnd* mRNA transcripts were

detected during embryogenesis and in the brains of newborn mice, arguing for a possible function of Dpl in brain development (Li et al. 2000).

To probe the function of Dpl, the *Prnd* gene was inactivated in embryonic stem (ES) cells (Behrens et al. 2001). Similarly to mice lacking PrP^C, mice devoid of Dpl survive to adulthood and do not show obvious phenotypical alterations, suggesting that Dpl is dispensable for embryogenesis and postnatal development (Behrens et al. 2002). The similarities between PrP^C and Dpl in primary amino acid sequence, structure, and subcellular localization suggest related biological functions: therefore a possible role of PrP^C and Dpl during development may be masked by functional redundancy. To address this question it will be necessary to generate mice lacking *Prnp* as well as *Prnd* and to study whether the lack of both PrP^C and Dpl will result in an exacerbation of the mutant phenotypes. These double-mutant mice may finally reveal the true physiological function of PrP^C and Dpl, and pave the way for the long-awaited understanding of these proteins.

ES cells carrying a homozygous null mutation of the *Prnd* locus, and a normal *Prnp* locus, were found to be capable of giving rise to all neural cell lineages when transplanted into host brains. The Dpl protein resembles an N-terminally truncated PrP^C protein lacking the octamer repeats. But the latter version of PrP^C is actually capable of supporting PrP^{Sc} propagation (Flechsig et al. 2000), suggesting that the Dpl protein may in principle be susceptible to conversion into 'Dpl^{Sc}.' However, presently there is no evidence whether upon scrapie inoculation Dpl can be converted into a misfolded β-strand-rich, protease-resistant conformation. After inoculation with scrapie prions, Dpl-deficient neural grafts showed spongiosis, gliosis, and unimpaired accumulation of PrP^{Sc} and infectivity similar to wild-type neuroectodermal grafts (Behrens et al. 2001). Therefore in neural grafts *Prnd* deficiency does not prevent prion pathogenesis. It is important to note, however, that this experimental approach does not rule out a role for Dpl in peripheral prion pathogenesis and in PrP^{Sc} transport to the brain. The latter possibility is worth studying, because *Prnd* is expressed in the spleen, a major peripheral reservoir of PrP^{Sc} and prion infectivity (Li et al. 2000).

Four polymorphisms in human *Prnd* were detected, but no strong association was found between any of these polymorphisms and human prion diseases (Mead et al., 2000; Peoc'h et al., 2000). These findings further argue against an important function of Dpl in neurons during prion disease, at least in genetically determined forms of these diseases.

Phenotypes of *Prnp*-deficient mice

Although this was not realized for several years, a Dpl-associated phenotype had been accidentally produced in knock-out mice lacking PrP^C. In the course of the 1990s, several mouse lines with targeted disruptions of *Prnp* were independently generated in various laboratories. All mutant mouse lines lacked significant portions of the *Prnp* ORF and did not produce PrP^C protein, but showed two strikingly different phenotypes. Zrch $Prnp^{0/0}$ and Edbg $Prnp^{-/-}$ (termed after the city of origin) showed only minor defects (Büeler et al. 1992; Collinge and Palmer 1994; Manson et al. 1994a; Tobler et al. 1996) whereas Ngsk $Prnp^{-/-}$, Zürich II and Rcm0 mice develop cerebellar Purkinje cell degeneration causing ataxia with advancing age (Sakaguchi et al. 1996; Moore et al. 1999; Rossi et al. 2001). This conundrum was solved when David Westaway and colleagues realized that in the brain of ataxic, but not of healthy *Prnp*-mutant mice, *Prnd* mRNA was up-regulated (Moore et al. 1999; Li et al. 2000). An intergenic splicing event places the Dpl locus under the control of the *Prnp* promoter, probably due to the deletion of the *Prnp* intron 2 sequence including its splicing acceptor (Moore et al. 1999). This intergenic splicing event could be detected at very low levels also in wild-type mice, but was greatly enhanced by the absence of the intron 2 splice acceptor (Moore et al. 1999). Whereas the *Prnp* promoter is strongly expressed in neuronal cells, the *Prnd* promoter is not (Moore et al. 1999; Li et al. 2000; Rossi et al. 2001) and therefore *Prnd* expression from the *Prnp* promoter results in overproduction of Dpl in the brain (Moore et al. 1999; Li et al. 2000; Rossi et al. 2001). Further experiments have demonstrated an inverse correlation between the mRNA levels of *Prnd* and the onset of ataxia. Disease progression was accelerated by increasing *Prnd* levels, supporting the notion that ectopic Dpl expression, but not functional loss of PrP^C, may be responsible for neuronal degeneration in ataxic *Prnp*-deficient mice (Rossi et al. 2001).

The function of the Doppel protein

Behrens and colleagues inactivated the *Prnd* gene. However, they have not been able to generate any progeny from intercrosses of $Prnd^{-/-}$ mice. Female $Prnd^{-/-}$ mice, when crossed to $Prnd^{-/-}$ or $Prnd^{+/-}$ mice, yielded litter sizes similar to those of wild-type. By contrast, male $Prnd^{-/-}$ mice were infertile. Their sexual activity was similar to that of controls, as shown by a normal number of copulation plugs. However, the number of spermatozoa in the cauda epididymis of $Prnd^{-/-}$ males was reduced, and motility of mutant sperm was decreased. Therefore, sterility of Dpl-mutant males is not due to behavioral abnormalities, but may be due to a spermatogenesis defect. Indeed, $Prnd^{-/-}$ sperm heads were severely malformed and lacked a discernible well-developed acrosome (Figure 9). As the acrosome is essential for sperm/egg interaction, this defect could explain the sterility of *Prnd*-deficient males.

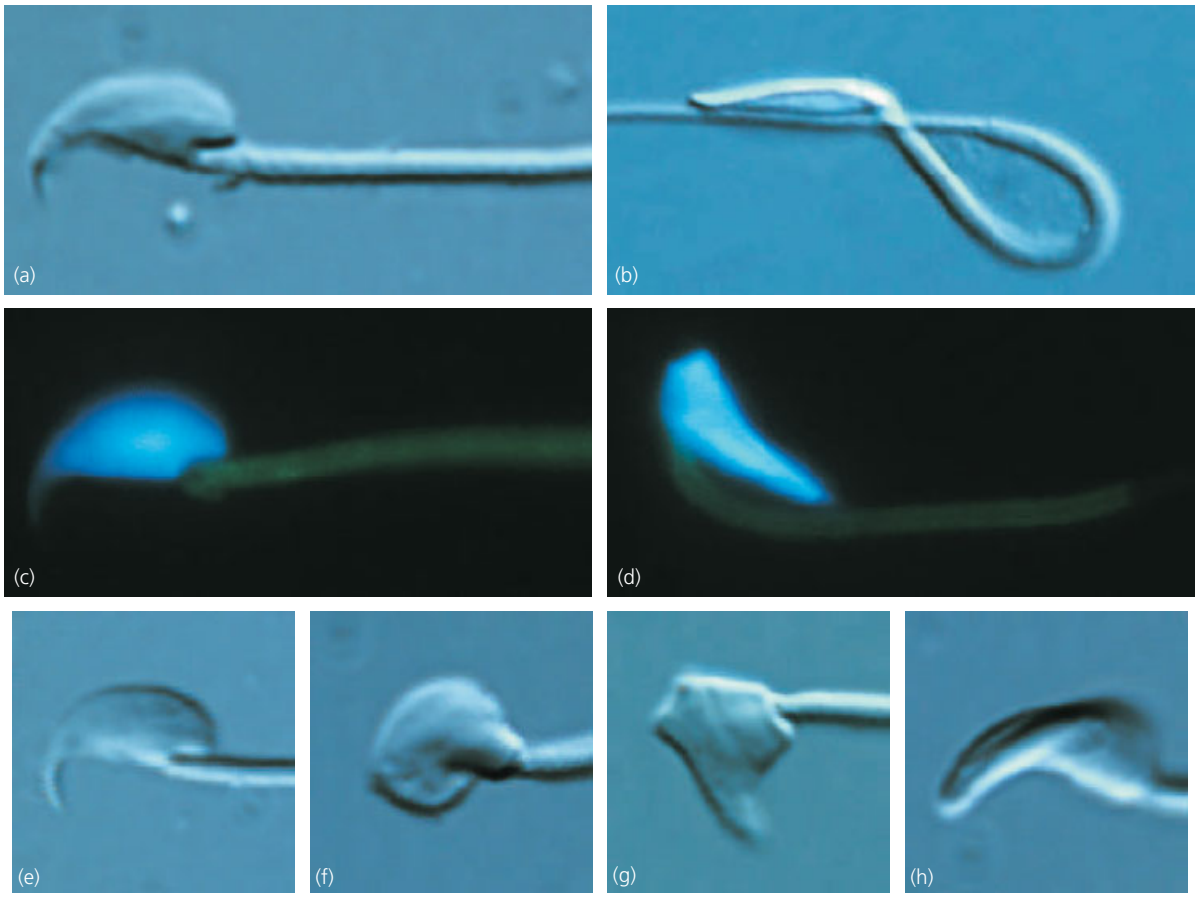

Figure 9 *Morphology of sperms in mice lacking the Doppel gene. Spermatozoa from Dpl-deficient mice are often heavily malformed. (a, b) Photographs of bright field images of spermatozoa isolated from wild-type* **(a)** *and* $Prnd^{-/-}$ *mice* **(b)**. **(c, d)** *Spermatozoa isolated from* $Prnd^{+/+}$ **(c)** *and* $Prnd^{-/-}$ *mice* **(d)** *were stained with mitotracker to detect mitochondria (in green) and with the DNA stain Hoechst (in blue). (***e, h** *Photographs of bright-field images of sperm heads from* $Prnd^{+/+}$ **(e)** *and* $Prnd^{-/-}$ *mice* **(f–h)**

In vitro fertilization (IVF) experiments confirmed that spermatozoa isolated from $Prnd^{-/-}$ males were unable to fertilize wild-type oocytes. Spermatozoa of $Prnd^{-/-}$ males never penetrated the zona pellucida. However, if the zona pellucida was partially dissected and IVF was performed with sperm suspension from $Prnd^{-/-}$ males, fertility was partially rescued. These data indicate that $Prnd^{-/-}$ spermatozoa are capable of oocyte fertilization, albeit at a lower frequency than controls, but that they cannot overcome the barrier imposed by the zona pellucida (Figure 10).

A significant amount of PrP^{C} is expressed in mature spermatozoa. The PrP protein found in testes was truncated in its carboxy terminus in the vicinity of residue 200 (Shaked et al. 1999). A protective role for PrP against copper toxicity has been proposed: sperm cells originating from *Prnp*-ablated mice were found to be more susceptible to high copper concentrations than wild-type sperm. However, male $Prnp^{0/0}$ knockout mice are not sterile and produce normal litter sizes. PrP expressed in testes is clearly not capable of compensating for the absence of Dpl, suggesting nonredundant functions for the two proteins. However, Dpl may mask a minor function of PrP^{C} in testes development or spermiogenesis; therefore, it will be very interesting to explore whether males lacking both PrP^{C} and Dpl might display a more severe defect than $Prnd^{-/-}$ single mutant mice. Also, it will be interesting to determine whether testicular expression of amino terminally truncated PrP^{C} restores the fertility of Dpl-deficient mice: if so, this would finally provide a long-sought experimental window into structure–function relationships affecting the physiology of PrP^{C}.

At present the molecular mechanism by which Dpl regulates acrosome development is unclear. Dpl may be present on the acrosomic vesicles through its GPI-anchor, and possibly participate in acrosome morphogenesis. Alternatively, Dpl may regulate acrosome function in a more indirect way. We have also observed that sperm isolated from $Prnd^{-/-}$ mice is greatly impaired in sperm/egg interaction and that $Prnd^{-/-}$ spermatozoa fail to trigger the acrosomal reaction. Oligosaccharides have been implicated in sperm binding

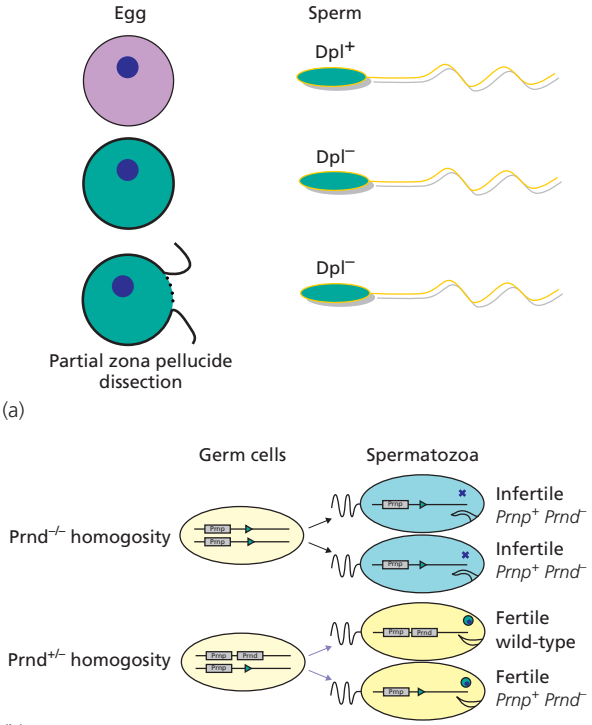

Figure 10 *A possible model for the function of the Doppel protein in fertilization.* **(a)** *Male sterility in mice that lack the Doppel protein appears to come about because Doppel-deficient sperms are incapable of fertilizing eggs. The process that appears to be disturbed relates to the acrosomal reaction and to the penetration of the zona pellucida. In fact, mechanical dissection of the zona pellucida restores, at least in part, fertility.* **(b)** *Interestingly, haploid spermatozoa lacking the Doppel gene (*Prnd^{-}*) are perfectly fertile when generated in the context of a heterozygous* Prnd$^{+/-}$ *mouse. Instead,* Prnd^{-} *sperms are infertile when generated in a* Prnd$^{-/-}$ *mouse. This may be because sperms spend much of the maturation time in the form of syncytia, with maturing cells connected to each other by cytoplasmic bridges. This may allow sufficient amounts of Doppel protein to be transferred from* Prnd^{+} *to* Prnd^{-} *spermatids, and rescue fertility in trans.*

and signaling for the acrosome reaction, but the composition and structure of the essential carbohydrate moieties remain controversial (Wassarman et al. 2001). Dpl is a highly glycosylated protein located at the outside of the plasma membrane (Moore et al. 1999; Silverman et al. 2000); therefore, it is possible that Dpl present on the sperm plasma membrane is directly involved in sperm/egg interaction. With regard to the keen interest in the development of new methods of contraception (in particular those targeting the male), the phenotype of *Prnd*$^{-/-}$ mutant mice suggests that inhibition of Dpl function may provide a novel target for contraceptive intervention (Behrens and Aguzzi 2002).

In this context, it is interesting to mention that the laboratory of David Melton in Edinburgh has reported a slightly different phenotype of Dpl-deficient mice (communicated by D. Paisley and D. Melton). In contrast to the mice generated in Zurich, Doppel-deficient homozygous males in Edinburgh appear to be able to fertilize eggs. In a series of IVFs, the Melton laboratory has reported that progression to the early cleavage divisions occurred, but was soon thereafter followed by death of the embryos at the preimplantation stage. Instead, there were no obvious malformations of sperms. Whether this discrepancy is related to a divergent genetic background of the mice utilized, whether it points to slightly different targeting strategies, or finally, whether it might uncover yet another surprising phenomenon in the genetics of prion-related genes, is – at the time of writing – wholly unclear.

CLINICAL AND PATHOLOGICAL ASPECTS OF PRION DISEASES

Scrapie

EXPERIMENTAL SCRAPIE

For decades, studies of experimental scrapie were performed exclusively with sheep and goats, which required incubation periods of 1–3 years. A crucial methodological advance in experimental studies of scrapie was created by the demonstration that scrapie could be transmitted to mice (Chandler 1961, 1963) which could be used for endpoint titrations of particular samples. In addition, pathogenesis experiments were performed directed at elucidating factors governing incubation times and neuropathological lesions (Eklund et al. 1967; Dickinson et al. 1968).

NATURAL SCRAPIE IN SHEEP AND GOATS

Even though scrapie was recognized as a distinct disorder of sheep with respect to its clinical manifestations as early as 1738, the disease remained an enigma, even with respect to its pathology, for more than two centuries (Parry 1983). Some veterinarians thought that scrapie was a disease of muscle caused by parasites, whilst others thought that it was a dystrophic process (M'Gowan 1914). An investigation into the etiology of scrapie followed the vaccination of sheep for louping-ill virus with formalin-treated extracts of ovine lymphoid tissue unknowingly contaminated with scrapie prions (Gordon 1946). Two years later, more than 1 500 sheep developed scrapie from this vaccine.

Scrapie of sheep and goats share with CWD of deer and elk a unique property among prion diseases: they seem to be readily communicable within flocks. Although the transmissibility of scrapie seems to be well established, the mechanism of the natural spread of scrapie among sheep is puzzling. The placenta has been implicated as one source of prions, accounting for the horizontal spread of scrapie within flocks (Pattison and Millson 1961a; Pattison 1964; Pattison et al. 1972;

Onodera et al. 1993). Whether this view is correct remains to be established. In Iceland, scrapie-infected flocks of sheep were destroyed and the pastures left vacant for several years; however, reintroduction of sheep from flocks known to be free of scrapie for many years eventually resulted in scrapie (Palsson 1979). The source of the scrapie prions that attacked the sheep from flocks without a history of scrapie is unknown. Transmission through mites has been advocated (Wisniewski et al. 1996), but its significance on the field remains somewhat anecdotal.

GENETICS OF SHEEP

Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease which could be eradicated by proper breeding protocols (Parry 1962, 1983). He considered its transmission by inoculation to be of importance primarily for laboratory studies and communicable infection of little consequence in nature. Other investigators viewed natural scrapie as an infectious disease, and argued that host genetics only modulates susceptibility to an endemic infectious agent (Dickinson et al. 1965). The incubation time gene for experimental scrapie in Cheviot sheep, called *Sip*, is said to be linked to a PrP gene (Hunter et al. 1989); however, the null hypothesis of nonlinkage has yet to be tested and this is important, especially in view of earlier studies which argue that susceptibility of sheep to scrapie is governed by a recessive gene (Parry 1962, 1983).

Polymorphisms at codons 136 and 171 of the PrP gene in sheep that produce amino acid substitutions have been studied with respect to the occurrence of scrapie in sheep (Clouscard et al. 1995). In Romanov and Ile-de-France breeds of sheep, a polymorphism in the PrP ORF was found at codon 136 (A→V) which correlates with scrapie (Laplanche et al. 1993b). Sheep homozygous or heterozygous for Val at codon 136 were susceptible to scrapie whereas those that were homozygous for Ala were resistant. Unexpectedly, only one of 74 scrapied autochthonous sheep had a Val at codon 136; these sheep were from three breeds denoted Lacaune, Manech, and Presalpes (Laplanche et al. 1993a).

In Suffolk sheep, a polymorphism in the PrP ORF was found at codon 171 (Q→R) (Goldmann et al. 1990a, 1990b). Studies of natural scrapie in the USA have shown that ca. 85 percent of the afflicted sheep are of the Suffolk breed. Only those Suffolk sheep homozygous for Gln (Q) at codon 171 had scrapie, although healthy controls with QQ, QR, and RR genotypes were found (Westaway et al. 1994b). These results argue that susceptibility in Suffolk sheep is governed by the PrP codon 171 polymorphism. Whether the PrP codon 171 or 136 polymorphisms in Cheviot sheep have the same profound influence on susceptibility to scrapie as has been found for codon 171 in Suffolks is unknown (Goldmann et al. 1991a; Hunter et al. 1991).

Bovine spongiform encephalopathy

EPIDEMIC OF MAD COW DISEASE

In 1986, an epidemic of a previously unknown disease appeared in cattle in Great Britain (Wells et al. 1987): bovine spongiform encephalopathy (BSE) or 'mad cow' disease. BSE was shown to be a prion disease by demonstrating protease-resistant PrP in brains of ill cattle (Hope et al. 1988; Prusiner et al. 1993). Based mainly on epidemiological evidence, it has been proposed that BSE represents a massive common-source epidemic which has caused more than 180 000 cases to date (Weissmann and Aguzzi 1997). In Britain, cattle, particularly dairy cows, were routinely fed MBM as a nutritional supplement (Wilesmith et al. 1988, 1992a, b; Wilesmith and Wells 1991). The MBM was prepared by rendering the offal of sheep and cattle using a process that involved steam treatment and hydrocarbon solvent extraction. The extraction process produced a protein and fat-rich fractions; the protein or greaves fraction contained about 1 percent fat from which the MBM was prepared. In the late 1970s, the price of tallow prepared from the fat fraction fell, making it no longer profitable to use hydrocarbons in the rendering process. The resulting MBM contained about 14 percent fat: perhaps the high lipid content protected scrapie prions in the sheep offal from being completely inactivated by steam.

Since 1988, the practice of using dietary protein supplements for domestic animals derived from rendered sheep or cattle offal has been forbidden in the UK. Curiously, almost half of the BSE cases have occurred in herds where only a single affected animal has been found; several cases of BSE in a single herd are infrequent (Wilesmith et al. 1988; Dealler and Lacey 1990; Wilesmith and Wells 1991; Weissmann and Aguzzi 1997). In 1992, the BSE epidemic reached a peak, with over 35 000 cattle afflicted (Figure 11). In 1993, fewer than 32 000 cattle were diagnosed with BSE and in 1994 the number was approximately 22 000. In 2003, BSE had become a rare disease in British and European cattle, but regrettably (and quite unexplicably), it still had not completely disappeared.

TRANSMISSION OF BSE TO EXPERIMENTAL ANIMALS

Brain extracts from BSE cattle have transmitted disease to mice, cattle, sheep, and pigs after intracerebral inoculation (Fraser et al. 1988; Dawson et al. 1990a, b; Bruce et al. 1993). Transmissions to mice and sheep suggest that cattle preferentially propagate a single 'strain' of prions: seven BSE brains all produced similar incubation times as measured in each of three strains of inbred mice (Bruce et al. 1993). However, this notion was

recently challenged on the basis of transgenetic studies (Asante et al. 2002). Incontrovertible evidence for the existence of several BSE strains, in the opinion of the author, has yet to be provided.

Of particular importance to the BSE epidemic is the recent transmission of BSE to the nonhuman primate marmoset after intracerebral inoculation followed by a prolonged incubation period (Baker et al. 1993). The potential parallels with kuru of humans, confined to the Fore region of New Guinea (Gajdusek et al. 1966; Gajdusek 1977), are worthy of consideration. Once the most common cause of death among women and children in that region, kuru has almost disappeared with the cessation of ritualistic cannibalism (Alpers 1987). Although it seems likely that kuru was transmitted orally, as proposed for BSE among cattle, some investigators argue that other routes of transmission were important because oral transmission of kuru prions to apes and monkeys has been difficult to demonstrate (Gajdusek 1977; Gibbs et al. 1980).

ORAL TRANSMISSION OF PRIONS

Besides BSE, four other animal diseases seem to have arisen from ingestion of prions. It has been suggested that an outbreak of transmissible mink encephalopathy in 1985 arose from the use for feed of a cow with a sporadic case of BSE (Will et al. 1996). The source of prions in CWD of mule, deer and elk is unclear (Williams and Young 1980, 1982). The prion-contaminated MBM thought to be the cause of BSE is also most likely the cause of feline spongiform encephalopathy (FSE) and exotic ungulate encephalopathy. FSE has been found in domestic cats throughout Europe, as well as in a puma and a cheetah (Willoughby et al. 1992). Three cases of FSE in domestic cats have been transmitted to laboratory mice and PrP^{Sc} has been identified in their brains by immunoblotting (Pearson et al. 1992). Whether FSE may have transmitted to a human (Zanusso et al. 1998) remains anectodal and unproven. Prion disease has been found in the brains of the nyala, greater kudu, eland, gembok, and Arabian oryx in British zoos; all of these animals are exotic ungulates. Of eight greater kudu born into a herd maintained in a London zoo since 1987, five have developed prion disease. Except for the first case, none of the other four kudu was exposed to feeds containing ruminant-derived MBM (Kirkwood et al. 1993). Brain extracts prepared from a nyala and a greater kudu have been transmitted to mice (Kirkwood et al. 1990; Cunningham et al. 1993). PrP of the greater kudu differs from the bovine protein at four residues; Arabian oryx PrP differs from the sheep PrP at only one residue (Poidinger et al. 1993).

Chronic wasting disease of deer and elk

As the only prion disease of wildlife, CWD was initially reported in 1980 as a TSE in captive research deer in Colorado and Wyoming – the disease origin still remains completely obscure (Williams and Young 1980). Since 1980, free-ranging mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginanus)*, and Rocky Mountain elk (*Cervus elaphus nelsoni*) have been detected in the same region of Colorado and Wyoming (Spraker et al. 1997), but recently increased surveillance efforts across the USA and Canada have startlingly revealed CWD in adjacent states (Nebraska, New Mexico, and Utah) but also distant (Wisconsin, Illinois, Canada) from this original endemic region. Additionally, game-ranched elk have not escaped the disease, with CWD being first detected in 1997. The staggeringly high transmission apparent in captive deer (up to 90 percent infected in one facility, (Williams and Young 1992)) and in free-ranging deer (up to 15 percent, (Miller et al. 2000)), occurs by unknown mechanisms, but it is likely to be a horizontal route; saliva or feces could potentially harbor infectious prions and contaminate grazing areas.

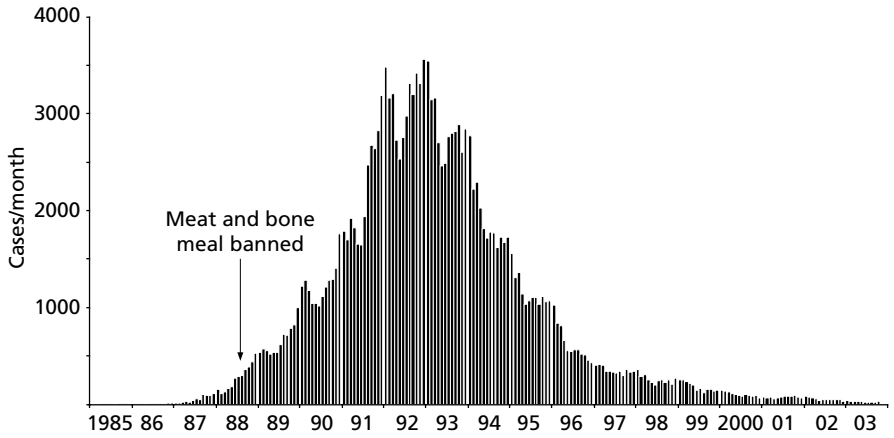

Figure 11 *Annual incidence of bovine spongiform encephalopathy in Great Britain. All cases were confirmed clinically and neuropathologically. (Statistics compiled by John Wilesmith, Central Veterinary Laboratory at Weybridge, United Kingdom)*

Clinical signs of CWD are remarkably subtle and nonspecific, characterized by lethargy, weight loss, flaccid hypotonic facial muscles, polydipsia/polyuria, excessive salivation, and behavioral changes, such as loss of fear of humans (Williams and Young 1980). Of the TSEs, histological brain lesions are most similar to BSE and scrapie, and include large single or septate intraneuronal vacuoles prominent in the parasympathetic vagal nucleus of the medulla oblongata, and also evident in the thalamus, hypothalamus, pons, midbrain, and olfactory cortex (Williams and Young 1993; Spraker et al. 1997). Similar to scrapie, PrP^{CWD} is abundant in secondary lymphoid tissues, and can be detected in tonsil, Peyer's patches, and ileocecal lymph node by immunohistochemistry as early as 12 weeks after experimental oral CWD inoculation (Sigurdson et al. 1999).

The capacity for CWD transmission to other species is clearly an area of great concern. Unfortunately, very little is known about the risk for other wildlife species, domestic ruminants, or humans contracting the disease. Cattle have been inoculated intracerebrally with CWD, and by 2 years post-inoculation, three of 13 had developed a TSE (Hamir et al. 2001). No cattle which have been orally inoculated have shown clinical signs of a TSE at 63 months post-inoculation (M.W. Miller and E.S. Williams, unpublished findings). Thus, cattle appear to be highly resistant to CWD by a natural route of exposure. The ability of PrP^{CWD} to convert human PrP^{c} in vitro was determined to be inefficient, but similar to the efficiency of PrP^{BSE} or ovine PrP^{Sc} to convert human PrP^{c} (Raymond et al. 2000).

Human prion diseases

The human prion diseases are manifest as infectious, inherited, and sporadic disorders, and are often referred to as kuru, sCJD, familial CJD, vCJD, GSS syndrome, and FFI, depending upon the clinical, genetic, and neuropathological findings (Table 1).

DIAGNOSIS OF HUMAN PRION DISEASES

Human prion disease should be considered in any patient who develops a progressive subacute or chronic decline in cognitive or motor function. Typically adults between 40 and 70 (or more) years of age, patients often exhibit clinical features helpful in providing a premorbid diagnosis of prion disease, particularly sCJD (Roos et al. 1973; Brown et al. 1986a). There is as yet no specific diagnostic test for prion disease in the cerebrospinal fluid: the concentration of the proteins 14-3-3 (Hsich et al. 1996), S-100, and neuron-specific enolase is often increased, but these represent general markers of neuronal breakdown and do not allow for definite diagnosis of prion encephalopathy. A definitive diagnosis of human prion disease, which is invariably fatal, can often be made from the examination of brain tissue. Since 1992, knowledge of the molecular genetics of prion diseases has made it possible, using peripheral tissues, to diagnose inherited prion disease in living patients by sequencing the prion alleles.

A broad spectrum of neuropathological features in human prion diseases precludes a precise neuropathological definition. The classic neuropathological features of human prion disease include spongiform degeneration, gliosis, and neuronal loss in the absence of any significant inflammatory reaction (Budka et al. 1995). When present, amyloid plaques that stain with α-PrP antibodies are diagnostic.

The presence of protease-resistant PrP (PrP^{Sc} or PrP^{CJD}) in the infectious and sporadic forms, and in most of the inherited forms of these diseases, implicates prions in their pathogenesis. The absence of PrP^{CJD} in a biopsy specimen may simply reflect regional variations in the concentration of the protein (Serban et al. 1990). In some patients with inherited prion disease, PrP^{Sc} is barely detectable or undetectable (Little et al. 1986; Brown et al. 1992b; Manetto et al. 1992; Medori et al. 1992b); this situation seems to be mimicked in transgenic mice that express a mutant PrP gene and spontaneously develop neurological illness indistinguishable from experimental murine scrapie (Hsiao et al. 1990, 1994).

In humans and transgenic mice that have no detectable protease-resistant PrP but express mutant PrP, neurodegeneration may, at least in part, be caused by abnormal metabolism of mutant PrP (Hegde et al. 1998, 1999). Because molecular genetic analyses of PrP genes in patients with unusual dementing illnesses are readily performed, the diagnosis of inherited prion disease can often be established where there was little or no neuropathology (Collinge et al. 1990), atypical neurodegenerative disease (Collinge et al. 1990) or misdiagnosed neurodegenerative disease (Heston et al. 1966; Azzarelli et al. 1985), including Alzheimer's disease.

Since the prion is defined as the transmissible agent causing spongiform encephalopathies, horizontal transmission of neurodegeneration to experimental hosts should be considered the 'gold standard' of prion disease. However, transmission of inherited prion diseases from humans to experimental animals is frequently negative when using rodents, despite the presence of a pathogenic mutation in the *PRNP* gene (Tateishi et al. 1992b). New lines of transgenic mice with enhanced susceptibility to human or animal prions (Telling et al. 1994; Weissmann et al. 1998) are starting to enable transmission studies that were not practical in apes and monkeys (Brown et al. 1994b).

Stanley Prusiner advocates that the hallmark common to all prion diseases, whether sporadic, dominantly inherited or acquired by infection, is that they involve the aberrant metabolism of the prion protein (Prusiner 1991). The author of this chapter, however, contends that nontransmissible diseases involving aberrant states

of PrP may differ significantly in their pathophysiology from TSE proper, and cautions that their classification can only be provisional given the current state of knowledge. The term 'prion' is used here in an operational meaning: it is not meant to be necessarily identical with PrP^{Sc}, but it simply denotes the infectious agent, whatever this agent exactly consists of (Aguzzi and Weissmann 1997). If one takes this viewpoint, PrP^{Sc} defined originally as the protease-resistant moiety associated with prion disease would be a mere surrogate marker for the prion. Even if this position may seem all too negativistic in view of the wealth of compelling evidence in favor of the protein-only hypothesis, we still do not understand the molecular details of prion replication.

Because protease resistance and infectivity of PrP^{Sc} may not necessarily be congruent, it is desirable to expand the range of reagents that will interact specifically with disease-associated prion protein, but not (or to a lesser degree) with its counterpart, PrP^{C}. One IgM monoclonal antibody termed 15B3 has been described a few years ago to fulfill the above conditions (Korth et al. 1997), but this antibody has not become generally available, and no follow-up studies have been published. Disappointingly, expression of the complementarity-determining regions of 15B3 in the context of a phage, as well as expression of its heavy chain variable region in transgenic mice, failed to reproduce the specificity of PrP^{Sc} binding (Heppner et al. 2001b).

The puzzling phenomenon was described that a prevalent constituent of blood serum, plasminogen, captures efficiently PrP^{Sc} but not PrP^{C} when immobilized onto the surface of magnetic beads (Fischer et al. 2000; Maissen et al. 2001). While the significance of this phenomenon in vivo has yet to be addressed, plasminogen binds to PrP^{Sc} from a variety of species and genotypes (Maissen et al. 2001), and the captured PrP^{Sc} retains the glycotype profile characteristic of the prion strain from which it was isolated (M. Maissen and A. Aguzzi, unpublished data).

In practical terms, however, a definitive diagnosis of human prion disease can be rapidly accomplished if PrP^{Sc} can be detected by western immunoblot analysis of brain homogenates in which samples are subjected to limited proteolysis to remove PrP^{C} before immunostaining (Bockman et al. 1985, 1987; Brown et al. 1986b; Serban et al. 1990). Because of regional variations in PrP^{Sc} concentration, methods using homogenates prepared from small brain regions may give false-negative results. Alternatively, PrP^{Sc} may be detected in situ in cryostat sections bound to nitrocellulose membranes followed by limited proteolysis to remove PrP^{C}, and guanidinium salt treatment to denature PrP^{Sc}, and thus enhance its accessibility to α-PrP antibodies (Taraboulos et al. 1992a). Denaturation of PrP^{Sc} in situ prior to immunostaining has also been accomplished by autoclaving fixed tissue sections (Kitamoto et al. 1992).

In the familial forms of the prion diseases, molecular genetic analyses of PRNP can be diagnostic and can be performed on DNA extracted from blood leukocytes antemortem. Unfortunately, such testing is of little value in the diagnosis of the sporadic or infectious forms of prion disease. Although the first missense PrP mutation was discovered when the two PrP alleles of a patient with GSS were cloned from a genomic library and sequenced (Hsiao et al. 1989), all subsequent novel missense and insertional mutations have been identified in PrP ORFs amplified by polymerase chain reaction (PCR) and sequenced. The 759 base pairs encoding the 253 amino acids of PrP reside in a single exon of the PrP gene, providing an ideal situation for the use of PCR. Amplified PrP ORFs can be screened for known mutations using one of several methods, the most reliable of which is automated sequencing. The list of disease-associated *PRNP* mutations is unlikely to be complete yet, and novel mutations may be found when the PrP ORF is sequenced. It is thinkable, but unproven, that mutations and/or polymorphisms in regulatory and intronic regions may contribute to CJD susceptibility.

When PrP amyloid plaques in brain are present, they are diagnostic for prion disease as noted above. Unfortunately, they are present in only ca. 10 percent of CJD cases but, by definition, in all cases of GSS. The amyloid plaques in CJD are compact (kuru plaques). Those in GSS are either multicentric (diffuse) or compact. The amyloid plaques in prion diseases contain PrP (Kitamoto et al. 1986; Roberts et al. 1986, 1988). The multicentric amyloid plaques that are pathognomonic for GSS may be difficult to distinguish from the neuritic plaques of Alzheimer's disease except by immunohistology (Ghetti et al. 1989; Nochlin et al. 1989; Ikeda et al. 1991). In the GSS kindreds, the diagnosis of Alzheimer's disease was excluded because the amyloid plaques failed to stain with β-amyloid antiserum, but stained with PrP antiserum. In subsequent studies, missense mutations were found in the PrP genes of these kindreds.

In summary, the diagnosis of prion disease may be made in patients on the basis of:

- the presence of PrP^{Sc}
- mutant PrP genotype or
- appropriate immunohistology,

and should not be excluded in patients with atypical neurodegenerative diseases until one, or preferably two, of these examinations have been performed.

Infectious prion diseases

IATROGENIC CREUTZFELDT–JAKOB DISEASE

Accidental transmission of CJD to humans seems to have occurred by corneal transplantation (Duffy et al. 1974), contaminated EEG electrode implantation (Bernoulli et al. 1977), and surgical operations using

contaminated instruments or apparatus (Table 2) (Masters and Richardson 1978; Kondo and Kuroina 1981; Will and Matthews 1982; Davanipour et al. 1984). A cornea unknowingly removed from a donor with CJD was transplanted to an apparently healthy recipient who developed CJD after a prolonged incubation period. Corneas of animals have significant levels of prions (Buyukmihci et al. 1980), making this situation seem quite probable. The same improperly decontaminated EEG electrodes that caused CJD in two young patients with intractable epilepsy caused CJD in a chimpanzee 18 months after their experimental implantation (Bernoulli et al. 1979; Gibbs et al. 1994).

Surgical procedures may have resulted in accidental inoculation of patients with prions during their operations (Gajdusek 1977; Will and Matthews 1982; Brown et al. 1992c), presumably because some instrument or apparatus in the operating theatre became contaminated when a CJD patient underwent surgery. Although the epidemiology of these studies is highly suggestive, no proof for such episodes exists.

Since 1988, 11 cases have been recorded of CJD after implantation of dura mater grafts (Otto 1987; Thadani et al. 1988; Masullo et al. 1989; Nisbet et al. 1989; Miyashita et al. 1991; Willison et al. 1991; Brown et al., 1992c; Martinez Lage et al. 1993). All the grafts were thought to have been acquired from a single manufacturer whose preparative procedures were inadequate to inactivate human prions (Brown et al. 1992c). One case of CJD occurred after repair of an eardrum perforation with a pericardium graft (Tange et al. 1989). Thirty cases of CJD in physicians and healthcare workers have been reported (Berger and David 1993); however, no occupational link has been established (Ridley and Baker 1993). Whether any of these cases represents infectious prion diseases contracted during care of patients with CJD or processing specimens from these patients remains uncertain. It is interesting to note that surgical operations of any kind appear to constitute a mild risk factor for subsequent CJD (Collins et al. 1999).

Table 2 *Transmission of prion diseases among humans (kuru and iatrogenic cases), as compiled from published reports and various national surveillance centers*

Diseases	No. of cases
Kuru (1957–82)	
Adult females	1 739
Adult males	248
Children and adolescents	597
Total	2 584
Iatrogenic Creutzfeldt–Jakob disease	
Electroencephalography electrodes	2
Corneal transplants	2
Human pituitary hormones	143
Dura mater grafts	114
Neurosurgical procedures	5
Total	266

HUMAN GROWTH HORMONE THERAPY

The possibility of transmission of CJD from contaminated human grown hormone (HGH) preparations derived from human pituitaries has been raised by the occurrence of fatal cerebellar disorders with dementia in >80 patients ranging in age from 10 to over 60 years (Brown et al. 1985b; Buchanan et al. 1991; Fradkin et al. 1991; Brown 1992). Although one case of spontaneous CJD in a 20-year-old woman has been reported (Packer et al. 1980; Brown et al. 1985a; Gibbs et al. 1985), CJD in people under 40 years of age is extremely rare. These patients received injections of HGH every 2–4 days for 4–12 years (Gibbs et al., 1985; Koch et al., 1985; Powell Jackson et al. 1985; Tintner et al. 1986; Croxson et al. 1988; Marzewski et al. 1988; New et al. 1988; Anderson et al. 1990; Billette de Villemeur et al. 1991; Macario et al. 1991; Ellis et al., 1992). It is interesting that most of the patients presented with cerebellar syndromes that progressed over periods varying from 6 to 18 months. Some patients became demented during the terminal phases of their illnesses. This clinical course resembles kuru more than ataxic CJD in some respects (Prusiner et al. 1982c). Assuming that these patients developed CJD from injections of prion-contaminated HGH preparations, the possible incubation periods range from 4 to 30 years (Brown et al. 1992c). The longest incubation periods are similar to those (20–30 years) associated with recent cases of kuru (Gajdusek 1977; Prusiner et al. 1982c; Klitzman et al. 1984). Many patients received several common lots of HGH at various times during their prolonged therapies, but no single lot was administered to all the American patients. An aliquot of one lot of HGH has been reported to transmit CNS disease to a squirrel monkey after a prolonged incubation period (Gibbs et al. 1993). How many lots of the HGH might have been contaminated with prions is unknown.

CJD has an annual incidence of 1–1.5/10^6 (Masters and Richardson 1978). Updated numbers can be found at the web site www.eurocjd.ed.ac.uk/euroindex.htm. About 1 percent of the population dies each year and most CJD patients die within one year of developing symptoms. Thus, one per 10^4 dead people may be infected with the CJD agent. Since 10 000 human pituitaries were typically processed in a single HGH preparation, the possibility of hormone preparations contaminated with CJD prions is not remote (Brown et al. 1985b). The concentration of CJD prions within infected human pituitaries is unknown; it is interesting that widespread degenerative changes have been observed in both the hypothalamus and pituitary of sheep with scrapie (Beck et al. 1964). The forebrains from scrapie-infected mice have been added to human pituitary suspensions to determine if prions and HGH co-purify (Lumley Jones

et al. 1979). Bioassays in mice suggest that prions and HGH do not co-purify with currently used protocols (Taylor et al. 1985). Although these results seem reassuring, the relatively low titer of the murine scrapie prions used in these studies may not have provided an adequate test (Brown 1985). The extremely small size and charge heterogeneity exhibited by scrapie (Alper et al. 1966; Prusiner et al. 1978, 1980b, 1983; Bolton et al. 1985) and presumably CJD prions (Bendheim et al. 1985; Bockman et al. 1985) may complicate procedures designed to separate pituitary hormones from these slow infectious pathogens. Fortunately, the advent of recombinant protein production has rendered this problem obsolete – however, it is possible that a number of HGH-treated persons are incubating prions, and may pass it to others and/or develop iCJD in the future.

Molecular genetic studies have revealed that most patients developing iCJD after receiving pituitary-derived HGH are homozygous for either Met or Val at codon 129 of the PrP gene (Collinge et al. 1991; Brown et al. 1994a; Deslys et al. 1994). Homozygosity at the codon 129 polymorphism has also been shown to predispose individuals to sCJD (Palmer et al. 1991). It is interesting that valine homozygosity seems to be over-represented in these HGH cases compared to the general population.

Five cases of CJD have occurred in women receiving human pituitary gonadotropin (Cochius et al. 1990; Cochius et al. 1992; Healy and Evans 1993).

Inherited prion diseases

FAMILIAL PRION DISEASE

The recognition that ca. 10 percent of CJD cases are familial (Kirschbaum 1924; Meggendorfer 1930; Stender 1930; Davison and Rabiner 1940; Jacob et al. 1950; Friede and DeJong 1964; Rosenthal et al. 1976; Masters and Gajdusek 1979; Masters et al. 1981a, b) posed a perplexing problem once it was established that CJD is transmissible (Gibbs et al. 1968, 1969). Equally puzzling was the transmission of GSS to nonhuman primates (Gibbs et al. 1968, 1969; Masters et al. 1981a) and mice (Tateishi et al. 1979), because most cases of GSS are familial (Gerstmann et al. 1936). Like sheep scrapie, the relative contributions of genetic and infectious etiologies in the human prion diseases remained a conundrum until molecular clones of the PrP gene became available to probe the inherited aspects of these disorders.

PRP MUTATIONS AND GENETIC LINKAGE

The discovery of the PrP gene and its linkage to scrapie incubation times in mice (Carlson et al. 1986) raised the possibility that mutation might feature in the hereditary human prion diseases. A proline (P)→leucine (L) mutation at codon 102 was shown to be linked genetically to development of GSS with a logarithm of odds (LOD) score exceeding 3 (Hsiao et al. 1989). This mutation may be due to the deamination of a methylated CpG in a germline PrP gene resulting in the substitution of a thymine (T) for cytosine (C). The P102L mutation has been found in ten different families in nine different countries, including the original GSS family (Doh-ura et al., 1989; Goldgaber et al. 1989; Goldhammer et al. 1993). Amyloid plaques isolated from patients with GSS (P102L) were composed of PrP molecules with an L at residue 102 based on protein sequencing of purified peptides (Kitamoto et al. 1991b). Patients with GSS who have a P→L substitution at PrP codon 105 have also been reported (Kitamoto et al. 1993a).

Some patients with a mutation at codon 117 have a dementing or telencephalic form of GSS (Doh-ura et al., 1989; Hsiao et al. 1991b), whereas others have an ataxic form of the disease (Mastrianni et al. 1995). In both forms of GSS (A117V), PrP amyloid plaques were found as well as spongiform degeneration. The factor or factors that determine the different phenotypes of this disease are unknown.

Patients with PrP mutations at codons 198 and 217 (Hsiao et al. 1992) were once thought to have familial Alzheimer's disease, but are now known to have prion diseases on the basis of PrP immunostaining of amyloid plaques and PrP gene mutations (Farlow et al. 1989; Ghetti et al. 1989; Nochlin et al. 1989; Giaccone et al. 1990). A genetic linkage study of this family produced a LOD score exceeding 6 (Dlouhy et al. 1992). Patients with the codon 198 mutation resulting in a phenylalanine (F)→serine (S) substitution (Hsiao et al. 1992) have numerous neurofibrillary tangles (NFT) that stain with antibodies to tau (τ) protein and have amyloid (Farlow et al. 1989; Ghetti et al. 1989; Nochlin et al. 1989; Giaccone et al. 1990) that are composed largely of a PrP fragment extending from residues 58 to 150 (Tagliavini et al. 1991). Because the F198S mutation is not contained within the major PrP peptide of the amyloid plaques, patients heterozygous at codon 129 were chosen to determine whether this peptide is derived from the mutant protein. Like the results of studies with GSS (P102L) (Kitamoto et al. 1991b) and GSS (Y145Stop) (Kitamoto et al. 1993b), protein sequencing revealed that the PrP peptides are derived exclusively from the mutant protein (Tagliavini et al. 1994). Similar results were found with PrP peptides from a patient of Swedish ancestry with the codon 217 mutation resulting in a glutamine (Q)→arginine (R) substitution (Hsiao et al. 1992). The neuropathology of patients with the codon 217 mutation was similar to that of patients with the codon 198 mutation (Ikeda et al. 1991).

One patient with a prolonged neurological illness spanning almost two decades who had PrP amyloid plaques and NFTs had an amber mutation of the PrP gene resulting in a stop codon at residue 145 (Kitamoto et al. 1993b; Kitamoto and Tateishi 1994). Staining of the plaques with α-PrP peptide antisera suggested that they might be composed

exclusively of the truncated PrP molecules. That a PrP peptide ending at residue 145 polymerizes into amyloid filaments is to be expected since an earlier study noted above showed that the major PrP peptide in plaques from patients with the F198S mutation was an 11 kDa PrP peptide beginning at codon 58 and ending at ca. 150 (Tagliavini et al. 1991). A synthetic PrP peptide containing residues 90–145 was found to adopt an α-helical or β-sheet structure depending on the solvent as determined by two-dimensional magnetic resonance imaging, FTIR spectroscopy and fiber diffraction (Nguyen et al. 1995; Zhang et al. 1995).

An insert of 144 bp at codon 53 containing six octarepeats has been described in patients with CJD from four families all residing in southern England (Owen et al. 1989, 1990b, 1991; Crow et al. 1990; Collinge et al. 1992; Poulter et al. 1992). This mutation must have arisen through a complex series of events, because the human PrP gene contains only five octarepeats, indicating that a single recombination event could not have created the insert. Genealogical investigations have revealed that all four families are related, arguing for a single founder born more than two centuries ago (Crow et al. 1990). The LOD score for this extended pedigree exceeds 11. Studies from several laboratories have demonstrated that two, four, five, six, seven, eight, or nine octarepeats in addition to the normal five are present in individuals with inherited CJD (Owen et al. 1989, 1990b, 1992; Goldfarb et al. 1991a; Brown 1993), whereas deletion of one octarepeat has been identified without the neurological disease (Laplanche et al., 1990; Vnencak Jones and Phillips 1992; Palmer et al., 1993).

For many years the unusually high incidence of CJD among Israeli Jews of Libyan origin was thought to be due to the consumption of lightly cooked sheep brain or eyeballs (Herzberg et al. 1974; Kahana et al. 1974, 1991). In reality, some Libyan and Tunisian Jews in families with CJD have a PrP gene point mutation at codon 200 resulting in a glutamate (E)→lysine (K) substitution (Goldfarb et al. 1990b; Hsiao et al. 1991a). One patient was homozygous for the E200K mutation but her clinical presentation was similar to that of heterozygotes (Hsiao et al. 1991a), arguing that familial prion diseases are true autosomal dominant disorders. The E200K mutation has also been found in Slovaks originating from Orava in north central Czechoslovakia (Goldfarb et al. 1990c), in a cluster of familial cases in Chile (Goldfarb et al. 1991b), and in a large German family living in the USA (Bertoni et al. 1992). It is likely that the E200K mutation has arisen independently multiple times through the deamidation of a methylated CpG as described above the codon 102 mutation (Hsiao et al. 1989, 1991a). In support of this hypothesis are records of Libyan and Tunisian Jews, indicating that they are descendants of Jews who settled on the island of Jerba off the southern coast of Tunisia around 500 BC and not from Sephardim (Udovitch and Valensi 1984).

Many families with CJD have been found to have a point mutation at codon 178, resulting in an aspartate (D)→asparagine (N) substitution (Fink et al. 1991; Goldfarb et al. 1991c, 1992a; Haltia et al. 1991; Brown et al. 1992a). In these patients as well as those with the E200K mutation, PrP amyloid plaques are rare; the neuropathological changes generally consist of widespread spongiform degeneration. A prion disease called fatal familial insomnia (FFI) was described in three Italian families with the D178N mutation (Lugaresi et al. 1986; Medori et al. 1992a). The neuropathology in these patients with FFI is restricted to selected nuclei of the thalamus. It is unclear whether all patients with the D178N mutation or only a subset present with sleep disturbances. It has been proposed that the allele with the D178N mutation encodes a methionine (M) at position 129 in FFI whereas a valine (V) is encoded at position 129 in familial CJD (Goldfarb et al. 1992b). The discovery that FFI is an inherited prion disease clearly widens the clinical spectrum of these disorders and raises the possibility that many other degenerative diseases of unknown etiology may be caused by prions (Johnson 1992; Medori et al. 1992b). The D178N mutation has been linked to the development of prion disease with a LOD score exceeding 5 (Petersen et al. 1992). Studies of PrP^{Sc} in FFI and familial CJD caused by the D178N mutation reveal that, after limited proteolysis, the molecular mass of the FFI PrP^{Sc} is ca. 2 kDa smaller (Monari et al. 1994). Whether this difference in protease resistance reflects distinct conformations of PrP^{Sc} that give rise to the different clinical and neuropathological manifestations of these inherited prion diseases remains to be established.

A valine (V)→isoleucine (I) mutation at PrP codon 210 produces CJD with classic symptoms and signs (Pocchiari et al. 1993; Ripoll et al. 1993). The V210I mutation is thought to be incompletely penetrant, like the E200K mutation was previously thought to be (Chapman et al. 1994; Spudich et al. 1995). It seems likely that, if a sufficiently large number of people with the V210I mutation could be analyzed, complete penetrance that is age-dependent would be found.

Other point mutations at codons 208 and, possibly, 232 also segregate with inherited prion diseases (Kitamoto et al. 1993c; Kitamoto and Tateishi 1994; Mastrianni et al. 1995). The codon 208 mutation results in the substitution of arginine (R) for histidine (H). A patient with this mutation presented with a progressive dementia and widespread myoclonus was subsequently observed. The diagnosis of CJD was confirmed at autopsy. Patients with the codon 232 mutation present with dementia; this mutation is particularly notable, because it lies within the C-terminal signal sequence that is removed from PrP when the GPI anchor is attached (Stahl et al. 1992). Table 3 summarizes all mutations within the human *PRNP* gene that were found to cosegregate with inherited prion disease.

Table 3 *Synopsis of all* PRNP *mutations and polymorphism, and their association with inherited prion diseases*

Presentation	Mutation in *PRNP*
Creutzfeldt–Jakob phenotype	D178N-129V, V180I, V180I-M232R, T183A, T188A, E196K, E200K, V203I, R208H, V210I, E211Q, M232R, 96 bpi, 120 bpi, 144 bpi, 168 bpi, 48 bpd
Gerstmann–Sträussler–Scheinker phenotype	P102L, P105L, A117V, G131V, F198S, D202N, Q212P, Q217R, M232T, 192 bpi
Fatal familial insomnia phenotype	D178N-129M
Vascular amyloid depositions	Y145STOP
Proven but unclassified prion disease	H187R, 216 bpi
Neuropsychiatric disorder, no proven prion disease	I138M, G142S, Q160s, T188K, T188R, M232R, P238S, 24 bpi, 48 bpi,
Polymorphisms, established influence on phenotype	M129V
Polymorphisms, suggested influence on phenotype	N171S, E219K
Silent polymorphisms	P68P, A117A, G124G, V161V, N173N, H177H, T188T, D202D, Q212Q, R228R, S230S

The wild-type human PrP gene contains five octarepeats [P(Q/H)GGG(G/–) WGQ] from codons 51–91. Deletion of a single octarepeat at codon 81 or 82 is not associated with prion disease (Laplanche et al. 1990; Puckett et al. 1991; Vnencak Jones and Phillips 1992); whether this deletion alters the phenotypic characteristics of a prion disease is unknown. There are common polymorphisms at codons 117 (Ala→Ala) and 129 (Met→Val); homozygosity for Met or Val at codon 129 seems to increase susceptibility to sCJD (Palmer et al. 1991). Octarepeat inserts of 16, 32, 40, 48, 56, 64, and 72 amino acids at codons 67, 75, or 83 are designated as bpi (base pair insertion). Point mutations are designated by the wild-type amino acid preceding the codon number and the mutant residue follows, e.g. P102L. These point mutations segregate with the inherited prion diseases and significant genetic linkage (underlined mutations) has been demonstrated where sufficient specimens from family members are available. The single letter codes for amino acids is as follows: A, Ala; D, Asp; E, Glu; F, Phe; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

Along with the demonstration that PrP-deficient mice are resistant to prion infections, the linkage of *PRNP* mutations with hereditary prion disease provide a very solid piece of evidence in favor of Prusiner's protein-only hypothesis.

Variant Creutzfeldt–Jakob disease

The most recently recognized form of CJD in humans, new variant CJD (vCJD), was first described in 1996 and has been linked to BSE (Will and Zeidler 1996). vCJD represents a distinct clinicopathological entity that is characterized at onset by psychiatric abnormalities, sensory symptoms, and ataxia, and eventually leads to dementia along with other features usually observed in sCJD. What distinguishes vCJD from sporadic cases is that the age of patients is abnormally low (vCJD, 19–39 years; sCJD, 55–70 years) and duration of the illness is rather long (vCJD, 7.5–22 months; sCJD, 2.5–6.5 months). Moreover, vCJD displays a distinct pathology within the brain characterized by abundant 'florid plaques,' decorated by a daisy-like pattern of vacuolation. Most cases of vCJD have been observed in the United Kingdom (Will et al. 2000). For several years, it has been thought that prions are distributed much more broadly within the body of vCJD victims than of sCJD patients (Wadsworth et al. 2001). However, this view has recently been challenged by the finding of PrP^{Sc} in muscle and spleen of sCJD patients (Glatzel et al. 2003a).

What is the evidence that the agent causing vCJD may be identical with that of BSE when transmitted to humans? None of the arguments to date is conclusive, yet each – and particularly when they are all considered together – is tantalizing. Much effort has gone into characterizing the 'strain properties' of the agent that affects cows and humans. Because the molecular substrate that underlies the nature of prion strains (which are heritable phenotypic traits that can be reproduced upon serial passage through experimental animals) is not known, strain-typing of prions has to rely on surrogate markers.

Two such markers have been particularly useful. One is the distribution of neuronal vacuoles in the brains of affected animals: for example, whereas some strains target the cortical cerebral ribbon, others mainly affect the midbrain (Fraser and Dickinson 1967). The BSE prion strain attacks the dorsal medulla and the superior colliculus (a part of the optical pathway) virulently and consistently (Bruce et al. 1997). Worryingly, BSE prions (extracted from the brains of affected cows) and vCJD prions derived from the brains of British patients produce the same lesional patterns when transmitted to panels of susceptible mice (Aguzzi 1996; Aguzzi and Weissmann 1996; Bruce et al. 1997).

The second marker for strain typing of prions comes from analyzing the biochemical properties of disease-associated PrP recovered from the brains of cattle and humans. Different steric conformations of PrP (which, according to the most popular hypothesis, account for the phenotypic strain properties) expose different sites to the action of proteolytic enzymes. These sites, in turn, can be identified by the different molecular masses of the resulting fragments. When used in conjunction with the ratio of diglycosylated to monoglycosylated PrP –

another parameter that seems to correlate with strain properties – these traits were again found to be indistinguishable between BSE and human vCJD prions (Collinge et al. 1996; Hill et al. 1997a). A third line of argument relating BSE and human vCJD concerns epidemiology of the disease. To date, the total number of definite or probable cases of vCJD is 136 in the UK (Table 4), but only a few cases in other countries including Ireland, France (Chazot et al. 1996), and Italy. Assuming that the quality of the epidemiologic surveillance is similar in these countries and in the rest of Europe (which has not reported cases of vCJD), the unavoidable conclusion is that the incidence of vCJD correlates with the prevalence of BSE.

One of the most powerful arguments is the study of pathogenesis in primates. In a classical experiment, Lasmezas and colleagues inoculated brain extracts from BSE-affected cows into cynomolgus macaques. After about 3 years, all inoculated primates (two adults and one infant) developed spongiform encephalopathy. The histopathological appearance of the disease was identical to that of vCJD and included characteristic 'florid plaques,' which have been recognized in every case of vCJD (Lasmezas et al. 1996b). These are spherical conglomerates of fibrillary birefringent material that stains positive with 'amyloidotropic' dyes such as Congo red. Characteristically, vCJD plaques are surrounded by a rim of microvacuolated brain tissue – a feature which they share with the deposits in sheep scrapie, but that is not seen in classical CJD, nor in any other human spongiform encephalopathy.

Impressive as all of these arguments may seem, however, each is phenomenological rather than causal. Distribution of histopathological lesions, as well as morphology of plaque deposits, is downstream of the molecular events responsible for prion strain specificity. Measurements of the 'glycotype ratio' may be more directly related to the essence of strains, but there is still no way to tell whether they might simply be surrogate markers. It would be desirable to measure the conformation of disease-associated PrP more directly. Some inroads have been made with a method that exploits the relative affinities of anti-PrP^C antibodies (Aguzzi 1998; Safar et al. 1998), but, to our knowledge, this possibility is restricted to differentiation of mouse and hamster PrPs, and has not yet been applied to investigating BSE and vCJD.

Given that a large fraction of the European population may have been exposed to BSE prions, yet only a minute minority developed vCJD, there can be hardly any doubt that additional genetic modifiers exist, other than *PRNP* polymorphisms. It was originally claimed that specific allelotypes of the major histocompatibility complex may represent such modifiers (Jackson et al. 2001), but this was not confirmed by later studies (Pepys et al. 2003).

So what will the numbers of vCJD victims be in the future? Terrible as the disease has been for patients, we

Table 4 *The Department of Health's latest information about the numbers of known cases of Creutzfeldt–Jakob disease*

Referral of suspect CJD			Deaths of definite and probable CJD					
Year	Referrals	Year	Sporadic	Iatrogenic	Familial	GSS	vCJD[a]	Total deaths
1990	53	1990	28	5	0	0	–	33
1991	75	1991	32	1	3	0	–	36
1992	96	1992	45	2	5	1	–	53
1993	78	1993	37	4	3	2	–	46
1994	118	1994	53	1	4	3	–	61
1995	87	1995	35	4	2	3	3	47
1996	134	1996	40	4	2	4	10	60
1997	161	1997	60	6	4	1	10	81
1998	154	1998	63	3	4	1	18	89
1999	170	1999	62	6	2	0	15	85
2000	178	2000	49	1	2	1	28	81
2001	179	2001	56	3	2	2	20	83
2002	163	2002	72	0	4	1	17	94
2003[a]	107	2003[a]	35	4	1	0	15	55
Total	1 753	Total deaths	667	44	38	19	136	904

The Department of Health is issuing the latest information about the numbers of known cases of Creutzfeldt–Jakob disease. This includes cases of variant Creutzfeldt–Jakob disease (vCJD) – the form of the disease thought to be linked to BSE (as of September 2003). For updated information, see the following link: www.doh.gov.uk/cjd/cjd_stat.htm

Referrals: a simple count of all the cases which have been referred to the National CJD Surveillance Unit for further investigation. About half the cases referred in the past have turned out not to be CJD. Cases are notified to the Unit from neurologists, neuropathologists, neurophysiologists, general physicians, psychiatrists, EEG departments, etc.

Deaths: all columns show the number of deaths that have occurred in definite and probable cases of all types of CJD and GSS in the year shown.

Definite cases: the diagnosis was pathologically confirmed, in most cases by postmortem examination of brain tissue.

Probable vCJD cases: those who fulfill the 'probable' criteria and are either still alive, or have died and await postmortem pathological confirmation.

a) As at 1st September 2003.

have not yet seen a large-scale epidemic. Although many mathematical models have been generated (Ghani et al. 1998, 2000), the number of cases is still too small to predict future developments with any certainty. Since the year 2001, the incidence of vCJD in the UK appears to be stabilizing and may actually be even falling (Figure 12). One may argue that it is too early to draw any far-reaching conclusions, but each month passing without any dramatic rise in the number of cases increases the hope that perhaps the total number of vCJD victims will be limited (Valleron et al. 2001).

POSSIBLE TRANSMISSION OF ADDITIONAL PRION EPIZOOTICS TO HUMANS

There is no example of zoonotic transmission of prions from sheep and goat to humans: many epidemiologic studies have failed to implicate scrapie prions from sheep as a cause of CJD (Malmgren et al. 1979; Harries Jones et al. 1988; Cousens et al. 1990). However, there is uncertainty surrounding the danger of transmission to humans represented by CWD. Actually, even transmissibility of BSE to humans relies on circumstantial evidence. Epidemiology and biochemistry favor the link between BSE and vCJD, but are not really conclusive. The Koch postulates (which would unambiguously assign an infectious agent to a disease) have never been fulfilled, and experimental inoculation of humans was never performed. Also, accidental exposure to BSE infectivity of a sizable collective at a precisely defined time point has never occurred, or did not result in disease. For these reasons, speculations that variant Creutzfeldt–Jakob disease may not be due to BSE have never completely subsided.

Likewise, we do not know whether scrapie is just a veterinary problem that only affects sheep and goats, or whether it can cross species barriers and affect humans. Finally, it is unknown whether BSE, upon transmission to sheep, remains as dangerous for humans as cow-derived BSE, or whether it becomes attenuated and acquires the (allegedly) innocuous properties of bona fide sheep scrapie.

Another question relates to the possibility of chronic subclinical disease or a permanent 'carrier' status in cows as well as in humans. Evidence that such a carrier status may be produced by the passage of the infectious agent across species was first reported by Race and Chesebro (Aguzzi and Weissmann 1998; Race and Chesebro 1998), and has been confirmed by others (Hill et al. 2000) – at least for the passage between hamsters and mice. Immune deficiency can also lead to a similar situation in which prions replicate silently in the body, even when there is no species barrier (Frigg et al. 1999). So the problem of animal TSEs could be more widespread than is assumed, and may call for drastic measures in farming. Moreover, people carrying the infectious agent may transmit it horizontally (Aguzzi 2000), and the risks associated with this possibility can be met only if we know more about how the agent is transmitted and how prions reach the brain from peripheral sites.

PrP gene polymorphisms

POLYMORPHISMS AT CODONS 129 AND 219

At PrP codon 129, an amino acid polymorphism for M→V has been identified (Owen et al. 1990a). This polymorphism seems able to influence prion disease

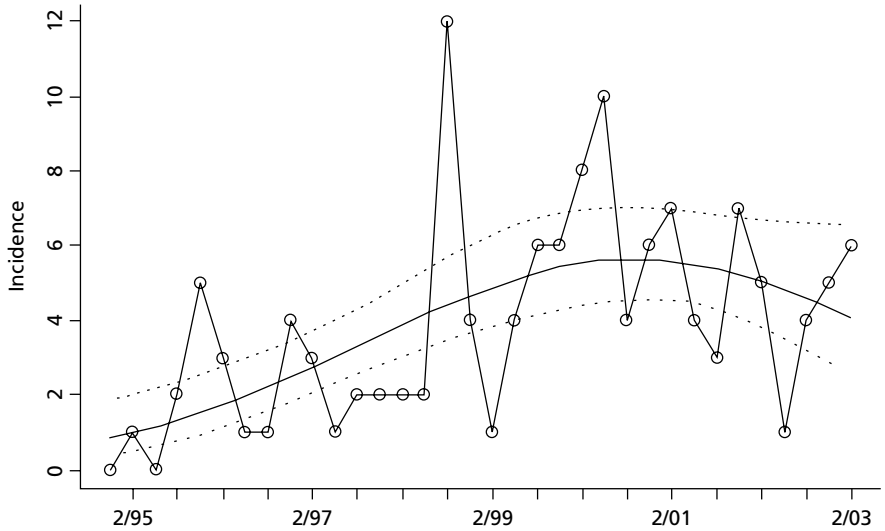

Figure 12 *The course of the vCJD epidemic in the UK. Observed numbers of vCJD deaths in the UK by a quarter (empty circles), and fitted quadratic trend (curved line) with 95 percent confidence interval for the trend (dotted line). The fitted trend shows a good fit to the data with a significant quadtratic term (P=0.002), which would indicate a departure from a constant exponential increase. This model estimates that the current quarterly incidence of deaths is 3.9 and shows that the epidemic reached a peak for deaths in about December 2000 (January 2000 to May 2003 with 95 percent confidence intervals). Regular updates are published by Mr N.J. Andrews (Communicable Disease Surveillance Centre, UK) at the web site: www.cjd.ed.ac.uk/vcjdq.htm*

expression, not only in inherited forms but also in iatrogenic and sporadic forms of prion disease. A second polymorphism resulting in an amino acid substitution at codon 219 (E→K) has been reported in the Japanese population, in which the K allele occurs with a frequency of 6 percent (Kitamoto and Tateishi 1994).

DOES HOMOZYGOSITY AT CODON 129 PREDISPOSE TO CJD?

Studies of caucasian patients with sporadic CJD have shown that most are homozygous for M or V at codon 129 (Palmer et al. 1991). This contrasts with the general population, in which frequencies for the codon 129 polymorphism in caucasians are 12 percent V/V, 37 percent M/M and 51 percent M/V (Collinge et al. 1991). By contrast, the frequency of the V allele in the Japanese population is much lower (Doh-ura et al. 1991; Miyazono et al. 1992) and heterozygosity at codon 129 (M/V) is more frequent (18 percent) in CJD patients than the general population, in whom the polymorphism frequencies are 0 percent V/V, 92 percent M/M and 8 percent M/V (Tateishi and Kitamoto 1993).

Although no specific mutations have been identified in the PrP gene of patients with sCJD (Goldfarb et al. 1990a), homozygosity at codon 129 in sCJD (Hardy 1991; Palmer et al. 1991) is consistent with the results of transgenic mouse studies. The finding that homozygosity at codon 129 predisposes to CJD supports a model of prion production that favors interactions between PrP molecules that are homologous in the H1 and H2 regions (Telling et al. 1995). However, there appears to be considerable evolutionary pressure for maintaining the prion gene. Protective polymorphisms in the human prion gene were heavily selected for, possibly because of evolutionary pressure from cannibalism-propagated prions (Mead et al. 2003). But if prion diseases were so frequent to skew the distribution of *Prnp* alleles, why were $Prnp^{-/-}$ individuals not selected for? This argues for an important, hitherto unidentified function of the cellular prion protein (Aguzzi and Hardt 2003).

BRAIN DAMAGE IN PRION DISEASES

An interesting question regards the molecular mechanism underlying neuropathological changes, in particular cell death, resulting from prion disease. Depletion of PrP^C is an unlikely cause, in view of the finding that abrogation of PrP does not cause scrapie-like neuropathological changes (Büeler et al. 1992), even when elicited postnatally (Mallucci et al. 2002). It is more likely that toxicity of PrP^{Sc} through some PrP^C-dependent process is responsible for neuronal dysfunction and death (Brandner et al. 1998, 1996a).

To address the question of neurotoxicity, brain tissue of $Prnp^{0/0}$ mice was exposed to a continuous source of PrP^{Sc}. Telencephalic tissue from transgenic mice overexpressing PrP (Fischer et al. 1996) was transplanted into the forebrain of $Prnp^{0/0}$ mice and the 'pseudochimeric' brains were inoculated with scrapie prions. All grafted and scrapie-inoculated mice remained free of scrapie symptoms for at least 70 weeks; this exceeded at least sevenfold the survival time of scrapie-infected donor mice (Brandner et al. 1996a). Therefore, the presence of a continuous source of PrP^{Sc} and of scrapie prions does not exert any clinically detectable adverse effects on a mouse devoid of PrP^C. On the other hand, the grafts develop characteristic histopathological features of scrapie after inoculation. The course of the disease in the graft is very similar to that observed in the brain of scrapie-inoculated wild-type mice (Brandner et al. 1998). Since grafts had extensive contact with the recipient brain, prions could navigate between the two compartments, as shown by the fact that inoculation of wild-type animals engrafted with PrP-expressing neuroectodermal tissue resulted in scrapie pathology in both graft and host tissue. Nonetheless, histopathological changes never extended into host tissue, even at the latest stages (>450 days), although PrP^{Sc} was detected in both grafts and recipient brain, and immunohistochemistry revealed PrP deposits in the hippocampus, and occasionally in the parietal cortex, of all animals (Brandner et al. 1996a). Thus, prions moved from the grafts to some regions of the PrP-deficient host brain without causing pathological changes or clinical disease. The distribution of PrP^{Sc} in the white matter tracts of the host brain suggests diffusion within the extracellular space (Jeffrey et al. 1994) rather than axonal transport.

These findings suggest that the expression of PrP^C by an infected cell, rather than the extracellular deposition of PrP^{Sc}, is one of the critical prerequisites for the development of scrapie pathology. Perhaps PrP^{Sc} is inherently nontoxic and PrP^{Sc} plaques found in spongiform encephalopathies are an epiphenomenon rather than a cause of neuronal damage. This hypothesis appears to be supported by the recent data (Ma and Lindquist 2002; Ma et al. 2002), indicating that exaggerated retrograde transport of the prion protein from the endoplasmatic reticulum into the cytosol, or functionally equivalent inhibition of proteasome function, might induce a self-propagating, extremely cytotoxic cellular form, which may ultimately be responsible for neuronal damage. These results are very novel and exciting, but they have been challenged (Roucou et al. 2003) and it is not clear whether they are universally valid (Drisaldi et al. 2003). Therefore it will be important to validate them by investigating whether the aggregated self-propagating material can also be transmitted between individual animals in a classic transmission experiment.

One may therefore propose that availability of PrP^C for some intracellular process elicited by the infectious agent, perhaps the formation of a toxic form of PrP (termed PrP* and discussed in Weissmann 1991a) other than PrP^{Sc} is responsible for spongiosis, gliosis, and neuronal death. This would be in agreement with the

fact that in several instances, and especially in fatal familial insomnia, localized spongiform pathology is detectable although very little PrP^{Sc} is present (Aguzzi and Weissmann 1997).

PERIPHERAL PRION PATHOGENESIS

The fastest and most efficient method for inducing spongiform encephalopathy in the laboratory is intracerebral inoculation of brain homogenate. Inoculation of 1 000 000 ID_{50} infectious units (defined as the amount of infectivity that will induce TSE with 50 percent likelihood in a given host) will yield disease in approximately 6 months; a remarkably strict inverse relationship can be observed between the logarithm of the inoculated dose and the incubation time (Prusiner et al., 1982b, Figure 13).

The above situation does not correspond to what typically happens in the field. There, acquisition of prion infectivity through any of several peripheral routes is the rule. However, prion diseases can also be initiated by feeding (Wells et al. 1987; Kimberlin and Wilesmith 1994; Anderson et al. 1996), by intravenous and intraperitoneal injection (Kimberlin and Walker 1978b) as well as from the eye by conjunctival instillation (J.R. Scott et al. 1993), corneal grafts (Duffy et al. 1974) and intraocular injection (Fraser 1982). Two routes of infection have suggested for a long time that immune cells might be of importance for this phase of prion pathogenesis: oral challenge and administration by scarification (Aguzzi 2003). Therefore, these routes are discussed in some detail in the following paragraphs.

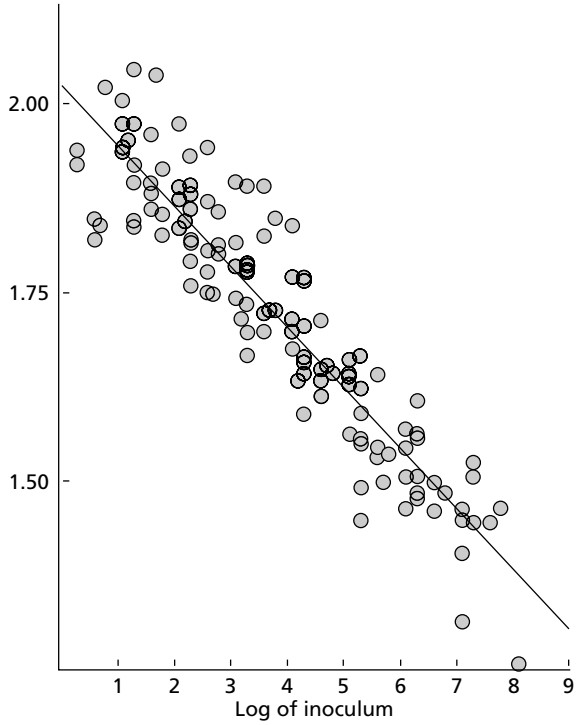

Figure 13 *Prion bioassay by the incubation time method. This figure is reproduced from an important study by Prusiner and colleagues that demonstrates an inverse logarithmic relationship between size of infectious inoculum and latency period of scrapie in experimental animals (Prusiner et al. 1982b). The relationship is so precise that incubation time can actually be used to back-calculate the prion titer of a test article, e.g. for inactivation studies. Measurement of prion titers by the incubation time method has become a standard procedure which can be used for both wild-type and transgenic animals.*

The pathway of orally administered prions

Upon oral challenge, an early rise in prion infectivity can be observed in the distal ileum of infected organisms. This is true for several animal species, but was most extensively investigated in the sheep (Wells et al. 1994; Vankeulen et al. 1996). There, Peyer's patches acquire strong immunopositivity for the prion protein. Immunohistochemical stains with antibodies to the prion protein typically reveal a robust signal in primary B-cell follicles and germinal centers, which roughly colocalizes with the complement receptor, CD35, in a wide variety of secondary lymphoid organs including appendix and tonsils (Hill et al. 1997b). Although conventional light microscopy does not allow differentiating between PrP^{C} and PrP^{Sc}, western blot analysis has not left any doubt about the fact that Peyer's patches do accumulate the disease-associated form of the prion protein.

The latter is true also in the mouse model of scrapie, which is being used as a convenient experimental paradigm by many laboratories including ours. Administration of mouse-adapted scrapie prions (Rocky Mountain Laboratory (RML) strain, originally derived from the Chandler sheep scrapie isolate) induces a surge in intestinal prion infectivity as early as a few days after inoculation (Prinz et al. 2003c). All of the above evidence conjures the suggestion that Peyer's patches may represent a portal of entry for orally administered prions on their journey from the luminal aspect of the gastroenteric tube to the central nervous system. However, the question as to whether the same applies for BSE-affected cattle has been answered less unambiguously.

In a monumental study of BSE pathogenesis in cattle carried out at the UK Veterinary Laboratory Agency, cows of various ages were fed with 100 g, 10 g, 1 g, or 100 mg of brain homogenate derived from BSE-sick cows (Bradley 2000). A large variety of tissues was taken at various points in time, homogenized, and transmitted intracerebrally to indicator organisms in order to assess their prion content. This study was designed to be performed over a time frame of more than a decade and is still under way at the time of writing: it has uncovered a transient surge in infectivity in the distal ileum of cows at approximately 6 months post-infection. Infectivity then subsides, but it appears to return to the terminal ileum at the end stages of disease, perhaps by means of

some sort of retrograde transport (Wells et al. 1998). Although this was not formally confirmed, it appears likely that Peyer's patches are the sites of prion accumulation in the gastrointestinal tract of cattle challenged orally with prions.

Oral prion susceptibility correlates with number but not structure of Peyer's patches

Recruitment of activated B lymphocytes to Peyer's patches requires $\alpha_4\beta_7$ integrin as an essential homing receptor: Peyer's patches of mice that lack β_7 integrin are normal in number, but are atrophic and almost entirely devoid of B cells (Wagner et al. 1996). Therefore, it seemed interesting to investigate the susceptibility to orally administered prions of $\beta_7^{-/-}$ mice. Surprisingly, minimal infectious dose and disease incubation after oral exposure to logarithmic dilutions of prion inoculum were similar in $\beta_7^{-/-}$ and wild-type mice (Prinz et al. 2003c). Despite their atrophy, Peyer's patches of both $\beta_7^{-/-}$ and wild-type mice contained 3–4 $\log LD_{50}$/g prion infectivity at 125 or more days after challenge.

Why does reduced mucosal lymphocyte trafficking not impair, as expected, the susceptibility to orally initiated prion disease? One possible reason may relate to the fact that, despite marked reduction of B cells, M cells were still present in $\beta_7^{-/-}$ mice. In contrast, mice deficient in both tumor necrosis factor (TNF) and lymphotoxin (LT)-α (TNF-$\alpha^{-/-}$ × LT-$\alpha^{-/-}$) or in lymphocytes (RAG-$1^{-/-}$, μMT), in which numbers of Peyer's patches are reduced in number, were highly resistant to oral challenge, and their intestines were virtually devoid of prion infectivity at all times after challenge. Therefore, lymphoreticular requirements for enteric and for intraperitoneal uptake of prions differ from each other and that susceptibility to prion infection following oral challenge correlates with the number of Peyer's patches, but is independent of the number of intestinal mucosa-associated lymphocytes (Prinz et al. 2003c).

Transepithelial enteric passage of prions: a role for membranous epithelial cells (M cells)?

The requirements for transepithelial passage of prions are of obvious interest to prion pathogenesis. Membranous epithelial cells (M cells) are key sites of antigen sampling for the mucosal-associated lymphoid system (MALT) and have been recognized as major ports of entry for enteric pathogens in the gut via transepithelial transport (Neutra et al. 1996). Interestingly, maturation of M cells is dependent on signals transmitted by intraepithelial B cells. The group of Jean-Pierre Kraehenbuhl (Lausanne) has developed efficient in vitro systems, in which epithelial cells can be instructed to undergo differentiation to cells that resemble M cells by morphological and functional–physiological criteria. Therefore, it became feasible to investigate whether M cells are a plausible site of prion entry in a coculture model (Kerneis et al. 1997). Colon carcinoma cells (line CaCo-2) were seeded on the upper face of transwell filters and cultured until confluency was reached. Next, B-lymphoblastoid cells Raji B cells were added onto the lower side of the filters. Lymphoid cells migrated through the pores of the filter and settled within the epithelial monolayer (Figure 14), inducing differentiation of some CaCo-2 cells into M cells. Successful conversion was monitored by measuring transport of fluorescein-conjugated latex beads in cocultures that exhibited a high transepithelial resistance and were therefore tight. Active transepithelial transport of beads, but not passive leakage, was blocked at 4°C. Scrapie prions were administered to the apical compartment of cocultures that combined integrity and active transport of beads. After 24 hours, infectivity was determined within the basolateral compartment by bioassay with *tga20* mice, which overexpress a *Prnp* transgene and develop scrapie rapidly after infection (Fischer et al. 1996). Upon challenge with 5 $\log LD_{50}$ scrapie prions, prions were consistently recovered in the basolateral compartment of co-cultures containing M cells, suggesting transepithelial prion transport (Figure 14). Even at low prion doses (3 $\log LD_{50}$), infectivity was found in at least one M cell-containing co-culture. By contrast, there was hardly any prion transport in CaCo-2 cultures without M cells (Heppner et al. 2001a).

These findings indicate that M cell differentiation is necessary and sufficient for active transepithelial prion transport in vitro. M cell-dependent uptake of foreign antigens or particles is known to be followed by rapid transcytosis directly to the intraepithelial pocket, where key players of the immune system, e.g. macrophages, dendritic cells, and lymphocytes (Neutra et al. 1996), are located. Therefore, prions may exploit M cell-dependent transcytosis to gain access to the immune system (Aguzzi and Heppner 2000). While these findings suggest that M cells are a plausible candidate for the mucosal portal of prion infection, it still remains to be established whether the pathway delineated above does, indeed, represent the first portal of entry of orally administered prions into the body. This will necessitate in vivo experimentation, for example by ablation of M cells through suicide transgenetic strategies, or by M cell-specific expression of *Prnp* transgenes.

Uptake of prion through the skin

Even less well understood, yet possibly much more efficient than oral administration of prions, is challenge by scarification. Application of one droplet of scrapie inoculum to skin abrasions and subsequent delivery of

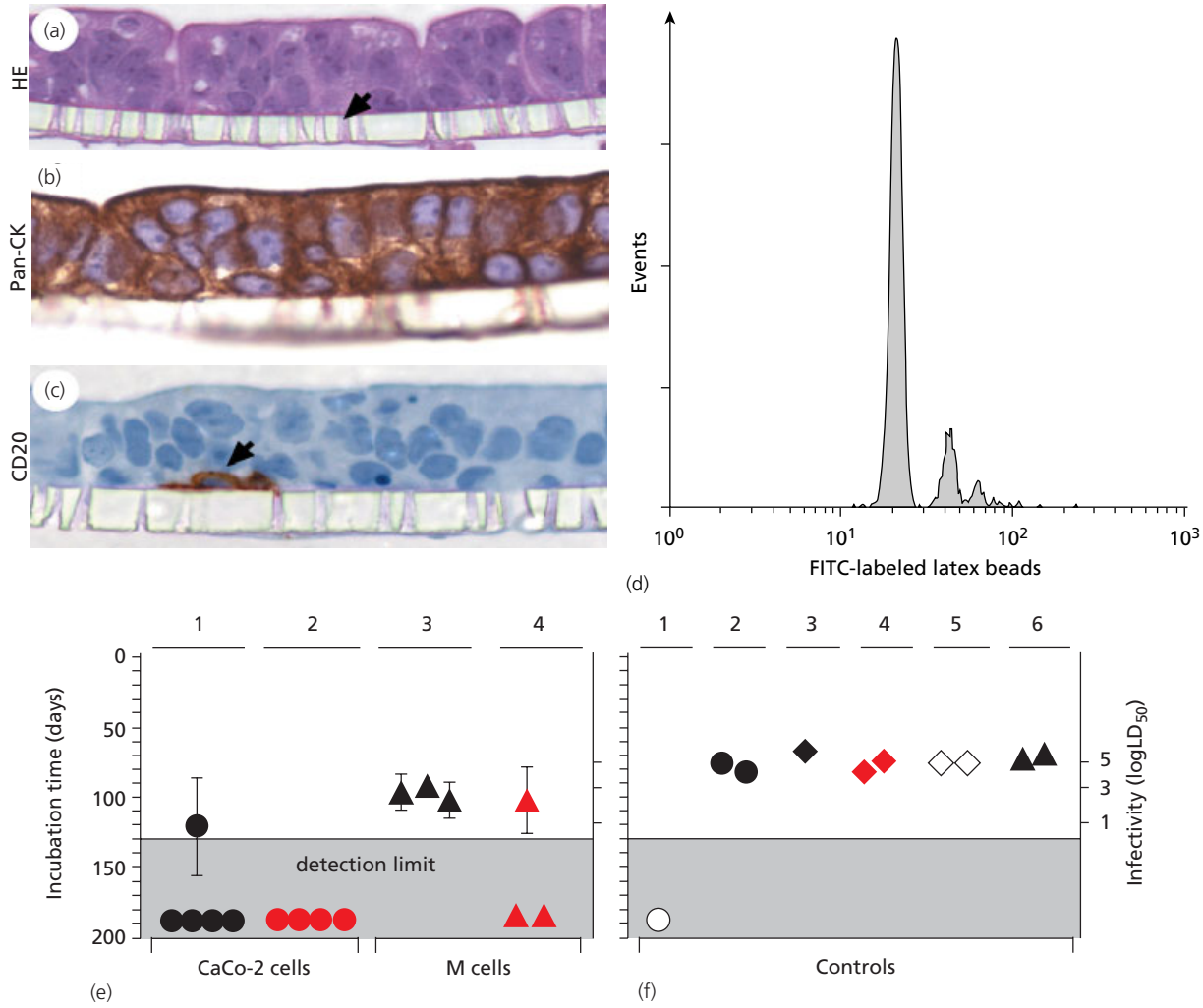

Figure 14 *Transepithelial transport of prions via M-cells. Filter membranes with 3 μm pores were overlaid with epithelial cells (colon carcinoma, line CaCo-2). Human B-lymphoblastoid Raji cells were then cultured on the back side of the membrane.* **(a)** *Morphology of co-cultures on filters, visualized by hematoxylin and eosin (HE) stains.* **(b)** *Immunohistochemical stain for cytokeratins (pan-CK) visualizing human CaCo-2 epithelial cells and* **(c)** *for the B-cell marker CD20, displaying B cells that have successfully migrated through the pores of the filter (arrow).* **(d)** *Flow cytometric analysis demonstrating active (i.e. temperature-dependent) transport of FITC-conjugated latex beads from the apical to the basolateral chamber compartment upon integration of B cells within pouches of the epithelial CaCo-2 cell layer, and differentiation of M cells. Intactness of co-cultures was assessed by measuring transepithelial resistance at 37°C, which was typically >200 Ωcm^2. The presence of additional peaks corresponds to aggregates of several latex beads.* **(e)** *Recovery of prion infectivity upon challenge with different prion inocula [5 (black) or 3 (red) logLD_{50} input infectious units] was only visible in co-cultures containing M cells (triangles, lanes 3, 4). No prion transport was observed in CaCo-2 cultures (circles, lanes 1, 2) without M cells, except in one case in which traces of infectivity were present. Controls: mock inoculum (lane 1), CaCo-2 culture (Ca) after slight mechanical manipulation resulting in a transepithelial resistance <50 Ωcm^2 (lane 2). Prion infectivity dilutions as indicated before (positive control, lanes 3, 4) and after incubating with either CaCo-2 (lane 5) or M cell containing (M) cultures (lane 6). Each symbol represents the mean incubation time in days (ordinate) of four* tga20 *indicator mice until terminal disease.*

sweeping strokes until blood is leaking has been shown to be a highly efficacious method of inducing prion disease (Carp 1982; Taylor et al. 1996). It is thinkable that dendritic cells in the skin may become loaded with the infectious agent by this method, and in fact recent work has implicated dendritic cells as potential vectors of prions in oral (Huang et al. 2002) and in hematogenous spread (Aucouturier et al. 2001) of the agent. It is equally possible (and perhaps more probable), however, that scarification induces direct neural entry of prions into skin nerve terminals. The latter hypothesis has not yet been studied in much detail, but it would help explain the remarkable speed with which central nervous system pathogenesis follows inoculation by this route: dermal inoculation of scarified mice yields typical latency periods of the disease that are similar to those obtained by intracerebral inoculation. The possibility of direct neural spread of the agent has been brought up also by a series of elegant experiments in the laboratories of Oldstone and Chesebro: transgenic mice that

lack the endogenous prion gene but express a *Prnp* transgene exclusively in nervous tissue (under transcriptional control of the neuron-specific enolase regulatory elements) can be efficiently infected by the oral route despite lack of prion protein expression in lymphoid organs (Race et al. 1995). Of course, these experiments do not exclude that dendritic or other mobile cells may participate in neuroinvasion even if they do not express endogenous PrP^{C}. Rapid neuroinvasion was reported following intralingual inoculation, and may also exploit a direct intraneural pathway (Bartz et al. 2003).

LYMPHOCYTES AND PRION PATHOGENESIS

More than a decade ago the normal prion protein was found to be consistently expressed, albeit at moderate levels, in circulating lymphocytes (Cashman et al. 1990). Subsequently, it was clearly shown in a wealth of experimental paradigms that innate or acquired deficiency of lymphocytes would impair peripheral prion pathogenesis, whereas no aspects of pathogenesis were affected by the presence or absence of lymphocytes upon direct transmission of prions to the central nervous system (Kitamoto et al. 1991a; Lasmezas et al. 1996a). Klein and colleagues pinpointed the lymphocyte requirement to B cells (Aguzzi 1997; Klein et al. 1997): at first sight this was very surprising, since there had been no suggestions that any aspect of humoral immunity would be involved in prion diseases. In the same study, it was shown that T-cell deficiency brought about by ablation of the T-cell receptor (TCR)-α chain did not affect prion pathogenesis (Table 5).

TCR-α-deficient mice, however, still contain TCR-γδ T lymphocytes. Although the latter represent a subpopulation of T cells, the experiments described did not allow excluding a role for TCR-γδ T lymphocytes. Therefore, we challenged also TCR-β/δ-deficient mice with prions. Incubation times after intracerebral and intraperitoneal inoculation of limiting or saturating doses of prions, however, elicited disease in these mice with the same kinetics as in wild-type mice (M. Klein and A. Aguzzi, unpublished data). Also, accumulation of PrP^{Sc} and development of histopathological changes in the brain were indistinguishable in these two strains of mice. We therefore conclude that the complete absence of T cells has no measurable impact on prion diseases. Therefore, it is unlikely that the T-cell infiltrates which have been reported to occur in the central nervous system during the course of prion infections (Betmouni et al. 1996; Betmouni and Perry 1999; Perry et al. 2002) represent more than an epiphenomenon.

In 1996–97, when the results of the studies described above were being collected, there was no precise mechanistic model for the role of B cells in prion pathogenesis. It had become rather clear, on the other hand, that lymphocytes alone could not account for the entirety of prion pathogenesis, and an additional sessile compartment had to be involved: adoptive transfer of $Prnp^{+/+}$ bone marrow to $Prnp^{0/0}$ recipient mice did not suffice to restore infectability of *Prnp*-expressing brain grafts, indicating that neuroinvasion was still defective (Blättler et al. 1997).

In an immediately subsequent series of bone marrow transfer experiments, it emerged that peripheral prion pathogenesis required the physical presence of B cells, yet intraperitoneal infection occurred efficiently even in B-cell-deficient hosts that had been transferred with B cell from *Prnp* knock-out mice (Klein et al. 1998).

Table 5 *Susceptibility of various strains of immunodeficient mice to intracerebrally or intraperitoneally administered prions (Klein et al. 1997)*

		Intracerebral route		Intraperitoneal route	
Defect	**Genotype**	**Scrapie**	**Time to terminal disease**	**Scrapie**	**Time to terminal disease**
T	$CD4^{0/0}$	7/7	159±11	8/8	191±1
T	$CD8^{0/0}$	6/6	157± 15	6/6	202±5
T	$\beta_2\text{-}\mu^{0/0}$	8/8	162±11	7/7	211±6
T	$Perforin^{-/-}$	3/4	171±2	4/4	204±3
T and B	SCID	7/8	160±11	6/8	226±15
T and B	$RAG\ 2^{0/0}$	7/7	167±2	0/7	healthy (>504)
T and B	$RAG\ 1^{0/0}$	3/3	175±2	0/5	healthy (>58)
T and B	$AGR^{0/0}$	6/6	184±10	0/7	healthy (>425)
B	μMT	8/8	181±6	0/8	healthy (>510)
IgG	t11μmt	5/5	170±3	4/4	223±2
FDC	$TNFR1^{0/0}$	7/7	165±3	9/9	216±4
Controls	129Sv	4/4	167±9	4/4	193±3
	C57BL/6	4/4	166±2	4/4	206±2

We measured the latency of scrapie (days from inoculation to terminal disease) upon delivery of a standard prion inoculum. All mice developed spongiform encephalopathy after intracerebral inoculation. By contrast, all mice that carried a defect in B-cell differentiation stayed healthy after intraperitoneal inoculation of RML scrapie prions. Intriguingly, in this and in several subsequent studies, $TNFRI^{-/-}$ developed disease with the same kinetics as wild-type mice, although there are no morphologically detectable follicular dendritic cells in the spleens of $TNFRI^{-/-}$ mice.

Therefore, the presence of B cells – but not expression of the cellular prion protein by these cells – is indispensable for pathogenesis upon intraperitoneal infection in the mouse scrapie model (Aguzzi et al. 2001a).

The above results have been reproduced and confirmed several times over the years by many laboratories in various experimental paradigms: the requirement for B cells in particular appears to be very stringent in most instances investigated. However, it has emerged that not all strains of prions induce identical patterns of peripheral pathogenesis, even when propagated in the same, isogenic strain of host organism. One quite intriguing exception to the B-cell requirement appears to have been recently identified: Manuelidis and colleagues have reported efficient peripheral infection of B-cell-deficient mice with a mouse-adapted strain of human CJD prions (Shlomchik et al. 2001). Despite its somewhat selective interpretation of previously published reports, this study is interesting in that it identifies the unique situation of a prion strain that induces a highly anomalous peripheral pathogenesis.

Straining the lymphocytes

Another interesting discrepancy that remains to be addressed concerns the actual nature of the cells that replicate and accumulate prions in lymphoid organs. In the RML paradigm, four series of rigorously controlled experiments over 5 years (Aguzzi et al. 2000b; Kaeser et al. 2001; Klein et al. 2001; Prinz et al. 2002) unambiguously reproduced the original observation by Thomas Blättler and colleagues that transfer of wild-type ($Prnp^{+/+}$) bone marrow cells (or fetal liver cells) to PrP-deficient ($Prnp^{0/0}$) mice restored accumulation and replication of prions in spleen (Blättler et al. 1997). By contrast, Karen Brown and colleagues reported a diametrically opposite outcome of similar experiments when mice were inoculated with prions of the ME7 strain (K.L. Brown et al. 1999). Since trivial experimental artifacts appear to have been excluded, it is enticing to speculate that this discrepancy may identify yet another significant difference in the cellular tropism of different prion strains.

It is important to note that the Blättler results do not necessarily indicate that lymphocytes are the primary splenic repository of prions: in fact, other experiments suggest that this is quite unlikely. Instead, bone marrow transplantation may

- transfer an ill-defined population with the capability to replenish splenic stroma and to replicate prions, or
- less probably, donor-derived PrP^C expressing hematopoietic cells may confer prion replication capability to recipient stroma by virtue of 'GPI painting,' i.e. the post-translational cell-to-cell transfer of glycophosphoinositol linked extracellular membrane proteins (Kooyman et al. 1998).

Some evidence might be accrued for either possibility: stromal splenic follicular dendritic cells have been described by some authors to possibly derive from hematopoietic precursors, particularly when donors and recipients were young (Szakal et al. 1995; Kapasi et al. 1998). Conversely, instances have been described in which transfer of GPI-linked proteins occurs in vivo with surprisingly high efficiency (Kooyman et al. 1995). Most recently, GPI painting has been described specifically for the cellular prion protein (Liu et al. 2002).

While it has been known for a long time that specific strains of prions may preferentially affect specific subsets of neurons, the Blättler/Brown paradox may uncover an analogous phenomenon in peripheral prion pathogenesis. The latter question may be much more important than it may appear at face value, and its clarification may warrant the investment of significant resources, since the molecular and cellular basis of peripheral tropism of prion strains is likely to be directly linked to the potential danger of BSE in sheep (Bruce et al. 2002; Glatzel and Aguzzi 2001; Kao et al. 2002), as well the potential presence of vCJD prions in human blood (Aguzzi 2000).

PRION HIDEOUTS IN LYMPHOID ORGANS

As mentioned above, prion infectivity rises very rapidly (in a matter of days) in the spleen of intraperitoneally infected mice. Although B lymphocytes are crucial for neuroinvasion, a series of prion titrations in *Prnp*-expressing and *Prnp*-ablated mice, as well as reciprocal bone marrow chimeras thereof, has virtually ruled out the hypothesis that the bulk of infectivity might be contained in lymphocytes. Instead, all evidence points to the fact that most splenic prion infectivity resides in a 'stromal' fraction. Therefore, lymphocytes may be important for trafficking prions within lymphoid organs, but they do not appear to represent a major hideout for the infectious agent. Follicular dendritic cells (FDC) are a prime candidate prion reservoir, since they express large amounts of cellular prion protein, and PrP accumulations tend to co-localize with FDCs in light and electron microscopical analyses of prion-infected spleens. More recently, elegant immunoelectron microscopic studies have evidenced that strong PrP immunoreactivity is situated in the immediate neighborhood of iccosomes (Jeffrey et al. 2000).

A definitive assessment of the contribution of FDCs to prion pathogenesis continues to be problematic since the histogenesis and the molecular characteristics of these cells are ill-defined. In particular, there is a dearth of molecular markers for FDCs. FDCs express S-100 proteins, as well as the complement receptors 2 (CD35) and 4 (identical to the marker FDC-M2). All of these markers, however, are also expressed by additional cell types, even within lymphoid organs (Bofill et al. 2000). The FDC-M1 marker recognized by hybridoma clone 4C11 appears to be more

specific, but it stains also tingeable body macrophages, which are most likely of hematopoietic origin. In addition, the antigen recognized by 4C11 has not been molecularly characterized.

Identification of FDC-specific genes would be extremely important and useful to this effect, since it would allow for transgenic studies of FDC histogenesis using fluorescent marker proteins. Other studies on the immediate wish list that would depend on FDC-specific transcription might include:

- transgenetic expression of *Prnp*-restricted to FDCs
- lineage ablation experiments by expressing conditional suicide genes – such as diphtheria toxin receptor or herpes simplex virus thymidine kinase – in FDCs.

Gene deletion experiments in mice have shown that signaling by both TNF and lymphotoxins is required for FDC development (Fu et al. 1997; Koni et al. 1997; Endres et al. 1999). Membrane-bound LT-α/β (LT-α_1/β_2 or LT-α_2/β_1) heterotrimers signal through the LT-β receptor (LT-βR) (Ware et al. 1995), thereby activating a signaling pathway required for the development and maintenance of secondary lymphoid organs (Mackay et al. 1997; Matsumoto et al. 1997). Membrane LT-α/β heterotrimers are mainly expressed by activated lymphocytes (Browning et al. 1993, 1995). Maintenance of preexisting FDCs in a differentiated state requires continuous interaction with B lymphocytes expressing surface LT-α/β (Gonzalez et al. 1998). Inhibition of the LT-α/β pathway in mice by treatment with LT-βR-immunoglobulin fusion protein (LT-βR-IgFcγ) (Crowe et al. 1994) leads to the disappearance of mature, functional FDCs (defined as cells that express markers such as FDC-M1, FDC-M2, or CD35) within one day, both in spleen and in lymph nodes (Mackay et al. 1997; Mackay and Browning 1998). Prolonged administration of LT-βR-IgFcγ leads to disruption of B-cell follicles.

All of the above prompted a study of the effect of selective ablation of functional FDCs on the pathogenesis of scrapie in mice. FDC depletion was maintained by weekly administration of LT-βR-IgFcγ for 8 weeks. Histological examination of spleen sections revealed that FDC networks had disappeared 1 week after treatment (Figure 15), as expected (Montrasio et al. 2000).

In mice, following peripheral inoculation, infectivity in the spleen rises within days and reaches a plateau after a few weeks (Bruce 1985; Büeler et al. 1993; Rubenstein et al. 1991). Even after intracerebral inoculation, spleens of C57BL/6 animals contain infectivity already 4 days post-infection (Büeler et al. 1993). However, western blot analysis (Figure 16) revealed that 8 weeks after inoculation spleens of control mice showed strong bands of protease-resistant PrP, whereas mice injected weekly with LT-βR-IgFcγ, starting either 1 week before or 1 week after inoculation, showed no detectable signal (less than one-fiftieth of the controls).

Prion infectivity in three spleens for each time point was assayed by intracerebral inoculation into indicator mice (Fischer et al. 1996). In spleens of mice treated with LT-βR-IgFcγ 1 week before intraperitoneal inoculation,

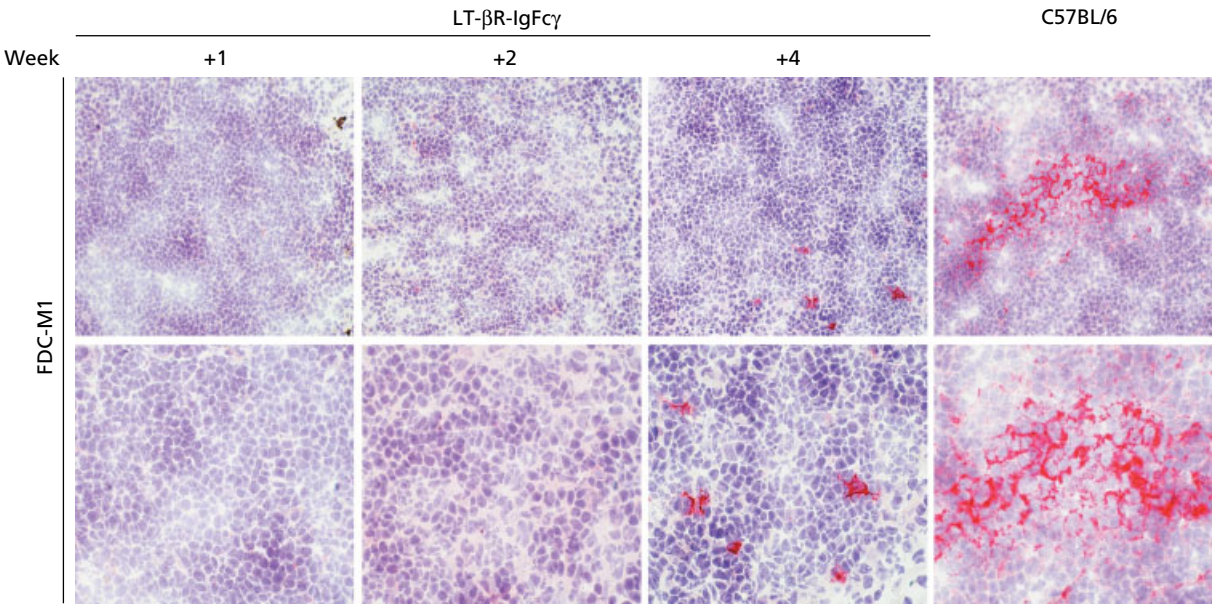

Figure 15 *Depletion of follicular dendritic cells by pharmacological inhibition of lymphotoxin signaling. Time course of FDC depletion in spleen of LT-βR-IgFcγ-treated mice. Left to right: spleen histology at 1 week, 2 weeks, and 4 weeks after administration of lymphotoxin antagonist. Right hand column shows administration of human immunoglobulin for control. Frozen sections of treated (left) and control mice (right) immunostained with follicular dendritic cell specific antibody FDC-M1 at different times points after injection of LT-βR-IgFcγ (original magnifications: upper row 8×25; lower row 8×63). Germinal centers FDC networks were depleted 1 week after treatment as described (Mackay and Browning 1998; Montrasio et al. 2000). Some FDC-M1 positive cells, which may represent residual FDCs or tingeable body macrophages, were still detectable in the spleens of treated mice.*

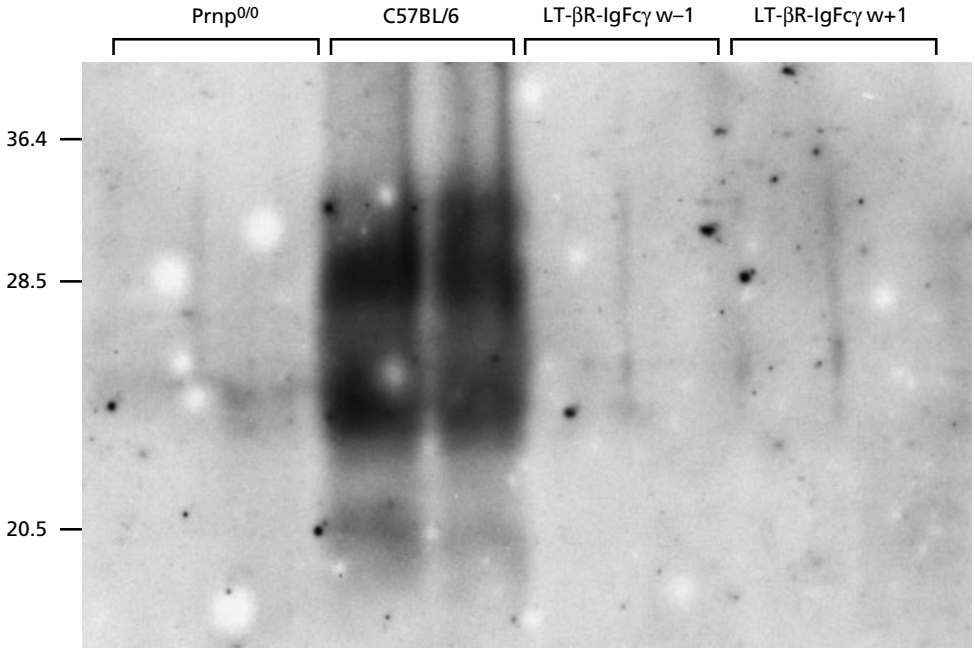

Figure 16 *Inhibition of lymphotoxin signalling blocks prion replication in lymphoid organs. Accumulation of PrP^{Sc} in spleens of LT-βR-IgFcγ-treated mice 8 weeks after inoculation. Immunoblot analysis of spleen extracts (200 μg total protein) from LT-βR-IgFcγ-treated and control mice sacrificed 8 weeks after inoculation. All spleen samples were treated with proteinase K. Mice analyzed (left to right):* $Prnp^{0/0}$*; untreated, LT-βR-IgFcγ-treated C57BL/6 mice either 1 week before (LT-βR-IgFcγ w−1) or after (LT-βR-IgFcγ w+1) prion inoculation. Spleens of two mice for each group were analyzed. Immunoreactive PrP was detected using a rabbit antiserum against mouse-derived PrP (1B3) (Farquhar et al. 1994) and enhanced chemiluminescence. The position of the molecular mass standards (in kDa) is indicated on the left side of the fluorogram. The three diagnostic bands of PrP^{Sc} are only recognized in untreated mice. LT-βR-IgFcγ treatment led to complete disappearance of the signal (reduction >50-fold).*

no infectivity could be detected after 3 or 8 weeks (<1.0 $logID_{50\backslash p}$units/ml 10 percent homogenate). Traces of infectivity, possibly representing residual inoculum, were present in the 1-week samples. In mice treated with LT-βR-IgFcγ 1 week after inoculation, the titers were about 2.2 and 4.1 $logID_{50}$ units/ml 10 percent homogenate at 3 weeks and at borderline detectability at 8 weeks after infection, suggesting that some prion accumulation took place in the first weeks after inoculation but was reversed under treatment with LT-βR-IgFcγ by 8 weeks. Spleens of scrapie-inoculated control mice treated with unspecific pooled human immunoglobulins (huIgG) 1 week after inoculation had 4.5 and 5.5 $logID_{50}$ units/ml 10 percent homogenate 8 weeks after intraperitoneal inoculation.

To assess whether prolonged depletion of mature FDCs would perturb the progression of the disease, C57BL/6 mice were subjected to weekly administration of the fusion protein up to 8 weeks post-inoculation and observed for more than 340 days. Mice receiving LT-βR-IgFcγ starting 1 week after inoculation developed the disease with about 25 days delay as compared to control mice. Whenever FDC depletion was initiated 1 week before inoculation, the effect on incubation time was even more pronounced: Two out of three mice developed scrapie symptoms 60 days later than huIgG-treated controls and one mouse survived >340 days (Montrasio et al. 2000). Double-color immunofluorescence analysis of spleen sections of terminally sick animals revealed that the FDC networks were reconstituted after interruption of LT-βR-IgFcγ administration at 8 weeks and that PrP^{C} and/or PrP^{Sc} colocalized with FDCs. Immunoblot analysis showed that PrP^{Sc} accumulation was restored upon reappearance of FDCs in the spleens of terminal sick LT-βR-IgFcγ-treated mice.

These results show that follicular dendritic cells are essential for the deposition of PrP^{Sc} and generation of infectivity in the spleen and suggest that they participate in the process of neuroinvasion. Therefore, the requirement for B cells for these processes might be explained by their essential role in the maturation of FDCs. PrP knock-out mice expressing PrP transgenes only in B cells do not sustain prion replication in the spleen or elsewhere, suggesting that prions associated with splenic B cells (Raeber et al. 1999a) may be acquired from FDCs. Finally, these results suggest that strategies aimed at depleting FDCs might be envisaged for post-exposure prophylaxis (Aguzzi and Collinge 1997) of prion infections initiated at extracerebral sites. The distance between follicular dendritic cells and sympathetic nerve terminals in lymphoid organs appears to control the velocity of neuroinvasion, since juxtaposition of FDCs and sympathetic nerve terminals increases the velocity of neuroinvasion (Prinz et al. 2003a).

THE CELLULAR BASIS OF PRION LYMPHOTROPISM

As discussed above, inhibition of the LT-β signaling pathway with a soluble receptor depletes FDCs (Mackay and Browning 1998) and abolishes prion replication in spleens, thereby prolonging the latency of scrapie after intraperitoneal challenge (Montrasio et al. 2000). This suggests that B-cell-deficient μMT mice (Kitamura et al. 1991) may be resistant to intraperitoneal prion inoculation (Klein et al. 1997) because of impaired FDC maturation (Klein et al. 1998; Montrasio et al. 2000). However, additional experimentation appears to indicate that PrP^C-expressing hematopoietic cells are required in addition to FDCs for efficient lymphoreticular prion propagation (Blättler et al. 1997; Kaeser et al. 2001). This apparent discrepancy called for additional studies of the molecular requirements for prion replication competence in lymphoid stroma. Therefore peripheral prion pathogenesis was studied in mice lacking TNF-α, LTα/β, or their receptors. After intracerebral inoculation, all treated mice developed clinical symptoms of scrapie with incubation times, attack rates, and histopathological characteristics similar to those of wild-type mice, indicating that TNF/LT signaling is not relevant to cerebral prion pathogenesis. Upon intraperitoneal prion challenge, mice defective in LT signaling (LT-$\alpha^{-/-}$, LT$\beta^{-/-}$, LT-β$R^{-/-}$, or LT-$\alpha^{-/-}$TNF-$\alpha^{-/-}$) proved virtually non-infectible with $\leqslant 5$ $\log LD_{50}$ scrapie infectivity, and establishment of subclinical disease (Frigg et al. 1999) was prevented. By contrast, $TNFR1^{-/-}$ mice were almost fully susceptible to all inoculum sizes, and TNF-$\alpha^{-/-}$ mice showed dose-dependent susceptibility. $TNFR2^{-/-}$ mice had intact FDCs and germinal centers, and were fully susceptible to scrapie. Unexpectedly, all examined lymph nodes of $TNFR1^{-/-}$ and TNF-$\alpha^{-/-}$ mice had consistently high infectivity titers (Prinz et al. 2002). Even inguinal lymph nodes, which are distant from the injection site and do not drain the peritoneum, contained infectivity titers equal to all other lymph nodes. Therefore, TNF deficiency prevents lymphoreticular prion accumulation in spleen but not in lymph nodes.

Why is susceptibility to peripheral prion challenge preserved in the absence of TNFR1 or TNF-$\alpha^{-/-}$, while deletion of LT signaling components confers high resistance to peripheral prion infection? After all, each of these defects (except $TNFR2^{-/-}$) abolishes FDCs. For one thing, prion pathogenesis in the lymphoreticular system appears to be compartmentalized, with lymph nodes (rather than spleen) being important reservoirs of prion infectivity during disease. Second, prion replication appears to take place in lymph nodes even in the absence of mature FDCs.

Bone marrow chimeric mice were generated in order to determine whether hematopoietic components are involved in prion propagation in $TNFR1^{-/-}$ lymph nodes. In $Prnp^{0/0}$ mice grafted with $TNFR1^{-/-}$ hematopoietic cells, high infectivity loads were detectable in lymph nodes but not spleens, indicating that TNFR1-deficient, PrP^C-expressing hematopoietic cells may support prion propagation within lymph nodes (Prinz et al. 2002). These findings are in line with previous studies, which showed that chimeras of PrP-deficient hosts with PrP-expressing hematopoietic cells can accumulate prions chronically (Blättler et al. 1997; Kaeser et al. 2001). The PrP signal colocalized with a subset of macrophages in $TNFR1^{-/-}$ lymph nodes. Since marginal zone macrophages are in close contact to FDCs and also interact with marginal zone B cells, this cell type is certainly a candidate supporter in prion uptake and replication. On the other hand, it has been reported that in short-time infection experiments depletion of macrophages appears to enhance the amount of recoverable infectivity, implying that macrophages may degrade prions rather than transport them (Beringue et al. 2000). The finding that a cell type other than mature FDCs is involved in prion replication and accumulation within lymph nodes may be relevant to the development of post-exposure prophylaxis strategies.

SYMPATHETIC NERVES: A NEUROIMMUNE LINK?

In the last several years, a model has emerged that predicts prion neuroinvasion to consist of two distinct phases (Aguzzi et al. 2001c). The details of the first phase are discussed above: widespread colonization of lymphoreticular organs is achieved by mechanisms that depend on B lymphocytes (Klein et al. 1997, 1998), follicular dendritic cells (Montrasio et al. 2000), and complement factors (Klein et al. 2001). The second phase has long been suspected to involve peripheral nerves, and possibly the autonomic nervous system, and may depend on expression of PrP^C by these nerves (Glatzel and Aguzzi 2000). Glatzel and colleagues have been attempting to test the requirement for expression of PrP^C in peripheral nerves for several years, and have developed a gene transfer protocol to spinal ganglia aimed at resolving this question (Glatzel et al. 2000); however, they were never able to recover infectivity in spinal cords of $Prnp^{0/0}$ mice whose spinal nerves had been transduced by *Prnp*-expressing adenoviruses (M. Glatzel and A. Aguzzi, unpublished results). Also, fast axonal transport does not appear to be involved in prion neuroinvasion, since mice that are severely impaired in this transport mechanism experience prion pathogenesis with kinetics similar to that of wild-type mice (Künzi et al. 2002).

There is substantial evidence suggesting that prion transfer from the lymphoid system to the CNS occurs along peripheral nerves in a PrP^C-dependent fashion

(Blättler et al. 1997; Glatzel and Aguzzi 2000; Race et al. 2000). Studies focusing on the temporal and spatial dynamics of neuroinvasion have suggested that the autonomic nervous system might be responsible for transport from lymphoid organs to the CNS (Clarke and Kimberlin 1984; Cole and Kimberlin 1985; Beekes et al. 1998; McBride and Beekes 1999).

The innervation pattern of lymphoid organs is mainly sympathetic (Felten and Felten 1988). Glatzel and colleagues have shown that denervation by injection of the drug 6-hydroxydopamine (6-OHDA), as well as 'immunosympathectomy' by injection of antibodies against nerve growth factor (NGF) leads to a rather dramatic decrease in the density of sympathetic innervation of lymphoid organs (Figure 17), and significantly delayed the development of scrapie (Glatzel et al. 2001) (Figure 18). Sympathectomy appears to delay the transport of prions from lymphatic organs to the thoracic spinal cord, which is the entry site of sympathetic nerves to the CNS. Transgenic mice overexpressing NGF under control of the K14 promoter, whose spleens are hyperinnervated, developed scrapie significantly earlier than nontransgenic control mice. No alteration in lymphocyte subpopulations was detected in spleens at any time point investigated. In particular, no significant differences in content of FDCs were detected between treated and untreated mice, which negates the possibility that the observed protection may be due to modulation of FDC microanatomy.

While the sympathetic nervous system may represent a major component of the second phase of prion neuroinvasion, many details remain to be elucidated. It is not known whether prions can be transferred directly from FDCs to sympathetic endings, or whether additional cell types are involved. The latter possibility is particularly enticing, since FDCs have not been shown to entertain physical contact with sympathetic nervous system terminals.

Moreover, it is unclear how prions are actually transported within peripheral nerves. Axonal and non-axonal transport mechanisms may be involved, and non-neuronal cells (such as Schwann cells) may play a role. Within the framework of the protein-only hypothesis, one may hypothesize a 'domino' mechanism, by which incoming PrP^{Sc} converts resident PrP^{C} on the axolemmal surface, thereby propagating spatially the infection. While speculative, this model is attractive since it may accommodate the finding that the velocity of neural prion spread is extremely slow (Kimberlin et al. 1983) and may not follow the canonical mechanisms of fast axonal transport. Indeed, recent studies may favor a non-axonal transport mechanism that results in periaxonal deposition of PrP^{Sc} (Hainfellner and Budka 1999; Glatzel and Aguzzi 2000).

The fact that denervated mice eventually developed scrapie may be due to an alternative, low-efficiency route of entry that may become uncovered by the absence of sympathetic fibers, or because of incomplete sympathectomy. Entry through the vagal nerve has been proposed in studies of the dynamics of vacuolation

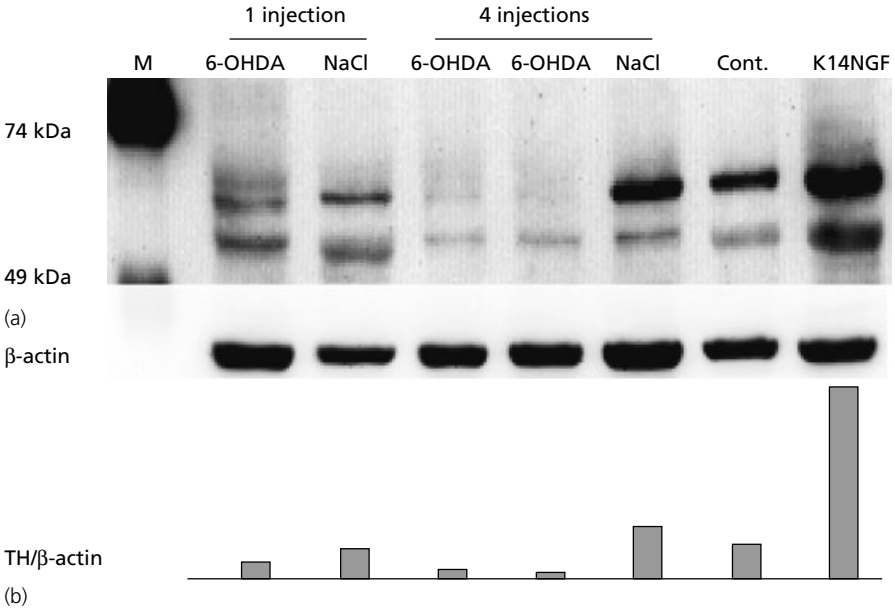

Figure 17 *The sympathetic innervation of the spleen can be ablated by 6-hydroxydopamine (6-OHDA).* **(a)** *The density of sympathetic innervation correlates with the concentration of tyrosine hydroxylase, which was measured by western blot analysis in spleens of adult 6-OHDA-treated mice, K14NGF transgenic mice, and controls. The two main tyrosine hydroxylase bands are visible at ca. 55 kDa. While there is a certain increase in sympathetic innervation with age, it is obvious that 6-OHDA treatment depletes, and the K14NGF transgene increases sympathetic innervation.* **(b)** *Quantification of tyrosine hydroxylase content by chemiluminescence scanning. The bars represent the ratio between signal intensity of the tyrosine hydroxylase band and that of the corresponding β-actin bands (arbitrary units).*

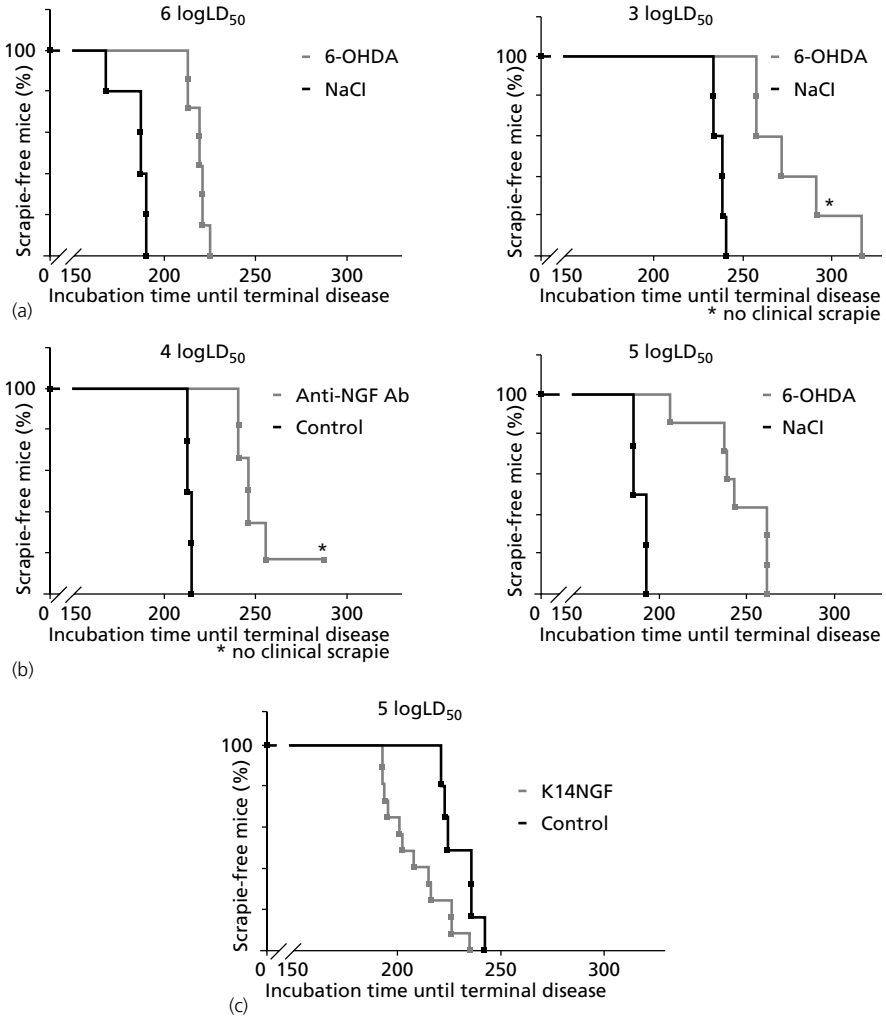

Figure 18 *Modulation of sympathetic nerve density in lymphoid organs affects the efficiency of peripheral prion pathogenesis. Survival plots displaying the incubation time (days) until development of terminal scrapie in C57BL/6 mice inoculated intraperitoneally with prions* **(a–c)** *and of* tga20 *indicator mice inoculated intracerebrally with spinal cord and spleen samples* **(d)**. *Mice injected with 6-OHDA in their adulthood* **(a)**, *as well as mice injected neonatally with anti-NGF antibodies or with 6-OHDA* **(b)** *developed terminal scrapie significantly later than their respective controls. Instead K14NGFmice, which have hyperinnervated spleens, developed terminal scrapie earlier than matched wild-type siblings of the same genetic background* **(c)**. *The size of inoculum is expressed in infectious units (logLD_{50}).*

following oral and intraperitoneal challenge with prions (Baldauf et al. 1997; Beekes et al. 1998). There was no evidence for transport along the vagal nerve in sympathectomized mice, since vagal nuclei were affected similarly to other regions of the brainstem unrelated to the vagal system. This supports the hypothesis that delayed neuroinvasion in denervated mice may occur because of residual innervation of lymphoreticular organs.

The surprising finding that infectious titers in hyperinnervated spleens are at least two logs higher and show enhanced PrP^{Sc} accumulations compared to control mice suggests that sympathetic nerves, besides being involved in the transport of prions, may also accumulate and replicate prions in lymphatic organs (Clarke and Kimberlin 1984). Obviously, this finding has implications related to the permanence and possibly eradication of prions in subclinically infected hosts.

Spread of prions within the central nervous system

Ocular administration of prions has proved particularly useful to study neural spread of the agent, since the retina is a part of the central nervous system and intraocular injection does not produce direct physical trauma to the brain, which may disrupt the blood–brain barrier and impair other aspects of brain physiology. The assumption that spread of prions occurs axonally rests mainly on the demonstration of diachronic spongiform changes along the retinal pathway following intraocular infection (Fraser 1982).

To investigate whether spread of prions within the CNS is dependent on PrP^{C} expression in the visual pathway, PrP-producing neural grafts were used as sensitive indicators of the presence of prion infectivity in

the brain of an otherwise PrP-less host. Following inoculation with prions into the eye of grafted *Prnp*$^{0/0}$ mice, none of the grafts showed signs of scrapie. Therefore, it was concluded that infectivity administered to the eye of PrP-deficient hosts cannot induce scrapie in a PrP-expressing brain graft (Brandner et al. 1996b).

Engraftment of *Prnp*$^{0/0}$ mice with PrP^{C}-producing tissue might lead to an immune response to PrP which, in turn, was shown to be in principle capable of neutralizing infectivity (Heppner et al. 2001b). In order to definitively rule out the possibility that prion transport was disabled by a neutralizing immune response, *Prnp*$^{0/0}$ mice were rendered tolerant by expressing PrP^{C} under the control of the *lck* promoter. These mice overexpress PrP on T lymphocytes, but are resistant to scrapie and do not replicate prions in brain, spleen, and thymus after intraperitoneal inoculation with scrapie prions (Raeber et al. 1999b). Engraftment of these mice with PrP-overexpressing neuroectoderm did not lead to the development of antibodies to PrP after intracerebral or intraocular inoculation, presumably due to clonal deletion of PrP-immunoreactive T lymphocytes. As before, intraocular inoculation with prions did not provoke scrapie in the graft, supporting the conclusion that lack of PrP^{C}, rather than immune response to PrP, prevented prion spread (Brandner et al. 1996b). Therefore, PrP^{C} appears to be necessary for the spread of prions along the retinal projections and within the intact CNS.

These results indicate that intracerebral spread of prions is based on a PrP^{C} -paved chain of cells, perhaps because they are capable of supporting prion replication. When such a chain is interrupted by interposed cells that lack PrP^{C}, as in the case described here, no propagation of prions to the target tissue can occur. Perhaps prions require PrP^{C} for propagation across synapses: PrP^{C} is present in the synaptic region (Fournier et al. 1995) and certain synaptic properties are altered in *Prnp*$^{0/0}$ mice (Collinge et al. 1994; Whittington et al. 1995). Perhaps transport of prions within (or on the surface of) neuronal processes is PrP^{C}-dependent. Within the framework of the protein-only hypothesis (Griffith 1967; Prusiner 1989b), these findings may be accommodated by a 'domino-stone' model in which spreading of scrapie prions in the CNS occurs per continuitatem through conversion of PrP^{C} by adjacent PrP^{Sc} (Aguzzi 1997).

INNATE IMMUNITY AND ANTIPRION DEFENSE

Macrophages and Toll-like receptors

Cells of the monocyte/macrophage lineage typically represent the first line of defense against an extremely broad variety of pathogens. In the case of prions, it might be conceivable that macrophages protect against prions. However, it would be equally conceivable that macrophages, by virtue of their phagocytic properties and of their intrinsic mobility, may function as Trojan horses that transport prion infectivity between sites of replication within the body. This interesting question has not yet been fully resolved.

In a short-term prion infection paradigm, Beringue and colleagues administered dichloromethylene disphosphonate encapsulated into liposomes to mice: this eliminates for a short period of time all spleen macrophages. Accumulation of newly synthesized PrP^{Sc} was accelerated, suggesting that macrophages participate in the clearance of prions, rather than being involved in PrP^{Sc} synthesis (Beringue et al. 2000).

On the basis of the results presented above, Beringue and colleagues have suggested that activation or targeting of macrophages may represent a therapeutic pathway to explore in TSE infection. This suggestion was taken up by Sethi and Kretzschmar, who recently reported that activation of Toll-like receptors (TLR), which function as general stimulators of innate immunity by driving expression of various sets of the immune regulatory molecules, can effect post-exposure prophylaxis in an experimental model of intraperitoneal scrapie infection (Sethi et al. 2002). In this experimental paradigm, administration of prions intraperitoneally elicited disease after approximately 180 days, whereas the administration of CpG oligodeoxynucleotides 7 hours after prion inoculation and daily for 20 days led to disease-free intervals of 'more than 330 days' – although it appears that all inoculated mice died of scrapie shortly thereafter (communicated by H. Kretzschmar at the TSE conference in Edinburgh in September, 2002).

This finding is very surprising, since most available evidence indicates that general activation of the immune system would typically sensitize mice to prions, rather than protect them. The mechanism by which activation of TLRs can result in post-exposure prophylaxis is wholly unclear at present, particularly in view of the fact that mice lacking MyD88 (Adachi et al. 1998), which is an essential mediator of TLR signaling, develop prion disease with exactly the same sensitivity and kinetics as wild-type mice (Prinz et al. 2003b). Moreover, repeated administration of CpG oligonucleotides (ODN) was shown to produce a profound depletion of follicular dendritic cells, which are prominent peripheral prion reservoirs (Heikenwälder et al. 2004). This finding suggests that the alleged protective effect of CpG-ODN administration relies on indiscriminate destruction of immune organs rather than any specific anti-prion effect.

The complement system

Another prominent component at the crossroads between innate and adaptive immunity is represented by the

complement system. Opsonization by complement system components also appears to be relevant to prion pathogenesis: mice genetically engineered to lack complement factors (Klein et al. 2001), or mice depleted of the C3 complement component by administration of cobra venom (Mabbott et al. 2001), exhibit a remarkable resistance to peripheral prion inoculation. This phenomenon may, once again, be related to the pathophysiology of FDCs, which typically function as antigen traps. Trapping mechanisms essentially consist of capture of immune complexes by Fcγ receptors, and binding of opsonized antigens (linked covalently to C3d and C4b complement adducts) to the CD21/CD35 complement receptors.

Capture mediated by Fcγ receptors does not appear to be very important in prion disease: for one thing, knockout mice lacking Fcγ receptors (Takai et al. 1994, 1996; Hazenbos et al. 1996; Park et al. 1998) are just as susceptible to intraperitoneally administered scrapie as wild-type mice. Furthermore, introduction into μMT mice of a generic immunoglobulin μ chain fully restored prion neuroinvasion irrespective of whether this heavy chain allowed for secretion of immunoglobulins, or only for production of membrane-bound immunoglobulins. We therefore conclude that circulating immunoglobulins are certainly not crucial to prion replication in lymphoid organs and to neuroinvasion.

A second mechanism exploited by FDCs for antigen trapping involves covalent linking of proteolytic fragments of the complement components C3 and C4 (Szakal and Hanna 1968; Carroll 1998). The CD21/CD35 complement receptors on FDCs bind C3b, iC3b, C3d, and C4b through short consensus repeats in their extracellular domain. Ablation of C3, or of its receptor CD21/CD35, as well as C1q (alone or combined with BF/C2$^{-/-}$), delayed neuroinvasion significantly after intraperitoneal inoculation when a limiting dose of prions was administered. These effects suggest that opsonization of the infectious agent may enhance its accessibility to germinal centers by facilitating docking to FDCs.

Very large prion inocula (>10^6 infectious units) appear to override the requirement for a functional complement receptor in prion pathogenesis. This is similar to systemic viral infections and coreceptor-dependent retention within the follicular compartment, whose necessity can be overridden by very high affinity antigens (Fischer et al. 1998) or adjuvants (Wu et al. 2000). Additional retention mechanisms for prions may therefore exist in FDCs, which are not complement-dependent, or which may depend on hitherto unidentified complement receptors.

ADAPTIVE IMMUNITY AND PRE-EXPOSURE PROPHYLAXIS AGAINST PRIONS

For many conventional viral agents, vaccination is the most effective method of infection control. But is it at all possible to induce protective immunity in vivo against prions? Prions are extremely sturdy and their resistance against sterilization is proverbial. Preincubation with anti-PrP antisera was reported to reduce the prion titer of infectious hamster brain homogenates by up to 2 log units (Gabizon et al. 1988) and an anti-PrP antibody was found to inhibit formation of PrP^{Sc} in a cell-free system (Horiuchi and Caughey 1999). Also, antibodies (Klein et al. 2001) and F(ab) fragments raised against certain domains of PrP (Peretz et al. 2001) can suppress prion replication in cultured cells. However, it is difficult to induce humoral immune responses against PrP^{C} and PrP^{Sc}. This is most likely due to tolerance of the mammalian immune system to PrP^{C}, which is an endogenous protein expressed rather ubiquitously. Ablation of the *Prnp* gene (Büeler et al. 1992), which encodes PrP^{C}, renders mice highly susceptible to immunization with prions (Brandner et al. 1996b), and many of the best available monoclonal antibodies to the prion protein have been generated in *Prnp*$^{0/0}$ mice (Prusiner et al. 1993). However, *Prnp*$^{0/0}$ mice are unsuitable for testing vaccination regimens since they do not support prion pathogenesis (Büeler et al. 1993).

It was therefore asked whether genes encoding high-affinity anti-PrP antibodies (originally generated in *Prnp*$^{0/0}$ mice) may be utilized to reprogram B-cell responses of prion-susceptible mice that express PrP^{C}. Indeed, the introduction of the epitope-interacting region of heavy chain of 6H4, a high-affinity anti-PrP monoclonal antibody (Korth et al. 1997), into the germline of mice sufficed to produce high-titer anti-PrP^{C} immunity. The 6H4μ heavy chain transgene induced similar anti-PrP^{C} titers in *Prnp*$^{0/0}$, *Prnp*$^{0/+}$ and *Prnp*$^{+/+}$ mice, indicating that deletion of autoreactive B cells does not prevent anti-PrP immunity. The buildup of anti-PrP^{C} titers, however, was more sluggish in the presence of endogenous PrP^{C}, suggesting that some clonal deletion is actually occurring.

How can these observations be interpreted? The total anti-PrP^{C} titer results from pairing of one transgenic μ heavy chain with a large repertoire of endogenous κ and λ chains: some pairings may lead to reactive moieties, while others may be anergic (Figure 19a). Maybe the B-cell clones with the highest affinity to PrP^{C} are being eliminated by the immune tolerization machinery, and only clones with medium affinity are retained (Figure 19a). This would explain the delay in titer buildup in the presence of PrP^{C}, and would be in agreement with our affinity measurements – indicating that the total molar avidity of 6H4μ serum is approximately 100-fold lower that that of the original 6H4 antibody from which the transgene was derived (Figure 19b). But most intriguingly, expression of the 6H4μ heavy chain sufficed to block peripheral prion pathogenesis upon intraperitoneal inoculation of the prion agent (Heppner et al. 2001a).

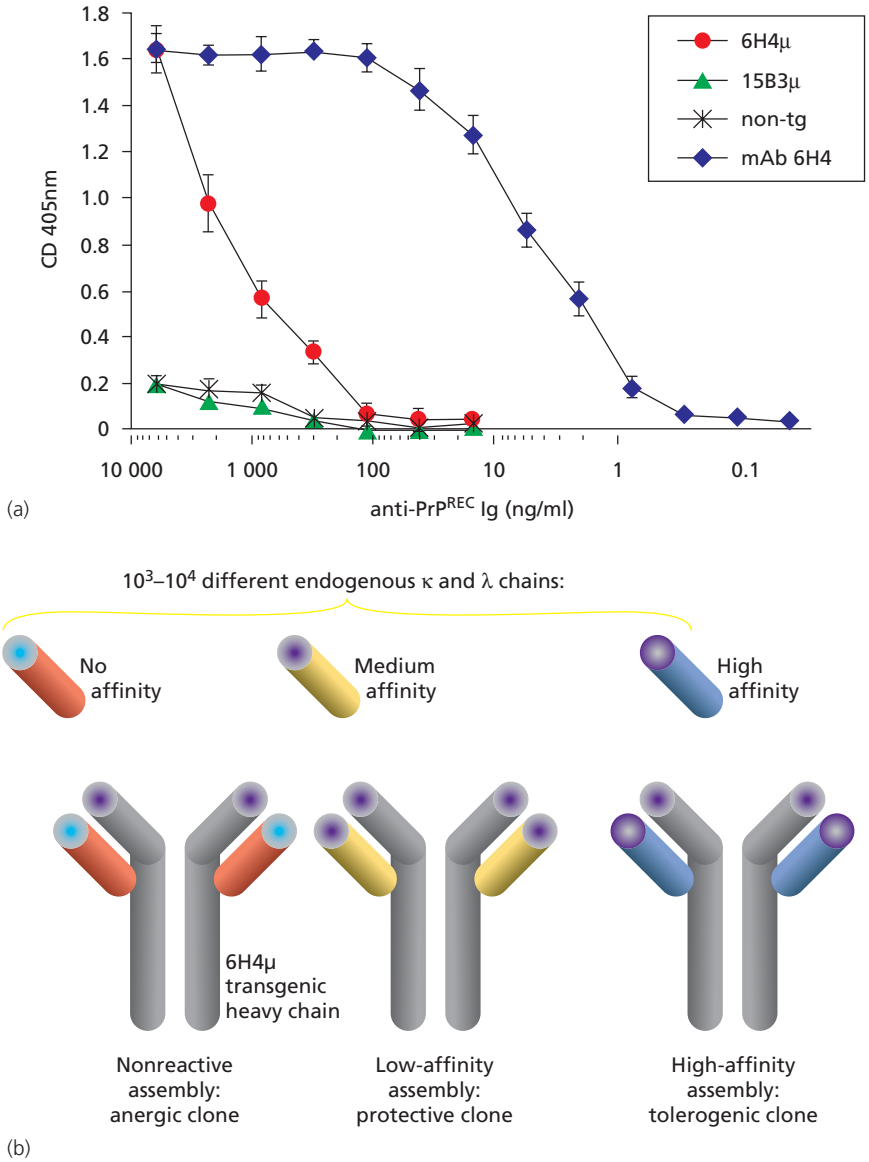

Figure 19 *Affinity and avidity of antibodies against the prion protein.* **(a)** *In order to gain some indication on the relative avidity of the immune response against PrP^C in 6H4 transgenic mice, we compared the binding of serum from transgenic mice to a dilution series of the 6H4 original monoclonal antibody. When normalized against the immunoglobulin concentration, taking into account that the anti-PrP^C antibodies 6H4 transgenic mice are pentameric, we found that the total avidity of the transgenic serum is approximately 2 log units lower than that of the monoclonal antibody from which the transgene was derived. A possible explanation for this phenomenon is depicted in* **(b)**. *While the heavy chain is kept constant in the transgene, it may pair with a large repertoire of endogenous light chains. Some of the pairs will yield very high affinity antibodies, others will have low affinity, but the majority may have no affinity at all for the prion protein. It is possible that some degree of clonal deletion occurs, and that combinations with the highest affinity are eliminated.*

PrP^C is a normal protein expressed by most tissues of the body. Therefore an anti-PrP immune response may conceivably induce an autoimmune disease, and defeat any realistic prospect for prion vaccination. We did not observe any blatant autoimmune disease as a consequence of anti-prion immunization – unless PrP^C was artificially transgenically expressed at nonphysiological, extremely high levels.

The strategy outlined above delivers proof-of-principle that a protective humoral response against prions can be mounted by the mammalian immune system, and suggests that B cells are not intrinsically tolerant to PrP^C. If the latter is generally true, lack of immunity to prions may be due to T-helper tolerance. The latter problem is not trivial, but may perhaps be overcome by presenting PrP^C to the immune system along with highly active adjuvants. These findings, therefore, encourage a reassessment of the possible value of active and passive immunization (Westaway and Carlson 2002), and perhaps of reprogramming B-cell repertoires

by μ-chain transfer, in prophylaxis or in therapy of prion diseases.

Prion immunization and its reduction to practice

As described above, monoclonal antibodies and F(ab) fragments recognizing PrP were shown to prevent de novo scrapie infection, and to abolish PrP^{Sc} as well as prion infectivity in chronically scrapie-infected neuroblastoma cells (Enari et al. 2001). Furthermore, transgenic expression of anti-PrP antibodies in mice arrested peripheral scrapie pathogenesis (Heppner et al. 2001b). In line with these results, White and colleagues confirmed the efficiency of anti-PrP antibodies in preventing prion disease by injecting such antibodies into wild-type mice upon peripheral prion challenge (White et al. 2003).

The prionostatic efficacy of anti-PrP antibodies is highest in extraneural compartments: transgenic 6H4μ mice expressing anti-PrP-specific IgM molecules were not protected when prions were administered intracerebrally (Aguzzi and Heppner, unpublished observations), and passive transfer of PrP-specific IgG immunoglobulins (White et al. 2003) was inefficient when started after the onset of clinical signs. This may be due to the limited influx of immunoglobulins into the central nervous system (CNS) and to the high prion load of clinically symptomatic animals.

By providing stable, sustained titers, active immunization may obviate to some of the problems listed above. However, host tolerance to endogenous PrP^{C} remains a major obstacle to devising active immunization regimens. Nevertheless, several recent studies suggest that the induction of anti-PrP antibodies in wild-type mice is, in principle, feasible (Souan et al. 2001; Koller et al. 2002; Sigurdsson et al. 2002; Arbel et al. 2003; Schwarz et al. 2003; Rossett et al. 2004). While anti-PrP immunoglobulin titers could be measured in most of these studies, the titers were rather low. Accordingly, the biological efficacy of these immunization series, if evaluated at all, was limited.

A systematic exploration of the efficacy of active immunization strategies against PrP (Heikenwälder et al. 2004) found that it was exceedingly difficult to elicit antibody titers to native cell-bound PrP^{C} as displayed on the cell surface of PrP^{C}-overexpressing splenocytes (Raeber et al. 1999b). This suggests that host tolerance to endogenous PrP^{C} is non-permissive to generating high-affinity anti-PrP B-cell clones, or leads to deletion or anergy of the cognate T-cell clones. Expression of PrP^{C} within the thymus did not prevent humoral immune responses to recombinant bacterially expressed PrP completely. However, extrathymic and extraneural PrP^{C} – even if expressed in very small amounts – blocked all immune responses to both PrP^{C} and bacterially expressed PrP. One must conclude, therefore, that while antibodies recognizing cell-surface PrP^{C} interfere with prion pathogenesis in two independent paradigms, humoral immune responses may not be of much value if their affinity does not extend to bona fide eukaryotic PrP^{C} as displayed on cell surfaces.

PROGRESS IN THE DIAGNOSIS OF PRION DISEASES

As with any other disease, early diagnosis would significantly advance the chances of success of any possible intervention approaches. Unfortunately, the situation in prion diagnosis continues to be rather primitive. Presymptomatic diagnosis is virtually impossible, and the earliest possible diagnosis is based on clinical signs and symptoms. Hence, prion infection is typically diagnosed after the disease has considerably progressed.

A significant advance in prion diagnostics was accomplished in 1997 by the discovery that protease-resistant PrP^{Sc} can be detected in tonsillar tissue of vCJD patients (Hill et al. 1997b). It was hence proposed that tonsil biopsy may be the method of choice for diagnosis of vCJD (Hill et al. 1999). Furthermore, there have been reports of individual cases showing detection of PrP^{Sc} at preclinical stages of the disease in tonsil (Schreuder et al. 1996) as well as in the appendix (Hilton et al. 1998), indicating that lymphoid tissue biopsy may represent a potential test for asymptomatic individuals. These observations triggered large screenings of human populations for subclinical vCJD prevelance, using appendectomy and tonsillectomy speciments (Glatzel et al. 2003b). PrP^{Sc}-positive lymphoid tissue were long considered to be a vCJD-specific feature which would not apply to any other forms of human prion diseases (Hill et al. 1999). However, a recent survey of peripheral tissues of patients with sCJD has identified PrP^{Sc} in as many as one-third of skeletal muscle and spleen samples (Glatzel et al. 2003a). In addition, PrP^{Sc} was found in the olfactory epithelium of patients suffering from sCJD (Zanusso et al. 2003). These unexpected findings raise the hope that minimally invasive diagnostic procedures may take the place of brain biopsy in intravital CJD diagnostics.

The sensitivity of PrP^{Sc} detection was significantly improved by the sodium phosphotungstic (NaPTA) precipitation method (Safar et al. 1998). By concentrating PrP^{Sc} prior to western blot analysis, this procedure improves the sensitivity of diagnostic assays by as much as four orders of magnitude (Wadsworth et al. 2001). An interesting development was brought about by the conformation-dependent immunoassay (CDI), in which conformational differences of PrP isoforms are mapped by quantitating the relative binding of antibodies to denatured and native protein (Safar et al. 1998). Rather than relying on protease resistance, the CDI measures a variety of misfolded PrP isoforms,

which may increase its sensitivity (Safar et al. 2002; Bellon et al. 2003).

Be that as it may, all techniques described above suffer from the fact that PrP^{Sc} continues to represent a surrogate marker for prion infectivity – since:

- PrP^{Sc} has not been incontrovertibly shown to be congruent with the prion
- several manipulations in vitro and in vivo can render PrP^{C} protease-resistant without bestowing infectivity on it.

Therefore, determination of prion infectivity by bioassay remains the golden standard: as in Pasteur's age, the concentration of the infectious agent is determined by inoculating serial dilutions of the test material into experimental animals, and the dilution at which 50 percent of the animals contract the disease (termed ID_{50}) is determined. Naturally, this system is riddled with inconveniences: scores of animals need to be sacrificed, and the incubation times are lengthy (transgenetic overexpression of PrP^{C} can help, but only to some extent). Also the method tends to be breathtakingly inaccurate: the inoculation schemes used in most studies typically suffer from standard errors of ± 1 order of magnitude!

A radical improvement of this situation is likely to be brought about by the use of prion-susceptible cell lines (Bosque and Prusiner 2000). The determination of prion infectivity endpoints combines the sensitivity and intrinsic biological validity of the bioassay (i.e. direct measurement of the infectivity) with the speed and convenience of an in vitro methodology amenable to medium-throughput automation (Klohn et al. 2003).

PERSPECTIVES IN PRION IMMUNOLOGY

Until approximately 10 years ago, it was thought that prion diseases are completely independent of the immune system. The lack of overt inflammation in spongiform lesions lent support to this viewpoint. It is fascinating to note that this view has been turned completely upside down, and it is now obvious that the relationships between the infectious agent and various components of the immune system are intimate, complex, and multifaceted. While several crucial questions are still open, notably regarding the true portal of entry of BSE prions into the human body, and the mechanism by which they cause vCJD, a fascinating multitude of unexpected findings has been reported, some of them in extraordinary detail.

In the long run, one would hope that this accrued knowledge will be put into useful practice. To that effect, it is encouraging to note that a sizable number of the steps in prion transport which have been discussed above appears to be rate-limiting. Because of that, these steps lend themselves as a target for interventions, which may be therapeutic or prophylactic (Aguzzi et al. 2001b).

A number of substances appear capable of influencing the outcome of a contact of mammalian organisms with prions: a non-exhaustive list includes compounds as diverse as Congo red (Caughey and Race 1992), amphotericin B (Pocchiari et al. 1987), anthracyclin derivatives (Tagliavini et al. 1997), sulfated polyanions (Caughey and Raymond 1993), pentosan polysulfate (Farquhar et al. 1999), soluble lymphotoxin-β receptors (Montrasio et al. 2000), porphyrins (Priola et al. 2000), branched polyamines (Supattapone et al. 2001), and β-sheet breaker peptides (Soto et al. 2000). However, it is sobering that none of the substances have yet made it to any validated clinical use: quinacrine appears to represent the most recent unfulfilled promise (Collins et al. 2002). On the other hand, the tremendous interest in this field has attracted researchers from various neighboring disciplines, including immunology, genetics, and pharmacology, and therefore it is to be hoped that rational and efficient methods for managing prion infections will be developed in the future.

CONCLUDING REMARKS

Do prions exist in lower organisms?

In *Saccharomyces cerevisiae*, *ure2* and [*URE3*] mutants were described that can grow on ureidosuccinate under conditions of nitrogen repression such as glutamic acid and ammonia (Lacroute 1971). Mutants of *URE2* exhibit mendelian inheritance, whereas [*URE3*] is cytoplasmically inherited (Wickner 1994). The [*URE3*] phenotype can be induced by UV irradiation and by overexpression of ure2p, the gene product of ure2; deletion of ure2 abolishes [*URE3*]. The function of ure2p is unknown but it has substantial homology with glutathione-*S*-transferase; attempts to demonstrate this enzymic activity with purified ure2p have been unsuccessful (Coschigano and Magasanik 1991). Whether the [*URE3*] protein is a post-translationally modified form of ure2p which acts upon unmodified ure2p to produce more of itself remains to be established.

Another yeast prion is the [*PSI*] phenotype (Wickner 1994). [*PSI*] is a non-mendelian inherited trait that can be induced by overexpression of *Sup35* (Cox et al. 1988; Chernoff et al. 1993). The production of [*PSI*] has recently been shown to require the molecular chaperone Hsp104, suggesting that this protein functions in producing an altered form of *Sup35* (Chernoff et al. 1995). Both [*PSI*] and [*URE3*] can be cured by exposure of the yeast to 3 mm GdnHCl. The mechanism responsible for abolishing [*PSI*] and [*URE3*] with a low concentration of GdnHCl is unknown. In the filamentous fungus *Podospora anserina*, the *het-s* locus controls the vegetative incompatibility; conversion from the S^{s} to the s state

seems to be a post-translational, autocatalytic process (Deleu et al. 1993). If any of the examples above cited can be shown to function in a manner similar to prions in animals, many new, more rapid and economical approaches to prion diseases should be forthcoming.

Unresolved problems in prion science

The study of prions has taken several unexpected directions over the past few years. The discovery that prion diseases in humans are uniquely both genetic and infectious has greatly strengthened and extended the prion concept. However, the areas that are still obscure do not relate only to the details: some of these concern the core of the prion concept (Chesebro 1998). In my opinion, there are five large groups of questions regarding the basic science of prion replication and of development of TSE diseases that deserve to be addressed with a vigorous research effort:

- Which are the molecular mechanisms of prion replication? How does the disease-associated prion protein, PrP^{Sc}, achieve the conversion of its cellular sibling, PrP^{C}, into a likeness of itself? Which other proteins assist this process? Can we inhibit this process? If so, how?
- What is the essence of prion strains, which are operationally defined as variants of the infectious agent capable of retaining stable phenotypic traits upon serial passage in syngeneic hosts? The existence of strains is very well known in virology, but it was not predicted to exist in the case of an agent that propagates epigenetically.
- How do prions reach the brain after having entered the body? Which molecules and which cell types are involved in this process of neuroinvasion? Which inhibitory strategies are likely to succeed?
- The mechanisms of neurodegeneration in spongiform encephalopathies are not understood. Which are the pathogenetic cascades that are activated upon accumulation of disease-associated prion protein, and ultimately lead to brain damage?
- What is the physiological function of the highly conserved, normal prion protein, PrP^{C}? The *Prnp* gene encoding PrP^{C} was indentified in 1985 (Oesch et al 1985; Basler et al. 1986), *Prnp* knock-out mice were described in 1992 (Büeler et al., 1992), and some PrP^{C}-interacting proteins have been identified (Oesch et al. 1990; Rieger et al. 1997; Yehiely et al. 1997; Zanata et al. 2002). Yet the function of PrP^{C} remains unknown.

GLOSSARY OF PRION TERMINOLOGY

Prion Agent of transmissible spongiform encephalopathy (TSE), with unconventional properties. The term does not have structural implications other than that a protein is an essential component.

'Protein-only' hypothesis Maintains that the prion is devoid of informational nucleic acid, and that the essential pathogenic component is protein (or glycoprotein). Genetic evidence indicates that the protein is an abnormal form of PrP (perhaps identical with PrP^{Sc}). The association with other 'non-informational' molecules (such as lipids, glycosaminoglycans, or perhaps even short nucleic acids) is not excluded.

PrP^{C} The naturally occurring form of the mature *Prnp* gene product. Its presence in a given cell type is necessary, but not sufficient, for replication of the prion.

PrP^{Sc} An 'abnormal' form of the mature *Prnp* gene product found in tissue of TSE sufferers, defined as being partly resistant to digestion by proteinase K under standardized conditions. It is believed to differ from PrP^{C} only (or mainly) conformationally, and is often considered to be the transmissible agent or prion.

These definitions (Aguzzi and Weissmann 1997) describe our terminology which is, however, not agreed on by convention and is not necessarily used by others.

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Infections of the central nervous system

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INTRODUCTION

Viral infections of the central nervous system (CNS) represent clinically important, often life-threatening complications of systemic viral infections. Except for rabies, they do not result per se from a pathogen-specific tropism for neural tissue, as most viruses associated with CNS disorders frequently infect humans without involving this organ. Some of these viruses cause clinically significant diseases only when invasion of the CNS takes place, for example certain entero- or arthropod-borne viruses. Other agents, such as herpes simplex or mumps viruses, usually induce a mild illness, which may follow a more severe course when the CNS is infected. The establishment of a CNS viral infection depends not only on the biological properties of the invading agent but also on the breakdown of the host defense mechanisms that normally protect CNS cells from infection. Moreover, the degree of damage to CNS tissues is influenced by several special features that set the CNS apart from other tissues. Brain cells are not unusual in their fine structure, but they are unique in the high degree of cell differentiation and the interaction between cell types, as revealed by the synapses between neurons and the relationship between myelin sheath and axons. These peculiarities, together with the existence of a blood–brain barrier (BBB) in the absence of lymphatic tissue in the CNS, play a major role in the development of neurological disease.

Virological, immunological, and molecular biological studies have provided important information on the etiology and pathogenesis of many acute and chronic disorders of the CNS associated with viral infections of animals and humans. In addition, information obtained from in vitro studies of infected neural cells in culture has helped our understanding of the processes involved in a wide range of virus-induced neurological diseases. Although many unresolved problems still remain, great progress has been made in molecular virology and neuroimmunology. Many sensitive techniques have been developed that allow a better characterization of the virus–cell and virus–host interactions occurring in CNS infections.

Rather than give a complete account of all virus infections of the CNS, this chapter is intended to give an overview of the most common human viral CNS disorders and to promote insights that may emerge by comparing this aspect of human diseases. The single viral agents and their corresponding (peripheral) diseases are dealt with separately in specific chapters.

INTERACTIONS OF VIRUSES WITH THE HUMAN CNS

Pathways of virus spread to the nervous system

Successful infection of the CNS by virus depends on a number of factors, and it is the direct interplay between the host's immune system in controlling or attenuating

infection on the one hand, and the neurotropism and neurovirulence of the infecting virus on the other, that has profound consequences for the host. Virus invasion of the CNS can be established via any of the commonly recognized routes for infection: vector-borne, fecal–oral, direct contact and aerosol.

VECTOR-BORNE

The most common route of infection associated with severe disorders of the CNS is via the bite of an infected arthropod vector which delivers virus inoculum through the dead keratinized layer of the skin into the dermis or bloodstream. Viruses belonging to the families *Alphaviridae*, *Bunyaviridae*, and *Flaviviridae* are usually transmitted to humans through the bite of an infected mosquito or tick, after the virus has replicated to high titers in the salivary glands of the insect. A number of the arthropod-borne viruses (arboviruses) are highly pathogenic and the CNS is often involved. Of the viruses commonly associated with CNS disease, Japanese encephalitis virus, a Flavivirus, is the most important, with thousands of reported infections each year and an associated case fatality rate of about 10 percent (see section on Japanese encephalitis). Infection with arboviruses is limited geographically and seasonally to conditions that support the virus vector.

FECAL–ORAL

The fecal–oral route has long been established as an important mode of transmission of numerous enteroviruses, and classically with epidemics of poliomyelitis. Enteric viruses, which replicate primarily in the alimentary tract, are shed in fecal matter, which in turn may contaminate food and water supplies, particularly in areas of poor sanitation. These viruses are able to resist and survive gastric acidity. Enteric viruses are responsible for large epidemics of disease which, unlike the arboviruses mentioned above, are not restricted geographically. In Europe enteroviruses are the leading cause of CNS disturbances, although 90 percent of these infections are clinically inapparent. Of the remainder, symptoms may range from paralytic poliomyelitis to nonparalytic meningitis, as well as the more rare encephalitides.

DIRECT CONTACT

Direct contact (sexual, prenatal, or by mechanical means, e.g. by needle, bite), as a mode of transmission for a number of viruses that cause CNS disease, is by nature a particularly emotive subject. Infection by direct contact involves the transfer of virus-infected cells or body fluids. These viruses include human immunodeficiency virus (HIV), herpes simplex virus (HSV), rabies virus, and *Cytomegalovirus* (CMV). More rarely, infection of the (CNS) with rabies virus or CMV has been reported following corneal tissue grafts. Vertical transfer of virus from mother to fetus, commonly HSV, CMV, rubella, human T-lymphotropic virus type I (HTLV-I) and HIV, may lead to severe CNS disease in newborns. In addition, breastfeeding by infected mothers is associated with HTLV-I, HIV and CMV infections in newborns.

AEROSOL

Establishment of CNS infection via the respiratory tract is not widely recognized as a common event. The exception to this generalization is mumps virus, when, during epidemics of parotitis, about 0.1–10 percent of infected individuals develop quite severe meningitis. Infection of the CNS by rabies virus has been documented following the inhalation of virus-laden aerosols by visitors to bat caves, where the virus is maintained in the bat population, or in laboratory workers exposed to high concentrations of virus. Aerosols may also be associated with the transmission of other potentially neurotropic pathogens, such as measles virus and varicella-zoster virus.

Regardless of the site of entry, for successful infection of the host by a virus a period of replication at this initial site is required, and the virus must also evade the local immune response. The possible exception to the above concerns arthropod-borne viruses, which are often inoculated directly into the bloodstream and initial replication is usually established in lymphatic tissue. Replication of virus at the site of entry or in lymphatic tissue increases the viral load and allows the establishment of viremia. Once in the bloodstream, virus is able to disseminate throughout the host and infection of susceptible secondary organs and tissues can take place. The ability of the immune system to terminate virus infection rapidly before significant viremia levels are reached largely dictates the outcome of a number of potential viral diseases of the CNS. Two main routes for the invasion of the CNS by virus are recognized: hematogenous and neural.

Entry into the CNS

THE HEMATOGENOUS ROUTE

The bloodstream, with its extensive network of vessels serving every part of the body, forms the perfect dissemination vehicle for invading viruses. The extent to which tissues and organs become exposed to virus depends on the length of time that a high viral load is maintained in the bloodstream and the efficiency with which the immune system can clear virus. Whether infection will be established by the virus depends on a number of factors, including the presence of specific cellular receptors for that virus. Before virus can enter the CNS, the so-called blood–brain barrier must be

circumvented (Zhang 1999). The BBB comprises endothelial cells of the cerebral microvasculature, pericytes, astrocyte foot processes, and the basal lamina, which together are a formidable barrier (Pardridge 1999; Miller 1999). However, the CNS is accessible to activated lymphocytes and macrophages which, if infected with virus, afford access to this normally protected environment (Persidsky 1999). Circumvention of the BBB by the transport of virus within infected cells is thought to be highly significant for CNS infection with mumps and measles viruses (Wolinsky et al. 1976; Fournier et al. 1985) and HIV (Haase 1986). Direct measles virus infection of the endothelial cells of the BBB is commonly found in patients suffering from subacute sclerosing panencephalitis (SSPE) (Allen et al. 1996). The choroid plexus is widely believed to be an important portal of entry for bloodborne virus to the CNS. This is based largely on the fine structure analysis of the choroid plexus itself, in which fenestrated endothelium and a sparse basement membrane combine to form a more permeable region. The choroid plexus is a major target of replication or transport of mumps and arboviruses, which subsequently infect ependymal cells lining the ventricles and finally the underlying brain tissue.

THE NEURAL ROUTE

A number of viruses establish CNS infection by directly infecting nerves of the CNS. Viruses such as HSV, poliovirus, and rabies (Sabin 1956; Martin and Dolvio 1983; Iwasaki et al. 1985) infect peripheral nerve endings and, by retrograde axonal transport, spread through the CNS. Entry into neurons is believed to occur via receptor-mediated endocytosis, after which virus is transported in vesicles. The exact mechanism of transport of virus within nerves remains to be established, although movement is known to occur via fast axonal transport (Kristensson 1982), which involves the passage of viral material in vesicles along microtubules. Viral replication occurs once virus particles reach the neuronal cell body and newly synthesized virion components accumulate at postsynaptic sites. The site of inoculation can influence the ultimate distribution of lesions in the CNS, especially for viruses that spread along neural routes. For example, HSV infection of the genitourinary tract establishes latency of virus in the sacral ganglia, whereas infection with HSV orally establishes latency in the trigeminal ganglia. At the time of primary infection, HSV is transported from the mucous membrane by retrograde axonal transport, whereas during periods of reactivation virus is transported to the periphery by anterograde flow. Once the CNS has been invaded, advancement of infection by many neurotropic viruses occurs through cell-to-cell spread (trans-synaptic transport).

The factors influencing the release of virus from presynaptic nerve terminals and the uptake of progeny at postsynaptic terminals are unknown. It is important to note that direct infection of peripheral nerve endings by virus may also take place during the viremic phase of an infection. For example, during the high-titer viremia found in clinically apparent infections of humans with the *Flavivirus* Japanese encephalitis virus (JEV), the possibility that neurons of the olfactory bulb will be infected is enhanced. Retrograde transport of virus within specialized neurons is then possible. The olfactory mucosa itself offers a unique pathway for the spread of viruses into the CNS, where the apical processes of receptor cells penetrate beyond the surface of the epithelium as olfactory rods. However, olfactory uptake and spread is not a common route of virus infection of the CNS (Johnston 1994). Once in the CNS, the cellular tropism and neurovirulence of virus and its ability to avoid the immune response will largely determine the outcome of disease.

Tropism and neurovirulence

CELLULAR RECEPTORS AND HOST FACTORS

The ability of a virus to infect specific cell populations of the CNS defines its neurotropism, which is influenced either by host cell expression of receptors or by genes important for viral replication. In general, the distribution of specific cellular receptors for viruses within a host governs the susceptibility of tissue(s) or organs to virus infection. In recent years, many cell surface proteins have been identified as viral receptors that normally serve various physiological functions (Schneider-Schaulies 2000; Schweighardt and Atwood 2001) (Table 1).

Besides the viral receptor the virus attachment protein itself plays a central role in the initial interaction of the virus with its target cell. Numerous virus–cell attachment proteins have been identified by analysis of genetic recombinants, genetic reassortants, and mutant viruses (Kennedy 1990). This approach has been used, for example, to define the neurotropism of serotype 3 reovirus, which has been mapped to the *S1* gene that encodes the σ1 protein (Tyler 1991). Similarly, neutralization escape mutants of rabies virus, with a single amino acid substitution in the envelope glycoprotein G that serves in viral cell attachment, revealed a lower virulence than the parent virus (Tyler 1990). A more detailed molecular view of a ligand in virus receptor–molecular interactions was obtained with the X-ray crystallographic analysis of the poliovirus virion. The analysis revealed that a series of peaks in the VP1 capsid proteins surrounded by a broad valley composed of VP1 and VP3 forms the receptor-binding pocket (Almond 1991). It is thought that other picornaviruses may have cell attachment proteins of similar topology. An exception to this is foot-and-mouth disease virus (FMDV), an aphthovirus, for which the virus attachment protein

Table 1 *Examples of viruses associated with CNS disease and their receptors*

Virus (family)	Receptor	References
Flaviviridae		
Tick-borne encephalitis virus (TBEV)	Heparan sulfate	Mandl et al. 2001
Lentiviridae		
HIV-1, 2	CD4	Dalgleish et al. 1984; Klatzmann et al. 1984
HIV-1	Galactosylceramide	Harouse et al. 1991
	Chemokine receptors	Berger et al. 1999
Herpesviridae		
CMV	Heparan sulfate	Compton et al. 1993
HSV	Heparan sulfate, HveA, -B, -C	WuDunn and Spear 1989; Montgomery et al. 1996; Geraghty et al. 1998
Human herpesvirus-6	CD46	Santoro et al. 1999
Paramyxoviridae		
Measles virus	Membrane cofactor protein (CD46) Signaling lymphocytic activation molecule (CD150)	Dörig et al. 1993; Naniche et al. 1993; Tatsuo et al. 2000
Picornaviridae		
Polioviruses	PVR (CD155) (immunoglobulin-like)	Koike et al. 1990; Mendelsohn et al. 1989
Echovirus 1, 8	VLA-2 (α-chain, integrin)	Bergelson et al. 1992; Bergelson and Finberg 1993
Echovirus 22	$\alpha_v\beta_3$ (integrin)	Roivainen et al. 1991
Echovirus 7 (6, 11, 12, 20, 21?)	Decay-accelerating factor (CD55)	Bergelson et al. 1994
Rhabdoviridae		
Rabies virus	Acetylcholine receptor (α-$_1$) Neural cell adhesion molecule (NCAM) Nerve growth factor receptor (NGFR)	Lentz 1990; Thoulouze et al. 1998; Tuffereau et al. 1998

(VAP) is exposed on VP1 (Mason et al. 1994). Mutations occurring in the VAP may be responsible for altered tropism of the virus, or even defective virus that is no longer able to bind to its receptor. This may be manifested by attenuation of virus infectivity, and is thought to be important in relation to the immunogenicity of vaccines.

In further attempts to unravel which factors govern the neurovirulence of poliovirus, a combination of nucleotide sequencing and gene cloning identified the genetic differences between wildtype and live attenuated vaccine strains (Almond 1991). Of the changes detected, the most significant were localized in the 5′ nontranslated region (NTR) at positions 480, 481, and 472 of poliovirus serotypes 1, 2, and 3, respectively. These mutations were associated with the ability of the virus to replicate in the brains of mice or neuroblastoma cells in culture (La Monica et al. 1987). A more detailed investigation of both the structure and the related function of mutated 5′ NTR regions of poliovirus revealed that attenuating mutations in vaccine strains act through the disruption of a stem–loop structure in the region of nucleotide positions 470–540. Computer-generated analysis has hinted that the greater the disruption of the system structure, the greater the temperature sensitivity and the degree of attenuation. Further analysis of the intracellular protein interactions associated with this poliovirus stem–loop structure may provide an explanation of why motor neurons are the specific targets of these viruses.

In addition to viral factors, host factors also influence viral tropism and virulence and modulate the viral pathogenesis of CNS infections. In this context, a lack of enzymatic activity required for maturational cleavage of viral proteins, or completion of the virus replicating cycles, will lead to incomplete infection of target cells, thereby limiting tropism and spread of the virus. On the other hand, this may facilitate the establishment of a persistent infection, as has been demonstrated in the case of SSPE (Billeter et al. 1994). Moreover, it has been observed that many neurotropic viruses more readily invade the CNS of the young, owing to an immature immune response, reduced capacity to produce interferon, and dependence of the susceptibility of viral infection on the stage of cell differentiation, as well as the age-specific nature and distribution of receptor proteins (Coyle 1991). Bias of infection for a particular gender has also been noted. The CNS disorder tropical spastic paraparesis/HTLV-I associated myelopathy (TSP/HAM), for which HTLV-I is the proposed

etiological agent, has a prevalence in females, with a female:male ratio of 1.6:1.

Neurovirulence and viral gene expression

Neurovirulence is the capacity of a virus to multiply and to extend infection after it has invaded the nervous system. This property, together with the effectiveness of the host immune response, determines the degree of tissue damage. Point mutations in envelope proteins of several viruses have been shown to influence neurovirulence in animal models. Some examples are Sindbis virus (Lee et al. 2002), murine coronavirus (Philips et al. 2002), measles virus (Moeller et al. 2001), and yellow fever virus/Japanese encephalitis virus chimera (Arroyo et al. 2001).

Many viruses contain nontranscribed genetic elements, referred to as promoters or enhancers, that regulate the transcription of specific genes. It is interesting that virus enhancers may exhibit cell or tissue specificity, and possibly species specificity, thus promoting or limiting virus infection. Important roles for viral enhancer elements in determining cell type-specific gene expression have also been described for picornaviruses. In the poliovirus-induced pathogenesis the internal ribosomal entry site (IRES) plays an important role for neurovirulence (Ohka and Nomoto 2001).

The available data suggest that, in general, the outer capsid or envelope glycoproteins of neurotropic viruses are probably important determinants of virulence. This assumption is based, for instance, on the observation that amino acid sequence changes – of proteins, or genetic reassortments between virulent and avirulent strains of a given neurotropic virus – can lead to an attenuated infection. On the other hand, not all changes in viral genes result in attenuation, as mutations causing reversions to neurovirulence have been seen in poliovirus isolates from vaccine recipients (Almond 1987). Moreover, when one region of a viral protein is linked to neurotropism and neurovirulence, changes in that protein could have dramatic effects on the resulting infections, as documented in studies of Theiler's murine encephalomyelitis virus infections (Nash 1991). Usually not only one gene contributes to the neurovirulence of a virus, as found for example for Theiler's virus, in which mutations of the envelope proteins, as well as the polymerase, influence the outcome of the disease (Reddi and Lipton, 2002; van Eyll and Michiels 2000). Taken together, the interplay of host and viral factors determines the tropism and virulence of viruses that infect the CNS.

Virus–cell interactions in the CNS

The interaction of infectious viruses with susceptible cells leads in general to cell destruction, and is largely determined by the genetic constitution of the host cell and by the type of viral agent. This destruction can occur following cytolysis, persistent or even latent infection, cell transformation, or be caused by infection-independent mechanisms. After infectious virus has reached the CNS, disease develops only if viral spread is accomplished and sufficient numbers of susceptible cells are infected, resulting in brain dysfunction. In this respect, a number of unique features of the CNS are noteworthy. First, the CNS contains a highly differentiated cell population, with complex functionally integrated cell-to-cell connections and highly specialized cytoplasmic membranes. This probably results in the great variability in virus receptor sites and their density on cells. Second, CNS tissue is unique in its high metabolic rate and a low regenerative capacity. Although persistent infection by a noncytopathogenic virus in cells of tissue with a low energy requirement and a high rate of regeneration may be tolerated, in CNS tissue such infections may interfere with normal cell functions, especially when neurons are affected. Third, the brain's relative isolation from the immune system is another feature that plays an important role in the establishment and pathogenesis of CNS infections.

ACUTE INFECTIONS

Acute infections lead to cell death and the release of progeny virus. Cell destruction is induced by the products of the viral genome or their effect on the regulatory mechanism of the cell. For some viruses, such as herpes and polio viruses, the molecular events during a lytic infection have been well described for tissue culture systems, and it may be inferred that similar processes occur during CNS infections. One of the most severe acute viral infections of the CNS is caused by herpes simplex virus (HSV), which results mainly from the primary infection. Productive replication in brain tissue leads to cell destruction, which in its extent and pathology depends on the neuroinvasiveness and neurovirulence of the virus and on the effectiveness of the host defense mechanisms. Extensive molecular biological studies have defined several regions on the viral genome as specific contributors to these processes (Stevens 1993). Among the HSV genes, 37 seem not to be necessary for HSV replication in tissue culture, and are referred to as 'supplemental essential genes.' They may be linked to the properties of neuroinvasiveness and neurovirulence by allowing the virus to invade the CNS, to replicate in a variety of different brain cells, and to spread efficiently from cell to cell. Another destructive disease of nervous tissue occurs after poliovirus infection (Melnick 1990). Following primary replication of poliovirus in the tonsils, the lymph nodes of the neck, and Peyer's patches in the small intestine, the virus spreads along the axons of peripheral nerves, and may invade the spinal cord and brain. Here, neuronal tissue can be damaged or

completely destroyed as a consequence of intracellular replication. The anterior and, in severe cases, the posterior horn cells of the spinal cord are most prominently involved, resulting in a flaccid paralysis (see Picornaviruses).

PERSISTENT INFECTIONS

Within the group of viruses that can establish persistent infections, a variety of virus–cell and virus–host interactions exist that lead to a number of different disease processes:

- Latent viral infections are characterized by long periods of pretended viral absence followed by intermittent episodes of viral replication and the formation of infectious virus (recurrence). This may either remain clinically silent or result in clinical disease (recrudescence). During latency, the virus remains within the host in a quiescent form.
- In chronic viral infections virus can be reproducibly and continuously recovered from the host, with or without the development of clinical disease. Disease may be caused by the replication of virus or by immunopathological mechanisms.
- In slow virus infections, a long incubation period of months to years is followed by a slow, progressive disease course that is usually fatal. The concentration of virus in the CNS increases until disease becomes clinically apparent.

The establishment and maintenance of persistent infections in the CNS may be governed by viral factors or host cell factors, or both, depending on the individual virus–host-cell relationships. As a general mechanism, virus-induced apoptosis may be an important pathogenic mechanism of CNS damage. On the other hand, virus-mediated inhibition of the apoptotic pathway has been linked to the establishment of persistent infections in brain cells (Levine et al. 1991). In the human nervous system, only the latent and the slow virus type of infection are found. The classic latent infection is caused by HSV types 1 and 2. HSV is transmitted from human to human by skin or mucous membrane contact, and is able to remain latent in the nervous system for the lifetime of the host. Its occurrence is not restricted to sensory neurons, and it has also been detected in neurons of peripheral nerve ganglia, the adrenal medulla, the retina and the CNS (Stevens 1993). The establishment of the latent phase is controlled and executed by the neuron rather than by the virus itself, and HSV gene expression is almost completely abolished except for the expression of a set of so-called latency-associated transcripts (LAT)(Taylor et al. 2002). No infectious virus can be isolated during latency. Although the underlying molecular events that trigger viral reactivation are still unknown, clinical observations indicate that recurrence is associated with physical or emotional stress, immune suppression, UV light, or nerve damage. HSV in latently infected neurons is refractive to the immune system and cannot be cleared from those cells. The role of the immune system in controlling HSV infection is not entirely clear, because recurrences can be observed even in the presence of an apparently normal cell-mediated and humoral immune response. Although there seems to be no impact on nerve function during silent periods of latency, reactivation of HSV leads to the destruction of infected neurons.

The best-studied slow virus disease of humans associated with a conventional virus is subacute sclerosing panencephalitis SSPE (ter Meulen et al. 1983). The disease develops after the establishment of a persistent measles virus infection in brain cells months to years after acute measles. How and when the virus reaches the CNS, and the mechanisms that trigger the disease, remain unknown. In brain material obtained post mortem, the accumulation of viral nucleocapsids in virtually any CNS cell population, together with a general failure to reisolate infectious virus, points to the presence of a defective viral replication cycle (see below). In general, gene functions required for intracellular amplification of the viral genome are functionally maintained, whereas those associated with viral maturation and budding are highly restricted. This allows the virus to survive and replicate intracellularly while evading immune surveillance. In fact, an exceedingly high humoral immune response against measles virus proteins, except for the matrix (M) protein, in both serum and CSF of patients with SSPE is pathognomonic.

Infection-independent mechanisms

Experimental evidence suggests that some viruses may trigger apoptosis in neural cells, thereby leading to rapid destruction of the host cell before the virus is efficiently replicated (Levine et al. 1991). Viral invasion of the CNS may not always lead to infection of susceptible cells, but it may trigger pathological changes in the absence of viral spread. This is particularly important in parainfectious conditions, such as acute measles encephalitis, in which the presence of the infectious agent cannot be unequivocally demonstrated, or HIV, in which the infected CNS cells can support only minimal viral replication. As is apparent from tissue culture studies and animal experiments, HIV gene products such as gp120, tat, and nef, as well as soluble factors and nitric oxide released from virus-infected microglial cells, may contribute to the pathogenesis of HIV brain infections (Patrick et al. 2002). This occurs by the induction of neuronal damage and the functional impairment of both neuronal and macroglial cells that, per se, seem not to be susceptible to viral infection.

Viral impact on specific neural cell functions

The interaction of a virus with the CNS may cause severe dysfunction of the infected cell, even if only restricted areas of the CNS are involved. In rat astrocytoma cells persistently infected with measles virus, a strong reduction in the cAMP response to catecholamines was observed, the density of β-adrenergic receptors was decreased by 50 percent and their G protein coupling was affected (Halbach and Koschel 1979; Koschel and Münzel 1980). The endothelin-1-induced Ca^{2+} signal was absent in the same cells, 95 percent of the binding sites for endothelin-1 being lost (Tas and Koschel 1991).

A further example of the direct disturbance of brain cell functions is infection with rabies virus (RV). This virus causes a nonlytic infection of brain cells that rapidly eads to the death of the infected individual. In contrast to the limited cytopathology, death seems to result from interference of the virus with neuronal cell functions in vital centers of the brain that regulate sleep, body temperature, and respiration (Tsiang 1993). The uptake and release of γ-aminobutyric acid (GABA) was reduced by 45 percent in the cortical neurons of rabies-infected embryonic rats (Ladogana et al. 1994), and binding of 5-hydroxytryptamine to serotonin receptor subtypes was reduced by 50 percent in infected rat brains (Ceccaldi et al. 1993). It is conceivable that disturbances of specialized receptor sytems for neurotransmitter and neurohormone turnover, as well as for the generation of chemical signals and electrical potentials, might be the main cause of death in this viral infection, rather than cytopathic effects or immunopathogenesis.

Induction of cytokine and chemokine expression in the brain

Cytokines are produced in response to viral infection in the CNS and are secreted either from infected brain cells or from mononuclear cell infiltrates. Virus-induced cytokine patterns in CSF and in brain tissue of both humans and experimentally infected animals have been intensively studied. Although individual variations may be observed between different virus infections, there seems to be a common set of cytokines induced in the CNS, as in the periphery. These include type I (IFN-α/β) and type II (IFN-γ) interferons, the proinflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α, IL-6, and, generally in lower amounts, IL-2 (Maudsley et al. 1989; Plata-Salaman 1991). For example, in the CSF of SSPE patients elevated levels of IFN-α/β are observed, and brain cells staining for IFN-γ and TNF-α have been detected in autopsy material (Neumann 2001; Joncas et al. 1976; Cosby et al. 1989; Hofman et al. 1991). In the brains of HIV-1 infected patients, where CD4+ microglial cells are the major cell population supporting viral replication, these cells are the source of intrathecal synthesis of IL-1, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), and TNF-α (Merrill and Chen 1991). In addition to the cytokines, a set of infection-induced chemokines attracts inflammatory mononuclear cells across the BBB. Besides lymphoid cells and endothelial cells, as neural cells microglia and astrocytes are the main producers of chemokines such as macrophage inflammatory proteins (MIP-1α, 1β, and 2), macrophage chemotactic proteins (MCP-1, -2, -3), interferon-γ-inducible protein (IP-10), IL-8, growth-related oncogene (GRO α and β), and regulated upon activation of normal T cell expressed and secreted (RANTES). Together with cytokines, which induce or enhance the expression of adhesion molecules on endothelial cells of the BBB, these chemokines promote the migration of monocytes, lymphocytes, eosinophils, and basophils across the BBB into the brain (Persidsky 1999). Viral infection of the CNS parenchyma strongly stimulates local chemokine expression. In simian immunodeficiency virus (SIV)-induced neuropathogenesis in primates the chemokines MIP-1α, MIP-1β, RANTES, MCP-3, and IP-10 play a major role in recruting inflammatory cell infiltrates (Sasseville et al. 1996). In tissue culture it has been shown that the HIV transactivator tat stimulates the expression of MCP-1 by astroctyes. In addition, this chemokine was detected in the brains and CSF of patients with HIV-associated dementia (Conant et al. 1998).

The multiple effects of cytokines in the CNS may be beneficial in stimulating immunological surveillance and intracellular viral resistance (mainly type I and II IFNs and TNF-α) (Schijns et al. 1991; Shankar et al. 1992). This occurs by recruitment and activation of cells of the immune system as well as by induction of MHC molecules (Campbell 1991; Neumann 2001; Plata-Salaman 1991) or antiviral proteins such as MxA, RNA-dependent protein kinase R (PKR), or 2′,5′-A synthetase in brain cells that directly interfere with intracellular viral replication (Staeheli 1990). Triggering of immune responses and inflammatory reactions by proinflammatory cytokines such as IL-1 and TNF-α, together with the induction of neurotoxins (Giulian et al. 1990) and reactive oxygen intermediates (Nathan 1992), may, however, also contribute directly to the immunopathology observed in most viral CNS conditions. Proinflammatory cytokines such as IL-1 or TNF-α produced by immune cells or by CNS cells in response to infection may be essential to the process of neuronal destruction (Quagliarello et al. 1991; Selmaj 1992), as indicated by the meningitis and BBB damage that followed injection of these cytokines into rat brains. Moreover, it has been shown for experimental allergic encephalitis that neurospecific T cells recruit activated inflammatory cells through the action of TNF-α and IFN-γ (Ruddle et al. 1990). In addition, these cytokines can prime macrophages for the production of inducible

reactive oxygen and nitrogen intermediates (Ding et al. 1988), which are thought to play an important role in cytotoxicity (Nathan 1992).

Direct analysis of cytokine expression in human CNS diseases is often difficult to perform, because the production of cytokines may be localized, the levels may be very low, and cytokines normally are rather unstable once secreted. Moreover, the role of cytokines in the development of viral CNS infection and in viral neuropathogenesis cannot be studied in humans for obvious reasons, and so suitable animal models must be used. For example, experimentally induced CNS infections with borna disease virus (BDV) in rats represent a recent example of an animal model to study the role of cytokines in neurological disorders (Bode 1995; Rott and Becht 1995; Stitz et al. 1995; Briese et al. 1999). Induction of IL-1α, IL-2, IL-6, TNF-α, and IFN-γ mRNA synthesis has been found in rat brains 2 weeks after intranasal infection, and IL-2 as well as IFN-γmRNA expression correlated well with the appearance of $CD4^+$ and $CD8^+$ lymphocytes during the early stages of BDV infection (Shankar et al. 1992). Moreover, mRNA levels for inducible nitric oxide synthetase (iNOS) were upregulated (Koprowski et al. 1993). It is interesting that the levels of iNOS mRNA correlated not only with the degree of neurological involvement and CNS inflammation but also with the levels of TNF-α, IL-1, and IL-6 mRNA (Selmaj 1992), potential mediators of iNOS expression (Nathan 1992). In the chronic phase of BDV infection of the CNS, levels of IL-1, IL-6, and TNF-α decreased dramatically, whereas mRNA levels for IFN-γ were greatly elevated, probably indicating that IFN-γ may act to reduce inflammation and to improve neurological signs during the transition phase from acute to chronic disease. In fact, IFN-γ has a pronounced synergistic effect in the TNF- and IL-1-mediated induction of manganese superoxide dismutase, which has been linked to the protection of uninfected cells from reactive oxygen toxicity during immune responses (Harris et al. 1991). To date, BDV has not been identified as a clear etiological agent of any known human disease (Carbone 2001).

MHC expression in the brain and its relation to viral infection

An effective immunological response to viral infections of the CNS is undoubtedly hampered by the relative lack of MHC and costimulatory molecule expression in this tissue. MHC class I expression is believed to be virtually absent from the surface of neurons, whereas MHC class II expression is confined to microglia and pericytes at the BBB (Pardridge 1999). Not only is MHC class I 'passively' absent, but surface contact with neurons also suppresses its expression on the surface of microglial cells (Hoek et al. 2000). In tissue culture, astrocytes and endothelial cells have also been found to express MHC class II after induction. Much attention has been focused on the ability of certain cytokines, such as IFN-γ and TNF-α, to induce or upregulate MHC class I and II, and adhesion molecules such as ICAM-1 on glial cells (Frohman et al. 1989). However, the presence of these factors in the CNS indicates that an inflammatory process had already been initiated, which is not likely to occur soon after a virus has entered the CNS. In support of this notion, infection-dependent upregulation of MHC class I expression on a variety of cell types (reviewed in Maudsley et al. 1989), and also MHC class II induction on otherwise negative cells (including astrocytes), has been described (Massa et al. 1986, 1987). Interestingly, infected neurons can be an intrathecal source of IFN-γ and express MHC class I after inhibition of their electrical activity, either experimentally or after infection (Neumann et al. 1997).

Although it is certain that induction or augmentation of immunoregulatory surface molecules on the surface of brain cells is a prerequisite for an efficient immune response, their role in neuropathogenesis is unclear. For instance, MHC class II expression was readily inducible in the SJL and CBA mouse strains, which are susceptible to experimentally induced CNS infections with Theiler's virus, but not in the resistant BALB/c mice (Nash et al. 1985). In contrast, BN rats, which live well with experimental *Coronavirus* JHM or MV infections, showed a constitutive high MHC class II expression in the brain, whereas in Lewis rats, in which MHC class II expression occurs as a consequence of the viral infection, immunopathological (autoimmune) processes are observed in a significant proportion of animals. This illustrates that the early presence of immunoregulatory molecules in the brain may provide protection, whereas the same process could lead to pathology and disease when MHC molecules are expressed several days or weeks later (Sedgwick and Dörries 1991). Induction of MHC molecules on neural cells is, however, not sufficient to result in elimination of virus, as illustrated by observations in brain tissue from patients with progressive multifocal leukoencephalopathy (PML), a slow papovavirus infection predominantly involving oligodendrocytes. Here, both MHC class I and II are expressed in the lesions, yet viral clearance does not occur, probably because no reactive T cells are generated by the immunosuppressed patient. This may allow the establishment of persistence and ultimately chronic viral infection with progressive disease (Achim and Wiley 1992).

The immune response to viral infections in the CNS

PASSAGE OF EFFECTOR LYMPHOCYTES INTO THE CNS

The BBB is the sentinel in the development of an inflammatory response in the CNS. Adhesion of lympho-

cytes and their subsequent migration through the capillary endothelium is essential for the development of an inflammatory reaction. Adhesion molecules expressed on the brain capillary endothelium act as important ligands to receptors on the surface of lymphocytes, and mediate both adhesion and subsequent migration (Cross 1992) (Figure 1). Two families of molecules on the endothelium are well known in the initiation of adhesion. The initial binding seems to be mediated by those belonging to the selectin (CD62) family (E-selectin, L-selectin, and P-selectin). The second family comprises the Ig superfamily of adhesion molecules such as ICAM-1 (CD54) and VCAM-1 (CD106), which bind to integrin molecules on lymphocytes (Springer 1994; Risau et al. 1998; Pardridge 1999).

Migration is mediated by the secretion of chemoattractant molecules released by the endothelium, as well as by astrocytes and microglial cells (Ransohoff et al. 1993; Persidsky 1999; Zhang 1999). Because adhesion to the capillary endothelium is an important step before cell migration, it follows that factors influencing the expression of the receptor molecules on the capillary endothelium or on the lymphocyte surface are likely to influence the extent and severity of the inflammatory response. Proinflammatory cytokines such as IL-1, TNF-α, and IFN-γ increase the expression of the Ig superfamily of adhesion receptors. In fact, ICAM-1 and -2 and VCAM-1 are expressed on CNS microvessels in experimental allergic encephalomyelitis (EAE), and its expression coincides with inflammatory cell infiltration (Engelhardt 1998; Cannella et al. 1991). Furthermore, in the human brain, ICAM-1 is expressed on vessels in viral encephalitic lesions (Sobel et al. 1990). Studies in vitro have shown that antibodies to proinflammatory cytokines, or cytokines that have an antagonistic effect, such as transforming growth factor-β_2 (TGF-β_2), alter the adhesion of lymphocytes to the capillary endothelium (Fabry et al. 1995). The anti-inflammatory properties of TGF-β have been exploited in the amelioration of two distinct T cell-mediated EAEs (Racke et al. 1991, 1993), and it can interfere with the adhesion of lymphocytes to endothelial cells, possibly inhibiting the entry of effector cells into the CNS (Cai et al. 1991; Gamble and Vadas 1991). In support of this suggestion, TGF-β treatment in EAE does not influence the appearance of sensitized cells in peripheral blood and lymph nodes, but does prevent the accumulation of T cells in the brain and spinal cord. This suggests that the protective effect of TGF-β is exerted at the level of the target organ, the CNS, its vascular endothelium, or both (Santambrogio et al. 1993). It has been shown that T cells require prior activation to cross the BBB (Hickey et al. 1991). However, once the BBB was breached, no activation-dependent differences in the homing pattern of T lymphocytes were observed (Trotter and Steinman 1985).

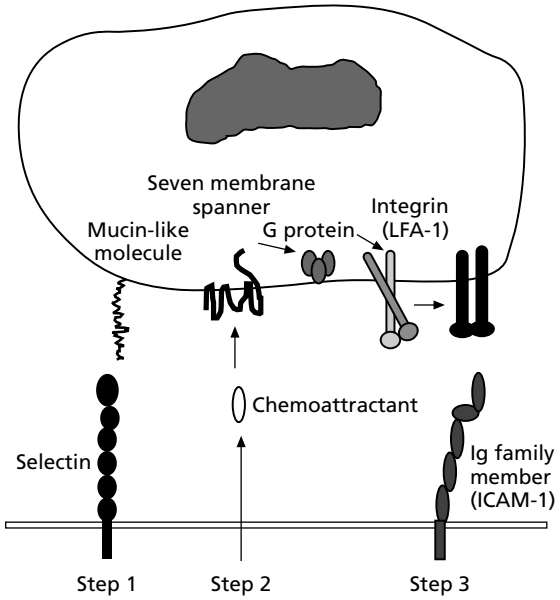

Figure 1 *Sequential steps in the traffic of leukocyte adhesion to the vascular endothelium. Selectins (CD62) on the surface of vascular endothelial cells initially interact with carbohydrate residues, often displayed on mucin-like molecules on flowing leukocytes to mediate a labile adhesion (Step 1). This interaction brings leukocytes into close proximity with chemoattractants (released from the endothelial cells lining the vessel wall) which bind to receptors that span the membrane seven times on the surface of leukocytes. Binding of chemoattractants to their receptors subsequently triggers the activation of G proteins, which then transduce signals to activate integrin adhesiveness (e.g. by activating leukocyte function antigen 1, LFA-1) (Step 2). The interaction of the latter with surface proteins of the Ig superfamily (expressed on the endothelial cells, such as intercellular adhesion molecule 1, ICAM-1) mediates strong adhesion, leading to an arrest of the rolling leukocyte (Step 3). Following directional cues from chemoattractants, and using integrins for traction, leukocytes then cross the endothelial lining of the blood–brain barrier and enter the CNS.*

THE HUMORAL IMMUNE RESPONSE IN THE CNS

The control of virus infection depends on the efficient generation of both humoral and cell-mediated immune responses. Antibodies, which attack predominantly extracellular virus particles released from infected cells, are required to limit the spread of some viruses in the host. For example, it seems that the humoral immune reaction is essential in controlling poliovirus infections. For infections with other viruses, such as varicella-zoster, *Cytomegalovirus*, or measles virus, T-cell responses may be more important than antibody. In this context it is important to remember that the immune response to viral infections of the CNS is initiated in peripheral lymphoid tissue, with subsequent entry of activated (end-differentiated) T cells into the meninges, brain parenchyma, and cerebrospinal fluid (Sedgwick et al. 1991a, 1991b).

Inhibition of virus entry into the brain and blockade of intracerebral viral spread

Specific antibodies are the main tool of the infected host in preventing extracellular viral spread from primary

sites of infection to other organs during viremia (Sissons and Oldstone 1985). Virtually all viral CNS infections are preceded by primary peripheral infection, and so it is obvious that virus neutralization and opsonization during viremia comprise one of the most efficient defense reactions preventing viral entry into the CNS. Agammaglobulinemic patients suffer more frequently from persistent CNS infections than do fully immunocompetent hosts (Smith et al. 1992), and peripheral virus-specific antibodies usually prevent neuroinvasion in perinatal viral hosts that do not display full immune competence. For example, lethal encephalomyelitis experimentally induced by the murine *Coronavirus* JHM (JHMV) in newborn or suckling rats is prevented by nursing the babies from JHMV-immunized mothers (Wege et al. 1983), and teratogenic effects induced by lymphocytic choriomeningitis virus (LCMV) in neonates are not observed when they are nursed by immunized mothers or injected with LCMV-specific neutralizing monoclonal antibodies (Baldridge and Buchmeier 1992; Baldridge et al. 1993). Moreover, experimental virus infections of the CNS in immunocompetent hosts usually remain subclinical, provided high titers of virus-neutralizing antibodies are mounted (Jubelt et al. 1991; Rima et al. 1991).

Nevertheless, in some instances viruses escape extracerebral neutralization and invade the CNS. If retrograde transport occurs along the axons of peripheral nerves, the virus is inaccessible for an immune system. Neither virus-specific antibodies nor cytotoxic T lymphocytes (CTL) can prevent the axonal transport of the virus to the CNS, particularly because nerve cells are thought to be unable to upregulate MHC class I molecules (Momburg et al. 1986). Using leukocytes as 'Trojan horses' is another way to escape neutralization by antibodies, although this hypothesis has so far not been supported by direct experimental evidence. Viral infection of peripheral recirculating monocytes may enhance invasion of the perivascular space in the CNS, because, in contrast to ramified microglia, the perivascular type of this cell is frequently exchanged by peripheral monocytes (Sedgwick et al. 1991a, 1991b, 1993).

Once in its target cell, the virus continues replicating and spreading in the CNS until immune effector cells are recruited into the brain parenchyma. Plasma cells of the B-lymphocyte lineage home to virus-infected sites in the tissue (Dörries et al. 1991), where they secrete virus-specific antibodies (Schwender et al. 1991), and cytotoxic T lymphocytes provide an early virus-specific defense mechanism in the brain parenchyma. Although the latter may efficiently lyse virus-infected cells, the formation of secondary virus-infected foci in the tissue by extracellular spread of the virus is prevented only when virus-specific antibodies are secreted intracerebrally in close proximity to infected sites. This has been confirmed in several animal models of virus-induced encephalitis. In addition to extracellular spread, virus-specific antibodies may inhibit dissemination from cell to cell, for example by interfering with cell fusion (Dietzschold et al. 1992). Moreover, antiviral antibodies may be directly involved in attenuating intracellular viral replication in vivo and in vitro. This concept, known as antibody-induced antigenic modulation (Fujinami and Oldstone 1980), has been established for MV infections in brain cells and mice with severe combined immunodeficiency (SCID) infected with Sindbis virus (Levine et al. 1991).

Although it is generally assumed that the protective effect of a virus-neutralizing antibody in the CNS is mainly due to binding of its Fab part to viral epitopes necessary for infection of the target cell, the Fc part of the antibody molecule may also contribute. A monoclonal antibody specific for the yellow fever virus (YFV) nonstructural protein NS1 protects mice from encephalitis only when given as an intact molecule, but not as a F(ab)2 fragment, which normally neutralizes YFV efficiently in vitro. Moreover, only monoclonal antibodies of the IgG_{2a} subclass efficiently prevented acute encephalitis in this virus system, indicating that only binding of a distinct IgG subclass to NS1 allows recognition by FcR expressing cells. Similar observations have been made for Semliki Forest virus (SFV) infection of mice, in which IgG_{2a} is the dominant subclass in the CSF, as well as for LCMV-induced encephalitis, which can be prevented with an IgG_{2a} monoclonal antibody (Baldridge and Buchmeier 1992). In rabies virus-infected mice, however, protection from lethal disease is achieved by both IgG_1 and IgG_{2a} subclasses (Dietzschold et al. 1992).

Successful action of the humoral immune response requires very rapid migration of virus-specific plasma cells into the brain parenchyma. This is usually achieved if viral CNS infection occurs concomitantly with the acute peripheral infection and pre-existing plasma cells can immediately enter the brain tissue. When viral CNS infection occurs late after primary infection, the immune response has to be initiated in local secondary lymphatics, such as the cervical lymph nodes. The period until humoral effector systems reach the brain parenchyma may be determined by the genetic background of the host, as documented for *Coronavirus* JHM-induced encephalomyelitis in two genetically different rat strains (Schwender et al. 1991).

The effect of a late or a rather unspecific recruitment of humoral immunity to the CNS facilitates viral spread within the CNS. Consequently, large infected areas will result in a vigorous infiltration of virus-specific T cells, leading to enhancement of neurological disease. This intimate relation between virus-specific humoral response and T cell-mediated immunopathology was shown in acute LCMV-induced encephalitis of mice. In this model, mice injected with neutralizing antibodies before and shortly after viral infection were protected from lethal encephalitis, and CTL responses were diminished compared with unprotected mice (Wright and Buchmeier 1991).

Enhancement of viral pathogenesis by humoral effector systems

Humoral immunity is not generally thought to contribute to the pathology of viral CNS infection. Nevertheless, there are theoretical considerations and experimental data obtained in vitro that do not strictly rule out disease-enhancing properties of humoral immune responses. As in the periphery, intrathecal antibody synthesis could help to augment viral pathogenesis by antibody-dependent enhancement (ADE): i.e. antibody-complexed viral particles can be taken up by cells either by binding to Fc receptors (FcR) or, in the case of activation of the complement cascade, by attachment via the C3 receptor. In both cases, cells of the monocyte/macrophage lineage, such as microglial cells, are prime targets for ADE in the CNS (Aloisi 2001; Homsy et al. 1989) In addition, the engagement of FcR triggers activation of macrophages or microglia, which may lead to unspecific tissue destruction. Binding of immune complexes to the FcR can stimulate the release of toxic substances, causing the destruction of healthy cells surrounding the infected areas. This assumption is supported by the observation in vitro of macrophage-dependent oligodendroglia cell degeneration in mixed glial cell cultures when they are treated with immune complexes formed by canine distemper virus (CDV) and CDV-specific antibodies (Botteron et al. 1992).

The presence of virus-specific antibodies in the CNS opens one pathway allowing a complement-dependent immunological effector mechanism to operate: the antibody-activated cytolytic action of complement (Speth et al. 2001). Activation of the complement cascade in the CSF has been demonstrated in patients suffering from HIV-1 infection (Reboul et al. 1989). Opsonized virions will bind to cellular complement receptors, an interaction which can amplify the infection of cells with HIV-1 rather than induce lysis of the virus (Stoiber et al. 2001). There is, however, no evidence for antibody-mediated tissue destruction in the CNS of AIDS patients (Lenhardt and Wiley 1989). Although not proven, there is some reason to suggest that complement-mediated lysis could contribute to the pathogenesis of SSPE, caused by a persistent measles virus infection of the human CNS. One of the cellular receptors for measles virus, CD46, was found to be absent in CNS lesions of SSPE patients, whereas it is present on a fraction of cells in uninfected regions of the brain (McQuaid and Cosby 2001; Ogata et al. 1997). Because the normal function of CD46 is to protect cells from autologous lysis by activated complement (Liszweski et al. 1991), the sera and CSF of SSPE patients reveal high complement activity (Oldstone et al. 1975), and CD46 down-regulation enhances the complement-mediated lysis of cells in tissue culture (Schnorr et al. 1995), it is tempting to speculate that this mechanism may contribute to the pathology of SSPE.

Viral persistence and virus-specific antibodies

Incomplete elimination of virus from the CNS may result in persistent infection that is usually accompanied by a long-lasting intrathecal antibody synthesis with specificity for viral proteins (Rammohan et al. 1983; Tyor et al. 1992). Over time there is selection of high-avidity antiviral antibodies, and the respective clones are preferentially recruited to the CNS. In this case, isoelectric focusing of CSF specimens will show a restricted 'oligoclonal pattern' of antibody clones, compared to the polyclonal distribution detectable in paired serum specimens. The presence of these oligoclonal bands can therefore be used as a diagnostic marker in viral infections of the CNS (Felgenhauer and Reiber 1992).

In addition to intrathecal virus-specific antibody synthesis being a reliable indicator of viral infection of the CNS, the long-lasting presence of antiviral antibodies at high titers may contribute to the selection of virus variants. In fact, changes in neural cell tropism have been shown for neurotropic JHMV grown in the presence of virus-neutralizing monoclonal antibodies (Buchmeier et al. 1984).

THE CELL-MEDIATED IMMUNE RESPONSE

T cell-mediated immune protection is usually mediated by cell destruction. Because a timely T-cell immune response encounters a small number of infected cells, the disease should be limited in most acute viral encephalitides, and the benefits will outweigh the harmful effects of the cell-mediated immune response. Paradoxically, the host's immune response to viral infection, which is usually protective outside the CNS, can be destructive when operating within this isolated compartment with such a low regenerative capacity. Cell lysis and toxic cytokines may themselves cause pathological changes while helping to clear virus, particularly during a long-lasting persistent infection.

The role of $CD4^+$ and $CD8^+$ T cells

Although both $CD4^+$ and $CD8^+$ T cells can be relatively easily isolated and grown in culture from the CSF of patients with viral encephalitis and meningoencephalitis, their relative contributions to antiviral defense are uncertain. As is apparent in some animal models, the presence and function of $CD4^+$ cells are more important than $CD8^+$ T cells in viral CNS infections. This is in contrast to the situation in peripheral tissues in which, for example, during acute and chronic viral hepatitis cytotoxic MHC class I-restricted $CD8^+$ T cells attack virus-infected hepatocytes and thus mediate protection as well as cell destruction (Almond et al. 1991).

Although there is still some controversy, results obtained in the Theiler's virus model in mice indicate that the ability to generate $CD8^+$ T cells is not essential for viral clearance. In both C57BL/10 mice, which develop an acute encephalitis followed by subsequent clearance of

virus from the CNS, and SJL mice, which experience a persistent viral infection with demyelination lesions, there are $CD8^+$ CTLs in the CNS (Lindsley et al. 1991). Furthermore, β_2-microglobulin-deficient transgenic mice that lack MHC class I and, therefore, functional $CD8^+$ cells, develop persistent encephalitis with extensive demyelination. However, neither antibody titers nor viral persistence was significantly affected (Pullen et al. 1993).

Consistent with these findings, $CD4^+$ cells proved indispensable in achieving viral clearance from the CNS of rats after transfer into animals experimentally infected with MV, whereas $CD8^+$ cells were not vital for recovery from the acute infection. It must be remembered, however, that in different species different virus-specific immune effector cells are generated in the control of viral infections in the periphery; even within a single species there is no unique T-cell subset used for antiviral defense. In many murine virus infections, including HSV, influenza A virus, LCMV, and mouse poxvirus, $CD8^+$ CTLs are important in combating infection (Moskophidis et al. 1987; Nash et al. 1987; Ahmed et al. 1988; Askonas et al. 1988). However, the clearance of murine hepatitis virus from the brain by $CD8^+$ T cells depends on $CD4^+$ help (Williamson and Stohlman 1990), and in the protective immunity to retroviruses both $CD8^+$ and $CD4^+$ T cells were only partially effective, whereas the combination of both led to full protection (Hom et al. 1991). In contrast, recovery from acute murine CMV infection can proceed in the absence of the $CD8^+$ subset, and it is mediated by $CD4^+$ T cells that develop a compensatory protective activity absent from normal mice (Jonjic et al. 1990). $CD4^+$ T cells seem to be required for the maintenance of spontaneous recovery from friend murine leukemia virus-induced leukemia (Robertson et al. 1992). These examples illustrate that there is no general assignment of a determinative role in vivo to either T-lymphocyte subset in the recovery from viral infections. It seems, however, that in the CNS antiviral cell-mediated activity is largely dependent on $CD4^+$ T cells.

Mechanisms of T cell-mediated antiviral activity in the brain

The mechanism of the antiviral activity of $CD4^+$ T cells in vivo has not been completely elucidated. A detailed characterization in vitro revealed that all protective T-cell lines produce large amounts of IL-2, IFN-γ, and TNF-α, but not IL-4 or IL-6, defining them as Th1 cells (Liebert and Finke 1995). If cytokines secreted by Th1 cells are important, two requirements should be met: virus-primed T cells have to invade the CNS and home in on sites of infection; and blocking cytokine function should abolish protection. Adoptive transfer experiments in MV-infected rats as well as in mice, using a genetic marker, revealed that MV-specific $CD4^+$ T cells from a donor animal enter the brain of the host. These cells accumulated in infected areas, where they represented $\leqslant$5 percent of all infiltrating T cells in immunocompetent animals. Furthermore, the neutralization of IFN-γ by the administration of antibodies rendered all mice susceptible to MV-induced acute encephalitis, suggesting that cytokines may indeed play an important role in the immune surveillance of the CNS. The mechanism of cytokine activity may be to assist in recruiting effector cells into the CNS (see also under Virus cell interaction in the CNS). The main source of IFN-γ and TNF-α in MV infection of the murine CNS seems to be $CD4^+$ T cells.

In the Theiler's murine encephalomyelitis virus (TMEV) encephalitis model in mice, susceptibility to infection is associated with MHC and maps to the class I locus H-2D, which is highly upregulated in the CNS, in contrast to the H-2K locus (Nash et al. 1987). In susceptible strains $CD8^+$ T cells apparently fail to recognize viral antigens in the context of MHC class I, so the virus persists and eventually causes disease. After depletion of $CD8^+$ T cells in vivo, virus clearance is delayed and demyelinating disease develops, indicating that $CD8^+$ T cells are not involved in demyelination and are not vital for recovery from acute infection. In contrast, depletion of $CD8^+$ cells after the acute phase does reduce disease. Observations made in β_2-microglobulin-deficient transgenic mice, however, suggest that $CD8^+$ T cells may play a role in clearing viral persistence from glial cells (Pullen et al. 1993). Depletion studies of $CD4^+$ cells in the TMEV model suggest that the major role of $CD4^+$ T cells in picornavirus infections is probably to control the early stages of infection by providing B-cell help and thus enable the production of neutralizing antibody (Virelizier 1989). TMEV-specific proliferation of $CD4^+$ MHC class II-restricted T cells was found in both resistant and susceptible strains (Clatch et al. 1987).

There is a strong correlation in a number of susceptible strains between demyelination and a $CD4^+$ T cell-mediated DTH response, and one DTH T-cell epitope in susceptible mice has been mapped to the VP2 protein (Geretry et al. 1994). The incidence of demyelinating disease was reduced following suppression of MHC II restricted $CD4^+$ T-cell function after viremia. After TMEV infection and initial T-cell infiltration into the CNS, MHC class II induction on astrocytes is a key step in allowing local antigen presentation and amplification of immunopathological responses within the CNS, and hence the development of demyelinating disease (Borrow and Nash 1992). A bystander effect caused by the induction of mononuclear cell infiltration, and activation of macrophages, which in turn can lead to damage on myelin sheaths, is probably responsible for the observed immunopathology.

Virus-induced cell-mediated autoimmune reactions against brain antigens

Another possibility involves T cells reactive against brain cell antigens, which could be induced during the

course of infection and may exacerbate current pathology or initiate new lesions. There are several possibilities of how a virus could induce strong cell-mediated immunity (CMI) responses to brain antigen:

- When enveloped viruses multiply in living cells, they may incorporate host antigens into their envelope. In this context these antigens might then be recognized by the host and thus elicit an immune reaction.
- Viruses with a tropism for lymphocytes and macrophages might be involved in the destruction of lymphocyte subpopulations or the generation/expansion of autoreactive lymphocyte clones.
- Immune responses raised against certain viral antigens may cross-react with normal host cell antigens and lead to autoimmunity by molecular mimicry (Oldstone 1989; Fujinami and Oldstone 1985). Synthetic peptides derived from common viruses (based on motifs required for MHC class II binding and T-cell receptor recognition) can activate human T-cell lines specific for MBP (Wucherpfennig and Strominger 1995).

The frequency of induction of T cells reactive against brain antigens, and their role in the pathology of human viral CNS infections, are unclear. One study suggests that a significant proliferative response of isolated peripheral lymphocytes occurs against myelin basic protein (MBP) in patients with acute measles encephalomyelitis (Johnston et al. 1984). Moreover, MBP was detected in the CSF of such patients as a consequence of myelin breakdown. MBP-specific lymphoproliferative responses have also been seen after postinfectious encephalomyelitis following rubella virus, varicella-zoster virus, and respiratory infection, and in patients with postexposure rabies immunization (Johnston and Griffin 1986). The latter disorder is probably the human equivalent of EAE, because these patients received rabies vaccine prepared in brain tissue. Continuing the analogy to EAE, it is not surprising that an MBP-specific lymphoproliferative response in these virus infections is considered to be of pathogenic importance. In experimental CNS infections autoimmune T cells do not seem to be involved in the induction of demyelination in Theiler's virus in mice, as tolerance induced to myelin antigens blocked the induction of EAE but did not affect the development of demyelinating disease (Kennedy et al. 1990). However, in rats infected with JHMV or MV, MBP-reactive $CD4^+$ T cells were detected that could transfer EAE to naive uninfected animals (Watanabe et al. 1983; Liebert et al. 1988). These observations support the hypothesis that viruses could be involved in the initiation of autoimmune processes.

Consequences of antiviral mechanisms in the CNS

In summary, immune responses generated in the periphery encounter great difficulties when combating CNS virus infections. If neurons are infected, elimination of the cells may not be of any advantage to the host as the immune response, although beneficial in the periphery, would inflict enormous damage when attacking cells that lack the capacity to regenerate. Hence, rapid elimination of infected cells is necessary to prevent virus amplification. In contrast, the development of a delayed immune response may allow the virus to spread in the CNS and, even if ultimately effective against the virus, may be destructive to the host. Thus, precautions are built into the system to prevent potentially damaging and disease-inducing immune responses during persistent infections in which the virus, at least temporarily, does not destroy its host cell.

The fine regulation of intracerebral immune surveillance has yet to be elucidated. However, it is clear that a delicate balance is normally maintained between the morphological and functional integrity of the CNS and the ability of the immune system to combat virus and eliminate infected cells. At present the evidence indicates that:

- The immune response to viral infections of the CNS is initiated in peripheral lymphoid tissues, followed by entry of activated end-differentiated T and B cells into the CSF, meninges, and brain parenchyma.
- During viral infections different sets of cytokines and chemokines are induced.
- Together with interferon-induced proteins, these factors contribute to the establishment of persistent infections, which may depend on downregulation of replication of certain viruses by lack of certain factors, or the restriction of viral gene expression, or both.
- During viral infections, MHC class I or II antigens are expressed extensively on microglial cells which present viral antigen produced by infected cells, and also on astrocytes oligodendrocytes, and damaged neurons.
- Full development of the inflammatory response requires virus-specific T cells; natural killer (NK) cells, γ/δ T cells, mononuclear phagocytes, B cells, and plasma cells participate in a bystander response.
- In many viral systems T cells are required for viral elimination, but clearance of virus may also depend on the timely presence of virus-specific antibodies.
- Immunopathology and autoimmunity may result from inopportune or inefficient T-cell responses generated after the viral agent has succeeded in establishing a persistent infection in brain cells as a result of immune-mediated damage during attempted viral clearance.

VIRUSES AND ASSOCIATED CNS DISORDERS IN HUMANS

As mentioned previously, except for rabies virus, human pathogenic viruses do not per se reveal a tropism for the

CNS, but rather cause CNS diseases in the course of an infection in the periphery. A summary of the relative frequencies of the ability of human pathogenic viruses to cause CNS diseases is given in Table 2. For example, CNS involvement is quite frequent with mumps virus infections, whereas the proportion of patients with measles developing CNS complications is generally small. The same applies to EBV infections, which are ubiquitous and normally clinically inapparent, CNS complications being observed only rarely. The following sections thus focus on viral infections of the CNS that are of major clinical interest and which have been investigated in more detail.

Polyomaviruses

Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the CNS resulting from infection of oligodendrocytes with JC virus (JCV), a *Polyomavirus* (Richards 1988, Dörries 2000) (see Polyomaviruses). Until the AIDS epidemic, experience with this disease was limited. Once regarded by most neurologists as a clinical curiosity, PML has now been recognized as an AIDS-associated disorder and the HIV-encoded transactivator Tat has been found to be an activator of the JCL promoter (Remenick et al. 1991). Current estimates suggest that 1–4 percent of all HIV-infected people will develop PML (Berger et al. 1987; Berger and Major 1999). In AIDS patients, as in those with other underlying diseases, PML usually progresses inexorably to death within a mean of 4 months. More than 80 percent succumb within 1 year of diagnosis. However, on rare occasions individuals with PML experience both clinical and radiographic recovery, including full neurological recovery, in the absence of specific therapeutic intervention.

The spread of JCV is thought to occur via the respiratory route (Shah 1990). To date, no disease has been convincingly associated with the acute infection. Usually, asymptomatic persistent infections with JCV (and the closely related BK virus) are established; consequently, 80–90 percent of the healthy adult population have IgG antibodies against JCV (Padgett and Walker 1983). Kidney, CNS, and in some cases peripheral blood mononuclear cells, have been identified as sites of persistent infection (Houff et al. 1988; Arthur et al. 1989; Elsner and Dörries 1992; Dörries et al. 1994). It is not yet entirely clear how these persistent infections are established and maintained. Although heterogeneity within the JCV control regions in brain isolates could be defined, these changes could be linked neither to the persistent phenotype of the infection nor to the efficiency and frequency of reactivation (Elsner and Dörries 1992). On the other hand, it has been suggested that the cell type specificity of JCV in vitro and in vivo is controlled by host and viral transcription factors that actively regulate JCV gene expression by interacting with the viral control region (Feigenbaum et al. 1987; Chowdhury et al. 1990; Lynch and Frisque 1991; Ranganathan and Khalili 1993). Reactivation of viral replication leading to PML is almost exclusively confined to immunosuppressed individuals (Walker and Frisque 1986), strongly suggesting that the host's immune system plays a major role in controlling viral replication in persistence. However, there is no evidence as to how the immune response controls virus persistence and prevents reactivation. Because only a minority of patients with underlying cellular immunodeficiency ultimately develop PML, it is possible that the presence of JCV and immunosuppression alone is not sufficient for its development.

The AIDS epidemic has dramatically altered the epidemiology of PML (Holman et al. 1991). Instead of affecting chiefly elderly people, PML has become a disease of the young and middle-aged affected with AIDS. It is interesting that this disease rarely occurs in immunosuppressed children with HIV infection (Berger et al. 1992), perhaps as a result of the smaller percentage of children infected with JCV. The high prevalence of antibodies in the adult population and the rarity of PML in children supports the contention that PML results from reactivation of JCV in individuals who have become immunosuppressed. Until the early 1980s (before the AIDS epidemic), the vast majority of patients with PML had lymphoproliferative disorders leading to immunosuppression, such as Hodgkin's disease, chronic lymphocytic leukemia and lymphosarcoma, tuberculosis, lupus erythematosus, or sarcoidosis (Brooks and Walker 1984). In contrast, AIDS has been estimated to be the underlying disease behind PML in 55–85 percent of all current cases in which PML was reported within 1 year

Table 2 *Viruses causing acute meningoencephalitis and acute encephalitis in humans*

Frequent	Infrequent	Rare
Herpes simplex virus 1 and 2	Varicella-zoster virus	Adenovirus
Mumps	Measles	Epstein–Barr virus
Enteroviruses	Rubella	Reovirus
Arboviruses	Influenza virus A and B	Parainfluenza virus 1–3
Rabies	Cytomegalovirus	Respiratory syncytial virus
		Human parvovirus
		Lymphocytic choriomeningitis virus

after the initial diagnosis of AIDS (Gullota et al. 1992; Kaye et al. 1992; Major et al. 1992).

The clinical hallmark of PML is the presence of focal neurological changes associated with radiographic evidence of white matter lesions, depending on the location of the CNS infection. Cardinal features of PML, apparent on clinical examination, include an insidiously progressive psychomotor slowing, impaired memory, and apathy. The most common presentations include weakness, visual deficits, and cognitive abnormalities, which occur in approximately one-third of patients (Brooks and Walker 1984). Weakness is typically a hemiparesis, but monoparesis, hemiplegia, and quadriparesis may be observed as the disease progresses. Other motor disturbances include limb and trunk ataxia, resulting most often from cerebellar involvement. Nearly one-third of patients have cerebellar signs at the time of diagnosis (von Einsiedel et al. 1993). Extrapyramidal disease is rare at onset, but bradykinesia and rigidity may be detected in a substantial minority of patients with advanced disease. Dystonia and severe dysarthria have also been observed as a consequence of lesions in the basal ganglia. Neuro-ophthalmic symptoms occur in 50 percent of patients and are the presenting manifestation in 30–45 percent. The most common visual deficits are homonymous hemianopia or quadrantanopia due to lesions of the optic tracts. The spectrum of cognitive changes observed is quite broad. Unlike the slowly evolving global dementia of the HIV-associated dementia complex, the mental impairments of PML often advance more rapidly and typically occur in conjunction with focal neurological deficits.

Neuropathologically, the cardinal feature of PML is demyelination, i.e. the loss of oligodendroglial cells and myelin sheaths (Figure 2). Demyelination is typically multifocal, but on rare occasions may also be unifocal. Although these lesions may develop in any location in the white matter, they have a predilection for the parieto-occipital regions. Not infrequently, lesions involve the cerebellum, brainstem and, less commonly, the spinal cord (Bauer et al. 1969; Kuchelmeister et al. 1993; von Einsiedel et al. 1993).

Other histopathological hallmarks of PML include hyperchromatic, enlarged oligodendroglial nuclei, and enlarged bizarre astrocytes with lobulated hyperchromatic nuclei (Figure 2). The abnormal astrocytes may undergo mitosis and have quite a malignant appearance. In situ hybridization for JCV DNA allows the detection of virions in the nuclei of infected oligodendroglial cells. Virions are detected less frequently in reactive astrocytes, and only rarely in macrophages engaged in removing the affected oligodendrocytes (Mazlo and

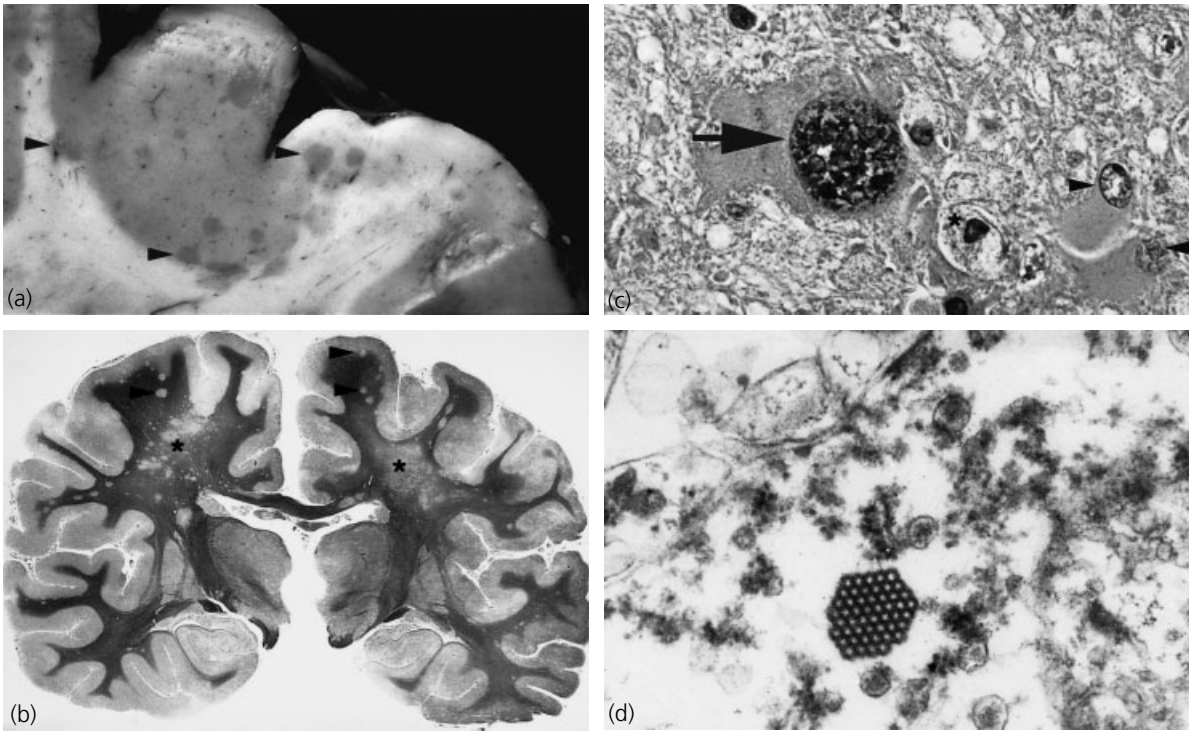

Figure 2 (a) *Close-up of the cortex of a patient who died from PML. Several small foci of demyelination are situated mainly at the border between the gray and the white matter.* **(b)** *Whole mount of the cerebrum of the same patient, stained with a myelinotropic dye. Again, there are several round areas of demyelination, as well as larger polycyclical (several circular confluent) regions of confluent foci in the central white matter.* **(c)** *Swollen, grotesquely deformed oligodendrocyte with a very large nucleus (×400). For comparison, see the two reactive astrocytes at the right of the picture (arrowed).* **(d)** *Electron micrograph showing papovaviruses within the affected oligodendrocytes. (Courtesy of Dr Adriano Aguzzi.)*

Tariska 1992). The subcellular distribution of virions, particularly in cells usually not involved in papovavirus infection (the nucleus and cytoplasm of neurons), has been reported in two cases (Boldorini et al. 1993). Although neuropathological findings do not reveal fundamental differences between cases of AIDS and non-AIDS PML, the former more frequently present with extensive lesions of a particularly destructive, necrotizing character.

The application of quantitative PCR to CSF samples enables premortem diagnosis with less invasive procedures than brain biopsy (Dörries 2000). Confirmation of PML requires the demonstration of typical histopathological changes and the detection of JCV in brain samples obtained from biopsies or at autopsy using conventional techniques, including electron microscopy, virus isolation, immunocytochemistry, and in situ hybridization. Recently, it was found that the newly approved cidofovir diphosphate, a structural analog of deoxycytidine trisphosphate, is not only a potent inhibitor of human herpes and papillomaviruses, but also a selective antipolyomavirus agent.

Alphaherpesviruses

Of the numerous herpesviruses so far characterized, eight have been isolated from humans. Among these, three members of the subfamily *Alphaherpesvirinae*, herpes simplex virus-1 and -2, and varicella-zoster virus (VZV), have been identified as etiological agents in the pathogenesis of human disease processes in both the peripheral and the central nervous system. HSV is responsible for the viral encephalitis with the highest fatality rate. Alphaherpesviruses have a variable host range and short replication cycles and spread rapidly in infected cell cultures, resulting in complete cell destruction. Paradoxically, they can establish latent, lifelong infections in their natural hosts, including those with natural or vaccine-induced immunity. These latent infections occur primarily, but not exclusively, in neural tissue of sensory and autonomic nerve ganglia and the CNS.

Human herpesviruses -6, -7, and -8 have been isolated from human peripheral blood mononuclear cells (PBMC) in recent years. Their association with human CNS diseases has not yet been fully defined, although a pathogenic role of HHV-6 has been suggested for multiple sclerosis (Challoner et al. 1995; Gutierrez and Vergara 2002).

HERPES SIMPLEX VIRUS

Following infection, HSV-1 and HSV-2 replicate at the mucocutaneous surface before entering nerve axons. Virus then moves centripetally via retrograde fast axonal transport to cell bodies of neurons in sensory and autonomic nerve ganglia, and in some cases to the CNS (Figure 3). Following retrograde transport, the virus replicates in the sensory ganglia that innervate the site of infection. In experimental work in animals there is evidence of lytic virus infection in neurons and accessory cells, and virus can be recovered from ganglionic homogenates. Once replication in the sensory ganglia is complete, antiviral drugs cannot eliminate the virus if it enters its latent state. In acutely infected ganglia two types of virus–neuron interaction can occur, resulting in either lytic or latent infection. Cellular factors involved in repressing immediate early gene expression may be paramount in this. After acute ganglionic infection subsides, virus persists in neurons, probably for the lifetime of the host. Infectious virus cannot be recovered from ganglionic homogenates during the latent phase of infection, but can be isolated from ganglionic explant cultures. Periodically, latent virus is reactivated either spontaneously or by a variety of stimuli. During this reactivation process, virus (or perhaps subviral particles) is transported centrifugally via the nerve cell axon to the original peripheral infection site, where replication can then recur.

The exact role of the cell in establishing latent HSV-1 infections is still unclear, because of the small number of latently infected cells present in vivo as well as the difficulty of establishing authentic in vitro latency systems. The dependence of latency on the presence of nerve growth factor (NGF) in primary cultures of sympathetic and sensory neurons has been described (Wilcox and Johnson 1988; Wilcox et al. 1990). Moreover, neurons in culture express factors that inhibit the expression of HSV-1 immediate–early genes (Ash 1986; Kemp et al. 1990; Wheatley et al. 1991; Lillycrop et al. 1993) and therefore arrest HSV-1 replication at an early stage, before irreversible cell damage.

Some molecular aspects of HSV latency and reactivation have been described (for review see Millhouse and Wigdahl 2000). The genome persists as a circularized or a linear concatenated structure that is not integrated into the cellular DNA in human and mouse tissue. The viral DNA is not extensively methylated, and estimates suggest that 20–30 copies of the viral genome may be present in individual cells. A set of RNAs, predominantly stable introns of 1.5 and 2 kb length, is produced in latently infected neurons, where they are the only viral transcripts detectable (latency-associated transcripts (LAT)) (Figure 4). They extend from a region in IR_L (large internal repeat region) without an assigned coding role into an oppositely orientated coding region. Mutations in the region encoding LATs have been reported as affecting entry into or reactivation from the latent state. It seems that LAT elements have been conserved in evolution, thus strongly suggesting that there are important functions that may include the silencing of genes such as *ICP0*, an activator of the viral replication, or the expression of proteins contributing to reactivation. Recently, an anti-apoptotic function of the

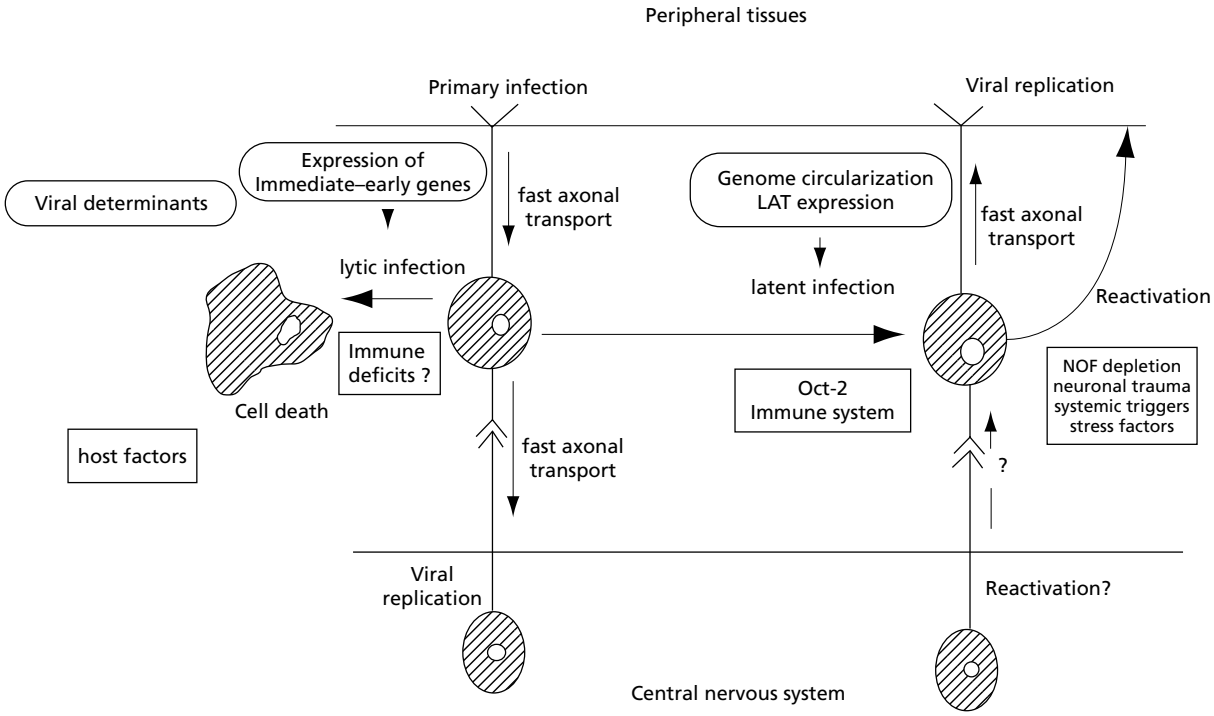

Figure 3 *Schematic representation of mechanisms of HSV-1 replication in the nervous system. After primary infection at peripheral tissues, the virion travels via fast axonal transport to peripheral sensory ganglia (PSG). Under conditions favoring viral replication (e.g. immune disruption), expression of HSV-1 immediate–early genes, followed by a complete viral replication cycle, will ensue, leading to neuronal cell death. Again by fast axonal transport, the virus may seed the CNS, causing an acute lytic infection (primary HSV-1 encephalitis). By contrast, in the presence of an effective immune system, viral replication will be prevented in PSG and probably CNS neurons (due at least in part to host cell factors such as neuronal-specific Oct-2 transcription factor). This may lead to either an abortive (not reactivatable) or a latent infection that is characterized by the circularization of the viral genomic DNA and a marked restriction of viral gene expression where no immediate–early genes, but only latency-associated transcripts (LATs), are expressed. Under specific systemic (e.g. menstruation) and local triggering conditions, the latent genome can reactivate in PSG, travel to the periphery, and replicate there. The ability of reactivated HSV-1 to invade the CNS is uncertain, as is reactivation from latent infections established in the CNS. NGF, neuronal growth factor.*

LATs has been described which promotes neuronal survival (Perng et al. 2001).

Primary CNS infection with HSV

Infection with HSV-1, generally limited to the oropharynx, can be transmitted by respiratory droplets or through direct contact of a susceptible individual with infected secretions. Thus, initial replication of virus occurs in the oropharyngeal mucosa. The trigeminal ganglia become colonized and harbor latent virus. HSV-2 infection is usually the consequence of transmission via a genital route. In these circumstances, virus replicates in the vaginal tract or on penile skin and eventually seeds the sacral ganglia. After recovery from the primary infection only a small percentage of individuals experience recurrent infections. HSV encephalitis is mainly associated with HSV-1 infection, whereas ascending myelitis or meningitis is linked mainly to HSV-2 (Shyu et al. 1993). Of these clinical entities, encephalitis is the most common and most severe HSV infection of the CNS. The clinical course is variable, depending on the location as well as the acuteness of the infection.

HSV infections of the newborn can be aquired in utero, intrapartum (75–80 percent of all cases), or postnatally. The clinical presentation of infection reflects the site and extent of viral replication. Neonatal HSV infection is almost invariably symptomatic and frequently lethal. Intrauterine infection is apparent at birth and is characterized by severe manifestations, such as microcephaly or hydrocephaly. Intrapartum or postnatally acquired HSV infections may lead to encephalitis or disseminated infection that involves multiple organs, including acute brain infections. In infants with disseminated infections the virus probably seeds the brain by a blood-borne route, resulting in multiple areas of cortical hemorrhagic necrosis. In contrast, infants who present only with encephalitis probably develop brain disease as a consequence of retrograde axonal transmission of virus to the CNS. Clinical manifestations of encephalitis with or without associated disseminated disease include seizures (both focal and generalized), lethargy, irritability, tremors, poor feeding, temperature instability, a

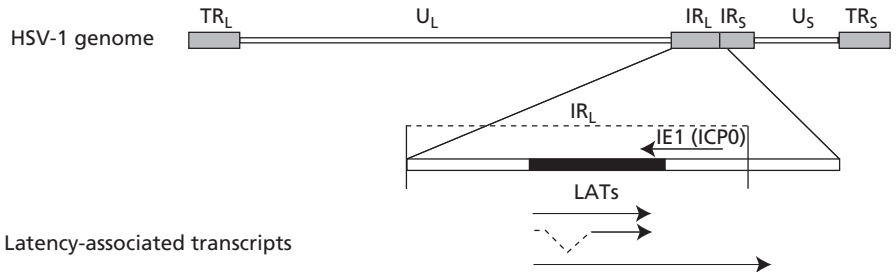

Figure 4 *The HSV-1 genome is a linear DNA molecule containing unique long (U_L) and short (U_S) regions bounded by the terminal and internal repeat regions (TR and IR, respectively). The region encoding the major species of latency-associated transcripts (LATs) that are expressed during viral latency is located within the IR_L region (as indicated by the black bar in the enlarged section). As also indicated, some LATs extend from the LAT locus into the IR_S region. The major LAT species expressed partly overlap with viral genes encoded in the opposite direction on the genome, as exemplified for the immediate–early 1 gene (*IE1 *or* ICP0*). LATs may interfere with the expression of these genes because they are antisense to their mRNAs, leading to the formation of dsRNA molecules.*

bulging fontanelle, and pyramidal tract signs. Cultures of CSF yield virus in 25–40 percent of cases. Likely findings on CSF examination include pleocytosis and proteinosis, which increase with progressive disease. Death occurs in 50 percent of untreated infants with localized CNS disease, and is usually related to brainstem involvement. With rare exceptions, survivors are left with neurological impairment.

Clinical manifestations of HSV encephalitis in older children and adults reflect the areas of pathology in the brain. These include primarily a focal encephalitis associated with fever, altered consciousness, bizarre behavior, disordered mentation, and localized neurological signs. These clinical findings are generally associated with neurodiagnostic evidence of localized temporal lobe disease. Histopathological findings include widespread areas of hemorrhagic necrosis, mirroring the area of infection, with oligodendrocytic involvement, gliosis and, frequently, astrocytosis late in the disease course (Figure 5). Primary HSV-2 infections can, independent of gender, be associated with fever, dysuria, localized inguinal adenopathy, and malaise. The severity of primary infection and its association with complications is statistically higher in women than in men, for unknown reasons. Systemic complaints are common in both sexes, approaching 70 percent of all cases. The most common complications include aseptic meningitis (approximately 20 percent). Following primary genital herpetic infection, sacral radiculomyelitis (which can lead to neuralgias) and meningoencephalitis can also occur. The relationship between HSV infections of the brain and chronic degenerative disease and psychiatric disorders requires further investigation.

Latent infection by HSV

Despite the wide range of anti-HSV immune responses to the initial infection, the nonreplicating latent virus can persist in the ganglia for the lifetime of the patient. Whether latency per se causes any neurological injury is unknown, but it is well recognized that latent HSV is the source of virus responsible for recurrent infections in the peripheral nervous system. It is assumed that latent virus is reactivated to a replication-competent form and transported via sensory nerves to cutaneous or ocular sites, where further replication results in recurrent HSV infections. The pathological changes induced by the replication of HSV are similar in both primary and recurrent infection.

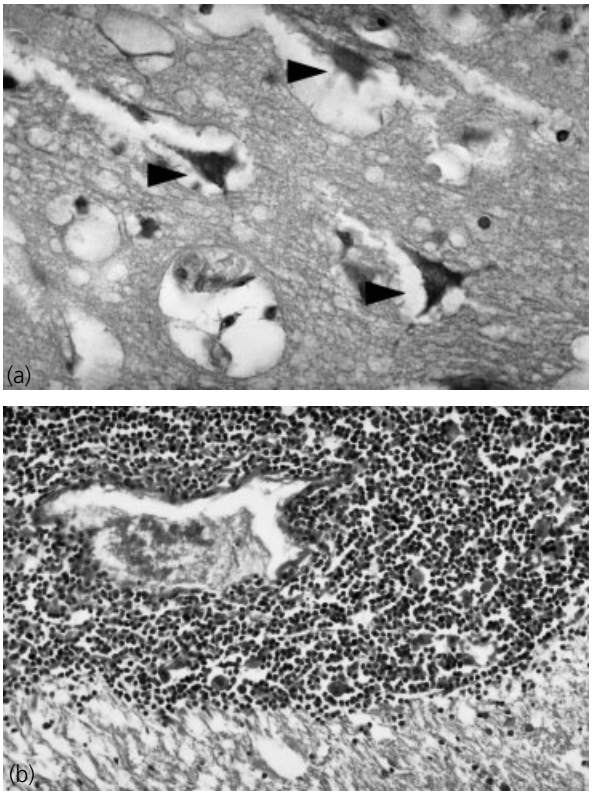

Figure 5 *Herpes simplex type 1 encephalitis.* **(a)** *Strongly positive intraneuronal staining with anti-HSV-1 immunostain (arrowed) (×400).* **(b)** *Massive accumulation of inflammatory cells (predominantly lymphocytes) in the neighborhood of small vessels ('cuffing') (×50). (Courtesy of Dr Adriano Aguzzi.)*

HSV encephalitis in immunocompetent individuals is about 1 million times less frequent than the peripheral disease. The source of virus behind HSV-1 encephalitis in immunocompetent individuals is unclear, as the ability of HSV-1 to reactivate in the CNS seems to be extremely limited. Despite the presence of HSV-1 nucleic acid in the human brain (Fraser et al. 1981; Steiner and Kennedy 1995), there is no convincing evidence of recurrent encephalitis induced by HSV-1, and attempts to reactivate it from explanted CNS tissue have generally failed. On rare occasions reactivated virus may be transported from the trigeminal ganglia to the cerebral cortex, resulting in HSV encephalitis. However, not all cases of HSV encephalitis are caused by the strain responsible for cold sores in an individual. Therefore, it is assumed that, in about half of patients, HSV encephalitis occurs as a primary infection (Whitley 1990). Moreover, even with a history of a prior HSV-1 infection, a second primary infection with another HSV-1 strain may be responsible for the encephalitis.

Diagnosis and treatment of HSV encephalitis

The severity of HSV-associated CNS complications warrants the application of rapid and specific diagnostic approaches in order to achieve effective treatment. Seroconversion or an increase in serum antibody titer are not diagnostic of CNS infection. Serological diagnosis requires proof of intrathecal synthesis of virus-specific antibodies, which is evident only when the quantity of specific antibodies in the CNS cannot be explained by transport of antibodies from the periphery across the BBB into the CNS. Classically, CNS HSV IgG antibody is distinguished from peripheral blood-derived HSV antibody appearing in the CSF by the calculation of an antibody index (CSF HSV IgG:serum HSV IgG/CSF albumin:serum albumin); HSV antibody index values >1.5 are considered indicative of intrathecal Ig synthesis. Several approaches are available to determine intrathecal antibody synthesis, including techniques such as enzyme linked immunosorbent assay (ELISA), isoelectric focusing (IEF) or Western blotting using protein antigens (virus purified, or expressed as recombinant proteins) (Klapper and Cleator 1992). For the detection of viral antigens or nucleic acids, brain biopsies stained with virus-specific antibodies or hybridized with virus-specific probes have been used successfully (with a sensitivity of 80–85 percent and a specificity of 100 percent). More recently, PCR-based analysis of CSF samples has been widely used as a less invasive, highly sensitive method more appropriate for clinical application for the diagnosis of HSV encephalitis, particularly in the acute phase of disease in the absence of virus-specific antibodies and in suspicious relapses (Calvario et al. 2002).

The currently accepted antiviral treatment is acyclovir, and new therapeutic drugs, i.e. penciclovir and oral prodrugs, valacyclovir, and Famciclovir (Naesens and De Clercq 2001), potent therapeutic antiviral drugs that specifically interfere with HSV polymerase function and are highly effective in reducing both mortality and morbidity. In fact, the early and systemic application of aciclovir has reduced the mortality of HSV encephalitis from 70 to 20 percent of affected patients.

The use of steroid treatment in HSV encephalitis is a matter of controversy. As brain edema is believed to represent the major cause of mortality associated with herpes encephalitis, reduction of intracranial pressure is another important consideration in the overall treatment.

VARICELLA-ZOSTER CNS COMPLICATIONS

As the name implies, varicella-zoster virus (VZV) causes two distinct clinical symptoms: chickenpox and shingles (for review see Wood 2000). Chickenpox (varicella) is a ubiquitous, highly contagious disease that spreads rapidly in a susceptible population, and is seen predominantly in childhood as the manifestation of primary infection with this virus. In contrast, shingles (herpes zoster) is the manifestation of a recurrent VZV infection. It usually occurs in the elderly or in immunocompromised individuals, and is characterized by a painful vesicular eruption, generally limited to a single dermatome. A live attenuated vaccine against VZV infection is available (Takahashi et al. 1974; Black et al. 1999).

As with HSV, a primary infection with VZV is thought to pass centripetally from skin and mucosal lesions to the corresponding sensory ganglia via contiguous sensory nerve endings and sensory nerve fibers. At the same time, some seeding of ganglia might also occur through direct spread of the virus owing to a varicella-associated viremia. Once in the ganglion, the virus sets up a latent infection in the neuronal cells without replicating or damaging them.

Immune responses to VZV may play a role in maintaining the latent state, and certainly seem to be responsible for preventing clinically apparent reactivation. Reactivation of VZV occurs only rarely, usually once in an individual's lifetime. The main factors that trigger reactivation include generalized immunosuppression, such as HIV infection, or certain cancers (e.g. Hodgkin's disease), suggesting that alteration in the host immunity to VZV may allow the development of zoster. Accumulating evidence points to CMI as the principal factor in the appearance of zoster. Zoster is seen most frequently in cases where CMI is lost (e.g. in bone marrow transplantation), and is accompanied by acute inflammation of the corresponding sensory nerve and ganglion. Acute lymphocytic inflammation, focal hemorrhage and neuronal destruction have also been described. In addition, the sensory nerve shows degeneration and demyelination, both peripherally and centrally, and the presence of viral antigen in neuronal and satellite cells and within the corresponding sensory nerves has been described.

VZV latency and reactivation are quite different from HSV latency and reactivation (reviewed by Hay and Ruyechan 1994). In contrast to HSV, VZV seems able to establish ganglionic latency in nonneural cells (satellite cells that surround the neurons) and to express several lytic cycle viral genes while latent; these genes probably represent very early events in viral multiplication. LAT-like molecules have not been described in these infections, and VZV has no genomic region directly corresponding to LAT. The proposed block at the molecular level, however, may be quite weak, allowing small amounts of virus to be produced on a regular basis.

CNS involvement in varicella is seen in about one case per 1 000. It occurs most often in children aged 5–14 years. The pathogenesis of the neurological complications of varicella is not well understood. Direct invasion of the CNS is seen in some cases, particularly with disseminated visceral infection, whereas other CNS manifestations seem to be immune mediated in origin. Involvement of the ganglia and nerves precedes the development of cutaneous lesions. Similar changes are often observed in adjacent ganglia. The inflammatory and degenerative changes can be traced peripherally to the cutaneous lesions and centrally into the adjacent segments of the spinal cord or brainstem. The myelitis is generally unilateral and predominantly involves the posterior horns. Degeneration with neuronal necrosis and neuronophagia may also involve the posterior columns and the gray matter. A mild lymphocytic meningitis is frequently present and is most intense in the involved area. Anterior nerve root degeneration can be associated with a motor neuron radiculitis. VZV can be isolated from CSF and nerve tissue, which suggests that these changes represent direct viral invasion. CNS involvement is responsible for 20 percent of admissions to hospital. The most common presentation is acute cerebellitis, which appears towards the end of the first or the beginning of the second week after the onset of the rash and is usually transient. A more serious form of encephalitis occurring earlier during the course of varicella is a progressive encephalopathy with loss of conciousness and convulsions. If the brain swelling cannot be reduced the patient may assume a decorticate position. Less common neurological presentations seen in varicella include meningoencephalitis, transverse myelitis, peripheral neuritis, and Reye's syndrome.

In contrast to neuralgia, the most common and important clinical manifestation of herpes zoster encephalitis is a rare complication. Typically, the rash involves the cranial or upper cervical nerves, and little is known about its pathogenesis. Lesions of disseminated herpes zoster are identical to those of fatal varicella. In zoster meningoencephalitis there are lesions ranging from mononuclear infiltration of the meninges to necrotizing encephalitis with axonal degeneration, macrophage infiltration, and characteristic intranuclear inclusions and viral particles in the glial cells.

Enteroviruses

The genus *Enterovirus* belongs to the family *Picornaviridae* (see Picornaviruses). It contains a number of viruses that give rise to infections of the CNS, notably polioviruses, enteroviruses, coxsackie, and echoviruses. Epidemics of poliovirus, for instance, were once a worldwide scourge, but with widespread vaccination and the eradication program since 1988 the number of annual cases could be reduced from approximately 350 000 to 497 in 2001, mostly in Africa and Asia. Of the 853 confirmed polio cases in 2002, 651 occured in India. Aseptic meningitis due to other enteroviruses is now the most frequent neurological infection of viral origin. It may occur sporadically or in epidemic form, with significant morbidity and, depending on specific host factors, mortality. Enteroviruses multiply in the lymphoid tissue of the alimentary tract and cause a wide range of syndromes. The extent to which enteroviruses are responsible for CNS disease is very difficult to gauge because of both underreporting and the subclinical nature of infection in approximately 90 percent of cases. Children are the main targets of enterovirus infection, and also serve as a mode for virus dissemination. In general, gender does not seem to predispose to a certain type of disease, although cases of childhood paralytic poliomyelitis are twice as common in males as in females.

POLIOMYELITIS

Natural infection with poliovirus occurs via the oral route, whereupon virus replicates in the tonsils, the lymphatic tissue which drains the neck, Peyer's patches, and the small intestine. Whether infection of lymphoid tissue such as the tonsils is achieved after initial replication of virus in the gut and subsequent viremia, or during the initial infection following ingestion, is a topic of debate. It is generally held that, for successful invasion of the CNS by poliovirus, a viremic phase is required, although some evidence suggests that direct infection of the CNS can take place via nerve endings in the gut.

Once poliovirus has gained access to the CNS the outcome of disease can be devastating, and is based largely on the highly cytopathic nature of poliovirus replication. The majority of infections (90–95 percent) are, however, silent, although virus may be recovered in the stools or from throat swabs of infected persons. Abortive infections (4–8 percent) include self-limiting forms of poliovirus infection and may present as an upper respiratory tract infection, gastroenteritis, or an influenza-like illness. Nonparalytic poliomyelitis (1–2 percent) presents initially as an abortive infection that is followed by invasion of the CNS by virus. Accompanying the assault on the CNS by virus are symptoms of aseptic meningitis with back pain and muscle spasms.

Paralytic poliomyelitis/polio encephalitis (0.1–2 percent), such as nonparalytic poliomyelitis, will generally present as an abortive infection during the prodromal stages, although there is often no evidence of an early phase of infection. The illness may be biphasic (often seen in children), with meningeal irritation and, finally, the onset of asymmetric flaccid paralysis (when whole muscle groups are involved) or paresis (when muscle group involvement is somewhat limited). Poliomyelitis can be divided into three basic types (spinal, bulbar, and bulbospinal), depending on the site of nerve cell involvement. Bulbar paralysis is more common in children and generally does not involve the limbs, whereas adults presenting with bulbar poliomyelitis typically have limb paralysis.

The gross pathology of poliovirus infection has been widely studied (Bodian and Horstmann 1965). Classically, there is extensive damage to the large anterior horn cells of the spinal cord. When paralysis is associated with poliomyelitis the cervical and lumbar areas (which serve limbs) are particularly affected. In fatal cases of poliomyelitis there may also be involvement of the brainstem motor nuclei, as seen in bulbar poliomyelitis, or of nerve cells of the motor cortex or thalamus and hypothalamus. After infection of cells of the CNS by poliovirus, the earliest histological change is vascular engorgement, rapidly followed by perivascular infiltration. The infiltrate comprises mainly lymphocytes and, to some degree, polymorphonuclear neutrophils, plasma cells and microglia. Cell damage is largely due to cytolytic replication of poliovirus within cells. Infected nerves swell and undergo satellitosis before being phagocytosed by mononuclear cells. Viral destruction of cells results in degeneration of axons and axon sheaths, followed by atrophy of the affected area and astrocytic scarring. Flaccid paralysis accompanies these changes, followed by a widespread atrophy of muscle no longer innervated by affected nerves. Death is often due to respiratory failure as a result of respiratory paralysis or secondary complications (e.g. bacterial pneumonia). People affected by paralytic poliomyelitis may develop new muscle weakness and prominent midday fatigue 25–30 years later as part of the postpolio syndrome (PPS) (Munsat 1991; Stone 1994; Dalakas 2002). The basis for the muscle weakness is an ongoing motor neuron deterioration related to the extensive motor unit remodeling during recovery from poliomyelitis, leading to post-poliomyelitis progressive muscular atrophy, whereas the basis for midday fatigue may be related to the abnormal presence of cytokines, possibly owing to persistence of poliovirus in the CNS.

ENTEROVIRUS

Of the numerous serotypes of enteroviruses that are recognized, two deserve special recognition with respect to disease involvement of the CNS: enterovirus serotypes 70 and 71 (for review, see Yin-Murphy 1994). Both are transmitted by the fecal–oral route, although ocular infections with enterovirus 70 may be mediated by facecloths and bath towels, etc. Although enterovirus 70 has a marked predilection for epithelial cells of the conjunctiva, the fact that patients may exhibit signs of CNS involvement suggests neurotropism. However, enterovirus 70 has yet to be isolated from the CSF or CNS of infected patients.

The three forms of CNS disease caused by enterovirus 70 are the spinal form, the cranial form, and a combination of the two. The spinal form is by far the most commonly encountered and is characterized by an asymmetrical flaccid paralysis or paresis, with one or more limbs involved. Paralysis is the manifestation of infection of the anterior horn cells of the spinal cord by enterovirus 70. Atrophy of the affected muscle groups resembles poliomyelitis. In the cranial form, acute motor cranial nerve palsy is seen which may involve single nerves or groups of nerves. In the spinal cord there is marked degeneration of anterior horn cells and neuroglial cell proliferation. Immunofluorescence staining for enterovirus 70 antigen reveals the presence of virus in the microglial or neuronal cells, or both.

Although expression of clinical disease of the CNS by enterovirus 70 is somewhat rare, enterovirus 71 has caused epidemics of aseptic meningitis and encephalitis (Minn 2002). Originally described in California, the virus has been associated with neurological disease on a worldwide scale. The major clinical manifestation is that of aseptic meningitis, which may often be concomitant with encephalitis, bulbospinal disturbances, and polyneuritis. Children under 5 years of age are particularly susceptible to the most severe forms of enterovirus 71-associated neurological disease, including aseptic meningitis, brainstem and/or cerebellar encephalitis, and acute flaccid paralysis. Because the majority of patients are hospitalized for possible bacterial infection, a rapid test such as RT-PCR for the diagnosis of enteroviral meningitis may reduce hospitalizations and unnecessary treatments (Stellrecht et al. 2002). Studies in primates with strains of virus isolated from patients with CNS disease show degeneration and necrosis of the neurons and neuronophagia by polymorphonuclear and mononuclear cells. Analysis of the CNS reveals predominantly perivascular cuffing in the lumbar and cervical cord, with minor involvement of specific areas of the brain (including the medulla oblongata and midbrain).

COXSACKIE VIRUSES

Coxsackie viruses A and B also enter the host primarily via the oral route and follow essentially the same path as poliovirus, with replication in the Peyer's patches of the alimentary tract. Most infections are clinically silent, although some individuals exhibit greater susceptibility than others (a male:female infection ratio of 1.5:2.0 is observed for clinical disease). The reasons for this

discrepancy remain elusive. Nervous system involvement has been documented for coxsackie viruses A7 and A9, and for B1–B6, which induce an aseptic meningitis and poliovirus-like paralysis. coxsackie virus B may also cause chronic meningitis and/or encephalitis in immunocompromised persons. Encephalitis, ataxia, and paralysis of the cranial nerves are rare. Both coxsackie viruses A and B are responsible for aseptic meningitis, although echoviruses (see below) are more frequently involved. Pathological changes with encephalitic and poliomyelitis cases in laboratory animals include brown fat necrosis and myocarditis, which is widespread in animals infected with coxsackie B virus (for review see Whitton 2002).

ECHOVIRUSES

Echoviruses have been associated with a number of syndromes, including aseptic meningitis, encephalitis, paralysis, ataxia, and Guillain–Barré syndrome, to list those affecting the CNS. Humans are the only recognized host for echoviruses, and several epidemics of disease have been attributed to them. Assigning a particular serotype to a specific syndrome has not been possible, although Guillain–Barré syndrome has been linked to infection with echovirus 9. In addition, echovirus 4 has been isolated from patients with bilateral limb paralysis. Similar syndromes have been noted for adults and children infected with echovirus serotypes 6, 9, 14, 17, 19, and 30. Primary infection results in replication of virus in the alimentary tract, after which other organs are seeded following an initial viremia. The nature of the secondary target is largely random. Occasional peripheral neuropathies have been documented following expression of the virus in the CNS. Initial clinical symptoms of CNS invasion by echoviruses can mimic bulbospinal poliomyelitis, although the major clinical manifestation is that of lymphocytic meningitis. Neonatal infection with echoviruses may lead to encephalitis with white matter sclerosis. In general, infection of newborns and younger children with echovirus is more severe than in older people.

Flaviviruses

There are currently about 70 registered flaviviruses (see Flaviviruses), of which about 50 percent are associated with human disease (Heinz et al. 2000). Of those that cause significant CNS disease, Japanese encephalitis virus (JEV), which causes epidemics over much of Asia, carries the highest mortality rate of any flavivirus. In epidemics, mortality rates range from 10 to 20 percent in most outbreaks to >30 percent (Umenai et al. 1985; Bu' Lock 1986). tick-borne encephalitis virus (TBEV) has long been recognized as a public health problem in eastern Europe. In Russia, the virus caused high mortality in the 1930s, prompting a great effort to isolate the causative agent and to produce a vaccine (Silber and Soloviev 1946). A number of subtypes of TBE have been defined in different geographical locations, including eastern subtypes such as Sofyn, the agent of Russian spring–summer encephalitis (RSSE) (Silber and Soloviev 1946), and western subtypes, such as Hypr (Blaskovic et al. 1967) and Neudörfl (Ackerman et al. 1986). Mosquito-borne JEV continues to threaten large populations, even though massive vaccination campaigns have been undertaken. Vaccination may, however, be undermined to a large degree by the high antigenic variation in different subtypes of JEV. Other mosquito-borne flaviviruses that tend to involve the CNS include St. Louis encephalitis (SLEV) and Murray Valley encephalitis (MVEV). SLE became a serious public health problem in the USA in the 1960s and continues to cause concern during sporadic outbreaks (Kokernot et al. 1969). MVE causes epidemics in Australia, disease occurring intermittently in areas that have experienced periods of high rainfall. Epidemics in the southern hemisphere are usually recorded from January to March and coincide with high population levels of the mosquito vector. Recently, the emergence of JEV and West Nile virus (WNV) in new geographic areas several thousand kilometres from their known habitats has caused considerable concern. JEV appeared in the Australian zoogeographic region in 1995 about 3 000 km from its nearest known focus in Bali, and WNV jumped from the Middle East to the United States in 1999 (Mackenzie et al. 2002b; Roehrig et al. 2002). The JEV group of the genus *Flavivirus* comprises several viruses, including *Japanese encephalitis*, *West Nile*, *St. Louis encephalitis*, and *Murray Valley viruses*.

JAPANESE ENCEPHALITIS VIRUS

Japanese encephalitis (JE) occurs in epidemic form in temperate areas of Asia, the northern parts of tropical southeast Asia, and northern parts of Australia. With respect to number of cases (30 000–50 000 annually) and associated morbidity and mortality, JE is the most important of the arbovirus encephalitides, which is transmitted to humans via the bite of an infected mosquito (*Culex* spp.) and the case fatality rate of which is up to 35 percent (Solomon and Vaughn 2002). JEV has a major mammalian amplifying host in the pig, and indeed most epidemic activity is driven by mosquito–pig transmission cycles.

After humans become infected, virus is thought to replicate in the skin and is transported to local lymph nodes, probably in migrating dendritic cells.

How JEV invades the CNS is unknown. After a variable incubation period of 6–16 days the disease may be asymptomatic or fulminant in course. Death may be rapid, occurring 10 days after onset in some cases. For patients who develop encephalitis, the disease pattern begins with headache, cough, coryza, nausea, vomiting, and gastroenteritis, proceeding to the onset of high fever

and alterations in sensory perception (including confusion or delirium, which may result in coma). Other patients present with aseptic meningitis and have no encephalitic features. Seizures are uncommon in adults, but are often (20 percent or more) seen in children. Facial motor weakness is often observed and upper motor neuron paralysis and paresis are common. Neurological sequelae are common (up to 70 percent) in survivors of symptomatic JEV infection, and are particularly severe in children. Such sequelae include convulsive disorders, motor abnormalities, impaired intellect, and emotional disturbances.

The areas of the brain infected by JEV include the cerebral cortex, the cerebellum, and the spinal cord. The highest concentration of infected neurons is in the thalamus and brainstem. Once in the CNS JEV virus replicates very efficiently in neurons, and it is the destruction of these cells by virus that is directly related to the manifestation of encephalitis. The brain and meninges of JE patients show edema, congestion, and focal hemorrhages. Microscopically, there is degeneration and necrosis of neurons, with accompanying neuronophagia in the cerebral cortex, cerebellum, and spinal cord. There is marked destruction of Purkinje cells in the cerebellum, as well as perivascular cuffing and inflammatory infiltrates in surrounding neural tissue. JE antigen is usually not detected in glial cells.

Vaccination against JEV ideally should be routinely practiced in all areas where the virus causes human disease (Monath 2002). For a laboratory confirmation of JE, rapid diagnostic kits based on ELISA techniques are available. There is currently no specific antiviral treatment.

WEST NILE VIRUS

Although mosquitos are the vector for WNV, birds are instrumental in its spread, acting both as a viral reservoir and in geographical spread. Geographically, WNV infections have been described in Egypt (1951–1975), Israel (1951–1980), France, Spain, Africa, India, Pakistan, since 1996 in Romania, and since 1999 in the eastern states of the USA (Murgue et al. 2002; Roehrig et al., 2002).

After an incubation period of 2–6 days patients develop a sudden onset of high fever with chills, malaise, headache, backache, arthralgia, myalgia, and retro-orbital pain, which is made worse by eye movement (Solomon and Vaughn 2002). Other nonspecific features are common. A rash appears from the second to the fifth day of illness in about 50 percent of patients, and is more common in young children. Neurological manifestations of WNV infection are similar to those of JEV infection, including aseptic meningitis, encephalitis, myelitis, or combinations of the three. After 1–7 days of a febrile prodrome, with headache, weakness, and gastrointestinal symptoms, patients become drowsy, confused, and disorientated. Although in most cases the prodrome is nonspecific, up to 16 percent of patients may have features suggestive of WN fever, including eye pain, facial congestion, pharyngeal or conjunctival hyperemia, lymphadenopathy, arthralgia, or cutaneous eruptions. In some patients the illness is biphasic, with two fever peaks. Focal neurological signs include upper motor neuron weakness, lower cranial nerve palsies, tremor, and ataxia. WNV infection may also cause a flaccid paralysis of the limbs and respiratory muscles, which may require ventilation. Urinary incontinence or retention may also occur. The case fatality rate for patients with symptoms of the CNS due to WNV infection is 5–10 percent. Whereas younger patients are more likely to present with WN fever or aseptic meningitis, older patients are more likely to develop encephalitis and to die.

WNV infections are most frequently diagnosed by assessment of the antibody response in an IgM antibody capture ELISA. There is currently no specific antiviral treatment.

ST. LOUIS ENCEPHALITIS VIRUS

St. Louis encephalitis virus is a mosquito-borne *Flavivirus* that occurs in both endemic and epidemic forms in the USA and represents the most important arbovirus disease of North America. The virus is transmitted by *Culex* spp.

Clinically, infection with SLEV can range from inapparent infections to fulminant encephalitis and death. Patients usually present with or progress rapidly to one of three established syndromes: febrile headache, aseptic meningitis, or encephalitis. The febrile headache is often accompanied by vomiting and nausea. Analysis of the CSF may reveal pleocytosis. There are no apparent signs of meningeal involvement or neurological abnormalities. Aseptic meningitis presents as an acute febrile illness with signs of meningeal irritation and pleocytosis in the CSF. Encephalitis attributed to SLEV infection also includes meningoencephalitis and encephalomyelitis. Alterations in the state of consciousness or localized neurological abnormalities, or both, may be seen. Brinker and co-workers (Brinker et al. 1979) analyzed data from 18 reports of patients with SLE. It is estimated that 70–80 percent of patients suffer from headache, nuchal rigidity, and an altered level of consciousness. Almost 50 percent display tremors, pathological reflexes, and confusion. Some 20–30 percent of patients exhibit multiple cranial nerve abnormalities, hypoactive deep tendon reflexes, positive Babinski signs, or myalgia. More significantly, 11–20 percent of those infected have VIIth cranial nerve lesions, lower motor neuron lesions, hyperactive deep tendon reflexes, or myoclonus, and progress to coma. Up to 10 percent exhibit convulsions, paresis, photophobia, nystagmus, or ataxia. There is a striking increase in case fatality rates for SLEV infection, depending on age. A fatality rate of approximately 2 percent is observed for young adults, rising to 22 percent for the elderly. Of those infected

with SLEV, approximately 50 percent with fatal disease die within 1 week after the onset of symptoms.

MURRAY VALLEY ENCEPHALITIS

Murray Valley encephalitis virus is the major *Flavivirus* in Australia and Papua New Guinea (Mackenzie et al. 2002a).

Antigenically, it is closely related to both JE and SLE viruses. In a number of respects the clinical presentation of patients suffering from MVE is similar to that of JE infection. Clinically, the disease begins with a prodrome of fever, headache, photophobia, myalgia, nausea, and vomiting. There are often early signs of CNS involvement that include drowsiness, speech difficulties, disorientation, and ataxia. Patients who do not progress to coma generally have a favorable prognosis, but may display sequelae including tremors, incontinence, stiffened limbs and neck, and speech impediments. Patients who enter coma often develop respiratory paralysis, necessitating mechanical aid for breathing. Fatalities are usually the result of secondary infection or extensive brain destruction, accompanied by decerebrate rigidity.

TICK-BORNE ENCEPHALITIS

The tick-borne encephalitis (TBE) complex of the family *Flaviviridae* comprises 14 antigenically related viruses, eight of which are significant human pathogens (Heinz et al. 2000). The two most important are Russian spring–summer encephalitis (RSSE) and Central European encephalitis (CEE). These differ in pathogenicity, the former being associated with much higher rates of morbidity and mortality.

RSSE is characterized by a gradual onset following 10–14 days' incubation after the transmission of virus from the bite of an infected tick. The patient suffers a prodromal phase of severe headache, nausea, photophobia, weakness, fever, and chills. The body temperature may rise as high as 41°C and the fever lasts 5–7 days. These symptoms are followed by neck stiffness, sensory changes, visual disturbances, and variable neurological dysfunction, including paresis, paralysis, sensory loss, and convulsions (Silber and Soloviev 1946). The more severe cases can develop central paralysis or involve the brainstem or spinal cord, which may lead to bulbospinal or spinal paralysis. In fatal cases death occurs within the first week after onset, with case fatality rates of approximately 30 percent (Gresikova and Beran 1981). Disease is more severe in children than in adults. Recovery from infection is protracted, with neurological sequelae in 30–69 percent of survivors, manifested by residual flaccid paralysis of the shoulders and arms. These sequelae probably reflect permanent damage to neurons.

In comparison to RSSE, infection with CEE subtypes of virus is much milder, with a number of abortive infections. Classically, CEE is a biphasic illness with a prodromal period resembling influenza that either resolves completely or is followed by the sudden onset of encephalitis. Onset of illness is often rapid, with fever, severe headache, photophobia, nuchal rigidity, nausea, and vomiting. Patients who present with brainstem involvement or have ascending paralysis generally have a poor prognosis. Although case fatality rates range between 1 and 5 percent, 20 percent of survivors suffer minor neuropsychiatric sequelae (Radsel-Medvescek et al. 1980). It is interesting that a small percentage of patients who recover from CEE have a progressive chronic encephalitis with remissions and relapses (Silber and Soloviev 1946).

Clinical signs are swelling, congestion, and petechial hemorrhages in the brain, similar to other flavivirus encephalitides. There is also meningeal and perivascular inflammation, with neuronal degeneration and necrosis, neuronophagia, and glial nodule formation in the cerebellar cortex, brainstem, basal ganglia, and spinal cord. Far Eastern subtypes have a tropism for neurons of the gray matter of the medulla oblongata and upper cervical cord. The predominance of lower motor neuron paralysis of the upper extremities, observed in many cases of encephalitis, may be explained by the vulnerability of the anterior horn cells of the cervical cord to virus infection.

Retroviruses

In this section, two neurotropic human pathogenic retroviruses, HIV and human T-lymphotropic virus (also known as human T-cell leukemia virus-1 (HTLV-1)), that cause distinct syndromes affecting the CNS are considered (see also Retroviruses and associated diseases in humans and Human immunodeficiency virus), Both viruses have a prolonged incubation period (average of 8–10 years for HIV, and up to 40 years for HTLV-I-associated myelopathy). Some similarities exist between modes of transmission via the transfer of body fluids containing free virus or, more often, infected cells. HIV and HTLV-I both have a primary tropism for lymphocytes.

HIV-ASSOCIATED DEMENTIA

Exactly how HIV gains access to the CNS is unknown. In early acute infections HIV can be detected as free virus in the CSF of patients with no apparent neurological disturbances. Crossing of the BBB is believed to occur through passage of the virus in infected peripheral blood macrophages or activated T cells (Gartner 2000; Patrick et al. 2002). In the brain, HIV infects mainly microglial cells. Additional evidence points to possible infection of brain capillary endothelial cells, which are CD4-negative, but chemokine receptor and SIGNR-positive (Mukhtar et al. 2002; Soilleux et al. 2002). Furthermore, galactosylceramide (GalC) has been proposed to fulfill a receptor function in brain-derived

cells (Harouse et al. 1991). Differences in tropism between strains of HIV could be demonstrated. In general, macrophage-tropic strains replicate more efficiently in cells of neural origin than strains of a T-cell tropic nature.

AIDS itself is often characterized by a high prevalence of marked and extensive neurological disturbances. In numerical terms, it is suggested that as many as 80 percent of AIDS patients will have neuropathological abnormalities at postmortem, and that as many as half of these will have suffered neurological disturbances prior to death. Transient neurological disturbances can also be detected at the time of seroconversion, and may include aseptic meningitis (characterized by headache, fever, and cellular pleocytosis of the CSF), acute encephalopathy, and myelopathy. All these disturbances are usually self-limiting during early infection. As HIV infection progresses to AIDS a new spectrum of neurological complications may emerge, which are globally described as the AIDS dementia complex or HIV-associated dementia (HAD) (Cortegis 1994). The primary stages of HAD show mild impairment of memory, as well as loss of concentration and the ability to process information. Symptoms may then progress to severe cognitive dysfunction and possibly paralysis. Morphologically, features associated with HAD include either a diffuse leukoencephalopathy with severe reduction in myelin, or areas of discrete demyelination and multinucleated giant cells (Figure 6). Clinically, HAD is suspected in AIDS patients presenting with memory loss, disturbances in concentration, depression, and motor complaints. Specific afflictions associated with HAD include peripheral neuropathy, cerebral tumors, and vascular lesions. Peripheral neuropathies are often observed in severely immunosuppressed patients who present with painful paraesthesiae. These are due to axonal degeneration of small myelinated and unmyelinated fibers as a result of exposure to toxic factors (see below). It is interesting to note that the inflammatory neuropathies, including demyelinating polyneuropathy, are identical in clinical course to Guillain–Barré syndrome or chronic inflammatory demyelinating polyneuropathy (Cornblath et al. 1987; Tyor et al. 1995). In the latter condition the nerves show perivascular lymphocytic and monocytic infiltrates, believed to be mediated by T cells and macrophages. Cerebrovascular

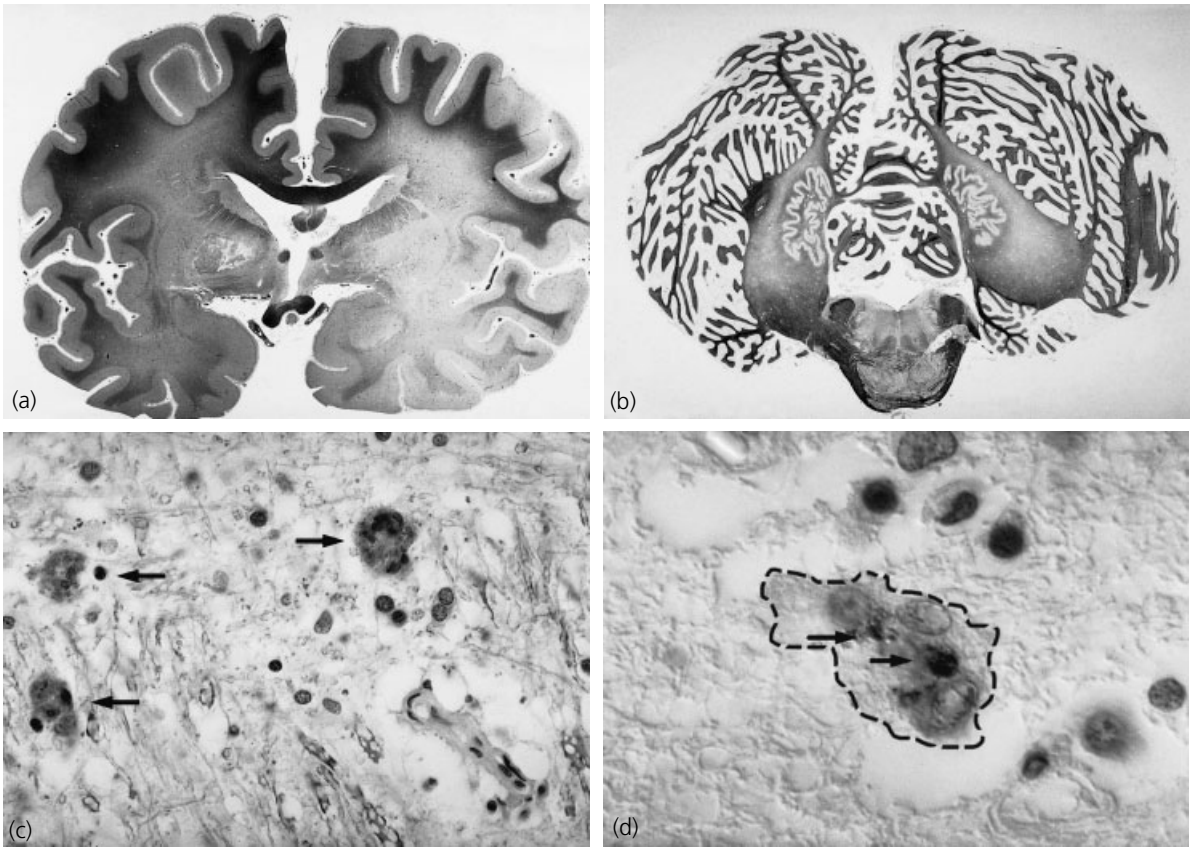

Figure 6 *HIV encephalopathy.* **(a)** *Whole mount of a brain slice, stained with a myelinotropic dye (Luxol fast blue). The hallmark of HIV leukoencephalopathy is diffuse reduction of myelin stain in the central white matter.* **(b)** *Whole mount of the cerebellum of the same patient. Leukoencephalopathy is evident in the deep cerebellar white matter.* **(c)** *High-power micrograph of HIV leukoencephalopathy, showing loss of myelin and typical multinucleated giant cells (arrows) (×200).* **(d)** *Immunostaining of a multinucleated giant cell for the HIV protein p24, showing granular immunoreactivity (arrows) (×400). (Courtesy of Dr Adriano Aguzzi.)*

complications such as vasculitis and hemorrhages are often found in areas of cerebral tumor or demyelination. Hemorrhages may occur as a result of alterations in coagulation (Snider et al. 1983).

Mechanism of CNS disturbances in neuro-AIDS

Despite the observation that HAD is associated with HIV infection of microglia/macrophages, the number of HIV-infected cells and the amount of viral antigen in the CNS do not correlate well with measures of cognitive decline (Glass et al. 1995). Direct viral infection of susceptible cells of the CNS may induce syncytia formation, producing multinucleated giant cells. Multinucleated giant cells are usually formed by macrophages or microglial cells and are considered a hallmark of subacute encephalitis in HIV-infected brains and spinal cord, but not in peripheral nerves (Cornblath et al. 1987) (Figure 6). As only a restricted number of cells of the CNS are susceptible to infection by HIV, secondary mechanisms of cellular injury are paramount in producing neurological disturbances in AIDS patients.

The release of neurotoxic soluble factors by cells in response to viral assault or to binding of virus proteins is an important facet of the disease process of HIV.

Infection of monocytes or macrophages by HIV is known to stimulate the synthesis and release of a number of cytokines, including chemokines, IL-1β, TNF-α, interferons, and nitric oxide (NO) (Garden 2002; Ryan et al. 2002; Wang et al. 2002). Possibly the most notable change associated with HIV dementia with AIDS is the upregulation of TNF-α. TNF-α levels were higher in patients with advanced dementia and those with multinucleated giant cells or diffuse myelin pallor (Wilt et al. 1995). The release of cytokines makes the BBB more permeable to inflammatory cells, which may then infiltrate the CNS to establish additional neurological sequelae. Although significant levels of quinolinic acid have been reported in the CNS of HIV-1 seropositive patients, whether there is a direct causal relationship with the observed neurological findings remains questionable. Induction of TGF-β by macrophages and astrocytes has also been linked to CNS disorders (Wahl et al. 1991). Studies examining levels of specific chemokines in patients with HAD have shown that β-chemokines (RANTES and MIP-1α) are elevated in the CSF of patients, but higher levels were associated with the preservation of cognitive function (Letendre et al. 1999). Microglia also release excitatory amino acids and related substances that most likely contribute to the neuronal disfunction in HAD, including glutamate, quinolinate, L-cysteine, and the amine Ntox (Garden 2002). These substances can induce N-methyl-D-aspartate (NMDA) receptor-mediated neuronal apoptosis.

Autoimmunity is also thought to be important in the process of HIV-induced neurological disease, and may be based on the partial identity between viral antigens and antigenic determinants on cells (i.e. molecular mimicry). An autoimmune process was proposed following the observation that small demyelinated regions of the brain seem similar to those in post-infectious encephalomyelitis or experimental allergic encephalitis (Kumar et al. 1989). AIDS patients with peripheral neuropathy have autoantibodies to an unidentified protein on the surface of neurons. In addition, fluorescence studies have shown antibody attached to the perineurium in tissues from AIDS patients. AIDS patients suffering from peripheral neuropathy who undergo subsequent plasmapheresis obtain relief of symptoms similar to that observed in the autoimmune conditions of Guillain–Barré syndrome and thrombocytopenic purpura. Autoantibodies to peripheral nerves are associated with peripheral neuropathy. A likely target in the nervous system is the myelin sheath of nerves. Antibody levels to myelin basic protein have been described in CSF of AIDS patients that correlate directly with the severity of dementia (Liuzzi et al. 1994). Cross-reactivity of anti-gp41 antibodies with certain proteins of astrocytes could compromise the function of these cells. Moreover, cross-reaction of anti-V3 antibody with human brain proteins may also be an important part of the pathogenic process. Further brain-reactive proteins in the sera of infected patients may lead to immune complex disease.

A number of HIV products (e.g. gp120) are toxic to cultured human brain cells in vitro. NMDA antagonists have been shown to reduce toxic effects attributed to gp120 interaction (Lipton et al. 1991). The same viral protein can also affect the permeability of the cell membrane, leading to ion influx/efflux and loss of electrical potential. Inhibitors of the p38 MAP kinase have been shown to abrogate neuronal apoptosis due to HIV gp120 exposure or α-chemokine (SDF-1) toxicity (Kaul and Lipton 1999). The HIV proteins gp41 and Tat have been shown to be toxic for cultured cells: Tat exhibits some homology with a neurotoxin and induces cellular aggregation and fascicle formation in primary rat cortical brain cells (Garry and Koch 1992). In addition, Tat may induce differentiation of these cells (Kolson et al. 1993). The clinical relevance of these findings, however, remains unclear. When discussing HIV infection, it is also important to consider that neurotropic effects may reflect infection with an additional agent. A number of studies have shown the presence of viruses such as *Cytomegalovirus* (Figure 7), or polyomaviruses in the CNS of AIDS patients, and have led to the suggestion that secondary infections are the primary cause of neurological disturbances.

HTLV-I ASSOCIATED MYELOPATHY/TROPICAL SPASTIC PARAPARESIS

Human T-lymphotropic virus type I infects 10–20 million people worldwide, and is endemic in southern Japan, the

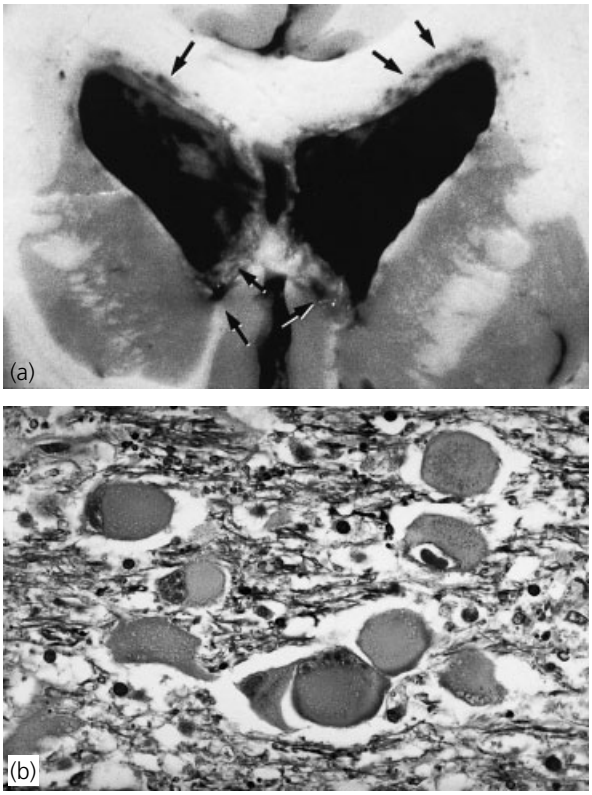

Figure 7 Cytomegalovirus *encephalitis in an AIDS patient.* **(a)** *Typical CMV-induced damage with areas of necrosis and microhemorrhage located in subependymal positions (arrows).* **(b)** *Focus of CMV encephalitis within the corpus callosum (x400). Characteristic cytomegalic cells with large inclusion bodies representing viral products. (Courtesy of Dr Adriano Aguzzi.)*

Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa.

HTLV-I was established as an etiologic agent of acute T-cell leukemia (ATL). A correlation between tumor cells and a monoclonal or oligoclonal pattern of viral genome integration, infection of lymphocytes in vitro resulting in immortalized T cells, and the establishment of HTLV-I oncogenic animal model systems has been reported (Cann and Chen 1990). In 1985 it was discovered that a number of HTLV-I-seropositive patients in the Caribbean and Japan were suffering from a chronic myelopathy. Since then, increasing evidence has accumulated linking HTLV-I infection with what is now termed HTLV-I-associated myelopathy (HAM) or tropical spastic paraparesis (TSP), collectively known as HAM/TSP, which is developed by 0.25–3 percent of virus carriers (Jacobson 2002).

HAM/TSP presents as a clinical disease very distinct from ATL. The disorder is characterized by weakness and spasticity of the extremities, hyperreflexia, peripheral sensory loss, and chronic inflammation. The development of HAM/TSP correlates with the presence of lesions in the white matter of the spinal cord, including demyelination and axonal changes. In the subacute disease there is progressive paralysis of the lower extremities and associated impotence, incontinence, and sensory symptoms, paradoxically often with minimal sensory findings on examination. Lesions are localized primarily in the thoracic spinal cord, with little, if any, cerebral involvement. In cases of late disease, hyalinization of vessels with necrosis and demyelination of the spinal cord, particularly in the throracic region, is observed. Because many patients are infected through breastfeeding from infected mothers or via sexual transmission, the virus is thought to be transmitted by infected lymphocytes. The incubation period is particularly long, with onset often in the fourth or fifth decade of life.

Gross examination of the brain reveals no apparent abnormalities, although the majority of cases exhibit severe loss of myelin and atrophy of the spinal cord, and some thickening of the meninges. These clinical findings are associated with HAM/TSP patients with a long history of illness, whereas in cases with a clinical history of 1 year or less the spinal cord often seems normal (Fukura et al. 1989). The major finding in HAM/TSP cases is a chronic inflammatory process, with perivascular infiltrates involving the gray and, more often, the white matter of the spinal cord. Numerous inflammatory cells, including $CD4^+$ and $CD8^+$ T cells, B cells, and foamy macrophages, are present in damaged areas of the spinal cord parenchyma (Wu et al. 1993). Later in the disease, immunohistochemical analysis of the affected lesions shows the predominance of $CD8^+$ T cells (Umehara et al. 1993). HTLV-I sequence analysis of PBMCs of HAM/TSP patients demonstrates a pattern of polyclonal integration, in contrast to an oligoclonal or monoclonal pattern associated with ATL patients. Most HAM/TSP patients have higher titers of anti-HTLV-I antibodies than do asymptomatic HTLV-I patients. The amount of proviral DNA in HAM/TSP patients correlates with the number of CTL. HTLV-I-specific sequences have been localized to astrocytes in affected areas of the CNS (Lehky et al. 1995). An autoimmune basis for HAM/TSP has been proposed after the isolation of circulating MHC class I-restricted CTLs from HAM/TSP patients. The CTLs show specific activity against a nine amino acid peptide of the HTLV-I tax protein, and are restricted to the HLA-A201 haplotype (Elovaara et al. 1993; Koenig et al. 1993). The presence of HTLV-I-specific CTL correlates with the production of several cytokines and chemokines, such as IFN-γ, TNF-α, IL-2, MIP-1α/β, and matrix metalloproteinases (Biddison et al. 1997; Kubota et al. 1998; Giraudon et al. 2000). In HLA-A201 patients the increased HTLV-I proviral DNA loads in CSF in comparison to PBMC were propotional to the frequency of tax-specific $CD8^+$ T cells. This observation supports the hypothesis that the HTLV-I proviral load may drive the increased virus-specific immune responses that have been suggested to be immunopathogenic in HAM/TSP (Soldan 2001). The

data led to the following immunopathogenesis model of HAM/TSP: processing and presentation of HTLV-I-specific peptides in association with high proviral loads and viral protein expression in $CD4^+$ and $CD8^+$ T cells may lead to the activation and expansion of antigen-specific T-cell responses in the CNS. Numbers of inflammatory $CD8^+$ T cells in the spinal cord lesions of HAM/TSP patients tend to increase with disease progression, and together with inflammatory cytokines and chemokines, immunopathogenesis may be enhanced.

To help in understanding the disease process involved in HAM/TSP, an animal model using Wistar–King–Aptekman (WKA) rats has been developed in which the characteristics and distribution of lesions in the rat CNS are similar to those observed in human cases (Ishiguro et al. 1992; Kasai et al. 1999). After an icubation period of approximately 15 months the rats develop a chronic progressive myelopathy with paraparesis of the hind limbs. HTLV-I proviral DNA was localized in some microglia/macrophages in spinal cord lesions. In addition, provirus was evident not only in ED-1-negative lymphoid cells, but also in ED-1-positive macrophages from lymph nodes. These infected microglia/macrophages may relate to cause the myeloneuropathy through neurotoxic cytokine synthesis. The expression of the antiapoptotic gene *bcl-2* was strongly downregulated in oligodendrocytes of infected rats. This may increase the susceptibility to TNF-α-induced apoptosis of oligodendrocytes and result in the development of the characteristic disease (Jiang et al. 2000).

Paramyxoviruses

The two paramyxoviruses frequently liable to cause CNS diseases are mumps and measles viruses, both members of the subfamily *Paramyxovirinae*.

MUMPS VIRUS INFECTIONS OF THE CNS

Mumps is a highly communicable and generally self-limited infection caused by mumps virus (MuV). It occurs in epidemics among susceptible school-aged children and is characterized clinically by nonsuppurative salivary gland enlargement. Although parotitis is the most obvious manifestation, mumps is clearly a systemic infection that can involve virtually all organs, including the CNS. Indeed, CNS involvement during acute mumps infection occurs with such high frequency that it should be considered a primary manifestation, rather than a complication. The spectrum of CNS infections caused by mumps virus ranges from aseptic meningitis, which is very common but usually mild, to fulminant encephalitis, which is very rare but potentially fatal. The term 'mumps meningoencephalitis' is often used to designate overlap syndromes that have features of both these typical forms. Mumps vaccine has successfully been used by many countries, and high coverage has shown a rapid decline in mumps morbidity (Galazka et al. 1999).

Mumps is a highly contagious infection that is transmitted from human to human (the only known natural host) by droplets. Infected individuals may transmit the virus for about 10–16 days. During the incubation period (which averages 18 days), primary viral replication takes place in epithelial cells of the upper respiratory tract, followed by spread of the virus to regional lymph nodes and subsequent viremia, with systemic dissemination.

Symptomatic CNS involvement occurs in 10–20 percent of cases (Russell and Donald 1958; Galazka et al. 1999). The vast majority of cases of CNS MuV infections are uncomplicated aseptic meningitis, whereas symptomatic encephalitis occurs in >0.1 percent of cases (Klemola et al. 1965; Levitt et al. 1970). The incidence of CNS inflammation as evidenced by pleocytosis in the CSF (observed in only 60 percent of cases) is actually much higher than the incidence of clinical meningoencephalitis (Bang and Bang 1943).

Although differences in neurotropism among MuV isolates have been demonstrated in animal models, there is no direct evidence that cases of human mumps encephalitis are caused by virus strains with enhanced neurotropism or virulence.

Patients with symptomatic mumps CNS infection most often present with fever, vomiting, and headache. Other frequent findings include nuchal rigidity, lethargy, and abdominal pain (possibly from pancreatitis). Defervescence is usually the first sign of clinical recovery, which is normally complete within 7–10 days. Patients with simple mumps aseptic meningitis have signs of meningeal irritation, however, with normal cortical function. Cases of mumps encephalitis present with seizures, markedly depressed levels of consciousness, and focal neurological signs. Typical findings also include psychiatric disturbances, lethargy, and stupor. CNS symptoms appear about 5 days after the onset of parotitis, although they may accompany or even precede the development of salivary gland enlargement. No association between the parotitis and the frequency or severity of CNS manifestations has been established. Other neurological manifestations of mumps include myelitis and polyneuropathy.

Confirmation of CNS involvement in patients with mumps is based on examination of the CSF. CSF findings do not differ significantly between mumps patients with meningitis and those with encephalitis. MuV can be isolated from CSF in 30–50 percent of patients with CSF pleocytosis (Björvatn and Wolontis 1971; Wolontis and Björvatn 1973). The spinal fluid opening pressure is normal. Infiltrating cells are predominantly lymphocytes, but significant amounts of polymorphonuclear leukocytes may be seen in the early stages of infection. CSF pleocytosis resolves slowly, and complete normalization of the spinal fluid may require several weeks (Azimi et al. 1975). No correlation has been established

between the magnitude of the CSF pleocytosis and the severity of clinical illness.

MuV spreads readily to the CNS, either as free plasma virus or within infected mononuclear cells (Fleischer and Kreth 1982). Initially, viral replication occurs in choroidal epithelial cells, with shedding of progeny virus into the CSF (Herndon et al. 1974). The development of mumps encephalitis probably results from a direct extension of the virus from ependymal cells into neurons within the brain parenchyma, as has been demonstrated in experimentally induced mumps encephalitis in hamsters (Wolinsky et al. 1974). Because of the low case:fatality ratio, autopsy reports describing the pathological findings in mumps meningoencephalitis are rare and variable (reviewed in Gnann 1991). Histological changes are seen in the white matter of the cerebral hemispheres and cerebellum, and in the white and gray matter of the brainstem and spinal cord. The most commonly recorded features are diffuse edema of the brain, limited mononuclear cell infiltration of the meninges, perivascular infiltration with mononuclear cells, glial cell proliferation, focal areas of neuronal cell destruction, and localized demyelination. A few cases of purported chronic mumps encephalitis have been described, but available data are insufficient to support a role for mumps virus in chronic CNS infection (Ito et al. 1991).

MEASLES VIRUS INFECTIONS OF THE CNS

Measles virus (MV) is a highly contagious agent that leads to a well-defined acute disease in unvaccinated individuals, followed by seroconversion and a lifelong immunity against reinfection. As hallmarks of acute measles, a transient, marked lymphopenia and immunosuppression are observed which favor the establishment and fatal outcome of opportunistic infections, and are the main reasons for the annual death toll of approximately 1 million children worldwide, particularly in developing countries (Clements and Cutts 1995; Schneider-Schaulies and ter Meulen 2002). Before the advent of vaccination, approximately 8 million deaths were due to measles annually. Vaccination protects not only from complications such as otitis media, pneumonia, and diarrhea, but also from acute and subacute encephalitis (Duclos and Ward 1998). Patients with a history of acute measles develop a lifelong immunity.

MV infections have also been linked etiologically to the establishment of CNS disease processes that include an early acute measles encephalitis and two late complications: subacute sclerosing panencephalitis and measles inclusion body encephalitis (MIBE). Moreover, some studies have suggested a pathogenic role of MV for other disease processes, including multiple sclerosis (ter Meulen and Katz 1997), chronic liver disease (Robertson et al. 1987; Andjaparidze et al. 1989), Paget's disease (Basle et al. 1986), otosclerosis (McKenna and Mills 1989) and, more recently, Crohn's disease (Wakefield et al. 1993). However, none of these diseases has been linked unequivocally to measles virus.

Acute measles encephalitis

CNS involvement in the course of measles is fairly common, as EEG and CSF changes are found in half of all patients with acute measles (Gibbs et al. 1959; Reinicke et al. 1974). Acute encephalitis is observed in 0.5–1 per 1 000 cases (Katz 1995). In general, about 15 percent of cases are fatal, and 20–40 percent of those who recover are left with lasting neurological sequelae. Encephalitis usually develops within about 8 days after the onset of measles. Occasionally, it may also occur during the prodromal stage. Measles encephalitis is characterized by a resurgence of fever, headache, seizures, cerebellar ataxia, and coma. As is commonly seen in postinfectious encephalitis induced by other viruses, demyelination, perivascular cuffing, gliosis, and the appearance of fat-laden macrophages near the blood vessel walls are detectable histopathologically. Petechial hemorrhages may be present, and in some cases inclusion bodies have been observed in brain cells. CSF findings in measles encephalitis usually consist of mild pleocytosis and the absence of MV-specific antibodies. Long-term sequelae include selective brain damage, with retardation, recurrent convulsive seizures, hemiplegia, and paraplegia. It is not entirely clear what pathogenic role MV plays in the development of acute measles encephalitis. MV has been occasionally recovered from CSF samples (McLean et al. 1966) and brain tissue of patients (ter Meulen et al. 1972; Ogura et al. 1997), including the detection of MV-specific nucleic acids by PCR techniques (Nakayama et al. 1995). Because there is no evidence that MV is regularly present in the nervous system, and because there is an aberrant immune reaction to brain antigens, acute measles encephalitis is considered an autoimmune disease (Griffin 1995).

Measles inclusion body encephalitis

MIBE has been recognized only recently and is confined to immunosuppressed children with, for example, leukemia undergoing axial irradiation therapy. The incubation period ranges from weeks to several months. The condition commences with convulsions, myoclonic jerks as seen in SSPE. The seizures are often focal and localized to one site; other findings include hemiplegia, coma, or stupor. However, the disease course is more rapid than that of SSPE, and proceeds to death within weeks or a few months. Characteristically, no or only low titers of MV-specific antibodies are mounted in the serum and CSF of patients.

Subacute sclerosing panencephalitis

SSPE is a rare, fatal, slowly progressive degenerative disease of the CNS. It is generally seen in children and young adults aged 5–15 years after acute measles, with an incidence of approximately 1 in 100 000 cases – after infection with wildtype virus, and not after vaccination (Duclos and Ward 1998). Boys are more likely than girls to develop SSPE, and half the patients have contracted measles before the age of 2 years. No unusual features of acute measles have ever been demonstrated. The course of SSPE is variable and usually starts with a generalized intellectual deterioration, which may last for weeks or months, until definite neurological signs or motor dysfunctions such as dyspraxia, generalized convulsions, aphasia, visual disturbances, and mild repetitive simultaneous myoclonic jerks appear. Viral invasion of the retina leads, in 75 percent of cases, to a chorioretinitis, often affecting the macular area, followed by blindness. Finally, the disease proceeds to progressive cerebral degeneration, leading to coma and inevitable death. The illness generally lasts for 1–3 years, but more rapid forms have been described. Neuropathological findings include a diffuse encephalitis affecting both the gray and the white matter, characterized by perivascular cuffing and diffuse lymphocytic infiltrations. Gliosis is usually observed. Neurons, oligodendroglial cells, fibrous astrocytes, and endothelial cells contain large aggregates of intranuclear inclusion bodies that contain MV nucleocapsid structures, visible on immunohistological examination (Allen et al. 1996). Giant cell formation or membrane changes consistent with virus maturation have not been detected.

Characteristic EEG changes are usually the first diagnostic hint and are regarded as characteristic and even pathognomonic for SSPE. They consist of periodic high-amplitude slow-wave complexes that are synchronous with myoclonic jerks recurring at 3.5–20-second intervals. These periodic complexes (Radermecker) are remarkably stereotypical in an individual patient, in that their form remains identical in any given lead. They are bilateral, usually synchronous, and symmetrical. Moreover, they usually consist of two or more δ waves and are biphasic, triphasic, or polyphasic in appearance. This EEG pattern, however, is variable within the course of the disease and from one patient to another, and may disappear as the disease progresses. Other important pathognomonic findings are the very high titers of measles antibodies in serum – except those against the M protein – and a pronounced increase in the γ-globulin in the CSF. In the CSF, the humoral immune response is oligoclonally restricted, as revealed by isoelectric focusing (Dörries and ter Meulen 1984). This indicates that antibodies are synthesized intrathecally by a restricted number of plasma cells that have invaded the CNS.

VIRUS–HOST INTERACTIONS

Acute MV infections are characterized by the marked tropism of the virus for PBMCs, including lymphocytes, monocytes/macrophages, and dendritic cells (Borrow and Oldstone 1995; Ohgimoto et al. 2001). The virus is present in a highly cell-associated form and is efficiently spread via infected PBMCs, in which both viral nucleic acid and proteins can easily be detected and from which infectious virus can be recovered. Subsequently, endothelial cells and underlying tissue in various organs are infected. Infected PBMCs have been proposed as potential carriers, but have not been positively identified. Exactly how and when the virus reaches the brain in SSPE is not known. Some PCR-based studies described MV-specific nucleic acids in autopsy brain material obtained from patients with no history of measles-associated CNS illness (Katayama et al. 1995), suggesting that persistent MV infections of the brain may be more frequent than initially thought and may stay clinically inapparent throughout life. It is also not known which cellular receptors mediate the infection of neural cells, as the known receptors – CD150 for wildtype and vaccine MV (Tatsuo et al. 2000; Erlenhoefer et al. 2001) and CD46 only for vaccine strains (Dörig et al. 1993; Naniche et al. 1993) – owing to their cell type-specific expression, are not sufficient to explain the CNS infection (Ogata et al. 1997; McQuaid and Cosby 2002). Virus spread in the brain occurs directly from cell to cell, most likely involving local microfusion events (summarized in Schneider-Schaulies et al. 2001).

No specific defects of cell-mediated immunity have been detected in SSPE, yet in both CNS diseases, SSPE and MIBE, large amounts of MV nucleocapsid structures are present within infected cells (ter Meulen et al. 1983). However, rescue of infectious virus in tissue culture is rarely possible (Ogura et al. 1997), indicating that the viral replication cycle is defective. As with other persistent infections, MV gene expression is markedly restricted during persistence. Viral envelope proteins are generally expressed at extremely low levels, whereas internal proteins required for intracellular viral reproduction (N, P) can be detected easily. As characterized mainly in autopsy material, this is generally achieved by particular restrictions of the viral envelope-specific mRNAs (M, F, and H), and by mutations within the envelope-specific genes that interfere with a functional expression of the corresponding proteins (reviewed in Billeter et al. 1994) (summarized in Figure 8). It is significant for the pathogenesis that mutations introduced into the envelope gene reading frames are of two types: point mutations, caused by the naturally high infidelity of the viral polymerase during transcription/replication, and clustered hypermutations, ascribed to a cellular unwinding/modifying enzyme complex. Functional alterations or complete abolition of the MV envelope proteins seem to readily explain why the

formation of infectious virus particles, viral budding, and the formation of giant cells are not encountered in persistent MV CNS infections (Cathomen et al. 1998). Moreover, important antigenic structures are mainly lacking on the surface of infected cells, which thus escape detection by the MV-specific humoral immune response.

Although it is not understood what triggers the development of a MV CNS disease, some progress has been made in understanding which factors lead to and maintain the establishment of a persistent MV infection in brain cells. Increasing evidence suggests that MV that infects brain cells is primarily nondefective, and that host factors initially attenuate viral gene expression on both transcriptional and translational levels (reviewed by Schneider-Schaulies et al. 1994). In addition, MV-specific double-stranded RNA intermediates, as formed accidentally during viral transcription and replication, serve as targets for a cellular enzyme (dsRNA adenosine deaminase activity (DRADA)) present in large amounts in brain cells in vitro. This enzyme introduces up to 50 percent of nucleotide transitions within a given double-stranded target RNA, thus profoundly altering the coding sequences (Billeter et al. 1994) (Figure 9, p. 33). There is still, however, some controversy about whether mutations within the viral genome are a prerequisite for the establishment of persistent MV infection in brain cells, whether mutant viruses have a selective advantage in replication and spread in the brain, or if mutations are important for the long-term maintenance of MV persistence. The last possibility is weakened by the high variability of the mutations encountered in individual patients with SSPE and MIBE, and also by the finding that, at least in an animal model, persistent MV CNS infections have been established in the absence of detectable mutations. In summary, the IFN response and its possible lack in neurons, a steep viral expression gradient, the accumulation of point and hypermutations within envelope genes, the antibody-induced antigenic modulation, and the observed cell-to-cell spread of nucleocapsids support the persistent brain infection, with failure of the immune response to eliminate the virus (for review see Schneider-Schaulies et al. 1995).

In newborn rodents the hamster neurotropic strain HNT, or the rat brain-adapted MV strain CAM/RB, spread efficiently, causing an age-dependent lethal acute encephalitis or subacute encephalitis (for review see Liebert and Finke 1995). Resistance and susceptibility to MV-induced encephalitis correlate with the MHC haplotype of the respective inbred strain. In resistent mouse strains depletion of the $CD4^+$ T cell subset by mAb led to a breakdown of resistance, whereas depletion of $CD8^+$ T cells had no effect. A breakdown of resistance is also observed after neutralization of IFN-γ, leading to the generation of a Th2 response. Further investigation of this measles encephalitis model revealed that $CD4^+$ T cells are able to protect either alone (resistant mice), through cooperation with $CD8^+$ T cells (intermediate susceptible), or after immunization as secondary T cells (susceptible mice), and $CD8^+$ T cells are able to protect alone after immunization if they are cytolytic (Weidinger et al. 2000). As found earlier in nontransgenic mice, IFN-γ has also a critical role in the protection of CD46-transgenic mice against MV encephalitis (Patterson et al. 2002). Interestingly, this protection functions in a noncytolytic manner without neuronal loss.

CONCLUSIONS AND OUTLOOK

The observation that viruses can induce slowly progressive neurological diseases with diverse pathologies has led to speculations that other neurological diseases of unknown cause may have a viral etiology, the prime candidate being multiple sclerosis. This potential, together with epidemiological, neuropathological, immunological, and clinical findings, has encouraged extensive virological studies in pursuit of relevant infectious agents. Although several claims have been made for viruses isolated from multiple sclerosis patients, no infectious agent has yet been linked to the disease. Other diseases, such as amyotrophic lateral sclerosis, continuous focal epilepsy, or Alzheimer's disease, also reveal certain features that would be compatible with a viral infection. Recently, borna disease virus, which has been linked to a variety of neurological diseases in several animal hosts, has also been associated with certain psychiatric disorders in humans, such as schizophrenia and manic depression. No direct evidence is yet available to support the possibility that viruses cause these diseases, but their involvement cannot be ruled out.

A better understanding of the pathogenesis of viral CNS infections is a prerequisite to obtaining new and more effective means of prevention. However, each virus has different protection requirements. On the other hand, neurotropic virus infections also offer interesting possibilities for studying the structure and function of the nervous system, as in studies using neurotropic rabies or HSV for tracing neural pathways (Kruypers and Ugolini 1990). Moreover, neurotropic viruses are also used as vehicles to bring foreign genes to the CNS. Gene therapy approaches have been used in animal experiments to reduce tumor growth in brain tissue (Tapscott et al. 1994). It is therefore conceivable that a controlled viral infection of the CNS might be used for the selective expression of, for example, pharmacological agents or neurotransmitters in order to influence functional aspects of the CNS. Thus, studies of viral infections of the CNS offer new perspectives, leading to a better understanding of the pathogenesis of disease.

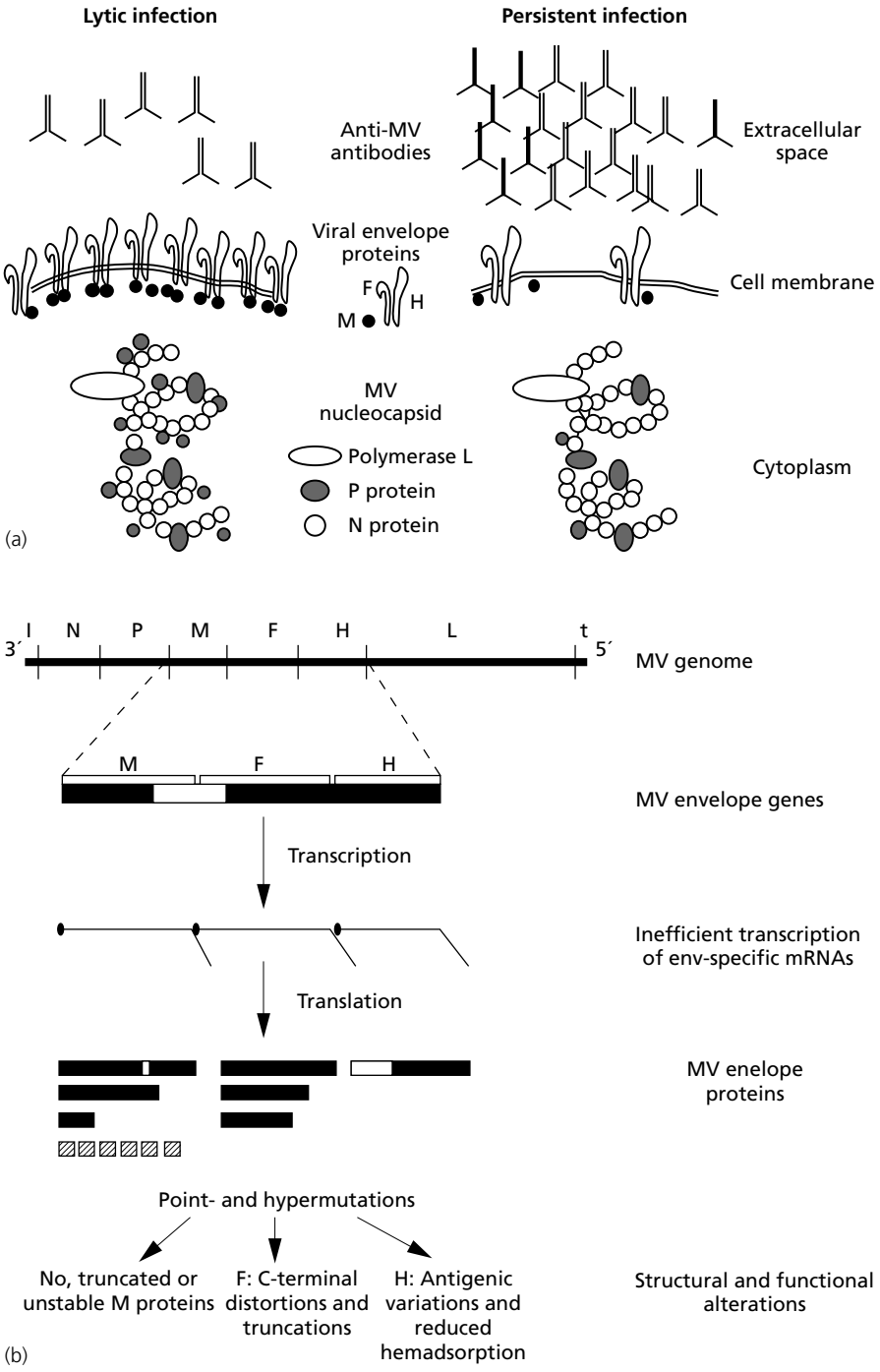

Figure 8 *Mechanisms of Measles virus persistence in the human CNS.* **(a)** *During acute infection the viral ribonucleocapsid particle (RNP) consisting of the genomic RNA encapsidated by the N protein and the transcriptase complex (L and P proteins) is amplified intracellularly. Concomitantly, the viral glycoproteins F and H (expressed as complexes) are expressed at the surface of the infected cell, with the M protein underlying their integration sites. In persistent infections the RNP structures are easily detected in large amounts in the cytoplasm (and the nucleus) of the infected brain cell. The expression of both the glycoproteins and the M protein is generally low or even absent. Pathognomonic for SSPE, a humoral hyperimmune reaction to viral antigens (mainly to N and H protein) is observed both in CSF and in serum.* **(b)** *The structural genes of MV are encoded in a linear arrangement along the MV genome: l represents the leader, t the trailer region that contains the viral promoter sequences and encapsidation sites. In persistent infections, the expression of the MV envelope genes* M, F, *and* H *(shown enlarged, the white region indicates a 1-kb noncoding region separating the* M *and* F *reading frames) is greatly reduced by different mechanisms: (1) the frequency of the corresponding mRNAs is very low, and (2) their reading frames are more or less affected by mutations that lead to complete abolition or truncated or distorted expression of the corresponding gene product. Most mutations have been encountered within the* M *gene, and all F proteins characterized so far have truncated C termini. Both point mutations (introduced by the viral polymerase) and hypermutations (clustered transitions most probably introduced by a cellular enzyme complex; see Figure 9) have been described.*

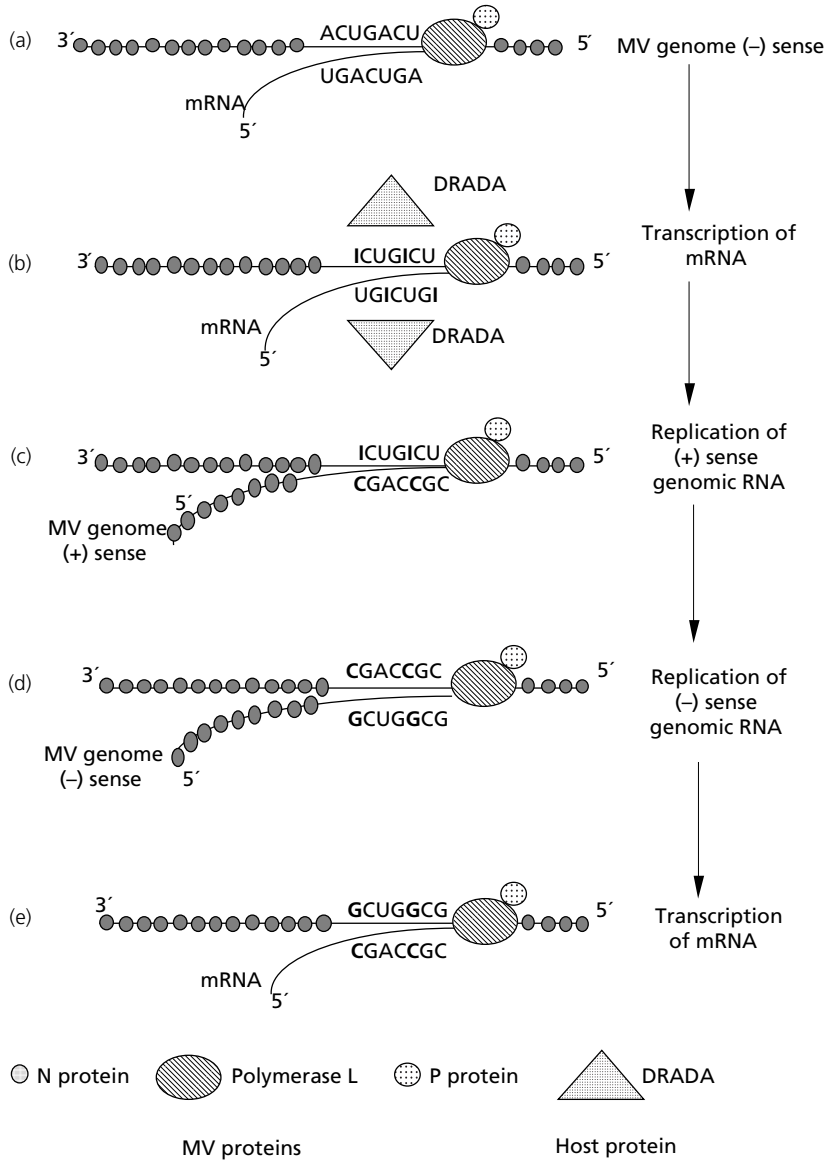

Figure 9 *DRADA (dsRNA adenosine deaminase activity): a cellular enzyme altering viral sequences. DRADA, initially detected and described in vitro in extracts of frog oocytes, is able to introduce clustered transitions into dsRNA structures both in vivo and in vitro. Increasing evidence suggests that unencapsidated viral dsRNAs (step a), accidentally formed during transcription/replication of negative-strand RNA viruses, may serve as targets for this cellular enzyme activity (step b) that deaminates adenosine (A) to inosine (I) residues. During replication of the positive-strand genome a cytidine (C) residue will be introduced instead of the expected uridine (U) into the positive-strand intermediate (step c); upon replication of this altered (+) genome, G will be introduced into the (–) genomic RNA (step d), which will in turn lead to the introduction of C into the mRNA (step e), replacing the initial U (step a). Although DRADA is mainly found in the nucleus, some activity has been consistently detected in the cytoplasm, in particular of neural cells.*

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Viral infections of the fetus and neonate, other than rubella

GISELA ENDERS

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INTRODUCTION

General factors in transmission of infection

The virus infections that may be transmitted from the mother during gestation or perinatally to the fetus or neonate are listed in Table 1. The importance of the various infections depends both on the frequency of maternal infection in the epidemiological situations prevailing in different geographic areas and on the frequency and seriousness of the infections in the embryo, the fetus, and the neonate. In this chapter, the various infections with known consequences for the fetus and infant are discussed more in order of importance than on a taxonomic basis. Details of the viruses themselves are given in their respective chapters in this volume. Virus infections in pregnancy with possible or suspected consequences for the fetus and infant (measles virus, mumps virus, Epstein–Barr virus, influenza a virus, human herpesvirus 6, human herpesvirus 7, and human herpesvirus 8, molluscum contagiosum virus, hantavirus, denguevirus, adenovirus, human respiratory syncytial virus, West Nile virus, TT virus, SEN virus) cannot be discussed on account of shortage of space. Full description of infections in pregnancy is published elsewhere (Enders 2005).

Transmission of a virus to the products of conception may occur within the uterus (congenital or prenatal infection), and during delivery or shortly thereafter (perinatal infection) (Table 2). Some viruses are transmitted only when the mother has an acute primary infection in pregnancy, others from chronically infected mothers. All such infections can be transmitted if maternal primary infection occurs shortly before delivery.

In pregnancy, the immune response is modified by a shift towards a Th2-type profile, which is associated with immunological tolerance (Reinhard et al. 1998). It has been hypothesized that this allows protection of the fetal allograft but may also influence the immune defense against microorganisms (Wegmann et al. 1993). However, data from animals and humans fit the Th1/Th2 concept, but newer results (e.g. the role of NK cells) suggest that the Th1/Th2 paradigm in pregnancy is an oversimplification and the situation is more complex (Chaouat et al. 2004).

In certain viral infections, though with some exceptions, the presence of antibodies in the female before conception protects both her and the fetus against infection and disease. In the presence of certain vaccine-acquired antibodies (e.g. rubella, measles, mumps, varicella) silent reinfection of the mother may occur, but usually without causing infection or disease of the fetus. Antibodies to viruses that establish latent or chronic infection

Table 1 *Virus infections in pregnancy with known consequences for the fetus and infant*

Virus	Consequences for the fetus and infant	
	Congenital infection	Perinatal infection
Rubella virus	+	–
Cytomegalovirus	+	+
Varicella-zoster virus	+	+
Herpes simplex viruses 1, 2	+/–	+
Parvovirus B19	+	–
Human immunodeficiency viruses 1, 2	+/–	+
Hepatitis virus A (*Picornavirus*)	+/–	+/–
Hepatitis viruses B, C, D, G	+/–	+
Hepatitis virus E	+	+
Enteroviruses: coxsackie/ echoviruses (*Picornavirus*)	+/–	+
Lymphocytic choriomeningitis virus	+	–
Genital human papillomaviruses	+/–	+
Hemorrhagic fever viruses[a, b]	+	+
Vaccinia virus[b]	+	–

+, common; +/–, less common; –, none.
a) Imported or in the respective geographic area.
b) Not described in detail.

(e.g. herpesviruses, human immunodeficiency viruses) before conception cannot prevent reactivation or recurrence, with consequent risks of fetal or neonatal infection.

The consequences of maternal infection for the embryo, fetus, or neonate are mainly determined by the gestational age and additional factors, such as the type of virus and its pathogenicity for the developing fetus. In the first few weeks of gestation many organisms may infect the pregnant woman and may lead to death and resorption of the embryo. Therefore the incidence of such infections is not known. The effect of embryonic or fetal infection may be apparent as early as the 6th to 8th week of gestation (WG) (WG 6–8). Infection acquired in utero may result in resorption of the embryo, abortion (until WG 20); intrauterine death (after WG 21); stillbirth (after WG 37); intrauterine growth retardation; prematurity (defined as the birth of a viable infant before the 37th week of gestation); malformation and other systemic abnormalities, and the sequelae of chronic postnatal infection. Signs of disease may be absent at birth, but serious sequelae may become apparent months or years later. Structural defects may occur following early maternal infection at the time of organogenesis (WG 3–8), for example caused by rubella. Maternal infection after WG 9 may lead to systemic abnormalities due to inhibition of organ development. Neonatal disease may also be caused by late intrauterine or perinatal infection.

The total rate of pregnancy loss after implantation due to all causes amounts to 31 percent (Wilcox et al. 1988). In cases with later implantation (>8–10 days after ovulation) the risk of early pregnancy loss markedly increases (Wilcox et al. 1999). Among the factors – mostly of unknown nature – that can affect the outcome of pregnancy, only 5–10 percent are attributable to maternal microbial infections and, of these, approximately 2 percent lead to congenital malformation or systemic abnormalities. These low rates are due to the existence of complex mechanisms of protection. The placenta is a powerful barrier with a wide variety of macrophages and lymphocytes. It allows the transit of maternal IgG, while preventing a large number of infective agents from reaching the embryo or the fetus. Furthermore, cellular immunity develops by the 12th week of gestation when antigen-presenting cells are detectable and the expression of MHC class I and II molecules by a variety of fetal tissues is evident (Lewis and Wilson 2001).

Diagnostic procedures

For the laboratory diagnosis of maternal, intrauterine, and perinatally acquired infections, new techniques for detecting both viruses and their antibodies have become available. The traditional methods for detecting viruses and viral antigens have been replaced or supplemented by detection of specific nucleic acid by various molecular biological techniques, such as the polymerase chain

Table 2 *Transmission of viral infections to embryo, fetus, and neonate*

Transmission	Mode	Time of gestation	Infections
Transovarian	Infected sperm	Early	Seldom: CMV, HIV-1
Intrauterine	Hematogenous transplacental	1st to 39th week	Rubella virus, CMV, parvovirus B19, VZV, coxsackie and echoviruses, HIV-1,[a] HBV,[a] HCV[a]
	Local extension from foci of infection (e.g. ovaries)		
	Ascending from vaginal tract	Late, after rupture of membranes	CMV, HSV, HIV-1
Perinatal	Infected birth canal	Delivery	CMV, HSV, HIV-1, HBV, HCV, HPV
Early postnatal	Breast milk/environment	After birth	CMV, HSV, HBV, HIV-1, HCV?

CMV, cytomegalovirus; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomaviruses; HSV, herpes simplex virus; VZV, varicella-zoster virus.
a) Possible, but rare.

reaction (PCR). For serology, basic and newly developed supplementary tests are now available. Whether the virological or serological route for diagnosis is pursued depends on the particular agent and on the availability of sensitive and specific assays for detecting it.

Unless stated otherwise, the techniques for diagnosing the various viral infections are the same as those used in nonpregnant people and are described in the relevant chapters in this volume.

Direct tests on the fetus commenced in 1983 (Daffos et al. 1983); the methods include ultrasound screening at low and high levels, detection of fetal infection by identification of the agent in chorionic villi biopsies, amniotic fluid, and fetal blood (Table 3) and by tests for specific IgM/IgA antibodies and cellular immunity in fetal blood. Methods for ensuring the purity of fetal blood samples are essential, and nonspecific biological, biochemical, and immunological factors reflecting fetal functions are also applied (Forestier et al. 1988). The optimal time for chorionic villi sampling (CVS) is at about WG 11, when the risk of spontaneous abortion is lowest. In case of positive results, CVS has the advantage of early decision on further management of the pregnancy, however there is a risk of false-positive results due to contamination with maternal blood still containing the infectious agent. The added risk of fetal complications after amniocentesis is 0.2–1 percent (Hanson et al. 1990; Blackwell et al. 2002; Scott et al. 2002) and the risk linked to fetal blood sampling by experienced teams is 0.5–1 percent in healthy fetuses (Maxwell et al. 1991).

Invasive prenatal diagnosis has been applied to pregnancies complicated by infections with, for example, rubella virus, cytomegalovirus, parvovirus B19, varicella-zoster virus, *Toxoplasma gondii* and *Borrelia burgdorferi* infections (Enders and Jonatha 1987; Daffos et al. 1988; Enders 1994; Hohlfeld et al. 1994; Tercanli et al. 1996; Enders and Miller 2000; Enders et al. 2001, 2004). Prenatal diagnosis has proved helpful in rescuing many unaffected fetuses from being lost by termination of pregnancy.

To interpret the results obtained by tests on the fetus (Table 3), the pathogenicity of the agent for mother and fetus, the time of maternal infection during gestation, and the specificity and sensitivity of the various methods must be considered. The ultrasound findings at high level are of major importance when deciding to continue or terminate a pregnancy. The predictive value of tests on the fetus can be fully estimated only by follow-up investigations: in cases of therapeutic abortion by detection of the virus or other agent in fetal tissue; or, if pregnancy continues, by tests for agents and specific antibodies in the infant.

In pediatric diagnosis, congenital infection can be assumed if either virus or IgM antibodies are detected in samples collected shortly after birth; perinatal or early postnatal infection is diagnosed if tests for virus and IgM antibody were negative soon after birth, but positive in samples obtained 3–4 weeks later (Tables 4 and 5).

It should be remembered that, even though clinically apparent congenital virus infections are infrequent, there are some important ones whose risk can now be reduced by preventive measures (vaccination in childhood and before pregnancy, safe sexual behavior before and during pregnancy, hygienic measures, and for certain infections passive prophylaxis in case of contact or therapy). Great progress has been made in achieving a correct laboratory diagnosis followed by intervention, such as prenatal diagnosis, therapy in utero, e.g. red cell intrauterine transfusion or therapeutic abortion if appropriate. The need and value of the formerly so-called TORCH or STORCH screening (Editorial 1990), now known under the acronym TORCHES-CLAP (*Toxoplasma gondii*, rubella virus, cytomegalovirus,

Table 3 *Prenatal diagnosis of fetal infection*

Week of gestation	Investigation	Sample	Object of detection
⩾11	Biopsy	Chorionic villi	Virus: by nucleic acid (PCR), antigen, culture
18–23	Amniocentesis	Amniotic fluid	Virus: by nucleic acid (PCR), antigen, culture
17–39	Ultrasonography of high level		Anatomical abnormalities
22–23	Cordocentesis and amniocentesis	Fetal blood (ensure purity of sample)	Specific IgM
			Total IgM (mg/dl)
			Other nonspecific biological, biochemical markers
		Fetal blood/amniotic fluid	Virus: by nucleic acid (PCR), antigen, culture
⩾24	If ultrasound is abnormal, investigation as for weeks 22–23		

PCR, polymerase chain reaction.

Table 4 *Virological differentiation between congenital and perinatal infections*

Type of infection	Transmission	Viral Infection		
		At birth	Neonate	Infant
Congenital	Intrauterine	+	+	+/–
Perinatal/early postnatal	Extrauterine	–	+/–	+

Adapted from Griffiths (1990).+, positive; +/–, positive or negative; –, negative.

Herpes simplex virus, enteroviruses, syphilis (*Treponema pallidum*), chickenpox (varicella-zoster virus), Lyme disease (*Borrelia burgdorferi*), acquired immune deficiency syndrome (AIDS) (human immunodeficiency virus), parvovirus B19, not to mention hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis G virus, hepatitis E virus, papillomavirus, and lymphocytic choriomeningitis virus) should be re-evaluated from time to time (Sutherland 1993; Klein and Remington 2001). This appears necessary with respect to changing epidemiology due to increasing vaccination and progress in preventing certain infections important for pregnancy. Moreover, globalization provides opportunities for the emergence/re-emergence of infectious diseases. The role of newly recognized viral pathogens in pregnancy, imported or in the respective geographic area, has to be elucidated.

CYTOMEGALOVIRUS

Cytomegalovirus (CMV), a gammaherpesvirus, is the most frequent viral cause of congenital infection in humans, with symptomatic disease at birth, and short- and long-term sequelae. CMV establishes latency and persists so for life, periodically reactivating from this latent state. In immunocompetent persons, including pregnant women, primary CMV infection is usually asymptomatic and only a small portion (<10 percent) presents with clinical symptoms such as mononucleosis-like illness. Primary maternal CMV infection is the major risk for congenital disease. Recurrent infection, which quantitatively accounts for most of the congenital infections in highly immune populations worldwide is always asymptomatic and clinical manifestations in the infected fetus or newborn infant are rare.

Infection in pregnancy

EPIDEMIOLOGY OF MATERNAL CMV INFECTION

CMV antibody prevalence differs in various populations according to socioeconomic factors, race, geographic location, and lifestyle. In the USA and western Europe, the antibody prevalence rates in women aged 20–40 years from the higher socioeconomic group ranges from <40–60 percent (mean 45 percent) compared to 85 percent in the lower socioeconomic group. In developing countries more than 90 percent of young women are positive for CMV antibodies (Yow et al. 1988; Istas et al. 1995; Enders et al. 2003).

CMV is not very contagious and requires close or intimate contact with infectious secretions. No data have indicated CMV transmission through respiratory droplets. Infections in adolescent women (aged 14–20) are acquired mainly by sexual/oral contact with saliva, genital secretions, and semen (Shen et al. 1994), particularly in those of lower socioeconomic groups who change sex partners frequently (Istas et al. 1995; Coonrod et al. 1998). Women aged ⩾25 years in the middle and upper socioeconomic classes may acquire infection predominantly by close contact with asymptomatic infants and toddlers excreting CMV in saliva and urine (Stagno and Cloud 1994; Sobaszek et al. 2000).

Primary infection, as well as recurrent or secondary infection, can lead to vertical transmission and congenital infection. The annual rate of primary infection in susceptible pregnant women ranges in British, American, Swedish, and German studies generally from 0.4 to 4.1 percent (Ades 1992; Stagno et al. 1986; Enders et al. 2003). As shown in the US study primary infection is lower (2 percent) in the higher income group as compared to the lower income group with 6 percent (Stagno et al. 1986). Furthermore, it was documented that the annual seroconversion rate increased with the

Table 5 *Serological differentiation between congenital and perinatal infections*

Type of infection	Transmission	Virus-specific antibodies		
		At birth	Neonate <4 weeks	Infant >4 weeks to 1 year
Congenital	Intrauterine	IgM+/–	IgM+	IgM–
		IgG+	IgG+	IgG+
Perinatal/early postnatal	Extrauterine	IgM–	IgM+/–	IgM+
		IgG+	IgG+	IgG+
None	None	IgM–	IgM–	IgM–
		IgG+	IgG+	IgG–

Adapted from Griffiths (1990).+, positive; +/–, positive or negative; –, negative.

number of pregnancies from about 2 to 3 percent in the first pregnancy to 4 to 7 percent between the second and third pregnancy (Stagno et al. 1986; Yow et al. 1988). It also became evident that seronegative mothers whose children acquired the infection in daycare centers have a 30 percent annual risk for seroconversion. On average these mothers acquired the infection within 4.2 months (range 3–7 months) after their children became infected (Adler 1988). The route of transmission among children in group daycare is caused by horizontal transmission from child to child. This is supported by analysis of the restriction enzyme digestion patterns of CMV-DNA of the virus isolates of the infected children (Adler 1988). The highest risk is posed by toddlers in their second year of life. These children are an important potential source for infection of susceptible parents or caregivers. The risk for seroconversion among seronegative caregivers ($n = 202$) was 11 percent per year in contrast to seronegative women employed in a local hospital ($n = 229$) with 2.0 percent (Adler 1992). Over all, approximately 25 percent of serious congenital infections occurring each year in the USA can be attributed to exposure in daycare centers.

PRIMARY INFECTION AND EFFECTS ON THE FETUS

Primary infection is defined as first contact with the virus. Characteristic symptoms (lymphadenitis with fever and hepatic dysfunction) occur in only 6.5 percent and suspicious symptoms (lymphadenitis with flulike symptoms) in about 19.4 percent (Daiminger et al. 2004). Therefore diagnosis of infection and immune status relies on laboratory tests. The association between primary CMV infection and fetal loss is not clear. In one early study, the number of spontaneous abortions after primary infection in early gestation was higher (4/26; 15 percent) than in the control group (16/744; 2 percent) (Griffiths and Baboonian 1984). A further low numbered study could not confirm an association between primary infection in early gestation and abortion (Yow et al. 1988). Although some studies published in the 1990s detected CMV DNA in abortive or placental tissue, up to now no correlation between active CMV infection and fetal loss was found, supporting the fact that CMV is not a major abortion-related factor (Spano et al. 2002).

Primary infection in pregnancy, regardless of socioeconomic status, accounts for 24–75 percent (mean 40 percent) of congenital infection (Stagno et al. 1986). These data were confirmed by newer studies on prenatal diagnosis in which intrauterine transmission rates varied between 23.5 and 68 percent (Revello et al. 1995; Ruellan-Eugene et al. 1996; Bodus et al. 1999; Lazzarotto et al. 2000; Liesnard et al. 2000; Azam et al. 2001; Enders et al. 2001).

Most studies reported a constant transmission rate throughout pregnancy following maternal primary infection, but some workers found a higher rate in late pregnancy. However all authors agree that the risk for congenital disease is strongly connected with infection in the first 12 weeks of pregnancy (Stagno et al. 1986; Enders et al. 2001; Daiminger et al. 2004). The risk of fetal damage following preconceptional maternal infection (8 to 2 weeks before last menstrual period) appears to be low, but more data are needed to confirm this observation (Revello et al. 2002; Daiminger et al. 2004). In this respect, the study of Fowler et al. (2004) is of interest, reporting that maternal infection up to 2 years before renewed pregnancy may still pose a risk for congenital infection (Plotkin 2004).

RECURRENT INFECTION AND EFFECTS ON THE FETUS

Recurrent (or secondary) infections are defined as either reactivation of endogenous virus or reinfection with a new strain. The most frequent mechanism for recurrent infection during pregnancy seems to be reactivation of latent virus. However, reinfection by CMV strains other than the original infecting strain, particularly in women with multiple sexual partners, has been demonstrated by restriction enzyme analysis (Chou 1990) and indirectly by measuring antibody specificity of glycoprotein H against CMV laboratory strains and isolates (Boppana et al. 2001).

Recurrent infections occur in the presence of both humoral and cellular immunity, but in the absence of a viremic phase. In contrast to primary infection the transmission rate is very low and amounts to only 1 percent (Fowler et al. 2003), however quantitatively recurrent infections account for most of the subclinical infections worldwide (Stagno et al. 1986; Fowler et al. 2003; Griffiths and Baboonian 1984). It is generally recognized by the presence of specific IgG antibodies before conception or by the presence of IgG antibodies in the first 12 weeks of gestation in the absence of IgM antibodies and by detection of congenital infection in the offspring (Fowler et al. 1992).

Recurrent infection occurs most frequently in the late second and third trimester, when a marked transient depression of CMV-specific cellular immunity was observed (Gehrz et al. 1981; Baboonian et al. 1989). With advancing gestational age the rate of viral shedding from the uterine cervix and urine increases (Shen et al. 1993) suggesting that productive CMV infection in nonpregnant women and in women during early gestation is under the control of the cellular immune system. In relation to CMV and pregnancy it is interesting that the rate of viral shedding from urinary or genital secretions of seropositive pregnant women is inversely related to age. For genital secretion, in one study, the high level of 15 percent at the age of 11–14 years falls to undetectable levels in women aged 31 years and older. For urinary excretion, the rate falls from a peak of 8 percent in the younger age group to zero in women

>26 years old. No excretion occurred from either site in postmenopausal women (Knox et al. 1979). Less well investigated is the influence of age in males on CMV excretion in semen (Shen et al. 1994). The relationship between maternal age and congenital infection and disease for maternal primary and recurrent infection in different populations is not yet well defined.

Between 1970 and 1973, it became evident that congenital infection can occur in consecutive pregnancies. In three independent studies, the first infant was severely affected or died and the second born in each case was subclinically infected. Thereafter, several case reports on congenital infection with disease in infants of seropositive mothers with and without immunosuppression have been published (Stagno 2001). Some recent publications indicate that symptomatic infection at birth in infants born to immune mothers may be more frequent than previously recognized (Ahlfors et al. 1999; Boppana et al. 1999; Casteels et al. 1999). However, it should be remembered that, in immune mothers, the rate of vertical transmission is about 1 percent, the rate of defects at birth in newborns less than 1 percent and the risk of late sequelae 5–8 percent (unilateral hearing loss approximately 5 percent or microcephaly 2 percent).

PATHOGENESIS OF FETAL INFECTION

In primary maternal infection, it is assumed that intrauterine infection results from maternal viremia with subsequent placental infection and hematogenous dissemination to the fetus. It is still undefined whether cell-free or leukocyte-associated virus is required for the spread from the mother to the fetus through the placenta. In detail, infected leukocytes may transmit the infection to uterine microvascular endothelial cells which are in direct contact with the cytotrophoblasts of anchoring villi with subsequent infection of underlying tissues of villous cores including fibroblasts, fetal macrophages, and endothelial cells (Sinzger et al. 1993; Gabrielli et al. 2001). Alternatively infected maternal leukocytes may directly spread the infection to the villous stroma through breaches of the syncytiotrophoblast layer (Hemmings et al. 1998). As a further hypothesis, transportation of antibody-coated CMV virions by transcytosis through the intact syncytiotrophoblast has been discussed (Fisher et al. 2000).

Following direct infection of placental and chorionic tissue, the virus may also spread to amniotic cells that are then ingested by the fetus. In this case the virus replicates in the oropharynx and is then carried through the fetal blood circulation to target tissues such as the kidney. The renal tubular epithelium seems to be a major site of replication. By either mechanism of intrauterine infection the fetus would excrete CMV via urine into amniotic fluid, which seems therefore to be the optimal specimen for prenatal diagnosis of CMV fetal infection.

Other modes of intrauterine transmission may include transovarian infection by infected semen (Shen et al. 1994) and in late pregnancy, by ascending infection from the vagina after premature rupture of the membranes (Ohyama et al. 1992).

The pathogenesis of fetal infection in twin pregnancies is not yet systematically evaluated with respect, for example, to type (primary or recurrent) and time of maternal infection. From the literature it appears, that in the case of monozygotic/monoamniotic pregnancies both fetuses are infected, whereas in dizygotic/diamniotic pregnancies one or both fetuses can be infected. The infected fetuses show various degrees of symptoms at birth. Gabrielli et al. (2003) presented the possibility of horizontal (fetus-to-fetus) transmission for a dichoriotic, diamniotic twin pregnancy with fused placentas. They found that only one fetus was infected at WG 21, whereas both fetuses were infected at birth. Altogether, because intrauterine transmission occurs in only 30–40 percent of pregnant women with primary infection, a mechanism that is not understood but is generally referred to as placental barrier must operate to prevent fetal infection.

In recurrent infection, the mode of CMV transmission in immune mothers is not well known. It is unclear how the virus evades the immune system under these circumstances. Latent CMV reactivates from sites that are less accessible to virologic examination, like placental tissue, ovaries, endometrium, or cervix (Mocarski et al. 1990; Reddehase et al. 1994). Reactivation of CMV especially from macrophages during local immunosuppression in the uterus may be a possible mechanism (Revello and Gerna 2004). As shown by dot-blot hybridization of PCR products CMV DNA was detected in 30 percent of placentas of seropositive mothers, localized mostly to the villi, the extravillous trophoblasts, and decidual cells. The frequency of CMV DNA detection in placentas increased from 18.2 percent for deliveries in the second trimester to 32.9 percent in the third trimester (Kumazaki et al. 2002). This is compatible with the increasing rate for cervical and urinary shedding with advancing gestational age.

In the fetus, the fetal cellular and humoral immune responses to infection are reduced compared to that of the adult (Lewis and Wilson 2001). Cellular immunity such as CMV-specific CD8 cells could be detected in congenitally infected fetuses as soon as WG 22, but may not be fully functional as shown by IFN-γ secretion (Elbou Ould et al. 2004). Humoral immune responses are triggered by immunoglobulin-secreting plasma cells, which are detectable by the 15th week of gestation (Toivanen et al. 1969; Gathings et al. 1981).

CONGENITAL DISEASE

Congenital CMV infection, affecting worldwide approximately 0.2–2 percent of all live-born infants (in the USA, mean approximately 1 percent), is one of the leading causes of mental deficiencies. Up to one in ten

newborns has signs at birth commonly associated with congenital CMV disease and about half of these have the classic stigmata of cytomegalic inclusion disease (CID). Another 5 percent present with milder or atypical involvement (e.g. hydrocephalus, hemolytic anemia, pneumonitis, and inguinal hernia) and approximately 90 percent are born with subclinical, but chronic, infection (Demmler 1994). Table 6 presents the clinical manifestations of congenital CMV disease in the newborn period. Most of the data come from the USA National Congenital CMV Disease Registry, the worldwide greatest analysis of congenital disease (n = 786 babies in the 11th year of investigation) (Demmler 2002).

Congenital CMV infection is the leading nongenetic cause of sensorineural hearing loss (SNHL) in childhood, causing 20–30 percent of all deafness cases (Barbi et al. 2003). By universal otoacoustic emission (OAE) screening of neonates as practiced, for example, in the USA, only about half of all SNHL caused by congenital CMV can be detected and late onset hearing loss, which generally occurs up to 6 years of age will pass unnoticed (Fowler et al. 1999). Ocular disease, which manifests principally by chorioretinitis, is detected more frequently in infants symptomatic at birth (Table 6). Because 23 percent of infants with chorioretinitis have either progression of lesions or delayed onset after the first year of life, serial eye examinations are recommended (Anderson et al. 1996; Coats et al. 2000). SNHL usually passes unnoticed for the first 1–2 months of life, as does the less common chorioretinitis. Among the most infected infants mortality ranges between 10 and 12 percent mainly in the neonatal period. More than 90 percent of the survivors have major long-term sequelae. The severity of neurologic impairment is highly variable. In infants with symptomatic infection, normal cranial computer tomographic scan (CT) and normal head circumference proved to be a predictor of favorable cognitive and neurologic outcome (Noyola et al. 2001). However, in symptomatically infected infants presenting with abnormal CT (about 70 percent) and microcephaly, subsequent neurologic abnormalities and mental retardation (IQ <70) may be expected in 90 and 56 percent, respectively (Boppana et al. 1997). Still no factors are identified for predicting progression of hearing loss. Table 7 compares the sequelae in congenital CMV infection in infants symptomatic and asymptomatic at birth. Asymptomatically infected infants have a 10 to 15 percent risk of abnormal development, such as SNHL, chorioretinitis, and microcephaly with varying degrees of mental retardation and neuromuscular defects. Except for SNHL, which may be detected even at school age, these sequelae usually present during the first 2 years of life (Table 7).

Encouraging aspects are that infants with asymptomatic infection at birth seem to have no differences in IQ and other neuropsychological tests compared with a healthy control collective (Temple et al. 2000; Kashden et al. 1998; Demmler 2004). Furthermore, children with congenital CMV infection, including those with systemic signs at birth, are unlikely to be at increased risk of subsequent neurodevelopmental or intellectual impairment if they show normal development at 12 months of age (Ivarsson et al. 1997).

Table 6 *Clinical manifestations and abnormalities in the newborn period with congenital CMV infection*

Abnormalities	Percentage (approx.)
Prematurity (<38 weeks)	34
Small for gestational age	47
Petechiae and purpura	54
Jaundice	36
Hepatomegaly	47
Splenomegaly	44
Pneumonia	11
Thrombocytopenia	54
Hemolytic anemia	13
Elevated liver enzyme values	46
Neurological findings	
One or more of the following	72
Microcephaly	40
Intracranial calcification	43
Lethargy/poor feeding	25
Convulsions	7
Hearing impairment	41
Chorioretinitis	11
Death in the first 6 weeks	12

Table 7 *Sequelae in children with congenital cytomegalovirus infection*

Sequelae	Symptomatic at birth (%)	Asymptomatic at birth (%)
Sensorineural hearing loss	41–58	7.4
Bilateral hearing loss	37	2.7
Speech difficulties	27	1.7
Chorioretinitis with/ without optic atrophy	20	2.5–3.9
Visual impairment	22	1.2
IQ <70	55	3.7
Microcephaly with convulsions or paresis/ paralysis	52	2.7
Microcephaly	37	1.8
Convulsions	23	0.9
Paresis/paralysis	13	0
Death after the newborn period	6	0.3

According to/adapted from: Stagno (2001); Anderson et al. (1996); Coats et al. (2000); Dahle et al. (2000).

PERINATAL AND EARLY POSTNATAL INFECTION

Perinatal infection is acquired by passage through the infected birth canal and early postnatal infection mainly by infected breast milk of a CMV-IgG-positive mother as a result of primary or recurrent infection. The source of both types of infection is due to local reactivation of the virus in the cervical and mammary gland.

The correct rate of perinatal infection is more difficult to determine because it can only be assumed in vaginally delivered infants of seropositive mothers with detection of virus in their cervicovaginal secretion at the time of delivery and in throat or ear swabs of the newborns. In an earlier study of this kind, the rate of perinatal infection was found to be 57 percent; in a similar more recent study restricted to preterm infants it was 22 percent (Hamprecht et al. 2001).

The CMV excretion rate in breast milk of seropositive mothers using highly sensitive diagnostic methods for detection of CMV such as DNA with PCR amounts to 88–96 percent (Mosca et al. 2001; Hamprecht et al. 2001). Instead of the less sensitive virus culture detection of CMV pp65 late mRNA was used to indicate replicating virus. Viral shedding in breast milk was seen as early as 2–3 weeks after delivery, peaks in activity by 3–6 weeks, and ending in most individuals by 8–10 weeks (Hamprecht et al. 2001; Numazaki et al. 2001). It is known that there is a considerable variability in perinatal and early postnatal CMV transmission throughout the world, which is due to maternal factors such as age, practice of breast-feeding and socioeconomic, geographic, and ethnic background.

Breast-feeding in premature infants is beneficial. However, breast-feeding of a high-risk group of preterm infants may be associated with symptomatic CMV infection (Hamprecht et al. 2001; Maschmann et al. 2001). This is shown in a 3-year prospective study of 176 preterm infants weighing <1 500 g (<32nd week of gestation) with a high infection rate of 38 percent (33/87 infants). The mean incubation period between positive DNA detection in breast milk and virus or viral DNA detection in the infant was 42 days (range 28–69 days). Half (16 of 33) of the infected infants had symptoms compatible with CMV disease: hepatomegaly, neutropenia, thrombocytopenia, and four infants had sepsis-like deterioration in the early phase of CMV infection. All infants recovered and thus far, no negative effect on neurodevelopment or hearing was found at follow-up of 22 CMV-infected preterm infants at 2–4.5 years of age and matched controls. Besides the relatively favorable outcome of infected preterm infants by breast-feeding, prevention of CMV infection through contaminated breast milk was and still is a challenge. The aim is removing CMV from breast milk without destroying nutritional–immunological factors (Forsgren 2004).

Perinatal and early postnatal CMV infection are as chronic as congenital infection with virus shed into urine up to 6 years (mean 3.9 years) and into saliva up to 2–4 years with the highest quantity excreted during the first 6 months of life. However, the quantity of virus excreted is less than in infants with symptomatic congenital infection.

Transfusion-acquired early postnatal CMV infection and disease is hardly observed due to the use of CMV antibody-negative blood available through more sensitive screening methods and improved pathogen inactivation methods (Roback 2002).

Diagnosis

DIAGNOSIS IN PREGNANCY

Primary CMV infection in pregnancy is usually detected by chance, as primary infection is generally asymptomatic or may cause nonspecific symptoms.

With the basic serological tests, primary infection can best be detected by seroconversion or rising IgG and IgM antibody titers to high level within 2–4 weeks of onset. Medium- to low-level IgM antibodies can be detected in less recent primary infection, as well as in persistent (chronic) and recurrent infection. This makes IgM an insufficient marker for diagnosing and timing primary infection, particularly since the various commercial IgM tests greatly differ in sensitivity and specificity (Landini 1993; Daiminger et al. 1999). Therefore, sera with positive IgM results should be retested in supplementary assays such as the IgG avidity enzyme immunoassay (EIA) (Eggers et al. 2000), the microneutralization assay (Eggers et al. 1998), and the gB/gH recombinant immunoblot (Eggers et al. 2001). Thus, primary maternal infection may be diagnosed with a single serum sample up to WG 20 by negative or low values in the IgG avidity assay and the microneutralization test (Enders et al. 2003, personal communication).

The cellular immune response determined by CMV-specific lymphoproliferative or cytotoxic assays in women with recent primary CMV infection is not routinely assessed, for example, for the prediction of intrauterine transmission. The first study of this kind (Stern et al. 1986) revealed that newborns of women with a good lymphocyte response were not infected ($n = 8$), whereas mothers whose lymphocytes did not respond delivered infected babies (4/6). In a later study there was a fair, but not complete correlation between a strong lymphocyte response and birth of noninfected infants (Fernando et al. 1993).

The detection of virus shedding in cervical secretion and urine in pregnant women is of little value for distinguishing primary from recurrent maternal infection, because CMV may be excreted intermittently in primary as well as recurrent infection.

Breast milk of lactating seropositive mothers of preterm infants may be investigated for presence of CMV DNA by PCR. However, negative results are of minor value for deciding whether or not to breast feed as excretion may be delayed or often intermittent.

Altogether there is presently no serologic or immunologic test that can predict, for example, protection against intrauterine infection and disease in the fetus (Weinberg 2002).

DIAGNOSIS IN THE FETUS

Prenatal invasive diagnosis of fetal CMV infection is increasingly used in pregnant women with serological suspicion of acute or recent primary infection and in those with abnormal findings by ultrasound examination, whose CMV antibody status is mostly unknown. A variety of sonographic findings has been reported from a number of authors: ascites, fetal growth retardation, cerebral ventriculomegaly, microcephaly, hydrocephalus internus, and oligohydramnion are the most common features. A wide range of brain abnormalities (e.g. intracranial calcification) and hyperechogenic fetal bowel has also been documented in addition to cardiomegaly, hepato- and splenomegaly, hydrops fetalis, or placentomegaly as reviewed by Crino (Crino 1999; Enders et al. 2001). In one study, more than two fetal abnormalities were recorded in 46 percent (27/58) and only one sign in 22 percent (13/58). Fetal abnormalities were first observed between WG 18 and 22 in 40 percent and beyond WG 23 to 32 in 60.3 percent at which time termination of pregnancy is a difficult option (Enders et al. 2001 and update 2001–2004). For detection of fetal intracranial abnormalities transvaginal sonography in WG 22–37 (mean gestational age of 27.5 weeks) provided additional information for all fetuses and fetal magnetic resonance imaging (MRI) in one of two cases (Malinger et al. 2003). In counseling the parents it is necessary to mention not only what can be seen of the characteristic manifestations of congenital infection but also what cannot be seen, like hearing defects or chorioretinitis.

Prenatal diagnosis relies on the detection of viral DNA by PCR and infectious virus by the rapid shell vial culture in amniotic fluid and on the detection of DNA and CMV-specific IgM antibodies in fetal blood (Table 3). Detection of CMV in chorionic villi was more rarely attempted. Amniotic fluid is the preferred specimen not only because of the relatively low technical risk of fetal loss with amniocentesis in undamaged fetuses (0.2–1 percent) but also because CMV is excreted into fetal urine, which accumulates in amniotic fluid as pregnancy proceeds. For avoiding false-negative results, amniotic fluid should be obtained after WG 21. In all instances an interval of at least 6 weeks between presumed onset of maternal infection and invasive procedure is recommended (Revello et al. 1995; Bodus et al. 1999; Liesnard et al. 2000; Enders et al. 2001; Gouarin et al. 2001).

In fetal blood obtained after WG 20 CMV DNA detection by PCR was found to be more sensitive (92.6 percent) than detection of IgM antibodies with a sensitivity of approximately 50–80 percent, especially in fetuses with overt infection (Enders et al. 2001 and update 2001–2004; Lipitz et al. 1997; Hagay et al. 1996; Revello et al. 1995; Liesnard et al. 2000).

In one study, the predictive value of prenatal diagnosis on fetal infection, as estimated by the outcome of pregnancy, for positive results (obtained with at least two detection methods) was 100 percent (74/74) and for negative results 96.1 percent (149/155) (Enders et al., 2001 and update 2001–2004). Generally, isolated positive PCR results in only one sample must be considered with caution. Therefore additional cell culture-based methods (shell vial assay or isolation in cell culture) or alternative DNA and RNA detection methods (e.g. nucleic acid sequence based amplification (NASBA), quantitative PCR), PCR in another sample, for example, fetal blood, or in addition a second sampling may be performed for a safe prediction of fetal infection.

The following virological markers may be useful for the prognosis of fetal outcome: viral load in amniotic fluid (Lazzarotto et al. 2000; Guerra et al. 2000; Enders et al. 2001; Gouarin et al. 2002) or fetal blood (Revello et al. 1999b) and presence of CMV-specific IgM antibodies in fetal blood (Revello et al. 1999b; Enders et al. 2001). Also nonspecific biological, biochemical, and immunological markers for fetal function (Azam et al. 2001; Forestier et al. 1988) may provide supportive evidence of congenital disease.

The present experience suggests that prenatal diagnosis carried out with well-controlled serological and virological techniques and ultrasound screening at high level saves a considerable number of pregnancies, since fetal infection can be excluded in about 75 percent of primary maternal infections in pregnancy (Enders et al. 2001 and update 2001–2004, personal communication).

DIAGNOSIS IN INFANTS

The diagnosis of suspected congenital infection in newborns is confirmed by detection of virus in saliva, urine, blood, and by detection of IgM antibodies in cord blood or blood of the neonate.

All the different CMV genotypes (four gB and four gN variants) can be vertically transmitted and produce congenital disease. However, up to now, it seems that the 'potential of infectivity' and ability to produce severe disease is not related to a single polymorphic gene alone (Barbi et al. 2001; Pignatelli et al. 2003).

The virus detection is carried out today mainly by detection of CMV DNA by PCR using commercial and 'in-house' tests of various sensitivities and by detection of infectious virus in the shell vial culture or less frequently in conventional cell culture. Virological tests must be initiated within 2 weeks after birth to distinguish congenital from perinatal or early postnatally

acquired infection. It is of interest that higher CMV DNA levels are detected in serum (Nelson et al. 1995) and peripheral leukocytes (Revello et al. 1999a) of symptomatic infants than in asymptomatic infants and that in the former the mean duration of leuko-DNAemia was longer (156 ± 50 versus 40 ± 8 days).

More recently a simple and reliable diagnosis of congenital infection can be accomplished by demonstration of CMV DNA in dried blood spots (DBS) on Guthrie cards (Barbi et al. 2000). The DBS test could be used as an alternative to viral culture. The main benefit comes from the fact that congenital infection may retrospectively be diagnosed beyond the neonatal period or to link morbid conditions like SNHL or white matter abnormalities in brain MRI to congenital infection. However the sensitivity of the DBS test, especially when used retrospectively has to be further investigated.

Antibody testing for detecting prenatal infection is less informative. After birth, approximately 70 percent of symptomatic newborns are IgM antibody positive in contrast to only 30 percent of asymptomatic infected neonates; IgM antibodies are usually detectable for 3–4 months in declining levels (Revello et al. 1999a; Enders et al. 2001). IgG antibodies measured in the cord blood or in the blood of the newborn are mostly of maternal origin. An initial decrease of IgG antibody is followed by an IgG antibody increase and lifelong persistence at medium to low titers. The presence of IgG antibody in asymptomatic infants ⩾8 months of age can be due to congenital or perinatal/early postnatal infection (see also Table 5). In this respect the IgG avidity test is helpful in identification of the type of infection (G. Enders, personal communication).

Management

MANAGEMENT IN PREGNANCY

Preventing primary CMV infection by hygienic measures and altering their lifestyle is difficult to achieve and only effective in women who want to become pregnant or are pregnant as they may be highly motivated to follow hygienic guidelines (Adler et al. 1996).

Seronegative pregnant women with significant exposure to CMV at home or at work should be serologically controlled in 2–3-weekly intervals up to 12 weeks after first exposure and at delivery to demonstrate that no infection has occurred (Enders 2003).

For passive prophylaxis, several intravenous hyperimmunoglobulin preparations (CMVIG) with known neutralizing antibody titers are available. They may be used for forensic aspects in seronegative pregnant women with close occupational contact to newborns, infants, or toddlers known to excrete CMV with the goal of preventing maternal infection. From our own experience, passive prophylaxis is less important or needed than observing correct hygienic measures. Antenatal care directives for CMV seronegative pregnant caregivers differ from country to country from reinforcement of hygienic measures to (for example, in Germany) removal from the workplace for the whole of the pregnancy or restriction to work with infants more than 3 years of age.

In cases of suspected primary infection in the first and second trimester of pregnancy, ultrasound screening at high level and prenatal diagnosis for assessing fetal infection is recommended up to WG 23. This is a better option than the often unnecessary immediate termination of pregnancy.

Treatment of pregnant women with suspected primary CMV infection with the antiviral drugs ganciclovir, foscarnet, and recently valganciclovir (all of which cross the placenta) is not recommended due to their potential teratogenic and embryonic toxicity according to animal studies. Thus far, ganciclovir was applied only in single pregnancies in which the mother had preceding organ transplantation or suffered from severe disease. Whether CMV-infected fetuses benefit from intravascular administration of ganciclovir or CMVIG preparations cannot be finally assessed since only a few case reports with diverse outcome have been published (Revello et al. 1993; Negishi et al. 1998; Nigro et al. 1999; Matsuda et al. 2004, G. Enders personal communication). New drugs for treatment of CMV, e.g. non-DNA polymerase inhibitors, are in development and some of them will hopefully enter clinical trials for safety and efficiency in the near future (Emery and Hassan-Walker 2002).

Delivery: cesarean section is not recommended in case of presumed maternal primary and fetal congenital CMV infection – unless the fetus is highly damaged.

Breast-feeding of seropositive mothers in uninfected and congenital infected newborns is not contraindicated. Recommendations for breast-feeding of very low birth weight (VLBW) preterm infants (WG <32, body weight <1 500 g) born to IgG-seropositive mothers vary from country to country between breast-feeding after counseling the parents for possible risks and feeding breast milk following various virus inactivation methods (Holder pasteurization or a new procedure for gentle virus inactivation up to corrected WG 34). Other intervention strategies for seronegative women are avoidance of iatrogenic transmission in in vitro fertilization (IVF) programs by using screened semen or screened blood products for seronegative pregnant women in case of emergency or seronegative donors in organ transplantation.

MANAGEMENT OF THE NEONATE

In infants of mothers with suspected CMV infection in pregnancy, screening for CMV congenital infection should be performed by detection of virus in urine or saliva within 14 days after birth or by DBS test on Guthrie cards followed by auditory screening of infected infants up to school age, so that affected infants can receive intervention (e.g. cochlea implants) as promptly as possible (Fowler et al. 1999).

An increasing number of neonates with hearing defects will now be identified due to the more universally practised auditory screening in industrialized countries by otoacustic emissions or auditory brain stem response. It should be remembered that about half of all cases with sensorineural hearing loss caused by CMV will be missed due to the late onset of hearing loss in formerly asymptomatic newborns.

In infants with congenital disease at birth antiviral therapy is an option and its aim is the suppression of virus replication in order to prevent the progression of auditory, visual, and mental impairment and the occurrence of further or late manifestations. From a number of studies it is known that there is a reduction of virus load during therapy, but no complete virus elimination. After cessation of therapy, virus shedding usually returns to preceding or even higher levels especially when treatment was suspended too early (Barbi et al. 1996). According to several studies – using different treatment regimes – i.v. ganciclovir is fairly well tolerated even in preterm infants. Acute symptoms (pneumonia, hepatitis, chorioretinitis) seem to resolve by early treatment (Hocker et al. 1990; Stronati et al. 1995; Nigro et al. 1994; Liberek et al. 2002; Barampouti et al. 2002). In the USA, a large-randomized controlled multicenter clinical trial (with and without 6 mg/kg i.v. twice a day for 6 weeks) was performed in infants with congenital disease at birth and central nervous system (CNS) involvement and showed very promising results with respect to safety and antiviral effects (phase I (Trang et al. 1993); phase II (Whitley et al. 1997)). The results from phase III indicate that i.v. ganciclovir therapy in symptomatically infected neonates prevented hearing deterioration at 6 months and might prevent it thereafter ($\geqslant$1 year) (Kimberlin et al. 2003).

The availability of oral therapy (oral ganciclovir and most recently valganciclovir) would be a great advance in long-term maintenance therapy usually following the initial i.v. treatment. Thus far, there are only few pharmacokinetic studies on oral ganciclovir application in infants (Michaels et al. 2003). In infants dosage should be related to body surface or body weight and controlled by drug monitoring in blood plasma. The effect of oral valganciclovir with a ten times higher bioavailability than oral ganciclovir is still under investigation. Preliminary results in seven infants show that valganciclovir is well tolerated, effective in a transient reduction of the viral load, and effective in resolving acute symptoms (G. Enders, personal communication).

There is good agreement that asymptomatic congenitally infected infants should not be treated with ganciclovir so far, but should be closely surveilled for onset of late manifestations, which may then justify treatment. Early after birth, e.g. via breast milk, infected preterm infants with symptomatic disease seem to benefit from early therapy in preventing severe disease (G. Enders, personal communication).

The overall effectiveness of ganciclovir i.v. treatment is difficult to judge from the multicenter study and from the increasing number of observational studies, due to the erratic natural course of the congenital disease.

With regard to childhood vaccination, it has been observed that the recently used sixfold vaccine combination (DTPa, hepatitis B, poliovirus 1, 2, 3, HiB), measles, mumps, and rubella (MMR), and lately varicella is well tolerated in asymptomatic and symptomatic congenitally infected infants producing satisfactory antibody levels against the various vaccine components (G. Enders, personal communication).

FUTURE MEASURES AND PUBLIC HEALTH ASPECTS

Opinions vary on the value and cost-effectiveness of screening programs for CMV. Universal antenatal CMV antibody screening is not officially recommended or has not been introduced into any prenatal programs in industrialized countries (Revello and Gerna 2002; Enders et al. 2003). Such programs have shown to be helpful for the guidance of pregnant seronegative women in commonsense measures for preventing the acquisition of primary infection and for reassuring IgG-positive/IgM-negative women that they are protected against primary infection. In case of recurrent infection there is only a low risk of the birth of a child with congenital disease. However, to such women a closer surveillance in the following pregnancy by serology and ultrasonography (WG 21–24) should be recommended.

For active prophylaxis, several vaccines are under investigation (Pass and Burke 2002) and currently four of them have reached clinical trials: Towne live-attenuated virus, subunit glycoprotein B (gB/MF59) (Mitchell et al. 2002), canary pox, and an improved Towne (chimeric CMV) vaccine. Further vaccines consisting of plasmid DNA, dense bodies, or synthetic peptides are in development (Plotkin 2001). To be effective, these vaccines should induce persisting titers of serum-neutralizing and mucosal antibodies comparable to those found after natural infection. Induction of cellular immunity (cytotoxic lymphocyte response of importance for protection) would also be beneficial, but will not be achieved with subunit vaccines alone. Potential vaccine recipients would be young boys and girls before puberty. Encouraging aspects come from a study by Griffiths (Griffiths et al. 2001) showing by a mathematic modeling approach that in developed countries a vaccine applied to 1-year old infants with only modest efficiency (60 percent protective effect against primary infection) would nevertheless be sufficient in eradication of CMV from the population.

The public health impact of congenital CMV infection is enormous, especially when the late sequelae of congenital CMV infection are more definitely associated with CMV congenital infection (Istas et al. 1995), which is presently the case (2004). The annual costs of supporting children

with congenital CMV in the USA approaches two billion US dollars (Griffiths 2002; Prober and Enright 2003).

VARICELLA-ZOSTER VIRUS

Varicella-zoster virus (VZV) is an alphaherpesvirus that causes two main clinical syndromes: primary infection gives rise to varicella (chickenpox) and recurrent infection to herpes zoster (shingles). Varicella is highly contagious, whereas herpes zoster is less so. Chickenpox in pregnancy may be associated with severe maternal disease, spontaneous abortion, and infrequently, during the first two trimesters, with congenital varicella syndrome (CVS). Maternal varicella around term carries the risk of serious neonatal disease. Zoster at any time during pregnancy does not result in congenital anomalies or neonatal disease.

Infection in pregnancy

INCIDENCE

Varicella in pregnancy is relatively uncommon because in densely populated areas of the northern hemisphere, approximately 95 percent women of childbearing age are seropositive. However, this figure may be smaller in some populations (e.g. black women in the USA and in countries of the southern hemisphere) (Gray et al. 1990; Dworkin 1996; Seward et al. 2000). Reliable data on the incidence of varicella in pregnancy are not available, but extrapolation from the consultation rates for chickenpox in adults aged 15–44 years in the UK suggest an infection risk of approximately 2–3 per 1 000 with an average mortality rate of 1/1 million pregnancies (Department of Health 1996a; Fairley and Miller 1996). In Germany, the seroprevalence to varicella was studied annually from 1984 to 1997 in more than 26 000 pregnant women aged 20–40 years; the seronegative rate was about 5–6 percent, with no significant change over the period (Enders 1984; Enders and Miller 1994; Enders and Miller 2000, and update 2004). Based on the suggested infection risk of 2 per 1 000 in the UK in all adults aged 15–44 years, the frequency of varicella in pregnancy was calculated to be 8 000 in the USA, 1 600 cases per year in Germany, and 1 400 in England and Wales (Department of Health 1996a; Enders and Miller 2000). Asymptomatic reinfection is possible and second attacks of varicella have also been reported (Martin et al. 1994, Enders et al. personal communication). An upward shift of incidence to adults aged 15–44 years has been recognized in England (Miller et al. 1993a) and in the USA (Gray et al. 1990; Choo et al. 1995). Zoster in pregnancy may have an annual incidence of 2 per 1 000, similar to that in nonpregnant adults aged 15–40 years (Miller et al. 1993b; Schmader 2001). There is no evidence that the incidence in adults has changed over the last few decades.

ADVERSE EFFECTS IN PREGNANCY

Anecdotal evidence suggests that varicella is more severe in pregnant women than in other adults, particularly the risk of varicella pneumonia. However, there are no reliable population-based prospective studies to confirm this impression (Nathwani et al. 1998). Among 1 721 pregnant women with varicella followed up prospectively by the author between 1980 and 1999, no cases of pneumonia nor deaths were recorded (Enders and Miller 1994, 2000). However, outside this study, we investigated three very severe cases of VZV pneumonia between WG 22 and 33 (1992–1997). All three women survived and two of the three infants were born healthy and uninfected. In the third pregnancy, intrauterine death and spontaneous abortion occurred in WG 23. This indicates that varicella pneumonia associated with pregnancy is rare, although mortality data from the UK suggest that the risk of fatal varicella appears to be fivefold higher in pregnant than in nonpregnant immunocompetent adults (Miller et al. 1993b).

VZV pneumonia is the most common serious maternal complication in pregnancy (Haake et al. 1990; Katz et al. 1995). It usually develops within 1 week of the rash. The predominant signs and symptoms are fever, cough, dyspnea, and tachypnea. The outcome is unpredictable and there may be rapid progress to hypoxia and respiratory failure. Pneumonia is regarded as a medical emergency requiring prompt diagnosis and treatment. Studies reporting the outcome of varicella pneumonia in pregnancy show an excess of cases in the third trimester, suggesting that the risk to the mother may be greatest towards term. For example, 28 of 34 cases reported in 1989 and 1991 occurred in the third trimester (Esmonde et al. 1989; Smego and Asperilla 1991). Of the nine deaths reported as due to varicella in pregnancy in England and Wales between 1985 and 1998, seven occurred between 27 and 32 weeks of gestation.

In pregnancies complicated by varicella, spontaneous abortion, stillbirth, and prematurity do not seem to be significantly increased (Paryani and Arvin 1986; Balducci et al. 1992). In a prospective study of a total of 1 721 VZV-complicated pregnancies, the incidence of fetal loss due to spontaneous abortion during the first 20 weeks was 2.9 percent ($n = 49$) and of intrauterine death after the 20th week 0.6 percent ($n = 11$) of 1 612 live-born infants (Enders and Miller 1994, 2000). In pregnancies complicated by localized zoster rarely has dissemination been reported in immune-competent women.

Effects on the fetus

CONGENITAL VARICELLA SYNDROME

The risk of congenital varicella syndrome is an important concern. Since its first description by Laforet and Lynch (1947) more than 112 such cases have been

reported in the English and German medical literature (Sauerbrei and Wutzler 2003). A causal association with maternal infection has been confirmed in only a few cases in live-born infants by isolation of VZV or detection of viral DNA from skin lesions at birth and in some more cases in tissues following therapeutic abortion or intrauterine death (e.g. Da Silva et al. 1990; Scharf et al. 1990; Puchhammer-Stöckl et al. 1994; Sauerbrei et al. 1996; Sauerbrei 1998; Hartung et al. 1999; Schulze-Oechtering et al. 2004).

CLINICAL MANIFESTATIONS

The clinical manifestations of CVS range from severe multisystem involvement, resulting in death in the neonatal period, to dermatomal skin scarring, limb hypoplasia, or both as the only defects. Table 8 shows the clinical features of CVS and their relative frequencies of 48 well-documented cases from the world literature, as well as the 13 prospective and the 12 retrospective cases investigated by Enders and Miller (2000).

PATHOGENESIS OF CVS

The precise mechanism of infection with VZV in utero is unknown. It is generally accepted that transplacental transmission of VZV may take place during the viremic phase, resulting in congenital infection (Trlifojova et al. 1986). The pattern of defects in CVS, particularly the limb hypoplasia and scarring, suggests that they are due to intrauterine zoster. The extremely short period between reactivation and fetal infection may be the result of an inadequate cell-mediated immune response in early gestation (Higa et al. 1987).

The clinical consequences of congenital VZV infections depend on the stage of pregnancy at which maternal infection occurs (Table 9).

Intrauterine varicella infection can, however, occur without clinical sequelae at any stage of pregnancy, the proportion rising from 5–10 percent in the first and second trimester to 25 percent towards WG 36 and reaching approximately 50 percent when maternal varicella occurs 1–4 weeks before delivery (Miller et al. 1989). It seems possible that fetal infection early in pregnancy may result in clinical manifestations with healing of lesions before birth. Localized zoster in pregnancy could theoretically result in fetal infection, if the dermatomes involved were T10 to L1, as sensory nerves to the uterus originate from these segments; but no such cases have yet been documented (Miller et al. 1993b; Enders and Miller 2000).

The risk of zoster in infancy and childhood following maternal varicella in the second and third trimesters is 0.6 percent (4/618) and 1.6 percent (7/431), respectively, and the overall risk 1 percent (Enders and Miller, 1994, 2000, and update 2004). In the prenatal diagnosis study of Mouly et al. (1997) the rate of localized early herpes zoster in infants born to mothers with varicella before the 24th week of pregnancy was 3.8 percent (3/78).

RISK OF CONGENITAL VARICELLA SYNDROME

Several workers undertook prospective studies to estimate the risk of CVS following maternal varicella infection at various stages of pregnancy (Enders 1984; Paryani and Arvin 1986; Balducci et al. 1992; Jones et al. 1994; Pastuszak et al. 1994). In the largest reported prospective study (Enders and Miller, 1994, 2000, and update 2004), including 1 795 pregnant women with acute varicella with known outcome the overall risk during the first 20 weeks of gestation is 1.4 percent, the highest (3.0 percent) being observed between weeks 13 and 20. The latest gestational age at which CVS occurred was WG 19, which is consistent with the findings in one retrospective report (Alkalay et al. 1987). In a recent prospective study in 2002, involving 347 pregnant women with primary VZV infection only one case

Table 8 *Congenital varicella syndrome*

	Number of infants (%)	
Main signs	***n* = 48[a]**	***n* = 25[b]**
Skin: dermatomal cicatricial skin lesions, contractures	39	18
Skeleton: Limb hypoplasia associated with reduction deformaties	21	18
Eye: microphthalmia, chorioretinitis, cataract, Horner syndrome	26	11
CNS: microcephaly, brain atrophy, paralysis, convulsions, encephalitis, mental retardation	26	14
Other organ defects (e.g. gastrointestinal, genitourinary)	5	5
Multiorgan involvement (e.g. hemorrhagic rash and dystrophy)	ND	6
Death postpartum and later	ND	9
Female	ND	13
Male	ND	12

ND, no data.
a) From world literature.
b) Enders and Miller (1994, 2000); 13 prospective and 12 retrospective cases.

Table 9 *Clinical manifestations of congenital varicella infection following chickenpox in pregnancy*

Stage of maternal infection	Sequelae
First and second trimester	Congenital varicella syndrome
Second and third trimester	Herpes zoster in infancy or childhood
Perinatal	Disseminated neonatal varicella

From Miller et al. (1993b).

of CVS with typical skin scars and left retinal macular lesion occurred, however, after maternal varicella at WG 24 (Harger et al. 2002). In zoster in pregnancy involving 504 women with localized zoster there was no serological evidence of intrauterine infection and no congenital varicella syndrome (CVS) symptoms in the infants (Enders and Miller 1994, 2000, Enders update 2004). Various congenital defects have been reported after maternal zoster (Webster and Smith 1977; Higa et al. 1987), but only one infant with characteristic limb hypoplasia and skin scarring has been described following disseminated maternal zoster at WG 12 with presumed maternal viremia (Higa et al. 1987).

Based on the estimate of risk, together with the incidence of chickenpox in pregnancy and the annual rate of births, the number of CVS cases, which could be expected to occur each year is 44 in the USA, eight in England/Wales (UK), and nine in Germany (Enders and Miller 2000). The incidence of the CVS in Australia has been reported as 1–2 of 100 000 pregnancies per year which is 2.3 cases per year (Forrest et al. 2000). This confirms the low frequency of CVS.

The possibility of reinfection with adverse consequences for the fetus, has been raised (Martin et al. 1994). In our investigation on asymptomatic reinfection in 347 seropositive pregnant women with close exposure a significant rise of IgG antibodies was detected in 203 women and a rise of IgG and IgM antibodies in 144 women. In one case of reinfection at WG 37 varicella-like symptoms were noted together with a very high rise of IgG and IgM antibodies. Reinfection could be recognized by the already high avidity index in the first low-level IgG serum sample. Pregnancy outcome was documented in about 70 percent of the cases. In the newborns, no abnormal serology nor defects or illness consistent with CVS or neonatal varicella were observed (Enders, personal communication).

Re-exposure to VZV also induces a VZV-specific cellular immune response with an expansion of antigen-specific $CD4^+$ T cells and changes in cytotoxic $CD8^+$ T cells and NK cells. This T-cell response of VZV-specific $CD4^+$ memory T cells largely resembles the primary immune response to VZV (Vossen et al. 2004).

Neonatal varicella

Severe neonatal disease is generally attributed to intrauterine infection and lack of maternal antibody. If the mother's rashes appears more than 6 days before delivery, the infant invariably has maternal antibodies. When it is <6 days before delivery, only some infants have significant titers of antibody, whereas those born <3 days after onset of maternal rash, or infants whose mothers develop varicella after delivery are VZV IgG-negative. Infants at greatest risk of severe or fatal illness are those whose mothers' rashes appears 5 days before to 2 days after delivery (Gershon 1975; Miller et al. 1989). The interval between the onset of rash in the mother and infant is usually 12–13 days, but may be as brief as 2 days, suggesting transplacental infection. In one retrospective series, the clinical attack rate was estimated to be 17 percent (Meyers 1974) and the fatality rate 30 percent (Gershon 1975). According to a more recent large prospective study the commonly cited fatality rate of 30 percent seems to be an overestimate due to selective reporting of fatal outcomes (Miller et al. 1989). The annual incidence of neonatal varicella is only known from Australia, which is 5.8 per 100 000 live births or one in 17 000 pregnancies. Of interest is that all these 44 infants recovered without reported sequelae (Forrest et al. 2000).

A severe outcome of neonatal varicella is not limited to transplacentally acquired infection, but occasionally occurs in early postnatally acquired infection (minimum incubation period 8 days after birth) despite the use of varicella immunoglobulin (VZIG) even at high dosage.

Most of the concern surrounding neonatal varicella relates to infants whose mothers develop varicella. But it should be borne in mind that maternal immunity to varicella does not always protect the neonate exposed postnatally at home. There have been occasional reports of varicella in neonates of mothers with a past history of chickenpox (Bendig et al. 1998; Baba et al. 1982). All the cases (two and five) were of moderate severity or mild. Miller et al. (1989) followed up 36 infants who had a home exposure to varicella during the first 4 weeks of life. Six infants (17 percent) were infected, three with symptoms (mild to severe) and three without clinical features. Clearly, passively acquired VZV antibody provides incomplete protection, particularly under conditions of close exposure. Following neonatal varicella reactivation is not uncommon giving rise to zoster within a few months or years of the primary infection. Further zoster episodes have not been reported in these children, suggesting that good cell-mediated immunity develops after reactivation.

Despite the potential for horizontal transmission of VZV from zoster cases, neonates do not seem to be at risk of infection if maternal zoster occurs around the time of delivery (Miller et al. 1993b; Enders and Miller 2000). The reason for this is the lower infectivity of zoster compared to chickenpox; furthermore, if the onset of maternal zoster is $\geqslant$4 days before delivery, the infant passively acquires antibody from the mother, whose own pre-existing immunity is reinforced as a result of her recent reactivation.

Diagnosis

DIAGNOSIS IN PREGNANCY

In typical cases of varicella and zoster a clinical diagnosis is usually sufficient. In pregnancy VZV infection

should be verified serologically. To detect specific IgG, IgM, and IgA antibodies, the enzyme-linked immunosorbent assay (ELISA) with whole virus has become the most widely used method, and for a very rapid determination of the immune status in case of exposure the latex agglutination (LA) test is also employed. Results with both tests correlate well with cell-mediated immunity as determined by lymphoproliferative assays (Weinberg et al. 1996). It should be known that in the majority of adults (e.g. pregnant women) following primary infection VZV IgG antibodies are detected before VZV IgM antibodies due to strong cross-reaction with herpes simplex virus type 1 (Enders and Miller 2000). More recently the IgG avidity test – which is investigated for its diagnostic value since 1990 (Thomas et al. 1990) – proved in our in-house version very useful for assuring protection against VZV disease in persons with low-level IgG antibodies, for diagnosing recent infection in the absence of IgM antibodies and for differentiation of asymptomatic or symptomatic reinfection from primary infection (G. Enders, personal communication).

Virus detection is unnecessary in uncomplicated varicella, but is used in cases complicated by pneumonia or encephalitis to guide antiviral therapy and in prenatal diagnosis. Rapid diagnosis can be achieved in smears of vesicle fluid or respiratory secretion by immunofluorescence applying monoclonal antibodies for identification. Virus detection with the conventional methods such as cell culture is only successful with varicella or zoster vesicular fluid containing free virus, whereas cell-associated virus can, apart from the rapid diagnosis, be demonstrated by detection of VZV DNA. Initially VZV DNA has been detected in amniotic fluid and fetal blood by dot-blot hybridization (Gottard et al. 1991), but now detection by single round and nested PCR is the method of choice. However, positive results with n-PCR only must be judged with caution.

DIAGNOSIS IN THE FETUS

Invasive prenatal diagnosis (PD) to exclude fetal infection with VZV is feasible, but not generally recommended. PD should only be performed in women with varicella up to WG 21–22, since the risk of CVS is limited to this period. In cases where VZV DNA is detected in the amniotic fluid and the ultrasonographic scan is considered normal, the majority of infants have no clinical manifestation at birth (Mouly et al. 1997; G. Enders personal communication 1996–2004). Amniotic fluid taken at WG 18–21 and >4–6 weeks after the onset of maternal varicella is technically easy to obtain and yields the best results. In case of varicella infection and genetic indication for prenatal diagnosis the latter could be done earlier, but not before all vesicles have disappeared in order to avoid iatrogenic infection of the fetus. High-level ultrasound screening is recommended between WG 19 and 23. If the findings are abnormal, both amniotic fluid and fetal blood should be tested for VZV DNA. Tests for fetal VZV-specific IgM are not helpful, because VZV IgM antibodies are usually not detected in fetal blood. Skeletal defects may be evident by ultrasound earlier, but cerebral or ocular abnormalities are seldom recognized before WG 22–23. They may not become evident until much later in pregnancy, when therapeutic abortion is not an option. The combined use of ultrasound and magnetic resonance imaging (MRI) may document earlier the extent of tissue damage, particularly involvement of the CNS (Verstraelen et al. 2003). These findings are substantiated by the author's own prospective series on invasive prenatal diagnosis of 195 women with acute varicella up to WG 24 between 1991 and July 2004. In nine cases, detection of VZV DNA in amniotic fluid and fetal blood was associated with suspicious finding for CVS by high-level ultrasound and in some cases by additional MRI between WG 20 and 27. Following termination of pregnancy in six cases, VZV DNA could also be demonstrated by PCR in fetal blood and various types of fetal tissues and also in fluids and tissues in three cases who died shortly after birth (Enders and Miller 2000; G. Enders personal communication 2000–2004).

DIAGNOSIS IN INFANCY

The criteria for the diagnosis of fetal varicella syndrome in the infant include maternal symptomatic varicella infection during early pregnancy, presence of dermatomal cicatrial skin lesions, limb hypoplasia, and some of the other signs and symptoms associated with CVS recognizable at birth (see Table 8). Laboratory confirmation of intrauterine VZV infection is more important in cases with less typical CVS features. Concerning the immunological evidence of in utero VZV infection, IgM antibody detection, and detection of cellular immune response are unreliable markers, because IgM antibodies are only exceptionally detected with the presently used commercial ELISA IgM tests even in infants with CVS. Also the detection of a positive cellular immune response over the age of 1 month by the standard lymphocyte proliferation (^{3}H-thymidine) test has been rarely accomplished (Paryani and Arvin 1986). Attempts with the newly developed interferon-γ ELISPOT assay have not been made thus far (Smith et al. 2001). Detecting persistent VZV IgG antibodies in the infant seems to be more indicative of congenital VZV as shown in CVS cases assessed prospectively and retrospectively since 1980–2003 (Enders and Miller 1994, 2000). An even later criterion of intrauterine VZV infection is the occurrence of zoster in early infancy. Therefore, attempts to detect VZV DNA by PCR in body fluids or skin biopsies and EDTA blood should be undertaken. Positive results are limited (Mehraein et al. 1991; Puchhammer-Stöckl et al. 1994; Schulze-Oechtering et al. 2004). In contrast, DNA detection in

tissues of various organs obtained postmortem is the method of choice in cases of early death and the demonstration of miliary calcified necrosis by histopathological techniques may further support intrauterine varicella infection (Hartung et al. 1999).

Management

MANAGEMENT IN PREGNANCY

Passive prophylaxis with varicella-zoster immunoglobulin

The value of passive prophylaxis is difficult to assess because of the differences in quality of the various varicella-zoster-immunoglobulin (VZIG) preparations used worldwide. This concerns IgG antibody concentration and the test used for establishing the international units (IU). Passive antibody prophylaxis with a standardized preparation of VZIG for intramuscular application is available in the USA (CDC 1996), in the UK, France, Scandinavian countries, and Australia for exposed susceptible pregnant women to prevent or attenuate varicella.

The English preparations have a relatively low IgG antibody concentration (700 IU per adult dose) and administration of VZIG is recommended within 4–10 days of a close (e.g. home) exposure. Their effect is to attenuate disease rather than to prevent infection (Miller et al. 1993b).

In Germany, two preparations of VZV immunoglobulins with defined antibody concentrations are available for passive prophylaxis, one for intramuscular and the other for intravenous administration, with an IgG antibody concentration of approximately 2 000 IU per adult dose. The dosage for example for a 70-kg woman is 20 ml Varicellon i.m. (2 000 IU) or 70 ml Varitect i.v. (1 750 IU). It should be given within 24–72 (96) hours after exposure. According to the experience of Enders and Miller (2000) in 212 seronegative pregnancies, even if VZIG is given in the recommended dosage and within 24–72 (96) hours of exposure, infection is prevented in only 54 percent. Of the remaining cases VZIG administration results in subclinical infection (5 percent) or in modified to normal disease (41 percent). When VZIG is given 4–5 days after exposure, there is still a mitigating effect in 20 percent of patients.

In the UK and Germany, postexposure prophylaxis with VZIG is recommended only for seronegative pregnant women with significant exposure to an acute case of varicella (e.g. household contact, face-to-face contact, contact indoors with a case of chickenpox for >1 h). As a consequence of the findings in the largest prospective study (Enders and Miller 1994), that no case of CVS occurred in maternal varicella after WG 20, VZIG is no longer recommended for women exposed after WG 21. However, VZIG prophylaxis for women exposed later in pregnancy is officially recommended, also in Germany by the RKI, to reduce the risk of rare maternal complications of varicella such as pneumonia. In the UK, however, VZIG is only issued for women exposed after WG 20 if national supplies permit (Department of Health 1996b). These recommendations differ from the US recommendation, which includes all susceptible pregnant women with close contact throughout pregnancy.

It is clear that seropositive women do not need VZIG. They can be assured that they are protected against clinical disease but not completely against reinfection. In the case of exposure to zoster, passive prophylaxis is only recommended for seronegative women up to WG 21 intimately exposed to persons with extensive zoster lesions.

Whether VZIG has any benefit in preventing intrauterine infection and of CVS is not known. A certain benefit of VZIG administration during pregnancy in case of contact is deduced from the prospective studies of Enders et al. (Enders and Miller 1994 and 2000 with update). There were no CVS cases in 108 pregnancies in which maternal varicella occurred despite VZIG prophylaxis and no persistent IgG antibodies could be detected at the age of 7 months in eight infants tested.

One other study (Pastuszak et al. 1994) indicates that in order to prove such a benefit a prospective study would be needed, which includes the following: a large cohort of VZV-seronegative women with confirmed close contact to VZV who all receive the same in neutralizing units of a standardized preparation of VZIG.

Besides postexposure prophylaxis with VZIG, oral acyclovir (ACV) given at the 9th day of incubation or within 24 h after onset of exanthema (5 × 800 mg/day for 5 days) and vaccination with live-varicella vaccine within 72 h after contact may be effective. In the UK and Australia in contrast to Germany, oral ACV is offered to persons presenting within 24 h of onset of rash and given for 5 days; pregnant women over 20 weeks of gestation with varicella are treated with this regimen. However, the use of oral ACV during the incubation period or vaccination in early pregnancy is not established.

The recommendations in the UK and Germany for postexposure prophylaxis to varicella in early and late pregnancy are summarized in Table 10.

The medical advice for acute varicella at various stages in pregnancy is summarized in Table 11.

Management of exposure to zoster and zoster in pregnancy is presented in Table 12.

Active prophylaxis with vaccine

In the USA, universal vaccination of children, adolescents, and young adults including all nonpregnant women

Table 10 *Recent exposure to* varicella *in pregnancy – high contagiousness*

VZV exposure in pregnancy

Ask for previous varicella:
If yes: no testing
If no or doubtful
↓
Quick antibody test (ELISA, LA test)
AB positive: previous infection
AB low or equivocal: repeat test in 2–3 weeks
AB negative: susceptible

First and second trimester: risk for CVS	**End of third trimester around delivery: risk for severe neonatal varicella**
VZIG: until WG 21 within 24–72 (96 h) after exposure[a]	No VZIG after WG 37, because it may shift onset of exanthem to delivery, better to induce labor
New: >20 WG: ACV oral within 24 h after onset of exanthem	
Check after 2–3 weeks for preventive effect	Check antibody status in 2–3 weeks

AB, varicella-zoster antibodies; ACV, acyclovir; CVS, congenital varicella syndrome; ELISA, enzyme-linked immunosorbent assay; LA test, latex agglutination test; VZIG, varicella immunoglobulin; WG, week of gestation.
a) Personal recommendations by G. Enders; official German recommendations also include women exposed beyond 21 WG.

of childbearing age with no history of varicella has been recommended since 1996 (CDC 1996). The effectiveness of this universal childhood vaccination in the USA is indicated by a significant decline of varicella cases in the total population and, in particular, in the vaccinated age group of 1–4 years. Also the rate of hospitalization in all age groups has decreased within the first 3 years since the beginning of routine childhood vaccination. Furthermore, a reduction in pediatric hospitalizations for VZV-related invasive group A streptococcal infections

Table 11 *Acute varicella throughout pregnancy*

Management for acute varicella throughout pregnancy		
First and second trimester		
1. Counsel		Risk of CVS is low
2. Mention		Prenatal diagnosis possible in case of acute varicella in WG 1–20, ~4 weeks after onset of varicella: VZV-DNA detection with PCR/n-PCR in amniotic fluid (WG >18–21) Advocate: Ultrasound control of high level (MRI) in WG 19–24, if abnormal: PCR/n-PCR in amniotic fluid and fetal blood in WG 23/24; IgM antibodies not detected
3. Observe		VZV pneumoniae occurring mainly late second and third trimester: advice immediate i.v. acyclovir therapy at any stage of pregnancy and intensive medical support
Third trimester		
More than 6 days prior to delivery	→	No VZIG
4–5 days prior to delivery	→	If birth cannot be delayed: VZIG to mother Instead of VZIG – new: ACV oral within 24 h of onset of rash (5 × 800 mg/day for 5 days) not yet practisized VZIG to neonate and ACV i.v. before or at onset of prodromal signs
2–4 days after delivery	→	VZIG to neonate
		No isolation of mother/child (rooming-in). Breast-feeding possible, in case of florid exanthem, pump off breast milk. Observe neonate ⩾12 days in hospital and until age of 28 days at home

ACV, acyclovir; CVS, congenital varicella syndrome; n-PCR, nested polymerase chain reaction; VZIG, varicella immunoglobulin; VZV, varicella zoster virus; WG, week of gestation.

Table 12 *Exposure to zoster and zoster in pregnancy – low contagiousness, no risk for CVS or perinatal infection*

Management for exposure to zoster and zoster in pregnancy		
Recent exposure to zoster lesion		
Determination of VZV IgG antibody status:		
VZV IgG antibody positive	→	no VZIG
VZV IgG antibody negative	→	VZIG only if close contact until WG ±21
Onset of zoster		
1st–3rd trimester	→	No VZIG
(1st), 2nd, 3rd trimester	→	Acyclovir if severe manifestations, i.v., oral
±4 days prior to delivery	→	No VZIG to newborn Cover maternal zoster lesions Breast-feeding: yes, if no vesicles in area of nipples; otherwise pump off breast milk
3–4 days after delivery	→	VZIG for newborn 0.5 ml/kg weight, since maternal antibody level is still low

CVS, congenital varicella syndrome; VZIG, varicella immunoglobulin; VZV, varicella-zoster virus; WG, week of gestation.

parallel to the increase of vaccination rates has been observed. In most European countries, the universal childhood vaccination is still controversial. In July 2004, Germany, however, introduced the US childhood vaccination program encouraged by the favorable US experience (Wagenpfeil et al. 2004). It is hoped that the shift in the proportion of cases to older persons will be minimized by catch-up vaccination of adolescents and adults. Selective vaccination is recommended for seronegative risk-patients, their family contacts, healthcare workers, women who want to become pregnant, and also adolescents at the age of 12–15 years without varicella history. Children aged 1–12 years generally receive one dose of vaccine, whereas from 13 years on two doses of vaccine with an interval of 6 weeks are recommended.

Pregnancy is a contraindication for administration of live-attenuated varicella vaccine. However, the virulence of the vaccine virus is less than wild-type VZV, so the risk to the fetus, if any, is even lower and therefore inadvertent administration is no reason for termination of pregnancy. As a precaution, however, nonpregnant women who are vaccinated should avoid becoming pregnant for 1 month following each injection. A varicella vaccine in the pregnancy registry in the USA has been established, similar to that for rubella vaccine. Between 1995 and 2000, 362 women were inadvertently exposed to varicella during pregnancy or within 3 months of conception. In this group 58, of 92 seronegative women received their first dose of vaccine (Varivax) during the first or second trimester. No cases of congenital varicella syndrome were identified in 56 live-births (Shields et al. 2001). With respect to transmitting vaccine virus from vaccinees, there is one report in which the vaccinated child transmitted vaccine virus to its pregnant mother (Long 1997). Despite this observation recommendation of the German Vaccine Commission (STIKO) does not consider pregnancy of the mother as a contraindication to the vaccination of her own child (Robert Koch Institut 2004).

Furthermore, postpartum vaccination of varicella-susceptible women need not be delayed because of breast-feeding. In a study of 217 postvaccination breast milk specimens, varicella DNA was not detected by PCR. In addition, none of the breast-fed infants became seropositive (Bohlke et al. 2003).

Antiviral therapy

For antiviral chemotherapy of VZV infection, acyclovir (which has an excellent safety record), valacyclovir, brivudin, and famciclovir are available.

The efficacy of intravenous acyclovir in the treatment of chickenpox in immunocompromised people is well established. By oral application, valacyclovir, brivudin, and famciclovir have a higher bioavailability than ACV. All these drugs in intravenous and oral form are not licensed for use in pregnancy. However, in varicella complicated by, for example, pneumonia or signs of dissemination, early treatment with intravenous acyclovir at any stage of pregnancy is essential and life-saving (Haake et al. 1990; Smego and Asperilla 1991). The prospective follow-up of a total of 1 207 women treated by oral and intravenous application during the first, second, and third trimester with ACV did not show an increase in the number of birth defects when compared with those expected in the general population, or any consistent pattern of defects. Similar data have been reported on the outcome of 94 pregnancies in which oral valacyclovir had been given (Enders and Miller 2000). The use of oral ACV following exposure as a postprophylactic measure has already been discussed. For cases of CVS antiviral therapy is recommended, particularly, in those in which VZV DNA in fluids and lesions is detected (ACV 30–60 mg/kg/day i.v., divided into three single doses).

MANAGEMENT OF THE NEONATE

For neonates whose mothers have varicella around the time of delivery, prophylaxis with VZIG is recommended to prevent neonatal infection or to modify the disease. In the USA and Germany, prophylaxis is only recommended for neonates whose mothers develop varicella between 5 days before, to 2 days after, delivery (CDC 1996); while in the UK prophylaxis is recommended for any infant whose mother develops varicella in the 14-day period centered on delivery (Department of Health 1996b). In the UK, VZIG is also recommended for infants with nonmaternal postnatal exposure during the first 7 days of life and who lack VZV antibodies. In the USA and UK, susceptible infants can be identified by testing a stored antenatal blood sample from mothers with a negative history of chickenpox. In the USA and Germany, prophylaxis for postnatal exposure is restricted to premature infants. However, all preterm infants less than 28 weeks or infants weighing less than 1 000 g exposed to acute cases should be given VZIG (0.5 ml/kg i.m.) regardless of a positive maternal history of varicella (Deutsche Gesellschaft für pädiatrische Infektiologie 2003) and regardless of low-level IgG antibodies in the infant.

If severe varicella develops in the infant despite treatment with VZIG, high-dose intravenous acyclovir should be given (Reynolds et al. 1999). Fatal outcomes have been reported despite VZIG prophylaxis and treatment with ACV (Holland et al. 1986; King et al. 1986). All were in infants whose mothers developed varicella within the period 4 days before, to 2 days after, delivery. Early treatment with intravenous ACV of infected neonates in this high-risk period is therefore recommended (Nathwani et al. 1998; Reynolds et al. 1999). Prophylactic use of intravenous ACV in this group has also been advocated (Sills et al. 1987), but there is no evidence of efficacy. For limitation of the existing symptoms and further symptoms by interrupting possibly still ongoing virus replication, ACV therapy is carried out.

At present, there is no convincing information about whether mothers who develop varicella in the high-risk period around the time of delivery should breastfeed or be isolated from their infants. Not unexpectedly, VZV DNA has been detected by PCR in breast milk (Yoshida et al. 1992), but whether VZV was transmitted to the newborn via breast milk could not be determined because the mother was breast-feeding in the 24 h prior to rash onset. Furthermore, neonates whose mothers have varicella shortly before delivery may have already been infected transplacentally at the time of birth.

For those infants whose mothers develop varicella in the puerperium, transmission during the infectious period before rash onset is likely. Therefore isolation of the infant from the mother is questioned (Trompeter et al. 1986; Stephenson 1993).

Management of nosocomial exposure

Despite the concern raised when varicella exposure occurs in an antenatal clinic or neonatal intensive care unit, documented nosocomial transmissions in these groups are rare. This is probably due to the low intensity of exposure, particularly in neonatal units. Furthermore, the majority (approximately 90–95 percent) of the neonates, also premature and low birth weight infants, have low or medium maternal antibody titers which may be protective. The index case is often a healthcare worker or visitor and control measures may require extensive VZV antibody testing of patients and staff contacts. To avoid unnecessary use of VZIG for the newborn only neonates of seronegative mothers should receive VZIG.

Newborns with CVS have to be isolated from other neonates although there is no report of an infection transmitted by the respiratory route or by intimate contact. Asymptomatic newborns whose mothers develop varicella 5 days before to 2 days after delivery do not need to be isolated from other babies.

For more than 20 years it has been stipulated that medical and healthcare workers should know their immunity status and since the availability of live vaccine in 1995, vaccination of susceptible healthcare workers is recommended.

Recommendations for limiting nosocomial spread of varicella are listed in Table 13.

HERPES SIMPLEX VIRUS TYPES 1 AND 2

Maternal herpes simplex virus (HSV), primary and recurrent genital infection, which is frequently asymptomatic, may cause neonatal herpes. Although infrequent, neonatal herpes is a devastating disease with a high rate of morbidity and mortality despite early and appropriate therapy. Because its incidence is significantly higher in the USA than elsewhere, special efforts are being made in that country to establish a reliable and cost-effective strategy for prevention.

Infection in pregnancy

EPIDEMIOLOGY OF MATERNAL HSV INFECTION

In the past, approximately 90 percent of herpetic genital infections were caused by HSV-2, but more recently HSV-1 has been implicated as the cause of up to 40 percent of cases of primary genital herpes in seronegative adults. However, the reactivation rate for genital HSV-2 is at least twice as high as for HSV-1, and so too are the risks of transmission by sexual intercourse and of neonatal herpes infection (Forsgren 1992; Koelle et al. 1992; Corey et al. 1996). This was confirmed in a recent study in the UK. The prevalence of HSV-1 antibodies among attenders of a sexually transmitted disease

Table 13 *Limitation of nosocomial varicella transmission*

Group	Action
For hospital staff	
Test for VZV IgG antibody	In personnel before working in maternity/newborn wards and wards for immunosuppressed patients
In case of exposure, test for IgG antibody if immunity status is unknown	Seropositive staff: remain on ward
	Seronegative staff: removal from work 8–21 days after exposure to a case of varicella or immediate vaccination with live vaccine
	Vaccinees: develop IgG antibody in low titer within 10–12 days. Should avoid close association with susceptible high-risk individuals if they develop some vesicles
For pregnant women	
Exposed to cases with acute varicella shortly before and after delivery	Determine IgG antibody titer Mother antibody-positive → no VZIG to neonate Mother antibody-negative → VZIG to neonate
For infants in neonatal care	
In case of exposure in the first 7 days of life	Determine IgG antibody titer Infants VZV-IgG positive → no VZIG[a] Infants VZV-IgG negative → VZIG
Newborn with CVS	Isolation mother and baby (rooming-in), contagiousness low

VZIG, varicella immune globulin; VZV, varicella-zoster virus.
a) Except premature neonates born before 25 weeks gestational age and/or with birth weight of less than 1 000 g.

(STD) clinic was 60 percent and among the blood donors 46 percent (n = 1 494). The increasing genital herpes infection due to HSV-1 was strongly associated with early age of first sexual intercourse (Cowan et al. 2002).

Seroprevalence studies with HSV type-specific antigens have demonstrated rates of HSV-1 infection of approximately 30 percent in people aged 20–40 years in higher socioeconomic groups and approximately 80 percent in those of lower socioeconomic groups (Corey and Spear 1986a, 1986b; Johnson et al. 1989). Thus people with higher living standards now acquire HSV-1 infection less frequently in childhood than they did in the past. The consequent lack of partial cross-protection may be one of the reasons for the higher frequency and severity of primary HSV-2 and HSV-1 genital infection (Koutsky et al. 1990; Bernstein 1991).

Most surveys of seroprevalence have shown a relatively low but increasing rate of HSV-2 infection during the last decade in the general adult population in the developed world; for example, in the USA from 16 percent in 1976–1980 to approximately 22 percent in 1989–1991 (Johnson et al. 1989, 1994). HSV-2 seroprevalence in women of childbearing age in the USA varied from 10 to 35 percent in white women and from 35 to 60 percent in black women (Johnson et al. 1989; Arvin and Prober 1990). In other surveys, the HSV-2 seroprevalences in pregnant women in various countries ranged between 10 and 33 percent, and in most of the HSV-2-seropositive women, a large proportion (55–87 percent) had asymptomatic infections (Slomka 1996). This finding led to alterations to the preventive strategies previously suggested (Prober et al. 1988; Randolph et al. 1993). A global review of type-specific HSV seroepidemiologic surveys shows that HSV-2 prevalence is higher in Africa and the Americas (South America), lower in western and southern Europe than in northern Europe and north America, and lowest in Asia (Smith and Robinson 2002).

CLASSIFICATION OF GENITAL HERPES INFECTION

Genital herpes infections are defined as primary first episode, nonprimary first episode, or recurrent infections. Primary first episode infections are those with seroconversion for either type 1 or type 2; in nonprimary first episode infection, seroconversion occurs to one type in the presence of antibody to the other type; and in recurrent – also called reactivated – infection, homologous antibodies to the type in question are already present.

Primary infection

Initial genital infection due to herpes may be either asymptomatic or associated with severe symptoms. With

symptomatic primary infection, lesions may occur on the vulva, vagina, or cervix, or on all three between 2 and 14 days following exposure to infectious virus. These lesions are larger in number and size than those observed in patients with recurrent disease and patients who have had prior infection with HSV-1.

When systemic symptoms (malaise, myalgia, and fever) occur (low risk), they are most commonly restricted to presumed primary herpetic infections. These symptoms reflect the viremia that occurs more likely with primary infection. Subclinical cervical and vulvar shedding occur at a rate of approximately 2.3 percent in women with HSV-2 infection and 0.65 percent in women with HSV-1 infection (ACOG, 2000a).

Nonprimary first episode

Prior infection with HSV-1 does not fully protect a patient from initial infection with HSV-2 in the genital tract. There are fewer systemic manifestations, less pain, a briefer duration of viral shedding, and a more rapid resolution of the clinical lesions in the nonprimary infection. These episodes are usually thought to be the result of an initial HSV-2 infection in the presence of partially protective HSV-1 antibodies.

Recurrent infection

Shedding of the virus from the genital tract without symptoms or signs of clinical lesions (subclinical shedding) is episodic and lasts an average of 1.5 days (ACOG 2000b). The clinical signs of genital herpes are present in approximately 25–30 percent of infected women (Table 14).

Table 14 *Classification of genital herpes simplex infection and viral shedding*

Infection	Clinical characteristics
Primary first-episode infection: HSV-2 (HSV-1)	Seroconversion for either type 1 or 2 Characteristic genital lesions, sometimes with systemic illness Low risk of disseminated severe disease Virus shedding in the cervix (ca. 3 weeks) and in high concentration, often continuing after clinical healing
Nonprimary first-episode infection: HSV-2, (HSV-1)	Seroconversion to one type in the presence of antibody to the other type Significant to moderate local symptoms, but little systemic illness Virus shedding often similar to that in primary infection
Recurrent infection: HSV-2 (HSV-1)	Homologous pre-existing antibody Usually minor or no local symptoms Viral shedding in low concentration during the very early phase for 2–5 days

(HSV-1), less common.

EFFECT ON PREGNANCY

The form of genital infection at the time of delivery is of major importance, whereas primary or recurrent oropharyngeal type 1 infections are of little significance. Severe disease after primary oropharyngeal or genital infection in pregnancy is rare. The first case of disseminated HSV infection in pregnancy was reported by Flewett et al. (1969). Since then 25 cases have been described in the English language literature (Kang and Graves 1999; Frederick et al. 2002), which occurred between WG 22 and 38 with an average gestational age of 36 weeks. Both HSV serotypes have been implicated with type 2 more prevalent accounting for 63 percent of cases. HSV hepatitis presents as anicteric hepatic dysfunction with highly elevated transaminases, fever, and abdominal tenderness. Symptoms of central nervous system involvement ranging from lethargy to seizures and coma may be observed in 50 percent of cases. Maternal mortality from 24 reported cases is 39 percent. Three of those received ACV therapy and six were not or inadequately treated. Early diagnosis and early initiation of antiviral therapy with i.v. acyclovir plays a major role in improving survival. Only three patients (20 percent) on antiviral acyclovir died and these three received antivirals late in their clinical course, whereas the mortality rate in patients without antiviral therapy was 67 percent. The overall perinatal mortality, excluding terminated gestations, was 39 percent, but evidence of HSV infections by virus detection in the tissues of these infants was very limited. The review of the reported cases shows that fever and anicteric hepatitis in the third trimester should prompt an investigation for disseminated herpes simplex infection and early treatment with acyclovir will reduce maternal and fetal mortality and morbidity.

Primary genital infections with HSV-2 up to midgestation have been associated with an increased rate of abortion (Nahmias et al. 1971). However, these findings were not confirmed in another study of 94 women with seroconversion during pregnancy (Brown et al. 1997). In the second or third trimester, intrauterine growth retardation and prematurity can occur (Brown et al. 1987). Furthermore, maternal primary, and less often nonprimary first episode infection during the first or second trimester, may lead to congenital infection with severe disease evident at birth (Hutto et al. 1987; Hoppen et al. 2001).

RISK FACTORS IN MOTHER–INFANT TRANSMISSION

Most infants with neonatal herpes acquire infection by passage through an infected birth canal. Mothers with a known history of genital herpes and those with unrecognized genital herpes infection (approximately 70 percent) are at risk of transmitting HSV to their infants. For the intrapartum route of transmission, the risk of neonatal infection depends on the form of maternal genital infection and is directly related to viral shedding into the cervicovaginal secretion at the onset of labor.

Brown and colleagues (Brown et al. 1991) made a prospective study of the very important group of women who are asymptomatic at onset of labor. Viral shedding in the genital tract at the beginning of labor occurred in 56 of 15 923 women (0.35 percent). Of these, 51 shed HSV-2 and five HSV-1. Serological tests revealed that 35 percent of the women with HSV shedding at the onset of labor had recently acquired primary infection or first episode genital infection. Vertical transmission occurred in 12.5 percent in women with positive HSV cultures and in 0.02 percent of those with negative cultures, indicating that viral shedding is not always detectable. Neonatal herpes developed in 33 percent of the babies born to women with recent primary or first episode genital infections, but in only 3 percent of those born to mothers with recurrent asymptomatic infection. The incidence of neonatal herpes in this study population was one per 2 000 live births. In a new prospective study of Brown et al. (2003) the outcome of 58 632 pregnancies with an incidence of neonatal herpes of 1:3 200 per live births. One of the novel finding in this study was that the neonatal HSV infection rate was reduced by cesarean delivery (1.2 percent) versus vaginal delivery (7.7 percent). Only one woman with subclinical nonprimary first-episode HSV-2 infection infected her child after undergoing a cesarean delivery because of failure to progress 19 h after rupture of the membranes. Another novel finding was the high efficiency of transmission of HSV-1 (45 percent) from mother to infant, both from primary infection and reactivation of genital HSV-1, among women with genital shedding.

The results of this study indicate that the risk of virus transmission to vaginally delivered infants is approximately 44 percent (3/3 with HSV-1; 1/6 with HSV-2) in women with primary infection 23 percent (0/1 with HSV-1; 4/16 with HSV-2) in those with nonprimary first-episode HSV genital infection shortly (<2 weeks) before delivery, 1.3 percent (2/11 with HSV-1; 0/140 with HSV-2) in women with recurrent lesions and <3 percent in those with recurrent asymptomatic infection (Brown et al. 2003).

ADDITIONAL RISK FACTORS IN MOTHER–INFANT TRANSMISSION

Additional risk factors for virus transmission and neonatal disease are prolonged ruptured or leaky membranes (>6 h before delivery), increasing the risk of intrauterine infection by the ascending route. Fetal scalp electrode monitoring, forceps delivery, or vacuum extraction may provide portals of virus entry in the newborn (Brown et al. 1991; Malm et al. 1991; Forsgren 1992). Other risk factors for neonatal HSV not statistically proven were younger maternal age (Brown et al. 2003).

Effects on the neonate

INCIDENCE OF NEONATAL DISEASE

Neonatal herpes in the USA occurs at an estimated annual rate of one per 3 500–5 000 deliveries, but may be as high as one per 2 000 in certain population groups with high HSV-2 seroprevalence (e.g. Whitley 1994). For unknown reasons, the annual incidence is much lower in some other countries, for example, the UK (1/40 000–60 000) and Sweden (1/15 000), even though the seroprevalences of HSV-1 and HSV-2 in European countries are comparable with those in the USA. In general, a larger proportion of neonatal infections in these countries, as well as in Germany (where no incidence rates are available) has been predominantly caused between 1993 and 1994 by HSV-2 than HSV-1, thereafter up to now by HSV-1 (1995–2004). A nationwide survey in Japan also indicated that twice as many neonatal infections are due to HSV-1 (Morishima et al. 1996). Similar observations have been reported from the Netherlands, where 73 percent of neonatal herpes cases (2.4 cases per 100 000 neonates) are caused by HSV-1 with a seroprevalence rate for HSV-1 of 61–75 percent and for HSV-2 of 11–35 percent in three different cities (Amsterdam, Nijmegen, Rotterdam) (Gaytant et al. 2002).

PATHOGENESIS OF NEONATAL INFECTION

Of the reported neonatal herpes cases in the USA, approximately 4 percent are acquired in utero, 86 percent intrapartum, and 10 percent postnatally (Whitley 1993; Arvin and Whitley 2001).

Transmission of infection in utero occurs transplacentally in <2 percent of cases, most probably in early to mid-gestation, because physical signs are present at birth and include some unusual stigmata (e.g. hydrocephalus, chorioretinitis); a further 2–3 percent arise late in gestation by the ascending route. In intrapartum transmission, the higher infection rate in infants born to mothers with primary or primary first-episode genital herpes shortly before delivery, causing most cases of neonatal herpes, is related both to viral shedding in high concentration and to the absence of maternal neutralizing antibodies (see, for example, Yeager et al. 1980; Brown et al. 1991; Whitley 1994). This is in contrast to recurrent infection, in which virus is shed for a shorter period in low amounts and maternal antibodies against the infecting type are passed to the newborn. Postnatal infections are acquired from maternal sources (oral, genital, and breast

lesions), or close contact with people with herpetic lesions (e.g. of the mouth or fingers), or by nosocomial spread from other infected babies.

IMMUNE RESPONSE IN THE INFECTED NEWBORN

In infected newborns the cellular immune response is suppressed and delayed as indicated by an absent or delayed T-lymphocyte proliferative response and decreased interferon-α and -γ production in response to herpes simplex antigen (see, for example, Kahlon and Whitley 1988; Cederblad et al. 1989). The IgG antibody response is initially not recognizable because, in the first 6–8 weeks, it cannot be distinguished from maternal antibody. Specific IgM antibodies develop within the first 2–3 weeks of illness and remain detectable for approximately 6–8 weeks. Specific IgG antibodies persist at medium to low titers for life. Transplacentally transferred neutralizing and antibody-dependent cell-mediated cytotoxic (ADCC) antibodies modify the severity of neonatal disease (Kohl et al. 1989).

CLINICAL MANIFESTATIONS

Neonatal herpes is almost invariably overt and frequently lethal. The most severely affected infants are those with in utero infection acquired early in pregnancy (<2 percent) with physical signs apparent at birth. Lesions include skin vesicles or scarring, chorioretinitis, microcephaly, and hydrocephaly (Hutto et al. 1987). So far, more than 30 such cases have been identified but the estimated incidence (one per 200 000 live births) is low. Infants with intrauterine infections acquired late by the ascending route (2–3 percent) usually present only with skin or eye lesions at birth, without multiorgan involvement (Baldwin and Whitley 1989; Whitley 1993; Arvin and Whitley 2001). Yet, there are also reports on disseminated neonatal herpes caused by late maternal ascending infection (Vasileiadis et al. 2003).

In a scheme based on large prospective studies, neonatal herpes cases are classified according to the three general patterns of infection, each occurring in about one third of cases (Forsgren 1992; Whitley 1994; Harrison 1995; Arvin and Whitley 2001) (Table 15).

In general, neonatal disease caused by HSV-1 seemed to have a better prognosis than that caused by HSV-2 (Forsgren 1992). However, in the national Japanese survey on neonatal herpes, although similar patterns of disease were evident, the type of virus had no significant effects on mortality, morbidity, and response to treatment (Morishima et al. 1996). This was also shown by Enders et al. (unpublished).

Diagnosis

DIAGNOSIS IN PREGNANCY

Maternal infection with typical mucocutaneous lesions in the oropharynx and the genital tract is easily diagnosed but less typical, minor genital lesions need laboratory confirmation.

PCR is the most sensitive method for detecting both HSV-1 and HSV-2, and can be used at the onset of labor for a rapid decision on the mode of delivery (Schalasta et al. 2000), high levels of HSV DNA in the maternal secretion increasing the probability of transmission to the infant. Assessment of the type-specific antibodies to both HSV-1 and HSV-2 in late pregnancy is essential for identifying mothers at risk of infecting their infants during delivery. The risk is highest in women with primary or nonprimary first-episode infection who seroconvert during pregnancy. ELISA and immunofluorescence tests with nontype-specific antigens detect seroconversion for IgG and IgM antibodies in primary oropharyngeal and genital infections and rises in specific IgG in genital first episodes; in recurrent infections (symptomatic or asymptomatic), there is no rise in specific IgG level and IgM is not detectable. The more cumbersome neutralization test, although not routinely used, is employed for determining the amount of protective antibodies in maternal and newborn blood.

None of the conventional tests, however, can accurately distinguish between type 1- and type 2-specific antibodies. This is possible only with assays (e.g. immunoblot, immunodot, gG2 indirect ELISA) employing recombinant expressed or native purified glycoprotein G antigen (gG-1 for HSV-1, gG-2 for HSV-2) (Svennerholm et al.

Table 15 *Classification, pattern, and outcome of neonatal HSV disease*

Disease	Onset of signs after birth	Mortality		Prognosis in survivors
		No therapy	With therapy	
Disseminated CNS, lung, liver, adrenals, SEM	6–12 days[a]	>80%	>50%	40% develop normally
CNS encephalitis/meningitis in 37–48% without SEM	16–18 days	>50%	15%	In ca. 56% neurological and ophthalmological impairment and recurrent skin lesions
SEM skin/eye/mouth	7–10 days	ND	0%	In $\geqslant$20% neurological impairment, recurrent skin lesions

ND, no data.
a) Onset at 12 days is less frequent.

1984; Johnson et al. 1989; Forsgren 1992; Slomka 1996; Enders et al. 1998; Ashley 2002). HSV-2 type-specific IgM antibodies may also be detected more easily by such assays (Ho et al. 1993). However, certain problems with immunoblot versus ELISA tests with regard to low values have to be recognized (Cherpes et al. 2003). It should be noted that the interval between onset of a primary or first episode genital infection to the detection of type-specific antibodies may range from 2 to 3 weeks for HSV-1 and from 2 to 4 weeks for HSV-2 (Ashley et al. 1988; Ho et al. 1993). The influence of immediate ACV therapy on prolonging seroconversion has to be considered (Ashley and Corey 1984).

DIAGNOSIS IN NEONATE AND INFANT

Cases with skin vesicles are easily recognized; neonatal herpes should also be considered in the differential diagnosis of severe generalized disease or of encephalitis within the first 6 weeks of life.

The most direct means of laboratory diagnosis is viral detection, especially by PCR, which is particularly useful for tests on cerebrospinal fluid (CSF). Serology is of little help in the first weeks of life. Testing the maternal serum together with acute phase and later sera of the infant is essential. A presumptive retrospective diagnosis of a child damaged by HSV-2 is obtained by demonstrating persistent IgG antibodies to HSV-2 or by isolating the virus from recurrent vesicles, because HSV-2 infections are rare before the onset of sexual activity (Malm et al. 1991).

Management in pregnancy and of the neonate

At the first antenatal visit, a careful history should be taken of genital infections in the woman herself and her sexual partner, together with visual inspection of her genital tract both at this visit and at the onset of labor. If lesions are seen, swabs should be taken for virus detection, but a decision on cesarean section rests mainly on the presence of visible lesions. If serological tests are used, seroconversion to HSV-2 antibody-positive during pregnancy may be an indication for rapid virus detection by PCR and possible treatment; this approach might reduce the need for cesarean section.

Cesarean sections, considered at present as the only effective means of preventing neonatal herpes infection, are performed on up to 70 percent of women in the USA with a history of genital herpes, because of possible fetal infection and of medicolegal considerations. It is recommended generally for women with visible cervicovaginal lesions at onset of labor, irrespective of the antibody status or viral shedding, and is best performed before rupture of the membranes. The procedure will, however, not always prevent neonatal herpes, even if performed before rupture of the membranes, and especially not after they have been ruptured for more than 4 h (Stone et al. 1989; Randolph et al. 1993).

Prenatal diagnosis is not recommended because the fetus is rarely transplacentally infected until mid-gestation. Termination of pregnancy in women with disseminated disease is not an option.

For immunoprophylaxis, specific immunoglobulins, humanized murine and human monoclonal antibodies, and hyperimmunoglobulin with high neutralizing activity (but never commercially available) have been developed for administration to neonates lacking maternal antibodies. Vaccines for prevention and treatment, particularly of HSV-2 genital infections, have also been developed and a candidate subunit vaccine is still on trial (Stanberry et al. 2002; Whitley and Roizman 2002).

Acyclovir or its derivatives are the mainstay of antiviral therapy for both topical and systemic application. Although this drug is not licensed for use in pregnancy, no association has so far been identified between its use in pregnancy and adverse effects in mother or child (Kroon and Whitley 1995). This was shown by a large registry of acyclovir use in pregnancy for both HSV and varicella pneumonia which was established in the USA (1984–1998). Up until its closure in 1998, no evidence of any increase in adverse fetal effects related to drug exposure in any trimester, in comparison with the general population, was reported (Enders and Miller 2000).

In maternal primary disseminated infection, early intravenous treatment with acyclovir (i.v. 10 mg/kg every 8 h for 10–14 days) at any stage of pregnancy is recommended. Oral treatment with acyclovir (400 mg three times daily for 14 days) is considered beneficial for women with recognized primary or non-primary first-episode genital infection in the later stages of pregnancy. This is also the case in maternal primary oral infection (e.g. gingivostomatitis) shortly before term, at delivery, or in the neonatal period and in primary genital herpes in the neonatal period. To suppress viral shedding during labor in women with recurrent lesions in late pregnancy, prophylactic oral use (400 mg three times daily) approximately 10 days before delivery (Stray-Pedersen 1990; Haddad et al. 1993) is still controversial for various reasons, such as potential renal toxicity for the fetus, occasional virus shedding despite suppressive treatment (Katz et al. 1995), and the question of its value. The need for suppressive therapy in the above-mentioned target group is limited, because there is only a low prospective risk to babies born to women with recurrent lesions, in contrast to those born to mothers with unrecognized primary and non-primary first-episode genital infections (Kroon and Whitley 1995; Arvin and Whitley 2001). Hence a recently published double-blind, randomized, placebo-controlled trial of acyclovir in late pregnancy shows that acyclovir significantly reduced, but did not eliminate, herpes simplex virus lesions in the genital tract and detection of HSV

DNA by PCR in genital secretion in late pregnancy (Watts et al. 2003).

The prophylactic administration of acyclovir to vaginally delivered asymptomatic newborns at risk of infection depends mainly on the form of maternal infection and the virus load in the maternal secretion. Follow up for clinical and laboratory signs of infection is recommended up to 4 weeks after delivery (e.g. Forsgren 1992; Whitley 1994; Arvin and Whitley 2001). Opinions on administration of acyclovir to exposed vaginally delivered infants who have no signs of infection are controversial. However, prophylactic treatment should be considered for infants of mothers with high-risk markers for primary or primary first-episode genital infection.

B19 VIRUS (PARVOVIRUS B19)

The *B19 virus* (B19), belonging to the genus *Erythrovirus*, family *Parvoviridae*, was discovered in the plasma of healthy blood donors in 1975, and recognized in 1983 as the cause of erythema infectiosum. In 1984, its causative role in hydrops fetalis and fetal death became evident and in 1993 blood group P antigen (glycolipide-globoside) was identified as the cellular receptor for parvovirus B19 (Brown et al. 1993). Since 1995, parvovirus B19 has become associated with a wide spectrum of hematological and nonhematological complications.

Recently, a second human erythrovirus, the V9 isolate, was detected in the serum and bone marrow of a child with transient aplastic anemia (Nguyen et al. 1999; Servant et al. 2002). The prevalence of V9 and its association with clinical disease and significance for pregnancy remains to be evaluated.

Postnatal infection

Postnatal infection of parvovirus B19 is acquired through aerosol droplets by the respiratory route by close person-to-person contact, but may also occur through blood-derived products administered parenterally. Transmission via respiratory secretions is greatest during viremia and before clinical signs are apparent, complicating efforts to control transmission. The incubation time ranges between 8 and 18 days (Anderson et al. 1985; Joseph 1986), the contagious period lasts from 5 to 7 days before, and until 2 to 4 days after, onset of symptoms. First-phase illness appears approximately 8 days after infection and second-phase illness with typical symptoms about 17/18 days. All immunocompetent people with or without characteristic symptoms develop antibodies to parvovirus B19 and presumably lasting immunity that protect them against infection with the virus in the future. The seroprevalence rate in childbearing age, in adults and in pregnancy ranges from 30 to 60 percent in individual countries and with regional differences (Cohen and Buckley 1988; Enders and Biber 1990; Kelly et al. 2000).

Infections in temperate climates are common in late winter, spring, and the early summer months. Rates of infection may rise to an epidemic level every 3–4 years. During epidemic periods, women of childbearing age show an annual seroconversion rate of 13 percent compared with 1.5 percent in endemic periods (Koch and Adler 1989; Valeur-Jensen et al. 1999; Jensen et al. 2000). During outbreaks, secondary spread to seronegative contacts is very common. The infection rate in susceptible children and adults is up to 50 percent within families and approximately 20 percent for care workers and teachers in primary school being highest for those in contact with younger and greater numbers of infected children (Gillespie et al. 1990). During school outbreaks, 10–60 percent of students may get fifth disease (CDC 2000). A serological study on the occupational risk in employees of hospital and elementary schools during an endemic period revealed that those persons in daily contact with school-age children had a fivefold increased annual occupational risk for infection (Adler et al. 1993).

In pregnancy, approximately 60 percent of infections are asymptomatic or clinically uncharacteristic, for example, without the typical slapped-cheek rash and the lacy red rash on the trunk and limbs, but often associated with joint swelling which lasts usually for 1–2 weeks, and more rarely for several months. Most frequently affected are the joints of the hands, wrists, and knees (Woolf et al. 1989; Enders and Biber 1990).

Effects on the fetus

PATHOGENESIS OF FETAL INFECTION

The main cellular receptor for B19 virus (the blood group P antigen, a glycolipid-globoside), is expressed on mature erythrocytes and erythroid progenitor cells. Abundant productive infection with cell lysis has only been demonstrated in the nucleated, rapidly dividing erythroid progenitors (burst-forming units erythroid (BFU-E) and colony-forming units erythroid (CFU-E)) in bone marrow and fetal liver (Morey and Fleming 1992). The inhibition of fetal erythropoiesis occurs mainly in the late pronormoblast stage in which intranuclear inclusions (Lantern cells) are commonly detected. Direct toxic cell injury, as well as B19-induced apoptosis and cell cycle arrest may be involved in the pathogenesis of erythroid aplasia (Chisaka et al. 2003). The damage of the erythroid progenitor cells, e.g. in the bone marrow, spleen, and liver during the time of a rapidly expanding red-cell volume combined with a shortened fetal red-cell life span of 45–70 days produces a profound aplastic crisis in the fetus with a resultant anemia culminating in cardiovascular decompensation, hydrops, ascites, and fetal death.

P antigen is also presented on nonerythroid cells, for example, megakaryocytes, endothelial cells, placental villous trophoblast cells, and cardiac myocytes. However, none of these cells are fully permissive for B19 replication. Various reasons for the nonpermissiveness are discussed and include incomplete virus replication (Liu et al. 1992), the need for a second receptor for viral entry (Weigel-Kelley et al. 2003) or a viral phospholipase A2 which appears to be necessary for B19 infectivity (Zadori et al. 2001; Heegaard and Brown 2002). Nevertheless, B19 was found to infect fetal myocardial cells and was associated with fetal myocarditis (Soulie 1995; von Kaisenberg et al. 2001). Hence, not only anemia but also myocarditis may be implicated in the pathogenesis of hydrops fetalis.

The presence of the globoside receptor on trophoblast cells may play a role in transmission of the virus across the placenta. It was observed that the immunoreactivity for globoside was strongest in the villous trophoblast cells of first trimester placentas with diminished reactivity in second trimester placentas and a near lack of staining for the antigen in those of third trimester. The observed change found in globoside immunoreactivity correlates well with the fact that fetal outcome is worse when maternal infection occurs during the first or second trimester as compared with an infection occurring near term (Jordan and DeLoia 1999). Furthermore, an enhanced apoptotic activity within placental villous trophoblast cells in pregnancies complicated by B19 infection was described. Damage to the protective trophoblast layer may compromise the integrity of the placenta, and cause fetal loss not necessarily associated with fetal infection (Jordan and Butchko 2002).

FETAL COMPLICATIONS

The virus may cross the placenta at any time during pregnancy and infection is possible as early as 6 weeks of gestation (Nunoue et al. 2002). The overall transplacental transmission rate was estimated to be 33 percent based on an adverse outcome, parvovirus B19 IgM in cord or neonatal blood, and on the persistence of specific IgG antibodies (Miller et al. 1998; Public Health Laboratory Service Working Party on Fifth Disease 1990) and others have reported similar rates (Gratacós et al. 1995; Koch et al. 1998).

The major risks are spontaneous abortion, stillbirth, and hydrops fetalis (HF). The interval between onset of maternal infection and fetal complications may range from 2 to 17 weeks (Miller et al. 1998), 2–6 weeks (Yaegashi et al. 1998), and 2–20 weeks (Nunoue et al. 2002).

Fetal loss

The rates of fetal loss varied in some small numbered prospective studies between 1.66 percent (one of 60 infected mothers) (Gratacós et al. 1995), 5 percent (2/39) (Rodis et al. 1990), 15 percent (7/48) Yaegashi et al. 1999), and 0 percent (0/52) (Harger et al. 1998). In two prospective studies from the UK, 427 pregnant women with acute B19 virus infection were investigated (Public Health Laboratory Service Working Party on Fifth Disease 1990; Miller et al. 1998). The excess rate of fetal loss was confined to the first 20 weeks of gestation and averaged 9 percent. In our prospective study, 1 018 pregnant women with B19 infection were enrolled and the excess rate of fetal loss was also confined to the first 20 WG, but slightly smaller (5.6 percent). The median interval between maternal infection and diagnosis of fetal death was 2 weeks (Enders et al. 2004). Although the majority of B19 infections associated with fetal death occur in the first 20 WG, intrauterine death in late pregnancy without signs of fetal hydrops has been reported by different authors (Skjöldebrand-Sparre et al. 2000; Tolfvenstam et al. 2001; Norbeck et al. 2002). According to our observations and to the findings of other studies (e.g. Miller et al. 1998), there seems to be no such association.

Nonimmune hydrops fetalis

In 1984, the first case of hydrops fetalis associated with confirmed parvovirus B19 infection was reported by Brown et al. (1984). The risk of fetal hydrops in the two English studies (Public Health Laboratory Service Working Party on Fifth Disease 1990; Miller et al. 1998) was diagnosed to be 2.9 percent. In our investigation, hydrops fetalis was diagnosed in 3.9 percent (40/1018) throughout pregnancy with a maximum of 7.1 percent (23/322) when maternal infection occurred between WG 13 and 20. The rate was lower than 1 percent when maternal B19 infection occurred after WG 32. The median interval between maternal infection and diagnosis of hydrops was 21 days. In the rates of hydrops, there were no significant differences between asymptomatic and symptomatic maternal infection (Enders et al. 2004). It should be noted that hydrops can resolve spontaneously without apparent ill-effects on the infant (Morey et al. 1991; Pryde et al. 1992, Sheikh et al. 1992; Zerbini et al. 1993; Tercanli et al. 1996). In the large study of Rodis et al. (1998a) with 539 cases of parvovirus B19 induced hydrops 34 percent resolved spontaneously.

DISEASES POSSIBLY DUE TO FETAL PARVOVIRUS B19 INFECTION

In addition to the fetal complications described above, B19 virus infection has been suspected to lead, if the fetus survives, to specific and permanent organ defects. Most of those findings have been made in aborted fetuses with B19 infection and involvement of myocardial cells (Porter et al. 1988), eye anomalies, and damage to other tissues (Weiland et al. 1987) or with bilateral cleft lip and palate, micrognathia, and webbed joints (Tiessen et al. 1994). Systematic studies, however,

have failed to substantiate congenital malformations caused by parvovirus B19.

Brown and colleagues (1994) described three cases with chronic anemia closely resembling congenital red cell aplasia (Diamond–Blackfan anemia). In all three, the sera lacked B19 DNA, but viral DNA was found in bone marrow. In one instance hepatic disease was associated with intrauterine B19 infection in a premature newborn (Metzman et al. 1989). Furthermore, a case of 'prune belly' after maternal B19 infection and hydrops fetalis has been reported (Walther et al. 1994). There is a more important preliminary report on three live-born infants with severe nervous system anomalies following serologically confirmed maternal B19 infection (Conry et al. 1993), but a follow-up on these children is missing. One report concerns fetal B19 infection and meconium peritonitis (Schild et al. 1998) and another one fetal parvovirus B19 myocarditis with terminal cardiac heart failure and perinatal heart transplantation (von Kaisenberg et al. 2001).

IMMEDIATE AND LONG-TERM OUTCOME OF B19 INFECTION IN PREGNANCY

In the study of Miller et al. (1998), no abnormalities attributable to parvovirus B19 infection were found at birth in surviving infants and no late effects were diagnosed at 7–10 years. The congenital infection rate was estimated with 22 percent based on the detection of IgG antibodies in children at least 1 year of age. It was concluded that the maximum possible risk of congenital abnormality is under 1 percent and the long-term development will be normal (Miller et al. 1998). In another study, the favorable long-term outcome in 108 children exposed in utero to maternal parvovirus B19 infection at the median age of 4 years (range from 6 months to 7 years) was described (Rodis et al. 1998b). Our observations on the outcome of live-born infants of mothers with serological proven B19 infection at birth and at age 8 to 24 months also indicate that there are no parvovirus B19 attributable immediate or long-term clinical consequences (G. Enders, unpublished 2004).

Diagnosis

DIAGNOSIS IN PREGNANCY

B19 virus infection should be excluded in pregnant women who are contacts to patients with erythema infectiosum. In pregnant women with exanthems, lymphadenopathy, or arthralgia, B19 infection as well as rubella must be considered. Anamnestic data about type and time of contact are helpful. In pregnant women with suspected hydrops fetalis or fetal anemia on sonography, parvovirus B19 infection should be confirmed or excluded.

Detection of specific IgM with or without IgG antibodies indicates acute B19 infection. This should be confirmed by testing a second serum. IgG antibodies in the absence of IgM antibodies indicate previous infection ($\geqslant$4 months) or protection (Searle et al. 1997; Ferguson et al. 1997). However, depending on the serological assay used, IgM antibodies may decline to undetectable levels within 4–6 weeks. The significance of detecting NS1-specific IgG has been continuously discussed and contested since it was suggested that this antibody was suggested to be associated with an altered course of disease (von Poblotzki et al. 1995). Conversely, others have found no evidence of NS1 IgG antibodies representing a marker of persistent infection or contributing to pathogenesis. Most of the studies find that, irrespective of the underlying disease, NS1-specific IgG antibodies appear late in infection (>6 weeks), and the NS1 antibody test may, therefore, be used to exclude very recent infections in patients with an otherwise unclear serology (Hemauer et al. 2000; Searle et al. 1998). Neither raised levels of alpha-1 fetoprotein (AFP) nor human chorionic gonadotropin (hCG) levels in maternal serum have been found to be regular prognostic markers for fetal complications in pregnant women with parvovirus B19 infection (Saller et al. 1993; Komischke et al. 1997). However, both markers are frequently elevated when fetal complications were already evident.

Maternal B19 infection can be confirmed by detection of B19 DNA using PCR when diagnostic interpretation of serological results is difficult. Large amounts of B19 DNA are present in the blood shortly before and after onset of symptoms and can persist at low titers for 2–6 months or even longer (Musiani et al. 1995; G. Enders et al. unpublished). This, however, also depends on the sensitivity of the PCR employed with refined PCR-based techniques such as automated sample preparation and real-time quantitative PCR with a detection limit of 234 IU/ml (Schorling et al. 2004).

DIAGNOSIS IN THE FETUS

Fetal infection is suspected most frequently when hydrops is diagnosed by ultrasonography after a serologically confirmed B19 virus infection in the mother or at routine scan without knowledge of maternal serology. The most reliable way to diagnose fetal infection is to detect B19 DNA by PCR in amniotic fluid, body effusions, or fetal blood. The severity of hydrops fetalis has been graded according to the degree of fetal anemia or ascites (Fairley et al. 1995; Schild et al. 1999). Mild hydrops is not usually associated with significant anemia (Hb 7.2–11.5 g/dl), and may resolve spontaneously over time (category I). Moderate hydrops fetalis is associated with Hb values of 5.7–7.2 g/dl (category II). In cases with generalized hydrops, fetal Hb values range between 1.9 and 5.4 g/dl (category III). Without very early intervention, most cases end in intrauterine death (Tercanli et al. 1996). In prenatal diagnostic centers more recently,

Doppler sonography is employed to recognize fetal anemia by measuring the peak systolic velocity (PSV) in the middle cerebral artery (MCA) (Delle Chiaie et al. 2001; Cosmi et al. 2002). According to the work of Cosmi et al., it seems to be a reliable predictor of parvovirus-induced fetal anemia.

In the case of pathologic PSV values, fetal blood sampling should be considered to determine fetal Hb and reticulocyte count, as well as to confirm B19 infection by detecting viral DNA, IgM antibodies, or both. To detect B19 DNA in fetal blood by PCR, it is important that heparin is not used for moistening the needle employed for cordocentesis, because this anticoagulant may inhibit *Taq* polymerase by binding to DNA (Beutler et al. 1990; Schwarz et al. 1992) and can cause false-negative results. Fetal parvovirus B19-IgM antibodies are a less reliable marker for fetal infection than the detection of B19 DNA in fetal blood or amniotic fluid (Schild et al. 1999). This is confirmed by our results performing the laboratory investigation of fetal specimens obtained at prenatal diagnosis in 349 B19-affected pregnancies (G. Enders, personal observation). In addition, B19 can be identified by histopathology, immunohistochemistry, and in situ hybridization in fetal tissues (Wright et al. 1996).

Management in pregnancy and of the neonate

When acute infection is diagnosed in pregnancy, weekly ultrasound monitoring at low level up to 8–12 weeks after onset of maternal infection is recommended to detect signs of fetal infection such as increased volume of amniotic fluid, skin or placental edema, ascites, and pleural and pericardial effusions early in the course of disease. In such cases the patient should be referred immediately to a prenatal tertiary diagnostic center for further tests and, if necessary, for intrauterine therapy (Schild et al. 1999).

In case of late maternal B19 infection after the 32nd week of pregnancy, fetal complications are less likely. If such an exceptional case occurs near term, the induction of labor or cesarean section may be performed. After delivery, no special precautions (e.g. isolation mother/child) are needed and breastfeeding is not contraindicated. No observations have been reported that late intrauterine or perinatally infected newborns pose an occupational risk to medical personnel or care workers.

TREATMENT OF THE HYDROPIC FETUS

Intrauterine transfusion as a new therapy regimen in B19-infected hydropic fetuses was first reported from Germany (Schwarz et al. 1988; Schwarz et al. 1990). Initially, this treatment was controversial, since the case of intrauterine transfusion rests against the spontaneous resolution of fetal hydrops. However, the findings in a study conducted in the UK during the epidemic year of 1993/1994 indicated that timely intrauterine transfusion of fetuses with severe hydrops fetalis reduces the risk of fetal death. In this study, the surviving fetuses had no abnormalities related to B19 infection (Fairley et al. 1995). In the meantime, an increasing number of studies on invasive prenatal diagnostics and intrauterine transfusion in fetuses mainly with hydrops fetalis and their favorable immediate and long-term outcomes were reported worldwide (e.g. Rodis et al. 1998a; Schild et al. 1999; Eis-Hübinger et al. 1998; Swain et al. 1999; Forestier et al. 1999; Grab et al. 2002). From all these studies there is little evidence of damage to fetuses infected with parvovirus B19, treated by intrauterine transfusion, and born alive. This is important for the justification of intrauterine treatment of infected fetuses.

PROPHYLAXIS AND THERAPY

Screening in pregnancy for B19 virus antibodies is not recommended and exclusion from work of pregnant women in, for example, childcare centers and elementary schools during the highest risk period between the 9th and 24th week of pregnancy may be considered, but is not officially recommended (Crowcroft et al. 1999). Efforts to prevent contact with infected individuals are of very limited effect, since people are contagious before they develop the rash (CDC 2000).

Passive prophylaxis with immunoglobulin may be effective, since all immunoglobulin preparations for i.v./i.m. application tested contain parvovirus B19-IgG antibodies which can be measured in international units per milliliter and neutralizing antibodies detectable by using a reverse-transcriptase polymerase chain reaction (Ballou et al. 2003). Postexposure prophylaxis with immunoglobulins in pregnancy is presently not recommended; however, pre-exposure prophylaxis has been proven to be beneficial in patients with chronic parvovirus infection and in nosocomial outbreaks in hospitals.

Antiviral drugs are not available for treatment and a parvovirus B19 vaccine is presently not licensed for use. A recombinant human parvovirus B19 vaccine has been developed and is composed of the VP1 and VP2 capsid proteins and formulated with a special adjuvant has recently been evaluated in a randomized, double-blind phase 1 trial in healthy B19-seronegative adults. The vaccine seems to be safe and immunogenic, since all volunteers developed neutralizing antibody titers that peaked after the third immunization and were sustained thus far through 1 year (Ballou et al. 2003).

It is now evident from all the available information that approximately 94 percent of parvovirus B19-affected pregnancies have a normal outcome, that the risk of fetal damage is low and no malformations were observed, and that in case of moderate to severe fetal anemia intrauterine transfusion(s) reduce the risk of fetal death. Therefore a pregnancy does not need to be terminated when B19 infection occurs. Even so, although studies on

immediate and long-term outcome of surviving infants following maternal infection in pregnancy have increased recently, further such studies are needed.

HUMAN IMMUNODEFICIENCY VIRUS

AIDS is caused by the lentiviruses human immunodeficiency virus 1 and human immunodeficiency virus 2 (HIV-1 and HIV-2). At the end of 2003, more than 40 million people were living worldwide with human immunodeficiency virus (HIV), including about 19 million women (estimated by UNAIDS); 700 000 children under the age of 15 years are infected annually, most of them by mother-to-child transmission. Prenatal care is an important means of detecting HIV infection in pregnancy. Progress in diagnosis, therapy, and interventional measures has drastically reduced the risk of vertical transmission in industrialized countries.

Effects on pregnancy

Pregnancy does not seem to accelerate progression of HIV-1 disease as demonstrated in studies from the USA and Europe (Burns et al. 1998; Weisser et al. 1998; Saada et al. 2000). Data from developing countries suggest a weak association between pregnancy and an adverse maternal outcome related to HIV infection (French and Brocklehurst 1998). Furthermore, a greater frequency of spontaneous abortion, preterm birth, low birth weight, intrauterine growth retardation, stillbirth, and infant death was shown among HIV-infected women (Brocklehurst and French 1998).

Mother–infant transmission

The main factor associated with increased risk of vertical transmission is the maternal plasma HIV-1 RNA level (e.g. Cooper et al. 2002). In former studies a critical threshold for transmission was estimated to be 100 000 RNA copies/ml of plasma (Fang et al. 1995), but later studies could not confirm this threshold (European Collaborative Study 1999). A lower copy number decreases the risk of transmission significantly (Ioannidis et al. 2001), whereas in pregnant women with undetectable virus copies the transmission rate might be reduced completely (Mofenson et al. 1999). Further factors associated with increased risk of vertical transmission as low maternal $CD4^+$ cell counts, advanced clinical HIV disease, increased levels of beta-2-microglobulin and race/ethnicity are also assumed to reflect the viral load (Cunningham et al. 2004). Other factors are, for example, co-existing sexually transmitted diseases, drug abuse, low vitamin A, chorioamnionitis, rupture of the membranes >4 h before birth regardless of the mode of delivery, and premature delivery (e.g. Minkoff et al. 1995; Landesman et al. 1996). Also immunological factors contribute to the risk of vertical transmission. A mother–child HLA class I concordance, several HLA-B alleles, maternal SDF1 chemokine receptor ligand polymorphism, and infant homozygosity in a mutation of the regulatory region of chemokine receptor 5 gene increase the risk of perinatal HIV-1 transmission (Polycarpou et al. 2002; Winchester et al. 2004; John et al. 2000; Kostrikis et al. 1999), whereas the presence of maternal neutralizing antibodies decreases the risk (Scarlatti et al. 1993). Also some MHC class II alleles of the infant are associated with reduced or enhanced risk of vertical HIV infection (Winchester et al. 1995).

ROUTES OF TRANSMISSION

Mother-to-child transmission may occur in utero, intrapartum, or by breast-feeding. Intrauterine infection in early pregnancy seems possible, but is more likely to occur shortly before delivery. It is usually assumed to occur by hematogenous spread across the placenta. However, it may also spread from pelvic organs in which replication is occurring, and in late pregnancy by ascending infection from the vagina after rupture of the membranes.

Intrapartum transmission causes a substantial proportion of the cases of vertical transmission (Chouquet et al. 1999). Possible mechanisms include transfusion of the mother's blood to the fetus during labor contractions, infection after the rupture of membranes, and direct contact of the fetus with infected secretions or blood from the maternal genital tract.

The mainly postnatal route of HIV transmission is by breast-feeding. Breast-feeding is associated with an additional 15 to 20 percent risk of HIV transmission (Nduati et al. 2000). Modifying infant feeding practices with early cessation of breast-feeding at 4–6 months or inactivation of HIV in breast milk reduces the risk of postnatal transmission (Rollins et al. 2004).

RATES OF PERINATAL TRANSMISSION

Transmission rates for HIV-1 range today from <2 up to 40 percent depending on geographic areas, socioeconomic conditions, therapeutic and obstetrical interventions. With optimal management of the mother and the newborn, a transmission rate below 2 percent can be achieved (Buchholz et al. 2002).

HIV-2 infections in pregnant women are still rarely reported. However, in the few studies the perinatal transmission rates range form 0 to 4 percent (Adjorlolo-Johnson et al. 1994; De Cock et al. 1994). The much lower rate of transmission of HIV-2 compared with HIV-1 is possibly due to a 37-fold lower maternal RNA level in HIV-2-infected women (O'Donovan et al. 2000).

Effect on infant

Two patterns of disease have been described for infants vertically infected with HIV-1. In one, infection

progresses rapidly to AIDS within the first year of life in approximately 25 percent. In the remainder it progresses more slowly and, of these, approximately 25 percent remain asymptomatic until pre-adolescence (Grubman et al. 1995). In two European prospective studies an estimated one-fifth of infected children will have been diagnosed with AIDS or have died by 12 months of age, rising to a third by 6 years of age (Blanche et al. 1997). In a study from Malawi, the mortality rate of HIV-infected children up to the age of 3 years was 45 percent, the major causes of death were wasting and respiratory conditions (Taha et al. 1999).

The more rapid progression of disease in vertically infected infants compared with adults is explained by the relative immaturity of the immune system, the infectious dose, the route of infection, and infections with opportunistic organisms. In children infected with HIV-1, the wide range of clinical manifestations includes features of severe immunodeficiency, nonspecific symptoms and AIDS-related diseases. The prospects for survival of children with opportunistic infections, neurological disease, and lymphoma are worse than for those with lymphocytic interstitial pneumonitis or bacterial infections.

Diagnosis

DIAGNOSIS IN PREGNANCY

Prenatal HIV testing offers the best opportunity for the prevention of perinatal HIV infection. Voluntary HIV screening in pregnancy has been implemented in the USA, Canada, and western European countries in gynecological, prenatal, and other obstetric clinics. For instance in Germany, HIV testing has been included since 1987 in routine prenatal care if the pregnant woman gives her consent. Over recent years, substantial increasing prenatal testing rates have been observed in the USA and Canada. Data from the USA indicate that in 2000, 93 percent of HIV-infected women knew their HIV status before delivery. However, an estimated 280–370 perinatal HIV transmissions continue to occur in the USA each year (CDC 2002a). In western countries, pregnant women remain at low risk of acquiring HIV infection. In Germany, from 1987 to 2004, the seroprevalence of HIV has been surveyed in 325 000 pregnant women resulting in a HIV prevalence of 0.03 percent (G. Enders 2004, unpublished results).

HIV screening in pregnancy is performed with sensitive antibody detecting enzyme immunoassays (EIA). Newer assays include the detection of p24 antigen which improves the diagnosis of a primary HIV infection when p24 antigen is present, but antibodies are not yet synthesized. Reactive HIV screening assays have to be confirmed serologically, for example, with western blot or immunofluorescence assays. For the management of HIV-1-infected women, the viral load in the blood is determined by quantifying RNA and the cellular immune status by counting the $CD4^+$ T cells.

DIAGNOSIS IN THE FETUS

Tests on the fetus of an HIV-infected woman are not recommended, because most vertical transmissions of HIV occur intrapartum and only occasionally during late pregnancy. Furthermore, as in the case of hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, contamination with maternal blood during amnio- or cordocentesis may transmit the virus to the fetus (Enders and Braun 2000; Geipel et al. 2001). An invasive prenatal diagnostic, for example, for the detection of chromosomal anomalies should be performed only when strictly indicated and with antiretroviral therapy/prophylaxis (Buchholz et al. 2002; Maiques et al. 2003).

DIAGNOSIS IN NEONATAL AND INFANT

Early diagnosis of HIV infection in infants born to an HIV-positive mother is not recommended by conventional serological tests, because IgG antibodies reflect maternal serology and IgM antibody determination lacks sensitivity and specificity (Schupbach et al. 1994). Although tests for HIV-specific IgA antibodies are less sensitive compared with virological tests especially for early diagnosis of HIV infection in the first 3 months of life, they may be useful in countries without facilities for viral culture or PCR detection (Livingston et al. 1995). In general, HIV infection can be definitively diagnosed in most infected infants by the age of 1 month and in virtually all infected infants by the age of 6 months by using viral diagnostic assays. A positive virological test (i.e. detection of HIV by culture or polymerase chain reaction) indicates possible HIV infection and should be confirmed by a repeat virological test on a second specimen as soon as possible. Pediatric HIV testing is recommended by the age of 48 h, at age 1–2 months, and at age 3–6 months. At birth only about 40 percent of infected children had positive viral assays. Then the sensitivity increases rapidly during the first week, with over 90 percent of infected children testing positive by the age of 1 month (Peckham and Gibb 1995). HIV DNA and/or RNA PCR is the preferred virological method; HIV culture has a sensitivity similar to that of HIV PCR, but HIV culture is more complex, expensive, and time-consuming to perform compared to HIV PCR. The use of HIV p24 antigen testing alone is not recommended for diagnosis, because the sensitivity and specificity of this assay is less compared with other HIV virological tests. HIV infection is diagnosed by two positive HIV virological tests performed on separate blood samples. HIV infection can be reasonably excluded among children with one negative virological test at the age of 1–4 month, and one at age $\geqslant$4 months. Definitively excluded is the HIV infection at the age of 18 months, if HIV IgG antibody is negative in the

absence of hypogammaglobulinemia and if the child has neither clinical symptoms of HIV infection nor negative HIV virological assays (CDC 1998a).

Previously cases of HIV clearance in children were described in several studies (e.g. Newell et al. 1996). Later investigations of 42 suspected transient HIV-1-infected children could not confirm a cleared HIV infection in any of these cases (Frenkel et al. 1998). Most cases suggest mislabeling of specimens or contamination in the laboratory, therefore rare cases of HIV clearance may be possible but have to be proven carefully.

Management

MANAGEMENT IN PREGNANCY

The great progress over the past few years for treatment of HIV infection has also dramatically influenced the management of HIV-infected pregnant women (European Collaborative Study 2001; Friese 2003).

Prevention

Most HIV infections are acquired sexually, the risk of transmission is associated with the viral load in genital secretions which can be different from the viral load in the peripheral blood (Robert Koch Institut 2003). The optimal preventive measure is sexual contact only with a partner known to be uninfected and the avoidance of unprotected sexual intercourse. Despite extensive worldwide research activities, a successful HIV preventive vaccine is not yet available (Stratov et al. 2004). After occupational exposure (e.g. accidental needlestick of healthcare workers), an antiretroviral postexposure therapy reduces the risk of HIV transmission (Cardo et al. 1997). For HIV exposures relating to sexual or drug injection, the post-exposure therapy can be offered under strict indication when the probability of HIV infection is high, therapy can be initiated promptly and adherence to the regime is likely. This therapy should never be considered as a routinely used form of primary HIV prevention (Robert Koch Institut 2002a).

Chemotherapy

In 1996, the ACTG 076 trial demonstrated a reduction of the vertical transmission after administration of zidovudine monotherapy throughout pregnancy and to the newborn for the first 6 weeks of life (Sperling et al. 1996). Shorter courses of therapy and treatment with other drugs, e.g. nevirapine or zidovudine/lamivudine also reduced the rate of vertical transmission. Studies with highly active antiretroviral therapy (HAART) resulted in the lowest transmission rates (Cooper et al. 2002). When combination therapy is administered during pregnancy, zidovudine should be included whenever possible. Maternal indication for therapy is based on the clinical, immunological, or virological status as published in current guidelines for the treatment of nonpregnant women (e.g. NIH 2004). To reduce the risk of vertical transmission, antiretroviral therapy is recommended even for pregnant women with a HIV-1 viral load below 1 000 copies/ml (Ioannidis et al. 2001). The possible risks of teratogenic drug effects of antiretroviral therapy during the first trimester of pregnancy are so far unknown. Exposure to maternal antiretroviral therapy was not associated with prevalence or pattern of congenital abnormalities, but was associated with reversible anemia in the newborn. However, treatment with some drugs, e.g. efavirenz, should be avoided because teratogenic effects occurred among animal species. The data on the teratogenic potential of antiretroviral agents are limited, currently approved agents and selected information regarding their use in pregnancy are available (US Department of Health and Human Services 2002). New antiretroviral agents, e.g. the 36-amino-acid peptide enfuvirtide, which prevents the fusion of the virus with the cell membrane, may improve the therapy response even in pretreated patients (Lazzarin et al. 2003).

Mode of delivery

Cesarean section before onset of labor or rupture of membranes effectively reduces the rate of transmission (European Mode of Delivery Collaboration 1999). In a meta-analysis of 15 prospective cohort studies, the elective cesarean section decreased the risk by approximately 50 percent as compared with other modes of delivery and independently of the effects of treatment with zidovudine (International Perinatal HIV Group 1999). In women with viral loads below 1 000 copies/ml, a benefit of cesarean section has not yet been demonstrated (Minkoff 2001), but generally cesarean section is recommended when possible.

MANAGEMENT OF THE NEONATE

Among the infants of HIV-infected women, no malformations or an increase in birth defects relating to HIV infection was observed (Brocklehurst and French 1998). Breast-feeding is not recommended in countries where appropriate milk substitutes are available. The risk of HIV transmission through breast-feeding is greatest in early infancy (before 6 months of age), and persists as long as breast-feeding continues (Nduati et al. 2000). In developing countries, however, breast-feeding is encouraged because artificial feeding in a poor hygienic environment greatly increases morbidity and mortality from diarrheal diseases and respiratory infections (Van de Perre 1995). In these countries, a threefold higher mortality rate in HIV-infected mothers who breast-fed their infants was reported compared with those who fed their infants with formula (Nduati et al. 2000). However, these limited results did not warrant any change in current policies on breast-feeding nor on infant feeding by HIV-infected women (World Health Organization

2001). After delivery, zidovudine is administered in general to the newborn for up to 6 weeks according to PATCG protocol 076. In developing countries the single dose nevirapine given to pregnant women in labor and to neonates shortly after birth effectively reduced the rate of perinatal HIV transmission and appears to be one of the most cost-effective options for the prevention of perinatal HIV transmission (Jackson et al. 2003). Management of infants and children is rapidly evolving and becoming increasingly complex, for example, considering possible early and late side-effects. Therefore the management of these children should be directed to a specialist wherever possible.

HEPATITIS VIRUSES

The list of hepatitis viruses continues to expand. Of those so far identified, types B, C, and E are of known significance in pregnancy and for the newborn (Table 16).

Hepatitis A virus

Hepatitis A virus (HAV) (enterovirus 72) is acquired by the fecal–oral route, and has an incubation period of approximately 4 weeks.

INFECTION IN PREGNANCY AND IN NEONATES

Hepatitis A virus has no apparent adverse effect on the outcome of pregnancy. Transmission during birth (e.g. by exposure to maternal feces or by breastfeeding) is very rare (e.g. Zhuang 1989; CDC 1990; Ye 1990; Zhang et al. 1990; ACOG 1998). In one investigation, neonatal infection was diagnosed in an infant born to a mother with hepatitis A 10 days after premature labor, the evidence indicating that he was infected by his mother before or during birth. In another case, vertical transmission of HAV resulted in a symptomatic neonatal disease. After a trip to India, the mother developed symptomatic hepatitis A infection during the 4th month of pregnancy (Fagan et al. 1999). HAV may spread within the neonatal intensive care unit, causing asymptomatic infection in neonates, and symptomatic or asymptomatic infection in staff members as a result of lapses in infection control precautions (Watson et al. 1993). Neonates, even when infected parenterally by transfusion with hepatitis A-contaminated blood (a rare event) rarely have biochemical evidence of hepatitis (Azimi et al. 1986). They are, however, a potential source of infection for nonimmune staff members, as the virus is excreted in the stools of infected neonates and infants for up to 5 months (Rosenblum et al. 1991). This is much longer than in older children, except those in whom icteric hepatitis relapses, as has been reported in 3–20 percent of children (Sjogren et al. 1987).

CONTROL MEASURES

Infection can be controlled by adopting simple hygienic measures and by the sanitary disposal of excreta. Strict isolation of cases is not effective, as fecal shedding of the virus peaks during the prodromal phase. For pre- and postexposure prophylaxis, human normal immunoglobulin preparations (HNIG) are in use and may be given to neonates born within 2 weeks of maternal illness, to neonates in an intensive care unit if a case occurs on the ward, and to nonimmune staff members (Zhang et al. 1990). Inactivated vaccines are effective, and will replace immunoglobulin for pre-exposure prophylaxis; they seem also to be useful for post-exposure prophylaxis as documented in a randomized study (Sagliocca et al. 1999). Neither HNIG nor inactivated hepatitis A vaccines are contraindicated in pregnancy.

Hepatitis B virus

Hepatitis B virus (HBV), a hepadnavirus, is transmitted primarily by the parenteral route, by blood, by sexual contact through contaminated secretions from acutely infected patients or carriers, and by vertical transmission. Perinatal infection and infection during the first year of life have important consequences because 90 percent of these infants become chronic carriers, compared with only approximately 5–10 percent of those infected after the age of 12 years. Such chronicity increases the risk of cirrhosis and hepatocellular carcinoma. Since the commencement of the hepatitis B vaccination approximately 20 years ago, the prevalence of chronic HBV infections has been reduced substantially among populations whose infection rates were previously high. CDC estimates that perinatal HBV infections in the USA declined 75 percent during the period 1987–2000 (CDC 2002b).

INFECTION IN PREGNANCY

The annual incidence of acute hepatitis B cases in pregnant women is not known because pregnancy status is not recorded in most countries. Pregnancy does not appear to influence the severity or mortality rates of acute HBV infections in countries under good living conditions (e.g. Shalev and Bassan 1982). No teratogenic effects have been associated with maternal HBV infection.

MOTHER–CHILD TRANSMISSION

About 90 percent of vertical transmission occurs as a consequence of intrapartum exposure to contaminated blood and genital secretions. In approximately 10 percent infection results from hematogenous transplacental transmission.

The main risk factors for intrauterine HBV infection are maternal serum HBeAg positivity (Wang and Zhu

Table 16 *Effects of hepatitis A–G infection on pregnancy, on the fetus, and the newborn*

Hepatitis	Increased morbidity/ mortality after acute infection	Increased incidence of abortion or IUD	Congenital defects	Vertical transmission			Postnatal disease[d]	
				Intrauterine	Perinatal	Early postnatal infection	Early	Late
A (HAV)	No	Controversial	No	Possible[c]	No	Possible[b]	No	No
B (HBV)	No	No	No	Possible[b]	Yes	Yes	No	Yes
C (HCV)	No	No	No	Possible[b]	Yes	Possible	No	Yes
D (HDV) co-superinfection with HBV	No	No	No	Possible[b]	Yes, only in HBsAg and HDV RNA positive mothers	N/A	No	N/A
E (HEV)	Yes mortality ±20% 3rd trimester	Yes ±12%	No	Yes	Yes	N/A	Yes with increased mortality	N/A
G (HGV)	No[a]	No[a]	No[a]	Possible	No	Possible	No[a]	N/A

IUD, intrauterine death; N/A, data not yet available.
a) Provisional.
b) Possible, but rare.
c) One case reported.
d) Following congenital infection.

2000), a history of threatened preterm labor, HBV in the placenta especially in the villous capillary endothelial cells (Xu et al. 2001, 2002), a high HBV viremia level in pregnant women (Zhang et al. 1998) which is particularly elevated in women acutely infected in the third trimester, and transplacental leakage of maternal blood to the fetus (Lin et al. 1987; Ohto et al. 1987). As reported by Xu et al. (2002), the intrauterine infection rate increased linearly and significantly with maternal HBV DNA concentration. Results of the pathology study showed that HBV infection rates decrease gradually from the maternal side to the fetal side in the placental cell layers.

Recently, it has been found that breast-feeding of infants of chronic HBV carriers poses no additional risk for transmission of the hepatitis B virus (Hill et al. 2002). The early postnatal HBV infections result from close contact between infant and the infected parent(s). In hyperendemic areas, most HBV-related complications occur during adulthood, but nearly half of the primary infected persons becoming chronic HBV carriers have acquired their infection during the perinatal period through the transmission from hepatitis B e antigen (HBeAg)-positive mothers. The other half is from horizontal transmission mainly through intrafamilial spread or injection using unsterilized needles (Chang 1998).

RATE OF TRANSMISSION

The transmission rate from asymptomatic carrier mothers who are positive only for HBsAg is approximately 10–20 percent in the absence of immune prophylaxis. Mothers positive for both HBsAg and HBeAg, and thus highly infectious, transmit the virus in 80–90 percent of cases (see, for example, Stevens et al. 1979). For predicting persisting infection in infants and a possible failure of neonatal hepatitis B passive/active vaccination in earlier studies, the HBV DNA viral load in the maternal serum of the HBeAg-positive mothers seemed to be the most important factor (e.g. Burk et al. 1994; Del Canho et al. 1994). In a more recent case–control study conducted to find out why some infants born full-term to HBeAg-seropositive mothers became infected despite full passive-active immunoprophylaxis, allelic base changes in maternal HBV genotype were determined as risk factors (Ngui et al. 1998).

OUTCOMES OF PERINATAL AND POSTNATAL INFECTION

Most infants born to carrier mothers are HBsAg-negative at birth and, without immunoprophylaxis, seroconvert in the first 3 months after delivery. Perinatally and postnatally infected newborns and infants are asymptomatic. Fulminant hepatitis secondary to perinatal HBV infection has been reported in infants aged 2–6 months, particularly if the mother is anti-HBe-positive and the infecting virus has undergone mutations in the pre-core region of its DNA (see for example, Carman et al. 1990; Omata et al. 1991; Terazawa et al. 1991; Waters et al. 1992; Schödel 1994; von Weizsäcker et al. 1995; Hsu et al. 1999; Ogata et al. 1999; Zuckerman 2000). A recent publication, however, indicates that HBV genomic heterogeneity is not primarily involved either in the initiation of infection nor the failure of neonatal HBV immunoprophylaxis (Cacciola et al. 2002). The risk of chronicity in perinatally and early postnatally infected infants and the rate of progression to liver disease or hepatocellular carcinoma are currently under observation. In earlier studies of almost 350 Chinese and Italian children followed from 1 to 10 years, no progression of liver disease was noted, although almost 50 percent had histological findings of chronic active hepatitis or cirrhosis at presentation (Bortolotti et al. 1986; Lok and Lai 1988). Some more recent observations (Chang 1998) confirm that children with chronic hepatitis B virus infection are mostly asymptomatic. They are generally active and growing well with very rare exceptions. Even with acute exacerbation of liver function and active inflammation, jaundice, or growth failure is uncommon. However, hepatocellular carcinoma may also follow perinatal or childhood HBV infection (Hsu et al. 1987). This is demonstrated in a Japanese long-term follow-up study of 3 to 22 years (mean, 11 years) in 52 adolescents with perinatally acquired HBV infection where hepatocellular carcinoma has been diagnosed in two cases (a 21-year-old and a 16-year-old). All children carrying hepatitis B surface antigen should be observed carefully to monitor the possible development of hepatocellular carcinoma, especially in the anti-HBe-positive phase after spontaneous seroconversion or even after interferon treatment (Fujisawa et al. 2000). It is known that hepatitis B pathology is a dynamic process between the virus, the infected cell, and the host's immune response. In this context, more studies are needed to clear the function of the soluble core gene product (HBeAg) of hepatitis B virus. It has been proposed that transplacentally passed HBeAg might induce fetal immunotolerance to HBeAg (Milich et al. 1990; Wang and Zhu 2000).

DIAGNOSIS IN PREGNANCY

Screening in pregnancy for HBsAg detects both acutely infected and asymptomatic carrier women whose babies are at risk of perinatal infection. Since the beginning of the late 1980s, in most European countries, universal antenatal screening is carried out and is advocated in the USA and in Canada (CDC 1991; ACOG 1993) because selective screening has been of low efficiency (Boxall 1995). Antenatal screening is usually done early in pregnancy together with screening tests for blood group and syphilis (Grosheide et al. 1995). In Germany, selective HBsAg screening was introduced in 1987, but since 1994 has formed part of the obligatory prenatal care program. Testing is performed in the third trimester with the same

blood sample as provided for the second obligatory indirect Coombs' test (Kassenärztliche Bundesvereinigung 1994).

The screening procedures in pregnancy are as follows. If the test for HBsAg is negative, no further testing is done unless there is a clinical indication of hepatitis or suspicion of behavioral risk factors (then testing anti-HBc is carried out). If the HBsAg result is positive, anti-HBc IgM testing and, if this test is positive, quantification of HBV DNA may also be carried out. For results notification requirement to healthy authorities exists. The HBsAg result is documented in the prenatal care notes and, if positive, the obstetrician is advised to administer active/passive hepatitis B immunization to the neonate within 12 h after birth. If antenatal HBsAg screening is not documented, it will be done at delivery. In Germany, universal antenatal HBsAg screening was estimated to be cost-effective over a wide range of assumption (Kassenärztliche Bundesvereinigung 1994). Tests for anti-HBc only in the case of negative results for HBsAg have not been included in the German strategy of antenatal screening. Although an anti-HBc prevalence of 8.71 percent was calculated in the middle-aged adult German population, 1.40 percent of the individuals had anti-HBc, only 7.7 percent of them or 0.1 percent of the whole population were DNA positive in the absence of HBsAg indicating infectiousness (Jilg et al. 2001). However, some countries, e.g. Switzerland, include anti-HBc testing in the antenatal screening algorithm. As a consequence, newborns of the solely anti-HBc-positive mothers also obtain active-passive hepatitis B immune prophylaxis shortly after delivery (personal communication, Professor Zimmermann, Universitätsspital Zürich, Department für Frauenheilkunde und Geburtshilfe, Switzerland).

MANAGEMENT IN PREGNANCY

Preventive measures are designed to prevent transmission by transfusion, transplantation, syringe-borne infection, frequent changes of sex partner, and nosocomial infection between patients. Pregnant women in sexual or household contact with HBV-infected individuals should be offered active/passive immunization after their HBsAg, anti-HBc, and anti-HBs seronegativity is established. The specific antiviral drugs lamivudine, adefovir, entecavir, and pegylated interferon-α are not recommended for pregnant women (Balfour 1999; Anonymous 2002; Schalm et al. 2002). During pregnancy, acute HBV infection is treated by supportive measures as in nonpregnant patients.

Invasive prenatal diagnosis in the second and third trimester is not indicated since transmission occurs primarily during delivery. If genetic reasons are the indication for prenatal diagnosis, a potential risk of iatrogenic infection of the fetus in viremic mothers has to be considered (Enders and Braun 2000; Delamare et al. 1999).

Termination of pregnancy is contraindicated.

Cesarean section is not usually recommended, but may be performed in women with high serum levels of HBV DNA and HBeAg and with acute infection in late pregnancy (Schalm and Pit-Grosheide 1989; Del Canho et al. 1994; Wang et al. 2002).

MANAGEMENT OF NEONATES

In the USA, all newborns are vaccinated at birth regardless of the maternal HBsAg status, with anti-HBs testing of infants at age of 7 months born to HBsAg-positive mothers (Committee on Infectious Diseases 1994). The detailed vaccination procedure is given by the Advisory Committee on Immunization (www.cdc.gov/nip/acip). In most western European countries (term and preterm) babies of mothers with positive HBsAg test or unknown HBsAg status are immunized within 12 hours after birth simultaneously with hepatitis B immunoglobulin (HBIG) and a hepatitis B low-dose vaccine, and again with hepatitis B vaccine only at 1 and 6 months of age; 1 month later, a test for anti-HBs is recommended (Robert Koch Institut 2002b). Infants of mothers with negative HBsAg status are vaccinated within the routine infant program starting at age of 2 months (Robert Koch Institut 2002b).

Perinatal immune prophylaxis has been effective in western populations, protecting 93–95 percent of infants 1 month after the third dose (antibody levels $\geqslant$10 mIU/ml). This was confirmed by a recent study carried out in a population at high risk of infection by examining 522 children in southern Italy born to HBsAg-positive mothers 5–14 years after postpartum immunization. Four hundred of 505 (79.2 percent) children had protective anti-HBsAg titers $\geqslant$10 mIU/ml. Thus, HBV vaccination of children born to HBsAg carrier mothers seems to provide immediate and long-term protection against HBV infection. There was no evidence that the emergence of HBV escape mutants secondary to the immune pressure against wild-type HBV is of concern (Mele et al. 2001). In areas with low HBV endemicity, for vaccination of infants low-dose vaccine (5 μg) is used, whereas in areas with high HBV endemicity, normal dose of vaccine (10 μg) seems to be more effective in perinatal and infant vaccination (Lee 1995; Milne et al. 1995).

In 1991, recognizing the difficulty of vaccinating high-risk adults and the substantial burden of HBV-related disease acquired from infections in childhood, a comprehensive strategy to eliminate HBV transmission in the USA was recommended (CDC 1991, 1995, 2002b). It includes universal childhood vaccination, antenatal screening, vaccination of all nonvaccinated young adults, and vaccination of groups at risk because of their occupation, behavior, contacts, travel, etc. This strategy was taken up by the majority of western countries, including Germany (Robert Koch Institut 1995) and various Asian (Ruff et al. 1995) and African countries.

For elimination of HBV transmission, it is important to sustain high vaccine-coverage rates among infants, children, adolescents, and adults at increased risk for HBV infection. However, if the efforts to vaccinate the latter group are not greatly expanded, complete elimination of HBV transmission might take another 20 years to achieve (CDC 2002b; Robert Koch Institut 2002b).

Hepatitis C virus

Hepatitis C virus (HCV), with >6 genotypes and >70 subtypes, is the etiological agent of most cases of non-A, non-B post-transfusion and sporadic hepatitis (Simmonds 1995; Maertens and Stuyver 1997). Acute HCV infections are asymptomatic in 75 percent of cases and in the remainder symptoms are mild, but >50 percent of infected adults develop chronic liver disease. Information on the perinatal implications of HCV is still constantly expanding. Based on observational data of more than 77 studies carried out in approximately 18 countries, vertical transmission has been confirmed to occur generally at a low rate (1–5 percent) and is favored mainly by a high maternal virus load in the blood. Maternal risk factors such as coinfection with HIV increase the vertical transmission rate to more than 20 percent (Roberts and Yeung 2002).

INFECTION IN PREGNANCY

The important risk factors for infection in pregnancy are intravenous drug abuse, sexual contact with intravenous drug users, high promiscuity, and infection with other sexually transmitted diseases. Other risk factors are previous transfusions, transplantation, and administration of blood products.

In pregnant women at normal risk, antibody prevalence is 0.7–2.5 percent, being higher than in blood donors (approximately 0.2–0.5 percent) in the same geographical area (Bohman et al. 1992; Marranconi et al. 1994; Pipan et al. 1996). In a UK study, the HCV seroprevalence in mothers born in the UK was 0.13–0.15 percent and 0.81 percent in mothers born elsewhere in Europe (Ades et al. 2000). In pregnant women from high-risk populations, antibody prevalence rates are >2.5 percent, irrespective of the geographic region.

It appears that pregnancy has no impact on the viral load and the clinical course of acute hepatitis C or the activity of chronic infection (Reth et al. 1995). In addition, abortions or intrauterine deaths are not increased in acute or chronic HCV infection (Dinsmoor 2001).

MOTHER–INFANT TRANSMISSION

Routes of transmission

Hepatitis C can be transmitted in utero and during parturition. Intrauterine transmission is suggested in some reports because viral RNA was detected in cord or neonatal blood (e.g. Kurauchi et al. 1993; Kojima and Yamanaka 1994), but not always in follow-up sera of the infants. As in HBV infection, intrauterine transmission may occur late in pregnancy due to placental leakage in cases of threatened abortion and preterm labor (e.g. Lin et al. 1987). The major route, however, is intrapartum transmission, which probably occurs by contact with contaminated blood. This route is verified by detection of viral RNA in nonbreast-fed infants >1 month old (e.g. Resti et al. 1995; Zanetti et al. 1995). Several studies of mother–infant pairs with follow-up of the infants show that the risk of vertical transmission is higher in mothers with acute infection in late pregnancy (Kuroki et al. 1991; Lynch-Salamon and Combs 1992) or chronic hepatitis (Degos et al. 1991); in high-risk mothers co-infected with HIV-1, but not with HBV; and particularly if a high level of HCV RNA is present in maternal blood, with (Zanetti et al. 1995) or without (e.g. Ohto et al. 1994; Tajiri et al. 2001) behavioral risk cofactors.

There is no evidence of transmission by breast milk in asymptomatic carrier mothers, but it cannot be fully excluded in symptomatic mothers (Kumar and Shahul 1998; Mast 2004).

Rates of vertical transmission and infection

In low risk groups In prospective studies of the world literature from 1994 to 2001 on vertical HCV transmission in low-risk, HIV-negative mothers a mean transmission rate of 5.5 percent (range, 0–11.1 percent) was reported (Ohto et al. 1994; Moriya et al. 1995; Giacchino et al. 1995; Meisel et al. 1995; Pipan et al. 1996; Kumar et al. 1997; Resti et al. 1998; Xiong et al. 1998; Conte et al. 2000; Ceci et al. 2001; Tajiri et al. 2001; Lima et al. 2004). All these studies indicate that infants are infected only by HCV-RNA-positive mothers, particularly those with a high viral load. One investigation reports that vertical transmission of HCV can occur irrespective of the maternal viral load (Pipan et al. 1996).

Older studies from 1990–1992 on vertical transmission in anti-HCV-positive and HIV-1-negative mothers indicate that, on the basis of antibody persistence the infection rate in infants followed up for 10–12 months was 4.5 percent (4/88) (Lynch and Ghidini 1993). In one of these older studies, in which both HCV RNA detection and antibody persistence were used as markers of infection of infants, RNA was found not only in infants who seroconverted or who had persisting antibody, but also in those who became and remained antibody negative (Thaler et al. 1991). This puzzling observation was also made in later studies (e.g. Giacchino et al. 1995; Paccagnini et al. 1995) and may indicate false-positive PCR results or seroreversion with clearance of infection, as has been found in a few infants born to HIV-1-positive mothers (Newell et al. 1996).

In high risk groups Several studies tried to establish the rate of vertical transmission of HCV in mothers

of high-risk groups with and without HIV-1 coinfection. In studies (1995–2002) from various countries, vertical transmission rates in children born to HCV-RNA-positive mothers with HIV infection ranged from 44 to 8 percent, whereas the transmission rate in children born to HCV-RNA-positive mothers without HIV infection range from 10 to 0 percent (Zanetti et al. 1995; Zuccotti et al. 1995; Paccagnini et al. 1995; Tovo et al. 1997; Mazza et al. 1998; Thomas et al. 1998; Papaevanegelou 1998; Zanetti et al. 1998; Gibb et al. 2000; Resti et al. 2002).

The investigations of Zanetti and colleagues (1995, 1998) and Zuccotti and co-workers (1995) show clearly a higher maternal HCV RNA positivity in HIV-1 coinfected mothers than in those with HCV RNA alone, and a higher rate of vertical transmission in the group positive for HIV-1 and HCV RNA. The study of Paccagnini et al. (1995) further indicates that HCV and HIV transmission, alone or in combination, can occur in infants born to coinfected mothers.

Some authors (Kudesia et al. 1995; Fischler et al. 1996; Spencer et al. 1997) recorded no vertical transmission in HCV-infected pregnant women engaged in injection drug use (IDU). In contrast to these studies, Resti et al. (1998) have shown that intravenous drug use itself and post-transfusional hepatitis is an important risk factor for transmission of hepatitis C virus (Resti et al. 1998). In their more recent study Resti et al. (2002) have postulated that transmission occurs mainly in those women who are former or current injection drug users and not in HIV-coinfected women. However, it has been questioned why transmission would be associated with a history of IDU in women who do not currently engage in IDU (Armstrong et al. 2003). Furthermore, Yeung et al. (2001) have examined the results of prior studies and have confirmed that transmission is more strongly associated with HIV coinfection than with IDU.

The outcomes of HCV-infected children in four studies of maternal low risk groups and in five studies of maternal high risk groups are summarized in Table 17. Nearly all the infected infants showed signs of liver involvement during the follow-up period. Increased, but fluctuating, values of alanine aminotransferase (ALT) were usually detected as well as histological evidence of liver disease. Tests for HCV RNA were repeatedly positive in all children aged ⩾6 months; HCV antibodies persisted beyond 14 months of age in 12 infants; there was temporary seroreversion in nine infants and loss of HCV antibodies in six infants aged 6–9 months despite persistence of HCV RNA.

These studies in high- and low-risk groups which have already been discussed above and some further studies reflect various courses of the infection in apparently vertically infected infants (especially in the first year of life), e.g. transient viremia without real infection, acute self-limited clinically inapparent infection, or clearance of HCV-RNA (Bortolotti et al. 1997; Sasaki et al. 1997; Ketzinel-Gilad et al. 2000). Long-term observations are needed to clarify if chronic hepatitis in infants will spontaneously resolve or progress to chronic liver disease.

Although transmission via breast milk has been suggested in one study (Gürakan et al. 1994), the majority of studies that have examined transmission rates with breastfeeding have not noted any significant increase in transmission in infants who were breast-fed, compared with those who were bottle-fed (Kumar and Shahul 1998; Lin et al. 1995; Zanetti et al. 1995; Zimmermann et al. 1995; Tovo et al. 1997; Resti et al. 2002; European Paediatric Hepatitis C Virus Network

Table 17 *Outcome of hepatitis C-infected infants born to mothers of low and high risk groups*

			Infants age ⩾6 to 20 months at follow-up		
Study	**Country**	**Risk group**	**HCV RNA pos.**	**Anti-HCV pos.**	**Abnormal ALT value**
Ohto et al. (1994)	Japan	Low	3	2/3	3/3
Giacchino et al. (1995)	Italy	Low	2	2/2	2/2[a]
Ceci et al. (2001)	Italy	Low	2	2/2	2/2
Tajiri et al. (2001)	Japan	Low	9	9/9	9/9
Zanetti et al. (1995)	Italy	High	8[b]	6/8	8/8
Paccagnini et al. (1995)	Italy	High	14[c]	11/14	10[d]/14
Spencer et al. (1997)	Australia	High	6[e]	6/6	6/6
Mazza et al. (1998)	Italy	High	6	6/6	6/6
Zanetti et al. (1998)	Italy	High	17	15/17	16/17

ALT, alanine aminotransferase; HCV, hepatitis C.
a) Histological signs of liver disease: 2/2.
b) Three HIV co-infected.
c) Four HIV co-infected.
d) Histological signs of liver disease: 4/10.
e) Two of six infants initially HCV RNA-positive were negative at age of 30 months.

2001). In recent studies, the authors were unable to find HCV RNA in any breast milk samples tested (Kage et al. 1997; Polywka et al. 1997).

According to the knowledge that vertical HCV transmission occurs like HIV transmission around the time of delivery, the effect of the mode of delivery on transmission was of interest. Of the various studies (Paccagnini et al. 1995; Spencer et al. 1997; Tovo et al. 1997; Resti et al. 1998; Thomas et al. 1998; Conte et al. 2000; European Paediatric Hepatitis C Virus Network 2001) only two reported significantly lower transmission rates with elective cesarean section. One study reported that in mothers with high viral load, vaginal delivery resulted in a vertical transmission rate of 44 percent (7/16) (Okamoto et al. 2000). In contrast, the eight patients with a high viral load who underwent elective cesarean section (prior to labor) did not transmit HCV to their ten neonates. In the study of Gibb et al. (2000) in 441 HCV-infected women in the UK, there were no cases of neonatal HCV following elective cesarean delivery in 31 patients. This transmission rate was significantly lower than that for vaginal delivery and emergency cesarean section combined. Unfortunately, data on maternal HCV RNA levels were not available for the patients in this study.

A viral factor which influence the risk of mother-to-infant transmission may be the HCV genotype (e.g. Zuccotti et al. 1995), but this could not be substantiated in a number of further investigations (e.g. Mazza et al. 1998).

At present, the guidelines from the American Academy of Pediatrics, the Centers for Disease Control and Prevention (CDC), and the American College of Obstetricians and Gynecologists (ACOG) do not prohibit breast-feeding in infants of HCV-positive mothers (CDC 1998b; American Academy of Pediatrics 1998; ACOG 2000c). It is likely that most of the individual studies done thus far lack the statistical power to detect differences in transmission following interventions such as cesarean section or formula feeding. The current guidelines from the CDC and ACOG recommend cesarean section only for obstetric indications in patients who are infected with hepatitis C (CDC 1998b).

It might be of interest that mothers who have already delivered an HCV-infected child can be advised that this event does not increase the probability of infecting the second child (Resti et al. 2000).

The contribution of vertical transmission to community-acquired HCV infection in western populations seems to be low (e.g. Alter et al. 1992; Goto et al. 1994; Yoshii et al. 1999), but may be important among children living in areas of high HCV prevalence (Chang et al. 1994).

DIAGNOSIS IN PREGNANCY

Laboratory tests for HCV infection in pregnancy are the same as for nonpregnant people. With regard to vertical transmission, a maternal viral load of more than 10^{6-7} IU/ml is considered high, 10^{4-6} as moderate and $<10^4$ as low or negative values. Commercially available qualitative and quantitative HCV RNA assays provide good sensitive, specific, and reproducible results today.

DIAGNOSIS IN INFANCY

Infants born to anti-HCV-positive and, particularly, to HCV-RNA-positive mothers should be tested at birth, at 6–8 weeks and at the age of 10–12 months for HCV RNA and, facultatively, for anti-HCV persistence. Liver function tests should be performed on HCV-RNA-positive infants at 2–3-monthly intervals and also on infants with antibodies persisting beyond 12 months of age.

MANAGEMENT IN PREGNANCY

Screening tests

Universal screening of pregnant women with anti-HCV antibodies is not recommended (CDC 1991). Selective screening should be performed in women with behavioral risk factors, with known positive HIV or HBsAg status, in those with signs of hepatitis or with a history of transfusion or transplantation and in nonpregnant and pregnant women with an HCV-positive partner. In cases of anti-HCV positivity, PCR for HCV RNA should be performed; if positive, the viral load should be determined toward the end of pregnancy.

Avoiding infection

The possibility of sexual transmission exists, predominantly in cases of high promiscuity. In contrast to previous findings, HCV RNA has been detected recently in seminal plasma by using highly sensitive PCR techniques (Hsu et al. 1991; Terada et al. 1992; Levy et al. 2000; Bourlet et al. 2003).

Although the risk of virus transmission is low, the presence of HCV RNA in seminal plasma suggest a possible risk of sexual transmission. It is noteworthy that French legislation has introduced a screening for HCV RNA in seminal fluids of HCV-infected men who are involved in programs of assisted reproductive techniques (Bourlet et al. 2003).

At present there is no contraindication to pregnancy for HCV-positive women or for anti-HCV-negative women with an HCV-positive partner.

Invasive prenatal diagnosis in the second and third trimester is not indicated since transmission occurs primarily during delivery and since, for example, genetic reasons for prenatal diagnosis, a potential risk of iatrogenic infection of the fetus in viremic mothers has to be considered (Enders and Braun 2000; Delamare et al. 1999).

Termination of pregnancy is not indicated.

Passive/active prophylaxis and therapy

The efficacy of passive prophylaxis for HCV by intravenous immunoglobulin seems limited (e.g. Krawczynski et al. 1996), and a vaccine is not yet available (Choo et al. 1994; Zanetti et al. 2003). Treatment with IFN-α or pegylated IFN-α alone or in combination with ribavirin (Di Bisceglie et al. 1995; Collier and Chapman 2001) is not indicated in pregnancy (McDonnell and Lucey 1995). However, more recently some reports suggest continuing pregnancy if they were exposed inadvertently at the beginning of pregnancy to interferon (Hiratsuka et al. 2000; Trotter and Zygmunt 2001; Ozaslan et al. 2002), but less so in case of therapy with ribavirin (Mishkin and Deschenes 2001; Hegenbarth et al. 2001).

Mode of delivery

If the viral load in the maternal serum is high, elective cesarean section has been suggested, to reduce the rate of vertical transmission (Paccagnini et al. 1995; Gibb et al. 2000), but should rather be reserved for cases acquiring acute infection during the second and third trimester (Lin et al. 1996). After delivery no special precautions (e.g. isolation) are needed.

Breast-feeding

Breast-feeding by HCV-positive mothers is not contraindicated, because of the generally low risk of early postnatal transmission, unless they have a high viral load in the serum. In this case, breast milk may be quickly assessed for HCV RNA before routine breast-feeding is recommended (Pipan et al. 1996).

MANAGEMENT OF THE NEONATE

Prolonged follow-up of infants (up to 24 months) born to anti-HCV and HCV-RNA-positive mothers is of great importance. Treatment of chronic hepatitis C in infants and children is usually started not before the age of more than 2 years. Treatment with interferon-α alone yielded poor results, but the combination therapy of interferon-α 2b with ribavirin is an important advance (Wirth et al. 2002).

Hepatitis D virus

Hepatitis D virus (HDV) is also known as delta agent. HDV is a defective single-stranded RNA virus that requires the helper function of HBV for viral replication since the envelope of HDV consists of HbsAg and a unique internal antigen, the delta antigen. HDV is transmitted principally by blood and blood products, but also by sexual contact or injecting drug use. There is considerable regional variation in its prevalence. The prevalence of HDV is generally low among both asymptomatic HBV carriers and among patients with chronic HBV-related liver disease in countries with low prevalence of chronic HBV infection. In countries with moderate and high levels of chronic HBV prevalence, the prevalence of HDV infection is highly variable (CDC 2001a). Vertical transmission is rare.

INFECTION IN PREGNANCY AND IN THE NEONATE

A study in northern Italy demonstrated an incidence of anti-HDV antibody among HBsAg-positive pregnant patients lower than that in a group of HBsAg-positive patients with chronic liver disease (7 versus 20 percent) (Zanetti et al. 1982). Exacerbation of HDV infection during pregnancy has not been reported. Vertical transmission seems possible either during late pregnancy or intrapartum (Ramia and Bahakim 1988; Riongeard et al. 1992). Perinatal infection has been described in an infant born to a mother positive for both HBeAg and anti-HDV, but in none whose mothers were anti-HBe-positive (Smedile et al. 1981). Prevalence rates of HDV as estimated by HDAg or anti-HDV antibody in hepatitis B-infected children range from 1.6 percent in Taiwan to 12.5 percent in parts of Italy, China, and the Balkans, and up to 25 percent in the Amazon basin in Brazil (e.g. Chen et al. 1990; Torres and Mondolfi 1991).

PROPHYLAXIS

Measures to control the spread of HBV are also effective in preventing transmission of HDV infection to the infant. Administration of HBIG and HBV vaccine within 12–24 h after birth to newborns of HBsAg-positive mothers seems to prevent both HBV and HDV infection (Silverman et al. 1991). Zanetti and colleagues (1982) reported serological evidence of HDV infection only in newborns who did not receive HBV immunoprophylaxis.

Hepatitis E virus

Hepatitis E virus (HEV) a currently unclassified probably zoonotic RNA-virus (HEV-like viruses) is transmitted enterically and is responsible for waterborne epidemics and for sporadic outbreaks in tropical and subtropical countries (Mast and Alter 1993; Emerson and Purcell 2003).

In contrast to hepatitis A, HEV affects mainly young adults (15–40 years old) and has considerable implications in pregnancy. The death rate due to hepatitis E in the general population is 0.5–3 percent, in pregnant women as high as 15–25 percent (Mast and Krawczynski 1996).

INFECTION IN PREGNANCY AND IN THE NEONATE

During epidemics, the incidence of HEV infection in pregnant women is nine times higher than in nonpregnant women or in men (Khuroo et al. 1981; Khuroo and Kamili 2003). Both in epidemic and in sporadic outbreaks, HEV causes fulminant hepatitis and maternal

death, especially among women in the third trimester (Nanda et al. 1995). Mortality in pregnant women is as high as 25 percent compared with the overall mortality of 0.5–3 percent (Krawczynski 1993; Purdy and Krawczynski 1994; Kumar et al. 2004). The reasons remain unknown, but it seems unlikely that HEV is the sole cause of liver failure in pregnant women (Fagan et al. 1994).

According to the early observations of Khuroo and co-workers (1981), even mild HEV infection during pregnancy seems to cause a high rate of abortion and intrauterine death and increased perinatal mortality in babies born to women with fulminant hepatitis. Another report suggests that, unlike other hepatitis viruses, HEV often causes intrauterine infection, as well as substantial perinatal morbidity and mortality (Khuroo et al. 1995) and a more recent report suggests significant vertical transmission of HEV among HEV-RNA-positive mothers with appreciable perinatal morbidity and mortality (Kumar et al. 2001, 2004). Excluding mothers with acute hepatic disease, breast-feeding appears to be safe in HEV seropositive mothers (Kumar et al. 2001).

PROPHYLAXIS

Fecally contaminated drinking water is the most common vehicle of transmission. Hygienic precautions (e.g. adequate heating of drinking water and food) are necessary in areas where HEV is endemic. Immunoglobulin prepared from plasma collected in industrialized countries does not prevent HEV infection, immunoglobulin prepared from donors in endemic areas is of unknown efficacy (CDC 2001b). Until now, no effective antiviral drugs exist. A recombinant capsid vaccine is under evaluation in Nepal with adult volunteers receiving three doses of HEV vaccine or placebo over 6 months (Purcell et al. 2003).

Hepatitis G virus

An additional agent that may be responsible for as yet unexplained liver disease was discovered in 1995–1996 by molecular amplification and named hepatitis G virus (HGV) also known as GB virus C (Simons et al. 1995; Linnen et al. 1996). At present, HGV infection is mainly detected by reverse-transcriptase polymerase chain reaction (RT-PCR), but serological assays are now also in use. The clinical importance of this agent is as yet unclear. No causal relationship between HGV and hepatitis has been established (Alter 1996; Alter et al. 1997). Mother-to-infant transmission has been demonstrated without evidence of disease manifestations.

INFECTION IN PREGNANCY AND IN THE NEONATE

Few data are yet available on vertical mother-to-child transmission and infection of the infant. Feucht and colleagues (1996) studied mothers belonging to a behavioral-risk group; 61 mother–infant pairs were investigated. In six of 30 mothers coinfected with HCV and three of 17 coinfected with HIV-1, HGV viremia was detected by RT-PCR. Vertical transmission of HGV occurred in three of the nine infants, but co-transmission with HIV-1 or HCV could not be demonstrated. The rate of vertical transmission for HIV-1 was 11.8 percent (2/17) and for HCV 6.7 percent (2/30), corresponding with the reported vertical transmission rates of these viruses.

During follow-up of all 61 infants for vertical transmission of HGV, HCV, and HIV-1 every 3 months for >1 year, none of the three HGV-infected infants or the two with HCV infection became icteric or showed other biochemical or clinical signs of hepatitis.

In another report on vertical transmission of HGV, three HGV-positive pregnant women (one coinfected with HCV, but all three negative for HIV-1) and their three infants were studied. All three women had elective cesarean delivery because of obstetric indications, and their infants were not infected with HGV or HCV during the 12 months of observation. These negative results may indicate that mother-to-infant transmission does not occur in women from low-risk populations or that cesarean delivery minimizes infection because there is less microtransfusion from mother to fetus compared with other modes of delivery (Lin et al. 1996). Viazov et al. demonstrated a mother-to-infant transmission of 56 percent. During follow-up studies, none of the GBV-C/HGV-infected infants had elevated levels of ALT or clinical signs of liver disease. Prospective epidemiological and clinical studies are further needed to determine the long-term outcome of this infection (Viazov et al. 1997).

ENTEROVIRUSES

There is no convincing evidence that maternal infection in early pregnancy with any of the picornaviruses is associated with teratogenic defects. Maternal poliomyelitis caused by wild-type poliovirus is now largely of historical interest. In the prevaccination era, poliovirus infections in pregnancy could result in abortion, stillbirth, and, rarely, in neonatal disease. Transplacental transmission of the virus to the fetus was demonstrated especially in maternal poliomyelitis in late pregnancy, but seldom in early pregnancy (Bates 1955; Carter 1956).

Coxsackie virus and ECHO viruses

Maternal enterovirus infections with various coxsackie and ECHO viruses may be followed by spontaneous abortion, intrauterine death, placentitis, and multiorgan fetal infection, as demonstrated in various prospective studies (Basso et al. 1990; Garcia et al. 1991; Frisk and

Diderholm 1992; Dommergues et al. 1994). In our investigations, during the past 14 years on the untoward effects of coxsackie/ECHO virus in pregnancy, involving 288 women with serologically suspected or virologically proven coxsackie/ECHO virus infections during pregnancy, fetal loss of 6.5 percent, but no teratogenic defects in the newborns were reported. In addition, no enterovirus RNA (virus culture was not attempted) could be detected in amniotic fluid of 30 women with symptomatic infection, who underwent prenatal diagnosis (18–24 WG) (G. Enders 2004, unpublished). A recent case report shows that a late intrauterine enterovirus infection (coxsackie virus B3) could be diagnosed antenatally in amniotic fluid by virus culture, but surprisingly not by enterovirus RNA detection (Ouellet et al. 2004). Late intrauterine, intrapartum, or early postnatally acquired infections are of importance for the newborns.

CONGENITAL AND PERINATAL INFECTION

Clinical manifestations in the neonate or infant often include sepsis, meningoencephalitis, hepatitis, myocarditis, and coagulation disorders (Kulhanjian 1992; Bendig et al. 2003). Meningitis is a common manifestation of neonatal enterovirus infection, and enteroviruses seem to be responsible as often as bacteria for meningitis in the first month of life (Shattuck and Chonmaitree 1992). Onset 1–2 days after birth suggests intrauterine or intrapartum transmission and disease is severe in approximately 80 percent of cases. Onset 5–6 days after birth suggests that the infant has acquired infection by contact with infected mothers, siblings, or an infected neonate in the maternity unit. The course of illness is less severe in such cases (Simoes and Abzug 1993).

More recently it was described, that in utero coxsackie virus infection of the placenta was associated with the development of severe respiratory failure and central nervous system sequelae in the newborn (Euscher et al. 2001). This underscores the importance of detailed pathologic and viral examination of the placenta in cases of systemic illness in the newborn. Data on the long-term prognosis of neonates with enteroviral meningitis are still incomplete and contradictory. Children <2 years of age with enteroviral meningitis do not have long-term sequelae (Rorabough et al. 1992). Encephalitis occasionally occurs and in young infants may have neurological sequelae. Furthermore, it was reported that varicella-like congenital skin lesions in the newborn may be one of the leading manifestations of intrauterine infection with coxsackie B viruses (e.g. CVB3) (Sauerbrei et al. 2000).

MANAGEMENT IN PREGNANCY AND OF THE NEONATE

Enterovirus infections in early pregnancy occasionally cause abortion but are not teratogenic, and the mother should be reassured accordingly. Prenatal diagnosis should be considered in the differential diagnosis of nonimmune *hydrops fetalis* with pericardial effusion and fetal tachycardia, particularly in women with suspicion of recent enterovirus infection. Acute symptomatic or asymptomatic infections towards the end of pregnancy carry the risk of intrauterine infection causing severe neonatal disease. In case of known infection of the mother, the child should be isolated at delivery and the baby should be fed with expressed maternal breast milk. The use of intravenous immunoglobulin up to the age of 14 days as a preventive measure for neonates exposed to infected infants did not confer significant benefit (Abzug et al. 1993). Pleconaril may be used as an emergency medication (Ouellet et al. 2004). In hospitals, adequate hygienic measures must be observed because of the ready spread of these viruses in neonatal units.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS

Lymphocytic choriomeningitis virus (LCMV) is a rodent-borne *Arenavirus*. The infected animals may be asymptomatic, but may excrete the virus for months. The illness in infected people is influenza-like and accompanied by fever, headache, nausea, and myalgia lasting 5–10 days; a small proportion develop meningitis or choriomeningitis, which in adults usually resolves without sequelae.

LCM virus infection is an often undiagnosed cause of congenital disease with features similar to those of cytomegalovirus or *Toxoplasma gondii*. Infection with LCMV in the first trimester of pregnancy may cause abortion (Ackermann et al. 1975; Biggar et al. 1975; Deibel et al. 1975), and infection of the fetus can result in congenital hydrocephalus, chorioretinitis, severe hyperbilirubinemia, and myopia (Sheinbergas 1975; Sheinbergas 1976; Enders et al. 1999). Although the number of published studies on intrauterine and perinatal infections is limited, the incidence of serious sequelae in infants seems to be high (Barton et al. 1995; Enders et al. 1999; Barton and Mets 2001).

Because healthy mice and hamsters shed LCMV chronically, pregnant women should avoid direct contact with these animals and their excreta.

HUMAN PAPILLOMAVIRUSES

Juvenile laryngeal papillomatosis (JLP) is associated most often with human papillomavirus (HPV) types 6 and 11; it is an uncommon but potentially serious problem, which is best treated by prevention if possible.

Infection in pregnancy

Growth of HPV warts is stimulated by several factors, including pregnancy. Some of the lesions may grow markedly during pregnancy, only to recede postpartum.

This phenomenon is thought to be related to hormonal influences or to changes in local cellular immunity. Rarely, the lesions become so large as to obstruct the birth canal and extensive formation of HPV condylomata may lead to premature rupture of the membranes, chorioamnionitis, and intrapartum infection of the fetus (Young et al. 1973).

Effects on the fetus and the neonate

Overall, the risk of perinatal transmission seems to be low, and it is still unclear how frequent it progresses to clinical lesions (Watts et al. 1996; Syrjanen and Puranen 2000). Nevertheless, transmission from mother to fetus and to the newborn may occur both in clinically apparent and subclinical maternal infection, and may lead to intrauterine infections, perinatal infections, or both. Approximately 50 percent of children with JLP are born to mothers with genital HPV (Quick et al. 1980; Nikolaidis et al. 1985), and HPV types 6 and 11, which are the most common in anogenital tract infections (Ferenczy 1989), are identified in >80 percent of JLP cases. Gastric and nasopharyngeal aspirates of babies born to mothers with genital tract HPV have been reported to contain HPV DNA (Ferenczy 1989; Sedlacek et al. 1989). The possibility of perinatal transmission is supported by the detection of DNA from HPV types 6, 11, 16, and 18 in foreskin specimens from neonates (Roman and Fife 1986).

Association with JLP

The long latent period from birth to development of JLP (>7 years) and the observation that HPV types found in newborns often differs from those detected in their mothers make association with intrauterine or perinatal infection difficult. Postnatally acquired infection from a variety of sources may account for numerous cases of JLP. For example, HPV may be transmitted on fomites after contact with individuals with warts (Patsner et al. 1990) and sexual abuse may also result in postnatal infection (Fleming et al. 1987). Recent data from Scandinavia indicate that despite the increasing rate of genital HPV infections in pregnant women, the incidence of JLP remains very low (Lindbergh and Elbrond 1990).

Diagnosis

The HPV infection may be latent without evident warts or manifest with warts. The diagnostic methodology extends from gynecological examinations, PAP smear tests, and colposcopy to the direct detection of the viral nucleic acids with molecular biology techniques such as PCR or hybrid capture (Arena et al. 2002). HPV antibodies are detectable in children of infected mothers, but there is generally no correlation between seropositivity and the detection of DNA in either the oral or the genital mucosa (Syrjanen and Puranen 2000; Burd 2003). Women under treatment for cytologically and virologically proven HPV infection should be examined at intervals until lesions and HPV DNA are no longer detectable. Testing their partners is recommended.

Management

The treatment of pregnant women with HPV-induced genital warts should be started as early as possible and directed at elimination of the virus near term to diminish risk of the infant's acquiring HPV infection by passage through an infected birth canal. Laser vaporization is proposed as an effective invasive technique for treatment of warts in pregnant women (Arena et al. 2001). As noninvasive methods, topically applicable immune modifiers have been reported. Prophylactic cesarean section in pregnant women with genital HPV infection is not advocated except in the rare event of large condylomata obstructing the birth canal.

Preventive and therapeutic vaccines, usually composed of recombinant viruslike particles directed against HPV, are under development (Hines et al. 1995; Borysiewicz et al. 1996). Beside this more classical technology, a new strategy based on genetic immunization with naked HPV DNA is now evaluated (Moniz et al. 2003; Burd 2003; Roden et al. 2004).

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Virus infections in immunocompromised patients

ANTHONY SIMMONS

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Perturbations of immunity can influence the pathogenesis of viral infections in several ways. Prominent among these is increased frequency or severity of disease, exemplified par excellence by the debilitating manifestations of many herpesvirus infections in immunocompromised hosts. However, not all viral infections are exacerbated by immunosuppression and the acute stages of some diseases, such as hepatitis B, may be unusually mild. This is presumably because immunologically mediated lysis of infected cells contributes more than virally induced cell damage to the pathogenesis of some diseases in normal hosts.

Diagnosis of viral infections in immunocompromised patients can be difficult. Total and differential leukocyte counts tend to be unhelpful, clinical signs and symptoms of infection are often atypical, and symptoms produced by the host's immune response (e.g. the rash of measles) may be absent. Serology has little role in diagnosis because patients with dysfunctional immunity do not usually mount appropriate immune responses rapidly enough to influence decisions about management. The mainstay of diagnosis is detection of virus in appropriate clinical specimens: rapid procedures, such as direct antigen detection, nucleic acid detection and culture amplified enzyme immunoassays, have increased the usefulness of laboratory diagnosis enormously (Simmons 1996). In some situations (e.g. post-transplantation), regular surveillance cultures may be helpful: virus shedding may precede the onset of symptoms, and initiation of therapy as soon as virus is detected may prevent extensive tissue damage.

The AIDS pandemic has created an explosive increase in the number of profoundly immunocompromised people worldwide. The clinical manifestations of viral infections in human immunodeficiency virus (HIV)-infected individuals often differ significantly from the signs and symptoms caused by the same viruses in patients with other types of immune deficiency. Accordingly, a significant part of this chapter is devoted specifically to patients infected with HIV.

HIV-INFECTED ADULTS

The progressive decline in immunological function associated with HIV infection is usually monitored by measuring the absolute $CD4^+$ T-cell count. In general, significant opportunistic viral infections (Table 1) are not seen until $CD4^+$ T cells fall below $200/mm^3$ and the most severe problems are associated with $CD4^+$ T-cell counts of $<100/mm^3$. Herpesviruses, particularly cytomegalovirus (CMV) and herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), are common causes of serious disease in profoundly immunosuppressed HIV-infected individuals and therefore herpesviruses receive particular attention here. Infections caused by papillomaviruses and enteric viruses are also given special consideration. Importantly, in locations where highly active antiretroviral therapy (HAART) is available, the prevalence in patients with AIDS of severe complications caused by viruses has been reduced considerably. Where appropriate, the discussion includes diagnosis and treatment of first-episode disease, prophylaxis against recurrence and, finally, measures to prevent acquisition of infection in previously unexposed individuals.

Table 1 *Opportunistic viral infections in HIV-infected patients*

Virus	Prominent clinical manifestations	References
Adenovirus	Gastroenteritis	Khoo et al. 1995
Retrovirus	Gastroenteritis	Grohmann et al. 1993
BK virus	Nephropathy, encephalitis	Vallbracht et al. 1993
Herpesviruses		
Cytomegalovirus	Retinitis, gastrointestinal disease, encephalitis, adrenalitis	Drew 1988; Peters et al. 1991
Epstein–Barr virus	Oral hairy leukoplakia, lymphoma	Andersson 1991
Herpes simplex virus type 1	Mucocutaneous lesions, keratoconjunctivitis	Stewart et al. 1995
Herpes simplex virus type 2	Genital and perianal ulceration	Stewart et al. 1995
Human herpesvirus 6	Pneumonitis, disseminated infection	Knox and Carrigan 1994
Human herpesvirus 8	Kaposi's sarcoma?	Chang et al. 1994
Varicella-zoster virus	Retinitis, recurrent dermatomal zoster, disseminated zoster, pneumonia, prolonged varicella	Buchbinder et al. 1992; Kelly et al. 1994
Hepatitis B virus	Increased rate of chronic infection	Hadler et al. 1991
Human papillomavirus	Recurrent and persistent anogenital warts, premalignant and malignant tumors, oral lesions	Palefsky 1991
Human parvovirus B19	Persistent anemia, red cell aplasia	Frickhofen et al. 1990
Influenza virus	Increased severity of illness	Safrin et al. 1990
JC virus	Progressive multifocal leukoencephalopathy	Berger et al. 1987
Measles virus	Encephalitis, pneumonia	Mustafa et al. 1993
Molluscum contagiosum virus	Disseminated cutaneous lesions	Epstein 1992
Parainfluenza virus	Exacerbation of respiratory symptoms	Hague et al. 1992
Picobirnavirus	Gastroenteritis	Grohmann et al. 1993
Respiratory syncytial virus	Increased mortality, prolonged viral shedding	Chandwani et al. 1990

Herpesvirus infections

Several genetically distinct herpesviruses have been isolated from humans. Some, namely HSV-1 and HSV-2, varicella-zoster virus (VZV), Epstein–Barr virus (EBV) and CMV have been recognized for decades. By contrast, human herpesviruses types 6 and 7 (HHV-6 and HHV-7) are relatively recent discoveries and the latest addition to the list, referred to here as *Human herpesvirus 8* HHV-8, is of particular interest in the present context because it is found in Kaposi's sarcoma (KS) cells (Chang et al. 1994).

Most well-characterized herpesviruses establish latent infections from which disease can periodically reactivate. In some cases, there are prolonged periods of asymptomatic virus shedding. Human herpesvirus (HHV) infections are generally acquired in early childhood and therefore most of the clinical problems associated with herpesviruses in adults are the result of reactivations. With the exception of varicella and herpes zoster, primary and recurrent HHV infections are often clinically unapparent and transmission of infection commonly occurs during periods of asymptomatic virus shedding.

The herpesviruses that cause greatest morbidity among HIV-infected people are HSV, CMV, VZV, and EBV. Occasional disease has been associated with HHV-6 and HHV-8 is thought to have a causal relationship with KS.

HERPESVIRUSES AS COFACTORS IN PROGRESSION OF HIV-RELATED DISEASE

Aside from their direct clinical impact, it has been suggested that herpesviruses could be cofactors in the development of AIDS, i.e. herpesviruses might accelerate progression toward immunodeficiency and death by influencing the pathogenesis of HIV infection at the molecular or cellular level. Epidemiologic data supporting this hypothesis are limited. A study of HIV-infected hemophiliacs suggested more rapid progression to AIDS in CMV-seropositive compared with CMV-seronegative patients (Webster et al. 1989), but this result could not be confirmed in a similar study elsewhere (Rabkin et al. 1993). Probably the best way to address the hypothesis is to study the effect of herpesvirus-specific antiviral drugs on HIV progression. There have been several clinical trials in which patients have been treated prophylactically with the anti-herpes compound aciclovir, in addition to zidovudine. Two groups (Cooper et al. 1993; Youle et al. 1994) showed, independently, that combination therapy increased survival time compared with zidovudine alone. However, neither study showed a decrease in deaths related to any of the known human herpesviruses and

the explanation for the apparent benefit of combination therapy remains obscure.

Although the proposal that herpesviruses promote the development of AIDS is in the realms of speculation, several molecular mechanisms can be envisaged by which HIV replication, or reactivation of latent provirus, could be enhanced.

For instance; all HHVs encode proteins called transactivators, which up-regulate viral gene expression during replication. Some herpesvirus transactivators are 'promiscuous,' i.e. they can upregulate expression not only of herpesvirus genes but also of genes belonging to other viruses or host cells. In vitro, several herpesvirus transactivators have been shown to interact with the HIV long terminal repeat, which is the main region of the HIV genome responsible for controlling gene expression. Thus, co-infection of a cell with a herpesvirus and HIV could, in principle, result in enhanced HIV replication. Alternatively, reactivation of latent HIV might be stimulated by superinfection with a herpesvirus. The best candidates for herpesviruses that might enhance HIV replication are CMV and HHV-6 because these viruses are commonly found in the same tissues as HIV in autopsy specimens (Emery et al. 1999). Therefore, coinfection of, for example, lymphocytes with HIV and either CMV or HHV-6 is a theoretical possibility. Furthermore, HHV-6 infection has been shown to transactivate the HIV *tat* gene in vitro (Garzino-Demo et al. 1996).

Promiscuous transactivation of various cellular genes could also, in principle, promote the spread of HIV. For example, virus induced up-regulation of CD4, the main cell surface receptor for HIV, might facilitate the spread of HIV from cell to cell. Similarly, up-regulation of cell surface Fc receptors, a property of most herpesviruses, could enable antibody-coated HIV particles to enter $CD4^+$ cell types, thereby expanding the pool of HIV-infected cells.

Coinfection of a cell with more than one type of virus raises the possibility of phenotypic mixing and the formulation of virus pseudotypes. This phenomenon is well established in vitro and can be manipulated to the extent that HIV genomes can be packaged into the capsids of vesicular stomatitis viruses. Formation of pseudotypes comprising HIV genomes packaged into particles containing herpesvirus glycoproteins has not been documented in vivo, but nevertheless, pseudotype formation remains a theoretical way in which HIV genomes could gain entry into a wide variety of otherwise resistant cell types.

HERPES SIMPLEX VIRUSES TYPES 1 AND 2

Herpes simplex is one of the commonest infections of humans. HSV-2 is transmitted almost exclusively by sexual contact; consequently, HSV-2 seropositivity is rare before the onset of sexual activity. The epidemiology of HSV-1 is more diverse; infection is commonly acquired during infancy or childhood from infected oral secretions, but sexual transmission of HSV-1 is becoming increasingly common. HSV latently infects primary sensory neurons innervating the portal of entry, creating a reservoir of infection that can periodically give rise to recrudescent disease. The generally early acquisition of HSV means that most episodes of herpes simplex seen in the clinic are recrudescences.

In the context of HIV infection, HSV causes a wide spectrum of disease, including genital herpes, perioral and facial herpes, perianal lesions, proctitis, conjunctivitis and keratitis, aseptic meningoencephalitis, herpetic Whitlow, autonomic nervous system dysfunction and acute necrotizing retinitis. In profoundly immunocompromised patients, with $CD4^+$ T-cell counts of $<100/mm^3$, extensive and chronic mucocutaneous ulceration is a frequently encountered and troublesome clinical problem, particularly in the genital and perianal regions (Safrin et al. 1991). In the absence of a normal inflammatory response, the lesions tend to respond slowly or poorly to antiviral therapy and they are often indolent or atypical in appearance.

In addition to prolonged mucocutaneous ulceration, there are several other severe manifestations of HSV infections in AIDS patients that merit further consideration. HSV may invade the esophagus or other parts of the gastrointestinal tract, giving rise to symptoms that reflect the region of the gut that is infected. Disseminated infection is rare but life-threatening. Many organs may be involved, including the lungs, liver, and adrenals. Prolonged ulcerative lesions and visceral disease are AIDS-defining illnesses.

There is evidence to suggest that genital ulceration facilitates sexual transmission of HIV and, in industrialized nations, the commonest cause of genital ulceration is HSV. By extrapolation, it has been proposed that genital herpes promotes the spread of HIV and there is some evidence that indirectly supports this contention (Holmberg et al. 1988; Stamm et al. 1988; Keet et al. 1990; Hook et al. 1992; Gwanzura et al. 1998).

Prevention of exposure

Strictly speaking, strategies designed to prevent exposure to HSV apply only to those rare individuals who are HSV-seronegative. However, first episode genital infections caused by HSV-2 in people previously exposed only orally to HSV-1 may be symptomatic; i.e. prior HSV-1 infection is, at best, only partially cross-protective against HSV-2 (Xu et al. 2002). Furthermore, in HIV-infected people, exogenous reinfection of the genital tract with different strains of HSV-2 is a possibility. With these considerations in mind, it is recommended that all HIV-infected people use latex condoms during every sexual contact. Condom usage is essential irrespective of whether the sexual partner has noticeable

herpetic lesions, because HSV is frequently shed from the genital tract asymptomatically.

Treatment and suppression of symptoms

HSV replication is inhibited by aciclovir, its pro-drug valaciclovir, and famciclovir. Aciclovir is available in oral and intravenous formulations; the appropriate route of administration and dose depend on the severity of lesions and the degree of immunological impairment. Primary genital herpes can be managed with oral aciclovir or valaciclovir unless immune function is profoundly impaired or lesions are unusually severe, in which case intravenous aciclovir is required. Recurrent genital herpes in patients with early HIV infection generally resolves spontaneously. In this group of patients, the approach to management is the same as for immunocompetent people: symptomatic relief of mild, infrequent outbreaks or, in selected patients who experience clear prodromal symptoms, episodic valaciclovir (Bodsworth et al. 1997) or famciclovir. Continuous administration of valaciclovir should be considered for patients with frequent episodes (Patel et al. 1997) or when lesions tend to heal slowly. In advanced HIV infection, when immune suppression is profound, herpetic lesions often require prolonged therapy with high doses of an antiviral compound; treatment failure is not uncommon, even when the drug is administered intravenously (Whitley and Gnann 1992). In this patient population, emergence of aciclovir-resistant viral strains, which are also resistant to the related nucleoside analogue ganciclovir is an increasingly recognized problem (Chatis and Crumpacker 1992). There are few avenues available for the treatment of recalcitrant lesions caused by aciclovir-resistant virus, the main option being intravenous foscarnet or perhaps cidofovir (Kopp et al. 2002).

Aciclovir resistance

Aciclovir is a nucleoside analogue which, when phosphorylated, inhibits DNA replication. The drug is phosphorylated by an HSV-encoded enzyme, thymidine kinase (TK), hence it is active only in HSV-infected cells. Emergence of HSV strains resistant to aciclovir is rare in immunocompetent hosts, even after long-term suppressive therapy. The situation is different in immunocompromised hosts, particularly patients with advanced HIV infection, in whom aciclovir resistance is a significant clinical problem.

HSV becomes resistant to aciclovir by way of viral TK mutations that decrease the ability of the virus to phosphorylate the drug. Such mutations can also affect the ability of TK to phosphorylate nucleosides required for virus replication. However, TK-altered HSV strains are able to grow in most cell types, because cellular kinases substitute for viral TK. Consequently, aciclovir-resistant viruses may cause aggressive mucocutaneous lesions, However, non-dividing cells, notably neurons, express unusually low amounts of endogenous kinases and, in these cells, replication of aciclovir-resistant HSV strains is, fortuitously, inhibited. This has important practical consequences: HSV must replicate in neurons in order to reactivate from latency and, therefore, the latency/reactivation cycle is interrupted for aciclovir-resistant viruses. The subtle influence of viral TK on the pathogenesis of herpes simplex may explain, in part, why aciclovir resistance is largely confined to patients treated over long periods for recalcitrant cutaneous lesions.

VARICELLA-ZOSTER VIRUS

Varicella is a common disease of childhood that is generally benign in immunocompetent hosts. In industrialized nations, seropositivity to VZV is >90 percent by the age of 15 years and therefore varicella is uncommon in adults. In the USA varicella vaccination is commonplace, further enhancing immunity among the young. This is fortunate, because the clinical impact of varicella in adults with impaired cellular immunity is high. Clinical manifestations include extensive, often hemorrhagic, cutaneous lesions and pneumonitis.

VZV establishes latency in sensory ganglia in much the same way as herpes simplex, although it has been suggested that the virus may be dormant in satellite glia rather than in neurons (Croen et al. 1988). However, despite controversy about the cell specificity of latent VZV for over a decade, it is now widely accepted that the virus is latent mainly in neurons, with only a small proportion of glia infected (Kennedy 2002). Recurrent VZV infection (herpes zoster, or shingles) is characterized by a blistering dermatomal rash and pain. HIV infection has a substantial impact on the incidence of VZV recurrence. HIV-infected adults are nine times more likely than the general population to develop herpes zoster (Holmberg et al. 1995) and the annual incidence of the disease is seven times higher in HIV-infected adults than in the general population. HIV-positive homosexual men are 17 times more likely to develop zoster than demographically matched HIV-negative subjects (Buchbinder et al. 1992).

The clinical manifestations of recurrent VZV infections in HIV-infected patients depend on the degree of immunological impairment. In the early stages of HIV infection, the signs and symptoms resemble herpes zoster in immunologically normal hosts. By contrast, repeated episodes of severe, prolonged, and sometimes atypical disease (Nikkels et al. 1999) are characteristic of advanced HIV infection. A few patients develop VZV retinitis, which has a particularly poor prognosis if the $CD4^+$ T-cell count is $<50/mm^3$. In some cases, atypical herpes zoster may require laboratory tests for confirmation of the diagnosis, in which case material from a recently erupted lesion should be sent for virus culture,

rapid antigen detection or, if available, culture amplified enzyme immunoassay. Antemortem diagnosis of herpes zoster is particularly difficult when atypical manifestations of the disease, such as retinitis, meningoencephalitis, optic neuritis, and visceral involvement, occur in the absence of cutaneous lesions.

Prophylaxis and treatment

Most adults with no history of chickenpox have antibodies to VZV, and serological tests are useful for determining whether an HIV-infected patient is truly at risk of acquiring primary VZV infection. Seronegative patients should avoid contact with varicella- and herpes zoster; if contact is documented, they should receive varicella-zoster immunoglobulin (VZIG) within 96 hours of exposure. In addition, household contacts should be vaccinated against VZV if they have no history of chickenpox or are known to be seronegative provided they themselves do not have advanced HIV infection. The prophylactic effect of varicella vaccine, administered to susceptible HIV-infected people before $CD4^+$ T cells become depleted, has yet to be fully evaluated. However, varicella is not a cofactor for progression to AIDS (Aronson et al. 1992) and is a generally benign illness early in the course of HIV. As a consequence, varicella vaccine (Oka strain) should be considered for non-immunocompromised HIV-positive children.

Most cases of herpes zoster respond well to valaciclovir, famciclovir, or high-dose oral aciclovir. Disseminated, recurrent, or unusually severe cases should be treated intravenously. Aciclovir-resistant VZV strains have been isolated from severely immunocompromised people on long-term suppressive therapy (Lyall et al. 1994). The usual cause of resistance is mutation of the viral TK gene; aciclovir-resistant VZV remains sensitive to intravenous foscarnet (Balfour et al. 1994).

CYTOMEGALOVIRUS

CMV infection is extremely common among men who have sex with men (MSM) and intravenous drug users (IVDU) and in these populations it is generally acquired before HIV. Therefore primary CMV infections are rare among HIV-infected MSM and IVDU. Other HIV-infected people should be informed that CMV is shed in body fluids including semen, saliva, and cervical secretions and advised to use latex condoms during sexual contact. Reactivation of CMV does not cause illness in immunocompetent adults. By contrast, when cellular immunity is impaired, reactivation has many potential clinical manifestations, including retinitis, esophagitis, colitis, pneumonitis, and CNS disease. The spectrum of disease caused by recurrent CMV infection is significantly different in HIV-infected people compared with transplant recipients. For instance, necrotizing retinitis and CNS disease, common complications of CMV infection in advanced AIDS (Drew 1988; Jacobson and Mills 1988), are very unusual in the transplant setting. Conversely, CMV pneumonitis is unusual in HIV-infected adults but is not uncommon in transplant recipients.

CMV is frequently present in body fluids of AIDS patients and it is often difficult to establish a causal relationship between CMV and disease. Detection of CMV in affected organs provides circumstantial evidence that disease is CMV-related, but this approach frequently fails. CMV is often detected in bronchoalveolar lavage specimens from AIDS patients but this alone is not predictive of CMV pneumonitis; histological examination of cells or tissue for characteristic cytomegaly is required to establish the diagnosis.

Prevention, pre-emptive therapy, and treatment

HAART has had a profound impact on the previously high incidence of CMV disease in HIV-infected persons. However, not all patients are able or ready to take HAART and in some cases HAART may have failed. CMV remains therefore a significant problem. Unfortunately, immunization is not an option currently available for protection against primary CMV infection in the immunocompromised but general prophylactic measures are appropriate, including counseling about the risks of sexual transmission and the increased risk of CMV acquisition in the child care setting. In the latter situation, good hygienic practices including hand washing can reduce the risk of acquisition. In reality, measures to prevent exposure apply to only a small proportion of HIV-infected adults but it is essential that patients understand that CMV is present in genital secretions. Finally, if CMV-seronegative patients require transfusion, blood products should be obtained from CMV-seronegative donors.

Many adults are already infected with CMV when HIV infection is diagnosed and reconstituting immunity with HAART is the most effective way of preventing CMV disease from developing in seropositive persons. Ways to predict and prevent development of CMV disease (primary prophylaxis) are being explored, allowing pre-emptive therapy to be commenced before irreversible damage has been done. CMV viremia per se is no more predictive of CMV disease than $CD4^+$ T-cell counts (Salmon-Ceron et al. 2000); most patients who develop CMV disease have <200 $CD4^+$ T cells/mm^3, and the risk increases as the $CD4^+$ T-cell count falls. The greatest risk is in patients with <50 $CD4^+$ T cells/mm^3, in whom the median time to development of retinitis is 6 months. Anti-CMV drugs are generally too toxic for continuous administration to profoundly immunocompromised persons ($CD4^+$ T-cell count <50/mm^3) and therefore true prophylaxis in AIDS patients awaits the development of safer, orally active compounds. Oral ganciclovir has been investigated for

primary prophylaxis of CMV in AIDS but neutropenia, anemia, cost, and lack of proven efficacy have made its use a generally unpopular option.

The mainstays of treatment for established disease are intravenous ganciclovir, foscarnet, cidofovir, and fomivirsen, given intravitreally for retinitis. These drugs are toxic and treatment is not curative, so it is usual to reduce the dosage to maintenance levels after an initial period of induction. Without maintenance, relapse is inevitable; even on maintenance, relapse is common, necessitating periods of reinduction.

EPSTEIN–BARR VIRUS

HIV infection promotes the development of a variety of EBV-related diseases. Oral hairy leukoplakia is an EBV-associated hyperkeratotic disease of the tongue that is very common in HIV-infected patients but very rare otherwise. It responds to aciclovir but relapses if therapy is stopped. Lymphomas occur with greatly increased frequency in HIV-positive individuals, and a high proportion of the tumors, which tend to be monoclonal, contain cells expressing EBV antigens. Despite intensive chemotherapy, the prognosis is poor, especially in patients with low $CD4^+$ T-cell counts.

HUMAN HERPESVIRUSES 6 AND 7

HHV-6 and HHV-7 are ubiquitous, closely related betaherpesviruses, and most humans are infected with these agents in early childhood. Primary infection may be asymptomatic or cause roseola infantum. It is probable that both viruses establish latent infections in lymphoid tissues; in immunocompromised people, latent infection may reactivate and disseminate. HHV-6 is also known to be resident in the brain. In AIDS patients, HHV-6 has been detected in a wide variety of tissues postmortem and may cause fatal pneumonitis (Knox and Carrigan 1994). Further work is required to clarify its clinical impact.

HUMAN HERPESVIRUS 8 (KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS)

HHV-8 is found in KS but not in normal skin. It is also associated with primary effusion lymphomas and some cases of multicentric Castelman's disease. On the basis of nucleotide sequence analysis, this agent is a gamma-2 herpesvirus, related to herpesvirus saimiri of squirrel monkeys and newly identified viruses of chimps and gorillas (Lacoste et al. 2000). Several lines of evidence suggest that HHV-8 plays an etiologic role in KS. First, antibodies against the virus are found in the sera of virtually all KS patients. Second, HHV-8 encodes several homologs of human proteins that could modulate cellular behavior, including a variety of cytokines (IL-6, MIPs, IRFs), cyclin D and a G-protein-coupled receptor (GPCR). Retinoblastoma protein and p53 are inhibited by viral cyclin D and the latency-associated nuclear antigen respectively and mice transgenic for GPCR have Kaposi-like regions in the skin. Finally, HHV-8 viral load in lymphocytes (Campbell et al. 2000) and skin lesions (Boivin et al. 2000) have been shown to increase with increasing disease burden.

Prevention and treatment

There is evidence to suggest that acquisition of HHV-8 after being infected with HIV may be associated with accelerated progression to KS, making prophylaxis against HHV-8 a priority. Counseling can be based on the three known routes of transmission, which are kissing (virus in saliva), sexual (virus in semen), bloodborne, and mother to child (Plancoulaine et al. 2000). Avoidance of deep kissing with persons with KS is currently accepted as advisable to reduce the risk of oral transmission. There is no evidence to support the supposition that latex condoms reduce the transmission of HHV-8 but their use is advisable for protection against not only HHV-8 but many other sexually transmissible infections. Owing to the lack of routine serological tests for HHV-8, screening of blood has not been possible and finally, there are no current recommendations for preventing mother-to-child transmission.

The prevalence of KS is lower among AIDS patients treated for retinitis with either ganciclovir or foscarnet, both of which inhibit HHV-8 replication in vitro, suggesting that these compounds might be useful for prevention or treatment of KS. However, the efficacies of antiviral therapies for HHV-8 infection have not been established and this remains a research priority. Finally, restoration of immune function with HAART, where it is available, has greatly reduced the prevalence of KS.

Human papillomavirus infections

Of the 70 or so known Human papillomavirus (HPV) infections, more than 25 have been detected in the genital region. Some HPVs, notably types 16 and 18, are associated with precancerous intraepithelial neoplasias of the cervix, vagina, vulva, penis, and anus. HIV-induced immune dysfunction increases the prevalence of detectable genital HPV infection and HPV-associated intraepithelial neoplasia (Northfelt and Palefsky 1992), as does iatrogenic immunosuppression (Penn 1986). However, the association between HIV and HPV is not explicable solely on the basis of immunosuppression, because HPV DNA is detectable with increased frequency in the genital tracts of women with early HIV infection, before there is a measurable decline in the $CD4^+$ T-cell count. Both direct interaction between HIV and HPV and the common risk factors for their acquisition may contribute to the association between HIV infection and HPV-related disease.

In HIV-infected women, cervical intraepithelial neoplasia (CIN) recurs more frequently than expected after treatment (Maiman et al. 1990), and recurrence is

related to severity of immunosuppression. Although CIN does not seem to progress unusually rapidly to invasive cancer, the latter is an AIDS-defining condition according to the Centers for Disease Control and Prevention guidelines. It is recommended that women have a pelvic examination and Papanicolaou (Pap) smear twice during the first year after HIV diagnosis and once per year thereafter. The usefulness of cytological screening of HPV-positive MSM for early detection of anal high-grade intraepithelial neoplasia is currently unclear (Piketty et al. 2003).

Hepatitis C

HIV infection and hepatitis C share some common risk factors (primarily injecting drug use and hemophilia) and therefore it is prudent to screen all HIV-positive patients for hepatitis C virus (HCV). This is important for helping in the interpretation of abnormal liver function tests and also because in patients with HIV infection the course of hepatitis C may be accelerated (Thomas et al. 1996). The safety of treatment for hepatitis C in HIV-infected patients is currently under investigation.

Enteric infections

Diarrhea is very common among HIV-infected patients. Potential viral causes include astroviruses, adenoviruses, picobirnavirus, and caliciviruses (Grohmann et al. 1993), on the basis that they are detected more frequently in patients with diarrhea than in patients without diarrhea. CMV is also associated with gastrointestinal disease in HIV-infected adults (Laughon et al. 1988).

PICOBIRNAVIRUSES

Picobirnaviruses are small, bisegmented, double-stranded RNA viruses that have been detected in a wide variety of vertebrates, including pigs, in which they are associated with diarrhea (Gatti et al. 1989). They can be grown in mammalian cell cultures, suggesting that they are vertebrate viruses rather than viruses of other intestinal organisms such as protozoa. It is not known with certainty whether picobirnaviruses cause diarrhea in humans (Glass et al. 2001) but their detection in a significant proportion of fecal specimens from HIV-infected patients with diarrhea raises this possibility (Giordano et al. 1998, 1999). Development of serological tests might help to determine the prevalence of picobirnavirus infections in the human population.

ENTERIC ADENOVIRUSES

Reported rates of faecal carriage of adenoviruses among patients with advanced HIV infection are very high (Cunningham et al. 1988). Adenovirus infections of the gastrointestinal tract are frequently asymptomatic, and when symptoms are noted they are often mild. In immunocompetent people, adenoviruses associated with diarrhea (Liste et al. 2000; Pollok 2001; Trevino et al. 2001) usually belong to subgenera A, C, or F, but in HIV-antibody-positive patients at least one report suggests that the most predominant fecal isolates belong to subgenus D (Khoo et al. 1995). There is some suggestion that prolonged infections in patients with $CD4^+$ T-cell counts $<200/mm^3$ may allow antigenic drift and genetic recombination between different serotypes (Hierholzer et al. 1988).

JC polyoma virus and progressive multifocal leukoencephalopathy

Progressive multifocal leukoencephalopathy (PML) is a neurological disease caused by JC polyoma virus (JCV). It is characterized by multiple foci of demyelination throughout the CNS. PML has a global distribution and, in patients who do not have AIDS, it is most common in people over the age of 50. The disease is seen in patients with a wide variety of disorders associated with impaired immune function but very rarely in immunocompetent hosts. JCV is found in the nuclei of oligodendrocytes in affected areas of the brain, and viral DNA is present in the cerebrospinal fluid (Gibson et al. 1993). The clinical features of PML are gradual deterioration of cerebral function, progressing to dementia, blindness, paralysis, and eventual coma and death, usually within 6 months of onset.

No treatment is curative and currently, the best initial approach to therapy for PML is improving the underlying immunodeficiency. Addition of cidofovir to HAART has been reported to improve clinical outcome in PML patients and reduce JCV DNA load in cerebrospinal fluid, indicating control of JCV replication (De Luca et al. 2000).

HIV-INFECTED CHILDREN

The impact of opportunistic viral infections on HIV-infected children has yet to be fully determined. However, as in adults, herpesviruses are a major problem.

Herpesvirus infections

There are marked differences in the clinical manifestations of herpesvirus infections in HIV-infected children compared with adults. In part, the differences depend on whether HIV infection precedes primary infections with herpesviruses.

HERPES SIMPLEX VIRUSES

In children, herpes simplex is almost always limited to the oropharynx. Esophagitis is rare, as is dissemination

or invasion of the CNS. Furthermore, there is no evidence suggesting that neonatal herpes is more common among the offspring of HIV-infected mothers. The diagnostic method of choice is detection of virus or viral antigens in material collected from newly erupted lesions. Treatment follows guidelines similar to those applied to herpes simplex in adults: oral or intravenous aciclovir according to the severity of disease and suppressive therapy with oral aciclovir for frequently recurrent episodes.

VARICELLA-ZOSTER VIRUS

A small number of cases of varicella in HIV-positive children have been reported in the literature, including a recent outbreak at a summer camp for infected children. Some cases run an uneventful course whereas others may be prolonged or complicated by sepsis, pneumonia, or cerebral vasculitis/aneurism. Recurrent varicella- and herpes zoster have been documented. The severity of complications seems to be inversely related to the $CD4^+$ T-cell count (Jura et al. 1989). Diagnosis is usually possible on clinical grounds. Atypical disease may require laboratory confirmation by detection of VZV in material collected from a freshly erupted vesicle.

Prophylaxis and treatment

Serological tests may be required to identify children who are susceptible to primary infection with VZV. Seronegative children should avoid contact with chickenpox and herpes zoster; when exposure is documented, VZIG may be useful. However, even when VZIG is given within 96 hours of exposure, protection is not guaranteed (Srugo et al. 1993). Varicella vaccine has recently been reported to be beneficial and safe in mildly immunocompromised HIV-infected children (ACIP 1999).

Aciclovir is effective in the treatment of varicella in HIV-infected children (Jura et al. 1989). Intravenous therapy is recommended, particularly for children with poor immune function.

CYTOMEGALOVIRUS

CMV infection is very common among infants whose mothers are HIV-antibody-positive. Furthermore, maternal HIV infection seems to increase the prevalence of congenital CMV. CMV-related disease is an important AIDS indicator event in HIV-infected children, and dissemination of CMV in children with advanced HIV infection carries a high mortality. There is evidence to suggest that HIV-related disease develops up to three times more rapidly in CMV-infected infants compared with uninfected infants. Therefore prophylaxis is a desirable but currently unattainable goal.

There are differences in the clinical manifestations of CMV infection in HIV-infected children and adults that most probably represent the different outcomes of primary and recurrent infections, respectively. Pneumonitis and hepatitis, the commonest diseases caused by CMV in HIV-infected children, are probably the result of primary infection. By contrast, CMV retinitis, which is common in adults and rare in children, is thought to follow reactivation of latent virus in the immune host.

Diagnosis of CMV disease is notoriously difficult, owing to the high prevalence of asymptomatic infection by the age of 2 years. Detection of CMV in urine is common and not indicative of CMV disease. Detection of CMV in the diseased organ [by culture, viral antigen detection, culture amplified antigen detection or polymerase chain reaction (PCR)] is currently the best diagnostic approach but the predictive value of a positive result is not absolute.

Prophylaxis and treatment

Ganciclovir, foscarnet, and cidofovir have all been used to treat CMV disease in children. Toxicity is less of a problem in children compared with adults but the drugs must be given intravenously, creating problems with compliance. Realistically, neither drug is suitable for prophylactic use.

EPSTEIN–BARR VIRUS

EBV DNA has been detected in the lungs of HIV-infected patients with lymphoid interstitial pneumonitis (LIP). LIP is rare in HIV-infected adults but is one of the commonest complications of HIV infection in infants under the age of 2 years. Clinical manifestations of LIP include lymphadenopathy, parotitis, bronchospasm, tachypnea, and sleep apnea. Serum immunoglobulin levels may be unusually high, with an associated hyperviscosity syndrome. Chest infections may complicate the disease and therefore immunization against bacterial pathogens is recommended. Diagnosis rests on sequential clinical assessments and chest X-ray changes over a period of 2 months, together with lung biopsy if necessary. Further work is merited to determine whether EBV, the host response to it, or other viruses, plays an etiologic role in LIP because, if this is the case, specific therapy may be possible. Corticosteroids and zidovudine have been reported to be useful in the management of the disease.

Respiratory syncytial virus infections

Pneumonitis, rather than bronchiolitis or wheezing, seems to be a prominent clinical manifestation of respiratory syncytial virus (RSV) in HIV-infected children (Chandwani et al. 1990). Bronchiolitis is thought to be the result of immune-mediated damage to the lung, which might explain its rarity in immunocompromised hosts. In infants with low $CD4^+$ T-cell counts, RSV pneumonia may be life-threatening as a result of

secondary bacterial infection (Chandwani et al. 1990). Pre-emptive antibacterial therapy may therefore be worthwhile in severely immunocompromised children who fail to respond promptly to ribavirin.

IMMUNE DEFICIENCIES NOT CAUSED BY HIV

Malignant tumors, cytotoxic chemotherapy or radiotherapy, and congenital disorders may all profoundly compromise the immune system, predisposing patients to unusually severe or prolonged infections with a wide range of viruses.

Herpesvirus infections

It has been known for a long time that herpesviruses are important opportunistic pathogens in patients with disorders of cellular immunity. The potential manifestations of infection are diverse and sometimes life-threatening.

HERPES SIMPLEX

The frequency and severity of recurrent episodes of herpes simplex are increased in patients with dysfunctional immunity, particularly allograft recipients (Naraqi et al. 1977; Rand et al. 1977; Meyers et al. 1980), those with malignancy or those receiving anti-tumor chemotherapy (Muller et al. 1972) and patients with Wiskott–Aldrich syndrome (St Geme et al. 1965). In allograft recipients, herpes simplex most commonly presents in the second week after transplantation.

Shedding of HSV may be asymptomatic or may present as vesicles or ulcers. Lesions may be widespread and persistent, and may involve the esophagus or rectum, causing pain and gastrointestinal disturbances. Ulcers may become indolent and, in immobile patients, perianal ulcers may be confused with pressure sores. Major manifestations of visceral infection are pneumonia and hepatitis.

Virus detection is the mainstay of laboratory diagnosis. In the case of a superficial infection, the specimen required is material collected from the base of the lesion. To diagnose HSV pneumonia, bronchoalveolar lavage fluid or, preferably, a lung biopsy specimen is necessary. Virus culture is probably the most sensitive diagnostic procedure in routine use but it can take up to 7 days before a characteristic cytopathic effect (CPE) develops. More rapid procedures include direct detection of viral antigens and culture-amplified enzyme immunoassays, in which inoculated cell cultures are tested for the presence of viral antigens after 24–48 hours, in order to prevent the development of cytopathology.

Prophylaxis and treatment

To control reactivation of HSV caused by iatrogenic immunosuppression, bone marrow recipients are often given aciclovir prophylactically, commencing at the start of induction chemotherapy. However, some transplant units believe it is more cost effective to treat episodes as they arise.

Intravenous aciclovir is recommended for the treatment of all HSV disease in profoundly immunocompromised patients, irrespective of whether the infection is localized or disseminated at the time of diagnosis. Oral aciclovir or valaciclovir may be appropriate for localized herpes simplex in patients who are not severely immunocompromised, with the caveat that intestinal absorption of the drug may be reduced by irradiation of the gut or systemic chemotherapy.

VARICELLA

Varicella is an aggressive and potentially fatal disease in people with severe cellular immune defects. The rash may be florid or hemorrhagic, and visceral manifestations include hepatitis, pneumonitis, and encephalitis. Most cases of varicella can be diagnosed on clinical grounds but, occasionally, virus culture or antigen detection tests are required, particularly to distinguish the disease from disseminated herpes simplex.

Treatment and prophylaxis

Intravenous aciclovir is of proven efficacy for the treatment of visceral disease caused by VZV in children with malignant disease (Schulmann 1985). The drug is beneficial in immunocompromised children presenting at the time of emergence of rash (Nyerges et al. 1988) and treatment should begin as soon as possible after rash eruption to be effective in reducing the incidence of visceral complications.

For prevention of varicella, live-attenuated varicella vaccine (Oka strain) is safe and effective when given to children with leukemia (in remission) or other malignancies, provided that anticancer chemotherapy is suspended from 1 week before to 1 week after vaccination. Passive immunization with VZIG is useful for seronegative people known to have been exposed to varicella. VZIG is potentially beneficial when given within 96 hours of exposure but protection is not a certainty (Kavaliotis et al. 1998).

HERPES ZOSTER

Herpes zoster (recrudescent VZV infection) is common in VZV-seropositive immunocompromised children and adults and, unlike zoster in immunocompetent people, episodes may be recurrent. After bone marrow transplantation, 30 percent of patients develop herpes zoster within a year, with a peak incidence in the fourth month (Locksley et al. 1985). The disease is also common in renal transplant recipients (Naraqi et al. 1977) and in patients with Hodgkin's disease (Schulmann 1985).

Infection may be confined to a single dermatome or may involve overlapping or noncontiguous dermatomes. Cutaneous dissemination of infection is not uncommon and, in severely immunocompromised patients, life-threatening visceral dissemination may occur.

Treatment and prophylaxis

Oral aciclovir, valaciclovir, or famciclovir may be used to treat localized herpes zoster in patients who are not severely immunocompromised (e.g. those on maintenance chemotherapy). Treatment should be commenced as soon as possible after eruption of the rash. In profoundly immunocompromised patients and in patients with signs of disseminated disease, intravenous aciclovir is currently the treatment of choice.

At the present time it is not feasible or cost effective to prevent herpes zoster in immunocompromised patients by continuous administration of antiviral compounds. This situation might change in the future if safe and inexpensive oral compounds with high activity against VZV become available. There is interest in determining whether herpes zoster can be prevented in immunocompetent adults by vaccination with the Oka strain of VZV. If this approach proves to be effective, it may be applicable to some immunocompromised patients, given that vaccination of leukemic children during periods of remission safely protects them against varicella.

CYTOMEGALOVIRUS INFECTIONS

CMV infection is usually asymptomatic in the immunocompetent host. Primary infection is acquired at any time throughout life; by 40 years of age, 50–70 percent of the general population are infected, with the lower prevalence correlated with higher socioeconomic class. Primary infection or reinfection of an immune host may be acquired as a result of transfusion of CMV-seropositive blood or blood products, or receipt of a kidney from a seropositive donor. However, reactivation of latent virus is the cause of most CMV infections in immunocompromised hosts. CMV is a major cause of morbidity and mortality in allograft recipients (Peterson et al. 1980; Meyers et al. 1982). Characteristically, reactivation is detected in the second and third months after bone marrow transplantation.

CMV causes a wide range of clinical syndromes in people with dysfunctional immunity, prominent among which are hepatitis, gastrointestinal disease, and pneumonitis. The last is thought to be immunologically mediated (Grundy et al. 1987) and responds poorly to specific antiviral therapy. In bone marrow recipients, graft-versus-host disease is a risk factor for development of CMV pneumonitis. Reactivation of CMV may be manifest as fever without localizing signs of infection, creating considerable difficulty in diagnosis. Surveillance cultures of urine, saliva, and blood, taken twice weekly, are useful in this respect, because virological evidence of active infection precedes disease. In addition, quantification of CMV load in whole blood or plasma has been shown to be a useful predictor of CMV disease in solid organ transplant recipients.

Diagnosis of CMV disease requires detection of virus in material from the affected organ. For diagnosis of CMV pneumonitis, a transbronchial biopsy is a suitable specimen. Detection of virus in buffy coat cells may be a useful adjunct to diagnosis when material cannot be obtained from the affected organ. In order to be useful in patient management, virus detection must be rapid. On average, 16 days are required to identify CMV in conventional cell cultures, making virus culture impractical when decisions have to be made about therapy. Rapid diagnosis depends on direct immunohistochemical detection of viral antigen in biopsy material (Volpi et al. 1983) or culture amplified antigen detection methods. The latter approach is both rapid and sensitive and is based on the principle that a few cells in an inoculated monolayer express immediate early CMV antigens, which can be detected, for instance, by enzyme immunoassay, within 24–48 hours of infection.

Quantification of CMV in plasma or leukocytes by PCR is a rapid and potentially useful diagnostic tool in solid organ transplant recipients (Ferreira-Gonzalez et al. 1999).

EPSTEIN–BARR VIRUS INFECTIONS

Primary EBV infection is very common in early childhood and adolescence, and more than 90 percent of the adult population is EBV-seropositive. EBV replicates in B lymphocytes and pharyngeal epithelial cells and the virus is transmitted in saliva. In the normal host, lytic infection is kept under control by cytotoxic T lymphocytes (CTL) with specificity for virally encoded antigens. After recovery from primary infection, EBV persists in a latent form in B lymphocytes. In renal allograft recipients and other profoundly immunocompromised patients, lytic infection is not adequately controlled, leading to frequently recurrent or persistent virus shedding and an unusually high virus load in the blood. In one report, EBV was detected in throat washings from more than 80 percent of seropositive renal transplant patients (Chang et al. 1978).

There has been considerable debate as to whether EBV can persist in epithelial cells in addition to lymphoid tissue. Current evidence suggests that lymphocytes are the only true reservoir of EBV in vivo. Carriage of EBV is lost following ablation of lymphoid tissue in EBV-seropositive patients undergoing bone marrow transplantation (Gratama et al. 1990). Furthermore, the characteristic pattern of viral gene expression associated with latency is not evident in epithelial cells at sites of oral hairy leukoplakia (Sandvej et al. 1992).

Primary EBV infections may be asymptomatic or cause infectious mononucleosis (IM). The clinical

features of reactivated infection are poorly characterized: fever and hepatitis have been implicated as potential manifestations of recrudescent disease but undoubtedly most reactivations must be asymptomatic. Primary and recurrent infections are associated with B-cell lymphomas in transplant recipients (Hanto et al. 1982; Ho et al. 1985), and EBV nuclear antigen and EBV DNA can be detected in the tumor cells. The incidence of lymphomas in transplant patients has increased substantially with the use of more aggressive immunosuppressive regimens, and recipients of bone marrow, heart, and liver grafts are most at risk. Rapidly progressive lymphomas that develop early after transplantation are most commonly the result of primary rather than recurrent EBV infections, acquired either from the graft or from transfused blood. Lymphoma tends to be a late complication of recurrent EBV infection. Post-transplant lymphomas are resistant to standard antitumor chemotherapies, and aciclovir is not beneficial.

All post-transplantation lymphomas contain EBV DNA. The higher the virus load in the blood, the greater is the risk of developing lymphoma (Riddler et al. 1994; Savoie et al. 1994), consistent with the hypothesis that failure to control lytic infection predisposes to the outgrowth of malignant cells. Tumors are often multifocal and particularly affect the gastrointestinal tract, liver, and CNS. They may progress from a polyclonal to a monoclonal phenotype, presumably representing dominance by the most rapidly growing cell in the original tumor. Different tumors in the same patient commonly have different clonal origins.

The lymphoma cells have several properties in common with EBV-transformed lymphoblastoid cell lines, suggesting that EBV may be the only factor underlying tumor development in vivo. Chromosomal translocations of the Burkitt's lymphoma type and c-*myc* rearrangements have been reported but are rare. Tumors may regress with improved immune function (Starzl et al. 1984), suggesting that they remain sensitive to attack by EBV-specific CTLs. The possibility of treating post-transplant lymphomas by adoptive transfer of CTL is being explored.

X-linked lymphoproliferative syndrome

X-linked lymphoproliferative syndrome (XLPS) is a rare form of immunodeficiency that selectively predisposes affected males to life-threatening EBV-related disease (Purtilo et al. 1975). About 75 percent of those affected die within a few weeks of primary EBV infection. The remainder has a greatly increased risk of developing lymphomas or hypogammaglobulinemia.

Many patients with XLPS fail to control the initial proliferation of EBV-transformed B cells associated with acute IM, despite the presence of reactive T cells. This may result in a disease resembling immunoblastic lymphoma in transplant patients. Infiltration of organs by lymphocytes is a common cause of death in these individuals. Alternatively, the initial proliferation of B cells and reactive T cells may abate, to be followed by widespread infiltration and destruction of lymphoid tissues and bone marrow by phagocytic cells (Sullivan and Woda 1989). This type of pathology is indistinguishable from the virus-associated hemophagocytic syndrome.

The genetic defect responsible for XLPS is closely linked to two X-chromosome markers, DXS42 and 37. Several phenotypic defects have been identified in XLPS patients and carriers, including failure to develop antibodies to EBV nuclear antigens. IgM to IgG switching is defective and the ability of T cells to produce interferon-gamma (IFN-γ) is reduced. It has been postulated that patients with XLPS have a critical defect in their ability to support the activity of EBV-specific CTLs.

Papillomavirus infections

Warts are common in patients with dysfunctional cellular immunity, including those with leukemia or lymphoma and renal transplant recipients (Koranda et al. 1974). Most infections probably represent reactivations of latent virus.

EPIDERMODYSPLASIA VERRUCIFORMIS

Epidermodysplasia verruciformis (EV) is a rare disease that is first manifest in infancy or childhood by the development of multiple warts that do not regress spontaneously. Patients with EV have a poorly understood immunological defect that predisposes them, apparently quite specifically, to papillomavirus disease. There is often a family history of the disease but its pattern of inheritance is not clear. Parental consanguinity is common, suggesting that the trait is recessive.

A mixture of typical flat warts and reddish-brown plaques is characteristic. The lesions are caused by a wide variety of papillomavirus types, most of which rarely cause warts in the general population. Some of the warts, particularly those in areas of the body exposed to the sun, progress to intraepithelial or invasive squamous carcinomas (Jablonska and Majewski 1994).

Enteric viruses

Many viruses have been associated with gastroenteritis. In immunocompetent hosts, illness is usually mild and shedding of virus in the feces is transient. By contrast, enteric viruses cause significant morbidity in immunocompromised patients. Rotaviruses, adenoviruses, astroviruses, caliciviruses, and small round viruses have all been associated with severe or persistent diarrhea in patients with perturbed immune function (Chrystie et al. 1982; Pedley et al. 1984; Oishi et al. 1991; Cox et al.

1994; Romberg 1994), bone marrow recipients being particularly at risk. A nosocomial outbreak of rotavirus infection, with severe diarrhea, has been documented in renal transplant recipients (Peigue-Lafeuille et al. 1991). Although enteroviruses are not usually associated with gastrointestinal disease, coxsackie A viruses have been implicated as causes of diarrhea in the contexts of hypogammaglobulinemia (Johnson et al. 1982) and bone marrow transplantation (Townsend et al. 1982).

ENTEROVIRAL MENINGOENCEPHALITIS

Severe and persistent enterovirus infections are seen in patients with congenital hypogammaglobulinemia, despite regular immunoglobulin replacement therapy. This finding is unusual in that hypogammaglobulinemic patients respond normally to other viral infections. The most frequent complication is chronic, often fatal, meningoencephalitis. Patients may present with neck stiffness, headache, lethargy, weakness or seizures, with or without fever. These features are often associated with a dermatomyositis-like syndrome, characterized by edema of the extremities, sometimes with an erythematous or violaceous rash overlying groups of muscles. Echovirus types 2, 3, 5, 9, 11, 17, 19, 24, 25, 30, and 33, and coxsackie virus B3 have been isolated from the cerebrospinal fluid or brain tissue, and occasionally from muscle, in these patients (Mease et al. 1981: Cooper et al. 1983). Respiratory illness caused by enteroviruses has also been described, and evidence of disseminated infection has been found in fatal cases.

Measles

Dysfunctional cellular immunity predisposes to persistence of measles virus with potentially lethal consequences. Two complications, namely giant cell pneumonia and measles inclusion body encephalitis (MIBE), are recognized, neither of which is seen in immunocompetent hosts. Giant cell pneumonia usually presents 2–3 weeks after exposure to measles. The characteristic measles rash may be absent, in which case the diagnosis may remain obscure until postmortem. Laboratory approaches to diagnosis include detection of virus or viral antigens in nasopharyngeal fluid, bronchoalveolar lavage fluid or lung tissue, and demonstration of multinucleated giant cells with characteristic inclusion bodies in histological sections of lung.

MIBE usually arises within 6 months of exposure to measles virus and is occasionally accompanied by giant cell pneumonia or retinopathy. The disease may be rapidly progressive and has a poor prognosis; rapid deterioration leads to death within a few weeks of onset. The underlying pathology is one of gliosis. Intracytoplasmic and intranuclear inclusion bodies are present in glial cells and neurons, and inflammatory cell infiltration of the brain is minimal. Defective measles virus has been detected in the brains of MIBE patients and it is possible that the disease is an accelerated version of subacute sclerosing panencephalitis (Ohuchi et al. 1987).

Measles vaccine contains live attenuated virus and is not generally recommended for administration to immunocompromised patients. The current vaccination program for normal children reduces the number of susceptible patients among those who subsequently develop dysfunctional immunity, and may reduce the chance of exposure in non-immune individuals. Measles in previously vaccinated hosts has, however, been reported. Administration of normal human immunoglobulin after exposure to measles virus has some beneficial effect, but may not prevent complications, particularly MIBE (Kay and Rankin 1984). There is as yet no specific antiviral therapy with proven efficacy for the treatment of measles or its complications in immunocompromised hosts, but sporadic case reports suggest that aerosolized ribavirin might be of some benefit to patients with pneumonitis.

Polyomavirus infections

BK virus (BKV) and JCV are human polyomaviruses which, according to serological studies, are ubiquitous. It was thought that active, persistent infections were rare in immunocompetent hosts, but recent data suggest that urinary excretion of JCV is common in older people and pregnant women.

About half of renal transplant recipients have serological or other evidence of prior exposure to *Polyomavirus* (Hogan et al. 1980; Gardner et al. 1984), in keeping with the high seropositivity rate in the general population. BKV and JCV are commonly detected in the urine of transplant patients, suggesting that immunosuppression allows latent infections to reactivate. Primary and reactivated infections are usually asymptomatic, although ureteric stenosis has been reported and there may be an association with impaired graft function. Infections are frequent in the second month after renal transplantation, but late infections, occurring several months or even years later, are not unusual.

Urinary excretion of BKV and JCV is also common after bone marrow transplantation (O'Reilly et al. 1981) and has been reported in association with leukemia, lymphoma, solid tumors and Wiskott–Aldrich syndrome. Infection is generally asymptomatic, although deterioration in renal and hepatic function has been observed; BK viruria following bone marrow transplantation may be associated with hemorrhagic cystitis (Rice et al. 1985).

Polyomavirus infections of the urinary tract may be diagnosed by electron microscopy or by demonstrating characteristic intranuclear inclusions in exfoliated urinary epithelial cells. The presence of Polyomavirus antigens within these cells can be confirmed by immunofluorescence. BKV can be isolated in cell cultures, such as human embryo lung fibroblasts.

A minority of non-AIDS immunocompromised patients infected with JCV may develop PML. The factors predisposing to PML are unclear and virus has been detected in the brains of normal individuals. It has been suggested that JCV may adapt itself for growth in the nervous system during the course of infection. In support of this hypothesis, structural alterations in the archetypal regulatory region of the JCV genome have been found in JCV DNA from PML brains. Structural variations in the regulatory region have also been reported among viral DNAs from different organs in the same infected patient (Loeber and Dorries 1988). However, the issue of virus adaptation to different tissues is controversial because different organs have been shown to be infected with the same JCV subtype and PML isolates can be the same subtype as urinary isolates (Myers et al. 1989).

Diagnosis of PML is based on the characteristic clinical picture, and should be confirmed by brain biopsy. The histological appearances are typical and polyomavirus particles can be demonstrated by electron microscopy. Immunofluorescence microscopy of acetone-fixed brain tissue may reveal the presence of polyomavirus antigens. Virus may also be isolated in primary cultures of human fetal glial cells.

No agent has proven efficacy for the treatment of polyomavirus infections; reduction of immunosuppressive therapy, when practicable is, however, beneficial.

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Safety in the virology laboratory

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INTRODUCTION

Both the emergence and re-emergence of new virological agents and the perceived threats associated with bioterrorism are requiring scientists, engineers, and biosafety professionals to design safe working environments and ensure the protection of the community. In addition, disease eradication programs, such as the World Health Organization (2003) plan for the elimination of wild polioviruses, calls for the establishment of limited holdings and inventories of the agent, possibly raising future containment requirements. Most current safety legislation is based on the principles laid down in a variety of government regulations, which may differ slightly from country to country. Examples of such regulations include the Health and Safety at Work Act 1974 and Heath and Safety Control of Substances Hazardous to Health 2002 (Health and Safety Executive 2002) in the UK, the occupational safety and health laws in the USA, European Directive 1993, and Labour Canada regulations, all of which place a duty of care on employers in relation to the safety of their employees and others. International and national legislation increasingly demand employees to co-operate with employers and place a duty on them to avoid putting themselves, colleagues, or others at potential risk. Therefore, overall safety in the virology, or any other, laboratory is the responsibility of all laboratory workers. People in a supervisory or managerial position are responsible and increasingly accountable for the health, safety, and welfare of all staff and visitors in laboratories under their jurisdiction (Health and Safety Executive, 2000). The emphasis in this chapter, though, is the unique subset of safety issues resulting from the operation of a microbiology laboratory using pathogenic or potentially pathogenic agents. The ability to perform accurate risk assessments (job hazard analysis), based on our understanding of science, is of key importance.

In his review of laboratory-acquired infections, Pike (1979) concluded 'the knowledge, the techniques, and the equipment to prevent most laboratory infections are available.' Over the last decade safety-related technology has advanced significantly, developing biosafety systems that provide a safer working environment. Despite these advances, laboratory accidents still occur at a disturbing rate; unfortunately, as in the past, the most common cause of such accidents is human error. New, sophisticated biosafety systems and improved training methods are still necessary to cope with the increased challenges raised by the emergence and re-emergence of significant pathogenic viruses such as arenaviruses, dengue viruses, Ebola virus, hantavirus, the new hepatitis viruses, severe acute respiratory syndrome (SARS), influenza strains (H5N1), and human immunodeficiency virus (HIV). The fundamental concepts on which to construct a laboratory safety

program must therefore ensure minimal risk to both laboratory workers and the community. These concepts include both the proper use and maintenance of new safety equipment and, especially, a greater appreciation of good laboratory practice and proper risk assessment.

A safety program must be based on a sound understanding of the microbiological properties of the agent to be manipulated; a microbiological risk assessment programme (Advisory Committee on Dangerous Pathogens 1996); an understanding of the protective potential of scientific equipment and facilities; comprehensive personnel training and development appropriate to the studies to be undertaken; and use of guidelines and protocols, with the understanding that both must be flexible and dynamic. Any understanding of the properties of a micro-organism must include its virulence, means of spread, tropism, infectious dose, and environmental stability. For a safety program to be effective, it must have the support of laboratory workers and managers. Laboratory management have the ultimate responsibility of providing a safe working environment as described above.

This chapter discusses the typical components of a prudent virology laboratory safety programme. Topics include facilities, containment criteria, work practices, biohazard surveillance, waste management, and training. It must be repeated, though, that the cornerstone of any worthwhile safety program is accurate risk assessment and management that is based on full understanding of the infectious and pathogenic potential of the agents under investigation.

Information on laboratory safety and biosafety may be found in a variety of national and international guidelines or codes of practice (National Institute of Health 1987 (Japan); Ministry of Health 1988 (Sweden); Association Française de Normalisation 1990; Standards Australia/Standards New Zealand 2002, US Department of Health and Human Services 1999 (being updated); World Health Organization 2004; UK Advisory Committee on Dangerous Pathogens 1995a, b, 2001; Health Canada 2004.

HISTORICAL PERSPECTIVE – LABORATORY-ACQUIRED INFECTIONS

The risk of infection is higher in virology laboratories than in the general population, although the risk of exposure to infectious agents is somewhat less than among other groups of healthcare workers.

To determine the essential components of a laboratory safety program, it is first necessary to assess the 'risk' of infection associated with working in a virus laboratory. Pike (1979) compiled the best collection of data to date dealing with accidental infections of laboratory workers with pathogenic micro-organisms. The analysis was based on global data relating to laboratory-acquired infections recorded from the beginning of the century. Although much of the data covers the period before the age of chemotherapy and biosafety cabinets, it still gives some idea of the potential risk to people working in laboratories. The study concluded that most of the reported infections occurred mainly in research rather than clinical laboratories. However, the data did not provide any information about the changing pattern of laboratory techniques or the relative importance of different groups of infectious agents responsible for the infections.

Examination of these data, as well as analyzing more recent anecdotal and unpublished information, has demonstrated a major shift away from bacteria and rickettsiae as the chief causes of laboratory-acquired infections. Most such infections reported since the 1970s have been of viral origin especially in research environments working with high threat or newly emerged viruses (see Table 1). The trend is generally a decrease in aerosol infections and an increase in bloodborne diseases associated with technique failures and work associated with animal studies.

The overall decrease in aerosol infections in the laboratory is probably due to the development and introduction of engineering controls (i.e. microbiological safety cabinets); increased awareness of the routes of infection and of the need for safe working practices in the laboratory. The increasing availability of antiviral drugs, as well as the increase in the number of vaccines available, has also played a role in the decrease in illness due to laboratory infections. Despite these general trends, though, laboratory-acquired infections still occur regularly; however, because of the lack of a worldwide reporting system and the absence of adequate medical surveillance, their true extent may never be known. In some countries, for example the UK (Health and Safety Executive 1986), microbiological accidents must be reported.

Table 1 lists some of the ongoing concerns relating to laboratory-acquired infections in virology laboratories reported since 1970. Unfortunately, these are merely examples that emphasize the need for a strong and continuing biosafety program in all virus laboratories. The Subcommittee of Arbovirus Laboratory Safety (1980) collated a number of reports relating to arbovirus incidents involving laboratory workers: Crimean-Congo hemorrhagic fever virus (eight cases, one death); Chikungunya virus (39 cases); Japanese encephalitis virus (22 cases); Argentinian hemorrhagic fever virus (21 cases); Kyasanur Forest disease virus (133 cases); Marburg virus (31 cases, five deaths); Rift Valley fever virus (47 cases); West Nile virus (18 cases); western equine encephalitis virus (seven cases, two deaths); yellow fever virus (38 cases, eight deaths); and numerous others.

The use of vaccinia virus in laboratories has increased in recent years, partly as a consequence of international concerns about the potential use of variola virus (smallpox) as a bioterrorist weapon. Although the vaccine is considered safe, it can produce mild to

Table 1 *Examples of laboratory acquired viral infections*

Agent	References	Country/Date	Laboratory type	Exposure details
Marburg virus	Martini et al. (1969)	Germany 1968 Yugoslavia 1968	Tissue culture	Direct
Lassa virus	Leifer et al. (1970)	USA 1969	Research	Direct
Ebola virus	Emond et al. (1977)	UK 1976	Research	Needlestick while inoculating guinea pigs
Hantaan virus	Lloyd and Jones (1986)	UK 1976	Research	Aerosol
Junin virus	Weissenbacher et al. (1978)	Argentina 1978	Research	Aerosol (?)
Hantaan virus	Desmyter et al. (1983)	Belgium 1983	Animal house	Aerosol
Dengue virus	(Kiley, unpublished)	USA 1988	Research	Direct
Marburg virus	International Society for Infectious Disease (2001)	Russian Federation 1990	Research	Direct
Sabià virus	Ryder and Gordsman (1995)	USA 1994	Research	Aerosol, centrifugation incident
Herpes 'B' virus	Centers for Disease Control and Prevention (1998)	USA 1998	Research	Fatal mucocutaneous exposure
West Nile virus	Campbell and Lanciotti (2002)	USA 2002	Animal house	Scalpel injury during bird necropsy
West Nile virus	Cambell and Lanciotti (2002)	USA 2002		Needlestick while harvesting mouse brains
Severe acute respiratory syndrome (SARS)	World Health Organization (2004a)	Singapore 2002	Research	PhD student working on WNV at BSL3, sample also contained SARs. CoV exposure not identified
Severe acute respiratory syndrome (SARS)	Taiwan Department of Health (2003)	Taiwan 2003	Research	Researcher working on SARS in P4 laboratory. Source of infection unknown
Severe acute respiratory syndrome (SARS)	World Health Organization, (2004)	China 2004	Research	2 research workers (7 associated, 1 fatal) in BSL3 laboratory, exposure incident/s not identified
Ebola virus	(International Society for Infectious Disease 2004a)	USA 2004	Research	Needlestick
Ebola virus	(International Society for Infectious Disease 2004b)	Russian Federation 2004	Research	Needlestick during guinea pig inoculation at BSL4, fatal

moderate disease in vaccines and can be disseminated to their close contacts (Fenner et al. 1988; Spencer and Lightfoot 2001; Centers for Disease Control and Prevention 2002) or through auto-inoculation causing vaccinia keratouveitis (Lee et al. 1994). There have been 18 previously reported cases of laboratory-related vaccinia infection, of which at least three were accidental infections of laboratory workers with recombinant vaccinia (Jones et al. 1986; Openshaw et al. 1991; Mempel et al. 2003) and through the manipulation of vaccinia virus-infected cells (Moussatche et al. 2003). This re-emphasizes the potential risks of human infection associated with the increasing volume of recombinant work.

It should be noted that most of the incidents could have been prevented by the use of common, readily available, biosafety practices and validated training schemes. An example of this was seen during the investigation into the cause of several laboratory-acquired dengue virus infections which occurred over a brief period in the late 1980s. It was determined that each infection was due to entry of the virus through a skin abrasion or wound and that these infections could have been prevented by the use of common safety practices such as covering exposed skin or by wearing gloves and greater appreciation of infectious disease transmission.

COMPONENTS OF A BIOSAFETY PROGRAM

Rules and regulations about the use, management, and disposal of hazardous and carcinogenic chemicals in the laboratory have been in place for some time. The US Fire Protection Agency, US Occupational Safety and Health Administration (OSHA) regulations, UK Health and Safety Executive, and Labour Canada are agencies that enforce the regulations which are backed by law. Before 1989, the criteria for working safely with biological materials consisted of a series of guidelines and recommendations issued by such organizations as the US Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH), the UK Public Health Laboratory Service (PHLS) or professional groups. The Canadian MRC/HWC guidelines were published in 1990.

The recent concern for laboratory safety was generated by the appearance of HIV, as well as the continuing problem of hepatitis B infections among healthcare workers. In addition potential infections from emergent and re-emergent pathogens have led to the adoption of regulations to reduce occupational exposure to bloodborne pathogens. These regulations have taken various forms, such as the UK Management of Health and Safety at Work Regulations 1992, Control of Substances Hazardous to Health Regulations (COSHH) 2002, Categorisation of Biological Agents According to Hazard and Categories of Containment 1995 and 2000, Workplace (Health, Safety and Welfare) Regulations 1992, Safe Working and the Prevention of Infection in Clinical Laboratories 2003; the European Directives to Member States, relating to the protection and exposure of workers to biological agents 1990; the US OSHA Bloodborne Pathogens Standard (29 CFR part 1910.1030); and the Canadian WHIMIS Biosafety Guidelines. They include most of the information in earlier guidelines with recommendations that stress the importance of employee training and education, the use of safety equipment and the duty of care to be provided by an employer ensuring a safe working environment. Although the new regulations cover many areas relating to laboratory safety, they should be viewed only as a starting point. A comprehensive laboratory safety program relating to work with biological agents should ensure that:

- Senior management actively support and resource it.
- Full and proper risk assessment policies and procedures are in place, and take into account the agent (virulence, transmissibility, routes of infection); the work being considered; and the codes of practice needed to prevent exposure.
- Laboratories, their equipment, and other facilities are properly maintained for the safe conduct of the work.
- Laboratories undergo regular safety audits to ensure compliance with agreed protocols and the identification of new hazards.
- Laboratory workers are provided with suitable safety information, safety instruction, and training as part of their continuing professional development.
- An effective infection control program, including monitoring of exposure, health surveillance, and listing of employees working with class 3 and 4 organisms, is developed and implemented.

All staff, regardless of their position, have a duty to protect themselves, their colleagues, and the community from any hazard arising from their work.

Developing a laboratory safety program

Each safety program, while having similar objectives, will vary according to the type of institution involved and any unique features present in the local regulations. Factors to be considered include the scope of the work being undertaken, the level of technical expertise of laboratory staff, the availability of personal protective equipment (laboratory coats and gowns, gloves, eye protection, respiratory protective equipment, ear defenders), and laboratory hardware designed to ensure a safe work environment. Of equal importance is engineering support to ensure the proper installation and maintenance of biosafety cabinets and fume hoods, eye washes, and the proper air balance. Equally important is the establishment of a continually defined program of facility and equipment validation undertaken after the maintenance program that ensures equipment safety standards are being maintained. It is also crucial that staff understand, and use, the correct procedures for operating laboratory equipment.

MICROBIOLOGICAL RISK ASSESSMENT AND INFECTION CONTROL

The exact incidence of occupational infection among laboratory workers is unknown. A number of published reports detail individual or small group outbreaks of laboratory-acquired infections, retrospective studies, and anecdotal information but there has been no report that has succeeded in determining the population at risk for any of these incidents. The overall mortality of reported cases is approximately 4 percent, but this number is probably very high considering the unknown denominator and the great number of unreported illnesses.

All virology laboratories should provide safe working conditions for all staff. In order to accomplish this goal it is essential to understand and determine the possible risks involved in the manipulation and propagation of viruses (including genetically manipulated viruses), the

propagation of viruses in cell cultures, and undertaking viral immunoassays. In addition, the potential hazards of chemicals and radiological products used must be taken into account during the risk assessment to reduce exposure to nonmicrobiological hazards.

It is increasingly recognized that a structured microbiological risk assessment (MRA) is necessary to identify and characterize microbiological hazards associated with laboratory working practices. Such formal risk assessments underpin current legislation in the UK (Advisory Committee on Dangerous Pathogens 1996, Health and Safety Executive 1986); Europe (EC Directive 89/391 2000); Canada (Workplace Hazardous Materials Information System (WHIMIS), 1987), and the USA (OSHA). Although such legislation requires employees to make suitable assessments of risk, they do not offer any formal guidelines on conducting risk analysis. Several agencies have begun to produce risk assessment guidelines on specific viruses: for example, HIV ((UK) Department of Health 1990); hepatitis ((UK) Public Health Service 1992); and bovine spongiform encephalitis ((UK) Health and Safety Executive 1996). For the process to be meaningful, it is important that formal risk analysis be undertaken on laboratory work practices, including the following:

- Risk assessment that identifies the source(s) of the hazard(s) and quantifies the risk of each hazard within the conditions examined.
- Identification of the gravity of the risk, to guide the management of that risk.
- A formal record of the assessment and recommendation of working practice that is communicated to all staff.
- A regular review and corrective action process.
- A corrective action plan.

For any risk assessment to be acceptable, some attempt at measurement or quantification of the risk(s) must be made. This relies on available experimental data, case and epidemiological information, and population studies including mathematical modeling, all of which relate to different aspects of risk.

It is important when establishing an infection control/medical surveillance program that the institution's authorities understand the relative risks that their employees face. This requires information about infectious agents and other potential sources of risk that are likely to be found at their institution. Laboratories should be especially alert if dealing with large numbers and wide varieties of clinical, environmental, or veterinary materials received for laboratory diagnosis or research.

Knowledge of infectious disease processes is the cornerstone of an infection control program. People must be identified whose duties include routine and anticipated tasks involving or potentially involving exposure to infectious material. Particular duties must be evaluated for their 'infectious potential.' It is the job that is rated and not the individual, although anyone who is highly susceptible to infection should be excluded from duties that are not a risk to the normal population. Especially vulnerable are people whose immune system has been compromised, for whatever reason. The infectious potential of any job is usually determined by asking a series of questions about the work involved. For example, does the individual routinely come in contact with virus-containing blood or blood products? Does the individual routinely handle incoming shipments of potentially infectious material? What is the level of experimental animal handling? Other information required may include the type of laboratory equipment that is used, the nature of the materials used, and whether test samples are inactivated.

Once it has been determined that a worker is at risk, a written infection control plan for that position must be considered. This plan should contain an exposure determination (the criteria used to evaluate the position), a schedule, and method for meeting any relevant safety standards and the content of any training to be undertaken. A well-structured and recorded training programme is vitally important to overall laboratory safety. Training should include familiarization with the nature of laboratory pathogens, and proper and safe use of laboratory equipment. A laboratory safety manual (or code of laboratory practice) must be developed and made available to all employees and should include all approved laboratory protocols, as well as a list of duties and responsibilities. One of the responsibilities of any laboratory worker should be to report any incident that might have the potential for an occupational exposure, according to a defined incidence policy.

Any infection control policy should include medical surveillance. This should incorporate a medical evaluation including an occupational/medical history and a physical examination covering conditions that a physician feels might interfere with a worker's ability to use personal protective equipment. It is also essential to ensure that all workers are adequately immunized.

A major goal of an effective medical surveillance policy is to educate workers of the need, and indeed the requirement, to report an occupational exposure. Employees should also have the right to a confidential medical evaluation and follow-up after reporting such an incident. Elements of such a follow-up include documentation of the events surrounding the exposure, the source (if known), and the route of exposure. For incidents involving possible exposure to HIV or Lassa virus, a quick decision may be necessary as to whether to begin azidothymidine (AZT) or other treatment regimen, or ribavirin treatment. It is therefore prudent to have an established policy covering the various possibilities within any establishment handling viruses. Continuing follow-up, including counseling and illness reporting, should also be part of the program.

A surveillance policy designed for reporting illnesses away from the work site should also be developed.

A typical plan should include employees carrying an information card that will notify treating physicians that the patient may be at risk of certain diseases because of his or her occupation. The card would also contain a means for contacting the employee's institution if it is deemed necessary, such as a physician noting symptoms compatible with a disease related to that person's work. The system is important for two reasons:

1. It is essential for an employer to know if more than one employee has contracted a similar disease, because this may be an indication of a common occupational source.
2. Maintenance of a good reporting system helps to determine any source of infection. Experience has taught us that examination of most laboratory accident reports seldom pinpoints the source of occupationally acquired illnesses.

It is evident that a good infection control/medical surveillance program requires well designed and clearly written procedures and policies. It is also essential that the policy be made known to all employees at their induction and any modifications circulated as part of a continuing process of awareness. Results of investigations of laboratory incidents, with a guarantee of anonymity, should be widely published for educational purposes. Laboratory workers should be made to appreciate the ultimate goal and benefit of policy and not be made to fear it.

All workers in microbiology laboratories should provide a baseline serum sample; consideration should be given to establishing a system whereby employees are tested at quarterly intervals for evidence of infection by any agents used in the laboratory, the purpose being to detect silent infections. Although this policy has not been adopted in all parts of the world, knowledge of such subclinical infections can lead to examination of safety procedures, which may result in changes that will eliminate the potential for infections with clinical, and therefore possibly political, consequences. A major laboratory has recently been able to detect silent infections with a virus with known pathogenic relatives that were being studied there. Procedural and protocol changes have eliminated the potential for what could have been a very serious incident.

Definition of hazard groups

The classification of microbial pathogens is based on the inherent hazard of the organism. The corresponding minimum levels of containment set out in Tables 2 and 3 are intended to compensate for microbial risks encountered when working with pathogens as defined in the various hazard groups. 'Hazard' is taken as expressing the level of danger associated with the nature of the organism and 'risk' as the probability that in some circumstances the dangers will be manifested as an infection. The more inherently infectious the microbe, the higher the risk. In reality there are different levels of risk arising from work with viruses, depending on such factors as the immune status of the individual, the dose, the route, and the site of infection.

Categorizing any virus into the currently recognized four hazard groups depends on whether the organism is:

- infectious for people
- hazardous to laboratory workers
- transmissible to the community; and
- susceptible to effective prophylaxis or treatment.

All pathogenic viruses (and other microorganisms) are assigned to one of the higher hazard groups according to the infective hazard they pose for healthy workers (Table 3). The categorization does not allow for any additional risks that an organism can present to people who may be more severely affected for other reasons, such as pre-existing disease, compromised immunity, pregnancy, or the effects of medication.

The hazard group assigned to a particular organism indicates the minimum level of containment under which it must be handled. It should be noted that viruses listed under one category may require a containment level different from that normally assigned. The decision may be influenced by the techniques used (animal infection, virus concentration procedures, or production of an aerosol). It should also be noted that some organisms are subject to monitoring by national departments of agriculture and fisheries.

STANDARD MICROBIOLOGICAL CODE OF SAFETY PRACTICES

The importance of establishing good laboratory practices cannot be overstated. Previous investigations of laboratory accidents indicate that failures in good laboratory practice are frequently the direct cause of accidents. Information derived from the investigation of laboratory accidents, practical experience obtained from working in microbiological laboratories and the application of informed risk assessment have contributed to the development of a list of good working practices.

- Management practice should ensure that all laboratory personnel are competent and understand the biological and other hazards found in a laboratory, and are fully trained to the biohazard level required before undertaking any work.
- The laboratory director should be the individual responsible for granting or limiting access to the laboratory. In general, people who are at increased risk of infection or for whom infection may be particularly dangerous should be excluded from the laboratory. Some other situations, such as a pregnancy, will have to be considered on an individual basis. Children under the age of 16 years should not

Table 2 *Comparative categorization of biological agents and categories of containment*

Country/Organization	Biological hazard classification	Facility designation
Australia	Biosecurity level 1	Biosecurity level 1
	Biosecurity level 2	Biosecurity level 2
	Biosecurity level 2(E)	Biosecurity level 2(E)
	Biosecurity level 3	Biosecurity level 3
	Biosecurity level 3(E)	Biosecurity level 3(E)
	Biosecurity level 3(Z)	Biosecurity level 3(Z)
	Biosecurity level 4	Biosecurity level 4
Canada	Risk group 1	Containment level 1
	Risk group 2	Containment level 2
	Risk group 3	Containment level 3
	Risk group 4	Containment level 4
European Directive	Group 1	Containment level 1
	Group 2	Containment level 2
	Group 3	Containment level 3
	Group 4	Containment level 4
UK	Hazard group 1	Laboratory containment 1
	Hazard group 2	Laboratory containment 2
	Hazard group 3	Laboratory containment 3
	Hazard group 4	Laboratory containment 4
USA	Class 1	Biosafety 1
	Class 2	Biosafety 2
	Class 3	Biosafety 3
	Class 4	Biosafety 4
	Class 5[a]	Biosafety 3 or 4[b]
WHO	Risk group 1	Basic biosafety level 1
	Risk group 2	Basic biosafety level 2
	Risk group 3	Containment biosafety level 3
	Risk group 4	Maximum containment 4

E, exotic; Z, zoonotic.
a) Non-indigenous pathogens.
b) Facility use dependent upon virus and work involved.

be allowed in the laboratory. Animals not involved in laboratory procedures should never be allowed in the laboratory.

- A laboratory code of practice should be available, read, and signed before a new member of staff commences work.
- Laboratories should have a designated biological safety officer and/or a safety committee whose responsibilities include ensuring that all work is carried out in accordance with established safety practices.
- Laboratories must be kept clean, neat, and orderly, and all materials or equipment not pertinent to the work should be kept to the minimum.
- Access to the laboratory must be strictly limited to authorized personnel especially biosafety level

Table 3 *Summary of agent hazard category and associated basis risk assessment*

Hazard group	Agent risk categorization
1	Biological agent unlikely to cause human disease
2	Biological agent that may cause disease and which may be a hazard to laboratory workers, but is unlikely to spread to community. Laboratory exposure rarely produces infection and effective prophylaxis or treatment is usually available. Examples: herpes viruses, paramyxoviruses, picornaviruses, adenoviruses
3	Biological agent can cause severe human disease, and presents a serious hazard to employees. It may present a threat of community spread. Usually an effective prophylaxis or treatment available. Examples include hepatitis B virus (HBV), hantaviruses, Japanese B encephalitis, Rift Valley fever virus, yellow fever virus, rabies virus
4	Biological agent causes severe human infections, serious threat to employees, high risk of community spread, no effective prophylaxis or treatment available. Examples Lassa virus, Ebola virus, smallpox virus, Crimean-Congo hemorrhagic fever virus, Russian spring–summer encephalitis virus

3 (BL3) and 4 laboratories. The laboratory door should remain closed and an international biohazard sign should be prominently displayed on the door, indicating that the room contains potentially infectious material. When an infectious agent (or agents) used in the laboratory requires special provisions for entry (e.g. vaccination), the relevant information must be included in the sign, together with the name of the individual responsible for the laboratory.

- A disinfection policy should be available, regularly reviewed, and displayed.
- Work surfaces should be decontaminated daily, and immediately following any spill involving potentially infectious material.
- All procedures should minimize the production of aerosols. This usually requires the development of standard operating practice and risk assessments for all laboratory procedures, and the timely review of these procedures to ensure that they are abreast of current technology and take advantage of the latest innovations in safety.
- Procedures that have the potential for producing an infectious aerosol should be undertaken within a biosafety cabinet. It is essential that all people who use such cabinets understand the principles of their operation.
- Laboratory coats, gowns, smocks, or uniforms should be worn in the laboratory, and removed before leaving the laboratory for other areas. Although a regular laboratory coat is sufficient for most clinical/diagnostic laboratory situations, a rear-closing, solid-front gown is required when practicing BL3 procedures.
- Face and eye protection or other such devices must be worn when it is necessary to protect from splashes, impacting objects, harmful substances, especially if the work is carried out on the open bench.
- Gloves must be worn for all procedures that might involve contact between broken skin or mucous membranes and potentially infectious material, toxins, or infected animals. This usually means wearing gloves for most laboratory work, including drawing blood. Gloves used in the laboratory are generally made of latex or a vinyl compound (usually polvinylchloride (PVC)), and there seems to be no difference in barrier effectiveness between gloves made of either material. When choosing gloves, consideration should be given to the toxic products (e.g. vinyl chloride and hydrochloric acid) produced when PVC gloves are incinerated.
- Mechanical pipetting devices should be used; mouth pipetting should never be permitted. Modern mechanical pipetting devices are available for almost any laboratory situation.
- Eating, drinking, smoking, application of cosmetics, or storage of food should be strictly prohibited in laboratory work areas.
- Staff must wash their hands immediately after handling any infectious materials or animals and when leaving the laboratory. For this purpose sinks, preferably with foot or elbow controls and located near the exit door, should be provided in each laboratory. Because of the number of times hands are washed it is probably best to use a mild soap with a lanolin base; although these soaps are not disinfectants, their use should remove most surface microbes from the skin.
- All contaminated or infectious liquid or solid materials must be decontaminated before disposal or cleaning for reuse. Contaminated materials that are autoclaved or incinerated at a site away from the laboratory must be packaged and labeled properly for transport and have the outside of the container disinfected chemically.
- Accidents and incidents should be immediately reported to and recorded by the person responsible for the work and to the proper safety and occupational health authorities.
- Appropriate medical surveillance and treatment should be provided.
- Hypodermic needles, syringes and needles, and scalpels, collectively referred as 'sharps,' are the main source of accidents. It is therefore especially important that laboratory workers, as well as others, use and dispose of these items with extreme caution. Needles and syringes should be restricted to parenteral injection and aspiration of fluids from laboratory animals. Only needle-locking syringes or disposable syringe–needle units should be used. Needles should not be recapped or removed by hand; attempts to perform these procedures are a major cause of needlestick injuries. Whenever a person's free hand is near a syringe and there is a needle in the other hand, the potential for an accident is very high. Several recent needlestick injuries have occurred when used syringes, capped or uncapped, were placed in the pocket of a laboratory coat and either the individual wearing the coat or a colleague was subsequently stuck. Hypodermic needles and syringes should not be used as substitutes for pipetting devices in the manipulation of infectious fluids. Once used, needles should be promptly placed in a puncture-proof container and decontaminated, preferably by incineration or autoclaving, before disposal.
- Any soiled 'sharp,' except nondisposable items, should be placed in a sharps disposal container. The sharps container itself must be of rigid construction and leak-proof. The top opening should be large enough so that the used sharps can be dropped into the container without the need to overfill it. These containers should have a top that closes easily and tightly when it is full, and they should be placed at convenient locations throughout the laboratory. When full, a container should be decontaminated by

autoclaving and then it and its contents should be incinerated.

- In modern laboratories instrumentation is a potential source of laboratory accidents. These accidents may involve chemical or electrical hazards or the possibility of infection with a biological agent. Centrifuges – ultra, high speed, or clinical – are always a potential source of aerosols. Care should be taken to load and unload centrifuge tubes containing infectious or harmful material in a biosafety cabinet. It is also prudent to use sealed tubes or safety heads while centrifuging such dangerous material. Automated analyzers are another potential source of danger in the laboratory. Blood or serum samples are often introduced into the instrument under pressure, which can increase the potential for aerosolization. The level of containment of such equipment will depend on the risk assessment and the infectious nature of the material.
- A program for insect and rodent control should be in place.
- If an autoclave is not present in the laboratory, all potentially infectious material for decontamination must be taken out of the laboratory in containers that preclude both aerosolization and leakage of the contents.

BIOSAFETY LABORATORIES

The design or refurbishment of virology laboratories increasingly has to respond to an increasingly national and global regulatory environment, and advances in scientific, technical, and industrial biosafety capabilities (Advisory Committee on Dangerous Pathogens 2001; Health Canada 2004; US Department of Health and Human Services 1999; World Health Organization 2004). Primary and secondary containment practices are increasingly more complex and their introduction varies throughout the world. There is a need to establish a common system for the implementation of common standards for future design and operation of virology laboratories that ensures worker and public confidence in biocontainment and biosafety strategies employed. Laboratory operation should also introduce a detailed record system for the monitoring and revalidation of facilities, equipment, and plant which should become more detailed the higher the biosafety level (BSL) category.

Working with potentially infectious viruses in the microbiological laboratory requires an appreciation both of work practices and of the facilities needed to make such laboratories a safe environment. Guidelines on the safe handling of pathogens have evolved over the past decade and have reached the point where there is general agreement on the basic principles of biosafety in the laboratory (Table 4). Four biosafety levels have been described (BL1–BL4), which comprise combinations of laboratory practices and procedures, safety equipment, and laboratory facilities appropriate for the operations performed and the hazard posed by the infectious agent. Essentially, the numerical designation of a safety level increases with an increase in the risks involved in working with an agent.

The levels of containment are intended to reflect the risks encountered when working with biological agents as categorized in the various hazard groups. All laboratories in which there is likely to be exposure to human

Table 4 *General summary of relation between hazard groups and biosafety level, work practices, and containment level*

Hazard group	Biosafety level (BL)	Working practices and procedures (use)	Primary engineering control requirements
1	BL1	Standard microbiological practices (SMP)	No special engineering requirements; containment achieved by adherence to standard laboratory practice
2	BL2	BL1 work practice, plus:	
		Limit access. Limit aerosols. Biohazard signs. Laboratory code of practice. Protective personal equipment (PPE): gloves, laboratory coats, face protection as needed. Infectious waste decontaminated	Procedures that produce potentially infectious aerosols performed in a biological safety cabinet (BSC). On-site autoclave required
3	BL3	BL2 work practice, plus:	
		All virus material contained. Restricted access. PPE: gloves, protective laboratory clothing, respiratory protection as needed	Inward airflow. Air-lock facility. Exhaust air discharged from laboratory via HEPA filtration (optional). On-site/ integrated autoclave required
4	BL4 (maximum containment)	BL3 work practice, plus	
		Change of clothing before entry. Shower on exit. All waste autoclaved before leaving facility. Regular engineering and biosafety maintenance	HEPA-filtered supply and exhaust air. Work undertaken in BSC class III cabinets or BSC II using an air-supplied positive full-body pressure suit in an air-tight laboratory. Integrated laboratory required. All waste decontaminated

pathogens (whether or not they are to be propagated or concentrated) require a minimum of BL2 containment. When there is a likelihood or strong indication that material (specimens or samples) contains BL3 or BL4 agents, additional controls are necessary.

Many national guidelines list the essential laboratory procedures that are basic to good laboratory practice (Table 5). It must be emphasized that good microbiological practice is fundamental to laboratory safety and cannot be replaced by specialized equipment or safety barriers, which can only supplement it.

It should be noted that although these classifications may be reasonable for clinical and research laboratories, they are not applicable to many industrial, food science, and agricultural laboratories.

Rules associated with animal facilities parallel those for laboratories. Additional considerations should be given to the requirements of individual species that take into account behavior, husbandry requirements, housing standards, zoonoses, parasites, and size (Advisory Committee on Dangerous Pathogens 1997; Richmond and Quimby 1999).

Biosafety level 1

BL1 practices, safety equipment, and laboratory facilities require no special engineering features for work with organisms that present no danger to humans. Examples of laboratories that may be operated at this level include undergraduate training and teaching laboratories. In these laboratories work is undertaken with well-characterized agents that are most unlikely to cause disease in normal healthy adult humans. Many agents not normally able to produce disease in healthy individuals may produce severe illness in elderly people, the immunodeficient, or the immunosuppressed, and can cause allergic or toxogenic responses, so appropriate precautions must be taken. Also, vaccine strains that, because they are attenuated in virulence, do not usually produce disease in healthy individuals may produce severe disease in some individuals. The latter two categories of organisms should be manipulated under BL2 conditions.

Work is generally conducted on the bench, and specialized containment equipment is not normally

Table 5 *Exposure controls: summary of minimum containment requirements for laboratories and animal rooms*

	Containment levels			
Containment requirements	**1**	**2**	**3**	**4**
Laboratory site: separated from other activities in the building	No	No	Yes	Yes
Facility: sealable	No	No	Yes	Yes
Ventilation				
Workplace maintained at air pressure negative to atmosphere	Opt	Opt	Yes	Yes
Through safety cabinet	No	Opt	Opt	No
Mechanical: direct	No	Opt	Opt	No
Mechanical: independent	No	No	Opt	Yes
Supply and extract air HEPA filtered	No	No	Yes, on extract	Yes on input and double on extract
Airlock	No	No	Yes	Yes
Airlock: with shower	No	No	No/Opt	Yes
Effluent treatment	No	No	Yes	Yes
Autoclave site				
On site	No	No	No	No
En suite	No	Yes	Yes	Yes
In laboratory: free-standing	No	No	Opt	No
In laboratory: double-ended	No	No	Rec	Yes
Microbiological safety cabinet/enclosure	No	Opt	Yes	Yes
Biosafety cabinet	NR	I	I/III	III I/II[a]
Facility to contain own equipment	NR	No	Yes as far as reasonably practical	Yes
Safe storage of biological material		Yes	Yes, secure storage	Yes, secure storage
Efficient vector control, e.g. rodents and insects		Yes, for animal containment	Yes, for animal containment	Yes

NR, not required; Opt, optional; Rec, recommended.

a) Class I and II biological safety cabinets permitted with use of positive-pressure suits.

required. Laboratory personnel should have specific training in the procedures undertaken within the laboratory and be supervised by experienced senior staff. The following laboratory facilities and basic work practices apply to viruses assigned to BL1.

LABORATORY FACILITIES

In the interests of safety, floors should be slip resistant, seamless, be impermeable to liquids, and resistant to most if not all chemicals. Laboratories should be designed so that the floors, walls, and ceilings are easy to clean. Benchtops should be impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat. Laboratory furniture should be of similar resistant materials and be arranged so as not to obstruct workers while performing their duties. Equipment should be placed where it is easily accessible for cleaning and, if it is likely to produce an aerosol, in the safest possible location. Attention should be paid to door swings because a door opening in the wrong direction has the potential to knock items from someone's hands. Equipment must not be placed near thermostats, because it may interfere with temperature control. Floors should be nonskid, to reduce the likelihood of slipping. Hand-washing facilities must be provided, preferably near the point of exit.

Biosafety level 2

Conditions described for BL2 are usually applicable to clinical, diagnostic, research, and teaching facilities in which work is done with a wide variety of microorganisms that cause human disease and may be a hazard to laboratory workers but unlikely to spread to the community. Laboratory exposure rarely produces infection, and effective prophylaxis or treatment is usually available. Many of the agents that require BL2 containment are already present in the community, and community vaccination programs for them are routine.

Hepatitis A virus, herpes simplex types 1 and 2, measles virus, Epstein–Barr virus, and certain viral vaccines are among those that can be manipulated at the BL2 level. Generally the agents that are assigned to this level cause bloodborne diseases and do not have a history of infection via the respiratory route. It is not surprising, then, that the usual routes of laboratory-acquired infections with agents in this group are auto-inoculation, ingestion, and mucous membrane exposure to the infectious agent. Procedures with agents assigned to this level that have a high probability of aerosol production should be conducted in primary containment equipment (e.g. Class 2 biosafety cabinet) or similar devices.

The competence expected of laboratory workers is greater than that of a newly qualified graduate. Personnel at this level need to be familiar with the institution's safety policy, to have received training in the handling of the viruses and be supervised by a competent senior member of staff.

LABORATORY DESIGN

The laboratory requiring BL2 containment is generally similar in construction and design to a BL1 facility. It also incorporates criteria for microbiological practices in association with additional primary and secondary containment facilities (Figure 1).

The equipment is selected according to the following principles:

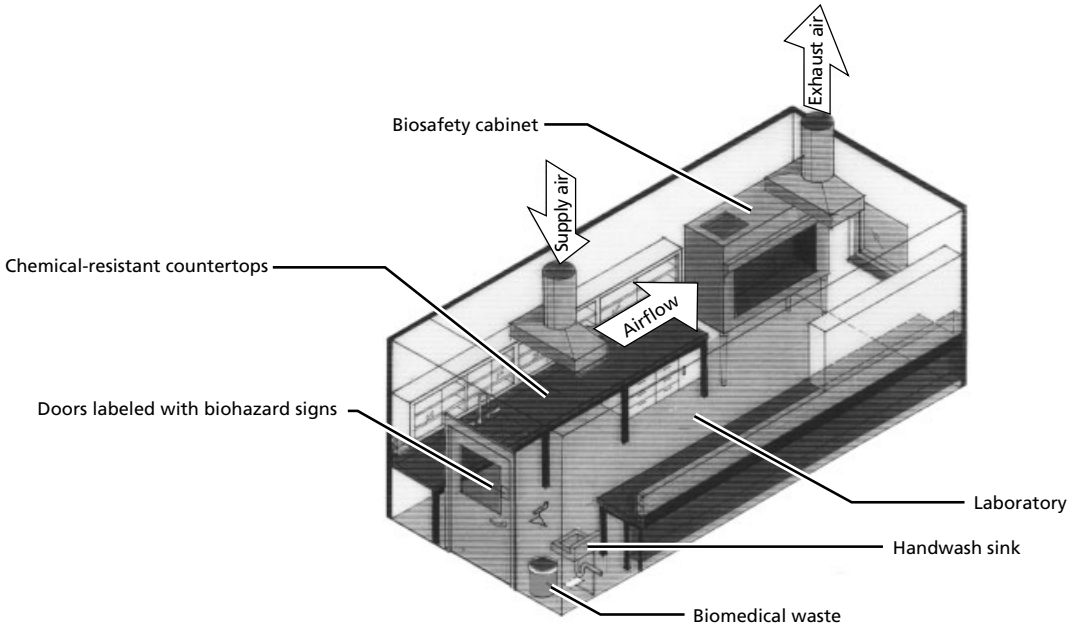

Figure 1 *A schematic of a generic BL2 laboratory design (courtesy of Smith Carter, architects, engineers, planners, interior designers).*

- Designed to prevent or limit exposure of the operator to infectious material.
- Constructed of materials that are resistant to corrosion and meet structural requirements.
- Installed to aid operation, maintenance, cleaning, decontamination, and safety testing.
- Complies with safety specifications.

Laboratories that anticipate working with potentially infectious material should use a biosafety cabinet, isolator, or other suitable containment. Standard laboratory working practice is undertaken at this level, with additional features that reflect the higher containment level (see Tables 3, 4, and 5). Procedures with the potential for creating infectious aerosols should be conducted within a biosafety containment cabinet. These include grinding, blending, vigorous shaking or mixing, sonic disruption, and opening containers of infectious materials. Intranasal inoculation of animals and harvesting infectious tissues from animals or eggs should also be done in a biosafety cabinet or equivalent. Any work with high concentrations of infectious agents should be carried out in a containment cabinet. The reduction of aerosols is also a consideration when working with many of the analytical machines found in any virology laboratory. Special precautions should be taken with machines such as cell sorters and when loading analytical machines where the sample may be under pressure and more likely to produce an aerosol. In many cases a splash-guard may suffice.

There may be occasions when agents routinely manipulated at BL2 may require BL3 containment, or at least BL3 practices. This usually occurs when the procedures produce concentrations significantly exceeding the physiological concentration of the agent in nature. A higher biosafety level should also be considered for studies involving animals.

Biosafety level 3

All the laboratory design features and working practices required for BL2 are applicable to BL3, with special emphasis on some of them. The work practices, safety equipment and facilities for BL3 apply when working with indigenous or exotic agents and when aerosol infection is a significant possibility resulting in disease that may have serious, or even lethal, consequences. Agents for which BL3 safeguards are routinely recommended include *Hantavirus* (Hantaan virus, Seoul virus, Sin Nombre virus) and St. Louis encephalitis virus. Two of the more exotic agents that require BL3 containment are Rift Valley fever virus and yellow fever virus, hemorrhagic fever viruses from Africa that can be worked with at this level provided workers are immunized.

Although auto-inoculation, ingestion, and mucous membrane contamination are hazards with these agents, the major concern is aerosol infection. The requirements for BL3 are more stringent than those for BL2 and are aimed at controlling aerosol production and possible spread into the community.

The laboratory staff must understand the safety procedures, as well as the risks involved in working with the agents used in their laboratory. Training, including the use of specialized equipment, should be done by competent scientists who have experience of working with the viruses contained at this level.

LABORATORY DESIGN

The laboratory has engineering and design features and physical containment equipment additional to those found at BL2 (see Tables 4 and 5). Principal differences from BL2 requirements are that the laboratory must be separated from other laboratory space by an anteroom and that personnel access is strictly controlled. The laboratory must be maintained at an air pressure negative to the atmosphere (maintain a verifiable inward directional air flow) and extracted air must be high efficiency particulate air (HEPA)-filtered (in some countries) and discharged to the outside so that it is dispersed clear of adjacent buildings or air intakes (Figure 2). Additional aerosol protection when working with animals is afforded by a variety of newly designed flexible-film isolators (Figure 3).

LABORATORY PRACTICES

All work involving infectious material is undertaken within biosafety cabinets by staff wearing full frontal gowns, gloves, and eye protection. Alternatively, some laboratories prefer to use class III cabinets. Contaminated solid materials that are to be decontaminated at a site away from the laboratory are sealed in a leak-proof container before being removed to an autoclave and then incineration. Contaminated liquids are autoclaved or disinfected in the laboratory area. An autoclave should be available in the laboratory or laboratory suite.

Biosafety level 4

This containment level is to be used when working with viruses that cause severe human disease (some with a high mortality rate), and present a serious hazard to laboratory workers for which there is no vaccine or generally no reliable treatment. A variety of agents causing hemorrhagic fevers, including Lassa virus, Marburg virus, Ebola virus, Crimean-Congo hemorrhagic fever virus, and related viruses, are those most commonly studied at BL4.

LABORATORY DESIGN

The laboratory is either a separate building or an isolated zone equivalent to a separate building within a building. Mainly because of their high construction and

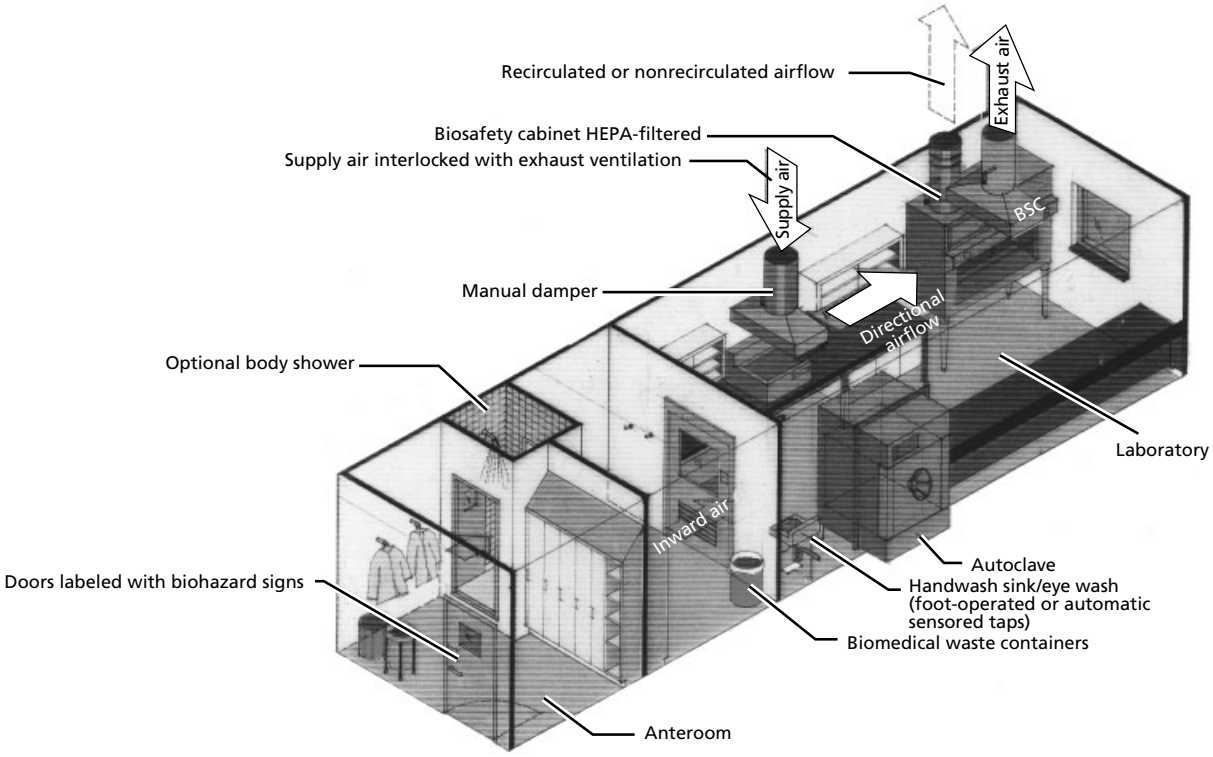

Figure 2 *A schematic of a generic BL3 laboratory design (courtesy of Smith Carter, architects, engineers, planners, interior designers).*

operational costs, there are fewer than ten BL4 laboratories in the world.

There are two operational designs that provide efficient primary containment. The first and oldest system is based on protecting the worker from exposure by containing the agent within a series of interconnecting airtight class III biosafety cabinets (Figure 4).

Each cabinet is equipped with its own HEPA-filtered air supply and extracts exhaust air via HEPA filters in series, and can be independently sealed and fumigated without interference with the rest of the system. All equipment (refrigerators, freezers, microscopes, centrifuges, and incubators) is built as an integral part of the system. All staff has a complete change of clothing and shower after each day's work. All work is conducted through glove ports. The cabinet system is connected to a double-ended high-containment autoclave, through which all liquid and solid waste is decontaminated before being removed for incineration. A chemical 'dunk tank' or formaldeclave is located at the other end of the system, through which suitably packaged material can be taken in or out of the system. The whole system is incorporated within a laboratory providing environmental protection and operating an interlocking HEPA-filtered supply and exhaust air system.

The alternative system, 'the suit laboratory' isolates the workers from the laboratory environment by means of fully encapsulating suits supplied with air from outside the laboratory (Figure 5). By the use of special construction techniques (impermeable surfaces and welded ductwork) the laboratory facility provides the biosafety barrier that ensures the protection of the exterior environment. Work with infectious material is carried out within class II biosafety cabinets. Air from the room and biosafety cabinets is removed via HEPA filtration, the HEPA filters are also an integral component of the supply air ductwork. A special chemical shower must be provided to decontaminate the surface of the suit before the worker leaves the area. A double-doored autoclave is provided for decontaminating waste materials to be removed from the suit area. When necessary, appropriate material may be removed from the laboratory, without heat treatment, by proper packaging and removal through the chemical shower or a dunk tank.

Laboratory practices

Basic work practices are undertaken in the same manner in both systems. Because of the complexity of the work, a detailed code of practice should be developed and form part of an individualized training program. On entering, staff should put on a complete change of clothing; before leaving, they must shower before putting on their own clothing. Entry and exit of personnel and supplies must only be through an airlock or pass-through system. All fluid effluents from the facility, including shower water, must be rendered safe before final release.

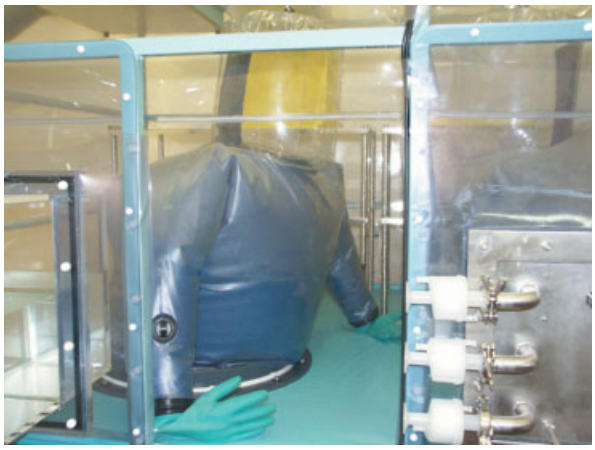

Figure 3 *Flexible-film isolator design for animal containment level 3 and 4 work (courtesy of Health Protection Agency, UK).*

Figure 4 *Cabinet-line BL4 laboratory (courtesy of Health Protection Agency, UK).*

A regular, rigorous, preventative maintenance program for the engineering controls, air flow, autoclave, safety cabinets, etc., should be developed to ensure continued integration of the facilities and suitability of the safety procedures. Within the UK the Health and Safety Commission institute regular safety inspections of the facilities, safety management, and procedures.

LABORATORY WASTE

The general principle involved in the treatment of laboratory waste is that all infectious or potentially infectious laboratory waste must be rendered noninfectious before leaving the laboratory. An alternative is to develop a system whereby infectious material can be safely delivered to an off-site disposal facility. A robust disinfection and waste policy is regarded as an operational necessity at this level.

In general, local regulations cover the disposal of infectious waste. There are different interpretations of what constitutes 'infectious waste,' so this must be considered when developing a waste management program. A program designed to minimize the production of regulated waste will reap cost savings because disposal of regulated waste is far more costly.

PACKING AND SHIPPING OF INFECTIOUS MATERIALS

The safe shipment of infectious substances or diagnostic specimens within or between countries is important for everyone involved with the process, basic guidance being provided by the World Health Organization (1997). Although no reports of illness have been attributed to the transportation of such materials, there are numerous areas of conflicting guidelines throughout the world. Because these packages have the potential for harboring infectious agents and move in public places during their journey, their transportation is strictly regulated. Postal and transport authorities have always been aware of the potential hazards to their employees of infectious material transported and handled by them.

In many countries such material is sent through the mail, and local regulations apply; guidelines are often issued (e.g. the *Post Office Guide* in the UK and the *Postal Service Manual* in the USA). The USPS requirements for the mailing of 'diseased tissue, blood, serum, and cultures of pathogenic microorganisms' are listed in their Postal Service Manuals.

The US Department of Transportation, the UK's Health and Safety Executive, and Canada's Transport Canada have major responsibility for safe transportation and provide the authorization for the movement of hazardous materials within their respective countries and to foreign countries. The importation of infectious agents that are predominately pathogenic to animals also needs to take into account various national regulations and permit requirements (UK: Importation of Animal Pathogens Order 1980, 1998; Canada: Animal Disease Protection Act; USA: USDA, APHIS 9 CFR Parts 94 and 95). Workers sending infectious material to other laboratories should ensure that these laboratories have the facilities to work with the agent. This applies particularly to the transfer of viruses in hazard groups 3 and 4. In response to the anthrax attacks in the USA during 2001, the USA (2002 42 CFR Part 73) and UK (Home Office 2001) introduced antiterrorism legislation which made laboratories and their personnel accountable and responsible for possessing, working with, and transferring high-threat pathogens known as 'selected agents.'

An agreement between the Universal Postal Union (UPU), the International Air Transport Association (IATA), and the International Civil Aviation Organization (ICAO) led to new regulations for overseas mail in the 1990s. These are currently being modified and due for publication in 2004/5. The IATA/ICAO guidelines and procedures also should be considered because they were designed to facilitate the safe shipment of

Figure 5 *BL4 suit laboratory (courtesy of Lyon CL4 facility, France).*

infectious substances while ensuring the safety of the transport personnel and the general public. The regulations define the terms 'diagnostic specimen,' 'biological product' and 'etiological agent,' and give minimum packaging requirements and volume limits for each.

The regulations define a diagnostic specimen as 'any human or animal material including, but not limited to, excreta, secretions, blood and its components, tissue, and tissue fluids, being shipped for the purposes of diagnosis (excluding live infected animals).' These must be transported under ICAO/IATA packing instruction 602. Infectious substances are 'substances containing viable microorganisms or toxins known or suspected to cause diseases in animals or humans (including HIV)' and are transported according to ICAO/IATA packing instruction 650.

With regard to packaging, all guidelines indicate that no one may knowingly transport or cause to be transported, directly or indirectly, any diagnostic specimens or biological product which they reasonably believe may contain an etiological agent unless such material is packaged to withstand leakage of contents, shocks, pressure changes, and other conditions incident to ordinary handling in transportation. The major decision to be made, then, when determining the transport requirements for any sample is whether it contains an etiological agent. It is prudent to be conservative in this matter and assume that each specimen may contain an agent unless it is certain that it does not.

The current packaging requirement is for triple packaging. A securely closed, watertight primary container is enclosed in a second, durable watertight container, with enough material in the space between them to absorb all the contents of the primary container in the event of leakage. Each set of primary and secondary containers is then enclosed in an outer shipping container constructed of fiberboard, cardboard, wood, or other material of equivalent strength. An etiological agent/biomedical material sticker is required for the outer mailing package. Any packaging requiring solid carbon dioxide (dry ice) should ensure that it permits the release of carbon dioxide gas to prevent the build-up of pressure.

Although on 1 January 1995, IATA (a voluntary organization of air carriers) issued requirements for packaging, and has established a certification procedure for packaging companies who wish to provide packages to ship 'infectious material' on aircraft, the issues of updated guidelines is expected in 2004/5. Certification of packaging will continue to be a requirement. With the increased concern surrounding the transport of infectious material internationally and nationally, individuals should recognize that the importation and export of infectious materials requires the approval of a number of government departments and biosafety and biosecurity agencies.

SUMMARY

An increasing variety of publications already covers the many topics involved in general safety, laboratory safety, and biosafety. In this chapter we have tried to make virologists aware of the range of guidelines and regulations that affect work in a virology laboratory. We have also attempted to present the issue of biosafety from the view of the scientist. It is increasingly recognized that virological scientific programs need to be conducted in an efficient, safe, and secure manner. Because it is the scientists who should have the best knowledge of the nature of the agents under investigation, including factors such as pathogenic potential, they should play a pivotal role in the development of continuing biosafety policy.

The biosafety professionals have knowledge of the guidelines and regulations that apply to the operation of a virology laboratory. They also have knowledge of the latest in validated biosafety technology and practices that have been developed around the world. In addition, occupational safety and health professionals generally have information in such areas as transportation of specimens and requirements for importation of agents, which can help the laboratory function more efficiently. Increasingly it is recognized that scientists need to be responsible and accountable for the agents they work with and provide both the public and regulatory agencies evidence-based information that work is conducted within appropriately designed containment systems.

With the current expansion of high containment laboratories throughout the world it is essential that international cooperation and consensus is established in facility design, commissioning, operation, and maintenance in accordance with our understanding of infectious disease processes. Equally the establishment of well-managed biosafety training programs address the 'duty of care' required. The operational integrity of

these facilities will be dependent upon the establishment of good working relationships between scientists, specialist engineers, and biosafety professionals being established. This will ensure that the primary mission of the laboratory, the research, developmental and detection programs, can be conducted efficiently, safely, and securely.

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The laboratory diagnosis of viral infections

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During the past three decades the diagnostic virology laboratory has emerged as an important adjunct to patient care. This is a result of several factors, including enhanced technology that has allowed for more accurate, useful and rapid detection of viruses, improvements in patient management resulting from increased understanding of viral pathophysiology, and the development of a growing arsenal of antiviral chemotherapeutic agents. Molecular biology and antigen detection techniques have emerged as the methods of choice for rapid viral detection in many cases. This often allows detection and quantitation of virus in a matter of a few hours, resulting in direct application of collected results to patient healthcare. The viral diagnostic laboratory now has a vast array of methods to assist in diagnosis, including cytology, cell culture, antigen detection, electron microscopy, nucleic acid detection, measurement of antibodies, and, most recently, antiviral susceptibility testing. These techniques are facilitated by the development of specific reagents, test kits, and automated instruments, many of which are commercially available, as well as published algorithms for how to test for specific viral agents.

In an age when administrators are examining all costs, the performance of viral testing is often driven by outcomes assessment. The laboratory is now able to provide data that lead to cost savings related to:

- number of hospital days saved due to rapid and accurate diagnosis
- decrease in unnecessary antibiotic use
- improved hospital infection control
- application of specific antiviral therapy or other patient management issues.

These all have become valid reasons for performing testing, in addition to the age-old standards of collecting epidemiologic information and screening the blood supply.

Thus, this chapter strives to assess the methods currently in use for their importance in achieving viral diagnoses, with little comment on the actual manner in which these tests are performed. For this reason many of the historically important procedures may only receive passing mention.

PRINCIPLES OF DIAGNOSIS

In the course of virus infection it is possible to detect virus, viral antigens, nucleic acids, or antibodies to the virus as a means of identification of the etiologic agent. A good understanding of viral pathophysiology and host response is needed in order to determine the most appropriate specimen(s) to collect and the best type of testing to perform. During the viral incubation period, which can last from 1 day to months or even years depending on the virus, virus or viral subunits are the best targets for detection methods. This is also a period when virus may be found in blood or other body fluids

in some infections. By the time signs or symptoms of disease become apparent, virus is usually present in the target tissues but may be more readily isolated or otherwise detected from other tissues. For example, enteroviruses are more easily detected in stool than in the cerebrospinal fluid (CSF) in infections of the central nervous system (CNS). Collection of a serum sample early in infection is also important since the rise in antibody titers in later specimen(s) is often a useful way to identify a cause of infection. For serological identification of virus it is necessary to either test for IgM antibodies as an indicator of recent infection or to look for a significant titer rise. This must be at least a two-dilution increase to limit the possibility that technical error is a factor (most commonly a fourfold rise, since use of twofold dilutions is mostly performed). For persistent infections or late infection it may be difficult to isolate virus; thus, detection of viral antigen or nucleic acids may be easier than virus.

Specimens

Collection and submission to the laboratory of an appropriate sample is a vital factor in successful diagnosis. Ideally one should know exactly what is necessary to collect and submit specimens to a particular laboratory to optimize results (Smith 2000) (Table 1). This is best accomplished by contacting the laboratory to which the specimen will be submitted for direction.

Specimens should be collected and submitted to the laboratory as rapidly as possible after the recognition of a clinical problem. Failure to get the laboratory a good specimen is the greatest contributor to improper results. The suspected etiology(ies) should help guide the choice of specimen site(s). Not all specimens are directly related to the clinical presentation and knowledge of the most appropriate specimen(s) for each condition as well as the laboratory that is going to be used are important. It is best to err on the side of too much sample, if the amount of material needed is in question, as most laboratories do not complain about too much material as compared to too little. Of similar importance is the submission of vital supportive data on the label of the container for the specimen including patient name, identification number, specimen source, date and time of collection, and ordering physician. Each specimen should be accompanied by a requisition form with identical information as the specimen label plus other useful information such as clinical diagnosis and suspected virus(es).

Another important factor for successful laboratory diagnosis is transport of the specimen to the laboratory. A number of commercial media are available to preserve specimens during transport including Culturettes, Micro Test Multi-Microbe (M4-3), Virocult, Viral Transport Medium (VTM) and ViraTrans. While these all-purpose transport media are usually sufficient for the transport of most specimens, occasionally a specific specimen type may require special medium (Johnson 1990).

Table 1 *Specimens useful for viral studies*

Specimen type	Disease category (examples)
Nasopharyngeal wash or aspirate	Respiratory tract infection Certain exanthems CNS infections (especially enteroviruses)
Throat wash or swab	Respiratory tract infection
Sputum	Respiratory infection (HSV, CMV)
Bronchoalveolar lavage	Lower respiratory infection (CMV, influenza virus, parainfluenza virus)
Rectal swab or stool (5 mg)	Gastroenteritis and enterovirus infections
Cerebrospinal fluid	Meningitis, encephalitis
Brain biopsy	Meningitis, encephalitis (HSV, *Rabies virus*)
Blood	CMV, HIV, HBV, HCV
Vesicle fluid or lesion scraping	HSV, VZV, HPV
Endocervical swab	Genital infections (HPV, HSV)
Swab (eye)	Ocular infections (adenovirus, HSV)
Urine	CMV, BK virus, mumps virus
Serum	Antibody studies (most viruses)

DETECTION OF VIRIONS OR THEIR COMPONENTS

Cytopathology

One of the oldest and most rapid methods of detecting viruses is to directly examine stained clinical material using light microscopy. Cytological alterations that are pathognomonic for specific viruses can be readily visualized in virus-infected tissues and cells (Table 2). More often, the examination of material by microscopy may only raise a suspicion of viral infection or may limit detection to a viral family, requiring more specific methods for viral identification. Nevertheless, these rapid examinations may quickly rule in or out viral infection. Cytological staining using Giemsa (Tzanck preparation), Papanicalaou (Pap) or Wright stain, while rapid, has relatively low sensitivity when compared with modern methods of antigen or nucleic acid detection. Inability to distinguish herpes simplex virus (HSV) from varicella-zoster virus (VZV) somewhat limits the utility of Tzanck preparations.

The most commonly used viral cytology procedure is the Pap stain of cervical scrapings to detect premalignant lesions. Since its implementation nearly 50 years ago, Pap staining has helped to dramatically decrease the incidence of cervical cancer. The most common

Table 2 *Cytopathology of viral infections*

Virus	Clinical presentation	Inclusion bodies/Cytologic finding
Adenovirus	Hemorrhagic cystitis	Intranuclear, basophilic
	Pneumonia, upper respiratory tract infection	Intranuclear, eosinophilic (early); basophilic (late)
	Acute keratoconjunctivitis	Intranuclear, eosinophilic (early); basophilic (late)
BK polyoma virus	Urinary tract	Intranuclear, mucoid (early); cytoplasmic, dense, full, basophilic (late)
CMV	Urinary tract	Intranuclear, basophilic surrounded by halo (owl eye)
		Intracytoplasmic, eosinophilic
	Pneumonia	Cytomegaly, small intracytoplasmic, eosinophilic
HSV	Tracheobronchitis	Large ground-glass nucleus (early); intranuclear, eosinophilic (late); multinuclear cells
	Local cystitis, generalized infection	Large ground-glass nucleus (early); intranuclear, eosinophilic (late)
	Herpes genitalis	Multinuclear cells
HPV	Cervical dysplasia, condyloma accuminatum	Enlarged nucleus (hyperchromatic), intranuclear, basophilic (rare)
		Koilocytes (perivascular cytoplasmic clearing and vacuolar degeneration)
Measles virus	Nasal secretions in prodrome	Lymphocytic nuclei in mulberry-like clusters
	Pneumonia	Intranuclear and intracytoplasmic, eosinophilic; multinucleated giant cells
	Exanthem	Intracytoplasmic; multinucleated giant cells
Molluscum contagiosum virus	Genital lesions	Intracytoplasmic, large, dense staining, nucleus displaced bean-shaped squamous cells
	Papule on eyelid or conjunctiva	Intracytoplasmic, basophilic, dense; nucleus displaced
Parainfluenza virus	Bronchiolitis, pneumonia	Intracytoplasmic, eosinophilic; cytomegaly; single or multiple nuclei
RSV	Tracheobronchitis, pneumonia	Intracytoplasmic, basophilic, large multinucleated cells
VZV	Chickenpox, shingles	Intranuclear, eosinophilic, multinucleated cells

abnormal findings using the Pap test are atypical squamous cells of undetermined significance (ASCUS) and atypical glandular cells of undetermined significance (AGUS), which are associated with human papillomavirus (HPV) infection. In follow-up, HPV DNA testing can be useful for identifying women who are more likely to have underlying high-grade squamous intraepithelial lesions (Solomon et al. 2001). The management of patients with equivocal Pap tests is a clinically debated topic and has led to the re-evaluation of the need for this test and development of new clinical paradigms, which may soon preclude the common use of this procedure in favor of molecular tests for HPV. Smith and Yassin (2000) have written a chapter elsewhere that details many of the uses for cytology in viral diagnosis.

Electron microscopy

Electron microscopy (EM) can be used to directly examine specimens for the presence of viral particles. Most commonly, negative-staining methods are used to directly visualize viruses. EM is particularly helpful when trying to detect non-cultivatable or fastidious viruses, although high concentrations are required (Chrystie 1996; Petric and Szymanski 2000). It is rapid and relatively inexpensive, provided an electron microscope is available to the laboratory. In recent times, the emergence of new viral agents has emphasized the value of EM, notably for Ebola virus, hantaviruses, and other agents causing viral hemorrhagic fevers. It has also been suggested that EM could play an important part in early identification of an etiologic agent in a biological warfare or bioterrorist attack. The major limitations of EM are the high cost of the microscope, the expertise needed, and the frequent lack of precise identification of the virus detected, as well as low sensitivity and specificity. Successful detection requires the presence of 10^5 or greater particles/ml. EM is effective for identification of virus morphologically by family, which may be sufficient clinically in many cases, and can be enhanced by the use of antibodies (immune EM), which permits identification of specific viruses.

The rapidity of EM and ability to visualize the actual virus particle are the major advantages of this technique.

Negative staining of liquid specimens can be accomplished in under an hour and specimens that need concentrating by ultracentrifugation, agar diffusion, or pseudoreplica methods can be done in about 2 hours. In solid tissues the specimens would require thin sectioning and this would result in a 2-day process to accommodate the steps of fixation, staining, embedding, and then sectioning. EM is particularly useful for gastrointestinal infections, as virions are often in high concentration. Other specimens, such as urine or CSF, usually require concentration.

Detection and quantitation of viral antigens

Immunological methods are highly effective for detection of viral antigens. They offer a high degree of sensitivity and specificity, are rapid, and the costs are reasonable. Availability of commercial reagents facilitates the detection of many agents. Virtually all methods use antibodies that are tagged with a fluorochrome, enzyme, or radiolabel. Radioimmunoassay is used to a much lesser extent today due to issues of exposure to radioisotopes and the high cost of disposal of radioactivity.

IMMUNOFLUORESCENCE

The direct staining of clinical specimens using monoclonal or polyclonal antibodies bound to fluorescent dye is extensively used in many laboratories for the detection of a wide variety of viral antigens. This may be accomplished using either direct or indirect techniques. In direct immunofluorescence (IF), a single fluorochrome-labeled antibody is used. Indirect IF uses two antibodies, one specific for the antigen under scrutiny and a second fluorochrome-labeled antibody to the immunoglobulin (species specific) used as the first antibody. Indirect IF offers a few advantages, including increased sensitivity and one reagent (the second antibody) that can be used for testing for many viruses. There are many uses for IF including direct staining of smears, cells present in body fluids, tissues, and applications of flow cytometry or cell sorting (McSharry 2000; Schutzbank and McGuire 2000; Madeley and Peiris 2002).

IF has several distinct advantages: it is rapid, simple to perform, and does not require live virus. Results in some cases can be obtained in 1–2 hours, thus impacting on patient management, need for hospitalization, length of hospital stay, and mortality. Specimen requirements are simpler than those for viral culture since maintenance of live virus is not needed. In addition, the visualization of results allows for determination of specificity, examination of the appropriateness of the specimen, and specimen quality. The quality of a specimen is a critically important factor in viral diagnosis and has come under greater scrutiny by regulatory agencies in recent years. Determining that specimens contain sufficient numbers of cells, proper cell types, etc., can be very important in reflecting on the predictive value of the test result.

The IF methods also have their limitations: they require special microscopes, which can be expensive, specially trained microscopists who can interpret results accurately, and reagents that are specific for a particular virus. Thus, multiple smears would be needed to test for several viruses and a new virus would not be detected. It is therefore advisable to use IF in combination with culture methods for detection of viruses. Diagnosis is dependent on the quality of specimens as with all tests, so it is important that uniform, well-made smears be submitted for IF testing.

ENZYME IMMUNOASSAYS

Enzyme immunoassays (EIA) can be performed to detect either antigens or antibodies in clinical specimens. This versatility has resulted in widespread use of EIA in diagnostic virology. Most commonly these assays are performed using a solid-phase format, referred to as enzyme-linked immunosorbent assay (ELISA), with the particular target reagent bound to plastics in a microtiter well (Leland 2000). Many reagents are commercially available for detection of viruses and this form of testing is routine in many laboratories. EIAs are generally highly specific, have high sensitivity, and can be performed rapidly, within minutes to hours. Some solid-phase assays have been adapted to a disposable membrane format for use in physicians' offices, notably for influenza and respiratory syncytial virus (RSV). Also, EIA can be used for the detection of antigens in tissues using immunoperoxidase staining (Docherty et al. 2000).

There are some limitations to EIA, including lack of availability of reagents for some viruses, lack of cost-effectiveness for viruses that have a large number of serotypes (e.g. rhinoviruses) that are not cross reactive, and less sensitivity compared to molecular techniques.

RADIOIMMUNOASSAYS

Radioimmunoassays (RIA) have generally fallen out of use for most viral assays due to the expense of disposal and the potential of radiation exposure, and have generally been replaced by EIA. RIA rivals EIA in specificity and sensitivity and could be used for the same antigens as EIA if desired. Details on use of RIA in viral diagnosis can be found elsewhere (Mushahwar and Brawner 2000).

Detection and quantitation of viral nucleic acids

Recent years have witnessed an exponential growth in the use of diagnostic methods based on the search for

viral nucleic acids in clinical samples. The advantages of this approach are, in fact, several-fold (Table 3). In particular, rapid turnaround times for all viruses, including the ones that are fastidious or noncultivable (Table 4) and do not accumulate sufficient amounts of antigenic material in the host's tissues, or express unusual, not readily detected antigens (Ireland et al. 2000) to be detectable with current immunoassays, and relative ease of automation represent important features in the clinical virology setting since they permit significant improvements in the management of viral diseases without weighing unbearably on work loads. The common denominator of all genome-based methods is the use of stretches of nucleic acids (oligonucleotides, cDNA, RNA) that are complementary to, and thus capable of hybridizing with, segments of the viral genome being targeted. It follows that, although the many, variably sophisticated methods that have been designed around this basic principle vary greatly, choosing the correct segment of viral genome to be targeted is always very critical. In fact, because genome detection methods are extremely sensitive to sequence variation, selected sequences should be as conserved as possible in order for the method to detect and quantify all the possible variants of the virus with equal efficiency. This may sometimes be hard to achieve, as exemplified by the difficulties encountered in developing diagnostic methods that reveal with sufficient efficiency all the lentiviruses that may cause AIDS, including all subtypes of group M human immunodeficiency virus type 1 (HIV-1), group O HIV-1, and human immunodeficiency virus type 2 (HIV-2).

For several years, the only practical means for revealing the presence of viral genetic material in clinical samples was by nucleic acid hybridization techniques, which, however, were limited in sensitivity or required radioactive reagents. The hybridization-based assays that were designed for the diagnostic laboratory routine were therefore relatively few and found limited application. The advent of amplification techniques, which increase the copy numbers of selected segments of nucleic acids (nucleic acid amplification) or the signals generated by these when coupled to appropriate detectors or probes (signal amplification) has permitted the development of assays with exquisitely high levels of sensitivity and tremendously expanded the diagnostic potential of viral genome detection. Indeed, currently the detection of viral genomes is considered to be the most accurate indicator of infection for several viruses. Consequently, a large share of the virological tests carried out in an average diagnostic laboratory is presently based on this approach, albeit the high cost of many commercial reagents often remains a critical issue.

HYBRIDIZATION TESTS

In certain viral infections, selected clinical specimens can contain enough viral genetic material to be detectable directly, i.e. without the need for amplification technologies. In these cases, genome detection may be successfully carried out by reacting the specimens with nucleic acid probes of appropriate specificity and checking whether these have bound to target viral DNA or RNA. Technical variations are numerous. Examining thin-tissue sections or smears (in situ hybridization) may provide clinically useful histological information in addition to virus detection, but requires too much expertise and is too cumbersome for routine use. Thus, hybridization tests are usually performed on extracted nucleic acids in solution or, more frequently, immobilized on a solid support (e.g. dot-(spot)-blot hybridization). In the latter instance, extracted nucleic acid can be digested with restriction enzymes and electrophoresed (e.g. Southern-blot hybridization), thus allowing for much more specificity. The probes can be either DNA or RNA, may vary extensively in length, covering the entire viral genome or segments of it, and can be labeled with a variety of signal-generating systems (biotin, digoxigenin, etc.), although only ^{32}P and other radioactive tracers guarantee maximum sensitivity. Some hybridization tests also provide a semi-quantitative estimate of sample content in viral nucleic acids.

In past years, hybridization tests have found large practical application for detection of certain viruses,

Table 3 *Advantages and limits of genome detection methods in the laboratory diagnosis of viral infection*

Advantages	Limits
High sensitivity and specificity	Risk of cross-contamination
Detection of fastidious or noncultivable viruses	Risk of cross-reactions with nonrelevant nucleic acids
Virus detection also in clinical samples from which virus isolation can be difficult (patients treated with antivirals, CSF, etc.)	Risk of false negatives and false positives due to inhibitors or degradation of primes, probes, and templates
Quantitation of viral load	Viral phenotype not analyzable
Ease of automation	Comparatively high cost of commercial methods
High throughput	Detection of clinically irrelevant viruses
Rapid turnaround time	
Specimen deterioration less critical than for virus isolation	

Table 4 *Slow, difficult or impossible to isolate in tissue culture from clinical specimens*

Human virus
Coronaviruses
Enteric adenoviruses (serotypes 40 and 41)
Epstein–Barr virus (EBV)
GB virus-C (GBV-C)
Hepatitis A virus (HAV)
Hepatitis B virus (HBV)
Hepatitis C virus (HCV)
Hepatitis delta virus (HDV)
Hepatitis E virus (HEV)
Human herpesvirus 8 (HHV-8)
Human immunodeficiency virus (HIV)
Human metapneumovirus
Human papillomavirus (HPV)
Human parvovirus
Human polyomaviruses BK virus and JC virus
Human T-lymphotropic virus (HTLV)
Norwalk virus and other caliciviruses
Rotaviruses
Selected enteroviruses (most coxsackie A viruses)
Torque-teno-virus (TTV)

such as HPV in anogenital specimens and hepatitis B virus (HBV), hepatitis delta virus (HDV), and parvovirus B19 in plasma, but have now been almost completely replaced by more sensitive amplification-based techniques. As examples, the lower limit of sensitivity of commercial hybridization assays for HBV and parvovirus B19 detection in plasma ranged around 10^5–10^7 genome copies/ml whereas current amplification methods consistently yield a positive result with only a few hundred copies (Bendinelli et al. 2000; Heegaard and Brown 2002).

AMPLIFICATION METHODS

In the large majority of viral infections, no matter whether acute or chronic, the amount of genetic material of viral origin present in patient tissues is below the detection threshold of even the most sensitive among the direct hybridization tests discussed above. This explains the great success of many amplification methods that have been introduced in diagnostic virology in the last decade. In fact, these are capable of specifically and reproducibly detecting 10–100 viral genomes, thus performing at levels that were previously achievable only by the isolation of viruses capable of growing with great efficiency in tissue culture. Another important reason is that several amplification-based methods have permitted the development of quantitative assays, which precisely determine the number of viral genomes (viral load) in plasma and other specimens. This, in conjunction with the recent recognition that viral load determinations reflect and reliably measure active infection, has led to widespread use of quantitative assays for monitoring the levels of virus in the plasma of patients infected with chronic viremia inducing viruses, such as HIV, HBV, hepatitis C virus (HCV), and human cytomegalovirus (CMV). Viral loads are in fact now considered important parameters for treatment decision-making. Thanks to expanded availability of suitable reagents coupled with improved understanding of other technical issues, viral load assays have rapidly become increasingly reliable and user friendly and have acquired an important role in the clinical management of the above infections, contributing significantly to improved patient care.

Signal amplification methods

In essence, these methods are evolved formats of the hybridization tests discussed above, which have been designed to increase manifold – and make easily measurable – the strength of the signals generated by DNA or RNA probes that have formed hybrids with template viral nucleic acids. Several stratagems have been conceived for this purpose and exploit different technologies representing the basis for a number of widely used commercial kits. These include EIA technologies using monoclonal antibodies to specific nucleic acid hybrids, chemiluminescent technologies using triggerable substrates, and the recently developed branched-chain DNA (bDNA) technology. The latter consists of an intricate network of simultaneous hybridization events between multiple sets of oligonucleotide probes (capture probes, extender and amplifier secondary probes, labeled tertiary probes), some of which contain unnatural bases to optimize their complementarity performance characteristics. Since the intensity of amplified signals is proportional to the number of template sequences present in the specimens being tested, these assays are valuable not only for detecting but also for quantitating viral nucleic acids over a wide range of concentrations. For example, the HIV bDNA assays currently on the market can detect as few as 50 viral genomes per ml plasma. However, in general signal amplification assays tend to be somewhat less sensitive than methods based on nucleic acid amplification. On the other hand, they are less prone to technical problems generated by carryover contamination (see below).

Nucleic acid amplification methods

These methods are based on the use of enzymatic machineries that replicate nucleic acids under controlled conditions in vitro, increasing their copy number to a point where detection is possible by conventional or nonconventional techniques. The template amplified can be either viral DNA or RNA (target amplification) or, less frequently, the detectors that have interacted with viral nucleic acids (probe amplification). The polymerase

chain reaction (PCR) developed by Mullis and colleagues at the Cetus Corporation in the late 1980s (Mullis et al. 1986) is the conceptual progenitor of all methods in this category and still represents a widely exploited technology in a variety of nested, non-nested, monoplex, multiplex, and quantitative configurations, that in many instances have become standard laboratory practice. For amplification of RNA viruses, PCR-based methods require that target RNA is first converted into cDNA by a reverse transcriptase (RT) or by a bifunctional enzyme having both RT and polymerase activities [reverse transcriptase polymerase chain reaction (RT-PCR)]. However, several other methods have been developed that achieve viral nucleic acid amplification by using different principles and enzymatic systems. These include nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), and strand displacement amplification (SDA), which use a variety of enzyme combinations and amplification schemes. An example of probe amplification methods are the ligase chain reaction (LCR), the cycling probe technology (CPT), and the cleavase invader assay. Some such techniques require that the reaction mixtures undergo cycles of defined temperature changes, thus needing specific instrumentation, while others are isothermal and need no special instruments (Table 5).

Nucleic acid amplification methods are at high risk of carryover contamination, the source being mainly the sequences amplified (amplicons) in previous runs, which readily become dispersed in the environment and generate false-positive results, but also other specimens. Control of cross-contamination requires the implementation of rigorous physical precautions, the description of which is beyond the scope of this chapter, as well as the need to include in each assay appropriate numbers of negative control samples interspersed with test samples. It is also important to note that the enzymatic reactions at the basis of nucleic acid amplification assays are variably prone to inhibition by a multiplicity of substances, including heme, heparin, urine, calcium alginate, polysaccharides, and polyamines. The latter problem is usually overcome by the use of extensive nucleic acid purification procedures or of systems that specifically capture the target template.

Table 5 *Nucleic acid amplification methods used in the laboratory diagnosis of viral infections*

Method	Template amplified	Thermal cycling required
PCR	Target DNA	Yes
RT-PCR	Target RNA	Yes
NASBA	Target RNA	No
TMA	Target RNA	No
SDA	Target DNA	No
LCR	Detector DNA	Yes
CPT	Detector chimeric DNA-RNA	No
Cleavase invader	Detector DNA	No

Microarrays

Microarrays are amplification/hybridization methods miniaturized in such a way as to make it possible to assay, in a single run, specimens against a large number (thousands) of probes and consist of slides or microchips that carry microspots of cDNA or oligonucleotides bound to their surface. In perspective, this technology is expected to allow not only the search of many different viruses in a single test but also the simultaneous recognition of clinically and epidemiologically useful characteristics of detected virus (genotype, susceptibility to drugs, etc.) that at present need to be investigated separately (see below) as well as cellular gene alterations characteristic of a specific type of pathology. Although offering much promise, microarrays have yet to find wide application in the diagnosis of viral infections.

Real-time methods

The term real-time refers to methods in which the two major phases of amplification technologies, i.e. amplification and amplified product detection, occur simultaneously in a single, closed tube or well. These methods require intercalating dyes, probes, or molecular beacons labeled in such a way that the signal emitted changes concomitant with amplicon formation and dedicated instruments with precision optics that reveal such change as soon as it has occurred. They considerably shorten assay times, have an extremely high throughput and minimize cross-contamination problems (Mackay et al. 2002).

Commercial kits and automation

Virus detection and quantitation using commercial assays in kit format and with an increasing level of standardization and automation have gradually invaded most diagnostic laboratories. In fact, the performance characteristics of well standardized commercial assays having built-in carryover protection systems are generally superior to those of the in-house-developed PCR and NASBA assays (many of the other methods are too complex to be developed independently in the user laboratory), that are, however, still in use for testing clinical samples for the presence of uncommon viruses and for cost containment. Commercial kits often come with automated or semiautomated platforms. Automated steps may include sample preparation, nucleic acid extraction, amplification reaction, reading both sample and appropriate control values, data processing, and report generation. Automation can dramatically decrease laboratory-to-laboratory differences as well as labor requirements. The automation level of real-time techniques is often quite high. Rapid point-of-care viral

genome detection methods may also become available in the future, possibly as a spinoff of the portable amplification systems that are being developed for screening the environment for pathogens considered potential biological weapons. A note of caution regarding these advances is that a loss of the technical skills needed for handling and characterizing viruses is also anticipated.

DETECTION OF VIRAL INFECTIVITY

Animal inoculation

The use of animals to grow viruses for laboratory identification has virtually disappeared from the diagnostic laboratory. This has resulted more from the development of sensitive techniques to detect viruses, as well as the health risks and the costs involved in handling the animals. It is likely that animal inoculation will be limited to specialized laboratories who might investigate suspected new pathogens for which other techniques have not yet been developed. A detailed description of animal inoculation is provided in Landry and Hsiung (2000).

Virus isolation in embryonated eggs

Similar to the use of animal inoculation, use of embryonated eggs has yielded to cell culture and direct detection methods for viral diagnosis. The embryonated hen's egg, however, was a very useful vessel for growing viruses, which could be cultivated on the chorioallantoic membrane or in the allantoic or amniotic sacs. Eggs are still used for the large-scale production of some viruses in vaccine production processes (e.g. influenza viruses).

Eggs used for virus isolation must be obtained from a facility that raises them in specific pathogen-free conditions and dedicated equipment is required. Eggs are incubated at 37°C in a humidified atmosphere (40–70 percent) and rotated several times every day to ensure the health of the developing embryo. Eggs are examined by candling, a visual inspection process using a light box with a hole at the top, to make certain that the embryos are developing properly. A detailed description of inoculation and evaluation procedures is described by Landry and Hsiung (2000).

Virus isolation in cell culture

Work in the 1930s pioneered the use of cell culture for the propagation of viruses. Since that time the numbers and types of cultured cells have expanded tremendously and variations of standard uses of cell culture have enhanced the ability to detect and identify viruses (Landry and Hsiung 2000). As a result of these changes, viruses can be isolated more rapidly and in many cases viral identification also results.

STANDARD CELL CULTURE

Three types of culture are routinely used to isolate viruses: primary cells, diploid cell lines, and heteroploid cell lines. Cells may be used as monolayers or cell suspensions to isolate viruses. Lymphocytes for cultivation of HIV and certain herpesviruses, such as Epstein–Barr virus (EBV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7), have been especially useful in suspension culture. Each cell type has distinct characteristics that make it susceptible to a limited number of viruses (Table 6). Thus, it is often important to use in parallel several cell types in order to cover the array of viruses that might be associated with pathology in a particular illness or organ system. For example, respiratory tract infection during the traditional season for such infections might include human diploid fibroblasts as well as HEp-2 cells, primary monkey kidney cells, and A549 cells, which can support growth of almost all of the common causes of respiratory tract infections and can help differentiate among them (Landry and Hsiung 2000). In addition to susceptibility, sensitivity and the speed with which cells begin to show infection are factors to be considered in selecting cell lines. Other important factors to consider in setting up a cell culture laboratory are:

- whether or not to prepare and maintain cell lines in-house or purchase them commercially, which must be based on cost and availability
- what are the common specimens encountered so that appropriate numbers of the most needed cells are readily available (this may be seasonally adjusted)
- what is the availability of experienced skilled technologists.

Cell cultures must be carefully maintained and replaced regularly to ensure that cells will be capable of replicating virus when needed, as older cultures often have reduced susceptibility and contaminated cultures cannot yield useful results. This includes visual inspection prior to inoculation and addition of antibiotics and antifungal agents to specimens in the initial processing steps. Typically, tubes for standard cell culture are 16×125 mm screw-cap tubes and they are held at 35°C. When inoculated, cultures may be maintained in stationary position or in roller tubes and incubated at the same temperature except for certain viruses (e.g. rhinoviruses that are incubated at slightly lower temperatures). Tubes are usually kept at a slight angle (5–7°C). Cells are observed daily for evidence of infection.

DETECTION OF VIRAL PRESENCE

Classically, detection of viral infection is dependent upon light microscopy of unstained cells for visualization of evidence of cytopathology. Cytopathic effect (CPE) is the term used for morphological changes noted as a result of viral replication in the cell culture. The CPE of

Table 6 *Cell lines sensitive to common viral agents*

Cell line	Viruses
A549 cells	Adenovirus, HSV
HeLa cells	HSV
HEp-2 cells	Adenovirus, HSV, RSV
Human embryonic kidney	Adenovirus, BK virus, HSV
Human diploid fibroblasts	CMV, HSV, VZV, enteroviruses, rhinoviruses
Madin–Darby canine kidney (MDCK)	Influenza viruses
NCI-H929	Adenovirus, enteroviruses, MuV, measles virus, parainfluenza viruses, RSV
Primary monkey kidney cells	Enteroviruses, influenza viruses
Primary rabbit kidney cells	HSV
Engineered cell lines	
BHK-21 (baby hamster kidney-inducible β-galactosidase gene with HSV-inducible promotor)	HSV
Super E-mix (CaCo-2 and BGMK-high expression of decay accelerating factor)	Enteroviruses
Mixed cell cultures	
E-mix A (RD and NCI H292)	Enteroviruses
E-mix B (BGMK and A549)	Enteroviruses
Super E-mix (CaCo-2 and BGMK) high expression of decay accelerating factor)	Enteroviruses
H and V mix (CV-1 and MRC5)	CMV, HSV, VZV
R-mix (A549 and ML)	Adenoviruses, influenza A and B viruses, parainfluenza viruses, RSV

cells may be described as clumping, destruction, granulation, rounding or vacuolation, giant cell or syncytia formation, or refractile cells. A presumptive identification of the etiologic agent may often be made, with a high degree of accuracy, based on the type of CPE in the cell line(s) used, specimen source, and information on the clinical presentation. It is important to examine cells at least once every 2 days and to maintain sham-inoculated controls for each cell line inoculated. The presence of adventitious agents in cells, especially simian viruses in primary monkey cells, is not uncommon.

In some circumstances, viruses will not cause a visible CPE and it is necessary to resort to other methods to detect their presence. Mumps virus (MuV) will not normally cause CPE but addition of guinea pig red blood cells (RBC) to infected primary monkey kidney cells will result in adherence of the RBC to the cells (hemadsorption), thus demonstrating the presence of infection. This results from a viral glycoprotein (hemagglutinin) being inserted into the cell membrane as part of the viral replicative cycle. Alternatively, viruses that do not cause CPE following infection may be detected using an interference assay. In this regard, rubella virus (RUBV) isolation may be detected by interference with echovirus 11 to replicate and cause cell destruction of African green monkey kidney cells.

Whilst all the assays described will isolate virus and may provide presumptive evidence, they do not provide definitive evidence of the identification of the etiologic agent. Identification can be achieved by using specific antiviral antibodies, most effectively monoclonal antibodies, in conjunction with cell culture. This can be achieved by using antibodies labeled with a fluorochrome (IF) or an enzyme (EIA) as described above.

Enhanced cell culture

Over the past few decades several advances have been introduced that have allowed for more rapid, sensitive and cost-effective use of cell culture. The first of these involved low-speed centrifugation of cell cultures to enhance the infectivity of viruses. Subsequently, the use of labeled antibodies for early detection of viral antigen allowed for greatly decreased time to detection as compared to awaiting development of CPE. Thereafter, cell culture has been enhanced by the identification of genetic regulatory elements that are specific to particular viruses, allowing for cell lines to be engineered to yield highly specific detection of a virus. More recently, the admixture of different cell types into a single culture vessel has allowed for a broader array of viruses to be identified in a single tube.

CYTOSPINNING (SHELL VIALS)

The use of the shell vial assay was adapted for detection of CMV as reported by Gleaves et al. (1984). The assay used MRC-5 cells (human diploid fibroblasts) grown on a coverslip in a vial, infected with a specimen, and submitted to low-speed centrifugation followed by overnight incubation. Labeled antibody to an early antigen of CMV is used to stain cells after 24 or 48 hours and

often yields a positive result a week or more sooner than waiting for the development of CPE. When used in combination with standard cell culture, the shell vial assay can result in more rapid detection and increases the overall rate of detection for CMV. The success of this assay for CMV has stimulated development of shell vial assays for other viruses and testing can now be done for adenovirus, enteroviruses, respiratory viruses, HSV and VZV. Depending on the virus suspected, an increased number of shell vials was inoculated, the time of incubation was extended and the variety of cell lines used was increased (Olsen et al. 1993). In the case of detection for a variety of viruses, such as respiratory viruses, a cocktail of antibodies can be used to determine if infection is present. The antibodies are used in such a way that the staining pattern is suggestive of a particular virus. Subsequent testing of a second shell vial containing the positive specimen using a monospecific antibody can then identify the etiologic agent.

ENGINEERED CELL LINES

Principles of histochemistry, genetic engineering, and cell culture were combined to enhance the development of a rapid, specific method for detection of HSV by Stabell and Olivo (Olivo 1996). They used BHK-21 (baby hamster kidney) cells that had been stably transformed with a β-galactosidase from *Escherichia coli* attached to an HSV-inducible promoter. When these cells are infected with either herpes simplex virus 1 (HSV-1) or herpes simplex virus 2 (HSV-2), the β-galactosidase is induced and then an X-gal colorimetric substrate is added, infected cells turn blue, while other viruses do not induce the enzyme and cells remain colorless. This method has been incorporated into a commercial kit that has been marketed as the enzyme-linked virus-inducible system (ELVIS) (Olivo 1996). When compared to standard isolation in cell culture that takes considerably longer at times, this test was highly sensitive and specific for both herpes simplex viruses. There are advantages to this method including its rapidity, use of relatively inexpensive substrate as compared to antibodies, and the ability to use cells to look for both the color change and CPE. The method is adaptable for other viruses, both DNA and RNA viruses, and a format for antiviral susceptibility testing for HSV (Olivo 1996) may further broaden the usefulness of this approach.

MIXED CELL CULTURES

The most recent significant development in virus isolation methodology is the use of a mixture of cells in a shell vial format. The choice of cells has been focused on the ability to isolate and identify viruses that have a common pathological presentation, such as enteroviruses or respiratory viruses. The cell mixture allows for a broader range of viruses to be isolated in a single culture. The mixtures have been designated by the cell types included in the culture and the specimen type submitted. R-mix uses a combination of A549 cells and mink lung cells and is useful for the detection of adenoviruses, influenza viruses, parainfluenza viruses, and RSV (Huang et al. 2000; St George et al. 2002). There are three combinations used for enteroviruses, designated E-mix A (RD and NCI-H292) and E-mix B (BGMK and A549) and Super E-mix (CaCo-2 and BGMK cells that have been genetically engineered to express a receptor for enteroviruses), which will also detect some additional viruses (Buck et al. 2002; Huang et al. 2002). The choice would depend on specimen type and clinical diagnosis since enteroviruses are seen in a variety of disease entities. In addition, there are H and V mix cells for the detection of herpesviruses (CMV, HSV, and VZV) that use CV-1 and MRC-5 cells (Huang et al. 2000). Preliminary results using these cell mixtures indicate that they perform similarly to or better than conventional cell culture or single-cell shell vial cultures and are more cost effective. By contrast, these cell mixtures do not appear to be as sensitive as PCR in comparison testing.

CHARACTERIZATION OF DETECTED VIRUSES

Viruses detected in clinical specimens by one or more of the methods discussed above generally need to be further investigated in order to achieve their precise identification (typing). In addition, they may need to undergo supplemental analyses that can provide clinically useful information, including antiviral drug susceptibility testing.

Typing

A precise identification of detected viruses is often necessary because viral agents belonging to different species or types within a same family or genus may vary greatly in pathogenic potential, and most virus detection methods do not allow such differentiation. Typing is also essential for epidemiologic surveillance purposes. Criteria that may be of guidance in typing a virus detected in a clinical specimen are numerous, including nature of the specimen, epidemiologic data, virion morphology and physical–chemical properties, electrophoretic pattern of segmented viral genomes, presence or absence of a lipid envelope, range of infectable cell types or animals, type of CPE or pathological manifestations produced, ability to agglutinate the RBC of different animal species, etc. However, generally such elements only provide presumptive evidence that permits one to narrow, sometimes substantially, the number of type-specific tests the laboratory will eventually need to perform. For example, assessing the

hemagglutination properties of an adenovirus isolated in tissue culture may assign it to one of the subgenera A–F but does not obviate the need for further typing. In fact, ultimate identification is based on an accurate definition of their antigenic makeup (serotyping) and/or nucleotide sequence analysis (genotyping) of detected viruses, the choice between the two being mainly dictated by how detection has occurred.

SEROTYPING

Viruses demonstrated by isolation in tissue culture, embryonated eggs, or experimental animals, or revealed by antigen detection methods are generally typed serologically. This is achieved by investigating how selected biological properties of the isolate behave when this is probed with type-specific immune sera. Although neutralization remains the most reliable means of serotype classification due to its exquisite specificity, it is technically demanding and labor-intensive. Also, it may be complicated by the poor neutralizability of certain fresh isolates by antisera raised against prototype viral strains or by the concomitant presence of more than one virus in the sample. In the case of viruses for which a profusion of different serotypes exist, performing the neutralization tests with pooled antisera represents a considerable help since it reduces the number of immune sera that need to be individually used in final identification. For example, serotyping of enteroviruses can be facilitated by the use of standardized antiserum pools provided by international agencies.

Additional frequently used methods for serotyping are inhibition of hemagglutination or hemadsorption and examination of virus-infected cells by IF or other immunostaining techniques. Finally, especially when the virus is difficult to cultivate, dissection of intertypic antigenic differences can be carried out with enzyme immunoassays, immune EM (which relies on the antibody-mediated agglutination of viral particles), immunochromatography, and other serological tests.

GENOTYPING

Although applicable to any virus, genotyping is most frequently used for the ones that have been demonstrated by nucleic acid detection and consists in analyzing segments of the viral genome that present unequivocally distinct type-specific nucleotide sequences. Genome regions suitable for typing can be the same used for virus detection as in the case of HCV genotyping (Zein 2000; Nolte 2001). However, because detection methods often target highly conserved genomic segments, genotyping frequently requires the prior amplification of additional carefully selected segments of the viral genome. For example, while human T-lymphotropic virus (HTLV) detection by PCR in Ficoll-purified peripheral blood mononuclear cells (PBMC) is usually carried out with primer sets targeted to the *tax* gene, differentiation of human T-lymphotropic virus type 1 (HTLV-1) and human T-lymphotropic virus type 2 (HTLV-2) is achieved by amplifying positive samples in a region of the *pol* gene (Schüpbach and Gallo 2000). Amplified segments are then sequenced, and obtained sequences compared to those existing in data banks with the help of special computer algorithms. Alternatively, the viral genotype can be deduced from the patterns generated by the amplicons when sized or examined for restriction fragment length polymorphism (RFLP), electrophoretic mobility following duplex formation with reference amplicons (heteroduplex mobility assay), denaturation at different temperatures (melting curve analysis; Whiley et al. 2001), or binding to carefully chosen genotype-specific oligonucleotide detectors immobilized on solid supports, such as membrane strips (line probe assay). The latter method has been exploited in several commercial kits since it is technically the least demanding. However, sequencing is regarded as the 'gold standard' since it permits pairwise comparisons, calculation of genetic identities on the basis of percent homology, and performance of phylogenetic investigations that may be useful for documenting epidemiologic links and other purposes. In addition, if the tracts of viral genome analyzed are sufficiently long, sequencing has the potential of revealing viral hybrids that may form as a result of recombinational events between viruses belonging to different genotypes. For some viruses, recombinants are a common occurrence. For example, recombinants of HIV-1 not only are frequently found in multiply exposed patients but also have the ability to be transmitted from patient to patient (circulating recombinant forms; Thomson et al. 2002).

As an alternative molecular strategy to classification, proposed, for example, for differentiating among orthopoxviruses (Ropp et al. 1999) and HCV genotype identification (Schröter et al. 2002), group- or type-specific PCR assays can be constructed which provide a positive result only when a specific viral group or type is present in the clinical specimen. Of note, this strategy has the potential to give a definitive diagnosis based on a single assay without the need for additional testing but is practicable only when the number of possible genotypes is minimal. Examples are the detection of the polyomaviruses BK virus and JC virus in urine (Biel et al. 2000) and of coxsackie virus A16, a frequent cause of hand-foot-and-mouth disease (Bendig et al. 2001).

Differentiating between wild-type and attenuated vaccine virus strains

This differentiation is necessary whenever a virus suspected to be responsible for a clinical form is detected in a patient who has recently been immunized against the same virus by means of an attenuated

vaccine or has been in contact with vaccinees. The assays involved are generally conducted in reference laboratories and may be phenotypic and/or genotypic. For example, discrimination between vaccine and wild-type strains of poliovirus can be based on in vitro growth behavior at different temperatures, intratypic serodifferentiation using strain-specific antibodies, RFLP patterns, or sequencing of PCR products. Molecular methods have found application also for discriminating the vaccine strains of the measles virus (Bellini and Rota 1998) and varicella virus (Loparev et al. 2000) from their wild-type counterparts.

ANTIVIRAL DRUG-SUSCEPTIBILITY TESTING

Patient treatment with antiviral agents frequently – if not invariably, especially if therapy is sufficiently prolonged – determines the emergence of virus mutants that show a decreased susceptibility to the drug(s) used and often also to other drugs having mechanisms of action similar to the ones in use. Because this usually leads to reduced patient response to therapy, revealed by rising viral loads and/or clinical deterioration, monitoring patients for the appearance of drug-resistant mutants is gradually becoming routine in clinical practice for evaluating whether and how antiviral regimens have to be modified. This is in turn impacting on diagnostic virology laboratories to an increasing extent. Currently, viruses obtained from antiviral naive patients are generally drug sensitive. However, as with antibacterial drugs, increased availability and use of powerful antiviral agents is likely in the future to lead to the circulation in the community of viruses that present baseline drug susceptibility characteristics that diverge to a variable extent from naive viruses. This development, which already has been observed with HIV-1 (Boden et al. 1999), is bound to extend the need for drug-susceptibility testing of viruses prior to initiation of treatment.

Phenotypic drug-resistance assays

Ideally, for better reflecting what is happening in patients, drug-resistance profiles should be based on the phenotype characteristics of detected viruses. This implies that the virus is isolated in tissue culture, grown at sufficiently high titers, titrated, and finally tested for the ability to replicate in the presence of graded concentrations of each of the drugs of interest. Thus, conventional phenotypic assays are laborious and, with the almost sole exception of HSV-susceptibility testing, take weeks to be completed. In addition, concerns exist that in vitro passage may result in the selection of an altered virus population having drug-susceptibility characteristics different from the one in the patient. In the case of HIV-1, an effort to circumvent these shortcomings has been the development of recombinant virus-based phenotypic assays, in which the relevant genes are directly PCR amplified from the patient and inserted into engineered HIV-1 clones from which such genes have been deleted, and the resulting chimeras subjected to phenotype analysis. It should be noted, however, that even the latter assays do not resolve the other major pitfall of phenotypic resistance tests, namely that attempts to standardize protocols and interpretation guidelines have met with limited success, due to the many technical and interpretational variables that may influence assay results. These include assay format (plaque reduction, dye uptake, yield reduction, flow cytometry, etc.), virus dose, type of cell substrate, viral strains adopted as drug-susceptible and resistant controls, duration of incubation, readout (CPE, accumulation of viral components, infectivity, etc.), and endpoint (50 versus 90 or 99 percent inhibition).

Genotypic drug-resistance assays

Due to the problems inherent to phenotypic assays, for certain viruses (HIV-1, HBV, CMV, influenza), these have been largely replaced by tests that analyze the genome of detected viruses. The procedure most often used consists in amplifying and sequencing the entire genetic region(s) known to control drug susceptibility. Alternatively, if the mutations involved are limited in number or only the most common need to be detected, PCR amplification can be confined to short genome segments, and the resulting amplicons examined by restriction enzyme digestion, melting curve analysis, or line probe assays, as well as by automatic or hybridization sequencing. Genotypic assays are much faster and less labor intensive than phenotypic assays but may miss resistant mutants that are still in the minority in the patient's total viral population. A further limitation stems from the fact that the multiple effects of individual mutations can make it extremely difficult to infer how different combinations of mutations, generated, for example, as a result of multidrug therapies, can impact on actual viral phenotype (level of resistance, cross-resistance, multidrug resistance, etc.). Of note, for HIV-1, an interesting solution has been to scrutinize the nucleotide sequences against constantly updated databases that embrace large numbers of data correlating HIV-1 genotype to phenotype (virtual phenotype). For a recent review of genotypic testing for HIV-1 drug resistance see Shafer (2002).

Viral parameters of poorly defined clinical utility

As discussed in the above sections, viral load and drug-susceptibility determinations have become an integral part in the management of several viral diseases. In recent years, however, there have been numerous efforts to identify further virological parameters that may help at formulating precise prognoses and effective antiviral treatments. One avenue has consisted in attempts to

correlate disease severity with variably subtle differences that may exist among different strains of a given virus or may evolve as a result of virus–host or virus–drug(s) interplay. For example, in HIV-1 the features considered have included genetic complexity, tropism for lymphocytes or macrophages, co-receptor usage, cytopathicity, syncytium-inducing ability, sensitivity to antibody-mediated neutralization, and replicative capacity. In particular, the latter parameter has received attention especially following the introduction of highly active antiretroviral therapy (HAART) due to the observation that certain drug-resistant HIV-1 isolates with mutated enzymes exhibit impaired replication rates (Brenner et al. 2002), leading to controversy regarding whether failing drugs should be stopped or rather continued in order not to remove the force that provides selective advantage to such unfit mutants (Deeks et al. 2001).

Similar studies have also involved several hepatitis viruses, although the range of parameters considered has been more restricted due to failure of these viruses to grow in vitro. HCV quasi-species composition has received particular attention in the hope that the swarm of viral variants found in patients may provide a recapitulation of the selective forces that have impacted and are impacting on the virus and of virus ability to evolve and resist host's defense mechanisms (Maggi et al. 1997). Although the findings in chronically infected patients have been inconclusive (Farci et al. 2002), recent evidence suggests that, when studied in the viral hypervariable region 1 at the time of seroconversion, quasispecies composition can predict the outcome of acute HCV infection (Farci et al. 2000).

A second approach has been trying to find correlations between disease progression and status of the infecting viruses in patients tissues. An early example was the correlation that was established between HBV replication activity and HBeAg detection in plasma (Bendinelli et al. 2000). More recently, suggestions have been put forward that detection of CMV DNA in plasma and not only in the peripheral blood leukocytes of bone marrow transplant recipients correlates with increased likelihood of progression to clinical disease (Boeckh et al. 1997) and that detecting viral mRNA, particularly late rather than early transcripts, in the tissues of transplanted patients is an indication of persistent infections reactivation (Lam et al. 1998; Gerna et al. 1999; Blok et al. 2000; Van den Bosch et al. 2001). Also, determining the physical state (integrated versus episomal) of HPV DNA in genital samples has been proposed as a measure of the risk of oncogenic progression (Kalantari et al. 2001).

To date, however, the overall practical product of these studies has been disappointing, since few, if any, of the parameters proposed have reached general acceptance and none has found extensive application in everyday clinical and laboratory practice. It should also be noted that, at least as currently performed, many of the parameters considered are too laborious for most clinical virology laboratories and are susceptible to artifacts. As an example, viral quasispecies analysis implies the amplification of selected regions of the viral genome followed by cloning or separation by gel mobility of the resulting amplicons and sequencing of the different variants (Gomez et al. 1999) or, as suggested in a recent report, mass spectroscopy of the peptides encoded by such amplicons (Ayers et al. 2002). Similarly, discriminating between viral genomic DNA and the cDNA formed as a result of mRNA reverse transcription and amplification can be extremely arduous. It is therefore to be expected that, even if eventually validated, many of the proposed parameters will remain in the realm of clinical research for a long time.

ANALYSIS OF THE HOST'S IMMUNE RESPONSE TO THE INFECTING VIRUS

Cell-mediated immune responses

Early attempts to exploit cell-mediated immune (CMI) responses, in particular delayed hypersensitivity skin tests, for the diagnosis of viral infections led to the conclusion that they could at most have some utility in epidemiologic surveys. The measurement of CMI responses has had a resurgence in the past two decades as a tool for monitoring patients who are virus infected or at risk of becoming infected. This is related to our recognition of the pathogenetic significance of cellular immune function and cytokines in viral infections. HIV infection and AIDS are perhaps the circumstances where this is most thoroughly used but other states of immunocompromise, notably bone marrow transplantation, have seen benefits from such testing. Testing of CMI function has included measurement of a variety of functional and nonfunctional parameters (Table 7), including circulating lymphocyte subset counts, cytotoxic T lymphocytes (CTL) and natural killer cell activities, response to specific antigens and to polyclonal lymphocyte phytomitogens, and cytokine production and profiles. Mawle (1996) has written a useful review that details many of the assays used to measure cellular immunity.

The determination of total T lymphocytes as well as the number of helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells has achieved its greatest importance in HIV-infected individuals as this is used for staging of disease, evidence of progression to AIDS, and as an indication of

Table 7 *Cell-mediated immunity used for clinical virology*

Cell numbers	Cell functions	Cytokines
CD4, CD8, CD4:CD8 ratios	Cytotoxic T cells	T helper cell populations
NK cells	NK cells	Th1 – IL-2, IFN-γ Th2 – IL-4, IL-1

efficacy of antiviral therapy. Absolute cell numbers as well as CD4:CD8 cell ratios are used for these purposes. The availability of monoclonal antibodies to measure these cell numbers has made this testing easy to perform in virtually any laboratory; however, it is more precisely accomplished using flow cytometry.

Testing of cellular cytotoxicity is usually performed using a radioactivity label release (^{51}Cr) assay. This testing requires a step to separate and collect PBMCs and then test them under conditions that are optimal for measuring virus-specific CTL. This testing has been used in evaluating AIDS patients and individuals with herpesvirus infections but is still not routinely used in the clinical laboratory.

Nonspecific or antigen-specific blastogenic responses as a measure of immune competence provide insight into CMI function, either generally or regarding a specific disease. More recently there has been an association of subsets of helper T cells with immune status, such that production of specific cytokines is associated with a predominance of CMI [Th1 cells, which produce interferon-gamma (IFN-γ) and interleukin (IL)-2] or antibody responses (Th2 cells, which produce IL-4, IL-5 and IL-10). Th1 responses are considered to favor a better prognosis in HIV infection and many virus infections, whereas Th2 responses would be considered to be important in other types of infections.

Antibody responses

Although at present somewhat overshadowed by the recent success story of molecular methods, serological tests for antiviral antibodies (serodiagnosis) have had and still have an important position in the diagnosis of viral infections. The reasons are several-fold. First, certain serological tests (in particular, detection of antiviral IgM) represent a rapid means for identifying several acute viral infections. Second, the technologies most frequently used are within reach of most laboratories since they do not demand the expertise and the equipment that are instead essential for most other virological procedures. Third, serology is theoretically valuable for establishing an etiologic diagnosis in all viral diseases, regardless of whether the etiologic virus is easily demonstrable by direct means or not. Fourth, serological tests are generally inexpensive in labor and reagents. Fifth, the market offers a vast selection of high-quality serological reagents, many of which are available in kit format and are suitable for automation. Finally and possibly most importantly, if the implicated virus is one of the many that tend to persist for prolonged periods of time or indefinitely in their hosts, serodiagnosis represents the only means, together with viral load determination when available, by which it can be demonstrated that a primary infection or a reactivation of a chronic infection is ongoing at the time of disease. When clinical, anamnestic, and epidemiologic data are compatible, this represents strong evidence that the disease under scrutiny is indeed caused by that virus.

Classically, the procedures exploitable for serodiagnosis include virus neutralization (which for decades has been the standard against which the other test systems were evaluated), complement fixation (CF), hemagglutination inhibition (HI), passive agglutination of latex particles or RBC, immunoblotting, indirect IF, and RIA. These methods are still in use for some viruses (e.g. neutralization for poliovirus and CF for uncommon agents for which no other assay is available), or for selected purposes (e.g. immunoblots for confirmation of HIV or HCV seropositivity assessed with other methods). However, currently, the scene is dominated by solid-phase EIAs, that have become enormously popular in diagnostic virology as well as in other fields due to their sensitivity (comparable to RIA), speed, ability to measure antibodies of different isotypes, use of nonhazardous reagents, and aptitude to be automated. The antigens used are purified viral preparations and, increasingly, recombinant or synthetic peptides. It is, however, possible that in the future, other practical methods will be adapted for viral serology. For example, flow-cytometry-based serological methods are being advocated for their ability to detect antibodies to more that one virus in a single assay (Álvarez-Barrientos et al. 2000).

For viruses that constantly cause chronic infections (HIV-1 and HIV-2, HTLV-1 and HTLV-2) or are very uncommon (rabies in unvaccinated individuals), the interpretation of serological data is unique since the detection of antibodies of any isotype virtually always denotes infection. For all other viruses, demonstration of ongoing infection by serology requires that the antiviral antibody response is shown to have been elicited (boosted in the case of virus reactivations) recently. This is a consequence of the long duration of most antiviral antibodies, often with little or no reduction in total titer for many years or lifelong, particularly if they have been generated by systemic infections. Thus, mere seropositivity reveals that the individual has been exposed to the tested virus but is not informative of whether this has occurred recently or more or less remotely or even as the result of artificial immunization. In a sense, seronegativity may be more informative because it excludes the infection or indicates that antibody production has not yet started.

Demonstration that an antiviral antibody response has been triggered recently can be achieved with one or more of the approaches below.

TITRATION OF PAIRED SERUM SAMPLES

Two serum samples, one collected as early after disease onset as possible (acute-phase serum) and the second at least 1 week later (convalescent-phase serum), are titrated for antiviral antibodies simultaneously and in the same run. Although seroconversion is rarely

observed especially in systemic diseases with an incubation period of weeks, this approach often permits the observation of a significant rise (fourfold or greater) in total or IgG antibody titer between the first and the second serum. Major pitfalls of this procedure are:

- if the acute-phase serum is drawn too late, antibodies may be already reaching peak levels and a significant rise in titer can be missed
- the result of testing is very often retrospective and, therefore, of little use for patient care.

DETERMINATION OF ANTIVIRAL IGM IN SERUM

This approach is currently the most widely used. Typically, during acute primary virus infections virus-specific IgM antibodies appear in serum between 5 and 10 days after initiation of infection, reach peak concentrations at 2–4 weeks, and then usually decline to undetectable levels at 2–4 months, although they may last considerably longer in some infections. In the course of persistent infections, IgM antibodies are usually negative, although they can briefly reappear during episodes of intense reactivations of virus replication. Thus, detecting IgM to a given virus, with or without the corresponding IgG, even in a single serum sample obtained during the acute phase of illness, denotes current or very recent primary infection or reactivation of a persistent infection by that virus. By contrast, the presence of virus-specific IgG but not IgM signifies past infection. Examples of acute viral infections in which specific IgM tests are particularly useful for diagnosis include hepatitis A, rubella, measles, CMV and EBV infections. Also, because IgM as well as IgA do not cross the placental barrier, detecting antibodies of these classes in a newborn is strongly diagnostic for a congenital infection.

The development of sensitive, specific, and reproducible tests for antiviral IgM that can be used on a routine basis has taken considerable effort, mainly due to the possible presence of IgM rheumatoid factors (RF) which, by binding to the Fc portion of IgG, can generate false positives and/or abundant IgG which can compete for antigen binding and produce false negatives. Methods used in the past have relied on IgM physicochemical separation or inactivation by reducing agents and on IgG removal with certain bacterial cell wall proteins (*Staphylococcus aureus* protein A, streptococcal protein G) or other means. However, the ones presently employed are almost exclusively variations around the use of highly selective class-specific anti-IgM antibodies to detect and/or to capture the IgM. The commercial kits that utilize the latter technology are numerous, varied for the use of different solid-phase supports, have different indicator labels to signal IgM–anti-IgM complex formation, and different inbuilt systems for RF and IgG removal (not needed for capture assays), and are generally dependable.

MEASUREMENT OF ANTIBODIES AGAINST DIFFERENT VIRAL EPITOPES

The various subsets of antibodies generated in response to an infecting virus and directed to diverse epitopes of the structural and nonstructural viral proteins may present consistent and marked differences in the kinetics of appearance, decline, and disappearance that are utilizable for staging the infection. This approach has wide application in HBV and EBV serology, where measuring separately the antibodies directed to different antigens has considerable clinical utility. As an example, Table 8 provides a key to the interpretation of the serological profiles to EBV that are most frequently encountered.

DETERMINATION OF ANTIVIRAL IGA IN SERUM

Appearance, level, and duration of serum IgA are less predictable than either IgG or IgM. In general, however, IgA antibodies are made acutely, like IgM antibodies although they tend to last longer. Albeit no IgA assay is widely used in routine diagnostic testing, detection of virus-specific serum IgA has been suggested to have practical utility in several situations. These include identification of HIV-1 infections in newborns (Moodley et al. 1997), rapid diagnosis of influenza (Voeten et al.

Table 8 *Interpretation of serological test results for EBV infection*

Anti-VCA[a] IgM	Total anti-VCA	Total anti-EBNA[b]	Total anti-EA[c]	Interpretation
–	–	–	–	No infection
+	±	–	±	Acute primary infection
+	+	+	–	Recent infection
–	+	–	+	Recent infection in immunocompromised
+	+	+	+	Resolution of acute primary infection or reactivation
–	+	+	+	Past infection or reactivation
–	+	+	–	Past infection

a) VCA, viral capsid antigen.
b) EBNA, EBV nuclear antigen.
c) EA, early antigen.

1998) and dengue fever (Talarmin et al. 1998), and diagnosis of EBV-associated nasopharyngeal carcinoma (Hsu et al. 2001).

DETERMINATION OF ANTIVIRAL ANTIBODY AVIDITY

This approach exploits the fact that the strength of antibody binding to antigens increases with time after initiation of immune responses. The parameter used (avidity index) is the ratio of the antibody titers obtained in a standard serological assay (generally EIA) with and without treating the formed antigen–antibody complexes with an appropriate concentration of a protein denaturant, such as urea or guanidine, before addition of the secondary antibody. The test has been successfully used in the diagnosis of a number of viruses, including several herpesviruses, hantaviruses, encephalitis viruses, HCV, and measles virus. In pregnant women, it has been found valuable, together with other assays, for measuring the risk of transmitting CMV infection to the fetus (Lazzarotto et al. 2001).

ANTIBODY IN ALTERNATIVE SPECIMENS

Due to the blood–brain barrier, normally the CSF is devoid of IgM and has an IgG content 250–500-fold lower than serum. Thus, in the absence of concomitant signs of barrier damage, finding specific IgM or increased CSF/serum ratios of specific IgG is indicative of intrathecal antibody synthesis resulting from CNS infection. For alphaviruses, bunyaviruses, and flaviviruses, the presence of specific IgG in the CSF is highly suspicious, although only the presence of specific IgM is truly diagnostic. For more common viruses, CSF antibody analysis provides diagnostic hints only if evidence of intrathecal synthesis is obtained. Currently, it is almost always preferred to examine the CSF for viral nucleic acids.

Studies have also advocated the testing of urine and oral, ocular, or genital fluids for antiviral antibodies as easily obtainable, noninvasive alternatives to serum or plasma or as a means for obtaining additional information in selected clinical conditions. In general, these specimens have proven valuable for epidemiologic purposes but are not commonly used for diagnosis. Methods need to be developed specifically or validated for use with each type of specimen and some are commercially available. For maximum sensitivity, assays may be designed to recognize IgA as well as IgG and IgM antibodies (Zmuda et al. 2001).

CAUTIONARY NOTE

Serodiagnosis of viral infections can be complicated by a number of factors (Table 9), thus calling for considerable judgment in the interpretation of results. Whenever possible, serological diagnoses should be confirmed with virus detection methods.

QUALITY ASSURANCE/QUALITY CONTROL

Quality assurance affects all aspects of the diagnostic laboratory through a comprehensive program that strives toward constant improvement (Warford 2000). Oversight of services related to the virology laboratory includes specimen handling (collection, transport, storage, and testing), monitoring of procedures, reporting of results and their interpretation. The importance of laboratory participation in this process is well documented (Nutting et al. 1996). Laboratories in the USA are currently highly regulated under the Clinical Laboratory Improvement Act, instituted in 1992, which provides strict standards for quality improvement. These regulations address personnel qualifications, assessment of competence and defined responsibilities, written and approved procedures, quality control for test reagents and equipment (includes maintenance), test method verification and validation, proficiency testing of all analytes and staff members, analysis and improvement of all laboratory services, and a well-documented reference section for accreditation requirements, methods,

Table 9 *Considerations for interpreting serological data for diagnosis of viral infections*

Considerations
Superficial infections may elicit no or very weak systemic antibody responses
Antibody production may be delayed or absent in newborns, elderly, and immunocompromised individuals
IgM antibodies may persist for protracted periods after primary infections or may be stimulated in reactivation of latent viruses
False-positive IgM tests are a common occurrence in certain lymphoproliferative disorders
Reinfections and reactivations may not produce significant rises in total and IgG antibodies
Antibodies may cross-react within certain virus groups (e.g. alphaviruses, herpesviruses, parainfluenza viruses)
Passively acquired IgG antibodies are common finding in newborns and in individuals recently treated with blood transfusions or immunoglobulins
Antibody appearance in the CSF may be delayed by 2–4 weeks
Defects in the blood–brain barrier produced by any nonviral neurological disease can cause leakage of serum antibodies into the CSF

and quality assurance literature. Five major areas have been identified where quality assurance is mandated:

1. documentation of competency testing, staff duties and training
2. a well-maintained procedures manual that conforms to National Committee for Clinical Laboratory Standards (NCCLS) formatting
3. proficiency testing
4. quality control
5. a specimen log complete that includes test requisitions (Warford 2000).

The goal of these quality assurance efforts should be the constant improvement of laboratory services to the benefit of patient care. In other countries, regulations are more or less detailed and enforced but there is no doubt that a stringent quality control surveillance is of great help in keeping good patient care standards.

A comprehensive program for quality control that is well documented and regularly reviewed must include all procedures performed in the diagnostic virology laboratory. When appropriate, corrective actions must be taken in a timely manner and must be documented in writing. Quality control is documented by daily checking of all materials and equipment with weekly review by a supervisor. Excellent quality control practices are essential to establish the reliability of clinical laboratories and for the provision of good patient care; this is dependent on attention to all details for all aspects of the laboratory. A particularly important issue for the clinical virology laboratory is prevention of contamination, which is critical to successful performance of both molecular diagnostic techniques and cell culture. Nearly all issues related to quality control measures and scheduling are covered in documents prepared by the NCCLS and can be found at their website (www.nccls.org).

FROM LABORATORY FINDINGS TO DIAGNOSIS

Demonstration of a virus by cell cultures or EM is virtually always a definitive indication that virus is present in a disease state. Whilst this usually is suggestive of an etiologic role for the particular virus, this is not always the case. Results may be complicated by mixed infections with more than one organism or an organism might be isolated that does not have a pathogenic role. Recently, it has been discovered that many respiratory tract infections result from infection with human metapneumovirus. In several cases this virus and other human respiratory pathogens have been detected concurrently and it is not yet clear whether one or both viruses contribute to disease pathology. Alternatively, certain small round viruses can be routinely visualized in the gastrointestinal tract but association with any pathology has been far more difficult to establish. The same can presently be said of TT virus and related anelloviruses, which are found, often at high titers, in the circulation of many diseased and healthy individuals, with no apparent relation to disease.

Presence of virus as determined by detection of viral nucleic acid is indicative of current or recent viral infection but does not necessarily reflect that live virus is present nor that the virus is responsible for the ongoing clinical manifestations. There has been extensive evaluation of the detection of quantities of nucleic acid to determine viral load and its relationship to disease state, progression, and the efficacy of antiviral therapy (Johanson et al. 2001). It is clear that viral load determination in plasma is very important in infections by CMV, HCV, HBV, and HIV.

Serological tests are more complex to interpret but have the potential of demonstrating that the virus is sustaining a primary or reactivated infection at the time clinical symptoms are present (see above).

In all cases, detection of virus, viral antigens, or viral nucleic acid is preferable to antibody detection as an indicator of current or recent viral infection. In many cases, direct testing for virus rather than antiviral antibody can provide rapid diagnosis that can be vital in affecting the management of the patient or patient contacts. Improved technology, automation, and reagents have allowed rapid, cost-effective viral detection, which has contributed significantly to better patient care.

Thus, the viral diagnostic laboratory has become a most important adjunct to patient care. The physician can be provided data on rapid, sensitive, and accurate testing that can identify a virus, indicate its genotype, phenotype, and/or serotype as is appropriate. In this regard, in an increasing number of cases the physician can select an antiviral agent and can even tailor drug combinations to avoid specific patterns of antiviral resistance. It is exciting to recognize the advances we have witnessed over these past 30 years in viral diagnosis and therapy, and to project to the future when even more effective tools can be expected to impact on the ability of physicians to help their patients.

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Immunoprophylaxis of viral diseases

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INTRODUCTION

Vaccines are among the most effective and cost-effective means for the prevention of disease. The development of viral vaccines predates the recognition of viral agents as causes of disease by over a century; their use has resulted in marked decreases in the incidence or eradication of diseases that were important causes of human mortality. Vaccines are now available to prevent 13 viral diseases, and new generations of vaccines being developed through molecular techniques hold the promise of providing protection against others.

The modern era of vaccine development began with the demonstration by Jenner that inoculation with cowpox virus provided protection against smallpox and the subsequent wide use of variolation to protect against this disease. The effective use of variola vaccine worldwide culminated, in 1980, in the certification of eradication of smallpox after a decade-long campaign coordinated by the World Health Organization (Fenner et al. 1988). By 1977, viral vaccines to prevent rabies, influenza, yellow fever, poliomyelitis, measles, rubella, and mumps had also been developed and used with striking success to reduce the disease burden due to these viruses. Following the eradication of smallpox, global immunization programs to prevent six childhood diseases, including poliomyelitis and measles, were implemented (Galazka 1994). These programs have achieved 70–75 percent global coverage with these vaccines, and are now being expanded to include hepatitis B vaccine (all countries) and yellow fever vaccine where this disease is endemic (World Health Organization and UNICEF, 2002; Vaccines and Biologicals 2002a). A goal for global eradication of poliomyelitis was established for the year 2000; to date, disease incidence has been reduced by 99 percent, with disease endemic in only six countries and eradication certified in the Americas (1994), the Western Pacific (2001), and Europe (2002) (Vaccines and Biologicals 2004). The global strategy, which includes achieving high routine infant vaccination coverage for oral polio vaccine, mass vaccination campaigns with house-to-house vaccination to interrupt remaining chains of transmission, and intensive surveillance for acute flaccid paralysis in children, is being implemented in the remaining countries where polio is endemic (Hull et al. 1994). A regional campaign to eliminate measles has interrupted disease transmission in the Americas; the key elements of this strategy are now being implemented in Africa and elsewhere through a global partnership whose goal is to reduce global measles mortality by 50 percent by 2005 (de Quadros et al. 2003; Strebel et al. 2003).

Viral vaccination has also seen new challenges, including a return to the use of smallpox vaccine in response to the threat of bioterrorism, withdrawal of the use of effective rotavirus vaccines in the USA due to rare adverse events (intussusception), and public concern over safety of viral vaccines including measles, mumps, rubella (MMR), and hepatitis B (Centers for Disease Control and Prevention 2003c; Murphy et al. 2001; Institute of Medicine 2001a, 2002a).

IMMUNOPROPHYLAXIS OF VIRAL INFECTIONS

The primary objectives of viral immunoprophylaxis are to prevent viral infection or to modify viral disease. Active immunoprophylaxis with viral vaccines stimulates the immune system to develop antibodies, cell-mediated immunity, or both, to prevent infection (e.g. measles, polio), and is usually given before exposure to disease. Vaccines (e.g. rabies, hepatitis B, and hepatitis A) may be effective in preventing disease after exposure, because the immune system is stimulated during the long incubation periods of these infections. Vaccines intended to modify the course of previously established infection (e.g. human immunodeficiency virus (HIV) infection, varicella vaccine to prevent or modify herpes zoster) are currently undergoing clinical trials.

Passive immunoprophylaxis, using pooled immunoglobulins prepared from human or animal serum or monoclonal antibodies from cell culture, can provide temporary protection against infection (pre-exposure prophylaxis for hepatitis A, respiratory syncytial virus) or can prevent or modify infection after exposure (disease-specific immunoglobulins to prevent rabies, hepatitis B, varicella).

TYPES OF VIRAL VACCINES

The two classic approaches to active viral immunization include inactivated viruses or their purified components, and live-attenuated vaccines. For some viral diseases, both approaches have been used (poliomyelitis, measles, influenza).

Inactivated viral vaccines

Inactivated vaccines may consist of whole virus particles, purified antigenic surface proteins or smaller peptide epitopes (Table 1). Inactivated whole virus vaccines are produced by the multiplication of virus in other animal species (e.g. rabies in mouse brain); in eggs, chick embryos or allantoic fluid (influenza, measles, mumps); or in cell culture (rabies in human diploid cells, polio, rubella), followed by purification and inactivation. Whole virus vaccines have the advantage of producing immune responses not only to surface proteins but also internal components, which may help eliminate viral infection. In the past, purified antigenic protein vaccines have been produced either by disruption of whole virus and purification of component proteins (split virus influenza) or by purification from the blood of virus carriers (plasma-derived hepatitis B vaccine). Newer generations of inactivated vaccines have been developed by genetic engineering, inserting DNA that encodes antigenic viral proteins into other micro-organisms (*Escherichia coli*, yeast, CHO cells, other viruses), which produce recombinant proteins that are subsequently purified (hepatitis B surface antigen, human papilloma virus (HPV) capsid polypeptides, and herpes simplex virus (HSV) glycoproteins in yeast). Most inactivated vaccines are combined with an adjuvant such as aluminum hydroxide (alum) to increase the immunogenicity of the vaccine. However, vaccines

Table 1 *Currently available inactivated viral vaccines*

Vaccine	Vaccine type	Uses
Inactivated polio	Killed whole virus	Universal childhood immunization (some developed countries); immunization of immunocompromised people
Hepatitis B	Purified HBsAg produced by recombinant yeast, mammalian cells or from plasma	Routine childhood or adolescent immunization; immunization of high risk adults; postexposure immunization with HBIG
Influenza	Killed whole virus, subvirion or purified surface antigen preparations	Vaccination of high risk individuals, elderly, children 6–23 months
Rabies	Killed whole virus	Postexposure vaccination of individuals, with HRIG; pre-exposure: veterinarians/travelers
Hepatitis A	Killed whole virus	Pre-exposure vaccination: high risk people and travelers
Japanese B encephalitis	Killed whole virus	Pre-exposure vaccination of travelers to endemic areas; routine or mass vaccination in endemic areas
Tick-borne encephalitis	Killed whole virus	Pre-exposure vaccination of travelers to endemic areas; (?) routine or mass vaccination in endemic areas
Under development (late stage)		
Human papillomavirus (prophylactic)	Virus-like particles prepared from surface capsid proteins (L1) produced in yeast	Pre-exposure vaccination of at-risk persons
Herpes simplex	Subunit glycoproteins D and B producd in yeast	Pre-exposure vaccination of at-risk persons

adjuvanted with alum lose potency when the vaccine is frozen.

New approaches to development

Other methods for producing inactivated vaccines include the use of synthetic peptides and idiotypic antibodies. Synthetic peptides, initially produced in 1963, were thought to have great promise as vaccines, especially as DNA sequencing technologies permitted identification of the precise sequences of antigenic sites of viral proteins (Brown 1988). The antigenic sites of viruses may be identified by determining the three-dimensional structure of the virus, comparing sequences of mutant and wild-type viruses or identifying hydrophilic regions of surface proteins and constructing peptides duplicating these sequences. Production of highly purified peptides is feasible, potentially inexpensive and results in a well-defined product without risk of containing adventitious agents. The feasibility of inducing protection in an immunized animal model has been demonstrated by use of an icosapeptide of the surface protein of the foot-and-mouth disease virus (Murphy and Channock 1990). However, other efforts with human disease viruses have met with less success. Peptides are often poorly immunogenic, may stimulate development of binding but not neutralizing antibodies, do not generally induce T-cell responses unless coupled to larger proteins, and cannot mimic conformational epitopes. Efforts to overcome these limitations include coupling the peptide genetically to a fusion protein (e.g. HBsAg), coupling to peptide carriers containing T-cell helper epitopes, and incorporating antigens into aggregates or self-assembling particles (Ellis 2004). Although experimental vaccines have been produced (influenza, foot-and-mouth disease) and found to be protective in animals (canine parvovirus and mink enteritis virus), at present it is uncertain whether viral vaccines for use in humans will follow (Meloen et al. 1995; Ellis 2004).

Anti-idiotype vaccines are based on the principle that neutralizing antibodies to viral antigens contain the mirror image of these epitopes, known as idiotypes. Anti-idiotype antibodies can be generated that mimic the conformation of the original viral epitope, and can then be used to induce neutralizing antibodies in the host. Immunization with anti-idiotype antibody results in the development of neutralizing antibodies to several viruses (poliovirus, hepatitis B surface antigen (HBsAg), rabies) and protection against disease in animal models (Dalgleish and Kennedy 1988). However, the levels of antibodies induced have been less than those induced by the original antigen, and the practicality of this approach for viral vaccines, for which many alternative production methods are available, remains to be demonstrated (Ellis 2004).

Transgenic plants have been engineered to produce viral antigens (e.g. HBsAg), with the potential advantages of producing low cost vaccines which can be administered orally (fed to recipients). Studies to date have demonstrated production of specific mucosal and serum antibodies against antigens in mouse models and reduction in disease in a pig gastroenteritis model, but studies in humans are still at an early stage (Kapusta et al. 2001).

Inactivation of viral vaccines

Inactivated whole virus vaccines produced in culture, animal tissues, or human serum must be subjected to steps that can inactivate the original virus as well as any adventitious agents that may be present in the initial substrate. Formalin has been most commonly used to ensure inactivation of residual viral activity in such vaccines. A balance between the concentration of formalin and the duration of treatment is needed to eliminate residual infectivity of the virus without destroying immunogenicity. Inadequate inactivation of poliovirus by formalin resulted in an outbreak of paralytic poliomyelitis due to inactivated polio vaccine soon after its introduction in the USA (Nathanson and Langmuir 1963). Review of this incident subsequently led to improvement in methods of viral purification and quality control of formalin inactivation procedures. Other inactivating agents that may be used include heat, acetylethyleneimine, and β-propiolactone. These inactivating agents may also provide protection against adventitious agents which can replicate in the cell or animal substrates used to produce vaccines. However, this protection depends on the sensitivity of the agent to the inactivation process. The failure of formalin to inactivate SV40 virus contamination in inactivated polio vaccine originating from monkey kidney cell substrate between 1955 and 1963 raised concern about the safety of this vaccine (Melnick and Stinebaugh 1962). Although some genomic studies suggest SV40 infection may be associated with rare carcinomas, such as mesothelioma and ependymoma, multiple epidemiological studies have not documented increased risk of any cancers in recipients of inactivated poliovirus vaccine (IPV) produced during this period (Institute of Medicine 2002b).

DNA vaccines (plasmid vaccines)

DNA vaccines represent a highly promising strategy for vaccine development. DNA-encoding antigenic portions of viruses – envelope, core, or nucleoprotein – are inserted into a plasmid, which can be injected directly into the host as ‘naked’ DNA, free of protein or nucleoprotein complexes. Host cells take up the DNA and may express the encoded proteins. Because the proteins are produced intracellularly, they enter the major histocompatibility complex (MHC) class I pathway and stimulate a strong cell-mediated immune (CMI) response, in contrast to inactivated vaccines, which enter

the MHC class II pathway and produce, primarily, an antibody response (Fynan et al. 1995; McDonnell and Askari 1996). This predominantly cell-mediated immune response may assist in immune control of both acute and chronic viral infections. The method of vaccine delivery may determine the nature of the T-helper response; saline injections induce primarily Th1 response (CMI), whilst delivery by microprojectiles ('gene-guns') induces Th2 response (antibody). Multivalent vaccines can be constructed that include multiple antigens from a single organism or antigens from several different organisms, or include chimeric proteins (e.g. interleukin-2 immunoglobulin) constructs that enhance immune response. Although prototype naked DNA vaccines have generally elicited weak immune response, facilitation of immune response has been accomplished by incorporation into gene-guns and by coating with cationic lipids, lipospermines, and other molecules that facilitate transfer across membranes (Ellis 2004).

A prototype naked-DNA vaccine using the core nucleoprotein of influenza virus has been developed that protects mice from lethal doses of both homologous and heterologous strains of influenza virus. This vaccine induces both humoral antibody and CMI. Although antibodies to surface glycoproteins protect against infection from the homologous strain, it is likely that CMI provides protection against heterologous strains. DNA vaccines have been demonstrated to stimulate CMI to surface and internal proteins to HIV, hepatitis B virus, influenza virus, and other viruses in animal and primate models and in human trials (Srivastava and Liu 2003). DNA vaccines have also been shown to elicit antibodies against various viral proteins, including influenza, HIV, HBV, rabies virus glycoprotein, herpes simplex glycoproteins, papillomavirus, and hepatitis C virus. Secretory antibodies can be induced through formulation with cationic lipids, encapsulation in microparticles, and other approaches. Candidate vaccines to prevent hepatitis B virus, HIV, and influenza have reached phase 1 or 2 clinical trials in humans. Candidate HIV vaccines induced weak lymphoproliferative responses and interferon chemochines in naive subjects, but these did not persist. Similar trials in HIV-infected persons increased CMI response to rev, nef, and tat, but produced no changes in lymphocyte subsets. However, an HBV DNA vaccine delivered by gene gun has induced protective levels of antibody in naive subjects. Some candidate vaccines induce strong and long-lasting responses in infant experimental animals, indicating their potential use in routine childhood immunizations (Butts et al. 1998).

A promising new approach is to use DNA vaccines in a sequential vaccination strategy, in which the first dose is a DNA vaccine followed by a dose of a live recombinant vectored vaccine (e.g. vaccinia virus, canarypox virus, or adenovirus) carrying similar genes. This strategy is being pursued most actively for HIV vaccines. The combination of DNA vaccine containing multiple proteins of simian immunodeficiency virus (SIV) and HIV, boosted with modified Ankara vaccinia strain containing similar antigens, has induced strong cellular and weak antibody immune responses and protected against virulent SIV infection (Amara et al. 2001). Key concerns about DNA vaccines include possible mutagenesis if the DNA plasmid becomes inserted into the host genome; the potential for developing tolerance to the antigen because of ongoing production in host cells; and the potential for immune attack on the cells producing the encoded antigen.

Live-attenuated viral vaccines

Currently available live-attenuated virus vaccines have been prepared by two approaches: (1) from related species of virus (Jennerian approach: vaccinia to prevent smallpox; rhesus rotavirus), and (2) by serial passage of virus through live organisms or through one or more cell culture lines (Table 2). Examples of the latter include yellow fever vaccine, developed via passage of wild virus in mice and subsequently in chick embryos; and polio (types 1 and 3) and measles vaccines, initially passaged in monkey kidney cells and chick embryo fibroblasts, respectively. Concern about the presence of adventitious viruses in animal-derived cell lines (SV40 virus in monkey kidney cell lines) has led to increased use of human diploid cell lines for producing live virus vaccines (rubella, varicella, polio). Several cell lines (WI-38 and MRC-5) are now commonly used. The World Health Organization has established guidelines for assessing the safety of vaccines produced in human cell lines (World Health Organization 1987).

Live-attenuated vaccines may also be produced via genetic reassortment. Among viruses with multiple-strand genomes, reassortants that combine the genomes encoding antigenic surface proteins from wild virus strains with genomes from attenuated or related strains of virus (cold-adapted influenza; rhesus- or bovine-based rotavirus vaccines) have been developed and shown to be effective (Murphy 1993; Kapikian 1994).

New approaches to development

Other approaches to the development of live virus vaccines include directed mutagenesis, use of strains of viruses from related species, and use of viral vectors such as vaccinia, and canarypox. Methods for direct mutagenesis include growth in low temperatures (25 or 32–34°C) to select temperature-sensitive or cold-adapted strains which do not grow well at human body temperature (Murphy and Channock 1990). Molecular biological techniques, combined with better understanding of molecular aspects of viral replication, can be used to develop specific mutations, such as deletion mutants,

Table 2 *Currently available live-attenuated viral vaccines*

Vaccine	Vaccine type	Uses
Oral polio	Attenuated trivalent	Routine childhood immunization; mass campaigns
Measles	Attenuated (Schwarz, Moraten, others)	Routine childhood immunization; mass campaigns
Rubella	Attenuated (RA 27/3)	Routine childhood immunization; adolescent girls; susceptible women of childbearing age
Mumps	Attenuated (Urabe or Jeryl Lynn)	Routine childhood immunization
Measles, mumps, rubella (MMR)	Attenuated	Routine childhood immunization (one or two doses)
Varicella	Attenuated (Oka strain)	Routine childhood immunization; susceptible adolescents and adults
Influenza	Cold adapted/reassortants	Immunization of high risk children/adolescents/adults
Yellow fever	Attenuated (17D)	Routine immunization or mass vaccination in endemic areas; vaccination of travelers to endemic areas
Smallpox (limited availability)	Vaccinia virus	Vaccination of research workers, military, BT response teams
Under development		
Rotavirus	Rhesus reassortants (four serotypes). Bovine reassortants, human neonatal rotavirus strain	Routine childhood immunization
Herpes zoster	Attenuated (Oka strain)	Adults

which are unlikely to revert to wild strains. Vaccines developed from related animal viruses include rhesus and bovine rotavirus as candidate strains to prevent human rotavirus infection, including reassortants that incorporate the surface protein genes for human rotavirus types 1, 2, 3, and 4 (Kapikian 1994; World Health Organization 2003b). Bovine parainfluenza virus 3 induces neutralizing antibodies to human parainfluenza virus type 3, and is a candidate vaccine to prevent this disease.

Large viruses such as vaccinia, other orthopoxviruses, and adenovirus can incorporate DNA that encodes essential proteins from other viruses (e.g. hepatitis B, rabies, measles hemagglutinin, or fusion proteins), attached to viral promoters that ensure adequate production of these antigens (Perkus et al. 1985; Moss et al. 1988). Vaccination results in replication of the parent virus, production of the encoded viral proteins and stimulation of both humoral and cell-mediated immunity. Although there has been some success in inducing immune response to the encoded proteins (HBsAg, influenza A, herpes simplex glycoprotein, rabies) and protection has been induced in experimental animals, concern about the potential for serious adverse events following vaccinia vaccination and limited success in inducing a strong antibody response have hampered development of vaccinia recombinants for humans. The use of canarypox virus, which undergoes only abortive infection in humans, may overcome concerns about the safety of such vaccines, and candidate vaccines have induced high levels of antibody against rabies glycoprotein and the measles hemagglutinin gene in humans. Yellow fever vaccine virus is being used as a foundation for development of Japanese encephalitis virus (JEV) and West Nile virus vaccines, through substituting JEV and West Nile surface antigens for those of yellow fever virus (Langevin et al. 2003; Monath et al. 2003).

Sequential vaccination, either live vectored vaccines followed by protein or glycoprotein antigens, or with DNA vaccines followed by genetically engineered live vaccines, has produced stronger antibody and cell-mediated immunity than either antigen alone (Ellis 2004). Vaccination with a DNA-based HIV vaccine followed by canarypox HIV vaccine has produced promising results for preventing HIV infection (Amara et al. 2001), while vaccination with a DNA-based vaccine followed by adenovirus vaccine has provided protection against Ebola virus infection in animals (Sullivan et al. 2003).

RISK OF ADVENTITIOUS AGENTS

Possible contamination with adventitious agents is also a concern with live virus vaccines (Waters et al. 1972). A series of tests for potential contaminating agents is undertaken during vaccine development, safety testing, and postlicensing monitoring of vaccines (World Health Organization 1987). However, concerns regarding agents not detectable by current methods continue to arise despite the apparent safety of these vaccines.

Immunological basis of response to active immunization

Development of an immune response generally requires the interaction of T lymphocytes with antigen processing and presenting cells (dendritic or macrophages) (McDevitt 1980; Lanzavecchia 1985; Claman 1992). T-cell response is induced following the uptake of antigen by mononuclear phagocytes or dendritic cells, and can be enhanced by use of an adjuvant; the antigen is processed

and presented, in association with MHC antigens, to T-helper cells. T cells recognize polypeptide antigens of 8–20 amino acids in size, presented in association with specific MHC molecules; the type of MHC molecule with which the antigen is presented by antigen-processing cells depends on the source and processing of the polypeptide. These in turn determine the primary type of T-cell response, either cytotoxic or helper. Presentation to T-helper cells results in secretion of immune mediators (cytokines) that can stimulate the maturation of naive T cells and communicate between leukocytes (via interleukins) to regulate the immune response (Baker 1975; Reinherz and Schlossman 1980; Arai et al. 1990).

Antigens from inactivated vaccines are absorbed into vacuoles, and processed and presented with MHC class II antigens; antigens from live-attenuated vaccines or vectored vaccines, produced within the cell, are processed in microtubules and presented with MHC class I antigens. Depending on the antigen and its MHC presentation, T lymphocytes differentiate into either T-helper type 1 cells (Th1, stimulated by MHC I-associated antigen), which mediate CMI, or T-helper 2 (Th2, with MHC II), which assist B cells in developing antibody production. Each of these subsets produces different interleukins and other immune mediators responsible for modulating the immune response.

An antibody response to an initial dose develops 2–6 weeks after immunization with inactivated antigens but may be incomplete even after two doses; after effective priming, booster responses occur within 4–14 days. The initial response is usually IgM antibodies, followed within weeks by IgG antibodies. Response to live vaccines follows a cycle of replication of the vaccine virus, and a period of several weeks or months for full development of the immune response. Response to measles vaccination is usually maximal by 6 weeks, but in younger children antibody titers may continue to rise for several months after vaccination.

Immune response to inactivated and live-attenuated vaccines

INACTIVATED VACCINES

Inactivated or purified viral vaccines induce responses only to the components present in the vaccine. Multiple doses, usually three or more, are necessary to induce a satisfactory response that will persist for long periods; booster doses may be necessary to ensure lasting protection. Most inactivated vaccines are given parenterally, and induce either no mucosal antibody or lower levels than live-attenuated vaccines administered by the oral or nasal routes. Inactivated vaccines may have limited ability to induce T-cell-mediated immunity, which is induced more strongly by antigens produced intracellularly as occurs with replication of live viral vaccines. Microencapsulation of inactivated viral vaccine antigens in microparticles prepared from polylactide-coglycocide and polylactide polymers can allow delayed release of these antigens over many months, and shows promise for reducing the number of injections required to successfully immunize with inactivated antigens (e.g. hepatitis B) (Singh et al. 1997).

The advantages of inactivated vaccines are the absence of risk of disease due to the vaccine virus, given adequate inactivation; greater thermal stability and ease of handling in routine vaccination programs; and a lower risk of contamination with active adventitious agents than in live-attenuated vaccines. Nevertheless, examples of both inadequate inactivation and presence of active adventitious agents were documented in the past in inactivated polio vaccine (Nathanson and Langmuir 1963; Mortimer et al. 1982), although these problems have not been observed with hepatitis B, hepatitis A, or any other recently developed inactivated vaccines. Inactivated vaccines can be given safely to people with immunosuppressive conditions.

A disadvantage of inactivated vaccines is the possible potentiation of the disease that the vaccine is intended to prevent. The original killed measles vaccines induced a detectable immune response, but recipients exposed to wild measles virus developed atypical measles with systemic symptoms and giant cell pneumonia (Annunziato et al. 1982). Subsequent analyses indicated that the formalin treatment destroyed the antigenicity of the measles fusion protein, resulting in an unbalanced response (to hemagglutinin only). Similar accentuation of respiratory syncytial virus disease in recipients of a formalin-inactivated vaccine was observed in early trials, and later shown in experimental animals to result from stimulation of formation of binding, but not neutralizing, antibodies to the surface F and G glycoproteins (Murphy and Channock 1990).

LIVE-ATTENUATED VACCINES

Live-attenuated vaccines have the advantage of inducing a complex immunological response simulating natural infection. Replication of the vaccine organism and processing of antigens mimic those of the natural organism, and both humoral and cell-mediated responses are generated to a variety of antigens. Because these antigens are similar or identical to those of the wild organism, responses are usually more effective and cross-reactive than those induced by inactivated vaccines. In addition, mucosal antibody may be stimulated directly by such vaccines when administered through oral or nasal routes (McGhee et al. 1992). Administration of oral or nasal live vaccines is more convenient than injection of inactivated vaccines. Immunity induced by one dose of a live-attenuated vaccine is long lasting, possibly life-long. However, the strength of humoral response is usually less than that

of natural infection, and detectable antibodies may wane with time and result in some loss of protection. Induction of immunity by live vaccines can be inhibited by passively acquired antibody, whether from transplacental acquisition from the mother or from receipt of immunoglobulin-containing blood products; thus, achieving optimal response relies on ensuring that passive antibody (e.g. of maternal origin or acquired through administration of blood products) has declined to noninterfering levels. In addition, because the response may be incomplete (90–95 percent) after a single dose, two or more doses may be necessary to induce sufficient immunity to protect both the individual and the community.

The potential disadvantages of live vaccines include residual virulence of the vaccine in both healthy and immunocompromised hosts; possible contamination with adventitious agents; possible interference of response by other viral infections; and greater lability of the vaccine. Potential reversion to virulence has been well demonstrated with oral polio vaccine, paralytic poliomyelitis occurring in either vaccinees or their contacts at a rate of approximately 1 per 2.5 million vaccine doses distributed, with greatest risk after the first vaccine dose (Strebel et al. 1992). Persistent viral infection may occur in immunocompromised people after inadvertent use of measles and oral polio vaccines, and may rarely occur in healthy adults given rubella vaccine (Institute of Medicine 1991, 1993). However, measles vaccine has not been linked to the development of subacute sclerosing panencephalitis. Early lots of oral polio vaccine and yellow fever vaccine were contaminated with SV40 virus and avian leucosis virus, respectively. Although these were not linked with any long-term consequences (Waters et al. 1972), cell substrates used for vaccine production are now tested to ensure that these and other known pathogenic viruses are not present. Nevertheless, it may not be possible to ensure the absence of any adventitious agents in live virus vaccines. The stability of live virus vaccines depends on appropriate handling. Such vaccines require a sound cold chain, with storage at either 0–4°C (measles; measles, mumps, rubella (MMR)) or −15°C (polio, varicella), which can increase the cost of vaccination and impede their use in developing countries.

Determinants of response to viral vaccines

Vaccine and host characteristics are the primary determinants of vaccine response. Response may be affected by vaccine dosage, adjuvant, route and site of administration, number and timing of doses, and vaccine handling. Vaccine doses are determined before licensing to ensure a high level of response; adjuvants such as aluminum salts (hydroxide or phosphate), used only with inactivated antigens, stimulate a better response with a lower dose of antigen. New adjuvants based on improved understanding of immunology of vaccine response are now in clinical trials, and promise further to enhance the immune response in future inactivated viral vaccines (Jennings et al. 1998). An oil–water emulsion MF59 is now used in a split virion influenza vaccine that increases immunogenicity compared with that achieved with alum adjuvant (Ellis 2004). The route of administration – intradermal, subcutaneous, intramuscular, or mucosal – can determine both the strength and the nature of the immune response. Mucosal administration (intranasal or oral) stimulates higher levels of mucosal immunity (IgA antibodies), which may inhibit disease transmission with greater effectiveness than parenteral administration, which induces limited or no mucosal response (McGhee et al. 1992). Intradermal vaccination with low doses may induce antibody responses similar to those induced by intramuscular or subcutaneous administration of recommended doses, but is more difficult to deliver precisely and achieves less predictable responses. Intramuscular injections should be given in the anterior thigh (infants) or deltoid (older children and adults); injection into the buttocks produces a lower response in adults, probably owing to delivery of the vaccine into adipose tissue (Centers for Disease Control and Prevention 1991). The timing of vaccine doses is important: a minimum interval of 1 month between primary doses is usual for inactivated vaccines; delay of a third or reinforcing dose for 6 or more months after the first enhances the response and duration of antibody persistence, and is preferred unless high risk of disease necessitates shorter intervals. Intervals of 4–6 weeks are considered necessary to ensure optimal response to successive doses of live virus vaccines. The recommended routes and sites of administration and the timing of doses are devised to ensure optimal effectiveness in disease prevention and should therefore be used.

Host factors

Host factors that affect immune response include genetic factors (MHC polymorphism), age, nutritional and immune status, gender, pregnancy, and smoking. Although genetic factors, such as MHC polymorphism, are known to affect response at a molecular level, they have a limited effect on population response to available vaccines. Age is an important factor in response to immunization. Newborn infants generally do not develop as strong a response to inactivated vaccines as older infants or children (hepatitis B), and with certain vaccines maternal antibody may inhibit response (IPV, hepatitis A). For live vaccines such as measles, inhibition of the response by maternal antibodies determines the optimal timing for vaccination in early childhood.

The response to vaccines is excellent in young and adolescent children and in young adults, but decreases with increasing age. Male gender in adults and pregnancy have minor negative effects on antibody response of limited clinical significance; smoking decreases response to many antigens, and may increase the risk of nonresponse when other negative factors are present (Hadler and Margolis 1992). Extreme debilitation, acquired or congenital immunodeficiency disorders, diseases or treatments that cause immunosuppression, and some chronic diseases (renal disease, diabetes) can decrease immune response. For people with such conditions, inactivated vaccines are recommended despite lower effectiveness but may require higher or more frequent doses to achieve optimal response; live vaccines are often contraindicated owing to the risk of disseminated disease and possible death due to the vaccine organism (Centers for Disease Control and Prevention 2002a).

Measurement of response

Ideally, reliable laboratory tests should be available to measure the presence and strength of each of the major effectors of protection against the disease. In practice, tests for the presence of antibody are usually available (e.g. radioimmunoassay (RIA), enzyme immunoassay (EIA), complement fixation, immunofluorescent techniques), but these often do not measure the presence of functional (neutralizing or opsonizing) antibody. Tests for CMI are generally available only in research facilities. For some diseases, such as hepatitis B, polio, and measles, there are reliable tests for neutralizing antibodies and the levels needed to confer protection are known; however, only for hepatitis B are inexpensive tests widely available. For other diseases such as rubella and measles, commercial tests are available, often using EIA methods, but specificity is often less well defined, and sensitivity may be lower than that of neutralization assays. Development of better laboratory methods to measure protection and to permit rapid diagnosis of acute disease will remain a priority of disease control programs.

PRELICENSURE VACCINE EVALUATION

Licenses are granted only after extensive review of safety and efficacy data. Before licensing, vaccines are studied first in animals and then in small numbers of humans to determine safety, immunogenicity, and optimal dosages and schedules (phase I and II trials). Phase III trials examine safety and efficacy in larger numbers (1 000–10 000) of subjects. Nevertheless, prelicensing trials are limited in their ability to detect rare adverse reactions (frequency less than one per 1 000–10 000 doses) to vaccination, or adverse events in specific risk groups. Phase IV trials postlicensing may include larger numbers of vaccinees and can better define the frequency of uncommon adverse events. Linking the records of vaccination and of medical outcome in large numbers of children in health maintenance organizations (HMO) or other providers of comprehensive medical care can provide a way to assess causality of temporally related adverse events. In addition, postmarketing surveillance permits detection of new or unanticipated adverse events; reporting of such events observed after vaccination is required in many countries.

PRINCIPLES OF IMMUNIZATION PROGRAMS

The development of successful disease control programs requires safe and effective vaccines and effective public health strategies to prevent disease. Strategies for vaccine use are based on knowledge of epidemiology, consideration of whether eradication or reduction of disease is the primary goal, and the potential for implementing effective programs to deliver vaccines either to the entire population or to target groups (Table 3).
Among the critical aspects of disease epidemiology are: (1) whether the disease causes infection universally in humans (e.g. poliomyelitis, measles, rubella, varicella, and hepatitis B in some populations) or only in certain geographic areas or in specific groups (hepatitis B in developed countries, rabies, yellow fever); (2) the primary age groups affected; (3) the primary reservoir for infection, including whether an animal reservoir exists (rabies, yellow fever) or infection occurs naturally only in humans (hepatitis B, hepatitis A, measles, mumps, rubella, varicella, poliomyelitis); and (4) whether the virus causes only acute infection or results in chronic or latent infection. Strategies targeted at eradication may be considered for diseases without substantial animal reservoirs or chronic or latent infection in humans, whereas reduction must be the initial goal when animal (rabies) or human reservoirs (hepatitis B, varicella) exist (Centers for Disease Control and Prevention 1993c). Mathematical modeling of the possible impact of vaccines on disease incidence may be helpful for selecting immunization strategies (Fox et al. 1972; Anderson and May 1990; Halloran et al. 1994).

Considerations of cost-effectiveness of vaccination programs have also become increasingly important when developing vaccination strategies. Universal use of vaccines to prevent polio, measles, mumps, and rubella saves money when direct costs of health care are considered (Hatziandreu et al. 1994). Studies showing that universal use of varicella and hepatitis B vaccines resulted in savings in combined direct (health care) and indirect costs influenced the development of policy for universal use of these vaccines in the USA (Lieu et al.

Table 3 *Approaches to the control of viral diseases with vaccines*

Disease	Proportion of population infected	Primary age of infection[a]	Chronic carrier	Animal reservoir	Eradicable with vaccine	Type of vaccination program
Polio	High	Child	No[b]	No	Yes	Child; mass campaigns
Smallpox[c]	High	Child	No	No	Yes	Child; containment
Measles	Universal	Child	No	No	Yes	Child; mass campaigns
Rubella	Universal	Child/adult	No	No	Yes	Child; adolescent girls/women
Varicella	Universal	Child	Yes[d]	No	Possible[e]	Child; adolescent; adult (susceptible)
Hepatitis B	Variable	Child/adult	Yes	No	Possible[e]	Infant; adolescent; high risk adult
Influenza	High	All	No	H + A[f]	No	High risk persons (e.g. elderly, chronic medical conditions, infants 6–23 months)
Yellow fever	None to high	All	No	Yes	No	High risk; universal
Rabies	Very low	All	No	Yes	No	High risk; post-exposure

a) Prior to wide use of vaccine.
b) On rare occasions immunodefficient individuals have excreted poliovirus for several years (Kew et al. 1998).
c) Smallpox was certified as eradicated in 1980.
d) Latent infection.
e) Potentially eradicable after several generations of vaccination or if human carriage of virus can be eliminated, or latency-defective vaccine can be developed.
f) Human and animal.

1994; Margolis et al. 1995). More recently, cost-effectiveness studies of varicella vaccine considering both the effect of a vaccination program on varicella and estimated effects on herpes zoster morbidity from modeled data have suggested that a childhood vaccination program may not be cost saving if varicella vaccination leads to an increase in herpes zoster (Brisson and Edmunds 2002).

Programs based on universal vaccination of infants or young children have been implemented successfully (poliomyelitis, measles) in both developed and developing countries (Hinman et al. 1999). Alternative approaches include universal vaccination of adolescents (rubella, hepatitis B), universal vaccination of all or some adults (influenza), and vaccination only of certain target groups or exposed people (rabies, hepatitis B, influenza). Mass immunization campaigns targeted at specific age groups are important in efforts to reduce the incidence of disease rapidly and in eradication and elimination programs for poliomyelitis and measles (Hull et al. 1994; de Quadros et al., 2003).

Vaccine schedules

Childhood immunization programs are now recommended in all countries and have resulted in substantial declines in the occurrence of vaccine-preventable diseases in both developed and developing countries (Galazka 1994; Centers for Disease Control and Prevention 1999b,c; Vaccines and Biologicals 2002a). Childhood immunization schedules are usually established by national governments or advisory bodies. In the USA, policy is established by the Advisory Committee on Immunization Practices (ACIP) of the US Public Health Service and the Committee on Infectious Diseases of the American Academy of Pediatrics (AAP), in consultation with the American Academy of Family Physicians (Centers for Disease Control and Prevention 2002a; Committee on Infectious Diseases 2003; Zimmerman et al 2003; Centers for Disease Control and Prevention 2005a). With effect from July 2004, the unified childhood and adolescent immunization schedule recommends use of vaccines to prevent six viral diseases in all children and adolescents: measles, mumps, rubella, polio, hepatitis B, and varicella (Figure 1). In addition, influenza vaccine is recommended for all children 6–23 months of age and their contacts, as well as contacts of infants <6 months of age, because children in this age group are at substantially increased risk for influenza-related hospitalization. Two viral vaccines are also recommended for select groups: hepatitis A vaccine for children and adolescents in areas of the country with high disease incidence and influenza vaccine for children $\geqslant$6 months with certain risk factors (including asthma, cardiac disease, sickle-cell disease, diabetes). With recommended universal hepatitis B vaccination of children, immunization can start at birth or any time up to 2 months of age, although the schedule indicates a preference for administering the first HepB dose to all newborns soon after birth and before hospital discharge. Routine doses are scheduled at 2, 4, and 6 months of age (diphtheria and tetanus toxoids and acellular pertussis vaccine (DTaP); *Haemophilus influenzae* type b (Hib); polio vaccine (IPV only); and hepatitis B). MMR is recommended at 12–15 months of age and varicella vaccine at 12 to

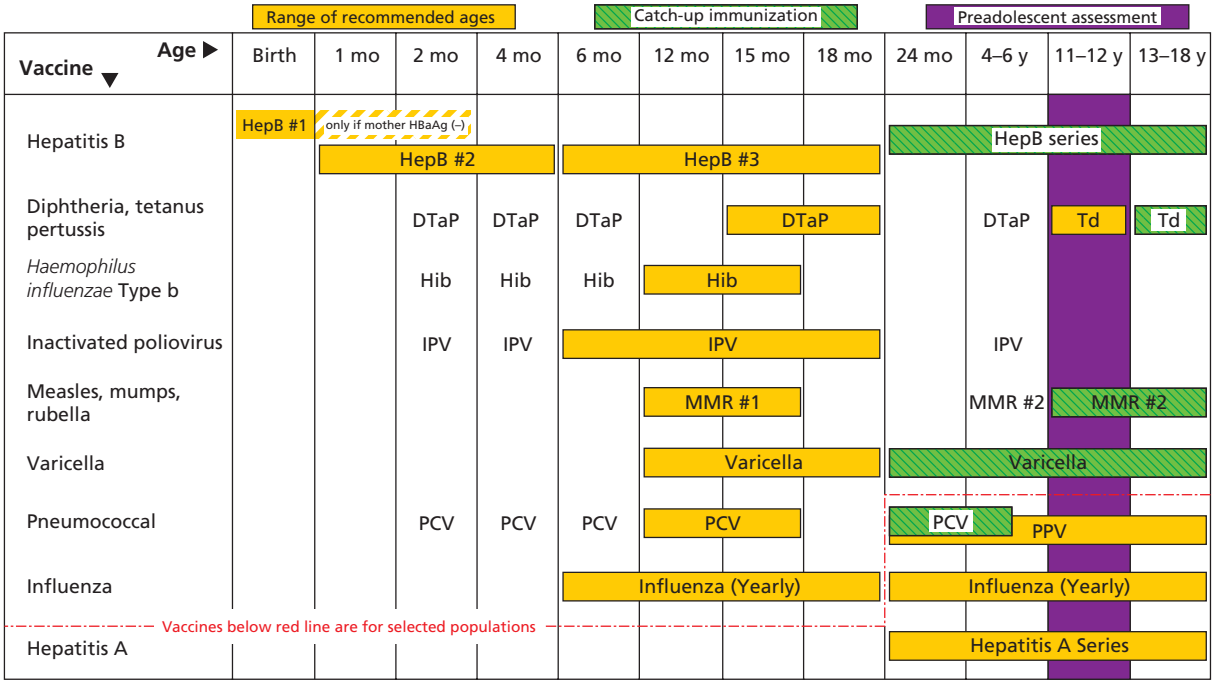

Figure 1 *Recommended immunization schedule, USA, 2005 (approved by the Advisory Committee on Immunization Practices, the American Academy of Pediatrics, and the American Academy of Family Physicians). This schedule indicates the recommended ages for routine administration of currently licensed childhood vaccines for children through age 18 years. Any dose not administered at the recommended age should be administered at any subsequent visit when indicated and feasible. Green bar with diagonal lines indicates age groups that warrant special effort to administer those vaccines not previously administered. Licensed combination vaccines may be used whenever any components of the combination are indicated and other components of the vaccine are not contraindicated. Providers should consult the manufacturers' package inserts for detailed recommendations. Readers should consult www.cdc.gov/nip/acip/recs/child-schedule.htm for footnotes and additional information.*

18 months. Booster doses of DTaP and polio vaccine are recommended at school entry, with the second MMR dose given at primary school entry (Centers for Disease Control and Prevention 1998).

During the early adolescent years (age 11–16 years), immunization status should be assessed to provide a Td booster to ensure that the second dose of MMR has been given, to provide the primary series of hepatitis B vaccine to children who have not already received it and to ensure immunity to varicella. More detail is provided in footnotes to the schedule available at: www.cdc.gov/nip/recs/child-schedule.htm#julydec. Another version of the schedule is provided for children and adolescents who start immunization late or who are at least 1 month behind.

In the UK, recommendations for immunization are issued and periodically updated by the Departments of Health (Joint Committee on Vaccination and Immunization 1996, update available at www.immunisation.nhs.uk. Differences between the USA and UK schedules include, in the USA, a Hib booster between 12–18 months, a dose of DTaP at 15–18 months, and routine use of hepatitis B, varicella, pneumococcal conjugate, and influenza (for children 6–23 months) vaccines, use of hepatitis A vaccine in high incidence areas and use of influenza vaccine in children >6 months with high risk conditions, and in the UK use of meningococcal C conjugate vaccine for all children, and bacillus Calmette–Guerin (BCG) vaccine, and an IPV booster dose at age 13–18 years (as Td/IPV).

Routine childhood vaccinations and schedules vary in other countries (Hinman et al. 1999; Salisbury and Olive 2004). Polio and measles vaccination are universal, and rubella, mumps, and hepatitis B vaccination are offered by most developed and some developing countries. The World Health Organization recommends that all countries provide immunization to prevent seven diseases, including polio and measles, vaccinations being given at birth, at 6, 10, and 14 weeks of age, and at 9 months of age, respectively (Table 4) (Vaccines and Biologicals 2003). Hepatitis B vaccination, beginning at birth or 6 weeks of age, is now recommended in all countries, with primary goal to prevent perinatal and infant HBV transmission in countries with high or intermediate HBV endemicity. Yellow fever vaccination is recommended at 9 months of age in areas where this disease occurs. The World Health Organization (WHO) recommends that a second opportunity for measles vaccination should be offered either through routine immunization or in a vaccination campaign. The Global Alliance for Vaccines and Immunization (GAVI) provides support for the poorest countries to introduce previously under-utilized

Table 4 *Vaccine schedule for Expanded Program on Immunization, World Health Organization*

Age	Vaccine(s)
Birth	TOPV, BCG, Hep B
6 weeks	TOPV, DTP, Hep B, Hib
10 weeks	TOPV, DTP, Hep B, Hib
14 weeks	TOPV, DTP, Hep B, Hib
9 months	Measles, yellow fever (in endemic areas)
After 9 months	Measles second opportunity

BCG, bacillus Calmette–Guérin; DTP, diphtheria and tetanus toxoids and pertussis vaccine; Hep B, hepatitis B; three-dose series beginning at birth; Hib, *Haemophilus influenzae* type b, recommended in high incidence countries; TOPV, trivalent oral polio vaccine dose at birth (optional). Measles second opportunity may be done either as part of the routine schedule or in a campaign.

hepatitis B, yellow fever, and *H. influenzae* type b vaccines.

Vaccination of adults is also recommended in many countries. In the USA, although the childhood immunization program has greatly reduced the burden of vaccine-preventable disease among children, substantial vaccine-preventable morbidity and mortality from diseases such as hepatitis A, hepatitis B, influenza, and pneumococcal infections continue to occur among adults. Each year the ACIP approves the schedule for the routine vaccination of persons aged >19 years (Figure 2). The Adult Immunization Schedule is a harmonized schedule accepted by the American Academy of Family Physicians (AAFP) and the American College of Obstetricians and Gynecologists (ACOG) (Centers for Disease Control and Prevention 2003a).

Influenza vaccination is recommended for high-risk groups and in some countries for all older people (>50 years in the USA) (Figure 2) (Centers for Disease Control and Prevention 2004; Hinman et al. 1999), and >65 years in many developed countries including UK, France, Canada (National Advisory Committee on Immunization, Canada (NACI) 2002 www.hc-sc.gc/pphb-dgspsp/naci-ccni) and Australia (www1.health.gov.au/immhandbook). In addition, vaccination of adults at risk for hepatitis B, hepatitis A, pneumococcal disease, meningococcal disease, measles, mumps, rubella, and varicella may be recommended on the basis of licensing of vaccines and the burden of disease in a risk group, as well as a lack of history of previous disease or immunization. A Td booster is recommended every 10 years.

Simultaneous administration of vaccines

All childhood vaccines may be given simultaneously if necessary, on the basis of data from many studies showing that most vaccines can be given at the same time without compromising safety or immunogenicity. Simultaneous administration of all vaccines for which a person is eligible increases the probability that a child will be fully immunized at the appropriate age. Use of combination vaccines can reduce the number of injections required at a single visit and is preferred over separate injection of their equivalent components (Centers for Disease Control and Prevention 1999d, 2002a). Thus, DTP, Hib, IPV, hepatitis B, MMR, and varicella vaccines may be given simultaneously (King and Hadler 1994). Interference between live virus vaccines other than OPV (e.g. MMR, varicella) can, theoretically, occur if they are given not simultaneously but within close intervals; therefore, live virus vaccines should either be given simultaneously or at least 28 days apart (Centers for Disease Control and Prevention 2002a). Data are limited concerning interference between live vaccines. Interference has been found between certain vaccines for travelers (cholera and yellow fever) (Centers for Disease Control and Prevention 2002a) and in a study conducted in two US health maintenance organizations, children who received varicella vaccine <28 days after MMR vaccination had a greater risk (2.5-fold higher) of varicella vaccine failure compared with those who received varicella and MMR vaccines simultaneously or ⩾28 days apart (Verstraeten et al. 2003). In contrast, one study did not show interference with seroresponse to yellow fever vaccine among persons who received monovalent measles vaccine 1–27 days earlier (Stefano et al. 1999). Immunoglobulins or blood products containing immunoglobulins inhibit the response to certain live virus vaccines (MMR, varicella). The duration of inhibition of response is related to the dose of immunoglobulins, and algorithms have been devised for calculating appropriate delays of MMR, measles, or varicella vaccination after administration of such products (Centers for Disease Control and Prevention 2002a; Siber et al. 1993). In general, MMR and varicella vaccines should be delayed at least 3 months after giving the usual doses of immunoglobulin (to prevent hepatitis A) or blood products, for 5 months after administration of measles immune globulin (IG) or varicella-zoster immune globulin (VZIG) and for longer after higher doses (e.g. ⩾11 months after 2 g/kg IGIV for treatment of Kawasaki disease) (Centers for Disease Control and Prevention 2002a).

Vaccination in specific situations

VACCINATION OF PREMATURE INFANTS

Infants born prematurely, regardless of birth weight, should be vaccinated at the same chronological age and with the same schedule as full-term infants. Some studies suggest that response to hepatitis B vaccine may be diminished in infants with birth weights below

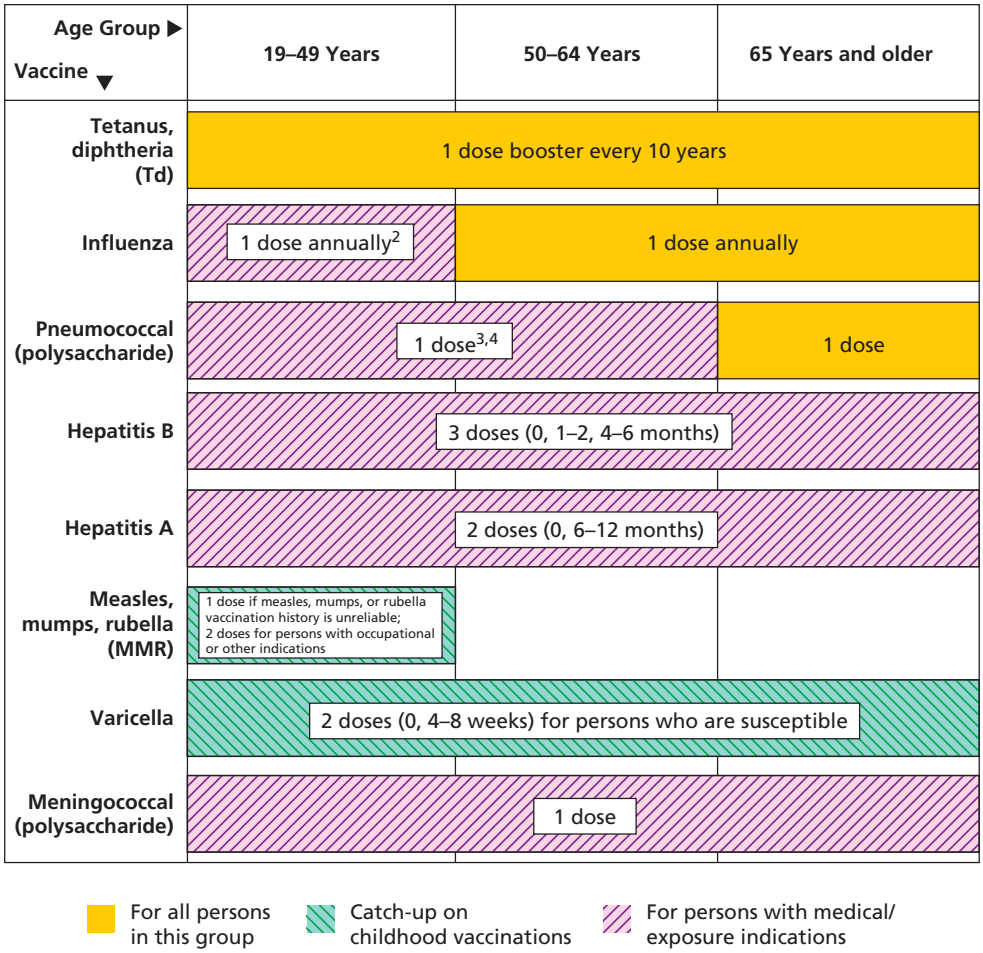

Figure 2 *Recommended Adult Immunization Schedule, 2003–2004 (approved by the Advisory Committee on Immunization Practices, and accepted by the American College of Obstetricians and Gynecologists, and the American Academy of Family Physicians) (Centers for Disease Control and Prevention, 2003a). Readers should consult www.cdc.gov/nip/acip/recs/child-schedule.htm for footnotes and additional information.*

2 000 g; in an infant born to a HBsAg-positive mother and mothers with unknown serostatus, the premature infant should receive hepatitis B vaccine and hepatitis B immune globulin (HBIG) $\leqslant$12 h after birth. If these infants weigh <2 000 g at birth, the initial vaccine dose should not count towards the completion of the hepatitis B vaccine series and three additional doses should be administered, beginning at 1 month of age. In an infant born to an HBsAg-negative mother, vaccination can be initiated either when the infant reaches 2 000 g or at 2 months of age with administration of other childhood vaccines (Centers for Disease Control and Prevention 2002a; Committee on Infectious Diseases 2003).

VACCINATION OF PREGNANT WOMEN

The risk from vaccination during pregnancy is largely theoretical and no evidence exists of risk from vaccinating pregnant women with inactivated virus or bacterial vaccines or toxoids. Hepatitis B and influenza vaccines may be given to those at high risk of these diseases. Because women in the second and third trimester of pregnancy have an increased risk for hospitalization due to influenza (Neuzil et al. 1998), influenza vaccine is recommended for all women who will be pregnant during the influenza season (Centers for Disease Control and Prevention 2004). Live viral vaccines, in general, should not be administered during pregnancy or to women who may become pregnant in the next 4 weeks. However in some circumstances, the benefit of vaccinating a pregnant woman with a live viral vaccine may outweigh the risk when the probability of exposure is high and when infection might cause harm to the mother or the infant, but the vaccine is unlikely to do so (Centers for Disease Control and Prevention 2002a). Live virus vaccines, such as MMR and varicella, are contraindicated in pregnant women on theoretical grounds; however, as no cases of congenital rubella syndrome or congenital varicella syndrome have been reported after MMR or varicella vaccination among over 200 susceptible women exposed during the first

trimester of pregnancy (for rubella vaccine) and >100 susceptible women (for varicella vaccine), inadvertent vaccination is not a reason to interrupt pregnancy (Centers for Disease Control 1989; Shields et al. 2001). Pregnant women may receive OPV (or IPV) and yellow fever vaccines if there is a risk of exposure. Vaccination of family members with MMR live virus vaccine does not pose a risk to pregnant women and because the risk of transmission of varicella vaccine virus is very low, MMR and varicella vaccine should be administered, as indicated, to the children and other household contacts of pregnant women (Centers for Disease Control and Prevention 1996, 1998). Breast-feeding does not adversely affect the responses to live or killed vaccines.

VACCINATION OF PEOPLE WITH ALTERED IMMUNOCOMPETENCE

People with altered immunocompetence require special consideration for vaccination (Centers for Disease Control and Prevention 1993b). Such individuals are often at increased risk of serious adverse consequences of disease; they may also be at increased risk of serious consequences of vaccination or of a poor response. Important groups of immunocompromised people include those with congenital immunodeficiency diseases; acquired immune deficiency syndrome (AIDS); hematopoietic or disseminated malignancy; immunosuppression because of administration of chemotherapy, systemic corticosteroids, radiation or other toxic drugs; and other chronic conditions, including splenectomy and diabetes. All such people can be vaccinated safely with killed, recombinant, subunit, and conjugate vaccines, which are usually recommended in the same doses and schedules as for immunocompetent individuals. Certain vaccines (e.g. influenza) may be specifically indicated for such people. The response to both killed and live vaccines, however, may be suboptimal, and higher doses or an increased number of doses may be needed to ensure protection. Live viral vaccines are generally not recommended for any of these groups, except for people with chronic medical conditions (e.g. diabetes), because of a known or theoretical risk of disseminated infection due to the vaccine. An exception is the recommendation of MMR vaccine for persons with HIV infection without severe immunocompromise, because of the high risk of serious consequences of wild measles infection (Onorato et al. 1988; Centers for Disease Control and Prevention 1998). Similarly, because of the high risk of serious consequences of wild varicella disease, varicella vaccine is licensed for use in immunocompromised people in Europe and in the USA is available under a research protocol for children with acute lymphoblastic leukemia in remission, can be used in persons with impaired humoral immunity, and should be considered for asymptomatic or mildly symptomatic HIV-infected children who should receive two doses of varicella vaccine, 3 months apart (Centers for Disease Control and Prevention, 1999e).

INTERNATIONAL TRAVELERS

International travelers may be at increased risk of exposure to vaccine-preventable diseases, even in developed countries. All children, adolescents, and adults planning international travel should be up to date for all routine childhood immunizations. For certain vaccines, children who travel overseas or live abroad should be vaccinated at an earlier age than recommended for children remaining in the USA. Before their departure, children aged $\geqslant$12 months should have received two doses of MMR vaccine separated by at least 28 days. Children aged 6–11 months should receive a dose of monovalent measles vaccine before departure or MMR if monovalent vaccine is not available (Centers for Disease Control and Prevention 1998). Other viral vaccines that should be considered before travel include hepatitis A and B vaccines, yellow fever, Japanese encephalitis, and rabies (if exposure to rabid animals is anticipated). The need for these vaccines should be determined by consulting specific guidelines for travelers (Centers for Disease Control and Prevention 2003b).

Vaccine safety and vaccine injury compensation

Viral vaccines, like other vaccines, usually cause only minor local and systemic adverse reactions, such as induration and pain at the injection site, fever, and malaise. Serious or life-threatening reactions can occur after some viral vaccines, such as paralytic poliomyelitis following oral polio vaccine, disseminated measles vaccine virus infection in immunocompromised children, or encephalitis or viscerotropic disease following yellow fever vaccine. The Institute of Medicine (IOM) in the USA has reviewed available information regarding the possible causality of serious adverse events following each of the licensed childhood vaccines (Institute of Medicine 1991, 1993, 2001a, 2002a), and continues to regularly review evidence regarding potential adverse events caused by vaccination. For many events, information is considered insufficient to determine causality. However, the panels have been able to classify some events definitively, as summarized in Table 5. These comprise the most comprehensive compilation of data on vaccine safety, although controversy still persists about some events. The panel has also examined broader issues of vaccine safety, concluding that evidence favors rejection of the hypothesis that multiple immunizations cause immune dysfunction or type 1 diabetes. Most recently, the panel rejected the hypothesis of a causal relationship between MMR vaccine or thimerosal-containing vaccines and autism (Institute of Medicine 2004). Based on a previous IOM report that

Table 5 *Summary of Institute of Medicine findings on the relationship of adverse events to individual vaccines*

Vaccine	Establishes causation	Favoring causation	Favoring rejection of causation
Measles (see also MMR)	Death from measles vaccine strain in immunocompromised people	Anaphylaxis	None
MMR (see also measles, mumps, and rubella)	Anaphylaxis/thrombocytopenia	None	Autism spectrum disorders
Mumps (see MMR)	None	None	None
OPV	Poliomyelitis in recipient or contact Death from polio vaccine strain in immunocompromised people	Guillain–Barré syndrome[a]	None
IPV	None	None	None
Hepatitis B	Anaphylaxis	None	Multiple sclerosis
Rubella[b] (see also MMR)	Acute arthritis	Chronic arthritis	None

a) A review of all additional information by the USPHS, which included a re-analysis of the Finnish data, concluded that the available data do not support a causal association.
b) Reviewed by an earlier committee. Initial report categories corresponding to those table headings were 'Evidence indicates a causal relationship,' 'Evidence is consistent with a causal relationship,' and 'Evidence does not indicate a causal relationship.'

found evidence inadequate to reject or accept a relationship between thimerosal preservative in some killed vaccines and neurodevelopmental disorders in children (attention deficit disorder and speech or language delays) (Institute of Medicine 2001b), the USA has removed thimerosal from childhood vaccines, although other countries and WHO have concluded there is insufficient evidence of risk to necessitate its removal from vaccines (see www.who.int/vaccine_safety/topics/thimerosal/statement200308).

Contraindications and precautions for each viral vaccine are usually described in the recommendations for use and in vaccine package inserts. For inactivated vaccines, anaphylactic reaction to a constituent or to a previous dose is usually the only contraindication, whereas, for live virus vaccines, any condition that results in immunocompromise (blood dyscrasias, HIV infection, steroid or chemotherapy, etc.) contraindicates use of the vaccine. Pregnancy is usually a contraindication for live vaccines that might be teratogenic (rubella, varicella), but not for inactivated vaccines. Potential recipients should be questioned regarding the presence of such conditions before any vaccine is administered.

Because viral vaccines may, rarely, cause serious adverse events, some countries have established programs to compensate people who develop permanent injury following vaccination (Centers for Disease Control 1988). A specific table of injuries may describe conditions for which compensation is routinely provided; in addition, anyone who can provide medical evidence of causality may be compensated. In the USA, this program is funded by a special excise tax on each dose of vaccine to which the program applies (DTP, OPV, MMR, Hib, hepatitis B, pneumococcal conjugate, and varicella vaccines). Assuring safe injection is also an essential part of immunization programs. Either single-use disposable or autodisable (AD) syringes should be used for each injection. However, improper reuse of disposable syringes, faulty sterilization of reusable syringes, and needlestick injuries pose a substantial risk of transmission of bloodborne infections (HIV, HBV, hepatitis C virus), and potentially add to the burden of these diseases in many developing countries (Hauri et al. 2004). United Nations International Children's Emergency Fund (UNICEF) and WHO now recommend use of AD syringes in all developing countries, to reduce the risk of transmission of bloodborne pathogens (Vaccines and Biologicals 2002b). GAVI is providing funds for purchase of AD syringes to assist the poorest countries to change from sterilized reusable or single-use syringes. WHO and UNICEF also recommend developing systems for the safe use and destruction of all injection equipment. Jet injectors eliminate the risk of needlestick injuries, but also may pose small risk of transmission of bloodborne diseases (Global Programme for Vaccines and Immunization, 1998).

Surveillance for vaccine-preventable diseases and vaccine supply, coverage, effectiveness, and adverse events

Monitoring the impact of vaccination programs and of the safety of vaccines is critical for refining immunization strategies and for assuring the public and medical community that vaccines are safe. Surveillance has shown the high effectiveness of universal childhood vaccines; compared with the prevaccine era, reductions of over 95 percent in the incidence of disease have been observed in the USA for polio, measles, mumps, and rubella (Centers for Disease Control and Prevention 1999b) and a reduction in varicella cases, hospitalizations and deaths of 70 to 80 percent by 2000 (Seward et al. 2002; Davis et al. 2004; Nguyen et al. 2005).

Surveillance for communicable diseases is maintained in each country, and may include any or all vaccine-preventable diseases (Wharton and Strebel 1995). These data should be monitored to assess the effectiveness of the vaccines and of the vaccination program. Surveillance needs vary according to the stage of the vaccination program. Early in implementation of a new vaccination program or before disease control has been achieved, aggregate reporting of total cases may be adequate. As the program progresses to the disease control stage, surveillance should become case-based; for each reported case of disease, confirmation by laboratory testing (virus isolation or serologic testing) and determination of vaccination status are essential to monitor whether continuing infections are due to failure to deliver vaccine or to low vaccine effectiveness. Information on age of cases is used to describe current epidemiology and to refine vaccine strategies. As the elimination of particular indigenous diseases approaches (achieved in the 1990s for measles in Finland, the UK and the USA), determination of chains of transmission, whether the case is indigenous or imported, and rapid implementation of control measures also become crucial (Peltola et al. 1994; Ramsay et al. 2003; Papania et al. 2004). At the elimination stage of a vaccination program, all healthcare providers are urged to report every suspected case of vaccine-preventable disease promptly to the local or national authorities. Monitoring the effectiveness of vaccines after licensing is essential, particularly during outbreaks in highly vaccinated populations, to reassess effectiveness in wide use and to maintain public confidence in vaccination (Orenstein et al. 1988). Monitoring vaccine coverage is also essential to track national and state vaccine coverage levels to identify pockets of unvaccinated people to target vaccination efforts and to maintain high levels of awareness for disease outbreaks (Vaccines and Biologicals 2002a). Documenting high vaccine coverage is also important; measles cases imported into the USA now have very limited spread due to the high levels of population immunity (Centers for Disease Control and Prevention 2002b).

Monitoring of adverse events after vaccination is also usually required in each country; in the USA it is the joint responsibility of the Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention (CDC). Physicians are required by law to report certain events that occur after vaccination, and should report all suspected adverse reactions after vaccination to the vaccine adverse events reporting system (VAERS) (Centers for Disease Control 1988; Chen et al. 1994). Within a year of licensure of live rotavirus vaccine in the USA in 1999, the VAERS received an unexpectedly high number of reported cases of intussusception clustered 3–7 days after rotavirus vaccine. These reports were the signal that led to the decision to conduct scientific studies to determine whether Rhesus–human reassortant rotavirus (RVV) was associated with intussusception. Based on the results from three studies (case–control, case series, and retrospective cohort) which demonstrated a $\geqslant$20-fold increase in risk of intussusception with use of RVV, the ACIP voted to withdraw their recommendation of use of this vaccine in the childhood vaccination program in the USA (Centers for Disease Control and Prevention 1999f).

Vaccine handling and storage

Vaccines are perishable products that require specific care in handling and storage. Ensuring that a vaccine maintains potency and safety is a responsibility shared by the manufacturer and everyone handling the vaccine. Live-attenuated vaccines, especially MMR, yellow fever, and varicella vaccines, are more susceptible to rises in temperature than are inactivated vaccines or toxoids. Vaccinia (smallpox) vaccine, the freeze-dried preparation available for the smallpox eradication program, is an exception and is extremely stable at high temperatures. All adjuvanted inactivated vaccines are sensitive to freezing and must be protected from freezing to ensure potency. Vaccines that are exposed to damaging environmental conditions may suffer loss of potency without a change in appearance. Live-attenuated virus vaccines should be administered promptly after reconstitution, $\leqslant$30 min for varicella vaccine, $\leqslant$1 h for yellow fever vaccine and $\leqslant$8 h after reconstitution for measles and MMR vaccine (Centers for Disease Control and Prevention 2002a). A vaccine quality control program should be established in each clinical practice, focusing on education of personnel, maintenance of equipment, and adherence to routine daily monitoring of vaccines (Committee on Infectious Diseases 2003).

PASSIVE IMMUNOPROPHYLAXIS

Passive immunoprophylaxis consists of the use of purified antibodies prepared from human or animal serum to prevent infection or to modify the course of infection, when given either before or after exposure. The use of such antibodies was developed in the late nineteenth century, when sera from hyperimmunized horses and rabbits were used to treat diphtheria and tetanus. Although the use of antibiotics has largely reduced the use of passive immunoprophylaxis for bacterial infections (except tetanus, diphtheria, and replacement prophylaxis for those who are agamma- or hypogammaglobulinemic or immunocompromised), passive prophylaxis has maintained a role in preventing the serious consequences of several viral infections.

Immunoglobulin preparations currently used for viral immunoprophylaxis or treatment are prepared from human serum by cold ethanol precipitation (Cohn et al.

1946; Oncley et al. 1949). This yields three fractions, enriched in cryoprecipitates, globulins, and albumin, respectively, determined by the specific ethanol concentration, temperature, pH, and ion and protein concentrations. Immunoglobulin prepared by Cohn fractionation consists of mainly IgG types 1, 2, and 3, but is relatively depleted in IgG4. Immunoglobulin preparations maintain the properties of complement activation and opsonization associated with Fc receptors, and may also have immunomodulatory functions. In 1998, a new type of immunoglobulin product – humanized monoclonal antibodies against respiratory syncytial virus – became available in the USA. This immunoglobulin is produced in the NSO cell line and purified by ion exchange and other chromatography. This product has no known adverse reactions and is free from potential contamination from bloodborne agents.

Types of immunoglobulin

Four types of immunoglobulin products are used for viral immunoprophylaxis: pooled human immunoglobulin (prophylaxis for hepatitis A, measles) for intramuscular use; specific immunoglobulins (hepatitis B immunoglobulin (HBIG), rabies immunoglobulin (HRIG), varicella-zoster immunoglobulin (VZIG), vaccinia immunoglobulin (VIG) used to prevent the respective diseases); intravenous immunoglobulin, used for immunoprophylaxis for persons with primary or acquired immunodeficiencies; and specific intravenous immunoglobulin (IVIG) (RSV-IVIG, CMV-IVIG) used to prevent specific diseases in persons at risk (Table 6) (Finlayson 2003). Pooled human immunoglobulin for both intramuscular and intravenous use is generally prepared from large pools of donors (>1 000) who are not screened for specific antibodies; however, the final immunoglobulin preparation is required to have minimal levels of antibodies to measles and polio (neutralizing), diphtheria (antitoxin), and to hepatitis B surface antigen (anti-HBs). Antibodies to other viral pathogens, including enteroviruses, hepatitis A (HAV) and respiratory syncytial virus (RSV), are also present, although titers may vary widely in different lots. Owing to the relatively high frequency of hepatitis A infection in the general population, anti-HAV titers in immunoglobulin lots produced in the USA have remained relatively constant, although they have decreased in recent years in the UK.

Specific immunoglobulins are produced from a smaller donor pool that has either been screened for high titers of the specific antibody (RSV, cytomegalovirus (CMV)) or been boosted by vaccination of donors (hepatitis B, rabies, varicella-zoster) to ensure high levels of antibody. RSV monoclonal antibody is produced in cell culture, in which the human IgG1 gene has had grafted variable regions of murine monoclonal antibody directed against the F glycoprotein of RSV.

Intravenous immunoglobulin preparations are suitable for administering the larger doses needed by certain groups of immunodeficient patients. To prevent the occurrence of immunoglobulin aggregates, which could activate complement and cause serious reactions if given intravenously, techniques to break them down have been developed, including pepsin or plasmin digestion, reduction and alkylation, β-propiolactone treatment, or ultrafiltration and diafiltration (Waldvogel 1981). These are intended to ensure that >80 percent of IgG is present in monomeric form and that minimal aggregates of dimeric or larger forms exist. Since the mid-1990s, IVIG preparations also undergo steps to inactivate any residual viruses, either the solvent-detergent or heat inactivation or nanofiltration (Finlayson 2003). Manufacturing techniques vary among manufacturers and are continually evolving; package inserts should be consulted for each preparation.

Uses of passive immunoprophylaxis

Immunoglobulin preparations may be used as pre-exposure prophylaxis, given before anticipated exposure to a specific viral agent or as postexposure prophylaxis, either to prevent clinical disease (measles, hepatitis B, rabies) or to modify the course of infection (varicella) (Table 6).

PRE-EXPOSURE PROPHYLAXIS

Pre-exposure prophylaxis with immunoglobulin (NHIG) is now limited to hepatitis A prevention. Immunoglobulin is about 80 percent effective when given in a dose of 0.02 ml/kg for short-term protection to travelers to countries where HAV is endemic, or 0.06 ml/kg for protection for up to 6 months. In developed countries, hepatitis A vaccine is now preferred for pre-exposure prevention of hepatitis A.

IVIG is the treatment of choice for preventing bacterial and viral infections in people with primary immunodeficiency disorders associated with low serum IgG levels (Committee on Infectious Diseases 2003). Doses are usually given every 3–4 weeks, but the frequency of treatment may be tailored to the patient's clinical course and trough levels of IgG. Although IVIG treatment is most valuable in preventing bacterial infections, IVIG also reduces the risk of viral infections, including measles, enteroviruses, and RSV. IVIG is recommended to prevent infection in children with HIV infection with hypogammaglobulinemia (AIDS), people with chronic lymphocytic leukemia, and bone marrow transplantation, and is being studied for use in multiple myeloma.

Specific CMV IVIG is indicated for preventing serious outcomes of CMV infection in recipients of solid organ or bone marrow transplants. A 6-week course of therapy beginning within 72 h of transplantation may be used for

Table 6 *Immunoglobulins currently available for use in viral diseases*

Preparation	Route of injection	Uses
Human immunoglobulin (NHIG)	Intramuscular	Pre- and postexposure prophylaxis for hepatitis A; postexposure prophylaxis for measles; treatment of hypogammaglobulinemia
Hepatitis B immunoglobulin (HBIG)	Intramuscular	Postexposure prophylaxis for hepatitis B (with hepatitis B vaccine): perinatal, sexual, blood/body fluid exposure
Human rabies immunoglobulin (HRIG)	Intramuscular + intralesion	Postexposure prophylaxis for rabies (with rabies vaccine)
Varicella-zoster immunoglobulin (VZIG)	Intramuscular	Postexposure prophylaxis for susceptible infants, immunocompromised people, adults
Vaccinia immunoglobulin (VIG)	Intramuscular	Treatment of complications of vaccinia (disseminated vaccinia)
Immunoglobulin (IVIG)	Intravenous	Treatment of agamma- and hypogammaglobulinemia; prevention of viral infections in immunocompromised
Respiratory syncytial immunoglobulin (RSV IVIG)	Intravenous	Prevention of respiratory syncytial virus infection in premature infants and children with pulmonary dysplasia
RSV monoclonal antibody	Intramuscular	Uses same as RSV-IG
Cytomegalovirus immunoglobulin (CMV IVIG)	Intravenous	Prevention of serious CMV infection in transplant patients (experimental)

kidney, liver, lung, pancreas, or heart transplants. CMV IVIG may be used in conjunction with drugs (gancyclovir) to reduce risk of illness when an organ from a CMV-positive donor is transplanted into a CMV-negative recipient.

Two products, high titer RSV IVIG and RSV monoclonal antibodies, are now available in the USA to prevent RSV infection in children with bronchopulmonary dysplasia and in premature infants (<32 weeks of gestation) (Committee on Infectious Diseases 1998, 2003). These products are 40–55 percent effective in preventing the most serious complications of RSV disease in these children. RSV monoclonal antibody is given intramuscularly monthly in doses of 15 mg/kg during the RSV season (November–April in USA) to children up to 24 months of age, and is generally preferred because of ease of administration and because it does not interfere with live virus vaccines. RSV IVIG is given intravenously monthly in doses of 750 mg/kg, and may be preferred in infants with severe pulmonary disease in whom respiratory infections other than those caused by RSV may be important.

POSTEXPOSURE PROPHYLAXIS

Immunoglobulin (0.02 ml/kg) is recommended for postexposure prophylaxis to prevent hepatitis A in susceptible people with household or other close exposure if it can be given within 2 weeks of exposure. Immunoglobulin prophylaxis may modify hepatitis A disease without preventing virus excretion, and therefore should only be given to food handlers with the caution that strict hygienic precautions should be followed for 6 weeks. Some experts prefer not to give immunoglobulin to food handlers. Immunoglobulin may also be given to prevent measles (0.5 ml/kg) in susceptible people at high risk of severe measles (<12 months of age, immunocompromised) if it is given within 1 week of exposure. Postexposure prophylaxis with specific immunoglobulins is recommended to prevent hepatitis B, rabies, and varicella infection. Hepatitis B immunoglobulin (HBIG) is usually given with hepatitis B vaccine to provide passive–active prophylaxis, which may enhance short-term protection and ensure long-lasting immunity: indications for HBIG include perinatal exposure to an HBsAg-positive mother (given as soon as possible after birth); sexual exposure to an HBsAg-positive person; and needlestick, blood, or other percutaneous exposure to HBsAg-positive blood or body secretions (Centers for Disease Control and Prevention 1991). Rabies immunoglobulin is also given as passive–active immunization with rabies vaccine after exposure to a rabies-infected animal; part of the HRIG dose is administered at the wound site and part at a separate site (Centers for Disease Control and Prevention, 1999i). VZIG is indicated for susceptible people at high risk of severe varicella who have household or other sustained contact (at least 1 h direct contact) with an infected individual. Those with highest priority include neonates exposed to maternal varicella (manifest between 2 days before and 5 days after birth), susceptible immunocompromised people, and premature infants born to

varicella-susceptible mothers (Centers for Disease Control and Prevention 1996). It may also be used for exposed adults, although pre-exposure use of varicella vaccine should greatly reduce the use of VZIG over all and particularly in this population.

THERAPEUTIC USES

Immunoglobulin products may, rarely, be used to treat diseases. Vaccinia immunoglobulin (VIG) is the recommended treatment for people with serious complications of vaccinia immunization (disseminated disease, vaccinia necrosum). IVIG has also been used to successfully treat parvovirus B19 aplastic crises and RSV infection (Finlayson 2003).

Immune plasma is still used very rarely to treat established infections with highly lethal infections such as those due to hemorrhagic fever viruses (Maiztegui et al. 1979; Peters et al. 1991). Such experimental treatment employs convalescent serum from a previously infected patient, but poses the risk of serious adverse events, including immune complex deposition and serum sickness.

Adverse reactions

Immunoglobulin products for intramuscular use are very safe, with few adverse events and no risk of transmission of bloodborne diseases. Adverse reactions to intramuscular immunoglobulin are uncommon and usually mild, consisting of local pain or low-grade fever (Barundun and Morell 1981; Finlayson 2003). Allergic reactions may, rarely, occur; the risk is greater in people with isolated IgA deficiency. The risk of systemic reactions is greater (5–20 percent) with IVIG, and is highest in those with agamma- or hypogammaglobulinemia who are beginning therapy. The risk of reaction may be reduced by decreasing the rate of administration or by premedication with antihistamines, analgesics (aspirin or paracetamol/acetaminophen), or steroids. Infrequent serious reactions may include aseptic meningitis, acute renal failure, and anaphylaxis.

Immune globulins may interfere with response to live virus vaccines, particularly measles vaccine. Measles, MMR, and varicella vaccination should be delayed for 3 or more months after HNIG or IVIG, depending on dose.

Intramuscular IgG produced in developed countries has never been implicated in the transmission of bloodborne diseases, such as hepatitis B, HIV infection, or hepatitis C, even before the screening of donor blood for these agents. This elimination of infectious agents is probably due to a combination of exposure to high concentrations of ethanol (20–25 percent) and the presence of antiviral antibodies that neutralize activity or enhance the removal of virus during precipitation and centrifugation (Wells et al. 1986; Cuthbertson et al. 1987). All plasma donors in developed countries are now screened for these agents. Several incidents of transmission of hepatitis C virus (HCV) infection by intravenous immunoglobulin preparations have been reported, including from one US manufacturer after screening of plasma donors for HCV antibodies was initiated (Rousell, 1988; Williams et al. 1988b; Centers for Disease Control and Prevention 1994). Although this step was intended to eliminate most HCV-positive plasma donors, the removal of HCV antibodies may have permitted HCV not complexed with antibodies to remain in the final product. To eliminate this potential hazard, methods of manufacture have been modified using solvent-detergent, heat treatment, or nanofiltration to eliminate the risk. Rarely, immunoglobulin produced in less developed countries has transmitted bloodborne disease, possibly owing to inadequate quality control (Finlayson 2003).

VACCINES RECOMMENDED FOR ROUTINE USE IN CHILDREN

Measles, mumps, and rubella

Live-attenuated measles vaccine is a universally recommended childhood vaccine and is administered most commonly as a single vaccine according to the WHO/EPI schedule at 9 months of age to children in countries following the basic expanded program on immunization (EPI) schedule and as a combined MMR vaccine to children 12–15 months in countries with expanded immunization programs, which include rubella and mumps. The immune response to each component vaccine is similar whether a combined or single product vaccine is administered. Simultaneous administration of MMR vaccine (or individual component vaccines) and other childhood vaccines does not alter the immune responses to any of them. Vaccination with MMR and its component vaccines is contraindicated for pregnant women and for people with moderate or severe febrile illnesses, with a previous anaphylactic reaction to any of the components of the vaccine, or with immunocompromising conditions (Centers for Disease Control and Prevention, 1998). Recent studies suggest that anaphylactic reactions to eggs are not contraindications to vaccination with measles, mumps, or MMR vaccines (James et al. 1995).

In addition to universal immunization of children with a first dose, the World Health Organization recommends that all children should be given a second opportunity for measles vaccination, either through routine vaccination or in a vaccination campaign (Vaccines and Biologicals 2003). Most developed countries have also implemented a schedule calling for a second dose of MMR vaccine The first dose is most commonly administered between 12 and 18 months with some countries administering the second dose before school entry (4–6 years) and others in early adolescence (11–13 years) (Hinman et al. 1999; Salisbury and Olive 2004). The primary aims are to assure that all

children receive at least one dose of measles vaccine and to induce protective immunity in children who have not responded to the first dose of the measles component, but there is the added benefit of increasing the proportion of people immune to mumps and rubella (Centers for Disease Control and Prevention 1998).

MEASLES

Within a few years of the initial isolation of the measles virus by Enders and Peebles (1954), an attenuated vaccine was developed by serial passage of the virus in chick embryo fibroblasts. The first measles vaccine was approved for use in 1963. The most common strains of measles vaccine in current use include the Moraten, Schwarz, and Edmonstron–Zagreb strains (Strebel et al. 2004). An inactivated measles vaccine was withdrawn from production following reports of an atypical measles syndrome characterized by high fever, pneumonia, and an unusual rash that occurred after exposure to wild-type measles virus (Annunziato et al. 1982).

In different countries, measles vaccine routinely administered at 8 months (China), 9 months (EPI schedule), 12–15 months of age (USA, Australia, New Zealand, and many countries in Europe), although some countries administer the vaccine at 18 months (Sweden) or even 2 years (Slovenia, Croatia) (Salisbury and Olive 2004). The maternal antibodies to measles transferred to the fetus across the placenta are sufficient to prevent or reduce the immune response to vaccination in young children (Albrecht et al. 1977). Selecting the most appropriate age for vaccination against measles requires balancing the likelihood of a child retaining maternal antibodies against the possibility of being without antibody protection and being exposed to measles. The rates of seroconversion range from 80–85 percent in 9-month-old children to 95 percent and higher in 15-month-old children (Halsey 1983). However, as mothers acquire immunity from vaccination rather than from natural disease, the levels of maternally acquired antibodies in their infants are lower and they decline earlier (Strebel et al. 2004). Measles vaccine is extremely safe, although 5 percent of children who receive it have a raised temperature of $\geqslant$103°F ($\geqslant$39.4°C) 7–12 days after immunization (Peltola and Heinonen 1986; Centers for Disease Control and Prevention 1998). A smaller proportion of children develop a rash, usually about 10 days after immunization. Rarely, the raised temperature provokes a febrile convulsion. Serious adverse events that are even less common include thrombocytopenia (one per 25 000–40 000 doses) and anaphylaxis (Institute of Medicine 1993).

The worldwide administration of measles vaccine has sharply reduced both the number of cases and the number of deaths attributed to measles. However, in 2001, the use of measles vaccine among children aged 12–23 months had remained between 70 and 75 percent for the last decade. It is estimated that in 2000, there were 40 million cases of measles and 777 000 deaths from measles (Stein et al. 2003) and that measles remained the fifth leading cause of child mortality, accounting for 5 percent of deaths among children <5 years). In the prevaccine era, as many as 130 million cases and 5.8 million deaths may have occurred globally (World Health Organization 1998b). In 2002, the United Nations Special Session for Children endorsed a measles mortality reduction goal of reducing measles deaths by 50 percent by 2005, compared with 1999 estimates. The main strategies for achieving this goal are to achieve high coverage with both the first dose of vaccine and with the second opportunity for measles vaccination. In developing countries, the best strategy for the second opportunity is through supplementary immunization campaigns targeting children 9 months to 14 years, with follow-up campaigns in those aged 9–59 months every 3–5 years. In addition, increased routine coverage to 90 percent or higher will be needed to reduce and maintain absence of measles deaths in these countries (Otten et al. 2003). These strategies were developed in the Americas and are now being implemented in sub-Saharan Africa and many countries in other regions. In the period 2001–2002, 60 million African children in 13 countries received measles vaccine through supplementary immunization activities, and the measles partnership is on target to achieve the 50 percent mortality reduction goal by 2005.

Because measles infection results in lifelong protective immunity, there is no carrier state and humans are the only host, measles is theoretically eradicable (Centers for Disease Control and Prevention 1993c). The availability of an effective vaccine and the successful control of measles in the Americas and some countries in Europe and Asia make measles a potential candidate for eradication after polio (Centers for Disease Control and Prevention 1999g). In 1967, elimination of measles was achieved in the Gambia, and sustained for a 4-year period (Foege 1971). Since that time, strategies for its elimination have evolved that deliver two doses of vaccine using either routine two-dose vaccination, routine vaccination with supplemental mass vaccination campaigns or routine vaccination with a single mass catch-up campaign followed by a routine two-dose schedule. Finland and the USA have achieved elimination based on a routine two-dose program (Peltola et al. 1994; Papania et al. 2004). Most countries in the Americas have used supplemental mass vaccination against measles and by 2003, measles cases had declined 99 percent compared with 1990 and indigenous transmission had been interrupted (de Quadros et al. 2003). The UK, Australia, and some other countries have achieved or are close to achieving elimination using a combination of single mass catch-up campaigns and a routine two-dose schedule (Ramsay et al. 2003; McFarland et al. 2003). Further progress in reducing

mortality and morbidity from measles depends on other countries implementing accelerated activities to achieve its elimination. Molecular epidemiological techniques will take on increasing importance in tracking the transmission of the measles virus and identifying importations (Rota et al. 1996). The World Health Organization has developed a standardized nomenclature to characterize the genetic variability in wildtype measles viruses (World Health Organization 1998a).

MUMPS

The mumps virus was first cultured in 1945 in chick embryos, leading to the development of vaccines that were first licensed in the Soviet Union and the USA in the 1960s (Plotkin 2004a). Several strains of mumps virus have been used to produce vaccine; until recently, the most widely used have been the Jeryl Lynn, Rubini, Urabe, Leningrad-3, L-Zagreb, and Hoshino. All produce seroconversion rates of 90–95 percent. Protective efficacy in clinical trials ranged from 92 to 96 percent. Post-licensure, vaccine effectiveness studied in outbreak investigations have been lower, from approximately 65 to 90 percent (Cheek et al. 1995; Wharton et al. 1988; Plotkin 2004a).

Mumps vaccine is well tolerated and, except for aseptic meningitis, few adverse events have been directly associated with vaccination. Aseptic meningitis has been associated mainly with vaccination with the Urabe and Leningrad strains of mumps vaccine (Plotkin 2004a; Ki et al. 2003; Dourado et al. 2000). When MMR vaccine has been used in mass campaigns, clusters of meningitis cases have been reported, highlighting that a specific vaccine strategy may influence the visibility of relatively rare adverse events and undermine public confidence in the vaccine (Dourado et al. 2000). Many developed countries, including Japan, UK, and other European countries, have removed the Urabe mumps vaccine from the market. These adverse events have had a detrimental effect on vaccination programs, in particular reducing acceptance of the combined MMR vaccine in routine vaccination programs, as well as in mass campaigns. The Jeryl Lynn strain has not been associated with aseptic meningitis and remains the mumps vaccine of choice throughout the world.

Widespread vaccination has greatly reduced the reported incidence of mumps in every country that has introduced mumps vaccine. As a disease without a carrier state and without a nonhuman host, the highly efficacious vaccine makes mumps, like measles, potentially eradicable (Centers for Disease Control and Prevention 1993c). It is not yet known whether a single dose can provide adequate population immunity to eradicate mumps, but it is unlikely given experience with single dose vaccine effectiveness under field conditions. Countries that use MMR in their childhood immunization schedule commonly have a two-dose policy for measles vaccine that also results in two doses of mumps being administered. Russia recommends two doses of mumps-containing vaccine at 12–15 months and 6 years. Using a two-dose schedule of MMR at 14–18 months and 6 years since 1983, Finland has eliminated mumps (Peltola et al. 1994, 2000).

RUBELLA

The mild febrile rash illness due to rubella is important because of the congenital rubella syndrome (CRS). Women infected with rubella during the first trimester of pregnancy frequently give birth to babies with characteristic birth defects. The association between rubella and this congenital syndrome was first recognized as a triad of ocular abnormalities, cardiac defects, and deafness in newborns in Australia in 1941 (Gregg 1941). Subsequent studies have expanded the list of abnormalities in the CRS to include mental retardation, hepato- and splenomegaly, as well as thrombocytopenia, hypothyroidism, and diabetes mellitus. Infection in the first trimester of pregnancy results in the highest frequency of, and most devastating, abnormalities, although infection in the third trimester may cause cataracts (Centers for Disease Control and Prevention 1998).

In the years following the isolation of the virus in 1962, several vaccines were developed. The RA27/3 vaccine strain is now the most commonly used throughout the world (replacing the Cendehill and HPV 77 vaccines in the USA), although the T0-336 and BRD-2 strains are used in Asia and have potency similar to the RA27/3 strain (Plotkin and Reef 2004). Rubella vaccine induces an effective immune response in >95 percent of recipients, which seems to be less affected by maternal antibody than is the response to measles vaccine. Vaccine effectiveness with a single dose, studied during outbreak settings for the RA27/3, as well as some Japanese vaccine strains, has most commonly been >94 percent (Plotkin and Reef 2004).

Rubella vaccination is usually well tolerated, although arthritis or arthropathy may occur. This side-effect is more common in susceptible adult women than in children or adult men (Institute of Medicine 1991; Centers for Disease Control and Prevention 1998). Joint symptoms are usually self-limited, although chronic arthritis associated with rubella vaccine has been reported (Tingle et al. 1986). Symptoms may be less common following vaccination with RA27/3 than after vaccination with other strains. Some workers have found no association with rubella vaccination and the subsequent development of persistent joint symptoms. Inadvertent rubella vaccination during pregnancy has not been associated with congenital defects, but vaccination is contraindicated in pregnancy (Centers for Disease Control, 1989, 1998) and women who receive rubella vaccine should be counseled to avoid pregnancy for 28 days after vaccination (Centers for Disease Control and Prevention, 2001b).

Several strategies have been implemented to control rubella and CRS. Some countries have opted to immunize only women of childbearing age, in practice girls 12–13 years of age, just before their reproductive years. With complete implementation of the program, virus continues to circulate among unvaccinated children and men, providing periodic boosting of immunity in vaccinated women. In theory, the control of CRS is achieved by protecting all women who might become pregnant. In the UK, an immunity level of 97 percent was found to be inadequate to prevent all cases of CRS (Miller 1991). The second strategy is universal vaccination of children. The ultimate aim here is the elimination of rubella transmission and the reduction of CRS by vaccination-induced immunity and herd immunity (Centers for Disease Control and Prevention 1998). Currently, most developed countries combine the two strategies, through universal immunization of children at 12–15 months of age with MMR vaccine or measles, rubella vaccine and immunization of susceptible women with rubella vaccine, and 119 countries have included rubella in routine immunization programs (Salisbury and Olive 2004; Vaccines and Biologicals 2002a). Some countries have conducted mass vaccination campaigns of adults of reproductive age in conjunction with initiating universal child vaccination against rubella (Hinman et al. 1998). Experience with various vaccination strategies in the Pan American Health Organization (PAHO) region in the late 1990s has lead to adoption of one-time mass vaccination of males and females (sometimes ranging from 5–39 years and administered as a combined MR campaign), in addition to introducing MMR in the childhood vaccination program as the most effective way to rapidly reduce virus transmission and more rapidly prevent congenital rubella syndrome. (Castillo-Solórzano et al. 2003). Cuba was the first country to eliminate rubella and CRS through two mass campaigns during 1985 and 1986 that initially targeted women aged 18–30 years followed by children 1–14 years (World Health Organization 2000).

Rubella, like measles, has only a human host and no chronic carrier state, so can theoretically be eradicated. Mathematical modeling suggests that a single dose of vaccine, given universally, should be sufficient to eliminate or even eradicate rubella. To date, Finland and Cuba have declared success in this effort, although the elimination of transmission in the USA is suggested by prolonged periods of no reported disease since the late 1990s, as well as by the epidemiology of CRS between 1997 and 1999, where 41 percent of mothers with CRS births were infected outside the country (Peltola et al. 2000; Reef et al. 2002). Molecular epidemiological methods, similar to those used in measles elimination efforts, in which the genetic information of wildtype viruses is characterized, will aid efforts to track and eliminate rubella virus transmission (Frey et al. 1998; Reef et al. 2002). The major constraints to eradication are a perceived lack of importance because of the mild clinical syndrome and the difficulty in maintaining surveillance for a disease in which half the cases may lack any clinical symptoms. These constraints are changing, however, as experience has been gained with combining rubella with measles surveillance mainly in the Americas (Irons et al. 2003). In developing countries, inadequate surveillance for CRS has meant that its importance is not known. Although rubella is not included in the World Health Organization's Expanded Program on Immunization (Robertson et al. 1997), there has been considerable progress in rubella control and CRS prevention especially in the region of the Americas where almost all countries are now using MMR vaccine in their routine childhood vaccination programs, are integrating measles and rubella surveillance including laboratory testing, and some of which have also initiated mass vaccination campaigns to accelerate rubella control/CRS prevention (Castillo-Solórzano et al. 2003; Irons et al. 2003). The European region has established a target for reducing incidence of CRS to <1 per 100 000 births by 2010. Strategies to achieve the goal of measles elimination by 2007 in this region have included mass campaigns using measles vaccine which have also provided opportunities for countries to launch or accelerate rubella and CRS prevention programs (Spika et al. 2003; Dayan et al. 2003).

Poliomyelitis

Poliomyelitis was a widespread cause of childhood morbidity and mortality in the prevaccine era (Strebel et al. 1992). In 1949, the discovery that poliovirus could be propagated in nonnervous human tissue permitted the large-scale production necessary for vaccine manufacture (Enders et al. 1949). Two standard approaches were pursued: inactivation and attenuation of poliovirus.

Following large-scale field trials of inactivated poliovirus vaccine (IPV) developed by Jonas Salk, IPV became, in 1955, the first poliovirus vaccine to be licensed in the USA (Francis et al. 1957; Salk et al. 1994). IPV contains formalin-inactivated poliovirus types 1, 2, and 3. In 1961, monovalent oral poliovirus vaccines (OPV) developed by Albert Sabin were licensed. Two years later, a balanced formulation of the three monovalent vaccines (trivalent OPV) became available and rapidly replaced IPV as the vaccine of choice for preventing poliomyelitis in the USA and in most other countries. OPV contains live-attenuated polioviruses of the three poliovirus serotypes formulated ratio 10:1:6, with 10^6 median tissue culture infective dose ($TCID_{50}$) of poliovirus type 1, 10^5 $TCID_{50}$ of poliovirus type 2, and $10^{5.8}$ $TCID_{50}$ of poliovirus type 3 (Centers for Disease Control 1982). In the 1980s, enhanced-potency IPVs (eIPV) were developed; eIPV containing 40:8:32

D-antigen units of poliovirus types 1, 2, and 3, respectively, was licensed in the USA in 1987 (Centers for Disease Control 1987).

In most industrialized countries, including the USA before 1997, and virtually all developed countries, the vaccine of choice for the prevention of poliomyelitis is OPV (Centers for Disease Control 1982; Hinman et al. 1999). The primary series of OPV usually consists of three doses in the first 6 months of life, followed by one or more additional doses between 1–2 years of age or at school entry. In the United States prior to 1997, doses were given at 2, 4, and 6–18 months of age, followed by a supplemental dose of OPV at school entry (i.e. 4–6 years). The primary series of IPV consists of between four and seven doses, with two to three doses given in the first year of life and at least one booster dose 6–12 months after the primary series. In the USA, doses are given at 2, 4, and 6–18 months of age, followed by a fourth dose of IPV at school entry. Until 1997, IPV in the USA was indicated primarily for immunologically abnormal infants and their family contacts, and for unvaccinated adults (Centers for Disease Control 1987). Following primary reliance on OPV between 1961 and 1996, the USA revised its poliomyelitis prevention policy with effect from early 1997. Recognizing the absence of indigenous wild poliovirus circulation since 1979, the occurrence of eight to nine cases of vaccine-associated paralytic poliomyelitis (VAPP) annually in the USA and the rapid progress in global polio eradication, the USA revised its policy first to increase the use of IPV, the first two doses of poliovirus vaccine as IPV given at 2 and 4 months of age followed by two doses of OPV at 12–18 months and 4–6 years of age (Centers for Disease Control and Prevention 1997). In 2000, the policy was revised to an IPV-only schedule (Centers for Disease Control and Prevention 2000). OPV is no longer recommended, except in special circumstances such as a child who will be traveling to a polio-endemic area. This schedule ensures development of good humoral immunity, while eliminating the risk of VAPP (McBean and Modlin 1987; Centers for Disease Control and Prevention 2000). IPV has been used exclusively in many northern European countries, including Finland, Sweden, and the Netherlands, and has led to eradication in these countries. Currently, 22 developed countries use exclusive IPV, and an additional eight countries use a sequential schedule of IPV followed by OPV (World Health Organization 2003a).

The Expanded Program on Immunization of the World Health Organization recommends a schedule of four OPV doses administered at birth (zero week), 6, 10, and 14 weeks to ensure earlier protection against poliomyelitis in infants residing in the developing world. Many developing countries also recommend a dose of OPV in the second year of life.

The objective of the routine series of poliovirus vaccine is to induce humoral immunity for individual protection against poliomyelitis and mucosal immunity for community protection against its circulation. In industrialized countries, three doses of OPV or IPV result in seroconversion or seroprevalence levels of >90 percent to all three poliovirus serotypes. In developing countries, the immunogenicity of OPV is substantially lower, with median seroconversion levels of 72, 95, and 65 percent (Patriarca et al. 1991) to poliovirus types 1, 2, and 3, respectively, following the administration of three doses of OPV. The lower immunogenicity of OPV in developing countries may be due to interference by concurrent enterovirus infection, diarrhea, nonspecific inhibition, or other factors. In addition, the immunogenicity of OPV may be lower under program conditions because of suboptimal storage, handling, and administration. When given by the WHO/EPI schedule, the immunogenicity of IPV has varied widely, from 67–99 percent for type 1, 65–99 percent for type 2, and 91–100 percent against type 3 (World Health Organization 2003a). The response to early administration of IPV may be inversely affected by maternally derived type-specific antibody (WHO Collaborative Study Group 1996). Whereas OPV and IPV induce a similarly high-level humoral immune response in infants residing in industrialized countries, the intestinal mucosal immunity induced by OPV is clearly superior to that induced by IPV in limiting the replication and excretion of circulating poliovirus (Sutter and Patriarca 1993).

The widespread use of IPV, starting in 1955, led to a major reduction in the incidence of poliomyelitis in the USA, from 57 879 cases in 1952 to 5 485 cases by 1957. The widespread use of OPV led to the elimination of indigenous circulation of wild poliovirus in the 1960s. The last endemic and epidemic cases of poliomyelitis were reported in 1979. Between 1980 and 1997, aside from a few imported cases of poliomyelitis, approximately eight cases of VAPP were reported each year in the USA (Strebel et al. 1992). Of these eight VAPP cases, three each occurred in OPV recipients and in people in contact with OPV recipients and two cases in immunologically abnormal people. Since the adoption of the IPV schedule, no VAPP cases have been reported in the USA.

The occurrence of VAPP is the most serious adverse event following use of OPV and has been reported from most industrialized countries (Esteves 1988; Joce et al. 1992; Strebel et al. 1992). Among immunocompetent people, the highest risk of VAPP is in recipients of the first dose of OPV (one VAPP case per 1.5 million infants) and in unvaccinated contacts (one VAPP case per 2.2 million contacts) of recently vaccinated infants. However, the risk of VAPP is highest in the presence of immunological abnormality (either congenital or acquired), particularly people with B-cell deficiencies. In Romania, a country that had reported very high rates of VAPP for many years, a case–control study suggested

that the apparently increased incidence was primarily due to multiple intramuscular injections of antibiotics following administration of OPV (Strebel et al. 1995). An estimated 250–500 VAPP cases occur annually. An additional risk of OPV use is circulating vaccine-derived polioviruses (cVDPV), in which vaccine-derived polioviruses have reverted to wild-type virulence and developed the ability to circulate widely in the population. Since 1988, four outbreaks of cVDPV have occurred, causing between three and 30 cases of paralytic poliomyelitis (World Health Organization, 2003a). These outbreaks have generally originated in populations with low vaccine coverage, allowing vaccine viruses to circulate for long periods and reattain virulence. IPV is not known to cause serious adverse reactions.

Progress toward poliomyelitis control in industrialized and developing countries prompted the member countries of the WHO region of the Americas to adopt the goal, in 1985, of regional elimination of poliomyelitis from the western hemisphere by the year 1990. Following the implementation of key strategies, the last case of poliomyelitis associated with wild poliovirus isolation was reported from Peru in September 1991, and the region was certified free of indigenous wild poliovirus by an International Certification Commission in September 1994 (Pan American Health Organization 1994). In 1988, the World Health Assembly, the governing body of WHO, adopted the goal of global eradication of poliomyelitis by the year 2000 (World Health Organization 1988). A unique partnership of governmental and private organizations is supporting this initiative; Rotary International, a private service organization, has provided more than US$500 million and immeasurable volunteer time to achieve the goal of eradication.

The poliomyelitis eradication initiative relies on four strategies:

1. high routine coverage with OPV;
2. supplemental immunization, including national immunization days (NID) twice a year to vaccinate all children in order to eliminate the remaining poliovirus reservoirs;
3. enhanced epidemiological and virologic surveillance to detect cases immediately, institute control measures, and eventually assist in eliminating wild poliovirus circulation;
4. house-to-house campaigns to interrupt remaining chains of transmission (Hull et al. 1994). Additional house-to-house campaigns have become the key strategy in the remaining polio-endemic countries.

At present, use of IPV in developing countries is not recommended because of its inferior protection against virus transmission (mucosal immunity), potential lower response to vaccination with the EPI schedule, higher cost, and greater complexity of administration (e.g. injection, combination vaccines) (World Health Organization 2003a). This recommendation is being reviewed as progress toward eradication continues.

Progress towards eradication can be measured both by outcome evaluation (e.g. the incidence of poliomyelitis) and by process evaluation (e.g. the number of countries conducting NIDs). Worldwide, the number of polio cases has decreased by over 99 percent, from over 200 000 cases in 1980 to 1 250 in 2004. Eradication of polio has been certified in the American (1994), Western Pacific (2001), and European (2002) regions. However, the virus remains endemic in six countries (Nigeria, India, Pakistan, Niger, Afghanistan, Egypt), and in 2004 polio cases caused by wild poliovirus originating from northern Nigeria were reported in ten sub-Saharan countries in Africa and in Saudi Arabia, re-establishing transmission (for more than 6 months) in five countries bordering or near Nigeria (Vaccines and Biologicals 2004; Centers for Disease Control and Prevention 2005b). During this period, NIDs were conducted in up to 96 countries, and supplemental doses were given to 500 million of the world's children < 5 years of age during 266 rounds of NIDs, supplemental immunization days, or mopping up activities in 2002. During 2004, supplemental immunization was conducted in 23 contiguous countries in Africa, and up to 6–8 rounds of vaccination were conducted in the remaining polio-endemic countries.

The progress toward global eradication of poliomyelitis suggests that this objective may be within reach in Asia and Egypt by the end of 2005, and in Africa soon thereafter. However, increased efforts are necessary to ensure that adequate resources are made available to eliminate the remaining poliovirus reservoirs, particularly those in southern Asia and Africa.

Hepatitis B vaccine

Hepatitis B vaccines initially consisted of HBsAg particles purified from the plasma of HBV carriers. Plasma-derived vaccines were highly effective in preventing hepatitis B, were used in developed countries throughout the early 1980s, and continue to be used in a few developing countries (Szmuness et al. 1981; Kane 1993). In 1985, the first recombinant HB vaccines became available. These consist of purified HBsAg produced by recombinant DNA technology in yeast or in mammalian cells. Newer generation vaccines, which may include the pre-S1 or pre-S2 antigens, or both, in addition to HBsAg have also been developed but have not replaced the vaccines based solely on HBsAg (Hadler and Margolis 1992).

Hepatitis B vaccine is usually given as a three-dose series. The licensed schedule in developed countries includes doses at 0, 1, and 6 months, with an alternative schedule of four doses given at 0, 1, 2, and 12 months. However, schedules that vary the timing of the second

and third doses to permit integration into the routine childhood immunization schedule have been highly immunogenic (Centers for Disease Control and Prevention 1991). Dosages vary by age, vaccine, and whether the child is born to an HBsAg-positive mother; in the USA, dosages for infants and children are one-half those required for adults.

The response to a three-dose series is excellent in all age groups, with anti-HBs produced in 85–99 percent of vaccinees; response is highest in children aged 2–19 years, and decreases with increasing age (Hadler and Margolis 1992). People with immunocompromising conditions such as renal failure have poorer responses, and higher dosages are recommended. Placebo-controlled trials both in high-risk children in HBV-endemic countries and in high risk adults in the USA and elsewhere have shown short-term efficacy of 80–95 percent (Szmuness et al. 1981; Stevens et al. 1992). Long-term follow up of both children and adults for up to 14 years has demonstrated protection against serious consequences of infection (chronic carriage and chronic liver disease) in virtually all people who respond to the initial series (anti-HBs $\geqslant$10 miu/ml) (Hadler and Margolis 1992; West and Calendra 1996; Mast et al. 2004). Booster doses are not currently recommended for any age group, but the need for them continues to be evaluated.

Hepatitis B vaccine causes few adverse reactions, mainly fever and soreness at the injection site. Rarely, anaphylaxis can occur, and contraindicates additional doses of vaccine. Serious neurological injuries such as Guillain–Barré syndrome and other demyelinating conditions (multiple sclerosis, optic neuritis, and transverse myelitis) have been reported, rarely, following hepatitis B vaccination, but available information favors rejection of a causal link with multiple sclerosis, and is insufficient to accept or reject a relation between other demyelinating conditions and hepatitis B vaccine (Centers for Disease Control and Prevention 1991; Institute of Medicine 1993, 2002a).

In developed countries, including the USA and Europe, hepatitis B vaccine was initially recommended only for infants of HBV-carrier mothers and high risk adults. However, in the USA, the lack of impact on disease incidence led to implementation of a comprehensive strategy for eliminating hepatitis B transmission (Centers for Disease Control and Prevention 1991). This strategy includes: (1) universal screening of pregnant women for HBsAg, and providing hepatitis B immunoglobulin and initiating hepatitis B vaccination within 12 h of birth for infants born to hepatitis B-carrier mothers; (2) universal vaccination of all infants, preferentially beginning at birth, but at least by 2 months of age when other routine childhood vaccines are initiated; (3) vaccination of all previously unvaccinated children at 11–12 years of age; and (4) vaccination of children, adolescents and adults in high risk groups, including people who live in households with HBsAg carriers; children $\leqslant$11 years old of immigrants from areas of high HBV endemicity; people who abuse drugs parenterally; the sexually active, whether homosexual or heterosexual; and those at occupational risk. The recommendation for universal vaccination of infants was based on several considerations: the low dose of vaccine required for this age group; the feasibility of integration into the routine childhood immunization schedule; the need to prevent chronic infection in high risk children; and data showing that protection persists to the age when high-risk behaviors begin.

Vaccination of all previously unvaccinated adolescents at 11–12 years of age is the primary strategy for hepatitis B control in Canada and some countries in Europe (Committee on Infectious Diseases 2003; Hinman et al. 1999). This approach has the advantage of ensuring that adolescents will be protected before beginning high-risk behaviors, that immunity will be high and that a booster dose of vaccine will not be needed. A key challenge is providing the three-dose vaccine series to this age group in the absence of regular preventive healthcare visits. The vaccination of adolescents has been implemented successfully in British Columbia via school-based programs (Dobson et al. 1995).

The World Health Organization now recommends that all countries should include HB vaccine in their routine immunization schedules, with emphasis on routine infant immunization and prevention of perinatal HBV transmission (Vaccines and Biologicals 2003). In all developing countries, universal vaccination of infants is the recommended strategy for disease control (Kane 1993). The initial dose should be given as soon after birth as possible, particularly in countries in which perinatal disease transmission is predominant (e.g. eastern Asia). Infant immunization has been shown to be highly effective in reducing the frequency of HBV carriers among children in many high risk countries (Mast et al. 2004), and has been associated with reduced risk of liver cancer among children in Taiwan (Chang et al. 2003). Introduction of hepatitis B vaccination in the poorest countries is now supported by funding from the Global Alliance for Vaccines and Immunization, and by 2003, 151 countries had hepatitis B programs, based mainly on universal vaccination of infants (World Health Organization 2003c). A few countries with extremely low HBV endemicity (Scandinavia, UK) continue to rely only on vaccination of high-risk adults.

Varicella vaccine

Live-attenuated varicella-zoster virus vaccine was developed in Japan in the 1970s, has been available in Japan and Korea and some European countries for many years, and is now widely available throughout the world. In 1995, it was licensed for primary prevention of varicella in healthy American children and adults. Clinical

efficacy trials conducted with this vaccine, developed from the Oka strain of varicella virus, used varying vaccine potencies and formulations that were not necessarily comparable. For the formulation of vaccine most similar to the currently licensed product, the vaccine is approximately 70–90 percent effective in prevention of varicella disease and over 95 percent effective in providing protection against severe varicella disease (Centers for Disease Control and Prevention 1996; Seward 2001). Moreover, the 1–4 percent of children and adults who experience breakthrough infection annually develop mild disease with an average of $\leqslant$50 lesions, compared with 300 lesions in typical varicella (Bernstein et al. 1993). Post-exposure vaccination, if given within 5 days of exposure, appears effective in preventing or reducing severity of varicella in household contacts of cases (Salzman and Garcia 1998; Centers for Disease Control and Prevention 1999e).

For primary prevention, varicella vaccine is given as a single dose for children age either 9 or 12 months (depending on vaccine manufacturer directions) to 12 years, and two doses separated by at least 1 month for older children and adults (Centers for Disease Control and Prevention 1996; Arvin 2002). Varicella vaccine may be given simultaneously with all other childhood vaccines, but should not be administered within 4 weeks of receipt of another live, viral vaccine. For adolescents and adults, serological screening of people without a history of varicella is likely to be cost-effective, because 70–91 percent of them will be immune to varicella.

Varicella vaccine induces a mild varicelliform rash and fever in 5–10 percent of vaccinees. However, these data are from uncontrolled clinical trials. In the only two placebo-controlled trials conducted among children, one trial reported no difference in rash or fever between vaccine and placebo recipients (Varis and Vesikari 1996). The second trial found no difference in fever and a small difference in rash: 3 percent of vaccine recipients developed noninjection site rashes compared with 2 percent of placebo recipients and 1 percent of vaccine recipients developed a generalized varicella-like rash compared with no placebo recipients (Weibel et al. 1984). Among adults, rash and fever may be more common, but there are no placebo-controlled trial data available. No unanticipated rare, serious adverse effects have occurred postlicensure (Wise et al. 2000). In the USA, varicella vaccine is contraindicated in people who are immunocompromised due to cell-mediated immunodeficiency (any malignant condition, including blood dyscrasias, leukemia, lymphomas of any type, or other malignant neoplasms affecting the bone marrow or lymphatic systems), in pregnant women and in anyone who has an anaphylactic reaction to varicella vaccine or any component, including gelatin. However, vaccine is available to any physician through an investigational drug protocol for use in patients who have acute lymphoblastic leukemia (ALL) in remission who meet specified criteria) (Centers for Disease Control and Prevention 1996). Vaccination should be delayed for 5 months after the administration of immunoglobulin or blood products, because the response to vaccine administered within this interval is unknown. The risk of herpes zoster after varicella vaccination appears to be lower than after natural varicella infection.

Strategies for the use of varicella vaccine include universal vaccination of children at 12–18 months of age (with or without a catch-up program) and/or targeted vaccination for high-risk groups for severe disease or those in contact with such persons. The latter groups could include children with leukemia, as well as older susceptible children, adolescents and adults, healthcare workers, and family contacts of immunocompromised persons. Targeted programs, although they will provide protection to the individual or their close contacts, will have little impact on varicella epidemiology. Universal vaccination is intended to reduce disease transmission greatly and provide herd immunity to those who remain unvaccinated, and is the strategy adopted by the USA (Centers for Disease Control and Prevention 1996). Varicella vaccine is recommended for all children at 12–18 months of age and for all susceptible older children (with no prior history of varicella). All children who have not had natural varicella infection should be vaccinated at 11–12 years of age. Varicella vaccine is also recommended for susceptible healthcare workers and others in close contact with immunocompromised people who might experience severe varicella if infected, for susceptible adults at high risk for exposure or transmission, such as teachers, childcare center workers, and adults who live in households with children. It should be considered for any adolescents and adults with no prior history of varicella (Centers for Disease Control and Prevention 1999e).

In the USA where experience has been gained with the universal childhood program, varicella cases and hospitalizations and deaths had declined approximately 70 to 80 percent 6 years after implementation of the vaccination program (Seward et al. 2002; Davis et al. 2004; Nguyen et al. 2005). Although the greatest decline in incidence is among children 1–4 years, incidence has declined in all age groups including infants <12 months and adults indicating reduced disease transmission.

OTHER VIRAL VACCINES

Hepatitis A vaccine

Both killed and live-attenuated hepatitis A vaccines have been under development since the 1980s (D'Hondt 1992). Killed vaccines produced in cell culture, purified and inactivated with formalin first became commercially

available in 1992. Inactivated hepatitis A vaccines are highly immunogenic (94–100 percent) and over 94 percent effective in preventing hepatitis A infection when given as a two- to three-dose series to children or a two-dose series to adults (Werzberger et al. 1992; Innis et al. 1994). The duration of protection is not known but, on the basis of high antibody levels induced by the vaccine, is expected to exceed 20 years (Van Damme et al. 1994). This vaccine may also provide protection post-exposure, and has been shown to be effective in controlling hepatitis A outbreaks in well-defined populations (Centers for Disease Control and Prevention 1999h). Development of live attenuated vaccines has been impeded by difficulty in achieving an appropriate balance of attenuation and protection, but one Chinese vaccine (H-2 strain) has been reported to be both immunogenic and effective, and has been licensed and used in China since 1992. This vaccine is reported to induce high rates of seroconversion and have high efficacy when given as a single dose subcutaneously to school-age children (Mao et al. 1997).

Licensed inactivated hepatitis A vaccine is given as a two- or three-dose series, with doses at 0 and 6–12 months (or 0, 1, and 6–12 months) to children $\geqslant$2 years of age, and as a two-dose series given 6–12 months apart to adults (Centers for Disease Control and Prevention 1999h). The known adverse reactions are limited to pain at the injection site. Rare serious adverse events (e.g. Guillain–Barré syndrome) have been reported following hepatitis A vaccination, but rates of such events have not exceeded those expected in the absence of vaccination. Hepatitis A vaccine may be given simultaneously with other childhood vaccines or vaccines for international travelers. The administration of passive immunoglobulin or maternal antibody interferes with response to the first dose of vaccine, but differences in final antibody response after the multidose series seem unlikely to be of clinical importance.

In developed countries, hepatitis A vaccine is recommended for people at high risk of infection, and is most widely used in those traveling to or working in countries where hepatitis A is highly endemic. In the USA, vaccine is recommended for native Americans and other groups that experience cyclic hepatitis A epidemics, for children age 2–18 years in states that historically had high incidence of hepatitis A, as well as for others at risk such as people with clotting disorders, homosexual men, users of illicit drugs, those at occupational risk, and people who have chronic liver disease (Centers for Disease Control and Prevention 1999h). Universal vaccination of children at age 2 years, with or without catch-up vaccination of susceptible older children, has resulted in marked decline in incidence in Native Americans and in states that previously had high disease incidence. Vaccination also may be considered for the prevention or control of community-wide outbreaks, although the data on effectiveness in this situation are limited. Ultimately, universal vaccination would be the best option to reduce the burden of disease in countries with substantial clinical disease burden such as the USA; however, this will probably require a less costly preparation and its incorporation into combination childhood vaccines.

Influenza virus vaccines

Influenza viruses continue to cause considerable mortality and morbidity throughout the world on an annual basis with the potential for much greater mortality and morbidity during pandemics. The 1918–19 pandemic is estimated to have caused more than 20 million deaths worldwide, more than 500 000 of which occurred in the USA. Recent estimates of influenza-associated deaths in the USA for the seasons 1990–91 through 1998–99 range from 36 000 underlying respiratory and circulatory deaths to 51 000 all-cause deaths (Thompson et al. 2003). The increase in influenza-associated deaths since the 1970s is believed to be partly due to the aging of the US population.

INACTIVATED INFLUENZA VACCINE

Influenza virus was first isolated from humans in 1933 and the first effective vaccine was administered in studies in Pennsylvania soon after that time. Since the 1940s, influenza virus has been grown in chick embryo allantoic sacs; this medium greatly increased yield of virus and promoted vaccine production. Because of the frequent antigenic changes in influenza viruses, the antigenic content of both types of vaccine is changed annually to optimize protection against the influenza type A and B strains expected to circulate during the following winter and spring. Influenza viruses of type A (H1N1 and H3N2) and B are selected according to their immunological match with circulating strains, grown in chicken embryos, inactivated with formalin and combined into vaccine. In the USA, inactivated vaccine is either subvirion vaccine, prepared by disrupting the lipid-containing membrane of the virus, or purified surface-antigen vaccine; whole-cell influenza vaccine is no longer available. The efficacy of vaccine is related to the degree of match between the vaccine and the circulating influenza viruses, as well as age and immunocompetence of the vaccine recipient. In recent years, effectiveness has been estimated to be 70–90 percent in adults <65 years, but lower in older adults (Centers for Disease Control and Prevention 2004; Bridges et al. 2000). Protection against serious complications, including hospitalization and death, is higher than against uncomplicated influenza (Nichol et al. 1994; Gross et al. 1995). Among healthy children, inactivated influenza vaccine efficacy in various studies has ranged from 77 to 91 percent for prevention of influenza

respiratory illness and 66 percent for prevention of culture-confirmed influenza. Inactivated influenza vaccine has also been reported to reduce influenza-associated otitis media in children by approximately 30 percent (Centers for Disease Control and Prevention 2004; Neuzil et al. 2001). When major antigenic shifts occur, vaccine effectiveness may be substantially less in all age groups. Annual vaccination is necessary to ensure protection against the current strains.

For previously unvaccinated children 6 months to 8 years of age, two doses of vaccine (0.25 ml), given at 4-week intervals, are recommended (Centers for Disease Control and Prevention 2004). For other groups, only a single dose (0.5 ml) is necessary. About 3–5 percent of vaccinees experience local tenderness or fever after vaccination. The occurrence of Guillain–Barré syndrome within 6 weeks of influenza vaccination was observed in adults after the swine influenza vaccine campaign in 1976, but was not observed subsequently until 1990, when a case–control study suggested a slightly elevated risk in 18–64-year-olds, but not in those over 65 years of age (Schonberger et al. 1979; Chen et al. 1992). A similar small elevated risk was shown in adults age 18 years and older during the 1992–93 and 1993–94 influenza vaccine seasons (Lasky et al. 1998). Given the substantial benefits of influenza vaccination among the target populations, the risk of Guillain–Barré syndrome, if any, is greatly exceeded by the benefits. A previous anaphylactic reaction to egg protein is the only contraindication to influenza vaccination.

Vaccination is intended to reduce the complications and mortality due to influenza infection. Annual immunization is recommended for those at highest risk of these complications (Centers for Disease Control and Prevention 2004). In most developed countries, vaccination is recommended for people with certain chronic medical conditions, including cardiovascular and pulmonary disorders, asthma, other medical disorders such as metabolic disease (including diabetes mellitus), renal disease, hemoglobinopathies (including sickle-cell disease), and for people with immunosuppressive disorders or treatments, and children on long-term aspirin therapy. In many countries, annual vaccination is recommended for all adults aged $\geqslant$65 years. Although immunogenicity and efficacy have not been established in all these high-risk groups, the risk from vaccine virus is extremely low because vaccine is inactivated. Healthcare workers and people caring for or living with those at high risk should also be vaccinated. In the USA, annual vaccination is also recommended for women who will be pregnant during the influenza season, and for adults 50–64 years. Because children aged 6–23 months are at substantially increased risk for influenza-related hospitalizations, vaccine policy setting groups in the USA (ACIP, American Academy of Pediatrics, American Academy of Family Physicians), recommend vaccination of all children in this age group (Centers for Disease Control and Prevention 2004; Committee on Infectious Diseases 2003). Many countries have developed plans to prepare for pandemic influenza, which occurs every 10–40 years and can have devastating mortality. Such plans include the rapid production and availability of vaccine to ensure the vaccination either of people at highest risk or of the entire population.

LIVE-ATTENUATED INFLUENZA VACCINE

Cold-adapted live-attenuated influenza vaccines (LAIV) have been used in Russia since 1977 (Murphy 1993; Maassab et al. 1994). These vaccines incorporate influenza virus strains attenuated through growth at 25°C, but with reduced growth at 37°C. The genes for relevant circulating influenza strains can be incorporated into these vaccines by reassortment, and the vaccine is administered intranasally. In controled trials, these vaccines have similar efficacy to inactivated influenza vaccines in older children and adults (Belshe et al. 1998; Nichol et al. 1999; Treanor et al. 1999). In children, where the vaccine was studied during two influenza seasons, vaccine efficacy was also high (86 percent) in the season where the vaccine and circulating virus strains were not well matched (Belshe et al. 2000). In 2003, a LAIV was licensed in the USA and is currently approved for use among healthy persons age 5–49 years (Centers for Disease Control and Prevention 2004).

Japanese encephalitis vaccine

Three types of Japanese encephalitis (JE) vaccines have been developed and used to substantially reduce disease incidence in Asia where the disease is endemic. Inactivated Japanese encephalitis vaccine, currently available in Japan, Taiwan, and other developed countries, is a purified preparation of whole virus (Beijing-1 or Nakayama strain) grown in mouse brain and inactivated with formalin. When given as a three-dose series, the vaccine has 91 percent efficacy in adults (Hoke et al. 1988). Antibody persists for at least 2 years; the need for booster doses is uncertain. Primary immunization consists of three doses given at 0, 7, and 30 days; the third dose may be given at 14 days if the schedule does not permit the longer interval (Centers for Disease Control and Prevention 1993a). Vaccination schedules vary in different countries; some countries use a primary series of two doses 4 weeks apart followed by a booster dose after 1 year. The optimal interval for boosting is not defined, but some countries recommend booster doses every 3 years (Vaccines and Biologicals 2003). The dose for young children (1–3 years) is 0.5 ml, and that for older children and adults is 1.0 ml. No data are available on safety and immunogenicity in children <1 year of age. Adverse events include local reactions (20 percent) and minor systemic symptoms (10 percent). Generalized allergic reactions (angioedema, urticaria)

may occur in 1–6 per 1 000 vaccinees; more rarely, anaphylaxis, respiratory distress, and hypotension have been reported. Rare neurological events such as fatal acute encephalitis have been reported. China also produces an inactivated vaccine (Beijing P-3 strain) grown in hamster kidney cell cultures, which has 85 percent efficacy for a primary series. This vaccine has been widely used in China, but is being replaced with the live-attenuated vaccine.

A live-attenuated vaccine (SA 14-14-2 strain), produced in primary hamster kidney cells, has been developed and widely distributed in China since 1989. Recent studies have confirmed the safety in 13 000 vaccinated children, and demonstrated 80 percent efficacy of one dose and 98 percent efficacy of two doses in children in China (Chambers et al. 1997). The cost of this vaccine (US$0.06 per dose) is substantially lower than that of the inactivated vaccine, and this vaccine is being investigated as a candidate for inclusion in the Expanded Program on Immunization in JE endemic countries. Developing a vaccine schedule compatible with the EPI childhood schedule, and assuring quality of manufacturing are two challenges for this goal.

JE vaccine is recommended for travelers or residents in areas where the disease is endemic (south and east Asia), who potentially will be exposed during the transmission season particularly in rural areas when traveling for 30 or more days (Centers for Disease Control and Prevention 1993a). JE vaccine is used routinely in many of these countries, but use should be expanded to all endemic countries. Assuring a sufficient supply of effective and inexpensive vaccine will be necessary to meet these disease control objectives (Vaccines and Biologicals 2003).

Rabies vaccine

The first attenuated and inactivated rabies vaccines were developed by Pasteur from desiccated infected rabbit brains. Subsequent vaccines were developed from virus harvested from the brains of rabbits, sheep, or goats, but caused neuroparalytic disease in about one per 2 000 recipients, due to residual myelin derivatives. Nerve tissue-derived vaccines continue to be locally produced and used in many developing countries. Vaccines developed from duck embryos reduced the risk of serious adverse events about tenfold (Hattwick 1974). However, in developed countries these vaccines have now been replaced by those grown in diploid human cell lines (World Health Organization 1992). Rabies virus is harvested from cell culture and inactivated with β-propiolactone. This vaccine (human diploid cell strain (HDCS)) is highly immunogenic when given as a multidose series, either alone (pre-exposure) or with human rabies immunoglobulin (HRIG) (postexposure). The vaccine schedule for pre-exposure prophylaxis is a three-dose series at weekly intervals. For people at high continuing risk of infection (e.g. veterinarians, travelers to endemic areas), testing for the presence of neutralizing antibody should be done at 2-year intervals and booster doses given if inadequate antibody is present. Alternatively, booster doses may be given at 2-year intervals without testing. Post-exposure prophylaxis includes a five-dose series: the first dose with HRIG, half infiltrated at the site of the bite and half given intramuscularly elsewhere, followed by additional vaccine doses 3, 7, 14, and 28 days later (Centers for Disease Control and Prevention 1999i). Although episodes of rabies have rarely followed the use of HDCS alone, there have been no reports of vaccine failure with HDCS given with HRIG (Devriendt et al. 1982; Centers for Disease Control and Prevention 1999i). The WHO strongly encourages that more potent and safe cell-derived vaccines should replace nerve tissue-derived vaccines (Vaccines and Biologicals 2003).

HDCS causes few adverse events. Local and mild systemic symptoms may occur in 5–40 percent of recipients. Type III hypersensitivity similar to serum sickness can occur with repeated doses. Although cases of neurological disease, such as Guillain–Barré syndrome, have been reported following HDCS, these are not conclusively linked to the vaccine. New vaccines under investigation include recombinant vaccines containing the surface glycoprotein produced in *E. coli*, mammalian or yeast cells, or vectored into vaccinia and canarypox viruses.

Smallpox (vaccinia) vaccine

Variola virus is the causative agent of smallpox. The mortality and morbidity caused by smallpox lead to development of the first vaccine to prevent human disease. In 1796, Edward Jenner demonstrated that material from a human cowpox lesion could be inoculated into the skin of another person to produce a similar infection and this person was later protected from inoculation with smallpox virus. Smallpox vaccine was initially administered by arm-to-arm inoculation. Advances in vaccine development occurred in the 1800s when growth of vaccinia virus on the flank of a calf provided an adequate and safer supply of vaccine material (Fenner et al. 1988; Henderson et al. 2004). However, the vaccine did not remain viable for more than 1 or 2 days at room temperature. Finally, a process for production of a stable freeze-dried vaccine was perfected in the late 1940s. Though Jenner is believed to have used cowpox virus in vaccination, vaccinia virus strains are neither cowpox nor variola and neutralizing antibodies induced by the vaccine are crossprotective for other orthopoxviruses, including monkeypox virus, cowpox virus, and variola virus (Henderson et al. 2004).

Many vaccinia virus strains were used in vaccine production. In the USA, the New York City Board of

Health strain was used exclusively. Other strains used globally included Lister, Temple of Heaven, Tashkent, Copenhagan, and Patwadanger. Attempts to produce less reactogenic vaccines (e.g. Rivers, MVA (modified vaccinia virus Ankara), CVI-78, and LC 16m8) were successful, but some resulted in lowered immunogenicity. The most satisfactory attenuated strain, the LC16m8 from Japan, did produce a satisfactory immune response in humans and had markedly lower rates of adverse reactions compared with other strains. However, the eradication of smallpox precluded testing this vaccine for efficacy (Henderson et al. 2004).

Smallpox vaccine is administered by intradermal inoculation most commonly using a bifurcated needle, though high pressure jet injectors were also used during the smallpox eradication effort. The vaccine is extremely stable even when stored at extremes of temperature. When the vaccine was being used in routine vaccination programs, it was recommended for healthy infants $\geqslant$12 months of age. Adverse reactions varied by vaccine strain and included severe reactions, including generalized vaccinia, eczema vaccinatum, progressive vaccinia, postvaccinial encephalitis, and vaccinial keratitis, and mild to moderate reactions, such as inadvertent inoculation, urticarial rashes, and Steven's Johnson syndrome (Lane et al. 1970; Centers for Disease Control and Prevention 2001a). Reported complications were more common among primary vaccinees and resulted in death in approximately one per million primary vaccinees.

Smallpox was a major cause of mortality worldwide with overall mortality rates of 20–30 percent. Variola major had case-fatality rates of approximately 30 percent and people who survived the infection were commonly left with residual facial pockmarks. Other serious sequelae included blindness and limb deformities. Variola minor produced less severe illness with case-fatality rates of 1 percent or less (Fenner et al. 1988). In the 1950s, when the disease had been eliminated in developed countries, 50 million cases a year were estimated to still occur globally resulting in 6 million deaths. By the 1960s, 15 million cases and 2 million deaths were estimated to occur per year. This stimulated efforts to eradicate the disease globally which were successful; the last naturally occurring case was in Somalia in 1977 and the World Health Assembly certified global eradication in 1980. Smallpox eradication is one of the greatest public health triumphs of the twentieth century.

After smallpox eradication, the vaccine was withdrawn from routine use globally and recommended only for selected persons at high risk for exposure (Centers for Disease Control 1980). In the USA, the vaccine was recommended for laboratory workers who directly handle cultures or animals contaminated or infected with non-highly attenuated vaccinia virus, recombinant vaccinia viruses derived from non-highly attenuated vaccinia strains, or other Orthopox viruses that infect humans (e.g. monkeypox virus, cowpox virus, vaccinia virus, and variola virus) (Centers for Disease Control and Prevention, 2001a). Vaccination could also be offered to healthcare workers who have contact with contaminated materials from non-highly attenuated vaccinia viruses. In 2002–2003, because of heightened concerns over the use of smallpox virus as a bioterrorism agent, the USA and some other countries reinstituted smallpox (vaccinia) vaccination for national and state teams of clinical and public health workers who were designated to respond to and care for smallpox cases if such cases were to occur (Centers for Disease Control and Prevention 2003c) and for military personnel. Through August 2003, approximately 450 000 military personnel and 37 000 clinical and public health workers had been vaccinated. Serious adverse events following vaccination have been very rare with no cases reported of progressive vaccinia, eczema vaccinatum, or transmission of vaccine virus in a healthcare setting. This may be due to the stringent screening procedures that were in place for this vaccination program among both vaccinees and their close contacts. Unexpected serious adverse events, previously described only in occasional case reports with the NYBOH vaccine strain, have included myo/pericarditis seen among both civilian and military vaccinees (Grabenstein and Winkenwerder 2003; Centers for Disease Control and Prevention 2003d; Cassimatis et al. 2004; Eckart et al. 2004).

New generation smallpox vaccines, grown in cell culture, have been developed. Several of these vaccines have produced major cutaneous reactions (takes) and evoked neutralizing antibody and cell-mediated immune responses in the vast majority of subjects with a reactogenicity profile similar to that of Dryvax (Weltzin et al. 2003; Monath et al. 2004; Greenberg et al. 2005). Other smallpox vaccines under development include more attenuated and replication-deficient vaccinia strains including modified vaccinia Ankara and NYVAC vaccinia viruses. These vaccines hold promise as safer vaccines against smallpox or against complications from vaccinia virus and may also have application for immunization of immunocompromised persons (Belyakov et al. 2003).

Yellow fever vaccine

Yellow fever (YF) vaccine, available since the 1930s, is the live-attenuated 17D strain of yellow fever virus. A single dose, given subcutaneously, is highly effective in inducing protection that lasts for 10 or more years; booster doses are recommended only at 10-year intervals (Centers for Disease Control and Prevention 2002c). YF vaccine causes local and limited systemic symptoms in 2–5 percent of recipients 5–10 days after vaccination. Serious reactions including encephalitis are rare; the risk is highest in children <9 months of age, and the vaccine is not recommended for children <9 months of age and contraindicated in those younger than 6 months. During recent

years, serious vaccine-associated viscerotropic and neurologic adverse events have been reported globally and have resulted in deaths, primarily in the elderly (Barwick et al. 2003; Kitchener 2004; Yellow Fever Vaccine Safety Working Group 2004). Serious viscerotropic disease has been identified in 23 persons, with >60% case fatality, at an estimated rate of 3 per million vaccines. Serious neurologic disease cases, totaling 19 to present, have included encephalitis, encephalomyelitis, and Guillain Barre syndrome, with an estimated incidence of 4 per million vaccines. Both systemic and neurologic reactions are more likely after the first vaccine dose and do not appear due to mutation of the vaccine virus. Risk appears to be elevated in persons with prior thymectomy (Barwick et al. 2003). YF vaccine should not be administered to pregnant women; however, if a pregnant woman is traveling to an endemic area and cannot avoid potential exposure, YF vaccine may be given. YF vaccine is contraindicated in people with anaphylactic allergy to egg or who are immunocompromised. The response to YF vaccine can be inhibited by cholera vaccine; these vaccines should be given at least 3 weeks apart. Because cholera vaccine is rarely indicated, YF should be given first.

Vaccine is recommended for travelers 9 months of age and older going to areas where YF is endemic, and may be required for entry into such countries. Routine YF vaccination at 9 months of age is recommended in all endemic areas; vaccine may also be given in mass campaigns, which are often initiated by the occurrence of outbreaks. The incorporation of YF vaccine into the routine childhood immunization schedule at 9 months of age was initially recommended by the Expanded Program on Immunization in 1988, and its need reinforced by resurgence of yellow fever outbreaks in West Africa during the 1990s (Monath 2001). Through 2002, 26 of 44 endemic countries have implemented the recommendation, but coverage remains low in many of the highest-risk African countries (Global Programme for Vaccines and Immunization 2003).

VACCINES IN DEVELOPMENT

Rotavirus vaccines

Rotavirus is the most common cause of severe, dehydrating gastroenteritis in children throughout the world. Virtually every child becomes infected with rotavirus at least once in the first 5 years of life. Rotavirus has been estimated to cause 20 percent of all diarrhea deaths in children less than 5 years of age resulting in about 500 000 global deaths annually, most of them in developing countries. Even in developed countries, rotavirus infections cause significant morbidity and some mortality. In the USA, approximately 50 000 children are hospitalized with rotavirus gastroenteritis each year and there are 20–40 deaths due to this infection (Centers for Disease Control and Prevention 1999a). Studies in developed countries have found incidence per 100 000 children ranging from 250 (Spain) to >1 000 (Ireland) (Cunliffe et al. 2002).

Rotavirus is a double-stranded RNA virus composed of three concentric shells, the outer capsid contains two structural proteins: VP7, the glycoprotein (G protein) and VP4, the protease-cleaved protein (P protein). These two proteins define the serotype of the virus and are considered critical to vaccine development because they are the target of neutralizing antibodies that are considered important for protection. The first natural infection with rotavirus results in a predominantly homotypic response to the virus; however, subsequent infections elicit a broader, heterotypic response. After a single natural infection, 40 percent of children are protected against any subsequent infection with any serotype, 75 percent are protected against severe rotavirus infection and 88 percent are protected against severe diarrhea (Velazquez et al. 1996).

Live-attenuated rotavirus vaccines have been developed from human rotaviruses, as well as from rotaviruses of other species (rhesus, bovine, and lamb). All rotavirus vaccines in development are live, orally administered vaccines that aim to mimic the protection acquired from naturally occurring rotavirus infection (Cunliffe et al. 2002). Vaccines based on animal viral strains are developed using a Jennerian approach enhanced with modern virological techniques. Animal rotaviruses are considered naturally attenuated for humans. Human rotaviruses recovered from children with acute rotavirus infection have been attenuated by passage in tissue culture and rotaviruses isolated from asymptomatic neonates are considered to be less virulent and have also been tested in clinical trials.

Taking advantage of the segmented genome of rotavirus, reassortants have been developed that retain most of the genome of the parent virus, but include one or both of two key surface glycoproteins, VP7 and VP4, of the human viruses. Multivalent vaccines were developed following the recognition that rotavirus disease is caused by four major serotypes (G1, G2, G3, G4, and P1a). Monovalent vaccines also appear to confer protection against other serotypes (Offit 2002).

In 1998, a tetravalent, rhesus-human reassortant rotavirus vaccine (RRV-TV) was licensed and recommended for routine childhood immunization in the United States (Centers for Disease Control and Prevention 1999a). However, within a year of use, the recommendations for vaccine use were withdrawn due to the risk of a rare but serious adverse event, intussusception, that occurred in approximately 1 in 10 000 infants vaccinated (Centers for Disease Control and Prevention 1999f; Kramarz et al. 2001; Murphy et al. 2001). WHO has strongly encouraged the rapid development of new and safe rotavirus vaccines (World Health Organization 2003b) and the Global Alliance for Vaccines and Immunization (GAVI)

and The Vaccine Fund have supported a project to accelerate development of rotavirus vaccines for the developing world (McCarthy 2003).

In January 2005, a new vaccine for prevention of rotavirus infections was licensed in Mexico. This monovalent live-attenuated human rotavirus vaccine (G1) produced by GlaxoSmithKline (derived from strain 89–12 which was isolated from a rotavirus-infected child in Cincinnati, Ohio, USA) is administered to infants using a two-dose schedule six to ten weeks apart. The first dose is administered between 6 and 14 weeks of age and the second dose between 14 and 24 weeks of age. Vaccine efficacy data from Finland and Latin America show 70–73 percent protection against rotavirus gastroenteritis, 85–86 percent protection against severe rotavirus gastroenteritis and 93 percent protection against rotavirus illness requiring hospitalization (Bernstein et al. 2002, De Vos et al. 2004, Vesikari et al, 2004). Vaccine efficacy was observed against severe rotavirus gastroenteritis caused by G1 and non-G1 types including the emerging G9 type in Latin America. Safety data available on 5024 immunized infants was reassuring with no increases in any solicited symptoms (including fever, vomiting, diarrhea) compared with placebo, however, the sample size from the combined trials was not intended to draw conclusions about the risk for intussusception which was 0.05 percent in the vaccine group and 0.08 percent in the placebo group (De Vos et al. 2004). The other licensed rotavirus vaccine is a lamb rotavirus vaccine, which is available for use in China, however there are few data available on efficacy and safety of this vaccine.

Several other rotavirus vaccines are currently in development. A pentavalent vaccine based on a bovine rotavirus strain (WC-3) reassorted with the common *VP7* and *VP4* genes of human rotaviruses (G1, G2, G3, G4, and PIa) has been well tolerated in phase I and phase II studies (World Health Organization 2003b). Various bovine reassortant vaccines have been studied as three-dose series in infants; a monovalent WC3 reassortant vaccine was 64–100 percent effective and a quadrivalent WC3 reassortant vaccine was 67 percent protective against all rotavirus disease with essentially complete protection against more serious disease (Clark et al. 1996). This protection is similar to that afforded by RRV-TV, which, when given during infancy in trials in the USA, Finland, and Venezuela, had demonstrated 49–68 percent efficacy against any rotavirus diarrhea and 69–91 percent efficacy against severe diarrhea (Rennels et al. 1996; Joensuu et al. 1997; Perez-Schael et al. 1997). The bovine pentavalent vaccine is currently undergoing large-scale safety trials to examine risks for intussusception, the rare serious adverse event of primary concern. Bovine rotaviruses do not replicate well in the infant small intestine and have lower first dose side-effects than the RRV-TV vaccine. Other vaccine candidates under development include human neonatal RV strains and other human-bovine reassortant vaccines. Additional approaches to the development of rotavirus vaccines include DNA vaccination and subunit vaccines (Barnes et al. 1997; Cunliffe et al. 2002; World Health Organization 2003b).

Other viral vaccines

Vaccines for prevention or treatment of human papillovirus (HPV) infections, herpes simplex virus infections (types 1 and 2), and cytomegalovirus (CMV) are in various stages of development and testing. An Institute of Medicine committee to assess vaccine needs for the twenty-first century placed two viral vaccines in its highest priority category (cost savings and QALYs) for vaccine development in the USA: CMV vaccine administration for 12 year olds and influenza virus vaccine administered to the general population and four viral vaccines in the second priority category for vaccine development (costs <$10 000 per QALY saved): hepatitis C virus vaccine for infants, herpes simplex virus vaccine for 12 year olds, HPV vaccine for 12 year olds and RSV vaccine for infants and 12-year-old females (Institute of Medicine 2000). Among these, HPV and HSV are in the late stages of development and with stage III clinical efficacy trials already conducted.

HUMAN PAPILLOMAVIRUS

HPV infection is the most common sexually transmitted disease; the spectrum of disease caused by this virus ranges from benign anogenital warts to cervical and anal cancer. Cancer of the uterine cervix is the third most common cancer in women. Worldwide, approximately 450 000 new cases are diagnosed each year with a 50 percent mortality rate. More than 100 types of HPV have been identified, approximately 30 of these infecting the anogenital region and about 15 of these types causing almost all cervical cancer. HPV-16 accounts for 50 percent of cases of cervical cancer. Thus effective vaccines to prevent and/or treat HPV infection would have enormous global public health impact.

Prophylactic vaccines are designed to prevent infection; the most common type of prophylactic vaccine is made up of viruslike particles (late structural proteins that make up the viral capsid) which are easily synthesized. Animal studies have provided convincing evidence that neutralizing antibodies can block new infection. A number of prophylactic HPV vaccines have been developed and are currently being tested in phase II and phase III trials, including monovalent HPV-16 and HPV-11 vaccines, multivalent HPV-16 and HPV-18 vaccine and a quadrivalent vaccine against types 6, 11, 16, and 18. Titers achieved following vaccination have frequently been 50 times higher than the titers seen in natural infection (Harro et al. 2001; Berry and Palefsky 2003; Schiffman and Castle 2003; Galloway 2003; Kahn and Bernstein 2003). A large placebo-controlled trial of

a HPV-16 L1 VLP vaccine with aluminum hydroxyphosphate sulfate adjuvant administered as three doses at 0, 2, and 6 months in >2 000 young adult HPV-16 seronegative women followed for a median duration of 17.5 months demonstrated 100 percent efficacy for prevention of persistent HPV infection (Koutsky et al. 2002). All nine cases of HPV-related cervical intraepithelial neoplasia occurred among the placebo recipients. The authors concluded that immunizing HPV-16-negative women may eventually reduce the incidence of cervical cancer. Large phase III trials of vaccines targeted against multiple oncogenic types are under way.

Therapeutic vaccines are designed to eradicate or reduce infected calls and are therefore aimed at stimulating cytotoxic T lymphocytes and are directed against early proteins (E6 and E7) which are thought to be responsible for the oncogenic potential of HPV. HPV theraupeutic vaccines have been tested in phase I and phase II trials against cervical cancer and anal dysplasia and include subunit, recombinant vaccinia virus, recombinant fusion protein, and plasmid DNA vaccines (Berry and Palefsky 2003; Stanley 2003).

HERPES SIMPLEX VIRUS

The herpes simplex virus has two biologically distinct serotypes, HSV-1 and HSV-2. HSV-2 infection is acquired either by sexual contact causing herpes genitalis (300 000 new infections per year in the USA) or may be vertically transmitted from a maternal genital infection to a newborn resulting in severe neurological sequelae and death (Morrison 2002; Schleiss 2003; Koelle and Corey 2003; Cunningham and Mikloska 2001). Vaccines to protect against or modify established genital herpes infections have been under development for decades. Many types of vaccines have been evaluated in clinical trials of immunogenicity and prophylactic and therapeutic efficacy (Morrison 2002; Koelle and Corey 2003). Two similar subunit vaccines prepared from recombinant glycoproteins with different adjuvants have completed phase III trials using a three-dose schedule at 0, 1, and 6 months. The HSV-2 glycoprotein B and D subunit vaccine combined with MF59 adjuvant was immunogenic, but not effective in preventing HSV-2 infections (Corey et al. 1999). In contrast, a glycoprotein D-subunit vaccine with alum and MPL adjuvant was 73–74 percent effective in prevention of HSV-2 infections among women who were seronegative for both HSV-1 and HSV-2. The vaccine was not efficacious in women who were seropositive for HSV-1 and seronegative for HSV-2 prior to the study or in men (Stanberry et al. 2002). A prophylactic plasmid vaccine using the gene for gD of HSV-2 has completed phase I studies. Other prophylactic and therapeutic HSV-2 vaccines are in various stages of development and preclinical and clinical testing. Several therapeutic vaccines (plasmid, subunit, and live replication-deficient virus (DISC)) have completed phase I and phase II trials (Koelle and Corey 2003; Morrison 2002).

CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is the most important cause of congenital viral infection, affecting 0.5–1.5 percent of births in the USA. Though most babies with congenital CMV infection have no abnormalities detected at or after birth, 15–25 percent of affected births have abnormalities with sensorineural hearing loss, mental retardation, cerebral palsy, or impaired vision detected at birth or on follow up (Pass and Burke 2002). A vaccine to prevent congenital CMV infection has been designated a top priority for development in the USA by the Institute of Medicine of the National Academy of Sciences (Institute of Medicine 2000). For vaccine development, the major barrier has been the knowledge that naturally acquired infection does not prevent reinfection with a new strain of CMV and that immunity acquired before conception does not always prevent transmission to the infant. These and other issues relevant to development of an effective CMV vaccine have been reviewed (Plotkin 2004b).

An effective vaccine will need to stimulate high levels of neutralizing antibody and cytotoxic lymphocytes and would ideally also stimulate mucosal antibodies (Plotkin 2004b). Vaccines tested to date in phase 1 or phase 2 trials or preclinical studies include various formulations of a live-attenuated vaccine (Towne strain) (phase 1 and phase 2 trials), a recombinant glycoprotein B subunit vaccine (phase 1 and phase 2 trials), chimeric live virus vaccine (Towne and Toledo strains, phase 1 trials), a canarypox vector vaccine with recombinant gB or pp65 (phase 1 trials) and DNA vaccines (preclinical studies) (Pass and Burke 2002; Temperton et al. 2003; Plotkin 2002).

VZV zoster vaccine for prevention of herpes zoster and its complications

Herpes zoster and its complications including postherpetic neuralgia cause a significant public health burden among the aging population in the USA and other developed countries. The incidence of herpes zoster increases with age and it is estimated that about 20 percent of people will develop herpes zoster during their lifetime, resulting in about 600 000 cases of herpes zoster in the USA each year (Hope-Simpson 1965; Schmader 2001). Declines in VZV-specific cell-mediated immunity are associated with increased risk and severity of herpes zoster (Arvin 1996; Oxman 1995). Early studies on administration of a live VZV vaccine (with higher titers than the licensed varicella vaccine) to older adults 55+ have shown that vaccinated persons experience increased VZV-specific cell-mediated immunity responses to levels typical of those observed in younger

persons, in whom the incidence and severity of herpes zoster are lower (Levin 2001). A booster dose of VZV vaccine administered ⩾5 years after the first dose resulted in boosting in VZV-specific cell-mediated immunity (Levin et al. 2003). A large (ca. 37 000) placebo-controlled clinical trial has been conducted in the USA to test whether a VZV 'zoster' vaccine in persons ⩾60 years will prevent or reduce the risk or severity of herpes zoster and its complications. Results of this trial are expected in late 2005. This may lead to a future zoster vaccine to reduce the incidence of herpes zoster or reduce/attenuate the severity of post-herpetic neuralgia and other herpes zoster complications.

SOURCES OF INFORMATION ON VACCINES

Important sources for information on vaccines include the following:

World Health Organization. Provides extensive information on vaccines, vaccine preventable diseases, and global and national statistics on immunization programs and vaccine preventable diseases. Additional information about the World Health Organization and international vaccine programs may be found at www.who.int/vaccines/. Training materials may be found at www.who.int/vaccines-diseases/epitraining.

United Nations Children's Fund. Provides information about state of the world's children and women of child-bearing age, including statistics on immunization coverage and progress of initiatives to eliminate neonatal tetanus and measles, and eradicate polio. Websites include www.unicef.org/ and www.childinfo.org.

Global Alliance for Vaccines and Immunization. Information on the Global Alliance for Vaccines and Immunization may be found at the website www.vaccinealliance.org.

National Health Service, UK. Provides information about immunization recommendations for the UK (www.immunisation.nhs.uk or www.immunisation.org.uk).

National Immunization Program, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA. Extensive resources provide specific information on immunization and vaccine preventable diseases (www.cdc.gov/nip/).

The Report of the Committee on Infectious Diseases of the American Academy of Pediatrics (Red Book). The full report containing recommendations on all licensed vaccines is usually updated every 3 years. The most recent *Red Book* was published in 2003. It can be ordered from the American Academy of Pediatrics, 141 Northwest Point Blvd., PO Box 927, Elk Grove Village, IL 60009-0927, USA or from the AAP website (www.aap.org).

Morbidity and Mortality Weekly Report. This report is published weekly by the Centers for Disease Control and Prevention (CDC) and contains vaccine recommendations, reports of specific disease activity, policy statements, and regular and special recommendations of the ACIP. The MMWR is available online at www.cdc.gov/mmwr/ or www.cdc.gov/nip/acip.

Allied Vaccine Group. A partnership of six independent websites providing science-based, reliable information about immunization (www.vaccines.org).

Immunization Action Coalition. The Immunization Action Coalition promotes physician, community, and family awareness of and responsibility for appropriate immunization of all children and adults against all vaccine-preventable diseases. This US-based site has a wealth of education materials (www.immunize.org).

Official Package Circular. Manufacturers provide product-specific information for each vaccine. Some of these are reproduced in their entirety in the *Physicians' Desk Reference* (PDR) and are dated.

Control of Communicable Diseases in Man. The American Public Health Association publishes a manual at approximately 5-year intervals. The 18th edition (2004) is currently available. The manual contains valuable information concerning infectious diseases; their occurrence worldwide; immunization, diagnostic, and therapeutic information; and up-to-date recommendations on isolation and other control measures for each disease presented. It can be ordered from the American Public Health Association, 800 St. NW, Washington, DC 20001, USA.

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Viral vectors for gene therapy

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INTRODUCTION

Our increased knowledge of the human genome and the understanding that a large number of human illnesses have a genetic basis have led to a desire to correct the genetic defects underlying these diseases. Human gene therapy has two basic requirements: 1) the wild type gene sequence, and 2) a means of delivering the wild type sequence to the cell so that it may be expressed in an appropriate manner. In the case of a deleterious mutation, which is dominant when heterologous, the therapeutic gene may encode a product which selectively inactivates the deleterious mutant product. While we know and have in hand large numbers of potentially corrective genes, development of successful delivery vehicles or vectors is still at a relatively early stage. To be successful, a vector should be able to introduce the corrective gene (transgene) into the target cell, in most cases transport it to the nucleus, establish a stable state in the nucleus, and express the desired levels of the product encoded by the transgene for extended periods of time. (The latter need not be the case when the intent of the therapy is to engender a protective immune response, i.e. a vaccine or a product intended as a lethal, anticancer agent.) Finally, the vector should not elicit an inflammatory or immune response against the cells that contain the vector.

A variety of delivery systems have been tried including viruses, artificial lipid envelopes, and direct injection of naked DNA (e.g. via the 'gene gun'). Viruses have been the favored instrument since they naturally deliver genes into human cells. The viral genes are expressed in a regulated manner and in many instances persist for extended periods of time. However, there have been only a moderate number of clinical trials and only one has offered good evidence of therapeutic benefit (see below). Several significant problems have been encountered. Either the level of transgene expression has been inadequate or of insufficient duration. The latter is a common problem and is thought to relate to the host immune response to the vector. An immune response to structural proteins in the vector virion leads to inhibition of successful vector delivery on subsequent administration. However, often there is an immune response to expression of the viral genes remaining in the vector. This leads to cell death and frequently is an inflammatory response. It was such a response which led to the only death of a patient in a gene therapy clinical trial after administration of a high dose of an adenovirus vector (see below). Thus, development of vectors which allow sustained expression of the transgene is a common goal; success in this implies the absence of deleterious host response. As an aside, if the patient has a null mutation, it is conceivable that expression of the wild type gene product would elicit an immune response. This is of concern and must be carefully monitored in each case. Another potential concern of gene therapy is the location of the vector within the nucleus. If it remains extrachromosomal and is not able to replicate in synchrony with cell DNA, then it is likely to be diluted out if the cell divides; extrachromosomal vectors are not useful if the goal is introduction of the transgene into progenitor cells. Thus, it would be advantageous to develop vectors that can integrate into the

cell genome so that the transgene can be replicated in synchrony. Almost all of the current vectors do integrate to an extent; retroviral-based vectors do so frequently, other current viral vectors integrate at a low frequency. However, of great significance is the possibility of insertional mutagenesis. If the vector integrates into an important cellular gene, it may inactivate the gene or convert its product into one that is deleterious to the cell. As indicated below, in the only clinically successful use of gene therapy a retroviral vector derived from Moloney murine leukemia virus was used to treat patients with severe combined immunodeficiency disease. However, two of the patients developed a leukemia-like disease because of the site of integration of the vector. While these two have been successfully treated for the complication, the risks inherent in vector integration at random sites remain (Hacein-Bey-Abina et al. 2003).

Although human gene therapy is in its infancy, significant progress has been made in the development of viral vectors. In this chapter we will describe such work with the four viruses that are most commonly used as vectors: adenovirus, herpes simplex virus, retrovirus, and adeno-associated virus.

RETROVIRAL VECTORS

Both in vitro and in vivo gene delivery strategies were pioneered using retroviral vector systems. In the early 1970s the concept of gene transfer using retroviruses for a variety of purposes had been put forward. Although nearly 35 years have passed since, the successful application of this system has mainly been limited to a range of laboratory uses. Only recently has clinical investigation resulted in unambiguous evidence that a therapeutically beneficial result can be achieved. Notwithstanding the long development phase, it must be noted that retroviruses have served as a guiding frontrunner in the development of a range of vector systems that are built on many different animal viruses.

Due to the large amount of research involving retroviruses the aim of this section is not to represent a comprehensive review of the virus and all possible applications, but rather to limit the discussion to those characteristics of retroviruses that are essential to understanding the complexities inherent in this promising gene transfer system.

Retrovirus Biology

The *Retroviridae* represent a large group of viruses that are widely distributed throughout the vertebrates (Coffin et al. 1997). The taxonomy of this virus family has recently been adjusted to reflect our understanding of the molecular biology of the different genera. In general, retroviruses can be divided into two groups, the 'simple' retroviruses among which the alpha-, beta-, and gamma retroviruses are counted and the 'complex' retroviruses with the genera delta-, epsilon-, spuma-, and lenti-retroviruses. Representative members of the retrovirus genera are avian leukosis virus (ALV) for alpha viruses, *Mouse mammary tumor virus* for beta-, and *Murine leukemia virus* (MLV) for the gamma retroviruses. The genera of complex viruses are represented by human T-lymphotropic viruses (HTLV) (delta virus), *Human foamy virus* (spumaviruses) and human immunodeficiency virus (HIV) (lentiviruses). Epsilon viruses are exclusively found in fish and reptiles and will not be further discussed. Based on the observation that the alpha-, beta-, and gamma retroviruses have oncogenic potential these genera have been also collectively called onco-retroviruses. There are several potential mechanisms that can contribute to the transforming potential of these viruses (Suzuki et al. 2002; Mikkers et al. 2002; Lund et al. 2002). First, these viruses are known to undergo recombination at high frequencies resulting in the generation of defective genomes that can be complemented by wild type retroviruses. These contaminating defective genomes have been shown to be able to acquire host cell sequences that, in very rare events can be biologically active. Among those sequences that have been identified to have a significant (detectable) effect on the cell cycle are a number of proto-oncogenes. A second mechanism is inherent to the viral life cycle through the retroviral strategy of integrating a copy of the cDNA into the host genome in a manner that is nonspecific with respect to the chromosomal locus (Stocking et al. 1993). Theoretically the viral cDNA can integrate into or close to a tumor suppressor or a proto-oncogene, respectively. We will discuss the events that have made this theoretical possibility a reality which, in the context of gene therapy, needs consideration.

The members of all retrovirus genera share three coding regions, *gag* (group-specific antigen), *env* (envelope), and *pol* (polymerase) (Goff 2001). The *env* region encodes the envelope protein, the *pol* region the reverse transcriptase, integrase, and a viral protease, and the *gag* region encodes the nucleoproteins as well as the matrix and capsid proteins. The complex retroviruses distinguish themselves from the 'simple' viruses by the presence of additional accessory genes that significantly alter the viral life style.

In general, retroviruses are diploid in that they contain two identical copies of the viral single-stranded RNA. The sizes of positive-strand virus genomes range from seven to 13 kb. As the retrovirus genome is generated by cellular transcription complexes it resembles cellular RNA through the presence of both a 5′ cap and a poly(A) sequence. Additional sequences that are essential to the viral life cycle include the packaging signal (ψ) and the *att*-sites that are required for provirus integration.

Retrovirus life cycle

The life cycle of retroviruses is somewhat complex and in addition to receptor binding, fusion, and uncoating involves reverse transcription (hence *Retro*viruses), nuclear import (either passive through cell division for onco-retroviruses or active in the case of lentiviruses), and integration of the proviral DNA. In order to generate daughter virions, the integrated provirus is transcribed by cellular proteins, followed by splicing and nuclear export. The cytoplasmic RNA is then translated into precursor proteins, and the life cycle is concluded by packaging of full length RNA into the assembled virions. A new round of infection can commence after budding of the virion and proteolytic processing and maturation of the virion. Each of these steps had to be taken into account for the development of retrovirus-based gene transfer strategies. Early problems with this vector system included the contamination by replication competent virus (in this class of viruses a certainly significant concern), low titers of the vectors, only low levels of expression by the transgene, and promoter down-regulation after successful transduction. Overall, the retrovirus gene therapy field has been able to overcome these hurdles to a large extent through many variations of vector design and production. One of the hurdles was the efficiency of receptor binding by retroviruses. All retroviruses that are used for gene therapy purposes are enveloped by a glycolipid membrane. This envelope contains proteins that are responsible for receptor binding and thereby defines the tropism of the viruses and vectors. In general, this receptor-mediated fusion triggers all of the subsequent steps in the viral entry process that are necessary for successful transduction. However, the viral glycoproteins of the envelope also define – and in some cases restrict – the efficiency of gene transfer. A significant development in this regard was the introduction of pseudotyping, a strategy that allowed for the replacement of the native glycoprotein with one of a broader tropism. In the early clinical trials that utilized retroviruses and that were aimed at the transduction of hematopoietic stem cells (HSC), the vectors were pseudotyped with the MLV amphotropic envelope (Chien et al. 1997). A disadvantage of these amphotropic viruses, however, is the relatively low amount of this specific receptor on HSCs. This observation possibly explains the relatively low efficiency transduction of HSCs by amphotropic retroviruses. The protein of choice for the expansion of the tissue tropism was the glycoprotein G from vesicular stomatitis virus (VSV-G) (Yang et al. 1995). While only replacing a single envelope protein, this variation shifts the mode of vector uptake from receptor-mediated fusion to endocytosis that, in addition, requires endosome escape as a result of vesicle acidification. The advantage of this pseudotyping strategy is several-fold. First, the receptor for VSV-G is believed to be a membrane lipid that is ubiquitous in all cells. Second, VSV-G itself mediates pH-dependent endosome escape in addition to its receptor attachment function (Yamada and Ohnishi 1986). As a result, this simple envelope modification conferred a broad tissue tropism and efficient cellular trafficking to many retroviruses, including the increasingly utilized lentiviruses. Conceptually, the introduction of pseudotyping has opened the door to many applications ranging from the broadening of tissue tropism to strategies aimed at the targeting of specific cell types.

Subsequent to membrane fusion, or endocytosis and endosome escape, are several steps that are of importance with respect to retrovirus gene transfer and vector production. Several viral gene products are required for these steps, including reverse transcriptase, integrase, and protease. Although in current vectors the genes encoding these proteins are not present, these, as well as other proteins, are part of the virion and, therefore, do not need to be encoded by the recombinant transfer vector genome.

The reverse transcriptase step during viral replication is of importance with respect to vector design and quality control based on its biochemical properties. The enzyme is characterized by a comparatively slow incorporation rate of <100 nucleotides per second, a poor processivity, a relatively low fidelity with a mis-incorporation rate of approximately one in 104 and the absence of a proof-reading activity (Kerr and Anderson 1997; Oude Essink and Berkhout 1999; Harrison et al. 1998). Although these values have been determined in vitro they do correspond somewhat to the high mutation rate in vivo of ca. one mutation per genome (Burns and Temin 1994). This error rate has been associated to the unique capacity of lentiviruses to evade either host cell responses or the effects of antiviral pharmaceuticals and is therefore biologically significant. With respect to vectors, however, the mis-incorporation rate is somewhat problematic, particularly since it has been documented that the error rate is template-dependent and can therefore not be predicted (Bebenek et al. 1993). An additional characteristic of retroviruses is their diploid genome that, theoretically, could provide a certain genetic stability to the viruses. It has, however, been documented that when genetically different genomes are present the reverse transcription process utilizes both RNA strands resulting in a high degree of recombination.

Nuclear import

In the field of retrovirus gene transfer a frequently discussed ‘roadblock’ is the transport of the viral cDNA into the nucleus. The onco-retroviruses do not encode for a machinery that would actively facilitate nuclear import. On the level of gene transfer application this

reliance on passive import translates into the requirement for cell division prior to transduction (Roe et al. 1993; Lewis and Emerman 1994). Although the majority of applications to date focus on HSC this dependency defines a limitation for the ultimate usefulness of 'simple' retroviruses as it has been demonstrated that even primitive HSC cells are predominantly in a quiescent, nondividing state. This roadblock has been central to the efforts of developing vectors that are based on the complex retroviruses such as the spumavirus human foamy virus (HFV) and, most importantly, lentiviruses. Possibly the most widely explored lentivirus remains HIV-1 (Weinberg et al. 1991; Blomer et al. 1997). Although it might appear counter-intuitive that a virus with such devastating effects on human health would be pursued as an ultimately nonpathogenic gene transfer vector, it has been exactly this phenomenon that resulted in the accumulation of an impressive body of information about the biology of the virus, which has allowed the accelerated development of this class of gene transfer vectors. Among the viruses explored in this class are both HIV-1 and 2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), and equine infectious anemia virus (EIAV) and visna virus. Despite this reorientation of the field towards the complex retroviruses that is mainly based on their nuclear import potential, the underlying mechanisms have not yet been identified and the subject is somewhat plagued by controversy. Similarly, foamy virus nuclear import is not understood, although a pathway that involves different cellular components (and is therefore distinct from lentiviruses) has been proposed (Saib et al. 1997). It must be pointed out that aspects of these unknown mechanisms possibly underlie the observation that in some differentiated tissue in vivo transduction by lentiviruses might require cell division (Park et al. 2000).

Provirus integration

The subsequent step in viral infection, cDNA integration, is one of the clearly attractive features of retroviruses. This step is an essential component of the viral life cycle and it is thus not surprising that the mechanism is quite efficient. Consistent with the life cycle of retroviruses in vivo the integration allows persistence of the viral genome in dividing cells.

Mechanistically, after formation of the double-stranded cDNA and nuclear import, the generation of circular molecules has been observed. To date, it is believed that the presence of certain circular species can be used to detect nuclear import, but it also has become clear that these molecules are poor substrates for the integration process (Lobel et al. 1989). This leaves unanswered the question of function of these circularized retroviral genomes. It is reasonable to assume that these structures represent a side product of integration rather than an essential part of the mechanism. However, of some concern is the possibility that the circular molecules can – transiently – contribute to transduction efficiency thereby skewing the estimated gene transfer rate (Haas et al. 2000).

A key concern, however, with respect to retroviral gene transfer, has been the potential of insertional mutagenesis. In general, the risk for tumor transformation as a result of a single insertion event could be estimated to be rather low in light of the well-documented notion that the establishment of malignancy in differentiated tissue requires multiple 'hits'. Nevertheless, the application of vectors that integrate in a nonspecific manner into the genome in rapidly dividing cells adds the additional level of selection to the consideration. That is, if virus genomes randomly integrate in cells that are first expanded (either in vivo through growth advantage over nontransduced cells or ex vivo through selective pressure) and then differentiated, it is feasible to hypothesize that cells with integration events into a tumor suppressor gene or close to a proto-oncogene are likely to be enriched. In this 'selection' scenario, a single-hit, vector-induced mutation might be sufficient to contribute to the subsequent establishment of malignancies. In this context it must be emphasized that in nearly 15 years of clinical experimentation with retroviruses, no integration-related cancer has been reported. Recently, however, in what might represent the first clinically successful gene therapy trial to date, two patients developed a T-cell leukemia that was correlated to the insertion of the retroviral vector. The trial was designed to treat X-linked severe combined immunodeficiency (X-SCID) using an MLV-based vector expressing the γ-c chain cytokine receptor. In the affected children the absence of this receptor function results in the inability to respond to cytokine signaling and therefore the absence of functional T- and natural killer (NK) cells (Hacein-Bey-Abina et al. 2002; Fischer 2000; Soudais et al. 2000). Approximately 30 months after the engraftment of retrovirally transduced cells, two of the patients developed a leukemia-like condition that required chemotherapeutic treatment. Since, it has been determined that in both patients the retroviral cDNA had integrated into and close to the LMO2 locus, respectively. Understandably, these adverse side effects have caused considerable anxiety within the gene therapy community, the government regulators, and beyond (Hacein-Bey-Abina et al. 2003; Check 2002a,b). However, this case, that was characterized by the unique willingness of the investigators to share the pertinent information with the scientific community and the public, deserves further analysis. First, among the more than ten treated patients, only two have fallen ill with leukemia. Second, a third patient is said to have insertion into the *LMO2* locus but did not develop leukemia within a comparable time frame. Third, within the

context of numerous clinical and preclinical experiments employing retroviruses these are the first serious adverse effects documented to result from insertional mutagenesis by a retrovirus. Taken together, these observations indicate that several parameters came together in this case. It is unlikely that random integration (in these cases into the *LMO2* oncogene that has previously been reported to play a role in leukemia development) by the vectors alone caused the development of the malignancies. It is more likely that the cancer in these two patients has resulted from a combination of factors that include the *LMO2* alterations, the unregulated transgene expression (note that the γ-chain of the cytokine receptor for IL2, 4, 7, 9, 15, and 21 naturally confers a selective advantage for the transduced cell) and the nature of this particular treatment strategy that is based on the proliferation of a few cells to repopulate this component of the hematopoietic system. Therefore, the importance of these events to retrovirus-mediated gene therapy in general is unclear and will likely only be assessed through further pre-clinical and clinical trials.

The further steps in the retroviral life cycle, transcription of the provirus, packaging and maturation are mainly important for considerations regarding the vector production and purification. Although most clinical trials to date have been conducted ex vivo, increasingly efforts are underway to develop strategies that employ in vivo delivery. These approaches confront the retrovirus gene therapy with issues related to anti-virus immunity. For example, it has been shown that the glycoprotein G of VSV can serve as an efficient antigen through which the vector can be inactivated (DePolo et al. 2000) by human serum. In order to address this concern, alternative envelopes or PEGylation of these vectors have been proposed (Croyle et al. 2004).

Vector design strategies

Both simple and complex retrovirus vector development have undergone numerous generations, including many variations in the strategy to provide the helper functions necessary to generate infectious virions (for a review of these developments see Brenner and Malech (2003)). Of particular concern in this regard has been, and still are, the attempts to minimize and exclude the risk of generating replication-competent viruses through recombination events. In brief, the approaches can be summarized by two different strategies: first, the use of stable cell lines that contain the genes encoding all necessary helper functions stably integrated within the genome of the producer cell line. A construct containing the transfer vector is then transiently transfected into the producer cell line and the recombinant virus is shed into the medium. Notably, only the essential retrovirus components are retained within the transfer vector, including the packaging signal ψ and the viral LTRs. In lentiviruses these elements also include the rev responsive element (RRE). The second strategy, transient transfection of both the helper functions and the transfer vector has been more employed during the development of lentivectors. The overall theme during this development process, that has now undergone more than three generations, has been to delete as many of the accessory factors as possible. This was particularly important since some of these factors are toxic to cells and enhanced the likelihood of eliminating the generation of replication competent virus. In exchange, however, both titers and transgene expression levels after gene transfer have been reduced. This problem has then been addressed by the addition of different promoters driving the transgene and the incorporation of post-transcriptional regulatory elements such as the one identified in Woodchuck hepatitis B virus (WHV). An additional safety element has been introduced through the use of self-inactivating (SIN) vectors (Zufferey et al. 1998). In these vectors there is a deletion in the 3′LTR including the TATA box. This deletion is transferred to the 5′ LTR resulting in its inactivation. While this modification does not result in lower titers or reduced transduction efficiency it considerably adds to the safety of these vectors.

Finally, retrovirus based systems have been the frontrunners of viral vector development and are likely to contribute significantly to both experimental and therapeutic gene transfer in the future. Several hurdles, however, will need to be overcome. These include the moderate titers achieved with current production schemes as well as regulatory restrictions with regard to the use of a vector that is derived from a serious human pathogen.

ADENOVIRAL VECTORS

The development of gene transfer for the future treatment of a variety of diseases has been accompanied by a number of breakthroughs and prominent setbacks. Due to their early presence in the gene therapy field, adenovirus-based vectors have been at the center of some of the most widely publicized events. In the following section we will outline the basic aspects of adenovirus biology, the early contributions to gene transfer, the culmination of extensive research, and vector-development efforts in the serious adverse effects leading to the death of Jessie Gelsinger and the redefinition of the entire gene therapy field as a direct result.

Adenovirus biology

Adenoviruses were discovered in the 1950s as pathogenic agents found in human adenoid tissue (Rowe et al. 1953) and from military recruits suffering from respiratory illness (Hilleman and Werner 1954). Soon thereafter, these agents were named *adenoviruses.* Since then

many viruses with similar characteristics have been isolated from humans and animals resulting in more than a hundred members of this virus group, 47 of which are classified as human adenoviruses. To date the family of *Adenoviridae* is divided into four genera, the *Mastadenoviruses* to which the human viruses belong, those limited to bird infections, the *Aviadenoviruses* (Norrby et al. 1976), the ovine *Atadenoviruses* (Both 2004), and the *Siadenoviruses* that are found in frogs and turkey (Davison 2000). During the early studies on adenovirus biology a key observation was made in 1962 when Trentin found that a human serotype of adenovirus (Ad12) could induce tumors in newborn hamsters (Trentin et al. 1962). Although to date no evidence has been found that adenoviruses are capable of inducing cancer in humans, this first notion of DNA viruses as tumor-inducing agents set precedence for numerous subsequent discoveries in the field of DNA tumor viruses. Furthermore, the use of adenoviruses as model systems to study cellular phenomena led to a number of highly significant findings related to cell cycle regulation, DNA replication, and gene expression. The most prominent discovery was made by Sharp and colleagues and, independently, the laboratory of Roberts who demonstrated the presence of spliced mRNA segments in adenovirus late genes (Berget et al. 1977). This discovery led to the identification of introns and ultimately to our current understanding of splicing and was awarded the Nobel Prize in 1993.

The capsids of adenovirus are not enveloped and form an icosahedral structure of approximately 100 nm in diameter. Each capsomer (one of 252 capsid subunits) contains hexons (at the six-fold axis), pentons (at the five-fold axis), a penton base, and an extending fiber that gives adenoviruses their characteristic appearance (Table 1).

The genome of adenoviruses (Shenk 2001) is flanked by inverted terminal repeats (TR) of approximately 160 nucleotides that contain origins of DNA replication. The TRs are covalently attached to terminal protein that is essential to the virus replication cycle. In addition, the left-end DNA contains a number of signals between nucleotides 200 to 400 that are essential for the packaging of the viral genome into preformed empty capsids (Grable and Hearing 1992; Hearing et al. 1987). Together, the packaging elements, the TRs and the 5′ covalently attached terminal proteins are sufficient *cis* elements for efficient packaging. This has enabled the development of a new generation of adenoviral vector (gutless vectors) that contain only these minimal *cis* elements flanking the gene cassette of interest (potential therapeutic gene). The viral genome is divided into two regions, the early region (encoding the E1, E2, E3, and E4 transcription units) with two delayed early transcripts (IV and IX) and the late region (L1–L5). In addition, the only RNA polymerase III product, VA, from the adenovirus genome is also expressed in the late phase. In general, the transcription units of adenovirus encode proteins of related functions. E1 units encode proteins involved in transcription (E1A) and the inhibition of apoptosis (E1B), while E2 products are involved in DNA replication. E3 products have functions involved in overcoming host defense mechanisms. It might be noteworthy that a generation of viral vector constructs has omitted the E3 region in order to gain larger capacity for transgene cassettes. The exception to the functional unity within the transcriptional units is provided by the E4 region that encodes proteins that are involved in a range of different functions, including transcription, replication, RNA trafficking, and apoptosis (Leppard 1997). Finally, the family of late genes is responsible for the generation and maturation of the viral capsids.

Vector design strategies

The rich history in adenovirus research has left us with a wealth of tools for the generation of recombinant Ad-based vectors. Strategies have been developed that allowed the generation of mutated and deletion variants

Table 1 *Selected characteristics of the most frequently used gene delivery vectors*

Vector	Genome capacity	Tropism	Immune response	Genome persistence	Limitations
Onco-retrovirus	8 kb RNA	Dividing cells	Limited	Integrated, long-term	Require cell division, insertional mutagenesis
Lentiretrovirus	8 kb RNA	Dividing and arrested cells	Limited	Integrated, long-term	Limited titers and some applications, insertional mutagenesis
Herpes virus	40–150 kb dsDNA	Particularly suited for neurons	Strong	Episomal, long-term possible in neurons	Immune rejection of infected tissue (except neurons)
Adenovirus	8–30 kb dsDNA	Broad	Strong	Episomal, transient	Innate and cellular immunity
AAV	5 kb ssDNA	Broad	Limited	Episomal, long-term	Limited packaging capacity, slow onset of transduction

of the adenovirus genome that could still be grown to appreciable titers of recombinant viruses. One of the essential reagents to this end is the human embryonic kidney cell line 293 (HEK293) (Graham et al. 1977). This cell line contains the E1 genes of Ad5 and thus allows the production of high titer stock of E1 deleted adenoviruses. Many of the recently employed recombinants simply replaced the E1 region by the transgene of interest. A corollary of the E1 deletion is, that because of the transcriptional activation functions of E1A, the remaining genes of the Ad-vectors cannot be expressed to appreciable amounts. A second aspect of adenovirus biology that has facilitated the development of viral vectors is the capacity of the virus for genome recombination and thus the possibility for insertion of transgenes into the approximately 36 000 bp genome. Wild type adenovirus undergoes recombination at high efficiency in cells that are co-infected with different serotypes (Williams et al. 1975). The precise mechanism of these recombination events is not entirely dissected to date. However, it has been demonstrated that DNA replication is required for these events to occur (Young et al. 1984). This requirement hints at DNA replication intermediates as likely substrates for the extraordinarily efficient recombination reaction. The proposed model for adenovirus DNA replication predicts that replication initiates at both origins within the TRs and proceeds in a unidirectional manner resulting in the displacement of the strand that is opposite of the replicated strand. This strand displacement mechanism results in generation of a single-stranded genome that can form a panhandle structure through hydrogen bonds between the terminal repeats (Lechner and Kelly 1977). It is this single-strand replication intermediate that is hypothesized to be involved in recombination with other serotypes through either strand-invasion of double-stranded genomes or simple annealing to single-strand intermediates of the other serotype followed by DNA repair (Flint et al. 1976). In order to conclude the DNA replication cycle, both products generated in the first round of replication, the double-stranded genome as well as the single-stranded displaced strand, can serve as templates for further replication.

Adenovirus pathogenesis

Much is known about the pathology and pathogenesis of adenovirus infection in humans and it would exceed the purpose of this chapter to summarize the extensive literature addressing these aspects. However, in view of the application of adenovirus as a gene transfer tool, several observations are noteworthy. With respect to preclinical evaluation of the safety of recombinant adenovirus, a key feature is the species dependent replication cycle of these viruses. Although there is some cross-permissivity in closely related species, no frequently used animal model is suited, e.g. to evaluate the replication of human adenoviruses. This species specificity is illustrated by the requirement of SV40 co-infection to achieve productive replication of human adenovirus 2 even in monkey cells (Klessig and Anderson 1975). Possibly the only model that was able to faithfully replicate the clinical consequences of wild type adenovirus infection in both the respiratory tract as well as in the eye was the cotton rat (Prince et al. 1993; Tsai et al. 1992). Host range mutants have also been described that allow replication of Ad2 and 5 in simian cells (Klessig and Grodzicker 1979; Klessig and Hassell 1978). Although it appears of limited importance with respect to toxicity evaluation of replication deficient recombinant adenoviruses, the availability of faithful animal models would facilitate the evaluation, e.g. of contaminating wild type particles.

An additional aspect of the infectious cycle of adenovirus is the general absence of toxins. Possibly the only exception to this rule is the capsid component penton. It has been demonstrated that, when added to cells in culture, penton leads to a clear cytopathic effect, even though the mechanism of this phenomenon is not yet fully understood (Pettersson and Hoglund 1969; Seth et al. 1984).

A more complex phenomenon of adenovirus infection is the host immune response to infection. Although a comprehensive description of the immunity to adenoviruses is certainly beyond the scope of this chapter, certain aspects deserve further elaboration in light of the death of Jessie Gelsinger, a patient at the University of Pennsylvania who died of the consequences of adenovirus mediated gene transfer that resulted in an overwhelming immune response and ultimately fatal multiple organ failure. It should be pointed out that this event resulted in profound and lasting changes in the then emerging field of gene therapy research.

Much like many viruses, wild type adenovirus encodes a number of immunomodulatory functions and it must be highlighted that recombinant adenoviruses that are devoid of the expression of viral genes are consequently vulnerable to the effects of capsid-mediated host responses. It has been known for more than a decade now that non-permissive infection by adenovirus results in an activation of cytokine networks (including IL1, IL6, and TNF responses) (Ginsberg et al. 1991; Ginsberg and Prince 1994). Immediately after infection the product of the early gene E1A inhibits interferon-controlled gene regulation while VA-RNA blocks PKR activity. Subsequently, the products of the early region 3 counteract apoptosis and the pro-inflammatory actions of TNF-α. Due to the deletion of E1 (involved in the regulation of both cellular and viral gene expression) in most recombinant adenoviral vectors, there is no controlled expression of the adenovirus 'stealth factors' that in fact make up approximately one third of the wild type genome. It can therefore be of no surprise that the

high-dose delivery of recombinant adenovirus (deleted for E1 and E4) could result in severe adverse effects observed in one of the patients enrolled in the study.

In general, a major hurdle in the development of future gene therapy is represented by the multifaceted host responses against viral infections. Adenovirus vectors have demonstrated this problem starting from the observation that transgene expression in all tissues was of transient nature in immune competent animals and culminating in the death of Jessie Gelsinger that is now understood to have resulted from the innate immunity to the viral vector (Marshall 1999, 2002).

Adenovirus vectors

To date, several strategies have been employed to engineer adenovirus vectors. It is noteworthy that most of the more recent strategies were designed to minimize the immune-mediated toxicity of the vectors. While first-generation vectors were deleted for E1 and E3 in order to both prevent late-gene expression and to allow for deficient cloning capacity, subsequent modifications such as the deletion of E2 and E4 were designed to lower the toxicity in animal models (Marshall 1999, 2002). The most recent variation on adenoviral vector design represents a more conceptual development in that all of the adenoviral genes are deleted, leaving only the terminal repeats and a packaging signal. These vectors are termed 'gutless' or helper dependent (HD) adenovirus vectors and clearly resemble the design of first-generation adeno-associated virus vectors (Morsy and Caskey 1999; Cregan et al. 2000; Zhou et al. 2002; Palmer and Ng 2003; Imperiale and Kochanek 2004; Mitani et al. 1995; Parks et al. 1996). Although a number of studies clearly demonstrated that transgene expression as a result of HD-adenovirus delivery, it has become evident that transduction is not persistent for the life span of the animal. It remains to be addressed whether the, albeit delayed, loss of transgene expression is due to vector genome loss, residual immune response to capsid components or to contaminating replication competent helper adenovirus. Although a number of strategies have been designed in order to purify the HD vectors, the biophysical similarity between HD-vectors and their helper virus have made this attempt challenging (Palmer and Ng 2003).

Finally, a different strategy takes advantage of the toxicity of replicating adenoviruses and has been proposed for use in cancer gene therapy. The first strategy proposed involved ONYX015, an adenovirus vector that was deleted in E1B (Bischoff et al. 1996). It was hypothesized that the deletion of the inactivating p53 binding partner would render this virus conditionally permissive in p53 deficient cells. Preclinical studies using this construct were sufficiently promising for clinical studies to be conducted (Nemunaitis et al. 2000; Lamont et al. 2001; Nemunaitis and O'Brien 2002a,b). This study, however, that is in progress in patients suffering from head and neck cancers, has not yet been able to demonstrate a clinical benefit although tumor-specificity was documented (Lamont et al. 2001). It is not clear what additional, possibly cellular, factors are required in order to convert tumor selectivity into clinical benefit. A number of current preclinical and clinical studies are underway to address this question and to validate this promising approach.

HERPES SIMPLEX VIRUS (HSV) VECTORS

Herpes simplex virus is a large DNA virus that has been well characterized (Roizman and Knipe 2001). It has a predilection for infection of neural tissue; for this reason it has been of special interest as a potential vector for gene therapy of the central nervous system. The genome is a linear, double stranded DNA composed of 152 kb which contains two unique segments, each bounded by an inverted terminal repeat. The two segments can be arranged in either orientation with respect to one another so that there are four possible sequence arrangements. The overall genome is bounded by two short terminal repeats. The capsid is icosahedral and enveloped in a trilaminar lipid membrane into which are embedded ten virally encoded glycoproteins, some of which are involved in cellular attachment. A protein matrix, the tegument, separates the nucleocapsid from the envelope. Functions of proteins in the tegument include regulation of induction of viral gene expression and the shutoff of cellular protein synthesis.

HSV life cycle

HSV gene expression is temporally regulated. Induction of immediate early (IE) gene expression requires VP16, a structural protein in the virion tegument. Only two (ICP 4 and 27) of the five IE proteins are required to turn on later gene expression, the other three (ICP 0, 22, and 47) are not required for productive infection of cell cultures. (One of the non essential genes, ICP 47, is involved in immune evasion in the intact host.) Early (E) gene expression products are required for viral DNA replication, while late (L) genes encode the structural proteins. Overall, the HSV genome encodes 84 proteins (Roizman and Knipe 2001). While deletion of the nonessential early genes helps reduce potential pathogenicity in the intact host, deletion of the required IE genes renders the mutant virus unable to replicate and, therefore, absolutely defective. In this manner it is possible to construct HSV vector viruses which should be nonpathogenic, as long as they do not elicit a toxic immune response in the host. The ability to delete significant regions of the genome and the overall large size of the DNA means that the space available for transgene insertion is large. Vector production can be

carried out in cell lines which constitutively express the IE genes which have been deleted from the vector construct. In practice, high titer preparations of recombinant HSV can be made (Advani et al. 2002).

The HSV life cycle is notable for the ability of the virus to establish persistent infections in various ganglia. Primary infection occurs through the skin or mucosal surface where the initial round of viral replication occurs. Progeny virions enter neural axons and are transported in a retrograde manner to the cell body where the virus can establish a latent state in which the viral genome is maintained as an extrachromosomal, duplex circular molecule. Latency can be maintained for periods of months or years until activation, usually induced by some form of stress to the host (e.g. superinfection or exposure to toxic stimuli). Activation leads to production of infectious virus, an event typically manifested in the case of HSV as a cold sore. To the extent that persistent infection can be maintained without the threat of multiplication during the primary infection or during reactivation, HSV represents a good potential vector for human gene therapy.

HSV vectors

An alternative approach to the construction of HSV-based vectors, known as amplicons, is to create plasmids which contain both bacterial and HSV origins of DNA replication, viral genomic packaging, and cleavage signals. All of the HSV functions which are required for DNA replication, structural proteins, etc. can be supplied in trans. If the complementing genes have none of the signals required for replication or packaging and no sequences homologous to the amplicon sequences, only the amplicon should be replicated and packaged into vector virion particles. Thus, amplicons are potentially capable of containing extremely large transgenic sequences. However, despite the lack of homology between amplicon and the HSV helper sequences, in practice it has proven difficult to produce vector preparations devoid of wild type virus.

All of the IE genes, with the exception of ICP 47, are cytotoxic; thus deletion of the other four would be desirable. However, it is difficult to achieve high titer preparations if all of the IE genes are deleted. ICP 0, which is a potent transactivator, appears to greatly facilitate vector production. ICP 0 is metabolized differentially in neurons and glial cells; it appears to be degraded in the former. If so, it is possible that vectors containing ICP 0 could have two potential benefits. They could be used for persistent infection of neurons without toxicity, yet they would retain cytotoxic properties which would make them of value in the treatment of central nervous system tumors of glial cells.

There is extremely limited transcription of the HSV genome during a latent infection. Overlapping transcripts are produced from two promoters, LAP1 and LAP2. One approach to vector construction is to create hybrid promoter enhancers which would incorporate a latency promoter so that expression would occur in the latent state. This has proven possible to do. Transgenes inserted into the latency transcript have been found to be expressed in dorsal root ganglia and motor neurons, as well as in the brainstem motor nuclei of mice.

In a primary HSV infection, virus enters at an epithelial surface and undergoes a round of replication before entering the axon and being transported to the body of the neuron in a ganglion. A consideration was whether replication-defective vectors could be expected to get to the axon and be transported in a retrograde fashion. The potential requirement for an initial round of replication at the site of entry can be overcome by introducing a sufficient amount of the vector. Another way of enhancing vector uptake by axons is to inject the vector intramuscularly in which case it is in proximity to the synapse between the axon and muscle. Of course, it is possible to inject virus directly into the brain in cases where the severity of the disease (i.e. glial cell tumors) would warrant a neurosurgically mediated method of introduction of the vector.

ADENO-ASSOCIATED VIRUS VECTORS

The human parvovirus adeno-associated virus (AAV) has been appreciated to have considerable promise as a vector for human gene therapy (Samulski 2003). AAV is a small, nonencapsidated, icosahedral virus with a linear single-stranded DNA genome (Linden and Berns 2000). The virion has a diameter of ~26 nm. The species type virus AAV2 genome contains 4.68 kb. Although the small size of the genome presumably constrains the size of any transgene, the biological properties of the virus have prompted its development as a gene therapy vector. Additionally, recent experiments have suggested that the constraints on transgene size may be overcome (see below). AAV is widespread in nature throughout vertebrate species. There are at least six human serotypes. The most common are the closely related AAV2/3; about 90 percent of adult humans are seropositive (Blacklow et al. 1971). In spite of this evidence for very common infection by the virus, there has been no evidence of association of the infection with any human disease. Indeed there have been several studies which suggest that infection by AAV may protect women against developing cervical carcinoma (Georg-Fries et al. 1984; Mayor et al. 1976; Sprecher-Goldberger et al. 1971).

AAV life cycle

AAV does not undergo productive infection in healthy cells in culture unless there is concomitant infection with

a helper virus, such as an adenovirus or a herpesvirus (Berns and Linden 1995). Several types of the latter have been shown to help AAV multiplication. It is possible to render cells permissive for low levels of AAV replication by treatment of the cells with various genotoxic agents (e.g. UV or gamma irradiation, or various chemical carcinogens) (Yakobson et al. 1987). If AAV infects a healthy cell in the absence of a helper virus coinfection, the viral DNA is uncoated, but undergoes minimal, barely detectable conversion into the double-stranded form and is then integrated into a specific site on the q arm of chromosome 19. As long as the cell remains healthy the viral genome remains relatively quiescent in the integrated form; rescue and replication seems to be inhibited by a very low level of expression of a viral regulatory protein. If the cell is stressed by superinfection with an adenovirus or a herpesvirus or by exposure to certain genotoxic agents, the integrated genome is 'activated,' rescued, replicated, and infectious virus made. As described here, AAV is close to a perfect parasite in that it uses the host to perpetuate the viral genome while doing no harm to the host. If, for unrelated reasons, the host is stressed, the virus activates its genome to form infectious particles to find a new host to infect. The facts that AAV causes no apparent disease, yet can persist for extended periods of time in an integrated state, were significant in alerting investigators to the potential use of AAV as a vector for human gene therapy (Hermonat and Muzyczka 1984).

AAV genetics

The linear, single stranded DNA genome contains two open reading frames bounded by inverted terminal repeats (ITR) of 145 nucleotides (nt). The outer 125 nt constitute an overall palindrome which contains two smaller palindromic sequences, one on either side of the midpoint of the overall palindrome (Lusby et al. 1980). Thus, when the terminal 125 nt sequence is folded on itself to maximize potential base pairing, a T- or Y-shaped structure is formed. The open reading frame in the left half of the genome encodes four regulatory proteins (Rep 78, 68, 52, 40) which have overlapping sequences and which regulate all phases of the AAV life cycle. The four proteins are the consequence of two promoters (P5, P19) and an intron (Lusby and Berns 1982; Mendelson et al. 1986). Both spliced and unspliced forms of the two mRNAs are translated, hence four Rep proteins.

The open reading frame in the right half of the genome encodes three structural proteins (P85, P75, P60), again with overlapping sequences (Srivastava et al. 1983). The three coat proteins are the consequence of alternative splicing and the use of an unusual ACG start codon for translation of the middle protein (Muralidhar et al. 1994).

Site specific integration

AAV integration into the human genome is unique in that it is the only known example of a human virus integrating at a specific site (Kotin et al. 1990, 1992; Samulski et al. 1991). The site (at 19q 13.4) is just upstream from the gene for one of the myosin binding proteins (MBS85) and downstream from the troponin I gene (Dutheil et al. 2000, 2004). The integration site sequence has been dubbed *AAVS1* and is a potential promoter. It is likely that the sequence has a relatively open chromatin structure that would render it susceptible to AAV integration. Within the AAV ITR there is a Rep binding site (RBS) and a terminal resolution sequence (TRS) that is nicked by Rep in a sequence-specific manner. A similar sequence arrangement is present in *AAVS1*. Genetic analyses have shown that both the RBS and the TRS are required for site-specific integration (Linden et al. 1996a,b). Interestingly, the length of the intervening sequence is not absolutely fixed, but the sequence significantly influences the frequency of integration (Meneses et al. 2000). About half of the junctions between viral and cellular DNA occur within the viral ITR; the other half occur just upstream of the viral promoter for Rep at map position 5 (p5 promoter) on the genome (Samulski et al. 1991; Giraud et al. 1995). This is of potential significance because studies of plasmid based integration suggest that the sequence from the end of the ITR to the p5 promoter greatly facilitates the frequency of site specific integration. In model systems where *AAVS1* is on a plasmid, the specific integration site clusters around the *AAVS1* RBS. Integration into the genome does not appear to be as tightly clustered. At the cellular level, integration into the q arm of chromosome 19 has been estimated to occur with a relative frequency of 70–100 percent. More recent studies have trended toward the higher figure. At the cellular level the AAV genome is integrated as a tandem repeat which undoubtedly facilitates rescue. Both head-to-head and head-to-tail repeats have been reported (Cheung et al. 1980; McLaughlin et al. 1988). Whether the tandem arrangement is the consequence of limited viral replication or recombination has not been determined.

AAV vectors

Despite the fact that one of the attractive features of the AAV life cycle, which suggested its use as a vector for human gene therapy, was its ability to persist in a latent form by site-specific integration, all of the AAV vectors which have been tested to date lack the Rep gene. The typical AAV vector consists of the transgene(s) with appropriate regulatory sequences flanked at the ends by the AAV ITR. Omission of Rep has been a consequence of two considerations: 1) the Rep gene occupies

half of a small genome, so omission doubles the transgene capacity of the vector; 2) Rep is a potent regulatory protein which might be toxic under certain conditions. Since almost all vectors also lack the p5 promoter sequence which has been found to enhance the frequency of site specific integration (Philpott et al. 2002), the current AAV vectors do not have a propensity for site specific integration and most often persist as extrachromosomal elements. The extrachromosomal elements are quite stable and can persist for a year in the mouse model. It seems likely that structures, which can be assumed by the AAV ITR protect the vectors from exonucleolytic degradation. One year after introduction of AAV vectors into skeletal muscle in the mouse a significant fraction of the vector DNA was found to be integrated at apparently random sites in the SCID mouse genome (Song et al. 2001).

AAV virion vector production requires three elements: 1) the vector construct, a transgene flanked by AAV ITRs within a plasmid vector; 2) the AAV genes for Rep and the structural proteins, either in a plasmid or integrated into the genome of a cell line; 3) required adenovirus helper functions, again either on a plasmid or integrated into the genome of the cell line. The appropriate combination of plasmids are transfected into cells in culture to produce vector virions. The major concern is vector production without contamination with either wild type AAV or adenovirus. Using only the adenovirus helper genes, which constitute a small fraction of the adenovirus genome, means that contamination with adenovirus is not a significant concern. Contamination with wild type AAV, on the other hand, is a major concern. Recombination between the vector construct and the AAV helper construct can occur readily if any homology remains between the two DNAs. Even in the absence of overlap homology illegitimate recombination can occur; hence, it is difficult to produce AAV vectors in which no contamination with wild type virus is detectable. In the past it was difficult to generate high titer preparations of AAV vectors. However, development of new cell lines, better helper plasmids, the use of cell factories, and column-based methods of purification have greatly improved the ability to generate sufficient amounts of clinical grade virus so that human gene therapy trials are feasible.

Several AAV serotypes have been tested with respect to their relative abilities to transduce various tissues. Hybrid vectors have been used in which an AAV2 vector genome is packaged into capsids of various AAV serotypes. Striking differences have been observed with respect to the frequency of transduction observed with the different hybrids. Rabinowitz et al. (2002) observed that in vivo Type 1 was the most efficient in transducing liver and muscle, followed by types 5, 3, 2, and 4, in that order. However, when transduction of the rat retina was attempted, types 4 and 5 were the most efficient, followed by type 1. Although others have found some relative differences in the ability of specific serotypes to transduce various tissues, the general principle is that there are significant differences and that these differences should be taken into account when it is desirable to transduce a specific tissue. All of the available data are consistent with the notion that the differences in efficiency of transduction reflect the fact that different AAV serotypes use different cell receptors. No evidence has emerged to suggest that the differences reflect regulatory variability within the transfected cells. Additional efforts to modify tissue specificity have involved chemical linkage of cell-specific ligands to vector virions or genetic modification of the coat proteins. Limited success has been achieved so far and, with the ever-increasing number of AAV serotypes which are becoming available, the need for specificity may be able to be met naturally. One likely exception to this conclusion would involve vectors designed to kill tumor cells. In this case, a likely vector would be linked to a monoclonal antibody to a specific tumor surface antigen.

Animal models

There have been numerous reports of successful transductions by AAV vectors in animal models. Most often these have been rodent models, especially genetically defined mice, but other species have been tested, including dogs, rabbits, and nonhuman primates. Among the many genetic lesions reported to have been corrected in mice or rats are a model for asthma, hemophilia B, retinopathy of prematurity, erectile dysfunction, phenylketonuria, diabetes, obesity, a model for acute macular degeneration, and Fabry's disease (Samulski 2003). The hemophilia B model is of particular interest because part of the transgene was in one AAV vector and the other was in a second vector construct. Apparently the two polypeptides were able at either the RNA or protein level to come together to form a functional protein (Duan et al. 2001). In the canine model, AAV has been used to correct Factor IX hemophilia and Leber's disease. The latter is of particular interest. Leber's disease, which also occurs in people, involves a defect in a protein, RPE65, affecting the retinal pigmented epithelium, so that puppies are born blind (as are the human patients). However, the disease is potentially reversible if treatment is instituted early enough. Acland et al. (2001) were able to treat blind puppies with an AAV vector containing a wild type RPE gene by injection into the eye. The treated eye gained visual function, but the contralateral eye did not.

A genetic disease which is relatively common, cystic fibrosis (CF), has been modeled in mice and safety tested in both rabbits and nonhuman primates. The AAV vectors have persisted for a long time and there has been no significant toxicity noted.

There have been several general conclusions noted from these animal studies. The first is regarding the relative safety of the vectors. There has been one report of tumor formation subsequent to AAV-mediated liver transduction (Daly et al. 2001), but analysis of the tumors did not reveal the presence of vector sequences and such tumor formation has not been observed in other experiments (Nakai et al. 2003). The affected animals had a specific genotype. The immune response elicited by the vectors has been limited. There has been at least one report of an immune response to the transgene product, but again this is a rare occurrence. Different strains of mice show variable immune responses to the vector coat proteins, thus the ability to re-administer the original vector is also variable. Recent reports of novel serotypes isolated from nonhuman primates (Gao et al. 2004) opens the possibility of a large increase in the number of vector serotypes, so that immune resistance to readministration may be largely mitigated. Various routes of administration have been used, in part depending on the target tissue. All of the routes, including direct injection, iv injection, and inhalation have been successful in specific cases. Of particular note are the high levels of transgene expression achieved in many instances, of ten at therapeutic levels, and the length of time which expression persists. In many cases the duration of expression has approached the normal life span of the mouse.

Clinical trials

Clinical trials using an AAV vector have been carried out with a limited number of diseases. The first studies concerned patients with CF with moderate symptoms. The first trial involved bronchoscopic administration to 19 patients (Flotte et al. 2003). The major finding was the lack of any detectable toxicity over a wide range of dosages. No significant enhancement in clinical status was noted. A second Phase I trial involving 12 patients was conducted by another group who administered the vector by an aerosolized spray. The experiment was monitored by bronchoscopy to gather experimental samples. Vector transfer was dose related: after nebulization involving 1 013 infectious particles, 0.6 copies per brushed cell were noted after 14 days and 0.1 vector copies were found after 30 days. No vector could be detected by 90 days. Vector was noted to the fourth airway generation after the nebulization administration. Although the conclusion from this experiment was that the protocol had been safe, there were six 'serious' adverse events, three of which might have been related to the vector. In a Phase II trial involving CF patients, vector was administered by spray to the maxillary sinus on one side, the other side serving as the control. No statistically significant differences were noted when relapse of clinically defined sinusitis was measured, or when sinus transepithelial potential difference, histopathology, or sinus fluid IL-8 measurements were made. Only the level of IL-10 was significantly increased at day 90. However, again the safety of the vector was noted. Trials have been carried out on three patients with Factor IX hemophilia. Since adequate serum levels of Factor IX are the goal, the site of protein synthesis is not critical. In this trial intramuscular injection was used. No evidence for toxicity was noted. Vector sequences were detectable in muscle and there was immunohistochemical evidence for expression of Factor IX, along with evidence for a moderate increase in serum levels (Margaritis et al. 2004). A clinical trial has been announced to test the use of AAV to deliver the aspartoacylase gene to patients with Canavan Disease, a neurodegenerative disease characterized by failure to cleave N-acetyl-aspartate leading to inhibition of normal myelination in the central nervous system. Finally, a clinical trial to see whether an AAV vector can lead to therapeutic levels of alpha-1-antitrypsin in serum is close to initiation. It seems likely that an increasing number of trials involving AAV will take place in the near future.

AAV has demonstrated significant promise as a vector for human gene therapy. To date, in both animal and human studies there has been minimal toxicity observed. The major question has been the extent to which humoral antibody to the virion coat might inhibit subsequent administration of the original vector. Occasional reports of possible AAV vector toxicity in animal models either have not been reproducible or have not been demonstrated to have been caused by the vector. There have been no reports of significant toxicity in several human trials. On the positive side, high levels of transgene expression have been noted in a large number of animal trials. This has not yet been achieved in any of the human trials, although lower levels have been observed. In the animal models, expression occurs rapidly and is sustained at potentially therapeutic levels for more than a year in several instances (essentially the life span of the mice used). This is in distinction to the results observed for either retrovirus or adenovirus vectors. The reason for this difference is not known, but may be a reflection of the fact that there is no expression of AAV genes.

Results to date are highly encouraging for the potential of AAV to be used as a vector for human gene therapy. However, before its utility in this context can be considered established, there are a number of questions that need to be answered. Long-term safety will have to be ascertained. The propensity for the vector to integrate in an apparently random manner after long periods of time raises the question of insertional mutagenesis and its potential consequences. Will the results using a Moloney murine leukemia virus-based vector to treat children with severe combined immunodeficiency (i.e. a leukemoid reaction similar to leukemia which was observed more than a year after therapy) occur? Would

it be better to develop AAV vector systems which involve Rep expression like that seen with wild type infection so that site-specific integration would occur? Would such systems have toxicity associated either directly with Rep expression or as a consequence of site-specific integration? If the experience with natural infection is a reasonable model, neither of these concerns would appear to have a high probability, but until there is direct testing of vectors, one cannot be sure. A related question is whether integration into *AAVS1* leads to expression at levels comparable to those observed with the vector as an extrachromosomal element. Another issue to be resolved is whether there is a preferred route of administration or does the optimal route depend upon the specific use of the vector. While the latter would seem to be the more likely answer, the extent to which there may be a preferred general route of administration remains to be determined. Animal models have shown that different AAV serotypes have differing levels of effectiveness depending on the cells to be infected. While this stands to reason, the details of tissue and cell specificity remain to be worked out. Also to be refined is the extent to which vector coat proteins can be selectively modified to both enhance and narrow the specificity of tissue or cellular susceptibility to infection by the vector.

Thus, while the effective application of AAV to human gene therapy has not yet been achieved, AAV remains one of the most promising of the viral vectors.

CONCLUSION

Considerable progress has been achieved in the development of viral vectors. After an early rush to clinical trials with an attendant lack of success, it has become clear that the key to success in human gene therapy is a detailed understanding of the basic biology of the viruses relevant to their use as vectors. Areas of continuing concern include tissue and cell specificity, persistent expression, host response, the possibility of insertional mutagenesis, and maintenance of the vector in dividing cells. Because use of gene transfer for therapeutic purposes is not a major philosophical concern, but altering the germ line is, vectors are monitored for their presence in germ cells. Although most viral vectors cannot replicate and often are delivered locally, systemic spread might be considered to be infrequent; but this is not the case. Much of the time at least some of the vector does reach the circulation and is distributed throughout the body. One way to avoid the possibility of germ line contamination would be to develop viral vectors whose tissue tropism was sufficiently narrow that infection of germ cells would not occur. Another concern stated above is insertional mutagenesis. Potentially, it would be possible to minimize this problem by designing vectors which would integrate at specific sites in the human genome which would not be deleterious. Wild type AAV is the only human virus which is known to do this. However site-specific integration in this case requires the *rep* gene product and cis-active sites on the vector DNA. Work is proceeding to develop such vectors. Another approach would be to develop a system that can lead to efficient homologous recombination between the resident gene and the transgene to correct the inherent defect. Work is progressing in this direction as well. Each of the viruses discussed in this chapter has inherent drawbacks. Three of the four are significant human pathogens. The challenge is to modify such viruses so that they are no longer virulent but retain the capabilities required of a vector. Although not known to be a pathogen, AAV has a limited transgene capacity and the Rep protein required for site specific integration is a powerful transactivator. Hence it must be demonstrated that AAV-based vectors designed to integrate site-specifically are both safe and effective. A similar issue bedevils retrovirus vectors; we have already seen one instance where the use of what was thought to be a 'disarmed' retroviral vector led to the development of a leukemia-like disease of the type naturally caused in mice by the virus used.

In summary, use of viral vectors for human gene therapy retains its considerable promise. We do have one instance in which a lethal disease has been 'cured,' albeit with some therapeutic complications. However, it is clear that a greater understanding of the biology involved with vector administration and more sophisticated engineering of vectors will be required.

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Antiviral chemotherapy

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INTRODUCTION

The science of antiviral chemotherapy has developed over the last 40 years; in the last two decades the rate of progress has accelerated markedly and more new chemical therapeutics to treat viral diseases have been introduced since 1990 than during the whole of the preceding 30 years. Viruses are obligate intracellular parasites that are completely dependent on an intact host cell to provide the ribosomal machinery for protein synthesis. As such, many scientists claimed that 'selective toxicity' towards viruses was unattainable and that the cost of interfering with virus replication would necessarily be unacceptable damage to the host. Historically, lack of susceptibility to antibiotics was considered to be one of the hallmarks of a virus. This gloomy prospect was transformed by a series of discoveries that has now led to effective and safe systemic chemotherapy for many different virus infections. The mechanisms of action and mode of application of some of the best known are covered in this chapter.

Achieving selective toxicity against human viruses

Although the potential toxicity of antiviral compounds remains important, over the last three decades the prescription of safe, systemic antiviral chemotherapy has gradually come to be taken for granted. This revolution was largely brought about by the development of the nucleoside analogue, acyclovir (also known as aciclovir), which became one of the world's best known medicines with more than 33 million patients treated by the end of the millennium (Darby 1995). Such is the confidence in its safety that it has been administered prophylactically for over 20 years to individuals, apparently without ill-effects, to suppress recurrences of genital herpes (Whitley and Gnann 1992; Tilson et al. 1993). The confidence generated by this one compound provided a completely new background to our current thinking about the development of antiviral chemotherapy and the encouraging prospects for future therapeutic strategies. The emergence of human immunodeficiency virus (HIV) during the 1980s provided a monumental stimulus to the antiviral field; driving research which led to the many new antiretroviral drugs currently available or in clinical development. Herpesviruses, which were historically the most important targets for chemotherapy are currently attracting less interest (Field and Fitzmaurice 2001), while alternative virus targets such as influenza and hepatitis viruses have come to the fore.

The importance of specific diagnosis and early therapy

Useful inhibitors are generally specific for one family of virus and, in some cases, to individual members of that family (e.g. particular members of the *Herpesviridae*). Initiating the appropriate therapy therefore depends on rapid and accurate diagnosis. In fact, often, significant tissue damage occurs before the etiology of virus infection is determined. For many acute respiratory infections

that have both short incubation periods and similar clinical signs early in the infection (e.g. caused by rhinoviruses, myxoviruses, or paramyxoviruses) there is a narrow window of opportunity for intervention by chemotherapy following specific diagnosis and this is an important barrier to the development of new compounds. However, experience with aciclovir demonstrated that therapy can be beneficial, even when the infection has progressed for several days, although, as defined below, early therapy is of paramount importance in determining the outcome when treating herpes zoster, herpes simplex encephalitis, and the complications of cytomegalovirus infection. With several virus infections, however, the slow development of clinical signs over many weeks or months allows ample opportunity, following specific diagnosis, to provide benefit to patients with such diseases as HIV, hepatitis B virus (HBV), hepatitis C virus (HCV) and Papillomavirus (HPV). Furthermore, rapid 'bedside tests' to confirm diagnosis of many different virus infections are becoming available and will be increasingly reliable.

The importance of an intact immune system

Another widely held view at the outset of antiviral chemotherapy was that an intact immune system would be a prerequisite for clearing infection. Experience with several excellent inhibitors of viral replication has shown that this is untrue. Indeed, some of the most spectacular successes with antiviral chemotherapy and prophylaxis have been obtained using aciclovir in severely immunosuppressed patients suffering from herpesvirus infections (Meyers et al. 1982) and subsequently highly active antiretroviral therapy (HAART) administered to HIV-infected patients who have severely compromised immunity (Jain et al. 2003). A particular problem in such patients, however, is their tendency to develop infections resistant to antiviral drugs and the approaches to countering this problem are discussed below.

DISCOVERY AND ASSESSMENT OF NEW ANTIVIRAL COMPOUNDS

Molecular targets for inhibition

The obligate intracellular parasitic mode of viral replication determines that it must pass through a series of stages common to all viruses: adsorption; entry; uncoating of the nucleic acid; expression of the genome; transcription and translation of proteins; replication of nucleic acids; assembly and release of mature progeny (Figure 1).

So far, almost all the useful inhibitors of viral replication are targeted to one or other of these steps. The mechanism of action of many compounds in current use involves interference with nucleic acid metabolism; they include the many nucleoside analogues that inhibit herpesviruses and nucleoside and nonnucleoside analogues that interact with HIV reverse transcriptase (reviewed by De Clercq 1992). Several of the early influenza virus inhibitors affect the disassembly of particles by blocking a virus protein complex that acts as an ion channel (Wang et al. 1993). Most viruses induce one or more proteases involved in processing virus polypeptides into functional components. The HIV protease was the first such enzyme to be targeted, and novel inhibitors of the proteases of other viruses are now being developed (Mills 1993). Finally, a series of inhibitors of picornaviruses act by binding directly to the virion capsid, thus blocking the interaction between the virion and the receptor on the cell surface that facilitates its entry and disassembly (Rossmann 1990).

Now that many compounds have been discovered that possess potent selective toxicity against particular viruses, it is ironic that there is renewed interest in compounds that appear to depend upon a cellular function that has a role in virus replication, e.g. the cyclin-dependent kinases as targets for the inhibition of herpes simplex virus (HSV) (Schang 2001). Furthermore, there is considerable interest in compounds that modify the host response to virus infection such as the induction of cytokines or the accelerated apoptosis of virus-infected cells. Examples of such immune response modifiers are imiquimod, which is reported as a treatment for HSV (Harrison et al. 1988) and HPV (Beutner et al. 1998), and resiquimod, which is also used to treat HSV (Tomai et al. 1995).

Drug discovery

SERENDIPITOUS DISCOVERY

In the early days, most new antiviral compounds were discovered by chance (often referred to as serendipity); indeed, several of the earliest antiviral compounds (e.g. idoxuridine) originated from cancer research where there was an interest in using nucleoside analogues to interfere with DNA synthesis and hence to inhibit the rapid division of tumor cells (Darby 1994).

RATIONAL DRUG DESIGN

With thorough understanding of the molecular basis of the replication of many important viruses, the availability of the entire nucleotide sequences of their genomes and the three-dimensional protein structure derived from X-ray diffraction analysis, compounds can be designed to interact with specific targets involved in the virus replication cycle. Among the first molecular targets were virus-induced enzymes which have properties differing from those of counterpart enzymes induced by the host cell. The best known examples are neuraminidase (NA) induced by orthomyxoviruses;

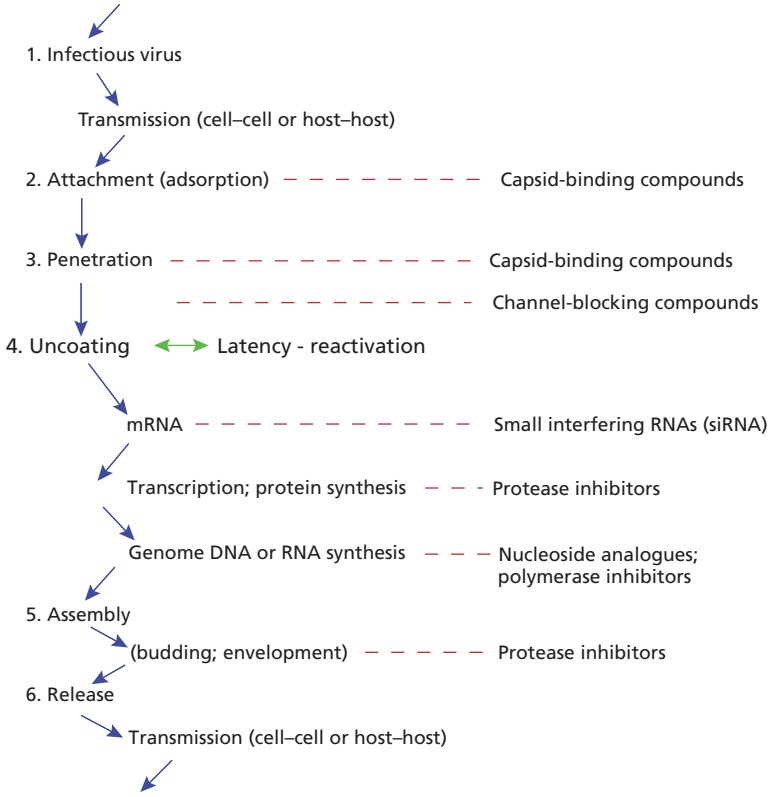

Figure 1 *Sites of action for several important classes of virus inhibitors. The classical progression of the virus replication cycle is denoted by the blue arrows. The sites at which the current successful antiviral compounds act are indicated by red dashed lines.*

thymidine kinase (TK) and DNA polymerase (DNApol) induced by some herpesviruses; transcriptase and reverse transcriptases induced by hepadnaviruses and retroviruses; and proteases induced by retroviruses and herpesviruses. Knowledge of such enzymes enables the establishment of biochemical screens to test hundreds of thousands of potential inhibitors and to develop structure–activity relationships. When the enzyme concerned can be crystallized and its three-dimensional structure solved, the synthesis of appropriate molecules to interact with particular sites on that enzyme can be attempted (Laver and Air 1990). For example, the crystal structure of two members of the herpesvirus proteinase family (human cytomegalovirus (CMV) and varicella-zoster virus (VZV)) revealed that they belong to a new class of proteinase with a completely novel protein fold and catalytic site. In some cases, potent inhibitors have been discovered that bind in a region away from the enzyme active site. Such is the case with several useful inhibitors of HIV, the so-called nonnucleoside reverse transcriptase inhibitors (NNRTI) and these will be elaborated on in the HIV section below. This molecular approach to the rational design of new inhibitors is being pursued in relation to HIV reverse transcriptase and protease; influenza neuraminidase and some potential nonenzymatic virus targets, e.g. the picornavirus capsid-binding molecules (Jones 1998).

Although the discovery of future antiviral compounds will rely heavily on these methods, the development of successful drugs still depends on finding molecules with the appropriate pharmacokinetic properties and lack of toxicity, and which can be synthesized inexpensively in large quantities from available precursors; thus, even with the best efforts of the medicinal chemist, relatively few new antiviral agents are likely to become successful antiviral drugs.

Methods for detecting the inhibition of molecular targets directly

STRATEGIES FOR ANTIVIRAL DRUG DISCOVERY

The methods for discovering new antiviral drugs have evolved over the past half century to a high degree of sophistication. The general principles, however, are unchanged. The first or most promising member of a family of related chemical structures that shows antiviral activity is referred to as a ‘lead’ compound. The overall process of developing an antiviral compound for clinical evaluation can be broken down into the stages of lead generation, lead optimization, and lead development. The virus genome sequence and the study at a molecular level of virus proteins and their functions enables potential molecular targets for attack to be identified.

Methods have been developed in the pharmaceutical industry for screening literally hundreds of thousands of compounds against particular targets. Much use is now made of X-ray crystallographic data obtained from target-inhibitor complexes in drug design and the optimization of lead compounds, in particular the ever-expanding databases of chemical structures such as the Cambridge Structural Database (CSD) for small organic and metallo-organic compounds and the Protein Database (PDB) for polypeptides and polysaccharides. To date, virus-induced enzymes have been particularly useful targets for attack by this means.

VIRUS ENZYME SCREENS

Viruses induce enzyme activities in the infected cells and there are now many examples of the assay of such enzymes in vitro and the screening of potential inhibitors for activity (Öberg 1983b). DNA and RNA polymerases were originally the best-studied systems, particularly in herpes and retroviruses, respectively. The herpesvirus TK was one of the earliest viral enzymes to be studied, because, as well as being a target for inhibition itself (Wright 1994), it is responsible for the phosphorylation of many nucleoside analogues that then become virus DNApol inhibitors. HIV research has largely been responsible for revolutionizing the study of enzyme-inhibitors at the molecular level and the classes of enzyme under investigation has extended beyond the conventional nucleic acid polymerases. In recent years the list of virus-induced enzymes to become targets for effective inhibitors includes retrovirus protease, herpesvirus protease, and helicase primase and orthomyxovirus neuraminidase, and there is no doubt that these will soon be joined by many others.

EFFECTIVE CONCENTRATION (EC_{50}) AND TOXIC CONCENTRATION

Antiviral compounds are usually assessed at an early stage for their ability to inhibit viral growth in tissue culture. The virus is generally titrated by means of cytopathic effect, plaque production or some other measure (e.g. infectious center assay). The percentage reduction is usually plotted against the $\log_{10}$ of the drug concentration. The concentration of compound that reduces the titer of virus by 50 percent is measured and is usually expressed as the effective dose 50 concentration (ED_{50}), inhibitory concentration (IC_{50}) or something similar (Figure 2). Although the ED_{50} provides the most reproducible value, in some cases the ability to reduce virus yield by 90 percent (ED_{90}) is regarded as a more realistic measurement and is sometimes quoted. When expressing results it is important to state the type of cell culture used for the test and the multiplicity of infection because the species of origin, the tissue from which the cells are derived, and whether the cells are resting or dividing can all influence the result. The multiplicity of infection may also be important: plaque reduction tests

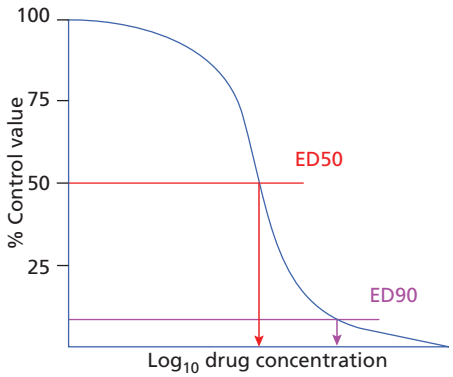

Figure 2 *Determination in cell culture of effective dose (ED_{50}; ED_{90}) for an inhibitor*

are necessarily conducted at low multiplicity but those based on reduction of cytopathic effect or virus yield may be carried out at different multiplicities and this can profoundly affect the result (Harmenberg et al. 1980; Harmenberg et al. 1985b).

A number of important human viruses that are targets for chemotherapy are extremely difficult to grow in convenient tissue culture systems, presenting a major hurdle in the identification and early development of inhibitors. Examples are HPV (Stanley 1993; Stanley et al. 1997; Phelps et al. 1998) and HBV and HCV (Murphy 1993; Main et al. 1998; Walker et al. 2003; Yuen and Lai 2003).

The quantification of compound toxicity is very much more difficult to assess in tissue culture and many methods have been employed (Dayan and Anderson 1988; Newton 1988). They include effects on cell division; cell generation time over several rounds of replication (Field and Reading 1987); cell replication measured by thymidine uptake; cell volume; and various other more subjective measurements of cytopathic effect. Several assays employ vital dyes to assess cell viability, and in these cases colorimetric apparatus can be used to automate the readings (McLaren et al. 1983). Further tests have been devised to assess genetic damage (Evans 1983) and effects on DNA repair systems (Collins et al. 1984) The problems of assessing potential long-term genetic damage by nucleosides is reviewed well by Wutzler and Thust (2001).

An important consideration for both toxicity and antiviral activity in cell culture is that cells from species other than humans are often employed and may give different results (Harmenberg et al. 1985a). Furthermore, cells adapted for tissue culture may provide misleading data in comparison with those actually involved in the natural pathogenesis (Perno and Calio 1994). Experimental work with differentiated cells (e.g. human macrophages or neuroblastomas) or organ fragments (e.g. tracheal rings or ganglion explants) have been used in attempts to circumvent these difficulties. Two important examples of the latter exist. For HPV,

raft cultures have been developed Similarly, the trimeric mouse has been utilized to study compounds directed against HCV. Although these methods can provide additional information the results may be difficult to interpret (Bartenschlager 2002a, b; Bartenschlager and Lohmann 2001)

Structure–activity relationships

Having established that a particular chemical inhibitor (lead compound) has a useful selective index against a particular virus or family of viruses, many closely related chemical analogues are synthesized, if possible. These are then screened for antiviral and toxic activity. It often emerges that particular features of the molecule are important and further modifications may be attempted in order to improve or refine the properties of the leading drug for further development. The analysis of such series led to the concept of structure–activity relationship (SAR) and this has been most important in the development of many new drugs. Examples of such series are the 5-substituted 2′-deoxyuridine inhibitors of herpesviruses (De Clercq et al. 1982) and the non-nucleoside reverse transcriptase inhibitors and peptidomimetic protease inhibitors of HIV (Roberts et al. 1990).

ANIMAL MODELS

Animal infection models were essential to the programme of development of most major antiviral compounds in clinical use. This is less true of compounds active against HIV because there is a paucity of suitable models (Koch and Ruprecht 1992).

Infection models for HSV dating back to the 1920s (Goodpasture and Teague 1923) have been developed in mice, rabbits, and guinea-pigs. There is thus a very large amount of published information on the pathogenesis of experimental infections, which aids the interpretation of the different models and the extent to which the results can be extrapolated to human disease (reviewed by Field and Brown 1989). Table 1 indicates some of the most important models for studying HSV as a cause of labial, genital, and ocular lesions, and of encephalitis and disease in the immunocompromised host. Such models have been used to provide further evidence of efficacy, to compare different compounds and to study therapeutic regimens. However, their particular strength is the ability to investigate complex features of pathogenesis and their interaction with potential inhibitors. For example, the effects of antiviral compounds on the establishment, maintenance, and reactivation of latency are extremely difficult to study in humans (reviewed by Darby and Field 1984).

Many viruses, including other herpesviruses (e.g. human cytomegalovirus (HCMV) and VZV), have a restricted host range and cannot establish infections in laboratory animals comparable with those in humans. It is, however, sometimes possible to do so using a different member of the same family of viruses, for example murine CMV (Sandford et al. 1985) and feline immunodeficiency virus (North et al. 1993). Clearly, the range of questions that may be asked of such models may be limited, but they can provide powerful systems for studying both the dynamics of drug resistance developing during therapy and counter-measures (e.g. the use of combinations or alternations of drugs).

Table 1 *Examples of the many animal models used to study and evaluate inhibitors of herpes simplex virus*

Type of disease	Animal species	Route of inoculation
Orofacial herpes	Guinea-pig	Flank
	Mouse	Ear pinna
		Flank
		Lip
		Foot pad
	Hairless mouse	Flank
	Nude mouse/human skin	Skin
Genital herpes	Guinea-pig (female)	Vagina
Ocular herpes	Rabbit	Cornea
	Mouse	Cornea
Herpes encephalitis	Mouse	Nares
		Vagina
		Cerebrum
		Tail vein
		Peripheral nerve (sciatic)
		Peritoneal cavity
	Rabbit	Olfactory bulb
Disease in immunocompromised host	Mouse (X-irradiated, cyclosporin-treated; nude transgenic knockout, etc.)	Various routes

Animals also play a vital role in the study of toxicity, and a number of statutory tests, both short and long term, are performed in several species in attempts to evaluate the risks of side effects before new compounds enter clinical trials. An important component of evaluation of any drug in animal species, generally two, is the assessment of adsorption, distribution, metabolism and elimination.

Selective index and therapeutic index

The ratio of the 50 percent toxic concentration to the 50 percent virus inhibitory concentration for the compound is often termed the *selective index*. When this is close to unity, the compound is generally taken to be toxic and is very unlikely to become useful. Conversely, a high selective toxicity index suggests a potentially useful compound. It should be stressed, however, that this ratio can be extremely misleading. For example, trifluorothymidine (Kaufman and Heidelberger 1964) has a selective index close to unity yet has been licensed as a topical agent against ocular HSV, for which it has enjoyed some success (Kaufman 1979). There are, of course, many compounds with high selective indices in vitro that demonstrate toxicity only when tested in vivo. Thus, when possible, compounds are tested in animals infected with the target virus itself or a suitable relative for comparison. If inhibition of virus growth and of clinical signs are observed with doses that are tolerated by the host, an estimate of *therapeutic index* (the ratio of the toxic dose to the therapeutic dose) can be made (Field 1988a). This value is generally obtained in suitable animal models; it may remain theoretical for humans.

CLINICAL TRIALS

The evaluation of a potential antiviral drug in humans generally proceeds through several phases, numbered I to IV (Rees and Brigden 1988). Although the boundaries are not always exact, this terminology is widely employed.

Phase I studies

The evaluation begins with the administration of the drug to a limited number of healthy volunteers. It is normally given in small single doses at first, increasing by stages to multiple doses in an attempt to mimic the probable clinical use of the compound. During these studies the pharmacokinetics, pharmacology, and metabolism of the compound are closely monitored. If these results are favorable the investigation may proceed to studies on virus-infected patients.

Phase II studies

The compound is administered to patients with disease, and data similar to those in phase I are collected. This is important because the metabolism of the drug in patients with disease may differ from that in healthy subjects. Phase II studies usually employ controls in order to provide 'proof of principle' data. These data are essential in order to estimate the extent of the drug's effect – information essential for the design of Phase III trials. Limited numbers of patients (<100) are utilized. One should be wary of claims of therapeutic benefit derived from such studies.

Phase III studies

Phase III studies are designed for registration whereby the drug is usually compared against placebo or standard licensed therapies. The main aim is to establish the therapeutic spectrum of the drug and the benefits to be gained from therapy compared with the risks associated with its use (the risk/benefit ratio). These studies require 100–1000 patients.

Phase IV studies

These are generally defined as investigations conducted after obtaining marketing approval. Phase IV studies increase experience of treating patients and provide more information about safety and efficacy. Different formulations, dosages, durations of therapy, drug interactions, and comparisons or combinations with other drugs may be evaluated and special problems such as the selection of drug-resistant mutants detected.

There are many reports of antiviral compounds that demonstrate good selective indices against particular viruses in cell culture and, in some cases, high therapeutic indices in animal models. However, in the absence of appropriate pharmacokinetic properties in humans, only a minority have proved clinically useful.

In practice, most compounds described during the preclinical investigation as active in enzyme screens, cell culture, and animal models, fail to pass through all the phases of clinical studies and many are withdrawn during these evaluations. Nevertheless, for severe and life-threatening diseases, the degree of toxicity allowed to certain compounds may be quite high, including zidovudine for therapy of HIV infections and ganciclovir for treating CMV in immunosuppressed patients.

The remainder of this chapter is confined to the relatively small, but rapidly growing, group of compounds that are currently used for chemotherapy of virus infections or are likely to become clinically useful in the near future.

MECHANISM OF ACTION OF IMPORTANT ANTIVIRAL COMPOUNDS

Of the innumerable compounds known to inhibit one or other of the viruses, only very few have proved useful for treating human infections. The early antiviral

compounds that were licensed for use in one or more indications were dominated by those for use against herpesviruses, especially HSV. Subsequently, this emphasis moved to the many new therapies for patients infected with HIV. Currently, the viruses attracting most attention for novel therapies include not only HIV but especially those causing hepatitis. Renewed interest exists for antiviral therapy for influenza. Interest in herpesviruses has, of course, continued in the background and there has been particular progress with the development of treatments for HCMV in immunocompromised patients and newborns.

Inhibitors of herpesviruses

HSV offered one of the most attractive targets for chemotherapy, and thousands of compounds have been discovered that inhibit it selectively. The infections are extremely common and a 1997 survey showed that 62 percent and 16 percent of adolescents in the USA were seropositive for HSV-1 and HSV-2, respectively (Rosenthal et al. 1997). Latent infection is lifelong and is associated with both symptomatic and asymptomatic recurrences with viral shedding on between 15 percent and 25 percent of all days (Wald et al. 1997). With this disease, clinical signs are characteristic, and specific diagnosis (including self-diagnosis) is possible and in many but not all cases can be made when prodromal symptoms occur (Griffiths 1995; Whitley and Roizman 2001). Ocular lesions offer the possibility of topical application. In the normal host, mucocutaneous lesions are almost always self-limiting; thus, it can be difficult to prove a clinical benefit or, indeed, a lack of benefit from a particular therapy.

ACYCLOVIR; ACICLOVIR; A PARADIGM FOR SELECTIVE TOXICITY

Acyclovir (first published under the name acycloguanosine and now as aciclovir (ACV), Zovirax) is a nucleoside analogue structurally related to the natural nucleoside guanosine (Figure 3) (Elion et al. 1977; Schaeffer et al. 1978, reviewed by Elion 1993). To become active, the nucleoside must be converted by three phosphorylation steps to ACV monophosphate (ACV-MP), ACV di- and then ACV triphosphate (ACV-TP) (Figure 4) (Fyfe et al. 1978).

ACV is a poor substrate for cellular enzymes, and uninfected human cells convert very little ACV to its triphosphate (ACV-TP). In contrast, the HSV-induced enzyme TK can, albeit with relatively low efficiency, convert ACV to ACV-MP. Thus the HSV TK recognizes ACV as thymidine but the fact that cellular enzymes convert ACV-MP successively to the di- and triphosphate only within virus-infected cells accounts for the extremely high selective index of the compound (Darby 1995). ACV-TP enters the cellular nucleotide pool where it competes with guanosine triphosphate as a substrate for HSV DNApol. ACV-TP is a particularly potent inhibitor of HSV DNApol (the inhibition constant, K_i, versus the natural substrate, deoxyguanosine, is ca. 0.01 μm) and this appears to provide further selectivity, because cellular DNApols are much less sensitive to

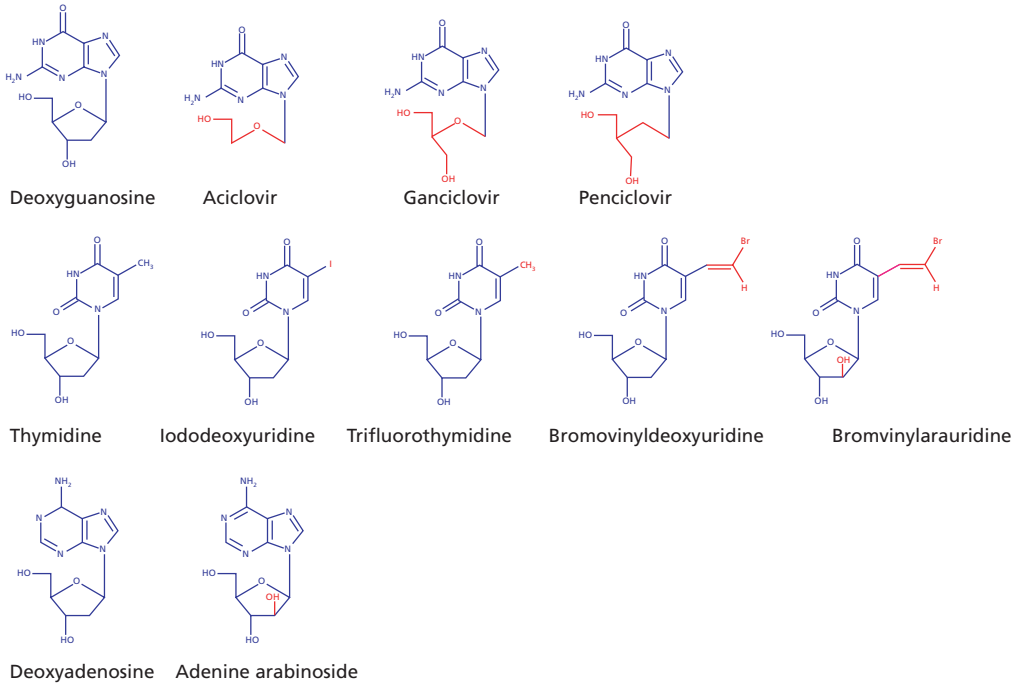

Figure 3 *Nucleoside analogues related to the natural nucleosides, deoxyguanosine, thymidine, and deoxyadenosine. The natural molecules are shown in blue. Red denotes those parts of the synthetic compounds that vary from the natural structures.*

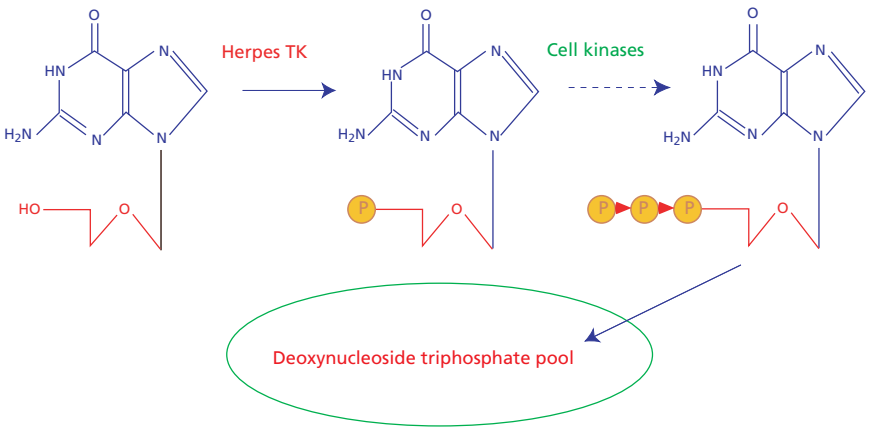

Figure 4 *The mechanism of action of aciclovir: activation*

inhibition. As the ACV residue is linked to the growing chain of virus DNA, it forms a chain terminator since there is no 3′-OH group on the ACV sugar moiety to link to the next residue in the growing chain of virus DNA (Figure 5) (Furman et al. 1979). ACV may thus be described as an obligate chain terminator.

In summary, ACV is taken up and phosphorylated in significant amounts only by HSV-infected cells and inhibits virus DNA synthesis, thus preventing the production and release of infectious progeny virus.

Clinically, ACV is licensed worldwide in topical, oral, and intravenous formulations. Generally, the drug is effective when given systemically for primary and recurrent genital HSV infections (Bryson et al. 1983; Mertz et al. 1984; Goldberg et al. 1993), mucocutaneous HSV in immunocompromised hosts and life-threatening infections, e.g. encephalitis and neonatal disease (Whitley et al. 1986, 1991). It is also an effective therapy for chickenpox (Balfour et al. 1990; Dunkle et al. 1991; Wallace et al. 1992) and herpes zoster in normal (Crooks et al. 1991) and immunocompromised hosts (Griffiths 1995).

NUCLEOSIDE ANALOGUES RELATED TO ACV

Following the discovery and development of ACV, many other nucleoside analogues with similar modes of

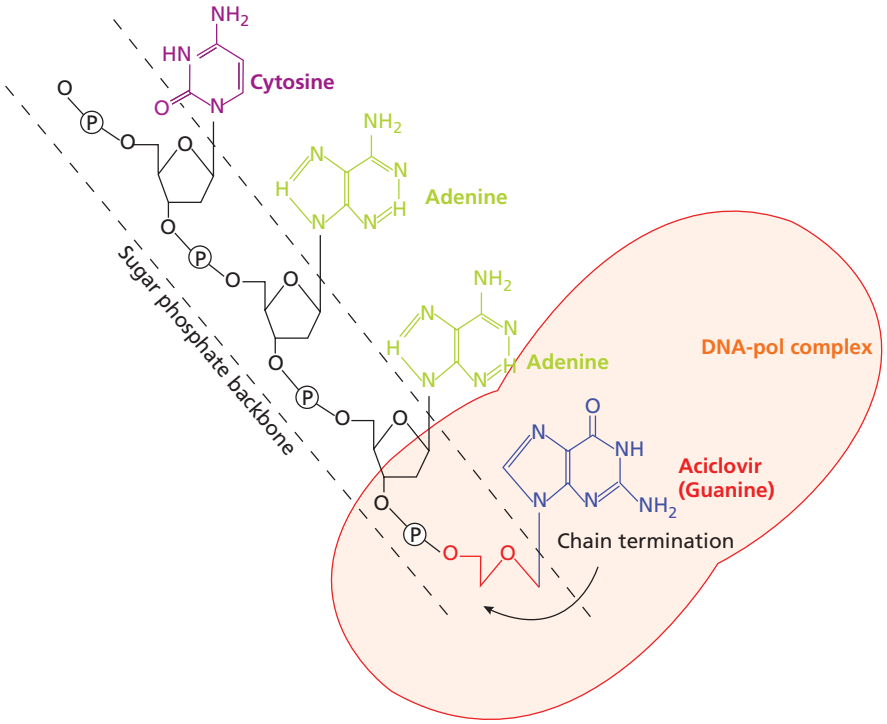

Figure 5 *The mechanism of action of aciclovir: inhibition of DNA synthesis and chain termination*

action were described. Some have high selective indices similar to or higher than that of ACV and several have been developed for clinical use. Each new compound, even those with close structural similarities with ACV (e.g. ganciclovir and penciclovir), has individual properties that differ from those of ACV, although the differences may be very subtle (Darby 1995). It is most important that this is kept in mind when comparing the antiviral efficacy and potential toxicity of such compounds with those of ACV (Wutzler and Thust 2001).

Ganciclovir

Figure 3 shows that ganciclovir (GCV), Cymevene or Cytovene has a structure similar to ACV, with an additional carbon atom in the sugar moiety and the presence of a 3′-OH group (Smee et al. 1983). The compound therefore differs from ACV in that further extension of the DNA chain is possible after incorporation of GCV triphosphate (GCV-TP). Although the compound shows significant toxicity in humans, its spectrum of action is broader than that of ACV and includes HCMV (Freitas et al. 1985; Matthews and Boehme 1988). Unlike HSV, HCMV does not encode a TK. Studies of viruses with acquired resistance to GCV have, however, revealed that a previously unsuspected virus enzyme is responsible for the metabolic conversion from GCV to GCV monophosphate (GCV-MP) (Littler et al. 1992; Sullivan et al. 1992). The enzyme is a product of CMV gene *UL97*, a discovery that focused attention on this gene which codes for a protein with close homology to several eukaryotic protein kinases. The precise function of the virus enzyme is thought to concern export of HCMV nucleocapsids from the nucleus (Krosky et al. 2002). Once formed in the CMV-infected cells, GCV-TP inhibits CMV DNApol and virus replication is prevented.

Clinical use of GCV has focused on patients at high risk for CMV disease (Fan-Havard et al. 1989; Levinson and Jacobson 1992; Balfour 1999). Thus, individuals with AIDS benefit from GCV therapy of retinitis and gastrointestinal disease. Oral administration of GCV delays onset of end-organ disease in patients with AIDS who have CD4 counts <200/μl. Similarly, GCV delays the onset of CMV disease in organ transplant recipients. Poor bioavailability and the problem of toxicity limit the value of the compound. The prodrug of GCV, val-ganciclovir (Figure 6), has been proven to provide higher oral bioavailability and is now licensed for the treatment of CMV retinitis. However, toxicity remains a problem.

Recently, intravenous GCV has been shown useful for the treatment of symptomatic congenital CMV infection (Kimberlin et al. 2003).

Penciclovir

Penciclovir (PCV), Vectavir or Adenovir, is another nucleoside analogue in which the base, guanine, is normal but the sugar moiety has a structural modification (Boyd et al. 1987). The structural similarity to ACV is apparent (see Figure 3); there is, however, no oxygen atom in the acyclic 'sugar' moiety although an OH group exists in the position equivalent to that of the 3′-OH group in the normal nucleoside, guanosine. Like

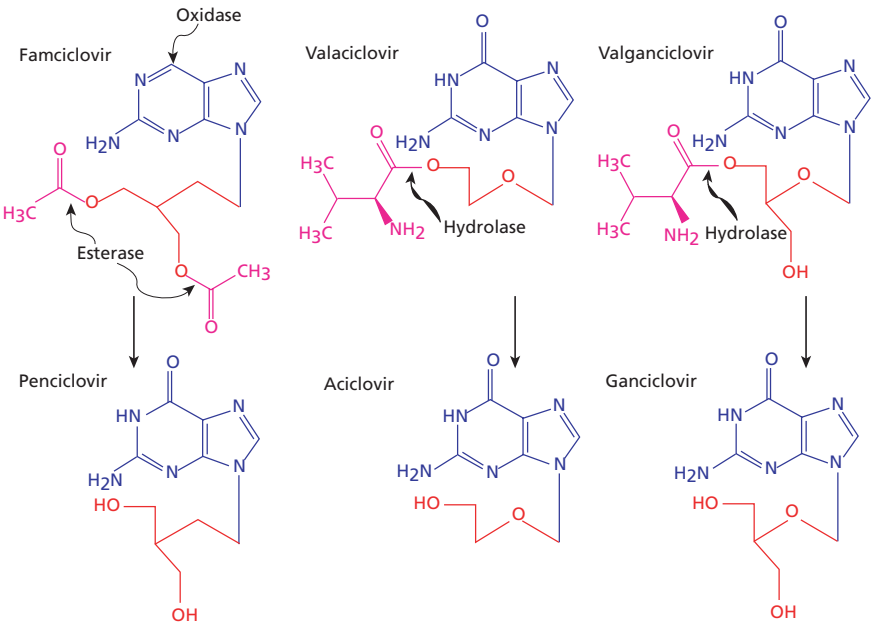

Figure 6 *The nucleoside analogue prodrugs famciclovir, valaciclovir, and ganciclovir showing their conversion to the active metabolites,* penciclovir, *aciclovir, and ganciclovir, respectively. The modifications that are subject to enzyme attack in vivo, providing oral bioavailability are shown in purple*

ACV, PCV is converted to PCV-monophosphate (PCV-MP) by the HSV or VZV TK (Vere Hodge and Cheng 1993), and PCV-triphosphate (PCV-TP) inhibits the viral DNApol but with the possibility of internal incorporation of PVC residues into viral DNA and further chain extension (Vere Hodge and Perkins 1989). The initial conversion of PVC to PCV-MP is more efficient than the phosphorylation of ACV, but the PCV-TP formed in infected cells is less active than ACV-TP as an inhibitor of HSV DNApol (Table 2) (Earnshaw et al. 1992).

PCV-TP has a significantly longer intracellular half-life than ACV-TP (Vere Hodge and Perkins 1989). Although the full implications of this observation have yet to be elucidated, it does seem that the compound may have more long-lasting inhibitory effects in infected tissues; this supposition is supported by tests in animals that compared famciclovir (FCV) and valaciclovir (VACV) (Sutton and Kern 1993; Field et al. 1995). It remains to be seen whether this biochemical feature translates into clinical benefit in humans. Although the compound seems to have a very high selective index, the bioavailability is poor (<5 percent in man) and the drug chosen initially for clinical development is a prodrug of PCV termed FCV, increasing the bioavailability of PCV to >70 percent.

As well as good activity against members of the *Herpesviridae*, PCV has limited activity against HBV (Boker et al. 1994; Locarnini et al. 1994). Thus far, no mechanism of action for this unexpected activity has been reported; it is, however, likely to involve specific inhibition of the HBV-induced DNApol although there is no explanation as yet for the phosphorylation of PCV in the infected hepatocytes.

Brivudin; bromovinyl deoxyuridine

Historically, brivudin, or bromovinyl deoxyuridine (BVDU), Helpin or Zostrex was discovered at about the same time as ACV (De Clercq et al. 1979) and, in vitro, appeared to be even more active than ACV against HSV-1 and VZV (De Clercq et al. 1980). However, the drug proved less useful than was anticipated. It is much more closely related to the natural substrate for TK (i.e. thymidine) with the modification of the bromovinyl group at the five position of the pyrimidine ring (see Figure 3).

The mechanism of action resembles that of ACV except that the conversion of BVDU-monophosphate (BVDU-MP) to BVDU-diphosphate (BVDU-DP) seems also to be achieved by the HSV TK (Descamps et al. 1982). The virus (but not cellular) TK thus possesses a thymidylate kinase activity. BVDU is significantly less active against HSV-2, probably because of the lack of conversion of BVDU-MP to BVDU-DP by the HSV-2 TK (De Clercq et al. 1981). A limitation of the compound in vivo is attack by phosphorylases that convert BVDU to bromovinyl ara-uracil, which is inactive. This, and concern about toxicity, has limited the development of the compound for clinical use; even so, the principles discovered during the development of this drug and SAR studies have led to several compounds (e.g. bromovinyl ara-uridine) that seem more promising. For a comprehensive review of brivudin see De Clerq (2004).

Sorivudine; bromovinylarabinosyl-uracil; brovavir

The mechanism of action of sorivudine, or bromovinylarabinosyl-uracil (BVaraU), Usevir (see Figure 3), seems similar to that of BVDU (Yokota et al. 1989; Machida 1990); its spectrum of action is, however, different and it seems to be some 2 000-fold more active than ACV or BVDU against VZV replication in cell culture (ED_{50} = 0.001 μm, compared with 14 μM for ACV) (Machida and Nishitani 1990). Like BVDU, however, the compound is poorly active against HSV-2. It is an extremely good substrate for VZV TK and its antiviral activity is attributed to the inhibition by BVaraU-TP of VZV DNApol; the compound is thought not to be incorporated into virus DNA. Unfortunately, one of the metabolites is bromovinylarabinosyl-uracil (BVU), a potent inhibitor of dihydrothymine dehydrogenase which is responsible for degrading drugs such as 5-fluorouracil that are used in cancer chemotherapy. Although this interaction was predicted, a number of deaths have occurred as a result of co-administration of the two drugs (Meeting Report 1995) (see section entitled The problem of toxicity of antiviral compounds). As a consequence, the compound will not be licensed in the USA. However, clinical benefit has been demonstrated against VZV disease in a

Table 2 *Comparison of key biochemical properties of the guanosine analogues aciclovir and penciclovir which are produced in vivo by their respective prodrugs, valaciclovir and famciclovir*

	Famciclovir	Valaciclovir
Oral absorption and blood pharmacokinetics parental compounds and production of PCV and ACV are similar		
	Penciclovir	**Aciclovir**
HSV1 thymidine kinase	High affinity (K_i = 1.5 μ m)	Low affinity (K_i = 173 μ m)
HSV1 DNApol	Low affinity (K_i = 8.5 μ m)	High affinity (K_i = 0.07 μ m)
Stability of triphosphate	Long ($t_{1/2}$ = 10 h)	Short ($t_{1/2}$ = 0.7 h)
DNA chain terminating	Rapid	Obligate

well-controlled clinical trial in comparison with ACV (Gnann et al. 1998).

Vidarabine; adenine arabinoside

Vidarabine, or adenine arabinoside (AraA), was an important landmark in the development of antiviral chemotherapy (Schabel 1968), being among the early compounds that achieved proven clinical benefit with relatively little acute toxicity, particularly in the systemic treatment of severe HSV infections (Whitley et al. 1977). The compound is a close analogue of adenine except that the OH group in the 2′ position on the 'sugar' moiety is in the unnatural 'arabinosyl' configuration (see Figure 3). The nucleotides formed from the phosphorylation of AraA have the potential to interfere with many different steps in the metabolism of DNA and its precursors, for example blocking of S-adenosyl homocysteine hydrolase (Hersfield 1979). The relative importance of these effects is unknown but, as with the other nucleoside analogues, the primary target for AraA-triphosphate (AraA-TP) seems to be the HSV-induced DNApol. The compound has low toxicity and may be used systemically, but its poor solubility necessitates the intravenous administration of large quantities of fluids. Secondly, its activity in vivo is limited by rapid deamination of AraA by adenosine deaminase, yielding arabinosyl-hypoxanthine which has no antiviral activity (Whitley et al. 1980a). Attempts to circumvent this problem by the administration of AraA-monophosphate (ARA-MP) have enjoyed little success (Whitley et al. 1980b). Today, vidarabine has largely been replaced in the physician's armamentarium for systemic therapy by more active and less toxic medications.

Idoxuridine

The pyrimidine analogue, idoxuridine (IDU), (e.g. Herpid, Kerecid, Virudox, Idurin, Stoxil) was the first specific antiviral compound to be discovered (Prusoff 1959). and is the best known example of the 5-substituted thymidine series; it is similar to BVDU. However, IDU does not possess the high selectivity associated with the more modern drugs. This 'first generation' nucleoside analogue is readily phosphorylated by cellular enzymes and thus can be converted to the active nucleotide in normal, as well as in virus-infected, cells although the conversion is much more efficient in the latter. The IDU triphosphate (IDU-TP) inhibits viral (and cellular) DNA synthesis, but a major part of the antiviral action may be due to incorporation of the analogue into viral nucleic acids and subsequent perturbation of virus gene expression (De Clercq 1982). The exact mechanism of action is unknown but some selectivity is assumed to result from the comparatively greater accumulation of IDU-TP in infected cells. IDU is, however, severely toxic upon systemic administration and is useful only as a topical agent, for which it continues to be useful both for herpes zoster and HSV keratitis. Skin penetration is a problem for topical administration. While formulations have been prepared in dimethyl sulfoxide (DMSO) for treating zoster (Dawber 1974; reviewed by Spruance 1994), they have been replaced by oral therapy with ACV, VACV, and FCV. Notwithstanding its traditional use for treating HSV in humans, the potential long-term genetic toxicity of this compound remains a major concern.

Trifluridine; trifluorothymidine

Like IDU, trifluridine (TFT), or trifluorothymidine Viroptic, (see Figure 3), synthesized in 1964 (Heidelberger et al. 1964), is also highly toxic and is unsuitable for systemic use. Even to demonstrate its selective antiviral effect in cell culture requires the use of cells lacking TK that are resistant to its toxic effects (Field et al. 1981). However, the compound is very soluble and has found a particular use in the topical treatment of ocular infections (Wellings et al. 1972). The very rapid hydrolysis of TFT to harmless metabolites probably accounts for the lack of toxicity in this application (Dexter et al. 1972). It is the treatment of choice for HSV keratoconjunctivitis (reviewed by Bigar 1979). The compound is reported to be effective against drug-resistant strains and remains in the armoury of anti-herpes drugs despite its toxicity in vitro.

NUCLEOSIDE ANALOGUE PRODRUGS

An important limitation of many nucleoside analogues is their poor bioavailability following oral administration. The development of orally bioavailable compounds that are converted in vivo to yield the relevant nucleoside analogues thus represents a major advance. The first compounds of this type were developed for herpes chemotherapy and the first to be licensed for use in humans were derived from ACV and PCV. Following this paradigm, a similar strategy has now been successfully applied to many different compounds including nucleosides active against alternative virus targets. Note that, technically, ACV itself is a 'prodrug' since the mechanism of action involves conversion of absorbed ACV to the active metabolite (ACV-TP) in vivo. However, in the antiviral field, the term prodrug is usually reserved for compounds where the first chemical conversion in vivo is primarily concerned with significantly improving bioavailability.

Valaciclovir

VACV Valtrex, Zelitrex is the L-valine ester of ACV (Figure 6) which, following oral administration, is cleaved in the gastrointestinal tract by an enzyme referred to as VACV hydrolase to yield ACV and the natural amino acid L-valine, little VACV being detected

in plasma (Beauchamp et al. 1992). Multiple oral doses of VACV (>1 g four times daily) result in plasma ACV concentrations similar to those achieved with intravenous ACV (5 or 10 mg/kg three times daily) but without the sharp peak concentrations (Carrington 1994; Crooks 1995). Once formed and distributed to the infected tissues, the mode of action is, of course, identical to that of simple ACV. This compound offers a formulation that is more convenient to administer for the treatment of herpes zoster than ACV (Murray 1995).

Famciclovir

The concept that led to VACV also resulted in FCV (Vere Hodge et al. 1989), the diacetyl ester of 6-deoxy-penciclovir (Figure 6). In this case two enzymes are involved, first in removing one ester preferentially, then the second, followed by oxidation by aldehyde oxidase to yield PCV (Vere Hodge 1993). The only degradation products from this reaction are harmless carboxylic acid and PCV and, as with VACV, little FCV is detected in plasma. Ease of administration provides a distinct advantage over ACV in the treatment of herpes zoster (Carrington 1994).

Have the nucleoside analogue prodrugs VACV and FCV met the clinical challenge? Following a decade of clinical experience with these nucleoside prodrugs, have the hoped-for improvements in antiviral efficacy been realized? The consensus is that both compounds do offer significant clinical benefits over oral ACV (Field 1996; Baker and Eisen 2003). However, the relative merits of the two new compounds in comparison with one another remain controversial (Carrington 1994, 1997). The disruption to the preclinical and clinical development teams associated with these drugs caused by the merger of the two giant pharmaceutical companies, GlaxoWellcome and SmithKlineBeecham, in the year 2000 may help to explain why there is still a paucity of data from clinical trials on the relative advantages and disadvantages of the two compounds under circumstances where they may be compared directly (Field and Fitzmaurice 2001).

NUCLEOTIDE ANALOGUES

A series of acyclic purine and pyrimidine nucleoside phosphonates was first described in 1986 (De Clercq et al. 1986). Several of these compounds showed a broad spectrum of activity against viruses, including both DNA and RNA viruses. Unlike the natural nucleotides, the acyclic nucleoside phosphonates (ANP) resist phosphorolytic cleavage by cellular esterases. The negative charge on the phosphate moiety impairs their cellular uptake which may occur by an endocytosis-like process. Once inside the cells, the ANP compounds need to be activated by cellular enzymes to catalyse their conversion to di- and tri-phosphorylated forms. The virus inhibitory effects then result from a selective interaction of their diphosphate metabolites with virus DNA pol (Votruba et al. 1987), thus their ultimate antiviral target is the same as that described above for the regular nucleoside analogues. A general feature of ANPs is the long intracellular half-life of the diphosphate metabolite, which may help to explain their long-lasting antiviral effects allowing infrequent dosing. The mechanisms of action of this series of compounds are thoroughly reviewed by De Clercq (1997)

Cidofovir (Vistide)

Cidofovir, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC, Forvade) (Figure 7). As mentioned above, HPMPC viz. has a very long tissue half-life; it has been shown in many animal models that long intervals between doses can be allowed without loss of antiviral effect. Thus, once weekly dosing is employed in humans with apparent success (Snoeck et al. 1993). The compound has significant toxic side effects, particularly nephrotoxicity, although this can be minimized by co-administration of probenecid (which competitively inhibits the transport of drugs through the glomeruli) and intravenous hydration. Intravenous HPMPC is licensed for treatment of HCMV retinitis and topical gel used for the treatment of refractory HSV lesions in AIDS patients. Its spectrum of action extends beyond herpesviruses and includes poxviruses, HPV and HBV (see below). Other important compounds in this series include 9-(2-phosphonylmethoxyethyl) adenine (PMEA) (adefovir), which is active against herpes- retro- and hepadnaviruses, and R-9-(2-phosphonylmethoxypropyl) adenine (PMPA), which is active against retro- and hepadnaviruses. Following the prodrug strategy outlined above for the guanosine nucleosides, the oral prodrug of PMEA, bis(pivaloyloxymethyl) PMEA or adefovir dipivoxil (Naesens et al. 1996) is a very promising compound for the treatment of HBV (see section entitled Inhibitors of hepatitis viruses below)

PYROPHOSPHATE ANALOGUES

A series of compounds has been known for many years whose members interact directly with herpesvirus DNApol at the pyrophosphate binding site. This was first shown with phosphonacetic acid (Purifoy et al. 1977), a compound too toxic for humans. However, the closely related compound phosphonoformate (Figure 8) has been developed for use in man in the treatment of CMV disease in immunocompromised hosts. Its administration is associated with toxicity as evidenced by electrolyte imbalances (reviewed by Öberg 1983a).

Phosphonoformic acid

Phosphonoformate (PFA), foscarnet, foscavir (Figure 8) seems to have a relatively simple mode of action, being a

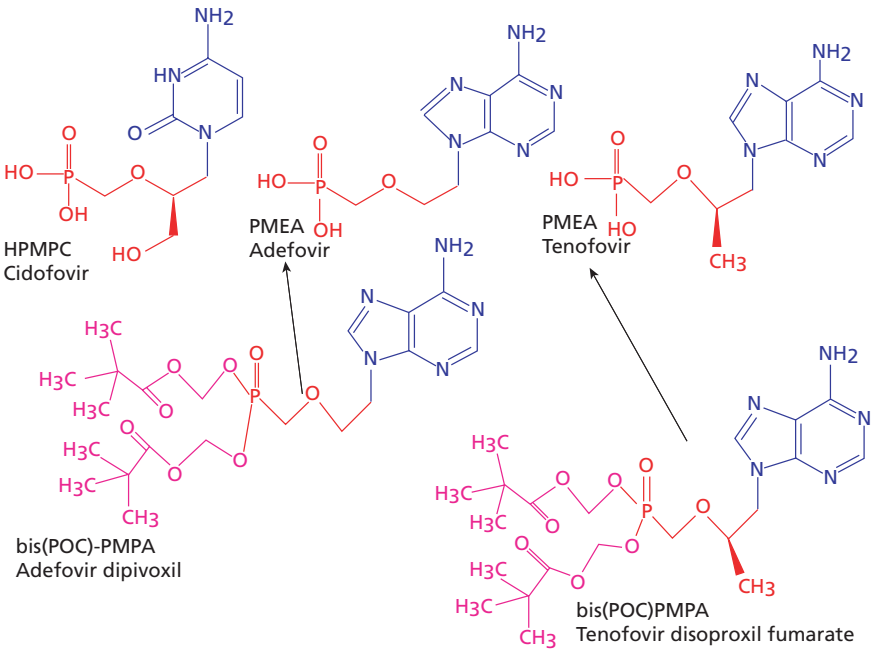

Figure 7 *Examples of effective inhibitors of virus polymerase: phosphonyl compounds cidofovir, adefovir, and tenofovir and two prodrugs that yield adefovir and tenofovir, respectively. The modifications to the compounds that provide oral bioavailability are shown in purple.*

direct inhibitor of HSV DNApol (Leinback et al. 1976). The compound suffers from some toxic features, and its tendency to accumulate in bone (Crisp and Clissold 1991) is of particular concern. It does, however, offer an alternative strategy for treating both infections with ACV-resistant strains of HSV and CMV retinitis in AIDS patients (Fanning et al. 1990).

ALTERNATIVE ENZYME TARGETS FOR HERPESVIRUS INHIBITORS

Most of the successful antiherpetic agents, to date, target the virus DNApol. Among the more than 80 distinct genes in the HSV genome, there are several other obvious enzyme targets for inhibition including ribonucleotide reductase (RR), protease, and helicase primase. There has been much effort to target herpes RR including synthetic oligopeptides directed to the enzyme active site. To date, none of these approaches has resulted in useful therapies. Given the success of compounds directed to the HIV protease (see section entitled Inhibitors of human immunodeficiency virus, below). It is likely that compounds designed to interfere with the herpes proteases will be effective (reviewed by Waxman and Darke 2000), but to date, none have been developed for clinical use. Another function that has been exploited is the HSV helicase primase. This is a virus enzyme that promotes the ATP-dependent unwinding of the DNA duplex during replication and it appears that compounds directed to it can inhibit virus replication at nanomolar concentrations.

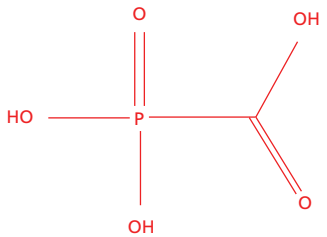

Figure 8 *An inhibitor of herpes DNA polymerase: the pyrophosphate analogue, foscarnet*

BAY-57-1293

The thiazolylsulfonamide, BAY-57-1293 (N-[5-(aminosulfonyl)-4-methyl-1,3-thiazol-2-yl)-N-methyl-[4-(2pyridinyl)phenyl]acetamide) (Figure 9) is an example of an inhibitor of herpes helicase primase (Kleymann et al. 2002, 2004). The compound is highly selective and extremely active in vitro with an IC_{50} concentration of approximately 20 nM (i.e. some 500-fold more active than ACV), furthermore, it is reported to be remarkably effective in several different laboratory animal HSV infection models. Other similarly potent molecules from different chemical series have also been described (Field and Fitzmaurice 2001). Although these particular compounds may not progress to the clinic, the experimental data clearly show that inhibition of this target has considerable potential and it is likely that effective

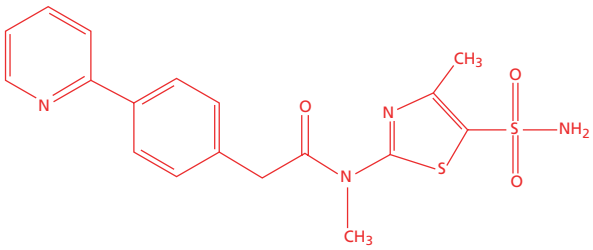

BAY-57-1293

Figure 9 *An inhibitor of the herpes simplex virus enzyme, helicase primase: BAY-57-1293*

compounds suitable for clinical development will emerge in the foreseeable future (Kleymann 2003, 2004).

DRUG COMBINATIONS TO MANAGE HERPESVIRUSES

Historically, there has been little enthusiasm for the use of drugs in combination against herpesviruses. Recently, the introduction of combination therapy has revolutionized the management of HIV and viral hepatitis (see sections below). Given the success obtained with different viruses, the possibility of this approach for HSV is currently being assessed – it is the turn for herpes virologists to learn from the younger areas of antiviral research! Actually, there is a long history of anti-inflammatory agents being used in combination with specific antiviral agents to manage severe lesions in recurrent herpes keratitis. One problem with this approach is that some effective anti-inflammatory agents also up-regulate and prolong virus shedding. This can be controlled by using a specific virus inhibitor (Harmenberg et al. 2003), thus a recent trial reported beneficial effects in patients with experimentally induced cutaneous HSV lesions using a combination of topical 5 percent ACV and 1 percent cortisone (Evans et al. 2002). There is no doubt that the further use of combinations of two or more specific antiviral drugs will be the subject of future studies and are likely to become more common in the future.

IMMUNOMODULATORS AS ANTIVIRAL TREATMENTS FOR HERPES

Compounds have been discovered that inhibit HSV replication by interference with a cellular target involved in virus replication (e.g. cyclin-dependent kinases) yet appear to behave as selective inhibitors of virus replication (Schang 2002). It remains to be seen whether such compounds will have a value either alone or in combination with conventional inhibitors of virus functions. Another approach is to stimulate the host to react more effectively to the reactivating virus so as to minimize the clinical signs generally referred to as immunomodulators.

Resiquimod

One compound of this type that is claimed to be effective is known as resiquimod (4-amino-2-ethoxymethyl-α,α-dimethyl-1H-imidazo[4,5-c]quinoline-1-ethenol). The compound (Figure 10) is described as an immune response modifier which induces cytokines including interferons (Tomai et al. 1995), however clinical trials as a topical therapy for genital herpes were recently abandoned.

HCMV INFECTION

HCMV is another member of the *Herpesviridae* that is very widespread among human populations worldwide. Infection is rarely associated with clinical signs in otherwise healthy individuals except when primary infection occurs during pregnancy when congenital disease can result. However, when the immune system is compromised, e.g. associated with allograft transplantation, clinical signs of infection often become apparent and may be extremely damaging. The coming of HIV heightened awareness of the condition and HCMV retinitis became one of the most feared complications of the retrovirus infection. The introduction of highly active antiretroviral therapy has decreased the incidence of this complication substantially, but this and other manifestations of HCMV infection still account for considerable morbidity and there is much interest in developing improved drugs to treat or prevent these conditions (Field 1999). Currently there are five compounds that have been officially licensed for the treatment of HCMV infections. These are ganciclovir and phosphonoformic acid and cidofovir (see above) and two further compounds.

Formivirsen

There have been many unsuccessful attempts to employ antisense oligonucleotides in an effort to block or

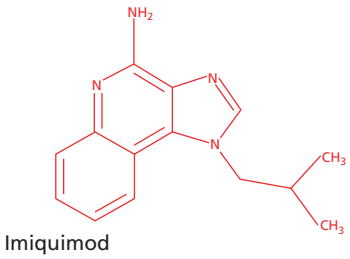

Imiquimod

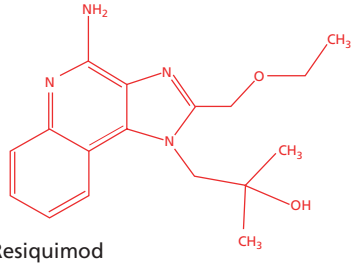

Resiquimod

Figure 10 *Examples of antiviral compounds whose primary activity is thought to be as immunomodulators: imiquimod and resiquimod*

5′-d-[G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G]3′

sodium salt

*: racemic phosphothorioate antisense oligonucleotide

Formivirsen sodium, Vitravene

Figure 11 *Formisvirsen, an antisense oligonucleotide active against human cytomegalovirus*

attenuate virus replication. There have been some more optimistic results, however, and formivirsen (ISIS 2922), vitravene is an antisense oligodeoxynucleotide (Figure 11) comprising 21 phosphorothioate-linked nucleosides that is complementary to the RNA of the HCMV major immediate–early region (Azad et al. 1993) and is one of the most promising compounds of this type.

Valganciclovir

Using the same oral prodrug strategy that led to valaciclovir and FCV, the orally bio-available valine ester of ganciclovir was synthesized and found to be effective. Valganciclovir (Valcyte) (Pescovitz et al. 2000; Reusser 2001) (Figure 6), when administered orally (two 450 mg tablets twice daily), gives equivalent systemic ganciclovir levels as intravenous ganciclovir at 5 mg/kg twice daily. Oral valganciclovir is expected to replace intravenous ganciclovir in both the therapy and prevention of CMV infections.

1263W94, Maribavir

There are currently many new compounds in development to combat HCMV (briefly reviewed by De Clercq 2003). Currently, one of the most promising of these is 1263W94 or maribavir. This is a novel benzimidazole compound, 5,6-dichloro-2-(isopropylamino)-1,β-L-ribofuranosyl-1-*H*-benzimidazole. (Figure 12). The compound has been demonstrated to potently selectively inhibit HCMV in vitro and to have favorable safety profiles in animal species and it is currently undergoing

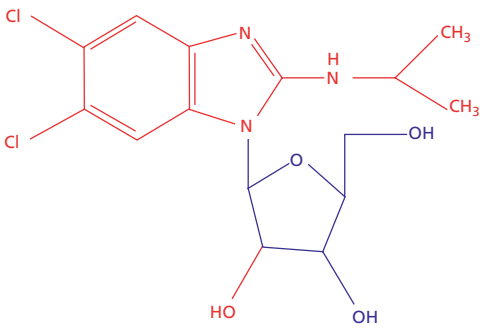

1263W94 Maribavir

Figure 12 *A new inhibitor, 1263W94,* maribavir, *which targets the human cytomegalovirus protein kinase*

trials in man (Wang et al. 2003). Its mechanism of action is mediated through inhibition of the protein kinase activity of the *UL97* gene product (Wang et al. 2003). The precise function of this virus product has recently been elucidated, it is thought to concern export of HCMV nucleocapsids from the nucleus (Krosky et al. 2002) and it is the same gene product that is responsible for phosphorylation of ganciclovir to its monophosphate (see above).

Inhibitors of human immunodeficiency virus

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTI)

The emergence of HIV as a major pathogen resulted in an unprecedented effort to develop new antiviral agents. The earliest compounds to enjoy limited therapeutic success were all nucleoside analogues; the first to be widely prescribed was 3′-azido-3′-deoxythymidine (azidothymidine).

Zidovudine; azidothymidine

Zidovudine, or azidothymidine (AZT), is an analogue of thymidine (Figure 13) (Mitsuya et al. 1985), and is readily phosphorylated in actively dividing cells. The drug is initially phosphorylated by cellular TK, the rate-limiting step being its conversion to AZT diphosphate (AZT-DP) by cellular thymidylate kinase. AZT triphosphate (AZT-TP) competitively inhibits HIV reverse transcriptase with respect to thymidine triphosphate (TTP) and, because the 3′-azido group prevents the formation of 5′,3′-phosphodiester linkages, AZT-TP acts as a chain terminator of DNA synthesis (Darby 1995). The compound, via its metabolites, also inhibits a variety of cellular enzymes, including DNA-polymerase-γ, which undoubtedly contributes to the range of toxic effects associated with prolonged clinical use of the drug (Fischl et al. 1987). It was the first drug to be used to treat HIV, and is still accepted as conferring at least transient benefit.

Stavudine; didehydrodideoxyuridine

Stavudine, or 2′,3′-didehydro-2′-deoxythymidine (D4T), is similar to AZT in that it is a thymidine nucleoside analogue (Figure 13) (Baba et al. 1987). It readily enters cells but, in contrast to AZT, the rate-limiting step is the initial phosphorylation by cellular TK. Once formed, D4T triphosphate (D4T-TP) is rapidly converted to D4T diphosphate (D4T-DP) and triphosphate derivatives. The mechanism of action at the level of reverse transcriptase seems to be similar to that of AZT (reviewed by Hitchcock 1991); toxicity in the form of peripheral neuropathy is, however, more common. The clinical use of D4T is variable worldwide.

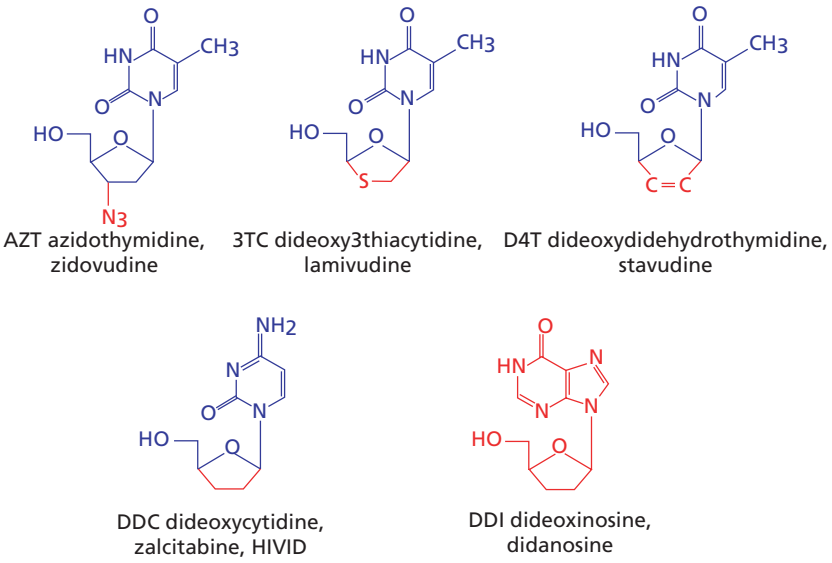

Figure 13 *Nucleoside analogue inhibitors of HIV reverse transcriptase (NRTI)*

Didanosine; dideoxyinosine (Videx)

Didanosine, or 2′,3′-dideoxyinosine (DDI), is a purine nucleoside analogue (Figure 13) (Mitsuya and Broder 1986) which is taken up intracellularly and converted by 5′ nucleotidase to DDI monophosphate (DDI-MP) and further metabolized by cellular enzymes to DDA triphosphate (DDA-TP), which has a prolonged intracellular half-life (8–24 h). Again, the molecular target for the drug action is the HIV reverse transcriptase (reviewed by McLaren et al. 1991). Clinical trials have been conducted in AIDS patients (Darbyshire and Aboulker 1992; Kahn et al. 1992) but the clinical use of DDI has been limited by its oral formulation which may cause gastrointestinal discomfort.

Zalcitabine; dideoxycytidine (HIVID)

Zalcitabine, or 2′,3′-dideoxycytidine (DDC) (Figure 13), is active against HIV-1 and 2 and against some strains that are resistant to AZT (Mitsuya and Broder 1986). The drug seems to be similar in potency to AZT (Bozzette et al. 1995).

Lamivudine; thiacytidine (Epivir)

Next, in this series of nucleoside analogue inhibitors of reverse transcriptase, lamivudine, or 2′-deoxy-3′-thiacytidine (3TC) (Figure 13), is synergistic with AZT in vitro and is active against HIV-resistant strains (Soudeyns et al. 1991). It seems to be relatively nontoxic but is less active than AZT in monotherapy. This drug is of particular interest for combination studies with AZT and a protease inhibitor. Lamivudine is also an active inhibitor of HBV DNApol in vitro (Doong et al. 1991) and against HBV infection in vivo (Honkoop and de Man 1995). Lamivudine has been coformulated with AZT and marketed as combivir.

Carbovir, Abacavir (Ziagen)

Abacavir is a carbocyclic synthetic nucleoside analogue (Figure 14). Intracellularly, abacavir is phosphorylated by cellular enzymes to its active metabolite, carbovir triphosphate, which is an analogue of deoxyguanosine-5′-TP. Carbovir-TP then inhibits the activity of HIV reverse transcriptase both by competing with the

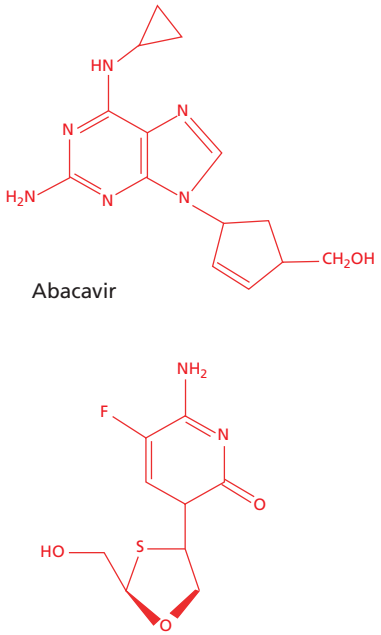

Figure 14 *Further examples of HIV nucleoside reverse transcriptase inhibitors: NRTI*

natural substrate dGTP and by its incorporation into viral DNA. The lack of a 3′-OH group in the incorporated nucleoside analogue prevents the formation of the 5′ to 3′ phosphodiester linkage essential for DNA chain elongation, producing chain termination. Abacavir is often used in combination with lamivudine and zidovudine, as well as with either a nonnucleoside reverse transcriptase inhibitor or protease inhibitor.

Adverse reactions associated with abacavir therapy include nausea, headache, stomach pain, diarrhea, insomnia, rash, fever, and dizziness. Importantly, fatal hypersensitivity reactions have been associated with abacavir use.

Abacavir resistance is conferred by mutations in the HIV reverse transcriptase gene that resulted in amino acid substitutions at positions K65R, L74V, Y115F, and M184V. M184V and L74V are the most frequently observed mutations among clinical isolates. Multiple reverse transcriptase mutations conferring abacavir resistance exhibit cross-resistance to lamivudine, didanosine, and zalcitabine in vitro.

Emtricitabine (FTC) (Emtriva)

Emtricitabine is a synthetic nucleoside analogue of cytosine (Figure 14) active against the reverse transcriptase of HIV-1. Following triphosphorylation by cellular enzymes, it competes with the natural substrate deoxycytidine 5′-triphosphate and, following incorporation into the nascent viral DNA, results in chain termination.

Other related compounds, e.g. Entecavir, have been found to be more useful against HBV infection and are under investigation for the treatment of chronic HBV infection (see section on Inhibitors of hepatitis viruses, below). Entecavir is a promising new drug that has entered a Phase III clinical trial for efficacy. The compound is a cyclopentile guanosine nucleoside analogue (Figure 15) with selective activity against hepatitis B viral DNApol. It is orally bioavailable, and due to a long terminal elimination half life of approximately 110 h, is clearly suitable for once daily therapy. Similarly, a series of L-nucleosides is advancing through Phase II and III clinical trials. Hepatitis viruses are considered in more detail and a description of further inhibitors is dealt with in the section entitled Inhibitors of hepatitis viruses.

NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTI)

The intensive screening of compounds for anti-HIV activity led to the discovery of several families of inhibitors that, although they differ from one another in structure, share the property of inhibiting reverse transcriptase by binding to sites other than that which normally interacts with the nucleosides. Some act at concentrations in the nanomolar range and are cytotoxic only at 10 000- to 100 000-fold higher concentrations. HIV-1 (but, notably, not HIV-2) reverse transcriptase is

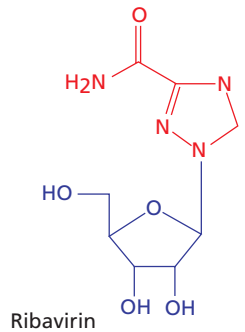

Ribavirin

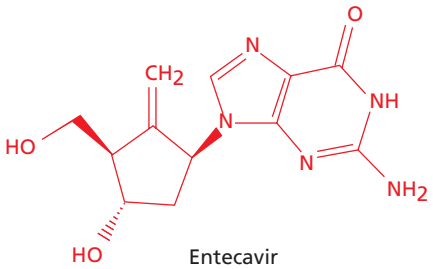

Entecavir

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2-

T-20, Enfuvirtide

Figure 15 *The broad spectrum nucleoside inhibitor, ribavirin, entecavir, and the synthetic peptide, T-20 which is anti-HIV fusion inhibitor*

the target sensitive to inhibition. Enzyme studies, however, indicated that such compounds inhibit reverse transcriptase in a noncompetitive fashion (De Clercq 1992). The site on the enzyme at which these compounds bind has been termed the TIBO site (Pauwels et al. 1990) and certain other compounds (e.g. HEPT, an acyclic uridine derivative) seem to interact with the same site (Baba et al. 1989) (TIBO and HEPT are discussed below). These compounds have been collectively described as allosteric inhibitors. The rapid selection of drug-resistance seems to be a major limitation of the success of these compounds, which, however, are likely to have an important role when used in combination both with other nonreverse transcriptase inhibitors and with other classes of inhibitor.

TIBO, nevirapine and HEPT

A series of nonnucleoside inhibitors was discovered that are tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2H (1H)-thione derivatives (Figure 16) (Debyser et al. 1991). This almost unpronounceable name is usually shortened to TIBO. This benzodiazepine-like drug was the first of the NNTRI to be identified (De Clercq 1992). Early trials showed a large but transient reduction in viral

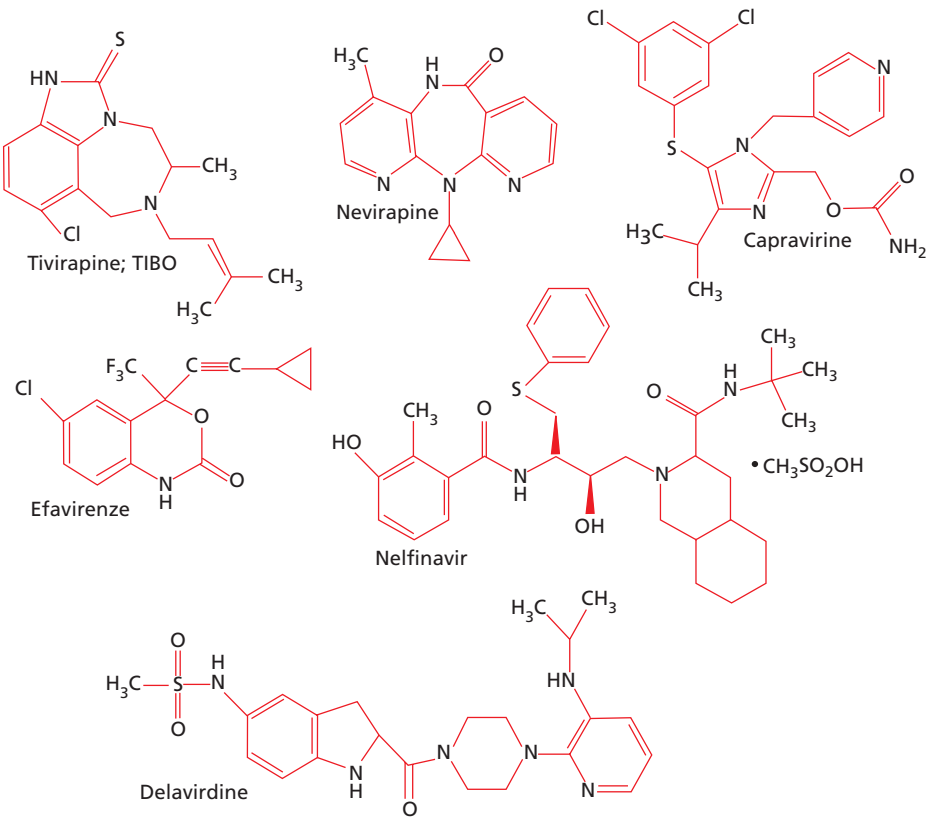

Figure 16 *Nonnucleoside analogue inhibitors of HIV reverse transcriptase (NNRTI)*

load. The drug is of relatively low toxicity but its oral bioavailability is poor. Nevirapine (Figure 16) is another noncompetitive inhibitor of reverse transcriptase that interacts with tyrosine residues on the enzyme (Merluzzi et al. 1990) and yet another series of compounds of this general type, hydroxyethoxymethylphenylthiothymine derivatives, has been given the acronym HEPT (Figure 16) (Miyasaka et al. 1989).

Nevirapine (Viramune)

Nevirapine is a nonnucleoside (dizyridodiazepinone) reverse transcriptase inhibitor that is structurally similar to benzodiazepines (Figure 16) (Merluzzi et al. 1990). Binding of nevirapine to reverse transcriptase is noncompetitive. It selectively inhibits HIV-1, but not HIV-2, reverse transcriptase without significant inhibition of other cellular polymerases. It is currently used in combination with nucleoside reverse transcriptase inhibitors and/or protease inhibitors. Because of the rapid and certain appearance of resistance, it is not used as monotherapy. The major side effect of nevirapine is the development of a cutaneous rash including 'Stevens Johnson syndrome' which is a severe rash, often drug-induced. Other less common side effects include altered liver function tests, fever, and myalgia. Since nevirapine is metabolized by the liver, there is the potential for interaction with other hepatically metabolized medications metabolized by this organ, including rifabutin, rifampin, ketoconazole, and the protease inhibitors.

Resistance to nevirapine occurs at codons 181, 188, 103, 106, 108, and 190, all of which result in a significant reduction in nevirapine susceptibility to treatment. A nevirapine-associated mutation at codon 181, concurrent with the presence of the most common zidovudine resistant mutation (codon 215), appears to restore susceptibility to zidovudine.

Delavirdine (Rescriptor)

Delavirdine binds directly to reverse transcriptase and blocks RNA-dependent and DNA-dependent DNApol activities (Figure 16). HIV-2 reverse transcriptase and human cellular DNApol are not inhibited by delavirdine. In addition, HIV-1 group O, a group of highly divergent strains that are uncommon in North America, may not be inhibited by delavirdine. Delavirdine is frequently used as combination therapy with two nucleoside reverse transcriptase inhibitors, such as zidovudine and didanosine, with or without a protease inhibitor. Adverse reactions associated with delavirdine therapy include rash (which may be maculopapular or pruritic), headache, increased transaminases, nausea, diarrhea, fatigue, and, rarely, Stevens Johnson syndrome. Delavirdine increases concentrations of the protease inhibitors saquinavir, nelfinavir, and indinavir when given in combination.

Mutations conferring resistance to delavirdine occur predominantly at position 103 and less frequently at positions 181 and 236. Combination therapy with delavirdine and zidovudine may result in increased zidovudine sensitivity of patient isolates after 24 weeks on therapy. Mutations at positions 103 and 181 have been associated with resistance to other NNRTI.

Efavirenz (Sustiva)

Efavirenz is mediated predominantly by noncompetitive inhibition of HIV-1 reverse transcriptase (Figure 16). HIV-2 reverse transcriptase and human cellular DNApol are not inhibited by efavirenz. Efavirenz is frequently used in combination with two or more nucleoside reverse transcriptase inhibitors, with or without a protease inhibitor. Side effects include headache, dizziness, diarrhea, skin rash, impaired concentration, anxiety, visual disturbances, nausea, vomiting, insomnia, fatigue, and flulike symptoms. Efavirenz has produced numerous birth defects in animal models, and is contraindicated in pregnancy. No clinically significant drug interactions occur between efavirenz and nucleoside reverse transcriptase inhibitors. Efavirenz decreases indinavir concentrations, necessitating an increase in indinavir dosing when given concomitantly with efavirenz. Saquinavir concentrations are diminished to such a degree by efavirenz that the two should not be used in combination.

Mutations at the reverse transcriptase amino acid positions 98, 100, 101, 103, 106, 108, 188, 190, and 225 confer efavirenz resistance. The mutation at position 103 (lysine to asparagine) is the most frequently observed. Efavirenz-resistant isolates are frequently resistant to others in the same class.

Capravirine

Capravirine (Figure 16) is the last of this series of NNRTIs currently under investigation. It has potent activity in vitro against HIV variants but RT substitutions are detected including K103N that confer broad cross resistance to the other drugs in this class.

PROTEASE INHIBITORS

HIV encodes an enzyme that specifically cleaves protein precursors in the maturation of gag and pol polyproteins (Pearly and Taylor 1987). Inhibition of this enzyme results in the production of noninfectious virus (Kohl et al. 1988). This virus-induced enzyme has no known cellular function, but a number of highly specific inhibitors of HIV whose action is based on inhibition of the HIV protease have been reported (De Clercq 1995).

Saquinavir (Fortovase, Invirase)

This was the first active virus protease inhibitor to be described. It is a peptide-based transition-state mimetic of an Asn.Phe.{ }Pro. substrate sequence, in which { } indicates the cleavage site (Roberts et al. 1990).

Saquinavir (Figure 17) is an extremely active inhibitor of HIV replication in cell culture, with a very high selective index and was the first licensed protease inhibitor worldwide. Its pharmacokinetic properties are, however, not ideal for use in vivo (Roberts 1995). Furthermore, viruses of many other families encode proteases, including pathogens as diverse as polio, HCV, influenza, and HSV, against which a similar approach is being directed. The potential for these compounds to be valuable inhibitors seems to be great but their ultimate success in clinical use cannot yet be predicted. Adverse reactions include diarrhea, abdominal discomfort, and nausea. Photosensitization can occur. Saquinavir is metabolized by the liver enzyme system: cytochrome P-450; thus, ketoconazole and itraconazone increase the plasma levels of saquinavir, as does ritonavir, a fact that has been utilized in the development of combination protease inhibitor therapies.

Saquinavir-resistant HIV occurs most commonly at the HIV protease L90M site. Saquinavir is effective against ritonavir- and indinavir-resistant mutants.

Ritonavir (Norvir)

Ritonavir (Figure 17) is a peptide-like inhibitor of HIV proteases and one that renders them incapable of processing the gag–pol polyprotein precursors required for assembly of progeny virions. Ritonavir is used in combination with NRTI and/or NNRTI. Ritonavir is also metabolized by the cytochrome P450 system, as is saquinavir. Ritonavir increases plasma levels of macrolides, particularly clarithromycin and erythromycin, anticonvulsants, tricyclic antidepressants, and the azole antifungals. Ritonavir also increases levels of saquinavir, and, as such, low doses of ritonavir are sometimes used in combination with saquinavir to achieve higher saquinavir concentrations. Adverse reactions include nausea, vomiting, anorexia, abdominal pain, taste perversion, and circumoral and peripheral paresthesia. Furthermore, there are alterations in triglyceride metabolism and elevation of liver function tests have also been described. Clinically, fat redistribution can appear.

Ritonavir resistance occurs with mutations in HIV protease at codons 84, 82, 71, 46, 54, and 36. The development of mutations is stepwise, occurring first at codon 82. Cross-resistance with indinavir but not saquinavir is common.

Indinavir (Crixivan)

Indinavir (Figure 17) binds to cleavage sites of HIV protease; thereby, it prevents cleavage of viral polyprotein precursors into individual proteins required for progeny virions assembly. Adverse reactions associated with indinavir therapy include nephrolithiasis, hematuria, and occasionally hyperbilirubinemia.

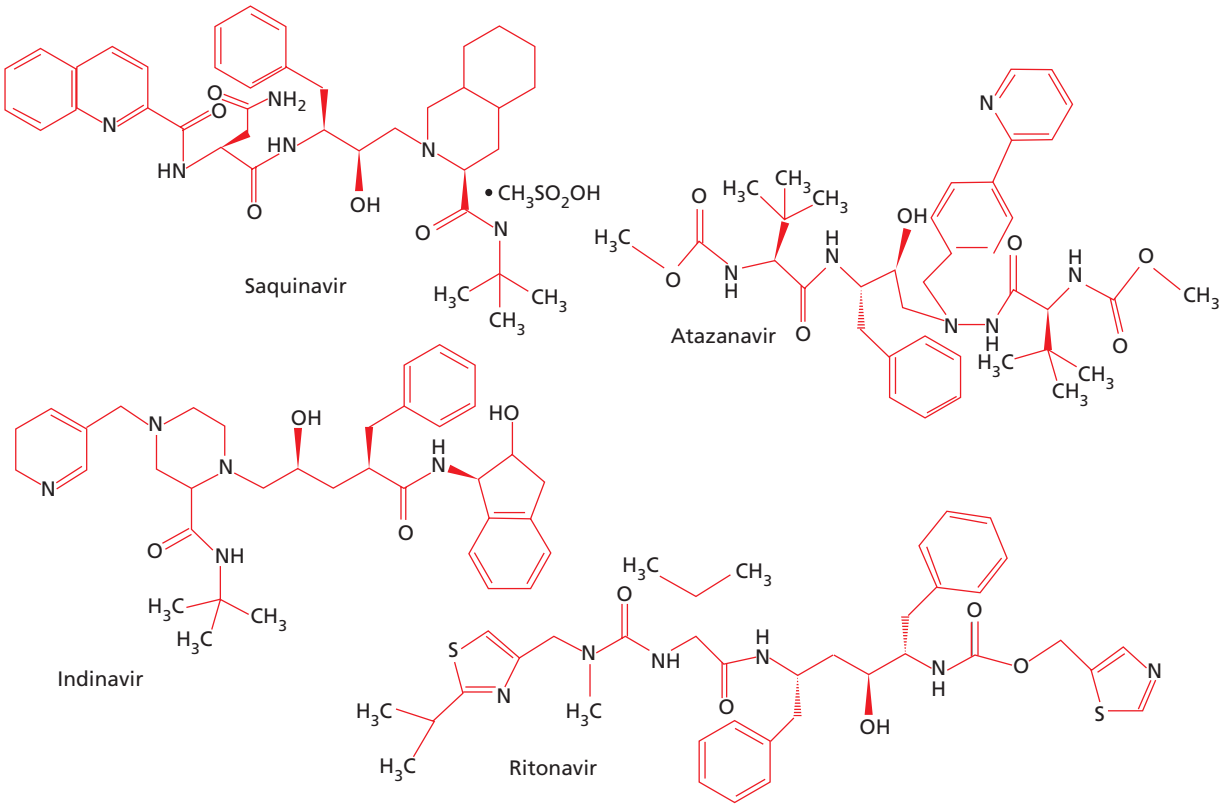

Figure 17 *Highly active inhibitors of the HIV protease (PI)*

Administration of indinavir selects for resistant mutants with alterations in HIV protease at a variety of different amino acid sites. Increased resistance is a function of stepwise acquisition of multiple mutations in HIV protease.

Nelfinavir (Viracept)

Nelfinavir (Figure 18) is a nonpeptidic inhibitor of HIV protease that inhibits or interferes with processing of viral polyprotein precursors, resulting in noninfectious progeny virions. It is given in combination with NRTI and/or NNRTI. Nelfinavir is metabolized by the cytochrome P450 system. Adverse events associated with nelfinavir administration include diarrhea, rash, leukopenia, and neutropenia.

Resistance to nelfinavir occurs by mutations in HIV protease, particularly at codons 30, 35, 36, 46, 71, 77, and 88. Importantly, these sites are all different from those that confer resistance to other protease inhibitors. However, once resistance to a protease inhibitor develops, it is likely that cross-resistance will occur.

Amprenavir (Agenerase)

Amprenavir (Figure 18) acts by binding to the active site of HIV-1 protease, preventing the processing of viral gag and gag–pol polyprotein precursors and results in the formation of immature non-infectious viral particles. In vitro, amprenavir has synergistic anti-HIV-1 activity in combination with abacavir, zidovudine, didanosine, or saquinavir, and additive anti-HIV-1 activity in combination with indinavir, nelfinavir, and ritonavir. Adverse effects associated with amprenavir administration include rash, nausea, vomiting, diarrhea, abdominal pain, and perioral paresthesias. Amprenavir is metabolized by the P450 system.

Resistance to amprenavir is conferred by amino acid substitutions primarily at positions M46I/L, I47V, I50V, I54L/V, and I84V, as well as mutations in the viral protease p1/p6 cleavage site. Cross-resistance between amprenavir and the other protease inhibitors is possible.

Lopinavir/Ritonavir (Kaletra)

Lopinavir/ritonavir combination interferes with processing of viral polyprotein precursors, resulting in noninfectious progeny virions (Figure 18). The addition of ritonavir enhances the concentrations of lopinavir that can be achieved following oral administration. It is given in combination with NRTI and/or NNRTI. Side effects of lopinavir/ritonavir include diarrhea, nausea, abdominal pain, and headache.

As lopinavir/ritonavir is a relatively new addition to the protease inhibitors; a complete understanding of resistance profiles will await its widespread utilization.

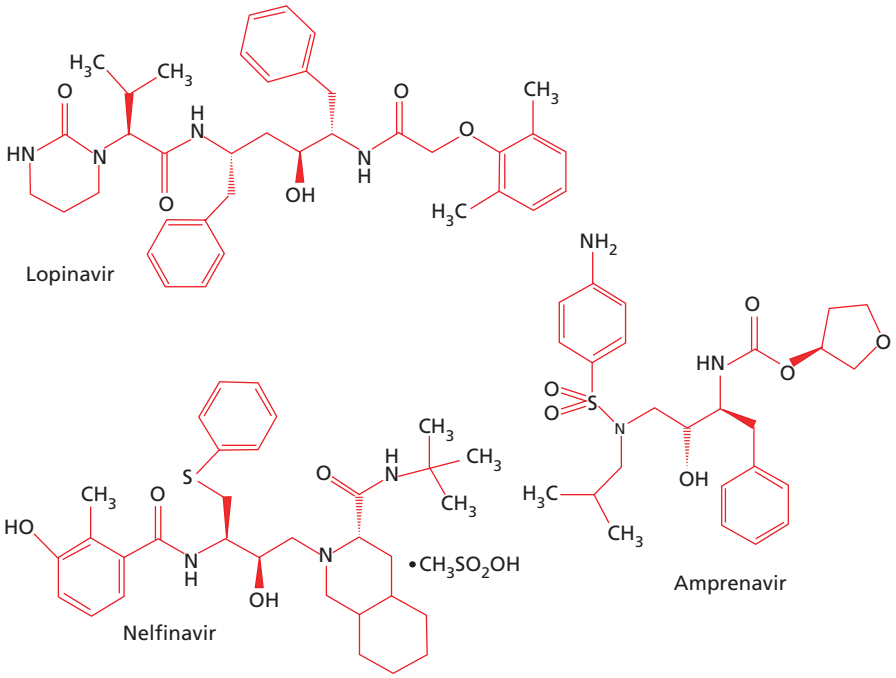

Figure 18 *Further examples of PI which are highly active against HIV*

Atazanavir (Reyataz)

Atazanavir (Figure 17) is a protease inhibitor. It prevents cells infected by HIV from producing new virus, reducing the amount of virus in the patient. It must be used in combination with at least two other anti-HIV drugs (Prober and Kimberlin 2005).

NUCLEOTIDE ANALOGUES

Tenofovir (tenofovir disoproxil fumarate) (Viread)

Tenofovir or disproxil fumarate salt (Figure 7) is an acyclic nucleoside phosphonate diester analogue of adenosine monophosphate. In vitro, the 50 percent inhibitor concentration for HIV is 0.04–8.5 μmol. After diester hydrolysis, tenofovir is phosphorylated to tenofovir-DP that then inhibits HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5-TP and, after incorporation into DNA, by DNA chain termination. Tenofovir DP is a weak inhibitor of mammalian DNApols α, β, and mitochondrial DNApol γ. Additive or synergic anti-HIV activity with nucleoside analogue, nonnucleoside analogue and protease inhibitors has been demonstrated in vitro. Side effects include lactic acidosis, hepatomegaly with steatosis, and diarrhea, (De Clercq 2002). Resistance is uncommon and occurs at codon 65.

Entecavir

Entecavir (Figure 19) is a cyclopentile guanosine nucleoside analogue with selective activity against hepatitis B viral DNApol. It is orally bioavailable, and due to a long terminal elimination half life of approximately 110 h is clearly suitable for once daily therapy.

HIV FUSION INHIBITORS

Enfuvirtide (Fuzeon)

The recent licensing of a fusion inhibitor introduces a new class of antiviral compounds for the treatment of HIV. T-20n (Figure 15) (Lalezari et al. 2003) is an inhibitor of fusion of HIV-1 with $CD4^+$ cells that consists of a 36 amino acid synthetic peptide with the N terminus acetylated and the C terminus is a carboxamide. Medication is administered subcutaneously.

Inhibitors of orthomyxo- and paramyxoviruses

EARLY PROGRESS WITH ION CHANNEL BLOCKERS

Amantadine and rimantadine

The compound amantadine (Figure 20) has long been known as a specific inhibitor of influenza virus A; influenza B and C are not affected. Clinical experience with amantadine and the chemically related rimantadine, dates from the early 1960s (Davies et al. 1964) and, although these compounds have lately been replaced, they represent major milestones in the history of the development of antiviral chemotherapy. The mechanisms of action of amantadine, and subsequently of rimantadine, which is very similar but with superior

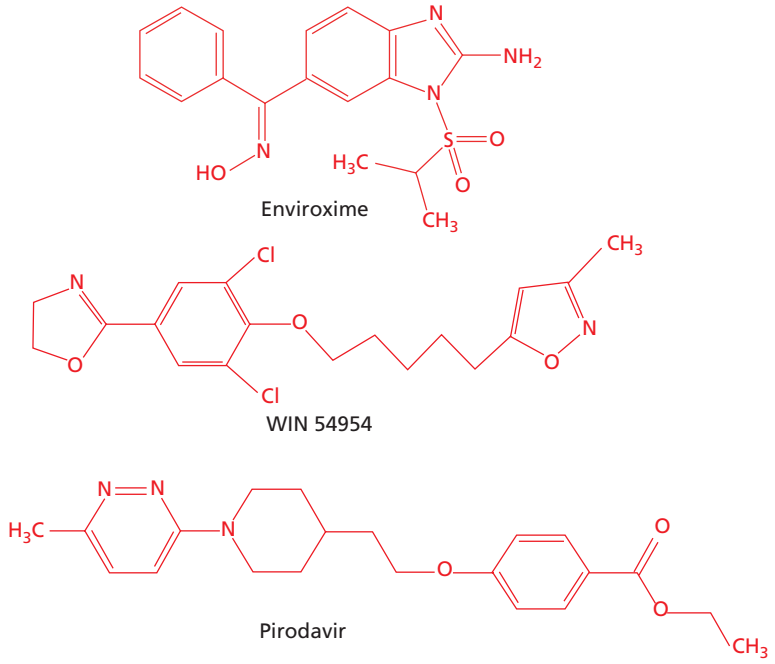

Figure 19 *Compounds with activity against the common cold – picornavirus inhibitors*

pharmacological properties (Hayden et al. 1981, 1985), were not elucidated until many years after they had been introduced into the clinic (Hay 1992).

Following attachment to host-cell plasma membrane receptors by means of the influenza envelope glycoprotein spikes, or hemagglutinin (HA), virus is taken up by cells into clathrin-coated pits. The virus, at this early stage of its replication cycle, is contained within lysosomes. Low pH within the lysosome results in conformational changes in the HA which exposes the amino-terminus region of the HA2; this region is hydrophobic and triggers a fusion between the endosomal membrane and the virus envelope, releasing the nucleocapsid into the cytoplasm of the host cell. Originally, amantadine was thought to work simply by raising lysosomal pH, thereby interfering with the conformational change leading to virus fusion with membrane. It now appears that the process of inhibition is more subtle. The M2 protein in the nucleocapsid seems to form a polymeric tube-like structure which behaves as an ion channel through which protons pass. Amantadine and rimantadine seem to act by binding to M2, possibly being inserted into the channel itself, and thus interfering with the penetration of hydrogen ions into the virion; a process essential for uncoating the single-stranded RNA genome (Wang et al. 1993). Resistant mutations map to the *M2* gene and such variants may ultimately limit the success of chemotherapy (Hayden 1994). Both amantadine and rimantadine have a basketlike hydrocarbon structure and further active compounds of this type are under study. The knowledge of how these compounds interact with the M2 proton channel should enable the rational design of further influenza inhibitors of this type.

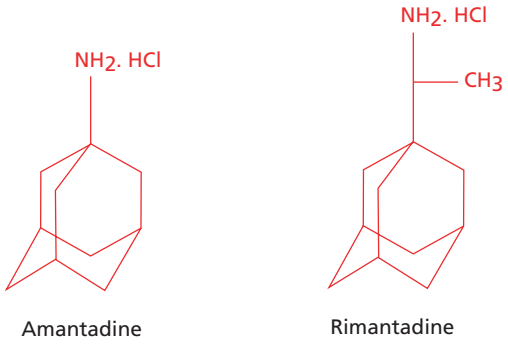

Figure 20 *Amantadine and rimantadine, early inhibitors of influenza which target the M2 protein*

The clinical efficacy of the prophylaxis and treatment of influenza virus infections with amantadine and rimantadine has been reviewed by Hayden (1997). The effectiveness of both compounds is limited by the adverse side effects, lack of activity against influenza B viruses, and rapid emergence of transmissible and pathogenic resistant viruses that remain transmissible and pathogenic emerge readily during clinical use (Hayden and Couch 1992; Monto and Arden 1992).

NEURAMINIDASE INHIBITORS

Zanamivir (Relenza) and Oseltamivir (Tamiflu)

While the study of HA dominated the early molecular characterization of influenza, it became apparent that

the influenza envelope is decorated with a second glycoprotein spike, much less numerous than HA, but with a distinctive long-stalked 'mushroom' shape. Neuraminidase (NA) was discovered to possess an enzymatic ability to cleave sialic acid from sialylated glycoproteins to yield free sialic acid. Known at first as sialidase, the term neuraminidase is now used universally. Sialic acid (Figure 21) is the receptor for the influenza virus on mammalian cells and interference with NA by mutations, anti-NA antibody or chemical compounds that bind to NA, all inhibit release of virus from infected cells. NA is also thought to have a role in the passage of virus through mucus to facilitate its colonization of respiratory mucosal cells.

An important molecule in research is Neu5Ac2en (Figure 21); this is a dehydrated neuraminidase acid derivative that mimics the geometry of the transition state during the enzymatic reaction. In the early 1990s the high resolution crystal structure of sialic acid and the transition state analogue Neu5Ac2en bound to influenza A and B NAs was solved. The NA active site contains some well-formed, large, and relatively rigid pockets that enable the rational design of molecules to interact with this virus component (reviewed by Kim et al. 1999). Several important inhibitors emerged from such studies. The guanidinyl group was substituted for a hydroxyl carbon atom of Neu5Ac2en to produce the compound 4-Guanidino-Neu5Ac2en (von Itzstein et al. 1993) or zanamivir (Relenza®; Figure 21). The polar zwitterionic properties of zanamivir give low oral bio-availability and, with rapid excretion, the compound must be administered intranasally or by inhalation. Another active compound introduced a cyclohexene ring and replaced a polar glycerol with lipophilic side chains (Tai et al. 1998; Sidwell et al. 1998; Mendel et al. 1998) to produce oseltamivir (Tamiflu®; Figure 22). This is an ethyl ester that is orally bio-available (30–100 percent) and is readily converted to the active carboxylate by esterases in the liver (Hitchcock 1996; Figure 22). Laboratory animal studies using infected mice and ferret models of influenza showed good dose-dependent antiviral effects and subsequently, both oseltamivir and zanamivir have proved successful when used prophylactically or therapeutically in clinical trials (reviewed by Oxford et al. 2002). Relatively few side-effects have been reported although a concern about zanamivir has been exacerbation of reactive airway disease (Cheer and Wagstaff 2002), however, compliance with the inhaler has been good. Oseltamivir causes some gastro-intestinal effects such as nausea. Several studies of prophylaxis among contacts in the workplace and among family members suggest a protective efficacy for zanamivir of 60–70 percent. The therapeutic use of either drug has been encouraging, and they have been shown to significantly alleviate the clinical signs of influenza (Oxford et al. 2002). Drug resistance mutations are detected following passage in cell culture. The mutations occur in both NA and HA hinting at functional interdependence between the two virus glycoproteins. However, the clinical data to date allow optimism. The incidence of resistance has been low compared to previous experience with rimantadine and where it does occur, resistance is

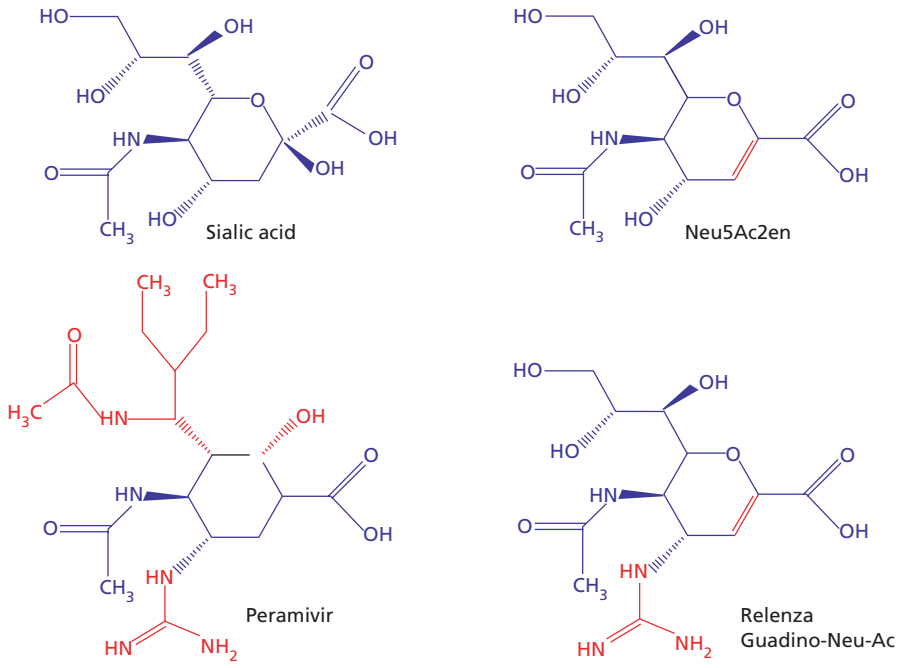

Figure 21 *The natural compound sialic acid showing its relationship to Neu5Ac2en and the first effective influenza virus neuraminidase inhibitor, relenza. Chemical modifications that vary from the natural compound are shown in red.*

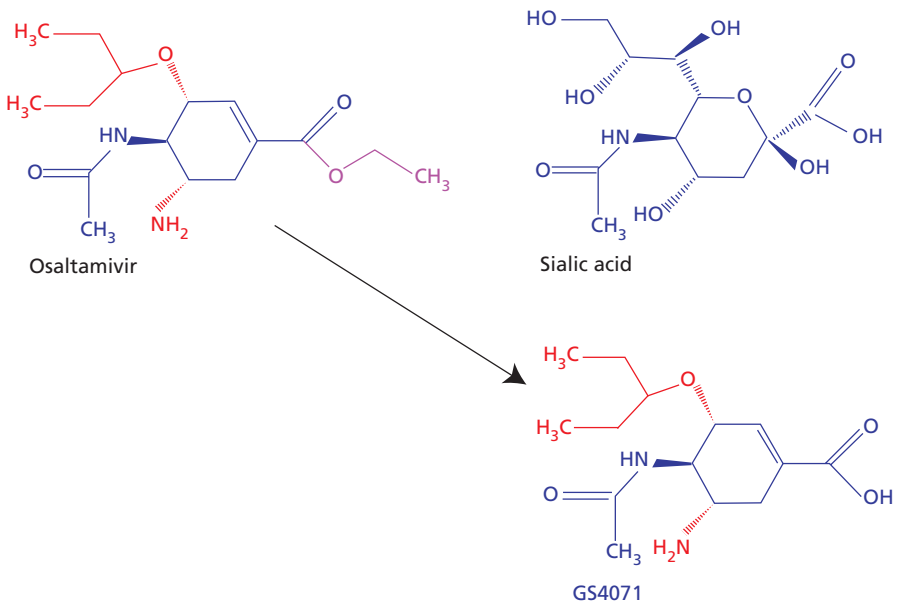

Figure 22 *Sialic acid and an orally bioavailable neuraminidase inhibitor, oseltamivir, showing its conversion to the active metabolite. The parts of the molecule shown in purple are removed by host enzyme attack to release the NA inhibitor in vivo.*

detected only transiently, arising late in infection and being cleared normally (Treanor et al. 2000). There is evidence that some of the mutants, at least, are attenuated in mice and ferret models (Herlocher et al. 2002). Thus early clinical data are very encouraging, however, the true potential of zanamivir, oseltamivir, and similar NA inhibitors now awaits the test of the next pandemic influenza outbreak.

Currently, the search for alternative NA inhibitors is active and it is likely that improved drugs will emerge. One novel compound is a dimerised zanamivir molecule that was suggested to be 100-fold more potent than zanamivir in vitro. It was shown to be retained longer in laboratory animal lungs compared with zanamivir, permitting much longer intervals between doses. Another Neu5Ac2en-derived analogue, peramivir (Figure 21) was also claimed to be more potent than zanamivir or oseltamivir in cell culture (Boivin and Goyette 2002). Phase II trials were promising, although Phase III less so, and currently development has stopped. Although these particular compounds may not be progressed, the preliminary results strongly suggest that the NA inhibitors presently in use have not achieved maximum efficacy and that more potent drugs in this class will become available in due course.

Ribavirin

Ribavirin 1-β-D-ribofuranosyl-1H-1,2,4-tiazole-3-carboxamide is an inhibitor of paramyxoviruses, particularly respiratory syncytial virus; however, clinical application has been controversial since its discovery was reported in 1972 (Sidwell et al. 1972). The compound is a guanosine analogue (Figure 15); evidence has been obtained for at least three distinct modes of action but the important molecular targets have yet to be confirmed (reviewed by Sidwell et al. 1985). It is a structural analogue of guanosine and is converted successively to ribavirin-MP, -DP, and -TP by cellular enzymes. The inhibition of virus is not, however, reversed by adding guanosine, suggesting a more complicated mode of action (Gilbert and Knight 1986). The enzyme inosine-MP dehydrogenase is effectively inhibited in vitro by ribavirin-MP, with a resulting decrease in pool size of GTP. However, this is not thought to be the single inhibitory factor. Ribavirin-resistant mutants have been very difficult, if not impossible, to isolate in the laboratory. This may be because the compound interferes with multiple virus targets; alternatively, there may be one or more important cellular effects that are not virus-specific. Clinically, aerosolized ribavirin has been reported to be efficacious for treating respiratory syncytial virus infection of infants (Sidwell et al. 1985); however, the results of more recent clinical trials cast doubt on the value of this agent (Wald et al. 1988). Ribavirin has proved to be a useful antiviral drug against several disparate virus families, e.g. hepatitis (see below). Paramyxoviruses cause a wide variety of human and animal diseases and there is a constant threat from new and dangerous viruses to emerge with pandemic potential. In molecular terms, the family has many similarities to the *Orthomyxoviridae* which includes influenza with similar potential targets for inhibition yet, to date, apart from ribavirin, to be screened there remains a paucity of effective compounds in development to combat members of the *Paramyxoviridae*.

As noted below, ribavirin is used in combination with interferon to treat HCV.

Inhibitors of hepatitis viruses

Several distinct viruses infect the liver and cause hepatitis. Furthermore, virus infections of this organ are prone to become persistent leading to chronic hepatitis and pathological sequelae including cirrhosis and liver cancer. Of the many hepatitis viruses discovered, to date, the most important are HBV and HCV. The former is a member of the *Hepadnoviridae*, a family of enveloped icosahedral viruses that contain a double-stranded, circular DNA genome and reverse transcriptase step in the replication cycle analogous to that in HIV. By contrast, HCV particles are nonenveloped and contain a small, positive single-stranded RNA genome. HCV is classified in the genus *Hepacivirus* within the family *Flaviviridae*. HCV does not replicate in conventional tissue culture systems and this has severely hampered research on potential inhibitors.

Both these viruses are major causes of human disease; there are estimated to be 300–400 million carriers of HBV worldwide (reviewed by Main et al. 1998) and some 170 million chronic HCV carriers (Walker et al. 2003), causing an estimated 10–12 000 deaths per annum in the USA alone. In both cases, chronic infection can lead to progressive liver disease, especially cirrhosis, with the increased risk of hepatocellular carcinoma. There is an effective subunit vaccine for HBV but this has no benefit for those already infected. The aim of antiviral chemotherapy is to clear the virus from the liver and thus prevent the life-threatening complications of chronic virus hepatitis and reduce the risk of transmission to others.

INTERFERONS

Since the early 1970s, interferon-α (IFN-α) has been used to treat chronic HBV and success rates of up to 40 percent have been reported for those who acquired infection as adults although treatment of carriers who acquired infection perinatally is less successful. Leucocyte and lymphoblastoid interferon (IFN), of both human and recombinant origin, have been reported as effective in decreasing viral replication in patients (Hoofnagle 1994). The mechanism of action is uncertain but may include the effects of increasing natural killer cell activity and enhancing MHC class-1 activity bearing in mind that hepatocytes naturally have little MHC -1 display. A typical regimen is 5–10 MU/m^2 three times weekly for 3–4 months. IFN-α treatment of Asian patients does not prevent the occurrence of cirrhosis-related complications and hepatocellular carcinoma (Yuen et al. 2001). For the treatment of HCV, like HBV, IFN-α monotherapy has enjoyed some success. Recently, PEG–IFN is now the preferred IFN molecule which is chemically modified form using mono-methoxy polyethylene glycol (PEG) or a branched PEG molecule. This modification has the effect of slowing IFN absorption, decreasing the rate of systemic clearance and produces an approximately 5–10-fold increase in serum half-life. For the treatment of HCV infection, PEG-IFN is used only in combination with ribavirin.

NUCLEOSIDE AND NUCLEOTIDE ANALOGUES

Apart from IFN-γ, there are currently several antiviral compounds in clinical use for the treatment of HBV. While treatment with several nucleoside analogues (e.g. fialuridine, ribavirin, and lamivudine) have been investigated (Honkoop and de Man 1995) only two are licensed: lamivudine and adefovir dipivoxil (Figures 7 and 13). The L-isomer of 3TC is more active against HBV replication and less cytotoxic than the D-isomer and is assumed to act on the HBV reverse transcriptase step and it may also inhibit the completion of double-stranded DNA. The drug is remarkably free of side effects but there is a high relapse rate, thus, after 6–9 months therapy, drug resistance develops due to mutations in the catalytic domain of the HBV polymerase gene. There is some evidence that the resistant viruses may be less replication-competent compared with the parental virus, however, resistant virus is thought to be responsible for the flare-up of hepatitis. Trials showed a rapid decrease in levels of HBV DNA in treated patients but a return to baseline at the end of therapy.

The nucleotide analogue adefovir dipivoxil (9-2-phosphonylmethoxyethyl) adenine (bis(POM)PMEA) (Figure 7) appears to be less susceptible to the development of resistance. The dose of adefovir is significantly lower than that used in studies of HIV infection where renal toxicity was observed. Whether this poses a problem on long-term administration remains to be determined.

COMBINATION THERAPY

Treatment of chronic HBV infection is associated with relapse on cessation of therapy and the emergence of drug-resistance. Following the success of combination therapy for HIV, the use of combinations of drugs to treat HBV and HCV have become common. IFN-α plus lamivudine or adefovir or the use of lamivudine and adefovir combinations are currently under investigation (Yuen and Lai 2003).

The combination of ribavirin with PEG–IFN-α-2a (rebetron) or PEG–IFN-α-2b, typically for 12–24 weeks, results in sustained virologic responses in the range of 40 to 50 percent, however, outcome is phenotype dependent.

Animal models for studying hepatitis virus chemotherapy

HBV and HCV are extremely difficult viruses to manipulate in tissue culture. Stably transfected lines of HepG2 cells can be used to study the replication of HBV; it is tetracyclin regulated. These cells can be used to screen and study inhibitors. However, much development work

for antiviral therapy has depended on the use of laboratory animal infection models. Chimpanzees provide reliable HBV and HCV models for evaluation of vaccines and therapeutic agents (Farci and Purcell 1993; Caselmann 1994; Purcell 1994) but severe restrictions are imposed on the use of nonhuman primates including ethical concerns, welfare considerations, and simply the very high cost of maintaining primates. Tree shrews (*Tupaia belangeri*) can be infected by inoculation with human serum positive with HBV and duck hepatitis has been studied in Pekin ducks. A virus related to HBV was discovered in woodchucks (WHV) that mimics human disease, including the progression of chronic liver infection to hepatocellular carcinoma. HBV is also studied in specially prepared mice: the HBV-Trimera mouse in which mice irradiated lethal total whole body irradiation reconstituted with severe combined immunodeficiency (SCID) mouse bone marrow cells. Human HCV- or HBV-infected liver tissue can be engrafted under the murine kidney capsule which then models the chronically infected tissue in vivo and is available for the study of therapeutic regimens (Ilan et al. 1999). Finally, the pestivirus bovine diarrhea virus (BVDV) is genetically related to HCV and has been used as a surrogate in cell culture system for evaluating anti-HCV compounds. This is currently an extremely active area of research among many academic institutions and pharmaceutical research laboratories. Likely progress will occur with the production of improved tissue culture systems and more convenient and reliable in vivo models to study the prevention and therapy of virus hepatitis; one of the most exciting major growth areas in antiviral therapy today.

Inhibitors of picornaviruses

PIRODAVIR AND PLECONARIL

The *Picornaviridae* are among the smallest of the RNA viruses and cause a broad spectrum of human and animal diseases. Among the *Picornaviridae* are the rhinoviruses. Scientists seeking to develop antiviral chemotherapy are often reproached with failure to 'cure the common cold.' An increasing number of compounds has been reported as potent inhibitors of rhinoviruses; they comprise an assorted collection that fall into two classes, with different antiviral targets. The first class (noncapsid binding) includes compounds such as enviroxime (Figure 19) (De Long 1984). Compounds of this type seem to act shortly after uncoating, inhibiting the formation of the virus RNA-dependent RNA polymerase complex. These compounds are no longer under investigation.

The second class of compounds (capsid binding) has a quite different target of inhibition. Picornaviruses possess a capsid comprising three types of protein subunit (VP1, VP2, and VP3) assembled into an icosahedral shell. The virions are not enveloped and therefore rely on receptor sites on their protein shell to engage and dock on to protein receptor molecules in the plasma membrane of the host cell. X-ray crystallographic studies revealed that attachment of the virus is mediated by a canyon-shaped receptor site which encircles each of the 12 pentameric vertices of the icosahedral capsid. Selective inhibitory compounds, e.g. pirodavir and WIN-54954, work by binding into a hydrophobic pocket (the 'win pocket') situated in VP1 beneath the canyon floor (Figure 23) (Rossmann 1990). Likely, this pocket forms a natural molecular hinge in the protein which may give flexibility to the molecule during disassembly and assembly of the icosahedral structure required when uncoating, and again during production of new virus particles. When the inhibitor is bound into the pocket it may have two effects. First it can interfere with virus attachment to the cell receptor. Secondly, and more important, it may interfere with uncoating (Woods et al. 1989; Andries et al. 1992).

The most promising antipicornavirus compound to progress into clinical development is known as pleconaril. It is a capsid-binding agent, structurally related to WIN 54954 (Pleconaril) (Figure 19) I n vitro, pleconaril inhibits the replication of most rhinoviruses (responsible for >50 percent of all common colds) and enteroviruses. Oral treatment has been studied in randomized double-blind, placebo-controlled trials and was found to produce significant benefits (Hayden et al.

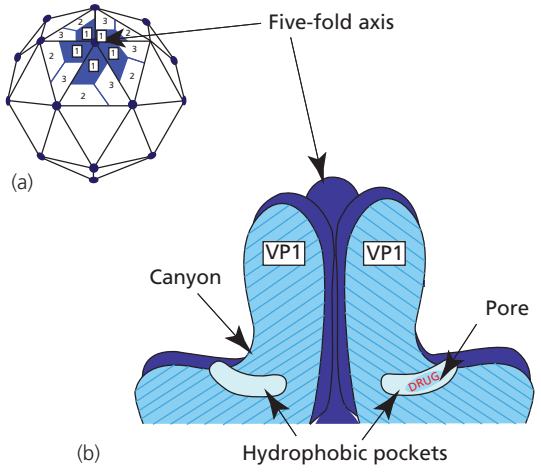

Figure 23 *The proposed mechanism of action for capsid-binding inhibitors of picornaviruses, including rhinoviruses.* **(a)** *Icosahedral capsid of a rhinovirus, depicting the arrangement of the three capsid proteins about a five-fold axis.* **(b)** *Three of the five VP1 proteins at the five-fold axis. A typical rhinovirus inhibitor is binding into the hydrophobic pocket at the base of the canyon, probably reducing the flexibility of the VP1 protein and affecting receptor binding and uncoating. An amino acid substitution in the region forming the pocket may lead to resistance because of the lost ability to bind drug. (Field and Goldthorpe (1985), with permission.)*

2003). Phase III clinical trials involving over 2 000 patients who took the drug showed early and substantial reduction in symptom severity and a 1 day reduction in disease duration. However, an unexpected problem was encountered; namely an interaction with oral contraceptives. Breakthrough bleeding was evident in 3 percent of women taking oral contraceptives during a 5 day course of pleconaril.

During 2002, the US Food and Drug Administration (FDA) rejected pleconaril (Picovir) as an antiviral drug being developed to treat the common cold on the grounds that this may encourage the development of resistant strains and may reduce the effectiveness of oral contraceptives (Senior 2002). Furthermore, a more recent 6 week prophylaxis study found that pleconaril induces cytochrome P-450 3A enzymes which metabolize a variety of drugs (Hayden et al. 2003).

Despite the encouraging early clinical data on the antiviral effects, the compound is unlikely to be widely used for the less severe upper respiratory virus infections at least until the pharmacological phenomena have been elucidated. It was hoped that the drug may be beneficial for the treatment of more serious picornavirus infections, however early data are less encouraging and a double-blind, placebo-controlled trial of pleconaril in infants suffering from enterovirus meningitis was inconclusive (Abzug et al. 2003).

Inhibitors of other DNA viruses

POXVIRUSES

Smallpox was officially declared to have been eradicated from the world in 1980 after a 10-year campaign of intensive surveillance and vaccination. Although smallpox has not afflicted humans for more than 20 years now, large quantities of the virus are thought to remain in storage and there is concern that this virus might be employed as a weapon of terrorism or biowarfare (Baker et al. 2003). The *Poxviridae* comprise several genera including *Orthopoxvirus* which contains variola (smallpox), vaccinia and monkeypox; *Capripoxvirus* which contains lumpy skin disease; and *Parapoxvirus* which contains orf of sheep and pseudocowpox of cattle. All these infections can infect humans and produce severe disease, especially when the subject is immunocompromised. For all these reasons there is a perceived need for effective antiviral chemotherapy. Historically, 1-methyl-isatin-β-thiosemicarbazones methisazone, Marboran (Bauer et al. 1969) (Figure 24) was among the first selective antiviral agents to be used in humans. It was used with some success during the 1960s to treat smallpox and the severe complication of vaccinia, namely *excema vaccinatum*. The mechanism of action was thought to involve interference with late virus protein synthesis (Hovi 1988). The further development of

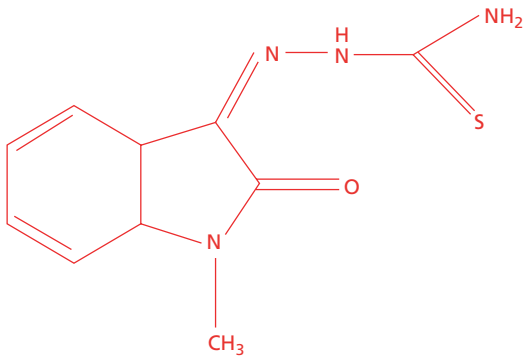

Methisazone

Figure 24 *An very early inhibitor of poxviruses – methisazone*

thiosemicarbazones was overtaken by the eradication of smallpox. Furthermore, modern criteria for laboratory assessment of compounds suggests that the potency of marboran is, at best, modest. Members of the acyclic nucleoside phosphonate family of compounds (see Herpesvirus section above) are found to be much more promising; in particular cidofovir and adefovir derivatives (Whitley 2003; Kern 2003). Beneficial clinical responses in severe orf infections in immunocompromised patients have been claimed in single patient studies (Geerinck et al. 2001). The potential for these compounds as inhibitors of poxviruses and a comprehensive catalogue of alternative antiviral strategies for prophylaxis and therapy of poxviruses is documented by Baker et al. (2003).

PAPILLOMAVIRUSES AND ADENOVIRUSES

Human papillomavirus infections most often cause genital warts or juvenile laryngeal papillomatosis. Interferons have been extensively evaluated for treating such infections (see Viral evasion of the host immune response) The local (intralesional) therapy of genital warts refractory to cytodestructive therapy is sometimes effective. The association between genital warts and cervical carcinomas provides further impetus for the development of antiviral agents to treat diseases associated with these viruses. The broad spectrum of action of the acyclic nucleoside phosphonates includes both HPV and adenoviruses. Success has been claimed in small studies (Stragier et al. 2002) providing some optimism for the clinical utility of this family of compounds in such severe cases that are relatively rare, but clinically very difficult to manage. The potential molecular targets offered by HPV for potential antiviral attack are reviewed by Phelps et al. (1998). There has also been much interest in the compound, 1-(2-methylpropyl)-1H-imidazo[4,5-C]quinolin-4-amine), given the generic name imiquimod (Figure 10) (Miller et al. 1995). Similar to resiquimod, the antiviral activity seems to result from interferon-α induction and, if so, would place this compound among the immunomodulators.

PROBLEMS IN ANTIVIRAL CHEMOTHERAPY

Virus evolution and resistance

A DEFINITION OF VIRUS DRUG RESISTANCE

As for all forms of microorganism, including eukaryotic cells, viruses can readily become resistant to inhibitors. This may be defined as: 'An acquired genetic change such that the virus is relieved from inhibition at drug concentrations that are normally inhibitory in the therapeutic range'. For viruses, this is always the result of one or more mutations in the virus genome.

Thus, drug resistance in viruses implies the emergence of strains for which the ED_{50} significantly exceeds the inhibitory concentration normally achieved in vivo (Field 1988b). When possible, a drug-resistant variant is compared directly with its drug-sensitive parent (Figure 25), but in clinical practice the latter may not be available. Criteria have been established to define acquired drug resistance which are particular to each family of virus and are related to the appropriate laboratory tests. Assays which detect mutations known to be associated with resistance are called 'genotypic tests' whereas 'phenotypic tests' are applied to infectious virus (Table 3). There are advantages and disadvantages associated with each type of test (Blaise et al. 2002). Virus families that are naturally insensitive to a compound should not properly be included within the definition of drug resistance.

REASONS FOR STUDYING DRUG RESISTANCE IN VIRUSES

Clearly, drug resistance is an important medical problem which may subvert otherwise successful therapy. However, the phenomenon also has some uses. The detection of resistant mutants is a very important criterion for demonstrating that a particular drug is selective for the target virus. Once drug-resistant mutants have been obtained, the location and nature of drug resistance mutations enables the identification of the target gene(s) in the virus and functional domains in target proteins to be defined at a molecular level. In turn, this enables patterns of cross resistance to be predicted and is often important in the development of further compounds with improved antiviral characteristics. Thus, the selection and study of drug-resistant variants plays a vital part in the development process of all new compounds

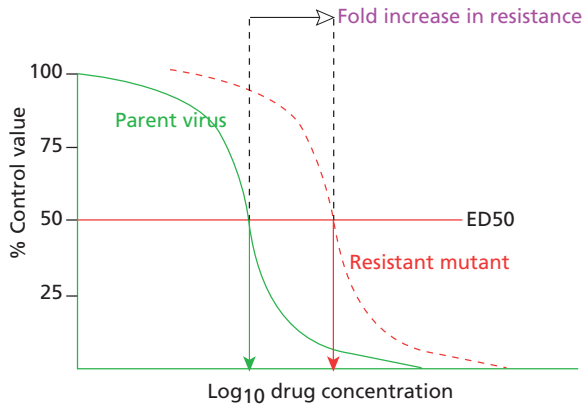

Figure 25 *Measurement of increase in resistance, e.g. when the ED_{50} of the mutant is 10 μg/ml and the parental strain is 0.1. This would represent an increased resistance of 100-fold.*

VIRUS EVOLUTION

Point mutations, or small groups of mutations, can readily lead to substantial increases in resistance to various specific antiviral drugs (Coen 1986; Field and Owen 1988; Mellors et al. 1995). Viruses are susceptible to natural mutations which may occur as a result of

- damage due to chemical mutagens, ionizing radiation cosmic rays etc
- enzymatic modification after replication
- errors by the polymerase during replication.

Of the three possibilities, the last is the most important. Some antiviral drugs are potential chemical mutagens, notwithstanding, most mutants appear to arise naturally and have a selective advantage in the presence of the inhibitor. Several types of mutation are possible: transition (A↔G, C↔T); transversion (A↔C↔G, A↔T↔G), insertion; and deletion. Insertions and deletions are particularly common when there are runs of identical nucleotides such as several consecutive guanosine residues, sometimes referred to as a 'G-string'. Insertions or deletions into such a sequence may introduce a frame-shift and disruption of the normal polypeptide.

VIRUS POLYMERASES ARE ERROR-PRONE

DNA viruses have a relatively low intrinsic error rate (1×10^{-7} to 1×10^{-8} errors per nucleotide). In double-stranded DNA viruses, a 3′–5′ exonuclease proofreading function can edit out errors giving an overall rate of $<1 \times 10^{-9}$. However, given the large number of viruses in an infected host, this is still sufficiently high for DNA viruses to evolve very rapidly relative to higher life forms.

RNA viruses have very high intrinsic mutation rates and there are no proofreading or repair mechanisms available. This high rate of mutation ($\geqslant 1 \times 10^{-4}$), coupled with a large population size, can quickly lead to enormous genetic diversity within a single infected host. For example, HIV has a single-stranded RNA genome of approximately 9 000 nucleotides. The replication rate in an infected individual has been estimated to be approximately 10^9 daily, thus, $10^{-4} \times 9\,000 \times 10^9 = 9 \times 10^8$ mutants occur each day. This means that, in theory, every point mutation occurs 10^5 times per day in an HIV-infected individual and every double mutant ten

Table 3 *Methods for detecting resistance in clinical specimens*

Advantages and disadvantages	
Phenotypic[a]	**Genotypic**[b]
Slow	More rapid
Cumbersome	Less cumbersome
Expensive	Less expensive
Clinically relevant	May not correlate with phenotype
May underestimate reResistance in vivo	May exaggerate resistance in vivo
May not detect mixtures	Insensitive to minor species

a) Usually tissue-culture based
b) Usually PCR-based

times per day! As a result, HIV actually exists as a 'quasi species' or 'swarm' around a particular consensus sequence.

PRIMARY, SECONDARY, AND COMPENSATING DRUG RESISTANCE MUTATIONS

Mutations that give rise to amino acid substitutions in, or close to, the site that interacts with the inhibitor and that are clearly associated with a biochemical change in the interaction leading to resistance, are termed 'primary mutations.' Such mutations characterize variants that arise early upon exposure to the inhibitor. Further mutations may accumulate – and these are termed 'secondary mutations' and these may contribute to the overall level of resistance. However, yet further mutations may appear which are apparently unrelated to the sites on proteins that interact with the drug. Such mutations may have no direct effect on biochemical drug sensitivity but may increase enzyme efficiency so as to compensate for the deleterious effects of the primary and secondary mutations. Another example has been observed in HIV where a mutation may change the protease cleavage site in the target protein that compensates for a change in the substrate specificity of the HIV protease itself due to a primary resistance mutations. Many compensating mutations are suspected but, in most cases, their precise role has yet to be elucidated (Swanstrom and Erona 2000).

BIOCHEMICAL VS CLINICAL DRUG RESISTANCE

From the earliest attempts at therapy of ocular herpesvirus, it was realized that treatment failure did not necessarily correlate with biochemical resistance in virus isolates. Conversely, patients carrying HIV with multiple drug resistance mutations may still respond to therapy; virus replication remains suppressed and immune function near normal despite the presence of highly resistant variants. There are likely to be several different explanations for these observations. First, the assay methods to measure resistance may not accurately reflect the conditions in the host, e.g. cell type (including the species/tissue of origin), the growth rate of cells (resting or multiplying), the complexity of media components (that may interfere with specific drugs), the multiplicity of infection, heterogeneous virus populations, and the existence of latently infected cells. Other possible host factors include noncompliance or changes in the patient that effect metabolism of the drug. Furthermore, the fact that many mutations may result in attenuation is probably a factor in resistance development by all types of virus (see below).

MATHEMATICAL MODELS TO PREDICT FUTURE RESISTANCE TRENDS

Based upon virus mutation rates (see above) alone, it would follow that virus infections would evolve to resist any specific inhibitor within a few days, rendering any drug useless. However, experience shows that this is rarely the case because the possession of drug resistance mutations usually has a biological price. For example, many mutations in the reverse transcriptase nucleotide binding site or protease active site in HIV or neuramidase of influenza are thought to reduce the catalytic activity of these enzymes and negatively affect replication and/or transmission efficiency. Similarly, HSV TK^{-ve} mutants are highly resistant to aciclovir; while such mutants replicate efficiently in tissue culture, normal TK activity is required for virus replication in neurons and to enable HSV to reactivate from latency. In some cases the attenuation may be reduced by compensating mutations although such effects are likely to be difficult to define.

VIRUS FITNESS

Some drug-resistant mutant viruses are clearly attenuated or 'less fit.' The biochemical changes resulting from mutations in the virus genome may, of course, be extremely subtle and the biological effects very difficult to measure. Much effort has been directed to the establishment of relevant laboratory tests to estimate attenuation (e.g. Weber et al. 2003). Certain constellations of changes may give rise to more successful drug-resistant viruses that will then dominate the population. These effects have been quantified by mathematical modelers. With increased understanding of virus pathogenesis and the mechanism of action of virus inhibitors, the

theoretical models have become increasingly sophisticated. Some predictions are optimistic, e.g. the likely future development of aciclovir resistance in genital herpes (Gershengorn et al. 2003), and influenza virus resistance to neuraminidase inhibitors (Ferguson et al. 2003) are both expected to remain low. However, for HIV, the predictions are more pessimistic and the expectation from the models is that, despite combination therapy, HIV drug resistance will eventually overtake the therapeutic effort. (Blower and Volberding 2002). However, this is a dynamic situation and viruses will continue to evolve. Resistance thus remains a threat for all types of antiviral therapy. The key general features of virus drug resistance are summarized in Table 4 and the methods for isolating and study of resistance in Tables 5 and 6.

DRUG RESISTANCE IN HERPES SIMPLEX AND VARICELLA-ZOSTER VIRUS

In the immunologically normal host

Historically, HSV was among the first viruses where the phenomenon of drug resistance was studied. A single passage of virus in the presence of a subinhibitory concentration of the compound (e.g. aciclovir, penciclovir or brivudin) results in significant decrease in sensitivity to the respective compound (Larder and Darby 1984). Clinical isolates, including those obtained from normal patients who have never received chemotherapy, contain a small proportion (perhaps 0.1 percent) of pre-existing drug-resistant mutants (Paris and Harrington 1982) that can continue to multiply when selective pressure is applied.

Table 4 *Acquired drug resistance*

Summary of general features
Results from one or more mutations in genes coding for target proteins
Develops spontaneously due to high mutation rates in viruses
Found in all viruses, for almost all classes[a] of specific inhibitor
Resistance is the hall-mark of selectivity for antiviral compound
Useful for elucidation of mechanism of drug action
Many resistance mutations alter virus pathogenicity (fitness)
Variants may be present among heterogeneous populations
Clinical importance varies among virus families
Incidence predicted from mathematical models
Especially important in immunocompromised host

a) Exceptions are immunomodulators and compounds with a cellular target for virus inhibition. It is arguable whether such compounds are true 'antiviral' compounds.

Table 5 *Methods for obtaining laboratory drug-resistant strains*

Methods
Passage of virus in presence of subinhibitory concentrations
Selection by using alternative drug with same target e.g. TK
Genetic recombination with resistant virus
Obtain molecular clone with induced mutation; recombine with wild-type
Obtain virus strains from clinical isolates following therapy

Table 6 *Sequence for the study of drug resistance to a new compound*

Sequence
Virus selected for resistance
Resistance demonstrated in tissue culture and, if possible, in animal model
Mutations mapped to specific gene
DNA sequence reveals: deletion, insertion, point mutation (homopolymers in TK)
Protein is analyzed and single amino acid substitutions/ truncations predicted
Enzyme shown to have lower affinity for substrate or be insensitive in vitro
Site-directed mutagenesis generate new mutants; then shown to be resistant
Patterns of cross-resistance to alternative drugs explored
Clinical isolates from treated patients shown with identical mutations

Selection for resistance in the laboratory

Aciclovir and similar nucleoside analogues were found to select for resistance mutations in two loci: DNApol and TK (Larder and Darby 1984; Coen 1986) (Table 7). Several laboratory mutants of each type shown in this table have been fully characterized (Larder and Darby 1984; Coen 1986). The mutant proteins (TK or DNApol) undergo changes in their enzymatic properties and DNA sequencing has identified the specific base substitutions of other changes that give rise to the drug-resistant phenotype; thus, the origin of resistance is fully explained (Collins and Darby 1991). The most common mutations are probably single G insertions into the G-string motif (Sasadeusz et al. 1997; Harris et al. 2003). Furthermore, several drug-resistant strains obtained from clinical isolates (almost all from immunocompromised patients following drug failure) proved to have genotypes identical to the previously characterized laboratory-selected strains (Larder and Darby 1984).

Herpesvirus DNApol is essential for virus replication and resistance mutations are generally single base substitutions that alter the affinity of the enzyme for nucleoside analogue triphosphates. Pyrophosphate analogues (e.g. foscarnet) also select for resistance mutations in DNApol. In contrast to DNApol, HSV TK is not essen-

Table 7 *Nature of herpes virus drug-resistant mutants so far isolated*

Genetic locus	Genetic change	Resistance	Frequency in clinical isolates
TK (TK^-)	Base: substitution, insertion or deletion	Little or no TK enzyme to phosphorylate thymidine or thymidine analogue	Relatively common (in mixtures) but usually attenuated
Mutations in promoter site			
TK (TK^r)	Base substitution	Altered TK substrate specificity; reduced phosphorylation of inhibitor but whole or partial phosphorylation of thymidine analogue	Quite rare
$DNApol^r$ ($DNApol^r$)	Base substitution	Low affinity for nucleoside analogue triphosphate	Very rare

tial for replication and TK^{-ve} viruses grow with normal kinetics in actively dividing cell cultures. In resting cells, however, TK^{-ve} viruses are less efficient and have restricted ability to replicate in neurons. Mutants with drug-resistance mutations in the TK gene may produce full-length polypeptides with base substitutions leading to altered enzyme substrate specificity (TK-altered) or the enzyme activity may be absent (TK-defective or TK^{-ve}). In laboratory animal infection models both DNApol and TK mutants of both types have been shown to be attenuated and, TK^{-ve} mutants show particularly marked reductions in neuropathogenicity.

In clinical practice, the incidence of resistant viruses is very low (<1 percent) in virus isolates obtained from immunocompetent patients. Large surveys measuring the sensitivity of virus isolates to aciclovir have been carried out regularly from 1979 to the present day and these show no significant changes in the mean sensitivity of isolates (ED_{50} approximately 1 μM). There is no evidence of increased incidence of resistance after more than 20 years use including oral prescription and widespread availability of topical formulations over the counter. Furthermore, daily administration to individual patients for more than 10 years for suppression of their recurrent disease has not been associated with resistance development. Recent surveys for penciclovir sensitivity among clinical isolates also show no evidence for increase in resistance (Bacon et al. 2002; Shin et al. 2003)

The explanation for the lack of resistance to HSV antiviral drugs in clinical practice appears to be that the majority of resistance mutations are of reduced pathogenicity. In cell culture, the kinetics and yields of such mutants are similar to those of their parental strains, but they are usually more or less attenuated when tested in animal models (Field et al. 1980, reviewed by Field 1989). It may be that the early establishment of neuronal latency with sensitive virus is another important factor (Field 2001).

Occasionally, TK^{-ve} viruses have been isolated that appear to be highly pathogenic in animal models. One such virus was recently elucidated. Sequencing showed that the TK^{-ve} phenotype resulted from a double G insertion into a G-string giving rise to a truncated polypeptide. However, this virus underwent a high frequency mutation event inserting a further G into the G-string thus restoring the open reading frame. Passage of this virus through the nervous system of mice resulted in rapid selection of the TK^{+ve} variant (Grey et al. 2003). Such viruses are likely to remain rare in nature, but this work reminds us of the potential for new and unexpected possibilities in the future.

Varicella-zoster virus resistance appears to follow the same pattern as HSV although there are no convenient rodent laboratory infection models to explore the pathogenicity of resistant strains as has been the case for HSV (Kimberlin et al. 1995). Currently, drug resistance is not regarded as a problem for either of these viruses except in immunocompetent patients.

Resistance to herpes simplex in the immunocompromised patient

Drug resistance to ACV has been encountered among immunocompromised patients, of whom several large surveys suggest that about 5 percent shed resistant virus (Wade et al. 1982; Englund et al. 1990). All three mechanisms of drug resistance have been observed (Table 3) and, in some cases, clinical isolates comprise mixtures of sensitive and resistant viruses, perhaps with more than one type of resistant virus present (Martin et al. 1985; Christophers and Sutton 1987; Sacks et al. 1989). Viruses that are resistant through lack of TK are, naturally, cross-resistant to other drugs that depend on virus TK for activation, whereas viruses of the TK^r phenotype show varying patterns of cross-resistance. Foscarnet selects for resistance only at the DNApol locus and is a possible alternative for therapeutic use when resistance develops to the nucleoside analogues. However, multiple drug resistance has occurred in some patients (Crumpacker et al. 1982). In summary, although drug resistance in the immunocompetent patient does not seem to be a problem at present, it may prove to be a very serious matter in the individual patient with defective immunity.

DRUG RESISTANCE IN HCMV

HCMV infection is one of the major virological problems in the immunocompromised patient and, unfortunately, drug resistance has emerged as a significant additional problem in these cases (Drew et al. 1991). Viruses that have acquired resistance to GCV have been encountered, and analysis of these strains led to the discovery of a hitherto unsuspected site of action of the drug (Drew et al. 1991; Drew et al. 1993). The drug-resistance mutations were traced to gene *UL97*, which, as mentioned under 'Ganciclovir', encodes a protein kinase function (Littler et al. 1992; Sullivan et al. 1992). Although this finding confirms a serious problem in these patients that renders the therapy ineffective, at a biochemical level it was an exciting discovery because it revealed a novel potential target for the design of inhibitors against this and other viruses (Birch and Mills 1994; Biron 1994). Relevantly, documentation of GCV resistance in transplant recipients may occur as frequently as 20 percent of the time.

Drug resistance in HIV

In contrast to the earlier experience with herpesviruses, antiviral drug resistance has emerged as one of the major problems in the management of HIV. Indeed, the study of resistance has had a central role in HIV drug development; it is the subject of numerous research communications and thus the subject has necessarily been covered in the consideration of HIV as a target for chemotherapy (see section on Inhibitors of human immunodeficiency virus above). HIV drug resistance is considered to be the most important factor limiting the continued effectiveness of all three classes of anti-HIV drug currently in use (RT inhibitors, nonnucleoside RT inhibitors, and protease inhibitors (Bean et al. 1987; Ljungman et al. 1990; Safrin et al. 1994) in patients undergoing monotherapy. This problem led to the introduction of the successful strategy of drug combinations in HAART.

At first it was difficult to show the development of resistance to the early drugs directed against HIV such as AZT. Unfortunately, this turned out to be due to a technical problem and, once the methods for detection were improved, virus drug resistance was revealed as a universal feature of all chemotherapeutic strategies so far employed against this infection.

Drug resistance in HIV results from point mutations leading to alterations in the affinity of reverse transcriptase for the inhibitor (the most important mutations are documented for individual HIV drugs in the section on Inhibitors of human immunodeficiency virus above) although this has, in many cases, been very difficult to prove by testing the enzyme functions for reduced sensitivity to inhibition in vitro. Development of resistance was first shown for zidovudine (Larder et al. 1989; Larder and Kemp 1989), and subsequently for all other nucleoside inhibitors. Resistance to the NNRTI apparently occurs even more readily and the resistant strains are cross-resistant (Richman et al. 1991; Condra et al. 1992). Resistance to the various proteinase inhibitors has also been clearly demonstrated (Otto et al. 1993; Kaplan et al. 1994; Mellors et al. 1995). Several hundred resistance mutations have now been documented and the sequential patterns of mutations are well understood (D'Aquila et al. 2003; Swanstrom and Erona 2000).

Particular mutations (and in some cases sequences of mutations) lead to progressively more resistant virus populations in the patient. Knowledge of resistance patterns to individual drugs enables rational strategies for combinations of drugs. The monitoring for resistance in individual patients has led to the possibility of tailoring therapeutic regimens to the patient. However, the success of triple or quadruple drug regimens has produced long-term suppression of virus growth in the patient and sustained normality of T-cell populations. Furthermore, it has come to light that among the heterogeneous population of virions in the patient, highly drug-resistant variants are present. The fact that the patient appears to be able to tolerate their presence may be explained by the fact that the resistant variants are of lower pathogenicity, i.e. less fit (discussed above).

A North American study (Little et al. 2000) showed that between 1–11 percent HIV infections were already resistant to one or more compounds when acquired and the proportion of new HIV infections involving resistance is increasing. Even taking into account the reduced fitness of some drug-resistant variants, mathematical models remain pessimistic, predicting that the long term future for the continued success of current antiviral agents is fairly bleak

DRUG RESISTANCE IN ORTHOMYXOVIRUSES

Influenza A virus has long been known to develop resistance to amantadine (Cochran et al. 1965; Oxford et al. 1970). Resistance was mapped first to the hemagglutinin and subsequently to the *M2* gene (Lamb et al. 1985; Hay et al. 1986) which codes for a component of the nucleocapsid. Of the two sites, changes in the M2 protein are the more important (Hay et al. 1985). Analyses of such drug-resistant mutants were of paramount importance in defining the mechanism of action of amantadine and similar compounds (Field and Owen 1988) (see section on Inhibitors of orthomyxo- and paramyxoviruses).

The rapid development of resistance following transmission to susceptible birds was reported following the experimental infection and therapy of chickens with highly virulent avian influenza virus causing fowl plague (Webster et al. 1986), and more recently a similar rapid development of drug resistance has been observed in humans infected with influenza A (Belshe et al. 1989;

Hayden et al. 1989, 1991; Hayden 1994). Although drug resistance is readily selected for in cell culture, there has been much debate on the significance of published surveys to assess the impact of resistance in humans to amantadine (Stilianakis et al. 1998).

The neuraminidase inhibitors zanamivir and oseltamivir select for resistance mutations in the neuraminidase gene and polymorphisms in the hemagglutinin may be important in determining the sensitivity of isolates (Gubareva et al. 2001a, 2001b). The new inhibitors were introduced into clinical practice in various parts of the world between 1999 and 2002. In order to monitor the potential development of resistance, the 'Neuraminidase Inhibitor Susceptibility Network' was established to coordinate testing of clinical isolates (McKimm-Breschkin et al. 2003). This has established a base line susceptibility of over 1 000 clinical isolates. It will enable accurate monitoring of resistance emerging in the future, particularly in the inevitable widespread use of anti-influenza virus therapy during an influenza pandemic. To date, resistance to the neuraminidase inhibitors appears to be limited in clinical practice and taken with the predictions from mathematical models (Ferguson et al. 2003), this allows cautious optimisms regarding the future impact of resistance in the treatment and prevention of influenza.

DRUG RESISTANCE IN HEPATITIS VIRUSES

Although there are relatively few data on clinical resistance compared to that for HIV, the development of resistance during HBV therapy appears to parallel that observed among retroviruses. Lamivudine treatment is associated with the rapid development of drug-resistance variants with specific polymerase mutations. Similarly, adefovir dipivoxil therapy is associated with the selection of mutations in the HBV polymerase, however, at a very low rate of occurrence (Angus et al. 2003). Compared with the monotherapy of HIV, resistance development is slower to emerge during HBV treatment, possible reflecting the slow rate of infected hepatocyte turnover which is significantly slower than infected lymphocyte turnover during HIV (Mutimer 1998). Similar to HIV, the most important approach is thought to be the introduction of combination therapy using two or more different drugs simultaneously. Treatment failures are also observed in HCV therapy by means of combinations of an antiviral drug with pegylated interferon. These failures are suspected to involve drug resistance although the mechanisms are, to date, unknown.

DRUG RESISTANCE IN PICORNAVIRUSES

The action of capsid-binding molecules that interact with rhinoviruses is readily reversed by the growth of mutants with point mutations in the genes coding for the capsid proteins concerned (Ninomiya et al. 1985). The patterns of cross-resistance have been widely studied (Yasin et al. 1990a) and in some cases, cross-resistance to the effects of neutralizing antibodies occurs (Dutko and Diana 1990; Rossmann 1990). Such antibodies also depend on capsid-binding at a nearby site in order to achieve neutralization. Although the study of such resistance is very useful in elucidating precise virus-inhibitor mechanisms, its implication for the future use of anti-picornavirus compounds of this type is far from clear. Resistance has the potential to arise very rapidly in vitro, and the unresolved question is whether such resistant variants will have impaired pathogenicity or reduced ability to spread. One trial suggested that this may indeed be the case (Dearden et al. 1989; Yasin et al. 1990b); however, there is as yet far too little information to predict the possible impact of drug resistance in the clinic of such compounds becoming widely used (Al-Nakib 1993). Pleconaril readily selects for drug resistant variants in tissue culture. There has been too little experience with the compound to determine whether or not this will be important in clinical practice. However, concern about resistance development has been cited among the reasons for the 2002 US Food and Drug Administration ruling that pleconaril should be rejected as an antiviral drug being developed to treat the common cold.

VIRUS DRUG RESISTANCE – CODA

Although drug resistance may be seen as a setback after many years of otherwise successful development of new drugs, it does have useful aspects: the isolation of resistant variants defines the compound as having a virus-specific mode of action; it enables the molecular targets for drug action to be identified with certainty; and, particularly when such targets are unsuspected, it may reveal hitherto unknown targets for the design of further inhibitors. Finally, when the precise mechanisms of drug resistance are elucidated, this information may provide a rationale for alternative therapeutic strategies to minimize or circumvent the impact of drug resistance in the patient.

The problem of toxicity of antiviral compounds

As stated at the outset of this chapter, the principal obstacle to the development of antiviral compounds has been to obtain selective toxicity. Having established both in in vitro and animal models that a compound has a high selective index, problems may be encountered when the compounds are administered to humans. In some cases the toxic effects may be caused by minor metabolites of the original compound.

Nucleoside analogues often cause a variety of acute effects, including malaise, weight loss, tremors, mutism,

peripheral neuropathy, acute liver and kidney damage as well as the more common complaints of nausea and vomiting. In some cases these effects have been related to the inhibition of cellular enzymes, particularly mitochondrial enzymes, by the antiviral compounds. (The problems encountered when treating patients with particular HIV drugs are considered in the section entitled Inhibitors of human immunodeficiency virus, above). Amantadine administration is associated with reversible neurological change (rimantadine is used successfully in the control of Parkinson's disease) and these effects are thought to relate to the release and uptake of dopamine in the central nervous system (Mizoguchi et al. 1994). To these problems must be added the long-term concern regarding possible teratogenicity and genetic damage caused by agents that interact with DNA (Griffiths 1995). The latter can theoretically occur when nucleoside analogues are incorporated into host-cell DNA or, indirectly, as a result of inhibition of DNA repair systems (Darby 1995).

'Familiarity breeds contempt' and there have been two incidences of toxicity in patients that resulted in deaths, reminding us of the need for constant attention to the potential that antiviral agents have for producing serious side-effects.

TWO EXAMPLES OF SEVERE TOXICITY PROBLEMS IN HUMANS

Fialuridine toxicity

Fialuridine (1-2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl-5-iodouracil; FIAU) is an orally bioavailable compound active against HBV (Fried et al. 1992). Pilot studies with a four-week course were encouraging and showed that significant reduction in virus replication could be achieved. However, further trials with a longer treatment period were stopped after 12 weeks when patients developed myopathy, lactic acidosis, peripheral neuropathy, pancreatitis and liver failure; there were several deaths (Meeting Report 1995). The mechanism for this unexpected delayed toxicity seems to be a consequence of FIAU incorporation into mitochondrial DNA.

Sorivudine, BVaraU: toxic interactions with 5-fluoruracil

Sorivudine, or BVaraU, is a potent inhibitor of VZV (see section on Inhibitors of herpesviruses) and the drug is currently undergoing clinical trials. It was licensed in Japan in 1993 but the license was withdrawn when several deaths occurred in patients also receiving 5-fluorouracil. The toxicity resulted from the formation of the metabolite bromovinyluracil (Ashida et al. 1993) which inhibits dihydrothymine dehydrogenase, normally responsible for degrading 5-fluorouracil. This drug interaction led to the accumulation of 5-uracil in cancer patients receiving both drugs, and thus to fatal bone marrow suppression.

The problem of virus latency

HERPESVIRUS LATENCY

Several viruses are capable of establishing latency in infected cells. This is a particular feature of all the herpesviruses, and both HSV and VZV can remain latent in neurons for the lifetime of the infected individual (Wildy et al. 1982). Periodic reactivations may cause clinical recurrences. In some cases they are subclinical but, whether or not disease is apparent, the infection may be transmitted during the episode to a susceptible contact. Because, during the latent phase, the virus does not express the genes coding for virus proteins, including TK and DNApol, it is unaffected by any of the conventional nucleoside analogues or drugs that rely on viral protein targets. Experimental infection in animal models suggests that latency is established early, and foci of latently infected neurons are detected a few hours after the first round of virus replication at the mucosal site of infection (Thackray and Field 2000). In man, the process of establishing latency may take a little longer, but the implication is that chemotherapy is unlikely to be started early enough to prevent the establishment of latency during a primary infection. Even if chemotherapy is successful in limiting the clinical signs of disease, the virus will persist, to reactivate later (Darby and Field 1984). However, both famiclovir and aciclovir do suppress recurrent infections and patients have taken suppressive therapy for this purpose over periods up to 10 years (Douglas et al. 1984; Straus et al. 1984; Goldberg et al. 1993; Mertz et al. 1997). Furthermore, prophylaxis can prevent episodes of herpes resulting from reactivation in immunosuppressed patients. The problem of eradicating herpesvirus DNA harbored in latently infected cells is one for which there remains no obvious solution however, the early establishment of latency and the subsequent role of the nervous system in the reactivation and recurrence of infection may underlie the very low incidence (discussed above in section entitled The problem of toxicity of antiviral compounds), of HSV drug resistance (Field 2001).

RETROVIRUS LATENCY

HIV can establish a different kind of latency in which the viral DNA, always formed as an intermediate during replication, may become stably integrated into the chromosome of the host cell. In some cases the cells survive infection and continue to harbor the viral genes, which are not expressed, or are expressed at a very low level, with the potential to become active later. Furthermore, the stimulation of resting lymphocytes is associated with the activation of latent HIV (Hermankova et al. 2003). HAART has been effective in reducing the plasma

levels of HIV-1 RNA below the limits of detection of most clinical assays. However, the infection has not been eradicated by HAART and this is likely due to latent HIV-1 replication-competent provirus in resting $CD4^+$ T-lymphocytes (Pomerantz 2003).

COMBINATION CHEMOTHERAPY FOR VIRAL INFECTIONS

As with the treatment of some bacterial diseases, especially mycobacterial infections, combinations of drugs have proven exceedingly valuable. The recognition that monotherapy of HIV infections has not resulted in a cure has led to numerous clinical trials evaluating various combinations of antiretroviral therapy (e.g. nucleoside analogues combined with nonnucleoside analogues and/or protease inhibitors) (Schinazi 1991, 1995). This has led to the concept of HAART. This strategy has proved extremely successful with suppression of virus to below the level of detection and a marked and sustained benefit (De Clercq 1998). However, the toxic effects of the chronic use of the current compounds remains a limiting factor in the medium to long term. Similarly, for life-threatening herpesvirus infections (e.g. encephalitis or neonatal herpes), combination therapies will be tested, including the combined use of drugs and immunoglobulins or cytokines, including interferons.

CONCLUSIONS AND FUTURE PROSPECTS

The rapid application of molecular biology to the study of viruses most assuredly will lead to the development of novel antiviral drugs. Already, the identification of protease in a variety of viruses other than HIV has led to a great increase in research directed toward the synthesis of peptidomimetics that inhibit these enzymes. Hepatitis C, HSV, CMV etc. are prime targets. For HSV alone, many genes provide targets for debilitation of viral replication; they include both those essential for viral replication and others that are not. The application of antisense RNA, triplex-forming oligonucleotides, ribozymes and direct DNA targeting, although intellectually appealing, poses drug delivery problems that have yet to be overcome in clinical practice.

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The emergence and re-emergence of viral diseases

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INTRODUCTION

The emergence of new diseases

New or previously unrecognized viral diseases are constantly being identified. In most cases, there is no way to predict when or where the next important new viral pathogen will emerge. Neither is there any way to predict the ultimate importance of a virus as it first emerges: it may be the cause of a geographically limited curiosity, of intermittent outbreaks of disease or of a new epidemic. New viral diseases seem to be emerging globally with increasing frequency, as suggested by published reports of cases, outbreaks, and epidemics and by the rate of identification of new pathogenic viruses (Mahy 2000). The list of newly emergent viruses of humans and animals is impressive (Figure 1; Tables 1 and 2).

DETERMINANTS CONTRIBUTING TO THE EMERGENCE OF NEW DISEASES

Many different factors can contribute to the emergence of a new viral disease. These include: (1) virological determinants such as mutation, recombination, reassortment, natural selection, fitness adaptation, and evolutionary progression; (2) individual host determinants such as specific risk behaviors, innate resistance, acquired immunity, and physiological factors such as age, nutritional status, and pregnancy; (3) host population determinants such as community, behavioral, societal, transport, commercial, and iatrogenic factors; (4) environmental determinants such as ecological and zoonotic influences; (5) viruses intentionally used for harm – the threat of bioterrorism. These determinants need to be understood if we are to develop control measures to prevent the occurrence and spread of specific diseases within human and/or animal populations. In this chapter we consider the underlying causes for emergence and re-emergence and illustrate them with selected examples.

Initial recognition of new, emerging diseases

Since 1986, following upon the development of the polymerase chain reaction (PCR) and other very sensitive, very specific, high-throughput detection and diagnostic

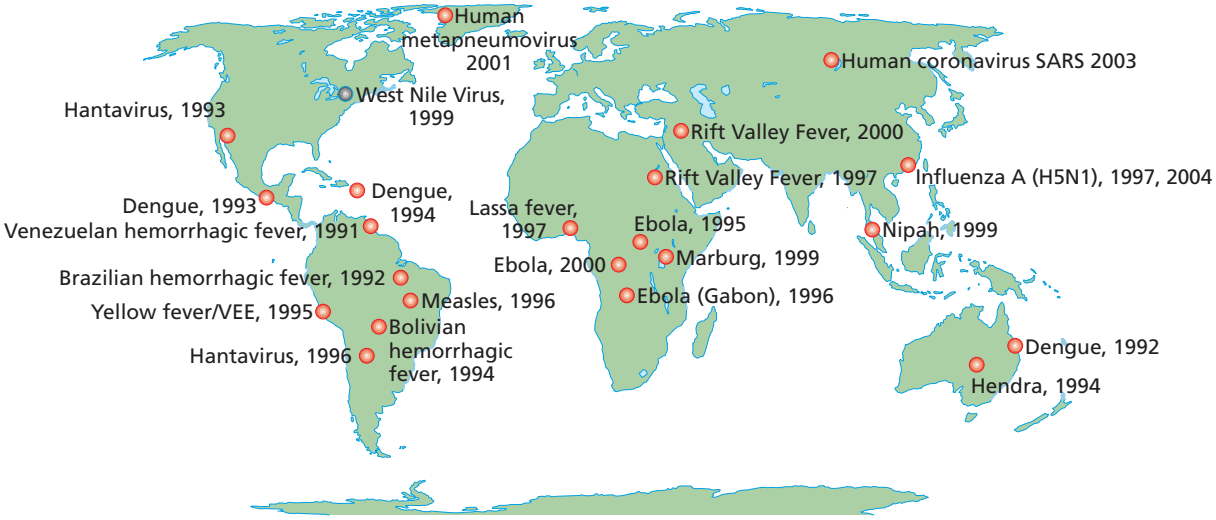

Figure 1 *Recent emergence and reemergence of human viral diseases, examples.*

methods, we have seen a dramatic increase in the direct detection of viruses, whether in diseased tissues, in cultured cells inoculated with clinical materials or in appropriate environmental samples. As a consequence, an impressive number of new, important viruses have been recognized (Table 3). In fact, some new viruses have been detected, described, and named solely on the basis of genomic sequences amplified by reverse transcription and polymerase chain reaction from postmortem tissues. For example, Bayou virus (a hantavirus distantly related to Sin Nombre virus, and an etiologic agent of hantavirus pulmonary syndrome in North America) is a case in point (Khan et al. 1995) – its significance as a human pathogen was determined entirely by indirect molecular methods.

As another example, the diversity of human papillomaviruses has been recognized solely by genomic nucleotide sequencing. The 100 or more human papillomaviruses have never been cultivated in cell culture or animals, and serotyping has not proven useful, so their pathogenic significance was not appreciated for many years. Since the advent of genomic sequence analysis, our appreciation of the importance of papillomaviruses has changed dramatically.

Human papillomaviruses can now be differentiated by sequence analysis, and exist in more than 100 recognized types in the human population (De Villiers et al. 2004) There is a clear causal relationship between certain papillomavirus types and genital cancers, especially cervical cancer (see Papillomaviruses). Papillomavirus genome DNA is a small circular molecule that is easily cloned from biopsy specimens of infected tissues. Originally these viruses were classified by DNA hybridization, and considered to be different types if they shared less than 50 percent homology. It was soon realized that certain types were frequently associated with cervical and other forms of genital cancer (for example types 16, 18, and 31), and other types were found in malignant respiratory papillomas (for example, types 6 and 11) so it was important to determine the human papillomavirus type as an aid in diagnosis. With the advent of simpler methods for DNA sequencing, some of the original hybridization data proved to be inconsistent, and a human papillomavirus type is now defined as new when the genome DNA is more than 10 percent dissimilar in the combined nucleotide sequences of 3 genes, E6, E7, and L1. This requires determination of 2.4 kbp of sequence (one-third of the genome) for each new isolate. More than 100 human papillomaviruses have now been recognized as types by this method, and divided into supergroups. For example, 54 papillomavirus types of genital origin form one supergroup (A) by sequence analysis, and another includes 24 types associated with epidermodysplasia verruciformis and other cutaneous lesions (Chan et al. 1995). Those human papillomavirus types that are presently known to be associated with a high risk of genital cancer are types 16, 18, 31–35, 51–52, 56, 58, 61, 66, 68, 70, and 73.

Another major advance in public health which stemmed directly from the application of molecular methods was the identification of hepatitis C virus. In 1989, as a result of a collaboration between CDC and Chiron Corporation to search for the cause of non-A non-B hepatitis, a new hepatitis virus, hepatitis C virus, was cloned from the blood of a chimpanzee that had been experimentally infected with blood factor VIII that was known to transmit non-A non-B hepatitis (Choo et al. 1989; Matsuura and Miyamura 1993). RNA extracted from the chimpanzee blood was reverse-transcribed to make cDNAs using random primers. The DNA was cloned in the bacteriophage gt 11 expression vector, and the resultant bacterial colonies were screened using sera from patients with non-A non-B hepatitis. After screening thousands of clones in this

Table 1 *Some of the most important new, emerging and re-emerging human viral pathogens*

Virus	Association of the virus(es) with human disease
Alkhurma virus[a]	Newly recognized member virus of the family *Flaviviridae*; the etiologic agent of severe hemorrhagic fever with a 25 percent mortality rate; tick-borne; first recognized in 1995 in Saudi Arabia where it has caused substantial morbidity and mortality
Australian bat lyssavirus	Newly recognized member virus of the genus *Lyssavirus*, family *Rhabdoviridae*; the etiologic agent of fatal rabies-like disease in humans and domestic animals; reservoir host fruit bats; found throughout eastern Australia
B-virus	Member virus of the family *Herpesviridae*; the common herpesvirus of macaques, especially rhesus macaques; the etiologic agent of severe, often lethal, encephalitis in humans; increasing as a threat because of increasing use of these animals in medical research
Crimean–Congo hemorrhagic fever virus[a]	Member virus of the genus *Nairovirus*, family *Bunyaviridae*; etiologic agent of severe hemorrhagic fever in humans with 10 percent mortality; tick-borne; reservoir in wildlife, sheep, and goats; widespread across Africa, the Middle East and Asia
Dengue viruses[a]	Member viruses of the family *Flaviviridae*; the etiologic agent of febrile disease with rash; mosquito-borne; the cause of millions of cases of febrile disease in the tropics and subtropics, and of dengue hemorrhagic fever (dengue shock syndrome), a life-threatening disease, especially in children
Ebola virus and Marburg virus[a]	Member viruses of the family *Filoviridae*; the etiologic agents of the most lethal hemorrhagic fevers known; zoonotic, but natural reservoir(s) unknown; there have been several recent substantial epidemics of Ebola hemorrhagic fever in central Africa
GB virus-C/hepatitis G virus	Newly recognized member viruses of the family *Flaviviridae*; disease association unproven, but there is a lifelong infection in humans and non-human primates world-wide
Guanarito virus[a]	Member virus of the family *Arenaviridae*; etiologic agent of Venezuelan hemorrhagic fever; rodent-borne
Hantaan virus and other Old World hantaviruses[a]	Member viruses of the genus *Hantavirus*, family *Bunyaviridae*; etiologic agents of hemorrhagic fever with renal syndrome; rodent-borne; an important cause of severe disease and death in Asia and Europe
Hendra virus	Newly recognized member virus of the genus *Henipavirus*, family *Paramyxoviridae*; etiologic agent of acute, often fatal, respiratory distress syndrome in humans and horses; zoonotic, reservoir host fruit bats (flying foxes); found in eastern Australia
Hepatitis C virus	Member virus of the family *Flaviviridae*; etiologic agent of hepatitis; associated with severe, chronic liver disease leading to hepatocellular carcinoma
Hepatitis delta virus	Member virus of the genus *Deltavirus*; etiologic agent is an unusual 'helper' virus; its presence makes hepatitis B more lethal
Hepatitis E virus	Member virus of the family *Picornaviridae*; etiologic agent of epidemic hepatitis, especially in Asia; recently recognized as widespread along the US–Mexico border; infection is associated with a high mortality rate in pregnant women
Human herpesvirus 8 (Kaposi sarcoma-associated herpesvirus)	Member virus of the family *Herpesviridae*; etiologically associated with Kaposi sarcoma; serologic studies show that HHV8 infection is tightly linked to KS risk; infection also associated with several uncommon lymphoproliferative syndromes in AIDS patients, including multicentric Castleman's disease and primary effusion lymphoma
Human herpesviruses 6 A and B and 7	Member viruses of the family *Herpesviridae*; etiologic agents of febrile illnesses in infants; HHV6B is the etiologic agent of roseola infantum (exanthem subitum); lifelong persistent/latent infections which may reactivate to cause disease in immunosuppressed people, typically AIDS patients and transplant recipients, sometimes culminating in rejection of transplanted organs and death
Human immunodeficiency viruses (HIV-1 and HIV-2)	Member viruses of the family *Retroviridae*; the etiologic agents of AIDS; the most important emerging infectious disease globally; still expanding in many parts of the world
Human metapneumovirus	Newly recognized member virus of the family *Paramyxoviridae*; the etiologic agent of respiratory disease in young children; first recognized in the Netherlands in 2001, now known to be distributed worldwide
Human papillomaviruses	Member viruses of the family *Papillomaviridae*; over 100 viruses, some etiologically associated with cervical, esophageal and rectal carcinomas

(*Continued over*)

Table 1 *Some of the most important new, emerging and re-emerging human viral pathogens (Continued)*

Virus	Association of the virus(es) with human disease
Human parvovirus B19	Member virus of the family *Parvoviridae*; etiologic agent of erythema infectiosum in children and a possible cause of fetal damage when pregnant women become infected
Human T-lymphotropic viruses (HTLV-I and HTLV-II)	Member viruses of the family *Retroviridae*; the etiologic agents of adult leukemia and neurological disease, especially in the tropics
Influenza viruses	Member viruses of the family *Orthomyxoviridae*; etiologic agents of influenza, causing thousands of deaths every winter in the elderly; the cause of the single most deadly epidemic ever recorded – the worldwide epidemic of 1918, in which 20–40 million people died; since 1997, highly pathogenic strains of avian influenza virus (H5N1, H7N1, and H9N2) have caused severe influenza in humans, with many fatalities, mainly in Asia, in persons in close contact with poultry
Japanese encephalitis virus	Member virus of the family *Flavivirus*; the etiologic agent of severe, often lethal, encephalitis; mosquito-borne; now spreading across southern Asia; potential for great epidemic and geographic expansion
Junin virus[a]	Member virus of the family *Arenaviridae*; etiologic agent of Argentine hemorrhagic fever; rodent-borne
Lassa virus[a]	Member virus of the family *Arenaviridae*; etiologic agent of Lassa fever; rodent-borne; a very important disease in West Africa; imported into a Chicago hospital in 1990
Machupo virus[a]	Member virus of the family *Arenaviridae*; etiologic agent of Bolivian hemorrhagic fever; rodent-borne
Measles virus	Member virus of the family *Paramyxoviridae*; the etiologic agent of the systemic disease measles; re-emerging in adolescents and young adults in many countries due to waning immunity; re-emerging in children in some countries due to poor vaccine coverage and infection in infancy before vaccination
Monkeypox virus	Member virus of the family *Poxviridae*; etiologic agent of vesiculo-pustular disease resembling a mild form of smallpox; repeated outbreaks in west Africa; reservoir in African squirrels; in 2003 mammals imported from Ghana into the USA transmitted the virus to prairie dogs from which an outbreak of monkeypox occurred in humans who had contact with infected prairie dogs
Nipah virus	Newly recognized member virus of the genus *Henipavirus*, family *Paramyxoviridae*; etiologic agent of severe, often fatal, encephalitis; zoonotic, reservoir in fruit bats; epidemic in Malaysian peninsula in 1999 with more than 100 human fatalities; since 2003 has caused outbreaks with high fatality rates in India and Bangladesh
Noroviruses	Member viruses of the family *Caliciviridae*; etiologic agents of explosive outbreaks of diarrhea and enteric disease throughout the world; increasingly associated with contaminated drinking water in institutional settings (schools, child care facilities, nursing homes) or other group settings (banquet halls, cruise ships, campgrounds)
Polioviruses	Member viruses of the family *Picornaviridae*; etiologic agents of poliomyelitis; still an important problem in developing countries of Africa and Asia; targeted by WHO for worldwide eradication by the year 2006
Rabies virus	Member virus of the genus *Lyssavirus*, family *Rhabdoviridae*; the etiologic agent of fatal encephalitis in humans and many animals; zoonotic, transmitted by the bite of rabid animals; raccoon epidemic still spreading across eastern USA; still an important human disease with ~30 000 human deaths per year globally
Rift Valley fever virus[a]	Member virus of the genus *Phlebovirus*, family *Bunyaviridae*; etiologic agent of febrile disease with hepatitis and hemorrhagic fever; mosquito-borne; the cause of one of the most explosive epidemics ever seen in Africa; recently spread from Africa to the middle East
Ross River virus	Member virus of the family *Togaviridae*; etiologic agent of febrile disease and epidemic polyarthritis; mosquito-borne; has moved from Australia across the Pacific region several times
Rotaviruses (Group A, B, and C rotaviruses)	Many member viruses of the genus *Rotavirus*, family *Reoviridae*; etiologic agents of diarrhea / enteric disease; the second leading cause of death among infants in the world; emergent when sanitary conditions deteriorate
Sabía virus[a]	Member virus of the family *Arenaviridae*; etiologic agent of Brazilian hemorrhagic fever; rodent-borne; high mortality rate, including two laboratory-acquired cases

(*Continued over*)

Table 1 *Some of the most important new, emerging and re-emerging human viral pathogens (Continued)*

Virus	Association of the virus(es) with human disease
SARS coronavirus	Newly recognized member virus of the family *Coronaviridae*; etiologic agent of severe acute respiratory distress syndrome; worldwide epidemic originating in China in 2002
Sin Nombre virus and other New World hantaviruses	Member viruses of the genus *Hantavirus*, family *Bunyaviridae*; Sin Nombre virus is the etiologic agents of hantavirus pulmonary syndrome; rodent-borne
Variant Creutzfeldt-Jakob disease prion	BSE prion; etiologic agent of invariably fatal spongiform encephalopathy; zoonotic, originating as the BSE prion; more than 150 cases in the United Kingdom in recent years
Venezuelan encephalitis virus	Member virus of the family *Togaviridae*; etiologic agent of febrile disease and encephalitis; mosquito-borne; the cause of recent major epidemics in Central and South America
West Nile virus	Member virus of the family *Flaviviridae*; etiologic agent of febrile disease and encephalitis; mosquito-borne, introduced into New York in 1999, now spread throughout the USA, north to Canada and south to Mexico and beyond
Yellow fever virus[a]	Member virus of the family *Flaviviridae*; etiologic agent of hepatitis and hemorrhagic fever; mosquito-borne; one of the most deadly diseases in history

a) The viruses that cause hemorrhagic fever in humans.

way, one was found that reacted with antibodies in the sera of infected patients but not controls. DNA from the positive clone was then used to screen other clones by DNA hybridization, and eventually the full length sequence of hepatitis C virus was determined. This enabled the development of specific diagnostic tests, including enzyme immunoassays that could be used to screen the blood supply, largely eliminating transfusion-associated transmission of hepatitis. We now recognize multiple genotypic variants of the original hepatitis C virus (see Hepatitis C virus).

Since the discovery of hepatitis C virus, additional viruses with similar characteristics (member viruses of the family *Flaviviridae*), have been found using molecular techniques (Muerhoff et al. 1995; Linnen et al. 1996). These viruses, known as GB virus A, GB virus B, and GB virus C/hepatitis G virus, appear to be widespread in human and nonhuman primate populations (Robertson 2001) but so far no human disease has been definitively ascribed to their presence (Mushahwar 2000). The search for possible new viruses causing hepatitis has revealed yet another new class of viruses, currently termed anelloviruses and typified by TT virus (Nishizawa et al. 1997). These viruses are ubiquitous in human and nonhuman primate populations, and consist of a single-stranded DNA genome of negative sense, about 3 800 nucleotides in length (Mushahwar et al. 1999; Hino 2002). So far, no specific disease association has been found for these viruses, but infection may alter the severity of other infectious diseases, such as gastritis caused by *Helicobacter pylori* (Maggi et al. 2003) (see TT virus and other Anelloviruses).

It should be emphasized that although molecular techniques may be crucial to the initial discovery of a new, emerging virus, classical techniques still have their place. For example, virus isolation in cell culture or even in experimental animals followed by characterization by histopathology, electron microscopy, immunofluorescence, and other specialized microscopy technologies still find their place in the discovery and initial characterization of new viruses. A case in point is the epidemic of severe acute respiratory syndrome, known as SARS (see Coronaviruses, toroviruses, and arteriviruses), which first appeared in China in November 2002 and then spread via Hong Kong to many countries in 2003. The importance of this epidemic and the interactive methods used to identify its cause provides lessons worthy of more detail.

An outbreak of severe pneumonia was first recognized in Guangdong Province, China, in November 2002, initially with 300 cases of unknown etiology and five deaths. This was reported to the World Health Organization (WHO) in February 2003, and initially it was thought that these pneumonia cases might be due to a novel influenza virus.

Within the next two weeks, a WHO official working in Hanoi, Vietnam reported an unusual case of severe acute respiratory disease to WHO, and this disease was soon recognized in a large number of sick health care workers in Hong Kong and Vietnam. WHO issued a global alert about these cases, and on March 14th cases of a similar disease syndrome were reported from Canada.

Over the next few weeks it became clear that all these cases of severe respiratory disease could be linked to a single hotel in Hong Kong where the index case, a 65-year old doctor from Guangdong province in China, had stayed one might before his admittance to a hospital. Eventually the disease spread from this hotel to eight countries worldwide. These countries included Vietnam, Singapore, Canada, Hong Kong, USA, Ireland, Germany, and Thailand. Cases were also exported to France from Vietnam. Virus isolated from patients was grown in Vero (African Green monkey kidney) cells, and when examined by electron microscopy was found

Table 2 *Some of the most important new, emerging and re-emerging viral pathogens of animals*

Virus	Association of the virus(es) with animal disease
African horse sickness viruses	Member viruses of the genus *Orbivirus*, family *Reoviridae*; etiologic agents of acute respiratory distress syndrome, myocarditis and massive edema in horses with a 95 percent mortality rate; zoonotic with some human cases of febrile disease; mosquito-borne; an historic problem in southern Africa; a major threat to horses world-wide
African swine fever virus	Member virus of the family *Asfarviridae*; etiologic agent of systemic febrile disease with very high mortality rate in swine; tick-borne and also spread by contact; endemic in Africa and recently has been present in Europe and South America; a major threat to commercial swine industries globally
Avian influenza viruses	Member viruses of the family *Orthomyxoviridae*; etiologic agents of avian influenza; very high mortality rate associated with some virus strains; spread by wild birds; a major threat to commercial poultry industries globally
Bluetongue viruses	Member viruses of the genus *Orbivirus*, family *Reoviridae*; etiologic agents of systemic febrile disease; culicoides-borne; the isolation of several strains in Australia became an important non-tariff trade barrier issue in the 1980s
Bovine spongiform encephalopathy prion	BSE prion; etiologic agent of invariably fatal spongiform encephalopathy; first recognized in 1986; the cause of a major epidemic in cattle in the UK, resulting in major economic loss and trade embargo; zoonotic, causing fatal variant Creutzfeld-Jakob disease (vCJD) in humans
Canine parvovirus	Member viruses of the family *Parvoviridae*; etiologic agent of systemic disease, with diarrhea and enteric disease, cardiomyopathy; a new virus in the late 1970s, originating by mutation from feline panleucopenia virus; the virus rapidly swept round the world in the 1980s causing a pandemic of severe disease in dogs; now has become endemic globally
Chronic wasting disease of deer and elk prion	CWD prion; etiologic agent of invariably fatal spongiform encephalopathy; discovered in captive animals in the USA and now spreading in captive and wild deer and elk in the USA and Canada; the question of whether this disease might be zoonotic is raising concern and prompting appropriate research
Dolphin, porpoise, and phocine (seal) morbilliviruses	Member viruses of the family *Paramyxoviridae*; the etiologic agents of epidemic respiratory disease in several species of marine mammals; first identified in 1988 in European seals; emerging pathogens endangering several marine species
Feline immunodeficiency virus and Simian immunodeficiency viruses	Member viruses of the family *Retroviridae*; etiologic agent of AIDS-like syndrome; important new viruses, the one affecting cats in nature and all serving as important models in AIDS research
Foot-and-mouth disease viruses	Member viruses of the family *Picornaviridae*; systemic vesicular disease; still considered the most dangerous disease of livestock in the world because of the great capacity for rapid transmission and great economic loss; still entrenched in Africa, the Middle East, and Asia; caused a devastating epidemic in the United Kingdom in 2001
Hendra virus	Newly recognized member virus of the genus *Henipavirus*, family *Paramyxoviridae*; etiologic agent of acute, often fatal, respiratory distress syndrome in horses and humans; reservoir host fruit bats (flying foxes); found in eastern Australia
Malignant catarrhal fever virus (Alcelaphine herpesvirus 1)	Member virus of family *Herpesviridae*; etiologic agent of systemic disease with fever and acute inflammation of nasal and oral membranes and involvement of pharynx and lungs; usually fatal; etiologic agent is silently carried by certain African wildlife species such as gnu, wildebeest, and hartebeest; it can occur in zoos, and its presence is an important non-tariff trade barrier issue throughout the world
Monkeypox virus	Member virus of the family *Poxviridae*; etiologic agent of vesiculo-pustular disease resembling a mild form of smallpox in wild and captive monkeys in contact with squirrels and other small rodents in Central and West Africa; reservoir in African squirrels; in 2003 mammals imported from Ghana into the USA transmitted the virus to prairie dogs from which an outbreak of monkeypox occurred in humans who had contact with infected prairie dogs

(*Continued over*)

Table 2 *Some of the most important new, emerging and re-emerging viral pathogens of animals (Continued)*

Virus	Association of the virus(es) with animal disease
Myxoma virus	Member virus of the family *Poxviridae*; etiologic agent of systemic exanthematous disease in rabbits; had been used to control rabbits in Australia, but with diminishing success; a damaging virus in commercial rabbit industries in some countries
Nipah virus	Newly recognized member virus of the genus *Henipavirus*, family *Paramyxoviridae*; etiologic agent of encephalitis and respiratory disease in swine; reservoir in fruit bats; massive epidemic in swine in southern Asia in 1999; zoonotic, with often fatal encephalitis in humans in contact with infected swine
Porcine reproductive and respiratory syndrome virus (PRRS virus)	Member virus of family *Arteriviridae*; etiologic agent of respiratory disease and reproductive tract disease; an important disease in commercial swine industries of several countries, with major economic impact
Rabbit hemorrhagic disease virus	Member virus of family *Caliciviridae*; etiologic agent of universally fatal hemorrhagic disease in rabbits; being used to control rabbits in Australia
Rinderpest virus	Member virus of the family *Paramyxoviridae*; etiologic agent of systemic disease with very high mortality rate in cattle and many other species of cloven footed animals; still considered very dangerous, with potential for causing great economic loss; still entrenched in limited areas in Africa and Asia; target of ongoing global eradication campaign
West Nile virus	Member virus of the family *Flaviviridae*; etiologic agent of febrile disease and encephalitis in birds and mammals, especially horses; mosquito-borne; in 1999 a particularly virulent strain from Israel moved to the USA, where it has caused thousands of deaths in crows and other passerine birds as well as horses; zoonotic, has become very important human pathogen

to resemble a coronavirus. Cultures of the virus showed immunofluorescence when reacted with serum from recovered patients (Ksiazek et al. 2003; Drosten et al. 2003). Subsequently, knowing that it was a coronavirus by electron microscopy, consensus primers were used in PCR amplification, and the complete genetic sequence of the virus was determined by several groups (Rota et al. 2003; Marra et al. 2003; Ruan et al. 2003; Thiel et al. 2003). Despite the availability of this sequence information, numerous studies have so far not determined the precise origin of the SARS coronavirus, although it is speculated that it arose from an animal reservoir, perhaps in palm civets (Guan et al. 2003).

THE VIRUS: VARIATION AND THE EMERGENCE OF NEW VIRAL PHENOTYPES

The variety and diversity of viruses

As knowledge of the molecular structure and replication of viruses has increased, we have learned that the viruses that we know to be responsible for human and animal diseases represent only a small fraction of a large, diverse global virus population. Currently, some 3 600 virus species are recognized taxonomically, but international reference centers and culture collections keep track of more than 30 000 viral strains (Murphy et al. 1995). This number continues to increase because each time a potential host species or a new disease is studied in detail, new viruses are found. In addition, clues to the existence of new viruses may sometimes be found in other species (ecological niches). For example, the picobirnaviruses, with a genome of two segments of double-stranded RNA, 2.6 and 1.5 kb in length, were first found in rat feces (Pereira et al. 1988), and only later in humans, where they may be a cause of diarrhea (Grohmann et al. 1993; Gallimore et al. 1995).

Polygenic basis for viral variation

There are several genetic mechanisms that drive virus evolution: discussed below are mutation, recombination, and reassortment of genes. However, many of the most important variances among viruses are polygenic, and of ancient derivation, the result of the natural selection of the most fit variants (Nathanson et al. 1993). A classic example of such variation was the definition many years ago of smallpox virus variants, variola major (Indian subcontinent and Europe, mortality up to 30 percent), and variola minor (South America, mortality about 1 percent) (Fenner et al. 1988). Another example was naturally occurring attenuated poliovirus variants, some of which inspired the development of Sabin live-attenuated polio vaccines (Sabin 1981). Only recently has it been possible to begin to explain such phenotypic differences from a molecular genetic perspective

Mutation

The replication of viruses involves copying the genome nucleic acid millions of times; mistakes in the copying process introduce mutations (see The origin

Table 3 *Emerging human viral diseases: recognition by molecular techniques*

Virus	Disease	Method
Human papillomaviruses (more than 100 types)	Warts, anogenital carcinomas, laryngeal papillomas, and carcinomas	Cloning and restriction fragment analysis or sequencing of viral genomic DNA from infected tissues
Hepatitis C virus	Hepatitis, often chronic, leading to hepatocellular carcinoma	Cloning, sequencing, expression, and immunoselection with specific sera
Hepatitis E virus	Acute epidemic hepatitis	Cloning, sequencing, expression, and immunoselection with specific sera
Human herpesvirus 8	Kaposi sarcoma	Representational DNA difference analysis (RDA) of normal vs. sarcoma tissue
Hepatitis G virus/GB virus-C	Unknown	Cloning, sequencing, expression, and immunoselection with specific sera
Human metapneumovirus	Respiratory disease in children	Random primer polymerase chain reaction
GBV-A, GBV-B	Unknown	Representational difference analysis
GBV-C – Hepatitis G virus	Unknown	Cloning, sequencing, expression, and immunoselection with specific sera
Sin Nombre virus	Hantavirus pulmonary syndrome	Reverse transcription and consensus sequence polymerase chain reaction amplification from infected human and rodent tissues
Bayou virus	Hantavirus pulmonary syndrome	Reverse transcription and polymerase chain reaction amplification from infected human tissues and sequence analysis
TT virus	Not known	Representational DNA difference analysis of human sera

and evolution of viruses). When such mutations lead to new phenotypic characters that enable the virus to replicate in a new host or cell type, to replicate to a higher titer or at a faster rate, or to better escape host defenses, the potential exists for emergence of a new viral disease. Genetically, mutational changes affecting phenotype may be minimal, involving one or a few nucleotides, or they may be large and complex, involving the addition of new genes into viral genomes by recombination or reassortment (see below). In any case, it is the expression of genotypic change as a new phenotype that is important in nature, and usually it is phenotypic change affecting transmission that is at the heart of the emergence of a new disease. Mutations that affect antigenic determinants on the viral surface proteins may be selected for, especially when viruses replicate in the presence of antibody. This has been clearly documented in the case of influenza. New antigenic variants of influenza viruses occur so pervasively that reformulation of vaccine is required on an annual basis.

The emergence of influenza virus variants by the accumulation of point mutations is termed genetic drift (see Orthomyxoviruses: influenza). Such mutations may dramatically alter viral pathogenicity, but more importantly they may result in new viral surface epitopes that the population does not recognize, when it is termed antigenic drift. An example of genetic drift occurred in 1983, when an outbreak of avian influenza A virus (H5N3) in Pennsylvania spread through the poultry industry of the region, leading to the destruction of 17 million fowl and a loss of about US$60 million. When the virus was sequenced, it was found to have acquired a single point mutation that altered the cleavability of its hemagglutinin, thereby greatly increasing its pathogenicity.

A dramatic example of the emergence of a new virus by mutation is afforded by canine parvovirus (Parrish 1990, 1994). Serological evidence suggests that this virus made its first appearance as a new pathogen of dogs in 1976. The virus was isolated in 1978 as the cause of severe enteritis, sometimes fatal, that occurred in dogs in North America, Europe, and Australia. Outbreaks of sudden death in puppies due to myocarditis were also linked to the new virus. Soon after its isolation, it became clear from antigenic analysis and restriction endonuclease mapping that the canine virus was closely related to feline panleukopenia virus, a pathogen that had been recognized since the 1920s. Sequence analysis of isolates of the canine virus revealed that it differed from feline panleucopenia virus in only six nucleotides, and probably originated as a result of two amino-acid changes in the viral capsid, which for the first time allowed the feline virus to replicate in dog cells. The rapidity with which canine parvovirus disease spread round the world has not been explained, but the extreme physical stability of the virus probably made fomite carriage by humans very efficient.

The special case of high frequency mutation and quasispecies formation in RNA viruses

Given their great potential for generating new genotypes, the RNA viruses seem to represent an enormous

and continuous risk with regard to the emergence of new epidemic diseases, but in fact the frequency of emergence of such new diseases is lower than might be expected (see The origin and evolution of viruses). New genotypes are tested severely by natural selection and few represent a better fit than current wild types. Better fit and better adaptation to an econiche require that a new genotype or a new virus has certain improved traits, such as improved transmissibility, and perhaps improved capacity for surviving 'lean times' such as those presented by a highly immune population or even a harsh physical environment. There is no doubt that the mode of transmission is the critically important element in this regard; viruses transmitted by the respiratory route fit their econiches very well indeed, and represent the threats to our civilization of major epidemic or pandemic disease (Mims 1991). However, as we are learning from the insidious spread of human immunodeficiency virus (HIV) in Africa and Asia, other transmission patterns can be equally deadly.

Recombination

Genetic recombination involves an interaction between two or more viral genomes during mixed infection of a cell, giving rise to progeny having a genome with characteristics derived from two or more parental genotypes. Recombination is observed with both DNA and RNA viruses, but surprisingly few examples have been documented of emergence of new viruses through this mechanism. For example, although experimental evidence exists for recombination in vivo among herpesviruses, and many individuals are dually infected by more than one herpesvirus, no herpesvirus disease manifestation in humans has ever been attributed to recombinational events (Javier et al. 1986).

There is one major exception to the dearth of evidence that recombination is important in the emergence of viral diseases: Western equine encephalitis virus (WEE) is a recombinant, its two glycoprotein genes being derived from a Sindbis-like virus progenitor and the remainder of its genome derived from Eastern equine encephalitis virus (Hahn et al. 1988). It is estimated that two cross-over events were required to produce WEEV from its progenitors, and that this probably occurred during persistent infection in a mosquito host more than 1 000 years ago (Strauss and Strauss 1994). Recombination is also well documented between serotypes of polioviruses, for example after administration of trivalent Sabin vaccine to infants, recombinant variant viruses are shed in the stool of some individuals (Liu et al. 2003). This confirms the potential for generation of recombinant viruses with altered pathogenicity or altered host range, yet it is surprising that so few examples have been found.

Reassortment

Closely related viruses that have segmented genomes often undergo genetic recombination during dual infections. This special case of recombination in which whole genome segments are recombined is termed reassortment. Different influenza A viruses can reassort their genome segments, forming viable progeny; when this results in replacement of the hemagglutinin or neuraminidase genes it is termed 'antigenic shift'. No reassortment occurs between influenza A and influenza B viruses; the basis for this restriction is not known. Each of the major human influenza pandemics of this century (1918, 1957, and 1968) was caused by reassortment of genome segments between an existing human virus and an avian virus (Webster and Kawaoka 1994). Several new strains of human influenza arose by reassortment between the prevailing human influenza virus and genes from the avian influenza virus gene pool that exists in wild ducks. For example, in 1957 the virus causing a worldwide pandemic of influenza (the Asian influenza pandemic) acquired three genes (PB1, HA, and NA) by reassortment with the influenza gene pool in ducks, and kept five other genes from the circulating human strain (Kawaoka et al. 1989). Swine may provide an important intermediate host in the stabilization of reassortants between avian and human influenza viruses. In 1997, reassortment between avian influenza virus genomes resulted in an avian influenza virus with a highly cleavable hemagglutinin. The hemagglutinin gene was apparently acquired from influenza A/Goose/Guandong/1/96 virus (Xu et al. 1999). This change allowed the variant virus to infect 18 humans, six of whom died. Fortunately this emergent virus did not readily transmit from human to human, but the virus has remained in poultry in Eastern Asia, causing many outbreaks of disease in birds and a significant number of influenza cases and deaths in humans. Clearly the danger is that the viruses now established in poultry will continue to mutate and could acquire the necessary phenotype for efficient human to human transmission through mutation, or through reassortment with a prevailing human influenza virus. Such an event would undoubtedly result in an influenza pandemic, as the H5 hemagglutinin molecule has not previously circulated widely in the human population (Li et al. 2004; Stephenson et al. 2004).

Reassortment between animal and human viruses also seems to be important in generating new pathogenic strains of rotaviruses (Gentsch et al. 1993; Iturriza-Gómara et al. 2001). Such novel viruses have been isolated as predominant outbreak strains in the USA (Ramachandran et al. 1998) and in the UK (Iturriza-Gomara et al. 2002).

Epidemic potential of newly evolved viruses

The epidemic potential of newly emergent viruses varies, depending upon the mode of transmission, the immunological and genetic susceptibility of the host population, the size of the population at risk, and other epidemiological and pathogenetic factors. Epidemic potential is greatest for an agent that is readily transmitted from host to host, particularly via the respiratory route. Conversely, zoonotic agents and arthropod-borne agents are usually limited in their geographic range, although the latter are certainly not limited in their capacity for causing very large epidemics.

Certain kinds of phenotypic change are most notable as the bases for the epidemic potential of particular viruses. An extension in host range can permit a virus to spread into a new species with devastating consequences. An increase in virulence (based on any of several mechanisms) can convert a nonpathogenic or trivially pathogenic virus into one with devastating pathogenic qualities. A change in antigenic signature can permit a virus to infect a population already immune to parental strains of the same virus.

THE INDIVIDUAL HOST: SUSCEPTIBILITY/RESISTANCE FACTORS FAVORING THE EMERGENCE OF VIRAL DISEASES

In many ways, the influence of the host on the emergence of viral diseases is more important than influences attributed to the virus per se. The host brings a much more complex genome to the battle between virus and host that involves the qualities of the virus that are crucial for its transmission and survival, and the resistance of the host. These host factors are usually categorized under (1) innate resistance, mostly dependent on poorly understood nonspecific resistance factors, (2) acquired resistance (e.g. macrophages and the cellular and humoral immune responses), and (3) physiological factors affecting resistance (e.g. age, nutritional status, hormonal effects especially in pregnancy, fever). When host resistance is optimal, infection may be subclinical or abortive, but when host response is inadequate qualitatively or quantitatively, disease is the usual outcome. Furthermore, when an overly exuberant immune response occurs there can be immunopathological disease.

The host immune response

Studies with inbred mice have identified a large repertoire of genes that confer survival potential on the host. Most of these genes are specific for a single family of viruses, although a few map to the major histocompatibility locus and encode proteins that influence host immune responses to multiple agents. Polygenic resistance characteristics are less well understood, but seemingly are even more important. For example, linebreeding and inbreeding experiments led to the development of the classic strains of mice that are exquisitely sensitive to certain viruses: these mice have been used for many years to isolate arboviruses, rabies virus, and picornaviruses. The genes selected for in these mice may be unknown, but the implication is that many genes may affect host resistance to viral infections.

The age of the host

The age of the host may also affect viral pathogenesis. This is true of poliomyelitis, a disease which emerged concurrently with improved sanitation and hygiene. It is likely that poliomyelitis occurred as a sporadic disease since ancient times. During the eighteenth and first half of the nineteenth centuries a few clusters of cases were reported, but from about 1905 onwards epidemics were reported annually in the USA. The same pattern of emergence of poliomyelitis as an epidemic disease has been repeated many times since, first in developed and then in developing countries. What accounts for this emergence of epidemic poliomyelitis? There is no evidence that it was due to the appearance of virus strains of increased virulence. Epidemiological data suggest that there is a correlation between age at the time of infection and risk of paralytic disease. Before vaccines were used to control the disease, it was apparent that in virgin soil epidemics in isolated communities without prior experience of the virus, most severe cases and deaths occurred in adults. It was proposed by Nathanson and Martin (1979) that the appearance of epidemic poliomyelitis was due, paradoxically, to improvements in public sanitation and personal hygiene, which led to a reduction in virus transmission. A delay in the age of initial infection, beyond the age when infants are protected by passively acquired maternal antibody, is postulated to have increased the risk of clinical disease. However, at the present time the phenomenon of increased disease severity of poliovirus infection in adults is still not completely understood.

Persistent infection and chronic shedding

The dynamics of emergence of a new viral disease can vary greatly, depending on the incubation period, whether the infection is rapidly self-limiting or persistent and whether the resulting disease (and shedding pattern) is acute or chronic. When associated with a short incubation period and acute disease, emergence can be a dramatic event. However, when the emerging agent is associated with a long incubation period, the resulting epidemic may rise over a period of years

before it reaches a peak. Furthermore, a long interval between infection and disease occurrence may obscure the identification of the causal agent and its mode of transmission and may thereby delay intervention actions. Finally, if the disease is chronic, the impact on the healthcare system may spread over decades rather than weeks, with major economic consequences beyond those ordinarily associated with an acute epidemic.

HIV AND ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

The premier example of the importance of a long incubation period disease in the emergence of a new disease is AIDS. In fact, the length of the incubation period, which averages 8–10 years, repeatedly led to underestimates of the true extent of spread and penetration of the epidemic (Figure 2). Associated with this was uncertainty regarding the proportion of infections that would lead to death. The long incubation period led to the initial assumption that only a small proportion of infections, perhaps no more than 10 percent, would be fatal; projections of total AIDS incidence were correspondingly low. Also important was a failure to appreciate the epidemiological impact of lifelong persistence of infection and virus shedding. It proved difficult to devise a model of the number of potentially infectious individuals in the population at any given time, and their impact on transmission dynamics. Finally, it was not appreciated that the chronic nature of AIDS would produce a vast new burden upon the healthcare system.

Coincident change in viral virulence and host resistance

The best documented example of coincident change in host and virus leading to the emergence of a variant disease involves the rabbit and myxoma virus. The disease, myxomatosis of rabbits, is caused by the poxvirus, myxoma virus; it occurs naturally as a mild infection of rabbits in South America and California (*Sylvilagus* spp.) where it produces a skin tumor from which virus is transmitted mechanically by biting insects. However, in the European rabbit (*Oryctolagus cuniculus*), myxoma virus causes a lethal infection, a finding that led to its use for biological control of wild rabbits in Australia. The European rabbit was introduced into Australia in 1859 for sporting purposes and rapidly spread to become the major animal pest of agricultural and pastoral industries. Myxoma virus was successfully introduced into this rabbit population in 1950; when originally introduced the virus produced case-fatality rates of over 99 percent. This highly virulent virus was readily transmitted by mosquitoes. Farmers operated 'inoculation campaigns' to introduce the virus into wild rabbit populations.

It might have been predicted that the disease, and with it the virus, would disappear at the end of each summer, owing to the greatly diminished numbers of susceptible rabbits and mosquitoes during the winter. This must have occurred often in localized areas, but it did not happen over the continent as a whole. The capacity of virus to survive the winter conferred a great selective advantage on viral mutants of reduced lethality: during this period,

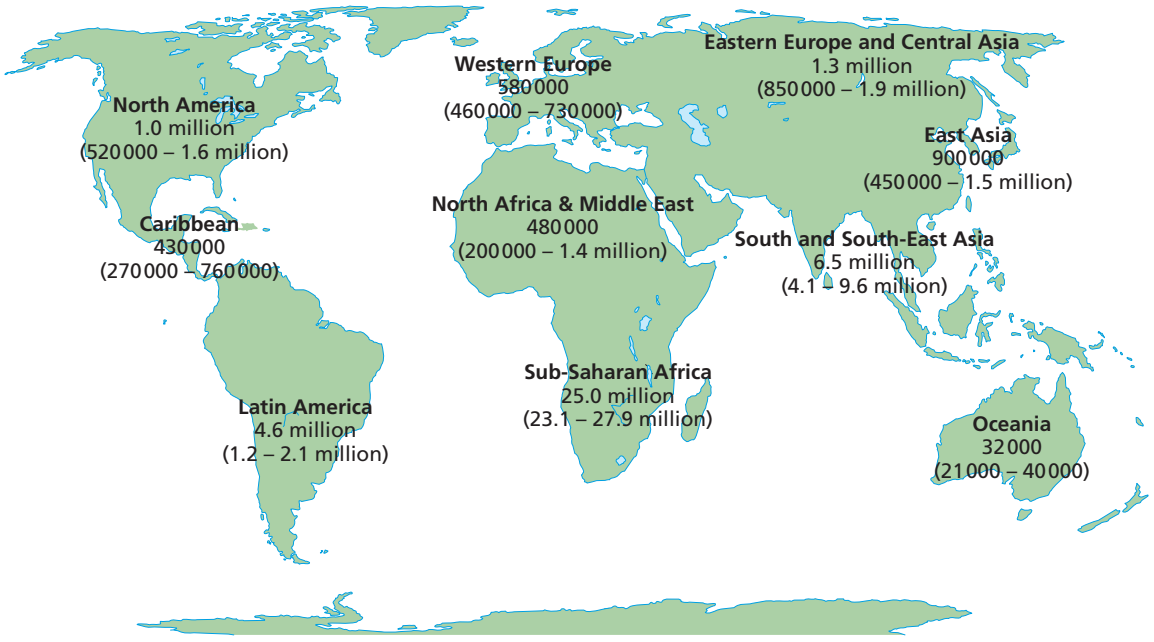

Figure 2 *Adults and children estimated to be living with HIV as of the end of 2003. Source WHO UNAIDS Programme.*

Table 4 *Emerging viral diseases: examples of probable crossings of the species barrier*

Year	Virus	Disease	Species Involved	Cause
1931	HIV-1	AIDS	Chimpanzees to humans	? Mutation
1940	HIV-2	AIDS	Sooty Mangabies to humans	? Mutation
1978	Canine parvovirus	Pandemic enteritis, myocarditis	Cat to dog	Mutation of feline panleucopenia virus
1988	Phocid distemper virus 1	Fatal respiratory disease (distemper)	Harp seals to harbor seals	Migration of harp seals due to climatic conditions
1989	Phocid distemper virus 2	Fatal respiratory disease (distemper)	Dogs to Siberian seals	Contact between different marine species or contact with terrestial animals (dogs)
1994	Hendra virus	Acute respiratory distress syndrome (ARDS), and encephalitis	Fruit bats to horses and humans	Unknown
1999	Nipah virus	Severe respiratory disease and encephalitis	Fruit bats to pigs and humans	Unknown

when mosquito numbers were low, rabbits infected by such mutants survived in an infectious condition for weeks instead of a few days and thereby contributed disproportionately to the progeny pool. Within 3 years such 'attenuated' mutants became the dominant strains throughout Australia. Thus, the original highly lethal virus was progressively replaced by a heterogeneous collection of strains of lower virulence, but most of them were still virulent enough to kill 70–90 percent of genetically unselected rabbits.

Rabbits that recover from myxomatosis are immune to reinfection. However, because most wild rabbits have a life-span of less than a year, herd immunity is not an important factor in the epidemiology of myxomatosis. Selection for more genetically resistant animals operated from the outset of the 'inoculation campaigns'. In areas where repeated outbreaks occurred, the genetic resistance of surviving rabbits increased progressively. The early appearance of viral strains of lower virulence, which allowed 10 percent of genetically unselected rabbits to recover, was an important factor in allowing the number of genetically resistant rabbits to increase. In areas where annual outbreaks occurred, the case-fatality rate fell from 90 percent to 25 percent within 7 years. Subsequently, in areas where there were frequent outbreaks, somewhat more virulent strains of virus became dominant, because they produced the kind of disease that was best transmitted in populations of genetically resistant rabbits. Thus, the ultimate balance struck between myxoma virus and Australian rabbits involved adaptations of virus and host populations, reaching a dynamic equilibrium that finds rabbits still greatly reduced compared with their pre-myxomatosis numbers, but too numerous for the wishes of farmers and conservationists.

'Species-jumping' – crossing the species barrier

The ability of animal viruses to cross the species barrier as a result of mutations has been documented in several cases (Mahy and Brown, 2000; Table 4) but in many cases involving humans, further transmission does not occur, and humans are a 'dead-end ' host (e.g. hantavirus pulmonary syndrome) (Mims 1991). Among important human viral pathogens, HIV-1 and HIV-2 provide the best examples of crossing the species barrier (Korber et al. 2000).

HIV-1 AND HIV-2

Perhaps the most significant example of species-jumping occurred when HIV entered the human population from a simian reservoir. Although the disease went unrecognized in the human population until 1977, careful molecular dating analysis suggests that HIV-1 entered the human population around 1931 (Korber et al. 2000) and HIV-2 around 1940 (Lemey et al. 2003).

Both HIV-1 and HIV-2 viruses have a narrow host range, seemingly limited to humans. The viruses can experimentally infect certain nonhuman primates, in which replication usually occurs without apparent disease. However, simian lentiviruses (simian immunodeficiency viruses (SIV)) can be recovered from a range of African monkey species, and phylogenetic analysis has confirmed the simian origins of both HIV-1 and HIV-2 (Myers et al. 1992; Korber et al. 2000). HIV-1 seems to have arisen as a cause of immunodeficiency in humans by species-jumping from the chimpanzee, and HIV-2 came from sooty-mangabeys in West Africa (Hahn et al. 2000); there is clear overlap between the areas in Africa where HIV-2 was first detected and the range of known SIV-infected monkey species (Smallman-Raynor and Cliff 1991). Humans in close contact with nonhuman primates are susceptible to infection with SIVs (Gao et al. 1992; Khabbaz et al. 1994), but these infections are usually asymptomatic and the precise mutational changes that led to the emergence of HIV as an epidemic disease spreading in the human population are unknown.

HENDRA VIRUS AND NIPAH VIRUS

The emergence of Hendra virus provides yet another lesson-filled example of the importance of species-jumping. In 1994 a horse trainer and a stablehand in Hendra, Queensland, Australia, became ill with interstitial pneumonia (acute respiratory distress syndrome (ARDS)) while nursing a sick horse that had recently been brought onto the property. The disease spread to other horses in the stables, and 14 of 21 infected horses died from a pulmonary disease with hemorrhagic manifestations that was caused by a previously unknown virus. Although the stablehand survived the infection, the trainer died. A virus was isolated from both equine lung tissue and human kidney tissue taken at autopsy, and from structural and antigenic studies was named an 'equine morbillivirus' (Murray et al. 1995), though subsequently it was renamed 'Hendra virus'. One year later a horse farmer 600 miles away, in Mackay, Queensland died of encephalitis from the same virus. He had assisted at the necropsy of two of his horses that had died (one with acute respiratory disease, and the other with neurological symptoms). Although he became ill initially with mild meningitis, one year elapsed before the farmer became severely ill and died, when at autopsy Hendra virus was found in his brain (O'Sullivan et al. 1997).

In an investigation as to the origin of Hendra virus, an extensive serological survey involving over 5 000 animals was carried out. Of all species tested, only fruit-eating bats (flying foxes) of the genus *Pteropus* were found seropositive (Young et al. 1996). Subsequently, Hendra virus was directly isolated from pteropid bats, and a sampling over a wide geographic range found that almost 50 percent of pteropid bats had serological evidence of infection (Field et al. 2001).

Hendra virus clearly belongs to the *Paramyxoviridae,* but has a longer genome (19 kb in length) than any previously studied members of the family, and genome sequence analysis clearly showed many unique features (Gould 1996; Wang et al. 2000). In 1999, a related virus was discovered in Malaysia following a major outbreak of respiratory disease in pigs and severe neurological disease in humans in close contact with diseased pigs. More than 100 humans died, and in a successful effort to control the disease 1.1 million pigs were slaughtered. A virus was isolated from brain tissue from a fatal human case – the patient had lived in Nipah River Village, and so the virus was named Nipah virus (Chua et al. 2000). Hendra virus and Nipah virus cross-react antigenically, and once the two viruses were compared by sequence analysis it was obvious that they belong to the same genus, which has been named '*Henipavirus*'. Although all human cases from the original Nipah virus outbreak had close contact with pigs, a 'mystery disease' recognized in India in 2003, and seen subsequently in Bangladesh in 2004 (Hsu et al. 2004), seems to involve direct infection of humans by Nipah virus without an intermediate domestic animal host. These new outbreaks seem to be due to direct transmission between Nipah virus-infected pteropid bats or their excreta and humans, most of whom have been young boys who climb trees in which infected bats can be found.

Spread in the human population

Many viruses can cross the species barrier and infect humans, but transmission to humans represents a 'dead end' for the virus and no further transmission occurs (Mims 1991). On the other hand, some are regularly or irregularly transmitted from human-to-human, but without establishing an independent long-term exclusively human reservoir. All of these variations define the terms 'zoonosis' and 'zoonoses'. Many of these zoonotic viruses are arthropod-borne, including alphaviruses such as Eastern equine encephalitis virus and Western equine encephalitis virus, flaviviruses such as West Nile virus and Japanese encephalitis virus and St. Louis encephalitis virus, and bunyaviruses such as La Crosse virus and Rift Valley fever virus. Other viruses are transmitted directly to humans from zoonotic animal reservoirs, most commonly from rodent reservoirs. Examples include bunyaviruses such as Hantaan virus (the etiologic agent of hemorrhagic fever with renal syndrome), Seoul virus, Puumala virus (the etiologic agent of nephropathia epidemica), and Sin Nombre virus (the etiologic agent of hantavirus pulmonary syndrome), and arenaviruses such as Machupo virus (the etiologic agent of Bolivian hemorrhagic fever), Junin virus (the etiologic agent of Argentine hemorrhagic fever) and Lassa virus (the etiologic agent of Lassa fever). Historically, each of these viruses has been responsible for the emergence of a 'new' human disease.

THE HOST POPULATION: INDIVIDUAL AND SOCIETAL BEHAVIORAL FACTORS FAVORING THE EMERGENCE OF VIRAL DISEASES

Several influences pertaining to human behavior (individual and societal behavior) have favored the emergence of new virus diseases; all of these influences seem to have accelerated over the last century. The global human population has continued to grow inexorably, bringing increasingly larger numbers of people into close contact. There have been successive revolutions in the speed of transportation, making it possible to circumnavigate the globe in less than the incubation period of most viral infections (Murphy and Nathanson 1994). For this reason, viral diseases occurring anywhere in the world can no longer be presumed to stay confined to their country or continent of origin. The death of a patient from Lassa fever in a hospital in Chicago in 1989

provided sobering testimony to the distance and speed that exotic viruses can move around the world (Holmes et al. 1990). The patient, an American citizen who had visited Nigeria to attend his mother's funeral, became ill 2 days after his return to Chicago. A total of 102 people had contact with him in the hospital. Fortunately, none of these contacts became infected. However, the incident illustrates the potential vulnerability of populations remote from the normal locale of geographically limited diseases. More recently, at least four cases of Lassa fever virus infection in European persons who had visited West Africa have been recorded, raising concern regarding the clinical management of such cases in diverse European settings (Crowcroft 2002).

The influence of individual behavioral factors

There are diverse influences pertaining to the behavior of individuals that can lead to the emergence of new diseases or new patterns of disease transmission. Many diseases that depend upon such factors are emergent or re-emergent. Behavioral influences include: (1) risk factors leading to sexually transmitted diseases (e.g. multiple sex partners, homosexual risk behaviors); (2) behavioral risk factors associated with day care and school; (3) behavioral risk factors favoring the transmission of childhood diseases in the community; (4) risk factors pertaining to food preparation and storage in the home; (5) risk factors associated with keeping pet animals in the home; and (6) viruses intentionally used for harm by bioterrorists.

SEXUALLY TRANSMITTED DISEASES

After declining over many years in developed countries, sexually transmitted diseases are now increasing at epidemic rates in urban populations in Europe, the USA, and many developing countries. Changes in sexual attitudes and behavior, combined with injectable drug use, have led to rapid amplification of certain viral diseases that are spread sexually. This is especially disturbing because many of the sexually transmitted diseases enhance the risk of transmission of each other (via new bacteria and viruses entering through established lesions). Thus, the risk of transmission is increased beyond expected rates and 'curable' diseases, such as gonorrhea and syphilis, support the spread of 'incurable' diseases, such as genital herpes and HIV infection/AIDS. The major societal failing in this regard stems from declining support for public health programmes, but in some instances emergence reflects advances in our ability to detect new viruses. One example is the emergence of human papillomavirus infection, genital papillomatosis, and cervical cancer. Multiple risk factors contribute to cervical cancer; these include behavioral (sexual behavior), dietary, hormonal, and viral risk factors (zur Hausen 2002). There are several theories but no real proof as to how these risk factors, in concert, lead to cervical neoplasia. Now that one risk factor for cervical cancer is known to be infection with certain types of human papillomaviruses, DNA probes for these viruses are being added to cytological screening programmes.

DAY CARE AND VIRAL INFECTIONS

Shifts in the structure of the family in all developed countries have resulted in a dramatic increase in the proportion of children in day care; currently in the USA, 35 million children under 14 years with working mothers spend 22–40 hours per week in day care. This trend is likely to continue, and in the year 2001 it was estimated that more than nine million families included working mothers with children under 5 years of age. Viral diseases represent the most important problems in day care, respiratory and diarrheal illnesses being most common. Children attending day care facilities may also become silent reservoir hosts for some agents of disease, such as hepatitis A virus and several enteroviruses. Depending on the disease, children attending day care have a 2- to 18-fold increased risk of becoming ill compared with children at home. The most common viral diarrheal pathogens acquired in day-care centers are rotaviruses. Rotaviruses, first discovered in 1973, infect every child in its first 3–4 years of life; this leads, in the USA, to an estimated 3 million cases of diarrhea, 500 000 doctor visits, 70 000 hospitalizations for 300 000 inpatient days and 75–125 deaths, and the costs for hospital care total US$200–400 million per year. Worldwide, some 600 000 children die each year of rotavirus diarrhea (Bern et al. 1992).

KEEPING PET ANIMALS

There have been many episodes of the introduction of new pathogens, such as exotic *Salmonella* types, via reptiles and amphibians kept as pets, but until recently viruses received little attention in this regard. The introduction in 2003 of monkeypox virus into the USA from Africa changed all this (CDC 2003b). A pet supplier in Texas, USA imported a shipment of rodents from Accra, Ghana which included giant Gambian rats, tree squirrels, rope squirrels, dormice, and several other African rodent species. One or more of these species were infected with monkeypox virus, which is endemic in Africa. These animals were housed by the importer together with prairie dogs, an American ground squirrel (*Cynomys* sp.) that is very popular as a family pet. The prairie dogs readily became infected with monkeypox virus, and when sold as pets human cases of monkeypox began to emerge in the mid-western United States (Guarner et al. 2004b). As a result, more than eighty human cases of monkeypox occurred in the USA, and there was a real danger that monkeypox virus could

become endemic in wild rodent populations. Following this episode, regulation of imports and interstate movement of exotic mammals have been upgraded, but it seems likely that we will see more of this kind of threat from imported pathogens in the future.

VIRUSES INTENTIONALLY USED FOR HARM

Since the terrorist attacks of September 11th 2001 in the USA, and the subsequent anthrax attacks through the US mail system, the possible use of viruses as weapons of bioterrorism must be considered (Smolinski et al. 2003). Although smallpox was eradicated in 1980, the possibility that the virus could be deliberately reintroduced into the world population remains the subject of considerable concern (Henderson 1999; Mahy 2003) and several countries are taking steps to prepare for such an event. Many other viral pathogens are also considered to be possible agents of biological warfare, including viruses causing severe hemorrhagic fever such as filoviruses (e.g. Ebola virus and Marburg virus), arenaviruses (e.g. Lassa fever, Machupo virus), and viruses causing severe encephalitis (e.g. Venezuelan equine encephalitis virus, Eastern equine encephalitis virus, Western equine encephalitis virus). The possible use of a virus to cause harm to the agricultural economy must also be considered, and in this context foot-and-mouth disease virus must be a prime candidate.

These threats also include the potential which now exists for deliberate genetic engineering of an existing virus so as to alter properties like pathogenicity or susceptibility to control by vaccination. The future emergence of a harmful virus disease by deliberate human action remains uncertain, but only by recognizing the possibilities can we prepare for, and minimize, the impact of such an event.

The influence of societal, commercial, and iatrogenic factors

There are diverse societal, commercial, and medical care factors that lead to the emergence of new diseases or new disease patterns. Again, many diseases that depend on such factors are emergent or re-emergent. Such risk factors include: (1) those associated with the commercial food industries, from sources on the farm to processing, transportation, wholesaling, and retailing; (2) those associated with water supply, (3) those associated with pharmaceuticals and biologicals; (4) those causing iatrogenic diseases.

DISEASES ASSOCIATED WITH ADVANCED MEDICAL CARE

Diseases such as those associated with immunosuppressive drug therapy, organ transplantation (including xenotransplantation), blood banking, and kidney dialysis are all increasing substantially in all developed countries. Since the early 1980s there has been an unprecedented rise in opportunistic infections associated with these medical care practices. In particular, the increase in organ transplantation and cancer chemotherapy, with their associated immunosuppressive drug therapies, has combined with the emerging AIDS epidemic to create a large, highly susceptible population at risk for a variety of viral diseases (see Reoviruses, orbiviruses and coltiviruses). In many cases viruses cause diseases of greatly increased severity in such individuals. These include diseases caused by DNA viruses, such as cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, human herpesvirus 6, human papillomaviruses, human parvovirus B19, human polyomaviruses JC and BK, adenoviruses, hepatitis B virus, and molluscum contagiosum virus, and RNA viruses such as measles virus, hepatitis C virus, influenza, respiratory syncytial virus and parainfluenza virus.

Human herpesvirus 8, which is strongly associated with and may be the cause of Kaposi's sarcoma, is an example of an emerging virus that might not have been recognized except for its association with HIV and its immunosuppressive effects (Chang et al. 1994). As the number of naturally and therapeutically immunosuppressed people continues to expand throughout the world, it is likely that more and more new viral diseases, or new manifestations of old viral diseases, will continue to be seen.

MODERN AGRICULTURAL AND FOOD INDUSTRY PRACTICES AND VIRAL INFECTIONS

Changes in every aspect of the food industry, from on-the-farm technologies to processing technologies, favor the emergence of new viral disease problems. Animal husbandry has changed in ways that increase stress and promote viral transmission and endemic infection cycles in livestock and poultry. Large numbers of animals are being confined in limited space and at very high density, cared for by a few, inadequately trained workers. Elaborate housing systems are used, with good evaluation of cost:benefit, but with inadequate evaluation of health effects of systems for ventilation, feeding, waste disposal, and cleaning. In addition, some diseases are favored by the global expansion of agricultural markets, involving the global transport of animals, animal products, and animal semen and embryos. Yet, other diseases can emerge as a consequence of changes in processing systems and distribution systems for finished foods.

In 1997 there was an outbreak of hepatitis A which was first noticed in Michigan schoolchildren and later in Maine. Investigation of these two outbreaks linked them to the consumption of frozen strawberries commercially prepared by the same processor. By genetic sequence analysis, the virus causing illness in 126 patients from Michigan and Maine was identical. Later investigation found virus of the same sequence in patients with hepatitis A in Arizona, Louisiana, and Wisconsin, all of whom had eaten strawberries supplied by the same food processor

(Hutin et al. 1999). In 2003, an outbreak of hepatitis A in Pennsylvania was linked to the consumption of green onions in a Mexican restaurant where they were served in salsa. As a result of this food-borne outbreak immune globulin was provided to approximately 9 000 persons. Traceback investigations showed that the green onions consumed in Pennsylvania came from one or more farms in Mexico. By genetic sequence analysis, this outbreak was linked to similar hepatitis A outbreaks in Tennessee, Georgia, and North Carolina that also were associated with eating green onions (CDC 2003c).

Hepatitis A virus outbreaks provide good examples of the ease with which a pathogen can be widely dispersed in the population due to current food processing and distribution systems (Tauxe 1997).

Nipah virus, which emerged in Malaysia in an intense pig farming area, and then spread with movement of infected animals to several other parts of Malaysia and even to Singapore, may have stemmed in part from certain newly-introduced farming practices. The disease caused severe respiratory distress and some encephalitis in the pigs, but when a large number of workers on the pig farms developed encephalitis, and more than 100 died, it was decided to slaughter more than a million pigs, and this stopped the disease spread to humans. Fortunately, as noted above, humans were a dead-end host for Nipah virus infection, and no secondary spread was noted in the human community. The virus was identified because of its close relationship to Hendra virus. Using antiserum against Hendra virus, cell cultures growing the Nipah virus fluoresced, and this soon led to complete characterization and sequencing of the Nipah virus (Chua et al. 2000).

LIVESTOCK DISEASES

We usually think of the importance of livestock diseases in terms of financial losses, because the capacity of the commercial livestock food and fiber industries of developed countries are such that surpluses are a greater problem than are shortages. However, in developing countries this is not the case: livestock diseases, especially new, emerging or re-emerging diseases, cause immediate human suffering by substantially compromising scarce human food resources, especially the supply of high-quality protein.

Epidemiological considerations

Perpetuation of a virus in nature depends on the maintenance of serial infections, i.e. a chain of transmission; the occurrence of disease is neither required nor necessarily advantageous. Although clinical cases may be somewhat more productive sources of virus than inapparent or mild infections, the latter are generally more numerous and do not restrict the movement of infectious individuals, and thus provide a major mechanism of viral dissemination and emergence.

Epidemics are classically divided according to their means of spread into two major categories, propagated and common source (Nathanson 1996).

PROPAGATED EPIDEMICS AND CRITICAL COMMUNITY SIZE

In propagated epidemics, spread from host to host continues to expand as long as each infection gives rise to more than one new infection. The rate of transmission depends on a number of variables, such as the density of the population, the proportion of the population that is susceptible, the degree and duration of contagiousness, and the frequency of contacts leading to transmission. The classic large epidemics of viral diseases have all been propagated epidemics.

In most instances, for viruses that produce acute self-limiting infections to survive, the susceptible host population must be both large and relatively dense. Such viruses may disappear from a population because they exhaust the supply of susceptible hosts as they acquire immunity to reinfection. Persistent viruses, on the other hand, may survive in very small populations, sometimes by spanning generations. Depending on the duration of immunity and the pattern of virus shedding, the critical community size varies considerably with different viruses.

MEASLES

Much was learned about the dynamics of the viral transmission chain by Panum, who studied the devastating measles epidemic of 1846 in the Faroe Islands; this was at a time when even the concept of a virus was unknown (Panum 1939). Nevertheless, Panum described the incubation period of measles and the lifelong immunity that follows infection. With an incubation period of about 12 days, maximum viral excretion for the next 6 days and solid immunity to reinfection, between 20 and 30 susceptibles would need to be infected in series to maintain measles transmission for a year. Since nothing like such precise one-to-one transmission occurs, many more than 30 susceptibles are needed to maintain endemicity. Analyses of the incidence of measles in different size communities have shown that a population of about half a million people is needed to ensure a large enough annual input of new susceptibles, provided by the annual birth cohort, to maintain measles indefinitely as an endemic disease. Because infection depends on close contact, the duration of epidemics of measles is correlated inversely with population density. If a population is dispersed over a large area, the rate of spread is reduced and the epidemic will last longer, so the number of susceptible persons needed to maintain endemicity is reduced. On the other hand, in such a situation, a break in the transmission cycle is much more likely. If a large proportion of the population is initially susceptible, the intensity of the epidemic builds up very quickly, often reaching almost 100 percent. Virgin-soil epidemics in

isolated communities have had devastating consequences owing to lack of medical care and the disruption of work capacity. In large urban communities, before the era of vaccination, measles epidemics occurred every 2–3 years, each time exhausting available susceptibles; this epidemic cycle occurred on a continental scale. Before the introduction of vaccine, the cyclic occurrence of measles epidemics was influenced by several variables besides the build-up of susceptibles; these included the dynamics of reintroduction of the virus and seasonal influences.

COMMON SOURCE EPIDEMICS

Common source epidemics occur when a virus is disseminated from a single focus; they usually result from contamination of air, food, water, drugs, biomedical devices or the like. If a virus is introduced from a common source into a large population, disease can emerge on an epidemic scale. An excellent example is provided by the unwitting introduction of hepatitis B virus during immunization against yellow fever.

In the early stages of World War II, it was decided to immunize large numbers of American troops with 17D yellow fever vaccine. In order to stabilize the infectivity of the live attenuated 17D virus, protein was added to the final vaccine formulation. To prevent serum sickness, human serum was selected as the protein source. Almost 1 000 donors were used; unfortunately, at least one donor was a carrier of hepatitis B virus. Consequently, in the spring of 1942 over 400 000 troops received contaminated vaccine, causing about 20 000 cases of hepatitis (Sawyer et al. 1944). Although the onset of disease was spread over a considerable time, when plotted according to the interval from administration of the vaccine the timeline formed a classic log-normal curve, thereby providing statistical proof of the association of the epidemic with the administration of the vaccine. This epidemic clearly established hepatitis B as a distinct entity, separable from hepatitis A (e.g. different mean incubation periods: 3 months for hepatitis B, one month for hepatitis A).

THE ENVIRONMENT: ECOLOGICAL AND ZOONOTIC FACTORS FAVORING THE EMERGENCE OF VIRAL DISEASES

Ecological factors pertaining to unique environments and geographic isolation often underpin the emergence of new viruses and thereby new viral diseases. Such factors favor the adaptation of viruses to new econiches. When ecosystems are altered, viral infections of humans and animals follow. Population movements and the intrusion of humans and domestic animals into new arthropod habitats have resulted in many new emergent disease episodes. The classic example of this was the emergence of yellow fever when susceptible humans entered the Central American jungle to build the Panama Canal; there are many contemporary examples suggesting that similar events will continue to happen, in most cases in unanticipated circumstances. Deforestation has been the key to the exposure of farmers and domestic animals to new arthropods and the viruses they carry. The occurrence of Mayaro virus disease in Brazilian wood-cutters as they cleared the Amazonian forest in recent years is a case in point. Increased long-distance transportation facilitates the carriage of exotic arthropod vectors around the world. The carriage of the Asian mosquito, *Aedes albopictus*, a vector for dengue viruses and California encephalitis virus, into the USA in the water contained in imported used tyres, represents an unsolved problem of this kind. Increased long-distance transportation of livestock facilitates the carriage of viral agents and arthropods (especially ticks) around the world. The introduction of the tick-borne agent, African swine fever virus, from Africa into Portugal (1957), Spain (1960), and South America (1960s and 1970s) is thought to have occurred in this way; it is just a matter of time until this virus makes further international forays.

A recent dramatic example is provided by West Nile virus (WNV), a flavivirus which appeared for the first time in the Western hemisphere in 1999 (Briese et al. 1999; Lanciotti et al. 1999). Although originally isolated in Uganda in 1937, from where it spread to Egypt, Israel, India, and other parts of Africa, the virus caused occasional outbreaks of human disease in the 50 years following its discovery, but was not considered a serious public health problem. However in the mid-1990s, the virulence of WNV apparently changed (Hubalek and Halouzka 1999; Murgue et al. 2001a,b) and epidemics associated with severe neurological disease in humans and high mortality in birds began to occur in the Mediterranean basin and surrounding countries before WNV appeared in New York in 1999 (Ostlund et al. 2001). Following its isolation and characterization, the New York virus was found to be virtually identical in nucleotide sequence to an isolate of WNV made in Israel (Lanciotti et al. 1999). The mode by which the virus moved across the Atlantic ocean is unknown, but transport of an infected mosquito or bird in the hold of a jetliner are reasonable possibilities. An infected human or other mammal seems unlikely to have seeded the virus in the US, because the level of viremia would be rather low compared to that in a bird or carried in a mosquito. From its origin in New York, the virus has spread at an alarming rate throughout the continent, and reached California by August 2003, also spreading north into Canada and south into Mexico and the Caribbean. (Petersen et al. 2003). Many of the human cases have involved severe encephalomyelitis (Guarner et al. 2004a), but this represents the tip of the iceberg, and many asymptomatic infections also occur. Direct human-to-human transmission through blood has been documented with WNV; this is the first time that the US

blood supply has had to be checked for a mosquito-borne virus (Armstrong et al. 2003; CDC 2003a).

Changing routes of long-distance bird migrations, brought about by new water impoundments, represent an important yet still untested additional risk of introduction of arboviruses into new areas. This may be a key to some of the movement of Japanese encephalitis virus into new areas of Asia. Ecological factors pertaining to environmental pollution and uncontrolled urbanization are contributing to many new, emergent disease episodes. Arthropod vectors breeding in accumulations of water (tin cans, old tyres, etc.) is a problem worldwide. Environmental chemical toxicants (herbicides, pesticides, residues) can also affect vector–virus relationships directly or indirectly. For example, mosquito resistance to all licensed insecticides in parts of California is a known direct effect of unsound mosquito abatement programmes, augmented indirectly by poorly regulated pesticide usage against crop pests. Ecological factors relating to water use (i.e. increasing irrigation and the expanding reuse of water) are becoming important factors in the emergence of viral diseases. The problem with primitive water and irrigation systems that are developed without attention to arthropod control is exemplified in the emergence of Japanese encephalitis in new areas of southeast Asia and the Indian subcontinent. Global warming, affecting sea level, estuarine wetlands, swamps, and human habitation patterns, may be affecting arthropod vector relationships throughout the tropics; however, data are scarce and too many programmes to study the effect of global warming have not included the participation of viral disease experts.

Dengue

Dengue is one of the most rapidly emerging diseases in the tropical parts of the world, millions of cases occurring each year. For example, Puerto Rico had five dengue epidemics in the first 75 years of the 20th century, but more recently has had epidemics nearly every year, at an estimated cost of over US$150 million. At the same time, a record number of cases have occurred elsewhere in the Americas; Brazil, Bolivia, Colombia, Paraguay, Ecuador, Venezuela, Nicaragua, Cuba, and Mexico have experienced major dengue epidemics. These epidemics have involved multiple virus types: all four dengue virus types are now circulating in the Caribbean region. These are the circumstances that lead to dengue hemorrhagic fever (dengue shock syndrome): the lethal end of the dengue disease spectrum. Dengue hemorrhagic fever first occurred in the Americas in 1981; since then, most countries have reported cases, and in 1998 more than a million cases of dengue/dengue hemorrhagic fever were reported to WHO. By the beginning of the 21st century it was estimated that, globally, between 50 million and 100 million cases of dengue fever and several hundred thousands of cases of dengue hemorrhagic fever occur each year, the latter accompanied by case fatality rates of 1–10 percent, depending on the country (Gubler 2002).

Why is dengue emerging, especially in the Americas? The answer is simple: urban mosquito habitats are expanding (the vector mosquito, *Aedes aegypti*, is extremely well adapted to human proximity), mosquito density is increasing, and mosquito control is failing. This is occurring not just in the least developed countries but also in many developed countries. Financial resources for public health are severely limited and, too often, mosquito control, which is very expensive, falls off the bottom of the priority list. Meanwhile, mosquito control is becoming more expensive as older, cheaper chemicals lose effectiveness or are banned as damaging to the environment. As mosquito control fails, dengue follows quickly.

Yellow fever

An even more frightening situation associated with failing mosquito control is that yellow fever virus, which is transmitted by the same mosquito vector as dengue, *Aedes aegypti*, might also re-emerge. Where dengue occurs, the conditions are also appropriate for yellow fever (initiated by importation via an infected person or an infected mosquito). It is one of the mysteries of tropical medicine that yellow fever has not occurred more often where vector density and a susceptible human population coexist (Monath 2001). However, since the 1980s there has been a resurgence of yellow fever both across Africa and in South America: this is a potential risk to the USA, since *Aedes aegypti* is present in urban areas in the South (Robertson et al. 1996).

In fact, no one knows where, when or even if yellow fever virus will re-emerge in the kinds of epidemics that were the scourge of tropical and subtropical cities of the western hemisphere and Africa throughout the seventeenth, eighteenth, and nineteenth centuries; however, because this virus is so dangerous, the possibility is constantly on the mind of national and international health officials.

Hantaviruses and hemorrhagic fever with renal syndrome

Hantaviruses provide an example of a virus species that no doubt has existed for a long time in the same habitats, but was only recognized relatively recently. The first well characterized hantavirus disease was Korean hemorrhagic fever, which emerged during the Korean war of 1950–52. Thousands of United Nations troops developed a mysterious disease marked by fever, headache, hemorrhage, and acute renal failure; the mortality rate was 5–10 percent. Despite much research, the agent of this disease remained unknown for 26 years; then, in

1976, a new virus, named Hantaan virus, was isolated in Korea from fieldmice. The discovery of this virus was, however, just 'the tip of an iceberg.' In subsequent years, related viruses have been found in many parts of the world in association with different rodents and as the cause of human diseases with more than 150 different local names (Schmaljohn and Hjelle 1997). It has been found in recent years that, from an ecological perspective, there are seven or eight different subgroups of viruses and three different transmission patterns: rural, urban, and laboratory acquired. From a clinical perspective, two disease patterns are described, one marked by severe disease (hemorrhagic fever with renal syndrome, with significant mortality) and the other by mild disease (febrile disease, without mortality). The rural, severe disease is widespread in the Far East (e.g. Korean hemorrhagic fever in Korea; 'epidemic hemorrhagic fever' in China, causing more than 100 000 cases per year). A similar rural, severe disease is emerging in the Balkans (mortality rate c. 20 percent).

Hantaviruses and acute respiratory distress syndrome

In May 1993, a new hantavirus disease was recognized in the southwestern region of the USA. The disease appeared as an acute respiratory distress syndrome. Clinical signs include fever, headache, and cough, followed by acute pulmonary congestion and edema leading to hypoxia, shock, and, in many cases, death. Within a short time, cases were found in 23 states and eventually throughout the USA. By July 2004, 366 cases of hantavirus pulmonary syndrome have been confirmed in the United States, with an overall case fatality rate of 38 per cent.

The causative virus was shown to be a previously unknown hantavirus (Nichol et al. 1993), now named Sin Nombre virus, that has a reservoir in the deer mouse (*Peromyscus maniculatus)*. Horizontal transmission, via biting, is believed to be an important route of transmission between rodents. Human infection occurs following inhalation of virus particles from rodent urine, feces, and saliva, from contact of broken skin or mucous membranes with infectious virus, or by rodent bite. Viral RNA amplified from patients' specimens by PCR, when sequenced and compared to sequences obtained from specimens from different areas, indicates that several different variant viruses, all new and previously unknown, are active in different rodent species and subspecies in the USA, some causing human disease and others merely present in rodent populations without any human disease association. Hantavirus sequences have been used in expression systems to produce homologous antigens for further studies and diagnostic services. The same serological and molecular biological methods were applied to large numbers of rodents collected in the areas where patients lived; this proved that at least eight species of rodents were involved, the primary reservoir host in the southwest being *Peromyscus maniculatus*, the deer mouse (c. 30 percent of this species were found to harbor the viruses in several areas of southwestern USA) (Morzunov et al. 1998). The viruses have very likely always been present in the large area of the western region of the USA inhabited by *Peromyscus* species; they were recognized in 1993 only because of the number and clustering of human cases, which in turn were probably caused by a great increase in rodent numbers consequent on an increase in piñon seeds and other rodent food. As a result of this kind of rapid field and laboratory investigation, the public is being advised about reducing the risk of infection, mostly by reducing rodent habitats and food supplies in and near homes and taking precautions when cleaning rodent-contaminated areas. Fortunately, human-to-human transmission has not been observed with hantaviruses in North America. Humans become infected by contact with or inhalation of rodent excreta, but are then dead-end hosts.

In 1996 it was recognized that hantaviruses are also present in rodent populations in South America, and here there has been some evidence for human-human transmission of Andes virus, which occurs mainly in Argentina and Chile (Wells et al. 1997). As in North America, there are both pathogenic and nonpathogenic hantaviruses in South America, and since the first report several hundred cases of human hantavirus pulmonary syndrome have been recorded. Fortunately, the rodents which transmit hantavirus pulmonary syndrome are confined to the S*igmodontinae*, a subfamily of the *Muridae*, members of which are only found in the Western hemisphere (Figure 3). The other murid subfamilies that transmit hantaviruses are the *Murinae,* (e.g. Hantaan virus, Dobrava-Belgrade virus, and Seoul virus) and the *Arvicolinae* (e.g. Puumala virus) both associated with hemorrhagic fever with renal syndrome in Europe and Asia. Interestingly, although several hantavirus species are associated with arvicoline rodent species in North America, none has been associated with human disease (Table 5).

Rabies

Rabies can serve as an example of ecological factors relating to the adaptation and emergence of viruses in new econiches. The most dramatic illustration of this in recent years has been the appearance of epidemic raccoon rabies in eastern USA. The epizootic has been traced to the importation of raccoons from Florida to West Virginia in 1977. This epidemic demonstrates dramatically how human disturbance of the environment, in this instance involving the transportation of wild animals, can lead to emergence of a disease in a previously unaffected area. A key to our understanding of this episode was the discovery that rabies virus is not one virus; rather, it is a set of

Figure 3 *Geographical distribution of New World hantaviruses recognized as of 2004. Viruses named in Roman face have not been associated with human disease – those named in bold face are known to cause hantavirus pulmonary syndrome. (Courtesy of James Mills, CDC).*

different genotypes, each transmitted within a separate reservoir host niche. In North America, there are about six terrestrial animal genotypes, one involving the skunk in the north-central states, one the skunk in the south-central states, one the Arctic fox and red fox in Alaska and Canada, one the grey fox in Arizona, one the coyote and feral dog in southern Texas and northern Mexico, and one the raccoon in eastern states. 'Raccoons-bite-raccoons-bite-raccoons' and after some time their virus becomes a distinct genotype, highly adapted to the host cycle and inefficient if introduced into another host cycle. When this discovery was made (first using monoclonal antibody panels and more recently partial viral genomic sequencing), many mysteries of rabies ecology were clarified.

THE PREVENTION AND CONTROL OF NEW, EMERGING, AND RE-EMERGING VIRAL DISEASES

Since 1991 we have witnessed the emergence or re-emergence of a large number of new human viruses (Figure 4). When a new virus disease is suspected, a complex continuum of prevention and control activities may be called into action, but, given financial and resource constraints, decisions must be made and priorities must be set. The full continuum comprises the following activities and resources, which may be divided into investigative and interventional phases.

The investigative phase

- The disease must be characterized (usually the work of clinicians and pathologists).
- There is nearly always need for epidemiological field investigation to assess the risk to the population (usually the work of medical epidemiologists).
- The agent must always be sought. When a new virus is suspected, it must be isolated, identified, characterized.
- There is usually need for diagnostics development, to provide and authenticate primary, reference, and confirmatory tests.
- An integrated research programme is usually needed (but not always implemented), covering sciences as diverse as molecular virology, pathogenesis, pathophysiology, immunology, ecology, epidemiology, sociology and behavior, vector biology, etc.

Table 5 *Recognized hantaviruses in the world, their hosts, the recognized geographic occurrence of the virus, and the diseases caused*

Virus	Host species (subspecies)[a]	Distribution of virus	Disease
	Order *Rodentia*, Family *Muridae*, Subfamily *Murinae*		
Hantaan virus (HTNV)[b]	*Apodemus agrarius (mantchuricus)*	Eastern Russia, Northern Asia, Balkans	Severe HFRS
Seoul virus (SEOV)[b]	*Rattus norvegicus*	Nearly Worldwide	Mild/Moderate HFRS
Dobrava-Belgrade virus (DOBV)[b]	*Apodemus flavicollis*	Balkans	Severe HFRS
Thailand virus (THAIV)[b]	*Bandicota indica*	India	NR
Saarema virus (SAAV)[b]	*Apodemus agrarius (agrarius)*	Europe	Mild HFRS
Amur virus (AMRV)	*Apodemus peninsulae*	Far Eastern Russia	HFRS
	Order *Rodentia*, Family *Muridae*, Subfamily *Arvicolinae*		
Puumala virus (PUUV)[b]	*Clethrionomys glareolus*	Europe, Scandinavia, Russia, Balkans	Mild HFRS
Prospect Hill virus (PHV)[b]	*Microtus pennsylvanicus*	North America	NR
Bloodland Lake virus (BLLV)	*Microtus ochrogaster*	North America	NR
Isla Vista virus (ISLAV)	*Microtus californicus*	North America	NR
Tula virus (TULV)	*Microtus arvalis/ M. rossiaemeridionalis*	Russia, Slovakia	NR
Khabarovsk virus (KBRV)	*Microtus fortis*	Eastern Russia	NR
Topografov virus (TOPV)	*Lemmus sibericus*	Siberia	NR
	Order *Rodentia*, Family *Muridae*, Subfamily *Sigmontinae*		
Sin Nombre virus (SNV)[b]	*Peromyscus maniculatus*	North America	HPS
New York virus (NYV) [b]	*Peromyscus leucopus*	Eastern USA and Central USA	HPS
Black Creek Canal virus (BCCV)[b]	*Sigmodon hispidus*	Southeastern USA	HPS
Bayou virus (BAYV)[b]	*Oryzomys palustris*	Southeastern USA	HPS
Muleshoe virus (MULEV)	*Sigmodon hispidus (texianus)*	Southern USA	HPS
Monongahela virus (MONV)	*Peromyscus maniculatus (nubiterrae)*	Eastern USA and Canada	HPS
Limestone Canyon virus (LSCV)	*Peromyscus boylii*	Southwestern USA	NR
Blue River virus (BRV)	*Peromyscus leucopus*	Central USA	NR
El Moro Canyon virus (ELMCV)[b]	*Reithrodontomys megalotis*	Western USA and Mexico	NR
Río Segundo virus (RIOSV)	*Reithrodontomys mexicanus*	Costa Rica	NR
Caño Delgadito virus (CDGV)[b]	*Sigmodon alstoni*	Venezuela	NR
Juquitiba virus (JUQV)	Unknown	Southeastern Brazil	HPS
Araraquara virus (ARAV)	Unknown	Southeastern Brazil	HPS
Castelo dos Sonhos virus (CASV)	Unknown	Central Brazil	HPS
Anajatuba virus (AJV)	*Oligoryzomys fornesi*	Northern Brazil	NR
Río Mearim virus (RMV)	*Holochilus sciureus*	Northen Brazil	NR
Río Mamoré virus (RIOMV)	*Oligoryzomys microtus*	Bolivia, Peru	NR
Laguna Negra virus (LNV)[b]	*Calomys laucha*	Western Paraguay and Bolivia	HPS
Andes virus (ANDV)[b]	*Oligoryzomys longicaudatus*	Southern Argentina and Chile	HPS
Lechiguanas virus (LECV)[b]	*Oligoryzomys flavescens*	Central Argentina	HPS
Bermejo virus (BMJV)	*Oligoryzomys chacoensis*	Northwestern Argentina, Southern Bolivia	HPS
Orán virus (ORNV)	*Oligoryzomys longicaudatus*[c]	Northwestern Argentina, Southern Bolivia	HPS
Maciel virus (MACV)	*Bolomys obscurus*	Central Argentina	NR
Hu39694	Unknown	Central Argentina	HPS
Pergamino virus	*Akodon azarae*	Central Argentina	NR

(*Continued over*)

Table 5 *Recognized hantaviruses in the world, their hosts, the recognized geographic occurrence of the virus, and the diseases caused (Continued)*

Virus	Host species (subspecies)[a]	Distribution of virus	Disease
Choclo virus	*Oligoryzomys fulvescens (costaricensis)*	Southwestern Panama	HPS
Calabazo virus	*Zygodontomys brevicauda (cherriei)*	Southwestern Panama	NR
Maporal virus	*Oecomys bicolor*	Central Venezuela	NR
	Order *Insectivora*, Family *Soricidae*		
Thottapalayam[b]	*Suncus murinus*	India	NR

HFRS = hemorrhagic fever with renal syndrome; HPS = hantavirus pulmonary syndrome; NR = None Recognized.
a) Taxonomy follows Wilson and Reeder (1992).
b) Virus isolated in cell culture; others identified from genetic sequence.

The interventional phase

- There is always a need for comprehensive communications systems.
- There is often need for technology transfer (transfer of diagnostics technology to local agencies and transfer of information pertinent to vaccine and drug development to the commercial sector).
- There is usually need for training and clinical continuing education.
- Comprehensive public health systems may need to be adapted to the disease at hand. This may involve new public education programmes, new vital record and disease register systems, rapid case reporting, new surveillance systems, expanded laboratory diagnostics services, new staffing and staff support, new logistical systems (facilities, equipment, supplies, transport), and the like.
- There may be a need for new policy decisions, new risk management decisions. These may involve new legislation and regulations, new law enforcement action, and even new administrative and management systems.
- There may be need for new clinical medical systems (isolation of cases by quarantine, barrier nursing, physical biocontainment, and extra disinfection and sterilization). There may be need for new patient management systems and even need for improving the general health of the population at risk (better nutrition, housing, primary medical care).
- There may be need for specialist systems, such as vector control, reservoir host control, environmental control (water, animal, and arthropod habitats, etc.) and sanitary engineering (to provide safe food and water, sewage treatment, etc.).
- In the largest epidemics there are also global issues to be dealt with, such as control of international movement of people and animals, establishment of international disease control task forces, and the like. Finally, there is the global issue of adequate funding for such programmes.

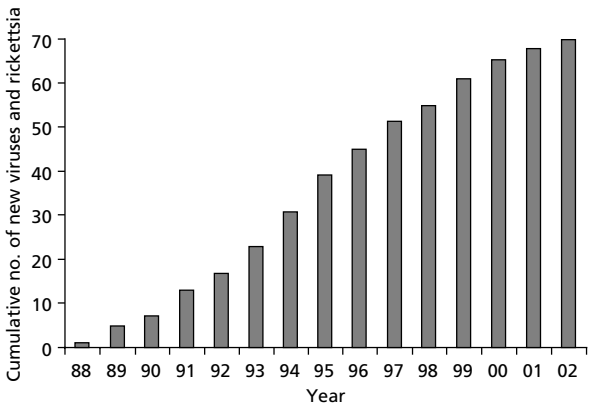

Figure 4 *Emergence of newly recognized viruses affecting humans over the period 1988–2002. Cumulative plot showing the annual increase in numbers of new viruses. (Courtesy of CDC)*

All these activities must be integrated to construct a comprehensive view of the immediate problem at hand and strategy and programmes for its control. Today, this kind of integrated investigative, problem-solving activity is usually thought to be the sole responsibility of government agencies, but, in fact, many professionals from throughout the health sector have played central roles in recent episodes. As it turns out, assessing risk and guiding intervention involve quite diverse activities; for example, in some cases complex field studies of the incidence of infection in the general population or in a selected subpopulation are necessary to determine risk factors for infection, mode of transmission, targets for intervention, etc., whereas in other cases, studies of the pathogenetic mechanisms underpinning the clinical presentation in the individual patient might hold the key. Today, in nearly every instance, important clues lie in characterizing the molecular structure and replication strategy of the virus and the cellular pathobiology of the infection.

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